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RESEARCH ARTICLE

FREE RADICAL SCAVENGING ACTIVITY OF METHANOLIC EXTRACT OF *DIPLAZIUM ESCULENTUM*- A WILD FERN OF WESTERN GHATS

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ABSTRACT

An attempt was made to investigate the antioxidant activity of the methanolic extract of *Diplazium esculentum*. Antioxidant activity of *D. esculentum* was evaluated using DPPH (1, 1- Diphenyl -2-picrylhydrazyl) and Ferric reducing power along with superoxide, hydroxyl and nitric oxide radical scavenging assays. The methanol extract showed the most potent radical-scavenging activity in DPPH with 85.43 % at a concentration of 12.5µg/ml, whereas the reducing power of the standard ascorbic acid was found to be only 58.3 %. The IC_{50} value of the extract was found to be 25.72 µg/ml. The extract manifested significant reducing power (0.98) which exceeded even that of ascorbic acid (0.772) at a concentration of 200 µg/ml. At a maximum concentration of 200 µg/ml, the extract showed 62.4 % inhibition of hydroxyl radical, 51.8% inhibition of superoxide radicals and 87.6 % of nitric oxide radicals respectively. The present investigation suggest that the methanolic extract of *D.esculentum* has significant antioxidant activity, so can be used as a promising food supplement or it may be utilized as a potential source of therapeutic agent.

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INTRODUCTION

Oxidative stress is among the major causative factors in induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases and others (Young and Woodside, 2001). A great number of aromatic, medicinal, spice and other plants contain chemical compounds exhibiting antioxidant properties. Oxidative process is one of the most important routes for producing free radicals in foods, drugs and even in living systems (Halliwell, 1994). The most effective path to eliminate and diminish the action of free radicals, which cause the oxidative stress, is antioxidative defense mechanisms. Antioxidants are those substances, which possess property to break free radical chain reaction. Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants in reducing oxidative stress-induced tissue injury (Pourmorad *et al*., 2006).

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Among the numerous naturally occurring antioxidants; ascorbic acid, carotenoids and phenolic compounds are more effective (Duh *et al*., 1999). They are known to inhibit lipid peroxidation (by inactivating lipoxygenase), scavenge free radicals and active oxygen species by propagating a reaction cycle and to chelate heavy metal ions (Sudarajan *et al*., 2006). *Diplazium esculentum* (Retz.) Sw (Family Athyriaceae) found in the rural areas of Western Ghats and is amongst the leafy vegetables consumed by Paniya and Chetti tribes. They are used for haemoptysis, cough, asthma, phthisis, fever, dyspepsia, stomach ache, diarrhea and antidysenteric. Hence, the present study aims to evaluate the antioxidant activities of methanolic extract of *D. esculentum* by free radical scavenging ability and reducing power.

MATERIALS AND METHODS

Methanol extraction

The *D. esculentum* was collected from Kalpetta, Waynadu and maintained in the Botanical garden of University College, Trivandrum. Plant materials were air dried in shade and powdered. About 40 g of the powdered *D.esculentum* was extracted with 200 ml of 95% methanol using a Soxhlet apparatus and filtered through muslin cloth.

The filtered extract was evaporated under reduced pressure and vacuum drier to get the viscous residue and used for the estimation of antioxidant activities.

DPPH Assay

The DPPH scavenging effect was assayed in the methanol extract of *D. esculentum* according to the method of Sreejayan and Rao, 1996. DPPH scavenging activity was measured by the spectrophotometric method. To a methanolic solution of DPPH (20 µm), 0.05 ml of the test compound dissolved in ethanol was added at different concentration (100 - 500µg). An equal amount of ethanol was added to the control. After twenty minutes, the decrease in the absorbance of test mixture (due to quenching of DPPH free radicals) was read at 517 nm and Inhibitory concentration (IC_{50}) of the extract that caused 50% inhibition was calculated. The experiment was performed in triplicate.

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay procedure described by Benzie and Strain, 1996 was followed to evaluate methanol extract of *D. esculentum*. The FRAP reagent contained 5 ml of a (10 $mmolL^{-1}$) TPTZ (2, 4 ,6- tripyridyl- S- triazine) solution in 40 $mmolL^{-1}$ HCL and 5 ml of $FeCl_3$ (20 $mmolL^{-1}$) and 50 ml of acetate buffer, (0.3 $molL^{-1}$, pH 3.6) and was prepared freshly and warmed at 37ºC. The sample extracts (0.5-2.5 µg/ml) were mixed with 3 ml of FRAP reagent and the absorbance of reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37ºC for ten minutes. The IC_{50} values of the methanol extract of *D. esculentum* and standard ascorbic acid were calculated.

Hydroxy Radical Scavenging Activity

The hydroxy radical scavenging activity was assayed in the methanol extract of *Diplazium esculentum* according to the method of Rajeshwar *et al*. (2005). The reaction mixture contains deoxyribose (2.8mM), $FeCl_3$ (0.1mM), EDTA (0.1mM), H_2O_2(1mM), ascorbate (0.1mM), KH_2PO_4 - KOH buffer (20mM pH 7.4) and various concentration of sample extracts in a final volume of 1.0ml. The reaction mixture was incubated for one hour at 37 ºC. Deoxyribose degradation was measured by TBA (Thiobarbituric acid) method. One ml of TBA 1% (w/v) was added to the mixture and heated in a water bath at 100ºC for twenty minutes. The absorbance of the resulting solution was measured spectrophotometrically at 530nm. The inhibition of degradation was calculated according to the equation $I = A_0\text{-}A_1/A_0 x100$, where A_0 is the absorbance of the control reaction, A_1 is the absorbance of test compound. Inhibitory concentration (IC_{50}) of the extract that caused 50% inhibition was calculated. The experiment was performed in triplicate.

Superoxide Radical Scavenging Activity

The superoxide radical scavenging effect was assayed in the methanol extract of *Diplazium esculentum* according to the method of Liu *et al*. (1997).

Superoxide radical was generated by the photo reduction of riboflavin and was detected by NBT (Nitro Blue Tetrazolium) reduction method. The reaction mixture contained EDTA (6µm), with 3µg NaCN, riboflavin (2 µM), NBT (2µM), KH_2PO_4 - Na_2HPO_4buffer (67mM, pH 7.8) and various concentrations of the extracts in a final volume of 3.0 ml. The tubes were illuminated under incandescent lamp for fifteen minutes. The optical density at 530 nm was measured before and after illumination. The inhibition of superoxide radical was determined by comparing the absorbance values of the control with those of treatments. Ascorbic acid was used as standard and experiment was performed in triplicate.The IC_{50} values of the methanol extract of *D. esculentum* and standard ascorbic acid were measured.

Nitric Oxide Radical Scavenging Activity

The nitric oxide radical scavenging effect was assayed in the methanol extract of *D. esculentum* according to the method of Madan *et al*., 2005). Nitric oxide was generated from sodium nitroprusside and measured by Greiss reaction. Sodium nitroprusside in standard phosphate buffer solution was incubated with different concentration (100 – 500 µg) of the methanol extract dissolved in phosphate buffer (0.025 M, pH 7.4) and the tubes were incubated at 25°C for five hour. Control experiments without the test compound but with equivalent amount of buffer, were run in an identical manner. After five hours, 0.5 ml of incubation solution was removed and diluted with 0.5 ml of Griess reagent (1% sulphanilmide, 2% orthophoshoric acid and 0.1% naphthylethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthylethylene diamine was read at 546 nm. The experiment was repeated in triplicates and IC_{50} value was calculated.

RESULTS AND DISSCUSSION

DPPH Assay

DPPH radical [Di (phenyl)-(2, 4, 6-trinitrophenyl) iminoazanium] is considered to be a model of lipophilic radical. In this mode, scavenging activity is attributed to hydrogen donating ability of antioxidants (Philips *et al*., 2010). Methanol extract of *D. esculentum* possess good DPPH scavenging activity and it was evident that the extract could serve as free radical inhibitor or scavenger. A reducing power is an indicative of reducing agent having the availability of atoms which can donate electron and react with free radicals and then convert them into more stable metabolites, finally terminating the radical chain reaction (Ganu *et al*., 2010). Accordingly, *D. esculentum* might contain a sizable amount of reductants, which may react with the free radicals to stabilize and terminate free radical chain reaction. The reductive capabilities of the *D. esculentum* were compared to ascorbic acid as standard. The reducing power of the plant extract was observed to be 85.43 % at a concentration of 12.5µg/ml, whereas the reducing power of the standard ascorbic acid was found to be only 58.3 % (Fig.1). IC_{50} value in methanol extract in *D. esculentum* (25.72 µg/ml) was more potent than ascorbic acid (38.29 µg/ml).

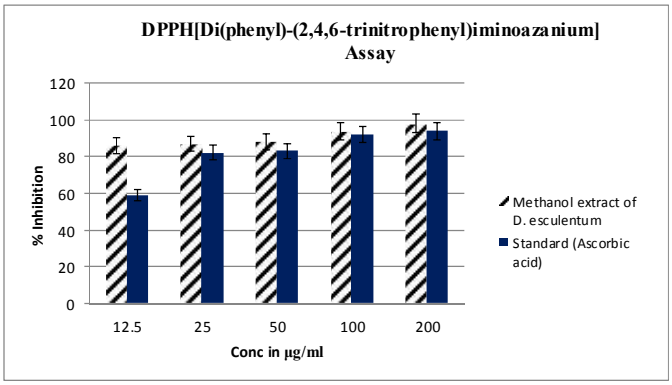

Fig. 1. DPPH Assay in the methanol extract of *Diplaziumesculentum*

FRAP Assay

In FRAP (Ferric Reducing Antioxidant Power) assay, non-enzymatic antioxidants react with prooxidants and inactivate them. In a redox reaction, antioxidants act as 'reductants'. In this context, the antioxidant power can be referred to as 'reducing ability'. In the FRAP assay, an easily reducible oxidant, Fe III, was used and there was a reduction of Fe III-TPTZ complex by antioxidants (Devasagayam *et al*., 2005). The decrease in the concentration of FRAP was a measure of the antioxidant activity of *D. esculentum*. The methanolic extract of *D. esculentum* exhibited a higher FRAP activity of 98 % at a concentration of 200 µg/ml than ascorbic acid and hence its scavenging potency is found to be higher than standard (Fig.2). IC_{50} value in methanol extract of *D. esculentum* was 36.11 µg/ml, showing high antioxidant potentiality.

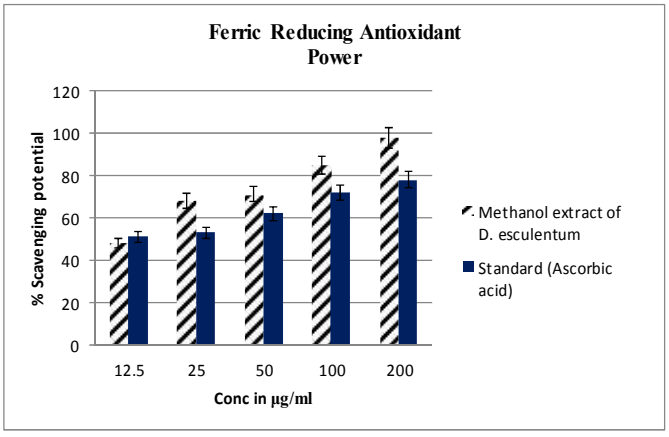

Fig. 2. FRAP Assay in the methanol extract of *iplaziumesculentum*

Hydroxyl Radical Scavenging Activity

Hydroxyl radicals are the major active oxygen species that cause lipid oxidation and immense biological damage (Kumar *et al*., 2008). It can react with lipids, polypeptides, saccharides, nucleotides, and organic acids, especially thiamine and guanosine, thereby causing cell damage (Halliwell *et al*., 1987). The deoxyribose method is a simple assay to determine the rate constants for reactions of hydroxyl radicals (Jiao *et al*., 2005).

When the mixture of $FeCl_3$-EDTA, H, and ascorbate were incubated with deoxyribose in a phosphate buffer (pH 7.4) the hydroxyl radicals attacked the deoxyribose and resulted in a series of reactions that caused the formation of MDA (Malondialdehyde). Any hydroxyl radical scavenger added to the reaction would compete with the deoxyribose for the availability of hydroxyl radicals, thus reducing the amount of MDA formation. In the present study, the methanolic extract of *D. esculentum* exhibited a strong scavenging capacity towards the hydroxyl radicals generated by the Fenton reaction in the test of various concentration range and the scavenging effects were increased with increasing concentration of the extract than the standard. The methanolic extract of *Diplazium esculentum* showed a significant dose-dependent hydroxyl radical scavenging activity and it reached up to 58.874 % at the concentration of 50µg/ml of methanolic extract. However, vitamin C which was used as a positive control showed less radical scavenging effect of 29.22 % at the concentration of 50µg/ml (Fig. 3). The hydroxyl radical scavenging activity of methanol extract of *D. esculentum* had an IC_{50} value of 23.61 µg/ml, more potent than ascorbic acid (IC_{50}=72.4 µg/ml).

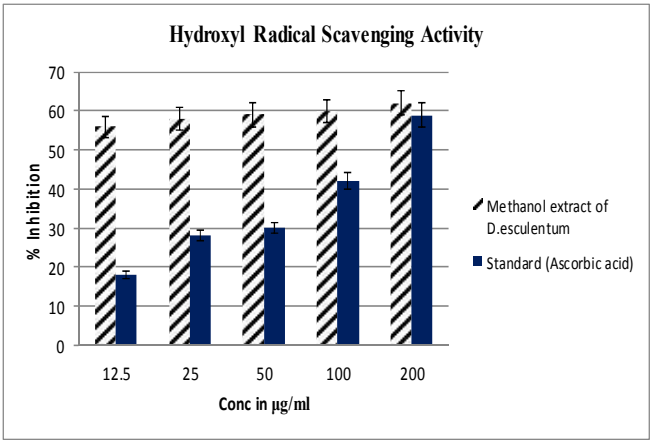

Fig. 3. Hydroxyl Radical Scavenging Activity in the methanol extract of *Diplaziumesculentum*

Super Oxide Free Radical Scavenging Activity

Superoxide anion is an initial free radical and plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radicals, or singlet oxygen in living systems (Stief, 2003). It can also react with nitric oxide and from peroxynitrite, which can generate toxic compounds such as hydroxyl radicals and nitric dioxide (Halliwell, 1997). The superoxide anion radical scavenging activity of the methanolic extract was assayed using an illuminating riboflavin system.

Superoxide anion radicals reduce NBT to a blue coloured formazan that was measured at 560 nm. The decrease of absorbance at 560 nm with antioxidants indicated the consumption of superoxide anions in the reaction mixture. The methanolic extract of *D. esculentum* had strong superoxide radical scavenging activity. The methanolic extract of *D. esculentum* scavenges the superoxide radicals up to 47.89 % at 50µg/ml concentration, whereas standard ascorbic acid at the same concentration scavenges 63 %.

In 12.5 and 25µg/ml concentration, the superoxide free radical scavenging activity in high in methanol extract of *D. esculentum*. The abilities of the plant extract and ascorbic acid to quench superoxide radicals from reaction mixture is reflected in the decrease of the absorbance (Fig.4). IC_{50} value of methanol extract of *D.esculentum* (55.28 µg/ml) were higher than ascorbic acid (28.34 µg/ml). As lower IC_{50} values indicated higher scavenging activity, superoxide free radical scavenging activity of *D. esculentum* is less compared to standard ascorbic acid.

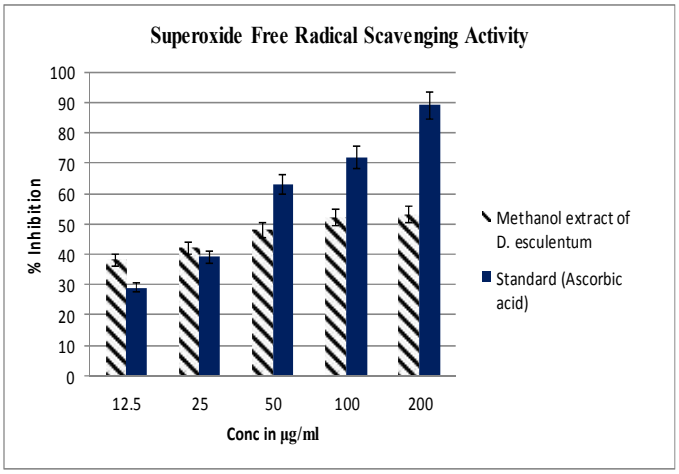

Fig. 4. Super Oxide Free Radical Scavenging Activity in the methanol extract of *Diplaziumesculentum*

Nitric Oxide Scavenging Activity

Nitric oxide is a free radical produced in mammalian cells, involved in the regulation of various physiological process including neurotransmission, vascular homeostasis, antimicrobial and antitumor activities. However, excess production of NO is associated with several diseases. It would be interesting to develop potent and selective inhibitors of NO for potential therapeutic use.

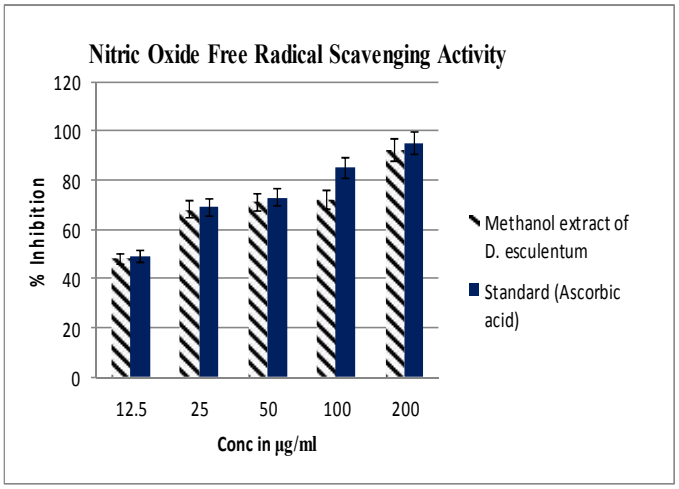

Fig. 5. Nitric Oxide Free Radical Scavenging Activity in the methanol extract of *Diplaziumesculentum*

In the present study, methanol extract of *D. esculentum* exhibited potent nitric oxide radical scavenging activity, which competes with oxygen to react with nitric oxide and thus inhibits the generation of nitrite. At 50 µg/ml concentration both *D. esculentum* extract and standard showed somewhat same scavenging activity. Nitric oxide free radical scavenging activity of 93.44 % was evident in the methanol extract of *D. esculentum* at 200µg/ml, meanwhile the standard ascorbic acid exhibited 94.92 % inhibition at the same concentration (Fig.5). The nitric oxide free radical scavenging activity of methanol extract of *D. esculentum* had an IC_{50} value of 48.56 µg/ml. The results obtained from this study strongly suggested that the methanolic extract of *D. esculentum* has significant antioxidant activity, could serve as an easily accessible item of natural rich antioxidant food which may enhance the immune system against oxidative damage, or it may be utilized as a potential source of therapeutic agent.

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