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Research Article

IN VITRO FREE RADICAL SCAVENGING ACTIVITY OF AQUEOUS EXTRACT FROM THE MYCELIA OF *VOLVARIELLA VOLVACEA* (BULLIARD EX FRIES) SINGER

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ABSTRACT

The aim of this research was to investigate the antioxidant activity of hot water extracts prepared from the mycelia of the paddy straw mushroom, *Volvariella volvacea*. The ability of the extract to scavenge DPPH, ABTS and DMPD radicals were assessed and the EC_{50} values were found to be less than 1mg/ml. The antioxidant potential of the extract was also determined by reducing power assay, ferrousion chelation and FRAP assay. The EC_{50} were found to be 0.51±0.03 mg/ml, 0.88±0.02 mg/ml and 0.77± 0.04 mg/ml, respectively. The extract was observed to contain significant amount of phenols and flavonoids. The free radical scavenging and antioxidant characteristics of the extract may be due to the presence of polyphenols in the extract This antioxidant and protective effect of the aqueous extract of mycelia could be harnessed in the management and prevention of degenerative diseases associated with oxidative stress.

Keywords: Mushrooms, *Volvariella volvacea*, Total phenols, Flavonoids, Antioxidant, Free radical scavenging activity.

INTRODUCTION

Oxidative stress is the steady level of oxidative damage in a cell, tissue, or organ, caused by reactive oxygen species (ROS). This damage can affect a specific molecule or the entire organism. Reactive oxygen species (ROS) production occurs during normal cell metabolism, both in animals and plants. Excess of ROS leads to oxidative stress, resulting in oxidative DNA damage which is implicated in the pathogenesis of numerous disorders, e.g. cardiovascular, atherosclerosis, reperfusion injury, cataractogenesis, rheumatoid arthritis, inflammatory disorders and cancer [1,2]. Many synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) have been used to retard the oxidation process; however, the use of synthetic antioxidants must be under strict regulation due to potential health hazards[3]. The search for natural antioxidants as alternatives is therefore of great interest among researchers. The consumption of a diet rich in fresh fruits and vegetables has been associated with a number of health benefits including the prevention of chronic diseases. This beneficial effect is believed to be due, at least partially, to the action of antioxidant compounds, which reduce oxidative damage in the body [4].

Mushrooms have been widely used since ancient times, not only as foods or food-flavouring materials but also for medicinal or functional purposes. Some common edible mushrooms, which are widely consumed in Asian culture, have currently been found to possess antioxidant activity which is well correlated with their total phenolic content [5]. The paddy straw mushroom, *Volvariella volvacea* (Bulliard ex Fries) Singer, is widely cultivated and is extensively used in Asian cuisines.

In the present study, the antioxidant efficacy of the aqueous extract from the mycelia of *V.volvacea* was determined by *in vitro* methods, including DPPH, ABTS, DMPD, hydroxyl radical scavenging assay, reducing power, chelating activity, FRAP and total molybdenum assay and for its total phenolic and flavonoid contents.

MATERIAL AND METHODS

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), DMPD (N,N-dimethyl-p-Phenylenediamine) and rutin were obtained from Sigma Co. (St. Louis, MO, USA). Ferrozine, gallic acid 2,2azino-bis-(3-ethylbenzothiazoline- 6-sulphonic acid) (ABTS), 2, 4, 6-tripyridyl-s-triazine (TPTZ), and ascorbic acid were obtained from Himedia, Mumbai. Potassium ferricyanide, ferric chloride, trichloroacetic acid, aluminium chloride, potassium persulphate, ammonium persulphate, ferrous suphate, sodium salicylate, ammonium molybdate, sodium carbonate, aluminium chloride, sodium nitrate, sodium hydroxide, Folin-Ciocalteu's phenol reagent, ferrous chloride, sodium hydroxide and solvents were obtained from Merck, Mumbai. All the other chemicals used in the study purchased commercially were of analytical grade.

Sample extraction

The mycelia of *Volvariella volvacea* were collected in petri plates from Tamilnadu Agricultural University, Coimbatore. The mycelia were subcultured and propogated in conical flasks in potato dextrose broth medium (20 g of glucose, 5 g of protease peptone, 1.5 g of $MgSO_4.7H_2O$, 0.2 g of K_2HPO_4, and 0.4 g of KH_2PO_4 in 1L of water with 200 g of boiled potatoes)[6]. The liquid media (70 ml) was dispensed in 250 ml conical flasks, sealed with cotton plugs and autoclaved in 15 lbs atm pressure at 11°C for 15 min. Solid cultures of 2 cm^2 sizes were cut from petriplates and placed in each flask, they were kept in static culture in a temperature-controlled incubator at 25°C for 18-20 days and the mycelia were separated from the filtrate and washed with deionized water. The mycelia were dried, powdered and extracted with water. 10g of the fine powder was boiled in 100ml water for 4 hours and filtered in Whatman filter paper no-1 and the filtrate was vacuum evaporated. The residue was preserved at 4^0C until use.

Phytochemicals

Estimation of total flavonoid content

Total flavonoid content was determined as described by Jia et al[7]. 0.25 ml of various extracts was diluted with 1.25 ml of distilled water. 75 µl of a 5% NaNO2 solution were added and after 6 min 150 µl of a 10% AlCl3.H2O were added and mixed. After 5 min, 0.5 ml of 1 M NaOH was added. The absorbance was measured immediately against the prepared blank at 510 nm. Rutin was used as a standard and the results were expressed as mg of rutin equivalents (RE) per g of dry extract.

Determination of total phenolic content

Total phenol content was determined by the method adapted from Singleton and Rossi [8] with some modifications using the Folin-Ciocalteu reagent. 1 ml of the extract was mixed with 1 ml of Folin-Ciocalteu's phenol reagent. After 3 min, 1 ml of saturated Na_2CO_3 (35%) was added to the mixture and it was made up to 10 ml by adding deionised distilled water. The mixture was kept for 90 min at room temperature in the dark. The absorbance was measured at 725 nm against the blank. The total phenolic content is expressed as mg of gallic acid equivalents (GAE) per gram of dry extract.

Total antioxidant capacity by Phosphomolybdenum assay

The antioxidant activity of the sample was evaluated by the phosphomolybdenum method according to the procedure Prieto et al[9]. An aliquot of 0.1 ml of sample solution was mixed with 1 ml of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated at 95°C for 90 min. The tubes were cooled to room temperature and the absorbance of aqueous solution was measured at 695 nm against a blank. Ascorbic acid was used as a standard. Total antioxidant capacity was expressed nM gallic acid equivalents (GAE) per gram of dry extract.

DPPH radical scavenging activity

The scavenging effect of the aqueous extract of mycelia on DPPH radicals was determined according to the method of Shimada et al[10]. Various concentrations of sample (4 ml) were mixed with 1 ml of methanolic solution containing DPPH radicals, resulting in the final concentration of DPPH being 0.2 mM. The mixture was shaken vigorously and left to stand for 30 min, and the absorbance was measured at 517 nm. The percentage inhibition was calculated according to the formula: $(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the control and A_1 was the absorbance of the sample.

Determination of reducing power

The reducing power of the extract was determined according to the method of Oyaizu[11]. 2.5 ml of various concentrations of the extract, 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml and 2.5 ml of 1% potassium ferricyanide were mixed and incubated at 50°C for 20 min and centrifuged for 10 min at 5000 g after addition of 2.5 ml of 10% trichloroacetic acid. 2.5 ml aliquot of supernatant was mixed with 2.5 ml of deionised water and 0.5 ml of 0.1% ferric chloride. After 10 min of incubation, the absorbance was measured at 700 nm against a blank.

Chelating effects on ferrous ions

The ability of the extracts to chelate ferrous ions was estimated by the method of Dinis et al[12]. Briefly, 2 ml of various concentrations of the extracts in methanol were added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml). The mixture was then shaken vigorously and left at room temperature for 10 min. The absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozineFe^{2+} complex formation was calculated as $[(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the control and A_1, the absorbance of the mixture containing the extract or the standard.

ABTS radical cation scavenging activity

The ABTS radical cation scavenging activity was performed with slight modifications described by Re et al[13]. The $ABTS^{\cdot+}$ cation radicals were produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate, stored in the dark at room temperature for about 12 h. Prior to use, the solution was diluted with ethanol to get an absorbance of 0.700 ± 0.025 at 734 nm. Free radical scavenging activity was assessed by mixing 10 µl of test sample with 1.0 ml of ABTS working standard in a microcuvette. The decrease in absorbance was measured exactly after 6 min. The percentage inhibition was calculated according to the formula: $[(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the control without the sample , and A_1 was the absorbance of the sample.

DMPD assay

The principle of the DMPD assay is that at an acidic pH and in the presence of a suitable oxidant solution DMPD can form a stable and colored radical cation ($DMPD^{\cdot+}$). The assay was performed as described by [14,15] . $DMPD^{\cdot+}$ has a maximum absorbance at 505 nm. DMPD. The discolourisation of the chromophore is indicative of the scavenging ability of the antioxidant. This assay is based on the capacity of the extract to inhibit the $DMPD^{\cdot+}$ radical cation. $DMPD^{\cdot+}$ was obtained by adding 0.3 ml ferric chloride (0.05 M) to DMPD in acetate buffer. The reaction mixture contained different concentrations (0.2mg to 1mg/ml) of the extract, water and 1ml of $DMPD^{\cdot+}$ solution .These samples were vortexed, and incubated in dark at room temperature for 15 min. Absorbance was measured at 505 nm. Buffer solution was used as blank instead of sample and distilled water used for control. The percentage inhibition was calculated according to the formula: $[(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the control, and A_1 was the absorbance of the sample.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was used to estimate the reducing capacity of fruit extracts, according to the method of Benzie and Strain[16]. The FRAP reagent contained 2.5 ml of a 10 mM TPTZ solution in 40 mM HCl, 2.5 ml of 20 mM $FeCl_3$.6H2O and 25 ml of 300 mM acetate buffer (pH 3.6). It was freshly prepared and warmed at 37°C. 900 µl FRAP reagent was mixed with 90 µl water and 30 µl of the extract. The reaction mixture was incubated at 37°C for 30 minutes and the absorbance was measured at 593 nm.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of fruit extracts was assayed by the method of Smirnoff and Cumbes[17]. The reaction mixture 3.0 ml contained 1.0 ml of 1.5 mM FeSO4, 0.7 ml of 6 mM hydrogen peroxide, 0.3 ml of 20 mM sodium salicylate and varied concentrations of the extracts. After incubation for an hour at 37°C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. The scavenging activity of hydroxyl radical effect was calculated as follows : $[1-(A_1 - A_2) / A_0] \times 100$, where A_0 is absorbance of the control (without extract) and A_1 is the absorbance in the presence of the extract, A_2 is the absorbance without sodium salicylate.

Statistical analysis

All assays were carried out in triplicates and results are expressed as mean ± SD.

RESULTS AND DISCUSSION

The extraction yield, total phenolic content, total flavonoid content and total antioxidant activity of the aqueous extract of the mycelium of *V. volvacea* is presented in Table 1.

Percent yield of the aqueous extract was found to be 40 .03 ± 1.2.

Polyphenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity. The phenolic compounds may contribute directly to antioxidative action [18].

The total phenol and flavonoid content of the aqueous extract of the mycelium was found to be 19.08 ± 0.45 mg GAE/g dry weight, 8.23 ± 0.27mg RE/g dry weight respectively. The phosphomolybdenum method is based on the reduction of molybdenum by the antioxidants and the formation of a green molybdenum (V) complex, which has an absorption at 695 nm. The total antioxidant capacity observed in the aqueous extract of mycelium was 231.49 ± 14.70 nM GAE/g respectively (Table 1).

Table 1: Extraction yield, total phenols, flavonoids contents and total antioxidant capacity of aqueous extract of *V. volvacea* mycelia

Extraction yield (%)	Total Phenols (mg GAE/g)	Total Flavonoids (mg RE/g)	Total antioxidant capacity (nM GAE/g)
40 .03 ± 1.2	19.08 ± 0.45	8.23 ± 0.27	231.49 ± 14.70

Values are expressed as mean ± SD (n =3);

GAE - Gallic acid equivalents; RE- Rutin equivalents

The antiradical activity of flavonoids and phenols is principally based on the structural relationship between different parts of their chemical structure. [19, 20] Natural polyphenols are capable of quenching free radicals, chelating metal ions and activating antioxidant enzymes, reducing α-tocopherol radicals, and inhibiting oxidases [21,22]

The free radical scavenging efficiency of the aqueous extract was evaluated by different *in vitro* methods viz., DPPH, DMPD, ABTS radical scavenging activity, reducing power, hydroxyl radical scavenging assay, FRAP and chelation activity.

DPPH radical scavenging activity

DPPH is a stable free radical frequently used to determine radical scavenging activity of natural compounds. In its radical form, DPPH absorbs at 517 nm, but upon reduction with an antioxidant, its absorption decreases due to the formation of its non-radical form, DPPH–H[23]. Thus, the radical scavenging activity in the presence of a hydrogen donating antioxidant can be monitored as a decrease in absorbance of DPPH solution[24]

Figure 1 shows free radical scavenging activity of the aqueous extract at different concentrations. The radical scavenging activity was observed to increase with increasing concentrations, with. The EC_{50} value was found to be 0.69 ± 0.05mg/ml .The results indicated that the extract exhibited the ability to quench the DPPH radical, which indicated that extract was good antioxidant with radical scavenging activity.

Reducing power

The reducing power of a compound is related to its electron transfer ability and may serve as a significant indicator of its potential antioxidant activity. In this assay, the yellow color of the test solution changes to green and blue depending on the reducing power of test specimen. Greater absorbance at 700 nm indicated greater reducing power.

Figure 2 presents the reductive capabilities of the aqueous extract of the mycelia of the paddy straw mushroom. The extract demonstrated reducing power that increased linearly with concentration. The EC_{50} value was found to be 0.51±0.03 mg/ml.

The reducing power of various extracts might be due to its hydrogen-donating ability, as described by Shimada et al [10]. Therefore, the extracts might contain reductones, which could react with free radicals to stabilize and terminate radical chain reactions.

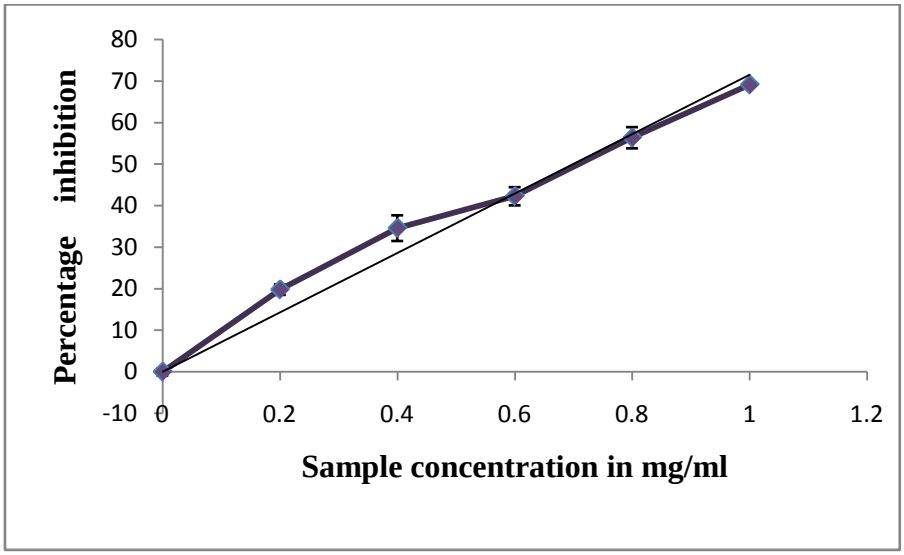

Fig. 1: DPPH Radical scavenging activity of aqueous extract of *V.volvacea*

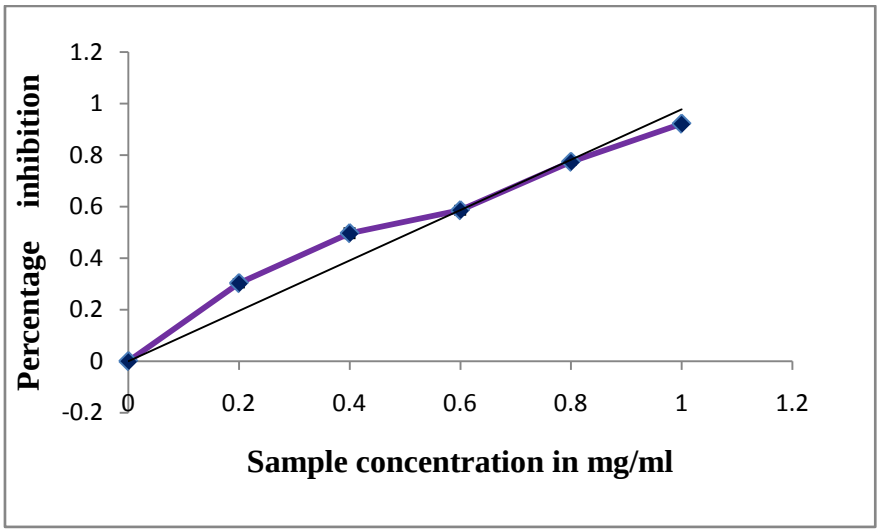

Fig. 2: Reducing power of aqueous extract of *V.volvacea*

Metal chelating activity

The chelating of ferrous ions by aqueous extract of the mycelia was estimated. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted and eventually that the red color of the complex fades. Measurement of color reduction therefore allows estimation of the chelating activity of the co-existing chelator [25] Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. Metal ion chelating capacity plays a significant role in antioxidant mechanism, since it reduces the concentration of the catalyzing transition metal in LPO[26].

The chelating effects of the mycelia on ferrous ions increased with increasing concentrations (**Figure 3**). The EC_{50} value was found to be 0.88±0.02 mg/ml. The data obtained from Figure 3 revealed that the extract demonstrates a marked capacity for iron binding, suggesting that their action as peroxidation protector may be related to its iron binding capacity.

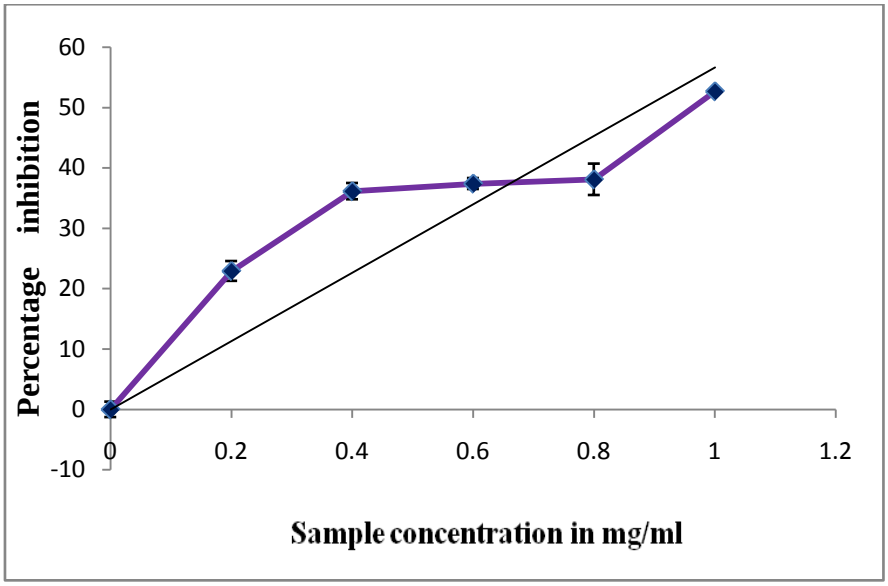

Fig. 3: Metal chelating ability of aqueous extract of *V.volvacea*

ABTS radical scavenging activity

ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants and of chain-breaking antioxidants [27]. ABTS assay is often used in evaluating total antioxidant power of single compounds and complex mixtures of various plants [28-30]. In this assay, ABTS radical mono cation was generated directly in stable form from potassium peroxodisulfate. (**Figure 4)** The activity was found to be increased in a dose-dependent manner from at a concentration of 0.2mg/ml to 1mg/ml. The extract exhibited an EC_{50} value of 0.68 ± 0.03mg/ml . Therefore, the ABTS radical scavenging activity of aqueous extract of mycelia indicates its ability to scavenge free radicals, thereby preventing lipid oxidation via a chain -breaking reaction.

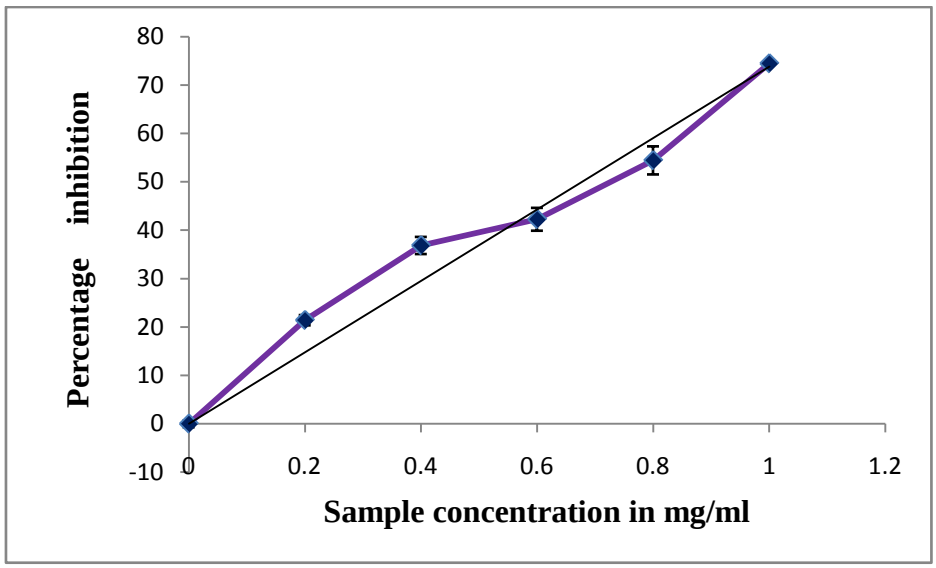

Fig. 4: ABTS Radical scavenging activity of aqueous extract of *V.volvacea*

DMPD Radical scavenging activity

The aqueous extracts (0.2mg/ml to 1.0mg/ml) were analysed for the ability to scavenge the DMPD radicals. The discoloration of the DMPD solution was found to increase with an increase in the concentration of the extract. Dark color of DMPD$^{\bullet+}$ radical cation solution becomes lighter and absorbance of solution becomes lower, in the presence of an antioxidant compound. The DMPD•+ radical cation solution shows a maximum absorbance at 505 nm. Antioxidant compounds which are hydrogen donors to DMPD$^{\bullet+}$ quench the color of DMPD•+ solution[15] The DMPD$^{\bullet+}$ radical quenching was found to increase with an increase in concentration of the extract and the EC_{50} value was found to be 0.61 ± 0.02mg/ml (figure 5)

Phenolic compounds are known to be powerful chain-breaking antioxidants[31]. It had been reported that the antioxidant activity of plant materials is well correlated with the content of their phenolic compounds[18]. So, it is important to consider the effect of the phenols present in the mushroom extract is responsible for its antioxidant activity. Thus the DMPD radical scavenging capacity of the extract could be attributed to that of the phytochemicals harbored in the extract.

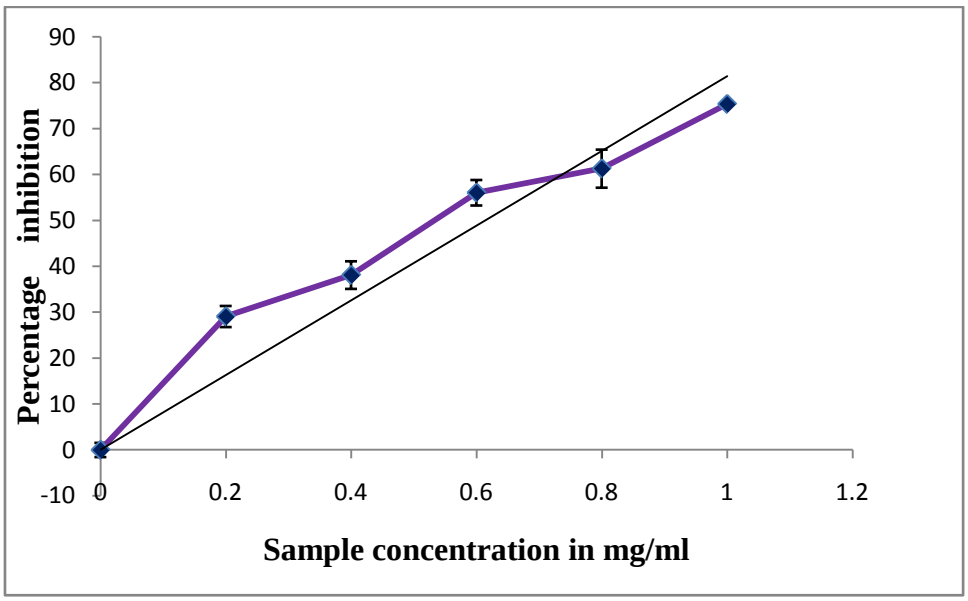

Fig. 5: DMPD Radical scavenging activity of aqueous extract of *V.volvacea*

Ferric reducing antioxidant power (FRAP)

The antioxidant capacity of the aqueous extract of mycelia is determined by the ability of the antioxidants in these extracts to reduce ferric iron to ferrous in FRAP reagent, which consists of 2,4,6-tris (1-pyridyl)-5-triazine (TPTZ) prepared in sodium acetate buffer, pH 3.6. The reduction of ferric iron in FRAP reagent will result in the formation of a blue product (ferrous – TPTZ complex) whose absorbance can be read at 593 nm. FRAP assay was initially developed to assay the plasma antioxidant capacity, but could also be used to measure the antioxidant capacity of a wide range of biological samples, pure compounds, fruits, wines, and animal tissues[28]

The absorbance decrease is proportional to the antioxidant content[16]. The ferric ions reducing activities of the extract at different concentrations are shown in Figure 6. The EC_{50} value was found to be 0.77± 0.04 mg/ml. Our results showed significant ferric reducing power which indicated the hydrogen donating ability of the extract.

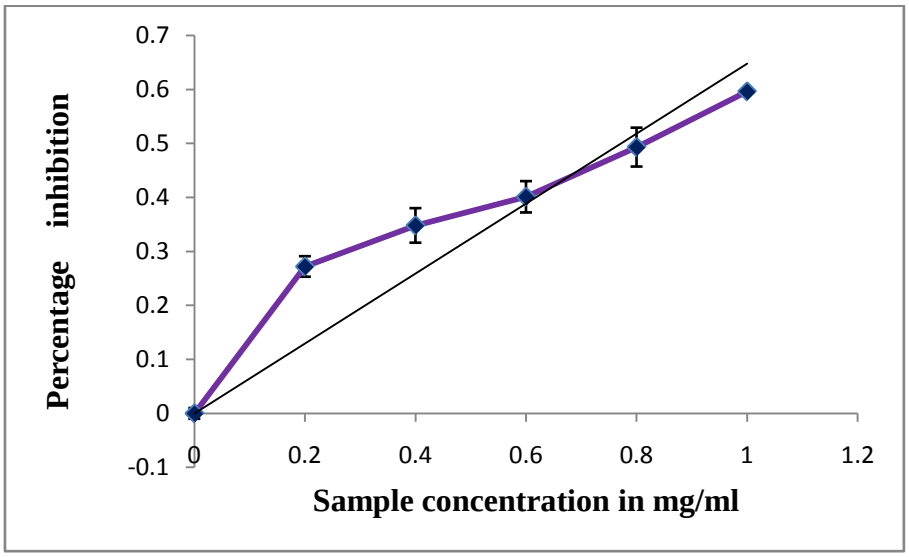

Fig. 6: FRAP assay of aqueous extract of *V.volvacea*

Hydroxyl radical scavenging activity

The hydroxyl radical is an extremely reactive in biological systems and has been implicated as highly damaging species in free radical pathology, capable of damaging biomolecules of the living cells. The hydroxyl radical can cause oxidative damage to DNA, lipids and proteins[33]. The •OH scavenging` activity of mushroom extracts was assessed by its ability to compete with salicylic acid for •OH radicals in the •OH generating/detecting system[34]. In the present study, the hydroxyl radical-scavenging effect of the aqueous extract of mycelia, in a concentration range of 0.2 mg/ml to 1mg/ml was determined (Figure 7). The scavenging capacity was found to increase with concentration. The EC_{50} value was found to be 0.68 ± 0.03 mg/ml.

The results indicate the effective scavenging potential of the extract against hydroxyl radicals.

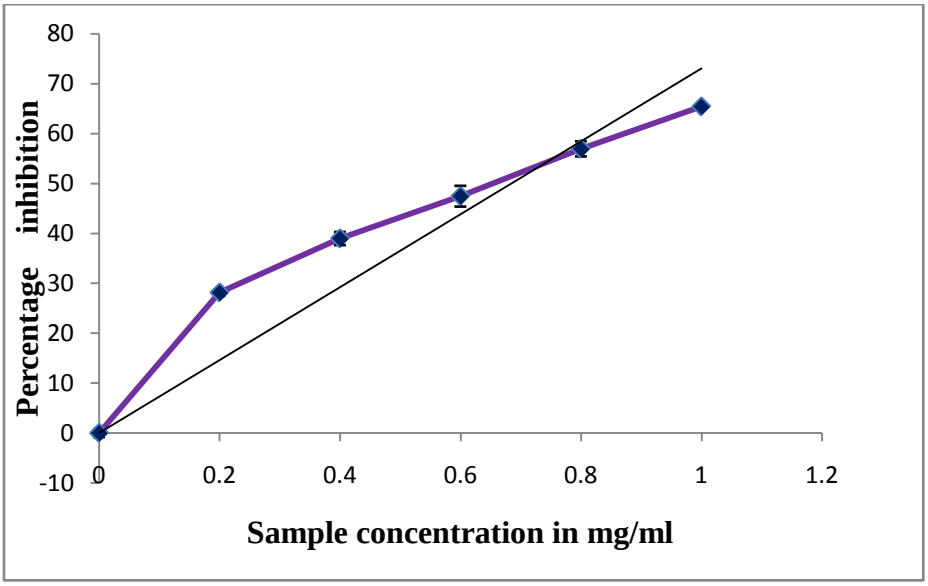

Fig. 7: Hydroxyl Radical scavenging activity of aqueous extract of *V.volvacea*

CONCLUSION

In present study, antioxidant activities of the aqueous extract from the mycelia of *V. volvacea* was investigated. The extracts were found to possess radical scavenging and antioxidant activities, as determined by scavenging effect on the DPPH, ABTS, DMPD and hydroxyl radicals, reducing power, chelating effect on ferrous ions, FRAP and total antioxidant activity. Generally, EC_{50} values of lower than 10 mg/ml indicated that the extracts were effective in antioxidant properties.

In the present study it is found that the aqueous extract of the mycelia contains substantial amount of phenolics and flavonoids and it is the extent of phenolics present in this extract being responsible for its marked antioxidant activity as assayed through various *in vitro* models. Thus, it can be concluded that aqueous extract of *V volvacea* mycelia could be used as an accessible source of natural antioxidants with consequent health benefits.

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