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Research Article

ANTIOXIDANT ACTIVITIES OF METHANOLIC EXTRACTS FROM TEN *PLEUROTUS* SPECIES

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ABSTRACT

The antioxidant activities of methanolic extract from mycelia of ten *Pleurotus* species were investigated. The main aim of this study was to evaluate and compare the antioxidant activities of methanolic extracts of mushroom mycelium of ten *Pleurotus* species by three different methods. The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity, reducing power, chelating effect on ferrous ion and phenolic content of mycelial extract of ten edible mushrooms were analyzed. *P. sajor-caju* showed highest free radical scavenging activity (92.05 %) and reducing power (1.082 %) at 8 mg/ml concentration. *P. citrinopileatus* showed highest percentage of chelating effect on ferrous ion (90.66 %). The lowest EC_{50} value of free radical scavenging activity was found in *P. sapidus* which indicated strongest ability of the mycelial extract to act as DPPH radical scavenger. The lowest chelating effect on ferrous ion was noticed in *P. sajor-caju* but EC_{50} of reducing power was much lower than the synthetic antioxidant (BHT). The revealed data showed that mycelia of all studied *Pleurotus* species possessed potent antioxidant activity and their inclusion in the diet may help to prevent diseases caused by oxidative damage.

Keywords: Antioxidant activity, EC_{50} value, edible mushroom, *Pleurotus* species, total phenol.

INTRODUCTION

Mushrooms have been reported as therapeutic foods that are useful in preventing diseases such as hypertension, hypercholesterolemia and cancer due to their chemical composition. The genus *Pleurotus* species comprises about 40 species[1], they are ubiquitous, being found in both temperate and tropical part of the world and are now considered to be the second most important cultivated mushroom in the world[2]. *Pleurotus* mushrooms (oyster mushrooms) are quite easily cultivated artificially, most often in liquid medium[3] and they are also appreciated as a food due to flavor along with medicinal and bioremediation properties.

Almost all organisms possess antioxidant defense and repair systems that have evolved to protect them against oxidative damage, these systems are not sufficient to prevent the damage entirely[4]. Natural antioxidants are being extensively studied for their capacity to protect organisms and cells from damage brought on by oxidative stress due to ageing and degenerative diseases[5]. Wild mushrooms are traditionally used in many Asian countries and also in other continents as both food and medicine[6,7]. Wild mushrooms, cultivated mushrooms are becoming more important in our diet for their nutritional and pharmacological properties[8] and mycelial mushrooms are also important for their secondary metabolite production which contains medicinal property. There are many researches of antioxidant activity on wild and cultivated mushroom in the world.

Our objective was to evaluate and compare the antioxidant activities of methanolic extracts of mushroom mycelium of ten *Pleurotus* species viz. *P. sapidus* (Schulzer) Sacc. (DMRP-4), *P. sajor-caju* (Fr.) Singer (DMRP-112), *P. membranaceus* Massee. (DMRP-189), *P. ostreatus* (Jacq. ex Fr.) Kummer (DMRP-262), *P. flabellatus* (Berk. & Broome) Sacc (DMRP-5), *P. florida* (Mont.) Singer (DMRP-88), *P. hypsizygus ulmarius* (Bull.) Redhead (DMRP-115), *P. djamor* (Rumph. ex Fr.) Boedijn (DMRP-205), *P. eryngii* (DC.) Quel (DMRP-135) and *P. citrinopileatus* Singer (DMRP-10) by three different methods (DPPH method, Reducing power method and Chelating effect on ferrous ion).

MATERIALS AND METHODS

Sample

Fungal mycelia cultures of ten *Pleurotus* species *viz*. *P. sapidus* (Schulzer) Sacc. (DMRP-4), *P. sajor-caju* (Fr.) Singer (DMRP-112), *P. membranaceus* Massee. (DMRP-189), *P. ostreatus* (Jacq. ex Fr.) Kummer (DMRP-262), *P. flabellatus* (Berk. & Broome) Sacc (DMRP-5), *P. florida* (Mont.) Singer (DMRP-88), *P. hypsizygus ulmarius* (Bull.) Redhead (DMRP-115), *P. djamor* (Rumph. ex Fr.) Boedijn (DMRP-205), *P. eryngii* (DC.) Quel (DMRP-135) and *P. citrinopileatus* Singer (DMRP-10) were procured from Directorate of Mushroom Research, Chambaghat, Solan, India.

Mushroom Mycelia Production

Pure cultures of all the *Pleurotus* species was carried out in Potato Dextrose Agar (PDA) medium. To study antioxidant activity, mushroom mycelium was grown sterile conical flask (250 mL) containing 50 ml of Basal Synthetic (BSL) Medium. The BSL Medium contained glucose (30 g/L), yeast extract (2 g/L), peptone (2.5 g/L), $MgSO_4$, $7H_2O$ (0.5 g/L), $Ca(NO_3)_2$ (0.5 g/L), $(NH_4)_2SO_4$ (0.25 g/L), KH_2PO_4 (0.25 g/L), $FeCl_3$

(0.01g/L), $ZnSO_4$ (0.0001 g/L), Inositol (0.05 mg/L), Thiamine (100 µg/L), Biotin (50 µg/L), Folic acid (100 µg/L), CaCl2 (0.1 M in 5 mL/L) and distilled water (1 L)[9]. The medium was inoculated with disk of 6 mm diameter of mushroom mycelia obtained from six to eight days old grown culture on Potato Dextrose Agar (PDA) plate. The growth was carried out under stationary condition at 28°C (Shaking Incubator: LSI 4018R). After 30 days incubation in the dark, the liquid medium was filtered and the mycelium separated from the liquid[10].

Mushroom Mycelia Extraction

Preparation of methanolic extracts of mushroom
was done by slightly modified method of Mau et al.[11]. The dried powdered mycelium (5g) was extracted by grinding with 50 mL of methanol with the help of pastle and morter. After filtering through Whatman No.4 filter paper, the mycelium was then extracted twice with addition of 50 mL of methanol in each. The methanolic extract was then evaporated at 40ºC to dryness in rotary evaporator (Rotavap: PBV-7D). The dried extract was used directly for determination of antioxidant activities.

Free Radical Scavenging Activity (DPPH)

Free radical scavenging activity (FRS) activity was measured by a little bit modified method of Shimada et al.[12]. 4 ml dried mushroom methanolic extract (0.25- 16 mg/ml) was mixed with 1 ml of (0.0002 M) methanolic solution containing 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (Sigma). The mixture after shaking vigorously was allowed to stand for 30 min and the absorbance was measured at 517 nm against a blank in a spectrophotometer (Eppendorf AG 22331Hamburg). EC_{50} (mg/mL) is the valuable concentration at which DPPH radical were scavenged by 50% (w/v) and was obtained by interpolation from linear regression analysis. BHT and ascorbic acid were used as a control. Inhibition of free radical by DPPH in percent was calculated as follows: Percentage of inhibition: $[(A_{Blank} - A_{Sample}) / A_{Blank}] \times 100$, Where, A_{Blank} and A_{Sample} denotes the absorbance of control and test compound respectively.

Reducing Power

Reducing power was determined by the slightly modified method of Oyaizu[13].Each mycelial extract (0.5-8.0 mg/mL) in methanol (2.5 mL) was mixed with 2.5 mL of 0.2 M sodium phosphate buffer and 2.5 mL of 1% potassium ferricyanide and the mixture was incubated at 50°C for 20 min. Then 2.5 mL of 10 % (w/v) trichloroacetiic acid (TCA) were added. After centrifugation at 200 g for 10 min, 5 mL of upper layer was mixed with 5 mL of deionised water and 1 mL of ferric chloride (0.1 %). The absorbance was measured against a blank in 700 nm in spectrophotometer (Eppendorf AG 22331Hamburg). EC_{50} (mg/mL) is the effective concentration at which the absorbance was 0.5 for reducing power. BHT was used as control.

Chelating Effect on Ferrous Ion

It was determined by the method of Decker & Welch[14]. 2 ml of each methanolic extract at various concentration (0.05-1.5 mg/ml) various concentrations of extract in the methanol was added to a solution of 0.002 M $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 0.005 M ferrozine (0.2 ml). The total volume was made to 5 ml with methanol. Then, the mixture after shaking vigorously was allowed to stand for 10 min in room temperature and the absorbance was measured at 562 nm in spectrophotometer (Eppendorf AG 22331Hamburg). A mixture without extract was used as the control. Ethylenediaminetetraacetic acid (EDTA) was used as a standard. The inhibition percentage of Ferrozine Fe^{2+} complex formation was then calculated: Metal Chelating effect (%) = $\{A_0\text{-}A_1/A_0\} \times 100$ %, Where, A_0 and $A_1$is the absorbance of the control and the sample respectively. EC_{50} (mg/ml) value was calculated from the graph of ferrous ion inhibition percentage against extract concentration.

Fungal Phenol Estimation

Total phenols was determined according to the method reported by Swain and Hillis, 1959[15] (Folin ciocalteau reagent method) with the Folin-Ciocalteu reagent, using tannic acid in ethanol (80%, w/v) as standard.

Statistical Analysis

For each one of the mushroom species, the assays were carried out in triplicate form. The average data recorded for each replica were subjected to the one way ANOVA technique using Origin 7 followed by Tukey's Least Significant Differences (LSD). The results were expressed as mean values ± standard deviation (SD) and the significance was tested.

RESULTS AND DISCUSSION
Free Radical Scavenging (FRS) Activity

The methanolic extract of mycelia was subjected to screening for possible antioxidant activity by the DPPH free radical scavenging method. Scavenging the stable DPPH radical is widely used method to evaluate the antioxidant activity in comparison to other method because this method is simple, requires short period of time and sensitive. DPPH is a stable free radical that shows a characteristic absorbance at 517 nm, which decreases significantly when exposed to radical scavengers by providing hydrogen atom or electron to be a stable diamagnetic molecule.

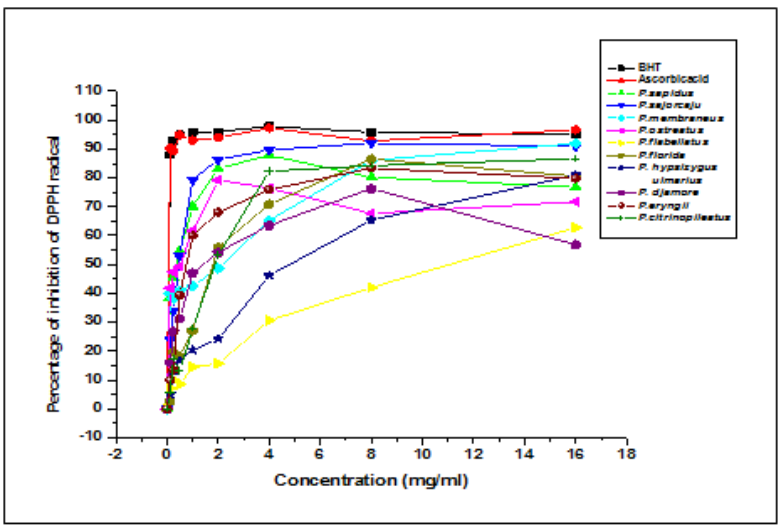
Methanolic extracts of mushroom mycelia of *Pleurotus* species showed varying scavenging ability. Among the ten *Pleurotus* species, *P. sajor-caju* showed highest free radical scavenging effect (92.05±1.37%) at 8.0 mg/ml concentration and followed by *P. membranaceus* (91.92±0.46 %), *P. citrinopileatus* (86.63±0.07), *P. florida* (86.58±0.52 %), *P. eryngii* (83.33±2.32%), *P. hypsizygus ulmarius* (80.98±3.78 %), *P. sapidus* (80.20±1.87 %), *P. ostreatus* (79.29±1.25 %), *P. djamor* (76.31±1.79 %) and *P. flabellatus* (62.75±1.87%). However, highest FRS activities of both the synthetic antioxidants BHT, ascorbic acid were 97.83% at 0.125 mg/ml concentration and 97.25% at 4 mg/ml concentration, respectively. Variations in FRS activities by various *Pleurotus* species were graphically represented in Graph 1.

EC_{50}, is the effective concentration at which the antioxidant activity was 50% and DPPH radicals were scavenged by 50%. EC_{50} was obtained by interpolation from linear regression analysis. The strongest EC_{50} of FRS activity had been found in *P. sapidus* at 0.352 mg/ml concentration in comparison to other *Pleurotus* species. Synthetic antioxidant (BHT and ascorbic acid), which was used as a standard, had a superior ($p<0.05$) EC_{50} (0.049 mg/ml) value in comparison to ten *Pleurotus* species. Table 1. represented the EC_{50} of FRS activity, reducing power, chelating effect on ferrous ion and total phenol content of all the *Pleurotus* species along with standard.

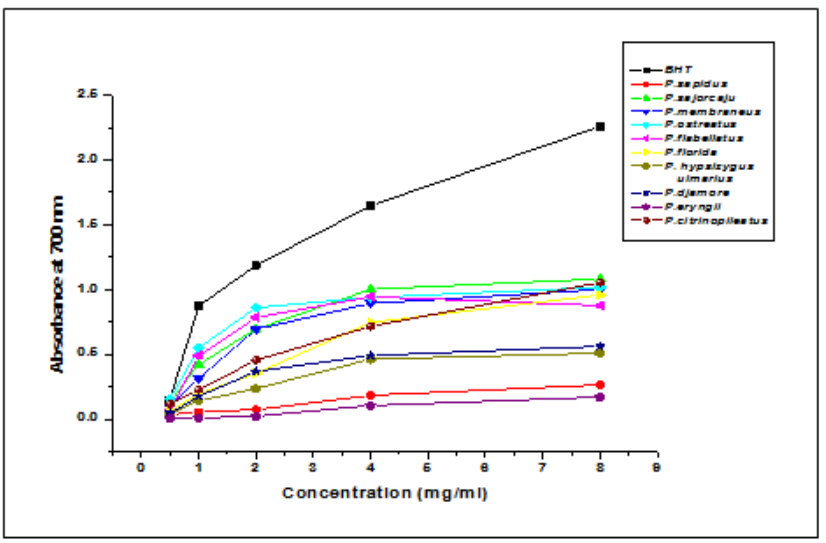
Table 1: EC_{50} of FRS Activity, Total Phenol Content of all the *Pleurotus* species and Standard

Mushroom samples	EC_{50} value (mg/ml)			Total phenol (mg/g)
	Free Radical Scavenging (FRS) activity	Reducing power	Chelating effect of ferrous ions	
BHT	0.049	1.847	-	-
Ascorbic acid	0.049	-	-	-
EDTA	-	-	0.033	-
P. sapidus (DMRP-4)	0.352	< BHT	0.349	3.87±0.08
P. sajor-caju (DMRP-112)	0.498	< BHT	0.031	3.87±0.09
P. membranaceus (DMRP-189)	2.187	< BHT	0.104	1.80±0.05
P. ostreatus (DMRP-262)	0.496	< BHT	0.041	4.33±0.05
P. flabellatus (DMRP-5)	11.190	< BHT	0.349	0.50±0.00
P. florida (DMRP-88)	1.792	< BHT	0.032	2.78±0.01
P. hypsizygus ulmarius (DMRP-115)	4.820	< BHT	0.041	1.48±0.17
P. djamor (DMRP-205)	1.440	< BHT	0.274	2.03±0.03
P. eryngii (DMRP-135)	0.741	< BHT	0.188	2.55±0.07
P. citrinopileatus (DMRP-10)	1.910	< BHT	0.052	1.25±0.01

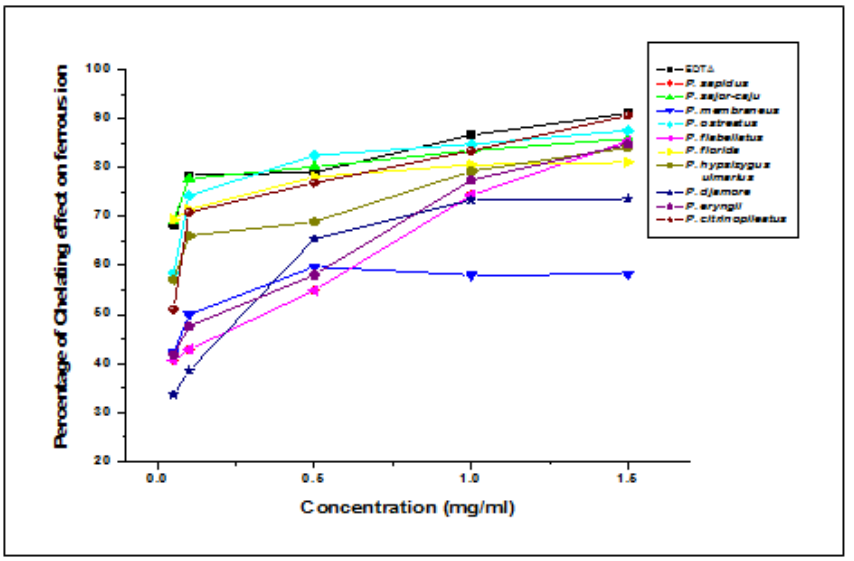
All values are expressed as Mean ± SD (n = 3). Values bearing different letters are significant t-test at P<0.05.



Graph 1: Free Radical Scavenging (FRS) activity of methanolic extract of mycelial mushroom on DPPH at various concentrations. Values express as mean ± SD of triplicate experiments.



Graph 2: Reducing power of methanolic extract of mycelial mushrooms at various concentration. Values express as mean ± SD of triplicate experiments.



Graph 3: Chelating effect on ferrous ion of methanolic extract of mycelial mushrooms at various concentrations. Values express as mean ± SD of triplicate experiments.

The DPPH scavenging activities of methanol extract was more effective in comparison to ethyl acetate and hot water extract[16]. Tsai[17], Mau et al.[18] at 10 mg/ml, the methanolic extracts of *Agrocybe cylindracea, Ganoderma tsugae* and *Morchella esculenta* mycelia scavenged 91.4%, 95.6% and 94.1% of DPPH radicals, respectively and showed close similarity with the finding of *P. sajor-caju*. Kalyoncu et al.[19] found that ethanolic extract of *Pleurotus eryngii* and *Pleurotus ostreatus* had potent free radical scavenging activity. The Free Radical Scavenging (FRS) activity of standard was higher than all mushroom extracts, which was reported previously[8,18]. The finding showed that the antioxidant activity of mushroom was strongly correlated with the phenolic content which was similar with the work of Velioglu et al.[20]. FRS ability of *P. djamor* was 76.31% at 8 mg/ml concentration which closely matched with the finding of Lee et al.[21] on *Hypsizigus marmoreus* mycelium. *P. ostreatus* showed highest FRS activity in different organic and inorganic extract was ranged 58.76 % to 89.93 % at a concentration of 20 mg/ml[3]. Crude Extract from *Lentinus squarrosulus* mycelial culture showed highest FRS activity of 86 % at 20 mg/ml concentration[22]. Highest percentage of DPPH scavenging activity at 4 mg/ml concentration of *Pleurotus pulmonarius* showed 87.17±0.86; *Pleurotus eryngii* showed 75.00±1.88 and EC_{50} was 1.21, 2.67 mg/ml, respectively[23] but Ferreira et al.[24] found EC_{50} value of *Pleurotus eryngii* was 9.21 mg/ml. Somasundaram et al.[25], reported that IC_{50} value of water and methanolic extract of *P. sajor-caju* was 1.80 and 2.50 mg/ml, where as in *P. djamor* it was 3.00 and 1.90 mg/ml, respectively. The EC_{50} value of DPPH radical of methanol extract of *P. eous* was 4.2 mg/ml[16]. The findings showed similarity with those of *P. djamor, P. florida* and *P. membranaceus.* Synthetic antioxidants showed higher inhibition in comparison to test samples which showed similarity with this finding[26]. The synthetic antioxidants ascorbic acid at 0.5 mg/ml, gave the scavenging activity of 90.93±2.16% with the IC_{50} values of 0.09±0.01 mg/ml. This result revealed that methnolic extract of the mushrooms mycelia were free radical scavengers and possibly acting as primary antioxidant. However, the active components in the mycelial extract was responsible for the observed antioxidant activity, are unknown. Further work is necessary on the isolation and purification of the active components.

Reducing Power Assay

The assay of reducing power, the antioxidant compounds convert into the oxidized form of iron in ferricyanide (Fe3+) ion to ferrous (Fe2+) ion. The reducing power is related to its electron transfer ability and may serve as a significant indicator of potential antioxidant activity. Here, the change of coloure of the test solution from yellow to green is dependent on the reducing power of the sample and absorbance at 700 nm indicates greater reducing power ability.

Reducing powers of methanolic extracts from mushrooms mycelia were excellent and increased steadily increased with lower to higher concentrations (0.5-8 mg/ml), which were shown in Graph 2. The highest reducing powers were found in *P. sajor-caju* (1.082±0.03), *P. citrinopileatus* (1.054±0.01), *P. ostreatus* (1.017±0.02) and *P. eryngii* (1.000±0.08) at 8 mg/ml concentration. Out of ten species, *P. djamor* showed lowest reducing power of 0.171±0.03 at highest concentration. However, the reducing power of BHT maintained the level of 0.153 to 2.258 at the concentration of 0.5 to 8.0 mg/ml. The significant (p<0.05) EC_{50} values of BHT were 1.847 mg/ml of concentration which were more than the maximum values of *Pleurotus* species.

Jeena et al.[27] found that cultivated species of *P. sajor-caju* and *P. ostreatus* had an excellent reducing power of 1.980 at 10 mg/ml and 1.780 at 10 mg/ml concentration respectively, which showed close similarity with our finding but *P. sapidus* showed 1.970 at 10 mg/ml concentration which was much more than our finding. The reducing powers of ethyl acetate, methanolic and hot water extracts of *P. eous* showed an excellent reducing power of 1.950, 1.132, and 1.632 at 10 mg/ml, respectively[16]. According to Arbaayah & Kalsom[28], the greatest ability of ethanolic extract for reducing the ferricyanide complex to ferrous form was observed in different flush of cultivated *P. djamor var. djamor* was 0.47-1.23, *P. djamor var. roseus* was 0.48-0.96, *P. pulmonarius* was 0.35-0.53 and *P. osteatus* was

0.29-0.57, respectively at concentration 10 mg/ml. However, the reducing power of BHT (2.258±0.09) at 8 mg/ml concentration which was much higher than the *Pleurotus* species and it also indicated that the synthetic antioxidant had superior reducing power ability compared to antioxidant from *Pleurotus* species[27].

Chelating Effect on Ferrous Ion

Chelating effects of methanolic extract from mycelia of ten *Pleurotus* species on ferrous ions increased with increased concentration except *P. membranaceus* (Graph 3). This assay analyzed that mycelial extract of *Pleurotus* species interfered with the formation of ferrous and ferrozine complex, suggesting their ability of chelating activity. Chelating effects of ferrous ion were highest in EDTA of 91.11% in comparison to *Pleurotus* species and lowest in *P. membranaceus* (59.78±2.21%) at 1.5 mg/ml concentration.

Metal ion chelating capacity played a significant role in antioxidant mechanism[29]. Since ferrous ion were the most effective pro-oxidants in food systems[30], the higher chelating effect of methanolic extracts from mycelial mushrooms would be more effective in comparison to DPPH and reducing power method. However, Yen & Wu[31] used the method of Decker and Welch[14] to determine the chelating effect instead of the method of Shimada et al.[12]. Methanolic extracts from ear mushrooms were good chelators for ferrous ions of 85.1–96.5% at 5 mg/ml concentration[32]. As compared with wild and commercial[33] mushrooms, 0methanolic extracts from mycelial mushrooms would be good chelators for ferrous ions at higher concentrations. The highest chelating activity of 74.88% was found in macerated ethanol extracts of *P. eryngii* with the IC_{50} value of 1.00 mg/ml[34].

Total Phenolic Content

Total phenol content was highest in *P. ostreatus* (4.33±0.05 mg/g) and lowest in *P. flabellatus* (0.50±0.00 mg/g) showed in Table 1. The mycelia of *Pleurotus* species contained phenolic compounds. It can therefore be concluded that these phenolic components might be involved in these antioxidative properties as mentioned in previous studies[3]. Many studies on spices, vegetables, fruits, and plants extracts had shown a good relationship between phenolic content and antioxidant activity[35]. According to published data with respect to their relationships between the concentration of phenolic compounds and the antioxidant activity there was much controversy.

CONCLUSION

On basis of the findings, it showed that *Pleurotus* species had low reducing power, moderate free radical scavenging activity and chelating effect of ferrous ion. But *Pleurotus* species had high chelating effect of ferrous ion in comparison to free radical scavenging activity. Out of ten *Pleurotus* species *P. sapidus, P. sajor-caju, P. ostreatus, P. eryngii* and *P. citrinopileatus* showed excellent antioxidant properties. Therefore, on the basis of the outcomes of study it can be concluded that the consumption of mushrooms i.e., *Pleurotus* mushrooms, their assumed antioxidant properties might be beneficial to the antioxidant protection system of the human body against oxidative damage.

The present comparative study of antioxidant activity of different species of *Pleurotus* documented for the first time. This result revealed that methanolic extract of the mushrooms mycelia were free radical scavengers possibly by acting as primary antioxidant. Thus *Pleurotus* mushroom consumers might be benefited by protecting themselves from oxidative damaged. However, the active components of the mycelial extract responsible for the antioxidant activity are unknown. Further work is necessary on the isolation and purification of the active components.

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