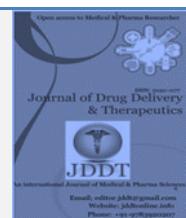


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Research Article

Evaluation of in vitro antioxidant activity of edible Basidiomycetes mushroom fungi

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Abstract

Introduction: Basidiomycetes mushroom fungi have been known for their nutritional values they can be considered as functional foods which can provide health benefits beyond the traditional nutrients. They are the excellent source of natural antioxidants because some synthetic antioxidants are now suspected to be potentially harmful to human health. As free radicals can cause inflammatory diseases, cancer, atherosclerosis, and aging, Consuming dietary antioxidant supplements can inhibit oxidative chain reactions. **Materials and Methods:** The antioxidant potential of the basidiomycetes mushroom fungal strains was established by total flavonoid content, FRAP assay, ABTS assay, Metal chelating activity, Phosphomolybdenum assay, Assay of superoxide radical scavenging activity, Free radical scavenging activity on DPPH along with the determination of total phenolic and tannin contents in the mushroom mycelial extracts. **Results:** Different Basidiomycetes mushroom fungi extracts (Hot water, Acetone and Hexane) were tested for antioxidant activities. Among these different extracts of Basidiomycetes mushroom fungi the hexane extract of *Pleurotus pulmonarius* showed potential antioxidant activity. **Discussion and Conclusion:** The results of this study suggest the possibility of using Basidiomycetes mushroom fungi as a natural antioxidant source for the pharmaceutical industry and could act as safe and cost-effective with potential antioxidant activities. These findings encourage studying these fungal strains further for their potential biological applications.

Keywords: Flavonoid, Tannin, FRAP, ABTS, DPPH and Free Radical

INTRODUCTION:

Carotenoids, vitamins and phenolic compounds are present naturally in vegetables, fruits, grains and pulses and they possess the ability to reduce oxidative damage associated with many diseases, including cancer, cardiovascular diseases, cataracts, atherosclerosis, diabetes, arthritis, immune deficiency diseases and ageing.^[1,2,3,4] Recently, the ability of phenolic substances including flavonoids, phenolic acids and lignans to act as potential antioxidants has been extensively investigated.^[5,6] Many synthetic antioxidants such as butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) are very effective and are used for industrial processing but they may possess some side effects and toxic properties to human health.^[7] Hence, compounds especially from natural sources capable of protecting against ROS mediated damage may have potential application in prevention and/or curing of diseases.

Mushrooms have been known for their nutritional values and used as medicines by humans for ages. In modern terms, they can be considered as functional foods which can provide health benefits beyond the traditional nutrients. Mushrooms

are used for a variety of biotechnological applications, particularly for the production of food, enzymes, dietary supplements, pharmaceutical compounds, feed supplements. Basidiomycete medicinal properties originate from various cellular components and secondary metabolites that can be isolated and identified in the fruiting body, vegetative mycelia, or in the culture broth.^[8]

Reactive oxygen species (ROS) are generated in the body as by-products of several cellular metabolic reactions; they consist of radical and non-radical oxygen species formed by the partial reduction of oxygen. Low levels of ROS are necessary for cellular processes such as intracellular signaling, cell progression and cell defense. Conversely, high levels of the ROS or the inability of the antioxidant system to regulate ROS levels efficiently results in oxidative stress. Oxidative stress results in direct or indirect ROS-mediated damage of nucleic acids, proteins, and lipids. Consuming dietary antioxidant supplements to fight diseases, especially cancer, has become popular among the general public.^[9]

Many studies have concluded that edible mushrooms possess potent antioxidants. It was found that the crude ethanol

extract of 150 mushrooms shows the antioxidant activity. An extensive search for traditional plant treatments for diabetes has concluded that recognized edible mushrooms are an ideal food for the dietetic prevention of hyperglycemia.^[10] Antioxidative materials are now thought to be prospective protective agents also against wood degrading organisms. Although natural antioxidants such as a-tocopherols and L-ascorbic acid are widely used, investigations are being carried out to discover more potent, safer antioxidants.^[11]

An overview of the literature on the medicinal properties of Basidiomycetes mushroom fungi indicates that there are gaps in knowledge which remain to be explored and there is a lot of scope for isolating new compounds with significant bioactivity. Keeping this perspective, the present investigation was designed to study the anti-oxidant activity of Basidiomycetes mushroom fungi.

MATERIALS AND METHODS

Chemicals

Butylated hydroxyanisole (BHA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potassium ferricyanide, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), potassium persulfate, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS^{•+}), 2,4,6-trypyridyl-s-triazine (TPTZ), linoleic acid, ferrous chloride, ammonium thiocyanate, hydrogen peroxide, ferrous ammonium sulfate, ethylenediamine tetraacetic acid (EDTA) disodium salt, hydroxylamine hydrochloride, nitro blue tetrazolium (NBT), riboflavin, thiobarbituric acid (TBA), trichloroacetic acid (TCA), Tween 40, ferric chloride, sulphanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride were obtained from Himedia, Merck and Sigma. All other reagents used were of analytical grade.

Estimation of total flavonoid content

The total flavonoid content of sample extracts was determined by the use of a slightly modified colorimetric method described previously.^[12] A 0.5 ml extract was mixed with 2 ml of distilled water and subsequently with 0.15 ml of a 5% NaNO₂ solution. After 6 min, 0.15 ml of a 10% AlCl₃ solution was added and allowed to stand for 6 min, then 2 ml of 4% NaOH solution was added to the mixture. Immediately distilled water was added to bring the final volume of 5 ml, and then the mixture is thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was determined at 510 nm versus a prepared water blank. Rutin was used as a standard compound for the quantification of total flavonoid. All the values were expressed as gram of rutin equivalent (RE) per 100 gram of extract.

Ferric-reducing/antioxidant power (FRAP) assay

The antioxidant capacity of phenolic extracts of samples was estimated according to the procedure described previously.^[13] FRAP reagent (900 µl), prepared freshly and incubated at 37 °C, was mixed with 90 µl of distilled water and 30 µl of test sample or methanol (for the reagent blank). The test samples and reagent blank were incubated at 37 °C for 30 min in a water bath. The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5 ml of 20 mmol/l TPTZ solution in 40 mmol/l HCl plus 2.5 ml of 20 mmol/l FeCl₃.6H₂O and 25 ml of 0.3 mol/l acetate buffer (pH 3.6) as described by Siddhuraju and Becker (2003). At the end of incubation the absorbance readings were taken immediately at 593 nm, using a spectrophotometer. Methanolic solutions of known Fe (II) concentration, ranging from 100 to 2000 µmol/l, (FeSO₄.7H₂O) were used for the

preparation of the calibration curve. The parameter Equivalent Concentration (EC₁) was defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mmol/l FeSO₄.7H₂O. EC₁ was calculated as the concentration of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 mmol/l concentration of Fe (II) solution, determined using the corresponding regression equation.

Antioxidant activity by the ABTS^{•+} assay

The total antioxidant activity of the samples was measured by ABTS radical cation decolorization assay according to the method described previously.^[14] ABTS^{•+} was produced by reacting 7 mM ABTS aqueous solution with 2.4 mM potassium persulfate in the dark for 12–16 h at room temperature. Prior to assay, this solution was diluted in ethanol (about 1:89 v/v) and equilibrated at 30 °C to give an absorbance at 734 nm of 0.700 ± 0.02. The stock solution of the sample extracts were diluted such that after introduction of 10 µl aliquots into the assay, they produced between 20% and 80% inhibition of the blank absorbance. After the addition of 1 ml of diluted ABTS solution to 10 µl of sample or Trolox standards (final concentration 0–15 µM) in ethanol, absorbance was measured at 30 °C exactly 30 min after the initial mixing. Appropriate solvent blanks were also run in each assay. Triplicate determinations were made at each dilution of the standard, and the percentage inhibition was calculated of the blank absorbance at 734 nm and then was plotted as a function of Trolox concentration. The unit of total antioxidant activity (TAA) is defined as the concentration of Trolox having equivalent antioxidant activity expressed as µmol/g sample extracts on dry matter.

Metal chelating activity

The chelating of ferrous ions by various extracts of *H. herbacea* and *N. alata* was estimated by the method previously.^[15] Briefly the extract samples (250 µl) were added to a solution of 2 mmol/l FeCl₂ (0.05 ml). The reaction was initiated by the addition of 5 mmol/l ferrozine (0.2 ml) and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The chelating activity of the extracts was evaluated using EDTA as standard. The results were expressed as mg EDTA equivalent/g extract.

Phosphomolybdenum assay

The antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation according to the method described previously.^[16] An aliquot of 100 µl of sample solution (in 1 mM dimethyl sulphoxide) was combined with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in a 4 ml vial. The vials were capped and incubated in a water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. The results reported (Ascorbic acid equivalent antioxidant activity) are mean values expressed as g of ascorbic acid equivalents/100 g extract.

Assay of superoxide radical scavenging activity

The assay was based on the capacity of various extracts to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system.^[17] Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 µg riboflavin, 12 mM EDTA, 0.1 mg NBT and 100 µl sample solution. Reaction was started by illuminating the reaction mixture with sample extract for 90

seconds. Immediately after illumination, the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture kept in the dark served as blanks. The percentage inhibition of superoxide anion generation was calculated as: % Inhibition = $[(A_0 - A_1) / A_0] \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample extract/standard.

Determination of total phenolic and tannin contents

The total phenolic content was determined according to the method described previously.^[18] Aliquots of each extracts were taken in test tubes and made up to the volume of 1 ml with distilled water. Then 0.5 ml of folin-ciocalteu phenol reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was recorded at 725 nm against the reagent blank. The analysis was performed in triplicate and the results were expressed as the tannic acid equivalents (TAE). Using the same extract the tannins were estimated after treatment with polyvinyl polypyrrrolidone (PVPP). One hundred milligrams of PVPP was weighed in a 100 × 12 mm test tube and to this 1.0 ml of distilled water and then 1.0 ml of tannin containing phenolic extract were added. The content was vortexed and kept in the test tube at 4 °C for 4 h. Then the sample was centrifuged (3000 g for 10 min at room temperature) and the supernatant was collected. This supernatant has only simple phenolics other than tannins (the tannins would have been precipitated along with the PVPP). The phenolic content of the supernatant was measured, as

monitored above and expressed as the content of non-tannin phenolics on a dry matter. From the above results, the tannin content of the sample was calculated as follows: Tannin (%) = Total phenolics (%) - Non-tannin phenolics (%).

Free radical scavenging activity on DPPH

The antioxidant activity of the extracts was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH, according to the method described previously.^[19] Sample extracts at various concentrations was taken and the volume was adjusted to 100 µl with methanol. 5 ml of a 0.1 mM methanolic solution of DPPH was added and shaken vigorously. The tubes were allowed to stand for 20 min at 27 °C. The absorbance of the sample was measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula: % DPPH radical scavenging activity = (Control OD - Sample OD / Control OD) × 100.

RESULTS AND DISCUSSION

Flavonoid compounds were reported as RE equivalent by reference to standard curve (Y = 0.0036 X, R² = 0.9887). Among mushroom mycelial extracts it has been found that the scavenging effect of hexane extract of *Pleurotus pulmonarius* has the highest value of 66.67 mg RE equivalent / g of extract. The least RE equivalent scavenging effect was observed in hot water extract of *Pleurotus ostreatus* with 5.32 mg RE equivalent / g of extract.

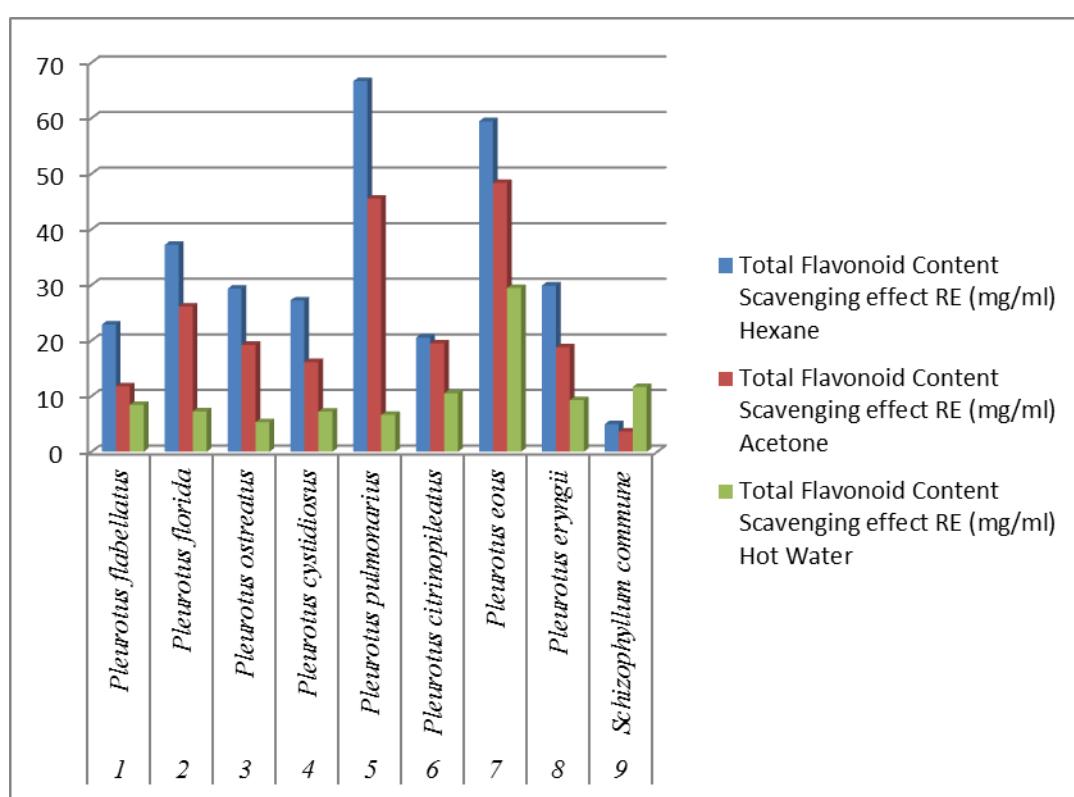


Figure 1: Total flavonoid content of selected mushroom fungi

Ferric reducing antioxidant power were reported as Fe (II) equivalent by reference to standard curve (Y = 0.456 X, R² = 0.9887). Among mushroom mycelial extracts it has been found that the scavenging effect of hexane extract of *Schizophyllum commune* has the highest value of 623.23 mg FE (II) equivalent / g of extract followed by hexane extract of *Pleurotus pulmonarius* with the value of 615.65 mg FE (II) equivalent / g of extract. The least Fe (II) equivalent scavenging effect was observed in acetone extract of *Pleurotus ostreatus* with 195.29 mg FE (II) equivalent / g of extract.

/ g of extract followed by hexane extract of *Pleurotus pulmonarius* with the value of 615.65 mg FE (II) equivalent / g of extract. The least Fe (II) equivalent scavenging effect was observed in acetone extract of *Pleurotus ostreatus* with 195.29 mg FE (II) equivalent / g of extract.

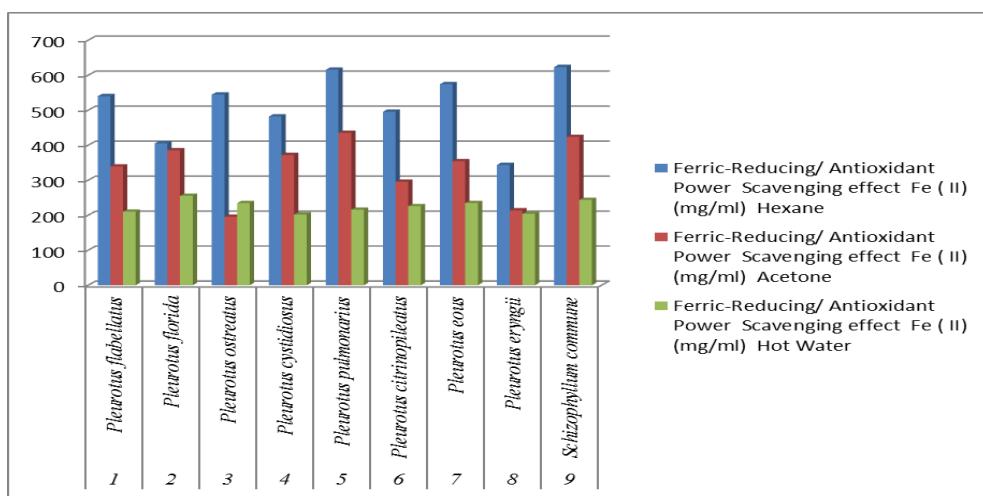


Figure 2: Ferric-Reducing/ Antioxidant Power of selected mushroom fungi

ABTS assay were reported as equivalent by reference to standard curve ($Y = 0.702 X$, $R^2 = 0.9951$). Among mushroom mycelial extracts it has been found that the scavenging effect of hexane extract of *Pleurotus pulmonarius* has the highest

value of 992.65 mg equivalent /g of extract. The least equivalent scavenging effect was observed in hot water extract of *Pleurotus florida* with 258.74 mg equivalent /g of extract.

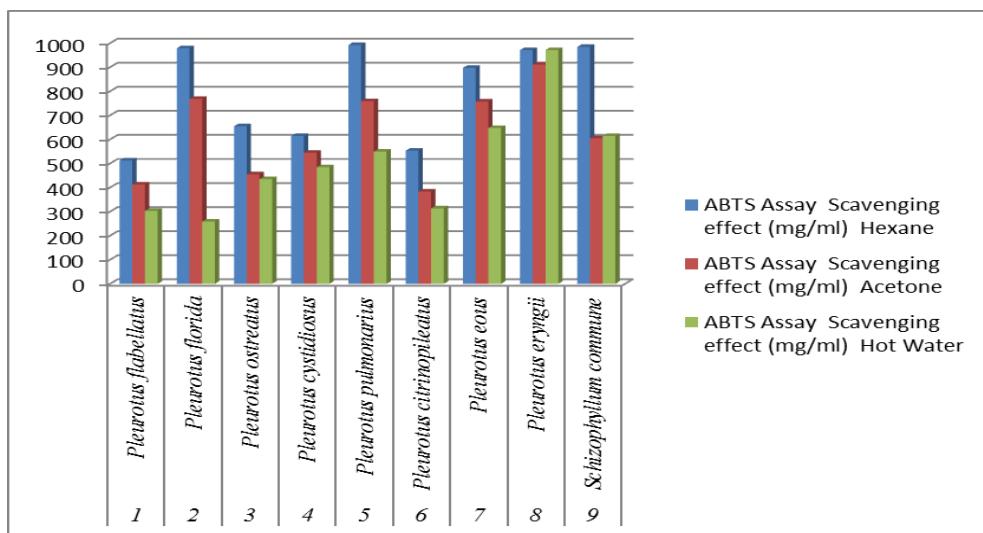


Figure 3: ABTS assay of selected mushroom fungi

Metal chelating activity were reported as EDTA equivalent by reference to standard curve ($Y = 0.0299 X$, $R^2 = 0.9887$). Among mushroom mycelial extracts it has been found that the scavenging effect of hexane extract of *Pleurotus citrinopileatus*

has the highest value of 5.68 mg EDTA equivalent / g of extract. The least EDTA equivalent scavenging effect was observed in acetone extract of *Pleurotus eous* with 1.81mg EDTA equivalent /g of extract.

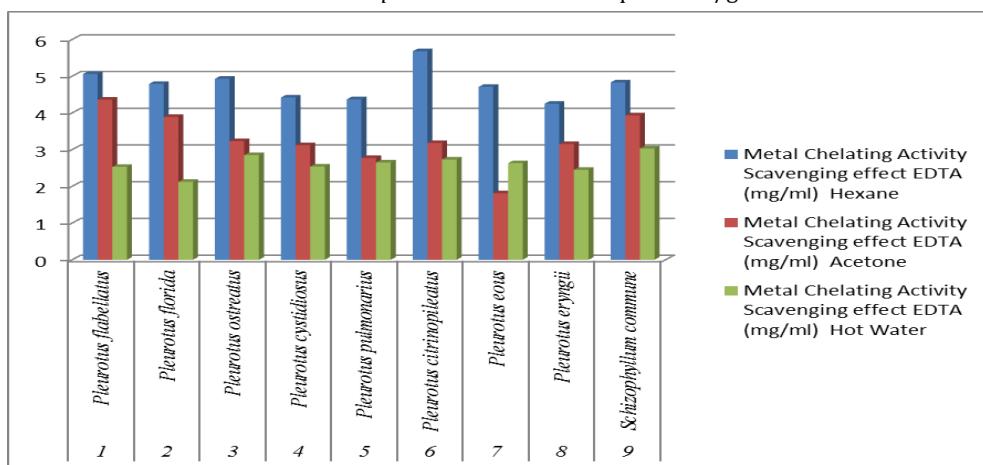


Figure 4: Metal chelating activity of selected mushroom fungi

Phosphomolybdinum compounds were reported as ascorbic acid equivalent by reference to standard curve ($Y = 0.187 X$, $R^2 = 0.9951$). Among mushroom mycelial extracts it has been found that the scavenging effect of hexane extract of *Pleurotus*

flabellatus has the highest value of 13.17mg AAE equivalent / g of extract. The least AAE equivalent scavenging effect was observed in acetone extract of *Pleurotus cystidiosus* with 1.23mg AAE equivalent /g of extract.

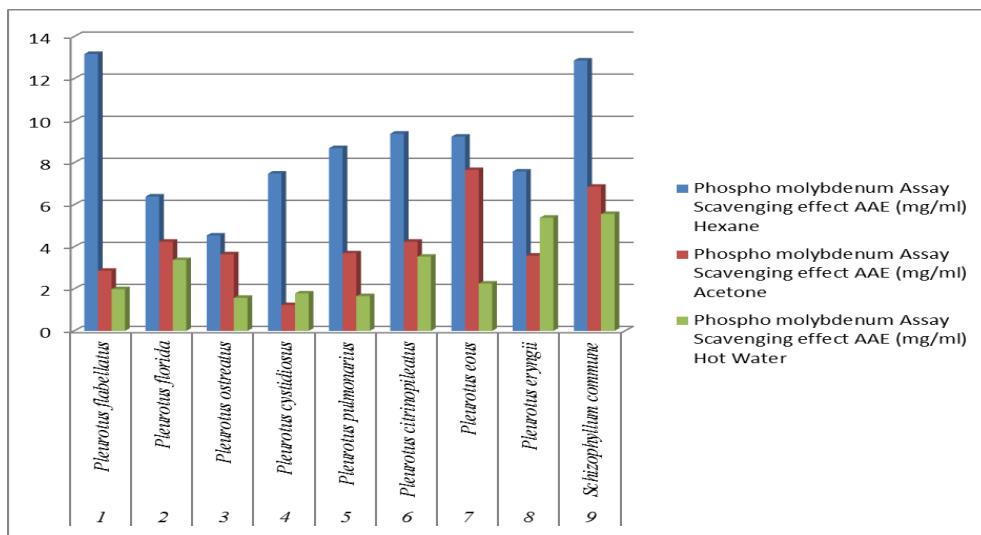


Figure 5: Phospho molybdenum assay of selected mushroom fungi

Superoxide Radical scavenging activity were reported equivalent by reference to standard curve ($Y = 0.053 X$, $R^2 = 0.9951$). Among mushroom mycelial extracts it has been found that the scavenging effect of hexane extract of *Pleurotus*

pulmonarius has the highest value of 98.70mg equivalent /g of extract. The least equivalent scavenging effect was observed in hot water extract of *Pleurotus citrinopileatus* with 13.87mg equivalent /g of extract.

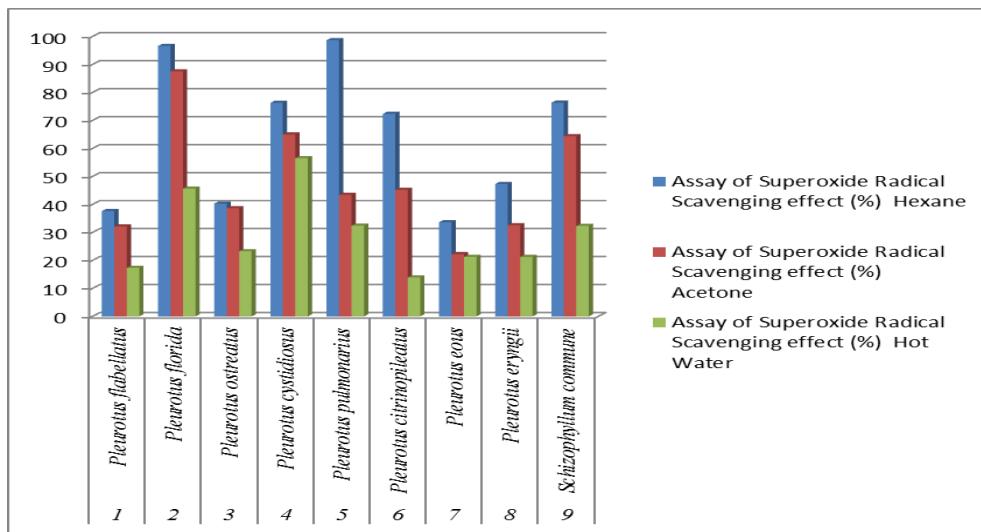


Figure 6: Assay of superoxide radical activity of selected mushroom fungi

Phenolic compounds were determined by folin ciocaltaue's method were reported as Gallic acid equivalents by reference to standard curve ($Y = 0.1075 X$, $R^2 = 0.9887$). Among mushroom mycelial extracts it has been found that the scavenging effect of acetone extract of *Pleurotus pulmonarius*

has the highest value of 80.54 mg Gallic acid equivalent / g of extract. The least Gallic acid equivalent scavenging effect was observed in acetone extract of *Schizophyllum commune* with 11.64 mg Gallic acid equivalent /g of extract.

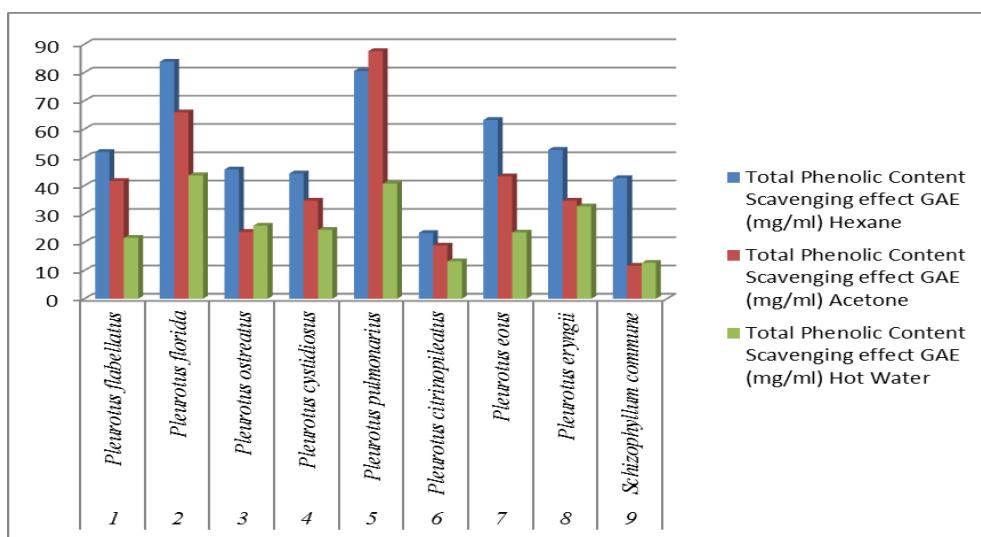


Figure 7: Total phenolic content of selected mushroom fungi

Tannin compounds were determined by folin ciocaltaue's method were reported as Gallic acid equivalents by reference to standard curve ($Y = 0.1075 X$, $R^2 = 0.9887$). Among mushroom mycelial extracts it has been found that the scavenging effect of acetone extract of *Pleurotus eryngii* has

the highest value of 82.25 mg Gallic acid equivalent / g of extract. The least Gallic acid equivalent scavenging effect was observed in hot water extract of *Pleurotus florida* with 13.29 mg Gallic acid equivalent /g of extract.

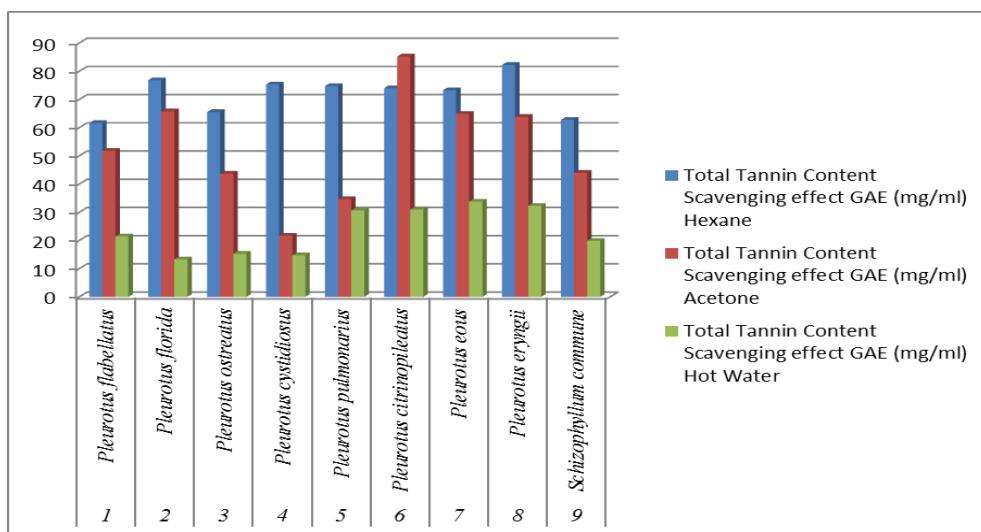


Figure 8: Total tannin content of selected mushroom fungi

Free radical scavenging activity on DPPH were reported equivalent by reference to standard curve ($Y = 0.389 X$, $R^2 = 0.9951$). Among mushroom mycelial extracts it has been found

that the hexane extract of *Pleurotus pulmonarius* showed the significant scavenging effect.

Table 1: DPPH assay of the selected mushroom fungal strains

Concentration of the Sample used (µg/ml)	<i>Pleurotus citrinopileatus</i>		<i>Pleurotus eous</i>		<i>Pleurotus cystidiatus</i>		<i>Pleurotus ostreatus</i>		<i>Pleurotus eryngii</i>		<i>Pleurotus flabellatus</i>		<i>Pleurotus florida</i>		<i>Pleurotus pulmonarius</i>		<i>Schizophyllum commune</i>	
	Hexane	Acetone	Hexane	Acetone	Hexane	Acetone	Hexane	Acetone	Hexane	Acetone	Hexane	Acetone	Hexane	Acetone	Hexane	Acetone	Hexane	Acetone
20	1.21 ± 0.01	0.58 ± 0.26	1.18 ± 0.01	5.38 ± 0.26	1.78 ± 0.01	8.52 ± 0.26	1.73 ± 0.01	- 35.89 ± 0.26	1.63 ± 0.01	- 62.01 ± 0.26	1.32 ± 0.01	17.02 ± 0.26	1.54 ± 0.01	-4.73 ± 0.26	1.87 ± 0.01	17.97 ± 0.26	1.205 ± 0.01	6.75 ± 0.26
40	1.47 ± 0.01	0.16 ± 0.26	1.11 ± 0.01	7.96 ± 0.26	1.84 ± 0.01	-4.92 ± 0.26	1.85 ± 0.01	-7.62 ± 0.26	1.80 ± 0.01	- 82.42 ± 0.26	1.48 ± 0.01	4.167 ± 0.26	1.75 ± 0.01	-4.22 ± 0.26	1.97 ± 0.01	14.29 ± 0.26	1.297 ± 0.01	3.49 ± 0.26
60	1.57 ± 0.01	7.47 ± 0.26	1.68 ± 0.01	5.65 ± 0.15	1.86 ± 0.01	- 11.86 ± 0.26	1.81 ± 0.01	-5.82 ± 0.26	1.85 ± 0.01	- 124.1 ± 0.26	1.49 ± 0.01	- 43.04 ± 0.26	1.76 ± 0.01	-6.49 ± 0.26	1.98 ± 0.01	15.12 ± 0.26	1.36 ± 0.01	5.22 ± 0.26
80	1.63 ± 0.01	1.29 ± 0.26	1.83 ± 0.01	3.25 ± 0.39	1.92 ± 0.01	1.82 ± 0.26	1.85 ± 0.01	2.16 ± 0.26	1.91 ± 0.01	- 122.0 ± 0.26	1.92 ± 0.01	- 138.0 ± 0.26	1.84 ± 0.01	-9.57 ± 0.26	1.99 ± 0.01	5.55 ± 0.26	1.47 ± 0.01	7.36 ± 0.26
100	1.68 ± 0.01	-4.02 ± 0.26	1.88 ± 0.01	-0.25 ± 0.26	1.95 ± 0.01	- 27.54 ± 0.26	1.87 ± 0.01	2.12 ± 0.26	1.92 ± 0.01	- 126.8 ± 0.26	1.98 ± 0.01	- 33.01 ± 0.26	1.88 ± 0.01	- 11.63 ± 0.26	1.99 ± 0.01	3.03 ± 0.26	1.68 ± 0.01	0.24 ± 0.26

CONCLUSION

The present study demonstrates the antioxidant potential of the basidiomycetes mushroom fungal strains which was established by total flavonoid content, FRAP assay, ABTS assay, Metal chelating activity, Phosphomolybdenum assay, Assay of superoxide radical scavenging activity, Free radical scavenging activity on DPPH along with the determination of total phenolic and tannin contents in the mushroom mycelial extracts. Different extracts of Basidiomycetes mushroom fungi (Hot water, Acetone and Hexane) were tested for its potential biological activities. These extracts of Basidiomycetes mushroom fungi were tested for *in vitro* antioxidant activities. The present study explored that the mushrooms which are efficient could act as safe and cost-effective with potential *in vitro* antioxidant activities. Among these different extracts of *Basidiomycetes* mushroom fungi the hexane extract of *Pleurotus pulmonarius* showed potential *in vitro* antioxidant activity. These findings encourage studying these fungal strains further for their potential biological applications.

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Conflict of Interest

None to declare

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