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ANTIOXIDANT ACTIVITY OF CULTURED MYCELIUM OF TEN DIFFERENT SPECIES OF OYSTER MUSHROOM, *PLEUROTUS*: A COMPARATIVE STUDY

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ABSTRACT: The antioxidant activity of cultured mycelia of ten species of the oyster mushroom, *Pleurotus* species namely *P. ostreatus*, *P. flabellatus*, *P. sapidus*, *P. citrinopileatus*, *P.roseus*, *P.sajor-caju*, *P.florida*, *P.cystidouses*, *P.fossulatus*, and *P.eryngii* was examined. The extracts were screened for their ability to scavenge DPPH, hydroxyl, superoxide radicals and also for ferric reducing power and lipid peroxidation inhibiting activity. Among the ten species studied, all were found to possess DPPH radical scavenging and lipid peroxidation inhibiting properties. *P florida* scavenged DPPH radicals (94.02%) and *P.citrinopileatus* inhibited the lipid peroxidation (76.65%) more efficiently than others. Five among the ten *Pleurotus* species showed ferric reducing power, and SOD activity. *P.ostreatus* efficiently reduced ferric radicals (51.06%) and scavenged super oxide radicals (74.61%) when compared to others. From the ten species studied all except *P.roseus* scavenged hydroxyl radicals and the maximum activity was showed by *P. sajor-caju* (76.17%). The present study thus reveals the profound free radical scavenging activity of cultured mycelia of oyster mushrooms. The findings suggest the potential use of cultured mycelia of *Pleurotus* species as a dietary antioxidant supplement.

INTRODUCTION: Oxygen, essential for aerobic life forms, becomes toxic by inappropriate metabolism, which leads to the generation of free radicals or reactive oxygen species (ROS). ROS such as superoxide radical $O_2^{-\cdot}$, hydroxyl radical ($^{\cdot}OH$) and non-free radical species, such as H_2O_2 , singlet oxygen ($^1O_2$), and reactive nitrogen species (RNS) are generated in the body by exposure to sunlight, ultraviolet, ionizing radiation, or by chemical reactions and metabolic processes [1].

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Oxidative stress, an excessive production of reactive oxygen species (ROS) that outstrips antioxidant defense mechanisms, has been implicated in wide variety of pathological effects, such as DNA damage, cellular and metabolic injury, carcinogenesis, cardiovascular diseases, neurodegenerative diseases, inflammation and cellular degeneration related to ageing and compounds that can scavenge free radicals have great potential in ameliorating these disease process [2, 3].

Removing ROS/RNS is probably one of the most effective defenses of a living body against diseases. Most living species have efficient defense systems to prevent themselves against oxidative stress

induced by ROS [4]. When these natural defenses are overwhelmed by excessive generation of pro-oxidants, a situation of oxidative stress evolves and cellular macromolecules suffer oxidative damage [5]. The potential oxidative damage caused by free radicals might be prevented or limited by dietary antioxidants. These antioxidants preserve an adequate function of immune cells against homeostatic disturbances and can counteract these injurious and carcinogenic changes caused by oxidative stress [6].

Mushrooms are long been esteemed for their medicinal properties. They are traditionally used in China and Japan for medicinal purposes [7]. Mushrooms are nutritional food as well as source of physiologically beneficial and non-toxic medicines. [8] Mushrooms contain a large number of biologically active components that offer health benefits and protection against many degenerative diseases. Mushroom metabolites are increasingly being utilized to treat a wide variety of diseases, particularly as they can be added to the diet and used orally, without the need to go through phase-I/II/III trials as a synthetic drug, and they are considered as safe and useful for disease treatment. [9] A number of medicinal mushrooms have recently been reported to possess significant antioxidant activity.[10, 11, 12, 13, 14] Some of the most recently isolated and identified substances from mushrooms have been reported to possess significant cardiovascular, antiviral, antibacterial, antiparasitic, hepatoprotective and antidiabetic activities.[15]

Pleurotus species popularly known as oyster mushrooms are widespread throughout the hardwood forests of the world that host the most diverse varieties of climates. *Pleurotus* species are now widely consumed as food in the East and increasingly in the West.[16] Oyster mushrooms are delicious and nutritious, and rank second in among the commercially cultivated mushrooms in the world.[17] Unlike the local use of many medicinal plants and fungi, the beneficial effects of *Pleurotus* were discovered independently on different continents and locations. Knowledge of their medicinal properties comes not only from Asia, but from the folklore of Central Europe,[18] South America [19] and Africa.[20] This fact strongly supports the notion that *Pleurotus* indeed contains substances that may help to cure or mitigate certain diseases or may improve the quality of life for the needy.

Approximately 80% of mushroom products are produced from fruiting bodies. The cultivation of mushroom for fruiting body production is a long-term process taking one to several months depending upon species and substrates. In contrast, production of mushroom mycelium in submerged culture would allow to accelerate the growth and to obtain high yield of biomass with constant composition. Hence, cultured mycelium of mushrooms is an ideal source for developing healthcare products.[21]

In this communication we report the anti-oxidant activity of aqueous extracts of cultured mycelia of ten different species of *Pleurotus, P. ostreatus, P. flabellatus, P. sapidus, P. citrinopileatus, P.roseus, P.sajor-caju, P.florida, P.cystidouses, Pfossulatus,* and *P.eryngii*.

MATERIALS AND METHODS:

Production of *Mushroom mycelium:*

Cultures of different Pleurotus species, obtained from Microbial Type Culture Collection, Institute of Microbiology; Chandigrah were used for the studies. The fungi were grown in submerged culture on Potato-Dextrose Broth (PDB) for the production of mycelial biomass. After ten days of growth at 24-25^{0}C in submerged culture, the fungal biomass was harvested, washed thoroughly and dried at 40 –50^{0}C.[22]

Preparation of the extract:

The dried mycelia were powdered and 100g powder was extracted with hot aqueous-ethanol (ethanol/ water 50/50 v/v) for 8 -10 hours. The extract was concentrated and solvent completely evaporated under vacuum. The residue thus obtained was employed in the experiments for antioxidant assays.

Chemicals:

1,1-diphenyl 2-picryl hydrazyl (DPPH), 2,4,6-tripyridyl-*s*-triazine (TPTZ), Ascorbic acid, 2-Deoxy-D-ribose were purchased from Sigma Chemicals Co. (St.Louis, MO, USA), nitroblue tetazolium (NBT) and thiobarbituric acid (TBA)

were purchased from BDH laboratories, England, riboflavin and sodium azide from SRL, Mumbai, Hydrogen peroxide (H_2O_2), EDTA, *n*-butanol, ascorbic acid and pyridine from Merck India Ltd, Mumbai, India. All other chemicals and reagents used were of analytical grade.

Analysis of *in vitro* antioxidant activity:

DPPH Radical scavenging assay:

In this method, a commercially available and stable free radical ($DPPH^+$, 2, 2-diphenyl-1-picrylhydrazil), soluble in methanol, was used.[23] DPPH in its radical form has an absorption peak at 515nm, which disappeared on reduction by an antioxidant compound. An aliquot (25µl) of the extract was added to 1ml of freshly prepared DPPH solution (0.25g/l in methanol). Absorbance was measured 20 min after the reaction was started. The absorbance of test was compared with that of control.

Ferric reducing antioxidant power assay:

The ferric reducing ability was measured at low pH.[24,25] The stock solution of 10mM 2,4,6-tripiridyl-*s*-triazine (TPTZ) in 40mM Hcl,20mM $FeCl_3.6H_2O$, and 0.3M acetate buffer (pH 3.6) were prepared. The FRAP reagent contained 2.5ml TPTZ solution, 2.5ml ferric chloride solution and 25 ml acetate buffer. It was freshly prepared and warmed to 37^0C.Then 900µl FRAP reagent was mixed with 90µl water and 30µl test sample/ methanol/ distilled water/ standard antioxidant solution.

The reaction mixture was then incubated at 37^0 for 30 minutes, and absorbance was recorded at 595nm.An intense blue coloured complex was formed when ferric tripyridyl triazine (Fe^{3+} TPTZ) complex was reduced to the ferrous (Fe^{2+}) form, and the absorbance at 595nm was recorded. The ferric reducing power of the extract was determined by comparing the O.D of test with that of control.

Inhibition of lipid peroxidation:

Lipid peroxidation induced by Fe^{2+}-ascorbate system in rat liver homogenate was estimated by TBA reaction method by Ohkawa et al (1979).[26] The reaction mixture contained rat liver homogenate 0.1ml (25 (w/v)%) in Tris-HCl buffer (20mM,pH 7.0); KCl (30mM);$FeSO_4$ $(NH_4)_2SO_4.6H_2O$ (0.16mM); ascorbate (0.06mM); and various concentrations of the extract in a final volume of 0.5ml.The reaction mixture was incubated for 1 hr at 37^0C.After the incubation period, 0.4ml was removed and treated with 0.2ml SDS (8.1%);1.5ml TBA(0.8%); and 1 ml acetic acid (20%, pH 3.5).

The total volume was made up to 4ml by distilled water and then kept in a water bath at 95-100^0 C for 1 hr. After cooling, 1.0ml of distilled water and 5.0ml of *n*-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10min.The organic layer was removed and its absorbance at 532nm was measured. Inhibition of lipid peroxidation was determined by comparing the O.D of treatments with that of control.

Assay of hydroxyl radical scavenging activity:

Hydroxyl radicals generated from Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction) was estimated by its degradation of deoxyribose that resulted in thiobarbituric acid reactive substance (TBARS).[27] The reaction mixture contained deoxyribose (2.8mM); FeCl3 (0.1mM); KH2PO4-KOH buffer 20mM, pH 7.4) EDTA (0.1mM); H_2O_2 (1.0mM); ascorbic acid (0.1mM) and various concentrations of the extract in a final volume of 1 ml. The reaction mixture was incubated at 37^0C for 60 min. The TBARS formed was estimated by TBA method of Ohkawa et al (1979).[25] The hydroxyl radical scavenging activity was determined by comparing absorbance of control with that of treatments.

Superoxide radical scavenging activity:

Superoxide radical ($O_2^{\cdot}$) generated from the photo reduction of riboflavin was detected by NBT reduction method of Mc Cord and Fridovich (1969).[28] The reaction mixture contained EDTA (6mM): 3µg NaCN; riboflavin (2µM); NBT (50µM); KH_2PO_4-Na_2HPO_4 buffer (67mM, pH 7.8) and various concentrations of extract in a final volume of 3ml.The tubes were illuminated under incandescent lamp for 15 minutes. The optical density (O.D) at 560 nm was measured before and after illumination. The inhibition of super oxide radical generation was determined by comparing

the absorbance values of the control with that of treatments.

RESULTS AND DISCUSSION:

Antioxidant activity of the extracts:

DPPH Assay:

Table 1 shows the DPPH radical scavenging activity of ten different *Pleurotus* species at concentrations of 100 µg, 500 µg and 1000 µg. Etracts of all the *Pleurotus* species scavenged DPPH radicals. Among the ten *species* studied *P. florida* showed highest activity (94.02%) and *P.sajor-caju* showed lowest activity (58%) at a concentration of 1000 µg of the extract. The activity was compared with that of standard antioxidant quercetin, which at a concentration of 10 µg/ml scavenged 95.6% DPPH radicals (**Table 6**). This assay showed second line of defense to scavenge free radicals by suppressing chain initiation and/or by breaking the chain propagation reaction.

TABLE 1: DPPH RADICAL SCAVENGING ACTIVITY OF TEN DIFFERENT SPECIES OF *PLEUROTUS*

Concentration (µg/ml)					Activity (%)	
P.roseus	***P.ostreatus P.sapidus***	***P.flabellatus P.fossulatus***	***P.citrinopileatus P.eryngii***	***P.cystidouses***	***P.sajor-caju***	***P.florida***
	13.4±1.51	18.52±2.56	10.2± 0.98	22.7±3.74	7.00±0.86	22.79± 3.12
100 30.2±2.89	40.5±2.83	51.84±3.91	34.00±1.64			
500 65.51±2.75	45±2.13	43.6±4.24	32.3±3.41	51.7±2.71	48.12±3.55	74.12±5.30
	51.2±3.26	78.13±5.36	61.25±3.35			
1000 86.54 ±2.43	70.00±3.60	6.9.00±2.23	69.25±1.50	65.80±2.45	58.00±1.32	94.02± 3.76
	67.50±3.56	88.01±5.60	74.00±2.62			

All values are mean ± SD

FRAP Assay:

The aqueous extracts of *Pleurotus* species showed significant ferric reducing antioxidant power (**Table 2**). Non-enzymatic antioxidants react with prooxidants and inactivate them. In this 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^{3+} is used in excess. Thus on reduction of Fe^{3+}-TPTZ complex by antioxidant, Fe^{2+}-TPTZ is formed, which can be measured spectrophotometrically at 595nm. Among the ten different species studied, only five possessed Ferric reducing ability. *P.osteatus* showed higher activity (58%) and *P.sajor-caju* showed lowest activity (39.38%) at a concentration of 1000 µg. The standard antioxidant quercetin, at a concentration of 20µg/ml reduced 89.3% ferric radicals (**Table 2**). Our results indicate the hydrogen donating capacity of five different *Pleurotus* species such as *P.ostreatus, P.flabellatus, P.citrinopileatus, P.cystidouses* and *P.sajor-caju*.

TABLE 2: FERRIC REDUCING ACTIVITY OF TEN DIFFERENT SPECIES OF *PLEUROTUS*

Concentration (µg/ml)					Activity (%)	
P.roseus	***P.ostreatus P.sapidus***	***P.flabellatus P.fossulatus***	***P.citrinopileatus P.eryngii***	***P.cystidouses***	***P.sajor-caju***	***P.florida***
	35.03±2.43	34.94±3.12	34.71±4.16	36.30±2.36	23.20±2.85	-----
100 -	-----	-----				
500 -	52.37±3.75	45.00± 3.11	43.11±4.12	48.77±4.3	34.71±1.16	-----
	-----	-----				
1000 -	56.16±6.12	51.06 ±2.35	49.13±3.11	48.52±2.83	39.38±5.12	-----
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All values are mean ± SD

Inhibition of lipid peroxidation:

Aqueous-ethanol extract of all the ten *Pleurotus* species significantly inhibited the lipid peroxidation induced by Fe^{2+}- ascorbate system in rat liver homogenate (**Table 3**). The generation of malondialdehyde (MDA) and related substances that react with thiobarbituric acid (TBARS) was found to be inhibited by the extract. *P.citrinopileatus* showed maximum inhibition (76.65%) at a concentration of 1000µg and *P.flabellatus* showed minimum inhibition at the same concentration (40.06%).

The results obtained were compared with that of standard antibiotic quercetin, which inhibited the lipid peroxidation by 78.5 % at a concentration of 200µg/ml (**Table 6**).

The liver microsomal fractions undergoes rapid non-enzymatic peroxidation when incubated $FeCl_3$ and ascorbic acid. The use of Fe (III) in the presence of a reducing agent such as ascorbate produces $^{\cdot}OH$ and they attack the biological material. This leads to the formation of MDA (malondialdehyde and other aldehydes), which form a pink chromogen with TBA absorbing at 532nm. The extract exhibited a strong scavenging effect of hydroxyl radical which could inhibit membrane damage at different concentrations.

TABLE 3: INHIBITION OF LIPID PEROXIDATION BY TEN DIFFERENT SPECIES OF *PLEUROTUS*

	Concentration (µg/ml)					Activity (%)	
	P.roseus	***P.ostreatus*** ***P.sapidus***	***P.flabellatus*** ***P.fossulatus***	***P.citrinopileatus*** ***P.eryngii***	***P.cystidouses***	***P.sajor-caju***	***P.florida***
100	37.37± 1.86	48.32±2.88 54.5± 3.49	19.87±1.13 11.26±4.31	22.29±2.73 36.07± 1.11	50.95±2.44	40.74±3.22	53.28± 1.04
500	59.67±3.44	52.95±3.13 64.5±2.22	31.36±2.43 27.19±2.31	52.26±1.97 50.22± 2.95	67.57±3.47	51.56± 5.06	64.55± 4.3
1000	74.13±1.24	62.72±3.52 67.5±C3.97	40.06±2.11 54.53±4.53	76.65±4.11 66.18± 3.83	73.29±4.51	60.68±2.01	70.9± 5.21

All values are mean ± SD

Hydroxyl radical scavenging activity:

The degradation of deoxyribose to TBARS by hydroxyl radical generated from Fe^{2+}-EDTA-H_2O_2 system was markedly decreased by the extract (**Table 4**). This indicated the significant hydroxyl radical activity of the extract. At a concentration of 1000µg/ml *P.sajor-caju* showed higher activity (76.17%) and *P.eryngii* showed lesser activity (35.87%). In this assay the standard antioxidant, quercetin scavenged the hydroxyl radicals by 86.4 % at a concentration of 100µg/ml (**Table 6**). Ferric-EDTA was incubated with H_2O_2 and ascorbic acid to generate hydroxyl radical, and was detected by their ability to degrade 2-deoxy-2-ribose into fragments, that on heating with TBA at low pH form a pink chromogen. Addition of mushroom extract to the reaction mixture efficiently removed the hydroxyl radicals and prevented the degradation of 2-deoxy-2-ribose in a dose dependent manner.

TABLE 4: HYDROXYL RADICAL SCAVENGING ACTIVITY OF TEN DIFFERENT SPECIES OF *PLEUROTUS*

	Concentration (µg/ml)					Activity (%)	
	P.roseus	***P.ostreatus*** ***P.sapidus***	***P.flabellatus*** ***P.fossulatus***	***P.citrinopileatus*** ***P.eryngii***	***P.cystidouses***	***P.sajor-caju***	***P.florida***
100	-	32.82±2.31 50.26± 2.54	28.55± 3.45 16.5± 1.96	26.14± 2.49 35.66± 1.32	33.19± 1.95	53.46± 2.33	43.86± 2.84
500	-	45.66±1.86 57.16± 2.99	35.35± 2.99 33.98± 2.76	36.73± 1.05 21.06± 2.54	35.65± 3.07	58.48± 1.87	61.06± 3.1
1000	--	58.26± 1.55 60.22±4.65	41.18± 1.07 59.39± 3.65	58.95 ± 3.73 35.87± 1.07	52.04± 4.11	76.17± 3.72	73.96± 3.48

All values are mean ± SD

Superoxide radical scavenging activity:

The aqueous-ethanol extract of five different species of *Pleurotus* effectively scavenged superoxide radicals generated by the photoreduction of riboflavin. (**Table 5**). Addition of different concentrations of extract to the reaction mixture significantly decreased the absorbance and hence significantly scavenged the superooxide radicals in a dose dependent manner. Among the ten species studied, five such as, *P.citrinopileatus, P.florida, P.fossulatus, P.eryngii* possessed superoxide radical scavenging activity. *P.ostreatus* scavenged about 74.61% of superoxide radicals at concentration of 1000µg which is found to be the highest activity. *P.eryngii* showed the lowest activity (38.60 %) at same concentration. Quercetin at a concentration of 20µg/ml scavenged the superoxide radicals by 81.7% (**Table 6**).

The genus *Pleurotus* comprises a group of edible ligninolytic mushrooms with medicinal properties and important biotechnological and environmental

applications. The cultivation of *Pleurotus* sp. is economically important in food industry worldwide which has expanded in the past few years. Nutritionally *Pleurotus* sp. have unique flavour and aromatic properties, and are rich in protein, fiber, carbohydrates, vitamins and minerals. Phytochemical analysis of *Pleurotus* species showed the presence of terpenoids, tannins, steroidal glycosides and carbohydrates. [29]

TABLE 5: SUPEROXIDE RADICAL SCAVENGING ACTIVITY OF TEN DIFFERENT SPECIES OF *PLEUROTUS*

	Concentration (µg/ml)					Activity (%)
	P.roseus	***P.ostreatus*** ***P.sapidus***	***P.flabellatus*** ***P.fossulatus***	***P.citrinopileatus*** ***P.eryngii***	***P.cystidouses***	***P.sajor-caju***	***P.florida***
100		15.62±1.03	-----	1.46± 0.43	-----	-----	14.72± 1.23
	-		16.50±1.34	16.48± 0.86			

500	-		-----	28.36± 1.78	-----	-----	28.57±1.58
		------	33.98±3.27	22.30± 1.40			
1000	--		-----	58.84± 2.61	-----	-----	49.89± 2.8
		------	59.39±3.02	38.60± 2.19			

All values are mean ± SD

TABLE 6: ANTIOXIDANT ACTIVITY OF STANDARD COMPOUND QUERCETIN

Assay	Quercetin (µg/ml)	% inhibition
DPPH	10	95.6 ± 0.68
FRAP	20	89.3 ± 1.24
Superoxide	20	81.7 ± 0.75
Lipid Peroxidation	200	78.5 ± 2.1
Hydroxyl Radical	100	86.4 ± 1.6

All values are mean ± SD

The present study reveals the significant antioxidant activity the aqueous-ethanol extract of the mycelia of ten different *Pleurotus* species. The fortification of medicinal mushrooms, which possesses antioxidant properties, in the human diet would potentially be useful to help the human body to reduce oxidative damage. Antioxidant activity of these edible mushrooms has significant importance because this activity greatly contributes to their neutraceutical properties thus enhancing their nutritive value.

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