COMPARATIVE EVALUATION ON THE FREE RADICAL SCAVENGING ACTIVITY OF ELEVEN INDIAN CULTIVATED STRAINS OF *PLEUROTUS OSTREATUS*

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Summary

The antioxidant potential of the ethanolic extracts from 11 different strains of *Pleurotus ostreatus* were comparatively investigated against hydroxyl radical, DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical, superoxide radical, reducing power and ferrous ion chelating ability. Total phenolic contents were also determined. Significant correlation was found between the total phenolic content and the antioxidant capacity of the ethanolic extracts of all the strains. Cellular damage caused by reactive oxygen species has been implicated in several diseases and hence antioxidants have significant importance in human health. Results of this study showed that, ethanolic extract of the strain MTCCNo.1804 has maximum antioxidant property and may be utilized as a promising source of therapeutics.

Key Words: Antioxidant activity, Chelating ability, Phenolics, Reducing power.

Introduction

The search of new products with antioxidative properties is very active domain of research. Recently, mushrooms have become attractive as functional foods and as a source of physiologically beneficial medicines, while being devoid of undesirable side effects [1].

Mushrooms are considered as rich food because they contain protein, sugar, glycogen, lipid, vitamins, amino acids and crude fiber [2, 3]. They also contain important minerals required for normal functioning of the body [3]. Edible mushrooms are highly nutritious and used as medicines for treatment of cancer, heart ailments, diabetes, high blood pressure, etc [4, 5]. Over two third of cancer related death could be prevented through lifestyle modification particularly through dietary means and mushroom consumption may contribute to minimize cancer risk through antioxidant input [6]. Phenolic compounds in mushroom are reported to be excellent antioxidants and synergists that are not mutagenic [7].

Oxidation is essential to many living organism for production of energy to fuel biological processes. Although, humans and other organisms possess antioxidant defence systems that protect against oxidative damage, such systems are reported to only partially prevent such damage [8]. But, uncontrolled production of reactive oxygen species (ROS) is responsible for various pathophysiological processes [9, 10]. ROS or reactive oxygen species produced by sunlight, ultraviolet (UV), ionizing radiation, chemical reaction and metabolic processes have a wide variety of pathological effects, such as causing DNA damage, carcinogenesis and cellular degeneration related to aging [11]. Majority of disease conditions like atherosclerosis, hypertension, ischemic disease, alzheimer's disease, parkinsonism, cancer, diabetes mellitus and inflammatory conditions are being considered to be preliminary due to the imbalance between prooxidant and antioxidant homeostasis [12]. Superoxide and hydroxyl radicals are the two most representative free radicals. In cellular oxidation reactions, superoxide radical is normally formed first, and its effects can be magnified because it produces other kinds of cell-damaging free radicals and oxidizing agents. However, the damaging action of the hydroxyl radical is the strongest among free radicals. Synthetic compounds are found to be strong radical scavengers but usually they have side effects [13]. Neutralization of this radical activity by naturally occurring substances mainly by supplementation of food having antioxidant property is becoming one of the most acceptable modes of modern therapy. Amongst them, mushroom or their derivatives or extracts occupy an elite position to perform this function [5, 14, 15, 16].

Pleurotus ostreatus, commonly known as the oyster mushroom, is a popular, easily recognized, edible mushroom. It is primarily found in northern temperate zones and grows on woods in cluster wherever conditions are warm and wet. They are whitish, large and tough when they are old. It is one of easier mushroom to cultivate and grows on a variety of substrates making it suitable for commercial exploitation [17]. *Pleurotus ostreatus* is reported to contain higher concentrations of cystine, methionine and aspartic acid than other edible mushrooms, such as *Agaricus bisporus* and *Lentinus edodes* [18]. *P. ostreatus* shows potent antitumor activity against Ehrlich's ascites carcinoma (EAC) [19]. Ethyl acetate and methanolic extracts of *Pleurotus florida* have found to exhibit potent scavenging of hydroxyl radicals and inhibition of lipid peroxidation activities [20]. Extracts of *Pleurotus squarrosulus* demonstrated potent *in vitro* free radical scavenging activity [21]. The present study was conducted to evaluate the antioxidant activity of eleven different strains of *Pleurotus ostreatus* including their reducing power, chelating effects on ferrous ion. The total phenolic content of all the strains were also determined.

Materials and methods

Sample collection and preparation

Pleurotus ostreatus strain no. Pl-20, Pl-350, Pl-500, Pl-560 were purchased from Directorate of Mushroom Research, Solan, India and MTCC No.1801, MTCC No.1802, MTCC No.1803, MTCCNo.1804 from Microbial Type Culture Collection and Gene Bank, Chandigarh, India. *P. ostreatus* strain no. N1, N2 and N3 were collected from the field, subsequently cultured. All the

strains were maintained in Molecular and Applied Mycology and Plant Pathology Laboratory, Department of Botany, University of Calcutta by periodic subculturing in PDA (Potato Dextrose Agar). For fresh fruit bodies from each strain, spawn production and cultivation were performed at Mushroom Cultivation Unit, Ramakrishna Mission Ashrama, Narendrapur, India using standard cultivation techniques.

Fresh mushrooms were randomly selected into three samples of 150 g each and air-dried in an oven at 40^0C for 48 h. Dried powdered mushroom samples were extracted by stirring with 200 ml of ethanol at 30^0C for 24 h at 150 rpm and filtering through Whatman No. 4 filter paper. The residue was then extracted twice with another 200 ml of ethanol as described above. The total extract was then rotary evaporated to dryness at 40°C and redissolved in distilled water at a concentration of 10 mg/ml and stored at -20°C for further use [22].

Assay of hydroxyl radical (OH^-) - scavenging activity

Hydroxyl radicals (OH^-) are generated from Fe^{2+}- ascorbate- EDTA- H_2O_2 system (Fenton's reaction) which attack the deoxyribose and set off a series of reactions that eventually result in the formation of malondialdehyde (MDA), measured as a pink MDA-TBA chromogen at 535 nm [23]. Reaction mixture (1 ml) contained deoxyribose (2.8 mM), KH_2PO_4- KOH (20 mM; pH 7.4), $FeCl_3$ (100 mM), EDTA (104 µM), H_2O_2 (1 mM) and ascorbate (100 µM). Reaction mixture was incubated at 37^0C for 1 h and colour developed as described above. Hydroxyl radical scavenging activity of ethanolic extract of all the strains of *P. ostreatus* were compared at a concentration of 1 mg/ml.

DPPH radical scavenging assay

The hydrogen atom or electron donation abilities of the ethanolic extracts of all the strains and a pure compound were measured from the bleaching of the purple colour methanol solution of 1, 1-diphenyl-2- picryl hydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent [24, 25]. 500 µl of the extracts in ethanol were added to 2ml of 0.004 % methanol solution of DPPH (w/v). After 30 min. incubation period at room temperature in dark, the absorbance was read against a methanol blank at 517 nm. Inhibition of free radical of DPPH by the ethanolic extract of the strains of *P. ostreatus* at a concentration of 1 mg/ml was calculated in percent (I %) in the following way:

$$I\ \% = (A_{blank} - A_{sample} / A_{blank}) \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound.

Assay of superoxide radical (O_2^-) - scavenging activity

The method used by Martinez *et al*. 2001 [26] for determination of the superoxide dismutase was followed with modification [27] in the riboflavin-light-nitrobluetetrazolium (NBT) system [28]. Each 3 ml reaction mixture contained 50 mM sodium phosphate (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM EDTA, 75 µM NBT and 500 µl sample solution of ethanolic extracts of the different strains of *P. ostreatus* at a concentration of 1 mg/ml. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 10 min of illumination of a fluorescent lamp. Identical tubes with the reaction mixture were kept in the dark and served as blanks.

Chelating ability of Ferrous ions

Chelating ability was determined according to the method of Dinis *et al.* (1994) [29]. Ethanolic extracts (1 mg/ml) in water was mixed with 3.7 ml of methanol and 0.1 ml of 2 mM ferrous chloride. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine. After 10 min at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. The percentage of inhibition of ferrozine- Fe^{++} complex formation is given by this formula:

% inhibition = $\{(A_0-A_1)/ A_0\} \times 100$.

Where, A_0 was the absorbance of the control and A_1 the absorbance in the presence of mushroom extracts.

Determination of reducing power

The reducing powers of the ethanolic extracts of different strains of *P. ostreatus* were determined according to the method of Oyaizu (1986) [30]. Each ethanolic extract (1 mg/ml) in 1ml of methanol was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min, and then 2.5 ml of trichloroacetic (10%) was added to the mixture which was then centrifuged for 10 min at 12,000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. A higher absorbance indicates a higher reductive capability.

Determination of total phenolic compounds

The total phenolic content of the ethanolic extracts of all the strains were determined by Folin-Ciocalteu method [31]. 1 ml of sample solution was mixed with 1 ml of Folin-Ciocalteu reagent. After 3 min of incubation at room temperature, 1 ml of saturated Na_2CO_3 (35% aqueous solution) was added to the mixture, followed by the addition of 7 ml of distilled water. The mixture was kept in the dark for 90 min and its absorbance at 725 nm was measured, using gallic acid as standard. The total phenolic content of the samples was expressed as gallic acid equivalents (GAE), which reflected the phenolic content as the amount of gallic acid (μg) in 1 mg of sample.

Analysis of data

In all the cases results is the mean ± SD (standard deviation) of at least three individual experimental data.

Results

Hydroxyl radical scavenging activity

Ferric-EDTA was incubated with H_2O_2 and ascorbic acid at pH 7.4. Hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy-2-ribose into fragments that formed a pink chromogen upon heating with TBA at low pH [32]. When ethanolic extracts were added to the reaction mixture, they removed hydroxyl radical from the sugar and prevented their degradation. Ethanolic extracts of all the strains of *Pleurotus ostreatus* showed potential hydroxyl radical scavenging activity [Figure 1]. At a concentration of 1 mg/ ml, the hydroxyl radical scavenging activity when compared were found in order of MTCC No.1804 (73.77%) > Pl-500 (71.86%) > MTCC No.1801 (71.76%) > MTCC No.1802 (68.02%) > Pl-20 (59.2%) > Pl-560 (53.86%) > Pl-350 (52.58%) > N1 (51.96%) > N2 (49.71) > N3 (37.6%) > MTCC No.1803 (31.14%).

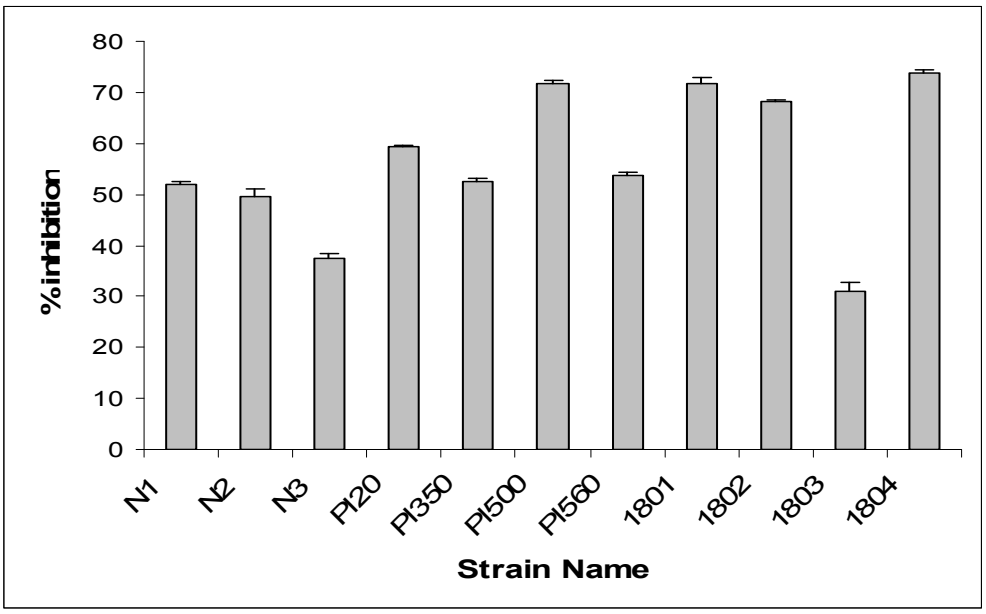

Figure 1 Hydroxyl Radical scavenging activity of ethanolic extract (1 mg/ml) of 11 Indian cultivated stains of *P. ostreatus*. Results are mean ± SD of three separate experiments, each in triplicate.

DPPH radical scavenging activity

DPPH radical scavenging activity of ethanolic extracts of all the strains were presented in Figure 2. At a concentration of 1 mg ethanolic extract/ ml, the DPPH radical scavenging activities were in the descending order: N2> MTCC No.1804> MTCC No.1802> Pl-500> MTCC No.1801> Pl-350> MTCC No.1803> Pl-20> Pl-560> N1> N3.

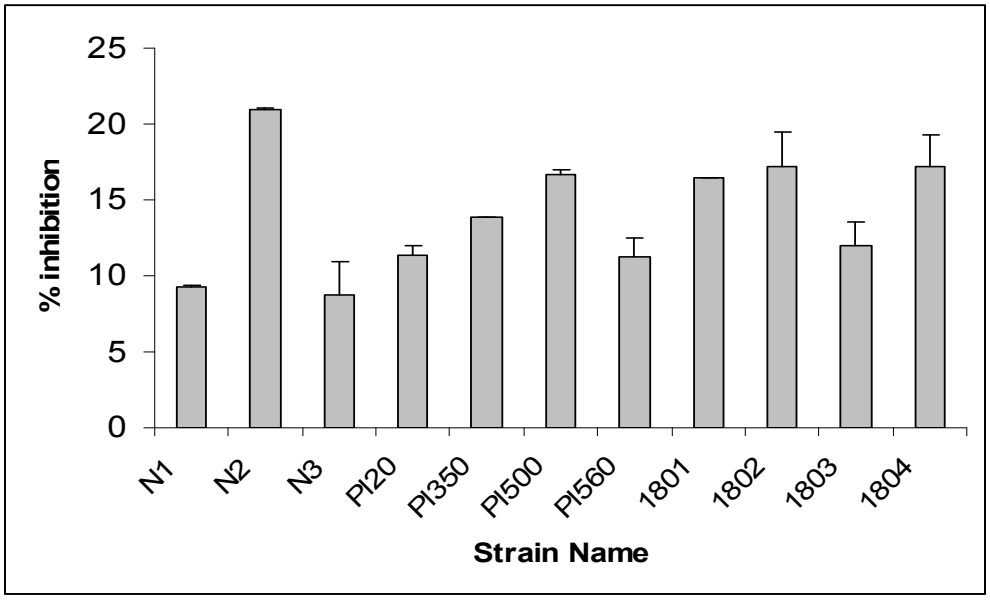

Figure 2 DPPH Radical scavenging activity of ethanolic extract (1 mg/ml) of 11 Indian cultivated stains of *P. ostreatus*. Results are mean ± SD of three separate experiments, each in triplicate.

Assay of superoxide radical scavenging activity

Figure 3 represented the superoxide radical scavenging activity of the ethanolic extract of *P. ostreatus* strains, as measured by the riboflavin- light system *in vitro*. In the present study, the ethanolic extracts were found to be a notable scavenger of superoxide radicals generated in riboflavin- NBT light system. The ethanolic extract of strain MTCC No.1804 at a concentration of 1 mg/ml, inhibited the formation of blue formazan upto 56% to 58% in comparison to other strains at same concentration. The other strains showed the activity in the order of N1> MTCC No.1801> Pl-500> Pl-20> MTCC No.1802> Pl-560> Pl-350> N2> N3. Strain MTCC No.1803 showed lowest activity (20.75%).

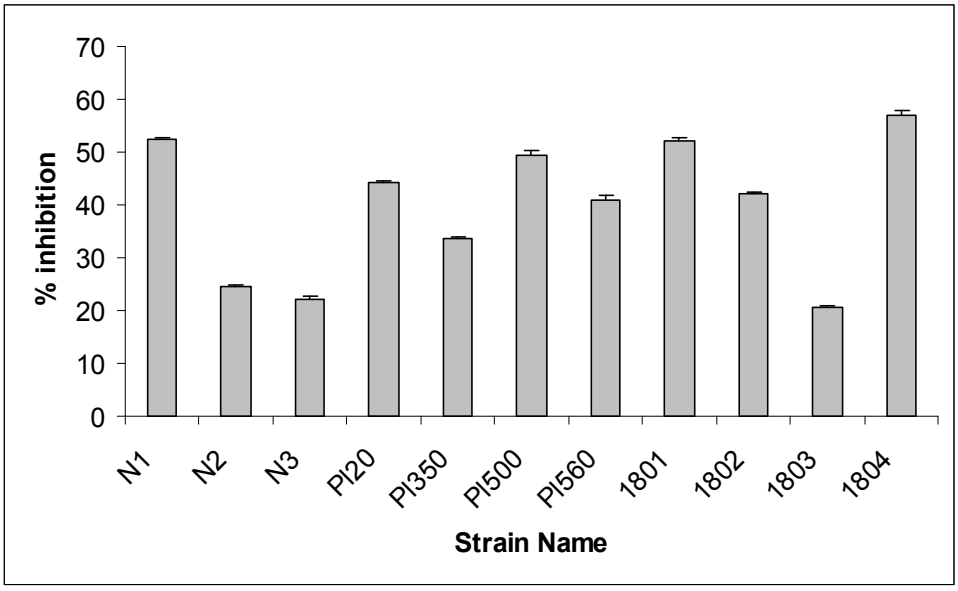

Figure 3 Superoxide radical (O_2^-) - scavenging activity of ethanolic extract (1 mg/ml) of 11 Indian cultivated stains of *P. ostreatus*. Results are the mean ± SD of three separate experiments, each in triplicate.

Chelating ability of ferrous ion

Ferrozine quantitatively forms complexes with Fe^{2+}. In the presence of chelating agent, the complex formation is disrupted, thus resulting in the reduction of red color. Reduction therefore allows estimation of the chelating ability of the coexisting chelator [33]. The range and the mean of Fe^{2+} chelating capacities of the ethanolic extracts at a concentration of 1 mg/ml among 11 different strains of *P. ostreatus* showed a higher range of variation from 26% to 76% [Figure 4]. As the ferrous ions are effective pro oxidants in food system [34], so the moderate to high ferrous ion chelating ability of the ethanolic extract of MTCC No.1801, MTCC No.1804, Pl-350, Pl-500, Pl-20 and Pl-560 would be beneficial.

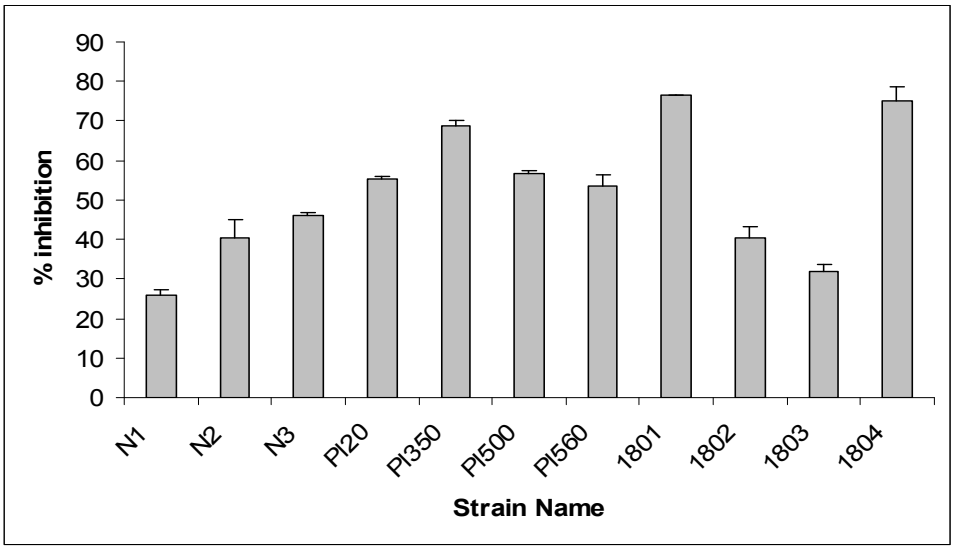

Figure 4 Chelating ability of ethanolic extract (1 mg/ml) of 11 Indian cultivated stains of *P. ostreatus*. Results are the mean ± SD of three separate experiments, each in triplicate.

Determination of reducing power

Comparative reducing powers of the ethanolic extracts at a concentration of 1 mg/ml among 11 strains were presented in Figure 5. It seems that the reducing power of 11 strains were in descending order: N2> N1> Pl-500> MTCC No.1804> MTCC No.1801> Pl-560> MTCC No.1802> Pl-350> Pl-20> N3> MTCC No.1803. The antioxidant activity has been reported to be concomitant with the development of reducing capacity [35]. The variation of the reducing among the extract might be due to its hydrogen-donating ability as described by Shimada et al. [36]. Therefore, the extract might contain reductones, which could react with free radicals to stabilize and terminate radical chain reaction.

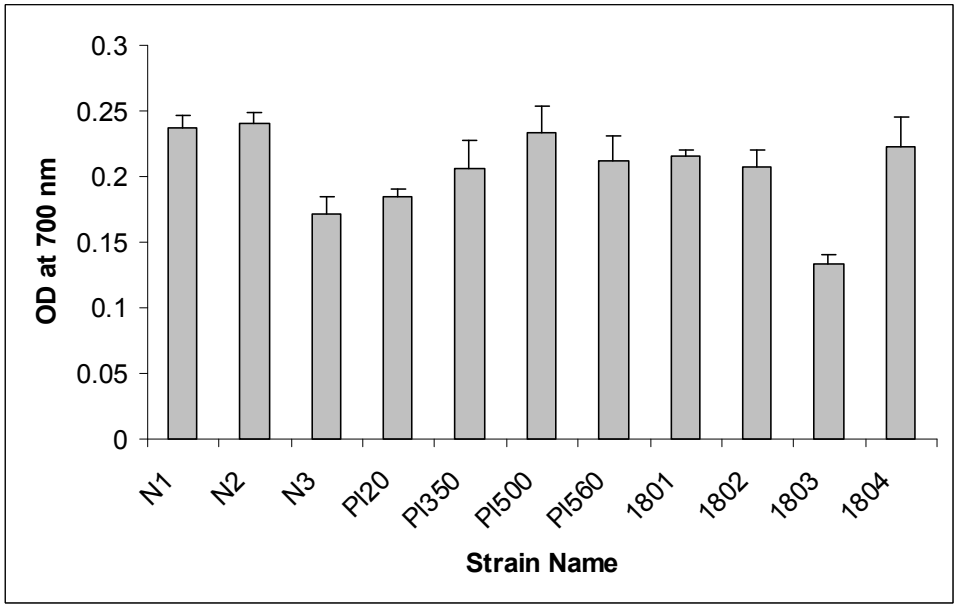

Figure 5 Assay of Reducing Power of ethanolic extract (1 mg/ml) of 11 Indian cultivated stains of *P. ostreatus*. Results are mean ± SD of three separate experiments, each in triplicate.

Determination of total phenolics

In the present study, the total phenolic contents in the mushroom strains ethanolic extract was expressed as µg of gallic acid equivalent (GAEs)/mg of extract [Figure 6]. The highest and the lowest phenolic content were measured in the ethanolic extract of MTCC No.1804 and MTCC No.1803 respectively.

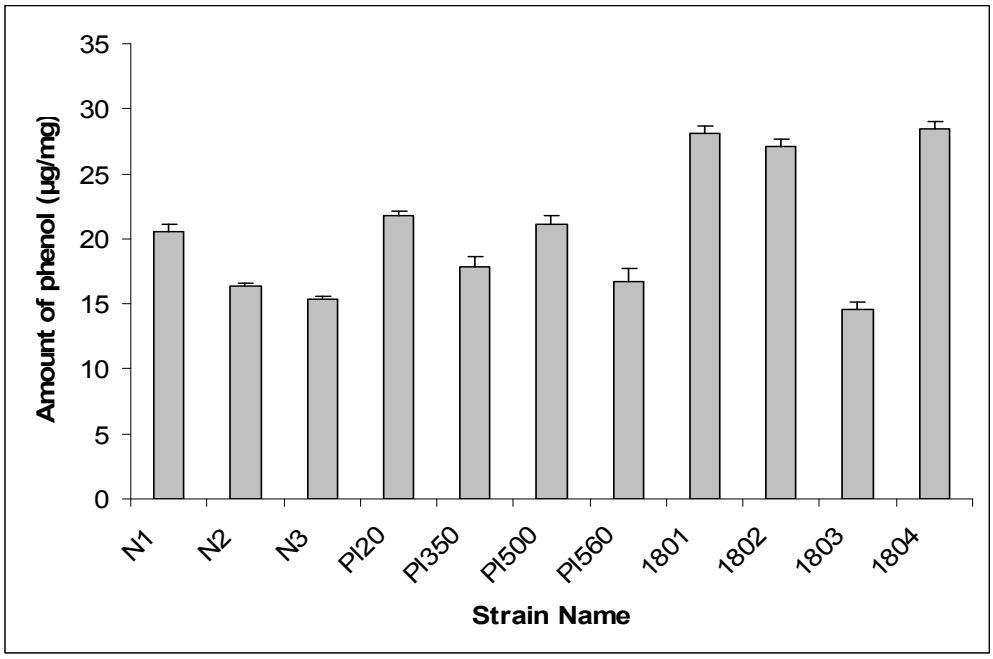

Figure 6 Phenolic content (µg/mg of ethanolic extract) of different strains of *P. ostreatus*. Results are mean ± SD of three separate experiments, each in triplicate.

Discussion

Results of the investigation revealed that the ethanolic extract of all the strains have potential free radical scavenging activity. The hydroxyl radical is the most reactive and it induces severe damage in adjacent biomolecules, which includes DNA, lipids and proteins [37, 38]. In the present study MTCC No.1804, Pl-500 and MTCC No.1801 showed higher range of hydroxyl radical scavenging activity. DPPH radical is a stable free radical and possess a characteristic absorbance at 517 nm, which decreases significantly on exposure to radical scavengers by providing hydrogen atom or electron to become a stable diamagnetic molecule [39]. The use of stable DPPH radical has the advantage of being unaffected by side reactions, such as enzyme inhibition and metal chelation [40]. One risk of superoxide generation is related to its interaction with nitric oxide to form peroxinitrite [41], which is a potent oxidant that causes nitrosative stress in the organ systems. Strains MTCC No.1804, N1, MTCC No.1801 showed the higher range of inhibition. Transition metals have been proposed as the catalysts for the initial formation of radicals. Chelating agents may stabilize transition metals in living systems and inhibit generation of radicals, consequently reducing free radical induced damage. To better estimate the antioxidant potential of the mushroom extract, its chelating activity was evaluated against Fe^{2+} [42]. Among 11 different strains of *Pleurotus ostreatus*, MTCC No.1801, MTCC No.1804 showed the highest range of ferrous ion chelating activity. Hence, the ethanolic extract of above strains shows higher interference with the formation of ferrous and ferrozine complex and can be considered as good chelator of ferrous ion. In the assay for determination of reducing power, the yellow colour of the

test solution was changed to various shades of green and blue depending on the reducing power of each compound. The higher reducing power of the ethanolic extract of N2, Pl-500, MTCC No.1804 were might be due to presence of reducers (i.e., antioxidants) causes the reduction of Fe^{3+}/ Ferrocyanide complex to ferrous form. Phenolics compounds are known to be powerful chain breaking antioxidants and they possess scavenging ability due to their hydroxyl groups [43]. Previous studies have shown that polyphenols found in dietary and medicinal plants could inhibit oxidative stress by antioxidative mechanisms [44]. The higher polyphenol contents of MTCC No.1804 might be correlated with the better results found in the scavenging of hydroxyl, DPPH, superoxide radicals, chelating ability of ferrous ion and reducing power of the same strain. In conclusion, the ethanolic extract of the strain 1804 could serve as a possible food supplement or even as a pharmaceutical agent, thus encouraging cultivation of this strain.

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