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Phytochemical screening and antioxidant capacity of polyphenol-rich fraction of *Pleurotus flabellatus*

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ABSTRACT

Polyphenol-rich fraction from edible mushroom, Pleurotus flabellatus, was tested for in vitro antioxidant activity in terms of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and chelating effect of ferrous ions, reducing power, total antioxidant capacity assay and estimation of bioactive components namely, total phenol, flavonoid, β-carotene, lycopene and ascorbic acid. Findings showed that EC_{50} values were below 1 mg /ml. Estimated putative antioxidant components are in order of phenol > flavonoids > ascorbic acid > β-carotene > lycopene. Result implies that P. flabellatus can be a potential source of natural antioxidant which may be used as food supplement to treat various oxidative stress related diseases.

Keywords: Chelating effect, DPPH, free radical, mushroom, reducing power.

INTRODUCTION

The presence of free radicals in the biological systems was not generally considered likely until the discovery of superoxide dismutase in 1969. Oxyradicals are now widely accepted as being very important, not only in the aging process but also in numerous human diseases and disorders such as cancer, heart ailments, diabetes, inflammation, gastric ulcer, hepatic damage etc., where they have either a primary or secondary role [1]. All living organisms are equipped with stress-response systems that regulate the processes of somatic maintenance and repair, but they often fall short, necessitating administration of dietary supplements to help combat oxidative stress and related ailments, which in turn leads to the search for antioxidants [2].

In the past few decades several antioxidants have been sought out, both natural and synthetic, which are being used as food additives; but the reported carcinogenic effects of synthetic antioxidants [3] have expedited extensive research for naturally occurring antioxidants. Whilst plants are considered as a major source of natural antioxidants due to elevated levels of phenols and flavones, many researchers are being fascinated by the nutritive and medicinal properties of various edible mushrooms. Mushrooms have long been used as medicine by the Chinese, and recently, a lot of attention is being paid to the therapeutic value of mushrooms based on their role in human health. Mushrooms accumulate a variety of secondary metabolites including phenolic compounds, polyketides, terpenes, and steroids. Some of these compounds have tremendous importance to humankind displaying a broad range of useful antibacterial, antiviral, and pharmaceutical activities as well as less toxic effects [4]. There is a plethora of mushrooms in the world of fungi, only a handful of which has been studied, leaving a huge uncharted stretch to be explored and exploited; and vast knowledge to be shared.

Owing to its geo-climatic exclusiveness, a large variety of mushrooms are found in the state of West Bengal, many of which are eaten by the local natives [5]. A number of wild edible mushrooms of West Bengal have been extensively studied and found promising for the treatment of diseases like cancer [6], microbial diseases [7, 8],

cardiovascular diseases [9], ulcer [10], diabetes [11], hepatic injury [12, 13] etc. In our present study, we demonstrate the antioxidative properties of Polyphenol-rich extract of a wild edible mushroom, *Pleurotus flabellatus* Sacc. and estimate the putative compounds that render the antioxidant properties to this fraction.

EXPERIMENTAL SECTION

MUSHROOM SAMPLING

Basidiocarps of *P. flabellatus* were collected in the month of August from the local markets of Darjeeling and Sikkim and identified using standard literature [14, 15]. A voucher specimen (AMF - 397) was deposited in the Mycological Herbarium of University of Calcutta, Kolkata, West Bengal, India.

CHEMICALS

Ammonium molybdate, BHA (butylated hydroxyanisole), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethylenediaminetetraacetic acid (EDTA), ferrous chloride, Folin-Ciocalteu reagent, gallic acid, L-ascorbic acid, Methionine, nitroblue tetrazolium (NBT), potassium ferricyanide, quercetin, riboflavin and trichlroacetic acid (TCA), were purchased from Sigma chemicals Co. (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade.

PREPARATION OF EXTRACT

Polyphenol rich fraction of *Pleurotus flabellatus* was extracted according to the method of Khatua et al (2013) [16] with a slight modification. The Basidiocarps of *Pleurotus flabellatus* were dried and powdered and kept in ethanol at 25˚C for 2 days to eliminate triterpenoids, steroids and other alcohol soluble compounds. It was then filtered and the residue was similarly re-extracted. After filtration, the residue was air-dried and boiled in distilled water for eight hours to extract the water soluble biomolecules and filtered. Polysaccharides were removed from the filtrate by ethanol precipitation and centrifugation. The supernatant was then dried in a rotary evaporator, and the dried material was re-dissolved in distilled water to obtain different concentrations and stored at 4˚C for analysis.

The percentage yield extracts were calculated based on dry weight as:

Yield (%) = (W1 × 100)/ W2

Where W1 = weight of extract after solvent evaporation; W2 = Weight of the grinded mushroom powder.

DETERMINATION OF TOTAL PHENOLS

Total phenols in the extract was measured directly using Folin-Ciocalteu reagent [17], where 1ml of the extract (100 mg/ml) was mixed with 1ml Folin-Ciocalteu reagent and incubated for 3 min at room temperature. After incubation, 1ml of 35 % saturated Na_2CO_3 solution was added in the reaction mixture, volume adjusted to 10 ml with distilled water and incubated in the dark for 90 min, after which the absorbance was monitored at 725 nm with a spectrophotometer. Gallic acid was used as standard. Total phenol content of the sample was expressed as mg of gallic acid equivalents per gram of extract.

DETERMINATION OF TOTAL FLAVONOID CONTENT

Flavonoid concentration was determined by the described method [18]. The extract (100 mg/ml) was diluted with 4.3 ml of 80% methanol and 0.1 ml of 10% aluminium nitrate and 0.1 ml of 1 M aqueous potassium acetate were added to it. After 40 min at room temperature, the absorbance was determined spectrophotometrically at 415 nm. Total flavonoid concentration was calculated using quercetin as standard.

DETERMINATION OF TOTAL ß-CAROTENE AND LYCOPENE CONTENT

ß-carotene and lycopene was determined by the following process [19]. 100 ml of the extract (10 mg/ml) was vigorously shaken with 10 ml of acetone-hexane mixture (4:6) for 1 min and absorbance of the mixture was measured at 453, 505 and 663 nm. ß-carotene and lycopene contents were calculated using the following equations:

Lycopene (mg/100mg) = $-0.0458A_{663} + 0.372A_{505} - 0.0806A_{453.}$

ß-carotene (mg/100mg) = $0.216A_{663} - 0.304A_{505} + 0.452A_{453}$

DETERMINATION OF ASCORBIC ACID CONTENT

Ascorbic acid content was determined by a method as described [20] with a little modification. Standard ascorbic acid (100 µg /ml) was taken in a conical flask and made up to 10 ml by 0.6% oxalic acid. It was titrated with a dye, 2, 6-dichlorophenol indophenol (21 mg sodium bicarbonate and 26 mg of dye in 100 ml water). The amount of dye

consumed (V_1 ml) is equivalent to the amount of ascorbic acid. The extract (w µg/ml) was similarly titrated with the dye (V_2 ml). The amount of ascorbic acid was calculated using the following formula,

Ascorbic acid (µg /mg) = [{(10 µg /V_1ml) × V_2 ml} × w µg] × 1000.

TOTAL ANTIOXIDANT CAPACITY ASSAY

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH [21]. The tubes containing the extract (1 mg/ml) and reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) were incubated at 95˚C for 90 min. After the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm against a blank. The antioxidant capacity was expressed as ascorbic acid equivalent (AAE).

2,2-DIPHENYL-1-PICRYLHYDRAZYL (DPPH) RADICAL-SCAVENGING ACTIVITY

Different concentrations of PfPre (0.5 to 2 mg/ml) were added to 0.004% solution of DPPH [22]. The mixture was shaken vigorously and left to stand for 30 min in the dark. Absorbance was measured at 517 nm against a blank. EC_{50} value is the effective concentration of extract that scavenged 50% DPPH radicals and it was obtained by interpolation from linear regression analysis.

REDUCING POWER

Reducing power of PfPre was determined following the method of Oyaizu, 1986 [23]. Variable concentrations of PfPre were added to 2.5 ml 0.2 M phosphate buffer (pH 6.6) and 2.5 ml 1% potassium ferricyanide. The mixture was incubated at 50˚C for 20 min. 2.5 ml of 10% trichloroacetic acid was added to the mixture and was centrifuged at 12000 rpm for 10 min. 2.5 ml of the supernatant was mixed with 2.5 ml distilled water and 0.5 ml 0.1% ferric chloride and absorbance was measured at 700 nm. An increase in absorbance of the reaction mixture was taken to mean an increase in reducing power of the sample.

CHELATING EFFECT ON FERROUS IONS

The ability of the extract of PfPre to chelate ferrous ions was estimated by the method of Dinis et al, 1994 [24]. Briefly, the extract was 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), and the mixture was then shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine-Fe^{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 of the mixture containing the extract or the absorbance of a standard solution.

SUPEROXIDE RADICAL SCAVENGING ASSAY

Superoxide radical scavenging activity was studied using the riboflavin-light-nitrobluetetrazolium (NBT) system, suggested by Martinez et al. (2001) [25] with a little modification. Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, sample solution of various concentrations of *Pleurotus flabellatus*, 100 µM EDTA, 75 µM NBT and 2 µM riboflavin. One set of reaction mixtures were exposed to light for 10 min to activate the riboflavin-NBT and the absorbance of each mixture was measured at 560 nm against identical mixtures from another set kept in the dark for the same duration. Butylated hydroxyanisole (BHA) was used as standard.

RESULTS AND DISCUSSION

RECOVERY PERCENT AND ANTIOXIDANT COMPONENTS

Table 1 summarises the percent yield, total phenol, flavonoids, ascorbic acid, beta-carotene and lycopene content in the Polyphenol-rich extract of *Pleurotus flabellatus* (PfPre). Data shows that total phenols, flavonoids and ascorbic acid are the major antioxidant components, whereas, β-carotene and lycopene were found in vestigial amounts.

	Flavonoids	Total phenols	Ascorbic acid	β- carotene	Lycopene
Yield %	(µg/mg)				
8.25%	6.304 ± 0.9	13.12 ± 0.633	0.559 ± 0.015	0.075 ± 0.005	0.017 ± 0.002

Table 1: yield percentage and antioxidant components of polyphenol-rich extract of *Pleurotus flabellatus* (PfPre). Values are the mean ± standard deviation of three separate experiments, each in triplicate. Total phenols are expressed in gallic acid equivalent (GAE) and flavonoids as quercetin equivalent (QE).

Phenolic compounds are known to be powerful chain-breaking antioxidants and they possess free radical scavenging ability due to their hydroxyl groups. The phenolic compounds contribute directly to the antioxidative action. In the present study, the total phenolic content of PfPre (13.12 µg/mg) was found to be much higher than that of the hot

water extract of *P. eous* [26], which was reported to be 8.77 µg/mg, *R. laurocerasi* (7.05 µg/mg) [15] and *P. citrinopileatus*, that being 8.62 µg/mg [27], while it was found to be a little lower than that of *P. Squarrosulus* which was 18.1 µg/mg [28].

Ascorbic acid is reported to interact directly with radicals such as O_2^- and OH^- in plasma, thus preventing damage to red cell membranes, it also assists α-tocopherol in inhibition of lipid peroxidase by recycling the tocopherol radical [29]. In the present study, the ascorbic acid content of *P. flabellatus* extract was relatively higher (0.559 µg/mg) when compared to the values reported from alcoholic extracts of *P. ostreatus* (25 ng/1 mg) [30] and *P. citrinopileatus* (31 ng/mg) [27], but was comparatively lower than the polyphenol-rich extract of *Russula laurocerasi* (1.03 µg/mg) [16]. Many other naturally occurring antioxidant components, including *β*-carotene, lycopene and flavonoids are known to possess strong antioxidative characteristics [31]. In this study *β*-carotene was found in vestigeal amounts, i.e. 0.075 µg/mg while lycopene content was significantly lower (0.017 µg/mg), which are higher than that of the methanolic extract of *P. Squarrosulus*, which were 570 ng and 225 ng per mg respectively [28], and polyphenol-rich extract of *R. Laurocerasi* (0.01 and 0.007 µg/mg respectively) [16]. The estimated flavonoid content of PfPre is 6.304 µg/mg, which is much higher than *R. laurocerasi*, where it was reported to be 2.4 µg/mg [16]. Total phenols, flavonoids and *β*-carotene were the major naturally occurring antioxidant components estimated in this study. The higher amounts of these components in this extract might explain its more effectiveness in antioxidant activities.

TOTAL ANTIOXIDANT CAPACITY

Total antioxidant capacity of PfPre was determined by the formation of green phosphomolybdenum complex. The phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of green phosphate/Mo (V) complex with the maximal absorption at 695 nm. Total antioxidant activity of the extract was estimated, using ascorbic acid as standard. On analysis, it was found that 0.5 mg of PfPre is as functional as approximately 34 ± 3.7 µg of ascorbic acid, expressed as 34 µg ascorbic acid equivalents (AAE). The total antioxidant capacity of PfPre may be attributed to their chemical composition and phenolic content. A recent study [32] showed that some bioactive compounds from citrus fruits had strong total antioxidant activity, which was probably due to the presence of flavonoids, carotenoids and ascorbic acid.

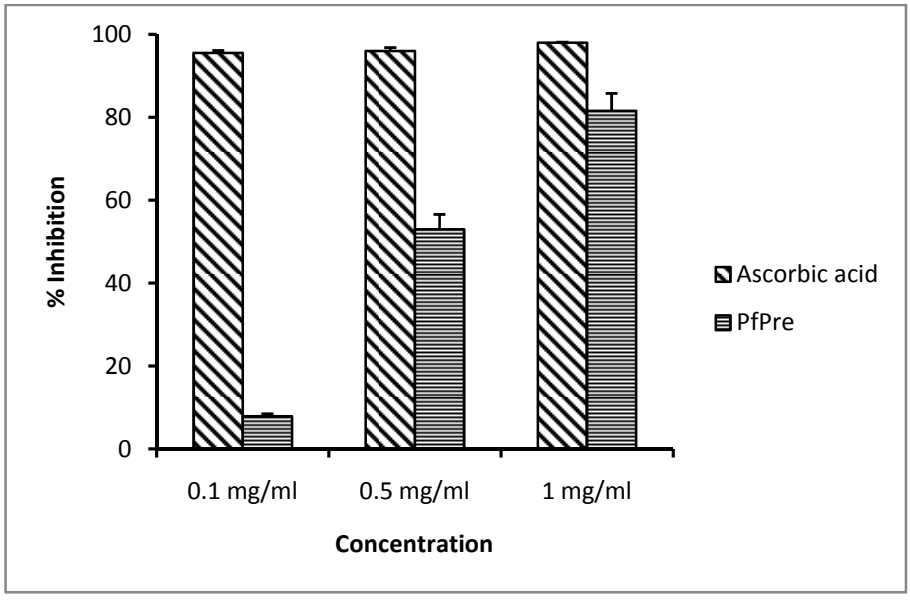

Fig. 1: DPPH radical scavenging activity of PfPre compared with that of the standard, ascorbic acid
Values are the mean ± standard deviation of three separate experiments, each in triplicate

2, 2-DIPHENYL-1-PICRYLHYDRAZYL (DPPH) RADICAL-SCAVENGING ACTIVITY

DPPH is a stable free radical and possesses 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. The use of stable DPPH radical has the advantage of being unaffected by side reactions, such as enzyme inhibition and metal chelation [33]. Such reactivity has been widely used to test the ability of a compound or extract as free radical scavengers. Upon treatment with an increasing concentration of the PfPre, a marked decrease in absorption was observed, indicating a potent DPPH scavenging ability of the extract (Fig. 1). EC_{50} of DPPH radical scavenging activity was 0.48 ± 0.02 mg/ml. When compared with our earlier investigations, the EC_{50} value of polyphenol-rich

fraction of *Pleurotus flabellatus* (PfPre) was lower than the polyphenol-rich extract of *Russula laurocerasi* [16] and *Amanita vaginata* [34]. On the other hand, the same fraction of *Russula albonigra* had almost similar activity [35]. *Ganoderma tsugae* was reported to have a lower EC_{50} value than PfPre [36] while that of *Pleurotus eous* was much higher [26]. Therefore, the DPPH radical scavenging activity of the polyphenol-rich extract from different basidiocarps were in decending order *G. Tsugae* [36] > *R. albonigra* [35] ~ *P. flabellatus* > *R. laurocerasi* [16] > *A. vaginata* [34] > *P. eous* [26]. Thus it can be said that the polyphenol-rich extract of *Pleurotus flabellatus* has significant DPPH radical scavenging ability.

REDUCING POWER

Reducing power of a compound may serve as a significant indicator of its potential antioxidant activity [24]. The presence of reducers (i.e. antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. In the reducing power assay the presence of antioxidants in the PfPre would effect in the reduction of Fe^{3+} to Fe^{2+} by the donation of an electron. The increasing absorbance at 700 nm by measuring the formation of Perl's Prussian Blue indicates an increase in reducing ability. A steady increase in reducing power was observed (fig 2). Results showed that EC_{50} for the reducing power of PfPre was of 0.87 ± 0.06 mg/ml. PfPre was found to be a potent reducing agent, with an EC_{50} value of 0.87 mg/ml. Compared with the reducing powers of previously studied edible mushrooms from our previous reports, the polyphenol-rich extract of *Pleurotus flabellatus* was an excellent reducer of ferric ions. The reducing power of polyphenol-rich extracts of different edible mushrooms in descending order are *P. flabellatus* > *R.* albonigra [35] > *A. vaginata* [34] > *Russula laurocerasi* [16]. In similar studies, it was reported that the hot water extract of *Ganoderma tsugae* [36] and *P. eous* [26] had a lower reducing power compared to that of *Pleurotus flabellatus* (EC_{50} 1.12 mg/ml). Apparently the polyphenol-rich extract of *P. flabellatus* is an excellent reducing agent.

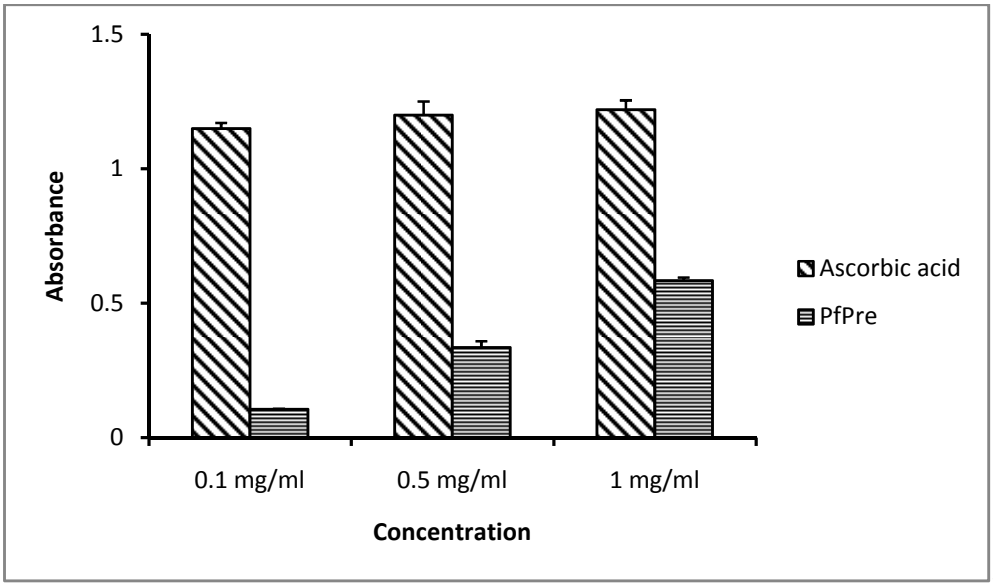

Fig 2: Reducing power of PfPre with respect to that of ascorbic acid used as standard
Values are the mean ± standard deviation of three separate experiments, each in triplicate

CHELATING EFFECT ON FERROUS IONS

Iron can stimulate lipid peroxidation by the Fenton reaction and accelerate peroxidation by decomposing lipid peroxide into peroxyl and alcoxyl radical that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation. The main mechanism of the ferrous ion chelating activity is the ability to deactivate and/or chelate Fe^{2+} which can promote Fenton reaction and hydroperoxide decomposition. Iron toxicity is associated with an increased risk of free radical damage and cancer. Chelation therapy may possibly reduce iron related free radical damage and increase the overall survival in cardiovascular diseases [37]. At 100 – 500 µg/ml the chelating effects of the PfPre was between 20% and 60% (fig 3). It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilising the oxidised forms of metal ions [38]. The ferrous ion chelating ability of PfPre was effective and the EC_{50} value was found to be 0.72 mg/ml. Our previous investigations have shown that the EC_{50} value of the polyphenol-rich extract for *R. laurocerasi* [16] was 0.58 mg/ml, which is comparatively higher, while the EC_{50} value for *A. vaginata* [34] was much higher than that of PfPre. PfPre showed a similar EC_{50} to that of *R. albonigra* [35]. Hence, the studied mushroom extract shows higher interference with the formation of ferrous and ferrozine complex and can be considered as a good chelator of ferrous ions.

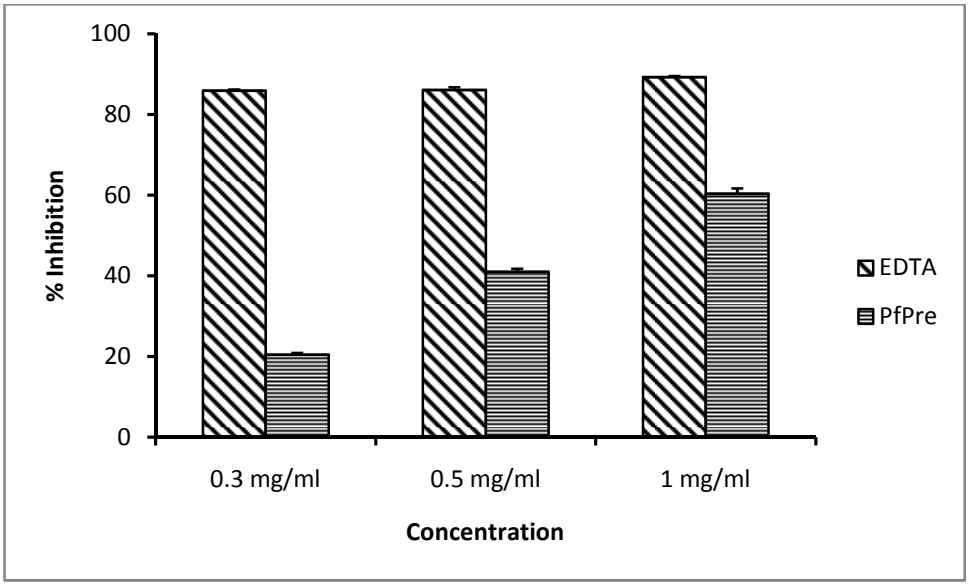

Fig 3: Chelating effects of PfPre on ferrous ions compared with that of EDTA, used as standard
Values are the mean ± standard deviation of three separate experiments, each in triplicate

SUPEROXIDE RADICAL SCAVENGING ASSAY

Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive species. One risk of the superoxide generation is related to its interaction with nitric oxide to form peroxynitrite which is a potent oxidant that causes nitrosative stress in the organ systems [39]. In the present study, the polyphenolic rich fraction of *Pleurotus flabellatus* was found to be a notable scavenger of superoxide radicals generated in riboflavin-nitrobluetetrazolium (NBT) light system (fig 4). In the present study, the EC_{50} value of the fraction was 0.71 mg/ml. Compared with an earlier investigation, the EC_{50} value of PfPre was lower than the polyphenol-rich extract of *Russula laurocerasi* [16] (1.56 mg/ml). It was also significantly lower than the EC_{50} value of *Pleurotus squarrosulus* [29] (8.63 mg/ml) and *Amanita vaginata* [34] (1.2 mg/ml). In comparison, *Tricholoma gigantium* [39] and *R. albonigra* [35] showed similar (0.735 and 0.74 mg/ml respectively) superoxide anion scavenging activity. Thus it can be inferred that PfPre is an effective scavenger of superoxide radicals.

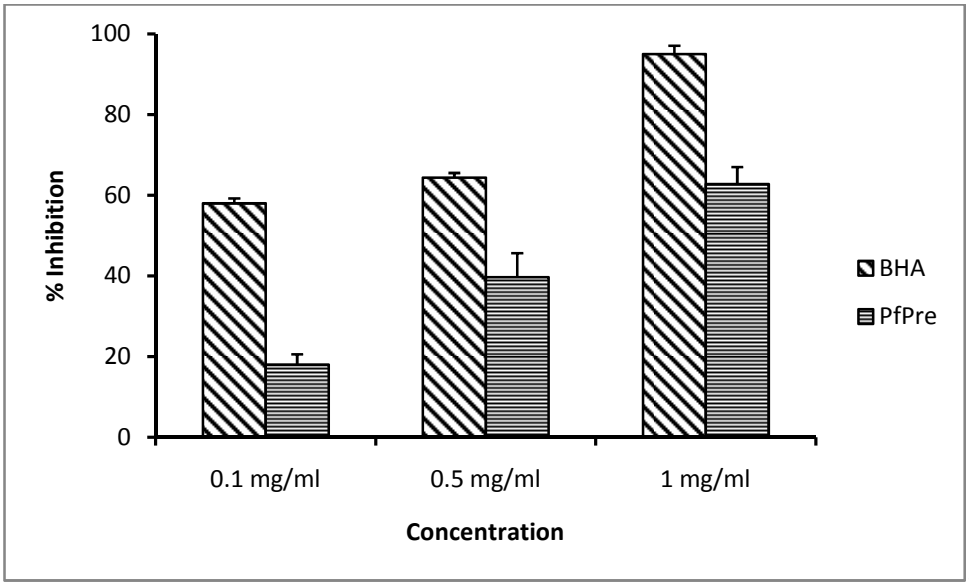

Fig 4: Superoxide radical scavenging activity of PfPre with respect to that of BHA, used as standard
Values are the mean ± standard deviation of three separate experiments, each in triplicate

Table 2 summarises the EC_{50} values of PfPre for the different antioxidant activities, which gives a view of its pontential as an antioxidant

	Chelating	DPPH	Reducing	SOD
EC_{50} (mg/ml)	0.72 ± .007	0.48 ± .02	0.87 ± .021	0.71 ± 0.012

Table 2: EC_{50} values of the polyphenol-rich extract of *Pleurotus flabellatus* for different antioxidant activities. Values are the mean ± standard deviation of three separate experiments, each in triplicate.

CONCLUSION

As a result of the study, the polyphenol-rich extract of *P. flabellatus* (PfPre) was found to be an effective antioxidant in different *in vitro* assays, with significant amounts of bioactive components. PfPre was an excellent chelator of Ferrous iron, scavenger of DPPH free radicals, reducer of ferric iron and superoxide anions. The EC_{50} values for these tests were all well below 1 mg/ml. Thus, it can be suggested as a natural additive in food and pharmaceutical industries.

REFERENCES

[1] M Rai; G Biswas; SC Mandal; K Acharya. Free radicals and human diseases In Herbal drugs: A modern approach to understand them better, ed SC Mandal, New Central Book Agency (P) Ltd., India, **2011**; 479-496.
[2] B Sultana; F Anwar; R przybylski, *Food Chem*., **2007**, 104(3), 1106-1114.
[3] YF Sasaki; S Kawaguchi; A Kamaya; M Ohshita; K Kabasawa; K Iwama; K Taniquchi; S Tsuda, *Mutat. Res-Gen. Tox. En.*, **2002,** 519(1-2), 103-109.
[4] MD Asatiani; V Elisashvili; G Songulashvili; AZ Reznick; SP Wasser, *Progress in Mycology*, **2010**, 311-326.
[5] P Pradhan; AK Dutta; A Roy; SK Basu; K Acharya, *Biodivers.*, **2012**, 13(2), 88-99.
[6] S Chatterjee; G Biswas; S Chandra; GK Saha; K Acharya, *Bioproc. Biosyst. Eng.*, **2013**, 36, 101-107.
[7] S Giri; G Biswas; P Pradhan; SC Mandal; K Acharya, *Int. J. Pharmtech Res.*, **2012**, 4, 1554-1560.
[8] TK Lai; G Biswas; S Chatterjee; A Dutta; C Pal; J Banerji; N Bhuvanesh; JH Reibenspies; K Acharya, *Chem. Biodivers.*, **2012**, 9, 1517-1524.
[9] G Biswas; S Rana; S Sarkar; K Acharya, *Pharmacologyonline*, **2011**, 2, 808-817.
[10] A Chatterjee; S Khatua; S Chatterjee; S Mukherjee; A Mukherjee; S Paloi; K Acharya; SK Bandyopadhyay, *Glycoconjugate J*., **2013**, 30(8), 759-768.
[11] G Biswas; K Acharya, *Int. J. Pharm. Pharm. Sci*., **2013**, 5, 391-394.
[12] K Acharya; S Chatterjee; G Biswas; A Chatterjee; GK Saha, *Int. J. Pharm. Pharm. Sci*., **2012**, 4, 285-288.
[13] S Chatterjee; R Datta; A Dey; P Pradhan; K Acharya, *Res. J. Pharm. Technol*., **2012**, 5, 1034-1038.
[14] DN Pegler. Pleurotus (Agaricales) in India, Nepal and Pakistan, Kew Bulletin, **1976**; 31, 501-510.
[15] B Manjula, A revised list of the agaricoid and boletoid basidiomycetes from India and Nepal, *Proc. Indian Acad. Sci.(Plant Sci.)*, **1983**, 92(2), 81-213.
[16] S Khatua; T Roy; K Acharya, *Asian J. Pharm. Clin. Res.*, **2013**, 6(4), 156-160.
[17] VL Singleton; JA Rossi, *Am. J. Enol. Vitic*., **1965**, 16, 144-158.
[18] EA Adebayo; JK Oloke; AA Ayandele; CO Adegunlola, *J. Microbiol. Biotech. Res.*, **2012**, 2(2), 366-374.
[19] M Nagata; I Yamashita, *Nippon Shokuhin Kogyo Gakkaishi*, **1992**, 39(10), 925-928.
[20] C Rekha; G Poornima; M Manasa; V Abhipsa; DJ Pavithra; KHT Vijay; KTR Prashith, *Chem. Sci. Trans*., **2012**, 1(2), 303-310.
[21] P Prieto; M Pineda; M Aguilar, *Anal. Biochem.*, **1999**, 269, 337-341.
[22] K Shimada; K Fujikawa; K Yahara; T Nakamura, *J. Agric. Food Chem*., **1992**, 40(6), 945-948.
[23] M Oyaizu, *Jpn. J. Nut.*, **1986**, 44, 307-315.
[24] TCP Dinis; VMC Madeira; LM Almeida, *Arch. Biochem. Biophys*., **1994**, 315, 161-169.
[25] AC Martinez; EL Marcelo; AO Marco; M Moacyr, *Plant Sci*., **2001,** 160, 505-515.
[26] G Sudha; S Vadivukkarasi; BIS Rajan; P Lakshmanan, *Food Sci. Biotechnol*., **2012**, 21(3), 661-668.
[27] YL Lee; GW Huang; ZC Liang; JL Mau, *LWT - Food Sci. Technol*., **2007**, 40(5), 823-833.
[28] J Pal; S Ganguly; KS Tahsin; K Acharya, *Indian J. Exp. Biol.*, **2010**, 48(12), 1210-1218.
[29] WN Beyer; E Conner; S Gerould, *J. Wildl. Manage.*, **1994**, 58, 375-382.
[30] SZ Dziedzic; GJF Hudson, *Food Chem*., **1983**, 12, 205-212.
[31] GK Jayaprakasha; B Girennavar; BS Patil, *Bioresour. Technol.*, **2008**, 99(10), 4484-4494.
[32] M Waltasinghe; F Shahidi, *Food Chem*., **1999**, 63, 399-414.
[33] S Khatua; S Paul; A Chatterjee; D Ray; A Roy; K Acharya, *Int. J. Pharm. Sci. Rev. Res.*, **2013**, 23(2), 368-373.
[34] S Paloi; K Acharya, *Int. J. PharmTech Res.*, **2013**, 5(4), 1645-1654.
[35] A Dasgupta; D Ray; A Chatterjee; A Roy; K Acharya, *Res. J. Pharm. Biol. Chem. Sci*., **2014**, 5(1), 510-520.
[36] JL Mau; SY Tsai; YH Tseng; SJ Huang, *LWT-Food Science and Technol*., **2005**, 38, 589-597.
[37] B Halliwell; JMC Gutteridge, *Methods Enzymol.*, **1990**, 86, 1-85.
[38] K Sowndhararajan; JM Joseph; S Manian, *Int. J. Food Prop*., **2013**, 16(8), 1717-1729.
[39] K Acharya; S Chatterjee, S Ghosh, *Pharmacologyonline*, **2011**, 1, 440-450.