Original Research

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Safety Evaluation and *In-vitro* Antioxidant Activity of Exopolysaccharide Produced by an Indigenous Species of *Pleurotus pulmonarius* RDM9

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Abstract

Higher fungus is capable of producing exopolysaccharides of different and unique sugar moieties. This property offers the use of EPS in various food and cosmetic applications. This study was designed to ensure that the EPS obtained from *Pleurotus pulmonarius* is safe for human consumption and is proposed to be used as a bioemulsifier and bioflocculant in food applications. Toxicological examination showed no sign of toxicity and mortality after treating BALB/c mice for consecutively 14 days by administering respective dose of EPS (100µl of sterilized PBS for control group and a dose of 50–400 mg EPS/kg in a constant volume of 100 µl PBS for the test groups) via orogastric gavage. No significant change was recorded in body mass, morphology and behaviour of animals. Both ALT and AST activity were not affected by EPS in treated mice and hence the liver physiology was found to be normal. Histopathological examination of liver, kidney and small intestine showed no significant change in the morphology of the organs. The antioxidant capacity of purified EPS from *P. pulmonarius* was studied in *in-vitro* conditions by studying different biochemical methods and the results revealed that the test EPS showed a strong total antioxidant capacity and H_2O_2 scavenging activity.

Keywords: Antioxidant Activity, Exopolysaccharide, *Pleurotus pulmonarius*, White Rot Fungi

1. Introduction

To introduce new food products in food industries, a continuous research is required on new ingredients from different sources. To cop up with the growing demand for quality food products, polysaccharides are now one of the widely explored food ingredients because of their diversity in structural and chemical properties making them as a suitable candidate for many prospective applications. Recently, there is a growing interest in the isolation of microbial exopolysaccharide. Exopolysaccharides with linear or branched structures are composed of repeating units of different monosaccharides or their derivatives. Exopolysaccharides (EPS) are known to improve the rheological properties and sensory qualities of the final product[1]. They are known to possess antitumor, antioxidant, immunomodulating, hyperglycemic, antiviral, function as prebiotic, lowers blood cholesterol and sorption of heavy metals[2,3]. Considerable number of microorganisms of different taxonomy (bacteria, fungi, yeast) is capable of producing exopolysaccharides. EPS produced from white rot fungi offers greater advantage because of its fast and easy separation from the culture medium compared to bacteria. However, the information on the chemical and physical properties of EPS from white rot fungi is still scarce. To the best of our knowledge, there are no available reports on toxicological assessment of EPS produced by *Pleurotus pulmonarius* Hence, this study focused on safety evaluation of EPS being produced by the test fungus using *Pleurotus pulmonarius* RDM9 using BALB/c mice as animal model and characterization of its antioxidant properties.

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2. Materials and Methods

2.1 Preparation of Test Material for Toxicological Examination of Purified EPS

EPS was produced by cultivation of *Pleurotus pulmonarius* RDM9 (MTCC 12822) in potato dextrose broth supplemented with effective nitrogen (peptone) and mineral source (K_2HPO_4) with pH 4 and incubating at 28°C for 7 days under shaking conditions at 150 rpm as per Maziero et al[4]. EPS was recovered from the culture broth by centrifugation from the cell free culture broth after precipitation with ethanol (1:4), dialysed with distilled water for removal of ions and small molecules and further purified by DEAE cellulose-52 and Sephadex G-100 chromatography[5,6]. The EPS obtained was frozen and then lyophilized until a constant weight was observed. EPS dose was prepared by dissolving it in sterilized saline solution (50 mM PBS; pH 7) at a concentration of 1.5 mg/100 µl (Group II), 6.75 mg/100 µl (Group III) and 12 mg/100 µl (Group IV).

2.2 Test Animals

BALB/c mice strains were supplied by Central Animal House, Sector 14, Panjab University, Chandigarh (India). The animals (both males and females; 6–8 weeks old; 29–30 gm) were divided into four dose groups consisting of 5 animals each. The animals were housed five mice of either sex per cage bedded with clean rice husk, equipped with free access to feed and water *ad libitum* and were maintained at a temperature of 23°C ± 1. These animals were provided with standard pellet diet (M/S. Ashirwad Industries Pvt. Ltd., Punjab, India) and were acclimatized for at least one week before the experimental use. Before the experiment initiation, the initial body weight (X ± SD) of all the animals was taken on Vintage tanita retro scale. Animals belonging to all the groups were treated for consecutively 14 days by administering respective dose of EPS (100 µl of sterilized PBS for control group I, 50 mg EPS/kg for test group II, 225 mg EPS/kg for group III and a dose of 400 mg EPS/kg for group IV in a constant volume of 100 µl PBS for the test group) via orogastric gavage[7]. The animals were observed for 14 days consecutively for any sign of toxicity or mortality. The body mass, morphological and behavioural changes were observed daily.

2.3 Serum Biochemistry

Blood was collected from individual mice by cardiac puncture at the end of the study. The estimation of Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) was done by the methods recommended by the International Federation of Clinical Chemistry (IFCC) using ERBA test kits (ERBA Diagnostics, Manheim, Germany) for assessment of liver function. For this, serum separation was done by clotting the blood samples at room temperature for 1–2 h and was kept at 4°C for 4–5 h. After that the blood samples were centrifuged at 3000 rpm for 10 min. The clear supernatant obtained was stored at −60°C for further use 0.1 ml of serum samples was added to 1.0 ml of ALT and AST reagent respectively, mixed well and change in absorbance per min (ΔA/min) was read at 340 nm. Enzyme activity was expressed as international units/liter (IU/L). The activities of ALT and AST were calculated as following:

$$\text{Activity of ALT (IU/L)} = \frac{(\Delta A/\text{min}) \times \text{T.V.} \times 10^3}{\text{S.V.} \times \text{Absorptivity} \times \text{P}}$$

$$\text{Activity of AST (IU/L)} = \frac{(\Delta A/\text{min}) \times \text{T.V.} \times 10^3}{\text{S.V.} \times \text{Absorptivity} \times \text{P}}$$

Where:
T.V. = Total reaction volume
S.V. = Sample volume
Absorptivity = Millimolar absorptivity of NADH at 340 nm = 6.22
P = Cuvette light path (cm) = 1cm
Activity of AST (IU/L) = (ΔA/min) × Factor (1768)

2.4 Organ Weights and Histopathology

At the end of the experiment, all the animals were sacrificed by cervical dislocation. Vital organs i.e., kidney, liver and small intestine were removed from each mice in the groups and fixed in 10% buffered formalin[8]. The organs were weighed and then processed for histopathological examination as per Garg *et al*[9]. After 48 hours, the organs were sliced in their desired sections, transferred into fresh formaldehyde solution and stained using haematoxylin and eosin stains (H & E).

2.5 Antioxidant Activity of Test EPS

2.5.1 In vitro Evaluation of Antioxidant Property of EPS

The antioxidant activity of the test EPS was determined by performing the following assays.

2.6 Determination of Total Antioxidant Capacity

The total antioxidant capacity of the test EPS was determined by the phosphomolybdenum method according to the procedure described by Prieto *et al*[10]. In this assay, molybdenum (VI) is reduced to molybdenum (V) in the presence of a reducing agent (antioxidant) to form a green phosphomolybdate (V) complex at acidic pH, which is then evaluated spectrophotometrically at 765 nm. The assay demands an Electron Transfer (ET) mechanism. The phosphomolybdenum method is quantitative, since the antioxidant activity is expressed as the number of equivalents of ascorbic acid. The reaction mixture consisted of the test EPS (1–5 µg/ml), 3 ml of distilled water and 1ml molybdate reagent. The mixture was incubated at 95°C for 90 minutes and then at room temperature for 20–30 minutes. The absorbance was measured at 695 nm against blank after cooling to room temperature. Ascorbic acid was taken as the positive control. The antioxidant activity is expressed as the number of gram equivalent of ascorbic acid.

2.7 Assay of Reducing Power

The reducing power of the test EPS was determined as per the modified method of Oyaizu *et al*[11]. Reaction mixture containing 0.75 ml of test EPS (1–5 mg/ml), 0.75 ml of 1% 0.2 M sodium phosphate buffer (pH 6.6) and 0.75 ml of potassium ferricyanide (0.1%, w/v) was incubated at 50°C for 20 minutes. Then 0.75 ml of Trichloroacetic Acid (TCA) (10%, w/v) was added to stop the reaction. After that, the reaction mixture was centrifuged at 5000 rpm for 10 min. After centrifugation, the upper layer was mixed with 1.5 ml of distilled water and 0.1 ml of fresh ferric chloride ($FeCl_3$) (0.1%, w/v) was incubated at room temperature for 10 minutes. The absorbance was measured at 700 nm against a blank. A higher absorbance indicated a higher reducing power. Deionized water and ascorbic acid were used as the blank and control, respectively.

2.8 DPPH Free Radical Scavenging Assay

The DPPH free radical scavenging assay of the test EPS was measured as per Kao and Chen[12]. The reaction mixture consisted of 1ml of the test EPS (1–5 mg/ml) and 2 ml of ethanolic DPPH (0.05 mM). The contents were mixed vigorously and were incubated for 30 minutes at room temperature in dark. After incubation the mixture was centrifuged at 8000 rpm for 10 minutes. The upper layer was separated, and the absorbance was measured at 517 nm. Distilled water served as blank and ascorbic acid as positive control. The DPPH scavenging activity was calculated using following formula,

$$\% \text{ DPPH scavenging activity} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

2.9 Hydrogen Peroxide (H_2O_2) Scavenging Assay

The H_2O_2 scavenging assay of EPS was determined as per Gulcin *et al*[13]. The reaction mixture consisted of 1 ml of the test EPS (1–5 mg/ml) and 2 ml of 10 mM H_2O_2 in 0.1 M phosphate buffer saline (pH 7.4). After 10 minutes of incubation at 37°C, the absorbance was taken at 230 nm. Distilled water served as the control. The scavenging activity was expressed as,

$$\% \text{ Scavenging} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

2.10 Hydroxyl Radical (OH) Scavenging Assay

The hydroxyl radical scavenging activity of EPS was determined as per Jin *et al*., 1996[14]. The hydroxyl radical was generated in the mixture of 1 ml of 0.75 mM 1,10 phenanthroline, 1.5 ml of 0.15 M sodium phosphate buffer (pH 7.4), 1 ml of 0.75 mM Ferrous Sulphate ($FeSO_4$) and 1 ml of H_2O_2 (0.01%, v/v). To this mixture, 1 ml of test EPS in the concentration ranging from 1–3 mg/ml, was added the mixture was incubated at 37°C for 30 min. The absorbance was measured at 536 nm against a blank. Deionized water and ascorbic acid were used as the blank and control, respectively. The scavenging activity of EPS on hydroxyl radical was calculated as per formula

$$\text{Scavenging activity on hydroxyl radical (\%)} = (A_{sample} - A_{blank})/(A' - A_{blank}) \times 100$$

Where, A' is the absorbance of the deionized water.

3. Results

The result of the acute toxicity study of purified EPS of *P. pulmonarius* RDM9 showed no sign of toxicity and mortality was observed in any of the test groups upto

14 days of treatment period. No significant change was recorded in their body mass (Figure 1). Also no change was observed in their morphology and all mice appeared to be absolutely normal with no hair loss and swelling on/or in their body. No behaviour changes including dullness, dizziness, laziness or hyperactivness was seen. The test group mice were equally active to mice of the control group.

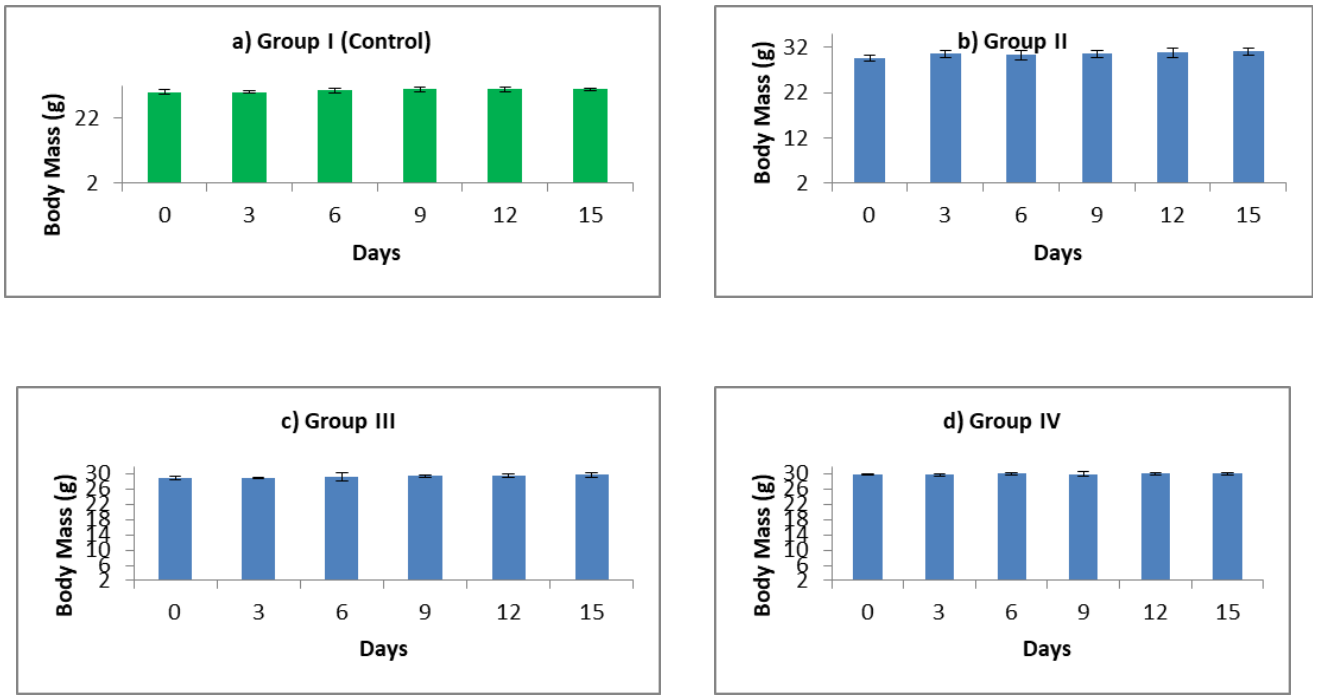


Figure 1. Effect of the purified EPS on body mass of the test group mice. a) Group I: Control (treated with PBS); b) Group II: Treated with 50 mg EPS/Kg c) Group III: Treated with 225 mg EPS/Kg d) Group IV: Treated with 400 mg EPS/Kg.

Table 1. Mean ALT and AST of the control and EPS treated group

Groups	Parameters (IU/L)	
	AST (IU/L)	ALT (IU/L)
CONTROL GROUP I	149 ± 0.2	151 ± 0.2
TEST GROUP II	144 ± 0.2	146 ± 0.2
TEST GROUP III	142.75 ± 1.0	144.27 ± 2.0
TEST GROUP IV	140.2 ± 1.2	147 ± 1.0

AST - Aspartate aminotransferase
ALT - Alanine aminotransferase
The values are a mean average of 5 mice. No significant difference between control and EPS- fed groups with $p > 0.05$ level of significance.

Further, the effect of EPS toxicity on the liver function was also assessed after oral administration of EPS for 14 days continuously. Two main liver enzymes, ALT and AST, are the well-known reliable markers for liver functioning. The results revealed that both ALT and AST activity were not affected by EPS in treated mice and hence the liver physiology was found to be normal (Table 1).

Though absolute weights of the organs of treated group varied from those of the control group, no statistically significant variations were observed when compared as relative weights, i.e., organ weight/body weight. Next, all the important vital organs, i.e., liver, kidney and small intestine, were examined for histopathological studies to study any toxic effect of EPS. The results showed no change in the morphology of these organs (Figure 2).

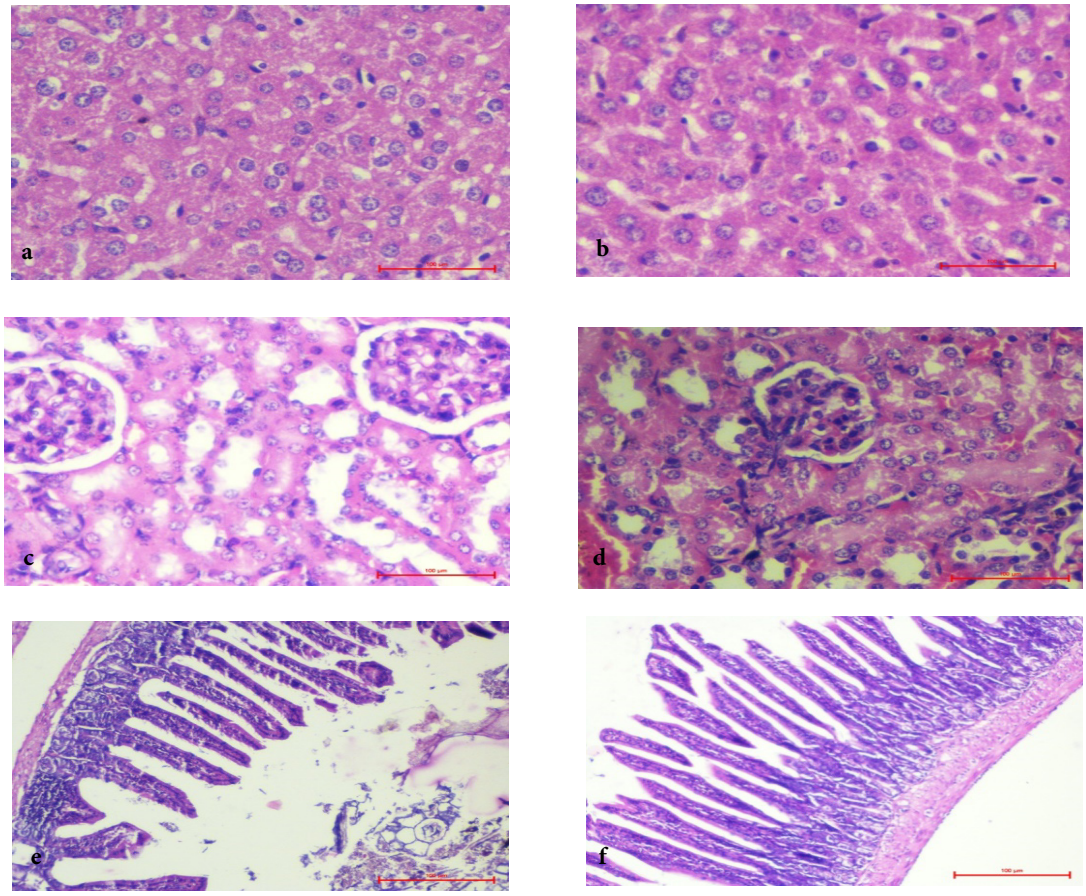


Figure 2. Effect of EPS on the organs of mice in a 14-day feeding test. (a and b) Sections of liver. (c and d) Sections of kidney. (e and f) Sections of small intestine. (a, c and e) PBS treated and (b, d and f) EPS treated (Group IV). The sections stained with Hematoxylin and Eosin (HE) were observed under light microscope.

3.1 *In vitro* Evaluation of Antioxidant Property of the Test EPS

The antioxidant capacity of purified EPS from *P. pulmonarius* was studied in *in-vitro* with different biochemical methods.

3.2 Total Antioxidant Capacity

Total Antioxidant Capacity (TAC) of the test EPS was analyzed using phosphomolybdate assay. It is used to evaluate the antioxidant level of the test samples and assesses the antioxidant reaction against free radicals produced during the reaction. It measures of the quantity of free radicals scavenged by the test solution. The total antioxidant capacity of EPS extracts of *P. pulmonarius* RDM9 along with standard ascorbic acid is depicted in Figure 3(a). The results obtained revealed that the total antioxidant capacity was concentration dependent, i.e., the test EPS contains as much quantity of antioxidants compounds as equivalents of ascorbic acid to effectively reduce the oxidant in the reaction matrix[15]. Ascorbic acid's antioxidant capacity was used as a reference standard for comparing the potential antioxidant activity of the test EPS[16]. However, limited information is provided, by measurement of only TAC, about the antioxidant status as TAC assays do not measure all antioxidant components. Therefore, the antioxidant capacity of the test EPS was measured by following methods too.

3.3 Assay of Reducing Power

The assay of reducing power shows the ability of exopolysaccharides to donate electrons. The reduction of Fe^{3+} to Fe^{2+} was observed spectrophotometrically by absorbance of Perl's Prussian blue formation at 700 nm. Greater the absorbance, higher was the reduction power. Ascorbic acid was used as a standard to compare the

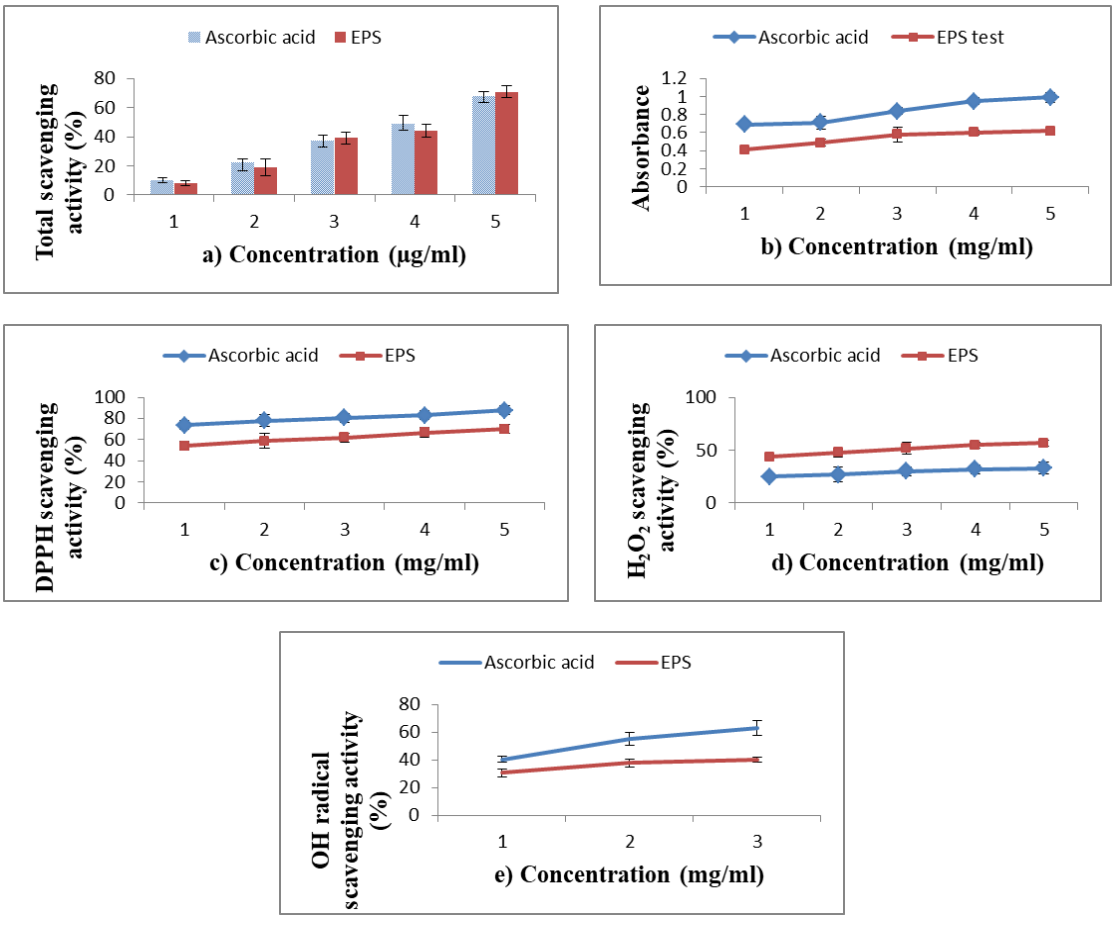
reduction capacity of the test EPS. The reducing power of the test EPS is presented in Figure 3(b). The result shows the reducing power capacity of EPS. The reducing power of the test EPS was directly proportional to its concentration. The maximum reducing power of the test EPS was read at absorbance of 0.62 at a concentration of 5 mg/ml. These results suggests the test EPS as a good electron donor which can cease the radical chain reactions by converting free radicals to more stable products. The reducing power of the test EPS was found almost at par with ascorbic acid.

3.4 DPPH Free Radical Scavenging Assay

DPPH is one of the compounds that has a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers[17]. Furthermore, it is well accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability. This test is based on change in colour of the substrate from violet to yellow, depending on the capacity of the product tested. The DPPH scavenging activity of the test EPS was directly correlated with its concentration (Figure 3(c)). The EPS sample showed a noticeable DPPH scavenging activity of 70% which suggests that it is somehow capable of donating hydrogen ions to react with the DPPH radical.

3.5 Hydrogen Peroxide Scavenging Assay

Hydrogen Peroxide (H_2O_2) is a biologically important, non-radical Reactive Oxygen Species (ROS) that can influence several cellular processes[18]. The test EPS produced by *P. pulmonarius* RDM9 was evaluated for its H_2O_2 scavenging ability. The results obtained revealed that the H_2O_2 scavenging activity of the test EPS was directly proportional to its concentration. EPS at a concentration of 5 mg/ml possessed a scavenging activity of 57%, respectively which was found even higher than ascorbic acid (Figure 3(d)).



Values are mean ±S.D. of three observations.

Figure 3. **(a)** Total antioxidant capacity. **(b)** Reducing power. **(c)** DPPH scavenging activity. **(d)** Hydrogen peroxide scavenging activity. **(e)** Hydroxyl radical scavenging activity of the test EPS produced by *P. pulmonarius* RDM9. Ascorbic acid was used as standard.

3.6 Hydroxyl Radical Scavenging Assay

Hydroxyl (OH) radical is one of the most reactive oxygen species and causes serious damage to the organisms. These radicals are an important reason for causing ageing and tissue damage. The ability of scavenging hydroxyl radicals by the test EPS was determined using the Fenton reaction and compared with that of ascorbic acid. The hydroxyl radical scavenging activity of the test EPS increased with increase in its concentration. The activity increased to 40.1% at a concentration of 3 mg/ml (Figure 3(e)). Although the test EPS was able to scavenge hydroxyl radicals at concentrations ranging from 1 to 3 mg/ml, but it was found to be lower than that of the ascorbic acid, used as reference, at every concentration point.

4. Discussion

The safety evaluation of the EPS showed that it did not cause any type of alterations in mice studied. Till date, there is no report on toxicological studies of EPS from this particular species of *Pleurotus*. Considering the results of this study, the EPS from *P. pulmonarius* RDM9, can be implied as safe when applied to food related applications. This finding opens a platform for further exploration of EPS with such potentials and discovery of novel products that would direct its potential application in food and pharmaceutical industries. The *in vitro* antioxidant assay showed strong antioxidant properties of the test EPS, especially the H_2O_2 scavenging ability. These results suggest that the EPS from *P. pulmonarius* RDM9 may be a natural potential alternative to conventionally used synthetic antioxidant products.

5. Acknowledgements

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6. Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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