INTRODUCTION

Reactive oxygen species (ROS) including superoxide anion radical, hydroxyl radical and hydrogen peroxide are produced through oxidative process within the mammalian body. They are degraded by many defence mechanisms against oxidative stress, including antioxidant enzymes and non-enzymatic compounds [1]. Under certain circumstances including synthetic (xenobiotic) chemicals, radiation, x-rays, pollution, stress etc the natural antioxidant defence mechanisms become insufficient and then the uncontrolled production of oxidative species can damage both the structure and function of cell membrane in a chain reaction leading to degenerative diseases and conditions such as Alzheimer, arteriosclerosis, cardiovascular disease, nephritis, diabetes mellitus, acute liver toxicity, rheumatism, inflammation, ageing process, cataracts and carcinogenesis [2,3]. In recent years, interest has augmented considerably in finding naturally occurring antioxidants for use in foods, cosmetics or medicinal materials to replace synthetic antioxidants, which are being restricted due to their carcinogenicity [4].

Nitrile oxide (NO), which is biosynthesized at the cellular level from L-arginine catalyzed by nitric oxide synthase (NOS), is a very important signalling molecule from the ground of pathophysiological condition of living entities. The omnipresence of NO in the living body suggests that NO plays an important role in the maintenance of health [5]. It is well studied in mammalian system and has been found to have numerous roles in pathophysiology [6,7]. Cellular production of NO below physiologic level causes initiation of different diseases like hypertension, atherosclerosis, diabetes mellitus, ischemia, stroke, myocardial infarction, heart failure, hypoxia, Alzheimer disease, fibrosis, cancer, renal failure, etc. [8]. In recent years, the NO signalling pathway has become a target for new drug development. The high level of flavonoids, catechins, tannins and other polyphenolic compounds present in vegetables, fruits, mushrooms, soy, tea and even red wine is believed to contribute to their beneficial health effects [5]. Thus, NOS activation by supplementation of food would find a new route of therapy.

Now-a-days, mushrooms have become attractive as functional foods and as a source of physiologically beneficial medicines, while being devoid of undesirable side effects [9]. Edible mushrooms are highly nutritious and having therapeutic potentiality for the treatment of cancer, heart ailments, diabetes, inflammation, hepatic damage, high blood pressure, microbial pathogens etc [10-17]. Recent investigations revealed that polysaccharides and extracts of mushrooms have strong antioxidant and NO synthase activation properties [18-24].

Therefore, the primary aim of this study was to isolate crude polysaccharide (POCP) from P. ostreatus basidiospores and to investigate its antioxidant activity and NOS activation property.

MATERIAL AND METHODS

Collection of Mushroom

Mushrooms, P. ostreatus, were purchased from mushroom cultivation unit of Narendrapur Ramakrishna Mission Ashrama, Narendrapur, West Bengal, India. Fruit bodies were dried in the oven for few days until all became desiccated.

Chemicals

Ferric chloride, L-methionine, nitro-blue tetrazolium (NBT), riboflavin, 2-Deoxy-D-ribose, hydrogen peroxide (H₂O₂), ferrozine, ferrous chloride, L-arginine, trichloro acetic acid (TCA), thio-barbituric acid (TBA), ascorbic acid, standards such as, ethylenediaminetetraacetic acid (EDTA), butylated hydroxyanisole (BHA), gallic acid, bovine serum albumin (BSA) were purchased from Sigma chemicals Co. (St. Louis, MO, USA). An analytical mushroom β-glucan kit was obtained from Megazyme Int. (Wicklow, Ireland). All other chemicals and reagents used were of analytical grade.

Sample Preparation

Dried and powdered fruit bodies of P. ostreatus were extracted with ten volume of 99% ethanol for two days at room temperature to remove phenolic compounds and lipid. Residue was filtered and re-extracted with ethanol. The air dried filtrate was suspended and re-extracted with distilled water. It was maintained at boiling condition for eight hours. The extract was filtered through gauze and was concentrated to one-tenth of the volume with a rotary evaporator at 80°C under vacuum. Four volumes of absolute alcohol were added to precipitate polysaccharide and was left at 4°C overnight. After centrifugation, the pellets were washed with 70% (v/v) ethanol and successively by ethyl acetate and acetone. The washed pellets were dissolved in water. At last it was lyophilized to yield crude polysaccharide. POCP was then redissolved in distilled water for further works.

Physico-Chemical Characterization

Determination of Polysaccharide Content

The total polysaccharide content was determined using phenolsulphuric acid method with D-Glucose as a reference [25]. Total
reaction mixture [4 ml] consisted of 100 μg of POCP, 0.5 ml of 6% phenol and 2.5 ml of concentrated sulphuric acid. It was incubated for 30 min at room temperature and absorbance was taken at 490 nm. Results were expressed as g of glucose equivalents/100 g of the dry polysaccharide.

**Determination of Glucan Content**

Contents of total and α-glucan were determined using the Mushroom and Yeast β-glucan Assay Procedure (Megazyme Int. Ltd.) kit. The enzyme kit contained exo-1, 3-β-glucanase, amyloglucosidase, invertase, β-glucosidase, glucose determination reagent and glucose standard solution. To estimate the total glucan content, HCl hydrolysed polysaccharide was subjected to enzymatic degradation by exo-β- (1-3) -β-glucanase and β-glucosidase. Free glucose was determined by addition of glucose oxidase/peroxidase reagent spectrophotometrically. The α glucan content was estimated upon enzymatic hydrolysis with amyloglucosidase plus invertase yielding glucose. Again glucose oxidase/peroxidase reagent was added to determine α-linked glucose. The β glucan content was calculated by subtracting α from total glucan content according to kit’s manual. All values of glucan content were expressed as g/100 g of dry weight of the polysaccharide.

**Measurement of Protein Content**

Protein content was measured according to the method of Bradford [26]. BSA (0.01 – 0.1 mg/ml) was used to produce standard calibration curve. Total protein content of the extracts was expressed as g of BSA equivalents/100 g of dry polysaccharide.

**Free Radical Scavenging Assays**

**Superoxide Radical Scavenging Activity**

According to Martinez et al. [28] each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, various concentrations (100 – 400 μg/ml) of POCP, 100 μM EDTA, 75 μM NBT and 2 μM riboflavin. Reaction started by illuminating sample with light and the absorbance was measured at 560 nm after 10 min of illumination. Identical tubes with the reaction mixture were kept in the dark and served as blank. BHA was used as a positive control. The degree of scavenging was calculated by the following equation:

\[
\text{Scavenging effect} (\%) = \left( \frac{A_b - A_s}{A_b} \right) \times 100
\]

Where As was the absorbance of the control and A_s was the absorbance in the presence of sample

**Hydroxyl Radical Scavenging Activity**

The method used by Halliwell et al. [29] was followed in this study. The reaction mixture (1ml) consisted of KH₂PO₄ - KOH buffer (20 mM, pH 7.4), 2-deoxy-D-ribose (2.5 mM), variable concentration (100 – 600 μg/ml) of POCP, FeCl₃ (100 mM), EDTA (104 μM), ascorbate (100 μM) and H₂O₂ (1 mM). It was incubated at 37°C for 1 h. 2ml thiobarbituric acid (TBA) and trichloroacetic acid (TCA) solution (0.375 w/v TBA, 15% TCA and 0.25 N HCl) was added and incubated at boiling water bath for 15 min. After cooling, absorbance was measured at 535 nm. EC₅₀ value in μg/ml expressed the effective concentration at which the it will be scavenging activity of free radical is 50%. BHT was used as positive control.

**Chelating Ability of Ferrous Ions**

Chelating ability was determined according to the method of Din et al. [30]. Reaction mixture [4 ml] contained different concentration of POCP (100 – 400 μg/ml) mixed with 3.7 ml of water 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 incubation at room temperature, the absorbance was determined at 562 nm against a blank. EDTA was used as positive control. The percentage of inhibition of ferrozine- Fe²⁺ complex formation is given by this formula:

\[
\% \text{ inhibition} = \left( \frac{(A_0 - A_s)}{A_0} \right) \times 100
\]

Where As was the absorbance of the control and A₀ the absorbance in the presence of mushroom

**Determination of NOS Activity**

NO was determined according to [31]. Typically, NO content was estimated by conversion of oxyhaemoglobin to methaemoglobin. The reaction mixture (2.5 ml) containing RBC (10⁶ cells) was incubated with L-arginine (10 μM), haemoglobin (30 µM) and POCP for different time periods at 37°C. After each incubation period, a portion of reaction mixture was centrifuged at 8,000 g for 5 min at 37°C and NO content of the supernatant was compared with an appropriate control set.

**Statistical Analysis**

Results were subjected to statistical analysis using Student's t test. Values are mean ± SD of 3 replications.

**RESULTS AND DISCUSSION**

**Physico-Chemical Characterization**

Estimation of crude polysaccharide from dried fruit bodies gave the yield of 5.24%, which appeared to be white powder and highly soluble in water. The hot water extraction yield of crude polysaccharides from Lentinus polyphorbus showed a similar yield from dried fruit bodies [32] which were clearly higher than Ganoderma tsugae, 1.7% [33] and Russula virens, 1.94% [34]. The chemical compositions of POCP were summarized in table-1. Total carbohydrate content of POCP was 62.67% which was more than that of Ganoderma lucidum, 56.6% [35]. Protein content of POCP when compared with few other samples was seen to be in the following increasing order POCP (15.75%) > Agaricus brasiliensis (7.3%) > Phellinus linteus (5.7%) > Ganoderma lucidum (2.6%) > Agaricus bisporus (9.9%) [36]. Total glucan content of POCP (43.9%) was also in a superior position than Ganoderma applanatum (22.4%), Ganoderma lucidum (3.6%), Lentinis edodes (42.6%), Agaricus brasiliensis (40.1%), Ganoderma lucidum (20.8%), and Phellinus linteus (24.5%) [35,36]. The α glucan and β glucan content of POCP were 3.9% and 40% respectively. Whereas α glucan content of Lentinus edodes (1.4%), and Trametes versicolor (2.9%) and the β glucan of Ganoderma applanatum (16%) was lower than POCP [35]. Very negligible amount of phenolic was detected from this crude polysaccharide though Kozarski et al. [35] reported that Ganoderma applanatum, Ganoderma lucidum, Lentinis edodes, Trametes versicolor had much higher amount of it.

| Table 1: Contents of total polysaccharides, protein, phenolics, total glucan, α glucan and β glucan of crude polysaccharide. |
|---|---|---|---|---|
| Total Polysaccharide (g/100gm) | Total Protein (g/100gm) | Total Phenol (g/100gm) | Glucan Content (g/100gm) |
| 62.67 ± 2.67 | 15.75 ± 0.75 | 4.5 ± 0.3 | 43.9 ± 1.2 |

**Superoxide Radical Scavenging Activity**

Superoxide radical is known to be very harmful to cellular components and plays a major role in the formation of other reactive oxygen species such as hydroxyl radical, hydrogen peroxide and singlet oxygen in living system. Regarding data presented in Figure

1. the scavenging activity of POCP increased with increasing concentration up to a certain level and reached a plateau. The EC₅₀ value was found to be 390 and 30 µg/ml for POCP and BHA, respectively. Recently, a pure heteroglycan was isolated from P. ostreatus showed EC₅₀ value of 553 µg/ml [22]. He et al. stated that the crude polysaccharide from Agaricus bisporus had EC₅₀ value of 1.17 mg/ml [37]. The results suggested that the crude
polysaccharide exhibited scavenging effect on superoxide anion radical generation that could help prevent or ameliorate oxidative damage.

**Figure 1: Superoxide radical scavenging activity of POCP. Results are the mean ± SD of three separate experiments, each in triplicate.**

**Hydroxyl Radical Scavenging Activity**

Hydroxyl radicals are the major active oxygen species capable of modifying almost every molecule in the living cells. This radical has the capacity to cause strand damages in DNA leading to carcinogenesis, mutagenesis, and cytotoxicity. Furthermore, this radical is also capable of stealing hydrogen atoms from unsaturated fatty acids leading to quick initiation of lipid peroxidation process [38, 39]. Ferric – EDTA was incubated with H2O2 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 which formed a pink chromogen upon heating with TBA at low pH [40]. Data presented in Figure 2 showed that the crude polysaccharide POCP was a potent scavenger of hydroxyl radicals which increased in a concentration dependent manner. The EC50 value of the POCP was 665 μg/ml. EC50 value of Agaricus bisporus and Lentinus edodes was seen to be 1.05 mg/ml and 1.90 mg/ml respectively [37]. Jayakumar et al. [41] found that the EC50 value of the ethanolic extract of P. ostreatus was 8 mg/ml. Patra and his co-workers [23], reported that EC50 value of a pure water soluble heteroglycan of P. ostreatus was 943 μg/ml, which was 1.5 fold higher than the present observation.

**Figure 2: Hydroxyl Radical scavenging activity of POCP. Results are the mean ± SD of three separate experiments, each in triplicate.**

**Chelating Ability of Ferrous Ion**

Like many transition metals Fe2+ can trigger process of free radical reaction such as hydroxyl radical formation. As the ferrous ions are effective prooxidants in food system, so ferrous ion chelating abilities to some extent would be beneficial [12]. The range and the mean of Fe2+ chelating capacity showed a marked capacity for iron binding ability. At 100 – 400 μg/ml, the chelating ability of this polysaccharide was between 12.85% and 52.55% (Figure 3). At the same concentration range, the chelating effect of the synthetic metal chelator, EDTA, was between 68.62% and 82.35%. The pure polysaccharide of P. ostreatus demonstrated iron binding ability of 54.82% at a concentration of 1 mg/ml [23] whereas ethanol extract of P. ostreatus was seen to have an ability of 50% chelation at 6 mg/ml [41].

**Figure 3: Ferrous ion chelating ability of POCP. Results are the mean ± SD of three separate experiments, each in triplicate.**

**Determination of NOS Activity**

NO is recognized to be an inter and intra- cellular mediator of several cell functions. It acts as a signal molecule in immune, nervous and vascular systems [42]. POCP showed more than 2 fold increase in NOS activity when compared with the control. Our findings suggested that the crude polysaccharide is a potent activator of NOS. Similar observations were made earlier where hot water extract of Astraeus hygrometricus [43], Ramaria aurea [20], Volvariella volvacea [21], Meripilus gigantium [22] were capable of inducing NOS in in vitro model system.

**Figure 4: NOS Activation property of POCP. Results are the mean ± SD of three separate experiments, each in triplicate.**

**CONCLUSION**

The data recorded in the above experiments showed that the crude polysaccharide of P. ostreatus had a good antioxidant property and NOS activation power, which might be good sources for the development of antioxidant food additives. Carbohydrate component mostly β-glucan seemed to be responsible for the antioxidant activity as the extract contained little amount of phenolic substances and protein. So, the crude polysaccharide of P. ostreatus could be used as a possible food supplement or in pharmaceutical industry.

**REFERENCE**

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37. He JZ, Ru QM, Dong DD, Sun PL. Chemical characteristics and antioxidant properties of crude water solubles polysaccharides from four common edible mushrooms. Molecules 2012; 17: 4373-87.