ORIGINAL ARTICLE

Antioxidant and nitric oxide synthase activation properties of water soluble polysaccharides from *Pleurotus florida*

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Context: Cellular damage caused by reactive oxygen species has been implicated in several diseases, and, at the same time, nitric oxide is recognized as an important messenger molecule for several pathophysiological conditions. Hence, a novel antioxidant and nitric oxide synthase (NOS) activator from natural sources have significant importance in human health. **Aims:** The present study was conducted to evaluate the free radical-scavenging activity and NOS activation properties of water-soluble crude polysaccharide (Floridan) from *Pleurotus florida.* **Materials and Methods:** Crude polysaccharide was precipitated from hot water extract of *P. florida*, and their physicochemical parameters were determined. Then, α and β glucan were estimated using mushroom and yeast β glucan assay kit and Fourier transform infrared spectroscopy (FT-IR). Floridan was analyzed for their free radical scavenging activity in different test systems, namely hydroxyl and superoxide radical scavenging activity, ferrous ion chelating ability, determination of reducing power and inhibition of lipid peroxidation. Floridan was also tested for NOS activation using oxyhaemoglobin method. **Statistical Analysis:** The results were statistically analyzed using the Student's *t*-test. **Results:** Results showed that Floridan was rich in water-soluble β glucan with very low amount of protein and phenols. The EC_{50} for hydroxyl and superoxide radical-scavenging activity were 140 and 320 µg/ml, respectively, 450 µg/ml for chelating ability, 300 µg/ml for inhibition of lipid peroxidation and 2 mg/ml for reducing power. Floridan also increased nitric oxide production significantly. **Conclusions:** The present results revealed that this mushroom polysaccharide may be utilized as a promising dietary supplement to combat several killer diseases.

Key words: Mushroom, nitric oxide, physicochemical parameters, polysaccharide, reactive oxygen species

INTRODUCTION

Oxidation is essential for the production of energy; however, as a consequence, harmful reactive oxygen species (ROS) are also produced,[1] which are involved in the pathogenesis of various diseases.[2,3] An organism possesses only partially efficient antioxidant defence systems;[4] therefore, incorporating external antioxidant agents is essential.

Nitric oxide (NO), an important signalling molecule, is produced by NO synthase (NOS).[5,6] The cellular production of NO below physiological level causes initiation of different diseases.[7] Thus, NOS activation can act as a therapeutic agent.

Consumption of mushroom is gaining much interest in recent years for their nutritional and medicinal properties.[2,3,8-14] In parallel, mushroom polysaccharides are potentially useful biologically active ingredients for pharmaceutical uses.[15] Therefore, this paper studied extraction and chemical characterization of water-soluble polysaccharide from *Pleurotus florida* for antioxidant and NOS activation properties.

Access this article online	
Quick Response Code:	Website: www.greenpharmacy.info
	DOI: 10.4103/0973-8258.120190

MATERIALS AND METHODS

Collection of Mushroom

P. florida was purchased from the mushroom cultivation unit of Narendrapur Ramakrishna Mission Ashrama, Narendrapur, West Bengal, India.

Chemicals

Ferric chloride, L-methionine, nitroblue tetrazolium (NBT), riboflavin, 2-deoxy-D-ribose, hydrogen peroxide (H_2O_2), ferrozine, potassium ferricyanide, trichloroacetic acid (TCA), thiobarbituric acid (TBA), L-arginine and standards such as ascorbic acid, ethylene diaminetetraacetic acid (EDTA), butylated hydroxytoluene (BHT), butylated hydroxylanisole (BHA), gallic acid and bovine serum albumin (BSA) were purchased from Sigma chemicals Co. (St. Louis, MO, USA). An analytical mushroom β-glucan kit was obtained from Megazyme

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Received: 19-03-2013; **Accepted:** 18-06-2013

Institute. (Wicklow, Ireland). All other chemicals and reagents used were of analytical grade.

Extraction of Polysaccharide

Air dried and powdered fruit bodies of *P. florida* was extracted with ten volume of 99% ethanol at room temperature for 2 days to remove the alcohol-soluble materials such as coloured materials, phenolic compounds and lipid. After filtration, the residue was re-extracted with ethanol and filtered. The filtrate was air dried, suspended and refluxed with distilled water in boiling condition for 8 h. The extract was filtered through gauze, and the filtrate was concentrated to one-tenth of the volume with a rotary evaporator at 80°C under vacuum. Polysaccharide was precipitated by addition of four volume of absolute alcohol and left at 4°C overnight. After centrifugation, the pellets were washed with 70% (v/v) ethanol and then successively washed with ethyl acetate and acetone.[16,17] The washed pellets were dissolved in water and lyophilized to yield crude polysaccharide, "Floridan."

Physicochemical Properties of Polysaccharide Floridan

The total sugar content was measured by phenol sulphuric acid method at 490 nm using glucose as standard. The protein contained of protein-bound polysaccharide was determined by Bradford reagent. Presence of starchy polysaccharide was carried out by iodine reaction. Total phenolic compounds present in the crude polysaccharide were determined using Folin–Ciocalteau reagent, where gallic acid was used as a standard. All values were expressed as gram of standard equivalents per 100 gram of crude dry polysaccharide.[18]

Contents of total and α-glucan of Floridan were determined using the mushroom and yeast β-glucan assay kit (Megazyme Int.). The kit contains exo-1, 3-β-glucanase, β-glucosidase, amyloglucosidase, invertase, glucose determination reagent and glucose standard solution. α-Glucan, β-glucan and total glucan were estimated as per the kit's manual. All values of glucan contained were expressed as gram of glucose equivalents per 100 gram of crude dry polysaccharide.

The FT-IR spectra were recorded on the Perkin Elmer Precirety Spectrum 100 Model. The crude polysaccharide were ground with potassium bromide powder and then pressed into pellets for FT-IR measurements in the frequency range 400-4000 cm^{-1}.

Evaluation of Free Radical-scavenging Activity

Hydroxyl radical scavenging assay

Scavenging activity for hydroxyl radical was done according to Halliwell *et al*.[19] The reaction mixture (1 ml) consisted of KH_2PO_4–KOH buffer (20 mM, pH 7.4), 2-deoxy-D-ribose (2.8 mM), variable concentration (0.05-0.2 mg/ml) of Floridan, $FeCl_3$ (100 mM), EDTA (104 μM), ascorbate (100 μM) and H_2O_2 (1 mM). It was incubated at 37°C for 1 h. Then, 2 ml TBA and TCA solution (TBA–TCA reagent: 0.375% w/v TBA; 15% w/v TCA and 0.25 (N) HCl) was added and incubated in a boiling water bath for 15 min. After cooling, absorbance was measured at 535 nm. EC_{50} value of deoxyribose degradation by the Floridan over the control was measured. BHA was used as a positive control.

Superoxide radical scavenging assay

The method used by Martinez *et al*.,[20] for determination of the superoxide dismutase was followed with modification in the riboflavin-light-NBT system. Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, 75 μM NBT and solution of various concentrations of Floridan (0.1-0.5 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 reaction mixture were kept in dark and served as blanks. BHA was used as control.

Chelating ability of ferrous ions

Chelating ability was determined according to the method of Dinis *et al*.[21] Different concentration of Floridan (0.1-0.5 mg/ml) in water was 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 at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. EC_{50} value is the effective concentration at which ferrous ions were chelated by 50% and was obtained by interpolation from linear regression analysis. EDTA was used as a standard. The percentage of inhibition of ferrozine–Fe^{2+} complex formation is given by this formula:

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

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

Determination of reducing power

The reducing power of the polysaccharide was determined according to the method of Oyaizu.[22] Various concentrations of Floridan (0.5-2 mg/ml) were 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 TCA (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. Ascorbic acid was used as a standard.

Inhibition of lipid peroxidation

Lipid peroxidation was induced by Fe^{2+}-ascorbate system in human red blood cells (RBC) and estimated as TBA-reacting substances (TBARS) by the method of Buege and Aust.[23] The reaction mixture (1 ml) contained RBC-packed cell (10^8 cells/ml) in Tris-HCl buffer (20 mM, pH 7), $CuCl_2$ (2 mM), ascorbic acid (10 mM) and various concentrations of Floridan (0.1-1 mg/ml). The reaction mixture was incubated at 37°C for 1 h. Lipid peroxidation was measured as malondialdehyde (MDA) equivalent using TBA–TCA solution. The incubated reaction mixture was mixed with 2 ml of TBA–TCA reagent and heated in a boiling water bath for 15 min. After cooling, the flocculent precipitate was removed by centrifugation at 10,000 g for 5 min. Finally, MDA concentration in the supernatant fraction was determined spectrophotometrically at 535 nm. BHT was used as a control.

Determination of NOS activity

NO was determined according to the method described by Jia *et al*.[24] Typically, NO content was determined by the conversion of oxyhaemoglobin to methaemoglobin. The reaction mixture (2.5 ml) containing RBC (10^8 cells) was incubated with L-arginine (10 µM), haemoglobin (30 µM) with 100 µg of Floridan 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

The results were subjected to statistical analysis using Student's *t* test. Values are mean ± SD of three replications.

RESULTS

Physicochemical Characterization of Floridan

Floridan appeared to be a white powder, which is highly soluble in water. The chemical composition of Floridan is summarized in Table 1. Total carbohydrate and protein content of Floridan was 68% and 4%, respectively. Very negligible amount of phenolics was detected from this crude polysaccharide. Iodine reaction showed negative result, demonstrating that there was no starch in the polysaccharide. Total glucan content was 11.79 gram/100 gram of polysaccharide. The α-glucan and β-glucan content were 1.42 gram and 10.37 gram/100 gram of polysaccharide, respectively. In most mushrooms, α glucan was found at levels <1%, whereas β glucan ranges from 4.71 to 46.2% on a dry weight basis.[17]

In order to investigate the functional groups of Floridan, the FT-IR spectra [Figure 1] showed a typical carbohydrate pattern. A strong band near the region of 3,400 cm^{-1} was characteristic of carbohydrate ring that indicated the presence of OH stretching in hydrogen bonds, revealing strong inter- and intra-molecular interaction of the polysaccharide chains.[25] Absorption at 2922 cm^{-1} indicated CH_2 stretching. The band at ca. 1148 cm^{-1} gave evidence of C-O-C stretching. The band between 1310 and 1410 cm^{-1}, i.e., 1372 cm^{-1} corresponds to OH group of phenolic compound. Peak of amide band at 1651, 1529 and 1417 cm^{-1} indicated the presence of some residual protein in the crude polysaccharide mixture. Absorption region at 1078 cm^{-1} was characteristic of the presence of β glucan due to O-substituted glucose residue.[17] The absorption peak at 914 and 871 cm^{-1} revealed the co-existence of α and β glucosidic bond.[26]

Evaluation of ROS-scavenging Activity

In our experiment, hydroxyl radicals ($OH^{\cdot}$) were generated from Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fenton's reaction), which attacked the deoxyribose and set off a series of reactions that eventually resulted in the formation of MDA, measured as a pink MDA–TBA chromogen at 535 nm. When test sample was added to the reaction mixture, they removed hydroxyl radicals and prevented sugar degradation. The Floridan showed potent hydroxyl radical-scavenging activity, which rose gradually with the increase of concentration [Figure 2]. The EC_{50} value of Floridan was found to be 140 µg/ml.

The method used by Martinez *et al*.,[20] is based on the generation of superoxide radical by auto-oxidation of

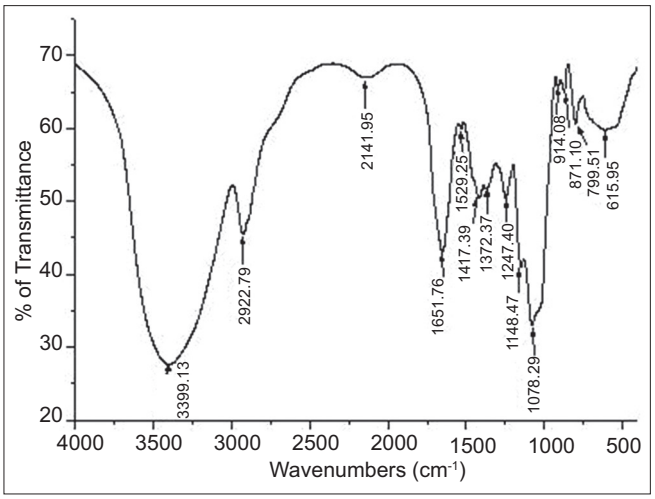

Figure 1: FT-IR spectra of Floridan

Table 1: Contents of total polysaccharides, protein, phenolics, total glucan, α glucan and β glucan of Floridan

Total polysaccharide (g/100 g)	Total protein (g/100 g)	Total phenol (g/100 g)	Glucan content (g/100 g)		
			Total glucan	α glucan	ß glucan
68±2	4±0.5	0.007±0.001	11.79±0.83	1.42±0.17	10.37±0.46

riboflavin in the presence of light, which in turn reduces yellow dye NBT to produce blue formazon. Intensity of colour is directly proportional to the concentration of superoxide anion. The superoxide radical-scavenging activity of Floridan was expressed as EC_{50} value, as presented in Figure 3. The result showed that the radical-scavenging activity of the polysaccharide increased with increasing concentration. The EC_{50} value of Floridan was 320 µg/ml, whereas the EC_{50} value of the standard BHA was 30 µg/ml.

Ferrozine quantitatively forms complexes with Fe^{2+}. In the presence of chelating agent, the complex formation is disrupted, resulting in the reduction of red colour. Reduction therefore allows estimation of the chelating ability of the coexisting chelator. The range and the mean of Fe^{2+} chelating capacity of Floridan showed a marked ability for iron binding [Figure 4]. EC_{50} for the extract revealed a value of 0.45 mg/ml. The concentration needed for the synthetic metal chelator (EDTA) was 0.04 mg/ml to obtain the same EC_{50}.

In determination of reducing power, the antioxidant compounds converted the oxidation form of iron (Fe^{+3}) in ferric chloride to ferrous (Fe^{+2}). Therefore, the yellow colour of the test solution changed from green to blue, as the reducing power of sample increased. Reducing power of Floridan increased readily along the increased concentrations [Figure 5]. EC_{50} value in reducing power was 2 mg/ml for the extract as compared to that for the standard ascorbic acid (0.08 mg/ml).

A free radical prefers to steal electron from lipid membrane of cell, initiating free radical attack and induces lipid peroxidation in polyunsaturated lipid rich areas such as brain and liver. The EC_{50} value of Floridan in inhibition of lipid peroxidation activity was 0.3 mg/ml as compared to standard BHT (0.20 mg/ml). The present result suggested that Floridan had strong inhibition effects of lipid peroxidation [Figure 6].

NOS Activation Property

NO is recognized to be the intra- and inter-cellular mediator of several cell functions. Further study was made

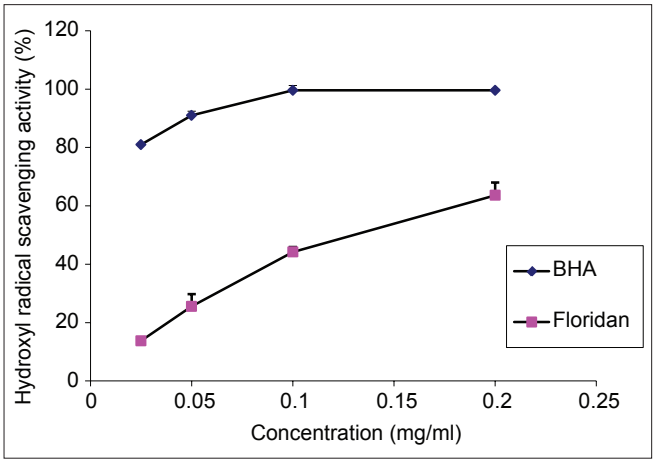

Figure 2: OH· radical-scavenging activity of Floridan. Results are the mean ± SD of three separate experiments, each in triplicate

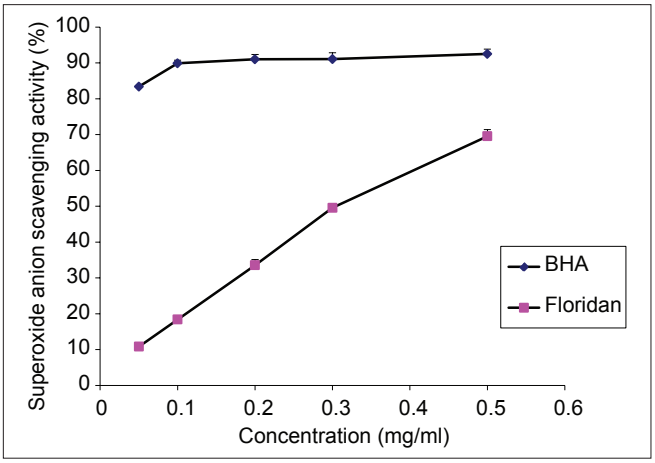

Figure 3: Superoxide radical scavenging activity of Floridan. Results are the mean ± SD of three separate experiments, each in triplicate

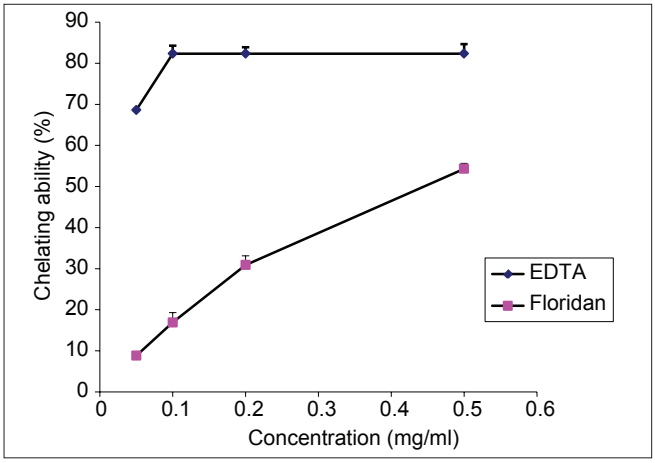

Figure 4: Ferrous ion-chelating ability of Floridan. Results are the mean ± SD of three separate experiments, each in triplicate

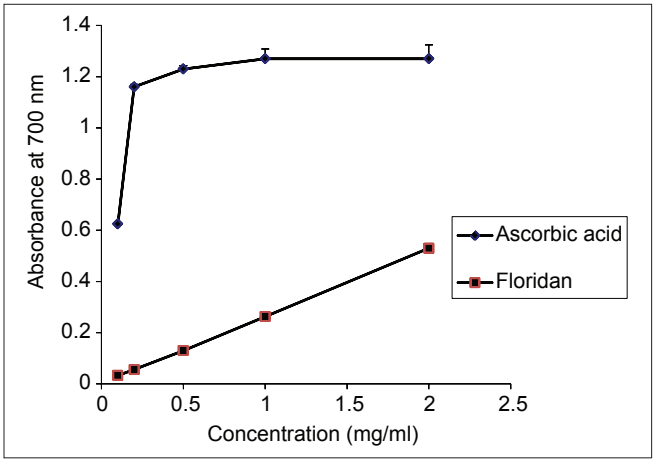

Figure 5: Reducing power of Floridan. Results are the mean ± SD of three separate experiments, each in triplicate

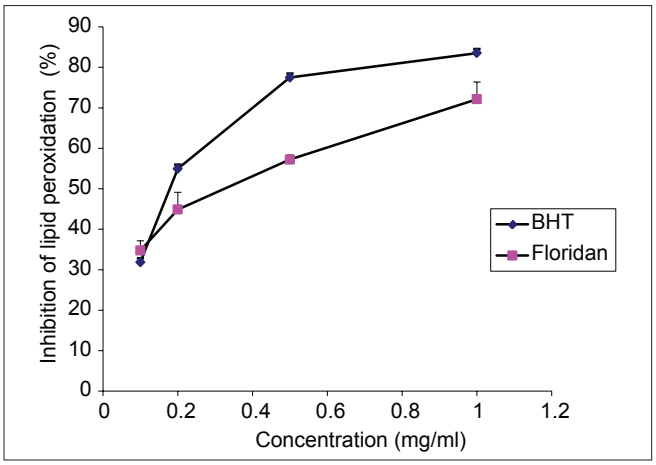

Figure 6: Inhibition of lipid peroxidation by Floridan. Results are the mean ± SD of three separate experiments, each in triplicate

to evaluate NOS activation properties of Floridan. The result showed a significant increase in NO production, i.e., 1.05 ± 0.18 nmol/mg Floridan/h over control (n = 3). Use of 10 µm N^G-methyl-L-arginine acetate ester (NAME), a competitive inhibitor of NOS, in the reaction mixture showed complete inhibition of NO production, which is indicated by the increase in production of NO due to activation of NOS.

DISCUSSION

In the presence study, water-soluble crude polysaccharide from fruiting bodies of *P. florida* was successfully isolated and its physicochemical parameters were documented. Measurement of antioxidant properties of the polysaccharide extract showed relatively high antioxidant and NO syntheses activation properties.

Hydroxyl radicals are formed by electron transfer from transition metals to H_2O_2 and interact immediately after formation. It can damage DNA by attacking purines, pyrimidines and deoxyribose. Therefore, removal of hydroxyl radicals is important for the protection of living systems. The results showed that Floridan was a good scavenger of hydroxyl radicals, which may be due to the active hydrogen-donating ability of polysaccharides. The EC_{50} value for hydroxyl radical-scavenging ability of Floridan was lower than that of the polysaccharide from another common edible mushroom, *Auricularia auricula*, which was 510 µg/ml.[27] However, polysaccharides of *Pholiota adipose* showed 50% scavenging activity at 0.042 mg/ml, which is much lower than that of Floridan.[28]

Superoxide anion is one of the six major reactive oxygen species that causes oxidative damage in the human body. It is considered as a primary ROS, as it is a relatively weak oxidant, but it can generate secondary ROS such as peroxynitrite (ONOO), peroxyl radicle (LOO), singlet oxygen, hydroxyl radical and hydrogen peroxide.[29] Therefore, superoxide radical-scavenging ability is of great importance to potential antioxidant activity. The EC_{50} of Floridan was found to be 320 µg/ml. In a related work, He *et al*., reported that the EC_{50} value of crude polysaccharide isolated from *Agaricus bisporus* was as 1.17 mg/ml.[30] A heteroglycan of *Pleurotus ostreatus* showed EC_{50} value of 553 µg/ml.[31]

Some transition metals, e.g., Fe^{2+}, Cu^{+}, Pb^{2+} and Co^{2+}, can trigger process of free radical reaction such as hydroxyl radical formation.[29] At 0.05-0.5 mg/ml, the chelating ability of Floridan was between 8.82% and 54.3%. At the same concentration range, the chelating effect of EDTA was between 68.62% and 82.35%. Ker *et al*., reported that the chelating ability of polysaccharide depends on the concentration of available hydroxyl group as well as the mean molecular mass of the polysaccharide.[32]

Reducing capacity of compounds could serve as an indicator of potential antioxidant properties. High potential in hydrogen-donating ability could react with free radicals to convert them to more stable products and thereby terminate radical chain reactions. Yang *et al*., reported that oyster mushrooms had a higher reducing power than Shiitake and golden mushrooms.[33] The EC_{50} value of Floridan was 2 mg/ml, which is higher than that of crude polysaccharide isolated from *Ganoderma applanatum* and *G. lucidum*, as they showed EC_{50} value <0.2 mg/ml. Kozarski *et al*., correlated the decrease of EC_{50} values with higher phenol and α glucan content as well as increase of EC_{50} with higher protein and polysaccharides contents of the extract.[18]

Lipid peroxidation is a complex process involving the interaction of oxygen-derived free radicals with polyunsaturated acids, resulting in a variety of highly electrophilic aldehydes. This phenomenon occurs through an ongoing free radical chain reaction until termination occurs. Free radicals attack an allylic carbon to form a carbon-centred radical. This radical reacts with O_2 to produce peroxyl radicals (O_2^-). These peroxyl radicals can react with adjacent lipids, forming a lipid hydroperoxide, repeating the cycle. Result showed that administration of Floridan decreased lipid peroxidation. Floridan has much higher inhibitory effect than three separate pure polysaccharides isolated form *Penicillium* sp. F23-2, whose EC_{50} value were >1 mg/ml.[34]

NO acts as a signal molecule in immune, nervous and vascular systems. Our findings suggested that Floridan is a potent activator of NOS. Similar observations were made earlier with hot water extract of *Auricularia auricula*, *Ganoderma applanatum*, *Astreus hygrometricus*, *Macrocybe*

giganteum and *Ramaria botrytis*. All were capable of inducing NOS in *in vitro* model systems.[35]

CONCLUSION

All the accumulated data suggested that crude polysaccharide extracts of *P. florida* act as a natural antioxidant and NOS activator, which may be a good source for the development of antioxidant food additives. Further investigations are necessary to verify these activities *in vivo*, which may ultimately lead to an inclusion of this medicinal mushroom in different pharmaceutical formulations.

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How to cite this article: Saha S, Khatua S, Paloi S, Acharya K. Antioxidant and nitric oxide synthase activation properties of water soluble polysaccharides from *Pleurotus florida*. Int J Green Pharm 2013;7:182-8.

Source of Support: Nil, **Conflict of Interest:** None declared.