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Antioxidant and immunostimulant β-glucan from edible mushroom *Russula albonigra* (Krombh.) Fr.

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ABSTRACT

A water soluble β-glucan (PS) with an average molecular weight ~1.95×10^5 Da was isolated from the alkaline extract of ectomycorrhizal edible mushroom, *Russula albonigra* (Krombh.) Fr. and found to consist of terminal, (1 → 3)-, (1 → 6)-, and (1 → 3,6)-linked β-D-glucopyranosyl moieties in a ratio of nearly 1:2:2:1. The structure of this PS was elucidated on the basis of total hydrolysis, methylation analysis, Smith degradation, partial hydrolysis, and 1D/2D NMR experiments. On the basis of these experiments, the repeating unit of the PS was found to contain a backbone of three (1 → 6)-β-D-glucopyranosyl residues, one of which was branched at *O*-3 position with the side chain consisting of two (1 → 3)-β-D-glucopyranosyl and a terminal β-D-glucopyranosyl residue. This PS showed *in vitro* macrophage activation by NO production as well as splenocytes and thymocytes proliferation. Moreover, it also exhibited potent antioxidant activities.

1. Introduction

Mushroom polysaccharides, especially, β-glucans are recognized as biological response modifier (BRM) and used for the treatment of cancer and various infectious diseases both in modern medicine and traditional chemotherapeutic drug (Chan, Chan, & Sze, 2009; Kidd, 2000; Wasser, 2002). They have drawn the attention of chemists and immunobiologists for their immunomodulatory, anti-tumor (Wasser, 2002) as well as antioxidant activities (Kozarski et al., 2011). Reactive oxygen species (ROS) which damage lipids, proteins, carbohydrates, and nucleic acids (Blokhina, Virolainen & Fagerstedt, 2003) are the roots of developing diseases like cancer, Alzheimer, and Parkinson (Papas, 1999). β-Glucans from mushrooms are well-known antioxidant material (Kofuji et al., 2012) which can neutralize the harsh effect of ROS (Papas, 1999). Modern research has been focused on utilizing the naturally occurring substances to neutralize the radical activities. Several linear (1 → 3) and branched (1 → 3)-, (1 → 6)-β-D-glucans (Ohno et al., 1993; Yoshioka, Tabeta, Saito, Uehara, & Fukuoka, 1985) are used as immunostimulating and antitumor materials. Some immunostimulating water soluble β-D-glucans (Maity et al., 2013; Sen et al., 2013) have been also reported by our group.

Russula albonigra (Krombh.) Fr., an ectomycorrhizal, edible and non-toxic fungus (Nandi et al., 2012) contained two water-soluble polysaccharides, α,β-glucan and a heteroglycan (Nandi et al., 2012, 2013) whereas alkali treated aqueous extract contained only one polysaccharide which was characterized as a β-glucan. The detailed structural investigation and study of immunostimulation as well as antioxidant activities of this β-glucan were carried out and reported herein.

2. Materials and methods

2.1. Isolation and purification of the polysaccharide

Fresh fruit bodies of mushroom *R. albonigra* (Krombh.) Fr. (500 g) were gently washed with distilled water and then boiled with 4% NaOH solution for 1 h. The alkaline extract was kept overnight at 4 °C and filtered through linen cloth. The crude polysaccharide was isolated and purified by the method described earlier (Maity et al., 2013; Sen et al., 2013) and it (30 mg) was purified by gel permeation chromatography on column of Sepharose 6B using water as eluant by Redifrac fraction collector. A single homogeneous fraction (test tube 20–32, Fig. 1a) was collected, and freeze-dried, yielding 20 mg of pure PS. The same procedure was repeated in several lots to yield

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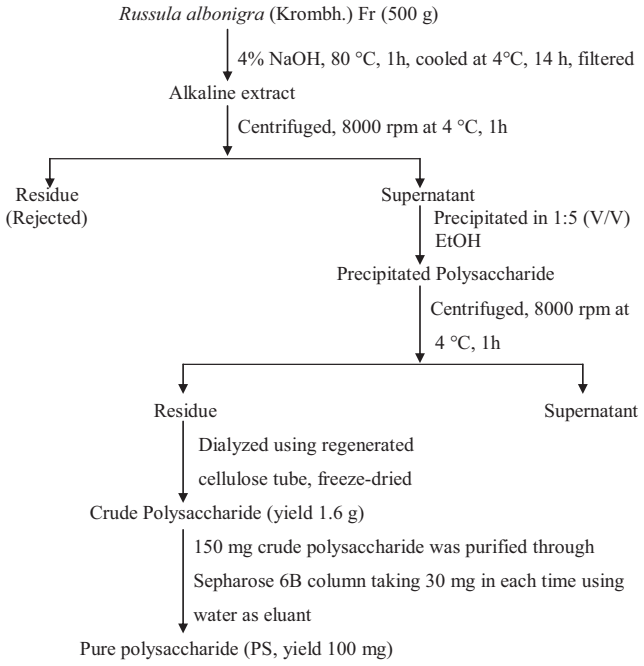
100 mg of pure polysaccharide. Isolation and purification steps are shown in the following diagram.

❖ **Flow diagram of isolation and purification of the polysaccharide**



2.2. Determination of molecular weight

The molecular weight of the polysaccharide was determined by gel-permeation chromatography. Standard dextrans (Hara, Kiho, Tanaka, & Ukai, 1982) T-200, T-70, and T-40 were passed through a Sepharose 6B column, and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of the polysaccharide was then plotted in the same graph, and molecular weight of polysaccharide was determined.

2.3. Absolute configuration of monosaccharides

The absolute sugar configuration was determined by the method of Gerwig, Kamerling, and Vliegenthart (1978). The polysaccharide (1.0 mg) was hydrolyzed with CF_3COOH, and then the acid was removed. A solution of 250 μL of 0.625 (M) HCl in R-(−)-2-butanol was added and heated at 80 °C for 16 h. Then the reactants were evaporated and TMS-derivatives were prepared with *N*,*O*-bis(trimethylsilyl)trifluroacetamide (BSTFA). The products were analyzed by GC using a capillary column SPB-1 (30 m × 0.26 mm), a temperature program (3 °C/min) from 150 to 210 °C. The 2,3,4,6-tetra-*O*-TMS-(+)-2-butylglycosides obtained were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides.

2.4. Optical rotation

Optical rotation was measured on a Jasco Polarimeter model P-1020 at 31 °C.

2.5. Constituent sugar, methylation, and periodate oxidation analysis

For sugar analysis, the PS (3.0 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) in a round-bottom flask at 100 °C for 18 h in a boiling water bath and the analysis was carried out as described in previous paper (Nandi et al., 2013). The PS (4 mg) was methylated according to the method of Ciucanu and Kerek method (1984) where distilled DMSO and finely grounded NaOH were used and then converted to alditol acetates as reported earlier (Nandi et al., 2013). Periodate oxidation experiment was carried out with this PS (5 mg) as described in the earlier report (Nandi et al., 2013). Alditol acetates of monosaccharides and the methyl sugar were analyzed by GC and GC–MS. A gas–liquid chromatography Hewlett-Packard 5730 A was used, having a flame ionization detector and glass columns (1.8 m × 6 mm) packed with 3% ECNSS-M (A) on Gas Chrom Q (100–120 mesh) and 1% OV-225 (B) on Gas Chrom Q (100–120 mesh). All GC analyzes were performed at 170 °C. Gas-liquid chromatography–mass spectrometric (GC–MS) analysis was performed on Shimadzu GC-MS Model QP-2010 Plus automatic system, using ZB-5MS capillary column (30 m × 0.25 mm). The program was isothermal at 150 °C; hold time 5 min, with a temperature gradient of 2 °C/min up to a final temperature of 200 °C.

2.6. Smith degradation

The polysaccharide (25 mg) was oxidized with 0.1 M sodium metaperiodate (20 mL) at 25 °C in the dark during 72 h. The oxidation was stopped by the addition of 1,2-ethanediol, and the solution was dialyzed against distilled H_2O. Thereafter, $NaBH_4$ was added and kept at room temperature for 15 h, with intermittent stirring. The mixture was neutralized with 50% AcOH and again dialyzed against distilled water, and freeze dried and was subjected to mild hydrolysis with 0.5 M TFA for 15 h at 25 °C. TFA was removed by repeated evaporation of water at 37 °C. Finally, it was purified by passing through a Sephadex G-25 column. A part of this material was subjected to methylation analysis and the remainder was used for ^{13}C NMR studies.

2.7. Partial acid hydrolysis

The polysaccharide (30 mg) dissolved in 0.1 M TFA (6 mL) was hydrolyzed at 100 °C for 1 h. Acid was removed by repeated evaporation of water at 37 °C. The residue was dissolved in water (4 mL), to which three volumes of ethanol were added. The precipitate was washed with ethanol and then freeze-dried (F1), was used for methylation and ^{13}C NMR analysis. The supernatant was dried by evaporation, and residue was dissolved in water, and reduced with $NaBH_4$ at 25 °C for 2 h. After neutralization with 1 M AcOH, it was desalted by passing through a Sephadex G-25 column. The carbohydrate containing eluate (F2) was collected, freeze-dried and subjected to methylation analysis.

2.8. NMR studies

The pure polysaccharide (PS) was kept over P_2O_5 under vacuum for several days, and then exchanged with deuterium by lyophilizing with D_2O (99.96% atom ^2H, Aldrich) for four times. Samples were dissolved in D_2O and NMR spectra were recorded on a Bruker Avance DPX-500 spectrometer at 30 °C. The ^1H and ^{13}C (both ^1H coupled and decoupled) NMR spectra were recorded at 30 °C. The ^1H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.70) using the WEFT pulse sequence. The 2D-DQF-COSY experiment was carried out using standard BRUKER software. The TOCSY experiment was recorded at mixing time of 150 ms, and complete assignment required several TOCSY experiments having mixing times ranging from 60 to 300 ms. The NOESY and ROESY mixing delay was 300 ms whereas the delay time in the HMBC experiment was 80 ms. The ^{13}C chemical shifts were measured using acetone as internal standard, fixing the methyl carbon signal at δ 31.05.

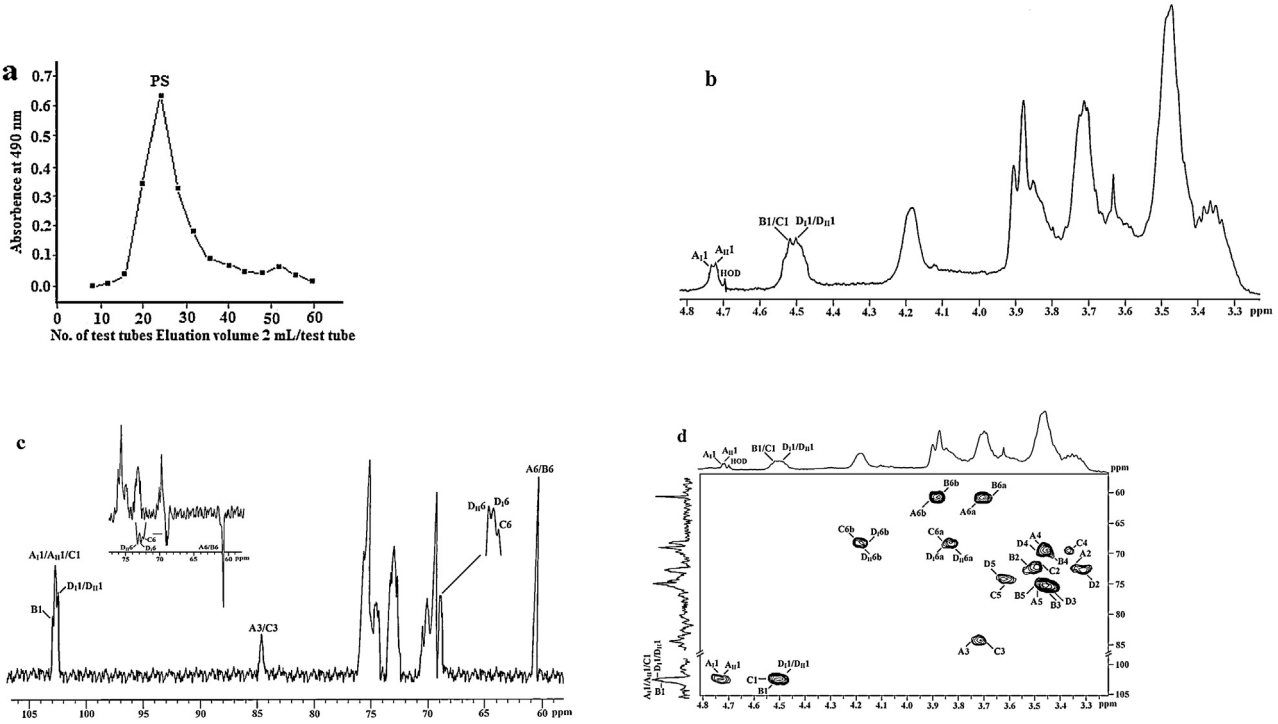


Fig. 1. (a) Gel permeation chromatogram, (b) ^{1}H NMR spectrum (500 MHz, D_2O, 30 °C), (c) combination of ^{13}C NMR and DEPT-135 spectrum (125 MHz, D_2O, 30 °C) and (d) part of HSQC spectrum (D_2O, 30 °C) of the β-glucan (PS) isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr.

2.9. Immunostimulating properties

2.9.1. Test for macrophage by nitric oxide (NO) assay

RAW 264.7, a murine macrophage cell line obtained from National Centre for Cell Sciences (NCCS), Pune, India, was grown in Dulbecco's modified Eagle's medium (DMEM) and seeded in 96 well flat bottom tissue culture plate at 5×10^5 cells/mL concentration (180 μL). Cells were left overnight for the attachment and then PS were treated with different concentrations (12.5, 25, 50, 100, and 200 μg/mL) to the wells. After 48 h of treatment culture supernatant of each well were collected and NO production was estimated using Griess Reagent (Green et al., 1982) at 540 nm (1:1 of 0.1% in 1-napthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid). Lipopolysaccharide (LPS), L6511 of *Salmonella enterica* serovar Typhimurium (Sigma, St. Louis, USA) was used as positive control (Nandi et al., 2012, 2013).

2.9.2. Splenocyte and thymocyte proliferation assay

A single cell suspension of spleen and thymus was prepared from Swiss Albino mice under aseptic conditions by homogenization in Hank's balanced salt solution (HBSS). The suspension was centrifuged to obtain cell pellet. The contaminating RBC was removed by hemolytic Gey's solution. After washing two times in HBSS the cells were resuspended in complete RPMI (Roswell Park Memorial Institute) with serum and antibiotics added. RPMI and fetal bovine serum (FBS) has been obtained from Gibco whereas antibiotics were obtained from Himedia. Cell concentration was adjusted to 1×10^6 cells/mL and viability of splenocytes and thymocytes (as tested by trypan blue dye exclusion) was always over 90%. The cells (180 μL) were plated in 96 well flat bottom tissue culture plates and incubated with 20 μL of various concentrations of the PS (12.5, 25, 50, 100, and 200 μg/mL). PBS (10 mM, Phosphate Buffer Saline, pH-7.4) was taken as negative control whereas LPS (4 μg/mL, Sigma) and Concavalin A (Con A, 10 μg/mL, Himedia) served as positive controls. All cultures were set up in triplicate for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. Proliferation of splenocytes indicated as Splenocyte Proliferation Index (SPI) and Thymocytes written as Thymocyte Proliferation Index (TPI) were checked by standard MTT assay method (Ohno et al., 1993). The data are reported as the mean ± standard deviation of different observations and compared against PBS control.

2.10. Antioxidant properties

2.10.1. Hydroxy radical scavenging activity

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 (100–400 μg/mL) of PS, $FeCl_3$ (100 mM), EDTA (104 μM), ascorbate (100 μM) and H_2O_2 (1 mM). It was incubated at 37 °C for 1 h. 2 mL thiobarbituric acid (TBA) and trichloroacetic acid (TCA) solution (100 mL contained 375 mg TBA, 15 mg TCA; 2 mL concentrated HCL added to 98 ml of TBA–TCA solution) was added and incubated at boiling water bath for 15 min. After cooling, absorbance was measured at 535 nm (Acharya, Chatterjee, & Ghosh, 2011). Butylated hydroxytoluene (BHT) was used as positive control.

2.10.2. Superoxide radical scavenging activity

The method by Martinez, Marcelo, Marco, and Moacyr (2001) for determination of the superoxide anion was followed with modification in the riboflavin-light-nitrobluetetrazolium (NBT) system. Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, various concentrations (100–400 μg/mL) of PS, 100 μM EDTA, 75 μM NBT and 2 μM riboflavin. Reaction started by illuminating sample with light and the increased 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. Butylated hydroxyanisole (BHA) was used as a positive control.

2.10.3. Chelating ability of ferrous ions

Chelating ability was determined according to the method of Dinis, Madeira, and Almeida (1994). Reaction mixture (4 mL) contained different concentrations of PS (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–Fe^{2+} complex formation is given by this formula:

$$\%\,\text{inhibition} = \left\{\frac{A_0 - A_1}{A_0}\right\} \times 100.$$

where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of mushroom fractions.

2.10.4. Determination of reducing power

Reducing power was determined according to the method of Oyaizu (1986). Various concentrations of PS (200–600 μg/mL) were mixed with 2.5 mL sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%). The mixture was incubated for 20 min and then 2.5 mL of trichloroacetic acid (10%) was added. 2.5 mL of this solution was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1%) and incubated for 15 min. The absorbance was measured at 700 nm against buffer. Ascorbic acid was used as standard. IC_{50} value is the effective concentration at which the absorbance was 0.5 for reducing power.

2.10.5. β carotene bleaching assay

Inhibition of β carotene bleaching was determined according to the method of Dapkevicius, Venskutonis, Van Beek, and Linssen (1998). Reaction mixture consisted of 0.5 mg β carotene in 1 ml HPLC grade chloroform, 25 μL linoleic acid and 200 mg Tween 40. Chloroform was completely evaporated. Then 50 mL distilled water saturated with O_2 was added with vigorous shaking. Aliquots (4 mL) of this emulsion were transferred into different tubes containing different concentrations of PS (100–400 μg/mL) and absorbance was read at 490 nm against water. The tubes were placed at 50 °C for 2 h and again absorbance was taken. Butylated hydroxyanisole (BHA) was used as positive control.

3. Results and discussion

3.1. Isolation, purification and chemical analysis

The fresh fruit bodies of edible mushroom, *Russula albonigra* (Krombh.) Fr. (500 g) were washed with distilled water, boiled with 4% NaOH solution, filtered, centrifuged, and the supernatant was precipitated in EtOH. The precipitated materials on dialysis followed by freeze drying yielded 1.6 g of crude polysaccharide. The water soluble crude polysaccharide on fractionation through Sepharose 6B column yielded one homogeneous fraction (PS). The molecular weight (Hara et al., 1982) of PS was estimated as $\sim 1.95 \times 10^5$ Da from a calibration curve prepared with standard dextrans. GC analysis of the alditol acetates of this polysaccharide revealed the presence of glucose only. The PS showed specific rotation $[\alpha]_D^{31}$ −19.5 (*c* 0.1, water). The negative optical rotation indicated that the glucosyl residues had β-anomeric configuration (Dong, Yao, Yang, & Fang, 2002). The absolute configuration of the monosaccharide present in the glucan was determined by the method of Gerwig et al. (1978) and it was found that glucose had D-configuration. The polysaccharide was methylated according to the method of Ciucanu and Kerek (1984) followed by hydrolysis and then converted to alditol acetate. The GC–MS analysis of partially methylated alditol acetates revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, 1,5,6-tri-*O*-acetyl-2,3,4,-tri-*O*-methyl-D-glucitol, 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a ratio of nearly 1:2:2:1, respectively. These results indicate the presence of nonreducing end, (1 → 3)-, (1 → 6)-, and (1 → 3,6)-linked D-glucopyranosyl residues in the β-glucan. According to this result, any of the three types of repeating unit is possible for this glucan: a (1 → 6)-linked backbone, a (1 → 3)-linked backbone or an alternatively (1 → 3)-, (1 → 6)-linked backbone. Therefore, periodate oxidation and mild hydrolysis were performed for determination of the backbone present in the polysaccharide. The GC analysis of the alditol acetates of the periodate-oxidised (Goldstein, Hay, Lewis, & Smith, 1965), reduced PS showed the presence of D-glucose along with glycerol and periodate-oxidised, reduced, methylated PS showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a ratio of nearly 2:1. Mild hydrolysis was carried out with the periodate-oxidised, reduced PS to get Smith degradation product (SDPS). The GC analysis of the alditol acetates of Smith degraded hydrolyzed product showed the presence of D-glucose and D-glycerol. The GC–MS analysis of the of methylated SDPS revealed the presence of 1,5-di-*O*-acetyl 2,3,4,6-tetra-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl 2,4,6-tri-*O*-methyl-D-glucitol in a ratio of nearly 1:2. Partial hydrolysis (Dong et al., 2002) of the β-glucan was carried out with 0.1 M TFA to know the backbone sequence of the β-glucan in the repeating unit. As a result of this hydrolysis, two fractions were obtained, *i.e.* partially hydrolysed polysaccharide (F1) and partially hydrolysed oligosaccharide (F2). The methylation analysis of F1 revealed the presence of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol only indicating the presence of (1 → 6)-linked backbone of the PS and F2 revealed the presence of (1 → 3)-linked, and terminal glucopyranosyl moieties present as oligosaccharide side chain. All the above chemical investigation proved that the repeating unit of the PS had a backbone consisting of three (1 → 6)-β-D-glucopyranosyl residues, one of which was branched at *O*-3 position with the side chain consisting of two (1 → 3)-β-D-glucopyranosyl and a terminal β-D-glucopyranosyl residue.

3.2. NMR and structural analysis of β-glucan (PS)

The ^{1}H NMR (500 MHz) spectrum (Fig. 1b and Table 1) at 30 °C showed four signals in the anomeric region at δ 4.74, 4.72, 4.52, and 4.50 in a ratio of nearly 1:1:2:2. They were designated as residues **A_I**, **A_{II}**, **B**, **C**, **D_I**, and **D_{II}** according to their decreasing proton chemical shifts. In the ^{13}C (125 MHz) spectrum (Fig. 1c and Table 1) at 30 °C three anomeric signals appeared at δ 103, 102.7, and 102.5 in a ratio of nearly 1:3:2. Based on the result of the HSQC experiment (Fig. 1d and Table 1), the anomeric carbon signal at δ 103.0 corresponded to **B**, whereas the signal at δ 102.7 corresponded to **A_I**, **A_{II}**, and **C** and the peak at δ 102.5 was correlated to **D_I** and **D_{II}** residues of the anomeric proton signals, respectively. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY, HSQC experiments. Coupling constants were measured from DQF-COSY spectrum. The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values of 8–10 Hz in residues **A**–**D** support the presence of the glucopyranosyl configuration in the polysaccharide. Residues **A**–**D** were established as β-anomers from the coupling constant values $J_{H\text{-}1,H\text{-}2} \sim 8$ Hz, and $J_{C\text{-}1,H\text{-}1} \sim 160$ Hz. In residues **A** (**A_I** and **A_{II}**), the downfield shift of C-3 (δ 84.5) with respect to standard value of methyl glycosides (Agrawal, 1992) indicated that they were (1 → 3)-linked β-D-Glc*p*. All the chemical shifts of residue **B** were nearly analogous with the standard values of methyl glycoside (Agrawal, 1992) of β-D-glucose. This observation clearly indicated that the residue **B** was non-reducing end β-D-Glc*p*. In residue **C**, the chemical shift values of C-3 (δ 84.5) and C-6 (δ 68.7) showed downfield shifts,

Table 1
^{1}H[a] and ^{13}C[b] NMR chemical shifts (δ in ppm) of PS from *Russula albonigra* (Krombh.) Fr. in D_2O.

Glucosyl residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,H-6b/C-6
→ 3)-β-D-Glc*p*-(1 →	4.74[d], 4.72[e]	3.35	3.73	3.46	3.47	3.72[c], 3.90[c]
A (**A**$_{I}$, **A**$_{II}$)	102.7	72.8	84.5	69.6	75.0	60.7
β-D-Glc*p*-(1 →	4.52	3.50	3.46	3.44	3.48	3.70[c], 3.88[c]
B	103.0	73.0	75.9	70.2	75.0	60.7
→ 3,6)-β-D-Glc*p*-(1 →	4.52	3.50	3.71	3.37	3.63	3.85[c], 4.19[c]
C	102.7	72.8	84.5	69.6	74.5	68.7
→ 6)-β-D-Glc*p*-(1 →	4.50	3.30	3.45	3.46	3.63	3.84[c,f], 4.17[c,f]
D	102.5	73.0	75.9	70.0	74.5	68.8[f]
						3.83[c,g], 4.18[c,g]
						69.0[g]

[a] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.70 at 30 °C.
[b] Values of the ^{13}C chemical shifts were recorded with reference to acetone as the internal standard and fixed at δ 31.05 at 30 °C.
[c] Interchangeable.
[d] For residue **A**$_{I}$.
[e] For residue **A**$_{II}$.
[f] For residue **D**$_{I}$.
[g] For residue **D**$_{II}$.

indicating the presence of (1 → 3,6)-linked β-D-Glc*p*. Two **D** residues (**D**$_{I}$ and **D**$_{II}$) were same in all chemical shift values except the values of C-6. The different downfield shifts of C-6 (δ 68.8 and 69.0) of two **D** residues supported the presence of (1 → 6)-linking in β-D-Glc*p* with different chemical environments. Among two **D** residues, one residue (**D**$_{II}$) was glycosidically attached to the rigid part (**C**) and other residue (**D**$_{I}$) was away from it. Between **D**$_{I}$ and **D**$_{II}$, C-6 of **D**$_{II}$ appeared slightly downfield in comparison to **D**$_{I}$ residue due to the neighboring effect (Maity et al., 2013; Yoshioka, Tabeta, Saito, Uehara, & Fukuoka, 1985) of rigid part **C** of the backbone. Consequently, the C-6 value of the rigid residue **C** also resonated at fairly upfield compared to the C-6 of the **D**$_{I}$ and **D**$_{II}$ for the same reason. The linking at C-6 of the residues **C** and **D** were further confirmed from DEPT-135 spectrum (Fig. 1c).

The sequences of glucosyl moieties were determined from NOESY (Fig. 2a) as well as ROESY experiments. A long range HMBC (Fig. 2b) experiment was carried out to confirm the NOESY connectivities. From both NOESY and HMBC experiment, the inter-residual contacts along with some intra-residual contacts were observed (Fig. 2c and Table 2). Thus, the HMBC and NOESY connectivities confirmed the repeating unit in the PS.

For further confirmation of the sequence of linkages in PS, the Smith degraded material (SDPS) was prepared and NMR experiment was carried out. The ^{13}C NMR (125 MHz) spectrum (Fig. 3 and Table 3) at 30 °C of SDPS showed two anomeric carbon signals

Table 2
NOESY and HMBC connectivities of PS from *Russula albonigra* (Krombh.) Fr. in D_2O at 30 °C.

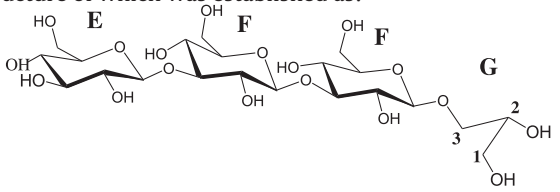
NOESY connectivities	HMBC connectivities
A$_{I}$H-1/**C**H-3	**A**$_{I}$H-1/**C**C-3
A$_{I}$H-1/**A**$_{I}$H-3	**A**$_{I}$C-1/**C**H-3
A$_{I}$H-1/**A**$_{I}$H-5	**A**$_{II}$H-1/**A**$_{I}$C-3
A$_{II}$H-1/**A**$_{I}$H-3	**A**C-1/**A**H-3
A$_{II}$H-1/**A**$_{II}$H-3	**A**C-1/**A**H-2
A$_{II}$H-1/**A**$_{II}$H-5	
BH-1/**A**$_{II}$H-3	**B**H-1/**A**$_{II}$C-3
BH-1/**B**H-3	**B**C-1/**A**$_{II}$H-3
BH-1/**B**H-5	**B**C-1/**B**H-2
CH-1/**D**$_{II}$H-6a; **D**$_{II}$H-6b	**C**H-1/**D**$_{II}$C-6
CH-1/**C**H-3	**C**C-1/**D**$_{II}$H-6a; **D**$_{II}$H-6b
CH-1/**C**H-5	**C**C-1/**C**H-2
	CC-1/**C**H-3
D$_{I}$H-1/**C**H-6a; **C**H-6b	**D**$_{I}$H-1/**C**C-6
D$_{II}$H-1/**D**$_{I}$H-6a; **D**$_{I}$H-6b	**D**$_{I}$C-1/**C**H-6a; **C**H-6b
DH-1/**D**H-3	**D**$_{II}$H-1/**D**$_{I}$C-6
DH-1/**D**H-5	**D**$_{II}$C-1/**D**$_{I}$H-6a; **D**$_{I}$H-6b
	DC-1/**D**H-2

Table 3
^{13}C NMR[a] chemical shifts (δ in ppm) of Smith-degraded glycerol-containing trisaccharide of PS from *Russula albonigra* (Krombh.) Fr. in D_2O.

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p*-(1 → **E**	102.7	73.52	76.0	70.69	75.68	60.77
→ 3)-β-D-Glc*p*-(1 → **F**	102.5	73.22	84.20	69.70	75.68	60.77
Gro-(3 → **G**	68.10	72.0	62.56			

[a] The values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 at 30 °C.

at δ 102.5 and 102.7 in a ratio of nearly 2:1, corresponding to → 3)-β-D-Glc*p*-(1 → (**F**) and β-D-Glc*p*-(1 → (**E**) residues respectively. The carbon signals C-1, C-2, and C-3 of the glycerol moiety were assigned as δ 68.10, 72.0, and 62.56, respectively. The nonreducing β-D-Glc*p*-(1 → (**E**) was generated from (1 → 3)-β-D-Glc*p* (**A**$_{II}$) due to complete oxidation of the β-D-Glc*p*-(1 → (**B**) and also one (1 → 3)-β-D-Glc*p* (**F**) was produced from the (1 → 3,6)-β-D-Glc*p* (**C**) due to oxidation followed by Smith degradation of the (1 → 6)-β-D-Glc*p* moiety (**D**$_{I}$) and the other (1 → 3)-β-D-Glc*p* (**F**) was retained from (1 → 3)-β-D-Glc*p* (**A**$_{I}$). The glycerol (**G**) moiety was generated from (1 → 6)-β-D-Glc*p* (**D**$_{II}$) after periodate oxidation followed by Smith degradation and be attached to (1 → 3)-β-D-Glc*p* moiety (**F**) as a gro part. Hence, Smith degradation resulted in the formation of an oligosaccharide unit from the parent polysaccharide and the structure of which was established as:



Therefore, the above result indicated that the (1 → 3)-linked β-D-glucose was present at the side chain, branching at *O*-3 of one backbone residue. This observation excluded the possibility of (1 → 3)-linked backbone. The ^{13}C spectrum was carried out with partially hydrolyzed polysaccharide (F1) and showed no C-3 signal for (1 → 3)-linked β-D-Glc*p* but a characteristic C-6 signal at δ 68.9 was observed. This result further proved that the glucan possessed (1 → 6)-linked backbone with (1 → 3)-linked moieties located at the branched point. This also excluded the possibility of alternatively (1 → 6) and (1 → 3)-linked moieties in the backbone. Hence, considering all the results of chemical investigations and NMR

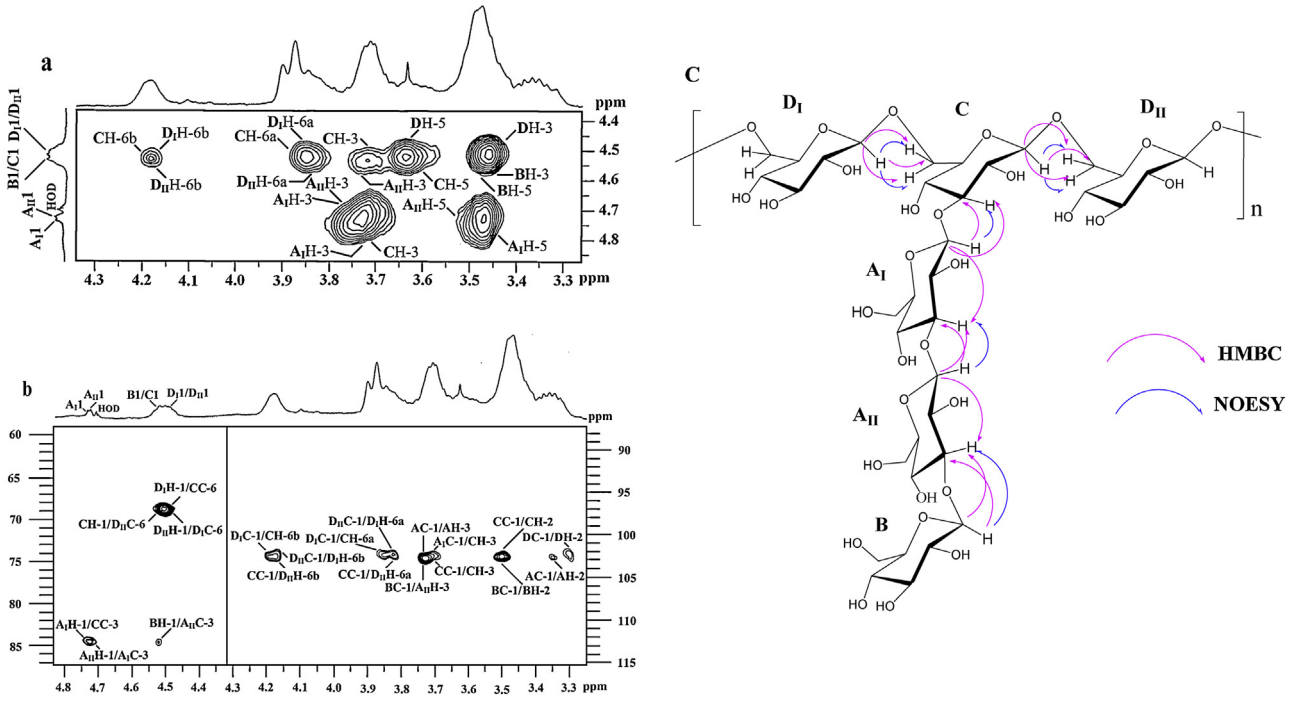
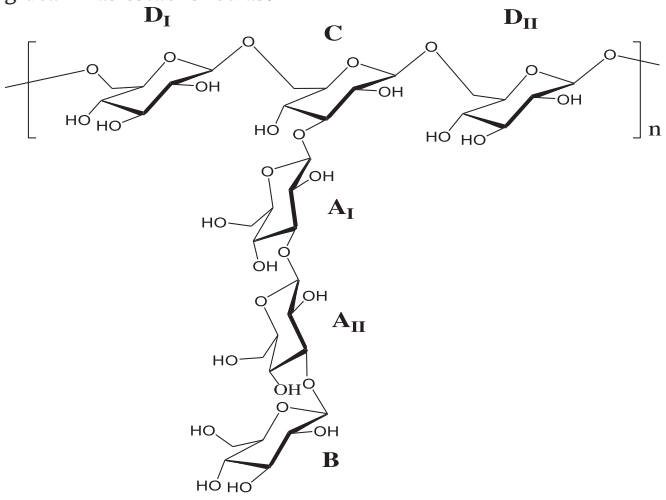


Fig. 2. (a) The part of NOESY spectrum of the β-glucan (PS) isolated an edible mushroom *Russula albonigra* (Krombh.) Fr. The NOESY mixing time was 300 ms. (b) The part of HMBC spectrum of the β-glucan (PS) isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr. The delay time in the HMBC experiment was 80 ms. (c) The key 2D NMR correlations of β-glucan (PS).

spectroscopic evidences, the structure of repeating unit of the β-glucan was established as:



3.3. Immunological studies of β-glucan (PS)

A negative (−) limulus amebocyte lysate (LAL) test with the PS was carried out adopting the procedure as reported in our previous publication (Nandi et al., 2013) and found that it was free from bacterial endotoxin. Immunological studies were also investigated with the PS. Macrophage activation by PS was observed *in vitro*. Upon treatment with different concentrations of the PS, enhanced production of NO was observed in a dose-dependent manner with optimum production of 22 μM NO per 5×10^5 macrophages at 100 μg/mL of the PS (Fig. 4a). The various types of β-glucan like lentinan inhibit tumor growth by stimulating the immune system (Suzuki, Takatsuki, Maeda, Hamuro, & Chihara, 1994) through activation of macrophages, T-helper, NK, and other cells.

Splenocytes are the cells present in the spleen that include T cells, B cells, and dendritic cells that stimulate the immune response in living organism. Thymocytes are hematopoietic cells in thymus which generate T cells. The splenocytes and thymocytes activation tests were carried out in Swiss Albino mice cell culture medium with the PS by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method (Ohno et al., 1993). Proliferation of splenocytes and thymocytes is an indicator of immunostimulation. The PS was found to stimulate splenocytes and thymocytes as shown in Fig. 4b and c, respectively and the asterisks on the columns indicate the statistically significant differences compared to PBS control. At 50 μg/mL of the PS, splenocyte proliferation index was found maximum as compared to other concentrations. Hence, 50 μg/mL of the PS can be considered as efficient splenocyte stimulators. Again 25 μg/mL of this same sample showed maximum effect on thymocyte proliferation. The splenocyte and thymocyte proliferation index as compared to Phosphate Buffered Saline (PBS) control if closer to 1 or below indicates low stimulatory effect on immune system.

3.4. Antioxidant activities of β-glucan (PS)

3.4.1. Assay of hydroxy radical scavenging activity

Hydroxy radical (OH•) has a very short life but it is considered to be the most toxic among all reactive oxygen species (ROS). It can damage DNA by attacking purines, pyrimidines and deoxyribose. Hydroxy radicals are formed by an electron transfer from transition metals to H_2O_2 and consequently interact with biomolecules (Ferreira, Barros, & Aberu, 2009). In our experiment, hydroxy radicals which are generated from Fe^{2+}–ascorbate–EDTA–H_2O_2 system (Fenton's reaction) attack the deoxyribose and eventually result in the formation of malondialdehyde (MDA). The formation of MDA is measured as a pink MDA–TBA chromogen at 535 nm (Aruoma, Laughton, & Halliwell, 1989). When test sample was added to reaction mixture, they removed hydroxy radicals and prevented sugar degradation. The PS showed potent hydroxy radical scavenging

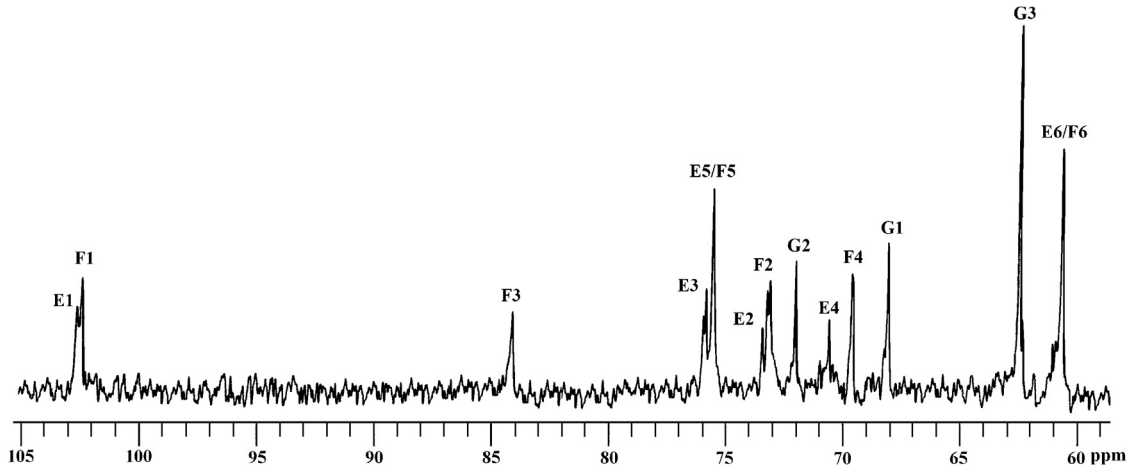


Fig. 3. The ^{13}C NMR spectrum of the Smith-degraded glycerol-containing trisaccharide of the β-glucan (PS) isolated from *Russula albonigra* (Krombh.) Fr. in D_2O at 30 °C.

activity which rose gradually with the increase of concentration (Fig. 5a). The IC_{50} value of the PS was found to be 265 μg/mL.

3.4.2. Assay of superoxide radical scavenging activity

Superoxide anion ($O_2^{\bullet}$) is one of the six major reactive oxygen species causing oxidative damage in the human body (Huang, Ou, & Prior, 2005). It is considered as primary ROS as it is a relatively weak oxidant but it can generate secondary ROS such as peroxynitrate ($ONOO^{\bullet}$), peroxy radical ($LOO^{\bullet}$), singlet oxygen, hydroxy radical and hydrogen peroxide (Chen, Ju, Li, & Yu, 2012; Huang, Ou, & Prior, 2005; Wootton-Beard & Ryan, 2011). Therefore, superoxide radical scavenging activity is of great importance to exhibit potential antioxidant property. The method used by Martinez, Marcelo, Marco, and Moacyr (2001) based on generation of superoxide radical by auto-oxidation of riboflavin in presence of light which in turn reduces yellow dye NBT to produce blue formazon. Intensity of color is directly proportional to the concentration of superoxide anion. In the present study, the PS was found to act as a notable scavenger of superoxide radicals (Fig. 5b). The IC_{50} value of the PS was determined 130 μg/mL, whereas Patra et al., 2013 reported that the IC_{50} value of the polysaccharide from *Pleurotus ostreatus* was 553 μg/mL.

3.4.3. Chelating ability of ferrous ion

Dietary nutrients containing metal chelators may act as preventive antioxidant because some transition metals, *e.g.* Fe^{2+}, Cu^{+}, Pb^{2+}, Co^{2+} and so on can trigger process of free radical reaction (Chen et al., 2012). Ferrozine quantitatively forms complexes with Fe^{2+}. In the presence of chelating agent, the complex formation is disrupted, resulting in the reduction of red color. Reduction therefore allows estimation of the chelating ability of the coexisting chelator. Fig. 5c reveals that the PS demonstrated a marked capacity for iron binding ability, where the 50% chelation was found at a concentration of 300 μg/mL.

3.4.4. Determination of reducing power

Reducing properties of a substance are generally associated with the presence of reductones or hydroxide groups. Such substance can exert antioxidant activity by donating hydrogen atom to break the free radical chain (Wootton-Beard & Ryan, 2011). In the reducing power assay, the more antioxidant compounds convert the

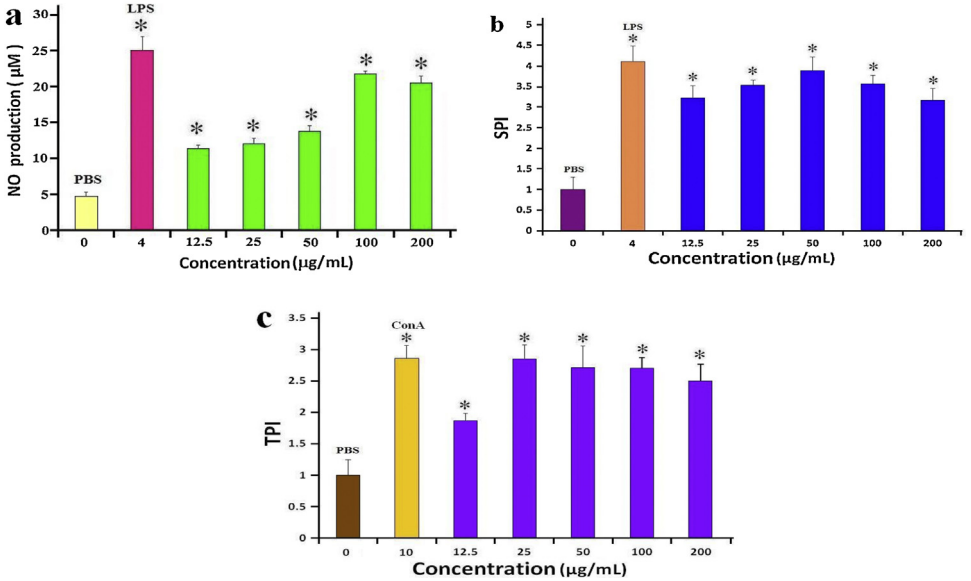


Fig. 4. (a) Activation of RAW 264.7 macrophage cells with different concentrations of β-glucan (PS) in terms of NO production. Effect of different concentrations of β-glucan (PS) on proliferation of (b) splenocyte and (c) thymocyte (asterisks indicate the statistically significant compared to the PBS control with $P < 0.05$).

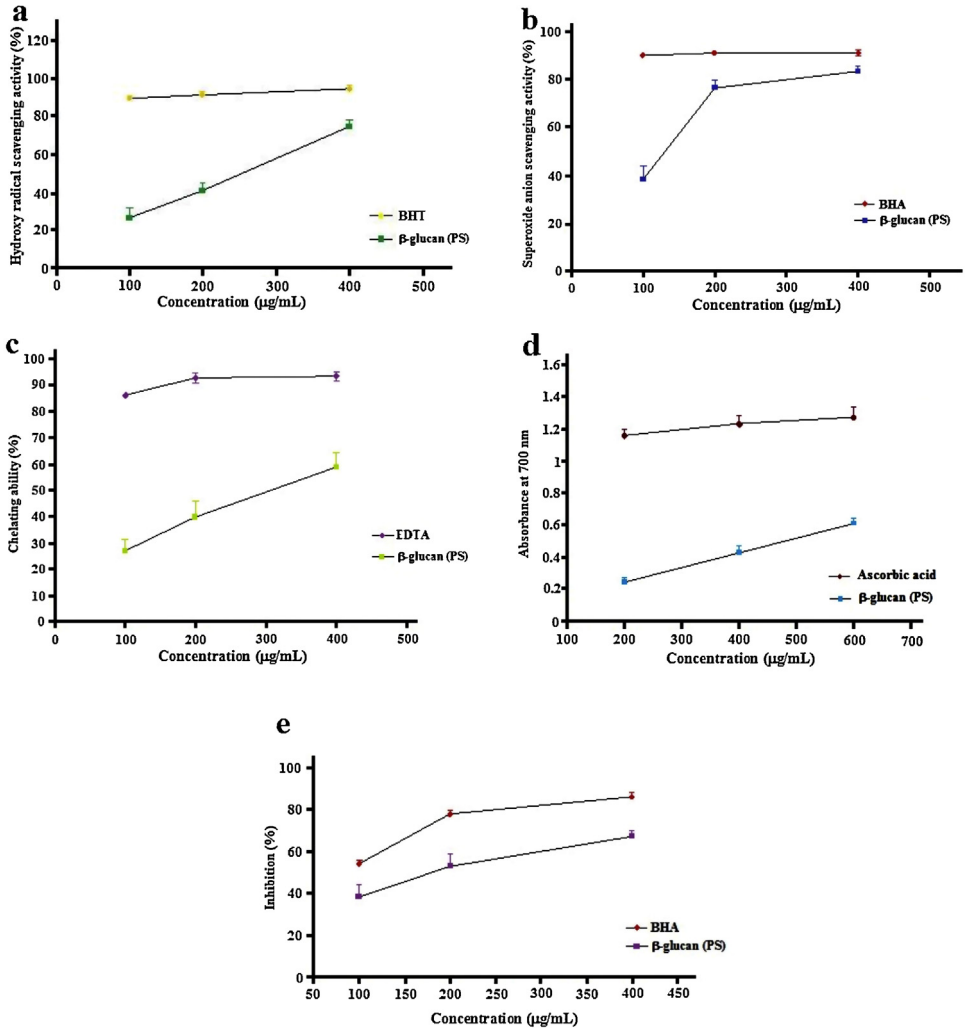


Fig. 5. (a) Hydroxy radical scavenging activity, (b) superoxide radical scavenging activity, (c) ferrous ion chelating ability, (d) reducing power and (e) inhibition of β carotene bleaching of β-glucan (PS) isolated from *Russula albonigra* (Krombh.) Fr. (All the above results are the mean ± SD of three separate experiments, each in triplicate.).

oxidation form of iron (Fe^{3+}) in ferric chloride to ferrous (Fe^{2+}). So the yellow color of the test solution changed from green to blue as the reducing power of sample increases. Fig. 5d reveals that at concentration of 500 μg/mL PS showed reducing power of 0.5.

3.4.5. *β carotene bleaching assay*

β carotene usually undergoes rapid discoloration in absence of antioxidant. Oxidation of β carotene and linoleic acid generate free radicals. Free radicals from linoleic acid are formed by the abstraction of a hydrogen atom from one of its diallylic methylene groups which attack the highly unsaturated β carotene molecule. Hence, β carotene is oxidized, and gradually losing its orange color which is then monitored spectrophotometrically (Okoh, Sadimenko, & Afolayan, 2011). Fig. 5e reveals that the PS had inhibition effect on β carotene bleaching. The PS showed 50% inhibition at a concentration of 180 μg/mL.

4. Conclusions

An immunoenhancing antioxidant water soluble β-glucan (PS) was isolated from the alkaline extract of an edible mushroom, *Russula albonigra* (Krombh.) Fr. The structure of this PS was elucidated on the basis of total hydrolysis, methylation analysis, Smith degradation, partial hydrolysis and 1D/2D NMR studies. These results indicated that the repeating unit of the PS contained a backbone of three (1 → 6)-β-D-glucopyranosyl residues, one of which was branched at *O*-3 position with the side chain consisting of two (1 → 3)-β-D-glucopyranosyl and a terminal β-D-glucopyranosyl residue. The PS activated the macrophages, splenocytes, and thymocytes and also showed several potent antioxidant activities. Hence, on the basis of these activities it could be used as a natural immunostimulant and antioxidant material, further, this mushroom can also be recommended as an excellent food for consumption.

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References

Acharya, K., Chatterjee, S., & Ghosh, S. (2011). Comparative evaluation on the free radical scavenging activity of eleven Indian cultivated strains of *Pleurotus ostreatus*. *Pharmacology Online*, *1*, 440–450.

Agrawal, P. K. (1992). NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. *Phytochemistry*, *31*, 3307–3330.

Aruoma, O. I., Laughton, M. J., & Halliwell, B. (1989). Carnosine, homocarnosine and anserine: Could they act as antioxidants *in vivo*? *Biochemistry Journal*, *264*, 863–869.

Blokhina, O., Virolainen, E., & Fagerstedt, V. K. (2003). Antioxidants, oxidative damage and oxygen deprivation stress: A review. *Annals of Botany*, *91*, 179–194.

Chan, G. C., Chan, W. K., & Sze, D. M. (2009). The effects of β-glucan on human immune and cancer cells. *Journal of Hematology & Oncology*, *2*, 25.

Chen, H., Ju, Y., Li, J., & Yu, M. (2012). Antioxidant activities of polysaccharides from *Lentinus edodes* and their significance for disease prevention. *International Journal of Biological Macromolecules*, *50*, 214–218.

Ciucanu, I., & Kerek, F. (1984). Simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research*, *131*, 209–217.

Dapkevicius, A., Venskutonis, R., Van Beek, T. A., & Linssen, P. H. J. (1998). Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania. *Journal of the Science of Food and agriculture*, *77*, 140–146.

Dinis, T. C. P., Madeira, V. M. C., & Almeida, L. M. (1994). Action of phenolic derivatives (acetaminophen, salicylate, and 5-amino salicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Archives of Biochemistry and Biophysics*, *315*, 161–169.

Dong, Q., Yao, J., Yang, X., & Fang, J. (2002). Structural characterization of a water-soluble β-D-glucan from fruiting bodies of *Agaricus blazei* Murr. *Carbohydrate Research*, *337*, 1417–1421.

Ferreira, C. F. R. I., Barros, L., & Aberu, R. M. V. (2009). Antioxidants in wild mushrooms. *Current Medicinal Chemistry*, *16*(12), 1543–1560.

Gerwig, G. J., Kamerling, J. P., & Vliegenthart, J. F. G. (1978). Determination of the D and L configuration of neutral monosaccharides by high-resolution capillary g.l.c. *Carbohydrate Research*, *62*, 349–357.

Goldstein, I. J., Hay, G. W., Lewis, B. A., & Smith, F. (1965). Controlled degradation of polysaccharides by periodate oxidation, reduction and hydrolysis. *Methods in Carbohydrate Chemistry*, *5*, 361–370.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., & Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. *Analytical Biochemistry*, *126*, 131–138.

Hara, C., Kiho, T., Tanaka, Y., & Ukai, S. (1982). Anti-inflammatory activity and conformational behavior of a branched (1 → 3)-β-D-glucan from an alkaline extract of *Dictyophora indusiata* Fisch. *Carbohydrate Research*, *110*, 77–87.

Huang, D., Ou, B., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, *53*, 1841–1856.

Kidd, P. M. (2000). The use of mushroom glucans and proteoglycans in cancer treatment. *Alternative Medicine Review*, *5*, 4–27.

Kofuji, K., Aoki, A., Tsubaki, K., Konishi, M., Isobe, T., & Murata, Y. (2012). Antioxidant activity of β-glucan. *ISRN Pharmaceutics*, http://dx.doi.org/10.5402/2012/125864

Kozarski, M., Klaus, A., Niksic, M., Jakovljevic, D., Helsper, J. P. F. G., & Griensven, L. J. L. D. V. (2011). Antioxidative and immunomodulating activities of polysaccharide extracts of the medicinal mushrooms *Agaricus bisporus*, *Agaricus brasiliensis*, *Ganoderma lucidum* and *Phellinus linteus*. *Food Chemistry*, *129*, 1667–1675.

Maity, K. K., Patra, S., Dey, B., Bhunia, S. K., Mandal, S., Behera, B., et al. (2013). A β-glucan from the alkaline extract of asomatic hybrid (*PfloVv5FB*) of *Pleurotus florida* and *Volvariella volvacea*: Structural characterization and study of immunoactivation. *Carbohydrate Research*, *370*, 13–18.

Martinez, A. C., Marcelo, E. L., Marco, A. O., & Moacyr, M. (2001). Differential responses of superoxide dismutase in freezing resistant *Solanum curtibolum* and freezing sensitive *Solanum tuberosum* subjected to oxidative and water stress. *Plant Science*, *160*, 505–515.

Nandi, A. K., Samanta, S., Sen, I. K., P.Devi, S. K., Maiti, T. K., Acharya, K., et al. (2013). Structural elucidation of an immunoenhancing heteroglycan isolated from *Russula albonigra* (Krombh.) Fr. *Carbohydrate Polymers*, *94*, 918–926.

Nandi, A. K., Sen, I. K., Samanta, S., Maity, K., P.Devi, S. K., Mukherjee, S., et al. (2012). Glucan from hot aqueous extract of an ectomycorrhizal edible mushroom, Russula albonigra (Krombh.) Fr.: Structural characterization and study of immunoenhancing properties. *Carbohydrate Research*, *363*, 43–50.

Ohno, N., Saito, K., Nemoto, J., Kaneko, S., Adachi, Y., Nishijima, M., et al. (1993). Immunopharmacological characterization of a highly branched fungal (1 → 3)-β-D-glucan, OL-2, isolated from *Omphalia lapidescens*. *Biological and Pharmaceutical Bulletin*, *16*, 414–419.

Okoh, O. O., Sadimenko, A. P., & Afolayan, A. J. (2011). Antioxidant activities of *Rosmarinus officinalis* L. essential oil obtained by hydro-distillation and solvent free microwave extraction. *African Journal of Biotechnology*, *10*(20), 4207–4211.

Oyaizu, M. (1986). Studies on products of browning reactions: Antioxidative activities of products of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition*, *44*, 307–315.

Papas, A. M. (1999). *Antioxidant status, diet, nutrition and health*. United Kingdom: CRC Press.

Patra, S., Patra, P., Maity, K. K., Mandal, S., Bhunia, S. K., Dey, B., et al. (2013). A heteroglycan from the mycelia of *Pleurotus ostreatus*: Structure determination and study of antioxidant properties. *Carbohydrate Research*, *368*, 16–21.

Sen, I. K., Maji, P. K., Behera, B., Maiti, T. K., Mallick, P., Sikdar, R. K., et al. (2013). Glucan of a somatic hybrid mushroom, *pfls 1h*: Structural characterization and study of immunological activities. *International Journal of Biological Macromolecules*, *53*, 127–132.

Suzuki, M., Takatsuki, F., Maeda, Y. Y., Hamuro, J., & Chihara, G. (1994). Antitumor and immunological activity of lentinan in comparison with LPS. *International Journal of Immunopharmacology*, *16*, 463–468.

Wasser, S. P. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology*, *60*, 258–274.

Wootton-Beard, P. C., & Ryan, L. (2011). Improving public health? The role of antioxidant-rich fruit and vegetable beverages. *Food Research International*, *44*, 3135–3148.

Yoshioka, Y., Tabeta, R., Saito, H., Uehara, N., & Fukuoka, F. (1985). Antitumor polysaccharides from *P. ostreatus* (Fr.) Que'l.: Isolation and structure of a β-glucan. *Carbohydrate Research*, *140*, 93–100.