Carbohydrate Polymers 114 (2014) 157–165

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Heteroglycan of an edible mushroom *Entoloma lividoalbum*: Structural characterization and study of its protective role for human lymphocytes

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ARTICLE INFO

Article history:
Received 21 May 2014
Received in revised form 22 July 2014
Accepted 29 July 2014
Available online 13 August 2014

Keywords:
Entoloma lividoalbum (Kühner & Romagn)
Kubička
Edible mushrooms
Heteroglycan
NMR studies
Biological activities

ABSTRACT

A water soluble heteroglycan (PS-II) of an average molecular weight ~5.2×10^4 Da was isolated from the alkaline extract of an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. Structural characterization of PS-II was carried out using sugar and methylation analysis, periodate oxidation study, and 1D/2D NMR experiments. Sugar analysis indicated the presence of glucose, mannose, galactose, and fucose in a molar ratio of nearly 5:1:2:1. The repeating unit of the PS-II had a backbone consisting of two (1 → 3)-β-D-glucopyranosyl, one (1 → 6)-β-D-glucopyranosyl, one (1 → 2)-α-L-fucopyranosyl, one (1 → 6)-α-D-glucopyranosyl, and two (1 → 6)-α-D-galactopyranosyl residues, out of which one (1 → 3)-β-D-glucopyranosyl residue was branched at *O*-6 position with terminal β-D-glucopyranosyl residue and one (1 → 6)-α-D-galactopyranosyl residue was branched at *O*-2 position with terminal β-D-mannopyranosyl residue. PS-II showed ameliorative activities at different concentrations (50, 100, 200, 400 μg/ml) and maintained the redox balance as well as reduced the lipid peroxidation to protect the cell destruction.

1. Introduction

Mushroom has been appreciated by humankind as an important edible and medical resource (Wasser & Weis, 1999) since ancient time. They are rich in nutritional value with high content of carbohydrates, proteins, vitamins, fats, and minerals (Wani, Bodha, & Wani, 2010). Mushroom polysaccharides are well known for their immunomodulatory (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007; Wasser & Weis, 1999), antitumor (Das, 2010; Wasser, 2002), and antioxidant activities (Maity et al., 2014). It was reported that several glucans such as (1 → 3)-β-D-glucan (Chakraborty, Mondal, Rout, & Islam, 2006; Ohno et al., 1993), (1 → 3)-, (1 → 6)-β-D-glucan (Bhanja et al., 2013), (1 → 6)-β-D-glucan (Kiho, Shiose, Nagai, & Ukai, 1992), and branched (1 → 3)-, (1 → 6)-α, β-D-glucan (Rout, Mondal, Chakraborty, Pramanik, & Islam, 2005) and heteroglycan (Dey et al., 2013; Maity et al., 2013; Nandi et al., 2013; Patra et al., 2012) are reported as antitumor and immunostimulating agents. *Entoloma lividoalbum* (Kühner & Romagn.) Kubička commonly called as 'Salle chyau' (Nepali), genus Entoloma, family Entolomataceae is a basidiomycetes fungus. This edible and non-toxic mushroom normally grows on Sikkim Himalayan region, India during July to August (Das, 2010). Two water soluble polysaccharides (PS-I & PS-II) have been isolated from the alkaline extract of this mushroom through Sepharose gel fractionation. The PS-I has been investigated and reported as a glucan (Maity et al., 2014) which showed antioxidant properties. The PS-II was characterized as heteroglycan containing α-L-fucose as one of the constituents which is used for human breast cancer and infertility treatment (Jay, Gene, & Catherine, 2011). In the present investigation, attempts were made to study the toxicological analysis of PS-II. Lymphocytes are the primary immune cells in the body. Destruction or

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http://dx.doi.org/10.1016/j.carbpol.2014.07.080
0144-8617/

strengthening of lymphocyte cells are directly linked to immunity. From this point of view the use of various natural and synthetic drugs has drawn the remarkable attention now a day. PS-I have been reported to possess antioxidant properties (Maity et al., 2014) and from that observation, the toxicological parameters including redox balances maintained by PS-II was investigated in the present study. Further, the protective effects of PS-II was also evaluated where nicotine was used as a potent cytotoxic agent which affects a variety of cellular processes from induction of gene expression to modulation of enzymatic activities (Mahapatra, Chakraborty, Majumdar, Bag, & Roy, 2009). Hence, the protective effects of PS-II on nicotine-induced human lymphocytes were studied.

The detailed structural investigation and biological activities of PS-II were carried out in the present investigation and reported herein.

2. Materials and methods

2.1. Collection and identification

Fruit bodies of the edible mushroom *E. lividoalbum* (Kühner & Romagn.) Kubička were collected from Sikkim Himalaya region, India. The voucher specimen was deposited with the accession code AMFH-608 in the Mycological Herbarium of Department of Botany, University of Calcutta, Kolkata, and West Bengal, India. One of the authors of this article Dr. Krishnendu Acharya, Department of Botany, University of Calcutta is a mycologist who identified the mushroom.

2.2. Isolation and purification of the polysaccharide

Fresh fruit bodies of the mushroom *E. lividoalbum* (Kühner & Romagn.) Kubička (600 g) were gently washed with distilled water several times, crushed and boiled with 4% NaOH solution for 1 h. The whole mixture was then kept overnight at 4 °C and then filtered through linen cloth, centrifuged and then precipitated in EtOH (1:5) to get crude polysaccharide. The detailed extraction procedure was described in previous communication (Maity et al., 2014). The crude polysaccharide was purified through Sepharose 6B gel permeation chromatography (GPC) to produce two fractions, PS-I (test tube no. 18–24, yield 13 mg) and PS-II (test tube no. 33–38, yield 9 mg). The structural characterization and study of antioxidant properties of PS-I were reported earlier (Maity et al., 2014). The isolation procedure was repeated several times to obtain 95 mg PS-II which was further purified by same procedure to get a homogeneous fraction, yield 80 mg. The structural and biological studies of only PS-II was carried out and presented herein.

2.3. Monosaccharide analysis

PS-II (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. The excess acid was completely removed by co-distillation with water. Then, the hydrolyzed product was reduced with $NaBH_4$ (9 mg), followed by acidification with dilute CH_3COOH, and then co-distilled with pure CH_3OH to remove excess boric acid. The reduced sugars were acetylated with 1:1 pyridine–acetic anhydride in a boiling water bath for 1 h to give the alditol acetates (Lindahl, 1970), which were analyzed by GLC Hewlett-Packard model 5730 A with 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 GLC analyses were performed at 170 °C.

2.4. Methylation analysis

PS-II (4.0 mg) was methylated using the procedure described by Ciucanu and Kerek (1984). The methylated products were isolated by making partition between $CHCl_3$ and H_2O (5:2, v/v) for four times. The organic layer containing products was collected and dried. The methylated product was then hydrolyzed with 90% formic acid (1 ml) at 100 °C for 1 h and excess formic acid was evaporated by co-distillation with distilled water. The hydrolyzed product was then reduced with sodium borohydride and acetylated with pyridine-acetic anhydride (1:1). The alditol acetates of methylated sugars were analyzed by GLC–MS. The gas–liquid chromatography–mass spectrometric (GLC–MS) analysis was performed on a Shimadzu GLC–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.

2.5. Periodate oxidation and Smith degradation study

PS-II (40 mg) was oxidized with 0.1 M sodium metaperiodate solution (20 ml) and the mixture was kept for 72 h in the dark at 25 °C. The excess periodate was destroyed by adding ethylene glycol and the solution was dialyzed against distilled water for 2 h. The volume of the dialyzed material was concentrated to 2–3 ml. This material was reduced with $NaBH_4$, 12 h, neutralized with 50% AcOH, and dialyzed with distilled water and finally freeze dried (19.0 mg). The periodate-reduced material was divided into three portions. One portion (2 mg) was hydrolyzed with 2 M CF_3COOH (1 ml) at 100 °C for 18 h and used for alditol acetate preparation and analyzed by GLC. The second portion (2.0 mg) was methylated by the method of Ciucanu and Kerek (1984), followed by preparation of alditol acetates which were analyzed by GLC–MS. Smith degradation experiment was performed with the third portion (15.0 mg). The mild hydrolysis of the periodate oxidized-reduced material was performed by the addition of 0.5 M CF_3COOH for 15 h at 25 °C to destroy the residual part of the oxidized sugars attached to the polysaccharide chain. The excess acid was removed by repeated freeze drying. The material was further purified by passing through a Sephadex G-25 column, freeze-dried and kept over P_2O_5 in vacuum for several days for ^{13}C NMR analysis.

2.6. Absolute configuration of monosaccharide

The absolute configuration of monosaccharide was determined by the method of Gerwig, Kamerling, and Vliegenthart (1978). PS-II (1.0 mg) was hydrolyzed with CF_3COOH, and then the acid was removed by co-distillation with water. A volume of 250 μl of 0.625 M HCl solution treated with R-(+)-2-butanol was added to the hydrolyzed product 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 GLC using a capillary column SPB-1 (30 m × 0.26 mm) with a temperature program (3 °C/min) from 150 to 210 °C. The resulting 2,3,4,6-tetra-*O*-TMS-(+)-2-butylglycosides were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides.

2.7. Optical rotation

Optical rotation was measured on a Jasco Polarimeter model P-1020 at 31.8 °C.

2.8. Determination of molecular weight

The molecular weight of the polysaccharide was determined by gel-permeation chromatography (Hara, kiho, Tanaka, & Ukai, 1982). Standard dextrans 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 PS-II was then plotted on the same graph and the average molecular weight of PS-II was determined.

2.9. NMR studies

PS-II was dried over P_2O_5 in vacuum for several days and then exchanged with deuterium (Dueñas-Chaso et al., 1997) followed by lyophilizing with D_2O (99.96% atom ^2H, Aldrich) for four times. The ^1H and ^{13}C NMR experiments were carried out at 500 MHz and 125 MHz, respectively with a Bruker Avance DPX-500 spectrometer. The ^1H, ^{13}C, TOCSY, DQF-COSY, ROESY, NOESY, and HSQC NMR spectra were recorded in D_2O at 30 °C. The ^1H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.70) using the WEFT pulse sequence (Hård, Zadelhoff, Moonen, Kamerling, & Vliegenthart, 1992) using acetone as internal standard fixing methyl proton signal at δ 2.19. Acetone was used as an internal standard (δ 31.05 ppm) for ^{13}C spectrum. The 2D-DQF-COSY experiment was performed 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.

2.10. Biological activities

2.10.1. Isolation of lymphocytes from peripheral blood mononuclear cells

Fresh blood samples were collected from all groups of individuals satisfying the Helsinki protocol. The lymphocytes were isolated from heparinized blood samples according to the method applied earlier by Chattopadhyay et al. (2013). Blood was diluted with phosphate-buffered saline (pH 7.0) in equal ratio and then layered very carefully on the density gradient (histopaque 1077) in a ratio of 1:2, centrifuged at 1400 rpm for 20 min and the white milky layer of mononuclear cells were carefully removed and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin, 4 mM L-glutamine under 5% CO_2 and 95% humidified atmosphere at 37 °C for 2 h. After 2 h, non adherent layer of the cultured cells were washed twice with the PBS and centrifuged at 2000 rpm for 10 min to get the required pellet of lymphocytes.

2.10.2. Cell viability

Normal human lymphocytes were seeded into 96 wells of tissue culture plates having 180 μl of complete media and were incubated for 48 h. PS-II was added to the cells at different concentrations (50, 100, 200, and 400 μg/ml) and incubated for 24 h at 37 °C in a humidified incubator (NBS) maintained with 5% CO_2. The cell viability was estimated by 3-(4,5-dimethylthiazol)-2-diphenyltetrazolium bromide (MTT) method as applied earlier (Chattopadhyay et al., 2012).

2.10.3. Cell lysate preparation

After treatment schedule, the cell suspension was collected in a centrifuge tube and centrifuged at 1500 rpm for 5 min. The supernatants were collected and stored at −20 °C. The cell pellets were re-suspended in ice cold phosphate buffered saline (PBS) at concentrations ranging from 2×10^5 cells/ml and subjected to four cycles of freeze-thaw cycles (alternating liquid nitrogen and 37 °C water bath treatment) followed by sonication for 20 s (Ultrasonic Processor, Tekmar, Cincinnati, OH, USA) on ice. Lysates were centrifuged at 12,000 rpm for 20 min at 4 °C to remove cellular debris as adopted earlier by Chattopadhyay et al. (2013). Protein content of lysate preparations was measured according to Lowry, Rosenbrough, Farr, and Randall (1951) using bovine serum albumin as standard.

2.10.4. Determination of reduced glutathione (GSH)

Reduced glutathione estimation in the cell lysate was performed by the method as applied in earlier publication (Mahapatra et al., 2009). The required amount of the cell lysate was mixed with 25% of trichloroacetic acid and centrifuged at $2000 \times g$ for 15 min to settle the precipitated proteins. The supernatant was aspirated and diluted to 1 ml with 0.2 M sodium phosphate buffer (pH 8.0). Later, 2 ml of 0.6 mM DTNB was added. After 10 min the optical density of the yellow-colored complex formed by the reaction of GSH and DTNB (Ellman's reagent) was measured at 405 nm. A standard curve was obtained with standard reduced glutathione. The levels of GSH were expressed as μg of GSH/mg protein.

2.10.5. Determination of oxidized glutathione level (GSSG)

The oxidized glutathione level was measured after derivatization of GSH with 2-vinylpyidine according to the method as applied earlier (Mahapatra et al., 2009). In brief, with 0.5 ml cell lysate, 2 μl 2-vinylpyidine was added and incubates for 1 h at 37 °C. Then the mixture was deprotenized with 4% sulfosalicylic acid and centrifuged at $1000 \times g$ for 10 min to settle the precipitated proteins. The supernatant was aspirated and GSSG level was estimated with the reaction of DTNB at 412 nm in spectrophotometer and calculated with standard GSSG curve.

2.10.6. Determination of lipid peroxidation (MDA)

Lipid peroxidation was estimated by the method of Ohkawa, Ohishi, and Yagi (1979) in cell lysate. Briefly, the reaction mixture contained Tris-HCl buffer (50 mM, pH 7.4), *tert*-butyl hydroperoxide (BHP) (500 μM in ethanol) and 1 mM $FeSO_4$. After incubating the samples at 37 °C for 90 min, the reaction was stopped by adding 0.2 ml of 8% sodium dodecyl sulfate (SDS) followed by 1.5 ml of 20% acetic acid (pH 3.5). The amount of malondialdehyde (MDA) formed during incubation was estimated by adding 1.5 ml of 0.8% TBA and further heating the mixture at 95 °C for 45 min. After cooling, samples were centrifuged and the TBA reactive substances (TBARS) were measured in supernatants at 532 nm by using $1.53 \times 10^5\ M^{-1}\ cm^{-1}$ as extinction coefficient. The levels of lipid peroxidation were expressed in terms of nmol/mg protein.

2.10.7. NO release assay

The NO concentration was measured by a microplate assay method with Griess reagent (1% sulfanilamide, 0.3% naphthylethylenediaminedihydrochloride, 7.5% H_3PO_4). Briefly, culture supernatants (100 μl) were mixed with 100 μl of the Griess reagent. The nitrite concentration in the culture supernatant was measured at an absorbance of 550 nm, 10 min after mixing (Hino et al., 2005).

2.10.8. Protective role

Normal human lymphocytes (4×10^6) were seeded into 96 wells plate and treated with 10 mM nicotine for 6 h at 37 °C, since this dose was found lethal as reported earlier (Mahapatra et al., 2009). After incubation, cells were washed with 1X PBS (50 mM) for 3 times and incubated with PS-II for 24 h at 37 °C. The cell viability was estimated by 3-(4,5-dimethylthiazol)-2-diphenyltetrazolium bromide according to the method as applied earlier (Chattopadhyay et al., 2012). The apoptotic cells were visualized by propidium iodide staining under phase contrast fluoresce microscope (50× magnifications).

2.10.9. Statistical analysis

The data were expressed as the mean ± the standard error of the mean ($n = 6$). Comparisons between the means of control and treated groups were made by one-way analysis of variance (using a statistical package; Origin 6.1, Origin Lab, Northampton, MA, USA) with multiple-comparison tests, with $p < 0.05$ as the limit of significance. The correlation analysis was performed using Statistica software version 8.0.

3. Results and discussion

3.1. Isolation, purification and chemical analysis of the PS-II

A water soluble polysaccharide was isolated from the fruiting bodies of *E. lividoalbum* (Kühner & Romagn.) Kubička (Maity et al., 2014). On fractionation through GPC it gave two fractions, PS-I and PS-II (Maity et al., 2014). The PS-II showed a specific rotation $[\alpha]_D^{31.8}$ +16.5 (*c* 0.09, H_2O). The molecular weight (Hara et al., 1982) of PS-II was estimated as $\sim 5.2 \times 10^4$ Da from a calibration curve prepared with standard dextrans. GLC analysis of the alditol acetates of the hydrolyzed product of PS-II revealed the presence of glucose, mannose, galactose, and fucose in a molar ratio of nearly 5:1:2:1. The absolute configuration of the monosaccharide was determined by the method of Gerwig et al. (1978) and found that glucose, galactose, and mannose had the D configuration but fucose was present in the L configuration. The mode of linkages of the sugar moieties present in the PS-II was determined by methylation analysis using the Ciucanu and Kerek (1984) method, followed by hydrolysis and preparation of alditol acetates. The GLC–MS analysis of the alditol acetates of methylated products have been presented in Table 1. These linkages (Table 1) were further confirmed by periodate oxidation experiment. GLC analysis of alditol acetates of the periodate-oxidized (Goldstein, Hay, Lewis, & Smith, 1965; Hay, Lewis, & Smith, 1965), $NaBH_4$-reduced, and hydrolyzed products showed the presence of only glucose, indicating that the D-galactose, D-mannose, and L-fucose moieties were consumed during oxidation. GLC–MS analysis of periodate-oxidized, reduced, methylated (Abdel-Akher & Smith, 1950) PS-II showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a molar ratio of nearly 1:1. These results clearly indicated that the (1 → 3)-linked and (1 → 3,6)-linked glucopyranosyl residues remain unaffected whereas all other residues were consumed during oxidation, which further confirmed the mode of linkages present in the PS-II.

3.2. NMR and structural analysis of the PS-II

The ^{1}H NMR spectrum (500 MHz; Fig. 1a, Table 2a) of PS-II at 30 °C showed the presence of nine signals in the anomeric region at δ 5.11, 5.04, 5.03, 4.99, 4.77, 4.73, 4.49, 4.48, and 4.47. The sugar residues were designated as **A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**, and **I** according to their decreasing anomeric proton chemical shifts. In ^{13}C NMR spectrum (125 MHz; Fig. 1b) at the same temperature, seven signals were observed in the anomeric region at δ 103.0, 102.9, 102.7, 102.5, 101.7, 101.6, and 98.3. From the HSQC spectrum (Fig. 1c, Table 2a), the anomeric carbon signals at δ 103.0, 102.9, 102.7, 102.5, 101.7 and 101.6 were correlated to the anomeric proton signals δ 4.47 (**I**), δ 4.73 (**F**), δ 4.48 (**H**), δ 4.49 (**G**), δ 4.77 (**E**), and δ 5.04 (**B**) respectively. Whereas, the anomeric carbon signal at δ 98.3 was correlated to the anomeric proton signals at δ 5.11 (**A**), δ 5.03 (**C**), and δ 4.99 (**D**). All the ^{1}H and ^{13}C signals (Table 2a) were assigned from DQF-COSY (Fig. S1a and b, Supplementary data), TOCSY (Fig. S2a and b, Supplementary data), and HSQC (Fig. 1c

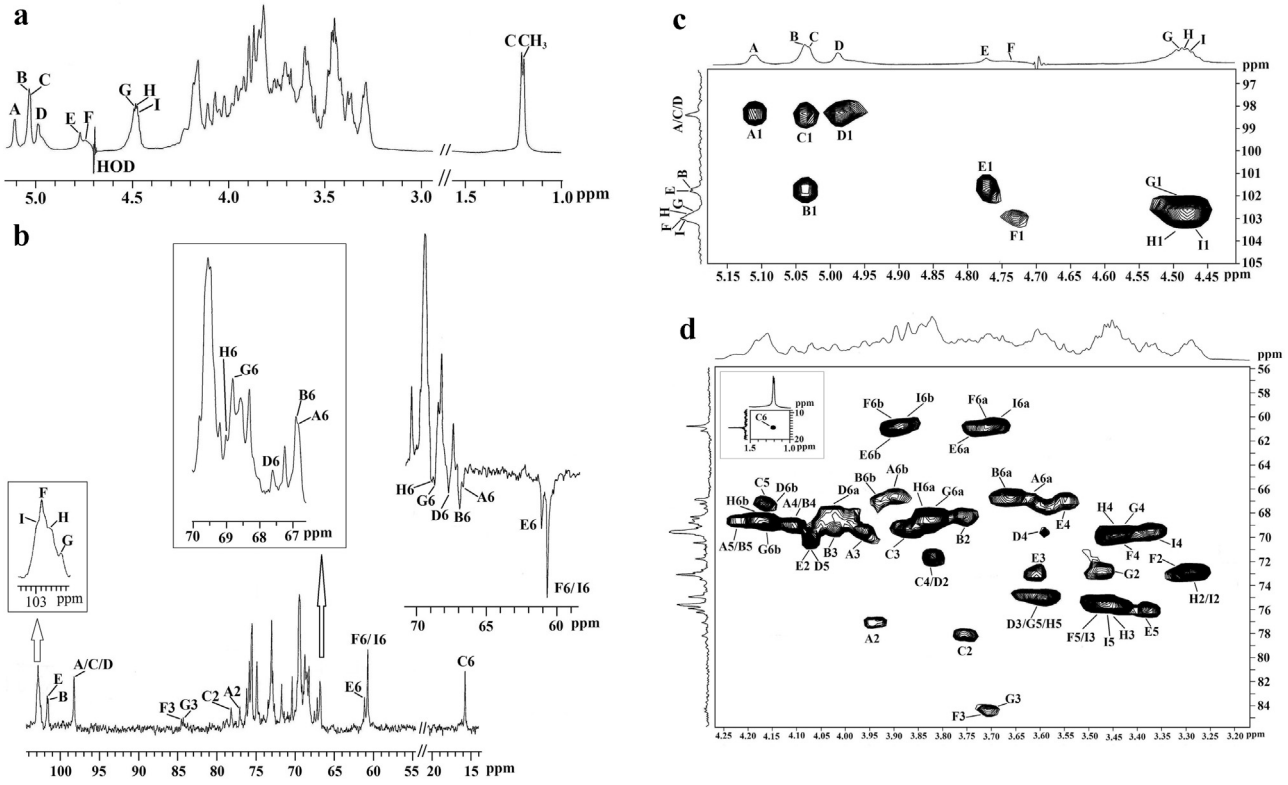


Fig. 1. The HSQC spectrum (D_2O, 30 °C) of (c) anomeric part and (d) other than anomeric part of the PS-II isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. (a) ^{1}H NMR spectrum (500 MHz, D_2O, 30 °C) of PS-II, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. (b) ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of PS-II, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. Part of DEPT-135 spectrum (D_2O, 30 °C) of PS-II, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička (inset).

Table 1
GLC–MS analysis of methylated polysaccharide (PS-II) isolated from *Entoloma lividoalbum* (Kühner & Romagn.) Kubička.

Methylated sugars	Molar ratio	Linkage type	Major mass fragments (*m*/*z*)
3,4-Me_2-Gal	1	→2,6)-Gal*p*-(1→	43,71,87,99,129,159,173,189,233
2,3,4-Me_3-Gal	1	→6)-Gal*p*-(1→	43,71,87,99,101,117,129,161,173,189,233
2,3,4,6-Me_4-Glc	1	Glc*p*-(1→	43,45,59,71,87,101,117,129,161,205
3,4-Me_2-Fuc	1	→2)-Fuc*p*-(1→	43,5971,89,99,115,129,131,173,189
2,3,4,6-Me_4-Man	1	Man*p*-(1→	43,45,59,71,87,101,117,129,161,205
2,4,6-Me_3-Glc	1	→3)-Glc*p*-(1→	43,45,71,87,101,117,129,143,161,173,203,217,233
2,4-Me_2-Glc	1	→3,6)-Glc*p*-(1→	43,58,87,101,117,129,139,159,189,201,233
2,3,4-Me_3-Glc	2	→6)-Glc*p*-(1→	43,45,58,71,87,99,101,117,129,161,173,189,233

and d) experiments. The proton coupling constants were measured from DQF-COSY experiment and one-bond C—H couplings were measured from proton coupled ^{13}C spectrum.

Residues **A** and **B** has coupling constant values of $J_{H\text{-}2,\,H\text{-}3}$ ~9 Hz and $J_{H\text{-}3,\,H\text{-}4}$ ~3.5 Hz and thus, they were confirmed as D-galactopyranosyl residues. The α-configuration of both **A** and **B** residues were assigned from the coupling constant values ($J_{H\text{-}1,H\text{-}2}$ ~3.1 Hz and $J_{C\text{-}1,H\text{-}1}$ ~171 Hz). The downfield shifts of C-2 (δ 77.1) and C-6 (δ 66.8) with respect to standard values of methyl glycosides (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that residue **A** was a (1 → 2,6)-linked α-D-galactopyranosyl. The downfield shifts of C-6 (δ 66.9) with respect to standard values of methyl glycosides (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that residue **B** was a (1 → 6)-linked α-D-galactopyranosyl. The linkage at C-6 of the both residues **A** and **B** were further confirmed from DEPT-135 spectrum (Fig. 1b). Hence, these observation confirmed that the residue **A** was (1 → 2,6)-α-D-galactopyranosyl and the residue **B** was (1 → 6)-α-D-galactopyranosyl moieties.

Residue **C** was assigned as an L-fucopyranosyl unit. This was strongly supported by the appearance of a proton signal at δ 1.21, carbon signal at δ 15.7 for a CH_3 group, and the relatively small $J_{H\text{-}3,H\text{-}4}$ (<3 Hz). The appearance of the anomeric proton and carbon signals for residue **C** at δ 5.03 and 98.3 respectively, as well as the coupling constant value $J_{H\text{-}1,H\text{-}2}$ ~3.75 Hz clearly indicated that **C** was α-anomer. The anomeric configuration was further confirmed by ^1H—^{13}C coupling constant $J_{C\text{-}1,H\text{-}1}$ ~171 Hz. The downfield shift of C-2 (δ 78.2) with respect to standard values of methyl glycosides indicated that the residue **C** was linked at C-2 position with residue **H** which further confirmed by the ROESY (Fig. 2, Table 2b) experiment. Thus, it may be concluded that the residue **C** was a (1 → 2)-α-L-fucopyranosyl moiety.

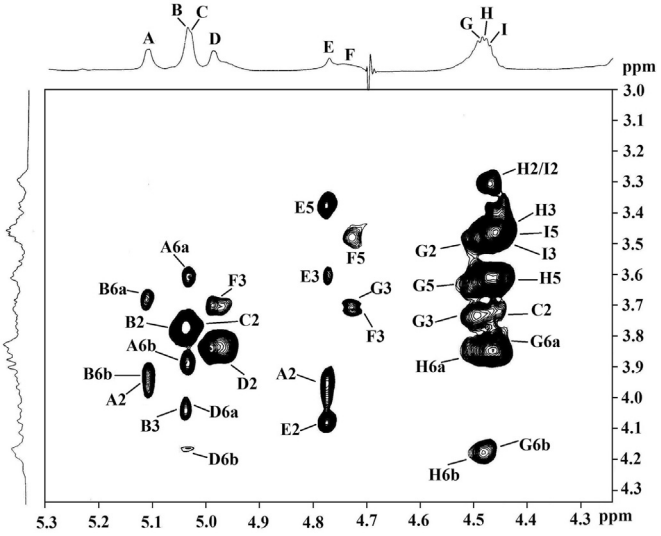


Fig. 2. Part of ROESY spectrum of PS-II, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. The ROESY mixing time was 300 ms.

The anomeric proton chemical shift (δ 4.99) and coupling constant values ($J_{H\text{-}1,H\text{-}2}$ ~3.0 Hz, $J_{C\text{-}1,H\text{-}1}$ ~171 Hz) confirmed that residue **D** was present in α-configuration. The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values (~10.0 Hz) confirmed that it was an D-glucopyranosyl moiety (Glc*p*). The downfield shifts of C-6 (δ 67.6) with respect to standard values of methyl glycosides indicated that residue **D** was linked at this position. The linkage at C-6 of the

Table 2a
The ^1H NMR[a] and ^{13}C NMR[b] chemical shifts for the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička in D_2O at 30 °C.

Glycosyl 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
→2,6)-α-D-Gal*p*-(1→	5.11	3.94	3.96	4.12	4.22	3.61[c], 3.90[d]
A	98.3	77.1	69.6	69.2	68.5	66.8
→6)-α-D-Gal*p*-(1→	5.04	3.76	4.02	4.12	4.22	3.68[c], 3.92[d]
B	101.6	68.3	69.6	69.2	68.5	66.9
→2)-α-L-Fuc*p*-(1→	5.03	3.75	3.87	3.82	4.16	1.21
C	98.3	78.2	69.6	71.8	67.2	15.7
→6)-α-D-Glc*p*-(1→	4.99	3.82	3.61	3.59	4.07	4.02[c], 4.16[d]
D	98.3	71.8	75.0	69.6	70.4	67.6
β-D-Man*p*-(1→	4.77	4.07	3.61	3.55	3.38	3.74[c], 3.90[d]
E	101.7	70.4	73.1	67.2	76.2	61.2
→3)-β-D-Glc*p*-(1→	4.73	3.30	3.71	3.43	3.47	3.70[c], 3.90[d]
F	102.9	72.9	84.5	69.6	75.6	60.8
→3,6)-β-D-Glc*p*-(1→	4.49	3.48	3.70	3.42	3.61	3.82[c], 4.16[d]
G	102.5	72.9	84.3	69.6	75.0	68.8
→6)-β-D-Glc*p*-(1→	4.48	3.29	3.45	3.44	3.61	3.84[c], 4.19[d]
H	102.7	73.1	75.6	69.6	75.0	69.0
β-D-Glc*p*-(1→	4.47	3.29	3.47	3.36	3.46	3.68[c], 3.87[d]
I	103.0	73.1	75.6	69.6	75.9	60.8

[a] The values of chemical shifts were recorded keeping HOD signal fixed at δ 4.70 ppm at 30 °C.
[b] The values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 30 °C.
[c] Interchangeable.
[d] Interchangeable.

Table 2b
ROESY data for the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička.

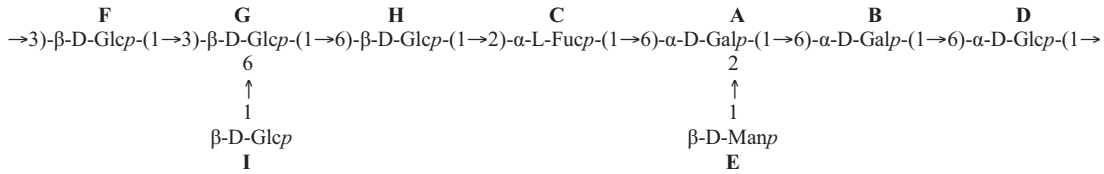
Glycosyl residue	Anomeric proton δ	ROE contact proton		
		δ	Residue	Atom
→2,6)-α-D-Gal*p*-(1→	5.11	3.68	**B**	H-6a
A		3.92	**B**	H-6b
		3.94	**A**	H-2
→6)-α-D-Gal*p*-(1→	5.04	4.02	**D**	H-6a
B		4.16	**D**	H-6b
		3.76	**B**	H-2
		4.02	**B**	H-3
→2)-α-L-Fuc*p*-(1→	5.03	3.61	**A**	H-6a
C		3.90	**A**	H-6b
		3.75	**C**	H-2
→6)-α-D-Glc*p*-(1→	4.99	3.71	**F**	H-3
D		3.82	**D**	H-2
β-D-Man*p*-(1→	4.77	3.94	**A**	H-2
E		4.07	**E**	H-2
		3.61	**E**	H-3
		3.38	**E**	H-5
→3)-β-D-Glc*p*-(1→	4.73	3.70	**G**	H-3
F		3.71	**F**	H-3
		3.47	**F**	H-5
→3,6)-β-D-Glc*p*-(1→	4.49	3.84	**H**	H-6a
G		4.19	**H**	H-6b
		3.48	**G**	H-2
		3.70	**G**	H-3
		3.61	**G**	H-5
→6)-β-D-Glc*p*-(1→	4.48	3.75	**C**	H-2
H		3.29	**H**	H-2
		3.45	**H**	H-3
		3.61	**H**	H-5
β-D-Glc*p*-(1→	4.47	3.82	**G**	H-6a
I		4.16	**G**	H-6b
		3.29	**I**	H-2
		3.47	**I**	H-3
		3.46	**I**	H-5

residue **D** was further confirmed from DEPT-135 spectrum (Fig. 1b). Thus, **D** was confirmed as (1 → 6)-α-D-glucopyranosyl residue.

The large coupling constant values ($J_{H\text{-}3,\,H\text{-}4}$ ~7.5 Hz and $J_{H\text{-}4,\,H\text{-}5}$ ~10 Hz) of residue **E** confirmed its mannopyranosyl configuration. The anomeric proton (δ 4.77) and anomeric carbon chemical shifts (δ 101.7) as well as the low coupling constant values ($J_{H\text{-}1,H\text{-}2}$ ~0, $J_{C\text{-}1,H\text{-}1}$ ~161) confirmed that residue **E** was present in β-configuration. All the proton and carbon chemical shifts of residue **E** corresponded nearly to the standard values of methyl glycosides of β-D-mannose. Thus, **E** was confirmed as terminal β-D-mannopyranosyl residue.

Residues **F**, **G**, **H,** and **I** were established as β-configuration from coupling constant values $J_{H\text{-}1,H\text{-}2}$ (~8.0 Hz), $J_{C\text{-}1,H\text{-}1}$ (~160–161 Hz) and the large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values (~10.0 Hz) of **F**, **G**, **H** and **I** confirmed their D-glucopyranosyl configuration (Glc*p*). The downfield shift of C-3 of **F** (δ 84.5) with respect to standard value of methyl glycoside (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that residue **F** was (1 → 3)-β-D-Glc*p*. The downfield shifts at C-3 (δ 84.3) and C-6 (δ 68.8) of residue **G** with respect to standard value of methyl glycoside indicated that it was (1 → 3,6)-β-D-Glc*p*. Since, the residue **G** was the rigid part its C-3 (δ 84.3) value appeared at up field region in comparison to the C-3 (δ 84.5) of the residue **F**. In case of residue **H**, the downfield shift of C-6 (δ 69.0) indicated that it was (1 → 6)-β-D-Glc*p* moiety. The linking at C-6 of the residues **G** and **H** were further confirmed by DEPT-135 spectrum (Fig. 1b). In residue **I**, all carbon chemical shifts values were found nearly to the standard values of methyl glycoside of β-D-glucose. Thus, residue **I** was confirmed as terminal β-D-Glc*p*.

The sequence of glycosyl residues (**A** to **I**) were determined from ROESY (Fig. 2, Table 2b) as well as NOESY (Fig. S3, Supplementary data). In ROESY experiment, the inter-residual contacts **A**H-1/**B**H-6a, 6b; **B**H-1/**D**H-6a, 6b; **C**H-1/**A**H-6a, 6b; **D**H-1/**F**H-3; **E**H-1/**A**H-2; **F**H-1/**G**H-3; **G**H-1/**H**H-6a, 6b; **H**H-1/**C**H-2; and **I**H-1/**G**H-6a, 6b along with other intra-residual contacts were also observed (Fig. 2). The above ROESY connectivities established the following sequences: **A** (1 → 6) **B; B** (1 → 6) **D; C** (1 → 6) **A; D** (1 → 3) **F; E** (1 → 2) **A; F** (1 → 3) **G**; **G** (1 → 6) **H**; **H** (1 → 2) **C**; and **I** (1 → 6) **G**. These data clearly indicated the positions of substitution and sequence of sugar residues in the polysaccharide. Hence, the structure of repeating unit in the PS-II was proposed as:



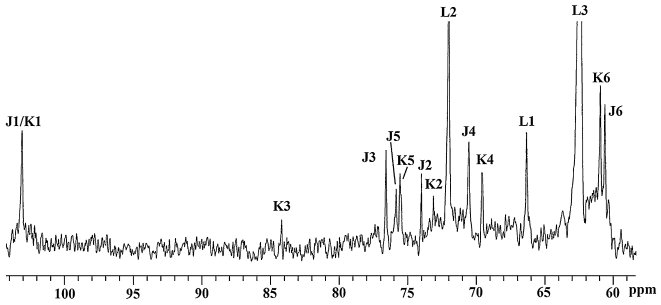


Fig. 3. ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of the Smith-degraded glycerol containing disaccharide isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička.

Table 3
The ^{13}C NMR[n] chemical shifts of Smith-degraded glycerol-containing disaccharide of the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička in D_2O at 30 °C.

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p*-(1→ **J**	103.1	74.0	76.6	70.6	75.8	60.7
→3)-β-D-Glc*p*-(1→**K**	103.1	73.1	84.3	69.6	75.6	60.9
Gro-(3→ **L**	66.4	72.1	62.5			

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

Finally, Smith degraded material (SDPS) from PS-II was prepared to confirm the linkages of the heteroglycan. The ^{13}C NMR (125 MHz) spectrum (Fig. 3, Table 3) at 30 °C of SDPS showed one anomeric carbon signals at δ 103.1 for one terminal β-D-Glc*p* (**J**) and one (1 → → 3)-β-D-Glc*p* (**K**) residues. The carbon signals C-1, C-2, and C-3 of the glycerol moiety (Gro) were assigned as δ 66.4, 72.1, and 62.5 respectively. The glycerol moiety (**L**) was generated from (1 → 6)-β-D-Glc*p* residue (**H**) after periodate oxidation followed by Smith degradation, and this moiety was attached to (1 → 3)-linked β-D-Glc*p* moiety (**K**). The residue (1 → 3)-β-D-Glc*p* (**F**) was converted to terminal β-D-Glc*p* unit (**J**) during Smith degradation. In ^{13}C NMR (Fig. 3), the carbon signal at δ 84.3 clearly indicated the presence of (1 → 3)-linked β-D-Glc*p* unit (**K**) which was generated from (1 → 3,6)-β-D-Glc*p* residue (**G**) during this reaction. Hence, Smith degradation results in the formation of a glycerol containing

disaccharide from the parent polysaccharide and the structure of which was established as:

J **K** **L**

β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-Gro

This result further confirmed the repeating unit present in the heteroglycan isolated from the edible mushroom *E. lividoalbum* (Kühner & Romagn.) Kubička.

3.3. Biological activities

The cell viability using PS-II was studied on human lymphocytes with increasing concentrations of PS-II ranging from 50 μg/ml to 400 μg/ml using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) method (Fig. 4a). It was observed that the cytotoxicity to normal lymphocytes by PS-II was insignificant. Cell proliferative activity was observed at 50 μg/ml of PS-II with respect to control. These results showed a lower level of cytotoxicity when lymphocytes were treated with PS-II up to 200 μg/ml but even at higher dose 400 μg/ml, the polysaccharide showed mild toxicity. Cell culture experiments were carried out and statistical calculations showed the IC50 value was 800 μg/ml (Fig. S5, Supplementary data), indicating that 200 μg/ml is safe with respect to the other higher doses.

Glutathione is an important antioxidant in cellular system. Hence to understand the glutathione level in cell, both reduced and oxidized form of glutathione were measured. The reduced glutathione level (GSH, Fig. 4b) was decreased and the mild augmentation of oxidized form of glutathione level (GSSG, Fig. 4b) was observed at the dose of 400 μg/ml. It was clearly observed that the alteration of redox ratio (GSH/GSSG) is fully correlated with alteration of drug concentrations (Pearson Co-efficient $r = 0.951$, Pearson correlation $p < 0.05$) (Fig. S6, Supplementary data). The redox ratio was found concentration dependent. When the dose of the PS-II was increased from 200 to 400 μg/ml, the redox ratio decreased from 1.01 to 0.499 compared to their respective control indicating that 400 μg/ml was toxic. These results indicated that 200 μg/ml is biologically safe and effective dose.

Study of lipid peroxidation is one of the important parameters to assess the cellular damage. It initiates inactivation of cellular components and protective enzymes, and thereby plays a crucial role of oxidative stress in biological systems (Samanta et al., 2013). Several toxic by-products especially malondialdehyde is released due to lipid peroxidation. Hence, lipid peroxidations in lymphocytes were measured in terms of the concentration of malondialdehyde (MDA) release (Fig. 4c). The present investigation showed slightly increase of MDA at the dose of 400 μg/ml in comparison to the previous doses indicating that 200 μg/ml is again biologically safe.

Stimulated lymphocytes secreted several factors like NO. The release of NO (Fig. 4d) clearly demonstrated that it was secreted by the lymphocytes when stimulated by PS-II. In presence of the PS-II, single culture of lymphocytes generated significant amount

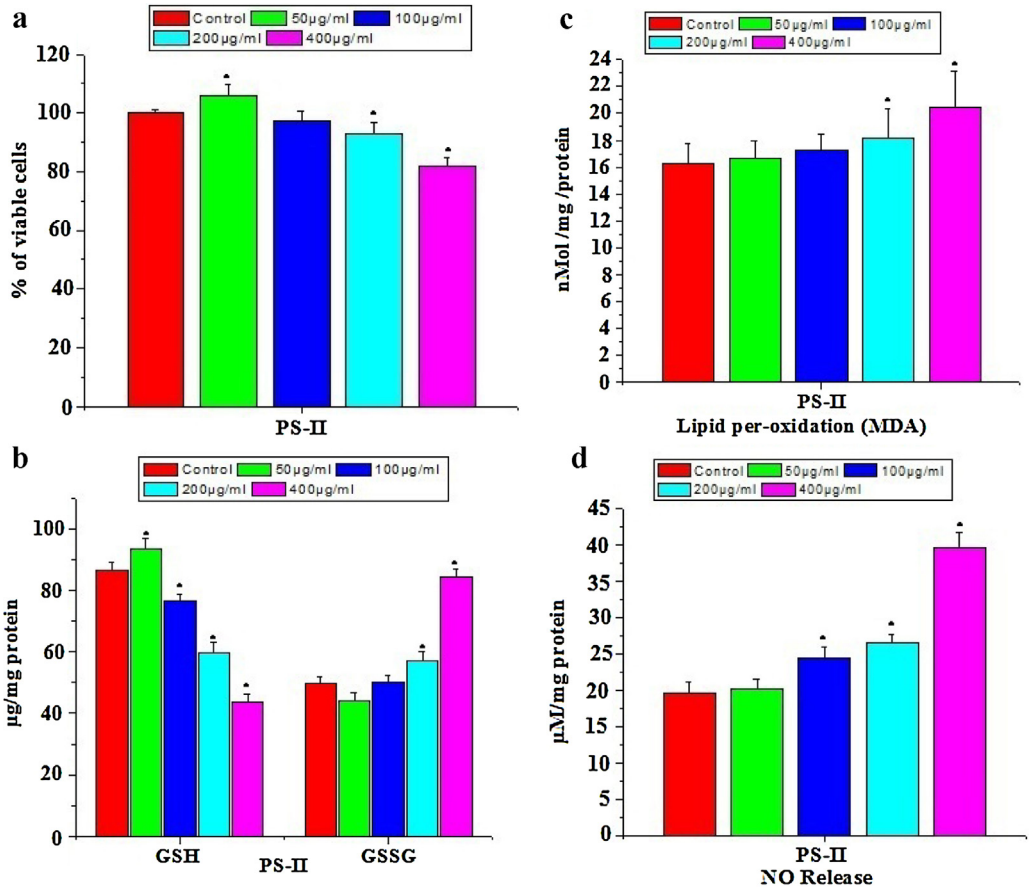


Fig. 4. (a) Cytotoxicity of PS-II against normal human lymphocytes. (b) Concentration of reduced glutathione (GSH) and oxidized glutathione of PS-II treated normal human lymphocytes. (c) Concentration of Lipidperoxidation in terms of MDA of PS-II treated normal human lymphocytes. (d) Concentration of nitric oxide release from PS-II treated normal human lymphocytes. ($n = 6$, values are expressed as mean ± SEM. * Indicates the significant difference as compared to control group).

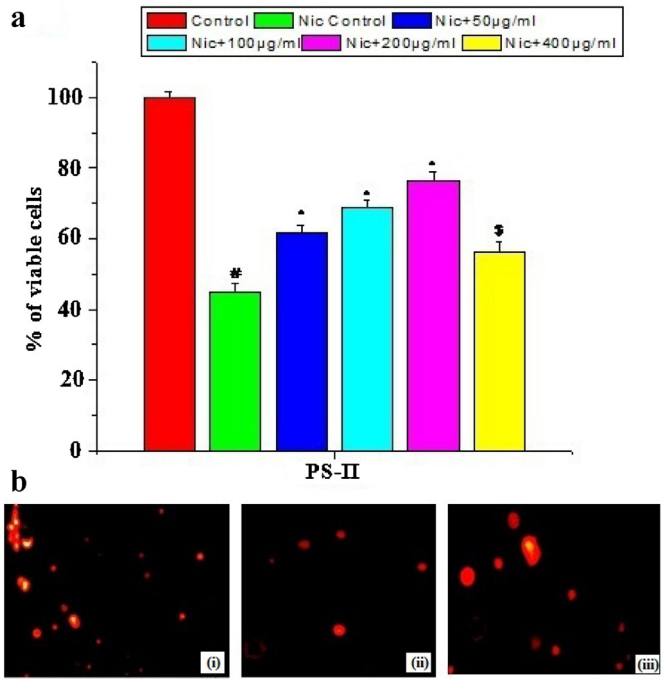


Fig. 5. (a) Cytoprotective role of PS-II was done by using nicotine treated normal human lymphocytes. $n = 6$; values are expressed as mean ± SEM. * Indicates the significant difference as compared to control group; (b) the uptake of PI stain by apoptotic cells were visualized by phase contrast fluorescent microscope under 50× mfg. Here, (i): lymphocytes were treated with 10 mM Nicotine, (ii): lymphocytes were pre-treated with 10 mM Nicotine and subsequently treated with PS-II (200 μg/ml) and (iii): lymphocytes were pre- treated with 10 mM Nicotine and subsequently treated with PS-II (400 μg/ml).

of NO ($p < 0.05$) into the medium after 24 h of incubation (Fig. 4d). The result showed the presence of a high concentration of NO in the co-culture medium of pulsed lymphocytes at 400 μg/ml indicating that this dose is cytotoxic. Hence, it is again established that 200 μg/ml is safe and effective dose.

To establish the protective role of PS-II against nicotine toxicity, lymphocytes were treated with nicotine (10 mM) as positive control and different concentrations of PS-II along with nicotine for 24 h in culture media. The significantly ($p < 0.05$) increased cell viability levels were observed up to 200 μg/ml. The fluorescent microscopic pictures established the result (Fig. 5a and b). The fluorescence images revealed that the PS-II was able to ameliorate the toxic effects of nicotine at the dose of 200 μg/ml, but when the dose was increased to 400 μg/ml, the PS-II lost its ameliorative effects on lymphocytes. The above result was confirmed by FACS, which established our findings that 400 μg/ml revealed the toxic effects synergistically with nicotine (Fig. 6).

It is evident from these experiments that, in vitro application of PS-II does not induce any cellular damage in lymphocytes associated with enhanced MDA level, NO level, GSSG level and decreased GSH level. The cytotoxic profile of PS-II in lymphocytes indicated 200 μg/ml safe and effective, whereas concentrations higher than 200 μg/ml showed significant increase of cytotoxicity. Administration of Nicotine to lymphocytes causes decrease in cell viability which is protected by supplementation of PS-II to nicotine treated cells. These findings suggest the potential use and beneficial role of PS-II for use as antioxidant as well as immunostimulant.

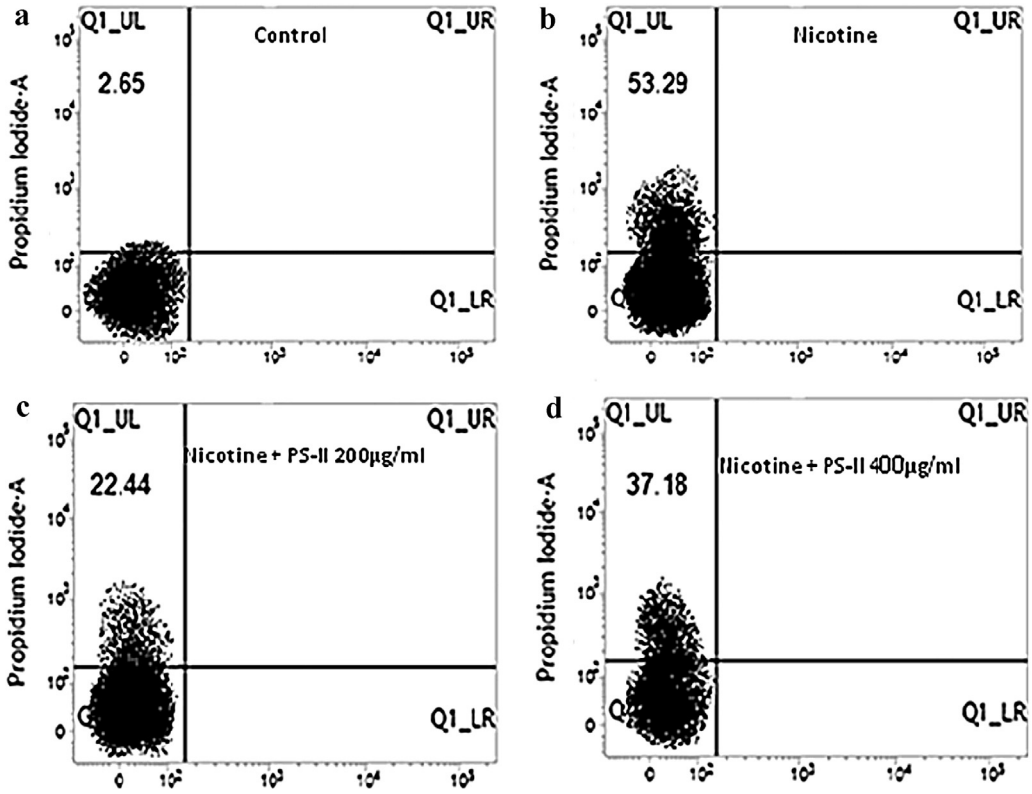
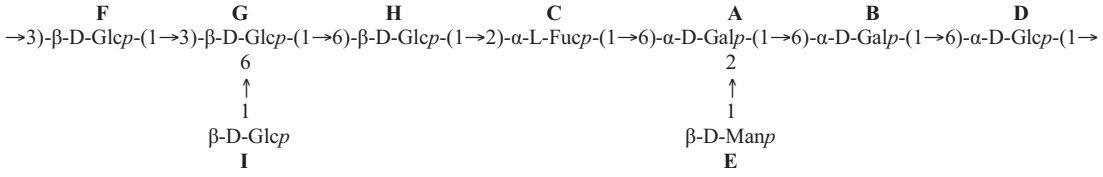


Fig. 6. FACS analysis of normal human lymphocytes at different concentration of PS-II. The propidium iodide was used for analysis of cell death and only PI (+) ve cells were counted.

4. Conclusion

A water soluble heteroglycan (PS-II), with the average molecular weight ~5.2×10^4 Da, was isolated from the alkaline extract of an edible mushroom *E. lividoalbum* (Kühner & Romagn) Kubička. The following structure was characterized by chemical analysis and 1D/2D NMR studies.



This material (PS-II) was observed as biologically non toxic and possess ameliorative role against toxic substances.

Acknowledgements

The authors are grateful to Dr. S. Roy, Director, IICB, Kolkata, for providing instrumental facilities. Mr. Barun Majumdar of Bose Institute, Kolkata, is acknowledged for preparing NMR spectra. One of the authors (P.M.) is thankful to CSIR, India for providing junior research fellowship (CSIR-09/599(0052)/2012-EMR-I).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.07.080.

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