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Structural elucidation of an immunoenhancing heteroglycan isolated from *Russula albonigra* (Krombh.) Fr.

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ABSTRACT

A water soluble heteroglycan (PS-II) of average molecular weight ~1.45×10^5 Da was isolated from the aqueous extract of an ectomycorrhizal edible mushroom, *Russula albonigra* (Krombh.) Fr. Structural characterization of PS-II was carried out using acid hydrolysis, methylation analysis, periodate oxidation, and 1D/2D NMR studies. Structural analysis revealed that PS-II was composed of terminal 2-O-methyl-Fuc*p*, terminal Man*p*, (1→2)-Fuc*p*, (1→3)-Glc*p*, (1→3,4)-Glc*p*, (1→6)-Gal*p*, and (1→2,6)-Gal*p* residues in a relative proportion of approximately 1:1:1:1:1:1:1. The proposed repeating unit of the PS-II had a backbone consisting of two (1→3)-β-D-glucopyranosyl, two (1→6)-α-D-galactopyranosyl, and one (1→2)-α-L-fucopyranosyl residues, out of which one (1→3)-β-D-glucopyranosyl residue was branched at *O*-4 position with terminal 2-*O*-methyl-α-L-fucopyranosyl and one (1→6)-α-D-galactopyranosyl residue was branched at *O*-2 position with terminal α-D-mannopyranosyl residue. This PS-II showed *in vitro* macrophage activation by NO production as well as splenocytes and thymocytes proliferation.

1. Introduction

Edible mushrooms have been used as a delicious food or food flavoring materials from ancient times. The important biologically active compounds like polysaccharides and polysaccharide–protein complexes from mushroom are being investigated widely for their immunomodulatory (Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Wasser & Weis, 1999) as well as anticancer activities (Ooi & Liu, 2000; Sun & Liu, 2009). Mushroom polysaccharides do not attack cancer cells directly but exhibit antitumor activity through the activation of T cells, B cells, NK cells and macrophage-dependent immune systems in living organism (Wasser, 2002). *Russula albonigra* (Krombh.) Fr., an ectomycorrhizal (Chakraborty, Mondal, Pramanik, Rout, & Islam, 2004; Lilleskov, Bruns, Dawson, & Camacho, 2009) edible and non-toxic (Pradhan, Banerjee, Roy, & Acharya, 2010) fungus, grows in symbiotic relationship with the roots of Sal (*Shorea robusta*) and other coniferous trees in the forest during rainy and autumn season throughout the world. Two water-soluble fractions (PS-I and PS-II) were isolated from the fruit bodies of the edible mushroom, *R. albonigra* (Krombh.) Fr. The first fraction, PS-I was identified as glucan which showed excellent immunoenhancing properties and reported recently (Nandi et al., 2012). The second fraction, PS-II was characterized as heteroglycan which contained α-L-fucose as an important bioactive natural compound. The important bioactive carbohydrate moiety α-L-fucose is essential for novel treatment approaches in human breast cancer (Jay, Gene, & Catherine, 2011) and also involved during *in vitro* fertilization (Jennifer, Jennifer, & Barry, 2010) for improvement of infertility treatments. Several heteropolysaccharides with α-L-fucose from different mushrooms like *Lentinus edodes* (Carbonero et al., 2008), *Hericium erinaceus* (Zhang, Fu, Xu, Sun, & Zhang, 2012), *Agaricus blazei* Murill (Liu & Sun, 2011), *Fomitella fraxinea* (Imaz.) (Cho, Koshino, Yu, & Yoo, 1998), *Agaricus bisporus* (Ruthes et al., 2013), *Phellinus baumii* Pilát (Ge, Zhang, & Sun, 2009), *Lentinus squarrosulus* (Mont.) Singer (Bhunia et al., 2010), *Calocybe indica* var. APK2 (Mandal et al., 2011) have been reported. Moreover, PS-II showed *in vitro* macrophage activation by NO production in RAW 264.7 cell line as well as splenocyte and thymocyte proliferations for which it can be used as immunostimulating material in future. The detailed structural investigation and study of immunostimulating properties of PS-II were carried out in the present investigation and reported herein.

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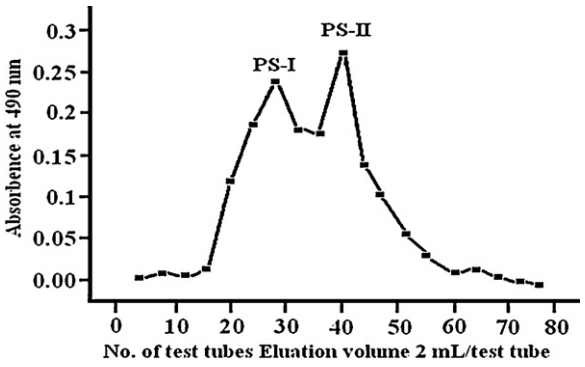


Fig. 1. Gel permeation chromatogram of crude polysaccharide isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr. using Sepharose 6B column.

2. Materials and methods

2.1. Isolation and purification of the crude polysaccharide

Fresh fruit bodies of the mushroom *R. albonigra* (Krombh.) Fr. (500 g) were washed, crushed and boiled with distilled water for 12 h. The aqueous extract was kept overnight at 4 °C and filtered through linen cloth. The crude polysaccharide was isolated and purified by the method described previously (Nandi et al., 2012). Finally, the crude polysaccharide (30 mg) was purified by gel permeation chromatography on a Sepharose 6B column and two homogeneous fractions (Fig. 1) were obtained *i.e.* PS-I (test tubes 20–32) and PS-II (test tubes 36–45), collected, and freeze-dried, yielding 10 mg and 7 mg of pure polysaccharide respectively. The PS-II was further purified by passing through Sepharose 6B column in several lots to yield 70 mg of pure polysaccharide.

2.2. General analyses

Optical rotation was measured on a Jasco Polarimeter model P-1020 at 28 °C. Paper chromatographic studies were performed on Whatmann Nos. 1 and 3 mm sheets. Solvent systems used were (X) BuOH–HOAc–H_2O (v/v/v, 4:1:5, upper phase) and (Y) EtOAc–pyridine–H_2O (v/v/v, 8:2:1). Silver nitrate in acetone (1.2%), methanol in sodium hydroxide solution, and 5% sodium thiosulphate solution were used as spray reagents (Hoffman, Lindberg, & Svensson, 1972). Alditol acetates of monosaccharides and the methyl sugar were analyzed by GC and GC–MS (Jansson, Kenne, Liedgren, Lindberg, & Lönngren, 1976). 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.

2.3. 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.

2.4. 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.

2.5. Constituent sugar analysis

The polysaccharide (PS-II, 3.0 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) in a round-bottomed flask at 100 °C for 18 h in a boiling water bath. The excess of acid was completely removed by co-distillation with water. The hydrolyzed product was divided into two parts. One part was examined by paper chromatography in solvent systems X and Y. Another part 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 (alditols) were acetylated with 1:1 pyridine–acetic anhydride in a boiling water bath for 1 h to give the alditol acetates, which were analyzed by GC and GC–MS.

2.6. Methylation analysis

The polysaccharide (PS-II) was methylated using the method described by Ciucanu and Kerek (1984). The polysaccharide (4.0 mg) was kept on P_2O_5 in a vacuum decicator for several days and then dissolved in 0.5 mL of distilled DMSO. Finely powdered anhydrous NaOH was added and stirring for 30 min. Then 1.0 mL CH_3I was added, stirring for 1.5 h. The methylated products were isolated by partitioning between $CHCl_3$ and H_2O (5:2, v/v). The organic layer containing products was washed with 3 mL water for three times and dried. The methylated products were then formolyzed with 90% formic acid (1 mL) at 100 °C for 1 h, and excess formic acid was evaporated by co-distillation with distilled water, and then reduced with $NaBH_4$, acetylated with (1:1) acetic anhydride–pyridine, and analyzed by GC and GC–MS.

2.7. Periodate oxidation

The polysaccharide (PS-II, 5 mg) was oxidized with 0.1 M sodium metaperiodate (2 mL) at 27 °C in the dark during 48 h. The excess periodate was destroyed by adding 1,2-ethanediol, and the solution was dialyzed against distilled water. The dialyzed material was reduced with $NaBH_4$ for 15 h and neutralized with acetic acid. The resulting material was obtained by co-distillation with methanol. The periodate-oxidized-reduced (Goldstein, Hay, Lewis, & Smith, 1965; Hay, Lewis, & Smith, 1965) material was divided into two portions. One portion was hydrolyzed with 2 M CF_3COOH and used for alditol acetate preparation. Another portion was methylated by Ciucanu and Kerek method (1984), and alditol acetate of the methylated product was prepared and analyzed by GC and GC–MS.

2.8. NMR studies

The pure polysaccharide (PS-II) was kept over P_2O_5 under vacuum for several days, and then exchanged with deuterium (Dueñas-Chaso et al., 1997) 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 ^{1}H and ^{13}C (both ^{1}H coupled and decoupled) NMR spectra were recorded at 30 °C. The ^{1}H 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). 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. The ^{13}C chemical shifts were measured using acetone as internal standard, fixing the methyl carbon signal at δ 31.05. The delay time in the HMBC experiment was 80 ms.

2.9. Preparation of LPS free polysaccharide for immunological studies

Prior to immunoactivation studies, LPS which may contaminate during isolation and purification process was removed from PS-II in order to discard the contribution of LPS in immunostimulation. The PS-II was passed through polymyxin-B agarose matrix (Sigma 160 and Aldrich, USA) packed in 2 mL column (1 cm × 2 cm), with 0.5 mL/min flow rate. It was equilibrated with 10 mM phosphate buffer, pH 7.4. The bacterial lipopolysaccharides (LPSs) were bound to the matrix and the unbound LPS free PS-II (LFPS-II) were eluted and collected for immunoenhancing studies.

2.10. Limulus amebocyte lysate (LAL) test

Limulus amebocyte lysate (LAL) test was carried out *in vitro* for detection of bacterial endotoxin. The test was performed using gel clot technique (Liu et al., 2009). Limulus amebocyte lysate (LAL) (G2125, sensitivity: 0.125 EU/mL) was purchased from Quantum Biotech, Mumbai, India. The control standard endotoxin (CSE) (code E0125) and water (code W1004) for the bacterial endotoxin test (BET) were provided by Quantum Biotech, Mumbai, India. Four tubes were taken, each containing 0.1 mL of LAL reagent. In two tubes, 0.1 mL LFPS-II aqueous solution were added, meanwhile 0.1 mL BET water and 0.1 mL CSE were added to the rest two tubes as negative control and positive control, respectively. All tubes were incubated for 1 h in a water bath at 37 °C. After the test tube was inverted 180° slowly, it is positive (+) if the gel in tube is not deformed and does not slip from the wall and a negative (−) test is characterized in the absence of a gel or by the formation of a viscous mass which does not hold when the tube is inverted. Test is invalid when positive control is (−) or negative control is (+).

2.11. 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 growing in Dulbecco's modified Eagle's medium (DMEM) and seeded in 96-well flat bottom tissue culture plate (Ohno, Hasimato, Adachi, & Yadomae, 1996; Sarangi, Ghosh, Bhutia, Mallick, & Maiti, 2006) at 5×10^5 cells/mL concentration (180 μL). Cells were left overnight for the attachment and then LFPS-II were treated with different concentrations (12.5, 25, 50, 100 or 200 μg/mL) to the wells. After 48 h of treatment culture supernatant of each well was 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* serotype typhimurium (Sigma, St. Louis, USA) was used as positive control.

2.12. 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). This study was approved by the ethics committee. 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 LFPS-II (12.5, 25, 50, 100 or 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 (Maiti et al., 2008; Mallick, Maiti, Bhutia, & Maiti, 2010; Shah et al., 2007).

3. Results and discussion

3.1. Isolation, purification, and chemical analysis of the polysaccharide

Five hundred grams of fresh mushroom fruit bodies were washed thoroughly with distilled water, boiled with water for 12 h followed by centrifugation, precipitation in EtOH, and finally freeze dried to yield 750 mg of crude polysaccharide. Fractionation of water soluble crude polysaccharide (30 mg) through Sepharose 6B column yielded two polysaccharides (Fig. 1). Fraction I (test tubes, 20–32) and fraction II (test tubes, 36–45) were collected and freeze dried, yielding purified polysaccharide PS-I (10 mg) and PS-II (7 mg), respectively. The pure PS-II showed a specific rotation $[\alpha]_D^{28}$ +34.5 (*c* 0.05, water). The molecular weight (Hara et al., 1982) of PS-II was estimated as ~1.45×10^5 Da from a calibration curve prepared with standard dextrans.

The sugar analysis of PS-II by paper chromatography and GC of alditol acetates showed that it was found to consist of glucose, galactose, manose, 2-*O*Me-fucose, and fucose in a molar ratio of nearly 2:2:1:1:1. The absolute configuration of the monosaccharides was determined by the method of Gerwig et al. (1978) and it was found that glucose, galactose, and manose had the D configuration but 2-*O*Me-fucose and fucose were present in the L configuration. The mode of linkages of the PS-II was determined by the methylation analysis using the method described by Ciucanu and Kerek (1984) followed by hydrolysis and alditol acetate conversion. The GC–MS analysis of partially methylated alditol acetates revealed the presence of 6-deoxy-2,3,4-Me_3-Fuc, 2,3,4,6-Me_4-Man, 6-deoxy-3,4-Me_2-Fuc, 2,4,6-Me_3-Glc, 2,6-Me_2-Glc, 2,3,4-Me_3-Gal, and 3,4-Me_2-Gal in a nearly equal molar ratio. The above result indicated that non reducing end 2-*O*Me-L-fucopyranosyl, terminal D-manopyranosyl, (1→2)-linked L-fucopyranosyl, (1→3)-linked D-glucopyranosyl, (1→3,4)-linked D-glucopyranosyl, (1→6)-linked D-galactopyranosyl, and (1→2,6)-linked D-galactopyranosyl moieties were present in the PS-II in a nearly equal molar ratio (Table 1a). These linkages were further confirmed by periodate

Table 1a
GC–MS analysis of methylated polysaccharide (PS-II) isolated from *Russula albonigra* (Krombh.) Fr.

	Methylated sugars	Linkage type	Major mass fragments (*m*/*z*)
Rasidue **A**	3,4-Me_2-Gal	→2,6)-α-D-Gal*p*-(1→	43,71,87,99,129,159,173,189,233
Rasidue **B**	2,3,4-Me_3-Gal	→6)-α-D-Gal*p*-(1→	43,71,87,101,117,129,161,173,189,233
Rasidue **C**	2,3,4,6-Me_4-Man	α-D-Man*p*-(1→	43,59,71,87,101,117,129,145,161,205
Rasidue **D**	2,3,4-Me_3-Fuc	α-L-Fuc*p*-(1→	43,72,89,101,115,117,131,161,175
Rasidue **E**	3,4-Me_2-Fuc	→2)-α-L-Fuc*p*-(1→	43,59,71,89,99,115,129,131,173,189
Rasidue **F**	2,6-Me_2-Glc	→3,4)-β-D-Glc*p*-(1→	43,58,74,87,101,117,129,143,159,173,189,233
Rasidue **G**	2,4,6-Me_3-Glc	→3)-β-D-Glc*p*-(1→	43,74,87,101,117,129,143,161,173,203,217,233

Table 1b
GC–MS analysis of methylated polysaccharide (PS-II) after oxidation of sodium periodate isolated from *Russula albonigra* (Krombh.) Fr.

	Methylated sugars	Linkage type	Major mass fragments (*m*/*z*)
Rasidue **F**	2,6-Me_2-Glc	→3,4)-β-D-Glc*p*-(1→	43,58,74,87,101,117,129,143,159,173,189,201,233
Rasidue **G**	2,4,6-Me_3-Glc	→3)-β-D-Glc*p*-(1→	43,58,74,87,101,117,129,143,161,173,203,217,233

oxidation experiment. GC analysis of alditol acetates of periodate-oxidized (Goldstein et al., 1965; Hay et al., 1965), reduced, and hydrolyzed products showed the presence of only D-glucose, indicating that the D-galactose, D-manose, 2-*O*-Me-L-fucose, and L-fucose moieties were consumed during oxidation. The GC and GC–MS analysis of periodate-oxidized and methylated (Abdel-Akher & Smith, 1950) PS-II showed the presence of 2,4,6-Me_3-Glc and 2,6-Me_2-Glc in a molar ratio of nearly 1:1 (Table 1b). This observation clearly indicated that (1→3)-linked and (1→3,4)-linked D-glucopyranosyl moieties remain unaffected whereas all other moieties were consumed during oxidation, which further confirmed the mode of linkages present in the PS-II.

3.2. NMR and structural analysis of the polysaccharide (PS-II)

The ^{1}H NMR (500 MHz) spectrum (Fig. 2a, Table 1c) at 30 °C showed five signals in the anomeric region at δ 5.10, 5.04, 4.97, 4.52, and 4.50 in a ratio of nearly 1:2:2:1:1. Hence, the signals at δ 5.10, 4.52, and 4.50 indicated the presence of only one residue while the signals at δ 5.04 and 4.97 corresponded to two residues. The sugar residues were designated as **A**–**G** according to their decreasing anomeric proton chemical shifts. In the ^{13}C (Fig. 2b, Table 1c) and DEPT-135 (Fig. 2c) NMR (125 MHz) spectrum at 30 °C five anomeric signals appeared at δ 102.5, 102.2, 100.8, 98.0, and 97.8 in a ratio of nearly 1:2:1:1:2. Based on the result of the HSQC experiment (Fig. 2d), the anomeric carbon signals at δ 102.5, 100.8, and 98.0 corresponded to the anomeric carbons of **G**, **A**, and **B** residues, respectively whereas the signal at δ 102.2 corresponded to the anomeric carbon of **C** and **F** residues while the peak at δ 97.8 was correlated to the anomeric carbon of **D** and **E** residues. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY, HSQC (Fig. 2d and e) experiments. Coupling constants were measured from DQF-COSY spectrum.

Based on the coupling constant, $J_{H\text{-}1,H\text{-}2} \sim 3.1$ Hz and $J_{C\text{-}1,H\text{-}1} \sim 171$ Hz the residues **A** and **B** were established as α-anomer. A large $J_{H\text{-}2,H\text{-}3}$ (~9 Hz) and small $J_{H\text{-}3,H\text{-}4}$ (<5 Hz) indicated that those were D-galactosyl unit. In residue **A**, the downfield shift of C-2 (δ 75.6) and C-6 (δ 66.5) with respect to standard values of methyl glycosides (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that the moiety **A** was (1→2,6)-linked unit. On the other hand, in residue **B**, the downfield shift of C-6 (δ 66.7) with respect to standard values of methyl glycosides indicated that it was (1→6)-linked unit. The linking at C-6 of the both residue **A** and **B** were further confirmed from DEPT-135 spectrum (Fig. 2c). Hence, these observations confirmed that residue **A** was a (1→2,6)-linked-α-D-galactopyranosyl moiety and the residue **B** was a (1→6)-linked-α-D-galactopyranosyl moiety.

The anomeric proton signal of residue **C** at δ 5.04 with low values of $J_{H\text{-}1,H\text{-}2}$, $J_{H\text{-}2,H\text{-}3}$ (~3.5 Hz) and $J_{C\text{-}1,H\text{-}1}$ of ~170 Hz clearly indicated that it was a α-linked mannopyranosyl moiety. This was further confirmed from the large coupling constant value $J_{H\text{-}3,H\text{-}4}$ ~7.5 Hz and $J_{H\text{-}4,H\text{-}5}$ ~10 Hz. The carbon chemical shifts of residue **C** from C-1 to C-6 corresponded nearly to the standard values of methyl

Table 1c
The ^{1}H[a] and ^{13}C[b] NMR chemical shifts for the polysaccharide (PS-II) isolated from *Russula albonigra* (Krombh.) Fr. 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	-*O*Me
→2,6)-α-D-Gal*p*-(1→	5.10	3.87	4.00	4.12	4.20	3.67[c], 3.67[d]	
A	100.8	75.6	69.8	69.5	69.0	66.5	
→6)-α-D-Gal*p*-(1→	5.04	3.82	3.97	4.12	4.20	3.64[c], 3.64[d]	
B	98.0	70.5	70.0	69.5	69.0	66.7	
α-D-Man*p*-(1→	5.04	4.05	3.86	3.54	3.82	3.71[c], 3.88[d]	
C	102.2	70.4	72.0	67.7	73.0	61.2	
α-L-Fuc*p*-2*O*Me (1→	4.97	3.80	3.88	4.06	4.21	1.24	3.43
D	97.8	78.2	69.5	70.2	67.1	15.6	56.0
→2)-α-L-Fuc*p*-(1→	4.97	3.80	3.88	4.06	4.21	1.24	
E	97.8	78.0	69.5	70.2	67.1	15.6	
→3,4)-β-D-Glc*p*-(1→	4.52	3.50	3.73	3.65	3.48	3.70[c], 3.90[d]	
F	102.2	73.2	84.5	75.2	75.6	60.8	
→3)-β-D-Glc*p*-(1→	4.50	3.35	3.74	3.39	3.46	3.70[c], 3.90[d]	
G	102.5	73.2	85.0	69.6	76.0	60.8	

[a] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.70 ppm 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 ppm at 30 °C.
[c] Interchangeable.
[d] Interchangeable.

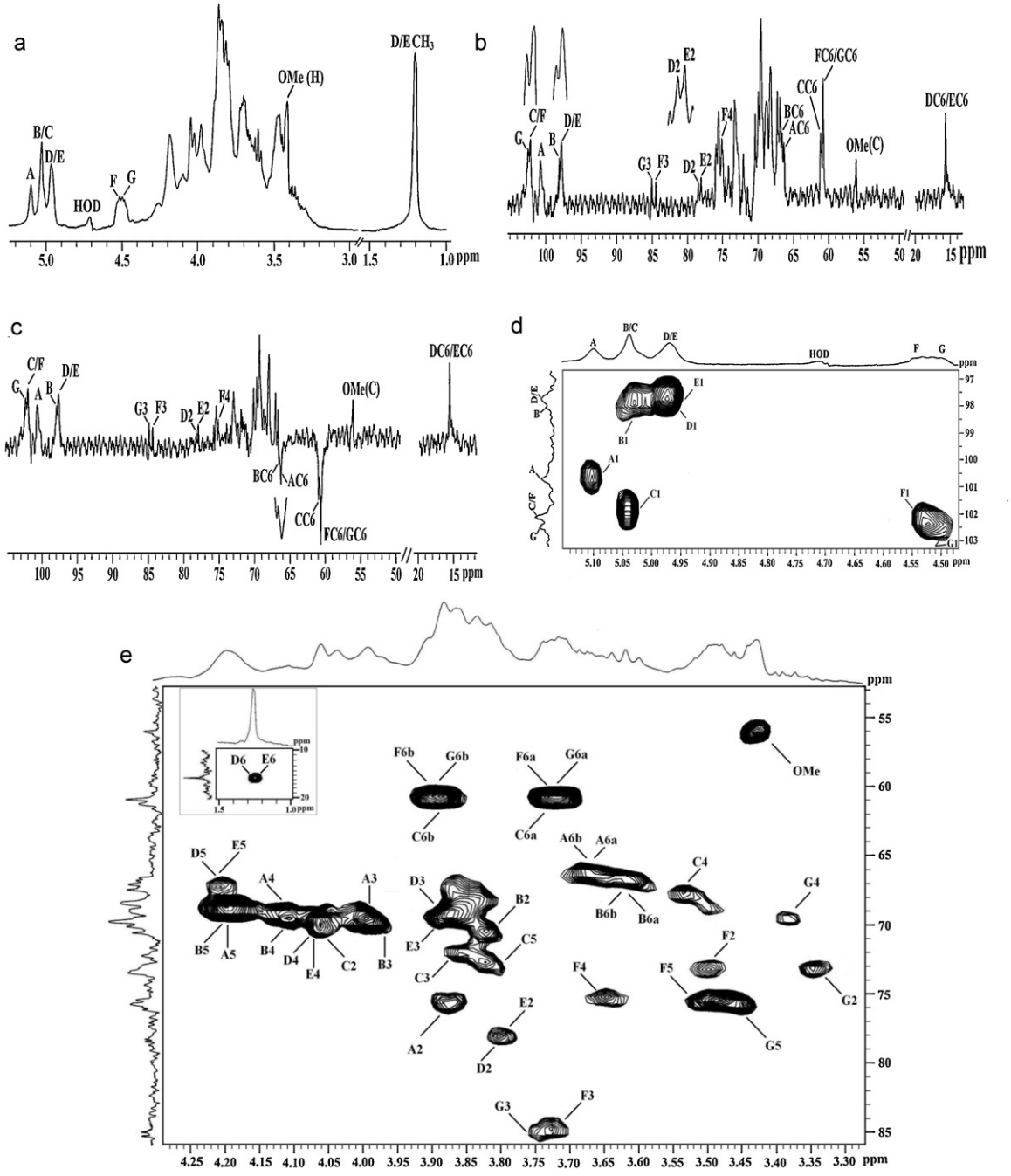


Fig. 2. (a) ^{1}H NMR spectrum (500 MHz, D_2O, 30 °C) of the PS-II isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr. (b) ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of the PS-II isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr. (c) DEPT-135 spectrum (D_2O, 30 °C) of the PS-II isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr. The HSQC spectrum (D_2O, 30 °C) of (d) anomeric part and (e) other than anomeric part of the PS-II isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr.

glycoside of α-D-mannose indicating residue **C** was terminal α-D-mannopyranosyl moiety.

Residues **D** and **E** were assigned to L-fucopyranosyl unit. This was strongly supported by the appearance of a proton signal at δ 1.24, carbon signal at δ 15.6 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 both residues at δ 4.97 and 97.8, respectively, as well as the coupling constant value $J_{H\text{-}1,H\text{-}2}$ ~3.75 Hz clearly indicated that those were α-anomer. The anomeric configuration was further confirmed by ^{1}H–^{13}C coupling constant $J_{C\text{-}1,H\text{-}1}$ ~171 Hz. In residue **D**, the downfield shift of C-2 (δ 78.2) with respect to standard values indicated that the moiety **D** was linked at C-2 position with –OCH_3 group. This was further confirmed by the appearance of cross coupling between the methoxy proton (δ 3.43) and the C-2 atom of residue **D** and between methoxy carbon (δ 56.0) and its H-2 atom in the HMBC experiment (Fig. 4, Table 3). On the other hand, the downfield shift of C-2 (δ 78.0) with respect to standard values of methyl glycosides (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that the residue **E** was also linked at C-2 position with residue **A** which further confirmed by the ROESY (Fig. 3, Table 2) as well as HMBC experiment (Fig. 4, Table 3). So the moiety **E** was (1→2)-linked unit. The C-2 chemical shift values of the residues

Table 2
ROE data for the polysaccharide (PS-II) isolated from *Russula albonigra* (Krombh.) Fr. in D_2O at 30 °C.

Glycosyl residue	Anomeric proton	ROE contact protons	
	δ	δ	Residue, atom
→2,6)-α-D-Gal*p*-(1→	5.10	3.87	**A**H-2
A		3.80	**E**H-2
→6)-α-D-Gal*p*-(1→	5.04	3.82	**B**H-2
B		3.74	**G**H-3
α-D-Man*p*-(1→	5.04	4.05	**C**H-2
C		3.87	**A**H-2
α-L-Fuc*p*-2*O*Me (1→	4.97	3.80	**D**H-2
D		3.65	**F**H-4
→2)-α-L-Fuc*p*-(1→	4.97	3.80	**E**H-2
E		3.64	**B**H-6a/6b
→3,4)-β-D-Glc*p*-(1→	4.52	3.73	**F**H-3
F		3.48	**F**H-5
		3.67	**A**H-6a/6b
→3)-β-D-Glc*p*-(1→	4.50	3.74	**G**H-3
G		3.46	**G**H-5
		3.73	**F**H-3

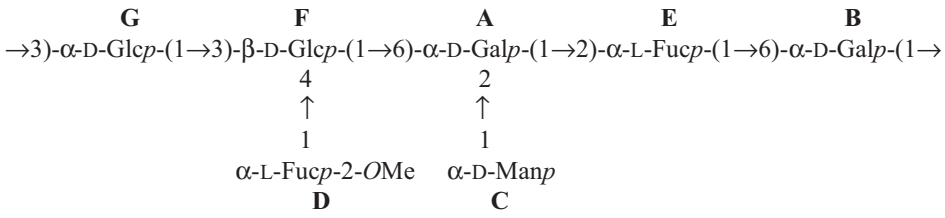
D and **E** were slightly different due to slight difference in chemical environment while other carbon signals remain almost same. Thus, it may be conclude that the residue **D** was a non reducing end 2-*O*Me-α-L-fucopyranosyl moiety and the residue **E** was a (1→2)-linked-α-L-fucopyranosyl moiety.

Residues **F** and **G** were established as β-anomer from coupling constant values $J_{H\text{-}1,H\text{-}2}$ (~8 Hz), and $J_{C\text{-}1,H\text{-}1}$ (~160 Hz) and the large coupling constant values $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ (~10 Hz) of the residues **F** and **G** confirmed their glucopyranosyl moiety. The downfield shift of C-3 (δ 84.5) and C-4 (δ 75.2) with respect to standard values indicated that moiety **F** was linked at C-3 and C-4. These observations indicated that **F** was (1→3,4)-linked-β-D-glucopyranosyl moiety. On the other hand, the downfield shift of C-3 (δ 85.0) with respect to standard values of methyl glycosides (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that moiety **G** was linked at C-3. Thus it may be concluded that **G** was (1→3)-linked-β-D-glucopyranosyl moiety. Since, the residue **F** was rigid part in comparison to that of residue **G**. So the C-3 (δ 84.5) value of residue **F** appeared at the upfield region than that of the C-3 (δ 85.0) of residue **G**.

The sequences of glycosyl moieties were determined from ROESY (Fig. 3, Table 2) as well as NOESY (not shown) experiments. In ROESY experiment, the inter-residual contacts **A**H-1/**E**H-2; **B**H-1/**G**H-3; **C**H-1/**A**H-2; **D**H-1/**F**H-4; **E**H-1/**B**H-6a, **B**H-6b; **F**H-1/**A**H-6a, **A**H-6b and **G**H-1/**F**H-3 along with some other intra residual contacts were also observed. The above ROESY connectivities established the following sequences:

A-(1→2)-**E**; **B**-(1→3)-**G**; **C**-(1→2)-**A**;
D-(1→4)-**F**; **E**-(1→6)-**B**; **F**-(1→6)-**A**; **G**-(1→3)-**F**;

A long range HMBC experiment was carried out to confirm the ROESY connectivities. In HMBC experiment (Fig. 4, Table 3), inter-residual couplings **A**H-1/**E**C-2, **A**C-1/**E**H-2, **B**H-1/**G**C-3, **B**C-1/**G**H-3, **C**H-1/**A**C-2, **C**C-1/**A**H-2, **D**H-1/**F**C-4, **D**C-1/**F**H-4, **E**H-1/**B**C-6, **E**C-1/**B**H-6a, **B**H-6b, **F**H-1/**A**C-6, **F**C-1/**A**H-6a, **A**H-6b, **G**H-1/**F**C-3, and **G**C-1/**F**H-3 along with some intra residual couplings were also observed. Thus, the HMBC and ROESY connectivities confirmed the presence of heptasaccharide repeating unit in the PS-II isolated from an edible mushroom *R. albonigra* (Krombh.) Fr. which is shown as:



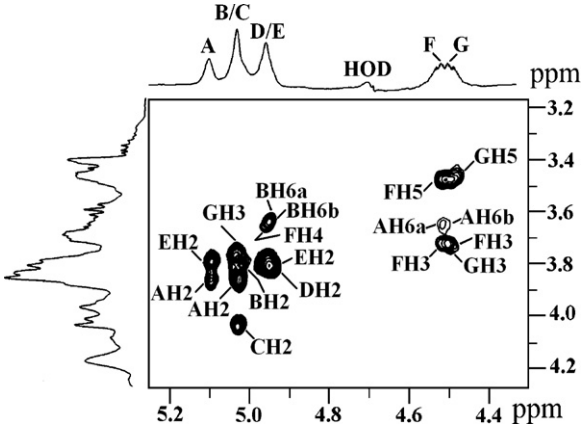


Fig. 3. The part of ROESY spectrum of the PS-II isolated an edible mushroom *Russula albonigra* (Krombh.) Fr. The ROESY mixing time was 300 ms.

3.3. Immunological studies of LPS free polysaccharide (LFPS-II)

A negative (−) LAL test indicated that LFPS-II which was obtained after passing the PS-II through polymixin-B matrix, was free from bacterial endotoxin. Immunological studies were also investigated with the LFPS-II. Macrophage activation by LFPS-II was observed *in vitro*. Upon treatment with different concentrations of the LFPS-II, enhanced production of NO was observed in a dose-dependent manner with optimum production of 18 μM NO per 5×10^5 macrophages at 100 μg/mL of the LFPS-II

Table 3
The significant $^3J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide (PS-II) isolated from *Russula albonigra* (Krombh.) Fr. in D_2O at 30 °C.

Residues	Sugar linkage	H-1/C-1	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	→2,6)-α-D-Gal*p*-(1→	5.10	78.0	**E**	C-2
			75.6	**A**	C-2
		100.8	3.80	**E**	H-2
			3.87	**A**	H-2
B	→6)-α-D-Gal*p*-(1→	5.04	85.0	**G**	C-3
			70.5	**B**	C-2
		98.0	3.74	**G**	H-3
			3.82	**B**	H-2
C	α-D-Man*p*-(1→	5.04	75.6	**A**	C-2
		102.2	3.87	**A**	H-2
D	α-L-Fuc*p*-2OMe (1→	4.97	75.2	**F**	C-4
		97.8	3.65	**F**	H-4
			3.80	**D**	H-2
E	→2)-α-L-Fuc*p*-(1→	4.97	66.7	**B**	C-6
			78.0	**E**	C-2
		97.8	3.64	**B**	H-6a/6b
			3.80	**E**	H-2
F	→3,4)-β-D-Glc*p*-(1→	4.52	66.5	**A**	C-6
			73.2	**F**	C-2
		102.2	3.67	**A**	H-6a/6b
			3.50	**F**	H-2
			3.73	**F**	H-3
			3.48	**F**	H-5
G	→3)-β-D-Glc*p*-(1→	4.50	84.5	**F**	C-3
		102.5	3.73	**F**	H-3
			3.74	**G**	H-3
			3.46	**G**	H-5
		-OCH_3 δ_H/δ_C			
D	α-L-Fuc*p*-2OMe(1→	3.43	78.2	**D**	C-2
		56.0	3.80	**D**	H-2

(Fig. 5a). Lentinan obtained from *L. edodes* (Berk.) Sing., inhibit the 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 LFPS-II 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

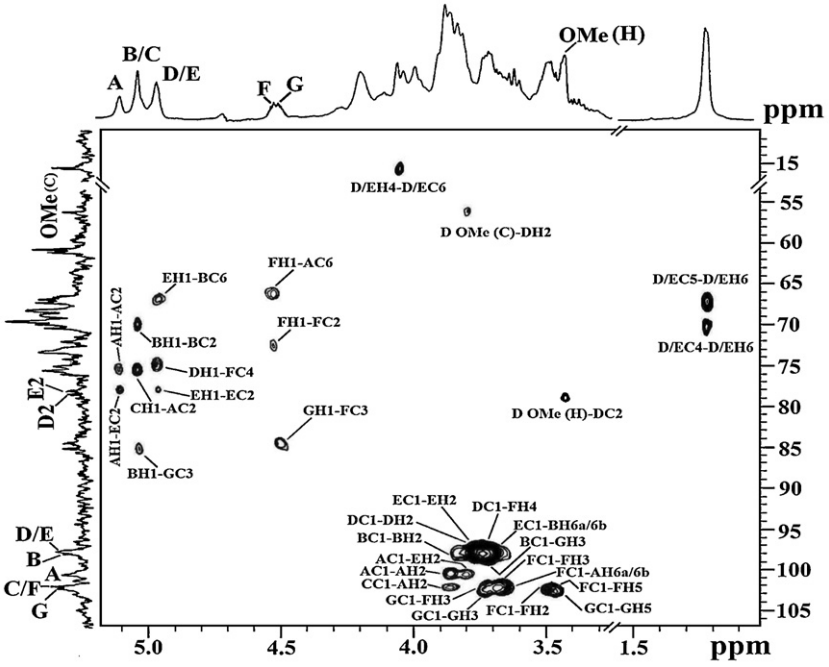
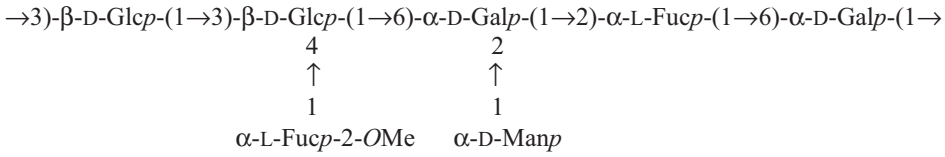


Fig. 4. The part of HMBC spectrum of the PS-II isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr. The delay time in the HMBC experiment was 80 ms.

immunostimulation. The LFPS-II was found to stimulate splenocytes and thymocytes as shown in Fig. 5b and c, respectively and the asterisks on the columns indicate the statistically significant differences compared to PBS control. At 25 μg/mL of the LFPS-II, splenocyte proliferation index was found to be maximum as compared to other concentrations. Hence, 25 μg/mL of the LFPS-II can be considered as efficient splenocyte stimulators. Again 50 μg/mL of this same sample showed maximum effect on thymocyte proliferation. The splenocyte and thymocyte proliferation index as compared to PBS control if closer to 1 or below indicates low stimulatory effect on immune system. It is noteworthy to mention that several α-L-fucose containing heteroglycan (Bhunia et al., 2010; Dey et al., 2013; Mandal et al., 2011) also showed immunoenhancing properties as reported earlier by our group.

4. Conclusions

A water-soluble heteroglycan (PS-II) was isolated from aqueous extract of an edible mushroom *R. albonigra* (Krombh.) Fr. This PS-II showed splenocyte, thymocyte, and macrophage activation. So, it may be used as a source of natural immunostimulant and it also contained naturally important bioactive carbohydrate moiety α-L-fucose which have large utility in medicinal chemistry. Architectural details of the repeating unit of the PS-II obtained from *R. albonigra* (Krombh.) Fr. was determined from chemical and NMR studies (^{1}H, ^{13}C, DEPT-135, TOCSY, DQF-COSY, NOESY, ROESY, HSQC, and HMBC) and the structure of the repeating unit of the PS-II was established as:



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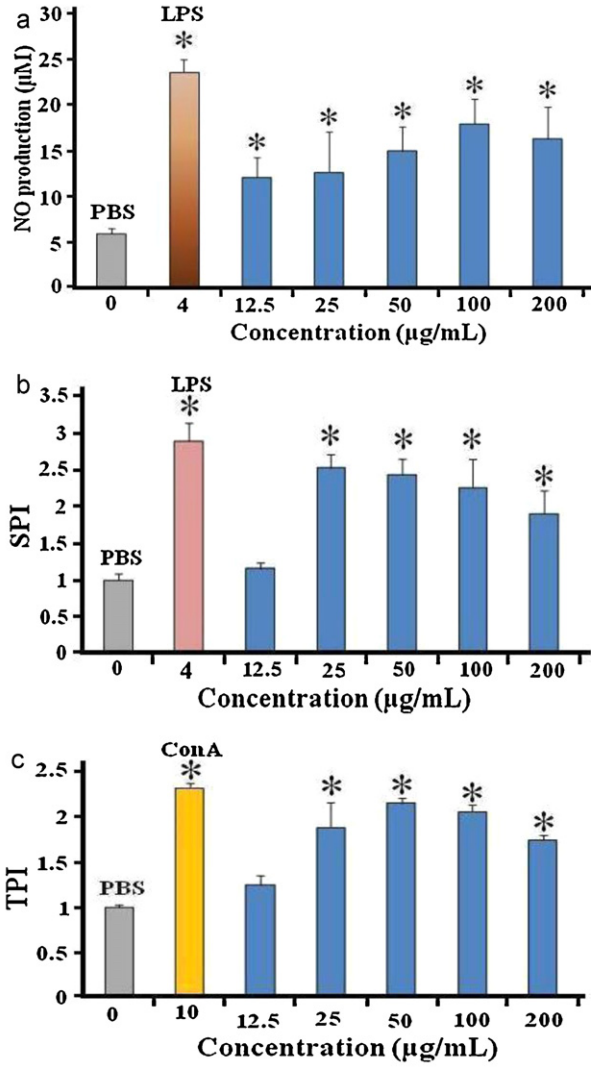

Fig. 5. (a) Activation of RAW 264.7 macrophage cells with different concentrations of the LPS free PS-II (LFPS-II) in terms of NO production. Effect of different concentrations of the LPS free PS-II (LFPS-II) on proliferation of (b) splenocyte and (c) thymocyte (asterisks indicate the statistically significant compared to the PBS control).

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