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Note

Chemical analysis of an immunoenhancing water-soluble polysaccharide of an edible mushroom, *Pleurotus florida* blue variant

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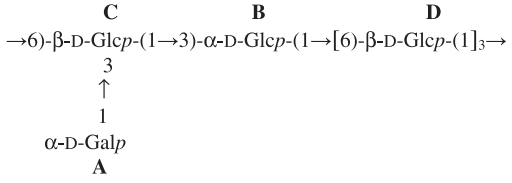
ABSTRACT

An immunoenhancing polysaccharide isolated from the aqueous extract of the fruit bodies of the mushroom, *Pleurotus florida* blue variant, was found to consist of D-glucose and D-galactose in a molar ratio of nearly 5:1. On the basis of sugar analysis, methylation analysis, periodate oxidation, Smith degradation, and NMR studies (^{1}H, ^{13}C, DEPT-135, DQF-COSY, TOCSY, NOESY, ROESY, HMQC, and HMBC), the structure of the repeating unit of the polysaccharide was established as:



The molecule activated macrophages, splenocytes, and thymocytes.

Mushrooms are used as food materials with unique flavor and texture, and are recognized as an important source of biologically active compounds,[1] especially polysaccharides for their immunomodulatory and anticancer properties.[2] Among different types of oyster mushrooms of the genus *Pleurotus*, mainly *Pleurotus sajor-caju*,[3–5] *Pleurotus ostreatus*,[6] *Pleurotus citrinopileatus*,[7] and *Pleurotus florida*,[8] are reported as commonly available edible mushrooms that contain immunomodulating and antitumor materials. The hot water extract of the edible mushroom *P. florida* was found to consist of different water-soluble and water-insoluble polysaccharides, which were isolated by our group and reported.[9–12] Water soluble and insoluble polysaccharides were also isolated from *P. florida*, cultivar (Assam Florida) and reported recently.[13,14] We report herein a new type of water-soluble polysaccharide isolated from the hot water extract of *P. florida*, blue variant. The main objective of this study was to investigate any differences that arise in the constituents of polysaccharide of this variant from its original mushroom *P. florida*. The present polysaccharide is a galactosyl-glucan consisting of D-glucose and D-galactose in a molar ratio of nearly 5:1. We report the detailed structural characterization of the molecule as well as some of its immunoenhancing properties, such as macrophage, splenocyte, and thymocyte activation.

The polysaccharide (PS) on acid hydrolysis by 2 M CF_3COOH, followed by GLC analysis showed the presence of glucose and galactose in a molar ratio of nearly 5:1. The absolute configuration[15] of the sugar residues was determined as **D**. Its molecular mass,[16] determined from a calibration curve prepared with standard dextrans, was found to be ~1.74×10^5 Da. The pure polysaccharide showed a specific rotation of $[\alpha]_D$ −4.11 (*c* 0.11, H_2O, 26.8 °C). The linkages in the polysaccharide were determined by methylation analysis using the method of Ciucanu and Kerek,[17] followed by hydrolysis and alditol acetate preparation. The GLC and GLC–MS analyses of partially methylated alditol acetates revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol (*m/z* 101, 117, 129, 161); 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol (*m/z* 43, 101, 117, 129, 161); 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol (*m/z* 99, 101, 117, 129); and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol (*m/z* 43, 87, 117, 129, 189) in a molar ratio of nearly 1:1:3:1. These results indicated that terminal D-galactopyranosyl, (1→3)-linked D-glucopyranosyl, (1→6)-linked D-glucopyranosyl, and (1→3, 6)-linked

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D-glucopyranosyl moieties were present in the polysaccharide in a molar ratio of nearly 1:1:3:1. Further, GLC analysis of the alditol acetates of the periodate-oxidized,[18,19] $NaBH_4$-reduced PS showed the presence of D-glucose only. The periodate-oxidized, $NaBH_4$-reduced, methylated PS exhibited the presence of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol in a molar ratio of nearly 1:1. These results indicated that (1→6)-linked D-glucopyranosyl and terminal D-galactopyranosyl moieties were destroyed during oxidation. Hence, these observations confirmed the mode of linkages of these sugar moieties present in the PS.

The proton NMR spectrum (500 MHz, Fig. 1) of the PS at 27 °C showed four signals in the anomeric region at δ 5.12, 4.98, 4.51, and 4.50 ppm in a molar ratio of nearly 1:1:1:3. The anomeric signals at δ 5.12, 4.98, 4.51, and 4.50 ppm were assigned as **A**, **B, C,** and **D**, respectively. In the ^{13}C NMR spectrum (125 MHz, Fig. 2) at 27 °C four anomeric signals appeared at δ 103.4, 103.2, 98.6, and 98.3 ppm in a molar ratio of nearly 3:1:1:1, corresponded to anomeric carbons of **D**, **C**, **A,** and **B** residues, respectively. The response of the signal at δ 103.4 ppm was almost thrice to those of other signals, which indicated the presence of three units of residue **D**. All the ^1H and ^{13}C signals (Table 1) were assigned using DQF-COSY, TOCSY, and HMQC experiments. The proton coupling constants were measured from DQF-COSY experiment.

Residue **A** was assigned as terminal D-galactopyranosyl unit. The *galacto* configuration was assigned from the large $J_{H\text{-}2,H\text{-}3}$ coupling constant (~8 Hz) and relatively small $J_{H\text{-}3,H\text{-}4}$ coupling constant (~3 Hz). The α-configuration of residue **A** (δ 5.12 ppm) was assigned from $J_{H\text{-}1,H\text{-}2}$ coupling constant (~3 Hz) and $J_{C\text{-}1,H\text{-}1}$ coupling constant (~171 Hz). The carbon signal at δ 98.6 ppm was assigned to C-1 of residue **A**. The signals from C-1 to C-6 corresponded nearly to the standard values of methyl glycosides.[20,21] These results indicated that residue **A** was an α-linked, terminal D-galactopyranosyl moiety.

The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values (~10 Hz) of residues **B**, **C**, and **D** confirmed their D-glucopyranosyl configuration. Residue **B** had an anomeric proton signal at δ 4.98 ppm, and the coupling constants $J_{H\text{-}1,H\text{-}2}$ (~3 Hz) and $J_{C\text{-}1,H\text{-}1}$ (~170 Hz), indicating its α-configuration. The carbon signal at δ 98.3 ppm was assigned to C-1 of residue **B**. The downfield shift of C-3 (δ 80.4 ppm) with respect to standard value indicated that it was (1→3)-linked residue. The other values of carbons corresponded nearly to the standard values. Thus, residue **B** was a (1→3)-linked-α-D-glucopyranosyl moiety.

The coupling constants $J_{H\text{-}1,H\text{-}2}$ (~8 Hz) and $J_{C\text{-}1,H\text{-}1}$ (~160 Hz) of residues **C** and **D** and in addition to their anomeric proton signals at δ 4.51 and 4.50 ppm, respectively, indicated their β-configuration. The anomeric carbon signals of residues **C** and **D** appeared at δ 103.2 and 103.4 ppm, respectively. The downfield shifts of C-3 (δ 85.0 ppm) and C-6 (δ 68.9 ppm) of residue **C** with respect to the standard values indicated that it was (1→3, 6)-linked moiety. The downfield shift of C-6 (δ 69.2 ppm) of residue **D** indicated that it was (1→6)-linked moiety. The linking of residues, **C** and **D** at C-6 was also confirmed from DEPT-135 spectrum (Fig. 3). These observations indicated that residue **C** was a (1→3, 6)-linked-β-D-glucopyranosyl moiety and residue **D** was a (1→6)-linked-β-D-glucopyranosyl moiety.

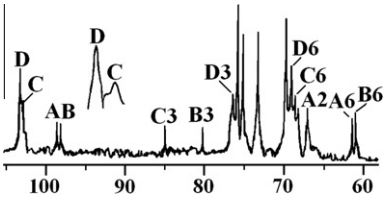


Figure 2. ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of the polysaccharide, isolated from *Pleurotus florida* blue variant.

The sequence of glycosyl residues of the PS was determined from NOESY as well as ROESY experiments followed by confirmation with an HMBC experiment. In NOESY experiment (Fig. 4, Table 2) the inter-residual contacts from **A**H-1 to **C**H-3, **B**H-1 to **D**H-6, **C**H-1 to **B**H-3, and **D**H-1 to **C**H-6 and **D**H-6 established the following sequences;

A **C**
α-D-Gal*p*-(1→3)-β-D-Glc*p*-(1→
6
↑

;

B **D**
→3)-α-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→

C **B**
→6)-β-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→
3
↑

;

D **C**
→6)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→
3
↑

D **D**
→6)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→

In the HMBC spectrum (Fig. 5, Table 3) the cross-peaks of both anomeric protons and carbons of each of the glycosyl residues were examined, and both intra and inter-residue connectivities were observed. Cross-peaks were found between H-1 of residue **A** and C-3 of residue **C** (**A**H-1, **C**C-3); C-1 of residue **A** and H-3 of residue **C** (**A**C-1, **C**H-3) with other intra-residue coupling between H-1 of residue **A** with its own C-2 atom (**A**H-1, **A**C-2). Similarly, cross-peaks between H-1 of residue **B** and C-6 of residue **D** (**B**H-1, **D**C-6); C-1 of residue **B** and H-6a, H-6b of residue **D** (**B**C-1, **D**H-6a; **B**C-1, **D**H-6b) were observed. Cross-peaks were also observed between H-1 of residue **D** and C-6 of residue **C** (**D**H-1**, C**C-6**);** C-1 of residue **D** and H-6a, H-6b of residue **C** (**D**C-1, **C**H-6a; **D**C-1, **C**H-6b) with other intra-residue coupling between H-1 and C-1 of residue **D** with its own C-2, C-6 atoms and H-2, H-3, H-6a

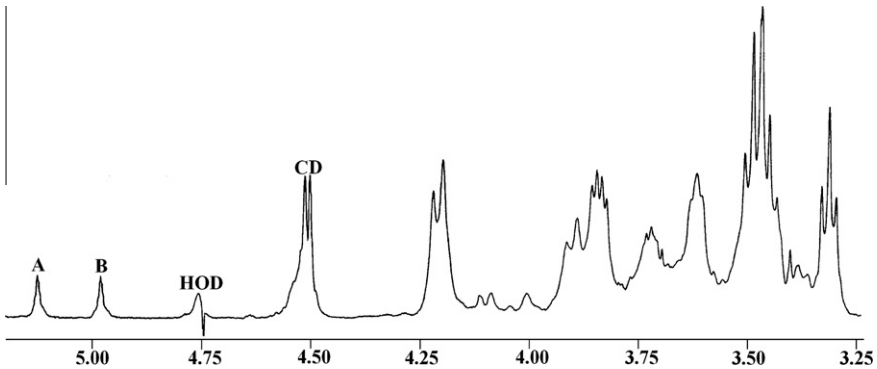


Figure 1. ^1H NMR spectrum (500 MHz, D_2O, 27 °C) of the polysaccharide, isolated from *Pleurotus florida* blue variant.

Table 1
The ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts for the polysaccharide isolated from *Pleurotus florida* blue variant in D_2O at 27 °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
α-D-Gal*p*-(1→	5.12	3.89	3.92	4.00	4.08	3.73[c], 3.92[d]
A	98.6	67.2	68.5	69.9	69.9	61.4
→3)-α-D-Glc*p*-(1→	4.98	3.83	3.86	3.82	3.89	3.69[c], 3.89[d]
B	98.3	69.7	80.4	69.2	73.4	61.1
→3,6)-β-D-Glc*p*-(1→	4.51	3.50	3.72	3.44	3.62	3.84[c], 4.22[d]
C	103.2	73.3	85.0	68.6	75.3	68.9
→6)-β-D-Glc*p*-(1→	4.50	3.31	3.48	3.46	3.61	3.83[c], 4.19[d]
D	103.4	73.5	76.6	69.9	75.9	69.2

[a] The values of chemical shifts were recorded keeping HOD signal fixed at δ 4.75 ppm at 27 °C.
[b] The values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 27 °C.
[c,d] Interchangeable.

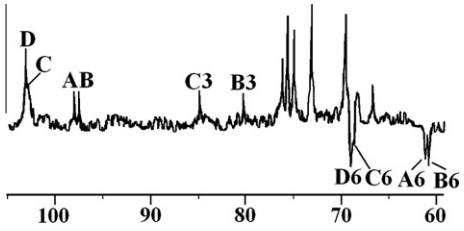


Figure 3. DEPT-135 spectrum (D_2O, 27 °C) of the polysaccharide, isolated from *Pleurotus florida* blue variant.

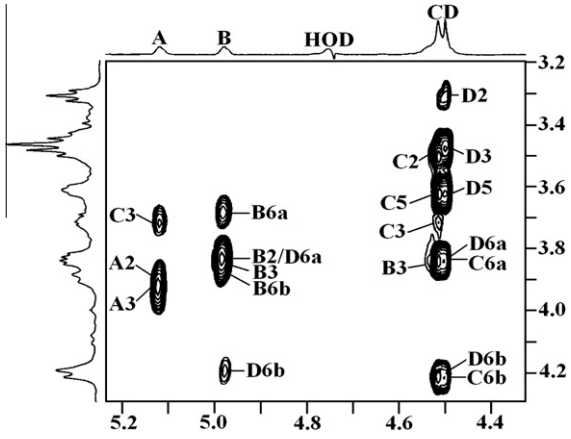
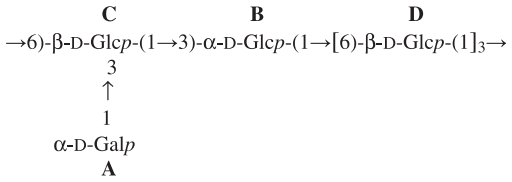


Figure 4. Part of NOESY spectrum of polysaccharide, isolated from *Pleurotus florida* blue variant. The NOESY mixing time was 300 ms.

atoms (**D**H-1, **D**C-2; **D**H-1, **D**C-6; **D**C-1, **D**H-2; **D**C-1, **D**H-3; **D**C-1, **D**H-6a), respectively. Cross-peaks between H-1 of residue **C** and C-3 of residue **B** (**C**H-1, **B**C-3); C-1 of residue **C** and H-3 of residue **B** (**C**C-1, **B**H-3) with other intra-residue coupling between H-1 and C-1 of residue **C** with its own C-2 atom and H-2, H-3 atoms (**C**H-1, **C**C-2; **C**C-1, **C**H-2; **C**C-1, **C**H-3), respectively, were also found. Thus, the HMBC and NOESY connectivities clearly supported the presence of the following repeating unit in the PS isolated from the mushroom, *P. florida* blue variant as:



To prove information on the sequence of the sugar residues in the repeating unit, the polysaccharide was subjected to Smith degradation[22–24] studies, and the products were separated on a Sephadex G-25 column using water as the eluant, resulting in one fraction (SDPS). GLC analysis of the alditol acetates of the acid-hydrolyzed product from SDPS showed the presence of D-glucose and glycerol in a molar ratio of nearly 2:1. The alditol acetates of the methylated product from SDPS were analyzed by GLC and these methylated sugars were also identified by GLC–MS analysis, which showed 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 molar ratio nearly 1:1. The 125-MHz ^{13}C NMR experiment (Fig. 6, Table 4) of SDPS showed two anomeric carbon signals at δ 102.8 and 98.7 ppm corresponding to β-D-Glc*p*-(1→ (**F**) and →3)-α-D-Glc*p*-(1→ (**B**) residues, respectively. The carbon signals C-1, C-2, and C-3 of the glycerol moiety were assigned as 66.2, 72.4, and 62.8 ppm, respectively. The nonreducing end β-D-Glc*p* unit (**F**) was generated from (1→3, 6)-β-D-Glc*p* (residue **C**) during Smith degradation of the terminal α-D-Gal*p* moiety (**A**). The (1→3)-α-D-Glc*p* residue (**B**) was retained and found attached to a glycerol moiety (**G**). The glycerol moiety was generated from (1→6)-β-D-Glc*p* (**D**) after periodate oxidation, followed by Smith degradation. Hence, Smith degradation results in the formation of an

Table 2
NOESY data for the polysaccharide isolated from *Pleurotus florida* blue variant

Glycosyl residue	Anomeric proton	NOE contact proton		
	δ	δ	Residue	Atom
α-D-Gal*p*-(1→	5.12	3.72	**C**	H-3
A		3.89	**A**	H-2
		3.92	**A**	H-3
→3)-α-D-Glc*p*-(1→	4.98	3.83	**B**	H-2
B		3.86	**B**	H-3
		3.89	**B**	H-6b
		3.69	**B**	H-6a
		3.83	**D**	H-6a
		4.19	**D**	H-6b
→3,6)-β-D-Glc*p*-(1→	4.51	3.62	**C**	H-5
C		3.50	**C**	H-2
		3.72	**C**	H-3
		3.86	**B**	H-3
→6)-β-D-Glc*p*-(1→	4.50	3.31	**D**	H-2
D		3.48	**D**	H-3
		3.61	**D**	H-5
		3.84	**C**	H-6a
		4.22	**C**	H-6b
		3.83	**D**	H-6a
		4.19	**D**	H-6b

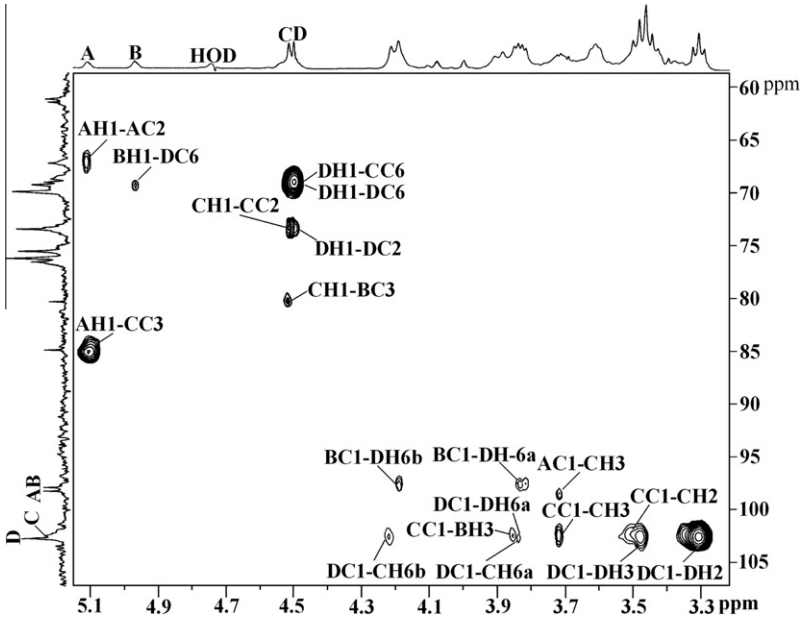


Figure 5. HMBC spectrum of polysaccharide isolated from *Pleurotus florida* blue variant. The delay time in the HMBC experiment was 80 ms.

Table 3
The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide isolated from *Pleurotus florida* blue variant

Residue	Sugar linkage	H-1/C-1	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	α-D-Gal*p*-(1→	5.12	85.0	**C**	C-3
			67.2	**A**	C-2
		98.6	3.72	**C**	H-3
B	→3)-α-D-Glc*p*-(1→	4.98	69.2	**D**	C-6
		98.3	3.83	**D**	H-6a
			4.19	**D**	H-6b
C	→3,6)-β-D-Glc*p*-(1→	4.51	80.4	**B**	C-3
			73.3	**C**	C-2
		103.2	3.86	**B**	H-3
			3.50	**C**	H-2
			3.72	**C**	H-3
D	→6)-β-D-Glc*p*-(1→	4.50	68.9	**C**	C-6
			73.5	**D**	C-2
			69.2	**D**	C-6
		103.4	3.84	**C**	H-6a
			4.22	**C**	H-6b
			3.31	**D**	H-2
			3.48	**D**	H-3
			3.83	**D**	H-6a

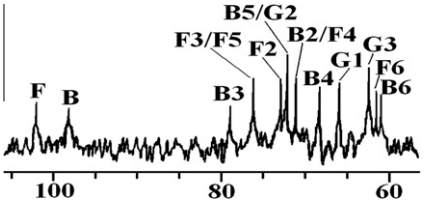


Figure 6. ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of the Smith-degraded Polysaccharide isolated from *Pleurotus florida* blue variant.

Table 4
The ^{13}C NMR[a] chemical shifts of Smith-degraded polysaccharide isolated from *Pleurotus florida* blue variant in D_2O at 27 °C

Glycosyl residue	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p*-(1→ **F**	102.8	73.5	76.5	70.9	76.5	61.5
→3)-α-D-Glc*p*-(1→ **B**	98.7	70.9	79.1	68.6	72.4	61.1
3)-Gr● **G**	66.2	72.4	62.8			

[a] The values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 27 °C.

oligosaccharide (SDPS) from the parent polysaccharide and the structure was established as:

F **B** **G**
β-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→3)-Gro

Therefore on the basis of above results, the structure of the repeating unit of the polysaccharide was confirmed.

Macrophage activation by the polysaccharide was observed in vitro. On treatment with different concentrations of this polysaccharide, an enhanced production of NO was observed in a dose dependent manner with optimum production of 8.1 μM NO per 5×10^5 macrophages at 10 μg/mL, and subsequently decreased with further increase in concentration (Fig. 7). Hence, the effective dose of this polysaccharide was observed at 10 μg/mL.

Proliferation of splenocytes and thymocytes is an indicator of immunoactivation. The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with the polysaccharide by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method.[25] The polysaccharide was tested to proliferate splenocytes and thymocytes and results are shown in Figure 8A and B, respectively, and the asterisks on the columns indicate the statistically significant differences compared to PBS control. The splenocyte proliferation index (SPI) as compared to PBS (Phosphate Buffered Saline) control closer to 1 or below indicates low stimulatory effect on immune system. At 50 μg/mL of the polysaccharide, splenocyte proliferation index was found maximum as compared to other concentrations. Hence, 50 μg/mL of the polysaccharide can be considered as efficient splenocyte

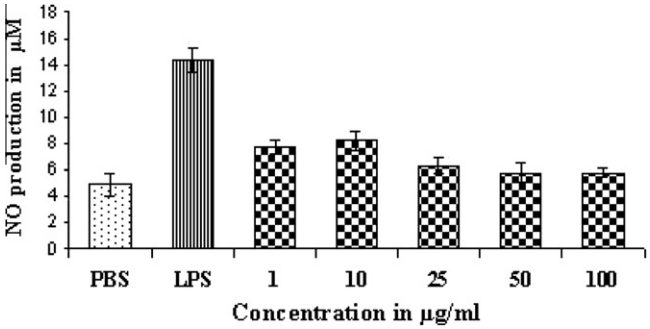


Figure 7. In vitro activation of peritoneal macrophage stimulated with different concentrations of the polysaccharides in terms of NO production.

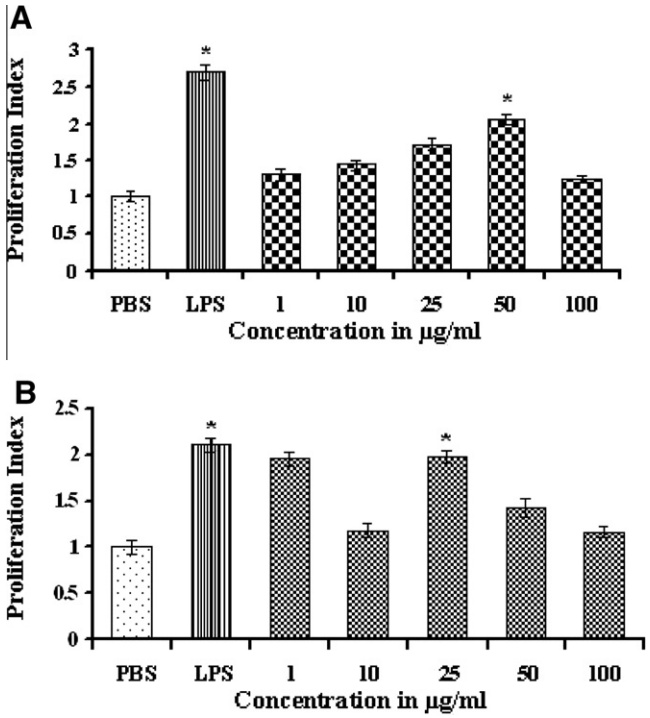


Figure 8. Effect of different concentrations of the polysaccharide on splenocytes (A) and thymocytes (B) proliferation. (* Significant compared to the PBS control.)

proliferator. Again, 25 μg/mL of the same sample showed maximum effect on thymocyte proliferation. It is noteworthy to mention that similar kind of activations was observed with mushroom polysaccharides[9,10,13] reported previously by our group.

1. Experimental

1.1. Isolation and purification of the polysaccharide

The fresh fruiting bodies of the mushroom, *P. florida* blue variant (500 g) were cultivated and collected from Falta experimental farm, Bose Institute. The material was first washed with water, then cut into pieces, and boiled with 250 mL distilled water for 6 h. The whole mixture was then kept overnight at 4 °C and filtered through a linen cloth. The filtrate was centrifuged at 8000 rpm (using a Heraeus Biofuge stratos centrifuge) for 30 min at 4 °C and the crude polysaccharide (wt 180 mg) was isolated as described in our previous papers.[26,27] The crude polysaccharide (25 mg) was purified by gel permeation chromatography on Sepharose 6B column. A single fraction (test tubes 18–45) was observed and collected through lypholyzation, yielding 15 mg of material. The purification process was carried out in seven lots (yield; 105 mg).

1.2. General methods

The optical rotation was measured on a Jasco polarimeter, model P-1020 at 26.8 °C. For monosaccharide analysis, the polysaccharide sample (3.0 mg) was hydrolyzed with 2 M CF_3COOH (2 mL), and the analysis was carried out as described earlier.[26] The molecular weight of the polysaccharide was determined as reported earlier.[16,26] The absolute configuration of the monosaccharide constituent was assigned according to Gerwig et al.[15] The polysaccharide was methylated according to Ciucanu and Kerek method.[17] Gas-liquid chromatography–mass spectrometric (GLC–MS) analysis was also performed on 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. Gas liquid chromatographic (GLC) analysis was done by using a Hewlett–Packard Model 5730 A, 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 analyses were performed at 170 °C. Periodate oxidation was performed with this polysaccharide as described in the earlier report.[18,19,26] NMR experiments were carried out as reported in our previous papers.[26,28,29] DEPT-135 NMR experiment was carried out at 27 °C.

1.3. Test for macrophage activity by nitric oxide assay

Peritoneal macrophages (5×10^5 cells/mL) after harvesting were cultured in complete RPMI (Roswell Park Memorial Institute) media in 96-well plates.[30,31] The purity of macrophages was tested by adherence to tissue culture plates. The polysaccharide was added to the wells in different concentrations. The cells were cultured for 24 h at 37 °C in a humidified 5% CO_2 incubator. Production of nitric oxide was estimated by measuring nitrite levels in cell supernatant with Greiss reaction.[32] Equal volumes of Greiss reagent (1:1 of 0.1% in 1-napthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid) and sample cell supernatant were incubated together at room temperature for 10 min. Absorbance was observed at 550 nm. Lipopolysaccharide (LPS), L6511 of *Salmonella enterica* serovar Typhimurium was used as positive control.

1.4. Splenocyte and thymocyte proliferation assay[31,33]

A single cell suspension of spleen and thymus was prepared from the normal mice under aseptic conditions by frosted slides in PBS (Phosphate Buffered Saline). The suspension was centrifuged to obtain cell pellet. The contaminating red blood cells were removed by hemolytic Gey's solution. After washing two times in PBS the cells were resuspended in complete RPMI medium. Cell concentration was adjusted to 1×10^5 cells/mL and viability of the suspended cells (as tested by trypan blue dye exclusion) was always over 90%. The cells (180 μL) were plated in 96-well flat-bottomed plates and incubated with 20 μL of various concentrations (1–100 μg/mL) of the polysaccharide. The same lipopolysaccharide as used in macrophage activation was also used here as positive control. The cultures were set-up for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. Proliferation was checked by MTT assay method.[25] The data are reported as the mean ± standard deviation of six different observations and compared against PBS control.

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