CHAPTER 3

POLYSACCHARIDE FROM *TERMITOMYCES HEIMII*

A part of this chapter has been published in the following article:

1. D. K. Manna, A. K. Nandi, M. Pattanayak, P. Maity,S. Tripathy, A. K. Mandal, S. Roy, S. S. Tripathy, N. Gupta, S. S. Islam. Carbohydrate Polymers; 134, 375–384, 2015.

3.1. Introduction and review of earlier works

Termitomyces heimii, an agaric fungus belongs to the family Lyophyllaceae, also known as "termite mushroom" [232]. It is a wild, edible and delicious mushroom that grows with termite's guts through symbiotic in the forests of laterite soil [17-19]. Isolation of several polysaccharides from the genus *Termitomyces* have been reported from our research group, namely *Termitomyces eurhizus* [233], *T. striatus* [234], *T. robustus* [235], *T. microcarpus* [236] and *T. clypeatus* [237].

Several linear (1→3) and branched (1→3)-, (1→6)-β-D-glucans [45-47,238] were reported as immunoactive materials from our laboratory. Some immune-modulating water soluble β-D-glucans have also been reported [58-61,239]. Two water soluble polysaccharides (PS-I & PS-II) have been isolated from the alkaline extract of this mushroom. The detailed structural investigation and biological activities of PS-I were investigated and presented in this chapter.

3.2. Present work

3.2.1. Isolation and purification of the polysaccharide from *T. heimii*

The dried fruit bodies (120 g) of mushroom *T. heimii* was boiled with 4% NaOH solution, followed by extraction from hot water, precipitation in alcohol, dialysis, centrifugation and freeze drying yielded crude polysaccharide (600 mg). On fractionation through Sepharose 6B column [203] two fractions, PS-I (12 mg) and PS-II (2 mg) were collected from the crude polysaccharide (30 mg) and freeze-dried. The repeatation of same procedure for 10 times yielded 116 mg of pure PS-I and 15 mg of pure PS-II. Further purification of the PS-I on Sepharose 6B column produced only a single homogeneous fraction, which established the homogeneous nature of PS-I.

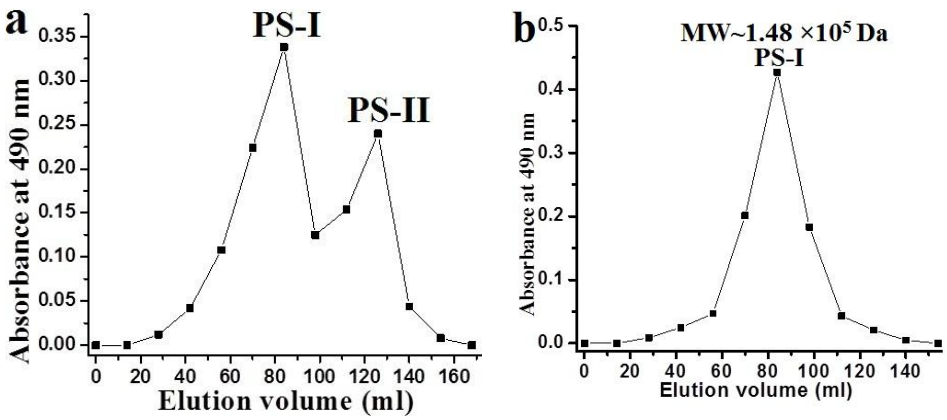


Figure 3.1. (a) Chromatogram of crude polysaccharide on Sepharose-6B column. (b) Chromatogram showing the homogeneous nature of PS-I isolated from mushroom *T. Heimii*

3.2.2. Optical rotation and molecular weight of PS-I

The specific rotation of PS-I was measured $[\alpha]_D^{31}$ -19.5 (*c* 0.2, water). The negative value of optical rotation indicates the presence of β-anomeric configuration [240]. The average molecular weight of PS-I was found to be as ~1.48 × 10^5 Da [204] (**Figure 3.2**).

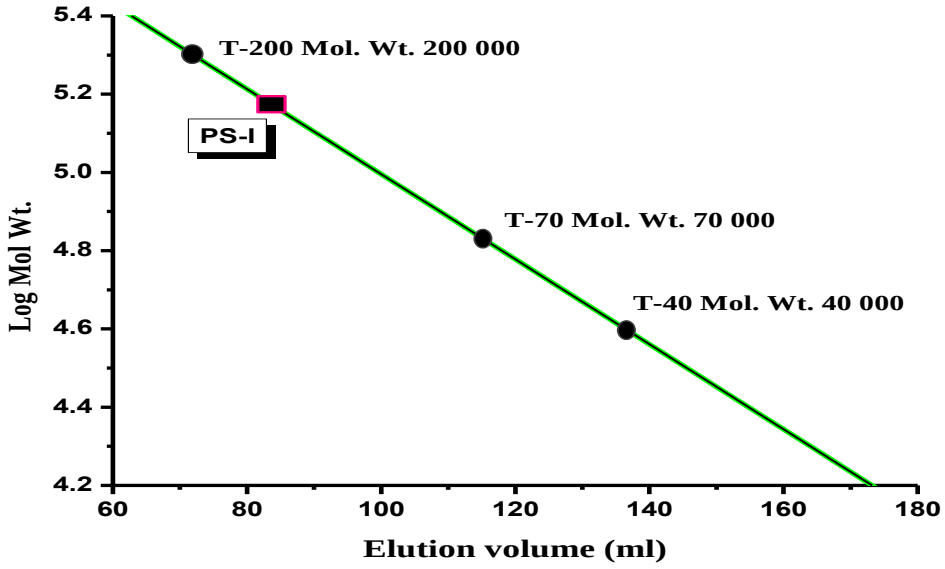


Figure 3.2. Determination of molecular weight of the PS-I isolated from *T. heimii*

3.2.3. Structural analysis of PS-I

3.2.3.1. Chemical analysis of PS-I

The GLC analysis of the alditol acetates of PS-I showed the presence of only glucose as monomeric unit and all the glucose units present as D-configuration [206]. The mode of linkages were determined by methylation using the method of Ciucanu and Kerek (1984) [207], followed by hydrolysis and alditol acetate preparation. The GLC-MS analysis showed the presence of 1,5-di-*O*-acetyl-2,3,4.6-tetra-*O*-methyl-D-glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol, and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a molar ratio of approximately 1:2:1:1 (**Table 3.1**, **Figure 3.3**). These results indicated the presence of terminal D-glucopyranosyl, (1→3)-D-glucopyranosyl, (1→6)-D-glucopyranosyl and (1→3,6)-D-glucopyranosyl residues in the PS-I as shown in the following table.

Table 3.1. GC-MS analysis of methylated PS-I isolated from *T. heimii.*

Methylated sugars	Molar ratio	Linkage type	Major Mass Fragments (m/z)
2,3,4,6-Me_4-Glc	1	β-D-Glc*p*-(1→	41,43,59,71,87,101,117,129,145,161,173,205
2,4,6-Me_3-Glc	2	→3)-β-D-Glc*p*-(1→	41,43,58,71,87,101,117,129,143,161,173,189,203.217,233
2,3,4,-Me_3-Glc	1	→6)-β-D-Glc*p*-(1→	41,43,58,71,87,101,117,129,145,161,173,189,203,217,233
2,4-Me_2-Glc	1	→3,6)-β-D-Glc*p*-(1→	40,43,58,74,87,101,117,129,143,159,173,189,201,233,245,305

Formation of the above fragments obtained from the methylation analysis is represented in the schematic diagram:

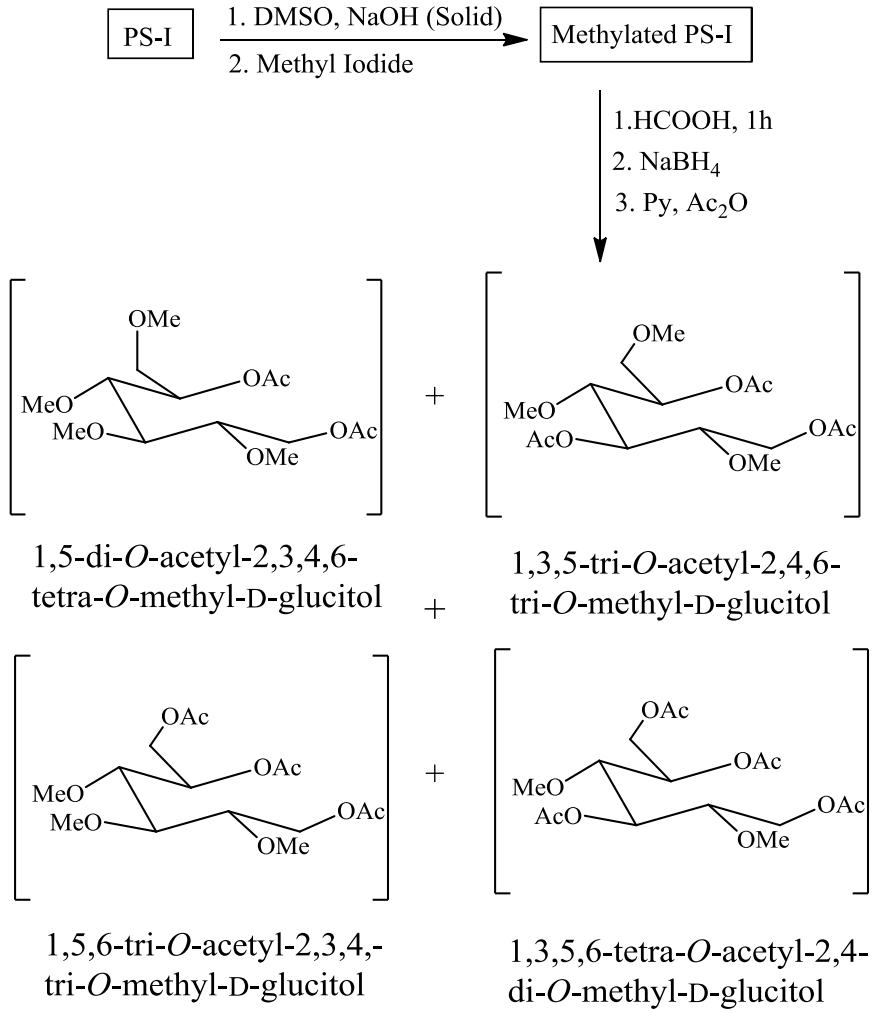


Figure 3.3. Schematic presentation of methylation experiment of PS-I.

According to these results there are possibilities of three types backbone as, a (1→6)-linked backbone, a (1→3)-linked backbone or an alternatively (1→3), (1→6)-linked backbone. So, to determine of the backbone chain present in the polysaccharide periodate oxidation and mild hydrolysis experiments were performed. The GLC analysis of the alditol acetates of the periodate-oxidised reduced [241], PS-I showed the presence of D-glucose along with glycerol and GLC-MS analysis of periodate-oxidised, reduced, methylated [242] showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol; 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a ratio of nearly 2:1, shown in **Table 3.2, Figure 3.4**.

Table 3.2. GLC-MS analysis of periodate-oxidised, reduced, methylated PS-I.

Methylated sugars	Molar ratio	Linkage type	Major Mass Fragments (m/z)
2,4,6-Me_3-Glc	2	→3)-β-D-Glc*p*-(1→	40,43,58,71,87,101,117,129,143,161,173,189,203.217,235
2,4-Me_2-Glc	1	→3,6)-β-D-Glc*p*-(1→	41,43,58,74,87,101,117,129,143,159,171,189,201,233,246

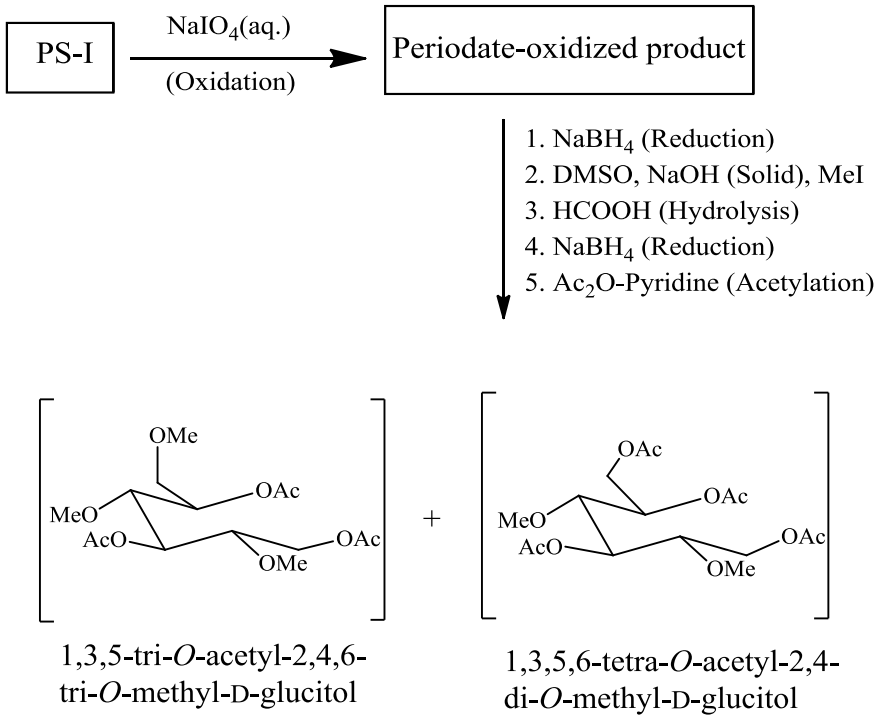


Figure 3.4. Schematic presentation of periodate oxidised reduced products of methylated PS-I.

Mild acid hydrolysis of the periodate-oxidised, reduced PS-I was carried out with 0.5 M TFA to get Smith degradation product (SDPS). The GLC analysis of the alditol acetates of Smith degraded hydrolyzed product showed the presence of D-glucose and D-glycerol. The GLC-MS analysis of the of methylated SDPS shows the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol in a ratio of nearly 1:2. Partial acid hydrolysis [59,155] of the β-glucan was performed with TFA (0.1 M) to confirm the backbone chain of the β-

glucan in the repeating unit. Two fractions were obtained after hydrolysis i.e. a polysaccharide (P1) and an oligosaccharide (O2). The methylation analysis of P1 showed the presence of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol only which indicates the presence of (1→6)-linked backbone of the PS, but the other fraction O2 showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol indicating the presence of (1→3)-linked and terminal glucopyranosyl moieties as oligosaccharide side chain. Hence, these studies established that the backbone PS-I consisted of two (1→6)-β-D-glucopyranosyl residues as repeating unit, one of which was branched at *O*-3 position with the side chain consisting of two (1→3)-β-D-glucopyranosyl and one terminal β-D-glucopyranosyl residues.

3.2.3.2 NMR and structural analysis of the PS-I

Five signals in the anomeric region at 4.72, 4.71, 4.47, 4.45 and 4.44 ppm were observed in the ^{1}H NMR (500 MHz) spectrum (**Figure 3.5**) at 30 ºC and designated as $\mathbf{A_I}$, $\mathbf{A_{II}}$, **B**, **C** and **D** residues according to their decreasing chemical shifts values. In the ^{13}C (125 MHz) spectrum (**Figure 3.6**) at 30 ºC five anomeric signals appeared at δ 102.8, 102.7, 102.5, 102.36 and 102.2.

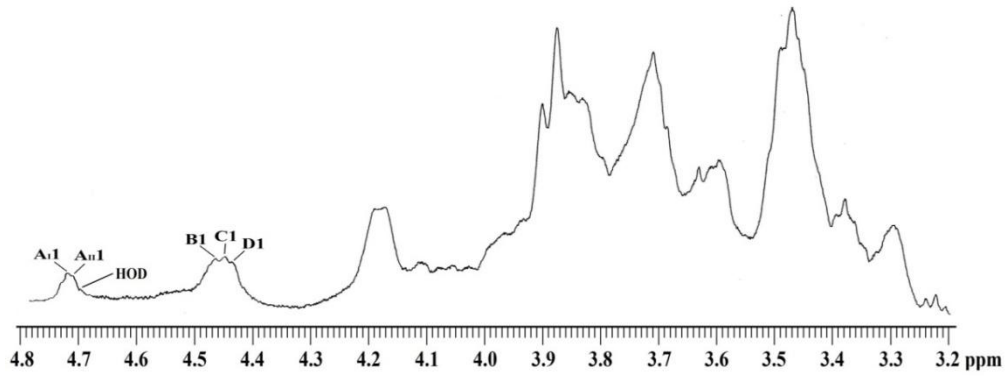


Figure 3.5. ^{1}H NMR spectrum (500MHz, D_2O, 30 ºC) of PS-I isolated from the mushroom *T. heimii*.

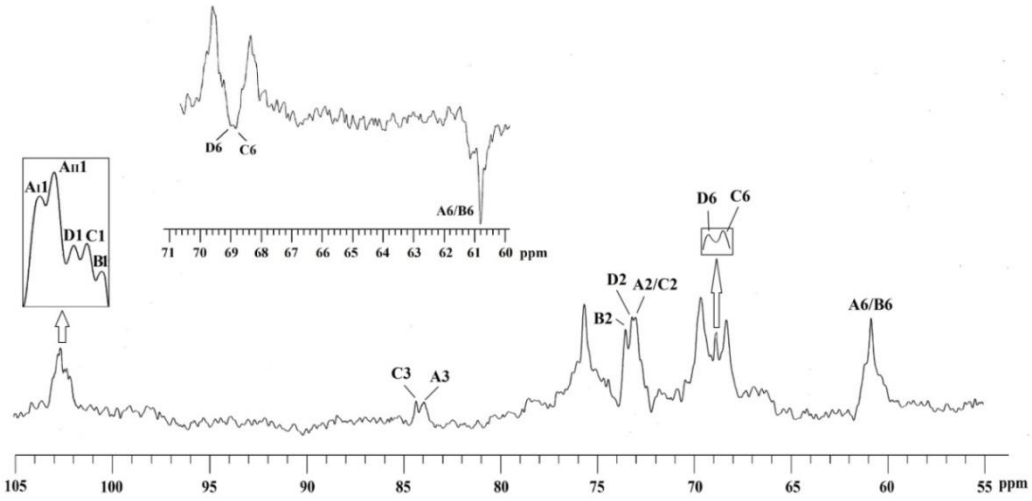


Figure 3.6. ^{13}C NMR spectrum (125MHz, D_2O, 30 ºC) and part of DEPT-135 spectrum (D_2O, 30 ºC) (inset) of PS-I.

Based on HSQC experiment (**Figure 3.7, Table 3.3**), the anomeric carbon signals at δ 102.8 corresponded to $\mathbf{A_I}$, 102.7 corresponded to $\mathbf{A_{II}}$, the signal at δ 102.5 corresponded to **D**, 102.36 corresponded to **C** and the peak at δ 102.2 was correlated to **B** residues of the anomeric proton signals, respectively. All the ^{1}H and ^{13}C signals (**Table 3.3**) were assigned using DQF-COSY, TOCSY, and HSQC experiments. Coupling constants were measured from DQF-COSY spectrum.

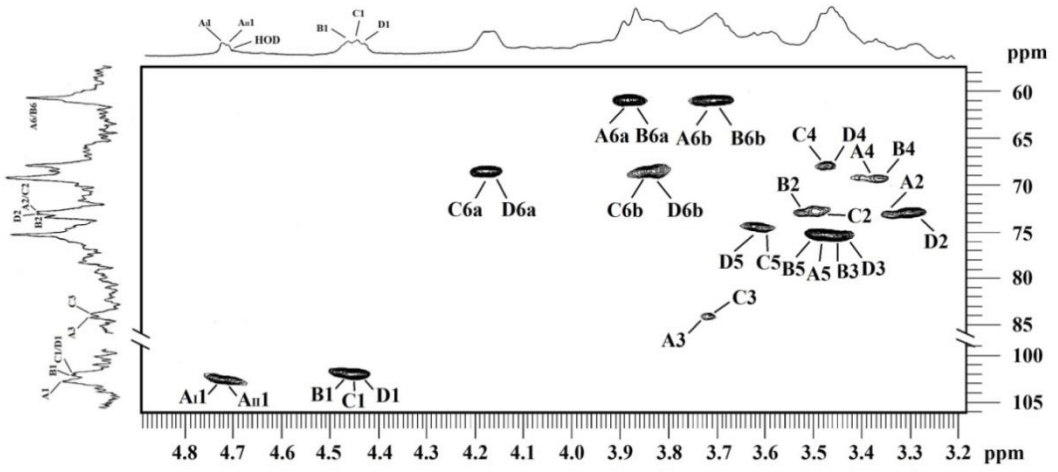


Figure 3.7. Part of HSQC spectrum of PS-I isolated from the mushroom *T. heimii*.

Table 3.3. The ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts of PS-I.

Glucosyl residue	H-1/ C-1	H-2/ C-2	H-3/ C-3	H-4/ C-4	H-5/ C-5	H-6a,H-6b/ C-6
→3)-β-D-Glc*p*-(1→	4.72[x], 4.71[y]	3.34	3.73	3.40	3.49	3.89[c], 3.73[d]
A (A_I, A_{II})	102.8[x] 102.7[y]	73.0	84.3	68.2	75.7	60.81
β-D-Glc*p*-(1→	4.47	3.51	3.44	3.36	3.51	3.87[c],3.70[d]
B	102.2	73.6	75.7	69.6	75.7	60.81
→3,6)-β-D-Glc*p*-(1→	4.45	3.50	3.71	3.49	3.60	4.18[c],3.85[d]
C	102.36	73.0	84.0	68.2	75.0	68.86
→6)-β-D-Glc*p*-(1→	4.44	3.29	3.43	3.46	3.62	4.16[c],3.83[d]
D	102.5	73.2	75.7	69.6	75.0	68.94

[a] The values of chemical shifts were recorded keeping HOD signal fixed at δ 4.70 at 30 ºC.

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

[c,d] Interchangeable; [x] for residue $\mathbf{A}_{I;}$ [y] for residue $\mathbf{A}_{II.}$

The large $J_{H\text{-}2,\,H\text{-}3}$ and $J_{H\text{-}3,\,H\text{-}4}$ coupling constant values ~8 Hz of **A**-**D** residues and $J_{C\text{-}1,\ H\text{-}1}$ ~ 160 Hz indicated the presence of β-configuration of the D-glucopyranosyl (Glc*p*) residues in the polysaccharide. The downfield shift of C-3 (δ 84.3) of residues **A** (**A_I** and **A_{II}**) with respect to standard value of methyl glycosides (Agrawal, 1992) indicated that they were (1→3)-linked β-D-Glc*p*. All chemical shifts of residue **B** were very close to the standard values of methyl glycoside (Agrawal, 1992) of β-D-glucose, evidently indicated that the residue **B** was non-reducing end. In residue **C**, the chemical shift values of C-3 (δ 84.0) and C-6 (δ 68.86) shifted downfield, indicating the presence of (1→3,6)-linked β-D-Glc*p*. Since, the residue **D** is glycosidically linked to the rigid part **C,** the C-6 value would show downfield shift (δ 68.94) in comparison to **C** residue (δ 68.86) due to the neighbouring effect (Nandi et al., 2014; Yoshioka et al., 1985) of rigid part **C.** The following DEPT-135 spectrum (**Figure 3.6**) further confirmed the linkage at C-6 of the residues **C** and **D**.

The ROESY experiment showed the inter-residual contacts $\mathbf{A_I}$H-1/**C**H-3; $\mathbf{A_{II}}$H-1/$\mathbf{A_I}$H-3; **B**H-1/$\mathbf{A_{II}}$H-3; **C**H-1/**D**H-6a,**D**H-6b; **D**H-1/**C**H-6a,**C**H-6b along with other intra-residual contacts $\mathbf{A_I}$H-1/$\mathbf{A_I}$H-3; $\mathbf{A_I}$H-1/$\mathbf{A_I}$H-5; **B**H-1/**B**H-2; **B**H-1/**B**H-3; **B**H-1/**B**H-5; **C**H-1/CH-2; **C**H-1/**C**H-3; **C**H-1/**C**H-5; **D**H-1/**D**H-2; **D**H-1/**D**H-3 and **D**H-1/**D**H-5 (**Figure 3.8 & Table 3.4**). Hence, the following sequences in PS-I were established as: $\mathbf{A_I}$ (1→3) **C**; $\mathbf{A_{II}}$ (1→3) $\mathbf{A_I}$; **B** (1→3) $\mathbf{A_{II}}$; **C** (1→6) **D** and **D** (1→6) **C**.

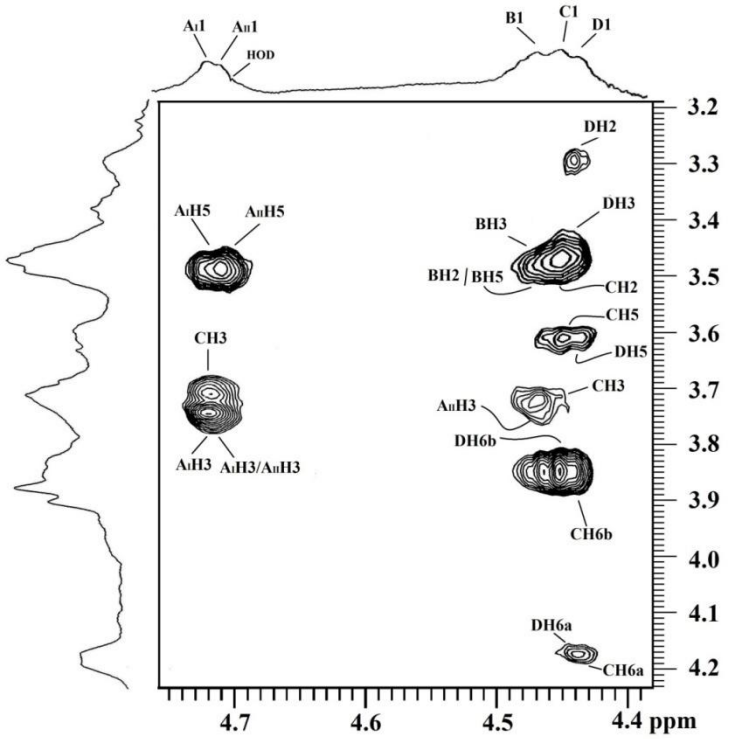


Figure 3.8. Part of ROESY spectrum The ROESY mixing time was 300 ms.

Table 3.4. ROESY data for the PS-I isolated from the mushroom *T. heimii*.

Glycosyl residue	Anomeric proton (δ)	ROE contact proton		
		δ	residue	atom
→3)-β-D-Glc*p*-(1→ **A_I**	4.72	3.71	**C**	H-3
		3.73	**A_I**	H-3
		3.49	**A_I**	H-5
→3)-β-D-Glc*p*-(1→ **A_{II}**	4.71	3.73	**A_I**	H-3
		3.73	**A_{II}**	H-3
		3.49	**A_{II}**	H-5
β-D-Glc*p*-(1→ **B**	4.47	3.73	**A_{II}**	H-3
		3.51	**B**	H-2
		3.44	**B**	H-3
		3.51	**B**	H-5
→3,6)-β-D-Glc*p*-(1→ **C**	4.45	4.16	**D**	H-6a
		3.82	**D**	H-6b
		3.50	**C**	H-2
		3.71	**C**	H-3
		3.60	**C**	H-5
→6)-β-D-Glc*p*-(1→ **D**	4.44	4.18	**C**	H-6a
		3.85	**C**	H-6b
		3.29	**D**	H-2
		3.43	**D**	H-3
		3.62	**D**	H-5

The ^{13}C NMR experiment, 125 MHz of Smith degraded material (SDPS) of PS-I was carried out at 30 ˚C for further confirmation of the sequence of linkages. The ^{13}C NMR spectrum (**Figure 3.9**) and their different values (**Table 3.5**) are presented below:

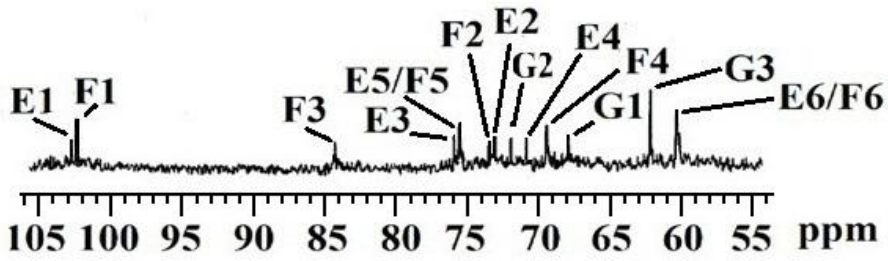


Figure 3.9. ^{13}C NMR spectrum (125MHz, D_2O, 30 ºC) of Smith-degraded glycerol-containing disaccharide of PS-I isolated from the mushroom *T. heimii*.

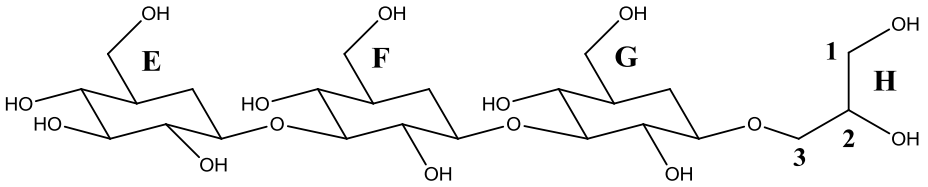
Table 3.5. The ^{13}C NMR[n] chemical shifts of Smith-degraded glycerol-containing disaccharide of the mushroom *T. heimii* in D_2O at 30 ºC.

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p*-(1→ **E**	102.8	73.58	76.0	70.70	75.67	60.75
→3)-β-D-Glc*p*-(1→ **F**	102.6	73.34	84.18	69.70	75.67	60.75
Gro-(3→ **G**	68.15	72.0	62.57			

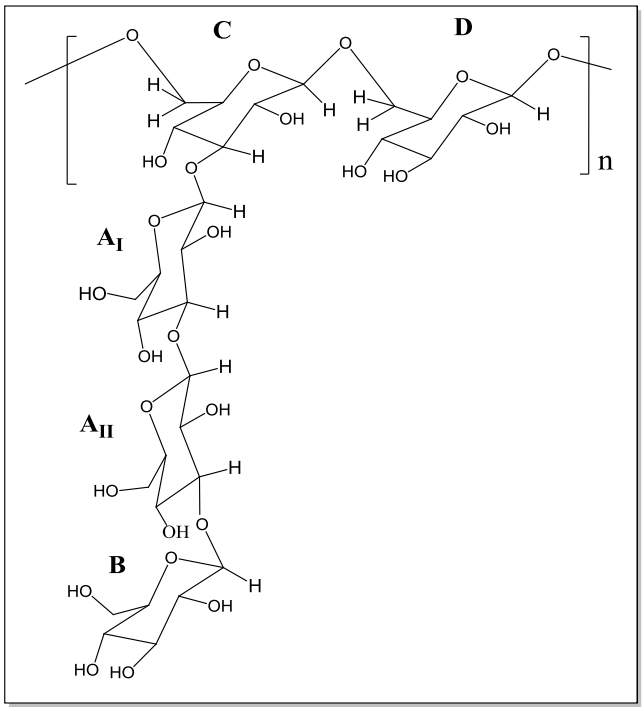
[n] The values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 at 30 ºC.

The ^{13}C NMR spectrum showed two anomeric carbon signals at δ 102.6 and 102.8 with a ratio of nearly 2:1 corresponding to →3)-β-D-Glc*p*-(1→ (**F**) and β-D-Glc*p*-(1→ (**E**) residues respectively. The carbon signals C-1, C-2, and C-3 of the glycerol moiety were assigned at δ 68.15, 72.0, and 62.57 respectively. The non-reducing β-D-Glc*p* (**E**) was produced from (1→3)-β-D-Glc*p* ($\mathbf{A_{II}}$) after complete oxidation of the β-D-Glc*p* (**B**) of the intact PS-I. One (1→3)-β-D-Glc*p* (**F**) was retained from (1→3)-β-D-Glc*p* ($\mathbf{A_I}$) and the other (1→3)-β-D-Glc*p* (**F**) was produced from the (1→3,6)-β-D-Glc*p* (**C**) due to oxidation followed by Smith degradation. The glycerol (**G**) moiety was produced from (1→6)-β-D-Glc*p* (**D**) after periodate oxidation followed by Smith degradation and be attached to (1→3)-β-D-Glc*p* moiety (**F**).

Hence, Smith degradation have resulted an oligosaccharide from polysaccharide and the structure was established as:



Therefore, the above discussions indicated that the backbone chain of the polysaccharide consisted of only (1→6)-linked β-D-glucose residues neither (1→3)-linked nor alternative (1→3)-, (1→6)-linked β-D-glucose. On the basis of all these above experiments, the β-glucan possessed a backbone of two (1→6)-linked β-D-glucopyronosyl residues, one of which was branched at *O*-3 positions with the side chain consisting of two (1→3)- linked and one terminal β-D-glucopyranosyl residues. Hence, the structure of the repeating unit of the polysaccharide was established and presented as:



3.2.4. Biological properties of PS-I

3.2.4.1. Cell viability study by MTT assay

The in-vitro cellular toxicity the PS-I isolated from *T. heimii* was studied on human blood lymphocytes at varied concentrations ranging from 25 µg/ml to 400 µg/ml. MTT assay suggested that the polysaccharide have no considerable cytotoxic effect on normal lymphocytes. Cell proliferative activity was observed significantly ($p<0.05$) up to 200 µg/ml but at the dose of 400 µg/ml the PS-I showed mild toxicity towards the lymphocytes (**Figure 3.10**). Earlier reports showed that polysaccharides extracted from mushroom *Entoloma lividoalbum* and bacterium *Klebsiella pneumoniae* PB12 showed cellular toxicity above 100 µg/ml of PS dosage [61,243].

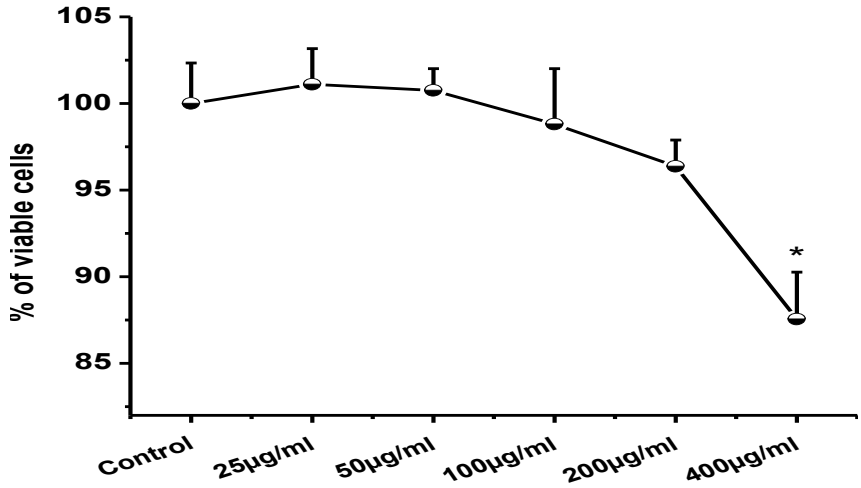


Figure 3.10. MTT assay showing in-vitro cytotoxicity of PS-I on peripheral blood lymphocytes. (n = 6, values are expressed as mean±SEM. * Indicates the significant difference as compared to control group).

3.2.4.2. Determination of reduced glutathione (GSH) and oxidizedglutathione (GSSG) level

Glutathione levels in both, oxidised and reduced form, were measured in cellular system with varied doses, D1-D5 (D1: 25 µg/ml, D2: 50 µg/ml, D3: 100 µg/ml, D4: 200 µg/ml, and D5: 400 µg/ml) of PS-I as shown below in **Figure 3.11 & 3.12**. The reduced glutathione (GSH) level significantly increased up to 200 µg/ml of PS-I, whereas at 400 µg/ml, it was moderately decreased exhibited slight increase in GSSG

level. It was clearly observed that the alteration of redox ratio (GSH/GSSG) is fully correlated with alteration in PS-I concentrations (Pearson Co-efficient r= 0.951, Pearson correlation $p < 0.05$).

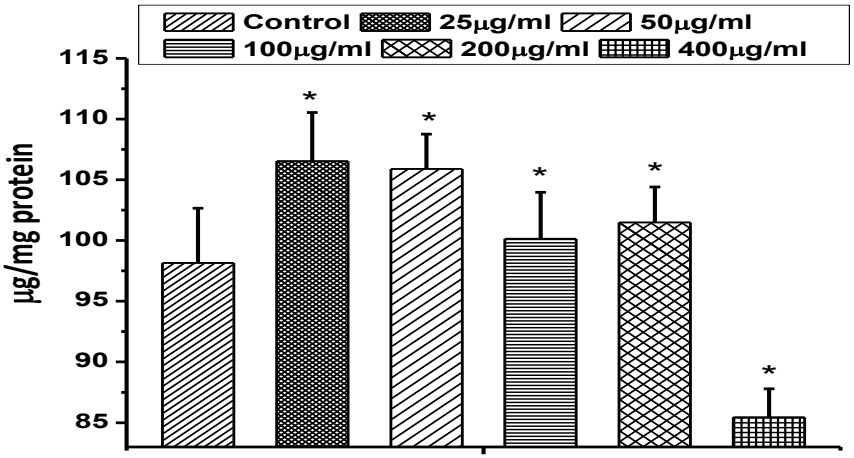


Figure 3.11. Concentration of reduced glutathione (GSH) level in normal human lymphocytes treated with different concentrations of PS-I. (n = 6, values are expressed as mean±SEM. * Indicates the significant difference as compared to control group).

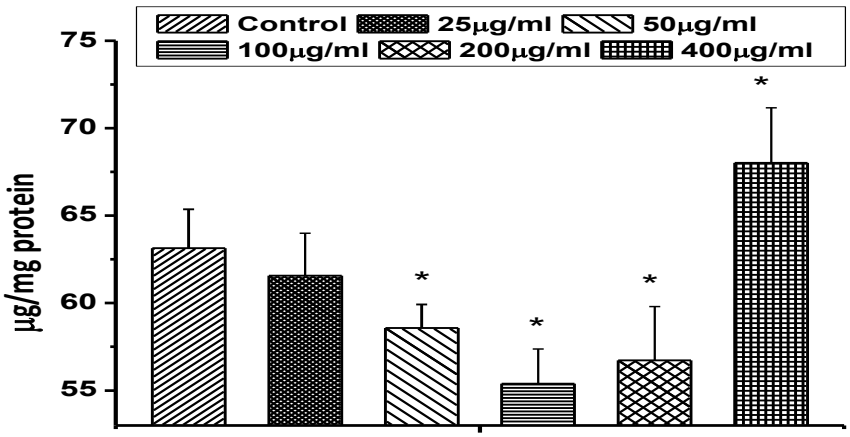


Figure 3.12. Concentration of oxidized glutathione (GSSG) level in normal human lymphocytes treated with different concentrations of PS-I. (n = 6, values are expressed as mean±SEM. * Indicates the significant difference as compared to control group).

3.2.4.3. Determination of lipid peroxidation (MDA)

Lipid peroxidation in lymphocytes was measured in terms of malondialdehyde (MDA) with varied doses, D1-D5 of PS-I. The result showed slight increase of MDA at the dose of 400 µg/ml of PS-I (**Figure 3.13**).

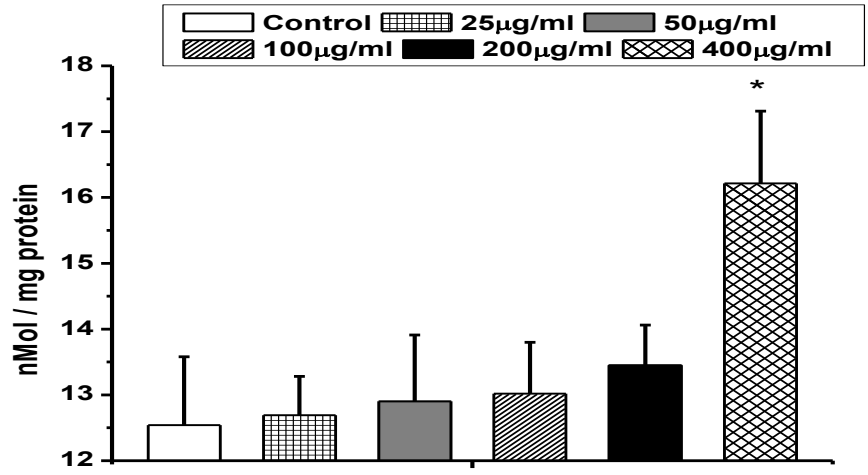


Figure 3.13. Concentration of MDA level of PS-I treated normal human lymphocytes toevaluate lipid peroxidation. (n = 6, values are expressed as mean±SEM. * Indicates the significant difference as compared to control group).

3.2.4.4. Protective role against nicotine toxicity

The lymphocytes were stimulated with nicotine (10 mM) as positive control and different concentrations (ranging from 25-400 µg/ml) of PS-I along with nicotine were treated for 24 h in culture media. The significant ($p < 0.05$) increase of the cell viability were observed up to 200 µg/ml, but when the dose was increased to 400 µg/ml, the polysaccharide lost their protective role which was shown in the following **Figure 3.14**.

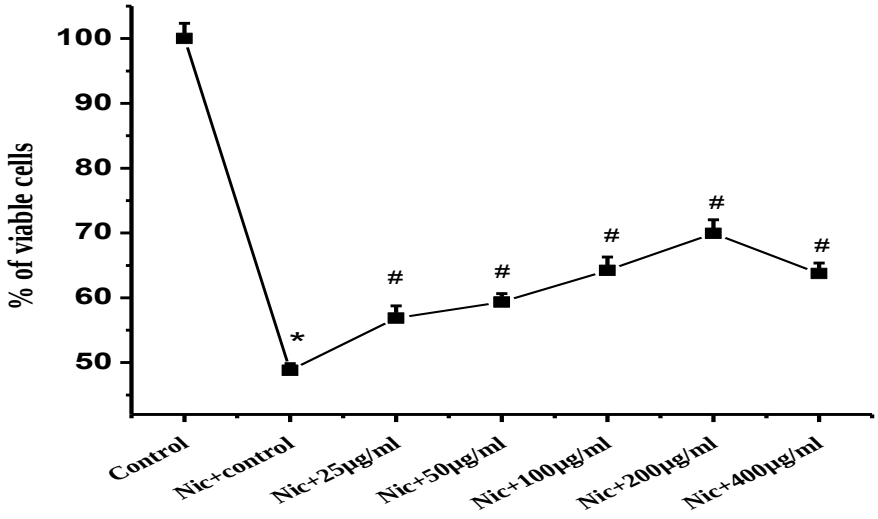


Figure 3.14. MTT assay showing in-vitro cell viability of PS-I on nicotine stimulated peripheral blood lymphocytes. (n = 6, values are expressed as mean±SEM. * Indicates the significant difference as compared to control group).

3.2.4.5. Role on nitric oxide (NO) and ROS generation

Nicotine stimulated lymphocytes secreted several factors like ROS and NO. The treatment of the PS-I with varied doses D1-D4 in the nicotine stimulated lymphocytes diminishes the NO (**Figure 3.15**) and ROS (**Figure 3.16**) respectively but at 400 µg/ml ($p < 0.05$) there is an increase in both ROS and NO production in PS-I treated lymphocytes. The fluorescence images revealed that the PS-I (at the dose of 200 µg/ml) was able to scavenge the ROS generated due to nicotine stimulation.

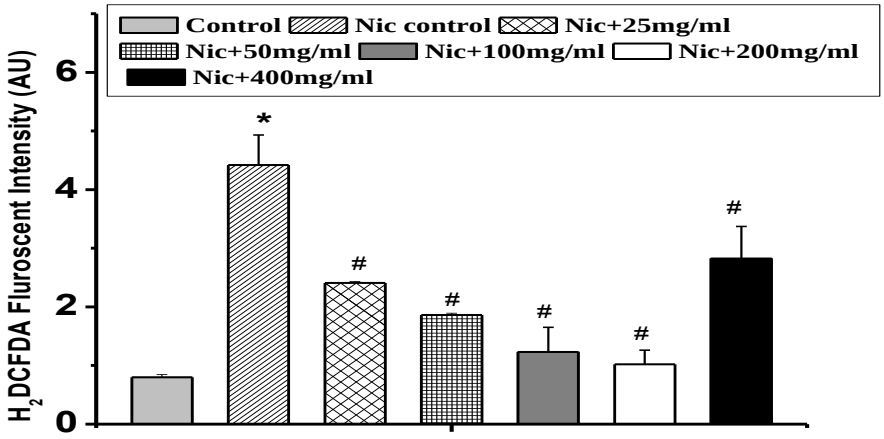


Figure 3.15. Concentration of nitric oxide (NO) release in nicotine stimulated human lymphocytes treated with PS-I. (n = 6, values are expressed as mean±SEM. * Indicates the significant difference as compared to control group).

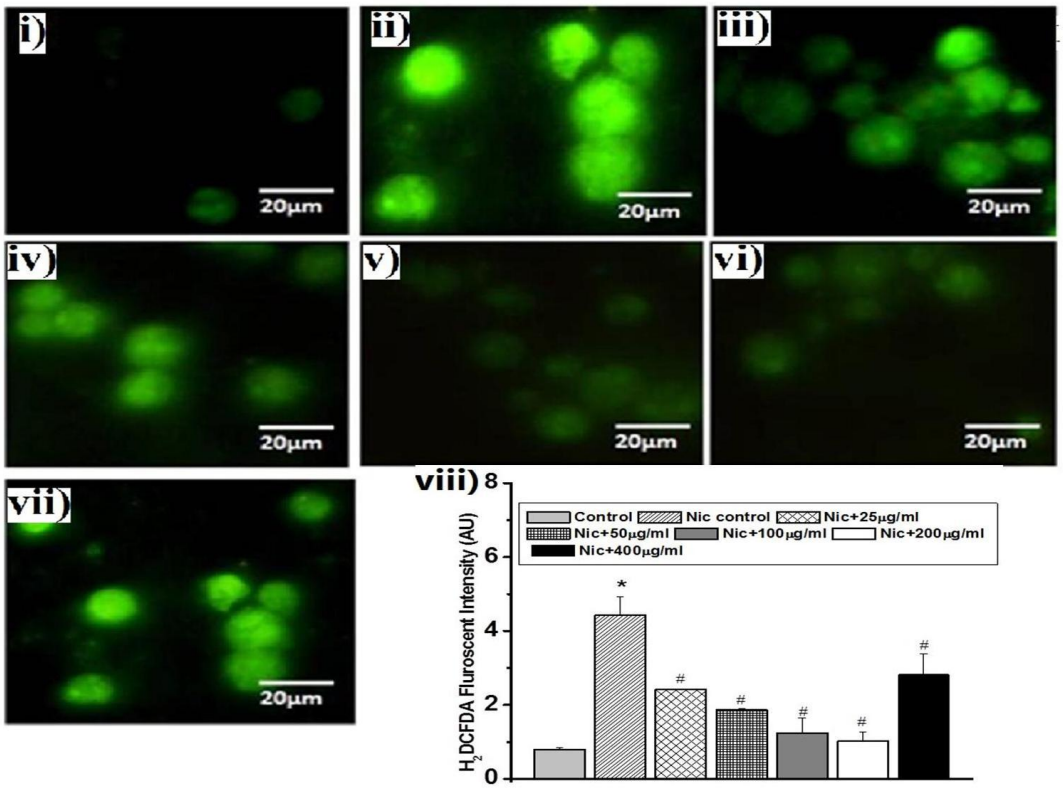


Figure 3.16. Fluorescence microscopic images (100X magnifications) showing intracellular ROS generation in lymphocytes. The scale bar represents 20µm (i) Control; (ii) Nicotine treated; (iii) Nicotine + 25 µg/ml of PS-I; (iv) Nicotine + 50µg/ml of PS-I. (v) Nicotine + 100 µg/ml of PS-I; (vi) Nicotine + 200 µg/ml of PS-I; (vii) Nicotine+400 µg/ml of PS-I; (viii) Histogram showing the mean fluorescent intensity. (n = 6, values are expressed as mean±SEM. * Indicates the significant difference as compared to control group).

This property of PS-I was also established using flow cytometry study. FACS analysis revealed that in nicotine treated lymphocytes due to lipid peroxidation cell membrane damages and ultimately causes disruption of membrane integrity as a result the fluorescence intensity of propidium iodide (PI) increases compared to that of control (**Figure 3.17**). Whereas, when treated with 200 µg/ml of PS-I along with nicotine there is a decrease in fluorescence intensity indicating the possible ROS scavenging property of PS-I. These are shown in the following **Figure 3.17**.

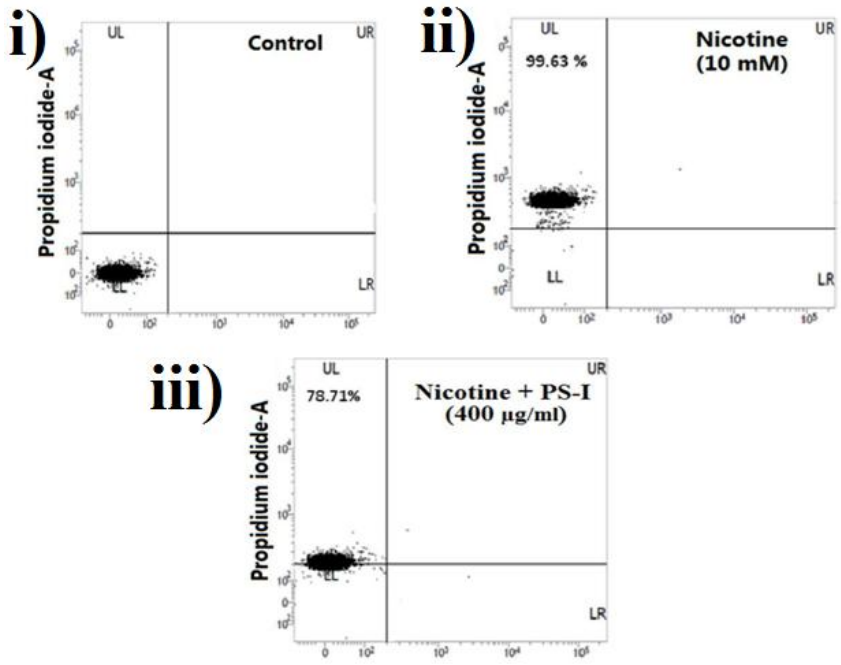
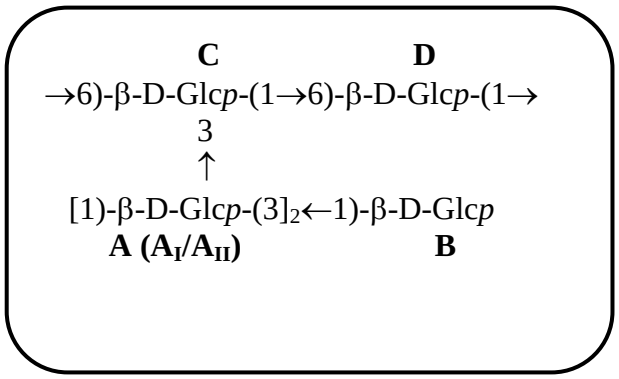


Figure 3.17. Flow cytometry of human lymphocytes: (i) without any treatment (control); (ii) Lymphocytes treated with 10 mM of Nicotine; and (iii) Lymphocytes treated with 10 mM of nicotine + 200 µg/ml of PS-I. The propidium iodide was used for analysis of cell death.

These results indicate that the polysaccharide possesses no adverse effect in lipid peroxidation, antioxidant property as well as beneficial role on cellular toxicity and reduction in GSSG level up to 200 µg/ml indicating the protective role of PS-I against oxidative stress.

3.2.6. Conclusion

A water soluble β-glucan (PS-I), with an average molecular weight ~1.48 x 10^5 Da, was isolated from the alkaline extract of an edible mushroom, *T. heimii* and the following structure was characterized by chemical analysis and 1D/2D NMR studies:



The polysaccharide showed no efficacy to induce lipid peroxidation upto 200 µg/ml. PS-I does not exhibit any cellular toxicity and play a beneficial role on cell proliferation and its protection against nicotine stimulated toxicity.