Chapter IV

Polysaccharide of an edible truffle *Tuber rufum*: Structural studies and effects on human lymphocytes

4.A. Introduction and review of earlier works

Tuber rufum (Pico) var., family *Tuberaceae* is an edible mushroom [92] distributed throughout the world. In India, this mushroom is found in the forest soil of Odisha and West Bengal and collected by digging the soil. *T. rufum*, an ectomycorrhizal [107] fungus grows in association with moist deciduous trees and conifers during the late autumn and winter. It belongs to the group of edible mushroom known as truffle. Recent investigations have shown that truffles possess bioactive compounds exhibiting antioxidant, antimicrobial, and anti-inflammatory activities [108]. Culinary experts regard truffles for their desirable flavours [109]. Different mushrooms of the genus *Tuber* like *T. aestivum, T. rufum, T. simonea, T. brumale, T. melanosporum, T. miesentericum* have been identified as delicious edible truffle [107]. Thirty six compounds have been detected from these six species, out of which the most common compounds present in them are ethanol, carbon dioxide, 2-butanone, ethyl acetate, acetaldehyde, butanoic acid, acetic acid, methyl ester, and 2-butanol. Different ester, acid, and amine compounds have also been identified from the truffle *T. rufum* and reported [110]. This mushroom tastes like goat meat after cooking [92]. Several desert truffles of the genus *Terfezia* and *Tirmania* [111] grow in the areas of the Mediterranean region, the Arabian Peninsula, and North-Africa during rainy season. Some species, like *Terfezia claveryi, Tirmania pinoyi*, and *Terfezia arenaria* grow in phosphate-rich soils [112]. Desert truffles are nutritious and rich in protein [113-115]. A peptide antibiotic was isolated from the aqueous extract of *Terfezia claveryi* which showed to inhibit the growth of *Staphylococcus aureus in vitro* [116]. The antioxidant properties of the desert truffle *Tirmania nivea* [117] and *Tuber*

indicum [118] were studied and reported. Study of antioxidant properties of a water soluble heteroglycan from *Tuber indicum* [119] and a heteroglycan and a glucan from Chinese truffle *Tuber huidongense* [120] were carried out but no detailed structural investigations of these polysaccharides were performed. Hence, detailed structural works relating to the polysaccharides of any truffle mushrooms including the desert truffle of the genus *Terfezia* and *Tirmania* are not reported in the literature. From this point of view through structural investigation of the polysaccharide constituents of the present truffle *Tuber rufum,* an ectomycorrhizal fungus deserves special attention. Several heteroglycans [86,121-123] and glucans [106,124,125] from the edible ectomycorrhizal fungus were reported to possess immunostimulating activities.

Two water soluble polysaccharides (PS-I and PS-II) have been isolated from the aqueous extract of this truffle through Sepharose gel fractionation. The PS-I containing mannose, glucose, and galactose is under investigation. Similar sugar compositions like PS-I was found in a polysaccharide isolated from *Astraeus hygrometricus* [121]. The PS-II was investigated as heteroglycan containing glucose, galactose, and fucose. A same sugar composition like PS-II was isolated from *Russula albonigra* (Krombh.) and characterized [123]. A few more heteroglycans [121-123] consisting of L-fucose were also reported by our research group. L-fucose was reported useful for treatment of infertility [126] and breast cancer [127]. In the present investigation, attempts were made to study the structure and cytotoxic effect of only PS-II. Lymphocytes are the primary immune cells in our body. The damage or intensification of the lymphocyte cells are related to immunity. The toxicological parameters like cell viability, malondialdehyde

(MDA), nitric oxide (NO), reduced glutathione (GSH), oxidized glutathione (GSSG), and reactive oxygen species (ROS) generation levels including redox balances maintained by PS-II were investigated. Hence, a detailed structural characterization and study of some biological activities of PS-II isolated from the edible truffle *T. rufum* have been carried out and discussed in this chapter.

4.B. Present work on PS-II

4.B.1. Isolation, fractionation, and purification of PS-II

The water soluble crude polysaccharide (700 mg) was isolated from the mushroom *T. rufum.* On fractionation through Sepharose gel it showed the presence of two fractions, PS-I and PS-II (**Fig. 1a**).

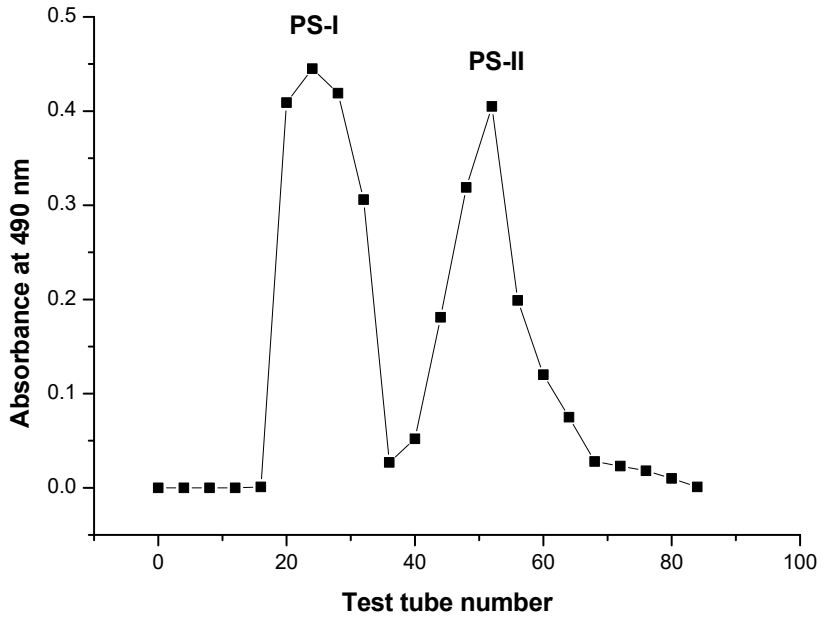


Figure 1(a): Gel permeation chromatogram of crude polysaccharide isolated from an edible mushroom *T. rufum* using Sepharose 6B column.

These two fractions were collected and freeze-dried to give PS-I (test tubes: 16-34), and PS-II (test tubes: 42-60) respectively. The PS-II was further purified by passing through Sepharose 6B column in several lots to obtain 95 mg of pure polysaccharide. The PS-II showed specific rotation $[\alpha]_D^{25.7}$ +16.8 (*c* 0.2, H_2O) .The molecular weight [55] of PS-II was estimated as ~7.27 $\times 10^4$ Da from a calibration curve (**Fig. 1b**; **Table 1**) prepared with standard dextrans.

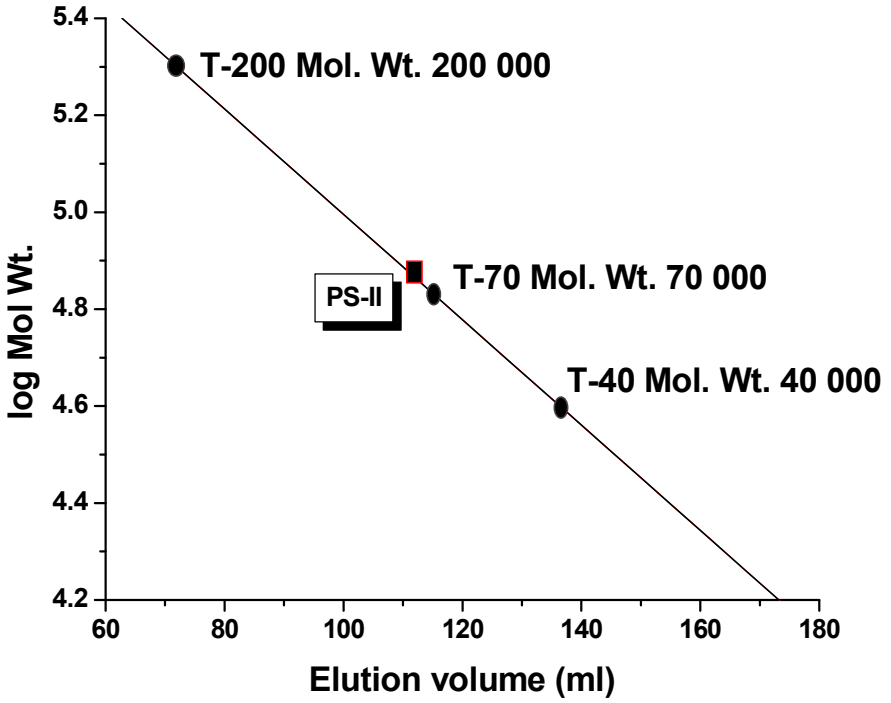


Figure 1(b): Determination of molecular weight of PS-II by gel permeation chromatography in Sepharose 6B column.

Table 1. Components of monosaccharide and properties of PS-II isolated from the mushroom *T. rufum*.

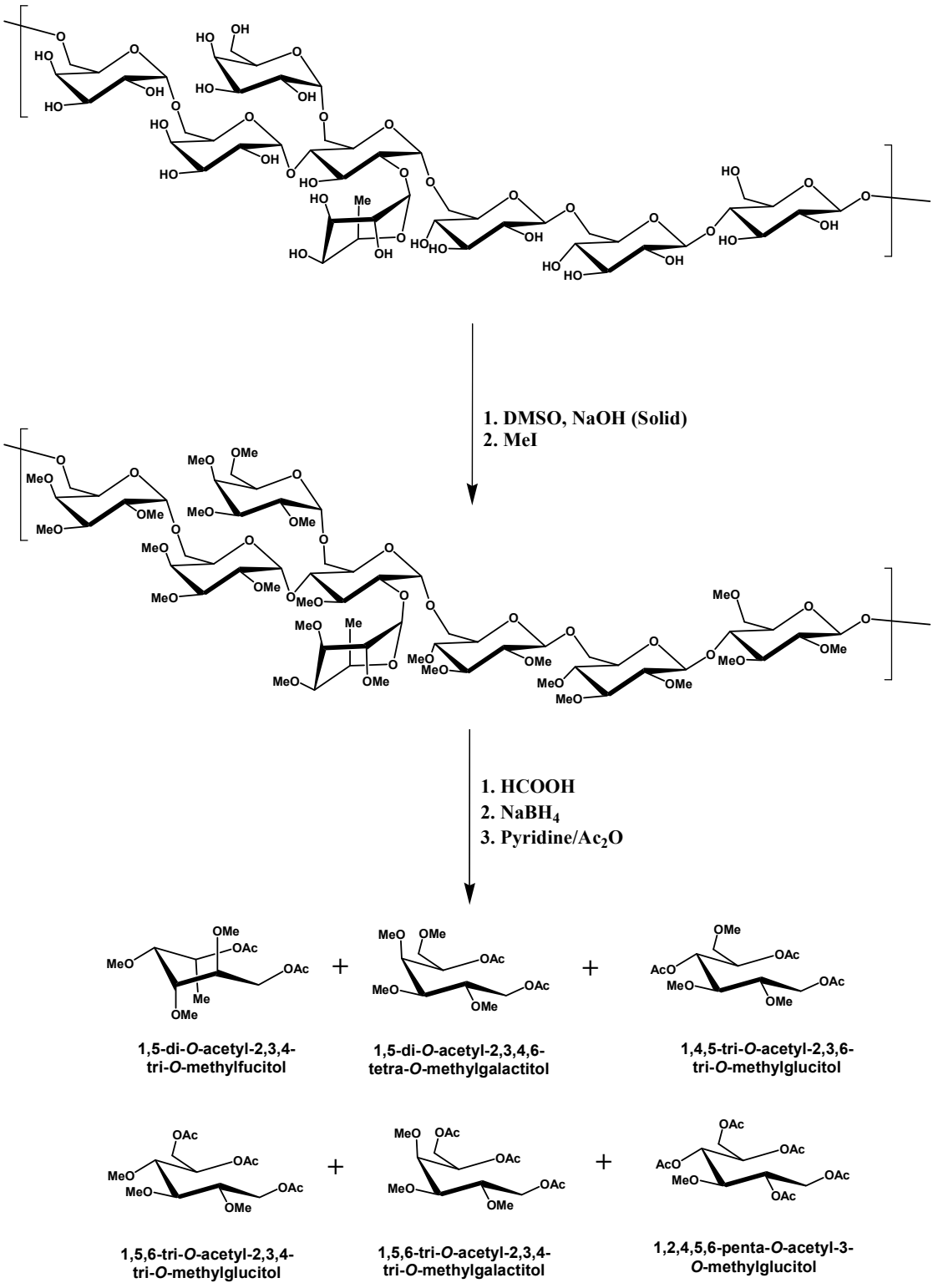
Properties	Data		
Monosaccharide component	Glucose	Galactose	fucose
Molar ratio	4	3	1
Absolute configuration	D	D	L
Specific rotation	$[\alpha]_D^{25.7}$ +16.8 (*c* 0.2, H_2O)		
Molecular weight	~7.27 ×10^4 Da		

4.B.2. Structural analysis of PS-II

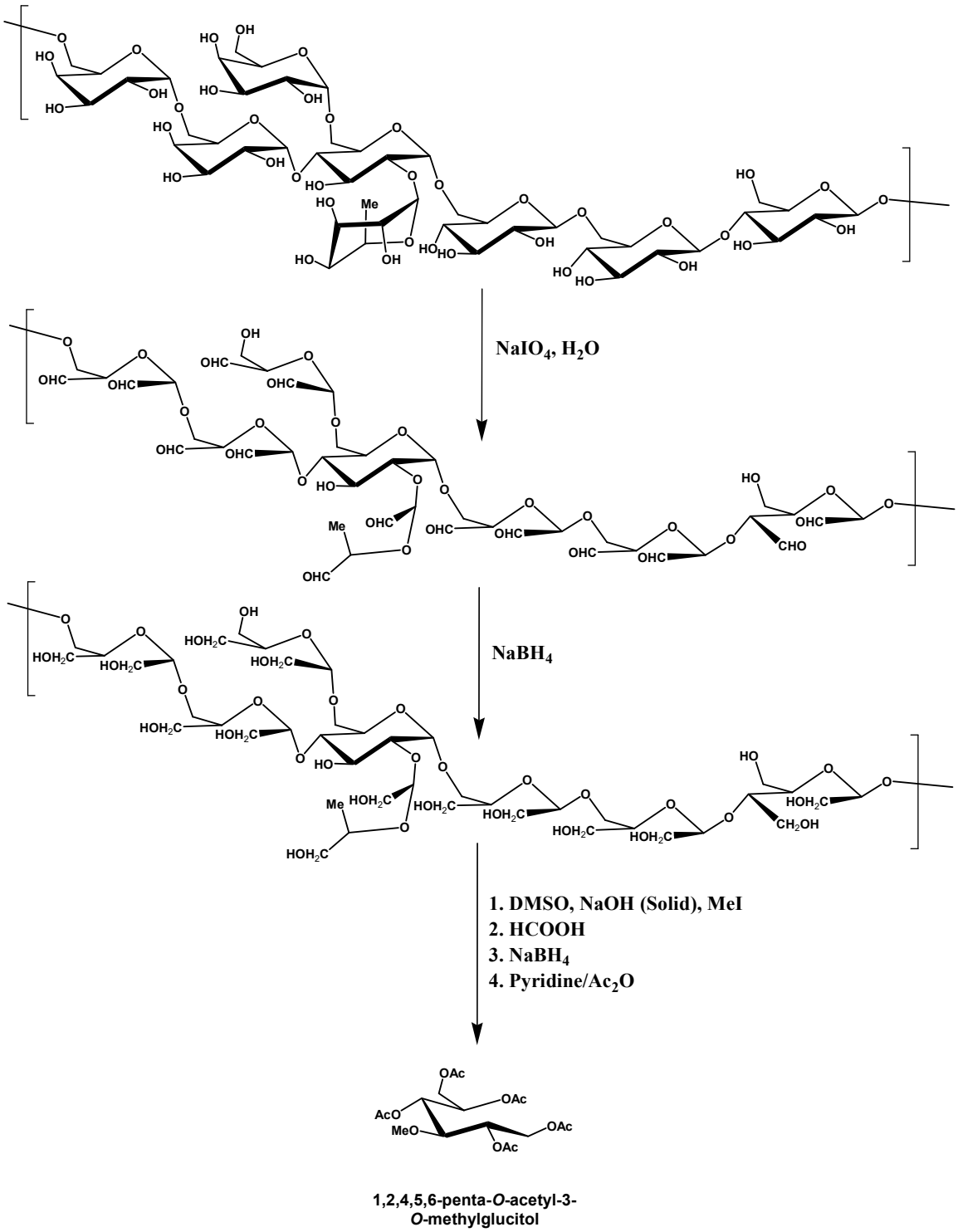
4.B.2.1. Chemical analysis

Gas liquid chromatographic (GLC) analysis of the alditol acetates of the hydrolyzed product of PS-II showed the presence of glucose, galactose and fucose in a molar ratio of nearly 4:3:1. GLC analysis of periodate oxidized reduced PS-II on hydrolysis followed by alditol acetate preparation showed the presence of glucose only. The absolute configuration of the monosaccharide was determined by the method of Gerwig et al. [61] and it was found that glucose and galactose had the D configuration but fucose was present in L configuration. The mode of linkage of the PS-II was determined by the methylation analysis using Ciucanu and Kerek method [62] followed by hydrolysis and alditol acetate preparation. The GLC-MS analysis of the alditol acetates of methylated product showed the presence of 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylfucitol; 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol; 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol; 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol; 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-

methylgalactitol; 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methylglucitol (**Table 2**) in a molar ratio of nearly 1: 1: 1: 2: 2: 1. These results indicated the presence of terminal L-fucopyranosyl, terminal D-galactopyranosyl, (1→4)-D-glucopyranosyl, (1→6)-D-glucopyranosyl, (1→6)-D-galactopyranosyl, and (1→2,4,6)-D-glucopyranosyl residues in the PS-II. GLC-MS analysis of periodate oxidized reduced methylated product of PS-II showed the presence of 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methylglucitol (**Table 3**) indicating the other residues were destroyed during oxidation. Methylation analysis and periodate oxidation reaction of PS-II are presented in the following diagrams:



❖ Schematic representation of methylation analysis of PS-II.



❖ Schematic representation of periodate oxidation reaction of PS-II.

Table 2. GLC-MS results of PS-II isolated from the mushroom *T. rufum*

Methylated sugars	Molar ratio	Linkage type	Major Mass Fragments (m/z)
2,3,4-Me_3-Fuc	1	Fuc*p*-(1→	43,72,89,101,115,117,131,161, 175
2,3,4,6-Me_4-Gal	1	Gal*p*-(1→	43,45,59,71,87,101,117,129,145, 161,205
2,3,6-Me_3-Glc	1	→4)-Glc*p*-(1→	43,45,58,71,87,99,101,113,117, 129,131,143,161,173,233
2,3,4-Me_3-Glc	2	→6)-Glc*p*-(1→	43,45,58,71,87,99,101,117,129, 143,161,173,189,233
2,3,4-Me_3-Gal	2	→6)-Gal*p*-(1→	43,45,58,71,87,99,101,117,129, 143,161,173,189,233
3-Me-Glc	1	→2,4,6)-Glc*p*-(1→	43,59,71,85,87,99,127,129,159, 189,201

Table 3. GLC-MS results of periodate oxidized methylated PS-II isolated from the mushroom *T. rufum*

Methylated sugars	Molar ratio	Linkage type	Major Mass Fragments (m/z)
3-Me-Glc	1	→2,4,6)-Glc*p*-(1→	43,59,71,85,87,99,127,129,159, 189,201

4.B.2.2. 1D and 2D NMR analysis

^{1}H NMR spectrum (500 MHz; **Fig. 2**, **Table 4**) of PS-II at 30 ^{o}C showed the presence of six signals in the anomeric region at δ 5.37, 5.05, 4.97, 4.75, 4.51, and 4.49. The peak at δ 5.37, 4.75, 4.51, and 4.49 were designated as **A**, **E**, **F_I**, and **F_{II}** respectively**.** The peak at δ 5.05 for **B** and **C**, and the peak at δ 4.97 were identified for **D_I** and **D_{II}** residues.

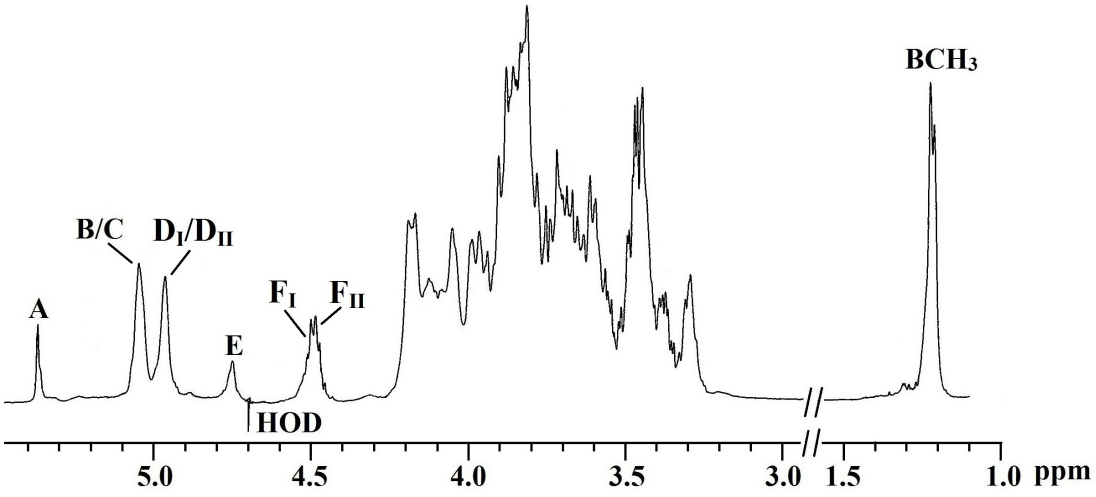


Figure 2: ^{1}H NMR spectrum (500 MHz, D_2O, 30 ºC) of PS-II, isolated from an edible mushroom *T. rufum*.

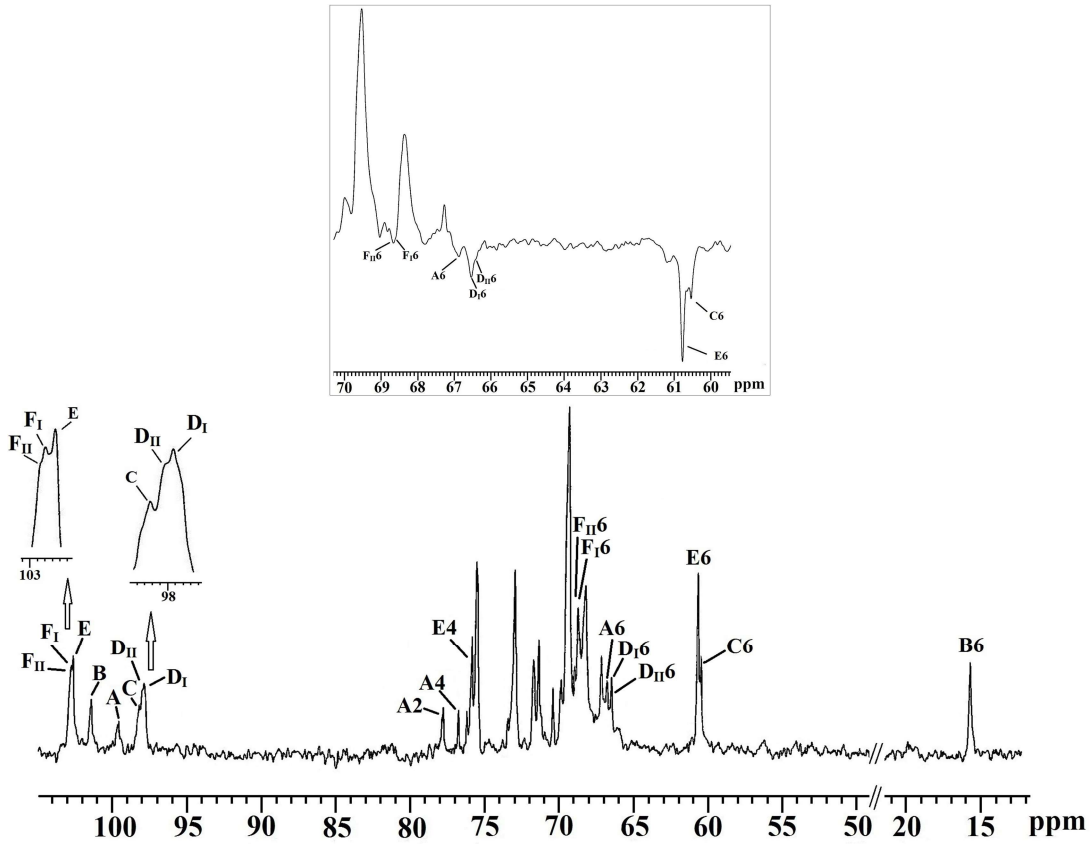


Figure 3: ^{13}C NMR spectrum (125 MHz, D_2O, 30 ^{o}C); (inset: Part of DEPT-135 spectrum (D_2O, 30 ^{o}C) of the PS-II, isolated from an edible mushroom *T. rufum*).

In ^{13}C NMR spectrum (125 MHz; **Fig. 3**) at the same temperature, eight signals were appeared in the anomeric region at δ 102.9, 102.8, 102.6, 101.4, 99.6, 98.3, 98.1, and 97.9. On the basis of HSQC spectrum (**Fig. 4a**; **Table 4**), the anomeric carbon signals at δ 102.9, 102.8, 102.6, 101.4, 99.6, 98.3, 98.1, and 97.9 were correlated to the anomeric proton signals at δ 4.49 **(F_{II})**, 4.51**(F_I)**, 4.75 **(E)**, 5.05 **(B)**, 5.37 **(A)**, 5.05 **(C)**, 4.97 **(D_{II})** and 4.97 **(D_I)** respectively. All the ^{1}H and ^{13}C NMR signals (Table 1) were assigned from DQF-COSY, TOCSY and HSQC 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.

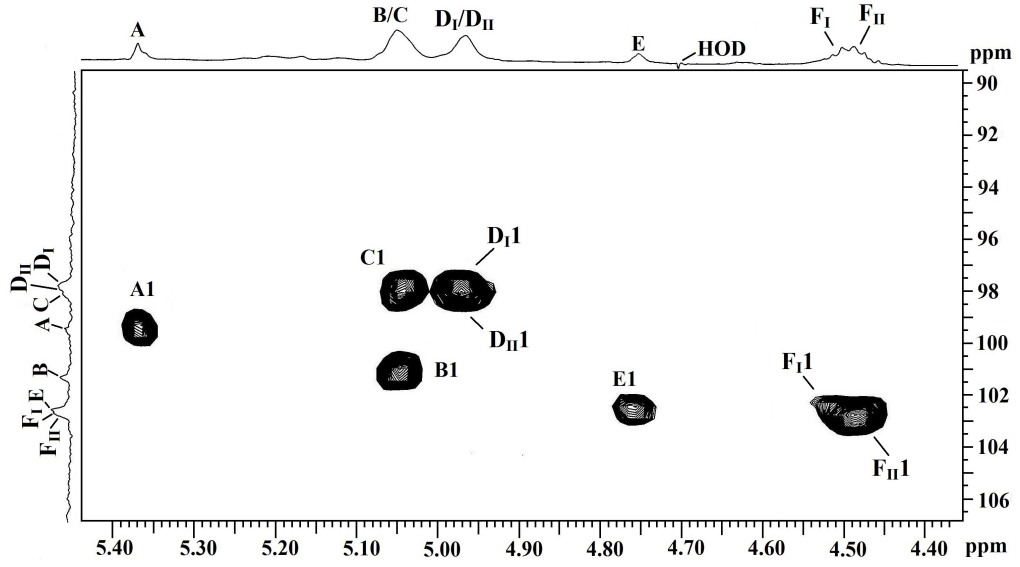


Figure 4(a): HSQC spectrum (D_2O, 30 °C) of anomeric part of PS-II isolated from an edible mushroom *T. rufum.*

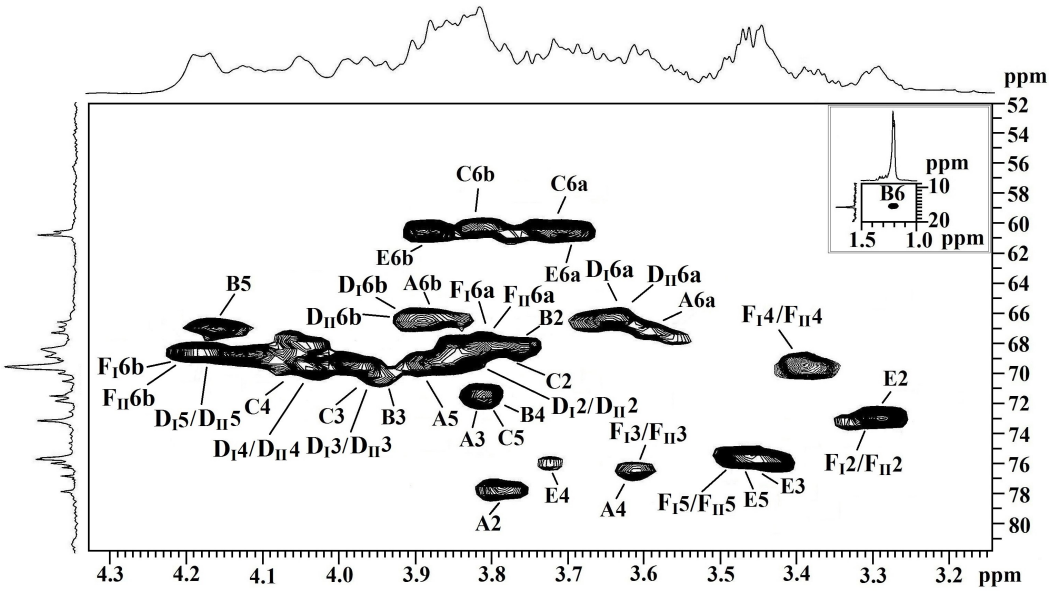


Figure 4(b): HSQC spectrum (D_2O, 30 °C) of other than anomeric part (inset: C-6/H-6 correlation of α-L-Fuc*p* moiety) of PS-II isolated from an edible mushroom *T. rufum.*

Table 4. The ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts of PS-II isolated from an edible mushroom *T. rufum* in D_2O at 30 ºC.

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
→2,4,6)-α-D-Glc*p*-(1→	5.37	3.78	3.81	3.61	3.90	3.61[c], 3.88[d]
A	99.6	77.7	71.8	76.8	69.9	66.9
α-L-Fuc*p*-(1→	5.05	3.76	3.94	3.81	4.17	1.22
B	101.4	68.3	69.5	71.4	67.3	15.7
α-D-Gal*p*-(1→	5.05	3.78	3.96	4.05	3.81	3.73[c], 3.83[d]
C	98.3	68.3	70.5	69.5	71.4	60.5
→6)-α-D-Gal*p*-(1→	4.97	3.84	3.97	4.03	4.19	3.64[c], 3.90[d]
D_I	97.9	69.0	70.0	69.5	68.3	66.5
→6)-α-D-Gal*p*-(1→	4.97	3.84	3.97	4.03	4.19	3.64[c], 3.90[d]
D_{II}	98.1	69.0	70.0	69.5	68.3	66.4
→4)-β-D-Glc*p*-(1→	4.75	3.29	3.44	3.73	3.46	3.71[c], 3.90[d]
E	102.6	73.1	75.6	76.0	75.9	60.8
→6)-β-D-Glc*p*-(1→	4.51	3.30	3.62	3.40	3.48	3.83[c], 4.19[d]
F_I	102.8	73.1	76.3	69.5	75.6	68.7
→6)-β-D-Glc*p*-(1→	4.49	3.30	3.62	3.40	3.48	3.83[c], 4.19[d]
F_{II}	102.9	73.1	76.3	69.5	75.6	68.8

[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.

Anomeric signals of residue **A** had the proton chemical signal at δ 5.37 and the carbon signal at δ 99.6 and the coupling constant values $J_{H\text{-}1,\ H\text{-}2}$ ~3.1 and $J_{C\text{-}1,\ H\text{-}1}$ ~171 Hz

indicated that the residue **A** was present in α-configuration. The large coupling constant values $J_{H\text{-}2,\ H\text{-}3}$ and $J_{H\text{-}3,\ H\text{-}4}$ (~10 Hz) indicated that the residue **A** was D-glucopyranosyl moiety. The downfield shift (Fig. 4b) of C-2 (δ 77.7), C-4 (δ 76.8), and C-6 (δ 66.9) of residue **A** with respect to the standard values of methyl glycoside [66,97] indicated that it was (1→2,4,6)-α-D-glucopyranosyl moiety. The linkage at C-6 of the residue **A** was further confirmed from DEPT-135 spectrum (**Fig. 3**; **Inset**).

Residue **B** was assigned as a terminal α-L-fucopyranosyl unit, which was strongly supported by the appearance of proton signal at δ 1.22, carbon signal at δ 15.7 (Fig. 4b) for an exocyclic-CH_3 group, and the relatively small $J_{H\text{-}3,\ H\text{-}4}$ (< 3 Hz). The anomeric proton and carbon chemical shifts appeared for residue **B** at δ 5.05 and 101.4 respectively. The coupling constant value $J_{H\text{-}1,\ H\text{-}2}$~3.8 Hz, $J_{C\text{-}1,\ H\text{-}1}$~171 Hz clearly indicated that it was in α-configuration. Thus, considering the results of methylation analysis and NMR experiments, it was concluded that residue **B** was α-linked terminal L-fucopyranosyl moiety.

The anomeric proton signal for residue **C** appeared at δ 5.05 and for residues $\mathbf{D_I}$ and $\mathbf{D_{II}}$ appeared at δ 4.97. The anomeric carbon signals for residues **C**, $\mathbf{D_I}$, and $\mathbf{D_{II}}$ appeared at δ 98.3, 97.9, and 98.1 respectively. Residues **C** and both the **D** had coupling constant values of $J_{H\text{-}2,\ H\text{-}3}$ ~8 Hz and $J_{H\text{-}3,\ H\text{-}4}$ ~3.5 Hz. These values indicated that residues **C**, $\mathbf{D_I}$ and $\mathbf{D_{II}}$ were D-galactopyranosyl moieties. The α-configuration of **C**, $\mathbf{D_I}$, and $\mathbf{D_{II}}$ residues were assigned from the coupling constant values $J_{H\text{-}1,\ H\text{-}2}$ ~3.1 and $J_{C\text{-}1,\ H\text{-}1}$ ~170 Hz. All the proton and carbon chemical shifts of residue **C** were found nearly to the standard values of methyl glycosides of α-D-galactose. Thus reisdue **C** was confirmed as terminal

α-D-galactopyranosyl moiety. The downfield shifts of C-6 (δ 66.5, 66.4) with respect to standard values of methyl glycoside [66,97] indicated that both the **D** residues were (1→6)-linked α-D-galactopyranosyl. The linkage at C-6 of the residues $\mathbf{D_I}$ and $\mathbf{D_{II}}$ were further confirmed from DEPT-135 spectrum (**Fig. 3**; **Inset**). Hence, these observations confirmed that the residue $\mathbf{D_I}$ and $\mathbf{D_{II}}$ were (1→6)-α-D-galactopyranosyl moiety.

Residue **E**, $\mathbf{F_I}$, and $\mathbf{F_{II}}$ were assigned as D-glucopyranosyl moieties due to their large coupling constant values $J_{\text{H-2, H-3}}$ (~9.5 Hz) and $J_{\text{H-3, H-4}}$(~10.0 Hz). The anomeric proton signals for residues **E**, $\mathbf{F_I}$, and $\mathbf{F_{II}}$ were appeared at δ 4.75, 4.51, and 4.49 respectively. The anomeric carbon signals for **E**, $\mathbf{F_I}$, and $\mathbf{F_{II}}$ were observed at δ 102.6, 102.8, and 102.9 respectively. The coupling constant values $J_{\text{H-1, H-2}}$ (~8.0 Hz) and $J_{\text{C-1, H-1}}$ (~160 Hz) indicated that residues **E**, $\mathbf{F_I}$, and $\mathbf{F_{II}}$ were in β-configuration. In the residue **E,** the downfield shift of C-4 (δ 76.0) with respect to standard values of methyl glycoside [28,29] indicated that it was (1→4)-β-D-glucopyranosyl moiety. The downfield shift of C-6 (δ 68.7 and 68.8) indicated that residues $\mathbf{F_I}$ and $\mathbf{F_{II}}$ were (1→6)-β-D-glucopyranosyl moiety. The linkage of residues $\mathbf{F_I}$ and $\mathbf{F_{II}}$ at C-6 was further confirmed from DEPT-135 spectrum (**Fig. 3**; **Inset**).

The sequence of the glycosyl residues were determined from ROESY (**Fig. 5**; **Table 5**) as well as NOESY (not shown) experiments. In ROESY experiment, the inter-residual contacts $\mathbf{D_I}$H-1/ $\mathbf{D_{II}}$H-6a, 6b; $\mathbf{D_{II}}$H-1/ **A**H-4; **A**H-1/ $\mathbf{F_I}$H-6a, 6b; $\mathbf{F_I}$H-1/ $\mathbf{F_{II}}$H-6a, 6b; $\mathbf{F_{II}}$H-1/ **E**H-4; **E**H-1/$\mathbf{D_I}$H-6a, 6b; **B**H-1/**A**H-2; **C**H-1/**A**H-6a, 6b along with other intra-residual contacts were also observed. Thus the ROESY connectivities established the following

sequences: **D$_I$** (1→6) **D$_{II}$**; **D$_{II}$** (1→4) **A**; **A** (1→6) **F$_I$**; **F$_I$** (1→6) **F$_{II}$**; **F$_{II}$** (1→4) **E**; **E** (1→6) **D$_I$**; **B** (1→2) **A**; and **C** (1→6) **A**.

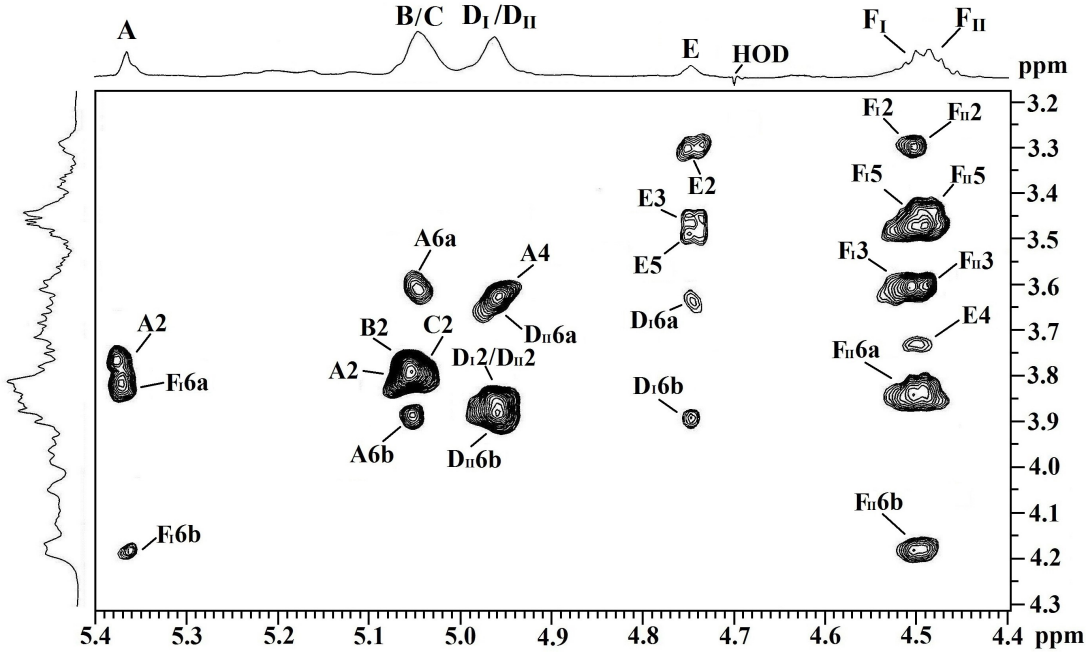


Figure 5: Part of ROESY spectrum of PS-II from an edible mushroom *T. rufum*. The ROESY mixing time was 300 ms.

Finally these connectivities were confirmed from HMBC spectrum (**Fig. 6a, 6b**; **Table 6**). In this spectrum the inter-residual cross-peaks were observed between **D$_I$**H-1/ **D$_{II}$**C-6; **D$_I$**C-1/ **D$_{II}$**H-6a, 6b; **D$_{II}$**H-1/ **A**C-4; **D$_{II}$**C-1/ **A**H-4; **A**H-1/ **F$_I$**C-6; **A**C-1/ **F$_I$**H-6a, 6b; **F$_I$**H-1/ **F$_{II}$**C-6; **F$_I$**C-1/ **F$_{II}$**H-6a, 6b; **F$_{II}$**H-1/ **E**C-4; **F$_{II}$**C-1/ **E**H-4; **E**H-1/**D$_I$**C-6; **E**C-1/**D$_I$**H-6a, 6b; **B**H-1/**A**C-2; **B**C-1/**A**H-2; **C**H-1/**A**C-6; and **C**C-1/**A**H-6a, 6b along with other intra-residual contacts.

Table 5. ROESY data of PS-II isolated from an edible mushroom *T. rufum.*

Glycosyl residue	Anomeric proton	ROE contact proton		
	δ	δ	residue	atom
→2,4,6)-α-D-Glc*p*-(1→	5.37	3.83	**F_I**	H-6a
A		4.19	**F_I**	H-6b
		3.78	**A**	H-2
α-L-Fuc*p*-(1→	5.05	3.78	**A**	H-2
B		3.76	**B**	H-2
α-D-Gal*p*-(1→	5.05	3.61	**A**	H-6a
C		3.88	**A**	H-6b
		3.78	**C**	H-2
→6)-α-D-Gal*p*-(1→	4.97	3.64	**D_{II}**	H-6a
D_I		3.90	**D_{II}**	H-6b
		3.84	**D_I**	H-2
→6)-α-D-Gal*p*-(1→	4.97	3.61	**A**	H-4
D_{II}		3.84	**D_{II}**	H-2
→4)-β-D-Glc*p*-(1→	4.75	3.64	**D_I**	H-6a
E		3.90	**D_I**	H-6b
		3.29	**E**	H-2
		3.44	**E**	H-3
		3.46	**E**	H-5
→6)-β-D-Glc*p*-(1→	4.51	3.83	**F_{II}**	H-6a
F_I		4.19	**F_{II}**	H-6b
		3.30	**F_I**	H-2
		3.62	**F_I**	H-3
		3.48	**F_I**	H-5
→6)-β-D-Glc*p*-(1→	4.49	3.73	**E**	H-4
F_{II}		3.30	**F_{II}**	H-2
		3.62	**F_{II}**	H-3
		3.48	**F_{II}**	H-5

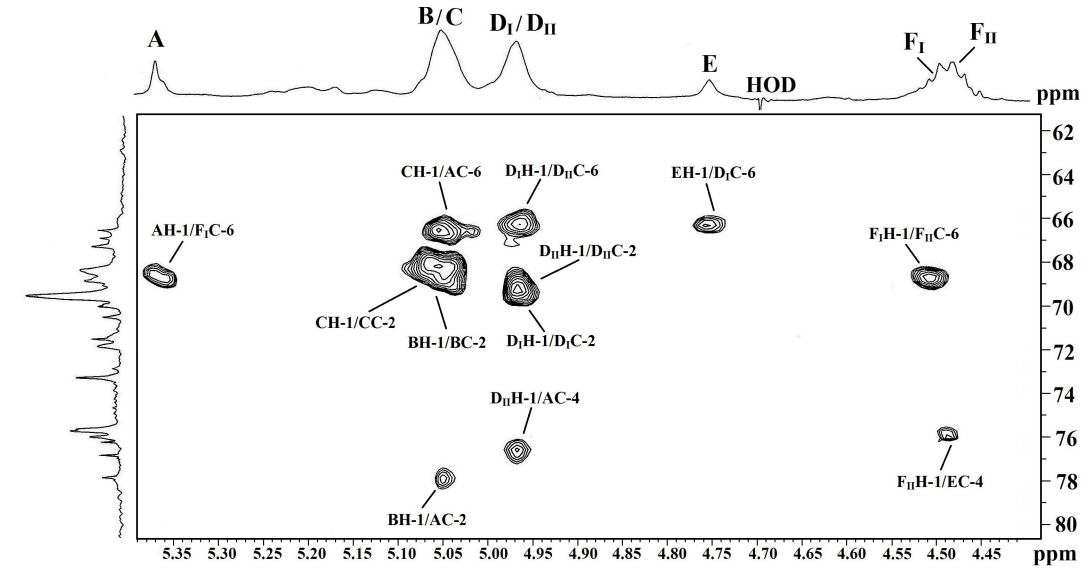


Figure 6(a): The part of HMBC spectrum for anomeric protons of PS-II isolated from an edible mushroom *T. rufum*.

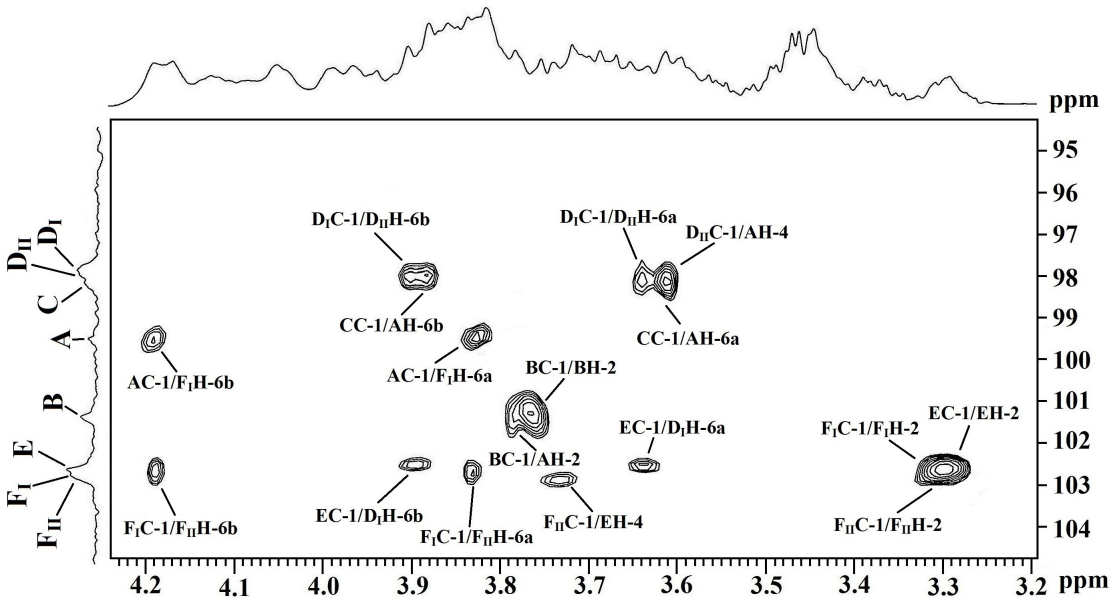
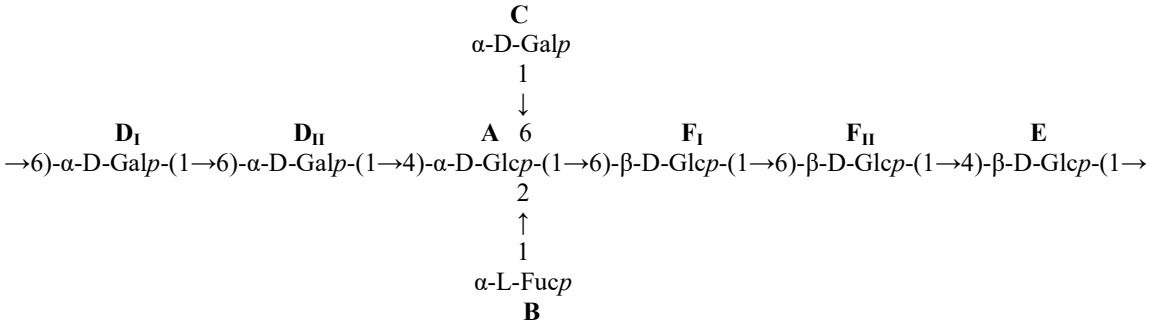


Figure 6(b): the part of HMBC spectrum for anomeric carbons of PS-II isolated from an edible mushroom *T. rufum*. The delay time in the HMBC experiment was 80 ms.

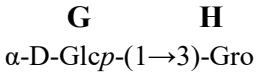
Table 6. The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the protons/carbons of the sugar residues of the PS-II isolated from the mushroom *T. rufum.*

Glycosyl residue	H-1/C-1	Observed connectivities		
	δ_H/δ_C	δ_H/δ_C	Residue	Atom
→2,4,6)-α-D-Glc*p*-(1→	5.37	68.7	**F_I**	C-6
A	99.6	3.83	**F_I**	H-6a
		4.19	**F_I**	H-6b
α-L-Fuc*p*-(1→	5.05	77.7	**A**	C-2
B		68.3	**B**	C-2
	101.4	3.78	**A**	H-2
		3.76	**B**	H-2
α-D-Gal*p*-(1→	5.05	66.9	**A**	C-6
C		68.3	**C**	C-2
	98.3	3.61	**A**	H-6a
		3.88	**A**	H-6b
→6)-α-D-Gal*p*-(1→	4.97	66.4	**D_{II}**	C-6
D_I		69.0	**D_I**	C-2
	97.9	3.64	**D_{II}**	H-6a
		3.90	**D_{II}**	H-6b
→6)-α-D-Gal*p*-(1→	4.97	76.8	**A**	C-4
D_{II}		69.0	**D_{II}**	C-2
	98.1	3.61	**A**	H-4
→4)-β-D-Glc*p*-(1→	4.75	66.5	**D_I**	C-6
E	102.6	3.64	**D_I**	H-6a
		3.90	**D_I**	H-6b
		3.29	**E**	H-2
→6)-β-D-Glc*p*-(1→	4.51	68.8	**F_{II}**	C-6
F_I	102.8	3.83	**F_{II}**	H-6a
		4.19	**F_{II}**	H-6b
		3.30	**F_I**	H-2
→6)-β-D-Glc*p*-(1→	4.49	76.0	**E**	C-4
F_{II}	102.9	3.73	**E**	H-4
		3.30	**F_{II}**	H-2

Thus, based on all these chemical and spectroscopic evidences, the structure of repeating unit of the polysaccharide was proposed as:



NMR studies were again carried out with Smith degraded product (SDPS) for further confirming the linkages. The ^{13}C NMR (125 Hz) spectrum (**Fig. 7**; **Table 7**) of SDPS at 30 ^{o}C showed one anomeric carbon signal at δ 99.8 for one terminal α-D-Glc*p* (**G**) residue. The C-1, C-2, and C-3 carbon signals of the glycerol moiety (Gro) [128,129] were assigned as δ 66.6, 72.1, and 62.6 respectively. The glycerol moiety **H** was generated from (1→6)-β-D-Glc*p* residue (**F$_I$**) after periodate oxidation followed by Smith degradation, and this moiety was attached to α-D-Glc*p* (**G**) which was generated from the (1→2,4,6)-α-D-Glc*p* (**A**) residue of the parent PS-II during Smith degradation. The other residues were destroyed during oxidation followed by mild hydrolysis. Hence, a glycerol containing monosaccharide was obtained from the parent PS-II after Smith degradation and the structure was established as:



Therefore, the Smith degraded oligomer further confirmed the repeating unit present in the PS-II of the truffle mushroom *T. rufum.*

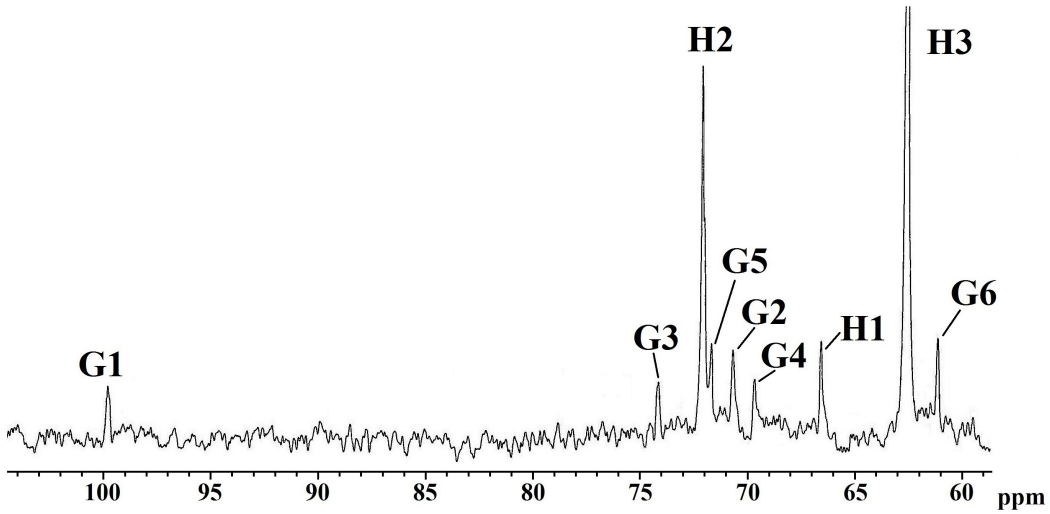


Figure 7: ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of the Smith-degraded glycerol containing monosaccharide of PS-II isolated from an edible mushroom *T. rufum*.

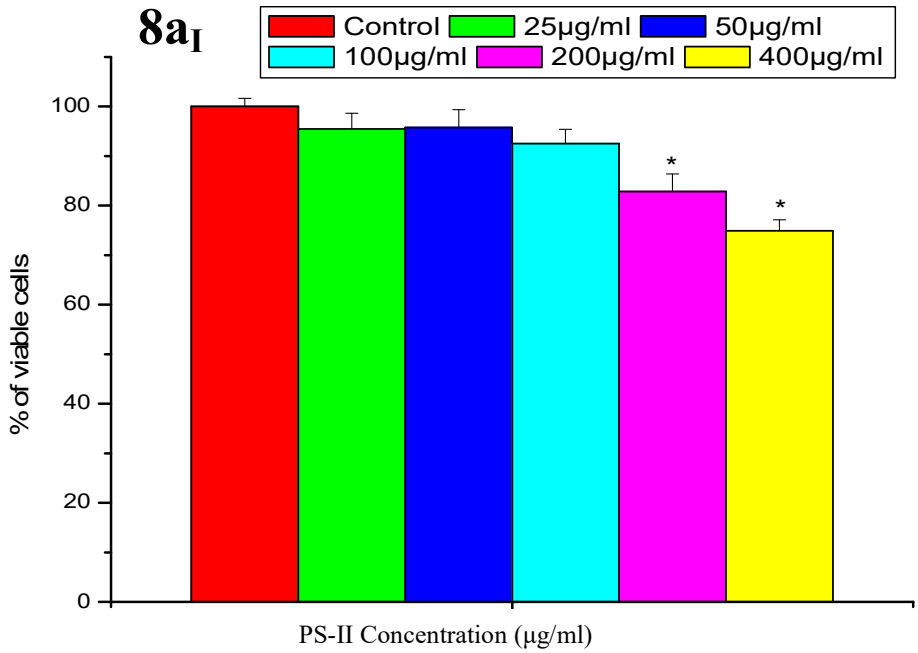
Table 7. The ^{13}C NMR[n] chemical shifts of Smith-degraded glycerol-containing monosaccharide of PS-II from an edible mushroom *T. rufum.* in D_2O at 30 °C.

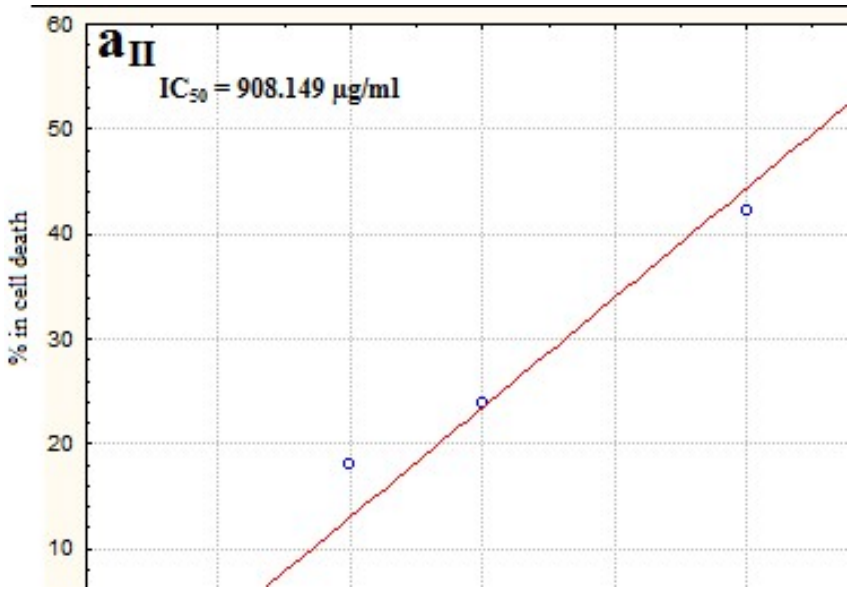
Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
α-D-Glc*p*-(1→ **G**	99.8	70.7	74.1	69.7	71.8	61.1
→3)-Gro **H**	66.6	72.1	62.6			

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

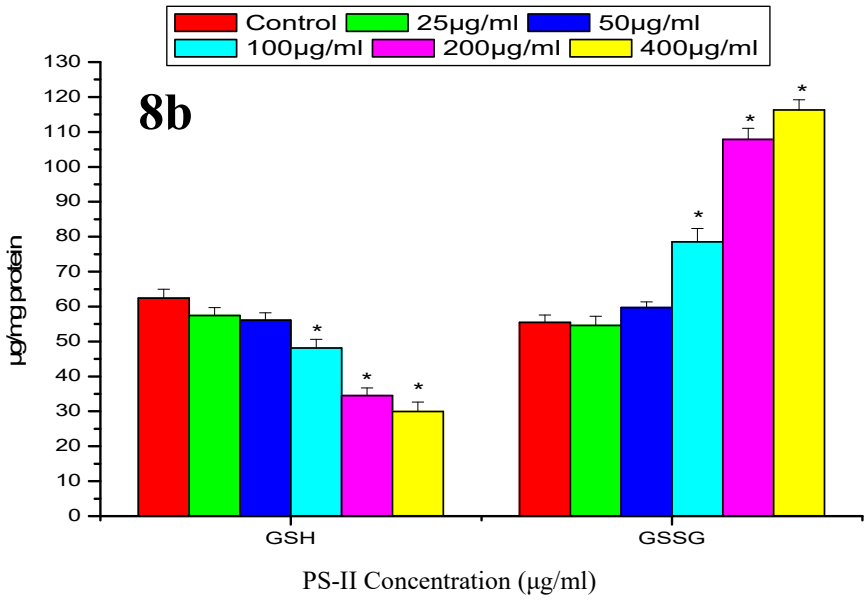
4.B.3. Biological activities of PS-II

The cytotoxic effect of the PS-II was studied in human blood 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. 8a$_I$**). The biological dose of PS-II on human lymphocyte culture showed no significant toxicity below 200 µg/mL. But from the dose 200 µg/mL onwards significant toxicity arises. The IC_{50} value (**Fig. 8a$_{II}$**) of PS-II determined 908.149 µg/mL indicates non toxicity of the polysaccharide which supports the cell viability results.

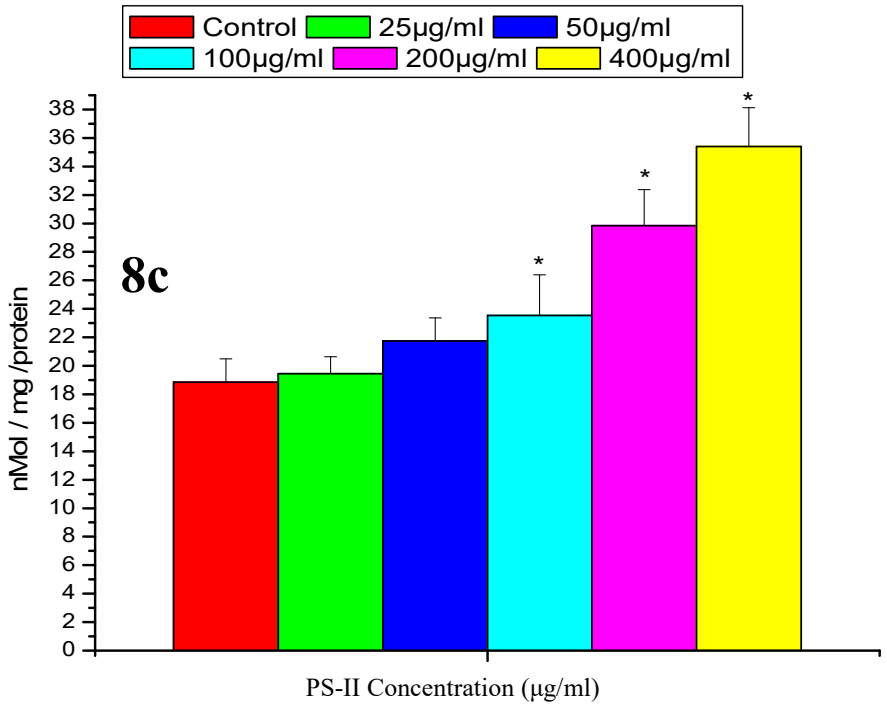




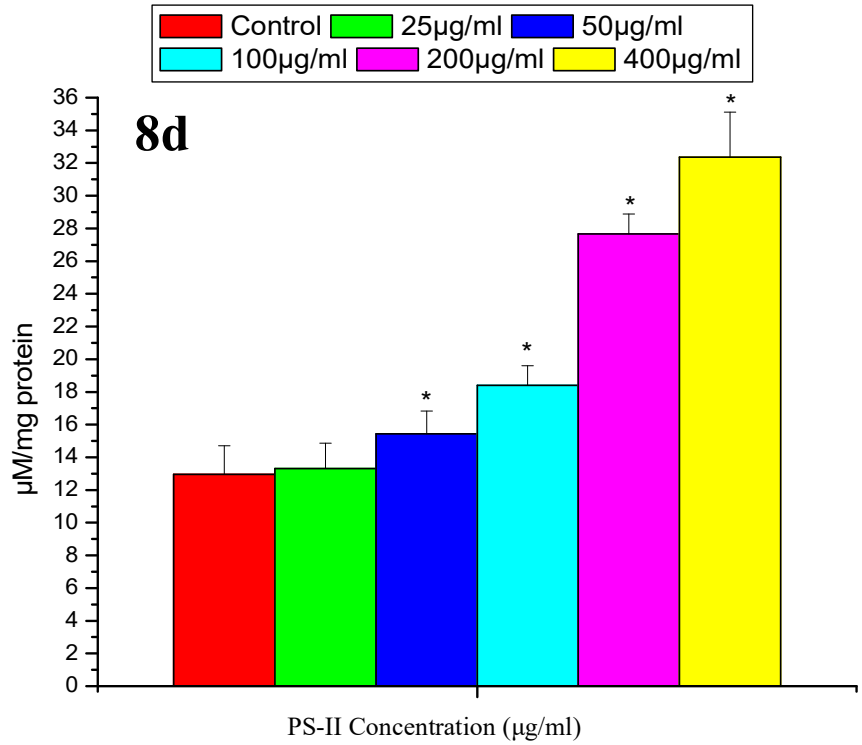
Glutathione is an important antioxidant in cellular system. Hence, both the reduced and oxidized form of glutathione was measured to understand the level of glutathione. The GSH level (**Fig. 8b**) was fairly decreased and the mild increase of GSSG level (**Fig. 8b**) was observed at the dose of 400 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 = 0.79, Pearson correlation $p < 0.05$). 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 0.311 to 0.241 compared to their respective control indicating that 400 µg/mL was toxic. These results further indicated that any dose below 200 µg/mL of PS-II is biologically safe and effective since it does not appreciably alter the redox state in lymphocytes.



Oxidative stress in biological system is induced due to lipid peroxidation which initiates inactivation of cellular components and protective enzymes. Hence, it can be regarded as an indicator to assess the cellular damage. Lipid peroxidation in lymphocytes was measured in terms of the release of MDA. The present result indicates slight increase of MDA level at 400 µg/mL. This observation confirms the dose dependent increment of MDA (**Fig. 8c**) which made the PS-II effective and safe below 200 µg/mL on lymphocytes.



Stimulated lymphocytes can release NO. The treatment of PS-II ranging from 50-200 µg/mL into the single culture of lymphocytes produced significant amount of NO ($p < 0.05$) after 24h of incubation. The present observation showed the presence of high concentration of NO (**Fig. 8d**; increase 2.53 fold in comparison to control) into the culture medium of pulsed lymphocytes at 400 µg/mL indicating that this dose is cytotoxic. The secretion of NO clearly demonstrated (**Fig. 8d**) that it was secreted by the lymphocytes when stimulated by PS-II. Hence, a connection between efficiency of the PS-II and its compatibility with healthy cells is established.



The generation of ROS was also studied using the PS-II on lymphocyte. The PS-II induced maximum ROS generation at higher concentration (400 µg/mL) into the lymphocytes. The excess generation of ROS can induce the damage of membrane lipid and alter the cellular antioxidant molecules like glutathione, followed by apoptosis mediated cell death. The dose dependent increase of ROS (**Fig. 8e**) at higher drug concentration is the responsible for cell death and these data again confirms that below 200 µg/mL is a safe dose for human lymphocytes. Thus the lower level of ROS generation by PS-II below 200 µg/mL prevents the cell damage from oxidative stress.

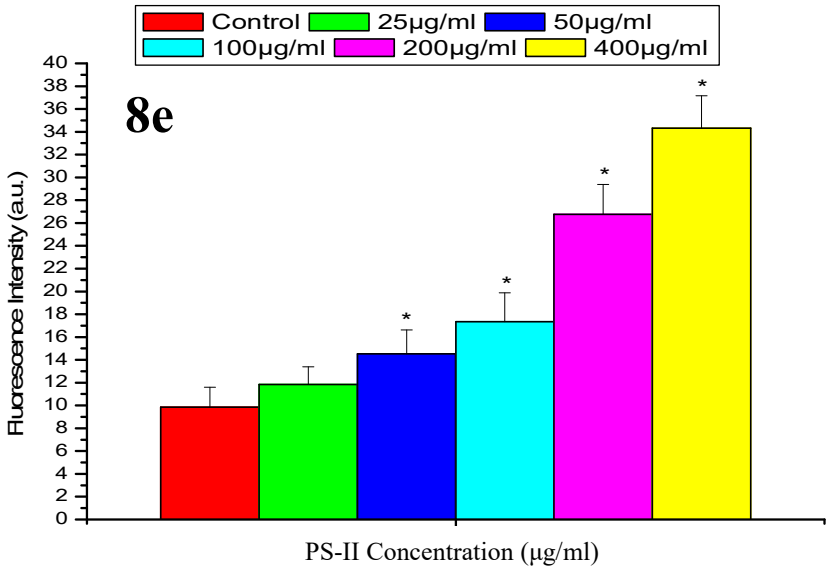
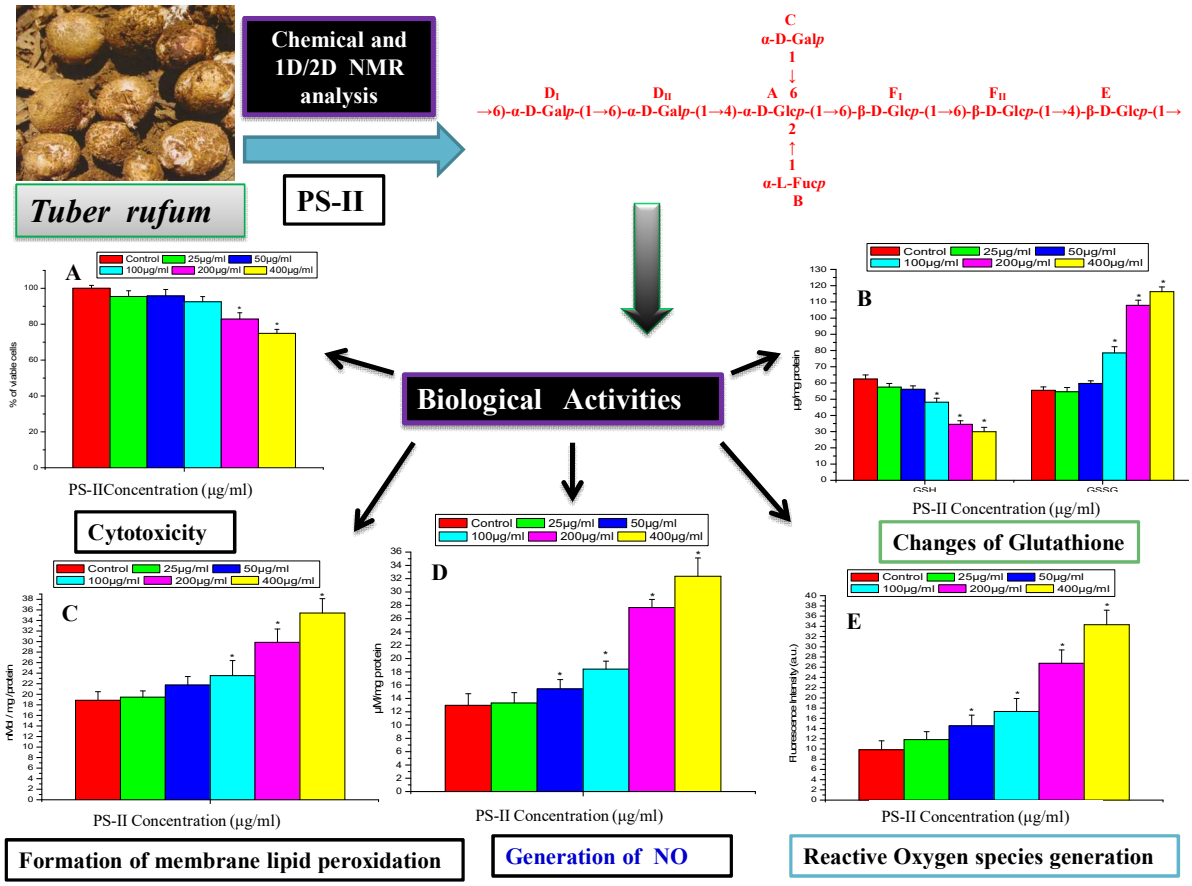


Figure 8(a_I): Cytotoxicity of PS-II against human lymphocytes, 8**(a_{II})**: IC_{50} value of PS-II against human lymphocytes **8(b)**: Changes of Glutathione (Reduced and Oxidised) of PS-II against human lymphocytes, **8(c)**: Formation of membrane lipid peroxidation in terms of Maloneldehyde (MDA) of PS-II against human lymphocytes, **8(d)**: Generation of nitric oxide (NO) of PS-II against human lymphocytes, **8(e):** Reactive Oxygen species (ROS) generation of PS-II against human lymphocytes. [*n* = 6; All values are expressed as mean ± SEM. Asterisks (*) indicates the significant difference as compared to control group].

It is evident from our study that, in vitro application of PS-II has good effect upto a certain level. This compound dose not induce cellular damage in lymphocytes associated with enhanced MDA level, NO level, GSSG level and decreased GSH level. It has been observed that the study of cytotoxic profile of PS-II in lymphocytes indicated 200 µg/mL is safe whereas the higher concentration of PS-II (400 µg/mL) showed significant

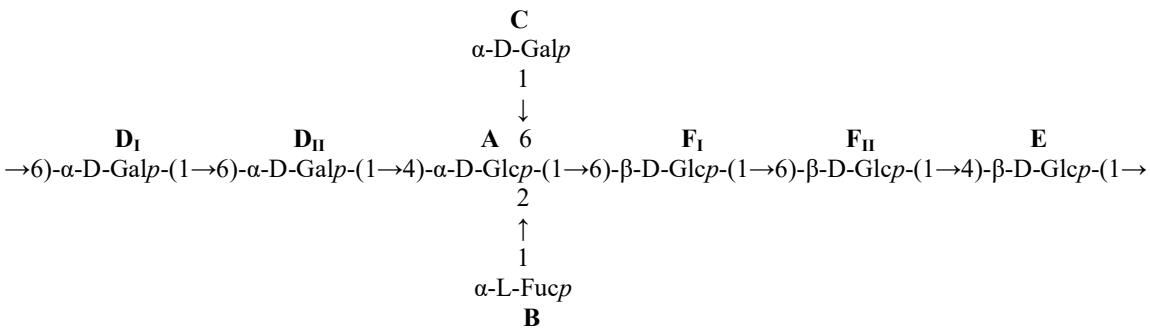
increase of cytotoxicity. These findings suggest that PS-II up to certain dose (below 200 µg/mL) may be used for therapeutic purposes.

❖ **All the structural and biological studies of a polysaccharide (PS-II) from edible mushroom *Tuber rufum* are presented in the following diagram:**



4.C. Conclusion

A water soluble heteroglycan (PS-II) with average molecular weight ~7.27 $\times 10^4$ Da was isolated from the fruiting bodies of an edible truffle mushroom *Tuber rufum* (Pico) Var. by hot water extraction. On the basis of all these experiments, the repeating unit of the PS-II was found to contain a backbone of two (1→6)-α-D-galactopyranosyl, one (1→4)-α-D-glucopyranosyl, two (1→6)-β-D-glucopyranosyl, and one (1→4)-β-D-glucopyranosyl residues, out of which (1→4)-α-D-glucopyranosyl residue was branched at *O*-2 position with terminal α-L-fucopyranosyl residue and at *O*-6 position with terminal α-D-galactopyranosyl residue. Based on chemical and spectroscopic evidences, the possible repeating motif of the polysaccharide was proposed as;



The isolated PS-II was nontoxic upto 200 μg/mL. At higher dose, it produces ROS which kills the cells. The PS-II may be used as an immunostimulant on the basis of further research.