Chapter III

Heteroglycan of an edible mushroom *Termitomyces clypeatus* : Structure elucidation and antioxidant properties

3.A. Introduction and review of earlier works

Mushrooms are consumed as delicious food and considered a source of biologically active compounds [83]. The polysaccharides of edible mushrooms have a lot of pharmaceutical applications in industries [84]. Several polysaccharides [85-90] from the edible mushrooms have been isolated, purified, and characterized. Reactive oxygen species (ROS) are produced during biochemical reactions in human body and uncontrolled production of the oxygen derived free radicals generate various diseases like cancer, coronary heart diseases, Alzheimer, and neurodegenerative disorders [91]. In recent years, the research work has been focused on the use of mushroom polysaccharides as neutralizing agents of the free radicals as well as its protective role for human lymphocytes [64].

Termitomyces clypeatus commonly called by local people as Bada bali chattu, Batki chattu and Bihiduni chattu in India belongs to the genus *Termitomyces*, family lyophyllaceae. The edible mushroom [92] normally grows in association with the termite's guts of the lateritic forest soil of South West Bengal and Odisha, India during September-October every year. Several polysaccharides from *Termitomyces striatus* [93], *T. eurhizus* [94], *T. microcarpus* [95], *and T. robustus* [96] have been isolated, and characterized. One water soluble polysaccharide (PS) has been isolated from the aqueous extract of the mushroom through gel permeation chromatography and characterized as heteroglycan. It showed promising antioxidant activities as evidenced from its ferrous ion chelating ability, superoxide radical scavenging activity, and high reducing power

property. The detailed structural investigations and antioxidant properties of the PS was carried out and discussed in this chapter.

3.B. Present work on PS

3.B.1. Isolation and purification of polysaccharide

The hot aqueous extract of fresh fruit bodies (500 g) of edible mushroom *T. clypeatus* was cooled, filtered, and precipitated in ethanol. The residue was dialyzed, centrifuged, and freeze dried, yield; 650 mg. A portion (25 mg) of this crude polysaccharide was purified by gel-permeation chromatography on Sepharose-6B column. One homogeneous fraction (**Fig. 1a**) was obtained (test tubes 16–34), collected, and freeze-dried, yield; 19 mg. It was further purified by passing through Sepharose 6B column in several lots to yield 110 mg of pure polysaccharide (PS). The pure polysaccharide (PS) showed specific rotation $[\alpha]_D^{25.7}$ +12.9 (*c* 0.11, H_2O) and the average molecular weight of the PS was determined as ~1.98×10^5 Da (**Fig 1b**).

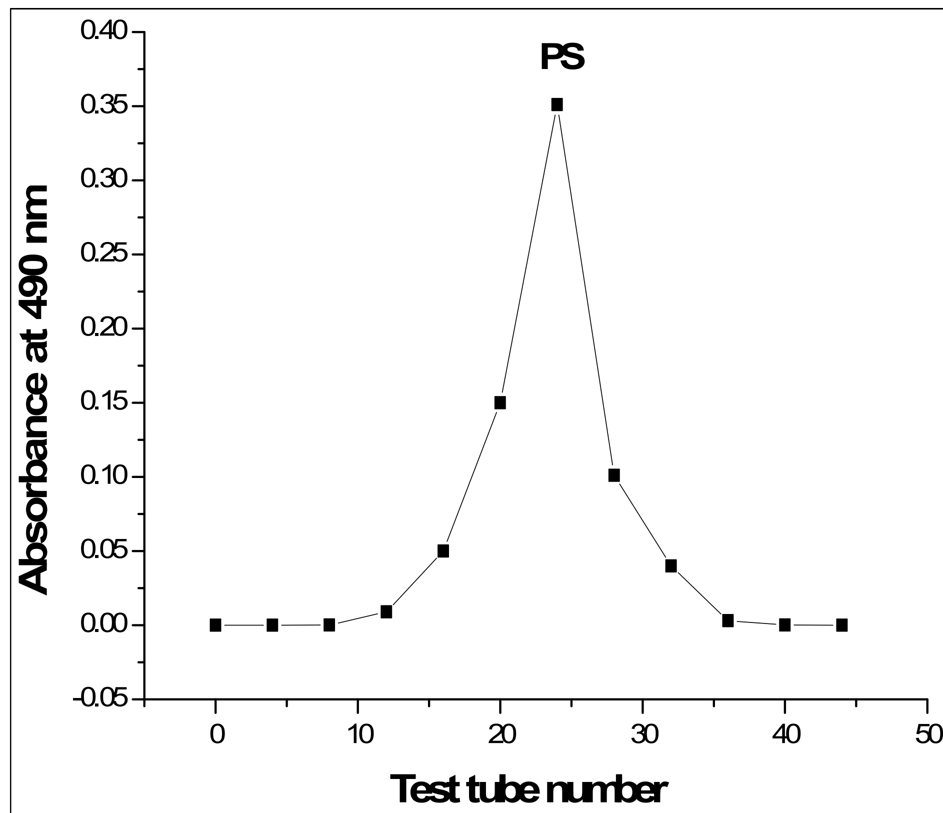


Figure 1(a): Gel permeation chromatogram of crude polysaccharide isolated from an edible mushroom *T. clypeatus* using Sepharose 6B column.

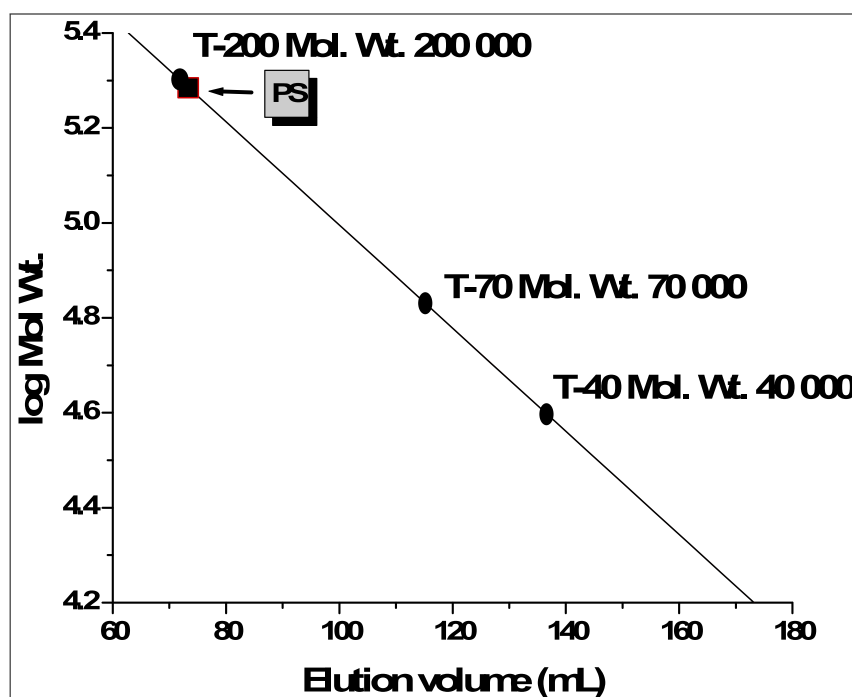


Figure 1(b): Determination of molecular weight of PS by gel permeation chromatography in Sepharose 6B column.

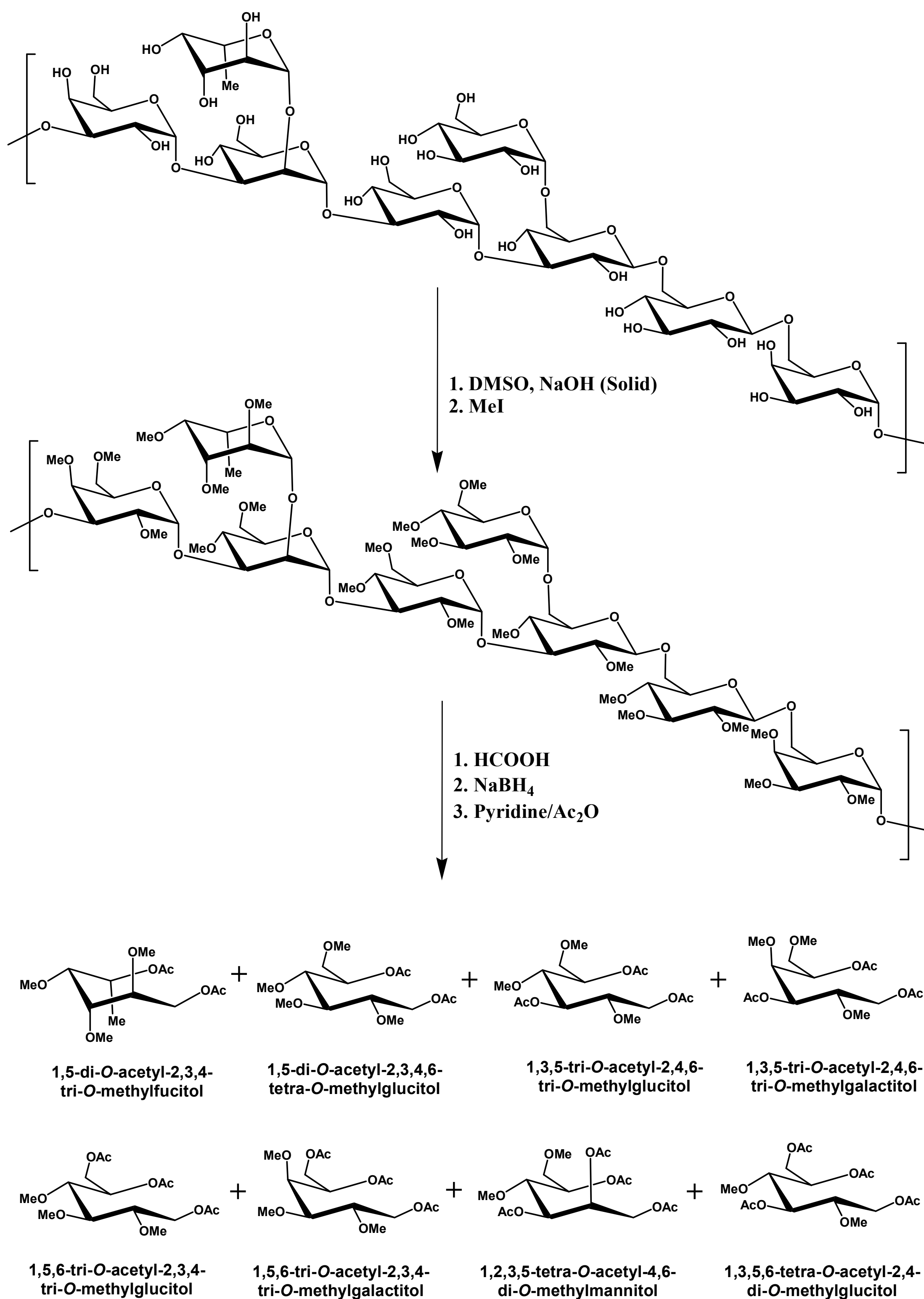
Table 1. Components of monosaccharide and properties of PS isolated from the mushroom *T. clypeatus*.

Properties	Data			
Monosaccharide component	Glucose	Galactose	Mannose	fucose
Molar ratio	4.10	1.95	1.0	0.95
Absolute configuration	D	D	D	L
Specific rotation	$[\alpha]_D^{25.7}$ +12.9 (*c* 0.11, H_2O)			
Molecular weight	~1.98 $\times 10^5$ Da			

3.B.2. Structural analysis of PS

3.B.2.1. Chemical analysis

The PS on acid hydrolysis by 2 M CF_3COOH, followed by alditol acetate preparation and analysis through gas-liquid chromatography was found to contain glucose, galactose, mannose, and fucose in a molar ratio of 4.10:1.95:1.0:0.95 respectively. The absolute configuration of the monosaccharide was determined by the method of Gerwig et al. [61] and found that glucose, galactose, and mannose had the D configuration but fucose was present in the L configuration (**Table 1**). The polysaccharide was methylated according to the method of Ciucanu and Kerek [62]. 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*-methylglucitol; 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol; 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol; 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol; 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol; 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methylmannitol; 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylglucitol in a ratio of 0.95:1.0:1.05:1.0:1.10:0.95:1.0:0.95 (**Table 2**). These results indicated the presence of terminal fucopyranosyl, terminal glucopyranosyl, (1→3)- glucopyranosyl, (1→3)-galactopyranosyl, (1→6)- glucopyranosyl, (1→6)- galactopyranosyl, (1→2,3)-mannopyranosyl, and (1→3,6)-linked glucopyranosyl moieties in the PS. Methylation analysis of the PS is presented in the following diagram:



❖ Schematic presentation of methylation analysis of PS.

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

Methylated sugars	Molar ratio	Linkage type	Major Mass Fragments (m/z)
2,3,4-Me_3-Fuc	0.95	Fuc*p*-(1→	43,72,89,101,115,117, 131,161, 175
2,3,4,6-Me_4-Glc	1.0	Glc*p*-(1→	43,45,59,71,87,101,117,129,145,161,205
2,4,6-Me_3-Glc	1.05	→3)-Glc*p*-(1→	43,45,58,71,87,99,101,117,129, 143,161,173,201,233
2,4,6-Me_3-Gal	1.0	→3)-Gal*p*-(1→	43,45,58,71,87,99,101,117,129, 143,161,173,201,233
2,3,4-Me_3-Glc	1.10	→6)-Glc*p*-(1→	43,45,58,71,87,99,101,117,129, 143,161,173,189,233
2,3,4-Me_3-Gal	0.95	→6)-Gal*p*-(1→	43,45,58,71,87,99,101,117,129, 143,161,173,189,233
4,6-Me_2-Man	1.0	→2,3)-Man*p*-(1→	43,45,85,87,99,101,127,129,161, 201,261
2,4-Me_2-Glc	0.95	→3,6)- Glc*p*-(1→	43,58,87,99,101,117,127,129, 139,159,173,189, 201,233

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

The proton NMR spectrum (500 MHz; **Fig. 2**; **Table 3**) of this PS at 30 ^{0}C contained seven signals in decreasing order at δ 5.19, 5.16, 5.09, 5.06, 4.97, 4.50, and 4.49 for anomeric protons. The peak at δ 5.09 is almost double in comparison to the other peaks; therefore it consists of two residues. Hence, they were designated as **A, B, C, D, E, F, G,** and **H** respectively (**Table 3**). In ^{13}C NMR spectrum (125 MHz; **Fig. 3a**) at the same temperature, seven signals were found in the anomeric region at δ 103.0, 102.7, 102.6, 102.2, 101.8, 100.1, and 97.9. From HSQC spectrum (**Fig. 4a**; **Table 3**), the anomeric carbon signals at δ 103.0, 102.7, 102.6, 101.8, 100.1, and 97.9 were correlated to the anomeric proton signals **E** (δ 5.06), **H** (δ 4.49), **G** (δ 4.50), **B** (δ 5.16), **A** (δ 5.19) and **F** (δ 4.97) respectively and the peak at δ 102.2 was correlated to the anomeric proton signals **C** (δ 5.09) and **D** (δ 5.09). All the ^{1}H and ^{13}C NMR signals (**Table 3**) were assigned from DQF-COSY, TOCSY, and HSQC experiments. From DQF-COSY experiment the proton coupling constants were measured and one-bond C-H coupling were measured from proton coupled ^{13}C spectrum.

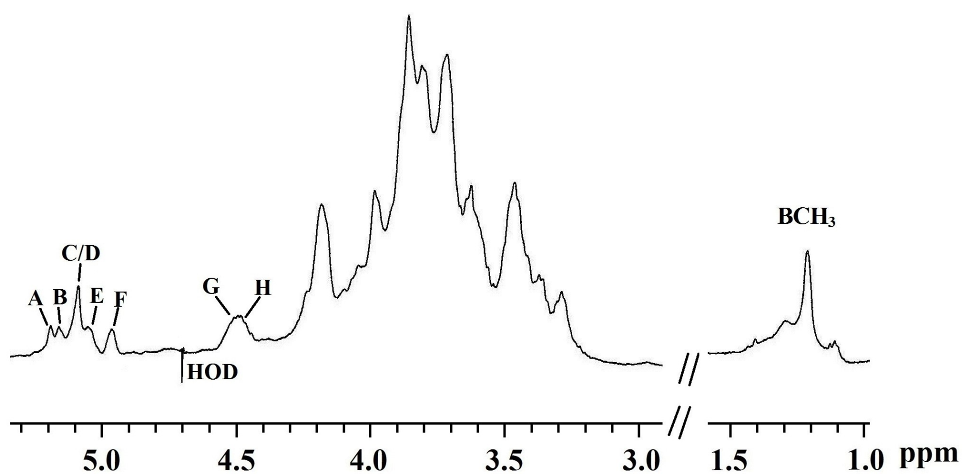


Figure 2: ^{1}H NMR spectrum (500 MHz, D_2O, 30 ^0C) of the PS isolated from the edible mushroom *T. clypeatus*.

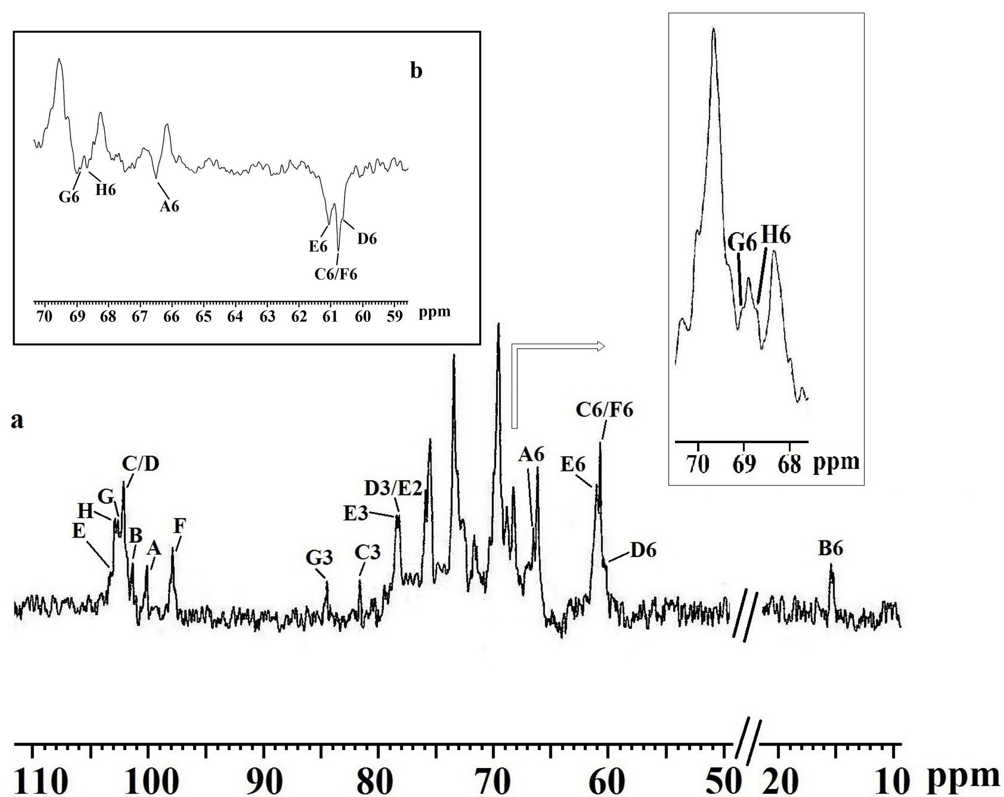


Figure 3: (a) ^{13}C NMR spectrum (125 MHz, D_2O, 30 ^0C) (b) with insert of the part of DEPT-135 spectrum (D_2O, 30 ^0C) of the PS isolated from the edible mushroom *T. clypeatus*.

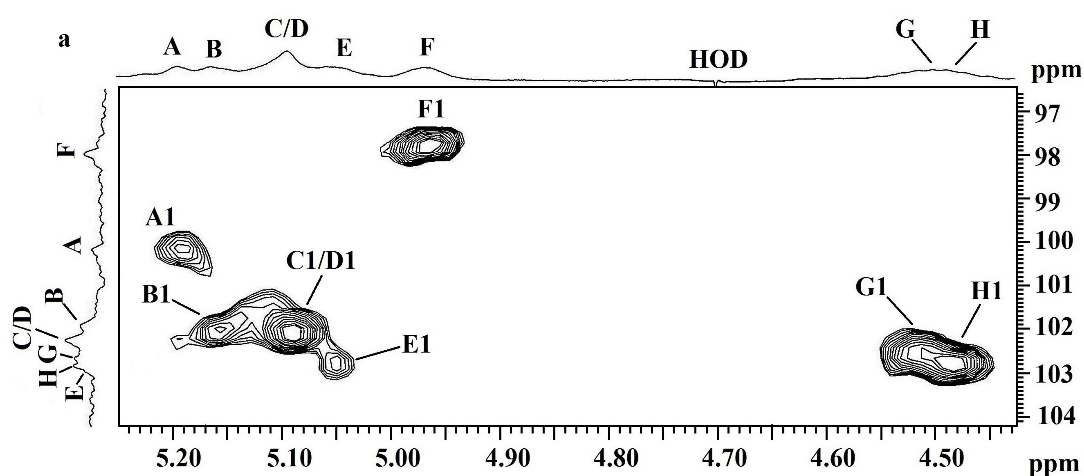


Figure 4(a): HSQC spectrum (D_2O, 30 ^{0}C) of anomeric part of the PS isolated from the edible mushroom *T. clypeatus*.

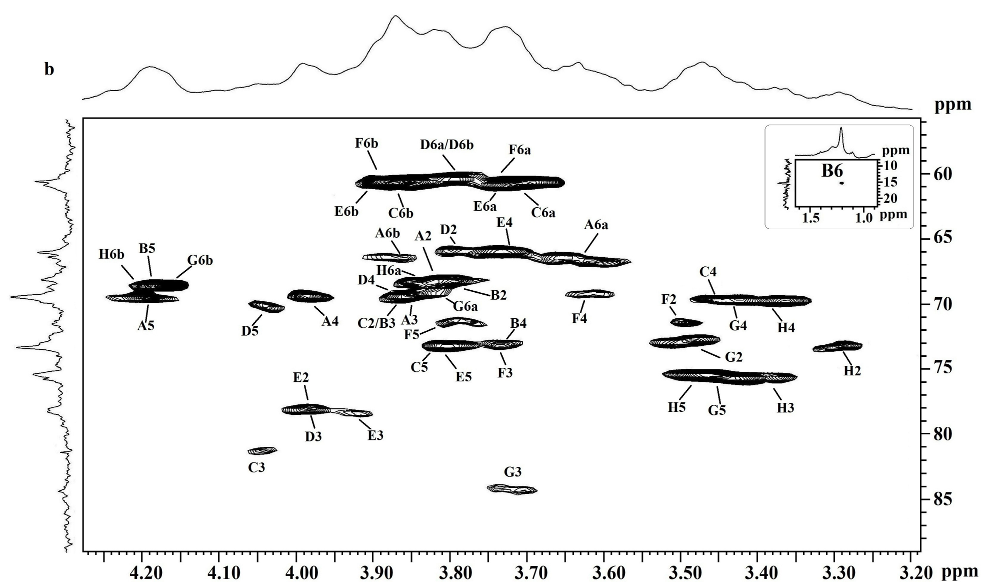


Figure 4(b): HSQC spectrum (D_2O, 30 ^{0}C) of other than anomeric part of the PS isolated from the edible mushroom *T. clypeatus*.

Residues **A** and **D** has large coupling constant $J_{H\text{-}2,H\text{-}3}$ ~9 Hz and relatively small $J_{H\text{-}3,H\text{-}4}$ ~3.5 Hz (**Table 4**) and thus, they were confirmed as D-galactopyranosyl moieties.

The α-configuration of both **A** and **D** residues were assigned from the coupling constant values $J_{H-1,H-2}$ ~3.1 and $J_{C-1,H-1}$ ~171 Hz. The downfield shift of C-6 (δ 66.5) of $-CH_2$ with respect to the standard values of methyl glycoside [66,97] indicated that the residue **A** was substituted at C-6 which was further confirmed from DEPT-135 spectrum (**Fig. 3b**). Hence, residue **A** was established as (1→6)-linked α-D-galactopyranosyl residue. The downfield shift of C-3 (δ 78.3) with respect to standard value of methyl glycoside indicated that residue **D** was (1→3)-linked α-D-galactopyranosyl [58].

Residue **B** was assigned as a terminal α-L-fucopyranosyl moiety, which was strongly supported by the appearance of proton signal at δ 1.22, and a carbon signal at δ 15.1 for a CH_3 group, and small coupling constant $J_{H-3,H-4}$ (< 3 Hz). The appearance of the anomeric proton signal for residue **B** at δ 5.16 and the coupling constant value of $J_{H-1,H-2}$ ~ 3.75 Hz clearly indicated that L-fucose was α-linked. This anomeric configuration was further confirmed by ^{1}H-^{13}C coupling constant $J_{C-1,H-1}$ ~ 171 Hz. The rest of carbon values in ^{13}C corresponded to the standard values of methyl glycosides indicating residue **B** was glycosidically linked terminal α-L-fucopyranosyl moiety.

Based on the anomeric proton chemical shift and coupling constant, $J_{H-1,H-2}$ ~3 Hz and $J_{C-1,H-1}$ ~171 Hz it was confirmed that residues **C** and **F** were present in α-configuration. The large $J_{H-2,H-3}$ and $J_{H-3,H-4}$ coupling constant values (~10.0 Hz) confirmed that residues **C** and **F** were D-glucopyranosyl moieties. The downfield shift of C-3 (δ 81.2) with respect to standard values of methyl glycosides indicated that residue **C** was linked at this position. Thus the residue **C** was (1→3)-linked α-D-glucopyranosyl [98,99] moiety. The

carbon signals from C-1 to C-6 of residue **F** corresponded nearly to the standard values of methyl glycosides. Thus the residue **F** was terminal α-D-glucopyranosyl moiety.

The anomeric proton signals of residue **E** at δ 5.06 with low values of $J_{H\text{-}1,H\text{-}2}$ (~1.6 Hz), $J_{H\text{-}2,H\text{-}3}$ (~3.5 Hz) and $J_{C\text{-}1,H\text{-}1}$ of 170 Hz clearly indicated that it was present in α-configuration. This was further confirmed from the large coupling values $J_{H\text{-}3,H\text{-}4}$ ~7.5 Hz and $J_{H\text{-}4,H\text{-}5}$ ~10 Hz. The downfield shifts of C-2 (δ 78.3) and C-3 (δ 78.6) with respect to standard values of methyl glycoside indicated that the moiety **E** was (1→2,3)-linked unit. Hence, these observations confirmed that residue **E** was (1→2,3)-α-D-mannopyranosyl moiety.

Residue **G** and **H** were established as β-configuration from coupling constant (**Table 4**) values $J_{H\text{-}1,H\text{-}2}$ (~8.0 Hz) , $J_{C\text{-}1,H\text{-}1}$ (~160 Hz) and the large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values (~10.0 Hz) of residues **G** and **H** confirmed their D-glucopyranosyl configuration. The downfield shifts of C-3 (δ 84.3) and C-6 (δ 69.0) of residue **G** with respect to standard values of methyl glycoside indicated that it was (1→3,6)-linked β-D-glucopyranosyl. In residue **H** the downfield shift of C-6 (δ 68.8) with respect to standard values of methyl glycoside indicated that it was (1→6)-linked β-D-glucopyranosyl. The chemical shift values of $-CH_2$ of both the residues of **G** and **H** indicated that the C-6 positions were substituted and further confirmed from DEPT-135 spectrum (**Fig. 3b**).

Table 3. The ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts for the PS isolated from the mushroom *T. clypeatus* in D_2O at 30 ^0C.

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
→6)-α-D-Gal*p*-(1→	5.19	3.82	3.85	3.98	4.19	3.64[c], 3.87[d]
A	100.1	68.9	69.6	69.3	69.6	66.5
α-L-Fuc*p*-(1→	5.16	3.81	3.87	3.73	4.19	1.22
B	101.8	68.3	69.6	72.7	68.9	15.1
→3)-α-D-Glc*p*-(1→	5.09	3.87	4.05	3.47	3.82	3.71[c], 3.87[d]
C	102.2	69.6	81.2	69.3	72.9	60.8
→3)-α-D-Gal*p*-(1→	5.09	3.80	3.98	3.87	4.05	3.74[c], 3.74[d]
D	102.2	66.2	78.3	69.6	70.1	60.7
→2,3)-α-D-Man*p*-(1→	5.06	3.98	3.92	3.73	3.82	3.73[c], 3.87[d]
E	103.0	78.3	78.6	66.2	73.5	61.1
α-D-Glc*p*-(1→	4.97	3.48	3.73	3.63	3.81	3.73[c], 3.89[d]
F	97.9	71.5	73.5	69.6	71.7	60.8
→3,6)-β-D-Glc*p*-(1→	4.50	3.47	3.73	3.43	3.46	3.81[c], 4.17[d]
G	102.6	72.7	84.3	69.6	75.6	69.0
→6)-β-D-Glc*p*-(1→	4.49	3.30	3.38	3.38	3.47	3.82[c], 4.19[d]
H	102.7	73.1	76.0	70.1	75.6	68.8

[a] The values of chemical shifts were recorded keeping HOD signal fixed at δ 4.70 at 30 ^0C.

[b] The values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 at 30 ^0C.

[c,d] Interchangeable.

Table 4. Coupling constant data for the PS isolated from the mushroom *T. clypeatus*.

Glycosyl residue	H-1/ C-1	Coupling constant values(Hz)
→6)-α-D-Gal*p*-(1→ **A**	5.19 100.1	$J_{H\text{-}1,H\text{-}2}$ ~3.1 $J_{H\text{-}2,H\text{-}3}$ ~9.0 $J_{H\text{-}3,H\text{-}4}$ ~3.5 $J_{C\text{-}1,H\text{-}1}$ ~171
α-L-Fuc*p*-(1→ **B**	5.16 101.8	$J_{H\text{-}1,H\text{-}2}$ ~3.75 $J_{H\text{-}3,H\text{-}4} <$ 3.0 $J_{C\text{-}1,H\text{-}1}$ ~171
→3)-α-D-Glc*p*-(1→ **C**	5.09 102.2	$J_{H\text{-}1,H\text{-}2}$ ~3.0 $J_{H\text{-}2,H\text{-}3}$ ~10.0 $J_{H\text{-}3,H\text{-}4}$ ~10.0 $J_{C\text{-}1,H\text{-}1}$ ~171
→3)-α-D-Gal*p*-(1→ **D**	5.09 102.2	$J_{H\text{-}1,H\text{-}2}$ ~3.1 $J_{H\text{-}2,H\text{-}3}$ ~9.0 $J_{H\text{-}3,H\text{-}4}$ ~3.5 $J_{C\text{-}1,H\text{-}1}$ ~171
→2,3)-α-D-Man*p*-(1→ **E**	5.06 103.0	$J_{H\text{-}1,H\text{-}2}$ ~1.6 $J_{H\text{-}2,H\text{-}3}$ ~3.5 $J_{H\text{-}3,H\text{-}4}$ ~7.5 $J_{H\text{-}4,H\text{-}5}$ ~10.0 $J_{C\text{-}1,H\text{-}1}$ ~170
α-D-Glc*p*-(1→ **F**	4.97 97.9	$J_{H\text{-}1,H\text{-}2}$ ~3.0 $J_{H\text{-}2,H\text{-}3}$ ~10.0 $J_{H\text{-}3,H\text{-}4}$ ~10.0 $J_{C\text{-}1,H\text{-}1}$ ~171
→3,6)-β-D-Glc*p*-(1→ **G**	4.50 102.6	$J_{H\text{-}1,H\text{-}2}$ ~8.0 $J_{H\text{-}2,H\text{-}3}$ ~10.0 $J_{H\text{-}3,H\text{-}4}$ ~10.0 $J_{C\text{-}1,H\text{-}1}$ ~160
→6)-β-D-Glc*p*-(1→ **H**	4.49 102.7	$J_{H\text{-}1,H\text{-}2}$ ~8.0 $J_{H\text{-}2,H\text{-}3}$ ~10.0 $J_{H\text{-}3,H\text{-}4}$ ~10.0 $J_{C\text{-}1,H\text{-}1}$ ~160

The sequences of glycosyl residue were determined from NOESY (**Fig.5**; **Table 5**) as well as ROESY (not shown) experiments. The NOESY experiment showed the inter-residual contacts: **A**H-1/ **D**H-3; **D**H-1/ **E**H-3; **E**H-1/ **C**H-3; **C**H-1/ **G**H-3; **G**H-1/ **H**H-6a, 6b; **H**H-1/ **A**H-6a, 6b; **B**H-1/ **E**H-2; **F**H-1/ **G**H-6a, 6b along with other intra-residual contacts. Thus, the NOESY connectivity established the sequences as, **A** (1→3) **D**; **D** (1→3) **E**; **E** (1→3) **C**; **C** (1→3) **G**; **G** (1→6) **H**; **H** (1→6) **A**; **B** (1→2) **E**; **F** (1→6) **G**.

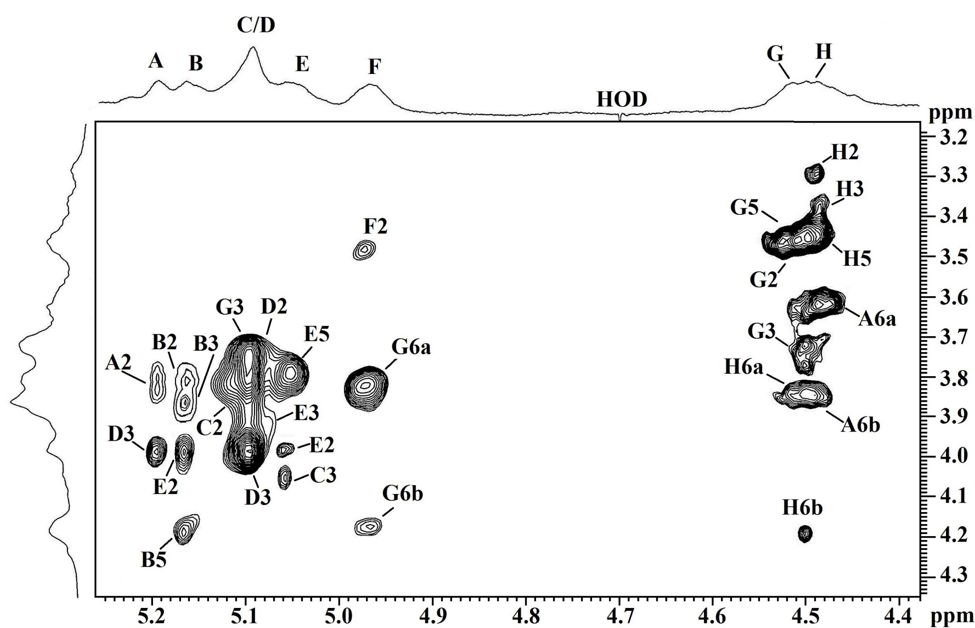


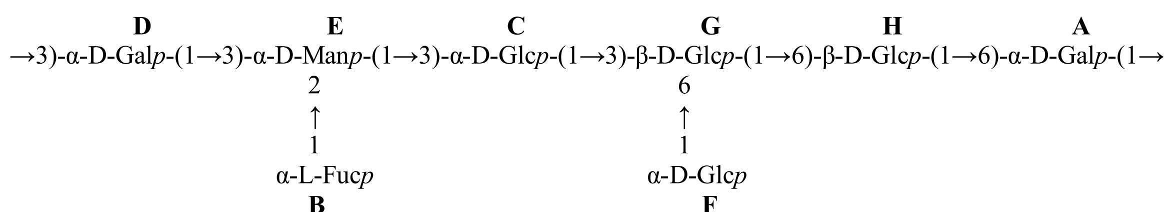
Figure 5: Part of NOESY spectrum of the PS of the edible mushroom *T. clypeatus*.

The NOESY mixing time was 300 ms.

Table 5. NOESY data for the PS isolated from the mushroom *T. clypeatus*.

Glycosyl residue	Anomeric proton	NOE contact proton		
	δ	δ	residue	atom
→6)-α-D-Gal*p*-(1→ **A**	5.19	3.98	**D**	H-3
		3.82	**A**	H-2
α-L-Fuc*p*-(1→ **B**	5.16	3.98	**E**	H-2
		3.81	**B**	H-2
		3.87	**B**	H-3
		4.19	**B**	H-5
→3)-α-D-Glc*p*-(1→ **C**	5.09	3.73	**G**	H-3
		3.87	**C**	H-2
→3)-α-D-Gal*p*-(1→ **D**	5.09	3.92	**E**	H-3
		3.80	**D**	H-2
		3.98	**D**	H-3
→2,3)-α-D-Man*p*-(1→ **E**	5.06	4.05	**C**	H-3
		3.98	**E**	H-2
		3.82	**E**	H-5
α-D-Glc*p*-(1→ **F**	4.97	3.81	**G**	H-6a
		4.17	**G**	H6b
		3.48	**F**	H-2
→3,6)-β-D-Glc*p*-(1→ **G**	4.50	3.82	**H**	H-6a
		4.19	**H**	H-6b
		3.47	**G**	H-2
		3.73	**G**	H-3
		3.46	**G**	H-5
→6)-β-D-Glc*p*-(1→ **H**	4.49	3.64	**A**	H-6a
		3.87	**A**	H-6b
		3.30	**H**	H-2
		3.38	**H**	H-3
		3.47	**H**	H-5

On the basis of the appearance of these cross peaks and NOESY connectivity, the structure of the repeating unit in the PS was proposed as:



Finally, Smith degraded material (SDPS) of PS was prepared to confirm the linkages of the heteroglycan. The ^{13}C NMR (125 Hz) spectrum (**Fig. 6**; **Table 6**) of SDPS at 30 ^{0}C showed three anomeric carbon signals at δ 103.2, 102.7, and 102.2. The anomeric carbon signals at δ 103.2 and 102.7 corresponded to (1→3)-α-D-Manp (**I**) and (1→3)-β-D-Glcp (**J**) respectively, whereas the carbon signal at δ 102.2 resembled both to the (1→3)-α-D-Glcp (**K**) and terminal α-D-Galp (**L**) residues. The carbon signals C-1, C-2, and C-3 of the glycerol moiety (Gro) were assigned as δ 66.2, 72.1, and 62.5 respectively. The terminal α-D-Galp (**L**) residue of SDPS was generated from the (1→3)-α-D-Galp (**D**) and (1→3)-α-D-Manp unit (**I**) from (1→2,3)-α-D-Manp (**E**) residue of parent PS during Smith degradation. The carbon signal at δ 78.2 clearly indicated the presence of **I** moiety. The (1→3)-α-D-Glcp residue (**K**) attached to **I** was retained during oxidation previously denoted as **C** of the parent PS**.** The carbon signal at δ 81.9 clearly confirmed the presence of **K** residue. Further, the (1→3)-β-D-Glcp residue (**J**) attached to **K** was formed from (1→3,6)-β-D-Glcp (**G**), during oxidation followed by degradation. The carbon signal at δ 84.3 clearly confirmed the presence of **J** residue in the SDPS. The glycerol moiety **M** was generated from (1→6)-β-D-Glcp residue (**H**) after periodate oxidation followed by Smith degradation, and this moiety was attached to **J**.

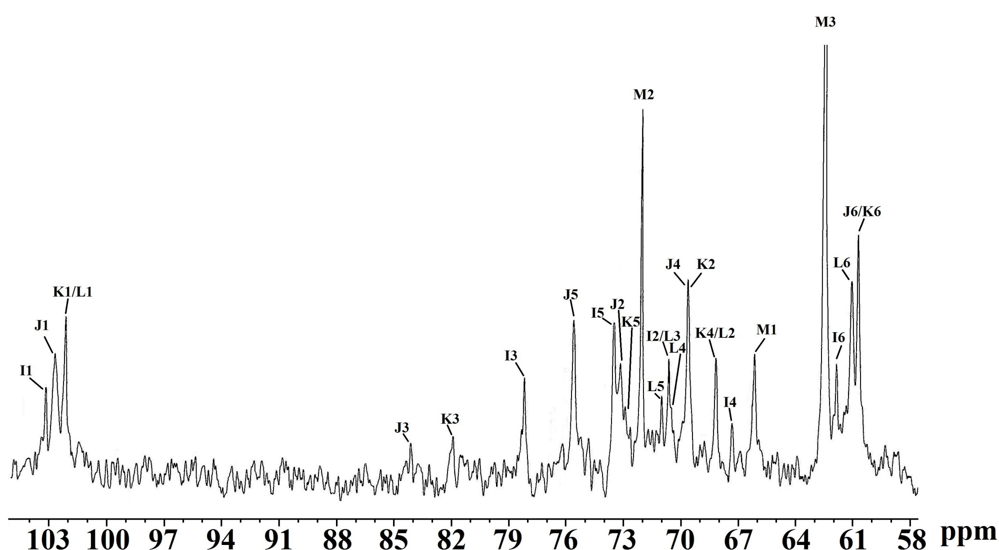


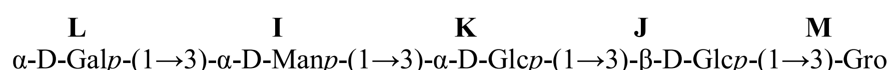
Figure 6: ^{13}C NMR spectrum (125 MHz, D_2O, 30 ^{0}C) of the Smith-degraded glycerol containing tetrasaccharide isolated from the edible mushroom *T. clypeatus*.

Table 6. The ^{13}C NMR^n chemical shifts of Smith-degraded glycerol-containing tetrasaccharide of the mushroom *T. clypeatus* in D_2O at 30 ^{0}C.

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
→3)-α-D-Man*p*-(1→ **I**	103.2	70.6	78.2	67.2	73.5	61.8
→3)-β-D-Glc*p*-(1→ **J**	102.7	73.2	84.3	69.7	75.6	60.8
→3)-α-D-Glc*p*-(1→ **K**	102.2	69.6	81.9	68.1	72.9	60.8
α-D-Gal*p*-(1→ **L**	102.2	68.1	70.6	70.5	71.1	61.1
→3)-Gro **M**	66.2	72.1	62.5			

[n] The values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 at 30 ^{0}C.

Hence, Smith degradation results in the formation of a glycerol containing tetrasaccharide obtained from the parent PS and the structure of which was established as:



These results further confirmed the repeating unit present in the PS of the edible mushroom *T. clypeatus.*

Similar kind of polysaccharide with same sugar composition but differing in ratios was isolated from another mushroom, *Termitomyces* striatus [93] belonging to the same genus. The present PS contains D-glucose, D-galactose, D-mannose, and L-fucose in a molar ratio of nearly 4:2:1:1 but in *T. striatus* the ratio is 4:2:2:2. In the present PS α-D-Manp and β-D-Glcp remain in the backbone chain terminated with α-L-Fucp and α-D-Glcp whereas, in *T. Striatus* α-D-Galp and α-D-Glcp remained in the skeleton chain and terminated with α-D-Glcp and β-D-Glcp respectively. The linkages and ^{1}H, ^{13}C signals are also different in both the molecules.

Another PS reported earlier from *Entoloma* lividoalbum [64] was found to contain same sugar composition but different ratios 5:2:1:1. The common fragments (1→3,6)-β-D-Glcp and (1→6)-β-D-Glcp were present in the skeleton chain of both the PS isolated from *T. clypeatus* and *E. lividoalbum*. But only the difference is that the branching terminates with α-D-Glcp in *T. clypeatus* (T.C) and β-D-Glcp in *E. lividoalbum* (E.L) respectively. The anomeric ^{1}H and ^{13}C NMR values for both fragments (1→3,6)-β-D-Glcp [T.C, δ 4.50, 102.6; E.L, δ 4.49, 102.5] and (1→6)-β-D-Glcp [T.C, δ 4.49, 102.7;

E.L, δ 4.48, 102.7] were found closer, but the other linkages and chemical shifts values are found different.

3.B.3. Antioxidant properties of PS

3.B.3.1. Chelating ability of ferrous ions

Dietary nutrients with metal chelators having transition metal ions e.g. Fe^{2+}, Cu^{+}, Pb^{2+}, Co^{2+} may act as preventive antioxidant [100]. The chelating ability of ferrous ions measures the ability of secondary antioxidants. Primary antioxidants prevent oxidative damage directly through scavenging the free radicals; while secondary antioxidants act indirectly by preventing the formation of free radicals [101]. Ferrozine quantitatively forms complexes with Fe^{2+}. In the presence of chelating agent, the complex formation is disrupted, thus resulting in the reduction of red color. Reduction therefore allows estimation of the chelating ability of the coexisting chelator [102]. Ferrous ion chelating ability of PS was found (**Fig. 7a**) to be 54.1 % at 500 μg/mL concentration.

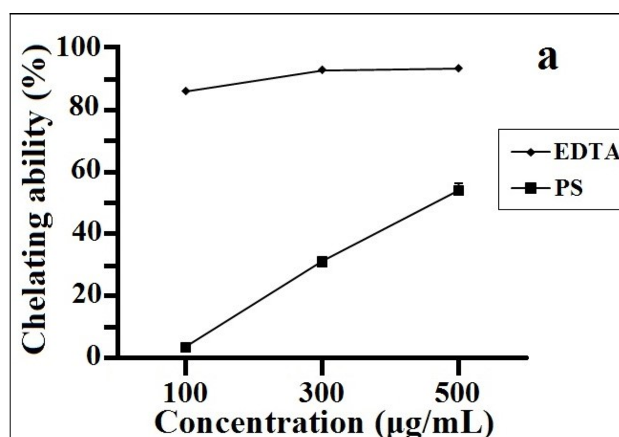


Figure 7(a): Ferrous ion chelating ability of the PS isolated from the edible mushroom *T. clypeatus*. All the results are the mean ± SD of three separate experiments, each in triplicate.

3.B.3.2. Reducing power

Reducing power of any compound is recognized as a parameter that can be considered as an antioxidant. The assay of the reducing power of the present PS revealed that it could reduce Fe^{3+} to Fe^{2+}. This change was monitored at 700 nm by measuring the intensity of the Perl's Prussian blue color. From figure 6b, the EC_{50} value of PS was observed 260 µg/mL (**Fig. 7b**).

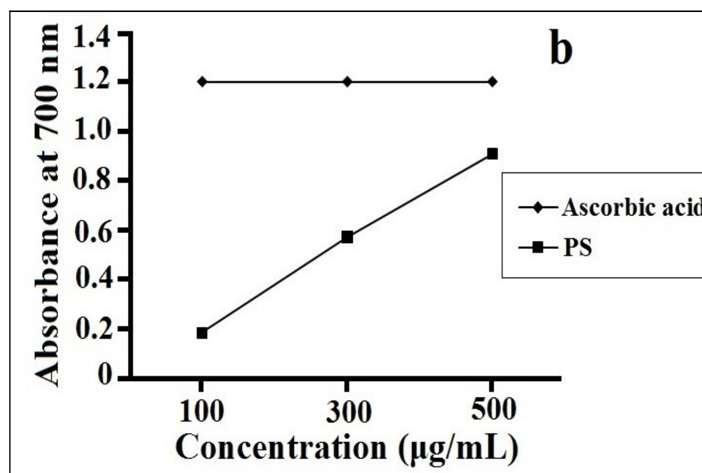


Figure 7(b): reducing power of the PS isolated from the edible mushroom *T. clypeatus*. All the results are the mean ± SD of three separate experiments, each in triplicate.

3.B.3.3. Superoxide radical scavenging assay

Superoxide radical is known to be very harmful for cellular components and plays a major role in the formation of other reactive oxygen species such as hydroxyl radical, hydrogen peroxide and singlet oxygen in living system. The data presented in figure 6c indicated the scavenging activity of PS increased with increasing concentration. The

EC_{50} value of the PS was found 180 µg/mL (**Fig. 7c**). These results suggested that the PS exhibited scavenging effect on the generation of superoxide anion radicals that could prevent or ameliorate oxidative cell damage.

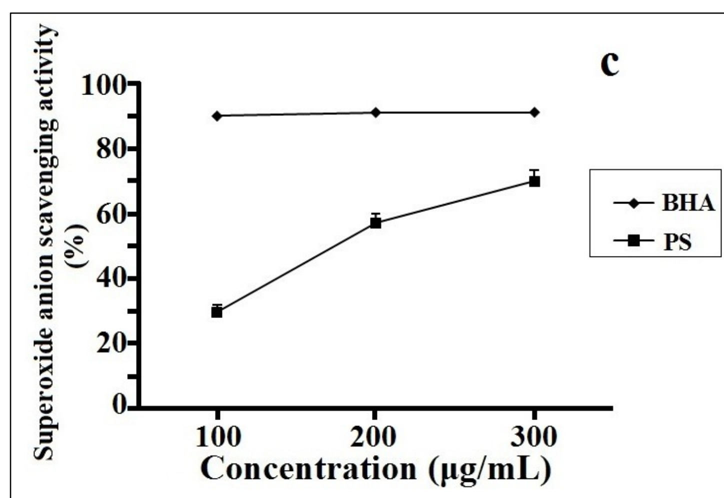
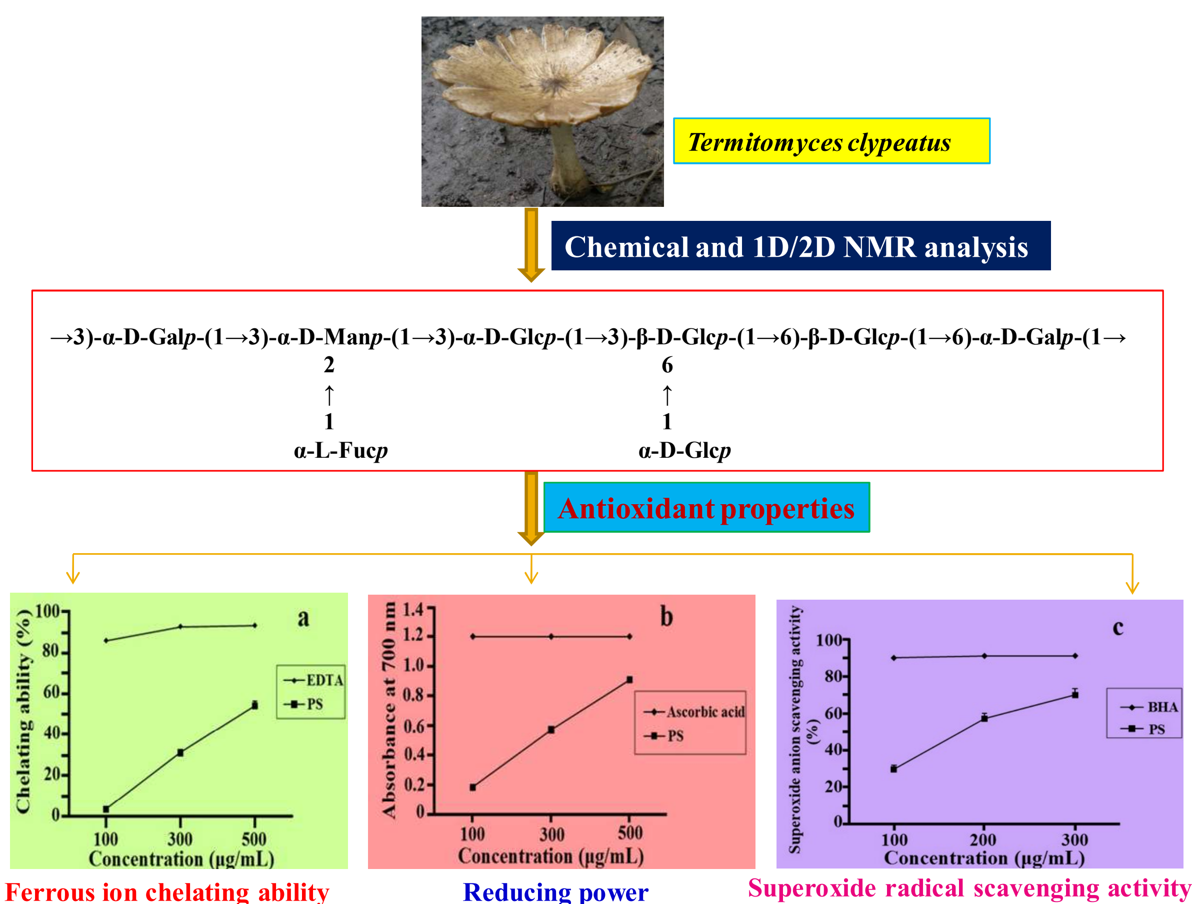


Figure 7(c): superoxide radical scavenging activity of the PS isolated from the edible mushroom *T. clypeatus*. All the results are the mean ± SD of three separate experiments, each in triplicate.

Earlier report established that reactive oxygen species (ROS), such as hydroxyl radicals, superoxide anions, and hydrogen peroxide, are frequently generated in the living cell during metabolism and play a vital role in cell signaling [103]. However, excessive amount of ROS induces oxidative stress causes damage to cellular macromolecules. Most of the antioxidants used to preserve adequate function of cells against homeostatic disturbances caused by oxidative stress, are usually synthetic and have been suspected of causing liver damage and carcinogenesis [104]. Therefore, there is an alarming need for the researcher to search natural and safe antioxidants. Earlier reports showed that

mushroom polysaccharide possess the potential to scavenge ROS [56,105,106]. Results showed that the polysaccharide isolated from *T. clypeatus* have the potential to scavenge intracellular ROS.

❖ **All the structural and biological studies of polysaccharides of *T. clypeatus* are presented in the following diagram:**



3.C. Conclusion

A water soluble heteroglycan (PS) was isolated from the aqueous extract of an edible mushroom *T. clypeatus*. The purified heteroglycan obtained by gel permeation chromatography with average molecular weight ~1.98×10^5 Da showed specific rotation $[\alpha]_D^{25.7}$ +12.9 (*c* 0.11, H_2O). The chemical and NMR analysis indicated the presence of a repeating unit with a backbone consisting of one each of the residues (1→3)-α-D-galactopyranosyl, (1→3)-α-D-mannopyranosyl, (1→3)-α-D-glucopyranosyl, (1→3)-β-D-glucopyranosyl, (1→6)-β-D-glucopyranosyl, and (1→6)-α-D-galactopyranosyl respectively. The (1→3)-α-D-mannopyranosyl residue was found branched at *O*-2 with terminal α-L-fucopyranosyl moiety and (1→3)-β-D-glucopyranosyl residue was branched at *O*-6 with terminal α-D-glucopyranosyl residue. The following structure was characterized by chemical analysis and 1D/2D NMR studies.

```
          D                   E                C                 G                 H                 A
→3)-α-D-Galp-(1→3)-α-D-Manp-(1→3)-α-D-Glcp-(1→3)-β-D-Glcp-(1→6)-β-D-Glcp-(1→6)-α-D-Galp-(1→
                              2                                  6
                              ↑                                  ↑
                              1                                  1
                          α-L-Fucp                           α-D-Glcp
                              B                                  F
```

The PS showed ferrous ion chelating ability, superoxide radical scavenging activities, and high reducing power. Thus, it would be considered as a source of natural antioxidant.