



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



Manabendra Pattanayak^a, Surajit Samanta^a, Prasenjit Maity^a, Ipsita K. Sen^a, Ashis K. Nandi^a, Dilip K. Manna^a, Payel Mitra^b, Krishnendu Acharya^b, Syed S. Islam^{a,*}

^a Department of Chemistry and Chemical Technology, Vidyasagar University, Midnapore 721102, West Bengal, India

^b Molecular and Applied Mycology and Plant Pathology Laboratory, Department of Botany, University of Calcutta, 35, Ballygunge Circular Road, Kolkata 700019, West Bengal, India

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ABSTRACT

A water-soluble heteroglycan (PS) of an average molecular weight $\sim 1.98 \times 10^5$ Da was isolated from the aqueous extract of an edible mushroom *Termitomyces clypeatus* (R. Heim). The structure of the polysaccharide (PS) was established using total hydrolysis, methylation analysis, Smith degradation, and 1D/2D NMR experiments. Total hydrolysis indicated the presence of D-glucose, D-galactose, D-mannose, and L-fucose in a molar ratio of 4.10:1.95:1.0:0.95, respectively. 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 PS exhibited antioxidant properties.

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1. Introduction

Mushrooms are consumed as delicious food and considered a source of biologically active compounds.¹ The polysaccharides of edible mushrooms have a lot of pharmaceutical applications in industries.² Several polysaccharides^{3–8} 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.⁹ 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.¹⁰

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 mushroom¹¹ 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*,¹² *Termitomyces eurhizus*,¹³ *Termitomyces microcarpus*,¹⁴ and *Termitomyces robustus*¹⁵ have been isolated, characterized and reported by our group. 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 reported herein.

2. Results and discussion

2.1. Isolation, purification and chemical analysis 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. One homogeneous fraction was obtained from the crude polysaccharide on separation through Sepharose-6B. The

* Corresponding author. Tel.: +91 03222 276558x437, +91 9932629971 (mobile); fax: +91 03222 275329.

E-mail address: sirajul_1999@yahoo.com (S.S. Islam).

pure polysaccharide (PS) showed specific rotation $[\alpha]_D^{25.7} +12.9$ (c 0.11, H₂O) and the average molecular weight of the PS was determined as $\sim 1.98 \times 10^5$ Da. The PS on acid hydrolysis by 2 M CF₃COOH, 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.¹⁶ and found that glucose, galactose, and mannose had the D configuration but fucose was present in the L configuration. The polysaccharide was methylated according to the method of Ciucanu and Kerek.¹⁷ 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. These results indicated the presence of terminal fucopyranosyl, terminal glucopyranosyl, (1 \rightarrow 3)-glucopyranosyl, (1 \rightarrow 3)-galactopyranosyl, (1 \rightarrow 6)-glucopyranosyl, (1 \rightarrow 6)-galactopyranosyl, (1 \rightarrow 2,3)-mannopyranosyl, and (1 \rightarrow 3,6)-linked glucopyranosyl moieties in the PS.

2.2. NMR and structural analysis of PS

The proton NMR spectrum (500 MHz; Fig. 1, Table 1) of this PS at 30 °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 1). In ¹³C NMR spectrum (125 MHz; Fig. 2a) 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 ppm. From HSQC spectrum (Fig. 3a; Table 1), 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 ¹H and ¹³C NMR signals (Table 1) 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 ¹³C spectrum.

Residues **A** and **D** has large coupling constant $J_{H-2,H-3} \sim 9$ Hz and relatively small $J_{H-3,H-4} \sim 3.5$ Hz (Table 2) 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 $J_{H-1,H-2} \sim 3.1$ and $J_{C-1,H-1} \sim 171$ Hz. The downfield shift of C-6 (δ 66.5) of –CH₂ with respect to the standard values of methyl glycoside^{18,19} indicated that the residue **A** was substituted at C-6, which was further confirmed from DEPT-135 spectrum (Fig. 2b). Hence, residue **A** was established as (1 \rightarrow 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 \rightarrow 3)-linked α -D-galactopyranosyl.²⁰

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 CH₃ 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 $J_{H-1,H-2} \sim 3.75$ Hz clearly indicated that L-fucose was α -linked. This anomeric configuration was further confirmed by ¹H–¹³C coupling constant $J_{C-1,H-1} \sim 171$ Hz. The rest of carbon values in ¹³C 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, $J_{H-1,H-2} \sim 3$ Hz and $J_{C-1,H-1} \sim 171$ 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 \rightarrow 3)-linked α -D-glucopyranosyl^{21,22} 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 $J_{H-1,H-2} (\sim 1.6$ Hz), $J_{H-2,H-3} (\sim 3.5$ Hz) and $J_{C-1,H-1}$ of 170 Hz clearly indicated that it was present in α -configuration. This was further confirmed from the large coupling values $J_{H-3,H-4} \sim 7.5$ Hz and $J_{H-4,H-5} \sim 10$ 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 \rightarrow 2,3)-linked unit. Hence, these observations confirmed that residue **E** was (1 \rightarrow 2,3)- α -D-mannopyranosyl moiety.

Residue **G** and **H** were established as β -configuration from coupling constant (Table 2) values $J_{H-1,H-2} (\sim 8.0$ Hz), $J_{C-1,H-1} (\sim 160$ Hz) and the large $J_{H-2,H-3}$ and $J_{H-3,H-4}$ 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)

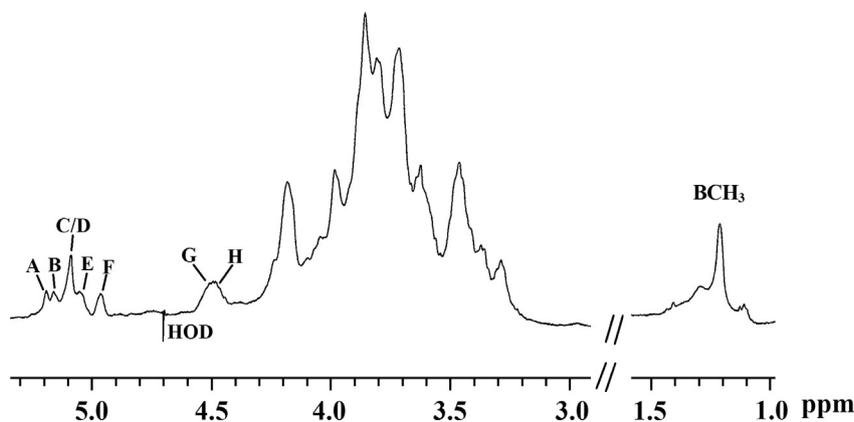


Fig. 1. ¹H NMR spectrum (500 MHz, D₂O, 30 °C) of the PS isolated from the edible mushroom *T. clypeatus*.

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

Glycosyl residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a, H-6b/C-6
→6)- α -D-Galp-(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-Fucp-(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-Glcp-(1→	5.09	3.87	4.05	3.47	3.82	3.71 ^c , 3.87 ^d
C ^{21,22}	102.2	69.6	81.2	69.3	72.9	60.8
→3)- α -D-Galp-(1→	5.09	3.80	3.98	3.87	4.05	3.74 ^c , 3.74 ^d
D ²⁰	102.2	66.2	78.3	69.6	70.1	60.7
→2,3)- α -D-Manp-(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-Glcp-(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-Glcp-(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-Glcp-(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

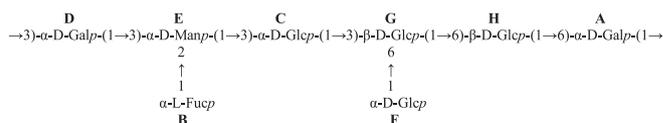
^{c,d} Interchangeable.

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

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

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 $-\text{CH}_2$ of both the residues of **G** and **H** indicated that the C-6 positions were substituted and further confirmed from DEPT-135 spectrum (Fig. 2b). The sequences of glycosyl residue were determined from NOESY (Fig. 4, Table 3) as well as ROESY (not shown) experiments. The NOESY experiment showed the inter-residual contacts: **AH-1/DH-3**; **DH-1/EH-3**; **EH-1/CH-3**; **CH-1/GH-3**; **GH-1/HH-6a, 6b**; **HH-1/AH-6a, 6b**; **BH-1/EH-2**; **FH-1/GH-6a, 6b** along with other intra-residual contacts (Fig. 2). 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**. 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. 5, Table 4) of SDPS at 30 °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,

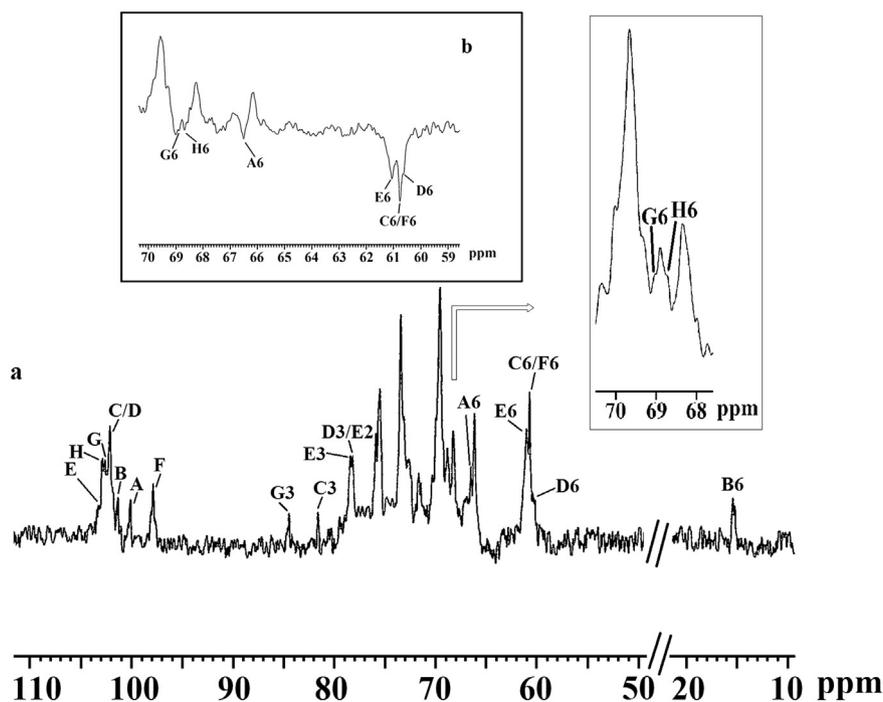


Fig. 2. (a) ^{13}C NMR spectrum (125 MHz, D_2O , 30 °C) (b) with insert of the part of DEPT-135 spectrum (D_2O , 30 °C) of the PS isolated from the edible mushroom *T. clypeatus*.

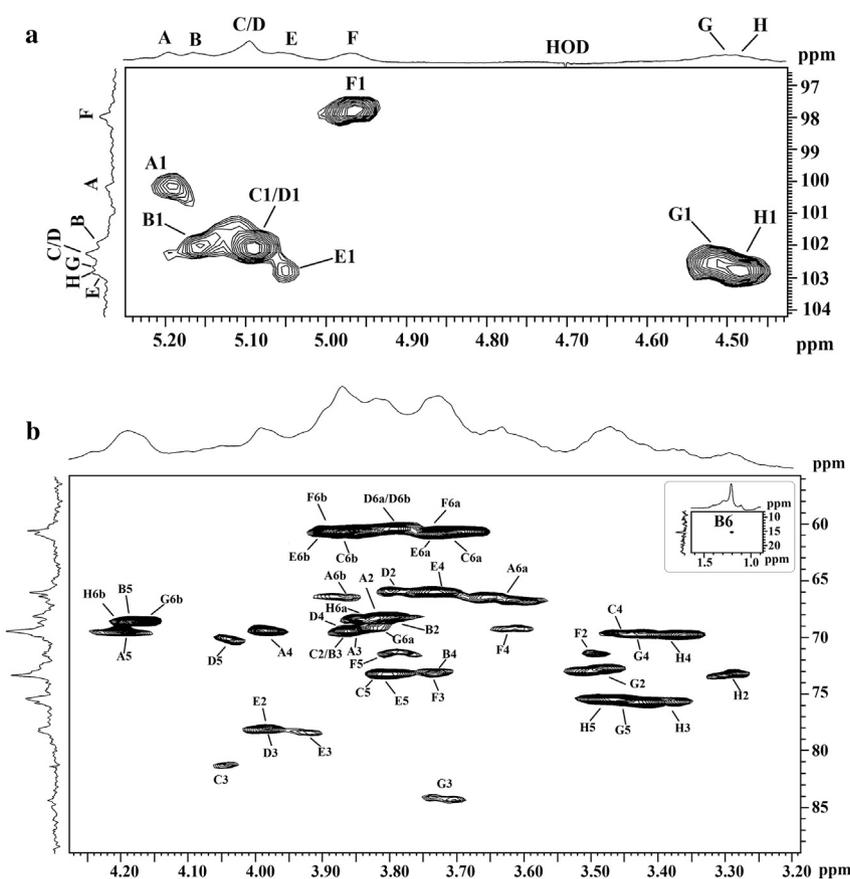
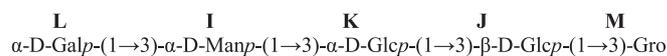


Fig. 3. HSQC spectrum (D_2O , $30\text{ }^\circ\text{C}$) of (a) anomeric part and (b) other than anomeric part of the PS isolated from the edible mushroom *T. clypeatus*.

Table 2
Coupling constant data for the PS isolated from the mushroom *T. clypeatus*

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

whereas the carbon signal at δ 102.2 resembled both to the (1 \rightarrow 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 \rightarrow 3)- α -D-Galp (**D**) and (1 \rightarrow 3)- α -D-Manp unit (**I**) from (1 \rightarrow 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 \rightarrow 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 \rightarrow 3)- β -D-Glcp residue (**J**) attached to **K** was formed from (1 \rightarrow 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 \rightarrow 6)- β -D-Glcp residue (**H**) after periodate oxidation followed by Smith degradation, and this moiety was attached to **J**. 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*¹² 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

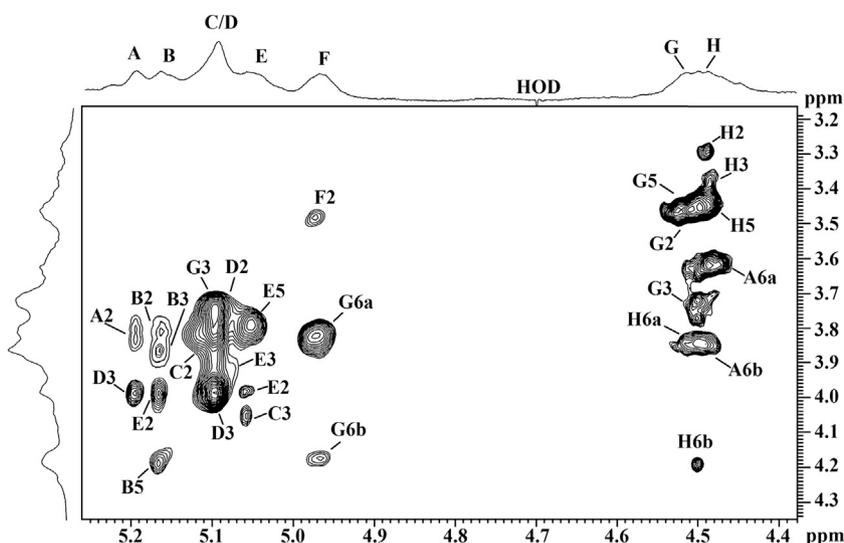


Fig. 4. Part of NOESY spectrum of the PS of the edible mushroom *T. clypeatus*. The NOESY mixing time was 300 ms.

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 ^1H , ^{13}C signals are also different in both the molecules.

Another PS reported earlier from *Entoloma lividoalbum*¹⁰ was found to contain same sugar composition but different ratios 5:2:1:1. The common fragments (1 \rightarrow 3,6)- β -D-Glcp and (1 \rightarrow 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 ^1H and ^{13}C

NMR values for both fragments (1 \rightarrow 3,6)- β -D-Glcp [T.C, δ 4.50, 102.6; E.L, δ 4.49, 102.5] and (1 \rightarrow 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.

2.3. Antioxidant properties

2.3.1. 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.²³ 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.²⁴ 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.²⁵ Ferrous ion chelating ability of PS was found (Fig. 6a) to be 54.1% at 500 $\mu\text{g}/\text{mL}$ concentration.

2.3.2. 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. The reducing power of PS was compared to that of butylated hydroxyanisole (BHA), a synthetic antioxidant. From Fig. 6b, the EC_{50} value of PS was observed 260 $\mu\text{g}/\text{mL}$.

2.3.3. 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 Fig. 6c indicated the scavenging activity of PS increased with increasing concentration. The EC_{50} value of the PS was found 180 $\mu\text{g}/\text{mL}$. These results suggested that the PS exhibited scavenging effect on the generation of superoxide anion radicals that could prevent or ameliorate oxidative cell damage.

Table 3
NOESY data for the PS isolated from the mushroom *T. clypeatus*

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

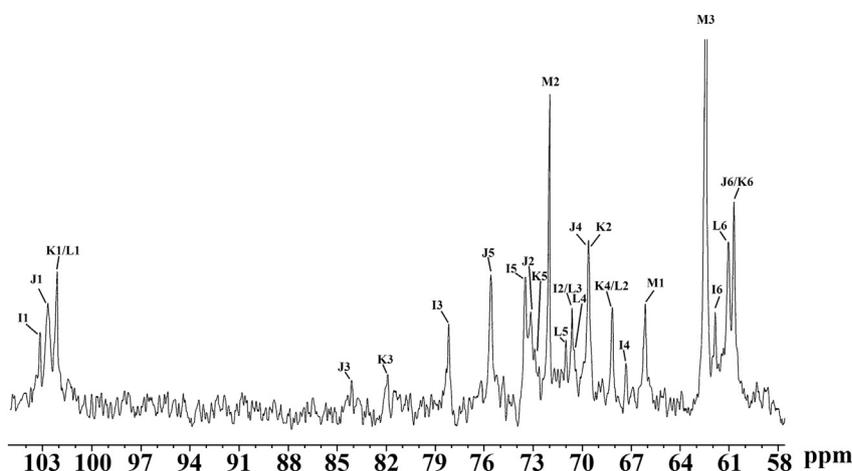


Fig. 5. ^{13}C NMR spectrum (125 MHz, D_2O , 30°C) of the Smith-degraded glycerol containing tetrasaccharide isolated from the edible mushroom *T. clypeatus*.

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.²⁶ 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.²⁷ 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.^{28–30} Results showed that the polysaccharide isolated from *T. clypeatus* have the potential to scavenge intracellular ROS.

3. Materials and methods

3.1. Isolation and purification of the polysaccharide

Fresh fruit bodies of the mushroom *T. clypeatus* (500 g) was washed, crushed and boiled in 500 mL of distilled water for 10 h, kept overnight at 4°C and filtered. The water-soluble crude polysaccharide (650 mg) was isolated by the method described previously.^{10,31,32} A portion (25 mg) of this crude polysaccharide was purified by gel-permeation chromatography on Sepharose-6B column and monitored by the phenol–sulfuric acid procedure³³ at 490 nm using Shimadzu UV–vis spectrophotometer, Model-1601. One fraction 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).

3.2. General methods

Optical rotation was measured by Jasco polarimeter (Model P-1020) at 25.7°C . The average molecular weight³⁴ of the polysaccharide was determined by gel chromatography as reported in

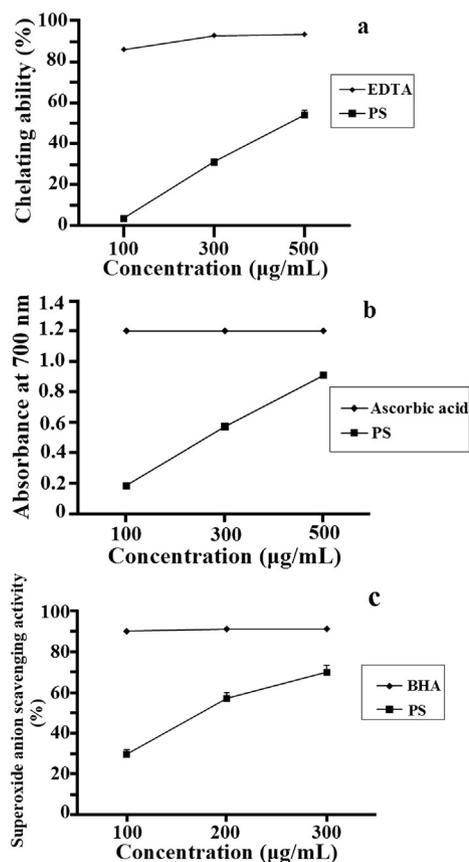


Fig. 6. (a) Ferrous ion chelating ability, (b) reducing power, (c) superoxide radical scavenging activity of the PS isolated from the edible mushroom *T. clypeatus*. All the results are the mean \pm SD of three separate experiments, each in triplicate.

Table 4

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

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
$\rightarrow 3$ - α -D-Manp-(1 \rightarrow)	103.2	70.6	78.2	67.2	73.5	61.8
I						
$\rightarrow 3$ - β -D-Glcp-(1 \rightarrow)	102.7	73.2	84.3	69.7	75.6	60.8
J						
$\rightarrow 3$ - α -D-Glcp-(1 \rightarrow)	102.2	69.6	81.9	68.1	72.9	60.8
K						
α -D-Galp-(1 \rightarrow)	102.2	68.1	70.6	70.5	71.1	61.1
L						
Gro-(3 \rightarrow)	66.2	72.1	62.5			
M						

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

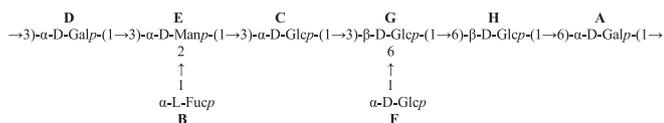
our earlier works.²⁸ For monosaccharide analysis, the PS (3 mg) was hydrolyzed with 2 M CF₃COOH (2 mL) in a round-bottom flask at 100 °C for 18 h in a water bath and the analysis was carried out as described in earlier publications.^{20,35,36} Methylation experiment was carried out by the method described previously.³⁷ The absolute configuration of monosaccharide was determined by the method of Gerwig et al.¹⁶ Smith degradation was carried out by the method described previously.¹⁰ 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 GLC analyzes were performed at 170 °C. Gas-liquid chromatography–mass spectrometric (GLC–MS) analysis was 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. Finally NMR experiments were carried out by a Bruker Avance DPX-500 spectrometer at 30 °C. as reported earlier. The ¹H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.70) using presaturation method. ¹H, ¹³C, 2D (DQF-COSY, TOCSY, NOESY, ROESY, HSQC) were performed as reported in earlier publications.^{10,37}

3.3. Antioxidant properties

Chelating ability of ferrous ions, reducing power and superoxide radical scavenging activity of the PS were measured adopting the procedures as described earlier.^{28–30}

4. 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 $\sim 1.98 \times 10^5$ Da showed specific rotation $[\alpha]_D^{25.7} +12.9$ (c 0.11, H₂O). The following structure was characterized by chemical analysis and 1D/2D NMR studies.



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.

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University of Calcutta has identified the mushroom. One of the authors (M.P.) is thankful to Council of Scientific and Industrial Research-UGC, India for providing junior research fellowship [UGC: Sr. No. 2061210344, Ref. No.:17-06/2012(i) EU-V].

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