Abstract

The present thesis entitled "**Studies of biologically active polysaccharides of mushrooms**" is mainly based on the elucidation of the structure as well as some biological activities and antioxidant properties of different polysaccharides, isolated from edible mushrooms *Termitomyces clypeatus* (R. Heim), *Tuber rufum* (Pico) var. and *Lentinus sajor-caju*. The whole thesis is divided into five chapters-

Chapter I:

Carbohydrates are essential constituents of all living organisms and have a variety of vital functions. Carbohydrates are generally classified into three classes: monosaccharides, oligosaccharides, and polysaccharides. Polysaccharides are polymers made up of many monosaccharides joined together by glycosydic bonds. They are therefore very large, often branched macromolecules and have relatively large molecular weights. Almost all living organism like fungi, bacteria etc. produces polysaccharides. Mushrooms are also fungi and many of them are edible, consumed as delicious food and considered as a source of biologically active compounds. The polysaccharides of edible mushrooms have a lot of pharmaceutical applications in industries. Several polysaccharides 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. 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. The antioxidant activities of polysaccharides are closely related to

their structural features such as molecular weight, monosaccharide composition, glycosidic bonds, degree of branching, and polymerization.

Chapter II:

This chapter describes the methodologies of isolation, purification and determination of the structure of pure polysaccharides and also their study of specific biological and antioxidant activity. The biological activities of polysaccharides depend on the molecular weight, linking sequences of the monosaccharide residues, branching pattern etc. So, it is very important to determine the exact structure of the repeating unit of the polysaccharides isolated from mushroom. Isolation of the crude polysaccharide is the first step for the determination of the structure of polysaccharides. The crude polysaccharide was purified by gel-permeation chromatography (GPC) technique using water as the eluant. The exact structure of the polysaccharides is determined using two types of methods:

1. Chemical method includes total acid hydrolysis, methylation, periodate oxidation, and smith degradation studies.
2. Spectroscopic method comprising of 1D (^{1}H, ^{13}C, DEPT-135) and 2D NMR (DQF-COSY, TOCSY, NOESY, ROESY, HSQC, HMBC).

Different biological studies were also carried out with different polysaccharide fractions. Lipid peroxidation, determination of reduced glutathione (GSH), oxidized glutathione level (GSSG), NO production, and ROS generation was carried out in human lymphocytes. The antioxidant activity of polysaccharide was evaluated through the

chelating ability of ferrous ions, reducing power, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, hydroxyl radical, ABTS radical, and superoxide radical scavenging activity.

Chapter III:

Structural analysis of polysaccharide isolated from the edible mushroom *Termitomyces clypeatus*

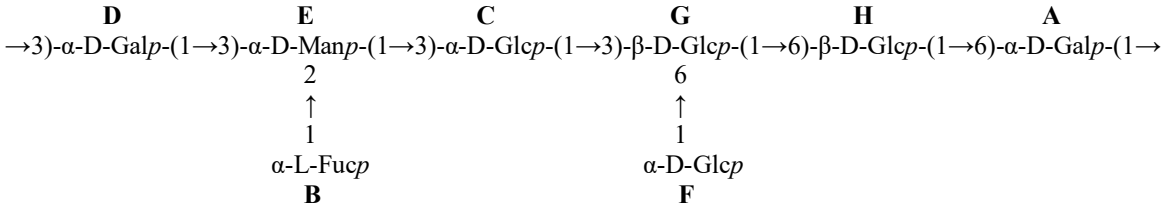
The hot aqueous extract of fresh fruit bodies (500 g) of edible mushroom *Termitomyces clypeatus* was cooled, filtered, and precipitated in ethanol. The residue was dialyzed, centrifuged, and freeze dried, yield; 650 mg. One homogeneous fraction was obtained from the crude polysaccharide on separation through Sepharose-6B. The 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 $\times 10^5$ Da. 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. 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. 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→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.

The proton NMR spectrum (500 MHz) 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. In ^{13}C NMR spectrum (125 MHz) 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, 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 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.

The sequences of glycosyl residue were determined from NOESY as well as ROESY 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/

EH-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**. On the basis of the appearance of these cross peaks and NOESY connectivity, the structure of the repeating unit in the pure polysaccharide (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 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**. 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:

L **I** **K** **J** **M**
α-D-Gal*p*-(1→3)-α-D-Man*p*-(1→3)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-Gro

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

Antioxidant properties

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. 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. Ferrous ion chelating ability of PS was found to be 54.1 % at 500 μg/mL concentration.

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 EC_{50} value of PS was observed 260 μg/mL.

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 EC_{50} value of the PS was found 180 μg/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.

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Chapter-IV:

Structural analysis of polysaccharide isolated from the mushroom *Tuber rufum*

The water soluble crude polysaccharide (700 mg) was isolated from the mushroom *Tuber rufum.* On fractionation through Sepharose gel it showed the presence of two fractions, PS-I and PS-II. The PS-II showed specific rotation $[\alpha]_D^{25.7}$ +16.8 (*c* 0.2, H_2O) .The molecular weight of PS-II was estimated as ~7.27 $\times 10^4$ Da from a calibration curve prepared with standard dextrans. 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. 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

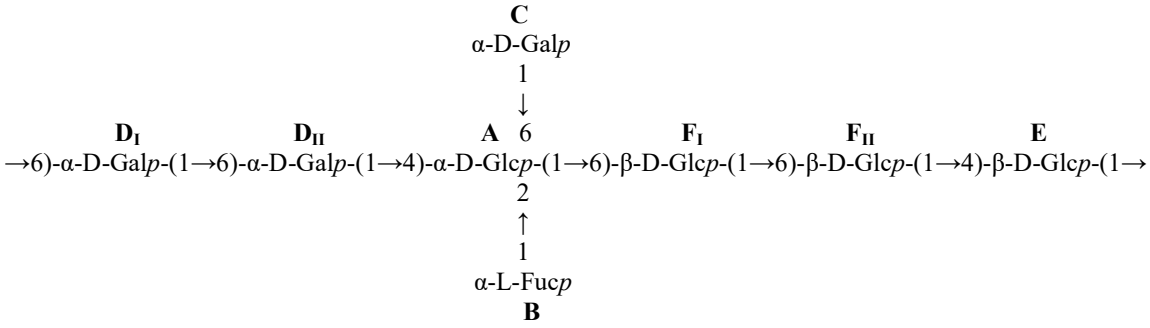
was determined by the methylation analysis using Ciucanu and Kerek method followed by hydrolysis and alditol acetate preparation. The GLC-MS analysis of the alditol acetates of methylated product showed the presence of 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylfucitol; 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol; 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 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 indicating the other residues were destroyed during oxidation.

^{1}H NMR spectrum (500 MHz) 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**, $\mathbf{F_I}$, and $\mathbf{F_{II}}$ respectively**.** The peak at δ 5.05 for **B** and **C**, and the peak at δ 4.97 were identified for $\mathbf{D_I}$ and $\mathbf{D_{II}}$ residues. In ^{13}C NMR spectrum (125 MHz) 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, 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 **($\mathbf{F_{II}}$)**, 4.51**($\mathbf{F_I}$)**, 4.75 **(E)**, 5.05 **(B)**, 5.37 **(A)**, 5.05 **(C)**, 4.97 **($\mathbf{D_{II}}$)** and 4.97 **($\mathbf{D_I}$)** respectively. All the ^{1}H and ^{13}C NMR signals (Table 1) were assigned from DQF-COSY, TOCSY and

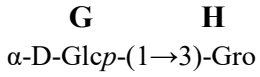
HSQC experiments. The proton coupling constants were measured from DQF-COSY experiment and one-bond C-H couplings were measured from proton coupled ^{13}C spectrum.

The sequence of the glycosyl residues were determined from ROESY as well as NOESY experiments. In ROESY experiment, the inter-residual contacts **D_I**H-1/ **D_{II}**H-6a, 6b; **D_{II}**H-1/ **A**H-4; **A**H-1/ **F_I**H-6a, 6b; **F_I**H-1/ **F_{II}**H-6a, 6b; **F_{II}**H-1/ **E**H-4; **E**H-1/**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**. Finally these connectivities were confirmed from HMBC spectrum. 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.

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 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) 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*.

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. 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 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 was fairly decreased and the mild increase of GSSG level 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 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 (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 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 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.

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.

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Chapter-V:

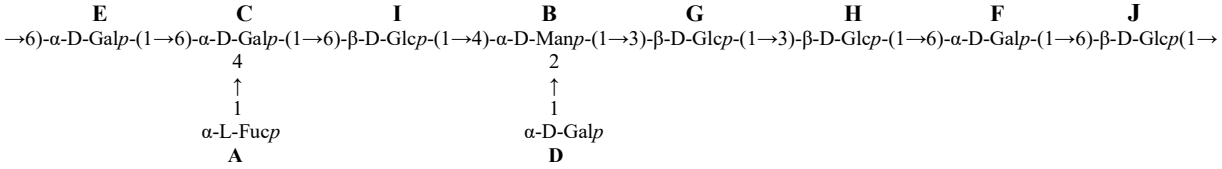
Structural characterization of polysaccharide isolated from edible mushroom *Lentinus sajor-caju*

The aqueous extract of edible mushroom (600 g) *Lentinus sajor-caju* was cooled, filtered, and precipitated in ethanol and 800 mg crude polysaccharide was isolated. Sepharose 6B gel filtration of crude polysaccharide (25 mg) yielded two fractions (fraction I; 14 mg and fraction II; 9 mg). In the present study, attempts have been made to investigate the structure of only the fraction I (PS-I). GLC analysis of the hydrolyzed product of PS-I revealed the presence of monosaccharides glucose, galactose, mannose, and fucose in a molar ratio of 4:4:1:1. GLC-MS analysis of the methylated product showed the presence of seven components. These results indicated that the repeating unit of PS-I consisted of terminal Fuc*p*, terminal Gal*p*, (1→3)-Glc*p*, (1→6)-Gal*p*, (1→6)-Glc*p*, (1→4,6)-Gal*p*, and (1→2,4)-Man*p* moieties. GLC analysis of periodate oxidized reduced material showed the presence of glucose and mannose in a molar ratio of nearly 2:1. GLC-MS analysis of alditol acetates of periodate oxidized reduced methylated product showed that (1→3)-Glc*p* and (1→2,4)-Man*p* moieties were unaffected during

oxidation while other residues were consumed, which further confirmed the mode of linkages present in the polysaccharide.

The proton NMR spectrum (500 MHz, in D_2O at 30 °C) of PS-I is shown in figure. The anomeric region (δ_H 5.25 - 4.51) contained ten signals. These ten signals in decreasing order at δ_H 5.25, 5.11, 5.09, 5.05, 5.01, 4.99, 4.78, 4.75, 4.53, and 4.51 in PS-I were arbitrarily labeled as **A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**, **I**, and **J** respectively. In ^{13}C NMR spectrum (125 MHz, in D_2O at 30 °C) the anomeric region (δ_C 102.8 - 97.6) contained nine signals. The anomeric signals of the residues **J** (δ_C 102.8), **I** (δ_C 102.6), **G** (δ_C 102.4), **H** (δ_C 102.4), **B** (δ_C 102.0), **C** (δ_C 101.3), **A** (δ_C 100.9), **E** (δ_C 98.3), **D** (δ_C 98.0), and **F** (δ_C 97.6) were correlated to the signals **J** (δ_H 4.51), **I** (δ_H 4.53), **G** (δ_H 4.78), **H** (δ_H 4.75), **B** (δ_H δ 5.11), **C** (δ_H 5.09), **A** (δ_H 5.25), **E** (δ_H 5.01), **D** (δ_H 5.05), and **F** (δ_H 4.99) respectively from HSQC spectrum. All the ^{1}H and ^{13}C NMR signals were assigned from DQF-COSY, TOCSY, and HSQC experiments. Proton coupling constants were measured from DQF-COSY experiment.

From ROESY and NOESY experiments the sequences of glycosyl residues were determined. The ROESY experiment showed the inter-residual contacts: **E**H-1/ **C**H-6a, 6b; **C**H-1/ **I**H-6a, 6b; **I**H-1/ **B**H-4; **B**H-1/ **G**H-3; **G**H-1/ **H**H-3; **H**H-1/ **F**H-6a, 6b; **F**H-1/ **J**H-6a, 6b; **J**H-1/ **E**H-6a, 6b; **A**H-1/ **C**H-4; **D**H-1/ **B**H-2 along with other intra-residual contacts. Thus, the ROESY connectivity established the sequences as, **E** (1→6) **C**; **C**(1→6) **I**; **I** (1→4) **B**; **B** (1→3) **G**; **G** (1→3) **H**; **H** (1→6) **F**; **F** (1→6) **J**; **J** (1→6) **E**; **A** (1→4) **C**; **D** (1→2) **B**. Hence, from all these chemical and NMR studies the repeating unit motif of PS-I was proposed as:



Finally, the structure of main chain of the heteroglycan was identified by Smith degradation, which produced an oligomeric product (SDPS) that was analyzed by ^{13}C NMR. The ^{13}C NMR (125 Hz; at 30 ^{o}C) spectrum of SDPS showed three anomeric signals at δ 103.2, 103.1, and 102.2. The anomeric carbon signals at δ 103.2 and 103.1 resembled both to the (1→3)-β-D-glucopyranosyl residues and arbitrarily labeled as **K** and **L** respectively. Whereas, the carbon signal at δ 102.2 corresponded to the terminal α-D-mannopyranosyl (**M**) residue. The carbon signals of the glycerol moiety (Gro) were assigned as C-1 (δ 66.5), C-2 (δ 72.2), and C-3 (δ 62.5). The terminal α-D-mannopyranosyl (**M**) residue of SDPS was generated from the (1→2,4)-α-D-mannopyranosyl moiety (**B**) of parent PS-I during Smith degradation. The downfield shifts of C-3 of residues **K** (δ 84.6) and **L** (δ 84.4) of SDPS indicated that residues **K** and **L** were (1→3)-linked β-D-glucopyranosyl. The (1→3)-β-D-Glc*p* residues of SDPS (**K** and **L)** were retained during oxidation previously denoted as **H** and **G** respectively of the parent PS-I. During Smith degradation, the glycerol moiety **N** was generated from (1→6)-α-D-Gal*p* residue (**F**). Thus, the residue **N** was attached to **K**. Therefore, a glycerol containing trisaccharide was formed from the parent PS-I through Smith degradation. Hence, the structure of SDPS was proposed as:

M **L** **K** **N**

α-D-Man*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)Gro

The above results further confirmed the repeating sugar unit in PS-I.

Antioxidant activities

The antioxidant activity of PS-I was determined as their scavenging capability against free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). In the present study, the PS-I was found as a notable scavenger of DPPH radicals. Results showed that the radical scavenging activity of the PS-I increased in concentration dependent manner. Figure revealed that 0.1, 0.5, 0.75, 1.0 and 1.5 mg/mL concentrations of polysaccharide exhibited radical scavenging activity at the rate of 6.14%, 21.88%, 30.56 %, 38.89 % and 52.5 %, whereas the standard ascorbic acid showed the activity at 98.6%, 98.9%, 99%, 98.7% and 99% respectively. The EC_{50} value of the PS-I was found 1.375 ± 0.02 mg/mL.

Hydroxyl radical is the most reactive oxygen centered species and can be generated in biological cells through the Fenton reaction. The PS-I showed potent hydroxyl radical scavenging activity which gradually increases with increasing concentration. The hydroxyl radical scavenging rate of PS-I at 0.25, 0.50, 0.75, 1.0, and 1.5 mg/mL were found as 18.7%, 28.5%, 36.5%, 43.06%, and 53.3% respectively. But BHT showed hydroxyl radical scavenging rate to that of corresponding concentrations at 98.7%, 99%, 98.6%, 99%, and 99%. The EC_{50} value of the PS-I was found to be 1.31 ± 0.048 mg/mL. These results indicated that PS-I has a moderate antioxidant activity and the activity of PS-I is weak compared to that of BHT.

The PS-I showed good reducing power ability in a dose dependent manner which was comparable to that of ascorbic acid, used as standard. At concentration of 0.1 mg/mL, the absorbance value of PS-I was 0.03 but in case of ascorbic acid the value was 1.2. The PS-I showed the absorbance value 0.196 at 0.5 mg/mL, whereas the ascorbic acid showed the value of 1.19 at the same concentration. Reducing power of the PS-I increases with the increase in concentration. At concentrations of 1.0, 1.5, and 2.0 mg/mL the absorbance value of ascorbic acid was fixed at 1.2 whereas the value of PS-I increases to 0.328, 0.443 and 0.539 respectively. Herein, PS-I could reduce Fe^{3+} to Fe^{2+} with 50 % inhibition capacity at a concentration of 1.75 ± 0.035 mg/mL. These results suggest that PS-I is a moderate electron donor and may terminate the radical chain reaction by converting free radicals to more stable product.

ABTS radicals possess blue green chromophore, formed by the reaction between ammonium persulphate and ABTS salt. The radical scavenging activity was compared with Trolox, commercially available water soluble vitamin E analog. In this assay, radical scavenging activity was evaluated and the results showed 501.3 ± 0.042 µM of Trolox equivalent/mg of polysaccharide antioxidant potentiality.

Chelating ability of any compounds may serve as a reflection of its antioxidant activity. In presence of any chelating agent, the ferrozine- Fe^{2+} formation is disrupted with decrease in color of the complex. Resultant color reduction demonstrates ferrous ion chelating efficacy of the sample. In this assay, polysaccharide (PS-I) and standard antioxidant compound EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity. At 0.5, 1, 1.5, 2 and 3 mg/mL

concentrations the polysaccharide showed chelation at the rate of 25.03 %, 33.6%, 38.2 %, 43.7 %, and 52.5%, whereas the used standard EDTA also chelated 99% at the same concentrations. This study revealed that the PS-I was good chelator of ferrous ions as evidenced by low EC_{50} value (2.67 ± 0.02 mg/mL).

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