BIOACTIVE POLYSACCHARIDES FROM EDIBLE MUSHROOMS

A THESIS

SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

(SCIENCE)

TO

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BY

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"Dedicated to

my beloved Parents"

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the research work presented in the thesis entitled "**Bioactive Polysaccharides from Edible Mushrooms**" has been carried out under my direct supervision and a bonafide research work of **Mr. Prasenjit Maity**. This work is original and has not been submitted for any degree or diploma to this or any other University or Institution.

Date:

(Syed Sirajul Islam)
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DECLARATION

I hereby declare that the research work embodied in this thesis has been carried out by me in the Department of Chemistry & Chemical Technology, Vidyasagar University, Midnapore-721 102, West Bengal, India, under the supervision of Professor Syed Sirajul Islam, Department of Chemistry & Chemical Technology, Vidyasagar University, Midnapore-721 102, West Bengal, India. I also affirm that this work is original and has not been submitted before in part or full for any degree/diploma or any other academic award to this or any other University or Institution.

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PREFACE

The present thesis entitled "**Bioactive Polysaccharides from Edible Mushrooms**" is divided into five chapters:

Chapter-I: It describes the general introduction to carbohydrates, polysaccharides including mushroom polysaccharides, and their biological activities.

Chapter-II represents the experimental methods, which were carried out during the thesis work.

Chapter-III: This chapter is one of the major parts of this thesis, which describes the isolation, purification, structural characterization, biological and antioxidant properties of two polysaccharides isolated from alkaline extract of an edible mushroom *Entoloma lividoalbum*.

These works have been published in

(i) ***International Journal of Biological macromolecules,* 2014**, *63*, 140-149

(ii) ***Carbohydrate Polymers,* 2014,** *114*, 157-165.

Chapter-IV: This chapter contains the structural, immunological, and antioxidant studies of β-glucan isolated from an edible mushroom *Entoloma lividoalbum.*

This work has been published in ***Carbohydrate Polymers,* 2015***,123*, 350-358.

Chapter-V: This chapter represents the isolation, purification, structural characterization and study of immunoactivation of a partially methylated mannogalactan isolated from hybrid mushroom *pfle 1p*.

This work has been published in ***Carbohydrate Research,* 2014**, 395, 1-8.

Each copy of the publications is attached at the end of the thesis.

ABBREVIATIONS

Ac	Acetyl
Ac_2O	Acetic anhydride
AcOH	Acetic acid
ADP	Adenosine diphosphate
ADCC	Antibody Dependent Cell mediated Cytotoxicity
AF	Antibody Formation
AIDS	Acquired immune deficiency syndrome
ATP	Adenosine triphosphate
BET	Bacterial endotoxin test
BHA	Butylated hydroxyanisole
BHP	Tert-butyl hydroperoxide
BHT	Butylated hydroxytoluene
BRM	Biological response modifier
BSTFA	N,O-bis(trimethylsilyl)trifluroacetamide
BuOH	Butanol
CF_3COOH	Trifluoro acetic acid
CH_3CO_2H	Acetic acid
$CHCl_3$	Chloroform
CH_3I	Methyl iodide
CH_3OH	Methanol
cm	Centimetre
CO_2	Carbondioxide
Con A	Concanavalin A
CSE	Control standard endotoxin
CTL	Cytotoxic T-Lymphocyte
°C	Degree centigrade
1D	1-Dimensional
2D	2-Dimensional

Da	Dalton
DEPT	Distortionless enhancement by polarization transfer
DEAE	Diethyl aminoethyl
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxy ribo nucleic acid
DQF-COSY	Double-quantum filtered correlation spectroscopy
D_2O	Deuterium oxide
E	*Entoloma*
EDTA	Ethylene diamine tetra acetic acid
ELPS	*Entoloma lividoalbum* Polysaccharide
EtOAc	Ethyl acetate
EtOH	Ethyl alcohol
FAD	Flavin adenine dinucleotide
FACS	Fluorescence assisted cell sorting
$FeCl_3$	Feric chloride
g	Gram
Glc	Glucose
GLC	Gas-liquid chromatography
GLC-MS	Gas liquid chromatography- mass spectrometry
GPC	Gel permeation chromatography
h	Hour(s)
HA	Hyaluronic acid
HBSS	Hank’s balanced salt solution
HCl	Hydrochloric acid
HCOOH	Formic acid
HDP	Host defense potentiator
HEC	Hydroxyethyl cellulose
HMBC	Hetero nuclear multiple bond correlation
HOD	Deuterated water

HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum coherence
Hz	Hertz
H_2O	Water
H_2O_2	Hydogen peroxide
H_3PO_4	Phosphoric acid
H_2SO_4	Sulfuric acid
IL-1	Interlukine-1
IL-2	Interlukine-2
J	Coupling constants
Kg	Kilogram
KOH	Potasium hydroxide
KH_2PO_4	Potasium hydrogen phosphate
LAL	Limulus amebocyte lysate
LFPS	LPS free polysaccharide
LPS	Lipopolysaccharide
M	Molar
M	Meter
MAF	Macrophage Activating Factor
MDA	Malondialdehyde
Me	Methyl
mg	Milligram
MHz	Mega hertz
min	Minute(s)
mL	Mililiter
mm	Milimeter
mM	Millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Mw	Molecular weight

m/z	Mass to charge ratio
NAD	Nicotinamide adenine dinucleotide
$NaBH_4$	Sodium borohydrate
$NaIO_4$	Sodium metaperiodate
NaOH	Sodium hydroxide
NBT	Nitroblue tetrazolium
NK	Natural killer cell
nm	Nanometer
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOE	Nuclear overhauser effect
NOESY	Nuclear overhauser enhancement spectroscopy
OD	Optical density
p	Pyranose
P_2O_5	Phosphorus pentoxide
PBS	Phosphate buffered saline
PC	Paper chromatography
PEG	Polyethyleneglycol
PG	propyl gallate
PMAA	Partially methylated alditol acetate
ppm	Parts per million
PS	Polysaccharide
PSK	Protein bound polysaccharide
PSP	Polysaccharide peptide complex
RNA	Ribo nucleic acid
ROE	Rotational nuclear overhauser effect
ROESY	Rotating frame overhauser enhancement spectroscopy
ROS	Reactive oxygen species
rpm	Rotation per minute
RPMI	Roswell Park Memorial Institute

SDPS	Smith degraded polysaccharide
SDS	Sodium dodecyl sulfate
SPI	Splenocyte proliferation index
TBA	Thiobarbituric acid
TCA	Trichloro acetic acid
TFA	Trifluoro acetic acid
TLC	Thin layer chromatography
TOCSY	Total correlation spectroscopy
TMS	Tetra methyl silane
TPI	Thymocyte proliferation index
UV	Ultraviolet
vis	Visible
v/v	Volume by volume ratio
α	Alfa
B	Beta
δ	Delta
μg	Microgram
μL	Microliter
μM	Micromolar

CONTENTS

ABSTRACT

The present thesis entitled "**Bioactive Polysaccharides from Edible Mushrooms**" is mainly based on the determination of the structure as well as some important biological and antioxidant activities of different polysaccharides isolated from the fruit bodies of edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička and hybrid mushroom *pfle 1p*. The entire thesis is divided into five chapters.

Chapter-I: It discusses the introduction of carbohydrates, mushroom, mushroom polysaccharides, and some of their important biological activities. Carbohydrates are essential constituents of all living organisms and have a variety of vital functions. It is the abundant source of dietary fiber and also serves as storage food for supply of energy. Based on number of sugar units present these are classified into four classes: monosaccharides, disaccharides, oligosaccharides, and polysaccharides. A great majority of carbohydrates of nature occur as polysaccharides. Polysaccharides are macromolecules consisting of a large number of monosaccharide units. Now-a-days mushrooms are popularly used as food flavouring substances due to their unique taste, flavor, high nutritive value (proteins, chitin, vitamins and minerals) and medicinal values. They contain a wide range of bioactive molecules including steroids, terpenoids, phenols, neucleotides, glycoproteins, and polysaccharides. Mushroom polysaccharides have drawn the attention of chemists and immunobiologists due to their immunomodulatory, antitumor, antimicrobial, antiflammatory, antiviral, antithrombotic, and antioxidant activities. Several mushroom polysaccharides such as lentinan, PSK, and sonifilan are clinically used as antitumor drugs throughout the world. Among the mushroom polysaccharides, β-D-glucan are most important polysaccharides with immunomodulating and antitumor activity and are known as biological response modifiers (BRM). The biological activities of polysaccharides depend on the molecular structure, molecular weight, size, branching frequency, structural modification, conformation, and solubility. Reactive oxygen species (ROS) are formed continuously as normal by-products of oxygen metabolism process. They play a major role in the development of several human diseases, such as cerebral ischemia, diabetes, Alzheimer, inflammation, rheumatoid arthritis, atherosclerosis and cancer. The antioxidant polysaccharides present in

mushrooms are of great interest as potential protective agents against oxidative damage. It is therefore important to determine the exact structure and biological activity of the polysaccharides isolated from mushrooms.

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 properties. The biological activities of polysaccharides depend on the molecular weight, linking sequences of the monosaccharide residues, and branching pattern. So, it is very important to determine the exact structure of the repeating unit of the polysaccharides isolated from mushroom. Isolation of the pure 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 eluent. The exact structure of the polysaccharides is determined using two types of methods: (1) chemical method that 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 the polysaccharide fractions. The macrophages activation induced by the polysaccharides was studied by nitric oxide (NO) production using Griess reagent. The activation of splenocyte and thymocyte tests were carried out in mouse cell culture medium with polysaccharide by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. Lipid peroxidation (MDA), the ratio of oxidized glutathione (GSSG) and reduced glutathione (GSH) levels were carried out in normal human lymphocytes. The antioxidant activity of polysaccharide was evaluated through the determination of reducing power, determination of total antioxidant capacity, hydroxyl and superoxide radical scavenging activity. The scavenging activity of the polysaccharide is calculated by the following equation:

$$\text{Scavenging activity (\%)} = \{(A_0-A_1)/ A_0\} \times 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of sample.

Chapter III: This chapter describes the isolation, purification, structural characterization, antioxidant and biological properties of two polysaccharides isolated from the edible mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička. Alkaline extract of fruit bodies of the edible mushroom *Entoloma lividoalbum* yielded two water soluble polysaccharides, PS-I and PS-II. The molecular weight of PS-I and PS-II were estimated as ~1.94×10^5 Da and ~1.48×10^5 Da respectively, from a calibration curve prepared using standard dextran.

Structural analysis of PS-I

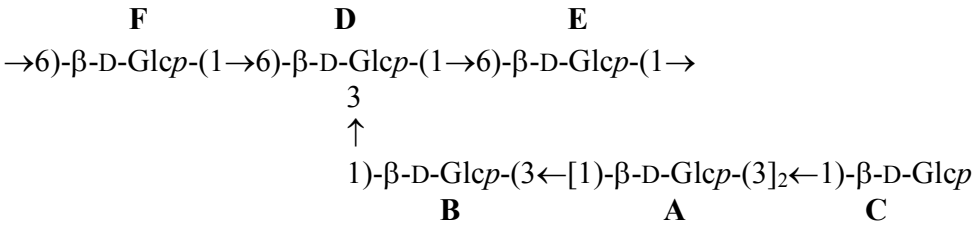
GLC analysis of the alditol acetates of PS-I revealed the presence of glucose only. Determination of absolute configuration of the monosaccharide showed that glucose was in D configuration. The GLC-MS analysis of the alditol acetates of methylated product showed the presence of 1,5-di-*O*-acetyl-2,3,4.6-tetra-*O*-methyl-D-glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol, and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a molar ratio of approximately 1:3:2:1. These results showed that the (1→6)-D-glucopyranosyl and terminal D-glucopyranosyl residues were consumed during oxidation whereas, (1→3,6)-D-glucopyranosyl and (1→3)-D-glucopyranosyl residues remain unaffected, further confirming the mode of linkages present in PS-I.

In the ^{1}H NMR spectrum (500 MHz) of PS-I, four anomeric signals at δ 4.77, 4.73, 4.50, and 4.49 were observed at 30 °C in a ratio of nearly 2:1:1:3. The peak at δ 4.77, 4.73, and 4.50 designated as **A**, **B**, and **D**, whereas the peak at δ 4.49 consists of **C**, **E**, and **F** residues. In ^{13}C NMR spectrum (125 MHz) at the same temperature, five signals were observed in the anomeric region at δ 103.0, 102.9, 102.8, 102.7, and 102.6. On the basis of HSQC spectrum, the anomeric carbon signal at δ 102.9 was correlated to both the proton signals δ 4.77 (**A**) and δ 4.73 (**B**) respectively. Again, the anomeric proton signal at δ 4.50 was correlated to the carbon signal at δ 102.6 (**D**) whereas the peak at δ 4.49 was correlated to the anomeric carbon signals at δ 103.0 (**C**), δ 102.8 (**E**), and δ 102.7 (**F**).

The sequence of glycosyl residues (**A** to **F**) were determined from NOESY as well as ROESY (not shown) studies. In NOESY experiment, the inter-residual contacts **A**H-1/**B**H-3; **B**H-1/**D**H-3; **C**H-1/**A**H-3; **D**H-1/**E**H-6a, 6b; **E**H-1/**F**H-6a, 6b; and **F**H-1/**D**H-6a, 6b along with other intra-residual contacts were also observed. The above NOESY connectivities established the following sequences: **F** (1→6) **D**; **D** (1→6) **E**; **E** (1→6) **F**; **B** (1→3) **D**; **A** (1→3) **B**; and **C** (1→3) **A**.

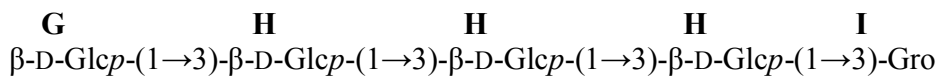
Finally, these NOESY connectivities were confirmed from HMBC spectrum. In this spectrum the inter-residual cross-peaks between **A**H-1/**B**C-3, **A**C-1/**B**H-3; **B**H-1/**D**C-3, **B**C-1/**D**H-3; **C**H-1/**A**C-3, **C**C-1/**A**H-3; **D**H-1/**E**C-6, **D**C-1/**E**H-6a, 6b; **E**H-1/**F**C-6, **E**C-1/**F**H-6a, 6b; **F**H-1/**D**C-6, **F**C-1/**D**H-6a, 6b along with some intra-residual peaks were also observed.

Hence NOESY and HMBC connectivities confirmed the structure of repeating unit presence in the PS-I as:



NMR experiments were again carried out with Smith degradation product (SDPS) of the PS-I for further confirming the linkages. Smith degradation results in the formation of a glycerol containing tetrasaccharide from the parent polysaccharide and the structure of SDPS were established as:

:



So, all these results indicated that the β-D-glucan (PS-I) isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička, is a branched glucan with (1→6)-linked backbone where branching occurred at *O*-3 of one unit followed by (1→3)-β-D-Glc*p* and terminal β-D-Glc*p*.

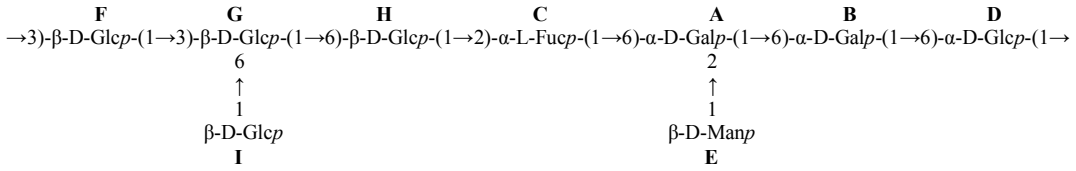
Structural analysis of PS-II

GLC analysis of the alditol acetates of the hydrolyzed product of PS-II revealed the presence of glucose, mannose, galactose, and fucose in a molar ratio of nearly 5:1:2:1. The absolute configuration of the monosaccharide was determined by the method of Gerwig, Kamerling, and Vliegenthart, (1978) and found that glucose, galactose, and mannose had the D configuration but fucose was present in the L configuration. The GLC-MS analysis of the alditol acetates of methylated product showed the presence of terminal mannopyranosyl, terminal glucopyranosyl, (1→2)-fucopyranosyl, (1→3)-glucopyranosyl, (1→6)-glucopyranosyl, (1→3,6)- glucopyranosyl, (1→6)-galactopyranosyl, and (1→2,6)-linked galactopyranosyl in a molar ratio of approximately 1:1:1:1:2:1:1:1. GLC analysis of alditol acetates of the periodate-oxidized, $NaBH_4$-reduced, and hydrolyzed products showed the presence of only glucose, indicating that the D-galactose, D-mannose, and L-fucose moieties were consumed during oxidation. GLC-MS analysis of periodate-oxidized, reduced, methylated PS-II showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a molar ratio of nearly 1:1. These results clearly indicated that the (1→3)-linked and (1→3,6)-linked glucopyranosyl residues remain unaffected whereas all other residues were consumed during oxidation, which further confirmed the mode of linkages present in the PS-II.

The ^{1}H NMR spectrum (500 MHz) of PS-II at 30 °C showed the presence of nine signals in the anomeric region at δ 5.11, 5.04, 5.03, 4.99, 4.77, 4.73, 4.49, 4.48, and 4.47. The sugar residues were designated as **A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**, and **I** according to their decreasing anomeric proton chemical shifts. In ^{13}C NMR spectrum (125 MHz) at the same temperature, seven signals were observed in the anomeric region at δ 103.0, 102.9, 102.7, 102.5, 101.7, 101.6, and 98.3. From the HSQC spectrum, the anomeric carbon signals at δ 103.0, 102.9, 102.7, 102.5, 101.7 and 101.6 were correlated to the anomeric proton signals δ 4.47 (**I**), δ 4.73 (**F**), δ 4.48 (**H**), δ 4.49 (**G**), δ 4.77 (**E**), and δ 5.04 (**B**) respectively. Whereas, the anomeric carbon signal at δ 98.3 was correlated to the anomeric proton signals at δ 5.11 (**A**), δ 5.03 (**C**), and δ 4.99 (**D**).

The sequence of glycosyl residues (**A** to **I**) were determined from ROESY as well as NOESY. In ROESY experiment, the inter-residual contacts **A**H-1/**B**H-6a, 6b; **B**H-1/**D**H-6a, 6b; **C**H-1/**A**H-6a, 6b; **D**H-1/**F**H-3; **E**H-1/**A**H-2; **F**H-1/**G**H-3; **G**H-1/**H**H-6a, 6b; **H**H-1/**C**H-2; and **I**H-1/**G**H-6a, 6b along with other intra-residual contacts were also observed. The above ROESY connectivities established the following sequences: **A** (1→6) **B; B** (1→6) **D; C** (1→6) **A**; **D** (1→3) **F; E** (1→2) **A; F** (1→3) **G**; **G** (1→6) **H**; **H** (1→2) **C**; and **I** (1→6) **G**. These data clearly indicated the positions of substitution and sequence of sugar residues in the polysaccharide.

Hence, the structure of repeating unit in the PS-II was proposed as:



Finally, Smith degraded material (SDPS) from PS-II was prepared to confirm the linkages of the heteroglycan. Hence, Smith degradation results in the formation of a glycerol containing disaccharide from the parent polysaccharide and the structure of which was established as:

J **K** **L**
β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-Gro

This result further confirmed the repeating unit present in the heteroglycan isolated from the edible mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička.

Antioxidant properties of PS-I

The hydroxyl radical scavenging activity of PS-I gradually increase with the increase of concentration. The hydroxyl radical scavenging rate of PS-I and BHT at 200 μg/mL were found to be 16.6 % and 91.2 % respectively, indicating that PS-I has a moderate antioxidant activity and the activity of PS-I is weak compared with that of BHT. The EC_{50} value of the PS-I was found to be 480 μg/mL. The hydroxyl radical scavenging activity

might be explained by hydrogen atom donation ability of the hydroxyl group in PS-I to terminate the free radical mediated oxidative chain reactions.

The scavenging effect of PS-I on superoxide radicals was found concentration-dependent. The percentage inhibition of superoxide generation by 200 µg/mL concentration of PS-I was found to be 64.47 % whereas of BHA was found as 91.01 %. They both showed a concentration dependent scavenging of superoxide radicals and the percentage inhibition of PS-I is close to that of synthetic standard drug BHA at a concentration of 400 µg/mL. The EC_{50} value of the PS-I was found to be 150 µg/mL.

The reducing power of PS-I was concentration-dependent. In this assay, reducing power of PS-I and ascorbic acid increased with increasing sample concentration. At 200 µg/mL, the reducing powers were 0.2765 and 1.16 for PS-I and ascorbic acid respectively. At 600 µg/mL, the reducing powers were 0.58 and 1.27 for PS-I and ascorbic acid respectively. At concentration of 480 µg/mL, PS-I showed reducing power 0.5. This result suggests that PS-I is a good electron donor and may terminate the radical chain reaction by converting free radicals to more stable product.

PS-I inhibited the phosphomolybdenum in the total antioxidant capacity assay. Result showed 1 mg of PS-I is equivalent to 70 ± 15 µg of ascorbic acid. Total antioxidant activity suggests that the electron donating capacity of PS-I and thus it may act as radical chain terminator, by transforming reactive free radicals into more stable non reactive products.

Biological activities of PS-II

The cell viability using PS-II was studied on human 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. It was observed that the cytotoxicity to normal lymphocytes by PS-II was insignificant. Cell proliferative activity was observed at 50 µg/ml of PS-II with respect to control. These results showed a lower level of cytotoxicity when lymphocytes were treated with PS-II up to 200 µg/ml but even at higher dose 400µg/ml, the polysaccharide showed mild toxicity. Cell culture

experiments were carried out and statistical calculations showed the IC50 value was 800 µg/ml, indicating that 200 µg/ml is safe with respect to the other higher doses.

Glutathione is an important antioxidant in cellular system. Hence to understand the glutathione level in cell, both reduced and oxidized form of glutathione were measured. The reduced glutathione level (GSH) was decreased and the mild augmentation of oxidized form of glutathione level (GSSG) 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= 0.951, 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 1.01 to 0.499 compared to their respective control indicating that 400 µg/ml was toxic. These results indicated that 200 µg/ml is biologically safe and effective dose.

Study of lipid peroxidation is one of the important parameters to assess the cellular damage. It initiates inactivation of cellular components and protective enzymes, and thereby plays a crucial role of oxidative stress in biological systems. Several toxic by-products especially malondialdehyde is released due to lipid peroxidation. Hence, lipid peroxidations in lymphocytes were measured in terms of the concentration of malondialdehyde (MDA) release. The present investigation showed slightly increase of MDA at the dose of 400 µg/ml in comparison to the previous doses indicating that 200 µg/ml is again biologically safe.

Stimulated lymphocytes secreted several factors like NO. The release of NO clearly demonstrated that it was secreted by the lymphocytes when stimulated by PS-II. In presence of the PS-II, single culture of lymphocytes generated significant amount of NO ($p< 0.05$) into the medium after 24h of incubation. The result showed the presence of a high concentration of NO in the co-culture medium of pulsed lymphocytes at 400µg/ml indicating that this dose is cytotoxic. Hence, it is again established that 200 µg/ml is safe and effective dose.

To establish the protective role of PS-II against nicotine toxicity, lymphocytes were treated with nicotine (10 mM) as positive control and different concentrations of PS-II along with nicotine for 24 h in culture media. The significantly ($p < 0.05$) increased cell

viability levels were observed up to 200µg/ml. The fluorescent microscopic pictures established the result. The fluorescence images revealed that the PS-II was able to ameliorate the toxic effects of nicotine at the dose of 200 µg/ml, but when the dose was increased to 400 µg/ml, the PS-II lost its ameliorative effects on lymphocytes. The above result was confirmed by FACS, which established our findings that 400 µg/ml reveled the toxic effects synergistically with nicotine.

It is evident from these experiments that, in vitro application of PS-II does not induce any cellular damage in lymphocytes associated with enhanced MDA level, NO level, GSSG level and decreased GSH level. The cytotoxic profile of PS-II in lymphocytes indicated 200 µg/ml safe and effective, whereas concentrations higher than 200 µg/ml showed significant increase of cytotoxicity. Administration of Nicotine to lymphocytes causes decrease in cell viability which is protected by supplementation of PS-II to nicotine treated cells. These findings suggest the potential use and beneficial role of PS-II for use as antioxidant as well as immunostimulant.

These works were published in

(i) ***International Journal of Biological macromolecules,* 2014**, *63*, 140-149

(ii) ***Carbohydrate Polymers,* 2014,** *114*, 157-165.

Chapter-IV: Fresh fruit bodies of the mushroom *Entoloma lividoalbum* (700 g) were gently washed with water, cut into pieces and boiled at 100 °C with distilled water for 10 h, cooled, centrifuged, supernatant was precipitated in EtOH (1:5) to get crude polysaccharide (900 mg). The crude polysaccharide (25 mg) was purified by gel-permeation chromatography (GPC) on column (90 cm × 2.1 cm) of Sepharose 6B using distilled water as the eluent with a flow rate of 0.5 mL min^{-1}. A single homogeneous fraction was collected and freeze-dried, yielding 15 mg pure polysaccharide. The average molecular weight of ELPS was estimated as ~2×10^5 Da on the basis of standard calibration curve prepared using standard dextrans. GLC analysis of the alditol acetates of this polysaccharide revealed the presence of glucose only. The absolute configuration of glucose was determined as D according to Gerwig et al. The GLC-MS analysis of the alditol acetates of methylated product showed the presence (1→3), (1→6), and (1→3,6)-

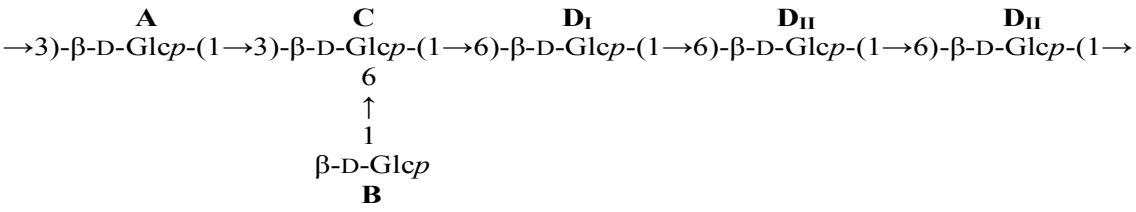
linked and terminal glucopyranosyl residues in a molar ratio of nearly 1:3:1:1 respectively.

In the anomeric region of the ^{1}H NMR spectrum (500 MHz) at 30 °C, three signals were observed at δ 4.78, 4.51, and 4.49. The peak at δ 4.78 corresponded to the anomeric proton of residue **A**. The signal at δ 4.51 corresponded to both the residues **B** and **C** and the signal at δ 4.49 corresponded to residue **D**. ^{13}C NMR spectrum (125 MHz) showed three signals in the anomeric region at δ 102.9, 102.7, and 102.5 at the same temperature. On the basis of HSQC spectrum, the anomeric proton signal at δ 4.51 was correlated to both the carbon signals δ 102.7 and δ 102.5, corresponded to anomeric carbons **B** and **C** respectively. Again, the anomeric proton signals at δ 4.78 and δ 4.49 were correlated to carbon signals at δ 102.7 and δ 102.9, corresponded to anomeric carbon of residues **A** and **D** respectively.

The different linkages that connected these residues (**A** to **D**) were determined from ROESY as well as NOESY experiment. In ROESY experiment, the inter-residual contacts **A**H-1/**C**H-3; **B**H-1/**C**H-6a, **C**H-6b; **C**H-1/**D$_{I}$**H-6a, **D$_{I}$**H-6b; **D$_{I}$**H-1/**D$_{II}$**H-6a, **D$_{II}$**H-6b; and **D$_{II}$**H-1/**A**H-3 along with other intra-residual contacts were also observed (Fig. 6a). The above ROESY connectivities established the following sequences: **A** (1→3) **C**; **B** (1→6) **C**; **C** (1→6) **D$_{I}$**; **D$_{I}$** (1→6) **D$_{II}$** and **D$_{II}$** (1→3) **A**.

Finally, the ROESY connectivities were confirmed from HMBC spectrum. In this spectrum the inter-residual cross-peaks between **A**H-1/**C**C-3, **A**C-1/**C**H-3; **B**H-1/**C**C-6, **B**C-1/**C**H-6a, **C**H-6b; **C**H-1/**D$_{I}$**C-6, **C**C-1/**D$_{I}$**H-6a, **D$_{I}$**H-6b; **D$_{I}$**H-1/**D$_{II}$**C-6, **D$_{I}$**C-1/**D$_{II}$**H-6a, **D$_{II}$**H-6b; **D$_{II}$**H-1/**A**C-3, **D$_{II}$**C-1/**A**H-3 along with some intra-residual peaks were also observed.

Thus, the HMBC and ROESY connectivities clearly supported the presence of the following repeating unit in the ELPS and the structure is proposed as:



Smith degradation was carried out with the ELPS where a disaccharide containing hydrated glyceraldehyde moiety is produced and the product was analyzed by ^{13}C NMR spectroscopy to confirm further the sequence of the sugar residues present in the repeating unit. The structure of hydrated glyceraldehyde containing disaccharide unit obtained from ELPS after Smith degradation was established as:

E **F** **G**
β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-Gro

Immunostimulating properties of ELPS

In vitro macrophage activation was observed with different concentrations of ELPS. Nitric oxide production was found to increase in dose-dependent manner with optimum production of 22 μM NO per 5×10^5 macrophages at 35 μg/mL of ELPS. Further increase in concentration of ELPS decreased the NO production implying that the effective dose of the ELPS was 35 μg/mL.

Splenocytes include T cells, B cells, dendritic cells, and macrophages that enhance the immunity in living systems. Thymocytes after maturation in thymus are designated as T cells. The splenocyte and thymocyte activation tests were conducted in mouse cell culture medium with the ELPS by the MTT assay method. Proliferation of splenocytes and thymocytes is an indication of immunostimulation. The ELPS was found to stimulate splenocytes and thymocytes. Maximum proliferation index of splenocytes and thymocytes by ELPS were found at 12.5 μg/mL and 100 μg/mL respectively. The decrease in the immunological activities of the polysaccharide after the optimum

concentration may be due to insufficient activation signal at the cellular surface. From these findings, it can be concluded that 35 µg/mL, 12.5 µg/mL and 100 µg/mL are the optimum concentration of ELPS for macrophage, splenocytes and thymocytes proliferation respectively.

Antioxidant properties of ELPS

Hydroxyl radical scavenging activity of ELPS was measured at different concentrations (100 to 800 µg/mL) taking butylated hydroxytoluene (BHT) as positive control. These results indicated that the activity of the ELPS gradually increases with the increase of concentrations. The hydroxyl radical scavenging activities of ELPS and BHT were respectively 20.48% and 91.2% at a dose of 200 µg/mL, indicating that antioxidant activity of ELPS is weak compared to BHT. The IC_{50} value of the ELPS was found to be 400 µg/mL.

The superoxide radical scavenging activities of ELPS and butylated hoydroxyanisole (BHA) were determined to be 56.58% and 89.88%, respectively at the dose of 100 µg/mL. At all concentrations, ELPS showed lower superoxide anion scavenging activity than synthetic standard drug BHA. The IC_{50} value of the ELPS was found to be 75 µg/mL.

The reducing power of ELPS and ascorbic acid increased with increasing sample concentration. The reducing power (absorbance at 700 nm) of ELPS and ascorbic acid were 0.2285 and 1.16 at 200 µg/mL, respectively. At concentration of 470 µg/mL, ELPS showed reducing power 0.5. This result suggests that ELPS has potency to donate electron to reactive free radicals, converting them into more stable non-reactive species and terminating the free radical chain reaction.

This work has been published in ***Carbohydrate Polymers,* 2015***,123*, 350-358.

Chapter-V: The hybrid mushroom *pfle 1p* was cultivated and collected from Falta experimental farm, Bose Institute, Kolkata. Aqueous extract of the fruit bodies of one of the hybrid mushroom strains, *pfle 1p* yielded two polysaccharides, PS-I and PS-II. The structural characterization and immunostimulating studies of PS-II have been discussed in

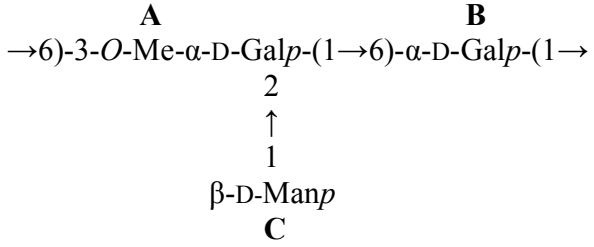
this chapter. The PS-II showed specific rotation $[\alpha]_D^{28.6}$ +54.7 (*c* 0.91, water). The apparent molecular weight of PS-II was estimated as ~1.65×10^5 Da from a calibration curve prepared with standard dextrans. The GLC analysis of alditol acetates of hydrolyzed product of PS-II confirmed the presence of mannose, galactose, and 3-*O*-methyl-galactose almost in a ratio of 1.0:0.99:1.1. The absolute configuration of all the sugar residues were determined as D. The GLC–MS analysis of partially methylated alditol acetates of PS-II revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol and 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-D-galactitol in a ratio of nearly 1:1:1. Thus, PS-II was assumed to consist of terminal D-mannopyranosyl, (1→6)-D-galactopyranosyl and (1→2,6)-D-galactopyranosyl moieties respectively. The GLC-MS analysis of the alditol acetates of periodate oxidized-reduced and methylated PS-II showed the presence of only 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-D-galactitol. These results clearly indicated that terminal D-mannopyranosyl and (1→6)-linked D-galactopyranosyl residues were consumed during oxidation while (1→2,6)-linked D-galactopyranosyl residue was unaffected by periodate since the C-3 position of the branched galactopyranosyl residue was already occupied by the -*O*Me group. Hence, the mode of linkages in the PS-II was confirmed.

The ^{1}H NMR spectrum showed three peaks in the anomeric region. The peaks were observed at δ 5.12, 4.98 and 4.78 in a ratio of nearly 1:1:1. Rest of the sugar protons were observed in the region of δ 3.37-4.18 and one -*O*CH_3 group signal at δ 3.43. The anomeric peaks were designated **A**, **B** and **C** according to their decreasing proton chemical shifts. In the ^{13}C NMR spectrum three peaks appeared in the anomeric region at δ 101.7, 98.2 and 97.9 in a ratio of nearly 1:1:1. The other carbon signals came in the region δ 79.1-61.1. In addition, there was a signal at δ 56.0, which was assigned for -*O*CH_3 signal. From HSQC spectrum anomeric proton signals at δ 5.12 (**A**), 4.98 (**B**) and 4.78 (**C**) were correlated to the carbon signals at δ 98.2, 97.9 and 101.7 respectively. The chemical shifts of -*O*CH_3 group (δ 3.43/ 56.0) were also assigned from HSQC spectrum. The different linkages that connected these three residues were determined from NOESY as well as ROESY spectrum. In NOESY spectrum, the inter-residual contacts were

observed between **A**H-1/**B**H-6a and **B**H-6b; **B**H-1/**A**H-6a and **A**H-6b; and **C**H-1/**A**H-2 along with other intra-residual contacts.

Finally, these links were confirmed from HMBC spectrum. In this spectrum the inter-residual cross-peaks were observed between **A**H-1/**B**C-6; **A**C-1/**B**H-6a and **B**H-6b; **B**H-1/**A**C-6; **B**C-1/**A**H-6a and **A**H-6b; **C**H-1/**A**C-2; and **C**C-1/**A**H-2 along with other intra-residual contacts.

Thus, the structural motif present in the polysaccharide,PS-II was established as:



Immunostimulating properties

Mushroom polysaccharides function as immunostimulator by activating the macrophages. On treatment with different concentrations of this PS-II, it was observed that 49% to 54% of NO production increased up to 50 µg/mL. This was further increased by 70% at 100 µg/mL, but decreased at 200 µg/mL. Hence, the effective dose of this PS-II was observed at 100 µg/mL with optimum production of 10.2 µM NO per 5×10^5 macrophages.

Splenocytes are the cells present in the spleen that include T cells, B cells, dendritic cells, and macrophages that stimulate the immune response in living organism. Thymocytes are hematopoietic cells in thymus which generate T cells. Proliferation of splenocyte and thymocyte is an indicator of immunostimulation. The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with the PS-II by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. The PS-II was tested to stimulate splenocytes and thymocytes respectively. Both the splenocyte and thymocyte proliferation indices were found maximum at 50 µg/mL, above

and below which it decreases. Hence, it can be concluded that 50 µg/mL is the optimum concentration of the PS-II for splenocyte and thymocyte proliferation.

This work has been published in ***Carbohydrate Research,* 2014**, 395, 1-8.

CHAPTER 1

Introduction to Carbohydrates and Bioactive Polysaccharides

1.A. Carbohydrates

Carbohydrates are the most abundant naturally occurring biomolecules belonging to class of organic compounds found in living organisms on earth. According to Robyt (1998) "Carbohydrates are polyhydroxy aldehydes or ketones or compounds that can be derived from them by oxidation, reduction or replacement of different functional groups". They can be synthesized in the laboratory or in living cells. In general carbohydrates have the empirical formula $C_x(H_2O)_{y,}$ that are composed of carbon, along with hydrogen and oxygen, usually with a hydrogen and oxygen atom ratio of 2:1 (as in water). Carbohydrates are of great importance in biology. The carbohydrates are the major source of metabolic energy, both for plants and for animals that depend on plants for food. They also serve as storehouse of chemical energy (glucose, starch, and glycogen). They play important roles in recognition between cells or recognition of cellular structures by other molecules. They also play an intimate role in the structure of nucleic acids (DNA and RNA), free nucleotides (as ATP and ADP), and coenzymes (as FAD and NAD). They contribute in a variety of vital functions that sustain life (Hiller, 1987). Certain carbohydrate derivatives are used as drugs, like cardiac glycosides or antibiotics. Finally, they provide flavor and texture in many processed foods.

❖ Classification of carbohydrates

Carbohydrates are classified into four groups: **monosaccharides, disaccharides, oligosaccharides, and polysaccharides**.

➢ Monosaccharides

The word "Monosaccharides" derived from the Greek word "*Mono*" means Single and "*saccharide*" means sugar. Monosaccharides are chiral polyhydroxy aldehyde or ketone which cannot be further hydrolysed to a smaller carbohydrate unit. The simplest and smallest unit of the carbohydrates is the monosaccharide, from which disaccharides, oligosaccharides, and polysaccharides are constructed. Monosaccharides contain carbon, hydrogen, and oxygen and have the general formula $C_x(H_2O)_x$, where *x* is a whole number

3 or higher. They are colorless, crystalline solids containing a single aldehyde or ketone functional groups that are freely soluble in water but insoluble in nonpolar solvents. Most have a sweet taste. The reactive centers of monosaccharides are the carbonyl and hydroxyl groups. A few examples of monosaccharides are glucose, mannose, galactose, fructose, ribose, and arabinose.

➢ Disaccharides

Disaccharides consist of two monosaccharide units bound together by a glycosidic linkage. Disaccharides are classified as homo- and heterodisaccharide according to the type of its monosaccharide units. Three most abundant disaccharides are sucrose, lactose, and maltose. Sucrose and lactose are heterosaccharides and maltose is homosaccharide. Sucrose is the sweetest of the disaccharides. It is composed of glucose and fructose. Sucrose is a non-reducing sugar as it contains no free aldehyde or keto group. Lactose, the disaccharide of milk, consists of galactose and glucose. It has a free aldehyde group. So it is a reducing sugar. In maltose, α (1→4)- glycosidic linkage joins two glucose units. It is also a reducing sugar due to the presence of a free aldehyde group.

➢ Oligosaccharides

Oligosaccharides are a class of carbohydrates that are composed of three to ten monosaccharide units joined by characteristics linkages called glycosidic bonds. According to the number of units, they are called trisaccharides, tetrasaccharides, pentasaccharides etc. Oligosaccharides can be hydrolyzed by acids or other hydrolytic enzymes to their monomeric units. Maltotriose, raffinose, and stachyose are the examples of oligosaccharides.

➢ Polysaccharides

Carbohydrates containing more than ten monosaccharide units joined together by glycosidic linkages are classified as polysaccharides.

1.B. Polysaccharides – the first biopolymer

Most carbohydrates found in nature occur as polysaccharides. Polysaccharides are relatively complex carbohydrates and considered as the first biopolymer formed on Earth (Tolstoguzov, 2004). They are composed of large numbers of monosaccharide units joined together by glycosidic bonds. They are, therefore, very large, often branched, macromolecules and high molecular weight. The structure of the polysaccharide is complicated due to its large size. Polysaccharides are capable of assuming only a limited number of conformations due to severe steric restrictions on the freedom of rotation of sugar units about glycosidic linkages. They yield monosaccharides on hydrolysis or monomeric units of sugars. They are amorphous, insoluble in water, and have no sweet taste (Varki et al., 2008). The polysaccharides serve two principal functions: 1) an energy storage 2) building materials for the cell or whole organism. Hence, polysaccharides are extremely important in organisms for the purpose of energy storage as well as structural integrity. They play important roles within the biology of life processes (Sharon & Lis, 1993). The most well known polysaccharides are starch, glycogen, cellulose, and chitin.

❖ Classification of polysaccharides

- **Based on the type of the monomers, polysaccharides are divided into two categories:**

(i) Homopolysaccharides: When all the monosaccharides in a polysaccharide are of the same type, the polysaccharide is called a homopolysaccharide. Starch, glycogen, and cellulose, which are made up of only glucose residues are the example of homopolysaccharides.

(ii) Heteropolysaccharides: Polysaccharides that are constituted of different type of monosaccharide units are known as heteropolysaccharides e.g. arabinoxylan, glucomannan, xyloglucan, galactomannan etc.

- **According to their charge, polysaccharides are mainly classified into three categories:**

(i) Cationic polysaccharides:

Natural: chitosan

Semi-Natural: cationic guar gum, cationic hydroxyethyl cellulose (HEC)

(ii) Anionic polysaccharides:

Natural: alginic acid, pectin, xanthan gum, hyaluronic acid, gum arabic, gum karaya, gum tragacanth

Semi-Natural: carboxymethyl-chitin, cellulose gum

(iii) Nonionic polysaccharides:

Natural: starch, dextrins, guar gum

Semi-Natural: cellulose ethers (hydroxyethylcellulose, methylcellulose, nitrocellulose)

- **Based on their function, polysaccharides are of two types:**

(i) Storage polysaccharides: The polysaccharides which serve as energy storage in plants and animals are called storage polysaccharides. E.g. starch, glycogen, and inulin etc

(ii) Structural polysaccharides: The polysaccharides which have structural roles in plant cell wall or exoskeleton of insects are called structural polysaccharides. E.g. cellulose, chitin etc.

- **Based on their structure polysaccharides are of two types:**

(i) Linear - cellulose, alginates, amylose etc

(ii) Branched – amylopectin, gum arabic, arabinoxylan etc.

❖ Structure and function of some common polysaccharides

➢ Starch

Starch is a storage polysaccharide composed entirely of glucose monomers. It is a polymer of α-D-Glc*p* units. They are water insoluble molecules. Most starches contain 20-25% amylose and 75-80% amylopectin (William, MacArdle, & Victor, 2006). Amylose is essentially a linear polymer consisting of (1→4)-linked α-D-glucopyranosyl units (**Figure 1**), whereas amylopectin is a branched polymer composed of (1→4)-α-D-Glc*p* and (1→4,6)-α-D-Glc*p* residues. Complete hydrolysis of both amylose and amylopectin yields only D-glucose. Starch is used for energy storage in plants. It is found in all plant seeds and tubers and is the form in which glucose is stored for later use. Human and other animals have amylase enzyme, so they can digest the potato, rice, wheat, and maize (corn) starch.

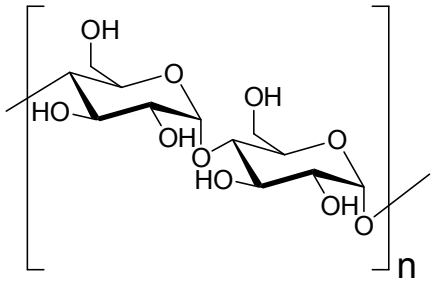


Figure 1. α-amylose, the linear component of starch.

➢ Glycogen

Animals store glucose in the form of polysaccharide called glycogen. It is present in all cells, but it is most prevalent in the liver and the muscles, and is called as 'animal starch'. Glycogen is a highly branched chain homopolysaccharide, like amylopectin in structure. It is a polymer of glucose molecules linked together with α-(1→4) glycosidic linkages, with α-(1→6)-linked branches points. Glycogen is made up of D-glucose residues. Upon hydrolysis, it yields D-glucose as the product. Glycogen plays an important role in the

glucose cycle. Animals use it for the short-term storage of food energy (Ingermann & Virgin, 1987; Miwa & Suzuki, 2002).

➢ Cellulose

Cellulose is the most abundant, renewable organic compound in nature and it is the chief constituent of the fibrous parts of plants. It is a major component of the cell walls of higher plants (Crawford, 1981; Updegraff, 1969), and is also produced by some bacteria, algae, fungi, amoebae, and sea-animals. Cellulose is a linear homopolymer made up of β-(1→4)-D-linked glucose residues (**Figure 2**). It is an unbranched polymer with about ten thousand glucose units per chain. The celluloses obtained from different sources differ in molecular size though they are all made up of glucose. It is a water insoluble polymer with a rigid linear structure. Upon hydrolysis, cellulose yields D-glucose as the product.

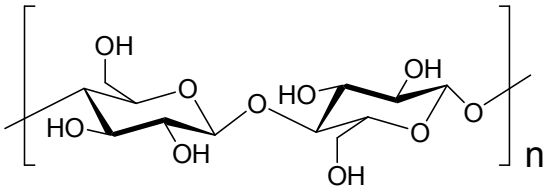


Figure 2. Cellulose, β-(1→4) linked glucose polymer.

➢ Chitosan

Chitosan is a versatile natural polymer. It is modified natural linear nitrogenous polysaccharides, a basic polysaccharide homo-polymer. Chitin is a water-insoluble, one of the most abundant naturally occurring polysaccharide materials in the world. Chitin is a linear homopolysaccharide composed of β-(1→4)-linked N-acetyl-D-glucosamine residues (Rianaudo, 2006). Removal of N-acetyl groups from chitin yields chitosan (**Figure 3**) which improves its solubility. Chitosan is soluble in most acids due to the free protonable amino groups present in the D-glucosamine units. It is derived from naturally occurring sources, which is the exoskeleton of insects, crustaceans and fungi. Chitosan is considered one of the most valuable polymers for biomedical and pharmaceutical

applications due to its biodegradability, biocompatibility, antimicrobial, non-toxicity, antioxidant, and anti-tumor properties (Aranaz et al., 2009). In pharmaceutical industry, chitosan has been employed in drug delivery systems due to its high biocompatibility.

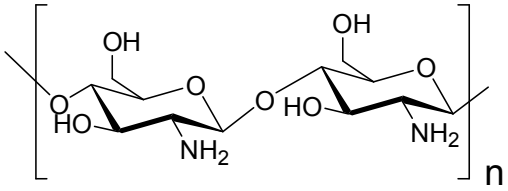


Figure 3. Chitosan, β-(1→4)-linked D-glucosamine.

➢ Pectin

Pectin is one of the main structural components of plant cell walls. It is an essentially linear polysaccharide and composed of a backbone of α-(1→4)-linked galacturonic acid or its ester units (**Figure 4**). Pectins are soluble in water. Pectins are divided into two major groups on the basis of their degree of esterification. Most plants, apples, citrus fruits (oranges, lemons, and grapefruits) and sugar beets are very rich in pectins and their by-products are the most important sources of pectin industry (Thakur, Singh, & Handa, 1997). Pectins are naturally occurring biopolymers that possess increasing applications in the pharmaceuticals, foods, and biotechnology industry. The most important use of pectin is its ability to form gels. These are widely used as food additives with gelling and stabilizing properties in jams, jellies, milks and confectionery products. It can reduce cholesterol levels in blood and exhibit anti-inflammatory activities (Attele, Wu, & Yuan, 1999).

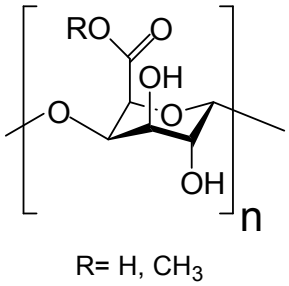


Figure 4. Pectins, α-(1→4)-linked galacturonic acid or its ester in the backbone.

➢ Heparin

Heparin is one of the oldest biological medicines in the field of thrombosis and haemostasis. Heparin is a highly sulfated polysaccharide consisting of (1→4)-linked hexosamine and uronic acid residues (**Figure 5**). This acidic polysaccharide is biosynthesized and stored in various animal tissues, particularly the liver, lungs, and gut. It is strongly acidic because of its content of covalently linked sulfate and carboxylic acid groups. It is the most widely used antithrombotic in clinical medicine due to its easy administration and availability, cost effective and known side-effect profile (Laremore et al., 2009).

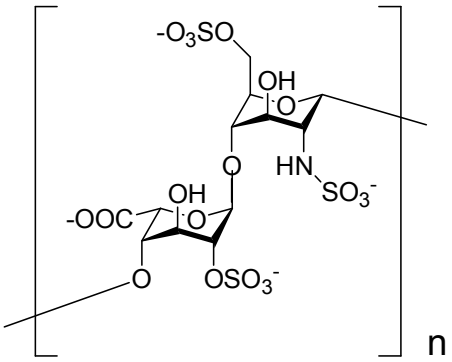


Figure 5. Heparin: sulfated (1→4)-linked hexosamine and uronic acid repeating unit.

➢ Hyaluronic acid

Hyaluronic acid (HA) is a linear, high molar mass, naturally occurring biopolymer found in every tissue of the body (**Figure 6**). It is composed of alternating units of glucuronic acid and N-acetyl glucosamine residues. It is found in most connective tissues including skin, umbilical cord, synovial fluid, and vitreous humor. Significant amounts of HA are also found in lung, kidney, brain, and muscle tissues. It is soluble in water and form viscous solution. HA has a variety of physicochemical properties. Clinically, HA has been used in several applications, including ophthalmology as a drug delivery system, in osteoarthritis for viscosupplementation, and as dermal filler. It is also used as a diagnostic marker for many diseases including cancer, rheumatoid arthritis, and liver dysfunction (Necas, Bartosikova, Brauner, & Kolar, 2008). Biocompatibility, non-

immunogenicity, biodegradability and viscoelasticity have proved that it is an ideal biomaterial for cosmetic, medical and pharmaceutical applications (Doughty & Glavin, 2009).

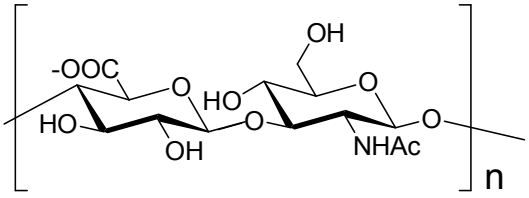


Figure 6. Hyaluronic acid: glucuronic acid and N-acetyl glucosamine repeating unit.

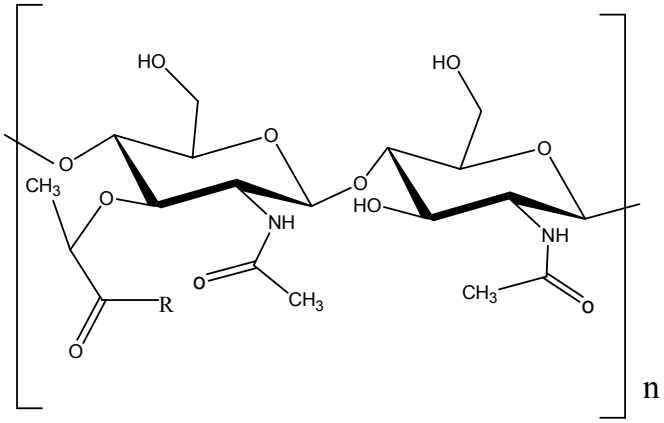
➢ Inulin

Inulin is the naturally occurring nondigestible carbohydrate found in many plants as a storage carbohydrate. It is present in many regularly consumed vegetables, fruits and cereals, including leek, onion, garlic, wheat, chicory, artichoke, and banana. Inulin is members of a larger group called fructans. It is a fructan consisting almost entirely of linearly (1→2)-β-fructose units with terminal glucosyl moieties. Inulins are increasingly used in food applications. In food formulations, inulin significantly improves the organoleptic characteristics, allowing an upgrading of both taste and mouthfeel in a wide range of applications. Inulin finds extensive applications as a low calorie fat replacer in dairy products, bakery foods, and ice creams.

➢ Murein

Murein (peptidoglycan) is a unique and essential cross linked, bag like macromolecule of the cell wall of most of the bacterial species like *Escherichia coli* etc. There is a high diversity in the composition and sequence of the peptides of the peptidoglycan of different species. The main structural features of Murein are linear glycan strands cross linked by short peptides. The glycan strands are made up of alternating *N*-acetyl glucosamine (GlcNAc) and *N*-acetyl-D-muraminic acid (MurNAc) residues linked by β-

(1→4) bonds (**Figure 7**). Its main function is to preserve cell integrity and maintaining cell shape (Vollmer & Bertsche, 2008).



R= Different peptide side chains

Figure 7. Murein (peptidoglycan), consisting of β-(1→4)-linked *N*-acetyl glucosamine (GlcNAc) and *N*-acetyl-D-muraminic acid (MurNAc) with different peptide side chains

➢ Galactomannan

Galactomannan is a polysaccharide that is present in the fungal cell wall component. It is built up of a β-(1→4)-mannopyranose backbone with α-(1→6) linked galactopyranose units attached as branched position (Mathur & Mathur, 2005). They are water soluble hydrocolloids which form highly viscous, stable aqueous solutions. Most galactomannans are used in different pharmaceutical, biomedical, cosmetics and food industries. Their mannose:galactose ratios (M:G) differ according to the species. Some major galactomannans of commercial importance in food and non-food industries are Guar gum (M/G ratio: 2:1), Tara gum (M/G ratio: 3:1), Locust bean gum or Carob gum (M/G ratio: 4:1) and Fenugreek gum (1:1) (Chatterjee, Sarkar, & Rao, 1982).

1.C. Mushrooms

1.C.1. Definition and description

Mushrooms are the fruiting bodies of higher fungi. The term 'mushroom' is defined by Chang and Miles (1992) as "a macro fungus with a distinctive fruiting body which can be hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand" (**Figure 8**). They typically produced above ground on soil, stumps, rotting wood or other organic substrates. Edible mushrooms were called the "food of the gods". Due to the absence of chlorophyll, it is unable to synthesize its own food by photosynthesis and hence, they need foods from the outer sources. The fruiting bodies of mushrooms possess different shapes, size and colour. The most common type of mushroom is umbrella shaped with pileus (cap) and stalk (stem), and some species additionally have an annulus (ring), volva (cup), gills, tubes, pores etc. They have a cap and a stalk and are frequently seen in fields and forests. Mushrooms can be roughly divided as edible mushrooms, medicinal mushrooms, and poisonous mushrooms.

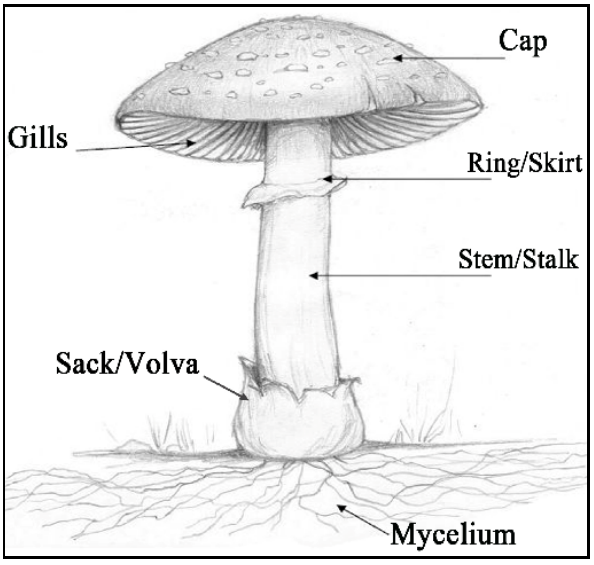


Figure 8. Different parts of Mushroom [Mushroom structure: Teacher's notes, www.fungi4school.org]

1.C.2. Chemical composition and nutritional value

The nutritional value of the mushroom originates from its chemical composition. The mushrooms are the good sources of dietary protein, carbohydrate, fats, vitamins, fibre and minerals (Wani, Bodha, & Wani, 2010). Mushrooms possess ~ 90% water by weight. The remaining 10% consists of 10-40% protein, 2-8% fat, 3-28% carbohydrate, 3-32% fiber and 8-10% ash (Breene, 1990). Mushroom proteins contain all the essential amino acids, especially rich in lysine and leucine (Mattaila et al., 2001) and considered as a good source of digestible proteins with protein content above most vegetables and somewhat less than most meats and milk (Fitzpatrick, Esselen, & Weir, 1946; Lintzel, 1941). These are the good source of several vitamins (A, B_1, B_2, B_3, B_{12}, C and D), and mineral elements (potassium, calcium, phosphorous, magnesium, iron, zinc and copper). They contain a wide range of bioactive molecules including steroids, terpenoids, phenols, neucleotides, glycoproteins, and polysaccharides besides actual nutrients and useful for maintaining health (Chang & Buswell, 1996; Zeisel, 1999). Mushrooms are used extensively as a food in many countries due to their good flavour, taste, and high nutritive values (Cheung, 2008; Ghorai et al., 2009; Sands, 2013; Wang et al., 2014).

1.C.3. Medicinal properties

Wild edible mushrooms provide a significant source of nutritional as well as medicinal compounds and used for the development of drugs (Brochers et al., 1999; Mizuno, 1996; Mizuno et al., 1999). Anticancer drugs (lentinan, PSK and sonifilan) were isolated from mushrooms such as *Lentinus edodes* (Taguchi et al., 1983)*, Coriolus (Trametes) versicolor* (Cui & Chisti, 2003), and *Schizophyllum commune* (Fujimoto et al., 1983) and clinically used throughout the world (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007). Nowadays mushroom have drawn the attention of chemists and immunobiologists due to their immunomodulatory, antimicrobial, antidiabetic, antitumor, anticancer, and antioxidant activities (Das, 2010; Mizuno, 1999; Tzianabos, 2000; Wani, Bodha, & Wani, 2010; Wasser & Weis, 1999a; Wasser & Weis, 1999b; Wasser, 2002). Different parts of

the mushroom are being used for the treatment of blood sugar, high blood pressure, as a preventive of ageing as well as for the beauty treatment (Wasser & Weis, 1999a).

1.C.4. Edible mushroom *Entoloma lividoalbum*

Edible and non-toxic mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička (**Figure 9**) is a basidiomycetes fungus, normally grows on Himalaya region during July to August (Das, 2010). It is edible and non-toxic. It contains minerals and nutrients such as aluminium, calcium, and iron (Stijve, Andrey, Lucchini, & Goessler, 2001). It was collected from Sikkim Himalaya region, India by the research group of Dr. Krishnendu Acharya, Professor, Department of Botany, University of Calcutta, and West Bengal. The voucher specimen was deposited with the accession code AMFH-608 in the Mycological Herbarium of department of Botany, University of Calcutta, Kolkata, West Bengal, India and identified by Dr. Acharya who is also a mycologist.

Figure 9. Photograph of the fruit bodies of the edible mushroom *Entoloma lividoalbum.*

1.C.5. Hybrid mushrooms

Both cultivated and wild edible mushrooms have been recognized as functional foods due to their excellent sensory characteristics, including their unique aroma and taste. But quality traits within the gene pool of any particular edible mushroom species are limited. A number of new hybrid strains have therefore been generated, with the aim of achieving pathogen resistance, high bio-efficacy, rapid maturation, high temperature tolerance, enhanced shelf life, higher yield, and better quality. Genetically improved strains not only increase the quality of cultivated mushrooms, but also reduce the costs of cultivation. Production of new hybrid mushroom strains through para-sexual mating is now well established. Protoplast fusion is an important approach for inter-specific and inter-generic somatic hybridization for strain improvement among edible mushrooms (Peberdy & Fox, 1999). There are number of reports on production of hybrid mushroom through inter-generic protoplast fusion. Several hybrid fruit bodies e.g. PC H8, PC H11, PC H17 and PC H18 were prepared through protoplast fusion between two edible strains *Pleurotus florida* and *Calocybe indica* (Chakraborty & Sikdar, 2010). Protoplast fusion of *Pleurotus florida* and *Volvariella volvacea* produced two new fruit bodies PfloVv1aFB and PfloVv5FB (Chakraborty & Sikdar, 2008). Intergeneric protoplast fusion between the strains of *Pleurotus florida* and *Lentinula edodes* produced nine new hybrid strains named as *pfle* were found to produce fruit bodies (Mallik & Sikdar, 2014). Twelve inter-generic somatic hybrids named as *pfls* were produced through PEG-mediated protoplast fusion between *Pleurotus florida* and *Lentinus squarrosulus* using double selection method (Mallik & Sikdar, 2015).

1.D. Mushroom polysaccharides

Mushrooms are potential source of different polysaccharides like chitin, hemicelluloses, glucans and heteroglycans. Bioactive polysaccharides from mushrooms consist of a β- or α- or both α- and β-linked glucan backbone displaying different patterns and degrees of branching (Bhanja et al., 2012; Bhanja et al., 2013; Chakraborty, Mondal,

Rout & Islam, 2006; Kiho, Shiose, Nagai & Ukai, 1992; Maity et al., 2013a; Ohno et al., 1993; Rout et al., 2004; Rout et al., 2005; Samanta et al., 2013; Wang, Deng, Li, & Tan, 2007; Yoshioka et al., 1985). However, other heteropolysaccharides have been also found in mushrooms, as well as protein-glucan complexes (Bhunia et al., 2010; Dey et al., 2013; Maity et al., 2013b; Mandal et al., 2011; Nandi et al., 2013; Patra et al. 2012a; Patra et al. 2012b). Several polysaccharides from mushrooms have been isolated and characterized, such as lentinan from *Lentinus edodes*, pleuran from *Pleurotus* mushrooms, schizophyllan from *Schizophyllum commune*, and PSK from *Coriolus (Trametes) versicolor*. All these polysaccharides exhibit anticancer activity and are used in cosmetics products (Wu et al., 2016).

1.D.1. Structures and biological activities

Natural polysaccharides are renewable. The pharmaceutical applications for natural polysaccharides are due to non-toxic, biodegradable, biocompatible, economical, and easy availability. A wide range of biologically active polysaccharides with different chemical structures have been investigated. Mushroom polysaccharides possess antitumor, immunomodulating, antioxidative, anti-inflammatory, antiviral, and antidiabetic effects (Hobbs, 2000; Smiderle et al., 2008; Zhang, Cui & Cheung, 2007). Among the mushroom polysaccharides, β-D-glucan are most important polysaccharides with immunomodulating and antitumor activity (Bohn & BeMillar, 1995; Fujimoto et al., 1983; Mansell et al., 1975; Morikawa, Takeda, Yamazaki, & Mizuno, 1985). The basic β-D-glucan is a repeating structure with the D-glucose units joined together in linear chains by β-glycosidic linkages. The physicochemical properties of β-glucans depend on their characteristics primary structure, including linkage type, degree of branching, molecular weight etc. The structures of the several biologically active linear and branched β-D-glucan have been reported. β-D-glucans, a well-known biological response modifiers (BRM), containing (1→3)-, (1→6)- linkages are the most active forms (Miura et al., 1996; Ruthes, Smiderle, & Iacomini, 2015). Glucans having α- or both α- and β- linkages are also isolated from different mushrooms (Mizuno et al., 1990; Rout et al., 2004; Wang, Deng, Li, & Tan, 2007; Zhang & Cheung, 2002). Some (1→3)-α-D-glucan derivative

have shown significant medicinal properties (Ghoneum et al., 1995; Kiho et al., 1994). Linear low molecular weight (1→4)-α-D-glucan is used as an immunomudulator and anti-cancer agent (Mizuno, Saito, Nishitoba, & Kawagashi, 1995; Matsushita et al., 1998). Besides glucans, heteroglycans (Gao, Seljelid, Chen, & Jiang, 1996; Mandal et al., 2011), β-glucan-protein complexes (Kawagishi et al., 1990), α-manno-β-glucan (Mizuno, Saito, Nishitoba, & Kawagashi, 1995), α-glucan-protein complexes (Mizuno, Saito, Nishitoba, & Kawagashi, 1995), and heteroglycan-protein complexes (Zhuang et al., 1993) have showed immunomodulating and antitumor activities.

The molecular structure, molecular weight, size, branching pattern, conformation, solubility, and intra- and intermolecular association of the polysaccharide chains are important for their biological responses (Fan, Li, Deng, & Ai, 2012; Tsiapali et al., 2001; Wasser, 2002). Polysaccharides that exhibit strong antitumor action differ greatly in their chemical structures. Structural features such as β-(1→3) linkages in the main chain of the glucan and additional β-(1→6) branch points are essential for antitumor action. β-glucans containing mainly β-(1→6)-linkages have less activity. Greater the molecular weight of the polysaccharides, higher the immunological and antitumor activity (Mizuno et al., 1999). The antitumor activity of mushroom polysaccharides depends on their conformational complexity which includes single helix, triple helix, and random coil structures. A triple-helical conformation of (1→3)-β-D-glucan is known to be important for their immune-stimulating activity. Lentinan, schizophyllan and PSK, all consist of triple helix structure. Polysaccharides that form triple-helical conformation have clinical applications for the treatment of cancers like human breast cancer (MCF-7), human promyelocytic leukemia (HL-60), and human liver cancer (HpG2) (Zhang, Cui & Cheung, 2007). In mushrooms, β-glucans are present either in their water soluble or insoluble form. Solubility in water of β-glucans plays significant role for biological activity of mushroom polysaccharides (Wasser, 2002). Chemical modifications sometime improve the biological activity of the mushroom polysaccharides (Mizuno et al., 1999). Smith degradation, formolysis, and carboxymethylation procedures have been designed for modification. A water insoluble, alkali soluble linear (1→3)-α-D-glucan derived from

Amanita muscaria and *Agrocybe aegerita* showed little antitumor activity, while carboxymethylated derived polysaccharides showed strong antitumor activity (Yoshida et al., 1996).

1.D.2. Some important antitumor and immunomodulating mushroom polysaccharides

Studies of mushroom polysaccharides are now great interest of research because of their immunomodulating and antitumor properties (Wasser & Weis, 1999b; Wasser, 2002). For medical purposes, mushrooms have been consumed to prevent cancer and cardiac diseases, to improve blood circulation and to reduce blood cholesterol level (Ooi & Liu, 2000; Wasser, 2002). Several mushroom polysaccharides such as Lentinan (from *Lentinus edodes*, Japan), Schizophyllan (from *Schizophyllum commune),* Krestin (from turkey tail mushroom *Trametes versicolor*), Agarican (from *Agaricus blazei*, USA), and Grifron-D (from *Grifola frondosa*, Japan) have been established as pharmaceutical agents and used as anticancer drugs (Cui & Chisti, 2003; Fujimoto et al., 1983; Mizuno, 2002; Taguchi et al., 1983; Zhuang et al., 1994).

➢ Lentinan

Lentinula edodes (shiitake mushroom) is one of the most widely cultivated edible mushrooms and is highly valued for its medical applications. Shiitake are traditionally well known edible mushrooms of high nutritional value. Lentinan isolated from medicinal mushroom Shiitake is recognized as being an effective biological response modifier (Chihara et al., 1969). The backbone structure of lentinan has been reported as a (1→3)-β-D-glucan backbone, branched with (1→6) β-D-glucan (**Figure 10**). Lentinan is used clinically as an antitumor, immunomodulating and antiallergy agent (Chihara et al., 1970; Chihara, 1978; Hobbs, 2000). Lentinan was shown to induce apoptosis in gastric cancer cells and could be used for the treatment of gastric cancer (Furue & Kitoh, 1981; Taguchi et al., 1985).

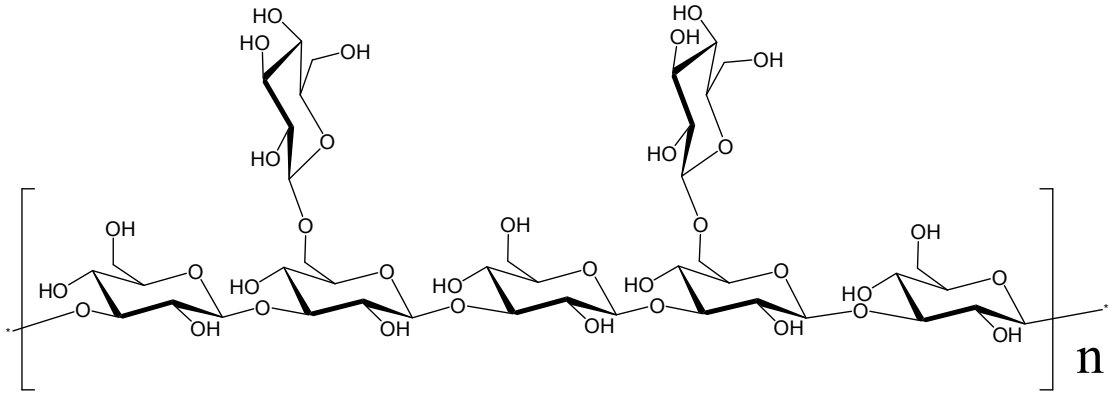


Figure 10. Lentinan β-glucan structure

➢ Schizophyllan

Schizophyllan is a jelly like slimy material which is non-ionic, soluble in water and a neutral extracellular polysaccharide. It was isolated from the mushroom *schizophyllum commune* (Zhang, et al., 2013). The repeating unit consists of three (1→3)-β-D-glucan residues and one (1→6)-β-D-glucan side group (**Figure 11**). Schizophyllan has great importance in the pharmaceutical and food industries (Reyes, Brabl, & Rau, 2009). In addition, schizophyllan has potential biomedical applications, such as anticancer therapies and as a bioactive cosmetics ingredient (Furue, 1985; Hobbs, 1995). Moreover, Schizophyllan (SPG) is found to be effective in the therapy of uterine cervix cancers (Okamura et al., 1986; Okamura et al., 1989).

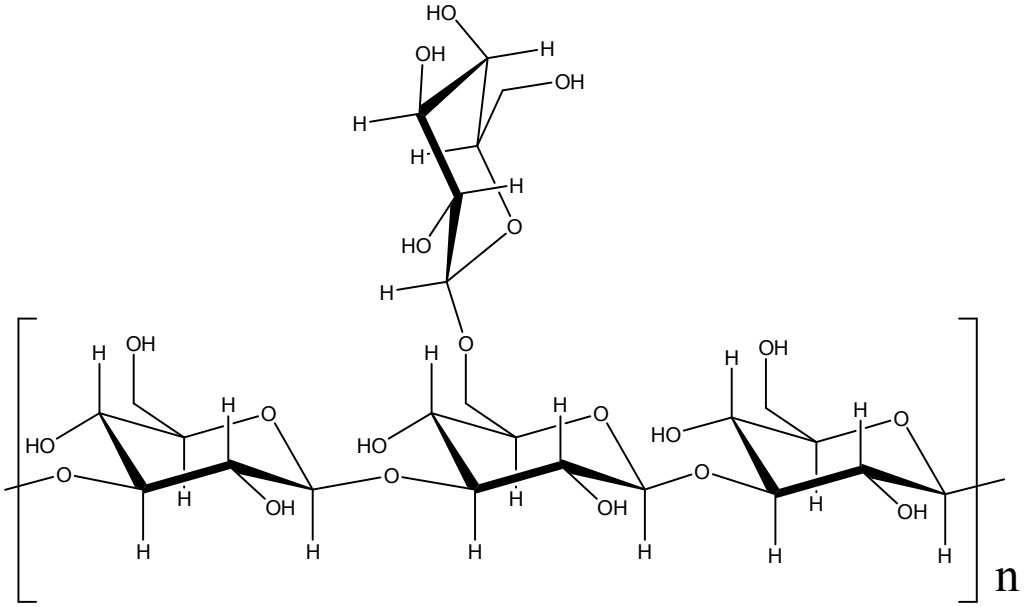


Figure 11. Repeating unit of Schizophyllan

➢ Krestin

Krestin (PSK) is a unique protein bound polysaccharide, obtained from turkey tail mushroom *Trametes versicolor.* It normally grows in wooded temperate zones year round on tree trunks, stumps, dead logs, and branches. The medicinal mushroom *Trametes versicolor* has a wide range of biological activities including antiviral, antitumor, and immunomodulatory effects (Cui & Chisti, 2003; Tsukagoshi et al., 1984). PSK contains 34-35% soluble carbohydrate (91-93% β-glucan), 28-35% protein, 7% moisture, 6-7% ash, and the remainders are free sugars and amino acids (Cui & Chisti, 2003). PSK has an accepted anti-tumor activity in various types of cancers, including colorectal, gastric, breast, liver, pancreatic, and lung cancer (Iguchi et al., 2001; Rai, Tidke, & Wasser, 2005; Tsang et al., 2003; Ying et al., 1987). It is also useful for hepatitis B (Mizoguchi et al. 1987; mizuno, 1995) and chronic active hepatites (Ying et al. 1987). These studies have suggested the efficacy of PSK as an immunotherapy or biological response modifier (BRM).

➢ Agarican

Agarican, the polysaccharide was isolated from *Agaricus blazei* mushroom. *A. blazei* is traditionally used as a health food source due to its medicinal properties. This mushroom has been commercialized and used clinically as antitumor agents (Ebina & Fujimiya, 1998; Kawagishi et al., 1989, 1990). It is also used to prevent various diseases including chronic hepatitis, allergies, and asthma (Biedron, Tangen, Maresz, & Hetland, 2012; Grinde, Hetland, & Johnson, 2006; Hsu, Hwang, Chiang, & Chou, 2008). The main bioactive substances of this mushroom are polysaccharides obtained from the fruiting bodies (Mizuno et al. 1990; Fujimiya et al. 1998), and its immunomodulatory activity is recognized mainly to β-glucans (Mizuno et al. 1990). Seven polysaccharide fractions obtained from *A. blazei* fruit bodies were demonstrated to have antitumor activity (Fujimiya et al., 1998; Mizuno, 2002).

➢ Grifron-D

Grifola frondosa (also known as Maitake) is a traditional medicinal mushroom used in enhancing the immune system. Several studies have shown that polysaccharides and polysaccharide-protein complexes from this mushroom have significant anticancer activity (Nishida, Nanba, & Kuroda, 1988). Maitake also protects cells with its antioxidant properties. Grifron-D is derived from Maitake mushroom (*Grifola frondosa*) and commonly named GD. It is a (1→3)-, (1→6)-β-D-glucan that demonstrates strong antitumor activity (Fulleroton & Samadi, 2000; Zhuang et al., 1994).

1.D.3. Mechanism of biological action

Mushroom polysaccharides have been proved to have a wide range of antitumor activity and perform their antitumor action mainly via activation of the immune response of the host organism. These substances are considered as biological response modifiers (Wasser & Weis, 1999a). BRM has been defined as those agents that modify the host's biological response by stimulation of the immune system, which may result in various therapeutic effects. Polysaccharides derived from mushrooms do not attack cancer cells

directly but they generate their antitumor effects via the activating the immune systems through stimulation of Natural Killer cells (NK-cell), T-cell, B-cell, and macrophage-dependent immune cells in the host. Several β-D-glucans (Wasser & Weis, 1999b) and α-D-glucans (Whistler et al., 1976) are widely used as antitumor and immunomodulating agents. β-D-glucans are recognized by human immune systems as foreign molecules since they are not synthesized by humans. These compounds can cause both innate and adaptive immune response (Bohn & BeMiller, 1995). A possible pathway of the biological action of β-D-Glucan has been presented in **Figure 12** (Mizuno, 2002).

Lentinan appears to act as a host defense potentiator (HDP) which is able to restore the responsiveness of host cells to lymphocytokines, hormones, and other biologically active substances by stimulating maturation, differentiation or proliferation of cells involved in host defense mechanism (Chihara et al., 1987; Chihara, 1992). HDP is functionally different from BRM. Lentinan is thus able to increase host resistance against various kinds of infectious diseases including AIDS. Lentinan is known to be able to restore the suppressed activity of helper T cells in the tumor-bearing host to their normal state, leading to complete restoration of humoral immune responses (Ooi & Liu, 1999). Lentinan has also been shown to inhibit suppressor T cells activity *in vivo* and to increase the ratio of activated T cells and cytotoxic T cells in the spleen when administered to gastric cancer patients undergoing chemotherapy. The biological action of Lentinan has been demonstrated by Chihara et al. (1970) and presented in **Figure 13**.

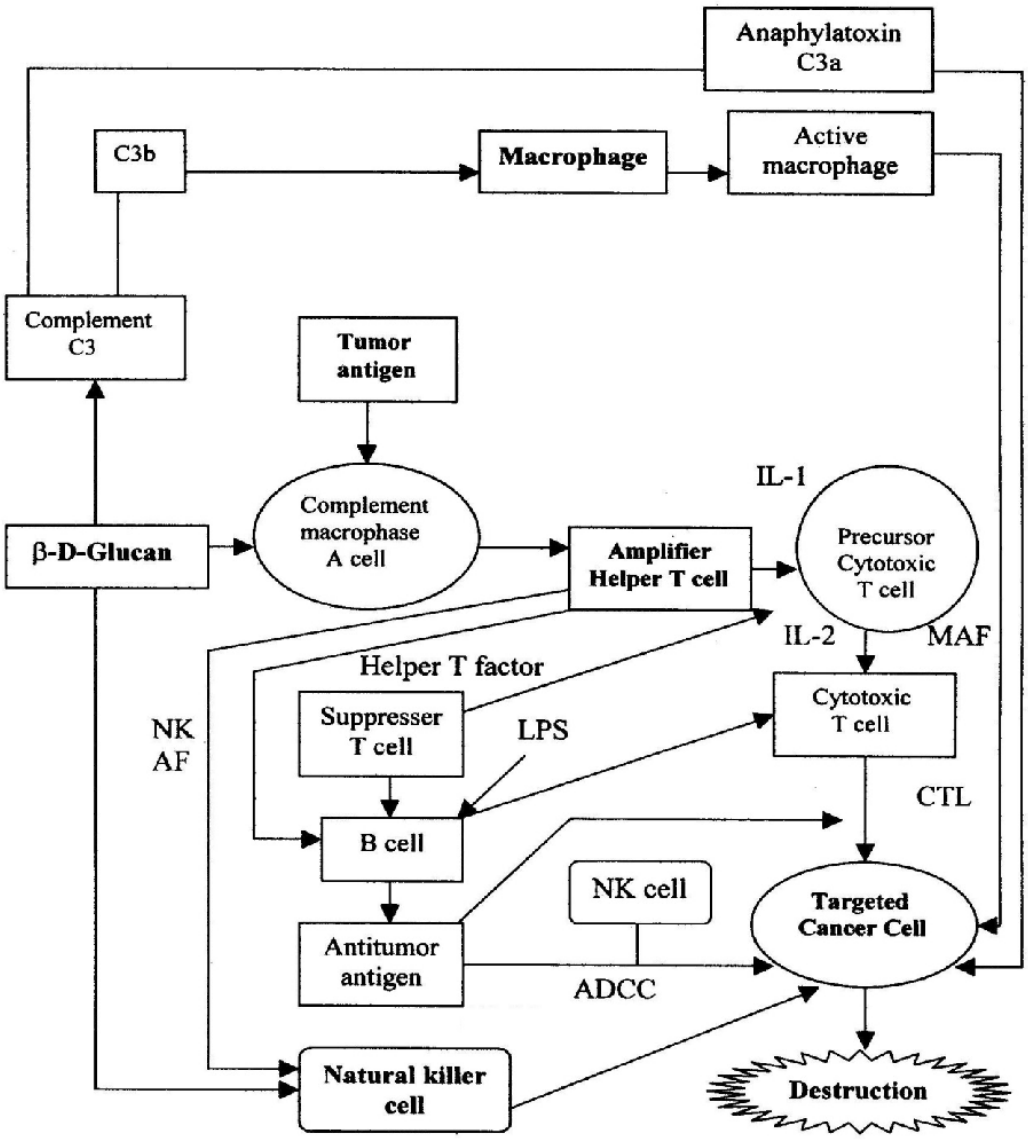


Figure 12. Possible immune mechanism: β-D-glucan as biological response modifier (BRM) to target cancer cells (Mizuno, 2002).

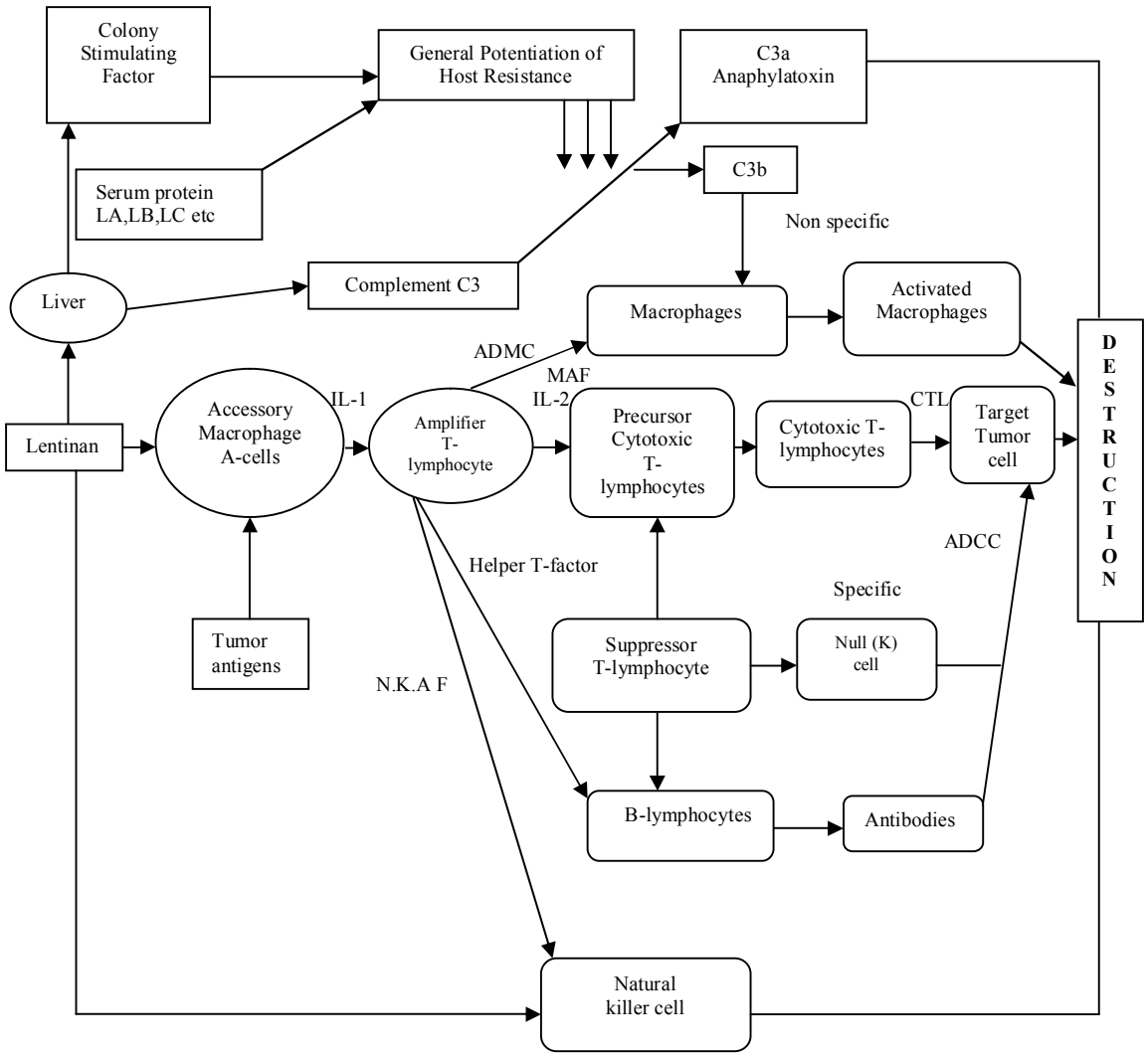


Figure 13. Possible pathways of lentinan action (Chihara et al., 1970; Chihara, 1992). NK: Natural Killer cell; AF: Antibody Formation; LPS: Liver Protein Serum; ADCC: Antibody Dependent Cell mediated Cytotoxicity; CTL: Cytotoxic T-Lymphocyte; MAF: Macrophage Activating Factor; IL-1: Interlukine 1; IL-2: Interlukine 2

1.D.4. Antioxidant properties

In all living organisms, oxidation is essential for the production of energy which maintains the biological processes such as utilization of nutrients, electron transport to produce ATP, and removal of xenobiotics (Blokhina, Virolainen, & Fagerstedt, 2003; Hemnani & Parihar, 1998). Oxidation process is one of the most important routes for producing free radicals in food, drugs and even living systems. Reactive oxygen species (ROS) are produced continuously as normal by-products of oxygen metabolism during mitochondrial oxidative phosphorylation (Oyaizu, 1986). Several biochemical reactions in our body also generate ROS. However, excessive amount of ROS can induce oxidative stress, resulting in significant damage to cell structures and macromolecules, including proteins, lipids, and nucleic acids. Free radicals derived from molecular oxygen are usually known as reactive oxygen species (ROS). The term ROS includes not only free radicals (superoxide radical, hydroxyl radical) but also molecules such as hydrogen peroxide, singlet oxygen and ozone. Oxidative stress plays a major role in the development of several human diseases, such as cerebral ischemia, diabetes, alzheimer, inflammation, rheumatoid arthritis, atherosclerosis and cancer, as well as aging processes (Halliwell & Gutteridge, 1984; Halliwell & Gutteridge, 1989). ROS also damage DNA, which has been associated with cancer, coronary heart diseases and many age related health problems (Stief, 2003; Wickens, 2001).

Antioxidants occur naturally in many foods and are essential for our health. Antioxidants may be defined as compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Aruoma, 1998; Tiwari, 2004). Antioxidants are substances that can react with ROS and reduce the risk of human chronic diseases (Aruoma, 1999; Wade, Jackson, Highton, & Van Rij, 1987). Human and other living organisms possess antioxidants which protect the oxidative damage, but these antioxidants are not sufficient to prevent them. The antioxidant activities proceed through various mechanisms. They can inhibit oxidizing chain reactions in several ways, including direct quenching of reactive oxygen species, inhibition of enzymes, hydrogen donating ability, the reductive capacity and chelating of

transition metal ions. There are two basic categories of antioxidants, namely, synthetic and natural (Nahm, Juliani, & Simon, 2012). Synthetic antioxidants are those created in laboratories, generally for use in the preservation of foods. The most commonly used synthetic antioxidants at the present time are butylated hyhroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate (PG). But uses of these antioxidants are restricted due to their side effects such as carcinogenesis and liver damage (Branen, 1975; Zhou, & Zheng, 1991). Thus, it is essential to discover new natural antioxidants that could protect the human body from free radicals without side effects (Yang, Guo, & Yuan, 2008). Hence, there is increasing interest in investigating naturally occurring antioxidants to replace synthetic antioxidants (Chanda & Dave, 2009; Patra et al., 2013). Recent studies have demonstrated that many antioxidants play important roles in the prevention of oxidative stress induced damage in living organisms as free radical scavengers. The most common natural antioxidants are Vitamin C (ascorbic acid), Vitamin E (tocopherols), Vitamin A (carotenoids), and various polyphenols (Preeti, Pushpa, Sakshi, & Jyoti, 2012). Natural antioxidants are those that can be found directly from natural sources such as cereals, fruits, vegetables, and beverages (Shebis et al., 2013). In this perspective, the antioxidants present in mushrooms are of great interest as potential protective agents against oxidative damage (Mau, Lin, & Chen, 2002; Pattanayak et al., 2015).

The main bioactive components of mushroom are phenolic and flavonoids compounds, tocopherols, ascorbic acid, and carotenoids. Several common and commercial mushrooms like *V. Volvacea, A. biporous, L. edodes, P. cystiodyosus,* and *P. ostreatus* showed the antioxidant properties (yang, Lin, and Mau, 2002). Polysaccharides from mushrooms exhibit strong antioxidant effects that are relevant to their health protecting functions (Kanagasabapathy, Malek, Kuppusamy, & Vikineswary, 2011; Liu et al., 2014; Nandi et al., 2014; Preeti, Pushpa, Sakshi, & Jyoti, 2012). It can be concluded that mushrooms have valuable therapeutic potential that can be used as a natural functional food ingredient and antioxidant agents.

1.E. Conclusion

Carbohydrates are the most abundant biomolecules belonging to class of organic compounds found in living organisms on earth. Mushrooms are the important natural renewable source of bioactive polysaccharides that exhibit immunostimulating and antitumor properties. Almost all living organism like fungi, bacteria etc. produces polysaccharides. β-D-glucans are useful to recover the impaired immune systems of humans and particularly against cancer and infectious diseases. The commercial pharmaceutical products such as schizophyllan, lentinan, grifolan, PSP (polysaccharide peptide complex) and PSK (polysaccharide protein complex) have shown potential clinical applications in cancer therapy. Mushroom polysaccharides activate immune systems as well as exhibit effective antioxidant activities. Hence, they have a great role for future application as drugs for immune and cancer therapy.

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CHAPTER 2

METHODOLOGY

(*Adopted during the whole course of the present work)*

2.A. Structural analysis of polysaccharides

The exact structure of the polysaccharides (PS) is determined using the combinations of two methods: (1) chemical method that 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). A schematic diagram of the methodology adopted during the whole course of the present work for structure determination of polysaccharides is presented in **Figure 1**.

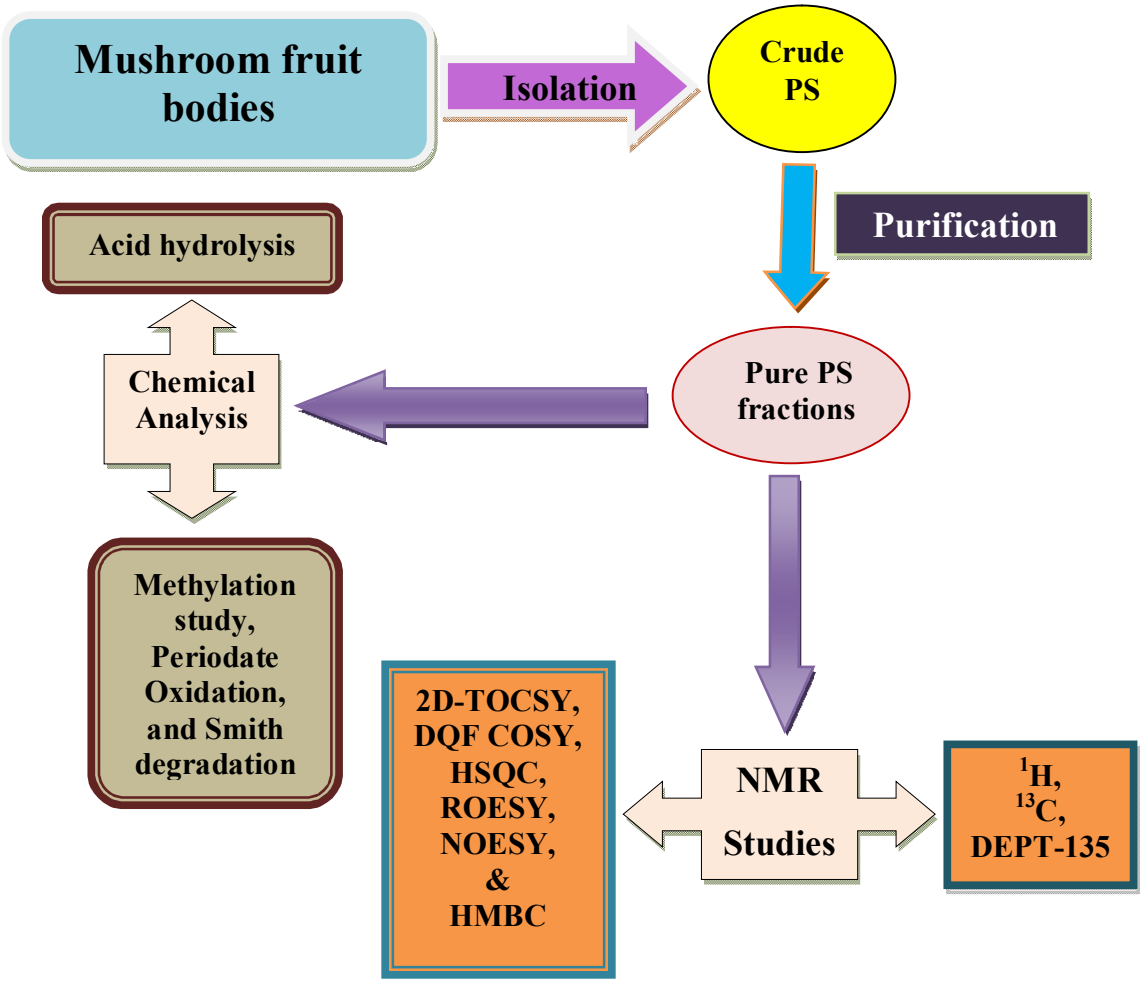

Figure 1. Schematic diagram of structural analysis of polysaccharides.

2.B. Isolation and Purification of polysaccharides from fruit bodies of mushroom

2.B.1. Polysaccharides (PS-I and PS-II) from alkaline extraction of an edible mushroom, *Entoloma lividoalbum*

Fresh fruit bodies of the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička (600 g) were gently washed with distilled water several times and the mushroom bodies were crushed and boiled with 4 % NaOH solution for 1 h. The whole mixture was then kept overnight at 4 °C and then filtered through linen cloth. The filtrate was centrifuged (using a Heraeus Biofuge stratus centrifuge) at 8000 rpm at 4 °C for 50 min to obtain a clear solution and then the supernatant was precipitated in EtOH. It was kept overnight at 4 °C and again centrifuged as above. The precipitated material was washed with ethanol for five times and then dissolved in water and freeze-dried. The freeze-dried material was dissolved in a minimum volume of distilled water and dialyzed through dialysis tubing of cellulose membrane (D9652, Sigma–Aldrich, retaining MW >12,400 Da) against distilled water for 24 h to remove low molecular weight materials. The water soluble part was then freeze-dried, yielding 700 mg crude polysaccharide. For the exact structural analysis, it is essential that the polysaccharide should be homogeneous. So, the purification of the crude polysaccharide is essential. The crude polysaccharide was purified by gel permeation chromatography (GPC). In this technique molecules are separated on the basis of size relative to the pores in the packing particles. This technique is also known as size exclusion chromatography (SEC). The crude polysaccharide was purified through Sepharose 6B column (fractionation range 10,000-10,00,000 Da) in water as eluant using a Redifrac fraction collector. The polysaccharide (30 mg) was passed through Sepharose 6B column (90 cm × 2.1 cm) using distilled water as the eluant with a flow rate of 0.5 mL min^{-1}. A total of 90 test tubes were collected and monitored spectrophotometrically at 490 nm using Shimadzu UV-vis spectrophotometer, model-1601 by the phenol-sulfuric acid method (York et al., 1986). A chromatogram of sample distribution was obtained by plotting the test tube number against absorbance. Two fractions, PS-I (test tube no. 18–24) and PS-II (test tube no. 33-38), were obtained,

collected and freeze-dried, yielding 13 mg and 9 mg of pure polysaccharide respectively. The purification procedure was repeated several times to get more pure PS-I and PS-II. The fractionation and purification steps are shown below (**Figure 2**).

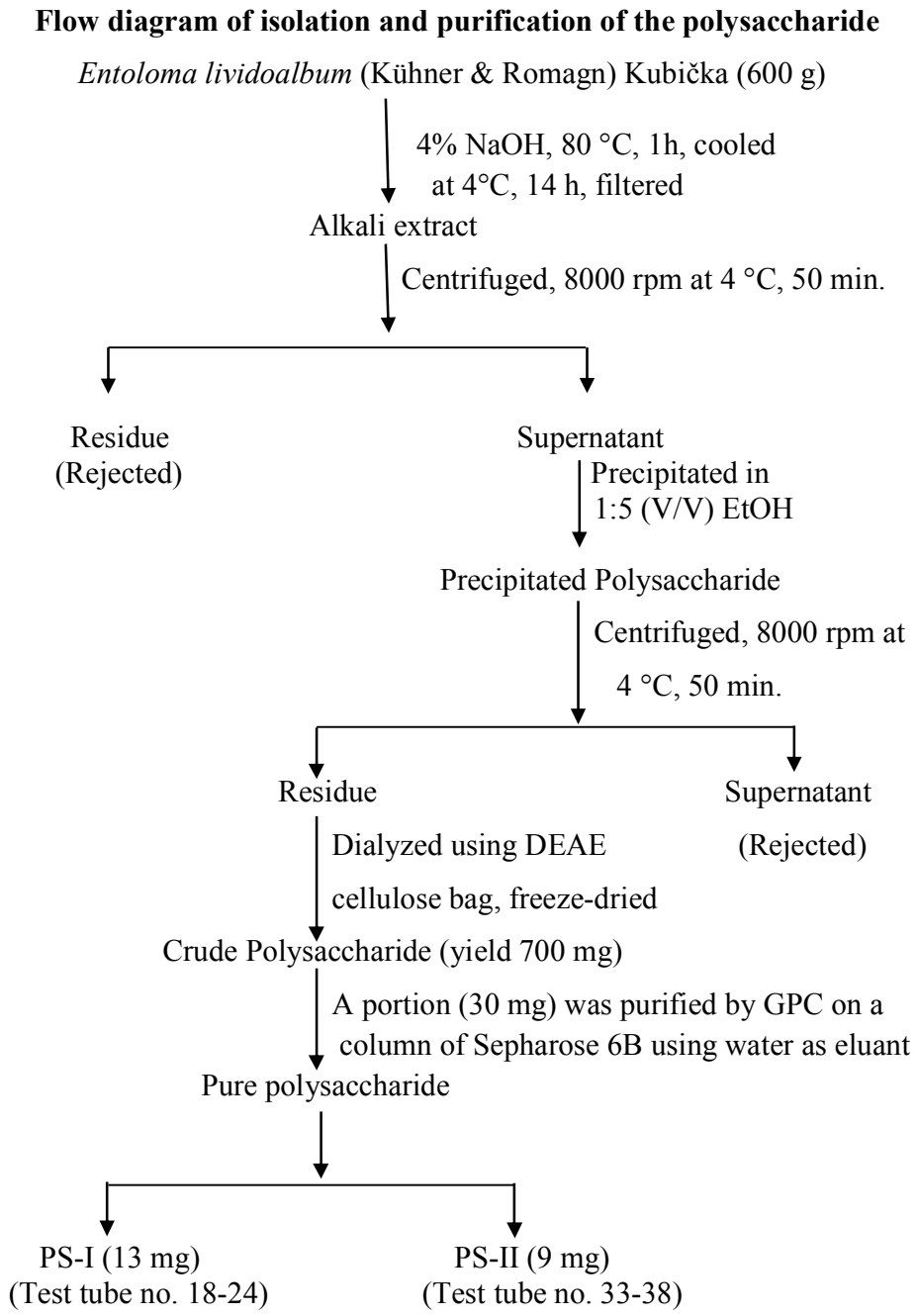

Figure 2. Schematic diagram of isolation and purification of polysaccharides from alkaline extract of the fruit bodies, *Entoloma lividoalbum.*

2.B.2. Polysaccharide (ELPS) from aqueous part of an edible mushroom, *Entoloma lividoalbum*

Fresh fruit bodies of the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička (700 g) were collected from Sikkim Himalayan region, India, gently washed with water, cut into pieces and boiled at 100 °C with distilled water for 10 h, cooled, centrifuged, supernatant was precipitated in EtOH (1:5) to get crude polysaccharide (900 mg). The crude polysaccharide (25 mg) was purified by gel-permeation chromatography (GPC) on column (90 cm × 2.1 cm) of Sepharose 6B using distilled water as the eluent with a flow rate of 0.5 mL min^{-1}. A total of 90 test tubes were collected and monitored by the phenol-sulfuric acid method (York et al., 1986) at 490 nm using Shimadzu UV-vis spectrophotometer, model-1601. A single homogeneous fraction (test tube 20 to 32) was collected and freeze-dried, yielding 15 mg pure polysaccharide. This experiment was repeated eight times and 110 mg of pure polysaccharide was collected and preserved for further analysis. The isolation and purification steps of polysaccharide from aqueous part of mushroom fruit bodies are shown in **Figure 3**.

Flow diagram of isolation and purification of the polysaccharide

Entoloma lividoalbum (Kühner & Romagn.) Kubička (700 g)

↓ Distilled water, boiled, 10 h

Aqueous Extract

↓ Centrifuged, 8200 rpm, 50 min.

Residue (Rejected)

Supernatant

↓ Precipitated in EtOH (1:5, v/v), kept overnight at 4 ºC

Precipitated Polysaccharide

↓ Centrifuged, 8200 rpm, 50 min.

Residue

↓ Dissolved in distilled water, dialyzed through cellulose bag, freeze dried

Supernatant (Rejected)

Crude Polysaccharide (900 mg)

↓ 25 mg crude polysaccharide dissolved in minimum volume of distilled water and then passed through Sepharose-6B column

Purified Polysaccharide

↓

ELPS (15 mg)
(Test tube no. 20–32)

Figure 3. Schematic diagram of isolation and purification of polysaccharide from aqueous extract of the fruit bodies, *Entoloma lividoalbum*.

2.B.3. Polysaccharides from aqueous part of the fruit bodies of a hybrid mushroom, *pfle 1p*

The hybrid mushroom *pfle 1p* was cultivated and collected from Falta experimental farm, Bose Institute, West Bengal, India. The fruit bodies (300 g) were washed thoroughly with distilled water, followed by boiling with distilled water for 10 h. Isolation and purification process are shown in **Figure 4**. The crude polysaccharide (25 mg) was passed through Sepharose 6B gel permeation column (90 × 2.1 cm) using water as eluant with a flow rate of 0.4 mL/min. Ninety five test tubes were collected using Redifrac fraction collector and monitored spectrophotometrically at 490 nm with phenol-sulfuric acid reagent (York et al., 1986) using a Shimadzu UV-vis spectrophotometer, model-1601. Two fractions of purified polysaccharide, PS-I and PS-II were collected. The purification process was carried out in several lots.

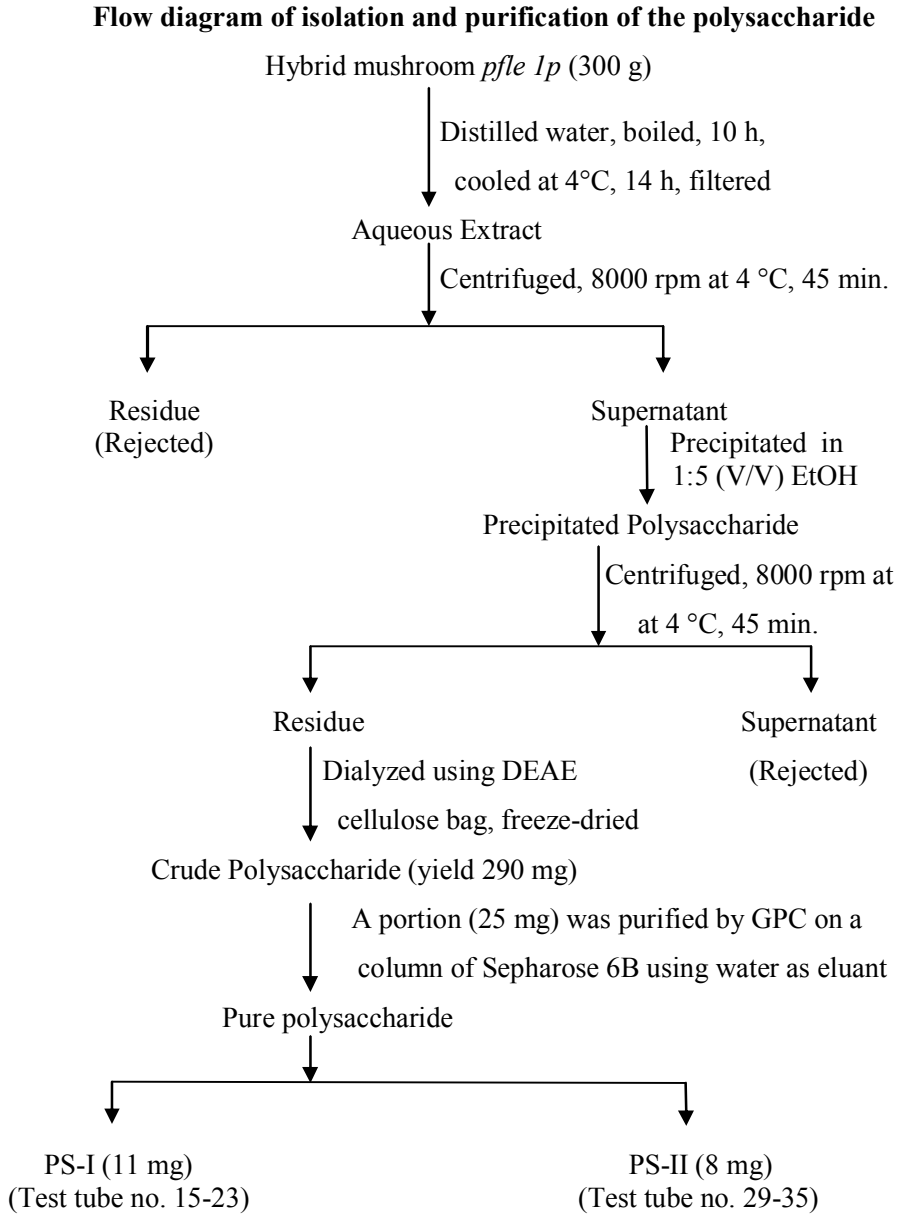

Figure 4. Schematic diagram of isolation and purification of polysaccharide from aqueous extract of the fruit bodies, *pfle 1p*.

2.C. Determination of physical properties

2.C.1. Optical rotation measurement

Optical rotation was measured on a Jasco Polarimeter Model P-1020 at room temperature.

2.C.2. Molecular weight determination

The molecular weight of the polysaccharides was determined by gel-permeation chromatography (Hara, kiho, Tanaka, & Ukai, 1982). Standard dextrans T-250, T-200, T-70, and T-40 were passed through a Sepharose 6B column, and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of the polysaccharide was then plotted on the same graph and the average molecular weight of polysaccharide was measured.

2.D. Chemical analysis

2.D.1. Monosaccharide analysis

The polysaccharides are composed of different monosaccharide units including pyranosidic or furanosidic configurations. The monosaccharide composition of these polysaccharides are analyze in different ways, such as thin layer Chromatography (TLC), Gas-liquid Chromatography (GLC), and high performance liquid Chromatography (HPLC). In the present investigation Gas-liquid Chromatography (GLC) was used for identification and quantification of the monosaccharide composition. Total acid hydrolysis of polysaccharide was carried out with 2 M CF_3COOH (2 mL) in a round-bottom flask at 100 °C for 18 h in a boiling water bath. The excess acid was completely removed by co-distillation with water. Then, the hydrolyzed product was reduced with $NaBH_4$ (9 mg), followed by acidification with dilute CH_3COOH, and then co-distilled with pure CH_3OH to remove excess boric acid. The reduced sugars were converted into the alditol acetates (Lindahl, 1970) by treatment with 1:1 pyridine–acetic anhydride in a

boiling water bath for 1 h (**Figure 5**). The excess pyridine-Ac_2O was removed by repeated co-distillation with toluene. Chloroform was used to extract the alditol acetates and then analyzed by Gas-liquid Chromatography (GLC). A gas-liquid chromatography Hewlett-Packard model 5730 A was used, with flame ionization detector and glass columns (1.8 m x 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 analyses were performed at 170 °C.

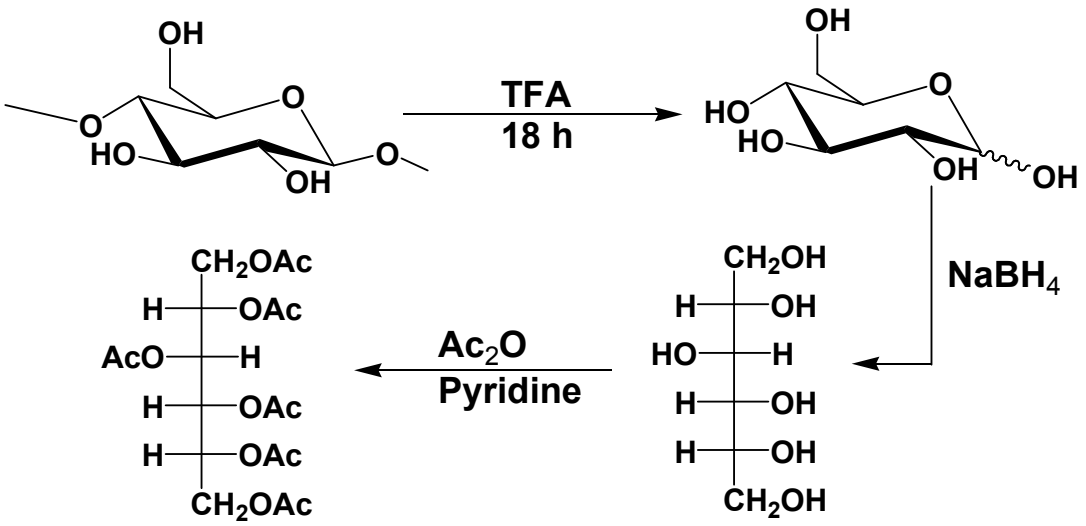

Figure 5. Schematic diagram of preparation of alditol acetates.

2.D.2. Paper chromatographic studies

Chromatography is a technique for separation of closely related groups of compounds. Paper chromatography is an example of liquid-liquid chromatography. Paper chromatographic studies are used for separation and identification of different monosaccharide present in the hydrolyzed polysaccharide. During the present thesis work, sugar mixture was separated by descending paper chromatography. In this type of paper chromatography, development of paper is done by allowing the solvent to travel down the paper and the mobile phase is present in the upper portion. The paper chromatographic studies (Martin & Synge, 1941; Partridge, 1946) were performed on Whatmann Nos. 1 (for small quantities) and Whatmann Nos. 3 mm (for large quantities

up to 30 mg) for the separation of monosaccharide. Solvent systems were used (i) BuOH-HOAc-H_2O (v/v/v, 4:1:5, upper phase) [Boggs et al., 1950] and (ii) EtOAc-pyridine-H_2O (v/v/v, 8:2:1) [Hamilton & Thompson, 1957]. The spray reagents for development of chromatograms were used as: (a) silver nitrate in acetone (1.2%), (b) methanol in sodium hydroxide solution, and (c) 5% sodium thiosulphate solution (Hoffman, Lindberg, & Svensson, 1972). The dried chromatogram paper was dipped into solution (a) and dried in air. It was then dipped into solution (b) for the spots to develop. The excess developing reagents were washed out with solution (c). Finally, the paper was washed with distilled water and dried in air.

2.D.3. Determination of absolute configuration

In carbohydrate chemistry, it is essential to determine the sugar composition as well as the absolute configuration of each monosaccharide units. Monosaccharides are the polyhydroxy carbonyl compounds and usually exist in cyclic hemiacetal form. The D and L- isomers constitute a pair of enantiomers that indicate the absolute configuration of the monosaccharide units. The absolute configuration of monosaccharide was determined by the method of Gerwig, Kamerling, and Vliegenthart (1978). The polysaccharide (1.0 mg) was hydrolyzed with CF_3COOH, and then the acid was removed by co-distillation with water. 250 μL of 0.625 M HCl solution treated with R- (-)-2-butanol was added to the hydrolyzed product and heated at 80 °C for 16 h. Then the reactants were evaporated and TMS-derivatives were prepared with N,O-bis (trimethylsilyl) trifluroacetamide (BSTFA). The products were analyzed by GLC using a capillary column SPB-1 (30 m × 0.26 mm) with a temperature program (3 °C/min) from 150 to 210 °C. The resulting 2,3,4,6-tetra-*O*-TMS-(+)-2-butylglycosides were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides.

2.D.4. Linkage analysis

The different mode of linkage of monosaccharide units in a polysaccharide is determined by methylation analysis which is further confirmed by periodate oxidation and smith degradation study.

2.D.4.1. Methylation analysis

Methylation experiment is a powerful tool for the determination of the structure of polysaccharide in carbohydrate chemistry. The different mode of linkage of each sugar residue in a polysaccharide is determined by well-known chemical method known as methylation analysis. Methylation analysis includes conversion of all free hydroxyl groups into methoxy groups followed by hydrolysis and then acid hydrolysis breaks only the inter-glycosidic linkages, keeping the methyl ether bonds intact. The hydrolyzed monomers are reduced and acetylated to produce volatile partially methylated alditol acetates (PMAA) as shown in **Figure 6**. The substitution pattern of the *O*-acetyl group of the PMAA reflects the linkage patterns of the corresponding sugars in the original polymer. Polysaccharide was methylated using the procedure as reported by Ciucanu and Kerek (1984). The polysaccharide (4.0 mg) was kept on P_2O_5 in vacuum desiccators for several hours. The dried polysaccharide was dissolved in 1 mL of anhydrous DMSO and then solid finely powdered sodium hydroxide (NaOH) was added and stirred for 30 minutes. The mixture was methylated with 1 mL of methyl iodide and stirred for another 1 hr. The methylated products were isolated by making partition between $CHCl_3$ and H_2O (5:2, v/v) for four times. The organic layer containing products was collected and dried. The methylated product was then hydrolyzed with 90% formic acid (1 mL) at 100 °C for 1 h and excess formic acid was evaporated by co-distillation with distilled water. After reduction with sodium borohydride, the methylated monosaccharides were acetylated with pyridine-acetic anhydride (1:1) adopting the procedures as applied earlier (2.D.1). The alditol acetates of the methylated sugars were extracted with chloroform and analyzed by GLC-MS. The Gas-liquid chromatography-mass spectrometric (GLC-MS) analysis was performed on a 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.

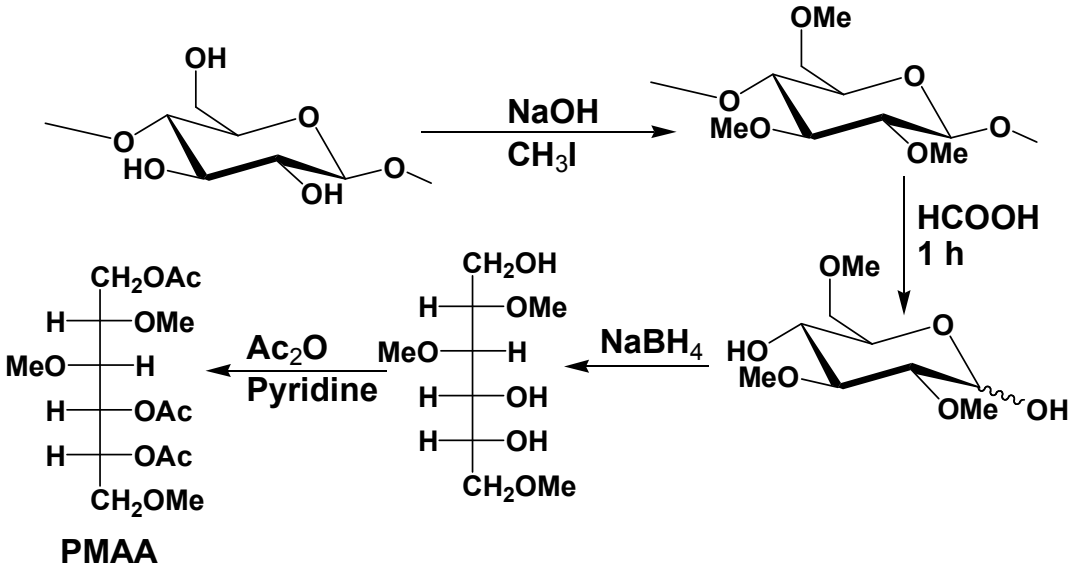

Figure 6. Schematic diagram of preparation of partially methylated alditol acetates (PMAA) of polysaccharides.

2.D.4.2. Periodate oxidation study

Periodate oxidation is a useful technique in structural studies and broadly used in carbohydrate chemistry to determine the pattern of substitutions. Due to the presence of free hydroxyl groups, polysaccharides can react with oxidizing agents such as periodic acid or its salts. Carbohydrates which contain at least three adjacent hydroxyl groups yield formic acid when oxidized with periodate. Non-reducing end sugar residues and (1→6)-linked hexopyranose residues consume two molar equivalent of periodate yielding one molar equivalent of formic acid and dialdehyde as shown in **Figure 7**. Non-terminal units like (1→2) and (1→4)-linked hexopyranose units consume one equivalent of periodate per mole yielding dialdehyde. But, (1→3)-linked hexopyranose residues will not be affected by this reaction because of absence of vicinal OH groups (**Figure 8**).

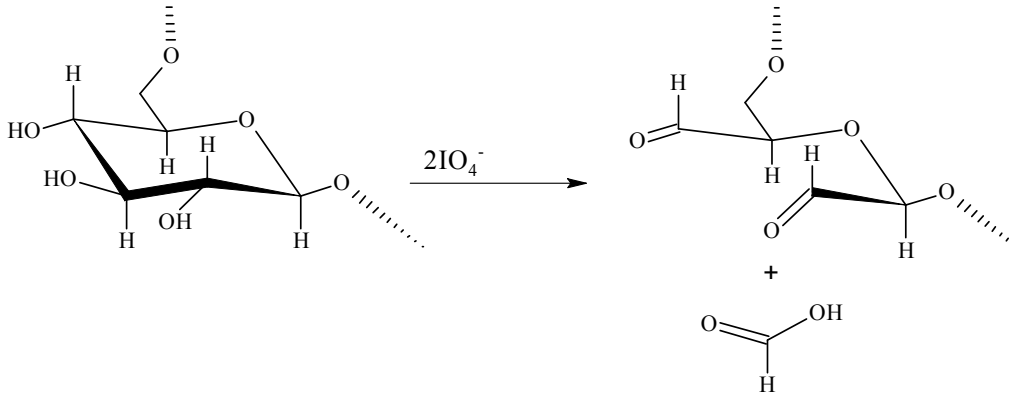

Figure 7. Schematic diagram of periodate oxidation of (1→6)-linked hexopyranoside.

The polysaccharide (20 mg) was swelled in 2 mL of distilled water, and then 20 mL of 0.1 M sodium metaperiodate was added into the solution. The mixture was kept in the dark for 72 h at 25 °C. Ethylene glycol was then added to consume the excess periodate and stop the oxidation reaction. The periodate product solution was dialyzed against distilled water for 2 h. The volume of the dialyzed material was concentrated to 2–3 mL. The dialyzed material was reduced with sodium borohydride ($NaBH_4$) for 12 h and neutralized with 50% acetic acid (AcOH). The resulting material was dried by co-distillation with methanol. The periodate-reduced material (Goldstein, Hay, Lewis, & Smith, 1965; Hay, Lewis, & Smith, 1965) was divided into two portions. One portion was hydrolyzed with 2 M CF_3COOH (1 mL) at 100 °C for 18 h and used for alditol acetate preparation as usual and analyzed by GLC. Another portion was methylated by the method of Ciucanu and Kerek (1984), followed by preparation of alditol acetates which were analyzed by GLC–MS.

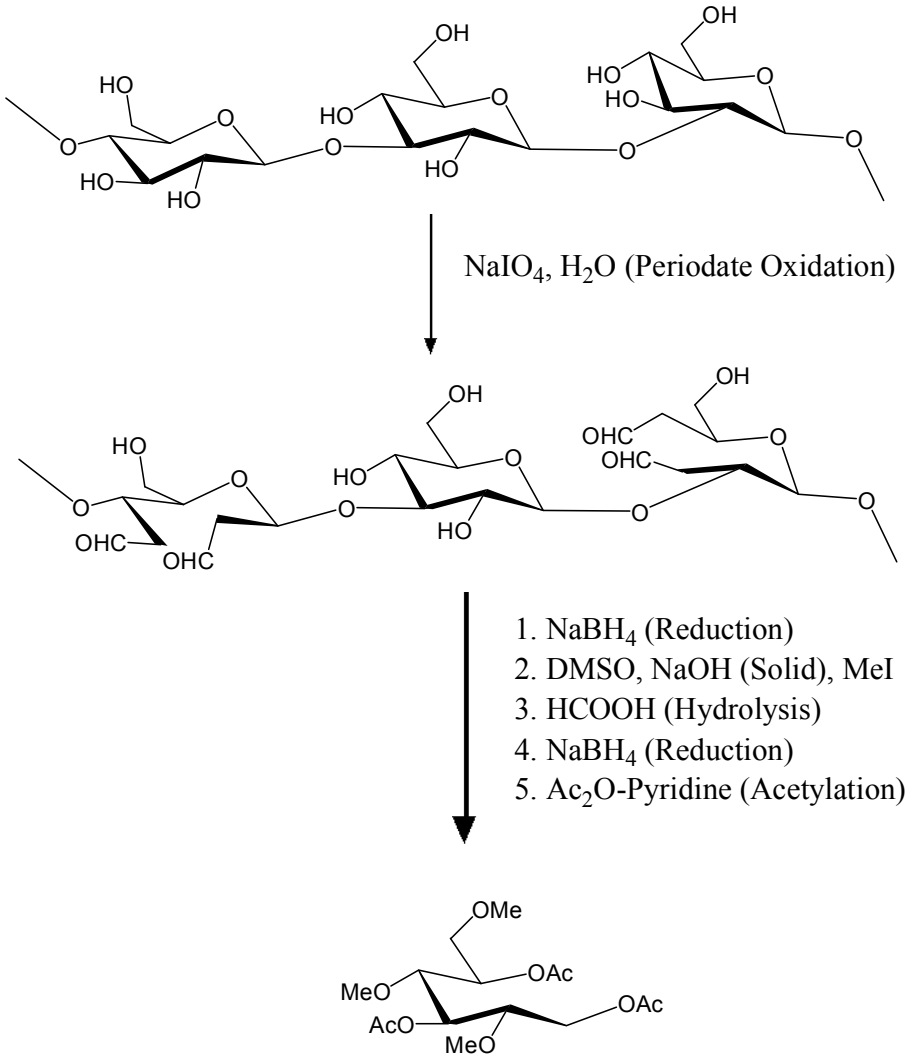

Figure 8: Schematic diagram of periodate oxidation study

2.D.4.3. Smith degradation

The combination of periodate oxidation, reduction, and mild acid hydrolysis is known as the Smith Degradation. The controlled smith degradation is a useful extension of periodate oxidation that provides a lot of information about the chemical structure of the polysaccharide. It can selectively degrade a polysaccharide to either an oligosaccharide or a polysaccharide with a smaller repeating unit. In the present thesis the controlled smith

degradation experiment was performed with the polysaccharide. The polysaccharide (25 mg) was oxidised with 0.1 M aqueous $NaIO_4$ (20 mL) at 25 °C in the dark during 96 h. The remaining periodate oxidised compound was treated with 1,2-ethanediol to decompose the excess of periodate. After stay for an hour the total solution was dialyzed against distilled H_2O for 6 h. After dialysis, the sample was freeze-dried. The freeze-dried material was dissolved in 2 mL of distilled water, and then $NaBH_4$ was added and kept at room temperature for 12 h, with occasional stirring. The mixture was neutralized with 50% AcOH and again dialyzed distilled water, and freeze-dried. The product was successively hydrolyzed with 0.5 M CF_3COOH, 15 h, 25 ºC and the excess acid was removed by repeated freeze drying. The material was further purified by passing through a Sephadex G-25 column, freeze-dried and kept over P_2O_5 in vacuum for several days. A part of this polymeric material (2 mg) was methylated and analysed as usual by GLC–MS and the remainder was used for ^{13}C NMR studies.

2.D.4.4. Sequence analysis: Partial acid hydrolysis

Partial acid hydrolysis of the polysaccharides gives detailed information about the sequence and linkage types of complex polysaccharide. Therefore, partial acid hydrolysis was performed for determination of additional structural features. In the present thesis the polysaccharide (35 mg) was partially hydrolyzed with 6 mL 0.1 M CF_3COOH at 100 °C for 1 h and the excess acid was removed by repeated evaporation of water at 37 °C. The residue was dissolved in water, to which three volumes of ethanol was added. The precipitate was washed with ethanol, freeze-dried and the fraction F2 was obtained which was used for methylation as well as ^{13}C NMR analysis. The supernatant was dried by evaporation, and the residue was dissolved in a minimum volume of water for reduction. After reduction with $NaBH_4$ at 25 ºC for 2 h, the product was neutralized with 1 M AcOH and it was desalted by passing through a Sephadex G-25 column. The carbohydrate containing eluate (F1) was collected, freeze-dried and subjected to methylation analysis.

2.E. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy is one of the most powerful analytical techniques that are often used in the structure elucidation of carbohydrates. NMR spectroscopy provides the detailed structural characterization of the polysaccharide, such as monosaccharide compositions, α- or β-anomeric configurations, establishment of linkage patterns, and sequences of the sugar moieties. Combination of 1D (^{1}H, ^{13}C, and DEPT-135) and 2D (DQF-COSY, TOCSY, NOESY, ROESY, HSQC and HMBC) NMR experiment (**Figure 9**) are performed in order to assign the structure of the polysaccharides.

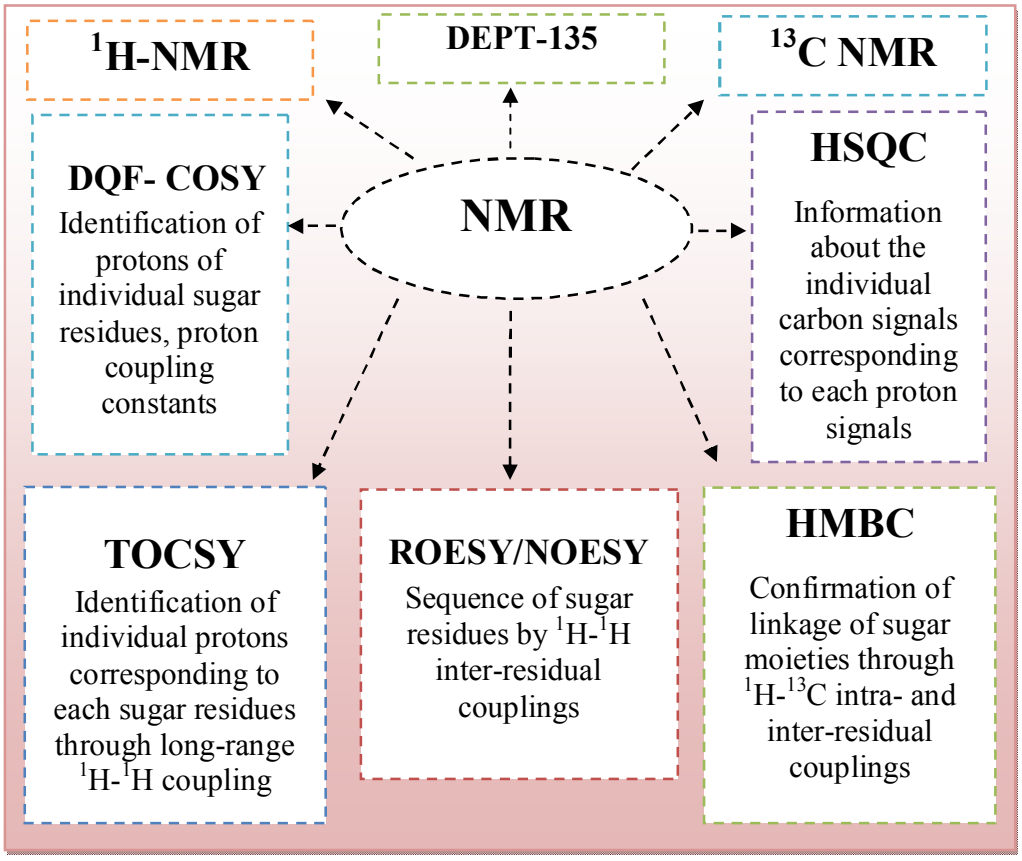

Figure 9. Schematic diagram of determination of structure of a polysaccharide by NMR spectroscopic methods.

➢ Preparation of NMR sample and instrumentation

Carbohydrates are structurally diverse but the hydroxyl group is common to all. A major problem in ^{1}H NMR is that the OH protons of sugar units and the proton signals from residual water appear in the region of the ring protons and creates a definite interference for peak identification. Proper sample preparation is very important before running an NMR spectrum. In the present thesis all NMR experiments were performed in D_2O. The polysaccharide was dried in a vacuum over P_2O_5 for several days and then exchanged with deuterium (Dueñas-Chaso et al., 1997) followed by lyophilizing with D_2O (99.96% atom ^{2}H, Aldrich) four times. After that, the sample was put in a 5 mm NMR tube and dissolved in 1.0 mL of 99.96% D_2O. The ^{1}H and ^{13}C NMR experiments were carried out at 500 MHz and 125 MHz, respectively with a Bruker Avance DPX-500 spectrometer. All one-dimensional (1D) and two-dimensional (2D) NMR spectra were recorded in D_2O at 30 ºC. The ^{1}H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.70) using presaturation method. The 2D DQF-COSY NMR experiment was performed using standard pulse sequence of BRUKER software. The TOCSY experiment was recorded at mixing time of 150 ms. The NOESY and ROESY mixing delay were 300 ms where as the delay time in the HMBC experiment was 80 ms. Acetone was used as an internal standard fixing the methyl carbon signal at δ 31.05 for ^{13}C spectrum.

➢ One-dimensional NMR

^{1}H and ^{13}C NMR spectroscopy (**Figure 10**) are important tools for the structural characterization of carbohydrates. The most useful nuclei in carbohydrate research are ^{1}H and ^{13}C. Every polysaccharide possesses a unique spectrum in both ^{1}H and ^{13}C NMR spectroscopy. This greatly helps in determining the number of different monosaccharide residues present in the polysaccharides and also in estimating their relative proportion. ^{1}H NMR signals are much more sensitive than ^{13}C signals due to their natural abundance. As a result, high ^{1}H NMR signals can be used for quantitative purposes in some applications.

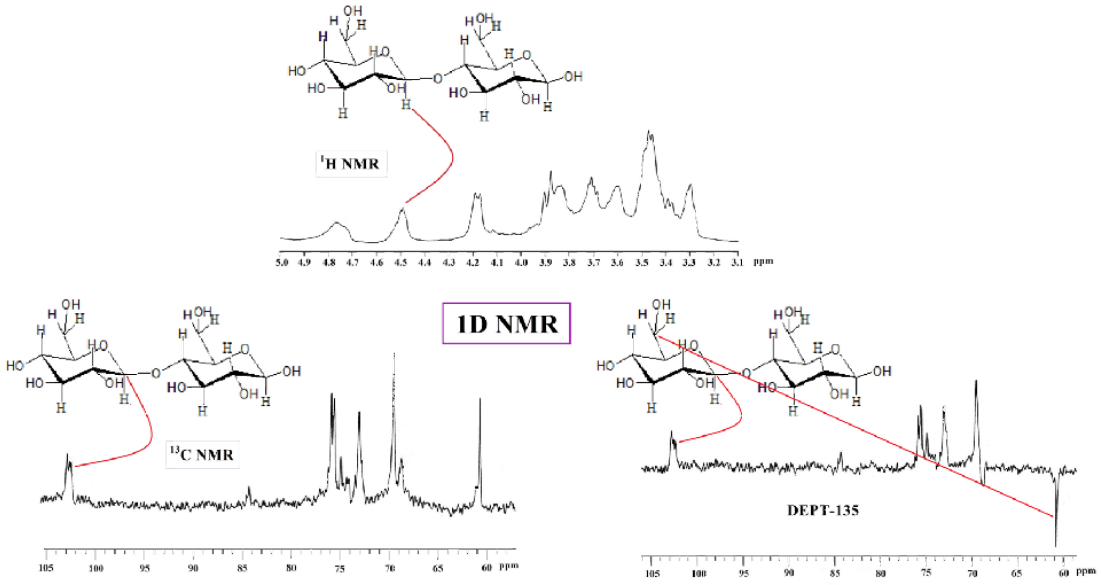

Figure 10. Introductions to 1D NMR

The carbohydrates, such as mono-, oligo-, and polysaccharides have proton chemical shifts in the range of δ 1.0-6.0. The anomeric proton regions are found in the range of δ 4.4-5.5. The remaining ring proton resonances are found in the range of δ 3.0-4.2. In case of deoxy sugars the methyl protons appear in the region of δ 1.1-1.3. The anomeric signals can be used to estimate the number of different monosaccharides present in the polysaccharide. The anomeric protons from each polysaccharide offer recognizable signals based on their α- or β-configurations. α-glycosides resonate at a downfield region by δ 0.3-0.5 compared to the β-glycosides in D-pyranoses conformation. Thus, the α-anomeric protons appear in the area of δ 4.8-5.5, while the β-anomeric protons appear in the δ 4.4-4.8 range. The vicinal coupling constant ($J_{1\text{-}2}$) between the anomeric H-1 and the H-2 protons indicates the relative orientation of two protons. If both of them are in an axial configuration in pyranose structures, a large coupling constant (7-8 Hz) is observed, whereas if they are equatorial-axial, the coupling constant observed is smaller at approximately 4 Hz (Jansson, Kenne, & Widmalm, 1987). Different sugars are identified from their characteristic coupling constants.

^{13}C NMR has larger chemical shift dispersion and can often provide more information. In the ^{13}C NMR spectra, signals from the anomeric carbons present in the region of δ 90-110 while the resonances for C-2 to C-5 can be found at δ 60-90. In case of deoxy sugars the methyl carbons appear in the region of δ 15-20. ^{13}C nuclei in furanose sugars are less shielded than in their configurationally related pyranose sugars. The α-anomeric carbon signals appear generally in the range of δ 95-103 whereas most of the β-anomeric carbons appear in the region of δ 101-105 for the glycosidically linked sugars present in pyranose form, but very high anomeric carbon chemical shifts are observed in the region of δ 103-112 for furanose form. In case of methoxy sugar the methyl carbons appear in the region δ 55-61. Signals for carbonyl carbons are generally observed between δ 165-185. Unsubstituted ring carbons usually appear in the region of δ 65-75 (Agarwal, 1992). If there is any linkage at any carbon, the signal for that carbon will suffer a downfield shift by δ 4-10 and the carbon adjacent to that one will tend to shift little upfield region (by δ 0.7-4.7) [Agarwal, 1992; Gruter et al., 1993].

From coupling constant $^{3}J_{H1,H2}$ the anomeric configuration of a glucopyranosyl or galactopyranosyl residue can be determined. The $^{3}J_{H1,H2}$ value is around 3-4 Hz for the α-form and 7-8 Hz for the β-form of this residue. Sometimes the ^{1}H J_{1-2} values are not sufficient to determine the anomeric configurations of the sugars. If the glycosyl residue has the manno configuration, the distinction between the two anomeric forms (α or β) is very difficult as the coupling constant values for α (J_{1-2}~ 1.8 Hz) and β (J_{1-2} ~ 1.5 Hz) are too close to be differentiated. One bond heteronuclear coupling constants ($^{1}J_{C1,H1}$) in pyranoses are useful for determination of the anomeric configuration of sugar residues (Bock, Pedersen, & Pedersen, 1984). For D sugars, α-anomeric configuration demonstrates a coupling constant of $^{1}J_{C1,H1}$ ~ 170 Hz whereas β-anomeric configuration exhibits a coupling constant of $^{1}J_{C1,H1}$ ~ 160 Hz (Bock & Pedersen, 1974). These values however are reversed for L-sugars. C-1, H-1 coupling constants were determined from proton coupled ^{13}C NMR experiment.

The DEPT-135 (Distortionless enhancement by polarization transfer-135) technique is a very useful adjunct to ^{13}C NMR spectroscopy. In a DEPT-135 spectrum the -CH_3 and -CH group both generate positive signals in the spectra whereas -CH_2 groups display a inverse signal. The signals for the C-6 atoms appear as -CH_2 and are therefore represented as negative signals between δ 60-70. The linking of residues at C-6 is supported from the DEPT-135 spectrum.

➢ Two-dimensional NMR

In two-dimensional (2D) NMR spectroscopy multipulse sequences are employed to provide additional information which is not easily obtained from one-dimensional (1D) spectra. In combination with the 1D-NMR analysis, selective 2D- experiments can be applied to determine the structure of the oligosaccharide unit by establishing how the monosaccharide units are linked together. In the present thesis different 2D NMR (**Figure 11**) experiments such as TOCSY, DQF-COSY, NOESY, ROESY, HSQC and HMBC have been used.

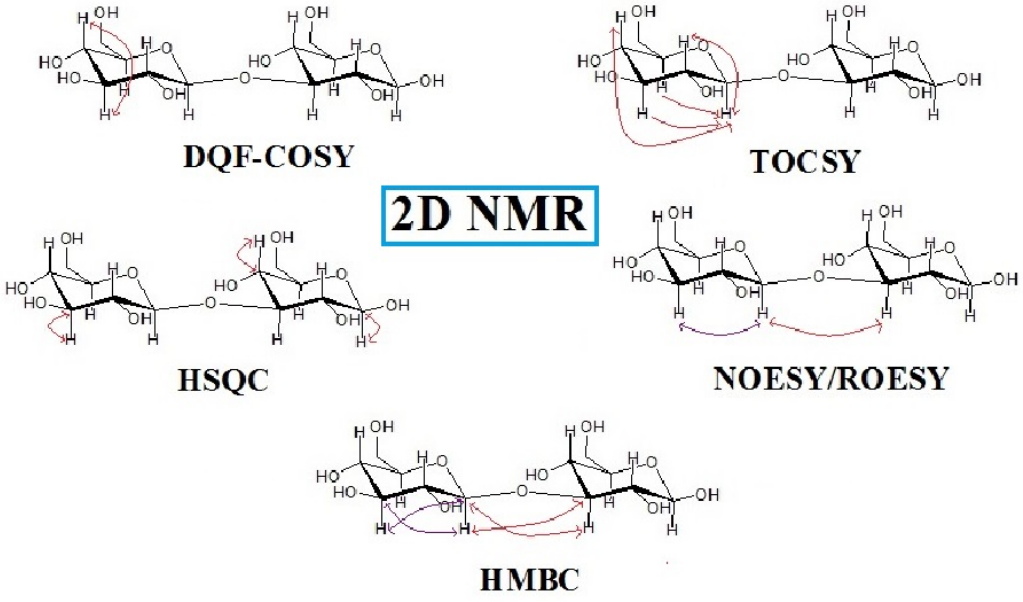

Figure 11. Introductions to 2D NMR

❖ TOCSY (Total Correlation Spectroscopy)

Total correlation spectroscopy (TOCSY) is also known as homonuclear Hartmann-Hahn spectroscopy (HOHAHA). TOCSY type experiments show correlations between all protons within a given spin system. The 2D ^{1}H-^{1}H-TOCSY is a very powerful technique which is used to confirm the assignments of ^{1}H spectrum. Depending on the mixing time of the experiment and the size of the coupling constants between the different protons, TOCSY make it possible to simultaneously correlate the entire spin system in a sugar residue. The TOCSY experiment was recorded at mixing time of 300 ms, and complete assignment required several TOCSY experiments having mixing times ranging from 60 to 300 ms.

❖ Double Quantum Filtered Correlation Spectroscopy (DQF-COSY)

DQF-COSY is a modified version of the basic COSY experiment. COSY spectrum indicates all the proton resonances correlated with itself (diagonal peaks) and with its geminal and vicinal neighboring protons. In DQF-COSY spectrum each proton is correlated with its germinal and vicinal neighboring protons. It gives us the information about the protons of an individual sugar residue through a three-bond coupling. The proton coupling constants can also be measured from the DQF-COSY spectrum. The anomeric configuration of the sugar residues was determined by the vicinal coupling constant value ($J_{H\text{-}1,H\text{-}2}$).

❖ NOESY (Nuclear overhauser enhancement spectroscopy)

^{1}H-^{1}H NOESY (nuclear overhauser enhancement spectroscopy) is used as a homonuclear ^{1}H technique. NOESY correlations are arising from the spatial proximity between protons. The NOESY spectrum provides information on space rather than through bond couplings. NOE connectivies are often observed between the anomeric protons of a particular sugar residue to protons of another sugar residue that is glycosidically linked to the former. Inter-residue NOEs are primarily used for

determination of the sequence of sugar residues and also in determining their linkages in a polysaccharide. The NOESY mixing delay was 300 ms.

❖ ROESY (Rotating frame Overhauser Enhancement Spectroscopy)

ROESY is also used to determine the glycosydic linkage position as well as the sequence information of the polysaccharide like NOESY spectra. Here also the signals arisen from protons of two different sugar residues, which are close in space but not closely connected by chemical bonds. A ROESY spectrum yields through space correlations via the Rotational nuclear overhauser effect (ROE). ROESY is especially useful for cases where NOESY signals are weak because they are near the transition between negative and positive. ROESY cross peaks are always negative. The ROESY experiment also yields cross peaks arisen from chemical exchange. The ROESY mixing delay was 300 ms.

❖ HSQC (Heteronuclear single quantum coherence)

In HSQC NMR spectrum each carbon is correlated with the proton(s) to which it is bonded directly. The number of monosaccharides can more easily be determined in an HSQC spectrum than in a ^{1}H spectrum. All the ^{13}C chemical shifts of the sugar residues are here assigned from the individual proton signals assigned from DQF-COSY and TOCSY spectrum as they are directly correlated.

❖ HMBC (hetero multiple bond coherence spectroscopy)

^{1}H-^{13}C HMBC shows cross-peaks between protons and carbons that are two or three bonds away. HMBC measures long range coupling between proton and carbon (2 or 3 bonds away) with great sensitivity. This very useful sequence provides information about the skeleton of a polysaccharide. The HMBC experiment is used to establish the linkage between monosaccharide units via the glycosidic bond. HMBC experiment confirms the NOESY and ROESY connectivities of a molecule.

2.F. Immunological Studies

2.F.1. Preparation of lipopolysaccharide (LPS) free polysaccharide (LFPS) for immunological studies

Prior to immunoactivation studies, LPS which may contaminate during isolation and purification process was removed from polysaccharide in order to discard the contribution of LPS in immunostimulation. The polysaccharide was passed through polymyxin-B agarose matrix (Sigma 160 and Aldrich, USA) packed in 2 mL column (1 cm×2 cm), with 0.5 mL/min flow rate. It was equilibrated with 10 mM phosphate buffer, pH 7.4. The bacterial lipopolysaccharides (LPS) were bound to the matrix and the unbound LPS free polysaccharide were eluted and collected for immunoenhanching studies.

2.F.2. Limulus amebocyte lysate (LAL) test

The Limulus amebocyte lysate (LAL) test is an alternative method for detection of bacterial endotoxins which are high-molecular-weight complexes associated with the outer membrane of gram-negative bacteria. They play an important role in occurrence and development of many diseases. So their detection is very important. In the present investigation the test was performed using gel clot technique (Liu et al., 2009). Limulus amebocyte lysate (LAL) (G2125, sensitivity: 0.125 EU/mL) was purchased from Quantum Biotech, Mumbai, India. The control standard endotoxin (CSE) (code E0125) and water (code W1004) for the bacterial endotoxin test (BET) were provided by Quantum Biotech, Mumbai, India. Four tubes were taken, each containing 0.1 mL of LAL reagent. In two tubes, 0.1 mL LFPS aqueous solution were added, meanwhile 0.1 mL BET water and 0.1 mL CSE were added to the rest two tubes as negative control and positive control, respectively. All tubes were incubated for 1 h in a water bath at 37 °C. After the test tube was inverted 180° slowly, it is positive (+) if the gel in tube is not deformed and does not slip from the wall and a negative (-) test is characterized in the absence of a gel or by the formation of a viscous mass which does not hold when the tube is inverted. Test is invalid when positive control is (-) or negative control is (+).

2.F.3. Test for macrophage activity by nitric oxide assay

RAW 264.7, a murine macrophage cell line growing in Dulbecco's modified Eagle's medium (DMEM) was seeded in 96 well flat bottom tissue culture plate at a concentration of 5×10^5 cells/mL (180 µL) [Mallick, Maiti, Bhutia, & Maiti, 2011]. Cells were left overnight for attachment and then incubated with different concentrations of PS (6.25, 12.5, 20, 25, 35, 50, 100 and 200 µg/mL) in a humidified atmosphere of 5% CO_2 at 37 °C for 48 h. After 48 h of treatment, culture supernatant of each well was collected and NO content was estimated using Griess reagent (Green et al., 1982) (1:1 of 0.1% in 1-napthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid). Lipopolysaccharide (LPS, L6511 of *Salmonella enterica* serotype typhimurium, Sigma, 4 µg/mL) was used as positive control and soluble starch (Merck, India, 100 µg/mL) as negative control.

2.F.4. Splenocyte and thymocyte proliferation assay

A single cell suspension of spleen and thymus was prepared from Swiss Albino mice under aseptic conditions by homogenization in Hank's balanced salt solution (HBSS) [Maiti et al., 2008]. The suspension was centrifuged to obtain cell pellet. The contaminating red blood cells were removed by hemolytic Gey's solution. After washing two times with HBSS, the cells were resuspended in complete Rose well Park Memorial Institute (RPMI) 1640 medium (Source- GIBCO, USA, Cat. No. 31800-022). Cell concentration was adjusted to 1×10^6 cells/mL and viability of splenocytes and thymocytes (as tested by trypan blue dye exclusion) was always over 90%. The cells (180 µL) were plated in 96 well flat bottom tissue culture plates and incubated with 20 µL of various concentrations of PS (6.5, 12.5, 25, 50, 100 and 200 µg/mL). PBS (10 mM, phosphate buffer saline, pH-7.4) was taken as carrier control and soluble starch (Merck, India, 100 µg/mL) as negative control. Lipopolysaccharide (LPS, L6511 of *Salmonella enterica* serotype typhimurium, Sigma, 4 µg/mL) served as positive control. All cultures were set up at 37 °C for 72 h in a humidified atmosphere of 5% CO_2. Proliferation of splenocytes (% Splenocyte Proliferation Index or % SPI) and thymocytes (% Thymocyte Proliferation

Index or %TPI) were determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay method (Mosmann, 1983; Sarangi et al., 2006). All the experiments were done twice with seven replicates and the data were reported as the mean ± standard deviation and compared against PBS control (Green et al., 1982; Maiti et al., 2008).

2.G. Antioxidant properties

2.G.1. Hydroxy radical scavenging activity

The reaction mixture (1mL) consisted of KH_2PO_4 - KOH buffer (20 mM, pH 7.4), 2-deoxy-D-ribose (2.8 mM), variable concentration (100-800 µg/mL) of PS, $FeCl_3$ (100 mM), EDTA (104 µM), ascorbate (100 µM) and H_2O_2 (1 mM). Following incubation at 37 ˚C for 1 h, 2 mL thiobarbituric acid (TBA) and trichloroacetic acid (TCA) solution (100 mL contained 375 mg TBA, 15 mg TCA, 2 mL concentrated HCl added to 98 mL of TBA-TCA solution) were added to the reaction mixture, which was then heated in a boiling water bath for 15 min. After cooling, absorbance was measured at 535 nm, with butylated hydroxytoluene (BHT) as positive control (Halliwell, Gutteridge, & Aruoma, 1987). The hydroxyl radical Scavenging activity of PS was calculated by the following equation:

$$\text{Scavenging activity (\%)} = \{(A0-A1)/ A0\} \times 100$$

Where A0 was the absorbance of the control and A1 was the absorbance in the presence of sample. The effect of hydroxyl radical Scavenging activity was expressed as IC_{50}: the amount of the sample needed to inhibit hydroxyl radical concentration by 50%.

2.G.2. Superoxide radical scavenging activity

The method by Martinez, Marcelo, Marco, and moacyr (2001) for determination of the superoxide radical was followed with modification in the riboflavin-light-nitroblue tetrazolium (NBT) system. Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 500 µM solution of various concentrations

(50 - 400 µg/mL) of PS, 100 µM EDTA, 75 µM NBT and 2 µM riboflavin. One set of reaction mixtures were exposed to light for 10 min to activate the riboflavin-NBT and the absorbance of each mixture was measured at 560 nm against identical mixtures from another set kept in the dark for the same duration. butylated hydroxyanisole (BHA) was used as a positive control. The superoxide radical Scavenging activity was calculated by the following formula:

$$\text{Scavenging activity (\%)} = \{(A0-A1)/ A0\} \times 100$$

Where A0 was the absorbance of the control and A1 was the absorbance in the presence of sample. IC_{50} value is the effective concentration at which the superoxide radicals were scavenged by 50%.

2.G.3. Determination of reducing power assay

The Reducing power of the samples was determined according to the method of Oyaizu (1986) with little modification. The reaction mixture contains various concentrations of ELPS (100-800 µg/mL), 1.5 mL sodium phosphate buffer (0.2 M, pH 6.6) and 1.5 mL of potassium ferricyanide (1%) solution. The mixture was incubated for 20 min. The reaction was terminated by adding 1.5 mL of trichloroacetic acid (10%). Then, 2.5 mL distilled water and 0.25 mL $FeCl_3$ (0.1%) were added to the reaction mixture (2.5 mL) and incubated for 15 min. The absorbance was measured at 700 nm. Ascorbic acid was used as standard. IC_{50} value represented the concentration of the compounds that providing 0.5 of absorbance. Increased absorbance of the reaction mixture indicates increased reducing power of the sample.

2.G.4. Determination of Total antioxidant capacity

The total antioxidant capacity was determined as described by Prieto, Pineda, and Aguilar (1999) with some modification. 0.3 mL of PS with varying concentration (0.1-1 mg) was added to 3 mL of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95 °C for 90 min. After cooling to room temperature, the absorbance of

the aqueous solution of each was measured at 695 nm against a blank. Ascorbic acid was used as standard and the total antioxidant capacity is expressed as equivalents of ascorbic acid.

2.H. Biological activities

2.H.1. Isolation of lymphocytes from peripheral blood mononuclear cells

Fresh blood samples were collected from all groups of individuals satisfying the Helsinki protocol. The lymphocytes were isolated from heparinized blood samples according to the method applied earlier by Chattopadhyay et al. (2013). Blood was diluted with phosphate-buffered saline (pH 7.0) in equal ratio and then layered very carefully on the density gradient (histopaque 1077) in a ratio of 1:2, centrifuged at 1400 rpm for 20 min and the white milky layer of mononuclear cells were carefully removed and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/mL penicillin and 100µg/mL streptomycin, 4 mM L-glutamine under 5% CO_2 and 95% humidified atmosphere at 37 °C for 2 hrs. After 2 hrs, non adherent layer of the cultured cells were washed twice with the PBS and centrifuged at 2,000 rpm for 10 min to get the required pellet of lymphocytes.

2.H.2. Cell viability

Normal human lymphocytes were seeded into 96 wells of tissue culture plates having 180 µL of complete media and were incubated for 48 h. PS-II was added to the cells at different concentrations (50, 100, 200, and 400 µg/mL) and incubated for 24 h at 37 °C in a humidified incubator (NBS) maintained with 5 % CO_2. The cell viability was estimated by 3-(4,5-dimethylthiazol)-2- diphenyltetrazolium bromide (MTT) method as applied earlier (Chattopadhyay et al. 2012).

2.H.3. Cell lysate preparation

After treatment schedule, the cell suspension was collected in a centrifuge tube and centrifuged at 1500 rpm for 5 min. The supernatants were collected and stored at −20 °C. The cell pellets were re-suspended in ice cold phosphate buffered saline (PBS) at concentrations ranging from 2×10^5 cells /mL and subjected to four cycles of freeze-thaw cycles (alternating liquid nitrogen and 37 °C water bath treatment) followed by sonication for 20 sec (Ultrasonic Processor, Tekmar, Cincinnati, OH, USA) on ice. Lysates were centrifuged at 12,000 rpm for 20 min at 4 °C to remove cellular debris as adopted earlier by Chattopadhyay et al. (2013). Protein content of lysate preparations was measured according to Lowry**,** Rosenbrough, Farr, and Randall **(**1951) using bovine serum albumin as standard.

2.H.4. Determination of reduced glutathione (GSH)

Reduced glutathione estimation in the cell lysate was performed by the method as applied in earlier publication (Mahapatra et al., 2009). The required amount of the cell lysate was mixed with 25% of trichloroacetic acid and centrifuged at 2,000X g for 15 min to settle the precipitated proteins. The supernatant was aspirated and diluted to 1 mL with 0.2 M sodium phosphate buffer (pH 8.0). Later, 2 ml of 0.6 mM DTNB was added. After 10 min the optical density of the yellow-colored complex formed by the reaction of GSH and DTNB (Ellman's reagent) was measured at 405 nm. A standard curve was obtained with standard reduced glutathione. The levels of GSH were expressed as μg of GSH/mg protein.

2.H.5. Determination of Oxidized glutathione level (GSSG)

The oxidized glutathione level was measured after derivatization of GSH with 2-vinylpyidine according to the method as applied earlier (Mahapatra et al., 2009). In brief, with 0.5 mL cell lysate, 2 μL 2-vinylpyidine was added and incubates for 1 hr at 37 °C. Then the mixture was deprotenized with 4% sulfosalicylic acid and centrifuged at 1,000X

g for 10 min to settle the precipitated proteins. The supernatant was aspirated and GSSG level was estimated with the reaction of DTNB at 412 nm in spectrophotometer and calculated with standard GSSG curve.

2.H.6. Determination of lipid peroxidation (MDA)

Lipid peroxidation was estimated by the method of Ohkawa, Ohishi, and Yagi, (1979) in cell lysate. Briefly, the reaction mixture contained Tris-HCl buffer (50 mM, pH 7.4), tert-butyl hydroperoxide (BHP) (500 μM in ethanol) and 1 mM $FeSO_4$. After incubating the samples at 37 °C for 90 min, the reaction was stopped by adding 0.2 mL of 8% sodium dodecyl sulfate (SDS) followed by 1.5 mL of 20% acetic acid (pH 3.5). The amount of malondialdehyde (MDA) formed during incubation was estimated by adding 1.5 mL of 0.8% TBA and further heating the mixture at 95 °C for 45 min. After cooling, samples were centrifuged and the TBA reactive substances (TBARS) were measured in supernatants at 532 nm by using $1.53 \times 10^5\ M^{-1}cm^{-1}$ as extinction coefficient. The levels of lipid peroxidation were expressed in terms of n mol/mg protein.

2.H.7. NO release assay

The NO concentration was measured by a microplate assay method with Griess reagent (1% sulfanilamide, 0.3% naphthylethylenediaminedihydrochloride, 7.5% H_3PO_4). Briefly, culture supernatants (100 μL) were mixed with 100 μL of the Griess reagent. The nitrite concentration in the culture supernatant was measured at an absorbance of 550 nm, 10 min after mixing (Hino et al. 2005).

2.H.8. Protective role

Normal human lymphocytes ($4X10^6$) were seeded into 96 wells plate and treated with 10mM nicotine for 6 h at 37 °C, since this dose was found lethal as reported earlier (Mahapatra et al., 2009). After incubation, cells were washed with 1X PBS (50 mM) for 3 times and incubated with PS-II for 24h at 37 °C. The cell viability was estimated by 3-(4,5-dimethylthiazol)-2- diphenyltetrazolium bromide according to the method as applied

earlier (Chattopadhyay et al. 2012). The apoptotic cells were visualized by propidium iodide staining under phase contrast fluoresce microscope (50X magnifications).

2.H.9. Statistical analysis

The data were expressed as the mean ± the standard error of the mean (n = 6). Comparisons between the means of control and treated groups were made by one-way analysis of variance (using a statistical package; Origin 6.1, Origin Lab, Northampton, MA, USA) with multiple-comparison tests, with $p < 0.05$ as the limit of significance. The correlation analysis was performed using Statistica software version 8.0.

2.I. Conclusion

The methodologies that have been adopted to determine the structure of polysaccharides and their biological and also some antioxidant studies have been discussed in this chapter. The structure of the repeating unit of the polysaccharides has been determined using two types of methods: (1) chemical method that includes total acid hydrolysis, paper chromatographic, methylation, periodate oxidation, Smith degradation, and partial acid hydrolysis studies, (2) spectroscopic method comprising of 1D and 2D NMR experiments.

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CHAPTER 3

Structural, antioxidant and biological studies of two polysaccharides isolated from the alkaline extract of the edible mushroom Entoloma lividoalbum (Kühner & Romagn.) Kubička.

3.A. Introduction and earlier works

Mushroom belongs to a special group of macroscopic fungi. The edible mushrooms are attractive because of their flavor, taste, delicacy, and healthy properties (Villares, Mateo-Vivaracho, & GuillamÓn, 2012). β-D-glucan, a well-known biological response modifier (BRM) (Bohn & BeMiller, 1995) is widely distributed in nature and used as food and medicine (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007; Wasser & Weis, 1999). The uncontrolled production of oxygen derived free radicals plays crucial role related to the pathological processes of various killer diseases, such as cancer, inflammation, atherosclerosis, coronary heart disease, alzheimer's disease, neurodegenerative disorders etc. (Halliwell & Gutteridge, 1989). Antioxidants are substances that delay or prevent oxidation by scavenging or preventing reactive oxygen species (ROS) generation. Synthetic compounds have strong radical scavenging activity but they have side effects also (Zhou & Zheng, 1991). Neutralization of activity of these radicals by naturally occurring substances is now most acceptable method of modern therapy. Amongst them, mushrooms occupy an elite position in this regards (Patra et al., 2013). Lymphocytes are the primary immune cells in the body. Destruction or strengthening of lymphocyte cells are directly linked to immunity. From this point of view, the use of various natural and synthetic drugs has drawn the remarkable attention now a day.

Entoloma lividoalbum (Kühner & Romagn.) Kubička is a basidiomycetes fungus. No works related to the polysaccharide of this mushroom are reported in literate. In the present investigation, two water soluble polysaccharides (PS-I & PS-II) have been isolated from the alkaline extract of this mushroom through Sephrarose gel filtration. The PS-I has been investigated as glucan and PS-II a heteroglycan. The detailed structural investigation and study of biological activities of PS-I and PS-II were carried out and discussed in this chapter.

3.B. Present work

3.B.1. Collection and identification of *Entoloma lividoalbum*

Fruit bodies of the edible mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička (**Figure 1**), commonly called as 'Salle chyau' (Nepali), were colleted from Sikkim Himalaya region, India (Das, 2010). These are consumed as food by the people of these areas for nutritional delicacy. Dr. Krishnendu Acharya, Department of Botany, University of Calcutta, West Bengal, India, is a mycologist who identified the mushroom.

Figure 1. Photograph of the fruit bodies of the edible mushroom *Entoloma lividoalbum.*

3.B.2. Isolation and purification of polysaccharides from *Entoloma lividoalbum*

Fresh fruit bodies of the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička (600 g) were gently washed with distilled water several times and the mushroom bodies were crushed and boiled with 4 % NaOH solution for 1 h. A water soluble crude

polysaccharide (700 mg) was isolated from the mushroom *Entoloma lividoalbum* followed by cooling, filtration, centrifugation and EtOH precipitation. 30 mg water soluble crude polysaccharide was passed through Sepharose 6B column in aqueous medium and two fractions, PS-I (13 mg) and PS-II (9 mg) were obtained (**Figure 2**).

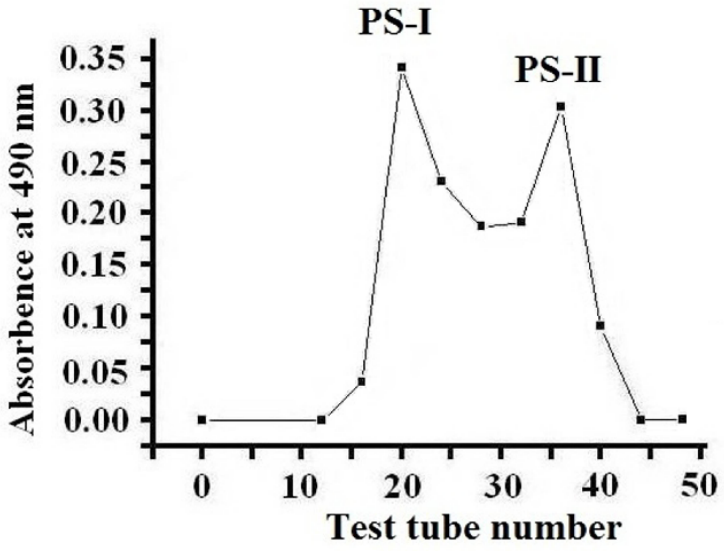


Figure 2. Gel permeation chromatogram of crude polysaccharide isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. using Sepharose 6B column.

3.B.3. Optical rotation and molecular weight of PS-I and PS-II

The specific rotation of PS-I was measured $[\alpha]_D^{32.1}$ -14.6 (*c* 0.054, H_2O) and that of PS-II was $[\alpha]_D^{31.8}$ +16.5 (*c* 0.09, H_2O). The molecular weight (Hara, Kiho, Tanaka, & Ukai, 1982) of PS-I and PS-II were estimated as ~2.1 × 10^5 Da and ~5.2 × 10^4 Da respectively, from a calibration curve prepared with standard dextrans (**Figure 3**).

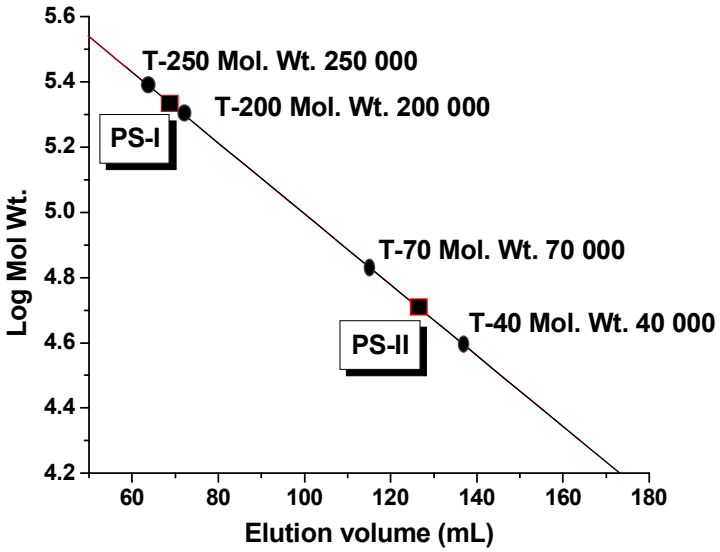


Figure 3. Determination of molecular weight of the polysaccharides isolated from hot alkaline extract of fruit bodies of edible mushroom *Entoloma lividoalbum.*

3.B.4. Structural analysis of PS-I

3.B.4.1. Chemical analysis of PS-I

GLC analysis of the alditol acetates (Lindahl, 1970) of PS-I revealed the presence of glucose only. The absolute configuration of the monosaccharide was determined by the method of Gerwig, Kamerling, and Vliegenthart, (1978) and it was found that glucose was present as D-configuration in the PS-I. The mode of linkages of the sugar moieties present in the PS-I was determined by methylation analysis using the Ciucanu and Kerek (1984) 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-2,3,4.6-tetra-*O*-methyl-D-glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol, and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a molar ratio of approximately 1:3:2:1 (**Table 1**, **Figure 4**). These results indicated the presence of terminal D-glucopyranosyl, (1→3)-D-glucopyranosyl, (1→6)-D-glucopyranosyl, and (1→3,6)-D-glucopyranosyl residues in the PS-I.

Table 1

GLC-MS analysis of methylated polysaccharide (PS-I) isolated from *Entoloma lividoalbum* (Kühner & Romagn.) Kubička

Methylated sugars	Molar ratio	Linkage type	Major Mass Fragments (m/z)
2,4,6-Me_3-Glc	3	→3)-β-D-Glc*p*-(1→	43,45,71,87,101,117,129,143, 161,173,203,217,233
2,3,4,6-Me_4-Glc	1	β-D-Glc*p*-(1→	43,45,59,71,87,101,117,129,161, 205
2,4-Me_2-Glc	1	→3,6)-β-D-Glc*p*-(1→	43,58,87,101,117,129,139,159, 189,201,233
2,3,4-Me_3-Glc	2	→6)-β-D-Glc*p*-(1→	43,45,58,71,87,99,101,117,129, 161,173,189,233

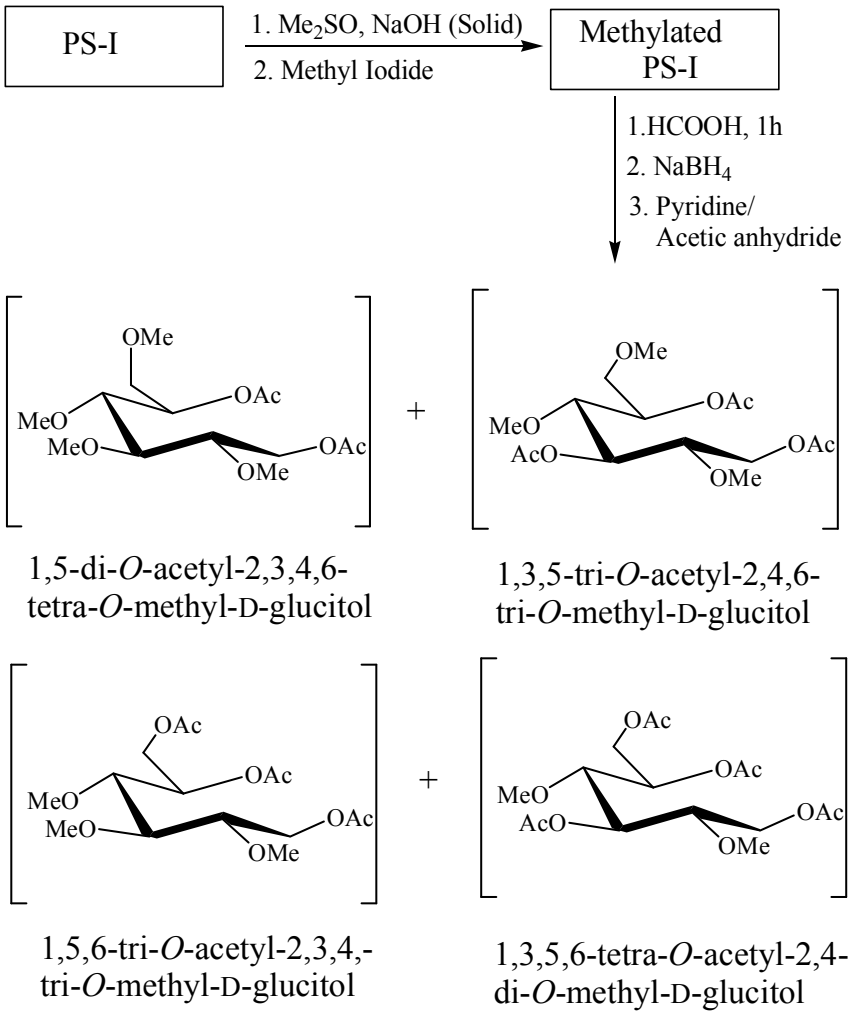


Figure 4. Schematic presentation of methylation experiment of PS-I isolated from *Entoloma lividoalbum.*

GLC analysis of alditol acetates of the periodate-oxidized (Goldstein, Hay, Lewis, & Smith, 1965; Hay, Lewis, & Smith, 1965), $NaBH_4$-reduced PS-I was found to contain glucose unit only and periodate-oxidized, reduced, methylated (Abdel-Akher & Smith, 1950) PS-I showed the presence of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol in a molar ratio of nearly 1:3 (**Table 2**, **Figure 5**). These results showed that the (1→6)-D-glucopyranosyl and terminal D-glucopyranosyl residues were consumed during oxidation whereas, (1→3,6)-D-

glucopyranosyl and (1→3)-D-glucopyranosyl residues remain unaffected. All these results indicated that the PS-I is a branched glucan and may have three possible repeating units: a (1→6)-linked backbone, a (1→3)-linked backbone or an alternatively (1→3)-, (1→6)-linked backbone. Therefore, Smith degradation and partial hydrolysis was performed for determination of the backbone present in the PS-I. Mild hydrolysis was carried out with the periodate-oxidised, reduced PS-I to get Smith degradation product (SDPS). The GLC analysis of the alditol acetates of Smith degraded hydrolyzed product showed the presence of D-glucose and D-glycerol. The GLC-MS analysis of the alditol acetates of methylated, reduced SDPS revealed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in a molar ratio of nearly 3:1. Partial hydrolysis of the PS-I was carried out with 0.1 M TFA to know the sequence of D-Glc*p* moieties in the repeating unit. As a result of this hydrolysis, two fractions were obtained; partially hydrolyzed oligosaccharide (F1) and partially hydrolyzed polysaccharide (F2). GLC-MS analysis of the methylated product of F1 revealed the presence of (1→3)-D-glucopyranosyl, and terminal D-glucopyranosyl moieties and methylation analysis of F2 revealed the presence of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol only. This result clearly indicated that F2 polysaccharide (backbone chain of the PS-I) consists of three (1→6)-D-glucopyranosyl residues, one of which was branched at *O*-3 position with the side chain consisting of three (1→3)-D-glucopyranosyl and a terminal D-glucopyranosyl residue.

Table 2

GLC-MS analysis of methylated polysaccharide (PS-I) isolated from *Entoloma lividoalbum* (Kühner & Romagn.) Kubička

Methylated sugars	Molar ratio	Linkage type	Major Mass Fragments (m/z)
2,4,6-Me_3-Glc	3	→3)-β-D-Glc*p*-(1→	43,45,71,87,101,117,129,143, 161,173,203,217,233
2,4-Me_2-Glc	1	→3,6)-β-D-Glc*p*-(1→	43,58,87,101,117,129,139, 159,189,201,233

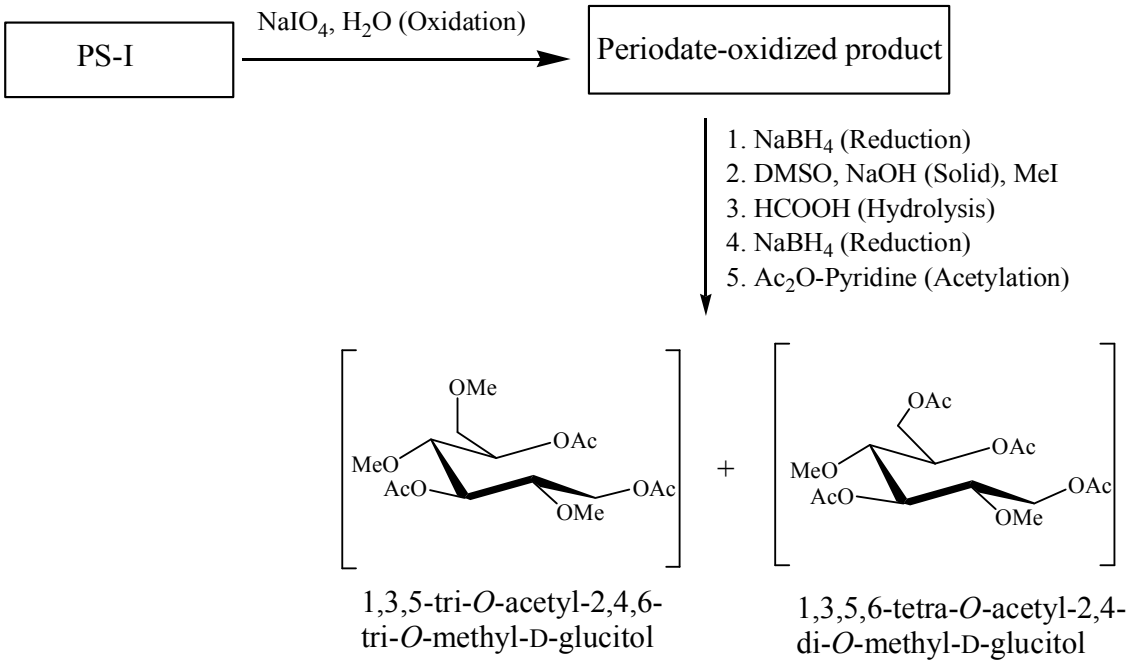


Figure 5. Schematic presentation of periodate oxidation reactions of PS-I isolated from *Entoloma lividoalbum.*

3.B.4.2 NMR and structural analysis of the PS-I

In the ^{1}H NMR spectrum (500 MHz; **Figure 6**, **Table 3**) of PS-I, four anomeric signals at δ 4.77, 4.73, 4.50, and 4.49 were observed at 30 °C in a ratio of nearly 2:1:1:3. The peak at δ 4.77, 4.73, and 4.50 designated as **A**, **B**, and **D**, whereas the peak at δ 4.49 consists of **C**, **E**, and **F** residues.

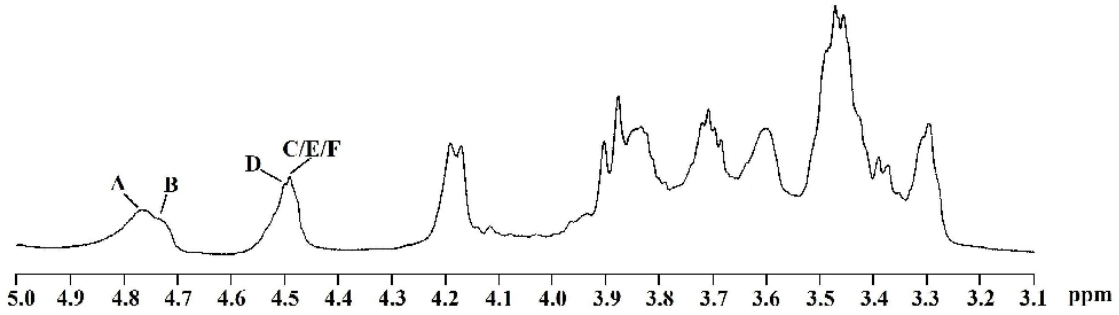


Figure 6. ^{1}H NMR spectrum (500 MHz, D_2O, 30 ºC) of PS-I, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička.

In ^{13}C NMR spectrum (125 MHz; **Figure 7**, **Table 4**) at the same temperature, five signals were observed in the anomeric region at δ 103.0, 102.9, 102.8, 102.7, and 102.6. On the basis of HSQC spectrum (**Figure 8**), the anomeric carbon signal at δ 102.9 was correlated to both the proton signals δ 4.77 (**A**) and δ 4.73 (**B**) respectively. Again, the anomeric proton signal at δ 4.50 was correlated to the carbon signal at δ 102.6 (**D**) whereas the peak at δ 4.49 was correlated to the anomeric carbon signals at δ 103.0 (**C**), δ 102.8 (**E**), and δ 102.7 (**F**). All the ^{1}H and ^{13}C signals (**Table 3** and **4**) were assigned from DQF-COSY, TOCSY, and HSQC experiments. The proton coupling constants were measured from DQF-COSY experiment.

Table 3

The ^{1}H NMR[a] chemical shifts for the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička in D_2O at 30 ºC.

Glycosyl residue	H-1	H-2	H-3	H-4	H-5	H-6a,H-6b
→3)-β-D-Glc*p*-(1→ **A**	4.77	3.31	3.71	3.43	3.48	3.71[c], 3.90[d]
→3)-β-D-Glc*p*-(1→ **B**	4.73	3.31	3.72	3.43	3.48	3.71[c], 3.90[d]
β-D-Glc*p*-(1→ **C**	4.49	3.30	3.47	3.37	3.43	3.68[c], 3.88[d]
→3,6)-β-D-Glc*p*-(1→ **D**	4.50	3.47	3.70	3.45	3.60	3.82[c], 4.17[d]
→6)-β-D-Glc*p*-(1→ **E**	4.49	3.30	3.45	3.43	3.60	3.83[c], 4.19[d]
→6)-β-D-Glc*p*-(1→ **F**	4.49	3.30	3.45	3.43	3.60	3.83[c], 4.19[d]

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

[c,d] Interchangeable.

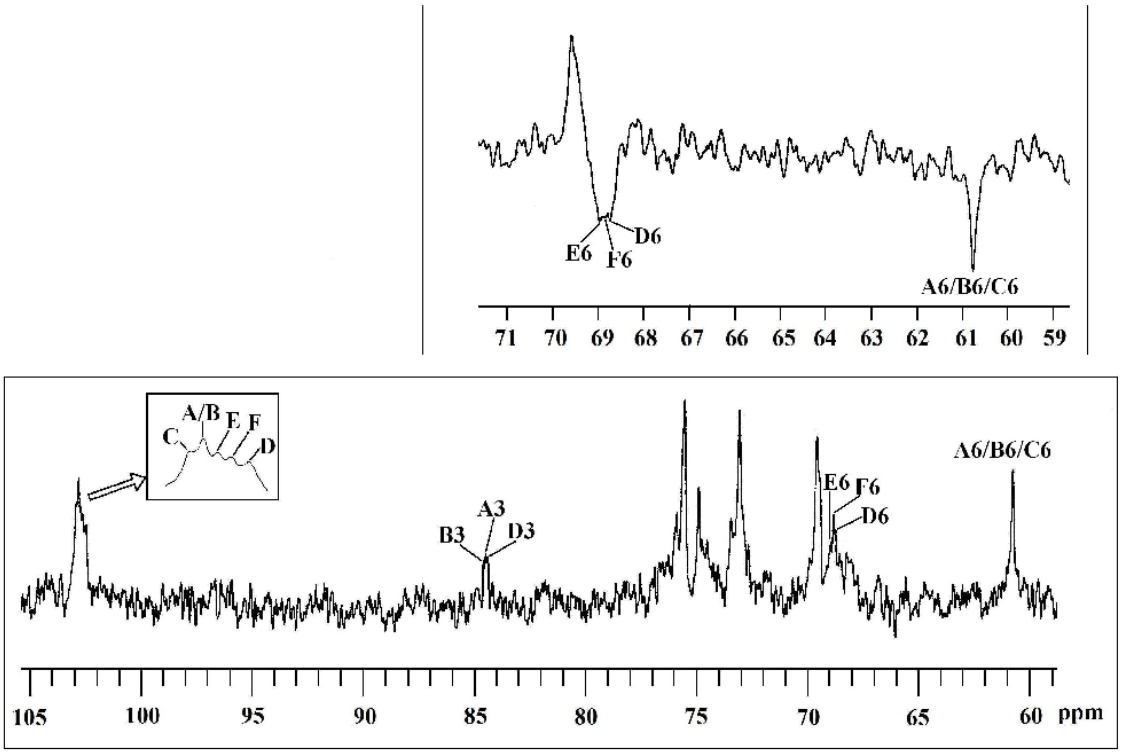


Figure 7. ^{13}C NMR spectrum (125 MHz, D_2O, 30 ºC) of PS-I, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. Part of DEPT-135 spectrum (D_2O, 30 ºC) of PS-I, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička (inset).

Table 4

The ^{13}C NMR[b] chemical shifts for the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička in D_2O at 30 ºC.

Glycosyl residue	C-1	C-2	C-3	C-4	C-5	C-6
→3)-β-D-Glc*p*-(1→ **A**	102.9	72.8	84.5	69.6	75.6	60.8
→3)-β-D-Glc*p*-(1→ **B**	102.9	72.8	84.6	69.6	75.6	60.8
β-D-Glc*p*-(1→ **C**	103.0	73.1	75.6	69.6	75.6	60.8
→3,6)-β-D-Glc*p*-(1→ **D**	102.6	72.8	84.4	69.6	75.0	68.8
→6)-β-D-Glc*p*-(1→ **E**	102.8	73.1	75.6	69.6	75.0	69.0
→6)-β-D-Glc*p*-(1→ **F**	102.7	73.1	75.6	69.6	75.0	68.9

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

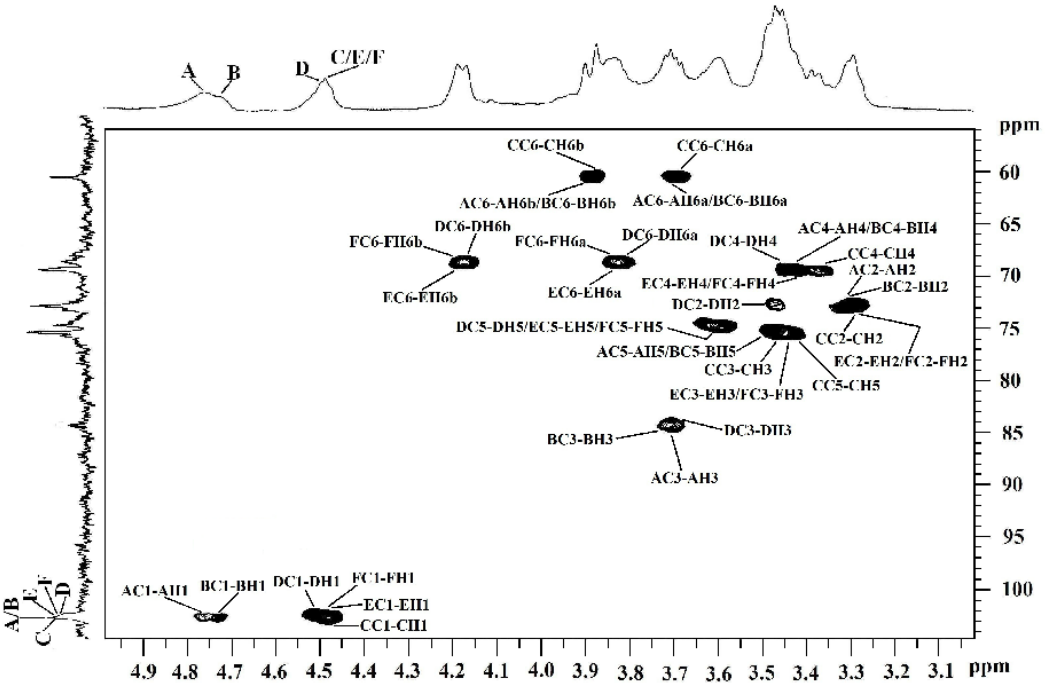


Figure 8. HSQC spectrum of PS-I, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička.

The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values (~10.0 Hz) confirmed glucopyranosyl configuration (Glc*p*) of all the residues from **A** to **F**. Anomeric proton chemical shifts (δ 4.77-4.49), anomeric carbon chemical shifts (δ 103.0-102.6), and the coupling constant values $J_{H\text{-}1,H\text{-}2}$ (~8.0 Hz), $J_{C\text{-}1,H\text{-}1}$ (~160-161 Hz) confirmed that the residues (**A** - **F**) were present in β-configuration. The downfield shift of C-3 of **A** (δ 84.5) and **B** (δ 84.6) with respect to standard value of methyl glycoside (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that residue **A** and **B** were (1→3)-β-D-Glc*p* moiety. In residue **C**, all carbon chemical shifts values were found nearly to the standard value of methyl glycoside (Agrawal, 1992; Rinaudo & Vincendon, 1982) of β-D-glucose. Thus, residue **C** was non-reducing end β-D-Glc*p*. The downfield shifts at C-3 (δ 84.4) and C-6 (δ 68.8) of residue **D** with respect to standard value of methyl glycoside (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that it was (1→3,6)-β-D-Glc*p* moiety. Since the residue **D** was the most rigid part of the backbone of this glucan, its C-3 (δ

84.4) appeared at slightly up field region compared to C-3 of (1→3)-linked residue **A** (δ 84.5) and **B** (δ 84.6). Consequently, the C-6 (δ 68.8) value of residue **D** appeared slightly up field region compared to that of the other (1→6)-linked residues (**E** and **F**). All the chemical shift values of **E** and **F** residues were same except the chemical shifts values of C-1 and C-6. The different downfield shifts of C-6 (δ 69.0 and 68.9) of **E** and **F** residues supported the presence of (1→6)-β-D-Glc*p* moiety with different chemical environments. Between **E** and **F** residues, **E** residue was glycosidically linked to the most rigid part **D**, hence, its C-6 signal (δ 69.0) showed δ 0.1 downfield shift with respect to C-6 signal of residue **F** (δ 68.9) due to neighboring effect (Bhanja et al., 2012) of the rigid part **D**. The linking at C-6 of the residues **D**, **E**, and **F** were further confirmed by DEPT-135 spectrum (**Figure 7**).

The sequence of glycosyl residues (**A** to **F**) were determined from NOESY (**Figure 9**, **Table 5**) as well as ROESY (not shown) studies followed by confirmation with HMBC experiment. In NOESY experiment, the inter-residual contacts **A**H-1/**B**H-3; **B**H-1/**D**H-3; **C**H-1/**A**H-3; **D**H-1/**E**H-6a, 6b; **E**H-1/**F**H-6a, 6b; and **F**H-1/**D**H-6a, 6b along with other intra-residual contacts were also observed (**Figure 9**). The above NOESY connectivities established the following sequences:

F (1→6) **D**; **D** (1→6) **E**; **E** (1→6) **F**; **B** (1→3) **D**; **A** (1→3) **B**; and **C** (1→3) **A**.

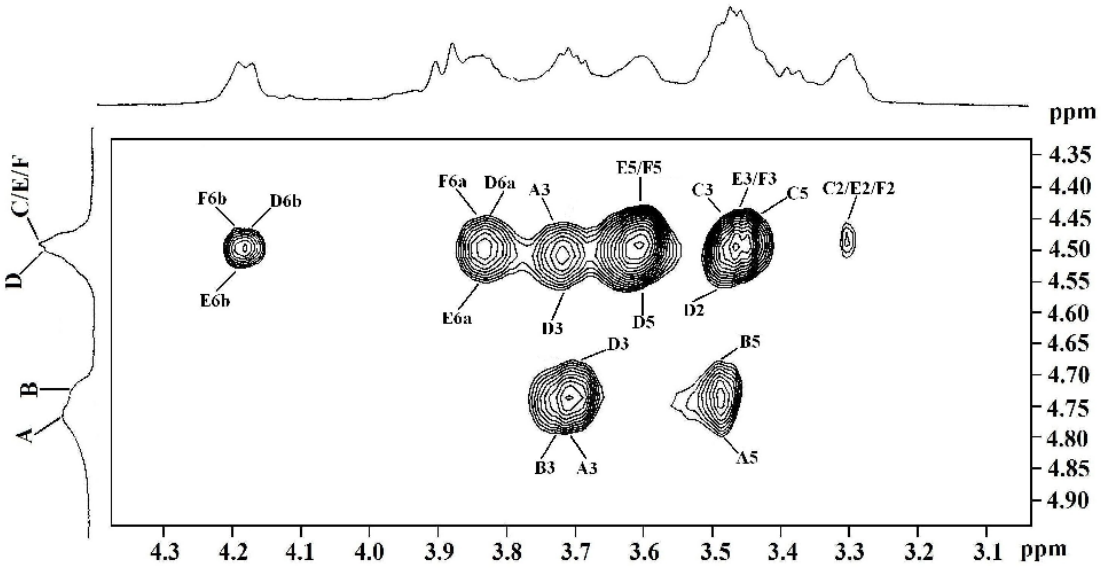


Figure 9. Part of NOESY spectrum of PS-I, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. The NOESY mixing time was 300 ms.

Table 5

NOESY data for the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička.

Glycosyl residue	**Anomeric proton**	**NOE contact proton**		
	δ	**δ**	**residue**	**atom**
→3)-β-D-Glc*p*-(1→	4.77	3.72	**B**	H-3
A		3.71	**A**	H-3
		3.48	**A**	H-5
→3)-β-D-Glc*p*-(1→	4.74	3.70	**D**	H-3
B		3.72	**B**	H-3
		3.48	**B**	H-5

β-D-Glc*p*-(1→	4.49	3.71	**A**	H-3
C		3.30	**C**	H-2
		3.47	**C**	H-3
		3.43	**C**	H-5
→3,6)-β-D-Glc*p*-(1→	4.50	3.83	**E**	H-6a
D		4.19	**E**	H-6b
		3.47	**D**	H-2
		3.70	**D**	H-3
		3.60	**D**	H-5
→6)-β-D-Glc*p*-(1→	4.49	3.83	**F**	H-6a
E		4.19	**F**	H-6b
		3.30	**E**	H-2
		3.45	**E**	H-3
		3.60	**E**	H-5
→6)-β-D-Glc*p*-(1→	4.49	3.82	**D**	H-6a
F		4.17	**D**	H-6b
		3.30	**F**	H-2
		3.45	**F**	H-3
		3.60	**F**	H-5

Finally, these NOESY connectivities were confirmed from HMBC spectrum (**Figure 10**). In this spectrum the inter-residual cross-peaks (**Table 6**) between **A**H-1/**B**C-3, **A**C-1/**B**H-3; **B**H-1/**D**C-3, **B**C-1/**D**H-3; **C**H-1/**A**C-3, **C**C-1/**A**H-3; **D**H-1/**E**C-6, **D**C-1/**E**H-6a, 6b; **E**H-1/**F**C-6, **E**C-1/**F**H-6a, 6b; **F**H-1/**D**C-6, **F**C-1/**D**H-6a, 6b along with some intra-residual peaks were also observed (**Figure 10**).

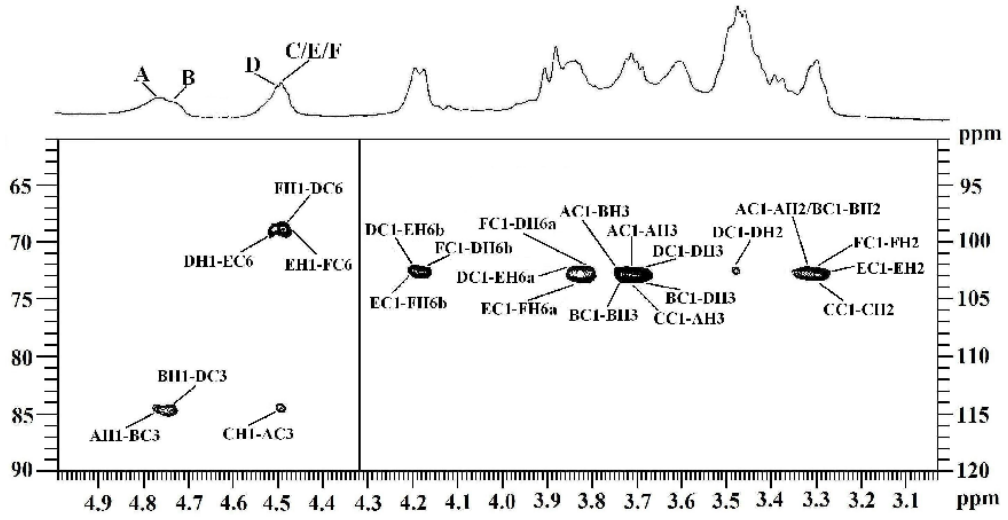


Figure 10. Part of HMBC spectrum of PS-I, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. The delay time in the HMBC experiment was 80 ms.

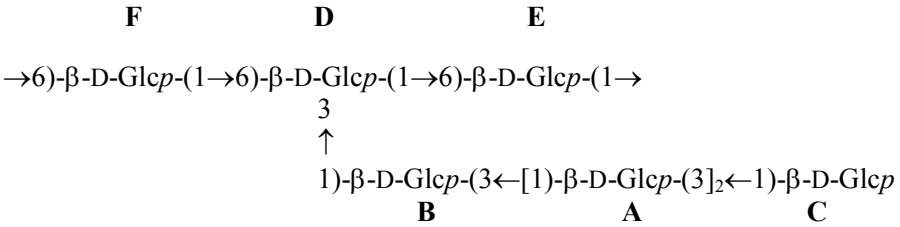
Table 6

The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička

Residues	Sugar linkage	H-1/C-1	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	→3)-β-D-Glc*p*-(1→	4.77	84.6	**B**	C-3
		102.9	3.72	**B**	H-3
			3.31	**A**	H-2
			3.71	**A**	H-3
B	→3)-β-D-Glc*p*-(1→		84.4	**D**	C-3
		4.74	3.70	**D**	H-3
		102.9	3.31	**B**	H-2
			3.72	**B**	H-3

C	β-D-Glc*p*-(1→	4.49	84.5	**A**	C-3
		103.0	3.71	**A**	H-3
			3.30	**C**	H-2
D	→3,6)-β-D-Glc*p*-(1→	4.50	69.0	**E**	C-6
		102.6	3.83	**E**	H-6a
			4.19	**E**	H-6b
			3.47	**D**	H-2
			3.70	**D**	H-3
E	→6)-β-D-Glc*p*-(1→	4.49	68.9	**F**	C-6
		102.8	3.83	**F**	H-6a
			4.19	**F**	H-6b
			3.30	**E**	H-2
F	→6)-β-D-Glc*p*-(1→	4.49	68.8	**D**	C-6
		102.7	3.82	**D**	H-6a
			4.18	**D**	H-6b
			3.30	**F**	H-2

Thus, the NOESY and HMBC spectrum analysis indicated that the PS-I is a branched glucan with (1→6)-β-D-glucopyranosyl backbone and branching at *O*-3 of **D** residue with (1→3)-β-D-Glc*p* (**B**) followed by (1→3)-linked **A** residue and non-reducing end **C** residue. Hence NOESY and HMBC connectivities confirmed the structure of repeating unit presence in the PS-I as:



NMR experiments were again carried out with Smith degradation product (SDPS) and partially hydrolyzed polysaccharide (F2) of the PS-I for further confirming the linkages. The ^{13}C NMR (125 MHz) spectrum (**Figure 11**, **Table 7**) at 30 ˚C of SDPS showed one anomeric carbon signal at δ 102.6 for one terminal β-D-Glc*p* (**G**) and three (1→3)-β-D-Glc*p* (**H**) residues. The C-1, C-2, and C-3 carbon signals of the glycerol moiety (Gro) were assigned as δ 68.1, 72.1, and 62.6 respectively. The glycerol moiety (**I**) was generated from (1→6)-β-D-Glc*p* residue (**E**) after periodate oxidation followed by Smith degradation which was attached to (1→3)-β-D-Glc*p* moiety (**H**). The terminal β-D-Glc*p* (**G**) was generated from one (1→3)-β-D-Glc*p* (**A**) due to complete oxidation of the terminal β-D-Glc*p* (**C**) and also one (1→3)-β-D-Glc*p* (**H**) was produced from the (1→3,6)-β-D-Glc*p* (**D**) due to oxidation followed by Smith degradation of the (1→6)-β-D-Glc*p* moiety (**F**) and the other two (1→3)-β-D-Glc*p* (**H**) were retained from (1→3)-β-D-Glc*p* (**A** and **B**).

Table 7

The ^{13}C NMR[n] chemical shifts of Smith-degraded glycerol-containing disaccharide of the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička in D_2O at 30 ºC.

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p*-(1→ **G**	102.6	73.3	75.7	70.7	75.7	60.8
→3)-β-D-Glc*p*-(1→ **H**	102.6	73.1	84.2	69.7	75.7	60.8
→3)-Gro **I**	68.1	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 ºC.

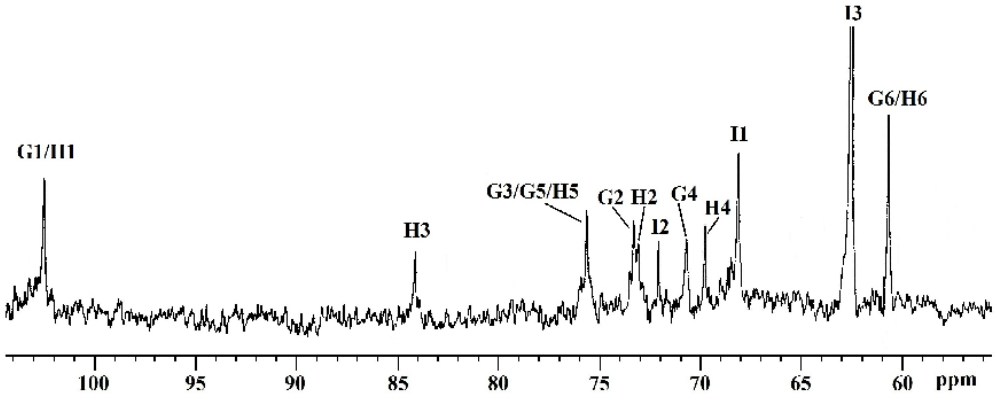


Figure 11. ^{13}C NMR spectrum (125 MHz, D_2O, 30 ºC) of the Smith-degraded glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička.

Hence, Smith degradation results in the formation of a glycerol containing tetrasaccharide from the parent polysaccharide and the structure of SDPS were established as:

G **H** **H** **H** **I**
β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-Gro

Therefore, the above result further confirmed that the branching occurred at *O*-3 of **D** with **B** residue.

The ^{13}C NMR (125 MHz, 30 °C) spectrum of F2, indicated that it was a polymeric chain of simple (1→6)-β-D-Glc*p* units as characteristic signal for C-6 at δ 69.0 was observed. This result further proved that the (1→3)-β-D-Glc*p* moieties were located at the branched point of the (1→6)-linked backbone of the repeating unit. So, all these results indicated that the β-D-glucan (PS-I) isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička, is a branched glucan with (1→6)-linked backbone where branching occurred at *O*-3 of one unit followed by (1→3)-β-D-Glc*p* and terminal β-D-Glc*p* (**Figure 12**).

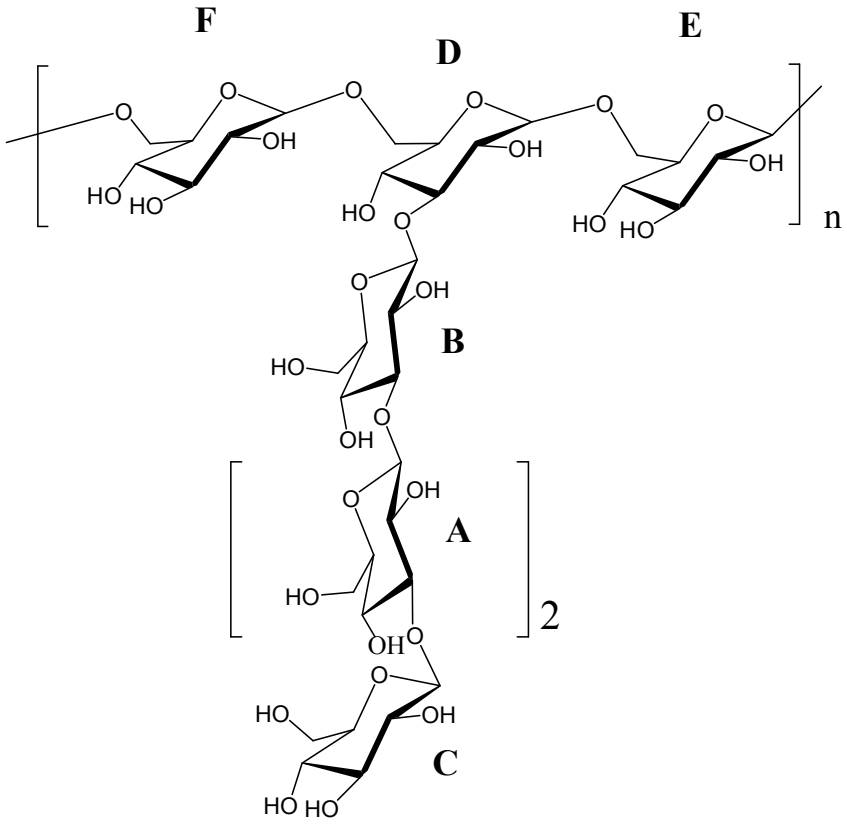


Figure 12. The structure of repeating unit of the β-glucan

3.B.5. Structural analysis of PS-II

3.B.5.1. Chemical analysis of PS-II

GLC analysis of the alditol acetates of the hydrolyzed product of PS-II revealed the presence of glucose, mannose, galactose, and fucose in a molar ratio of nearly 5:1:2:1. The absolute configuration of the monosaccharide was determined by the method of Gerwig, Kamerling, and Vliegenthart, (1978) and found that glucose, galactose, and mannose had the D configuration but fucose was present in the L configuration. The mode of linkages of the sugar moieties present in the PS-II was determined by methylation analysis using the Ciucanu and Kerek (1984) method, followed by hydrolysis and preparation of alditol acetates. The GLC-MS analysis of the alditol acetates of methylated products have been presented in **Table 8**. These linkages (**Figure 13**, **Table 8**) were further confirmed by periodate oxidation experiment. GLC analysis of alditol acetates of the periodate-oxidized (Goldstein, Hay, Lewis, & Smith, 1965; Hay, Lewis, & Smith, 1965), $NaBH_4$-reduced, and hydrolyzed products showed the presence of only glucose, indicating that the D-galactose, D-mannose, and L-fucose moieties were consumed during oxidation. GLC-MS analysis of periodate-oxidized, reduced, methylated (Abdel-Akher & Smith, 1950) PS-II showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a molar ratio of nearly 1:1(**Figure 14**, **Table 9**). These results clearly indicated that the (1→3)-linked and (1→3,6)-linked glucopyranosyl residues remain unaffected whereas all other residues were consumed during oxidation, which further confirmed the mode of linkages present in the PS-II.

Table 8

GLC-MS analysis of methylated polysaccharide (PS-II) isolated from *Entoloma lividoalbum* (Kühner & Romagn.) Kubička

Methylated sugars	Molar ratio	Linkage type	Major Mass Fragments (m/z)
3,4-Me_2-Gal	1	→2,6)-Gal*p*-(1→	43,71,87,99,129,159,173, 189,233
2,3,4-Me_3-Gal	1	→6)-Gal*p*-(1→	43,71,87,99,101,117,129, 161,173,189,233
2,3,4,6-Me_4-Glc	1	Glc*p*-(1→	43,45,59,71,87,101,117, 129,161,205
3,4-Me_2-Fuc	1	→2)-Fuc*p*-(1→	43,5971,89,99,115,129,131, 173,189
2,3,4,6-Me_4-Man	1	Man*p*-(1→	43,45,59,71,87,101,117, 129,161,205
2,4,6-Me_3-Glc	1	→3)-Glc*p*-(1→	43,45,71,87,101,117,129, 143,161,173,203,217,233
2,4-Me_2-Glc	1	→3,6)-Glc*p*-(1→	43,58,87,101,117,129,139, 159,189,201,233
2,3,4-Me_3-Glc	2	→6)-Glc*p*-(1→	43,45,58,71,87,99,101,117, 129,161,173,189,233

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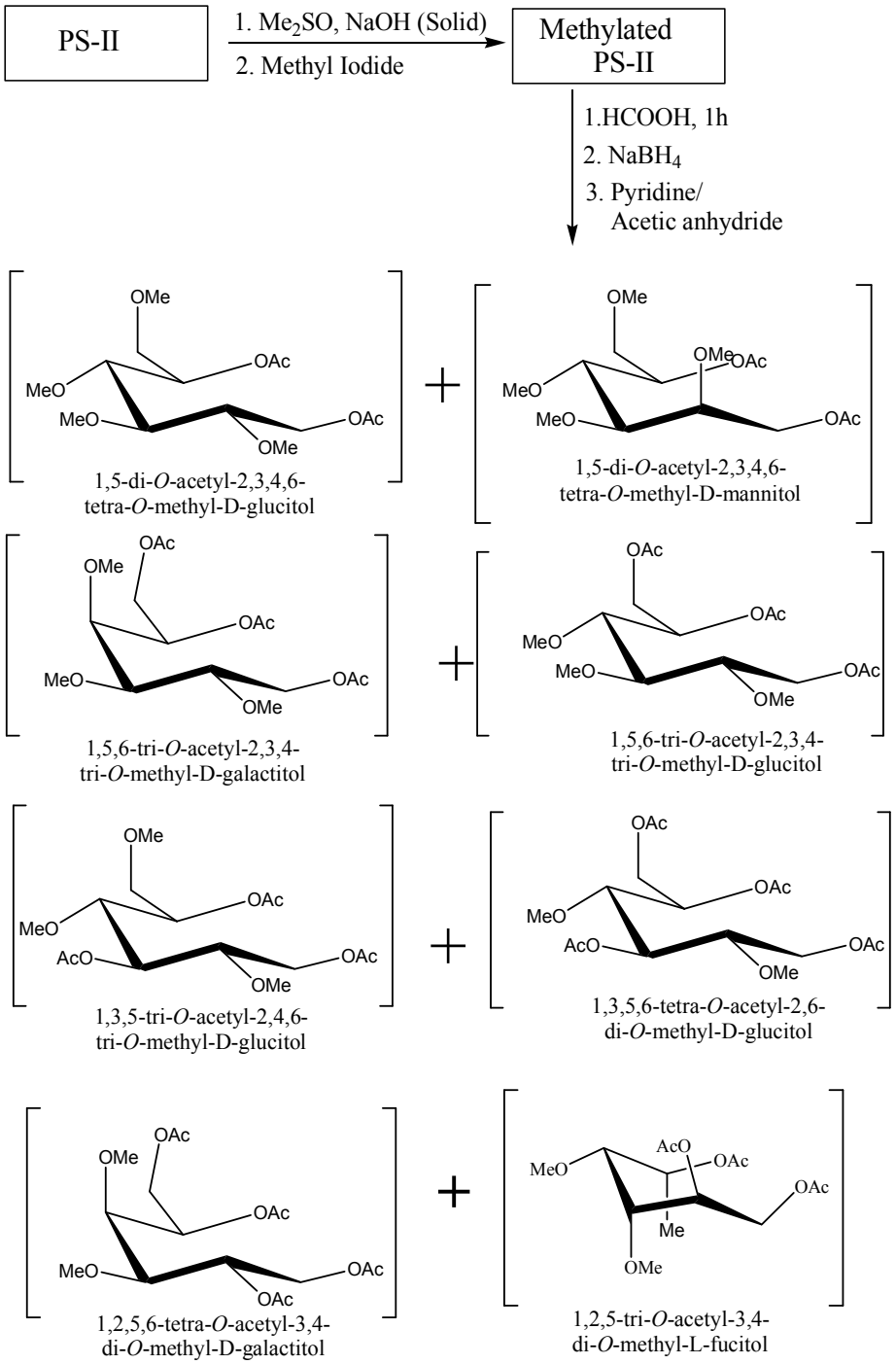


Figure 13. Schematic presentation of methylation experiment of PS-II isolated from *Entoloma lividoalbum.*

Table 9

GLC-MS analysis of methylated polysaccharide (PS-II) isolated from *Entoloma lividoalbum* (Kühner & Romagn.) Kubička

Methylated sugars	Molar ratio	Linkage type	Major Mass Fragments (m/z)
2,4,6-Me_3-Glc	1	→3)-β-D-Glc*p*-(1→	43,45,71,87,101,117,129,143, 161,173,203,217,233
2,4-Me_2-Glc	1	→3,6)-β-D-Glc*p*-(1→	43,58,87,101,117,129,139,159, 189,201,233

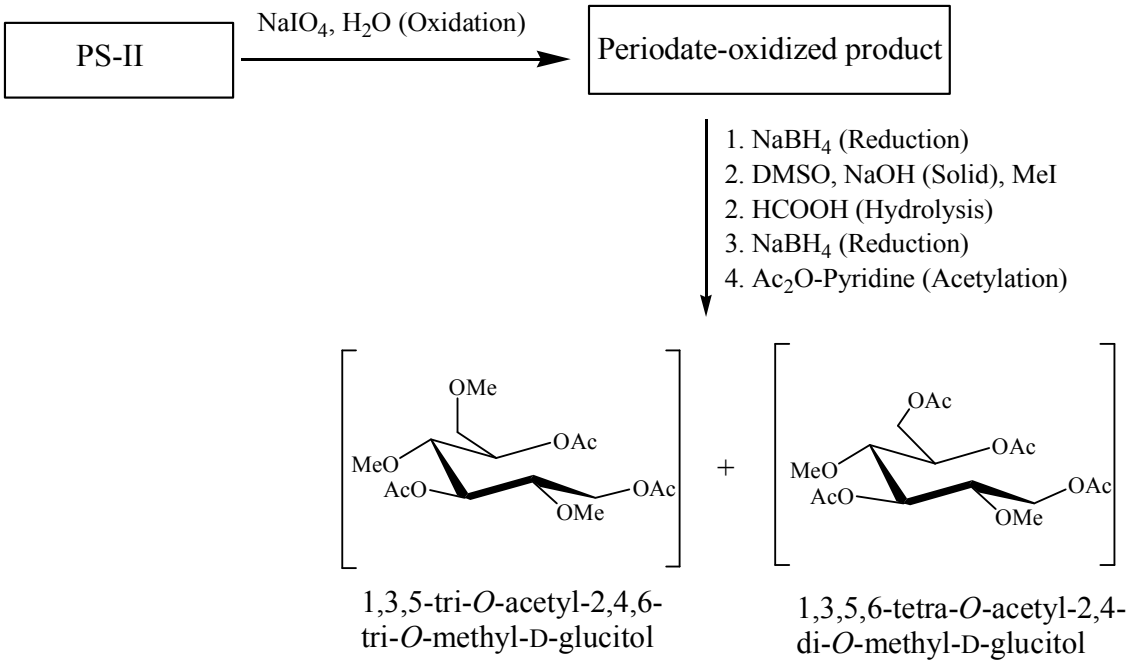


Figure 14. Schematic presentation of periodate oxidation reactions of PS-II isolated from *Entoloma lividoalbum.*

3.B.5.2 NMR and structural analysis of the PS-II

The ^{1}H NMR spectrum (500 MHz; **Figure 15**, **Table 10**) of PS-II at 30 °C showed the presence of nine signals in the anomeric region at δ 5.11, 5.04, 5.03, 4.99, 4.77, 4.73, 4.49, 4.48, and 4.47. The sugar residues were designated as **A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**, and **I** according to their decreasing anomeric proton chemical shifts.

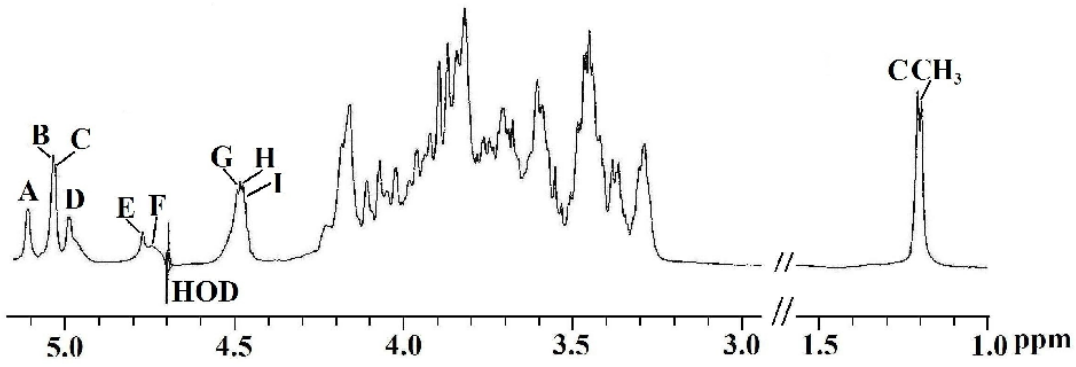


Figure 15. ^{1}H NMR spectrum (500 MHz, D_2O, 30 ºC) of PS-II, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička.

In ^{13}C NMR spectrum (125 MHz; **Figure 16**, **Table 10**) at the same temperature, seven signals were observed in the anomeric region at δ 103.0, 102.9, 102.7, 102.5, 101.7, 101.6, and 98.3. From the HSQC spectrum (**Figure 17a** and **b**, **Table 10**), the anomeric carbon signals at δ 103.0, 102.9, 102.7, 102.5, 101.7 and 101.6 were correlated to the anomeric proton signals δ 4.47 (**I**), δ 4.73 (**F**), δ 4.48 (**H**), δ 4.49 (**G**), δ 4.77 (**E**), and δ 5.04 (**B**) respectively. Whereas, the anomeric carbon signal at δ 98.3 was correlated to the anomeric proton signals at δ 5.11 (**A**), δ 5.03 (**C**), and δ 4.99 (**D**). All the ^{1}H and ^{13}C signals (**Table 10**) 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.

Table 10

The ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts for the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička 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
→2,6)-α-D-Gal*p*-(1→	5.11	3.94	3.96	4.12	4.22	3.61[c], 3.90[d]
A	98.3	77.1	69.6	69.2	68.5	66.8
→6)-α-D-Gal*p*-(1→	5.04	3.76	4.02	4.12	4.22	3.68[c], 3.92[d]
B	101.6	68.3	69.6	69.2	68.5	66.9
→2)-α-L-Fuc*p*-(1→	5.03	3.75	3.87	3.82	4.16	1.21
C	98.3	78.2	69.6	71.8	67.2	15.7
→6)-α-D-Glc*p*-(1→	4.99	3.82	3.61	3.59	4.07	4.02[c], 4.16[d]
D	98.3	71.8	75.0	69.6	70.4	67.6
β-D-Man*p*-(1→	4.77	4.07	3.61	3.55	3.38	3.74[c], 3.90[d]
E	101.7	70.4	73.1	67.2	76.2	61.2
→3)-β-D-Glc*p*-(1→	4.73	3.30	3.71	3.43	3.47	3.70[c], 3.90[d]
F	102.9	72.9	84.5	69.6	75.6	60.8
→3,6)-β-D-Glc*p*-(1→	4.49	3.48	3.70	3.42	3.61	3.82[c], 4.16[d]
G	102.5	72.9	84.3	69.6	75.0	68.8
→6)-β-D-Glc*p*-(1→	4.48	3.29	3.45	3.44	3.61	3.84[c], 4.19[d]
H	102.7	73.1	75.6	69.6	75.0	69.0
β-D-Glc*p*-(1→	4.47	3.29	3.47	3.36	3.46	3.68[c], 3.87[d]
I	103.0	73.1	75.6	69.6	75.9	60.8

[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.

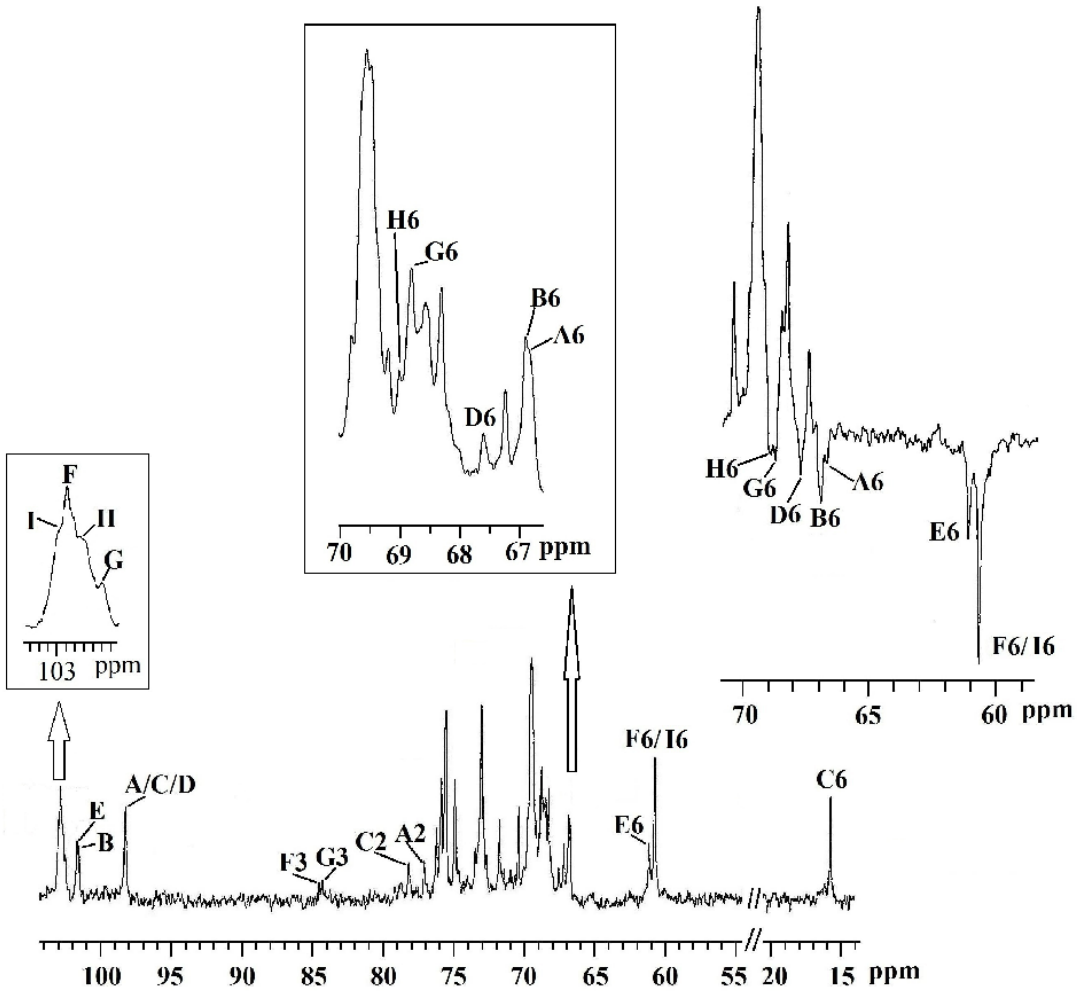


Figure 16. ^{13}C NMR spectrum (125 MHz, D_2O, 30 ºC) of PS-II, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. Part of DEPT-135 spectrum (D_2O, 30 ºC) of PS-II, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička (inset).

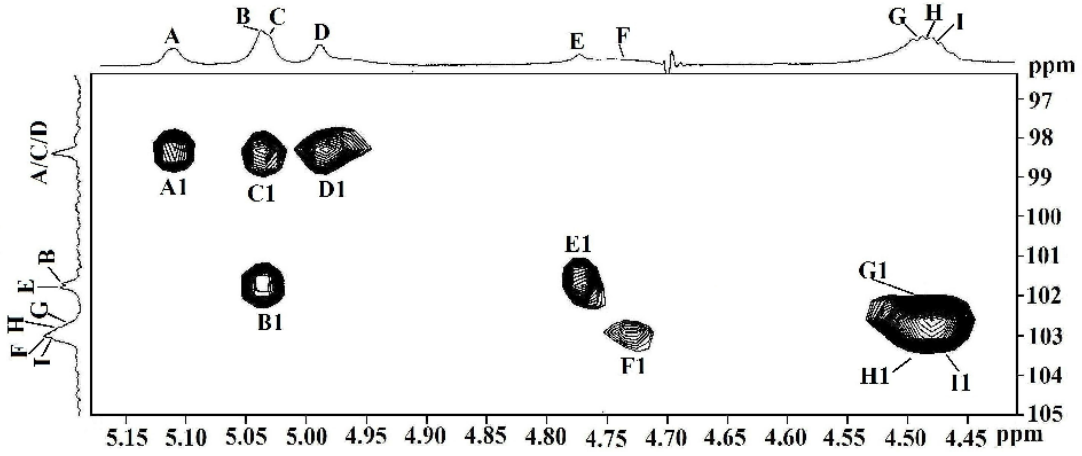


Figure 17a. The HSQC spectrum (D_2O, 30 ºC) of anomeric part of the PS-II isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička.

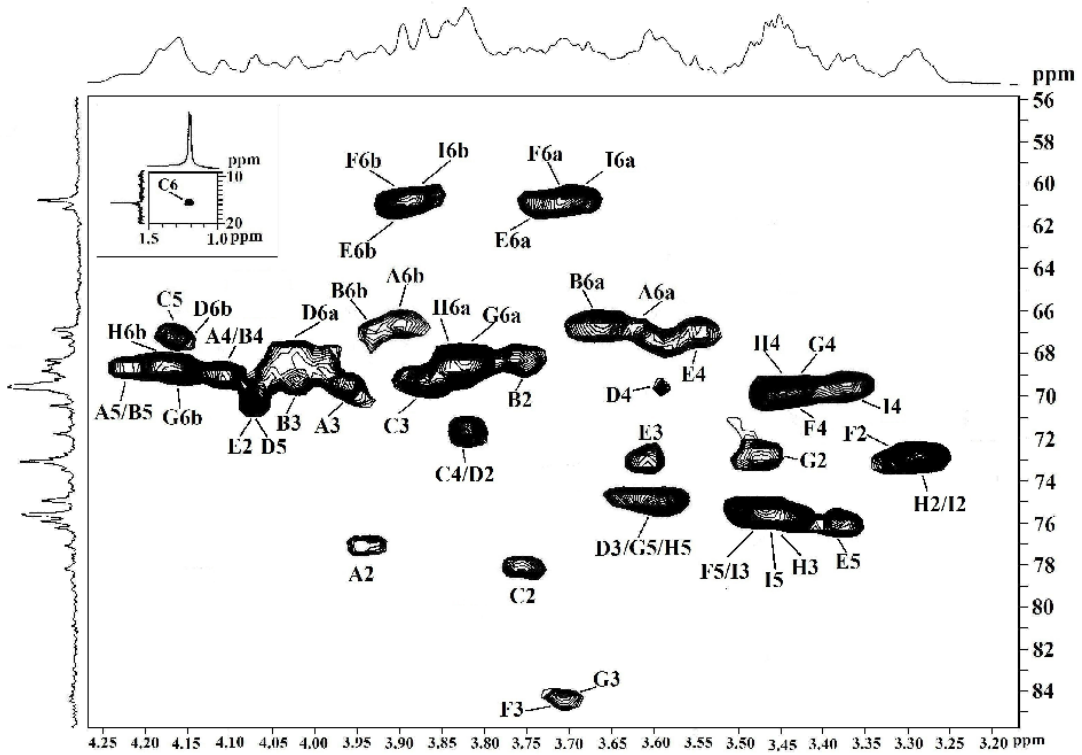


Figure 17b. The HSQC spectrum (D_2O, 30 ºC) of other than anomeric part of the PS-II isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička.

Residues **A** and **B** has coupling constant values of $J_{H\text{-}2,\,H\text{-}3}$ ~9 Hz and $J_{H\text{-}3,\,H\text{-}4}$ ~3.5 Hz and thus, they were confirmed as D-galactopyranosyl residues. The α-configuration of both **A** and **B** residues were assigned from the coupling constant values ($J_{H\text{-}1,H\text{-}2}$ ~3.1 Hz and $J_{C\text{-}1,H\text{-}1}$ ~171 Hz). The downfield shifts of C-2 (δ 77.1) and C-6 (δ 66.8) with respect to standard values of methyl glycosides (Agarwal, 1992; Rinaudo, and Vincendon, 1982) indicated that residue **A** was a (1→2,6)-linked α-D-galactopyranosyl. The downfield shifts of C-6 (δ 66.9) with respect to standard values of methyl glycosides (Agarwal, 1992; Rinaudo, and Vincendon, 1982) indicated that residue **B** was a (1→6)-linked α-D-galactopyranosyl. The linkage at C-6 of the both residues **A** and **B** were further confirmed from DEPT-135 spectrum (**Figure 16**). Hence, these observation confirmed that the residue **A** was (1→2,6)-α-D-galactopyranosyl and the residue **B** was (1→6)-α-D-galactopyranosyl moieties.

Residue **C** was assigned as an L-fucopyranosyl unit. This was strongly supported by the appearance of a proton signal at δ 1.21, carbon signal at δ 15.7 for a CH_3 group, and the relatively small $J_{H\text{-}3,H\text{-}4}$ (< 3 Hz). The appearance of the anomeric proton and carbon signals for residue **C** at δ 5.03 and 98.3 respectively, as well as the coupling constant value $J_{H\text{-}1,H\text{-}2}$ ~ 3.75 Hz clearly indicated that **C** was α-anomer. The anomeric configuration was further confirmed by $^{1}H\text{-}^{13}C$ coupling constant $J_{C\text{-}1,H\text{-}1}$ ~ 171 Hz. The downfield shift of C-2 (δ 78.2) with respect to standard values of methyl glycosides indicated that the residue **C** was linked at C-2 position with residue **H** which further confirmed by the ROESY (**Figure 18**, **Table 11**) experiment. Thus, it may be concluded that the residue **C** was a (1→2)-α-L-fucopyranosyl moiety.

The anomeric proton chemical shift (δ 4.99) and coupling constant values ($J_{H\text{-}1,H\text{-}2}$ ~3.0 Hz, $J_{C\text{-}1,H\text{-}1}$ ~171 Hz) confirmed that residue **D** was present in α-configuration. The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values (~10.0 Hz) confirmed that it was an **D**-glucopyranosyl moiety (Glc*p*). The downfield shifts of C-6 (δ 67.6) with respect to standard values of methyl glycosides indicated that residue **D** was linked at this position. The linkage at C-6 of the residue **D** was further confirmed from DEPT-135 spectrum (**Figure 16**). Thus, **D** was confirmed as (1→6)-α-D-glucopyranosyl residue.

The large coupling constant values ($J_{H-3, H-4}$ ~7.5 Hz and $J_{H-4, H-5}$ ~10 Hz) of residue **E** confirmed its mannopyranosyl configuration. The anomeric proton (δ 4.77) and anomeric carbon chemical shifts (δ 101.7) as well as the low coupling constant values ($J_{H-1,H-2}$ ~0, $J_{C-1,H-1}$ ~161) confirmed that residue **E** was present in β-configuration. All the proton and carbon chemical shifts of residue **E** corresponded nearly to the standard values of methyl glycosides of β-D-mannose. Thus, **E** was confirmed as terminal β-D-mannopyranosyl residue.

Residues **F**, **G**, **H,** and **I** were established as β-configuration from coupling constant values $J_{H-1,H-2}$ (~8.0 Hz), $J_{C-1,H-1}$ (~160-161 Hz) and the large $J_{H-2,H-3}$ and $J_{H-3,H-4}$ coupling constant values (~10.0 Hz) of **F**, **G**, **H** and **I** confirmed their D-glucopyranosyl configuration (Glc*p*). The downfield shift of C-3 of **F** (δ 84.5) with respect to standard value of methyl glycoside (Agarwal, 1992; Rinaudo & Vincendon, 1982) indicated that residue **F** was (1→3)-β-D-Glc*p*. The downfield shifts at C-3 (δ 84.3) and C-6 (δ 68.8) of residue **G** with respect to standard value of methyl glycoside indicated that it was (1→3,6)-β-D-Glc*p*. Since, the residue **G** was the rigid part its C-3 (δ 84.3) value appeared at up field region in comparison to the C-3 (δ 84.5) of the residue **F**. In case of residue **H**, the downfield shift of C-6 (δ 69.0) indicated that it was (1→6)-β-D-Glc*p* moiety. The linking at C-6 of the residues **G** and **H** were further confirmed by DEPT-135 spectrum (**Figure 16**). In residue **I**, all carbon chemical shifts values were found nearly to the standard values of methyl glycoside of β-D-glucose. Thus, residue **I** was confirmed as terminal β-D-Glc*p*.

The sequence of glycosyl residues (**A** to **I**) were determined from ROESY (**Figure 18**, **Table 11**) as well as NOESY (not shown). In ROESY experiment, the inter-residual contacts **A**H-1/**B**H-6a, 6b; **B**H-1/**D**H-6a, 6b; **C**H-1/**A**H-6a, 6b; **D**H-1/**F**H-3; **E**H-1/**A**H-2; **F**H-1/**G**H-3; **G**H-1/**H**H-6a, 6b; HH-1/**C**H-2; and **I**H-1/**G**H-6a, 6b along with other intra-residual contacts were also observed (**Figure 18**).

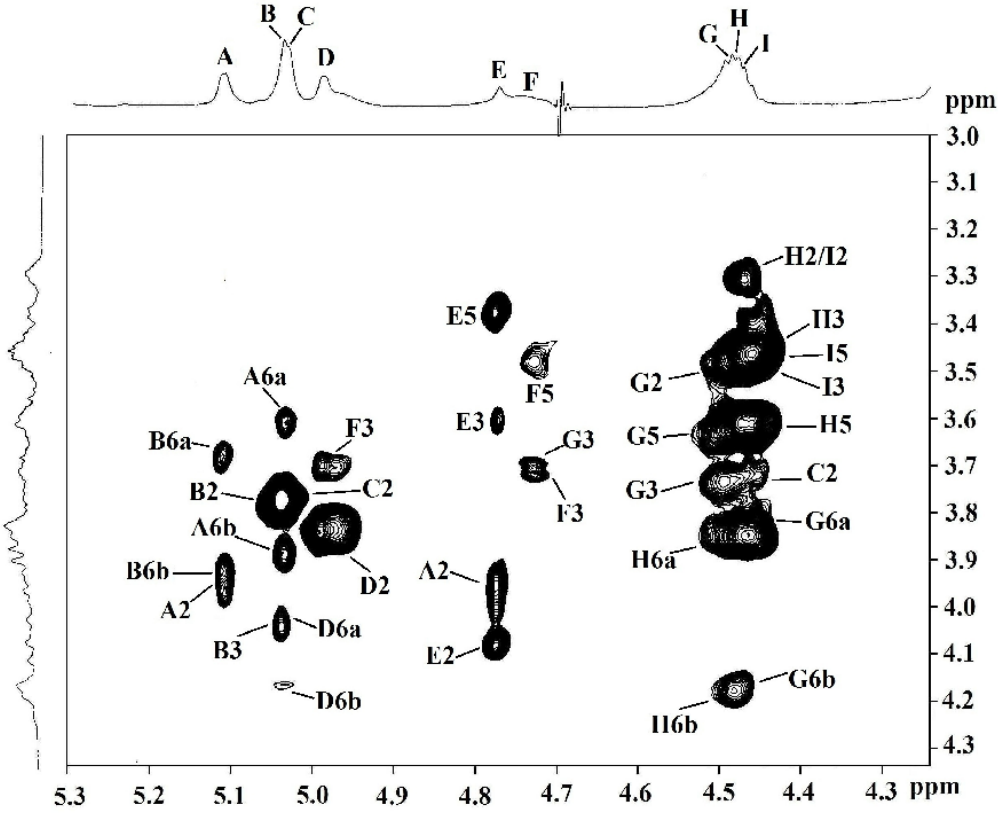


Figure 18. Part of ROESY spectrum of PS-II, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. The ROESY mixing time was 300 ms.

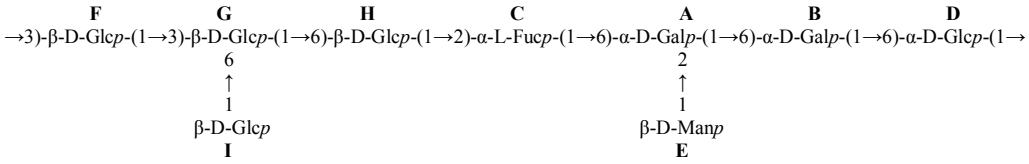
Table 11

ROESY data for the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička.

Glycosyl residue	Anomeric proton	ROE contact proton		
	δ	δ	residue	atom
→2,6)-α-D-Gal*p*-(1→	5.11	3.68	**B**	H-6a
A		3.92	**B**	H-6b
		3.94	**A**	H-2
→6)-α-D-Gal*p*-(1→	5.04	4.02	**D**	H-6a
B		4.16	**D**	H-6b
		3.76	**B**	H-2
		4.02	**B**	H-3
→2)-α-L-Fuc*p*-(1→	5.03	3.61	**A**	H-6a
C		3.90	**A**	H-6b
		3.75	**C**	H-2
→6)-α-D-Glc*p*-(1→	4.99	3.71	**F**	H-3
D		3.82	**D**	H-2
β-D-Man*p*-(1→	4.77	3.94	**A**	H-2
E		4.07	**E**	H-2
		3.61	**E**	H-3
		3.38	**E**	H-5
→3)-β-D-Glc*p*-(1→	4.73	3.70	**G**	H-3
F		3.71	**F**	H-3
		3.47	**F**	H-5
→3,6)-β-D-Glc*p*-(1→	4.49	3.84	**H**	H-6a
G		4.19	**H**	H-6b
		3.48	**G**	H-2
		3.70	**G**	H-3
		3.61	**G**	H-5
→6)-β-D-Glc*p*-(1→	4.48	3.75	**C**	H-2
H		3.29	**H**	H-2
		3.45	**H**	H-3
		3.61	**H**	H-5
β-D-Glc*p*-(1→	4.47	3.82	**G**	H-6a
I		4.16	**G**	H-6b

	3.29	**I**	H-2
	3.47	**I**	H-3
	3.46	**I**	H-5

The above ROESY connectivities established the following sequences: **A** (1→6) **B; B** (1→6) **D; C** (1→6) **A**; **D** (1→3) **F; E** (1→2) **A; F** (1→3) **G**; **G** (1→6) **H**; **H** (1→2) **C**; and **I** (1→6) **G**. These data clearly indicated the positions of substitution and sequence of sugar residues in the polysaccharide. Hence, the structure of repeating unit in the PS-II was proposed as:



Finally, Smith degraded material (SDPS) from PS-II was prepared to confirm the linkages of the heteroglycan. The ^{13}C NMR (125 MHz) spectrum (**Figure 19**, **Table 12**) at 30 °C of SDPS showed one anomeric carbon signals at δ 103.1 for one terminal β-D-Glc*p* (**J**) and one (1→3)-β-D-Glc*p* (**K**) residues. The carbon signals C-1, C-2, and C-3 of the glycerol moiety (Gro) were assigned as δ 66.4, 72.1, and 62.5 respectively. The glycerol moiety (**L**) was generated from (1→6)-β-D-Glc*p* residue (**H)** after periodate oxidation followed by Smith degradation, and this moiety was attached to (1→3)-linked β-D-Glc*p* moiety (**K**) . The residue (1→3)-β-D-Glc*p* (**F**) was converted to terminal β-D-Glc*p* unit (**J**) during Smith degradation. In ^{13}C NMR (Fig. 3), the carbon signal at δ 84.3 clearly indicated the presence of (1→3)-linked β-D-Glc*p* unit (**K**) which was generated from (1→3,6)-β-D-Glc*p* residue (**G)** during this reaction.

Table 12

The ^{13}C NMR[n] chemical shifts of Smith-degraded glycerol-containing disaccharide of the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička in D_2O at 30 ºC.

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p*-(1→ **J**	103.1	74.0	76.6	70.6	75.8	60.7
→3)-β-D-Glc*p*-(1→ **K**	103.1	73.1	84.3	69.6	75.6	60.9
→3)-Gro **L**	66.4	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 ºC.

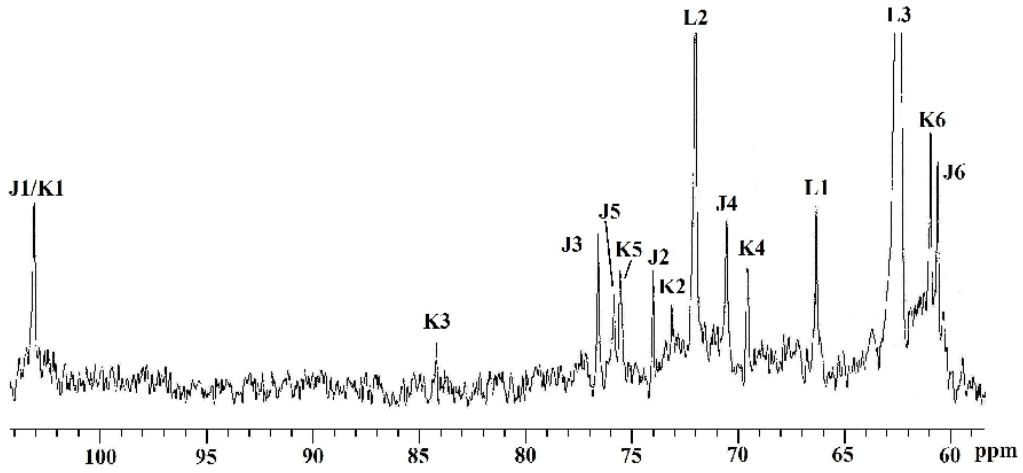


Figure 19. ^{13}C NMR spectrum (125 MHz, D_2O, 30 ºC) of the Smith-degraded glycerol containing disaccharide isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička.

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

J **K** **L**
β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-Gro

This result further confirmed the repeating unit present in the heteroglycan isolated from the edible mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička.

3.B.6. Antioxidant properties of PS-I

3.B.6.1. Assay of hydroxyl radical scavenging activity

The hydroxyl radical is considered to be the most reactive and poisonous free radicals among all reactive oxygen species (ROS) in biological systems and may react with almost all biomolecules, including cellular carbohydrates, proteins, lipids, and DNA causing tissue damage or cell death. Thus, removal of hydroxyl radical is very important for the protection of living systems (Yang, Guo, & Yuan, 2008). **Figure 20** shows that PS-I and BHT exhibited concentration-dependent scavenging activities against hydroxyl radicals generated in a Fenton reaction system. The hydroxyl radical scavenging activity of PS-I gradually increase with the increase of concentration (**Figure 20**). The hydroxyl radical scavenging rate of PS-I and BHT at 200 µg/mL were found to be 16.6 % and 91.2 % respectively, indicating that PS-I has a moderate antioxidant activity and the activity of PS-I is weak compared with that of BHT. The EC_{50} value of the PS-I was found to be 480 µg/mL. The hydroxyl radical scavenging activity might be explained by hydrogen atom donation ability of the hydroxyl group in PS-I to terminate the free radical mediated oxidative chain reactions.

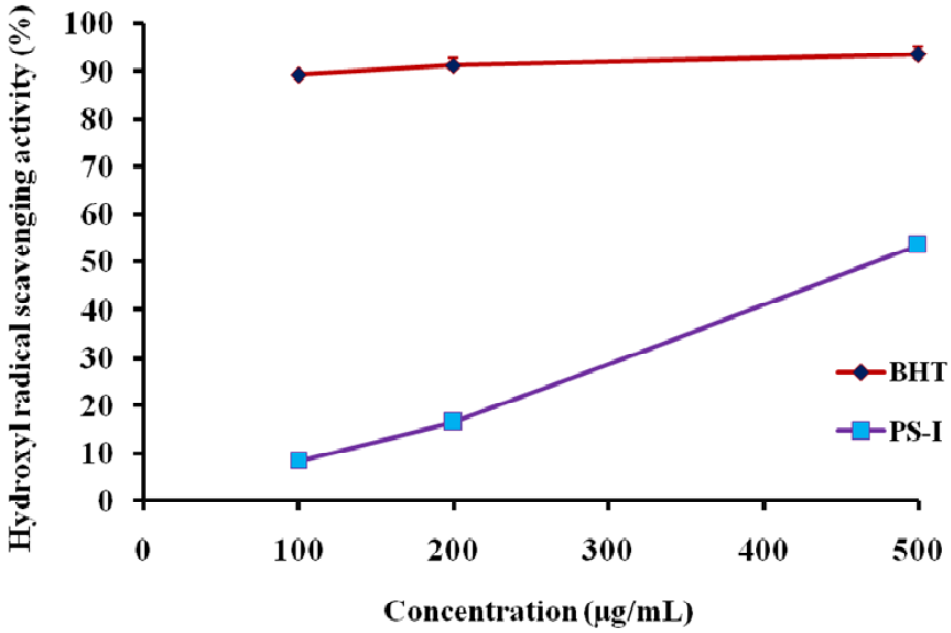


Figure 20. Hydroxyl radical scavenging activity of the PS-I, isolated from the *Entoloma lividoalbum* (Kühner & Romagn) Kubička. Results are the mean ± SD of three separate experiments, each in triplicate.

3.B.6.2. Assay of superoxide radical scavenging activity

Superoxide anion radical is known as an initial radical and precursor of active free radicals. It plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radicals, and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA (Stief, 2003; Wickens, 2001). In the present study, the PS-I was found to be a potent scavenger of superoxide radicals generated in riboflavin- nitroblue tetrazolium (NBT) light system. **Figure 21** illustrates the superoxide radical scavenging ability of 100 to 400 µg/mL of PS-I and comparison with the same doses of BHA. The percentage inhibition of superoxide generation by 200 µg/mL concentration of PS-I was found to be 64.47 % whereas of BHA was found as 91.01 %. They both showed a concentration dependent scavenging of superoxide radicals and the

percentage inhibition of PS-I is close to that of synthetic standard drug BHA at a concentration of 400 µg/mL. The EC_{50} value of the PS-I was found to be 150 µg/mL.

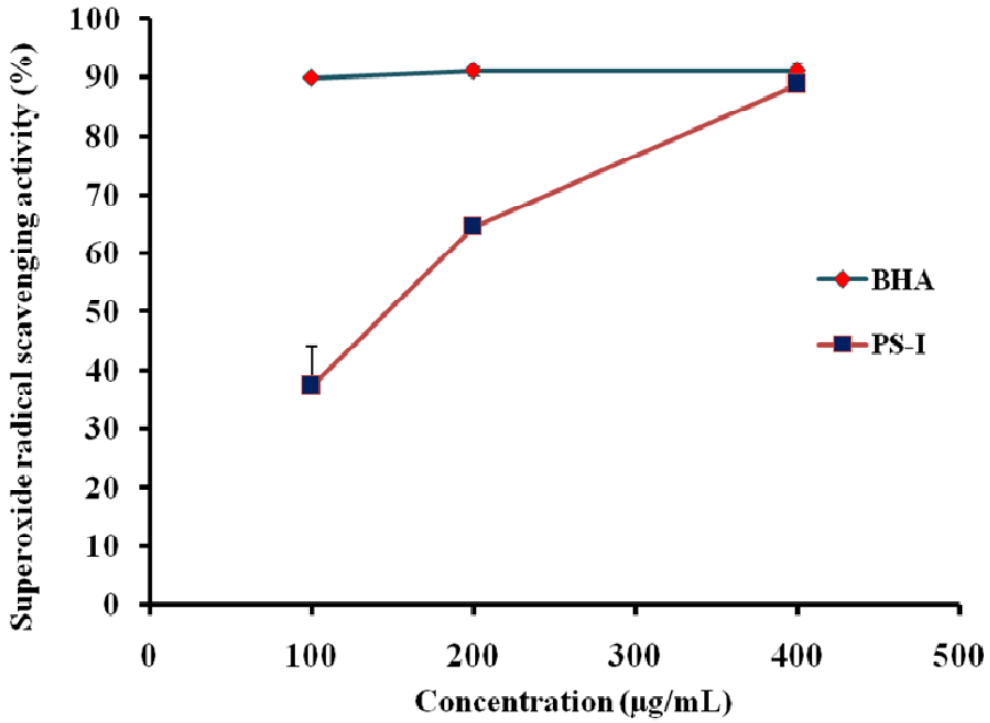


Figure 21. Superoxide radical scavenging activity of the PS-I, isolated from the *Entoloma lividoalbum* (Kühner & Romagn) Kubička. Results are the mean ± SD of three separate experiments, each in triplicate.

3.B.6.3. Determination of reducing power

The reducing power assay measures the electron-donating ability of antioxidants using the potassium ferricyanide reduction method. The reducing power of a compound could serve as an indicator of its potential antioxidant activity. Higher absorbance of the reaction mixture indicated greater reducing power. The reducing power of PS-I was concentration-dependent. In this assay, reducing power of PS-I and ascorbic acid increased with increasing sample concentration (**Figure 22**). At 200 µg/mL, the reducing

powers were 0.2765 and 1.16 for PS-I and ascorbic acid respectively. At 600 µg/mL, the reducing powers were 0.58 and 1.27 for PS-I and ascorbic acid respectively. At concentration of 480 µg/mL, PS-I showed reducing power 0.5. This result suggests that PS-I is a good electron donor and may terminate the radical chain reaction by converting free radicals to more stable product.

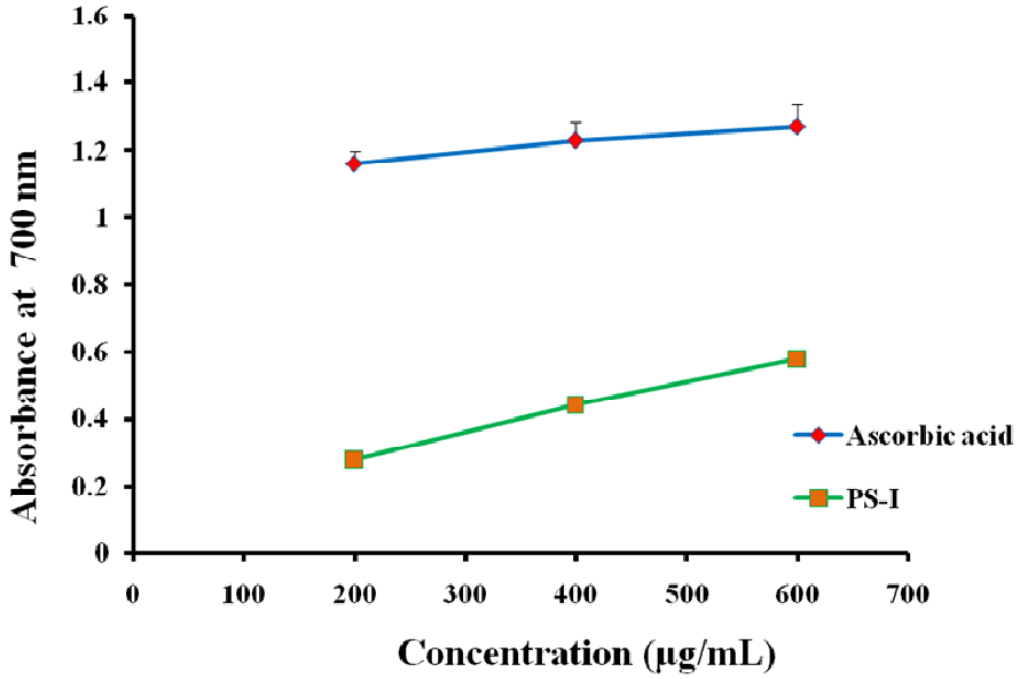


Figure 22. Determination of reducing power of the PS-I, isolated from the *Entoloma lividoalbum* (Kühner & Romagn) Kubička. Results are the mean ± SD of three separate experiments, each in triplicate.

3.B.6.4. Total antioxidant capacity assay

PS-I inhibited the phosphomolybdenum in the total antioxidant capacity assay. Result showed 1 mg of PS-I is equivalent to 70 ± 15 µg of ascorbic acid. Total antioxidant activity suggests that the electron donating capacity of PS-I and thus it may act as radical chain terminator, by transforming reactive free radicals into more stable non reactive products.

3.B.7. Biological activities of PS-II

The cell viability using PS-II was studied on human 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 (**Figure 23a**). It was observed that the cytotoxicity to normal lymphocytes by PS-II was insignificant. Cell proliferative activity was observed at 50 µg/mL of PS-II with respect to control. These results showed a lower level of cytotoxicity when lymphocytes were treated with PS-II up to 200 µg/mL but even at higher dose 400µg/mL, the polysaccharide showed mild toxicity. Cell culture experiments were carried out and statistical calculations showed the IC50 value was 800 µg/mL, indicating that 200 µg/mL is safe with respect to the other higher doses.

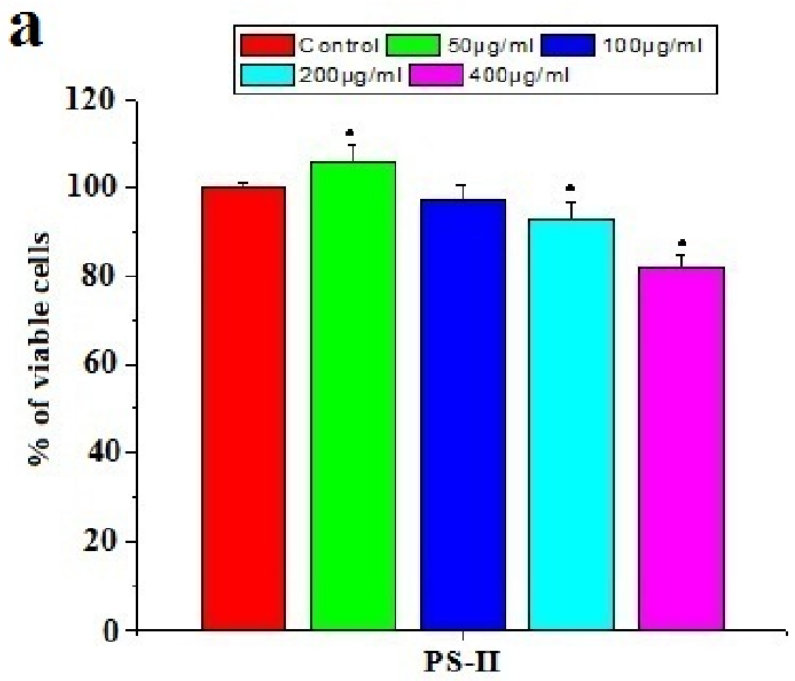


Figure 23. (a) Cytotoxicity of PS-II against normal human lymphocytes. (n=6, values are expressed as mean ± SEM. *indicates the significant difference as compared to control group).

Glutathione is an important antioxidant in cellular system. Hence to understand the glutathione level in cell, both reduced and oxidized form of glutathione were measured. The reduced glutathione level (GSH, **Figure 23b**) was decreased and the mild augmentation of oxidized form of glutathione level (GSSG, **Figure 23b**) 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= 0.951, 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 1.01 to 0.499 compared to their respective control indicating that 400 µg/mL was toxic. These results indicated that 200 µg/mL is biologically safe and effective dose.

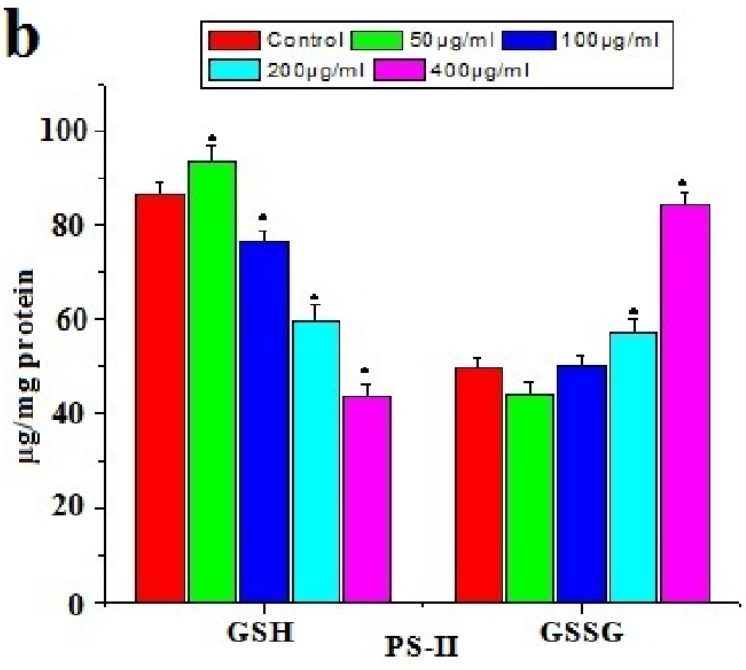


Figure 23. (b) Concentration of reduced glutathione (GSH) and oxidized glutathione of PS-II treated normal human lymphocytes. (n=6, values are expressed as mean ± SEM. *indicates the significant difference as compared to control group).

Study of lipid peroxidation is one of the important parameters to assess the cellular damage. It initiates inactivation of cellular components and protective enzymes, and thereby plays a crucial role of oxidative stress in biological systems (Samanta et al., 2013). Several toxic by-products especially malondialdehyde is released due to lipid peroxidation. Hence, lipid peroxidations in lymphocytes were measured in terms of the concentration of malondialdehyde (MDA) release (**Figure 23c**). The present investigation showed slightly increase of MDA at the dose of 400 µg/mL in comparison to the previous doses indicating that 200 µg/mL is again biologically safe.

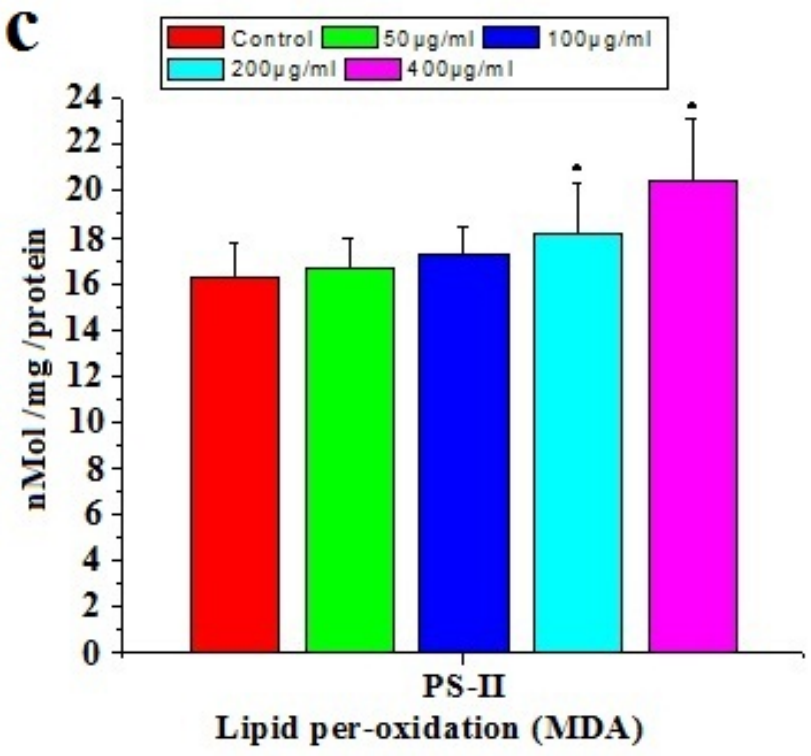


Figure 23. (c) Concentration of Lipidperoxidation in terms of MDA of PS-II treated normal human lymphocytes. (n=6, values are expressed as mean ± SEM. *indicates the significant difference as compared to control group).

Stimulated lymphocytes secreted several factors like NO. The release of NO (Figure 23d) clearly demonstrated that it was secreted by the lymphocytes when stimulated by PS-II. In presence of the PS-II, single culture of lymphocytes generated significant amount of NO ($p < 0.05$) into the medium after 24h of incubation (**Figure 23d**). The result showed the presence of a high concentration of NO in the co-culture medium of pulsed lymphocytes at 400µg/mL indicating that this dose is cytotoxic. Hence, it is again established that 200 µg/mL is safe and effective dose.

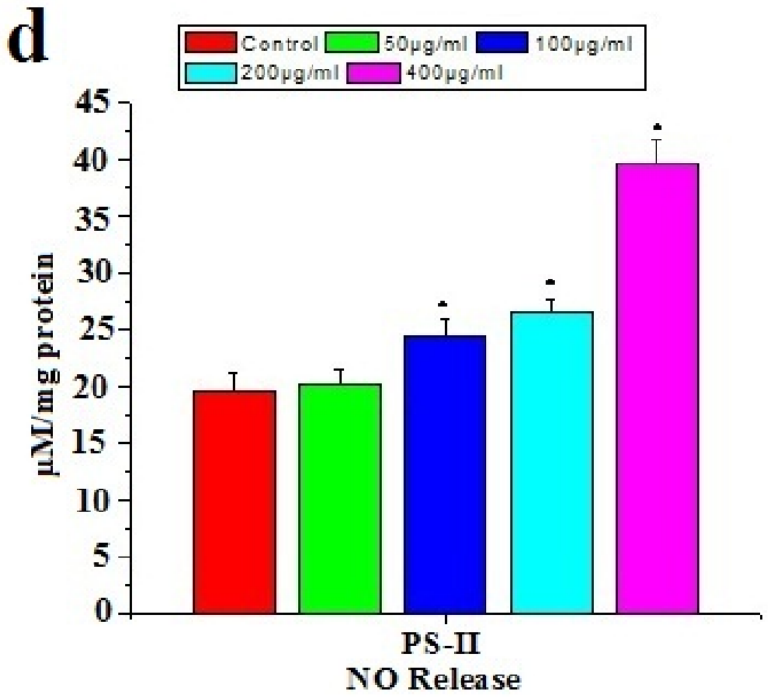


Figure 23. (d) Concentration of nitric oxide release from PS-II treated normal human lymphocytes. (n=6, values are expressed as mean ± SEM. *indicates the significant difference as compared to control group).

To establish the protective role of PS-II against nicotine toxicity, lymphocytes were treated with nicotine (10 mM) as positive control and different concentrations of PS-II along with nicotine for 24 h in culture media. The significantly ($p < 0.05$) increased cell

viability levels were observed up to 200µg/mL. The fluorescent microscopic pictures established the result (**Figure 24a** and **b**). The fluorescence images revealed that the PS-II was able to ameliorate the toxic effects of nicotine at the dose of 200 µg/mL, but when the dose was increased to 400 µg/mL, the PS-II lost its ameliorative effects on lymphocytes. The above result was confirmed by FACS, which established our findings that 400 µg/mL reveled the toxic effects synergistically with nicotine (**Figure 25**).

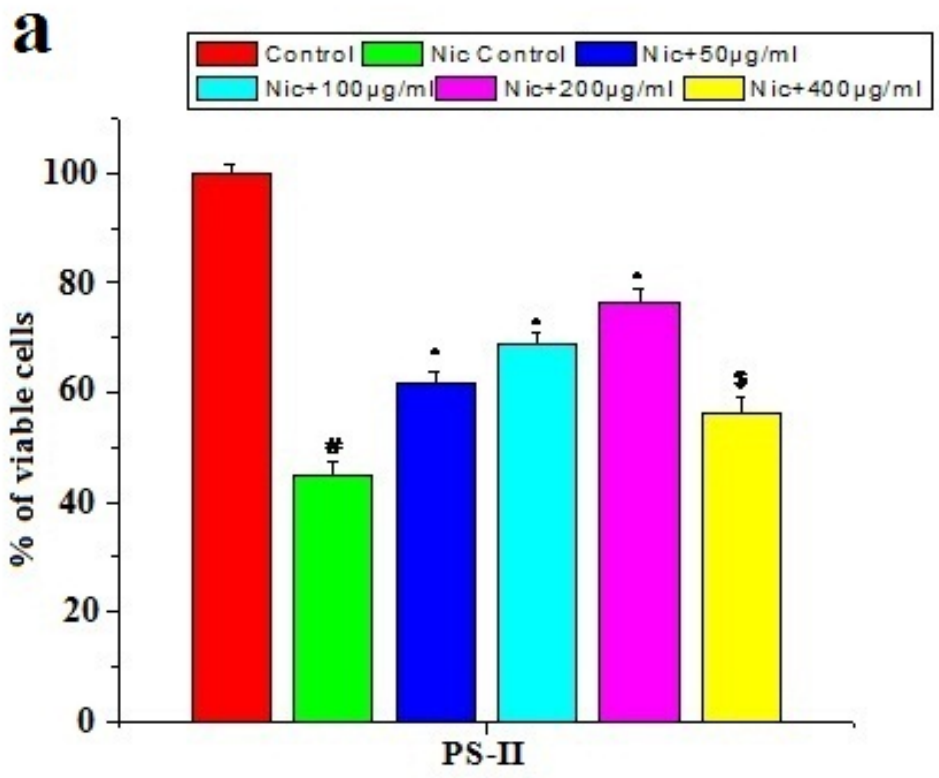


Figure 24. (a) Cytoprotective role of PS-II was done by using nicotine treated normal human lymphocytes. n=6; values are expressed as mean ± SEM. *indicates the significant difference as compared to control group.

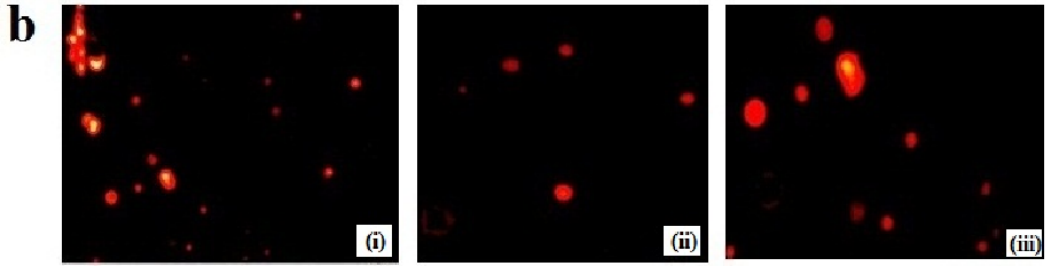


Figure 24. (b) The uptake of PI stain by apoptotic cells were visualized by phase contrast fluorescent microscope under 50X mfg. Here, (i): Lymphocytes were treated with 10 mM Nicotine, (ii): Lymphocytes were pre-treated with 10mM Nicotine and subsequently treated with PS-II (200 µg/ml) and (iii): Lymphocytes were pre- treated with 10mM Nicotine and subsequently treated with PS-II (400 µg/ml).

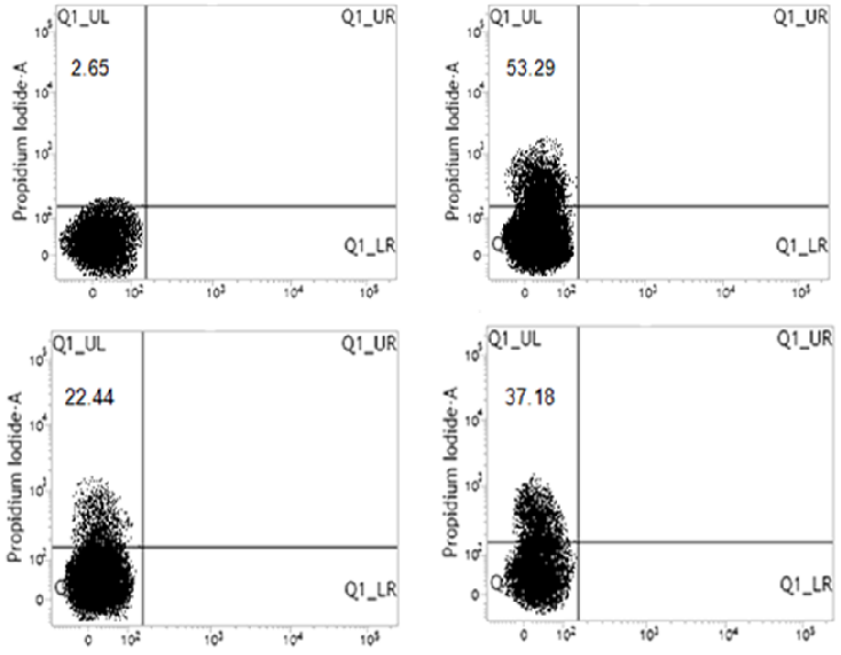


Figure 25. FACS analysis of normal human lymphocytes at different concentration of PS-II. The propidium iodide was used for analysis of cell death and only PI (+) ve cells were counted.

It is evident from these experiments that, in vitro application of PS-II does not induce any cellular damage in lymphocytes associated with enhanced MDA level, NO level, GSSG level and decreased GSH level. The cytotoxic profile of PS-II in lymphocytes indicated 200 µg/mL safe and effective, whereas concentrations higher than 200 µg/mL showed significant increase of cytotoxicity. Administration of Nicotine to lymphocytes causes decrease in cell viability which is protected by supplementation of PS-II to nicotine treated cells. These findings suggest the potential use and beneficial role of PS-II for use as antioxidant as well as immunostimulant.

3.C. Conclusion

Two water soluble polysaccharides, a glucan (PS-I) and a heteroglycan (PS-II) were isolated from the alkaline extract of the fruit bodies of an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. The repeating unit of the polysaccharide (PS-I) contains a backbone chain of three (1→6)-β-D-glucopyranosyl residues, one of which was branched at *O*-3 position with the side chain consisting of three (1→3)-β-D-glucopyranosyl and a terminal β-D-glucopyranosyl residues. PS-II is also structurally new. The repeating unit of the PS-II had a backbone consisting of two (1→3)-β-D-glucopyranosyl, one (1→6)-β-D-glucopyranosyl, one (1→2)-α-L-fucopyranosyl, one (1→6)-α-D-glucopyranosyl, and two (1→6)-α-D-galactopyranosyl residues, out of which one (1→3)-β-D-glucopyranosyl residue was branched at *O*-6 position with terminal β-D-glucopyranosyl residue and one (1→6)-α-D-galactopyranosyl residue was branched at *O*-2 position with terminal β-D-mannopyranosyl residue. These two polysaccharides (PS-I and PS-II) are biologically non toxic and should be considered as a source of natural antioxidants in future.

3.D. References

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CHAPTER 4

Structural, immunological, and antioxidant studies of β-glucan isolated from the aqueous extract of the edible mushroom Entoloma lividoalbum

4.A. Introduction and earlier works

Mushroom polysaccharides are potent renewable source for the development of several drugs. PSK from *Coriolus (Trametes) versicolor* (Cui & Chisti, 2003), lentinan from *Lentinus edodes* (Taguchi et al., 1983) and sonifilan (SPG) from *Schizophyllum commune* (Fujimoto et al., 1983) have been recognized as anticancer drugs throughout the world (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007). Polysaccharides derived from these medicinal mushrooms have been explored for several years and found to possess immunomodulatory and anticancer properties (Maity, Samanta et al., 2014; Patra et al., 2013; Wasser & Weis, 1999; Wasser, 2002). The anticancer activity of these polysaccharides is mediated mostly through the activation of immune cells such as B cells, T cells, macrophages and NK cells (Nandi et al., 2013; Wasser, 2002). It has been reported that in vivo administration of β-glucans (Kogan, 2000) can enhance immune reactions and up regulate the resistance of host against tumor cells. Furthermore, administration of glucans to macrophages activated by LPS can lead to increased production of cytokines like interleukin-1 and TNF-α which subsequently induce lymphocyte differentiation and proliferation to enhance immune responses (Adachi, Okazaki, Ohno, & Yadomae, 1994; Chihara, 1992). It is also noteworthy to mention the antioxidant activity of the polysaccharide which depends on monosaccharide composition and their different arrangements during polymerization (Tsiapali et al., 2001). Antioxidants prevent the chain reactions by hydrogen donation and interrupting the process of oxidation. As a result, stable free radicals cannot survive or propagate further oxidation (Aruoma, 1999; Wade, Jackson, Highton, & Van Rij, 1987). Different types of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radicals are closely involved in various human diseases (Halliwell & Gutteridge, 1989). Antioxidants play key role to prevent the generation of ROS or scavenge them and minimize oxidative tissue damage. Hence, ROS induced oxidative cell damage can be prevented by supplementation of naturally occurring biomolecules like polysaccharide, which is one of the most acceptable techniques for modern therapy.

Edible and non-toxic mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička showed antimicrobial activity (Rai, Sen, & Acharya, 2013), antioxidant properties (Maity, Samanta et al., 2014), and protective role in human lymphocytes (Maity, Nandi et al., 2014). From these points of views *Entoloma lividoalbum* may therefore be useful as a medicinal fungus with various immunostimulating and other protective effects. Two water soluble polysaccharides, PS-I (Maity, Samanta et al., 2014) and PS-II (Maity, Nandi et al., 2014) were isolated from the alkaline extract of the mushroom, characterized and reported in the previous chapter. In the present investigation another water soluble polysaccharide (ELPS) has been isolated from the aqueous extract of this mushroom and characterized as β-glucan. The detailed structural characterization, immunological studies and antioxidant properties of this β-glucan have been carried out and reported in this chapter.

4.B. Present work

4.B.1. Isolation and purification of polysaccharide from *E. lividoalbum*

Fresh fruit bodies of the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička (700 g) were collected from Sikkim Himalayan region, India, gently washed with water, cut into pieces and boiled at 100 °C with distilled water for 10 h, cooled, centrifuged, supernatant was precipitated in EtOH (1:5) to get crude polysaccharide (900 mg). A single fraction (15 mg) was obtained in the course of fractionation of water soluble crude polysaccharide (25 mg) through Sepharose 6B column using water as the eluent (**Figure 1**).

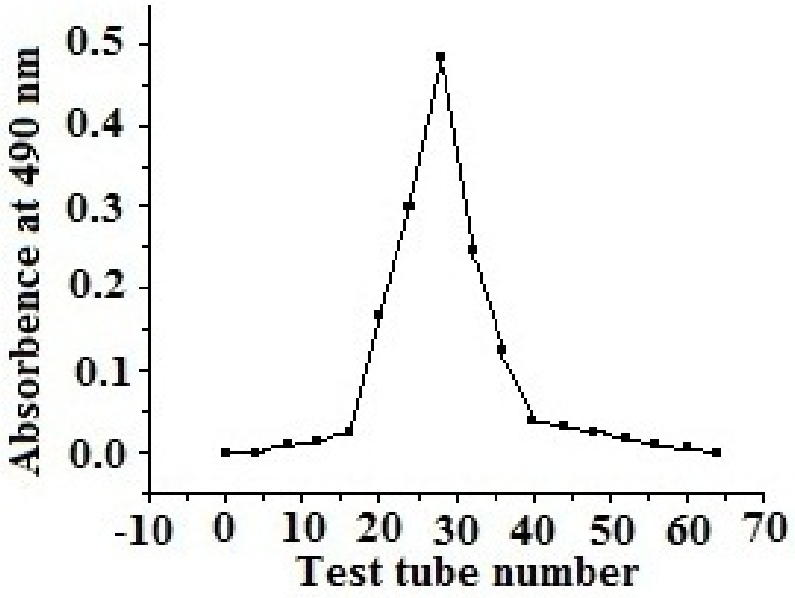


Figure 1. Gel permeation chromatogram of crude polysaccharide isolated from an edible mushroom *Entoloma lividoalbum* using Sepharose 6B column.

4.B.2. Optical rotation and molecular weight of β-glucan (ELPS)

The ELPS showed specific rotation $[\alpha]_D^{30}$ -6.9 (*c* 0.11, H_2O). The average molecular weight (Hara, Kiho, Tanaka, & Ukai, 1982) of ELPS was estimated as ~2×10^5 Da on the basis of standard calibration curve prepared using standard dextrans (**Figure 2**).

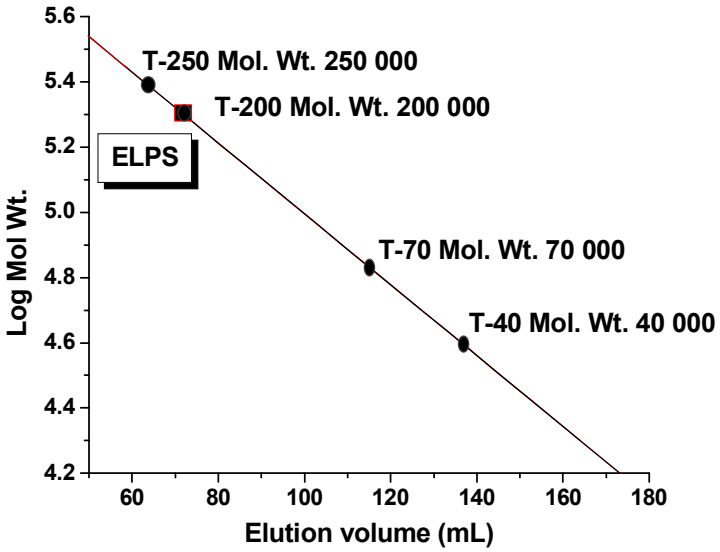


Figure 2. Determination of molecular weight of ELPS by gel permition chromatography in sepharose 6B column.

4.B.3. Structural analysis of ELPS

4.B.3.1. Chemical analysis of ELPS

GLC analysis of the alditol acetates (Lindahl, 1970) of this polysaccharide revealed the presence of glucose only. The absolute configuration of glucose was determined as D according to Gerwig, Kamerling, & Vliegenthart (1978). The glucan was methylated according to the Ciucanu and Kerek (1984) method, followed by hydrolysis and alditol acetate preperation. The GLC-MS analysis of the alditol acetates of methylated product showed the presence (1→3), (1→6), and (1→3,6)-linked and terminal glucopyranosyl residues in a molar ratio of nearly 1:3:1:1 respectively (**Table 1**, **Figure 3**).

Table 1

GLC-MS analysis of methylated polysaccharide (ELPS) isolated from *Entoloma lividoalbum* (Kühner & Romagn.) Kubička

Methylated sugars	Molar ratio	Linkage type	Major Mass Fragments (m/z)
2,4,6-Me_3-Glc	1	→3)-β-D-Glc*p*-(1→	43,45,71,87,101,117,129, 143,161,173,203,217,233
2,3,4,6-Me_4-Glc	1	β-D-Glc*p*-(1→	43,45,59,71,87,101,117,129, 161,205
2,4-Me_2-Glc	1	→3,6)-β-D-Glc*p*-(1→	43,58,87,101,117,129,139, 159,189,201,233
2,3,4-Me_3-Glc	3	→6)-β-D-Glc*p*-(1→	43,45,58,71,87,99,101,117, 129,161,173,189,233

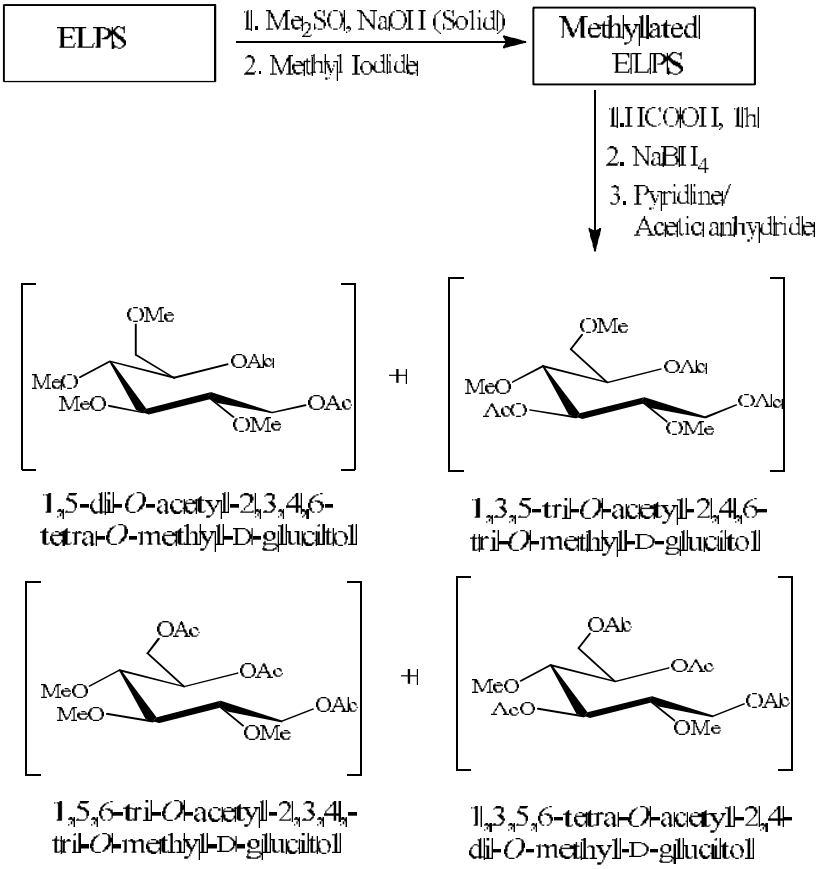


Figure 3. Schematic presentation of methylation experiment of ELPS isolated from an edible mushroom, *Entoloma lividoalbum.*

The periodate oxidation experiment was carried out with the ELPS for further confirming the linking information of the sugar moieties. GLC analysis of alditol acetates of the periodate-oxidized (Goldstein, Hay, Lewis, & Smith, 1965; Hay, Lewis, & Smith, 1965), $NaBH_4$-reduced, hydrolyzed products showed the presence of glucose unit only. GLC-MS analysis of periodate-oxidized, reduced, methylated (Abdel-Akher & Smith, 1950) products of ELPS showed the presence of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol in a molar ratio of nearly 1:1 (**Table 2**, **Figure 4**). These results showed that the (1→6)-linked and terminal glucopyranosyl residues were consumed during oxidation, whereas (1→3,6)- and (1→3)-

linked glucopyranosyl residues remained unaffected. Hence, the mode of linkages present in the ELPS was confirmed.

Table 2

GLC-MS analysis of periodate oxidized methylated ELPS of an edible mushroom, *Entoloma lividoalbum.*

Methylated sugars	Molar ratio	Linkage type	Major Mass Fragments (m/z)
2,4,6-Me_3-Glc	1	→3)-β-D-Glc*p*-(1→	43,45,71,87,101,117,129, 143,161,173,203,217,233
2,4-Me_2-Glc	1	→3,6)-β-D-Glc*p*-(1→	43,58,87,101,117,129,139, 159,189,201,233

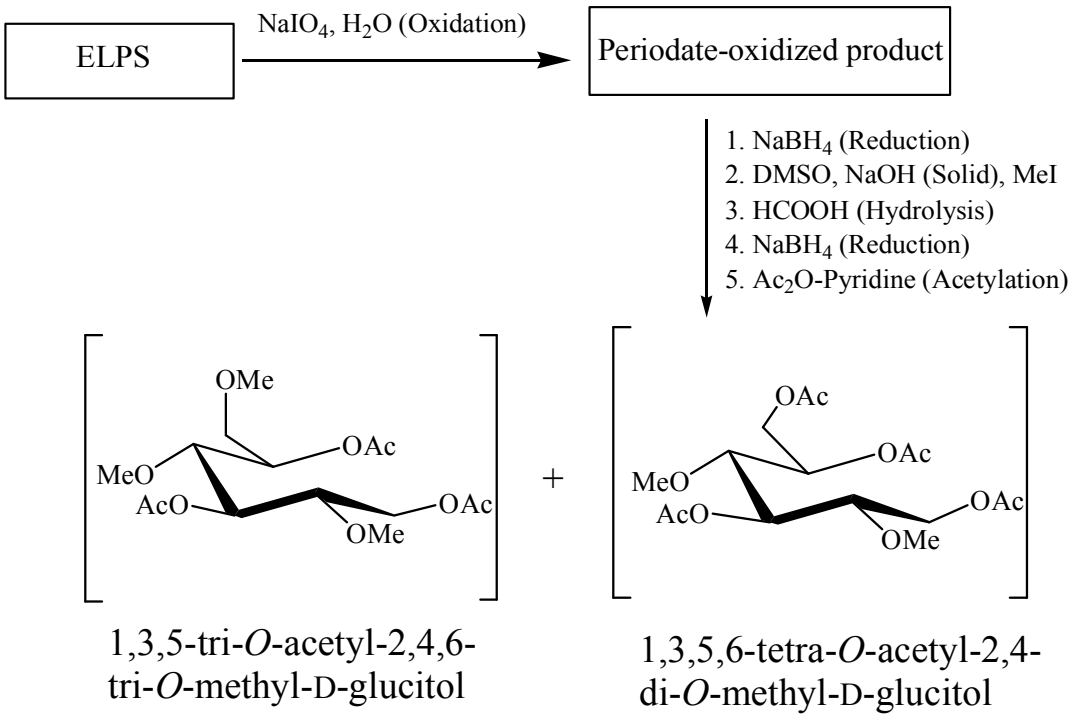


Figure 4. Schematic presentation of periodate oxidation reactions of ELPS isolated from an edible mushroom, *Entoloma lividoalbum.*

4.B.3.2. 1D and 2D NMR analysis of ELPS

In the anomeric region of the ^{1}H NMR spectrum (500 MHz; **Figure 5**, **Table 3**) at 30 °C, three signals were observed at δ 4.78, 4.51, and 4.49. The peak at δ 4.78 corresponded to the anomeric proton of residue **A**. The signal at δ 4.51 corresponded to both the residues **B** and **C** and the signal at δ 4.49 corresponded to residue **D** (**Figure 5**).

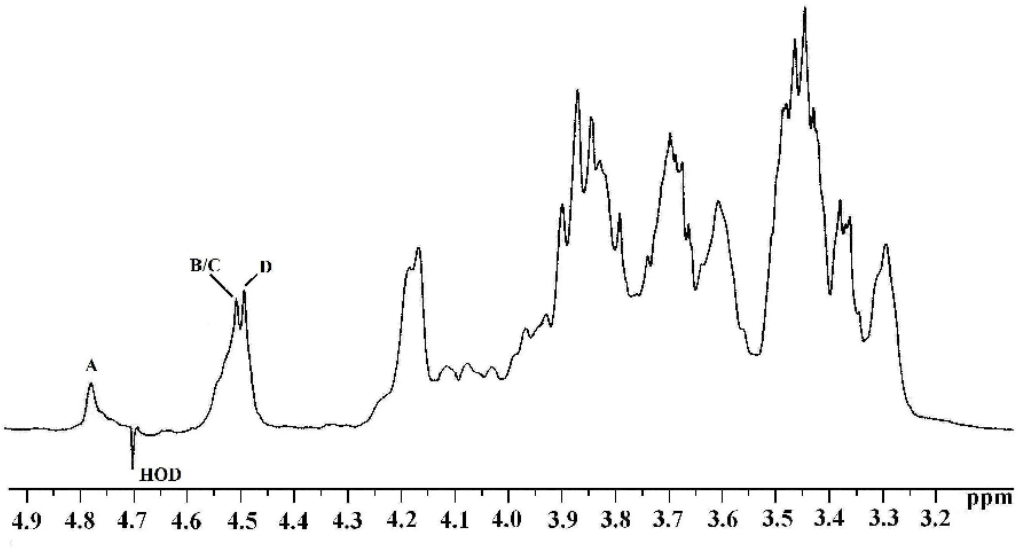


Figure 5. ^{1}H NMR spectrum (500 MHz, D_2O, 30 ºC) of a glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička.

Table 3

The ^{1}H NMR[a] chemical shifts for the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička in D_2O at 30 ºC.

Glycosyl residue	H-1	H-2	H-3	H-4	H-5	H-6a,H-6b
→3)-β-D-Glc*p*-(1→ **A**	4.78	3.38	3.70	3.45	3.48	3.70[c], 3.90[d]
β-D-Glc*p*-(1→ **B**	4.51	3.29	3.47	3.38	3.43	3.69[c], 3.87[d]
→3,6)-β-D-Glc*p*-(1→ **C**	4.51	3.49	3.69	3.45	3.61	3.83[c], 4.17[d]
→6)-β-D-Glc*p*-(1→ **D**	4.49	3.29	3.45	3.43	3.61	3.85[c], 4.19[d]

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

[c,d] Interchangeable.

^{13}C NMR spectrum (125 MHz; **Figure 6**, **Table 4**) showed three signals in the anomeric region at δ 102.9, 102.7, and 102.5 at the same temperature. On the basis of HSQC spectrum (**Figure 7**) the anomeric proton signal at δ 4.51 was correlated to both the carbon signals δ 102.7 and δ 102.5, corresponded to anomeric carbons **B** and **C** respectively. Again, the anomeric proton signals at δ 4.78 and δ 4.49 were correlated to carbon signals at δ 102.7 and δ 102.9, corresponded to anomeric carbon of residues **A** and **D** respectively. From DQF-COSY, TOCSY, and HSQC experiments, all the ^{1}H and ^{13}C signals (**Table 3** and **4**) were assigned. The proton-proton and one-bond C-H coupling constants were measured from DQF-COSY and proton coupled ^{13}C spectrum respectively.

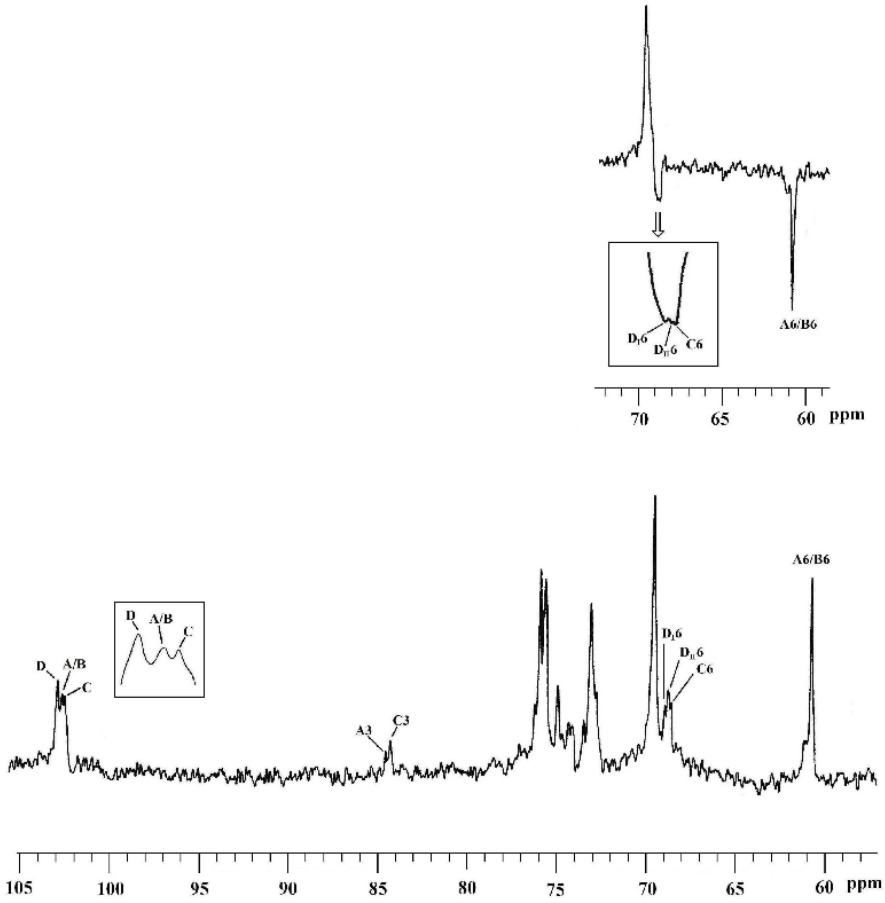


Figure 6. ^{13}C NMR spectrum (125 MHz, D_2O, 30 ºC) of a glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. Part of DEPT-135 spectrum (D_2O, 30 ºC) of a glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička (inset).

Table 4

The ^{13}C NMR[b] chemical shifts for the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička in D_2O at 30 ºC.

Glycosyl residue	C-1	C-2	C-3	C-4	C-5	C-6
→3)-β-D-Glc*p*-(1→ **A**	102.7	72.8	84.6	69.6	75.9	60.8
β-D-Glc*p*-(1→ **B**	102.7	73.1	75.7	69.6	75.9	60.8
→3,6)-β-D-Glc*p*-(1→ **C**	102.5	72.8	84.3	69.6	75.0	68.7
→6)-β-D-Glc*p*-(1→ **D**	102.9	73.1	75.7	69.6	75.0	68.9[e], 68.8[f]

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

[e] For residue $\mathbf{D_I}$.

[f] For residue $\mathbf{D_{II}}$.

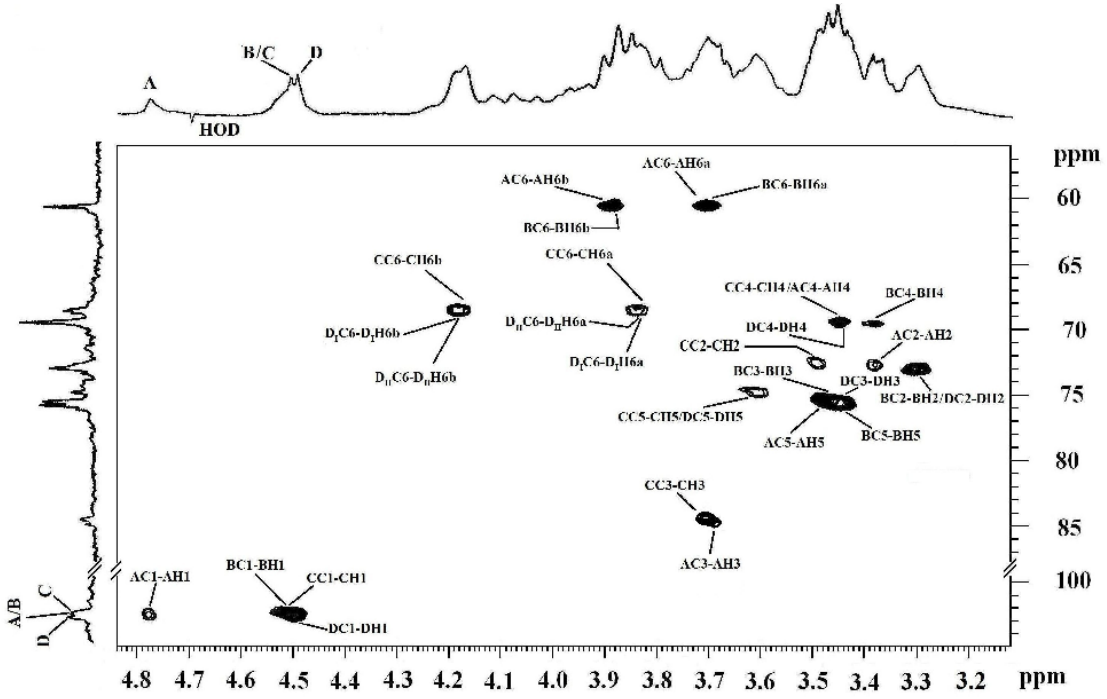
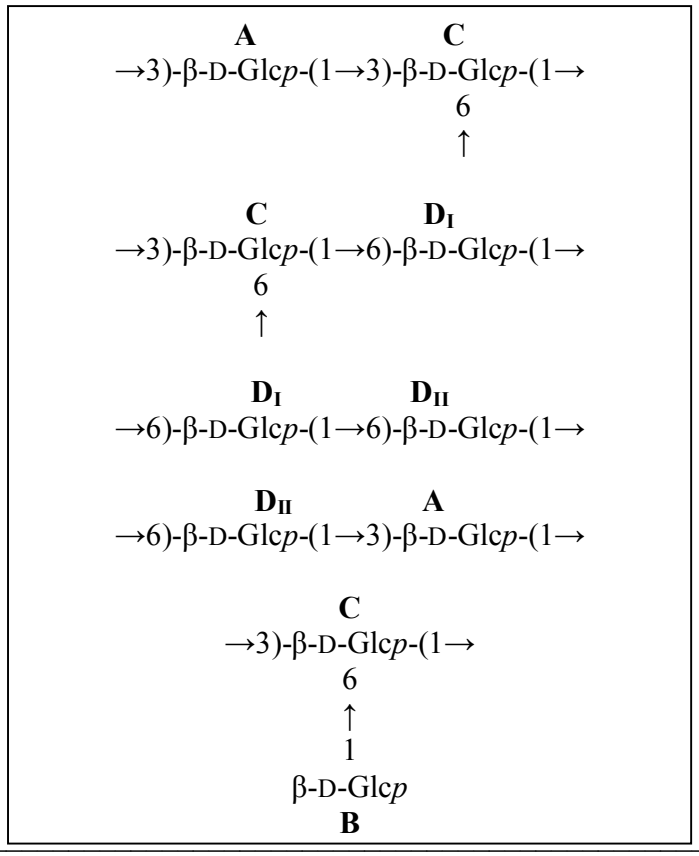


Figure 7. Part of HSQC spectrum of a glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička.

All the residues from **A** to **D** were present as D-glucopyranosyl configuration (Glc*p*) as confirmed by the large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values (~10.0 Hz). Anomeric proton chemical shifts (δ 4.78-4.49), anomeric carbon chemical shifts (δ 102.9-102.5), and the coupling constant values $J_{H\text{-}1,H\text{-}2}$ (~8.0 Hz), $J_{C\text{-}1,H\text{-}1}$ (~160-161 Hz) confirmed that the residues (**A** -**D**) were present in β-configuration. The downfield shifts of C-3 (δ 84.6) of residue **A** with respect to standard value of methyl glycoside (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that residue **A** was (1→3)-linked β-D-Glc*p*. In residue **B**, all carbon chemical shifts values were found nearly to the standard value of methyl glycoside (Agarwal, 1992; Rinaudo & Vincendon, 1982) of β-D-glucose. Thus, residue **B** was terminal β-D-Glc*p*. The downfield shifts at C-3 (δ 84.3) and C-6 (δ 68.7) of residue **C** with respect to standard value of methyl glycoside (Agarwal, 1992; Rinaudo & Vincendon, 1982) indicated that it was (1→3,6)- β-D-Glc*p*. Since the residue **C** was the most rigid part of the backbone of this glucan, its C-3 (δ 84.3) appeared at slightly up

field region (δ 0.3) compared to C-3 (δ 84.6) of the other (1→3)-linked residue **A.** Consequently, the C-6 (δ 68.7) value of residue **C** appeared slightly at the up field region compared to that of the other (1→6)-linked residues (**D**). All the chemical shift values of three **D** residues were same except the chemical shift values of C-6. Among the three **D** residues, one moiety ($\mathbf{D_I}$) was glycosidically linked to the most rigid part **C**, hence, its C-6 signal (δ 68.9) showed 0.1 ppm downfield shift with respect to another two residues of $\mathbf{D_{II}}$ (δ 68.8) due to neighboring effect (Bhanja et al., 2012) of the rigid part **C.** The linking at C-6 of the residues **C** and **D** were further confirmed by DEPT-135 spectrum (**Figure 6**).

The different linkages that connected these residues (**A** to **D**) were determined from ROESY (**Figure 8**, **Table 5**) as well as NOESY (not shown) experiment. In ROESY experiment, the inter-residual contacts **A**H-1/**C**H-3; **B**H-1/**C**H-6a, **C**H-6b; **C**H-1/$\mathbf{D_I}$H-6a, $\mathbf{D_I}$H-6b; $\mathbf{D_I}$H-1/$\mathbf{D_{II}}$H-6a, $\mathbf{D_{II}}$H-6b; and $\mathbf{D_{II}}$H-1/**A**H-3 along with other intra-residual contacts were also observed (**Figure 8**). The above ROESY connectivities established the following sequences:



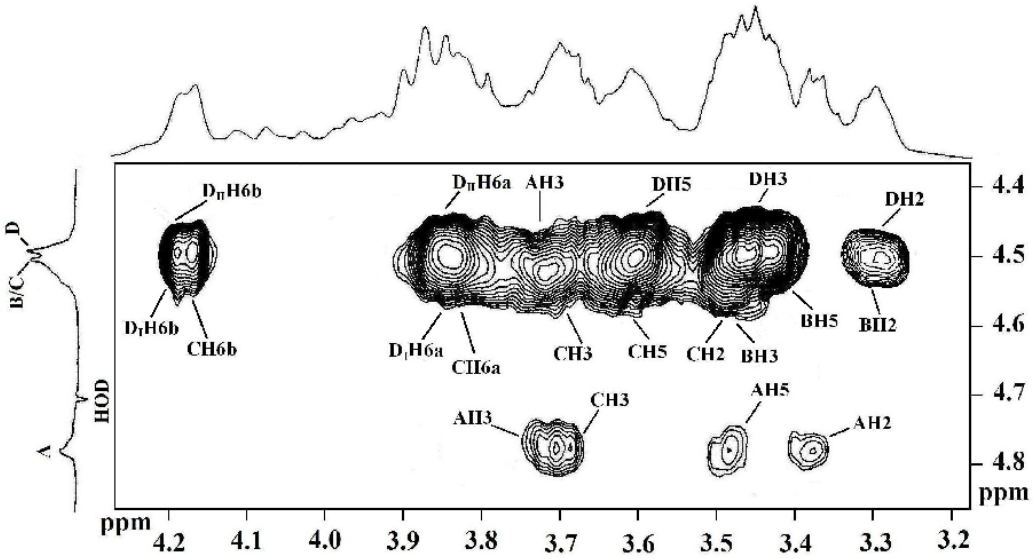


Figure 8. Part of ROESY spectrum of a glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. The ROESY mixing time was 300 ms.

Table 5

ROESY data for the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička.

Glycosyl residue	Anomeric proton	ROE contact proton		
	δ	δ	residue	atom
→3)-β-D-Glc*p*-(1→	4.78	3.69	**C**	H-3
A		3.38	**A**	H-2
		3.70	**A**	H-3
		3.48	**A**	H-5
β-D-Glc*p*-(1→	4.51	3.83	**C**	H-6a
B		4.17	**C**	H-6b
		3.29	**B**	H-2
		3.47	**B**	H-3

		3.43	**B**	H-5
→3,6)-β-D-Glc*p*-(1→	4.51	3.85	**D_I**	H-6a
C		4.19	**D_I**	H-6b
		3.49	**C**	H-2
		3.69	**C**	H-3
		3.61	**C**	H-5
→6)-β-D-Glc*p*-(1→	4.49	3.85[g]	**D_{II}**	H-6a
D		4.19[g]	**D_{II}**	H-6b
		3.70[h]	**A**	H-3
		3.29	**D**	H-2
		3.45	**D**	H-3
		3.61	**D**	H-5

[g] For **D_I** H-1 to both **D_{II}** H-6a and **D_{II}** H-6b contacts.

[h] For **D_{II}** H-1 to **A** H-3 contact.

Finally, the ROESY connectivities were confirmed from HMBC spectrum (**Figure 9**). In this spectrum the inter-residual cross-peaks (**Table 6**) between **A**H-1/**C**C-3, **A**C-1/**C**H-3; **B**H-1/**C**C-6, **B**C-1/**C**H-6a, **C**H-6b; **C**H-1/**D_I**C-6, **C**C-1/**D_I**H-6a, **D_I**H-6b; **D_I**H-1/**D_{II}**C-6, **D_I**C-1/**D_{II}**H-6a, **D_{II}**H-6b; **D_{II}**H-1/**A**C-3, **D_{II}**C-1/**A**H-3 along with some intra-residual peaks were also observed (**Figure 9**).

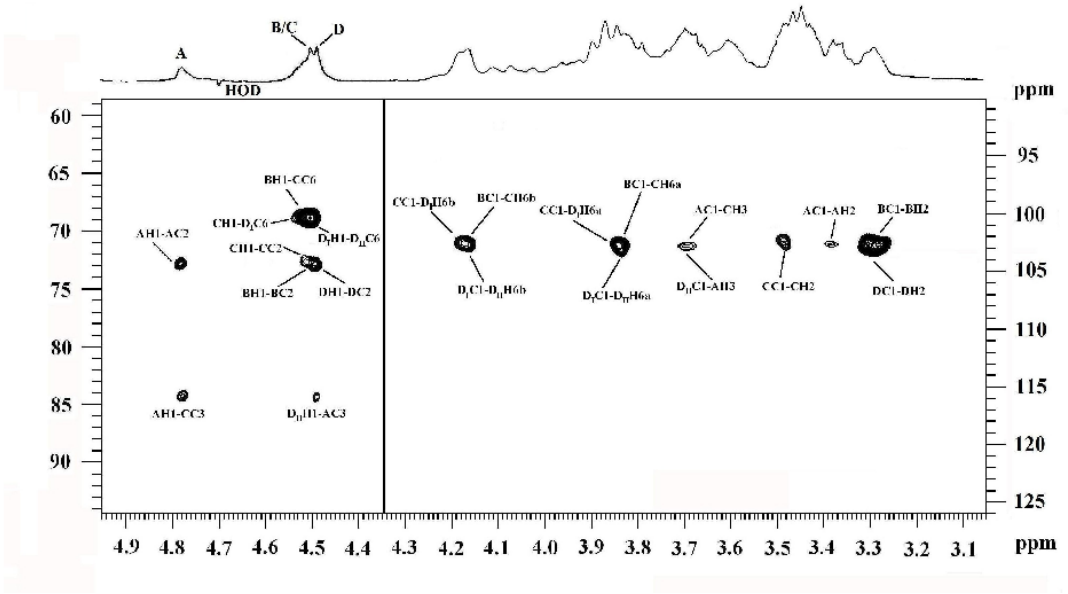


Figure 9. Part of HMBC spectrum of a glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. The delay time in the HMBC experiment was 80 ms.

Table 6

The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička

Residues	Sugar linkage	H-1/C-1	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	→3)-β-D-Glc*p*-(1→	4.78	84.3	**C**	C-3
		102.7	3.69	**C**	H-3
			3.38	**A**	H-2
			72.8	**A**	C-2
B	β-D-Glc*p*-(1→	4.51	68.7	**C**	C-6
		102.7	73.1	**B**	C-2
			3.83	**C**	H-6a

			4.17	**C**	H-6b
			3.29	**B**	H-2
C	→3,6)-β-D-Glc*p*-(1→	4.51	68.9	**D_I**	C-6
		102.5	3.85	**D_I**	H-6a
			4.19	**D_I**	H-6b
			3.49	**C**	H-2
			72.8	**C**	C-2
D	→6)-β-D-Glc*p*-(1→	4.49	68.8[a]	**D_{II}**	C-6
		102.9	84.6[b]	**A**	C-3
			3.85[c]	**D_{II}**	H-6a
			4.19[d]	**D_{II}**	H-6b
			3.70[e]	**A**	H-3
			3.29	**D**	H-2
			73.1	**D**	C-2

[a] For cross peak between **D_I** H-1 and **D_{II}** C-6.

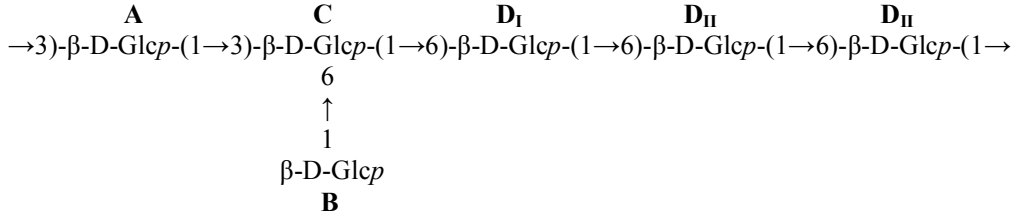
[b] For cross peak between **D_{II}** H-1 and **A** C-3.

[c] For cross peak between **D_I** C-1 and **D_{II}** H-6a.

[d] For cross peak between **D_I** C-1 and **D_{II}** H-6b.

[e] For cross peak between **D_{II}** C-1 and **A** H-3.

Thus, the HMBC and ROESY connectivities clearly supported the presence of the following repeating unit in the ELPS and the structure is proposed as:



The NMR experiment was conducted with Smith degraded material (SDPS) of ELPS for further confirmation of the linkages of this β-glucan. The ^{13}C NMR (125 MHz)

spectrum (**Figure 10**, **Table 7**) at 30 ˚C of SDPS showed two anomeric carbon signals at δ 102.7 and 103.2 corresponding to terminal β-D-Glc*p* (**E**) and (1→3)-β-D-Glc*p* (**F**) residues respectively. The carbon signals C-1, C-2, and C-3 of the glycerol moiety (**G**) was generated from (1→6)-β-D-Glc*p* residue (**D_1**) of the repeating unit after periodate oxidation followed by Smith degradation and assigned as δ 66.4, 72.1, and 62.5 respectively. The **G** moiety would be attached to (1→3)-linked β-D-Glc*p* moiety (**F**) as evidenced from the structure of the repeating unit. The residue (1→3)-β-D-Glc*p* (**A**) was converted to terminal β-D-Glc*p* unit (**E**) during Smith degradation and (1→3,6)-β-D-Glc*p* (**C**) was converted to (1→3)-β-D-Glc*p* (**F**) when the terminal β-D-Glc*p* (**B**) was consumed during oxidation.

Table 7

The ^{13}C NMR[n] chemical shifts of Smith-degraded glycerol-containing disaccharide of the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička in D_2O at 30 ºC.

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p*-(1→ **E**	102.7	73.4	75.9	70.6	76.7	60.8
→3)-β-D-Glc*p*-(1→ **F**	103.2	73.2	84.5	69.7	75.6	61.1
→3)-Gro **G**	66.4	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 ºC.

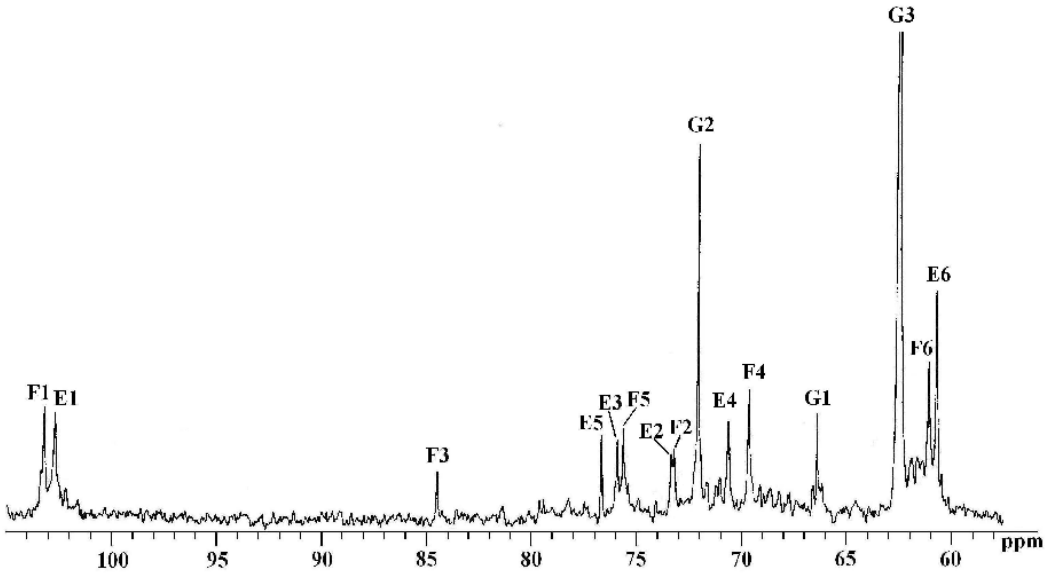


Figure 10. ^{13}C NMR spectrum (125 MHz, D_2O, 30 ºC) of the Smith-degraded glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička.

Hence, Smith degradation yielded an oligosaccharide from the parent polysaccharide and the structure was established as:

E **F** **G**
β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-Gro

Therefore, Smith degradation results further confirmed the repeating unit present in the glucan isolated from the edible mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička.

4.B.4. Immunostimulating properties of ELPS

LAL test showed negative results indicating that the ELPS was free from any endotoxin contamination. Thus, it can be concluded that the immunostimulatory effects were solely for the ELPS. *In vitro* macrophage activation was observed with different concentrations of ELPS. Nitric oxide production was found to increase in dose-dependent manner with optimum production of 22 μM NO per 5×10^5 macrophages at 35 μg/mL of

ELPS. Further increase in concentration of ELPS decreased the NO production (**Figure 11**) implying that the effective dose of the ELPS was 35 µg/mL.

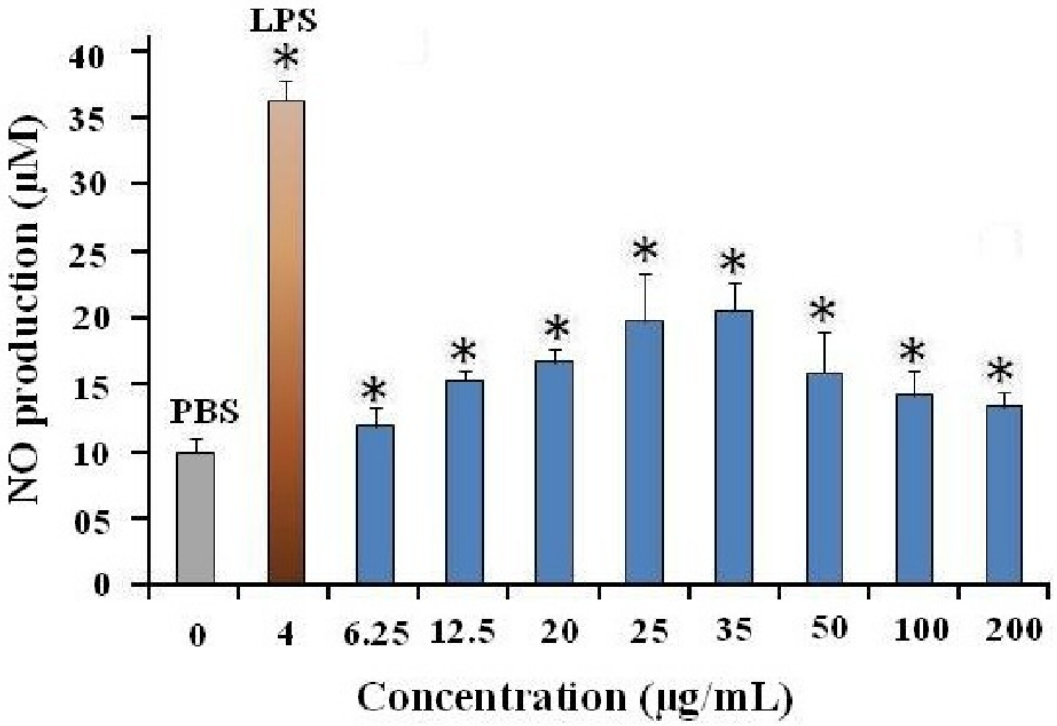


Figure 11. *In vitro* activation of macrophage stimulated with different concentrations of a glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička in terms of NO production. Results are the mean ± SD of n separate experiments, each in triplicate (n = 9 for NO production).

Splenocytes include T cells, B cells, dendritic cells, and macrophages that enhance the immunity in living systems. Thymocytes after maturation in thymus are designated as T cells. The splenocyte and thymocyte activation tests were conducted in mouse cell culture medium with the ELPS by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method (Ohno, Hasimato, Adachi, & Yadomae, 1996). Proliferation of splenocytes and thymocytes is an indication of immunostimulation (Ohno et al., 1993). The splenocyte proliferation index (SPI) and thymocyte proliferation index

(TPI) of ELPS were measured with respect to the lipopolysaccharide (LPS, 4 µg/mL, Sigma) and Concanavalin A (Con A, 10 µg/mL) taking as positive control respectively. Phosphate Buffered Saline (PBS) was chosen as negative control for both splenocyte and thymocyte studies. The ELPS was found to stimulate splenocytes and thymocytes and the results are shown in **Figure 12** and **13**, respectively. The asterisks on the columns indicated the statistically significant differences compared to PBS control. Maximum proliferation index of splenocytes and thymocytes by ELPS were found at 12.5 µg/mL and 100 µg/mL respectively.

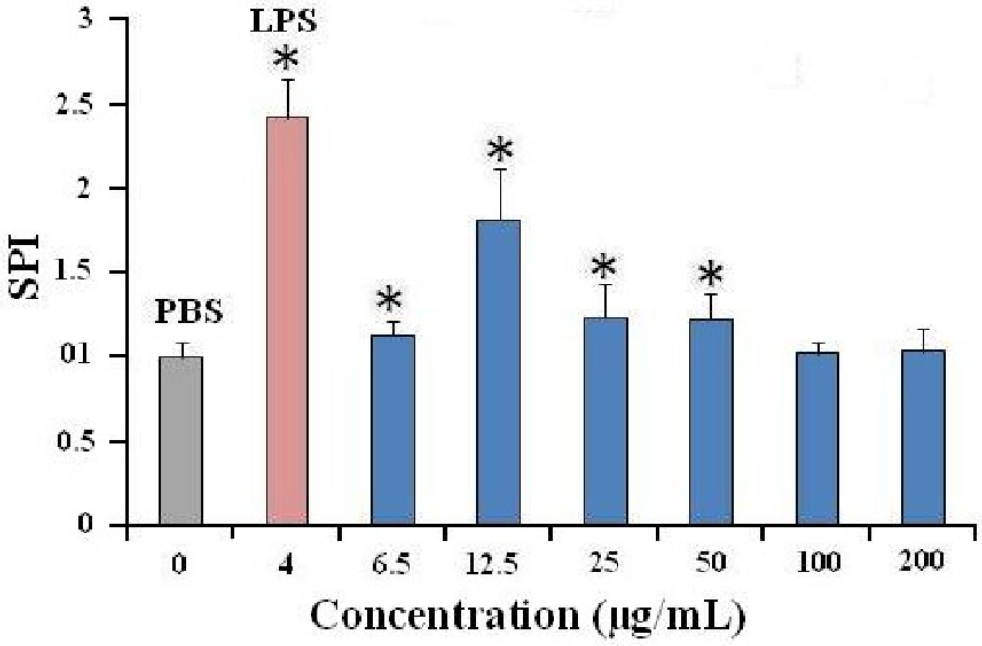


Figure 12. Effect of different concentrations of a glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička on proliferation of splenocyte. (*significant compared to PBS control). Results are the mean ± SD of n separate experiments, each in triplicate (n = 7 for splenocytes).

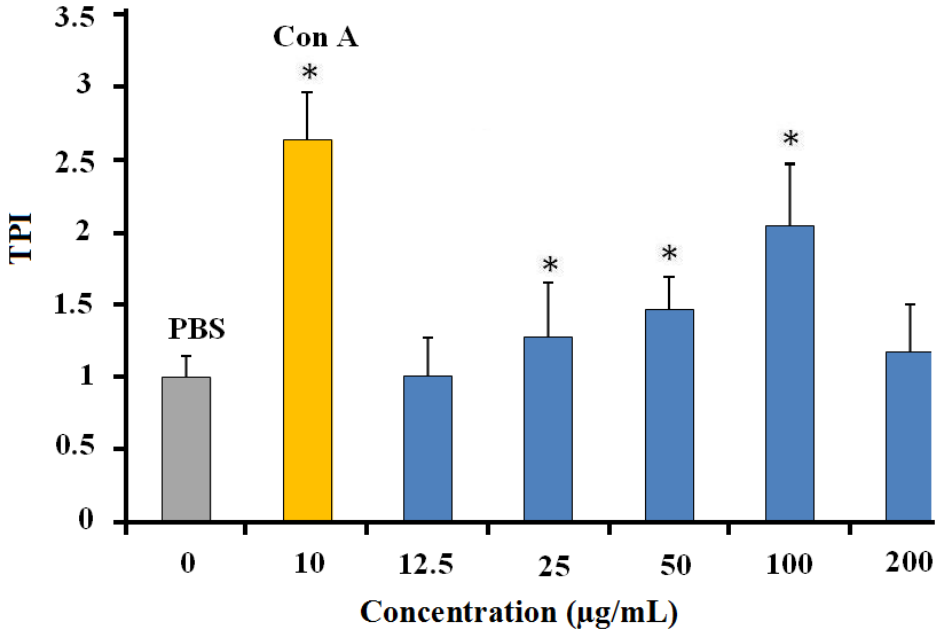


Figure 13. Effect of different concentrations of a glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička on proliferation of thymocyte. (*significant compared to PBS control). Results are the mean ± SD of n separate experiments, each in triplicate (n = 6 for thymocytes).

The decrease in the immunological activities of the polysaccharide after the optimum concentration may be due to insufficient activation signal at the cellular surface. This may happen due to various factors that include the type of cells, the number of receptors on the cells and the nature of the polysaccharide under investigation. Various physical factors of the stimulant (polysaccharide) such as the molecular weight, the number of branching side chains and their conformation in solution determine the extent of their biological activity (Bohn & BeMiller, 1995; Ohno, Miura, Nakajima, & Yadomae, 2000). From these findings, it can be concluded that 35 µg/mL, 12.5 µg/mL and 100 µg/mL are the optimum concentration of ELPS for macrophage, splenocytes and thymocytes proliferation respectively.

4.B.5. Antioxidant properties of ELPS

4.B.5.1. Assay of hydroxyl radical scavenging activity

The hydroxyl radicals are most toxic among all reactive oxygen species (ROS) and can easily cross cell membranes at specific sites, react with most biomolecules and furthermore cause tissue damage and cell death (Aruoma, 1998). It is therefore important to remove the hydroxyl radicals for protection of the biological systems (Yang, Guo, & Yuan, 2008). Hydroxyl radical scavenging activity of ELPS was measured at different concentrations (100 to 800 µg/mL) taking butylated hydroxytoluene (BHT) as positive control (Halliwell, Gutteridge, & Aruoma, 1987). These results indicated that the activity of the ELPS gradually increases with the increase of concentrations (**Figure 14**). The hydroxyl radical scavenging activities of ELPS and BHT were respectively 20.48% and 91.2% at a dose of 200 µg/mL, indicating that antioxidant activity of ELPS is weak compared to BHT. The IC_{50} value of the ELPS was found to be 400 µg/mL (**Figure 14**). The antioxidant mechanism may be due to the supply of hydrogen by the polysaccharide, which combines with radicals and forms a stable radical to terminate the radical chain reaction.

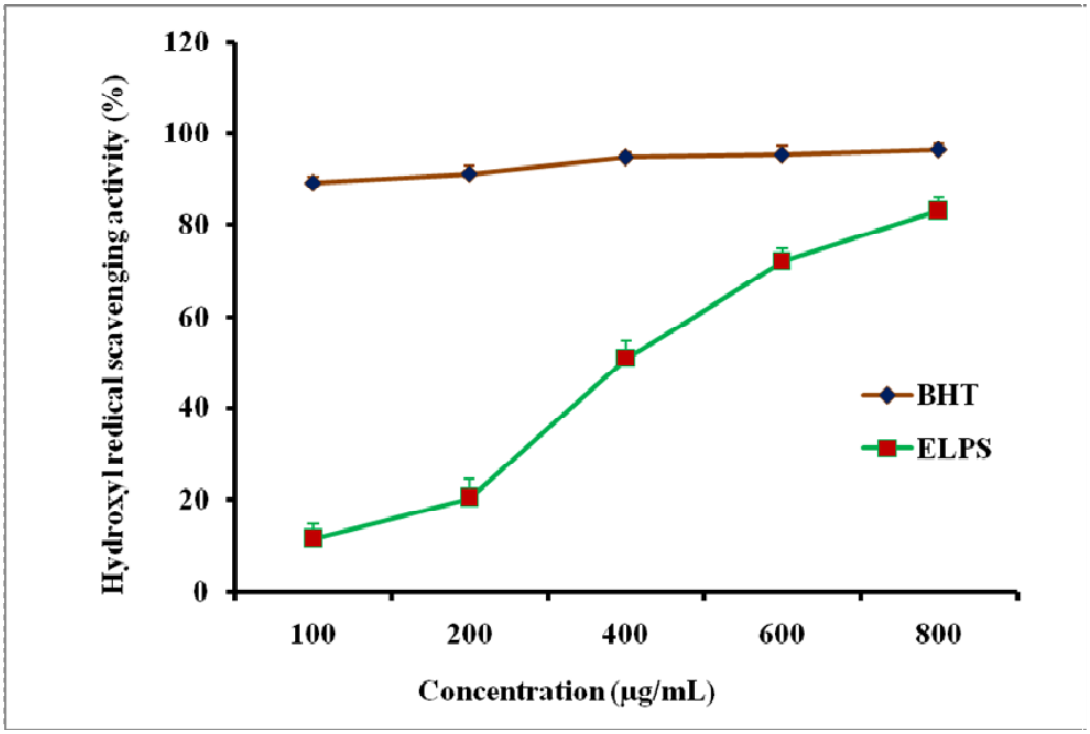


Figure 14. Hydroxyl radical scavenging activity of the ELPS isolated from the *Entoloma lividoalbum* (Kühner & Romagn) Kubička. Results are the mean ± SD of five separate experiments, each in triplicate.

4.B.5.2. Assay of superoxide radical scavenging activity

Superoxide radicals are harmful to cellular components as they are the precursor of several reactive species (Halliwell & Gutteridge, 1989). These are considered as the primary ROS which can further interact with other molecules to generate secondary ROS such as hydroxyl radicals, hydrogen peroxide, and singlet oxygen. As a result, the formation of superoxide radical could induce oxidative damage in lipids, proteins, and DNA (Stief, 2003; Wickens, 2001). The superoxide radical scavenging activities of ELPS and butylated hoydroxyanisole (BHA) were determined to be 56.58% and 89.88%, respectively at the dose of 100 µg/mL (**Figure 15**). At all concentrations, ELPS showed

lower superoxide anion scavenging activity than synthetic standard drug BHA. The IC_{50} value of the ELPS was found to be 75 µg/mL.

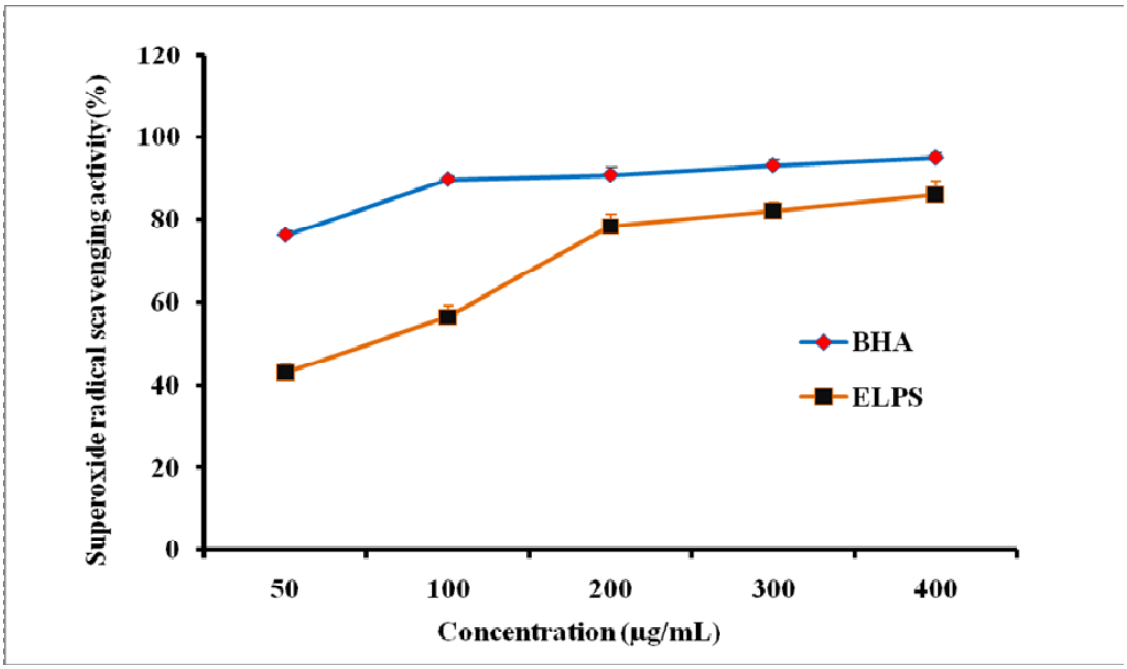


Figure 15. Superoxide radical scavenging activity of the ELPS isolated from the *Entoloma lividoalbum* (Kühner & Romagn) Kubička Results are the mean ± SD of five separate experiments, each in triplicate.

4.B.5.3 Determination of reducing power

The reducing capacity of a compound is regarded as the indicator of its potential antioxidant activity (Chanda & Dave, 2009). It is measured in terms of the absorbance at 700 nm of the reaction mixture (Maity, Samanta, et al., 2014) containing different concentrations of ELPS. **Figure 16** shows the reducing power of ELPS and ascorbic acid increased with increasing sample concentration. The reducing power (absorbance at 700 nm) of ELPS and ascorbic acid were 0.2285 and 1.16 at 200 µg/mL respectively. At concentration of 470 µg/mL, ELPS showed reducing power 0.5. This result suggests that

ELPS has potency to donate electron to reactive free radicals, converting them into more stable non-reactive species and terminating the free radical chain reaction.

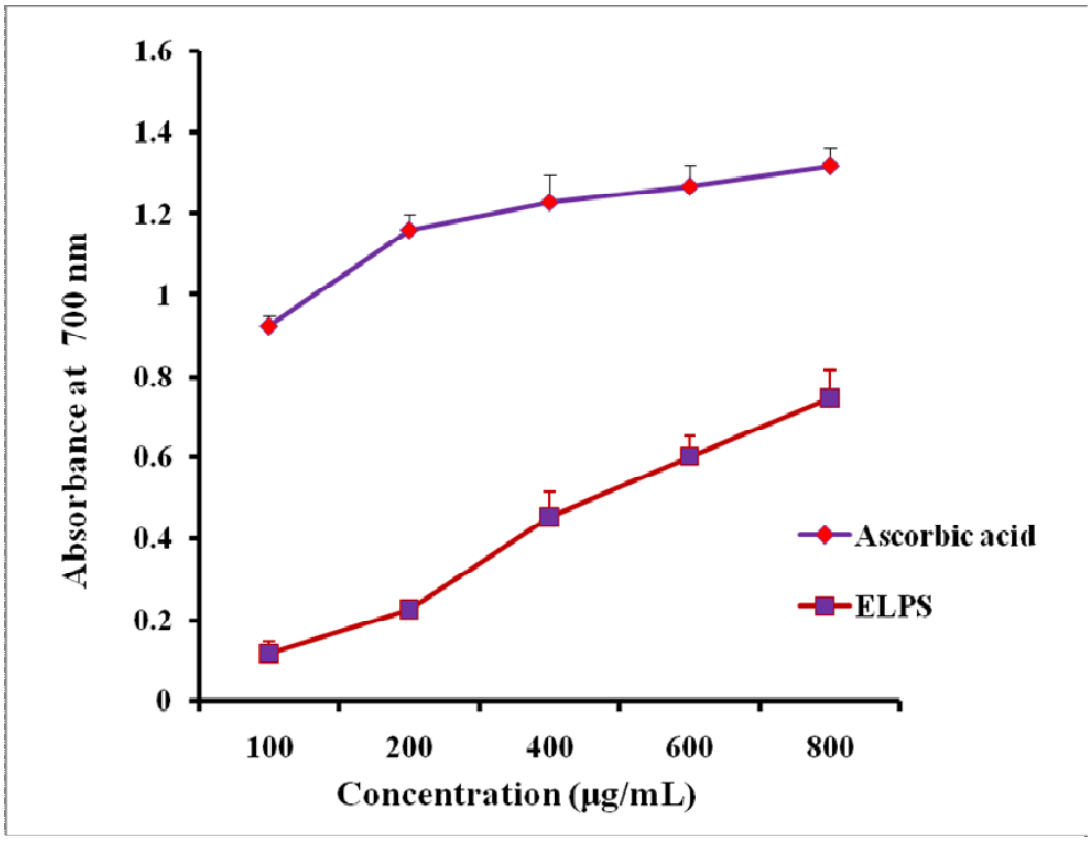


Figure 16. Determination of reducing power of the ELPS isolated from the *Entoloma lividoalbum* (Kühner & Romagn) Kubička. Results are the mean ± SD of five separate experiments, each in triplicate.

In the present study, ELPS showed immunostimulatory effects through the production of reactive nitrogen species which are strong oxidants and on the other hand these also behave as anti-oxidants. The explanation to such paradoxical observation may be referred to the dual function of ELPS. It has been established that the polysaccharides bind to beta-glucan receptors and exhibit immunomodulatory effects during macrophage activation through the production of reactive nitrogen species. The antioxidant ability of the carbohydrates depends on monosaccharide constituents and the scavenging activity

increases when they are in polymeric forms. In the present manuscript, optimum immunostimulating effects of ELPS were observed in cell culture medium at lower concentrations (12.5µg/mL for splenocyte proliferation and 35µg/mL for NO production during macrophage activation). Antioxidant properties were studied using chemical methods which showed the activities were maximum at higher concentrations (800 µg/mL for hydroxyl radical scavenging and reducing power and 400 µg/mL for superoxide radical scavenging). These results confirmed the low antioxidant activity of ELPS in comparison to other classical scavengers. Thus, the weak scavenging activities of ELPS obtained from chemical methods cannot be correlated to its immunomodulatory effects studied in cell culture medium. Similar observations were also reported by Tsiapali et al. (2001).

4.C. Conclusion

A water soluble β-glucan (ELPS) was isolated from the aqueous extract of an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. Chemical and spectroscopic analysis showed that the backbone of glucan consists of three (1→6)-β-D-glucopyranosyl and two (1→3)-β-D-glucopyranosyl residues, out of which one (1→3)-β-D-glucopyranosyl moiety was branched at *O*-6 with a terminal β-D-glucopyranosyl residue. ELPS showed macrophage activation in a dose-dependent manner with optimum production of NO at 35 µg/mL. Splenocyte and thymocyte proliferation indices were found maximum at the ELPS dosages of 12.5 µg/mL and 100 µg/mL respectively. Thus, from the above results ELPS can be considered as a potent immunostimulating agent. Moreover, the hydroxyl and superoxide free radical scavenging activity showed that the ELPS has IC_{50} value of 400 µg/mL and 75 µg/mL respectively and also the reducing power was found 0.5 at concentration of 470 µg/mL. Therefore, the polysaccharide of the edible mushroom *Entoloma lividoalbum* should also be explored as a natural antioxidant for use in functional foods or medicine.

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CHAPTER 5

A partially methylated mannogalactan from hybrid mushroom pfle 1p: purification, structural characterization and study of immunoactivation

5.A. Introduction and earlier works

Mushrooms are valuable foods and a source for the development of new drugs (Wani, Bodha, & Wani, 2010; Wasser & Weis, 1999). Mushroom polysaccharides, especially β-glucans are best known for their antitumor and immunostimulating properties (Mizuno et al., 1990; Wasser, 2002). Besides glucans, other polysaccharides such as galactans, fucans, xylans and mannans isolated from different mushrooms also exhibit significant biological activities (Zhang, Cui, Cheung, & Wang, 2007). Development of new hybrid mushrooms through para-sexual mating is now well established. Several somatic edible hybrid mushrooms were developed through polyethylene glycol-mediated protoplast fusion between *Pleurotus florida* and *Volvariella volvacea* (Chakraborty & Sikdar, 2008), *Pleurotus florida* and *Calocybe indica var.* APK2 (Chakraborty & Sikdar, 2010), *Pleurotues florida* and *Lentinus* squarrosulus (Mallik & Sikdar, 2015), *Pleurotus florida* and *Lentinula edodes* (Mallik & Sikdar, 2014). Hybridity of the fusant lines was set up on the basis of their colony morphology, mycelia growth rate and hypal traits, while the fruit body generating lines were demonstrated on the basis of nature of sporophores, isozymes, random amplified polymorphic DNA markers (Chakraborty & Sikdar, 2010). A few biologically active glucans and heteroglycans have been isolated and reported from hybrid mushrooms. Two immunostimulating and antioxidant polysaccharides from fruit bodies of the strain *PCH9FB* (hybrid of *P. florida* and *C. indica var.* APK2) were isolated and reported (Maity, Kar et al., 2011; Maity, Mandal et al., 2011). Two polysaccharides were isolated from aqueous (Nandan et al., 2011) and alkaline (Sarkar et al., 2012) extract of another hybrid mushroom obtained through back cross mating of a somatic hybrid mushroom *PfloVv12* (sterile line) with *V. volvacea*. Different immunoenhancing glucans from somatic hybrid *PfloVv5FB* (Das et al., 2010; Maity, Patra et al., 2013) and heteroglycan from somatic hybrid *PfloVv1aFB* (Bhunia et al., 2012; Patra et al., 2011) of *P. florida* and *V. volvacea* were isolated and reported by our group.

5.B. Present work

5.B.1. The hybrid mushroom *pfle 1p*

The oyster mushroom *Pleurotus florida* is cultivated in the sub-tropical climate (22–26 ºC). This mushroom is attractive because of their flavor, taste, and high nutritive values. Compositional analyses revealed that *P. florida* is highly rich in protein, carbohydrate, fibre, and essential fatty acids (Crisan & Sands, 1978). Four different immunostimulating polysaccharides (Rout et al., 2004; Rout et al., 2005; Rout, Mondal, Chakraborty, & Islam, 2006; Rout, Mondal, Chakraborty, & Islam, 2008), were isolated from *Pleurotus florida*.

Lentinula edodes, known as 'shiitake' is one of the widely used medicinal mushrooms of the East Asia, which is attributed not only to its nutritional value but also to possible potential for therapeutic applications. Lentinan, a water insoluble (1→3)-, (1→6)-β-glucan isolated from *Lentinula edodes* is well known for its high antitumor activity (Sasaki, & Takasuka, 1976). A water soluble glucan having prominent antitumor activity was also isolated from *Lentinula edodes* (Chihara et al., 1970; Xu, Chen, Zhang, & Ashida, 2012; Yu et al., 2010).

Intergeneric protoplast fusion between the strains of *Pleurotus florida* and *Lentinula edodes* produced nine new hybrid strains, out of which six strains *i.e.*, *pfle 1o, pfle 1p, pfle 1q, pfle 1r, pfle 1s,* and *pfle 1v* were found to produced fruit bodies (Mallik, & Sikdar, 2014). Recently, different immunostimulating polysaccharides have been isolated from the hybrid strain *pfle 1q, pfle 1r,* and *pfle 1v* (Maity, Mandal, et al., 2013; Maji et al., 2012; Maji et al., 2013a, Maji et al., 2013b). Two water soluble polysaccharides (PS-I and PS-II) were isolated from the fruit bodies of *pfle 1p* (**Figure 1**). PS-I (Maity, Bhunia, et al., 2013) was found to consist of D-glucose, D-galactose and D-mannose in a molar ratio of nearly 4:2:1 and PS-II consisted of mannose, 3-*O*-Me-galactose and galactose in a ratio of nearly 1:1:1. The detailed structural characterization and study of immunoactivation of PS-II was carried out and discussed in this chapter.

Figure 1. Photograph of the fruit bodies of the hybrid mushroom *pfle 1p*

5.B.2. Isolation and purification of polysaccharide from *pfle 1p*

The hybrid mushroom *pfle 1p* was cultivated and collected from Falta experimental farm, Bose Institute. A water soluble crude polysaccharide was isolated from the fruiting bodies of *pfle 1p* by hot water extraction followed by ethanol precipitation. The polysaccharide (25 mg) was purified by Sepharose 6B column using water as eluant and yielded two fractions (**Figure 2**). Fraction I and fraction II were collected and freeze dried, yielding purified polysaccharide of 11 mg PS-I and 8 mg PS-II, respectively.

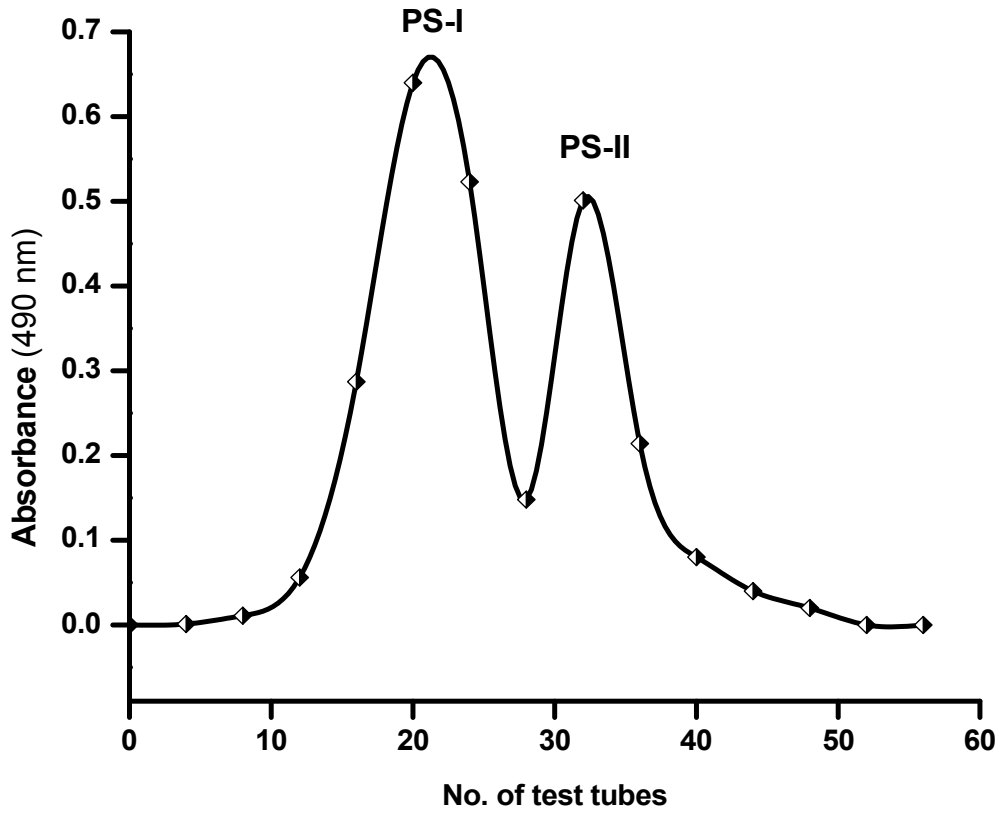


Figure 2. Gel permeation chromatogram of crude polysaccharide isolated from hybrid mushroom *pfle 1p* using Sepharose 6B column.

5.B.3. Optical rotation and molecular weight of PS-II

The PS-II showed specific rotation $[\alpha]_D^{28.6}$ +54.7 (*c* 0.91, water). The apparent molecular weight (Hara, Kiho, Tanaka, & Ukai, 1982) of PS-II was estimated as ~1.65 × 10^5 Da from a calibration curve prepared with standard dextrans (**Figure 3**).

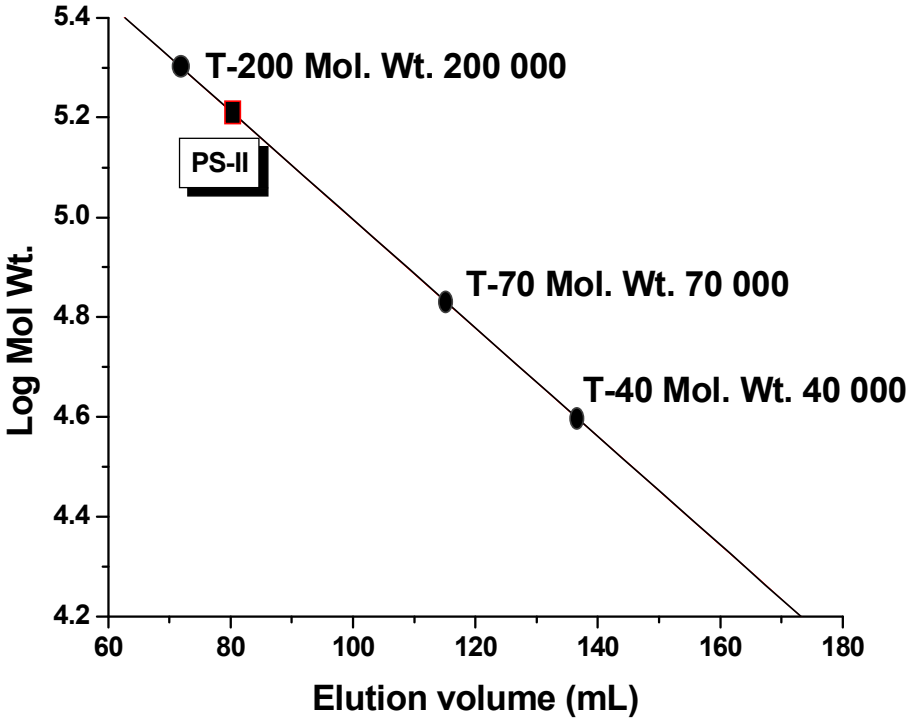


Figure 3. Determination of molecular weight of PS-II by gel permition chromatography in sepharose 6B column.

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

5.B.4.1. Chemical analysis of PS-II

The sugar analysis of PS-II by paper chromatography (Hoffman, Lindberg, & Svensson, 1972) and GLC analysis of alditol acetates (Lindahl, 1970) of hydrolyzed product of PS-II confirmed the presence of mannose, galactose, and 3-*O*-methyl-galactose almost in a ratio of 1.0:0.99:1.1. The absolute configuration (Gerwig, Kamerling, & Vliegenthart, 1978) of all the sugar residues were determined as D. The mode of linkages of the sugar moieties present in the PS-II was determined by methylation analysis using the Ciucanu and Kerek (1984) method followed by hydrolysis and alditol acetate conversion. The GLC–MS analysis of partially methylated alditol acetates of PS-II

revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol and 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-D-galactitol in a ratio of nearly 1:1:1 (**Table 1**, **Figure 4**). Thus, PS-II was assumed to consist of terminal D-mannopyranosyl, (1→6)-D-galactopyranosyl and (1→2,6)-D-galactopyranosyl moieties respectively. These linkages were further confirmed by periodate oxidation experiment. The GLC-MS analysis of the alditol acetates of periodate oxidized-reduced (Goldstein, Hay, Lewis, & Smith, 1965; Hay, Lewis, & Smith, 1965) and methylated (Abdel-Akher & Smith, 1950) PS-II showed the presence of only 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-D-galactitol (**Table 2**, **Figure 5**). These results clearly indicated that terminal D-mannopyranosyl and (1→6)-linked D-galactopyranosyl residues were consumed during oxidation while (1→2,6)-linked D-galactopyranosyl residue was unaffected by periodate since the C-3 position of the branched galactopyranosyl residue was already occupied by the -*O*Me group. Hence, the mode of linkages in the PS-II was confirmed.

Table 1

GLC-MS analysis of methylated polysaccharide (PS-II) isolated from *hybrid mushroom pfle 1p*

Methylated sugars	Linkages	Molar Ratio	Major Fragments (m/z)
2,3,4,6-Me_4-Man*p*	Man*p*-(1→	1	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
2,3,4-Me_3-Gal*p*	→6)-Gal*p*-(1→	1	43,45,71,87,101,117,129,161, 173,189,233
3,4-Me_2-Gal*p*	→2,6)-Gal*p*-(1→	1	43,71,87,99,129,159,173,189,233

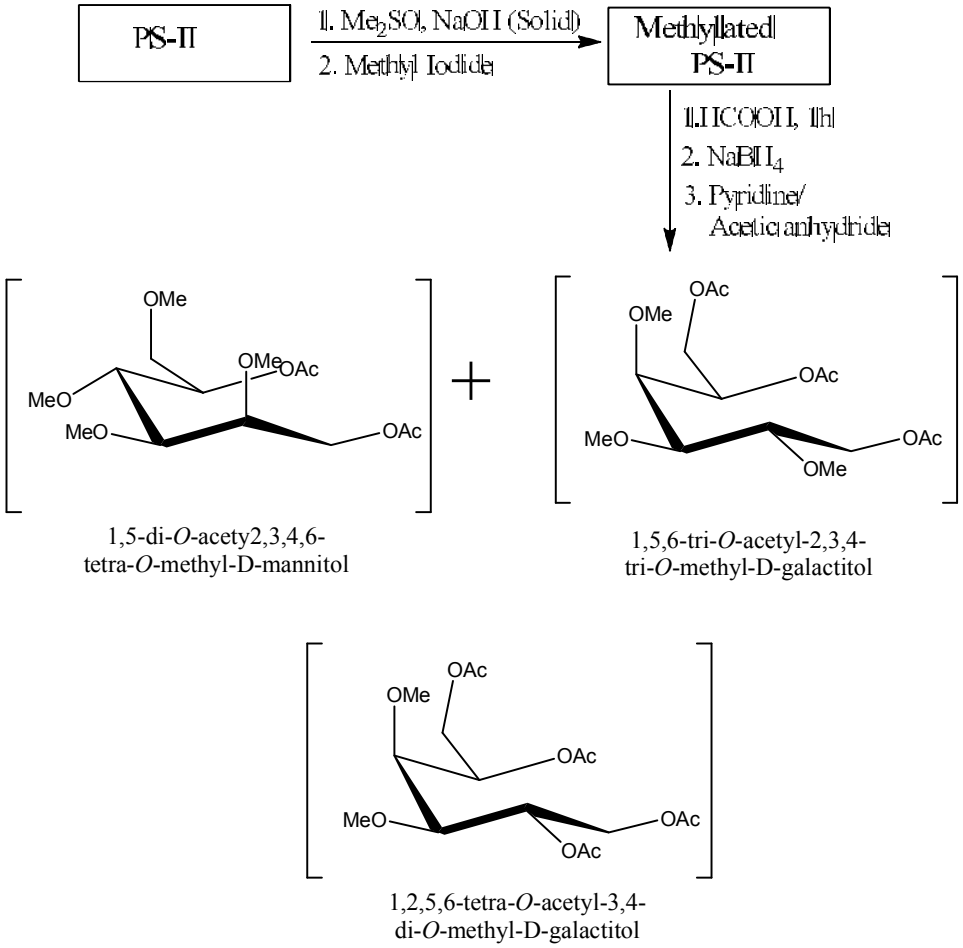


Figure 4. Schematic presentation of methylation experiment of PS-II isolated from hybrid mushroom *pfle 1p.*

Table 2. GLC-MS analysis of periodate oxidized methylated PS-II of hybrid mushroom *pfle 1p.*

Methylated sugars	Linkages	Molar Ratio	Major Fragments (m/z)
3,4-Me_2-Gal*p*	→2,6)-Gal*p*-(1→	1	43,71,87,99,129,159,173,189,233

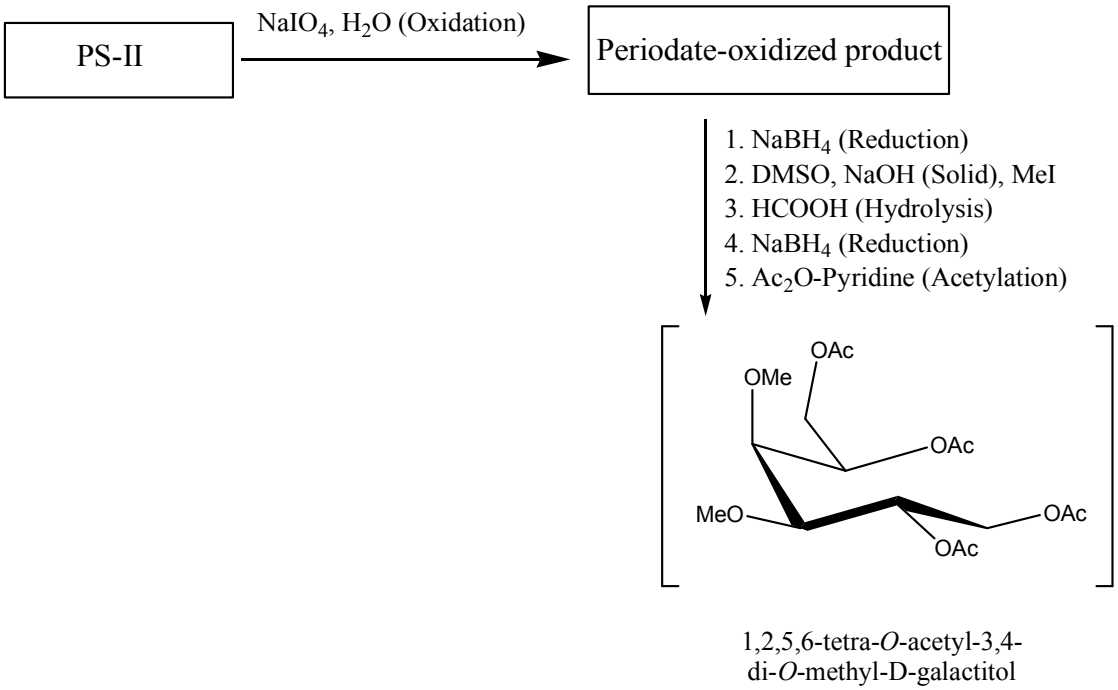


Figure 5. Schematic presentation of periodate oxidation reactions of PS-II isolated from hybrid mushroom *pfle 1p*.

5.B.4.2. 1D and 2D NMR analysis of PS-II

The ^{1}H NMR spectrum (500 MHz; **Figure 6**, **Table 3**) showed three peaks in the anomeric region. The peaks were observed at δ 5.12, 4.98 and 4.78 in a ratio of nearly 1:1:1. Rest of the sugar protons were observed in the region of δ 3.37-4.18 and one -*O*CH$_3$ group signal at δ 3.43. The anomeric peaks were designated **A**, **B** and **C** according to their decreasing proton chemical shifts.

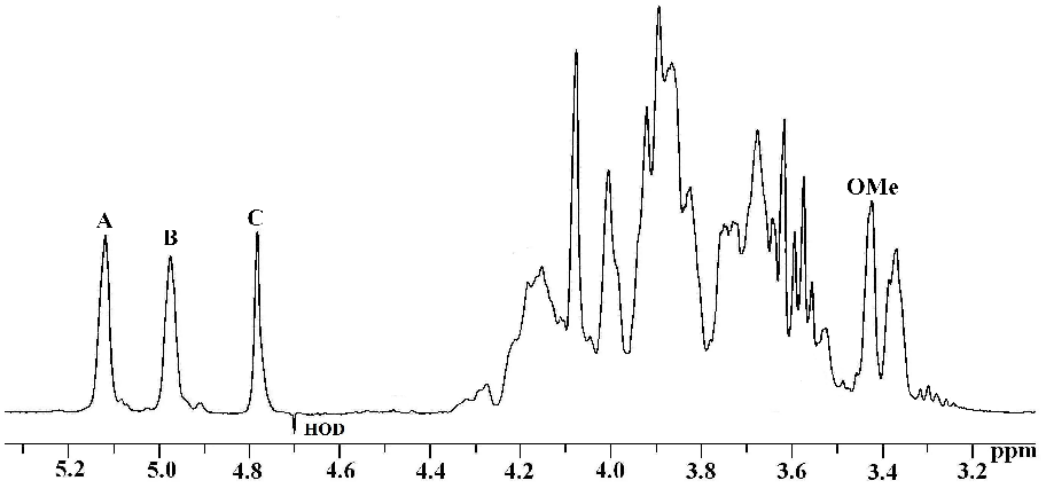


Figure 6. ^{1}H NMR spectrum (500 MHz, D_2O, 30ºC) of the PS-II isolated from hybrid mushroom (*pfle 1p*) of *Pleorutus florida* and *Lentinula edodes*.

Table 3

The ^{1}H and ^{13}C NMR chemical shifts for the PS-II isolated from *pfle 1p* [a,b] 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	*O*Me
→2,6)-3-*O*-Me-α-D-Gal*p*-(1→ **A**	5.12 98.2	3.94 77.0	3.53 79.1	4.15 68.5	3.99 68.3	3.86[c], 3.67[d] 66.7	3.43 56.0
→6)-α-D-Gal*p*-(1→ **B**	4.98 97.9	3.83 68.3	3.87 69.5	4.18 68.8	4.01 69.2	3.88[c], 3.68[d] 67.0	
β-D-Man*p*-(1→ **C**	4.78 101.7	4.08 70.5	3.63 73.0	3.57 66.8	3.37 76.3	3.90[c], 3.75[d] 61.1	

[a] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.70 at 30 °C.

[b] Values of the ^{13}C chemical shifts were recorded with reference to acetone as the internal standard and fixed at δ 31.05 at 30 °C.

[c,d] Interchangeable.

In the ^{13}C NMR spectrum (125 MHz; **Figure 7a**, **Table 3**) three peaks appeared in the anomeric region at δ 101.7, 98.2 and 97.9 in a ratio of nearly 1:1:1. The other carbon signals came in the region δ 79.1-61.1. In addition, there was a signal at δ 56.0, which was assigned for -*O*CH_3 signal. From HSQC spectrum (**Figure 8**, **Table 3**) anomeric proton signals at δ 5.12 (**A**), 4.98 (**B**) and 4.78 (**C**) were correlated to the carbon signals at δ 98.2, 97.9 and 101.7 respectively. The chemical shifts of -*O*CH_3 group (δ 3.43/ 56.0) were also assigned from HSQC spectrum. Rest of the ^{1}H and ^{13}C signals were assigned from DQF-COSY, TOCSY, NOESY, ROESY 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.

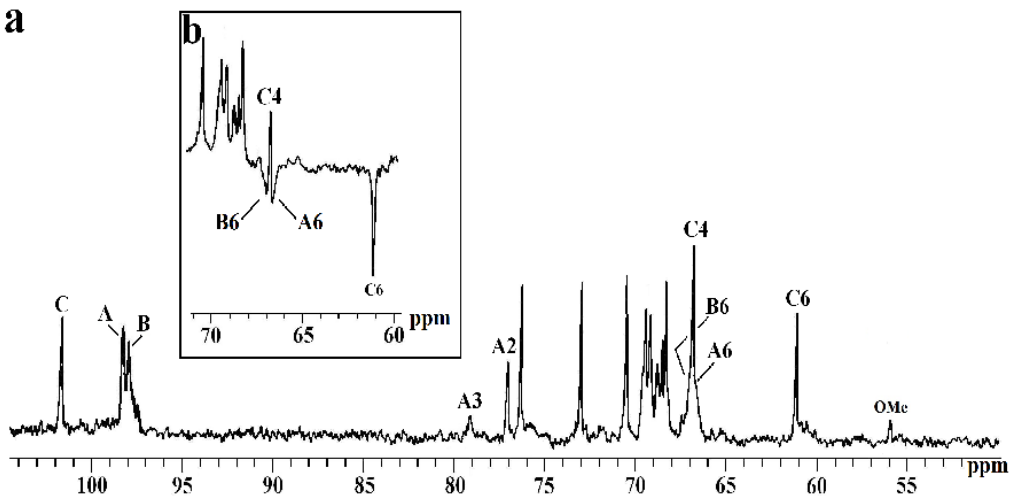


Figure 7. (a) ^{13}C NMR spectrum (125 MHz, D_2O, 30ºC) (b) with insert of the part of DEPT-135 spectrum (D_2O, 30ºC) of the PS-II isolated from hybrid mushroom (*pfle 1p*) of *Pleorutus florida* and *Lentinula edodes* (inset).

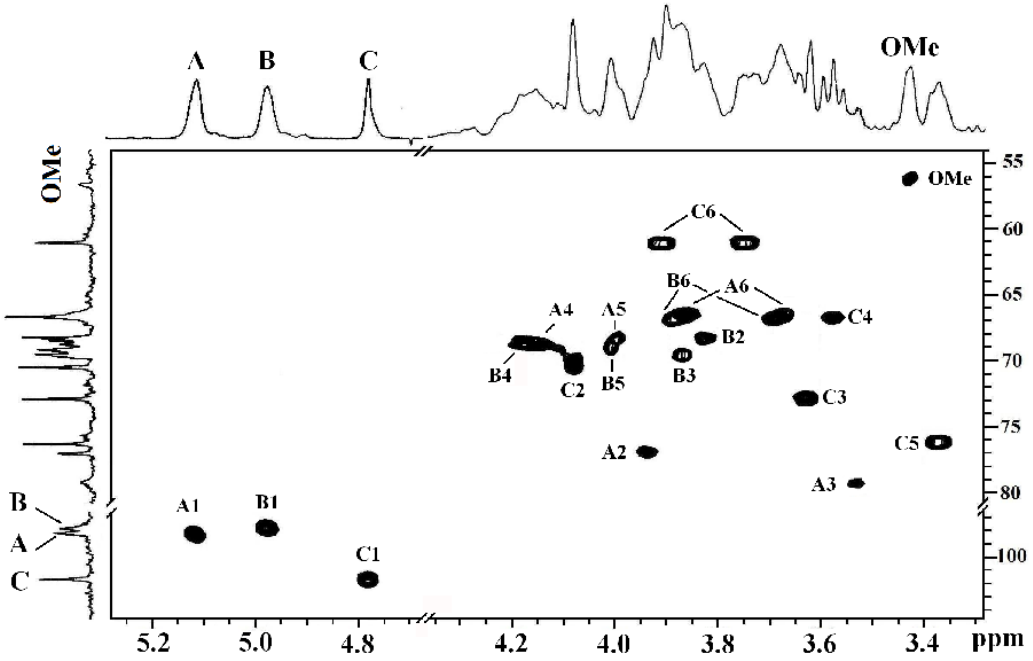


Figure 8. HSQC spectrum (D_2O, 30ºC) of the PS-II isolated from hybrid mushroom (*pfle 1p*) of *Pleorutus florida* and *Lentinula edodes*.

Among these three residues, residues **A** and **B** have coupling constant values of $J_{H-2,H-3}$ ~9 Hz and $J_{H-3,H-4}$ ~3.5 Hz and thus they are established as D-galactopyranosyl residues. The anomeric proton chemical shifts (δ 5.12 for **A** and δ 4.98 for **B**) as well as the coupling constant values ($J_{H-1,H-2}$ ~3.1 and $J_{C-1,H-1}$ ~171 Hz) confirmed that both residues were present as α anomer. Similarly, from the coupling constant values and the chemical shift of the anomeric proton, residue **C** was established as β-linked (δ 4.78; $J_{H-1,H-2}$ ~0, $J_{C-1,H-1}$ ~161) mannopyranosyl moiety ($J_{H-3,\,H-4}$ ~7.5 Hz and $J_{H-4,\,H-5}$ ~10 Hz).

In case of residue **A**, the anomeric carbon signal appeared at δ 98.2. All the proton and carbon chemical shifts except C-2, C-3 and C-6 matched nearly with the standard values of methyl glycosides (Agarwal, 1992; Rinaudo & Vincendon, 1982) where downfield shifts of C-2 (δ 77.0) and C-6 (δ 66.7) appeared. These observations confirmed that residues **A** was linked at C-2 and C-6. The linkage at C-6 was further confirmed from

DEPT-135 spectrum (**Figure 7b**). ^{1}H resonance of -*O*CH_3 correlated with C-3 (δ 79.1) (Poppera, Sadlerb & Frya, 2001) of residue **A** in the HMBC spectrum which confirmed that the -*O*CH_3 was located at C-3 position of residue **A**. Thus, **A** was confirmed as (1→2,6)-linked 3-*O*-methyl-α-D-galactopyranosyl residue.

Residue **B** had an anomeric carbon signal at δ 97.9. The downfield shift of C-6 (δ 67.0) of residue **B** with respect to standard methyl glycosides indicated that it was linked at C-6. The linkage at C-6 was further supported by DEPT-135 spectrum (**Figure 7b**). Therefore, **B** was confirmed as (1→6)-linked α-D-galactopyranosyl residue. In case of residue **C**, anomeric carbon appeared at δ 101.7. All the proton and carbon chemical shifts of this residue corresponded nearly to the standard values of methyl glycosides of mannose. Thus, **C** was confirmed as terminal β-D-mannopyranosyl residue.

From these observations the repeating unit may consist of a longer stretches of -A-A-A-A- and -B-B-B-B- (with **C** attached to **A**) but in that case the electronic environment of most of the **A** and **B** residues will be different and hence different chemical shift values would be observed both in ^{1}H and ^{13}C (Bhanja et al., 2012; Yoshika et al., 1985) but in the present NMR spectrum only three distinct anomeric proton peaks were observed which corresponded to the three anomeric carbon peaks as evident from HSQC spectrum (**Figure 8**). Hence, this kind of combination is discarded and also established that only three sugar residues were present in the repeating unit of the polysaccharide.

The different linkages that connected these three residues were determined from NOESY (**Figure 9** and **Table 4**) as well as ROESY spectrum (not shown). In NOESY spectrum, the inter-residual contacts were observed between **A**H-1/**B**H-6a and **B**H-6b; **B**H-1/**A**H-6a and **A**H-6b; and **C**H-1/**A**H-2 along with other intra-residual contacts (**Figure 9**). The above NOESY connectivities established the following sequences:

A **B**
→6)-3-*O*-Me-α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→

A
→6)-3-*O*-Me-α-D-Gal*p*-(1→
↑
1
β-D-Man*p*
C

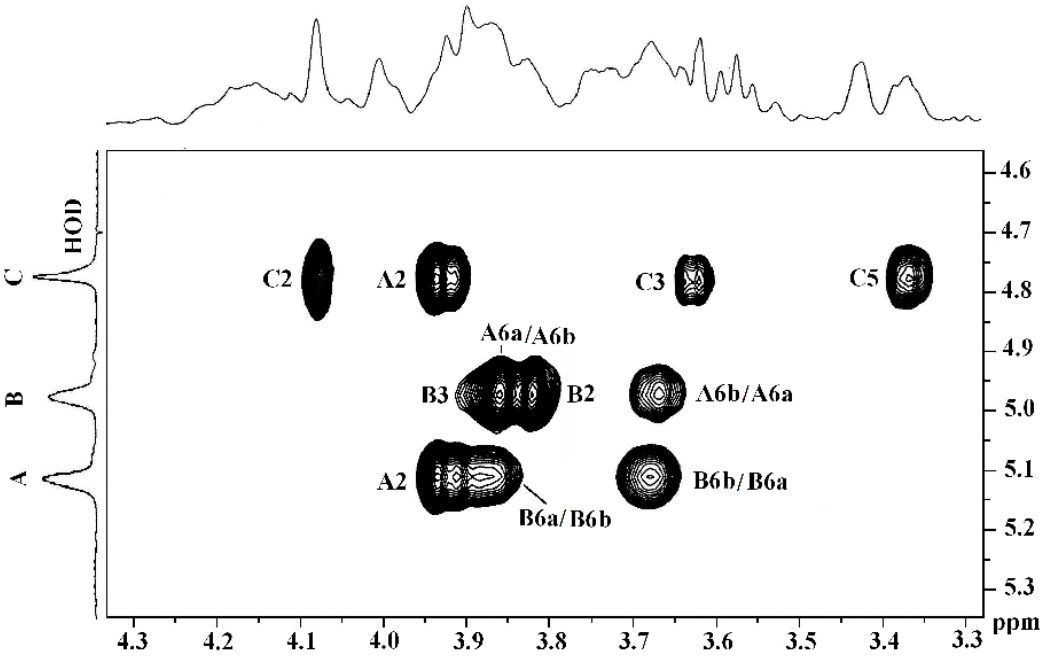


Figure 9. Part of the NOESY spectrum of the PS-II isolated from hybrid mushroom (*pfle 1p*) of *Pleorutus florida* and *Lentinula edodes*. The NOESY mixing time was 300 ms.

Table 4

NOESY data for the PS-II isolated from hybrid mushroom *pfle 1p*

Glycosyl residue	Anomeric proton	NOE contact protons	
	δ	δ	Residue, atom
→2,6)-3-*O*-Me-α-D-Gal*p*-(1→ **A**	5.12	3.94	**A** H-2
		3.88	**B** H-6a
		3.68	**B** H-6b
→6)-α-D-Gal*p*-(1→ **B**	4.98	3.83	**B** H-2
		3.87	**B** H-3
		3.86	**A** H-6a
		3.67	**A** H-6b
β-D-Man*p*-(1→ **C**	4.78	4.08	**C** H-2
		3.63	**C** H-3
		3.37	**C** H-5
		3.94	**A** H-2

Finally, these links were confirmed from HMBC spectrum (**Figure 10** and **Table 5**). In this spectrum the inter-residual cross-peaks (**Table 5**) were observed between **A**H-1/**B**C-6; **A**C-1/**B**H-6a and **B**H-6b; **B**H-1/**A**C-6; **B**C-1/**A**H-6a and **A**H-6b; **C**H-1/**A**C-2; and **C**C-1/**A**H-2 along with other intra-residual contacts.

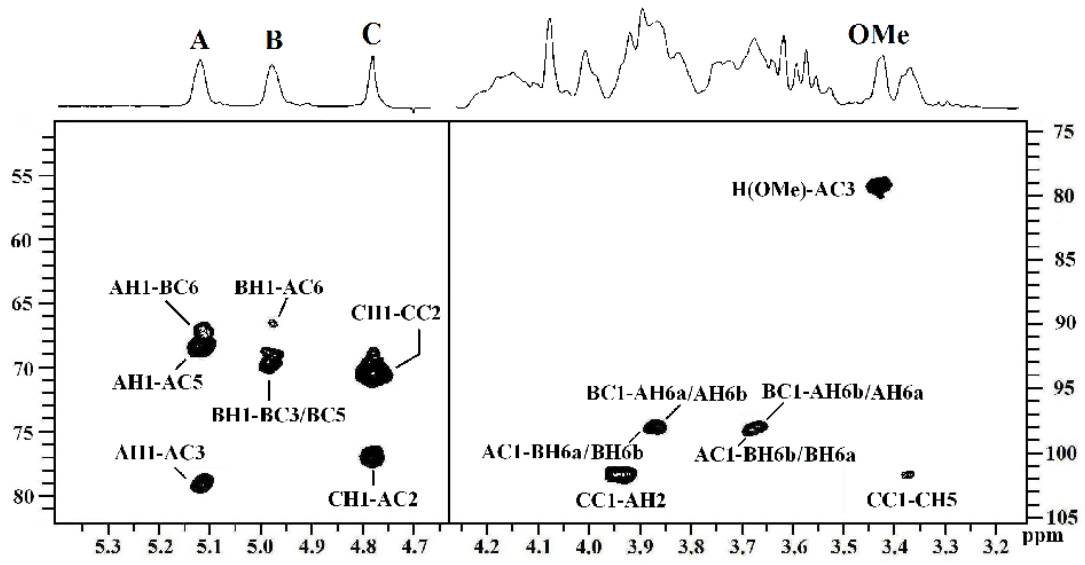


Figure 10. Part of the HMBC spectrum of the PS-II isolated from hybrid mushroom (*pfle 1p*) of *Pleorutus florida* and *Lentinula edodes*. The delay time in the HMBC experiment was 80 ms.

Table 5

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 hybrid mushroom *pfle 1p*

.

Residue	Sugar linkage	H/C	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	→2,6)-3-*O*-Me-α-D-Gal*p*-(1→	5.12 (H-1)	79.1	**A**	C-3
			68.3	**A**	C-5
			67.0	**B**	C-6
		98.3 (C-1)	3.88	**B**	H-6a
			3.68	**B**	H-6b
		3.43 H (*O*-Me)	79.1	**A**	C-3
B	→6)-α-D-Gal*p*-(1→	4.98 (H-1)	69.5	**B**	C-3
			69.2	**B**	C-5
			66.7	**A**	C-6
		97.9 (C-1)	3.86	**A**	H-6a
			3.67	**A**	H-6b
C	β-D-Man*p*-(1→	4.78 (H-1)	70.5	**C**	C-2
			77.0	**A**	C-2
		101.7 (C-1)	3.37	**C**	H-5
			3.94	**A**	H-2

Thus, the monosaccharide composition, methylation studies and NMR experiments indicated that the repeating unit of the PS-II has a backbone consisting of one (1→6)-α-D-galactopyranosyl and (1→6)-α-D-3-*O*Me-galactopyranosyl residues, out of which (1→6)-α-D-3-*O*Me-galactopyranosyl residue was branched at C-2 position with terminal β-D-mannopyranose residue and the structural motif present in the polysaccharide, PS-II was established as:

A **B**
→6)-3-*O*-Me-α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→
2
↑
1
β-D-Man*p*
C

5.B.5. Immunostimulating properties of PS-II

Mushroom polysaccharides function as immunostimulator by activating the macrophages (Wasser, 2002). Macrophages are white blood cells which play key roles in immune defense system. They usually engulf and destroy pathogens by phagocytosis. To test immunomodulatory effect of PS-II, murine macrophage was incubated with PS-II in a humidified atmosphere of 5% CO_2 at 37 °C for 48 h and the production of nitric oxide (NO) was measured. On treatment with different concentrations of this PS-II, it was observed that 49% to 54% of NO production increased up to 50 µg/mL. This was further increased by 70% at 100 µg/mL, but decreased at 200 µg/mL (**Figure 11**). Hence, the effective dose of this PS-II was observed at 100 µg/mL with optimum production of 10.2 µM NO per 5×10^5 macrophages.

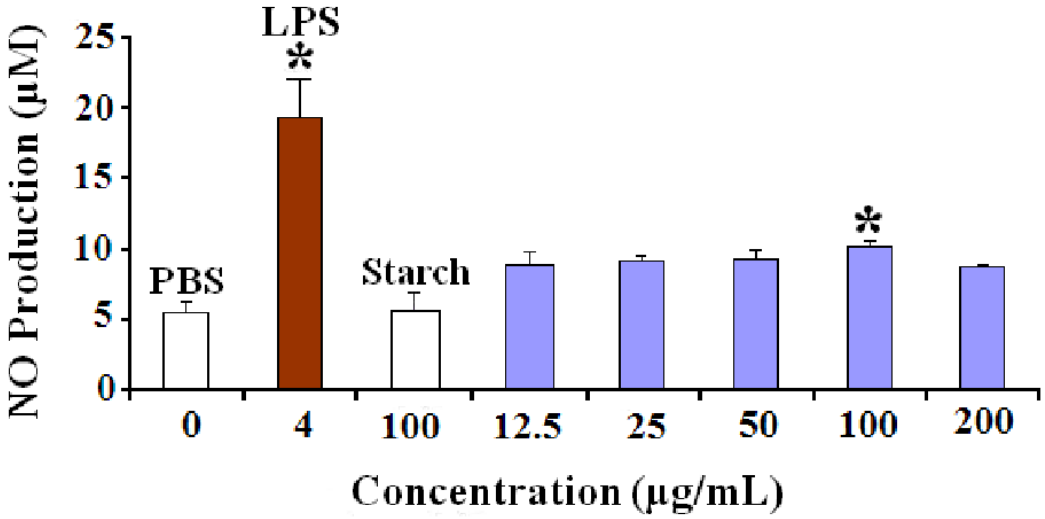


Figure 11. *In vitro* activation of macrophage stimulated with different concentrations of the PS-II isolated from hybrid mushroom (*pfle 1p*) of *Pleorutus florida* and *Lentinula edodes* in terms of NO production. (*significant compared to PBS control).

Splenocytes are the cells present in the spleen that include T cells, B cells, dendritic cells, and macrophages that stimulate the immune response in living organism. Thymocytes are hematopoietic cells in thymus which generate T cells. Proliferation of splenocyte and thymocyte is an indicator of immunostimulation (Ohno et al., 1993). The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with the PS-II by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method (Sarangi et al., 2006). The PS-II was tested to stimulate splenocytes and thymocytes and the results are shown in **Figure 12** and **13** respectively. The splenocyte and thymocyte proliferation indices as compared to PBS control if closer to 100 or below indicates low stimulatory effect on immune system. The asterisks on the columns indicated the statistically significant differences compared to phosphate buffer saline (PBS) control if $P < 0.05$. Both the splenocyte and thymocyte proliferation indices were found maximum at 50 µg/mL, above and below which it decreases. Hence, it can be

concluded that 50 µg/mL is the optimum concentration of the PS-II for splenocyte and thymocyte proliferation.

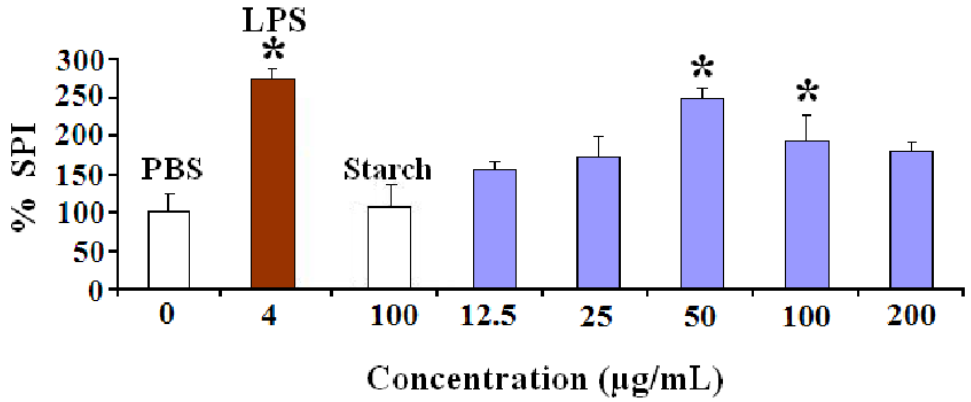


Figure 12. Effect of different concentrations of the PS-II isolated from hybrid mushroom (*pfle 1p*) of *Pleorutus florida* and *Lentinula edode* on proliferation of splenocyte. (*significant compared to PBS control).

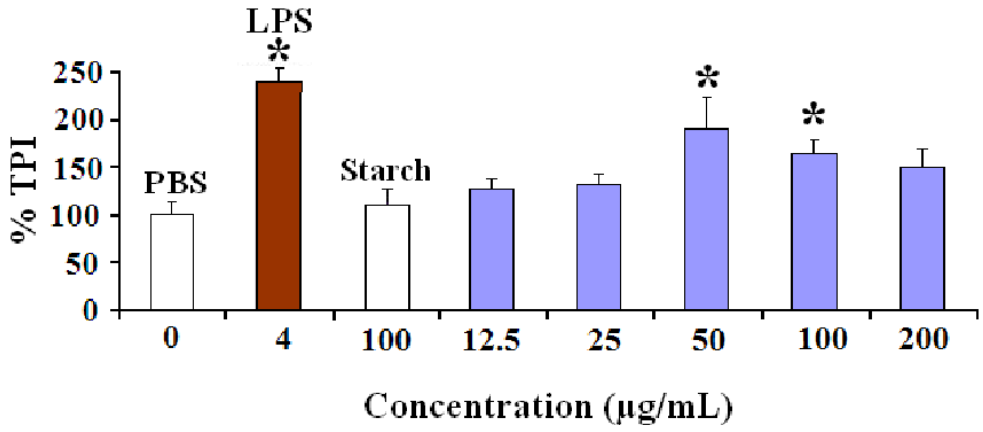
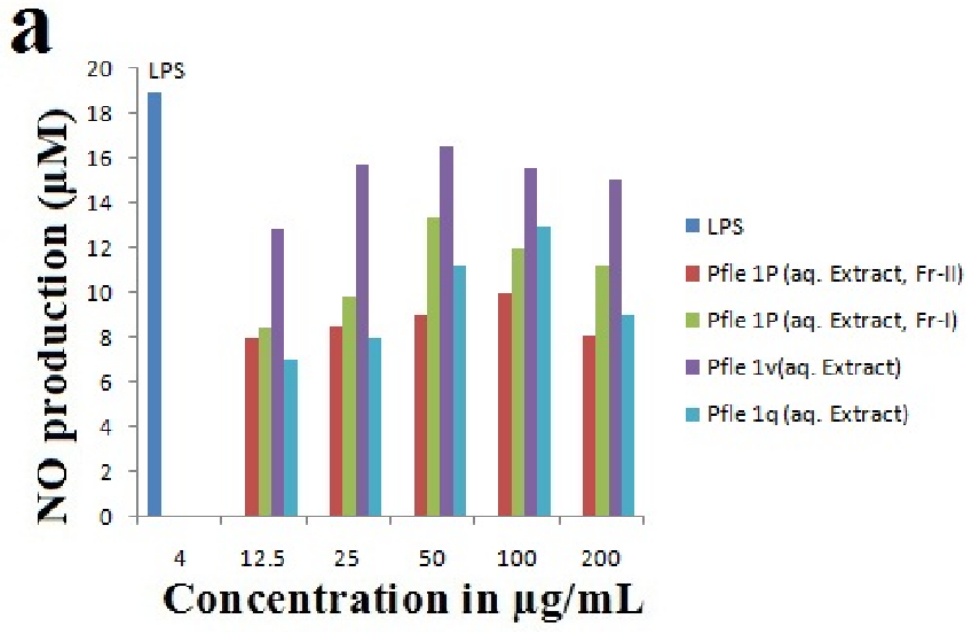


Figure 13. Effect of different concentrations of the PS-II isolated from hybrid mushroom (*pfle 1p*) of *Pleorutus florida* and *Lentinula edode* on proliferation of thymocyte. (*significant compared to PBS control).

Mushroom polysaccharides have been recognized as immunostimulators which are capable of interacting with the immune system to upregulate or downregulate the host immune response. They affect different cell type involving hematopoietic stem cells, innate (nonspecific) and adaptive (specific) immune systems and cytokine networks and signaling pathways and the activities also depend on their mechanism of action or the site of activity (Tzianabos, 2000). These studies like NO production by macrophages, splenocyte and thymocyte proliferations are preliminary steps to explore whether the PS-II has any immunomodulating property or not. However, further studies are needed in order to identify the specific cells or cytokine pathway through which the PS-II exerts its action.

The comparative study of macrophage, splenocyte, and thymocyte activations of the present PS-II with other polysaccharides (Maity, Bhunia, et al., 2013; Maity, Mandal, et al., 2013; Maji et al., 2013b) obtained from hybrids mushrooms has been compared and shown in the **figure 14a-c**.



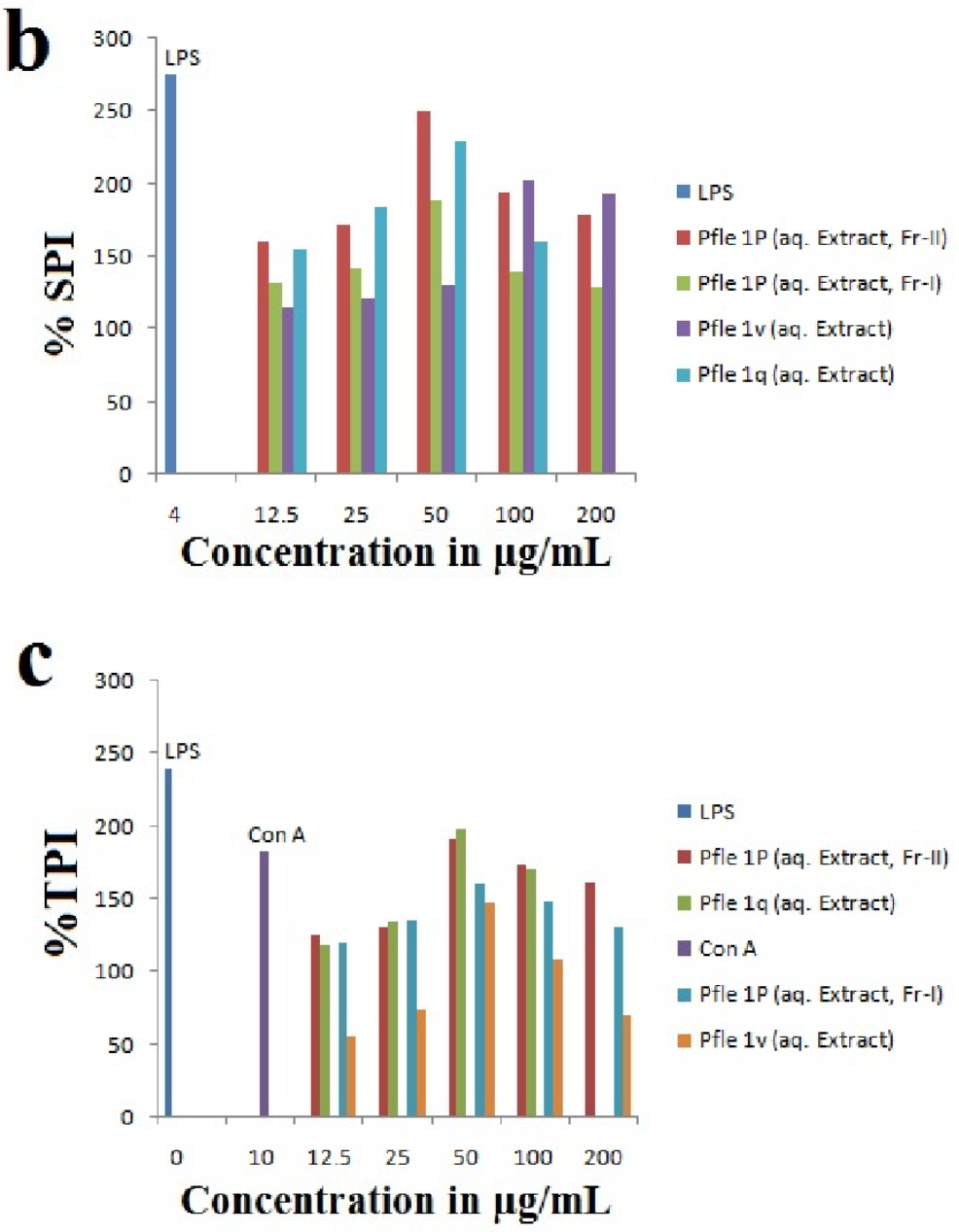


Figure 14. Comparative study of the (a) macrophage, (b) splenocyte, and (c) thymocyte activations of the present polysaccharide with the other polysaccharides of hybrids mushrooms. In (c) thymocyte activation LPS used as standard for *pfle 1p* (Fr-II) and *pfle 1q* and Con A used as standard for *pfle 1p* (Fr-I) and *pfle 1v*.

5.C. Conclusion

A water soluble partially methylated mannogalactan was isolated from hot water extract of the fruit bodies of the hybrid mushroom *pfle 1p*. The polysaccharide was purified through Sepharose 6B gel-filtration. Sugar analysis, methylation studies together with 1D/2D NMR analyses confirmed that the polysaccharide contained a backbone of (1→6) linked α-D-galactopyranosyl and (1→6) linked α-D-3-*O*Me-galactopyranosyl residues with a terminal D-mannopyranose linked to C-2 of the partially methylated galactopyranosyl residue. The polysaccharide showed significant macrophage, thymocytes and splenocytes activation and thus it can be concluded that this partially methylated mannogalactan can be used as a biological response modifier to combat microbial infection including tuberculosis, tumor cell eradication and autoimmune diseases.

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1. Structure elucidation and antioxidant properties of a soluble β-D-glucan from mushroom *Entoloma lividoalbum*. **Prasenjiy Maity**, Surajit Smanta, Ashis K. Nandi, Ipsita K. Sen, Soumitra Paloi, Krishnendu Acharya, Syed S. Islam. ***International Journal of Biological Macromolecules*, 2014,** *63*, 140-149.
2. A partially methylated mannogalactan from hybrid mushroom *pfle 1p*: purification, structural characterization and study of immunoactivation. **Prasenjit Maity**, Manabendra Pattanayak, Saikat Maity, Ashis K. Nandi, Ipsita K. Sen, Birendra Behera, Tapas K. Maiti, Pijush Mallick, Samir R. Sikdar, Syed S. Islam. ***Carbohydrate Research,* 2014**, *395*, 1-8.
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APPENDIX

1. Structure elucidation and antioxidant properties of a soluble β-D-glucan from mushroom *Entoloma lividoalbum*. **Prasenjiy Maity**, Surajit Smanta, Ashis K. Nandi, Ipsita K. Sen, Soumitra Paloi, Krishnendu Acharya, Syed S. Islam. ***International Journal of Biological Macromolecules*, 2014,** *63*, 140-149.

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Structure elucidation and antioxidant properties of a soluble β-D-glucan from mushroom *Entoloma lividoalbum*

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ABSTRACT

A water soluble branched β-D-glucan (PS-I) with an average molecular weight ~2.1×10^5 Da was isolated from alkaline extract of the fruit bodies of the edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička which consists of terminal β-D-glucopyranosyl, (1 → 3)-β-D-glucopyranosyl, (1 → 6)-β-D-glucopyranosyl, and (1 → 3,6)-β-D-glucopyranosyl moieties in a molar ratio of nearly 1:3:2:1. The structure of PS-I was elucidated using acid hydrolysis, methylation analysis, periodate oxidation study, partial hydrolysis, and 1D/2D NMR experiments. The repeating unit of the polysaccharide (PS-I) contains a backbone chain of three (1 → 6)-β-D-glucopyranosyl residues, one of which was branched at *O*-3 position with the side chain consisting of three (1 → 3)-β-D-glucopyranosyl and a terminal β-D-glucopyranosyl residues. Total antioxidant capacity of 1 mg PS-I was measured and found equivalent to 70 ± 15 μg of ascorbic acid. The PS-I was found to possess hydroxyl and superoxide radical-scavenging activities with EC_{50} values of 480 and 150 μg/mL, respectively. The reducing power of PS-I was determined 0.5 at 480 μg/mL.

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1. Introduction

Mushroom belongs to a special group of macroscopic fungi. The edible mushrooms are attractive because of their flavor, taste, delicacy, and healthy properties [1]. β-D-glucan, a well-known biological response modifier (BRM) [2] is widely distributed in nature and used as a food and medicine [3]. Mushroom polysaccharides have drawn the attention of chemists and immunobiologists due to their immunomodulatory, antitumor, antimicrobial, and antioxidant properties [4–7]. Reactive oxygen species (ROS) are formed continuously as normal by-products of oxygen metabolism during mitochondrial oxidative phosphorylation. Several biochemical reactions in our body also generate ROS. However, the uncontrolled production of oxygen derived free radicals plays crucial role related to the pathological processes of various killer diseases, such as cancer, inflammation, atherosclerosis, coronary heart disease, Alzheimer's disease, neurodegenerative disorders etc. [8]. Antioxidants are substances that delay or prevent oxidation by scavenging or preventing ROS generation through activating a battery of detoxifying proteins [9]. Synthetic compounds have strong radical scavenging activity but they have side effects also [10]. Neutralization of activity of these radicals by naturally occurring substances is now most acceptable method of modern therapy. Amongst them, mushrooms occupy an elite position in this regards [6,11]. Soluble fungal β-D-glucans such as Lentinan [12] and sonifilan (SPG) [13] have been clinically used for tumor immunotherapy. Several glucans such as (1 → 3)-β-D-glucan [2], (1 → 3)-, (1 → 6)-β-D-glucan [14], and (1 → 6)-β-D-glucan [15] are widely used as antitumor and immunostimulating agents. New branched (1 → 3)-, (1 → 6)-α, β-D-glucan [16], water soluble β-D-glucan [17], and water insoluble (1 → 3)-, (1 → 6)-β-D-glucan [18] were also reported by our group.

Entoloma lividoalbum (Kühner & Romagn.) Kubička is a basidiomycetes fungus. These mushrooms normally grow on Sikkim Himalayan region, India during July to August [5]. It is edible and non-toxic. It contains aluminum, calcium, major lanthanides, iron, and thorium [19]. These are consumed as food by the people of these areas for nutritional delicacy. Two water soluble polysaccharides (PS-I & PS-II) have been isolated from the alkaline extract of this mushroom through fractionation. The PS-I has been investigated as glucan and PS-II a heteroglycan. The present study has been focused on the structural investigation and antioxidant properties of the polysaccharide (PS-I).

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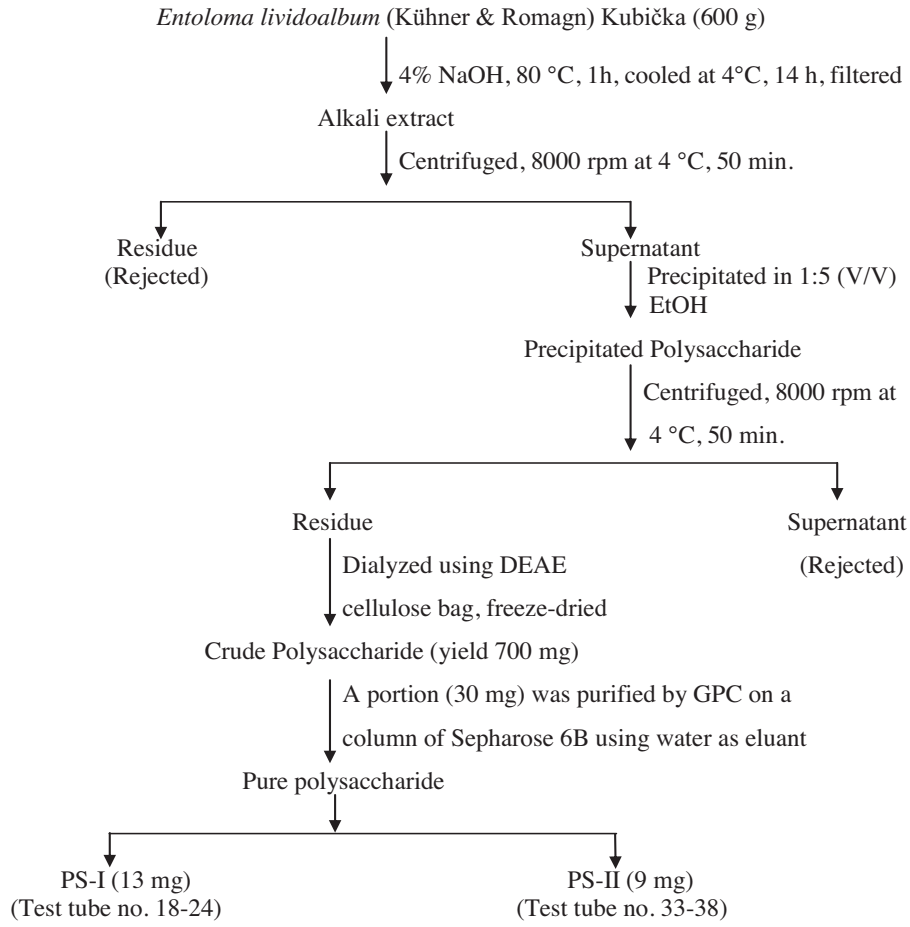
2. Materials and methods

2.1. Collection and identification

Fruit bodies of the edible mushroom *E. lividoalbum* (Kühner & Romagn.) Kubička, commonly called as 'Salle chyau' (Nepali), were colleted from Sikkim Himalaya region, India. The voucher specimen was deposited with the accession code AMFH-608 in the Mycological Herbarium of department of Botany, University of Calcutta, Kolkata, West Bengal, India. One of the authors of this article Dr. Krishnendu Acharya, Department of Botany, University of Calcutta, West Bengal, India, is a mycologist who identified the mushroom.

2.2. Isolation and purification of the polysaccharide

Fresh fruit bodies of the mushroom *E. lividoalbum* (Kühner & Romagn.) Kubička (600 g) were gently washed with distilled water several times and the mushroom bodies were crushed and boiled with 4% NaOH solution for 1 h. The whole mixture was then kept overnight at 4 °C and then filtered through linen cloth. The filtrate was centrifuged (using a Heraeus Biofuge stratus centrifuge) at 8000 rpm at 4 °C for 50 min to obtain a clear solution and then the supernatant was precipitated in EtOH. It was kept overnight at 4 °C and again centrifuged as above. The precipitated material was washed with ethanol for five times and then dissolved in water and freeze-dried. The freeze-dried material was dissolved in a minimum volume of distilled water and dialyzed through dialysis tubing of cellulose membrane (Sigma–Aldrich, retaining MW > 12,400 Da) against distilled water for 24 h to remove low molecular weight materials. The water soluble part was then freeze-dried, yielding 700 mg crude polysaccharide. The crude polysaccharide was purified by gel permeation chromatography (GPC). The polysaccharide (30 mg) was passed through Sepharose 6B column (90 cm × 2.1 cm) using distilled water as the eluant with a flow rate of 0.5 mL min^{-1}. A total of 90 test tubes were collected and monitored spectrophotometrically at 490 nm using Shimadzu UV–vis spectrophotometer, model-1601 by the phenol-sulfuric acid method [20]. Two fractions, PS-I (test tube no. 18–24) and PS-II (test tube no. 33–38), were obtained, collected and freeze-dried, yielding 13 mg and 9 mg of pure polysaccharide respectively. The present work deals with the PS-I only. The same procedure was repeated several times to get 90 mg pure PS-I. The fractionation and purification steps are shown below.



2.3. General methods

The optical rotation of the PS-I was measured on a Jasco Polarimeter model P-1020 at 32.1 °C. The average molecular weight of the PS-I was measured as reported earlier [21,22]. The PS-I (3 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) in a round-bottom flask at 100 °C for 18 h in a boiling water bath for monosaccharide analysis and the analysis was carried out as described earlier [21,22] by Gas-liquid-chromatography (GLC). The absolute configuration of the monosaccharide was determined by the method of Gerwig et al. [23]. Periodate oxidation and methylation experiments were carried out as described in our earlier reports [21,22]. A gas-liquid chromatographic analysis (GLC) was performed using 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) for monosaccharide analysis. All

GLC analyses were performed at 170 °C. 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 run at 150 °C; hold time 5 min, with a temperature gradient of 2 °C min^{-1} up to a final temperature of 200 °C. Finally NMR experiments [24] were carried out by a Bruker Avance DPX-500 instrument at 30 °C as reported in our previous papers [21,22].

2.4. Smith degradation [25–27]

The controlled smith degradation experiment was performed with this PS-I. The polysaccharide (25 mg) was oxidized with 0.1 M aqueous $NaIO_4$ (20 mL) at 25 °C in the dark during 96 h. The excess $NaIO_4$ was consumed and oxidation process was stopped by the addition of 1,2-ethanediol. After stay for an hour the total solution was dialyzed against distilled H_2O (500 mL × 3) for 6 h. The volume of the dialyzed material was reduced to 2–3 mL by the evaporation of water in a rotary evaporator. $NaBH_4$ was added and kept at room temperature for 12 h, with intermittent stirring. The mixture was neutralized with 50% AcOH and again dialyzed distilled water, and freeze-dried. The mild hydrolysis of this material was performed by the addition of 0.5 M CF_3COOH for 15 h at 25 °C to destroy the residues of oxidized sugars attached to the polysaccharide chain. The excess acid was removed and the product was freeze-dried. A part of this polymeric material (2 mg) was methylated and analyzed as usual by GLC–MS. The remainder was used for ^{13}C NMR studies.

2.5. Partial hydrolysis [27,28]

The polysaccharide (35 mg) was partially hydrolyzed with 6 mL 0.1 M CF_3COOH at 100 °C for 1 h and the excess acid was removed by repeated evaporation of water at 37 °C. The residue was dissolved in water, to which three volumes of ethanol was added. The precipitate was washed with ethanol, freeze-dried and the fraction F2 was obtained which was used for methylation analysis as well as ^{13}C NMR analysis. The supernatant was dried by evaporation, and the residue was dissolved in a minimum volume of water for reduction. After reduction with $NaBH_4$ at 25 °C for 2 h, the product was neutralized with 1 M AcOH and it was desalted by passing through a Sephadex G-25 column. The carbohydrate containing eluate (F1) was collected, freeze-dried and subjected to methylation analysis.

2.6. Antioxidant properties

2.6.1. Hydroxyl radical scavenging activity

For the assay of hydroxyl radical scavenging activity, a reaction mixture was prepared consisting of 1 mL KH_2PO_4–KOH buffer (20 mM, pH 7.4), 2-deoxy-D-ribose (2.8 mM), variable concentration (100–500 μg/mL) of PS-I, $FeCl_3$ (100 mM), EDTA (104 μM), ascorbate (100 μM) and H_2O_2 (1 mM). It was incubated at 37° C for 1 h. 2 mL thiobarbituric acid (TBA) and trichloroacetic acid (TCA) solution (0.375, w/v TBA, 15% TCA and 0.25 N HCl) was added and incubated at 100 °C for 15 min. Hydroxyl radicals are generated from Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fenton's reaction) which attack the deoxyribose and set off a series of reactions that eventually result in the formation of malondialdehyde (MDA) which develops a pink colored chromogen by adding TBA and measured at 535 nm in presence of varying concentration of PS-I [29]. EC_{50} value in μg/mL expressed the effective concentration at which the scavenging free radical activity is 50%. Butylated hydroxytoluene (BHT) was used as positive control. The degree of scavenging was calculated by the following equation:

$$\text{Hydroxyl radical scavenging rate (\%)} = \left\{\frac{A_0 - A_1}{A_0}\right\} \times 100$$

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of sample.

2.6.2. Superoxide radical scavenging activity

The superoxide radical scavenging activity was determined by the method Martinez et al. [30] followed by modification in the riboflavin-light-nitroblue tetrazolium (NBT) system. Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, various concentrations (100–400 μg/mL) of PS-I, 100 μM EDTA, 75 μM NBT and 2 μM riboflavin. Reaction was started by illuminating sample with light and the increased absorbance was measured at 560 nm after 10 min of illumination. Identical tubes with the reaction mixture were kept in the dark and served as blank. Butylated hydroxyanisole (BHA) was used as a positive control. The degree of scavenging was calculated by the following equation:

$$\text{Superoxide radical scavenging rate (\%)} = \left\{\frac{A_0 - A_1}{A_0}\right\} \times 100$$

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of sample.

EC_{50} value is the effective concentration at which the superoxide radicals were scavenged by 50%.

2.6.3. Determination of reducing power

The reducing power of PS-I was determined according to the method described by Oyaizu [31]. Various concentrations of PS-I (100–600 μg/mL) were mixed with 2.5 mL sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%). The mixture was incubated for 20 min and then 2.5 mL of trichloroacetic acid (10%) was added. 2.5 mL of solution mixture was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1%) and incubated for 15 min. The absorbance was measured at 700 nm against buffer. Ascorbic acid was used as standard. EC_{50} value is the effective concentration at which the absorbance was 0.5 for reducing power. A higher absorbance of the reaction mixture indicates a stronger reducing power of the sample.

2.6.4. Determination of total antioxidant capacity

The total antioxidant capacity was determined as described by Prieto et al. [32] with some modification. 0.3 mL of PS-I with varying concentration (0.1–1 mg) was added to 3 mL of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95 °C for 90 min. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. Ascorbic acid was used as standard and the total antioxidant capacity is expressed as equivalents of ascorbic acid.

3. Results and discussion

3.1. Isolation, purification and chemical analysis of the PS-I

A water soluble crude polysaccharide (700 mg) was isolated from the mushroom *E. lividoalbum* (Kühner & Romagn.) Kubička by hot alkaline extraction followed by cooling, filtration, centrifugation and EtOH precipitation. 30 mg water soluble crude polysaccharide was passed through Sepharose 6B column in aqueous medium and two fractions, PS-I (13 mg) and PS-II (9 mg) were obtained (Fig. 1). The PS-I showed specific rotation $[\alpha]_D^{32.1}$ −14.6 (*c* 0.054, H_2O) and this negative sign indicated that the glucosyl residues had β-anomeric configuration. The molecular weight [33] of PS-I was estimated as ~2.1×10^5 Da from a calibration curve prepared with standard dextrans. GLC analysis of

Table 1
GLC–MS analysis of methylated polysaccharide (PS-I) isolated from *Entoloma lividoalbum* (Kühner & Romagn.) Kubička.

Methylated sugars	Molar ratio	Linkage type	Major mass fragments (*m*/*z*)
2,4,6-Me_3-Glc	3	→3)-β-D-Glc*p*-(1→	43, 45, 71, 87, 101, 117, 129, 143, 161, 173, 203, 217, 233
2,3,4,6-Me_4-Glc	1	β-D-Glc*p*-(1→	43, 45, 59, 71, 87, 101, 117, 129, 161, 205
2,4-Me_2-Glc	1	→3,6)-β-D-Glc*p*-(1→	43, 58, 87, 101, 117, 129, 139, 159, 189, 201, 233
2,3,4-Me_3-Glc	2	→6)-β-D-Glc*p*-(1→	43, 45, 58, 71, 87, 99, 101, 117, 129, 161, 173, 189, 233

the alditol acetates of PS-I revealed the presence of glucose only. The absolute configuration of the monosaccharide was determined by the method of Gerwig et al. [23] and it was found that glucose was present as D-configuration in the PS-I. The mode of linkages of the sugar moieties present in the PS-I was determined by methylation analysis using the Ciucanu and Kerek [34] 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-2,3,4.6-tetra-*O*-methyl-D-glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol, and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a molar ratio of approximately 1:3:2:1 (Table 1). These results indicated the presence of terminal D-glucopyranosyl, (1 → 3)-D-glucopyranosyl, (1 → 6)-D-glucopyranosyl, and (1 → 3,6)-D-glucopyranosyl residues in the PS-I. GLC analysis of alditol acetates of the periodate-oxidized [35,36], $NaBH_4$-reduced PS-I was found to contain glucose unit only and periodate-oxidized, reduced, methylated [37] PS-I showed the presence of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol in a molar ratio of nearly 1:3. These results showed that the (1 → 6)-D-glucopyranosyl and terminal D-glucopyranosyl residues were consumed during oxidation whereas, (1 → 3,6)-D-glucopyranosyl and (1 → 3)-D-glucopyranosyl residues remain unaffected. All these results indicated that the PS-I is a branched glucan and may have three possible repeating units: a (1 → 6)-linked backbone, a (1 → 3)-linked backbone or an alternatively (1 → 3)-, (1 → 6)-linked backbone. Therefore, Smith degradation and partial hydrolysis was performed for determination of the backbone present in the PS-I. Mild hydrolysis was carried out with the periodate-oxidized, reduced PS-I to get Smith degradation product (SDPS). The GLC analysis of the alditol acetates of Smith degraded hydrolyzed product showed the presence of D-glucose and D-glycerol. The GLC–MS analysis of the alditol acetates of methylated, reduced SDPS revealed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in a molar ratio of nearly 3:1. Partial hydrolysis of the PS-I was carried out with 0.1 M TFA to know the sequence of D-Glc*p* moieties in the repeating unit. As a result of this hydrolysis, two fractions were obtained; partially hydrolyzed oligosaccharide (F1) and partially hydrolyzed polysaccharide (F2). GLC–MS analysis of the methylated product of F1 revealed the presence of (1 → 3)-D-glucopyranosyl, and terminal D-glucopyranosyl moieties and methylation analysis of F2 revealed the presence of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol only. This result clearly indicated that F2 polysaccharide (backbone chain of the PS-I) consists of three (1 → 6)-D-glucopyranosyl residues, one of which was branched at *O*-3 position with the side chain consisting of three (1 → 3)-D-glucopyranosyl and a terminal D-glucopyranosyl residue.

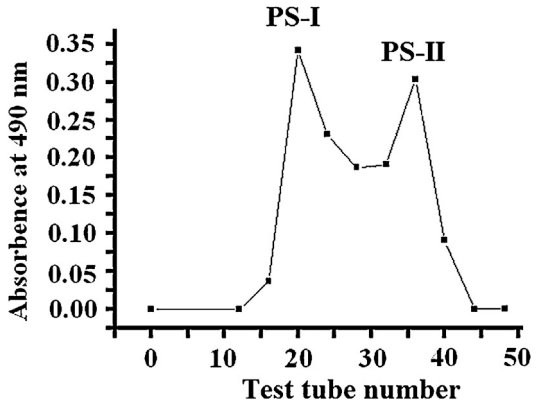


Fig. 1. Gel permeation chromatogram of crude polysaccharide isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. using Sepharose 6B column.

3.2. NMR and structural analysis of the PS-I

In the ^{1}H NMR spectrum (500 MHz; Fig. 2, Table 2) of PS-I, four anomeric signals at δ 4.77, 4.73, 4.50, and 4.49 were observed at 30 °C in a ratio of nearly 2:1:1:3. The peak at δ 4.77, 4.73, and 4.50 designated as **A**, **B**, and **D**, whereas the peak at δ 4.49 consists of **C**, **E**, and **F** residues. In ^{13}C NMR spectrum (125 MHz; Fig. 3) at the same temperature, five signals were observed in the anomeric region at δ 103.0, 102.9, 102.8, 102.7, and 102.6. On the basis of HSQC spectrum (Fig. 4, Table 2), the anomeric carbon signal at δ 102.9 was correlated to both the proton signals δ 4.77 (**A**) and δ 4.73 (**B**) respectively. Again, the anomeric proton signal at δ 4.50 was correlated to the carbon signal at δ 102.6 (**D**) whereas the peak at δ 4.49 was correlated to the anomeric carbon signals at δ 103.0 (**C**), δ 102.8 (**E**), and δ 102.7 (**F**). All the ^{1}H and ^{13}C signals (Table 2) were assigned from DQF-COSY, TOCSY, and HSQC experiments. The proton coupling constants were measured from DQF-COSY experiment.

The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values (~10.0 Hz) confirmed glucopyranosyl configuration (Glc*p*) of all the residues from **A** to **F**. Anomeric proton chemical shifts (4.77–4.49 ppm),

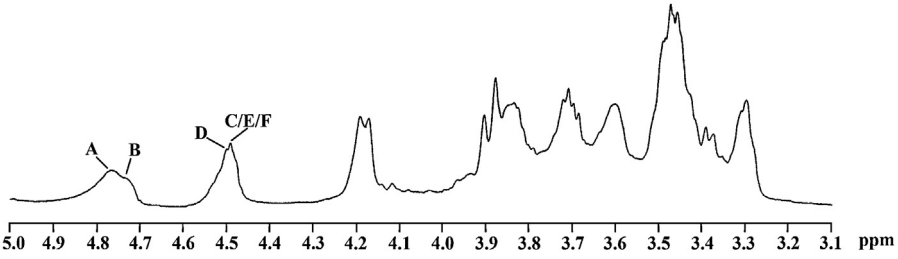


Fig. 2. ^{1}H NMR spectrum (500 MHz, D_2O, 30 °C) of PS-I, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička.

Table 2
The ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts for the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička 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
→3)-β-D-Glc*p*-(1→	4.77	3.31	3.71	3.43	3.48	3.71[c], 3.90[c]
A	102.9	72.8	84.5	69.6	75.6	60.8
→3)-β-D-Glc*p*-(1→	4.73	3.31	3.72	3.43	3.48	3.71[c], 3.90[c]
B	102.9	72.8	84.6	69.6	75.6	60.8
β-D-Glc*p*-(1→	4.49	3.30	3.47	3.37	3.43	3.68[c], 3.88[c]
C	103.0	73.1	75.6	69.6	75.6	60.8
→3,6)-β-D-Glc*p*-(1→	4.50	3.47	3.70	3.45	3.60	3.82[c], 4.17[c]
D	102.6	72.8	84.4	69.6	75.0	68.8
→6)-β-D-Glc*p*-(1→	4.49	3.30	3.45	3.43	3.60	3.83[c], 4.19[c]
E	102.8	73.1	75.6	69.6	75.0	69.0
→6)-β-D-Glc*p*-(1→	4.49	3.30	3.45	3.43	3.60	3.83[c], 4.19[c]
F	102.7	73.1	75.6	69.6	75.0	68.9

[a] The values of chemical shifts were recorded keeping HOD signal fixed at δ 4.70 ppm at 30 °C.
[b] The values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 30 °C.
[c] Interchangeable.

anomeric carbon chemical shifts (103.0–102.6 ppm), and the coupling constant values $J_{H\text{-}1,H\text{-}2}$ (~8.0 Hz), $J_{C\text{-}1,H\text{-}1}$ (~160–161 Hz) confirmed that the residues (**A**–**F**) were present in β-configuration. The downfield shift of C-3 of **A** (δ 84.5) and **B** (84.6) with respect to standard value of methyl glycoside [38,39] indicated that residue **A** and **B** were (1 → 3)-β-D-Glc*p* moiety. In residue **C**, all carbon chemical shifts values were found nearly to the standard value of methyl glycoside [38,39] of β-D-glucose. Thus, residue **C** was non-reducing end β-D-Glc*p*. The downfield shifts at C-3 (δ 84.4) and C-6 (δ 68.8) of residue **D** with respect to standard value of methyl glycoside [38,39] indicated that it was (1 → 3,6)-β-D-Glc*p* moiety. Since the residue **D** was the most rigid part of the backbone of this glucan, its C-3 (δ 84.4) appeared at slightly up field region compared to C-3 of (1 → 3)-linked residue **A** (δ 84.5) and **B** (δ 84.6). Consequently, the C-6 (δ 68.8) value of residue **D** appeared slightly up field region compared to that of the other (1 → 6)-linked residues (**E** and **F**). All the chemical shift values of **E** and **F** residues were same except the chemical shifts values of C-1 and C-6. The different downfield shifts of C-6 (δ 69.0 and 68.9) of **E** and **F** residues supported the presence of (1 → 6)-β-D-Glc*p* moiety with different chemical environments. Between **E** and **F** residues, **E** residue was glycosidically linked to the most rigid part **D**, hence, its C-6 signal (69.0 ppm) showed 0.1 ppm downfield shift with respect to C-6 signal of residue **F** (68.9 ppm) due to neighboring effect [18] of the rigid part **D**. The linking at C-6 of the residues **D**, **E**, and **F** were further confirmed by DEPT-135 spectrum (Fig. 3). The sequence of glycosyl residues (**A**–**F**) were determined from NOESY (Fig. 5, Table 3) as well as ROESY (not shown) studies followed by confirmation with HMBC experiment. In NOESY experiment, the inter-residual contacts **A**H-1/**B**H-3; **B**H-1/**D**H-3; **C**H-1/**A**H-3; **D**H-1/**E**H-6a, 6b; **E**H-1/**F**H-6a, 6b; and **F**H-1/**D**H-6a, 6b along with other intra-residual contacts were also observed (Fig. 5). The above NOESY connectivities established the following sequences: **F** (1 → 6) **D**; **D** (1 → 6) **E**; **E** (1 → 6) **F**; **B** (1 → 3) **D**; **A** (1 → 3) **B**; and **C** (1 → 3) **A**. Finally, these NOESY connectivities were confirmed from HMBC spectrum (Fig. 6). In this spectrum the inter-residual cross-peaks (Table 4) between **A**H-1/**B**C-3, **A**C-1/**B**H-3; **B**H-1/**D**C-3, **B**C-1/**D**H-3; **C**H-1/**A**C-3, **C**C-1/**A**H-3; **D**H-1/**E**C-6, **D**C-1/**E**H-6a, 6b; **E**H-1/**F**C-6, **E**C-1/**F**H-6a, 6b; **F**H-1/**D**C-6, **F**C-1/**D**H-6a, 6b along with some intra-residual peaks were also observed (Fig. 7). Thus, the NOESY and HMBC spectrum

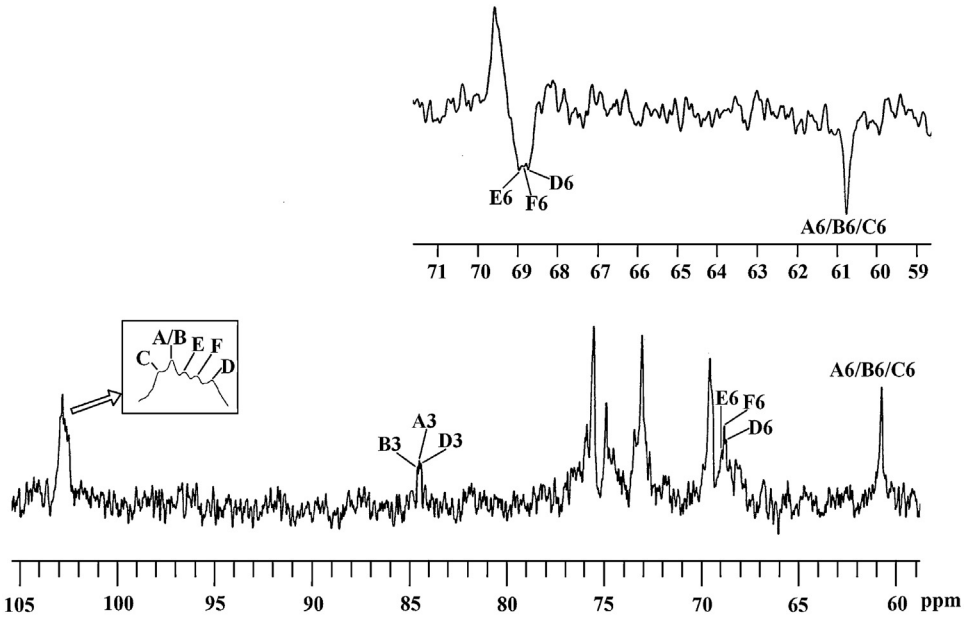


Fig. 3. ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of PS-I, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. Part of DEPT-135 spectrum (D_2O, 30 °C) of PS-I, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička (inset).

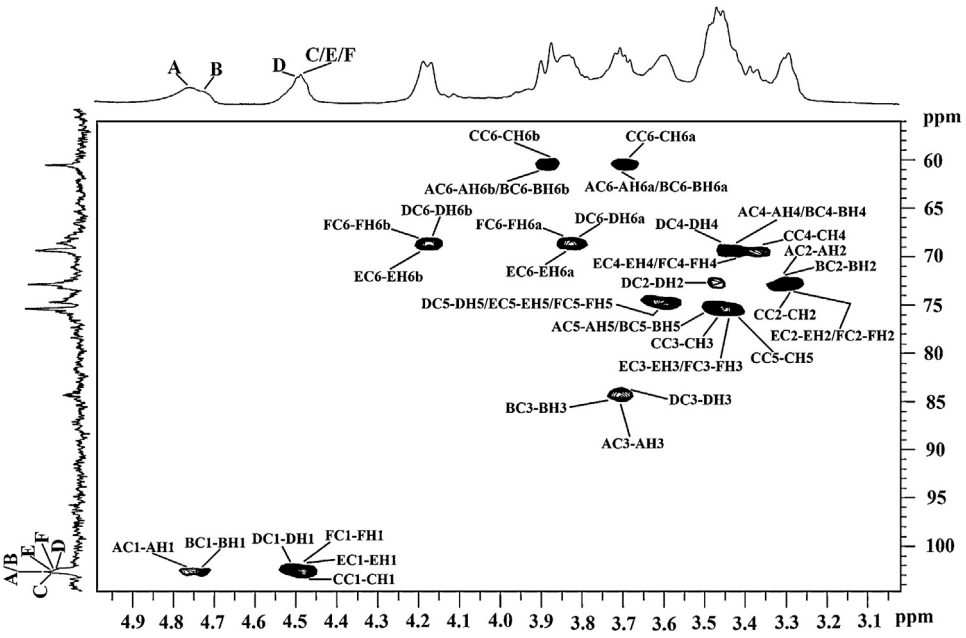


Fig. 4. HSQC spectrum of PS-I, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička.

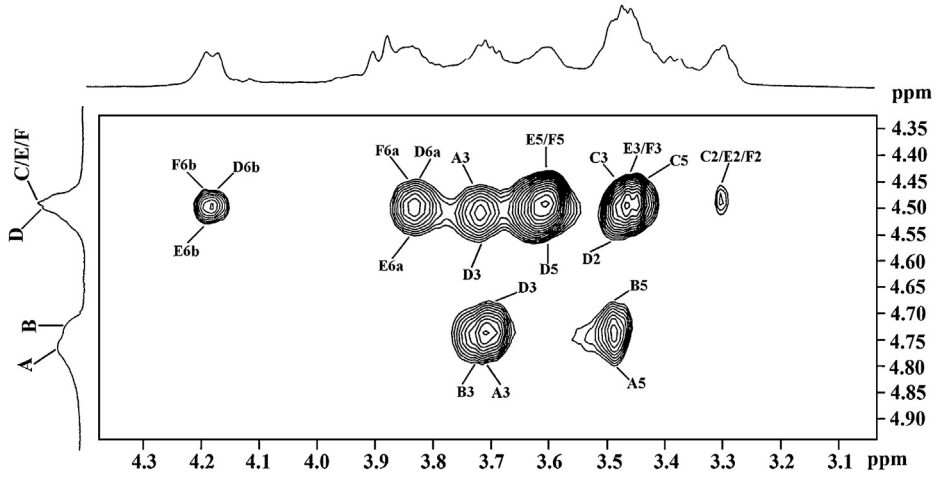


Fig. 5. Part of NOESY spectrum of PS-I, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. The ROESY mixing time was 300 ms.

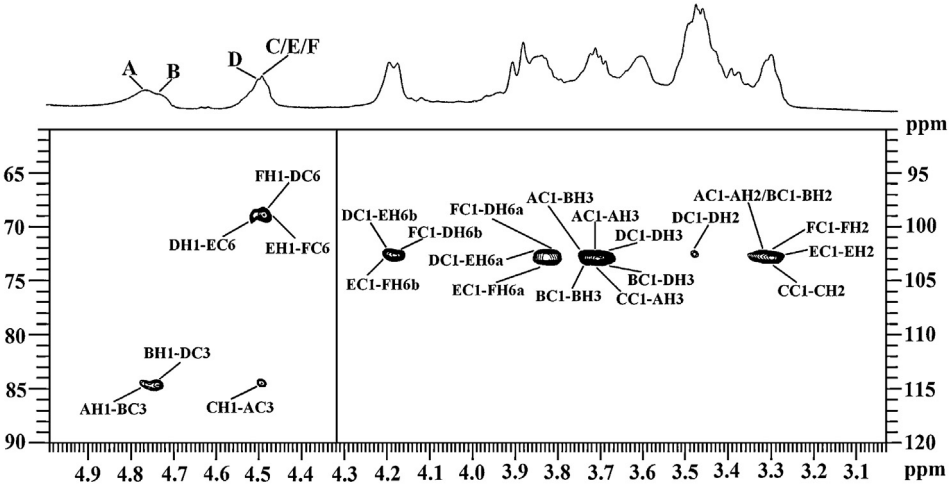


Fig. 6. Part of HMBC spectrum of PS-I, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. The delay time in the HMBC experiment was 80 ms.

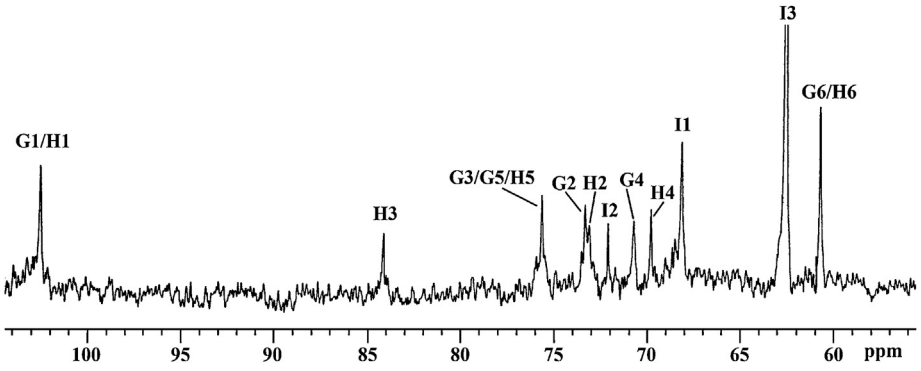


Fig. 7. ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of the Smith-degraded glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička.

analysis indicated that the PS-I is a branched glucan with (1 → 6)-β-D-glucopyranosyl backbone and branching at *O*-3 of **D** residue with (1 → 3)-β-D-Glcp (**B**) followed by (1 → 3)-linked **A** residue and non-reducing end **C** residue. Hence NOESY and HMBC connectivities confirmed the structure of repeating unit presence in the PS-I as:

F **D** **E**

→6)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→

3

↑

1)-β-D-Glc*p*-(3←[1)-β-D-Glc*p*-(3]$_2$←1)-β-D-Glc*p*

B **A** **C**

NMR experiments were again carried out with Smith degradation product (SDPS) and partially hydrolyzed polysaccharide (F2) of the PS-I for further confirming the linkages. The ^{13}C NMR (125 MHz) spectrum (Fig. 7, Table 5) at 30 °C of SDPS showed one anomeric carbon signal at 102.6 ppm for one terminal β-D-Glc*p* (**G**) and three (1 → 3)-β-D-Glc*p* (**H**) residues. The C-1, C-2, and C-3 carbon signals of the glycerol moiety (Gro) were assigned as 68.1, 72.1, and 62.6 ppm respectively. The glycerol moiety (**I**) was generated from (1 → 6)-β-D-Glc*p* residue (**E**) after periodate oxidation followed by Smith degradation which was attached to (1 → 3)-β-D-Glc*p* moiety

Table 3
NOESY data for the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička.

Glycosyl residue	Anomeric proton	ROE contact proton		
	δ	δ	Residue	Atom
→3)-β-D-Glc*p*-(1→	4.77	3.72	**B**	H-3
A		3.71	**A**	H-3
		3.48	**A**	H-5
→3)-β-D-Glc*p*-(1→	4.74	3.70	**D**	H-3
B		3.72	**B**	H-3
		3.48	**B**	H-5
β-D-Glc*p*-(1→	4.49	3.71	**A**	H-3
C		3.30	**C**	H-2
		3.47	**C**	H-3
		3.43	**C**	H-5
→3,6)-β-D-Glc*p*-(1→	4.50	3.83	**E**	H-6a
D		4.19	**E**	H-6b
		3.47	**D**	H-2
		3.70	**D**	H-3
		3.60	**D**	H-5
→6)-β-D-Glc*p*-(1→	4.49	3.83	**F**	H-6a
E		4.19	**F**	H-6b
		3.30	**E**	H-2
		3.45	**E**	H-3
		3.60	**E**	H-5
→6)-β-D-Glc*p*-(1→	4.49	3.82	**D**	H-6a
F		4.17	**D**	H-6b
		3.30	**F**	H-2
		3.45	**F**	H-3
		3.60	**F**	H-5

Table 4
The significant $^3J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička.

Residue	Sugar linkage	H-1/C-1	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	→3)-β-D-Glc*p*-(1→	4.77	84.6	**B**	C-3
		102.9	3.72	**B**	H-3
			3.31	**A**	H-2
			3.71	**A**	H-3
B	→3)-β-D-Glc*p*-(1→	4.74	84.4	**D**	C-3
		102.9	3.70	**D**	H-3
			3.31	**B**	H-2
			3.72	**B**	H-3
C	β-D-Glc*p*-(1→	4.49	84.5	**A**	C-3
		103.0	3.71	**A**	H-3
			3.30	**C**	H-2
D	→3,6)-β-D-Glc*p*-(1→	4.50	69.0	**E**	C-6
		102.6	3.83	**E**	H-6a
			4.19	**E**	H-6b
			3.47	**D**	H-2
			3.70	**D**	H-3
E	→6)-β-D-Glc*p*-(1→	4.49	68.9	**F**	C-6
		102.8	3.83	**F**	H-6a
			4.19	**F**	H-6b
			3.30	**E**	H-2
F	→6)-β-D-Glc*p*-(1→	4.49	68.8	**D**	C-6
		102.7	3.82	**D**	H-6a
			4.18	**D**	H-6b
			3.30	**F**	H-2

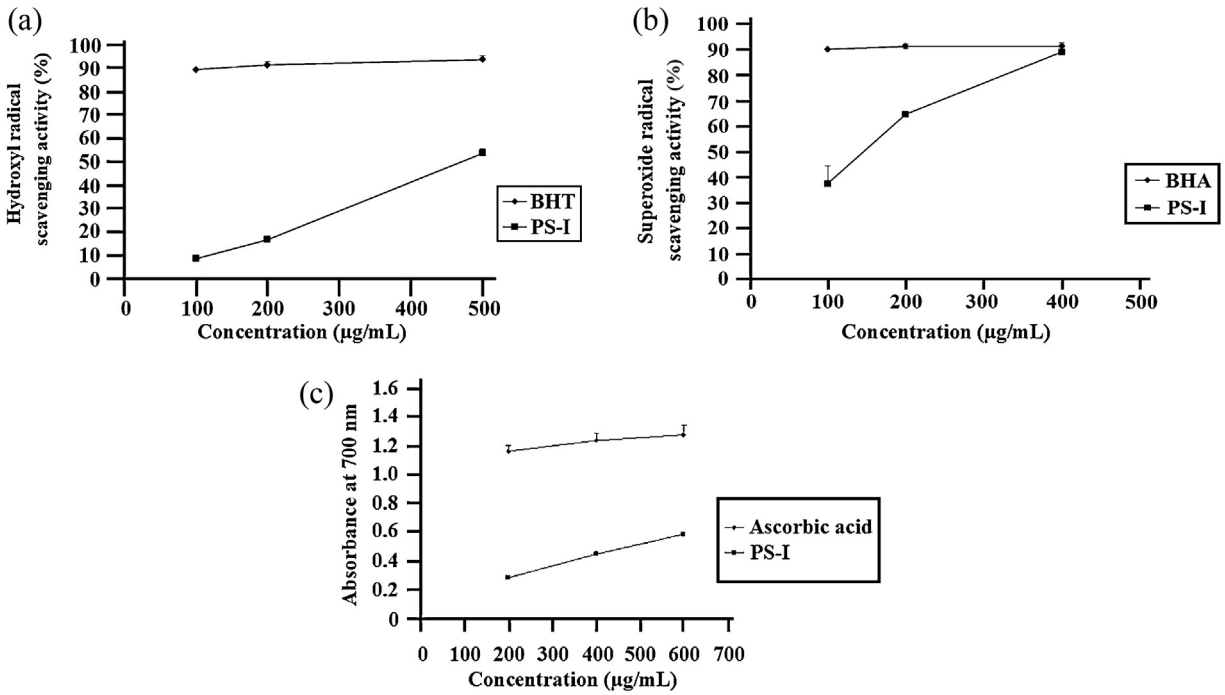


Fig. 8. (a) Hydroxyl radical scavenging activity of the PS-I, isolated from the *Entoloma lividoalbum* (Kühner & Romagn) Kubička. (b) Superoxide radical scavenging activity of the PS-I, isolated from the *Entoloma lividoalbum* (Kühner & Romagn) Kubička. (c) Determination of reducing power of the PS-I, isolated from the *Entoloma lividoalbum* (Kühner & Romagn) Kubička. Results are the mean ± SD of three separate experiments, each in triplicate.

(**H**). The terminal β-D-Glc*p* (**G**) was generated from one (1 → 3)-β-D-Glc*p* (**A**) due to complete oxidation of the terminal β-D-Glc*p* (**C**) and also one (1 → 3)-β-D-Glc*p* (**H**) was produced from the (1 → 3,6)-β-D-Glc*p* (**D**) due to oxidation followed by Smith degradation of the (1 → 6)-β-D-Glc*p* moiety (**F**) and the other two (1 → 3)-β-D-Glc*p* (**H**) were retained from (1 → 3)-β-D-Glc*p* (**A** and **B**). Hence, Smith degradation results in the formation of a glycerol containing tetrasaccharide from the parent polysaccharide and the structure of SDPS were established as:

G **H** **H** **H** **I**

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

Therefore, the above result further confirmed that the branching occurred at *O*-3 of **D** with **B** residue.

The ^{13}C NMR (125 MHz, 30 °C) spectrum of F2, indicated that it was a polymeric chain of simple (1 → 6)-β-D-Glc*p* units as characteristic signal for C-6 at δ 69.0 was observed. This result further proved that the (1 → 3)-β-D-Glc*p* moieties were located at the branched point of the (1 → 6)-linked backbone of the repeating unit. So, all these results indicated that the β-D-glucan (PS-I) isolated from an edible mushroom *E. lividoalbum* (Kühner & Romagn.) Kubička, is a branched glucan with (1 → 6)-linked backbone where branching occurred at *O*-3 of one unit followed by (1 → 3)-β-D-Glc*p* and terminal β-D-Glc*p*.

3.3. Antioxidant properties

3.3.1. Assay of hydroxyl radical scavenging activity

The hydroxyl radical is considered to be the most reactive and poisonous free radicals among all reactive oxygen species (ROS) in biological systems and may react with almost all biomolecules, including cellular carbohydrates, proteins, lipids, and DNA causing tissue damage or cell death. Thus, removal of hydroxyl radical is very important for the protection of living systems [40]. Fig. 8a shows that PS-I and BHT exhibited concentration-dependent scavenging activities against hydroxyl radicals generated in a Fenton reaction system. The hydroxyl radical scavenging activity of PS-I gradually increase with the increase of concentration (Fig. 8a). The hydroxyl radical scavenging rate of PS-I and BHT at 200 μg/mL were found to be 16.6% and 91.2% respectively, indicating that PS-I has a moderate antioxidant activity and the activity of PS-I is weak compared with that of BHT. The EC_{50} value of the PS-I was found to be 480 μg/mL. The hydroxyl radical scavenging activity might be explained by hydrogen atom donation ability of the hydroxyl

Table 5
The ^{13}C NMR[a] chemical shifts of Smith-degraded glycerol-containing disaccharide of the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička in D_2O at 30 °C.

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p*-(1→ **G**	102.6	73.3	75.7	70.7	75.7	60.8
→3)-β-D-Glc*p*-(1→ **H**	102.6	73.1	84.2	69.7	75.7	60.8
Gro-(3→ **I**	68.1	72.1	62.6			

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

group in PS-I to terminate the free radical mediated oxidative chain reactions.

3.3.2. Assay of superoxide radical scavenging activity

Superoxide anion radical is known as an initial radical and precursor of active free radicals. It plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radicals, and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA [41,42]. In the present study, the PS-I was found to be a potent scavenger of superoxide radicals generated in riboflavin-nitroblue tetrazolium (NBT) light system. Fig. 8b illustrates the superoxide radical scavenging ability of 100–400 μg/mL of PS-I and comparison with the same doses of BHA. The percentage inhibition of superoxide generation by 200 μg/mL concentration of PS-I was found to be 64.47% whereas of BHA was found as 91.01%. They both showed a concentration dependent scavenging of superoxide radicals and the percentage inhibition of PS-I is close to that of synthetic standard drug BHA at a concentration of 400 μg/mL. The EC_{50} value of the PS-I was found to be 150 μg/mL.

3.3.3. Determination of reducing power

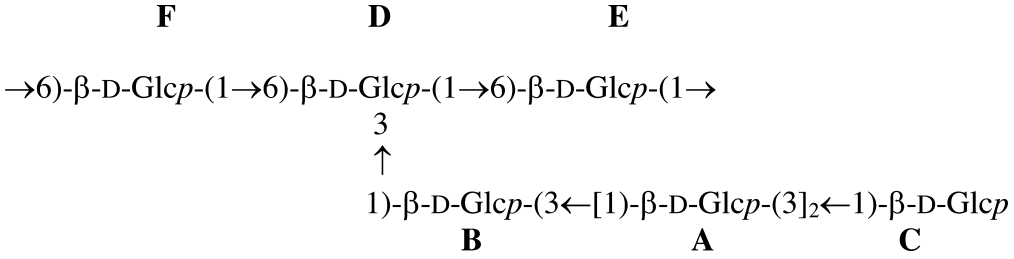
The reducing power assay measures the electron-donating ability of antioxidants using the potassium ferricyanide reduction method. The reducing power of a compound could serve as an indicator of its potential antioxidant activity. Higher absorbance of the reaction mixture indicated greater reducing power. The reducing power of PS-I was concentration-dependent. In this assay, reducing power of PS-I and ascorbic acid increased with increasing sample concentration (Fig. 8c). At 200 μg/mL, the reducing powers were 0.2765 and 1.16 for PS-I and ascorbic acid respectively. At 600 μg/mL, the reducing powers were 0.58 and 1.27 for PS-I and ascorbic acid respectively. At concentration of 480 μg/mL, PS-I showed reducing power 0.5. This result suggests that PS-I is a good electron donor and may terminate the radical chain reaction by converting free radicals to more stable product.

3.3.4. Total antioxidant capacity assay

PS-I inhibited the phosphomolybdenum in the total antioxidant capacity assay. Result showed 1 mg of PS-I is equivalent to 70 ± 15 μg of ascorbic acid. Total antioxidant activity suggests that the electron donating capacity of PS-I and thus it may act as radical chain terminator, by transforming reactive free radicals into more stable non reactive products.

4. Conclusion

A water soluble glucan (PS-I), with the average molecular weight ~2.1×10^5 Da, was isolated and purified from an edible mushroom *E. lividoalbum* (Kühner & Romagn) Kubička. The following structure was characterized by chemical analysis and 1D/2D NMR studies.



Total antioxidant capacity of 1 mg PS-I was equivalent to 70 ± 15 μg of ascorbic acid. IC_{50} value of the hydroxyl and superoxide free radical scavenging activity of PS-I was 480 and 150 μg/mL, respectively and also the reducing power was found 0.5 at concentration of 480 μg/mL. These results indicated that the present glucan should be considered as a source of a natural antioxidant in future.

Acknowledgements

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Heteroglycan of an edible mushroom *Entoloma lividoalbum*: Structural characterization and study of its protective role for human lymphocytes

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ABSTRACT

A water soluble heteroglycan (PS-II) of an average molecular weight ~5.2×10^4 Da was isolated from the alkaline extract of an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. Structural characterization of PS-II was carried out using sugar and methylation analysis, periodate oxidation study, and 1D/2D NMR experiments. Sugar analysis indicated the presence of glucose, mannose, galactose, and fucose in a molar ratio of nearly 5:1:2:1. The repeating unit of the PS-II had a backbone consisting of two (1 → 3)-β-D-glucopyranosyl, one (1 → 6)-β-D-glucopyranosyl, one (1 → 2)-α-L-fucopyranosyl, one (1 → 6)-α-D-glucopyranosyl, and two (1 → 6)-α-D-galactopyranosyl residues, out of which one (1 → 3)-β-D-glucopyranosyl residue was branched at *O*-6 position with terminal β-D-glucopyranosyl residue and one (1 → 6)-α-D-galactopyranosyl residue was branched at *O*-2 position with terminal β-D-mannopyranosyl residue. PS-II showed ameliorative activities at different concentrations (50, 100, 200, 400 μg/ml) and maintained the redox balance as well as reduced the lipid peroxidation to protect the cell destruction.

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1. Introduction

Mushroom has been appreciated by humankind as an important edible and medical resource (Wasser & Weis, 1999) since ancient time. They are rich in nutritional value with high content of carbohydrates, proteins, vitamins, fats, and minerals (Wani, Bodha, & Wani, 2010). Mushroom polysaccharides are well known for their immunomodulatory (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007; Wasser & Weis, 1999), antitumor (Das, 2010; Wasser, 2002), and antioxidant activities (Maity et al., 2014). It was reported that several glucans such as (1 → 3)-β-D-glucan (Chakraborty, Mondal, Rout, & Islam, 2006; Ohno et al., 1993), (1 → 3)-, (1 → 6)-β-D-glucan (Bhanja et al., 2013), (1 → 6)-β-D-glucan (Kiho, Shiose, Nagai, & Ukai, 1992), and branched (1 → 3)-, (1 → 6)-α, β-D-glucan (Rout, Mondal, Chakraborty, Pramanik, & Islam, 2005) and heteroglycan (Dey et al., 2013; Maity et al., 2013; Nandi et al., 2013; Patra et al., 2012) are reported as antitumor and immunostimulating agents. *Entoloma lividoalbum* (Kühner & Romagn.) Kubička commonly called as 'Salle chyau' (Nepali), genus Entoloma, family Entolomataceae is a basidiomycetes fungus. This edible and non-toxic mushroom normally grows on Sikkim Himalayan region, India during July to August (Das, 2010). Two water soluble polysaccharides (PS-I & PS-II) have been isolated from the alkaline extract of this mushroom through Sepharose gel fractionation. The PS-I has been investigated and reported as a glucan (Maity et al., 2014) which showed antioxidant properties. The PS-II was characterized as heteroglycan containing α-L-fucose as one of the constituents which is used for human breast cancer and infertility treatment (Jay, Gene, & Catherine, 2011). In the present investigation, attempts were made to study the toxicological analysis of PS-II. Lymphocytes are the primary immune cells in the body. Destruction or

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strengthening of lymphocyte cells are directly linked to immunity. From this point of view the use of various natural and synthetic drugs has drawn the remarkable attention now a day. PS-I have been reported to possess antioxidant properties (Maity et al., 2014) and from that observation, the toxicological parameters including redox balances maintained by PS-II was investigated in the present study. Further, the protective effects of PS-II was also evaluated where nicotine was used as a potent cytotoxic agent which affects a variety of cellular processes from induction of gene expression to modulation of enzymatic activities (Mahapatra, Chakraborty, Majumdar, Bag, & Roy, 2009). Hence, the protective effects of PS-II on nicotine-induced human lymphocytes were studied.

The detailed structural investigation and biological activities of PS-II were carried out in the present investigation and reported herein.

2. Materials and methods

2.1. Collection and identification

Fruit bodies of the edible mushroom *E. lividoalbum* (Kühner & Romagn.) Kubička were collected from Sikkim Himalaya region, India. The voucher specimen was deposited with the accession code AMFH-608 in the Mycological Herbarium of Department of Botany, University of Calcutta, Kolkata, and West Bengal, India. One of the authors of this article Dr. Krishnendu Acharya, Department of Botany, University of Calcutta is a mycologist who identified the mushroom.

2.2. Isolation and purification of the polysaccharide

Fresh fruit bodies of the mushroom *E. lividoalbum* (Kühner & Romagn.) Kubička (600 g) were gently washed with distilled water several times, crushed and boiled with 4% NaOH solution for 1 h. The whole mixture was then kept overnight at 4 °C and then filtered through linen cloth, centrifuged and then precipitated in EtOH (1:5) to get crude polysaccharide. The detailed extraction procedure was described in previous communication (Maity et al., 2014). The crude polysaccharide was purified through Sepharose 6B gel permeation chromatography (GPC) to produce two fractions, PS-I (test tube no. 18–24, yield 13 mg) and PS-II (test tube no. 33–38, yield 9 mg). The structural characterization and study of antioxidant properties of PS-I were reported earlier (Maity et al., 2014). The isolation procedure was repeated several times to obtain 95 mg PS-II which was further purified by same procedure to get a homogeneous fraction, yield 80 mg. The structural and biological studies of only PS-II was carried out and presented herein.

2.3. Monosaccharide analysis

PS-II (3.0 mg) was hydrolyzed with 2 M CF_3COOH (2 ml) in a round-bottom flask at 100 °C for 18 h in a boiling water bath. The excess acid was completely removed by co-distillation with water. Then, the hydrolyzed product was reduced with $NaBH_4$ (9 mg), followed by acidification with dilute CH_3COOH, and then co-distilled with pure CH_3OH to remove excess boric acid. The reduced sugars were acetylated with 1:1 pyridine–acetic anhydride in a boiling water bath for 1 h to give the alditol acetates (Lindahl, 1970), which were analyzed by GLC Hewlett-Packard model 5730 A with 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 analyses were performed at 170 °C.

2.4. Methylation analysis

PS-II (4.0 mg) was methylated using the procedure described by Ciucanu and Kerek (1984). The methylated products were isolated by making partition between $CHCl_3$ and H_2O (5:2, v/v) for four times. The organic layer containing products was collected and dried. The methylated product was then hydrolyzed with 90% formic acid (1 ml) at 100 °C for 1 h and excess formic acid was evaporated by co-distillation with distilled water. The hydrolyzed product was then reduced with sodium borohydride and acetylated with pyridine-acetic anhydride (1:1). The alditol acetates of methylated sugars were analyzed by GLC–MS. The gas–liquid chromatography–mass spectrometric (GLC–MS) analysis was performed on a 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.

2.5. Periodate oxidation and Smith degradation study

PS-II (40 mg) was oxidized with 0.1 M sodium metaperiodate solution (20 ml) and the mixture was kept for 72 h in the dark at 25 °C. The excess periodate was destroyed by adding ethylene glycol and the solution was dialyzed against distilled water for 2 h. The volume of the dialyzed material was concentrated to 2–3 ml. This material was reduced with $NaBH_4$, 12 h, neutralized with 50% AcOH, and dialyzed with distilled water and finally freeze dried (19.0 mg). The periodate-reduced material was divided into three portions. One portion (2 mg) was hydrolyzed with 2 M CF_3COOH (1 ml) at 100 °C for 18 h and used for alditol acetate preparation and analyzed by GLC. The second portion (2.0 mg) was methylated by the method of Ciucanu and Kerek (1984), followed by preparation of alditol acetates which were analyzed by GLC–MS. Smith degradation experiment was performed with the third portion (15.0 mg). The mild hydrolysis of the periodate oxidized-reduced material was performed by the addition of 0.5 M CF_3COOH for 15 h at 25 °C to destroy the residual part of the oxidized sugars attached to the polysaccharide chain. The excess acid was removed by repeated freeze drying. The material was further purified by passing through a Sephadex G-25 column, freeze-dried and kept over P_2O_5 in vacuum for several days for ^{13}C NMR analysis.

2.6. Absolute configuration of monosaccharide

The absolute configuration of monosaccharide was determined by the method of Gerwig, Kamerling, and Vliegenthart (1978). PS-II (1.0 mg) was hydrolyzed with CF_3COOH, and then the acid was removed by co-distillation with water. A volume of 250 μl of 0.625 M HCl solution treated with R-(+)-2-butanol was added to the hydrolyzed product and heated at 80 °C for 16 h. Then the reactants were evaporated and TMS-derivatives were prepared with *N*,*O*-bis (trimethylsilyl) trifluroacetamide (BSTFA). The products were analyzed by GLC using a capillary column SPB-1 (30 m × 0.26 mm) with a temperature program (3 °C/min) from 150 to 210 °C. The resulting 2,3,4,6-tetra-*O*-TMS-(+)-2-butylglycosides were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides.

2.7. Optical rotation

Optical rotation was measured on a Jasco Polarimeter model P-1020 at 31.8 °C.

2.8. Determination of molecular weight

The molecular weight of the polysaccharide was determined by gel-permeation chromatography (Hara, kiho, Tanaka, & Ukai, 1982). Standard dextrans T-200, T-70, and T-40 were passed through a Sepharose 6B column, and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of PS-II was then plotted on the same graph and the average molecular weight of PS-II was determined.

2.9. NMR studies

PS-II was dried over P_2O_5 in vacuum for several days and then exchanged with deuterium (Dueñas-Chaso et al., 1997) followed by lyophilizing with D_2O (99.96% atom ^2H, Aldrich) for four times. The ^1H and ^{13}C NMR experiments were carried out at 500 MHz and 125 MHz, respectively with a Bruker Avance DPX-500 spectrometer. The ^1H, ^{13}C, TOCSY, DQF-COSY, ROESY, NOESY, and HSQC NMR spectra were recorded in D_2O at 30 °C. The ^1H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.70) using the WEFT pulse sequence (Hård, Zadelhoff, Moonen, Kamerling, & Vliegenthart, 1992) using acetone as internal standard fixing methyl proton signal at δ 2.19. Acetone was used as an internal standard (δ 31.05 ppm) for ^{13}C spectrum. The 2D-DQF-COSY experiment was performed using standard BRUKER software. The TOCSY experiment was recorded at mixing time of 150 ms, and complete assignment required several TOCSY experiments having mixing times ranging from 60 to 300 ms. The NOESY and ROESY mixing delay was 300 ms.

2.10. Biological activities

2.10.1. Isolation of lymphocytes from peripheral blood mononuclear cells

Fresh blood samples were collected from all groups of individuals satisfying the Helsinki protocol. The lymphocytes were isolated from heparinized blood samples according to the method applied earlier by Chattopadhyay et al. (2013). Blood was diluted with phosphate-buffered saline (pH 7.0) in equal ratio and then layered very carefully on the density gradient (histopaque 1077) in a ratio of 1:2, centrifuged at 1400 rpm for 20 min and the white milky layer of mononuclear cells were carefully removed and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin, 4 mM L-glutamine under 5% CO_2 and 95% humidified atmosphere at 37 °C for 2 h. After 2 h, non adherent layer of the cultured cells were washed twice with the PBS and centrifuged at 2000 rpm for 10 min to get the required pellet of lymphocytes.

2.10.2. Cell viability

Normal human lymphocytes were seeded into 96 wells of tissue culture plates having 180 μl of complete media and were incubated for 48 h. PS-II was added to the cells at different concentrations (50, 100, 200, and 400 μg/ml) and incubated for 24 h at 37 °C in a humidified incubator (NBS) maintained with 5% CO_2. The cell viability was estimated by 3-(4,5-dimethylthiazol)-2-diphenyltetrazolium bromide (MTT) method as applied earlier (Chattopadhyay et al., 2012).

2.10.3. Cell lysate preparation

After treatment schedule, the cell suspension was collected in a centrifuge tube and centrifuged at 1500 rpm for 5 min. The supernatants were collected and stored at −20 °C. The cell pellets were re-suspended in ice cold phosphate buffered saline (PBS) at concentrations ranging from 2×10^5 cells/ml and subjected to four cycles of freeze-thaw cycles (alternating liquid nitrogen and 37 °C water bath treatment) followed by sonication for 20 s (Ultrasonic Processor, Tekmar, Cincinnati, OH, USA) on ice. Lysates were centrifuged at 12,000 rpm for 20 min at 4 °C to remove cellular debris as adopted earlier by Chattopadhyay et al. (2013). Protein content of lysate preparations was measured according to Lowry, Rosenbrough, Farr, and Randall (1951) using bovine serum albumin as standard.

2.10.4. Determination of reduced glutathione (GSH)

Reduced glutathione estimation in the cell lysate was performed by the method as applied in earlier publication (Mahapatra et al., 2009). The required amount of the cell lysate was mixed with 25% of trichloroacetic acid and centrifuged at $2000 \times g$ for 15 min to settle the precipitated proteins. The supernatant was aspirated and diluted to 1 ml with 0.2 M sodium phosphate buffer (pH 8.0). Later, 2 ml of 0.6 mM DTNB was added. After 10 min the optical density of the yellow-colored complex formed by the reaction of GSH and DTNB (Ellman's reagent) was measured at 405 nm. A standard curve was obtained with standard reduced glutathione. The levels of GSH were expressed as μg of GSH/mg protein.

2.10.5. Determination of oxidized glutathione level (GSSG)

The oxidized glutathione level was measured after derivatization of GSH with 2-vinylpyidine according to the method as applied earlier (Mahapatra et al., 2009). In brief, with 0.5 ml cell lysate, 2 μl 2-vinylpyidine was added and incubates for 1 h at 37 °C. Then the mixture was deprotenized with 4% sulfosalicylic acid and centrifuged at $1000 \times g$ for 10 min to settle the precipitated proteins. The supernatant was aspirated and GSSG level was estimated with the reaction of DTNB at 412 nm in spectrophotometer and calculated with standard GSSG curve.

2.10.6. Determination of lipid peroxidation (MDA)

Lipid peroxidation was estimated by the method of Ohkawa, Ohishi, and Yagi (1979) in cell lysate. Briefly, the reaction mixture contained Tris-HCl buffer (50 mM, pH 7.4), *tert*-butyl hydroperoxide (BHP) (500 μM in ethanol) and 1 mM $FeSO_4$. After incubating the samples at 37 °C for 90 min, the reaction was stopped by adding 0.2 ml of 8% sodium dodecyl sulfate (SDS) followed by 1.5 ml of 20% acetic acid (pH 3.5). The amount of malondialdehyde (MDA) formed during incubation was estimated by adding 1.5 ml of 0.8% TBA and further heating the mixture at 95 °C for 45 min. After cooling, samples were centrifuged and the TBA reactive substances (TBARS) were measured in supernatants at 532 nm by using $1.53 \times 10^5\ M^{-1}\ cm^{-1}$ as extinction coefficient. The levels of lipid peroxidation were expressed in terms of nmol/mg protein.

2.10.7. NO release assay

The NO concentration was measured by a microplate assay method with Griess reagent (1% sulfanilamide, 0.3% naphthylethylenediaminedihydrochloride, 7.5% H_3PO_4). Briefly, culture supernatants (100 μl) were mixed with 100 μl of the Griess reagent. The nitrite concentration in the culture supernatant was measured at an absorbance of 550 nm, 10 min after mixing (Hino et al., 2005).

2.10.8. Protective role

Normal human lymphocytes (4×10^6) were seeded into 96 wells plate and treated with 10 mM nicotine for 6 h at 37 °C, since this dose was found lethal as reported earlier (Mahapatra et al., 2009). After incubation, cells were washed with 1X PBS (50 mM) for 3 times and incubated with PS-II for 24 h at 37 °C. The cell viability was estimated by 3-(4,5-dimethylthiazol)-2-diphenyltetrazolium bromide according to the method as applied earlier (Chattopadhyay et al., 2012). The apoptotic cells were visualized by propidium iodide staining under phase contrast fluoresce microscope (50× magnifications).

2.10.9. Statistical analysis

The data were expressed as the mean ± the standard error of the mean ($n = 6$). Comparisons between the means of control and treated groups were made by one-way analysis of variance (using a statistical package; Origin 6.1, Origin Lab, Northampton, MA, USA) with multiple-comparison tests, with $p < 0.05$ as the limit of significance. The correlation analysis was performed using Statistica software version 8.0.

3. Results and discussion

3.1. Isolation, purification and chemical analysis of the PS-II

A water soluble polysaccharide was isolated from the fruiting bodies of *E. lividoalbum* (Kühner & Romagn.) Kubička (Maity et al., 2014). On fractionation through GPC it gave two fractions, PS-I and PS-II (Maity et al., 2014). The PS-II showed a specific rotation $[\alpha]_D^{31.8}$ +16.5 (*c* 0.09, H_2O). The molecular weight (Hara et al., 1982) of PS-II was estimated as $\sim 5.2 \times 10^4$ Da from a calibration curve prepared with standard dextrans. GLC analysis of the alditol acetates of the hydrolyzed product of PS-II revealed the presence of glucose, mannose, galactose, and fucose in a molar ratio of nearly 5:1:2:1. The absolute configuration of the monosaccharide was determined by the method of Gerwig et al. (1978) and found that glucose, galactose, and mannose had the D configuration but fucose was present in the L configuration. The mode of linkages of the sugar moieties present in the PS-II was determined by methylation analysis using the Ciucanu and Kerek (1984) method, followed by hydrolysis and preparation of alditol acetates. The GLC–MS analysis of the alditol acetates of methylated products have been presented in Table 1. These linkages (Table 1) were further confirmed by periodate oxidation experiment. GLC analysis of alditol acetates of the periodate-oxidized (Goldstein, Hay, Lewis, & Smith, 1965; Hay, Lewis, & Smith, 1965), $NaBH_4$-reduced, and hydrolyzed products showed the presence of only glucose, indicating that the D-galactose, D-mannose, and L-fucose moieties were consumed during oxidation. GLC–MS analysis of periodate-oxidized, reduced, methylated (Abdel-Akher & Smith, 1950) PS-II showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a molar ratio of nearly 1:1. These results clearly indicated that the (1 → 3)-linked and (1 → 3,6)-linked glucopyranosyl residues remain unaffected whereas all other residues were consumed during oxidation, which further confirmed the mode of linkages present in the PS-II.

3.2. NMR and structural analysis of the PS-II

The ^{1}H NMR spectrum (500 MHz; Fig. 1a, Table 2a) of PS-II at 30 °C showed the presence of nine signals in the anomeric region at δ 5.11, 5.04, 5.03, 4.99, 4.77, 4.73, 4.49, 4.48, and 4.47. The sugar residues were designated as **A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**, and **I** according to their decreasing anomeric proton chemical shifts. In ^{13}C NMR spectrum (125 MHz; Fig. 1b) at the same temperature, seven signals were observed in the anomeric region at δ 103.0, 102.9, 102.7, 102.5, 101.7, 101.6, and 98.3. From the HSQC spectrum (Fig. 1c, Table 2a), the anomeric carbon signals at δ 103.0, 102.9, 102.7, 102.5, 101.7 and 101.6 were correlated to the anomeric proton signals δ 4.47 (**I**), δ 4.73 (**F**), δ 4.48 (**H**), δ 4.49 (**G**), δ 4.77 (**E**), and δ 5.04 (**B**) respectively. Whereas, the anomeric carbon signal at δ 98.3 was correlated to the anomeric proton signals at δ 5.11 (**A**), δ 5.03 (**C**), and δ 4.99 (**D**). All the ^{1}H and ^{13}C signals (Table 2a) were assigned from DQF-COSY (Fig. S1a and b, Supplementary data), TOCSY (Fig. S2a and b, Supplementary data), and HSQC (Fig. 1c

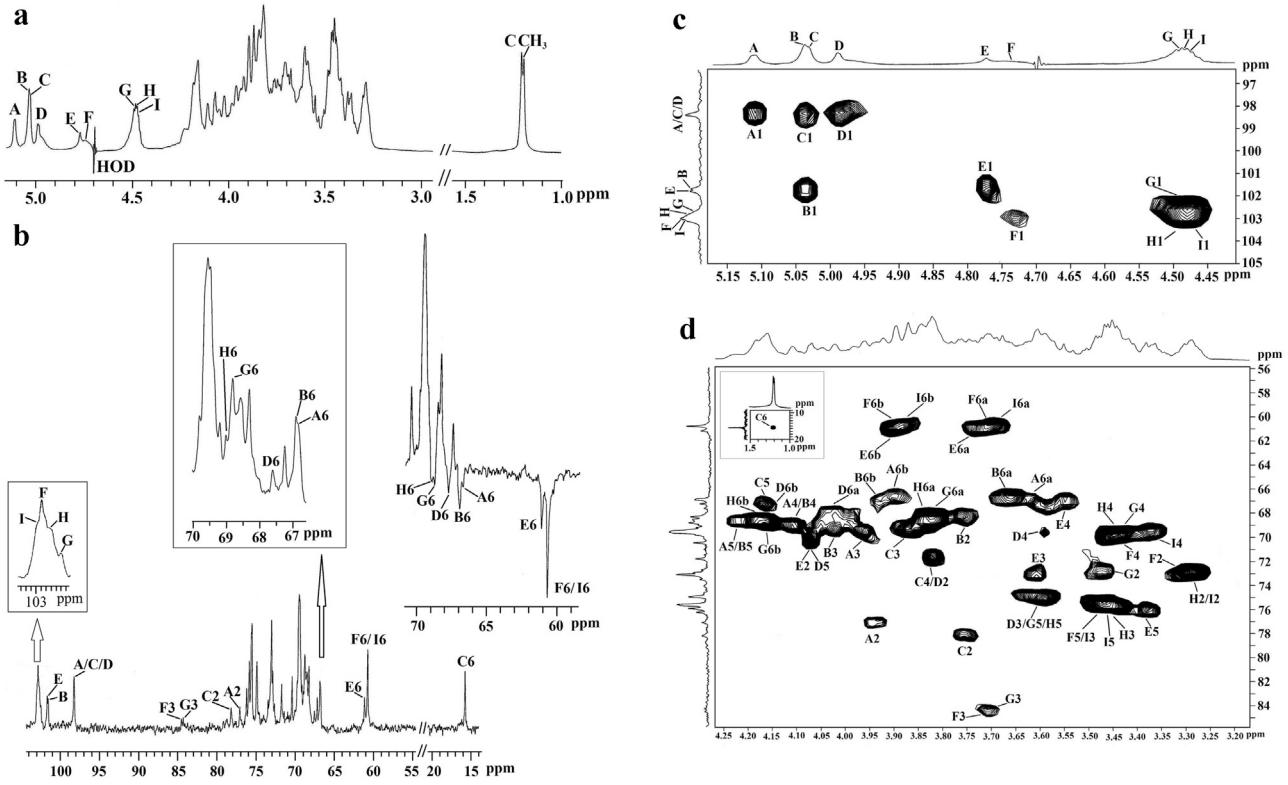


Fig. 1. The HSQC spectrum (D_2O, 30 °C) of (c) anomeric part and (d) other than anomeric part of the PS-II isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. (a) ^{1}H NMR spectrum (500 MHz, D_2O, 30 °C) of PS-II, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. (b) ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of PS-II, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. Part of DEPT-135 spectrum (D_2O, 30 °C) of PS-II, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička (inset).

Table 1
GLC–MS analysis of methylated polysaccharide (PS-II) isolated from *Entoloma lividoalbum* (Kühner & Romagn.) Kubička.

Methylated sugars	Molar ratio	Linkage type	Major mass fragments (*m*/*z*)
3,4-Me_2-Gal	1	→2,6)-Gal*p*-(1→	43,71,87,99,129,159,173,189,233
2,3,4-Me_3-Gal	1	→6)-Gal*p*-(1→	43,71,87,99,101,117,129,161,173,189,233
2,3,4,6-Me_4-Glc	1	Glc*p*-(1→	43,45,59,71,87,101,117,129,161,205
3,4-Me_2-Fuc	1	→2)-Fuc*p*-(1→	43,5971,89,99,115,129,131,173,189
2,3,4,6-Me_4-Man	1	Man*p*-(1→	43,45,59,71,87,101,117,129,161,205
2,4,6-Me_3-Glc	1	→3)-Glc*p*-(1→	43,45,71,87,101,117,129,143,161,173,203,217,233
2,4-Me_2-Glc	1	→3,6)-Glc*p*-(1→	43,58,87,101,117,129,139,159,189,201,233
2,3,4-Me_3-Glc	2	→6)-Glc*p*-(1→	43,45,58,71,87,99,101,117,129,161,173,189,233

and d) 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.

Residues **A** and **B** has coupling constant values of $J_{H\text{-}2,\,H\text{-}3}$ ~9 Hz and $J_{H\text{-}3,\,H\text{-}4}$ ~3.5 Hz and thus, they were confirmed as D-galactopyranosyl residues. The α-configuration of both **A** and **B** residues were assigned from the coupling constant values ($J_{H\text{-}1,H\text{-}2}$ ~3.1 Hz and $J_{C\text{-}1,H\text{-}1}$ ~171 Hz). The downfield shifts of C-2 (δ 77.1) and C-6 (δ 66.8) with respect to standard values of methyl glycosides (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that residue **A** was a (1 → 2,6)-linked α-D-galactopyranosyl. The downfield shifts of C-6 (δ 66.9) with respect to standard values of methyl glycosides (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that residue **B** was a (1 → 6)-linked α-D-galactopyranosyl. The linkage at C-6 of the both residues **A** and **B** were further confirmed from DEPT-135 spectrum (Fig. 1b). Hence, these observation confirmed that the residue **A** was (1 → 2,6)-α-D-galactopyranosyl and the residue **B** was (1 → 6)-α-D-galactopyranosyl moieties.

Residue **C** was assigned as an L-fucopyranosyl unit. This was strongly supported by the appearance of a proton signal at δ 1.21, carbon signal at δ 15.7 for a CH_3 group, and the relatively small $J_{H\text{-}3,H\text{-}4}$ (<3 Hz). The appearance of the anomeric proton and carbon signals for residue **C** at δ 5.03 and 98.3 respectively, as well as the coupling constant value $J_{H\text{-}1,H\text{-}2}$ ~3.75 Hz clearly indicated that **C** was α-anomer. The anomeric configuration was further confirmed by ^1H—^{13}C coupling constant $J_{C\text{-}1,H\text{-}1}$ ~171 Hz. The downfield shift of C-2 (δ 78.2) with respect to standard values of methyl glycosides indicated that the residue **C** was linked at C-2 position with residue **H** which further confirmed by the ROESY (Fig. 2, Table 2b) experiment. Thus, it may be concluded that the residue **C** was a (1 → 2)-α-L-fucopyranosyl moiety.

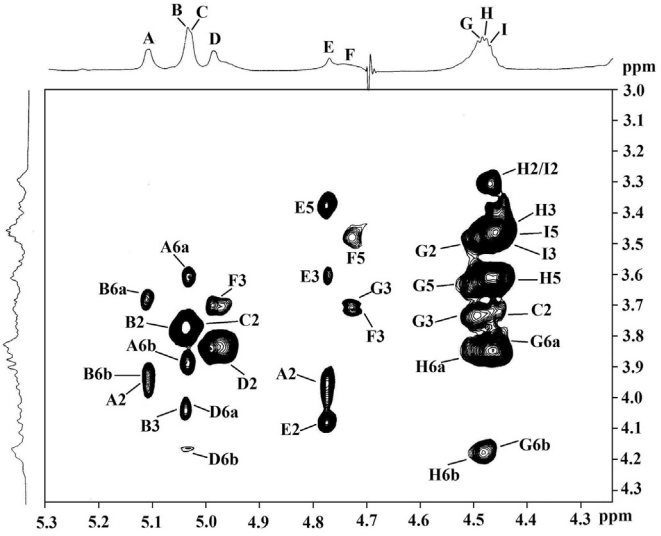


Fig. 2. Part of ROESY spectrum of PS-II, isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. The ROESY mixing time was 300 ms.

The anomeric proton chemical shift (δ 4.99) and coupling constant values ($J_{H\text{-}1,H\text{-}2}$ ~3.0 Hz, $J_{C\text{-}1,H\text{-}1}$ ~171 Hz) confirmed that residue **D** was present in α-configuration. The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values (~10.0 Hz) confirmed that it was an D-glucopyranosyl moiety (Glc*p*). The downfield shifts of C-6 (δ 67.6) with respect to standard values of methyl glycosides indicated that residue **D** was linked at this position. The linkage at C-6 of the

Table 2a
The ^1H NMR[a] and ^{13}C NMR[b] chemical shifts for the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička 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
→2,6)-α-D-Gal*p*-(1→	5.11	3.94	3.96	4.12	4.22	3.61[c], 3.90[d]
A	98.3	77.1	69.6	69.2	68.5	66.8
→6)-α-D-Gal*p*-(1→	5.04	3.76	4.02	4.12	4.22	3.68[c], 3.92[d]
B	101.6	68.3	69.6	69.2	68.5	66.9
→2)-α-L-Fuc*p*-(1→	5.03	3.75	3.87	3.82	4.16	1.21
C	98.3	78.2	69.6	71.8	67.2	15.7
→6)-α-D-Glc*p*-(1→	4.99	3.82	3.61	3.59	4.07	4.02[c], 4.16[d]
D	98.3	71.8	75.0	69.6	70.4	67.6
β-D-Man*p*-(1→	4.77	4.07	3.61	3.55	3.38	3.74[c], 3.90[d]
E	101.7	70.4	73.1	67.2	76.2	61.2
→3)-β-D-Glc*p*-(1→	4.73	3.30	3.71	3.43	3.47	3.70[c], 3.90[d]
F	102.9	72.9	84.5	69.6	75.6	60.8
→3,6)-β-D-Glc*p*-(1→	4.49	3.48	3.70	3.42	3.61	3.82[c], 4.16[d]
G	102.5	72.9	84.3	69.6	75.0	68.8
→6)-β-D-Glc*p*-(1→	4.48	3.29	3.45	3.44	3.61	3.84[c], 4.19[d]
H	102.7	73.1	75.6	69.6	75.0	69.0
β-D-Glc*p*-(1→	4.47	3.29	3.47	3.36	3.46	3.68[c], 3.87[d]
I	103.0	73.1	75.6	69.6	75.9	60.8

[a] The values of chemical shifts were recorded keeping HOD signal fixed at δ 4.70 ppm at 30 °C.
[b] The values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 30 °C.
[c] Interchangeable.
[d] Interchangeable.

Table 2b
ROESY data for the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička.

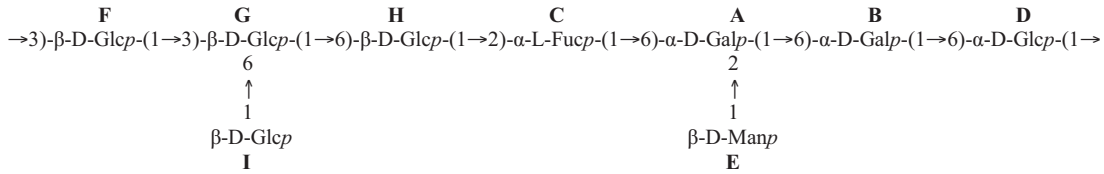
Glycosyl residue	Anomeric proton δ	ROE contact proton		
		δ	Residue	Atom
→2,6)-α-D-Gal*p*-(1→	5.11	3.68	**B**	H-6a
A		3.92	**B**	H-6b
		3.94	**A**	H-2
→6)-α-D-Gal*p*-(1→	5.04	4.02	**D**	H-6a
B		4.16	**D**	H-6b
		3.76	**B**	H-2
		4.02	**B**	H-3
→2)-α-L-Fuc*p*-(1→	5.03	3.61	**A**	H-6a
C		3.90	**A**	H-6b
		3.75	**C**	H-2
→6)-α-D-Glc*p*-(1→	4.99	3.71	**F**	H-3
D		3.82	**D**	H-2
β-D-Man*p*-(1→	4.77	3.94	**A**	H-2
E		4.07	**E**	H-2
		3.61	**E**	H-3
		3.38	**E**	H-5
→3)-β-D-Glc*p*-(1→	4.73	3.70	**G**	H-3
F		3.71	**F**	H-3
		3.47	**F**	H-5
→3,6)-β-D-Glc*p*-(1→	4.49	3.84	**H**	H-6a
G		4.19	**H**	H-6b
		3.48	**G**	H-2
		3.70	**G**	H-3
		3.61	**G**	H-5
→6)-β-D-Glc*p*-(1→	4.48	3.75	**C**	H-2
H		3.29	**H**	H-2
		3.45	**H**	H-3
		3.61	**H**	H-5
β-D-Glc*p*-(1→	4.47	3.82	**G**	H-6a
I		4.16	**G**	H-6b
		3.29	**I**	H-2
		3.47	**I**	H-3
		3.46	**I**	H-5

residue **D** was further confirmed from DEPT-135 spectrum (Fig. 1b). Thus, **D** was confirmed as (1 → 6)-α-D-glucopyranosyl residue.

The large coupling constant values ($J_{H\text{-}3,\,H\text{-}4}$ ~7.5 Hz and $J_{H\text{-}4,\,H\text{-}5}$ ~10 Hz) of residue **E** confirmed its mannopyranosyl configuration. The anomeric proton (δ 4.77) and anomeric carbon chemical shifts (δ 101.7) as well as the low coupling constant values ($J_{H\text{-}1,H\text{-}2}$ ~0, $J_{C\text{-}1,H\text{-}1}$ ~161) confirmed that residue **E** was present in β-configuration. All the proton and carbon chemical shifts of residue **E** corresponded nearly to the standard values of methyl glycosides of β-D-mannose. Thus, **E** was confirmed as terminal β-D-mannopyranosyl residue.

Residues **F**, **G**, **H,** and **I** were established as β-configuration from coupling constant values $J_{H\text{-}1,H\text{-}2}$ (~8.0 Hz), $J_{C\text{-}1,H\text{-}1}$ (~160–161 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 **F**, **G**, **H** and **I** confirmed their D-glucopyranosyl configuration (Glc*p*). The downfield shift of C-3 of **F** (δ 84.5) with respect to standard value of methyl glycoside (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that residue **F** was (1 → 3)-β-D-Glc*p*. The downfield shifts at C-3 (δ 84.3) and C-6 (δ 68.8) of residue **G** with respect to standard value of methyl glycoside indicated that it was (1 → 3,6)-β-D-Glc*p*. Since, the residue **G** was the rigid part its C-3 (δ 84.3) value appeared at up field region in comparison to the C-3 (δ 84.5) of the residue **F**. In case of residue **H**, the downfield shift of C-6 (δ 69.0) indicated that it was (1 → 6)-β-D-Glc*p* moiety. The linking at C-6 of the residues **G** and **H** were further confirmed by DEPT-135 spectrum (Fig. 1b). In residue **I**, all carbon chemical shifts values were found nearly to the standard values of methyl glycoside of β-D-glucose. Thus, residue **I** was confirmed as terminal β-D-Glc*p*.

The sequence of glycosyl residues (**A** to **I**) were determined from ROESY (Fig. 2, Table 2b) as well as NOESY (Fig. S3, Supplementary data). In ROESY experiment, the inter-residual contacts **A**H-1/**B**H-6a, 6b; **B**H-1/**D**H-6a, 6b; **C**H-1/**A**H-6a, 6b; **D**H-1/**F**H-3; **E**H-1/**A**H-2; **F**H-1/**G**H-3; **G**H-1/**H**H-6a, 6b; **H**H-1/**C**H-2; and **I**H-1/**G**H-6a, 6b along with other intra-residual contacts were also observed (Fig. 2). The above ROESY connectivities established the following sequences: **A** (1 → 6) **B; B** (1 → 6) **D; C** (1 → 6) **A; D** (1 → 3) **F; E** (1 → 2) **A; F** (1 → 3) **G**; **G** (1 → 6) **H**; **H** (1 → 2) **C**; and **I** (1 → 6) **G**. These data clearly indicated the positions of substitution and sequence of sugar residues in the polysaccharide. Hence, the structure of repeating unit in the PS-II was proposed as:



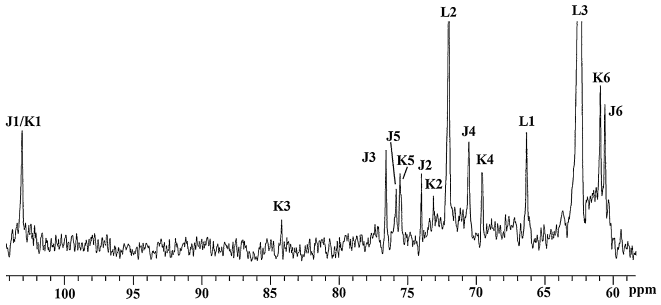


Fig. 3. ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of the Smith-degraded glycerol containing disaccharide isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička.

Table 3
The ^{13}C NMR[n] chemical shifts of Smith-degraded glycerol-containing disaccharide of the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička in D_2O at 30 °C.

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p*-(1→ **J**	103.1	74.0	76.6	70.6	75.8	60.7
→3)-β-D-Glc*p*-(1→**K**	103.1	73.1	84.3	69.6	75.6	60.9
Gro-(3→ **L**	66.4	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 ppm at 30 °C.

Finally, Smith degraded material (SDPS) from PS-II was prepared to confirm the linkages of the heteroglycan. The ^{13}C NMR (125 MHz) spectrum (Fig. 3, Table 3) at 30 °C of SDPS showed one anomeric carbon signals at δ 103.1 for one terminal β-D-Glc*p* (**J**) and one (1 → → 3)-β-D-Glc*p* (**K**) residues. The carbon signals C-1, C-2, and C-3 of the glycerol moiety (Gro) were assigned as δ 66.4, 72.1, and 62.5 respectively. The glycerol moiety (**L**) was generated from (1 → 6)-β-D-Glc*p* residue (**H**) after periodate oxidation followed by Smith degradation, and this moiety was attached to (1 → 3)-linked β-D-Glc*p* moiety (**K**). The residue (1 → 3)-β-D-Glc*p* (**F**) was converted to terminal β-D-Glc*p* unit (**J**) during Smith degradation. In ^{13}C NMR (Fig. 3), the carbon signal at δ 84.3 clearly indicated the presence of (1 → 3)-linked β-D-Glc*p* unit (**K**) which was generated from (1 → 3,6)-β-D-Glc*p* residue (**G**) during this reaction. Hence, Smith degradation results in the formation of a glycerol containing

disaccharide from the parent polysaccharide and the structure of which was established as:

J **K** **L**

β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-Gro

This result further confirmed the repeating unit present in the heteroglycan isolated from the edible mushroom *E. lividoalbum* (Kühner & Romagn.) Kubička.

3.3. Biological activities

The cell viability using PS-II was studied on human 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. 4a). It was observed that the cytotoxicity to normal lymphocytes by PS-II was insignificant. Cell proliferative activity was observed at 50 μg/ml of PS-II with respect to control. These results showed a lower level of cytotoxicity when lymphocytes were treated with PS-II up to 200 μg/ml but even at higher dose 400 μg/ml, the polysaccharide showed mild toxicity. Cell culture experiments were carried out and statistical calculations showed the IC50 value was 800 μg/ml (Fig. S5, Supplementary data), indicating that 200 μg/ml is safe with respect to the other higher doses.

Glutathione is an important antioxidant in cellular system. Hence to understand the glutathione level in cell, both reduced and oxidized form of glutathione were measured. The reduced glutathione level (GSH, Fig. 4b) was decreased and the mild augmentation of oxidized form of glutathione level (GSSG, Fig. 4b) 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 $r = 0.951$, Pearson correlation $p < 0.05$) (Fig. S6, Supplementary data). 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 1.01 to 0.499 compared to their respective control indicating that 400 μg/ml was toxic. These results indicated that 200 μg/ml is biologically safe and effective dose.

Study of lipid peroxidation is one of the important parameters to assess the cellular damage. It initiates inactivation of cellular components and protective enzymes, and thereby plays a crucial role of oxidative stress in biological systems (Samanta et al., 2013). Several toxic by-products especially malondialdehyde is released due to lipid peroxidation. Hence, lipid peroxidations in lymphocytes were measured in terms of the concentration of malondialdehyde (MDA) release (Fig. 4c). The present investigation showed slightly increase of MDA at the dose of 400 μg/ml in comparison to the previous doses indicating that 200 μg/ml is again biologically safe.

Stimulated lymphocytes secreted several factors like NO. The release of NO (Fig. 4d) clearly demonstrated that it was secreted by the lymphocytes when stimulated by PS-II. In presence of the PS-II, single culture of lymphocytes generated significant amount

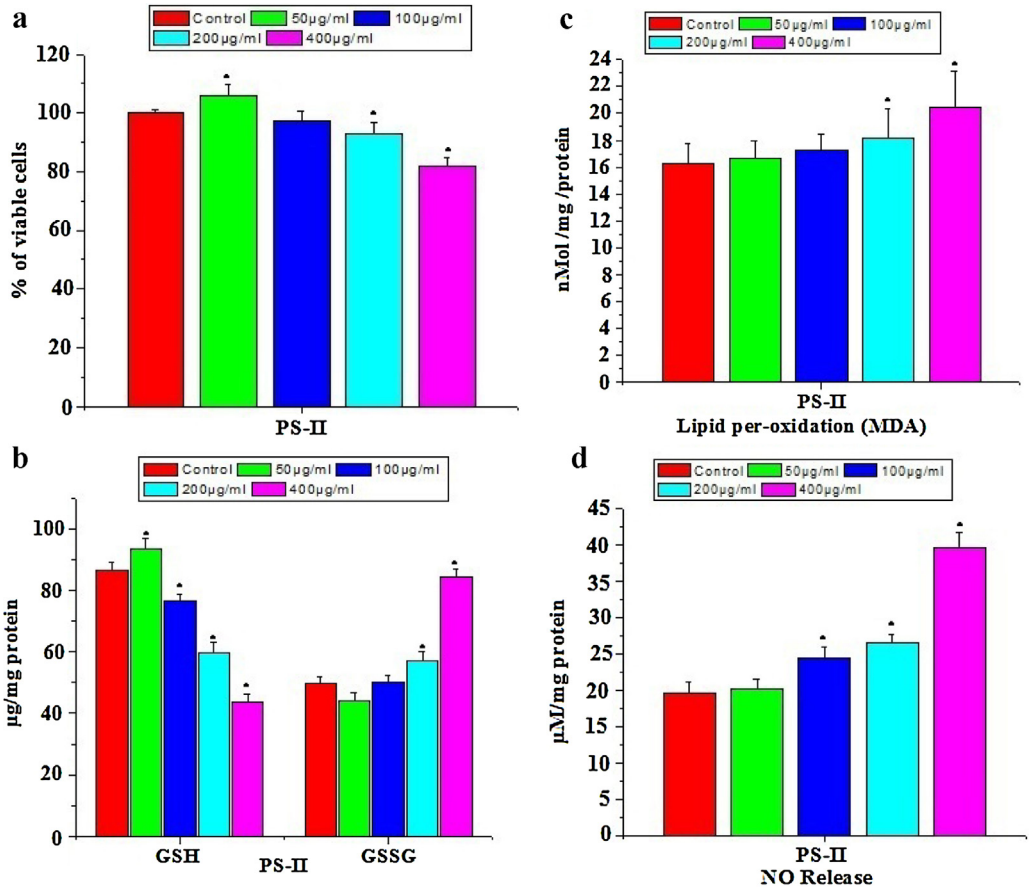


Fig. 4. (a) Cytotoxicity of PS-II against normal human lymphocytes. (b) Concentration of reduced glutathione (GSH) and oxidized glutathione of PS-II treated normal human lymphocytes. (c) Concentration of Lipidperoxidation in terms of MDA of PS-II treated normal human lymphocytes. (d) Concentration of nitric oxide release from PS-II treated normal human lymphocytes. ($n = 6$, values are expressed as mean ± SEM. * Indicates the significant difference as compared to control group).

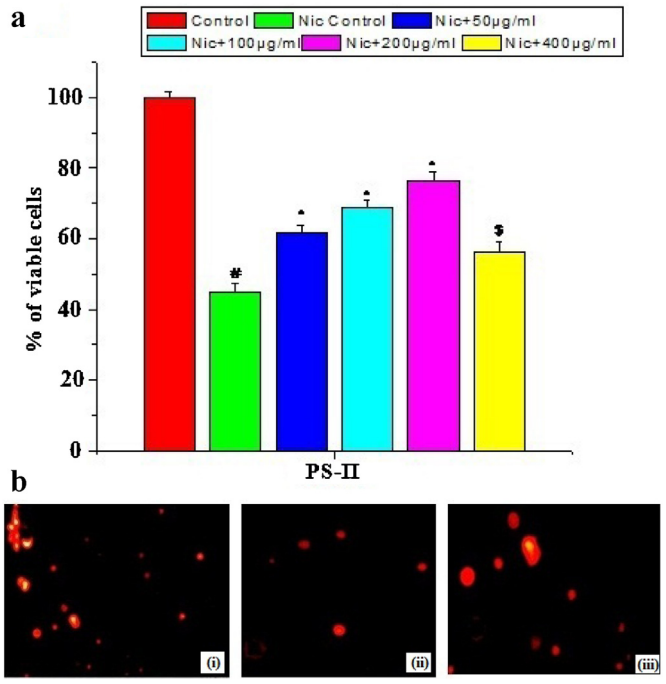


Fig. 5. (a) Cytoprotective role of PS-II was done by using nicotine treated normal human lymphocytes. $n = 6$; values are expressed as mean ± SEM. * Indicates the significant difference as compared to control group; (b) the uptake of PI stain by apoptotic cells were visualized by phase contrast fluorescent microscope under 50× mfg. Here, (i): lymphocytes were treated with 10 mM Nicotine, (ii): lymphocytes were pre-treated with 10 mM Nicotine and subsequently treated with PS-II (200 μg/ml) and (iii): lymphocytes were pre- treated with 10 mM Nicotine and subsequently treated with PS-II (400 μg/ml).

of NO ($p < 0.05$) into the medium after 24 h of incubation (Fig. 4d). The result showed the presence of a high concentration of NO in the co-culture medium of pulsed lymphocytes at 400 μg/ml indicating that this dose is cytotoxic. Hence, it is again established that 200 μg/ml is safe and effective dose.

To establish the protective role of PS-II against nicotine toxicity, lymphocytes were treated with nicotine (10 mM) as positive control and different concentrations of PS-II along with nicotine for 24 h in culture media. The significantly ($p < 0.05$) increased cell viability levels were observed up to 200 μg/ml. The fluorescent microscopic pictures established the result (Fig. 5a and b). The fluorescence images revealed that the PS-II was able to ameliorate the toxic effects of nicotine at the dose of 200 μg/ml, but when the dose was increased to 400 μg/ml, the PS-II lost its ameliorative effects on lymphocytes. The above result was confirmed by FACS, which established our findings that 400 μg/ml revealed the toxic effects synergistically with nicotine (Fig. 6).

It is evident from these experiments that, in vitro application of PS-II does not induce any cellular damage in lymphocytes associated with enhanced MDA level, NO level, GSSG level and decreased GSH level. The cytotoxic profile of PS-II in lymphocytes indicated 200 μg/ml safe and effective, whereas concentrations higher than 200 μg/ml showed significant increase of cytotoxicity. Administration of Nicotine to lymphocytes causes decrease in cell viability which is protected by supplementation of PS-II to nicotine treated cells. These findings suggest the potential use and beneficial role of PS-II for use as antioxidant as well as immunostimulant.

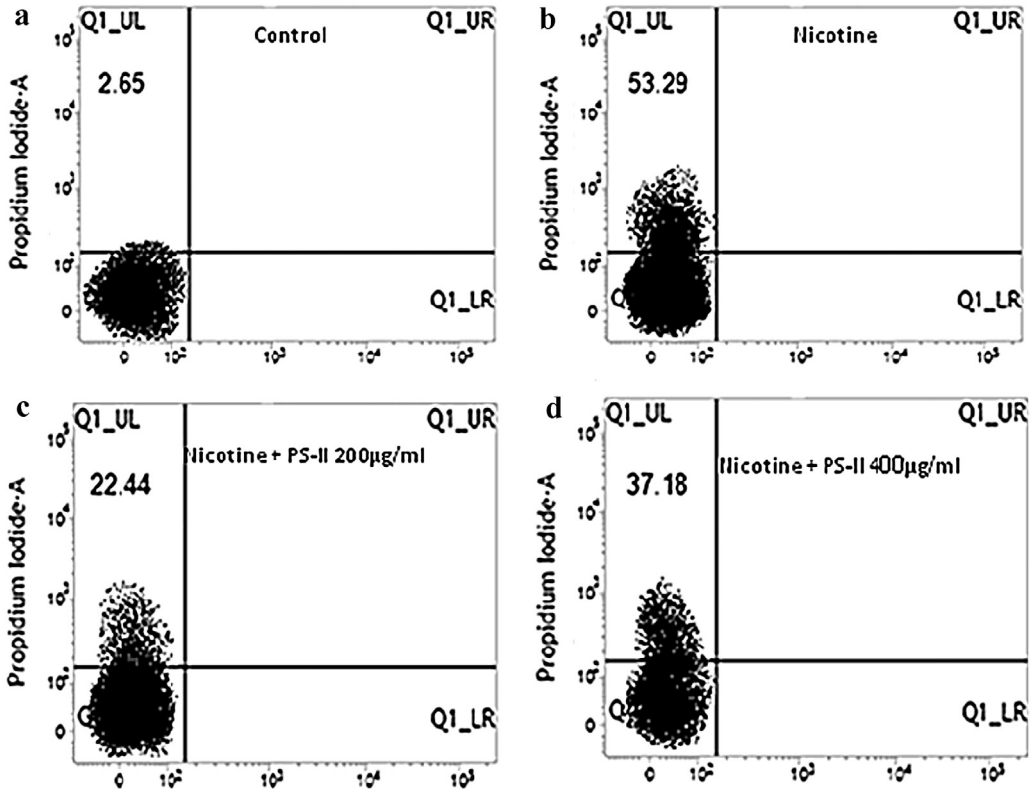
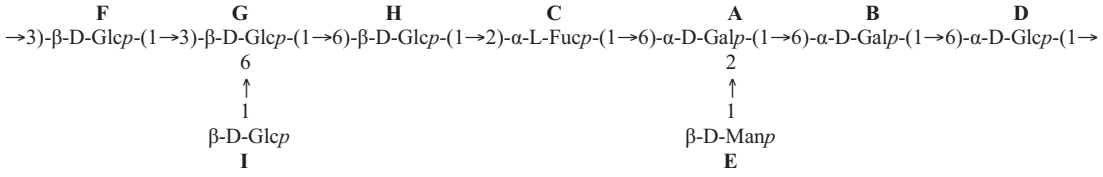


Fig. 6. FACS analysis of normal human lymphocytes at different concentration of PS-II. The propidium iodide was used for analysis of cell death and only PI (+) ve cells were counted.

4. Conclusion

A water soluble heteroglycan (PS-II), with the average molecular weight ~5.2×10^4 Da, was isolated from the alkaline extract of an edible mushroom *E. lividoalbum* (Kühner & Romagn) Kubička. The following structure was characterized by chemical analysis and 1D/2D NMR studies.



This material (PS-II) was observed as biologically non toxic and possess ameliorative role against toxic substances.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.07.080.

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Structural, immunological, and antioxidant studies of β-glucan from edible mushroom *Entoloma lividoalbum*

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ABSTRACT

A water soluble β-glucan having molecular weight ~2×10^5 Da was isolated from hot water extract of the fruit bodies of an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. This polysaccharide (ELPS) contains (1 → 3,6)-β-D-Glc*p*, (1 → 3)-β-D-Glc*p*, (1 → 6)-β-D-Glc*p*, and terminal β-D-Glc*p* moieties in a molar ratio of nearly 1:1:3:1. Chemical and spectroscopic analysis showed that the backbone of glucan consists of three (1 → 6)-β-D-glucopyranosyl and two (1 → 3)-β-D-glucopyranosyl residues, out of which one (1 → 3)-β-D-glucopyranosyl moiety was branched at *O*-6 with a terminal β-D-glucopyranosyl residue. This β-glucan exhibited macrophage, splenocyte, and thymocyte stimulations. It possesses promising antioxidant activities as evidenced from its hydroxyl and superoxide radical scavenging activities and reducing properties.

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1. Introduction

Mushroom polysaccharides are potent renewable source for the development of several drugs. PSK from *Coriolus (Trametes) versicolor* (Cui & Chisti, 2003), lentinan from *Lentinus edodes* (Taguchi et al., 1983) and sonifilan (SPG) from *Schizophyllum commune* (Fujimoto et al., 1983) have been recognized as anticancer drugs throughout the world (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007). Mushroom polysaccharides have drawn the attention of chemists and immunobiologists for their immunomodulating (Wasser & Weis, 1999), antioxidant (Maity, Samanta, et al., 2014; Patra et al., 2013), and antitumor activities (Wasser, 2002). Mushrooms have a long traditional history in curing various life threatening diseases. Polysaccharides derived from these medicinal mushrooms have been explored for several years and found to possess immunomodulatory and anticancer properties. The anticancer activity of these polysaccharides is mediated mostly through the activation of immune cells such as B cells, T cells, macrophages and NK cells (Nandi et al., 2013; Wasser, 2002). It has been reported that *in vivo* administration of β-glucans (Kogan, 2000) can enhance immune reactions and up regulate the resistance of host against tumor cells. Furthermore, administration of glucans to macrophages activated by LPS can lead to increased production of cytokines like interleukin-1 and TNF-α which subsequently induce lymphocyte differentiation and proliferation to enhance immune responses (Adachi, Okazaki, Ohno, & Yadomae, 1994; Chihara, 1992). It is also noteworthy to mention the antioxidant activity of the polysaccharide which depends on monosaccharide composition and their different arrangements during polymerization (Tsiapali et al., 2001). Antioxidants prevent the chain reactions by hydrogen donation and interrupting the process of oxidation. As a result, stable free radicals cannot survive or propagate further oxidation (Aruoma, 1999; Wade, Jackson, Highton, & Van Rij, 1987). Different types of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radicals are closely involved in various human diseases, such as cerebral ischemia, diabetes, alzheimer, inflammation, rheumatoid arthritis, atherosclerosis and cancer, as well as aging processes (Halliwell & Gutteridge, 1989). Antioxidants play key role to prevent the generation of ROS or scavenge them and minimize oxidative tissue damage. Hence, ROS induced oxidative cell damage can be prevented by supplementation of naturally occurring biomolecules like polysaccharide, which is one of the most acceptable techniques for modern therapy.

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Edible and non-toxic mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička normally grows on Sikkim Himalayan region, India during July to August (Das, 2010). It was reported that this mushroom contains minerals and nutrients such as calcium, aluminum and iron (Maity, Samanta, et al., 2014). The antimicrobial activity (Rai, Sen, & Acharya, 2013), antioxidant properties (Maity, Samanta, et al., 2014), and protective role in human lymphocytes (Maity, Nandi, et al., 2014) of *E. lividoalbum* were reported. From these points of views *E. lividoalbum* may therefore be useful as a medicinal fungus with various immunostimulating and other protective effects. Two water soluble polysaccharides, PS-I (Maity, Samanta, et al., 2014) and PS-II (Maity, Nandi, et al., 2014) were isolated from the alkaline extract of the mushroom, characterized and reported. In the present investigation another water soluble polysaccharide (ELPS) has been isolated from the aqueous extract of this mushroom and characterized as β-glucan. Similar type of β-glucan was isolated by Bhanja et al. (2012) but differing in the number of sugar moieties and linkages in the skeleton chain. The water insoluble β-glucan (PS-II) isolated by Bhanja et al. (2012) contained (1 → 3)-linked three glucose moieties in the skeleton chain with branching at C-6 by another glucose residue, where as the present material is a water soluble β-glucan (ELPS) consisting of five (1 → 3)-, (1 → 6)-linked glucose moieties in the skeleton chain with branching at C-6 by another one. In the present investigation detailed structural characterization, immunological studies and antioxidant properties of the ELPS have been carried out and reported herein.

2. Materials and methods

2.1. Isolation and purification of the polysaccharide

Fresh fruit bodies of the mushroom *E. lividoalbum* (Kühner & Romagn.) Kubička (700 g) were collected from Sikkim Himalayan region, India, gently washed with water, cut into pieces and boiled at 100 °C with distilled water for 10 h, cooled, centrifuged, supernatant was precipitated in EtOH (1:5) to get crude polysaccharide (900 mg) and purified using the procedure as described in previous publication (Maity, Samanta, et al., 2014). The crude polysaccharide (25 mg) was purified by gel-permeation chromatography (GPC) on column (90 cm × 2.1 cm) of Sepharose 6B using distilled water as the eluent with a flow rate of 0.5 mL min^{-1}. A total of 90 test tubes were collected and monitored by the phenol-sulfuric acid method (York, Darvill, McNeil, Stevenson, & Albersheim, 1986) at 490 nm using Shimadzu UV–vis spectrophotometer, model-1601. A single homogeneous fraction (test tube 20–32) was collected and freeze-dried, yielding 15 mg pure polysaccharide. This experiment was repeated eight times and 110 mg of pure polysaccharide was collected and preserved for further analysis.

2.2. General methods

The average molecular weight of the ELPS was measured as reported earlier (Maity, Nandi, et al., 2014). The optical rotation was measured on a Jasco Polarimeter model P-1020 at 30 °C. For monosaccharide analysis, the ELPS (3 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) in a round-bottom flask at 100 °C for 18 h in a boiling water bath and the analysis was carried out as described in previous paper (Maity, Nandi, et al., 2014). The absolute configuration of monosaccharide was determined by the method of Gerwig, Kamerling, and Vliegenthart (1978). Periodate oxidation, Smith degradation, and methylation experiments were carried out by the method described previously (Maity, Nandi, et al., 2014). A gas–liquid chromatographic analysis (GLC) was done using 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) for monosaccharide analysis. All GLC analyses were performed at 170 °C. 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^{-1} up to a final temperature of 200 °C. Finally NMR experiments (Dueñas-Chaso et al., 1997; Hård, Zadelhoff, Moonen, Kamerling, & Vliegenthart, 1992) were carried out by a Bruker Avance DPX-500 spectrometer at 30 °C as reported in our previous paper (Maity, Nandi, et al., 2014).

2.3. Immunostimulating properties

Limulus Amebocyte Lysate (LAL) assay was performed to confirm the presence of any endotoxin in the ELPS by the procedure as described previously (Nandi et al., 2013). Macrophage, splenocyte and thymocyte proliferation assays were determined by adopting the procedures as described previously (Bhanja et al., 2012; Nandi et al., 2013). The formulas to calculate the SPI and TPI are as follows:

$$\text{Splenocyte Proliferation Index (SPI)} = \frac{\text{O.D. value of glucan treated splenocytes}}{\text{O.D. value of negative control (PBS)}} \times 1$$

$$\text{Thymocyte Proliferation Index (TPI)} = \frac{\text{O.D. value of glucan treated thymocytes}}{\text{O.D. value of negative control (PBS)}} \times 1$$

The full form of O.D. is "Optical Density" or absorbance value of the dissolved formazon crystals during MTT assay, which gets reduced after interacting with the oxidoreductase enzymes present only in viable cells.

2.4. Antioxidant properties

2.4.1. Hydroxyl radical scavenging activity

The reaction mixture (1 mL) consisted of KH_2PO_4–KOH buffer (20 mM, pH 7.4), 2-deoxy-D-ribose (2.8 mM), variable concentration (100–800 μg/mL) of ELPS, $FeCl_3$ (100 mM), EDTA (104 μM), ascorbate (100 μM) and H_2O_2 (1 mM). Following incubation at 37 °C for 1 h, 2 mL thiobarbituric acid (TBA) and trichloroacetic acid (TCA) solution (100 mL contained 375 mg TBA, 15 mg TCA, 2 mL concentrated HCl added to 98 mL of TBA-TCA solution) were added to the reaction mixture, which was then heated in a boiling water bath for 15 min. After cooling, absorbance was measured at 535 nm, with butylated hydroxytoluene (BHT) as positive control (Halliwell, Gutteridge, & Aruoma, 1987). The hydroxyl radical Scavenging activity of ELPS was calculated by the following equation:

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of sample. The effect of hydroxyl radical Scavenging activity was expressed as IC_{50}: the amount of the sample needed to inhibit hydroxyl radical concentration by 50%.

2.4.2. Superoxide radical scavenging activity

The method by Martinez, Marcelo, Marco, and Moacyr (2001) for determination of the superoxide radical was followed with modification in the riboflavin-light-nitroblue tetrazolium (NBT) system. Each 3 mL reaction mixture contained 50 mM sodium phosphate

buffer (pH 7.8), 13 mM methionine, 500 μM solution of various concentrations (50–400 μg/mL) of ELPS, 100 μM EDTA, 75 μM NBT and 2 μM riboflavin. One set of reaction mixtures were exposed to light for 10 min to activate the riboflavin-NBT and the absorbance of each mixture was measured at 560 nm against identical mixtures from another set kept in the dark for the same duration. Butylated hydroxyanisole (BHA) was used as a positive control. The superoxide radical Scavenging activity was calculated by the following formula:

$$\text{Scavenging activity}\,(\%) = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of sample. IC_{50} value is the effective concentration at which the superoxide radicals were scavenged by 50%.

2.4.3. Determination of reducing power

The reducing power of the samples was determined according to the method of Oyaizu (1986) with little modification. The reaction mixture contains various concentrations of ELPS (100–800 μg/mL), 1.5 mL sodium phosphate buffer (0.2 M, pH 6.6) and 1.5 mL of potassium ferricyanide (1%) solution. The mixture was incubated for 20 min. The reaction was terminated by adding 1.5 mL of trichloroacetic acid (10%). Then, 2.5 mL distilled water and 0.25 mL $FeCl_3$ (0.1%) were added to the reaction mixture (2.5 mL) and incubated for 15 min. The absorbance was measured at 700 nm. Ascorbic acid was used as standard. IC_{50} value represented the concentration of the compounds that providing 0.5 of absorbance. Increased absorbance of the reaction mixture indicates increased reducing power of the sample.

3. Results and discussion

3.1. Isolation, purification and chemical analysis of the ELPS

A single fraction (15 mg) was obtained in the course of fractionation of water soluble crude polysaccharide (25 mg) through Sepharose 6B column using water as the eluent (Fig. 1a). The ELPS showed specific rotation $[\alpha]_D^{30}$ −6.9 (*c* 0.11, H_2O). The average molecular weight (Hara, Kiho, Tanaka, & Ukai, 1982) of ELPS was estimated as ~2×10^5 Da on the basis of standard calibration curve prepared using standard dextrans (Fig. 1b). GLC analysis of the alditol acetates (Lindahl, 1970) of this polysaccharide revealed the presence of glucose only. The absolute configuration of glucose was determined as D according to Gerwig et al. (1978). The glucan was methylated according to the Ciucanu and Kerek (1984) method, followed by hydrolysis and alditol acetate preperation. The GLC–MS analysis of the alditol acetates of methylated product showed the presence (1 → 3), (1 → 6), and (1 → 3,6)-linked and terminal glucopyranosyl residues in a molar ratio of nearly 1:3:1:1 respectively (Table 1). The periodate oxidation experiment was carried out with the ELPS for further confirming the linking information of the sugar moieties. GLC analysis of alditol acetates of the periodate-oxidized (Goldstein, Hay, Lewis, & Smith, 1965; Hay, Lewis, & Smith, 1965), $NaBH_4$-reduced, hydrolyzed products showed the presence of glucose unit only. GLC–MS analysis of periodate-oxidized, reduced, methylated (Abdel-Akher & Smith, 1950) products of ELPS showed the presence of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol in a molar ratio of nearly 1:1. These results showed that the (1 → 6)-linked and terminal glucopyranosyl residues were consumed during oxidation, whereas (1 → 3,6)-and (1 → 3)-linked glucopyranosyl residues remained unaffected. Hence, the mode of linkages present in the ELPS was confirmed.

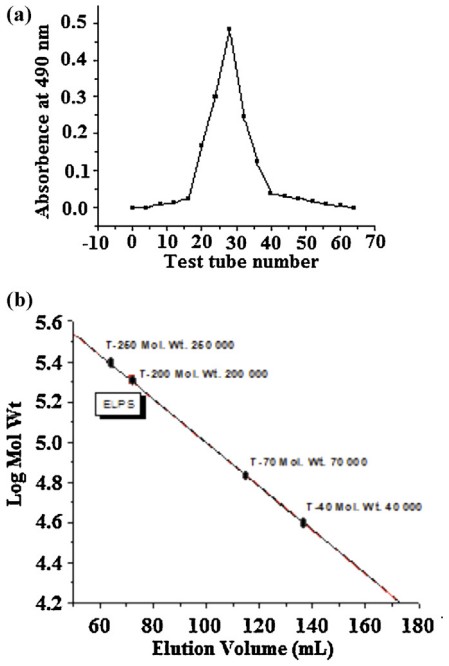


Fig. 1. (a) Gel permeation chromatogram of crude polysaccharide isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička using Sepharose 6B column. (b) Determination of molecular weight of ELPS by gel permition chromatography in sepharose 6B column.

3.2. NMR and structural analysis of the ELPS

In the anomeric region of the ^1H NMR spectrum (500 MHz; Fig. 2a, Table 2a) at 30 °C, three signals were observed at δ 4.78, 4.51, and 4.49. The peak at δ 4.78 corresponded to the anomeric proton of residue **A**. The signal at δ 4.51 corresponded to both the residues **B** and **C** and the signal at δ 4.49 corresponded to residue **D** (Fig. 2a). ^{13}C NMR spectrum (125 MHz; Fig. 2b) showed three signals in the anomeric region at δ 102.9, 102.7, and 102.5 at the same temperature. On the basis of HSQC spectrum (Fig. 2c, Table 2a) the anomeric proton signal at δ 4.51 was correlated to both the carbon signals δ 102.7 and δ 102.5, corresponded to anomeric carbons **B** and **C** respectively. Again, the anomeric proton signals at δ 4.78 and δ 4.49 were correlated to carbon signals at δ 102.7 and δ 102.9, corresponded to anomeric carbon of residues **A** and **D** respectively. From DQF-COSY, TOCSY, and HSQC experiments, all the ^1H and ^{13}C signals (Table 2a) were assigned. The proton-proton and one-bond C—H coupling constants were measured from DQF-COSY and proton coupled ^{13}C spectrum respectively.

All the residues from **A** to **D** were present as D-glucopyranosyl configuration (Glc*p*) as confirmed by the large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values (~10.0 Hz). Anomeric proton chemical shifts (δ 4.78–4.49), anomeric carbon chemical shifts (δ 102.9–102.5), and the coupling constant values $J_{H\text{-}1,H\text{-}2}$ (~8.0 Hz), $J_{C\text{-}1,H\text{-}1}$ (~160–161 Hz) confirmed that the residues (**A**–**D**) were present in β-configuration. The downfield shifts of C-3 (δ 84.6) of residue **A** with respect to standard value of methyl glycoside (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that residue **A** was (1 → 3)-linked β-D-Glc*p*. In residue **B**, all carbon chemical shifts values were found nearly to the standard value of methyl glycoside (Agrawal, 1992; Rinaudo & Vincendon, 1982) of β-D-glucose. Thus, residue **B** was terminal β-D-Glc*p*. The downfield shifts at C-3 (δ 84.3) and C-6 (δ 68.7) of residue **C** with respect to standard value of methyl glycoside (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that it was (1 → 3,6)-β-D-Glc*p*. Since the residue **C** was the most rigid part of the backbone of this glucan, its C-3 (δ 84.3) appeared at slightly up field region (δ 0.3) compared

Table 1
GLC–MS analysis of methylated polysaccharide (ELPS) isolated from *Entoloma lividoalbum* (Kühner & Romagn.) Kubička.

Methylated sugars	Molar ratio	Linkage type	Major Mass Fragments (*m*/*z*)
2,4,6-Me_3-Glc	1	→3)-β-D-Glc*p*-(1→	43, 45, 71, 87, 101, 117, 129, 143, 161, 173, 203, 217, 233
2,3,4,6-Me_4-Glc	1	β-D-Glc*p*-(1→	43, 45, 59, 71, 87, 101, 117, 129, 161, 205
2,4-Me_2-Glc	1	→3,6)-β-D-Glc*p*-(1→	43, 58, 87, 101, 117, 129, 139, 159, 189, 201, 233
2,3,4-Me_3-Glc	3	→6)-β-D-Glc*p*-(1→	43, 45, 58, 71, 87, 99, 101, 117, 129, 161, 173, 189, 233

Table 2a
The ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts for the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička 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
→3)-β-D-Glc*p*-(1→	4.78	3.38	3.70	3.45	3.48	3.70[c], 3.90[d]
A	102.7	72.8	84.6	69.6	75.9	60.8
β-D-Glc*p*-(1→	4.51	3.29	3.47	3.38	3.43	3.69[c], 3.87[d]
B	102.7	73.1	75.7	69.6	75.9	60.8
→3,6)-β-D-Glc*p*-(1→	4.51	3.49	3.69	3.45	3.61	3.83[c], 4.17[d]
C	102.5	72.8	84.3	69.6	75.0	68.7
→6)-β-D-Glc*p*-(1→	4.49	3.29	3.45	3.43	3.61	3.85[c], 4.19[d]
D	102.9	73.1	75.7	69.6	75.0	68.9[e], 68.8[f]

[a] The values of chemical shifts were recorded keeping HOD signal fixed at δ 4.70 ppm at 30 °C.
[b] The values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 30 °C.
[c] ,[d] Interchangeable.
[e] For residue $\mathbf{D_I}$.
[f] For residue $\mathbf{D_{II}}$.

to C-3 (δ 84.6) of the other (1 → 3)-linked residue **A.** Consequently, the C-6 (δ 68.7) value of residue **C** appeared slightly at the up field region compared to that of the other (1 → 6)-linked residues (**D**). All the chemical shift values of three **D** residues were same except the chemical shift values of C-6. Among the three **D** residues, one moiety ($\mathbf{D_I}$) was glycosidically linked to the most rigid part **C**, hence, its C-6 signal (δ 68.9) showed 0.1 ppm downfield shift with respect to another two residues of $\mathbf{D_{II}}$ (δ 68.8) due to neighboring effect (Bhanja et al., 2012) of the rigid part **C**. The linking at C-6 of the residues **C** and **D** were further confirmed by DEPT-135 spectrum (Fig. 2b).

Table 2b
ROESY data for the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička.

Glycosyl residue	Anomeric proton	ROE contact proton		
	δ	δ	Residue	Atom
→3)-β-D-Glc*p*-(1→	4.78	3.69	**C**	H-3
A		3.38	**A**	H-2
		3.70	**A**	H-3
		3.48	**A**	H-5
β-D-Glc*p*-(1→	4.51	3.83	**C**	H-6a
B		4.17	**C**	H-6b
		3.29	**B**	H-2
		3.47	**B**	H-3
		3.43	**B**	H-5
→3,6)-β-D-Glc*p*-(1→	4.51	3.85	$\mathbf{D_I}$	H-6a
C		4.19	$\mathbf{D_I}$	H-6b
		3.49	**C**	H-2
		3.69	**C**	H-3
		3.61	**C**	H-5
→6)-β-D-Glc*p*-(1→	4.49	3.85[a]	$\mathbf{D_{II}}$	H-6a
D		4.19[a]	$\mathbf{D_{II}}$	H-6b H-3
		3.70[b]	**A**	H-2
		3.29	**D**	H-3
		3.45	**D**	H-5
		3.61	**D**	

[a] For $\mathbf{D_I}$ H-1 to both $\mathbf{D_{II}}$ H-6a and $\mathbf{D_{II}}$ H-6b contacts.
[b] For $\mathbf{D_{II}}$ H-1 to **A** H-3 contact.

The different linkages that connected these residues (**A**–**D**) were determined from ROESY (Fig. 3a, Table 2b) as well as NOESY (not shown) experiment. In ROESY experiment, the inter-residual contacts **A**H-1/**C**H-3; **B**H-1/**C**H-6a, **C**H-6b; **C**H-1/$\mathbf{D_I}$H-6a, $\mathbf{D_I}$H-6b; $\mathbf{D_I}$H-1/$\mathbf{D_{II}}$H-6a, $\mathbf{D_{II}}$H-6b; and $\mathbf{D_{II}}$H-1/**A**H-3 along with other intra-residual contacts were also observed (Fig. 3a). The above ROESY connectivities established the following sequences: **A** (1 → 3) **C**; **B** (1 → 6) **C**; **C** (1 → 6) $\mathbf{D_I}$; $\mathbf{D_I}$ (1 → 6) $\mathbf{D_{II}}$ and $\mathbf{D_{II}}$ (1 → 3) **A**. Finally,

Table 2c
The significant $^3J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide isolated from the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička.

Residue	Sugar linkage	H-1/C-1 δ_H/δ_C	Observed connectivities		
			δ_H/δ_C	Residue	Atom
A	→3)-β-D-Glc*p*-(1→	4.78	84.3	**C**	C-3
		102.7	3.69	**C**	H-3
			3.38	**A**	H-2
			72.8	**A**	C-2
B	β-D-Glc*p*-(1→	4.51	68.7	**C**	C-6
		102.7	73.1	**B**	C-2
			3.83	**C**	H-6a
			4.17	**C**	H-6b
			3.29	**B**	H-2
C	→3,6)-β-D-Glc*p*-(1→	4.51	68.9	$\mathbf{D_I}$	C-6
		102.5	3.85	$\mathbf{D_I}$	H-6a
			4.19	$\mathbf{D_I}$	H-6b
			3.49	**C**	H-2
			72.8	**C**	C-2
D	→6)-β-D-Glc*p*-(1→	4.49	68.8[a]	$\mathbf{D_{II}}$	C-6
		102.9	84.6[b]	**A**	C-3
			3.85[c]	$\mathbf{D_{II}}$	H-6a
			4.19[d]	$\mathbf{D_{II}}$	H-6b
			3.70[e]	**A**	H-3
			3.29	**D**	H-2
			73.1	**D**	C-2

[a] For cross peak between $\mathbf{D_I}$ H-1 and $\mathbf{D_{II}}$ C-6.
[b] For cross peak between $\mathbf{D_{II}}$ H-1 and **A** C-3.
[c] For cross peak between $\mathbf{D_I}$ C-1 and $\mathbf{D_{II}}$ H-6a.
[d] For cross peak between $\mathbf{D_I}$ C-1 and $\mathbf{D_{II}}$ H-6b.
[e] For cross peak between $\mathbf{D_{II}}$ C-1 and **A** H-3.

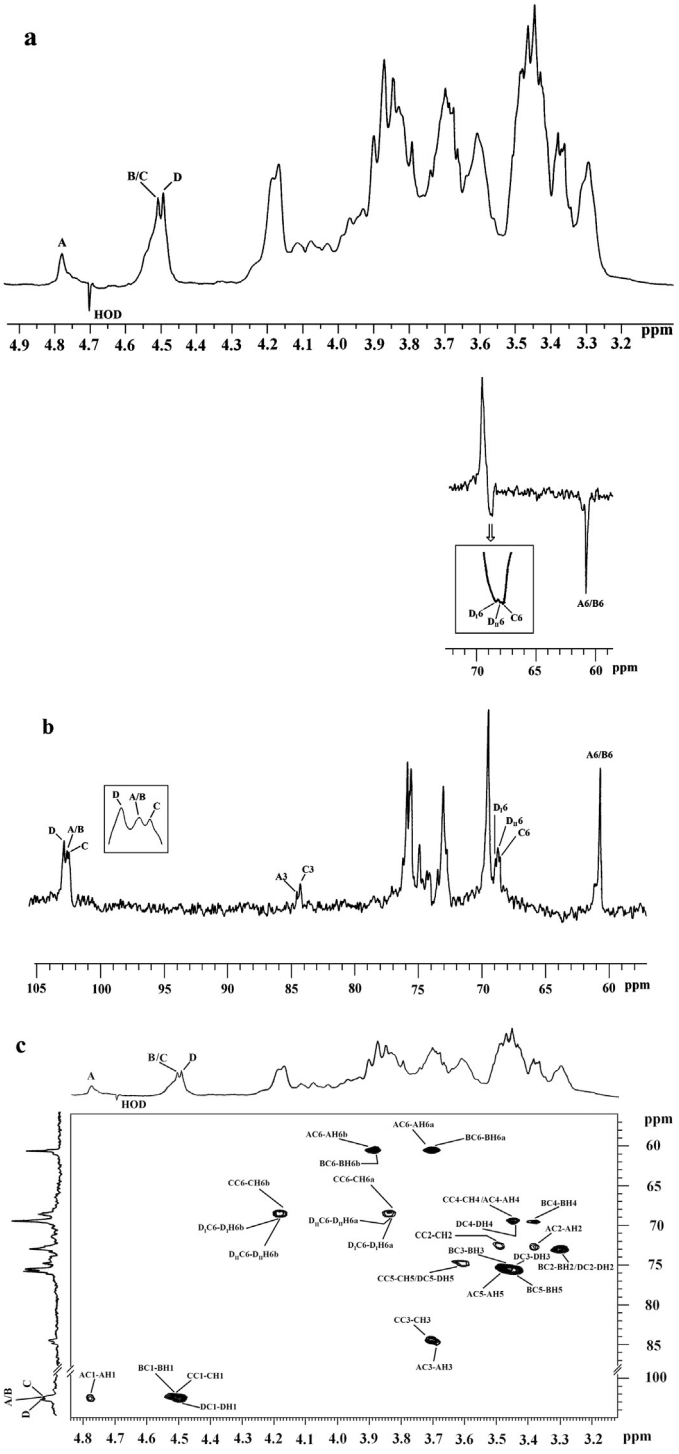


Fig. 2. (a) ^{1}H NMR spectrum (500 MHz, D_2O, 30 °C) of a glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. (b) ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of a glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. Part of DEPT-135 spectrum (D_2O, 30 °C) of a glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička (inset). (c) Part of HSQC spectrum of a glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička.

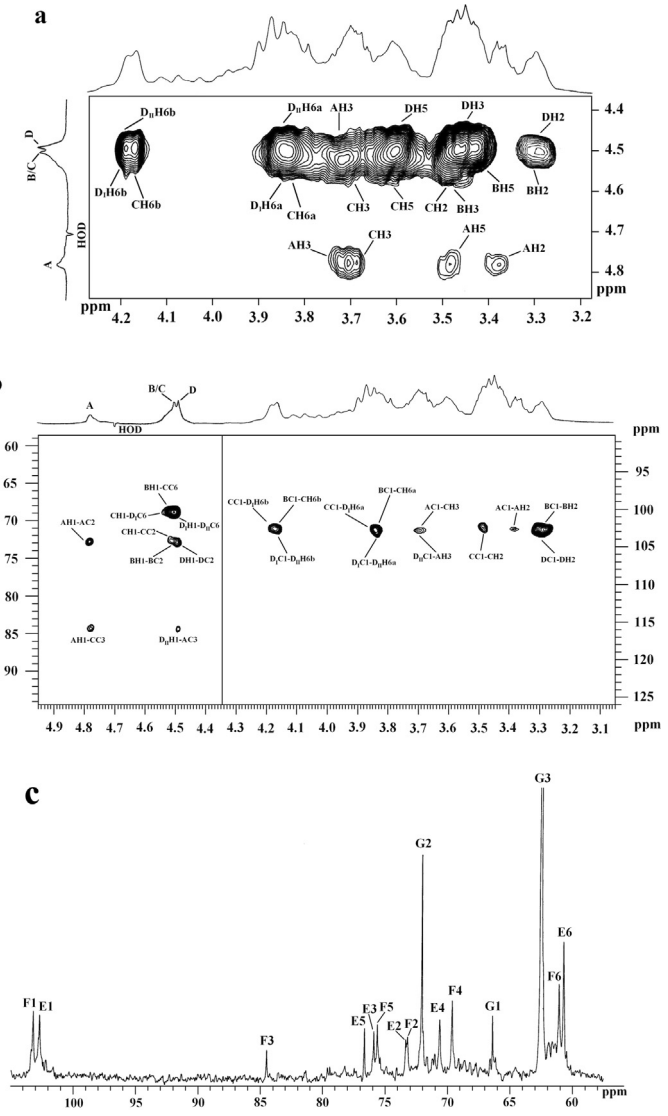


Fig. 3. (a) Part of ROESY spectrum of a glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. The ROESY mixing time was 300 ms. (b) Part of HMBC spectrum of a glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. The delay time in the HMBC experiment was 80 ms. (c) ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of the Smith-degraded glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička.

the ROESY connectivities were confirmed from HMBC spectrum (Fig. 3b). In this spectrum the inter-residual cross-peaks (Table 2c) between **A**H-1/**C**C-3, **A**C-1/**C**H-3; **B**H-1/**C**C-6, **B**C-1/**C**H-6a, **C**H-6b; **C**H-1/**D$_I$**C-6, **C**C-1/**D$_I$**H-6a, **D$_I$**H-6b; **D$_I$**H-1/**D$_{II}$**C-6, **D$_I$**C-1/**D$_{II}$**H-6a, **D$_{II}$**H-6b; **D$_{II}$**H-1/**A**C-3, **D$_{II}$**C-1/**A**H-3 along with some intra-residual peaks were also observed (Fig. 3b). Thus, the HMBC and ROESY connectivities clearly supported the presence of the following repeating unit in the ELPS and the structure is proposed as:

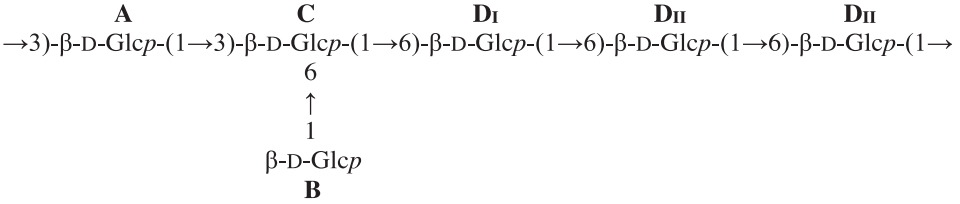


Table 3
The ^{13}C NMR[a] chemical shifts of Smith-degraded glycerol-containing disaccharide of the mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička in D_2O at 30 °C.

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p*-(1→ **E**	102.7	73.4	75.9	70.6	76.7	60.8
→3)-β-D-Glc*p*-(1→**F**	103.2	73.2	84.5	69.7	75.6	61.1
Gro-(3→ **G**	66.4	72.1	62.5			

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

The NMR experiment was conducted with Smith degraded material (SDPS) of ELPS for further confirmation of the linkages of this β-glucan. The ^{13}C NMR (125 MHz) spectrum (Fig. 3c, Table 3) at 30 °C of SDPS showed two anomeric carbon signals at δ 102.7 and 103.2 corresponding to terminal β-D-Glc*p* (**E**) and (1 → 3)-β-D-Glc*p* (**F**) residues respectively. The carbon signals C-1, C-2, and C-3 of the glycerol moiety (**G**) was generated from (1 → 6)-β-D-Glc*p* residue ($\mathbf{D_I}$) of the repeating unit after periodate oxidation followed by Smith degradation and assigned as δ 66.4, 72.1, and 62.5 respectively. The **G** moiety would be attached to (1 → 3)-linked β-D-Glc*p* moiety (**F**) as evidenced from the structure of the repeating unit. The residue (1 → 3)-β-D-Glc*p* (**A**) was converted to terminal β-D-Glc*p* unit (**E**) during Smith degradation and (1 → 3,6)-β-D-Glc*p* (**C**) was converted to (1 → 3)-β-D-Glc*p* (**F**) when the terminal β-D-Glc*p* (**B**) was consumed during oxidation. Hence, Smith degradation yielded an oligosaccharide from the parent polysaccharide and the structure was established as:

E **F** **G**
β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-Gro

Therefore, Smith degradation results further confirmed the repeating unit present in the glucan isolated from the edible mushroom *E. lividoalbum* (Kühner & Romagn.) Kubička.

3.3. Immunostimulating properties of ELPS

LAL test showed negative results indicating that the ELPS was free from any endotoxin contamination. Thus, it can be concluded that the immunostimulatory effects were solely for the ELPS. *In vitro* macrophage activation was observed with different concentrations of ELPS. Nitric oxide production was found to increase in dose-dependent manner with optimum production of 22 μM NO per 5×10^5 macrophages at 35 μg/mL of ELPS. Further increase in concentration of ELPS decreased the NO production (Fig. 4a) implying that the effective dose of the ELPS was 35 μg/mL.

Splenocytes include T cells, B cells, dendritic cells, and macrophages that enhance the immunity in living systems. Thymocytes after maturation in thymus are designated as T cells. The splenocyte and thymocyte activation tests were conducted in mouse cell culture medium with the ELPS by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method (Ohno, Hasimato, Adachi, & Yadomae, 1996). Proliferation of splenocytes and thymocytes is an indication of immunostimulation (Ohno et al., 1993). The splenocyte proliferation index (SPI) and thymocyte proliferation index (TPI) of ELPS were measured with respect to the lipopolysaccharide (LPS, 4 μg/mL, Sigma) and Concanavalin A (Con A, 10 μg/mL) taking as positive control respectively. Phosphate Buffered Saline (PBS) was chosen as negative control for both splenocyte and thymocyte studies. The ELPS was found to stimulate splenocytes and thymocytes and the results are shown in Fig. 4b and c, respectively. The asterisks on the columns indicated the statistically significant differences compared to PBS control. Maximum proliferation index of splenocytes and thymocytes by ELPS were found at 12.5 μg/mL and 100 μg/mL respectively. The decrease in the immunological

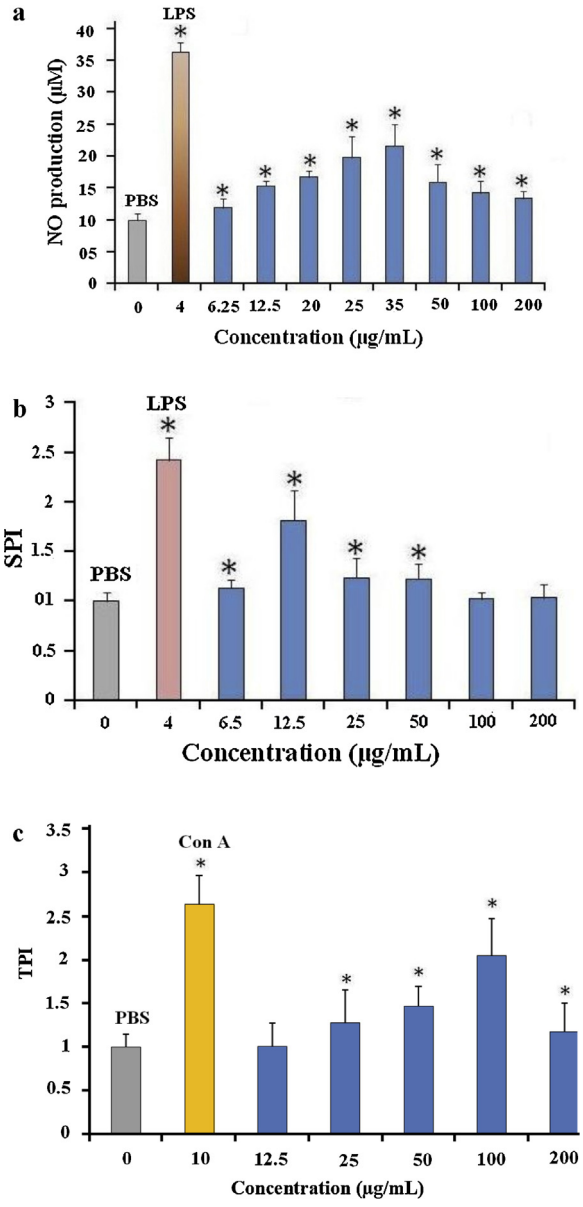


Fig. 4. (a) *In vitro* activation of macrophage stimulated with different concentrations of a glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička in terms of NO production. Effect of different concentrations of a glucan isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička on proliferation of splenocyte (b) and thymocyte (c). (* Significant compared to PBS control). Results are the mean ± SD of *n* separate experiments, each in triplicate (*n* = 9 for NO production, *n* = 7 for splenocytes, *n* = 6 for thymocytes).

activities of the polysaccharide after the optimum concentration may be due to insufficient activation signal at the cellular surface. This may happen due to various factors that include the type of cells, the number of receptors on the cells and the nature of the polysaccharide under investigation. Various physical factors of the stimulant (polysaccharide) such as the molecular weight, the number of branching side chains and their conformation in solution determine the extent of their biological activity (Bohn & BeMiller, 1995; Ohno, Miura, Nakajima, & Yadomae, 2000). From these findings, it can be concluded that 35 μg/mL, 12.5 μg/mL and 100 μg/mL are the optimum concentration of ELPS for macrophage, splenocytes and thymocytes proliferation respectively.

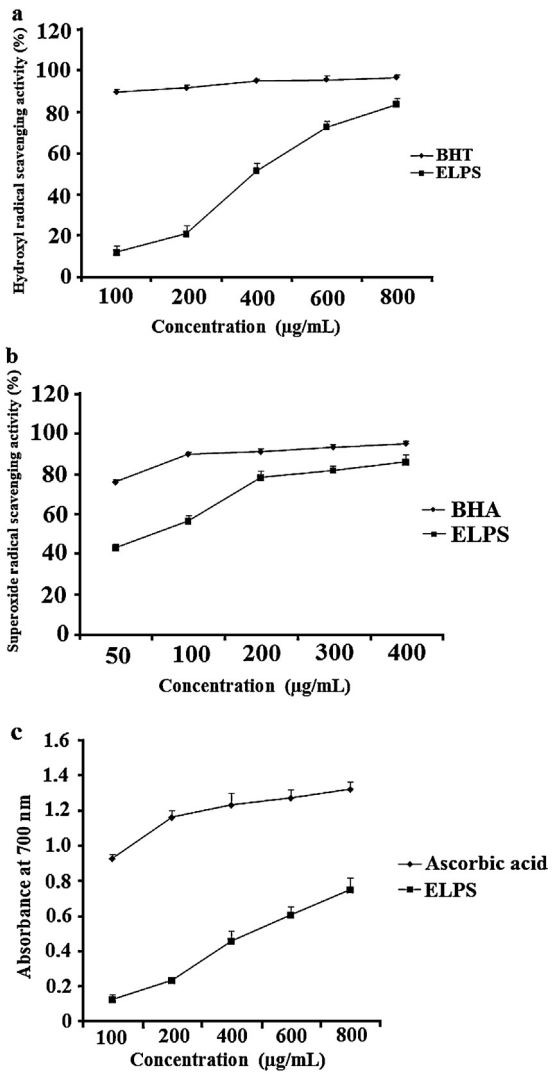


Fig. 5. (a) Hydroxyl radical scavenging activity of the ELPS isolated from the *Entoloma lividoalbum* (Kühner & Romagn) Kubička. (b) Superoxide radical scavenging activity of the ELPS isolated from the *Entoloma lividoalbum* (Kühner & Romagn) Kubička. (c) Determination of reducing power of the ELPS isolated from the *Entoloma lividoalbum* (Kühner & Romagn) Kubička. Results are the mean ± SD of three separate experiments, each in triplicate.

3.4. Antioxidant properties

3.4.1. Assay of hydroxyl radical scavenging activity

The hydroxyl radicals are most toxic among all reactive oxygen species (ROS) and can easily cross cell membranes at specific sites, react with most biomolecules and furthermore cause tissue damage and cell death (Aruoma, 1998). It is therefore important to remove the hydroxyl radicals for protection of the biological systems (Yang, Guo, & Yuan, 2008). Hydroxyl radical scavenging activity of ELPS was measured at different concentrations (100–800 μg/mL) taking butylated hydroxytoluene (BHT) as positive control. These results indicated that the activity of the ELPS gradually increases with the increase of concentrations (Fig. 5a). The hydroxyl radical scavenging activities of ELPS and BHT were respectively 20.48% and 91.2% at a dose of 200 μg/mL, indicating that antioxidant activity of ELPS is weak compared to BHT. The IC_{50} value of the ELPS was found to be 400 μg/mL (Fig. 5a). The antioxidant mechanism may be due to the supply of hydrogen by the polysaccharide, which combines with radicals and forms a stable radical to terminate the radical chain reaction.

3.4.2. Assay of superoxide radical scavenging activity

Superoxide radicals are harmful to cellular components as they are the precursor of several reactive species (Halliwell & Gutteridge, 1989). These are considered as the primary ROS which can further interact with other molecules to generate secondary ROS such as hydroxyl radicals, hydrogen peroxide, and singlet oxygen. As a result, the formation of superoxide radical could induce oxidative damage in lipids, proteins, and DNA (Stief, 2003; Wickens, 2001). The superoxide radical scavenging activities of ELPS and butylated hoydroxyanisole (BHA) were determined to be 56.58% and 89.88%, respectively at the dose of 100 μg/mL (Fig. 5b). At all concentrations, ELPS showed lower superoxide anion scavenging activity than synthetic standard drug BHA. The IC_{50} value of the ELPS was found to be 75 μg/mL.

3.4.3. Determination of reducing power

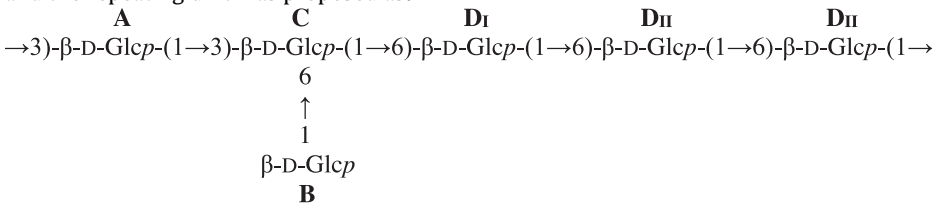
The reducing capacity of a compound is regarded as the indicator of its potential antioxidant activity (Chanda & Dave, 2009). It is measured in terms of the absorbance at 700 nm of the reaction mixture (Maity, Samanta, et al., 2014) containing different concentrations of ELPS. Fig. 5c shows the reducing power of ELPS and ascorbic acid increased with increasing sample concentration. The reducing power (absorbance at 700 nm) of ELPS and ascorbic acid were 0.2285 and 1.16 at 200 μg/mL respectively. At concentration of 470 μg/mL, ELPS showed reducing power 0.5. This result suggests that ELPS has potency to donate electron to reactive free radicals, converting them into more stable non-reactive species and terminating the free radical chain reaction.

In the present study, ELPS showed immunostimulatory effects through the production of reactive nitrogen species which are strong oxidants and on the other hand these also behave as antioxidants. The explanation to such paradoxical observation may be referred to the dual function of ELPS. It has been established that the polysaccharides bind to beta-glucan receptors and exhibit immunomodulatory effects during macrophage activation through the production of reactive nitrogen species. The antioxidant ability of the carbohydrates depends on monosaccharide constituents and the scavenging activity increases when they are in polymeric forms. In the present manuscript, optimum immunostimulating effects of ELPS were observed in cell culture medium at lower concentrations (12.5 μg/mL for splenocyte proliferation and 35 μg/mL for NO production during macrophage activation). Antioxidant properties were studied using chemical methods which showed the activities were maximum at higher concentrations (800 μg/mL for hydroxyl radical scavenging and reducing power and 400 μg/mL for superoxide radical scavenging). These results confirmed the low antioxidant activity of ELPS in comparison to other classical scavengers. Thus, the weak scavenging activities of ELPS obtained from chemical methods cannot be correlated to its immunomodulatory effects studied in cell culture medium. Similar observations were also reported by Tsiapali et al. (2001).

4. Conclusion

The structure of a water soluble β-glucan (ELPS) isolated from the aqueous extract of an edible mushroom *E. lividoalbum* (Kühner

& Romagn) Kubička was established by chemical and NMR studies and the repeating unit was proposed as:



ELPS showed macrophage activation in a dose-dependent manner with optimum production of NO at 35 μg/mL. Splenocyte and thymocyte proliferation indices were found maximum at the ELPS dosages of 12.5 μg/mL and 100 μg/mL respectively. Thus, from the above results ELPS can be considered as a potent immunostimulating agent. Moreover, the hydroxyl and superoxide free radical scavenging activity showed that the ELPS has IC_{50} value of 400 μg/mL and 75 μg/mL respectively and also the reducing power was found 0.5 at concentration of 470 μg/mL. Therefore, the polysaccharide of the edible mushroom *E. lividoalbum* should also be explored as a natural antioxidant for use in functional foods or medicine.

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A partially methylated mannogalactan from hybrid mushroom *pfle 1p*: purification, structural characterization, and study of immunoactivation

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Immunostimulation

ABSTRACT

A new water-soluble heteropolysaccharide (PS-II) with apparent molecular weight ~1.65 × 10^5 Da, was isolated from the fruiting bodies of hybrid mushroom *pfle 1p* by hot aqueous extraction. It is composed of D-mannose, D-galactose, and 3-*O*-Me-D-galactose in a molar ratio of 1.0:0.99:1.1. The structural investigation of PS-II has been carried out using acid hydrolysis, methylation analysis, periodate oxidation study, and 1D/2D NMR experiments. Based on the results of these experiments, it was established that PS-II contained a main chain of (1 → 6) linked α-D-galactopyranosyl residues, one of which was substituted at C-2 by a terminal mannopyranosyl residue and also methylated at C-3 position. This heteropolysaccharide (PS-II) exhibited macrophage activation by NO production as well as in vitro splenocyte and thymocyte stimulation.

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1. Introduction

Mushroom polysaccharides, especially β-glucans are best known for their antitumor and immunostimulating properties.[1,2] Lentinan, a (1 → 3)-, (1 → 6)-β-glucan isolated from *Lentinula edodes* has been found to possess prominent antitumor activity.[3,4] Different types of glucans from *Pleurotus florida* also exhibit immunomodulating properties.[5,6] Besides glucans, other polysaccharides such as galactans, fucans, xylans, and mannans isolated from different mushrooms also exhibit significant biological activities.[7] Several studies have been carried out on heterogalactans such as mannogalactan,[8] fucogalactan,[9] fucomannogalactan,[10] and fucoglucogalactan.[11] The heterogalactans are mostly composed of a main chain of (1 → 6) linked-α-D-Gal*p* with mainly fucose or mannose as non-reducing residues; although there are a few reports on the biological activities of the heterogalactans.[10,12] In addition to non-methylated heterogalactans, there are also several reports on partially O-methylated heterogalactans isolated from mushrooms.[13–16] Protoplast fusion between the strains of *Pleurotus florida* and *Lentinula edodes* produced nine new hybrid strains adopting the procedure as applied earlier,[17] out of which six strains, that is, *pfle 1o*, *pfle 1p*, *pfle 1q*, *pfle 1r*, *pfle 1s*, and *pfle 1v* produced fruit bodies. Recently, a (1 → 3)-, (1 → 6)-β-D-glucan has been isolated from the hybrid strain *pfle 1r*.[18] Two water soluble polysaccharides (PS-I and PS-II) were isolated from the fruit bodies of *pfle 1p*. PS-I[19] was found to consist of D-glucose, D-galactose, and D-mannose in a molar ratio of nearly 4:2:1 and PS-II consisted of mannose, 3-*O*-Me-galactose, and galactose in a ratio of nearly 1:1:1. In the present work, the detailed structural investigation and study of immunostimulating properties of PS-II was carried out and reported herein.

2. Results and discussion

2.1. Isolation, purification, and chemical analysis of PS-II

Water soluble crude polysaccharide (25 mg) obtained from the hybrid mushroom *pfle 1p* yielded two polysaccharides after fractionating through Sepharose 6B column. Fraction I (test tubes, 15–23) and fraction II (test tubes, 29–35) were collected and freeze dried, yielding, PS-I (11 mg) and PS-II (8 mg), respectively. The PS-II showed specific rotation $[\alpha]_D^{28.6}$ +54.7 (*c* 0.91, water). The apparent molecular weight[20] of PS-II was estimated as ~1.65 × 10^5 Da from a calibration curve prepared with standard dextrans. The sugar analysis of PS-II by paper chromatography[21] and GLC analysis of alditol

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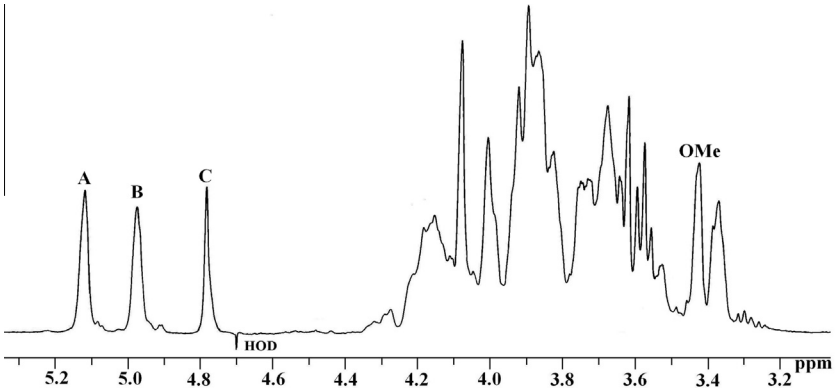


Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 30°C) of the PS-II isolated from hybrid mushroom (*pfle 1p*) of *Pleurotus florida* and *Lentinula edodes*.

acetates[22] of hydrolyzed product of PS-II confirmed the presence of mannose, galactose, and 3-*O*-methyl-galactose almost in a ratio of 1.0:0.99:1.1. The absolute configuration[23] of all the sugar residues were determined as D. The mode of linkages of the sugar moieties present in the PS-II was determined by methylation analysis using the Ciucanu and Kerek method[24] followed by hydrolysis and alditol acetate conversion. The GLC–MS analysis of partially methylated alditol acetates of PS-II revealed the presence of 1,5-di-*O*-acetyl-2, 3,4,6-tetra-*O*-methyl-D-mannitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol, and 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-D-galactitol in a ratio of nearly 1:1:1. Thus, PS-II was assumed to consist of terminal D-mannopyranosyl, (1 → 6)-galactopyranosyl and (1 → 2,6)-D-galactopyranosyl moieties, respectively. These linkages were further confirmed by periodate oxidation experiment. The GLC–MS analysis of the alditol acetates of periodate oxidized-reduced,[25,26] and methylated[27] PS-II showed the presence of only 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-D-galactitol. These results clearly indicated that terminal D-mannopyranosyl and (1 → 6)-linked D-galactopyranosyl residues were consumed during oxidation while (1 → 2,6)-linked D-galactopyranosyl residue was unaffected by periodate since the C-3 position of the branched galactopyranosyl residue was already occupied by the –*O*Me group. Hence, the mode of linkages in the PS-II was confirmed.

2.2. NMR and structural analysis of PS-II

The ^{1}H NMR spectrum (Fig. 1) showed three peaks in the anomeric region. The peaks were observed at δ 5.12, 4.98, and 4.78 ppm in a ratio of nearly 1:1:1. Rest of the sugar protons were observed in the region of δ 3.37–4.18 ppm and one –OCH_3 group signal at δ 3.43 ppm. The anomeric peaks were designated **A**, **B**, and **C** according to their decreasing proton chemical shifts. In the ^{13}C NMR spectrum (Fig. 2a) three peaks appeared in the anomeric region at δ 101.7, 98.2 and 97.9 ppm in a ratio of nearly 1:1:1. The other carbon signals came in the region δ 79.1–61.1 ppm. In addition, there was a signal at δ 56.0 ppm, which was assigned for –OCH_3 signal. From HSQC spectrum (Fig. 3 and Table 1) anomeric proton signals at δ 5.12 (**A**), 4.98 (**B**), and 4.78 (**C**) ppm were correlated to the carbon signals at δ 98.2, 97.9 and 101.7 ppm respectively. The chemical shifts of –OCH_3 group (δ 3.43/56.0 ppm) were also assigned from HSQC spectrum. Rest of the ^{1}H and ^{13}C signals were assigned from DQF-COSY, TOCSY, NOESY, ROESY, 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. Among these three residues, residues **A** and **B** have coupling constant values of $J_{H\text{-}2,H\text{-}3}$ ~9 Hz and $J_{H\text{-}3,H\text{-}4}$ ~3.5 Hz and thus they are established as D-galactopyranosyl residues. The anomeric proton chemical shifts (δ 5.12 for **A** and δ 4.98 ppm for **B**) as well as the coupling constant values ($J_{H\text{-}1,H\text{-}2}$ ~3.1 and $J_{C\text{-}1,H\text{-}1}$ ~171 Hz) confirmed that both residues were present as α anomer. Similarly, from the coupling constant values and the chemical shift of the anomeric proton, residue **C** was established as β-linked (δ 4.78 ppm; $J_{H\text{-}1,H\text{-}2}$ ~0, $J_{C\text{-}1,H\text{-}1}$ ~161) mannopyranosyl moiety ($J_{H\text{-}3,\ H\text{-}4}$ ~7.5 Hz and $J_{H\text{-}4,H\text{-}5}$ ~10 Hz).

In case of residue **A**, the anomeric carbon signal appeared at δ 98.2 ppm. All the proton and carbon chemical shifts except C-2, C-3 and C-6 matched nearly with the standard values of methyl glycosides[28,29] where downfield shifts of C-2 (δ 77.0 ppm) and C-6

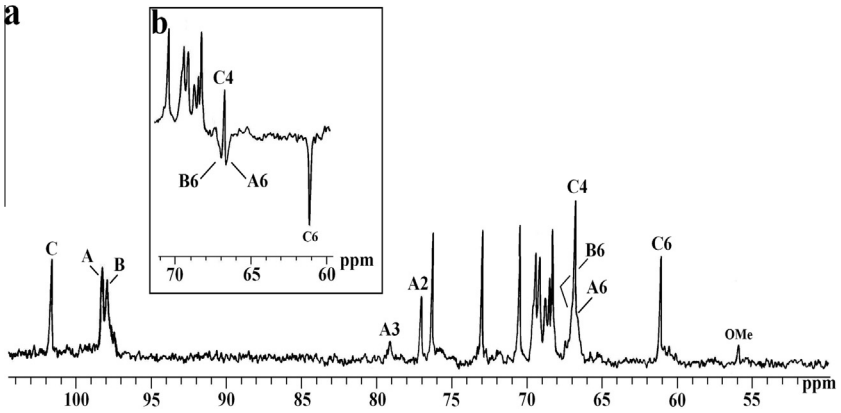


Figure 2. (a) ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C); (b) with insert of the part of DEPT-135 spectrum (D_2O, 30 °C) of the PS-II isolated from hybrid mushroom (*pfle 1p*) of *Pleurotus florida* and *Lentinula edodes*.

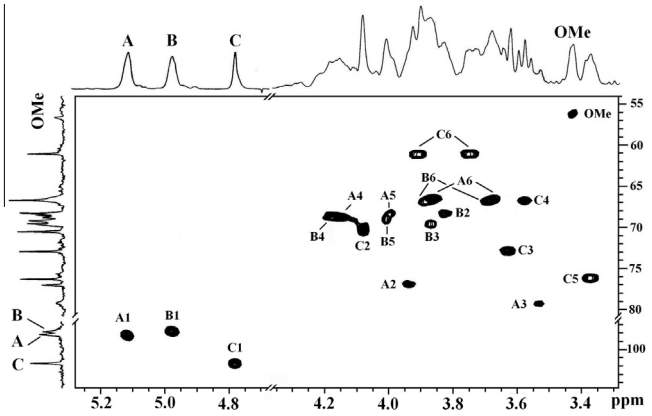


Figure 3. HSQC spectrum (D_2O, 30 °C) of the PS-II isolated from hybrid mushroom (*pfle 1p*) of *Pleurotus florida* and *Lentinula edodes*. Each cross-peak corresponds to a C–H pair.

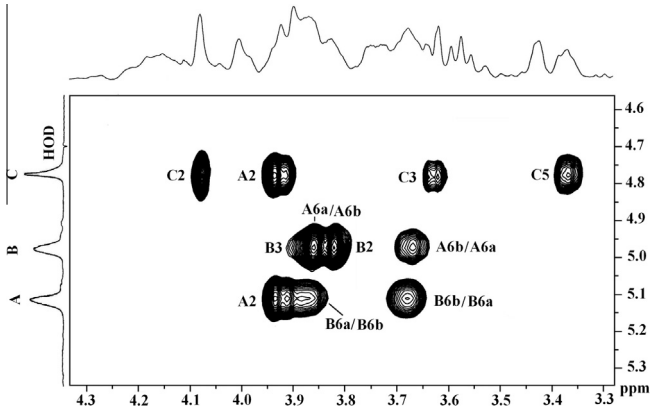


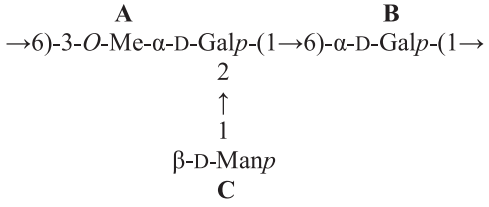
Figure 4. Part of the NOESY spectrum of the PS-II isolated from hybrid mushroom (*pfle 1p*) of *Pleurotus florida* and *Lentinula edodes*.

(δ 66.7 ppm) appeared. These observations confirmed that residue **A** was linked at C-2 and C-6. The linkage at C-6 was further confirmed from DEPT-135 spectrum (Fig. 2b). ^{1}H resonance of $-OCH_3$ correlated with C-3 (δ 79.1 ppm)[30] of residue **A** in the HMBC spectrum which confirmed that the $-OCH_3$ was located at C-3 position of residue **A**. Thus, **A** was confirmed as (1 → 2,6)-linked 3-*O*-methyl-α-D-galactopyranosyl residue.

Residue **B** had an anomeric carbon signal at 97.9 ppm. The downfield shift of C-6 (δ 67.0 ppm) of residue **B** with respect to standard methyl glycosides indicated that it was linked at C-6. The linkage at C-6 was further supported by DEPT-135 spectrum (Fig. 2b). Therefore, **B** was confirmed as (1 → 6)-linked α-D-galactopyranosyl residue. In case of residue **C**, anomeric carbon appeared at δ 101.7 ppm. All the proton and carbon chemical shifts of this residue corresponded nearly to the standard values of methyl glycosides of mannose. Thus, **C** was confirmed as terminal β-D-mannopyranosyl residue.

From these observations the repeating unit may consist of longer stretches of –A–A–A–A– and –B–B–B–B– (with **C** attached to **A**) but in that case the electronic environment of most of the **A** and **B** residues will be different and hence different chemical shift values would be observed both in ^{1}H and ^{13}C spectrum[31,32] but in the present NMR spectrum only three distinct anomeric proton peaks were observed which corresponded to the three anomeric carbon peaks as evident from HSQC spectrum (Fig. 3). Hence, this kind of combination is discarded and also established that only three sugar residues were present in the repeating unit of the polysaccharide.

The different linkages that connected these three residues were determined from NOESY (Fig. 4 and Table 2) as well as ROESY spectrum (not shown). In NOESY spectrum, the inter-residual contacts were observed between **A**H-1/**B**H-6a and **B**H-6b; **B**H-1/**A**H-6a and **A**H-6b; and **C**H-1/**A**H-2 along with other intra-residual contacts. Finally, these links were confirmed from HMBC spectrum (Fig. 5 and Table 3). In this spectrum the inter-residual cross-peaks (Table 3) were observed between **A**H-1/**B**C-6; **A**C-1/**B**H-6a and **B**H-6b; **B**H-1/**A**C-6; **B**C-1/**A**H-6a and **A**H-6b; **C**H-1/**A**C-2; and **C**C-1/**A**H-2 along with other intra-residual contacts. Thus, the monosaccharide composition, methylation studies, and NMR experiments indicated that the repeating unit of the PS-II has a backbone consisting of one (1 → 6)-α-D-galactopyranosyl and (1 → 6)-α-D-3-*O*Me-galactopyranosyl residues, out of which (1 → 6)-α-D-3-*O*Me-galactopyranosyl residue was branched at C-2 position with terminal β-D-mannopyranose residue and the structural motif present in the polysaccharide, PS-II was established as:



2.3. Immunostimulating properties

2.3.1. Assay for macrophage activity by NO

Mushroom polysaccharide function as immunostimulator by activating the macrophages.[2] Macrophages are white blood cells which play key roles in immune defense system. They usually engulf and destroy pathogens by phagocytosis. To test immunomodulatory effect of PS-II, murine macrophage was incubated with PS-II in a humidified atmosphere of 5% CO_2 at 37 °C for 48 h and

Table 1
The ^{1}H and ^{13}C NMR chemical shifts for the PS-II isolated from *pfle 1p* [a,b] 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	*O*Me
→2,6)-3-*O*-Me-α-D-Gal*p*-(1→	5.12	3.94	3.53	4.15	3.99	3.86[c], 3.67[d]	3.43
A	98.2	77.0	79.1	68.5	68.3	66.7	56.0
→6)-α-D-Gal*p*-(1→	4.98	3.83	3.87	4.18	4.01	3.88[c], 3.68[d]	
B	97.9	68.3	69.5	68.8	69.2	67.0	
β-D-Man*p*-(1→	4.78	4.08	3.63	3.57	3.37	3.90[c], 3.75[d]	
C	101.7	70.5	73.0	66.8	76.3	61.1	

[a] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.70 ppm at 30 °C.
[b] Values of the ^{13}C chemical shifts were recorded with reference to acetone as the internal standard and fixed at δ 31.05 ppm at 30 °C.
[c,d] Interchangeable.

the production of nitric oxide (NO) was measured. On treatment with different concentrations of this PS-II, it was observed that 49% to 54% of NO production increased up to 50 μg/mL. This was further increased by 70% at 100 μg/mL, but decreased at 200 μg/mL (Fig. 6a). Hence, the effective dose of this PS-II was observed at 100 μg/mL with optimum production of 10.2 μM NO per 5×10^5 macrophages.

2.3.2. Splenocyte and thymocyte proliferation assay

Splenocytes are the cells present in the spleen that include T cells, B cells, dendritic cells, and macrophages that stimulate the immune response in living organism. Thymocytes are hematopoietic cells in thymus which generate T cells. Proliferation of splenocyte and thymocyte is an indicator of immunostimulation.[33] The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with the PS-II by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method.[34] The PS-II was tested to stimulate splenocytes and thymocytes and the results are shown in Figure 6b and c, respectively. The splenocyte and thymocyte proliferation indices as compared to PBS control if closer to 100 or below indicate low stimulatory effect on immune system. The asterisks on the columns indicated the statistically significant differences compared to phosphate buffer saline (PBS) control if P <0.05. Both the splenocyte and thymocyte proliferation indices were found maximum at 50 μg/mL, above and below which it decreases. Hence, it can be concluded that 50 μg/mL is the optimum concentration of the PS-II for splenocyte and thymocyte proliferation.

Mushroom polysaccharides have been recognized as immunostimulators which are capable of interacting with the immune system to upregulate or downregulate the host immune response. They affect different cell types involving hematopoietic stem cells, innate (nonspecific) and adaptive (specific) immune systems and cytokine networks and signaling pathways and the activities also depend on their mechanism of action or the site of activity.[35] These studies like NO production by macrophages, splenocyte, and thymocyte proliferations are preliminary steps to explore whether the PS-II has any immunomodulating property or not. However, further studies are needed in order to identify the specific cells or cytokine pathway through which the PS-II exerts its action.

Table 2
NOESY data for the PS-II isolated from hybrid mushroom *pfle 1p*

Glycosyl residue	Anomeric proton	NOE contact protons	
	δ	δ	Residue, atom
→2,6)-3-*O*-Me-α-D-Gal*p*-(1→	5.12	3.94	**A** H-2
A		3.88	**B** H-6a
		3.68	**B** H-6b
→6)-α-D-Gal*p*-(1→	4.98	3.83	**B** H-2
B		3.87	**B** H-3
		3.86	**A** H-6a
		3.67	**A** H-6b
β-D-Man*p*-(1→	4.78	4.08	**C** H-2
C		3.63	**C** H-3
		3.37	**C** H-5
		3.94	**A** H-2

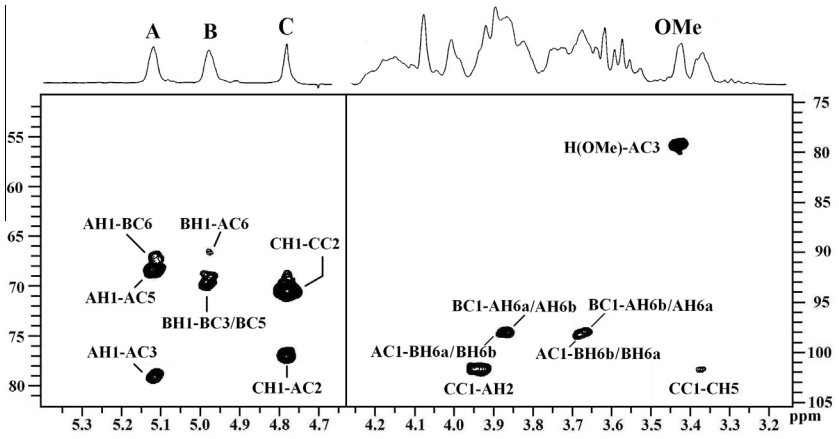


Figure 5. Part of the HMBC spectrum of the PS-II isolated from hybrid mushroom (*pfle 1p*) of *Pleurotus florida* and *Lentinula edodes*.

Table 3
The significant $^3J_{H,C}$ connectivities observed in an HMBC spectrum for the protons/carbons of the sugar residues of the PS-II isolated from hybrid mushroom *pfle 1p*

Residue	Sugar linkage	H/C	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	→2,6)-3-*O*-Me-α-D-Gal*p*-(1→	5.12	79.1	**A**	C-3
		(H-1)	68.3	**A**	C-5
		98.3	67.0	**B**	C-6
		(C-1)	3.88	**B**	H-6a
		3.43	3.68	**B**	H-6b
		H (*O*-Me)	79.1	**A**	C-3
B	→6)-α-D-Gal*p*-(1→	4.98	69.5	**B**	C-3
		(H-1)	69.2	**B**	C-5
		97.9	66.7	**A**	C-6
		(C-1)	3.86	**A**	H-6a
			3.67	**A**	H-6b
C	β-D-Man*p*-(1→	4.78	70.5	**C**	C-2
		(H-1)	77.0	**A**	C-2
		101.7	3.37	**C**	H-5
		(C-1)	3.94	**A**	H-2

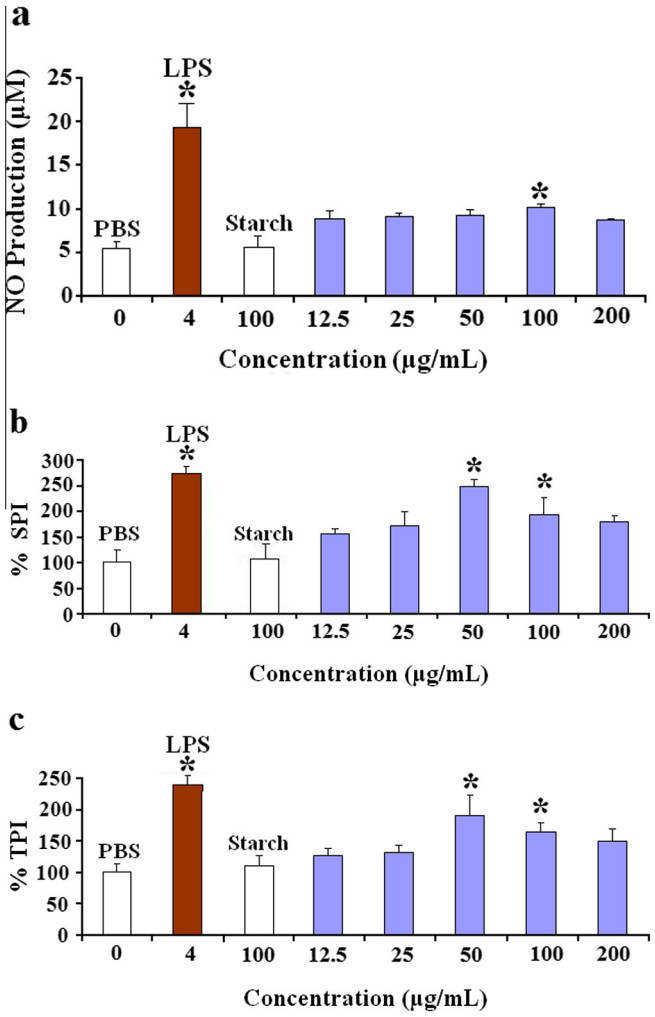


Figure 6. (a) In vitro activation of macrophage stimulated with different concentrations of the PS-II isolated from hybrid mushroom (*pfle 1p*) of *Pleurotus florida* and *Lentinula edodes* in terms of NO production. Effect of different concentrations of the PS-II isolated from hybrid mushroom (*pfle 1p*) of *Pleurotus florida* and *Lentinula edodes* on; (b) splenocyte and; (c) thymocyte proliferation. ('*' means data is significant compared to the PBS control).

The comparative study of macrophage, splenocyte, and thymocyte activations of the present PS-II with other polysaccharides[19,36,37] obtained from hybrid mushrooms has been compared and shown in the Figure 7a–c.

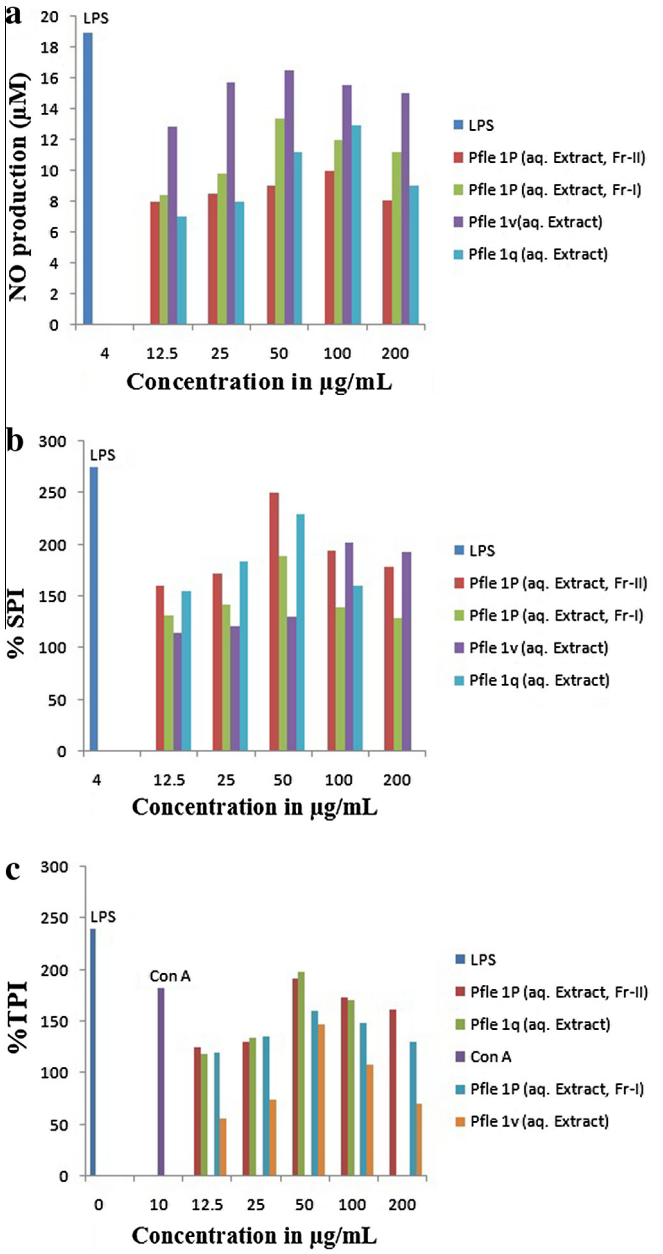


Figure 7. Comparative study of the (a) macrophage; (b) splenocyte, and; (c) thymocyte activations of the present polysaccharide with the other polysaccharides of hybrid mushrooms. In (c) thymocyte activation LPS was used as standard for *pfle 1p* (Fr-II) and *pfle 1q* and Con A was used as standard for *pfle 1p* (Fr-I) and *pfle 1v*.

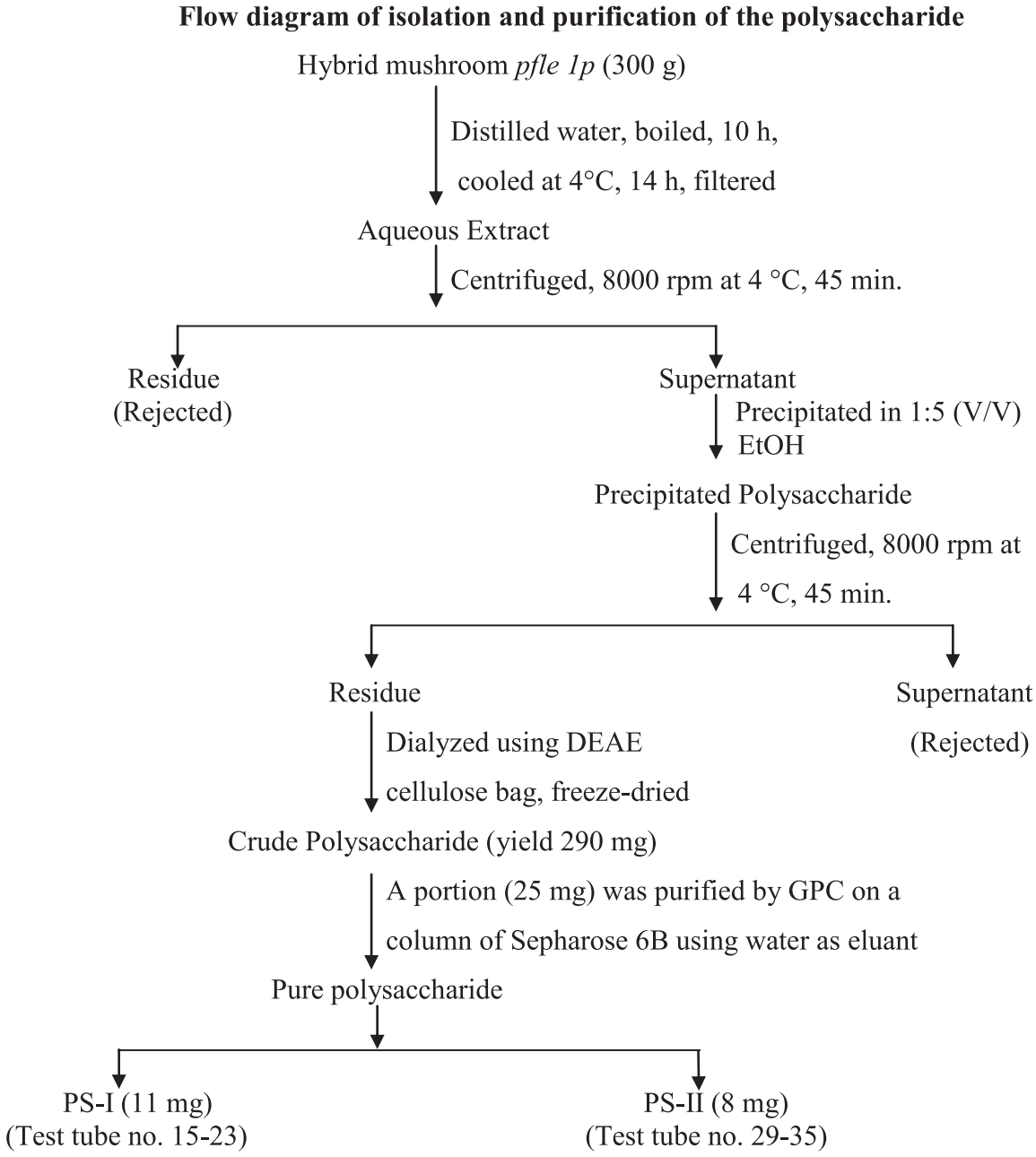
3. Materials and methods

3.1. Preparation of hybrid mushroom strain *pfle 1p*

The hybrid mushroom strain *pfle 1p* was produced through polyethyleneglycol (30% PEG, MW-3350)-mediated somatic protoplast fusion between *Pleurotus florida* and *Lentinula edodes*. Hybrid strains were selected based on double selection method and afterward maintained in Potato-Dextrose-Agar medium. Spawn of the hybrid strain was produced on paddy grain and mushroom was produced on paddy straw substrate.

3.2. Isolation, fractionation, and purification of the crude polysaccharide

The hybrid mushroom *pfle 1p* was cultivated and collected from Falta experimental farm, Bose Institute. The fruit bodies (300 g) were washed thoroughly with distilled water, followed by boiling with distilled water for 10 h. Isolation and purification process were carried out as reported in previous paper.[19] The crude polysaccharide (25 mg) was passed through Sepharose 6B gel permeation column (90 × 2.1 cm) using water as eluant with a flow rate of 0.4 mL/min. Ninety five test tubes were collected using Redifrac fraction collector and monitored spectrophotometrically at 490 nm with phenol-sulfuric acid reagent[38] using a Shimadzu UV–vis spectrophotometer, model-1601. Two fractions of purified polysaccharide, PS-I and PS-II were collected. The purification process was carried out in several lots. The fractionation and purification steps are shown below.



3.3. Monosaccharide analysis

PS-II (3.0 mg) was hydrolyzed with 2 mL (2 M) trifluoro acetic acid (CF_3COOH) in a round-bottom flask at 100 °C for 18 h in a water bath. The excess acid was completely removed by co-distillation with water. Then, the hydrolyzed product was divided into two parts. One part was examined by paper chromatography[21] in *n*-BuOH–AcOH–H_2O (v/v/v, 4:1:5, upper phase; X) and AcOEt–pyridine–H_2O (v/v/v, 8:2:1; Y). Another part was reduced with sodium borohydride ($NaBH_4$), 9 mg, followed by acidification with dilute acetic acid (CH_3COOH) and then co-distilled with pure methanol (CH_3OH) to remove excess boric acid. The reduced sugars were acetylated with 1:1 pyridine–acetic anhydride in a boiling water bath for 1 h to prepare the alditol acetates,[22] which were analyzed by GLC. A gas-liquid chromatograph Hewlett-Packard model 5730 A was used, with 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 analyses were performed at 170 °C. Quantization was carried out from the peak area, using response factors from standard monosaccharides.

3.4. Methylation analysis

PS-II (4.0 mg) was methylated using the procedure of Ciucanu and Kerek.[24] The methylated products were isolated by making a partition between chloroform and water (5:2, v/v) for four times. The organic layer containing products was collected and dried. The methylated product was then hydrolyzed with 90% formic acid (1 mL) at 100 °C for 1 h and excess formic acid was evaporated by co-distillation with distilled water. The hydrolyzed product was then reduced with sodium borohydride and acetylated with pyridine–acetic anhydride (1:1). The alditol acetates of methylated sugars were analyzed by GLC–MS. The Gas-liquid

chromatography–mass spectrometric (GLC–MS) analysis was performed on a 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.

3.5. Periodate oxidation

PS-II (5 mg) was added to 2 mL 0.1 M sodium metaperiodate solution and the mixture was kept in the dark for 48 h at room temperature. The excess periodate was destroyed by adding 1,2-ethanediol and the solution was dialyzed against distilled water for 2 h. The dialyzed material was reduced with sodium borohydride for 15 h and neutralized with acetic acid. The resulting material was dried by co-distillation with methanol. The periodate-oxidized material[25,26] was divided into two parts. One portion was hydrolyzed with 2 M CF_3COOH for 18 h and this hydrolyzed material was used for alditol acetate preparation as usual for GLC analysis. Another portion was methylated by the method of Ciucanu and Kerek,[24] followed by preparation of alditol acetates which were analyzed by GLC–MS.

3.6. Absolute configuration of monosaccharide

The absolute configuration of monosaccharide was determined by the method of Gerwig et al.[23] PS-II (1.0 mg) was hydrolyzed with CF_3COOH and then the acid was removed by co-distillation with water. 250 μL 0.625 M HCl solution treated with *R*-(+)-2-butanol was added and heated at 80 °C for 16 h. The reactants were evaporated and TMS (trimethylsilyl)-derivatives were prepared with *N,O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA). The products were analyzed by GLC using a capillary column SPB-1 (30 m × 0.26 mm) with a temperature program (3 °C/min) from 150 to 210 °C. The resulting (+)-2-butyl-2,3,4,6-tetra-*O*-TMS-glycosides were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides.

3.7. Optical rotation

Optical rotation was measured on a Jasco Polarimeter model P-1020 at 28.6 °C.

3.8. Determination of molecular weight

The average molecular weight of PS-II was determined by gel permeation chromatography.[20] Standard dextrans T-200, T-70, and T-40 were passed through a Sepharose-6B column and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of PS-II was plotted on the same graph and the average molecular weight of PS-II was determined.

3.9. NMR studies

PS-II was dried over P_2O_5 in vacuum for several days and then exchanged with deuterium[39] by lyophilizing with D_2O (99.96% atom ^2H, Aldrich) for four times. Then ^1H, ^{13}C, TOCSY, DQF-COSY, ROESY, NOESY, HSQC, and HMBC NMR spectra were recorded on a Bruker Avance DPX-500 spectrometer in D_2O at 30 °C. The ^1H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.70 ppm) using the WEFT pulse sequence.[40] The 2D-DQF-COSY experiment was carried out using standard Bruker software. The TOCSY experiment was recorded at mixing time of 150 ms and complete assignment required several TOCSY experiments requiring several mixing times ranging from 60 to 300 ms. The NOESY and ROESY mixing delay were 200 ms. The ^{13}C NMR experiments were carried out using acetone as internal standard, fixing the methyl carbon signal at δ 31.05 ppm. The delay time in the HMBC experiment was 80 ms.

3.10. Immunostimulating properties

3.10.1. Test for macrophage activity by nitric oxide assay

RAW 264.7, a murine macrophage cell line growing in Dulbecco's modified Eagle's medium (DMEM) was seeded in 96 well flat bottom tissue culture plate at a concentration of 5×10^5 cells/mL (180 μL).[41] Cells were left overnight for attachment and then incubated with different concentrations of PS-II (12.5, 25, 50, 100 and 200 μg/mL) in a humidified atmosphere of 5% CO_2 at 37 °C for 48 h. After 48 h of treatment, culture supernatant of each well was collected and NO content was estimated using Griess reagent (1:1 of 0.1% in 1-napthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid).[42] Lipopolysaccharide (LPS, L6511 of *Salmonella enterica* serotype typhimurium, Sigma, 4 μg/mL) was used as positive control and soluble starch (Merck, India, 100 μg/mL) as negative control.

3.10.2. Splenocyte and thymocyte proliferation assay

A single cell suspension of spleen and thymus was prepared from Swiss Albino mice under aseptic conditions by homogenization in Hank's balanced salt solution (HBSS).[43] The suspension was centrifuged to obtain cell pellet. The contaminating red blood cells were removed by hemolytic Gey's solution. After washing two times with HBSS, the cells were resuspended in complete Rose well Park Memorial Institute (RPMI) 1640 medium (Source- GIBCO, USA, Cat. No. 31800-022). Cell concentration was adjusted to 1×10^6 cells/mL and viability of splenocytes and thymocytes (as tested by trypan blue dye exclusion) was always over 90%. The cells (180 μL) were plated in 96 well flat bottom tissue culture plates and incubated with 20 μL of various concentrations of PS-II (12.5, 25, 50, 100 and 200 μg/mL). PBS (10 mM, phosphate buffer saline, pH-7.4) was taken as carrier control and soluble starch (Merck, India, 100 μg/mL) as negative control. Lipopolysaccharide (LPS, L6511 of *Salmonella enterica* serotype typhimurium, Sigma, 4 μg/mL) served as positive control. All cultures were set up at 37 °C for 72 h in a humidified atmosphere of 5% CO_2. Proliferation of splenocytes (% Splenocyte Proliferation Index or % SPI) and thymocytes (% Thymocyte Proliferation Index or %TPI) was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay method.[34,44] All the experiments were done twice with seven replicates and the data were reported as the mean ± standard deviation and compared against PBS control.[42,43]

4. Conclusion

A water soluble partially methylated mannogalactan was isolated from hot water extract of the fruit bodies of the hybrid mushroom *pfle 1p*. The polysaccharide was purified through Sepharose 6B gel-filtration. Sugar analysis, methylation studies together with 1D/2D NMR analyses confirmed that the polysaccharide contained a backbone of (1 → 6) linked α-D-galactopyranosyl and (1 → 6) linked α-D-3-*O*Me-galactopyranosyl residues with a terminal D-mannopyranose linked to C-2 of the partially methylated galactopyranosyl residue. The polysaccharide showed significant macrophage, thymocyte, and splenocyte activation and thus it can be concluded that this partially methylated mannogalactan can be used as a biological response modifier to combat microbial infection including tuberculosis, tumor cell eradication, and autoimmune diseases. Further biological studies are to be carried out to determine the mechanism of action.

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