STRUCTURAL AND BIOLOGICAL INVESTIGATION OF PLANT AND MUSHROOM POLYSACCHARIDES

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 family members"

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The research work presented in this thesis entitled "**Structural and Biological Investigation of Plant and Mushroom Polysaccharides**" has been carried out under my direct supervision and a bonafide research work of **Mr. Bibhash Chandra Panda**. This work is original and has not been submitted for any degree or diploma to this or any other University or Institution.

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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 and Dr. Soumitra Mondal, Assistant Professor, Panskura Banamali College, Panskura, Purba Medinipur, 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 "**Structural and Biological investigation of Plant and Mushroom Polysaccharides**" is divided into four chapters:

Chapter-I:

This Chapter describes the general introduction to carbohydrates, polysaccharides including plant and mushroom and their biological activities.

Chapter-II:

It represents the experimental methodologies which were adopted to carry out the work of the thesis.

Chapter-III:

This chapter is one of the major parts of this thesis, which describes the isolation, purification, structural characterization, immunoenhancing and antioxidant properties of the polysaccharide isolated from the green fruits of *Momordica charantia*, (Karela).

This work has been published in **Carbohydrate Research, 2015**, *401*, 24–31.

Chapter-IV:

This chapter is another part of the thesis which contains structural characterization and study of biological activities of the polysaccharide isolated from an edible mushroom, *Pleurotus cystidiosus*.

This work has been published in **International Journal of Biological Macromolecules**, **2017**, *95*, 833-842.

ABBREVIATIONS

Ac	Acetyl
Ac_2O	Acetic anhydride
AcOH	Acetic acid
ADCC	Antibody dependant cell mediated cytotoxicity
AIDS	Acquired immuno deficiency syndrome
BHA	Butylated hydroxyanisole
BRM	Biological response modifier
BSTFA	N,O-bis(trimethylsilyl)trifluroacetamide
CF_3CO_2H	Trifluoro acetic acid
CH_3COOH	Acetic acid
$CHCl_3$	Chloroform
CH_3I	Methyl iodide
CH_3OH	Methanol
cm	Centimeter
CO_2	Carbon dioxide
Con A	Concavalin A
CTL	Cytotoxic T-Lymphocyte
ºC	Degree centigrade
1 D	1-Dimensional
2 D	2-Dimensional
Da	Dalton
DEAE	Diethyl aminoethyl
DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxy ribo nucleic acid
DPPH	1,1-diphenyl-2-picryl hydrazyl
D_2O	Deuterium oxide

DQF-COSY	Double-quantum filtered correlation spectroscopy
EtOH	Ethyl alcohol
g	Gram
Gal	Galactose
GalA	Galacturonic acid
GalA6Me	Methyl galacturonate
Glc	Glucose
GLC	Gas-liquid chromatography
GLC-MS	Gas-liquid chromatography mass spectrometry
GPC	Gel permeation chromatography
h	Hour(s)
HCl	Hydrochloric acid
HCOOH	Formic acid
HDP	Host defence potentiator
HIV	Human immunodeficiency virus
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
H_2O	Water
HOAc	Acetic acid
HOD	Deuterated water
H_2SO_4	Sulphuric acid
HSQC	Hetronuclear single quantum coherence
Hz	Hertz
IL-1	Interlukine-1
IL-2	Interlukine-2
J	Coupling constants
KDa	Kilo Dalton
Kg	Kilogram
LPS	Lipopolysaccharide
M	Molar

MAF	Macrophage activating factor
Man	Mannose
Me	Methyl
MeI	Methyl iodide
CH_3OH	Methyl alcohol
mg	Milligram
MHz	Mega hertz
min	Minute(s)
mL	Mililiter
mm	Millimeter
mM	Millimolar
ms	Millisecond
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
$NaBH_4$	Sodium borohydride
NaCl	Sodium chloride
NaOH	Sodium hydroxide
$NaIO_4$	Sodium metaperiodate
$Na_2S_2O_3$	Sodium thiosulphate
NK cell	Natural killer cell
nm	Nanometer
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOE	Nuclear overhauser effect
NOESY	Nuclear overhauser enhancement spectroscopy
P	Pyranose
PBS	Phosphate buffered saline
P_2O_5	Phosphorus pentoxide
ppm	Parts per million

PS	Polysaccharide
PSK	Protein bound polysaccharide
RBC	Red blood cell
ROESY	Rotating frame overhauser enhancement spectroscopy
rpm	Rotation per minute
S 6B	Sepharose 6B
TFA	Trifluoroacetic acid
THF	Tetra hydro furan
TOCSY	Total correlation spectroscopy
UV	Ultra violet
vis	Visible
v/v	Volume by volume ratio
λ_{max}	Absorption maximum
µg	Microgram
µL	Microliter
µM	Micromolar

CONTENTS

ABSTRACT

The present thesis entitled "**Structural and Biological investigation of Plant and Mushroom Polysaccharides**" is divided into four chapters.

Chapter-I:

This chapter describes the general introduction to carbohydrates, polysaccharides including plant and mushroom and their biological activities. Polysaccharide chemistry is one of the branches of chemistry which stands at the interface of Organic Chemistry, Biochemistry and Polymer Chemistry. Carbohydrates are the integral constituents of all living organisms that are associated with a variety of vital functions which sustain life. These are the most abundant and diverse class of organic compounds occurring in nature.

Carbohydrates are the first group of bioorganic compounds which are also organic compounds found in biological systems. The structures of most bioorganic compounds are more complicated than the structures of many of the organic compounds. Carbohydrates are classified into four groups: monosaccharides, disaccharides, oligosaccharides and polysaccharides. Natural macromolecules containing carbohydrate units i.e. monosaccharides (the sugars) linked together by glycosidic bonds are known as polysaccharide. Actually polysaccharides are the complex carbohydrates.

Almost all living organism like fungi, plant, mushroom etc. produce polysaccharides. According to their structural features polysaccharides can be classified as: (1) homopolysaccharides which are constituted by one type of monosaccharide like starch, cellulose, dextran, glycogen etc. and (2) heteropolysaccharides which are composed of different monosaccharide units like xyloglucan, glucomannan etc.

In recent years polysaccharides from both mushroom and plant have drawn the attention of chemists and immunobiologists on account of their immunomodulatory, antitumor, antimicrobial and antioxidant properties. It has been observed that these bioactive polysaccharide exhibits anticancer activity mostly through the activation of immune cells such as B cells, Hep-2 cancer cells, macrophages etc. The antioxidant activities of polysaccharides are also closely related to their structural features such as

molecular weight, monosaccharide composition, glycosidic linkages, degree of branching, and polymerization.

Chapter II:

The methodologies that have been adopted for isolation, purification and determination of the structure of polysaccharides and their bioactivity studies have been discussed in this chapter. The biological activities of polysaccharides depend on the size of the molecule, molecular weight, linking sequences of the monosaccharide residues, branching pattern etc. So, it is very important to determine the exact structure of the repeating unit of the polysaccharides isolated either from the plant or the mushroom. The polysaccharide is purified using the techniques as revealed under. 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 methods consist of 1D (^{1}H, ^{13}C) and 2D NMR (DQF-COSY, TOCSY, NOESY, ROESY, HMQC, HSQC, HMBC etc), and Mass spectrometric (GLC-MS) experiment.

Different biological analysis was also carried out with different polysaccharide fractions. The macrophage 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. The antioxidant activity of polysaccharide was evaluated through the chelating ability of ferrous ions, hydroxyl and superoxide radical scavenging activity.

Chapter III:

This chapter describes the isolation, purification, structural characterization, immunoenhancing and antioxidant properties of the polysaccharide isolated from the green fruits of *Momordica charantia*, (Karela).

The fresh fruits of *M. charantia* (1.5kg) were washed with water, cut them into small pieces and boiled with distilled water for 10 h. The whole extract was kept overnight at 4

ºC and then filtered through linen cloth. The filtrate was then centrifuged at 8000 r.p.m. for 45 min at 4 ºC. The supernatant was precipitated in ethanol (1:5, v/v). The precipitated polysaccharide was collected through centrifugation, washed with ethanol, and freeze dried. The crude polysaccharide (1.416 g) was isolated. The polysaccharide (30 mg) was passed through Sepharose-6B column (90 cm x 2.1 cm) using distilled water as eluant with a flow rate of 0.5 mL min-1. A total of ninety test tubes were collected, monitored spectrophotometrically at 490 nm using Shimazdu UV-vis spectrophotometer, model – 1601 by phenol-sulphuric acid method. Single fraction (test tube no. 20 to 40) was obtained, collected yielding 16.2 mg pure polysaccharide (PS). The process was repeated several times to get 95 mg PS.

The specific rotation of the pure polysaccharide (PS) showed $[\alpha]_D^{29.8}$ +168.9 (*c* .098, H_2O). Molecular weight of the polysaccharide was found to be ~2.0 x 10^2 kDa, from a calibration curve prepared using standard dextran.

Structural analysis of PS

Paper chromatographic analysis of the hydrolyzed product of the PS showed the presence of galactose and galacturonic acid. The GLC analysis of the alditol acetates of the sugars showed the presence of galactose only. But the carboxyl-reduced polysaccharide on hydrolysis followed by GLC examination of the corresponding alditol acetates showed also the presence of only galactose. The absolute configurations of galactose and galacturonic acid was determined as D-cofiguration. The GLC-MS analysis revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol indicating that the D-galactopyranosyl moiety is present as non-reducing end in the polysaccharide. The carboxyl-reduced methylated polysaccharide on hydrolysis followed by GLC-MS analysis of corresponding alditols showed the presence of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol, 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-D-galactitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol in a molar ratio 3:1:1. The appearance of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol indicated the presence of (1→4)–linked D–galacturonic acid and 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-D-galactitol indicated (1→2,4)–linked D-galacturonic acid and the D-galactose is attached at the non reducing

end of the backbone of the polysaccharide. Thereafter, a periodate oxidation experiment was carried out with the polysaccharide. The periodate oxidized, reduced material on hydrolysis with trifluoroacetic acid followed by paper chromatography experiment showed the presence of D-galacturonic acid only. GLC-MS analysis of the alditol acetates of the periodate-oxidized, carboxyl-reduced methylated polysaccharide showed the retention of the peak corresponding to 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-D-galactitol, indicating further that D-galcturonic acid was retained as it was (1→2,4)-linked moiety and the non reducing end D-galactose and (1→4)-linked D-galacturonic acid were destroyed during periodate oxidation.

The ^{1}H NMR spectrum (500 MHz, 30ºC) of the PS showed three peaks in the anomeric region at δ 4.59, 4.94 and 5.06 and designated as **A**, **B**, and **C** respectively according to their increasing anomeric chemical shift values. In the ^{13}C NMR (125 MHz) spectrum three anomeric carbon signals appeared at δ 105.0, 100.8 and 100.0 were correlated to the residues **A**, **B** and **C** respectively from the HSQC spectrum. All the ^{1}H and ^{13}C signals were assigned using DQF–COSY, TOCSY and HSQC NMR experiments.

The sequence of glycosyl residues of the polysaccharide was determined on the basis of NOESY as well as ROSEY experiments, followed by confirmation with HMBC experiment. In NOESY experiment, the inter-residual contacts **A**H-1/**C**H-2; **B**H-1/**C**H-4; **C**H-1/**B**H-4 along with other intra-residual contacts were also observed. Thus from NOESY experiment the following sequences are established: A (1→2) **C**; B (1→4) **C**; **C** (1→4) **B.**

These sequences were further confirmed by ^{13}C-^{1}H correlation in HMBC spectrum. Inter residual cross peaks **A**H1/**C**C2, **A**C1/**C**H2; **B**H1/**C**C4, **B**C1/**C**H4; **C**H1/**B**C4 and **C**C1/**B**H4 along with other intra residual peaks were also observed. Therefore, based on the results obtained from monosaccharide composition, methylation studies and NMR experiments the following repeating unit of the pectic polysaccharide of *Momordica charantia* was assigned as:

B **C**
[→4) -α-D-Gal*p*A6Me-(1]$_3$→4)-α-D-Gal*p*A6Me-(1→
2
↑
1
β-D-Gal*p*
A

Immunostimulating properties of the PS

Macrophage activation by polysaccharide was observed in vitro. On treating different concentrations of polysaccharide, an enhanced production of NO was observed in a dose dependent manner with optimum production of 17 μM NO per 5 x 10^5 macrophages at 200 μg/mL. Hence, the effective dose of Polysaccharide was observed at 200 μg/mL.

Proliferation of splenocytes and thymocytes is an indicator of immunoactivation. The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with polysaccharide by the MTT assay method. Polysaccharide was tested to stimulate splenocytes and thymocytes and the asterisks on the columns indicate the statistically significant differences compared to PBS control. The splenocyte proliferation index (SPI) as compared to PBS control closer to 1 or below indicates low stimulatory effect on immune system. At 200 μg /mL of the PS, splenocyte proliferation index was found maximum as compared to other concentrations. Hence, 200 μg /mL of the PS can be considered as efficient splenocyte stimulator. Again 25 μg/mL of this same sample showed maximum effect on thymocyte proliferation.

Antioxidant properties of PS

Generation of reactive oxygen species beyond the body`s antioxidant capacity gives rise to oxidative stress. During this stress, hydroxyl radicals are mainly responsible for the oxidative injury of biomolecules since they can easily react with amino acids and DNA. It is also believed that they are the active initiators for lipid peroxidation. It is thus important to remove hydroxyl radicals for protection of the living systems.

Scavenging effects of the PS on hydroxyl radicals increased with increasing concentration. This shows percentage inhibition of hydroxyl radical generation by 0.1, 1, 2.5 mg/mL concentration of the PS and butylated hydroxyl anisole (BHA). Results indicated that PS have a noticeable effect on scavenging free radicals (EC_{50}= 2.22 mg/mL). Xu *et al* isolated three purified polysaccharides from flower of tea plant and all of them exhibited <25% hydroxyl radical scavenging activity at 4 mg/mL concentration. However, the scavenging effect of BHA was much higher than the PS (EC_{50}= <0.01 mg/mL). The results revealed that the PS was hydroxyl radical scavengers, acting possibly as primary antioxidant.

Cellular components such as polyunsaturated fatty acid residues of phospholipids are extremely sensitive to oxidation induced by reactive oxygen species. Major aldehyde products of this peroxidation method are malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE). HNE is thought to be highly toxic whereas malondialdehyde is carcinogenic in mammalian cell. Lipid peroxidation also results in breakdown of membrane integrity, affecting its fluidity and permeability. Therefore, inhibition capacity of lipid peroxidation is of great importance to show potential antioxidant activity.

Lipid peroxidation was prevented by the PS. The absorbance of malondialdehyde - thiobarbituric acid (MDA-TBA) complex was linearly decreased in a dose dependent path (0.2, 1, 2.5 mg/mL). The PS showed 50% lipid inhibition at 2.05 mg/mL, whereas the standard ascorbic acid showed much higher activity with EC_{50}= <0.1 mg/mL.

This paper has been published in **Carbohydrate Research, 2015**, *401*, 24–31**.**

Chapter-IV:

This chapter, another part of the thesis, contains structural characterization and study of biological activities of the polysaccharide, isolated from an edible mushroom, *Pleurotus cystidiosus*.

The fresh mushroom of *P. cystidiosus* (250 g) was washed, boiled, precipitated, centrifuged, dialyzed and freeze-dried to isolate the crude polysaccharide (1.414 g). The crude polysaccharide (30 mg) was passed through Sepharose-6B gel-permeation column,

single fraction (test tube nos. 22 to 45) of 20 mg pure polysaccharide (PCPS) was obtained. The process was repeated several times to get 120 mg of PCPS.

The PCPS showed specific rotation $[\alpha]_D^{30.8}$ +15.9 (*c* 0.10, water). Molecular weight of the polysaccharide was found to be ~1.46 x10^5 Da from a calibration curve prepared with standard dextran.

Structural analysis of PCPS

The GLC analysis of the alditol acetates of acid hydrolyzed PCPS showed the presence of glucose, galactose, and mannose in a molar ratio of nearly 6:2:1 respectively. The absolute configuration of the monosaccharides are determined as D-configuration. The GLC-MS analysis revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol; 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-D-mannitol; 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; 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol; 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-D-galactitol; 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a ratio of 1:1:2:2:1:1:1. These results indicated the presence of terminal glucopyranosyl, terminal mannopyranosyl, (1→3)-glucopyranosyl, (1→6)-glucopyranosyl, (1→6)-galactopyranosyl, (1→2,6)-galactopyranosyl, and (1→3,6)-linked glucopyrnosyl moieties. GLC-MS analysis of the alditol acetates of the periodate-oxidized methylated PCPS 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 2:1. 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 PCPS.

The ^{1}H NMR spectrum (500 MHz) of the PCPS at 30 ºC showed four broad peaks which represent nine ^{1}H resonances in the anomeric region at δ 5.11, 5.09, 4.97, 4.96, 4.77, 4.74, 4.52, 4.48, and 4.46 and designated as **A, B, C, D, E, F, G, H,** and **I** respectively according to their decreasing proton chemical shift values. In the ^{13}C NMR (125 MHz) spectrum at the same temperature, nine peaks appeared in the anomeric region at δ 102.8, 102.6, 102.3, 102.2, 101.8, 101.5, 98.2, 98.0, and 97.6. Based on the result of

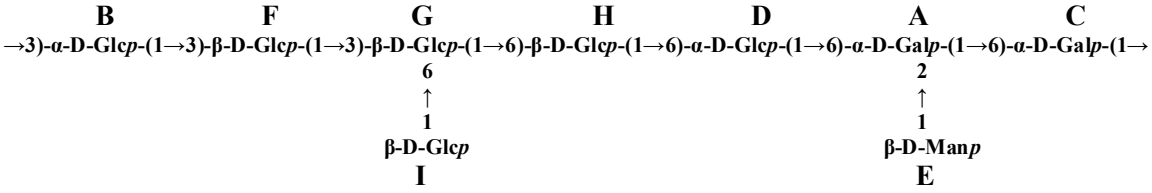
HSQC spectrum, the anomeric carbon signals at δ 102.8, 102.6, 102.3, 102.2, 101.8, 101.5, 98.2, 98.0, and 97.6 corresponded to the proton signals of δ 4.46 (**I**), δ 4.74 (**F**), δ 4.48 (**H**), δ 4.52 (**G**), δ 4.77 (**E**), δ 5.09 (**B**), δ 5.11 (**A**), δ 4.97 (**C**), and δ 4.96 (**D**) respectively.

The sequence of glycosyl residues (**A** to **I**) were established from ROESY as well as NOESY experiment. In ROESY experiment, the inter-residual contacts **A**H-1/**C**H-6a, 6b; **B**H-1/**F**H-3; **C**H-1/**B**H-3; **D**H-1/**A**H-6a, 6b; **E**H-1/**A**H-2; **F**H-1/**G**H-3; **G**H-1/**H**H-6a, 6b; **H**H-1/**D**H-6a, 6b; and **I**H-1/**G**-6a, 6b along with other intra-residual contacts were also observed. The above mentioned ROESY connectivity established the sequences as follows: **A** (1→6) **C**; **B** (1→3) **F**; **C** (1→3) **B**; **D** (1→6) **A**; **E** (1→2) **A**; **F** (1→3) **G**; **G** (1→6) **H**; **H** (1→6) **D;** and **I** (1→6) **G**.

Smith degraded product (SDPCPS) of PCPS was prepared and NMR experiments were again carried out to confirm the linkages of the heteroglycan. The ^{13}C NMR (125 MHz) spectrum at 30 ºC of SDPCPS showed three anomeric carbon signals at δ 103.2, 102.8, and 98.2. The anomeric carbon signals at δ 98.2 corresponded to α-D-Glc*p* (**J**), δ 103.2 corresponded to (1→3)-β-D-Glc*p* (**K**) and δ 102.8 corresponded to (1→3)-β-D-Glc*p* (**L**) residues respectively. The carbon signals at C-1, C-2, and C-3 of the glycerol moiety (Gro) were assigned as δ 66.4, 72.1, and 62.6 respectively. The glycerol moiety (**M**) was generated from (1→6)-β-D-Glc*p* (**H**) after periodate oxidation followed by Smith degradation which was attached to (1→3)-linked β-D-Glc*p* moiety (**L**) generated from (1→3,6)-β-D-Glc*p* (**G**). The terminal α-D-Glc*p* (**J**) of SDPCPS was generated from (1→3)-α-D-Glc*p* (**B**) and (1→3)-β-D-Glc*p* (**K**) was produced from unaffected residue (1→3)-β-D-Glc*p* (**F**). Thus, the Smith degradation results in the formation of a glycerol containing trisaccharide from the parent PCPS and the structure of which was established as:

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

These results further confirmed the presence of the repeating moiety in the heteroglycan (PCPS) isolated from the edible mushroom *Pleurotus cystidiosus* and the structure was proposed as:



Biological properties of the polysaccharide

The cytotoxic effect of the PCPS was studied on human blood lymphocytes with increasing concentrations ranging from 10 µg/mL to 400 µg/mL using 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) assay method. It was observed that PS has no considerable cytotoxic effect to normal lymphocytes up to 200µg/mL. But at higher dose 400µg/mL, the polysaccharide possesses mild levels of toxicity. It indicates that 200µg/mL is safe with respect to the other higher doses.

Glutathione is an important antioxidant in cellular system. Therefore, glutathione level in cell, both its reduced and oxidized states was measured. The reduced glutathione level (GSH) was increased significantly up to 200 µg/mL and the maximum level of increment was found at 200µg/mL. But at 400µg/mL, the reduced glutathione level (GSH) was moderately decreased and the mild augmentation of oxidized form of glutathione level (GSSG) was observed.

Study of lipid peroxidation is one of the important parameters to assess the cellular damage. It deactivates the cellular components and protective enzymes, and thereby plays a vital 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 malondialdehyde (MDA) release. The result showed no significant changes at different concentration except at 100 µg/mL, where it

decreases significantly. But it showed slightly increase of MDA level at the dose 400µg/mL.

It is evident from these experiments that, in vitro application of PCPS has good effect up to a certain level. It does not induce any cellular damage in lymphocytes associated with enhanced MDA level, GSSG level and decreased GSH level. The cytotoxic profile of PCPS in lymphocytes indicated 200µg/mL is safe and effective, whereas concentrations higher than 200 µg/mL showed slight increase of cytotoxicity. These findings suggest that the PCPS exhibits antioxidant and beneficial role on cellular system.

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CHAPTER 1

General introduction

1.1. Carbohydrates

Carbohydrates are considered as most abundant and essential organic molecules in the universe produced through photosynthesis, whose key role is to supply energy for living system. Carbohydrates are most essential constituents of all living organisms, and have a variety of different function. They play an important role in the structure of DNA and RNA and are also involved in cellular recognition processes. Carbohydrates contain of carbon (C), hydrogen (H) and oxygen (O) having the molecular formula $C_x(H_2O)_y$. Earlier, carbohydrates are called the hydrates of carbon because they contain hydrogen and oxygen in the same proportion as in water. But later structural studies revealed that these compounds are not hydrates since they do not contain any intact water molecules. It refers either to polyhydroxy aldehydes or polyhydroxy ketones. So, carbohydrates have been classified on the basis of their structures, not their formulas. Generally, carbohydrates are optically active compounds. The actual definition of the carbohydrate proposed by Robyt [1] is "Carbohydrates are polyhydroxy aldehydes or ketones or compounds that can be derived from them by oxidation, reduction or replacement of different functional groups".

1.1.1. Classification of carbohydrates

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

Monosaccharides are chiral carbohydrate with single unit of polyhydroxy aldehydes or ketones. They cannot be further hydrolyzed to a smaller one. They are usually colorless, crystalline and water soluble in nature. They are also called reducing sugar because they reduce Felling's and Tollen's reagents. Monosaccharides are called as trioses, tetroses, pentoses, hexoses etc. depending upon the presence of three, four, five or six numbers of carbon atom respectively [2]. A few examples of monosaccharides are glucose, mannose, galactose, fructose, ribose, and arabinose.

Disaccharides are composed of two monosaccharide units bound together by a covalent bond i.e. glycosidic linkage. It is the condensed product of two monosaccharides, formed by the elimination of water. According to the monosaccharide composition, disaccharides are of two types: homogeneous and heterogeneous. They can also be divided as reducing and non-reducing types depending upon whether they possess a free hydroxyl groups on anomeric carbon or not. The most important naturally occurring disaccharides are sucrose and lactose. Lactose and sucrose are made up of galactose and glucose, glucose and fructose respectively. Sucrose is the sweetest of the disaccharides. It is found in all plants and commercially obtained from sugar cane and sugar beets, where as the lactose is present in the milk of the mammals. Other examples of disaccharides are maltose and cellobiose.

Oligosaccharides are compounds that are consisting of three to ten monosaccharide residues joined together through glycosidic linkages. According to the number of monosaccharide units, they are called trisaccharides, tetrasaccharides, pentasaccharides etc. Such as maltotriose is composed of three glucose units whereas raffinose is formed by glucose, galactose, and fructose units. Stachyose is the example of tetrasaccharide.

Polysaccharides are considered as the first biopolymer on Earth [3]. These are macromolecules in which more than nine monosaccharides joined together by glycosidic linkages and on hydrolysis give the constituent monosaccharides or oligosaccharides. They have a general formula of $C_n(H_2O)_{n-1}$ where n is usually a large number between 200 and 2500. Polysaccharides may be linear or branched, with the exception of cyclic polysaccharide known as cycloamyloses. They may be amorphous or even insoluble in water [4]. The structural variation in combination with the heterogeneity complicates the structural determination of the polysaccharides. Certain structural features such as chain conformation and intermolecular hydrogen bonding influence the physicochemical properties of polysaccharides. Some of the polysaccharides, especially β-D-glucans containing large numbers of hydroxyl groups are often thought to be hydrophilic but they are hydrophobic due to greater number of internal hydrogen bonds. Polysaccharide shows important roles in biological recognition

processes [5], in development of diseases [6], and in many important areas like foods, pharmaceuticals, textiles, papers and biodegradable packaging materials [7].

1.1.2. Classification of polysaccharides

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

(i) Homopolysaccharides: Polysaccharides that are composed of only one kind of monosaccharide are known as homopolysaccharides e.g. starch, cellulose, glycogen, dextran etc.

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

Homopolysaccharides can further be divided to either α- or β- configuration by the types of glycosidic linkages between the monosaccharide units. Heteropolysaccharides can have the same kind of linkage diversity as homopolysaccharides.

According to their morphological location polysaccharides can also be divided into three major groups:

(i) Extracellular surface polysaccharides located outside the cell membrane.

(ii) Cell wall polysaccharide located on the cell wall.

(iii) Intracellular polysaccharides located inside the cytoplasmic membrane.

1.1.3. Structure and function of some common polysaccharides

➢ Storage polysaccharides

The polysaccharides which serve as energy storage in plants and animals are called storage polysaccharides. Starch, glycogen, inulin etc. are common storage polysaccharide.

❖ **Starch:** Starch is a polymer in which glucopyranosyl residues are joined by α-glycosidic linkages. They are water insoluble molecules. Starch generally contains 20-25% amylose and 75-80% amylopectin [8]. Amylose is a linear glucose polymer with α-(1→4) glycosidic linkages (**Fig. 1a and 1b**), whereas amylopectin is a branched glucan composed of (1→4)-α-D-Glc*p* and (1→4,6)-α-D-Glc*p* residues. Plants store glucose as amylose or amylopectin. Potato, rice, wheat, and maize (corn) are the major sources of starch in human diet.

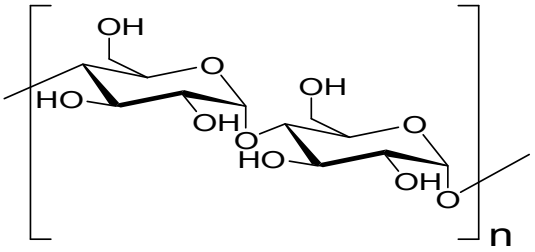

Fig. 1a. α-amylose

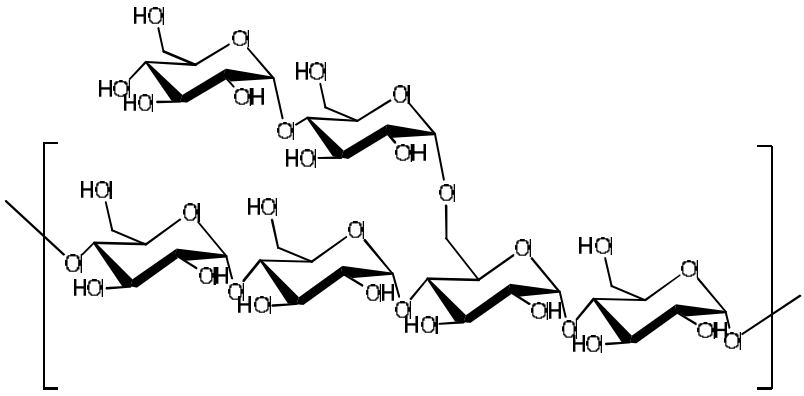

Fig. 1b: Amylopectin, α-(1→4), α-(1→6) glucose polymer

❖ **Glycogen:** Glycogen functions as energy storage in animals. It is a homopolysaccharide having similar structure to amylopectin with more extensive branching. It is stored in liver and muscles of animals, and called as 'animal starch'.

➢ Structural polysaccharides

The polysaccharides which have structural roles in plant cell wall or exoskeleton of insects are called structural polysaccharides. Such as cellulose, chitin, arabinoxylans etc.

❖ **Cellulose:** Cellulose is an unbranched chain polymer with about ten thousand β-(1→4)-linked-D-glucopyranosyl residues [9] in a chain (**Fig. 2**). It is a major constituent of plant cell walls [10]. It is odorless, hydrophilic, biodegradable, and insoluble in water and most organic solvents. Multiple hydroxyl groups on the glucose units form intra-chain and inter-chain hydrogen bonds that hold the chains firmly together side by side forming micro fibrils with high tensile strength. This strength confers rigidity to plant cell walls. About 33% of all plant matter is cellulose. Human beings and many other animals cannot digest cellulose due to lack of an enzyme that is able to break the β-linkages. Certain animals like cows, horses, sheep and goats are able to digest this polysaccharide due to presence of bacteria in their digestive tracts and active metabolic system.

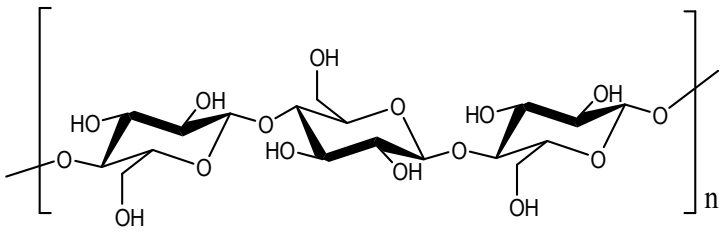

Fig. 2. Cellulose, a β-(1→4)-linked D-glucose

❖ **Chitin:** Chitin (**Fig. 3a**) is one of the most abundant naturally occurring polysaccharide materials in the world. It is composed of β-(1→4)-linked N-acetyl-D-glucosamine residues [11]. Removal of N-acetyl groups from chitin yields chitosan (**Fig. 3b**) which has improved solubility. It is the main constituent of exoskeletons of arthopods like crabs, lobsters, shrimps and insects. Chitosan is used in the treatment of patients of kidney failure, loss of strength and appetite, and trouble during sleeping (insomnia). In pharmaceutical industry, chitosan is used as filler in tablets as a carrier in controlled drug delivery. It is used as fertilizer and in water purification. Industrial separation membranes and ion-exchange resins can be made from chitin.

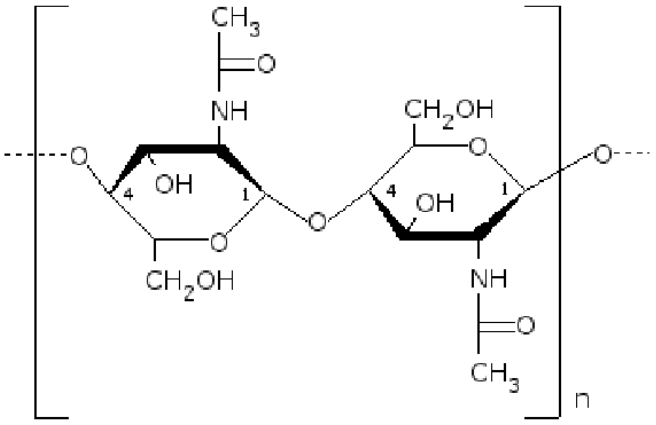

Fig. 3a. Chitin, a β-(1→4)-linked N-acetyl-D-glucosamine polymer

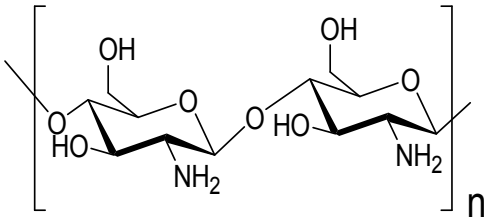

Fig. 3b. Chitosan, a β-(1→4)-linked D-glucosamine polymer

❖ **Arabinoxylan:** Arabinoxylan is a copolymers of two pentose sugars arabinose and xylose, are found in both primary and secondary cell walls of plants including woods and cereal grains [12]. They exhibit antioxidant activity [13], owing to their bound phenolic acids.

❖ **Heparin:** Heparin is an acidic mammalian polysaccharide consisting of highly sulfated (1→4)-linked hexosamine and uronic acid residues (**Fig. 4**). It is commonly extracted from animal tissues such as bovine lung and porcine intestine. It has a variety of biological functions including blood anticoagulation, mitogenesis, cell migration and anti-inflammation promotion of cell adhesion [14]. It helps to inhibit the clot formation in blood by the interaction with the protein antithrombin.

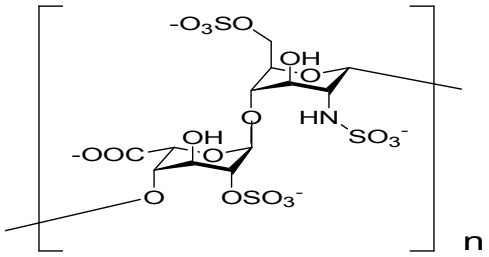

Fig. 4. Heparin, sulfated (1→4)-linked hexosamine and uronic acid.

➢ Pectic polysaccharides

❖ Pectin

Pectins are heterogeneous polysaccharides composed of galacturonic acid or its ester in the backbone (**Fig. 5**) was first isolated by Henri Braconnot [15]. They are important part of human diet but do not play a significant role to nutrition. The average molecular weight of pectins is in the order of 10^4-10^5 Daltons depending on fruit source [16]. Pectins are available in apples, guavas, quince, plums, gooseberries, oranges and other citrus fruits. Pectins are traditionally used as gelling and thickening agent for the

production of jams and jellies. It is also used in fillings, medicines, sweets, as a stabilizer in fruit juices and milk drinks, and as a source of dietary fiber. In fruits, pectin keeps the wall of adjacent cells joined together, helping them remain firm and hold their shape. Pectins possess anti-inflammatory and other pharmaceutical activities [17].The branching and acetyl groups on galacturonic acid may play an important role in bioactivities [18].

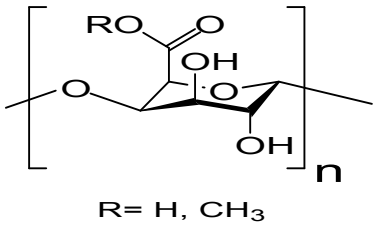

Fig. 5. Pectins contain α-(1→4)-linked D-galacturonic acid or its ester in the backbone

➢ Plant polysaccharides

Polysaccharides isolated from different fruits, vegetables and plants are now a matter of great interest to the chemist due to their diversity. Plant polysaccharides are mainly classified into neutral polysaccharides (e.g. arabinogalactan, dextran, gumguar, gumlocust bean, β-glucan, inulin, laminaran, mannan, starch etc) and acidic polysaccharides (carrageenan, dextran sulfate, gum ghatti, gum karaya, pectin etc.). Several plant polysaccharides such as neutral xyloglucans and glucuronic acid containing arabinogalactans or 4-*O*-methyl glucuronoxylans are capable to stimulate the macrophage functions [19]. These are very attractive source of additive for the food and drug industries because of their use in complementary medicinal supplement [20]. It has been observed that biologically active plant polysaccharides exhibit antioxidant [21], immunomodulatory as well as anti tumor activity [22,23]. Several plant polysaccharides isolated from *Aloe barbadensis* [24], fruit juice of *Morinda citrifolia* (noni) [25], *Morus alba, Chlamydomonas mexicana* and *Poria cocos* [26] exhibit immunomodulatory and antitumor activity. Several plant polysaccharides from *Moringa oleifera* (sajina) [27-30], *Cochlospermum religiosum* (Kartira gum) [31], *Capsicum annuum* [32], *Lycopersicon*

esculentum (tomato) [33], fruits of *Psidium guajava* (Guava) [34], *Lagenaria siceraria* [35], *Solenum melongena* (Bringal) [36], *Benincasa hispida* [37], leaves of *Catharanthus rosea* [38], seeds of *Caesalpinia bonduc* (Nata Karanja) [39], stems of *Amaranthus tricolor Linn. (Amaranthus gangeticus L.)* [40] etc. have already been isolated in our laboratory and reported. Isolation, chemical analysis, structural characterization, immune studies, and antioxidant properties of the polysaccharide from green fruits of *Momordica charantia*, (karela) is described in the present thesis.

1.2. Mushrooms - a miracle food

1.2.1. Definition and description

A mushroom is a fleshy spore bearing fruiting body of a fungus, typically produced above the ground on soil, on decomposing leaves, and in mulch and compost. Fungi are extraordinary organism, which are neither plants nor animals. The term 'mushroom' is defined by Chang and Miles [41] 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 up by hand". The fruiting body of mushroom is umbrella like with various shapes, size and color. Commonly, it consists of a cap or pileus and a stalk or stipe but others have additional structures like scale, volva, gills, tubes, pores, ring etc. The spores of the mushroom are called basidiospores, produced on the gills and fall in the form of powder from under the cap. The cap of the mushroom protects the spore produced in gills. The gills of the mushroom are thin and vertical. Stem is raised above the ground to disperse the spores. A mushroom and its various parts have been shown in **Fig. 6**. The cell wall of mushroom is encapsulated by an extracellular matrix consisting of rigid layer of glycoprotein and polysaccharide. The cell wall protects mushroom from osmotic pressure, environmental stress and also determines cell shape. A number of species of mushrooms are poisonous, although some are edible species. Wild mushrooms are risky to eat and should only be taken by knowing detailed information and identification. About 140,000 types of mushrooms are present on the earth, out of

which only around 10% are known. The 50% of the known species have varying degrees of edibility, more than 2,000 are safe, and about 700 species are known to possess significant pharmacological properties [42-44].

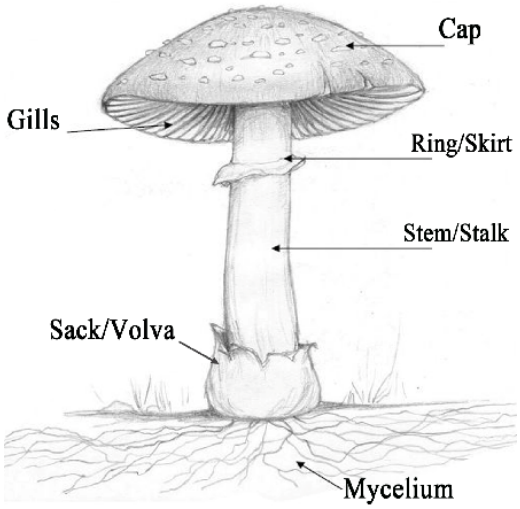

Fig. 6. A mushroom and its various parts [www.fungi4school.org]

1.2.2. Chemical composition and nutritional value

Mushrooms are valuable source of health food, edible and have medicinal resources. Mushrooms possess ~ 90% water by weight, and are very low in calories (only100 cal/oz). The rest 10% consists of 10-40% protein, 2-8% fat, 3-28% carbohydrate, 3-32% fiber and 8-10% ash including calcium, potassium, phosphorous, magnesium, iron, zinc and copper [45]. Most mushrooms contain some vitamins, particularly niacin, thiamine, riboflavin, biotin and vitamin C. Besides actual nutrients, mushrooms contain a wide variety of bioactive molecules including terpenoids, steroids, phenols, nucleotides, glycoproteins and polysaccharides.

Mushroom, which we use as tasty and nutritional food [46], is popularly known as miracle food. Mushrooms are being extensively used in many countries for food and fodder due to its flavor, taste and low-calorific value [47-50]. They are enriched with protein, minerals and vitamins but of low fat content. Presence of high protein content in

mushrooms is popularly known as “poor man’s protein” [51]. According to Robinson and Davidson [52], the efficiency of protein production from a given quantity of carbohydrates in mushrooms and other higher fungi is about 65% compared with about 20% for pork, 15% for milk, 5% for poultry, and 4% for beef. Chemical analyses have shown that the composition of amino and fatty acids, vitamins, minerals present in the commercially available edible mushrooms is comparable to meats and higher than fruits and vegetables. It contains large amount of well-balanced essential amino acids [53]. Nutritive values of mushrooms in 100 gm can be shown in **Table 1** [54].

Nutritive values of mushroom in 100 gm (dry weight)	
Vitamin B	Thiamine 0.10 mg
Riboflavin	0.44 mg
Niacin	4.9 mg
Vitamin C	5 mg
Calcium	9 gm
Iron	1 mg
Phosphorus	115 mg
Potassium	150 mg
Fat	0.3 gm
Carbohydrates	4 gm
Protein	2.4 gm
Calories	16

Table 1. Nutritive values of the mushroom

1.2.3. Mushroom polysaccharides

Mushrooms contain biologically active polysaccharides in fruit bodies, cultured mycelium and culture broth. Chemists and immunobiologists have been attracted by

mushroom polysaccharides for their immunomodulatory and anti-tumor properties [55, 56]. Mushrooms are potential source of different polysaccharides like chitin, hemicelluloses, glucans and heteroglycans. Among the various polysaccharides, β-glucans are the main active molecules. β-D-glucans are composed of D-glucose monomers and joined together by β-glycosidic linkages, such as (1→6)-β-D-glucan, (1→3)-, (1→6)-β-D-glucans, (1→3)-α-D-glucans, and some are true heteroglycans. (1→3)-β-D-glucans having a variety of biological and immuno-pharmacological properties, and used clinically as biological response modifiers (BRMs) [57]. The structures of the several biologically active linear and branched β-D-glucan have been reported. Glucans having α- or both α- and β- linkages are also isolated from different mushrooms [58-61].

1.3. Plant and mushroom polysaccharides- as immunomodulator and anti- tumor agents

A wide range of biologically active polysaccharides of different chemical structures from plants and mushrooms have been investigated. Different plant [22,23] and mushroom [62,63] polysaccharides possess immunomodulatory and anti-tumor properties. Mushrooms are source of physiologically beneficial and non-toxic medicines [43]. Immunological properties of polysaccharide were first reported by Dochez and Avery [64]. Polysaccharides are used currently in the treatment of cancer of the digestive organs, lung and breast, as well as cancer of the stomach and cervical cancer respectively. Mushroom polysaccharides are known to possess not only anti cancer activity but also anti-inflammatory, anti-viral (against AIDS), hypoglycemic and anti-thrombotic activities [65]. Plant polysaccharides showed both anti-genotoxic and anti-tumor activities in *in vitro* models [66]. Several polysaccharides are widely used and commercialized worldwide as anti cancer agents for therapeutic purposes. Polysaccharides have the highest capacity for carrying biological information than other biopolymers such as proteins and nucleic acids due to their structural variability (branched or linear structures) [67].

1.3.1. Some important immunomodulator and anti-tumor agents

Polysaccharide based immuno therapeutic agents like **Lentinan**, **Krestin**, **Schizophyllan, Agarican, Maitake**, and **Aloe barbadensis Miller** have been developed from mushroom and plant.

Lentinan, β-(1→3), β-(1→6)-glucan, was isolated from mushroom, *Lentinus edodes* [68]. Lentinan is a widely used drug and dietary supplement in Japan [69]. It was first isolated and studied by Chihara et al. who demonstrated that its antitumor effects were greater than other mushroom polysaccharides [70]. It has been successfully used in prolonging life span of the cancer patients, especially those with gastric and colorectal carcinomas [71,72]. It also stimulates the production of white blood cells in the human cell line U937 [73].

Krestin is a unique protein bound polysaccharide (PSK), which has been used as a chemoimmunotherapy agent in the treatment of cancer in Japan and other Asian countries for over 30 years. It was developed from turkey tail mushroom *Trametes versicolor*. It contains 75% glucan and 25% protein [74]. PSK has remarkable immunostimulating activity and exhibits a marked effect against different types of tumors. It prevents liver cancer [75] and is also useful for treating hepatitis B [76- 79]. PSK has great potential as an adjuvant cancer therapy agent, with positive results seen in the adjuvant treatment of gastric, esophageal, colorectal, breast cancers [80].

Schizophyllan, obtained from the mushroom *schizophyllum commune* showed anti-tumor activity against the solid and ascite forms of sarcoma 180, as well as solid form of Sarcoma 37, Erlich sarcoma, Yoshida sarcoma and Lewis lung carcinoma [81]. Various clinical trials have been carried out in Japan. Schizophyllan in combination with conventional chemotherapy (tegafur or mitomycin C and 5-fluorouracil) showed significant increase of survival of gastric cancer patients [82]. In randomized controlled study of schizophyllan in combination with radiotherapy, showed that it significantly prolonged the overall survival of stage II cervical cancer patients [83,84].

Agarican, the polysaccharide isolated from *Agaricus blazei***,** showed the increase of immune system to fight a variety of infectious agents and conditions including cancer. Seven polysaccharide fractions have been isolated from *Agaricus blazei* fruit bodies were demonstrated to have antitumor activity [85]. Water soluble fractions, having high anti-tumor activity, contained β-(1→6), β-(1→3)-glucan, β-(1→6), α-(1→4)-glucan and β-(1→6), α-(1→3)-glucan [59]. A new anti-tumor polysaccharide, β-(1→2); β-(1→3)-glucomannan [86], showed high activity against Sarcoma 180 was separated from liquid cultured mycelium of *Agaricus blazei*.

The β-(1→3), (1→6)-glucan (Grifron-D, GD) derived from the mushroom *Grifola frondosa* (also known as **Maitake**) have strong antitumor activity in xenographs [87,88]. GD also has cytotoxic affect on human prostate cancer cells (PC9) in vitro, possibly acting through oxidative stress, and causing 95 % cell death by an apoptosis [89].

Plant polysaccharide from ***Aloe barbadensis Miller*** is considered as potential agent for cancer chemo prevention [66]. The inhibitory effects of various plant polysaccharides on DNA restrictions (Hinds III and Eco RI) revealed that acidic polysaccharides (even at low concentrations) were very active than neutral polysaccharide [90].

1.3.2. Structures and biological activities

A wide range of biologically active polysaccharides of different chemical structures from mushrooms and plants have been investigated. Anti-tumor activity of polysaccharide generally depends of their chemical composition, configuration and physical properties. Differences in activity can be correlated with solubility in water, size of the molecules, branching rate and form. β-glucan containing (1→3)-linkages in the main chain with additional β-(1→6) branching have more anti-tumor activity than β-glucan containing mainly (1→6)-linkages. High molecular weight glucans are more effective than those of low molecular weight [56,91,92]. Linear low molecular weight α-(1→4)-D-glucans is used as an immunomudulator and anti-cancer agent [93,94].

Polysaccharides, having other chemical structures such as, hetero-β-glucans [93], hetero glycans [95], β-glucan-protein complexes [96], α-manno-β-glucan [93], α-glucan-protein complexes and hetero-glycan-protein complex [97] are used as anti-tumor agents.

A triple-helical conformation of (1→3)-β-D-glucans is known to be important for their immune-stimulating activity. 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) [63]. It has been found that higher degree of structural complexity is associated with more potent immunomodulatory and anti-cancer effects. Solubility in water is one of the more important characteristics of mushroom polysaccharides. In mushrooms, β-glucans are present either in their water soluble or insoluble form. The biological activity of water soluble form, however, has been shown to be much greater in humans and animals and to more pronounced effect on their immune systems [98]. The procedures used for modification of the polysaccharides are Smith degradation, formolysis, and carboxymethylation. The immune functions of polysaccharides apparently depend on their conformational complexity.

1.3.3. Mechanism of biological action of polysaccharides:

The mechanism of antitumor action of polysaccharide is still not completely clear. The polysaccharides do not attack cancer cells directly, but produce their anti-tumor effects by activating macrophages such as Natural Killer cells (NK-cell), T-cells, B-cell etc. in the host [98]. These substances are defined as biological response modifiers, BRM [99]. Biological response modifiers are those agents that modify the host's biological response by stimulation of the immune system, which may result in various therapeutic effects. BRMs mean that they (i) cause no harm and place no additional stress on the body, (ii) help the body to adapt to various environmental and biological stresses, and (iii) exert a nonspecific action on the body, supporting some or all of the major systems, including nervous, hormonal, and immune systems, as well as regulatory

functions [100]. A possible mode of action of β-D-glucan as biological response modifier (BRM) was established by T. Mizuno [101] and has been presented in the schematic diagram (**Fig. 7**).

Lentinan appears to perform as a host defence potentiator (HDPs) which is able to restore or augment the responsiveness of host cells to lymphocytokynes, hormones, and other biologically active substances by stimulating maturation, differentiation or proliferation of cells involved in host defence mechanism [102]. HDPs are functionally different from BRMs. Lentinan is thus able to increase host resistance against various kinds of infectious disease 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 [103]. The same effect is true for polysaccharide-protein complexes (PSK), while it has no substantial effect on immune responses of the host under normal conditions. The biological action of Lentinan has been demonstrated by Chihara et al. [104] and presented in the schematic diagram (**Fig. 8**).

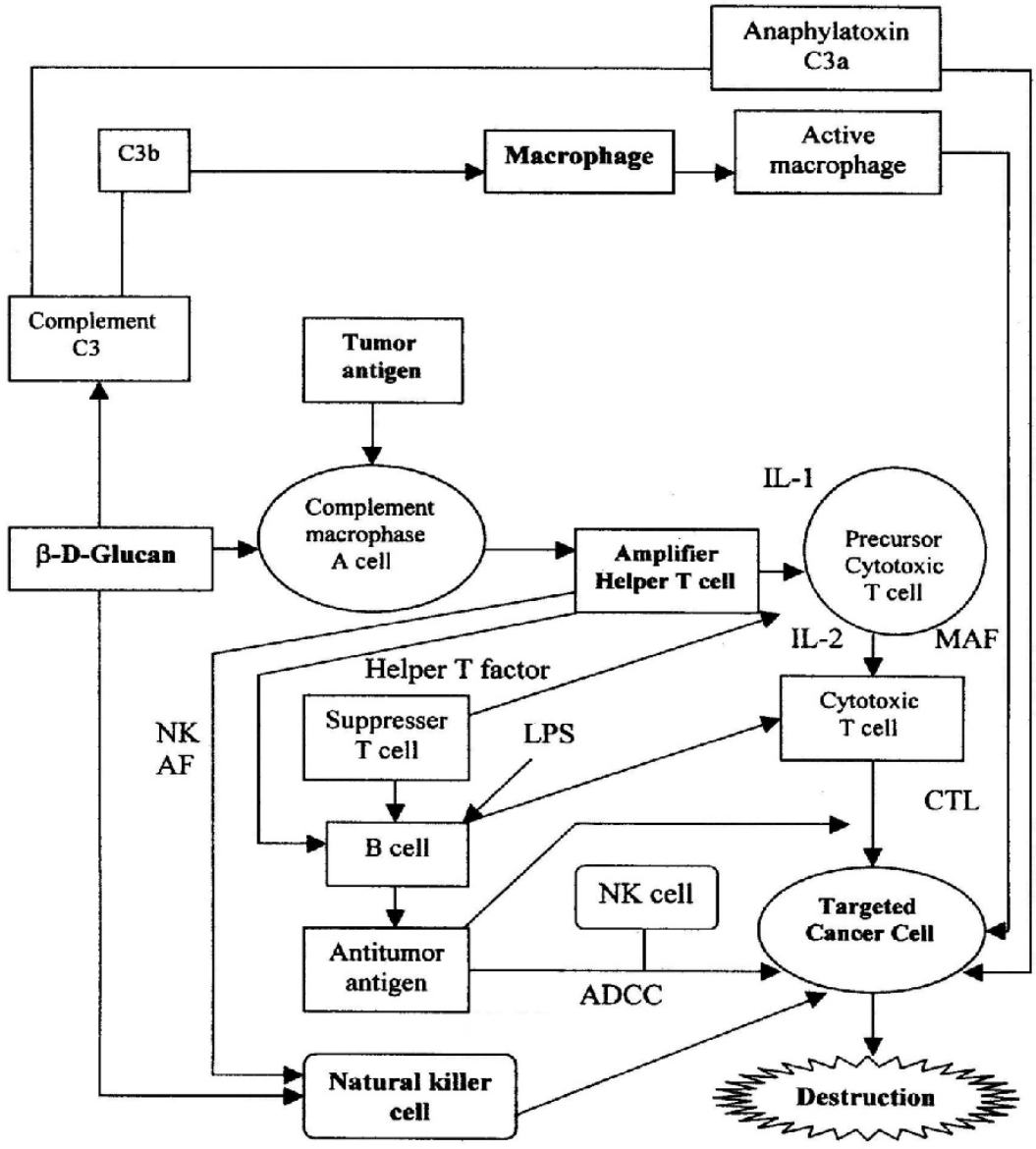

Fig. 7. Possible immune mechanism (Schematic diagram by T. Mizuno): β-D-glucan as biological response modifier (BRM) to target cancer cells.

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

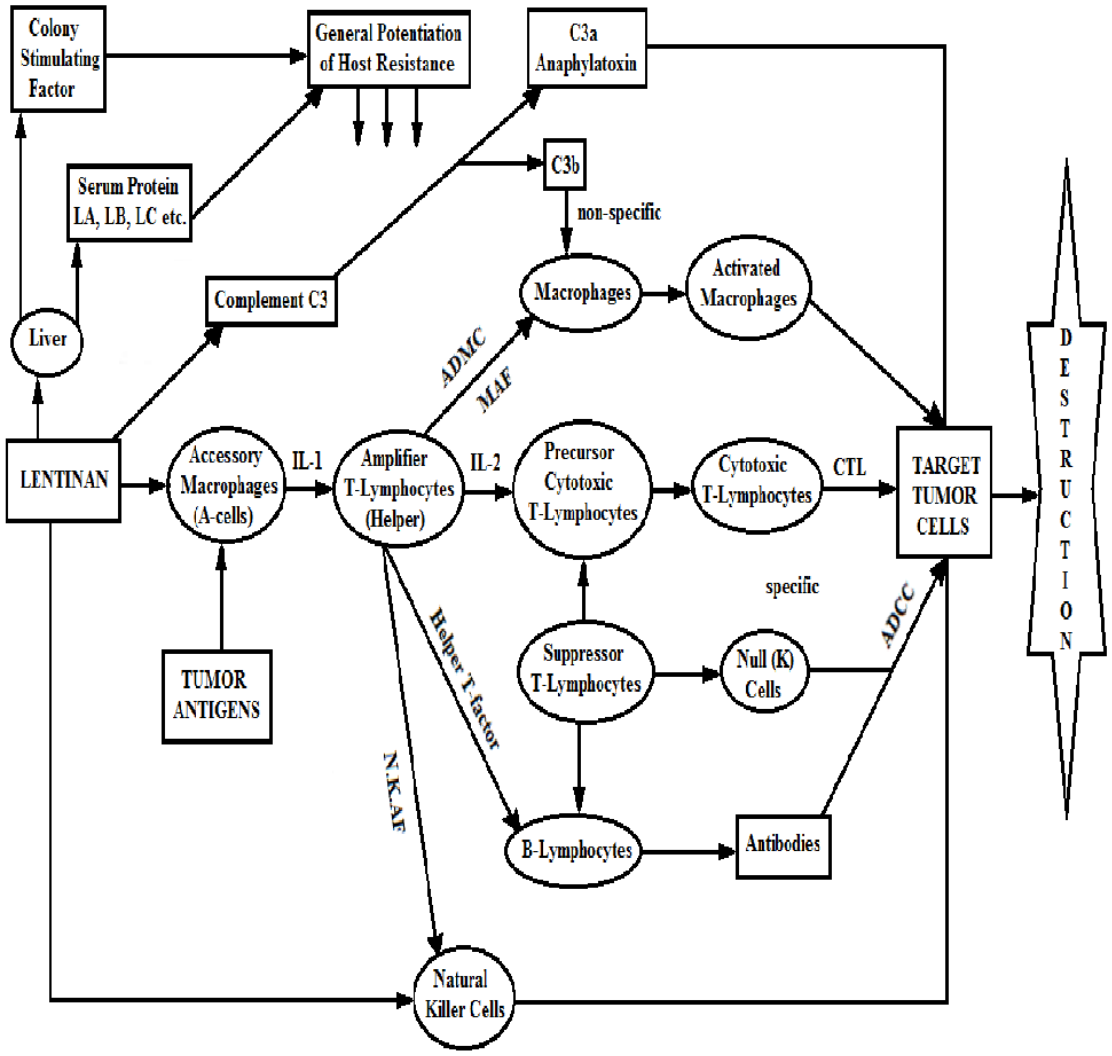

Fig. 8. The mode of action of Lentinan. Schematic diagram of Chihara et al.

1. 4. Polysaccharides as dietary fibers

Dietary fibers are the high molecular weight polysaccharides of different plants and mushrooms, which are excreted without digestion and absorption by human beings [105]. The dietary fibers such as cellulose, lignin, waxes, chitins, and pectins are present in plant. Mushroom contains dietary fibers belonging to β-glucans, chitin and hetero polysaccharides. Dietary fibers are generally divided into insoluble and soluble fibers. Insoluble refers to lack of solubility in water, with attracting properties that help to increase bulk, soften stools and shorten transit time through the intestinal tract. Soluble fiber undergoes active metabolic processing via fermentation that yields end products with broad, significant health effects. Oats, peas, beans, apples, citrus fruits, carrots, barley and psyllium [106] are the sources of soluble fibers, whereas wheat, corn bran, flax seed lignans and vegetables such as celery, green beans, potato skins and tomato skins and tomato peel [107] are the sources of insoluble fiber. Dietary fibers lower the risk of heart diseases and diabetes [108] and can inhibit cancer of colon and rectum [91]. One of the most versatile sources of dietary fiber is the husk of seeds from psyllium grain (*plantago ovata*) that can lower blood cholesterol when chronically included in human diets. Short chain fructo oligosaccharide [109-112] along with other dietary fibers occurs naturally in different tuber crop vegetables like onion (*Allium cepa*), khamalu (*Dioscorea alata*) and garlic (*Allium sativum*) etc.

1.5. Antioxidant properties

Oxidation is essential in living organisms for the production of energy to motivate biological processes. The oxidative properties of oxygen play a vital role in diverse biological functions such as utilization of nutrients, electron transport to produce ATP, and removal of xenobiotics [113,114]. However, oxygen derived free radicals such as superoxide radical (O^{2-}), hydroxyl radicals (OH^{-}), and hydrogen peroxide (H_2O_2) etc. are generated during the oxidative metabolism. Reactive oxygen species can react with all biological molecules such as DNA, proteins, lipids and carbohydrates. This may lead to the development of a wide variety of pathological effects [115]. Generation of

reactive oxygen species (ROS) is characteristic of oxidative stress. Free radicals are formed as part of body's normal metabolic process.

The oxidant status in humans reflects the dynamic balance between the antioxidant defense and pro oxidant conditions and this has been suggested as a useful tool in estimating the risk of oxidative damage [116]. The imbalance between pro oxidant and antioxidant due to xenobiotics, x-ray, radiation, pollution and even stress have been implicated in the pathogenesis of atherosclerosis, ischemic disease, hypertension, alzheimer's disease, parkinson, inflammation, rheumatoid arthritis, cancer and diabetes mellitus [117,118]. Oxidative stress is neutralized by naturally occurring substances mainly by supplementation of food having antioxidant property is becoming one of the most acceptable modes of modern therapy [119].

Consumption of food containing antioxidants has been found to offer protection against these diseases [120]. Polysaccharides extracted from plants are particularly good source of compounds with antioxidant properties. The structure and molecular mass of polysaccharide have been found to play an important role in their biological activities [121]. The polysaccharides from mushrooms and plant also showed antioxidant properties [122,123]. The antioxidant properties of polysaccharides include its scavenging capacities against hydroxy, superoxide radicals and hydrogen peroxide, and metal chelating ability as well as reducing power.

1. 6. Conclusion

Almost all living organism like fungi, bacteria etc. produces polysaccharides. Polysaccharides from plants and mushrooms exhibit immunostimulating and antitumor properties. The commercial pharmaceutical products such as schizophyllan, lentinan, and grifolan, have shown potential clinical applications. The immunomodulating action of mushroom polysaccharides is to stimulate NK cells, T-cells, B-cells, neutrophils, and macrophage dependent immune system *via* differing receptors involving dectin-1, the toll-like receptor-2 (a class of proteins that play a role in the immune system),

scavengers and lactosylceramides. β-D-glucans with various structures show distinct affinities toward these receptors to trigger different host responses. The antitumor activities of the polysaccharides are influenced by the molecular mass, branching configuration, conformational structure and chemical modification.

1.7. References

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CHAPTER 2

METHODOLOGY [Adopted in this thesis]

2.1. Structural analysis of polysaccharides

The structure determination of the polysaccharides depends on two methods: (i) chemical method that includes total acid hydrolysis, methylation, periodate oxidation studies, and Smith degradation, (ii) spectroscopic method comprising of 1D (^{1}H, ^{13}C, DEPT-135) and 2D (TOCSY, DQF-COSY, NOESY, ROESY, HSQC, HMBC) NMR analysis. A schematic diagram of the methodology adopted for structure determination of polysaccharides has been presented in **Fig. 1**.

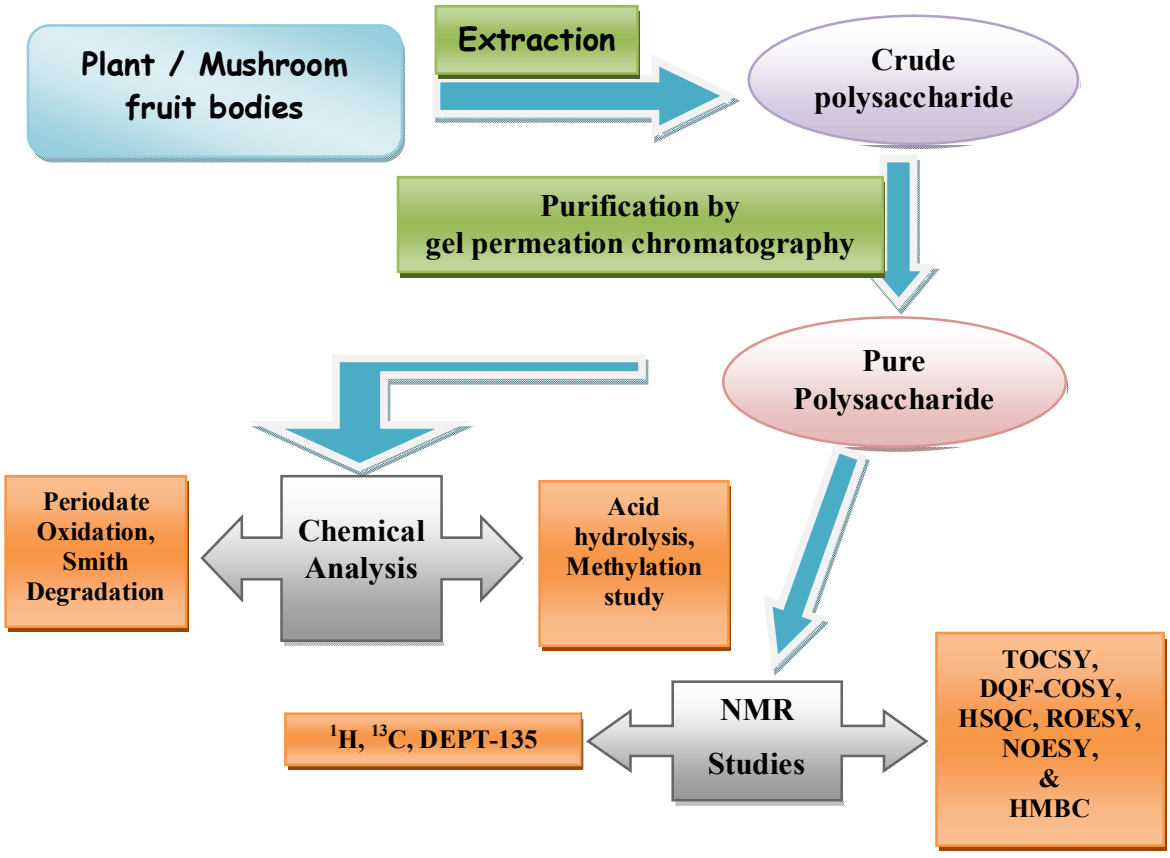

Fig. 1. Schematic diagram of structural analysis of polysaccharide

2.2. Isolation of polysaccharides

The fresh fruit bodies of *Momordica charantia (Karela)* and the fruit bodies of an edible mushroom of *Pleuritus cystidiosus* were washed with distilled water, cut them into small pieces, followed by boiling with hot water for 10-12 h. The whole extract was kept overnight at 4 °C and then filtered through linen cloth. The filtrate was centrifuged at 8000 rpm (using a Heraeus Biofuge stratus centrifuge) for 45 min at 4 °C. The supernatant was precipitated in 1:5 (v/v) Ethanol, kept overnight at 4 °C. The precipitated polysaccharide was collected through centrifugation and then dissolved in a minimum volume of distilled water and dialyzed through a dialysis tubing cellulose membrane (D9652, Sigma-Aldrich, retaining MW >12,000 Da) against distilled water for 2 days (**Fig. 2**). The material inside the cellulose bag was then centrifuged as above. The residue was rejected and the filtrate (water soluble part) was freeze dried and the crude polysaccharide was obtained.

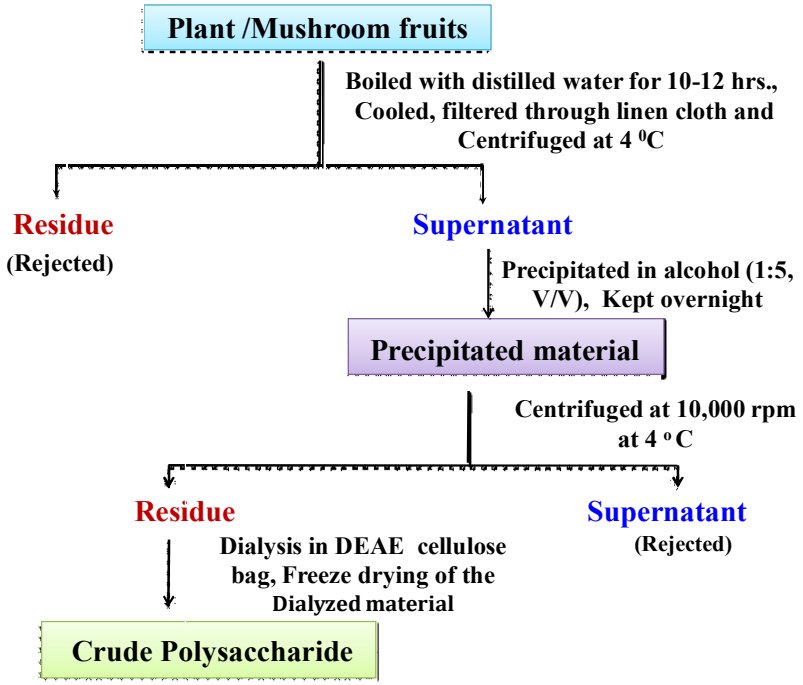

Fig. 2. Schematic diagram of isolation of polysaccharides

2.3. Purification of polysaccharides

The activity of the polysaccharide depends on the size of the molecules, branching rate, and solubility in water, the purification of crude polysaccharide is necessary. Purification of a polysaccharide is crucial for determination of the structure of its repeating unit. The technique of gel-permeation chromatography (GPC) is widely used to separate a mixture of polysaccharides based on their molecular weight hydrodynamic volume [1]. In this technique molecules are separated on the basis of their size relative to the pores of the packing materials. This technique is also known as size exclusion chromatography (SEC). The crude water-soluble polysaccharide was fractionated through Sepharose 6B column (92×2.1 cm, fractionation range 10,000-10,00,000 Da) using water as eluent (0.4 mL min^{-1}) by Redifrac fraction collector. Smaller molecules diffuse into the pores of the gel slowly and where as larger molecules move through the gel more quickly. Ninety five test tubes were collected and monitored spectrophotometrically at 490 nm with phenol-sulfuric acid reagent [2] using Shimadzu UV-vis spectrophotometer, model-1601. A chromatogram of sample distribution was obtained by plotting the test tube number against absorbance. The purification procedure was carried out in several times. Soluble fractions were collected and freeze dried.

2.4. Physical characterization

2.4.1. Measurement of optical rotation

Purified polysaccharide (5 mg) was dissolved in 5 mL distilled water and then optical rotation was measured on a Jasco Polarimeter (Model P-1020) at room temperature.

2.4.2. Determination of Molecular weight

The molecular weights of the polysaccharides were determined by gel-chromatographic technique [3]. Standard dextrans T-250, T-200, T-70, T-40 were passed through a Sepharose-6B column using water as an eluant, and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution

volumes of the polysaccharides were then plotted on the same graph and the average molecular weights were determined.

2.5. Chemical analysis

2.5.1. Monosaccharide analysis

The monosaccharide composition is essential to determine the structure of polysaccharide. Total acid hydrolysis of the polysaccharides was carried out to determine the monosaccharide constituents present in the polysaccharide. Polysaccharide (3 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) in a round-bottomed flask at 100 ºC 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 the excess boric acid was removed by co-distillation with pure CH_3OH. The reduced sugars (i.e. alditol) were acetylated with 1:1 pyridine-acetic anhydride in a boiling water bath for 1 h to give alditol acetates (**Fig. 3**). The excess pyridine- acetic anhydride was removed by co-distillation repeatedly with toluene. The alditol acetates were extracted with chloroform and then analyzed by Gas Chromatography (GLC), Hewlett-Packard, model 5730 A, 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) at 170 ºC.

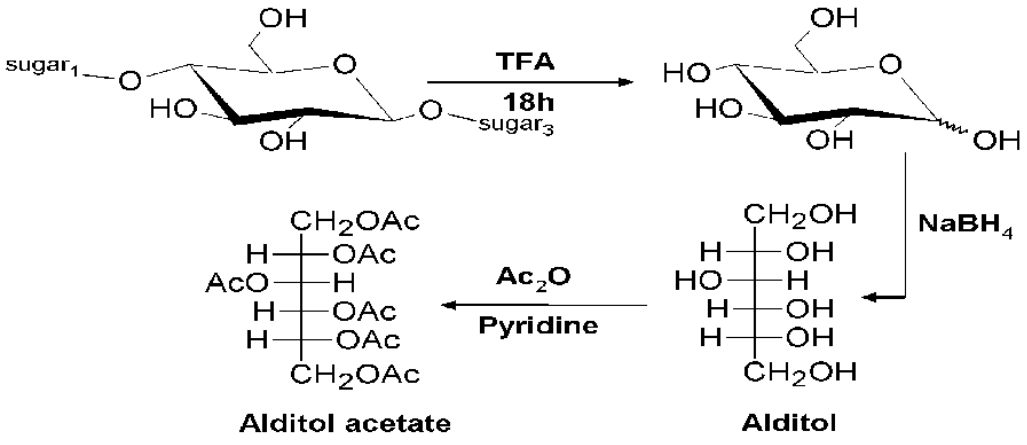

Fig. 3. Schematic diagram of preparation of alditol acetates.

Uronic acids or its derivatives (**Fig. 4**) are resistant to normal acid catalyzed hydrolysis due to the inductive effect of the carboxyl group [4]. Here carboxyl-methyl reduced polysaccharide [5] on hydrolysis followed by GLC examination of the corresponding alditol acetates provides the identification of the sugar residues.

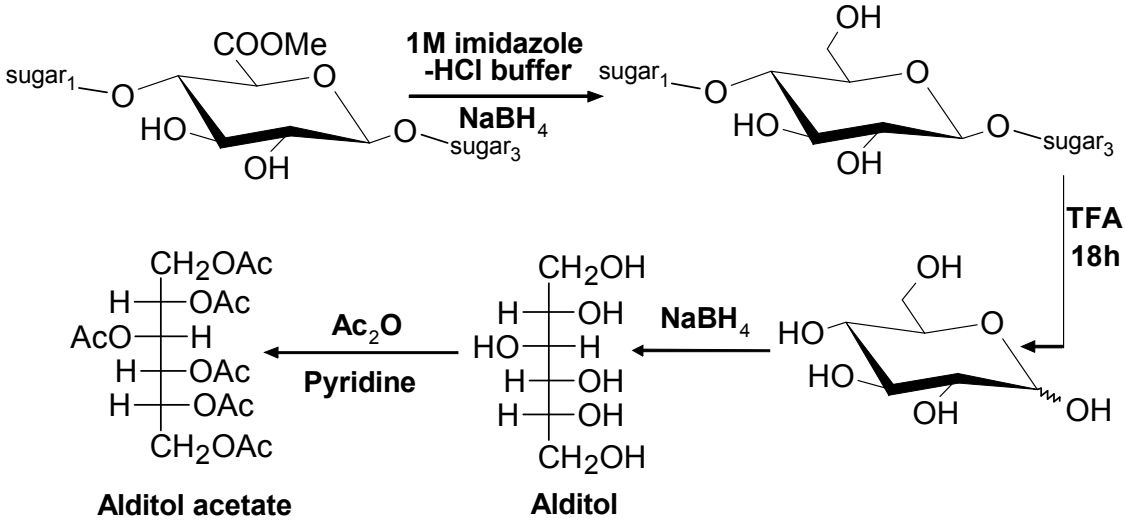

Fig. 4. Schematic diagram of preparation of alditol acetates.

2.5.2. Determination of absolute configuration

The absolute configuration of the monosaccharide was determined by the method of Gerwig et al. [6,7] Monosaccharide may present either in D or L configuration. The polysaccharide (1.0 mg) was hydrolyzed with CF_3COOH, and then the excess acid was removed by co-distillation with distilled water. A volume of 250 µL of 0.625 M HCl in R-(-)-2-butanol was added to the hydrolyzed product and the mixture was heated at 80 ºC for 16 h. 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 ºC /min) from 150 to 210 ºC. 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.

2.5.3. Paper chromatographic studies

Paper partition chromatographic studies [8,9] were performed on whatmann no.1 (for small quantities) and whatmann no. 3 MM papers (for large quantities up to 30 mg) for identification of different sugars in the hydrolyzed polysaccharides. A sugar mixture was separated by descending paper chromatography using following solvent systems (v/v):

(i) 1-butanol-acetic acid-water (4:1:5). [10]

(ii) Ethyl acetate-pyridine-water (8:2:1). [11]

The spray reagents, used for development of chromatograms were -

(a) Silver nitrate in acetone (1.2%). [12]
(b) Methanol in sodium hydroxide solution.
(c) 5% sodium thio sulphate ($Na_2S_2O_3$) solution.

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 chromatogram paper was washed with distilled water and dried in air.

2.5.4. 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.

2.5.4.1. Methylation analysis

Methylation analysis is a well-known chemical method for determination of the mode of linkages and the type of ring structure of the sugar moieties present in polysaccharide. Methylation analysis involves conversion of all free hydroxyl groups

into methoxy group by treating the material in presence of alkali with a suitable methylating agent like methyl halide and dimethyl sulphate. Acid hydrolysis of the resulting poly-methyl-ethers only breaks the inter-glycosidic linkages, keeping the methyl ether bonds intact. The hydrolyzed monomers are then reduced and acetylated to produce volatile partially methylated alditol acetates (PMAA) as shown in **Fig. 5**. The substitution pattern of the *O*-acetyl group of the PMAA indicates the linkage patterns of the corresponding sugars in the polysaccharide. PMAA [13] are separated and identified through GLC. On a specific column the retention times of PMAAs is highly reproducible. When methyl esters of galacturonic acid are present as a monomer unit in polysaccharide, carboxyl methyl reduced polysaccharide [5] is used for methylation. The methylated $LiAlH_4$ reduced polysaccharide [14] is also used. PMAA can be better identified by gas liquid chromatography equipped with a mass spectroscopic detector (GLC-MS). In GLC-MS volatile molecules are identified by their GLC-retention times and also by their EI mass spectra [15].

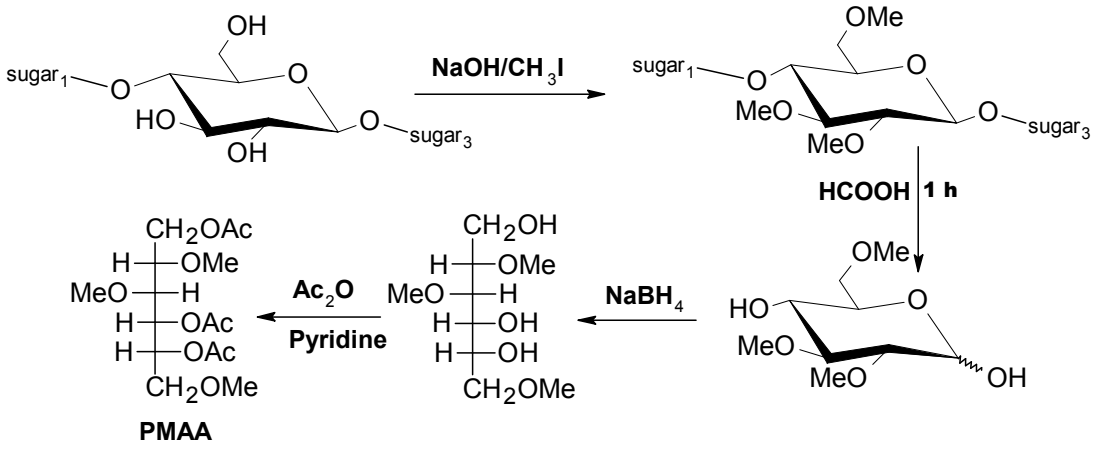

Fig. 5. Schematic diagram of Methylation analysis by Ciucanu and Kerek method.

Methylation analysis gives the information about the branching points, the type of ring structure (pyranose and furanose) of the monosaccharide units and the nature of the terminal units. So, it is important to carry out the complete methylation of the entire free hydroxyl groups in the polysaccharide.

There are several methods for methylation, but the most popular one is adopted in the present investigation i.e. Ciucanu and Kerek [16] method and incorporated in this thesis. According to this method the polysaccharides (3.0 mg) was kept overnight on P_2O_5 in a vacuum desiccator. Then it was treated with finely grounded sodium hydroxide (NaOH) in DMSO (1mL) to de-protonate the hydroxyl groups yielding polyanion (alkoxide ions) followed by addition of methyl iodide (1 mL) in stirring condition. The methylated polysaccharide was isolated by making a partition between $CHCl_3$ and water (5:2, v/v). The organic layer containing product was washed with water for several times and dried on water bath. The methylated product was hydrolyzed with 90% HCOOH (1 mL) at 100 ºC for 1 h, and excess HCOOH was evaporated by co-distillation with distilled water. The hydrolyzed product was then reduced with $NaBH_4$, acetylated with pyridine-Ac_2O (1:1) and the excess was removed by repeated co-distillation with toluene. The alditol acetates of the methylated sugars were extracted with chloroform and analyzed by Gas Chromatography-Mass Spectroscopy (GLC-MS). GLC-MS analysis was performed on Shimadzu GLC-MS Model QP-2010 Plus automatic system, using ZB-5MS capillary column (30 m x 0.25 mm). The GLC program was isothermal at 150 ºC; hold time 5 min, with a temperature gradient of 2 ºC min^{-1} up to a final temperature of 200 ºC.

2.5.4.2. Methylation of carboxyl reduced polysaccharide

The polysaccharide was dissolved in 1M imidazole-hydrochloric acid buffer, pH 7.0 (200 µL/mg) and cooled on ice. $NaBH_4$ (40 mg) was then added, and the reaction mixture was maintained on ice for at least 1 h. The excess $NaBH_4$ was decomposed by adding HOAc (100 µL/40 mg $NaBH_4$) slowly to the cooled sample. An equal volume of redistilled water was added, and the reduced polysaccharide was precipitated by adding 3-4 volumes of 95 % (v/v) EtOH (2 mL). The sample was reprecipitated two more times with 95 % ethanol and freeze-dried to yield carboxyl-reduced polysaccharide [**Fig. 6**]. Then carboxyl-reduced PS was methylated [5] by the Ciucanu and Kerek method [16].

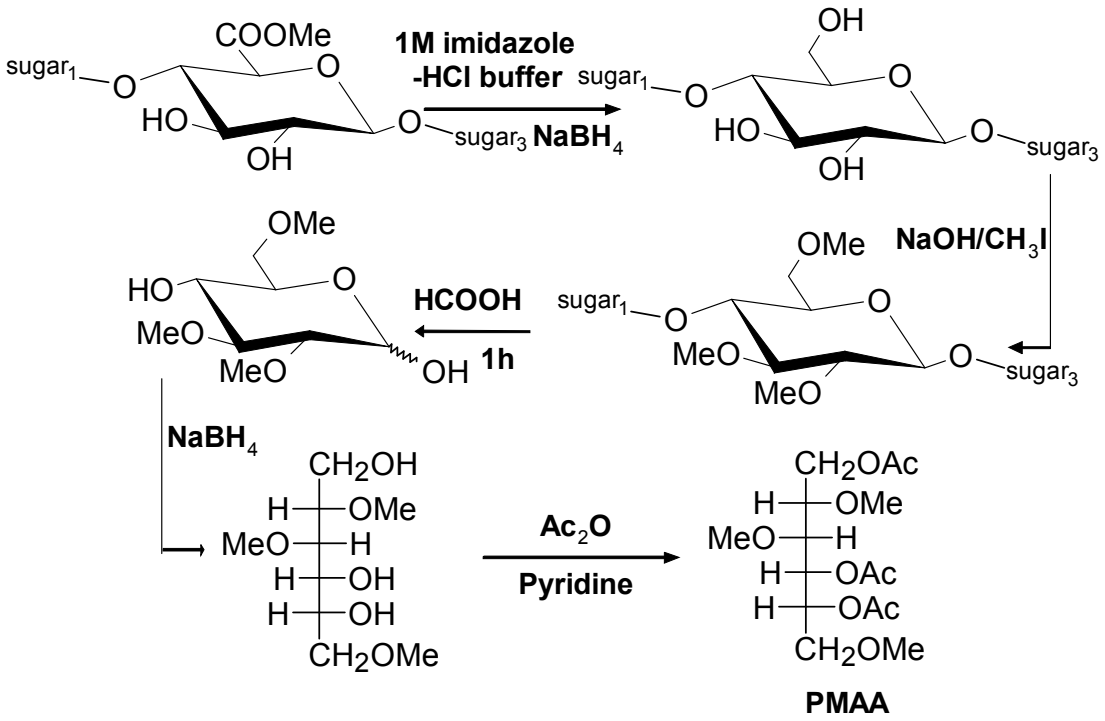

Fig. 6. Schematic diagram of Methylation of Carboxyl reduced polysaccharide.

2.5.4.3. Carboxyl reduction of methylated polysaccharide

The methylated product (2.0 mg) was dissolved in dry THF (2 mL) and refluxed with $LiAlH_4$ [14] (40 mg) for 5 h and kept overnight at room temperature. The excess of the reductant was decomposed by drop wise addition of ethyl acetate and aqueous THF. The inorganic materials were filtered off. The filtrate is evaporated to dryness giving the carboxy-reduced permethylated product. The product was hydrolyzed with formic acid as before and the alditol acetates of the reduced methylated sugars [**Fig. 7**] were prepared in the usual way and analyzed by GLC and GLC-MS experiments.

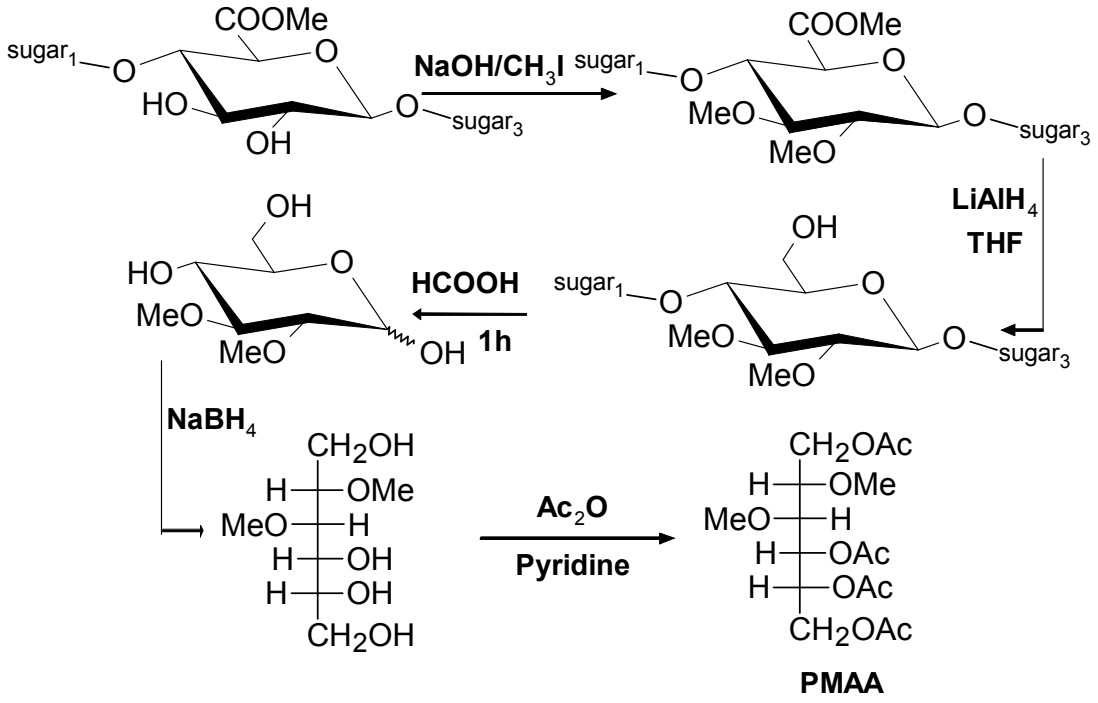

Fig. 7. Schematic diagram of Carboxyl reduction of methylated polysaccharide.

2.5.4.4. Periodate oxidation study

Polysaccharides have the affinity to react with oxidizing agents such as periodic acid or its salts due to the presence of free hydroxyl groups. Non-reducing end sugar residue and 1→6-linked hexopyranose residues have three adjacent hydroxyl groups, hence double cleavage will occur and the reaction will consume two equivalents of periodate and gives one molecular equivalents of formic acid as shown in **Fig. 8**. Non terminal units joined through 1→2 or 1→4 linkages take up one molar equivalent of periodate to yield a dialdehyde, but in this case no formic acid is liberated. Whereas (1→3), (1→2,3), (1→2,4), (1→3,4), and (1→3,6)-linked hexapyranose residues will not be affected by this reaction due to the absence of adjacent –OH groups.The products of the periodate oxidation reaction [17,18] are subjected to both hydrolysis and methylation, followed by reduction and conversion to alditol acetates. Alditol acetates are then identified by GLC and GLC-MS. The partially methylated alditol acetate corresponding to hexopyranose unit that contain (1→3) / (1→2,3) / (1→2,4 / (1→3,4) / (1→3,6)-

linkage will survive in the native polysaccharide after this reaction. So the periodate oxidation study further confirms the linkages of sugar units as determined by methylation experiments.

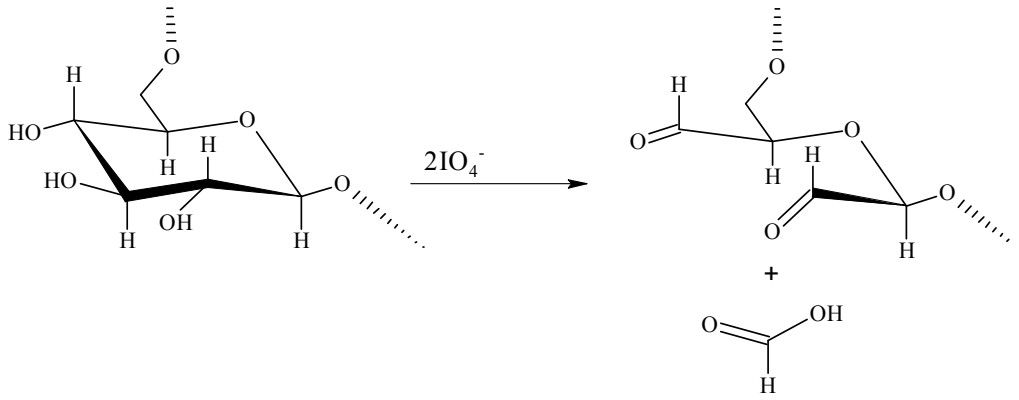

Fig. 8. Schematic diagram of periodate oxidation of (1→6)-linked hexopyranoside.

In this experiment, the polysaccharide (5 mg) was treated with 2 mL 0.1 M $NaIO_4$ (sodium metaperiodate), kept in dark for 48 hours at room temperature and the excess periodate was consumed by adding ethylene glycol. The solution was dialyzed against distilled water for 3-4 h followed by reduction with $NaBH_4$ for overnight and neutralized with AcOH. The periodate oxidized-reduced product was subjected to both hydrolysis and methylation (Ciucanu and Kerek method) [16], the same process described earlier and the related products were analyzed by GC and GC-MS respectively.

2.5.4.5. Smith degradation

Oligosaccharides are more easily characterized than polysaccharide with the help of NMR spectroscopy. Smith degradation [19,20] is another method to degrade polysaccharide to oligosaccharide or modified polysaccharide. This method is utilized to simplify the identification of the repeating unit by selective removal of some of the residue. This procedure can be applied when several overlaps of NMR signals or heterogeneity in the repeating unit is a hindrance in the structural studies. The Smith degradation involves sequential treatment of polysaccharide with $NaIO_4$ (sodium

metaperiodate), sodium borohydride, and dilute trifluoroacetic acid. The oxidation yields a product in which vicinal hydroxyl groups have been oxidized to aldehydes by cleavage of carbon-carbon bonds (**Fig. 9**).

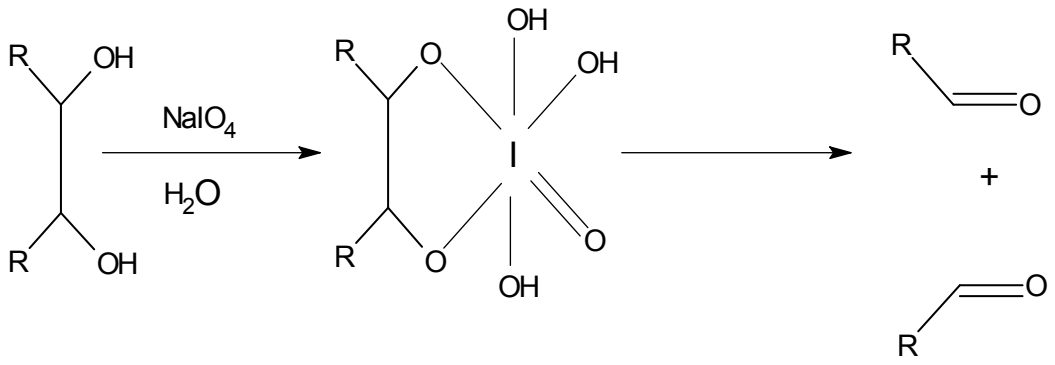

Fig. 9. Schematic diagram of Smith Degradation.

Residues without any vicinal hydroxyl groups remain unaffected. The reduction of aldehyde yields a polyalcohol. On mild acidic hydrolysis, these yield oligosaccharides or modified polysaccharides, that contains sugar residues and fragments have modified sugar residues. The polysaccharide in solution was oxidized with 0.02 M sodium metaperiodate in the dark at 48 h. The excess periodate was destroyed with ethylene glycol and the solution was dialyzed against distilled water and then freeze-dried. The dialyzed material was reduced with $NaBH_4$ for 16 h at 25 °C, neutralized with 50% acetic acid and again dialyzed against distilled water and freeze-dried. Then it was subjected to mild hydrolysis with 0.5 M trifluroacetic acid for 18 h at 25 °C to eliminate the residues of oxidized sugars attached to polysaccharide chain (Smith degradation). The excess acid was removed after repeated addition and evaporation of water. The product was purified by passing through Sephadex G-25 column.

2.5.5. Estimation of total carbohydrate

Phenol–sulfuric acid method [2] was adopted for the estimation of total carbohydrate of polysaccharide. Standard glucose solution of the different

concentrations (20 μg, 40 μg, 80 μg, 100μg and 200μg) were prepared. The 1000 μg solution of sample was prepared by dissolving 5 mg sample in 5 mL distilled water and two different concentrations (100 μg and 80 μg) from that solution were prepared. Then, 1 mL of each standard glucose solution was taken into five test tubes. 1 mL of different concentration sample solution was pipette out into two test tubes and 1ml water was added into a test tube for blank test. Now 1 ml of 5% phenol solution was added into each test tube, and then 5 ml of concentrated H_2SO_4 was also added to each test tube. All test tubes were shaken well by using cyclomixture and kept for 20 minutes. The absorptions of each test tube solution were recorded at 490 nm in Shimadzu UV-visible spectrophotometer, model 1601. Concentrations of standard solution were plotted against absorption in a graph and a straight line was obtained. The absorption values of the sample solution were plotted on the same graph paper and exact carbohydrate percentage of the sample was estimated.

2.6. Analytical methods

2.6.1. Gas-liquid chromatography

Gas-liquid chromatography (GLC) is a non-destructive method for resolving a mixture of components into individual components through equilibrium distribution between two phases. GLC consists of a mobile gas phase and a stationary liquid phase. The mobile gas phase is a carrier gas, usually an inert gas such as helium or inert gas such as nitrogen. Stationary phase is a microscopic layer of liquid that is coated on to either a solid matrix (e.g. diatomaceous earth) or the wall of a capillary tube. Stationary phase has a sufficiently low vapor pressure at the column temperature. So that it can be considered as non-volatile. The sample mixture in gaseous form is run through the column with a carrier gas (e.g. N_2). Separation can be achieved by the differences in the distribution ratios of the components of the sample between the mobile (gaseous) and stationary (liquid) phases causing them to move through the column at different rates and with different retention times. After elution, the components can be detected by a suitable detector. For GLC [21] analysis, sample must be volatile. As monosaccharide

units are not volatile, they must be converted into their alditol acetates. The essential part of the convertization procedure [22,23,24] is reduction of neutral sugars to alditol and subsequent acetylation.The alditol acetates are dissolved in suitable solvents such as chloroform or methylene chloride and injected into a GLC column.

The gas-liquid chromatography Hewlett-Packard model 5730 Å was used, having a flame ionization detector and glass columns (1.8m 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). The detector responds to all organic compounds except formic acid, the response being greatest for hydrocarbons. All GLC analyses were performed at 170 °C.

2.6.2. Gas-liquid chromatography-Mass spectrometry

Mass spectrometry is coupled with gas-liquid chromatography, operating in a high vacuum system has been reported to be a very useful tool in biological and chemical studies. GLC-MS [25] analysis of polysaccharide has advantages because it is very sensitive and a small amount of sample is required. After separation in capillary column in GLC, individual compounds are ionized in high vacuum chamber for mass fragmentation. The fragmentation patterns are compared with standard compounds and analyzed for identification of compounds. GLC-MS analysis of PMAA gives information about the linkage [26,27] of the monosaccharides present in the polysaccharide.

GLC–MS analysis was also 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.

2.7. Nuclear magnetic resonance (NMR) spectroscopy

Nuclear Magnetic Resonance (NMR) is a branch of spectroscopy in which radio frequency waves induce transitions between magnetic energy levels of nuclei of a molecule. NMR spectroscopy is widely used for structural analysis of polysaccharides.

It is most powerful and non-destructive technique for identification of monosaccharide composition, elucidation of α or β anomeric configurations, linkage patterns, and sequence of sugar units in the repeating unit of the polysaccharide. The structure of the polysaccharides were assigned by 1D and 2D NMR techniques, which includes ^{1}H, ^{13}C, DEPT-135, DQF-COSY, TOCSY, NOESY, ROESY, HSQC and HMBC experiments, as shown in the following diagram (**Fig. 10**).

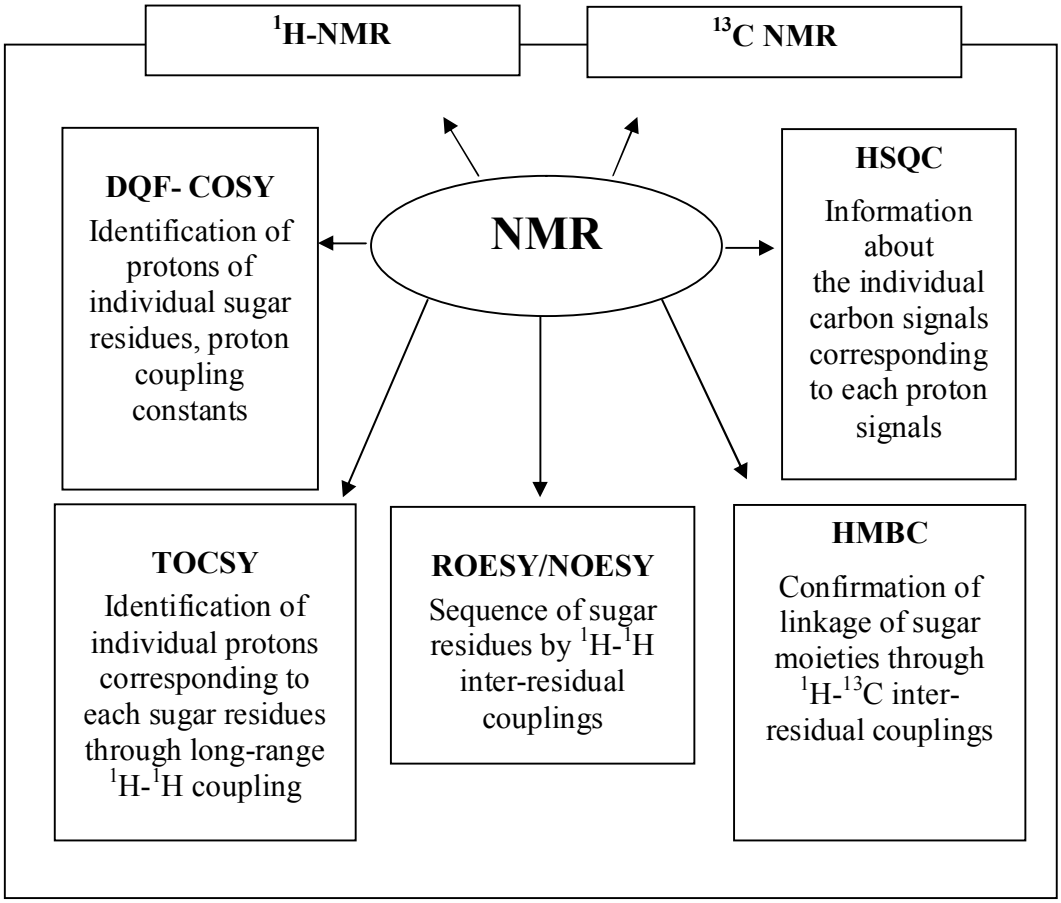

Fig. 10. Schematic diagram of determination of structure of a polysaccharide by NMR spectroscopic methods.

2.7.1. Preparation of NMR sample and instrumentation

Proper sample preparation is very important before running an NMR spectrum. In the present thesis all NMR experiments were performed in D_2O. The samples were made free from water by keeping over P_2O_5 in vacuum for several days, and deuterium exchanged by repeated lyophilization with D_2O (99.96%. atom ^2H, Aldrich). Then ^1H NMR and ^{13}C NMR experiments were performed with a Bruker Avance DPX-500 instrument. The signal for residual HOD was suppressed at δ 4.7 at 30 °C using pre saturation method. The 2D-(DQF-COSY) NMR experiment was carried out using standard pulse sequence at 30 °C. The NOESY and ROESY mixing delay were 300 ms. The delay time in the HMBC experiment was 80 ms. The ^{13}C NMR experiments of the polysaccharide were carried out taking acetone as the internal standard, fixing the methyl carbon signal at δ 31.05 by using D_2O as the solvent.

2.7.2. One-dimensional NMR

1D NMR spectroscopy of Two types is in common uses today are ^1H (proton ^1H NMR) and ^{13}C (carbon-13, ^{13}C NMR). The number of sugar residues (monosaccharide units) and their anomeric configuration can be determined by 1D ^1H-NMR. The polysaccharide has chemical shifts in the range of 1.0-6.0 ppm. The anomeric proton resonances are normally found in the range of 4.4-5.5 ppm. The remaining ring proton resonances are found in the range of 3.0-4.2 ppm. Signal of acetyl methyl proton appears in 2.0-2.2 ppm. The anomeric protons from each monosaccharides give signals depending on their α or β anomeric configurations. Normally the α-anomer resonates downfield compared to the β-anomer in D-pyranoses. The vicinal coupling constant between the anomeric H-1 and the H-2 ($J_{1\text{-}2}$) 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, this is smaller ($J_{1\text{-}2}$ ~ 4 Hz) [28]. Different sugars are identified from their characteristic coupling constants. From coupling constant $^3J_{H1,H2}$ the anomeric configuration of a glucopyranosyl residue can be determined. If the $^3J_{H1,H2}$ =3-4 Hz. the anomeric

configuration of this residue is α and if the value is 7-8 Hz. then the configuration is β. $J_{H1,H2}$ values are not always sufficient to determine the anomeric configuration of the sugars

The ^{13}C NMR signals are much weaker than ^{1}H. It has significant advantages over the ^{1}H-NMR spectroscopy, because in the former the signals are spread out over a wide range. In the ^{13}C NMR spectra, anomeric carbons signals lie in the region 90-110 ppm where as non-anomeric carbons in 60-90 ppm. In case of deoxy sugars the methyl carbons appear in the region of 15-20 ppm. The α-anomeric carbon signals appear generally in the range of 95-103 ppm whereas most of the β-anomeric carbons appear in the region of 101-105 ppm. In case of methoxy sugar the methyl carbons appear in the region 55-61 ppm. Acetyl methyl carbon appears in the region 18-22 ppm. Signals for carbonyl carbons are generally observed between 165-185 ppm. Unsubstituted ring carbons usually appear in the region between 65 ppm and 75 ppm [29]. If there is any linkage at any carbon, the signal for that carbon will show a downfield shift by 4-10 ppm and the carbon next to that one will appear in a little upfield region (by 0.7-4.7 ppm) [29,30].

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 α or β anomer is very difficult since 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. The one bond ^{13}C-^{1}H-coupling constants are useful for determination of the anomeric configuration of sugar residues [31-33]. For D sugar a $^{1}J_{C1,H1}$ ~ 170 Hz indicates an α-anomeric sugar configuration, where as $^{1}J_{C1,H1}$ ~ 160 Hz indicates a β-anomeric configuration [34]. This is reversed for the L sugars.

The linking of residues at C-6 is confirmed from the DEPT-135 (Distortion less enhancement by polarization transfer-135) spectrum.

2.7.3. Two-dimensional NMR

The conventional ^{1}H NMR spectrum has a frequency axis and an intensity axis, whereas the 2D-NMR spectra have two frequency axes and one intensity axis. Two-dimensional NMR can be applied to analyze complex spectra, which are difficult to predict, by conventional methods [35]. The connectivity between the nuclei in a residue and the cross connectivity between the nuclei of different residues can be obtained from 2D NMR. In the present thesis different 2D NMR experiments such as TOCSY, DQF-COSY, NOESY, ROESY, HMQC and HMBC have been used.

2.7.3.1. TOCSY (Total Correlation Spectroscopy)

TOCSY is also known as homonuclear Hartmann-Hann spectroscopy (HOHAHA). This spectrum correlates protons that are in the same spin system and yields both long range and short range correlations. It is useful for establishing the scalar connectivity if the proton signals are within a spin system, especially when the multiplets overlap, or there is extensive second order coupling. It is useful in the identification of individual monosaccharide 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.

2.7.3.2. DQF-COSY (Double Quantum Filtered Correlation Spectroscopy)

DQF-COSY gives the information about the protons of an individual sugar residue through a three-bond coupling. The strategy of assigning a COSY spectrum is to find one unmistakable characteristic signal from which to begin the tracing of a spin system or network. An anomeric proton is often chosen as the starting point because it is connected with the other proton atoms of that residue. It is a ^{1}H homonuclear shift correlation spectrum that provides information on spin coupling networks within a residue through the observation of the cross peaks off diagonal. Coupling constants can also be measured from DQF-COSY spectrum.

2.7.3.3. NOESY (Nuclear overhauser enhancement spectroscopy)

NOESY spectrum provides information through space rather than through bond couplings. NOE connectivities are often observed between the anomeric proton of a particular sugar residue to proton of the other sugar residues that is glycosydic linked to the former. The experiments give information on linkages and sequence of sugar residues in a polysaccharide. In NOESY spectrum numerous peaks are obtained. Therefore, the first step in analyzing NOESY spectrum is to eliminate those uninteresting peaks comparing them to DQF-COSY and TOCSY spectrum. The NOESY mixing delay was 300 ms.

2.7.3.4. ROESY (Rotating frame Overhauser Enhancement Spectroscopy)

ROESY spectroscopy technique is used to determine the glycosydic linkage position as well as the sequence information of the polysaccharide like NOESY spectra. Here also the signals arise 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.

2.7.3.5. HSQC (Heteronuclear single quantum coherence)

In **HSQC** NMR spectrum all signals in the spectra represent a direct correlation between a carbon and a proton. 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.

2.7.3.6. HMBC (Hetero multiple bond coherence spectroscopy)

An **HMBC** experiment detects long range coupling between proton and carbon (two or three bonds away) with high sensitivity. Once all the carbon signals are assigned, especially the anomeric and glycosidically linked carbons, unambiguous glycosidic linkages and sequences of the sugar residues can be established through the long-range

^{13}C-^{1}H correlations. HMBC experiments establish multiple-bond correlation through the glycosidic bonds, and this together with NOESY experiments provides necessary information on linkages and sequences of a polysaccharide. The delay time in the HMBC experiment was 80 ms.

2.8. Immunological studies

2.8.1. Test for macrophage activity by nitric oxide assay

RAW 264.7 growing in Dulbecco's modified Eagle's medium (DMEM) was seeded in 96 well flat bottom tissue culture plate at 5 x 10^5 cells/mL concentration (180 µL) [36]. Cells were left overnight for attachment and treatment of different concentrations (12.5, 25, 50, 100, 200, and 300 µg/mL) of polysaccharides. After 48 hrs 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) [37]. Lipopolysaccharide (LPS), L6511 of *Salmonella enterica* serotype typhimurium (sigma, St. Louis, USA) was used as positive control.

2.8.2. Splenocyte and thymocyte proliferation assay

A single cell suspension of spleen and thymus were prepared from the normal mice under aseptic conditions by homogenization in Hank's balanced salt solution (HBSS). The suspension was centrifuged to obtain cell pellets. The contaminating red blood cells (RBC) were removed by hemolytic Gey's solution. After washing two times in HBSS, the cells were resuspended in complete Roswell Park Memorial Institute (RPMI) medium. Cell concentration was adjusted to 1×10^6 cells/mL and the 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 (12.5, 25, 50, 100, 200, and 300 µg/mL) of polysaccharides. PBS (Phosphate Buffer Saline, 10 mM, pH-7.4) was taken as negative control, whereas lipopolysaccharide (LPS, L6511 of Salmonella enterica

serotype typhimurium, Sigma, 4 µg/mL) and Concanavalin A (Con A, 10 µg/mL) served as positive controls. 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 checked by MTT assay method [38]. The data are reported as the mean ± standard deviation of seven different observations and compared against PBS control [36,38].

2.9. Antioxidant activity

2.9.1. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the polysaccharide (from 0.1 to 2.5 mg/ml) was investigated using Fenton's reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^-$) [39]. 1 ml reaction mixture consisted of KH_2PO_4 - KOH buffer (20 mM, pH 7.4), 2-deoxy-D-ribose (2.8 mM), various concentrations of the polysaccharide, $FeCl_3$ (100 mM), EDTA (104 µM), ascorbate (100 µM) and H_2O_2 (1 mM). It was incubated at 37 ˚C for 1 h to allow hydroxyl radical generation which will attack deoxyribose to form malondialdehyde (MDA). 2ml thiobarbituric acid (TBA) and trichloroacetic acid (TCA) solution (0.375% (w /v) TBA, 15% (w /v) TCA and 0.25 N HCl) was added and incubated at boiling water bath for 15 min to form MDA-TBA chromogen at acidic pH. After cooling, absorbance was measured at 535 nm against identical set of reaction mixtures where TBA-TCA solution was added prior incubation to subtract background colour. Butylated hydroxyanisole (BHA) was used as positive control and results were expressed as the rate of inhibition. EC_{50} value expressed the effective concentration at which the scavenging free radical activity was 50%. The degree of scavenging was calculated by the following equation:

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

Where A0 was the absorbance of the control and A1 was the absorbance in the presence of sample.

2.9.2. Inhibition of lipid peroxidation by egg homogenate

The antioxidant activity of the polysaccharide was evaluated according to lipid peroxidation method using egg homogenate, as described in the literature [40]. The reaction mixture consisted of 0.5 ml of egg homogenate (10% v/v), polysaccharide of various concentrations (0.2 – 2.5 mg/ml) and 0.05 ml of $FeSO_4$ (0.07 M) and incubated to allow oxidation of polyunsaturated fatty acids. After 30 min, 1.5 ml acetic acid (20%, pH 3.5), 1.5 ml of 0.8% (w/v) TBA in 1.1% sodium dodecyl sulphate and 0.05 ml 20% TCA were added. Reaction mixture was heated at 95 °C for 1 h, during which pinkish red chromogen developed due to reaction between TBA and MDA, end product of oxidation. After cooling, 5 ml of butanol was added and centrifuged at 3000 rpm for 10 min. Absorbance of upper layer was measured at 532 nm. Ascorbic acid was used as positive control. EC_{50} value expressed the effective concentration at which the inhibition of lipid peroxidation was 50%. The degree of inhibition was calculated by the following equation:

$$\text{Inhibition effect (\%)} = \{(A0-A1)/ A0\} \times 100$$

Where A0 was the absorbance of the control and A1 was the absorbance in the presence of sample.

2.10. Biological activity

2.10.1. Chemicals and reagents

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT reagent), Histopaque 1077, and $DCFH_2$ DA were procured from Sigma (St. Louis, MO, USA). Minimum essential medium (MEM), RPMI 1640, fetal bovine serum (FBS), penicillin, streptomycin, doxorubicin, sodium chloride (NaCl), sodium carbonate (Na_2CO_3), sucrose, Hanks balanced salt solution were purchased from Himedia, India.

2.10.2. Cytotoxicity or cell viability

To evaluate the in vitro cellular toxicity of the polysaccharide on human peripheral blood lymphocytes, lymphocytes were seeded into 96 wells of culture plates having 180 µl of complete media and were incubated for 48 h. Polysaccharides were added to the cells at different concentrations (D1: 10, D2: 25, D3: 50, D4: 100, D5: 200 and D6: 400 µg/ml), were incubated for 24 h at 37°C in a humidified incubator (NBS) maintained with 5 % CO_2. The cell viability was estimated by MTT assay method [41].

2.10.3. Determination of reduced glutathione (GSH)

Reduced glutathione (GSH) assay was performed [42]. In brief, the required amount of the cell lysate was mixed with 25% of trichloroacetic acid and centrifuged at 2,000× 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 minutes 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 µ gm of GSH/mg protein.

2.10.4. Determination of Oxidized glutathione level (GSSG)

The oxidized glutathione (GSSG) level was measured after derevatization of GSH with 2-vinylpyidine [43]. Cell lysate of 0.5 ml was added with 2 µl 2-vinylpyidine, incubated for 1 hr at 37°C. The mixture was then deprotenized with 4% sulfosalicylic acid and centrifuged at 1,000× 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.

2.10.5. Determination of lipid peroxidation (MDA)

Lipid peroxidation of cell lysate was measured [44] with the reaction mixture containing Tris-HCl buffer (50 mM, pH 7.4), tert-butyl hydroperoxide (BHP) (500 μM in ethanol) and 1 mM $FeSO_4$. The samples were then incubated at 37°C for 90 min and 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 level of lipid peroxidation was expressed in terms of n mol/mg protein. The protein was estimated [45] by using bovine serum albumins as standard.

2.10.6. Statistical analysis

The data were expressed as mean ± SEM, n = 4. Comparisons of the means of control, and experimental groups were made by one way ANNOVA tests (using a statistical package, Origin 6.1, Northampton, MA 01060 USA), $P < 0.05$ as a limit of significance.

2.11. Conclusion

The methodologies that have been adopted to determine the structure of polysaccharides and their bioactivity studies have been discussed in this chapter. The structure of the repeating unit of the polysaccharides is determined using two types of methods: (1) chemical method that includes total acid hydrolysis, methylation and periodate oxidation, (2) spectroscopic method comprising of 1D and 2D NMR experiments.

2.12. References

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CHAPTER 3

Structural and Immunological Studies of a pectic Polysaccharide Isolated from the green fruits of Momordica charantia, (Karela)

3.1. Introduction and earlier work

Polysaccharides extracted from different fruits and vegetables [1,2] are found to exhibit immunoenhancing and antioxidant properties. The structure and molecular mass of the polysaccharide have been found to play an important role in their biological activities [3]. The active principles present in medicinal plants have been reported to possess pancreatic β cells regenerating; insulin releasing and fighting the problem of insulin resistance [4].The plant possesses over two hundred twenty five different medicinal constituents [5]. The fruit of Momordica charantia commonly known as bitter gourd or karela belongs to the family cucurbitaceae and economically important medicinal plant, widely grown in India and other parts of the Indian subcontinent. Two varieties of this plant found in India, called *M. charantia* var. *charantia* produce large shaped fruits and *M. charantia* var. *muricata*, give fruits of smaller size [6]. More than thousands herbal products have been used by diverse cultures of the world to treat hyperglycemia and among them bitter melon (*M. charantia*) is one of the most popular herbal resource [7]. The immature fruits are eaten as vegetables and the fruits contain high amounts of vitamin C, vitamin A, vitamin E, vitamins B1, B2 and B3, as well as vitamin B9 (folate) [8]. The fruit is also rich in minerals including potassium, calcium, zinc, magnesium, phosphorus and iron, and is a good source of dietary fiber. The caloric values for leaf, fruit and seed were 213.26, 241.66 and 176.61 Kcal/100 g respectively [9]. Fruits extracted from *M. charantia* possess anti-HIV, antimicrobial, antitumor and antidiabetic properties [10]. Reactive oxygen species can react with the biological molecules such as DNA, proteins, lipids and carbohydrates that may lead to the development of a wide variety of pathological disorders [11]. Consumption of food containing antioxidants has been found to offer protection against these diseases [12]. Today, processed *M. charantia* in the form of capsules or tablets are commonly sold in pharmaceutical shops. The products are marketed [13] under

the brand names Gourdin, Karela, and Glucobetic in Canada, India, the United Kingdom, the United States, and many other Asian countries.

3.2. Present work

3.2.1. The pectic polysaccharide

Aqueous extract of fruit bodies of the *M. charantia*, Karela (**Fig. 1**) yielded one water soluble polysaccharide (PS), consisting of methyl galacturonate and galactose. The PS showed splenocyte, thymocyte as well as macrophage activations. The detailed structural characterization and study of immunoenhancing and antioxidant properties of the PS were carried out and discussed in this chapter.

Fig. 1. Photograph of the fruit of *M. charantia*, (Karela).

3.2.2. Isolation and purification of polysaccharide from *Momordica charantia*, (Karela)

The fresh fruits of *Momordica charantia* (1.5 kg.) were washed with distilled water, boiled for 10 h. in water, kept overnight at 4º C, then filtered, centrifuged, precipitation

in ethanol, dialysis, centrifugation, washed with ethanol, and freeze drying to yield crude polysaccharide (1.416 g). The water soluble crude polysaccharide (30 mg) was fractionated through Sepharose-6B column [14]. Single fraction (test tubes, 20 - 40) was obtained (**Fig. 2**), collected and freeze dried, yielding pure 16.2 mg of polysaccharide. The process was repeated several times to get 95 mg polysaccharide.

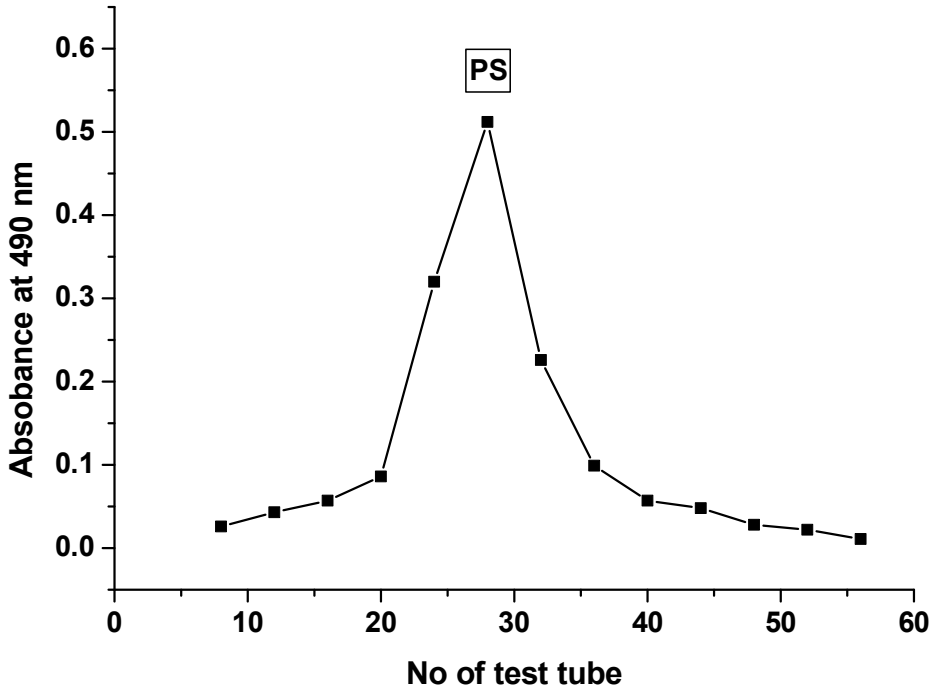


Fig. 2. The gel permeation chromatogram of the polysaccharides isolated from hot aqueous extract of the fruit bodies of *M. charantia*, (Karela).

3.2.3. Optical rotation and molecular weight of PS

The specific rotation of the PS was measured $[\alpha]_D^{29.8}$ +168.9 (*c* .098, H_2O). The average molecular weight of PS was estimated to be ~$2.0x10^2$ kDa, from a calibration curve (**Fig. 3**) prepared using standard dextran [15].

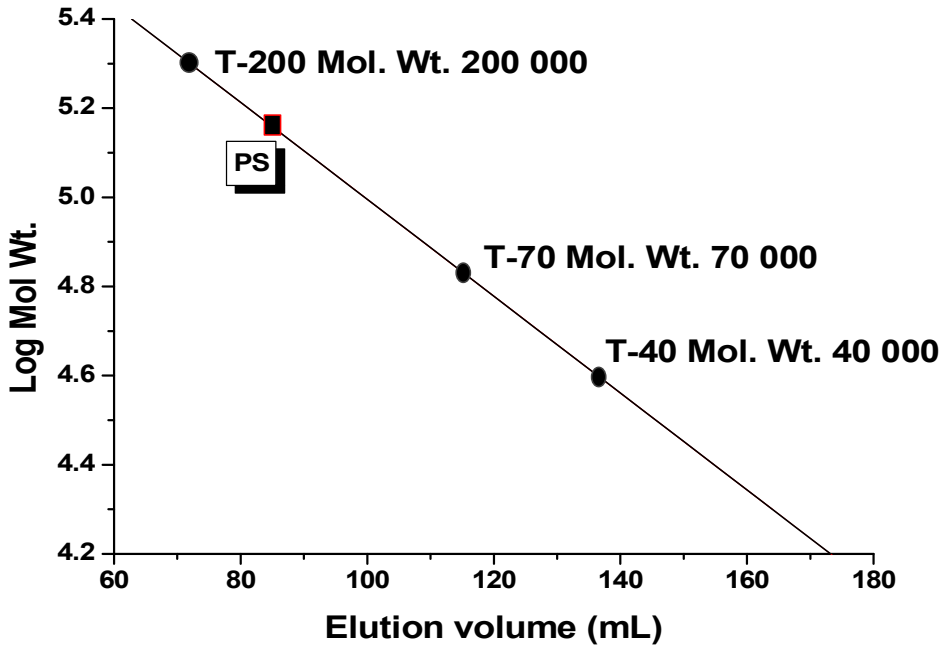


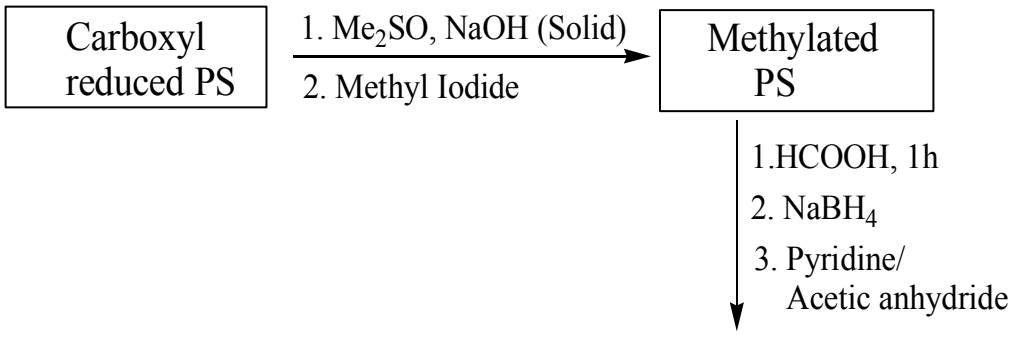
Fig. 3. Determination of molecular weight of the PS, isolated from hot aqueous extract of fruit bodies of the *M. charantia*, (Karela).

3.2.4. Structural analysis of PS

3.2.4.1. Chemical analysis of PS

Paper chromatographic analysis [16] of the hydrolyzed product of the PS showed the presence of galactose and galacturonic acid. The GLC analysis of the alditol acetates [17] of the sugars showed the presence of galactose only. But the carboxyl-reduced [18] polysaccharide on hydrolysis followed by GLC examination of the corresponding alditol acetates showed also the presence of only galactose. The absolute configurations [19] of galactose and galacturonic acid was determined as D-cofiguration. The PS was methylated [20] followed by hydrolysis and preparation of alditol acetates. GLC-MS analysis revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol indicating that the D-galactopyranosyl moiety is present as non-reducing end in the

polysaccharide. The carboxyl-reduced [21] methylated polysaccharide on hydrolysis followed by GLC-MS analysis of corresponding alditols showed the presence of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol, 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-D-galactitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol in a molar ratio 3:1:1 **[fig. 4].** The appearance of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol indicated the presence of (1→4)–linked D–galacturonic acid and 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-D-galactitol indicated (1→2,4)–linked D-galacturonic acid and the D-galactose is attached at the non reducing end of the backbone of the polysaccharide. Thereafter, a periodate oxidation [22,23] experiment was carried out with the polysaccharide. The periodate oxidized, reduced material on hydrolysis with trifluoroacetic acid followed by paper chromatography [16] experiment showed the presence of D-galacturonic acid only. GLC-MS analysis of the alditol acetates of the periodate-oxidized, carboxyl-reduced methylated polysaccharide showed the retention of the peak corresponding to 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-D-galactitol, indicating further that D-galcturonic acid was retained as it was (1→2,4)-linked moiety and the non reducing end D-galactose and (1→4)-linked D-galacturonic acid were destroyed during periodate oxidation [**fig. 5**].



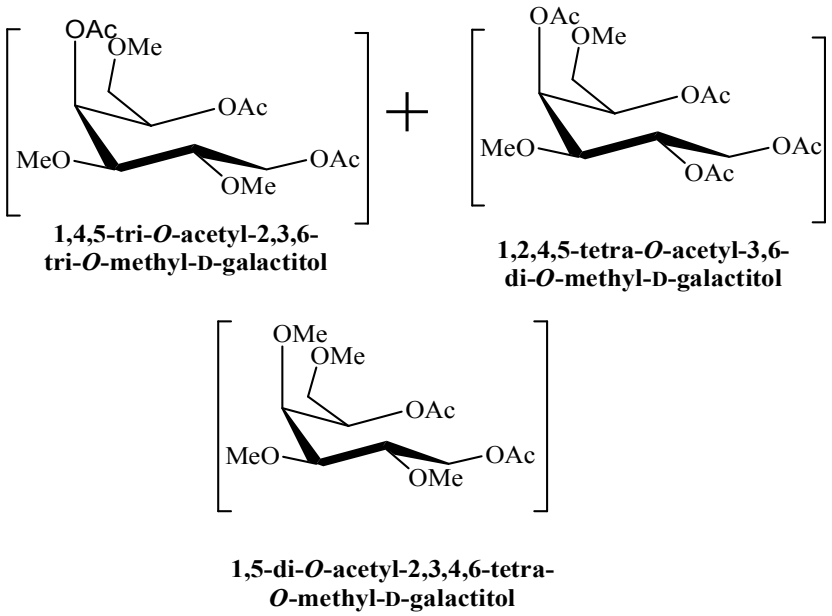


Fig. 4. Schematic presentation of the methylation experiment of PS.

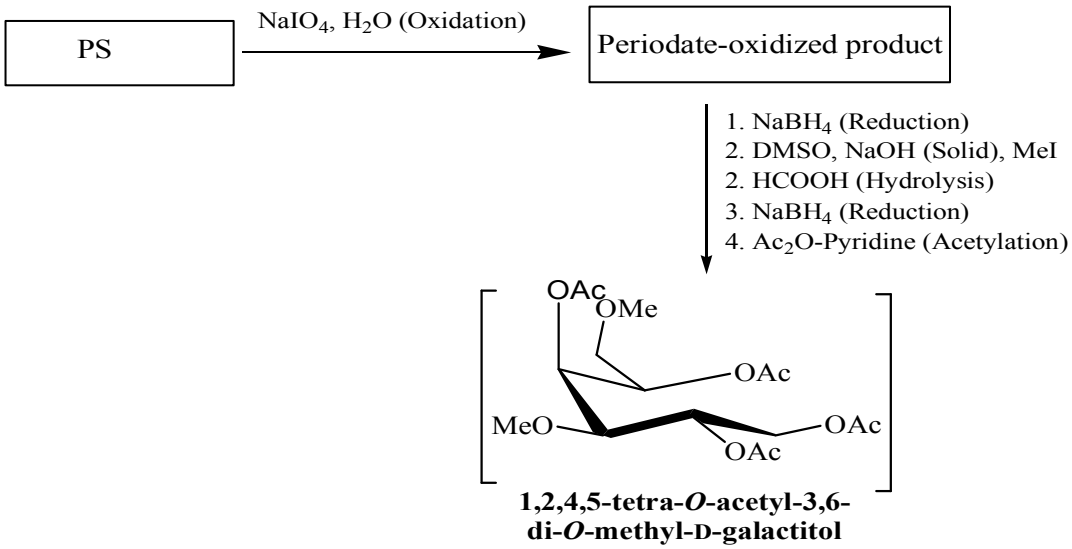


Fig. 5. Schematic presentation of periodate oxidation reactions of PS.

3.2.4.2. 1D and 2D NMR analysis of PS

The ^{1}H NMR spectrum (500 MHz, **Fig. 6**) of the PS at 30 ºC showed three signals in the anomeric region at δ 4.59, 4.94 and 5.06 and designated as **A**, **B**, and **C** respectively according to their increasing anomeric chemical shift values. In the ^{13}C NMR spectrum (125 MHz; **Fig. 7**) at the same temperature three anomeric carbon signals appeared at δ 105.0, 100.8 and 100.0 were correlated to the residues **A**, **B** and **C** respectively from the HSQC spectrum (**Fig. 8**). All the ^{1}H and ^{13}C signals (**Table 1**) were assigned using DQF–COSY, TOCSY and HSQC NMR experiments.

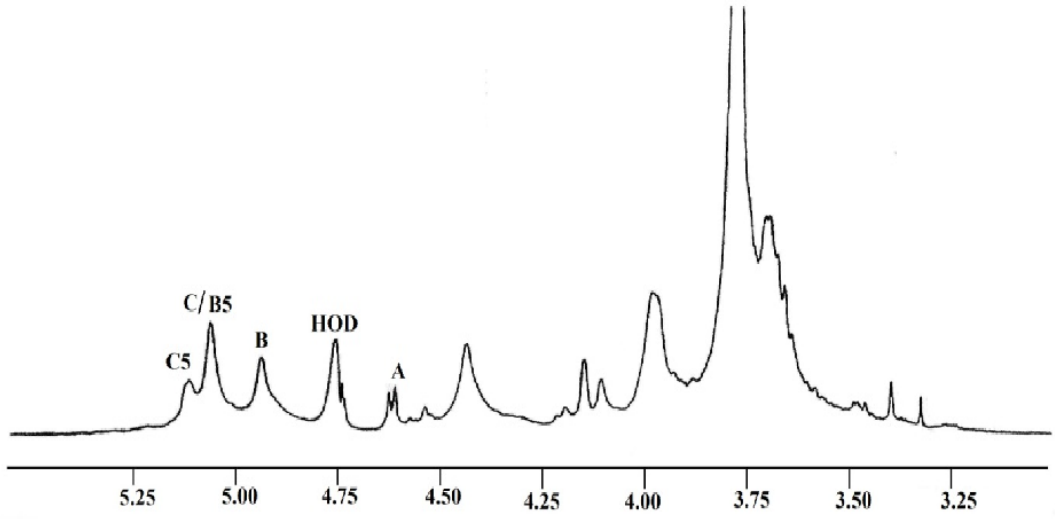


Fig. 6. ^{1}H NMR spectrum (500 MHz, D_2O, 30°C) of the polysaccharide (PS) isolated from the fruits of *M. charantia*, (Karela).

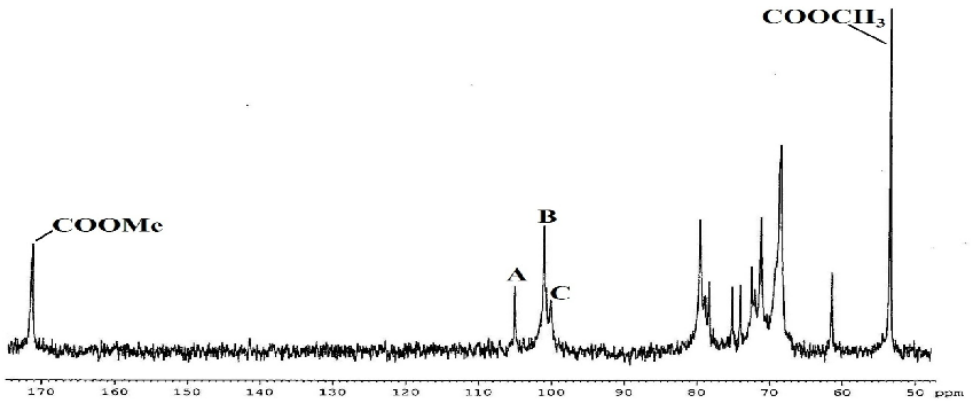


Fig. 7. ^{13}C NMR spectrum (125 MHz, D_2O, 30°C) of the polysaccharide (PS) isolated from the fruits of *M. charantia*, (Karela).

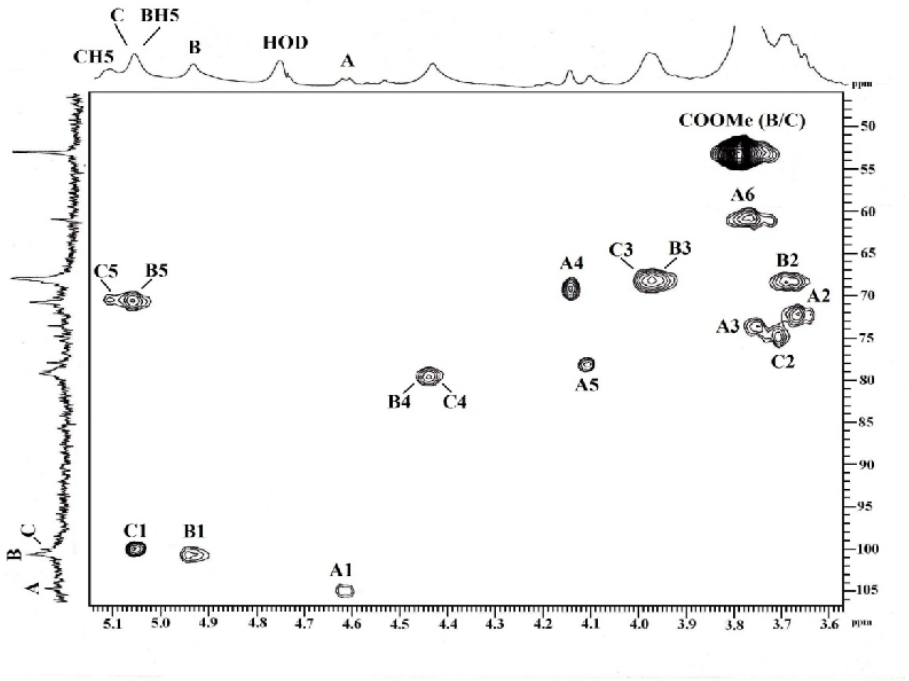


Fig. 8. The part of HSQC spectrum (D_2O, 30°C) of the polysaccharide (PS) isolated from the fruits of *M. charantia*, (Karela).

Table 1. ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts (ppm) of the PS isolated from the fruits of *Momordica charentia*, (Karela)[a,b] recorded 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	-COOMe
β-D-Gal*p*-(1 →	4.59	3.66	3.76	4.15	4.10	3.73[c], 3.78[d]	
A	105.0	72.8	73.9	69.5	78.1	61.03	
→4)-α-D-Gal*p*A6Me-(1→	4.94	3.70	3.96	4.44	5.05		3.79[e]
B	100.8	68.4	68.3	79.5	70.6	171.0[g]	53.3[f]
→2,4)-α-D-Gal*p*A6Me-(1→	5.06	3.73	3.99	4.44	5.11		3.79[e]
C	100.0	74.5	68.5	79.5	71.06	171.0[g]	53.3[f]

[a] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.74 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.

[e] Proton value of ester group.

[f] ^{13}C Chemical shift value of the methyl carbon in ester group.

[g] ^{13}C Chemical shift value of the carbonyl group of ester group.

Residue **A** has an anomeric chemical shift at δ 4.59 and a large coupling constant values $J_{H\text{-}1,\ H\text{-}2}$ ~8.4Hz and $J_{H\text{-}1,\ C\text{-}1}$~ 161 Hz indicating that it was β-linked residue. A large $J_{H\text{-}2,\ H\text{-}3}$ ~9.3 Hz and also small $^{3}J_{H\text{-}3,\ H\text{-}4}$ ~3.1 Hz indicated that **A** is β-D-galactosyl residue. The carbon signals from C-1 to C-6 of residue **A** corresponded nearly to the standard values of methyl glycosides [24] and therefore the residue **A** was established as terminal β-D-galactopyranosyl moiety which is attached at the C-2 position of residue **C**, confirmed from the cross peak of **A**C1 / **C**H2 in HMBC experiment (**Fig. 9, Table 2**).

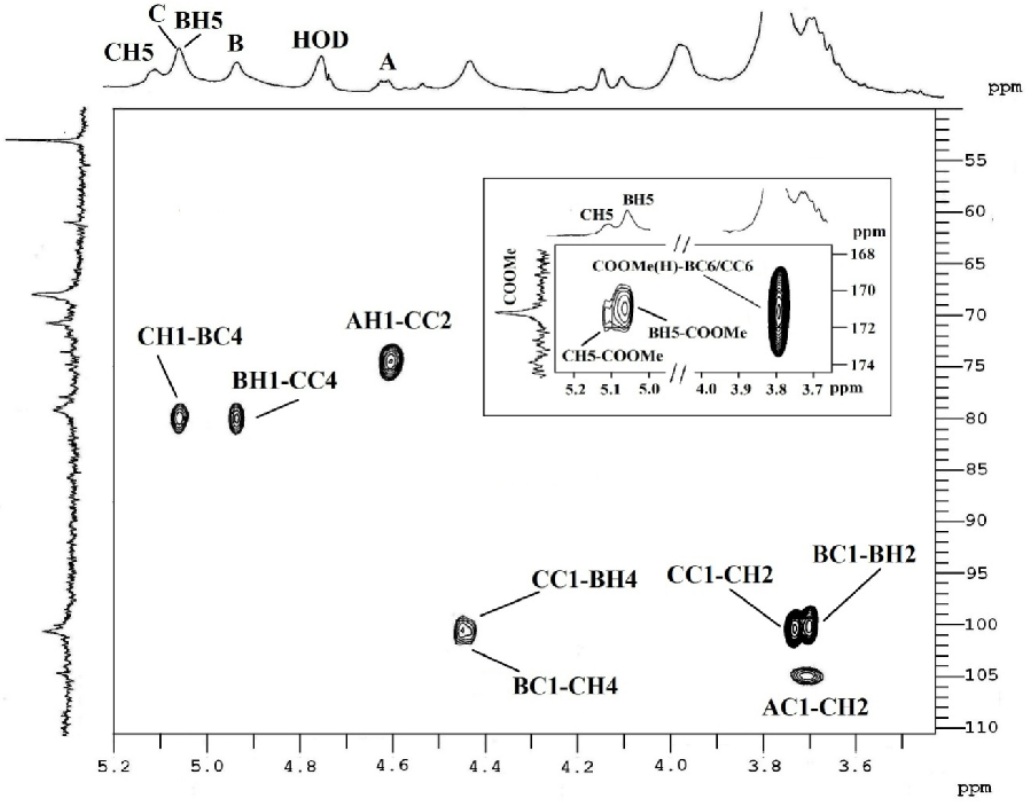


Fig. 9. The part of HMBC spectrum (D_2O, 30°C) of the polysaccharide (PS) isolated from the fruits of *M. charentia*, (Karela). The delay time in the HMBC experiment was 80 ms.

Table 2 The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/ carbons of the sugar residues of the PS isolated from the fruits of *M. charentia*, (Karela).

Residue	Sugar linkage	H-1/C-1 δ_H/δ_C (ppm)	Observed connectivities		
			δ_H/δ_C (ppm)	Residue	Atom
A	β-D-Gal*p*-(1 →	4.59 105.0	74.5 3.73	**C** **C**	C-2 H-2
B	→4)-α-D-Gal*p*A6Me-(1→	4.94 100.8	79.5 4.44 3.70	**C** **C** **B**	C-4 H-4 H-2
C	→2,4)-α-D-Gal*p*A6Me-(1 →	5.06 100.0	79.5 4.44 3.73	**B** **B** **C**	C-4 H-4 H-2
			Observed connectivities		
		COOMe(δ_H)	δ_C	Residues	Atom
B/C	→4)-α-D-Gal*p*A6Me-(1→	3.79	171.0	**B/C**	C-6
		COOMe(δ_C)	δ_H		
		171.0	5.05	**B**	H-5
			5.11	**C**	H-5

The residue **B** and **C** were established as α-linked residue from their coupling constant values $J_{H\text{-}1,\ H\text{-}2}$ (~3.1Hz and~3.0Hz) and $J_{H\text{-}1,\ C\text{-}1}$ (171Hz and 170Hz). **B** and **C** residues have relatively high chemical shift values of H-5 signal in anomeric region at δ 5.05 and 5.11 respectively [25] and weak coupling between H-3, H-4 and H-5 indicated that the residue **B** and **C** are α-D-galacturonosyl moieties.

The residue **B** has an anomeric proton chemical shift at δ 4.94 and anomeric carbon chemical shift at δ 100.8. The carbon signals of residue **B** were observed at δ 68.4, 68.3, 79.5, 70.6, and 171 corresponding to C-2, C-3, C-4, C-5, and C-6 (carbonyl carbon) respectively. The C-4 signal at δ 79.5 showed a downfield shift compared to that of standard methyl glycosides [24] due to α-glycosylasion effect indicating the (1→4)-linked β-residue. The appearance of intra residual couplings at δ_c171.0/ δ_H5.05 (**B**C6/ **B**H5 and δ_c171.0/ δ_H3.83 (**B**C6/Carboxy methyl proton) in the HMBC spectrum (**Fig. 9)** clearly indicates that caboxyl group of galactouronic acid is present as methyl ester. These indicated that the residue **B** is (1→4)-α-D-Gal*p*A6Me.

The residue **C** has an anomeric proton chemical shift at δ 5.06 and anomeric carbon chemical shift at δ 100.0. The carbon signals of C-2 (δ 74.5) and C-4 (δ 79.5) showed downfield shift with respect to standard value of methyl glycosides indicating that the residue **C** is (1→2, 4)-linked residue. In HMBC spectrum the C-6 carboxyl carbon signal (δ 171.0) couples with carboxy methyl proton (δ 3.79) and also **C**H-5 (δ 5.11) indicating that the residue **C** is also methyl ester of galacturonic acid. These results indicated that the residue **C** is (1→2,4)-α-D-Gal*p*A6Me.

The sequence of glycosyl residues of the polysaccharide was determined on the basis of NOESY (**Fig. 10, Table-3**) as well as ROSEY experiments, followed by confirmation with HMBC (**Fig. 9, Table 2**) experiment. In NOESY experiment, the inter-residual contacts **A**H-1/**C**H-2; **B**H-1/**C**H-4; **C**H-1/**B**H-4 along with other intra-residual contacts were also observed (**Fig. 10**).

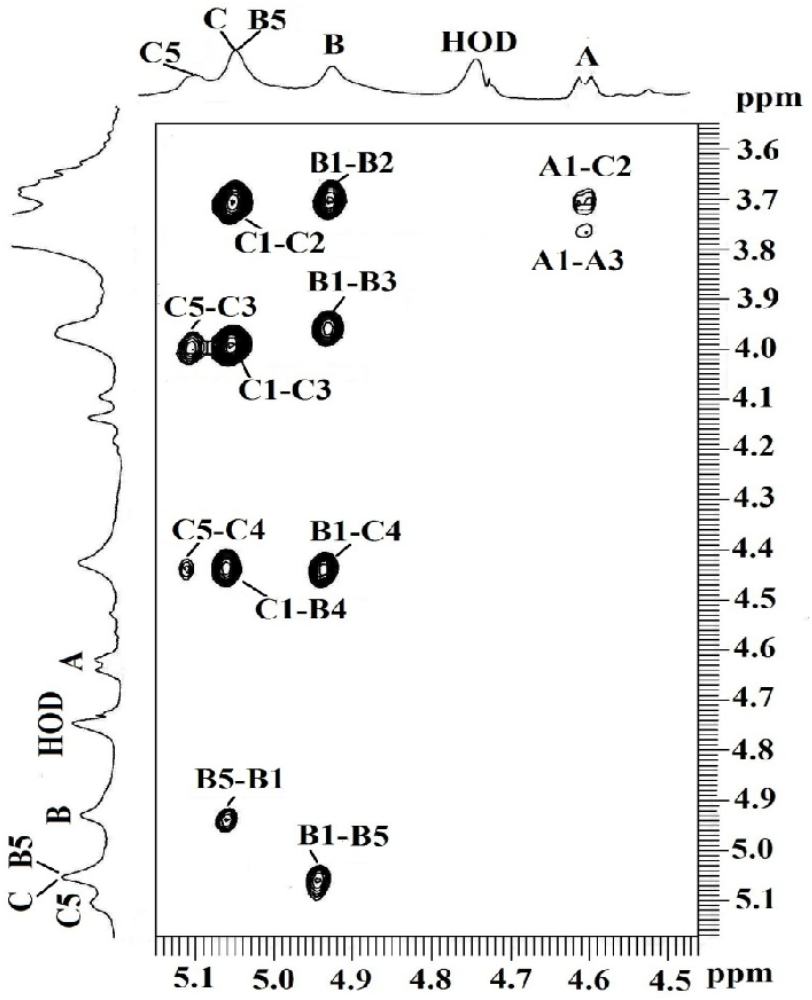


Fig. 10. The part of NOESY spectrum (D_2O, 30 °C) of the polysaccharide (PS) isolated from the fruits of *M. charentia*, (Karela).

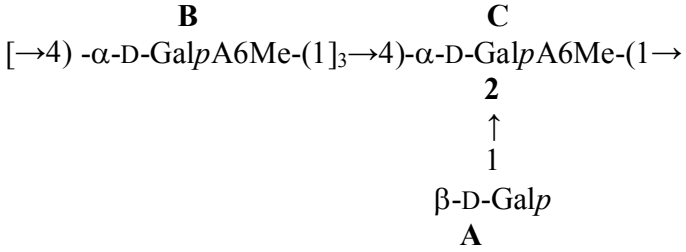
Table 3 NOE data for the PS isolated from the fruits of *M. charantia*, (Karela).

Anomeric proton		NOE contact protons		
Glycosyl residue	δ (ppm)	δ (ppm)	Residue	Atom
β-D-Gal*p*-(1 →	4.59	3.71	**C**	H-2
A		3.76	**A**	H-3
→ 4)-α-D-Gal*p*A6Me-(1 →	4.94	4.44	**C**	H-4
B		3.96	**B**	H-3
		3.70	**B**	H-2
		5.05	**B**	H-5
	5.05 (**BH-5**)	4.94	**B**	H-1
→2,4)-α-D-Gal*p*A6Me-(1 →	5.06	4.44	**B**	H-4
C		3.71	**C**	H-2
		3.99	**C**	H-3
	5.11 (**CH-5**)	3.99	**C**	H-3
		4.44	**C**	H-4

Thus from NOESY experiment the following sequences are established:

A (1→2) **C**; **B** (1→4) **C**; **C** (1→4) **B**

These sequences were further confirmed by ^{13}C-^{1}H correlation in HMBC spectrum (**Fig. 9, Table 2**). Inter residual cross peaks **A**H1/**C**C2, **A**C1/**C**H2; **B**H1/**C**C4, **B**C1/**C**H4; **C**H1/**B**C4 and **C**C1/**B**H4 along with other intra residual peaks were also observed. Therefore, based on the results obtained from monosaccharide composition, methylation studies and NMR experiments the following repeating unit of the pectic polysaccharide of *Momordica chrantia* was assigned as:



3.2.5. Immunostimulating properties of PS

Macrophage activation was tested with different concentrations of PS *in vitro*. Enhanced production of NO was observed in a dose dependent manner with optimum production of 17 μM NO per 5 x 10^5 macrophages at 200 μg/mL (**Fig. 11**). Hence, the effective dose was observed at 200 μg/mL of PS.

Proliferation of splenocytes and thymocytes is an indicator of immunoactivation. The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with the PS by the MTT assay method [26]. Polysaccharide was tested to stimulate splenocytes and thymocytes and the results are shown in **Fig. 12** and **13** respectively and the asterisks on the columns indicate the statistically significant differences compared to PBS control. The splenocyte proliferation index (SPI) as compared to PBS control closer to 1 or below indicates low stimulatory effect on immune system. At 200 μg /mL of the PS, splenocyte proliferation index was found maximum as compared to other concentrations. Hence, 200 μg /mL of the PS can be considered as efficient splenocyte stimulator. Again 25 μg/mL of this same sample showed maximum effect on thymocyte proliferation.

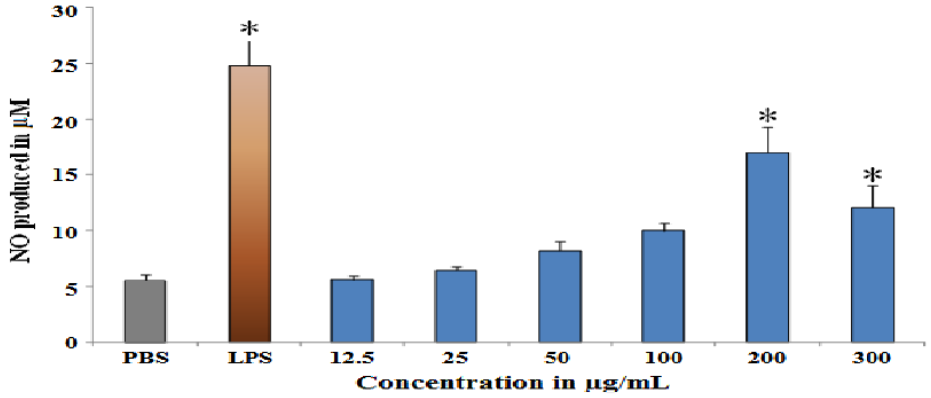


Fig. 11. Activation of Raw 264.7 macrophage cells stimulated with different concentrations of the PS isolated from the fruits of *M. charantia*, (Karela) in terms of NO production.

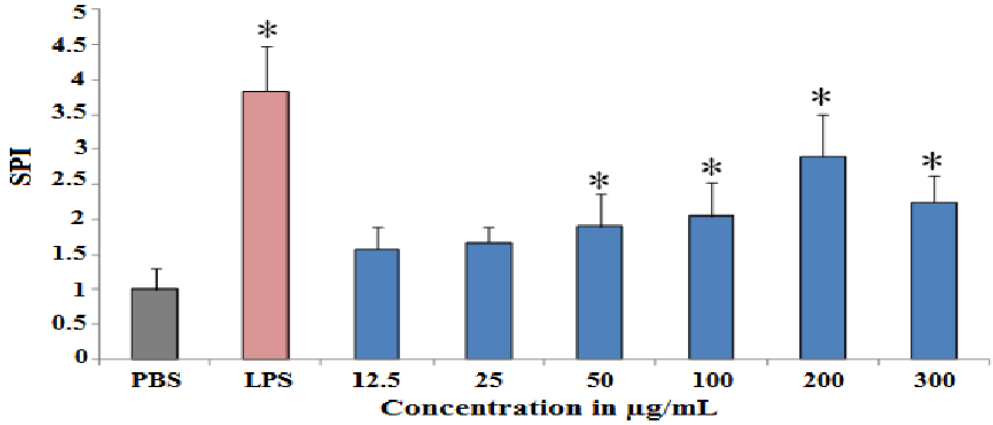


Fig. 12. Effect of different concentrations of the PS on proliferation of splenocyte (* indicate the statistically significant compared to the PBS as a positive control with $P<0.05$. Here LPS (4 µg/mL) are used as a negative control).

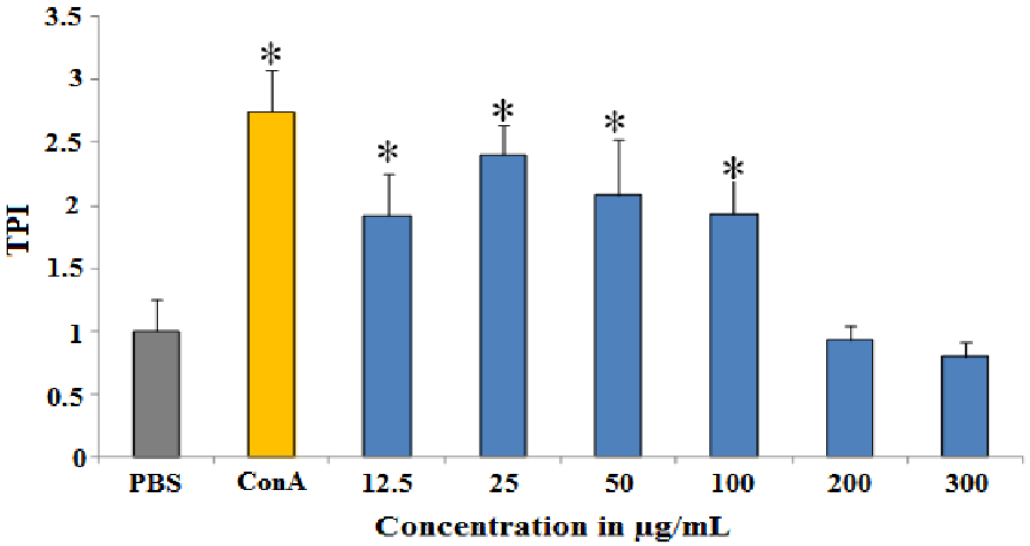


Fig. 13. Effect of different concentrations of the PS on proliferation of thymocyte (* indicate the statistically significant compared to the PBS as a positive control with $P<0.05$. Here conA (10 µg/mL) are used as a negative control).

3.2.6. Antioxidant properties of PS

3.2.6.1. Hydroxyl radical scavenging activity

Generation of reactive oxygen species beyond the body`s antioxidant capacity gives rise to oxidative stress. During this stress, hydroxyl radicals are mainly responsible for the oxidative injury of biomolecules since they can easily react with amino acids and DNA. It is also believed that they are the active initiators for lipid per oxidation [27]. It is thus important to remove hydroxyl radicals for protection of the living systems.

Scavenging effects of the PS on hydroxyl radicals increased with increasing concentration (**Fig 14**). The Figure shows percentage inhibition of hydroxyl radical generation by 0.1, 1, 2.5 mg/ml concentration of the PS and butylated hydroxyl anisole (BHA). Results indicated that PS have a noticeable effect on scavenging free radicals (EC_{50}= 2.22 mg/mL). Xu *et al* isolated three purified polysaccharides from flower of tea

plant [28] and all of them exhibited <25% hydroxyl radical scavenging activity at 4 mg/mL concentration. However, the scavenging effect of BHA was much higher than the PS (EC_{50}= <0.01 mg/mL). The results revealed that the PS was hydroxyl radical scavengers, acting possibly as primary antioxidant.

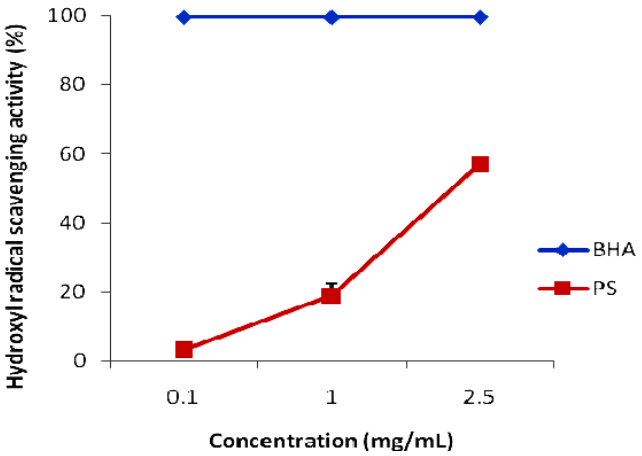


Fig. 14. Hydroxyl radical scavenging activity. (b)Inhibition of lipid peroxidation activity of the pectic polysaccharide (PS) isolated from the fruits of *M. charantia*, (Karela). (All the above results are the mean ± SD of three separate experiments, each in triplicate).

3.2.6.2. Lipid peroxidation by egg homogenate

Cellular components such as polyunsaturated fatty acid residues of phospholipids are extremely sensitive to oxidation induced by reactive oxygen species. Major aldehyde products of this peroxidation method are malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE). HNE is thought to be highly toxic whereas malondialdehyde is carcinogenic in mammalian cell [27]. Lipid peroxidation also results in breakdown of membrane integrity, affecting its fluidity and permeability. Therefore, inhibition capacity of lipid peroxidation is of great importance to show potential antioxidant activity.

As shown in **fig. 15**, lipid peroxidation was prevented by the PS. The absorbance of malondialdehyde - thiobarbituric acid (MDA-TBA) complex was linearly decreased in a dose dependent path (0.2, 1, 2.5 mg/mL). The PS showed 50% lipid inhibition at 2.05 mg/mL, whereas the standard ascorbic acid showed much higher activity with EC_{50}= <0.1 mg/mL.

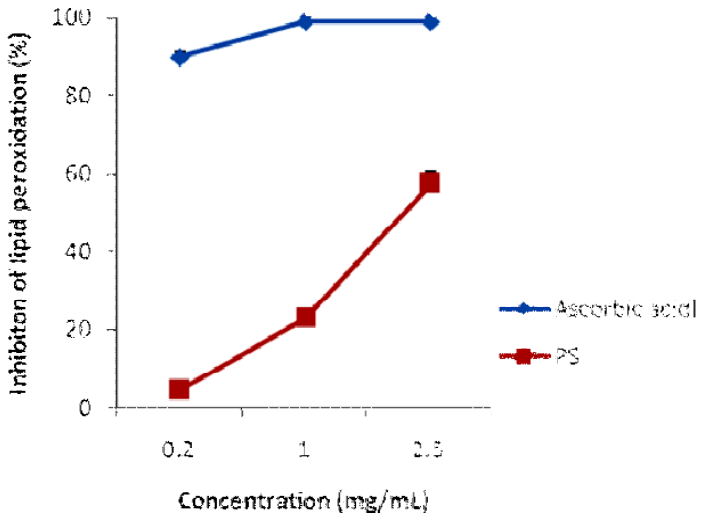


Fig. 15. Inhibition of lipid peroxidation activity of the PS isolated from the fruits of *M. charantia*, (Karela). (All the above results are the mean ± SD of three separate experiments, each in triplicate).

3.3. Conclusion

The pectic polysaccharide (PS) isolated from the aqueous extract of the green fruits of *Momordica charantia*, (Karela) contains D-galactose and D-methyl galacturonate in a molar ratio of nearly 1:4. The repeating unit of the PS contained a backbone of four (1→4)–linked D-methyl galacturonate residues, out of which one residue was branched at *O*-2 position with terminal β-D-galactopyranosyl residue. The PS activated the macrophages, splenocytes, and thymocytes and also showed several potent antioxidant

activities. Hence, on the basis of these activities it could be used as a natural immunostimulant and antioxidant material.

This work has been published in **Carbohydrate Research**, **2015**, *401*, 24–31.

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CHAPTER 4

Structural and Biological studies of a Heteroglycan isolated from an edible mushroom, Pleurotus cystidiosus

4.1. Introduction and earlier work

Edible mushrooms are considered highly nutritious, and delicious [1-3]. They possess several pharmaceutical constituents [4,5]. Chemists and druggists are interested in identifying the immunomodulating compounds of mushrooms which help improvement of the immune function of cancer patients [6] as well as lowering blood pressure and free cholesterol in plasma [7]. In recent years, some bioactive polysaccharides of the mushrooms [8,9] have attracted attention in the field of chemistry, biochemistry, microbiology, and pharmacology. The members of the genus *Pleurotus* (Jacq. Fr) P. Kumm form a heterogeneous group of edible species of high commercial importance [10,11]. The present mushroom *P. cystidiosus* belongs to the genus *Pleurotus* of the gilled mushrooms and cultivated on various agricultural lignocellulosic wastes, saw dust, rice husk, cotton waste, waste tea leaves, corn husks, waste papers etc. or in hardwood, but never on conifer wood [12,13]. *P. cystidiosus* is also known as brown oyster mushroom and has the longest storage life among all pleurotus [12,14]. The mushroom *Pleurotus cystidiosus* is distributed throughout the world and grows in warm climatic zone [10,11]. The energy value of the mushrooms of this genus varies according to the species [15]. *P. cystidiosus* extract shows antioxidant and cytotoxic activity against Hep-2-cancer cell line [16].

Several polysaccharides of the genus *Pleurotus* have been reported by the present research group. Branched (1→3)-, (1→6)-α,β-D-glucan [17], a heteroglycan [18], (1→3)-, (1→6)-β-D-glucan [19] was reported from *P. florida*. Several glucans of *P. florida*, cultivar (Assam florida) [20,21], *P. florida* blue variant [22] and heteroglycans [23] from *P. Sajor-caju* and cultivar Black Japan [24] were isolated and reported. The aim of the present work is to isolate polysaccharide from *P. cystidiosus*, another mushroom of the same genus and the detailed structural characterization with some biological activities have been carried out and discussed in this chapter.

4.2. Present work

4.2.1. Isolation and purification of the polysaccharides from *P. cystidiosus.*

The dry mushroom fruit bodies (250 g) were washed thoroughly with distilled water followed by extraction with hot water, filtration, precipitation in alcohol (83%), dialysis, centrifugation, and freeze drying to yield crude polysaccharide (1.414 g). One fraction (**Fig. 1**) of pure polysaccharide was obtained after fractionating water soluble crude polysaccharide (30 mg) through Sepharose 6B column. Fractions (test tubes, 22-45) positive to phenol/sulfuric acid test [25] were collected and freeze dried to yield 20 mg pure polysaccharide (PCPS). The process was repeated several times to get 120 mg of PCPS.

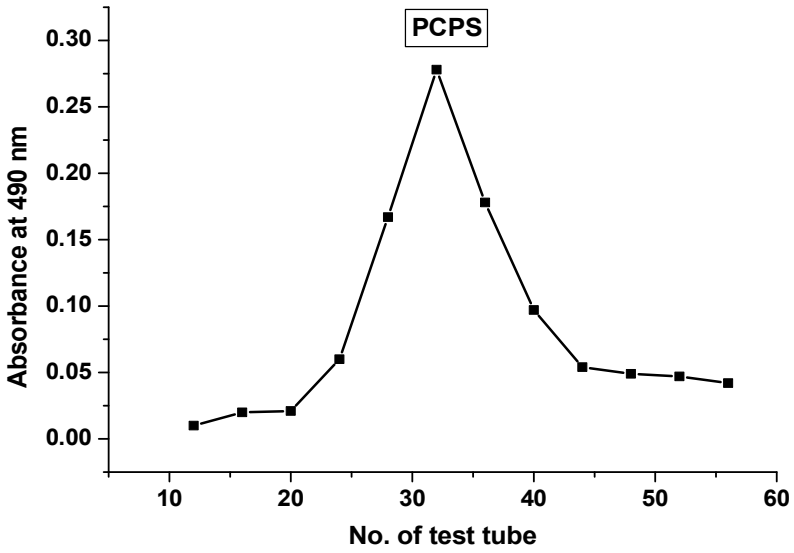


Fig. 1. The gel permeation chromatogram of the polysaccharides (PCPS) isolated from aqueous extract of an edible mushroom (*P. cystidiosus*).

4.2.2. Optical rotation and molecular weight of PCPS

The PCPS showed specific rotation $[\alpha]_D^{30.8}$ +15.9 (*c* 0.10, water). The molecular weight [26] of the PCPS was found to be ~1.46 x10^5 Da, from a calibration curve (**Fig. 2**) prepared with standard dextran.

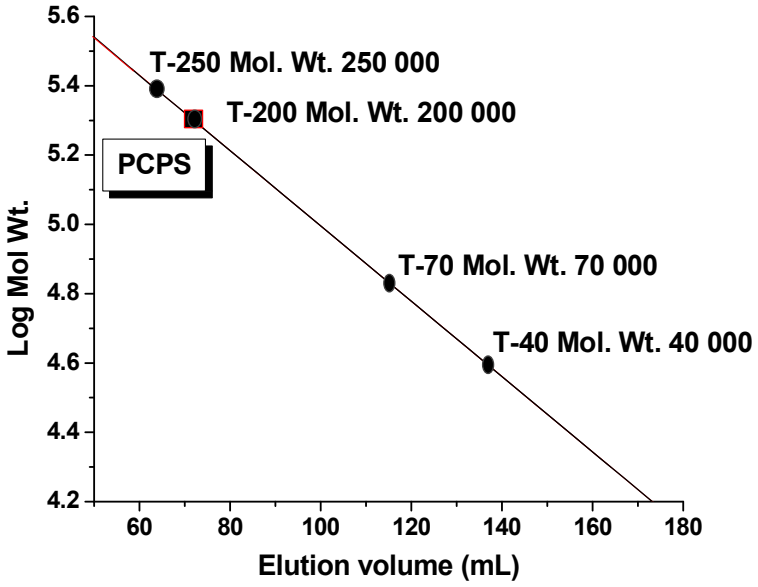


Fig. 2. Determination of molecular weight of the PCPS, isolated from aqueous extract of an edible mushroom (*P. cystidiosus*).

4.2.3. Structural analysis of PCPS

4.2.3.1. Chemical analysis of PCPS

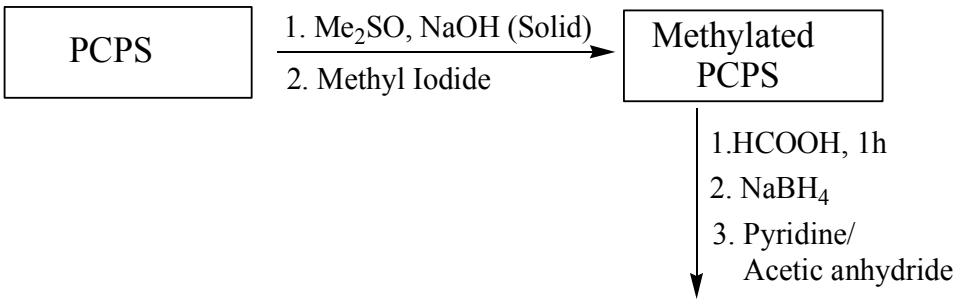
The GLC analysis of the alditol acetates [27] of acid hydrolyzed PCPS showed the presence of glucose, galactose, and mannose in a molar ratio of nearly 6:2:1 respectively. The absolute configuration of the monosaccharides are determined as D-configuration by the method of Gerwig et al. [28].

The PCPS was methylated using the Ciucanu and Kerek [29] method, followed by hydrolysis and preparation of alditol acetates. The GLC-MS analysis revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol; 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl- D-mannitol; 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; 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol; 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-D-galactitol; 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a ratio of 1:1:2:2:1:1:1 (**Table 1, Fig. 3**). These results indicated the presence of terminal glucopyranosyl, terminal mannopyranosyl, (1→3)-glucopyranosyl, (1→6)-glucopyranosyl, (1→6)-galactopyranosyl, (1→2,6)-galactopyranosyl and (1→3,6)-linked glucopyrnosyl moieties. GLC-MS analysis of the alditol acetates of the periodate-oxidized [30,31] methylated [32] PCPS 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 2:1 (**Table 2, Fig. 4**). 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 PCPS.

Table 1. GC-MS data for methylated polysaccharide (PCPS) of an edible mushroom *P. cystidiosus*

Methylated sugars	Linkage types	Molar ratio	Major fragments (m/z)
2,3,4,6-Me_4-Glc*p*	Glc*p*-(1→	1	43,45,59,71,87,101,117,129, 145,161,205
2,3,4,6-Me_4-Man*p*	Man*p*-(1→	1	43,45,59,71,87,101,117,129, 145,161,205

2,4,6-Me_3-Glc*p*	→3)-Glc*p*-(1→	2	43,45,58,74,87,99,101,117,129, 143,161,173,189,203,217, 233,277
2,3,4-Me_3-Glc*p*	→6)-Glc*p*-(1→	2	43,45,58,71,87,99,101,117,129, 143,161,173,189,233
2,3,4-Me_3-Gal*p*	→6)-Gal*p*-(1→	1	43,45,58,71,87,99,101,117,129, 142,161,173,189,207,233
3,4-Me_2-Gal*p*	→2,6)-Gal*p*-(1→	1	43,57,71,87,99,113,129,159, 189,233
2,4-Me_2-Glc*p*	→3,6)-Glc*p*-(1→	1	43,58,74,87,101,117,129,139, 143,159,173,189,233



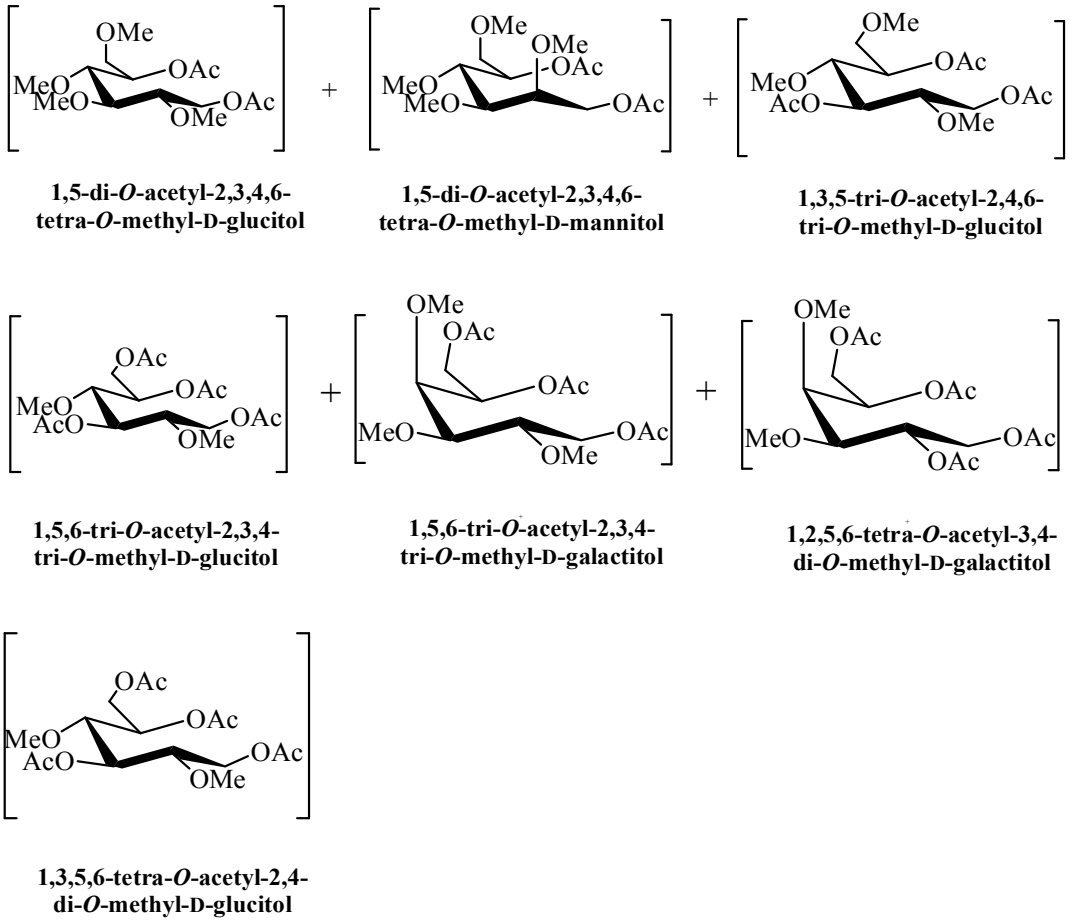


Fig. 3. Schematic presentation of methylation experiment of PCPS isolated from the mushroom *P. cystidiosus*.

Table 2. GC-MS data of periodate oxidized methylated polysaccharide (PCPS) of an edible mushroom *P. cystidiosus*

Methylated sugars	Linkage types	Molar ratio	Major fragments (m/z)
2,4,6-Me_3-Glc*p*	→3)-Glc*p*-(1→	2	43,45,58,74,87,99,101,117,129, 143,161,173,189,203,217, 233,277

2,4-Me_2-Glc*p*	→3,6)-Glc*p*-(1→	1	43,58,74,87,101,117,129,139,143, 159,173,189,233

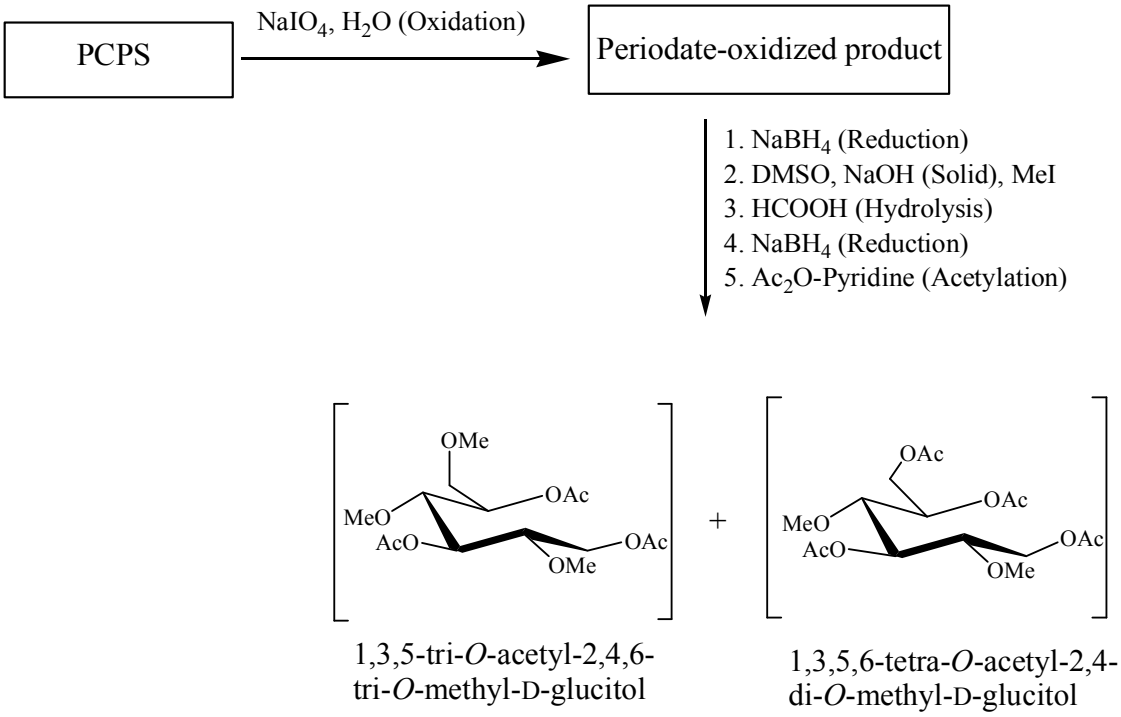


Fig. 4. Schematic presentation of periodate oxidation reactions of PCPS isolated from the mushroom *P. cystidiosus*.

4.2.3.2. 1D and 2D NMR analysis of PCPS

The ^{1}H NMR spectrum (500 MHz, **Fig. 5, Table 3**) of the PCPS at 30 ºC showed four broad peaks which represent nine ^{1}H resonances in the anomeric region at δ 5.11, 5.09, 4.97, 4.96, 4.77, 4.74, 4.52, 4.48, and 4.46 and designated as **A, B, C, D, E, F, G, H,** and **I** respectively according to their decreasing proton chemical shift values. In the ^{13}C (**Fig.**

6a, Table 3) NMR (125 MHz) spectrum at the same temperature, nine peaks appeared in the anomeric region at δ 102.8, 102.6, 102.3, 102.2, 101.8, 101.5, 98.2, 98.0, and 97.6. Based on the result of HSQC spectrum (**Fig. 7a and 7b, Table 3**), the anomeric carbon signals at δ 102.8, 102.6, 102.3, 102.2, 101.8, 101.5, 98.2, 98.0, and 97.6 corresponded to the proton signals of δ 4.46 (**I**), δ 4.74 (**F**), δ 4.48 (**H**), δ 4.52 (**G**), δ 4.77 (**E**), δ 5.09 (**B**), δ 5.11 (**A**), δ 4.97 (**C**), and δ 4.96 (**D**) respectively. All the ^{1}H and ^{13}C signals (**Table 3**) were assigned using DQF–COSY, TOCSY, and HSQC experiments. From DQF–COSY experiment the proton coupling constants were measured and one bond C–H couplings were measured from proton coupled ^{13}C spectrum.

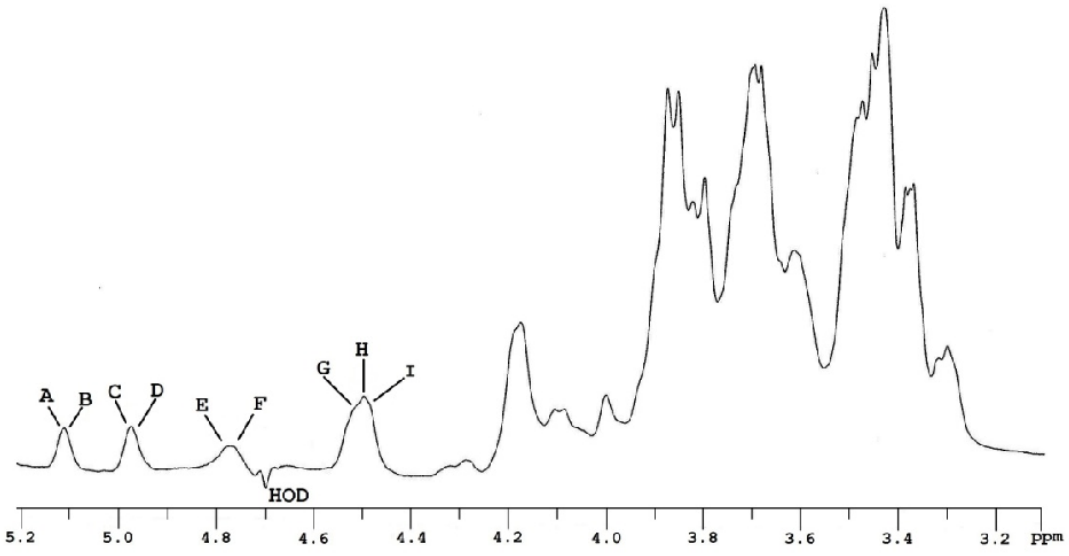


Fig. 5. ^{1}H NMR spectrum (500 MHz, D_2O, 30 °C) of the PCPS isolated from an edible mushroom *P. cystidiosus*.

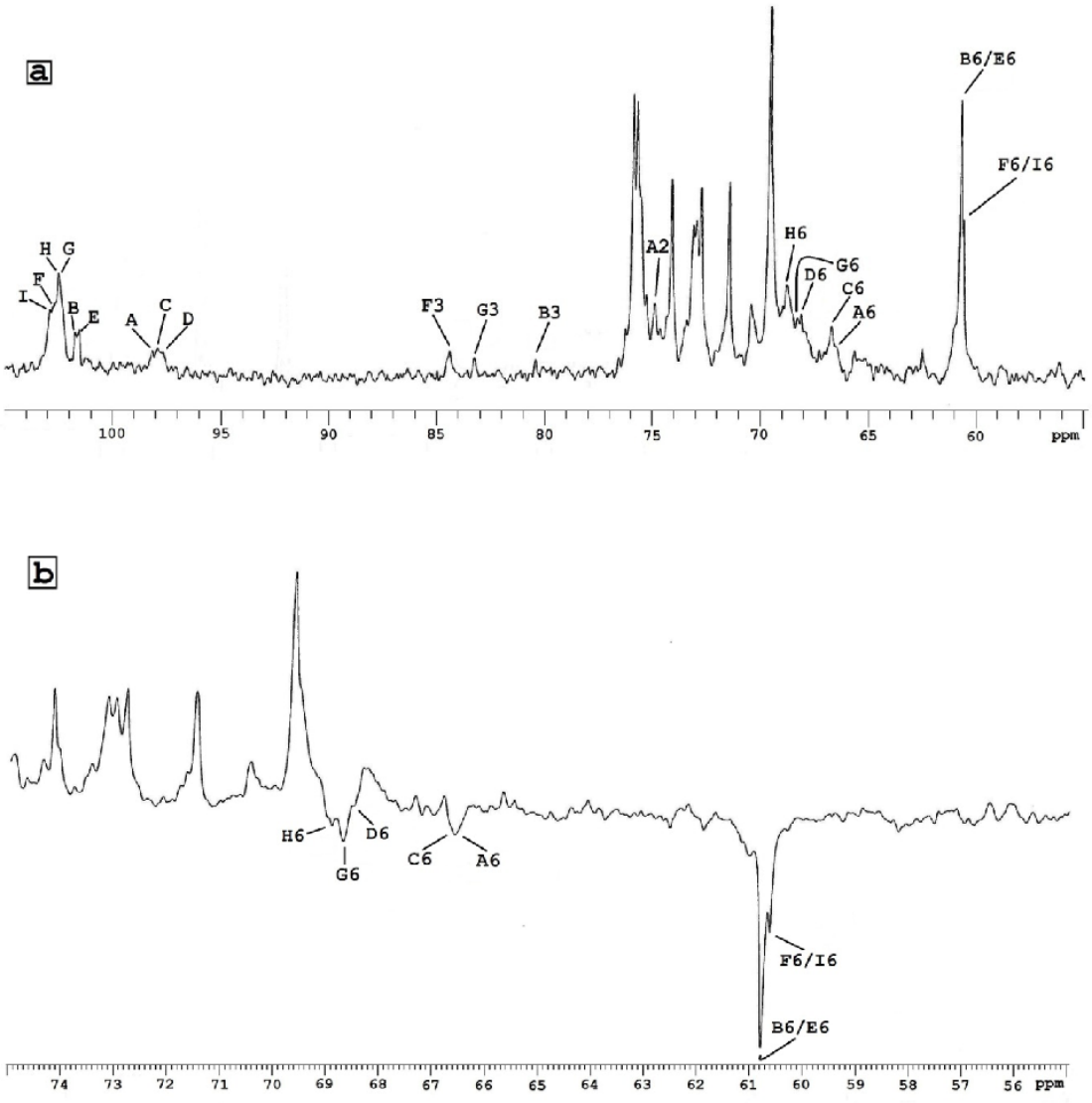


Fig. 6. (**a**) ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of the polysaccharide isolated from *P. cystidiosus*. (**b**) DEPT-135 spectrum (D_2O, 30 °C) of PCPS isolated from edible mushroom *P. cystidiosus*.

Table 3: The ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts of the PCPS isolated from the mushroom *P. cystidiosus* recorded 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	3.86	3.99	4.11	4.20	3.62[c], 3.87[d]
A	98.2	75.0	69.0	68.5	68.3	66.5
→3)- α-D-Glc*p*-(1→	5.09	3.79	3.68	3.49	3.65	3.70[c], 3.85[d]
B	101.5	71.4	80.5	71.4	72.5	60.8
→6)- α -D-Gal*p*-(1→	4.97	3.80	4.03	4.11	4.20	3.67[c], 3.90[d]
C	98.0	68.4	69.2	68.5	68.3	66.6
→6)- α-D-Glc*p*-(1→	4.96	3.81	3.40	3.59	4.07	4.02[c], 4.15[d]
D	97.6	71.4	72.9	69.8	69.9	68.4
β-D-Man*p*-(1→	4.77	4.05	3.69	3.55	3.37	3.73[c], 3.89[d]
E	101.8	69.9	75.3	67.2	75.9	60.8
→3)- β-D-Glc*p*-(1→	4.74	3.32	3.71	3.42	3.49	3.69[c], 3.85[d]
F	102.6	72.9	84.2	69.5	75.5	60.6
→3,6)- β-D-Glc*p*-(1→	4.52	3.49	3.77	3.40	3.59	3.84[c], 4.15[d]
G	102.2	72.8	83.2	69.5	74.2	68.6
→6)- β-D-Glc*p*-(1→	4.48	3.28	3.43	3.45	3.63	3.86[c], 4.19[d]
H	102.3	73.0	75.5	69.6	74.4	68.9
β-D-Glc*p*-(1→	4.46	3.28	3.47	3.36	3.45	3.67[c], 3.80[d]
I	102.8	73.0	75.5	69.5	75.6	60.6

[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.

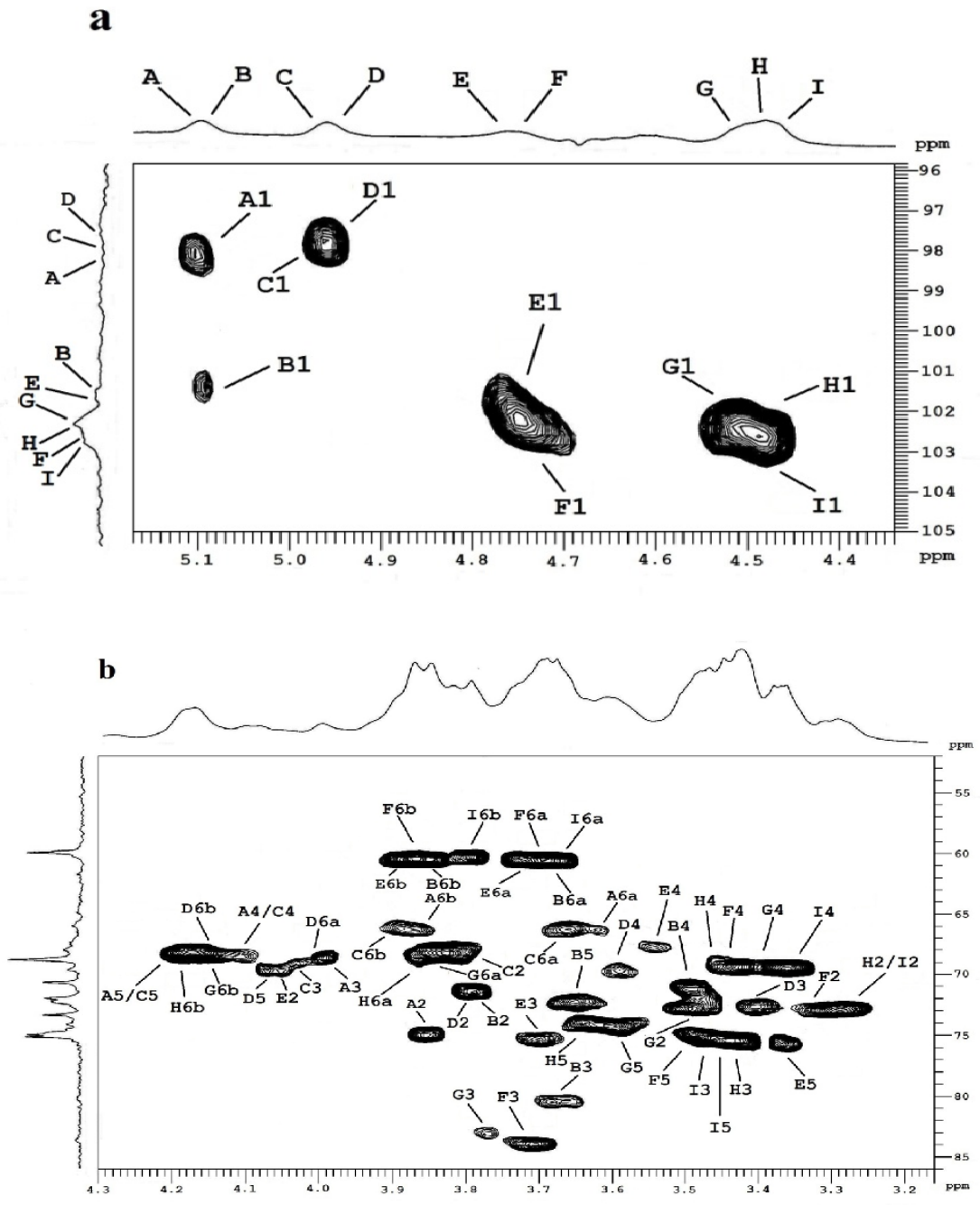


Fig. 7. The HSQC spectrum (D_2O, 30 °C) of (**a**) anomeric part and (**b**) other than anomeric part of the PCPS isolated from edible mushroom *P. cystidiosus*.

The galacto configuration of residues **A** and **C** were assigned from the large coupling constant values of $J_{H\text{-}2,\ H\text{-}3}$ ~9 Hz and small coupling constant value $J_{H\text{-}3,\ H\text{-}4}$ ~3.5 Hz. The α-configuration of both **A** and **C** 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 75.0) and C-6 (δ 66.5) with respect to standard values of methyl glycosides [33,34] indicate that residue **A** was a (1→2,6)-linked α-D-galactopyranosyl residue. The downfield shifts of C-6 (δ 66.6) with respect to standard values of methyl glycosides indicated that the residue **C** was a (1→6)-linked α-D–galactopyranosyl residue. The linkage at C-6 of the both residues **A** and **C** were further established from DEPT-135 spectrum (**Fig. 6b**). From these observations it can be concluded that the residue **A** was (1→2,6)-α-D-galactopyranosyl and **C** was (1→6)-α-D-galactopyranosyl.

The α-linked configuration of residue **B** and **D** was confirmed from the anomeric proton chemical shift and the coupling constant values at $J_{H\text{-}1,H\text{-}2}$ (~3.0 Hz) and $J_{C\text{-}1,H\text{-}1}$ (~171 Hz). 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 residues **B** and **D** were D-glucopyranosyl moieties (Glc*p*). The residues **B** showed downfield shift of C-3 (δ 80.5) with respect to standard values of methyl glycosides which indicated that the residue **B** was linked at this position. Thus, the residue **B** was confirmed as (1→3)-α-D-glucopyranosyl [35] moiety. In case of residue **D**, the downfield shift of C-6 (δ 68.4) with respect to standard values of methyl glycosides indicated that the residue **D** was linked at this position. The linkage at C-6 of the residue **D** was further confirmed from DEPT–135 spectrum (**Fig. 6b**). The other values for carbons and protons corresponded nearly to the standard values. So, **D** was confirmed as (1→6)-α-D-glucopyranosyl residue.

The manno configuration of residue **E** was supported from the large coupling constant values of $J_{H\text{-}3,H\text{-}4}$ (~7.5 Hz) and $J_{H\text{-}4,H\text{-}5}$ (~9.5 Hz). The anomeric proton and carbon signals at δ 4.77 and δ 101.8, as well as the low coupling constant values of $J_{H\text{-}1,H\text{-}2}$ (~0 Hz), $J_{C\text{-}1,H\text{-}1}$ (~160.0 Hz) confirmed that residue **E** was present in β-configuration. All the proton and carbon chemical shifts of residue **E** nearly corresponded to the standard values

of methyl glycosides of β-D-mannose. So, **E** was confirmed as terminal β-D-mannopyranosyl residue.

The β-configuration of the residues **F, G, H,** and **I** were established from the coupling constant values $J_{H\text{-}1,H\text{-}2}$ (~8.0 Hz), $J_{C\text{-}1,H\text{-}1}$ (~160 - 161 Hz) and their anomeric proton and carbon chemical shifts ranging from δ 4.46 – 4.74 and δ 102.2 – 102.8 respectively. The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values (~10.0 Hz) of the residues **F, G, H,** and **I** conformed their D-glucopyranosyl configuration (Glc*p*). The anomeric carbon signal of **F** at δ 102.6 and the downfield shifts of C-3 (δ 84.2) with respect to standard value of methyl glycoside [33,34] indicated that the residue **F** was (1→3)-β-D-glucopyranosyl moiety. The downfield shifts at C-3 (δ 83.2) and C-6 (δ 68.6) of residue **G** with respect to standard value of methyl glycoside indicated that it was (1→3,6)-β-D-Glc*p*. Since, residue **G** was one of the rigid part of this glucan, its C-3 (δ 83.2) value appeared at slightly upfield region compared to the C-3 (δ 84.2) of (1→3)-linked residue **F**. For the residue **H**, the downfield shift of C-6 (δ 68.9) 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. 6b**). In case of carbon chemical shifts of residue **I** from C-1 to C-6 corresponded nearly to the standard values of methyl glycosides of β-D-Glucose indicating **I** was terminal β-D-Glc*p*.

The sequence of glycosyl residues (**A** to **I**) were established from ROESY (**Fig. 8, Table 4**) as well as NOESY experiment. In ROESY experiment, the inter-residual contacts **A**H-1/**C**H-6a, 6b; **B**H-1/**F**H-3; **C**H-1/**B**H-3; **D**H-1/**A**H-6a, 6b; **E**H-1/**A**H-2; **F**H-1/**G**H-3; **G**H-1/**H**H-6a, 6b; **H**H-1/**D**H-6a, 6b; and **I**H-1/**G**-6a, 6b along with other intra-residual contacts were also observed (**Fig. 8**). From the ROESY spectrum the sequences of connectivity established as follows: **A** (1→6) **C**; **B** (1→3) **F**; **C** (1→3) **B**; **D** (1→6) **A**; **E** (1→2) **A**; **F** (1→3) **G**; **G** (1→6) **H**; **H** (1→6) **D;** and **I** (1→6) **G**. These data and connectivities clearly supported the positions of substitution and sequence of sugar residues in the polysaccharide.

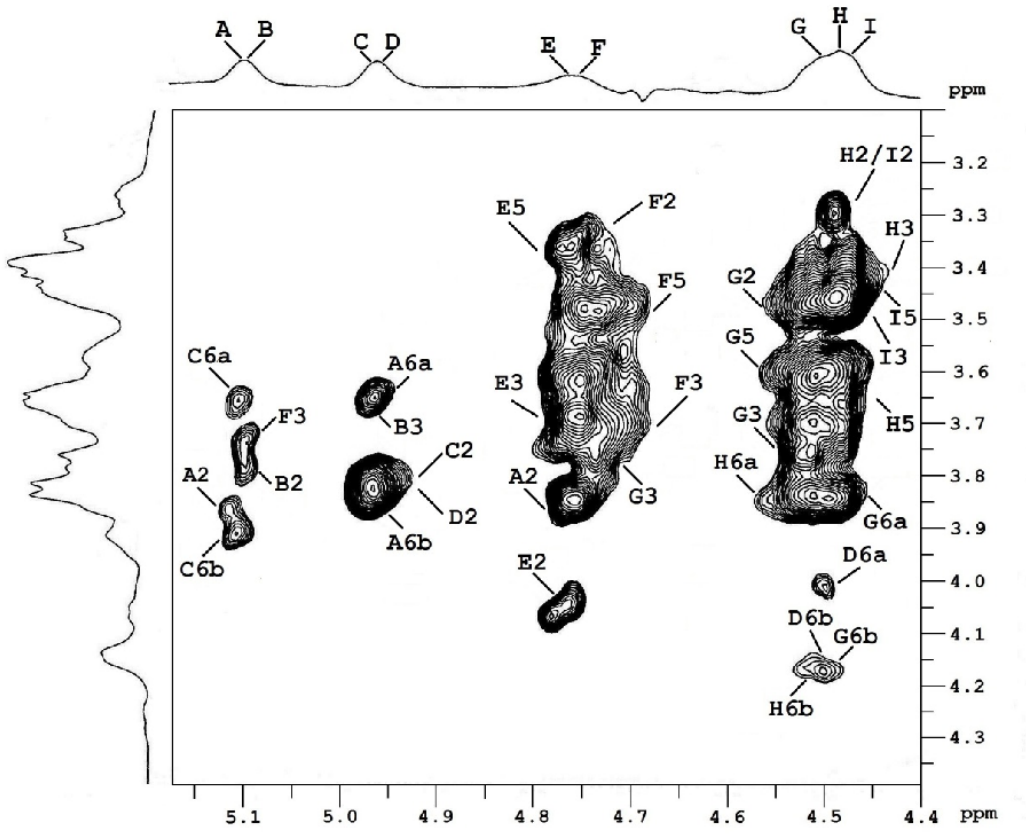


Fig. 8. The part of ROESY spectrum of PCPS, isolated from an edible mushroom *P. cystidiosus*. The mixing time was 300 ms.

Table 4: ROESY data for the polysaccharide (PCPS) isolated from the mushroom of *P. cystidiosus* in D_2O at 30 °C.

Glycosyl Residue	Anomeric proton	ROE contact proton		
	δ	δ	Residue	Atom
→2,6)- α-D-Gal*p*-(1→		3.67	**C**	H-6a
A	5.11	3.90	**C**	H-6b
		3.86	**A**	H-2
→3)- α-D-Glc*p*-(1→	5.09	3.71	**F**	H-3
B		3.79	**B**	H-2
→6)- α -D-Gal*p*-(1→	4.97	3.68	**B**	H-3
C		3.80	**C**	H-2
→6)- α-D-Glc*p*-(1→	4.96	3.62	**A**	H-6a
D		3.87	**A**	H-6b
		3.81	**D**	H-2
β-D-Man*p*-(1→	4.77	3.86	**A**	H-2
E		4.05	**E**	H-2
		3.69	**E**	H-3
		3.37	**E**	H-5
		3.77	**G**	H-3
→3)- β-D-Glc*p*-(1→	4.74	3.32	**F**	H-2
F		3.71	**F**	H-3
		3.49	**F**	H-5
		3.86	**H**	H-6a
		4.19	**H**	H-6b
→3,6)- β-D-Glcp-(1→	4.52	3.49	**G**	H-2
G		3.77	**G**	H-3
		3.59	**G**	H-5
		4.02	**D**	H-6a
→6)- β-D-Glcp-(1→	4.48	4.15	**D**	H-6b
H		3.28	**H**	H-2
		3.43	**H**	H-3
		3.63	**H**	H-5
		3.84	**G**	H-6a
β-D-Glcp-(1→	4.46	4.15	**G**	H-6b
I		3.28	**I**	H-2
		3.47	**I**	H-3
		3.45	**I**	H-5

Smith degraded product (SDPCPS) of PCPS was prepared and NMR experiments were again carried out to confirm the linkages of the heteroglycan. The ^{13}C NMR (125 MHz) spectrum (**Fig. 9, Table 5**) at 30 ºC of SDPCPS showed three anomeric carbon signals at δ 103.2, 102.8, and 98.2. The anomeric carbon signals at δ 98.2 corresponded to α-D-Glc*p* (**J**), δ 103.2 corresponded to (1→3)-β-D-Glc*p* (**K**) and δ 102.8 corresponded to (1→3)-β-D-Glc*p* (**L**) residues respectively. The carbon signals at C-1, C-2, and C-3 of the glycerol moiety (Gro) were assigned as δ 66.4, 72.1, and 62.6 respectively. The glycerol moiety (**M**) was generated from (1→6)-β-D-Glc*p* (**H**) after periodate oxidation followed by Smith degradation which was attached to (1→3)-linked β-D-Glc*p* moiety (**L**) generated from (1→3,6)-β-D-Glc*p* (**G**). The terminal α-D-Glc*p* (**J**) of SDPCPS was generated from (1→3)-α-D-Glc*p* (**B**) and (1→3)-β-D-Glc*p* (**K**) was produced from unaffected residue (1→3)-β-D-Glc*p* (**F**).

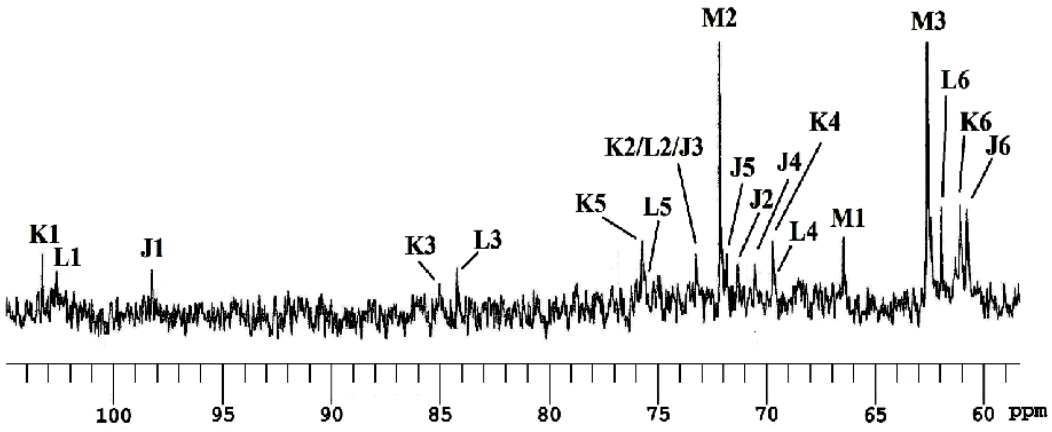


Fig. 9. ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of the Smith-degraded glycerol containing trisaccharide isolated from the edible mushroom *P. cystidiosus*.

Table 5. The ^{13}C NMR[n] chemical shifts of Smith-degraded glycerol-containing disaccharide of the mushroom *P. cystidiosus* in D_2O at 30 °C

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
α -D-Glcp-(1→ **J**	98.2	71.4	73.2	70.4	71.8	60.8
→3)-β-D-Glcp-(1→ **K**	103.2	73.2	85.0	69.7	75.8	61.1
→3)-β-D-Glcp-(1→ **L**	102.8	73.2	84.2	69.6	75.6	61.9
Gro-(3→ **M**	66.4	72.1	62.6			

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

Thus, the Smith degradation results in the formation of a glycerol containing trisaccharide from the parent PCPS and the structure of which was established as:

J **K** **L** **M**

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

These results further confirmed the presence of the repeating moiety in the heteroglycan (PCPS) isolated from the edible mushroom *P. cystidiosus* and the following structure of the PCPS was characterized and elucidated on basis of chemical analysis, and 1D/2D NMR studies as:

B **F** **G** **H** **D** **A** **C**

→3)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→

6 2

↑ ↑

1 1

β-D-Glc*p* β-D-Man*p*

I **E**

4.2.4. Biological properties of PCPS

The cytotoxic effect of the PCPS was studied on human blood lymphocytes with increasing concentrations ranging from 10 µg/mL to 400 µg/mL using 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) assay method (**Fig. 10**). It was observed that PS has no considerable cytotoxic effect to normal lymphocytes up to 200µg/mL. But at higher dose 400µg/mL, the polysaccharide possesses mild levels of toxicity. It indicates that 200µg/mL is safe with respect to the other higher doses.

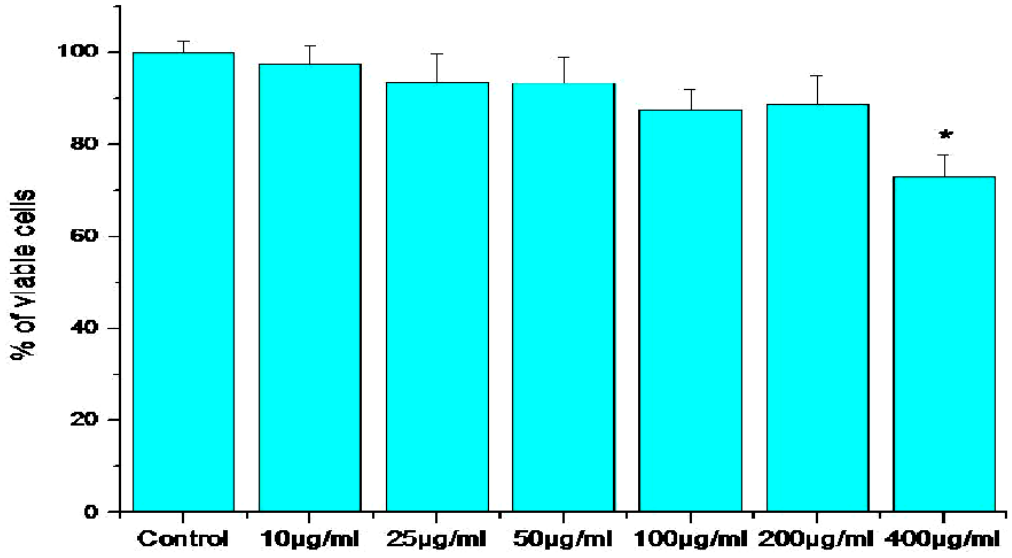


Fig. 10. Cytotoxicity of PCPS on peripheral blood lymphocytes by MTT assay. (Values are expressed as mean ± S.E.M., n=6. * indicates significant difference ($P<0.05$) compared to control group).

Glutathione is an important antioxidant in cellular system. Therefore, glutathione level in cell, both its reduced and oxidized states was measured (**Fig. 11a and b**). The reduced glutathione level (GSH, **Fig. 11a**) was increased significantly up to 200 µg/mL and the maximum level of increment was found at 200µg/mL. But at 400µg/mL, the

reduced glutathione level (GSH, **Fig. 11a**) was moderately decreased and the mild augmentation of oxidized form of glutathione level (GSSG, **Fig. 11b**) was observed.

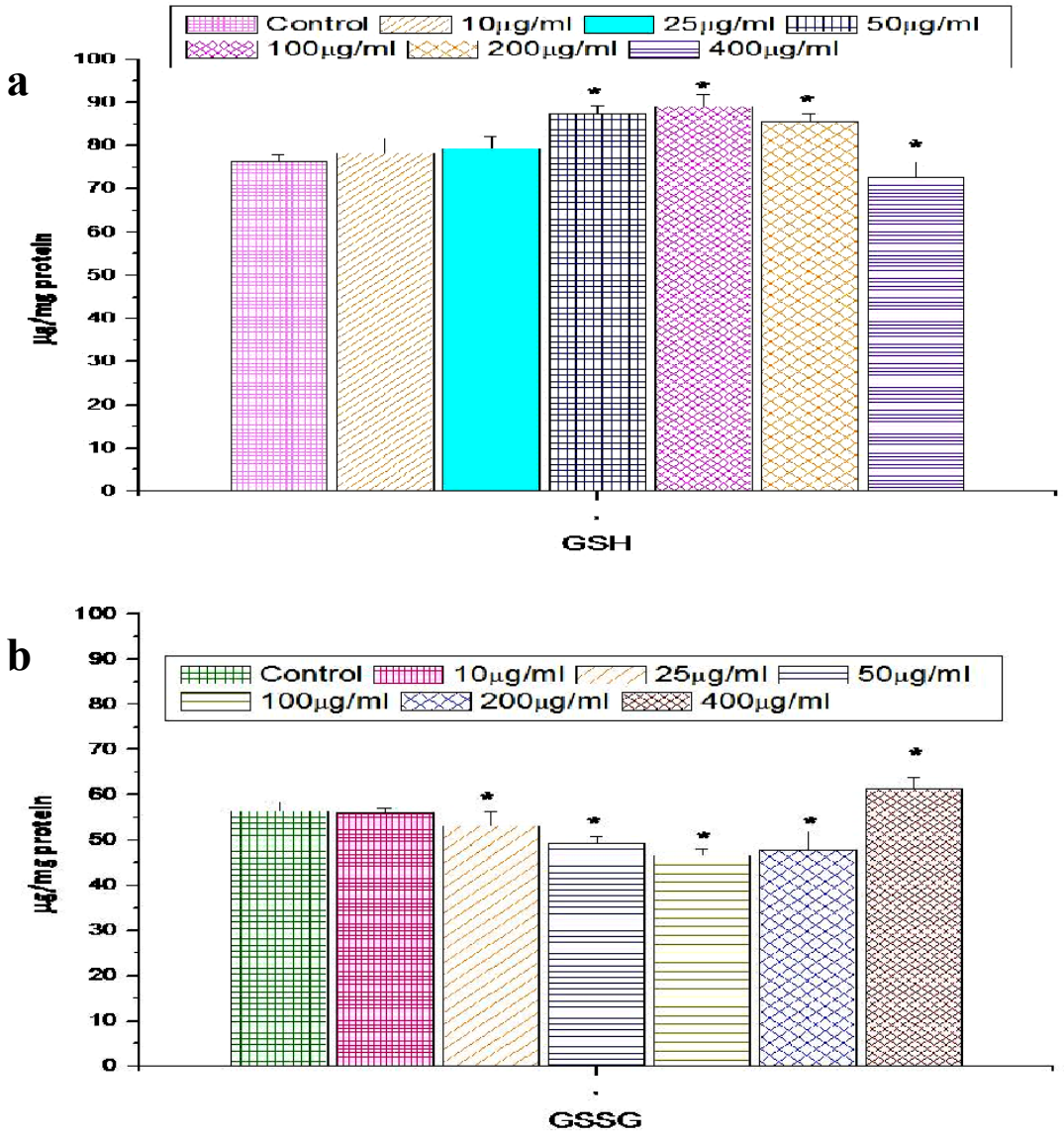


Fig. 11. (**a**) Concentration of reduced glutathione (GSH) level of PCPS treated in normal human lymphocytes. (**b**) Concentration of oxidized glutathione (GSSG) level of PCPS in normal human lymphocytes. (Values are expressed as mean ± S.E.M., n=6. * indicates significant difference ($P<0.05$) compared to control group).

Study of lipid peroxidation is one of the important parameters to assess the cellular damage. It deactivates the cellular components and protective enzymes, and thereby plays a vital role of oxidative stress in biological systems [36]. Several toxic by-products, especially malondialdehyde is released due to lipid peroxidation. Hence, lipid peroxidations in lymphocytes were measured in terms of malondialdehyde (MDA) release (**Fig. 12**). The result showed no significant changes at different concentration except at 100 µg/mL, where it decreases significantly. But it showed slightly increase of MDA level at the dose 400µg/mL.

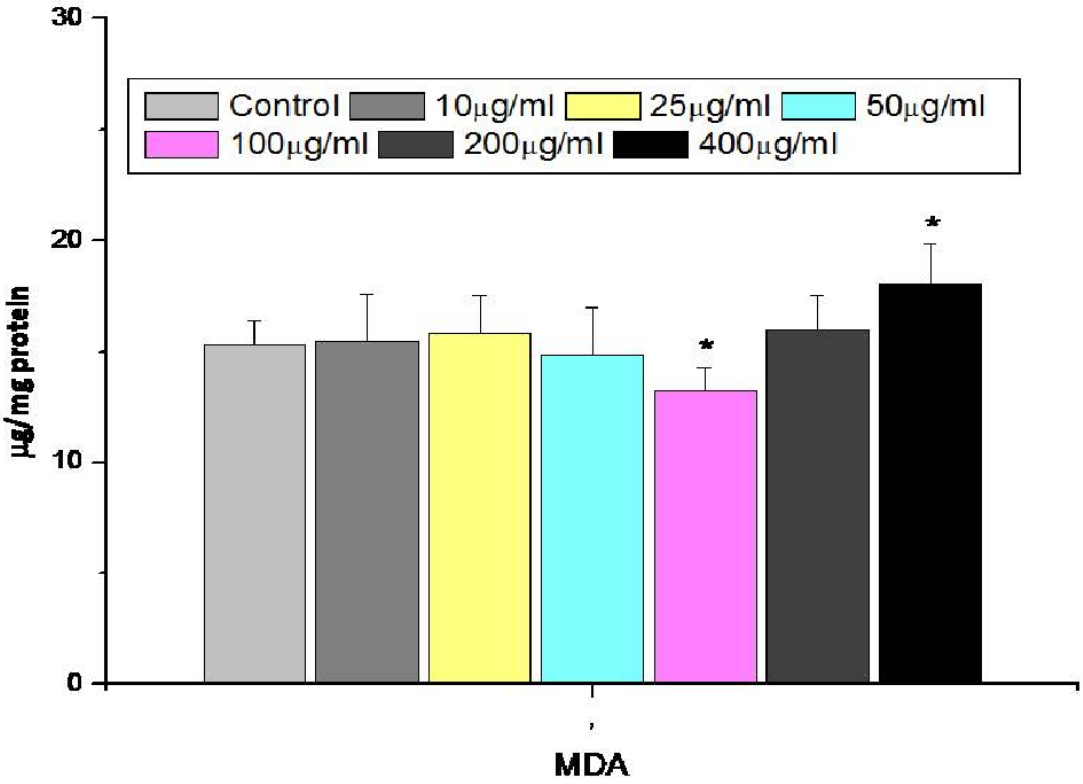


Fig. 12. Concentration of Lipid peroxidation in terms of Malonaldehyde (MDA) level of PCPS treated normal human lymphocytes. (Values are expressed as mean ± S.E.M., n=6. * indicates significant difference ($P<0.05$) compared to control group).

It is evident from these experiments that, in vitro application of PCPS has good effect up to a certain level. It does not induce any cellular damage in lymphocytes associated

with enhanced MDA level, GSSG level and decreased GSH level. The cytotoxic profile of PCPS in lymphocytes indicated 200µg/mL is safe and effective, whereas concentrations higher than 200 µg/mL showed slight increase of cytotoxicity. These findings suggest that the PCPS exhibits antioxidant and beneficial role on cellular system.

4.3. Conclusion

The polysaccharide, a water soluble heteroglycan (PCPS) was isolated from the aqueous extract of an edible mushroom *Pleurotus cystidiosus*. The PCPS indicated the presence of a repeating unit with a backbone consisting of one unit of (1→6)-β-D-glucopyranosyl, two (1→3)-β-D-glucopyranosyl, one (1→3)-α-D-glucopyranosyl, one (1→6)-α-D-glucopyranosyl, and two (1→6)-α-D-galactopyranosyl moieties respectively, out of which one (1→3)-β-D-glucopyranosyl residue was branched at *O*-6 with terminal β-D-glucopyranosyl and another (1→6)-α-D-galactopyranosyl residue was branched at *O*-2 with terminal β-D-mannopyranosyl moiety. The heteroglycan was found to exhibit cellular activities at different concentrations (10, 25, 50, 100, 200, 400 µg/mL) and maintained the redox balance as well as reduced lipid per oxidation which protect the cell destruction.

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3. Structural characterization and antioxidant activity of a glucan from *Meripilus giganteus*, Prasenjit Maity, Ashis K. Nandi, Dilip K. Manna, Manabendra Pattanayak,Ipsita K. Sen, Sunil K. Bhanja, Surajit Samanta, **Bibhash C. Panda**, Soumitra Paloi, Krishnendu Acharya, Syed S. Islam, **Carbohydrate Polymers**, **2017**, *157*, 1237–1245.

4. Polysaccharide of an edible truffle *Tuber rufum*: Structural studies andeffects on human lymphocytes, Manabendra Pattanayak, Surajit Samanta, Prasenjit Maity, Dilip K. Manna, Ipsita K. Sen, Ashis K. Nandi, **Bibhash C. Panda**, Sourav Chattopadhyay, Somenath Roy, Atish K. Sahoo, Nibha Gupta, Syed S. Islam, **International Journal of Biological Macromolecules**, **2017**, *95*, 1037–1048.

5. Structural elucidation and immunostimulating property of a novel polysaccharide extracted from an edible mushroom *Lentinus fusipes*, Dilip K. Manna, Prasenjit Maity, Ashis K. Nandi, Manabendra Pattanayak, **Bibhash C. Panda**, Amit K. Mandal, Satyajit Tripathy, Krishnendu Acharya, Atish K. Sahoo, Nibha Gupta, Somnath Roy, Syed S. Islam, **Carbohydrate Polymers**, **2017**, *157*, 1657–1665.

APPENDIX

1. Pectic polysaccharide from the green fruits of Momordica charantia, (Karela): structural characterization and study of immunoenhancing and antioxidant properties, **Bibhash C. Panda**, Soumitra Mondal , K. Sanjana P. Devi, Tapas K. Maiti, Somanjana Khatua, Krishnendu Acharya, Syed S. Islam, **Carbohydrate Research, 2015**, *401*, 24-31.

2. Heteroglycan of an edible mushroom *Pleurotus* cystidiosus: Structural characterization and study of biological activities, **Bibhash C. Panda**, Prasenjit Maity, Ashis K. Nandi, Manabendra Pattanayak, Dilip K. Manna, Soumitra Mondal , Satyajit Tripathy, Somenath Roy, Krishnendu Acharya, Syed S. Islam, **International Journal of Biological Macromolecules, 2017**, *95*, 833–842.

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Pectic polysaccharide from the green fruits of *Momordica charantia* (Karela): structural characterization and study of immunoenhancing and antioxidant properties

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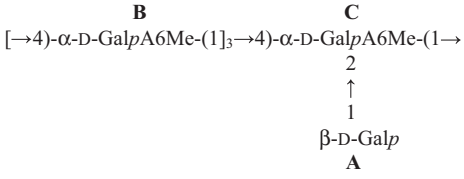
ABSTRACT

A water soluble pectic polysaccharide (PS) isolated from the aqueous extract of the green fruits of *Momordica charantia* contains D-galactose and D-methyl galacturonate in a molar ratio of nearly 1:4. It showed splenocyte, thymocyte as well as macrophage activations. Moreover, it exhibited potent antioxidant activities. On the basis of total acid hydrolysis, methylation analysis, periodate oxidation, and 1D and 2D NMR studies, the structure of the repeating unit of the pectic polysaccharide was established as:



1. Introduction

Polysaccharides isolated from different fruits and vegetables[1–3] have been found to exhibit immunoenhancing and antioxidant activities. The plant *Momordica charantia* commonly known as bitter gourd or karela belongs to the family Cucurbitaceae and economically important medicinal plant, widely grown in India and other parts of the Indian subcontinent. Two varieties of this plant found in India are *M. charantia* var. *charantia* which produces large size fruits and *M. charantia* var. *muricata* which gives fruits of smaller size.[4] Fruit and seed extracts of *M. charantia* possess anti-HIV, antimicrobial, antitumor, and antidiabetic properties.[5] Reactive oxygen species can react with the biological molecules such as DNA, proteins, lipids, and carbohydrates that may lead to the development of a wide variety of pathological disorders.[6] Consumption of food containing antioxidants has been found to offer protection against these diseases.[7] Polysaccharides extracted from plants are particularly good source of compounds with antioxidant properties. The structure and molecular mass of the polysaccharide have been found to play an important role in their biological activities.[8] The polysaccharide isolated from the fruits of *M. charantia* was reported to possess glucose, galactose, arabinose, rhamnose, and mannose with varying proportions and its sulfated derivative was found to exhibit antioxidant properties[9] but no detailed structural characterization of the polysaccharide was carried out. In the present investigation a pectic polysaccharide composed of methyl galacturonate and galactose was isolated

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0008-6215/

from hot water extract of the green fruits of *M. charantia* and therefore, a detailed structural characterization and study of its immunoenhancing and antioxidant properties were carried out and reported herein.

2. Results and discussion

2.1. Chemical analysis of the polysaccharide

The pure polysaccharide (PS) showed specific rotation $[\alpha]_D^{29.8}$ +168.9 (*c* .098, H_2O). Molecular weight[10] of the polysaccharide was found to be $\sim 2.0 \times 10^2$ kDa. Paper chromatographic analysis[11] of the hydrolyzed product of the PS showed the presence of galactose and galacturonic acid. The GLC analysis of the alditol acetates[12] of the sugars showed the presence of galactose only. But the carboxyl-reduced[13] polysaccharide on hydrolysis followed by GLC examination of the corresponding alditol acetates showed also the presence of only galactose. The absolute configurations of galactose and galacturonic acid were determined as D-configuration by Gerwig et al.[14] The PS was methylated using the Ciucanu and Kerek method[15] followed by hydrolysis and preparation of alditol acetates.[12] GLC–MS analysis revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol indicating that the D-galactopyranosyl moiety is present as non-reducing end in the polysaccharide. The carboxyl-reduced[16] methylated polysaccharide on hydrolysis followed by GLC–MS analysis of corresponding alditols showed the presence of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol, 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-D-galactitol, and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol in a molar ratio 3:1:1. The appearance of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol indicated the presence of (1 → 4)-linked D-galacturonic acid and 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-D-galactitol indicated (1→2,4)-linked D-galacturonic acid and the D-galactose is attached at the non reducing end of the backbone of the polysaccharide. Thereafter, a periodate oxidation[17,18] experiment was carried out with the polysaccharide. The periodate oxidized, reduced material on hydrolysis with trifluoroacetic acid followed by paper chromatography[11] experiment showed the presence of D-galacturonic acid only. GLC–MS analysis of the alditol acetates of the periodate-oxidized, carboxyl-reduced methylated polysaccharide showed the retention of the peak corresponding to 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-D-galactitol, indicating further that D-galacturonic acid was retained as it was (1 → 2,4)-linked moiety and the non reducing end D-galactose and (1 → 4)-linked D-galacturonic acid were destroyed during periodate oxidation.

2.2. NMR and structural analysis of polysaccharide

The ^{1}H NMR spectrum (500 MHz, Fig. 1) of the PS showed three peaks in the anomeric region at δ 4.59, 4.94, and 5.06 and designated as **A**, **B**, and **C**, respectively, according to their increasing anomeric chemical shift values. In the ^{13}C NMR (125 MHz) spectrum (Fig. 2) three anomeric carbon signals appeared at δ 105.0, 100.8, and 100.0 were correlated to the residues **A**, **B**, and **C**, respectively, from the HSQC spectrum (Fig. 3). All the ^{1}H and ^{13}C signals (Table 1)

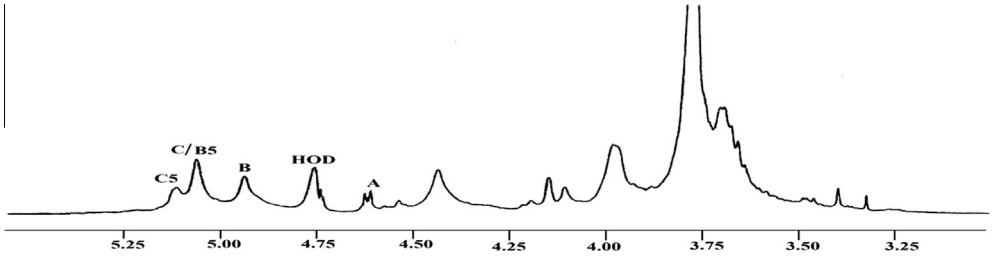


Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 30 °C) of the polysaccharide (PS) isolated from the fruits of *Momordica charantia* (Karela).

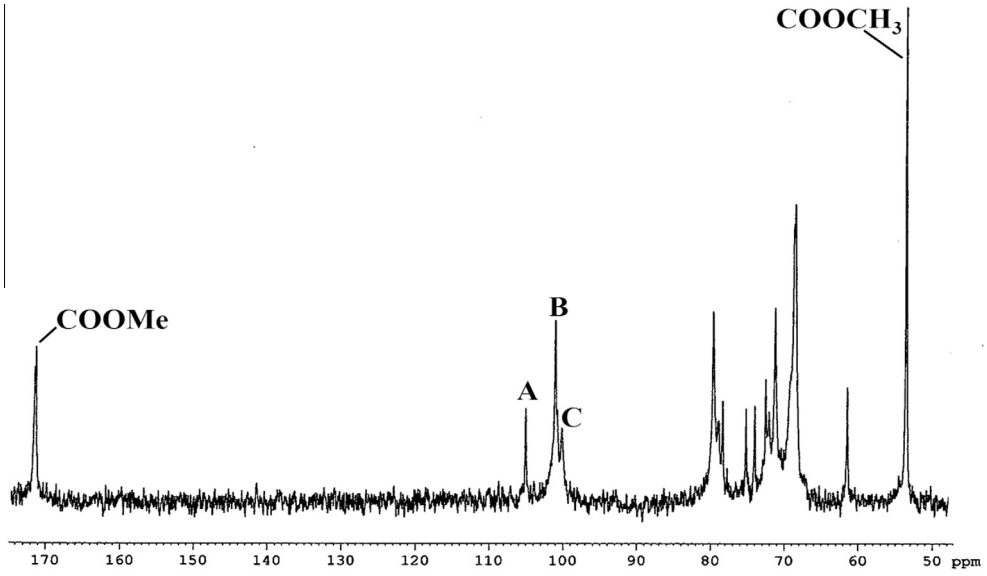


Figure 2. ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of the polysaccharide (PS) isolated from the fruits of *Momordica charantia* (Karela).

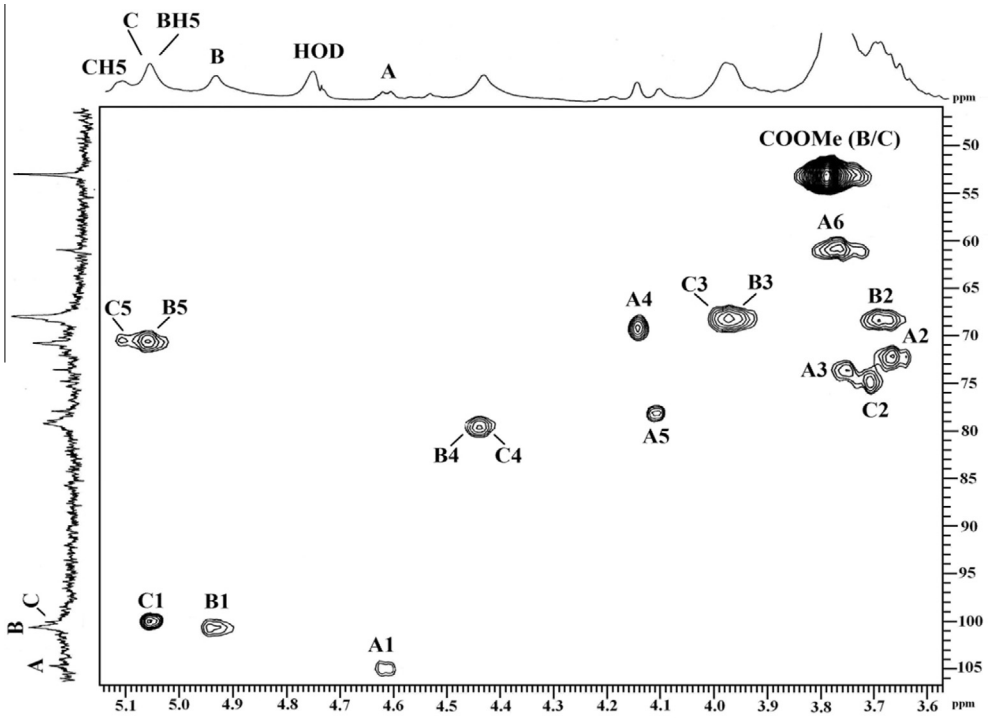


Figure 3. Part of HSQC spectrum (D_2O, 30 °C) of the polysaccharide (PS) isolated from the fruits of *Momordica charantia* (Karela).

were assigned using DQF–COSY, TOCSY, and HSQC NMR experiments.

Residue **A** has an anomeric chemical shift at δ 4.59 and large coupling constant values $J_{H\text{-}1,\ H\text{-}2}$ ~8.4 Hz and $J_{H\text{-}1,\ C\text{-}1}$ ~161 Hz indicating that it was β-linked residue. A large $J_{H\text{-}2,\ H\text{-}3}$ ~9.3 Hz and also small $^3J_{H\text{-}3,\ H\text{-}4}$ ~3.1 Hz indicated that **A** is β-D-galactosyl residue. The carbon signals from C-1 to C-6 of residue **A** corresponded nearly to the standard values of methyl glycosides,[19] and therefore the residue **A** was established as terminal β-D-galactopyranosyl moiety which is attached at the C-2 position of residue **C**, confirmed from the cross peak of **A**C1/**C**H2 in HMBC experiment (Fig. 4, Table 2).

The residues **B** and **C** were established as α-linked residue from their coupling constant values $J_{H\text{-}1,\ H\text{-}2}$ (~3.1 Hz and ~3.0 Hz) and $J_{H\text{-}1,\ C\text{-}1}$ (171 Hz and 170 Hz). **B** and **C** residues have relatively high chemical shift values of H-5 signal in anomeric region at δ 5.05 and 5.11, respectively,[20] and weak coupling between H-3, H-4, and H-5 indicated that the residues **B** and **C** are α-D-galacturonosyl moieties.

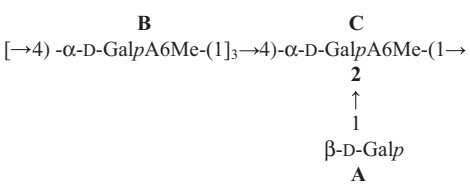
The residue **B** has an anomeric proton chemical shift at δ 4.94 and anomeric carbon chemical shift at δ 100.8. The carbon signals of residue **B** were observed at δ 68.4, 68.3, 79.5, 70.6, and 171 corresponding to C-2. C-3, C-4, C-5, and C-6 (carbonyl carbon) respectively. The C-4 signal at δ 79.5 showed a downfield shift compared to that of standard methyl glycosides[19] due to α-glycosylation effect indicating the (1 → 4)-linked β-residue. The appearance of intra residual couplings at δ_c 171.0/δ_H 5.05 (**B**C6/**B**H5 and δ_c 171.0/δ_H 3.83 (**B**C6/carboxy methyl proton) in the HMBC spectrum (Fig. 4) clearly indicates that the carboxyl group of galacturonic acid is present as methyl ester. These indicated that the residue **B** is (1 → 4)-α-D-Gal*p*A6Me.

The residue **C** has an anomeric proton chemical shift at δ 5.06 and anomeric carbon chemical shift at δ 100.0. The carbon signals of C-2 (δ 74.5) and C-4 (δ 79.5) showed downfield shift with respect to standard value of methyl glycosides indicating that the residue **C** is (1 → 2, 4)-linked residue. In HMBC spectrum the C-6 carboxyl carbon signal (δ 171.0) couples with carboxy methyl proton (δ 3.79) and also **C**H-5 (δ 5.11) indicating that the residue **C** is (1 → 2,4)-α-D-Gal*p*A6Me.

The sequence of glycosyl residues of the polysaccharide was determined on the basis of NOESY (Fig. 5, Table 3) as well as ROSEY (not shown) experiments, followed by confirmation with HMBC (Fig. 4, Table 2) experiment. In NOESY experiment, the inter-residual contacts **A**H-1/**C**H-2; **B**H-1/**C**H-4; **C**H-1/**B**H-4 along with other intra-residual contacts were also observed (Fig. 5). Thus from NOESY experiment the following sequences are established:

A(1 → 2)**C**; B(1 → 4)**C**; **C**(1 → 4)**B**

These sequences were further confirmed by ^{13}C–^{1}H correlation in HMBC spectrum (Fig. 4, Table 2). Inter residual cross peaks **A**H1/**C**C2, **A**C1/**C**H2; **B**H1/**C**C4, **B**C1/**C**H4; **C**H1/**B**C4, and **C**C1/**B**H4 along with other intra residual peaks were also observed. Therefore, based on the results obtained from monosaccharide composition, methylation studies, and NMR experiments the following repeating unit of the pectic polysaccharide of *Momordica charantia* was assigned as:



2.3. Immunostimulating properties of the polysaccharide

Macrophage activation by polysaccharide was observed in vitro. On treating different concentrations of polysaccharide, an

Table 1
^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts (ppm) of the PS isolated from the fruits of *Momordica charantia* (Karela) [a,b] recorded D_2O at 30 °C

Glycosyl residue	H-1/3/ C-1	H-2/ C-2	H-3/ C-3	H-4/ C-4	H-5/ C-5	H-6a, H-6b/C-6	–COOMe
β-D-Gal*p*-(1→	4.59	3.66	3.76	4.15	4.10	3.73[c]/ 3.78[d]	
A	105.0	72.8	73.9	69.5	78.1	61.03	
→4)-α-D-Gal*p*A6Me-(1→	4.94	3.70	3.96	4.44	5.05		3.79[e]
B	100.8	68.4	68.3	79.5	70.6	171.0[g]	53.3[f]
→2,4)-α-D-Gal*p*A6Me-(1→	5.06	3.73	3.99	4.44	5.11		3.79[e]
C	100.0	74.5	68.5	79.5	71.06	171.0[g]	53.3[f]

[a] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.74 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.
[e] Proton value of ester group.
[f] ^{13}C Chemical shift value of the methyl carbon in ester group.
[g] ^{13}C Chemical shift value of the carbonyl group of ester group.

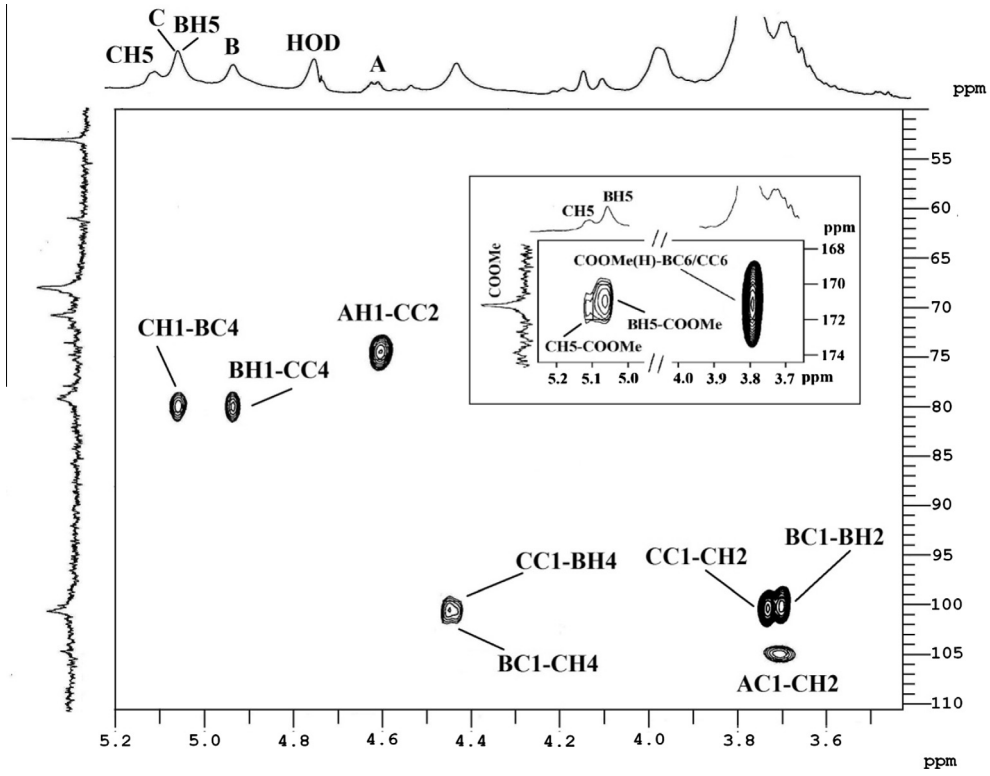


Figure 4. Part of HMBC spectrum (D_2O, 30 °C) of the polysaccharide (PS) isolated from the fruits of *Momordica charantia* (Karela). The delay time in the HMBC experiment was 80 ms.

enhanced production of NO was observed in a dose dependent manner with optimum production of 17 μM NO per 5×10^5 macrophages at 200 μg/mL (Fig. 6a). Hence, the effective dose of Polysaccharide was observed at 200 μg/mL.

Proliferation of splenocytes and thymocytes is an indicator of immunoactivation. The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with polysaccharide by the MTT assay method.[21] Polysaccharide was tested to stimulate splenocytes and thymocytes and the results are shown in Figure 6b and c, respectively, and the asterisks on the columns indicate the statistically significant differences compared to PBS control. The splenocyte proliferation index (SPI) as compared to PBS control closer to 1 or below indicates low stimulatory effect on the immune system. At 200 μg/mL of the PS, splenocyte proliferation index was found maximum as compared to other concentrations. Hence, 200 μg/mL of the PS can be considered as efficient splenocyte stimulator. Again 25 μg/mL of this same sample showed maximum effect on thymocyte proliferation.

2.4. Antioxidant properties

2.4.1. Hydroxyl radical scavenging activity

Generation of reactive oxygen species beyond the body's antioxidant capacity gives rise to oxidative stress. During this stress, hydroxyl radicals are mainly responsible for the oxidative injury of biomolecules since they can easily react with amino acids and DNA. It is also believed that they are the active initiators for lipid peroxidation.[22] It is thus important to remove hydroxyl radicals for protection of the living systems.

Scavenging effects of the PS on hydroxyl radicals increased with increasing concentration (Fig. 7a). Figure 7a shows percentage inhibition of hydroxyl radical generation by 0.1, 1, and

Table 2
The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the PS isolated from the fruits of *Momordica charantia* (Karela)

Residue	Sugar linkage	H-1/C-1 δ_H/δ_C (ppm)	Observed connectivities		
			δ_H/δ_C (ppm)	Residue	Atom
A	β-D-Gal*p*-(1→	4.59	74.5	**C**	C-2
		105.0	3.73	**C**	H-2
B	→4)-α-D-Gal*p*A6Me-(1→	4.94	79.5	**C**	C-4
		100.8	4.44	**C**	H-4
			3.70	**B**	H-2
C	→2,4)-α-D-Gal*p*A6Me-(1→	5.06	79.5	**B**	C-4
		100.0	4.44	**B**	H-4
			3.73	**C**	H-2
			Observed connectivities		
		COOMe (δ_H)	δ_C	Residues	Atom
B/C	→4)-α-D-Gal*p*A6Me-(1→	3.79	171.0	**B/C**	C-6
		COOMe (δ_C)	δ_H		
		171.0	5.05	**B**	H-5
			5.11	**C**	H-5

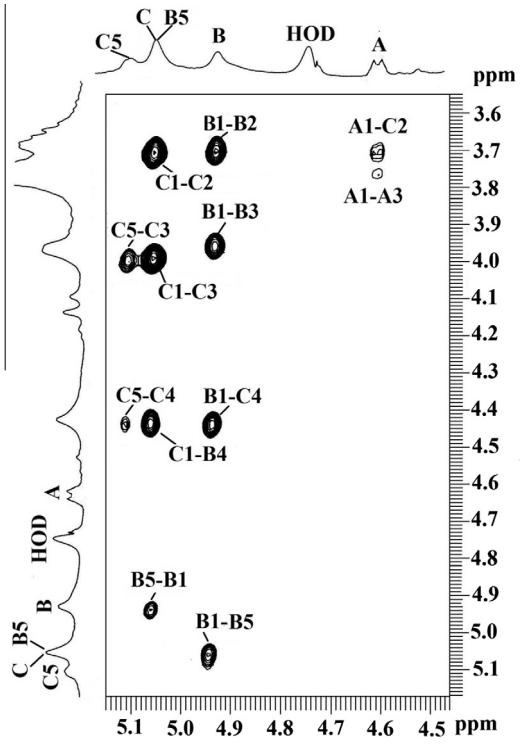


Figure 5. Part of NOESY spectrum (D_2O, 30 °C) of the polysaccharide (PS) isolated from the fruits of *Momordica charantia* (Karela).

2.5 mg/mL concentration of the PS and butylated hydroxyl anisole (BHA). Results indicated that PS has a noticeable effect on scavenging free radicals (EC_{50} = 2.22 mg/mL). Xu et al. isolated three purified polysaccharides from flowers of tea plant[23] and all of them exhibited <25% hydroxyl radical scavenging activity at 4 mg/mL concentration. However, the scavenging effect of BHA was much higher than the PS (EC_{50} = <0.01 mg/mL). The results revealed that the PS was hydroxyl radical scavengers, acting possibly as primary antioxidant.

2.4.2. Lipid peroxidation by egg homogenate

Cellular components such as polyunsaturated fatty acid residues of phospholipids are extremely sensitive to oxidation induced by reactive oxygen species. Major aldehyde products of this peroxidation method are malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE). HNE is thought to be highly toxic whereas malondialdehyde is carcinogenic in mammalian cell.[22] Lipid peroxidation also results in breakdown of membrane integrity, affecting its fluidity and permeability. Therefore, inhibition capacity of lipid peroxidation is of great importance to show potential antioxidant activity.

As shown in Figure 7b, lipid peroxidation was prevented by the PS. The absorbance of malondialdehyde–thiobarbituric acid (MDA–TBA) complex was linearly decreased in a dose dependent path (0.2, 1, 2.5 mg/mL). The PS showed 50% lipid inhibition at 2.05 mg/mL, whereas the standard ascorbic acid showed much higher activity with EC_{50} = <0.1 mg/mL.

Table 3
NOE data for the PS isolated from the fruits of *Momordica charantia* (Karela)

Anomeric proton		NOE contact protons		
Glycosyl residue	δ (ppm)	δ (ppm)	Residue	Atom
β-D-Gal*p*-(1→	4.59	3.71	**C**	H-2
A		3.76	**A**	H-3
→4)-α-D-Gal*p*A6Me-(1→	4.94	4.44	**C**	H-4
B		3.96	**B**	H-3
		3.70	**B**	H-2
		5.05	**B**	H-5
	5.05 (**BH-5**)	4.94	**B**	H-1
→2,4)-α-D-Gal*p*A6Me-(1→	5.06	4.44	**B**	H-4
C		3.71	**C**	H-2
		3.99	**C**	H-3
	5.11 (**CH-5**)	3.99	**C**	H-3
		4.44	**C**	H-4

3. Materials and methods

3.1. Isolation and purification of the polysaccharide

The fresh fruits of *Momordica Charantia* (1.5 kg) were washed with water, cut them into small pieces, and boiled with distilled water for 10 h. The whole extract was kept overnight at 4 °C and then filtered through linen cloth. The filtrate was then centrifuged at 8000 rpm for 45 min at 4 °C. The supernatant was precipitated in ethanol (1:5, v/v). The precipitated polysaccharide was collected through centrifugation, washed with ethanol, and freeze dried according to our previously reported papers. The crude PS (1.416 g) was isolated and purified (30 mg) through Sepharose-6B

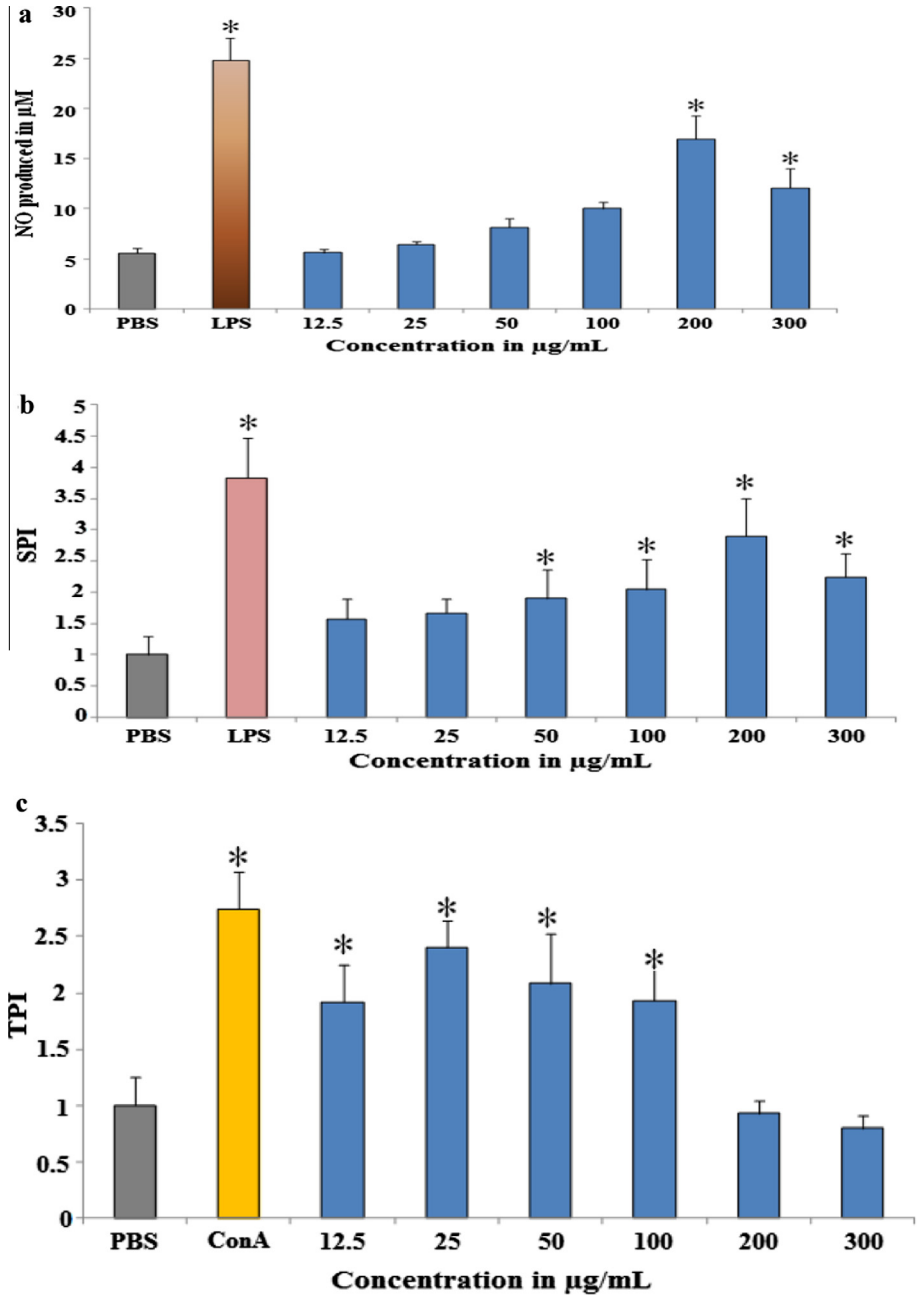


Figure 6. (a) Activation of Raw 264.7 macrophage cells stimulated with different concentrations of the pectic polysaccharide (PS) isolated from the fruits of *Momordica charantia* (Karela) in terms of NO production. Effect of different concentrations of the pectic polysaccharide (PS) on proliferation of (b) splenocyte and (c) thymocyte (asterisk indicates the statistically significant difference compared to the PBS as a positive control with P <0.05. Here LPS (4 µg/mL) and Con A (10 µg/mL) are used as a negative control).

as described in our previous papers.[24,25] Single fraction (test tube no. 20 to 40) was obtained, collected yielding 16.2 mg PS. The process was repeated several times to get 95 mg PS.

3.2. General methods

Optical rotation was measured on a Jasco Polarimeter model P-1020 at 30 °C. The polysaccharide sample (3.0 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) and analysis was carried out as described in our previous papers.[26,27] The average molecular weight was determined by adopting the method as described in our previous publications.[26,27] The absolute configuration of the monosaccharide was determined by the method of Gerwig et al.[14] Periodate oxidation and methylation experiments were carried out as described in our earlier reports.[24–27] The GLC instrument 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) for monosaccharide analysis. All GLC analyses were performed at 170 °C. 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^{-1} up to a final temperature of 200 °C. Finally NMR experiments[28,29] were carried out by a Bruker Avance DPX-500 instrument at 30 °C as reported in our previous papers.[24,25]

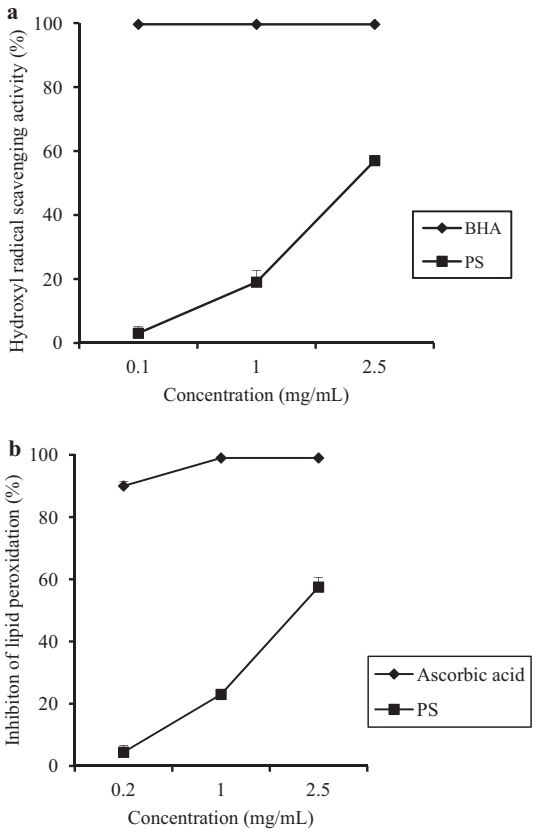


Figure 7. (a) Hydroxyl radical scavenging activity. (b) Inhibition of lipid peroxidation activity of the pectic polysaccharide (PS) isolated from the fruits of *Momordica charantia* (Karela). (All the above results are the mean ± SD of three separate experiments, each in triplicate).

3.3. Immunostimulating properties

3.3.1. Test for macrophage activity by nitric oxide assay

RAW 264.7 growing in Dulbecco's modified Eagle's medium (*DMEM*) was seeded in 96 well flat bottom tissue culture plates at 5×10^5 cells/mL concentration (180 μL).[30] Cells were left overnight for attachment and treatment of different concentrations (12.5, 25, 50, 100, 200, and 300 μg/mL) of PS. 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).[31] Lipopolysaccharide (LPS), L6511 of Salmonella enterica serotype typhimurium (sigma, St. Louis, USA) was used as positive control.

3.3.2. Splenocyte and thymocyte proliferation assay

A single cell suspension of the spleen and thymus were prepared from the normal mice under aseptic conditions by homogenization in Hank's balanced salt solution (HBSS). The suspension was centrifuged to obtain cell pellet. The contaminating red blood cells (RBC) were removed by hemolytic Gey's solution. After washing two times in HBSS the cells were further suspended in complete RPMI (Roswell Park Memorial Institute) medium. Cell concentration was adjusted to 1×10^6 cells/mL and the 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 (12.5, 25, 50, 100, 200, and 300 μg/mL). PBS (Phosphate Buffer Saline, 10 mM, pH-7.4) was taken as negative control whereas lipopolysaccharide (LPS, L6511 of *Salmonella enterica* serotype typhimurium, Sigma, 4 μg/mL) and Concanavalin A (Con A, 10 μg/mL) served as positive controls. All cultures were set up at 37 °C for 72 h in a humidified atmosphere of 5% CO_2. Proliferation of splenocytes (SPI) and thymocytes (TPI) was checked by MTT assay method.[32] The data are reported as the mean ± standard deviation of seven different observations and compared against PBS control.[30,32]

3.4. Antioxidant properties

3.4.1. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the PS (from 0.1 to 2.5 mg/mL) was investigated using Fenton's reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$).[33] 1 mL reaction mixture consisted of KH_2PO_4–KOH buffer (20 mM, pH 7.4), 2-deoxy-D-ribose (2.8 mM), various concentrations of the PS, $FeCl_3$ (100 mM), EDTA (104 μM), ascorbate (100 μM), and H_2O_2 (1 mM). It was incubated at 37 °C for 1 h to allow hydroxyl radical generation which will attack deoxyribose to form malondialdehyde (MDA). 2 mL thiobarbituric acid (TBA) and trichloroacetic acid (TCA) solution (0.375% (w/v) TBA, 15% (w/v) TCA and 0.25 N HCl) was added and incubated at boiling water bath for 15 min to form MDA–TBA chromogen at acidic pH. After cooling, absorbance was measured at 535 nm against identical set of reaction mixtures where TBA–TCA solution was added prior incubation to subtract background color. Butylated hydroxyanisole (BHA) was used as positive control and results were expressed as the rate of inhibition. EC_{50} value expressed the effective concentration at which the scavenging free radical activity was 50%. The degree of scavenging was calculated by the following equation:

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

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

3.4.2. Inhibition of lipid per oxidation by egg homogenate

The antioxidant activity of the PS was evaluated according to lipid per oxidation method using egg homogenate, as described in the literature.[34] The reaction mixture consisted of 0.5 mL of egg homogenate (10% v/v), PS of various concentrations (0.2–2.5 mg/mL), and 0.05 mL of $FeSO_4$ (0.07 M) and incubated to allow oxidation of polyunsaturated fatty acids. After 30 min, 1.5 mL acetic acid (20%, pH 3.5), 1.5 mL of 0.8% (w/v) TBA in 1.1% sodium dodecyl sulfate, and 0.05 mL 20% TCA were added. Reaction mixture was heated at 95 °C for 1 h, during which pinkish red chromate developed due to reaction between TBA and MDA, end product of oxidation. After cooling, 5 mL of butanol was added and centrifuged at 3000 rpm for 10 min. Absorbance of upper layer was measured at 532 nm. Ascorbic acid was used as positive control. EC_{50} value expressed the effective concentration at which the inhibition of lipid per oxidation was 50%. The degree of inhibition was calculated by the following equation:

$$\text{Inhibition effect}(\%) = \{(A_0 - A_1)/A_0\} \times 100$$

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

4. Conclusions

An immunoenhancing antioxidant water soluble pectic polysaccharide (PS) was isolated from the hot water extract of the green fruits, *Momordica charantia* (Karela). The structure of this PS was elucidated on the basis of total hydrolysis, methylation analysis, and 1D/2D NMR studies. These results indicated that the repeating unit of the PS contained a backbone of four (1 → 4)–linked

D-methyl galacturonate residues, out of which one residue was branched at *O*-2 position with terminal β-D-galactopyranosyl residue. The PS activated the macrophages, splenocytes, and thymocytes and also showed several potent antioxidant activities. Hence, on the basis of these activities it could be used as a natural immunostimulant and antioxidant material.

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Heteroglycan of an edible mushroom *Pleurotus cystidiosus*: Structural characterization and study of biological activities

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ABSTRACT

A water soluble heteroglycan (PCPS) was isolated from the aqueous extract of an edible mushroom *Pleurotus cystidiosus*. Structural characterization of the heteroglycan was carried out using total hydrolysis, methylation analysis, periodate oxidation, Smith degradation, and 1D/2D NMR experiments. Sugar analysis indicated the presence of glucose, galactose, and mannose in a molar ratio of nearly 6:2:1 respectively. The chemical and NMR analysis of the PCPS indicated the presence of a repeating unit with a backbone consisting of one unit of (1 → 6)-β-D-glucopyranosyl, two (1 → 3)-β-D-glucopyranosyl, one (1 → 3)-α-D-glucopyranosyl, one (1 → 6)-α-D-glucopyranosyl, and two (1 → 6)-α-D-galactopyranosyl moieties respectively, out of which one (1 → 3)-β-D-glucopyranosyl residue was branched at *O*-6 with terminal β-D-glucopyranosyl and another (1 → 6)-α-D-galactopyranosyl residue was branched at *O*-2 with terminal β-D-mannopyranosyl moiety. The polysaccharide was found to exhibit cellular activities at different concentrations (10, 25, 50, 100, 200, 400 μg/mL) and maintained the redox balance as well as reduced lipid per oxidation which protect the cell destruction.

1. Introduction

Edible mushrooms are considered highly nutritious, and delicious [1–3]. They possess several pharmaceutical constituents [4,5]. Chemists and druggists are interested in identifying the immunomodulating compounds of mushrooms which help improvement of the immune function of cancer patients [6] as well as lowering blood pressure and free cholesterol in plasma [7]. The members of the mushroom genus *Pleurotus* (Jacq. Fr) P. Kumm form a heterogeneous group of edible species of high commercial importance [8,9]. The present mushroom *P. cystidiosus* belongs to the genus *Pleurotus* of the gilled mushrooms and cultivated on various agricultural lignocellulosic wastes, saw dust, rice husk, cotton waste, waste tea leaves, corn husks, waste papers etc. or in hardwood, but never on conifer wood [10,11]. *P. cystidiosus* is also known as brown oyster mushroom and has the longest storage life among all pleurotus [10,12]. The mushroom *P. cystidiosus* is distributed throughout the world and grows in warm climatic zone [8,9]. The energy value of the mushrooms of this genus varies according to the species [13]. *P. cystidiosus* extract shows antioxidant and cytotoxic activity against Hep-2-cancer cell line [14].

Several polysaccharides of the genus *Pleurotus* have been reported by the present research group. Branched (1 → 3)-,(1 → 6)-α,β-D-glucan [15], a heteroglycan [16], (1 → 3)-,(1 → 6)-β-D-glucan [17] was reported from *P. florida*. Several glucans of *P. florida*, cultivar (Assam florida) [18,19], *P. florida* blue variant [20] and heteroglycans [21] from *P. Sajor-caju* and cultivar Black Japan [22] were isolated and reported. The aim of the present work is to isolate polysaccharide from *P. cystidiosus*, another mushroom of the same genus and the detailed structural characterization with some biological activities have been carried out and reported herein.

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2. Materials and methods

2.1. Isolation and purification of polysaccharide

The fresh mushroom of *P. cystidiosus* (250 g) were washed with water, cut them into small pieces and boiled with distilled water for 12 h. The whole extract was kept overnight at 4 °C and then filtered through linen cloth. The filtrate was then centrifuged (using a Heraeus Biofuge stratus centrifuge) at 8000 rpm for 45 min at 4 °C. The supernatant was precipitated in ethanol (1:5, v/v). It was kept overnight at 4 °C and again centrifuged as above. The precipitate was dissolved in a minimum volume of distilled water and dialyzed through tubing cellulose membrane (Sigma–Aldrich, retaining MW >12,400 Da) against distilled water for 2 days to remove low molecular weight materials. The dialyzed material was freeze-dried and the crude polysaccharide (1.414 g) was isolated [23,24]. The crude polysaccharide (30 mg) was passed through Sepharose–6B gel-permeation column (92 × 2.1 cm) using water as eluant with flow rate of 0.5 mL/min. Total ninety five test tubes were collected using Redifrac fraction collector and monitored spectrophotometrically (UV-vis spectrophotometer, model-1601) at 490 nm using phenol-sulphuric acid method [25]. Single fraction (test tube nos. 22–45) was obtained, collected, and freeze-dried, yield; 20 mg pure PCPS. The process was repeated several times to get 120 mg of PCPS.

2.2. Determination of optical rotation

The optical rotation of PCPS was measured by Jasco Polarimeter (Model P-1020) at 30.8 °C.

2.3. Monosaccharide analysis

The PCPS (3.0 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) at 100 °C for 18 h in a water bath, reduced with $NaBH_4$, followed by acidification with dilute CH_3COOH, and then co-distilled with pure CH_3OH. The reduced sugars were acetylated with 1:1 pyridine-Ac_2O in a to give the alditol acetates [26] and analyzed by Gas-liquid chromatography (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) at 170 °C [27,28]. The sugars were identified with respect to different standard sugars and quantitation was carried out taking inositol as standard.

2.4. Determination of molecular weight

The molecular weight of the polysaccharide was determined by gel-permeation chromatography [29] with reference to standard dextrans T-200, T-70, and T-40. The elution volumes of the standard dextrans were plotted against the logarithms of their respective molecular weights and the elution volume of PCPS on the same graph, and the average molecular weight of PCPS was determined [23].

2.5. Methylation analysis

The polysaccharide (PCPS, 3.0 mg) was methylated according to the Ciucanu and Kerek method [30]. The methylated product was hydrolyzed with 90% HCOOH (1 mL) at 100 °C for 1 h, reduced with $NaBH_4$. Following removal of excess boric acid by co-distillation with CH_3OH, the reduced sugars were acetylated with 1:1 pyridine-AC_2O in a boiling water bath for 1 h to give partially mehylated alditol acetates. Gas chromatography-mass spectrometric (GLC–MS) analysis was performed using ZB-5MS capillary column (30 m × 0.25 mm). The GC 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. The peaks were identified with respect to tetra methyl glucose (TMG) as standard and mass-spectra data of different methylated sugars.

2.6. Periodate oxidation study

Periodate oxidation experiment was carried out as described in earlier papers [31]. The periodate-reduced material 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 and analyzed by GLC. Another portion was methylated by the method of Ciucanu and Kerek [30], followed by preparation of alditol acetates and analyzed by GLC–MS.

2.7. Smith degradation study

The controlled Smith degradation [32,33] experiment was performed, where PCPS (25 mg) was oxidized with 0.1 M aqueous $NaIO_4$ (20 mL) at 25 °C in the dark for 96 h. Oxidation was stopped by the addition of 1, 2-ethanediol and then dialysis, neutralization, and hydrolysis were carried out as described earlier [32,33].

2.8. Absolute configuration of monosaccharide

The absolute configuration of the monosaccharide was determined by the method of Gerwig et al. [34]. PCPS (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 in R-(−)-2-butanol was added to the hydrolyzed product and the mixture was heated at 80 °C for 16 h. 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.

2.9. NMR studies

PCPS was dried over P_2O_5 in vacuum for several days and then exchanged with deuterium followed by lyophilization with D_2O (99.96% atom ^2H, Aldrich) for four times [35,36]. The ^1H and ^{13}C NMR experiments were performed 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 pre-saturation method. ^{13}C NMR experiment of PCPS was carried out taking acetone as an internal standard, fixing the methyl carbon signal at δ 31.05. The 2D-DQF-COSY NMR 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 properties

2.10.1. Chemicals and reagents

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT reagent), Histopaque 1077, and $DCFH_2$ DA were procured from Sigma (St. Louis, MO, USA). Minimum essential medium (MEM), RPMI 1640, fetal bovine serum (FBS), penicillin, streptomycin, doxorubicin, sodium chloride (NaCl), sodium car-

bonate (Na_2CO_3), sucrose, Hanks balanced salt solution were purchased from Himedia, India.

2.10.2. Cytotoxicity or cell viability

In vitro cellular toxicity of PCPS was studied on human peripheral blood lymphocytes. Normal human lymphocytes were seeded into 96 wells of culture plates having 180 μL of complete media and were incubated for 48 h. The PCPS of different concentrations (D1: 10, D2: 25, D3: 50, D4: 100, D5: 200 and D6: 400 μg/mL) were added to the cells and incubated for 24 h at 37 °C in a humidified incubator (NBS) having 5% CO_2. The cell viability was estimated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay method [37].

2.10.3. Determination of reduced glutathione (GSH)

Reduced glutathione (GSH) assay [38] was performed using the cell lysate. The required amount of the cell lysate was mixed with 25% of trichloroacetic acid and centrifuged at 2000 × g for 15 min to precipitate the 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 μ gm of GSH/mg protein.

2.10.4. Determination of oxidized glutathione level (GSSG)

The oxidized glutathione (GSSG) level was measured after derevatization of GSH with 2-vinylpyidine [39]. 2 μL 2-vinylpyidine was added to 0.5 mL cell lysate and incubated for 1 h at 37 °C. Then the mixture was deprotenized with 4% sulfosalicylic acid and centrifuged at 1000 × g for 10 min to precipitate the proteins. The supernatant was aspirated and GSSG level was estimated with the reaction of DTNB at 412 nm using spectrophotometer and calculated with standard GSSG curve.

2.10.5. Determination of lipid peroxidation (MDA)

Lipid peroxidation of cell lysate was estimated [40] using the reaction mixture containing Tris-HCl buffer (50 mM, pH 7.4), *tert*-butyl hydroperoxide (BHP) (500 μM in ethanol) and 1 mM $FeSO_4$. The samples were then incubated at 37 °C for 90 min and the reaction was stopped by adding 0.2 mL of 8% sodium dodecyl sulfate (SDS) followed by addition of 1.5 mL of 20% acetic acid (pH 3.5). The mixture was further heated at 95 °C for 45 min and then the amount of malondialdehyde (MDA) produced during incubation was estimated by adding 1.5 mL of 0.8% TBA. After cooling, the samples were centrifuged and the TBA reactive substances (TBARS) in supernatants were measured at 532 nm. The level of lipid peroxidation was expressed in terms of n mol/mg protein.

2.10.6. Statistical analysis

The data were expressed as the mean ± SEM, n = 4. Comparisons between the means of control, and experimental groups were made by one way ANNOVA tests (using statistical package; Origin 6.1, Northampton, MA 01060 USA), with $P < 0.05$ as a limit of significance.

3. Results and discussion

3.1. Isolation, purification and chemical analysis of PCPS

The mushroom fruit bodies (250 g) were washed thoroughly with distilled water followed by extraction with hot water, filtration, precipitation in alcohol (83%), dialysis, centrifugation, and freeze drying to yield crude polysaccharide (1.414g). One fraction of pure polysaccharide was obtained after fractionating water soluble crude polysaccharide (30 mg) through Sepharose 6 B column. Fractions (test tubes, 22–45) positive to phenol/sulfuric acid test [25] were collected and freeze dried to yield 20 mg pure polysaccharide (PCPS). The PCPS showed specific rotation $[\alpha]_D^{30.8}$ +15.9 (*c* 0.10, water). Molecular weight [29] of the polysaccharide was found to be ~1.46×10^5 Da from a calibration curve prepared with standard dextran. The GLC analysis of the alditol acetates [26] of acid hydrolyzed PCPS showed the presence of glucose, galactose, and mannose in a molar ratio of nearly 6:2:1 respectively. The absolute configuration of the monosaccharides are determined as D-configuration by the method of Gerwig et al. [34].

The PCPS was methylated using the Ciucanu and Kerek [30] method, followed by hydrolysis and preparation of alditol acetates. The GLC–MS analysis revealed the presence of 1,5-di-*O*-acetyl-

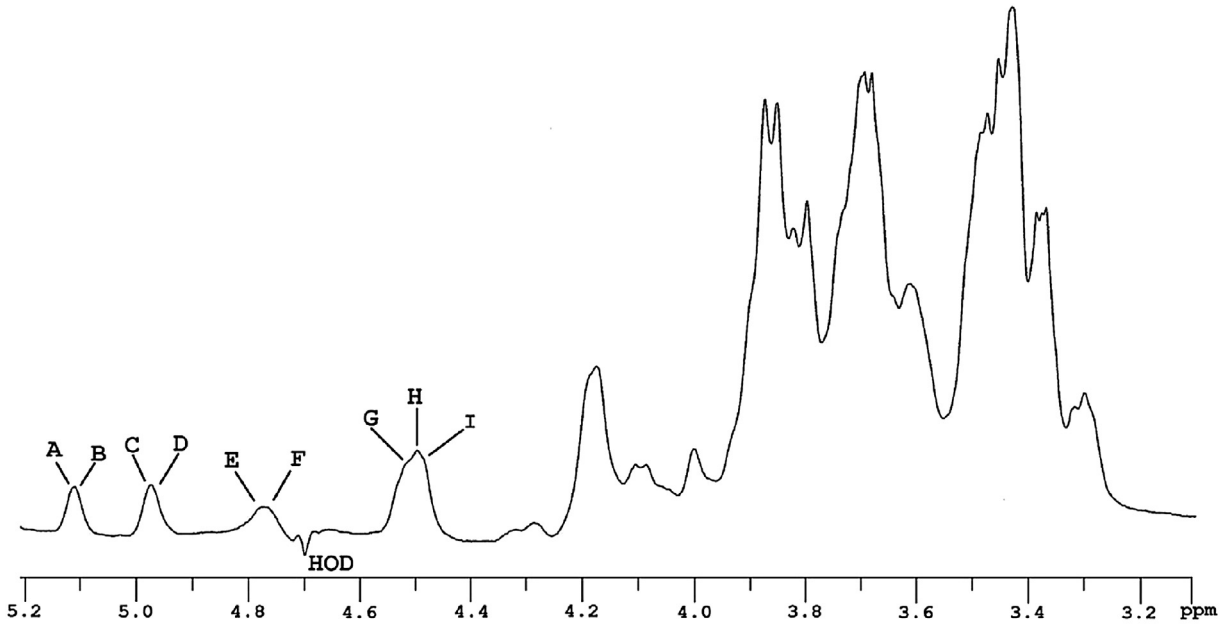


Fig. 1. ^{1}H NMR spectrum (500 MHz, D_2O, 30 °C) of the PCPS isolated from an edible mushroom *P. cystidiosus*.

Table 1
The ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts of the PCPS isolated from the mushroom *P. cystidiosus* recorded D_2O at 30 °C.

Glycosyl residue 3	H-1/C-1	H-2/C-2	H-3/C-	H-4/C-4	H-5/C-5	H-6a, H-6b/C-6
→2,6)- α-D-Gal*p*-(1→	5.11	3.86	3.99	4.11	4.20	3.62[c], 3.87[d]
A	98.2	75.0	69.0	68.5	68.3	66.5
→3)- α-D-Glc*p*-(1→	5.09	3.79	3.68	3.49	3.65	3.70[c], 3.85[d]
B	101.5	71.4	80.5	71.4	72.5	60.8
→6)- α −D-Gal*p*-(1→	4.97	3.80	4.03	4.11	4.20	3.67[c], 3.90[d]
C	98.0	68.4	69.2	68.5	68.3	66.6
→6)- α-D-Glc*p*-(1→	4.96	3.81	3.40	3.59	4.07	4.02[c], 4.15[d]
D	97.6	71.4	72.9	69.8	69.9	68.4
β-D-Man*p*-(1→	4.77	4.05	3.69	3.55	3.37	3.73[c], 3.89[d]
E	101.8	69.9	75.3	67.2	75.9	60.8
→3)- β-D-Glc*p*-(1→	4.74	3.32	3.71	3.42	3.49	3.69[c], 3.85[d]
F	102.6	72.9	84.2	69.5	75.5	60.6
→3,6)- β-D-Glc*p*-(1→	4.52	3.49	3.77	3.40	3.59	3.84[c], 4.15[d]
G	102.2	72.8	83.2	69.5	74.2	68.6
→6)- β-D-Glc*p*-(1→	4.48	3.28	3.43	3.45	3.63	3.86[c], 4.19[d]
H	102.3	73.0	75.5	69.6	74.4	68.9
β-D-Glc*p*-(1→	4.46	3.28	3.47	3.36	3.45	3.67[c], 3.80[d]
I	102.8	73.0	75.5	69.5	75.6	60.6

[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] Interchangeable.
[d] Interchangeable.

2,3,4,6-tetra-*O*-methyl-D-glucitol; 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol; 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; 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol; 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-D-galactitol; 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a ratio of 1:1:2:2:1:1:1. These results indicated the presence of terminal glucopyranosyl, terminal mannopyranosyl, (1 → 3)-glucopyranosyl, (1 → 6)-glucopyranosyl, (1 → 6)-galactopyranosyl, (1 → 2,6)-galactopyranosyl, and (1 → 3,6)-linked glucopyrnosyl moieties. GLC–MS analysis of the alditol acetates of the periodate-oxidized [41,42] methylated [43] PCPS 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 2:1. 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 PCPS.

3.2. NMR and structural analysis of the PCPS

The ^{1}H NMR spectrum (500 MHz, Fig. 1, Table 1) of the PCPS at 30 °C showed four broad peaks which represent nine ^{1}H resonances in the anomeric region at δ 5.11, 5.09, 4.97, 4.96, 4.77, 4.74, 4.52, 4.48, and 4.46 and designated as **A, B, C, D, E, F, G, H,** and **I** respectively according to their decreasing proton chemical shift values. In the ^{13}C (Fig. 2**a,** Table 1) NMR (125 MHz) spectrum at the same temperature, nine peaks appeared in the anomeric region at δ 102.8, 102.6, 102.3, 102.2, 101.8, 101.5, 98.2, 98.0, and 97.6. Based on the result of HSQC spectrum (Fig. 3**a and b,** Table 1), the anomeric carbon signals at δ 102.8, 102.6, 102.3, 102.2, 101.8, 101.5, 98.2, 98.0, and 97.6 corresponded to the proton signals of δ 4.46 (**I**), δ 4.74 (**F**), δ 4.48 (**H**), δ 4.52 (**G**), δ 4.77 (**E**), δ 5.09 (**B**), δ 5.11 (**A**), δ 4.97 (**C**), and δ 4.96 (**D**) respectively. All the ^{1}H and ^{13}C signals (Table 1) were assigned using DQF–COSY, TOCSY, and HSQC experiments. From DQF–COSY experiment the proton coupling constants were measured and one bond C—H couplings were measured from proton coupled ^{13}C spectrum.

The galacto configuration of residues **A** and **C** were assigned from the large coupling constant values of $J_{H\text{-}2,H\text{-}3}$ ~9 Hz and small coupling constant value $J_{H\text{-}3,H\text{-}4}$ ~3.5 Hz. The α-configuration of both **A** and **C** 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 75.0) and C-6 (δ 66.5) with respect to standard values of methyl glycosides [45,46] indicate that residue **A** was a (1 → 2,6)-linked α-D-galactopyranosyl residue. The downfield shifts of C-6 (δ 66.6) with respect to standard values of methyl glycosides [44,45] indicated that the residue **C** was a (1 → 6)-linked α-D–galactopyranosyl residue. The linkage at C-6 of the both residues **A** and **C** were further established from DEPT-135 spectrum (Fig. 2**b**). From these observations it can be concluded that the residue **A** was (1 → 2,6)-α-D-galactopyranosyl and **C** was (1 → 6)-α-D-galactopyranosyl.

The α-linked configuration of residue **B** and **D** was confirmed from the anomeric proton chemical shift and the coupling constant values at $J_{H\text{-}1,H\text{-}2}$ (~3.0 Hz) and $J_{C\text{-}1,H\text{-}1}$ (~171 Hz). 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 residues **B** and **D** were D-glucopyranosyl moieties (Glc*p*). The residues **B** showed downfield shift of C-3 (δ 80.5) with respect to standard values of methyl glycosides which indicated that the residue **B** was linked at this position. Thus, the residue **B** was confirmed as (1 → 3)-α-D-glucopyranosyl [46] moiety. In case of residue **D**, the downfield shift of C-6 (δ 68.4) with respect to standard values of methyl glycosides indicated that the residue **D** was linked at this position. The linkage at C-6 of the residue **D** was further confirmed from DEPT–135 spectrum (Fig. 2**b**). The other values for carbons and protons corresponded nearly to the standard values. So, **D** was confirmed as (1 → 6)-α-D-glucopyranosyl residue.

The manno configuration of residue **E** was supported from the large coupling constant values of $J_{H\text{-}3,H\text{-}4}$ (~7.5 Hz) and $J_{H\text{-}4,H\text{-}5}$ (~9.5 Hz). The anomeric proton and carbon signals at δ 4.77 and δ 101.8, as well as the low coupling constant values of $J_{H\text{-}1,H\text{-}2}$ (~0 Hz), $J_{C\text{-}1,H\text{-}1}$ (~160.0 Hz) confirmed that residue **E** was present in β-configuration. All the proton and carbon chemical shifts of residue **E** nearly corresponded to the standard values of methyl glycosides of β-D-mannose. So, **E** was confirmed as terminal β-D-mannopyranosyl residue.

The β-configuration of the residues **F, G, H,** and **I** were established from the coupling constant values $J_{H\text{-}1,H\text{-}2}$ (~8.0 Hz), $J_{C\text{-}1,H\text{-}1}$ (~160 − 161 Hz) and their anomeric proton and carbon chemical shifts ranging from δ 4.46–4.74 and δ 102.2–102.8 respectively. The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values (~10.0 Hz) of the

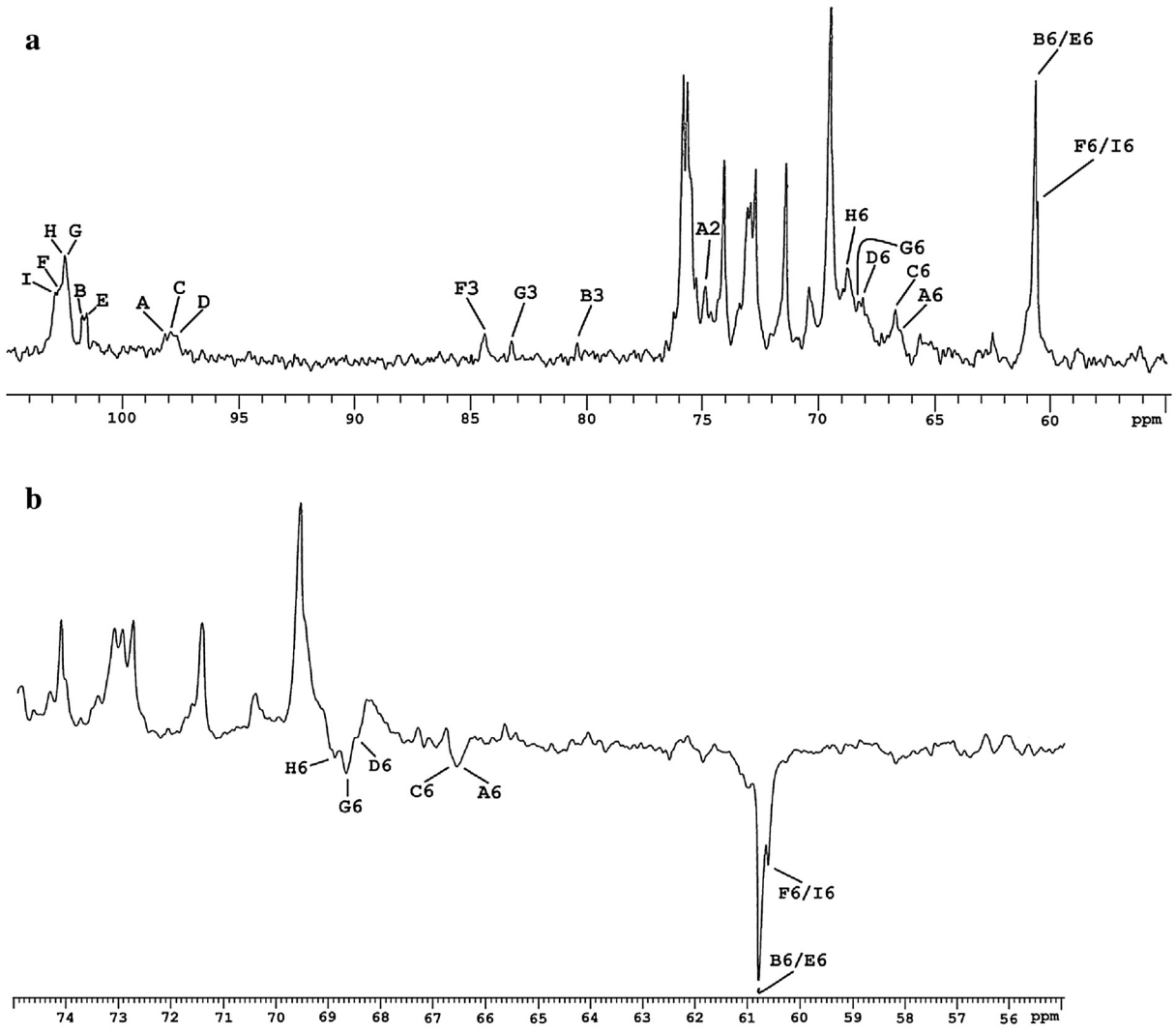


Fig. 2. (a) ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of the polysaccharide isolated from *P. cystidiosus*. (b) DEPT-135 spectrum (D_2O, 30 °C) of PCPS isolated from edible mushroom *P. cystidiosus*.

residues **F, G, H,** and **I** conformed their D-glucopyranosyl configuration (Glc*p*). The anomeric carbon signal of **F** at δ 102.6 and the downfield shifts of C-3 (δ 84.2) with respect to standard value of methyl glycoside [44,45] indicated that the residue **F** was (1 → 3)-β-D-glucopyranosyl moiety. The downfield shifts at C-3 (δ 83.2) and C-6 (δ 68.6) of residue **G** with respect to standard value of methyl glycoside indicated that it was (1 → 3,6)-β-D-Glc*p*. Since, residue **G** was one of the rigid part of this glucan, its C-3 (δ 83.2) value appeared at slightly upfield region compared to the C-3 (δ 84.2) of (1 → 3)-linked residue **F**. For the residue **H**, the downfield shift of C-6 (δ 68.9) 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. 2**b**). In case of carbon chemical shifts of residue **I** from C-1 to C-6 corresponded nearly to the standard values of methyl glycosides of β-D-Glucose indicating **I** was terminal β-D-Glc*p*.

The sequence of glycosyl residues (**A** to **I**) were established from ROESY (Fig. 4, Table 2) as well as NOESY (not shown) experiment. In ROESY experiment, the inter-residual contacts **A**H-1/**C**H-6a, 6b; **B**H-1/**F**H-3; **C**H-1/**B**H-3; **D**H-1/**A**H-6a, 6b; **E**H-1/**A**H-2; **F**H-1/**G**H-3; **G**H-1/**H**H-6a, 6b; **H**H-1/**D**H-6a, 6b; and **I**H-1/**G**-6a, 6b along with other intra-residual contacts were also observed (Fig. 4). The above mentioned ROESY connectivity established the sequences as follows: **A** (1 → 6) **C**; **B** (1 → 3) **F**; **C** (1 → 3) **B**; **D** (1 → 6) **A**; **E** (1 → 2) **A**; **F** (1 → 3) **G**; **G** (1 → 6) **H**; **H** (1 → 6) **D;** and **I** (1 → 6) **G**. These data and connectivities clearly supported the positions of substitution and sequence of sugar residues in the polysaccharide.

Smith degraded product (SDPCPS) of PCPS was prepared and NMR experiments were again carried out to confirm the linkages of the heteroglycan. The ^{13}C NMR (125 MHz) spectrum (Fig. 5, Table 3) at 30 °C of SDPCPS showed three anomeric carbon signals at δ 103.2, 102.8, and 98.2. The anomeric carbon signals at δ 98.2 corresponded to α-D-Glc*p* (**J**), δ 103.2 corresponded to (1 → 3)-β-D-Glc*p* (**K**) and δ 102.8 corresponded to (1 → 3)-β-D-Glc*p* (**L**) residues respectively. The carbon signals at C-1, C-2, and C-3 of the glycerol moiety (Gro) were assigned as δ 66.4, 72.1, and 62.6 respectively. The glycerol moiety (**M**) was generated from (1 → 6)-β-D-Glc*p* (**H**) after periodate oxidation followed by Smith degradation which was attached to (1 → 3)-linked β-D-Glc*p* moiety (**L**) generated from (1 → 3,6)-β-D-Glc*p* (**G**). The terminal α-D-Glc*p* (**J**) of SDPCPS was generated from (1 → 3)-α-D-Glc*p* (**B**) and (1 → 3)-β-D-Glc*p* (**K**) was produced from unaffected residue (1 → 3)-β-D-Glc*p* (**F**). Thus, the Smith degradation results in the formation of a glycerol containing trisaccharide

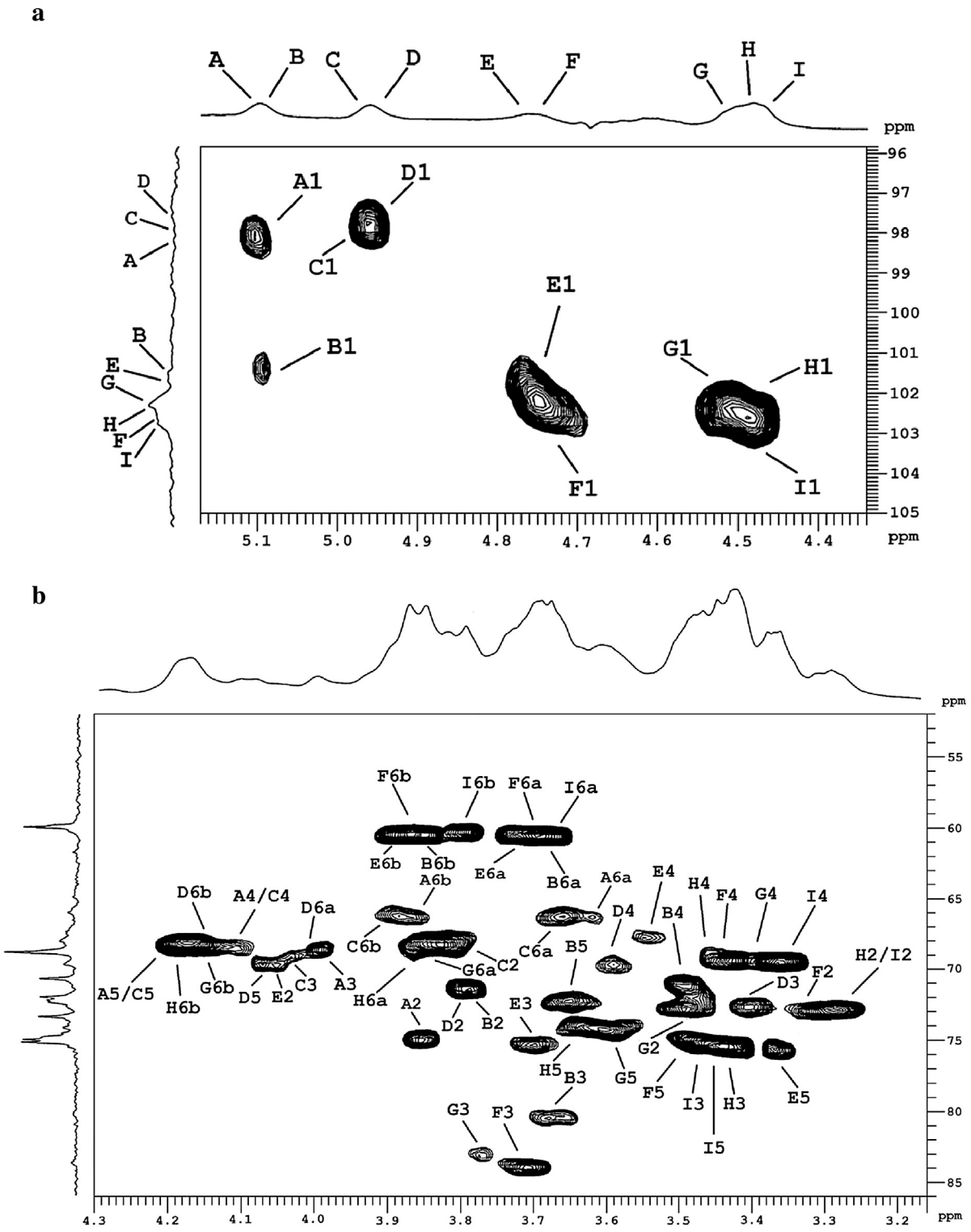


Fig. 3. The HSQC spectrum (D_2O, 30 °C) of (a) anomeric part and (b) other than anomeric part of the PCPS isolated from edible mushroom *P. cystidiosus*.

from the parent PCPS and the structure of which was established as:

J **K** **L** **M**

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

These results further confirmed the presence of the repeating moiety in the heteroglycan (PCPS) isolated from the edible mushroom *P. cystidiosus* and the structure was proposed as:

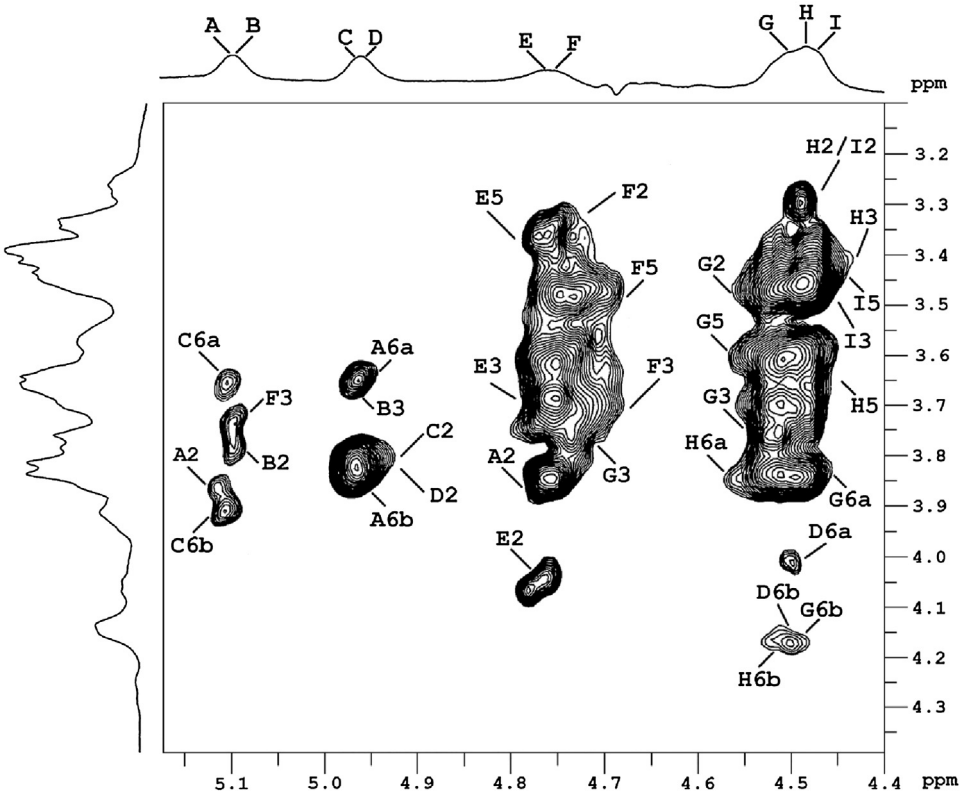


Fig. 4. The part of ROESY spectrum of PCPS, isolated from an edible mushroom *P. cystidiosus*. The mixing time was 300 ms.

Table 2
ROESY data for the polysaccharide (PCPS) isolated from the mushroom of *P. cystidiosus* in D_2O at 30 °C.

Glycosyl Residue	Anomeric proton δ	ROE contact proton		
		δ	Residue	Atom
→2,6)- α-D-Gal*p*-(1→	5.11	3.67	**C**	H-6a
A		3.90	**C**	H-6b
		3.86	**A**	H-2
→3)- α-D-Glc*p*-(1→	5.09	3.71	**F**	H-3
B		3.79	**B**	H-2
→6)- α −D-Gal*p*-(1→	4.97	3.68	**B**	H-3
C		3.80	**C**	H-2
→6)- α-D-Glc*p*-(1→	4.96	3.62	**A**	H-6a
D		3.87	**A**	H-6b
		3.81	**D**	H-2
β-D-Man*p*-(1→	4.77	3.86	**A**	H-2
E		4.05	**E**	H-2
		3.69	**E**	H-3
		3.37	**E**	H-5
→3)- β-D-Glc*p*-(1→	4.74	3.77	**G**	H-3
F		3.32	**F**	H-2
		3.71	**F**	H-3
		3.49	**F**	H-5
→3,6)- β-D-Glcp-(1→	4.52	3.86	**H**	H-6a
G		4.19	**H**	H-6b
		3.49	**G**	H-2
		3.77	**G**	H-3
		3.59	**G**	H-5
→6)- β-D-Glcp-(1→	4.48	4.02	**D**	H-6a
H		4.15	**D**	H-6b
		3.28	**H**	H-2
		3.43	**H**	H-3
		3.63	**H**	H-5
β-D-Glcp-(1→	4.46	3.84	**G**	H-6a
I		4.15	**G**	H-6b
		3.28	**I**	H-2
		3.47	**I**	H-3
		3.45	**I**	H-5

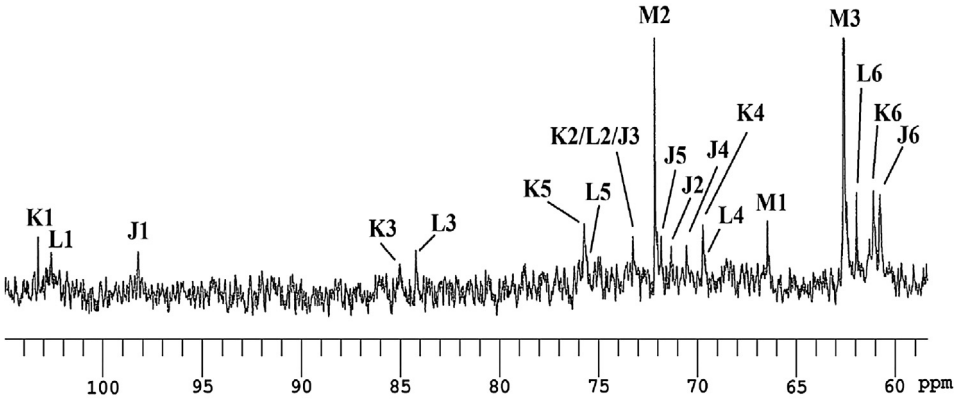


Fig. 5. ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of the Smith-degraded glycerol containing trisaccharide isolated from the edible mushroom *P. cystidiosus*.

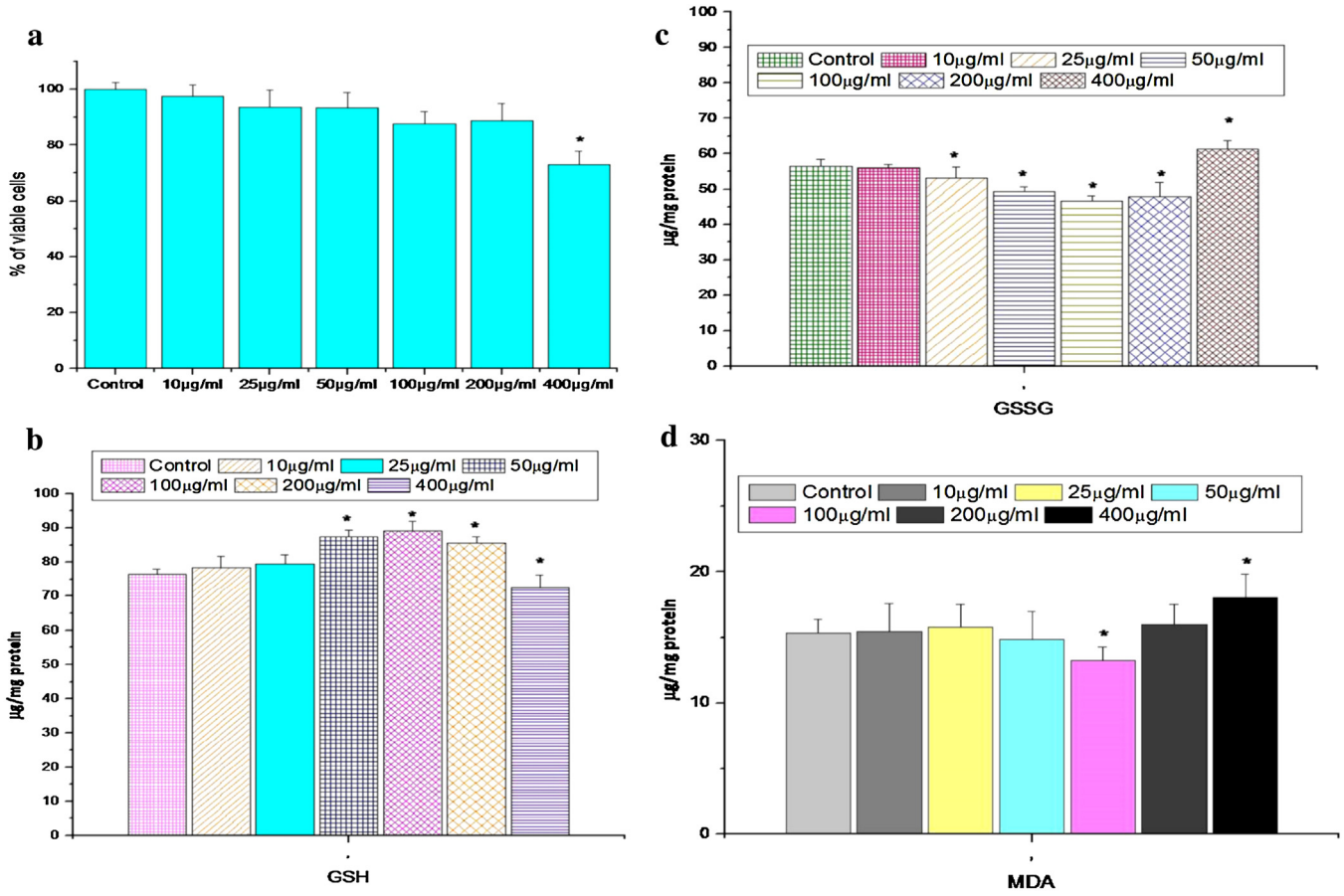
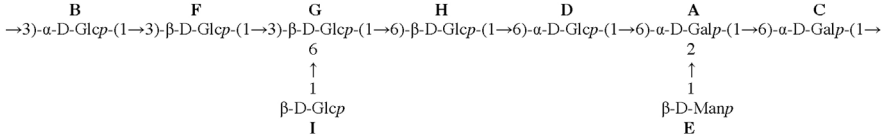


Fig. 6. (a) Cytotoxicity of PCPS on peripheral blood lymphocytes by MTT assay. (b) Concentration of reduced glutathione (GSH) level of PCPS treated in normal human lymphocytes. (c) Concentration of oxidized glutathione (GSSG) level of PCPS in normal human lymphocytes. (d) Concentration of Lipid peroxidation in terms of Malonaldehyde (MDA) level of PCPS treated normal human lymphocytes. (Values are expressed as mean ± S.E.M., n = 6. * indicates significant difference (P<0.05) compared to control group).



3.3. Biological properties of the PCPS

The cytotoxic effect of the PCPS was studied on human blood lymphocytes with increasing concentrations ranging from 10 µg/mL to 400 µg/mL using 3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) assay method (Fig. 6**a**). It was observed that PS has no considerable cytotoxic effect to normal lymphocytes up to 200 µg/mL. But at higher dose 400 µg/mL, the

Table 3
The ^{13}C NMR[a] chemical shifts of Smith-degraded glycerol-containing disaccharide of the mushroom *P. cystidiosus* in D_2O at 30 °C

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
α -D-Glcp-(1→ **J**	98.2	71.4	73.2	70.4	71.8	60.8
→3)-β-D-Glcp-(1→ **K**	103.2	73.2	85.0	69.7	75.8	61.1
→3)-β-D-Glcp-(1→ **L**	102.8	73.2	84.2	69.6	75.6	61.9
Gro-(3→ **M**	66.4	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 at 30 °C.

polysaccharide possesses mild levels of toxicity. It indicates that 200 μg/mL is safe with respect to the other higher doses.

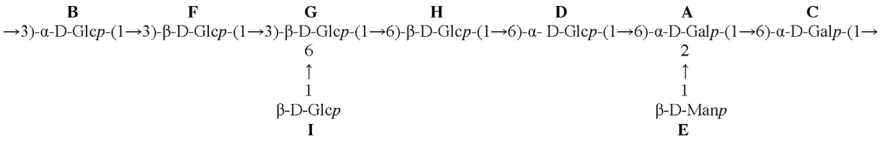
Glutathione is an important antioxidant in cellular system. Therefore, glutathione level in cell, both its reduced and oxidized states was measured (Fig. 6**b** and **c**). The reduced glutathione level (GSH, Fig. 6**b**) was increased significantly up to 200 μg/mL and the maximum level of increment was found at 200 μg/mL. But at 400 μg/mL, the reduced glutathione level (GSH, Fig. 6**b**) was moderately decreased and the mild augmentation of oxidized form of glutathione level (GSSG, Fig. 6**c**) was observed.

Study of lipid peroxidation is one of the important parameters to assess the cellular damage. It deactivates the cellular components and protective enzymes, and thereby plays a vital role of oxidative stress in biological systems [47]. Several toxic by-products, especially malondialdehyde is released due to lipid peroxidation. Hence, lipid peroxidations in lymphocytes were measured in terms of malondialdehyde (MDA) release (Fig. 6**d**). The result showed no significant changes at different concentration except at 100 μg/mL, where it decreases significantly. But it showed slightly increase of MDA level at the dose 400 μg/mL.

It is evident from these experiments that, in vitro application of PCPS has good effect up to a certain level. It does not induce any cellular damage in lymphocytes associated with enhanced MDA level, GSSG level and decreased GSH level. The cytotoxic profile of PCPS in lymphocytes indicated 200 μg/mL is safe and effective, whereas concentrations higher than 200 μg/mL showed slight increase of cytotoxicity. These findings suggest that the PCPS exhibits antioxidant and beneficial role on cellular system.

4. Conclusions

Biologically active water soluble heteroglycan (PCPS) was isolated from the hot water extract of the edible mushroom, *P. cystidiosus*. The following structure of the PCPS was characterized and elucidated on basis of chemical analysis, and 1D/2D NMR studies.



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