SYNOPSIS

The main objective of this thesis entitled "**Studies on bioactive polysaccharides from mushroom and plant**" is to determine the structure as well as some biological activity of different polysaccharides isolated from the fruiting bodies of two edible mushrooms *Pleurotus ostreatus* and *Pflo Vv5 FB* (hybrid mushroom of *Pleurotus florida* and *Volvariella volvacea*) and the leaves of *Azadirachta indica* (Neem). The entire thesis is divided into five chapters.

Chapter-I: Here the general introduction to carbohydrates, polysaccharides, plant and mushroom polysaccharides and their biological activities are discussed.

Carbohydrates are the most abundant and diverse class of compounds in the biological world, making up more than 50 % of the dry weight of the Earth's biomass. Carbohydrates are important constituents of all living organisms, and have a variety of different functions. The great bulk of the carbohydrates in nature are present as polysaccharides, which have relatively large molecular weights. Polysaccharides belong to a structurally diverse class of macromolecules, where polymers of monosaccharide residues are joined to each other by glycosidic linkages. The polysaccharides in mushrooms occur mostly as glucans, some of which are linked by β-(1→3), (1→6) glycosidic bonds and α-(1→3) glycosidic bonds but many are true heteroglycans. A wide range of glycans extending from homopolymers to highly complex heteropolymers exhibit antitumor activity. Differences in activity can be correlated with ability of the polysaccharide molecules to solublize in water, size of the molecules, branching rate and form.

Natural macromolecules containing carbohydrate units more than 10 are known as polysaccharide. Actually polysaccharides are the complex carbohydrates. They are made up of chains of monosaccharides (the sugars) which are linked together by glycosidic bonds.

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.

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. Polysaccharide Chemistry is a broad topic that

stands at the intersection of Organic Chemistry, Biochemistry and Polymer Chemistry. In recent years polysaccharides from both mushroom and plant have drawn the attention of chemist and immunobiologists on account of their immunomodulation and antitumor properties.

Chapter-II: This chapter describes the experimental methods, which were carried out during the thesis work.

The methodologies that have been used to determine the structure of polysaccharides have been discussed in this chapter. The biological activities of polysaccharides depend on the size of molecule, branching rate and form. So, it is very important to determine the exact structure of the polysaccharides isolated either from medicinal plant or from mushroom. The polysaccharide is purified using different chromatographic techniques. The exact structures of the polysaccharides are determined using two types of methods: (1) Chemical method that includes total acid hydrolysis, methylation, and periodate oxidation studies. (2) Spectroscopic method comprising of 1D (^{1}H, ^{13}C) and 2D NMR (DQF-COSY, TOCSY, NOESY, ROESY, HMQC, HMBC etc), and Gas-liquid chromatography-Mass spectrometry experiment (GLC-MS).

The immunoenhancing effect of the polysaccharides are examined by macrophage activation, splenocyte and thymocyte proliferation. The macrophage activation was tested on peritoneal macrophage, cultured in complete RPMI (Roswell Park Memorial Institute) media. After harvesting peritoneal macrophages (5×10^5 cells mL^{-1}) were cultured in complete RPMI media in 96-well plates. The polysaccharide was added to the wells in different concentrations. Production of nitric oxide was estimated by measuring nitrite levels in cell supernatant with Greiss reaction. Absorbance was observed at 550 nm. Lipopolysaccharide (LPS), L6511 of *Salmonella enterica* serotype typhimurium (sigma) was used as positive control.

A single cell suspension of spleen and thymus was prepared from the normal mice under aseptic conditions by frosted slides in Phosphate Buffer Saline (PBS). The contaminating RBC was removed by hemolytic Gey's solution. After washing two times in PBS the cells were further suspended in complete RPMI medium. Cell concentration was adjusted to 1×10^5 cells/mL and viability of the suspended cells (as tested by trypan blue dye exclusion) was always over 90%. The cells (180 μL) were plated in 96-well flat-bottomed plates and incubated with 20 μL of various concentrations (1-100 μg/mL) of the polysaccharide. Similar lipopolysaccharide (LPS) as used in macrophage activation was used here as positive control.

The cultures were set-up for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. Proliferation was checked by MTT assay method. The data are reported as the mean ± standard deviation of five different observations and compared against PBS control.

Chapter-III: It is one of the major part of the thesis. It represents the isolation, purification, determination of the structure, and as well as study of immunological activity of the polysaccharide isolated from the aqueous extract of the fruiting bodies of an edible mushrooms *Pleurotus ostreatus*. This work is published in *Carbohydrate Research*, **2011**, *346*, 2237-2243.

Pleurotus ostreatus cultivar was collected from Sangri La Mushroom, Jalpaiguri, West bengal, India. The pure polysaccharide (PS) showed a specific rotation of $[\alpha]_D^{23.0}$ +7.24 (c 0.1, water). The molecular weight of this PS was estimated from a calibration curve prepared with standard dextrans as ~ 2.0×10^5 Da. Paper chromatographic analysis of the TFA hydrolyzed product showed the presence of glucose and galactose as neutral sugars. The GLC analysis of alditol acetates of neutral sugars showed glucose and galactose in molar ratio of nearly 7:1. The absolute configuration of the monosaccharides was determined by the method of Gerwig et al. which showed that glucose and galactose were present in the polysaccharide with D-configuration. The PS was then methylated using the Ciucanu and Kerek method followed by hydrolysis and alditol acetate conversion. The GLC-MS analysis of alditol acetates of the methylated polysaccharide revealed the presence of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol; 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol; 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-galactitol; 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol; and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol in a molar ratio of nearly 2:2:2:1:1. These results confirmed the presence of (1→6)-linked-D-glucopyranosyl, (1→3)-linked-D-glucopyranosyl, (1→3,6)-linked-D-galactopyranosyl, terminal D-glucopyranosyl, and D-galactopyranosyl moieties in a molar ratio of nearly 2:2:2:1:1. The periodate oxidation reaction was carried out with the polysaccharide for further linking information of sugar moieties. The periodate-oxidized, $NaBH_4$ reduced material of PS upon hydrolysis with trifluoroacetic acid followed by GLC analysis showed that only galactose was retained during oxidation. GLC-MS analysis of periodate-oxidized, $NaBH_4$ reduced, and methylated PS showed the peak corresponding to 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-galactitol which confirmed the above results.

The ^{1}H NMR spectrum (500 MHz) recorded in D_2O at 27 °C showed the presence of five signals in the anomeric region at 5.11, 4.98, 4.51, 4.49, and 4.47 ppm in a ratio of nearly

1:1:2:2:2. The sugar residues were designated as **A**, **B**, **C, D,** and **E** according to their decreasing anomeric proton chemical shift values. The ^{13}C NMR (125 MHz) spectrum at 27 $^{\circ}$C showed that five anomeric signals appeared at 99.7, 98.6, 103.3, 103.1, and 103.2 ppm in a molar ratio of nearly 1:1:2:2:2. All ^{1}H and ^{13}C signals were assigned from DQF-COSY, TOCSY, and HMQC NMR experiments.

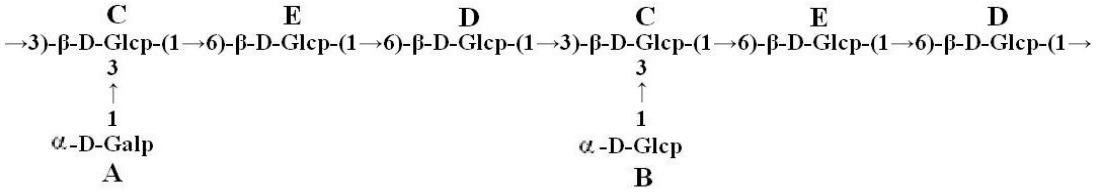
Residue **A** was assigned to terminal D-galactosyl unit. The galacto configuration was assigned from the large coupling constant $J_{H\text{-}2,H\text{-}3}$ (~9 Hz) and a relatively small coupling constant $J_{H\text{-}3,H\text{-}4}$ (~3.5 Hz). The anomeric proton chemical shift for residue **A** at 5.11 ppm and a carbon chemical shift at 99.7 ppm ($J_{C\text{-}1,H\text{-}1}$~170 Hz) indicated that the galactose was α-linked anomer. The carbon signals from C-1 to C-6 of residue **A** corresponded nearly to the standard values of methyl glycosides. Thus the residue **A** was an α-linked terminal D-galactopyranosyl moiety. In case of residues **B**, **C**, **D**, and **E** the large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ (9~10 Hz) indicating of their gluco configuration. Residue **B** had an anomeric proton signal at 4.98 ppm and the coupling constants $J_{H\text{-}1,H\text{-}2}$ (~3.2 Hz) and ($J_{C\text{-}1,H\text{-}1}$~171 Hz) indicating its α-configuration. The signals from C-1 to C-6 indicated that residue **B** was a terminal α-D-glucopyranosyl moiety. The coupling constants $J_{H\text{-}1,H\text{-}2}$ (~8-9 Hz) and $J_{C\text{-}1,H\text{-}1}$(~160-162 Hz) of residue **C**, **D**, and **E** and in addition to their anomeric proton signals at 4.51, 4.49, and 4.47 ppm respectively, indicating of their β-configuration. The anomeric carbon signals of residue **C**, **D**, and **E** appeared at 103.3, 103.1, and 103.2 ppm respectively. The downfield shift of C-3 (84.8 ppm) of residue **C** indicated that it was (1→3)-linked β-D-Glucopyranosyl moiety. The residue **D** was assigned as (1→6)-β-D-glucopyranosyl moiety as the presence of the downfield shift of C-6 (69.2 ppm). The downfield shifts of C-3 (84.9 ppm) and C-6 (69.3 ppm) of residue **E** indicated that **E** was (1→3,6)-linked β-D-glucopyranosyl moiety. The C-6 linking of residue **D** and **E** was confirmed from DEPT-135 spectrum.

The sequences of glycosyl residues of the polysaccharide were determined on the basis of the phase sensitive ROESY as well as NOESY experiments. The inter-residual contacts from **A**H-1 to **E**H-3, **B**H-1 to **E**H-3, **C**H-1 to **E**H-6a and **E**H-6b, **D**H-1 to **C**H-3, and **E**H-1 to **D**H-6a and **D**H-6b indicated the following sequences:

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

Long range ^{13}C-^{1}H correlation obtained from the HMBC experiment corroborated the assigned repeating unit established from the ROESY experiment. The cross peaks of both anomeric protons and carbons of each of the sugar residues were examined and the connectivities were observed from the HMBC experiment. Inter residual cross peaks **A**H-1 / **E**C-3; **A**C-1 / **E**H-3; **B**H-1 / **E**C-3; **B**C-1 / **E**H-3; **C**H-1 / **E**C-6; **C**C-1 / **E**H-6a; **C**C-1 / **E**H-

6b; **D**H-1 / **C**C-3; **D**C-1 / **C**H-3; **E**H-1 / **D**C-6; **E**C-1 / **D**H-6a; **E**C-1 / **D**H-6b with other intra residual peaks were observed. On the basis of the appearance of these cross peaks and ROESY connectivities, the structure of the repeating unit present in the polysaccharide (POPS) isolated from *P. ostreatus* cutivar was established as:



In order to obtain information on the sequence of the sugar residues in the repeating unit, the polysaccharide was subjected to Smith degradation studies, and the products were separated on a Sephadex G-25 column using water as the eluant, resulting in one fraction (SDPS). GLC analysis of the alditol acetates of the acid-hydrolyzed product from SDPS showed the presence of D-glucose and glycerol in a molar ratio of nearly 2:1. The alditol acetates of the methylated product from SDPS were analyzed by GLC and these methylated sugars were also identified by GLC–MS analysis using ZB-5MS capillary column which showed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol in a molar ratio of nearly 1:1. The ^{1}H NMR (500 MHz) experiment of SDPS showed two anomeric signals at 4.51 and 4.47 ppm in a molar ratio of nearly 1:1. The anomeric signals at 4.51 and 4.47 ppm corresponded to β-D-Glc*p*-(1→ (Residue **F**), and →6)-β-D-Glc*p*-(1→ (Residue **G**) respectively. The ^{13}C NMR (125 MHz) experiment (Table 4) of SDPS showed two anomeric carbon signals at 103.1 and 102.9 ppm corresponding to β-D-Glc*p*-(1→ (**F**) and another →6)-β-D-Glc*p*-(1→ (**G**) residues, respectively. The carbon signals C-1, C-2, and C-3 of the glycerol moiety were assigned as 66.8, 72.4, and 62.9 ppm, respectively. The terminal β-D-Glc*p* unit (**F**) was generated during Smith degradation of (1→3)-β-D-Glc*p* moiety (**C**) of the main chain. The (1→6)-β-D-Glc*p* (**G**) was produced from the (1→3,6)-β-D-Glc*p* (**E**) residue due to oxidation, followed by Smith degradation of the terminal α-D-Gal*p* moiety (**A**) and of the terminal α-D-Glc*p* moiety (**B**) on another side. The glycerol moiety (**H**) was generated from (1→6)-β-D-Glc*p* (**D**) after periodate oxidation, followed by Smith degradation. Hence, the structure of the Smith degraded product was established as

F **G** **H**

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

Therefore, these results supported the above mentioned structure of the repeating unit of the polysaccharide.

The PS was found to activate the macrophages. Macrophage activation was studied by nitric oxide (NO) production in culture supernatant in vitro. Upon treatment with different concentrations of this PS, enhanced production of NO was observed maximum value of 9.1 µM per 5 x 10^5 macrophages at 25 µg/mL but decreased at concentration 50 µg/mL and further increased at 100 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 the polysaccharide by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. At 25 µg/mL of the polysaccharide, both splenocyte and thymocyte proliferation indexes were maximum as compared to other concentrations. Hence, 25 µg/mL of the polysaccharide can be considered as efficient splenocyte and thymocyte proliferators.

Chapter-IV: It is another major part of the thesis, contains structural and biological investigation of the polysaccharide, isolated from alkaline extract of an edible mushroom, *Pflo Vv5 FB* (hybrid mushroom of *Pleurotus florida* and *Volvariella volvacea*).

A water soluble (1→6)-β-D-glucan from the hot water extract of the somatic hybrid (*Pflo Vv5 FB*) strains of *P. florida* and *V. volvacea* was reported. Here, the structural characterization and immunoenhancing properties of the polysaccharide isolated from the alkaline extract of the hybrid mushroom *Pflo Vv5 FB* is presented. The aim of this work is to investigate any difference arising in the constituent of polysaccharide of this strain from the parent mushrooms and their other hybrids. It has been observed that the present polysaccharide has not been obtained from any of the parent strains, *P. florida* and *V. volvacea* and its other hybrids.

The pure polysaccharide showed a specific rotation of $[\propto]_D^{25}$ +14.5 (c 0.094, water). The average molecular weight was estimated as ~ 1.87x10^5 Da compared with standard dextrans. Paper chromatographic analysis of the hydrolyzed product showed the presence of glucose only. The GLC analysis of the alditol acetates of PS also showed only the presence of glucose. The absolute configuration of the monosaccharides was determined by the method of Gerwig et al. All the sugar residues had D configuration. The mode of linkages of PVPS was determined by methylation analysis using Ciucanu and Kerek method. The GLC and GLC-MS of alditol acetates of the methylated product showed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol; 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol; and

1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a molar ratio of nearly 1:2:1. This result indicated the presence of terminal D-glucopyranosyl, (1→6)-linked-D-glucopyranosyl, and (1→3,6)-linked D-glucopyranosyl moieties in a molar ratio of nearly 1:2:1.

The ^{1}H NMR spectrum (500 MHz) recorded in D_2O at 27 °C showed the presence of three signals in the anomeric region at 4.50, 4.49, and 4.47 ppm in a ratio of nearly 1:2:1. The sugar residues were designated as **A**, **B**, and **C** according to their decreasing anomeric proton chemical shift values. The ^{13}C NMR (125 MHz) spectrum at 27 °C showed that four anomeric signals appeared at 102.75, 102.85, 102.9, and 103.2 ppm in a ratio of nearly 1:1:1:1. All ^{1}H and ^{13}C signals were assigned from DQF-COSY, TOCSY, and HSQC NMR experiments.

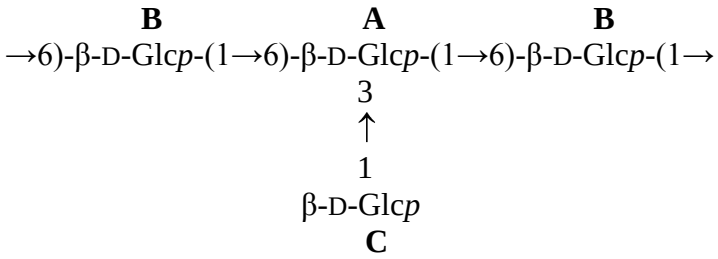
The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ (9~10 Hz) of all the residues indicated their gluco configuration. The coupling constants $J_{H\text{-}1,H\text{-}2}$ (~8-9 Hz) and $J_{C\text{-}1,H\text{-}1}$(~160-162 Hz) of the residues and in addition to their anomeric proton signals at 4.50, 4.49, and 4.48 ppm respectively, indicated their β-configuration. Residue **A** was assigned as (1→3,6)-linked β-D-glucopyranosyl moiety. The anomeric proton appeared at 4.50 ppm was correlated carbon signal at 102.75 ppm in HSQC spectrum. The downfield chemical shifts of C-3 (85.0 ppm) and C-6 (68.6 ppm) of residue **A** with respect to the standard methyl glycosides indicated that it was (1→3,6)-linked β-D-glucopyranosyl moiety. The other carbon signals of this residue corresponded nearly to the standard values of methyl glycosides. The anomeric proton signal of residue **B** appeared at 4.49 ppm was further correlated with two carbon signals at 102.85 ($\mathbf{B_{II}}$) and 102.9 ($\mathbf{B_{I}}$) ppm respectively. The downfield chemical shifts of C-6 of $\mathbf{B_{II}}$ at 68.7 and $\mathbf{B_{I}}$ at 68.8 ppm were due to α-effect of glycosylation. Since, residue **A** was the most rigid part of the backbone of this glucan, it's C-6 (68.6 ppm) appeared at the upfield region in comparison to that of the other (1→6)-linked residues (**B**). Among the two **B** residues, $\mathbf{B_{I}}$ is glycosidically linked to **A**, its C-6 (68.8 ppm) showed downfield shift due to neighbouring effect of the rigid part **A**, while $\mathbf{B_{II}}$ (68.7 ppm) reasonably appeared in the upfield region. $\mathbf{B_{I}}$ and $\mathbf{B_{II}}$ residues differ only in chemical shifts at C-1 (102.9 and 102.85 ppm) and C-6 (68.8 and 68.7 ppm), respectively where as the other carbon and all the proton chemical shifts remain unaltered. The C-6 linkages of residue **A** and **B** were further confirmed from DEPT-135 spectrum. Residue **C** was assigned as terminal β-D-glucopyranosyl moiety. The anomeric proton and carbon signals appeared at 4.47 and 103.2 ppm respectively was assigned from HSQC. The carbon signals from C-1 to C-6 of residue **C** corresponded nearly to the standard values of methyl glycosides. Thus, the residue **C** was established terminal β-D-glucopyranosyl moiety.

The sequences of glycosyl residues of the polysaccharide were determined on the basis of the phase sensitive ROESY as well as NOESY experiments. The inter-residual contacts from **A**H-1 to **B**H-6a and **B**H-6b, **B**H-1 to **A**H-6a and **A**H-6b, and **C**H-1 to **A**H-3 indicated the following sequences:

A (1→6) **B**; **B** (1→6) **A**; **C** (1→3) **A**

Long range ^{13}C-^{1}H correlation of the HMBC experiment established the assigned sequences of the ROESY experiment. Inter- residual cross peaks **A**H-1 / **B**C-6; **A**C-1 / **B**H-6a, **B**H-6b; **B**H-1 / **A**C-6; **B**C-1 / **A**H-6a, **A**H-6b; **C**H-1 / **A**C-3; **C**C-1 / **A**H-3 with other intra residual peaks were observed.

Thus, the monosaccharide composition, methylation studies, and NMR experiments indicated that the repeating unit of the polysaccharide has a backbone of three (1→6)-linked β-D-glucopyranosyl units, where one of them is substituted at *O*-3 position with β-D-glucopyranosyl moiety and the structure is proposed as:



The PS was found to activate the macrophages. Macrophage activation was studied by nitric oxide (NO) production in culture supernatant in vitro. On treatment with different concentrations of this PS, an enhanced production of NO was observed in a dose-dependent manner with optimum production of 33.24 µM NO per 5 x 10^5 macrophages at 50 µg/mL. The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with the polysaccharide by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. At 25 µg/mL of the polysaccharide, both splenocyte and thymocyte proliferation indexes were maximum as compared to other concentrations. Hence, 25 µg/mL of the polysaccharide can be considered as efficient splenocyte and thymocyte proliferators.

Chapter V: It is another part of the thesis, contains structural and biological investigation of the polysaccharide, isolated from hot water extract of the leaves of *Azadirachta indica* (neem).

Azadirachta indica (locally known as neem and nimba) belongs to the family Meliaceae. The aqueous extract of neem leaves is very effective to the viruses of small pox, polio, and HSV. Extract of neem leaves has been found to possess anticarcinogenic properties.

An attempt for the characterization of the water-extracted polysaccharide isolated from *A. indica* was performed and reported. But the detailed structural work has not yet been done. Here, we discuss the detailed structural characterization and also immunoenhancing properties of the polysaccharide isolated from the leaves of *A. indica*.

The hot aqueous extract of fresh leaves of *A. indica* (500 g) was cooled, filtered, and precipitated in alcohol and then centrifuged. The residue was dissolved in a minimum volume of distilled water, dialyzed, centrifuged, and then freeze dried to yield 1.5 g of material. The material on fractionation through DEAE-Cellulose in chloride form was eluted using distilled water as eluant followed by stepwise NaCl gradient (0 – 1.0 M) at a flow rate of 0.9 mL min^{-1} and monitored at 490 nm using phenol-sulphuric acid colorimetric assay method and a homogeneous peak was obtained. The material was collected and freeze-dried. The purified material was successively passed through sephacryl S-400 followed by sepharose-6B and a pure polysaccharide (PS) was obtained. Similar type of graph has been obtained for each type of purification. The total carbohydrate of this fraction was estimated to be 98.5% using phenol-sulfuric acid method.

The molecular weight of the PS was found to be ~2×10^2 kDa. It showed a specific rotation of $[\alpha]_D^{32.5}$ +8.96 (*c* 0.05, water). The polysaccharide was hydrolyzed with 2 M CF_3COOH for 18 h at 100 ^oC. Paper chromatographic analysis of the hydrolyzed PS showed the presence of galactose, and galactouronic acid and two slow moving spots nearer to arabinose and rhamnose. The GLC analysis of the alditol acetates of the hydrolyzed PS showed the presence of arabinose, rhamnose, and galactose in the molar ratio of nearly 1:1:1, but the carboxyl-reduced polysaccharide on hydrolysis followed by GLC analysis showed the presence of the above mentioned sugars in a molar ratio of nearly 1:1:2, which confirmed the presence of 1 mol of galacturonic acid in the PS. The absolute configuration of the sugar units present in the PS were determined by the method of Gerwig et al, taking intact and carboxyl-reduced polysaccharide, and it was found that arabinose and rhamnose had L-configuration and galactose and galacturonic acid had D-configuration. The modes of linkages of PS were determined by methylation analysis using Ciucanu and Kerek method followed by hydrolysis and alditol acetates preparation. The GLC and GLC-MS analysis of alditol acetates of methylated product revealed the presence of 2,3,5-Me_3-Ara, 3,4-Me_2-Rha, and 2,3,4-Me_3-Gal in a molar ratio of nearly 1:1:1. These results indicated the presence of terminal Ara*f*, (1→2)-Rha*p*, and (1→6)-Gal*p* moieties in the PS. The alditol acetates of methylated carboxyl-reduced polysaccharide were identified by GLC-MS analysis, which

showed the presence of the above peaks along with a new peak of 3-Me-Gal in a molar ratio of nearly 1:1:1:1. These result indicated that 1 mol of (1→2,4)-linked galacturonic acid was also present in the PS. The methylated sugars were identified with respect to their retention times and comparing with standard derivatives. Thereafter, these linkages were further confirmed by periodate-oxidation experiment. The periodate-oxidized reduced material upon hydrolysis with trifluoro acetic acid followed by GLC analysis showed no peaks corresponding to neutral sugars but paper chromatographic studies showed the presence of galacturonic acid only. Thus the periodate oxidation experiment confirmed that galacturonic acid was present as (1→2,4)-linked moiety.

The 500 MHz ^{1}H NMR spectrum of PS recorded at 27 ^{o}C, gave four anomeric signals at δ 5.23, 5.22, 4.50, and 4.44 in a ratio of nearly 1:1:1:1. The sugar residues were designated as **A-D** according to their decreasing anomeric proton chemical shifts. The ^{13}C NMR spectrum (125 MHz) of PS at 27 ^{o}C also revealed four signals at δ 98.8, 103.5, 103.7, and 107.9 which corresponded to the anomeric carbons of **A**, **C**, **D**, and **B**, respectively, from HSQC spectrum. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY, and HSQC NMR experiments. The proton-proton coupling constant values were determined from DQF-COSY experiments.

The spin system of residue **A** which consisted of only five protons with a relatively high chemical shift of the H-5 signal (δ 4.44) and weak coupling between H-3, H-4, and H-5 indicated that it was D-galacturanosyl residue. The anomeric proton at δ 5.23 ($J_{H\text{-}1,H\text{-}2}$ ~3.0 Hz) and $J_{C\text{-}1,H\text{-}1}$ ~170 Hz of this residue indicated that it was α-linked sugar residue. The C-2 (δ 75.2) and C-4 (δ 76.0) signals of residue **A** showed downfield shifts compared to the standard methyl glycosides. The appearance of a carbon signal at δ 171.2 due to the carboxylic acid (-COOH) group clearly indicated that the residue was galacturonic acid. These data confirmed that residue **A** was (1→2,4)-α-D-Gal*p*A.

Residue **B** with anomeric proton signal at δ 5.22 and very high anomeric carbon chemical shift at δ 107.9 and $J_{H\text{-}1,H\text{-}2}$ ~1.7 Hz indicated that it was α-linked sugar residue existing as a furanose structure. The signals at δ 81.3, 77.5, 83.9, and 61.1 corresponded to C-2, C-3, C-4, and C-5, respectively. All the carbon values with respect to the standard methyl arabinofuranosides indicated that the residue **B** was terminal-α-L-arabinofuranosyl moiety.

In the case of residue **C**, the anomeric proton chemical shift at δ 4.50, a large coupling constant value ($J_{H\text{-}1,H\text{-}2}$ ~8.1 Hz) and ($J_{C\text{-}1,H\text{-}1}$ ~162 Hz) indicated that it was β-linked sugar residue. The $J_{H\text{-}2,H\text{-}3}$ value (~9 Hz) and $J_{H\text{-}3,H\text{-}4}$ value (~3.4 Hz) for residue **C** indicated that it was β-D-galactopyranosyl residue. The downfield shift of C-6 (δ 66.4) with respect to the

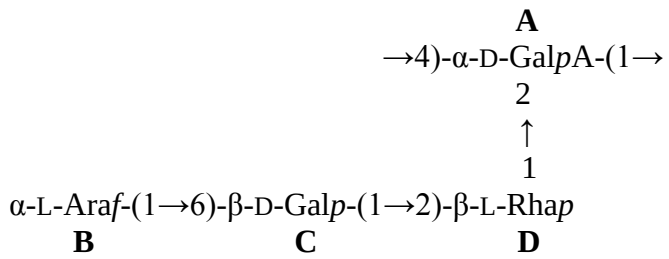
standard methyl glycosides indicated that it was present as (1→6)-β-D-galactopyranosyl moiety. The C-6 linkage was further confirmed from DEPT-135 spectrum.

For residue **D**, the anomeric proton chemical shift at δ 4.44 with $J_{C\text{-}1,H\text{-}1}$ ~160 Hz indicated that it was β-linked residue. The anomeric carbon signal appeared at δ 103.7. A very upfield carbon signal (δ 16.6) and proton signal (δ 1.24) of the exocyclic CH_3 group in residue **D** and weak coupling between H-1, H-2, and H-3 indicated that it was a β-L-rhamnopyranosyl residue. Downfield chemical shift of C-2 (δ 79.2) with respect to the standard value of methyl glycosides indicated that it was (1→2)-β-L-rhamnopyranosyl moiety.

The sequence of glycosyl residues of the polysaccharide was determined from ROESY as well as NOESY experiments. The inter-residual NOESY contacts between **A** H-1 to **A** H-4; **B** H-1 to both **C** H-6a and **C** H-6b; **C** H-1 to **D** H-2; and **D** H-1 to **A** H-2 were observed and the following sequences were established:

A-(1→4)-**A; B**-(1→6)-**C; C**-(1→2)-**D; D**-(1→2)-**A**

The sequence of glycosidic linkages was further confirmed by the HMBC experiment. The inter-residual cross couplings **A** H-1/**A** C-4; **A** C-1/**A** H-4; **B** H-1/**C** C-6; **B** C-1/**C** H-6a, H-6b; **C** H-1/**D** C-2; **C** C-1/**D** H-2; **D** H-1/**A** C-2; **D** C-1/**A** H-2 were observed in HMBC experiment. Thus, the NOESY and HMBC connectivities clearly supported the structure of the repeating unit of the pectic PS as:



In order to obtain information on the sequence of the sugar residues in the repeating unit, the polysaccharide was subjected to Smith degradation studies, and the products were separated on a Sephadex G-25 column using water as the eluant, resulting in one fraction (SDPS). GLC analysis of the alditol acetates of the acid-hydrolyzed product from SDPS showed no peaks corresponding to neutral sugars, but the alditol acetates of the acid-hydrolyzed carboxyl-reduced product from SDPS showed the presence of only galactose indicating that only the galacturonic acid is survived during oxidation. The alditol acetates of the methylated product from carboxyl-reduced SDPS were analyzed by GLC–MS analysis using ZB-5MS capillary column which showed the presence of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol indicating the presence of (1,4)- linked galacturonic acid in SDPS. The

^{13}C NMR (125 MHz) experiment of SDPS showed only one anomeric carbon signal at δ 99.5 corresponding to →4)-α-D-Gal*p*A-(1→ (**E**), indicating all the other residues were consumed during oxidation. The signals at δ 68.8, 70.9, 77.0, 71.6, and 171.1 corresponded to C-2, C-3, C-4, C-5, and C-6 respectively. Hence, from all these data the structure of the Smith-degraded homopolysaccharide is established as →4)-α-D-Gal*p*A-(1→ which further proves that the backbone of the polysaccharide is composed of galacturonic acid with rhamnogalactoarabinan side chain.

Macrophage activation by this polysaccharide has been studied by nitric oxide (NO) production in culture supernatant in vitro. Upon treatment with different concentrations of PS, an enhanced production of NO was observed with optimum production of 20.7 μM NO with initial seeded cell number 5×10^5 macrophages at 50 μg/mL and then decreases. Hence 50 μg/mL was the effective dose of PS for NO production. The cytotoxic effect of the PS was evaluated in peritoneal macrophages using the MTT method. It was found that, treatment of peritoneal macrophages up to 500 μg/ml for 24 hours had negligible or no toxic effect on cell survivability. Therefore, it can be concluded that up to 500 μg/mL PS concentration could be considered as nontoxic that did not damage peritoneal macrophage cell survivability.

The activation of splenocyte and thymocyte tests were carried out in mouse cell culture medium with PS by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. Both splenocyte and thymocyte proliferation index was found to be maximum at 50 μg/mL of the PS as compared to other concentrations. Hence, 50 μg/mL of the PS can be considered as the optimum concentration for both the splenocyte and thymocyte proliferation. Again the polysaccharide was nontoxic up to the concentration 500 μg/mL. Therefore, the biological immunoenhancing effect of the PS was nontoxic and highest at 50 μg/mL concentration. From the above observations it was clear that this PS can act as efficient immunostimulating agent.