Synopsis

The thesis entitled "**Structural characterization and biological investigation of mushroom polysaccharides**" comprises five chapters.

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

Chapter II deals with the methodologies to isolate, purify and determine the structure of pure polysaccharides and also to study their biological activities.

Chapter III represents the structural characterization of an immunoenhancing heteroglycan isolated from aqueous extract of an edible mushroom, *Lentinus squarrosulus* (Mont.) Singer and published in ***Carbohydrate Research, 345*** **(2010) 2542–2549**.

Chapter IV describes the isolation and characterization of an immunoenhancing glucan from alkaline extract of an edible mushroom, *Lentinus squarrosulus* (Mont.) Singer and published in ***Carbohydrate Research, 346*** **(2011) 2039–2044**.

Chapter V deals with the structural characterization and immunoenhancing properties of a heteroglycan isolated from an alkaline extract of a somatic hybrid mushroom (*PfloVv1aFB*) of *Pleurotus florida* and *Volvariella volvacea* and published in ***Carbohydrate Research, 354*** **(2012) 110–115.**

Chapter I: Carbohydrates are the most abundant and diverse class of organic compounds occurring in nature. Carbohydrate played a key role in the establishment and evolution of life on earth. These are classified into monosaccharide, disaccharide, oligosaccharide, and polysaccharide. The great bulk of the carbohydrates in nature are present as polysaccharides. These are composed of long chain of monosaccharide units bound together by glycosidic bonds. Polysaccharide is the major structural component for both plants and animals.

Mushrooms are a nutritionally beneficial foodstuff and a source of physiologically beneficial and nontoxic medicines. There have been reported that some mushrooms inhibit tumor growth and enhance aspects of the immune system and thus they have been a subject of research for approximately 50 years. The use of medicinal mushrooms in the fight against cancer is known from a very long time in Korea, China, Japan, Russia, USA and Canada. Polysaccharide is the most important

component of mushrooms that induces immunomodulation and plentiful medicinal properties including anti-tumor effects. Mushroom polysaccharides are not only used against cancers of stomach, esophagus, lungs, and colons but also act as anti-inflammatory, antiviral (against AIDS), hypoglycaemic and antithrombotic agents. Polysaccharides from *Lentinus edodes* (**Lentinan, Japan**), *Schizophyllum commune* (**Schizophyllan**), *Agaricus blazei* (**Agarican, USA**), *Ganoderma lucidum* (**Lingzhi, China**), *Grifola frondosa* (**Maitake, Japan**) have been used clinically as anti-tumor agents. The biological activities of mushroom 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 from mushroom.

Chapter II: This chapter illustrates the methodologies of isolation, purification and determination of the structure of pure polysaccharides and also the study of their biological activities. The crude polysaccharide was purified by gel-permeation chromatography technique using water as the eluent. The neutral sugars present in the polysaccharide were identified and estimated by gas-liquid-chromatography (GLC). The absolute configurations of sugars were identified using the method of Gerwig et al. The polysaccharide was methylated by the Ciucanu and Kerek method, followed by the GLC-MS analysis to know the mode of linkages. Periodate oxidation was carried out to confirm the mode of linkages of the sugar residues present in the polysaccharide. Besides the above chemical methods, NMR experiments (^{1}H, ^{13}C, DEPT-135, DQF-COSY, TOCSY, NOESY, ROESY, HMQC and HMBC) were also carried out to confirm the repeating unit present in the polysaccharide.

Chapter III: This chapter represents the structural characterization of an immunoenhancing heteroglycan isolated from aqueous extract of an edible mushroom, *Lentinus squarrosulus* (Mont.) Singer. The fresh fruit bodies of this mushroom, collected from the Falta experimental farm, Bose Institute were washed with distilled water, followed by extraction with hot water, precipitation in alcohol, dialysis, centrifugation, and freeze drying to yield crude polysaccharide. The crude polysaccharide was fractionated by passing through Sepharose 6B column to yield one homogeneous fraction. The molecular weight of this pure polysaccharide (PS) was estimated as ~1.96×10^5, as determined from a calibration curve using standard dextran. The PS showed a specific rotation of $[\propto]_D^{25.2}$ +7.97 (*c* 0.116, water). The

detailed structural investigation of the PS was carried out using chemical analysis and NMR experiments.

On hydrolysis with 2 M TFA, followed by alditol acetate preparation and GLC analysis, the PS was found to contain galactose, fucose, and glucose in a molar ratio of nearly 1:1:5. The absolute configuration of the monosaccharides was determined by the method of Gerwig et al. and it was found that galactose and glucose had D configuration but fucose was present with L configuration. The PS was then methylated using the method of Ciucanu and Kerek. The results of GLC-MS analysis indicated that non reducing end L-fucopyranosyl, terminal D-glucopyranosyl, (1→4)-linked D-glucopyranosyl, (1→6)-linked D-glucopyranosyl, (1→6)-linked D-galactopyranosyl, (1→4,6)-linked D-glucopyranosyl, and (1→3,6)-linked D-glucopyranosyl moieties were present in the PS in a molar ratio of nearly 1:1:1:1:1:1:1. These linkages were further confirmed by periodate oxidation experiment.

In the anomeric region of the ^{1}H NMR spectrum (500 MHz) at 27 °C, five signals were observed at δ 5.10, 5.03, 4.54, 4.52, and 4.50. The integral values of signal at δ 4.52 and 4.50 were almost double to those of other signals, which indicated that two protons were contained by each of the signals at δ 4.52 and 4.50. So five anomeric proton signals contained seven sugar residues, designated as **A**, **B**, **C**, **D**, **E**, **F**, and **G** according to their decreasing proton chemical shifts. In ^{13}C NMR spectrum (125 MHz) at 27 °C, seven signals were found in the anomeric region at δ 103.4, 103.3, 103.1, 102.9, 102.8, 101.8, and 98.2. On the basis of HMQC spectrum, the anomeric protons at δ 5.10, 5.03, and 4.54 were correlated to the anomeric carbons at δ 101.8, 98.2, and 103.4, designated as **A**, **B**, and **C** residues, respectively, whereas the proton signal at δ 4.52 was correlated to both the anomeric carbon signals at δ 103.3 and 103.1, assigned to the residues **D** and **E**, respectively. Again the proton signal at δ 4.50 was correlated to both the anomeric carbon signals at δ 102.9 and 102.8, designated as **F** and **G** residues, respectively. All the ^{1}H and ^{13}C signals were assigned from DQF-COSY, TOCSY, and HMQC experiments. The proton coupling constants were measured from DQF-COSY experiment. The sugar residues were identified by the values of $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$. The anomeric configuration of the sugar residues was determined by the values of $J_{H\text{-}1,H\text{-}2}$ and $J_{C\text{-}1,H\text{-}1}$. On the basis of

the above experimental data along with proton and carbon chemical shifts residues **A-G** were assigned as (1→6)-α-D-Gal*p*, terminal α-L-Fuc*p*, (1→3,6)-β-D-Glc*p*, (1→4,6)-β-D-Glc*p*, (1→4)-β-D-Glc*p*, (1→6)-β-D-Glc*p*, and terminal β-D-Glc*p*, respectively.

The sequences of glycosyl moieties were determined from NOESY as well as ROESY experiments. In NOESY experiment, the inter-residual contacts from **A**H-1 to **C**H-3, **B**H-1 to **D**H-4, **C**H-1 to **E**H-4, **D**H-1 to both **A**H-6a and **A**H-6b, **E**H-1 to **F**H-6a and **F**H-6b, **F**H-1 to H-6a and H-6b of residue **D**, and **G**H-1 to **C**H-6a and **C**H-6b established the following sequences;

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

;

B **D**
α-L-Fuc*p*-(1→4)- β-D-Glc*p*-(1→
6
↑

C **E**
→3)-β-D-Glc*p*-(1→4)- β-D-Glc*p*-(1→
6
↑

;

D **A**
→4)- β-D-Glc*p*-(1→6)-α-D-Gal*p*-(1→
6
↑

E **F**
→4)- β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→

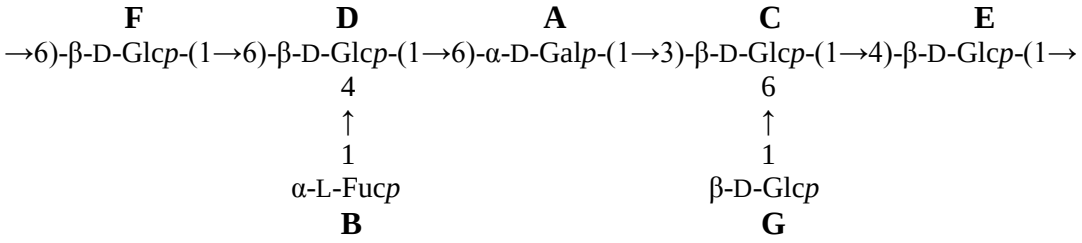
;

F **D**
→6)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→
4
↑

G **C**
β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→
3
↑

A long range HMBC experiment was carried out to confirm the NOESY and ROESY connectivities. The cross peaks of both anomeric protons and carbons of each of the sugar moieties were examined. In the HMBC experiment, inter-residual cross-peaks (**A**H-1/**C**C-3), (**A**C-1/**C**H-3), (BH-1/DC-4), (**B**C-1/**D**H-4), (**C**H-1/**E**C-4), (**C**C-1/**E**H-4), (**D**H-1/**A**C-6), (**D**C-1/**A**H-6a and **D**C-1/**A**H-6b), (**E**H-1/**F**C-6), (**E**C-1/**F**H-6a and **E**C-1/**F**H-6b), (**F**H-1/**D**C-6), (**F**C-1/**D**H-6a and **F**C-1/**D**H-6b), (**G**H-

1/**C**C-6), and (**G**C-1/**C**H-6a and **G**C-1/**C**H-6b) were found. On the basis of chemical analysis and NMR studies, the structure of the heptasaccharide repeating unit in the PS isolated from aqueous extract of *L. squarrosulus* (Mont.) Singer was established as;



Some biological studies were carried out with the PS. Macrophage activation of the PS was observed *in vitro*. On treatment with different concentrations of the PS an enhanced production of NO was observed in a dose-dependent manner with optimum production of 15.1 µM NO per 5×10^5 macrophages at 100 µg/mL of the PS. Splenocytes are the cells present in the spleen that include T cells, B cells, dentric cells, etc. that stimulate the immune response in living organism. Thymocytes are hematopoietic cells in thymus which generate T cells. The PS showed optimum activation of splenocytes and thymocytes at 20 µg/mL and 50 µg/mL, respectively.

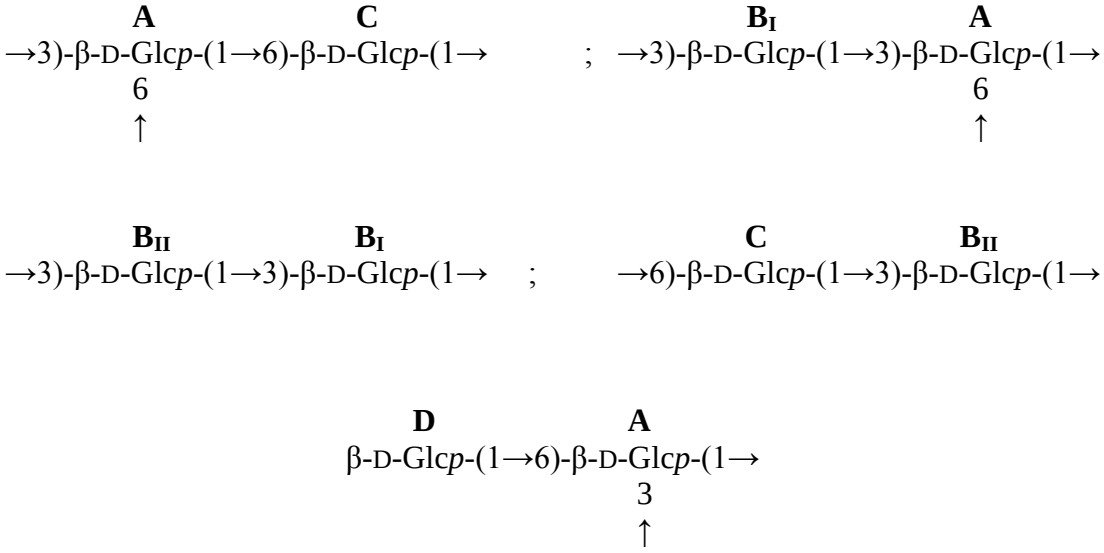
Chapter IV: It describes the isolation and characterization of an immunoenhancing glucan from alkaline extract of an edible mushroom, *Lentinus squarrosulus* (Mont.) Singer. Fruit bodies of the mushroom was washed with distilled water, followed by extraction with hot water for 12 h, and then the material was kept overnight at 4 °C and filtered through linen cloth. The residue part was boiled with 4% NaOH for 1 h, kept overnight, and again 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) EtOH. The residue was dialyzed, centrifuged and freeze dried to yield 2.1 g of crude polysaccharide, which on fractionation through Sepharose-6B column using water as eluant yielded only one fraction. The molecular weight of the pure polysaccharide (PS) was estimated as ~1.98×10^5, as determined from a calibration curve using standard dextran. The PS showed a specific rotation of $[\propto]_D^{25.5}$ –21 (*c* 0.1, water).

GLC analysis of the alditol acetates of the acid hydrolyzed PS showed the presence of glucose only. The absolute configuration of the monosaccharides was determined by the method of Gerwig et al. and it was found that glucose was present with D configuration. The results of GLC-MS analysis indicated that (1→3,6)-, (1→3)-, (1→6)-linked, and terminal glucopyranosyl moieties were present in the PS in a molar ratio of nearly 1:2:1:1. These linkages were further confirmed by periodate oxidation experiment.

In the anomeric region of the ^{1}H NMR spectrum (500 MHz) at 27 °C, two signals were observed at δ 4.52 and 4.50. In ^{13}C NMR spectrum (125 MHz) at the same temperature, four signals were found in the anomeric region at δ 103.4, 103.2, 102.9, and 102.7. So, two anomeric proton signals contained four sugar residues, designated as **A**, **B**, **C**, and **D**. On the basis of HMQC spectrum, the anomeric proton signal at δ 4.52 was correlated to both the carbon signals at δ 103.4 and 102.9, designated as **A** and **B** residues, respectively. Again, the proton signal at δ 4.50 was correlated to both carbon signals at δ 103.2 and 102.7, assigned to the residues **C** and **D**, respectively. The response of the signal at δ 102.9 was almost double to those of other signals, indicating the presence of two units of residue **B**. All the ^{1}H and ^{13}C signals were assigned from DQF-COSY, TOCSY, and HMQC experiments. The proton coupling constants were measured from DQF-COSY experiment. The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values (~10 Hz) of all residues from **A**-**D** confirmed their glucopyranosyl configuration. In case of all residues (**A**-**D**), the coupling constants $J_{H\text{-}1,H\text{-}2}$ (~8 Hz) and $J_{C\text{-}1,H\text{-}1}$ (~160-161 Hz) and in addition to their range of anomeric proton chemical shifts (4.52-4.50 ppm) and anomeric carbon chemical shifts (103.4-102.7 ppm) confirmed their β-configuration. The downfield shifts of C-3 and C-6 of residue **A** with respect to the standard values of methyl glycosides indicated that it was present in the PS as (1→3,6)-β-D-Glc*p*. Since, residue **A** was the most rigid part of the backbone of this glucan, it's C-3 appeared at the upfield region in comparison to that of the other (1→3)-linked residues (**B)**. Among the two **B** residues, one residue (**B$_{I}$**) was situated adjacent to the residue **A**, and other residue (**B$_{II}$**) was away from it. C-3 of **B$_{I}$** showed upfield shift due to neighbouring effect of the rigid part '**A**' while that of **B$_{II}$** reasonably appeared in downfield region. **B$_{I}$** and **B$_{II}$** residues differ only in chemical shifts of H-3 and C-3 while the other chemical shifts remain

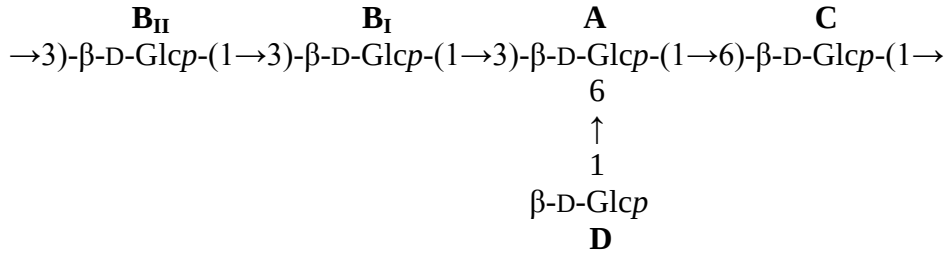
same. The downfield shift of C-6 of residue **C** indicated that it was (1→6)-linked moiety. The linking of residues of **A** and **C** at C-6 was further confirmed from DEPT-135 spectrum. The carbon chemical shifts of residue **D** from C-1 to C-6 corresponded nearly to the standard values of methyl glycoside of β-D-glucose. Thus, residue **D** was terminal β-D-Glc*p*.

The sequences of glycosyl moieties were determined from NOESY as well as ROESY experiments. In NOESY experiment the inter-residual contacts from **A**H-1 to both **C**H-6a and **C**H-6b, $\mathbf{B_I}$H-1 to **A**H-3, $\mathbf{B_{II}}$H-1 to $\mathbf{B_I}$H-3, **C**H-1 to $\mathbf{B_{II}}$H-3, and **D**H-1 to both H-6a and H-6b of residue **A** were observed. The above NOESY connectivities established the following sequences:



A long range HMBC experiment was carried out to confirm the NOESY and ROESY connectivities. In the HMBC spectrum the cross-peaks of both anomeric protons and carbons of each glycosyl residues were examined. The cross-peaks were found between **A**H-1 and **C**C-6; **A**C-1 with both H-6a and H-6b of residue **C**, $\mathbf{B_I}$H-1 and **A**C-3; $\mathbf{B_I}$C-1and **A**H-3, $\mathbf{B_{II}}$H-1 and $\mathbf{B_I}$C-3; $\mathbf{B_{II}}$C-1 and $\mathbf{B_I}$H-3, **C**H-1 and $\mathbf{B_{II}}$C-3; **C**C-1 and $\mathbf{B_{II}}$H-3, and **D**H-1 and **A**C-6; **D**C-1 with both H-6a and H-6b of residue **A**.

Thus, the HMBC and NOESY connectivities clearly supported the presence of the pentasaccharide repeating unit in the PS isolated from *L. squarrosulus* (Mont.) Singer as:



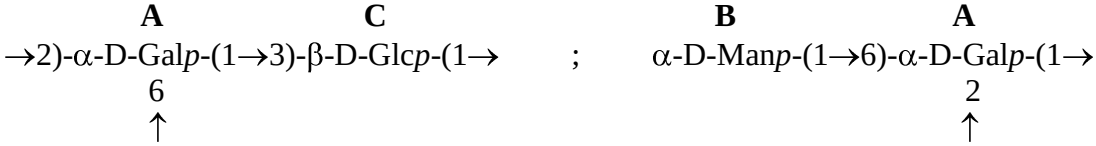
Some biological studies were carried out with this PS. This PS showed optimum activation of macrophages as well as splenocytes and thymocytes at 10 µg/mL.

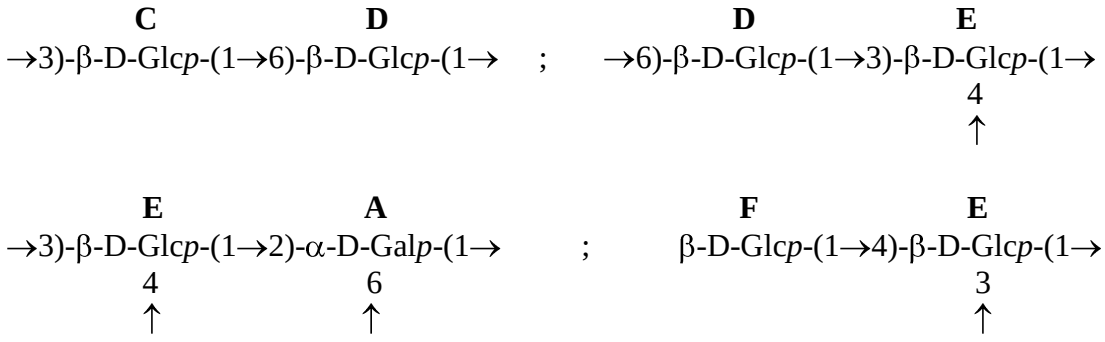
Chapter V: This chapter deals with the structural characterization and immunoenhancing properties of a heteroglycan isolated from an alkaline extract of a somatic hybrid mushroom (*PfloVv1aFB*) of *Pleurotus florida* and *Volvariella volvacea*. Fruit bodies of this mushroom were washed with distilled water, followed by extraction with 6% NaOH for 45 min. The crude polysaccharide was dialyzed through cellulose membrane against distilled water to remove low molecular weight materials and then fractionated by passing through Sepharose 6B column to yield one homogeneous fraction. The molecular weight of the pure polysaccharide (PS) was estimated as ~1.92×10^5, as determined from a calibration curve using standard dextran. The PS showed a specific rotation of $[\infty]_D^{25.5}$ + 8.95 (*c* 0.1, water).

The alditol acetates of acid hydrolyzed PS showed the presence of galactose, mannose, and glucose in a molar ratio of nearly 1:1:4 that were detected by GLC analysis. The absolute configuration of the monosaccharides was determined by the method of Gerwig et al. and it was found that all the monosaccharides were present with D configuration. The results of GLC-MS analysis indicated that (1→2,6)-galactopyranosyl, (1→3,4)-, (1→3)-, (1→6)-linked, and terminal glucopyranosyl as well as terminal mannopyranosyl residues were present in a molar ratio of nearly 1:1:1:1:1:1. These linkages were further confirmed by periodate oxidation experiment.

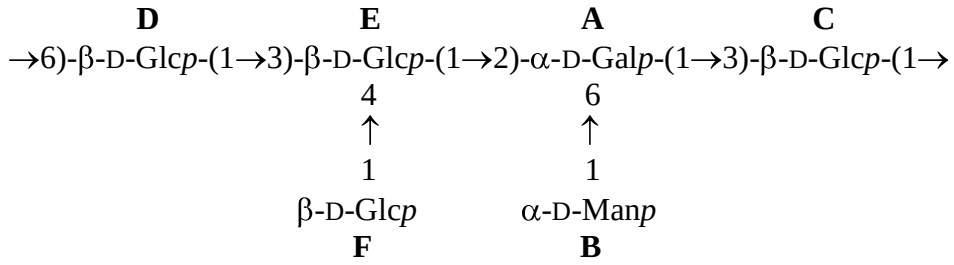
In the anomeric region of the ^{1}H NMR spectrum at 27 ^{o}C, four signals were observed at δ 5.12, 4.98, 4.52, and 4.50. The integral values of the signals at δ 4.52 and 4.50 were almost double to those of other signals, which indicated that two protons were contained by each of the signals at δ 4.52 and 4.50. In ^{13}C NMR spectrum five signals were observed in the anomeric region at δ 103.4, 103.0, 102.8, 102.0, and 99.8 where the signal at δ 103.4 was found almost double to those of other signals. So, four anomeric proton signals and five anomeric carbon signals clearly indicated that the PS contained six sugar residues, designated as **A**, **B**, **C**, **D**, **E**, and **F**. On the basis of the HMQC spectrum, the anomeric proton signals at δ 5.12 and 4.98 correlated to the carbon signals at δ 99.8 and 102.0, designated as **A** and **B** residues, respectively. The anomeric proton signal at δ 4.52 was correlated to both the carbon signals at δ 103.4 and 103.0, and assigned to the residues **C** and **D**, respectively. Again, the anomeric proton signal at δ 4.50 was correlated to both the carbon signals at δ 103.4 and 102.8, and assigned as **E** and **F** residues, respectively. All the ^{1}H and ^{13}C signals were assigned from DQF-COSY, TOCSY, and HMQC experiments. The proton coupling constants were measured from DQF-COSY experiment. **A-F** sugar residues were determined on the basis of proton coupling constants, measured from DQF-COSY experiment. The anomeric configuration of the sugar residues was determined from $J_{\text{H-1,H-2}}$ and $J_{\text{C-1,H-1}}$ coupling constant values. On the basis of the above experimental data along with proton and carbon chemical shifts the residues **A**-**F** were assigned as (1→2,6)-α-D-Gal*p*, terminal α-D-Man*p*, (1→3)-β-D-Glc*p*, (1→6)-β-D-Glc*p*, (1→3,4)-β-D-Glc*p*, and terminal β-D-Glc*p*, respectively.

The sequences of glycosyl moieties were determined from ROESY as well as NOESY experiments. In ROESY experiment, the inter-residual contacts from **A**H-1 to **C**H-3, **B**H-1 to both H-6a and H-6b of residue **A**, **C**H-1 to **D**H-6a and **D**H-6b, **D**H-1 to **E**H-3, **E**H-1 to **A**H-2, and **F**H-1 to H-4 of residue **E** were observed. Thus, the ROESY connectivities established the following sequences;





A long range HMBC experiment was carried out to confirm the ROESY and NOESY connectivities. In HMBC spectrum the cross peaks of both the anomeric protons and carbons of each glycosyl residues were examined. In the HMBC experiment, inter residual cross peaks **A**H-1/**C**C-3; **A**C-1/**C**H-3; **B**H-1/**A**C-6; **B**C-1/**A**H-6a; **B**C-1/**A**H-6b; **C**H-1/**D**C-6; **C**C-1/**D**H-6a; **C**C-1/**D**H-6b; **D**H-1/**E**C-3; **D**C-1/**E**H-3; **E**H-1/**A**C-2; **E**C-1/**A**H-2; **F**H-1/**E**C-4; and **F**C-1/**E**H-4 were found. Thus, the HMBC and ROESY connectivities clearly supported the presence of the hexasaccharide repeating unit in the PS as:



Some biological studies were carried out with this PS. Macrophage activation of the PS was observed *in vitro*. On treatment with different concentrations of the PS it was observed that there was an increase in NO production in varying concentrations and reaches maximum at 200 µg/mL but decreases at concentration, 400 µg/mL. Hence, an enhanced production of NO was observed with optimum production of 14.8 µM NO per 5×10^5 macrophages at 200 µg/mL. This result was further confirmed through Reactive Oxygen Species (ROS) production assay using different concentrations of the PS. In comparison to PBS control and LPS as positive control ROS production was found maximum at 200 µg/mL and subsequently decreases at

400 µg/mL, confirming the previous results obtained from NO production. Thus, from these two experiments it can be concluded that the macrophage activation is optimum at 200 µg/mL. The PS showed optimum activation of splenocytes and thymocytes at 10 µg/mL.