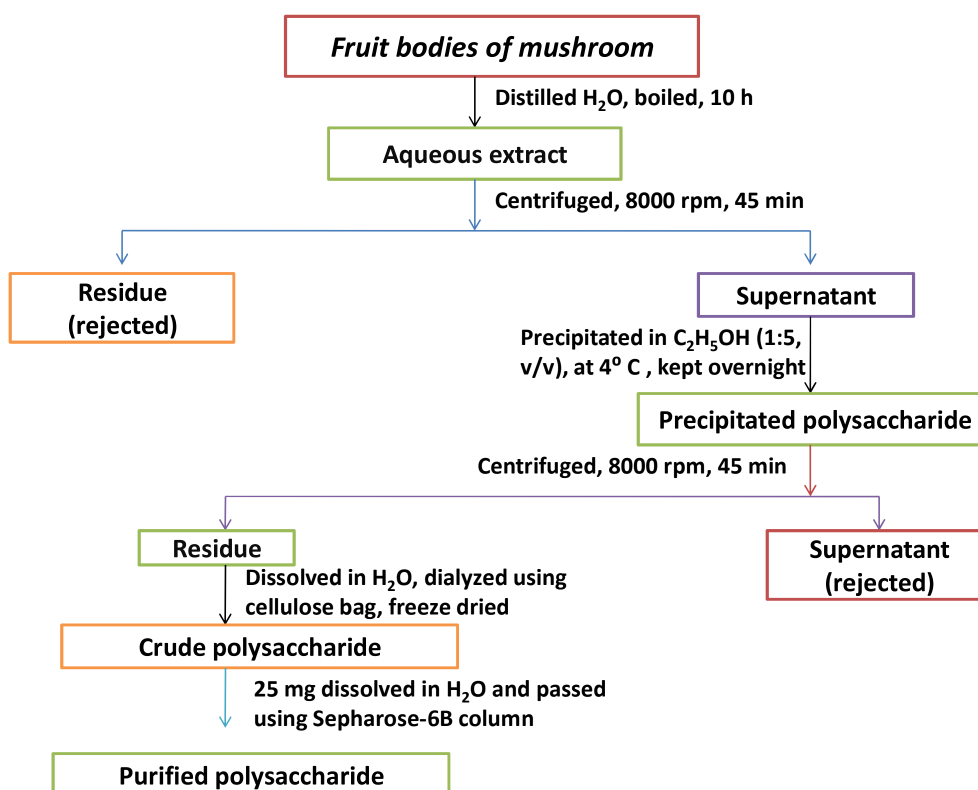
Chapter II

Methodology

2.A. Isolation and purification of polysaccharides from edible mushrooms

General schematic representation of isolation of purified polysaccharide from the mushroom are given below:



2.A.1. Polysaccharide from *Termitomyces clypeatus* (R. Heim)

Fresh fruit bodies of the mushroom *Termitomyces clypeatus* (R. Heim) (500 g) were collected, washed with H_2O, crushed, and boiled, 10 h in 500 mL of distilled H_2O, kept overnight at 4 °C, filtered, and centrifuged at 8000 rpm for 45 min at 4 °C. The

supernatant was precipitated in 1:5 (v/v) C_2H_5OH and the crude polysaccharide was freeze dried and collected. This crude materials (25 mg) was purified by using Sepharose 6B column in distilled H_2O as eluent by a Redifrac fraction collector. The fractionated material was collected and monitored by UV-vis spectrophotometer, Shimadzu, model-1601 using phenol-sulfuric acid procedure [54]. One fraction was obtained (PS) during fractionation process.

2.A.2. Polysaccharides from *Tuber rufum* (Pico) var.

Fruit bodies of the mushroom *Tuber rufum* (Pico) Var. (500 g) was washed with H_2O, boiled 10 h with distilled H_2O, and the crude polysaccharide was precipitated, and purified using the procedure as described earlier. Two fractions were obtained (PS-I and PS-II) during fractionation process.

2.A.3. Polysaccharides from *Lentinus sajor-caju*

Lentinus sajor-caju (600 g) was the gently washed with H_2O, and then boiled at 100 ^{o}C with distilled H_2O for 10 h. The mixture was cooled, centrifuged, and the supernatant was precipitated in C_2H_5OH (1:5, v/v) to get crude polysaccharide (800 mg) and purified using the procedure as described earlier. 25 mg of crude polysaccharide was fractionated using Sepharose-6B gel and two fractions were obtained, fraction I (test tube no.16-26, yield 14 mg) and fraction II (test tube no. 36-46, yield 9 mg) were collected and freeze-dried. The purification process was repeated several times to get pure polysaccharide of fraction I (PS-I, 120 mg) and preserved for further reactions and analyses.

2.B. Determination of physical properties

2.B.1. Optical rotation measurement

The optical rotation of the pure polysaccharide in aqueous medium was measured using Jasco Polarimeter (Model P-1020) at 25.7 ºC .

2.B.2. Molecular weight determination

The average molecular weight of the polysaccharide was estimated by gel-chromatographic technique [55] using standard dextrans T-200, T-70, and T-40 passing through a Sepharose 6B column, and the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of polysaccharide was then plotted in the same graph, and molecular weight of polysaccharide was determined [56,57].

2.C. Structural analysis of polysaccharides

The structural characterization of the polysaccharide was carried out by:

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

2.D. Chemical analysis

2.D.1. Monosaccharide analysis

Total acid hydrolysis of the polysaccharide was performed to investigate the monosaccharide constituents present in the polysaccharide. The Polysaccharide (3 mg)

was hydrolyzed [58-60] using 2 M trifluoroacetic acid (2 mL) in a 50 mL closed flask at 100 ºC, 18 h in water bath. The excess acid was totally removed by co-distillation with H_2O and the product was reduced with sodium borohydride (9 mg, 12h), acidified with dil. CH_3COOH, and co-distilled with CH_3OH to eliminate free boric acid and kept overnight in a vacuum desiccator over P_2O_5. The reduced sugars were acetylated with pyridine and acetic anhydride 1 mL, (1:1) in a boiling water bath for 1 h. The solvents were removed by co-distillation with toluene and the alditol acetates extracted with $CHCl_3$ was analyzed by Gas-liquid-chromatography (GLC), Hewlett-Packard, model 5730 A, at 170 ºC using 3% ECNSS-M (A) and 1% OV-225 (B).

2.D.2. Determination of absolute configuration

The absolute configuration of monosaccharide was analyzed as described by Gerwig, Kamerling, and Vliegenthart (1978) [61]. The polysaccharide (1.0 mg) was hydrolyzed with trifluoroacetic acid and the hydrolyzed product was treated with a volume of 250 µL of HCl (0.625 M) solution in R-(+)-2-butanol and heated at 80 ^{o}C for 16 h. Then the TMS-derivatives of dry reactants were prepared with *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA). The products were analyzed by GLC using a capillary column SPB-1 (30 m ×0.26 mm) with a temperature program (3 ^{o}C/min) from 150 to 210 ^{o}C. The (+)-2-butyl-2,3,4,6-tetra-*O*-TMS-glycosides were identified by comparison with those prepared from the D- and L-enantiomers of different monosaccharides.

2.D.3. Linkage analysis

2.D.3.1. Methylation analysis

Polysaccharide was methylated adopting the Ciucanu and Kerek [62] method. The polysaccharide (4.0 mg) was dissolved in DMSO (1 mL) and then treated with finely powdered NaOH in a closed vessel and stirred for 30 min. on a magnetic stirrer. Then added methyl iodide (1 mL) in stirring condition. The mixture was cooled and shaken vigorously with chloroform and water (5:2 ; v/v). The chloroform layer was collected, washed thrice with water and then dried. The methylated product was hydrolyzed 1 h, 100 ^{o}C with 90% formic acid (1 mL). The excess formic acid was then evaporated completely by co-distillation with distilled H_2O and dried over P_2O_5. The alditol acetate was prepared as described earlier, section 2.D.1 and analyzed by Gas-liquid chromatography-mass spectrometry (GLC-MS, capillary column, Shimadzu Model QP-2010) with the program 150 ºC; hold time 5 min, temperature gradient 2 ºC min^{-1} up to a final temperature 200 ºC.

2.D.3.2. Periodate oxidation study

Polysaccharide (8 mg) was dissolved in 0.1 M sodium metaperiodate solution (2 mL) and kept 48 h, 25 ^{o}C in dark. Then ethylene glycol (1 mL) was added, and dialyzed against repeated changing the distilled H_2O for 2 h to remove excess periodate and diol. The volume of the product was reduced to 2 mL through freezdrying and then reduced overnight using sodium borohydride followed by acidification with acetic acid and dried by codistillation with methanol [63]. The periodate-reduced material was divided into two parts. One part was hydrolyzed by 1 mL 2 M trifluoroacetic acid for 18 h, and the alditol

acetate of this hydrolyzed material was prepared as described earlier and analyzed by the GLC. Another part was methylated by the Ciucanu and Kerek [62] method followed by preparation of alditol acetates, which were analyzed by GLC and GLC-MS [64].

2.D.3.3. Smith degradation

Smith degradation [65] analysis was carried out using the polysaccharide (20 mg) to which 0.1 M aqueous sodium metaperiodate (4 mL) was added, kept at 25 ^{o}C, 72 h in dark. Then ethylene glycol (1 mL) was added to remove excess periodate and diol. The mixture was dialyzed four times with distilled H_2O. The dialyzed material was lyophilized to 2 mL and then reduced overnight with sodium borohydride, neutralized with 50% acetic acid, dialyzed again with distilled H_2O to eliminate small molecules, and freeze dried. The mild hydrolysis of this material was carried out by adding 0.5 M trifluoroacetic acid, 15 h, 25 ^{o}C. Then repeated co-distillation was carried out with distilled H_2O to eliminate excess acid at 37 ^{o}C and lyophilized, dried over P_2O_5 in vacuum. The freeze dried product was used for ^{13}C NMR studies.

2.E. Nuclear magnetic resonance (NMR) spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is used for identification of monosaccharide composition, elucidation of α or β anomeric configurations, the linkage patterns, and sequence of the sugar units in the repeating unit of the polysaccharide adopting 1D and 2D techniques (^{1}H, ^{13}C, DEPT-135, DQF-COSY, TOCSY, NOESY, ROESY, HSQC, and HMBC).

2. E.1. Preparation of NMR sample and instrumentation

The samples were kept over P_2O_5 in vacuum for a week to remove water completely and then dissolved in D_2O (99.96% atom ^2H, Aldrich) and lyophilized four times with D_2O for deuterium exchange. The ^1H, ^{13}C, DEPT-135, 2D (DQF-COSY, TOCSY, NOESY, ROESY, HSQC, and HMBC) NMR spectra were recorded in D_2O at 30 ^oC using Bruker Avance DPX-500 spectrometer.

2.E.2. One-dimensional (1D) NMR

2.E.2.1. ^1H NMR

The anomeric protons resonates in the chemical shift range of δ 4.4 to δ 5.5. The other ring protons are observed in the range δ 3.0 to δ 4.2. The monosaccharide units and their relative proportions are identified from the number of the signals present in anomeric region. Chemical shifts between δ 4.4 and δ 4.8 indicate the anomeric protons of β-linked residues and δ 4.9 and δ 5.5 [66,67] are for α-anomeric protons.

2.E.2.2. ^{13}C NMR

In the ^{13}C spectra, anomeric carbon signals resonate in the range of δ 90 to δ 112. The C-1 of reducing end residues usually appears in the region δ 90 to δ 98 and that of *O*-linked monosaccharides (non-reducing monosaccharides) appears at δ 98 to δ 112. This information helps greatly in determining the number of *O*-linked monosaccharides and in estimating their relative proportions. The rest of the methane and methylene resonate between δ 51 and δ 86. The methyl carbon of deoxy sugars shows in the range of δ 15 to δ 20.

The α- and β-anomer of mannose cannot be distinguish by coupling constant values of $J_{H-1,H-2}$ since they are too close. The one bond ^{13}C-^{1}H coupling constants in manno-pyranoses can be used to determine the anomeric configuration [68]. For D sugars the α- and β-anomer of mannose show coupling constant values $^{1}J_{C-1,H-1}$ ~170 Hz and $^{1}J_{C-1,H-1}$ ~160 Hz respectively [69].

2.E.2.3. DEPT-135

The linking of residues at C-6 is confirmed from the DEPT-135 (Distortion less enhancement by polarization transfer-135) spectrum. From this spectrum the methyl and methine group both produce positive signals whereas methylene group display an inverse signal appearing between δ 60 to δ 70.

2.E.3. Two-dimensional (2D) NMR spectroscopy

One-dimensional NMR methods yield limited information about the complete structure and stereochemistry of polysaccharide. Most of the proton signals of polysaccharide are observed within δ 3.0 to δ 4.2 in overlapping situation and therefore difficult to assign them. These difficulties can be overcome by the use of two-dimensional NMR techniques which have been adopted to identify the sugar units and repeating units of the polysaccharide.

2.E.3.1. Total correlation spectroscopy (TOCSY):

Total correlation spectroscopy (TOCSY) [70] as well as homonuclear Hartmann-Hahn spectroscopy (HOHAHA) [71] is used to identify each and individual protons present in each sugar moiety through long-range proton-proton coupling with mixing time

of 300 ms. The complete assignment of the protons present in each sugar unit was performed using several TOCSY experiments ranging mixing times from 60 to 300 ms.

2.E.3.2. Double quantum filtered correlation spectroscopy (DQF-COSY):

It gives an idea about protons of an individual sugar unit through a three-bond coupling. The proton coupling constants $J_{H\text{-}1,H\text{-}2}$, $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ etc are measured from the DQF-COSY spectrum.

2.E.3.3. Nuclear overhauser enhancement spectroscopy (NOESY):

Nuclear overhauser effect spectroscopy (NOESY) provides information on through space rather than through bond couplings. Cross-peaks are observed in 2D NOESY between proton pairs that are close in space (less than 5Å). In general, 1,3-diaxial and equatorial-axial proton pairs in pyranose rings produce intra NOESY cross-peaks, i.e. for β-glucopyranosyl residue crosspeaks are observed between H-1 and H-3 (and H-5) whereas a strong peak is observed between H-1 and H-2 in an α-glucopyranosyl configuration. Moreover, NOE connectivity is also observed between the anomeric proton of a particular sugar residue, and the relevant proton of the adjacent glycosidically-linked sugar residue. Inter-residue NOEs are used for determination of the sequence and linkage positions of sugar residues of a polysaccharide.

2.E.3.4. Rotating frame overhauser enhancement spectroscopy (ROESY):

ROESY is particularly useful for those cases where NOESY signals are weak. ROESY is used to find out signals arising from protons, which are close proximity by

chemical bonds. ROESY cross peaks give more accurate information about the linkage pattern of the sugar moieties present in the polysaccharide.

2.E.3.5. Heteronuclear single quantum coherence (HSQC):

All signals in HSQC spectrum gives a correlation between carbon and corresponding proton(s). All the ^{13}C chemical shifts of the sugar residues are thus assigned from the individual proton signals primarily detect from DQF-COSY and TOCSY.

2.E.3.6. Heteronuclear multiple bond correlation (HMBC):

The HMBC experiment assigned long range coupling between proton and carbon via more than one bond. HMBC experiment support the NOESY and ROESY connectivities and supply vital information about the pattern of linkages.

2.F. Antioxidant properties

2.F.1. Chelating ability of ferrous ions

The ferrous ion chelating ability of the polysaccharide was determined by the procedure as described by Dinis et al. [72] where 1 mL of each of the concentrations (0.5, 1, 1.5, 2, and 3 mg/mL) of polysaccharide were mixed with 0.1 mL 2 mM $FeCl_3$ solution. This reaction was carried out adding 0.2 mL 5 mM ferrozine with vigorous shaking, kept 10 min and absorbance was calculated at 562 nm. EDTA was taken as standard for comparison. A lower absorbance indicated a higher ferrous ions chelating ability. The percentage of inhibition of ferrozine- Fe^{2+} complex was measured using the standard equation:

$$\% \text{ of inhibition} = \{(A_0 - A_1)/ A_0\} \times 100.$$

[A_0, absorbance of control; A_1, absorbance in presence of sample]

2.F.2. Reducing power

The reducing ability was calculated as described by Oyaizu [73] with minor modification. One mL of various concentrations of polysaccharide (0.1, 0.5, 1.0, 1.5, and 2.0 mg/mL) was mixed with 2.5 mL of 1% potassium ferricyanide and 2.5 mL of 0.2 mol/L Na_3PO_4 buffer (pH 6.6), incubated 20 min and then 2.5 mL 10% CCl_3COOH was further added to stop the reaction followed by centrifugation at 3000 rpm for 10 min. 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride were added to 2.5 mL of the supernatant of the reaction mixture and incubated for 15 min. Ascorbic acid was used as standard for comparison. The absorbance of the mixture was measured at 700 nm against the blank phosphate buffer in distilled water.

2.F.3. Superoxide radical scavenging assay

The superoxide radical scavenging ability of the polysaccharide was determined by the procedure as described by Martinez et al. [74]. Reaction mixtures (3 mL) consisted of 13 mM methionine, 10 mM riboflavin, 75 µM NBT, 100 mM EDTA, 0.05 M phosphate buffer (pH 7.8), and 500 µM polysaccharide solution taken from various concentrations (50 - 400 µg/mL) and exposed to the light during 10 min. The absorbance of the solution was measured at 560 nm. Butylated hydroxyanisole (BHA) was taken as standard for comparison. This radical scavenging property was measured by the standard equation:

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

[A_0, absorbance of control ; A_1, absorbance in presence of sample]

2.F.4. Hydroxyl radical scavenging activity

The scavenging activity of hydroxyl radicals was measured by Fenton reaction as described by Halliwell et al. [75]. The reaction mixture (1 mL) consisted of KH_2PO_4 - KOH buffer (20 mM, pH 7.4), 2-deoxy-D-ribose (2.8 mM), polysaccharide with different concentrations (0.25, 0.50, 0.75, 1.0, and 1.5 mg/mL), 100 mM $FeCl_3$, 104 µM EDTA, 100 µM ascorbate, and 1 mM H_2O_2; incubated 1 h, 37˚C. Now 2 mL concentrated HCl was added to 98 mL of thiobarbituric acid (TBA) - trichloroacetic acid (TCA) solution (100mL TBA-TCA solution contained 375 mg of TBA and 15 mg of TCA). 2 mL of this solution (conc. HCl mixed TBA-TCA solution) was added to the reaction mixture and heated at boiling water bath for 15 min, cooled and absorbance was measured at 535 nm. Butylated hydroxytoluene (BHT) was used as standard for comparison. The scavenging power of the hydroxyl radicals was measured by the standard formula:

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

[A_0 , absorbance of control (without sample) ; A_1, absorbance in presence of sample]

2.F.5. DPPH radical scavenging activity

The free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) is a widely used method to estimate the free radical scavenging capability of natural compounds. The free radical scavenging activity of the polysaccharide was examined in vitro using purple colored

DPPH radical using the method as applied by Shimada et al. [76]. One mL of DMSO solution of DPPH (0.101 mM) was mixed with one mL of each of the concentrations (0.1, 0.5, 0.75, 1.0, and 1.5 mg/mL) of polysaccharide. The mixture was shaken vigorously and left to stand for 30 min in dark. The absorbance was calculated at 517 nm. For comparison Ascorbic acid was used. The concentration of samples that reduced the absorption of DPPH solution by 50% (EC_{50}) was calculated from the calibration curve. The lower absorbance of the reaction mixture indicated the higher free radical scavenging activity. The capability to scavenge the DPPH radical was measured by using the standard equation:

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

[A_0, absorbance of control (without sample); A_1, absorbance in presence of sample]

2.F.6. ABTS radical scavenging assay

Trolox Equivalent Antioxidant Capacity (TEAC) value is based on the ability of the antioxidant to scavenge the blue-green 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate ($ABTS^{\bullet+}$) radical cation relative to the $ABTS^{\bullet+}$ scavenging ability of the water-soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox).

The scavenging activity of polysaccharide against ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] radicals was determined using the method as applied in Peiyuan et al. [77] The stock solution of $ABTS^{\bullet+}$ was prepared by mixing 7 mM of ABTS with 2.45 mM of potassium persulfate and allowed to kept in dark at 30 ^{o}C during 10-14 h. The $ABTS^{\bullet+}$ solution was then diluted with 5 mM phosphate buffered

saline (pH 7.4). 100 µL of polysaccharide was added to 2 mL diluted $ABTS^{\cdot+}$ solution and absorbance was measured at 750 nm. The higher the TEAC value indicated the stronger antioxidant ability.

2.G. Biological activity

Human peripheral blood mononuclear cells (PBMCs) were collected from normal healthy donors with their consents. All the donors were from the same area, same economic status, non-smokers and non-alcoholic, and having same food habit. All of them received no medication during the period. The study protocol was in accordance with the declaration of Helsinki. PBMCs were separated by Histopaque density gradient centrifugation [78]. Cells were allowed to adhere to culture plates for 2 h. Non-adherent cells (lymphocytes) were collected and washed two times with PBS, centrifuged at 2400 rpm during 15 minutes to obtain the necessary pellet of lymphocytes.

2.G.1. Cell viability

96 wells of tissue culture plate containing 180 µL of complete media were seeded with normal human lymphocytes and incubated for 48 h. Different concentrations of the polysaccharide (25, 50, 100, 200, and 400 µg/mL) were added to the cells and incubated further 24 h at 37 ^{o}C in a humidified 5 % CO_2 incubator. The cell viability was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide according to the method as applied by Chattopadhyay et al [79].

2.G.2. Determination of reduced glutathione (GSH)

Estimation of reduced glutathione of the cell lysate was carried out using the method as adopted by Dash et al [78]. The required amount of the cell lysate was mixed with 25% trichloroacetic acid and centrifuged at 2,000 rpm 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) and then 2 ml 0.6 mM DTNB (Ellman's reagent) was added to it. After 10 minutes the optical density of the yellow-colored complex was measured at 405 nm. A standard curve was also prepared with the standard reduced glutathione. The levels of GSH were expressed as µg of GSH/mg protein.

2.G.3. Determination of Oxidized glutathione level (GSSG)

Estimation of oxidized glutathione level was carried out after derivatization of GSH with 2-vinylpyridine according to the method as applied by Dash et al [78]. 2 µL of 2-vinylpyidine was added to 0.5 mL cell lysate and incubated for 1 hr at 37°C. The mixture was then deprotenized with 4% sulfosalicylic acid and centrifuged at 1,000 rpm for 10 min to precipitate the 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.G.4. Determination of lipid peroxidation (MDA)

Estimation of lipid peroxidation of cell lysate was carried out using the method of Ohkawa et al [80]. The reaction mixture contained Tris-HCl buffer (50 mM, pH 7.4), tert-butyl hydroperoxide (BHP) (500 µM in ethanol) and 1 mM $FeSO_4$. After incubating the

samples at 37°C for 90 min, the reaction was stopped by adding 0.2 mL of 8% sodium dodecyl sulfate (SDS) followed by addition of 1.5 ml of 20% acetic acid. 1.5 ml 0.8% thiobarbituric acid (TBA) was added to the mixture and heated at 95 ^{o}C for 45 min. After cooling, the samples were centrifuged. The amount of MDA formed was estimated in terms of TBA reactive substances (TBARS) in supernatants measuring at 532 nm using 1.53 × 105 $M^{-1}cm^{-1}$ as extinction coefficient. The levels of lipid peroxidation were expressed in terms of nmol/mg protein.

2.G.5. NO release assay

The measurement of NO concentration was performed using the microplate assay method [81] with Griess reagent (1% sulfanilamide, 0.3% naphthyl ethylene diamine dihydrochloride, 7.5% H_3PO_4). The culture supernatants (100 µl) were mixed with 100 µL of the Griess reagent and kept for 10 min. The nitrite concentration in the culture supernatant was measured at an absorbance of 550 nm.

2.G.6. ROS generation

The production of intracellular ROS was measured using 2,7-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) according to the method as applied by Chattopadhyay et al. [82] The $DCFH_2$-DA passively enters into the cells where it reacts with ROS to form the highly fluorescent compound dichlorofluorescein (DCF). 10 mM $DCFH_2$-DA solution was diluted with methanol in the culture medium without serum to yield 100 µM working solution. At the end of exposure with different concentrations of the polysaccharide, cells were washed twice with PBS. Then the cells were incubated in

1.5 mL working solution of $DCFH_2$-DA at 37 ^oC for 30 min. Cells were lysed in alkaline solution and centrifuged at 2,300 rpm. 1 mL supernatant was transferred to a cuvette and fluorescence was measured at 485 nm excitation and 520 nm emission using a fluorescence spectrophotometer (Hitachi F-1700). The values were expressed as percent of fluorescence intensity relative to control wells.

2.G.7. Statistical analysis

The Statistical analysis was performed using Statistica software version 8.0.

2.H. Conclusion

This chapter describes the methodologies adopted to establish the structure of the polysaccharides, their antioxidant and biological properties. The repeating unit of the polysaccharides has been determined using chemical (acid hydrolysis, methylation, periodate oxidation, and Smith degradation studies) and spectroscopic methods (1D and 2D NMR experiments). Antioxidant activities such as ferrous ion chelating ability, superoxide radical scavenging activity, reducing power, hydroxyl radical scavenging activity, DPPH radical scavenging activity, and ABTS radical scavenging property of the polysaccharide were carried out. The toxicological parameters like cell viability, malondialdehyde (MDA), nitric oxide (NO), reduced glutathione (GSH), oxidized glutathione (GSSG), and reactive oxygen species (ROS) generation levels including redox balances maintained by polysaccharide were also investigated.