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Structural investigation of a water-soluble glucan from an edible mushroom, *Astraeus hygrometricus*

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Abstract—A water-soluble glucan, Fraction I, was isolated from the aqueous extract of the fruit bodies of the mushroom *Astraeus hygrometricus*. On the basis of total hydrolysis, methylation analysis, periodate oxidation, and NMR studies (^{1}H, ^{13}C, 2D-COSY, TOCSY, NOESY, and HSQC), the structure of the repeating unit of the glucan is determined as:

$$\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1 \rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1 \rightarrow$$
(A) (B)

This glucan shows strong splenocyte activation.

Keywords: *Astraeus hygrometricus*; Ectomycorrhiza; Polysaccharide; Structure; NMR spectroscopy; Splenocyte activation

1. Introduction

Astraeus hygrometricus is an ectomycorrhizal fungus,[1] growing in association with the roots of Chir pine (*Pinus roxburghii*) and sal (*Shorea robusta*) trees. It helps the plants in extracting nutrients, especially phosphorus, from very slightly soluble soil minerals and organic substances. Mycorrhizal fungi confer many attributes to plants such as growth stimulation due to increased nutrient uptake, tolerance of plants to odd conditions, and bio-control of root diseases. In return, mycorrhizal fungi, which are highly specific in their nutritional requirements, get simple sugars, amino acids, and other substances from the host plants for their growth and development. At the beginning of the rainy season (first week of June) the fruit body of the ectomycorrhiza develops along the roots of these plants just beneath the soil layer in the laterite forest soil of South Bengal. During this period these are available in the market, and local people consume them as delicious vegetables.

Several polysaccharides and polysaccharide–protein complexes have been isolated from fungi (mushrooms) and are being used as a source of therapeutic agents.[2] A number of β-glucans having (1 → 3)- and (1 → 6)-linkages[3,4] and α-glucans[5] with (1 → 4)-linkages are widely used as antitumor and immunomodulating agents. These materials also act as biological response modifiers.[4] The present glucan under study showed strong splenocyte activation. Splenocytes are the cells present in the spleen that include T cells, B cells, macrophages, etc. that are responsible for promoting the immune response in living systems. This glucan showed the stimulation in a dose-dependent way. Therefore, a detailed structural investigation was carried out with this fraction, and the results are reported herein.

2. Results and discussion

An earlier study showed the presence of heteroglycan in the aqueous fraction of *Astraeus hygrometricus*.[6] In the present case a thorough investigation was carried out by taking the fungi from different places, and the fraction was found to contain only glucans. The fruit bodies

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of the fungi were washed several times initially with water and then with distilled water, followed by extraction with boiling water for 6 h, and then the material was cooled. The solid residue was filtered with a linen cloth, and the liquid was then centrifuged at 4 °C at 4000 rpm for 1 h. The supernatant was collected and precipitated in 1:10 (v/v) ethanol and kept overnight at 4 °C. The precipitate was centrifuged at 10,000 rpm at 4 °C for 1 h, and the centrifugate was washed with ethanol several times. The precipitated material was dissolved in a minimum volume of water and reprecipitated with ethanol. Exhaustive dialysis of the precipitated material was carried out with a DEAE cellulose bag to remove small carbohydrate molecules. The dialyzed material was freeze dried and collected. The crude polysaccharide was again dissolved in water. It was then divided into two parts as an aqueous-soluble fraction (AQS) and an aqueous-insoluble fraction (AQINS). Both fractions were separately freeze dried, and AQS on fractionation through Sepharose-6B yielded two homogeneous polysaccharide fractions, Fraction-I (AQS-I) and Fraction-II (AQS-II). Total hydrolysis indicated that AQS-I is a glucan. Since AQS-I showed splenocyte activation, a detailed structural study was conducted with this polysaccharide fraction, and the results are reported in this paper.

AQS-I showed specific rotation of $[\alpha]_D$ −124 (*c* 0.8, water, 25 °C). The molecular weight of this polysaccharide fraction was determined by a gel-filtration technique[7] using different carbohydrate markers passing through a Sepharose-6B column and found to be $\sim 2.04 \times 10^5$ daltons. The total sugar content was determined by the phenol–sulfuric acid method[8] and was found to be 99.1%. AQS-I was hydrolyzed with 2 M trifluoroacetic acid, and the alditol acetates on analysis through GLC using columns A (3% ECNSS-M) and B (1% OV-225) indicated only glucose. One part of the hydrolyzate on paper chromatographic (PC) analysis showed only the spot for glucose. The absolute configuration of the monosaccharide was determined[9] by GLC analysis of trimethylsilylated (+)-2-butyl glycoside derivative, and the monosaccharide was found to have the D configuration.

AQS-I was then methylated using the method of Ciucanu and Kerek,[10] and then by the Purdie method,[11] followed by hydrolysis and alditol acetate preparation. The alditol acetates were then analyzed through GLC using columns A and B as well as by GLC–MS using HP-5 fused silica capillary column. The presence of 1,4,5-tri-*O*-acetyl 2,3,6-tri-*O*-methyl-D-glucitol (*m/z*: 43, 71, 87, 99, 101, 113, 117, 129, 131, 161, 173, 233) and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol (*m/z*: 43, 58, 71, 87, 99, 101, 117, 129, 161, 173, 189, 233) in a molar ratio of 1:1 (Table 1) were detected. This indicates that (1 → 4)-linked-D-glucopyranosyl and a (1 → 6)-linked-D-glucopyranosyl moieties are present in the glucan. Thereafter, a periodate oxidation experiment was carried out with AQS-I, and the GLC analysis of the methylated periodate oxidized–reduced AQS-I showed no peaks corresponding to 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol. Thus, the periodate oxidation study confirms the mode of linkages of glucose moieties present in the polysaccharide.

The 300-MHz ^{1}H NMR spectrum of AQS-I (Fig. 1) at 27 °C showed two anomeric proton signals at δ 4.83 ($J_{H\text{-}1,H\text{-}2}$ 3.9 Hz) and 4.39 ($J_{H\text{-}1,H\text{-}2}$ 8.5 Hz) ppm. These were assigned as (1 → 4)-D-Glc*p* (residue **A**) and (1 → 6)-D-Glc*p* (residue **B**), respectively, in a molar ratio of 1:1. All the proton signals of AQS-I (Table 2) were assigned on the basis of the correlation of 2D-COSY and TOCSY NMR experiments (Fig. 2, left panel). The anomeric carbon signals for the (1 → 4)-D-Glc*p* and (1 → 6)-D-Glc*p* residues were assigned at δ 98.35 and 103.42 ppm, respectively, from ^{13}C (75 MHz) and HSQC NMR experiments. The coupling constant values ($J_{H\text{-}1,H\text{-}2}$) of the anomeric protons and the appearance of the respective carbon signals indicate that the (1 → 4)-

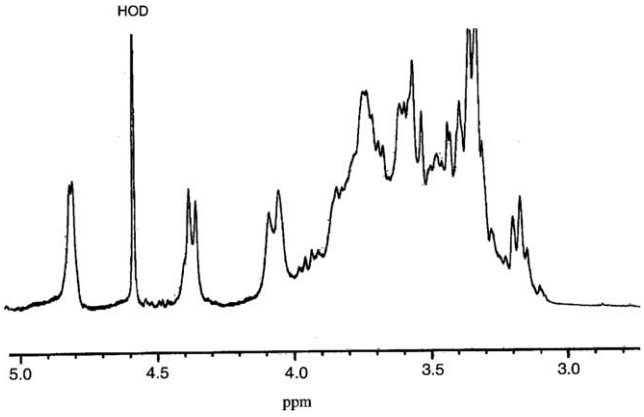

Figure 1. ^{1}H NMR (300 MHz, D_2O) spectrum of polysaccharide AQS-I isolated from *Astraeus hygrometricus* recorded at 27 °C.

Table 1. GLC and GLC–MS data for the alditol acetates derived from the methylated polysaccharide AQS-I isolated from *Astraeus hygrometricus*

Methylated sugars	t_R [a]	t_R [b]	Mass fragmentation (*m/z*)	Molar ratio	Mode of linkage
2,3,6-Me_3-Glc*p*	2.50	2.32	43, 71, 87, 99, 101, 113, 117, 129, 131, 161, 173, 233	1.0	→z ^{1}Glc*p*^{4} →
2,3,4-Me_3-Glc*p*	2.49	2.22	43, 58, 71, 87, 99, 101, 117, 129, 161, 173, 189, 233	1.05	→ ^{1}Glc*p*^{6} →

[a] Retention time with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a 3% ECNSSM column on gaschrom-Q at 170 °C.
[b] Retention time with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a 1% OV-225 column on gaschrom-Q at 170 °C.

Table 2. [1]H NMR chemical shifts of AQS-I isolated from *Astraeus hygrometricus* recorded in D_2O at 27 °C[a]

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6
AQS-I						
$(1 \rightarrow 4)$-α-D-Glc*p* (A)	4.83	3.41	3.57	3.36	3.59	3.75[b] 3.84[c]
$(1 \rightarrow 6)$-β-D-Glc*p* (B)	4.39	3.18	3.32	3.49	3.34	3.71[b] 4.09[c]

[a] The values of chemical shifts were recorded keeping HOD signal fixed at δ 4.60 ppm.
[b,c]Interchangeable.

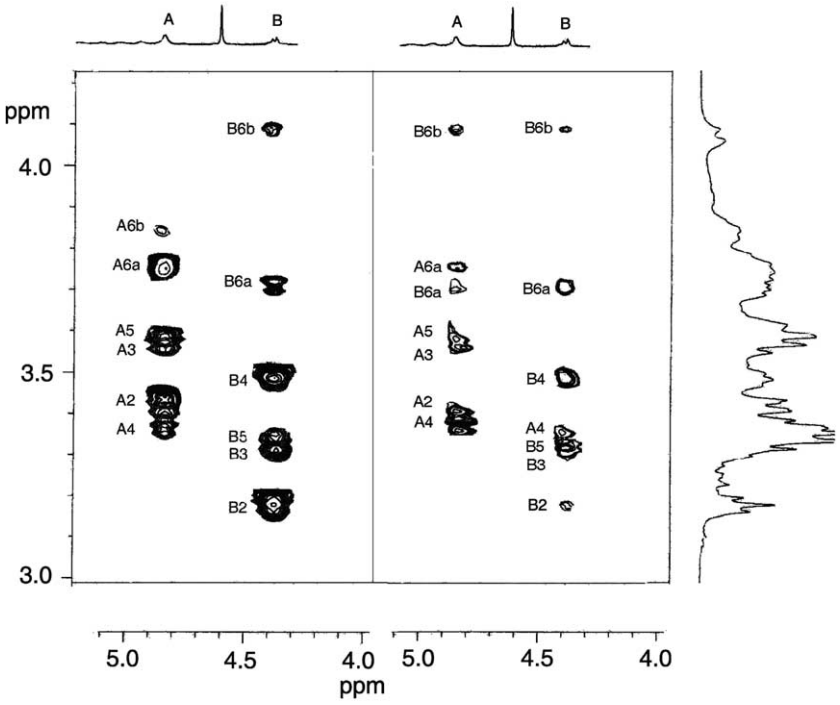

Figure 2. The TOCSY (left panel) and NOESY (right panel) spectra of polysaccharide, AQS-I isolated from *Astraeus hygrometricus*. The mixing time for the TOCSY spectrum shown was 150 ms. Complete assignment required several TOCSY experiments having mixing times ranging from 60–300 ms. The NOESY mixing delay was 200 ms.

linked-D-glucopyranosyl moiety (Residue **A**) is of the α-anomeric configuration and the $(1 \rightarrow 6)$-linked-D-glucopyranosyl moiety (Residue **B**) is of the β-anomeric configuration. The carbon signal at δ 65.98 ppm is due to C-6 of the $(1 \rightarrow 6)$-linked-D-glucopyranosyl moiety (Residue **B**), which is shifted 4.18 ppm downfield compared to the resonance of standard methyl glycosides due to the α effect of glycosylation.[12,13] Similarly, the C-4 signal at 75.30 ppm of $(1 \rightarrow 4)$-D-Glc*p* appears 4.70 ppm downfield compared to that of the standard methyl glycoside value. The other carbon signals for both the residues were assigned by comparing resonances with those generally accepted for standard methyl glycosides.[13] The C-3 and C-5 signals of residue **A** appear, respectively, 3.48 and 3.24 ppm upfield compared to the literature values of methyl glycosides due to the β-glycosylation effect.[13] For the same reason, the C-5 signal of residue **B** is shielded by 3.08 ppm compared to the standard value. The carbon signals at δ 71.81, 70.62, 69.26, and 61.16 ppm correspond, respectively, to C-2, C-3, C-5, and C-6 of $(1 \rightarrow 4)$-D-Glc*p*. The other signals for $(1 \rightarrow 6)$-D-Glc*p* are C-2 (73.48), C-3 (76.01), C-4 (69.94), and C-5 (73.78). These values were further corroborated from the HSQC experiment (Fig. 3, Table 3).

The sequence of the glycosyl residues was determined on the basis of a 2D-NOESY NMR experiment (Fig. 2, right panel). Since the anomeric proton (H-1) of residue **A** has a strong interresidue NOE contact to H-6a and medium to H-6b of residue **B** in addition to intraresidue NOE contacts to H-2, H-3, H-4, H-5, and H-6a, it is evident that residue **A** is linked at the 6-position of residue **B**. Therefore the following sequence is established:

$$\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1 \rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1 \rightarrow$$
(A) (B)

On the other hand, residue **B** has a strong interresidue NOE contact from H-1 to H-4 of residue **A** in addition to intraresidue NOE contacts to H-2, H-3, H-4, H-5,

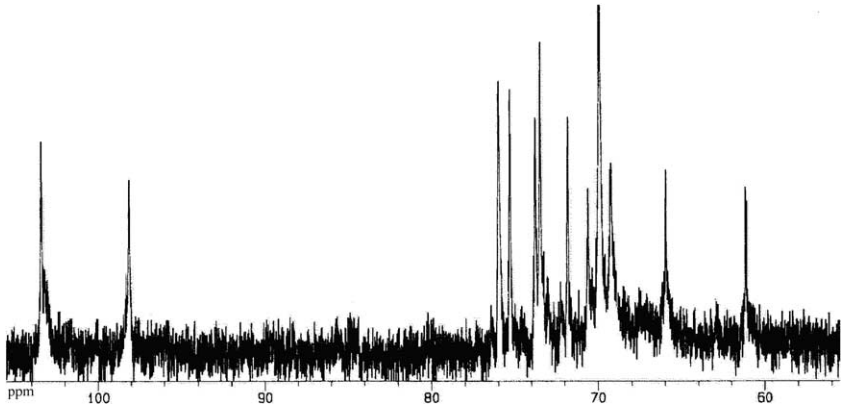

Figure 3. ^{13}C NMR (75 MHz, D_2O) spectrum of polysaccharide AQS-I isolated from *Astraeus hygrometricus* recorded at 27 °C.

Table 3. ^{13}C NMR chemical shifts of AQS-I isolated from *Astraeus hygrometricus* recorded in D_2O at 27 °C[a]

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
AQS-I						
(1 → 4)-α-D-Glc*p*	98.35	71.81	70.62	75.30	69.26	61.16
(1 → 6)-β-D-Glc*p*	103.42	73.48	76.01	69.94	73.78	65.98

[a] Values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 27 °C.

H-6a, and H-6b. This clearly indicates that residue **B** is linked at 4-position of residue **A**. Thus, the following sequence is established:

$$\rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1 \rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1 \rightarrow$$
$$\quad\ \ \mathbf{B} \qquad\qquad\qquad\quad \mathbf{A}$$

Since residue **A** and **B** are present in a molar ratio of 1:1, the following disaccharide repeating unit for AQS-I is assigned:

$$\rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1 \rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1 \rightarrow$$
$$\quad\ \ \mathbf{B} \qquad\qquad\qquad\quad \mathbf{A}$$

The splenocyte activation test was performed using a mouse cell culture medium with AQS-I, and it was found to show strong splenocyte stimulation at a dose of 10 ng/mL (Table 4). A similar kind of splenocyte activation[14] has been observed in the case of a highly branched fungal β-glucan isolated from *Omphalia lapidescens*. (1 → 6)-β-D-Glucans obtained from the fungi *Agaricus blazei*,[15] *Gyrofera esculenta*,[5] and (1 → 4)-α-D-glucan from *Cetraria islandica*[5] are reported as effective antitumor materials. The present polysaccharide (AQS-I) is composed of both of the fragments linked together, and hence it is a strong immunoenhancing material as evidenced from the splenocyte activation studies.

3. Experimental

3.1. Isolation and purification of the polysaccharide

The ectomycorrhiza *Astraeus hygrometricus* (2 kg) was collected from the local forest, and the fruit body was gently washed with water. It was then crushed and allowed to boil in water for 6 h. The liquid was filtered and then kept overnight at 4 °C. It was then centrifuged at 4000 rpm at 4 °C for 1 h. The supernatant was collected and freeze dried; the centrifugate was rejected. This freeze-dried material was then dissolved in water, and the polysaccharide was then isolated by precipitation in 1:10 (v/v) ethanol at 25 °C. This was kept at 4 °C for 48 h, followed by centrifugation at 4 °C at 10,000 rpm for 1 h. The centrifugate was then washed with dehydrated ethanol for several times. The precipitated material was dissolved in water and reprecipitated. This process was repeated several times. Finally the material was dialyzed through a DEAE cellulose bag for 16 h to remove small molecules. The dialyzed material was then freeze dried, and crude polysaccharide was obtained (2.57 g). When this crude PS was dissolved in water, one portion became completely soluble while

Table 4. Result of the splenocyte activation test by the MTT method using a mouse model in cell-culture medium[a]

Sample	Concentration of the sample				
	1 ng/mL	10 ng/mL	100 ng/mL	1 μg/mL	10 μg/mL
AQS-I	0.065 (133%)	0.256 (522%)	0.142 (289.7%)	0.125 (255%)	0.110 (224.4%)

[a] Control reading: 0.049 (100.0%).

another remained insoluble. These two parts, soluble (AQS) and insoluble (AQINS), were separated by centrifugation and freeze dried; AQS (1.9 g) and AQINS (0.65 g).

AQS was dialyzed using a DEAE cellulose bag against distilled water for 4 h to remove monosaccharides as well as low-molecular-weight materials. The material inside the dialysis bag was collected and freeze dried, yield 1.6 g. AQS was further purified by using a Sepharose 6B column. AQS (30 mg) was dissolved in water (2 mL) and passed through a column (85 cm × 2.4 cm) packed with Sepharose 6B with a flow rate of 12 min/mL. The eluant was collected in test tubes (2 mL each), fitted with a fraction collector. Color was developed using the phenol–sulfuric acid method and monitored by a spectrophotometer at 490 nm. Two homogeneous fractions (AQS-I and AQS-II) were found. The solutions inside the test tubes (30–65 and 75–105) were collected and freeze dried, yielding 14 mg of AQS-I and 11 mg of AQS-II. This fractionation was performed in several lots.

3.2. Determination of molecular weight

The molecular weight of AQS-I was determined by a gel-chromatographic technique. Standard dextrans T-200, T-70, and T-40 were passed through a Sepharose 6B column, and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of AQS-I was then plotted in the same graph, and the molecular weight of AQS-I was determined.

3.3. Total acid hydrolysis

AQS-I (1 mg) and inositol (0.5 mg, internal standard) were taken in a round-bottom flask, and 2 M CF_3COOH (1 mL) was added to it, followed by boiling in a water bath for 18 h. After the hydrolysis was complete, excess acid was removed by co-distillation with distilled water. The hydrolyzate was then divided into two parts. One part was examined by PC in solvent systems X and Y. Another part of the hydrolyzate was reduced by $NaBH_4$, followed by acidification with acetic acid. It was then co-distilled with MeOH to remove excess boric acid and dried over P_2O_5. Thereafter the whole mass was treated with pyridine (1 mL) and Ac_2O (1 mL) to prepare the alditol acetate, which was then analyzed by GLC using columns A and B.

3.4. Determination of absolute configuration[9]

The PS (AQS-I) was methanolyzed with 0.625 M methanolic HCl, treated with 0.625 M (+)-2-butanolic HCl under the same conditions, and then trimethylsilylated. The trimethylsilylated 2-butyl glycosides were analyzed by GLC–MS. The temperature program was isothermal at 130 °C, followed by a 2 °C/min gradient up to 250 °C.

3.5. Methylation analysis

AQS-I was methylated using the method of Ciucanu and Kerek,[10] and the product was isolated by partitioning between $CHCl_3$ and H_2O. It was methylated again by the Purdie method.[11] The product showed no band in the region 3600–3300 cm^{-1}. It was then hydrolyzed with 90% HCOOH for 1 h. Excess HCOOH was evaporated off by co-distillation with distilled water. The hydrolyzate was then reduced with $NaBH_4$, and the alditol acetate was prepared as usual. The alditol acetate of the methylated sugar was analyzed by GLC (using columns A and B) and by GLC–MS using an HP-5 fused silica capillary column.

3.6. Periodate oxidation study

AQS-I was added to 0.1 M $NaIO_4$, and the mixture was kept at 4 °C for 48 h in the dark. Excess periodate was destroyed by addition of ethylene glycol, and the solution was dialyzed against distilled water. It was then freeze dried. This material was divided into two portions. One portion was hydrolyzed by 2 M CF_3COOH for 16 h, and the alditol acetate was prepared. Another portion was methylated by the method of Ciucanu and Kerek,[10] and the alditol acetate of this methylated product was prepared. Alditol acetates were analyzed by GLC using columns A and B.

3.7. Paper chromatographic studies

Paper partition chromatographic studies were performed on Whatmann nos 1 and 3 mm sheets. Solvent systems used were: (X) 1-butanol–acetic acid–water (v/v/v, 4:1:5, upper phase) and (Y) ethyl acetate–pyridine–water (v/v/v, 8:2:1). The spray reagent used was alkaline silver nitrate solution.[16]

3.8. Optical rotation

Optical rotation was measured on a Perkin–Elmer model 241 MC spectropolarimeter at 25 °C.

3.9. Colorimetric estimations

Colorimetric estimations were carried out on a Shimadzu UV–vis spectrophotometer model 1601.

3.10. GLC experiments

All gas–liquid chromatography (GLC) analyses were performed on a Hewlett–Packard Model 5730 A gas chromatograph having a flame ionization detector and

glass columns (1.83 × 6 mm) packed with 3% ECNSS-M (A) on Gas Chrom Q (100–120 mesh) and 1% OV-225 (B) on Gas Chrom Q (100–120 mesh). All GLC analyses were performed at 170 °C.

3.11. GLC–MS experiments

All the GLC–MS experiments were carried out in a Hewlett–Packard 5970 MSD instrument using an HP-5 fused silica capillary column. The program was isothermal at 150 °C; hold time 2 min, with a temperature gradient of 4 °C min^{-1} up to a final temperature of 200 °C.

3.12. NMR studies

The ^{1}H and ^{13}C NMR experiments were, respectively, recorded at 300 and 75 MHz on a Bruker Avance DPX-300 spectrometer. For NMR measurements the sample (AQS-I) was dried in vacuum over P_2O_5 for several days and then exchanged with deuterium[17] by lyophilizing with D_2O for several times. The deuterium-exchanged polysaccharide (5 mg) was dissolved in 0.6 mL D_2O (99.96% atom ^{2}H, Aldrich). The ^{1}H and ^{13}C NMR spectra were recorded at 27 °C. Acetone was used as an internal standard (δ 31.05 ppm) for the ^{13}C spectrum. The ^{1}H NMR spectrum was recorded by fixing the HOD signal at δ 4.60 ppm at 27 °C. The 2D (COSY) NMR experiment was performed using standard Bruker software. The mixing time in the TOCSY experiment was 60–300 ms, and the NOESY mixing delay was 200 ms.

3.13. Splenocyte activation test by the MTT method[14] in cell-culture medium

A homogeneous spleen cell proliferation in RPMI (Roswell Park Memorial Institute) complete medium was prepared. The cell concentration was adjusted to $10^{6}\times$ cells/mL. The above cell suspension (180 mL) was plated in a 96-well flat-bottom cell-culture plate. Sample (20 μL, concentration range: 1 ng to 100 μg/mL) was added to each well. All samples were checked in triplicate. The plate was incubated for 72 h in a 5% CO_2, 37 °C humidified CO_2 incubator. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution was prepared in PBS (phosphate buffer solution) at 5 mg/mL concentration. After 72 h of incubation, the above MTT solution (20 μL) was added to each well including the control. It was further incubated for 8 h as above. Thereafter, the supernatant was aspirated carefully from each well, and the formazan crystals were dissolved in Me_2SO (100 μL). The plate was read at 570 nm. Then the % viability was calculated in comparison to control, taking control as 100% viable.

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