International Journal of Biological Macromolecules 168 (2021) 537–549

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Structure and biological properties of exopolysaccharide isolated from *Citrobacter freundii*

Indranil Choudhuri [a], Kalyani Khanra [a], Prasenjit Maity [b], Anutosh Patra [a], Gajendra Nath Maity [c], Bikas Ranjan Pati [d], Anish Nag [e], Soumitra Mondal [c,*], Nandan Bhattacharyya [a,*]

[a] Department of Biotechnology, Panskura Banamali College, P.O. – Panskura R.S., Purba Medinipur, West Bengal PIN-721152, India
[b] Department of Chemistry, Sabang Sajanikanta Mahavidyalaya, Sabang, Paschim Midnapore, West Bengal PIN-721166, India
[c] Department of Chemistry, Panskura Banamali College, P.O. – Panskura R.S., Purba Medinipur, West Bengal PIN-721152, India
[d] Dept. of Microbiology, Vidyasagar University, Medinipur, West Bengal PIN-721102, India
[e] Department of Life Sciences, CHRIST (Deemed to be University), Bengaluru PIN-560029, India

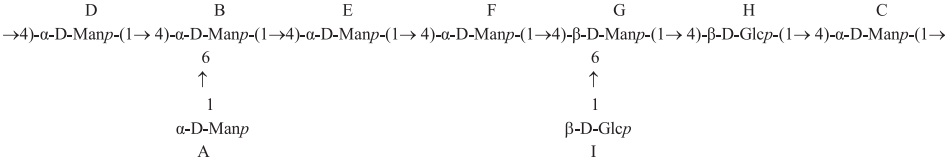
ARTICLE INFO

Article history:
Received 19 July 2020
Received in revised form 25 September 2020
Accepted 7 December 2020
Available online 11 December 2020

Keywords:
Citrobacter freundii
Exopolysaccharide
NMR studies
Structure
Antioxidant activity
Cytotoxicity

ABSTRACT

This study aimed to investigate the molecular characterization, antioxidant activity in vitro, cytotoxicity study of an exopolysaccharide isolated from *Citrobacter freundii*. Firstly, the culture conditions were standardized by the Design of experiments (DoE) based approach, and the final yield of thecrude exopolysaccharide was optimized at 2568 ± 169 mg L^{-1}. One large fraction of exopolysaccharide was obtained from the culture filtrate by size exclusion chromatography and molecular characteristics were studied. A new mannose rich exopolysaccharide (Fraction-I) with average molecular weight ~ 1.34×10^5 Da was isolated. The sugar analysis showed the presence of mannose and glucose in a molar ratio of nearly 7:2 respectively. The structure of the repeating unit in the exopolysaccharide was determined through chemical and 1D/2D- NMR experiments as:



Finally, the antioxidant activity, and the cytotoxicity of the exopolysaccharide were investigated and the relationship with molecular properties was discussed as well.

1. Introduction

Citrobacter freundii is a facultative anaerobic, Gram-negative bacillus within the family *Enterobacteriaceae*. *C. Freundii* is frequently found in the environment, including in the intestines of animals and humans. This bacterium was previously recognized as a low virulence microorganism, however, recent reports demonstrated it to be thecausative agent for a spectrums of infection, including pneumonia, diarrhea, and septicemia [1–6].

C. freundii was first isolated from soil, now it is known to exist in various natural habitats. Some *C. Freundii* strains isolated from various environments were reported for potential use in various fields. *C. freundii* isolated from industrial outlet streams in Maharashtra, India, was found to be electrochemically active [7]. *C. freundii* strain JPG1, isolated from a gold mining tailing in China, was reported to be resistant to heavy metals, and to have the capacity to remove copper. Thus, it has a good potential in the treatment of copper-rich industrial effluents [8]. Another strain (IFO 13545) of *C. Freundii* found to has the ability to produce bioflocculant, can be used in water supply system, to treat wastewater, and food production [9]. We found a Cu and Pb resistant strain [GenBankaccession no MT594116.1] of *C. freundii* from industrial wastewater ground of *Haldia*, India which is capable of producing exopolysaccharide. Basically bacteria have several mechanisms to tolerate heavy metal, including the production of extracellular polymers [10]. These polymers consisting mainly of polysaccharides and proteins with little portion of DNA, lipids, and humic substances [11], are secreted by microorganisms into their environments [12]. These polymers are anionic in nature, and are capable chelating certain metals and ions

* Corresponding author.
E-mail addresses: mondalsoumitra78@yahoo.com (S. Mondal), bhattacharyya_nandan@rediffmail.com (N. Bhattacharyya).

https://doi.org/10.1016/j.ijbiomac.2020.12.063
0141-8130/

[13–16].Thus biofilms are protecting the bacterial cells from heavy metals and other stresses [17].

Exopolysaccharide production is a common characteristic of ESKAPE (*Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiellapneumoniae*, *Acinetobacterbaumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*) pathogen [18]. However, the production of exopolysaccharide is less common to *C. freundii*. Hossain et al.(2017), isolated 47 virulent strains of *C. freundii* from pet turtle, six out of which strains were capableofproducing biofilm [19]. *C. freundii* Cf1 isolated from sewage sludge samples, was reported to form biofilm [20].

Natural polysaccharides including bacterial exopolysaccharides are diverse in structure, and most of them have a molecular weight ranging between 10^4–10^6 Da [21]. The structural complexity of polysaccharides enables them to interact with other molecules, and thus many of them exhibit biological activity. Recent studies have found that many microbial exopolysaccharides that have biological activities, including antitumor, antiviral, immunostimulatory, and anti-inflammatory activities [22–24]. It is considered that the polysaccharide ability to increase the host immune function, is one of the mechanisms of antitumor activity. [25,26]. Another widely reported property of exopolysaccharides isolated from different bacteria is antioxidant properties.Polysaccharides consist ofvarious organic group like –OH, which play an important role in exhibiting antioxidant activity [27–31].

Mannose is a biologically active compound. Mannose rich polysaccharides have anticancer [32,33], antitumor [34,35], and antioxidant [36] activity. In this study we isolated and purified a mannose rich exopolysaccharide from *C. freundii*, established the probable repeating unit structure, and evaluated its in-vitro antioxidant activity and cytotoxicity against HeLa cell line.

2. Materials and methods

2.1. Isolation and purification of the exopolysaccharide

The microorganism was isolated from industrial wastewater ground and identified as *Citrobacter freundii*. A design of experiment (DoE) approach was undertaken to optimize the yield (mg L^{-1}) of the exopolysaccharides with the target of minimum runs possible, however without compromising with statistical robustness. Design of Experiment (DoE) is an advanced technique, which enables optimization of the process parameters by using a robust multivariate statistical method to obtain desired performance in terms qualitative as well as quantitative outputs [37]. A customized two-factor interaction (2FI) model was adopted to evaluate the five numerical main effects (Concentration of Yeast Extract, pH, Temperature, Days of incubation, and NaCl concentration), two categorical main effects (Sugar: Glucose and Control) and fifteen interaction effects by using Design of Experiment® software. DoE experiments were executed according to the thirty-two random runs as given by design. The designs as well as the results of the experimental runs are shown in (**Table S1**). Finally, a design space was developed based on the optimized parameters.

To validate the design space, further experimentation was performed in triplicates by selecting the specific parameters within the established range. The microorganism was cultured in 250 mL Erlenmeyer flask in batch to produce the exopolysaccharide. The composition of the culture medium was 10 g L^{-1} of tryptone, 4 g L^{-1} of yeast extract, and 11 g L^{-1} of NaCl. The pH of the media was adjusted at 7.5. The culture was inoculated with 1% inoculum, and incubated at 30 °C for 120 h (5 days) with 120 rpm constant shaking. After the incubation, bacterial cells were separated from media by centrifugation at 8500 ×*g* for 15 min. To separate theexopolysaccharide, the supernatant was mixed with isopropanol (1:1 *v*/v), and incubated overnight at −20 °C. The polysaccharide was precipitated by centrifuging the resultant mixture at 20000 ×*g* for 30 min [38].

The crude exopolysaccharide was dialyzed by cellulose dialysis tube (Fisher Scientific, USA, molecular cutoff 12 kD - 14 kD) for 24 h against deionized water, and lyophilized. The exopolysaccharide was further purified by Gel filtration chromatography (GFC). For each batch 25 mg of exopolysaccharide was purified using a Seralose 6B (SRL, India) column. The column dimension was 50 cm × 1.8 cm, the flow rate was fixed at 0.5 mL per min, 90 fractions of 2 mL each were collected. The desiredfractions were collected after identification by monitoring phenol-sulphuric acid method [39], and lyophilized to obtain the purified polysaccharide (Fraction-I).

2.2. Estimation of carbohydrate and protein

The total sugar content of the Fraction-I was estimated by phenol-sulphuric acid method using glucose as the standard [40]. The protein content of the sample was determined by Lowery method [41] using Bovine serum albumin (BSA) as the standard.

2.3. Determination of molecular weight

The apparent molecular weight of the Fraction-I was determined by gel-permeation chromatography [42,43]. Standard dextrans T-110, T-40, and T-10 were passed through a Sepharose 6B column, and the elution volumes were then plotted against the logarithms of their respective molecular weights. The elution volume of Fraction-I was plotted in the same graph and the average molecular weight of Fraction-I was measured accordingly.

2.4. Monosaccharide analysis

The monosaccharide constituent of the purified sample was analyzed as follows. The sample was hydrolyzed by CF_3COOH, and reduced using $NaBH_4$ in NH_4OH. The monosaccharide residues were acetylated by Ac_2O. The sample was dissolved in chloroform, and 1 μl of the sample was injected into the GLC using autosampler (Perkin Elmer Clarus SQ 8). AnRTX-5MS 30 M column with a 0.32 mm inner diameter (ID)was used in this experiment. Helium was used as the carrier gas. The total run time for this experiment was 30 min. The initial holding was 1.5 min at 40 °C; the temperaturewas then increased up to 130 °C with a heating rate of 40 °C min^{-1}; subsequently the temperature was increased up to 290 °C with a heating rate of 8 °C min^{-1}, and the final holding spanned was 5 min.

2.5. Methylation analysis

Methylation analysis was carried out as follows. Five mg of Fraction-I in 0.5 mL Dimethyl sulfoxide (DMSO) was added in finely powdered NaOH (20 mg) and methyl iodide (0.1 mL), and kept at room temperature for a 1 and ½ h with continuous stirring. The methylated products were isolated by partition between $CHCl_3$ and H_2O (5:1, *v*/v). The product was hydrolysed with 90% HCOOH,and the excess acid was evaporated by co-distillation with distilled water. The hydrolysed product was dissolved in water (4 mL), to which $NaBH_4$ (9 mg) was added for the reduction and acetylated with 1:1 pyridine-Ac_2O in boiling water bath for 1 and ½ h [44]. The alditol acetates of the methylated products were analyzed by Gas-liquid chromatography–mass spectrometric (GLC-MS). GLC-MS analysis was performed on PerkinElmer Clarus 680 GC system, using Elite-5MS, 20 m × 0.32 mm ID, 1.0 μmdf column. The program was isothermal at 150 °C; the holding time was 5 min, with a temperature gradient of 2 °C min^{-1}, up to a final temperature of 200 °C.

2.6. NMR analysis

The polysaccharide sample was kept in a vacuum desiccator over P_2O_5 until the sample was completely dried. The sample was then dissolved in D_2O (Sigma), and lyophilized for three times to

exchange with deuterium [45,46]. The ^{1}H and ^{13}C NMR experiments were carried out at 700 MHz and 125 MHz, respectively with a Bruker Avance DPX-700 spectrometer. The ^{1}H, ^{13}C, DQF-COSY, NOESY, and HSQC NMR spectra were recorded in D_2O at 27 °C. The ^{1}H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.64) using the WEFT pulse sequence [46] with acetone as internal standard fixing methyl proton signal at δ 2.19. Acetone was used as an internal standard (δ 31.05 ppm) for ^{13}C spectrum. The 2D-DQF-COSY experiment was performed using standard BRUKER software. The NOESY mixing delay was 300 ms.

2.7. Biological properties

2.7.1. Total antioxidant capacity

The totalantioxidant capacity of Fraction-I was evaluated by the phosphomolybdenum method, and expressed as the number of equivalents of ascorbic acid. This assay was based on the reduction of Mo (V1) to Mo(V) by the sample analyte, which formed green phosphate/Mo (V) complex at acidic pH. In brief, 100 μL of different concentrations of the sample were mixed with 1 mL of reagent solution containing 0.6 M of Sulphuric acid, 28 mM of Sodium phosphate, and 4mMof Ammonium molybdate. The mixtures were incubated in water bath at 95 °C for 1 and ½ h, and cooled to room temperature. The absorbance of the mixture was measured at 695 [47].

2.7.2. Chelating effect on ferrous ions

To evaluate the chelating capacity of the polysaccharide, different concentration of sample solution was mixed with 0.2 mM ferrous chloride at1:5 ratios. 5 mMferrozine was added to the sample (two volume of the polysaccharide sample) to initiate the reaction, and the mixture was incubated at room temperature. After 10 min, the absorbance was measured at 562 nm, this decreasing absorbance being an indication of the chelation of Iron (II). Ethylenediaminetetraacetic acid (EDTA) was used as a positive control [48].

2.7.3. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of Fraction-I was performed as follows. Different concentration of Fraction-I (0–1000 μg mL^{-1}, 500 μL) were mixed with 500 μL of 9 mM $FeSO_4$,500 μL of 9 mM salicylic acid-ethanol, and finally 500 μL of 9 mMH_2O_2 to initiate reaction. The mixtures were incubated a37 °C for 30 min, and the absorbance of the reaction mixtures were recorded at 510 nm [49]. Ascorbic acid was used as the positive control. The ability to scavenge hydroxyl radical was calculated as follows:

$$\text{Scavenging rate } (\%) = [1-(A_1-A_2)/A_0] \times 100$$

where A_0indicates the absorbance of the blank,A_1 is the final absorbance of Fraction-I/ ascorbic acid, and A_2 is the backgroundvalue of the existing H_2O_2.

2.7.4. Cytotoxicity study

The cytotoxicity of the Fraction-I was tested by 3-(4, 5dimethylthiazol2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay [50]. HeLa and HEK-293 cell lines were obtained from the National Centre for Cell Science, Pune, India, and cultured in Dulbecco's Modified Eagle's medium (DMEM)with 10% Foetal bovine serum (FBS). The cells were seeded in a 96 well tissue culture plate at a density of 10^4 cells per well, and were allowed to attach in the surface. Next day, the cells were treated with different concentrations (0–500 μg mL^{-1}) of Fraction-I after 4 h of serum starvation, and wereincubated at 37 °C with 5% CO_2. 1% DMSO in complete medium was used as thesolvent control. Afterincubation, MTT was added to each well at a final concentration of 0.5 mg mL^{-1}, and the cells were incubated further at 37 °C for 3 and ½ h. The formazan was dissolved by adding 100 μl of DMSO into each well. The color changes were measured at 540 nm using an ELISA reader (Robonik, Readwell touch ELISA PLATE analyzer, India) [51]. The rate of survival of the treated cells was determined as follows,

$$\text{Cell viability } (\%) = (OD_{AT}/OD_{AC}) \times 100$$

where OD_{AT} = Absorbency of control cells and OD_{AC} = Absorbency of treated cells.

2.7.5. Anti-proliferation assay

HeLa cells were plated at a density of 4×10^5 cells mL^{-1} in 24-well plates and were incubated at 37 °C in 5% CO_2 incubator until they got confluent. A scratch was made by using a sterile pipette tip (p10) after 6 h serum starvation. The plates were then rinsed with Phosphate buffered saline (PBS) and grown in complete medium with 250 μg mL^{-1} of Fraction-I. After 0, 24, 48, and 72 h, the images were taken at 200× magnification and analyzed by ToupView 3.7 (ToupTek) software [52].

2.7.6. Statistical analysis

Experiments were done at least three times, and the results are here expressed as mean ± Standard deviation (SD). Variancesatless than 5% ($P < 0.05$) have been considered as significant results.

3. Results and discussion

3.1. Isolation, purification and chemical analysis

Four models, namely Linear, 2FI (Two Factor Interaction), Quadratic and Cubic were statistically compared, and 2FI model was found to be statistically significant in the current experimental design. Terms namely Yeast extract, Day, Sugar and interactions like Yeast extract X pH (**AB**), Yeast extract X Temperature (**AC**), Yeast extract X NaCl (**AE**), pH XNaCl (**BE**) and NaCl X Sugar (**EF**) were found to be statistically significant ($p \leq 0.05$). Therefore, it was evident that except day, all the parameters played a pivotal role in the yield of the exopolysaccharide through interactions (**Table S2**).

Based on the Yield criteria ≥2700 mg L^{-1}, we observed that although Glucose was an essential parameter for increasing the yield of exopolysaccharides, however, it was found that control condition (i.e. without Glucose) with other interacting variables could also produce a high amount of exopolysaccharides. Based on the statistical significance ($p \leq 0.05$) we screened AB, AC, AE, BE, and EF to establish a design space for the target Yield (≥2700 mg L^{-1}) with and without Glucose. The basis of selection of design space out of the available options was to provide a comparatively broad range of interacting significant variables (X1 and X2) as well as to identify the out of target specification (i.e. Yield ≤2700 mg L^{-1}) points. Since 'Day' did not have any statistical effect on interaction terms, it was kept at the highest point, i.e. five days. After the careful screening, we selected pH X NaCl (**BE**) as the interacting terms in the design space. Parameters were for the optimized design space was finalized as pH range: 7.3 to 7.9 (X1), NaCl: 8.6 to 12 mg L^{-1} (X2), Yeast extract: 4 g L^{-1}, and

Temperature: 30 °C without sugar (i.e. Control). In similar parameters, when Glucose was used it gave a broad range of pH (6.9 to 7.9) and NaCl (8 to 12 g L^{-1}) (**Fig. S1**).

The yield of the crude exopolysaccharide (2568 ± 169 mg L^{-1}) from the validation experiment was found to be close to the predicted design space value 2760.21 mg L^{-1}. 25 mg of thecrude polysaccharide was purified through Gel permeation chromatography (Fig. 1a) in batch. In total 90 fractions of 2 mL each were collected. One major fraction was obtained (>90%, Fraction I). The fraction was lyophilized, yielding 15.1 ± 0.3 mg of polysaccharide. This fraction contained 90.3 ± 0.6% of carbohydrate and 1.2 ± 0.2% of proteins. The molecular weight of the Fraction-I was estimated as 1.34 × 10^5 Da(Fig. 1b). GLC analyses of the alditol acetates of this polysaccharide revealed the presence of glucose and mannose in a molar ratio of nearly 2:7. The GLC-MS analysis of the alditol acetates of methylated product showed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-mannitol, and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-mannitol in a molar ratio of approximately 1:1:1:4:2. These results indicated the presence of terminal glucopyranosyl, terminal mannopyranosyl, (1 → 4)-glucopyranosyl, (1 → 4)-mannopyranosyl, and (1 → 4,6)-mannopyranosyl residues in the Fraction-I.

3.2. NMR and structural analysis of fraction-I

The ^{1}H NMR spectrum (700 MHz, 27 °C, Fig. 2a) of the Fraction-I showed nine anomeric signals at 5.14, 4.99, 4.96, 4.94, 4.90, 4.89, 4.75, 4.60, and 4.59 ppm. They were chosen as **A**, **B, C, D, E, F, G, H** and **I** according to their falling anomeric proton chemical shifts (Table 1a). In ^{13}C NMR spectrum (125 MHz, Fig. 2b) at 30 °C, eight signals were found in the anomeric region at 102.6, 101.9, 100.9, 100.5, 100.0, 99.5, 98.6, and 97.9 ppm. From the HSQC spectrum (Fig. 3, Table 1a), the anomeric carbon signals at 101.9, 100.9, 100.5, 100.0, 99.5, 98.6, and 97.9 ppm were correlated to the anomeric proton signals δ 4.99 (**B**), 4.59 (**I**), 5.14 (**A**), 4.89 (**F**), 4.75 (**G**), 4.96 (**C**), and 4.94 (**D**) respectively. Whereas the anomeric carbon signal at 102.6 ppm was correlated to both the proton signals 4.90 (**E**) and 4.60 (**H**) respectively. All the ^{1}H and ^{13}C signals (Table 1a) were assigned using DQF-COSY, and HSQC (Fig. 3) spectrum.

The anomeric chemical shift for residue **A** at δ 5.14 and 100.5 confirmed that the residue **A** was present in α-configuration. All the proton and carbon signals matched nearly to the standard values of methyl glycosides [53,54]. Thus, residue **A** was terminal α-D-mannopyranosyl moiety.

Residue **B** was assigned as (1 → 4, 6)-α-D-Man*p*. The H-1 (δ 4.99) and C-1 (δ 101.9) signals of **B** confirmed by HSQC experiment indicated that residue **B**was α-D-Man*p*. The downfield shift of C-4 (δ 78.4) and C-6 (δ 65.5) carbon signals with respect to the standard methyl glycosides [53,54] indicated that residue **B** was (1 → 4,6)-linked α-D-manopyranosyl moiety. The *O*-6 substitution was confirmed from the respective reverse peak in the DEPT-135 spectrum (Fig. 2b).

The α-configuration of the residues (**C**–**F**) was confirmed by H-1 (4.96–4.89 ppm) and C-1 (97.9–102.6 ppm) signals. The downfield shifts of C-4 (δ 78.7, 78.7, 78.1, and 78.4) with respect to the standard value of methyl glycoside indicated that residue **C, D, E** and **F** were (1 → 4)-linked α-D-Man*p*.

The downfield shifts at C-4 (δ 78.2) and C-6 (δ 65.8) with respect to the standard value of methyl glycoside indicated that residue **G** was (1 → 4,6)-β-D-Man*p*. The *O*-6 substitution of residue **G** was confirmed from the respective reverse peak in the DEPT-135 spectrum (Fig. 2b).

The β-configuration of residues (**H** and **I**) was confirmed by H-1 (4.59 and 4.60 ppm) and C-1 (102.6 and 100.9 ppm) signals [55,56]. The downfield shifts of C-4 (δ 77.0) with respect to the standard value of methyl glycoside indicated that residue **H** was (1 → 4)-linked β-D-Glc*p*. In residue **I**, all the proton and carbon chemical shifts values were found to be nearly to the standard value of methylglucosides. Thus, residue **I** was terminal β-D-Glc*p*.

The sequence of glycosyl residues (**A** to **I**) was determined from the NOESY experiment (Table 1b, Fig. 4). The NOESY experiment showed the inter-residual contacts: **A**H-1/**B**H-6a, **B**H-6b; **B**H-1/**E**H-4; **C**H-1/**D**H-4, **D**H-1/**B**H-4, **E**H-1/**F**H-4, **F**H-1/**G**H-4, **G**H-1/**H**H-4, **H**H-1/**C**H-4, **I**H-1/**G**H-6a, **G**H-6b along with other intra-residual contacts (Fig. 4). The above NOESY connectivities established the following sequences: **A** (1 → 6) **B**; **B** (1 → 4) **E**; **C** (1 → 4) **D**; **D** (1 → 4) **B**; **E** (1 → 4) **F**; **F** (1 → 4) **G**; **G** (1 → 4) **H**; **H** (1 → 4) **C**; **I** (1 → 6) **G** (Table 1b).

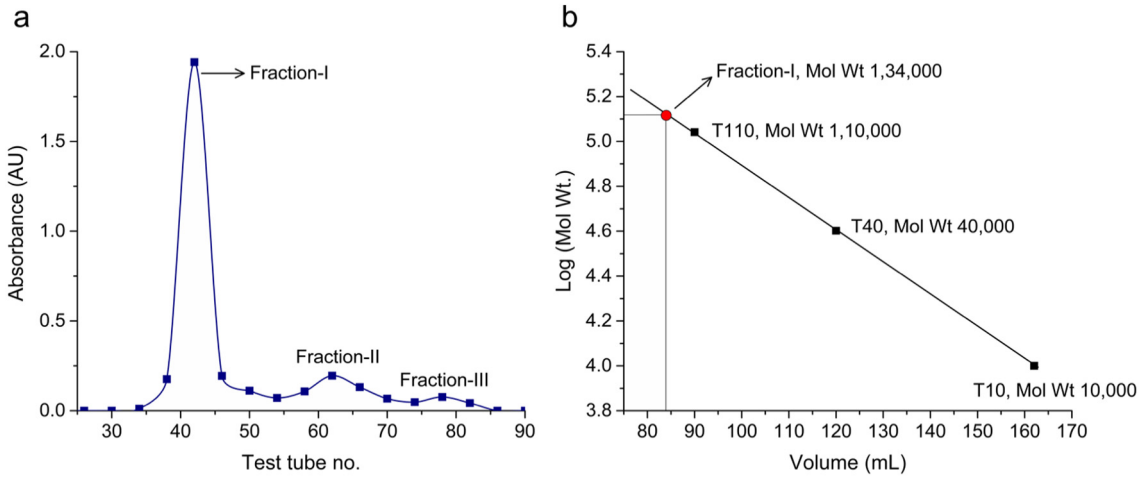


Fig. 1. (a) Gel permeation chromatogram of the crude exopolysaccharide isolated from *Citrobacter freundii* using Sepharose 6B column. (b) Determination of molecular weight of the Fraction-I by gel permeation chromatography in Sepharose 6B column.

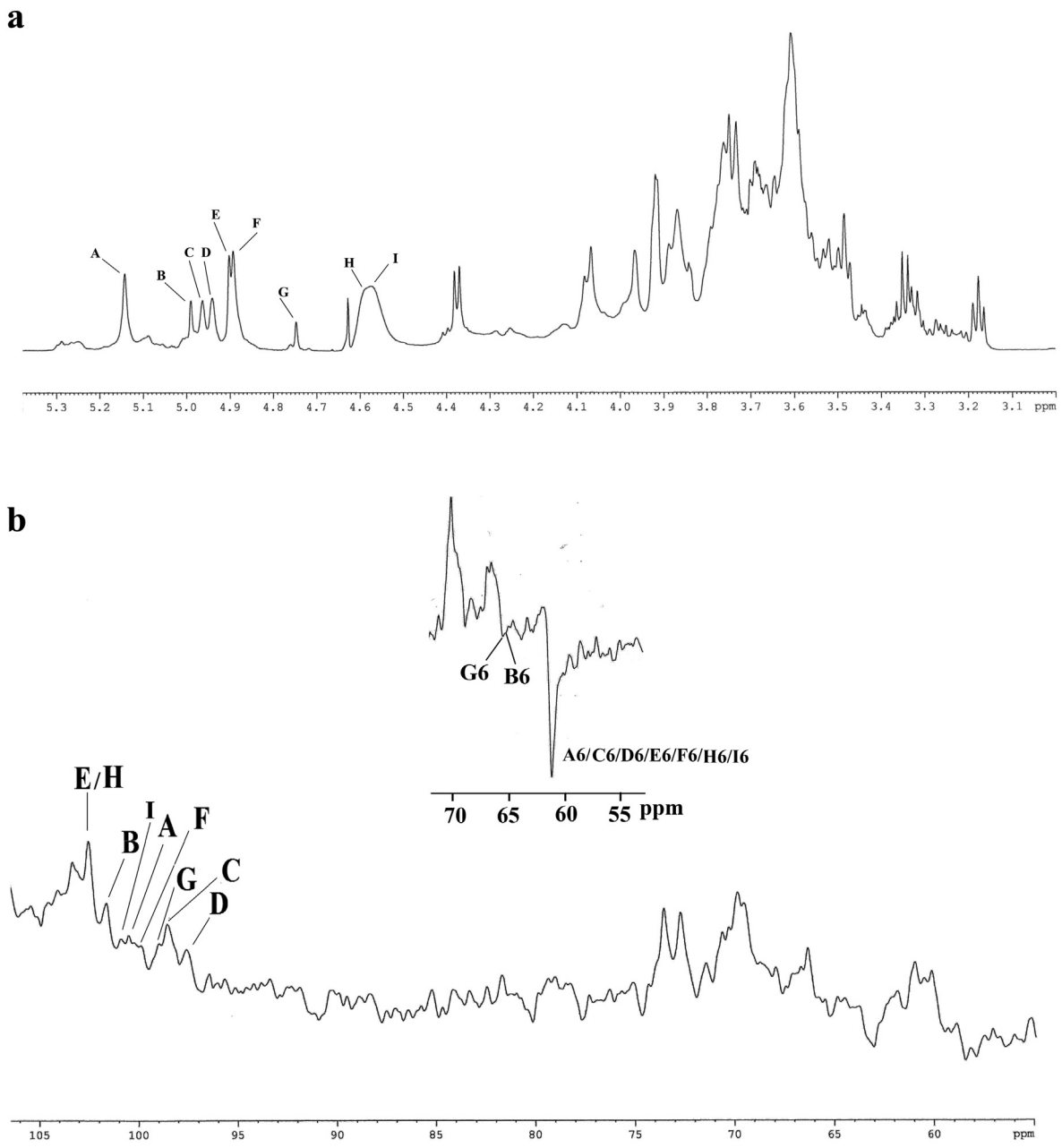
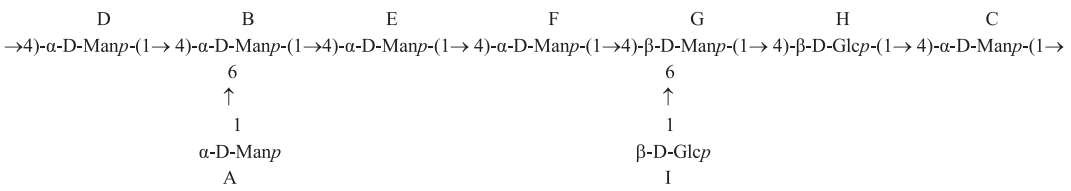


Fig. 2. (a) ^{1}H NMR spectrum (700 MHz, D_2O, 27 °C) of the Fraction-I isolated from the exopolysaccharide produced by *Citrobacter freundii*. (b) ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of a Fraction-I isolated from *Citrobacter freundii*. Part of DEPT-135 spectrum (D_2O, 27 °C) of a Fraction-I isolated from the exopolysaccharide produced by *Citrobacter freundii* (inset).

Based on the chemical and NMR spectral evidences the structure of repeating unit in the Fraction-I was proposed as:



3.3. Evaluation of biological properties

3.3.1. Total antioxidant capacity

Free radicals are generated in cells due to biochemical reaction within the cells, or by external agent present in environment like Methyl methanesulfonate (MMS), Benzo[*a*]pyrene (BAP), radiation etc. Cells can scavenge a certain amount of free radicals by their own mechanism.

Table 1a
The ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts of Fraction-I from *Citrobacter freundii.*

Glycosyl residue	H-1/ C-1	H-2/ C-2	H-3/ C-3	H-4/ C-4	H-5/ C-5	H-6a, H-6b/ C-6
α-D-Man*p*-(1→	5.14	3.93	3.86	3.58	3.66	3.61[c], 3.73[d]
A	100.5	70.0	69.8	66.7	73.2	61.0
→4,6)-α-D-Man*p*-(1→	4.99	4.08	3.78	3.97	3.45	3.63[c], 3.79[d]
B	101.9	69.1	70.3	78.4	74.2	65.5
→4)-α-D-Man*p*-(1→	4.96	4.08	3.69	3.87	3.50	3.61[c], 3.75[d]
C	98.6	69.1	70.4	78.7	74.8	61.0
→4)-α-D-Man*p*-(1→	4.94	4.08	3.73	3.87	3.50	3.61[c], 3.75[d]
D	97.9	69.1	68.9	78.7	74.8	61.0
→4)-α-D-Man*p*-(1→	4.90	4.08	3.67	3.82	3.50	3.61[c], 3.75[d]
E	102.6	69.1	68.6	78.1	74.8	61.0
→4)-α-D-Man*p*-(1→	4.89	4.08	3.66	3.97	3.50	3.61[c], 3.73[d]
F	100.0	69.1	70.4	78.4	74.8	61.0
→4,6)-β-D-Man*p*-(1→	4.75	4.09	3.64	3.81	3.37	3.63[c], 3.87[d]
G	99.5	69.1	73.1	78.2	75.6	65.8
→4)-β-D-Glc*p*-(1→	4.60	3.45	3.67	3.78	3.47	3.61[c], 3.75[d]
H	102.6	74.2	75.6	77.0	75.0	61.0
β-D-Glc*p*-(1→	4.59	3.24	3.49	3.34	3.36	3.61[c], 3.75[d]
I	100.9	73.1	75.1	69.6	75.6	61.0

[a] The values of chemical shifts were recorded keeping HOD signal fixed at δ 4.64 at 30 °C.
[b] The values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 at 30 °C.
[c] Interchangeable.
[d] Interchangeable.

Excessive numbers of free radicals generated within the cells result in an array of diseases including cancer. Antioxidants through their scavenging power can prevent those diseases. Many natural polysaccharides were found to possess free radical scavenging activities [57–59]. The total antioxidant activity of Fraction-I of the exopolysaccharide was estimated by the phosphomolybdenum assay method, using ascorbic acid as the standard. Since it was a quantitate method, 1 mg of Fraction-I was found as functional as approximately 92.50 ± 0.17 μg of ascorbic acid.

3.3.2. Chelating effect on ferrous ions

Some transition metals act as catalysts for the initial formation of radicals. Chelating agents might stabilize transition metals in living cells and inhibit free radical generation. Due to high reactivity of ferrous ions, they are the most effective pro-oxidants in the food content among the transition metals. Fe^{2+} catalyses oxidative changes of cellular components like proteins, lipids, and other. Thus, it is associated with an increased risk of free radical damage. Chelation of ferrous ions may possibly reduce this kind of free radical damage [60]. Molecule containing more than one of the functional groups, such as —OH, —COOH, —C═O, —PO_3H_2, —SH, —NR_2, —S—, and —O—, exhibit chelating ability [61]. Thus, one of the common properties of polysaccharides is ferrous ions chelating property. The Fe^{2+} ions chelating activity of Fraction-I is presented in Fig. 5a.The percentage of Fe^{2+} ions chelating capacity at 62.5–1000 μgmL^{-1} of the Fraction-I was found to be between 5.63% and 71.92% with a 479.5 μg mL^{-1}EC_{50} value.This value is higher than some other bacterial polysaccharide previously reported,where the EC_{50}values were found to be >1000 μg mL^{-1} [29,62].

3.3.3. Hydroxyl radical scavenging activity

Hydroxyl radical (OH•) is generated in living cells through Fenton reaction. It is considered as the most toxic of the reactive oxygen species. Though it has a very short life span, it is capable of damaging almost all biomolecules in the cellular system [63]. The hydrogen irons release fromthe hydroxyl groups present in the polysaccharide, neutralized the free radicals, thus terminating the chain reaction initiated by the hydroxyl radical. The hydroxyl radical scavenging activity of the Fraction-I was evaluated in different concentration between 62.5 and 1000 μg mL^{-1}, which scavenged 2.97 ± 0.09 to 60.3 ± 0.6% of the hydroxyl radical generated in the reaction (Fig. 5b),the EC_{50}value found at 525.9 μg mL^{-1}.The result was higher than some other polysaccharide isolated from bacterial and plant origin [64–66], where the EC_{50} value found to be >1000 μg mL^{-1}.

3.3.4. Cytotoxicity study

The Fraction-I, showed toxicity against HeLa cells after 48 h of treatment. It showed toxicity against HeLa cells in adose dependent manner at 62.5–500 μg mL^{-1}, killing 28.7 ± 7.2 to 63.9 ± 4.2% of the population (Fig. 6a). The LD_{50}value was calculated to be 262.8 μg mL^{-1}. Although the LD_{50} value is not comparable with Doxorubicin (Fig. 6c), it is comparable in many other reported polysaccharides isolated from natural sources [67–69], where the LD_{50} values after 48 h of treatment was found to be ~300–500 μg mL^{-1}. Apart from that, Doxorubicin possesses many adverse side effects [70,71], whereas in-vitro study showed that isolated polysaccharides were found to be less toxic against HEK-293 cell line, which is a non-cancer cell line. In the case of HEK-293, more than 75% cells survived up to 250 μg mL^{-1} of Fraction-I.It killed only around 32% HEK-293 cells at a concentration of 500 μg mL^{-1} after 48 h of incubations. Thus at that concentration, more than 68% of the cells survived (Fig. 6b).

3.3.5. Anti-proliferation activity

Fraction-I showed anti-proliferation activity against HeLa cells. With a treatment of 250 μg mL^{-1} of Fraction-I, which is less than LD_{50} valueit healed 33.1%, 42.2% and 56.8% of scratch in24, 48, and 72 h respectively. At the same time 48.9, 72.8, and 100% scratch was healed respectively in control (without any treatment)(Fig. 7). This result is justified bythe cytotoxicity study result.

The isolated exopolysaccharide (Fraction-I) from *C. freundii* exhibited Fe^{2+} chelation, and OH^{-} radical scavenging activity. Although, the activities are not best in the class, found to be higher than some previously reported bacterial exopolysaccharide. A comparison between antioxidant activities of different bacterial exopolysaccharides shown in Table 2. The antioxidant properties of polysaccharides are associated with several factors including monosaccharide constituent, molecular mass, structure and conformation of the polysaccharides [72,73]. It is considering that the molecular mass of

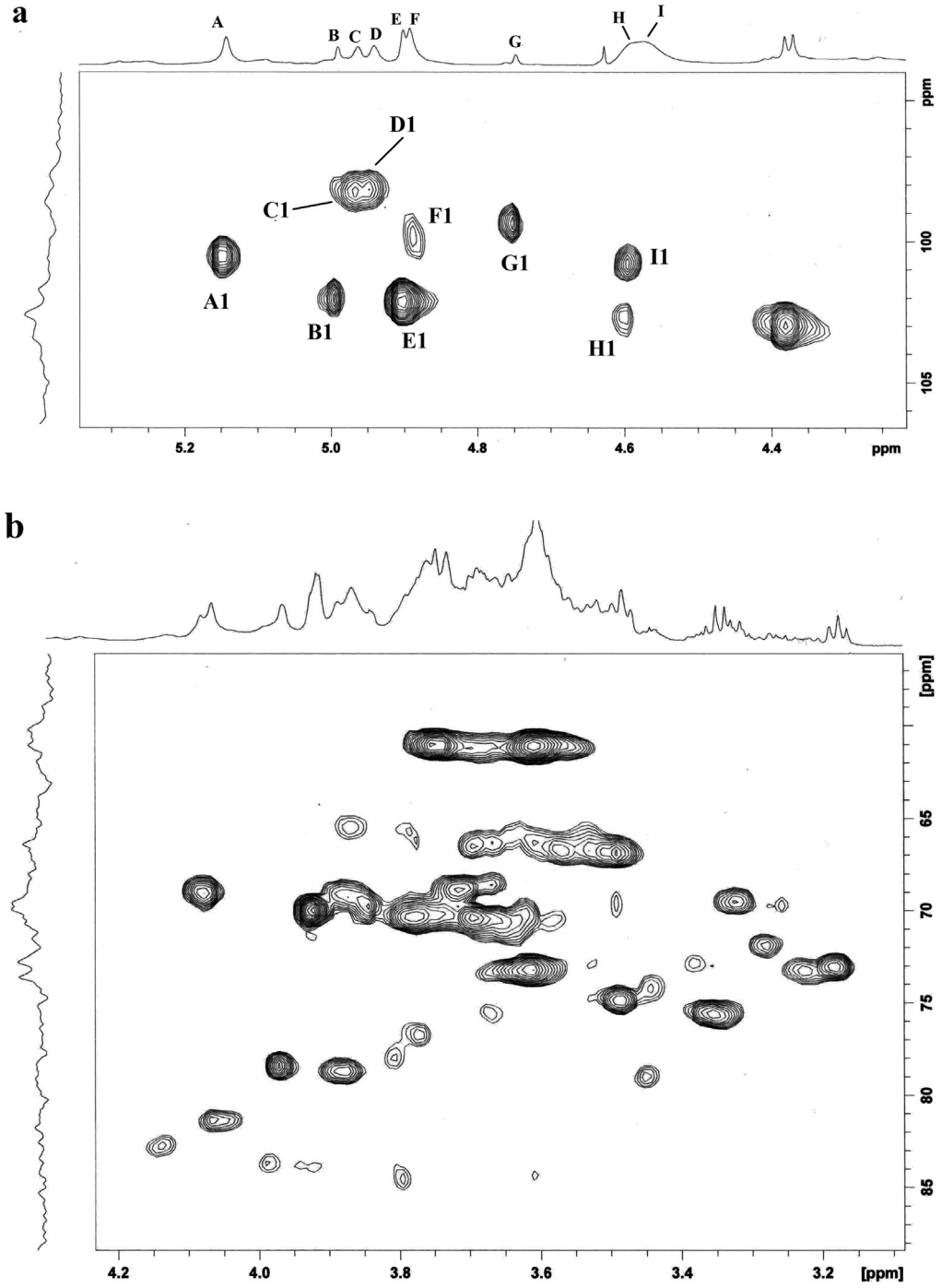


Fig. 3. The HSQC spectrum (D_2O, 27 °C) of (a) anomeric part and (b) other than anomeric part of Fraction-I isolated from the exopolysaccharide produced by *Citrobacter freundii*.

polysaccharides is one of the most important structural factors [74]. The polysaccharides with higher molecular weight, have large no of organic groups than the polysaccharides have lower molecular weight, which helps to increase antioxidant activity. The Fraction-I is a mannose rich large molecule (1.34×10^5 Da), exhibited Fe^{2+} chelation, and OH^- radical scavenging activity with an LC_{50} value of 479.5 μg mL^{-1}, and 525.9 μg mL^{-1} respectively. In contrast, exopolysaccharides isolated from *Edwardsiella tarda* (ETW1, ETW2) are also mannose rich but relatively small molecule (29 and 70 kDa respectively) [66]. The LC_{50} value of OH^- radical scavenging activity for both ETW1 and ETW2 reported >1000 μg mL^{-1}. The EPS-1 and EPS-2 isolated from *Paenibacilluspoly myxa* are fructose-rich and have large molecular weights (1.22×10^6 and 8.69×10^5 Da respectively). The corresponding LC_{50} values of OH^- radical scavenging activity were reported for the exopolysaccharide are ~400, and ~200 μg mL^{-1} respectively [31]. Similarly, exopolysaccharide isolated from *Pseudomonas* PF-6 has a molecular weight of 8.83×10^5 Da, which is almost four times higher than the molecular weight of Fraction-I. The LC_{50} value for Fe^{2+} chelation was reported as ~250 μg mL^{-1}, almost half of the Fraction-I (479.5 μg mL^{-1}) [28]. The above examples have validated the relationship between the size of the exopolysaccharide and their antioxidant activity. But the molecular weight is not the sole factor that affecting antioxidant property of the polysaccharides. As an example, EPS-1, EPS-2, and EPS-3 isolated from

Table 1b
NOESY data for the Fraction-I## from *Citrobacter freundii.*

Glycosyl residue	Anomeric proton	NOE contact proton		
	δ	δ	residue	atom
α-D-Man*p*-(1→	5.14	3.63	**B**	H-6a
A		3.79	**B**	H-6b
		3.93	**A**	H-2
		3.86	**A**	H-3
		3.66	**A**	H-5
→4,6)-α-D-Man*p*-(1→	4.99	3.82	**E**	H-4
B		4.08	**B**	H-2
		3.78	**B**	H-3
→4)-α-D-Man*p*-(1→	4.96	3.87	**D**	H-4
C		3.69	**C**	H-3
		3.50	**C**	H-5
→4)-α-D-Man*p*-(1→	4.94	3.97	**B**	H-4
D		3.72	**D**	H-3
		3.50	**D**	H-5
→4)-α-D-Man*p*-(1→	4.90	3.97	**F**	H-4
E		4.08	**E**	H-2
		3.67	**E**	H-3
		3.50	**E**	H-5
→4)-α-D-Man*p*-(1→	4.89	3.81	**G**	H-4
F		4.08	**F**	H-2
		3.66	**F**	H-3
		3.50	**F**	H-5
→4,6)-β-D-Man*p*-(1→	4.75	3.78	**H**	H-4
G		3.64	**G**	H-3
→4)-β-D-Glc*p*-(1→	4.60	3.87	**C**	H-4
H		3.45	**H**	H-2
		3.67	**H**	H-3
		3.47	**H**	H-5
β-D-Glc*p*-(1→	4.59	3.63	**H**	H-6a
I		3.87	**H**	H-6b
		3.24	**I**	H-2
		3.49	**I**	H-3
		3.36	**I**	H-5

Lactobacillus helveticus have a similar molecular weight (~2×10^5 Da) like the Faction-I, but all are reported less Fe^{2+} chelation, and OH^- radical scavenging activity than Fraction-I (>1500 μg mL^{-1}, and >2000 μg mL^{-1} respectively) [29].

Natural polysaccharides are considered safe to healthy cells; however, some of them exhibiting anticancer and antitumor properties [75]. Glucans are widely distributed in mushrooms and possess anticancer and antitumor activity, reported in several studies earlier [76]. Glucomannans are another natural polysaccharide, available in microorganisms and higher plants, which are known to possess anticancer activity [77]. Fraction-I consists glucose and mannose in 2:7 M ratios; the backbone structure consists β-(1 → 4) glycoside bond, that linked D-mannose and D-glucose, and only lightly branched through β-(1 → 6), which are the common characteristics of glucomannan [78]. Although the mode of action of Fraction-I on HeLa cells not studied yet, earlier studies suggested some probable mechanisms. The anti-cancer activity mechanism of polysaccharides is considered to be three approaches, (1) direct cytotoxicity, (2) immune-enhancement, and (3) synergistic effects in combination anti-cancer drugs [75] Earlier

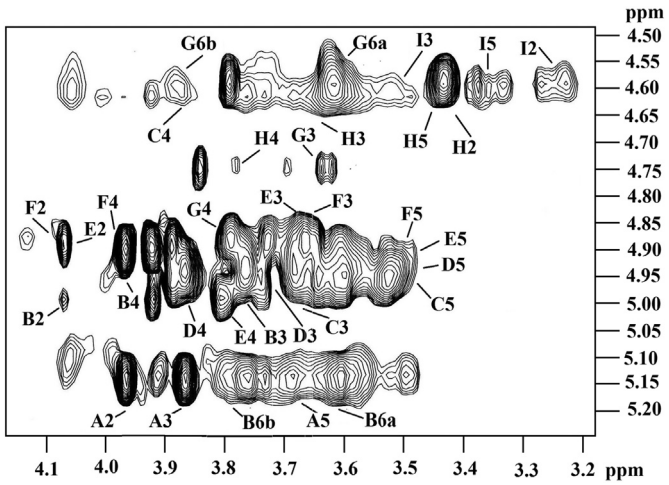


Fig. 4. Part of NOESY spectrum of a Fraction-I isolated from the exopolysaccharide produced by *Citrobacter freundii*. The NOESY mixing time was 300 ms.

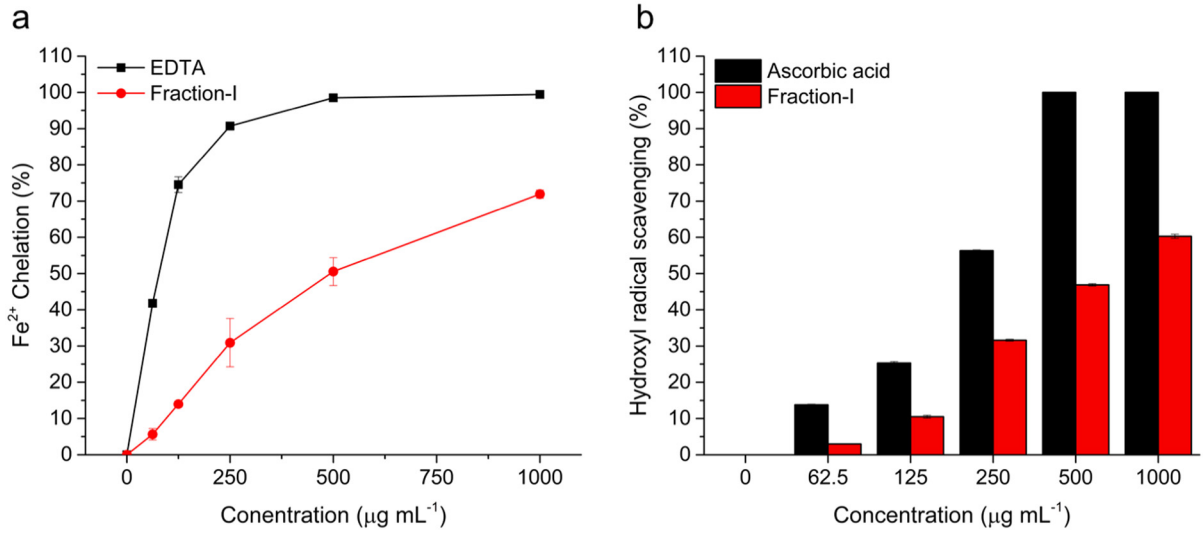


Fig. 5. (a) Chelating effects of Fraction-I on ferrous ions, EDTA used as standard (b) Hydroxyl radical scavenging activity of Fraction-I, Ascorbic used as standard. Data are presented as mean ± SD ($n = 3$).

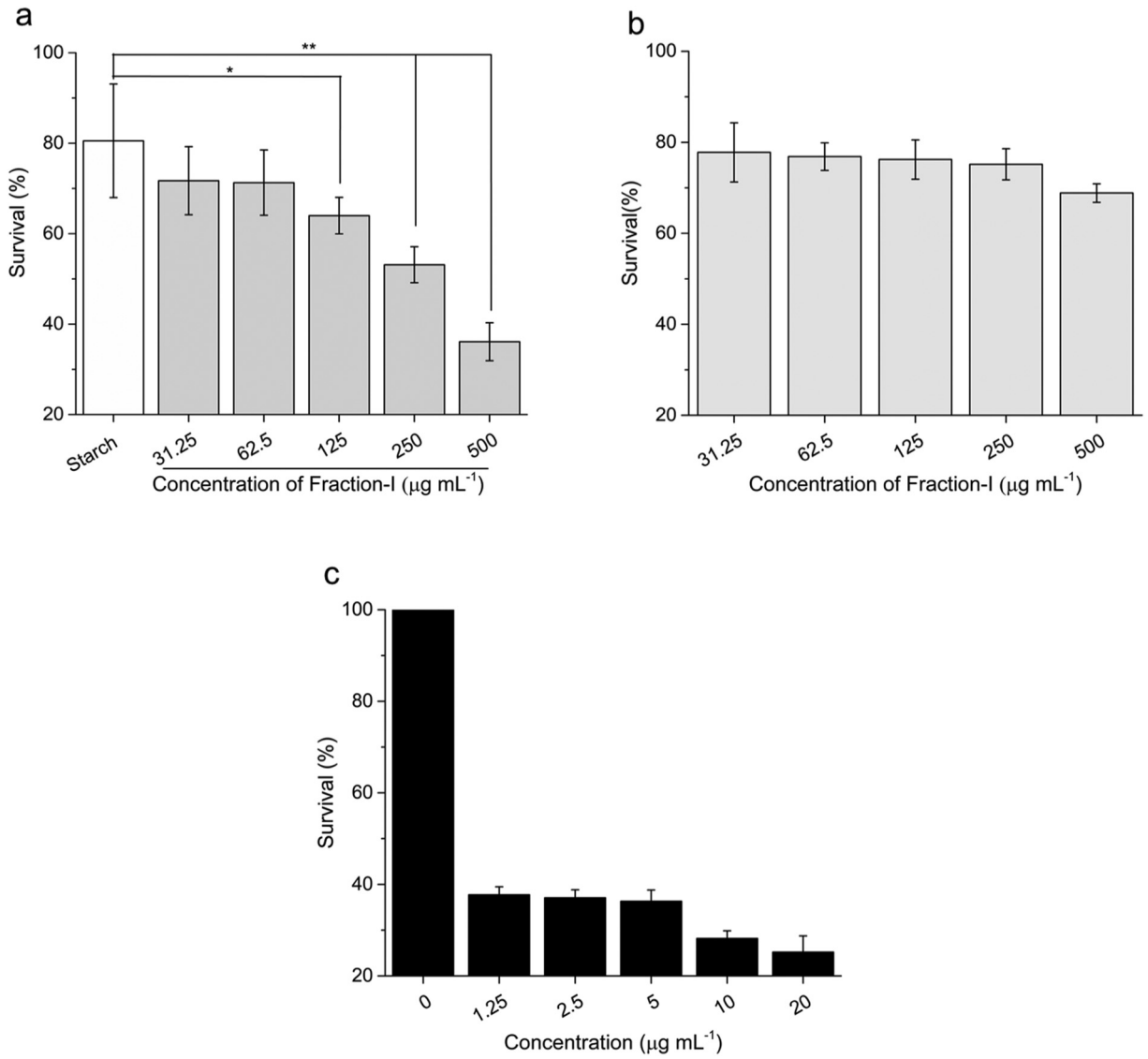


Fig. 6. (a) Cytotoxicity of the Fraction-I against HeLa cells at 48 h. The error bar indicating SD ($n = 6$). 500 μg mL^{-1} starch was used as negative control (* $P < 0.5$; ** $P < 0.001$). (b) Cytotoxicity of the Fraction-I against HEK293 cell line at 48 h (n = 6). (c) Cytotoxicity of Doxorubicin against HeLa at 48 h.

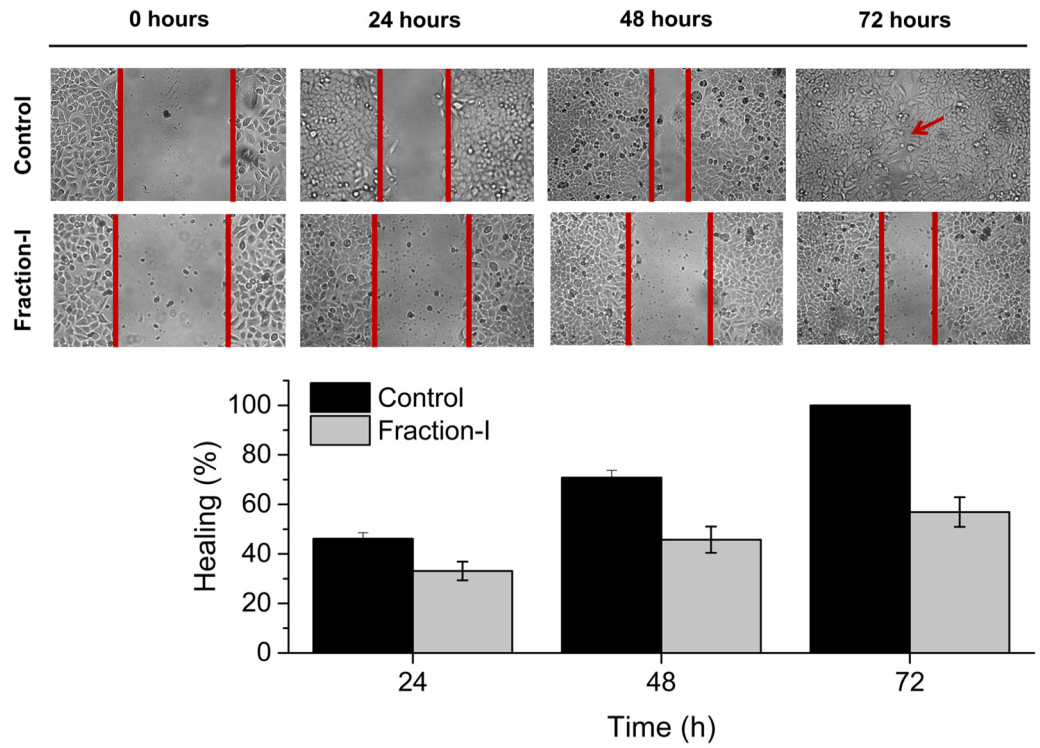


Fig. 7. Anti-proliferation activity of Fraction-I. Black column are % of wound heal in case of without treatment (control). Grey columns indicate wound heal for treatment with Fraction-I. Error bar indicating SD (n = 3).

reports revealed that glucomannan blocking the PI3K/AKT signalling pathway, and directly impact on survival of tumour cells [79,80]. Generally, after treatment with glucomannan, cells exhibit increased apoptosis, and proliferation capacity is decreased. During this time, the chemokine receptors (CCR7 and CXCR4) expression is reduced. As a result of that, the migratory ability of the tumour cells is decreased [81]. In this study, Fraction-I exhibits cytotoxicity against HeLa cells after 48 h, and almost nontoxic to HEK-293 cells after the same period of incubation. The prolong cytotoxicity of Fraction-I probably is the indication of apoptosis. The Fraction-I also inhibited proliferation of the HeLa cells. The results validated the above-mentioned phenomenon. The actual mode of action of the Fraction-I on carcinoma cells will be studied in future.

The researches on cancer treatment are focused on two areas, one is discovering a new anticancer drug, another is the development of an effective drug delivery system. A specific drug delivery system enhancing the efficiency of drug with a reduce side effect. In general, glucomannans are low toxic to normal cells, have modifiability, and non-immunogenic in nature [82]. These characteristics of glucomannan make it a suitable drug carrier. The isolated Fraction-I also exhibit low toxicity to noncancerous HEK-293 cells. The possibility of Fraction-I as a drug delivery system will be studied in future.

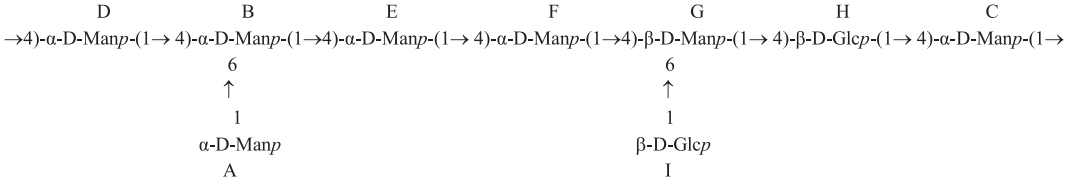
4. Conclusion

This study has explored the structure and the biological activity of the exopolysaccharide produces by the *Citrobacter freundii*, isolated from industrial wastewater ground.This is one of the few reports, where theexopolysaccharide producing *C. freundii* is reported. The work was reported

Table 2
Comparison of antioxidant activity between Fraction-I and some other bacterial exopolysaccharides.

Sl No	Origin of the exopolysaccharide	EC_{50} (μg mL^{-1})		References
		Ferrous ion chelating activity	Hydroxyl radical scavenging activity	
1	***Citrobacter freundii* (Fraction-I)**	**479.5**	**525.9**	This study
2	*Lactobacillus helveticus* (EPS-1, EPS-2, EPS-3)	>1500	>2000	29
3	*Paenibacillus polymyxa*	>1000	–	62
4	*Halolactibacillus miurensis* (HMEPS)	>5000	>1200	64
5	*Edwardsiella tarda* (ETW1, ETW2)	–	>1000	66
6	*L. paracasei* (01EP)	~10,000	–	27
7	*Pseudomonas PF-6*	~250	–	28
8	*Paenibacillus polymyxa* (EPS-2, EPS-1)	–	~200–400	31
9	*Streptococcus phocae*	–	>2400	82
10	*Streptococcus thermophiles* (SEPS, EPS)	–	~750, ~1000	83
11	*Bacillus thuringiensis*	–	~600	84

on *C. freundii* capable of producing 2568 ± 169 mg L^{-1} of exopolysaccharide, as optimized by DoE based method. The main fraction (Fraction I) had a molecular weight of ~1.34 × 10^5 Da. The monosaccharide composition of the Fraction I was mannose and glucose with a molar ratio of 7:2.The following structure was characterized by chemical analysis and 1D/2D NMR studies:



Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2020.12.063.

Authors' contributions

IC designed the study, conducted the experiments, and prepared the manuscript.

KK helped to collect samples, and isolate the strain.

PM analyzed NMR, GC–MS data, and prepared the manuscript.

AP helped to collect samples.

GM helped purify the sample.

BP designed the study.

AN designed, and analysis optimization experiment.

SM designed the study, analyzed NMR, GC–MS data, and prepared the manuscript.

NB designed and supervised the study, and prepared the final manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

The author Indranil Choudhuri expresses his thanks to the Council of Scientific and Industrial Research (CSIR) for his SRF direct fellowship (File no. 2018/(006)/EMR-I). The authors also acknowledge Mr. Barun Majumdar for the conducting of the NMR experiments, and Dr. Debojoy Chanda for editing the manuscript.

References

[1] B.A. Lipsky, E.W. Hook, A.A. Smith, J.J. Plorde, Citrobacter infections in humans: experience at the Seattle Veterans Administration Medical Center and a review of the literature, Rev. Infect. Dis. 2 (1980) 746–760, https://doi.org/10.1093/clinids/2.5.746.

[2] C. Pepperell, J.V. Kus, M.A. Gardam, A. Humar, L.L. Burrows, Low-virulence citrobacter species encode resistance to multiple antimicrobials, AAC 46 (2002) 3555–3560, https://doi.org/10.1128/AAC.46.11.3555-3560.2002.

[3] P. Kumar, S. Ghosh, D. Rath, A.K. Gadpayle, Multidrug resistant citrobacter: an unusual cause of liver abscess, Case Reports 2013 (2013)https://doi.org/10.1136/bcr-2013-008714 bcr2013008714–bcr2013008714.

[4] L. Liu, D. Chen, L. Liu, R. Lan, S. Hao, W. Jin, H. Sun, Y. Wang, Y. Liang, J. Xu, Genetic diversity, multidrug resistance, and virulence of citrobacter freundii from diarrheal patients and healthy individuals, Front. Cell. Infect. Microbiol. 8 (2018) 233, https://doi.org/10.3389/fcimb.2018.00233.

[5] M.T. Anderson, L.A. Mitchell, L. Zhao, H.L.T. Mobley, Citrobacter freundii fitness during bloodstream infection, Sci. Rep. 8 (2018), 11792, https://doi.org/10.1038/s41598-018-30196-0.

[6] J. Ouyang, F. Sun, D. Zhou, J. Feng, Z. Zhan, Z. Xiong, B. Yang, Z. Liu, T. Li, Y. Tong, P. Xia, Comparative genomics of five different resistance plasmids coexisting in a clinical multi-drug resistant Citrobacter freundii isolate, IDR Volume 11 (2018) 1447–1460, https://doi.org/10.2147/IDR.S165818.

[7] G. Gunasekaran, S. Chongdar, S. Naragoni, P.V. Rodrigues, R. Bobba, Microbial fuel cell constructed with micro-organisms isolated from industry effluent, Int. J. Hydrog. Energy 36 (2011) 14914–14922, https://doi.org/10.1016/j.ijhydene.2011.03.031.

[8] X. Wang, N. Huang, J. Shao, M. Hu, Y. Zhao, M. Huo, Coupling heavy metal resistance and oxygen flexibility for bioremoval of copper ions by newly isolated Citrobacter freundii JPG1, J. Environ. Manag. 226 (2018) 194–200, https://doi.org/10.1016/j.jenvman.2018.08.042.

[9] P. Baranwal, K. Kimura, S. Mayilraj, S. Negoro, M. Takeo, Draft genome sequence of a Bioflocculant-producing bacterium, *Citrobacter freundii* IFO 13545, Microbiol Resour Announc. 8 (2019), e00524-19, , /mra/8/29/MRA.00524-19.atom https://doi.org/10.1128/MRA.00524-19.

[10] O.D. Ianeva, Mechanisms of bacteria resistance to heavy metals, Mikrobiol. Z. 71 (2009) 54–65.

[11] R.M. Donlan, J.W. Costerton, Biofilms: survival mechanisms of clinically relevant microorganisms, CMR. 15 (2002) 167–193, https://doi.org/10.1128/CMR.15.2.167-193.2002.

[12] C. Staudt, H. Horn, D.C. Hempel, T.R. Neu, Volumetric measurements of bacterial cells and extracellular polymeric substance glycoconjugates in biofilms, Biotechnol. Bioeng. 88 (2004) 585–592, https://doi.org/10.1002/bit.20241.

[13] I.B. Beech, R.C. Tapper, Exopolymers of Sulphate-reducing Bacteria, in: J. Wingender, T.R. Neu, H.-C. Flemming (Eds.), Microbial Extracellular Polymeric Substances, Springer Berlin Heidelberg, Berlin, Heidelberg, 1999: pp. 119–126. doi:https://doi.org/10.1007/978-3-642-60147-7_6.

[14] W.P. Iverson, Microbial corrosion of metals, Advances in Applied Microbiology, Elsevier 1987, pp. 1–36, https://doi.org/10.1016/S0065-2164(08)70077-7.

[15] S. Ozturk, B. Aslim, Z. Suludere, Evaluation of chromium(VI) removal behaviour by two isolates of Synechocystis sp. in terms of exopolysaccharide (EPS) production and monomer composition, Bioresour. Technol. 100 (2009) 5588–5593, https://doi.org/10.1016/j.biortech.2009.06.001.

[16] V. Zinkevich, I. Bogdarina, H. Kang, M.A.W. Hill, R. Tapper, I.B. Beech, Characterisation of exopolymers produced by different isolates of marine sulphate-reducing bacteria, Int. Biodeterior. Biodegradation 37 (1996) 163–172, https://doi.org/10.1016/S0964-8305(96)00025-X.

[17] P.S. Stewart, J. William Costerton, Antibiotic resistance of bacteria in biofilms, Lancet 358 (2001) 135–138, https://doi.org/10.1016/S0140-6736(01)05321-1.

[18] P.M. Bales, E.M. Renke, S.L. May, Y. Shen, D.C. Nelson, Purification and characterization of biofilm-associated EPS exopolysaccharides from ESKAPE organisms and other pathogens, PLoS One 8 (2013), e67950, https://doi.org/10.1371/journal.pone.0067950.

[19] S. Hossain, S.H.M.P. Wimalasena, G.-J. Heo, Virulence factors and antimicrobial resistance pattern of Citrobacter freundii isolated from healthy pet turtles and their environment, Asian J. of Animal and Veterinary Advances. 12 (2017) 10–16, https://doi.org/10.3923/ajava.2017.10.16.

[20] L.J. Thompson, V. Gray, D. Lindsay, A. Von Holy, Carbon : nitrogen : phosphorus ratios influence biofilm formation by *Enterobacter cloacae* and *Citrobacter freundii*: influence of C : N : P ratios on biofilm formation, J. Appl. Microbiol. 101 (2006) 1105–1113, https://doi.org/10.1111/j.1365-2672.2006.03003.x.

[21] F. Freitas, V.D. Alves, M. Carvalheira, N. Costa, R. Oliveira, M.A.M. Reis, Emulsifying behaviour and rheological properties of the extracellular polysaccharide produced by Pseudomonas oleovorans grown on glycerol byproduct, Carbohydr. Polym. 78 (2009) 549–556, https://doi.org/10.1016/j.carbpol.2009.05.016.

[22] A. Arena, T.L. Maugeri, B. Pavone, D. Iannello, C. Gugliandolo, G. Bisignano, Antiviral and immunoregulatory effect of a novel exopolysaccharide from a marine thermotolerant Bacillus licheniformis, Int. Immunopharmacol. 6 (2006) 8–13, https://doi.org/10.1016/j.intimp.2005.07.004.

[23] D. De Stefano, G. Tommonaro, V. Simeon, A. Poli, B. Nicolaus, R. Carnuccio, A polysaccharide from tomato (*Lycopersicon esculentum*) peels affects NF-κB activation in LPS-stimulated J774 macrophages, J. Nat. Prod. 70 (2007) 1636–1639, https://doi.org/10.1021/np070168z.

[24] R. Weiner, S. Langille, E. Quintero, Structure, function and immunochemistry of bacterial exopolysaccharides, J. Ind. Microbiol. 15 (1995) 339–346, https://doi.org/10.1007/BF01569989.

[25] Y.-N. Feng, X.-F. Zhang, Polysaccharide extracted from Huperzia serrata using response surface methodology and its biological activity, Int. J. Biol. Macromol. 157 (2020) 267–275, https://doi.org/10.1016/j.ijbiomac.2020.04.134.

[26] X. Hanyu, L. Lanyue, D. Miao, F. Wentao, C. Cangran, S. Hui, Effect of Ganoderma applanatum polysaccharides on MAPK/ERK pathway affecting autophagy in breast cancer MCF-7 cells, Int. J. Biol. Macromol. 146 (2020) 353–362, https://doi.org/10.1016/j.ijbiomac.2020.01.010.

[27] C.-F. Liu, K.-C. Tseng, S.-S. Chiang, B.-H. Lee, W.-H. Hsu, T.-M. Pan, Immunomodulatory and antioxidant potential of lactobacillus exopolysaccharides, J. Sci. Food Agric. (2011)https://doi.org/10.1002/jsfa.4456 n/a-n/a.
[28] S. Ye, F. Liu, J. Wang, H. Wang, M. Zhang, Antioxidant activities of an exopolysaccharide isolated and purified from marine Pseudomonas PF-6, Carbohydr. Polym. 87 (2012) 764–770, https://doi.org/10.1016/j.carbpol.2011.08.057.
[29] W. Li, J. Ji, X. Chen, M. Jiang, X. Rui, M. Dong, Structural elucidation and antioxidant activities of exopolysaccharides from lactobacillus helveticus MB2-1, Carbohydr. Polym. 102 (2014) 351–359, https://doi.org/10.1016/j.carbpol.2013.11.053.
[30] R. Inturri, A. Molinaro, F. Di Lorenzo, G. Blandino, B. Tomasello, C. Hidalgo-Cantabrana, C. De Castro, P. Ruas-Madiedo, Chemical and biological properties of the novel exopolysaccharide produced by a probiotic strain of Bifidobacterium longum, Carbohydr. Polym. 174 (2017) 1172–1180, https://doi.org/10.1016/j.carbpol.2017.07.039.
[31] J. Liu, J. Luo, H. Ye, Y. Sun, Z. Lu, X. Zeng, Production, characterization and antioxidant activities in vitro of exopolysaccharides from endophytic bacterium Paenibacillus polymyxa EJS-3, Carbohydr. Polym. 78 (2009) 275–281, https://doi.org/10.1016/j.carbpol.2009.03.046.
[32] D. Zeng, S. Zhu, Purification, characterization, antioxidant and anticancer activities of novel polysaccharides extracted from Bachu mushroom, Int. J. Biol. Macromol. 107 (2018) 1086–1092, https://doi.org/10.1016/j.ijbiomac.2017.09.088.
[33] M. Lemieszek, W. Rzeski, Anticancer properties of polysaccharides isolated from fungi of the Basidiomycetes class, Wo 4 (2012) 285–289, https://doi.org/10.5114/wo.2012.30055.
[34] B. Ismail, K. Nampoothiri, Exposition of antitumour activity of a chemically characterized exopolysaccharide from a probiotic lactobacillus plantarum MTCC 9510, Biologia 68 (2013)https://doi.org/10.2478/s11756-013-0275-2.
[35] N. Sun, H. Liu, S. Liu, X. Zhang, P. Chen, W. Li, X. Xu, W. Tian, Purification, preliminary structure and antitumor activity of exopolysaccharide produced by Streptococcus thermophilus CH9, Molecules 23 (2018) 2898, https://doi.org/10.3390/molecules23112898.
[36] Y. Zhu, C. Wang, S. Jia, B. Wang, K. Zhou, S. Chen, Y. Yang, S. Liu, Purification, characterization and antioxidant activity of the exopolysaccharide from Weissella cibaria SJ14 isolated from Sichuan paocai, Int. J. Biol. Macromol. 115 (2018) 820–828, https://doi.org/10.1016/j.ijbiomac.2018.04.067.
[37] A. Ahmad, M.U. Rehman, A.F. Wali, H.A. El-Serehy, F.A. Al-Misned, S.N. Maodaa, H.M. Aljawdah, T.M. Mir, P. Ahmad, Box–Behnken response surface Design of Polysaccharide Extraction from Rhododendron arboreum and the evaluation of its antioxidant potential, Molecules 25 (2020) 3835, https://doi.org/10.3390/molecules25173835.
[38] S.R. Sudhamani, R.N. Tharanathan, M.S. Prasad, Isolation and characterization of an extracellular polysaccharide from Pseudomonas caryophylli CFR 1705, Carbohydr. Polym. 56 (2004) 423–427, https://doi.org/10.1016/j.carbpol.2004.03.008.
[39] Michel. DuBois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, Fred. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356, https://doi.org/10.1021/ac60111a017.
[40] W.S. York, A.G. Darvill, M. McNeil, T.T. Stevenson, P. Albersheim, Isolation and characterization of plant cell walls and cell wall components, Methods in Enzymology, Elsevier 1986, pp. 3–40, https://doi.org/10.1016/0076-6879(86)18062-1.
[41] G.L. Peterson, A simplification of the protein assay method of Lowry et al. which is more generally applicable, Anal. Biochem. 83 (1977) 346–356, https://doi.org/10.1016/0003-2697(77)90043-4.
[42] C. Hara, T. Kiho, Y. Tanaka, S. Ukai, Anti-inflammatory activity and conformational behavior of a branched (1→3)-β-d-glucan from an alkaline extract of dictyophora indusiata fisch, Carbohydr. Res. 110 (1982) 77–87, https://doi.org/10.1016/0008-6215(82)85027-1.
[43] P. Maity, M. Pattanayak, S. Maity, A.K. Nandi, I.K. Sen, B. Behera, T.K. Maiti, P. Mallick, S.R. Sikdar, S.S. Islam, A partially methylated mannogalactan from hybrid mushroom pfle 1p: purification, structural characterization, and study of immunoactivation, Carbohydr. Res. 395 (2014) 1–8, https://doi.org/10.1016/j.carres.2014.06.004.
[44] I. Ciucanu, F. Kerek, A simple and rapid method for the permethylation of carbohydrates, Carbohydr. Res. 131 (1984) 209–217, https://doi.org/10.1016/0008-6215(84)85242-8.
[45] M.T. Dueñas-Chasco, M.A. Rodríguez-Carvajal, P.T. Mateo, G. Franco-Rodríguez, JoséL. Espartero, A. Irastorza-Iribas, A.M. Gil-Serrano, Structural analysis of the exopolysaccharide produced by Pediococcus damnosus 2.6, Carbohydr. Res. 303 (1997) 453–458, https://doi.org/10.1016/S0008-6215(97)00192-4.
[46] K. Hard, G. Van Zadelhoff, P. Moonen, J.P. Kamerling, J.F.G. Vliegenthart, The Asn-linked carbohydrate chains of human Tamm-Horsfall glycoprotein of one male. Novel sulfated and novel N-acetylgalactosamine-containing N-linked carbohydrate chains, Eur. J. Biochem. 209 (1992) 895–915, https://doi.org/10.1111/j.1432-1033.1992.tb17362.x.
[47] P. Prieto, M. Pineda, M. Aguilar, Spectrophotometric quantitation of antioxidant capacity through the formation of a Phosphomolybdenum complex: specific application to the determination of vitamin E, Anal. Biochem. 269 (1999) 337–341, https://doi.org/10.1006/abio.1999.4019.
[48] T.C.P. Dinis, V.M.C. Madeira, L.M. Almeida, Action of phenolic derivatives (acetaminophen, salicylate, and 5-Aminosalicylate) as inhibitors of membrane lipid peroxidation and as Peroxyl radical scavengers, Arch. Biochem. Biophys. 315 (1994) 161–169, https://doi.org/10.1006/abbi.1994.1485.
[49] C. Gao, Y. Wang, C. Wang, Z. Wang, Antioxidant and immunological activity in vitro of polysaccharides from Gomphidius rutilus mycelium, Carbohydr. Polym. 92 (2013) 2187–2192, https://doi.org/10.1016/j.carbpol.2012.12.011.
[50] J. van Meerloo, G.J.L. Kaspers, J. Cloos, Cell sensitivity assays: The MTT assay, in: I.A. Cree (Ed.), Cancer Cell Culture, Humana Press, Totowa, NJ 2011, pp. 237–245, https://doi.org/10.1007/978-1-61779-080-5_20.
[51] G.N. Maity, P. Maity, I. Choudhuri, N. Bhattacharyya, K. Acharya, S. Dalai, S. Mondal, Structural studies of a water insoluble β-glucan from Pleurotusdjamor and its cytotoxic effect against PA1, ovarian carcinoma cells, Carbohydr. Polym. 222 (2019), 114990, https://doi.org/10.1016/j.carbpol.2019.114990.
[52] G.N. Maity, P. Maity, I. Choudhuri, G.C. Sahoo, N. Maity, K. Ghosh, N. Bhattacharyya, S. Dalai, S. Mondal, Green synthesis, characterization, antimicrobial and cytotoxic effect of silver nanoparticles using arabinoxylan isolated from Kalmegh, Int. J. Biol. Macromol. 162 (2020) 1025–1034, https://doi.org/10.1016/j.ijbiomac.2020.06.215.
[53] P.K. Agrawal, NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides, Phytochemistry 31 (1992) 3307–3330, https://doi.org/10.1016/0031-9422(92)83678-R.
[54] M. Rinaudo, M. Vincendon, 13C NMR structural investigation of scleroglucan, Carbohydr. Polym. 2 (1982) 135–144, https://doi.org/10.1016/0144-8617(82)90059-5.
[55] P. Maity, I.K. Sen, P.K. Maji, S. Paloi, K.S.P. Devi, K. Acharya, T.K. Maiti, S.S. Islam, Structural, immunological, and antioxidant studies of β-glucan from edible mushroom Entoloma lividoalbum, Carbohydr. Polym. 123 (2015) 350–358, https://doi.org/10.1016/j.carbpol.2015.01.051.
[56] A.K. Nandi, S. Samanta, S. Maity, I.K. Sen, S. Khatua, K.S.P. Devi, K. Acharya, T.K. Maiti, S.S. Islam, Antioxidant and immunostimulant β-glucan from edible mushroom Russula albonigra (Krombh.) Fr, Carbohydr. Polym. 99 (2014) 774–782, https://doi.org/10.1016/j.carbpol.2013.09.016.
[57] H. Wang, Y.M. Liu, Z.M. Qi, S.Y. Wang, S.X. Liu, X. Li, H.J. Wang, X.C. Xia, An overview on natural polysaccharides with antioxidant properties, Curr. Med. Chem. 20 (2013) 2899–2913, https://doi.org/10.2174/0929867311320230006.
[58] G. Huang, X. Mei, J. Hu, The antioxidant activities of natural polysaccharides, CDT 18 (2017)https://doi.org/10.2174/1389450118666170123145357.
[59] Y. Yu, M. Shen, Q. Song, J. Xie, Biological activities and pharmaceutical applications of polysaccharide from natural resources: a review, Carbohydr. Polym. 183 (2018) 91–101, https://doi.org/10.1016/j.carbpol.2017.12.009.
[60] B. Halliwell, Invited commentary:superoxide, Iron, vascular endothelium and reperfusion injury, Free Radic. Res. Commun. 5 (1989) 315–318, https://doi.org/10.3109/10715768909073413.
[61] İ. Gülçin, Antioxidant and antiradical activities of l-carnitine, Life Sci. 78 (2006) 803–811, https://doi.org/10.1016/j.lfs.2005.05.103.
[62] W. Raza, K. Makeen, Y. Wang, Y. Xu, S. Qirong, Optimization, purification, characterization and antioxidant activity of an extracellular polysaccharide produced by Paenibacillus polymyxa SQR-21, Bioresour. Technol. 102 (2011) 6095–6103, https://doi.org/10.1016/j.biortech.2011.02.033.
[63] O.I. Aruoma, Free radicals, oxidative stress, and antioxidants in human health and disease, J Amer Oil Chem Soc 75 (1998) 199–212, https://doi.org/10.1007/s11746-998-0032-9.
[64] J. Arun, S. Selvakumar, R. Sathishkumar, M. Moovendhan, G. Ananthan, T. Maruthiah, A. Palavesam, In vitro antioxidant activities of an exopolysaccharide from a salt pan bacterium Halolactibacillus miurensis, Carbohydr. Polym. 155 (2017) 400–406, https://doi.org/10.1016/j.carbpol.2016.08.085.
[65] Y.-T. Li, B.-J. Chen, W.-D. Wu, K. Ge, X.-Y. Wei, L.-M. Kong, Y.-Y. Xie, J.-P. Gu, J.-C. Zhang, T. Zhou, Antioxidant and antimicrobial evaluation of carboxymethylated and hydroxamated degraded polysaccharides from Sargassum fusiforme, Int. J. Biol. Macromol. 118 (2018) 1550–1557, https://doi.org/10.1016/j.ijbiomac.2018.06.196.
[66] S. Guo, W. Mao, Y. Han, X. Zhang, C. Yang, Y. Chen, Y. Chen, J. Xu, H. Li, X. Qi, J. Xu, Structural characteristics and antioxidant activities of the extracellular polysaccharides produced by marine bacterium Edwardsiella tarda, Bioresour. Technol. 101 (2010) 4729–4732, https://doi.org/10.1016/j.biortech.2010.01.125.
[67] W. Cao, X.-Q. Li, X. Wang, H.-T. Fan, X.-N. Zhang, Y. Hou, S.-B. Liu, Q.-B. Mei, A novel polysaccharide, isolated from Angelica sinensis (Oliv.) Diels induces the apoptosis of cervical cancer HeLa cells through an intrinsic apoptotic pathway, Phytomedicine 17 (2010) 598–605, https://doi.org/10.1016/j.phymed.2009.12.014.
[68] R. Zhao, X. Gao, Y. Cai, X. Shao, G. Jia, Y. Huang, X. Qin, J. Wang, X. Zheng, Antitumor activity of Portulaca oleracea L. polysaccharides against cervical carcinoma in vitro and in vivo, Carbohydr. Polym. 96 (2013) 376–383, https://doi.org/10.1016/j.carbpol.2013.04.023.
[69] J.-H. Xie, X. Liu, M.-Y. Shen, S.-P. Nie, H. Zhang, C. Li, D.-M. Gong, M.-Y. Xie, Purification, physicochemical characterisation and anticancer activity of a polysaccharide from Cyclocarya paliurus leaves, Food Chem. 136 (2013) 1453–1460, https://doi.org/10.1016/j.foodchem.2012.09.078.
[70] A.D. Hanna, A. Lam, S. Tham, A.F. Dulhunty, N.A. Beard, Adverse effects of doxorubicin and its metabolic product on cardiac RyR2 and SERCA2A, Mol. Pharmacol. 86 (2014) 438–449, https://doi.org/10.1124/mol.114.093849.
[71] C. Carvalho, R. Santos, S. Cardoso, S. Correia, P. Oliveira, M. Santos, P. Moreira, Doxorubicin: the good, the bad and the ugly effect, CMC 16 (2009) 3267–3285, https://doi.org/10.2174/092986709788803312.
[72] H. Qi, Q. Zhang, T. Zhao, R. Chen, H. Zhang, X. Niu, Z. Li, Antioxidant activity of different sulfate content derivatives of polysaccharide extracted from Ulva pertusa (Chlorophyta) in vitro, Int. J. Biol. Macromol. 37 (2005) 195–199, https://doi.org/10.1016/j.ijbiomac.2005.10.008.
[73] E. Tsiapali, S. Whaley, J. Kalbfleisch, H.E. Ensley, I.W. Browder, D.L. Williams, Glucans exhibit weak antioxidant activity, but stimulate macrophage free radical activity, Free Radic. Biol. Med. 30 (2001) 393–402, https://doi.org/10.1016/S0891-5849(00)00485-8.
[74] T. Zhao, Q. Zhang, H. Qi, H. Zhang, X. Niu, Z. Xu, Z. Li, Degradation of porphyran from Porphyrahaitanensis and the antioxidant activities of the degraded porphyrans with different molecular weight, Int. J. Biol. Macromol. 38 (2006) 45–50, https://doi.org/10.1016/j.ijbiomac.2005.12.018.

[75] A. Zong, H. Cao, F. Wang, Anticancer polysaccharides from natural resources: a review of recent research, Carbohydr. Polym. 90 (2012) 1395–1410, https://doi.org/10.1016/j.carbpol.2012.07.026.
[76] V. C.Ooi, F. Liu, Immunomodulation and anti-Cancer activity of polysaccharide-protein complexes, CMC 7 (2000) 715–729, https://doi.org/10.2174/0929867003374705.
[77] J. Li, F. Sun, H. Zhou, J. Yang, C. Huang, H. Fan, A systematic review exploring the anticancer activity and mechanisms of Glucomannan, Front. Pharmacol. 10 (2019) 930, https://doi.org/10.3389/fphar.2019.00930.
[78] W.-T. Wu, H.-C. Cheng, H.-L. Chen, Ameliorative effects of konjac glucomannan on human faecal β-glucuronidase activity, secondary bile acid levels and faecal water toxicity towards Caco-2 cells, Br. J. Nutr. 105 (2011) 593–600, https://doi.org/10.1017/S0007114510004009.
[79] S.M. Frisch, R.A. Screaton, Anoikis mechanisms, Curr. Opin. Cell Biol. 13 (2001) 555–562, https://doi.org/10.1016/S0955-0674(00)00251-9.
[80] Q. Guanen, S. Junjie, W. Baolin, W. Chaoyang, Y. Yajuan, L. Jing, L. Junpeng, N. Gaili, W. Zhongping, W. Jun, MiR-214 promotes cell meastasis and inhibites apoptosis of esophageal squamous cell carcinoma via PI3K/AKT/mTORsignaling pathway, Biomed. Pharmacother. 105 (2018) 350–361, https://doi.org/10.1016/j.biopha.2018.05.149.
[81] C. Wu, S. Qiu, P. Liu, Y. Ge, X. Gao, RhizomaAmorphophalli inhibits TNBC cell proliferation, migration, invasion and metastasis through the PI3K/Akt/mTOR pathway, J. Ethnopharmacol. 211 (2018) 89–100, https://doi.org/10.1016/j.jep.2017.09.033.
[82] X. Zhan, L. Jia, Y. Niu, H. Qi, X. Chen, Q. Zhang, J. Zhang, Y. Wang, L. Dong, C. Wang, Targeted depletion of tumour-associated macrophages by an alendronate–glucomannan conjugate for cancer immunotherapy, Biomaterials 35 (2014) 10046–10057, https://doi.org/10.1016/j.biomaterials.2014.09.007.