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STRUCTURAL CHARACTERIZATION OF CYTOTOXIC EXOPOLYSACCHARIDES PRODUCED BY *Bifidobacterium pseudocatenulatum* ATCC 27919 CULTIVATED IN ALOE VERA MEDIUM

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Abstract: Exopolysaccharides (EPS) produced by several probiotics, including *Bifidobacteria* strains, have various benefits, such as anticancer activity due to its cytotoxic property components. However, different media have an influence of the components of EPS produced during cultivation and lead to differences in benefits and functions. Aloe vera medium was selected as the cultivation medium for EPS production by *Bifidobacteria pseudocatenulatum* ATCC 27919 due to it being rich with carbon sources. The aim of this study was to a) determine the cytotoxicity effect of EPS produced by *B. pseudocatenulatum* strain ATCC 27919 cultivated in aloe vera medium against Caco-2 cell line using MTS assay, and b) study the component profile of EPS produced by *B. pseudocatenulatum* strain ATCC 27919 using CHNS elemental analysis, high-performance liquid chromatography (HPLC), flourier-transform infrared (FT-IR), and nuclear magnetic resonance (NMR) spectroscopy techniques. The EPS obtained from aloe vera medium was found to be toxic to Caco-2 cells at concentration of 1 mg/ml at both 20- and 40-hour incubation with percentage cell viability of 82% and 65%, respectively. The cytotoxic activity of EPS was justified by determining the presence of mannose and molecular properties of EPS through CHNS, HPLC, FTIR and NMR analyses. As a conclusion, EPS produced by *B. pseudocatenulatum* strain ATCC 27919 during cultivation in aloe vera medium has the potential to act as a cytotoxic compound against Caco-2 cells due to its biological components.

Keywords: Exopolysaccharide, EPS structure, extraction, *Bifidobacterium*, cytotoxicity, repeating unit.

Introduction

Many bacteria are able to produce cell-surface polysaccharides, also known as exopolysaccharides (EPS). The production of EPS by the cells is related to the prevention of desiccation, protection from environmental stresses, adherence to surfaces, pathogenesis and symbiosis (Caggianiello *et al.,* 2016). The unique properties of EPS lead to it having potential health benefits, such as having anticancer activity properties (Z. Liu *et al.,* 2017; Pan *et al.,* 2010). The effect of acidic EPS on colon cancer cell proliferation has been reported by Di *et al.* (2018). Exopolysaccharides are long-chain polysaccharides consisting of branched, repeating units of sugar or sugar derivatives, such as mannose arabinose glucose, fructose and galactose (Ismail & Nampoothiri, 2010).

Members of the genus *Bifidobacterium* are commensal microorganisms found in the human gastrointestinal tract and have been traditionally considered as beneficial to human health. Some of their strains are able to produce EPS with potential health benefits (Castro-Bravo *et al.,* 2018; Salazar *et al.,* 2008). EPS produced by the strains lay a great variety of structures and one of the factors is due to medium used during cultivation. The carbon source seems to be one of the most important factors influencing the

monomeric composition and variations in the glycosidic bonds of EPS (Lynch *et al.,* 2018) and this also leads to variations in the EPS functions and benefits (Imran *et al.,* 2016).

Several studies have reported that plant-based media are one potential medium that can be used to support EPS production by probiotic strain due to them being rich with carbon and nitrogen sources (Zhang *et al.,* 2014). However, studies on aloe vera medium as the cultivating medium for Bifidobacteria to produce EPS remain scarce. Aloe vera contains various polysaccharides, including an acetylated glucomannan, also known as acemannan, which is the most abundant polysaccharide. Mannose constitutes the backbone of the polysaccharide intercepted with glucose units. Mannose and glucose are linked by β-(1→4) glycosidic bonds (+8), which re believed to support EPS production in bacteria.

Thus, this study aims to 1) study the cytotoxic activity of EPS produced by *Bifidobacteria pseudocatenulatum* ATCC27919 cultivated in aloe vera medium, and 2) determine the characteristics of EPS produced using high-performance liquid chromatography (HPLC), flourier-transform infrared (FT-IR), nuclear magnetic resonance (NMR) spectroscopies techniques and CHNS elemental analysis.

Chemicals

Tri Fluoroacetic acid (TFA) (80% v/v), H2SO4 (Con), DMSO, human colon cancer cells (Caco-2), Dulbecco's Modified Eagle Medium (DMEM) media, de Mann, Rogosa, Sharpe (MRS) media, Trichloroacetic acid (TCA), D-Glucose monohydrate, D(+)-Xylose, D(+)-Galactose, D(-)-Ribose, D(-)-Arabinose, D-(+)-Manose, LAB-LEMCO powder (beef extract), Sodium chloride, Tryptone, L-Cysteine, Sephadex LH-20, KBr, deuterium water D2O, Butanol, 2-Propanol, ethanol, Sterile peptone water (0.1% w/v), Prewarmed phosphate-buffered saline (PBS; 37°C), (Invitrogen, Carlsbad, CA, USA), Annexin V-FITC/ Propidium iodide (PI) cell apoptosis kit (Ebioscience, San Diego, USA).

Methods

Inoculum Preparation

A total of 100 µl (10^7 CFU/ml) of *B. pseudocatenulatum* ATCC 27919 stock culture was added into a falcon tube and incubated for 24 hours at 37°C in anaerobic conditions. Later, the culture media was centrifuged at 7,000 rpm for 5 minutes at 4°C, and the pellet was then collected. Then, 10 ml of MRS culture was added to the same falcon tube and incubated for 48 hours at 37°C in anaerobic conditions. After an incubation period, the tube was centrifuged at 7,000 rpm for 5 minutes in 4°C and the pellet was then collected. About 10 ml of sterile water was added into the pellet and centrifuged twice. Subsequently, the inoculum was transferred to culture media for the fermentation process.

Medium Preparation for B. pseudocatenulatum ATCC 27919 Cultivation

Aloe vera medium was prepared by mixing 1% (w/v) pancreatic digest of casein (Trypton) (1 g), 1% (w/v) of beef extract (1 g), 0.5% (w/v) of NaCl (0.5 g), 0.05% (w/v) of L-cysteine (0.05 g) and 80 ml of sterile water in a 200 ml sterilised bottle. The mixture was autoclaved at 121°C for 20 minutes. A mass of 0.1 g of aloe vera (UV exposed for 15 minutes) was dissolved in 10 ml of sterile water and vortexed using a magnetic stirrer for 15 minutes. Then, about 3.65 ml (3.65% v/v) of aloe vera solution was transferred into the cooled autoclaved mixture. Subsequently, D- Glucose (Bio Basic, USA) was added into the mixture.

In this study, de Mann, Rogosa and Sharpe (MRS) (Basingstoke, Hampshire, England, UK) media were used as the comparative medium for EPS production. About 10% of inoculum *B. pseudocatenulatum* ATCC 27919 was inoculated to the media. This mixture is allowed to ferment for 24 hours at 37°C under anaerobic conditions for EPS production.

Extraction and Purification of Exopoly-saccharide (EPS)

A 100 ml of fermentation solution was added to the water bath at 100°C for 30 minutes and then allowed to cool down. After that, 10% (v/v) of Trichloroacetic acid (TCA provided by EMSURE, Germany) was added to the bacteria suspension. The suspension was incubated for 3 hours in the icebox before centrifuged at 7,800 rpm for 10 minutes at 4°C. Later, the supernatant was transferred into a separate tube. Cold ethanol was added to that supernatant according to a 1: 1 ratio. The mixture was incubated under low temperature conditions for 48 hours. Following incubation, the mixture was centrifuged at 7,800 rpm for 10 minutes at 4ºC.

The EPS was collected in a visking tube and retained for 48 hours. During the procedure, water was changed twice a day. After 48 hours, the extracted EPS was transferred to the falcon tube and allowed to freeze-dry for three days. Later, the sample was purified using gel filtration column chromatography packed with Sephadex LH 20 (Sigma, USA). Distilled water was used as the elution solvent, as suggested by Yang *et al.* (2019). Approximately 4 ml of the fraction was collected into a vial. Each fraction was freeze-dried for 48 hours and then transferred to a falcon tube until used.

Analysis

Cytotoxic Activity of Exopoly-saccharide (EPS)

For EPS cytotoxic activity, colon cancer Caco-2 cells were cultured in Roswell Park Memorial Institute (RPMI) 1,640 containing 5% FBS and 50 µM 2-ME (2-mercaptoethanol), under 5% CO_2 atmosphere at 37°C. The initial concentration of Caco-2 cells was maintained at 2×10^5 cells/ml using EPS treatment. Then, the Caco-2 cells were washed twice prior to being centrifuged at 300 × g for 5 minutes. Cell viability was determined using trypan blue exclusion and the cells were resuspended to the final concentration of 1×10^5 cells/ml in RPMI 1640 supplemented with 5% FBS and 50 µM 2-ME. Briefly, cancer cells were seeded into a 96-well plate with 1×10^5 cells per well. About 1mg/ml concentration of EPS was added to each well and further incubated at 37°C in a humidified condition with 5% CO_2 for 20 and 40 hours. After incubation, 20 µl of MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)] (Bajak *et al*., 2015) and an electron coupling reagent (phenazine ethosulfate, or PES) were added to the cells and further incubated again for 4 hours at 37°C. Finally, the absorbance was recorded at 490 nm using a 96 well plate reader. The inhibition ratio was calculated using Equation 1 as shown below. In this experiment, dexamethasone and RPMI 1640 were used as positive and negative control, respectively.

$$cell\ viabilty\ (\%) = \frac{absobance\ at\ time}{absobance\ at\ 0\ hrs}\ x\ 100$$

Equation 1

Exopolysaccharides Profile Analyses

CHNS Element Ratio Analysis of Exopoly-saccharide (EPS)

Initially, surfaces were cleaned by ethanol and air dried. Then, the pre-cleaned small tin tare containers were prepared. The empty tare containers were placed on a microbalance and were calibrated to zero weight. Then the desire value was set for this analysis. Later, the sample was added to the container until the pre-defined target weight range is reached, and for this process, a spatula was used. The container was removed from the balance using tweezers. A second pair of tweezers was used to pinch the container's top to close it and fold it over the edge twice. Then, this container was flattened and compressed gently to form a ball. This was placed in the 96 wells microtiter plate. The plate was transferred to an elemental analyser (a Flash 2000 organic elemental analyser was used to these experiments). An elemental analyser software was used to calculate the percentage of C, H, N, S (carbon, hydrogen, nitrogen and sulphur) ratio and it was calculated using Equation 2 as shown.

$$Element\ mole = \frac{percentage\ of\ the\ element}{molar\ mass\ (g/mol)}$$

$$number\ of\ atom\ present\ in\ the\ sample = \frac{element\ mole\ (mol)}{smallest\ element\ mole\ (mol)}$$

Equation 2

EPS Monosaccharides Analysis Using HPLC-RID

The monosaccharide composition of EPS was analysed qualitatively using high-performance liquid chromatography (HPLC-RID) on an Agilent Technologies 1100 series HPLC system (Boeblingen, Germany). The system was equipped with a refractive index detector (RID). The separation of EPS was carried out with a NH2 (ZORBAX 300, 5μM, 4.6 × 250 mm, USA) maintained at 30°C, mobile phase of 75% acetonitrile and eluted in isocratic mode at a flow rate of 1 mL/min for 15 minutes at 30°C. The injection volume was 10 ***μl*** for the standards and samples. For peak identification, the standard mixture containing glucose, mannose, arabinose, galactose, ribose and xylose were used. All the monosaccharide standards used were of highly purified analytical grade (≥99%).

Structural Identification of Exopolysaccharide Using FTIR

The functional groups in the EPS molecular structure were analysed using a PerkinElmer Spectrum One FT-IR spectrometer. The purified polysaccharides (1 mg) were grounded with KBr (Spectroscopic grade) powder and were then pressed into pellets using a mold for FT-IR. The measurements were carried out at a frequency range of 450 to 4,000 cm^{-1}.

Structural Identification of Exopolysaccharide Using NMR Spectroscopy

The structural identification of the exopolysaccharide produced by the *B.pseudocatenulatum* ATCC 27919 was mainly accomplished through NMR analysis. Deuterium oxide (D2O), aka "heavy water", was used as a solvent. This analysis was carried out through 1D and 2D NMR. For the 1D NMR, ^{1}H and ^{13}C experiments were applied, whereas the 2D NMR experiments include HMBC, HSQC and COSY. All spectrums were recorded at 47°C on BRUKER Ascend 600 Hz NMR machine Topspin software (powered by BURKER).

Statistical Analysis

All experiments were performed in triplicates, and the mean values are presented. Data were expressed as mean ± standard deviation by three biological replicates for each experimental data. The experimental data were processed using the MINITAB-14 statistical package (MINITAB Inc., PA, USA) to perform data analysis, experimental design matrix and optimisation procedure. One-way analysis of variance (ANOVA) was used for data analysis. Values of $P < 0.05$ represent statistical significance in terms of high correlation and fitting the predicted model with the experimental data. Values of P more than 0.05 indicate that the replicated experimental data were not significantly different, which is close to the predicted data and a low error percentage provide an indication of good performance prognosis of the optimal formulation.

Results and Discussion

Cytotoxic Activity of EPS Produced by B.pseudocatenulatum ATCC 27919 during Cultivation in Aloe Vera Medium

In this study, the cytotoxicity activity of EPS produced *by B. pseudocatenulatum* strain ATCC27919 cultivated in aloe vera medium against Caco-2 cells was examined. Glucose was supplemented into aloe vera medium prior to the *B. pseudocatenulatum* strain ATCC 27919 cultivation due to availability of only simple carbon sources in the medium, which provided more rapid support to cell growth. Rapid growth

of bacteria at optimum densities facilitates EPS production (Maria *et al.,* 2017). The extracted EPS (1 mg) was subsequently exposed to Caco-2 cells for 0, 20 and 40 hours, respectively. In this experiment, commercial (MRS) media was used as the comparative media. The dexamethasone drug was used as a positive control and Caco-2 cells alone were used as a negative control.

Based on the results, the viability of Caco-2 exposed to EPS from aloe vera medium was 82% after 20 hours of exposure (Figure 1). Cell viability was significantly reduced ($p<0.05$) to 65% after 40 hours of EPS exposure (Figure 1). Compared with EPS from MRS medium, the viability of the cells reduced significantly ($p< 0.05$) to 80% and 38% at 20 hours and 40 hours of exposure, respectively.

The reduction of Caco-2 cells viability to 65% after 40 hours of exposure to EPS produced by *B.pseudocatenulatum* ATCC 27919 in aloe vera medium indicate that EPS is able to act as an anticancer therapy and it is believed that prolonged exposure to EPS can lead to higher reduction of cancer cells. It has been reported that most of the cancer cells reached a 50% cell viability after 48 hours of EPS exposure, and exposure time of EPS to Caco-2 cells may affect the cytotoxic activity (Sevda *et al.,* 2015).

CHNS Elemental Analysis of EPS

From the elemental analysis, the C-N ratio of the EPS produced in aloe vera medium was 19.00, which is higher than the C-N ratio of EPS produced in MRS medium (Table 1). Based on the results of the elemental analysis, it can be observed that EPS is composed of mainly carbon, hydrogen, and nitrogen; of which carbon is the most abundant (29.08% in EPS produced in aloe vera medium compared with 41.34% in EPS produced in MRS medium) followed by sulphur. The carbon and hydrogen amount suggest the EPS may be a polymeric substance that is joined with many monosaccharides. Meanwhile, the presence of 1.53% of nitrogen in EPS produced in aloe vera medium indicates the presence of nitrogenous compounds in EPS,

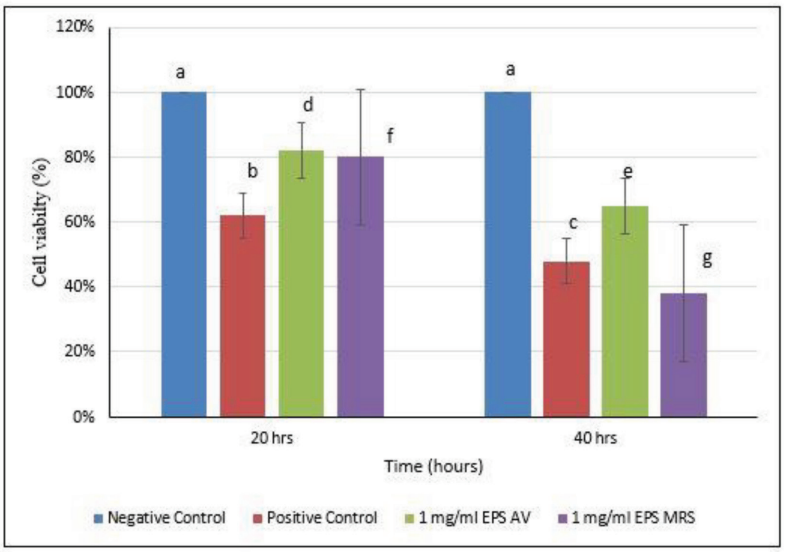

Figure 1: The viability of Caco-2 cells after 20 and 40 hours of treatment with Exopolysaccharides produced by *B. pseudocatenulatum* ATCC 27919 cultivated in aloe vera medium. MRS medium was used as the comparative medium for EPS production by *B. pseudocatenulatum* ATCC 27919. (Note: EPS-Exopolysaccharide, AV-Aloe vera, MRS-de man, Rogosa, Sharpe media. Positive control - dexamethasone, Negative control - Caco-2 cells alone). Data obtained was based on the average of the triplicate data. Symbol ± is the standard deviation of the data

Table 1: Elements present in the exopolysaccharide sample from aloe vera and commercial (MRS) media synthesised by *Bifidobacterium pseudocatenulatum* ATCC 27919 analysed using an elemental analyser

	Element (%)			
Sample Name	**Nitrogen**	**Carbon**	**Hydrogen**	**Sulphur**
EPS Aloe Vera	1.53	29.08	5.45	1.09
EPS MRS	12.60	41.34	6.21	0.73

which may be due to the presence of protein contaminants in the EPS. Microbes have used this nitrogen to produce free amino acids (Ismail & Nampoothiri, 2010; J. Liu *et al.,* 2009).

Monosaccharide Composition Analysis Using HPLC-RID

The monosaccharide composition analysis is used to determine the identities and quantities of the various monosaccharides in the carbohydrates and glycoproteins. The information can be used to analyse the structure of carbohydrates and play an important role in quantification. The composition of EPS was identified using HPLC-RID. Based on the results, the two main monosaccharides components detected were D-glucose and D-mannose. Additionally, fructose, arabinose, and N-acetylglucosamine were detected at different ratios (Table 2). The percentage of D-mannose and D-glucose were at 9.8% and 72%, respectively. It has been reported that the anticancer activity of EPS is related to mannose in EPS compositions (Vidhyalakshmi & Vallinachiyar, 2013). A study on the functional properties of EPS derived from yeast reported that EPS consisting of mannose at a percentage of more than 50% are classified as biologically active (Gientka *et al.,* 2015). Shao *et al.* (2004) showed that polysaccharides consisting of glucose and mannose can interact with toll-like receptors and activate host immunity. Vidhyalakshmi and Vallinachiyar (2013) reported that macrophages carry mannose- and glucose-specific receptors, which are important in triggering anti-cancer activities and suppressing cell proliferation in tumours.

Molecular Structure of EPS Using Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR uses the fact that a group of bonds vibrate in their own unique frequencies. It helps to identify the functional group and characterise covalent bonds in samples (Kanamarlapudi & Muddada, 2017). As shown in Figure 2, EPS from aloe vera medium indicated that the polysaccharides contain free hydroxy groups as it displayed broad and intense stretching absorption band of around 3,418 cm^{-1}. C-H antisymmetric starching vibration occurred in the 2,900 cm^{-1} region. The band at 1,049 cm^{-1} suggested C-O-C vibration is present in the structure. This was recommended as α-glycosidic bridge is present in the sample

Table 2: The monomer composition of the EPS produced by *B. pseudocatenulatum* ATCC 27919 cultivated in aloe vera medium

Monosaccharide's Composition (%)	**EPS by *B. pseudocatenulatum* ATCC 27919 during Cultivation in Aloe Vera Medium**
Glucose	9.8% ± 0.03
Mannose	72.1% ±
Fructose	3.2%±0.5
Arabinose	2.9%±0.35
N-acetylglucosamine	0.5%±1.3

ND: Not determined composition. The results presented in average

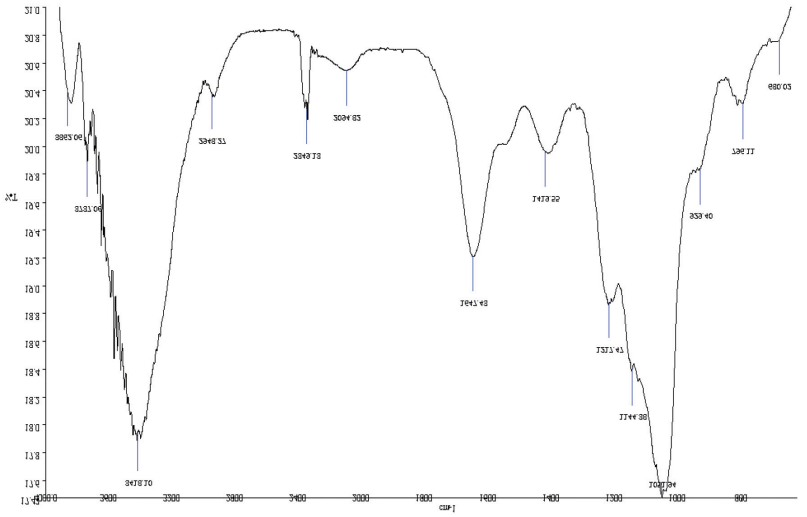

Figure 2: FT-IR spectra of the purified EPS from aloe vera medium synthesised by Bifidobacterium pseudocatenulatum ATCC 27919

(Ye *et al.,* 2009). The peak at 935 cm^{-1} provided evidence of glycosidic bond being present in the EPS from aloe vera medium. The broad peak at 1,031 cm^{-1} was relevant to the vibration of the cyclic C-O bond at the C-4 position of glucose residue. The band at 804 cm^{-1} also proved a pyran ring is present in the sample (Sheng *et al.,* 2007; Wei *et al.,* 2007).

Figure 3 of the EPS from the MRS media shows a similar absorption pattern with EPS from aloe vera medium. In this study, EPS from MRS medium contained a significant number of hydroxyl group as it displayed broad and intense stretching peaks at around 3,418 cm^{-1}. C-H starching vibration occurred in the 2,948 cm^{-1} region. This was an antisymmetric stretch. The band at 1,217 cm^{-1} suggested C-O-C vibration being present in the structure, and this was the reason for the α-glycosidic bridge. The peak at 935 cm^{-1} provided evidence of glycosidic bond being present in EPS from MRS medium. The broad peak at 1,051 cm^{-1} is proof of the vibration of C-O bond. This belongs to the C4 position of the carbon in the ring structure. The pyran ring is also confirmed by the peak at 769 cm^{-1} (Sheng *et al.,* 2007; Wei *et al.,* 2007).

Moreover, there was a band occurring at the 2,349 cm^{-1} region, which indicates nitrogen being present in the EPS from MRS media. This finding is in agreement with Ahluwalia and Goyal (2005). The EPS from aloe vera medium also have a small band in that region due to the nitrogen group. It also suggests nitrogen impurities mixed with commercial and aloe vera media. This nitrogen might constitute protein molecules in the sample. It was proven further by the CHNS data. However, the FT-IR spectra of EPS synthesised by *Bifidobacterium pseudocatenulatum* ATCC 27919 suggested α-1-6 and α-1-4 linkages present in the structure of the polysaccharide from aloe vera and MRS media.

It has been shown that molecular properties, including the type of linkages of the polysaccharides, strongly impact the interactions with proteins (Diemer *et al.,* 2012). Biological activities such as anti-tumour and apoptosis-inducing activity by polysaccharides are strongly associated with their structures. The variations in the ratio of monomers among *Bifidobacteria* spp. strains suggest that the compositional diversity of EPS isolated from *Bifidobacteria* spp. likely contributes strain-to-strain variation in their ability to inhibit proliferation and induce apoptosis.

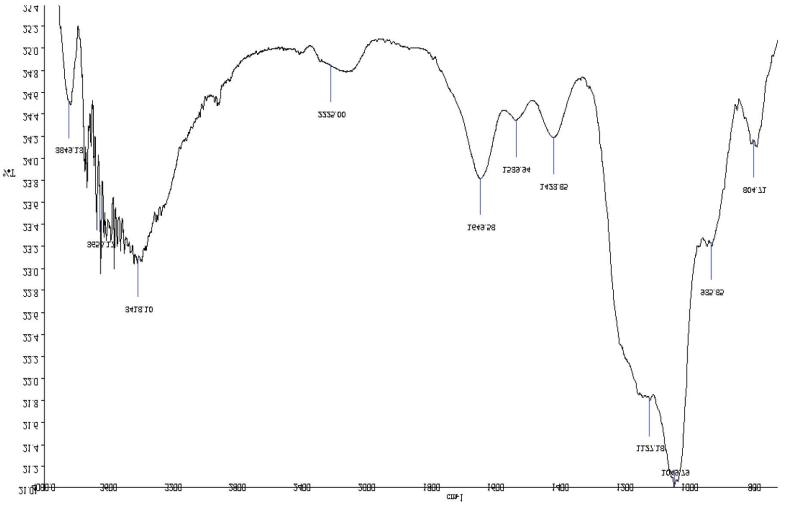

Figure 3: FT-IR spectra of the purified EPS from commercial medium (MRS) synthesised by *Bifidobacterium pseudocatenulatum* ATCC 27919

Nuclear Magnetic Resonance Data Analysis

NMR is fan efficient method to identify the structure, content purity, dynamics and interactions of biological macromolecules. It has been widely used in the fields of physics, chemistry, biology and medicine. The robust nature and specific detection of each element are displayed as a peak (Opella *et al.,* 2002). This analysis was carried out using the ^{1}H and ^{13}C NMR spectra for EPS from aloe vera and commercial media and compared with the literature value.

As seen in Table 3, the peaks in ^{1}H spectrum for EPS from aloe vera medium (Figure 4) are in the 3 ppm to 6 ppm range. Based on the range, it can be suggested the compound used was polysaccharide ^{1}H spectrum as EPS from aloe vera medium presented a peak in the 4.8-5.6 ppm region as compared with commercial medium (Figure 5), which was at between 5.54 and 5.60, and this suggest an α-glucose residue. This α-residue confirmed by peaks in the 102 ppm region in ^{13}C NMR, which proved that the EPS from commercial medium contained glycosidic bond with an α-configuration. There

Table 3: Assignments of 1^{H} and 13^{C} chemical shifts (ppm) for the spin systems in EPS extracted from *Bifidobacterium pseudocatenulatum* ATCC27919 using aloe vera and commercial (MRS) media determined by 1D experiments recorded at 600 MHz in D_2O at 47°C

NMR Data $^{1}H/^{13}C$ (ppm)	Aloe Vera Medium (^{1}H)	Commercial Medium (^{1}H)	Aloe Vera Medium (^{13}C)	Commercial Medium (^{13}C)
H-1/C-1	5.61	5.54	100.1	102.1
H-2/C-2	3.73	3.91	72.2	72.1
H-3/C-3	3.79	4.18	73.9	73.9
H-4/C-4	3.53	3.51	77.7	76.6
H-5/C-5	3.97	4.21	71.6	66.7
H-6/C-6	3.64	3.79	61.0	61.5
H-6'/C-6'	4.39	5.25	61.0	61.5

are some peaks in the 1 ppm to 3 ppm range, which may be due to impurity in the sample. The peaks occurred at 1.5 ppm and 2.2 ppm in ^{13}C NMR for EPS from commercial medium, which belong to CH_3 and O-(C=O)-CH_3.

Referring to the ^{13}C NMR spectrum, the peak range for EPS from commercial media is 60 ppm to 105 ppm (Figure 6). It represents the polysaccharide compound with glucose as a monomer (Dahech *et al.,* 2013). Based on this carbon profile, six main resonance shift are observed at δ61.5(C-6); δ66.7(C-5); δ76.6(C-4); δ72.1(C-2); δ73.9(C-3); δ102.1(C-1). An α anomeric carbon is present in the EPS from commercial media based on the peak between 95 ppm and 101 ppm region, which is 102.1 ppm in the ^{13}C NMR spectrum. There were no peaks between the 101 ppm and 105 ppm region in the ^{13}C NMR spectrum, which means that there are no β-carbon present in the EPS from commercial medium. The peaks belong to the C2-C4 carbon observed between the 70 ppm to 80 ppm region and the C6 carbon peak occurred at the 61 ppm region. It is the same NMR peak with dextran (Huang *et al*., 2011). Further purification is

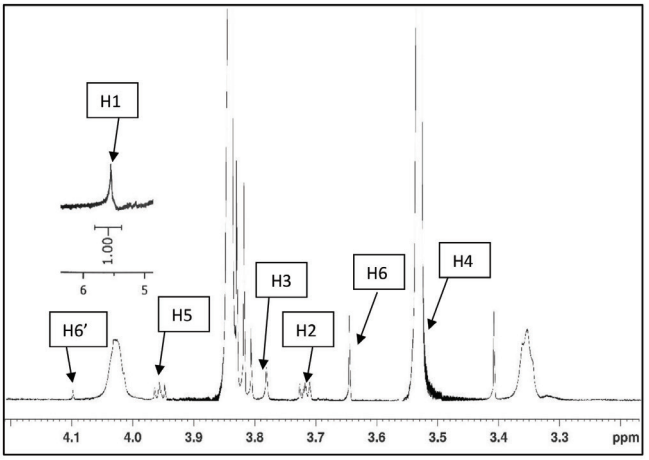

Figure 4: ^{1}H NMR spectra of the purified EPS extracted from *Bifidobacterium pseudocatenulatum* ATCC 27919 cultivated in aloe vera medium recorded at 600 MHz in D_2O at 47°C

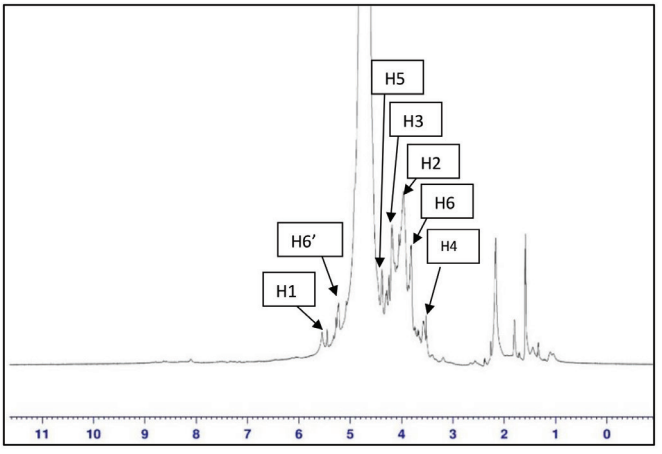

Figure 5: ^{1}H NMR spectra of the purified EPS extracted from *Bifidobacterium pseudocatenulatum* ATCC 27919 cultivated in commercial medium (MRS) recorded at 600 MHz in D_2O at 47°C

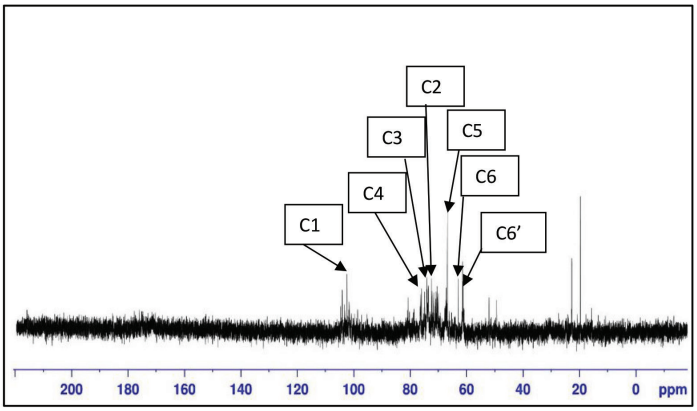

Figure 6: ^{13}C NMR spectra of the purified EPS extracted from *Bifidobacterium pseudocatenulatum* ATCC 27919 cultivated in commercial media (MRS) recorded at 600 MHz in D_2O at 47°C

needed for the EPS from both media to identify the structure better. However, the data from the FT-IR and HPLC analyses have confirmed the structure further.

Conclusion

In conclusion, the data obtained from the cytotoxic assay suggest that the EPS cultivated in aloe vera medium was able to kill Caco-2 cancer cells after 40 hours of exposure and was believed to have a better effect with prolonged exposure. This activity aligned with data obtained through CHNS and HPLC, which indicates that the cytotoxic activity by EPS from aloe vera medium was due to its mannose component, which has an impact on biological activities. Through FT-IR analysis, it was found that the molecular properties of EPS, including the type of linkages of the polysaccharides, lead to the strengthening of the structure. Moreover, the NMR peaks indicate that a α-glycosidic link is present in the EPS structure, which supports the cytotoxic activity against Caco-2 cells.

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