Biological Activity of Chemically Modified Levan Produced by Moderately Halophilic Chromohalobacter salexigens KT989777

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Introduction

Fructans are widely distributed as carbohydrate storage polymers in the vegetative tissue of many families of plants, bacteria and fungi (Hosono et al., 2003). According to the type of linkage, fructans are classified into three families, namely, inulin [(2-1)-linked β-D-fructofuranosyl units], levan [(2-6)-linked β-D-fructofuranosyl units], and graminan [both (2-1)-linked and 2-6]-linked β-D-fructofuranosyl units] (Roberfroid 2005). Levan is a homopolymer of fructose with many outstanding properties. Microbial levan is of commercial importance, which offers a variety of industrial applications in the fields of cosmetics, foods and pharmaceutic; it can be used as industrial gums, blood plasma extender, sweeteners, hypcholesterolemic agent and antitumor agent (Leibovici & Stark 1985; Yamamoto et al., 2000). Potential applications of levan have also been proposed as an emulsifier, formulation aid, stabilizer and thickener, surface-finishing agent, encapsulating agent, and carrier for flavor and fragrances (Han 1990; Jang et al., 2001). Production of levan from halophile microorganisms was reported by many researchers (Poli et al., 2009; Küçükkaşık et al., 2010). Natural polysaccharides derivatives studied recorded stronger antioxidant, or antitumor activities than their corresponding natural polysaccharides (Chen et al., 2009; Abdel-Fattah et al., 2012; Liu et al., 2012). Thus, polysaccharides chemical modifications provide an opportunity to develop new agents with possible therapeutic uses. Sulfated polysaccharides are polysaccharides containing high amounts of sulfate groups. They can be found in nature, but a lot of sulfated polysaccharides have been obtained by chemical modification.

Material and Methods

Microorganism

The strains was isolated from a hyper saline shallow bond near Suez canal –Al-Kantara Gharb- Esmailia-Egypt using enrichment technique in Sehgal and Gibbons complex medium (SGC medium) (Sehgal & Gibbons 1960) contains (g/L): casmino acids, 7.5, yeast extract, 10, starch, 5, KCl, 2.0, sodium citrate, 3.0 MgSO4.7H2O, 20, NaCl, 200, MnCl2.4H2O, 0.05 and FeCl3.nH2O. 0.01 The medium was adjusted to pH 7.0 by 0.5 M NaOH before autoclaving at 121°C for 15 min. For screening the isolates for EPS production SGC medium was supplemented with 50 g L-1 of sucrose. Isolates were identified on the basis of biochemical reaction and 16S rRNA.

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The DNA was extracted from bacterial cultures and 16S rDNA gene was isolated and amplified by SIGMA company for scientific services, Egypt. The PCR products were analyzed on 1% (w/v) agarose gels and sent to GATC (Germany) for sequencing using ABI 3730xl DNA sequencer. Sequence data were imported into the BioEdit version 5.0.9 sequence editor; base-calling was examined, and a contiguous sequence was obtained. The full sequence was aligned using the RDP Sequence Aligner program. Sequences used in the phylogenetic analysis were obtained from the RDP and Gen Bank databases. A dendrogram was constructed using the neighbor-joining method. Confidence in tree topology was determined.

Production of Exopolysaccharide (EPS)
Production medium of the following composition (g/L): sucrose, 30, yeast extract, 1, casmino acids, 0.75, (NH₄)₂SO₄, 1, tri sodium citrate, 3, KCl, 2, KH₂PO₄, 0.5 and MgSO₄,7H₂O, 20, NaCl 150, FeCl₃.nH₂O, 0.01 and MnCl₂.4H₂O, 0.05 (pH 8.0). 250 ml Erlenmeyer flask containing culture medium of 50 ml working volume was inoculated with 5 ml 24 old culture. The inoculated flasks were incubated on a rotary shaker at 200 rpm and 30 °C for 24 h. Cells were used thereafter to inoculate either 250 ml Erlenmeyer flasks with 5% (v/v) inoculum concentration and inoculum density of 1 OD 600 nm and incubated at 30 °C for 24 h then transferred to 4°C for 48 h to enhance EPS production.

Biofilm formation
The experiment was conducted according to (O’Toole and Kolter 1998) where the overnight cultures were diluted 1:100 into fresh media then 100 µl of the dilution were added to each well of microtitr plate and incubated for 24h under optimum temperature of each organism. After incubation cells were dumping out and the wells were washed gently by submerging in water.

Levan extraction and purification
The levan producing organisms were cultivated as described above then; culture was centrifuged at 5000g to remove cells. The culture filtrate was deproteinized by adding 40% (w/v) ammonium sulphate, the mixture incubated over night at 4°C then centrifuged at 5000g and dialyzed against deionized water for 48 h with dialysis membrane (Mr Cut off 104-12.103, diameter 60 mm) to remove unfermented sucrose, ammonium sulphate and any fermentation products with low molecular weight. The dialysate was frozen with liquid nitrogen and freeze dried to afford Lev- KT7. Total carbohydrate of the product was estimated by Dubois et al. (1956).

Chemical characterization of EPS
For sugar analysis, lyophilized samples (3 mg) were hydrolyzed using 0.1 N HCl in boiling water bath for 1 h. Hydrolysate was analyzed by descending spot test on paper-chromatography using Whatman No.1 and solvent system n-butanol: acetone:water (4:5:1, v/v/v) (Tanaka et al., 1978) and sprayed with aniline phthalate for identifying sugars in EPS samples (Block et al., 1955). The ATR-FTIR spectra from three samples (32 scans per sample, spectral resolution, 4 cm⁻¹; wave number range, 4000- 650 cm⁻¹ using a single reflection attenuated total reflectance (ATR) device (MIRacle, Pike Technologies, www.piketech.com) and a DLATGS detector) were recorded with a Bruker FT-IR spectrometer (Vertex 70). All samples used for infrared measurements were stored in a drying oven for three days at 50°C. Thereafter, they were stored in a desiccator overnight over silica gel before measurement. The average molecular weight (Mw) of the levan was measured by gel permeation chromatography (GPC Agilent 1100 series, Germany, Detector: Refractive Index. Deionized water was used as a solvent and column PL aquagel-OH 7.5 mm, 30µm & 50µm pore type, 8um particle size, in series. Sample 0.01 g was dissolved in 2ml of deionized water, and then filtrated by siring filter 0.45 um, the flow rate was set at 1.0mL/min and the column oven was at 50°C.

Modification of bacterial levan:
Carboxymethylation:
The carboxymethylation reaction was adopted according to the literature (Rahul et al., 2014) as follows: Levan-like polysaccharide of two studied bacterial strains (1.0 g, 6.17 mmol) was suspended in 20 ml isopropanol in a round bottom flask with constant stirring at room temperature. The resulting mixture was heated to 50 °C and purged with nitrogen for 1 h. Required amount of aqueous solution of sodium hydroxide was added to the above mixture to keep pH at 11 constant and was stirred for 30 min. Afterwards, the sodium chloroacetate (2.6 g, 22.3 mmol) was added in small batches under constant stirring and pH 11 and the reaction was continued for 4 h. The reaction mixture was cooled gradually and the excess alkali neutralized with dil. HCl bringing the pH to 7. The
solution was dialyzed against deionized water for 48 h with dialysis cellulose acetate membrane (M<sub>c</sub> cut off 10<sup>-3</sup>-13×10<sup>3</sup>, diameter 60 mm) to remove the salts. Dialysate was frozen with liquid nitrogen and freeze-dried to afford “CM-KT7”.

Sulphation

Levan-like polysaccharide was sulfated in dimethylformamide (DMF) according to literature Zhang et al., (2009) In brief, 1.0 g (6.17 mmol) of biopolymer was suspended in 20 ml anhydrous DMF at room temperature for 2 hrs. followed by addition of 3.5 g (22 mmol) SO<sub>3</sub>-pyridine complex in 10 ml DMF. The reaction mixture was maintained at 0 °C for 4 hrs. After the reaction, the mixture was adjusted to pH 7 by 1 M NaOH solution. The dialysate was dialyzed against deionized water for 48 h with dialysis cellulose acetate membrane (M<sub>c</sub> cut off 10<sup>-3</sup>-13×10<sup>3</sup>, diameter 60 mm). The dialysate was frozen with liquid nitrogen and freeze-dried to afford “SA-KT7”.

Biological activity of original and modified polymer.

Anti-tumor activity

Cell Culture

Human hepatocarcinoma cell line (Hep-G2), purchased from ATCC, USA, was used to evaluate the cytotoxic effect of levan produced by Chromohalobacter salexigens KT989777 and its sulphated and carboxymethylated derivatives. Cells were routinely cultured in DMEM (Dulbecco’s Modified Eagle’s Medium), which was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, containing 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulphate, and 250 ng/ml amphotericin B. Cells were maintained at sub-confluency at 37°C in humidified air containing 5% CO<sub>2</sub>. For sub-culturing, monolayer cells were harvested after trypsin/EDTA treatment at 37°C. Cells were used when confluence had reached 75%... All cell culture material was obtained from Cambrex BioScience (Copenhagen, Denmark). All chemicals were from Sigma/Aldrich, USA, except mentioned. All experiments were repeated three times, unless mentioned.

Anti-tumor activity measurement.

The MTT assay was carried out using a modification of the method of Mossman (1983). Cells (0.5X10<sup>4</sup> cells/ well), in serum-free media, were plated in a flat bottom 96-well microplate, and treated with different volumes of the tested samples to have concentration gradient (v/v) beginning with 100 then 50 then 25 and finally 12.5 µl ml<sup>-1</sup> for 48 h at 37º C, in a humidified 5% CO<sub>2</sub> atmosphere. After incubation, media were removed and 40 µl MTT solution / well were added and incubated for an additional 4 h. MTT crystals were solubilized by adding 180 µl of acidified isopropanol / well and plate was shacked at room temperature, followed by photometric determination of the absorbance at 570 nm using microplate ELISA reader. Triplicate repeats were performed for each concentration and the average was calculated. Data were expressed as the percentage of relative viability compared with the untreated cells compared with the vehicle control, with cytotoxicity indicated by <100% relative viability.

Calculation

Percentage of relative viability was calculated using the following equation:

\[ \text{Percentage of relative viability} = \left( \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \right) \times 100 \]

Then the half maximal inhibitory concentration (IC<sub>50</sub>) was calculated from the equation of the dose response curve.

Evaluation of fibrinolytic activity

Fibrinolytic activity of original levan and sulphated levan was performed as described by Helmy et al. (2007) Sets of 328 three-hard glass test tubes (31 mm× 100 mm) were cleaned by 329 immersions overnight in chromic acid. To each tube 0.8 ml of 0.9% saline solution, 1 ml plasma and 0.2 ml of 2% calcium chloride solution were added. After mixing, the tubes were placed in water bath at 37 °C and when clotting was complete, 2000 Ug of heamoclar or tested samples was added individually. After 30 minute of incubation at 37 °C. Lyses percentage of plasma clots was recorded by using measuring cylinder.

Determination of prebiotic activity

Prebiotic activity assay was proceeded according to Anprung &Sangthawan (2012). Lactobacillus lactis MCAIN B 01357 and Escherichia coli were used in this study. L. lactis was prepared by streaking onto MRS agar and streaking onto Tryptic soy agar (TSA) for E. coli. Then, incubated at 37°C for 24-48 h under aerobic condition. After that, one colony from each plate was transferred into 10 ml of MRS broth for L. lactis and Tryptic soy broth (TSB) for E. coli and incubated overnight. For L. lactis, an additional transfer of 1% (v/v) was transferred to MRS broth with 1% w/v glucose or 1% w/v samples. For E. coli, an additional transfer of 1% (v/v) was transferred to minimal medium broth with 1% w/v glucose or 1% w/v samples and incubated overnight.

The prebiotic activity score was determined using the following equation: Prebiotic activity score =
Data obtained from experiment were subjected to analysis of variance and treatments means were compared using the L.S.D method.

**Results**

**Microorganism**

Depending on the slimy appearance on solid SGC medium, two morphologically different isolates were selected as the most potent EPS producers. Biochemical (Table 1) and molecular identification proved that the two isolates were related to the genus *Chromohalobacter* sp with different identity; in addition further differentiation based on different susceptibility to antibiotics was also assayed, they have the accession numbers according to NCBI *Chromohalobacter saleigens* KT989776 and *Chromohalobacter saleigens* KT989777, and the later was used to complete this study.

**Table 1: Morphological and biochemical characters of the most potent EPS producer.**

<table>
<thead>
<tr>
<th>Character</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram stain</td>
<td>Negative</td>
</tr>
<tr>
<td>Spore formation</td>
<td>Negative</td>
</tr>
<tr>
<td>Cell shape</td>
<td>Single rods.</td>
</tr>
<tr>
<td>Colony morphology</td>
<td>Opaque, circular and about 1.5 ml in diameter.</td>
</tr>
<tr>
<td>Relation to oxygen</td>
<td>Strictly aerobic.</td>
</tr>
<tr>
<td>Catalase</td>
<td>Positive</td>
</tr>
<tr>
<td>Casein hydrolysis</td>
<td>Positive</td>
</tr>
<tr>
<td>Optimum NaCl concentration</td>
<td>5-10%</td>
</tr>
<tr>
<td>Anaerobic growth with nitrate</td>
<td>Positive</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>Positive</td>
</tr>
<tr>
<td>Anaerobic growth in presence of nitrate</td>
<td>Positive</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>Positive</td>
</tr>
<tr>
<td>Glucose</td>
<td>Positive</td>
</tr>
<tr>
<td>Growth on sucrose</td>
<td>Positive</td>
</tr>
</tbody>
</table>

**EPS production**

Levan production was carried out in shake flask culture under optimum conditions as described previously, where 10.2 g L\(^{-1}\) was produced.

**Biofilm formation**

One of the most important features of levan is its high biofilm forming ability so this feature was assayed to confirm the formation of levan. The result obtained from this experiment proved that *Chromohalobacter saleigens* KT989777 exhibit biofilm formation ability and it was determined quantitatively Where the absorption of crystal violet at 550 nm recorded (0.78) using acetic acid as blank.

**Chemical characterization of EPS**

**Paper chromatography**

Hydrolyzed EPS presented a spot attributable to fructose on paper-chromatography using Whatman No.1 and solvent system n-butanol: acetone:water (4:5:1, v/v/v) . Total carbohydrate of the deprotenized EPS was 90 %.

**Fourier Transform Infra-Red (FTIR) Spectroscopy**

In these spectra (Fig.1) the strong bands around 3,300 cm\(^{-1}\) were assigned to the hydroxyl (OH) stretching vibration of the polysaccharides, and the two bands existing around 2,950 and 2,910 cm\(^{-1}\) due to C-H stretching vibration of CH\(_2\) and CH groups, respectively which indicate the existence of fructose residue (Liu *et al.*, 2010). The bands in the region of 1,430 and 1,200 cm\(^{-1}\) were assigned to C-H plane deformation vibration combined with aromatic skeletal vibrations (Schwaninger *et al.*, 2004; Liu *et al.*, 2010). The bands at 1,020 cm\(^{-1}\) was dominated by the stretching vibrations of the glycosidic linkage contributions of C–O–C and C–O–H (Wu *et al.*, 2009). Characteristic absorption at 930 cm\(^{-1}\) and 820 cm\(^{-1}\) was also observed, indicating the presence of the furanoid ring.
of the sugar units. (Schwanninger et al., 2004). This results in addition to the result of paper chromatography and the high level of resemblance with the FT-IR observed in the other studies (Poli et al., 2009; Küçükaşik et al., 2011) suggested that the polysaccharide polymer produced in this study were levan-type polysaccharides.

![Fig 1: FT-IR spectrum of Lev-KT7 produced by Chromohalobacter salexigens KT989777.](image1)

Molecular mass determination

The Gel-Permeation chromatograms (GPC) of levan product from Chromohalobacter salexigens KT989777 showed two peaks, one has molecular weight greater than 114000 Da and represent the majority of the sample while the other has molecular weight around 720 Da.

Polymer modification

Preparation and characterization of carboxymethylated levan (CM-KT7)

Carboxymethylated levan (CM-KT7) have been synthesized by derivatization of bacterial expolysaccharides using carboxymethylation. In order to reduce the percentage of byproduct formed, the reaction was carried out in an oxygen-free inert atmosphere. The reaction follows Williamson’s etherification protocol and in the first step, in the presence of a base, alcoholic functionality is converted to an alkoxide. The base performs the dual role of a reactant as well as a catalyst and was taken in slight excess. The generated adduct, undergoes nucleophilic substitution with sodium monochloroacetic acid to give the carboxymethyl derivative.

![Fig. 2: FT-IR spectrum of carboxymethylated levan (CM-KT7) produced by Chromohalobacter sp.TA5 compared with Lev-Ta5](image2)

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The reaction temperature was maintained at 50°C. Although at relatively high temperature the reaction was faster, the chances of the formation of the by product (hydroxyl acid) increased. Evidence of synthesis of two Carboxymethyl bacterial polysaccharide (CM-KT7) from isolated polysaccharides can be explained by FTIR spectroscopy. As evident from (Fig. 2), spectrum of CM-KT7 has a broad peak centered at 3300 cm\(^{-1}\) from the O-H stretching of associated glucose and fructose units in the polysaccharide backbone. Two bands around 2950 cm\(^{-1}/2910\) cm\(^{-1}\) correspond to C-H stretch and peak at 1020 cm\(^{-1}\) can be assigned to glycosidic bond. The bands at 930 cm\(^{-1}\) and 820 cm\(^{-1}\) correspond to furanose ring. Apart from these, there are two additional characteristic peaks at 1590 and 1420 correspond to antisymmetric and symmetric stretching for carboxylate group, respectively. Also there are two peaks assigned at 1590 cm\(^{-1}\) and 1420 cm\(^{-1}\) respectively. The above peaks can be attributed to antisymmetric and symmetric stretching modes of the carboxylate group (COO\(^{-}\)) and are a strong proof for the addition of carboxymethyl groups to Lev-KT7 polysaccharides. Band corresponding to the polysaccharides skeleton, including the vibration of the glycosidic bonds, C-O-C at 1020 and absorption at 930 cm\(^{-1}\) and 820 cm\(^{-1}\) confirmed that the furanoid ring backbone of the synthesized derivative is still intact.

Preparation and characterization of sulphated levan (SA-KT7):

Chemical sulfation of polysaccharides is one of the most utilized procedures to produce heparin-like polymers. This type of chemical modification is reproducible and allows the employing of high amounts of sulphated polysaccharides. The studies with regard to the mechanism of sulfation reaction using pyridine\(\cdot\)SO\(_3\) complex had showed that the controlling transition state has only weak N–S and O–S bonds and is symmetrical. Sulfate group transfer between nitrogen of SO\(_3\)·Pyridine complex and oxygen is consistent with a concerted ‘in line’ sulfate group transfer or an open ‘exploded’ transition state (Hopkins et al., 1983). Thus the strong nucleophilicity of the oxygen atoms in the polysaccharides was necessary for high degree of sulfation. The goal of this study was to synthesize sulphated and carboxymethylated fructose-rich polysaccharides to evaluated their biological activities in vitro.

![FT-IR spectrum of sulphated levan SA-KT7 compared with Lev-KT7](image)

Fig. 3: FT-IR spectrum of sulphated levan SA-KT7 compared with Lev-KT7

The FT-IR spectrum of Lev-KT7 showed a broad stretching intense characteristic peak at around 3300 cm\(^{-1}\) for OH and a narrow stretching characteristic peak at around 2950 cm\(^{-1}\) for C-H (Fig.1). The peaks of 1134 and 1031 cm\(^{-1}\) were referred to C-O stretching vibration of ring ether C-O-C and O-H variable angle vibration of C-O-H, respectively. In comparison with the spectrum of SA-KT7 (Fig. 3), two new intense absorption peaks were observed at 1260 cm\(^{-1}\) and 817 cm\(^{-1}\) attributed to asymmetrical S=O stretching vibration and symmetrical C-O-S vibration, respectively. These above results are in agreement with other sulphated polysaccharides (Yang et al., 2002; Sun et al., 2009; Wei et al., 2012). Moreover, the absorption peak at 2933 cm\(^{-1}\) of C-H stretching vibration became weaker and moved to 2967 cm\(^{-1}\), suggested the sulfate substitution may occur on the position of C-6. The variable angle vibration absorption peak of 1089 cm\(^{-1}\) decreased significantly, which could be the result of hydroxyl group replaced partly by sulfate groups (Sudipta et al., 2012).

Biological activity (anti-tumor activity) of levan KT7 and its derivatives.

Anti-tumor activity of levan KT7 and its derivatives.

Using MTT assay, the effect of the samples on the proliferation of Hep-G2 cells were studied after 48 h of incubation. As shown in the figure (3A,3B &3C) the treatment of Hep-G2 cells with the samples Lev-KT7 and CM-KT7 did not show any cytotoxic effect against Hep-G2 cells as concluded from their high IC\(_{50}\) values equals to 1450 &1365.7 µg/ml. On the other hand their sulphated analogues SA-KT7 showed cytotoxic effect on the cells; their calculated IC\(_{50}\) values equals to 504.1 µg/ml. (Fig 4a, b &c).

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Fig 4: Cytotoxic effect against Hep-G2 cells using MTT assay (n=4), data expressed as the mean value of cell viability (% of control) ± S.E for (a) Lev-KT7, (b) CM-KT7, (c) SA-KT7.

Fibrinolytic activity analysis of levan and sulphated levan.

In vitro fibrinolytic activity analysis of Lev-KT7 and SA-KT7 was carried out and compared with standard fibrinolytic compound heamoclar (pentosan sulfuric polyester, product of ClinMidy, Paris). The results showed that the Lev-KT7 sulph. exhibited 64% fibrinolytic activity while standard heamoclar "pentosan sulfuric polyester recorded (57%) by the same amount (Fig. 5). The findings presented in this study demonstrated the capacity of lev-KT7 and its sulphated derivative to liquefy rapidly clotted fibrin of normal human plasma. This result introduced new approach in levan applications. Fibrinolytic activity of polysaccharides was reported in many studies (Al-Nahas et al., 2011 & Esawy et al., 2013).
Fig 5: Fibrinolytic activity analysis of lev-T5 and its sulphated derivative SA-KT7 compared with heamoclar.

**Prebiotic activity score**

Lactobacillus strain was chosen to test the prebiotic activity score because it is used in dairy foods and has good potential probiotic properties. From the determination of prebiotic activity of Lev-KT7 compared with its carboxymethylated derivative CM-KT7, the amount of *L. lactis* grown on media with CM-KT7 was higher than ones grown on media with Lev-KT7 and media with glucose. On the other hand, the amount of *E.coli* (representative of Enteric bacteria) grown on glucose (no prebiotic) was higher than other media with prebiotic. It was found that the highest prebiotic activity scores were *L. lactis* grown on media with CM-KT7.

Table 2: Probiotic activity of lev-KT7 and its carboxymethylated derivative CM-KT7.

<table>
<thead>
<tr>
<th>Bacterial culture</th>
<th>Cell density [log10(cfu/ml)]</th>
<th>Lev-KT7</th>
<th>CM-KT7</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. lactis</em></td>
<td>2.22 ±0.02 a</td>
<td>2.35 ±0.04 *</td>
<td>2.2 ±0.04 b</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>1.61 ±0.02 b</td>
<td>1.63 ±0.02 *</td>
<td>2.33 ±0.01 *</td>
<td></td>
</tr>
</tbody>
</table>

F Test *  
L.S.D 0.01 0.073

**Discussion**

Levan, primary found as microbial exopolysaccharide (EPS), is a beta-(2,6)-linked fructose homopolymer with beta-(2,1)-linked side chains, chemical modifications of polysaccharides provide an opportunity to develop new agents with possible therapeutic uses. There have been many reports on the bioactivities of polysaccharide derivatives (Yuan et al. 2005; Wang et al. 2009; Xu et al., 2009; Zhang et al., 2009). Levan derivatives consider a promising antioxidant and antitumor agents (Liu et al., 2012). The studies with regard to the mechanism of sulfation reaction using pyridine.SO$_3$ complex had showed that the controlling transition state has only weak N–S and O–S bonds and is symmetrical. Sulfate group transfer between nitrogen of SO$_3$·Py complex and oxygen is consistent with a concerted ‘in line’ sulfate group transfer or an open ‘exploded’ transition state (Hopkins et al., 1983). Thus the strong nucleophilicity of the oxygen atoms in the polysaccharides was necessary for high degree of sulfation. The goal of this study was to synthesize sulphated and carboxymethylated fructose-rich polysaccharides to evaluated their biological activities in vitro. The result of IR spectroscopy are in agreement with other sulphated polysaccharides (Yang Du and Huang 2002; Sun et al., 2009; Wei et al., 2012). Moreover, the absorption peak at 2933 cm$^{-1}$ of C H stretching vibration became weaker and moved to 2967 cm$^{-1}$, suggested the sulfate substitution may occur on the position of C-6. The variable angle vibration absorption peak of 1089 cm$^{-1}$ decreased significantly, which could be the result of hydroxyl group replaced partly by sulfate groups (Sudipta et al., 2012).

**Anti-tumor activity**

It has been reported that the addition of electron-donating substituents such as phosphated and sulphated groups could increase radical scavenging activity as a result of increased electron density on the heterocyclic ring of carbons (Tsiapali et al., 2001). Chemical sulfation of polysaccharides is one of the most utilized procedures to produce heparin-like polymers. This type of chemical modification is reproducible and allows the employing of...
high amounts of sulphated polysaccharides. Sulfated polysaccharides have been shown to possess immunomodulatory activities that may be of potential application in stimulating the immune response or in controlling immune cell activity to mitigate associated negative effects such as inflammation (Chen et al., 2008). Sulfated polysaccharides may affect multiple targets in the immune and inflammatory systems that can have impact on disease progression and outcome including tumor progression and metastasis (Groth et al., 2009).

**Fibrinolytic activity**

Heparin is highly sulphated, linear polysaccharide that is derived from animal tissues and is widely used as clinical anticoagulant (Toshihiko et al., 2003). As a result of the multiplicity of biological activities associated with heparin and its importance as a major pharmaceutical, substantial research effort has been dedicated to the discovery of heparin analogues. Heparinoids, defined as heparin analogues are usually obtained from animal, plant and synthetic sources. Heparinoids mimic biological functions of heparin by interacting with heparin-binding proteins. The interaction of heparin-binding proteins with heparinoids usually involves both ionic and hydrogen bonding interaction. Number of investigations suggests more than one mechanism of action including direct and indirect inhibition of thrombin through the activation of thrombin inhibitors (e.g antithrombin and heparin cofactor II) (Grauffel et al., 1989; Mauray et al., 1995; Pereira et al., 1999).

**Prebiotic activity**

Prebiotics are non-digestible food ingredients that stimulate the growth or activity of the beneficial to human health bacteria in the digestive system. Generally, the prebiotic should increase the number of potentially probiotic lactic acid bacteria if they could convert it. A fructan have beta (2→1) fructosylfructose glycosidic bonds, which gives levans its unique structural and physiological properties, allowing it to resist enzymatic hydrolysis by human salivary and small intestinal digestive enzymes. The molecular mass of levans produced in this study make it classified as fructooligosaccharides so it can be accepted as a prebiotic because it meets the following three criteria: 1. it resists gastric acidity, hydrolysis by mammalian enzymes, and intestinal absorption, 2. it is fermented by the intestinal microflora and 3. It selectively stimulates the growth of large intestinal bacteria associated with health and well-being (Benefits and Applications 2009)

**Conclusion**

Levan produced from a moderately halophilic bacterium *Chromohalobacter* salexigens KT989777 was modified into two derivatives, sulphated and carboxymethylated levan. Modification process was confirmed by Fourier Transform Infra-Red (FTIR) and resulted in increase of biological activity of the original polymer, where sulphated levan was a promising anti-tumor and fibrinolytic agent. On the other hand carboxymethylated levan gave a highest prebiotic activity compared with the original levan.

**References**


