



MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES
IN PURE AND APPLIED SCIENCES



BIOLOGICAL ACTIVITIES OF LEVAN BY
***HALOMONAS* sp.**

HANDE KAZAK SARILMIŞER

Ph.D. THESIS

Department of Bioengineering

Thesis Supervisor

Prof. Ebru TOKSOY ÖNER

ISTANBUL, 2016



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Hande KAZAK SARILMIŞER, a Doctor of Philosophy student of Marmara University Institute for Graduate Studies in Pure and Applied Sciences, defended her thesis entitled as "BIOLOGICAL ACTIVITIES OF LEVAN BY *HALOMONAS* sp.", on February 10, 2016 and has been found to be satisfactory by the jury members.

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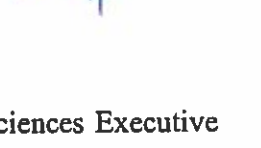
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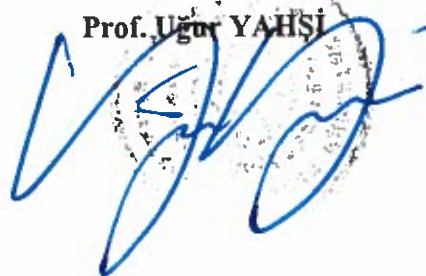
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APPROVAL

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HANDE KAZAK SARILMIŞER



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ÖZET

HALOMONAS sp. 'DEN ÜRETİLEN LEVANIN BİYOLOJİK AKTİVİTELERİ

Levan birçok bitkide bulunan ve mikroorganizmalarca üretilen fruktan tipi bir biyoaktif polimerdir. Suda çözünen, güçlü bir yapışkan ve film oluşturuucu biyopolimer olan levan polisakkariti, düşük viskozite, yağda yüksek çözünürlük, tuzlarla ve surfaktanlarla uyumluluk, sıcak, asidik ve bazik ortamlara dayanıklılık, su ve kimyasalları tutma kapasitesinin yüksekliği ve biyoyumluluk gibi değerli birçok özelliği ile diğer polisakkaritlerden ayrılmaktadır. Levan ve medikal endüstrideki potansiyel uygulama alanlarına olan ilginin giderek artması göz önünde bulundurularak ilk defa bu çalışma ile *Halomonas smyrnensis* AAD6^T tarafından üretilen lineer levanın biyolojik aktiviteleri incelenmiştir. İlk olarak pankreatik β -hücrelerinde ve yüksek şeker ortamında, CM-H₂DCFDA ve kaspaz 3/7 olmak üzere hücre bazlı iki farklı yöntem kullanılarak antioksidan aktivitesi çalışılmıştır. Sonuçlar doğrultusunda yüksek şeker konsantrasyonu şartlarında artan apoptoz değerlerini levanın düşürüp oksidatif stresi azalttığı ve antioksidan aktivitesi gösterdiği bulunmuştur. Aynı zamanda, levan polimeri oksitlenerek farklı oksidasyon derecelerine sahip aldehit grubu içeren türevleri oluşturulmuştur. Bu polimerin ve oksitlenmiş türevlerinin A549 (akciğer kanseri), HepG2/C3A (karaciğer kanseri), AGS (mide kanseri) ve MCF-7 (meme kanseri) gibi farklı kanser hücre hatlarında anti-kanser aktivitesi, sağlıklı fare fibroblast hücrelerinde (L929) sitotoksitesi ve RAW 264.7 makrofaj tipi hücrelerde ise immün düzenleyici aktivitesi incelenmiştir. Anti-kanser aktivite WST-1 hücre proliferasyonu analizi ile, kaspaz 3/7 aktivitesi ApoTox-Glo üçlü analiz yöntemi ile, immün düzenleyici aktivite ise Griess reaksiyonuna bağlı kolorimetrik yöntem ile değerlendirilmiştir. Sonuçlar doğrultusunda, örneklerin hiç birisi L929 hücrelerinin canlılığını olumsuz etkilememiştir. En yüksek oksidasyon derecesine sahip levan türevi tüm kanser hatlarına karşı en yüksek anti-kanser aktiviteyi göstermiştir. Herhangibir modifikasyona uğramamış levan makrofaj hücrelerine toksik etki göstermeyip NO oluşumunu stimule etmiştir. Sonuçlar levan türevlerinin gelecek vaadeden bir antioksidan, anti-kanser ve immune sistemi düzenleyici ajan olabileceğini öne sürmektedir. Yapılan çalışmalar, levan ve türevlerinin medikal uygulamaları açısından büyük önem taşımaktadır.

Ocak, 2016

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ABSTRACT

BIOLOGICAL ACTIVITIES OF LEVAN BY *HALOMONAS* sp.

Levan is a bioactive fructan type polymer found in many plants and produced by many microorganisms. As a water soluble, strongly adhesive and film-forming biopolymer, levan is distinguished from other polysaccharides by its valuable properties like low viscosity, high solubility in oil, compatibility with salts and surfactants, stability to heat, acid, and alkali media, high holding capacity for water and chemicals, and good biocompatibility. Considering the ever increasing interest in levan and its potential applications in medical industry, for the first time with the present study, biological activities of linear levan from *Halomonas smyrnensis* AAD6^T were evaluated. First, antioxidant activity by using two different cell-based methods; CM-H₂DCFDA assay and caspase 3/7 assay, was investigated under high glucose condition in pancreatic β -cell line. Levan was found to attenuate oxidative stress that is associated with decreased apoptosis in high glucose condition suggesting the antioxidant role of this polymer. On the other hand, levan was subjected to oxidation, producing the aldehyde derivatives with different degree of substitution. Anti-cancer activity of this polymer and its aldehyde activated derivatives were investigated in different cancer cell lines; such as A549 (human lung adenocarcinoma), HepG2/C3A (human liver hepatocellular carcinoma), AGS (human gastric adenocarcinoma), and MCF-7 (human breast adenocarcinoma), while demonstrating their cytotoxicity in the mouse fibroblast cell line L929 and immunostimulating activity in macrophage cell line RAW 264.7. The WST-1 assay was performed for evaluating anticancer activity of levan samples, ApoTox-Glo triplex assay was performed for detecting caspase 3/7 activity, and colorimetric assay based on the Griess reaction was performed for measuring NO production related to immunostimulating activity. As a result, samples didn't show cytotoxicity in L929 cell line. Oxidized form of levan with the highest oxidation degree showed the highest anticancer activity in all cancer cell lines. Pure levan showed no toxicity on macrophages while it increased production of NO in RAW264.7 cells. The results suggested that levan and its derivatives could be explored as promising antioxidant, anticancer, and immunostimulating agents. These studies hold great importance for the medicinal applications of levan and its chemical derivatives.

January, 2016

Hande

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CLAIM OF ORIGINALITY

BIOLOGICAL ACTIVITIES OF LEVAN BY *HALOMONAS* sp.

There is an extensive interest in characterizing polysaccharides that show biological activity both in healthy and pathologic conditions. It is well known that the molecular structure of polysaccharides is a very important issue in their biological activity since the bioavailability may be associated with structural patterns. The biological response is closely related to molecular weight, chemical structures, glycosidic bonds, backbone length, polymerization, degrees of branching, and 3D conformation. Detailed characterization of polysaccharides is critical for a better understanding of the structural and functional properties for their future nutritional and therapeutic applications. Levan-type fructan polymers are bioactive polysaccharides that are mainly associated with high-value applications. The ever-growing interest in these polysaccharides encouraged this work to investigate biological activities, such as antioxidant activity, anti-cancer activity, and immunostimulating activity, of linear levan by the producer halophilic strain *H. smyrnensis* AAD6^T, which is the first extremophilic source for this polymer. Moreover, in polysaccharide chemistry, there is a continuous interest in chemical modification of polysaccharides since it can improve their intrinsic biological properties and/or create new functional properties. For this purpose, levan from *H. smyrnensis* AAD6^T was subjected to oxidation, producing the aldehyde derivatives with different degree of substitution. Then, their biological activities were investigated as a function of the degree of oxidation and compared with those of the native one. *In vitro* anti-cancer activity of linear levan polysaccharide was greatly improved by introducing aldehydes in its structure. This effect became more apparent with the increasing degree of oxidation. The results of the present study suggested that levan derivatives could be explored as promising anti-cancer agents due to their high anti-cancer activity together with improved caspase 3/7 activity and low cytotoxicity to normal cells. This study will have high impact on research focused on biological activities of polysaccharides and additionally, it will play an important role in developing polysaccharide-based nanocarrier systems for anti-cancer drugs.

January, 2016 Prof. Ebru TOKSOY ÖNER Hande KAZAK SARILMIŞER

SYMBOLS

g	: gram
h	: hour
L	: liter
M	: Molar
min	: minute
v/v	: Volume per volume
w/v	: Weight per volume
μL	: Microliters
mL	: Milliliters
μg	: Micrograms
mg	: Milligrams
mM	: millimolar
rpm	: Round per minute
nm	: Nanometers
μm	: Micrometers
M_w	: weight average molecular weight
M_n	: number average molecular weight

ABBREVIATIONS

EPS	: Exopolysaccharide
P	: Phosphate
DP	: Degree of Polymerization
MW	: Molecular Weight
BSA	: Bovine Serum Albumin
LDW	: Laser Direct Writing
OD	: Optical density
HPLC	: High performance liquid chromatography
FT-IR	: Fourier transform-infrared
NMR	: Nuclear magnetic resonance
FADD	: Fas-associated death domain protein
CASPASE	: Cysteine aspartic acid specific proteases
MAPLE	: Matrix-assisted pulsed laser evaporation
PEO	: Polyethylene oxide
NO	: Nitric oxide
ANOVA	: Analysis of variance
PBS	: Phosphate Buffered Saline
ROS	: Reactive Oxygen Species
DMSO	: Dimethyl Sulfoxide
DMEM	: Dulbecco's Modified Eagle's Medium
FBS	: Fetal Bovine Serum
CM-H₂DCFDA	: 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate

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1. INTRODUCTION

1.1. General Background

1.1.1. Biopolymers

Biopolymers are polymers formed under natural conditions during the growth cycles of all organisms. Therefore, they are also known as natural polymers. They are formed within cells by complex metabolic processes as proteins, nucleic acids, and carbohydrates. Nucleic acids are responsible for storage, retrieval and copying of the building plan of a cell (genotype), proteins for function and metabolism (phenotype), while carbohydrates that decorate membranes and proteins play a dominant role in cellular signaling and cell–cell recognition events (Dawson and Leumann, 2004; Rao et al., 2014). There are different ways to produce biopolymers for different applications. Many biopolymers occur abundantly in nature and are isolated from natural environments such as plants and algae. Some biopolymers are synthesised *in vitro* with isolated enzymes in cell-free systems. Some others are produced by fermentation, e. g. polysaccharides. The biotechnological production of biopolymers may either occur intracellularly or extracellularly. The intracellular biopolymers are few and have very limited use. Glycogen, starch, cyanophycin, polyphosphate, and polyhydroxyalkanoates are examples of biopolymers, which are produced intracellularly in the cytoplasm of cells. The availability of space in the cytoplasm therefore limits the amount of polymer produced by a cell. Poly (β -D-glutamate) and many polysaccharides, such as chitosan, xanthan, alginates, dextran, pullulan, curdlan, and microbial cellulose are examples of biopolymers, which are produced as a result of extracellular synthesis or of excretion by the cells. For these biopolymers, breaking of cells is not required and separation of the biopolymer from the biomass is not very complex (Nwodo et al., 2012; Rao et al., 2014). Biopolymers have received considerable research attention over recent years since they are superior to petrochemical-derived polymers in several aspects that embrace biocompatibility and biodegradability. Biopolymers have potential applications in various fields of the economy such as food industry, packaging industry, chemical industry, agriculture and medicine with their both environmental and human compatibility. These naturally occurring polymers have many uses as adhesives, absorbents, lubricants, soil conditioners, cosmetic, drug delivery vehicles, textiles, high-strength materials, implantable biomaterials, controlled disease carriers or scaffolds for tissue engineering (Guo and Murphy, 2012; Ige et al., 2012). However, medical applications have been developed more

rapidly than others since high specialization and greater unit values of biopolymers. They have great potential use in a variety of diseases. For many years, pharmaceutical research focused mainly on the synthesis or discovery of new drugs. The use of biopolymers for drug delivery can minimize tissue reaction and allow drugs to be administered in nonconventional ways. Drug formulations including polymer drug delivery systems are currently undergoing registration and are likely to make an impact on the market in near future. The major area of application for these novel sustained release systems is in the treatment of cancers. Therefore, current research is focused on producing new biopolymers since there is an increasing attention (Rao et al., 2014).

1.1.2. Exopolysaccharides (EPSs)

Carbohydrates which are based on a ring unit containing 4 or 5 carbons and one oxygen are the most common type of biopolymers and they vary in size, from one to several thousand of these units. So they can be classified according to their molecular size or degree of polymerization (number of monosaccharide units combined), into monosaccharides, oligosaccharides or polysaccharides. Monosaccharides are the smallest with the general formula $C_nH_{2n}O_n$. According to IUBIUPAC nomenclature, oligosaccharides are defined as saccharides containing between 3 and 10 sugar moieties. Other authorities classify saccharides including anyone from 3 to 19 monosaccharide units in this group. However, there is not a rational physiological or chemical reason for setting these limits. Consequently, oligosaccharides are low molecular weight carbohydrates and they are often combined with proteins to form glycoproteins. Polysaccharides are increasingly larger molecules often referred to as complex carbohydrates. The monosaccharide units in oligosaccharides and polysaccharides can interconnect at several points to form a wide variety of branched or linear structures (Mussatto et al., 2007; Wang et al., 2013). Polysaccharides have the highest capacity for carrying biological information since they have the greatest potential for structural variability. The nucleotides in nucleic acids and the amino acids in proteins can connect in only one way whereas the monosaccharide units in polysaccharides can connect at several points to form a wide variety of branched or linear structures. This potential variability in polysaccharide structure gives the essential flexibility for the exact regulatory mechanisms of various cell-cell interactions in higher organisms (Wang et al., 2013; Ooi et al., 2000). They have several functions such as structural components of cell walls, energy storage, regulation of signaling, cell recognition, cell proliferation, cell differentiation, and immune responses (Dwek, 1996). Polysaccharides are industrially used as stabilisers, gelling agents, and thickeners in food

products. More recently, they are used as depollution agents and there is an increasing interest in their biological functions like anti-cancer, anti-oxidant and prebiotic activities (Donot et al., 2012). Polysaccharides have attracted increasing attention in a variety of biological activity studies as key biomolecules in essentially all living organisms. They have critical role in many fields of research. For example, in glycobiology, as potential drugs directed to enzymes or receptors involved in their function and metabolism, and as advanced materials due to their biocompatibility, structure-forming capacity, and environmentally friendly properties (Yip et al., 2006; Finkenstadt et al., 2005). On the other hand, polysaccharides act as key ingredients for the production of bio-based materials in various areas such as food, cosmetics, medical devices, and pharmaceuticals (García-González et al., 2011). In the molecular immune system, all the immunoglobulins and most of the complement components are glycosylated. The biological activities of polysaccharides have attracted increasing attention in biochemistry and medicine. Among various natural antioxidants, polysaccharides in general have strong antioxidant activities. The antioxidant effect is one of the main promising biopharmacological activities of polysaccharides (Wang et al., 2013).

Polysaccharides are derived from an extensive variety of sources such as bacterial, fungal, algal and plant. Despite the many sources of polysaccharides, the world market is dominated by polysaccharides from algae and higher plants. These polysaccharides are obtained by direct extraction from biomass and may be subjected to chemical hydrolysis or fermentation to obtain the smallest molecules able to be polymerised. Higher plants are the primary source of polysaccharides, which include cellulose, pectins, starch, and galactomannan. Polysaccharides come from plant cell walls in the form of cellulose or lignin. They can also be stored as starch for reserves. Cellulose, mainly from cotton plants (*Gossypium* sp.), is the most widely used biopolymer today. Galactomannan is another commonly used polysaccharide in the food industry. It is produced from Guar (*Cyamopsis tetragonolobus*) and Locust Beans (*Ceratonia siliqua*). Starch is extracted about 60 million tons per year from different cereal crops, including maize and wheat, and roots and tubers. Starch is used in several applications such as a stabiliser for soups and frozen foods, a paper covering, a pill coating, and as a raw material to produce ethanol. The main polysaccharides from red algae (Rhodophyceae) and brown algae (Phaeophyceae) are carrageenans, derived from *Kappaphycus alvarezii* and *Eucheima denticulum*; agar, derived from *Gelidium* sp. and *Gracilaria* sp.; alginates, derived from *Laminaria* sp., *Pelvetia* sp., *Sargassum* sp., *Ecklonia* sp. and *Undaria* sp.; and fucans, derived from *Stichopus* sp. and *Laminaria* sp. Polysaccharides produced by microorganisms, including bacteria, yeasts and moulds, represent an

unexploited market. Polysaccharide biosynthesis and accumulation generally occur after the growth phase of the microorganism. The polysaccharides derived from microorganisms is classified into three main groups according to their location in the cell: (1) cytosolic polysaccharides, which provide a carbon and energy source for the cell; (2) polysaccharides that make up the cell wall, including peptidoglycans, teichoic acids and lipopolysaccharides and (3) polysaccharides that are excreted into the extracellular environment in the form of capsules or biofilm, known as exopolysaccharides (EPSs). EPSs are divided into two groups: homopolysaccharides and heteropolysaccharides. Homopolysaccharides are composed of a single type of monosaccharide, like dextran or levan. Heteropolysaccharides are composed of several types of monosaccharides like xanthans or gellans, have complex structures and are usually synthesized inside the cell in the form of repeating units. EPS biosynthesis can be divided into three main steps: (1) assimilation of a carbon substrate, (2) intracellular synthesis of the polysaccharides and (3) EPS exudation out of the cell.

EPSs support the cell in various functions. They protect against biotic stress, like competition, and abiotic stresses that might include temperature, light intensity or pH. In the cases of acidophilic or thermophilic species and Archaea, EPSs help in adapting to extreme conditions (Donot et al., 2012). The structure of EPSs is very expanded. The building blocks (derived from sugar residues) of complex EPSs exhibit very similar structures, but their differentiation (diversified linkage style) is more comprehensive than that of amino acids. The primary structure of an EPS is defined by monosaccharide composition, configuration of glycosidic linkages, position of glycosidic linkages, sequence of monosaccharides, as well as the nature, number and location of appended non-carbohydrate groups. These macromolecules have long been known to possess a wide range of biological activities, such as antioxidant, anticoagulant, anti-mutant, immunostimulation, and tumor inhibition activities depending on their monomer compositions, molecular weights and degree of branching as well as on the presence of functional groups in their structure (Donot et al., 2012; Zong et al., 2012; Zhang et al., 2007). Besides these structural features, some physical characters such as charge, solubility and conformation of EPSs are also important factors affecting their bioactivities (Liu et al., 2012). More importantly, since the chemical and structural features of EPSs are highly determined by the type of the microbial producer and the production conditions (Kazak et al., 2010), EPSs of each specific microbial system call for individual studies to establish their bioactivities. On the other hand, many EPSs have remained classified as nonspecific

bioactive substances because their exact mode of action is unknown, and chain conformation of their active components is unclear.

1.1.3. Levan

One of the most widely distributed EPSs in nature, levan is a naturally occurring fructose polymer (fructan). Fructans are reserve carbohydrates comprising up to 70 units of fructose, linked to a terminal glucose molecule. They are classified into three families according to the type of linkage, namely, inulin [$(\beta 2 \rightarrow 1)\text{-D-Fruf}$] (Figure 1.1 a), levan [$(\beta 2 \rightarrow 6)\text{-D-Fruf}$] (Figure 1.1. b), and highly branched structures comprising of both $(\beta 2 \rightarrow 1)\text{-}$ and $(\beta 2 \rightarrow 6)\text{-}$ linked D-Fruf units such as graminans (Figure 1.1 c). Fructans are recognized as health-promoting food ingredients. They are found in a small number of mono- and dicotyledonous families of plants, such as Liliaceae, Amaryllidaceae, Gramineae, Compositae, Nolinaceae, and Agavaceae. Many fructan-containing plant species, including asparagus, garlic, leek, onion, Jerusalem artichoke, and chicory roots, are also found in nature as vegetables (Dong et al., 2015; Ferreira et al., 2015; Franco-Robles and Lopez, 2015). Though plant fructans are available, many microorganisms like *Acetobacter*, *Aerobacter*, *Azotobacter*, *Bacillus*, *Corynebacterium*, *Erwinia*, *Gluconobacter*, *Mycobacterium*, *Pseudomonas*, *Streptococcus* and *Zymomonas* also produce extracellular levan by the action of an extracellular enzyme with saccharose specificity named levansucrase, also known as sucrose 6-fructosyltransferase, $\beta\text{-(2-6)-fructosyltransferase}$, and $\beta\text{-(2-6)-fructan: D-glucose 1-fructosyltransferase}$ or EC 2.4.1.10 from sucrose-based substrates (Smelcerovic et al., 2008; Donot et al., 2012; Freitas et al., 2011; Nakaponga et al., 2013). In addition, halophilic bacterium *H. smyrnensis* AAD6^T has also been reported as a high level levan producer extremophile for the first time by our research group (Poli et al., 2009).

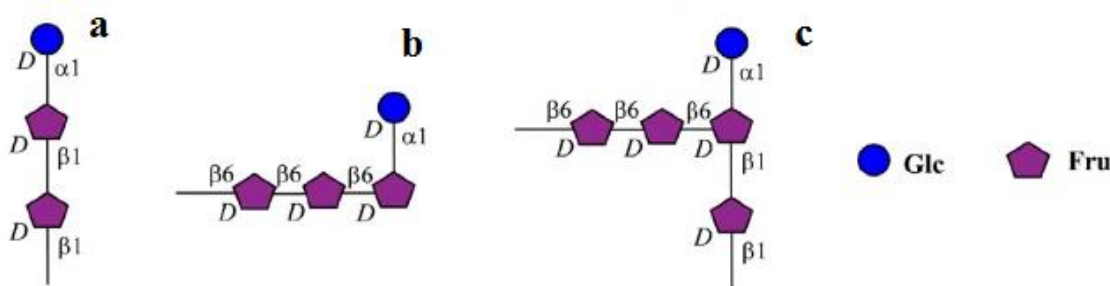


Figure 1.1. Chemical structure of fructans (Ferreira et al., 2015)

As a water soluble, non-toxic, self-assembling, strongly adhesive and film-forming biopolymer with many distinguishing properties like low viscosity, high solubility in oil, compatibility with salts and surfactants, stability to heat, acid, and alkali media, high holding capacity for water and chemicals, and good biocompatibility, levan is gaining escalating popularity as a functional biopolymer with diverse applications in medical and chemical industries (Kang et al., 2009).

Further researches on the potential use of levan by *H. smyrnensis* AAD6^T as a bioflocculating agent (Sam et al., 2011), its suitability for drug nanocarrier systems (Sezer et al., 2011; Sezer et al., 2015), and its production by use of cheap sucrose substitutes like molasses (Küçükaşık et al., 2011) were reported. Ternary blend films of chitosan and polyethylene oxide (PEO) with levan were prepared to evaluate the morphological, thermomechanical, surface and biological properties of the films (Sennaroğlu Bostan et al., 2014). Chemically modified forms of levan by *H. smyrnensis* AAD6^T were also reported such as the formation of adhesive multilayer films from phosphanate-modified levan (Costa et al., 2013) and uniform, homogeneous, nanostructured, thin films from oxidized levan (Sima et al., 2011; Sima et al., 2012).

1.1.3.1. Chemically modified levan

Different approaches to improve biological activity of polysaccharides by chemical modifications are described in the literature. The activities of the polysaccharides depend on their monosaccharide composition, glycosidic bonds, branch types, degree of polymerization, flexibility and configuration of the chains. So, the activities of the polysaccharides can be strengthened by modifying the structure by chemical methods. Chemical modifications increase bioactivities of the polysaccharides (Wang et al., 2013; Guo et al., 2013; Chen et al., 2009). Derivatives of natural polysaccharides may exhibit stronger biological activities than their corresponding natural polysaccharides and thus, they could provide an opportunity to develop new agents with possible therapeutic uses.

Besides naturally occurring levan, chemically modified levan is also synthesized because of its various new chemical and biological functions. Different derivatives from levan were prepared by means of sulfation (Abdel-Fattah et al., 2012), acetylation, phosphorylation and benzylation (Liu et al., 2012). Roberts and Garegg (1998) reported usages of levan derivatives such as levan phosphates, acetates, and sulfates in medicine as anti-AIDS agents; food processing as food additive with prebiotic and hypocholesterolemic effects, and as an environmentally friendly adhesive. Levan acetate and levan crosslinked with epichlorohydrin can be used to form thin

plastic films, while levan blended with ethylcellulose was casted into films (Cavalcanti et al., 2004).

In fact, in our earlier studies, nanostructured thin films of levan and its aldehyde-activated derivative were synthesized by Matrix Assisted Pulsed Laser Evaporation (MAPLE) and the aldehyde-activated levan thin films were found to induce an increase in cell proliferation as compared with the levan coatings (Sima et al., 2011). In a consecutive study, where gradient organic thin films of these two polymers were fabricated by combinatorial matrix-assisted pulsed laser evaporation (C-MAPLE), certain areas were found to exhibit distinctive influence on osteoblast cells spreading which in turn pointed to the importance of the amount of aldehydes for the observed biological activity (Sima et al., 2012).

Polysaccharides can be activated by oxidation with periodate due to the fact that aldehydes generated by the oxidation reaction provide an electrophilic carbonyl group. Because of this reactive functional group, aldehydes attach and react readily with nucleophilic functional groups present on the macromolecules such as proteins, lipids and DNA (Sanderson et al., 1971; Xie et al., 2013). This attachment has a variety of effects in biological systems, most often resulting in cytotoxicity (Canuto et al., 1994). Reaction of the aldehyde with an amine of a protein or other cellular component forms Schiff bases varying with cell type (Forman, 2010). Oxidized polysaccharides with aldehyde-functionalized groups may also be useful as tissue adhesives or sealants for medical applications that require a more rapid degradation time, such as the prevention of undesired tissue-to tissue adhesions resulting from trauma or surgery (Lu and Shuey, 2013).

1.1.3.2. Levan as a bioactive polysaccharide

The complexity and diversity of polysaccharides in their structures make them become one of the most important information carriers. They are not only used as a structural material or energy storage source, but also show some information functions comparable to those of nucleic acids or proteins, involved in the recognition among cells, the regulation of immune functions, the intercellular transport of substances, and the transformation and apoptosis process of cells (Jiang et al., 2016). Therefore, polysaccharides have a wide range of biological effects, such as antibiotic, antioxidant, anti-mutant, anticoagulant, antinociceptive, antiviral, anti-diabetic, hepatoprotective, cholesterol-lowering, anti-cancer, anti-inflammation, vaccination, anti-infection, anti-allergy, and immunostimulation activities (Ruthes et al., 2016; Wang et al., 2016; Vogt et al., 2015). With such

range of biological and pharmacological activities, a number of studies have reported the ability of many polysaccharides to induce apoptosis on cancer cells with low cytotoxicity on normal cells. The connection and importance of polysaccharides in cancer treatment was first recognized by Nauts et al. in 1946 when it was found that certain polysaccharides could induce complete remission in patients with cancer. Several polysaccharides and polysaccharide conjugates such as lentinan, schizophyllan, grifolan, PSP (polysaccharide–peptide complex) and krestin (polysaccharide–protein complex) have been commercialized for the clinical treatment of patients undergoing anti-cancer therapy (Zhang et al., 2007; Wang et al., 2016). Like other polysaccharides, there is a growing interest in elucidating the biological activities of levan from various microorganisms. Levan has potential use as plasma substitute, prolongator of drug activity, radio protector (Xu et al., 2006), immunostimulating (Dong et al., 2015), hypocholesterolemic (Belghith et al., 2012; Yamamoto et al., 1999), anti-diabetic (Dahech et al., 2011a; Dahech et al., 2011b), anti-inflammatory (Kim et al., 2005), and anti-cancer agents (Bayoumi et al., 2013; Esawy et al., 2013; Abdel-Fattah et al., 2012; Dahech et al., 2012; Yoo et al., 2004; Yoon et al., 2004). Calazans et al. (Calazans et al., 1997; Calazans et al., 2000) showed that the anti-cancer activity of levan from *Zymomonas mobilis* depended on its molecular weight. Yoon et al (2004) reported that the branching structure of levan from *Microbacterium laevaniformans* played an important role in its anti-cancer activity and they found that as the branching degree of levan decreased, the anti-cancer activity on a stomach carcinoma cell line linearly decreased. Esawy et al. (2013) showed cancer protective property of high molecular weight levan with branched structure from *Bacillus subtilis* M in murine hepatoma Hepa1c1c7 cells and their results suggested that the chemical structure, molecular weight, and branching of levan closely relate to its anti-cancer activity. Dong et al. (2015) reported that levan from *C. kwangsiensis* showed significant immunostimulatory activity based on its ability to stimulate macrophage proliferation and enhance phagocytosis. Park et al. (2008) examined the tumoricidal activity, phagocytosis and nitric oxide (NO) production in levan treated RAW264.7 cells and found that tumoricidal activity induced by levan was mediated by NO production. Dahech et al. (2011a) showed that levan from *Bacillus licheniformis* is efficient in inhibiting hyperglycemia and oxidative stress induced by diabetes in the alloxan-induced diabetic rats and they suggested that levan supplemented to diet may be helpful in preventing diabetic complications in adult rats. Liu et al. (2012) studied the antioxidant and anti-cancer activities of levan derivatives (acetylated, phosphorylated and benzylated) from *Paenibacillus polymyxa* EJS-3 in human gastric cancer

BGC-823 cells. And their results showed that levan derivatives could be explored as promising antioxidant and anti-cancer agents.

1.1.4. Oxidative stress

The formation of reactive oxygen species (ROS) is a natural consequence of aerobic metabolism and is vital for maintaining tissue oxygen homeostasis. Approximately 1–3% of oxygen consumed by the body is converted into ROS. Three of the major ROS are hydrogen peroxide (H_2O_2), superoxide ion (O_2^-), and hydroxyl radical ($\text{OH}^\cdot$). These compounds are normal metabolic byproducts that are generated continuously by the mitochondria in growing cells. Oxygen homeostasis, the balance between constitutive oxidants and antioxidants, is maintained through a natural series of reduction–oxidation (redox) reactions involving the transfer of electrons between two chemical species: compounds that lose electrons (oxidized) and those that gain electrons (reduced). When oxygen homeostasis is not maintained, the cellular environment becomes oxidatively stressed. Oxidative stress was first characterized by Sies (1991) as “a disturbance in the prooxidant to antioxidant balance in favor of the oxidant species, leading to potential damage”. Oxidative stress has been understood as an excessive amount of ROS that is the outcome of an imbalance between the generation and depletion of ROS. Hence, oxidative stress is the consequence of an enhanced free radical occurrence, but also of a reduced activity of the protective antioxidant defense system. When present at a high concentration, ROS damage cellular proteins and lipids or form DNA adducts that may promote carcinogenic activity. The purpose of antioxidants in a physiological setting is to prevent ROS concentrations from reaching a high level within a cell that damage may occur (Seifried et al., 2007; Pisoschi and Pop, 2015; Siti et al., 2015). Polysaccharides play an important role as free radical scavengers and antioxidants for the prevention of oxidative damage in living organisms (Wang et al., 2013).

1.1.5. Apoptosis

Apoptosis, unlike necrosis, is a tightly regulated type of cell death in which a cell effectively executes its own demise in a programmed manner. It is essential for normal tissue development and homeostasis (Correia et al., 2015; Khan et al., 2014; Lee and Pervaiz, 2007; Renehan et al., 2001; Hengartner, 2000). ROS and the resulting oxidative stress play an important role in

apoptosis (Chowdhury et al., 2014). Multicellular animals often need to eliminate cells that are damaged, unattached, mutant and aged. To this end, they use apoptosis that allows the organism to tightly control cell numbers and tissue size, and to protect itself from unneeded cells that threaten homeostasis (Arya and White, 2015; Hengartner, 2000). The process was discovered several times by various biologists and acquired a number of names over the past two centuries. It was first described in 1842 by Carl Vogt (Peter et al., 1997) and the term finally adopted is apoptosis, coined by Kerr and colleagues (1972) who described a series of morphological changes that were associated with the death of a range of tissues. These changes are initiated by various physiological and pathological stimuli such as compaction of nuclear chromatin, followed by condensation of the cytoplasm, DNA degradation, membrane blebbing and fragmentation of the cell into apoptotic bodies. The apoptotic bodies are taken up by surrounding cells and degraded in their lysosomes in the absence of inflammation. The biochemical changes include double stranded cleavage at the linker regions between nucleosomes, leading to the formation of multiple DNA fragments and a range of genes and protein expression changes. Biochemically, apoptosis typically involves the conversion of various signals into caspase-mediated intracellular protease activity (Correia et al., 2015; Khan et al., 2014). Apoptosis cascade is activated by intrinsic or extrinsic signals and occasionally by both resulting in cellular changes including chromatin condensation, DNA fragmentation and cytoplasmic budding leading to cell death (Yan and Shi, 2005). The central regulatory proteins in both pathways are the caspases (cysteine aspartic acid specific proteases). All caspases are synthesized as inactive zymogens that need activation to exert their function. Full activation is achieved through cleavage of a pro-domain generally by other caspases. The extrinsic death pathway induces apoptosis via cell surface death receptors and ligands like TNF (tumor necrosis factor) or Fas which result in the activation of caspases as the major executioners of apoptosis. It is initiated by the activation of death receptors, which leads to cleavage of caspase-8. The intrinsic death pathway is started in the mitochondria in response to DNA injury induced by various stresses such as heat, radiation or oxidative stress. It involves mitochondrial dysfunction, release of cytochrome c and subsequent activation of caspase-9 (Figure 1.2). Mitochondrial outer membrane permeabilization is a critical event in the intrinsic pathway which precipitates to cell death through release of pro-apoptotic proteins and loss of mitochondrial functions (Favaloro et al., 2012; Jin and El- Deiry, 2005; Chowdhury et al., 2014; Landes and Martinou, 2011).

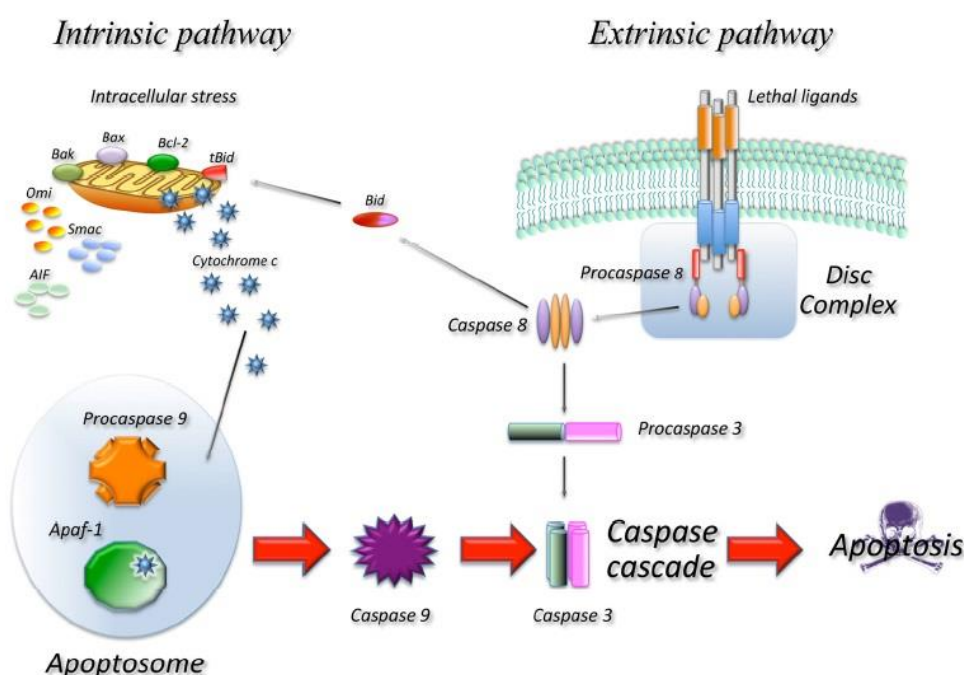


Figure 1.2. Schematic representation of the main molecular pathways leading to apoptosis.

In the extrinsic pathway upon ligand binding to specific receptors the Death Initiation Signalling Complex (DISC) is formed and caspase 8 activated. In the intrinsic pathway release of cyt c from the mitochondria result in the formation of the apoptosome and activation of caspase 9. Caspase 8 and 9 then activate downstream caspases such as caspase 3 resulting in cell death. The two pathways are connected through the cleavage of the BH3 only protein BID (Favaloro et al., 2012).

Complex regulatory systems participate in apoptosis adjustment. Abnormal regulation of this important biological phenomenon has been implicated in the pathogenesis of the various diseases. One of the important regulator molecules of the apoptosis process is Bcl-2 family proteins (Dewson and Kluck, 2009). The pro- and anti-apoptotic proteins of the Bcl-2 (B-cell lymphoma 2) family are reported to be the crucial regulators of cell signaling in the mitochondria-dependent intrinsic pathway of apoptosis. The Bcl-2 family, together with downstream proteins, maintains a dynamic balance between cell death and cell survival. Several synthetic and natural antioxidants are reported to influence Bcl-2 family proteins (Chowdhury et al., 2014). Natural products, such as quercetin, proanthocyanidin, curcumin, triterpenoids and silymarin, their active constituents and formulations are used in the treatment of a wide variety of clinical diseases. Their protective action is through antioxidant activity which modify multiple signal transduction pathways and proteins, including the Bcl-2 family members and the DNA damage/repair processes, thus

maintaining the pro-oxidant/antioxidant balance in the body (Yi et al., 2016). The activation of apoptosis is controlled at multiple checkpoints within the cell. Upstream, the proapoptotic Bax and antiapoptotic Bcl-2 are membrane-bound poreforming proteins that interact through heterodimerization. Together they regulate the mitochondrial transmembrane passage of cytochrome c, which in turn activates caspase proteins. The Bax/Bcl-2 ratio appears more important than the individual Bax or Bcl-2 levels in determining a cell's vulnerability to apoptosis; high Bax/Bcl-2 ratios lead to greater apoptotic activity (Jarskog et al., 2004).



2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Microorganism for levan production

H. smyrnensis AAD6^T (JCM 15723, DSM 21644) isolated from Camalti Saltern area (Izmir, Turkey) (Latitude: 38° 25' N, Longitude: 27° 08' E) was used as microbial source for levan production (Poli et al., 2013).

2.1.2. Culture medium for levan production

The production medium consisted of (per liter): 137.2 g NaCl; 50 g sucrose; 7 g K₂HPO₄; 2 g KH₂PO₄; 0.1 g MgSO₄·7H₂O; 1 g (NH₄)₂SO₄ and 0.5 g peptone, 0.8 mL Thiamine and 1 mL trace elements. Trace element solution (per 100 mL): 36 mg MnCl₂·4H₂O; 44 mg ZnSO₄·7H₂O; 230 mg FeSO₄·7H₂O; 5 mg CuSO₄·5H₂O was filter sterilized and added as 0.1% (v/v) to the medium. Filter sterilized Thiamine solution was added at 0.8 mg/L final concentration. The pH was adjusted to pH 7 by using 1 M NaOH or 1 M HCl.

2.1.3. Cell lines

The clonal β -cell line INS-1E derived from a rat insulinoma was kindly provided by Dr C. Wollheim, Switzerland. Human lung adenocarcinoma cell line A549 (ATCC, CCL-185) and human gastric adenocarcinoma cell line AGS (ATCC, CRL-1739) were kindly provided by Assist. Prof. Ömer Faruk Bayrak, Faculty of Medicine (Medical Genetics), Yeditepe University. Murine fibroblast cell line L929 (ATCC, CCL-1), human breast adenocarcinoma cell line MCF-7 (ATCC, HTB-22), and human liver hepatocellular carcinoma HepG2/C3A (ATCC, CRL-10741) cell line were kindly provided by Prof. Jülide Akbuğa, Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Marmara University. Murine macrophage-like cell line RAW 264.7 (ATCC, TIB-71) was kindly provided by Prof. Fikretin Şahin, Department of Genetics and Bioengineering, Yeditepe University.

2.1.4. Chemicals and kits

All chemicals and solutions used in this study were supplied by Merck (Germany), Sigma (USA), Difco (USA), Fluka (Switzerland), Thermo Scientific (USA), Invitrogen (USA), Chemicon (USA), Pan-Biotech (Germany), Gibco (USA), Hyclone (USA), Amresco (USA), and Bio-Rad (USA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) was purchased from Sigma (Oakville, ON, Canada). 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) cell proliferation reagent was purchased from Roche Applied Science (Germany). ApoTox-Glo triplex assay kit was purchased from Promega (USA). 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) general oxidative stress indicator kit was purchased from Molecular Probes (USA). CaspaTag Caspase 3,7 *In Situ* Assay Kit was purchased from Chemicon (USA). Precision Plus Protein Kaleidoscope Standards and DC Protein Assay Reagents were purchased from Bio-Rad (USA). Griess Reagent was purchased from Sigma (USA). The primary antibodies Bax, Bcl-2, and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Caspase-3 was purchased from Cell Signaling Technology (Danvers, MA, USA). The secondary antibody for Bax and Caspase-3 was anti-rabbit IgG HRP-linked Ab and was purchased from Cell Signaling Technologies (Danvers, MA, USA). The secondary antibody for Bcl-2 was anti-mouse IgG HRP-linked Ab and was purchased from Cell Signaling Technologies (Danvers, MA, USA).

2.1.5. Buffers and solutions

2.1.5.1. Buffers and solutions for levan production

Solutions Used in Preparing Stock Culture

Sea Water Stock Solution (30%)

NaCl	240 g
MgSO ₄ ·7H ₂ O	35 g
MgCl ₂ ·6H ₂ O	30 g
KCl	7 g

After the contents were dissolved in 1 L distilled water, pH was adjusted to 7.0. The solution was sterilized by autoclaving at 121°C 1.06 bar for 15 min and 5 mL of sterile $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1 M) was added to the sterile solution.

80% Glycerol, 6% Sea Water Solution

Glycerol	80 ml
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Sea Water Stock Solution (30%)	20 mL
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After the solution was sterilized by autoclaving at 121°C 1.06 bar for 15 min, 0.2 mL of sterile 0.5 M CaCl_2 was added to the solution aseptically.

Buffers Used for pH Adjustment

NaOH Buffer (1M)

NaOH	40 g
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ddH ₂ O	1 L
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NaOH was dissolved in 1 L distilled water. The buffer was sterilized by autoclaving at 121°C 1.06 bar for 3 min and stored at 4°C.

HCl Buffer (1M)

HCl (37%)	83 mL
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ddH ₂ O	917 mL
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83 mL of HCl (37%) was added to 917 mL of distilled water. The buffer was sterilized by autoclaving at 121°C 1.06 bar for 3 min and stored at 4°C.

Solutions Used in Titration

$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ (sodium thiosulfate) (0.01 N)

$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$	0.2482 g
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ddH ₂ O	100 mL
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$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ was dissolved in 100 mL distilled water.

Starch Indicator (1%)

Starch	0.1 g
ddH ₂ O	10 mL

Starch was dissolved in 10 mL hot distilled water.

2.1.5.2. Buffers and solutions for cell culture

1X PBS (Phosphate Buffered Saline)

NaCl	8 g
KCl	0.2 g
Na ₂ HPO ₄	1.44 g
KH ₂ PO ₄	0.24 g

After the contents were dissolved in 1 L of ddH₂O, pH was adjusted to 7.4. The solution was sterilized by autoclaving at 121°C 1.06 bar for 15 min.

Trypan Blue Solution (0.4 %)

Trypan Blue	0.4 g
PBS	100 mL

After trypan blue was dissolved in PBS, the solution was filtered first with 0.45 µm filter then with 0.22 µm filter to remove particles.

MTT Solution (5 mg/mL)

MTT powder	200 mg
PBS	40 mL

After MTT was dissolved in PBS, the solution was filtered to sterilize and to remove traces of insoluble residues present in some batches of MTT and then stored at 4°C for 4 weeks in dark.

Solutions Used in Total Protein Isolation

Cell Lysis Buffer:

NaCl	0.8766 g (150 mM)
NP-40	1 mL (1%)
Tris	0.6057 g (50 mM)

After the contents were dissolved in 100 mL ddH₂O, the pH was adjusted to 8.0 with HCl. The buffer was stored at 4°C. Prior to protein isolation, complete cell lysis buffer was prepared by adding protease/phosphatase inhibitor cocktail freshly.

Protease/Phosphatase Inhibitor Cocktail (for 10 mL Lysis buffer):

Aprotinin (1 mg/mL)	10 µL
Pepstatin (0.1 mg/mL)	100 µL
Leupeptin (1 mg/mL)	10 µL
Sodium orthovanadate (200 mM)	500 µL
Sodium fluoride (200 mM)	500 µL
Phenylmethylsulfonyl fluoride (PMSF) (200 mM)	50 µL

Solutions Used in Western Blotting

10%SDS

SDS	100g
ddH ₂ O	1L

1.5 M Tris-HCl (pH 8.8)

Tris Base	54.45 g
ddH ₂ O	300 mL

After pH was adjusted to 8.8 with 1N HCl, solution was stored at 4°C.

0.5 M Tris-HCl (pH 6.8)

Tris Base	6 g
ddH ₂ O	100 mL

After pH was adjusted to 6.8 with 1N HCl, solution was stored at 4°C.

Resolving gel (10 %)

ddH ₂ O	8.4 mL
Tris 1.5M (pH 8.8)	5.0 mL
Acrylamide 40%	5.0 mL
SDS 10%	0.2 mL
Glycerol 50%	1.35 mL
APS 10 %	300 µL
TEMED	26.4 µL

Stacking gel (10 %)

ddH ₂ O	5.08 mL
Tris 0.5M (pH 6.8)	2.0 mL
Acrylamide 40%	0.8 mL
SDS 10%	80.0 µL
APS 10 %	80 µL
TEMED	20 µL

2X SDS Loading Buffer (50 mL)

Bromophenol blue	1.2 mg
1 M Tris (pH 6.8)	8 mL
Glycerol	5 mL
10% SDS	20 mL
β-mercaptoethanol	1 mL
ddH ₂ O	16 mL

10X Running Buffer (1 L)

Glycine	144.1 g
Tris Base	30.3 g
10%SDS	50 mL

After the contents were dissolved in 1 L ddH₂O, solution was stored at room temperature until use.

1X Running Buffer (1 L)

10X Running Buffer	100 mL
ddH ₂ O	900 mL

10X Transfer Buffer (1 L)

Glycine	144.1 g
Tris Base	30.3 g

The contents were dissolved in 1 L ddH₂O.

1X Transfer Buffer (1 L)

10X Transfer Buffer	100 mL
ddH ₂ O	700 mL
Methanol	200 mL

10X Tris-buffer saline (TBS) Solution (1 L)

Tris Base	30.3 g
NaCl	87.6 g

After the contents were dissolved in 1 L ddH₂O, the pH was adjusted to 7.5 with HCl.

1X TBS-T Solution (1 L)

10X TBS solution	100 mL
ddH ₂ O	900 mL
Tween-20	1 mL

Blocking Solution (5% BSA in 1X TBS-T)

BSA	5 g
1X TBS-T	100 mL

Ponceau S Solution (100 mL)

Ponceau dye	10 g
Trichloroacetic acid	100 mL

Solution was stored in dark.

ECL Stock Solutions

Coumaric acid (90 mM)

p-Coumaric acid	0.15 g
DMSO	10 mL

Solution was stored in dark at -20°C.

Luminol (250 mM)

Luminol (3-aminophthalhydrazide)	0.44 g
DMSO	10 mL

Solution was stored in dark at -20°C.

Tris-HCl (1 M)

Tris Base	8.72 g
Tris-HCl	4.42 g

After the contents were dissolved in 100 mL ddH₂O, pH was adjusted to 8.5.

ECL Working Solutions

Soulution 1

1 M Tris-HCl (pH 8.5)	2 mL
H ₂ O ₂ (30%)	12 µL
ddH ₂ O	18 mL

Solution 2

1 M Tris-HCl	2 mL
Coumaric acid (90 mM)	88 μ L
Luminol (250 mM)	200 μ L
ddH ₂ O	18 mL

2.2. Laboratory Equipment

Antioxidant activity of levan in the pancreatic β -cell line INS-1E was investigated in the Northern Ontario School of Medicine (Lakehead University, Canada) research laboratories. The list of laboratory equipment exploited is listed below.

Autoclave:

- STERIS Amsco Century SV-120, USA
- VWR AS12, USA
- Market Forge Industries Inc. (Model: STMEL 120/208/240), USA

Inverted microscope:

- Nikon Eclipse TS100, Japan

Cell viability analyzer:

- Beckman Counter, Vi-Cell XR, Canada

CO₂ incubator:

- Thermo Electron Corporation, Forma Series II, Water Jacketed, USA

Biological safety cabinet:

- Thermo Electron Corporation, NAPFlow II, Type A2, USA

Microplate spectrophotometer: • BioTek Instruments, Inc. (Power Wave XS), USA

Flow cytometry: • FACScan, Becton Dickinson, Germany

Liquid nitrogen storage system: • Thermo Scientific, Thermolyne Cryobiological Storage Systems, USA

Deepfreeze: • Thermo Electron Corporation, USA

• VWR, USA

Laboratory fume hood: • AMS Air Master Systems Corp., USA

Water bath: • Fisher Scientific Isotemp Water Bath Systems, USA

pH meter: • Mettler Toledo, Switzerland

Balance: • Mettler Toledo, Switzerland

Centrifuge: • Beckman Coulter Centrifuge Systems, USA

Water purification system: • Millipore Milli-Q, USA

Western workflow: • Bio-Rad, USA

Heating Magnetic Stirrer: • VWR, USA

Belly dancer shaker: • Stovall Life Science, USA

Microliter pipette: • Finnpipette, Finland
• Eppendorf, Germany

Cytotoxicity, anti-cancer activity and immunostimulating activity experiments of levan and oxidized levan were carried out in the Industrial Biotechnology and Systems Biology (IBSB) Research Laboratory of Bioengineering Department at Marmara University. The list of laboratory equipment exploited is listed below.

Autoclave: • Mod3870 Elv, Systec, Germany
• Nüve OT 032, Turkey
• Nüve S-Class EN 13060, Turkey

Inverted microscope: • SOIF XDS – 1B, China

CO₂ incubator: • Thermo Scientific Model 370 Series Direct Heat with Sterilization Cycle, USA

Biological safety cabinet: • Thermo Scientific Safe 2020, USA (cell culture lab.)
• Heto Holten, Class II Safety Cabinet, Safe 2010, Denmark (production lab.)

Microplate reader: • Promega GloMax®-Multi+ Microplate Multimode Reader, USA

Liquid nitrogen storage system:	• Air Liquide GT38, France
Refrigerator	• +4°C Arçelik, Turkey • +4°C, Indesit, Turkey
Deepfreeze:	• -80°C Heto Holten, Denmark • -20°C Heto Holten, Denmark • -20°C Uğur Derindondurucu, Turkey
Water bath:	• Polyscience, USA
pH meter:	• Mettler Toledo, Switzerland
Balance:	• Mettler Toledo Ab204-S, Switzerland • Mettler Toledo Pg403-S, Switzerland • Mettler Toledo Pg40002-S, Switzerland
Centrifuge:	• Nüve NF400R, Turkey • Sigma 3K30, Germany • Sigma 8K10, Germany
Water purification system:	• Primary Grade Water Purification System, Purelab Prima 30 Ultrapure Water System, P.Maxima Ls, (Usf Elga, U.K.)

Heating Magnetic Stirrer:	• Heidolph Mr3003s, Germany
Microliter pipette:	• Finnpiquette, Finland
Multichannel microliter pipette:	• Rainin L8-200XLS+, 8-channel, USA
Automatic pipettor	• Witeg, Germany
Freeze dryer:	• Lyovac GT2, Steris, USA
Orbital shaker:	• B.Braun Certomat BS1, Germany • New Brunswick Scientific, Excella E24, USA
Oven:	• Binder, Germany
Spectrophotometer:	• Perkin Elmer UV/Vis, Lambda35, USA
Vortex:	• Heidolph, Germany
FT-IR Spectrometer:	• Thermo Scientific Nicolet 6700, USA
Fermenter:	• Sartorius, Biostat B, Germany
NMR:	• Varian, USA

2.3. Experimental Methods

2.3.1. Microbial levan production

2.3.1.1. Sterilization

Throughout this study, sterilization was carried out by killing the microorganisms using steam. The culture medium used was sterilized in an autoclave. This process, which is known as steam autoclave sterilization, has advantages of short cycle time and good penetration.

Sterilization of Medium

While preparing medium, NaCl and the rest of the medium components were dissolved in distilled water, respectively and then sterilized separately by autoclaving at 1.06 bar and 121°C for 15 min. Sucrose was also sterilized separately in the same manner but for 3 min to avoid its degradation. Sterile salt and sucrose solutions were combined to 1 L final volume aseptically.

Sterilization of Equipments

Micropipette tips, eppendorfs and glass equipments were sterilized by autoclaving at 1.06 bar and 121°C for 15 min.

2.3.1.2. Preparation of preculture

Sterile liquid medium in Erlenmeyer flask was inoculated with 1% (v/v) frozen glycerol culture and incubated at 37°C at 180 rpm for 24 h until its growth reached the early exponential phase in an orbital shaker (Certomat BS1, B. Braun, Germany). 1% (v/v) of this preculture was used as an inoculum.

2.3.1.3. Growth conditions

Microbial levan production by halophilic *H. smyrnensis* AAD6^T was performed under controlled bioreactor conditions using a semichemical medium. The BIOSTAT B fermenter of 3 L working volume was employed. During cultivation, the temperature and pH were kept constant at 37°C and pH 7, respectively. The aeration rate was 0.1 vvm, whereas the agitation was set to 200 rpm.

2.3.1.4. Isolation and purification of levan

Cells from the stationary phase cultures were harvested by centrifugation (10,000 rpm for 20 min). The supernatant phase containing the soluble levan polymer was treated with an equal volume of

ethanol, stirred vigorously and kept overnight at -18°C. The precipitate from the ethanol dispersion was collected by centrifugation at 12,000 rpm for 30 min, re-dissolved in hot distilled water, dialyzed against several runs of distilled water for at least 3 days. Levan was further purified by passing through a DEAE-Sepharose CL-6B column to remove the impurities. Fractions containing pure levan were collected, tested for total carbohydrate and protein and then dried by lyophilization (Kazak Sarılmışer et al., 2015).

2.3.1.5. Total carbohydrate analysis of levan

Carbohydrate content of levan was determined using phenol/sulfuric acid method using glucose as standard. Lyophilized levan samples were dissolved in distilled water to 1 mg/mL final concentration. According to the method, 50 µL of sample was pipetted into a glass tube, and 950 µL of distilled water, 0.25 µL of 80% phenol and 2.5 mL of concentrated sulfuric acid were added, respectively. The solution was vortexed gently in order to obtain good mixing and after incubation for 30 min at room temperature, optical density was measured at 490 nm using Lambda35 UV/Vis spectrophotometer (Perkin Elmer, USA). Blank was prepared by substituting distilled water for the sugar solution (Dubois et al., 1956). Spectroscopic reading directly gives the % carbohydrate concentration.

2.3.1.6. Protein analysis of levan

Protein concentration was determined by the Bradford test using Bovine Serum Albumin (BSA) as standard (Bradford, 1976). The Bradford dye assay is based on the equilibrium between three forms of Coomassie Blue G dye. Under strongly acidic conditions, the dye is most stable as a doubly-protonated red form. Upon binding to protein, however, it is most stable as an unprotonated, blue form. Levan sample was dissolved in distilled water to 1 mg/mL final concentration. To determine the protein concentration of samples, 0.3 mL of dye stock (BioRad) was added to 1.2 mL of dilutions of sample to be assayed and vortexed. After incubation at room temperature for at least 5 min, absorbance was measured at 595nm using Lambda35 UV/Vis spectrophotometer (Perkin Elmer, USA). A standard curve of absorbance versus protein concentration was prepared by using known amounts of Bovine Serum Albumin (BSA) (Appendix A).

2.3.2. Oxidation of levan

Pure levan samples were subjected to periodate oxidation by prolonged magnetic stirring in a beaker. Pure levan (1 g) was dissolved in 600 mL of distilled water and after the addition of 12 g KIO_4 , the solution was stirred at 300 rpm in dark oven at 50°C for 1 day (OL I), 3 days (OL II), and 6 days (OL III) to achieve different oxidation degrees (OxD). After periodate was eliminated from the solution by dialysis against distilled water for at least 48 h, the polymer solution was lyophilized (Kazak Sarılmışer et al., 2014).

2.3.3. Characterization of levan and its oxidized forms

2.3.3.1. Determination of oxidation degree

The amount of oxidized fructose residues (oxidation degree) in the levan molecule was evaluated by the iodometric method of Lindenbaum et al. (1977) involving the direct determination of aldehyde content in oxidized levan. The oxidation degree was calculated according to the following equation where V_u and V_m are the volume of 0.01 N thiosulfate solution in unmodified and modified levan titration, respectively (mL); m is the mass of levan in sample (g); N_T is normality of thiosulfate solution; 12.4 is the coefficient equal to the amount of aldehyde groups (mmol) under full (100%) oxidation of 1 g levan:

$$\text{OxD} = \frac{V_u - V_m}{2 \times 12.4 \times m} \times N_T \times 100\%$$

The detailed titration procedure is as follows: 10 mg of oxidized levan was dissolved in 40 mL ddH₂O. The same concentration of unmodified levan solution was used as a blank. 100 μL saturated KI solution and 500 μL concentrated HCl was added to the solutions, respectively. The solutions were put into dark place at 4°C for 30 min. Sample solutions were titrated with 0.01 N sodium thiosulfate until the solutions became pale yellow. Then, 75 μL starch indicator (1%) was introduced to the solutions and the solutions were retitrated with 0.01 N sodium thiosulfate until the disappearance of the dark blue colour. At this time, the consumed volume of sodium thiosulfate was noted as V_u and V_m for unmodified and modified levan, respectively.

2.3.3.2. Fourier transform-infrared (FT-IR) analysis

Fourier transform-infrared (FT-IR) spectra of levan and oxidized levan samples were obtained with Nicolet 6700 FT-IR Spectrometer equipped with a Smart Orbit high performance diamond attenuated total reflectance (ATR) accessories. Measurements were performed between 400 and 4000 wave numbers (1/cm).

2.3.3.3. Nuclear magnetic resonance (NMR) analysis

Nuclear magnetic resonance (NMR) analysis was also performed to provide more structural information of oxidized levan. The NMR spectrum of oxidized levan with the highest oxidation degree (OL III) was recorded on a Varian 600 MHz NMR at 25°C for ^1H NMR. The sample was freeze-dried and dissolved in a dimethyl sulfoxide (DMSO) solution before measurements of ^1H NMR.

2.3.4. Cell Culture

2.3.4.1. Thawing cells

Frozen cell line vial taken from the liquid nitrogen tank was placed in 37°C water bath until the external part of the cell solution was thawed. The cells were added slowly, drop by drop, into 10 mL of pre-warmed growth medium, resuspended gently and centrifuged at 500 rpm at 4°C for 5 min. Supernatant was discarded and the pellet was resuspended in 10 mL of culture medium and plated into T25 flask. The viability of cells was checked in the following days. A new vial of frozen cells from the liquid nitrogen tank was thawed in every month.

2.3.4.2. Culture conditions

The pancreatic β -cell line INS-1E was grown in monolayer cultures in RPMI-1640 medium supplemented with 2 g NaHCO_3 , 10 % fetal bovine serum (FBS), 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 10 mM HEPES, 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin at 37°C in a humidified (5% CO_2 , 95 % O_2) atmosphere. The medium was further supplemented with high glucose concentration (25 mM).

Human gastric adenocarcinoma cell line AGS was grown in RPMI-1640 medium, while human lung adenocarcinoma cell line A549, human liver hepatocellular carcinoma cell line HepG2/C3A, human breast adenocarcinoma cell line MCF-7, mouse fibroblast cell line L929, and murine

macrophage-like cell line RAW 264.7 were routinely cultured in DMEM (Dulbecco's Modified Eagle's Medium) at 37°C in humidified air containing 5% CO₂. Media were supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin.

All steps of the cell culture were performed only with gloved hands under sterile conditions, with sterile media, solutions and materials in a sterile hood to avoid any contamination. Cells were checked microscopically daily to ensure they were healthy and growing as expected. The maintenance culture was passaged every 3-4 days before cells became 80% confluent. For sub-culturing the cells, firstly fresh medium, PBS, and trypsin were warmed in 37°C water bath. The growth medium from a filter capped T75 flask (allowing gas exchange) was removed with a sterile pipette and discarded. The cultured cells were washed with 4-5 mL 1X PBS to get rid of any residual FBS and then PBS was discarded. 2-3 mL of trypsin-EDTA solution (0.25%) was added into the flask and the flask was rolled gently to ensure trypsin contacted with all cells. Cells were incubated for 3 min at 37°C in the CO₂ incubator. After incubation, cells were checked under an inverted microscope if they were detached and moving in solution. Fully trypsinized cells appeared rounded up and no longer attached to the surface of the flask. Trypsin was quenched by adding 8 mL of fresh growth medium to the flask (the FBS in the medium inactivated the trypsin). Cell solution was pipetted gently in order to obtain a single cell suspension. Then, cells were counted and the amount of cell solution required for a density of 4×10^4 cells/cm², *i.e.* 3×10^6 cells, was determined. Required volume was added to a new flask and fresh growth medium was added up to 10-15 mL if using a T75 flask (or up to 6-10 mL if using a T25 flask).

2.3.4.3. Cell counting

The diazo dye, Trypan Blue ((3Z,3'Z)-3,3'-[(3,3'-dimethylbiphenyl-4,4'-diyl)di(1Z)hydrazin-2-yl-1-ylidene] bis (5-amino-4-oxo-3,4-dihydronaphthalene-2,7-disulfonic acid)) is a stain used to discern living from dead cells. It is cell-impermeable and penetrates only damaged, non-viable cells with compromised membrane integrity.

Cell concentration and viability of pancreatic β-cell line INS-1E were assessed via automated Trypan Blue exclusion assay using a Vi-Cell XR cell viability analyzer (Beckman Coulter, Canada), wherein 0.5 mL of diluted, trypsinized cell suspensions were loaded, mixed with Trypan Blue reagent, and live/dead cell counts were assessed via 50 individual sub-samples measured using the Vi-Cell XR Cell Viability Analyzer software.

The number of all other cell lines was determined with Thoma cell counter by counting under the

inverted microscope. Cell solution was mixed with an equal volume of trypan blue solution. Then this suspension was loaded to the counting chamber of Thoma cell counter. Using the microscope with a 10X ocular, the numbers of unstained cells (viable cells) in the middle (composed of 16 small squares) with 1 mm^2 area were counted (Figure 2.1). Counting was repeated in order to check reproducibility.

The depth between coverslip and cell counting chamber is 0.1 mm. Thus, each 1 mm^2 area holds a volume of $0.1 \text{ mm}^3 (=10^{-4} \text{ mL})$. So, the total number of cells in 1 mL was calculated as follows:

$$\text{Total cell number / mL} = \text{cell number in 16 little square} \times 2 \text{ (dilution factor)} \times 10^4$$

↓
Number of cells per 0.1 mm^3

Dilution factor was usually 2 because of 1:1 dilution with trypan blue solution.

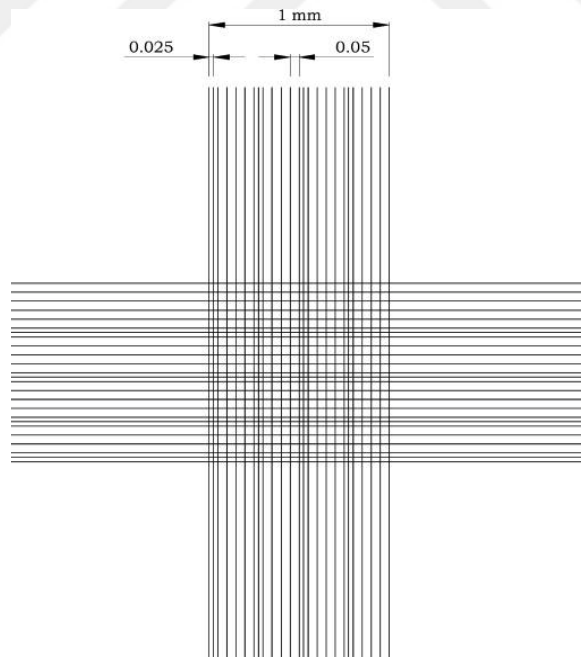


Figure 2.1. Schematic drawing of the Thoma chamber

2.3.4.4. Cryopreservation

Exponentially growing cells with 60-70% confluency were trypsinized and collected with growth medium. The cells were centrifuged at 500 rpm at 4°C for 5 min. The precipitated cell pellet was resuspended in 1X PBS and centrifuged again at 500 rpm at 4°C for 5 min. The supernatant was discarded and cell pellet was resuspended in a freezing solution (10% DMSO and 90% FBS) at a concentration of $2-3 \times 10^6$ cells/mL. 1 mL of this solution was quickly transferred into 1 mL screw capped-cryotubes. The tubes were left at -80°C overnight. The next day, the tubes were placed in the liquid nitrogen storage tank. It is important to freeze the cells quickly to minimize the amount of damage the DMSO can cause to the cells.

2.3.4.5. Preparation and sterilization of levan solutions

Levan samples (200 mg) were added in 100 mL distilled water, stirred overnight at 50°C, dissolved completely and sterilized by autoclaving at 121°C for 3 min. Sterile stock solutions were stored at 4°C. Different concentrations of levan solutions were prepared by using stock solutions (2 mg/mL).

2.3.5. Cell viability experiments

2.3.5.1. MTT assay

The metabolic activity of living INS-1E cells demonstrating their proliferation and viability at the end of the incubation period with levan in the presence of high (25 mM) glucose was assessed based upon the conversion of tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Mosmann, 1983). The yellow tetrazolium salt reduces to form a water-insoluble crystallized purple formazan via the cleavage of the tetrazolium ring by the succinate dehydrogenase within the mitochondria. The formazan product is impermeable to the cell membrane and thus accumulates in healthy cells whereas it is released from cells with damaged or leaky membrane (Ngamwongsatit et al., 2008; Ishiyama et al., 1996). The amount of formazan generated is directly proportional to the cell number. Briefly, INS-1E cells were trypsinized when they were grown to 90 % confluency, seeded onto sterile flatbottom 96-well plates (Corning, USA) at a density of 5×10^4 cells per well and then allowed to grow for 48 h at 37°C in humidified air containing 5% CO₂ to achieve the desired confluence. Plated cells were subjected to levan treatment (in serum- and antibiotic- free RPMI-1640 medium) with

different concentrations (10-1000 $\mu\text{g/mL}$). With $t = 4$ h of treatment time remaining, MTT reagent (Sigma, Canada) was added to wells to achieve a final concentration of 10 % (v/v), and cells were incubated at 37 °C for an additional 4 hours, during which time the MTT reagent was converted to purple formazan crystals in living cells according to their metabolic activity. Following this, the incubation media from each well was aspirated, replaced with 50 μL /well of DMSO to solubilize the formazan crystals. Following 10 min of agitation on a Belly Dancer shaker (Stovall Life Science, USA) at its highest setting at room temperature protecting from light, absorbance of the formazan product formed was measured at 570 nm using a PowerWave XS microplate spectrophotometer (BioTek, USA). Untreated cells were used as control. Viability of treated wells was assessed relative to control wells, which were considered to represent 100 % viability (Kazak et al., 2014).

2.3.5.2. WST-1 assay

In order to determine cell viability of L929, A549, HepG2/C3A, MCF-7, AGS, and RAW 264.7 cells, WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) cell proliferation and viability assay (Roche Applied Science, Germany) was performed. In principle, WST-1 works similarly to MTT by reacting with the mitochondrial succinate-tetrazolium reductase forming the formazan dye. The WST-1 reagent produces a water-soluble formazan rather than the water-insoluble product of the MTT assay. The WST-1 reaction product can be quantified in 0.5 to 4 h without an additional solubilization step (Ngamwongsatit et al., 2008; Berridge et al., 2005; Ishiyama et al., 1996). Briefly, 1.5×10^3 cells were seeded onto sterile flatbottom 96-well plates (TPP, Switzerland) and incubated overnight at 37°C in humidified air containing 5% CO_2 . Then the culture medium was removed and replaced with fresh FBS-free medium containing varying concentrations (10-1000 $\mu\text{g/mL}$) of levan samples (100 μL volume per well). After incubation with levan samples for 24 h, WST-1 reagent (10 μL) was added directly to culture wells and incubated. Incubation time with WST-1 reagent was 2 h for L929, HepG2/C3A, and MCF-7 cells, 1.5 h for AGS and RAW 264.7 cells, 1 h for A549 cells. The yellow-orange colored product, a stable soluble formazan, was quantified at 450 nm using a GloMax Multi+Microplate Multimode Reader (Promega, USA). Untreated cells were used as control. The control cells were considered 100 % viable.

2.3.6. Measurement of nitric oxide production

The concentration of nitric oxide (NO) in macrophage culture supernatant was determined by using colorimetric Griess reaction assay as described by Green et al. (1982). This assay relies on a diazotization reaction that was originally described by Griess in 1879. Briefly, RAW 264.7 cells were cultivated in a 96-well microplate (1×10^5 cells/well, in a volume of 100 μ L) and incubated for 24 h at 37°C in a 5% CO₂ incubator. The cultured cells were then treated with pure levan solution (100 μ L) at concentrations ranging from 10 to 1000 μ g/mL. After treatment for 24 h at 37°C, the cultured cell supernatant (100 μ L) was mixed with an equal volume of Griess reagent (1% [w/v] sulfanilamide and 0.1% [w/v] N-[1-naphthyl] ethylenediamine hydrochloride in 2.5% [v/v] phosphoric acid) (Sigma, USA) and incubated at room temperature for 15 min. The absorbance was measured at 540 nm using a GloMax Multi+Microplate Multimode Reader (Promega, USA). NO production from the RAW 264.7 cells was calculated with reference to a standard curve that was obtained with NaNO₂ (0–200 μ M in the culture medium) (Appendix B).

2.3.7. Measurement of ROS Generation

Intracellular ROS production of INS-1E cells was measured using a cell-permeable indicator CM-H₂DCFDA (5-(and-6)-chloromethyl - 2',7'-dichlorodihydrofluorescein diacetate, acetyl ester) as per the manufacturer's protocol. This molecule remains non-fluorescent until the acetate groups are removed by intracellular esterases as oxidation occurs within the cell. In addition, esterase cleavage of the lipophilic blocking groups yields a charged form of the dye that is much better retained by cells than the parent compound. Moreover, the chloromethyl derivative of H₂DCFDA used here allows for covalent binding to intracellular components, permitting even longer retention within the cell (Li et al., 2009; Brubacher et al., 2001). Briefly, 1.5×10^6 cells were seeded onto sterile flat-bottom T25 culture flasks (Corning, USA) and grown at 37°C in a humidified atmosphere for 48 h, then pretreated with levan in serum- and antibiotic- free RPMI 1640 medium. Treated and control cells were then incubated with 200 μ M H₂O₂ at 37°C for 1 h to induce apoptosis. Following H₂O₂ treatment, the medium was removed, the cells were washed with PBS, and then incubated with 1 mL of CM-H₂DCFDA solution (Molecular Probes, USA) at 37°C for 30 min. After this incubation period, the dye solution was removed by aspiration, labeled cells were washed with PBS, detached from the flask using trypsin, harvested by centrifugation ($500 \times g$ for 5 min at 4°C), and suspended in PBS for flow cytometric analysis using the FL1-H

channel of a BD FACSCalibur Flow Cytometer (Becton Dickinson, Germany) supported by BD CellQuest Pro Software. A minimum of 1×10^4 gated events were acquired per trial. Mean fluorescence (specifically: geometric mean fluorescence) was understood to be directly proportional to levels of intracellular ROS. The results were expressed as mean intensity of fluorescence. Untreated cells at the same cell density in PBS were used as the control, and the relative fluorescence intensity of treated cells was compared with the control.

2.3.8. Apoptosis assays

2.3.8.1. Flow-cytometric analysis of apoptosis

Apoptosis in INS-1E cells was analyzed by a fluorescence-based assay that detects active caspase 3/7 using the CaspaTag Caspase 3/7 *In Situ* Assay kit (Chemicon International, USA). The methodology is based on Fluorochrome Inhibitors of Caspases (FLICA). The inhibitors are cell permeable and non-cytotoxic. Once inside the cell, the inhibitor binds covalently to the active caspase (Ekert et al. 1999). This kit uses a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspase-3 (FAM-DEVD-FMK), which produces a green fluorescence. When the FAM-DEVD-FMK probe is added to a population of cells, the probe enters each cell and covalently binds to a reactive cysteine residue that resides on the large subunit of the active caspase heterodimer, thus inhibiting further enzymatic activity. The bound labeled reagent is retained within the cell, while any unbound reagent diffuses out of the cell and is washed away. The green fluorescent signal is a direct measure of the amount of active caspase 3/7 present in the cell at the time the reagent is added. Briefly, cells seeded at a density of 7×10^5 cells onto sterile flat-bottom 6-well plates (Corning, USA) were cultured at 37°C in a humidified atmosphere for 48 h, and then pretreated with levan (dissolved in serum- and antibiotic- free RPMI-1640 medium). After pretreatment with levan for 24 h, treated and control cells were incubated with 200 μ M H₂O₂ at 37°C for 1 h to induce apoptosis, followed by harvesting the cells by centrifugation ($350 \times g$ at 18°C for 5 min), resuspended in 300 μ L 1X PBS and incubated with 10 μ L freshly prepared 30X FLICA reagent at 37°C for 1 h under 5% CO₂, protecting from light (mixing gently twice during this incubation). After 1 h in reagent, FLICA was aspirated and the cells were washed three times for 5 min each at room temperature in the wash buffer supplied by the manufacturer. Active caspase-3/7 positive cells (containing the bound-labeled reagent) were immediately assessed by flow cytometry (FACScan, Becton Dickinson, Germany) supported by BD CellQuest Pro

Software on the FL1-H (FLICA) channel, acquiring a minimum of 1×10^4 gated events per trial. The activation of caspase-3/7 was expressed as the mean fluorescence.

2.3.8.2. ApoTox-Glo triplex assay

Fluorescent-based viability and luminogenic-based caspase-3/7 activity of MCF-7 cells were conducted using the ApoTox-Glo triplex assay (Promega, USA). The ApoTox-Glo Triplex Assay combines three assays to evaluate viability, cytotoxicity and caspase activation within a single assay well. The first part of the assay simultaneously measures two protease activities; cell viability and cytotoxicity. The live-cell protease activity is restricted to intact viable cells and is measured using a fluorogenic, cell-permeant, peptide substrate (glycyl-phenylalanyl-aminofluorocoumarin; GF-AFC). The substrate enters undamaged cells, where it is cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells. This live-cell protease becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium. A second, fluorogenic cell-impermeant peptide substrate (bis-alanylalanyl-phenylalanyl-rhodamine 110; bis-AAF-R110) is used to measure dead-cell protease activity, which is released from cells that have lost membrane integrity. Since bis-AAF-R110 is not cell-permeant, viable cells generate essentially no signal from this substrate. The live- and dead-cell proteases produce different products, AFC and R110, which have different excitation and emission spectra, allowing them to be detected simultaneously (Niles et al., 2007).

The second part of the assay provides a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity, luciferase activity and cell lysis. Adding the Caspase-Glo® 3/7 Reagent results in cell lysis, followed by caspase cleavage of the substrate and generation of luminescent signal produced by luciferase (Figure 2.2). Luminescence is proportional to the amount of caspase activity present.

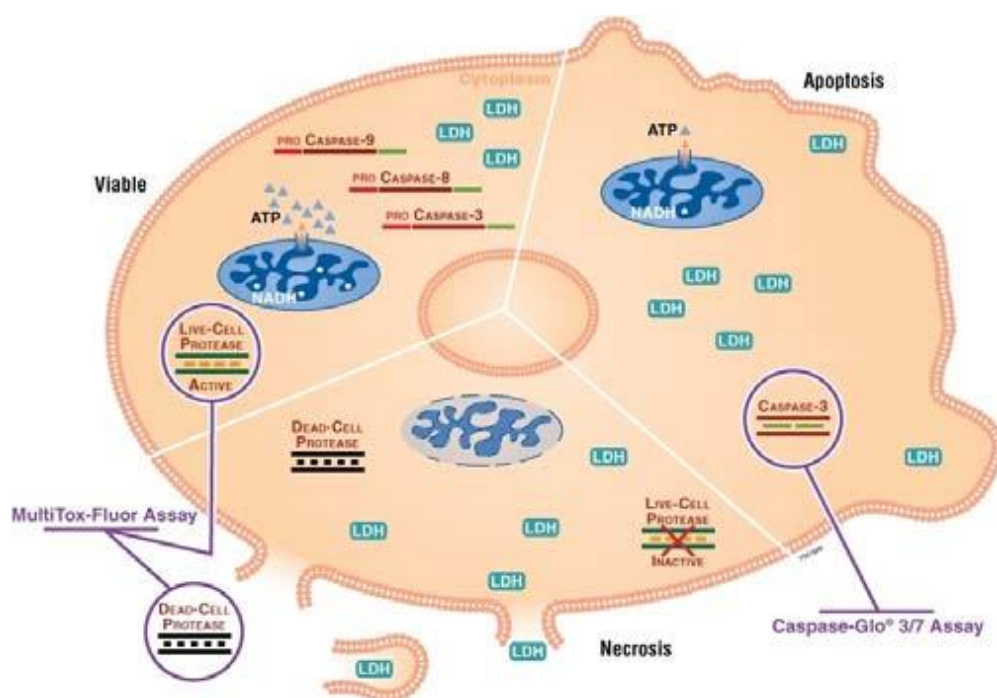


Figure 2.2. The ApoTox-Glo triplex assay principle using the MultiTox- Fluor™ and Caspase-Glo® 3/7 Assays (Shultz et al., 2009)

Fluorescent-based viability and luminogenic-based caspase-3/7 activity were conducted using the ApoTox-Glo triplex assay (Promega, USA) according to the manufacturer's instructions. For this purpose, MCF-7 cell line was treated with unmodified levan and OL III at the concentration of 1000 $\mu\text{g/mL}$. Briefly, cells were seeded onto sterile flatbottom 96-well opaque-walled tissue culture plates (Greiner Bio-One, Germany) at a density of 1.5×10^3 cells/well in 100 μL culture medium and allowed to grow for 24 h at 37°C in a humidified atmosphere. Then cultured cells were treated with levan samples in 100 μL of serum- and antibiotic- free medium and incubated for 24 h. After treatment with levan samples, 20 μL of viability/cytotoxicity reagent containing both GF-AFC substrate and bis-AAF-R110 substrate was added to all wells, and briefly mixed by orbital shaking (300–500rpm for ~30 seconds). After incubation for 1 h at 37°C , fluorescence was measured at the following two wavelength sets:

- 400_{Ex}/505_{Em} (Viability)
- 485_{Ex}/520_{Em} (Cytotoxicity)

This was followed by adding 100 μL of Caspase-Glo 3/7 reagent to the same sample wells, mixing by orbital shaking and incubating for 1 h at room temperature. Then luminescent signal was measured using GloMax Multi+Microplate Multimode Reader (Promega, USA) (Figure 2.3).

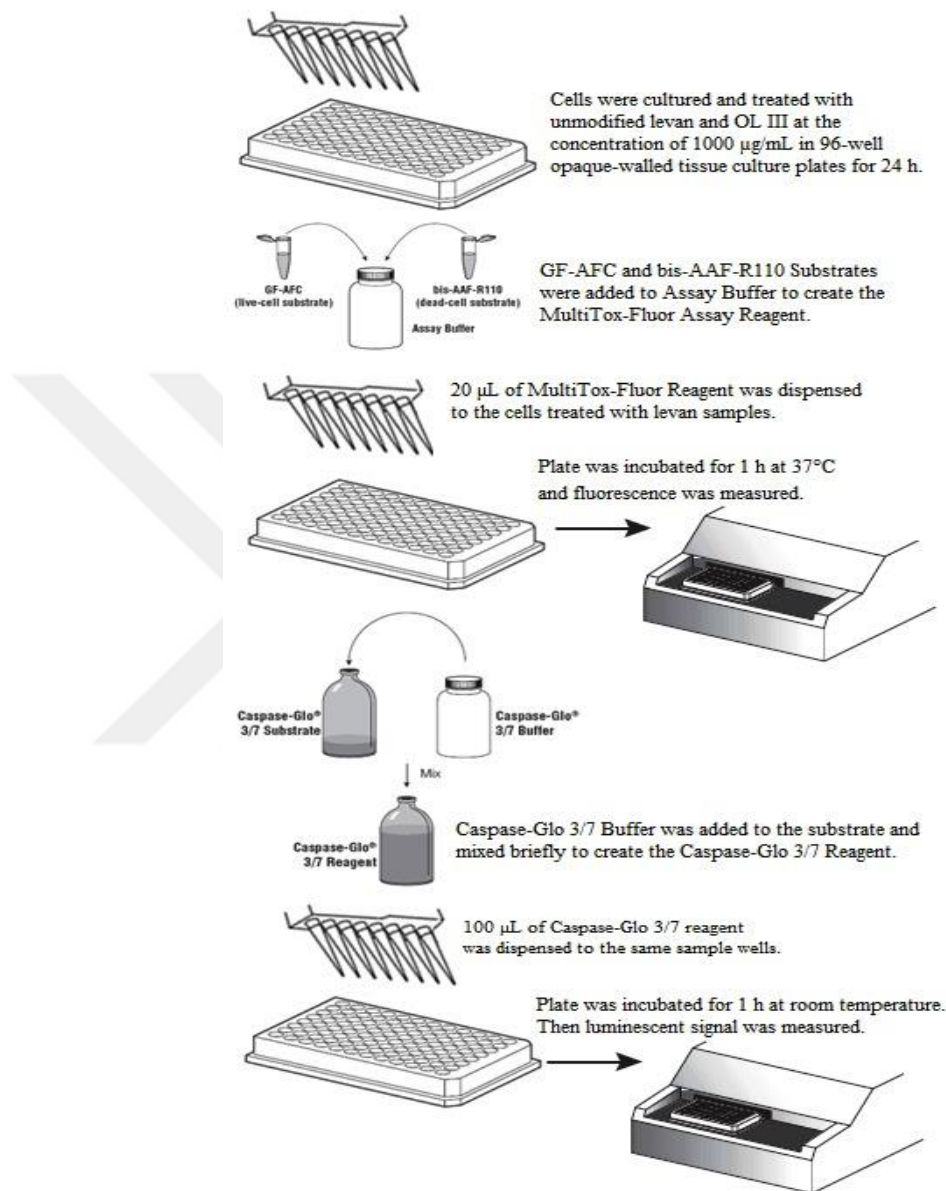


Figure 2.3. A schematic diagram of the ApoTox-Glo assay protocol (Shultz et al., 2009)

2.3.9. Total protein isolation

INS-1E cells were seeded at a density of 2×10^6 cells onto sterile flatbottom T25-flasks (Corning, USA) at 37°C in a humidified atmosphere for 48 h. Cultured cells were first pretreated with 100 µg/mL levan for 24 h and then treated with 200 µM H₂O₂ at 37°C for 1 h to induce apoptosis. Treated and control cells were trypsinized, harvested by centrifugation (380 × g at 4°C for 5 min), washed twice for 5 min each with ice-cold PBS, and lysed immediately on ice in lysis buffer. For lysis, the pellets were resuspended in 200 µL of lysis buffer supplemented with protease/phosphatase inhibitor cocktail and incubated on ice for 30 min. After cell lysis, the lysates were centrifuged at 10000 rpm for 30 min at 4°C to remove cellular debris. Supernatants were transferred to clean pre-labeled tubes, stored at -80°C, and protein concentrations were measured.

2.3.10. Measurement of protein concentration

Protein lysates were quantitated via a detergent-compatible, colorimetric, Lowry-based protein assay for equal loading to the gels using the DC Protein Assay (Bio-RAD, USA). Different concentrations (0-2 mg/mL) of bovine serum albumin (BSA) standards were prepared by serial dilutions with lysis buffer. 5 µL of standards and sample dilutions were put in 96-well plates in triplicates. 25 µL from solution A and 200 µL from solution B were added to each well, incubated for 15 min at room temperature and OD was measured at 750 nm using a PowerWave XS microplate spectrophotometer (BioTek, USA). A standard curve was prepared from the BSA standards, and the equation of the line of best fit (minimum accepted $R^2 = 0.99$) was used to calculate the sample protein concentrations. Stock sample lysates were kept on ice to minimize degradation during the course of the assay. As blank for samples, lysis buffer was used.

2.3.11. Western blotting

Effect of levan on apoptosis-related protein expression levels of INS-1E cells in high glucose condition was analyzed by Western blot analysis.

Sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis (PAGE) of proteins

Bio-Rad Mini PROTEAN 3 Cell System apparatus was used as protein electrophoresis. The glass plates were casted according to the user guide instructions. Resolving gel solution was prepared quickly, poured into the space between the glass plates using Pasteur pipette and overlaid with a thin layer of distilled water. After polymerization was complete (30 min to an hour), water was removed by tilting the gel and any remaining water was wicked away with a kimwipe. Stacking gel solution was prepared in a disposable plastic tube at an appropriate volume and desired concentration, then poured onto the surface of the resolving gel. The comb was immediately inserted into the stacking gel avoiding air bubble formation. The gel was placed in a vertical position at room temperature. While the gel was polymerizing, samples to be loaded were prepared. For this, 2x SDS sample loading buffer containing β -mercaptoethanol was added to the sample lysates, which were then placed in a 100 °C water bath for 5 min (with punctured tube lids to alleviate pressure) and then on ice for an additional 5 min. After the polymerization of the stacking gel was complete, the comb was removed carefully and each well was checked to discard any unpolymerized acrylamide. The gel was placed in the electrophoresis apparatus, 1X Running Buffer was poured into top and bottom reservoirs, and any bubble was removed carefully. Boiled lysates were quick-spun and loaded into gels at 40 μ g per well. 3-5 μ L of Precision Plus Kaleidoscope (Bio-Rad, USA) protein standard was loaded alongside the sample wells. The electrophoresis apparatus was attached to the power supply and gels were run at 80 V for 20 min or until the sample front cleared the stacking gel, followed by 100 V until the appropriate kDa range had migrated to the centre of the resolving gel, as indicated by reference to the protein standard indicator.

Transfer of proteins from SDS-PAGE to solid supports

Immediately upon completion of running process, power supply was turned off and the glass plates were removed from the apparatus. Plates were separated using a spatula and gels were removed from the electrophoresis assembly. Their edges trimmed and orientation marks added (i.e. diagonal edge cut at top-left), and then were soaked in 1X Transfer Buffer for ~10 min for equilibration. Extra thick blot papers (Bio-Rad, Canada) and one piece of Polyvinylidene fluoride (PVDF) transfer membrane (Pierce, Rockford, IL, USA) were cut to the exact size of the SDS-PAGE by wearing gloves. PVDF transfer membrane was left in methanol for ~15 min, washed with deionized water and soaked into the 1X Transfer Buffer for ~15 min along with extra thick blot papers. The transfer cassette was assembled as per the manufacturer's instructions, filled with 1X Transfer Buffer, and the transfer was performed at 100 V for 1 h 30 min on ice with ddH₂O ice blocks.

Protein immunoblot and antibodies

Following electrophoretic transfer, membranes were stained with Ponceau S solution for 5-10 min to verify transfer efficiency (i.e. substantial Ponceau S staining of the membranes), and Ponceau S stain was removed by successive washes in ddH₂O. The membranes were then soaked in TBS-T for 3 x 5 min (protein side up) to wash and equilibrate them before being blocked with 5 % BSA solution for 1 h 30 min at room temperature or 16 h at 4°C. Blocked membranes were incubated with primary antibodies (Table 2.1) in 5% BSA on a Belly Dancer shaker (Stovall, USA) for 1 h 30 min at room temperature or ~14 h at 4 °C. Membranes were then washed in TBS-T 3 x 10 min and secondary antibodies (Table 2.1) in 5 % BSA solution were used to probe the membrane on a shaker for 1 h 30 min at room temperature. Following this step, membranes were again washed with TBS-T 3 x 10 min.

Table 2.1. Antibodies used for Western Blotting analysis

Primary Antibody	Brand	Molecular Weight (kDa)	Dilution	Secondary Antibody	Dilution
β-Actin	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	45	1:5000	Anti-mouse IgG HRP-linked Ab	1:5000
Bax	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	23	1:100	Anti-rabbit IgG HRP-linked Ab	1:5000
Bcl-2	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	26	1:100	Anti-mouse IgG HRP-linked Ab	1:2000
Caspase-3	Cell Signaling Technology (Danvers, MA, USA)	35	1:1000	Anti-rabbit IgG HRP-linked Ab	1:5000

Chemiluminescent imaging and densitometry

Visualization of transferred proteins was done with Enhanced Chemiluminescence (ECL). ECL utilizes horseradish peroxidase enzyme (HRP) attached to the molecule of interest through labeling an immunoglobulin that specifically recognizes the molecule. Standard ECL was performed to detect protein-banding patterns on the immunoblots by virtue of the HRP-conjugated secondary antibodies. Chemiluminescent immunoblots were detected via 10-min High-Sensitivity Chemiluminescent exposures using a Chemidoc XRS imager (Bio-Rad, USA) supported by Quantity One software for Windows (Bio-Rad, USA). Brief Epi-White exposures were also collected in order to interpret chemiluminescent banding patterns with the membranes' visible protein ladders. Blots were analyzed via densitometry using the Quantity One software and all quantification was normalized to the β-actin level.

2.3.12. Statistical analysis

Data were presented as mean $\pm$ standard error of the mean (SEM), and all data presented here represents $n \geq 3$ independent experiments. Statistical analyses were performed using GraphPad Prism software. One-way ANOVA with post hoc Tukey's test were utilized when possible with $p < 0.05$ considered significant. Asterisks are used herein to denote significance according to the following scheme: */# = $p < 0.05$; **/## = $p < 0.01$; ***/### = $p < 0.001$.



3. RESULTS AND DISCUSSION

3.1. Biological activity of levan

For the first time with the present study, biological activities of linear levan from *Halomonas smyrnensis* AAD6^T and its aldehyde-activated form were evaluated. Antioxidant activity under high glucose condition in pancreatic β -cell line, anti-cancer activity in different cancer cell lines, cytotoxicity in the mouse fibroblast cell line, and immunostimulating activity in macrophage cell line were studied.

Levan was found to attenuate oxidative stress that is associated with decreased apoptosis in high glucose condition suggesting the antioxidant role of this polymer. It didn't show cytotoxicity in L929 cell line. Oxidized form of levan with the highest oxidation degree showed the highest anticancer activity in all cancer cell lines. Pure levan showed no toxicity on macrophages while it increased production of NO in RAW264.7 cells. Hence all these *in vitro* results suggest that levan and its derivatives could be explored as promising antioxidant, anticancer, and immunostimulating agents.

3.1.1. Bioactivity of levan on pancreatic INS-1E cell line

INS-1E cells were subjected to various concentrations of levan treatment (10, 100, 500, and 1000 $\mu\text{g/mL}$) for 24 hours at 37°C, 5 % CO₂. Cell viability was assessed via the colourimetric MTT assay, wherein MTT reagent was added to cultures at $t = 20$ h and incubated for the remaining 4 h before analysis at $t = 24$ h. At high glucose concentration (25 mM), cell viability with levan treatment (10, 100, 500, and 1000 $\mu\text{g/mL}$) was found to be 85%, 72%, 60%, and 62% respectively as compared to the untreated cells (Figure 3.1). As a result, levan showed good bioactivity on INS-1E cells and 100 $\mu\text{g/mL}$ was chosen as the appropriate concentration for all further experiments.

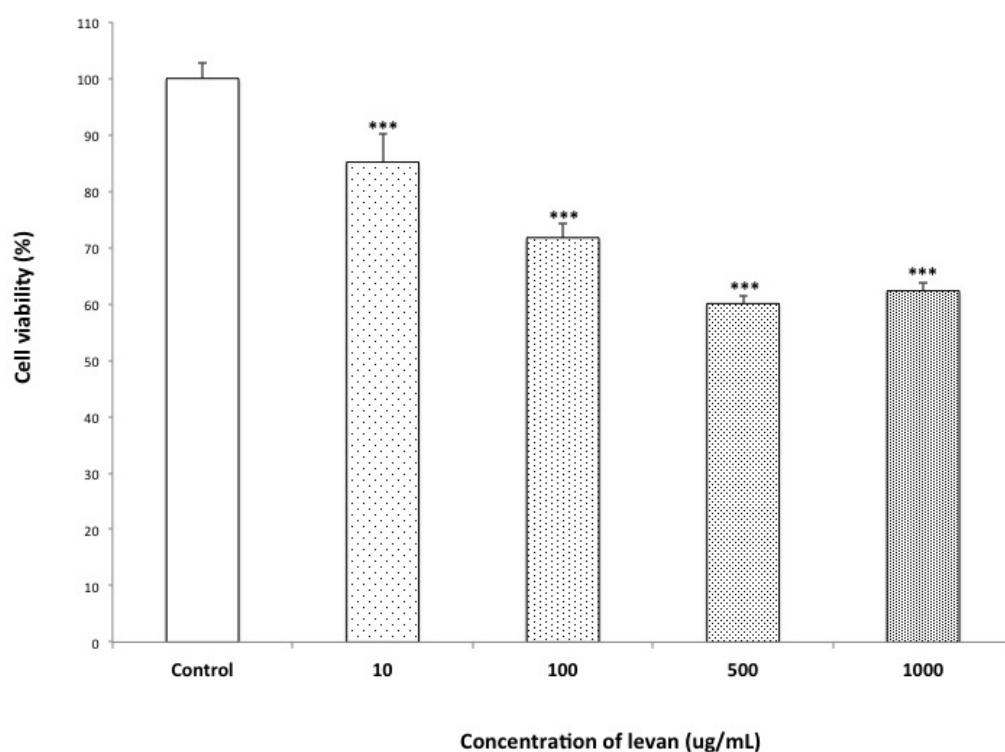


Figure 3.1. Effect of levam on INS-1E cell viability. The effect of levam on the viability of INS-1E cells in the presence of high (25 mM) glucose was assessed via measurement of mitochondrial dehydrogenase activity (MTT assay). Cells were treated in medium containing 10, 100, 500, and 1000 $\mu\text{g/mL}$ levam for 24 h. Untreated cells were used as control (G+H-). Bars represent mean $\pm$ SEM of 3 independent experiments. Data is expressed as % viability (***) = $p < 0.001$ versus Control).

3.1.2. Antioxidant role of levam

Antioxidants have a positive effect on human health by protecting human body against damage by reactive oxygen species (ROS), which attack macromolecules such as membrane lipids, proteins and DNA, lead to many health disorders such as cancer, diabetes mellitus, neurodegenerative and inflammatory diseases with severe tissue injuries (Butterfield et al., 2002). Moreover, deterioration of some foods is identified owing to oxidation of lipids or formation of undesirable secondary lipid peroxidation products. Lipid oxidation by ROS such as superoxide anion, hydroxyl radicals and H_2O_2 also causes a decrease in nutritional value of lipid foods, and affect their safety and appearance. Thus, in food and pharmaceutical industries, various synthetic commercial

antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) are used to delay the oxidation and peroxidation processes. Though, the use of these synthetic antioxidants must be under strict regulation due to potential health hazards. Hence, natural antioxidants as safe alternatives are important in the food industry. Recently, there is a considerable interest in the food industry as well as pharmaceutical industry for the development of antioxidants from natural sources. Antioxidant activity can be determined by various methods such as 1,1-diphenyl-2-picryl hydrazil (DPPH) radical scavenging, lipid peroxide inhibition, ferric reducing antioxidant power (FRAP), nitric oxide (NO) scavenging, ABTS radical scavenging, superoxide radical and hydroxyl radical scavenging assays (Wijesekara et al., 2011). In the present study, the antioxidant activity of levan from *H. smyrnensis* AAD6^T by using two different cell-based antioxidant methods; 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) assay and caspase 3/7 assay was investigated.

3.1.2.1. Effect of levan on H₂O₂-induced oxidative stress in INS-1E cells

H₂O₂-induced oxidative stress was investigated in cultured INS-1E cells. Intracellular ROS levels were assessed using the CM-H₂DCFDA assay and measured via flow cytometry. Both high glucose concentration and 1-h treatment with 200 μ M H₂O₂ (G+H+) caused a significant increase in ROS generation (Figure 3.2) as indicated by increased mean FL1 fluorescence versus control (G-H-). These data indicate a pronounced elaboration of ROS resulting from H₂O₂ treatment of the INS-1E cells in high glucose condition. Given the observed H₂O₂-induced ROS elaboration, the potential protective antioxidant effect of levan was investigated. 24-h pre-treatment with 100 μ g/mL levan abrogated the significant increase in H₂O₂-induced ROS generation. This finding indicates that the observed H₂O₂-induced increase in ROS was attenuated by pre-treatment with levan.

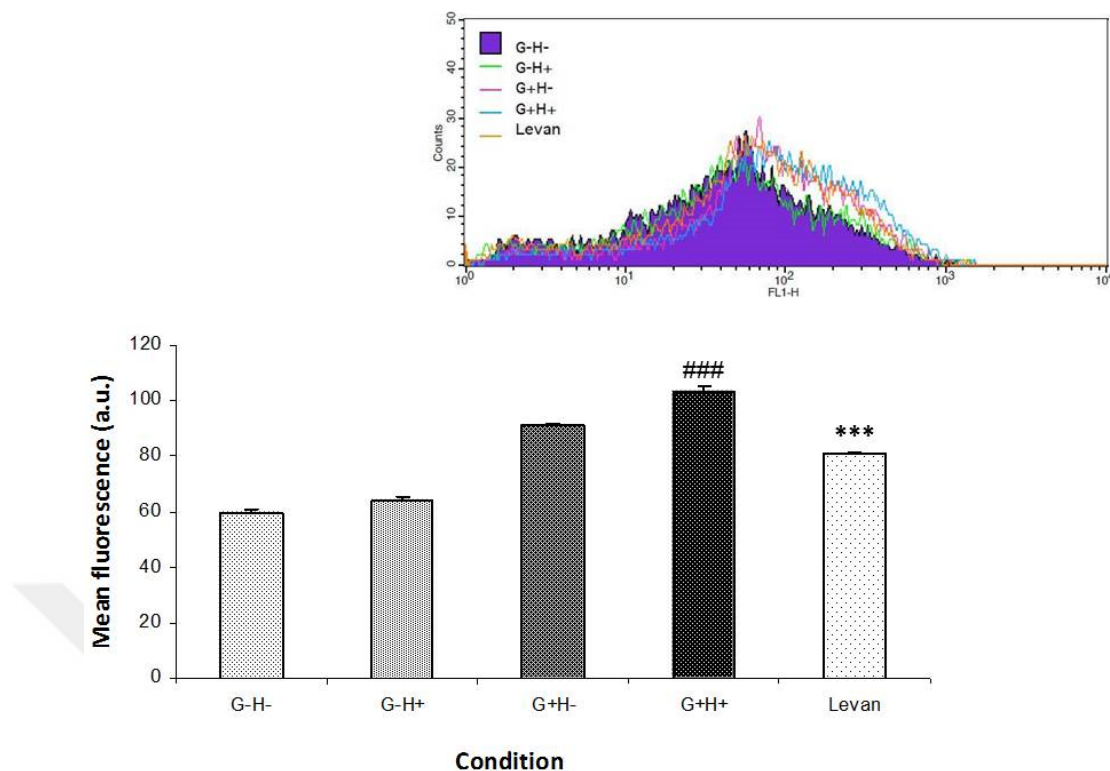


Figure 3.2. Effect of levan on H₂O₂-induced oxidative stress in INS-1E cells. The effect of H₂O₂ and levan on intracellular ROS levels in INS-1E cells was assessed via fluorescent detection of oxidation-induced esterase activity (CM-H₂DCFDA assay), and analyzed via flow cytometry. Bars represent mean $\pm$ SEM of 3 independent experiments. Data is expressed as mean fluorescence arbitrary units (a.u.) (### = $p < 0.001$ versus G-H-; *** = $p < 0.001$ versus G+H+).

3.1.2.2. Effect of levan on H₂O₂-induced INS-1E apoptosis

To investigate the potential cytotoxicity of the observed H₂O₂-induced ROS elaboration, the activity of apoptotic proteins, caspases 3 and 7, was assessed using the CaspaTag assay and measured via flow cytometry. H₂O₂ caused a significant increase in apoptosis (Figure 3.3) as indicated by increased mean FL1 fluorescence versus control. These data indicate that this concentration of H₂O₂ was cytotoxic to the INS-1E cells. Given the observed levan-mediated prevention of H₂O₂-induced ROS elaboration, the potential cytoprotective effect of levan was investigated. Pre-treatment with levan abrogated the significant increase in H₂O₂-induced apoptosis, maintaining it at near control levels (Figure 3.3). This finding suggests that H₂O₂-induced activation of effector caspases can be attenuated by treatment with levan.

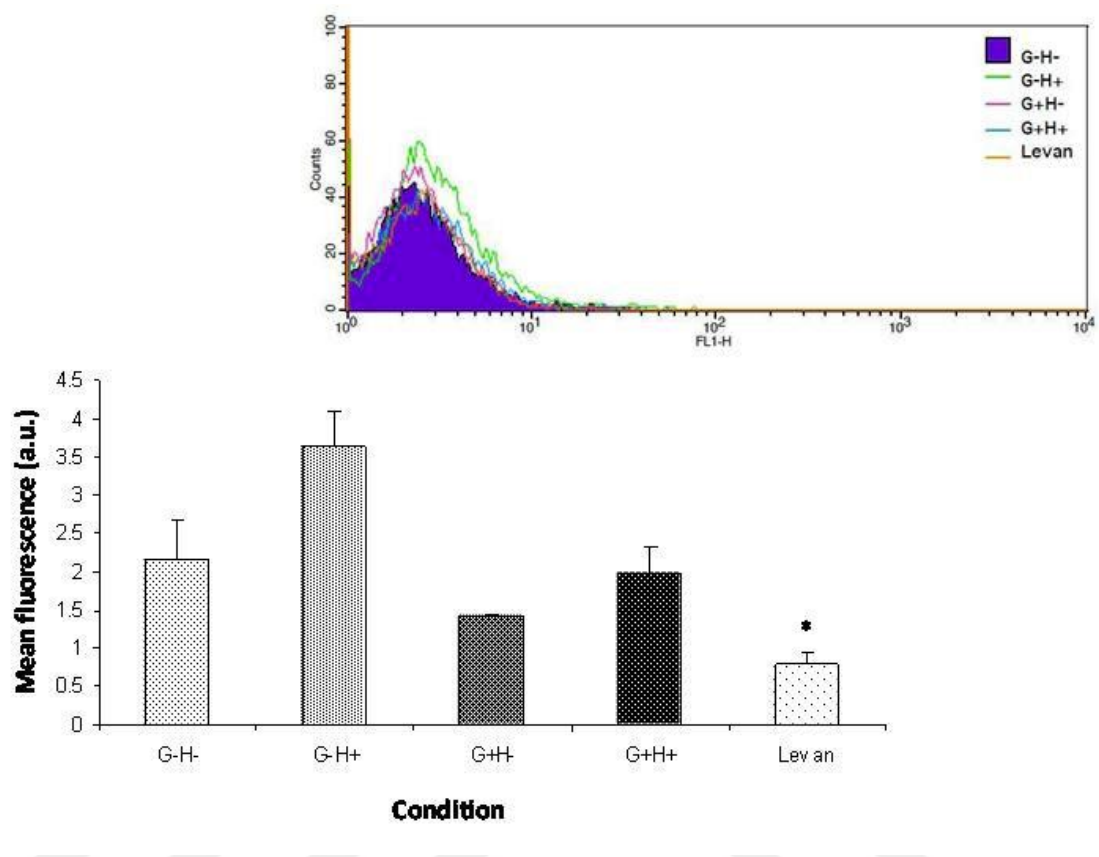


Figure 3.3. Effect of levan on H₂O₂-induced apoptosis in INS-1E cells. The effect of levan on INS-1E cellular apoptosis was assessed via the fluorescent detection of active caspase-3 and -7 (CaspaTag 3/7 assay), and analyzed via flow cytometry. Bars represent mean $\pm$ SEM of 3 independent experiments. Data is expressed as mean fluorescence arbitrary units (a.u.) (*= $p < 0.05$ versus G+H+).

3.1.2.3. Effect of H₂O₂ and levan on Caspase 3 activity and Bax/Bcl2 ratio

To further investigate the H₂O₂-induced apoptosis Caspase 3, Bax, and Bcl-2 protein expression was assessed via immunoblotting. Caspase 3 protein expression did not change significantly after pre-treatment of levan (Figure 3.4 a) when compared to control while levan decreased bax/bcl-2 ratio (Figure 3.4 b) when compared to control depending on antioxidant activity. The ratio between Bax and Bcl-2 was measured using band-densitometry. As shown in (Fig 3.4 b), H₂O₂-

induced stress increased the ratio (G+H+ compared with the control G-H-), whereas levan treatment for 24 h decreased the ratio (1.5 fold decrease compared with the G+H+).

Apoptosis is an essential process for maintaining cellular homeostasis under normal conditions. One of the best characterized mechanisms to induce cell death is the release of proapoptotic proteins from the mitochondria into the cytosol. Members of the Bcl-2 family, including Bcl-2 and Bax, are central regulators of apoptosis that promote (Bax) or inhibit (Bcl-2) cell death. Although there is a competition between Bax and Bcl-2, each is able to regulate apoptosis independently. Hence, the ratio of Bax/Bcl-2 is an important indicator of apoptosis. The Bax/Bcl-2 ratio appears more important than the individual Bax or Bcl-2 level in determining a cell's vulnerability to apoptosis; high Bax/Bcl-2 ratios lead to greater apoptotic activity (Jarskog et al., 2004).

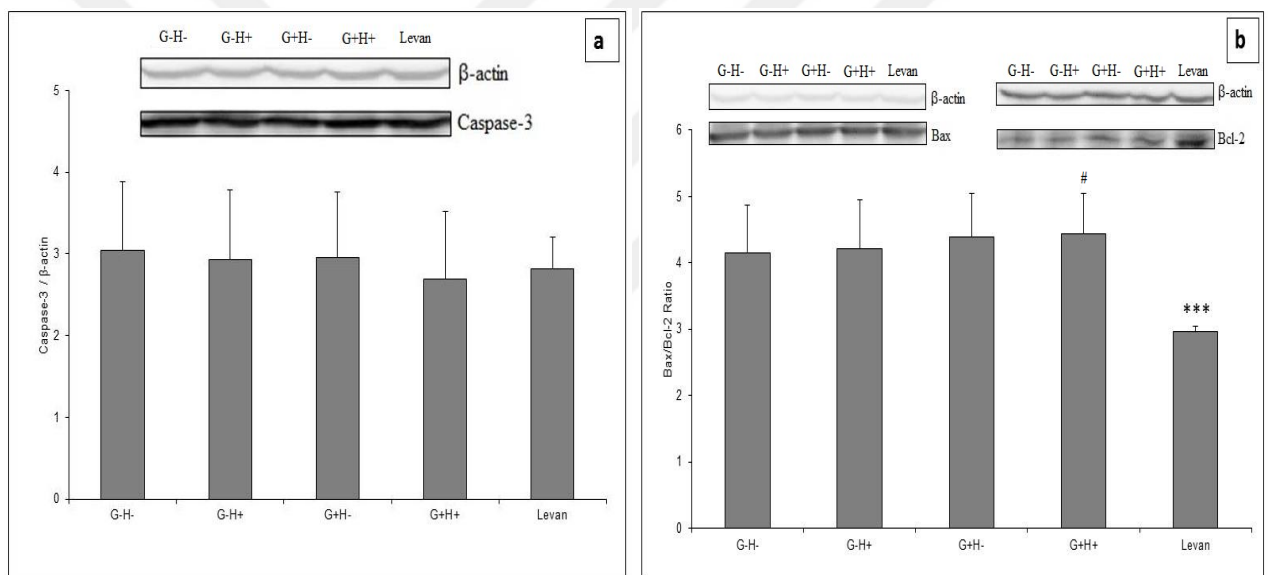


Figure 3.4. Western blot ratio analysis of caspase-3 and β-actin (a) bax/bcl-2 ratio (b) The effect of levan on Caspase 3, Bax, and Bcl-2 protein expressions in INS-1E cells was assessed via immunoblotting. Bars represent mean ± SEM of 3 independent experiments (# = $p < 0.05$ versus G-H-; *** = $p < 0.001$ versus G+H+).

In this study, we demonstrated that H_2O_2 treatment increased the Bax/Bcl-2 expression ratio, suggesting that H_2O_2 induces mitochondrial dysfunction by increasing the Bax/Bcl-2 ratio, followed by mitochondrial ROS accumulation in pancreas cells. In order to clarify the mechanism of levan protection, the expressions of key apoptotic related molecules was investigated. Levan increased the levels of antiapoptotic Bcl-2 protein level and the increase in Bcl-2 protein observed in pancreas cells of levan-treated cells might be a result of increasing expression of Bcl-2.

Bax/Bcl-2 ratio decreased with levan treatment. In conclusion, the present study demonstrates that oxidative stress was induced during the development of diabetic complications, and apoptosis was supposed to contribute to the loss of pancreas cells. The results also indicated that the levan effectively reduced oxidative injury and inhibited apoptosis by modifying bcl-2 expression and bax/bcl-2 ratio. The conclusions can open up new avenues in the search for natural antioxidants that can be employed successfully in further clinical trials.

3.1.3. Cytotoxicity of levan on L929 cell line

Cytotoxicity of levan was investigated in murine fibroblast cell line L929. Cells were subjected to various concentrations of levan treatment (10, 100, 500, and 1000 $\mu\text{g/mL}$) for 24 hours at 37°C , 5 % CO_2 . Cell viability was assessed via the colourimetric WST-1 assay. Cell viability with levan treatment (10, 100, 500, and 1000 $\mu\text{g/mL}$) was found to be 100%, 101%, 95%, and 78% respectively as compared to the control cells (Figure 3.5). As a result, samples didn't show cytotoxicity even when cells were treated with levan at a very high concentration (1000 $\mu\text{g/mL}$) while it was about 95-100% at the 10-500 $\mu\text{g/mL}$ concentration ranges.

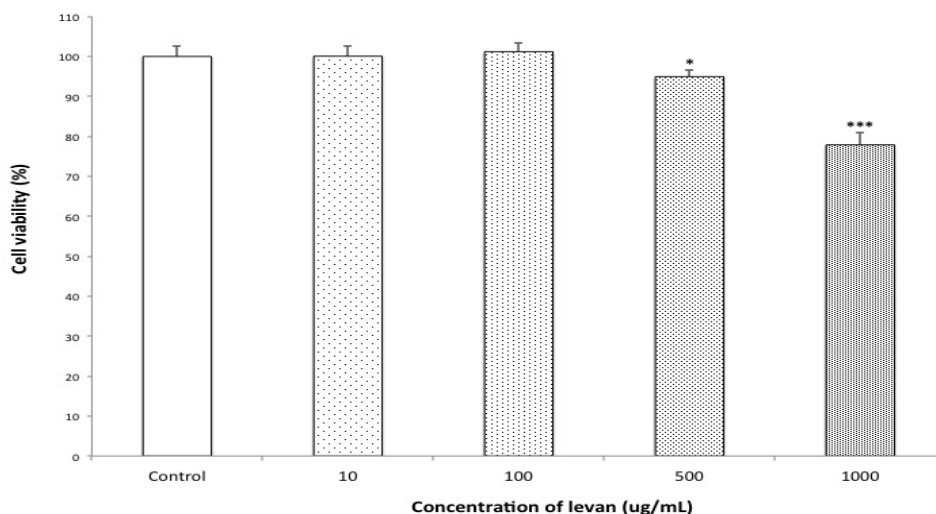


Figure 3.5. Effect of levan on L929 cell viability. The effect of levan on the viability of L929 cells was assessed via WST-1 assay. Cells were treated in medium containing 10, 100, 500, and 1000 $\mu\text{g/mL}$ levan for 24 h. Untreated cells were used as control. Bars represent mean $\pm$ SEM of 3 independent experiments. Data is expressed as % viability (* = $p < 0.05$ versus Control; *** = $p < 0.001$ versus Control).

3.1.4. Anti-cancer activity of levan on cancer cell lines

The anti-cancer activity of levan was investigated against human cancer cell lines such as A549, HepG2/C3A, AGS, and MCF-7 using WST-1 assay. In the literature, levan concentration for anti-cancer activity studies covers a wide range by varying from 3.15 $\mu\text{g/mL}$ (Dahech et al., 2012) up to 1000 $\mu\text{g/mL}$ (Yoon et al., 2004). Also in this study, all tested cells were treated with levan at a broad concentration range of 10-1000 $\mu\text{g/mL}$ for 24 hours at 37°C, 5 % CO_2 . Levan was found to display growth inhibition against cancer cell lines at the highest dose (1000 $\mu\text{g/mL}$). Anti-cancer activity with 1000 $\mu\text{g/mL}$ levan treatment against A549, HepG2/C3A, AGS, and MCF-7 was found to be 10%, 21%, 30%, and 16% respectively as compared to the control cells (Fig 3.6). As a result, levan showed the highest anti-cancer effect against AGS cells (Fig. 3.6 C) among the cancer cell lines while it showed no effect against A549 cells (Fig. 3.6 A).

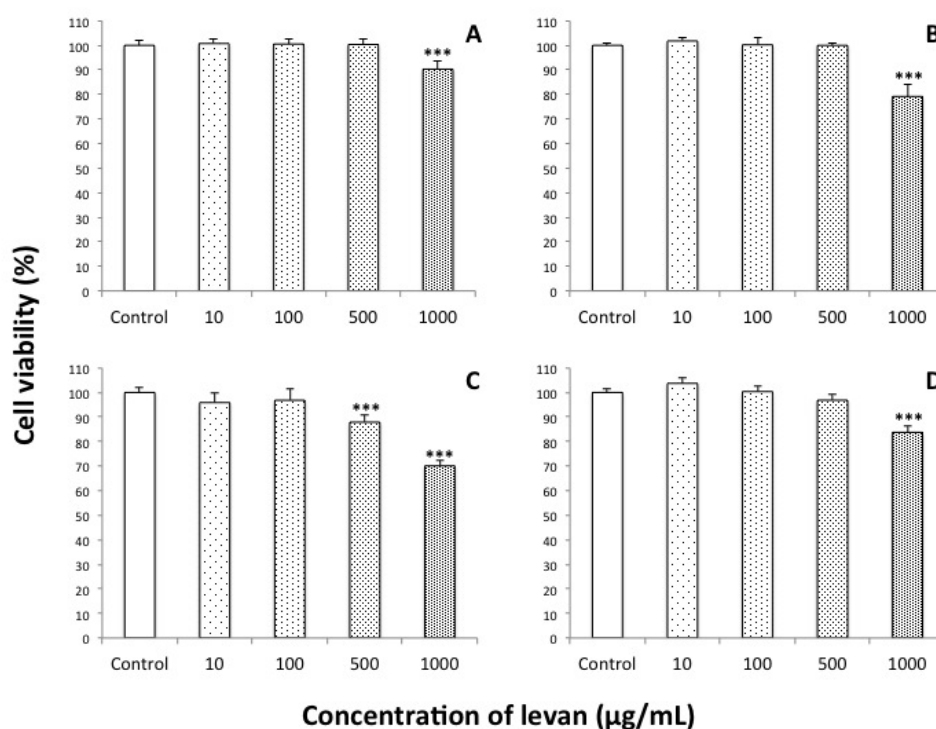


Figure 3.6. Effect of levan on A549 (A), HepG2/C3A (B), AGS (C), and MCF-7 (D) cell viability. The effect of levan on the viability of cancer cells was assessed via WST-1 assay. Cells were treated in medium containing 10, 100, 500, and 1000 $\mu\text{g/mL}$ levan for 24 h. Untreated cells were used as control. Bars represent mean $\pm$ SEM of 3 independent experiments. Data is expressed as % viability (*** = $p < 0.001$ versus Control).

Although recent developments in diagnosis and therapy have increased the survival of patients suffered from cancer, there is still no effective prophylactic or therapeutic method for cancer so far. Chemotherapy is one of the most frequently used therapeutic treatments for cancer, but most of the anti-cancer drugs currently used in chemotherapy are cytotoxic to normal cells, leading to multiple organ toxicity that includes hemopoietic suppression and immunotoxicity. Screening for natural anti-cancer agents with low toxicity and high efficacy is a growing interest in cancer inhibition and therapy. Nowadays, the advancing studies have confirmed the anti-cancer potential of the promising polysaccharides (Ren et al., 2016).

There are several reports in the literature on the anti-cancer effect of levan produced by other microbial systems that support the findings of the present study. Kyung et al. (2004) studied effect of molecular weight on anti-cancer activity of branched levan from *Microbacterium laevaniformans* against SNU-1 (human stomach carcinoma) and HepG2 cancer cell lines at the dosage level of 100-800 µg/mL. They found that levan with higher molecular weight showed the greatest cell growth inhibition (84.6%) on SNU-1 cells at 400 µg/mL while levan with lower molecular weight showed the greatest cell growth inhibition (46.32%) on HepG2 cells at 800 µg/mL. Bayoumi et al. (2013) showed anti-proliferative property of sulphated levan from *B. subtilis* against HepG2 cell line. They studied different doses of levan (12.5-100 µg/mL) and found that the greatest inhibition (95%) was achieved by the lowest dose (12.5µg/mL) of levan. Abdel-Fattah et al. (2012) tested the anti-cancer activity of levan from *B. subtilis* with different molecular weights and its sulfated forms against HepG2, HCT-116 (colon carcinoma), HeLa (cervical carcinoma), histiocytic lymphoma, MCF-7 and lymphoblastic leukemia cells and found that higher molecular weight levan and its sulfated form resulted in a remarkable growth inhibition at the concentration of 100 µg/mL. Sulfated levan with higher molecular weight showed variable cytotoxic effect against different cell lines in the following order: Hep-G2 > HeLa > HCT-116 > 1301 > MCF-7 cells. *In vitro* anti-cancer activity of levan from *B. licheniformis* against HepG2, CaCo2 (human colon adenocarcinoma cell line) and WRL68 tumor cell lines at varying concentrations (3.125-200 mg/mL) was studied and relatively high activity was observed against the HepG2 cell line at the highest concentration (200 mg/mL) (Dahech et al., 2012). Esawy et al. (2013) showed cancer protective property of high molecular weight levan with branched structure from *B. subtilis* M in murine hepatoma Hepa1c1c7 cells. Liu et al. (2012) successfully acetylated, phosphorylated and benzylated levan from *Paenibacillus polymyxa* EJS-3 and studied anti-cancer activities of the samples at different concentrations (5-400 µg/mL) *in vitro*. They found that

different derivatives of levan exhibited higher antiproliferative activity than the natural levan against human gastric cancer BGC-823 cells at a concentration of 400 $\mu\text{g/mL}$. Yoon et al. (2004) studied the effect of branching on anti-cancer activity of levan from *M. laevaniformans* at the concentration range between 200-1000 $\mu\text{g/mL}$ and the strongest inhibiting activities were shown at the highest dosage (1000 $\mu\text{g/mL}$) of the levan. As the branching degree decreased, the anti-cancer activity on SNU-1 cells linearly decreased while the activity rapidly dropped when the branching increased on HepG2 cells.

3.1.5. Immunostimulating activity of levan

3.1.5.1. Effect of levan on RAW 264.7 cell viability

As the health benefits of functional polysaccharides continue to be elucidated, research on fructans has received renewed interest. Fructans are well known to be endowed with the capacity to modulate the immunological properties of the immune system by direct or indirect mechanisms. Levan-type fructans have been noted as health-functional polysaccharides showing potential immune-stimulatory activity with similar modes of action. Given that macrophages perform key functions in the induction of both innate and adaptive immune responses, the effects of levan on RAW264.7 murine macrophage cells were preliminarily evaluated by measuring the rate of macrophage proliferation and phagocytosis. First, the influence of levan on macrophage viability was evaluated by WST-1 assay. With the viability of control wells considered to be 100%, the viability ratios of macrophages activated by levan was determined at the indicated concentrations. As shown in Figure 3.7, when RAW 264.7 macrophages were treated with levan, no significant change in cell viability was observed.

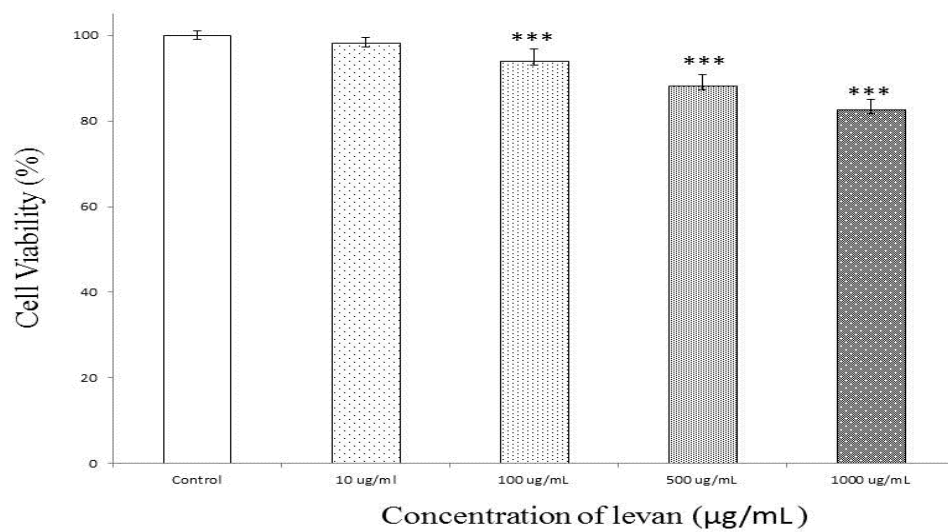


Figure 3.7. Effect of levan on RAW 264.7 cell viability. The effect of levan on the viability of RAW 264.7 cells was assessed via WST-1 assay. Cells were treated in medium containing 10, 100, 500, and 1000 µg/mL levan for 24 h. Untreated cells were used as control. Bars represent mean $\pm$ SEM of 3 independent experiments. Data is expressed as % viability (***) = $p < 0.001$ versus Control).

3.1.5.2. Effect of levan on nitric oxide production by macrophages

Macrophage is one of the most important antigen-presenting cells (APCs) of innate immunity. The first event of innate immune response in mammals is activation of macrophages; these cells engulf invading pathogens and secrete intercellular messengers, such as IL-6, TNF- α , and NO. Furthermore, macrophages may be the first line of tumor resistance, as they rapidly colonize and secrete cytokines that attack tumor cells and they activate dendritic cells and natural killer cells (Zong et al., 2012).

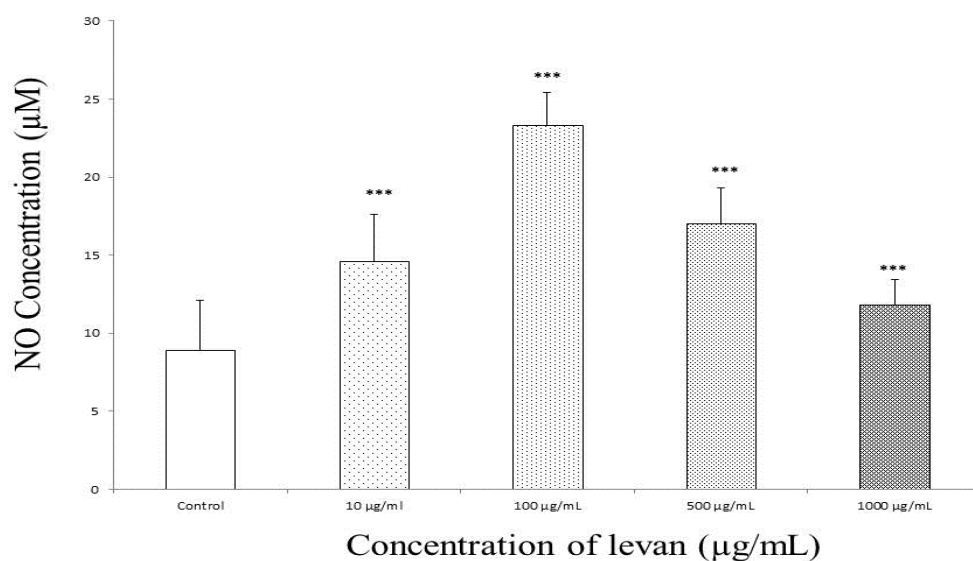


Figure 3.8. Effect of levan on NO concentration. The concentration of NO in macrophage culture supernatant was determined by using colorimetric Griess reaction assay. Cells were treated in medium containing 10, 100, 500, and 1000 μg/mL levan for 24 h. Untreated cells were used as control. Bars represent mean $\pm$ SEM of 3 independent experiments (*** = $p < 0.001$ versus Control).

As shown in Figure 3.8, a dose dependent change in the concentration of NO in macrophage culture supernatant was determined and at the concentration of 100 μg/mL levan, NO concentration was 2.6-fold higher than the control. Hence levan showed no toxicity on macrophages while it increased production of NO in RAW264.7 cells. All these data suggest that levan has strong immunomodulation effects *in vitro*. In conclusion, levan showed significant immunomodulating activity, such as inducing production of the inflammatory cytokine, NO. Moreover, this cytokine may be associated with the anti-cancer effects of supernatants of RAW264.7 cells induced by levan. Further understanding the signaling pathways and the activation effects of macrophage induced by levan might provide novel insights into the mechanisms of immunomodulation and new opportunities for rational application of levan.

Park et al. (2008) examined the tumoricidal activity, phagocytosis and nitric oxide (NO) production in levan treated RAW264.7 cells and found that the NO production, tumoricidal and phagocytic activities were significantly increased in treated cells. Their results suggested that tumoricidal activity induced by levan was mediated by NO production. Dong et al. (2015) studied *in vitro* biological activity of levan, from the medicinal plant *Curcuma kwangsiensis*, containing a

backbone composed of ($\beta 2 \rightarrow 6$)-linked D-Fruf residues and single β -D-Fruf residues as side chains branched at the O-1 position along the backbone on RAW 264.7 murine macrophage cells. Levan showed significant immunostimulatory activity based on its ability to stimulate macrophage proliferation and enhance phagocytosis. The branching degree of the polysaccharides is a structural feature associated with the presence of linked monosaccharide residues or linked chains to their backbone. Immunostimulatory polysaccharides may have a linear backbone without branches or have more or less complex branches linked to their backbone. Depending on this structural feature, biological activity may be affected (Ferreira et al., 2015). Immunostimulatory polysaccharides can interact directly or indirectly with the immune system, triggering several cellular/molecular events, leading to immune system activation (Figure 3.9). Monocytes, macrophages, and neutrophils are the main targets described responding to these molecules. When searching for reported immunostimulatory activities of polysaccharides, most studies addressed macrophage function. Several *in vivo* studies have shown an enhanced macrophage phagocytic function induced by treatment with immunostimulatory polysaccharides. Beyond phagocytosis, immunostimulatory polysaccharides may also enhance other macrophage functions, such as production of ROS and NO, secretion of pro-inflammatory cytokines, such as tumour necrosis factor (TNF- α), interleukin (IL)-1, IL-6, IL-8, IL-12, and interferon (IFN)- γ . Moreover, several immunostimulatory polysaccharides have also been reported to affect macrophage proliferation and differentiation (Ferreira et al., 2015). Cytokine secretion as a cellular response is characterized by coordinated activation of receptors and various signaling pathways. TLRs play a key role in innate immune responses to enhance the body's defense system against bacterial and viral infections (Kawai and Akira, 2006). Among TLRs, TLR4 is known to induce production of TNF- α and IL-6. Moreover, because many studies have showed that TLR4 mediates polysaccharide-induced cytokine production (Hsu et al., 2004; Li and Xu, 2011). Various natural polysaccharides, such as polysaccharides from *Ganoderma lucidum* (Hsu et al., 2004; Shao et al., 2004), *Polyporus umbellatus* (Li and Xu, 2011), safflower (Ando et al., 2002), and *Platycodon grandiflorum* (Yoon et al., 2003) induce activations of macrophages via TLR4 receptor. A previous study suggested that *Astragalus* polysaccharides may interact with TLR4 in affecting peritoneal macrophages (Shao et al., 2004).

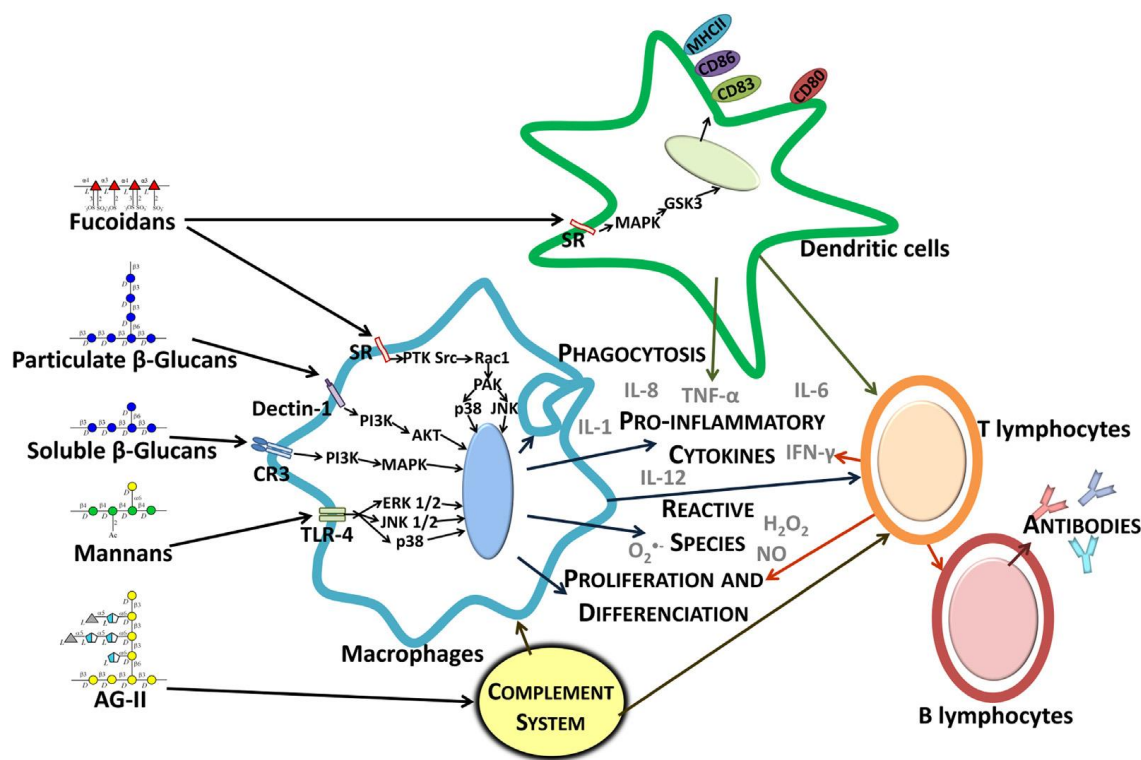


Figure 3.9. Illustration of immune system activation by immunostimulatory polysaccharides after interaction and trigger of several molecular/cellular events (Ferreira et al., 2015).

3.2. Oxidation of levan

Modified form of the linear levan from *H. smyrnensis* AAD6^T was synthesized by periodate oxidation and characterized. A schematical presentation of the periodate oxidation of levan is shown in Figure 3.10 A. Upon the oxidation reaction of levan with potassium periodate, selective scission of chemical bonds between two carbon atoms (C3 and C4) in the fructose ring occurs resulting in the formation of two reactive aldehyde groups. This aldehyde group shows strong, prominent, stake-shaped band around 1710 - 1720 cm⁻¹ due to the highly polar C=O bond. As shown in Figure 3.10 B, all samples exhibited the characteristic absorption of levan at 2890, 2940, and 3350 cm⁻¹, or 930, 1040, and 1110 cm⁻¹ (Sima et al., 2011). For oxidized derivative of levan, a new absorption appeared at around 1730 cm⁻¹ which was assigned to the highly polar C=O bond due to aldehyde groups. This peak demonstrated that the oxidized derivative was successfully synthesized. Increase in reaction time lead to an increase in the aldehyde concentration. To further verify the presence of aldehyde groups in the modified levan, the ¹H NMR spectrum of oxidized levan with the highest oxidation degree, OL III, was obtained (Figure 3.10 C). In this spectrum, the signal at ~ δ 9.40 ppm was assigned to the aldehyde proton.

Furthermore, the amount of aldehyde groups formed by the oxidation of levan was quantified by thiosulfate titration. As a result, the oxidation degrees were calculated to be 0.08, 0.16, and 0.32 (OL I, OL II, OL III, respectively).

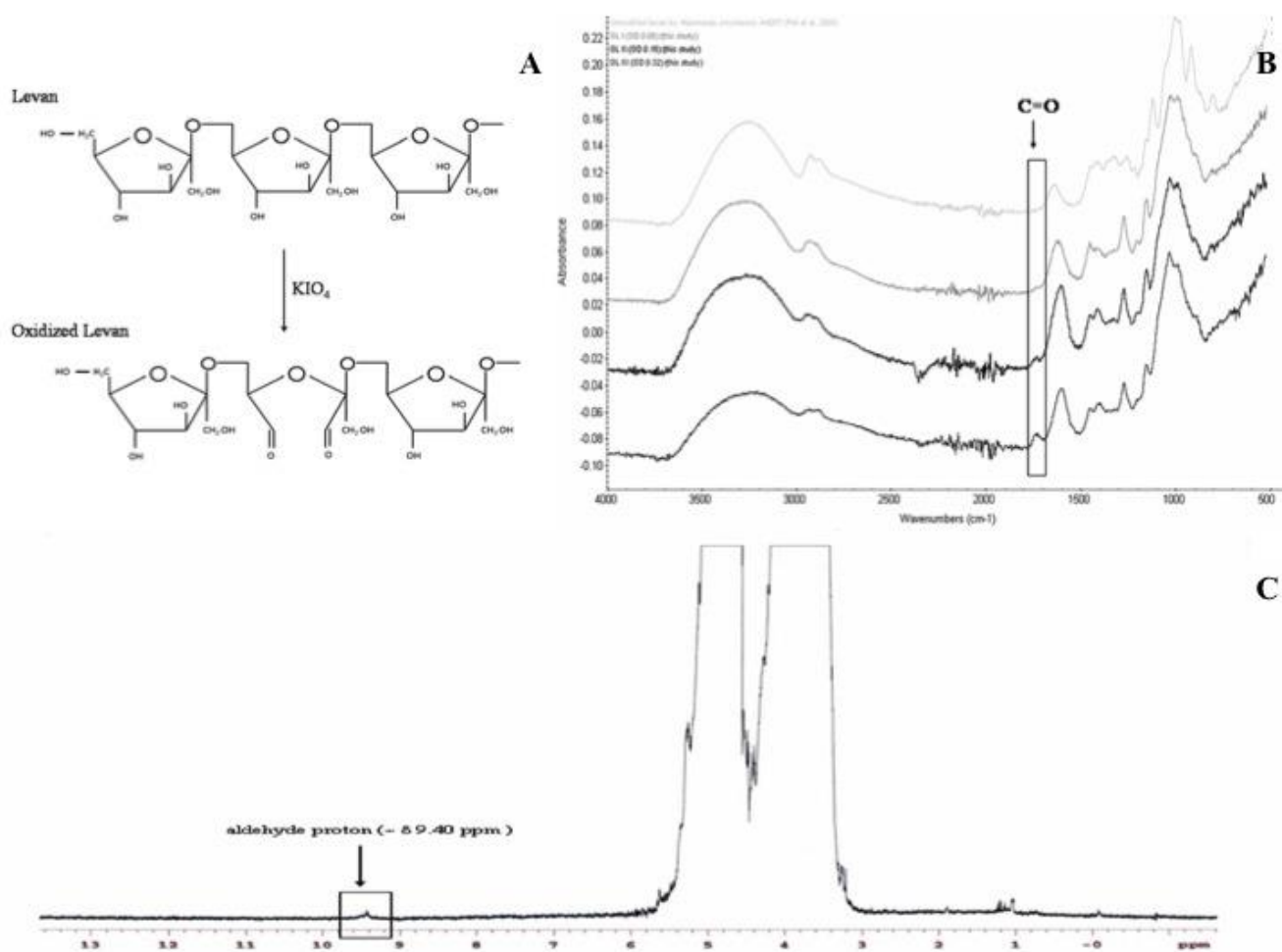


Figure 3.10. The structure of levan and its oxidized form (A) FTIR absorption spectra of unmodified levan and its oxidized derivatives, OL I, OL II, and OL III (from light to intense color) (B) ^1H NMR spectrum of OL III (C)

3.3. Biological activity of oxidized levan

3.3.1. Cytotoxicity of oxidized levan on L929 cell line

Cytotoxicity of oxidized levan was investigated in murine fibroblast cell line L929. Cells were subjected to various concentrations of levan treatment (10, 100, 500, and 1000 $\mu\text{g/mL}$) for 24 hours at 37°C, 5 % CO_2 . Cell viability was assessed via the colourimetric WST-1 assay. Cell viability with levan treatment between 10-500 $\mu\text{g/mL}$ was found to be around 100% while it was about 70% at the concentration of 1000 $\mu\text{g/mL}$ as compared to the control cells (Figure 3.11). As a result, oxidized levan samples didn't show cytotoxicity even when cells were treated with levan at a very high concentration (1000 $\mu\text{g/mL}$).

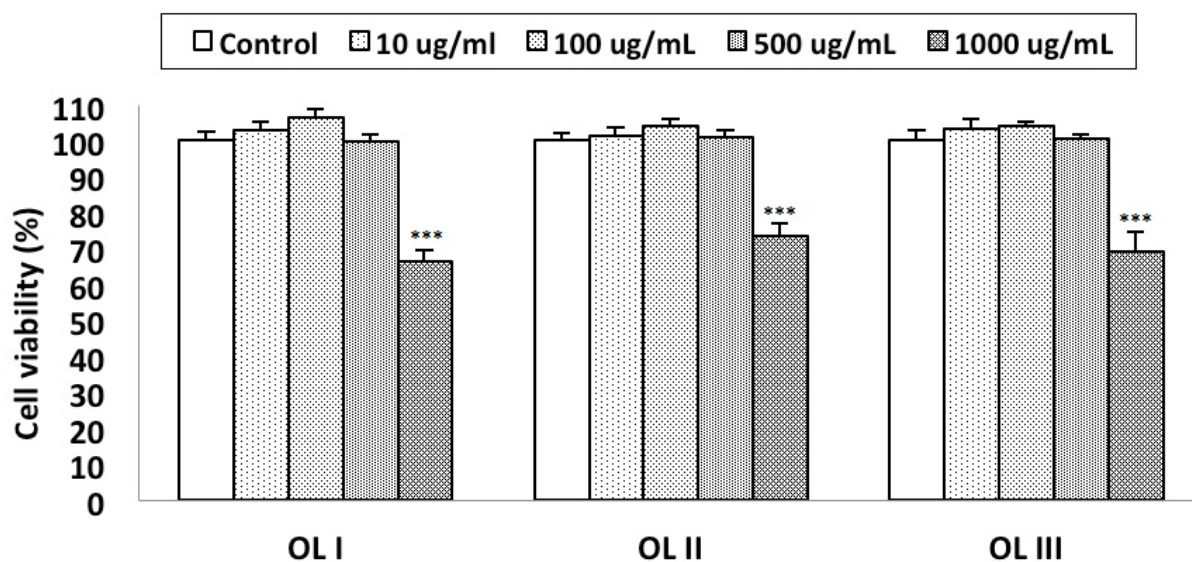


Figure 3.11. Effect of oxidized levan on L929 cell viability. The effect of oxidized levan samples on the viability of L929 cells was assessed via WST-1 assay. Cells were treated in medium containing 10, 100, 500, and 1000 $\mu\text{g/mL}$ oxidized levan for 24 h. Untreated cells were used as control. Bars represent mean $\pm$ SEM of 3 independent experiments. Data is expressed as % viability (***) = $p < 0.001$ versus Control).

3.3.2. Anti-cancer activity of oxidized levan on cancer cell lines

The anti-cancer activity of aldehyde-activated derivatives of levan was investigated against human cancer cell lines such as A549, HepG2/C3A, AGS, and MCF-7 using WST-1 assay. All tested cells were treated with levan samples at a broad concentration range of 10–1000 $\mu\text{g/mL}$. Results are shown in Figure 3.12. Generally, all samples were found to display growth inhibition against cancer cell lines at the highest dose (1000 $\mu\text{g/mL}$). Notably, aldehyde-activated levan showed higher anti-cancer activity than unmodified levan against all cancer cell lines. OL samples showed higher anti-cancer activity against A549 (Figure 3.12 A) and HepG2/C3A (Figure 3.12 B) cells than AGS (Figure 3.12 C) and MCF-7 (Figure 3.12 D) cells. By increasing the oxidation degree, the anti-cancer activity also increased. Hence, it was clearly demonstrated that the introduction of chemically modified group, aldehydes, into the linear levan molecule could significantly enhance the anti-cancer activity of levan.

When levan is oxidized, the distance between C3 - C4 atoms is increased and it leads to an increase in C5–O–C2 angle. It is suggested that this region can be connected with the immunological activity of levan (Gonta et al., 2004). Levan derivatives may exert their anti-cancer action via activation of the immune response. It is known in the literature that polysaccharides have the immunotherapeutic property to inhibit growth of cancer cells and exert carcinostatic action (Ooi et al., 2000). Some polysaccharides may possess direct cytotoxic effects on cancer cells even though some of them do not attack cancer cells directly, but produce their anti-cancer effects by activating different immune responses in the host which means that the anti-cancer activities of polysaccharides result primarily from their immunopotential effects (Wasser et al, 2002; Cha et al., 2012).

Chemical modification enhances the immunopotential and anti-cancer activities of polysaccharides, due to the change of substituent groups and chain conformation of polysaccharides. Polysaccharides can interact with receptors of immune cells to trigger immunological responses, including anti-cancer activity (Ooi et al., 2000; Cha et al., 2012). TNF- α , IL-1 β , and IL-6 are important regulators of host defense against tumor cells. The increase in production of cytokines would suggest an enhanced ability of the host to combat the growth of tumors. Macrophages can be activated by polysaccharides to kill tumor cells by production of TNF- α and interleukins. The bioactivities of polysaccharides and polysaccharide-protein complexes are dependent on binding on the surface receptor of immune cells (Cha et al., 2012).

Wang et al. (1997) showed in their study that a polysaccharide from *Ganoderma lucidum* had a strong stimulatory effect both on macrophages and on T lymphocytes in promoting the synthesis/release of various cytokines, including IL-1 β , IL-6, TNF- α and IFN- γ , which were known to play important roles in the regulation of immune responses and suppression of tumor-cell growth. These results were in agreement with those reported by Ooi et al. (2000) showing that polysaccharides from mushrooms exert anti-cancer effects via activation of different immune responses in the host rather than by directly attacking cancer cells.

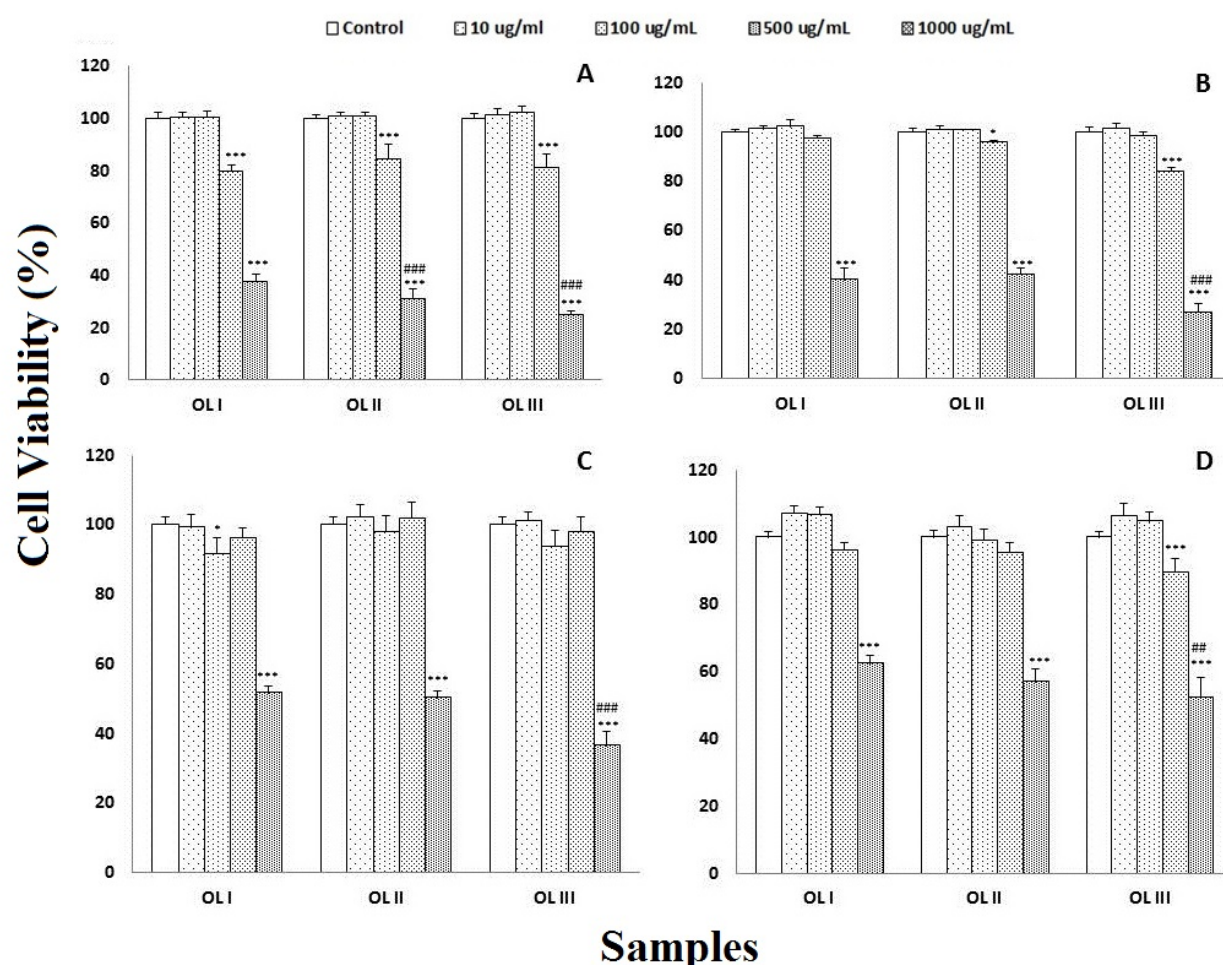


Figure 3.12. *In vitro* anti-cancer activity of oxidized derivatives of levan against **A549** (human lung adenocarcinoma) **(A)**, **HepG2/C3A** (human liver hepatocellular carcinoma) **(B)**, **AGS** (human gastric adenocarcinoma) **(C)**, and **MCF-7** (human breast adenocarcinoma) **(D)** Bars represent mean $\pm$ SEM of 3 independent experiments. Data is expressed as % viability. * = $p < 0.05$ versus Control; *** = $p < 0.001$ versus Control; ## = $p < 0.01$ versus OL I (1000 μ g/mL); ### = $p < 0.001$ versus OL I (1000 μ g/mL)

3.3.3. Viability and caspase 3/7 activity

To determine whether the observed anti-cancer activity was caused by apoptosis, viability and caspase 3/7 activity of MCF-7 cells treated with levan and OL III were determined via ApoTox-Glo triplex assay. According to WST-1 results, 1000 $\mu\text{g/mL}$ concentration of OL III reduced cell viability of MCF-7 cells three-fold when compared with unmodified levan which in turn was consistent with the fluorescent-based viability assay where the viability of MCF-7 cells treated with OL III was lower than those treated with unmodified levan (Figure 13A). As shown in Figure 13B, caspase 3/7 activity of MCF-7 cells treated with OL III was higher than those treated with unmodified levan. According to the luminogenic-based caspase-3/7 activity assay, one can suggest that this reduction in cancer cell viability may result from apoptosis. These results are in agreement with those reported by Abdel-Fattah (2012), showing that sulfated *B. subtilis* levan was a promising anticancer agent that induced intrinsic apoptosis pathway in liver cancer cells. Zhang et al. (2014) also showed that a novel *Ganoderma atrum* heteropolysaccharide had potential anti-tumor activity by inducing apoptosis and enhanced host immune system function. This novel polysaccharide had also efficient immunopotentiating activity and could boost the anti-tumor action by improving immune system function through Toll-like receptor (TLR) 4 mediated-NF- κB and MAPK signaling pathway. It also obviously inhibited the growth of CT26 tumor in CT26-bearing mice via TLR4-dependent signaling path-ways (Li et al., 2011; Zhang et al., 2013).

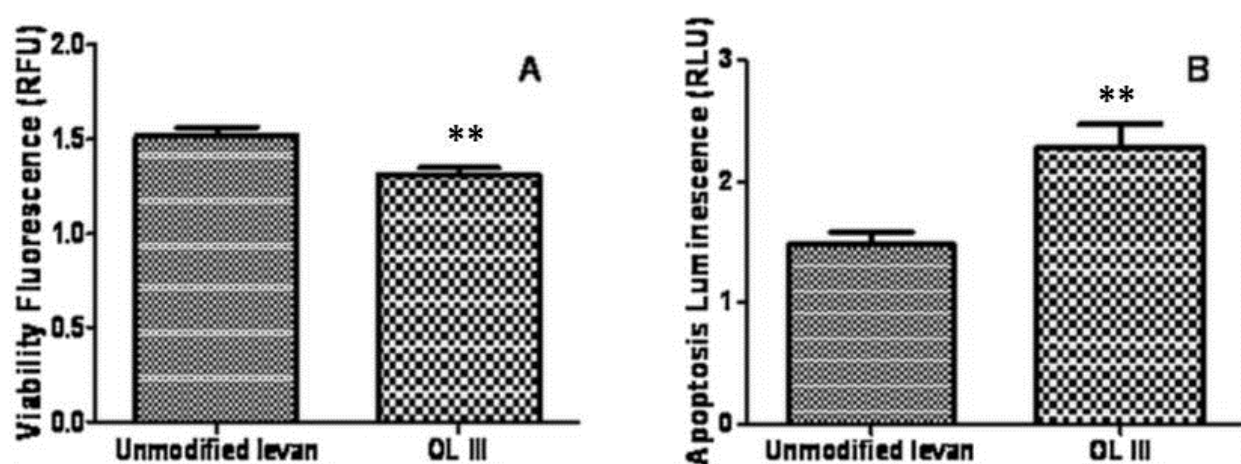


Figure 3.13. Fluorescent-based viability (A) and caspase 3/7 activity (B) of unmodified levan and OL III in MCF-7 cells. Fluorescent-based viability and luminogenic-based caspase-3/7 activity were conducted using the ApoTox-Glo triplex assay. MCF-7 cells were treated with

unmodified levan and OL III at the concentration of 1000 $\mu\text{g/mL}$ for 24h. Bars represent mean $\pm$ SEM of 3 independent experiments (***) = $p < 0.001$ versus Unmodified levan).



4. CONCLUSIONS AND RECOMMENDATIONS

Present study clearly established the antioxidant potential for levan in high glucose conditions in the INS-1E cells by demonstrating a correlation between reduction in oxidative stress and apoptosis with levan treatment. These findings suggest a promising potential for levan in alleviating some complications of diabetes. On the other hand, the importance of the chemical structure and treatment dose of levan samples on the biological activity was observed. *In vitro* anti-cancer activity of linear levan polysaccharide has been greatly improved by introducing aldehydes in its structure. This effect became more apparent with the degree of oxidation. The enhanced bioactivities of levan derivatives were probably caused by the increased electron donating ability and affinity with the receptors on immune cells. The results suggested that levan derivatives could be explored as promising anti-cancer agents due to their high anti-cancer activity together with improved caspase 3/7 activity and low cytotoxicity to normal cells. Understanding the involvement of the bioactive polysaccharide levan and its aldehyde-activated derivative in human cancer treatment is an attractive challenge and yet requires further investigation. Further investigations are needed to establish detailed molecular mechanisms of this linear polysaccharide-mediated apoptosis of human carcinoma cells that shows relationship between their structure and anti-cancer activity. In future studies, this molecular mechanism needs to be validated *in vivo*; a positive result, if confirmed, would provide priceless information to the development of new approaches for effective anti-cancer agents. Moreover, these levan-based polymer derivatives could be used in developing polysaccharide-based nanocarrier systems for anti-cancer drugs. Ultimately, the pharmaceutical industry may provide new markets for chemically modified levan and help developing new generations of polysaccharides with more beneficial biological activities.

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APPENDIX A

ABSORBANCE OD595 VERSUS PROTEIN CONCENTRATION CALIBRATION CHART

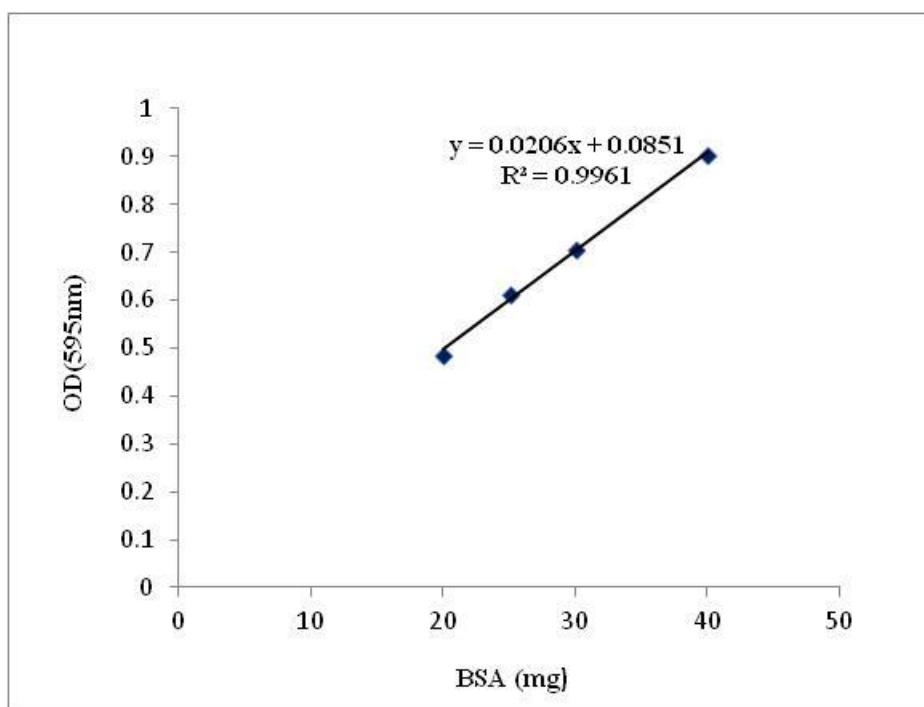


Figure A.1. Protein Concentration Calibration Chart

APPENDIX B

APPENDIX B

ABSORBANCE OD560 VERSUS NaNO_2 CONCENTRATION CALIBRATION CHART

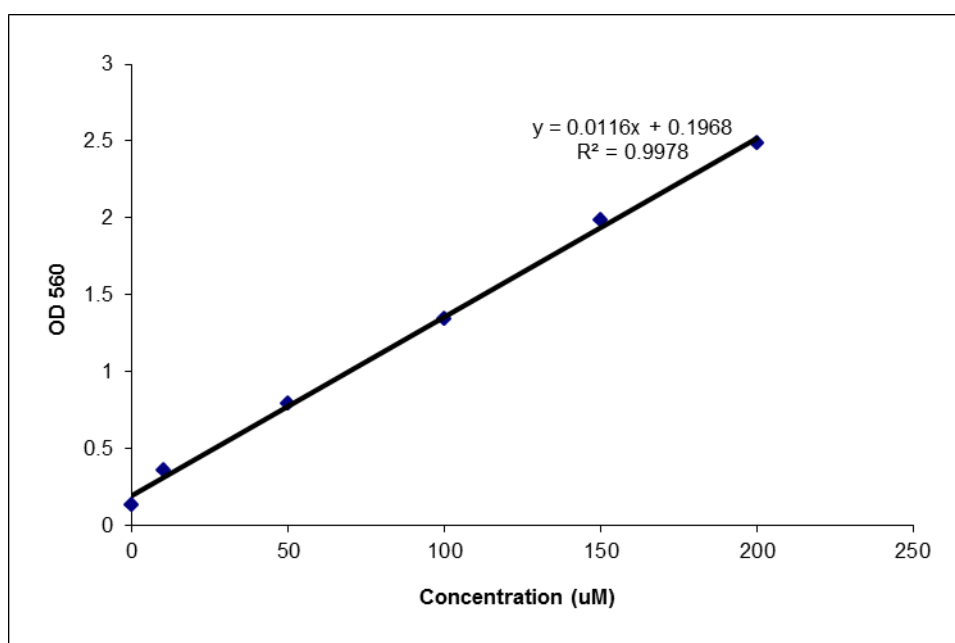
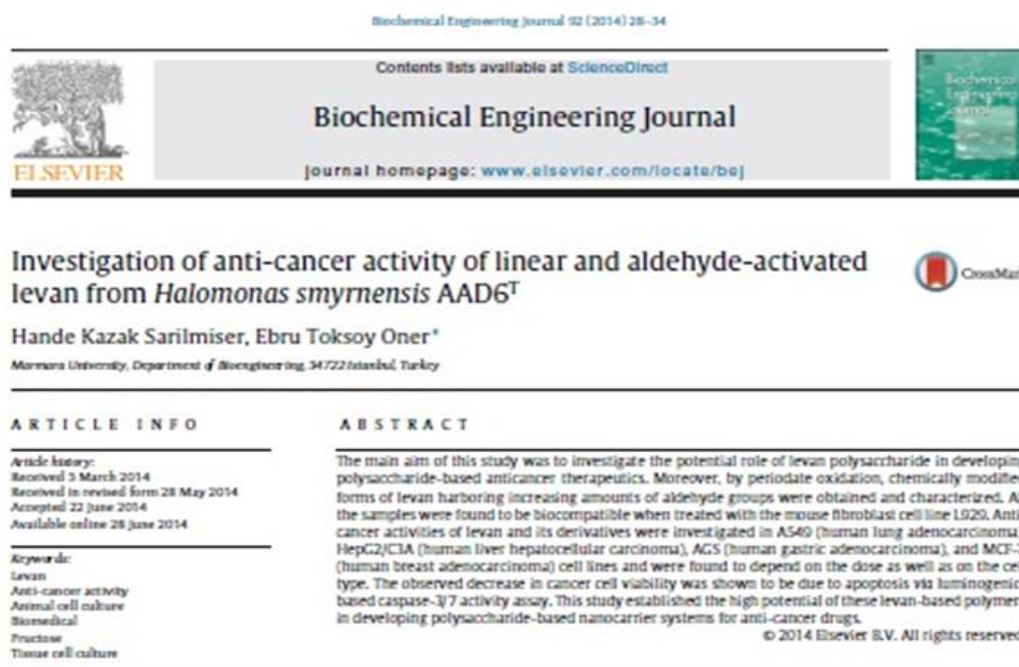


Figure B.1. NaNO_2 Concentration Calibration Chart

APPENDIX C

APPENDIX C

Investigation of anti-cancer activity of linear and aldehyde-activated levan from *Halomonas smyrnensis* AAD6^T



1. Introduction

Exopolysaccharides (EPSs) are polymers of sugar residues excreted by microorganisms into the surrounding environment. These macromolecules have long been known to possess a wide range of biological activities, such as antioxidant, anti-mutant, anticoagulant, immunostimulation, tumor inhibition and stimulation activities depending on their monomer compositions, molecular weights and degree of branching as well as on the presence of functional groups in their structure [1,2]. Besides these structural features, some physical characters such as charge, solubility and conformation of EPSs are also important factors affecting their bioactivities [3]. More importantly, since the chemical and structural features of EPSs are highly determined by the type of the microbial producer and the production conditions, EPSs of each specific microbial system call for individual studies to establish their bioactivities.

One of the most widely distributed EPSs in nature, levan, is a naturally occurring $\beta(2 \rightarrow 6)$ -linked fructose polymer (fructan). As a water soluble, non-toxic, self-assembling, strongly adhesive and film-forming biopolymer with many distinguishing properties like low viscosity, high solubility in oil, compatibility with salts and surfactants, stability to heat, acid, and alkali media, high holding

capacity for water and chemicals, and good biocompatibility, levan is gaining escalating popularity as a functional biopolymer with diverse applications in medical and chemical industries. Though plant fructans are available, many microorganisms like *Acetobacter*, *Aerobacter*, *Azotobacter*, *Bacillus*, *Corynebacterium*, *Erwinia*, *Gluconobacter*, *Mycobacterium*, *Pseudomonas*, *Streptococcus* and *Zymomonas* also produce extracellular levan by the action of levansucrase (EC 2.4.1.10) from sucrose-based substrates [4]. As such, halophilic bacterium *Halomonas smyrnensis* AAD6^T was reported as the first extremophilic producer of levan by our research group [5,6]. Further researches on the potential use of levan by *H. smyrnensis* AAD6^T as a bioflocculating agent [7], its suitability for drug nanocarrier systems [8], and its production by use of cheap sucrose substitutes like molasses [9] were reported. The antioxidant potential of this levan polysaccharide in high glucose condition in the pancreatic beta cells by demonstrating a correlation between reduction in oxidative stress and apoptosis with its treatment was also reported for the first time by our research group [10]. Ternary blend films of chitosan and polyethylene oxide (PEO) with levan were prepared to evaluate the morphological, thermomechanical, surface and biological properties of the films [11]. Chemically modified forms of levan by *H. smyrnensis* AAD6^T were also reported such as the formation of adhesive multilayer films from phosphonate-modified levan [12] and uniform, homogeneous, nanostructured, thin films from oxidized levan [13,14].

There is a growing interest in elucidating the biological activities of levan from various microorganisms where its potential

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use as plasma substitute, prolongator of drug activity, radio protector, immunostimulating [15], hypo-cholesterolemic [16,17], anti-diabetic [18,19], anti-inflammatory [20], and anti-tumor agents have been reported by several groups [21–26]. Calzans et al. [27,28] showed that the anti-tumor activity of levan from *Zymomonas mobilis* depended on its molecular weight. Yoon et al. [26] reported that the branching structure of levan from *Microbacterium laevaniformans* played an important role in its antitumor activity and they found that as the branching degree of levan decreased, the anti-tumor activity on a stomach carcinoma cell line linearly decreased. Esawy et al. [22] showed cancer protective property of high molecular weight levan with branched structure from *Bacillus subtilis* M in murine hepatoma Hepa1c17 cells. Hence the chemical structure, molecular weight, and branching of levan closely relate to its anti-tumor activity.

On the other hand, derivatives of natural polysaccharides may exhibit stronger biological activities than their corresponding natural polysaccharides and thus, they could provide an opportunity to develop new agents with possible therapeutic uses. For example, polysaccharides can be activated by oxidation with periodate due to the fact that aldehydes generated by the oxidation reaction provide an electrophilic carbonyl group. Because of this reactive functional group, aldehydes attach and react readily with nucleophilic functional groups present on the macromolecules such as proteins, lipids and DNA [29,30]. This attachment has a variety of effects in biological systems, most often resulting in cytotoxicity [31]. Reaction of the aldehyde with an amine of a protein or other cellular component forms Schiff bases varying with cell type [32]. Oxidized polysaccharides with aldehyde-functionalized groups may also be useful as tissue adhesives or sealants for medical applications that require a more rapid degradation time, such as the prevention of undesired tissue-to-tissue adhesions resulting from trauma or surgery [33]. In fact, in our earlier studies, nanostructured thin films of levan and its aldehyde-activated derivative were synthesized by Matrix Assisted Pulsed Laser Evaporation (MAPLE) and the aldehyde-activated levan thin films were found to induce an increase in cell proliferation as compared with the levan coatings [13]. In a consecutive study, where gradient organic thin films of these two polymers were fabricated by combinatorial matrix-assisted pulsed laser evaporation (C-MAPLE), certain areas were found to exhibit distinctive influence on osteoblast cells spreading which in turn pointed to the importance of the amount of aldehydes for the observed biological activity [14].

Considering the lack of knowledge on the potential anti-tumor activity of levan by *H. smyrnensis* AAD6^T, studies were initiated to systematically investigate the potential role of this biopolymer in developing polysaccharide-based anticancer therapeutics. For this, besides the linear levan from *H. smyrnensis* AAD6^T, its modified forms containing increasing amounts of aldehydes in their structure were also synthesized by periodate oxidation and characterized. Then, after ascertaining their biocompatibility using the mouse fibroblast cell line L929, their anti-cancer activities were investigated in A549 (human lung adenocarcinoma), HepG2/C3A (human liver hepatocellular carcinoma), AGS (human gastric adenocarcinoma), and MCF-7 (human breast adenocarcinoma) cell lines.

2. Materials and methods

2.1. Microbial levan production

Microbial levan production was performed with the halophilic *H. smyrnensis* AAD6^T under controlled bioreactor conditions using a semichemical medium with the following composition in (g/L): 137.2 NaCl; 50 sucrose; 7K₂HPO₄; 2KH₂PO₄; 0.1MgSO₄·7H₂O;

1(NH₄)₂SO₄, and 0.5 peptone [5,6]. The BIOSTAT B fermenter of 3 L working volume was employed. During cultivation, the temperature and pH were kept constant at 37 °C and pH 7. The aeration rate was 0.1 vvm, whereas the agitation was set to 200 rpm. Cells from the stationary phase cultures were harvested by centrifugation and levan in the supernatant phase was recovered by ethanol addition, followed by centrifugation. The levan pellet was resuspended in distilled water, dialyzed against distilled water for at least 3 days. Levan was further purified by passing through a DEAE-sepharose CL-6B column to remove the impurities. Fractions containing pure levan were collected, tested for total carbohydrate, protein, and nucleic acid contents, and then dried by lyophilization.

2.2. Oxidation of levan

Pure levan samples were subjected to periodate oxidation by prolonged magnetic stirring in a beaker. 1 g pure levan was dissolved in 600 mL of distilled water and after the addition of 12 g KIO₄, the solution was stirred at 300 rpm in dark oven at 50 °C for 1 day (OL I), 3 days (OL II), and 6 days (OL III) to achieve different oxidation degrees (OD). After periodate was eliminated from the solution by dialysis against distilled water for at least 48 h, the polymer solution was lyophilized.

The formation of aldehyde groups was ascertained by Nicolet 6700 Fourier transform infrared spectroscopy (FTIR) and measured by the iodometric method of Lindenbaum et al. [34] according to the following equation where V_u and V_m are the volume of 0.01 N thiosulfate solution in unmodified and modified levan titration, respectively (mL); m is the mass of levan in sample (g); N_2 is normality of thiosulfate solution; 12.4 is the coefficient equal to the amount of aldehyde groups (mmol) under full (100%) oxidation of 1 g levan [35]:

$$OD = \frac{V_u - V_m}{2 \times 12.4 \times m} \times N_2 \times 100\%$$

Nuclear magnetic resonance (NMR) analysis was also performed to provide more structural information of oxidized levan. The NMR spectrum of oxidized levan with the highest oxidation degree (OL III) was recorded on a Varian 600 MHz NMR at 25 °C for ¹H NMR.

2.3. Cell culture

Four cancer cell lines, namely A549 (human lung adenocarcinoma) (ATCC, CCL-185), HepG2/C3A (human liver hepatocellular carcinoma) (ATCC, CRL-10741), AGS (human gastric adenocarcinoma) (ATCC, CRL-1739), and MCF-7 (human breast adenocarcinoma) (ATCC, HTB-22) were used for anticancer screening of levan and its aldehyde activated derivatives while demonstrating their biocompatibility in L929 (mouse fibroblast cell line) (ATCC, CCL-1). AGS cells were grown in RPMI-1640 medium, while all other cells were routinely cultured in DMEM (Dulbecco's Modified Eagle's Medium) at 37 °C in humidified air containing 5% CO₂. Media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Monolayer cells were routinely harvested by treating with a trypsin-ethylene diamine tetraacetic acid (EDTA) solution (0.25%).

2.4. Biocompatibility of levan samples

To assess the biocompatibility of levan samples, WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) cell proliferation and viability assay (Roche Applied Science, Germany) was performed with the mouse fibroblast cell line L929. Briefly, cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated overnight at 37 °C in humidified air containing 5% CO₂. Then the cells were exposed

to varying concentrations (10–1000 µg/mL) of levan samples and further incubated for 24 h. Finally, WST-1 reagent was added directly to culture wells and incubated for 2 h. The absorbance was measured at 450 nm with a GloMax Multi + Microplate Multimode Reader (Promega, USA). Untreated cells were used as control. The control cells were considered 100% viable.

2.5. Anti-cancer activity of levan samples

The anti-cancer activity of levan samples on tumor cell lines was evaluated *in vitro* using WST-1 cell proliferation and viability assay. Briefly, 1.5×10^3 cells (100 µL volume per well) were inoculated into 96-well microtiter plates and incubated overnight at 37 °C in humidified air containing 5% CO₂. Then the culture medium was removed and replaced with fresh FBS-free medium containing varying concentrations (10–1000 µg/mL) of levan samples. After incubation with levan samples for 24 h, WST-1 reagent (10 µL) was added directly to culture wells and incubated. The absorbance was measured at 450 nm with a GloMax Multi + Microplate Multimode Reader (Promega, USA). Untreated cells were used as control. The control cells were considered 100% viable.

2.6. Viability and caspase-3/7 activity assay

Fluorescent-based viability and luminogenic-based caspase-3/7 activity were conducted using the ApoTox-Glo triplex assay (Promega, USA) according to the manufacturer's instructions. For this purpose, MCF-7 cell line was treated with unmodified levan and OL III at the concentration of 1000 µg/mL. Briefly, cells were cultured and treated as previously described in 96-well opaque-walled tissue culture plates where after 20 µL of the viability reagent was added to each well. The contents of the wells were mixed by orbital shaking (300–500 rpm for ~30 s), incubated for 30 min at 37 °C and fluorescence was measured. This was followed by adding 100 µL of Caspase-Glo 3/7 reagent to the same sample wells, mixing by orbital shaking and incubating for 30 min at room temperature. Then luminescent signal was measured using GloMax Multi + Microplate Multimode Reader (Promega, USA).

2.7. Statistical analysis

Statistical analyses were performed by one-way ANOVA followed by the Tukey test for multiple comparisons using the Prism analysis program (Graphpad, V 5.0). Data were presented as means and 95% confidence interval. A *p*-value of less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Oxidation of levan

A schematic presentation of the periodate oxidation of levan is shown in Fig. 1A. Upon the oxidation reaction of levan with potassium periodate, selective scission of chemical bonds between two carbon atoms (C3 and C4) in the fructose ring occurs resulting in the formation of two reactive aldehyde groups. As shown in Fig. 1B, all samples exhibited the characteristic absorption of levan at 2890, 2940, and 3350 cm⁻¹, or 930, 1040, and 1110 cm⁻¹ [13]. For oxidized derivative of levan, a new absorption appeared at around 1730 cm⁻¹ which was assigned to the highly polar C=O bond due to aldehyde groups. This peak demonstrated that the oxidized derivative was successfully synthesized. To further verify the presence of aldehyde groups in the modified levan, the ¹H NMR spectrum of oxidized levan with the highest oxidation degree, OL III, was obtained (Fig. 1C). In this spectrum, the signal at $\delta = 9.40$ ppm was assigned to the aldehyde proton.

Furthermore, the amount of aldehyde groups formed by the oxidation of levan was quantified by thiosulfate titration. As a result, the oxidation degrees were calculated to be 0.08, 0.16, and 0.32 (OL I, OL II, OL III, respectively).

3.2. Biocompatibility of levan samples

Biocompatibility of the samples was investigated in L929 cells. As a result, all samples showed good biocompatibility such that cell viability was at about 70% even when cells were treated with the samples at a very high concentration (1000 µg/mL) while it was 100% at the 10–500 µg/mL concentration range (Fig. 2).

3.3. Anti-cancer activity of levan samples

The anti-cancer activity of levan and its aldehyde-activated derivatives was investigated against human cancer cell lines such as A549, HepG2/C3A, AGS, and MCF-7 using WST-1 assay. In the literature, levan concentration for anti-cancer activity studies covers a wide range by varying from 3.15 µg/mL [24] up to 1000 µg/mL [26]. Also in this study, all tested cells were treated with levan samples at a broad concentration range of 10–1000 µg/mL. Results are shown in Fig. 3. Generally, all samples were found to display growth inhibition against cancer cell lines at the highest dose (1000 µg/mL). Unmodified levan showed the highest anti-cancer effect against AGS cells (Fig. 3C) among the cancer cell lines while it showed no effect against A549 cells (Fig. 3A). Notably, aldehyde-activated levan showed higher anti-cancer activity than unmodified levan against all cancer cell lines. OL samples showed higher anti-cancer activity against A549 (Fig. 3A) and HepG2/C3A (Fig. 3B) cells than AGS (Fig. 3C) and MCF-7 (Fig. 3D) cells. By increasing the oxidation degree, the anti-cancer activity also increased. Hence, it was clearly demonstrated that the introduction of chemically modified group, aldehydes, into the linear levan molecule could significantly enhance the antitumor activity of levan.

When levan is oxidized, the distance between C3–C4 atoms is increased and it leads to an increase in C5–O–C2 angle. It is suggested that this region can be connected with the immunological activity of levan [36]. Levan derivatives may exert their anti-tumor action via activation of the immune response. It is known in the literature that polysaccharides have the immunotherapeutic property to inhibit growth of cancer cells and exert carcinostatic action [37]. Some polysaccharides may possess direct cytotoxic effects on cancer cells even though some of them do not attack cancer cells directly, but produce their anti-tumor effects by activating different immune responses in the host which means that the antitumor activities of polysaccharides result primarily from their immunopotential effects [38,39].

Chemical modification enhances the immunopotentialization and antitumor activities of polysaccharides, due to the change of substituent groups and chain conformation of polysaccharides. Polysaccharides can interact with receptors of immune cells to trigger immunological responses, including antitumor activity [37,39]. TNF- α , IL-1 β , and IL-6 are important regulators of host defense against tumor cells. The increase in production of cytokines would suggest an enhanced ability of the host to combat the growth of tumors. Macrophages can be activated by polysaccharides to kill tumor cells by production of TNF- α and interleukins. The bioactivities of polysaccharides and polysaccharide–protein complexes are dependent on binding on the surface receptor of immune cells [39]. Wang et al. [40] showed in their study that a polysaccharide from *Ganoderma lucidum* had a strong stimulatory effect both on macrophages and on T lymphocytes in promoting the synthesis/release of various cytokines, including IL-1 β , IL-6, TNF- α and IFN- γ , which were known to play important roles in the regulation of immune responses and suppression of tumor-cell growth.

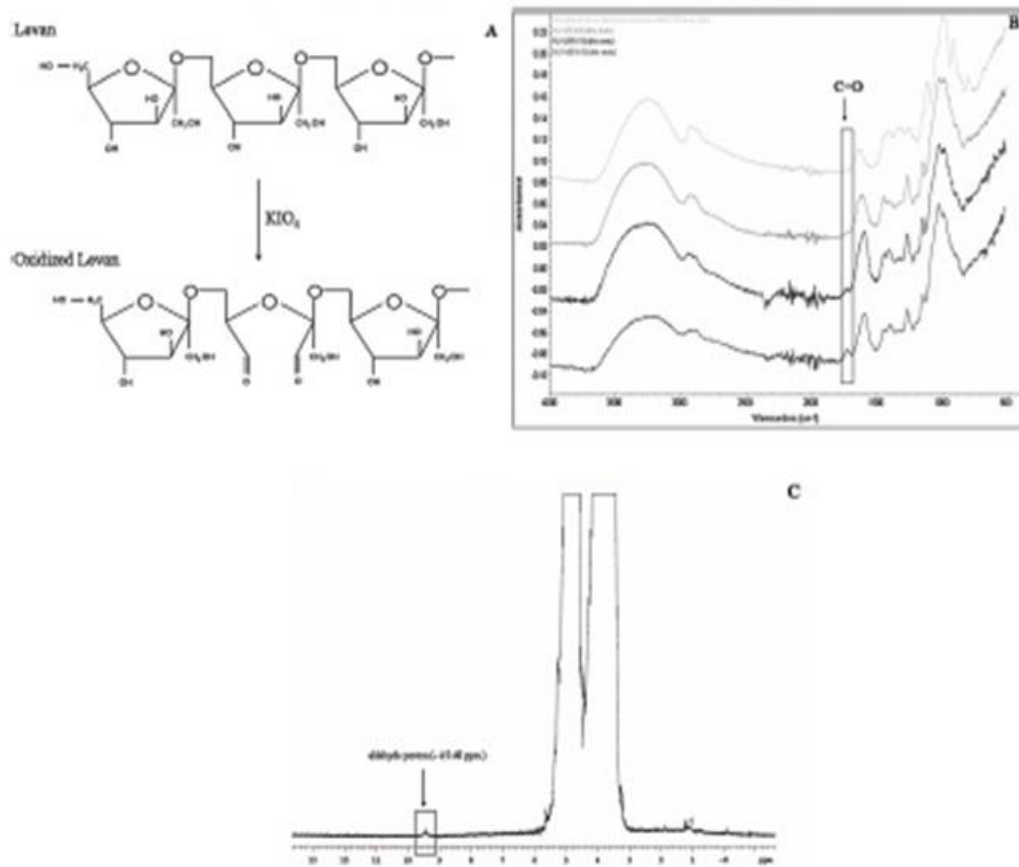


Fig. 1. The structure of levan and its oxidized form (A) FTIR absorption spectra of unmodified levan and its oxidized derivatives, OL I, OL II, and OL III (from light to intense color) (B) ¹H NMR spectrum of OL III (C).

These results were in agreement with those reported by Ooi et al. [37] showing that polysaccharides from mushrooms exert antitumor effects via activation of different immune responses in the host rather than by directly attacking cancer cells.

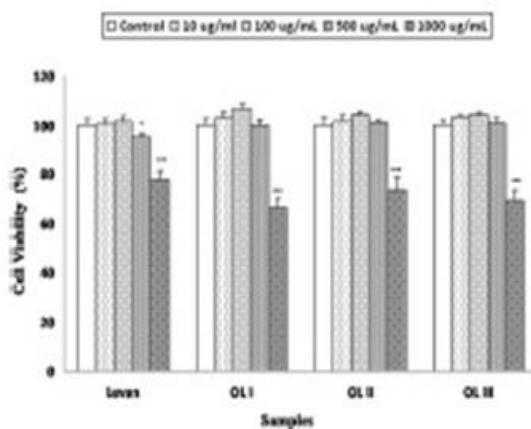


Fig. 2. Cell viability of LS29 cells after being cultured with levan samples for a period of 24 h (* $p < 0.05$ versus control; ** $p < 0.001$ versus control).

There are several reports in the literature on the anti-cancer effect of levan produced by other microbial systems that support the findings of the present study. Kyung et al. [41] studied effect of molecular weight on anti-cancer activity of branched levan from *M. laeviformans* against SNU-1 (human stomach carcinoma) and HepG2 cancer cell lines at the dosage level of 100–800 µg/mL. They found that levan with higher molecular weight showed the greatest cell growth inhibition (84.6%) on SNU-1 cells at 400 µg/mL, while levan with lower molecular weight showed the greatest cell growth inhibition (46.32%) on HepG2 cells at 800 µg/mL. Bayoumi et al. [21] showed anti-proliferative property of sulphated levan from *B. subtilis* against HepG2 cell line. They studied different doses of levan (12.5–100 µg/mL) and found that the greatest inhibition (95%) was achieved by the lowest dose (12.5 µg/mL) of levan. Abdel-Fattah et al. [23] tested the anti-cancer activity of levan from *B. subtilis* with different molecular weights and its sulfated forms against HepG2, HCT-116 (colon carcinoma), HeLa (cervical carcinoma), histiocytic lymphoma, MCF-7 and lymphoblastic leukemia cells and found that higher molecular weight levan and its sulfated form resulted in a remarkable growth inhibition at the concentration of 100 µg/mL. Sulfated levan with higher molecular weight showed variable cytotoxic effect against different cell lines in the following order: Hep-G2 > HeLa > HCT-116 > 1301 > MCF-7 cells. *In vitro* antitumor activity of levan from *Bacillus licheniformis* against HepG2, CaCo2 (human colon adenocarcinoma cell line) and WRL68 tumor cell lines at varying concentrations

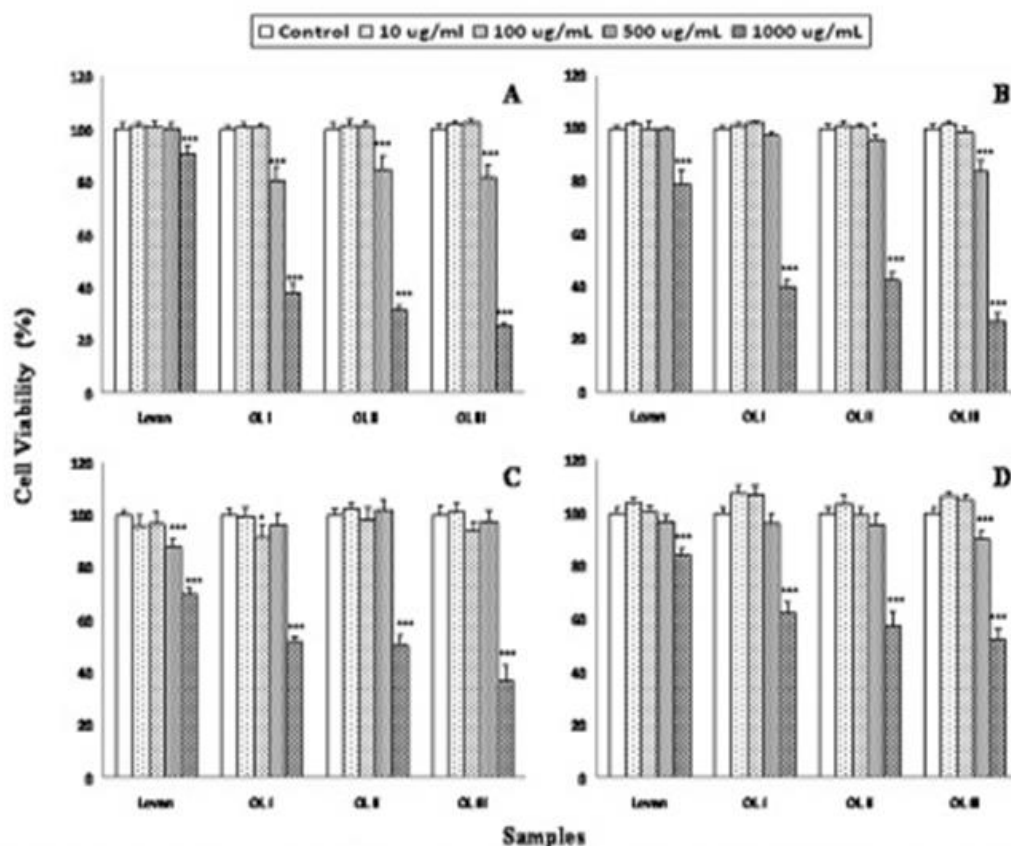


Fig. 3. In vitro anti-cancer activity of levan and its oxidized derivatives against A549 (human lung adenocarcinoma) (A), HepG2/C3A (human liver hepatocellular carcinoma) (B), AGS (human gastric adenocarcinoma) (C), and MCF-7 (human breast adenocarcinoma) (D) (*p < 0.05 versus control; ***p < 0.001 versus control).

(3.125–200 mg/mL) was studied and relatively high activity was observed against the HepG2 cell line at the highest concentration (200 mg/mL) [24]. Esawy et al. [22] showed cancer protective property of high molecular weight levan with branched structure from *B. subtilis* M in murine hepatoma Hepa1c1c7 cells. Liu et al. [3] successfully acetylated, phosphorylated and benzylated levan from *Pantoea agglomerans* EJS-3 and studied antitumor activities of the samples at different concentrations (5–400 µg/mL) in vitro. They found that different derivatives of levan exhibited higher antiproliferative activity than the natural levan against human gastric cancer BGC-823 cells at a concentration of 400 µg/mL. Yoon et al. [26] studied the effect of branching on anti-tumor activity of levan from *M. lachniformans* at the concentration range between

200 and 1000 µg/mL and the strongest inhibiting activities were shown at the highest dosage (1000 µg/mL) of the levan. As the branching degree decreased, the anti-cancer activity on SNU-1 cells linearly decreased while the activity rapidly dropped when the branching increased on HepG2 cells.

3.4. Viability and caspase 3/7 activity

To determine whether the observed anti-cancer activity was caused by apoptosis, viability and caspase 3/7 activity of MCF-7 cells treated with levan and OL III were determined via ApoTox-Glo triplex assay. According to WST-1 results (Fig. 3D), 1000 µg/mL concentration of OL III reduced cell viability of MCF-7 cells three-fold

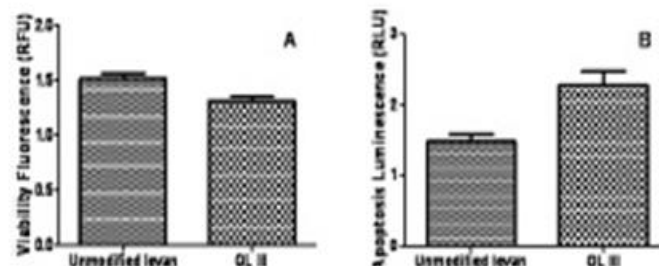


Fig. 4. Fluorescent-based viability (A) and caspase 3/7 activity (B) of unmodified levan and OL III in MCF-7 cells.

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APPENDIX D

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Biological Activities of Bacterial Levan and Three Fungal β -glucans, Botryosphaeran and Lasiodiplodan Under High Glucose Condition in the Pancreatic β -cell line INS-1E

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8. Biological Activities of Bacterial Levan and Three Fungal β -glucans, Botryosphaeran and Lasiodiplodan Under High Glucose Condition in the Pancreatic β -cell Line INS-1E

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Abstract

A comparison among four exopolysaccharides (EPSs), namely a levan type fructan produced by the halophilic bacterium, *Halomonas smyrnensis* sp. nov. AAD6T, two botryosphaerans produced on glucose (BOT-1) and fructose (BOT-2) by *Botryosphaeria rhodina* MAMB-05, and lasiodiplodan (LAS) by *Lasiodiplodia theobromae* MMPI was developed under high glucose condition in pancreatic β -cell line. In order to investigate the role of these EPSs on oxidative stress and apoptosis, the INS-1E cells were grown in RPMI-1640 medium supplemented with high (25mM) glucose concentration. The INS-1E cells were pretreated with EPS (100 μ g/mL) for 24h and then treated with 200 μ M hydrogen peroxide (H_2O_2) for 1h. Oxidative stress was detected by the CM-H2DCFDA assay and apoptosis was analyzed by caspase 3/7 assay by flow cytometry. Under high glucose concentration, and in the presence of H_2O_2 , ROS production was significantly increased. Levan, BOT-1, BOT-2 and LAS significantly decreased oxidative stress when compared to the control. Apoptosis was significantly increased in cells treated with H_2O_2 and treatment with levan, LAS and BOT-2 were found to attenuate apoptosis while there was no significant difference in cells treated with BOT-1. These data demonstrated that EPSs such as levan, BOT and LAS attenuated oxidative stress that is associated with decreased apoptosis in high glucose concentration suggesting an antioxidant role for these EPSs.

Keywords: exopolysaccharide, levan, fructan, *Halomonas smyrnensis*, beta-glucan, apoptosis.

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Introduction

Exopolysaccharides (EPSs) are carbohydrate biopolymers synthesized by bacteria and fungi and are found connected to the cell surface or are secreted extracellularly (1). They possess biological response modifying (BRM) activities that can be important in treating certain disease conditions (2). Levan is an extracellular homopolymer consisting of β -(2 $\rightarrow$ 6)-linked fructofuranoside residues with occasional β -(2 $\rightarrow$ 1)-branching (3), and this fructan type EPS has been described to be produced from sucrose based substrates by several bacteria such as *Bacillus subtilis* (4), *Zymomonas mobilis* (5), *Streptococcus salivares*, *S. mutans* (6, 7), *Lactobacillus reuteri* LB 121 (8) and the moderately halophilic bacterium, *Halomonas smyrnensis* sp. nov. AAD6^T (9).

Botryosphaeran (BOT) (10) and lasiodiplodan (LAS) (11) are fungal EPSs of the β -D-glucan type produced by *Botryosphaeria rhodina* MAMB-05 and *Lasiodiplodia theobromae* MMPI, respectively. Botryosphaeran consists of a main chain of a β -(1 $\rightarrow$ 3)-linked glucose units branched at C-6 with β -(1 $\rightarrow$ 6)-linked glucose and gentiobiose residues. The number of BOT branches varies according to the carbon source used to grow *B. rhodina*; approximately 22% on glucose, 21% on sucrose, and 31% on fructose (10, 12). Lasiodiplodan is an uncommon fungal EPS that presents a linear main chain constituted by β -(1 $\rightarrow$ 6)-linked D-glucopyranoside residues (11). Both EPSs exist as a triple-helix (11, 13) and this structural conformation is important in manifesting BRM activity (14).

Many common applications have been ascribed for β -glucans and levan that include their use as nutraceuticals as well as in cosmetic and pharmaceutical products (15). Recently, levan was shown to possess an anti-diabetic effect in alloxan-induced diabetic rats (16, 17) and also exhibited hypolipidaemic activity and antioxidant properties in cholesterol-fed rats (18). Similarly, the β -glucan botryosphaeran was demonstrated to have hypoglycaemic activity in streptozotocin-induced diabetic rats and exhibited hypocholesterolaemic properties in rats in hyperlipidemia conditions (19).

Diabetes mellitus is a chronic multifactorial metabolic disorder which is characterized by high blood glucose level, insulin insufficiency and increased oxidative stress (20). There is increased interest in the use of natural products (nutraceuticals) that possess anti-diabetic properties with minimal side effects. Dietary β -glucans are present in oat, barley bran and mushrooms. They are known to reduce diabetic risk factors and offer a treatment for diabetes (21). There have been no studies to date that have compared the effect of levan, BOT and LAS on oxidative stress and apoptosis in the pancreatic β -cell line INS-1E using high glucose conditions.

In this study, we report for the first time the antioxidant potential of the EPSs: levan from *H. smyrnensis* sp. nov. str. AAD6^T grown on sucrose, two BOTs from *B. rhodina* MAMB-05 grown on glucose (BOT-1) and fructose (BOT-2), and LAS from *L. theobromae* MMPI grown on glucose, in pancreatic INS-1E cells.

Materials and Methods

Production of Bacterial Levan and Fungal Beta-glucans

Levan was produced by *Halomonas smyrnensis* sp. nov. str. AAD6^T (GenBank Accession Number DQ131909) (9) under controlled fermentation conditions (37°C, pH 7.0, 0.1 vvm aeration rate, and

200 rpm agitation) on nutrient medium containing 137.2 g NaCl, 50 g sucrose, 7 g K_2HPO_4 , 2 g KH_2PO_4 , 0.1 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2SO_4$, 0.5 g peptone per 1 L of distilled water in a BIOSTAT Q Multi-fermenter (B. Braun-Sartorius, Germany). After 41 h of growth, the bacterial cells were harvested by centrifugation ($13,000 \times g/20$ min), and the levan precipitated from the supernatant solution with 1 vol. ethanol, and then left at $-18^\circ C$ overnight. Levan was recovered by centrifugation ($15,000 \times g/30$ min) and resuspended in distilled water and dialyzed against distilled water for 3 days at $4^\circ C$. Impurities in the levan preparation were removed by chromatography on DEAE-Sephacel CL-6B. Fractions containing levan were collected, assayed for total carbohydrate (22), protein (23) and then lyophilized (24).

Botryosphaeran was produced by *Botryosphaeria rhodina* MAMB-05 on 50 g/L glucose (BOT-1), or at the same concentration of fructose (BOT-2), as carbon sources. Inoculum was prepared by growing the fungal isolates on Vogel minimal salts medium (VMSM)-glucose-agar plates for 5 d at $28^\circ C$, and a standardized mycelial preparation for inoculation according to Steluti et al. (2004) (25). Ten mL aliquots of the standardized suspension were used to inoculate 2 L Erlenmeyer flasks containing 400 mL of VMSM and sole carbon sources (glucose or fructose). The fungal isolates were grown in submerged culture (180 rpm) for 72 h at $28^\circ C$. Mycelium was removed by centrifugation ($5500 \times g/10$ min/ $4^\circ C$), and the β -glucans were precipitated from the supernatant with 3 vol. absolute ethanol. The precipitate was recovered, dissolved in distilled water followed by extensive dialysis at $4^\circ C$ against frequent changes of distilled water, and then lyophilized.

Lasioidiplodan was produced by *Lasioidiplodia theobromae* MMPI on glucose (40 g/L) and nutrient medium containing mineral salts (2 g K_2HPO_4 , 2 g $MgSO_4 \cdot 7H_2O$), yeast extract (2 g) and water (1 L). The initial pH was adjusted to 6.0. The fungal isolate was cultivated in a stirred-tank bioreactor for 96 h at $28^\circ C$ according to Cunha et al. (2012) (26). Fungal biomass was separated from the fermentation broth by centrifugation ($1,500 \times g/30$ min), and the recovered supernatant containing the EPS was exhaustively dialyzed against distilled water over 48 h. EPS was precipitated from solution by the addition of 4 vol. absolute ethanol, and left to stand at $4^\circ C$ overnight. The precipitate was recovered by filtration, solubilized in water at $60^\circ C$ followed by dialysis against distilled water, and lyophilized.

Stock Solution of the Exopolysaccharides

A stock solution of the EPSs (levan, BOT-1, BOT-2, LAS) was made by dissolving each EPS in distilled water at a concentration of 2 g/L followed by stirring overnight at $50^\circ C$ and sterilized by autoclaving at $121^\circ C$ for 3 min. Different concentrations of the EPS solutions were prepared from each stock solution and used to treat pancreatic β -cells INS-1E.

Cell Line and Culture Conditions

The clonal β -cell line INS-1E derived from a rat insulinoma (kindly provided by Dr C. Wollheim, Switzerland) was grown in monolayer cultures in RPMI-1640 medium supplemented with 2 g $NaHCO_3$, 10% fetal bovine serum, 1 μM sodium pyruvate, 50 μM 2-mercaptoethanol, 10 μM HEPES, 100 U/mL penicillin, and 100 $\mu g/mL$ streptomycin, at $37^\circ C$ in a humidified (5% CO_2 , 95% O_2) atmosphere. The medium was further supplemented with glucose at a normal (5.6 mM) and high (25 μM) concentrations.

Cell Viability Assay

The metabolic activity of INS-1E cells demonstrating their proliferation and viability at the end of the incubation period was assessed based upon the conversion of tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan (27). Briefly, the cells were seeded in 96-well microplates at a density of 5×10^4 cells per well for 48 h. At different time-points after treatment with the EPS solutions (levan, BOT-1, BOT-2, LAS) of different concentrations (dissolved in serum- and antibiotic- free RPMI-1640 medium), MTT solution (5 $\mu\text{g}/\text{mL}$) was added to each well. The cells were then cultured at 37°C for 4 h, and the absorbance of the formazan product formed was measured at 570 nm on a microplate spectrophotometer (BioTek Power Wave XS). Untreated cells were used as control.

Measurement of ROS Generation

Intracellular ROS production was measured using an oxidation-sensitive fluorescent probe 2', 7'-dichlorofluorescein diacetate (DCFH-DA) as per the manufacturer's protocol. Briefly, 1.5×10^6 cells/flask were cultured at 37°C in a humidified atmosphere for 48 h, then treated with levan, BOT-1, BOT-2 and LAS in serum- and antibiotic- free RPMI 1640 medium. Treated and control cells were incubated with 200 μM H_2O_2 at 37°C for 1 h. The cells were then incubated with DCFH-DA (Molecular Probes, Eugene, USA) at 37°C for 30 min. Labeled cells were harvested by centrifugation (500 $\times$ g/5 min) following trypsinization, washed with PBS and suspended in PBS for detection of fluorescence. The DCF fluorescence intensity was measured by flow cytometry (BD Biosciences, Cell Quest Pro Software, US). Untreated cells at the same cell density in PBS were used as the control, and the relative DCF fluorescence intensity of treated cells was compared with the control.

Apoptosis Assay

Apoptosis was analyzed by a fluorescence-based assay that detects active caspase 3/7 using the CaspaTag Caspase 3/7 *In Situ* Assay kit (Chemicon International, Temecula, USA). Briefly, cells seeded at a density of 7×10^5 cells in 6-well plates were cultured at 37°C in a humidified atmosphere for 48 h, and then treated with levan, BOT-1, BOT-2 and LAS (dissolved in serum- and antibiotic- free RPMI-1640 medium). Treated and control cells were incubated with 200 μM H_2O_2 at 37°C for 1 h, followed by harvesting the cells by centrifugation, resuspended in PBS and incubated with FLICA reagent (fluorochrome inhibitors of caspases) at 37°C for 1 h. Cells containing the bound-labeled reagent were analyzed by flow cytometry.

Statistical Analysis

Data were presented as mean $\pm$ SEM. Statistical analyses were performed by one-way ANOVA followed by the Tukey test for multiple comparisons using the Prism analysis program (Graphpad, V 5.0).

Results

Cell Viability

The effect of levan, BOT-1, LAS and BOT-2 on INS-1E cell viability was evaluated by the MTT reduction assay. At high glucose concentration (25 μM), cell viability with levan, BOT-1, LAS and BOT-2 was found to be 72%, 67%, 62% and 70% respectively as compared to the untreated cells (Fig. 1).

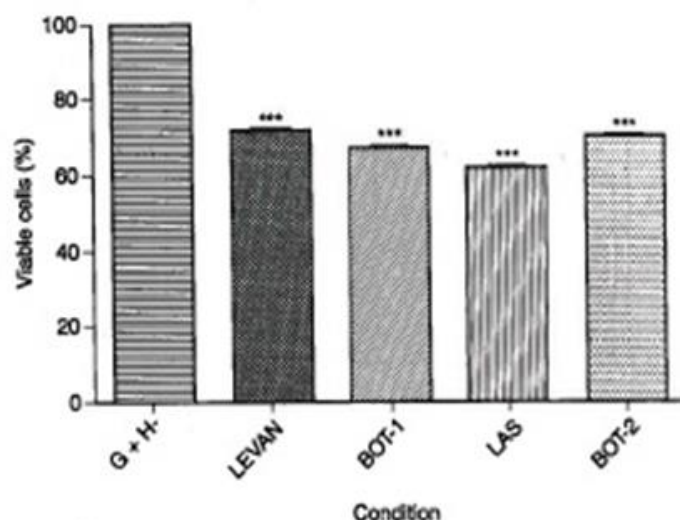


Fig. 1. Effect of EPS treatment on INS-1E cell viability. INS-1E cell viability was assessed in the presence (+) and absence (-) of 25 μ M D-glucose (G) and 200 μ M H_2O_2 (H). INS-1E cells were also pretreated with levan, two different botryosphaerans (BOT-1 and BOT-2) or lasiodiplodan (LAS) (100 μ g/mL, 24 h) in serum-free medium containing D-glucose. Cell viability was assessed by the MTT assay. Data is presented as mean $\pm$ SEM of 3 independent experiments. Data is expressed as percentage of viable cells (***= $p < 0.001$ versus G+H-).

Oxidative Stress

To investigate the effect of levan, BOT-1, BOT-2 and LAS on oxidative stress in the presence of high (25 μ M) glucose and H_2O_2 , INS-1E cells were pretreated with 100 μ g/mL of the EPSs for 24 h, and then treated with 200 μ M H_2O_2 for 1 h. ROS production was significantly increased in the presence of high glucose and H_2O_2 . Treatment with levan, BOT-1, BOT-2 and LAS has significantly decreased ROS production in the INS-1E cells as compared to the control (Fig. 2).

Apoptosis

The effect of levan, BOT-1, BOT-2 and LAS on high glucose and H_2O_2 -induced apoptosis was analyzed in INS-1E cells by caspase 3/7 assay. Cells were pretreated with 100 μ g/mL levan, BOT-1, BOT-2 and LAS for 24 h, and then with 200 μ M H_2O_2 for 1 h. Apoptosis was significantly increased in cells treated with H_2O_2 . Treatment with levan significantly attenuated apoptosis when compared to the control. A decrease in apoptosis was also observed in cells treated with BOT-2 and LAS as compared to cells treated with H_2O_2 alone (Fig. 3).

Discussion

The biological response modifying activities of EPSs have attracted more and more attention in the biomedical field due to their immunomodulatory, antioxidant and anti-cancer properties (28, 29). Recently, it has been demonstrated that levan supplemented in foods as a nutraceutical, might be helpful in preventing diabetic complications due to its efficiency in inhibiting hyperglycaemia and oxidative stress (16). High glucose condition is a known inducer of ROS (30). Chronic hyperglycaemia is known to deplete the antioxidant reserve resulting in an increased generation of ROS (31). Imbalance

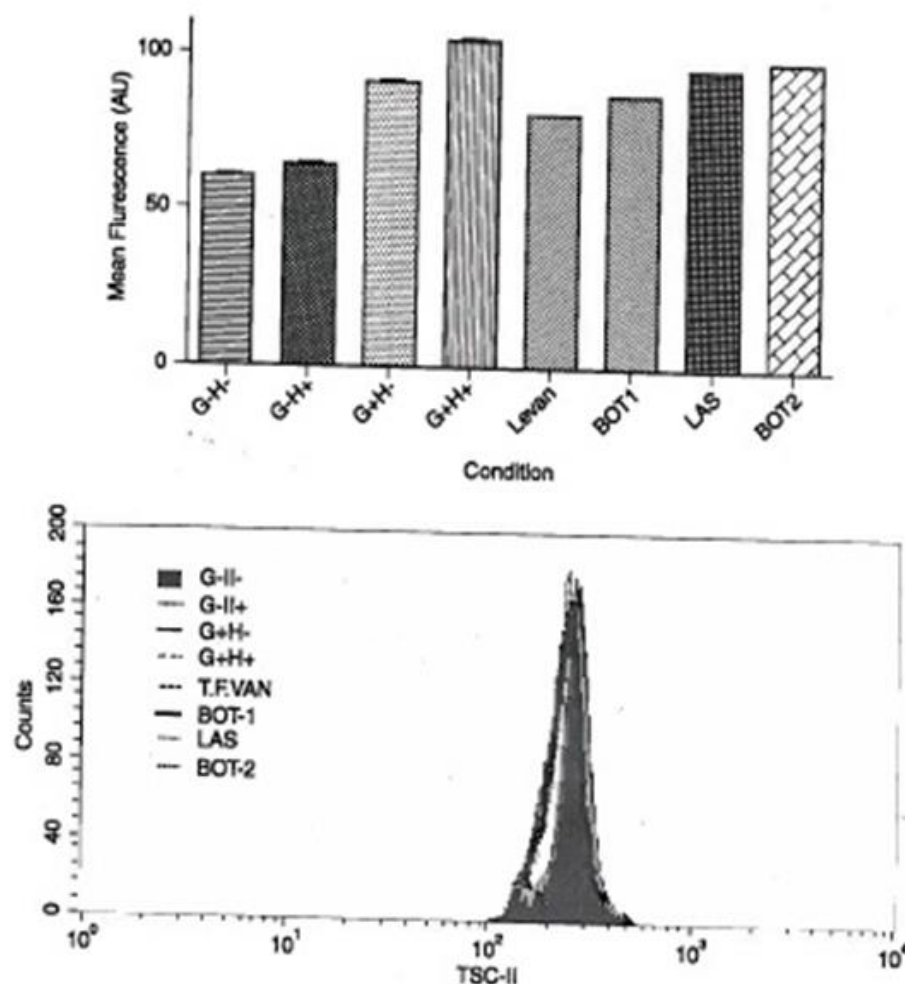


Fig. 2. Effect of EPS pretreatment on oxidative stress in INS-1E cells. Intracellular ROS levels in INS-1E cells, in the presence (+) and absence (-) of 25 μ M D-glucose (G) and 200 μ M H₂O₂ (H), were assessed via fluorescent detection of oxidation-induced esterase activity (CM-H₂DCFDA assay) and analyzed via flow cytometry. Cells were also pretreated with levan, two different botryosphaerans (BOT-1 and BOT-2) or lasiodiplodan (LAS) (100 μ g/mL, 24 h), in serum-free medium containing D-glucose. Data is presented as mean $\pm$ SEM of 3 independent experiments. Representative original histogram is also displayed. Data is expressed as mean fluorescence arbitrary units (AU). (* = $p < 0.05$ versus G+H+; *** = $p < 0.001$ versus G+H+).

between ROS production and cellular defense mechanisms results in oxidative stress, which can initiate apoptosis (30).

In the present study, we have demonstrated that treatment with levan, BOT-1, BOT-2 and LAS has significantly decreased ROS production in the INS-1E cells under high glucose concentration suggesting that these EPSs may possess antioxidant properties. To confirm the relationship between antioxidant effect of EPS and apoptosis, we investigated the levels of active caspase 3/7 in INS-1E cells. Based on our results, we have demonstrated that apoptosis was significantly increased in cells treated

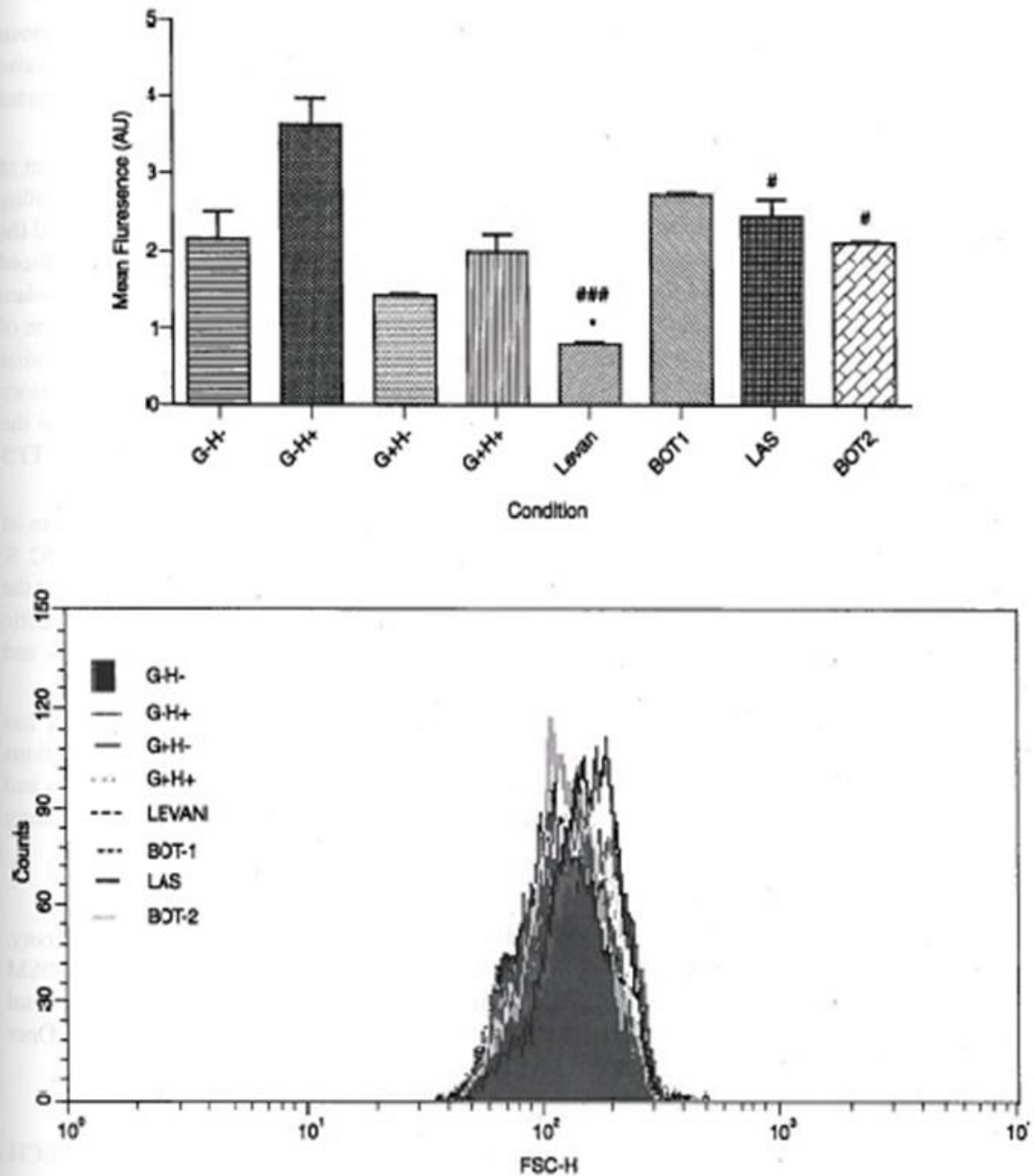


Fig. 3. Effect of EPS pretreatment on apoptosis in INS-1E cells. Apoptosis in INS-1E cells was assessed in the presence (+) and absence (-) of 25 μ M D-glucose (G) and 200 μ M H_2O_2 (H). INS-1E cells were also pretreated with levan, two different botryosphaerans (BOT-1 and BOT-2) or lasiodiplodan (LAS) (100 μ g/mL, 24 h), in serum-free medium containing D-glucose. The effect of EPS pretreatment on INS-1E cellular apoptosis was assessed using the CaspaTag Caspase 3/7 *In Situ* Assay kit and measured via flow cytometry. Data is presented as mean $\pm$ SEM of 3 independent experiments. Representative original histogram is also displayed. Data is expressed as mean fluorescence arbitrary units (AU). (* = $p < 0.05$ versus G-H+; # = $p < 0.05$ versus G-H-; ### = $p < 0.001$ versus G-H+).

with H_2O_2 by enhancing ROS production. In light of the results obtained, we also found that apoptosis was reduced in the cells treated with levan in the presence of high glucose and H_2O_2 . Apoptosis was also significantly decreased in cells treated with BOT-2 and LAS as compared to the control cells treated with H_2O_2 alone.

Previous studies have reported that EPSs could act as antioxidants and prevent the development of diabetes. Dahech et al. (16, 17) showed that levan from *Bacillus licheniformis* was efficient in inhibiting hyperglycaemia and oxidative stress in alloxan-induced diabetic rats. Fu et al. (2012) investigated the effects of *Acanthopanax senticosus* polysaccharide (ASP) on antioxidant status in alloxan-induced diabetic mice, and demonstrated that ASP alleviated oxidative stress and enhanced the antioxidant status (32). Hwang et al. (2008) investigated the stimulatory effects of EPS from submerged culture of an edible mushroom, *Laetiporus sulphureus* var. *miniatus*, on insulinoma cell (RINm5F) proliferation and insulin secretion. They found that EPS was able to increase proliferation and insulin secretory function in these cells in a dose-dependent manner. In addition, EPS also significantly reduced the streptozotocin-induced apoptosis in RINm5F cells further confirming the cytoprotective role of EPS (33).

Botryosphaeran, a β -(1 $\rightarrow$ 3; 1 $\rightarrow$ 6)-D-glucan, from *B. rhodina* MAMB-05 at a concentration of 12 mg/kg rat body weight has been shown to exhibit significant hypoglycaemic activity with a 52 % reduction in blood glucose in streptozotocin-induced diabetic rats (19). Ye et al. (2011) showed that the EPS from *Lachnum calyculiforme* had a significant hypoglycaemic effect on alloxan-induced diabetic mice with a dose-effect relationship by possibly repairing the damage of the pancreatic β -cells and promoting insulin production (34).

Despite structural differences between the several EPSs that we studied, the present study has demonstrated an antioxidant potential for levan, BOT-1, BOT-2 and LAS in high glucose conditions in the INS-1E cells, and has also demonstrated a correlation between reduction in oxidative stress and apoptosis with EPS treatment. These findings suggest a promising potential for EPSs in alleviating some complications of diabetes.

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Education

Degree	Department/Program	University/High School	Graduation Year
M.Sc	Department of Bioengineering	Marmara University, İstanbul	2009
B.Sc	Department of Bioengineering	Ege University, İzmir	2006
High School	Science	Atakent Anatolian High School, İzmir	2001

Experiences

Year	Company/Institution	Position
2014 - ...	Abdi Ibrahim Pharmaceutical Company <i>Istanbul, TURKEY</i>	R&D Analyst, Biotechnology
2012-2014	Marmara University, Faculty of Engineering, Department of Bioengineering <i>Istanbul, Turkey</i>	Scholar through Project TUBITAK-MAG-111M232
2011	Northern Ontario School of Medicine, Lakehead University <i>Thunder Bay, ON, CANADA</i>	Visiting researcher through TUBITAK-BIDEB 2214 International Research Fellowship Program
2008-2010	Marmara University, Faculty of Engineering, Department of Bioengineering <i>Istanbul, Turkey</i>	Scholar through Project TUBITAK-MAG-108M193
2007	Consiglio Nazionale delle Ricerche <i>Naples, ITALY</i>	Visiting researcher through joint Project TUBITAK-TBAG-U/122(104T263)

Research Projects

03.09.2012 - -	Marmara University Research Fund, FEN-C-DRP-030912-0306, "Biological Activities of Levan by <i>Halomonas</i> sp."
01.01.2012 – 01.09.2014	TUBITAK, 111M232, "In vitro and in silico Investigation of Biological Activities of Levan Type Polymers"
19.03.2011-19.12.2011	TUBITAK-BIDEB, 2214 International Research Fellowship Program, "Investigation of Anti-Inflammatory, Anti-Oxidant, Anti-Cancer and Insulin-Lowering Activities of Exopolysaccharides (Levan, Botryosphaeran, and Lasiodiplodan) in Cardiac and Pancreatic Cells"
1.11.2008-1.11.2010	TUBITAK, 108M193, "Investigation of Potential Industrial Applications of a Novel Microbial Biopolymer"
2006 – 2008	TUBITAK, 104T263, "Extremophiles as Sources of Polysaccharides"

Publications

Book Chapters

1. **Kazak H.**, Barbosa A.M., Baregzay B., Alves da Cunha M.A., Toksoy Öner E., Dekker R.F.H., Khaper N. "Biological Activities of Bacterial Levan and Three Fungal β -glucans, Botryosphaeran and Lasiodiplodan Under High Glucose Condition in the Pancreatic β -cell line INS-1E" **Adaptation Biology and Medicine (Volume 7: New Challenges)** (L.M. Popescu, A.R. Hargens, P.K. Singal Eds.) Narosa Publishing House, New Delhi, 105-115, 2014.
2. **Kazak H.**, Toksoy Öner E., Dekker R.F.H. "Extremophiles as Sources of Exopolysaccharides" **Carbohydrate Polymers: Development, Properties and Applications**, (F. Columbus Ed.) Nova Science Publishers, Inc. NY (USA), 605-619, 2010.

Papers

3. Sezer A.D, **Kazak Sarılmışer H.**, Rayaman E., Çevikbaş A., Toksoy Öner E., Akbuğa J. “Development and characterization of vancomycin-loaded levan-based microparticulate system for drug delivery”, *Pharmaceutical Development and Technology (IF: 1.202)*, **in press**, DOI: 10.3109/10837450.2015.1116564.
4. **Kazak Sarılmışer H.**, Ates O., Ozdemir G., Arga K.Y., Toksoy Oner E. “Effective stimulating factors for microbial levan production by *Halomonas smyrnensis* AAD6^T”, *Journal of Bioscience and Bioengineering (IF: 1.869)*, 119, 455-463, **2015**.
5. **Kazak Sarılmışer H.**, Toksoy Oner E. "Investigation of anti-cancer activity of linear and aldehyde-activated levan from *Halomonas smyrnensis* AAD6^T", *Biochemical Engineering Journal (IF: 2.579)*, 92, 28-34, **2014**.
6. Sennaroglu Bostan M., Cansever Mutlu E., **Kazak H.**, Keskin S.S., Toksoy Oner E., Eroglu M.S. “Comprehensive characterization of chitosan/PEO/levan ternary blend films”, *Carbohydrate Polymers (IF: 3.479)*, 102, 993-1000, **2014**.
7. Küçüktaşık F., **Kazak H.**, Güney D., Finore I., Poli A., Yenigün O., Nicolaus B., Toksoy Öner E. "Molasses as Fermentation Substrate for Levan production by *Halomonas* sp." *Applied Microbiology and Biotechnology (IF: 3.689)*, 89, 1729-1740, **2011**.
8. Sezer A.D., **Kazak H.**, Toksoy Oner E., Akbuğa J. "Levan-based nanocarrier system for peptide and protein drug delivery: Optimization and influence of experimental parameters on the nanoparticle characteristics" *Carbohydrate Polymers (IF: 3.479)*, 84, 358-363, **2011**.
9. Poli A., **Kazak H.**, Gürleyendağ B., Tommonaro G., Pieretti G., Toksoy Öner E., Nicolaus B. "High level synthesis of levan by a novel *Halomonas* species growing on defined media" *Carbohydrate Polymers (IF: 3.479)*, 78, 651-657, **2009**.

Conference Papers

10. **Kazak H.**, Ates Ö., Kucukasik F., Arga K.Y., Toksoy Öner E. "Levan Production by *Halomonas* sp." *Chem. Listy (IF: 0.453)*, 104, 881-902, **2010**.
11. **Kazak H.**, Arga K.Y., Toksoy Öner E. "Modeling of batch fermentation kinetics for

levan production by *Halomonas* spp." *New Biotechnology* (IF: 1.706), 25, pp. s115, **2009**.

- 12. Kazak H.**, Gürleyendag B., Poli A., Toksoy Öner E., Nicolaus B. "Exopolysaccharide production by *Halomonas* strains isolated from Turkey" *Journal of Biotechnology* (IF: 3.183), 131(2), pp. s163, **2007**.

Selected Presentations (among 21 presentations)

- 1. Kazak H.**, Toksoy Oner E. "Investigation of Anticancer Activity of Levan Polysaccharide", VI. Bioengineering Congress "Human Welfare" (BEC 2013), November 12-15, **2013**, Kuşadası, Turkey, *poster presentation*.
- 2. Sennaroglu Bostan M.**, Cansever Mutlu E., **Kazak H.**, Keskin S.S., Toksoy Oner E., Eroglu M.S. "Surface characterization of chitosan/PEO/levan ternary blend films", International Symposium on Surface and Interface of Biomaterials (ISSIB) – IV, September 24-28, **2013**, Rome, Italy, *oral presentation*.
- 3. Kazak H.**, Toksoy Oner E. "*In vitro* Anticancer Activity of Levan from *Halomonas smyrnensis* AAD6^T", 38th FEBS Congress, July 6-11, **2013**, St. Petersburg, Russia, *poster presentation*.
- 4. Kazak H.**, Söğütçü E., Ates O., Arğa K.Y., Toksoy Öner E. "Levan by *Halomonas* sp.: From Production to Application", 7th International Fructan Symposium, July 2 - 6, **2012**, Saint-Jean-Le-Thomas, France, *oral presentation*.
- 5. Sezer A.D.**, **Kazak H.**, Toksoy Öner E., Akbuğa J. "Levan-based nanocarrier system for peptide and protein drug delivery", 9th World Biomaterials Congress, June 1-5, **2012**, Chengdu, China, *poster presentation*.
- 6. Kazak H.**, Toksoy Öner E., Barbosa A.M., Dekker R.F.H., Khaper N. "Effects of levan, botryosphaeran and lasiodiplodan on oxidative stress and apoptosis in high glucose condition", 7th Meeting of the Canadian Oxidative Stress Consortium, May 10-12, **2012**, Thunder Bay, Ontario, Canada, *poster presentation*.
- 7. Kazak H.**, Toksoy Öner E., Barbosa A.M., Dekker R.F.H., Khaper N. "Biological significance of levan and glucan type exopolysaccharides in pancreatic cells", Winnipeg Heart

- International Conference 2011, October 13-16, **2011**, Winnipeg, Canada, *poster presentation*.
8. **Kazak H.**, Ates Ö., Kucukasik F., Arga K.Y., Toksoy Öner E. "Levan production by *Halomonas* sp." 6th International Conference on Polysaccharides-Glycoscience, 29 September - 1 October, **2010**, Prague, Czech Republic, *poster presentation*.
 9. **Kazak H.**, Gökmen B., Ates Ö., Güney D.B., Mutlu E., Gürleyendağ B., Küçükasik F., Toksoy Öner E. "Levan: a promising biopolymer from halophilic bacteria", 5th International Bioengineering Congress, June 16-19, **2010**, Izmir, Turkey, *oral presentation*.
 10. Sezer A.D., **Kazak H.**, Toksoy Öner E., Akbuğa J. "Levan as a promising biomaterial for protein delivery" 36th Annual Meeting and Exposition of the Controlled Release Society, July 18-22, **2009**, Copenhagen, Denmark, *poster presentation*.
 11. **Kazak H.**, Gürleyendağ B., Poli A., Toksoy Öner E., Nicolaus B. "Exopolysaccharide Production from Extremophiles", 15th National Biotechnology Congress, **2007**, Antalya, Turkey, *oral presentation*.
 12. **Kazak H.**, Gürleyendağ B., Poli A., Toksoy Öner E., Nicolaus B. "Exopolysaccharide Production by a Novel *Halomonas* Strain", 13th European Congress of Biotechnology (ECB13), September 16-19, **2007**, Barcelona, Spain, *poster presentation*.

Awards and Fellowships

- | | |
|-------------|---|
| 2013 | "38 th FEBS Congress", FEBS Fellowship , Saint Petersburg, Russia |
| 2012 | "7 th Meeting of the Canadian Oxidative Stress Consortium", Trainee Travel Award , Thunder Bay, Ontario, Canada. |
| 2010 | "6 th International Conference on Polysaccharides-Glycoscience", Young Glyco-Scientist Poster Award , Prague, Czech Republic. |
| 2010 | TUBITAK-B İ D E B 2214 International Research Fellowship (for PhD students), Canada, 9 months. |

Memberships

- European Federation of Biotechnology (EFB)
- Turkish Biochemical Society

Research Interests

- Biotechnology
- Bioprocess
- Biopolymers
- Biopharmaceuticals
- Bioassays
- Anti-oxidant, immunostimulating, anti-cancer, and antiviral activities of biomolecules