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Antitumor and antioxidant activities of levan and its derivative from the isolate *Bacillus subtilis* NRC1aza

Aza. M. Abdel-Fattah[a], Amira M. Gamal-Eldeen[b], Wafaa A. Helmy[a], Mona A. Esawy[a,*]

[a] Department of Chemistry of Natural and Microbial Products, National Research Centre, Dokki, Giza, Egypt
[b] Cancer Biology Laboratory, Excellence for Advanced Science, Department of Biochemistry, National Research Centre, Dokki, Giza, Egypt

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ABSTRACT

The novel levansucrase produced from *Bacillus subtilis* NRC1aza yielded two types of levan L1 and L2 with different molecular weights 85.23 kDa and 31.95 kDa, respectively. The levan identification was detected by paper chromatography and high-pressure liquid chromatography. The antioxidant activity of levan and their derivatives (SL1 and SL2) exhibited a strong free radical scavenging activity with DPPH. The antitumor activity of SL1 was tested against different human cancer cell lines. The cell death was explored mechanistically through evaluation of Apoptosis/necrosis ratio, DNA fragmentation, histone deacetylase activity, mitochondrial transmembrane potential ($\Delta\psi_m$), cytochrome *C* release, total caspases, caspase-3, and caspase-9, and cell cycle. SL1 showed high selective cytotoxicity against HepG2 cells. SL1 led to DNA damaging and fragmentation that was associated with induced apoptosis *via* mitochondrial pathway, which initiated by the impairment of $\Delta\psi_m$ and then increased mitochondria, released cytochrome *c*, that in turn activated caspase-9 and caspase-3 and induced apoptosis.

1. Introduction

Levansucrase, one of the fructosyltransferases or glycansucrases, is produced by various microorganisms (Ben Ammar et al., 2002; Kojima, Saito, Iizuka, Minamiura, & Ono, 1993; Lizuka, Tanaka, & Yamamoto, 1991). Bacterial levansucrases catalyze at least three different reactions: hydrolysis of sucrose, polymerization of fructose derived from sucrose and hydrolysis of levan. It is reported that levansucrase activity is involved in a variety of processes including survival of bacteria in soil (*B. subtilis*), phytopathogenesis (*Erwinia* and *Pseudomonas* species) and symbiosis (*Paenibacillus polymyxa*) of plant interactive bacteria (Hettwer, Gross, & Rudolph, 1995).

Levan is one of two main types of fructans, composed of a homopolymer of fructose and can be produced by the transfructosylation reaction of levansucrase (EC 2.4.1.10) from sucrose (Arvidson, Rinehart, & Gadala-Maria, 2006). It is a naturally occurring polymer of β-D-fructofuranose with β (2→6) linkages between repeating five-member fructofuranosyl rings and branching at C-1 (Barone & Medynets, 2007; Arvidson et al., 2006). Levan produced by different organisms differ in their molecular weight and degree of branching. Levan from plants generally have molecular weights about 2–33 kDa (Rhee et al., 2002). The molecular weight of levan, and the fraction of residues incorporated in side chains, depends on both the source and the growth conditions, with plant levan and microbially produced levan with different characteristics (Barone & Medynets, 2007; Kasapis & Morris, 1994; Kasapis, Morris, Gross, & Rudolph, 1994). Levan has a variety of applications in the fields of cosmetics, foods and pharmaceuticals (Belghith, Song, Kim, & Rhee, 1996), as a hypo-cholesterolemic agent (Yamamoto et al., 2000), and an anti-tumor agent (Yoo, Yoon, Cha, & Lee, 2004; Leibovich & Stark, 1985). Many studies on levan were motivated by its therapeutic role in dental caries (Arvidson et al., 2006), in plant pathogenesis (Kasapis & Morris, 1994; Kasapis et al., 1994), and cholesterol-lowering properties (Yamamoto et al., 1999). Levan is also shown to exert excellent cell-proliferating, antitumor, skin moisturizing, and skin irritation-alleviating effects as a blending component in cosmetics (Yoo et al., 2004; Calazans, Lima, deFranca, & Lopes, 2000). In addition, *in vitro* anti-tumor activity of levan produced from *Microbacterium laevaniformans*, *Rahnella aquatilisand* and *Zymomonas mobilis*, has been shown against eight different tumor cell lines (Yoo et al., 2004).

Roberts and Garegg (1998), reported novel uses of levan derivatives such as levan sulfates, phosphates, and acetates polymers in medicine as anti-AIDS agents; food processing as food additive with prebiotic and hypocholesterolemic effects, and as an environmentally benign adhesive (Kang et al., 2009; Combie, Steel, & Sweitzer, 2004). Levan acetate and levan crosslinked with epichlorohydrin

* Corresponding author. Tel.: +20 02 33335479; fax: +20 02 3370931.
E-mail address: mona_esawy@hotmail.com (M.A. Esawy).

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can be used to form thin plastic films, while levan blended with ethylcellulose was casted into films (Cavalcanti, Petenuci, Bedin, Pineda, & Hechenleitner, 2004).

In recent years, more interest has been paid to protect foods and human beings against oxidative damage caused by free radicals (Namiki, 1990). Due to the fact that reactive oxygen species (ROS) like superoxide anion radicals, hydrogen peroxide, hydroxyl radical and other free radicals can cause functional abnormalities and pathological changes leading to and exacerbating many chronic diseases like heart diseases, stroke, arteriosclerosis, gastric ulcer, cancer, as well as cause aging process and also to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity an interest in the antioxidant activity of bacteria has increased (Kirkinezosa & Moraesa, 2001). Recently, a number of natural polysaccharides and their derivatives have been demonstrated to possess potent antioxidant activities and potential applications as antioxidants (Ananthi, Ranjitha Kumari, & Ramachandran, 2010) Apoptosis, programmed cell death, plays an important role in developmental processes by eliminating unwanted cells so as to maintain homeostasis in healthy tissue (Brunelle & Letai, 2009). Perturbations in its regulation contribute to numerous pathological conditions, including cancer and autoimmune and degenerative diseases (Adams & Cory, 2007; Meier & Vousden, 2007). A wide variety of chemotherapeutic agents depend on causing the death of cancer cells by inducing apoptosis (Kaufmann & Earnshaw, 2000). Two principal signal pathways have been established for the induction of apoptotic cell death, the extrinsic pathway (the death receptor pathway), which is triggered following the activation of cell-surface-expressed death receptors, such as CD95 and tumor necrosis factor receptor, followed by the activation of caspase-8, which activates the downstream effectors caspases-3 and -7, and then -6. The intrinsic apoptotic pathway (the mitochondrion-mediated pathway) is initiated in response to a variety of stress signals (Dewson & Kluck, 2009; Willis & Adams, 2005) and a complex interplay of Bcl-2 proteins relays this signal to the mitochondrial outer membrane (OM) to initiate Bak and Bax activation, oligomerisation and OM damage. Breaching the mitochondrial OM releases apoptogenic factors, including cytochrome *c*, which activate a group of caspases (Dewson & Kluck, 2009; Youle & Strasser, 2008). Caspases, in turn, cleave several hundred cellular proteins to coordinate the destruction of the cell (Dix, Simon, & Cravatt, 2008; Lüthi & Martin, 2007). By contrast, the extrinsic apoptotic pathway can activate caspases without the participation of mitochondria (Dewson & Kluck, 2009; Zhang & Wu, 2007).

In this work, the optimized conditions for levansucrase production and levan yield were tested. Two types of levan were isolated from *B. subtilis* and subjected to sulfating process. The anticancer activity and the mechanistic role in the apoptosis pathway of the native and sulfated levan were tested.

2. Experimental

2.1. Isolation of bacterial strain

One hundred microliters of Mediterranean Sea shallow water in Alexandria was spread on sucrose medium plates (g/l): sucrose, 100; yeast extract, 2.0; Mg SO_4, 0.1; agar, 15.0, pH 7. After drying for 20 min in a laminar flow hood, the plates were incubated at 30 °C for 24 h or until the bacterial colonies were sufficient size for colony replication (approximately larger than 3–5 mm in diameter). The bacterial isolate was streaked onto agar plates and preserved at 4 °C. The purity of the bacterial isolate was assessed by colony morphology and microscopy.

2.2. Isolates identification

The spores were examined by microscopic observation, and identification was based on morphological and biochemical tests (API) in Serum and Vaccine Lab. Cairo, Egypt.

2.3. Media

Nutrient agar medium was used for the culture maintenance and stock cultures. It had the following composition (g/l): peptone 5.0; meat extract 3.0; agar 15.0; NaCl 5.0; the pH value was adjusted to 7.0. The following medium was used as cellular production medium (BM) (Yanase et al., 1991) for levansucrase production and had the following composition (g/l): yeast extract 2.5; sucrose 80; $MgSO_4$ 0.2; K_2HPO_4 5.5. The medium was completed by the addition of 1 l distilled water, and the pH was adjusted to 7.8 before autoclaving.

2.4. Cellular production

Cultivation was carried out in 250 ml Erlenmeyer flasks. Each flask contained 50 ml production medium and was autoclaved for 15 min. The flasks were then inoculated with 2.0 ml inoculums and incubated for 24 h at 30 °C. The culture broth was then centrifuged in a cooling centrifuge (K70; Janektzki, Germany) at 10,397 × *g* to separate the bacterial cells from the supernatant.

2.5. Optimization conditions

The parameters included initial incubation temperature (28, 30, 33 and 35 °C); incubation time (8–72 h); shaking speed ranged between 50 and 200 rpm; different concentrations of sucrose (60–160 g) were studied. In every test the BM medium was used.

2.6. Assay of levansucrase

Levansucrase assays were performed according to the method of Yanase et al. (1991) with some modifications.

2.7. Levan and levansucrase sedimentation from B. subtilis culture

Levan was isolated from culture filtrate of *B. subtilis* NRC1aza after the stage of fermentation using sedimentation by ethanol. Ethanol (96%) added to the culture filtrate (2:1, v/v) mixed for 24 h at room temperature. Sediment of EPS (L1) was then separated from the culture filtrate by decantation and used for levan extraction. exopolysaccharid (L2) was float in the surface; it was collected by small spoon.

2.8. Levan identification

Acid hydrolysis was done using 0.1 N HCL in boiling water bath for 1 h. The produced acid compound was analyzed by descending paper-chromatography using Whatman No.1 and solvent system n-butanol:acetone:water (4:5:1, v/v/v) (Tanaka, Oi Lizuka, & Yamamoto, 1978) and sprayed with aniline phthalate (Block, Vurrum, & Zweig, 1955). On the other hand, the acid hydrolyzed product was identified by HPLC (Model Hewlett Packard, 1050).

2.9. Determination of molecular weight

Different concentrations of levan and oligosaccharide were prepared, and the flow time of equal volumes for each concentration at 30 °C was determined in a U-shaped Ostwald viscometer. Flow time of the same volume of distilled water was also determined as control. Thus, specific viscosity/*C* (gsp) was estimated.

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A plot of levan and oligosaccharide concentration (C) against intrinsic viscosity (C) (gsp/C) therefore yielded a straight line.

2.10. Preparation and characterization of sulfated levan

Sulfation of levan was carried out with chlorosulfonic acid (Hussein, 1994). The formed product was isolated by precipitation with 3 vols. of MeOH. Purification of the sulfated derivatives was preformed with repeated dissolution in water and re-precipitation with MeOH.

2.11. Antioxidant activity

The antioxidant capacity of the extracts was studied through their scavenging activity against 1,1-diphenyl-2-pycryl-hydrazyl (DPPH) radicals, using the method of Gerhäuser et al. (2003), as modified from Van Amsterdam, Roveri, Maiorino, Ratti, and Ursini (1992). The percentage of DPPH bleaching utilized for SC_{50} (half maximal scavenging concentration) was calculated as being 0% is the DPPH absorbance and 100% is the absorbance in presence of ascorbic acid.

2.12. Cell culture

In preliminary studied the antitumor activity of the four types of levan (L1, L2, SL1 and SL2) were evaluated against human hepatocarcinoma cell line (HepG2). Then a variety of human cell lines were used in testing the anti-cancer activity of sulfated *B. subtilis* levan (SL1) including: hepatocellular carcinoma (HepG2), colon carcinoma (HCT-116), cervical carcinoma (HeLa), histiocytic lymphoma and breast adenocarcinoma (MCF-7) (ATCC, VA, USA) and lymphoblastic leukemia (1301) cells, a generous gift from The Training Center of DakoCytomation, Elly, UK. HCT-116 cells were grown in McCoy's medium, while all cells were routinely cultured in DMEM (Dulbeco's Modified Eagle's Medium) at 37 °C in humidified air containing 5% CO_2. Media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, containing 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate, and 250 ng/ml amphotericin B. Monolayer cells were harvested by trypsin/EDTA treatment, while and leukemia cells were harvested by centrifugation. The dilutions of *B. subtilis* levan were tested, before assays, for endotoxin using Pyrogent® Ultra gel clot assay, and they were found endotoxin free. All experiments were repeated four times, unless mentioned, and the data was represented as (mean ± S.D.). Cell culture material was obtained from Cambrex BioScience (Copenhagen, Denmark), and all chemicals were from Sigma (USA).

2.13. Cytotoxicity assay

The cytotoxic effect of four types of levan was screened by estimation its effect on the growth of human hepatocarcinoma cell line (HepG2). According to the results the cytotoxic effect of sulfated *B. subtilis* levan (SL1) on the growth of different human cancer cell lines was estimated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Hansen, Nielsen, & Berg, 1989). The yellow tetrazolium salt of MTT is reduced by mitochondrial dehydrogenases in metabolically active cells to form insoluble purple formazan crystals, which was dissolved by the addition of a detergent. Cells (5×10^4 cells/well) were incubated for 48 h with various concentrations of sulfated *B. subtilis* levan SL1 at 37 °C in a FBS-free medium, before submitted to MTT assay. The absorbance was measured with microplate reader (BioRad, München, Germany) at 570 nm. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The data are expressed as the mean percentage of viable cells as compared to untreated cells. The relative cell viability was expressed as the mean percentage of viable cells as compared to the respective untreated cells (control). The half maximal growth inhibitory concentration (IC_{50}) value was calculated from the line equation of the dose-dependent curve of sulfated *B. subtilis* levan (SL1). The results were compared with the cytotoxic activity of paclitaxel, a known anti-cancer drug.

2.14. Apoptosis and necrosis staining

The type of cell death was investigated in sulfated *B. subtilis* levan-(SL1) cells using acridine orange/ethidium bromide staining (33 and 34). In brief, a mixture of 100 μg/ml acridine orange and 100 μg/ml ethidium bromide was prepared in PBS. The cell uptake of the stain was monitored under a fluorescence microscope, and the apoptotic, necrotic, and viable cells were counted. The early apoptotic cells had yellow chromatin in nuclei that were highly condensed or fragmented. Apoptotic cells also exhibited membrane blabbing. The late apoptotic cells had orange chromatin with nuclei that were highly condensed and fragmented. The necrotic cells had bright orange chromatin in round nuclei. Only cells with yellow, condensed, or fragmented nuclei were counted as apoptotic cells in a blinded, nonbiased manner.

2.15. DNA fragmentation

DNA fragmentation was essentially assayed as reported previously (Messmer, Reed, & Brüne, 1998). Briefly, SL1 levan-treated HepG2 cells pellet was re-suspended in 250 μl 10 mM Tris, 1 mM EDTA, pH 8.0 (TE-buffer), and incubated with an additional volume lysis buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5% Triton X-100) for 30 min at 48 °C. After lysis, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation. Pellets were re-suspended in TE-buffer and samples were precipitated by 10% trichloroacetic acid at 48 °C. The sample pellets were added to 5% trichloroacetic acid and boiled. DNA contents were quantified using the diphenylamine reagent (Burton, 1956). The percentage of DNA fragmented was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

2.16. Mitochondrial transmembrane potential ($\Delta\psi_m$)

5,59,6,69-Tetrachloro-1,19,3,39-tetraethybenzimidazol carbocyanine iodide (JC-1, Molecular Probes) is a lipophilic, cationic dye that enters mitochondria in proportion to the membrane potential and forms J-aggregates at the high intramitochondrial concentrations induced by higher $\Delta\psi_m$ values (Kidd et al., 2002). Measurement of mitochondrial transmembrane potential ($\Delta\psi_m$) was carried out by JC-1 (Wadia et al., 1998). In 96 well plates, HepG2 cells were seeded and then preloaded with 10 μg/ml JC-1 dissolved in HBSS for 30 min, 37 °C. The JC-1 loaded cells were incubated with and without (SL1) at 37 °C for 2 h period. JC-1 exists as a monomer (emission 527 nm) at low $\Delta\psi_m$ but forms J-aggregates (emission 590 nm) at high $\Delta\psi_m$, which can be assessed by JC-1 by monitoring fluorescence emission ratios at (emission 590:527 nm). The ratio of red (590 nm) to green (527 nm) gives an index of the $\Delta\psi_m$: the higher the $\Delta\psi_m$ the greater proportion of JC-1 aggregates in the mitochondria, the greater the intensity of the red light signal and so with active mitochondria we see a relatively high ratio of 590/527 nm. Fluorescence values were monitored in plates at zero time and after 2 h by microplate fluorescence reader (FluoStarOptima, BMG). Values are expressed as percent of zero time reading. Carbonyl cyanide m-chlorophenylhydrazone (CCCP, 10 μM) was used as a positive control.

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2.17. Preparation of cell lysates

After harvesting of sulfated levan (SL1) HepG2 cells, they were washed and centrifuged for 10 min at 1000 × *g*. The cell pellet was lysed in 0.5 ml of ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP40, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 10 mg/ml leupeptin (pH 7.4)). The lysates were passed through a 21-gauge needle to break up cell aggregates. After centrifugation at 14,000 × *g* for 15 min at 4 °C, the supernatants (total cell lysates) were submitted to measure cytochrome *C* and caspases. The cellular total protein content of the lysates was measured according to Smith et al. (1985), by bicinchoninic acid (BCA) assay and bovine serum albumin (BSA), as a standard.

2.18. Immunoblotting of cytochrome C

Cytochrome *C* was assessed in cell lysate of from sulfated levan-SL1 HepG2 cells. Briefly, 20 μg of isolated soluble proteins was applied at dot blotting set using nitrocellulose membranes. The change in cytochrome *c* protein was detected by dot immunoblotting using cytochrome *C* Releasing Apoptosis Assay kit (# K257, BioVision, CA, USA). After being washed, bound antibody was detected using rabbit anti-goat antibody linked to horseradish peroxidase (Dako), and bound complexes were detected using O-phenylenediamine dihydrochloride (OPD) (Amersham-Pharmacia). The percentage of the enhanced color intensity was shown as fold induction after normalization of dot intensity with actin control. The dot photographing and analysis was performed using gel documentation system (Biometra, Goettingen, Germany).

2.19. Evaluation of caspases activity

The cell lysates of sulfated levan-SL1 HepG2 cells were submitted to different kits to measure the level of total caspases, caspase-3, and caspase-9, according to the manufacturer instructions. Red Multi-Caspase Staining Kit (# PK-CA577-K190), PromoKine, Heidelberg, Germany was used for analysis of total caspases in a black microtiter plate with fluorescence plate reader at Ex. = 540 nm and Em. = 570 nm. The assay utilizes the caspase family inhibitor VAD-FMK conjugated to sulfo-rhodamine (Red-VAD-FMK) as the fluorescent *in situ* marker. Caspase-3 Colorimetric Detection Kit (# 907-013), Stressgen biotechnologies, Canada, was used to measure caspase-3 at 405 nm. One unit of Caspase-3 activity is defined as the amount of enzyme needed to convert one picomole of substrate per minute at 30 °C (U/ml). Caspase-9 Colorimetric Assay Kit (# PK-CA577-K119), PromoKine Heidelberg, Germany, was used to evaluate caspase-9 activity. The assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labeled substrate LEHD-*p*NA. The *p*NA light emission can be quantified at 405 nm. In the assay, a constant substrate concentration (200 μM final concentration) was added to the assay reaction. A comparison of the fluorescence or absorbance readings of the treated and untreated-HepG2 cells allows determination of the fold increase in the total caspases, caspase-3, and caspase-9 activities.

2.20. Histone deacetylase activity

The activity of HDAC in the lysate of sulfated levan-(SL1) HepG2 cells was measured using a colorimetric assay kit (BioVision, Mountain View, kit no. K331-100). The procedure involves the use of the HDAC colorimetric substrate (Boc-Lys (Ac)-pNA), which comprises an acetylated lysine side chain and is incubated with a sample containing nuclear extract. Deactivation sensitizes the substrate, and treatment with the lysine developer produces a chromophore, which can be analyzed using a colorimetric plate reader. HeLa cell nuclear extract was used as a positive control. A standard curve was prepared using the known amount of the deacetylated standard (Boc-Lys-pNA) included in the kit. A similar volume of control sample was added to 100 ng/ml trichostatin A (TSA), as a known inhibitor of HDAC activity.

2.21. Statistical analysis

The Student's unpaired *t*-test was used to detect the statistical significance between treated and untreated cells in each measured activity, where a *P* value less than 0.05 was considered significant.

3. Results

3.1. Isolation and identification of bacterial strain

The levansucrase producer was isolated from Mediterranean Sea in shallow water Alexandria and identified as *Bacillus subtilis* NRC1aza based on API identification.

3.2. Studies on the optimum conditions for levansucrase production and exopolysaccarides (EPS) yield

The bacterial isolate could produce levansucrase and yield EPS in both static and shaken culture at 50 rpm with approximately no difference in enzyme production and EPS yield (61–59 U/ml, 29–27.5 (L1), 5–6 (L2) g static and shaken, respectively). The increase of rpm has a bad influence in EPS yield, at 100 rpm the minor EPS L2 completely disappeared and the major EPS decreased to (12 g/l). On the other hand the levansurase production approximately not affected till 200 rpm (Fig. 1a). The amount of produced levansucrase and EPS yield was measured at different temperatures, incubation times and sucrose concentrations. The results (Fig. 1b) showed that the maximum levansucrase production (65 U/ml) and EPS yield (30 g L1 and 6 g L2) were at 28 °C and the increase of temperature over 30 °C had adverse effect on both. Fermentation time had a noticeable effect on enzyme production and EPS yield. The result in Fig. 1c showed that the maximum enzyme activity (70 U/ml) obtained after 16 h and the highest EPS (34 and 6 g for L1 and L2, respectively) yield was achieved at 32 h. The amount of levansucrase and EPS yield is directly proportional to the sucrose concentrations and the maximum EPS and levansucrase production were achieved at 14–16% sucrose concentration (Fig. 1d).

3.3. Levan identification

The *B. subtilis* NRC1aza extracellular levansucrase yielded two types of exopolysaccharide (EPS) called (L1), its molecular weight = 85.23 kDa and (L2) with Mw = 31.95 kDa. The results of paper chromatography and HPLC analyses referred to the levan nature of those products where the main backbones of the hydrolysate were fructose with tiny traces of glucose, the ratio of glucose to fructose was 1–20.

3.4. Antioxidant property

3.4.1. Scavenging effect assay

Investigation of the antioxidant activity of different extracts against DPPH radicals indicated that they are strong antioxidants as concluded from the low SC_{50} values of extract, where L1 and SL1 were the best radical scavenger (Table 1).

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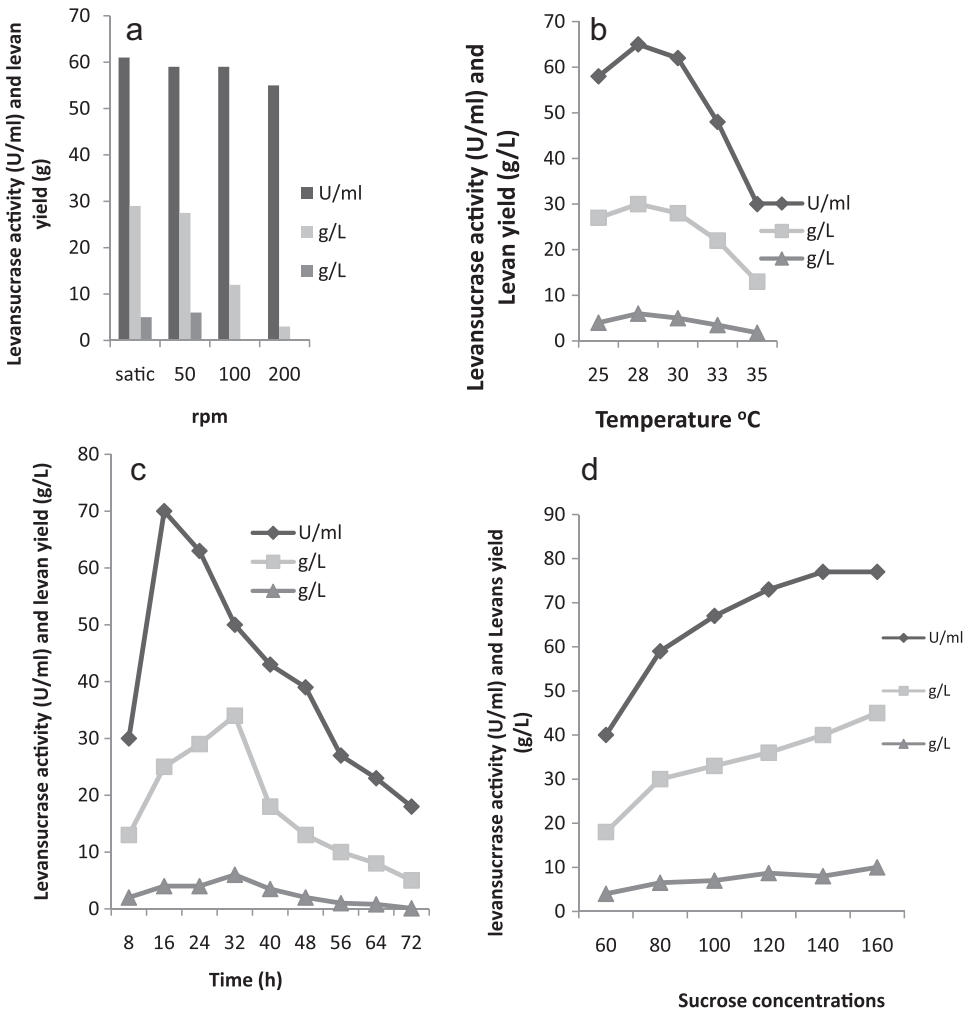

Fig. 1. Effect of different parameters on levansucrase production and levan yield (rpm a, temperature b, incubation period c and substrate concentrations d).

3.5. Cytotoxicity of levans

Preliminary study was done to test the anti-cancer activity of levan (L1 and L2) and levans sulfate (SL1 and SL2), a synthetic derivative of *B. subtilis* NRC1aza polyfructan levan to evaluate the anti-tumor activity of them against human hepatocarcinoma cell line (HepG2). The treatment with the L2 and SL2 samples lead to an insignificant change of the proliferation rate of Hep-G2 cells compared to the growth of the control cells, while treatment with both L1 and SL1 resulted in a remarkable growth inhibition at the high doses, as indicated from the cell growth rate (Fig. 2). According to this result the sulfated levan SL1 was chosen for the following investigations.

Screening of the cytotoxic effect of sulfated levan (SL1) against variable human cancer cell lines revealed that SL1 possessed a highly cytotoxic against Hep-G2 cells as indicated from its low IC_{50} value. The sulfated levan showed variable cytotoxic effect against different cell lines, as shown at Table 2, in the following order: Hep-G2 > HeLa > HCT-116 > 1301 > MCF-7 cells as concluded from their IC_{50} values.

Table 1
Antioxidant activity of native (L1 and L2) and sulfated levans 857 (SL1 and SL2).

Samples	DPPH scavenging activity (SC_{50} (μg/ml))
L1	3.5 ± 0.28
L2	9.1 ± 0.80
SL1	1.5 ± 0.16
SL2	6.4 ± 0.72

3.6. Apoptosis and necrosis analysis

According to the findings of the cytotoxicity experiment, sulfated levan possessed a variable cytotoxic effect against different cell lines. Apoptosis and necrosis are two different types of cell death with different pathways. To detect the type of cell death

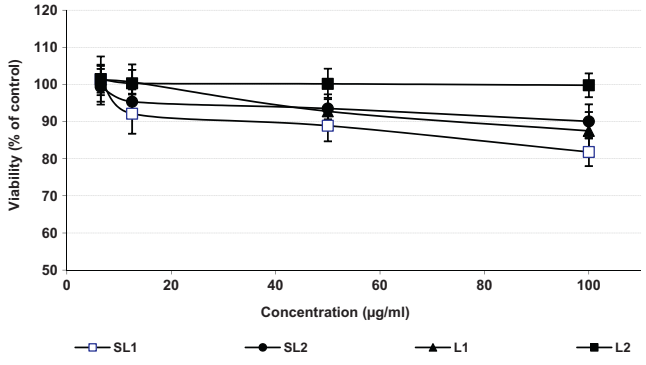

Fig. 2. Anti-proliferative activity: the viability percentage of HepG2 (circle), 1301 cells (square) and HCT-116 cells (triangle) treated with different concentrations of the tested extracts.

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Table 2
Cytotoxicity (IC_{50}, μg/ml) of different fractions of sulfated *B. subtilis* levan against human malignant cell lines after 48 h of incubation.

Sample	Cell line				
	Hep-G2	MCF-7	HCT-116	HeLa	1301
SL1	13.29 ± 0.61	43.18 ± 3.41	18.74 ± 2.14	14.05 ± 0.42	30.15 ± 0.77
Paclitaxel	0.47 ± 0.10	0.87 ± 0.20	0.38 ± 0.13	0.51 ± 0.08	0.68 ± 0.09

that induced by sulfated levan, cells were treated with the corresponding IC_{50} values of sulfated levan for only 6 h and the number of apoptotic and necrotic cells were recorded after acridine orange/ethidium bromide staining. sulfated levan was found to lead to apoptosis-dependant cell death up to 82.7% of the number of total dead cells in HepG2 cells and 71.6% of the number of total dead cells in HeLa cells, as shown in Fig. 3A. On the other hand, sulfated levan (SL1) was found to lead to necrosis-dependant cell death in HCT-116 and 1301 cells.

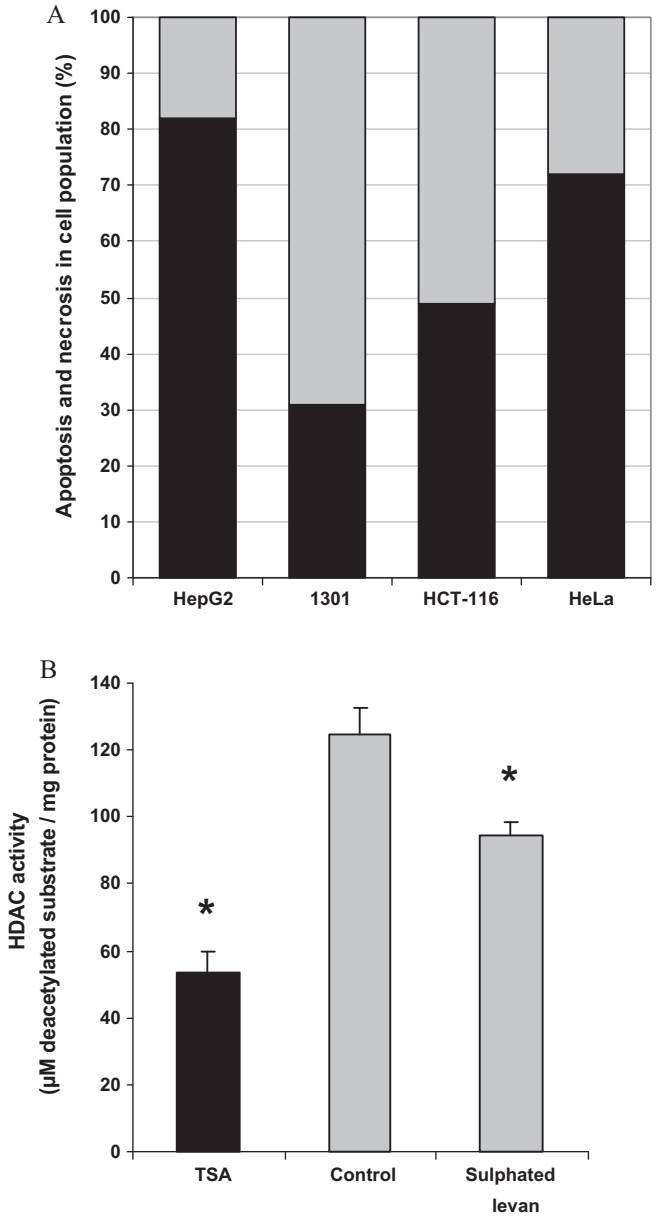

Fig. 3. (A) The type of cell death was investigated in different cell lines after the treatment with IC_{50} value of sulfated *B. subtilis* levan for 6 h, using acridine orange/ethidium bromide staining to compare between the percentage of necrotic cells (black segment) and the apoptotic cells (grey segment). (B) The effect of sulfated *B. subtilis* levan on HDAC activity of HepG2 cells after 48 h of treatment. The data was compared with the HDAC activity of the control cells pellet that was treated with TSA (100 ng/ml), as a known inhibitor. The data represent the mean of three readings ± S.D, where (*) represent the $P < 0.05$.

3.7. DNA fragmentation

Comparing the untreated cells with paclitaxel-treated HepG2 cells indicated that the later significantly induced the DNA fragmentation up to 51.1% ($P < 0.001$). Similarly the treatment of HepG2 cells with sulfated levan resulted in a high DNA fragmentation level (47.3%) that was significantly different from control ($P < 0.01$), while in HeLa cells sulfated levan resulted in a lower DNA fragmentation level (21.1%, $P > 0.05$).

3.8. Histone deacetylase activity

To determine if decreased deacetylation is involved in net increased DNA fragmentation and apoptosis in sulfated levan-treated HepG2 cells, we measured HDAC activity using the substrate Boc-Lys(Ac)-pNA and a colorimetric detection. TSA was used as a known inhibitor of HDAC activity. TSA resulted in 48.3% of HDAC activity ($P < 0.01$) (Fig. 3B) similarly the treatment of HepG2 cells with sulfated levan resulted in a drastic decline in HDAC activity of 24.3% ($P < 0.05$) of the control activity (Fig. 3B).

3.9. Mitochondrial transmembrane potential

Loss of $\Delta\psi_m$ is a crucial dysfunction of mitochondria, because loss of $\Delta\psi_m$ induces release of apoptogenic factors into cytoplasm and decrease of ATP generation, leading to cell death (37 and 40). In the present study, we have demonstrated that the preferential target of sulfate levan is the mitochondria and it induced the mitochondrial dysfunction. The mitochondrial dysfunction was assessed by measuring $\Delta\psi_m$ in HepG2 cells. Interestingly, the significant ability of sulfated levan to lower the $\Delta\psi_m$ was close to the reduction resulted from CCCP treatment, as a known inhibitor of $\Delta\psi_m$ (Fig. 4A).

3.10. Cytochrome C release

Cytochrome *c*, a component of the mitochondrial electron transfer chain that is present in the intermembrane space, is released into the cytosol during the early phases of apoptosis. Therefore, we assayed the accumulation of mitochondrial cytochrome *c* release into the cytosol by immunoblotting of the cell lysate of sulfated levan-treated HepG2 cells. Cytosol from untreated cells was found to contained low cytochrome *c* content, as concluded from the low intensity percentage (18.5%), compared to the normalized blotting membrane color intensity. In contrast, cytochrome *c* accumulated significantly in the cytosol of the HepG2 cells after the treatment with sulfated levan, as concluded from the high dot intensity percentage (83.2%).

3.11. Cell cycle

Exploring the cytotoxic effect of sulfated levan against HepG2 cells, we investigated their further effect on the cell cycle phases

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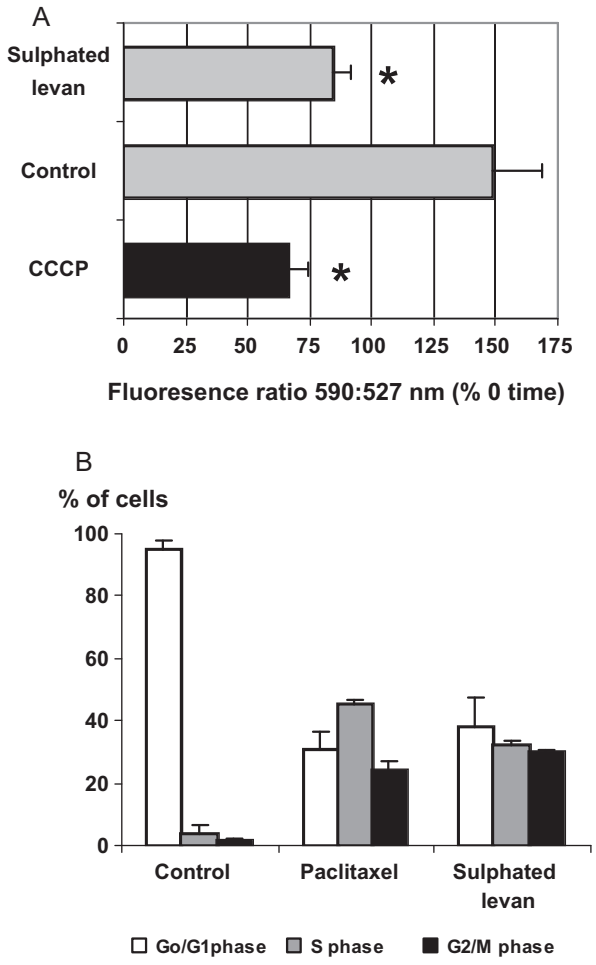

Fig. 4. (A) The effect of sulfated *B. subtilis* levan on mitochondrial membrane potential ($\Delta\psi_m$) of HepG2 cells. CCCP was the positive control. Values are expressed as percentage of zero time readings and represent mean of three readings ± S.D, where (*) represent the $P < 0.05$. (B) Cell cycle analysis in untreated (D) HepG2 cells and after treatment with sulfated *B. subtilis* levan or paclitaxel, using flow cytometry (a representative pattern of three trials for each). (C) The effect of sulfated *B. subtilis* levan on the level of total caspases, caspase-3 and caspase-9 in HepG2 cells. Data are expressed as fold of control untreated cells activity.

distribution. Our findings described that the treatment with sulfated levan (Fig. 4B) resulted in huge arrests in S- and G2/M phases as concluded from the high accumulated cell populations in these phases compared with the phases pattern of untreated cells (Fig. 4B).

3.12. Evaluation of caspases

In many apoptotic systems, release of cytochrome *c* into the cytosol results in the activation of the executioner caspases of apoptosis. To explore the possible involvement of caspases activation in the sulfated levan-associated apoptosis, caspases were investigated and revealed that there was a significant increase in the total caspases after the treatment with sulfated levan ($P < 0.01$), as shown in Fig. 4C. Moreover, the investigation of the individual caspases after the treatment of HepG2 cells with sulfated levan (SL1) indicated that the treatment led to a significant increase in caspase-3 and -9 activity ($P > 0.01$), as shown in Fig. 2C.

4. Discussion

Finding of new levan producer species is of great interest considering its potential as a multifunctional biopolymer. The chemical modification of polysaccharides may allow the preparation of derivatives with new properties and a variety of applications (Yang et al., 2005). Recently, the antivirus, antioxidant and antitumor activities of levan and its derivatives were evaluated (Liu et al., 2012; Esawy et al., 2011). Within this context, a levan hyperproducing *B. subtilis* NRC1aza was isolated from Mediterranean Sea in shallow water Alexandria. Considering the wide spread use of microbial polysaccharides in various industrial applications, microorganisms from marine environments was indented as the best levan producer (Poli et al., 2009). *B. subtilis* NRC1aza produced two types of levans with different molecular weights. Generally *B. subtilis* are well known as producers of a great variety of secondary metabolites such as EPS (Esawy et al., 2011; Abdel-Fattah, Mahmoud, & Esawy, 2005; Shih, Shieh, Yu, & Hsieh, 2005). It was reported in levan product consisted of two fractions with different molecular masses (1794 and 11 kDa) (Shih et al., 2005). The results indicated that levans were composed mainly of fructose, which is in agreement with the report by (Esawy et al., 2011; Poli et al., 2009; Han & Clarke, 1990). The optimum conditions for levansucrase production and levans yield was determined. In spite, the enzyme production not affected by the increasing of rpm the L2 was disappeared completely at 100 rpm. On contrary, the best levan production by *Zymomonas mobilis* was obtained using orbital agitation at 100 rpm (Melo, Pimentel, Lopes, & Calazans, 2007). The temperature above 30 °C has adverse effect on levansucrase production and levan yield. Similar results were reported by (Esawy, Mahmoud, & Abdel Fattah, 2008; Abdel-Fattah et al., 2005; Senthilkumar & Gunasekaran, 2005; Keith et al., 1991). The maximum *B. subtilis* NRC1aza levansucrase activity and levan yield were reached at 140 sucrose concentrations at 16–24 h. On the other hand, it was reported in production of *Zymomonas mobilis* CT2 levan at 200 g/l sucrose. The enzyme that is responsible for synthesizing levan was maximally produced at concentration of 20% sucrose for 48 h and pH 6 (Nasab, Layegh, Aminlari, & Hashemi, 2010). DPPH is a kind of stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule, which is widely used to investigate radical scavenging activity (Blois, 1958). The results obtained in this investigation reveal that all levan are free radical scavengers and able to react with the DPPH radical, which might be attributed to their electron donating ability. The sulfating levan increased the antioxidant activity to great instant. Chemically modified group, acetyl, phosphoryl or benzyl group, into levan molecule could significantly enhance the antioxidant and antitumor activities of levan (Liu et al., 2012). The following data suggest for that sulfating of polysaccharides significantly increases antitumor activity. Anti-proliferative activity against tumor cell lines human hepatocarcinoma (HepG2) was tested by using the four sulfated and nonsulfated levan. The sulfated sample (SL1) resulted in a remarkable growth inhibition at the high doses, as indicated from the cell growth rate; accordingly it was chosen for several tests. The activation of the effector caspase is regularly initiated by the amplification of death-inducing signaling complex signals by engagement of the cell-intrinsic pathway. An essential step in the cell-intrinsic pathway is the activation of Bax, leading to dissipation of mitochondrial transmembrane potential ($\Delta\psi_m$) and cytochrome c release into the cytosol (Kang et al., 2009). The agents that induce apoptosis may be implemented by the cell-intrinsic pathway, which regularly begins with the disruption of $\Delta\psi_m$ and the release of apoptogenic factors such as cytochrome c from the intermembrane space into the cytosol. These factors activate caspase-9, which in turn activates the executioner caspase-3. In this pathway, mitochondrion is the center of cell death control, and the mitochondrial membrane is the

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primary site of action by proapoptotic and antiapoptotic factors (Kang et al., 2009).

The sulfated levan-induced apoptosis may be implemented by the cell-intrinsic pathway (the mitochondrion-mediated pathway), which regularly begins with the disruption of $\Delta\psi_m$ and the release of apoptogenic factors (proteins) such as cytochrome c from the intermembrane space into the cytosol (Liu, Kim, Yang, Jemmerson, & Wang, 1996). These factors activate caspase-9, which in turn activates the executioner caspase-3. In this pathway, mitochondrion is the center of cell death control, and the mitochondrial membrane is the primary site of action by proapoptotic and antiapoptotic factors (Liu et al., 1996).

Mitochondria are subcellular organelles that are essential in the regulation of cellular bioenergetics as a major source of ATP, which is produced through oxidative phosphorylation by the mitochondrial respiratory chain (RC) that localized in the inner membrane of the mitochondria and includes cytochrome *c* as an electron carrier (Jia et al., 2001). The electron transport generates an electrochemical proton gradient across the inner membrane, measured as $\Delta\psi_m$, which drives ATP synthesis by the ATP synthase (Jia et al., 2001). Besides respiration, mitochondria also play important roles in the regulation of apoptosis. The mitochondrial pathway of apoptosis is initiated by the proapoptotic Bcl-2 family proteins, which form pores and induce mitochondrial outer membrane permeabilization. This leads to a release of cytochrome *c*, loss of $\Delta\psi_m$, and activation of various caspases. These caspases cleave specific substrates within the cell to produce changes associated with apoptosis ($\Delta\psi_m$ is important for ATP production and mitochondrial protein transport (Liu et al., 1996)). On the other hand, disruption of $\Delta\psi_m$ is also implicated in various apoptotic phenomena (Kwong, Henning, Starkov, & Manfredi, 2007). Mitochondrial dysfunction and dysregulation of apoptosis are implicated in many diseases such as cancer and neurodegeneration. Mutations in the mitochondrial DNA cause RC defects in some forms of mitochondrial disorders but have also been shown to accumulate in cancer (Kwong et al., 2007).

The important characteristic in any chemotherapeutic drugs is its ability to induce $\Delta\psi_m$ collapse and cytochrome c release, thereby induce the activation of the downstream caspases and apoptosis (Amarante-Mendes, Naekyung, Liu, Huang, & Perkins, 1998). In the present work, the treatment of HepG2 cells with sulfated levan resulted in DNA damaging and fragmentation that was associated with the induction of apoptosis *via* mitochondrial pathway. This pathway is initiated by the impairment of $\Delta\psi_m$ and in response to that besides the DNA damage and the mitochondria released cytochrome c that may in turn activated caspase-9 through auto-proteolysis (Jia et al., 2001). Active caspase-9 proteolyses activated caspase-3, which activates other downstream caspases causing the apoptotic phenotype.

Previously, levan was produced from *Microbacterium laevaniformans* KCTC 9732 (M-levan) was isolated and treated to produce its hydrolyzed form (Yoon, Yoo, Cha, & Gyu Lee, 2004). Strong levan-induced inhibition of cell growth was detected on SNU-1 and HepG2 tumor cell lines. As the branching degree of M-levan reduced, antitumor activity on SNU-1 linearly decreased ($r^2 = 0.96$). In HepG2, the antitumor activity rapidly dropped when the branching reached up to 9.3%, then slightly increased as the branching degree of M-levan further decreased. These results suggested that the branch structure would play a crucial role in levan's antitumor activity (Yoo et al., 2004). In another report, levans of different molecular weights were isolated from different microbial cultures of *Gluconoacetobacter xylinus, Microbacterium laevaniformans, Rahnella aquatilis, and Zymomonas mobilis.* In the *in vitro* antitumor activity test of the levans against eight different tumor cell lines, relatively stronger activity was observed from the SNU-1 and HepG2 (Yoo et al., 2004).

Previously Aerobacter levan was shown to have antitumor and immunostimulating activities. Zymomonas levan as a potential antitumor agent was studied with Sarcoma-180 cell (Yoo et al., 2004). To our knowledge no other reports investigated the mechanism of antitumor effect of the sulfated microbial levan.

5. Conclusion

This study focused in isolation of *B. subtilis* NRC1aza, the unique feature of this isolate its ability to produce two types of levan with different molecular weights. The optimization conditions for levan production affected greatly in levan yield. The antioxidant and anticancer activity of the levans and their derivatives (sulfated form) recommended all the tested levans as stronger antioxidants. The characterization of sulfated levan SL1 and investigated its anticancer activity and its mechanistic role in cell death was done. Investigations of the cytotoxic effect of sulfated *B. subtilis* against different human cell lines indicated that sulfated *B. subtilis* NRC1aza levan showed high selective cytotoxicity against hepatocellular carcinoma HepG2 cells. Sulfated *B. subtilis* levan led to DNA damaging and fragmentation that was associated with the induction of apoptosis *via* mitochondrial pathway. This pathway is initiated by the impairment of mitochondrial transmembrane potential ($\Delta\psi_m$) and in response to that the mitochondria released cytochrome c increased, that in turn activated caspase-9 and caspase-3 and induced apoptosis. Sulfated *B. subtilis* levan was a promising anticancer agent that induced intrinsic apoptosis pathway in liver cancer cells.

References

Abdel-Fattah, A. F., Mahmoud, D. A. R., & Esawy, M. A. (2005). Production of levansucrase from *Bacillus subtilis* NRC 33a and enzymic synthesis of levan and fructo-oligosaccharides. *Current Microbiology*, *51*, 402–407.

Adams, J. M., & Cory, S. (2007). Bcl-2-regulated apoptosis: Mechanism and therapeutic potential. *Current Opinion in Immunology*, *19*, 488–496.

Amarante-Mendes, G. P., Naekyung, K. C., Liu, L., Huang, Y., Perkins, C. L., et al. (1998). Bcr-Abl exerts its antiapoptotic effect against diverse apoptotic stimuli through blockage of mitochondrial release of cytochrome *C* and activation of caspase-3. *Blood*, *91*, 1700–1705.

Ananthi, P., Ranjitha Kumari, B. D., & Ramachandran, A. (2010). In vitro propagation of *Rorippa indica* L. from nodal and shoot tip explants. *International Journal for Biotechnology and Molecular Biology Research*, *2*, 50–55.

Arvidson, S. A., Rinehart, B. T., & Gadala-Maria, F. (2006). Concentration regimes of solutions of levan polysaccharide from *Bacillus* sp. *Carbohydrate Polymers*, *65*, 144–149.

Barone, J. R., & Medynets, M. (2007). Thermally processed levan polymers. *Carbohydrate Polymers*, *69*, 554–561.

Belghith, H., Song, K. B., Kim, C. H., & Rhee, S. K. (1996). Optimal conditions for levan production by an overexpressed recombinant levansucrase. *Biotechnology Letter*, *18*, 467–472.

Ben Ammar, B. Y., Matsubara, T., Ito, K., Iizuka, M., Limpaseni, T., Pongsawasdi, P., et al. (2002). Characterization of a thermostable levansucrase from *Bacillus* sp. TH4-2 capable of producing high molecular weight levan at high temperature. *Journal of Biotechnology*, *99*, 111–119.

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, *26*, 1199–1200.

Block, R. J., Vurrum, E. L., & Zweig, U. (1955). *A manual of paper chromatography and paper electrophoresis*. New York: Academic Press., p. 127.

Brunelle, J. K., & Letai, A. (2009). Control of mitochondrial apoptosis by the Bcl-2 family. *Journal of Cell Science*, *122*, 437–441.

Burton, K. A. (1956). Study of the conditions and mechanisms of the diphenylamine reaction for the estimation of deoxyribonucleic acid. *Biochemical Journal*, *62*, 315–323.

Calazans, G., Lima, R., deFranca, F., & Lopes, C. (2000). Molecular weight and antitumour activity of *Zymomonas mobilis* levans. *International Journal of Biological Macromolecules*, *27*, 245–247.

Cavalcanti, O. A., Petenuci, B., Bedin, A. C., Pineda, E. A. G., & Hechenleitner, A. A. W. (2004). Characterisation of ethylcellulose films containing natural polysaccharides by thermal analysis and FTIR spectroscopy. *Acta Farmaceutica Bonaerense*, *23*, 53–57.

Combie, J., Steel, A., & Sweitzer, R. (2004). Adhesive designed by nature (and tested at Redstone Arsenal). *Clean Technologies and Environmental Policy*, *6*, 258–262.

Dix, M. M., Simon, G. M., & Cravatt, B. F. (2008). Global mapping of the topography and magnitude of proteolytic events in apoptosis. *Cell*, *134*, 679–691.

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Dewson, G., & Kluck, R. M. (2009). Mechanisms by which Bak and Bax permeabilise mitochondria during apoptosis. *Journal of Cell Science*, *122*(Pt 16), 2801–2808.
Esawy, M. A., Mahmoud, D. A. R., & Abdel Fattah, F. A. (2008). Immobilisation of *Bacillus subtilis* NRC33a levansucrase and some studies on its properties. *Brazilian Journal of Chemical Engineering*, *25*, 237–246.
Esawy, M. A., Ahmed, E. F., Wafaa, A. H., Mansour, N. M., El-Senousy, W. M., & El-Safty, M. M. (2011). Production of levansucrase from novel honey *Bacillus subtilis* isolates capable of producing antiviral levans. *Carbohydrate Polymer*, *36*, 823–830.
Gerhäuser, C., Klimo, K., Heiss, E., Neumann, I., Gamal-Eldeen, A., Knauft, J., Liu, J. U., Sitthimonchai, S., & Frank, N. (2003). Mechanism-based in vitro screening of potential cancer chemopreventive agents. *Mutation Research*, *523–524*, 163–172.
Han, W. Y., & Clarke, A. M. (1990). Production and characterization of microbial levan. *Journal of Agricultural and Food Chemistry*, *38*, 393–396.
Hansen, M. B., Nielsen, S. E., & Berg, K. (1989). Re-examination and further development of aprecise and rapid dye method for measuring cell growth/cell kill. *Journal of Immunological Methods*, *119*, 203–210.
Hettwer, U., Gross, M., & Rudolph, K. (1995). Purification and characterization of an extracellular levansucrase from *Pseudomonas syringae* pv. *phaseolicola*. *Journal of Bacteriology*, *177*, 2834–2839.
Hussein, M. M. (1994). *Egyptian patents, No. 19381 August*. CO8G63100, A61K9100.
Jia, L., Patwari, Y., Srinivasula, S. M., Newland, A. C., Fernandes-Alnemri, T., Alnemri, E. S., & Kelsey, S. M. (2001). Bax translocation is crucial for the sensitivity of leukaemic cells to etoposide-induced apoptosis. *Oncogene*, *20*, 4817–4826.
Kang, et al. (2009). Levan: Applications and perspectives. In *Microbial production of biopolymers and polymer precursors*. Caister Academic Press., ISBN 978-1-904455-36-3.
Kasapis, S., & Morris, E. (1994). Conformation and physical properties of two unusual microbial polysaccharides: Rhizobium CPS and levan. *In K.*
Kasapis, S., Morris, E., Gross, M., & Rudolph, K. (1994). Solution polysaccharide from Pseudomonas syringe pv phaseolicola and its possible primary role as a blocker of recognition during pathogenesis. *Carbohydrate Polymer*, *23*, 55–69.
Kaufmann, S. H., & Earnshaw, W. C. (2000). Induction of apoptosis by cancer chemotherapy. *Experimental Cell Research*, *256*, 42–49.
Keith, J., Wiley, B., Ball, D., Arcidiacono, S., Zorfass, D., Mayer, J., et al. (1991). Continuous culture system for production of biopolymer levan using Erwinia herbicola. *Biotechnology and Bioengineering*, *38*, 557–560.
Kidd, J. F., Pilkington, M. F., Schell, M. J., Fogarty, K. E., Skepper, J. N., Taylor, C. W., & Thorn, P. (2002). Paclitaxel affects cytosolic calcium signals by opening the mitochondrial permeability transition pore. *The Journal of Biological Chemistry*, *277*, 6504–6510.
Kirkinezosa, I. G., & Moraesa, C. T. (2001). Reactive oxygen species and mitochondrial diseases. *Cellular and Developmental Biology*, *12*, 449–457.
Kojima, I., Saito, T., Iizuka, M., Minamiura, N., & Ono, S. (1993). Characterization of levan produced by Serratia sp. *Journal of Fermentation and Bioengineering*, *75*, 9–12.
Kwong, J. Q., Henning, M. S., Starkov, A. A., & Manfredi, G. (2007). The mitochondrial respiratory chain is a modulator of apoptosis. *Journal of Cell Biology*, *179*, 1163–1177.
Liu, J., Luo, J., Ye, H., & Zeng, X. (2012). Preparation, antioxidant and antitumor activities in vitro of different derivatives of levan from endophytic bacterium *Paenibacillus polymyxa* EJS-3. *Food and Chemical Toxicology*, *50*, 767–772.
Liu, X., Kim, C. N., Yang, J., Jemmerson, R., & Wang, X. (1996). Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome *c*. *Cell*, *86*, 147–157.
Lizuka, M., Tanaka, T., Yamamoto, S., et al. (1991). Synthesis of fructan and oligosaccharides by microbial and plant fructosyltransferases. *Denpun Kagaku*, *38*, 217–222.
Leibovich, J., & Stark, Y. (1985). Increase of permeability to aCytotoxic agent by the polysaccharide levan. *Cell and Molecular Biology*, *31*, 337–341.
Lüthi, A. U., & Martin, S. J. (2007). The CASBAH: A searchable database of caspase substrates. *Cell Death and Differentiation*, *14*, 641–650.
Meier, P., & Vousden, K. H. (2007). Lucifer's labyrinth—Ten years of path finding in cell death. *Molecular Cell*, *28*, 746–754.
Melo, I. R., Pimentel, M. F., Lopes, C. E., & Calazans, G. M. T. (2007). Application of fractional factorial design to levan production by Zymomonas mobilis. *Brazilian Journal of Microbiology*, *38*, 45–51.
Messmer, U. K., Reed, U. K., & Brüne, B. (1998). Bcl-2 protects macrophages from nitric oxide-induced apoptosis. *Journal of Biological Chemistry*, *271*, 20192–20197.
Namiki, M. (1990). Antioxidants/antimutagens in food. *Critical Reviews in Food Science and Nutrition*, *29*(4), 273–300.
Nasab, M. M., Layegh, B., Aminlari, L., & Hashemi, M. B. (2010). Microbial production of levan using date syrup and investigation of its properties. *World Academy of Science, Engineering and Technology*, *68*, 1238–1244.
Rhee, S., Song, K., Kim, C., Park, B., Jang, E., & Jang, K. (2002). Levan. In A. Steinbüchel (Ed.), *Biopolymers; polysaccharides I* (pp. 351–377). New York/Weinheim: Wiley/VCH.
Roberts, E. J., & Garegg, P. J. (1998). *Levan derivatives, their preparation, composition and applications including medical and food applications*. World Intellectual Property Organization Patent Application No. WO 98/03184.
Poli, A., Kazak, H., Gürleyendaı, B., Tommonaro, G., Pieretti, G., Öner Ebru, T., & Nicolaus, B. (2009). High level synthesis of levan by a novel Halomonas species growing on defined media. *Carbohydrate Polymers*, *78*, 651–657.
Senthilkumar, & Gunasekaran. (2005). Influence of fermentation conditions on levan production by *Zymomonas mobilis* CT2. *Indian Journal of Biotechnology*, *4*, 491–549.
Shih, I., Shieh, Y., Yu, C., & Hsieh, C. (2005). Selective production and characterization of levan by *Bacillus subtilis* (Natto) Takahashi. *Journal of Agriculture Food Chemistry*, *53*, 8211–8215.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, *150*, 76–85.
Tanaka, T., Oi Lizuka, M., & Yamamoto, T. (1978). Levansucrase of *Bacillus subtilis*. *Agriculture and Biological chemistry*, *42*, 323–326.
Van Amsterdam, F. T., Roveri, A., Maiorino, M., Ratti, E., & Ursini, F. (1992). Lacidipine: A dihydropyridine calcium antagonist with antioxidant activity. *Free Radical Biology and Medicine*, *12*, 183–187.
Wadia, J. S., Chalmers-Redman, R. M., Ju, W. J., Carlile, G. W., Phillips, J. L., Fraser, A. D., & Tatton, W. G. (1998). Mitochondrial membrane potential and nuclear changes in apoptosis caused by serum and nerve growth factor withdrawal: time course and modification by (-)-deprenyl. *Journal of Neuroscience*, *18*, 932–947.
Willis, S. N., & Adams, J. M. (2005). Life in the balance: How BH3-only proteins induce apoptosis. *Current Opinion in Cell Biology*, *17*, 617–625.
Yamamoto, Y., Takahashi, Y., Kawano, M., Iizuka, M., Matsumoto, T., Saeki, S., & Yamaguchi, H. (1999). In vitro digestibility and fermentability of levan and its hypocholesterolemic effects in rats. *Journal of Nutritional Biochemistry*, *10*, 13–18.
Yamamoto, Y., Takahashi, Y., Kawano, M., Iizuka, M., Matsumoto, T., Saeki, S., & Yamaguchi, H. (2000). Hypercholesterolemic effects of levan in rats. In K. Nishinari (Ed.), *Hydrocolloids (Part II)* (pp. 399–400). The Netherlands: Elsevier Science.
Yanase, H., Fukushi, H., Ueda, N., Maeda, Y., Toyoda, A., & Tonomura, K. (1991). Cloning, sequencing and characterization of the intracellular invertase gene from *Zymomonas mobilis*. *Agricultural and Biological Chemistry*, *55*, 1383–1390.
Youle, R. J., & Strasser, A. (2008). The BCL-2 protein family: Opposing activities that mediate cell death. *Nature Reviews Molecular Cell Biology*, *9*, 47–59.
Yoo, S., Yoon, E., Cha, E., & Lee, H. (2004). Antitumor activity of levan polysaccharides from selected microorganisms. *International Journal of Biological Macromolecules*, *34*, 37–41.
Yoon, E. J., Yoo, S. H., Cha, J., & Gyu Lee, H. (2004). Effect of levan's branching structure on antitumor activity. *International Journal of Biological Macromolecule*, *34*, 191–194.
Zhang., Q. Z., & Wu, J. Y. (2007). *Cordyceps sinensis* mycelium extract induces human premyelocytic leukemia cell apoptosis through mitochondrion pathway. *Experimental Biology and Medicine*, *232*, 52–57.

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