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Biological Activity of Levan Produced from Rhizospheric Soil Bacterium *Brachybacterium phenoliresistens* KX139300

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Abstract:

Levan is an exopolysaccharide produced by various microorganisms and has a variety of applications. In this research, the aim was to demonstrate the biological activity of levan which produced from *B. phenoliresistens* KX139300. These were done *via* study the antioxidant, anti-inflammatory, anticancer and antileishmanial activities *in vitro*. The antioxidant levan was shown 80.9% activity at 1250 µg/mL concentration. The efficient anti-inflammatory activity of 88% protein inhibition was noticed with levan concentration at 35 µg/mL. The cytotoxic activity of levan at 2500 µg/mL concentration showed a maximum cytotoxic effect on L20B cell line and promastigotes of *Leishmani tropica*. Levan has dose-dependent anticancer and antileishmanial activities. An addition to the antioxidant, anti-inflammatory and anticancer potential activities of levan, it can be concluded that levan produced from *B. phenoliresistens* can efficiently be applied as an antileishmanial agent.

Keywords: Anticancer; Anti-inflammatory; Antioxidant; Antileishmanial; *Brachybacterium phenoliresistens*; Levan.

Introduction:

Levan is natural homopolysaccharide of fructose, which is composed of monomers of D-fructose attached by β (2→6) linkages that carry a D-glucosyl residue at the end of the core chain. The biosynthesis of levan is commonly done by an enzyme called levansucrase which is also defined as a sucrose 6-fructosyl transferase (1). Levan is produced extracellularly by both gram-positive and gram-negative bacteria (2). Because of levan production depends on levansucrase, levan production is limited to bacteria that excrete levansucrase. Levansucrase have been found in some of gram-negative, plant-associated bacteria like *Acetobacter* (3). *Erwinia amylovora* (4), *Zymomonas mobilis* (5), and *Azotobacter* (6). As well as in gram-positive bacteria like *Bacillus amyloliquefaciens*, *Bacillus polymyxa* (7). According to Levan properties and depending on its molecular weight, levan is used in various fields such as food industry as industrial gums, flavors, sweetener, fragrances carrier emulsifier, thickener and stabilizer.

As well as pharmaceuticals, cosmetics, blood plasma extender and biomedical properties including antipathogenic, anti-inflammatory, antioxidant activity (8, 9, 10).

Levan has several biological properties that make it as an antitumor agent prevent infections and necrosis, tumor inhibition and increasing cell permeability to cytotoxic (11). Also, as an anti-inflammatory activity through the stimulation of interferon response (12).The activity of levan to inhibit the growth of pathogenic bacteria was described by (13). Also, leishmaniasis is a parasitic disease, which still public health problem in endemic countries, causes morbidity and economic losses. According to report of World Health Organization's (WHO), about 350 million people are living in regions with the risk of infection and 12 million people are infected by parasites (14).The objective of the current study amid to evaluating the biomedical potential of levan produced from Rhizospheric soil bacterium *B. phenoliresistens*as well as to evaluate the antileishmanial efficacy of levan against *Leishmania tropica*.

Material and Methods:

Chemicals and media utilized

Bovine serum albumin (BSA) and 2, 2-diphenyl-l-picrylhydrazyl (DPPH) were supplied from Sigma, Germany. Nutrient agar and peptone

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were obtained from Himedia, India. Diclofenac sodium and Ascorbic acid were obtained from Samarra Drug Industry (SDI), Iraq. The other chemicals used in these experiments were supplied from Merck Laboratories Pvt., Ltd. India.

Maintenance of Bacteria

B. phenoliresistens KX139300 strain was previously isolated by (Moussa *et al.*) (15), from legumes rhizosphere soil samples were collected from farms in Kana'an village (a region 60 km north-west the capital Baghdad), Iraq. The isolated strain was identified based on molecular identification (16Sr RNA) (15). For long term storage cultures were maintained on Luria-Bertani (LB) agar medium (6% sucrose, 1.5% peptone, 0.02% ammonium sulphate, 0.1% potassium dihydrogen phosphate, 0.1% magnesium sulphate heptahydrate and agar 20%, pH 6.8 at 30ºC for 18-24h, supplemented with 25% glycerol and stored at -20ºC. For working stock cultures were maintained at 4ºC (16).

Biopolymer production and purification

1. Inoculum preparation

Every experiment was conducted *via* two manner resuscitation of one glycerol vial in liquid media. The primary manner is used LB broth and then production medium which was prepared by dissolving (yeast extract, 2.5g; sucrose, 200g; Hydrated Magnesium Sulfate ($MgSO4\ 7H_2O$) 0.2g; Potassium Hydrogen Phosphate (K_2HPO_4) 5.5g in 1 liter of distilled water. Culture media was inoculated with 250 µL of glycerol culture to 250 mL Erlenmeyer flask of 50 mL working volume. The inoculated flasks were incubated on a rotary shaker (Sartorius Stedim, England) at 200 rpm and 30 ºC for 24 hours. Consequently, cells were used to inoculate 250 mL Erlenmeyer flask with 5% (v/v) inoculum concentration and inoculum density of OD 600 nm (17).

2. Batch fermentation and purification

Bacterial inoculum was prepared by the preparation of 50 ml of sterilized nutrient broth that contained in 250 ml in volume Erlenmeyer conical flask. This medium was inoculated by one loopful of bacterial culture, shaken well and incubated for 24 h at 30 ºC and 37 ºC. The content of these flasks were used as standard inoculum (1ml contained 7×10^6 viable cells) for shake flasks. At the end of the fermentation, the culture was centrifuged at 10000 rpm for 10 min to get cell-free supernatant. The culture filtrate was dialyzed against deionized water for 48 h with the dialysis membrane (Dialysis Membrane-110 Av. flat width - 31.13 mm, Av. diameter - 21.5 mm, capacity approx - 3.63 $mLcm^{-1}$, HiMedia Laboratories Pvt. Ltd.) to remove the residual sucrose and any low molecular weight fermentation products. The produced levan was characterized by previous study used TLC, FTIR, 1H NMR and 13C NMR spectroscopy technique (15) The dialysate was frozen with liquid nitrogen, freeze-dried and weighed (17).

Biomedical activity of B. phenoliresistens levan

1. In vitro antioxidant assay

The percentage of levan antioxidant activity was assessed by DPPH free radical scavenging trial. The DPPH solution (0.1 mM) was obtained by dissolving DPPH in ethanol. Levan solutions obtained by dissolving following concentrations of levan (0, 50, 100, 150, 200, 250, 500, 1000 and 1250 µg/mL) in distilled water. 300 µL of freshly prepared DPPH was added to 1 mL of the prepared Levan solution, then incubated in dark room for 30 min with ambient temperature. The change in the color was measured at 517 nm using UV-Vis spectrophotometer (Cecil, England). As a control, Ascorbic acid was used as a standard antioxidant for this trial (18, 19, 20), while a DPPH-ethanol mixture was represented as a control and a levan-ethanol mixture was indicated as a blank for the sample test. The percentage of antioxidant activity was determined according to the equation.

$$\text{DPPH Scavenging Activity}(\%) = (\text{AC} - \text{AS} / \text{AC}) \times 100$$

Where Ac and As are the peak intensity for DPPH and levan samples, respectively.

2. In vitro anti-inflammatory

Anti-inflammatory assay of *B. phenoliresistens* levan was estimated using inhibition of protein denaturation method (20, 21). Test solution (0.5 mL) consisting of 0.45 mL BSA (5% (w/v) aqueous solution) and 0.05 mL of different concentrations of levan test solution (0, 25, 50, 75, 100, 150,200, 250, 300 and 350µg/mL) in water was prepared. Test control solution (0.5 mL) was prepared with 0.45 mL BSA (5% (w/v) aqueous solution) and 0.05 mL of distilled water. Product control solution (0.5 mL) which consists of 0.45 mL distilled water and 0.05 mL levan solution was also prepared. All solutions were adjusted to pH6.3 using 1 N HCL. All samples were incubated at 37ºC for 30 min and at 57ºC for 3 min, respectively. The cold phosphate buffer saline (2.5 mL) was added to all solutions. The solution absorbance was estimated at 416 nm using UV-Vis spectrophotometer. Aliquots of Diclofenac sodium were served as a standard comparison. Inhibition percentage was determined as follows

$$\text{Inhibition}\ (\%) = 100 - (\text{AC} - \text{AS} / \text{AC}) \times 100$$

Where Ac and As are the peak intensity for Diclofenac sodium and levan samples, respectively.

3. In vitro anticancer activity

The anticancer efficacy was evaluated for levan from *B. phenoliresistens* against L20B cell line. According to (22, 23), the colorimetric cell viability MTT assay was used.

Briefly, 100 µL well^{-1} of L20B cells (10^6 cell mL^{-1}) were cultured in 96-well tissue culture plate. Different concentrations of levan test solution were prepared by dissolving (19, 39, 78, 156, 312, 625, 1250, and 2500 µg/mL) in water. Then, each well was treated with 100 µL of various concentrations and incubated at 37^{o}C for 24 h. After the incubation, 10 µL of MTT solution (5 mg/mL) was added to each well and incubation was continued for a further 4 hours. Finally, 50 µL of solubilization solution of DMSO (dimethyl sulfoxide) was added to each well and incubated for 10 min. L20B cells were cultured in complete medium without treated with levan solution as a positive control, and in a complete medium only as a blank. The experiment was performed in triplicate. The ELISA reader was used to measure absorbance for each well at 620 nm.The mean absorbance for each group of replicates was calculated. The live cells, percentage of viability and inhibition ratio were calculated according to the formula

$$\text{Inhibition}\,(\%) = (\text{AC} - \text{AS} \,/\, \text{AC}) \times 100$$

where Ac and As are the optical density for medium and levan samples, respectively.

4. In vitro antileishmanial activity

The antileishmanial was evaluated for levan from *B. phenoliresistens* against promastigote forms of *L. tropica*. The *L. tropica* was obtained and maintenance at Biotechnology Research Center/ Al-Nahrain University. *L. tropica* promastigotes were grown at 22–26 °C in RPMI-1640 (Gibco, BRL, Maryland, USA) plus L-glutamine (20 mM), supplemented with 10% fetal bovine serum (FBS) (Gibco, BRL, Maryland, USA) and 100 U/mL penicillin and 50 µg/ml streptomycin (Sigma-Aldrich,Saint Louis, USA). According to (24), the colorimetric cell viability MTT assay was used. Briefly, 100 µLwell^{-1} *Leishmania* promastigotes (10^6 cell mL^{-1}) were cultured in 96-well tissue culture plate. Prepare different concentrations of levan test solution by dissolving (19, 39, 78, 0.156, 312, 625, 1250, and 2500 µg/mL in water and added 100 µL of various concentrations to each well and incubated at 26ºC for 24 hours. After incubation, 10 µL of MTT solution (5 mg/mL) was added to each well and incubation was continued for a further 4 h.

Finally, 50 µL of solubilization solution of DMSO (dimethyl sulfoxide) was added to each well and incubated for 10 min. Promastigotes were cultured in complete medium without treated with levan solution as a positive control, and in a complete medium only as a blank. The experiment was performed in triplicate. The ELISA reader was used to measure absorbance for each well at 620 nm. The mean absorbance for each group of replicates was calculated. The live cells, percentage of viability and inhibition ratio were calculated according to the formula

$$\text{Inhibition}\,(\%) = (\text{AC} - \text{AS} \,/\, \text{AC}) \times 100$$

Where Ac and As are the optical density for medium and levan samples, respectively.

Results and Discussion:

1. Potential activity of antioxidant

DPPH act as free radical scavengers or hydrogen donors to become a stable diamagnetic molecule, which is an easier way to estimate radical scavenging activity (25). For this reason, antioxidant activity was investigated by using DPPH free radical scavenging assay. The results revealed that levan has an electron donating ability. The ascorbic acid, which is served as standard antioxidant was shown about 80% activity at 100 µg/mL (w/v), while at the same concentration the *B. phenoliresistens* levan was demonstrated 77.3% antioxidant activity. Whereas, 90 and 80.9% activity were noticed with 1250 µg/mL concentration of ascorbic and levan, respectively (Fig. 1). Levan was indicated to show antioxidant activity which might be attributed to the superoxide dismutase (SOD) and catalase (CAT) activities in the heart (11, 20, 26, 27). These strong antioxidant results revealed that the produced levan can be subjected as an antioxidant additive for pharmaceutical product, food processing and other biomedical uses.

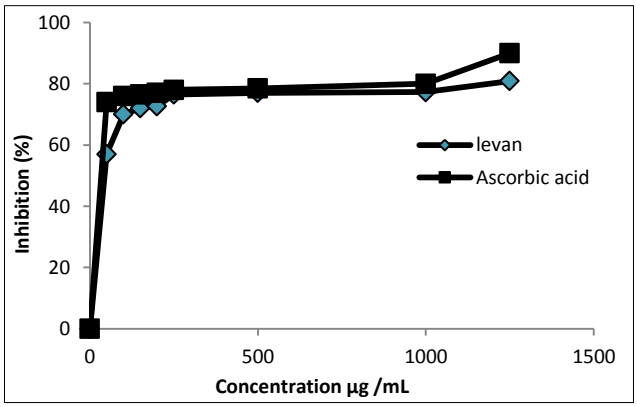

Figure 1. The antioxidant potential activity of *B. phenoliresistens* levan

2. Potential anti-inflammatory

Levan obtained from microorganism source was indicated to have potent action toward inflammation and has therapeutic instance in the medicinal cosmetic industry. So one of our interested goals, to determine the anti-inflammatory propriety of the levan produced from *B. phenoliresistens*. The *in vitro* anti-inflammatory assay was estimated using protein (BSA) denaturation protocol (21). Diclofenac sodium drug revealed a potent 98%inhibitory influence at

concentration 250 µg/mL, while a potency anti-inflammatory activity of 88% protein inhibition was noticed with the levan at concentration 350 µg/mL (Fig. 2). These results were agreement with the results of (21, 28). From these significant results of this trial, it can be deduced that the levan obtained from *B. phenoliresistens* have efficient prospective as an anti-inflammatory agent.

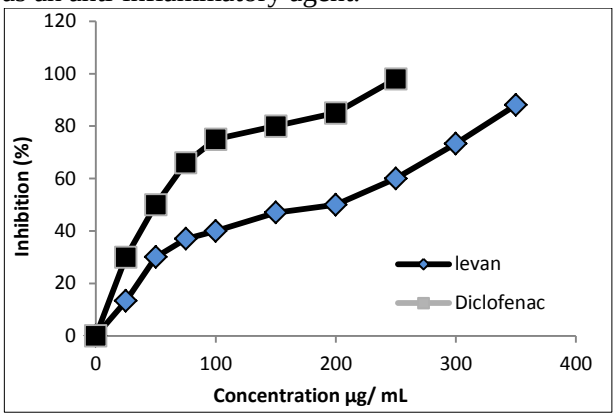

Figure 2. The anti-inflammatory potential activity of *B. phenoliresistens* levan.

3. Cytotoxic effect of Levan on L20B cell line by MTT assay

The cytotoxic effect of levan on L20B cell line was evaluated at eight concentrations (19, 39, 78, 156, 312, 625, 1250, and 2500 µg/mL) for 24 hours. The concentration of 2500 μgmL^{-1} showed a maximum cytotoxic effect on L20B cell line. The result showed, by increasing levan concentration, the cytotoxicity of cells will increase while viability will decrease (Fig. 3). The results of the present study showed that levan have dose-dependent anticancer activities and IC_{50} was 528 µg/mL at 24 hours. The majority of anticancer drugs presently used in clinical settings have been described to induce cell death by apoptosis (29), while the anticancer mechanism of levan is yet unknown. But, the levan anticancer effect may be has been suggested to be mediated through induction of p53 expression, which causes cell cycle arrest and apoptosis (30). Furthermore, MTT reduction is usually performed to study mitochondrial and or non-mitochondrial dehydrogenase activity as a cytotoxic test for a variety of chemical compounds (31). Therefore, levan may be potentially effective to change the enzymatic activity of mitochondria and initiate a preliminary injury that leads to cell death. On the other hand, similar results indicated that the levan has growth inhibition activity on various cancer cell lines such asSNU-1 and HepG2 tumor cell lines. These results suggested that the branch structure of levan would play a crucial role in levan's antitumor activity (32, 33).

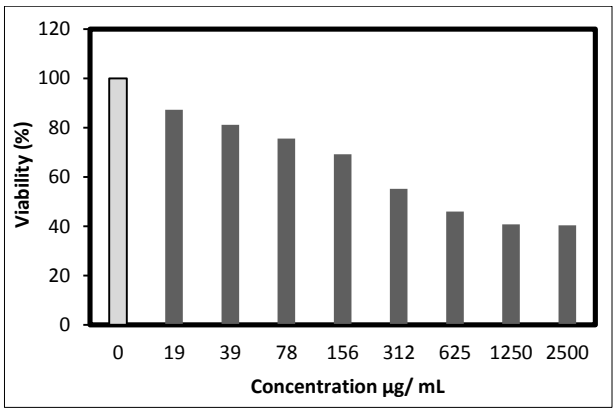

Figure 3. Cytotoxic effect of *B. phenoliresistens* levan on L20B cell line by MTT assay. Data is expressed as the Mean±S.D. of data obtained from triplicate experiment.

4. Cytotoxic effect of Levan on L. tropica by MTT assay

The cytotoxic effect of levan on promastigotes of *L. tropica* was evaluated at eight concentrations (19, 39, 78, 156, 312, 625, 1250, and 2500 µg/mL) for 24 hours. The concentration of 2500 µg/mL of levan showed the maximum cytotoxic effect on promastigotes of *L. tropica*. The result revealed, by increasing the levan concentration, the cytotoxicity of promastigotes will increase while viability will decrease (Fig. 4). The results of our study showed that levan has dose-dependent antileishmanial activities. The antileishmanial drugs like Miltefosine can induce apoptosis in *Leishmania* through mitochondrial membrane permeability reduction, after increasing cytochrome c releasing (34).While the antileishmanial mechanism of levan is yet unknown. From the results of this assay, it can be concluded that levan produced from *B. phenoliresistens* can efficiently be used as an antileishmanial agent.

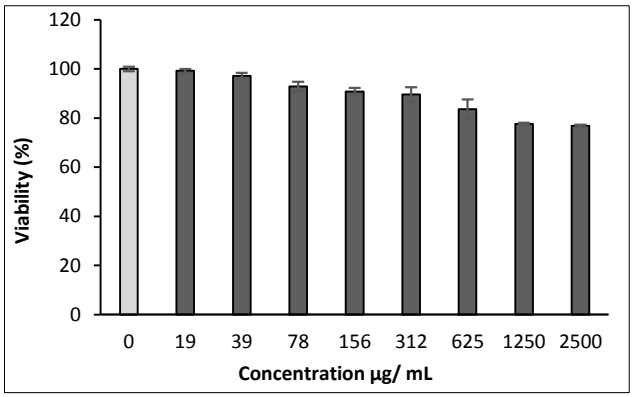

Figure 4. Cytotoxic effect of *B. phenoliresistens* levan on *L. tropica* by MTT assay. Data is expressed as the Mean±S.D. of data obtained from triplicate experiment.

Conclusion:

This study was conducted to evaluate biological activity form a new microorganism producing levan biopolymer *B. phenoliresistens*. The *B. phenoliresistens* levan exhibited a strong antioxidant activity. Levan is free radical scavengers and has the ability to react with the DPPH radical, which might be according to its electron donating ability. From the results of this assay, it can be concluded that levan have potential an anti-inflammatory agent. The results of our study showed that levan have dose-dependent anticancer activities and further study need to determine the mechanism of levan anticancer activity. This is the first study conducted to evaluate the cytotoxic effect of levan on promastigotes of *L. tropica*. Which was shown that levan have dose-dependent antileishmanial activities.

Conflicts of Interest: None

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الفعالية البايولوجية لليفان المنتج بواسطة البكتريا المعزولة من التربة المحيطة بالجذور *Brachybacterium phenoliresistant* KX139300

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الخلاصة:

الليفان هو عبارة عن سكريات متعددة خارجية تنتج بواسطة انواع مختلفة من الكائنات الحية المجهرية ويمتلك تطبيقات متنوعة. كان الهدف الرئيسي من هذه الدراسة هو اثبات الفعالية البايولوجية لليفان المنتج بواسطة البكتريا المعروفة بـ *B. phenoliresistens* KX139300، تم ذلك من خلال اختبار فعاليته كمضادة للأكسدة ، الفعالية التضادية للألتهابات، كمضاد للسرطان و مضاد لطفيلي اللشمانيا خارج الجسم الحي *in vitro*. اظهر الليفان فعالية مضادة للأكسدة تصل الى 80.9 عند تركيز 1250 ملغم/مل. اما فعاليته المضادة للألتهابات فقد بلغت 88% في تثبيط البروتين قيد الدراسة عند التركيز 35 مايكروغرام /مل. بلغت اعلى فعالية سمية للخلايا السرطانية عند التركيز 2500 مايكروغرام /مل تجاه خط الخلايا السرطانية قيد الدراسة والمعروف بـ L20B وكذلك تجاه طفيلي اللشمانيا الاستوائية. ولهذا يمكن اعتماده كمضاد للسرطان واللشمانيا. ويمكن الاستنتاج ان الليفان المنتج بواسطة البكتريا المعروفة بـ *B. phenoliresistens* يمكن تطبيقة واعتماده كمضاد لطفيلي اللشمانيا.

الكلمات المفتاحية: مضاد للسرطان، مضاد للألتهابات، مضاد للتأكسد، مضاد للشمانيا، *Brachybacterium phenoliresistens*، الليفان.