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The Effect of *Staphylococcus aureus* Exopolysaccharides of Biofilm Extract on Apoptosis Induction in LNCaP Prostate Cancer Cell Lines

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ABSTRACT

Background: One of the most prevalent solid tumors in men is prostate cancer which is caused by a lack of apoptosis, which leaves cancerous cells that refuse to die. Consequently, triggering apoptosis is a common approach for anticancer treatments to destroy tumor cells. The bacterial biofilm's EPS (Exopolysaccharides) is paying attention as a potential novel cancer treatment source.
Methods: This study targeted to determine the anti-cancer capabilities of EPS extract and its impact on the expression of the genes Bcl-2, Bax, p53, caspase3, and caspase9 in the prostate cancer cell line (LNCaP).
Results: Treatment of LNCaP cells with EPS extract caused a significant increase in the expression rate of the apoptosis genes and behavior LNCaP cells were driven toward cell death.
Conclusion: According to the obtained result the bacterial EPS shows promising results to be considered as an anticancer drug for prostate tumors.
Keywords: *Staphylococcus aureus*, Exopolysaccharides, EPS, LNCaP cells, Caspase3, Casapase9

INTRODUCTION

Prostate cancer (PCa) is the second most common solid tumor in men and the fifth cause of cancer mortality (1). Hormone-ablation therapy often has an initial positive effect on prostate cancer patients, but later, the disease becomes androgen-independent and resistant to further treatment (2). Although local surgery, chemotherapy, RT, endocrine therapy, and the use of monoclonal antibodies have improved the prognosis, there are still large numbers of patients at risk of relapse and death (3). Bacterium-mediated cancer therapy (BMCT) has attracted much attention because of its considerable benefits (4). Bacteria has been studied as a potential cancer treatment for over a century (5). William B. Coley, a bone surgeon, first reported that injection of a preparation of heat-killed Streptococcus pyogenes could induce tumor regression in patients with bone and soft tissue sarcoma could experience tumor regression by injecting a preparation of heat-killed Streptococcus pyogenes (6,7). These days,

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BMCT is receiving greater attention because of advancements in medical technology (8). Various bacteria species, including Streptococcus, Bifidobacterium, Clostridium, and Salmonella species have been studied (5). Bacterial biofilms are complex surfaceattached communities of bacteria held together by selfproduced polymer matrixes mainly composed of polysaccharides, secreted proteins, and extracellular DNAs (9). To prevent desiccation and maintain adhesions by building biofilms, bacteria release EPS into an environment in the capsules form or slime (10) Watersoluble Ionic or nonionic polymers can make up polysaccharides (10). Several factors and para meters influence the production of EPS among these are the composition of the medium, especially carbon and nitrogen sources, pH, temperature, and incubation time (10). EPS exhibit effectiveness as an antioxidant, anticancer, and immunestimulating activities attribute have lead to increase attention to EPS, which is regard as one of a top 10 global reasons of death as substances secrete from *S. aureus* biofilm might impair immune responses (11). Roca et al. (10) were considered the first who informed the production of EPS with high uronic acids. the EPS has intriguing qualities that make it suitable for applica tion in tissue engineering and regenerative medicine, much like heparin, due to its high uronic acid content. Priyanka et al. (10) showed that the EPS (650 mg l1) with 7.08% uronic acid containing sugars and sulphate functional group (2.68%); its molecular weight was 90 kDa produced from isolated Nitratireductor sp. strain PRIM31 is a promising drug for brain tumors through this anionic charged of EPS (the Presence of uronic acid, sulphate functional groups, and phosphate to this EPS assigns an overall anionic charge to the polymer) binding to the epidermal growth factor (EGF) secreted by the tumor and this coincides with the findings of Liu et al. (10) that anionically charge EPS be preventing EGF receptors phosphorylation. The EPS has a sulfur group, which is a crucial mineral needed in precise amounts for healthy metabolism and antioxidant defense mechanisms. Carcinogenic trans formation prevents cysteine from being biodegraded into sulfane sulfur metabolite, which leads to a disruption in cellular redox control. The triggering of apoptosis and the suppression of cell proliferation depend on the control of cellular redox (10).

Identifying and targeting apoptosis genes has been a major effort in developing effective therapeutic strategies for advanced cancer (8). In this regard mutations in the p53 gene is a common molecular abnormality in cancer (12). A consequence of the lack of normal function of p53 may be the failure to induce apoptosis in cells with damaged DNA, and it can also possibly impair a full apoptotic response to the administration of hormonal or chemotherapeutic interventions (13). Furthermore, the genes of the Bcl2 family have emerged as key regulators of apoptosis. Several members of the Bcl2 family, including bcl2 suppress apoptosis; whereas others, including Bax, induce apoptosis (14). Decreasing antiapoptotic Bcl2 levels with increasing tumor grade, it has been proposed that Bcl2 has an early role within the tumor by rescuing cells with otherwise lethal mutations. After additional oncogene activation, some cells would acquire additional ways to protect themselves against apoptosis (15). At that point, loss of Bcl2 might confer a growth advantage. In fact, Bcl2 is known to restrain cell proliferation (16)Thus, expression of Bcl2 would change from high levels in early or lowgrade tumors, characterized by low apoptotic indices, to low levels in advanced or highgrade tumors, characterized by high apoptotic indices (17).

In addition, during apoptotic cell death caspases genes also, play an essential role. The control of caspases is a key and central component of the biochemical pathway, At least three distinct pathways for caspase activation exist in mammalian cells; recruitmentactivation, transactivation, and auto activation. Trigger caspase activation for the treatment of disorders where insufficient apoptosis occurs (e.g. cancer), is one of the therapeutic strategies (18,19).

This study tested the cytotoxic effects of the EPS from S. aureus against Vero and LNCaP cells, then analyzed the apoptosisrelated genes in prostate cancers after treatment cells with extract EPS.

MATERIALS AND METHODS

Bacterial Strain and Culture Medium

S. aureus strains were grown in Tryptic-Soy-Broth (TSB) prepared as per the manufacturer's instructions and autoclaved (121 °C / 15 min).

Quantitative Biofilm Formation

The ability of *S. aureus* to create biofilm was assessed through adherence to polystyrene microtiter plates. Briefly, 100 µl of TSB plus supplements were placed into each of the 96 wells of flatbottom sterile polystyrene tissue culture plates. Each well received 100 µl of a diluted overnight bacterial culture (1:100 in TSB). In negative control wells, only TSB was present.

Following a 24hour incubation period, the plate contents were poured off, and the wells underwent three rounds of deionized water washing. After allowing the microtiter plates to air dry for half an hour, 0.1% w/v crystal violet stain was applied. After properly cleaning the plates with deionized water to remove any remaining stains, they were left to dry at room temperature for two hours. Biofilm generation was quantified by measuring the absorbance at a wave length of 570 nm (OD 570 nm) using a micro ELISA autoreader (20), *table 1*.

Table 1 - Layout of sample uninoculated medium as (control), and the samples of inoculated medium with *S. aureus* in micro ELISA.

	1	2	3	4	5	6
A	con1	con2	con3	con4	con5	con6
B	sample	sample	sample	sample	sample	sample
C	sample	sample	sample	sample	sample	sample
D	sample	sample	sample	sample	sample	sample

Congo Red Agar Method

The Congo red agar method is another method of screening biofilm formation, which requires the use of a specially prepared solid medium brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo red (21).

Extraction Crude Exopolysaccharides (EPS)

Following the formation of mature biofilms, the culture broth was centrifuged for 15 minutes at 4,500 g and 4°C, precipitating and discarding the cell pellets. The supernatant at night left in 4°C with 5% Trichloroacetic acid added then all cells were eliminated and no protein content remain. Then centrifuged at 3,500 g, using 0.1 M NaOH, and the pH of the clear supernatant was brought to 7.0. After adding 95% ethanol to each supernatant in three volumes and let it sit at 4 °C for at night, the precipitate of EPS was separated by centrifugation at 3,500 g for 20 min at 4°C. It was then twice cleaned with acetone and dehydrated with ether. Finally, dried in a desiccator then weighed and stored at room temperature (22).

Structural Characterization of the EPS

The dried EPS samples were analyzed by FTIR spectroscopy in ATR mode using an FTIR spectrometer. The spectrum was recorded in transmittance mode in the range of 400 – 4,000 cm^{1} (23).

Preparation of EPS Extract as Treatment

To prepare different concentrations of bacterial Exopolysaccharides (EPS) extract of *S. aureus* was dissolved in sterile RPMI. The obtained solution was filtered twice with the filter paper. Then the solution was sterilized by 0.22 micron filters and concentrations of 20,80, 160, 320, and 640 µg/ ml) were prepared by serially diluting of standard stock solution (640 µg/ ml).

Cell Culture and Treatment

Vero cells and human prostate carcinoma cell lines LNCaP cells were purchased from the Pasteur Institute of Iran and maintained in the proper conditions. The Vero cell was maintained in RPMI1640 90%, supplemented with fetal bovine serum (10%), and 1% (100 µg /ml Penicillin and 100 µg/ml streptomycin) medium. The LNCaP cells were cultured in 50% Dulbecco's modified Eagle's Medium (DMEM) and 50% RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 µg/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA), at 37 °C and maintained humidified incubator with 5 % CO2. The cells were harvested after trypsinization (0.025 % trypsin and 0.02 % EDTA) and washed twice with phosphatebuffered saline (PBS). When the cell density reached approximately 80%, cells were split for further culture. The experiments were made up in the growth phase of cells in the logarithmic (24).

Cytotoxicity Assay

The EPS effect on proliferative capacity (Viability) was determined using 3(4,5 dimethylthiazol2yl)2,5-diphenyltetrazolium bromide assay. Briefly, 1 × 104 cells per well (LNCAP, and Vero) were plated in 96well culture plates. After overnight incubation, the cells were treated with different concentrations of EPS (20, 80, 160, 320 and 640 µg/ml) for 24, 48, and 72 hours with three replicates. In this assay, the resulting formazan crystals that had formed dissolved in DMSO (150 µL). Absorbance was recorded at 540 nm with a reference at 650 nm serving as the blank. The EPS effect on cells viability was assessed as % cell viability compared with control on treatment cells, which were arbitrarily assigned 100% viability. This experiment was performed in triplicate and the data were obtained as mean±SD (24,25).

Primers Preparation

All primers used in this study were provided by Macrogen in a lyophilized form. as the manufacturer's instructions all lyophilized primers were in 300 µL of nucleasefree water to obtain a final concentration of 100 pmol/µL as a stock solution. Working solution was prepared by adding 10 µL of primer stock solution frozen at 20 °C to 90 µL of nucleasefree water to give a final concentration of 10 pmol/µL of working primer stock solution.

Primers

The sequences (forward and reverse primer) for RTPCR and Realtime PCR were selected according to previously published articles. The primers with reference are shown in *table 2*.

RNA Extraction and cDNA Synthesis

RNA extraction and cDNA synthesis were performed (30). In brief, the steps would be as follows: (1) extraction of total RNA by SinaPureTM RNA kit from Sinaclon company (2) quantization of RNA's concentration and purity by NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA), (3) comple mentary DNA (cDNA) synthesis by reverse transcription reaction, by SinaClon First Strand cDNA synthesis kit from Sinaclon company (4) quantization of cDNA concentration by NanoDrop 1000 Spectrophotometer.

Real-time PCR

Realtime PCR was carried out. GAPDH were used as housekeeping genes and were amplified by PCR using forward and reverse primers, *table 2* contains a list of all primer genes. Each primer and cDNA template was used with 2X SYBR Green dye, and qPCR Master Mix to create the PCR reactions. The 40 cycles of the PCR reaction were held for 30 seconds at each temperature level after the first cycle of initial denaturation at 94°C for 10 min was completed in the realtime PCR program. Finally, melting curve analysis was performed over a gradient extending from an annealing to a denaturation temperature. The expression was calculated by using the relative standard curve method of quantification and reported as a fold change in gene expression. Finally, melting curve analysis was performed over a gradient extending from an annealing to a denaturation temperature. The expression was calculated according to (31).

Statistical Analysis

The data were expressed as mean values ± SD. The difference between the control and treated groups was analyzed by Oneway ANOVA. P<0.05 was considered statistically significant.

RESULT

Tissue Culture Plate (TCP) Method or (Microtiter Plate Test)

TCP showed positive findings, with a high significant differences (p≤0.05). The average OD of *S. aureus* as (Positive Control B, C, D) was 0.310389 ± 0.015 and the average OD of the uninoculated medium as (negative control A) were 0.092167 ± 0.023 (*tables 3, 4*), (*figs. 1, 2*).

Congo Red Agar (CRA) Method

The CRA plate was inoculated with the bacteria (S. aureus) overnight culture plate and incubated at 37°C for 24-48 h. A positive result was indicated by black colonies with a dry crystalline consistency While the negative result did not change to black. The experiment was performed in triplicate (*fig. 3*).

Exopolysaccharides (EPS) Description

S. aureus showed a great growth on the TSB agar medium after 4 days of incubation at 37°C. and showed opaque, offwhite with mucoid texture colonies (*S. aureus* grampositive, nonmotile forms a

Table 2 - Forward and reverse primer sequences

Oligo Name	Primer seq (5-3)	Reference
GAPDH	F 5'-'TGAAGGTCGGTGTGAACGGATTTGGTC-3 R 5'-CATGTAGGCCATGAGGTCCACCAC-3'	(26)
Bcl-2	F 5'-ATCTTCTCCTTCCAGCCTGA-3' R 5'TGCAGCTGACTGGACATCTC3'	(27)
Bax	F 5'-CTGCAGAGGATGATTGCTGA-3' R 5'GAGGAAGTCCAGTGTCCAGC-3'	(27)
Caspase 9	F 5'-CGAACTAACAGGCAAGCAG C-3' R 5'-ACCTCACCAAATCCTCCAGAAC-3'	(28)
Caspase 3	F 5'-TGGTTCATCCAGTCGCTTTG-3 R 5'-TGGTTCATCCAGTCGCTTTG-3	(28)
p53	F 5'-GGCTCTGACTGTACC ACCATCCA-3' R 5'-GGCACAAACACGCAC CTCAAAG-3'	(29)

Table 3 - The OD of control samples (A), and (B,C,D) show the OD of *S. aurous* in micro ELISA.

(O.D.570 nm)	1	2	3	4	5	6
A	0.06	0.12	0.112	0.097	0.108	0.056
B	0.295	0.263	0.438	0.362	0.489	0.227
C	0.263	0.337	0.525	0.337	0.283	0.219
D	0.293	0.247	0.205	0.272	0.223	0.309

* OD of uninoculated medium as (negative control)
* OD above that OD considered positive if its OD was twice that of the negative control strain.

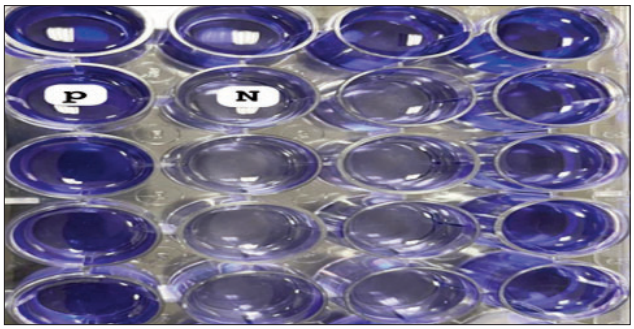


Figure 1 - Screening of biofilm producers by TCP method: high and non-slime producers differentiated with crystal violet staining in 96 well tissue culture plate.

Table 4 - The mean of OD for *Staphylococcus aureus* and Negative control

Group	Mean OD
Negative control	0.092167± 0.023
Staphylococcus aureus PTCC 1431	0.310389±0.015

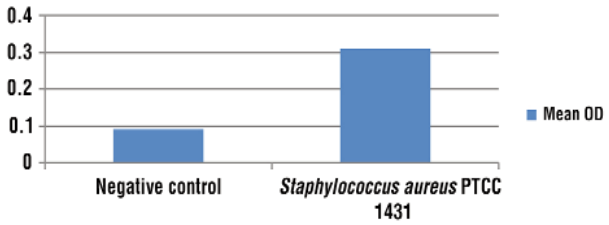


Figure 2 - Graph mean of OD for *Staphylococcus aureus* and Negative control.

biofilm on the bottom of the flask), *fig. 4*. The crude EPS was obtained from bacteria S. aureus grown under optimum conditions, in the stationary phase of growth, and extracted and purified. The amounts of the EPS produced by bacteria are shown in the *fig. 5*.

Characterization of EPS Extract

The FTIR spectrophotometer of the EPS *table 5*, showed many peaks to major functional parts of the EPS, specifically carbonyl compounds. The bands at 40003500 cm^{1} were referred to as the stretching vibration of OH residue in the EPS. The bands at 2800 3000 cm^{1} were a result of the stretching vibration of the

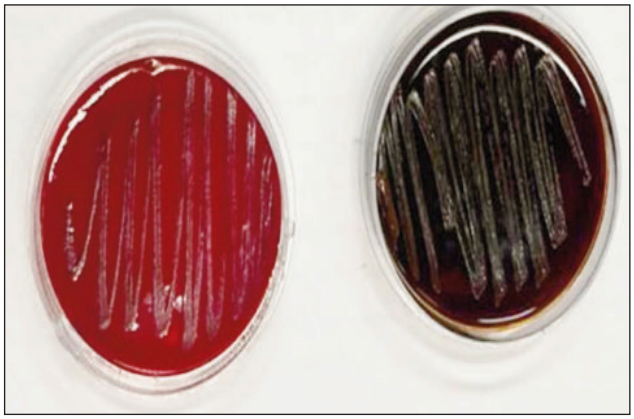


Figure 3 - Biofilm of *S. aureus* result on congo red agar plate.

Table 5 - Assignments of principal infrared vibrational signals of the (4000-500 cm^{-1}) region of the ART/FT-IR spectrum of the *S. aureus* biofilm.

Windows of IR spectra corresponding to EPS signals	Principal EPS	Main functional groups inbiomolecules
4000-3500 cm^{-1}	alcoholic group	O-H
2800-3000 cm^{-1}	lipids	CH,CH2,CH3
1500-1800 cm^{-1}	Proteins	C=O, N-H,C-N (amidel, amidell)
1800-1000 cm^{-1}	sulfate	SO4
900-125 cm^{-1}	Polysaccharides, nucleic acids	C-O,C-O-C,N-H (amide III)

Figure 4 - Heavy growth of biofilm after culture bacteria four days.

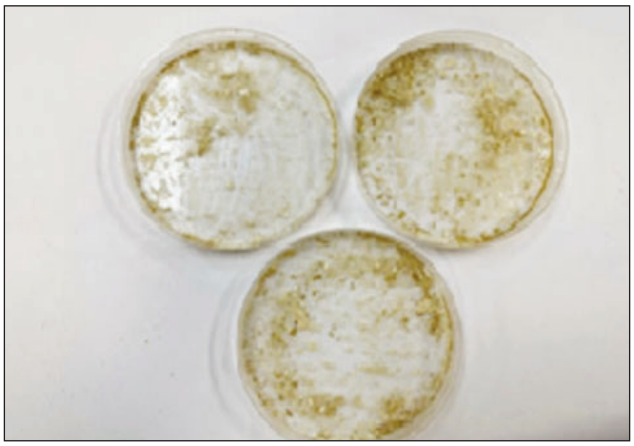


Figure 5 - The EPS after Extraction steps from biofilm.

Lipid (Ch,CH2,CH3). The bands at 15001800 cm^{1} were a result of the stretching vibration of the carboxyl group, and C=O. The bands 15001800 cm^{1} indicated the Proteins from C=O, NH, CN (amideI, amideII) residue in an EPS, sulfate active group SO4 is represent at 1800-1000 cm^{1} and 900125 cm^{1} indicate Polysaccharides, nucleic acids CO,COC,NH (amide III) residue in EPS. The major functional groups of the EPS were identified using an FTIR spectrophotometer. The EPS of *S. aureus* found to contain a sulfur functional group.

Cell Viability

The cell viabilities and survival rates of both cells were significantly declined in a dosedependent manner when they were treated with different concentrations of EPS extract, and significantly increased numbers of apoptotic cells.

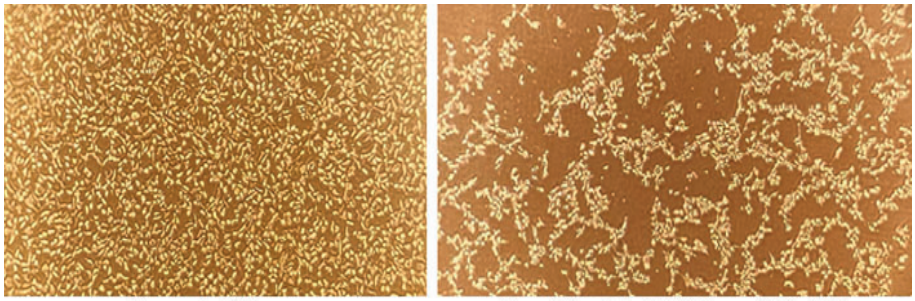
Cell Viability of Impact of S. aureus EPS on Vero Cell Line

Results showed there were significant differences in the Vero cell lines viability, dosedependent manner was observe in cells treat with a EPS of biofilm. The greater rates of Vero cell viability was at a concentration of 20 µg/ml and the lowest rate of viability was at a concentration of 640 µg/ml at different time treatment exposure (24-, 48-, 72- hrs), and there was remarkable inhibitions in cells viability represente by a marked inhibitory effects on the cell population *fig. 6*, also there were no significant differences between viability time-dependent manners, *table 6*.

Cell Viability of Impact of EPS on LNCaP Cell Line

Results showed significant differences in the viability of the LNCaP cell lines, dose dependent manner were observe in cells treat with EPS. The greater rate of viability LNCaP cell was at a concentration of 20 µg / ml and the fewer rate of viability was at a concentration of 640 µg/ml. At different times of treatment exposure (24, 48, 72 hrs) and there was

Figure 6 - Steady decline of the Vero cell population that noted after treated with IC50 dose of EPS at 48h as compared to the control (Untreated cells).



Vero control 4X

Vero 4X treated with IC50 dose of EPS at 48 h

Table 6 - Vero cells viability as determined by MTT, post treatment with different concentration of EPS for three times exposure.

Concentrations µg/ml	Viability percentage % Vero 24	Viability percentage % Vero 48	Viability percentage % Vero 72
0	100±.00	100±.00	100±.00
20	90.95±0.68	90.63±0.56	89.63±0.67
80	86.83±8.44	83.98±8.54	70.99± 8.34
160	79.75±11.80	59.30±10.54	59.30±9.57
320	76.33± 18.81	43.86±17.45	43.87±16.32
640	43.75±0.46	42.95±0.98	42.95±0.45

Values are mean ± SD (n-3)
*Represents a significant difference in comparison between treated groups and control ($p<0.05$)

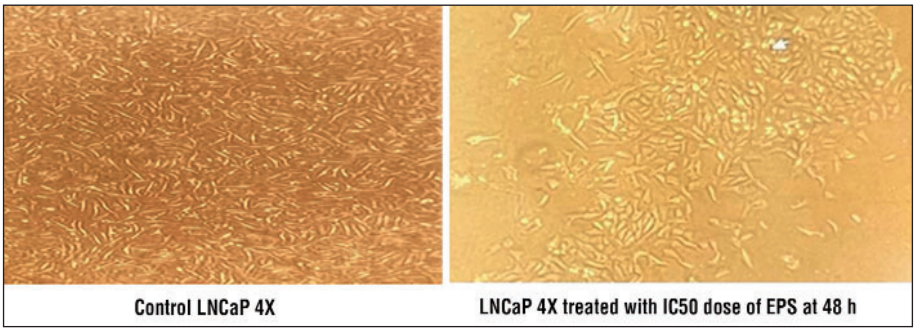


Figure 7 - Steady decline of the LNCaP cell population that noted after treated with IC50 dose of EPS at 48h as compared to the control (Untreated cells)

Concentrations µg/ml	Viability % LNCaP 24	Viability % LNCaP 48	Viability % LNCaP 72
0	100±.00*	100±.00	100±.00
20	82.83±7.63	89.99±7.66	74.72±6.67
80	74.74±1.70	71.34±1.80	73.27±1.79
160	63.71±6.21	51.81±5.59	60.86±6.95
320	55.77±7.41	47.25±7.64	41.00±6.59
640	38.97±0.67	38.32±0.56	39.67±0.61

Table 7 - Viability percentage of LNCaP cell lines

Values are mean ± SD (n-3)
* Represents a significant difference in comparison between treated groups and control (p<0.05)

remarkable inhibition in cell viability represented by a marked inhibitory effect on the cell population *fig. 7* and *table 7*. Also, there were no significant differences between the treatments base on time 48-h and 72-h, but there was a clear difference between them and time 24.

Assess the Half Maximal Inhibitory Concentration (IC50) of EPS

The IC50 values were calculated by using the MS Excel curve represented in *table 8* and the most active dose of EPS that inhibit growths and proliferation of the VERO and LNCaP cell lines were on 24-h. The IC50 value of Vero cell lines were 479.0123 µg/ml, 454.7238µg/ml and 428.9419µg/ml respectively. While IC50 of LNCaP cell lines value were 442.3609 µg/ml, 252.8518 µg/ml and 206.4241 µg/ml respectively. Also, there was significant difference in IC50 between the time of exposure.

Biochemical Analysis of Cell Death by Real Time PCR

The effect of the tested bacterial EPS on gene expression of cell lines was analyzed by qPCR in order to evaluate the genes expression (P53, BAX, BCL2, Caspase9, Caspase3) in Vero and LNCaP cells was treated with IC50 does of EPS for 48 h (*table 9*), the RNA samples that were extracted from cells treated

Table 8 - Comparison of IC50 of Vero and LNCaP Cell Lines at three time.

cell line	IC50 µg/ml		
	Exposure time – 24	Exposure time – 48	Exposure time – 72
Vero	479.0123±.784*	454.7238±1*	428.9419±1*
LNCaP	442.3609 ±1*	252.8518 ±1*	206.4241±1*

Values are mean ± SD (n-3)
*Represents a significant difference in comparison between treated groups and control (p<0.05)

Group cell line	P53 expression	BAX expression	Bcl-2 expression	Caspase-9 expression	Caspase-3 expression
Vero Treatment with EPS	1.47±.01*	1.23±.01*	1.05±.01*	2.34±.01*	1.47±.01*
LNCaP Treatment with EPS	2.21±.01*	1.79±.01*	0.84±.01*	1.15±.01*	2.13±.01*

Table 9 - Gene expression both Vero and LNCaP after treatment with EPS

Values are mean ± SD (n-3)
*Represents a significant difference in comparison between treated groups and control (p<0.05)

with EPS and were reverse transcribed to make the first strand of DNA were used as the templates. cDNA template was mixed with primers amplifying the gene to measure its expression using qPCR relative expression assay using SYBR green master mix. Data were analyzed by ΔΔ CTs and normalized to (GAPDH) housekeeping gene were ($P < 0.05$). The experiment was performed in triplicate.

Apoptosis Induction by Activation p53 Gene

The result found that EPS treatment enhanced the expression of P53 in both cell lines compared to its level in the corresponding controls. Expression was found to have the highest effect of EPS on treated LNCaP cell lines compared to VERO cell lines, *fig. 8*.

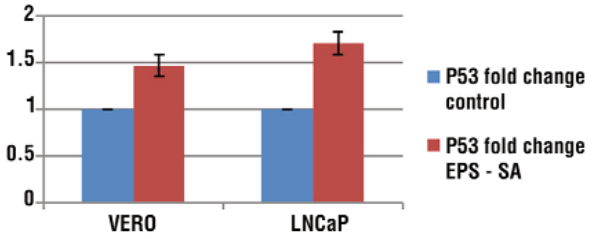


Figure 8 - The relative expression of P53 in Vero and LNCaP cell treated and untreated with EPS compare to control group.

Apoptosis Induction by Activation BAX Gene

The result found that EPS treatment enhanced the expression of BAX in both cell lines compared to its level in the corresponding controls. Expression was found to have the highest effect of EPS on the treated VERO and LNCaP, *fig. 9*.

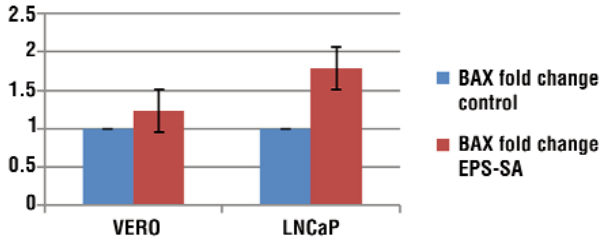


Figure 9 - The relative expression of BAX in Vero and LNCaP cell treated and untreated with EPS compare to control group.

Apoptosis Induction by Activation Bcl-2 Gene

The finding found that the expression of Bcl2 gene showed a significant decrease ($P < 0.05$) in cancer cell lines (LNCaP) compared to its level in the corresponding controls, On the other hand, treated Vero cell lines didn't show any significant decrease in bcl2 gene level, *fig. 10*.

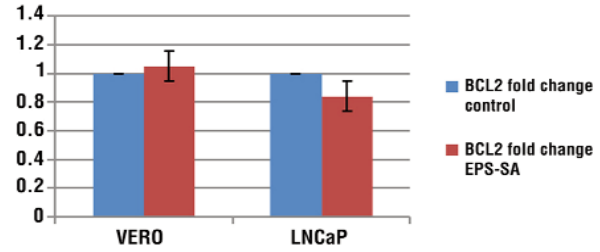


Figure 10 - The relative expression of Bcl2 in Vero and LNCaP cell treated and untreated with EPS compare to control group.

Ratio of BAX and Bcl2 Genes

TThe obtained data showed that the ratio of Bax/Bcl2 was increased significantly in LNCaP cells treated with IC50 at 48 hours, compared to the control group and the columnar chart for changes in the relative expression of Bax and Bcl2 genes in both groups untreated (control) and treated, on the other hand in Vero cell line didn't showed any significant difference between treated and untreated (control) group, *fig. 11*.

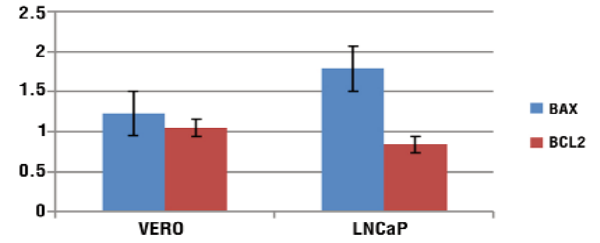


Figure 11 - The relative expression of BAX and Bcl2 ratio in Vero and LNCaP cell treated and untreated with EPS compare to control group.

Apoptosis Induction by Activation Caspase-3 Gene

The results found that the effect of the prepared EPS on the expression of Caspase3 gene was significantly increased in treated cancer cells LNCaP with at IC50 does at 48 h, compared to the control sample, while the VERO showed a slight increase in the gene, *fig. 12*.

Apoptosis Induction by Activation Caspase-9 Gene

The results of Caspase9 expression showed that the

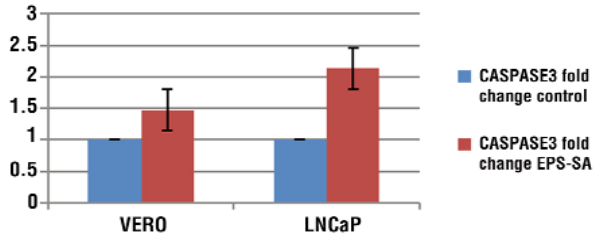


Figure 12 - The relative expression of Caspase-3 in Vero and MCF-7 cell treated and untreated with EPS compare to control group.

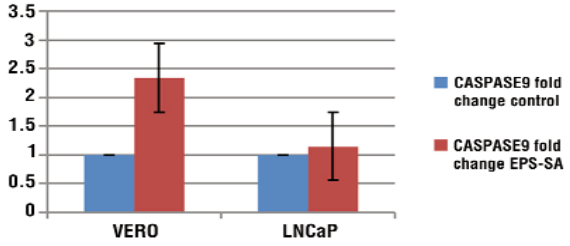


Figure 13 - The relative expression of Caspase-9 in Vero and LNCaP cell treated and untreated with EPS compared to control group.

gene was higher in normal cells (Vero) compared to the negative control sample and the cancer (LNCaP), the LNCaP showed a slight increase in the gene was observed compared to the negative control sample, *fig. 13*.

DISCUSSION

Bacterial biofilms are complex surfaceattached bacterial populations kept together by selfproduced polymer matrixes primarily consisting of Exopoly saccharides EPS, EPS accounts for around 50% to 90% of the total organic carbon in biofilms and can be regarded as the major matrix material of the biofilm. Although the chemical and physical properties of EPS vary, it is mostly made of polysaccharides. The (EPS) plays a critical role in bacterial, fungal, and algal defense systems (32). In recent years, EPSs have had great potential as antitumor drugs as recent treatment strategies, so the study considers it of interest to extract EPS from bacteria and used it as antitumor effects on prostate cancer carcinoma.

In current study's findings demonstrated that cells treated with EPS exhibited suppression of cell viability in a concentrationdependent manner and LNCaP was shown to be the most sensitive cell line to EPS, whereas VERO was the most tolerant.

Authors reported that EPS produced by bacteria strains has cytotoxic action on cancer cells by inhibiting growth and proliferation of the HepG2 cell line, these crude EPSs were obtained from bacteria grown under optimum conditions, in the stationary phase of growth, and extracted and purified (22). Others observed nearly the same result, they found that E. lactis IW5 secretions had no harmful effects on healthy cells (33).

Priya JN et al (34) showed that the EPS with 7.08% uronic acid containing sugars and sulfate functional group, is a promising drug for brain tumors through this anionic charged EPS (the Presence of uronic acid, sulfate functional groups, and phosphate to this EPS assigns an overall anionic charge to the polymer) binding to the epidermal growth factor (EGF) secreted by the tumor and this coincides with the findings of (33), that EPS with an anionic charge has the ability to stop the phosphorylation of the EGF receptor, with EPS (300µg ml) produced cytotoxicity against U87MG glioplastoma cells using the MTT assay, and the IC50 value (234.04 µg ml) was demonstrated.

Based on the current characteristics of biofilm and its antitumor effect, exopolysaccharides (EPS) are crucial components of defense systems in bacteria (34). Vidhyalakshmi and Vallinachiyar (35) also extracted exopolysaccharide from Bacillus that showed cytotoxicity against MCF7 breast cancer cells without any cytotoxicity against normal cells.

Authors evaluated the anticancer impact of L. acidophilus was performed with verification of the likely effect of its polysaccharides extract on the suppression of colon cancer cell proliferation (87.27%) and cell death (80.65%) with 1.96–51.3 colon cancer selectivity index (36). The utilized pentasaccharide demonstrated a strong cancer cell selectivity index, reaching 51.3, suggesting that the inhibitory effects of L. acidophilus LAEPS20079 on colon cancer cell proliferation could be accompanied by an increase in the proliferation of noncancerous cells.

The creation of treatments that encourage the efficient death of cancer cells by apoptosis has been a cornerstone and objective of clinical oncology for more than thirty years. There are two routes that mediate this process of programmed cell death (called intrinsic and extrinsic). Consequently, apoptosis is a mechanism that several anticancer medications used to killing tumor cells, make it a valuable targets to target (37).

The majority of human cancers, if not all of them, include TP53 (p53) gene dysfunction. Due to its nearly ubiquitous inactivation in cancer, p53 is a very desirable target for the development of novel anti-cancer medications. P53 can induce apoptosis through

both extrinsic and intrinsic mechanisms (38) and many cancer treatmentinduced apoptosis are mediate by the tumor protein p53 activation, Bax and sub sequent activation of caspases (39).

Normal cells that are not under stress have very low levels of p53 and very little of it active. Stress causes p53 to undergo a number of posttranslational changes that activate it and allow it to attach to particular DNA sequences (13). Numerous hundred genes that are variably regulated (induced or repressed) based on the type of cell include the very loose p53 recognition sequence (13). The kind of stress and the degree of harm, The findings demonstrated that the EPS extract had a significantly distinct effect on P53 expression; the treated LNCaP had the highest expression followed by treated Vero cells. Consequently, our findings suggested that EPS reduced the mortality of prostate cancer cell death by raising p53.

A study found that the mitochondrial pathway was implicated in the EPSinduced apoptosis due to the upregulation of Bax and p53 mRNA and the down-regulation of Bcl2 mRNA (40). These results shed new light on the possible application of EPS as an anticancer medication in the treatment of human colo-rectal cancers.

According to Sangour and By (2020), ZnsAg NPs treatment (25 µg/ml) for 48 hours boosts P53 expression levels in both treated cell lines (VERO and MCF7) when compared to the control, with treated samples of MCF7 showing a notable rise. Since treating three different types of cell lines with ZnS impacts their viability and increases the P53 mRNA level, P53 upregulates expression is consistent with Akhtar et al (35). On the other hand, P53 mRNA increases almost 35 times as opposed to twofold. This could be explained by the concomitant action of the utilized nanoparticles' Ag NPs.

P53 expression in MCF7 treatment is 16 times higher than in control, with relative increases in P53 in VERO cells, after 48 hours of treatment with an IC50 concentration of CdSAu NPS in VERO and MCF7 cells. According to a different study, reactive oxidizing species and nanoparticles damage DNA and activate P53 and other proteins involved in DNA repair (41). The p53 plays a controlling gene linked to apoptosis In addition to that it also seem to have a direct effect on mitochondria, potentially encourage the convergence of extrinsic and intrinsic pathways. It can interacting with members of the BCL2 family to lead to apoptosis and mitochondrial outer membrane permeabilization (35).

Apoptosis characteristics that have been studied to target cell death therapeutically include the release of cytochrome C from the mitochondria (42), which is controlled by the balance between proapoptotic BAX and antiapoptotic BCL2 family members (16). Our data found that the expression of Bax and Bcl2 genes was also associated with a significant increase in IC50 doses of EPS extract at 48 hours in treated LNCaP cell lines, compared to cells that were not treated with the EPS extract. On the other hand.

The Bax/Bcl2 ratio determines whether a cell lives or dies. Some chemicals can change the Bax/Bcl2 ratio, causing cancer cells to die (39). A study on anticancer pharmaceuticals discovered that these drugs change the Bax/Bcl2 ratio in the AGS cell line, causing malignant cells to die. Some plant compounds can induce cancer cells to die by increasing the Bax/Bcl2 ratio. Pyrethrin is a naturally occurring insecticide that boosts the Bax/Bcl2 ratio in HepG2 cells (a human liver cancer cell line) (43). in the present study when comparing untreated cells to treat MCF7 cell lines that expressed the Bax and Bcl2 genes had significantly more ($P< 0.05$) IC50 dosages of EPS extract after 48 hours. As a result, cancerous cells commit suicide.

Nowadays the development of anticancer drugs has been shifted toward cell cycle transduction signals, growth factors, DNA repair, and apoptosis. Apoptosis can occur via two pathways: the extrinsic pathway and the intrinsic pathway (42). When mitochondria are damaged, the intrinsic apoptotic pathway is activated. Caspase 9 acts as a starter in the intrinsic pathway of apoptosis activation, while caspase 3 acts as an "executor" enzyme. As a result, an increase in caspase3 leads to an increase in apoptosis. Furthermore, altered caspase 3 expression may represent an additional route of androgen resistance (44).

The study was conducted in vitro on prostate cancer cell lines (LNCaP), when comparing the treated LNCaP cell line to the control (untreated cells), the current study found a considerable rise in Caspase 3 and a slight increase in Caspase 9. These findings suggest that EPS may have therapeutic potential in preventing the growth of prostate tumors. However, there was also an increase in both Caspase 3 and Caspase 9 in the Vero cell lines that were treat compared to those not treated. Caspase is a mediatory enzyme involved in cellular apoptosis; thus, increased caspase expression is an indicator of an ongoing apoptosis process in cancer cells (45). Numerous proteins/enzymes mediate cell death by the intrinsic pathway (caspase9) and caspase executor (caspase3) (46). A study by Zhang et al. (29) demonstrated that caspase3 was a crucial component in the apoptotic signaling linked with the activation of internucleosomal

DNA fragmentation. A study demonstrated higher expression of caspase3, −8, and −9 that was associated with enhanced apoptosis in cancer cells (47).

A study showed that The increase of caspase3 can activate specific proteins in cells which will further induce the apoptosis process (48). The increase of caspase3 enzyme expression will increase the turnover of prostate cancer cell line PC3 in vitro through the intrinsic pathway. The research presented stated that Cytochrome C is released through an intrinsic mechanism that increases the ratio of Bax to Bcl2 expression (Bax/Bcl2) (17). As a result, caspase activation proteins, can either be effectors (caspases 3, 6, and 7) or initiators (caspases 8, 9, and 10) of apoptosis. The apoptosome forms when caspase activation occurs. This study presented similar results to those who stated that Initiator caspases trigger effector caspases, which cause apoptosis by cleaving 100s of proteins via proteolysis (19).

CONCLUSION

S. aureus extracellular polymer (EPS) has highly promising effects on prostate cancer. However, in vivo investigations are necessary to determine potential interactions between the bacterial EPS and the host immune system, ensuring the safety and efficacy of the EPS in the prevention or treatment of prostate cancer. Additional investigation is necessary to clarify.

Conflict of interests

All authors declared no any conflict of interesting.

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Ethics of approval

This work approved by Biology Department, College of Science, University of Basrah (no. 45 in December 2023).

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