

RESEARCH ARTICLE

Endotoxin Production by *Pseudomonas aeruginosa* ATCC 9027 with Potential Medical Applications

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Abstract

Four Gram-negative bacteria (*E. coli* ATCC 19404, *P. aeruginosa* ATCC9027, *A. hydrophila* ATCC 7966 and *V. damsela* ATCC 33539) were screened for production of endotoxin. *P. aeruginosa* recoded the highest endotoxin concentration (338.59×10^3 IU/ml). The optimization of endotoxin production resulted in the increase of endotoxin titer (25% at pH 7.0 and 26% at both 30°C and 90 rpm). The detection of lipid core complex (50 kDa) and O-specific polysaccharides (20, 25, 38 kDa) was performed by the application of SDS-PAGE. GC/MS analysis indicated the presence of 4 compounds namely 2H Pyran, 2,5 diethenyltetrahydro, Tetradecanoic acid, Bis(2 ethylhexyl) phthalate and Dodecanoic acid. The endotoxin was thermally stable (78%) at 120°C for 60 min. Reduction of endotoxin activity (45 and 69%) occurred when treated with 1N-NaOH or HCl for 60 min, respectively. The endotoxin showed a bio-toxicity against *Artemia salina*, (LC_{50} of 102.99 µg/ml). The endotoxin exhibited antitumor activity against colon cancer cells (Caco-2) and breast cancer cells (Mcf-7) with growth reduction amounted to 87% and 92%, respectively. On the other hand, a weak antiviral activity (5.2%) was recorded in Vero cells infected with HAV. In conclusion, the endotoxin with antioxidant property could be an alternative for the treatment of colon and breast cancer.

Keywords: *Pseudomonas aeruginosa* ATCC 9027, endotoxin, antitumor and antiviral activities, bio-toxicity.

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INTRODUCTION

Endotoxins, or bacterial lipopolysaccharides (LPS), are the main constituents (~75%) of the outer membrane of the cell wall of all Gram-negative bacteria such as; *Escherichia coli*, *Bordetella pertussis*, *Haemophilus influenzae*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria* and *Vibrio cholerae*^{1,2}. Endotoxins are heat-stable, non-proteinaceous, immunogenic compounds and can be divided into three covalently linked distinct domains: the hydrophobic "lipid A" portion, the "core" oligosaccharide and the hydrophilic "O-specific" polysaccharide region³. Lipid A is a highly conserved region and responsible for its biological toxic effect as well as beneficial activities, while the "core" is the connection part between lipid A and the O-specific chain⁴.

LPS are the causative agents for various inflammatory symptoms and pathophysiological disorders caused by Gram-negative bacterial infections^{2,5-7}. However, the beneficial activities of endotoxins are protection against atopic sensitization and asthma in children and adults, induction of tumor necrosis and adjuvant activity⁸.

P. aeruginosa is considered as one of the most important causative agents responsible for life-threatening systemic infections in the intensive care units and to the immuno compromised patients⁹⁻¹¹. However, only few reports have dealt with the antitumor and interferon-inducing activities of the LPS isolated from *P. aeruginosa*^{12,13}.

Thus, the current study was meant to investigate the ability of *P. aeruginosa* ATCC 9027 for endotoxin production potential under optimum cultural and environmental conditions, in addition to the characterization of the produced endotoxin. The possibility for the application potential of the produced endotoxin was also experimented.

MATERIALS AND METHODS

Screening for Endotoxin Production

Bacterial strains and culture conditions

Gram-negative bacterial isolates used for the screening for endotoxin production were; *Escherichia coli* ATCC 19404, *Pseudomonas aeruginosa* ATCC9027, *Aeromonas hydrophila* ATCC 7966 and *Vibrio damsela* ATCC 33539. The isolates were kindly provided by the Identification Unit of the Regional Center for Mycology and Biotechnology, Al-Azhar University, Egypt. Bacterial

isolates were inoculated in nutrient broth medium (NB) in a rotary shaker (120 rpm) at 37°C for 24hrs¹⁰.

Extraction, Detection and Measurement of endotoxin(s)

Bacterial cells were harvested by centrifugation at 4°C (5,000 'g, 10 min) and washed twice with sterile NaCl solution (0.85%). The S-type LPS was extracted from the collected cells by the hot phenol-water method according to Iihara *et al.*¹⁴. After extraction, S-type LPS was treated with RNase, DNase, and proteinase K as described by Rezanian *et al.*³. Finally, LPS were collected by ultracentrifugation (4°C) at 300,000 'g for 45 min, lyophilized and stored at 4°C for further use.

The detection and quantification of extracted LPS were carried out by the Limulus Amebocyte Lysate (LAL) assay using PYROGENT-5000 kit (Sigma-Aldrich, USA) according to the manufacturer instructions¹⁵.

Detection of Antimicrobial Activity

The extracted endotoxins were screened for their antimicrobial activity against selected pathogenic bacterial indicators; *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 19404, *Bacillus cereus* ATCC 33018 and *Klebsiella granulomatis* ATCC 43816, by well diffusion method¹⁶. Overnight cultures of the test pathogens were uniformly mixed with Mueller-Hinton agar medium. Wells of 5 mm inner diameter were filled with the endotoxin in concentrations ranging from 10 to 120 µg/l and incubated at 37°C for 24hrs. Then, the diameters of inhibition zones (mm) were measured.

Optimization of Fermentation Conditions

For maximum endotoxin production, fermentation conditions were optimized based including the following factors; pH, incubation temperature, rate of shaking and length of the incubation period. The fermentation medium was adjusted to different pH values ranging from 5 - 9, using either 1N HCl or NaOH. Furthermore, the production medium was incubated at different temperatures from in the range of 25°- 45°C with different rate of shaking; 90, 120 and 150 rpm. Similarly, the effect of incubation period on endotoxin production was studied at 3hrs time intervals up to 48hrs. For each treatment, samples were collected and analyzed for endotoxin concentration by LAL assay.

SDS-Page Analysis of LPS

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for the characterization of S-type LPS following the procedures of Doruk *et al.*¹⁷. LPS bands were stained and visualized by silver staining method¹⁰.

GC/MS Analysis

Extracted endotoxin was examined by Gas Chromatography/Mass Spectrophotometer (GC/MS-Agilent Technologies 7890A, USA) coupled to an Agilent 5975C Triple-Axis Detector, following the procedure described by Binding *et al.*¹⁸.

Factors Affecting Endotoxin Stability

Thermal stability of endotoxins

Thermal stability of endotoxins was evaluated upon exposure to the following temperatures: 120°C, 140°C and 200°C for 20, 30 and 60 min. Residual endotoxin concentration was measured by using the LAL assay and compared to a control set kept at 30°C at the same period¹⁹.

Chemical stability of endotoxins

Chemical stability of endotoxins upon treatment with 0.1, 0.5 and 1.0 N NaOH or HCl was evaluated after 20, 30 and 60 min of exposure. In addition, endotoxins were incubated with 70% and 100% ethyl alcohol for 20, 30 and 60 min and both were compared with endotoxin sets without treatment²⁰. Residual endotoxin concentration was measured by using the LAL assay.

Bio-Toxicity Assay of Endotoxins

The brine shrimp, *Artemia salina*, was used as a toxicity biomarker for endotoxins produced by *P. aeruginosa* ATCC9027, according to Barakat and Gohar²¹. Briefly, four different concentrations of endotoxin (25, 50, 75 and 100 µg/ml) were distributed in glass vials containing 10 ml sterile seawater. Ten live *A. salina* nauplii were equally distributed in each glass vial, incubated at room temperature and the biomarker viable count was monitored under a stereoscopic microscope after 24hrs. Negative control vials contained 10 nauplii in sterile seawater only. The mortality percentage was calculated according to Rajabi *et al.*²²:

$$\text{Mortality percentage (\%)} = \frac{\text{Survivors in the control well} - \text{Survivors in the test well}}{\text{Survivors in the control well}} \times 100$$

Furthermore, the half-lethal dose (LD₅₀) was calculated following Barakat and Beltagy²³.

Potential Applications of Endotoxin

Antiviral activity

The African green monkey kidney (Vero) cells were used as the host for hepatitis A viral (HAV) infection²⁴. Cytotoxicity of endotoxin (1500 µg/ml) was measured by MTT method following Bastos *et al.*²⁵ and the protection percentage (%) for each treated Vero cell was calculated according to the following formula:

$$\text{Protection Percentage (\%)} = \frac{\text{Absorbance of the treated cells} - \text{Absorbance of the control virus}}{\text{Absorbance of the cellular control} - \text{Absorbance of the control virus}} \times 100$$

Antitumor activity

Two tumor cell lines; colon cancer cells (Caco-2) and breast cancer cells (Mcf-7), were kindly provided from the Egyptian Company for Drugs and Veterinary Vaccines, VACSERA CO., Giza, Egypt. Antitumor activity of endotoxin (1500 µg/ml) against both tumor cell lines were evaluated using standard methods described by Ibrahim *et al.*¹⁴ and the percentage of cell viability was calculated as follows:

$$\text{Cell viability (\%)} = (\text{Mean Abs control cells} - \text{Mean Abs treated cells}) \times 100$$

Where; Abs is the absorbance at 490 nm

RESULTS AND DISCUSSION

Endotoxins produced by Gram-negative bacteria were mainly considered as potential components of bacteriological weapons. Recently, this consideration has been slightly changed as some of toxins have useful properties rather than harmful ones²⁶. In this context, the current study was conducted in an attempt to produce endotoxins from *P. aeruginosa* ATCC 9027 under optimum conditions with useful potential medical applications.

Screening for endotoxin production

Screening for endotoxins produced by the selected Gram-negative bacteria showed that *P. aeruginosa* ATCC 9027 recorded the highest concentration of endotoxins (338.59 × 10³ IU/ml) (Table 1). Thus, *P. aeruginosa* ATCC 9027 was selected as the most promising endotoxin-producing strain for further studies. The results (data not shown) showed that the lowest endotoxin concentration exhibiting antibacterial activity, against the test organisms, was 50 µg/l, which is considered as the minimum inhibitory

concentration (MIC) value of the endotoxin produced by *P. aeruginosa* ATCC9027.

Optimization of endotoxin production by *P. aeruginosa* ATCC 9027

The result revealed that the highest endotoxin concentration was detected after 18hrs of incubation. The endotoxin concentration decreased gradually after 48hrs of incubation and the endotoxin concentration produced under a shaken incubation condition was always higher than that of a stationary incubation condition.

The results showed that the highest endotoxin concentration, 451.3×10^3 IU/ml and 457.3×10^3 IU/ml were recorded at both pH 7.0 and 30°C incubation temperature, respectively and were selected for next experiments (Fig. 1a, b). Giotis *et al.*²⁷ suggested that low pH values lead

to conformational changes in the branch-chain fatty acids (FAs) of the endotoxin produced by *Listeria monocyete* that affect its biological activity and membrane fluidity. Kawahara *et al.*²⁸ studied the influence of growth temperature on LPS composition produced by *Yersinia pestis*. They found that tetra acyl lipid A is most abundant species at 25°C with an additional production of penta acyl and hexa acyl species. Further more, (Fig. 2) the maximum concentration of endotoxin produced by *P. aeruginosa* ATCC 9027 was 455.3×10^3 IU/ml when incubated at 90 rpm for 18hrs (Fig. 3). Mahamuni *et al.*²⁹ reported that above the optimum growth temperature, the conformation of the produced endotoxin is highly affected leading to the loss of the attachment of the endotoxin molecule. In addition,

SDS-PAGE analysis

Bands of endotoxin extracted from *P. aeruginosa* ATCC 9027 were visualized by silver staining of SDS-PAGE, as shown in Fig. 4. LPS was separated into two parts; "lipid A-core" complex (50 kDa) and "O-specific" polysaccharide chain consisting of three bands of 38, 25 and 20 kDa. Therefore, the lipid- A core is considered the fastest migrating component and the slower migrating components are O-antigens. Zankharia³⁰ analyzed and separated endotoxin extracted from *Salmonella enterica* serovar *typhi* by SDS-PAGE followed by silver staining. He showed that LPS molecules had a molecular weight ranging from 10 kDa to 55 kDa.

Table 1. Selected Gram-negative bacteria and their potential as endotoxin producers

Organism	Endotoxin conc. (IU/ml)
<i>Escherichia coli</i> ATCC 19404	125.86×10^3
<i>Pseudomonas aeruginosa</i> ATCC9027	338.59×10^3
<i>Aeromonas hydrophila</i> ATCC 7966	87.71×10^3
<i>Vibrio damsels</i> ATCC 33539	37.12×10^3

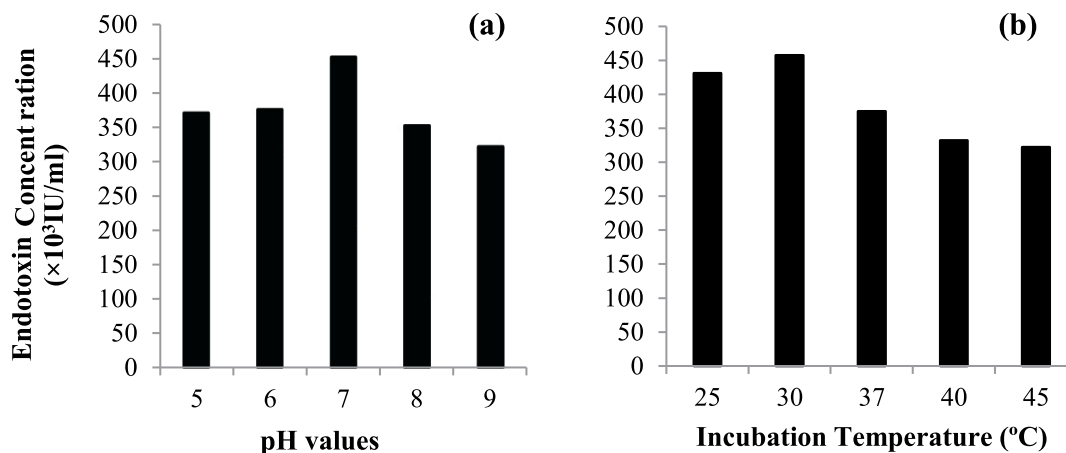


Fig. 1. Effect of different pH values (a) and incubation temperature (b) on the endotoxin production by *P. aeruginosa* ATCC 9027

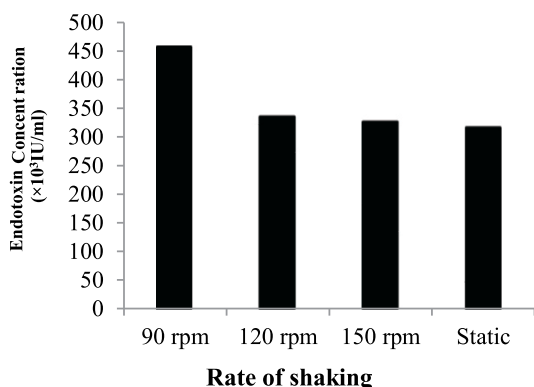


Fig. 2. Effect of rate of shaking on the endotoxin production by *P. aeruginosa* ATCC 9027

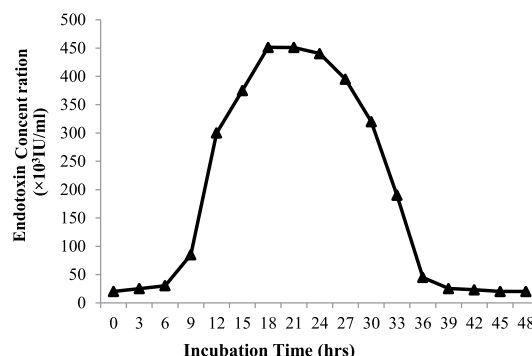


Fig. 3. Time course of the endotoxin production by *P. aeruginosa* ATCC 9027

GC/MS spectroscopy

The components of endotoxin extracted from *P. aeruginosa* ATCC 9027 were characterized by GC/MS. As shown in Fig. 5, four compounds were detected; 2H Pyran, 2,5 diethenyltetrahydro, Tetradecanoic acid, Bis(2 ethylhexyl) phthalate and Dodecanoic acid with a relative retention times of 1.38, 19.32, 23.18 and 25.68 min, respectively. The detected compounds are identical with the main components obtained from *Serratia rubidaea* and reported by Renukadevi *et al.*³¹. They reported the following components: 3-hydroxytetradecanoic acid (3-OH 14:0), 3- methoxytetradecanoic acid

(3-OMe 14:0) and tetradecenoic acid (14:1). Furthermore, dodecanoic (12:0), tetradecanoic (14:0) and hexadecanoic acid (16:0) were found in significant amounts.

Determination of endotoxin stability

The extracted endotoxin from *P. aeruginosa* ATCC 9027 showed high thermal stability upon exposure to 120°C for 60 min and the activity decreased (97%) with the progressive increase in temperature treatment at 200°C for 60 min (Data not shown). This was supported by the findings of Miyamoto *et al.*¹⁹, who reported the complete inactivation of endotoxins produced

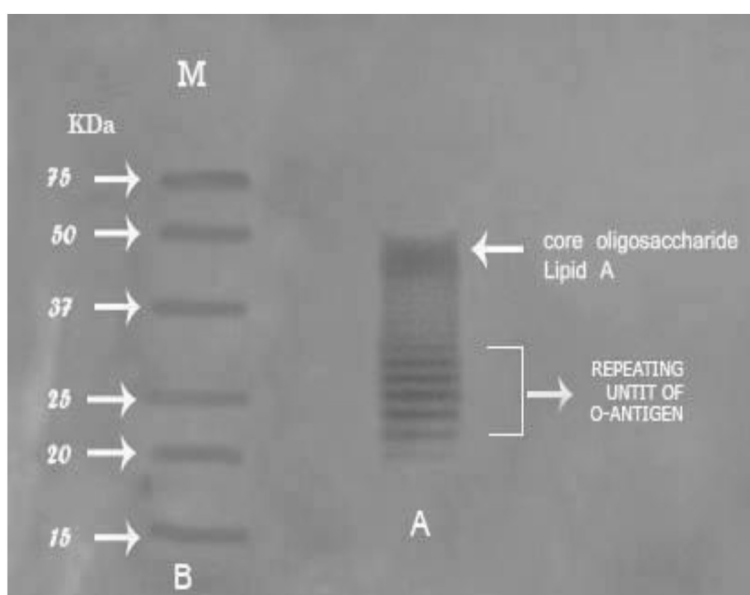


Fig. 4. SDS-PAGE profile of the purified endotoxin from *P. aeruginosa* ATCC 9027

Lane A: ladder pattern of purified endotoxin. **Lane B:** protein marker ranged from 18-75 kDa

by *E. coli* upon exposure to thermal treatment at 250°C for 60 min. In line with this concept, Gorbet and Sefton³² reported that endotoxins are highly heat-stable under normal sterilization conditions but the inactivation happened when exposed to 250°C for 30 min or 180°C for 3hrs. Thus, soft hydrothermal processing can inactivate endotoxins and this would be useful for the sterilization of

medical devices that cannot tolerate the exposure to dry heat at higher temperatures¹⁹. Gorbet and Sefton³² reported that, the endotoxins are highly heat-stable under normal sterilizing conditions but endotoxins can be inactivated upon exposure to 250°C for 30 min or 180°C for 3hrs.

Ibrahim *et al.*¹⁵ tested four temperature degrees (50, 100, 150, and 200°C) factors on the

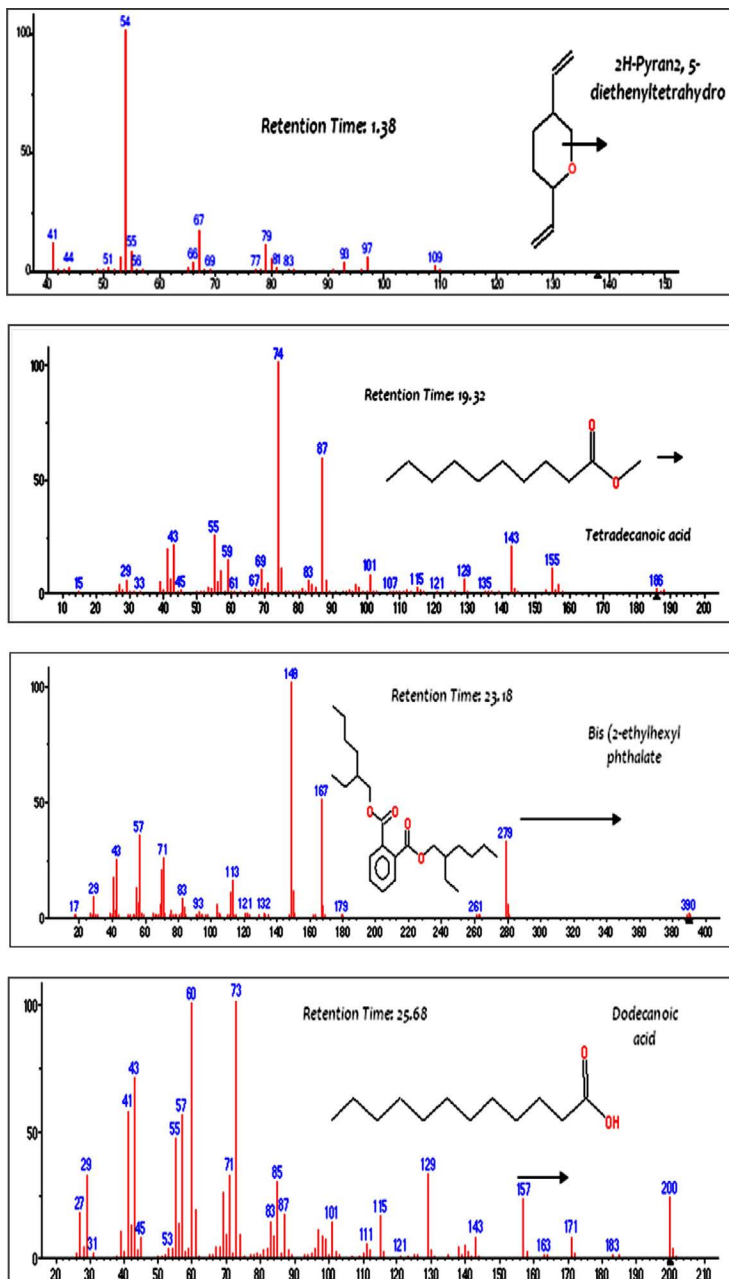


Fig. 5. Mass spectra of major constituents of the endotoxin produced by *P. aeruginosa* ATCC 9027

stability of endotoxin produced by *E. coli* AS10. Results revealed that the higher temperature, the lower endotoxin activity and consequently endotoxin concentration decreased upon the increase of temperature. The endotoxin lost about 88.6% and 94% of its activity/concentration at 150 and 200°C, respectively. Miyamoto *et al.*¹⁹ studied the mechanism of the hydrothermal treatment effect on the endotoxin stability. They suggested that inactivation occurs via hydrolysis of the ketosidic linkage between 2-keto-3-deoxyoctonic acid of the core oligosaccharide and lipid A, or glycosidic phosphate or fatty acids within lipid A. Schromm *et al.*³³ explained that the hydrothermal treatment effects the distribution of negative charges within the hydrophilic part of lipid A, which strongly influences its overall molecular conformation.

Chemical treatment of endotoxin extracted from *P. aeruginosa* ATCC 9027, either by 1N NaOH, HCl or ethyl alcohol (70% and 100%), resulted in detectable lowering in its activity (44.7%, 68.5%, 64.7% and 88.5%, respectively) after 60 min of treatment as reported by Magalhães *et al.*³⁴. Ibrahim *et al.*¹⁵ examined four NaOH concentrations (0.1, 0.2, 0.5, and 1.0 N) influencing the stability of endotoxin produced by *E. coli* AS10. They concluded that endotoxin activity and concentration decreased upon the increase of

NaOH normality and endotoxin lost about 69% and 81% of its activity/concentration at 0.5 N and 1 N, respectively. Further more, they emphasized that alkaline and acidic treatment changes irreversibly the endotoxin conformation which causes the loss of its activity and resulted in toxicity reduction and the deacylated LPS with the elimination of ester-linked fatty acids and changes occurred in the carbohydrate moiety of the lipid A component.

Endotoxin bio-toxicity and applications

For toxicity test toward *A. salina*, different concentrations of endotoxin from 25 to 100 µg/ml were used and the mortality percentage of nauplii was estimated after 24hrs. The results, presented in Table 2, indicated that the endotoxin produced by *P. aeruginosa* ATCC 9027 had a moderate toxicity level with LD₅₀ value of 102.99 µg/ml.

Table 2. Bio-toxicity of different endotoxin concentrations produced by *P. aeruginosa* ATCC 9027

Conc. (µg/ml)	Log Conc.	Mortality (%)	Log LD ₅₀	LD ₅₀ (µg/ml)
25	1.40	10	2.01	102.99
50	1.70	30		
75	1.88	40		
100	2.00	50		

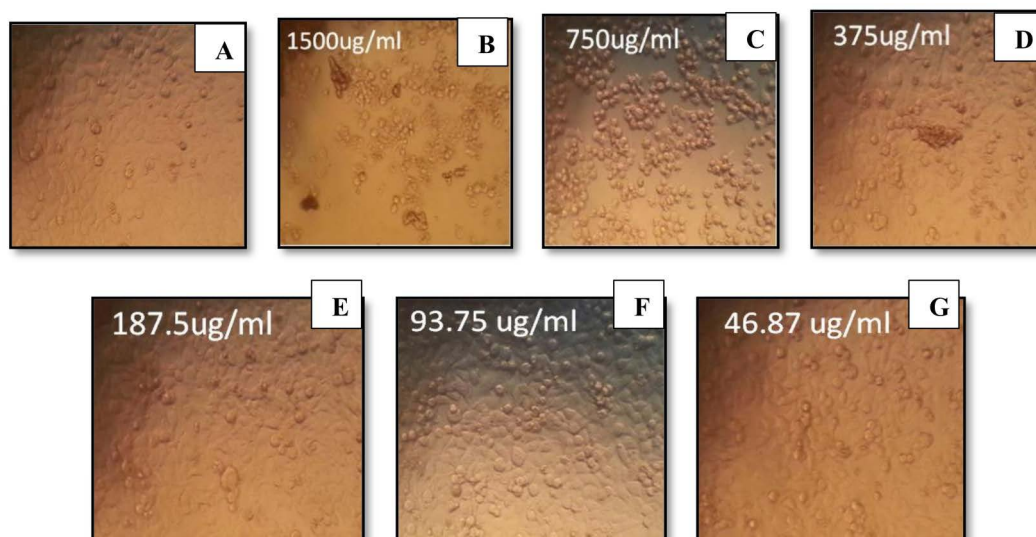


Fig. 6. Effect of different endotoxin concentrations on cell viability of Caco-2 (A) Control (untreated) Caco-2 cells. (B-G) Caco-2-treated cells with endotoxins

Antibacterial activity of the endotoxin produced from *P. aeruginosa* ATCC 9027 was determined. The results revealed its antibacterial potential against Gram-negative bacterial pathogens as *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 19404, and *Klebsiella granulomatis* ATCC 43816 and against Gram-positive bacterial pathogens as *Bacillus cereus* ATCC 33018. Ibrahim *et al.*¹⁵ evaluated the endotoxin produced by *E. coli* AS10 that showed potent antibacterial activity against *S. aureus* ATCC6538, *B. cereus* ATCC 33018 and *K. pneumonia* ATCC 43816. They highlighted the contribution of the antibacterial activity of endotoxin in driving the innate immune response, which is considered the first line of defense against pathogens through the core part in endotoxin signaling pathway produced LPS-binding protein (LBP).

A low antioxidant activity of the endotoxin from *P. aeruginosa* ATCC 9027 with LC₅₀ value of 688µg/ml. The endotoxin produced by Ibrahim *et al.*¹⁵ from marine *E. coli* AS10 showed a considerable antioxidant activity under the experimental conditions with LC₅₀ of 14.2µg/ml. Further more, the endotoxin (1500µg/ml) exhibited relatively weak antiviral activity (5.2%) against Vero cells infected with HAV. Campbell

and White³⁵ reported that bacterial endotoxin was unable to protect effectively the mice infected with lethal doses of Mengo virus that is related to the level of circulating interferon produced. However, El Tayeb and Hanson³⁶ reported that the titer of Newcastle disease virus (NDV) in infected chickens significantly ($P < 0.05$) decreased when inoculated with *E. coli* endotoxin before 3 days of exposure to NDV.

Furthermore, the cytotoxicity of endotoxin produced by *P. aeruginosa* ATCC 9027 was analyzed for its antitumor activity. *P. aeruginosa* endotoxin showed sharp decrease in the percentage of cell viability, 87% and 92%, of both Caco-2 and MCF-7 cell lines, respectively (Figs. 6, 7). Similarly, Sourek and Oravec³⁷ found that endotoxins of *Shigella dysenteriae* exhibited 76-92% tumoricidal effect against N-meth-Kellner mouse lymphoma cells in an *in vitro* assay. Nerkar and Bandekar³⁸ showed that endotoxin of *Salmonella typhimurium*, irradiated with gamma radiation, exhibited antitumor activity against S180 cells in Swiss mice and gave 90% survival of the infected mice. Bandekar and Nerkar³⁹ reported the antitumor activity of lipopoly-saccharide and radio-detoxified lipopoly-saccharide of *Vibrio parahaemolyticus*

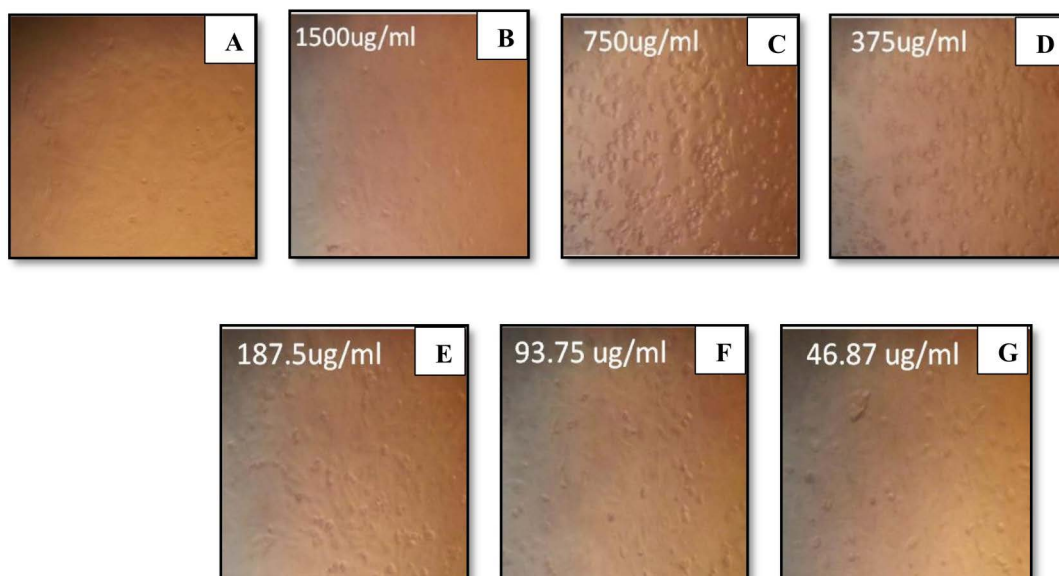


Fig. 7. Effect of different endotoxin concentrations on cell viability of MCF-7 (A) Control (untreated) MCF-7 cells. (B-G) MCF-7-treated cells with endotoxins

In conclusion, the present study showed the successful endotoxin production by *P. aeruginosa* ATCC 9027 and its use as an unconventional new anticancer therapeutic agent. This requires more clinical evaluation using animal model experiments to assess its pharmacological kinetics and usefulness for both colon and breast cancer treatment.

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CONFLICTS OF INTEREST

The author declares that there are no conflict of interest.

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