RESEARCH ARTICLE

In vitro Cytotoxic and Genotoxic of Lipopolysaccharide Isolated from *Klebsiella pneumoniae* AS1 on MCF-7 Human Breast Tumor Cell Line

Sura A. Abdulateef [1], Mohanad H. Hussein[2], Ali Z. Al-Saffar[2]*

[1]*Division of Biotechnology, Department of Applied Sciences, University of Technology, Baghdad, Iraq*
[2]*Department of Molecular and Medical Biotechnology, College of Biotechnology, Al-Nahrain University, Baghdad, Iraq*

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ABSTRACT

Lipopolysaccharide (LPS) from gram-negative bacteria induces many biological activities such as host defense, immunomodulation, antioxidant, and anti-proliferative activity. LPS extracted from the locally isolated *Klebsiella pneumoniae* (*K. pneumoniae*) AS1 was evaluated for the efficiency of *K. pneumoniae* AS1 LPS in inducing toxicity and genotoxic activity against breast tumor cell line MCF-7 *in vitro*. LPS from *K. pneumoniae* AS1 was extracted using a simple procedure of brief sonication and hot phenol. The crude LPS was subjected to Sepharose Cl-6B gel filtration chromatography column and two major peaks were eluted with the carbohydrate content of 41.42% and 21.65% for peak 1 and peak 2, respectively. The LPS collected from peak one was authenticated using high-performance liquid chromatography with purity of 90.53%. The cytotoxic effect of *K. pneumoniae* AS1 LPS was analyzed using methyl thiazole tetrazolium assay against MCF-7 cells compared with normal WRL68 cells. LPS exhibited a significant decrease in MCF-7 viability in a dose-dependent pattern with good cytocompatibility when applied LPS against normal WRL68 cells. As determined by DNA fragmentation assay, *K. pneumoniae* AS1 LPS induced an apparent deoxyribonucleic acid (DNA) fragmentation in MCF-7 cells compared with controls.

Keywords: Cytotoxicity, DNA Fragmentation, *K. pneumoniae*, Lipopolysaccharide, MCF-7 cells.

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INTRODUCTION

Despite advances in cancer treatment and diagnosis, cancer has remained a significant health concern and one of the leading causes of death worldwide. Every year, over eleven million people are diagnosed with cancer. This number is expected to rise to sixteen million by 2020.[1] Conventional treatments, such as chemotherapy, radiation, and combination therapy, are not sufficiently efficient to inherit more disadvantages due to weak bio-distribution and non-specific delivery of chemotherapy medications, high recurrence risk, minimal clinical efficacy, and distressing undesired results.[2] There is, therefore, a critical need for new, high-quality, and better-tolerated agents with chemotherapy activity of these agents in tumor cells.

In recent decades, the therapeutic role of bacteria has attracted interest in pharmaceutical and medicinal science. Although bacteria are considered among the primary cancer-causing agents, recent research has shown interesting results indicating that bacteria may be efficient cancer-treatment agents, the ideal vessels for targeted cancer therapy[3]—*Klebsiella* spp. a gram-negative bacteria that are part of the normal intestinal microbiota. Approximately one-third of humans bear asymptomatic *K. pneumoniae* in their gastrointestinal tract.[4] As such, these commensal microorganisms rarely cause infection in healthy individuals. Pathogenicity of the pathogen is due to lipopolysaccharides (LPS).[5]

K. pneumoniae has LPS bound to the bacterial membrane. The LPS structurally consists of three components–the lipid A component that anchors the entire structure in the bacterial membrane, the oligosaccharide center, and the terminal side chain called the O antigen. Lipid, A portion of LPS, is a hydrophobic moiety located in the outer leaflet of the outer membrane and is synthesized by a sequence of enzymes encoded by the *lpx* gene cluster. Lipid A plays an essential role in LPS host identification and binds strongly to toll-like receptor 4 (TLR4), leading to a robust immune response activation.[6] The waa locus contains genes that encode the core oligosaccharide that binds lipid A to the O antigen. The O antigen is covalently bound to the oligosaccharide center by the Waal ligase encoded by waaL. The O antigen consists of a polymer of repeated oligosaccharide units in the outermost part of the LPS structure. Various O antigens repeat the structural variations of the LPS.[7]

**Author for Correspondence: ali.btc80@gmail.com*

The LPS from different gram-negative bacteria have anti-tumor activity in animals due to their host-mediated immunostimulatory action, provided by cell-mediated cytotoxicity, including macrophage stimulation. LPS are, however, capable of controlling the gene expression of pro-inflammatory cytokines by binding. This leads to the death of tumor cells.[8] The LPS-induced immune activation is initiated by capturing the membrane-bound or released LPS molecules by LBP in the serum. These are then transferred to the soluble or membrane-bound CD14, a molecule that transforms LPS aggregates into monomeric forms and presents the latter to the membrane-bound TLR4–MD2 complex. Rough LPS molecules have been shown to interact easily with TLR4–MD2 and do not need LBP and CD14 pre-capture and processing.[9] After the interaction, the ternary complex is internalized into the host immune cells, resulting in two main signal cascades. The first cascade includes activation of MyD88 and ultimately contributes to the secretion of many different pro-inflammatory cytokines and chemokines through activation of NF-£B, IRF5 and AP-1. The second pathway, also known as the MyD88-independent pathway, is activated by TRIF activation, resulting in IFN type I secretion resulting from IRF3 activation.[10] LPS is also capable of activating $CD4^+$ and $CD8^+$ T cells in vivo, which are thought to be a by-product of a complex cytokine mixture secreted after TLR4 activation in monocytes.[11]

LPS from *K. pneumoniae* was found to cause apoptosis in certain human malignant cell lines such as HeLa, Jurkat (T-cell originating from acute T-cell leukemia), RJ2.25 (a type of Burkitt's lymphoma), and colorectal carcinoma cells with no effect on normal cells.[12] In this way, the purpose of this study was to determine the action of LPS extracted from locally isolated *K. pneumoniae* AS1 in cancer proliferation, viability, and DNA fragmentation against MCF-7 cell line.

MATERIALS AND METHODS

Materials

Chemicals including Luria Broth (LB), formalin, acetone, phenol, Sepharose Cl-6B, RPMI-1640, trypsin, RNase, and fetal bovine serum were obtained from Sigma-Aldrich (Germany). MTT kit was purchased from Intron Biotech (Korea). Cellomics® Multiparameter Cytotoxicity 3 kit was purchased from ThermoScientific (USA). Lipopolysaccharides standard of *Escherichia coli* O111 (Sigma-Aldrich, Germany).

Bacterial Isolate

Locally isolated *K. pneumoniae* AS1 was provided from the College of Biotechnology–Al-Nahrain University and was cultured in LB at 37ºC overnight.

Cell Line and Cell Maintenance

A human breast tumor cell line MCF-7 and normal WRL68 cell line were provided from Al-Nahrain Biotechnology Center of Al-Nharin University. Cells were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum 10^3 IU 100^{-1} mL penicillin G and 0.001 g 100^{-1} mL streptomycin. Cells were incubated at 37°C in a humidified incubator with 5% CO_2. For growth initiation, MCF-7 cells were seeded into T 25 cm^2 tissue culture flasks (ThermoScientific, USA) at density 2×10^4 cells mL^{-1}. When reached exponential growing phase (36–48 hours), cells were harvested after brief trypsinization (50 $mg.mL^{-1}$ of trypsin) and were seeded at desired concentration.[13]

Extraction of LPS

K. pneumoniae AS1 cells were cultured in 25 mL LB overnight at 37ºC. The culture was used to inoculate 2 L LB, the broth was incubated at 37ºC with shaking (120 rpm) for 24 hours. After incubation, bacterial cells (10^8 colony-forming unit mL^{-1}) were centrifuged at 3000 rpm for 15 min. The pellet was washed in 10 mL PBS (pH: 7.2) twice, sonicated in ice for 10 min, resuspended in PBS containing 0.5% formalin, and kept at 4ºC overnight. The mixture was cold centrifuged at 3000 rpm for 15 minutes and washed twice with PBS, then cells were dried using cold acetone (10 times the sample's volume) with vortexing.[14] The dried cells were suspended in prewarmed (65ºC) PBS with vigorous mixing and were added to an equal volume of hot 90% phenol. The mixture was incubated at 65ºC for 15 minutes, then centrifuged at 10000 g for 15 minutes. The aqueous phase was removed carefully, and the phenol phase was suspended with hot PBS. The centrifugation process was repeated, and the resulted aqueous phases were combined and dialyzed against distilled water. The dialyzed sample was lyophilized to obtain the final LBS powder.[15]

Purification and Chemical Characterization of LPS

The lyophilized crude LPS (30 mg mL^{-1}) was purified using Sepharose Cl-6B column (3×50 cm). After column equilibration with PBS (pH 7.2), a volume of 5 mL of LBS solution was poured gently on the surface of the resin; the column was washed with PBS at flow rate 30 mL/hour with fraction size of 3 mL. Fractions were collected, and absorbance at 280 and 260 nm was detected for protein and nucleic acid contamination, respectively. Carbohydrate content was measured according to the method described by Dubois *et al.,*[16] while protein content was estimated according to the method described by Bradford.[17] Fractions of interest were pooled, lyophilized, and kept at 4ºC for further studies.

Detection of LPS by HPLC

The purity of the extracted LPS was examined using high-performance liquid chromatography (HPLC) system. The configuration of HPLC separation included Nova-Pak C_{18} (250 x 4.6 mm) column supplied with a multichannel detector 490E, a Millennium data processor (Shimadzu), and a Rheodyne injector with 20 µL loop. The process was optimized at a flow rate of 1 mL min^{-1} and a mixture of water and acetonitrile (95:5 vol/vol) was used as mobile phase. Aliquots of 100 µL of both LPS sample and standard were injected, and peaks were detected at wavelength 210 nm.

Cytotoxicity Test (MTT Assay)

The cytotoxic effect of *K. pneumoniae* AS1 LPS was tested against MCF-7 and WRL-68 cells using MTT colorimetric

assay. In brief, aliquot of 200 µL of suspended cells (1 x 10^5 cells mL^{-1}) in culture medium were seeded per well in 96 flat plates and incubated at 37°C, 5% CO_2 for 24 hours. After incubation, the medium was removed, and a range of *K. pneumoniae* AS1 LPS (50, 100, 200, 300, 400, and 500 µg mL^{-1}) were added to the wells. Cells without treatment were used as controls (cells treated with serum-free medium), and different treatments were performed in triplicates. Plates were incubated at 37°C, 5% CO_2, for another 24 hours. Following incubation, 10 µL of MTT solution was added to each well, and plates were incubated at 37°C, 5% CO_2, for 4 hours. The media was carefully decanted off, and 100 µL of solubilization solution was added with five min waiting. The final reaction (formazan formation) was detected at 575 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Rad, USA). Viability (%) was calculated according to the following formula:

$$\text{Viability (\%)} = \text{OD control} - \text{OD sample/OD Control} \times k100$$

The cytotoxicity of *K. pneumoniae* AS1 LPS was expressed as IC_{50}.

DNA Fragmentation Assay

K. pneumoniae AS1 LPS was assayed for DNA fragmentation ability according to the method of Quadri *et al.*,[18] with modification. MCF-7 cells (2 x 10^6 cells mL^{-1}) treated with LBS were washed with PBS and resuspended in lysis buffer (40 mL of 0.5 M EDTA, 5 mL of 1 M Tris-HCL buffer pH 8.0, 5 mL of 100% Triton X-100 and 50 mL H_2O), cells were incubated in ice for 30 min. After incubation, the suspension was centrifuged at 12000 rpm for 30 minutes at 4ºC. The supernatant was carefully transferred into a fresh tube, and 50 µL of 3 M was added, and DNA was extracted with phenol-chloroform (vol/vol) solution with vortexing. DNA was pelleted by adding two volumes of cold ethanol, centrifuged at 12000 rpm for 30 minutes at 4ºC, and resuspended in 50 µL TE buffer. DNA samples were mixed with loading buffer (40% sucrose in TE, 1% SDS, 0.05% bromophenol blue, and 2.5 mg mL^{-1} RNase), incubated at 37ºC for 10 minutes and loaded into 1.5% agarose. Electrophoresis was carried out at 4-volt cm^{-1} for 4 hours DNA was detected by staining with ethidium bromide and visualized under UV light.

Statistical Analysis

All statistical analyses were performed using Graph Pad Prism version 6 (Graph Pad Software Inc., La Jolla, CA). A one-way analysis of variance (ANOVA) (Tukey's post hoc test) was applied to identify the differences between different groups. All data were expressed as mean ± standard deviation and statistical significance was defined as * $p \leq 0.05$ or ** $p \leq 0.01$. All experiments were independently performed in triplicate ($n = 3$).

RESULTS AND DISCUSSION

Isolation and Purification of LPS

LPS is considered as the main out membrane component of gram-negative bacteria. Bacterial LPS is composed of three regions, lipid A, core saccharide, and O-antigenic side chain with covalent attachment.[19] Due to the wide range of LPS biological activities as a membrane barrier, bacterial recognizing site, immuno-modular and therapeutic activity [20], many protocols were developed for bacterial LPS extraction depending on the chemical nature, bacterial species, and purpose of extraction.[21]

Out of 10 g of *K. pneumoniae* AS1 biomass, 72.53 mg of partially purified LPS was recovered after lyophilization. The extraction process is a simple and easy to run the procedure with a higher yield of LPS. Although many reports used different protocols for LPS extraction, the overall LPS yield was ranged from 0.5 to 2.5% of LPS of bacterial cell dry weight.[22] The application of hot phenol could adversely reduce the amount of yielded LPS; on the other hand, it will provide a high-quality product with minimum proteins and nucleic acid contaminants.[23] The partially purified LPS was chromatographed over a Sepharose Cl-6B column, and 50 fractions were monitored by detecting the carbohydrate and protein contents. Two major peaks were recorded (Figure 1). The first peak is a large peak and mostly contained high concentrations of carbohydrates. The spectrophotometric scanner of the LPS fractions showed maximum absorption at fractions 12 to 16, while the other small peak is containing LPS-associated proteins. It was reported that LPS elutes with protein contaminants in gel filtration chromatography based on size.[24] Our result confirmed previous suggestions that gel filtration chromatography separate two forms of LPS physically and chemically distinct based on their differences in size, one eluted in void volume and the second in relative retention after first peak. The high molecular weight LPS diffuse through the gel particles and elute in the void volume, which constitutes the highly concentrated LPS with less protein contaminants.[25] Chemical analysis showed that the carbohydrate contents was 41.42% and 21.65% for peak 1 and peak 2, respectively, while the protein content was 0.932% for peak 1 and 3.42% for peak 2.

The purity of LPS extracted from *K. pneumoniae* AS1 by HPLC. was assessed was detected by HPLC. Figure 2A shows one major peak detected at a retention time of 8.311 minutes.

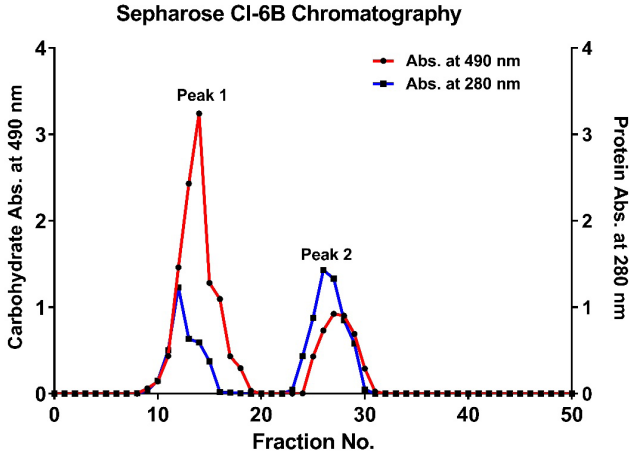

Figure 1: LPS purification by Sepharose Cl-6B gel filtration chromatography column (3 x 50 cm) equilibrated with PBS (pH 7.2) at flow rate 3 mL/fraction.

The major peak from the unknown sample was identified as LPS by comparing the standard's retention time (Figure 2B) with the retention time of the major peak. The concentration of purified LPS isolated from *K. pneumoniae* AS1 was 2.01 mg g^{-1} with purity of 90.53%.[26]

Cytotoxic Activity of LPS against MCF-7 Cell Lines *in vitro*

The anti-tumor activity of *K. pneumoniae* AS1 LPS was evaluated *in vitro* against MCF-7 cells using MTT test after 24 hours exposure. As shown in Figure 3, the inhibition rate of MCF-7 cells was ranged from 4.63 ± 1.86 to 64.54 ± 3.51%,

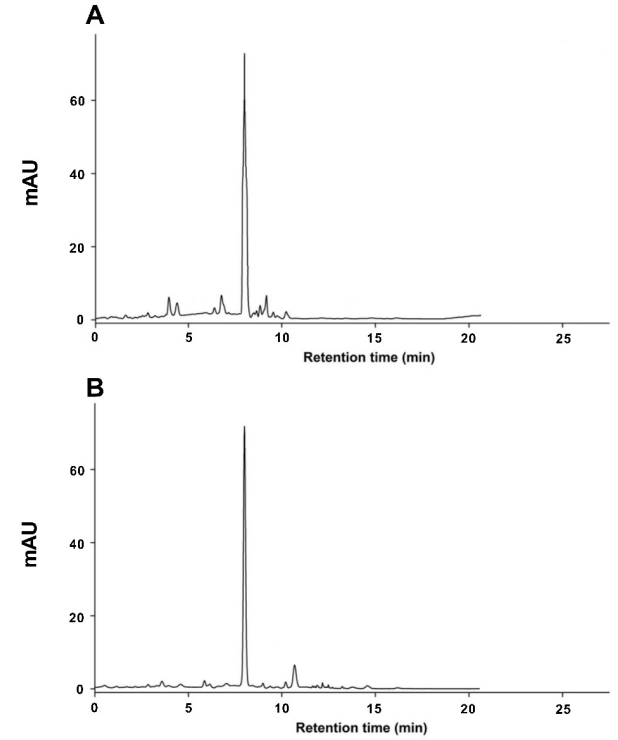

Figure 2: HPLC chromatogram for (A) test sample and (B) LPS standard at 210 nm.

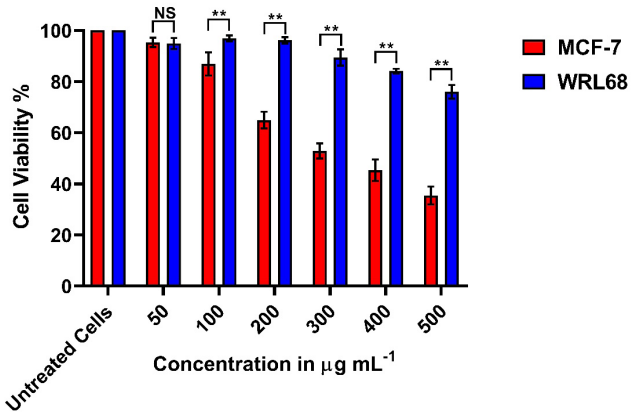

Figure 3: Mean percentage (± SD) of cell viability. MTT assay for *K. pneumoniae* AS1 LPS against MCF-7 and WRL68 cells at 37ºC for 24 hrs (n = 3). NS: Nonsignificant, **: p ≤ 0.01, SD: Standard Deviation

and *K. pneumoniae* AS1 LPS decreased the cell viability in a dose-dependent pattern. On the other hand, *K. pneumoniae* AS1 LPS showed a good cytocompatibility against WRL68 normal cells with a maximum inhibition rate of 24.02 ± 2.69% at concentration 500 µg mL^{-1}. By comparing the cytotoxic effect of *K. pneumoniae* AS1 LPS between MCF-7 and WRL68 cells, all applied concentrations displayed a significant difference ($p \leq 0.0001$) except 50 µg mL^{-1} which revealed no significant cytotoxic effect between MCF-7 and WRL68 cells. IC_{50} concentration was detected by a dose-response curve fitting of the cell viability rate. The IC_{50} value of LPS for MCF-7 and WRL68 cells were 267.5 and 737.9 µg mL^{-1}, respectively.

K. pneumoniae AS1 LPS induction actives transcription of genes encoding pro-inflammatory proteins, which leads to the release of cytokines and enzymes inducing leading to cell death.[27] One reason could be attributed to tumor necrosis factor (TNF) production; at significant concentration, *K. pneumoniae* AS1 LPS might trigger the overproduction of TNF, consequently being toxic to the cell, leading to cell death. On the other hand, cancer cells can be sensitized to *K. pneumoniae* AS1 LPS**,** that induces the production of reactive oxygen species. in contrast, the LPS can stimulate the clearance of immunogenic tumor cells promoting adaptive immune responses.[28]

Genotoxic Activity of LPS against MCF-7 Cell Lines *in vitro*

To further evaluate the ability of *K. pneumoniae* AS1 LPS in inducing DNA damage, the agarose gel electrophoresis assay was performed in MCF-7 cells. Figure 4 represented the DNA fragmentation in MCF-7 cells after treatment with

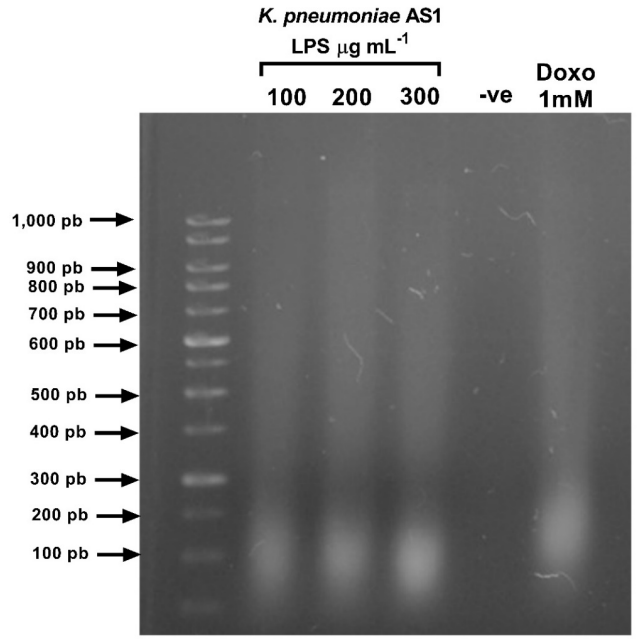

Figure 4: UV-Illumination images of fragmented DNA. First line: DNA marker with molecular weight ranged from 100 bp to 1000 bp. Three middle lines: MCF-7 cells treated with *K. pneumoniae* AS1 LPS (100, 200 and 300 µg mL^{-1}, respectively). The last two lines -ve control and +ve control (1 mM doxorubicin), respectively.

100, 200, and 300 µg mL^{-1} concentration of *K. pneumoniae* AS1 LPS and exhibited an apoptotic DNA smear, and the effect was dose-dependent and showed a similar pattern to that of doxorubicin treatment (positive vehicle). A proposed mechanism for cell death induced by *K. pneumoniae* AS1 LPS via DNA fragmentation may result through the activation of free radicals of reactive oxygen species (ROS). When the DNA exposed to ROS, this led to the production of 8-hydroxy-20-deoxyguanosine (8-OHdG); the presence of 8-OHdG alters the guanosine base and increasing the chance of G to T mutation during DNA replication. 8-OHdG considers as a marker of DNA damage, a site of increased hazard for mutagenicity, and a biomarker of oxidative stress.[29] Induction of apoptosis has been considered the major mechanism of anti-cancer drug discovery. The development of anti-cancer drugs that inhibit abnormal cancer cell proliferation and induce cell death through apoptosis is a fundamental objective of cancer research.[30,31]

CONCLUSIONS

In conclusion, LPS extracted from the local isolate *K. pneumoniae* AS1 showed significant cytotoxicity against tumor MCF-7 cells with good biocompatibility when normal WRL68 cells treated with *K. pneumoniae* AS1 LPS. Besides, *K. pneumoniae* AS1 LPS exhibited marked stimulation of DNA fragmentation in MCF-7 treated cells.

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