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Original Article

Antimicrobial Activity Assay of Liposomal Lipopolysaccharide Extracted from *Escherichia coli*

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Abstract

Escherichia coli (E. coli) is a bacterial bacillus known to be negative for the gram dye, and it is part of the normal flora inside the human body. The current study aimed to investigate the possibility of using lipopolysaccharides (LPS) as an antimicrobial agent. A total of 30 isolates of *E. coli* were collected from 100 specimens of urine isolated from patients with recurrent urinary tract infections (UTIs) referring to Al-Sadr Teaching Hospital. The samples were cultured on identification media; thereafter, they were diagnosed according to the phenotypic form, biochemical tests, and finally by VITK-2. The results of the prevalence of *E. coli* isolates illustrated that from 30 isolates of *E. coli* which were collected from 100 urine specimens, 14 (46.6%), 6 (20%), and 10 (33.3%) cases belonged to the urinary tract infections, kidney stones, and urinary catheter samples, respectively. The isolates displayed multiple drug resistance (MDR) to most of the antibiotics used in the study. Therefore, the lipopolysaccharide extracted from *E. coli* was used as an antimicrobial agent. The recorded data obtained from the polymerase chain reaction (PCR) for *WaaA*, *WaaC*, *wamB*, and *wabG* genes demonstrated that the isolates possessed biosynthesis genes for LPS. The results indicated that LPS at concentrations of 150, 250, and 500 µg/ml has as an anti-growth agent for *Klebsiella pneumonia, Proteus mirabilis, Salmonella typhi, Pseudomonas aeruginosa, Bacillus subtilis, Staph aureus, and candida albicans.* The highest inhibitory effect was observed on *K. pneumonia, P. mirabilis, S. typhi, B. subtilis, and C. albicans*, as compared to the control antimicrobials agents (Amoxicillin, Nystatin). The LPS loaded on liposome nanoparticles can open new horizons in medicine by its inclusion in the manufacture of broad-spectrum antibiotics.

Keywords: Antimicrobial, *E. coli*, Liposome, LPS, Nanoparticles

1. Introduction

Escherichia coli (E. coli) is a bacterial bacillus known to be negative for the gram dye, and it is part of the normal flora inside the human body. It can be observed inside and outside the intestinal tract, causing a wide array of diseases. Hundreds of its strains have been diagnosed over time; therefore, the severity of the infections they cause ranges from acute fatal to mild self-healing gastroenteritis. It has many virulence factors that enable it to attack the human immune system, cause serious infections, such as kidney failure and septic shock, and make it resistant to many antibiotics (1).

The *E. coli* is one of the common causes of intestinal infections in humans; nonetheless, it is also present outside the intestinal tract and causes urinary tract infections (UTI), pneumonia, bacteremia, infections of the peritoneum, and other infections (2). It is one of the leading causes of nosocomial infections, including catheters associated with urinary tract infections and ventilators associated with pneumonia (3). The *E. coli* is not confined to the human body but is also observed

in soil, vegetables, water, and undercooked meat since the pathogenic strains of *E. coli* cause intestinal diseases in the people who consume them. Bacterial lipopolysaccharides (LPS) are the main component of the bacterial wall of gram-negative species and constitute approximately 90% of the outer surface of the bacteria (the outer layer), where the phospholipid layer occupies the inner part of the membrane. As we know, the structure of LPS is the same in all gram-negative types since it consists of three parts. The hydrophobic part is lipid A, which is the main part of the outer membrane, while the oligosaccharides connected to the lipid A are considered the contact area for long chains of polysaccharides (O antigen; O chain) (4).

The majority of the genes of *Enterobacteriaceae* encode proteins responsible for the biosynthesis of LPS. The genera of *Enterobacteriaceae* share the presence of this set of genes, depending on the available genetic information (5). The LPS plays a crucial role in the protection of bacteria since it acts as a physical barrier from the environment surrounding bacteria. It is also a well-known part of the immune system through which bacterial species are distinguished as invading pathogens and is also responsible for increasing the inflammatory response (6).

The use of liposome nanoparticles has recently become an important drug conductive carrier. The liposome consists of a lipid bilayer spherical, hollow shape filled with the aqueous phase. Therefore, this molecule has two parts, a hydrophilic part and a hydrophobic part; therefore, any compound can be associated with the liposome. Therefore, most drugs can be carried on the liposome and at the same time provide protection from hydrolysis and the degradation that the compounds may encounter during their movement to the target site. Furthermore, targeting the surface proteins on the cells and ligand on lipid bilayer shell has another functional permitting targeted access of liposome into the cell by ligand receptors target. These ligands confer to cell receptors that are over-expressed in convinced diseased cells, allowing entry of the drug through the cell membrane (7).

Given the importance of this component, the current study aimed to investigate the possibility of using LPS as an antimicrobial agent alone and loaded with liposome nanoparticles by extracting it and applying it against some pathogenic microorganisms.

2. Materials and Methods

2.1. Bacterial Characterization

All specimens were taken under sterile conditions from patients with chronic urinary tract infections and kidney stones, as well as long-term urinary catheter users, attending Al-Sadr Teaching Hospital from March to June 2019. The specimens were grown on media MacConkey agar, Blood agar, and incubated at 37°C for 24 h for bacterial growth. The phenotypic characteristics of the growing colonies, such as shape, size, and color, were confirmed and the isolates suspected to be *E. coli* were subjected to biochemical tests and finally confirmed by using Vitek-2 Compact (Bio Mérieux, France,). An antibiotic sensitivity test was also carried out for all isolates using Vitek -2 system with AST-XN05 card.

2.2. Molecular Studies of *E. coli* Lipopolysaccharides Biosynthesis

2.2.1. Extraction of Bacterial DNA Genomic

The method was carried out according to Li, Carey (8) using Tm Mini DNA Bacteria Kit (Geneaid, Indonesia).

2.2.2. Polymerase Chain Reaction Assay

The *E. coli* isolates were subjected to genetic screening for four genes (*waaA*, *waaC*, *wamB* and *wabG*) to detect the pathway for lipopolysaccharides synthesis. The primers used in this study are presented in table 1.

Table 1. Primers synthesized by Bioneer (Korea)

Primer	Sequencing	Product size
waaA	F:CAGGCGCAAAAGTCCGTATC R : TTCCCGCCCATAAAACTTCG	618
waaC	F:TGTTCAGCATCGCCTTTACG R : AATCCGCTTTAGTGCCGTTC	691
wamB	F:ACCCCGTTTTCAGCAACTTT R : TAACTGAAGGTGAGCGTCGT	611
wabG	F : GTTTTGTTTCTCGCGCACTG R : ATCGCCTCAATAGCAGCTCT	619

2.2.3. Polymerase Chain Reaction Amplification

The method which was described by Joshi and Albert (9) was used in this study. The PCR products were evaluated on 1.5% agarose gels stained with 5 µl ethidium bromide, and photographed with UV illumination.

2.3. Extraction of Lipopolysaccharide

2.3.1. Cell Preparation

The *E. coli* isolates were cultured overnight in a 25 ml flask of Luria Broth (LB) for the purpose of activating bacteria at 37C° for 18 h. The active cultures were inoculated in 3.5 liters of LB and suspended in 500 ml flakes containing 200 ml of broth then incubated at 37C° for 24 h with shaking at 150 rpm. Following that, it was centrifuged at 3000 rpm for 15 min. The precipitate was washed twice with phosphate buffer. The cells were suspended in phosphate buffer containing 0.5% formalin (pH=7.2) and kept at 4C° for 18 h. The precipitate was centrifuged at 3000 rpm for 15 min and washed again with a phosphate purifier. Finally, the cells were dried using cold acetone, 10 times the sample volume (10).

2.3.2. Lipopolysaccharide Extraction

The LPS of *E. coli* was extracted from multidrug-resistant isolates by the hot EDTA method as previously described by Chandan, Fraser (11).

2.3.3. Partial Purification of Lipopolysaccharide by Gel Filtration (Sephacryl S-300)

Sephacryl S-300 gel was informed conferring to the evidence of the manufacturer company (Sigma, Germany). It was washed and suspended in 0.025 M of phosphate buffer (PB) (pH 7.2), degassed by using a vacuum pump then decanted with maintenance to avoid bubbles into a column with a dimension of 75×2 cm. The last volume of the column was 235.5 cm^3. Thereafter, the column was equilibrated with 0.025 M of phosphate buffer saline (PBS) (pH 7.2), and the stream rate was 75 ml/hour. The extracted sample was chemically analyzed to define the content of carbohydrates according to the method previously described by Dubois, Gilles (12), and the total protein was analyzed according to the method previously published by Bradford (13). Sterility was detected by culturing of LPS on blood and nutrient agar.

2.4. Nanoparticles- fractional Mixture Preparation

Nanoparticles Liposome Solution was prepared and loaded according to the method described by Suhad and Ruaa (14).

2.5. Antimicrobial Activity Assay

These were achieved for each nano-fractional and partially purified fractions of lipopolysaccharide extract as the antimicrobial activity that was determined by the agar diffusion method as previously described by Kavanagh (15). Petri plates containing 25 ml of sterile Muller-Hinton agar were inoculated along with. *Klebsiella pneumonia*, *Proteus mirabilis*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staph aureus*, and *candida albicans*. The final inoculums of these bacteria were adjusted to 1.5×10^8 (CFU)/ml and compared to the 0.5 McFarland standards tube. The cut wells have (6mm) in diameter in agar by using a sterile Pasteur pipette and removed the agar discs by a sterile forceps; subsequently, the wells were filled with 0.1 ml of each concentration of nano-fractional and partial purify fraction of lipopolysaccharide extract (150,250,500 µg/ml) then incubated in the upright position to keep the LPS extract in the wells at 37°C for 24 h. Measured inhibition zone diameter formed around each well assessed the antimicrobial activity of LPS and matched up against amoxicillin and nystatin control.

3. Results

The results of the prevalence of *E. coli* isolates demonstrated that from 30 isolates of *E.coli* which were collected from 100 urine specimens, 14 (46.6%), 6 (20%), and 10 (33.3%) cases belonged to the urinary tract infections, kidney stones, and urinary catheter samples, respectively (Table 2). The recorded data obtained from the PCR for *WaaA*, *WaaC*, *wamB*, and *wabG* genes illustrated that the isolates possessed biosynthesis genes for LPS (Figure 1). The LPS used in

the study was extracted from the most antibiotic-resistant *E. coli* isolates and was used to eradicate many pathogens. About 40 gm of the dry weight of bacteria produced 300 mg of Crude LPS. The partial purification results indicated that the amounts of carbohydrates and proteins were 94% and 4.4%, respectively; therefore, the amount of LPS was 1.6 gm.

Table 2. Distribution of *E. coli* isolates among urine specimens of patients with (urinary tract infections, kidney stones, and urinary catheters)

Specimens sources	No. *E. coli* isolates (%)
Urinary tract infections	14 (46.6%)
Kidney stones	6 (20%)
Urinary catheter	10 (33.3%)

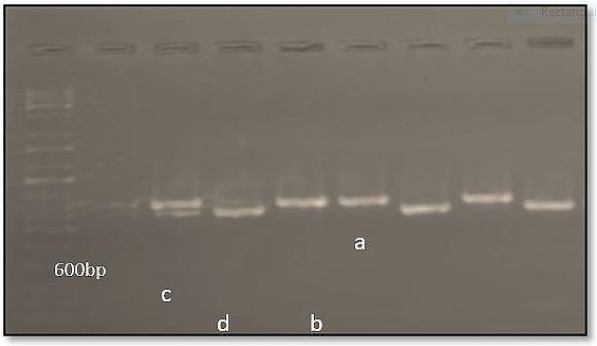

Figure 1. Gel electrophoresis and PCR product of LPS genes, Ladder (100-1000bp). a-waaC gene 691 bp, b-wamB gene 611 bp, c-waaA gene 618 bp and d-wabG gene 619 bp

The results of microbial antagonism using partially purified LPS only showed significant effectiveness in inhibiting the studied microbes, where the inhibition area ranged from 8.6-24.6) mm with three concentrations, 150, 250, and 500 µg/ml. The lowest effect was on bacteria (*P. aeruginosa* and *S. aureus*) and the highest inhibitory effect was on (*K. pneumonia*, *P. mirabilis*, *S. typhi*, *B. subtilis*, and *C. albicans*), compared to the control antimicrobials (Amoxicillin, Nystatin) which were bacteria and fungi resistant to it (Figure 2 and Table 3). Mixing LPS with the liposome significantly increased the inhibition activity, as the rate of the inhibition diameters increased and ranged between 18-33.6 mm. It was also noted that the activity of LPS with the liposome in the inhibition of *P. aeruginosa* and *S. aureus* growth was significantly improved.

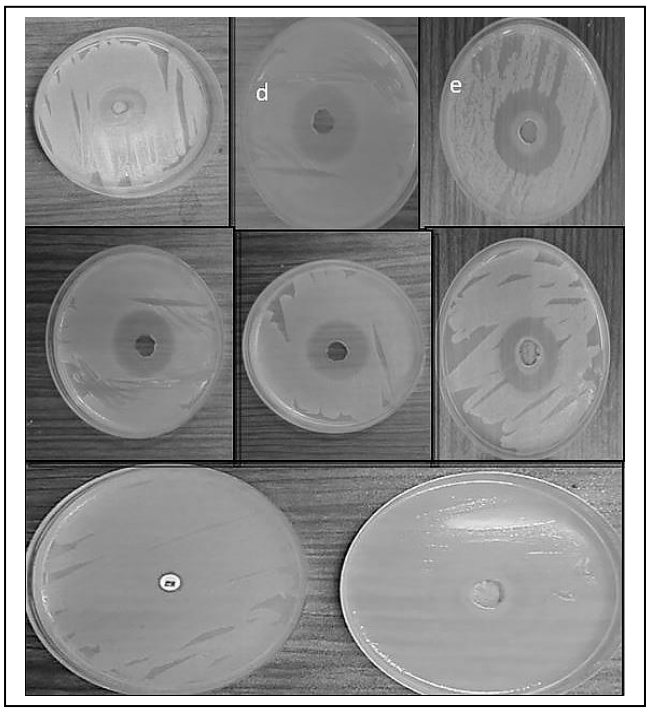

Figure 2. Inhibition zones (mm) of nano- partially purified LPS fraction against *a. Candida albicans b. P. aeruginosa c. B. subtilis d. S. typhi e. P. mirabilis f. S. aureus g. K. pneumonia* with amoxicillin

Table 3. Inhibition zones (mm) of nano- partially purified LPS fraction against studied microbes at three concentrations (150,250 and 500) µg/ml

Concentration µg/ml	*K. pneumonia*	*P.mirabilis*	*S. typhi*	*P. aeruginosa*	*B. subtilis*	*S. aureus*	*C. albicans*
150	19±1	21±3.2	24.1±1.7	18±3.6	23.6±2	19.6±1.5	22±3
250	27±3.6	25±4.5	28.6±1.1	21.3±5	29.3±2.5	27±4	23.3±8
500	24±8.6	26.6±5.6	29.3±2.3	27.6±0.5	33.6±1.1	26.3±7	28±1
Amoxicillin	7±3.4	4.1±3.3	1.3±2.3	1±1.7	4±4	0±0	-
Nystatin	-	-	-	-	-	-	4.6±5

*The values represented by means±sd

4. Discussion

Escherichia coli is considered a major component of the normal intestinal flora and is mostly a non-pathogenic organism. It also exists outside the living body in nature and is acquired as a pathogen from hospitals, causing urinary tract infections (16-17). According to a study conducted by De Francesco, Ravizzola (16) in Beirut, *E. coli* isolates were the most frequent, where the percentage of its presence reached 60.64% of the rest of the other isolates. Recently the number increased from 2.3%-16.8% from 2000 to 2009 (17).

The genes involved in LPS core biosynthesis in most *Enterobacteriaceae* are usually found clustered in a region of the chromosome, the *waa* (*rfa*) gene cluster as in *K. pneumia* (16). In other species, these genes may be non-clustered and distributed among several regions as in yersinia pestis or proteus mirabilis although one common core biosynthetic gene (*wabO*) was found outside this cluster (18). In their research, Zuhir and Alaubydi (19) pointed out that 1.4 g of LPS was produced from 10.5 dry cells. Proteins and carbohydrates in the crude extract were 2.3% and 0.9%, respectively, while they were 9.5% and 0.06, respectively, in the partial purification of LPS.

Along the same lines, Yossef (20) reported that the partial purification of LPS extract from *Proteus mirabilis* demonstrated that the first curve contains more effective LPS, while the second curve contains less effective LPS, while the standard bacteria are present in the second curve. The increasing carbohydrate quantity inside the purified pattern will be due to the elimination of a few impurities (21). Steimle, Autenrieth (22) determined that LPS in distinct concentrations (700,800,900,1000,1100, and 1200 Ug/ml) gave distinct inhibition zone to *E-coli* variety from 27-37mm. Zuhir and Alaubydi (19) reported that the inhibition zone of polysaccharides ranged from 2.7-11.5 mm against *E. coli* and *P. aeruginosa*. The specific shape of LPS is active against distinct bacterial strains (gram-positive and gram-negative) (23).

The susceptibility of Gram-negative cells to LPS is related to elements that facilitate the delivery of the LPS throughout the outer membrane (24). The O-antigen is probably a reliable indicator of virulence capability and those crucial capabilities frequently vary in the identical bacterial strain. This can assist in the designing of novel antibacterial as destiny therapeutics (24).

Given that most antimicrobial agents have a short half-life, and many of them exert adverse side effects on the human body, researchers resort to loading these agents onto manufactured nanoparticles ranging in size between 50-500 nm. The most important of these bodies is what is known as a liposome which consists of an outer membrane of phosphorylated lipids around an aqueous core (25), where the presence of these hydrophilic and hydrophobic layers allows the liposome to be used as a carrier and transporter for many medicinal compounds of different lipid profiles (26).

Microbial sensitivity *in vitro* studies indicated that the rate of inhibition of cefepime carried on the liposome on *E. coli* is similar to the effect observed when using free cefepime. It was also detected that the growth rate of *E. coli* is constant with or without the presence of the liposome without the antibiotics (27). On the other hand, other results indicated that the antibacterial activity of both cefepime and ceftazidime loaded on the manufactured liposome was effective in the inhibition of *P. aeruginosa*, and therefore, considered a treatment for the infections caused by them. Moreover, they can overcome the resistance of bacteria that increased significantly with the wrong use of antibiotics (28).

Authors' Contribution

Study concept and design: S. M.
Acquisition of data: S. M.
Analysis and interpretation of data: S. M.
Drafting of the manuscript: S. M.
Critical revision of the manuscript for important intellectual content: S. M.

Statistical analysis: S. M.
Administrative, technical, and material support: S. M.

Ethics

All procedures were approved by the ethics committee of the University of Kufa, Faculty of Science, Najaf, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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