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Effect of *Proteus* Lipopolysaccharide (LPS) on virulence factor of *Proteus mirabilis*

Abeer.I.Gazaa[1] , Najeeb.M.Hussen[2]
[1,2] Department of Biology, College of Science, University of Al-Anbar, Al-Anbar, Iraq.
abyr3873@gmail.c om , Dr.najeb@uoanbar.edu.iq

Abstract: Two hundred samples were collected from different samples (urine, ear and wounds swaps), from AL-Ramadi Teaching Hospital, Ramadi Maternity & Children Teaching Hospital. The results of cultural, microscopic, biochemical tests indicated that in urine samples *K. pneumonia* have high occurrence frequency 35 (29%) followed by Proteus mirabilis26(21.7%) and *Pseudomonas aeruginosa* 20 (16.7%),while in wounds samples *Pseudomonas aeruginosa*10(25%) followed by *Klebsiella pneumonia5* (12.5%) and *Proteus mirabilis* 3 (7.5%) , while Ear swaps samples revealed that *P.aeruginosa* 12(30%) was the major bacterium followed by *P.mirabilis* 4(10%)and *Klebsiella pneumonia* 2(5%) . Sensitivity test against twelve antimicrobial agents was done for all of the *Proteus mirabilis* isolates (33 isolates).The results displayed that most of the isolates were resistant to Nitrofurantion (100%) ,Cefepime 70% followed by each Trimethoprim-Sulfamethoxazole(60.6%) and Ceftriaxone(60.6%) while the most effective antimicrobial agent against *P.mirabilis* were Amoxicillin+Clavulanic acid (96.97%), Ofloxacin (78.79%), Levofloxacin (78.78 %) Ciprofloxacin (69.7%),

Amikacin (66.7%), Imipenem (66.67%), Gentamicin (54.5%) and Naldixic acid (54.55%). More resistant isolate was selected, and lipopolysaccharide was extracted by hot Phenol method this method was tedious, but yielded LPS of high purity and the yield was (93) mg LPS from (5)g dry weight cell of *P.mirabilis* After partial purification, chemical analysis of crud and partial purified LPS showed that the amount of carbohydrate and protein in crude and partial purified LPS extract were (69.375)mg ml^{-1}, and (7.107)mg ml^{-1}) respectively followed by, while the Carbohydrate in dialysis (85975 mg ml^{-1}) (1.21mg ml^{-1}) and in partial purification of LPS (93.011mg ml^{-1}) while protein 0.144mg ml^{-1}.

Keywords: Hot phenol method, lipopolysaccharide, *Proteus mirabilis,* Growth conditions of *Proteus mirabilis* ,Antimicrobial sensitivity, Sephadex G-200

1. INTRODUCTION

The bacterium *Proteus mirabilis* is Gram-negative, motile, flagellate, non-spore- forming, non-capsular, coccobacilli-forming, and has fimbriae. Both animals and humans can find it in soil, water, and their guts (1). Urinary tract infections are a pathological condition caused by this bacterium. It depends on how strong the biofilms are, how many virulence factors are present, and how resistant the bacteria are to different types of antibiotics (2). Such factors affect how severe the urinary tract infections are. *P. mirabilis* also has urease, hemolysin, protease, and lipopolysaccharides (LPS) that make it more dangerous (3). It also expresses flagella, toxins, fimbriae, adhesins, enzymes, and immune cell resistance (4). The primary component of gram-negative bacterial outer membrane is LPS. It increases the likelihood of the germs infecting humans and animals (5). The LPS from P. mirabilis is defined by three elements: 'Lipid A is an endotoxic glycolipid, while OS is an oligosaccharide that binds to both O-PS and lipid A (6). It has a number of advantageous effects, such as tumor necrosis factor production, adjuvant, and radioprotection, while producing a number of pathologic symptoms, such as fever, septic shock, and death (7). Despite being a non-toxic chemical on its own, LPS damages cells by stimulating immune-competent cells and activating neutrophils, monocytes, and macrophages. People think that these cells are primarily responsible for the development of gram-negative septicemia because they produce biologically active molecules such as prostaglandins, pro-inflammatory cytokines such as interleukin 1, interleukin 6, and tumor necrosis factor, and free radical mediators such

as superoxide, hydrogen peroxide, hydroxyl radicals, and nitric oxide (2,8).The purpose of the current work was to assess the functionality and purity of the LPS that was extracted from P. mirabilis using hot phenol and investigate its impact on a few virulence factors.

2. MATERIALS AND METHODS

2.1 Sample Collection

200 specimens have been collected from Al-Ramadi Teaching Hospital patients. Specimen have been collected from urine, wounds and ear swabs. The specimens have been streaked right away on MacConkey and blood agars and then incubated for 24 hours at 37C.

2.2 Identification of the Isolates

We found isolates using morphological and biochemical assays and we also used VITEK 2compact system (BioMérieux/France) as (8).

2.3 Antimicrobial sensitivity

A number of antibiotics prepared by Bioanalysis Company were used by using the disk diffusion method, where 3-5 colonies were transferred from the agar plate to a sterile tube containing 5 ml of normal saline solution. The tube was shaken well until a homogeneous bacterial suspension was obtained. Then, the turbidity was adjusted by comparing it with the standard McFarland tube, as the turbidity of this tube represents an approximate number of (108×1) cells/ml. Then, a sterile cotton swab was immersed in the bacterial suspension, and the excess suspension was removed by rotating the swab on the inner walls of the tube. Then, the bacterial suspension was spread on the nutrient agar medium, and the plates were left for 3-5 minutes to absorb the suspension until they were completely dry. After that, the antibiotic discs were fixed using sterile forceps. Then, the plates were incubated at 37°C for 24 hours, and the diameter of the inhibition zone for each disc was measured. We assessed the susceptibility of isolated

P. mirabilis using twelve antibacterial discs. Included among these medicines is Amoxicillin Combined with Clavulanic acid, Gentamicin, Amikacin, Trimethoprim- Sulfamethoxazole, Ceftriaxone, Cefepime, Imipenem, Levofloxacin, Ciprofloxacin, Nitrofurantoin, Nalidixic acid and Ofloxacin.

2.4 Growth conditions of *Proteus mirabilis* :

2.4.1. Effect of inoculum size on growth

This experiment was conducted using 250 ml conical glass flasks containing 100 ml of nutrient broth. These flasks were inoculated with the previously prepared inoculum at the following concentrations: 0.5, 1, 1.5, 2 ml/L for the four selected isolates, respectively. The cultures were incubated in a shaking incubator at a speed of 100 rpm at a temperature of 37°C for 24 hours, after which the absorbance was read at a wavelength of 600 nm to estimate growth (9).

2.4.2. Effect of pH on growth

According to the findings from the prior experiment, the vaccination size that yielded optimal growth for this trial was selected. The experiment used 250 ml conical glass flasks filled with 100 cc of nutritional broth. The flasks were inoculated with the produced inoculum at several pH levels of 5.5, 6.5, 7, 7.5, and 8 for the chosen isolates. The cultures were maintained at 37°C in a shaking incubator operating at 100 rpm for 24 hours. The absorbance was then measured at a

wavelength of 600 nanometers to assess growth (10).

2.4.3. Effect of temperature on growth

According to the findings from the preceding experiment, the vaccination size that yielded optimal development for this experiment was selected at pH 7. The experiment used 250 ml conical glass flasks filled with 100 cc of nutritional broth. The flasks were injected with the pre-prepared vaccine at a concentration of 0.5 ml/L at various temperatures of 25, 30, 37, and 42 degrees Celsius for the chosen isolates. The cultures were cultured in a shaking incubator at 100 rpm for 24 hours, following which absorbance was measured at 600 nm to assess growth (11).

2.4.4. Effect of incubation period on growth

This experiment was conducted under the same conditions as the previously mentioned experiment, where these flasks were inoculated with the previously prepared vaccine at the same concentration and pH 7 for the selected isolates, and the cultures were incubated at 37°C in a shaking incubator at a speed of 100 rpm with a change in the incubation period, as different periods were used, namely 6 hours, 12 hours, 18 hours and 24 hours, after which the absorbance was read at a wavelength of 600 nanometers to estimate growth (12)'.

3. EXTRACTION AND PARTIAL PURIFICATION OF LIPOPOLYSACCHARIDE

3.1. Culturing of bacteria

Inoculate 500 ml of Luria Bertani broth medium with 5 ml of overnight bacterial culture in Luria Bertani broth and incubate for 18 hours at 37° C. The cells were collected using a cooling centrifuge at '3000 rpm for 15 minutes at 4°C.The cell pellet is washed twice with phosphate-buffered saline at pH 7.2 and subjected to cooling centrifugation.

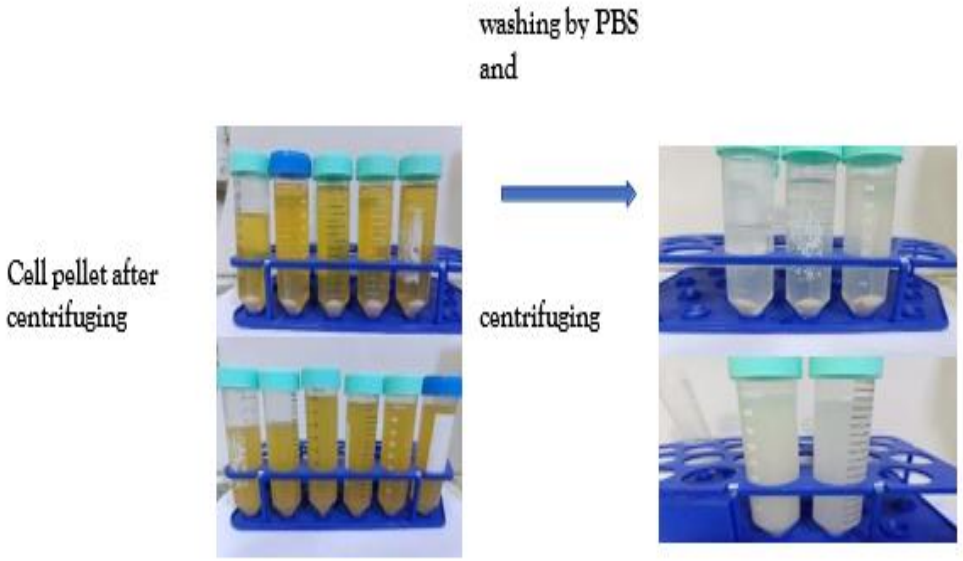
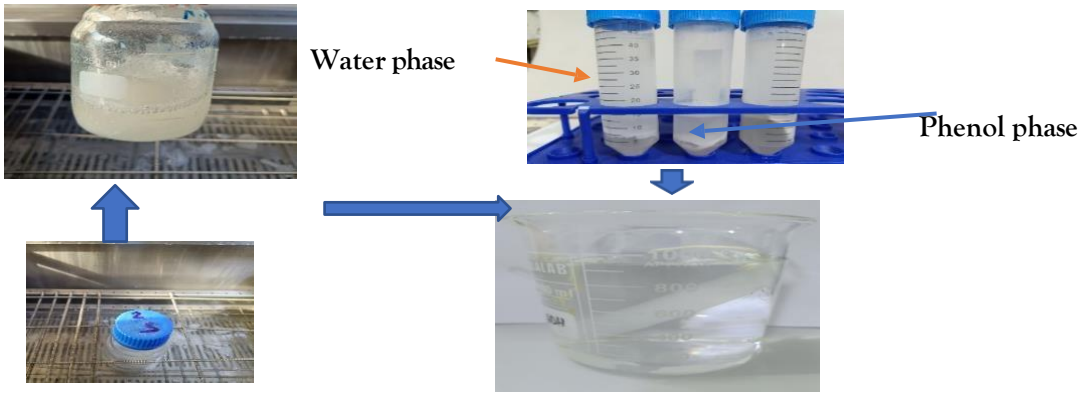


Figure (1)Cell pellet after centrifuging, washing by PBS and centrifuging

3.2. Hot Phenol technique lipopolysaccharide extraction

Resuspend the bacterial pellet in deionized water and place it in a boiling water bath for 30 minutes to lyse the cells. Add an equal volume of 90% phenol (pre-heated to 65°C) to the lysed cells. Shake or stir the mixture at 65°C for 15-20 min. Centrifuge the mixture at 8000 rpm for 30 minutes. Two phases will form: an aqueous phase containing LPS and a phenol phase. Carefully collect the upper aqueous phase containing the LPS and save it. Repeat the extraction with phenol for the lower phase to ensure maximum recovery of LPS Westphal and Jann (1965). Accordingly, Dialyze the aqueous phase against deionized water for 24–48 hours to remove phenol and smaller contaminants (14).



Figure(2) LPS was extracted by hot phenol method

Figure (3) Carbohydrate concentration was determined by using phenol - H2SO4 method

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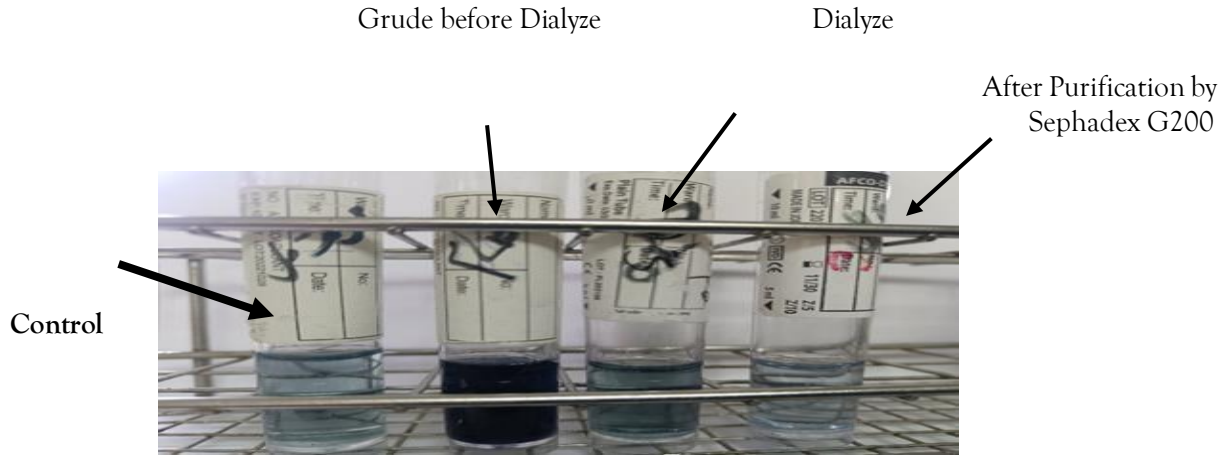


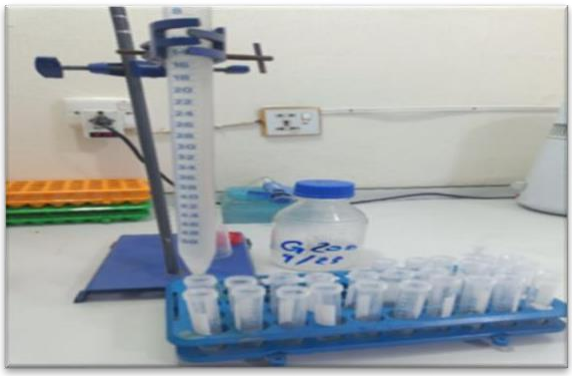
Figure (4) Protein concentration by lowery method

3.3. Partial purification by gel filtration Sephadex G200

In accordance with the recommendations of Pharmacia Fine Chemicals Company, Sephadex G-200 was suspended in phosphate-buffered saline at pH 7.2, degassed, and then packed onto a glass column (2×40 cm) at a flow rate of 1 ml min^{-1} (Figure 4). Elution was accomplished using the identical equilibration buffer, with the LPS sample introduced to the column, followed by washing with PBS buffer. Fractions were collected, and absorbance was measured at 280 nm to detect contaminating proteins, at

490 nm to estimate carbohydrate concentration, and at 600 nm for protein quantification.

Figure (5) Gel Filtration Sephadex G200

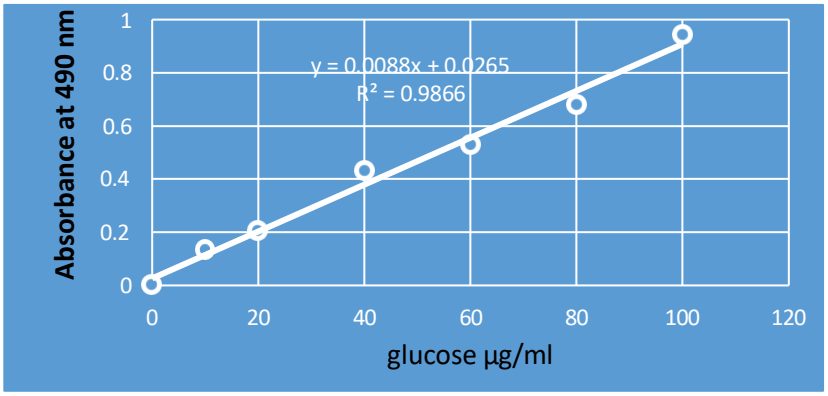


4.3. Determination of Carbohydrate Concentration

One milligram of glucose was dissolved in ten milliliters of distilled water to create the glucose stock solution. Distilled water is used to dissolve a 5% phenol solution. Solution of H2SO4 (98%) The phenol-H2SO4 technique, which was first reported by (14) as follows, was used to assess the content of carbohydrates. 1. A glucose stock solution with a final volume of 1 ml was used to create various concentrations (20, 40, 60, 80, and 100 µg/ml). After that, 1 milliliter of a 5% phenol

solution was mixed into each tube.2. After vigorously mixing in 5 ml of H2SO4, the liquid was allowed to cool to room temperature. The blank was made from 1 milliliter of distilled water, 1 milliliter of phenol solution (5%) and 5 milliliters of H2SO4. The absorbance at 490 nm was measured. 4. The quantities of glucose were plotted against the matching absorbance of the glucose concentrations using a standard curve. 5. Using 1 ml of a 1 mg/ml lipopolysaccharide solution diluted in distilled water, the identical prior addition was made, and the absorbance at 490 nm was measured to quantify the carbohydrate content of the lipopolysaccharide sample (16). The standard curve figure

(6) was used to compute the carbohydrate content.



Figure(6) Standard curve of glucose

3.5. Protein determination

These solutions were produced using the procedure outlined in (16), which included: Solution 1: Na2CO3 at 2%. 500 liters of 0.1M NaoH was used to dissolve 10g of Na2CO3. 2% sodium potassium tartarate is solution (2). To make it, 2 grams of sodium potassium tartarate were dissolved in a tiny amount of distilled water, and the volume was then increased to 100 milliliters. Solution 3: 1 percent CuSO4. One gram of CuSO4 was dissolved in one hundred milliliters of distilled water to create it. Solution 4 was made right away by combining 98 milliliters of solution (1) with one milliliter of solution (2). Bovine serum albumin (BSA) is the fifth solution. It was made by gradually dissolving 0.01g of BSA in 100 ml of distilled water, then adding more distilled water to reach the final amount. Folin-reagent is solution number six. One milliliter of Folin was diluted with two milliliters of distilled water (1:2 v/v) to create the reagent.

Using the solutions previously made in (5), the protein concentration was ascertained using the (16) approach, yielding the following results: The initial concentration of 100µg/ml of bovine serum albumin was used to create the necessary concentration, as shown in Table 1. After adding four milliliters of solution 4 to each tube, they were left for ten minutes. Each tube was filled with 400 microliters of folin reagent (solution 6), agitated well, and then left for half an hour. Tube number one served as a blank when the absorbance was measured at 600 nm.

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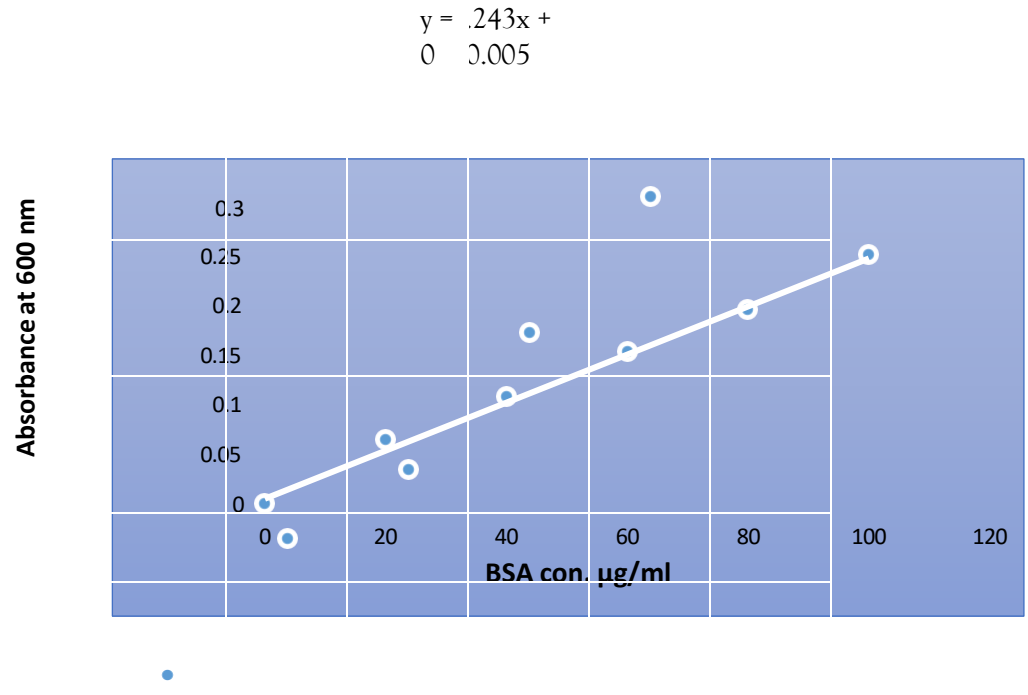
Table 1: Preparation of Bovine serum albumin (BSA) concentration from stock solution of BSA (100µg/ml)

Tube No.	Volume of BSA solution (ml)	Volume of D.W. (ml)	Final volume (ml)	Final concentration (µg/ml)
1	0	1.0	1	0
2	0.1	0.9	1	10
3	0.2	0.8	1	20
4	0.3	0.7	1	30
5	0.4	0.6	1	40
6	0.5	0.5	1	50
7	0.6	0.4	1	60
8	0.7	0.3	1	70
9	0.8	0.2	1	80
10	0.9	0.1	1	90
11	1.0	0	1	100



Figure(7)Standard curve of bovine serum albumin Polyacrylamide Gel Preparation

Table (2): Components of SDS Polyacrylamide gel

Stock solution	Stacking gel	Resolving gel 10%	Reservoir buffer
Acrylamide-Bisacrylamide (30:8)	2.5 ml	10 ml	-
Stacking gel buffer	5 ml	-	-
	-	3.7 ml	-
Resolving gel buffer	-	-	100 ml undiluted
Resolving buffer SDS 1.5% ammonium	1 ml	1.5 ml	-
Persulphate			
Water	11.3 ml	14.45 ml	**900ml**
TEMED	0.015 ml	0.015 ml	-
10% SDS	0.2ml	0.3ml	-

3.6. How to prepare samples

3.6.1. Sample Preparation as (Hames, 1985)

Six volumes of protein and one volume of 0.002% Bromophenol Blue (BPB) were combined to create a protein sample. Every stage of the native protein sample preparation process is carried out at 4°C. A concentrated stock solution was used to bring the protein sample for SDS-polyacrelamid to 2% (w/v) SDS, 5% (v/v) 2- mercaptoethanol, 10% (v/v) glycerol, and 0.002% (w/v) bromophenol blue after it had been replaced in 0.0625 M Tris-HCl (pH 6.8). For three minutes, the SDS-PAGE samples were heated in a boiling water bath. This guarantees that, upon heating, the protein will get denaturated. After allowing the sample to cool to ambient temperature (25°C), it was centrifuged for five minutes at 10,000 rpm.(16)

3.6.2. Sample Loading and Electrophoresis as (Hames, 1985)

Below is a summary of the liquid sample loading procedure for both native PAGE and SDS-PAGE. The electrophoresis equipment was used to install the gels, and reservoir buffer was added up to the top line. Applying a microsyringe or micropipette to the gel surface, the sample was carefully loaded. The power pack was attached to the electrophoresis device, and the gel was exposed to 100 volts for two hours (16).

3.6.3. Gel Staining

100 mg of silver nitrate was dissolved in 50 ml of a combination of water, methanol, and glacial acetic acid (5:5:2 by volume) while being stirred. Distilled water was added to bring the volume up to 1 L, and the liquid was filtered through Whatman No. 1 filter paper before being kept in a dark container at 4°C until it was needed.according to Tsai (1982) (15).

3.6.4. Method of Staining

The Chang et al. approach was followed in order to stain the protein in the native gel. By submerging the gel in a silver nitrate solution, protein bands were created in SDS- PAGE. Three hours later, the stain solution was rinsed with 5% acetic acid to halt the process (18).

3.7.1 Inhibition biofilm of proteus by LPS A 96-well microtiter plate test based on the crystal violet staining technique was used to assess the LPS extract's capacity to prevent the development of biofilms in isolates of Proteus sp. In short, 20 µL of suspended isolate of 0.5-0.7 McFarland (1.108 cfu/ml) was added to each 96-well flat-bottomed sterile polystyrene microplate well that contained 200 µL of Mueller-Hinton broth supplemented with 93, 46.5, 23.25, and

11.6 mg/ml for all isolates except positive control LPS extract. Microplates are incubated at 37°C for four hours. After discarding the liquid medium, the adhering cells underwent two rounds of washing in phosphate buffered saline (PBS), and the wells were dried for no more than an hour at 60°C. It was then stained for 15 minutes using 150 µL of 2% crystal violet. The crystal violet stain was then released from the well microplates by giving them two PBS washes. Following the microplate's air drying procedure, 150 µL of 95% ethanol was used to re-solubilize the dye of the biofilms that lined the microplate walls. A microplate reader measures the microplate spectrophotometrically at 490 nm after 5–1010 minutes. Fresh samples were used for the experiment at least three times.as Kerkeni and colleagues (19)'

3.7.2 Inhibition Hemolysin of proteus by LPS

Serial dilutions of the bacterial suspension in 1 ml of sodium phosphate buffer. Then add 80 µl of 5% RBC and incubate at 37°C for 3 h. Then the results read as follows After incubation, results are assessed based on hemolysis. Hemolysis is typically detected by observing color changes (clear red supernatant indicates complete hemolysis, while an intact pellet of RBCs suggests no hemolysis) The degree of hemolysis can be quantified using spectrophotometry at 540 nm (measuring released hemoglobin)(20).

4. RESULT AND DISCUSSION

Its illustrated from table that most given bacteria were highest in urine. Where, *K. pneumonia* was highest in urine samples of 35. Whereas, it was lowest in ear swaps of 2 count only. *P. mirabilis* achieved 26 counts and *P aeruginosa* of 20 counts. While *P. aeruginosa* gave highest count in ear swaps of 12 counts, followed by *P. mirabilis* of 4 counts. Contrarily, *K. pneumonia* had the lowest count of 2.

Table (2) detection of four bacteria in samples

Source	Samples no	P mirabilis	P aeruginosa	K pneumonia	Others
Urine	120	26 (21.7%)	20 (16.7%)	35 (29%)	39(32.5%)
Wounds	40	3 (7.5%)	10 (25%)	5 (12.5%)	22(55%)
Ear swaps	40	4 (10%)	12 (30%)	2 (5%)	22(55%)
Total	200	33(16.5%)	42 (21%)	42(21%)	83(41.5%)

The other 83 some isolate didn't grow on MacConkey or blood agar due to their anaerobic growth condition, or other causative agents or mix growth as Zuhir & Sattar (21).

4.1. Prevalence of Proteus mirabilis according to the source samples

According to the results of biochemical tests and the Vitek @2compact system, the proportion of Proteus in all samples and isolates was ‘33 (16.5%). The prevalence percentage of P. mirabilis in urine was 21.7%, which is greater than the rate in other samples. The rate for wounds was 7.5%, whereas the percentage for ear swaps was 10% (8).

4.2. Antimicrobial sensitivity of *Proteus mirabilis*

All 33 *P. mirabilis* isolates were 100% resistant to nitrofurantoin. Cefepime (70%), trimethoprim-sulfamethoxazole (60.6%), and ceftriaxone (60.6%) all showed high resistance. The most effective antibiotics were Amoxicillin + Clavulanic Acid (96.97% susceptibility) and Ofloxacin (78.79%) . Sensitivity test against twelve antimicrobial agents was done for all of the Proteus mirabilis isolates (33 isolates).The results displayed that most of the isolates were resistant to Nitrofurantion (100%) ,Cefepime 70% followed by each Trimethoprim-Sulfamethoxazole(60.6%) and Ceftriaxone(60.6%) while the most effective antimicrobial agent against P.mirabilis were Amoxicillin+Clavulanic acid (96.97%), Ofloxacin (78.79%), Levofloxacin (78.78

%) Ciprofloxacin (69.7%), Amikacin (66.7%), Imipenem (66.67%), Gentamicin (54.5%) and Naldixic acid (54.55%).

Depending on the study's prior findings and how they compare to those of other investigations, resistance to these antimicrobial drugs developed. This might be because of mutations that caused these isolates to become resistant and because antimicrobial agents were used at random (22).

4.3. Growth conditions of Proteus mirabilis

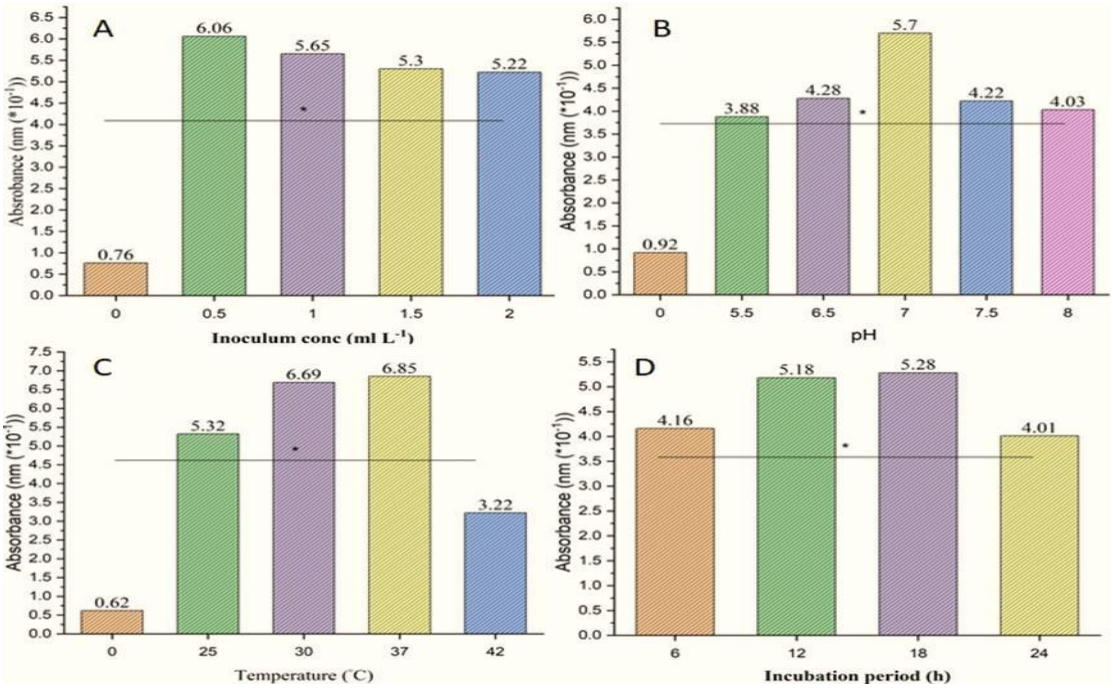


Figure (7)**Absorbance Measurements at Different Concentrations of bacterial inoculum (A), pH (B), Temperature (C) and incubation time Using a Spectrophotometer at 600 nm**

The present study had revealed significant findings regarding the optimal conditions for the growth of *Proteus mirabilis*. It had been determined that a vaccine concentration of 0.5 ml/L had resulted in the highest bacterial growth, indicating its effectiveness in enhancing proliferation under laboratory conditions. Furthermore, the study had shown that the optimal pH level for bacterial growth had been7,aligning with the natural physiological conditions favorable for this species. The temperature that had supported maximum growth had been 37°C, which corresponds to human body temperature and reflects the organism's pathogenic potential. Additionally, the highest growth rate had been observed after an incubation period of 18 hours, suggesting that this time frame is critical for achieving peak bacterial activity.These findings provide valuable insights into the environmental factors influencing *P.mirabilis* growth,which could be important for both clinical and research applications(11,12)

4.4. Partical purification of lipopolsacharide

The crude LPS used in this investigation was partly purified using Sephadex G200 gel filtration, which is very effective at separating complex sugars and high molecular weight proteins [27, 28].At a wavelength of 490 nm, 25 fractions were collected and evaluated for chosin-isolated bacteria by measuring the quantity of carbohydrates in accordance with (15). At 280 nm, the quantity of protein-linked LPS was determined. The absorbance and transmitted fraction were then compared in a blotted figure.The findings revealed a big peak of carbohydrates and two peaks that included protein components connected to lipopolysaccharide that were difficult to separate from the peak of carbohydrates. The elimination of some contaminants may be the cause of the purified sample's increased carbohydrate content (24). The proportion of carbohydrates after endotoxin purification has been shown to vary greatly, and these discrepancies in computations are ascribed to the kinds of bacteria that the LPS was extracted from as well as the extraction and purification procedures (24).However, the protein data showed that the isolates of P. mirabilis had a proportion of 0.1%. This outcome was consistent with the research (25), which found that the protein percentage ranged from 0.1% to 1.4%. The detrimental effects of circulating endotoxins may be considerably reduced by endotoxin binding proteins (26)The table indicates that partial purification of LPS (93.011 mg ml-1) and dialysis (85.976 mg ml-1) had a significant impact on carbohydrates. However, crude LPS produced the least amount of carbohydrates (69.375 mg ml-1). Using 5 g of dry cell weight of P. mirabilis, the hot phenol technique produced 93 mg of LPS. Compared to the crude LPS (69.375 mg/ml) table, the carbohydrate content increased during partial purification (93.011 mg/ml). Following purification, the protein level decreased (0.144 mg/ml in purified LPS vs. 7.107 mg/ml in crude extract).

Table (3) Amount of carbohydrates and protein in the crude and partial purified LPS extract

Lipopolysaccharide	Carbohydrate (mg ml^{-1})	Proteins (mg ml^{-1})
Crude LPS	69.375	7.107
Dialysis	85.975	1.21
Partial purification of LPS	**93.011**	**0.144**

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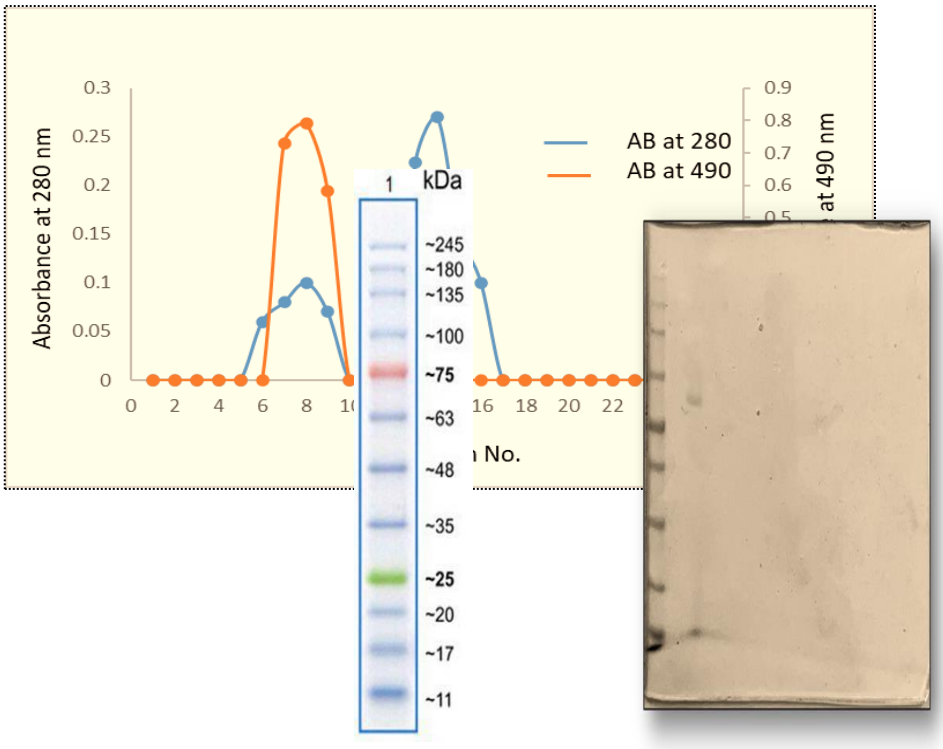

Figure(8)Gel filtration chromatography of *Proteus mirabilis* lipopolysaccharide by using Gel filtration Sephadex G200the column dimensions was (2 ×40 cm) and the elution was done with phosphate buffer saline pH 7.2 at flow rate 1 ml/1 min.5ml for each fraction.

Figure (9) Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) characterization of lipopolysaccharide(LPS) Determination of Lipopolysaccaride Molecular Weight

Lipopolysaccharide extracted by hot phenol method revealed typical ladder pattern on silver staining confirmed the purity as no protein contamination was observed between ~48 - ~63 kDa AND lps appear ~57.

The calculated molecular weight of LPS was equal to around 57 kDalton for the bacterial core oligosaccharide (24). This outcome almost with In the research, the estimated molecular weight of LPS was 63095 Dalton for the conventional bacterium and 70794 Dalton for the pathogenic one [30]. LPS's structure determines its size and molecular weight; for example, its molecular weight is determined by its core oligosaccharide. Additionally, there are two kinds of oligosaccharides: long and short, and no oligosaccharide (27). In addition to variations in the oligosaccharide repeating unit, all LPS preparations are heterogeneous because of variations in the replacement of heptose and KDO in the core unit (28).

Additionally, LPS suppressed hemolysis, as demonstrated by reduced hemoglobin release (OD_{540} nm) in erythrocyte lysis assays (Table 5).

Table (5) effect of LPS on hemolysin in proteus source isolated from urine, wounds and ear

Conc. of LPS (mg ml^{-1})	urine mean ± SD	wounds mean ± SD	Ear mean ± SD	P. value 0.00001
Control positive	1.32 ± 0.06	1.45 ±0.38	1.93± 0.02	
93	0.43± 0.12	0.47± 0.12	0.50± 0.10	
46.5	0.71 ±0.08	0.62 ±0.06	0.65± 0.10	
23.25	0.95± 0.06	0.88 ±0.01	0.88 ±0.01	
11.6	0.93± 0.06	0.97 ±0.05	0.96± 0.06	

Table(6) effect of LPS on biofilm in proteus isolated from urine, wounds and ear

Conc of LPS (mg ml^{-1})	urine mean ± SD	wounds mean ± SD	Ear mean ± SD	P. value 000001
Control positive	1.22 ± 0.01	1.44 ± 0.02	1.77 ± 0.04	
93	0.20 ± 0.10	0.21 ± 0.10	0.13 ± 0.05	
46.5	0.53 ± 0.05	0.46 ± 0.05	0.47 ± 0.06	
23.25	0.59 ± 0.07	0.61 ± 0.06	0.65 ± 0.07	
11.6	0.79 ± 0.07	0.78 ± 0.06	0.84 ± 0.06	

Polysaccharides, particularly extracellular polymeric substances (EPS) produced by Proteus mirabilis, play a pivotal role in bacterial pathogenesis by regulating adhesion, immune evasion, and biofilm formation. EPS can inhibit biofilm formation by altering the surface hydrophobicity and electrostatic properties of the bacterial cell envelope, interfering with fimbrial-mediated adhesion to biotic and abiotic surfaces. In addition, the dense polysaccharide matrix can obstruct the diffusion of quorum sensing autoinducers, impairing coordinated expression of biofilm-associated genes. This inhibition extends to hemolysin activity, where EPS may block hemolysin (HpmA) secretion or its access to host cell membranes via steric hindrance or modulation of gene regulation (30,31).

Furthermore, polysaccharides influence the expression of virulence-associated genes such as ureC, zapA, and hpmA, potentially through environmental signal modulation or direct interference with regulatory networks. The suppression of these virulence factors is often associated with persistent, chronic infection phenotypes, where attenuated virulence favors long-term colonization while evading host immune responses (32)

The intracellular second messenger cyclic di-GMP (c-di-GMP), a master regulator of the

transition between planktonic and sessile lifestyles, is primarily responsible for mediating these effects at the molecular level. Increased c-di-GMP levels inhibit the expression of motility and virulence genes while promoting the synthesis of exopolysaccharides and the development of biofilms. Intracellular c-di-GMP levels are controlled by enzymes including diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) in response to external stimuli like pH, osmolarity, and nutritional signals. By modifying the activity of sensor kinases in two-component systems such as RcsCDB and BarA/UvrY, which modify downstream transcriptional regulators connected to virulence and biofilm formation, polysaccharides may also have an indirect effect (33, 34)'.

Collectively, the inhibitory role of polysaccharides on biofilm formation, hemolysin activity, and virulence gene expression in P. mirabilis represents a complex regulatory mechanism that facilitates bacterial adaptation and persistence within the host. Understanding these interactions offers promising therapeutic avenues for anti- virulence drug development (35).

CONCLUSION

This study emphasizes the therapeutic significance of *Proteus mirabilis* in urinary tract, wound, and ear infections, especially given its high antibiotic resistance. The lipopolysaccharide (LPS) extracted using the hot phenol technique showed strong inhibitory effects on major virulence factors such as biofilm formation and hemolysin production. These data imply that LPS may influence bacterial pathogenicity by inhibiting adhesion and toxin-mediated host harm. The resistance profile emphasizes the need for alternate therapeutic approaches, such as targeting LPS-associated virulence pathways (36). Future study should look into the molecular pathways by which LPS affects bacterial behavior and evaluate its potential as a therapeutic or diagnostic target. Furthermore, enhancing LPS extraction techniques may improve yield and purity for broader biomedical applications.

Key Implications

- **LPS is** an important factor in *P. mirabilis* virulence and may be a target for anti- virulence therapy.
- **Antibiotic stewardship is** critical given the widespread resistance to frequently used medicines such as Nitrofurantoin.

Additional research is required to determine the structural and functional features of P. mirabilis LPS in infectious models.

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