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Extraction and Partial Purification of Lipopolysaccharide from Clinical *Proteus mirabilis* Isolate and Compared with Standard Bacteria

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Abstract

Ninety five samples were collected from different samples (urine, ear and wounds swaps), from hospitals in Baghdad city. The results of cultural, microscopic , biochemical tests indicated that in urine samples *E.coli* have high occurrence frequency 19 (47.5%) followed by *Proteus mirabilis* 18(45%) and *Klebsiella* species 1 (2.5%), while in wounds samples each of *Pseudomonas* spp. and *Proteus mirabilis* 10 (25%) , then followed by *E.coli* with 5 (12.5%) and *Klebsiella* species 3 (7.5%). Ear swaps samples revealed that *Pseudomonas aeruginosa* 7 (46%) was the major bacterium followed by *Proteus mirabilis* 4(26.6).Sensitivity test against eleven antimicrobial agents was done for all of the *Proteus mirabilis* isolates (32 isolates). The results displayed that most of the isolates were resistant to Methicillin (96.8%), and Rifampin (93.7%) followed by trimethoprim–sulfamethoxazole (71.8%), chloramphenicol (62.5%), and cefazoline (59.3%). while the most effective antimicrobial agents against *P. mirabilis* were Imipenem (96.9%), Azetronam (81.3%), Azithromycin (71.9%) ,Ciprofloxacin (69%).Whereas a moderate effect appeared against both gentamycin and tobramycin in a percentage of (53.2 and 62.5) % respectively. More resistant isolate was selected, and lipopolysaccharide was extracted by hot EDTA method and the yield was (150) mg LPS from (22)g dry weight cell of pathogenic *P.mirabilis* and (95) mg as LPS from (16) g dry weight cell of standard bacteria were obtained. After partial purification ,chemical analysis of crud and partial purified LPS showed that the carbohydrate percentages were (35 , 44.3) % and (49 , 62)%, while the protein percentage (0.98 ,0.1) % and (1.3 ,0.1)% for the standard and isolated bacteria respectively ,whereas both extract appeared free from nucleic acid . Molecular weight of LPS was estimated and it was equivalent to (63095 and 70794) Dalton for the standard bacteria and pathogenic one respectively.

Keywords: Bacterial endotoxin, lipopolysaccharide, *Proteus mirabilis*

الاستخلاص والتنقية الجزئية لمتعدد السكريد الشحمي للعزلة السريرية *Proteus mirabilis* ومقارنتها مع البكتريا القياسية

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الخلاصة

جمعت 95 عينة تضمنت عينات مختلفة (ادرار ومسحات من الاذن والجروح) ومن مستشفيات مختلفة في محافظة بغداد .اظهرت نتائج اختبارات عملية الزرع ، الفحص المجهري ، والاختبارات البايوكيمائية ان عزلة بكتريا *E.coli* في عينات الادرار سجلت النسبة الاعلى (47.5%) 19 وتبعتها عزلة *Proteus mirabilis* 18(45%) ، ثم جنس الـ *Klebsiella* (2.5%) 1 ، بينما في عينات الجروح سجل كل من عزلات جنس بكتريا *Pseudomonas*، و *Proteus mirabilis* (25%) 10، ثم تبعتها بكتريا *E.coli* (12.5%) 5 ،

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وجنس الـ *Klebsiella*. (7.5%) 3 . وأظهرت عينات مسحات الأذن ان بكتريا *Pseudomonas aeruginosa* (46%) 7 كانت الرئيسة و تبعتها *Proteus mirabilis* (26.6)4 . أجري أختبار الحساسية الدوائية لأحد عشر مضاد مايكروبي على عزلات *Proteus mirabilis* عددها (32 عزلة) ،وا ظهرت نتائج الفحص ان معظم هذه العزلات كانت مقاومة لـ Methicillin (96.8%) ، و Rifampin (93.7%) ، وتبعها trimethoprim–sulfamethoxazole (71.8%)، chloramphenicol (62.5%) ، وcefazoline (59.3%) . بينما المضادات المايكروبية التي اظهرت فعالية ضد *Proteus mirabilis* كانت Imipenem (96.9%)، Azetronam (81.3%) ، Azithromycin (71.9%)، و (69%). في حين أظهر كل من gentamycin و tobramycin تاثيرا معتدلا وبنسبة (53.2 و 62.5) على التوالي. وعلية أختيرت العزلة الأكثر مقاومة للمضادات المايكروبية، وتم استخلاص متعدد السكريد الشحمي منها ومن بكتريا *Proteus mirabilis* القياسية بطريقة EDTA الساخن ، وكان ناتج عملية الاستخلاص (95.150) ملغم كمتعدد السكريد الشحمي من (16.22) غم من الخلايا البكتيرية الجافة لكل من *Proteus mirabilis* القياسية والمرضية على التوالي .ثم اجريت عملية التنقية الجزئية لمتعدد السكريد الشحمي، واجري التحليل الكيمائي لكل من المستخلص الخام والمنقى جزئيا، واظهرت النتائج ان نسبة الكاربوهيدرات في النموذج الخام كانت (35 ، 49) لكل من العزلتين المرضيه والقياسيه على التوالي %, بينما نتائج الكاربوهيدرات للعزلتين المنقاه جزئيا (44.3 ، 62)%. نسبة البروتينات الخام كانت (0.98% للعزله القياسيه و 1.3 % للعزله المرضيه, اما نتائج التنقيه الجزئيه كانت (0.1)% لكل من البكتريا القياسية والمرضية ، في حين اظهر كلا المستخلصين خلوه من الاحماض النووية. ثم قدر الوزن الجزيئي لمتعدد السكريد الشحمي وكان (63095 و 70794) دالتون لكل من البكتريا القياسية والمرضية على التوالي .

Introduction

Proteus mirabilis is gram-negative, dimorphic, motile bacteria belong to enterobacteriaceae family [1]. These species are found in soil, water, and intestinal tract of many mammals, such as humans [2]. *P. mirabilis* is an opportunistic pathogen to human urinary tract. It is capable to pass-through capillaries causing more systemic infections [3], and it is consider as one of the most etiological agent associated with UTI infection in particular individual with structural abnormalities of urinary tract [4]. Its expresses several virulence factor involved in uropathogenesis like adhesions, flagella, toxins (Hemolysin), quorum-sensing, enzymes (urease) and immune invasion [5].

Lipopolysaccharide (LPS) is the main component of the external membrane of gram-negative bacteria and it's an important virulence factor for both humans and animals [6]. The LPS of *P. mirabilis* has three domains: O-polysaccharide chain or O-antigen (O-PS); lipid A an endotoxic glycolipid; and a core oligosaccharide (OS) domain, which bind lipid A and O-PS [7]. It may cause several pathophysiological symptoms such as fever, septic shock, and death, but it also has many beneficial activities, such as tumor necrosis factor production, adjuvant, and radioprotection effects [8]. LPS is non- toxic molecule by itself, but exerts the toxic effect through activation of the immune competent cells(monocytes, neutrophils and macrophages) these cells are believed to play a central role in the pathophysiology of gram-negative septicemia *via* the production of biologically active molecule such as: prostaglandins ;free radicals mediators (superoxide, hydrogen peroxide, hydroxyl radical, nitric oxide) and pro-inflammatory cytokines (interleukin 1, interleukin 6, tumor necrosis factor) [9-11].

The aim of this study is to isolate lipopolysaccharide from P.mirabilis and compared it characteristics with standard P. mirabilis bacteria.

Materials and Methods

Sample Collection

Total of 95 samples were collected from patients attending (Baghdad teaching hospital, Al-Yarmook, Al-Kindy and Central Child) hospitals. Samples were taken from wounds and ear swabs, and urine. The specimens were directly streaked onto MacConkey and blood agars (Himedia) and were incubated at 37ºC for 24 hours.

Identification of the Isolates

Isolates were identified depending on morphological and biochemical tests [12], and API 20E (Bio-merieux/France) and chromoagar as confirmatory tests.

Antimicrobial sensitivity test

Eleven antimicrobial disks were used to detect the sensitivity of isolated *P. mirabilis*. These antimicrobials include (Methicillin, Gentamycin, Tobramycin, Cefazolin, Imipenem, Rifampicin, trimethoprin/ sulphamethazol, Ciprofloxacin, Azithromycin, Chloramphenicol and Azitronam). As described by [13].

Extraction and Partial Purification of Lipopolysaccharide from isolated and standard *Proteus mirabilis* bacteria

1-Culturing of bacterial isolates

The selected bacterial isolate was cultured on nutrient agar medium (Himedia), incubated overnight at 37◦C under aerobic condition .Isolate growth was harvested by phosphate buffer saline pH 7.2 (Kallested/U.S.A) using spreader. Then the plates washed twice with the same buffer . Precipitation of cells was achieved by centrifugation with cooling centrifuge at 3000 rpm/min for15 min at 4°C. The cells pellet re-suspended in PBS buffer and centrifuged again (repeated twice) .Cells drying were accomplished by cooled acetone in a ratio of 1:10(v/v).

2- Extraction of lipopolysaccharide by Hot EDTA method (Chandan and Fraser, 1994)

The dried cells were suspended in PBS with a ratio of 1:1(w/v), 0.1 ml from EDTA solution (0.5) M was added to each 10ml of cell suspension, then autoclaved for 10 minutes. The mixture was left to cool at room temperature. DNase and RNase enzymes (Sigma) were added at a final concentration of 1µg/ml and incubated at 37◦C for 10 minutes, then proteinase K (Promega) added at a final concentration of 1µg/ml, incubated in water bath at 56◦C for 10 min, the temperature was raised to 60◦C for 10 min. The mixture was left to cool at room temperature.Cooling centrifuge was used at 10000rpm/min for 15 min to separate the mixture into two phases the upper aqueous phase which supposed to contain the LPS and the sediment phase in the bottom. Finally the upper phase (supernatant) was collected and dialyzed against D.W for 4 days.

Partial purification by gel filtration Sepharose CL-6B

1-Column preparation

One hundred milliliters of Sepharose CL-6B gel (Pharmacia). was washed with phosphate buffer saline pH 7.2, degassed under vacuum, subsequently the suspension was poured into a glass column (2.5× 80cm) and allowed the matrix to settle down .The gel was equilibrated with PBS pH 7.2 with flow rate 48ml/h (4ml/5min). LPS sample added to the column, washed with PBS buffer, the fractions were collected, and measured the absorbance at 280 nm for detecting of contaminating proteins within fractions [14], at 490 nm to estimate the carbohydrate concentration [15], and measuring the absorbance at 260 nm for detecting the nucleic acids [16]. Proteins were measured according to [17] at a wavelength of 595nm. Whereas the molecular weight of LPS was determined according to standard proteins (Lysozyme 14.4 KD, Trypsin 24 KD, Casein 31 KD, Lipase 50 KD and Bovine Serum Albumin 67 KD) which was added to column and the ratio of Ve/V_o was determined to the standard proteins, and they were used as molecular weight markers. Blue dextran was also used for the determination of the column void volume (v_o), and (Ve/V_o) was measured for both pathogenic and standard bacteria. The logarithm of the molecular weight of each standard protein were plotted to obtain standard curve.

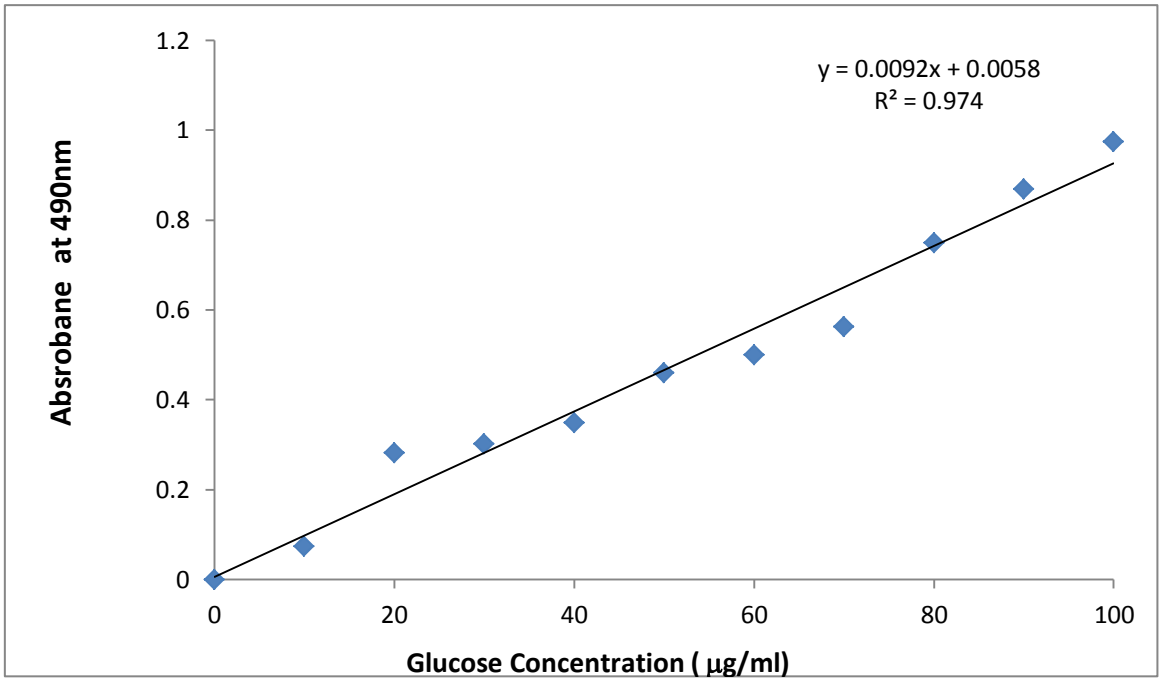

Figure 1- Standard curve of glucose

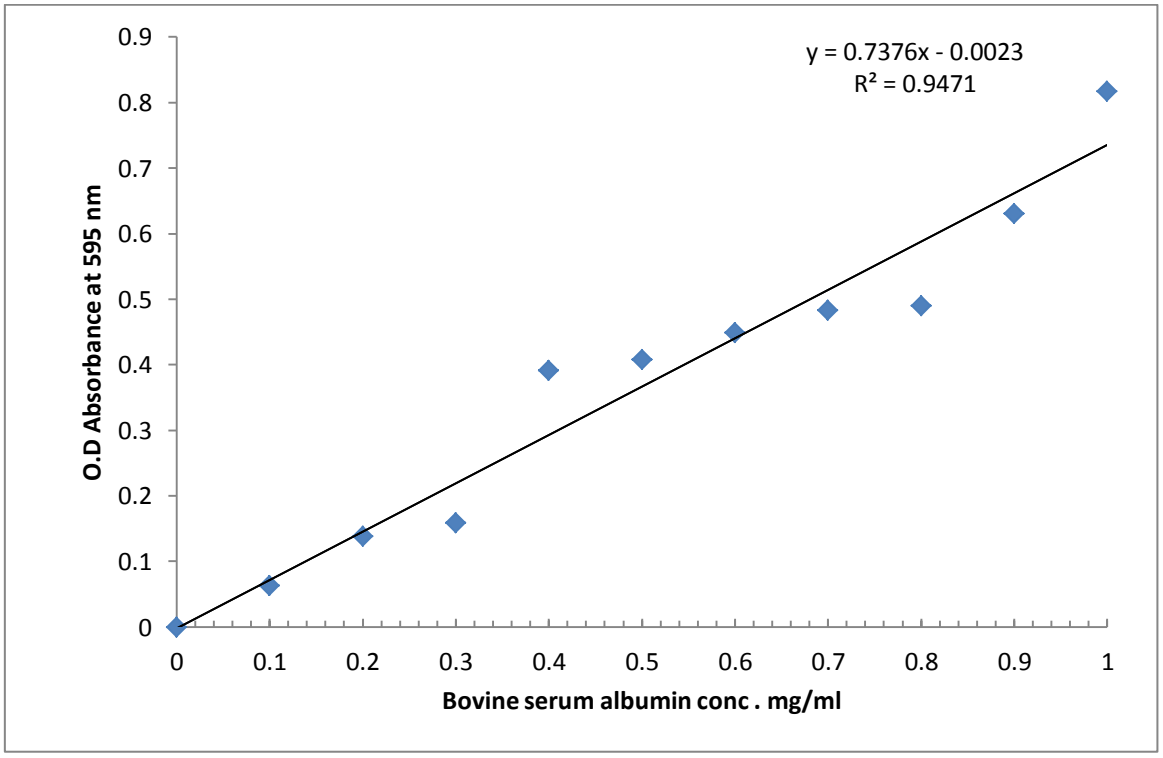

Figure 2- Standard curve of bovine serum albumin

Result and Discussion

Prevalence of different isolates percentage according to the source sample

The prevalence of bacterial isolates in different infectious specimen showed that there was variation in causative agents. In urine the major bacterium was *E.coli* with high occurrence frequency 19/40(47.5%) followed by *P. mirabilis* in a percentage of 18(45%), *Klebsiella* species 1/40(2.5%).While in wound isolates *Pseudomonas* spp. 10/40(25%) and *P. mirabilis*10/40 (25%) were the major isolates followed by *E.coli* with5/40(12.5%) and finally *Klebsiella* species 3/40(7.5%). On the other hand the ear swabs isolates indicated high level of *P. aeruginosa* 7/15(46%) which is similar to the result produced by Orji and Dike, (2015)[18] they indicated that the most common isolated bacteria from ear swab was *P. aeruginosa* ,followed by *P. mirabilis* Table-1.

Table 1- Number and percentage of bacteria isolated from different clinical sources

Source of sample	No. of sample	No. (%) *P. mirabilis*	No. (%) *P. Vulgaris*	No. (%) *E.coli*	No. (%) *Klebsiella* species	No. (%) *P. Aeruginosa*	*Other agent*
Urine	40	18(45)	2(5)	19(47.5)	1(2.5)	----	----
Wound	40	10(25)	2(5)	5(25)	3(7.5)	10(25)	10(25)
Ear swab	15	4(26.6)	----	----	----	7(46.6)	4(26.6)
Total	95	32(33.6)	4(4.2)	24(25.2)	4(4.2)	17(17.8)	14(14.7)

The other 14 isolate didn't grow on MacConkey or blood agar due to their anaerobic growth condition, or other causative agents.

Prevalence of *Proteus mirabilis* according to the source sample

The result of biochemical tests, chromo agar and APi 20E test indicated that *Proteus mirabilis* percentage was 32(33.6%) among the total samples and isolates. *P. mirabilis* prevalence percentage in urine was 45% which is the higher percentage in comparable with other samples. While the percentage in ear swap was 26.6%, whereas in wounds (30%) was indicated as Proteus species, and *P.mirabilis* was (25%) followed by *P.vulgaris* (5%).

Antimicrobial sensitivity of *Proteus mirabilis*

Thirty two selected isolates were tested for antimicrobial sensitivity against eleven antimicrobial agents. Depending on NCCLs (2007) guideline, most isolates were resistant to Methicillin (96.8%), and Rifampin (93.7%). As well the results showed an elevation in *Proteus mirabilis* resistant to chloramphenicol up to (62.5%), whereas moderate effect appeared for each of Tobramycin, Gentamycin and Cefazolin, the isolates showed sensitivity at a percentage (62.5, 53.2 and 40.7) % respectively. The most effective drugs against *P. mirabilis* were Imipenem, Azetronam, Azithromycin and Ciprofloxacin with a sensitivity percentage (96.9, 81.3, 71.9, and 68.8) % respectively.

Depending on the previous result of this study and its comparable with other studies, development of the resistance against these antimicrobial agent appeared, which may be due to mutation occurrence made these isolates became resistant and randomly use of antimicrobial agents [19].

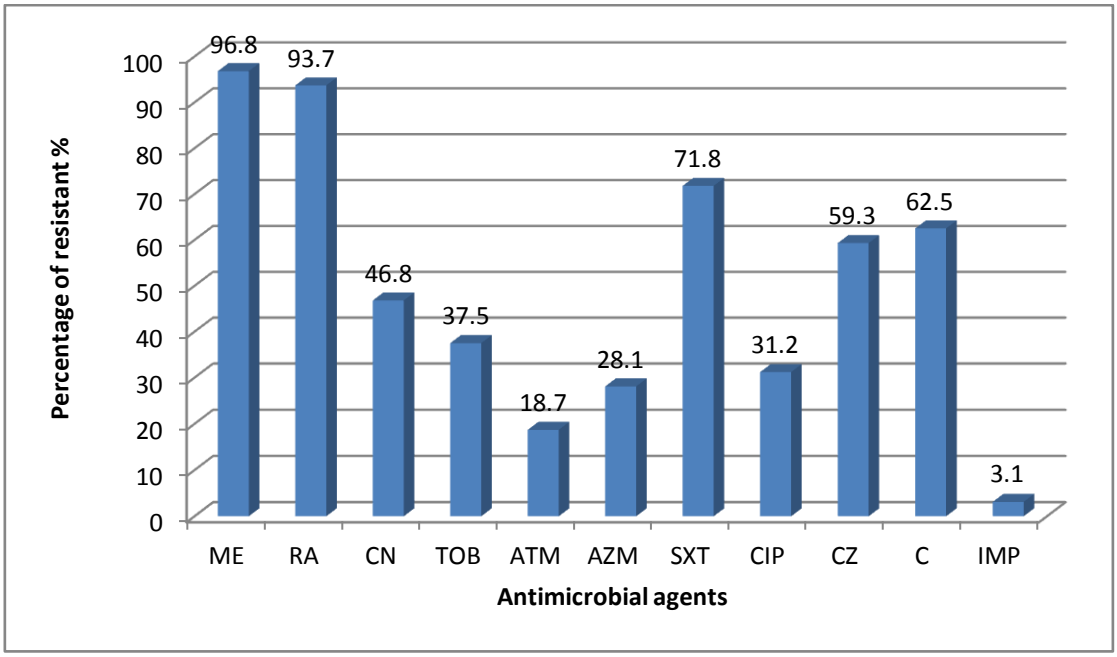

Figure 3- Susceptibility of *P.mirabilis* to antimicrobial agents

Aztreonam ATM (30mg), Trimethoprim/sulfamethoxazole SXT (25mg), Methicillin ME (10mg), Imepinem IMP (10mg), Tobramycin TOB (10mg), Rifampcin RA (5mg), Cefazolin CZ (30mg), Gentamycin CN (10mg), Chloramphenicol C (10mg), Azithromycin AZM (15mg), Ciprofloxacin CIP (5mg).

Extraction of lipopolysaccharide

According to Chandan and his coworker [20] method the extraction of LPS was done. The yield of obtained bacterial growth was (22) gm and (16) gm as dry weight of pathogenic and standard *P.mirabilis*. LPS was extracted by hot EDTA method, this procedure is suitable for extraction with low amount of contaminated proteins [27].Aliquot of 150mg and 95 mg of LPS were obtained from 22 g and 16 g of dry cells for pathogenic isolate and standard bacteria respectively, this result agreed with Gerhardt *et al*., 1981 [21] which reported that the LPS yield range between 100-500mg from 20g dry weight cells.

The percentage of protein and carbohydrate in crude extract were determined (1.3% and 49%), (0.98% and 35%) for pathogenic isolates and standard isolate respectively. These percentages were determined by measuring the concentration of carbohydrates and proteins in (1 ml) and calculate the concentration in (100ml). The elevation of carbohydrate content in the isolated bacteria may be related to the pathogenicity of *P.mirabilis* in compared with the standard one, as [22] reported about the amount of sugar composition of *P.mirabilis* LPS may either enhance or inhibit the crystallization of struvite and apatite, depending on its chemical structure and ability to bind cations in addition to the biofilm formation ability. These points increased importance of endotoxin in urinary tract infections and formation of stones. As well as the efficiency of the extraction method where the extract was treated with three enzymes DNase, RNase and Proteinase K. In addition to the ability of EDTA act as chelating agent which have a significance role in the fixation of LPS in the membrane. Thus this extraction method consider as both extraction and purification method [23- 25].

Furthermore, the nucleic acids that contaminate the extract were measured according to the method [16], also depending on the standard curve of deoxyribose and ribose sugar. The result appeared that there were no nucleic acids due to the effect of nuclease enzymes on the bacterial nucleic acids , and this results confirmed that results reported by [26].

Partial purification of lipopolysaccharide

In the present study, the crude LPS was partially purified by gel filtration chromatography using Sepharose CL-6B gel which is highly efficient in the separation of high molecular weight protein and complex sugar [27, 28].

Sixty fractions was collected and assessed for both pathogenic isolated and standard bacteria by measuring the carbohydrate amount according to (15) at a wavelength of 490nm. Whereas the amount

of protein linked LPS was measured at 280nm, subsequently the relationship between the absorbance and transmitted fraction was blotted Figure-4, -5.The result showed two peaks of carbohydrate large and small one, and both of them contain protein component linked to lipopolysaccharide which was difficult to separate.

The result revealed that in the pathogenic *P. mirabilis* the first peak contained the active LPS more than the second peak. While the standard bacteria the second peak contained the active LPS more than the first one. The increase in the carbohydrate amount in the purified sample could be due to the removal of some impurities [26]. It was indicated that the carbohydrates percentage following endotoxin purification may vary widely, and these differences in calculations are attributed to the types of bacterial species in which the LPS extracted from and extraction and purification process [29].

On the other hand the protein results indicated a percentage of 0.1 % for both the pathogenic and standard *P.mirabilis* isolates. This result agreed with the study [30], who reported the protein percentage between 0.1- 1.4 %. The endotoxin binding proteins can significantly minimize the harmful action of circulating endotoxin [29]. The nucleic acids content in the partial purified LPS results indicated 0 % percentage due to the efficiency of the nuclease enzymes that were used in the removal of nucleic acid Table-2, Figure-4,-5.

Table 2- Amount of carbohydrates, protein and nucleic acids in the crude and partial purified LPS extract for the standard and pathogenic *Proteus mirabilis*

Lipopolysaccharide	Carbohydrate (%)	Proteins (%)	Nucleic acids (%)
Crude for standard LPS	35	0.98	0
Partial purified for standard LPS	44.3	0.1	0
Crude LPS for pathogenic	49	1.3	0
Partial purified of LPS for pathogenic	62	0.1	0

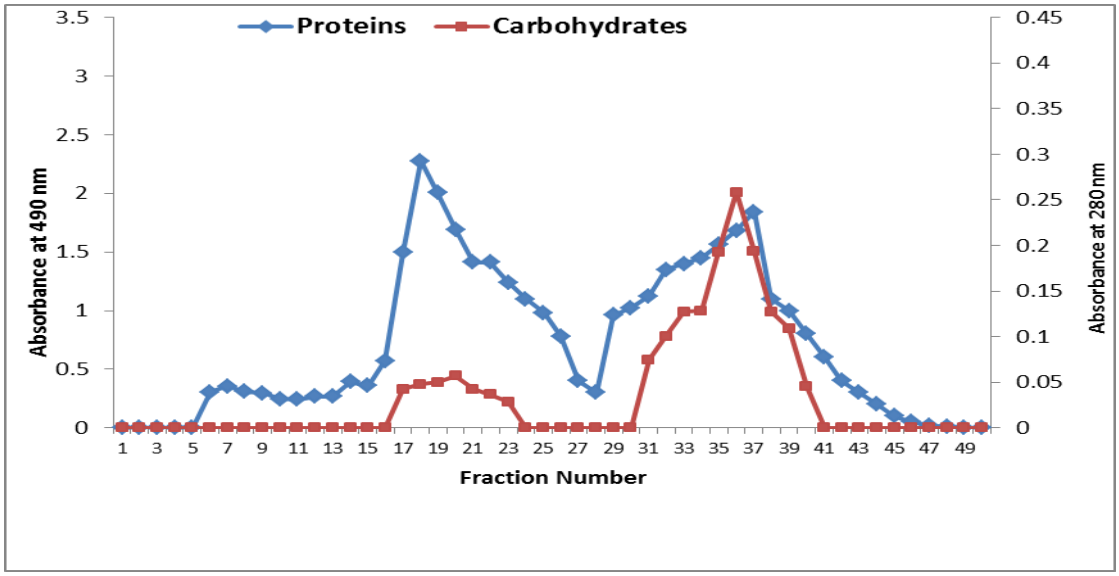

Figure 4- Gel filtration chromatography of *Proteus mirabilis* (standard) lipopolysaccharide by using sepharose Cl-6B, the column dimensions was (2.5 ×80 cm) and the elution was done with phosphate buffer saline pH 7.2 at flow rate 48 ml/h, 4ml for each fraction.

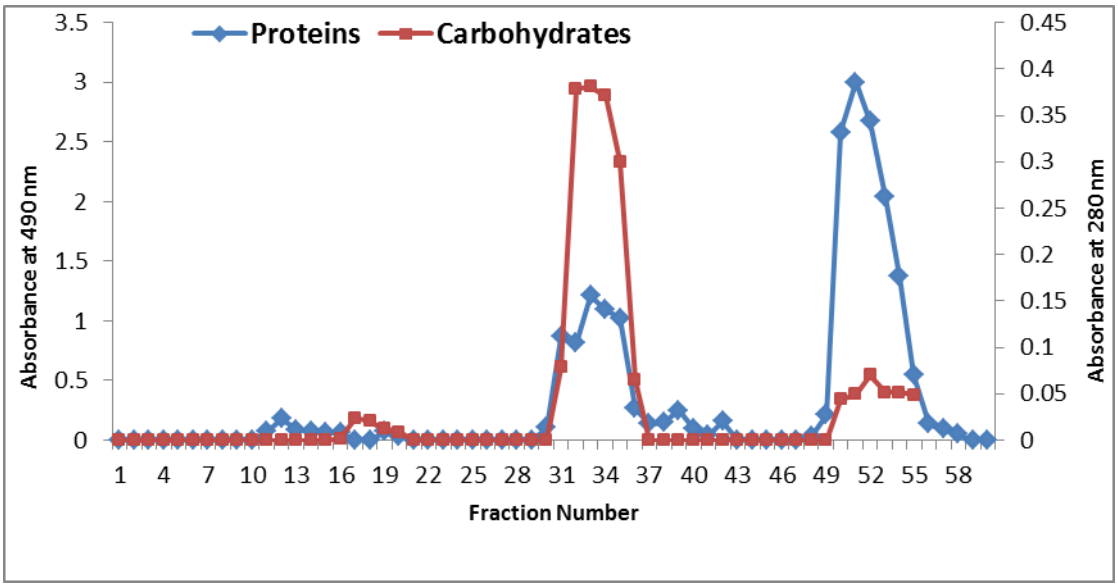

Figure 5-Gel filtration chromatography of *Proteus mirabilis* (pathogenic) lipopolysaccharide by using sepharose Cl-6B, the column dimensions was (2.5 ×80 cm) and the elution was done with phosphate buffer saline pH 7.2 at flow rate 48 ml/h, 4ml for each fraction.

Determination of lipopolysaccharide molecular weight

Lipopolysaccharide molecular weight was determined by gel filtration chromatography by Sepharose CL-6B .The void volume was determined by using Blue dextran 2000, which was equal to 78 ml, as well the elution volumes (Ve) to each standard proteins were measured .Ve for LPS was equivalent to (134) ml for pathogenic isolate and (140ml) for the standard bacteria.

Molecular weight of LPS was estimated and it was equivalent to (63095 Dalton) for the standard bacteria and (70794 Dalton) for the pathogenic one. The size and molecular weight of LPS depend on it structure, such as the molecular weight of LPS is depended on core oligosaccharide, in addition there are two types of oligosaccharide either (long or short) and no oligosaccharide [30]. All the LPS preparations are heterogeneous due to the variation in the substitution of heptose and KDO in the core unit, in addition to the differences in the oligosaccharide repeating unit [31].

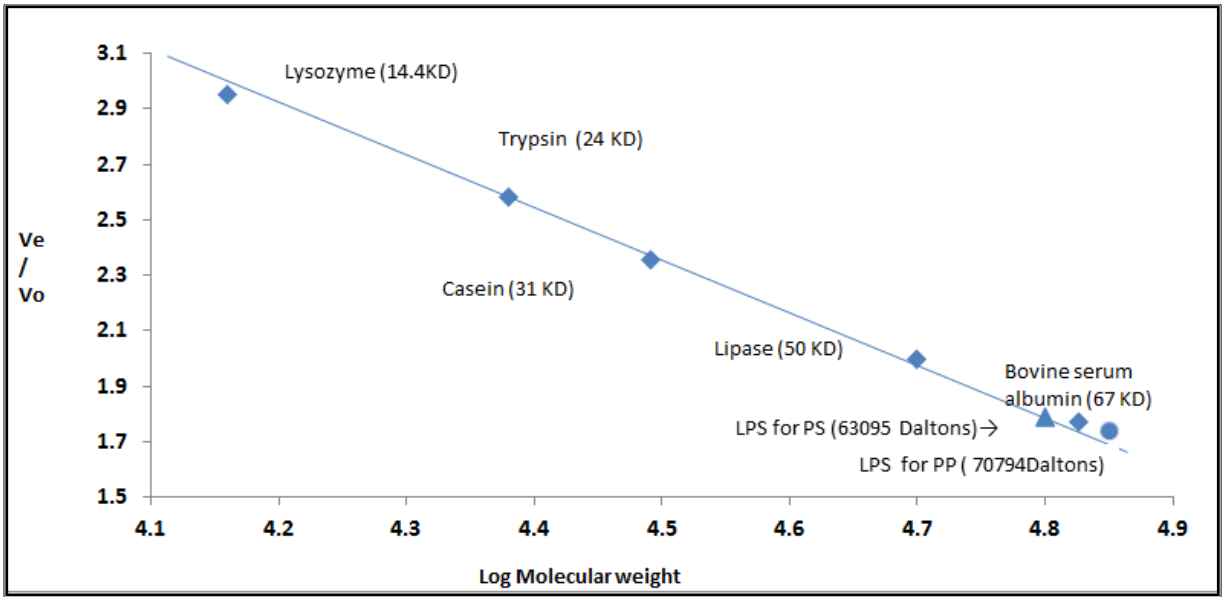

Figure 6- Molecular weight of lipopolysaccharide by using gel filtration chromatography (sepharose Cl-6B), the column dimensions was (2.5 ×80 cm) and the elution was done with phosphate buffer saline pH 7.2 at flow rate 48 ml/h, 4ml for each fraction.

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