Study the inhibitory effect of purified lipopolysaccharide extracted from *Proteus mirabilis* on *E.coli* O157 H7 isolate from diarrheal infected animals

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Received 30 July 2016 ; Accepted 26 September 2016

Abstract

Current study included four parts: the first part is the isolation and identification 4 isolates of *Proteus mirabilis* from 45 different samples include 20 samples of urine, 15 samples of feces , 10 samples from ear infection. These isolates were identified by morphological examination and biochemical tests and API20 E system .The second part was the isolation and identification 1 isolates of *E. coli* O157 H7 from 33 diarrheal infected animals, these isolates were identified by morphological examination , growing on selective media such as(sorbitol MacConkey agar and CHROMagar) ,biochemical tests and by latex agglutination test. In the third part of this study, lipopolysaccharide (LPS) was extracted from *Proteus mirabilis* by using hot phenol method ,and after that crude extracted LPS purified by gel filtration chromatography using Sepharose CL-6B gel . And the yield was (180) mg LPS from 26 g from dry weight cell of *P. mirabilis*. chemical analysis of LPS in 1 ml of crud and partial purified LPS showed that the carbohydrate percentages were (3.23, 5.92) % respectively ,while protein percentage were (0.53, 0.38) % respectively. The fourth part of this study ,determination antibacterial activity of LPS extracted from *Proteus mirabilis* in different concentration (700,800,900,1000,1100,1200) µg /ml against *E.coli* O157 H7, the mean of inhibitory zone diameter were (27.33 , 30 , 31.33 , 32 , 34 , 37) respectively .The present study provides evidence antibacterial effect of LPS extracted from *Proteus mirabilis* on the *E.coli* O157 H7 isolated from diarrheal infected animals. This study was conducted in University of Diyala /

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Aims of study : Study the effect of purified Lipopolysaccharide of *Proteus mirabilis* as an antimicrobial agent On pathogenic *Escherichia coli* isolated from diarrhea infection *in vitro*.

Key words : *Proteus mirabilis* , *E.coli* O157 H7, lipopolysaccharide

دراسة التأثير التثبيطي لمتعدد السكريد الشحمي على بكتريا الاشريشية القولونية الممرضة المعزولة من حالات الاسهال في الحيوانات

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الخلاصة

تضمنت الدراسة الحالية أربعة محاور :المحور الأول عزل وتشخيص اربعة عزلات من بكتريا *Proteus mirabilis* تم الحصول عليها من 45 عينة مختلفة تتضمن 20 عينة من الادرار، 15 عينة من البراز، 10 عينات من اصابات الاذن. وهذه العزلات شخصت بالاعتماد على الفحص الشكلي والفحوصات البيوكيماوية وعلى نظام الابي20 .اما المحور الثاني من الدراسة تضمن عزل وتشخيص عزلة واحدة من بكتريا *E. coli* O157:H7من (ثلاثة وثلاثون)عينة من العجول المصابة بالإسهال. أما المحور الثالث من الدراسة فتضمن استخلاص عديد السكريد الشحمي من بكتريا *Proteus mirabilis* بطريقة الفينول الساخن ثم نقى جزئيا بوساطة كروماتوكرافيا الترشيح الهلامي باستخدام هلام السيفاروس وبلغت نسبة الكربوهيدرات في المستخلص الخام والمنقى (5.92-3.23) على التوالي في حين كانت نسبة البروتين (0.38-0.53) % ،اما المحور الرابع شمل دراسة مدى فعالية عديد السكريد الشحمي كمضاد بكتيري ضد بكتريا *E. coli* O157 H7 وبالتراكيز (700,800,900,1000,1100,1200) ميكروغرام/مل وكانت معدل اقطار التثبيط (27.33,30,31.33,32,34,37)ملم على التوالي. هذه الدراسة اظهرت مدى فعالية مستخلص عديد السكريد الشحمي كمضاد بكتيري ضد بكتريا *E .coli* O157 :H7 المعزولة من العجول المصابة بالإسهال. تم اجراء هذه الدراسة جامعة ديالى /كلية الطب البيطري/مختبر البكتريولوجي و مركز الرازي للبحوث وانتاج العدد الشخصية الطبية من الفترة(2015/11/1 الى 2016/4/1)

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الهدف من الدراسة: دراسة تاثير عديد السكريد الشحمي المستخلص من بكتريا *proteus mirabilis* كمضاد بكتيري ضد بكتريا *E .coli* O157 :H7 المعزولة من العجول المصابة بالإسهال في المختبر .

الكلمات المفتاحية : المتقلبة الرائعة ،الاشريشيا القولونية النزفية ،عديد السكريد الشحمي

Introduction

The genus *Proteus* is a member of the family Enterobacteriaecae ,there are currently four recognized species of *Proteus : P. mirabilis, P. penneri , P. vulgaris, and P. myxofaciens* , from these species *P. mirabilis* is most often isolated from clinical infections, opportunistic infections (33). *Proteus mirabilis* has very simple nutritional requirements and presents in different natural source like soil, water and both of human and animal intestinal tract, often found as free-living organisms , its expresses several virulence factor such as production of urease and hemolysine, movement in waves (swarming) and the production of endotoxin (34). Lipopolysaccharide (LPS) molecules localized on the outer leaflet of the outer membrane constitute the major surface component of the Gram-negative bacterial cell envelope and it is an important virulence factor for both human and animal health (5). LPS is typically composed of three distinct regions: lipid A (endotoxic principle and anchoring molecule in the OM), a core oligosaccharide, and O-antigen Currently ,microbial LPS has received considerable consideration to their therapeutic uses of LPS against infection (36) . More interestingly some LPS could be plausible candidates to be developed into useful drugs for many diseases such as allergic illness, inflammatory bowel disease and demyelinizing pathology of CNS (35). The most important cause of diarrhea is *E. coli* , distribution of *E. coli* in the environment is determined by its presence in the bowel of human and animal, polysaccharide (highly variable O-antigenic polysaccharide consisting of repeating units) ,commonly, the basic structure of the LPS is similar however ,there is extensive variations in their chemical structures depending on bacterial genera, species and strains (36).

Material and method

Samples collection for isolate *Proteus mirabilis* :-

Samples were collected from suspected cases of cow suffering from different infection of both sexes in Baqubah city. The total 45 samples were collected in this study from period (October 2015 to February 2016).Which include 20 urine samples , 15 fecal samples , 10 samples from ear infection. Urine samples put in a sterile test tube and transfer to laboratory in less than two hours for examination (10) and other samples placed in a sterile test tube contain 5 ml of a sterile nutrient broth and put in ice box for bacteriological examination (11).

Isolation and identification of *Proteus mirabilis:*

The primary identification of the suspected isolates was done by biochemical tests and growing on selective media ,as well as by microscopical examination by using Gram stain ,the second step of identification was performed by using API 20 E system.

Sample collection for Isolation *E. coli* O157:H7

Thirty three rectal samples were collected from calves suffering from severe diarrhea, for the period from the of (November 2015 to April 2016) in the province of baquabah city, samples were transferred directly without delay to the laboratory Bacteriological / University of Diyala / College of veterinary medicine, by Aimes transport medium . feces were examined within 2 hours after sampling (12).

Identification of *E. coli* O157 H7:

Different biochemical methods were used for identification of *E.*coli growth while the special growth color in CHROMagar and sorbitol macCaonky agars with cefixime potassium tellurite (SMA-CT) were used for identification of *E.coli* O :157 H7 strain,also identified by latex agglutination test .(13).

Extraction and purification of Lipopolysaccharide from *Proteus mirabilis*:

Preparation of bacterial cells :

The selected bacterial isolate was cultured on brain heart infusion agar medium incubated overnight at 37◦C. Bacterial colonies were harvested. The cells were washed three times with PBS, then precipitated by cooling centrifuge at 3000 rpm/min for 15 min at 4˚C .The cells were dried by cooled acetone at 4˚C with ratio of 1:10 (14). after that 26g of dried cells were obtained. Then destruction of the bacterial cell by enzymes according to(23)

Lipopolysaccharide extraction:-

Extraction of LPS from *Proteus mirabilis* was done by the hot phenol-water method (15),Overheat suspension record in the previous step to the point (70) c^{o} in a water bath for 5 minutes. Added to an equal volume of solution 90% phenol 70ºC in a water bath. The mixture is put on a magnetic stirrer at a constant temperature at 70 ° C for 15 minutes. Put the mixture directly in the ice path for cooling the mixture. Centrifugation the mixture in cool centrifuge at 10000 rpm/min for 30 min, it was observed four separated phases arranged from top to bottom, (Aqueous phase , Interphase, phenolic phase and Sediment).The aqueous phase obtained were dialyzed against distilled water using a dialysis tube for a period of 4 days with the renewal of distilled water every day to the distinctive smell of phenol disappeared.

Partial Purification of LPS by gel filtration chromatography.

The preparation of Sepharose Cl -6B gel was done according to the manufacturer company instructions. PBS with pH 7.2 (0.1)M was used to be washed, vacuum pump was used for degassed. The gel was poured slowly and carefully to prevent air bubbles formation into a column with dimension of (2.5 *80 cm), equilibration of column was performed with PBS PH 7.2 with flow of columns were 5 ml per 5 minutes. The dialyzed LPS were poured slowly on the column , and eluted with PBS .The collection of fractions of 5 ml per tube were performed .The optical density was measured to each collected fraction at 280 nm to estimate the quantity

of total protein in the fractions (16), while at 490 nm to estimate the carbohydrate quantity according to phenol-sulfuric acid method (17).

***In–vitro* Activity of (lipopolysaccharide) extracts as Antibacterial:-**

six concentration from lipopolysaccharide extracts repared(700,800,900,1000,1100,1200 µg /ml) The antibacterial activity of lipopolysaccharide extract were determined by agar diffusion method according to (32). seeded petri plates containing Twenty five ml of sterile Muller – Hinton agar with *E. coli* O157H7 after adjusted final inoculums of *E.coli* O157 H7 to approximately 1.5 **x**10 ^8 (CFU)/ml, by comparison with the 0.5 McFarland standards tube (by swab dipped in broth of *E. coli* O157 H7). Cut wells have (6mm) in diameter in agar by using a sterile Pasteur pipette and removed the agar discs by a sterile forceps, after that filled wells with 0.1ml of each concentration of lipopolysaccharide extracts, filled one of the wells with distilled water as a control . The plates were then incubated in the (upright position) to keep the LPS extract in the wells at 37 o C for 24 hours. Measured inhibition zone diameter formed around each well assessment the antibacterial activity of LPS .

Results

The results of the present study showed obtaining of 4 isolates belong to *Proteus mirabilis* out of total 45 samples included 3 isolates obtained from urine, one isolate from fecal samples. The *Proteus mirabilis* isolates were identified depending upon culture characteristics which appears (swarming phenomena),microscopic examination , biochemical tests and API 20 E system . The result of isolation *E.coli* O157 H7 in the present study shows obtained one isolates out of 33 diarrheal infected calves, The diagnosis based on Culture characteristics on selective media sorbitol macConkey agar plus cefixime potassium tellurite (SMAC-CT), which appeared as colorless due to non-ability to ferment sorbitol culturing on chromo agar which Typical *E. coli* O157:H7colonies appeared as mauve color on Chromo agar while other bacteria appeared as blue colonies (20),biochemical tests and also confirmed by agglutination test.

According to the hot phenol-water method the extraction of LPS was done . Chemical characterizations of the crude endotoxin extracted from *Proteus mirabilis* were performed by estimating the carbohydrate contents according to (17) depending on the standard curve of glucose, and estimating the protein contents according [18] depending on the standard curve of bovine serum albumin. The crude LPS was partially purified by gel filtration chromatography using Sepharose CL-6B gel which is very effective in the separation of great molecular weight protein and complex sugar. Aliquot of 180 mg of partial purified LPS were obtained. The (52) collected fractions were first evaluated for the determination of protein by reading the absorbance of each fraction at (280) nm as suggested by (16). After that, each fraction was processed by a method of phenol-sulphuric acid (17) to determine carbohydrate content, and then the absorbance was read at a wave length of 490 nm. The relationship between absorbency and fraction number of each component (protein and carbohydrate) was drawn Fig (1).

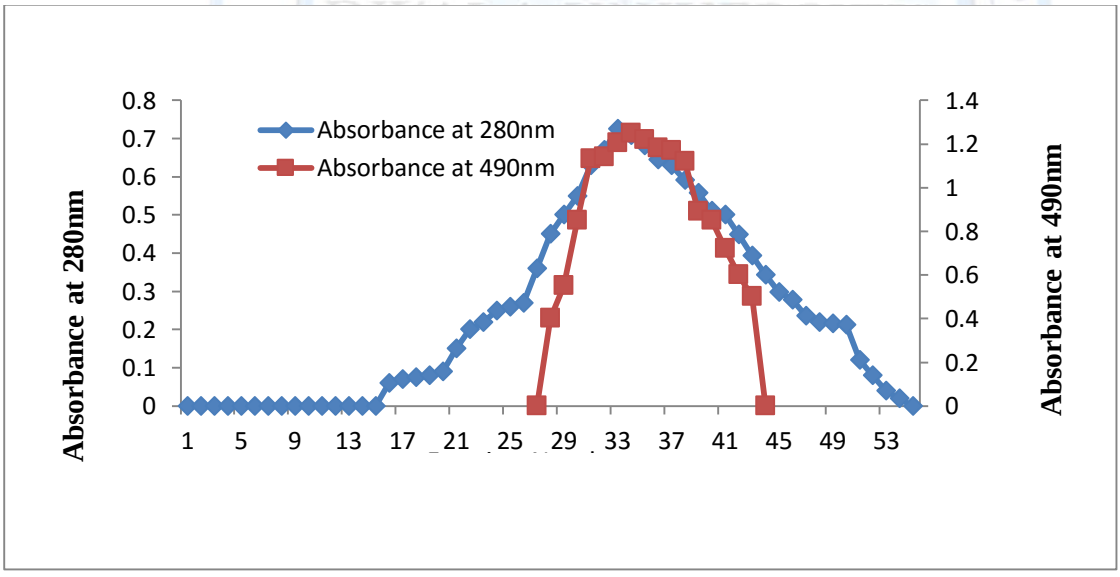

Figure (1) shows relationship between absorbency and fraction number of each component (protein and carbohydrate) .

The figure (1) demonstrates that at 490 nm one peak was observed for carbohydrate. This peak contain protein components linked with LPS and difficult to separation from it as show in figure (1) .The percentage of carbohydrate and protein in crud and partial purified LPS was determined as showed in table (1)

Table (1): Amount of carbohydrates, protein in the crude extract and partial purified LPS

LPS	Carbohydrate %	Protein%
crude	3.23	0.53
purified	5.92	0.38

The antibacterial activity assay of LPS extract of *Proteus mirabilis* were done against *E. coli* O157 H7 isolates. Antibacterial activity of LPS at different concentration (700,800,900,1000,1100,1200) µg /ml were evaluated by measuring the diameters of zones of growth inhibition on bacterial strain and the results are presented as shown in table (2) and figures (2).

Table (2)the inhibition zone diameter for different concentration of lipopolysaccharide extract against *E. coli* O157:H7 by agar well diffusion method.

Concentration Ug/ml	Size of inhibition zone diameter for three replicated (mm)			Means
700	28	27	27	27.33
800	29	30	31	30
900	30	31	33	31.33
1000	32	31	33	32
1100	34	33	35	34
1200	38	36	37	37
Control (D.W)	0	0	0	0

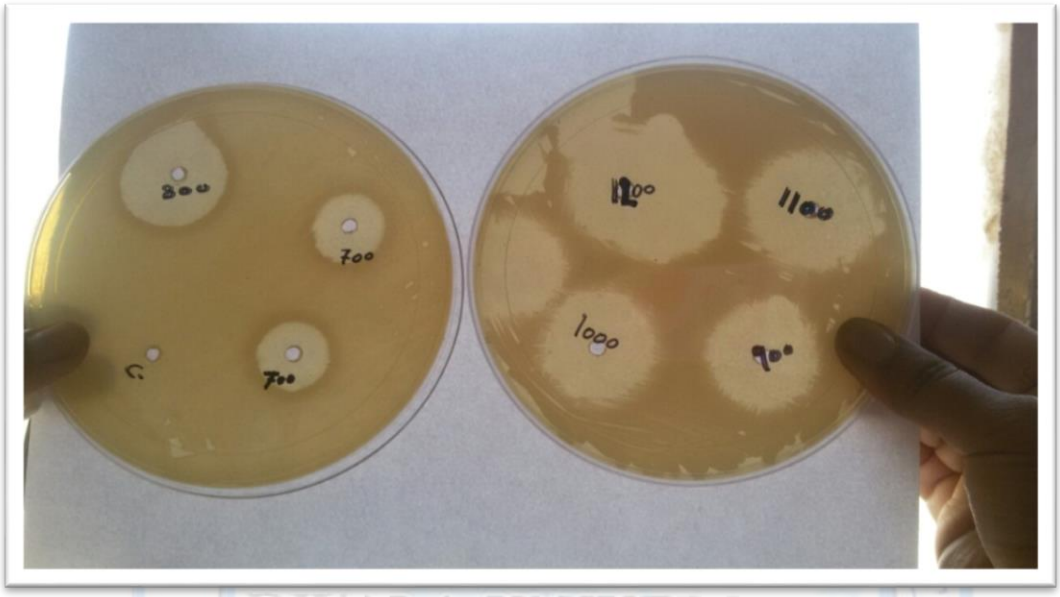

Figure (2): The inhibition zone diameter for different concentration of LPS extract against *E .coli* O157 H7 by agar well diffusion method.

Discussion

Proteus mirabilis rich sources of isolation from human and animal ,these finding well agree with those of (21),who recorded A total of 500 bacterial isolates were collected from different sources of human and animals, From a total of 70 *Proteus* isolates, 62 were identified as *Proteus mirabilis* and also agreement with (27) who was isolate 9 (23.1%) of *proteus mirabilis* from cows` urin samples. From results provided that *E.coli* O157 H7 found in diarrheal infected calves , the same reported by (22) Who that isolate 32 *E. coli* O157:H7 isolates 4 isolated from diarrheic calves and 28from non diarrheic calves and also agree with(28) who found that *E.coli* O157 H7 performed 1.3% of calves' fecal samples . The basic method for LPS extraction found by Westphalia, and still the most frequent procedure employed for LPS

extraction. Digestive enzymes have been used to reduce the level of contaminated proteins and nucleic acid in the extracted LPS .As the Ultra centrifuged alone is not enough to remove contaminants and get LPS Pure and free of contaminants(24) . We note that carbohydrate percentage in partial purified LPS was 3.23% while in crude extract was 5.92%. This evidence of the removing of some cellular components that contaminated LPS extraction and raised purity of LPS [25]. These result agreement with(26) that recorded the percentage of carbohydrate of *proteus* strain between (2.7-6.1)% and differs from other researches (29) were obtained 12-18% carbohydrate from partially purified LPS, The present finding protein percentage in the crude and partially purified LPS of local *P.mirabilis* was (0.53 %and 0.38%) respectively these finding constitute with (26) that recorded the percentage of protein of *proteus* strain between (0.4-1.8)%. while differs from that recorded by (19), who demonstrated that the protein percentage in the partially purified LPS of local *P. aeruginosa* isolate was(2%) The differences in the protein and carbohydrate percentages in the purified LPS may be related to the differences in the bacterial strains and their content of LPS, the differences in the methods used in extraction and purification of LPS and the experiments circumstances The current results revealed that the maximum susceptibility was associated with increasing concentration of LPS extract (mg /ml) ,this Inhibitory effect of these extracts may be due to LPS extract as immune modulators which stimulating the immune system and thus in the killing of these bacteria and limit the spread it . and also contain the effective compounds affect forward growth of bacteria,(30)mentioned that *In vitro* where is LPS extracts of gram negative bacteria that have ability to increase the functional and metabolic efficiency of phagocytosis in macrophages operations and kill germs ,these result agreement with (31) who showed that the lipopolysaccharide investigated had possesses a marked inhibitory effect, at different concentration on growth of the of *Leishmania tropica in-vitro*, that cause cutaneous leishmaniasis lesions.

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