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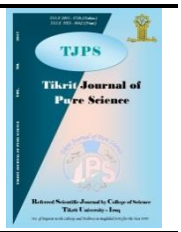

Extraction and purification of klebocin from urine isolate *Klebsiella pneumonia(k42)*

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Abstract

From September 2016 to January 2017 ninety isolates of *Klebsiella spp*. have been collected from clinical sample(urine,sputum, wound swab, burn swab,and ear swab). There are three *Klebsiella spp*. were identified: 53 isolates of *Klebsiella pneumoniae pneumonia*e, 29 isolates of *Klebsiella pneumoniae ozaenae*, and 8 isolates of *Klebsiella oxytoca*. Cup assay method was used to determine capability of *klebsiella spp.* to produce bacteriocin. The results showed (7/36) 19.4% of isolates produced bacteriocin. Isolates number 42 of *Klebsiella pneumoniae pneumonia*e, which selected to extract and purify klebocin. Molecular weight of klebocin was 95 kd determined through gel filtration by using cepharose 6B gel. In this study antimicrobial activity was tested against different pathogenic bacteria, such as *E. coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* in different inhibition zones range from 11mm to 17mm.

Introduction

Bacteriocins are proteinaceous antibiotics produced by bacteria. Bacteriocins that produced by *Klebsiella* is called klebocins or klebicins[1]. Klebocin is secret from *Klebsiella pneumoniae* and appear it have active against various Enterobacteriaceae [2]. Klebocins are toxic for *Klebsiella* species carrying a Klebocinogenic plasmid that bears the genetic determinants for Klebocin synthesis, immunity and release [3]. Klebocins were found also chromosomally encoded. Genetic analysis of Klebocin antibiotic system confirmed the protein content of this system, since it is expressed by specific regulation genes [4]. Klebocin differ from other bacteriocins produced by other bacterial strains which were related to its way of dealing with the target to their lethal action [5]. Klebocins would reach their target and act either by forming a voltage-dependent channel into the inner membrane or by using their endonuclease activity on DNA, rRNA or tRNA [6].

Materials and Methods

Bacterial isolates

Ninety *Klebsiella spp.* isolates were collected from different clinical samples obtained from, urine sample, sputum, wound swab, burn swab, ear swab and pus swab that were collected from Al-Yarmouc Hospital, Al-khadhimia Hospital, Al-kindey Hospital, Baghdad Hospital and Educational laboratories, for the period from September 2016 to January 2017.

Identification tests

Suspected *Klebsiella spp.* isolates were obtained from clinical specimens after culturing on MacConkey agar and incubated for 24 hour at 37˚C. All lactose fermenting and mucoid colonies were selected. Suspected bacterial isolates further identify by colony morphology, Gram stain, and biochemical test. The final identification was performed with analytic profile index for enterobacteriaceae API20E System. *Klebsiella pneumoniae* (k42) was selected from 36 local isolates of *Klebsiella spp.* for klebocin production as a highest klebocin producer.

Induction of *K. pneumoniae* K42 strain for Klebocin production

The induction of the *K. pneumoniae* K42 for producing Klebocin was done as following: preparing 500 ml of Tryptic Soy Broth medium according to the instructions of the manufacturer company (Himedia). *K. pneumonia* K42 strain was inoculated into tryptic soy broth and incubated at 37°C for 18 hours at 50 cycle/min in a shaker incubator [7]. Mitomycin C the

mutagenic agent (Kyowa) (0.25µg/ml) was dissolved in 1ml methanol and added to the broth medium after the incubation period. The broth was homogenized with Mitomycin C in order to ensure the complete diffusion of the Mitomycin C incubated for 90 minutes at 37°C and followed by separation step [8].

Separation of klebocin

After induction of klebocin production the separation step done according to Chhibber and Vadehra [7]. the broth media containing klebocin were separated by cooling centrifuge at 4^{o}C for 10000 rpm, the supernatant considered to be crude klebocin. The next separation step for crude Klebocin was separated by dialysis that having cut off (3 kDa). dialysis were applied for the crude klebocin which was also concentrated crude Klebocin solution from 400 ml to a volume of 30 ml by using sucrose.

Purification of the Klebocin

Ion-exchange Chromatography by DEAE-Cellulose

The DEAE cellulose ion-exchange chromatography column was done according to [9]. DEAE-cellulose was packed in a column with dimensions of 3×15cm. The column was equilibrated by adjusting its pH to approximately 7.5 through suspending in equilibration buffer (0.05M phosphate buffer) overnight Ten milliliters sample that resultant from dialysis step was passed through the DEAE-column with flow rate of 30 ml/hr. and elution was collected as 5 ml fraction. The elution was obtained by gradient NaCl (0-1M). Each fraction was measured absorbance at 280nm by UV-Visual Beam Spectrometer.

Gel-filtration Chromatography by Sepharose 6B

Sepharose 6B gel was prepared by following the instructions of the manufacturer company (Pharmacia Fine Chemicals) according to Chhibber and Vadehra [10]. It was packed in a column with dimensions of 1.5×81cm. The column was equilibrated with 1500 ml 0.05M phosphate buffer overnight. Fractions of 5 ml were collected with flow rate of 30 ml/hr. each fraction was measured absorbance at 280nm by UV-Visual Beam Spectrometer.

Detection of Klebocin antimicrobial activity

According to Baurenfiend and his colleagues [8]. this method were applied to detecting antimicrobial activity for the Klebocin after each step of klebocin purification (Ion exchange and gel filtration chromatography) by inoculating 20µl of klebocin in to the wells previously made with pasture pipette on streaked Muller-Hinton agar medium with indicator strains which included: *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acenitobacter baumanni*.

Results and Discussion

Klebocin was produced from *K. pneumoniae* (K42) in Tryptic Soy broth medium incubation for 18 hours at 37°C at 50cycle/min in shaker incubator in order to obtain suitable aeration and prevent primary metabolites from aggregating ,then Mitomycin C was added as an inducer (mutagenic agent) according to its specific action to induce SOS response in the bacterial cells to express Klebocin [3]. which was dissolved in one milliliter methanol that has high solubility in water according to its chemical structure [11]. in order to ensure the equal spread of the inducer in the broth medium then incubated at 37°C, which is the optimum temperature for bacterial growth for 90 min. which was the suitable time for the highest induction and to prevent proteases effect caused from induction of cell lyses [12].

After incubation period of induction, the Klebocin was released in to the medium and should be extracted depending on biological assay because using chemical assay would affect its activity [10]. Klebocin was separated by centrifugation with cooling centrifuge at 4°C to keep Klebocin from deterioration according to its proteinaceous composition [13].

Klebocin was dialysid before being exposed to purification techniques by using dialysis that having cut of (3000 dalton) that concentrated crude klebocin according to [10].

The Klebocins that had variable net charge properties depending on the induction step and their ionic interaction were also different according to its complex system of three proteins [3]. Anion exchanger DEAE-Cellulose (diethyl amino ethyl cellulose) was used [10]. Figure 1, showed a prominent peak of Klebocin activity and it was in fraction number from 24 to 29 that represented the maximum Klebocin activity.

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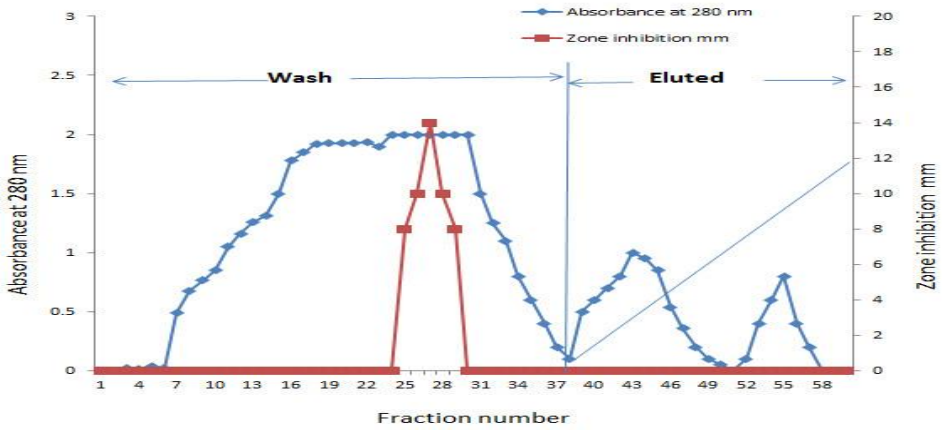


Figure (1): Ion-Exchange Chromatography for Klebocin purification from *K. pneumoniae* (K42) by DEAE-cellulose column (15×3)cm, column equilibrated with 0.05M phosphate buffer pH=7.5

Mitomycin C had induced the over-expression of group A Klebocins that associated with lysis and immunity complex proteins; the lysis protein would cause the death of the bacterial population, while the immunity protein still bounded to the active Klebocin protein and that were successfully extracted according to [14]. that used ion-exchange chromatography to separate immunity protein which bounded to the column; while the active protein appears in the wash step depending on antimicrobial activity. This step resulted in partial purification of Klebocin from its associated proteins that bounded to the gel. Other studies followed the same technique in their purification of Klebocin that it was the suitable assay in order to keep Klebocin's antimicrobial activity and separating the complex system of its associated proteins [10].

The ion exchange chromatography was used by Aggarwal [15]. for the Klebocin extracted as partial purification step from *K. oxytoca*. It was considered as a simple process that could be used for separating and purifying Klebocin extracted from different producing *Klebsiella* species, but its purification also depended on the inducer used for Klebocin production from the bacteria that might add contaminant proteins according to their random action [13].

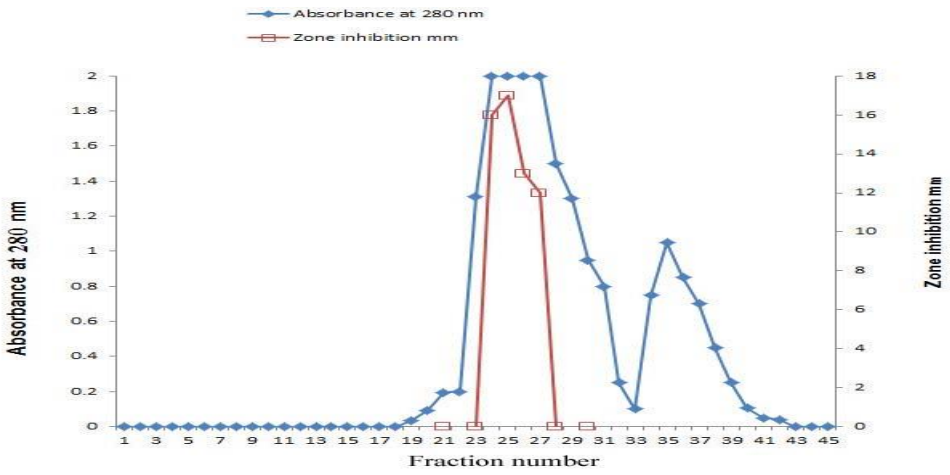
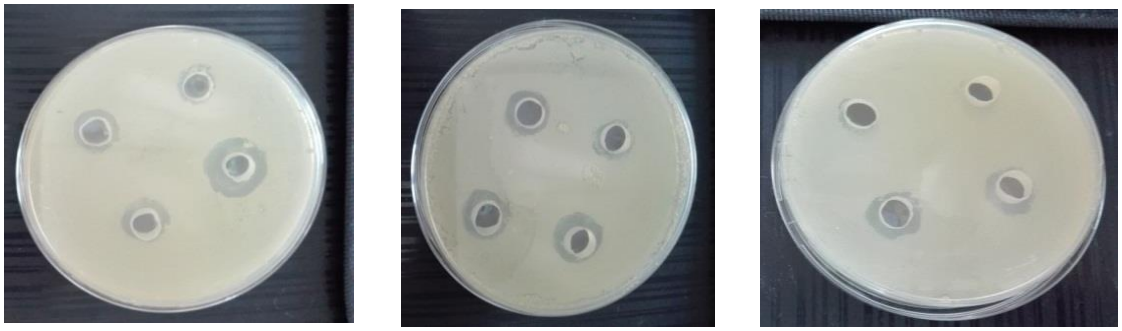


Figure (2): Gel-filtration Chromatography for Klebocin purification from *K. pneumoniae* using Sepharose-6B 81×1.5cm column equilibrated with 0.05M phosphate buffer pH=7.5

According to figure 2, four major peak of Klebocin activities resulted and it was in four active peaks resulted that represented the maximum Klebocin activity. This characteristic resulted in good separation in short well defined times depending on the size of the protein molecules without interfering with the filtration process besides preserving its biological activity [10]. These results were similar to other studies were done by using the same technique [16].

Figure 3(A-B) showed that Klebocin inhibited after two step of extraction (Ion exchange and gel filtration chromatography) the microbial growth of different pathogenic bacteria in different inhibition zones included: *Staphylococcus aureus* ,*Ecoli, Pseudomonas aeruginosa* and *Acenitobacter bawmannii*. The result showed that a highest zone inhibition of klebocin activity after ion exchange chromatography was against *Staphylococcus aureus, E. coli, Pseudomonas aeruginosa* which gave

(14,12,11mm) inhibition zone respectively, while *Acenitobacter bawmannii* not effect by klebocin. Klebocin activity after gel filtration showed that a highest inhibition zone of klebocin activity was against *Staphylococcus aureus, E. coli, Pseudomonas aeruginosa* which gave (17,16, 15mm) inhibition zone respectively, while *Acenitobacter bawmannii* not affected by klebocin. The result of present study same with other study which extracted klebocin by two step (ion exchange and gel filtration) and klebocin activity was against *S. aureus, E.coli, K. pneumoniae* [17].



a-activity of klebocin against *S.aureus* | b-activity of klebocin against *E.coli* | a-activity of klebocin against *P.aeruginosa*

Figure 3- A(a,b,c)klebocin activity after ion exchange chromatography

a-activity of klebocin against *S.aureus* | b-activity of klebocin against *E.coli* | c-activity of klebocin against *P.aeruginosa*

Figure 3 B (a,b,c)klebocin activity after gel filtration

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استخلاص وتنقية الكليبوسين من بكتريا (k42)*klebsiella pneumoniae* المعزولة من الإدرار

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الملخص

خلال الفترة من 2016/9/1 لغاية 2017/1/1 تم جمع 90 عزلة تعود لبكتريا الكليبسيلا من مصادر سريرية مختلفة شملت (الادرار,القشع , مسحات الحروق, مسحات الجروح, مسحات الاذن) توزعت على ثلاث انواع وهي كالتالي:53 عزلة من بكتريا *Klebsiella pneumoniae* و29عزلة *Klebsiella pneumoniae ozaenae* و 8 عزلات *Klebsiella oxytoca* . لتحديد قابلية بكتريا الكليبسيلا على انتاج البكتريوسين استخدمت طريقة اقراص الاكار, اذ اظهرت النتائج أن(7/36) 19.4% من عزلات بكتريا الكليبسيلا منتجة للبكتريوسين. تم اختيار العزلة رقم 42 المعزولة من الادرار كأفضل عزلة منتجة للبكتريويسين ليتم استخدامها فيما بعد في عملية استخلاص وتنقية البكتريوسين. عملية تنقية الكليبوسين تمت باستعمال كروماتوغرافيا التبادل الايوني باستعمال DEAE cellulose كما تمت عملية التنقية المتقدمة من خلال الترشيح الهلامي باستعمال كروماتوغرافيا الترشيح الهلامي. بعد كل مرحلة من مرحلتي التنقية يتم اختبار الفعالية ضد المايكروبية للكليبوسين تجاه عدد من الانواع البكتيرية الممرضة . إذ أظهرت النتائج فعالية الكليبوسين تجاه كل من *Staphylococus aureus* و *E.coli* و *Pseudomonas aeruginosa* في حين لم يظهر الكليبوسين فعالية تجاه بكتريا *Acenitobacter baumannii*.