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Antibiofilm activity of klebocin crude extract against some species of Enterobacteriaceae

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Abstract

Bacteriocins are antibacterial proteins created by bacteria and its effective against other strains of bacteria which are closely linked to the producing strains and a number of species from the same family. The aim of current study was to evaluate the activity of bacteriocin that extracted from clinical isolates of *Klebsiella pneumonia* against different species of enterobacteriaceae in both planktonic and biofilm state. The antibacterial activity of bacteriocins (klebocins) from *Klebsiella pneumoniae* isolates towards diverse pathogenic species from enterobacteriaceae (by well assay method) was demonstrated , however the antibiofilm activity against some species of enterobacteriaceae was studied also by Tissue culture plate method (TCP) in first and last stages of biofilm formation .The results revealed that klebocin with different concentrations was efficient against different pathogenic species by producing different inhibition zones. Results also showed that klebocins had a wide antibiofilm activity on some pathogenic species of Gram-negative bacteria .In addition to these finding it was observed that the antibiofilm activity of klebocin on premature biofilm of *Klebsiella* was higher than its effect towards other pathogenic species, while it was affected on mature biofilm of other bacterial species as well as *Klebsiella*.

Keywords: Klebocins, Biofilm, antibiofilm effect, *Klebsiella pneumoniae*.

نشاط مضاد حيوي لمستخلص كريبوسين الخام ضد بعض أنواع البكتريا المعوية

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الخلاصة

البكتريوسينات هي بروتينات مضادة للجراثيم تخلق بواسطة البكتيريا وفعاليتها ضد سلالات أخرى من البكتيريا التي ترتبط ارتباطًا وثيقًا بالسلالات المنتجة وعددًا من الأنواع من نفس العائلة. ان الهدف من الدراسة الحالية هو تقييم فعالية البكتريوسين المستخلص من العزلات السريرية لبكتريا*pneumoniae* *Klebsiella* ضد انواه مختلفة من العائلة المعوية في حالة كونها خلايا عالقة وفي حال تكوينها للغشاء الحيوي . اثبت النشاط المضاد للبكتيريا بواسطة البكتريوسين (الكليبوسين) المستخلص من عزلات *Klebsiella pneumoniae* تجاه الأنواع المسببة للأمراض المختلفة من العائلة المعوية بطريقة الحفر ، درست ايضا فعالية البكتريوسين ضد الغشاء الحيوي بواسطة طريقة الواح المزارع النسجية في المراحل الاوللى والاخيرة من تكوين الغشاء الحيوي لأنواع مختلفة. اظهرت النتائج أن الكليبوسين بتراكيز مختلفة كان فعالاً ضد الأنواع المسببة للأمراض المختلفة عن طريق إنتاج مناطق تثبيط مختلفة. أظهرت النتائج أيضا أن الكليبوسين كان له نشاط واسع ضد الغشاء الحيوي على العديد من الأنواع المسببة للأمراض من البكتيريا

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السالبة لصبغة كرام. بالإضافة إلى ذلك لوحظ أن نشاط الكليبوسين على الغشاء الحيوي الغير ناضج لبكتريا *Klebsiella pneumoniae* كان أعلى من تأثيره على الأنواع المسببة للأمراض الأخرى، بينما اثر على الغشاء الحيوي الناضج للأنواع البكتيرية الأخرى وكذلك بكتريا *Klebsiella pneumoniae*.

Introduction

Bacteriocins are a group of antimicrobial peptides that are created by Gram positive and Gram negative bacteria [1,2]. Bacteriocins have a constricted killing range and are generally only toxic to susceptible strains of the same or a closely related species so in this character was unlike traditional broad-spectrum antibiotics, [1**]**.

Klebsiella pneumoniae are present in all places and reported worldwide. In the present, *K. pneumonia* have become significant pathogens in nosocomial infections [3]. The magnitude of *K. pneumoniae* species in the constantly increasing number of gram negative aerobic bacillary nosocomial infections in the United States [4] and India [5] has been well recognized. Epidemic and endemic nosocomial infections caused by *K. pneumoniae* species are main causes of morbidity and mortality [6]. It causes infections of the alimentary tract like enteritis, appendicitis and cholycystitis, in addition to being the main cause of respiratory tract infections like pneumonia, rhinoscleroma, ozaena, sinusitis and otitis.

Bacteriocin of *Klebsiella* (Klebocins) are proteins produced and toxic for *Klebsiella* species that carry a Klebocinogenic plasmid which bears the genetic determinants for Klebocin production, immunity and liberate [7]. According to Foutus *et al*. [8], Klebocins were found also chromosomally encoded. Genetic analysis of Klebocin antimicrobial system confirmed the protein content of this system, since it is expressed by specific regulation genes [9, 10,11]. Biofilm is a microbial consequent sessile population characterized by cells that have the ability for attached to an abiotic or biotic surfaces and fixed in a matrix of extracellular polymeric substances that they have formed [12]. The persistent infections in the human body caused by the resistant characteristic of biofilms, as well as to difficult biofilms in industrial processes. Biofilms may be found inside the human body by aggregate the pathogenic bacteria inside the body, e.g. in lungs or on implant surfaces [13,14]. Due to antibiotic resistance for bacterial biofilm, this study aimed to extract the active Klebocin and then studying its antibiofilm activity against some bacterial species, especially the pathogenic one.

Materials and Methods

Collection of samples

Isolation and identification of *K. pneumoniae*:

In the laboratory within aseptic conditions, the urine samples were cultured directly on MacConkey agar (Bio-Rad Laboratories Ltd, France) , after that incubated for 24h at 37ºC. Pink mucoid colonies were picked and recultured on another MacConkey plates in order to obtain pure well isolated colonies. Further identification tests included the colonial morphology and additional identification such as IMViC tests, Triple Sugar Iron agar (TSI), Urease and Motility tests were done according to Forbes *et al* [15].

Detection klebocin producing isolates:

The *K.pneumoniae* indicator isolates used in this study was taken from different urine samples and each one of these isolates were tested against each other. Well diffusion method was used to investigate the ability of these bacteria for bacteriocin production, overnight cultures of tested bacteria were added in wells made on Muller hinton agar (Himedia/ India), that cultured previously with indicator bacteria so the results appeared as clear zones after 24 hrs from incubation.

Extraction of klebocin from producing isolates

Klebocin crude extract was achieved according to [16]. The overnight culture of bacterial isolates in volume 2.5 ml of Luria Bertani broth (LB) (Himedia/ India), was used to inoculate100 ml of sterile LB broth supplemented with 5 % glycerol in shaker incubater.At cell density of about $3x10^8$ (14hr incubation of late log phase), Mitomycin C was added at concentration of 2 µg / ml, incubation continued with shaking for another 3 hrs later. The cooling centrifuge (Beckman Coulter/ Germany), was used for separate the supernatant at 5000Xg for 30 min. The supernatant was taken for assay of klebocin activity and protein determination. For estimation of protein concentration in klebocin crude extract, the procedure was done according to Bradford method [17] were followed and the protein concentration was calculated depending on BSA standard curve.

Detection activity of klebocin crude extract:

Different dilutions were prepared from klebocin crude extract that achieved from producing isolates and then tested against indicator isolates such as *K.pneumoniae, Proteus mirabilis and Escherichia coli,* all these species were isolated from urine samples, the activity of klebocin extract was detected by using well diffusion method.

Biofilm assay

Method described by [18] was followed to achieve biofilm formation:

To study the ability of *K.pneumoniae, P.mirabilis* and *E.coli* to form biofilm , microtiter plate method was used by cultured the bacterial isolates in Tripticase soya broth (TSB) (Himedia/ India), containing 1% glucose or sucrose in tissue culture plates (BioTek/ USA), containing 96-well and incubated for 24 h at 37◦C under aerobic conditions. After that , the non adherent cells were washed three times with distilled water (D.W), and the adhering bacterial cells in each well were fixed with 200 µl of absolute ethanol for 20 min. The plates were emptied and left to dry overnight. Crystal violet was used for staining the adhering cells in volume 200 µl of 0.1% concentration for 15min, and excess stain was rinsed off. The plates were washed with D.W and crystal violate dye bound to the adherent cells was dissolved with 200 µl of 96% ethanol per well. Finally, the plates were read at 490 nm by using a spectrophotometer. The trial was performed in triplicates, and the absorbance of wells containing sterile TSB was used as the negative control the result calculated as in Table-1.

Table 1- Classification of bacterial adherence by tissue culture plate method(18)

OD values	Adherence	Biofilm formation
< OD c	Non	Non
OD < OD ≤ 2*ODc	Weakly	Weak
2*ODc < ODt≤ 2*ODc	Moderately	Moderate
4ODc<OD t	Strong	High

OD: optical density, c: Control, t: test

[Detection antibiofilm activity of klebocin crude extracts.

Method followed according to [19] for the estimate the antibiofilm activity of klebocin, the isolates of *K. pneumoniae, P.mirabilis* and *E.coli (indicator isolates),* were selected to be assayed according to inhibition zones of klebocin extract against planktonic cells of these species on plate agar.

Same protocol described earlier was followed to produce a biofilm but 100 µl of klebocin extract was added to each well. The plate was incubated for 24hr at 37 ºC, after incubation period all wells were washed and stained, then ELISA reader (Beckman coulter,Austria), was used for reading the absorbance at 490 nm. Controls were performed with wells containing crystal violet binding to the culture medium with bacteria.

Results and Discussion:

Detection klebocin producing isolates:

Thirteen bacterial isolates of *K.pneumoniae* were tested against each others, each one of these isolates testes as sensitive isolates in one experiment and indicator isolates in another and from which we can achieved the producing isolates. The results showed that producing isolates were (K 1, K 2, K5, K6,K8,K10, K11,K12 and K13), but klebocin was extracted from 5 isolates that gave highly inhibition zone and tested against different pathogenic isolates in both planktonic and biofilm states.see Table-2.

Table 2-The diameters of inhibiton zones of klebocin produced by *K.pneumoniae* against each to other

Indicator isolates	Testing isolates												
	K1	K2	K3	K4	K5	K6	K7	K8	K9	K10	K11	K12	K13
K 1	-	-	-	-	-	-	-	-	-	-	-	-	-
K 2	10mm	-	-	-	-	-	-	-	-	-	20mm	-	-
K 3	15mm	-	-	-	20mm	20mm	-	-	-	-	-	-	
K 4	-	-	-	-	-	9 mm	-	-	-	-	-	-	-
K 5	-	10mm	-	-	-	-	-	10mm	_	9mm	-	-	10mm
K 6	-	-	-	-	-	9mm	-	-	-	-	-	-	-
K 7	-	-	-	-	-	-	-	-	-	-	-	-	-
K 8	-	15mm	-	-	14mm	-	-	-	-	-	-	11mm	-
K 9	_	_	_	_	10mm	11mm	_	_	_	_	_	_	_
K 10	10mm	20mm	_	_	_	_	_	_	_	_	_	_	_
K 11	_	_	_	_	_	_	_	_	_	_	_	_	_
K 12													
K 13	_	_	_	_	_	_	_	10mm	_	_	_	_	_

There are a diversity of proteins in terms of size, microbial targets, mode of action, and immunity mechanism belong to bacteriocin family. Colicins considered the most extensively studied bacteriocin produced by *E. coli* [20]. Bacteriocins of *Klebsiella* (klebocin) was described first time by Hamon and Peron [21].Bauernfeind *et al*.[22] reported that *Klebsiella* bacteriocin has the ability to decrease or completely inhibit the growth of one or more of other strains but not the strain that produced it, that's mean that producing strain was not inhibited by their own bacteriocin.

Extraction of klebocin from producing isolates:

The bacteriocin was extracted from 5 bacterial isolates (K 1, K 2, K5, K6 and K11) and the results of klebocin concentrations were detected by Bradford assay. The concentration of klebocin crude extract was achieved by bradford assay and the results ranged from (36.31- 165.43 µg/ml), see Table-3. The crude extract of klebocin isolated from producer local isolate showed wide activity spectrum against other gram negative bacteria such as *E.coli* and *P.mirabilis* in different concentrations. For producing large amount of bacteriocin the most efficient ways used by inducing the bacteriocinogenic strain with suitable metabolic inhibitor, one of the most effective is mitomycin C [23, 24]. Several studies approved for stimulation of different colicins, have investigated that increased production of colicin occurs as result to a wide range of physical agents that caused DNA damag such as ultraviolet (UV) radiation [25]; antibacterial agents, such as Mitomycin C (MMC) [16]; all strains bearing a colicin gene detects virtually by phenotypic assay for mitomycin C-inducible bacteriocin production, [26,27,28]. In preceding studies by [29, 30], it was found that addition of 300 ng/ml of mitomycin increase colicin production. Glycerol was also used as an attractive agent where Asenio, [31] and Pugsely, [32] observed that the production of these antagonists was best by using minimal glycerol media.

Table 3-The concentration of klebocin crude extract that estimated by Bradford method

Producing isolates	Concentration µg/ml
K 1	**36.31873**
K 2	**165.4318**
K5	**135.4689**
K6	**49.93826**
K 11	**143.459**

Detection the klebocin activity on some species of enterobacteriaceae:

The results revealed that klebocin crude extract was effective against different bacterial isolates by producing clear zones around indicator isolates Figure-1.

The isolates that gave largely inhibition zones was choosed for using its extract against biofilm of *Klebsiella* and other bacterial species.see Table-4.

Table 4-Effect of klebocin crude extract against *P.mirabilis*, *E.coli* and *K.pneumoniae* by well diffusion method.

	Indicator isolates				
Producing isolates	***Proteus 1***	***Proteus 2***	***E.coli 1***	***Klebsiella 8***	***Klebsiella 10***
K 1	15 mm	30 mm	-	-	15 mm
K 2	**15**	**20 mm**	**15 mm**	**10 mm**	**10 mm**
K 5	15 mm	15 mm	-	-	15 mm
K 6	-	10 mm	-	-	-
K 11	-	20 mm	-	-	-

Al- Charrakh *et al*. [33] reported that klebocin of *Klebsiella* isolates had a wide antimicrobial range and active against many pathogenic species of Grm negative and and some Gram positive bacteria in addition to *Klebsiella* .

In other study, Musleh *et al*. [34] revealed that Klebocin repressed the microbial growth of different microorganisms included: bacteria (both Gram Positive and Gram negative), yeast and molds, these results were an agreement with the current study.

In addition to klebocin production that exert a specific lethal action against other competing microorganisms resulting in their prevail than other organisms [35, 36]. Other study also evaluated to study if the clinical isolates of *Klebsiella* differ from non clinical strains with value to klebocin susceptibility types , and their finding showed that nonclinical *Klebsiella* strains did not give other bacteriocin susceptibility pattern than those appeared by clinical isolates [37].

Another study reported that klebocins from *K. pneumoniae*, *K. ozaenae*, and *K. rhinoscleromatis* were effective on *Klebsiella, Enterobacter, Escherichia, Shigella* and *Proteus* while all cultures of *Agrobacterium , Corynebacterium , Micrococcus , Staphylococcus*,and *Streptococcus* were resistant to the activity of these klebocins [38].

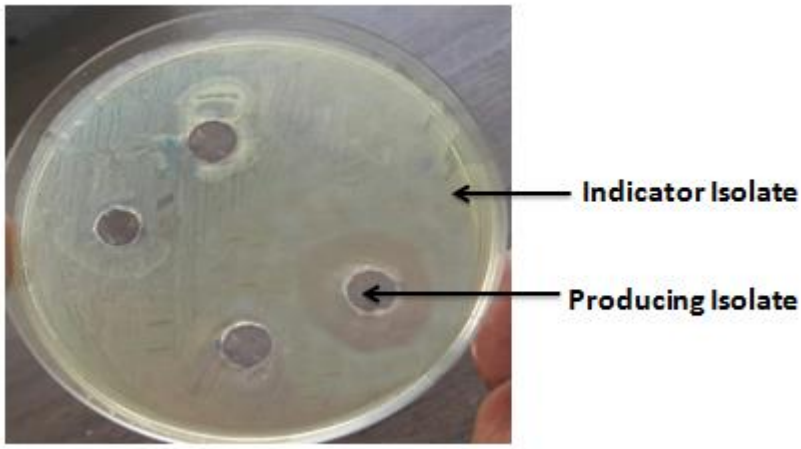

Figure1-Effect of klebocin against indicator isolates, all these wells contain different concentration of klebocin from producing isolate, while the indicator isolate was cultured on the agar plate and then wells were made

Detection antibiofilm activity of klebocin crude extracts against pre- formed biofilm

The results of present study showed that klebocin crude extract was affected against biofilm of some bacterial isolates Table-5 and Figure-2.

It was found in this research that klebocin extracted from isolate K2 gave significant effect (P< 0.05) against K8 biofilm in all concentrations of klebocin. The effect against K 10 biofilm observed in the lowest concentration at dilution (1/16).

There was no significant effect (P>0.05) against biofilm of these isolates (P1, P 2 and *E 1*).

From all these results it can observed that klebocin was effected against preformed biofilm of *Klebsiella* but there was no effect against *Proteus* and *E.coli* biofilm because the effects of it against intimately strains larger than other isolates in the same family.

Previous studies reported that the effect of bacteriocins on biofilm formation by allow for growing two organisms or more together and study the competing between them [39, 40].

Table 5-Effect of klebocin extract on bacterial biofilm at first stage of formation

Bacterial spp.	Treatments/ Concentration µg/ml					LSD value
	Control	82.71	41.35	20.67	10.33	
K 8	0.165 ±0.008	0.117 ±0.006	0.098 ±0.002	0.095 ±0.003	0.083 ±0.002	0.046 *
K 10	0.259 ±0.013	0.387 ±0.016	0.237 ±0.008	0.217 ±0.006	0.166 ±0.004	0.083 *
P 1	0.158 ±0.008	0.145 ±0.007	0.176 ±0.007	0.20 ±0.004	0.194 ±0.006	0.077 NS
P 2	0.148 ±0.006	0.111 ±0.006	0.135 ±0.005	0.14 ±0.008	0.128 ±0.003	0.061 NS
E1	0.113 ±0.004	0.112 ±0.002	0.101 ±0.002	0.087 ±0.004	0.095 ±0.002	0.069 NS
LSD value	0.062 *	0.115 *	0.072 *	0.069 *	0.066 *	---
* (P<0.05), NS: Non-significant.						

Detection antibiofilm activity of klebocin crude extracts against mature biofilm

The results of the present study showed that the klebocin extract gave highly effects against mature biofilm of different bacterial isolates, but the action of bacteriocin different according to the type of bacterial spp. and the dilution of bacteriocin.

There was significant effect of klebocin against bacterial mature biofilm of K8 biofilm was only in all its dilutions, while the effect against K10 in dilution 1/2 and 1/32.It was reported also that klebocin extract gave significant effect against P 1 (P<0.05), but there was no effect against P2.

The results indicated that klebocin extract gave significant effect against *E.coli* (P<0.05) in compare with the effect of it against preformed biofilm. see Table-6 and Figure-3.

Table 6-Effect of klebocin extract against bacterial mature biofilm

Bacterial spp.	Treatments/ Concentration µg/ml						LSD value
	Control	165.43	82.71	41.35	20.67	10.33	
Klebsiella 8	0.165 ±0.008	0.072 ±0.001	0.085 ±0.003	0.091 ±0.003	0.082 ±0.003	0.106 ±0.001	0.064 *
Klebsiella 10	0.387 ±0.017	0.149 ±0.004	0.345 ±0.009	0.428 ±0.006	0.590 ±0.006	0.209 ±0.003	0.139 *
Proteus 1	0.311 ±0.009	0.179 ±0.006	0.223 ±0.006	0.201 ±0.006	0.229 ±0.005	0.173 ±0.002	0.094 *
Proteus 2	0.227 ±0.008	0.166 ±0.002	0.160 ±0.004	0.221 ±0.004	0.196 ±0.003	0.233 ±0.004	0.079 NS
E. coli	0.672 ±0.037	0.154 ±0.002	0.164 ±0.004	0.195 ±0.006	0.208 ±0.007	0.200 ±0.002	0.237 *
LSD value	0.209 *	0.067 *	0.106 *	0.142 *	0.157 *	0.110 *	---
* (P<0.05), NS: Non-significant.							

There are few studies about the effect of klebocin on biofilm of different bacterial species, but many studies reported the effect of colicin (bacteriocin of *E.coli*) against biofilm. Khalaf and Flayyih [41] reported that crude colicin extracts from 6 isolates of *E.coli* were affected against all isolates biofilm as treatment, these results showed that colicin, was active against mature biofilm for different pathogenic isolates.

Lyon *et al.*, [42] reported in their patent that the colicin was had the ability to inhibit the growth of one or more species and/or strains of pathogenic Enterobacteriaceae, as for example, *Salmonella* spp. such as *S. typhi, S. typhimurium, S.paratyphi* A, *S. choleraesuis*, *Shigella* spp. such as *Shigella dysenteriae, Shigella flexneri, Shigella boydii, Shigella sonnei*; and the like; *Escheri chia* spp. such as *E.coli* strain 0157:H7, *E. afreundii*, and *Enterococcus* spp.

The limited number of researches demonstrated that biofilm-mediated bacterial infections can be treated or prevented by colicin like bacteriocins, For example, Trautner *et al.* [43] exposed that colonization by a colicin-susceptible *E. coli* clinical isolate on catheter can be prevented by pre-growth of colicin-producing *E. coli* K-12, demonstrating that colicin manufacturing may work as a strong inhibitor of *E. coli* biofilm development. In addition, it was found that other enteric bacterial biofilms in the murine gastrointestinal tract can be inhibited by colicin producing *E. coli* strains that have been revealed to continue for longer periods in this tract [44].

Furthermore, Saeidi *et al.* [45] also explained that bacteriocin of *Pseudomonas* (pyocins) could be used as anti-biofilm therapy. *Klebsiella pneumoniae* is an significant biofilm producing organism that causes a wide range of infections placing it among the eight most important nosocomial pathogens [46]. So using klebocin extract as effective antibacterial agent gave acandidate for using it as treatment for infections that caused by this pathogen and other multi-resistance organisms.

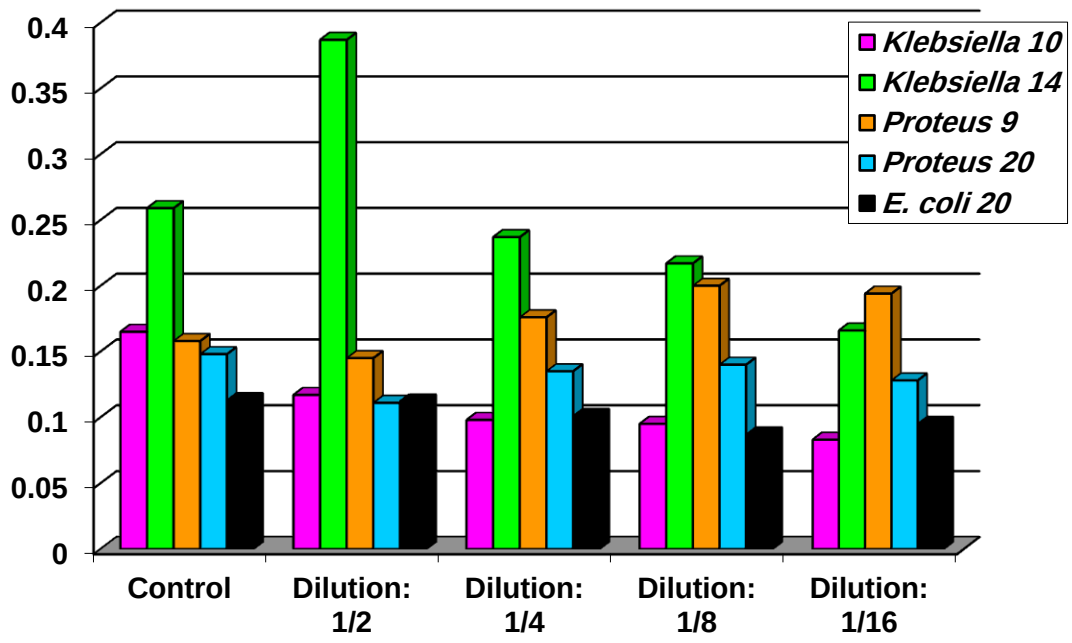

Figure 2-Effect of klebocin on pre-formed biofilm of Gram negative pathogenic bacteria

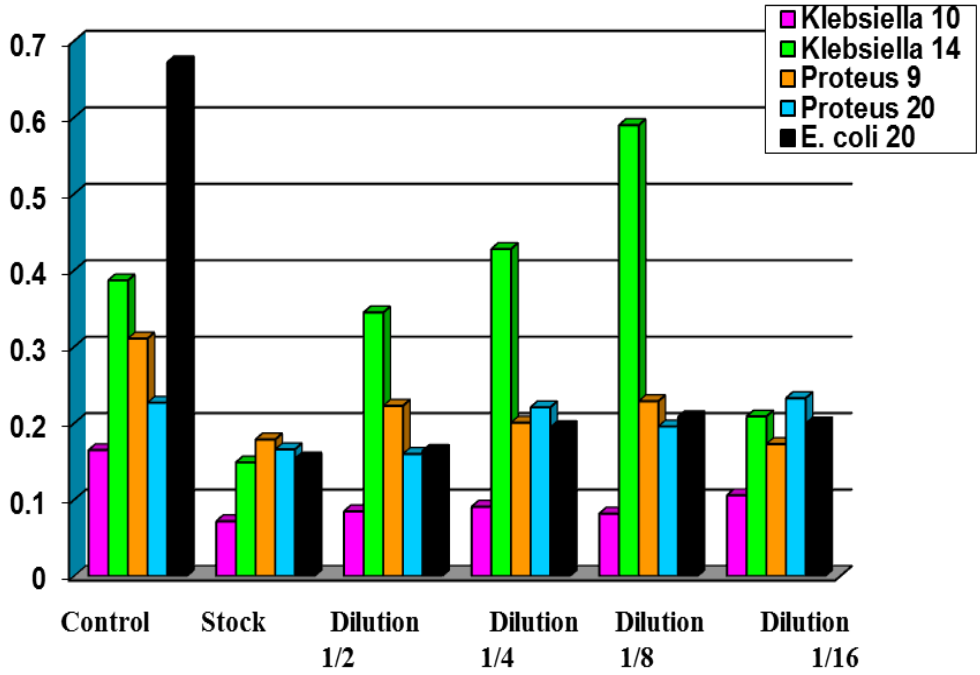

Figure 3-Effect of klebocin on mature biofilm of Gram negative pathogenic bacteria

Conclusions

The results of this research showed that clinical isolates of *K.pneumoniae* have the ability to produced bacteriocin (klebocin).Klebocin has highly antibacterial and antibiofilm activity against pathogenic isolates such as (other strains of *Klebsiella* , *E.coli* and *Proteus*). So these results may give a candidate for using it as antibacterial agents.

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