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Antibacterial And Antibiofilm Of Klebocin Extracted From Klesiella Pneumoniae On Two Pathogenic Bacteria

Zahraa Obaies Ali[1], Eman Mohammed Jarallah[2], Zahraa M. Al-Taee[3]
[1,2,3]Department of Biology, College of Science, University of Babylon, Iraq
sci105.zahraa.obaies@student.uobabylon.edu.iq[1], sci.eman.mohammad@uobabylon.edu.iq[2], sci.zahraa.mohammad@uobabylon.edu.iq[3]

Abstract
The main aim of the current study target to evaluate the anti-bacterial and anti-biofilm activity of Klebocin, a type of bacteriocin produced by some bacteria to attack competing bacteria. Bacteriocins are promising alternatives to conventional antibiotics due to their precise targeting and effectiveness against resistant bacteria.
Two hundred clinical samples were collected, 150 of which were confirmed to be infected with Klebsiella pneumoniae using selective culture media and biochemical tests. After isolating Klebocin from the confirmed samples, its biological activity was evaluated against Pseudomonas aeruginosa and Staphylococcus aureus, two clinically significant pathogenic microorganisms.Antibacterial activity was measured using a method known as agar-well diffusion, with Klebocin showing a concentration-dependent inhibitory effect. At the highest concentration tested (0.01 mg/ml), the average diameter of the inhibition zones was 16.2 mm against Pseudomonas aeruginosa and 16.25 mm against Staphylococcus aureus, indicating strong antimicrobial activity. In contrast, there was no activity observed in the control group (0 mg/ml).Biofilm inhibition assays dependent on using microplates also showed that Klebocin significantly decreased biofilm formation in both bacterial species, with the highest inhibition rate at 0.01 mg/ml.
Current results suggest that Klebocin holds promise as an alternative therapeutic option against antibiotic-resistant bacteria, given its potent antimicrobial and antibiofilm properties.
Keywords: *Bacteriocin, Klebocin, Klebsiella pneumonia,*

INTRODUCTION

Antibiotic-resistant bacteria are becoming more prevalent due to the extensive consumption of antibiotics in population. *Klebsiella pneumoniae* (*K. pneumoniae*) has become more virulent and progressed in its resistance to drugs as a result of the increasing frequency of functional gene acquisition through mobile components (Cai *et al.*, 2022). As a symbiotic bacterium, these bacterianaturally occurring Gram-negative member of the Enterobacteriaceae family that is frequently found in many environments. It can colonize the host's epidermis, nasopharynx, and intestinal mucosa (Podschun and Ullmann, 1998). Numerous infections in deferent site of body systems can be infected by it(Li *et al.*, 2023).
Bacteriocins are proteinaceous antibiotics synthesis by bacteria. Bacteriocins that are produced by *Klebsiella* are called klebocins ,secreted from *Klebsiella pneumoniae* and appears to be active against various Enterobacteriaceae ; (Hardegree & Tu, 1988;.Thomas *et al.*, 2004). Klebocinshas toxic effect for *Klebsiella* species carrying a Klebocinogenic plasmid that bears the genetic determinants for Klebocin synthesis, immunity, and release(Cascales et al., 2007).
Numerous disease-causing bacteria, including some types resistant to antibiotics, are inhibited by the component bacteriocins, indicating that they may be used to combat harmful infections. There are many bacteria in the human body, such as in the respiratory, gastrointestinal, cutaneous, and reproductive systems, and the host microbiota and host cell are always interacting. In the search for effective therapeutic alternatives to combat bacterial resistance to antibiotics, this study evaluated the inhibitory effect of a Klebsiella pneumoniae extract on the formation of biofilms of Staphylococcus aureus and Pseudomonas aeruginosa. A validated methodology was used to measure anti-biofilm activity. Biospecies were treated with the extract and incubated, and

absorbance was measured using an ELISA reader to determine the extent of the effect(Harry & Walker, 2013).

This study aimed to investigate the antibacterial and anti-biofilm potential of Klebocin, a bacteriocin protein extracted from Klebsiella pneumoniae, against two clinically significant pathogenic bacteria: Pseudomonas aeruginosa and Staphylococcus aureus. Given the rising threat of multidrug-resistant organisms, bacteriocins such as Klebocin represent a promising alternative due to their selective antimicrobial activity and ability to disrupt biofilm formation. The goal of this research was to assess the efficacy of Klebocin as a potential candidate for novel therapeutic strategies targeting resistant bacterial infections.

MATERIALS AND METHODS

Collection of specimens

A total of 200 clinical specimens, comprising urine, trachea(airway) and appendix were collected from both genders and various age groups between July and October2024. These specimens were obtained from several hospitals in Hillah city, including Imam Al-Sadiq Hospital, Hillah Surgical Teaching Hospital and Marjan Teaching Hospital, located in Babylon (Table 1).

Table 1: Distribution of distinct bacterial strains and their percentages

Sample Type	*Klebsiella Pneumoniae*		Others		Total
	Number	Percentage (%)	Number	Percentage (%)	
Urine	120	60	37	18.5	157
Trachea (Airway)	25	12.5	11	5.5	36
Appendix	5	2.5	2	1.0	7
Total	150	75	50	25	200

Isolation and Identification of *K.pneumoniae*

All clinical samples were cultured using the streak plate technique on all of the agar media (blood, MacConkey, Eosin Methylene Blue (EMB) , and CHROMagar), then incubated at 37°C for one day. For long-term preservation, bacterial isolates were stored in specific broth, such as Brain Heart Infusion Broth or Nutrient Broth, supplemented with 15% glycerol and stored at −20°C**.** The identification of bacterial isolates was performed by examining the general morphology of colonies and the microscopic appearance of cells, supported by biochemical tests, according to the criteria described by McFadden.

In this context, a series of biochemical tests were performed on the studied isolates to identify their featured metabolic and enzymatic characteristics, which contribute to their classification and confirmation of identity. These tests included urease enzyme activity in urea hydrolysis, sodium citrate as the only carbon source, glucose fermentation patterns utilizing the Methyl Red and Voges–Proskauer tests to detect acid or acetoin formation, and the analysis of indole production from tryptophan. The bacteria's ability to ferment multi-sugars (glucose, lactose, and sucrose) and produce gas was also evaluated using TSI medium, and hydrogen sulfide (H_2S) production was also monitored. Tests were also performed to detect the presence of oxidase and catalase enzymes, along with bacterial motility (Munita & Arias, 2016).

Extraction of Klebocin

According to a previously defined protocol, *Klebsiella pneumoniae* isolates that demonstrated klebocin production by the well diffusion assay were chosen for additional extraction processes (Khalaf & Hussein, 2018). The isolates were first grown in Luria-Bertani (LB) broth that had been enhanced with glycerol with a concentration of 5%, and they were then shaken for the duration of the incubation period. Mitomycin C was administered at a final dosage of 2 μg/ml to trigger the synthesis of klebocin once the cell density had reached about 3×10^8 CFU/ml. After that, the culture was continuously shaken for three more hours of incubation..Following induction, the bacterial suspension was subjected to centrifugation at 5,000 × g for 30 minutes

at 4°C using a refrigerated centrifuge (Beckman Coulter, Germany), and the resulting supernatant containing the crude klebocin extract was carefully collected. The concentration of Protein in the crude extract was determined by Bradford method, with bovine serum albumin (BSA) serving as the standard procedure (Bradford, 1976).

Antibacterial activity of Klebocin on two Pathogenic bacteria

Klebocins, naturally occurring bacteriocins from some *Klebsiella spp*., are characterized by their specialized ability to inhibit the growth of multiple pathogenic bacteria. Recent evidence indicates that crude extracts of Klebocin exhibit potent antibacterial activity, even against multidrug-resistant isolates. In the current results for evaluation, Klebocin demonstrated significant inhibitory activity against both Staphylococcus aureus and Pseudomonas aeruginosa, with a significant reduction in bacterial density compared to the control sample. This feature is attributed to Klebocin's mechanism of action, which involves selective binding to the target cell wall, disrupting the cell membrane, or inhibiting protein biosynthesis. This reinforces its very importance as a promising alternative to traditional antibiotics in light of the rising antibiotic resistance (Ali *et al*., 2022; Alattar *et al*., 2024).

Anti-Biofilm Activity of Klebaccin

The studied isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* were used to test the klebocin extract's inhibitory effect on biofilm formation. The anti-biofilm activity assessment procedure was modified from Harry and Walker's approach (2013). The preformed biofilm matrix in the wells of a 96-well microtiter plate was mixed with 100 µL of klebocin extract, and the mixture was then incubated for 24 hours at 37°C.To get rid of non-adherent cells, the wells were gently rinsed after incubation. To assess the biofilm biomass, the remaining biofilm was preserved, stained with crystal violet, and its absorbance was measured at 490 nanometers using an ELISA reader. Both bacterial species showed a significant decrease in formed biofilm, with the degree of inhibition varying according to the klebocin extract's protein concentration.Three concentrations (0.01, 0.005, and 0.001 mg/mL) were tested, with the highest inhibition observed at 0.01 mg/mL.

Biofilm inhibition percentage was calculatedaccording to the below formula:

$$\text{Biofilm inhibition (\%)} = [(\text{Control OD} - \text{Treated OD}) / \text{Control OD}] \times 100$$

RESULTS AND DISCUSSION

Isolation and Identification

A total of 150 *Klebsiella pneumoniae* isolates, numbered k1 to k150 were obtained from two hundred clinical specimens, which included urine, trachea, and appendix. A total of 120 specimens, accounting for80%, were obtained from urine, while 25 specimens , representing 16.67%were sourced from trachea, and 5 specimens or 3.34% were collected from the appendix. The identification of *Klebsiella pneumoniae* isolates included culture, microscopy, biochemical tests, Gram stain results showed that the bacterial isolates appeared as single or double rods when cultured on MacConkey medium, and were characterized by a distinct color resulting from their ability to ferment the lactose present in the medium. When cultured on blood medium, Klebsiella pneumoniae colonies were clearly visible, ranging in size from medium to large, and characterized by a light creamy-brown color. Occasionally, mild degrees of α-hemolysis were observed, but most colonies did not significantly change the color of the surrounding medium.

Klebsiella pneumoniae is classified as a Gram-negative bacterium, appearing under the microscope as red rods. Its ability to ferment lactose was confirmed by the color change on MacConkey medium, which changed from yellow to red. The Voges-Proskauer test also showed a positive result, indicating the conversion of glucose to acetoin, while the oxidase and citrate

tests were negative. Also, no hydrogen sulfide (H_2S) gas production was observed on Kligler's medium, indicating that this property is absent in the isolate studied.
Due to the aerobic nature of this species, an alkaline reaction was observed on the surface of the Kligler medium as a result of glucose consumption, with the color changing from red to pink. However, no change occurred at the bottom of the tube, indicating that glucose is not utilized under anaerobic conditions. Table (2) shows the detailed results of Biochemical Characterization of *K. pneumoniae* Isolates

Table (2): Biochemical Test Results of the *Klebsiella pneumoniae* Isolate

Test	**Expected Result**	**Brief Description**
Indole	Negative (–)	Detects the ability to produce indole from tryptophan via the enzyme tryptophanase.
Methyl Red (MR)	Negative (–)	Refer to glucose as fermenting with a mixture of acids, producing stable acidic byproducts.
Vogas–Pruskauer (VP)	Positive (+)	Detects acetoin production from glucose fermentation via the butylene glycol pathway.
Citrate Utilization	Positive (+)	Tests the have able to utilize sodium citrate as the sole carbon source.
Urease	Positive (+)	Identifies the hydrolysis action of urea to ammonia and carbon dioxide byacurate urease enzyme.
Triple Sugar Iron (TSI)	A/A, Gas, no H_2S	Indicates fermentation of glucose and lactose/sucrose with gas production; no H_2S.
Oxidase	Negative (–)	Determines the presence of cytochrome c oxidase; negative in Enterobacteriaceae.
Catalase	Positive (+)	Detects catalase enzyme that breaks down hydrogen peroxide into water and oxygen.
Motility	Negative (–)	Assesses bacterial motility; *K. pneumoniae* is non-motile.

The results showed a good match with the known biochemical profile of Klebsiella pneumonia.

Extraction of Klebocin from *Klebsiella pneumoniae* Isolates

Five *Klebsiella pneumoniae* isolates that exhibited inhibitory activity against pathogenic bacteria were selected for crude Klebocin extraction. Each isolate was inoculated into **Luria-Bertani (LB) broth** supplemented with 5% glycerol and incubated in a shaking incubator at 37°C for 18–24 hours until reaching an approximate cell density of 3×1083 \times 10^83×108 CFU/mL. To induce Klebocin production, **Mitomycin C** was added at a final concentration of 2µg/mL2 \mu g/mL2µg/mL, followed by further incubation for 3 hours with continuous shaking. After induction, the cultures were centrifuged at 5000 rpm for 30 minutes at 4°C using a refrigerated centrifuge to separate the cell debris.

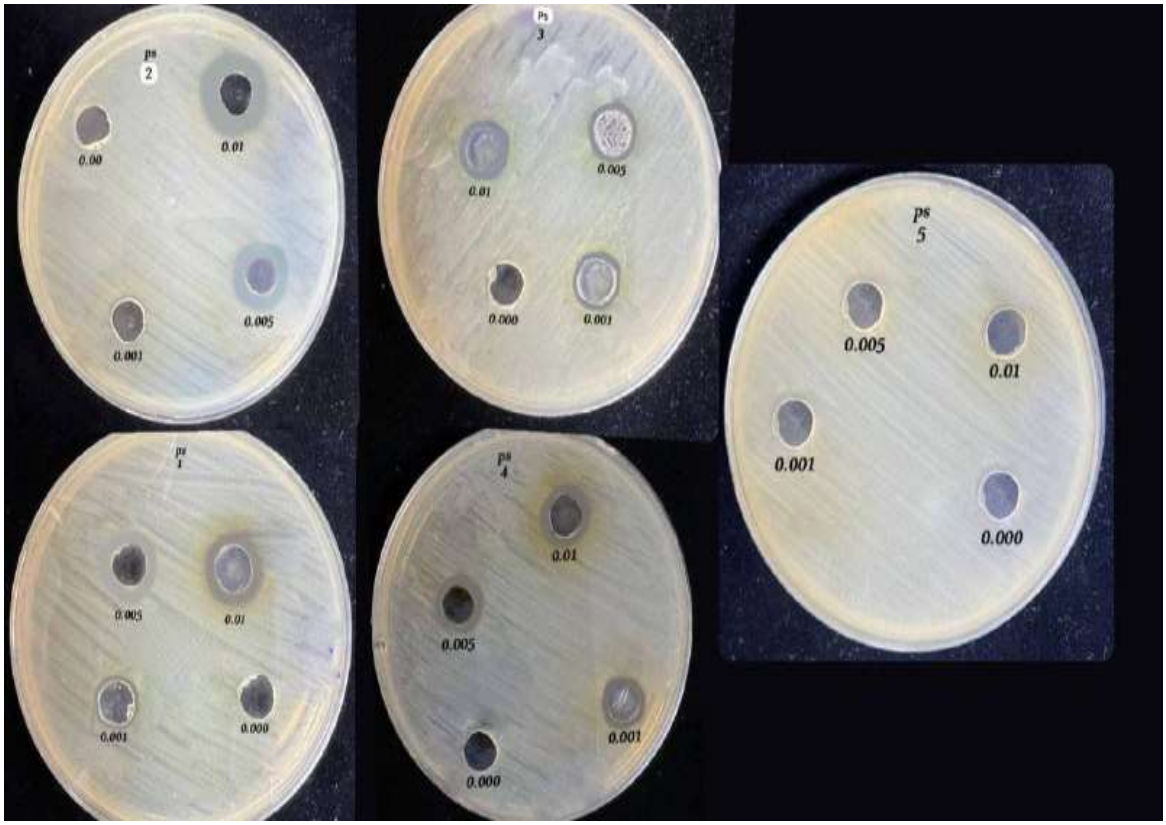
The **supernatant**, which contains the Klebocin, was collected, while the **pellet** was discarded. The collected supernatant was then subjected to **freeze-drying (lyophilization)** to obtain a powdered crude extract. The resulting powder was stored in sterile tubes at −20°C until further analysis. Using bovine serum albumin (BSA) as the reference, the Bradford test was utilized to quantify the crude Klebocin extract's total protein concentration.

Antibacterial Activity of Klebocin Extracts Against Pathogenic Isolates

The antibacterial effect of crude Klebocin extracts, obtained from five clinical isolates of *Klebsiella pneumoniae*, was evaluated against two clinically significant pathogens: *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Three different protein concentrations were tested (0.01 mg/mL, 0.005 mg/mL, and 0.001 mg/mL), in addition to a control group with no Klebocin. The results demonstrated a concentration-dependent inhibitory effect on both target pathogens. For *Pseudomonas aeruginosa*, the highest inhibition zone was observed at 0.01 mg/mL, reaching up to 17 mm in some samples, with a gradual decline in activity at lower concentrations. Similar trends were recorded for *Staphylococcus aureus*, with inhibition zones reaching 17 mm at the highest concentration.

Sample No. 5 in both studied pathogens, they consistently do not show inhibition across all concentrations, suggesting strain-specific resistance or absence of active Klebocin production in this isolate. Notably, no inhibitory effect was observed in the control groups also.These findings approve the potential of crude Klebocin extracts as promising antibacterial agents, particularly against multidrug-resistant bacteria. The clear non-dependent response highlights their potential use as alternatives or adjuncts to traditional antibiotics.

Fig. 1: Anti-bacterial acti



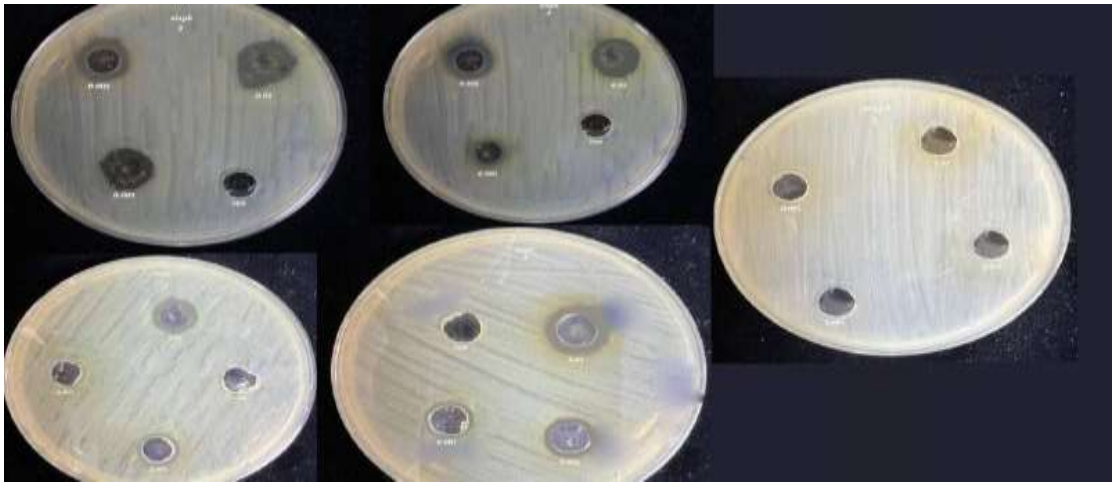


Fig. 2: Anti-bacterial activity for klebocin materials against bacteria(*S. aureus*)

Evaluation of the Antibiofilm Activity of Klebocin Crude Extract

With a few minor adjustments to Atshan et al.'s methods in (2012), the **96-well microtiter plate assay with crystal violet staining**, as previously reported in their research, was used to evaluate the antibiofilm impact of crude Klebocin extract. The evaluation was conducted using clinical isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. After bacteria being cultivated in **Tryptic Soy Broth (TSB)** with 1% glucose added, the bacterial cultures were transferred into sterile 96-well flat-bottom plates and incubated for 24 hr. at 37°C to promote the production of biofilms. Following incubation, non-adherent cells were eliminated by gently washing the wells three times with sterile distilled water. After adding 100 µL of crude Klebocin extract to each well at doses of 0.001, 0.005, and 0.01 mg/mL, the wells were incubated for a further 24 hours. Following 200 µL of 100% ethanol per well for fixing, biofilms were dyed for 15 minutes with 0.1% crystal violet and rinsed to get rid of extra color. After ethanol was used to dissolve the residual dye, which represents the mass of the biofilm, the optical density (OD) was determined using a microplate reader at 490 nanometers.

The inhibition concentration for biofilm was calculated according to the formula below.:

$$\textbf{Biofilm inhibition (\%)} = [(\textbf{Control OD} - \textbf{Treated OD}) / \textbf{Control OD}] \times 100$$

The results revealed a **clear inverse relationship** between Klebocin concentration and biofilm formation. As the protein concentration increased, the ability of both *S. aureus* and *P. aeruginosa* to form biofilm significantly decreased. The highest biofilm inhibition was recorded at the concentration of 0.01 mg/mL, indicating that Klebocin exhibits effective antibiofilm activity and holds potential as an alternative antimicrobial agent, especially against biofilm-forming pathogenic strains.

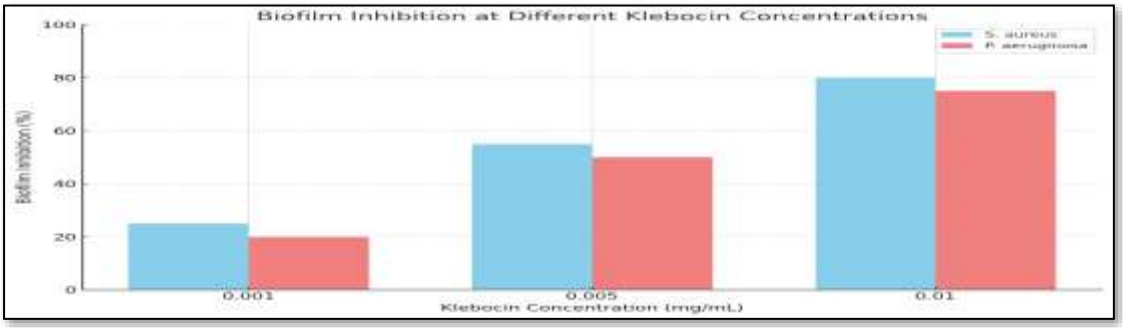


Fig. 3: Biofilm Inhibition at different Klebocin concentrations

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