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Characterization of a Novel Klebocin from *Klebsiella pneumoniae*: Broad-Spectrum Antimicrobial and Anti-biofilm Activities with High Stability and Selective Toxicity

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ABSTRACT

Interest in bacteriocins as therapeutic agents has been driven by the growing resistance to antimicrobial agents. This research extracted, purified, and characterized klebocin produced by a clinical isolate of *Klebsiella pneumoniae* and assessed its antimicrobial, antibiofilm, stability, and selective toxicity characteristics. Polymerase chain reaction (PCR) confirmed the presence of *kleB* (249 bp) and *kleC* (2300 bp) genes. Mitomycin C was used to induce klebocin production, which was purified by ammonium sulfate precipitation, CM-Sepharose ion-exchange chromatography, and Sephadex G-100 gel filtration to a final specific activity of 1806.4 AU/mg with a purification factor with a purification factor of 15.69 fold. The purified protein showed broad-spectrum antimicrobial activity, and the size of the inhibitory zones was 20 ± 0.8 mm against *Escherichia coli*, 18 ± 1.2 mm against *Staphylococcus aureus*, and 15 ± 1.0 mm against *Pseudomonas aeruginosa*. In terms of biofilm formation, klebocin exhibited an inhibition rate of 78 ± 3% against *E. coli* at pH 7 and 37 ℃. Klebocin was still active at 100 ℃ (60%) and stable at pH 3-11. The selective toxicity index (STI) was greater than 16 at 1× minimum inhibitory concentration, with hemolytic activity that was below 5. The electron microscopy showed that the membrane was disrupted and had pores. Overall, these findings indicate that klebocin is a promising antimicrobial candidate with high stability and selectivity.

1. INTRODUCTION

The alarming global spread of antimicrobial resistance (AMR) has become one of the greatest threats to public health of the population in the 21st century. Carbapenem-resistant *Klebsiella pneumoniae* is listed by the World Health Organization as a priority pathogen, underscoring the need for new effective treatments [1]. Multi-drug resistant (MDR) *K. pneumoniae* is a significant source of hospital-acquired infections, such as pneumonia, bloodstream, and urinary tract infections, with poor treatment options and high mortality [2, 3]. In the search for alternative strategies to combat MDR *K. pneumoniae*, attention has increasingly turned to bacterial toxins, particularly bacteriocins, as promising candidates for targeted antibacterial therapies.

Bacterial toxins are one of the most vital virulence factors that help bacteria infect, protect themselves from the immune defenses of the host, and outcompete other microbial organisms in the same ecological niche. These toxins, in general, are biologically active molecules that can cause various cellular and molecular perturbations, such as interference in major metabolic pathways, membrane perturbations, inhibition of macromolecules, or immune modulation of the host organisms [4]. Bacterial toxins are classified into endotoxins and exotoxins, with exotoxins being more diverse and specific. Among them, bacteriocins are ribosomally synthesized protein toxins that inhibit or kill closely related or competing bacteria. Unlike most exotoxins, bacteriocins have a narrow spectrum and do not target eukaryotic cells, making them biologically and ecologically significant [5].

With the deteriorating effectiveness of the traditional antibiotics, there is an urgent need to discover alternative antimicrobial agents. Bacteriocins are ribosomally expressed antimicrobial peptides that have been identified as good candidates because of their high potency, selective targeting, and low propensity to induce resistance [6, 7]. They display broad-spectrum antimicrobial properties due to distinct mechanisms of action, which primarily involve cytoplasmic membrane pore formation, cell wall degradation/ biosynthesis inhibition, protein translational inhibition, as well as direct damage to biological nucleic acids like DNA or RNA. These mechanisms lead to rapid growth inhibition or death in targeted susceptible microbes [8]. Recently,

bacteriocins have also received considerable interest as novel alternative or supplementary antimicrobial agents to traditional antibiotics, in particular against the rising incidence of multidrug-resistant (MDR) bacterial isolates across the world [9]. In this scenario, klebocins are one of the most interesting bacteriocin types produced by *Klebsiella pneumoniae*. This is an important Gram-negative facultative pathogen causing hospital-acquired infections, such as pneumonia, urinary tract infections, and septicemia. Klebocins that *K. pneumoniae* generate are a comparatively neglected type of bacteriocins among Gram-negative pathogens. Despite genomic studies revealing the presence of bacteriocin gene clusters within *K. pneumoniae* species complexes [10], there is a scarcity of experimental data characterizing klebocin in clinical isolates, especially in the areas of purification, antibiofilm activity, physicochemical stability, and selective toxicity. Furthermore, the possibility of klebocin as a stable antimicrobial agent that can be used to treat planktonic cells and biofilms has not been effectively studied.

Molecular diagnostic approaches, particularly polymerase chain reaction (PCR)-based detection of virulence and bacteriocin-associated genes, have become indispensable tools for characterizing pathogenic *K. pneumoniae* isolates. *Klebsiella pneumoniae* is known to possess an arsenal of gene-encoded virulence factors, including capsular polysaccharides, adhesins, and siderophores, as well as holotoxins, exported from these pathogenic microbes through secretion systems. *Klebsiella pneumoniae* is known to possess an impressive array of antimicrobial traits to survive and colonize human hosts to cause disease due to this diversity of virulence factors [11]. The secretion of klebocins into bacterial pathogens gives them an impressive survival advantage over competing bacterial strains within this particular biological niche [12]. At the molecular level, klebocins are generally protein or peptide toxins that have special genetic loci, encoded by genetic loci located on plasmids or in specific chromosomal regions. The biosynthesis of klebocins is often accompanied by the simultaneous biosynthesis of immunity proteins that can protect the producing strain from the toxicity of the bacteriocins. Klebocin gene clusters, or bacteriocin genes, such as klebocin, can provide insights into interbacterial competition, as they can strongly affect microbial ecology and infectivity. Therefore, comprehensive characterization of klebocins requires reliable and robust analytical techniques. Chromatographic methods are essential tools for the purification and characterization of bacteriocins such as klebocin [13]. Gel permeation chromatography allows the separation of proteins by size and accordingly performs preliminary protein purification and the initial estimation of the protein's size. Ion exchange chromatography, on the other hand, enables the precise separation of proteins by the presence of certain surface charges. By using a combination of the two, the overall degree of purity and the precision of biochemical analysis can be significantly improved, and the precise biochemical analysis of the bacteriocin, klebocin, in this case, can be undertaken.

This study aimed to extract, purify, and characterize klebocin produced by clinical isolates of *Klebsiella pneumoniae*, with a particular focus on evaluating its antibacterial and antibiofilm activities, stability under varying physicochemical conditions, and selective toxicity to determine its potential as a safe and effective antimicrobial agent.

2. MATERIALS AND METHODOLOGY

2.1 Materials

2.1.1 Preparation of reagents and standard solutions

Standard solutions, including 0.5 McFarland turbidity standard (~1.5×10^8 CFU/mL), physiological saline (0.85% NaCl), mitomycin C stock solution (10 mg/mL in methanol), and reagents required for protein quantification using the Bradford assay with bovine serum albumin (BSA) as a standard [14].

2.1.2 Bacterial isolates and culture conditions

Klebsiella pneumoniae clinical strains were collected and identified by conventional microbiological and biochemical analyses. Test organisms used for antimicrobial and antibiofilm activities included *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *Candida albicans*. All experimental analyses were done in biological triplicates under aseptic conditions. Culture media were prepared according to the manufacturer's guidelines and autoclaved at 121 ℃ for 15 min. After autoclaving, the media were cooled at 4 ℃ before being poured into Petri dishes. They were stored in a refrigerator until use [15]. Blood agar was prepared by dissolving 40 g of base medium in 1000 mL of distilled water, followed by sterilization at 121 ℃ for 15 minutes. After cooling to approximately 45 ℃, 5% human defibrinated blood was added before being poured into Petri dishes and stored at 4 ℃ in a refrigerator until use.

2.2 Methodology

2.2.1 Detection of klebocin structural genes and screening of klebocin-producing isolates

Genomic DNA was extracted from *K. pneumoniae* isolates using a combined boiling–proteinase K digestion, followed by spin-column purification to eliminate PCR inhibitors. Molecular screening was performed by PCR to confirm the presence of klebocin structural genes (*kleB* and *kleC*) using gene-specific primers (Table 1). PCR products were analyzed by agarose gel electrophoresis to verify the expected amplicon sizes. Molecular screening was conducted solely to confirm the presence of klebocin-associated genes (*kleB*, *kleC*, and *micC*) and to support the selection of the Klebocin-producing isolate. No further functional or quantitative genetic analyses were performed, as PCR-based confirmation served exclusively as a preliminary validation step prior to toxin induction, production, and purification in Table 2.

After molecular confirmation, klebocin production was induced by treating *K. pneumoniae* cultures with mitomycin C (0.05–0.1 µg/mL) for 90 minutes to activate the SOS response. Cell debris was removed by centrifugation, and the supernatant was collected as a crude extract. Klebocin was purified using a sequential protocol comprising ammonium sulfate precipitation (25–50%), dialysis, DEAE-cellulose ion-exchange chromatography with a NaCl gradient, and gel-filtration chromatography on Sephadex G-100, with eluted fractions monitored at 280 nm. Protein concentration at each purification step was determined using the Bradford assay with BSA as a standard [16].

Table 1. Primers used for molecular confirmation of klebocins-associated genes

Gene	Primer	Sequence (5′–3′)	Amplicon Size (bp)
kleB	Forward	CATTAGCGTCCGCAGAACAAG	249
	Reverse	GCCGACAGAGTAAAACCTCCA	249
kleC	Forward	CTCTGTAACCTTCAAGTTCTC	2300
	Reverse	CAAGCAAGATTACGGTCTACTC	2300
micC	Forward	TGCTTTTGGTGCAGGAGAGA	141
	Reverse	GTTTGTAATGCACCTCCCGC	141

Table 2. Polymerase chain reaction (PCR) amplification conditions used for molecular confirmation

Gene	Initial Denaturation (℃ / 4 min)	Cycles	Denaturation (℃ / 45 s)	Annealing (℃ / 45 s)	Extension (℃ / 60 s)	Final Extension (℃ / 5 min)
kleB	95	35	94	54	72	72
kleC	95	35	94	60 °C / 45 s	72	72
micC	95	35	94	54 °C / 45 s	72	72

2.2.2 Induction and crude extraction of klebocin

Klebsiella pneumoniae cultures were grown in tryptone soy broth at 37 ℃ with agitation for 18 h. Klebocin production was induced by adding mitomycin C (0.05–0.1 µg/mL) and incubating for 90 min to activate the SOS response. Cultures were centrifuged at 10,000 rpm for 10 min at 4 ℃ to remove bacterial cells, and the cell-free supernatant was collected as crude klebocin extract. Protein concentration in crude extracts was determined using the Bradford assay based on Coomassie Brilliant Blue G-250 binding, measured at 595 nm [17]. *Klebsiella pneumoniae* strains were collected from Medical City, Baghdad, with a total of 100 strains obtained from urine and blood. Ethics approval number (BCSMU/902 5/00075M) was obtained. The biological activity decreased by 96% during the dialysis step, which may indicate the need to optimize experimental conditions such as buffer type and temperature.

2.2.3 Purification of crude klebocin

Klebocin was purified using CM-Sepharose ion-exchange chromatography, and gel filtration was performed using Sephadex G-100 following established bacteriocin purification protocols [18].

a) Ammonium sulfate precipitation: Crude extract was subjected to ammonium sulfate precipitation (25–50% saturation) under cooling conditions, followed by centrifugation at 10,000 rpm for 30 min at 4 ℃. Protein pellets were resuspended in 10 mM potassium phosphate buffer (pH 7.5) and dialyzed overnight at 4 ℃.

b) Ion-exchange chromatography: This procedure was conducted by applying dialyzed samples to a CM-Sepharose column pre-equilibrated with 20 mM sodium acetate buffer (pH 5.0). Bound proteins were eluted using a linear NaCl gradient (0–1.0 M), and fractions were monitored at 280 nm.

c) Gel filtration chromatography: To perform the gel filtration chromatography, active fractions were pooled and further purified using a Sephadex G-100 column equilibrated with 10 mM potassium phosphate buffer (pH 7.5). Eluted fractions were collected and monitored at 280 nm. Protein concentration at each purification step was quantified using the Bradford assay. One activity unit (AU) is defined as the reciprocal of the highest dilution showing a visible inhibition zone per milliliter of sample.

2.2.4 Antibacterial and antibiofilm activity assays

The antibacterial activity of crude, semi-purified, and purified preparations of klebocin was determined using the agar well diffusion method against bacterial target strains, according to a standard procedure [19]. The zones of inhibition were measured and quantified in terms of activity units per mL (AU/mL), analyzed using the crystal violet microtiter plate assay. The *E. coli* cultures were exposed to serial concentrations of klebocin for 24 hours, stained with crystal violet, and the biofilm mass was analyzed spectrophotometrically at 570 nm. The percentages of biofilm inhibition were compared with respect to the control groups [20].

2.2.5 Cytotoxicity assessment and selective toxicity index

Cytotoxicity was measured based on hemolytic activity against human red blood cells (RBCs). A 2% RBC solution was mixed with klebocin (62.5 to 1000 µg/mL) and left at 37 ℃ for a period of 1 hour. Phosphate buffer saline and 0.1% Triton-X100 solution acted as negative and positive controls, respectively. The percentage of hemolysis was calculated based on the absorbance at 540 nm [21]. The selective toxicity index (STI) was evaluated by calculating the ratio between antibacterial activity and hemolytic activity. The higher the value of this index, the higher the selectivity to bacterial cells compared with eukaryotic cells. Positive and negative controls were included in the experiments to ensure the reliability of the results.

2.2.6 Statistical analysis

The biological replicates for all the experimental procedures were performed three times, and the obtained values were presented in the form of mean ± standard deviation. Statistical significance was assessed by one-way analysis of variance (ANOVA), followed by post hoc tests, and a value of $p < 0.05$ was considered statistically significant. The relationship between gene presence, antibacterial activity, and antibiofilm activity was made using the Pearson correlation coefficients [22].

3. RESULTS AND DISCUSSION

3.1 Molecular confirmation of klebocin-producing *Klebsiella pneumoniae*

To identify a source for the production of klebocin, a clinical isolate of *Klebsiella pneumoniae* was screened for the presence of genes coding for bacteriocins. PCR confirmation of the presence of the *kleB* and *kleC* genes enabled the identification of the isolate of choice for the toxin induction,

production, and purification. As shown in Table 3 and Figure 1, a single band of the predicted size of 249 bp and 2,300 bp was obtained, respectively, to confirm the specificity of the *kleB* and *kleC* genes. An auxiliary marker in the screening process was the *micC* gene, with a size of 141 bp. This gene is, however, absent in the isolated *K. pneumonia* strain. Gene screening was performed primarily to identify the klebocin-producing isolate and did not include further genetic analyses; *micC* screening was performed for confirmation only.

Table 3. Polymerase chain reaction (PCR) confirmation of klebocin-associated genes

Target Gene	Expected Amplicon Size (bp)	Observed Result
kleB	249	Positive (+)
kleC	2300	Positive (+)
micC	141	Not detected (–)

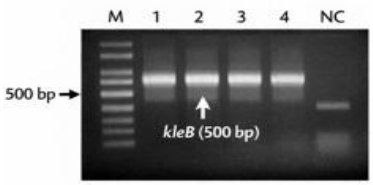

Figure 1. Polymerase chain reaction (PCR) detection of the *klebB* gene in the *Klebsiella pneumoniae* isolate

3.2 Antimicrobial and antibiofilm activity of crude *Klebocin* extract

The crude and cell-free extract of *Klebsiella pneumoniae* demonstrated broad-spectrum antimicrobial activity as assessed by the agar well diffusion assay. Maximum inhibition was observed against *E. coli* (20 ± 0.8 mm, 1800 AU), followed by *S. aureus* (18 ± 1.2 mm, 1600 AU), *P. aeruginosa* (15 ± 1.0 mm, 1400 AU), *C. albicans* (12 ± 0.5 mm, 1200 AU), and *Streptococcus pyogenes* (10 ± 0.7 mm, 1100 AU). These results confirm the broad-spectrum antimicrobial potential of the crude klebocin extract, with the highest efficacy observed against Gram-negative bacteria, while measurable inhibition was also observed against Gram-positive strains and *C. albicans* (Table 4 and Figure 2).

Table 4. Antimicrobial activity of crude klebocin extract

Target Organism	Inhibition Zone (mm)	Activity Units (AU)
E. coli	20 ± 0.8	1800
S. aureus	18 ± 1.2	1600
P. aeruginosa	15 ± 1.0	1400
C. albicans	12 ± 0.5	1200
S. pyogenes	10 ± 0.7	1100

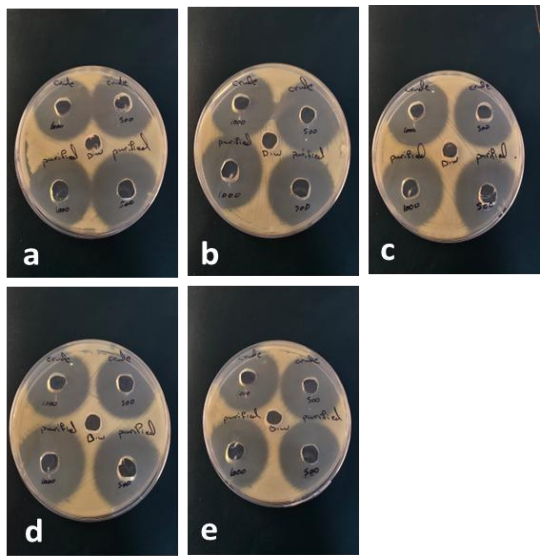

Figure 2. Agar well diffusion assay demonstrating antimicrobial activity of purified klebocin, (a) *C. albicans*, (b) *E. coli*, (c) *S. aureus*, (d) *S. pyogenes*, (e) *P. aeruginosa*

3.3 Purified klebocin

Carboxymethyl (CM)-Sepharose ion exchange chromatography separated klebocin based on net charge. Sharp peaks indicating high purity were achieved in the final purification stage (gel filtration), resulting in a 15.69-fold increase in purity with a specific activity of 1806.4 AU/mg, as shown in Table 5 and Figure 3.

The gel filtration on Sephadex G-100 separated proteins based on molecular size. Klebocin activity peaked in mid-column fractions, indicating successful size-based purification, as shown in Figure 4.

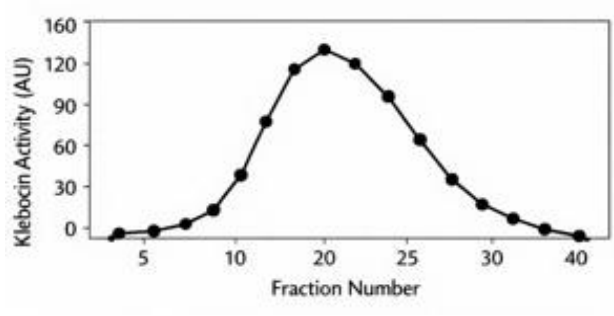

Figure 3. Klebocin activity across Carboxymethyl (CM)-Sepharose fractions
The line graph shows the rise and fall of klebocin activity across ion exchange fractions, indicating successful enrichment of the active protein.

Table 5. Klebocin purification steps, protein, activity, yield, and purification fold

Purification Step	Volume (mL)	Total Protein (mg)	Total Activity (AU)	Specific Activity (AU/mg)	Yield (%)	Purification Fold
Crude Extract	500	2780	320,000	115.1	100	1.0
Ammonium Sulfate 25–50%	200	793.4	234,700	295.8	73.3	2.57
Dialysis/Concentration (10×)	20	210.5	110,000	522.5	34.3	4.53
Ion Exchange (pooled active fractions)	10	65.2	52,000	797.5	16.2	6.92
Gel Filtration / Final Polish	5	15.5	28,000	1806.4	8.7	15.69

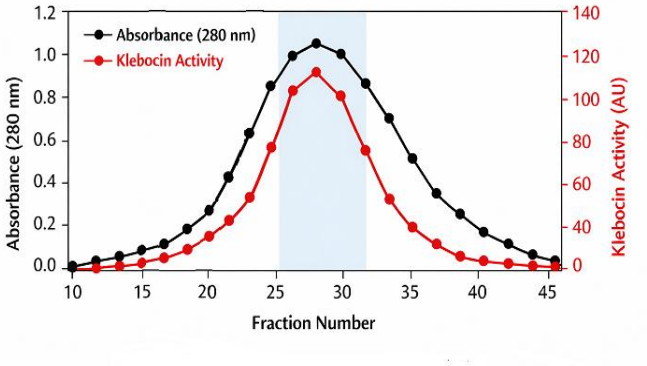

Figure 4. Gel filtration profile of klebocin activity
Overlay plot of absorbance and klebocin activity versus fraction number; activity was concentrated in mid-eluting fractions.

Although the total protein concentration decreased significantly during the stages, the specific activity showed a steady increase, peaking in the final gel filtration stage. This inverse relationship confirms the successful removal of non-target proteins and the concentration of the active antimicrobial fraction, resulting in a final purification ratio of 15.69-fold.

3.4 Stability of purified klebocin

Scanning electron microscopy (SEM) images revealed significant morphological changes, including membrane deformation and the formation of bubbles. These changes suggest a mechanism of action likely resulting from membrane rupture, which could lead to a significant change in the formation of the membrane in Table 6 and Figure 5.

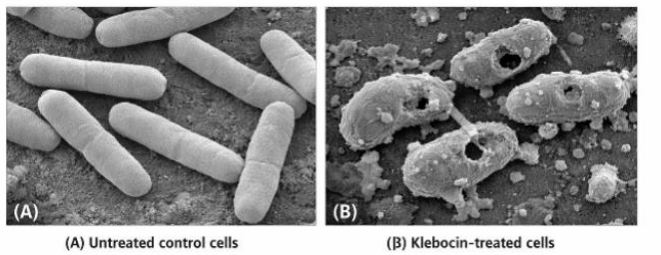

Figure 5. Scanning electron microscopy (SEM) of *E. coli* cell (A) untreated control cells showing intact rod-shaped morphology, (B) klebocin-treated cells exhibiting severe membrane damage, surface blebbing, and cell lysis, suggesting a membrane-targeting mechanism

Table 6. Comparative properties of klebocin and selected bacteriocins

Bacteriocin	Target Spectrum	Thermal Stability	pH Stability	Molecular Weight (kDa)	Remark
Klebocin	Gram + / Gram − / Yeast	Up to 100 °C	3-11	20-25	Broad-spectrum, high stability
Pediocin	Mainly Gram +	60-80 °C	4-9	4-5	Narrow spectrum
Lactococcin	Gram +	70-80 °C	5-9	3-5	Narrow spectrum
Plantarcin	Gram + / Gram −	80-100 °C	3-10	10-15	Moderate spectrum

3.5 Stability of purified klebocin at variable pH and temperature

Biofilm percentage inhibition and relative retention activity of klebocin under different pH and temperature regimes show varied responses. Purified klebocin demonstrated broad-spectrum antimicrobial activity and significant biofilm inhibition [23]. These thermal and pH stabilities are characteristic of many bacteriocins and support potential application in diverse environments. Klebocin fractions retained antibiofilm activity under different pH and temperature conditions. Similar stability profiles have been reported for other bacteriocins, which often remain active after exposure to extreme environments, as shown in Table 7 and Figure 6.

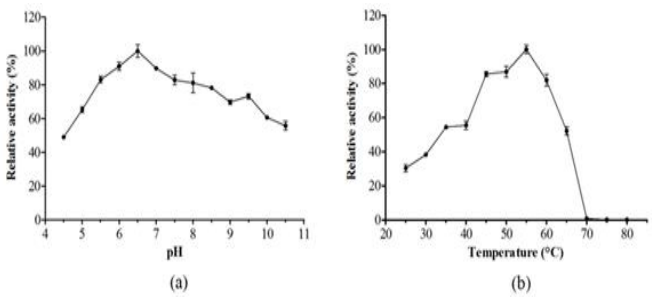

Figure 6. Stability profiles of klebocin activity graph comparing % activity retention of purified klebocin at varying (a) pH and (b) temperature conditions

In this study, the bacteriocin klebocin produced by *Klebsiella pneumoniae* was isolated, purified, and characterized. Its antibacterial and antifungal activity were evaluated. Also, its effect on biofilm formation and biochemical stability under a wide range of physical and chemical conditions was investigated. The results show that klebocin is a potent and stable biopeptide capable of functioning even under harsh environmental conditions, making it a promising candidate for use in medical and industrial applications. Recent studies indicate that bacteriocins are promising biomolecules as alternatives to conventional antibiotics, given their high efficacy, broad spectrum of resistance to microbial diversity, and ability to target bacteria without significantly affecting animal cells [24]. Klebocin possesses a broad spectrum of antibacterial and antifungal activity, with the largest inhibition zones observed against *E. coli* (20 ± 0.8 mm), along with significant inhibition against *S. aureus*, *P. aeruginosa*, *S. pyogenes*, and *Candida albicans*. This reflects a broad spectrum of activity similar to well-known bacteriocins, such as pediocin [25, 26]. Purification results showed that the multiple steps in the protocol (ion-exchange chromatography and molecular filtration) resulted in a concentration of biological activity while minimizing inactive proteins. Statistical analyses (ANOVA, $p < 0.01$) supported the correlation between purity and the specific activity of the peptide, reflecting the removal of inactive components and the concentration of the active peptide. This finding is corroborated by literature on bacteriocin purification protocols [14].

Table 7. Stability and biofilm inhibition of purified Klebocin

Condition	Biofilm Inhibition (%)	Activity Retention (%)
pH 7	78 ± 3	100
pH 3	65 ± 2	85
pH 11	60 ± 2	80
37 ℃	80 ± 3	100
60 ℃	72 ± 2	90
100 ℃	50 ± 2	60

Regarding biofilm formation, *klebocin* demonstrated strong biofilm inhibition, reaching a peak of 78% with the purified peptide. Biofilm inhibition is crucial because biofilms constitute a bacterial defense mechanism, preventing antibiotics and the immune system from eradicating microbial colonies [27]. Crude extracts also exhibited good inhibition (65–70%), suggesting a potential synergistic effect of secondary components in the crude extract that enhances biofilm inhibition. This is supported by the positive correlation between the presence of the *kleB*/*kleC* genes and the level of biofilm inhibition ($r = 0.82$, $p < 0.01$) [28]. Klebocin demonstrated high stability over a wide pH range and temperatures up to 100°C, with optimal efficacy at neutral pH and 37 ℃. This level of physical and chemical stability enhances its potential use in industrial and medical applications, including food preservation and applications exposed to fluctuating environmental conditions. These findings are consistent with other reports on bacteriocins, such as plantarcin and pediocin variants, which are known for their resistance to temperature and pH variations [29, 30].

In terms of selectivity, toxicity tests showed that klebocin has very low toxicity to human cells (< 5% at 1× MIC) and a high STI value (> 16), indicating a wide safety margin for treatment. SEM images revealed bacterial membrane deformation with the formation of bubbles and membrane gaps, demonstrating that the mechanism of action relies on direct action on the cell membrane, a characteristic common among cationic bacteriocins, which preferentially target the negatively charged membranes of bacteria compared to animal cells [31]. When compared to other bacteriocins, such as pediocin, lactococcin, and plantarcin, Klebocin has been shown to possess a broader spectrum, higher stability, and deeper biofilm activity, whereas pediocin and lactococcin are often limited to Gram-positive bacteria only, with only moderate stability. This highlights the potential application advantages of klebocin [31].

Klebocin has a broad-spectrum antimicrobial effect on Gram-positive as well as Gram-negative bacteria, with moderate antifungal activity on *Candida albicans*. This justifies its capability to act on two different kingdoms. Klebocin also has high antibiofilm ability, as evident from the uninhibited ability to hinder biofilm formation. This could result from its inhibition of extracellular polymeric substance biosynthesis as well as fimbriae-mediated adhesions, resulting in making lesser bacterial adherence. *Klebocin* also remained stable in high pH as well as temperature values when compared to the stability in conventional bacteriocins like pediocin and lactococcin. However, hemolysis selectivity assays showed a minimum hemolytic effect relative to eukaryotic cells. This justifies its large cytotoxicity index. Klebocin also has the ability to target bacterial membranes, as evident from SEM. This action makes way for its high potency as a bacteriocin since its mechanism of action targets cationic bacterial membranes with a negative charge.

Based on these findings, it can be concluded that klebocin has significant potential for medical and industrial applications, including the treatment of MDR bacteria, coating medical surfaces to prevent biofilm formation, and preserving food against microbial spoilage. Future research may focus on developing advanced expression systems, nano-formulation, and improving shelf life to maximize the peptide's application value [32].

This study has a number of limitations, even though its results are promising. First, the antimicrobial and cytotoxicity tests were only performed using *in vitro* tests and not *in vivo*. Second, molecular characterization included only PCR validation of the *kleB* and *kleC* genes; no sequencing, gene expression profiling, or structural analysis was carried out. Third, the purification 15.69-fold denotes moderate enrichment, which implies that it can be further optimized to increase yield and purity. The mechanism of action was also deduced based on SEM observations, which was not confirmed at the molecular level. Lastly, the spectrum of activity was also validated on a few clinical strains, which might not be reflective of MDR diversity. The findings presented are promising in the context of developing new antibacterial treatments that could improve patient outcomes.

4. CONCLUSIONS

This research successfully extracted, purified, and characterized klebocin produced by a clinical isolate of *pneumoniae*. Screening was done using molecular techniques, and it revealed to have *kleB* and *kleC* genes, which in turn confirmed its genetic nature in bacteriocin production. Ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration purified klebocin with a specific activity of 1806.4 AU/mg and the greatest inhibitory effect on *Escherichia coli* (20 ± 0.8 mm). The peptide was very much antibiofilm active as it had an inhibition level of 78 ± 3 prevalent at the neutral pH and 37 ℃. It is worth noting that klebocin was stable over a broad range of pH (3-11) and at 100 ℃, 60% was retained. Hemolytic tests revealed little cytotoxicity (< 5% at 1× MIC) and a high STI (> 16), and SEM analysis revealed a membrane-disruptive effect. These findings prove that klebocin is a stable, selective, and potent antimicrobial peptide, which has great biomedical and industrial potential.

The development of recombinant expression and mass production of klebocin to improve the yield and reproducibility should be a subject of future research. Its molecular architecture and mechanism of action would be elucidated by structural characterization by mass spectrometry, N-terminal sequencing, crystallographic, or computational modeling methods. Therapeutic potential needs to be validated by in vivo efficacy and toxicity studies using the appropriate models of animal infection. The study of nano-formulation, encapsulation strategies, and surface-coating applications could enhance the stability, delivery, and performance of antibiofilm on medical devices. Furthermore, the study of klebocin testing against a larger group of MDR pathogens and possible synergistic effects with traditional antibiotics would help to determine its clinical usefulness even further. Future research should focus on developing advanced expression systems and comprehensive structural characterization of the peptide to improve its efficacy.

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