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Infectious Diseases / Bulaşıcı Hastalıklar

In Vitro Antibacterial Activity of NET1 and NET3 Peptide Against Carbapenem-Resistant and Susceptible Klebsiella Pneumoniae

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ABSTRACT

Purpose: In recent years, antimicrobial resistance is one of the biggest and most important reasons for the difficulties in treating infections and threatening public health. One of the priority pathogens listed by the World Health Organization is carbapenem resistant Enterobacterales (Klebsiella pneumoniae). Antimicrobial peptides are considered as alternatives to antibiotics due to their broad-spectrum antibacterial properties and the difficulty of developing resistance. In this study, we investigated the antimicrobial effects of NET1 and NET3 peptides, which are known to have antimicrobial effects on different bacteria, on carbapenem-resistant and -susceptible K. pneumoniae control strains.

Methods: NET1 and NET3 peptides were synthesized and purified. In vitro antibacterial activity of the peptides against carbapenem-resistant and susceptible K. pneumoniae was demonstrated by minimum inhibitory concentration test and minimum bactericidal concentration test. In addition, the mechanism of action of the peptides on carbapenem-resistant K. pneumoniae cells was investigated by scanning electron microscopy.

Results: The minimum inhibitory concentration and minimum bactericidal concentration results of NET1 peptide are 4 µg/ml for carbapenem-resistant K. pneumoniae and 2 µg/ml for NET3 peptide. Scanning electron microscope imaging has shown that NET3 peptide exhibits antibacterial activity by damaging the carbapenem-resistant K. pneumoniae cell membrane and disrupting the permeability of the outer membrane.

Conclusion: When our results are evaluated, we show that NET1 and NET3 peptide has the potential to be a new generation broad-spectrum antibiotic candidate that can be used for the treatment of carbapenem-resistant K. pneumoniae infection.

Keywords: antimicrobial peptide, NET1, NET3, Klebsiella pneumoniae, carbapenem-resistance, antibacterial assay

ÖZET

Amaç: Son yıllarda halk sağlığını tehdit eden ve enfeksiyonların tedavisinde yaşanan zorlukların en büyük ve en önemli nedenleri arasında antimikrobiyal direnç gelmektedir. Dünya Sağlık Örgütü tarafından listelenen öncelikli patojenlerden biri de karbapenemlere dirençli Enterobacterales (ör: Klebsiella pneumoniae)'dir. Antimikrobiyal peptitler, geniş spektrumlu antibakteriyel özellikleri ve direnç gelişiminin zor olması nedeniyle antibiyotiklere alternatif olarak kabul edilmektedirler. Bu çalışmada, farklı bakteriler üzerinde antimikrobiyal etkisi bilinen NET1 ve NET3 peptidinin karbapenem dirençli ve duyarlı K. pneumoniae kontrol suşları üzerindeki antimikrobiyal etkisini araştırdık.

Yöntemler: NET1 ve NET3 peptitleri tarafımızca sentezlenip saflaştırılmıştır. Peptitlerin karbapenem dirençli ve duyarlı K. pneumoniae'ye karşı in vitro antibakteriyel aktivitesi minimum inhibitör konsantranyon testi ve minimum bakterisidal konsantrasyon testi ile gösterilmiştir. Ayrıca peptitlerin karbapenem dirençli K. pneumoniae hücresi üzerindeki etki mekanizması taramalı elektron mikroskobu ile araştırılmıştır.

Bulgular: NET1 peptidinin minimum inhibitör konsantrasyon ve minimum bakterisidal konsantrasyon sonucu karbapenem dirençli K. pneumoniae için 4 µg/ml, NET3 peptidinin 2 µg/ml'dir. Taramalı elektron mikroskobunda yapılan görüntülemede, NET3 peptidinin, karbapenem dirençli K. pneumoniae hücre zarına zarar vererek ve dış zarın geçirgenliğini bozarak antibakteriyel etki gösterdiği ortaya konmuştur.

Sonuç: Sonuçlarımız değerlendirildiğinde, NET1 ve NET3 peptidinin karbapenem dirençli K. pneumoniae enfeksiyonunun tedavisi için kullanılabilecek yeni nesil geniş spektrumlu antibiyotik adayı olma potansiyeline sahip olduğunu göstermektedir.

Anahtar Kelimeler: antimikrobiyal peptit, NET1, NET3, Klebsiella pneumoniae, karbapenem direnci, antibakteriyel test

Antimicrobial resistance (AMR) has emerged as one of the major public health threats in recent years (1–3). The spread of antimicrobial resistance among bacteria leads to treatment failure worldwide (4,5). AMR arises when bacteria undergo evolutionary processes that lead them to become resistant to antimicrobial drugs commonly used to treat infections (1,6,7). This widespread problem is largely attributed to the repercussions of overuse or irresponsible use of antibiotics (8). It has been reported that if preventive measures against AMR are not taken, AMR could potentially replace all other causes of death worldwide by 2050 (9).

This problem is common among people with *Klebsiella pneumoniae* infection. *K. pneumoniae* is a Gram-negative opportunistic pathogen and is frequently seen in nosocomial infections (10). The increasing prevalence of antimicrobial drug resistance is a major problem leading to high mortality and morbidity rates, especially in intensive care units (ICUs) (11,12). Carbapenems are among the antibiotics used to treat *K. pneumoniae* infections (13). The unconscious use of antibiotics is the most important reason for the emergence of carbapenem-resistant *K. pneumoniae* (CRKP) (14,15). Multidrug resistance (MDR) and carbapenem-resistant *K. pneumoniae* (CRKP) have limited treatment options for many infections (16,17). In the last decade, the worldwide spread of carbapenem-resistant *K. pneumoniae* (CRKP) has emerged as one of the most important crises due to difficulties in treatment (18). Infections caused by *K. pneumoniae* have a significant impact on mortality, especially in critically ill and immunocompromised patients (19). In conclusion, alternative strategies for the treatment of CRKP infections are needed.

In recent years, scientists have emphasized the importance of antimicrobial peptides (AMPs) produced by organisms living in nature as an alternative to antibiotics. AMPs are peptide molecules that organisms produce to protect themselves from infections. For the treatment of infections against antibiotic resistance, it is important to modify and develop existing natural AMPs to have broad-spectrum effects. In this context, many studies have shown that artificially produced molecules inspired by natural AMPs can be used to confirm this idea. These studies have shown that AMPs are suitable for the production of new antimicrobial drugs because they are natural molecules with low toxicity on human cells and broad-spectrum activity on microorganisms (20–22).

NET1 and NET3 peptides are 16 amino acid long, protease-resistant peptides that have been developed to resemble the three-dimensional structure of catalicidin LL-37, a natural AMP. In our previous study, we demonstrated that the NET1 and NET3 peptides exhibit antibacterial activity against clinically important bacteria, including *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *S. aureus* ATCC 25923, *E. coli* NTCC 13846, *E. coli* ER2566, and a vancomycin-resistant *Enterococcus* (VRE) clinical isolate.Toxicity tests also showed that the minimum inhibitory concentration results of these peptides were 5 times below the half maximal inhibitory concentration (IC50) and half maximal cytotoxic concentration (HC50) values, indicating that they were in the toxic safe range (22). There is no information about the antimicrobial effect of these AMPs on carbapenem resistant and susceptible *K. pneumoniae* ATCC BAA1705 and BAA1706. The *Klebsiella pneumoniae* strains ATCC BAA-1705 and ATCC BAA-1706 are frequently utilized as reference strains in antimicrobial resistance studies due to their well-characterized resistance profiles. ATCC BAA-1705 is a carbapenem-resistant strain that produces the KPC-2 (*Klebsiella pneumoniae carbapenemase*) enzyme, conferring high-level resistance to beta-lactam antibiotics, including carbapenems such as imipenem and meropenem (23). This strain is commonly used as a positive control in molecular and phenotypic assays for the detection of KPC-producing organisms. In contrast, ATCC BAA-1706 lacks the KPC gene and remains susceptible to carbapenems, making it an ideal negative control for comparative studies (24). Both strains were originally isolated from human clinical samples and serve as important tools in understanding resistance mechanisms and validating diagnostic methods for multidrug-resistant *K. pneumoniae* (25).

In this study, we investigated the antibacterial activity and mechanism of action of NET1 and NET3 peptides, especially against the carbapenem-resistant *K. pneumoniae* ATCC BAA1705 strain by *in vitro* studies. Our results showed that NET1 and NET3 peptides may be potential antibiotic alternatives for the treatment of carbapenem-resistant *K. pneumoniae* infections and are worth further investigation.

Material and Methods

Solid-Phase Chemical Synthesis and HPLC Analyses of AMPs

Peptide synthesis was conducted on the CEM Liberty Blue peptide synthesizer. The synthesis process was according to standard deprotection and coupling protocols. Following synthesis, peptides were cleaved using the Razor™ device according to the manufacturer's instructions, and peptides were purified as a powder. The sequences of the synthesized peptides are presented in Table 1. First, the synthesized peptide antibiotics were concentrated to 1 mg/mL for reversed-phase high-performance liquid chromatography (RP-HPLC) and were analyzed on an analytical column to estimate their relative hydrophobicity from peak retention times. Two peptides produced a single sharp peak on the HPLC chromatogram, as shown in Figure 1, which showed good purity and avoided purification. The levels of the antimicrobial peptides (AMPs) were quantified by the Pierce™ Quantitative Fluorometric Peptide Assay Kit (Thermo Fisher).

Bacterial Strains

K. pneumoniae ATCC BAA1705 and *K. pneumoniae* ATCC BAA1706 standard strains were used in the study. The strains were inoculated on blood agar and incubated at 37°C overnight.

In Vitro Antibacterial Testing

NET1 and NET3 peptide antibiotics were used in antimicrobial activity assays. For this purpose of the study, *K. pneumoniae* ATCC BAA1705 and *K. pneumoniae* ATCC BAA1706 bacterial strains were used.

For the minimum inhibitory concentration (MIC) assay, 0.5 McFarland bacterial suspension was prepared in Mueller Hinton Broth (MHB) medium. The final bacterial concentration was diluted to 10^6 cfu/ml. In 96-well plate, serial dilutions from 128 to 0.25 µg/mL for NET1 and NET3 peptides in MHB medium were made and 5 µl of bacterial suspensions were added. Incubated overnight at 37°C. The results were evaluated based on turbidity in the wells and real-time absorbance measurements taken every 30 minutes at a wavelength of 600 nm (22).

To determine the minimum bactericidal concentration (MBC) assay of NET1 and NET3 peptides, 10 microliter samples were taken from the wells that did not show growth in the MIC test and inoculated on blood agar. It was incubated at 37°C overnight. The next day, growth was evaluated. The lowest concentration with no growth at the end of the incubation period indicates the concentration of total cell death. The MIC and MBC value of each peptide is given in Table 1 and Figure 2.

Table 1: Peptide sequences

	Peptide Sequence	Amino acid content
NET1	RLLLRLLRRLLRLLLR-NH2	D- leucine, L- arginine
NET3	RLLLRLLRRLLRLLLR-NH2	L- leucine, D- arginine

SEM (Scanning Electron Microscope)

The mechanism of action of NET3 peptide with the lowest MIC value on *K. pneumoniae* ATCC BAA1705 cells was demonstrated by SEM. Peptide concentration of 16 µg/mL was used. NET3 peptide was mixed with 10^6 CFU/mL *K. pneumoniae* ATCC BAA1705 and incubated at 37°C for 4-5 hours. As reported in the literature, the incubation time was kept short due to the possibility of dissociation of the peptide and the bacteria. After incubation, 20 µL of the mixture containing peptide and bacteria was applied to a dialysis membrane, which was then fixed and visualized by SEM microscopy. As a negative control, only bacteria were used (21,22).

Results

Chemical Synthesis of AMPs and Purifications with HPLC

The peptides synthesized using the CEM Liberty Blue® peptide synthesizer were obtained with approximately 95% purity, supported by both instrument and manufacturer assurance. Analytical HPLC results showed a single distinct peak for the peptides. (Fig. 1).

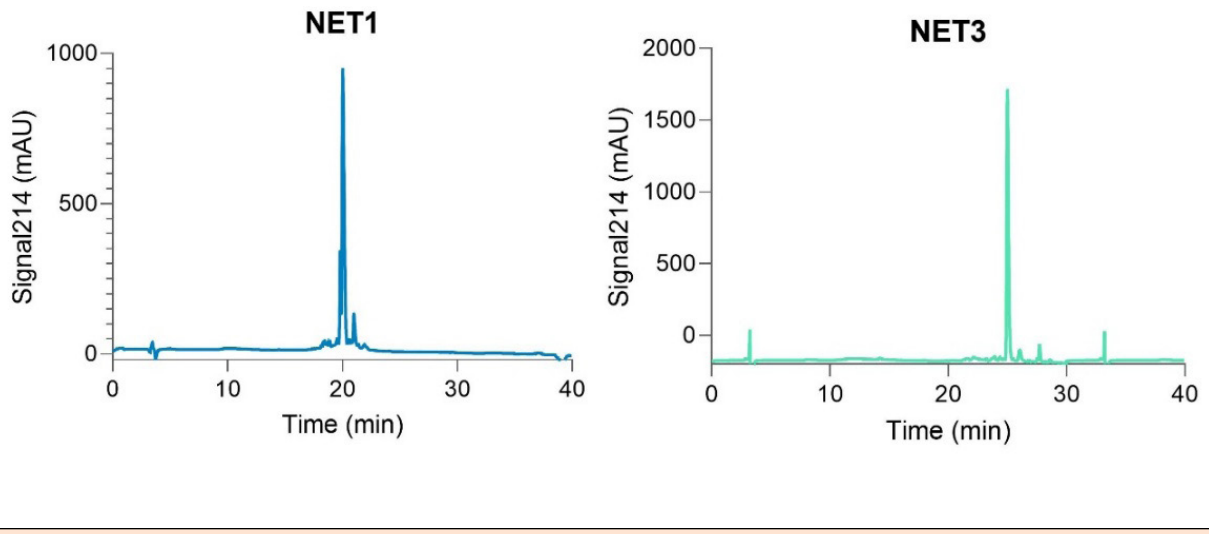

Figure 1: HPLC result of NET1 and NET3 peptides

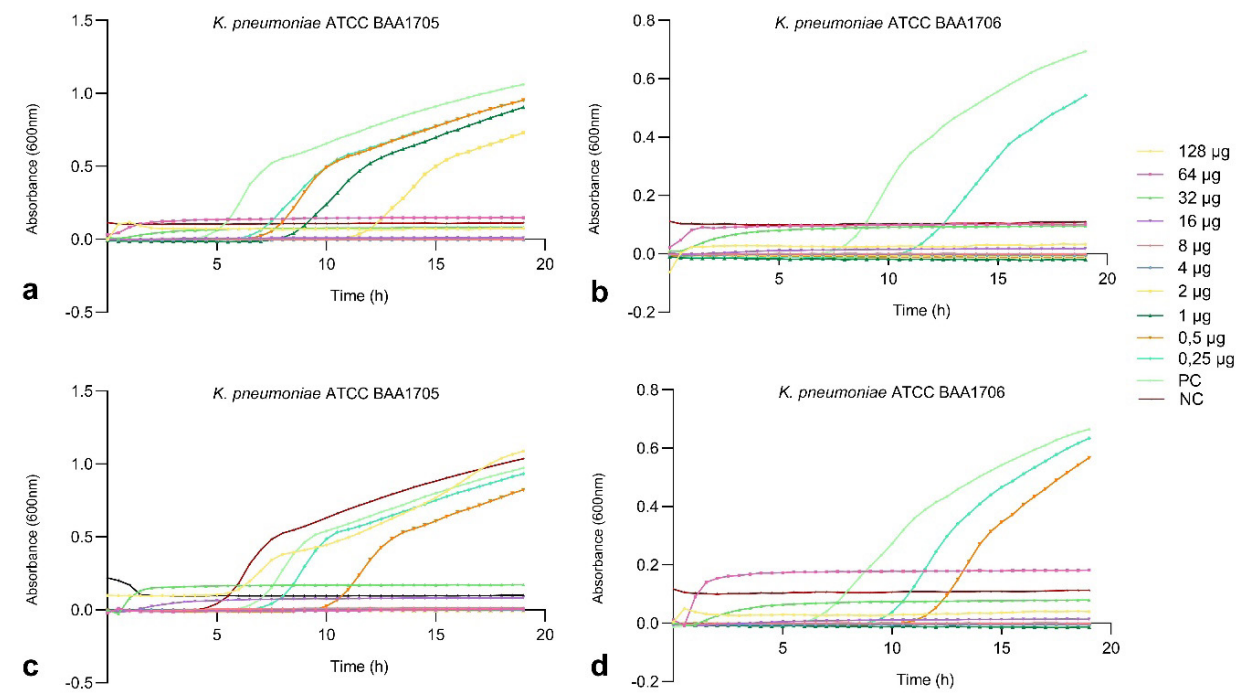

Figure 2: Real-time monitoring results of bacterial growth at 600 nm are shown in panels a and b for the NET1 peptide, and in panels c and d for the NET3 peptide. NC: Negative control (no bacteria); PC: Positive control (contains bacteria, no peptide).

Table 2: MIC and MBC results of NET1 and NET3 peptides

Bacterial strains	MIC µg/mL)		MBC µg/mL)	
	NET1	NET3	NET1	NET3
K. pneumoniae ATCC BAA1705	4 µg	2 µg	4 µg	2 µg
K. pneumoniae ATCC BAA1706	0.5 µg	1µg	0,5 µg	1µg

Scanning electron microscopy (SEM)

When SEM images were examined, it was seen that the *K. pneumoniae* ATCC BAA1705 strain used as control and not treated with NET3 peptide had a smoother cell surface morphology compared to *K. pneumoniae* ATCC BAA1705 treated with NET3 peptide (Figure 3). It was observed that the surface morphology of *K. pneumoniae* ATCC BAA1705 treated with NET3 peptide had changed and had different shaped blisters. Thus, it was shown that NET3 peptide was lethal for carbapenem-resistant *K. pneumoniae* ATCC BAA1705.

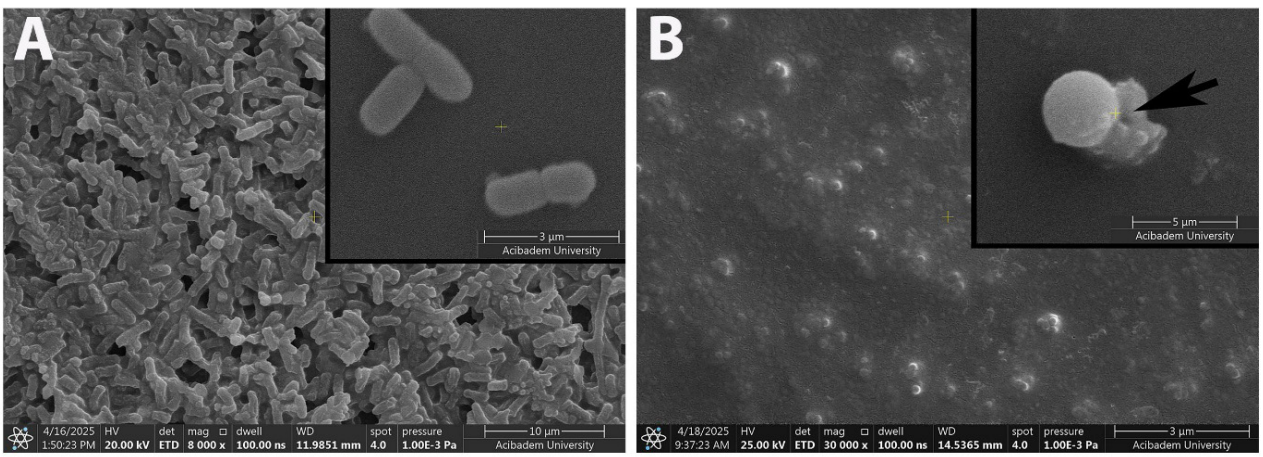

Figure 3: Representative SEM micrographs of experimental groups. Numerous bacteria with normal morphology were observed in the Control group (A, inset A). In contrast, the Peptide group (B, inset B) exhibited degenerated bacterial morphology, including craters and ruptured cells (arrow). Magnifications: A – 8,000×; inset A – 30,000×; B – 30,000×; inset B – 14,000×. Scale bars: A – 10 µm; inset A – 3 µm; B – 3 µm; inset B – 5 µm.

Discussion

Antimicrobial resistance has emerged as a serious problem affecting all living organisms in recent years. The most important reasons for this problem include the unconscious and excessive use of antibiotics, as well as mutations in microorganisms.

Antimicrobial resistance is considered to the most important problem in the treatment of carbapenem-resistant *K. pneumoniae* (CRKP) infections. This is because CRKP is resistant to many antibiotics, such as fluoroquinolones, aminoglycosides, and β-lactams (26).

Among the solutions, the development of new antibiotics and the use of AMPs are considered very promising. AMPs are naturally found in the immune systems of living organisms and can be designed and synthesized synthetically. AMPs have broad antibacterial activity and low resistance potential, giving them the potential to be new generation antimicrobial agents. Many AMPs cause microbial death by causing membrane interaction and disruption of membrane integrity. The presence of positively charged cationic amino acids in the structure of AMPs maximizes peptide membrane interaction (22).

NET1 and NET3 peptides have been shown to be protease-resistant, antibacterial, AMPs with 16 amino acids in their structures (22). However, the antibacterial activity of these peptides against *K. pneumoniae* is unknown. The aim of this study was to determine the in vitro antibacterial activity and potential mechanisms of NET1 and NET3 peptides, especially on carbapenem-resistant and susceptible *K. pneumoniae*.

In this study, NET1 and NET3 peptides were synthesized with a peptide synthesizer in Acibadem Mehmet Ali Aydinlar University research laboratories, purified by HPLC and lyophilized. The antimicrobial activities of NET1 and NET3 on carbapenem-resistant and susceptible *K. pneumoniae* were determined by MIC assay performed with microbroth dilution method and MBC assay performed to determine total cell death. When MIC and MBC results were evaluated, we found that NET1 and NET3 peptides exhibited strong antimicrobial activity on carbapenem-resistant and susceptible *K. pneumoniae*. In addition, we visually observed the effect of NET1 and NET3 peptides on bacterial morphology under electron microscope. We observed that NET3 peptide particularly disrupted the cell morphology of carbapenem-resistant *K. pneumoniae* and caused cell shrinkage, cell morphology crumpled, collapsed, and ruptured.

In the study conducted by Huijing Zhou and colleagues, it was stated that the MIC and MBC values of A20L on carbapenem-resistant and susceptible *K. pneumoniae* strains were 4 µg/ml (22). In another study by Deyi Zhao et al., when the antibacterial activity results of Hs02 peptide in carbapenem-resistant *K. pneumoniae* clinical isolates were evaluated, the lowest MIC value was 8µg/ml (27). Samantha J. Hitt et al. examined the antibacterial effect of peptides inspired by Komodo-dragon cathelicidin against carbapenem-resistant *K. pneumoniae* and reported that the MIC value was in the range of 4-8 µg/ml (28) While the MIC and MBC values of NET1, which are among the peptides we used in our study, were found to be 4 µg/ml on carbapenem-resistant and susceptible *K. pneumoniae,* the MIC and MBC values of NET3 peptide on carbapenem-resistant *K. pneumoniae* were found to be 2 µg/ml, and the MIC and MBC values on carbapenem-susceptible *K. pneumoniae* were found to be 0.5 µg/ml. While carbapenem-derived antibiotics are effective at a concentration of 4-16 µg/ml on *K. pneumoniae* ATCC BAA1705 (carbapenem-resistant) bacteria (29), the NET3 peptide is effective at a concentration of 2 µg/ml. This shows that NET3 peptide is more effective than currently used carbapenem antibiotics. In addition to carbapenems, ceftazidime-avibactam and colistin are among the last-resort antimicrobial agents used against carbapenem-resistant *K. pneumoniae*. However, there are also strains that exhibit resistance to these antibiotics. In such cases, peptides like NET3 may provide an alternative treatment option (30).

Although the clinical development of AMPs is limited by various factors such as possible toxicity and stability, reducing toxicity and improving their stabilization are important studies to be carried out in future research to develop new AMPs.

This study has several limitations. The effect of the peptides was tested only in carbapenem-resistant and susceptible reference *K. pneumonia* strains. Since carbapenem resistance and susceptibility profiles in clinical isolates may differ, studies with clinical isolates are required to fully understand the efficacy of the peptides and to determine MIC values.

Conclusion

In conclusion, this study showed that NET1 and NET3, which exhibit antibacterial peptide properties, have in vitro antibacterial activity against carbapenem-resistant *K. pneumoniae*, and this effect occurs through the membrane destruction mechanism, therefore, they have potential antibiotic properties.

Declarations

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Ethical Approval

This study involved only in vitro experiments using laboratory strains of Klebsiella pneumoniae. No human participants, human-derived materials, or animal subjects were involved. Therefore, ethical approval was not required.

Author Contributions

Concept: N.Ü, Literature Review: N.Ü, Design: N.Ü, Data acquisition: N.Ü, T.P, M.E and M.A, Analysis and interpretation: N.Ü, T.P and M.E, Writing manuscript: N.Ü, Critical revision of manuscript: N.Ü and M.A

Conflict of Interest

The authors have no conflicts of interest to declare.

Financial Disclosure

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