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Evaluation of the antibiofilm effects the related mechanisms on colistin-resistant *Pseudomonas aeruginosa*

Zinah Ayyed Habeeb [1*], Sanaa Khudhur Jameel [1], Mais Emad Ahmed [2]

[1] Medical Microbiology Department, College of medicine, AL-Iraqia University, Baghdad, Iraq.
[2] Department of Biology, College of Science, University of Baghdad, Jadriya, Baghdad, Iraq

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* Corresponding Author: Zinah Ayyed Habeeb
Email: zeina.a.habib@aliraqia.edu.iq

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Abstract

Pseudomonas aeruginosa is thought to be the third most frequent cause of catheter-associated UTIs. The production of urease greatly increases the effectiveness of catheter blockage caused by biofilm formation. Because biofilms are important virulence factors that make antibiotics less effective, there is an urgent need to develop novel antibiotic substitutes. Urinary tract infections have been mostly linked to *Pseudomonas aeruginosa* (*P. aeruginosa*). The final line of treatment for *P. aeruginosa* infections is colonistin. But, when it comes to treating individuals with colistin-resistant (COL-R) *P. aeruginosa*, colistin is losing its effectiveness. This study looked into how the antibiofilm, characteristics, and underlying mechanisms of COL-R *P. aeruginosa*. The results showed the sub–Minimum Inhibitory Concentration (MIC) at (62 µg/ml), with remarkable biofilm inhibitory outcome in wild type strains of multidrug resistant *P. aeruginosa*. Strong biofilm producer strains were incubated with 1ml of sub-MIC of colistin for 24 and 48 hours at 37C. The colistin was also examined for their ability to inhibit the biofilm-forming *P. aeruginosa*. Colistin-provide an innovative strategy for biomedical therapy of resistant bacteria because of their elevated antibacterial effect, but only for certain purposes.

Keywords: *Pseudomonas aeruginosa*, Biofilm, colistin, Antibiofilm

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that belongs to the γ-proteobacteria family. It is well known to cause pneumonia associated with ventilator usage and nosocomial infections. The need to find substitutes for traditional antibiotics is urgent due to the rise in the number of resistant germs. Plant-derived substances (PDSs) can modulate antibiotic resistance and act as antibacterial agents (1). Although *P. aeruginosa* seldom infects healthy individuals, it can cause serious infections in immunocompromised and cystic fibrosis patients, which are linked to high rates of morbidity and mortality (2). Various virulence factors in *Pseudomonas aeruginosa* contribute to its alarmingly high pathogenicity and resistance to commercially available antibiotics. Finding a fresh substitute for conventional antimicrobials is therefore

essential. Because of its antibacterial, anti-inflammatory, and antioxidant qualities, resveratrol is a well-known phytochemical with numerous positive health effects(3).
One of the 21st century's most potentially catastrophic problems is antimicrobial resistance. Due to its limited antibiotic treatment options, *Pseudomonas aeruginosa* (*P. aeruginosa*) is a clinically relevant pathogen that is the most resistant. The World Health Organization lists *P. aeruginosa* as one of the top three critically resistant bacteria, raising concerns about its increasing resistance. Since *P. aeruginosa* resistance is now known to be strongly correlated with the use of all antibiotics, utilization surveillance is essential (4). Since they are resistant to antibiotics, antibiotic-resistant bacteria pose a serious threat to public health today. Uro-pathogens, or antibiotic-resistant gram-negative bacteria, include *Escherichia coli* (UPEC), *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Enterococcus faecalis*. These bacteria cause severe, protracted UTIs that can be fatal and raise medical expenses (5). Urinary tract infections (UTIs), which include any infection of the kidneys, bladder, or urethra, are responsible for an estimated 400 million infections and billions of dollars in medical costs. Although many other infections, including *Klebsiella*, *Enterococcus*, *Pseudomonas*, *Staphylococcus*, and even yeast, such as *Candida* species, can cause UTIs, *Escherichia coli* is the most commonly linked bacterium to UTIs. Both men and women, as well as healthy and immunocompromised patients, can get UTIs. Nonetheless, some patient characteristics make them more susceptible to illness, such as being female, having a history of UTIs, or having a urinary catheter or other urinary tract abnormalities (6). Resistant to carbapenem, according to the World Health Organization, *Pseudomonas aeruginosa* is a "high priority" for developing novel antimicrobials. In fact, the risk of morbidity and death for infected individuals is increased when multidrug-resistant (MDR) or extensively drug-resistant (XDR) bacteria arise and proliferate. *P. aeruginosa* genomic variations exhibiting MDR/XDR characteristics have been classified as high-risk global clones. A few high-risk foreign clones possess β-lactamase genes, which can cause chronic colonization and raise the morbidity and fatality rates of infected patients (7). Worldwide, the resistant to multiple drugs gram-negative bacterial infections are a major source of morbidity and mortality. The clinical importance of these infections is further highlighted due to develop antimicrobial resistance (AMR). *Pseudomonas aeruginosa* is resistant to many drugs, *Enterobacterales* (such as *Escherichia coli* and *Klebsiella* species), and carbapenem-resistant the most troublesome and designated priority pathogens include *Acinetobacter baumannii*. Several novel diagnostic technologies—such as biochemical, molecular, genomic, and proteomic approaches—have been created to identify AMR (8) quickly. Antimicrobial resistance contributes significantly to global mortality and economic cost, making it a serious global health concern. The development of novel antimicrobial medicines is hampered by the big pharmaceutical corporations' cessation of research, and the problem is made worse by superbugs such as multidrug-resistant, extensively drug-resistant, and pan-drug-resistant varieties. Once thought to be harmful for therapeutic usage, colistin is now being re-examined as a "last resort" antibiotic to combat Gram-negative bacteria resistant to multiple drugs (9). Colistin is used as a last option, especially in patients who are very sick, to treat Gram-negative bacterial infections that are resistant to many drugs. However, it still poses a serious risk to public health. Previous study evaluated the percentage of colistin-resistant Gram-negative isolates from infections in intensive care units (ICUs) across various years, regions, pathogens, and antimicrobial susceptibility testing (AST) (10). The development of gram-negative isolates resistant to colistin is a major obstacle to infection control, especially in tertiary care settings. The multicellular, surface-associated growth mode known as biofilm formation is a crucial

bacterial activity impacted by sub-lethal antibiotic dosages. Biofilms are collections of bacteria in a matrix of extracellular polymeric substances, which include proteins, lipids, nucleic acids, and polysaccharides (11).

Numerous bacteria can transition between planktonic and biofilm forms. Although they have a lower chance of surviving, planktonic bacteria can adapt to live in different environments due to their comparatively rapid cell growth and reproduction rates. Bacteria naturally and primarily exist in biofilm conditions. Bacterial biofilm development is essential because it strengthens the bacteria's resilience to adverse environmental circumstances. By merely adhering to a surface or tissue, bacteria can resist being washed away by water flow or the bloodstream, and the EPS matrix shields the deeper layers of bacterium cells from antimicrobial agents. The initial contact or attachment to the surface is the first stage in forming a biofilm, microcolony formation, biofilm maturation and architectural formation, and biofilm detachment or dispersion (12). Additionally, epidemiological research has demonstrated that *P. aeruginosa* infections may considerably raise the rate of death, morbidity, surgical intervention, chronic care, and treatment expenses. Therefore, the current study focuses on effective treatment options to avoid biofilm infections linked to *P. aeruginosa*. Pediatric and critical care medicine have significant rates of resistance, particularly in neonatal patients. However, antibiotic resistance differs by department and age, requiring customized antimicrobial treatment. New-born babies' resistance to antibacterial drugs is alarming, and extra care is required during therapy.

METHODS

Sample collection

One hundred and two distinct specimens were collected from urine catheters patients who were referred to the Baghdad Medical City, Imam Ali (Jawader) and Martyr Sadr Hospitals. The samples were collected from Baghdad during October and December 2024. The samples were collected using sterile cotton swabs and kept in a sterile tube with transport media before being brought to the laboratory.

Isolation and identification

P. aeruginosais was validated by the VITIC 2 method after being identified mainly by growing on MacConkey and Blood agar at 37C for 24 hours.

Isolation and Identification of Pseudomonas aeruginosa

MacConkey agar (Lab/England) was used to culture the specimens, and aerobically incubated for 24 hours at 37°C. The former colonies of pale non-lactose fermenters were chosen, grown on Cetrimide agar (Himedia/India), and then incubated correspondingly. To perform the Gram-stain, oxidase, and catalase tests, one colony of *P. aeruginosa* has been administered on Cetrimide medium. To verify the identification of the bacterial isolate, the Vitek 2 Compact system was tested using a Gram-negative (GN) card that included 48 biochemical experiments.

Antibiotic susceptibility test

The Vitek 2- Compact system used the AST-N204 card for *P. aeruginosa* to conduct the antibiotic susceptibility test. For 33 clinical isolates, susceptibility, resistance, and MIC values and analyses were mechanically collected. Finding the isolates' Minimal Inhibitory Concentrations (MIC) and antibiotic sensitivity: Vitek 2-Compact performed the antibiotic susceptibility test for *P. aeruginosa* using the (AST-N204) card. MIC, susceptibility, and resistance measurements were also done. Automatic recording of interpretations was also recorded. These procedures were done in accordance with the manufacturer's instructions (Biomerieux, France), every step taken in this test was identical to the one used for Vitek 2-Compact's definite identification that was previously demonstrated.

Biofilm activity of *P. aeruginosa*

Briefly, 18-hour-old cultures of the chosen strains were added to a sterile BHI at a final cell concentration of 10^8 CFU/ml. Sterile polystyrene microtiter plates with 96 wells have been loaded with 180 µl of brain heart infusion broth that contained 1% glucose. A 20 µl of bacterial suspension was put into each of three sterile 96-well polystyrene microtiter plates. Six wells filled with bacterial-free BHI and acts as a negative control. To enable bacteria to develop biofilms, the prepared plates had been incubated for 24 hours at 37 °C. Following development, the intensity of the biofilms was verified using a crystal violet assay. After being safely disposed of, the cultures were thoroughly cleaned with 1X PBS. After fixing the adhering biofilms for 15 minutes with 150 µl of methanol, the extra was thrown away. With a 10-minute drying period at room temperature, 250 µl of a 0.2% (w/v) crystal violet solution was used to dye the plates for 15 minutes and wished up with distilled water. The conforming Crystal Violets was removed from the biofilms using 95% ethanol (v/v), and the biofilms were then cultured for 15 minutes for calculating absorbance at OD 630 nm (13) .

Antimicrobial susceptibility test

The minimum inhibitory concentration (MIC) of colistin was determined using the cationic adjusted Mueller–Hinton broth (CAMHB) microdilution method. Colistin Eas, acquired from Zhejiang, China's Ltd., was diluted twice and submitted to a series of two-fold dilutions ranging from 1000 to 62.5 µg/mL for colistin synthesized on CAMHB 96-well microtiter plates. Each well was filled with a final bacterial suspension of 1.5×10^6 CFU/mL, and the plate was then treated with colistin for 18 hours at 37°C. The antimicrobial susceptibility test was interpreted using the 2020 CLSI breakpoint for antibiotics (intermediate < 2 µg/mL; resistant > 4 µg/mL), and each MIC test was independently verified as a duplicate (14).

Screening for antibiofilm activity of Colistin

Each colonist's antibiofilm activity was assessed independently using a modified microtiter plate assay approach (15). Ten milliliters of bacteria was incubated for 24 hours on brain heart infusion broth and at kept 37°C for 24 hours. The cultures were subsequently diluted to 1.5×10^8 CFU/ml using Densicheck. Following that, 96-well microplates containing 180 microliters of BHI with 1% glucose were filled with 10

microliters of the diluted culture. Each microplate well received 200 µl of media, 10 µl of an incubated bacterial culture, and 10 µl of MIC-concentrated Colistin. Only the sterile liquid medium has been employed as a control. The wells were washed four times with 0.2 ml of phosphate-buffered saline (PBS, pH 7.2) after incubation. A 95% (v/v) methanol was used to fix the biofilms in the wells, and 0.2% crystal violet was used to dye them. Once the wells were dry, 200 µl of 95% (v/v) ethanol was added after any excess dye had been removed with distilled water. After measuring the optical density at 630 nm in a multi-plate reader, the biofilm inhibition activity was calculated using the following formula : % biofilm inhibition = 100- (OD of Colistin treated cells/ OD sample) *100

Results

P. aeruginosa was isolated from 102 bacterial specimens collected from patients referred to three hospitals in Baghdad, 33 (32.353%) were diagnosed as On MacConkey agar, P. aeruginosa forms circular, mucoid, and smooth pale colonies that had a sweaty grape odor. While bacterial colonies showed greenish yellow on Cetrimide agar (Figure.1 A& B). Gram staining revealed that they were Gram-negative bacilli. In addition, catalase and oxidase were positive. The Vitek 2-test indicated that all 33 isolates were confirmed as *P. aeruginosa*.

Table 1: Isolation percentage of bacteria according to specimen source

Specimen source	**Positive n. (%) from 33 Isolates**	**Positive n. (%) from 102 Specimens**
Urine	33 (100%)	(32.35%)

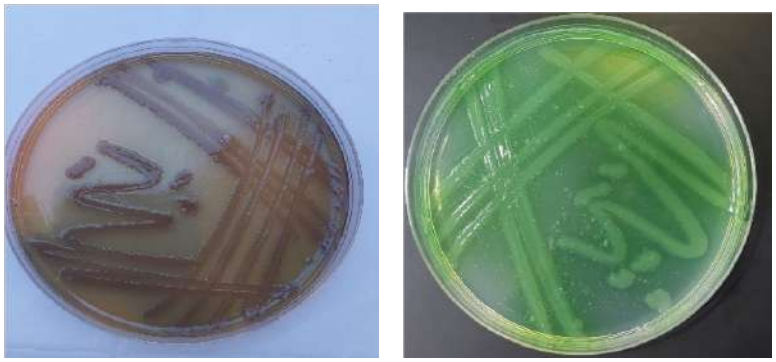
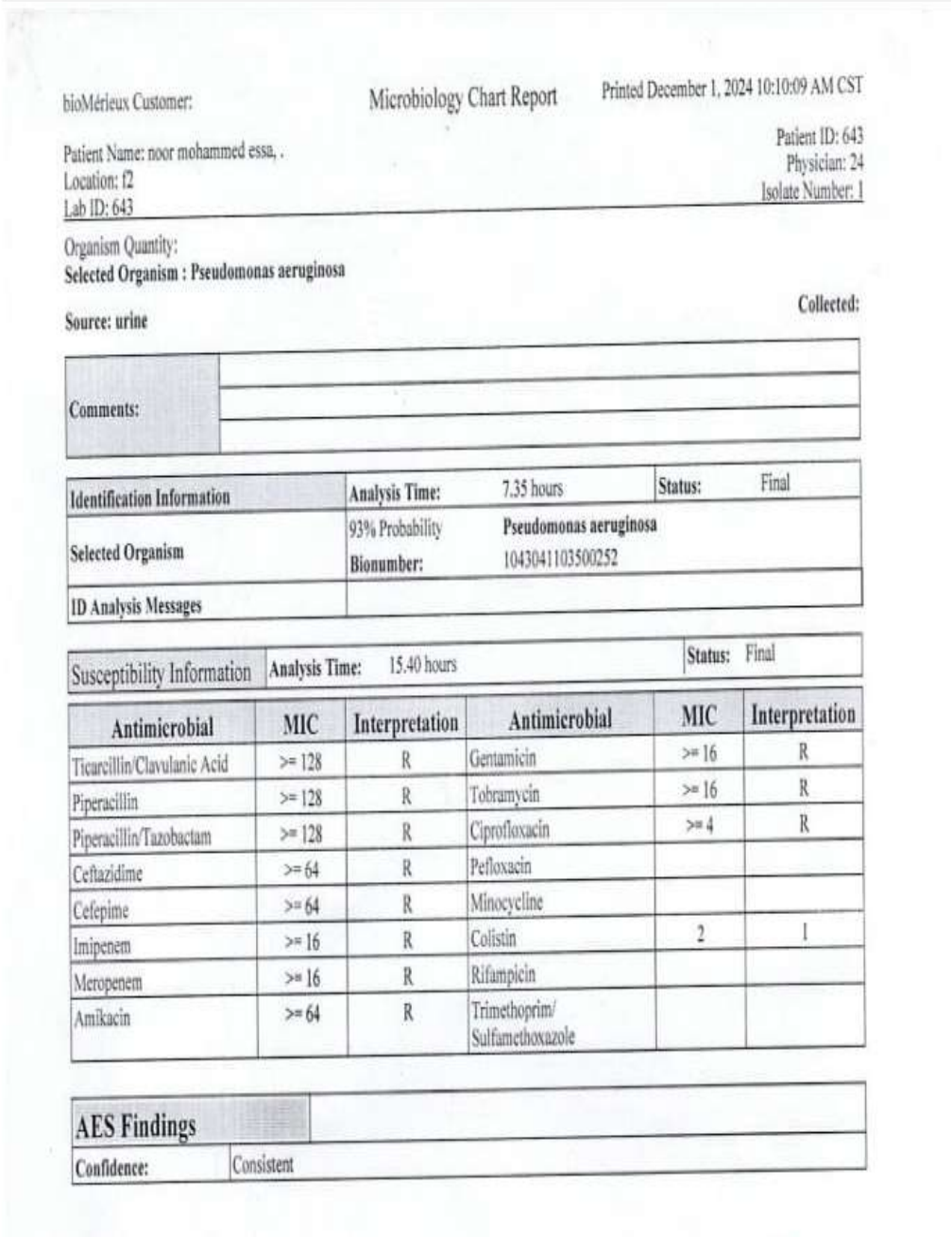


Figure. 1: *P. aeruginosa* on (a) MacConkey agar, (b) Cetrimide agar
Identification by VITEK 2-System

Compact system was used to assess the antibiotic susceptibility of all 33 *P. aeruginosa* i solates to nine different medications from four different antimicrobial groups.
The Clinical and Laboratory Standards Institute 2022 (CLSI) criteria were used to interp ret the results. After testing for oxidase and catalase activity, all of the bacterial isolates that grew on this medium were found to be oxidase and catalase positive. They were also tested for their ability to grow at 42 º C, and all of the isolates were able to do so. Using the VITEK 2 technology, 45 *Pseudomonas aeruginosa* isolates with a 98%–99% likelihood of identification were confirmed. **(**Figure -2).



bioMérieux Customer: Microbiology Chart Report Printed December 1, 2024 10:10:09 AM CST

Patient Name: noor mohammed essa, .
Location: f2
Lab ID: 643

Patient ID: 643
Physician: 24
Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source: urine

Collected:

Comments:	

Identification Information	**Analysis Time:** 7.35 hours	**Status:** Final
Selected Organism	93% Probability **Pseudomonas aeruginosa** **Bionumber:** 1043041103500252	
ID Analysis Messages		

Susceptibility Information	**Analysis Time:** 15.40 hours	**Status:** Final

Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Ticarcillin/Clavulanic Acid	>= 128	R	Gentamicin	>= 16	R
Piperacillin	>= 128	R	Tobramycin	>= 16	R
Piperacillin/Tazobactam	>= 128	R	Ciprofloxacin	>= 4	R
Ceftazidime	>= 64	R	Pefloxacin		
Cefepime	>= 64	R	Minocycline		
Imipenem	>= 16	R	Colistin	2	I
Meropenem	>= 16	R	Rifampicin		
Amikacin	>= 64	R	Trimethoprim/ Sulfamethoxazole		

AES Findings	
Confidence:	Consistent

Figure. 2: VITEK 2 -System *P. aeruginosa*

Multi drug resistant *P. aeruginosa* screening

Antibiotic susceptibility tests were performed for 33 isolates of *P. aeruginosa* and 20 isolates of *P. aeruginosa* from catheter urine infection, by the Vitek 2-compact system using cards containing different antibiotics AST-N222. Results of *P. aeruginosa* isolates exhibit complete resistance (100%) to the following antibiotics: Cefotaxime, Ceftazidime, Cefepime, Imipenem, Gentamicin, Ciprofloxacin, Norfloxacin, Meropenem, , Amikacin and Colistin. Figure (3)

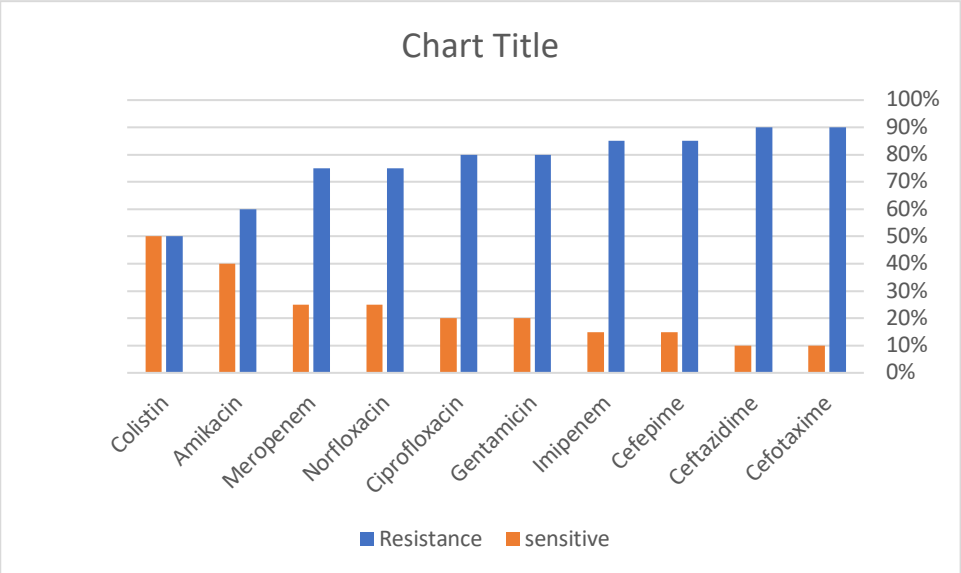


Figure. 3: Percentage of Antibiotic profile for *P. aeruginosa*

Determination of biofilm formation before Colistin treatment

A quantitative technique utilizing microtiter plates (MTP) was used evaluate the MDR *P. aeruginosa* isolates' capacity to generate biofilms (n = 33). As seen in Figures .4 and 5, the results showed that isolates of P. aeruginosa biofilm production are 15.1% non-producers, 17.8% generate weak biofilm, 35.7% produce moderate biofilm, and 46.4% produce robust biofilm.

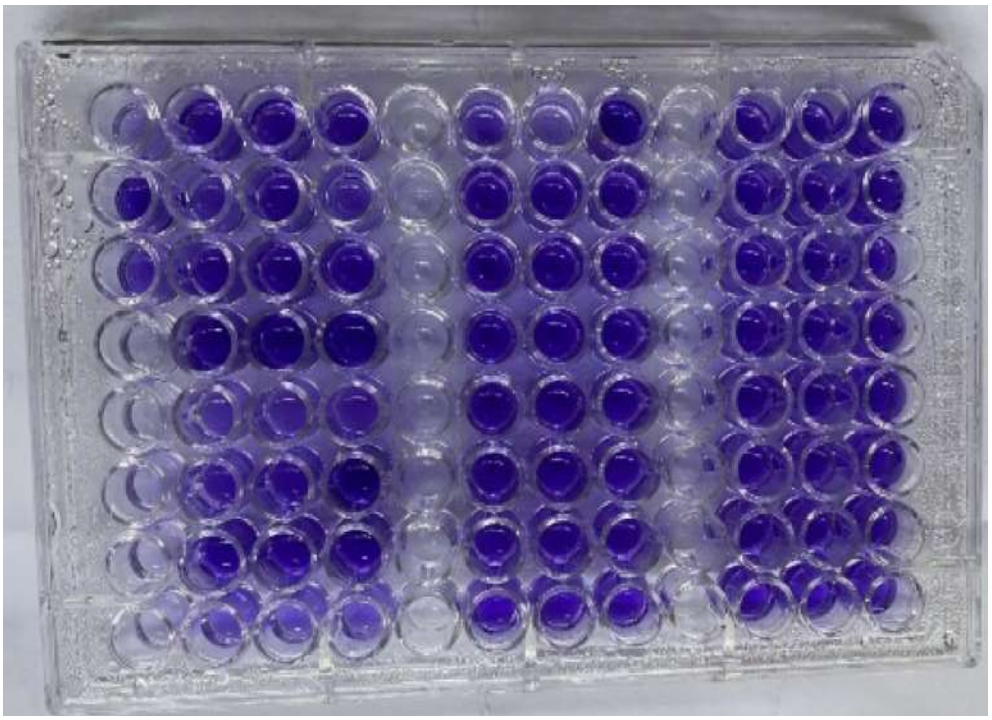


Figure. 4: Biofilm forming examination results of *P. aeruginosa* isolates by Micro-titer plate method

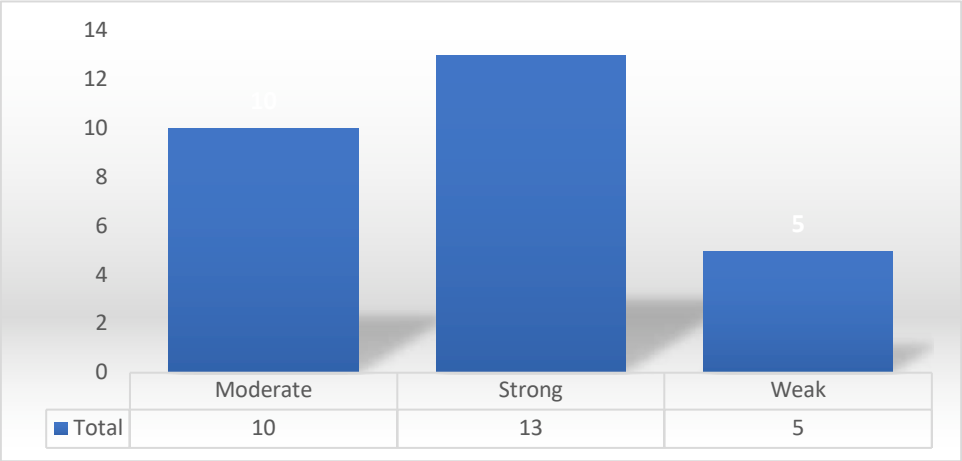


Figure. 5 : Percentage of biofilm values of *P. aeruginosa* isolates by Microtiter plate method

Minimum inhibitory concentration of Colistin

The lowest MIC of **Colistin** for ten *P. aeruginosa* isolates (Z1-Z10) was determined for each isolate as shown in Figure.6 , 7 and Table. 2.

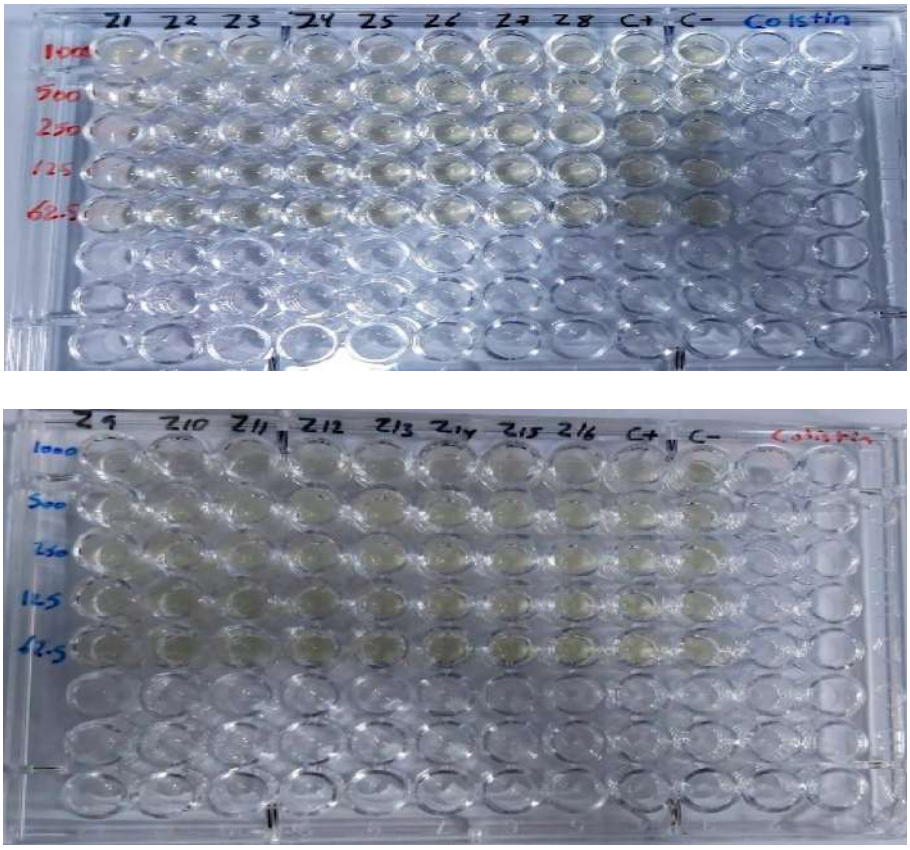
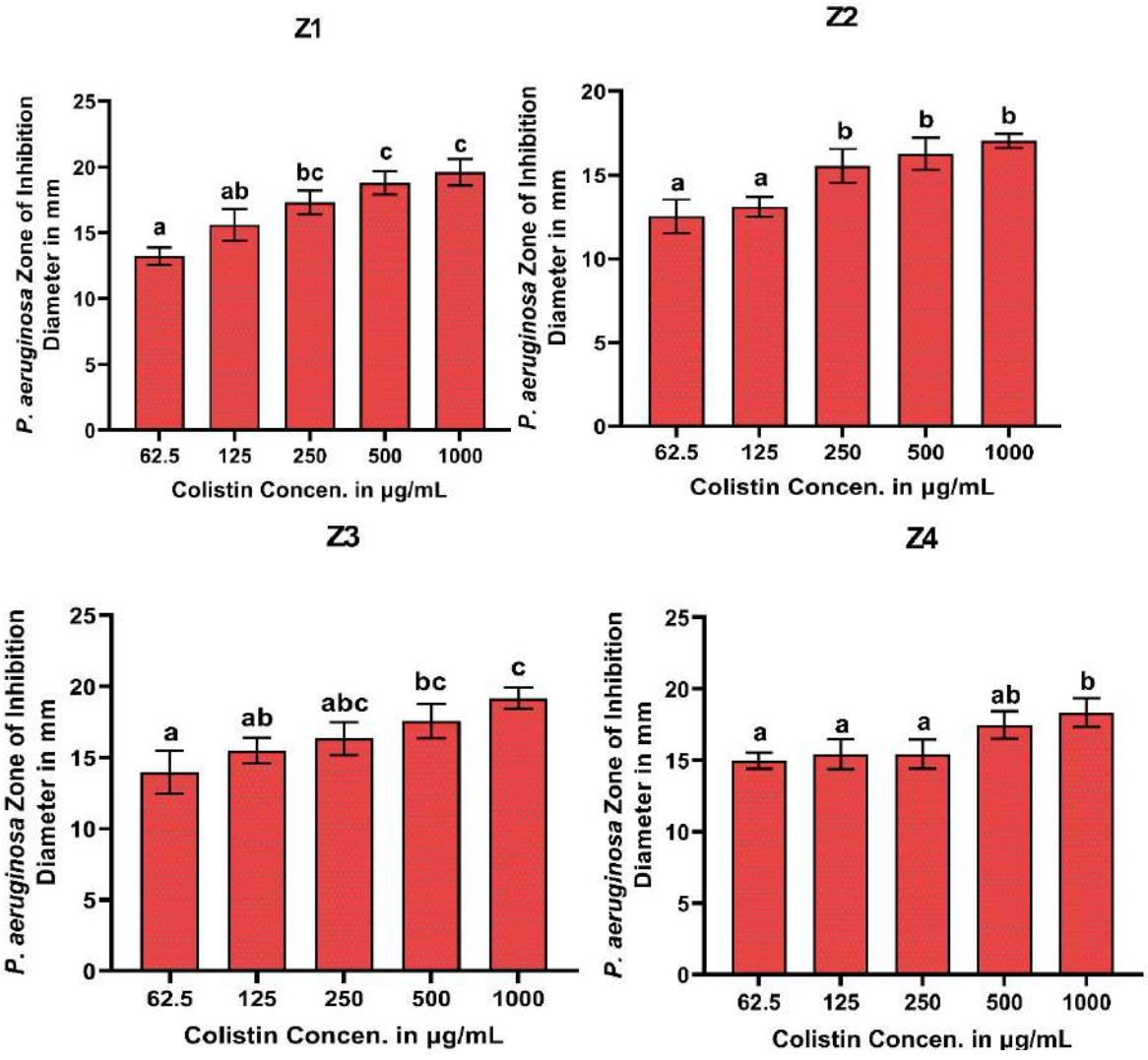
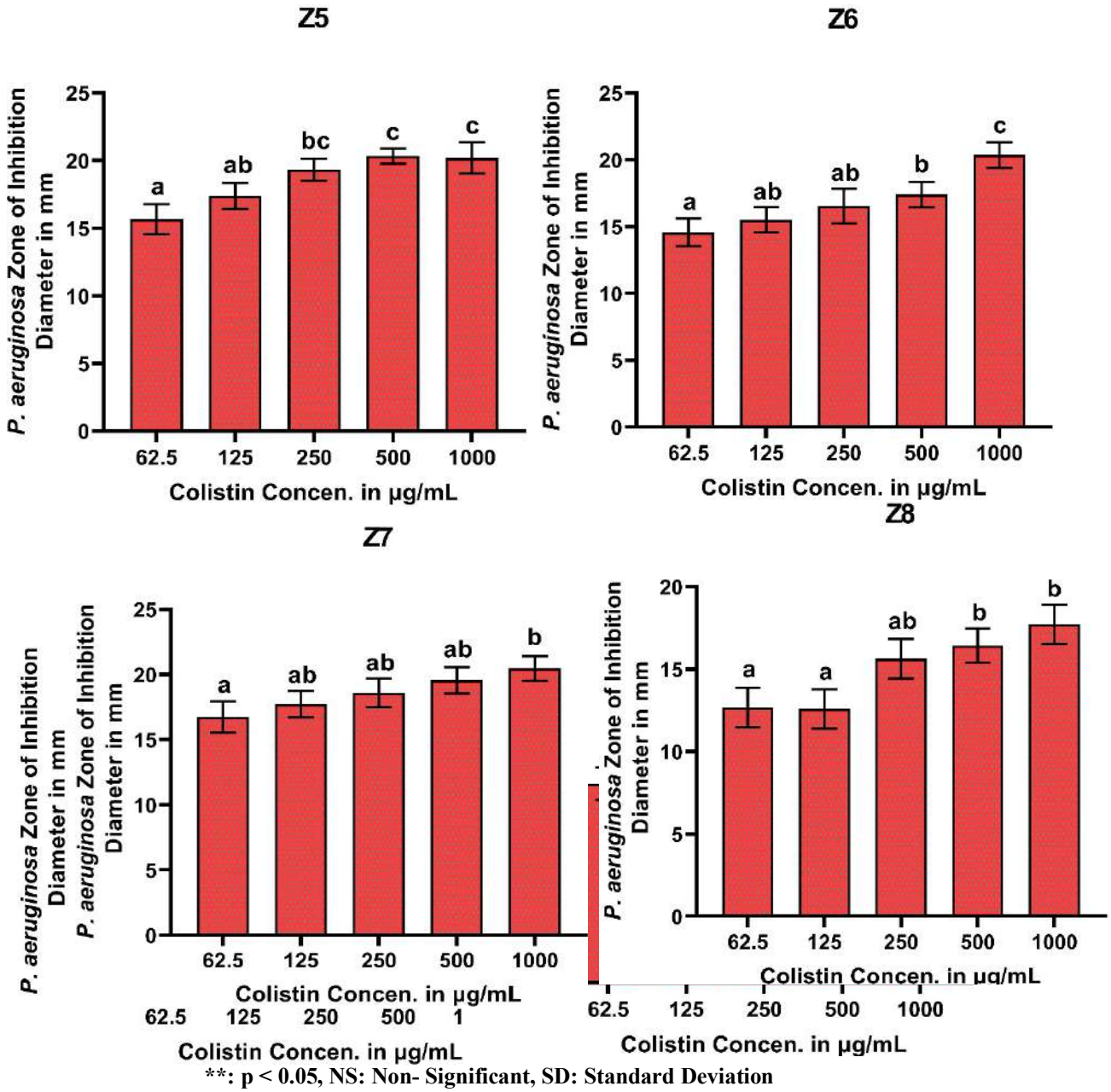


Figure. 6: Minimum inhibitory concentration of Colistin

Table.2: The minimal inhibitory concentration of Colistin

Bacterial isolate NO.	MIC of Colistin (µg/ml)	Bacterial isolate NO	MIC of Colistin (µg/ml)
Z1	125	Z6	62
Z2	250	Z7	125
Z3	62	Z8	125
Z4	250	Z9	250
Z5	62	Z10	62





****: p < 0.05, NS: Non- Significant, SD: Standard Deviation**

Figure. 7: Shows mean (± SD) Zone of Bacterial Inhibition in mm Treated with Different Concentrations (in µg/mL) of Colistin against *P. aeruginosa* Standard Deviation, (n = 3)

Determination of biofilm formation after Colistin treatment

The OD measurement of the identical *P. aeruginosa* strains indicated a drop from strong to weak production after 24 hours of incubation at 37°C with MIC of Colistin. The proportion of inhibition of biofilm formation attributed to Colistin was computed as follows: As indicated by table (3a&b) and Figure.8, the percentage inhibition is equal to 100 - [OD after treatment / OD before treatment x 100).

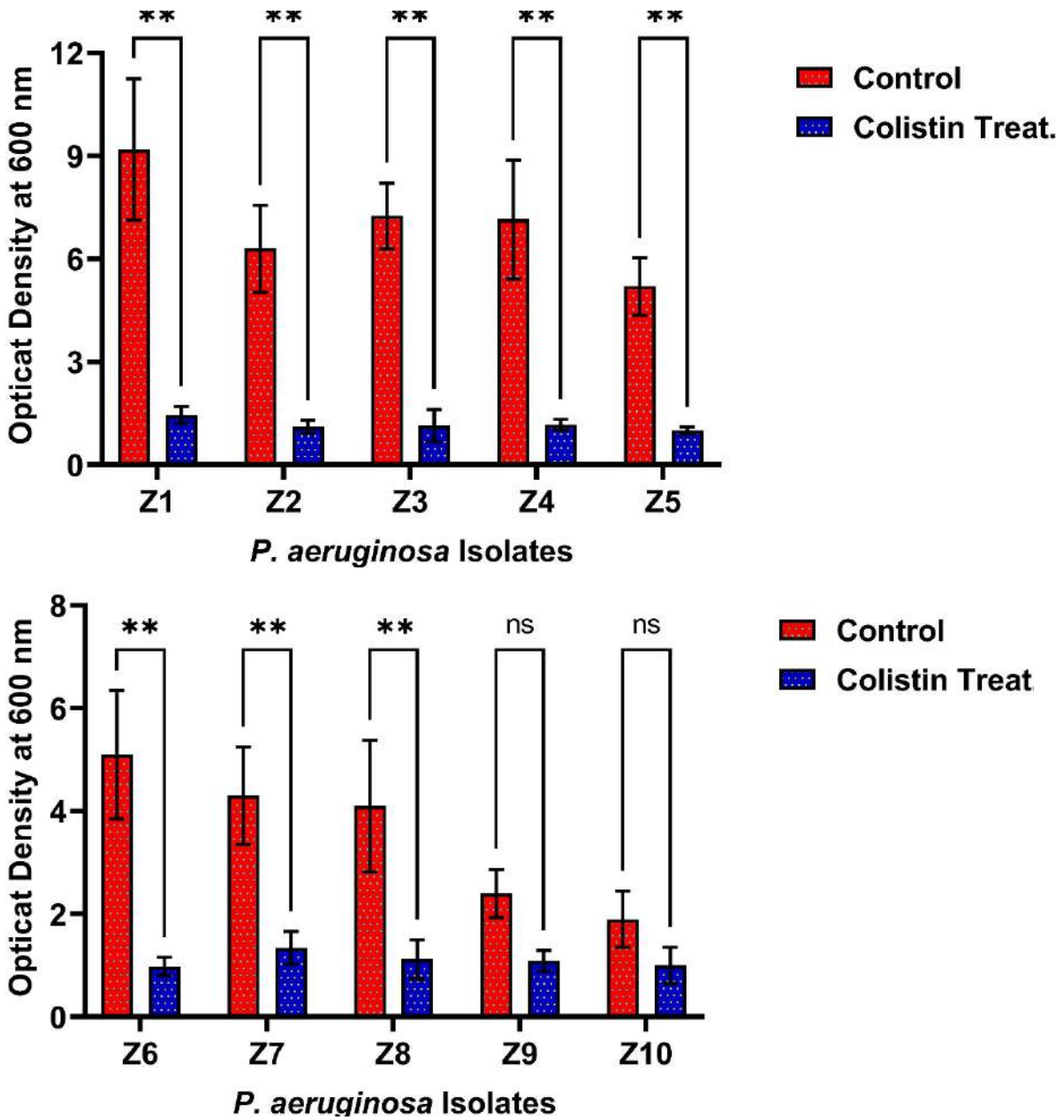


Figure.8: Shows antibiofilm activity of Colistin

Discussion

P. aeruginosa is a major cause of acute hospital infections and pneumonia, including chronic obstructive pulmonary disease (COPD), and healthcare-associated pneumonia, with substantial morbidity and death (16). *P. aeruginosa* which bacterial variables are necessary for this infection, virulence factors linked to *P. aeruginosa* isolated from the urinary tract, and UTI and CAUTI to better understand the infection dynamics and outcome in clinical settings. Knowing how *P. aeruginosa* can cause CAUTI and UTI can help one understand how these infections start and develop, as well as potentially offer measures to prevent them (17) emphasizes the critical need for focused surveillance systems to track and manage antibiotic-resistant infections in hospital settings. They suggested and customized antibacterial therapies to reduce the dangers of resistance in medical settings. The isolation and detection of pathogenic *P. aeruginosa* in semen is the

main goal of the current study. Additionally, the detection of resistance genes in the DNA of multidrug-resistant organisms (18). Antimicrobial resistance (AMR) is one of the biggest risks to global development and public health. A substantial contributor to both life-threatening acute infections and chronic, chronic disorders, Pseudomonas aeruginosa (*P. aeruginosa*) is directly linked to the problem of antimicrobial resistance (AMR). The growing incidence of *P. aeruginosa* that is resistant to carbapenem presents a serious risk to public health in Iraq. (19)

Carbapenem-resistant Pseudomonas aeruginosa has been identified by the WHO as a "high priority" for the creation of new antimicrobials. Indeed, the growth and spread of extensively drug-resistant (XDR) or multidrug-resistant (MDR) bacteria increases the possibility of morbidity and death for those who are infected. P. aeruginosa genomic variations exhibiting MDR/XDR characteristics have been classified as high-risk global clones. The β-lactamase genes found in some worldwide high-risk clones that can cause chronic colonization and raise the morbidity and mortality rates of infected (20).

However, multidrug-resistant organisms and their patterns of susceptibility reported in many medical environment. Before treating *P. aeruginosa* infections, particularly MDR strains, clinicians should regularly confer with a clinical microbiologist and select their treatment medications with a reserve. A different strategy to avoid infections linked to healthcare is required due to the introduction and ongoing growth of MDR *P. aeruginosa* and the lack of effective anti-pseudomonal drugs on the horizon. The infection control team and hospital infection control committee should create appropriate antibiotic policies and keep an eye on the precise prevalence of MDR strains and their pattern of susceptibility. (21)

In cases of persistent infections, microbes are most frequently detected as biofilm communities rather than as planktonic species. When a sizable population of microorganisms adheres to a biotic or abiotic surface, it is known as a biofilm. In this case, the primary causal agent for chronic and sometimes fatal infections in patients with cystic fibrosis and other lung conditions is the Gram-negative nosocomial bacteria *Pseudomonas aeruginosa*. When the bacteria cling to a surface that is conducive to the formation of a biofilm matrix, they can build a robust biofilm structure (22).

The process by which *P. aeruginosa* forms a biofilm is complicated. First, a free-floating bacterium adheres to a conditioned surface in a reversible manner. Next, surface adhesins bind the adherent bacteria irreversibly, and finally, an extracellular matrix forms to create a fully developed biofilm. Lastly, germs spread out from the matrix to infiltrate additional surfaces. Furthermore, *P. aeruginosa* infections may significantly increase the rate of death, morbidity, surgical intervention, chronic care, and treatment costs, according to epidemiological study. Therefore, the current focus is on effective treatment options to avoid biofilm infections linked to *P. aeruginosa*. A variety of polysaccharides, such as alginate, pel, and psl, influence the stability of *P. aeruginosa* biofilm structure. (**23**)

As a last resort for treating a variety of diseases brought on by extensively drug-resistant (XDR) and multidrug-resistant (MDR). *P. aeruginosa*, colistin was reintroduced into medical therapy. Nevertheless, colistin-resistant (COL-R) *P. aeruginosa* is growing as a result of the extensive usage of colistin. To prevent and treat COL-R P. aeruginosa infection, creative alternate approaches must be used**.** (24) has anti virulence, antibacterial, and antibiofilm properties that allow it to combat COL-R *P. aeruginosa*. In order to enhance the use of colistin in the treatment of infections, this study is to investigate the antibacterial, antibiofilm, anti-virulence, and mechanistic effects of COL-R *P. aeruginosa* isolated from sputum and wounds (25) .

With a high morbidity and mortality rate, *P. aeruginosa* is a major cause of acute hospital infections and pneumonia, including chronic obstructive pulmonary disease (COPD) and pneumonia linked to healthcare. Due to the extensive usage of colistin, COL-R *P. aeruginosa* is becoming more prevalent, which presents serious problems for clinical antiinfection prophylaxis and therapy. It is vinegar's active ingredient, and it has been shown to treat *P. aeruginosa* wound infections (26).

In conclusion, The majority of P. aeruginosa members are able to easily form biofilms and continue to act as the causative agent for biofilm-mediated illnesses, which can result in chronic infectious diseases and recurring infections. Accordingly, the opportunistic pathogen P. aeruginosa in its mucoid stage attracts interest in the field of study due to its correlation with the creation of biofilms. The broad spectrum of resistance to currently available antibiotic therapies is one of the main disadvantages in treating these biofilm-related diseases. Following its successful manufacturing employing an indirect, green, low-cost, high-yield method, colistin demonstrated exceptional antibacterial efficacy against strains of Pseudomonas aeruginosa. Colistin can be administered externally as an antibacterial agent by coating surfaces on a range of substrates to prevent biofilm-forming bacteria from sticking and growing in indwelling medical devices.

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Competing interest statement

None

Ethics statement

The authors declare that the author approved that this research follows the journal's Attach Ethic Approval guidelines as appeared on the journal's author guidelines page.

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