In Vitro Evaluation of Antibiotic Ertapenem Resistance of Extended Beta-Lactamase Producing Pseudomonas Sp.

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**Abstract:** This study evaluates the resistance of extended-spectrum beta-lactamase (ESBL) producing pathogenic isolate *Pseudomonas* species to the antibiotic Ertapenem, focusing on its impact on biofilm formation and hemolytic activity. Biochemical identification confirmed the pathogen as a *Pseudomonas* species, characterized by Gram-negative, rod-shaped, motile bacteria that tested positive for citrate, oxidase, catalase, urease, and starch hydrolysis. Biofilm assays revealed that untreated biofilms exhibited dense structures with strong cell-to-cell adhesion, while Ertapenem treatment significantly reduced bacterial density, disrupted biofilm architecture, and decreased extracellular matrix production, highlighting partial efficacy against biofilm integrity. Hemolytic activity assays demonstrated a dose-dependent increase in hemolysis, with concentrations ranging from 250 to 1000 µg/ml, indicating a strong cytotoxic effect of the cell lysate. Additionally, beta-hexosaminidase activity showed a marked increase with higher cell lysate concentrations, suggesting an enhanced enzymatic response involved in cellular processes. These findings emphasize the complexity of treating ESBL-producing *Pseudomonas* infections, particularly those involving biofilm formation, and underscore the potential need for combination therapies to fully eradicate resistant strains.

**Keywords:** Ertapenem; Pseudomonas; Extended Beta-Lactamase; Antibiotic Resistance

# Introduction

Antibiotics are antimicrobial agents which treat bacterial infections by either killing bacteria or inhibiting their growth and also significantly reduces morbidity and mortality associated with bacterial infections [(Chinemerem Nwobodo et al., 2022; Pushpaanjali et al., 2020)](https://paperpile.com/c/fkvTk7/rzoX+JXA2). Based on the chemical structure and mode of activity, antibiotics are classified into various classes, including beta-lactams, macrolides, tetracyclines, aminoglycosides, and others [(Begum et al., 2021)](https://paperpile.com/c/fkvTk7/kBKt2). Beta-lactams antibiotics, penicillins, cephalosporins, monobactams, and carbapenems, are widely used antibiotics due to their broad spectrum of activity and effectiveness against many pathogenic bacteria [(Aurilio et al., 2022)](https://paperpile.com/c/fkvTk7/yMWVU). Ertapenem, a carbapenem antibiotic with a crucial beta-lactam ring, is renowned for its broad-spectrum antibacterial activity, high potency, and convenient once-daily dosing due to its long half-life, enhancing patient compliance [(Girija et al., 2019; Herrera-Hidalgo et al., 2023)](https://paperpile.com/c/fkvTk7/6CYR4+axQwE). Ertapenem antibiotics inhibits the peptidoglycan and bacterial cell wall synthesis that cause cell lysis and further leads to cell death in susceptible bacteria [(Impey et al., 2020)](https://paperpile.com/c/fkvTk7/Su0M1). Bacteria play crucial roles in ecosystems, including nutrient cycling and decomposition, but some species are pathogenic that causes diseases in humans, animals, and plants [(Yadav et al., 2021)](https://paperpile.com/c/fkvTk7/VDDWe). Pathogenic bacteria cause diseases and a wide range of infections, leading to minimal skin infections to serious life-threatening allergies [(Qadri et al., 2022)](https://paperpile.com/c/fkvTk7/FUpPG). Extended-spectrum beta-lactamase (ESBL) producing *Pseudomonas* species present a significant threat in clinical studies due to their ability to hydrolyze a wide range of beta-lactam antibiotics which includes penicillins, aztreonam and cephalosporins [(Ferous et al., 2024)](https://paperpile.com/c/fkvTk7/zpuRu). The antibiotic resistance mechanisms in *Pseudomonas* spp. are multifaceted, involving the production of carbapenemases, efflux pumps, porin mutations, and biofilm formation [(Lorusso et al., 2022)](https://paperpile.com/c/fkvTk7/AhhkI). Capacity of Pseudomonas spp. to form biofilms on various surfaces, including medical devices and human tissues, significantly contributes to their persistence in clinical settings and resistance to antimicrobial treatments [(Devi et al., 2021; Verdial et al., 2023)](https://paperpile.com/c/fkvTk7/VNOLR+YUvlu). The resistance patterns and mechanisms of ESBL-producing Pseudomonas spp. is crucial for developing effective treatment strategies and for informing antibiotic stewardship programs [(Husna et al., 2023)](https://paperpile.com/c/fkvTk7/1c2Mr). The persistence of antibiotic resistance organisms required the need for regular observation, novel therapeutic approaches, and stringent infection control measures. This study aims to provide insights into the in vitro resistance profiles of ertapenem in ESBL-producing Pseudomonas spp., contributing to the broader understanding of antimicrobial resistance and aiding in the fight against these formidable pathogens.

# Materials and Methods

*Pseudomonas aeruginosa*, an oral pathogen, was collected from and cultured on blood agar and MacConkey agar. Then incubated for 24 hours at 37°C. All bacterial isolates were streaked on the media to obtain pure cultures. Slide smears prepared from these cultures were stained and examined under a microscope with a 100x objective lens for Gram staining. The isolates were further identified through various biochemical characteristics according to the guidelines provided in Bergey’s Manual: indole production test,Voges-Proskauer, methyl red tests, oxidase test, citrate utilization test, motility test, urease test, catalase test and, triple sugar iron (TSI) test, lactose test, hydrogen sulphide (H2S) production test, Inositol tests other fermentation of carbon sources will be performed. The effectiveness of Ertapenem in inhibiting biofilm formation was assessed using a microtiter plate assay. The bacterial pathogen was incubated in the microtiter plates at 37 °C for 24 hours, achieving a concentration of up to 10^9 cells/ml. Following biofilm formation, the samples were treated with various concentrations of Ertapenem, in conjunction with amoxiclav (100 µg/ml), and incubated at 37 °C for an additional 48 hours. Biofilm quantification was performed using crystal violet staining and also the optical density was measured at 570 nm. Controls included well-grown cultures without treatment, and sterile medium served as the blank. Biofilm inhibition was determined using the formula as mentioned in [(Wannigama et al., 2020)](https://paperpile.com/c/fkvTk7/wARko):

% Inhibition=100−(OD of sample/OD of control)/ OD of Control ×100

The bacterial isolates were cultured in tryptone soya broth (TSB) for 24 hours, and the cell lysate was collected by centrifugation. Various concentrations of the culture supernatant were mixed with RBCs and 10 mM CaCl₂ in a microtiter plate (96-well) and incubated for 60 minutes at room temperature (37°C). After incubation, button formation will be visualized then the optical density of the supernatant was observed at 540nm [(Sebastian & Mc, 2020)](https://paperpile.com/c/fkvTk7/Uw0Ny). Triton X-100 (1%) has been used as positive control and sterile TSB has been used as negative control. The percentage of hemolysis was calculated by following formula:

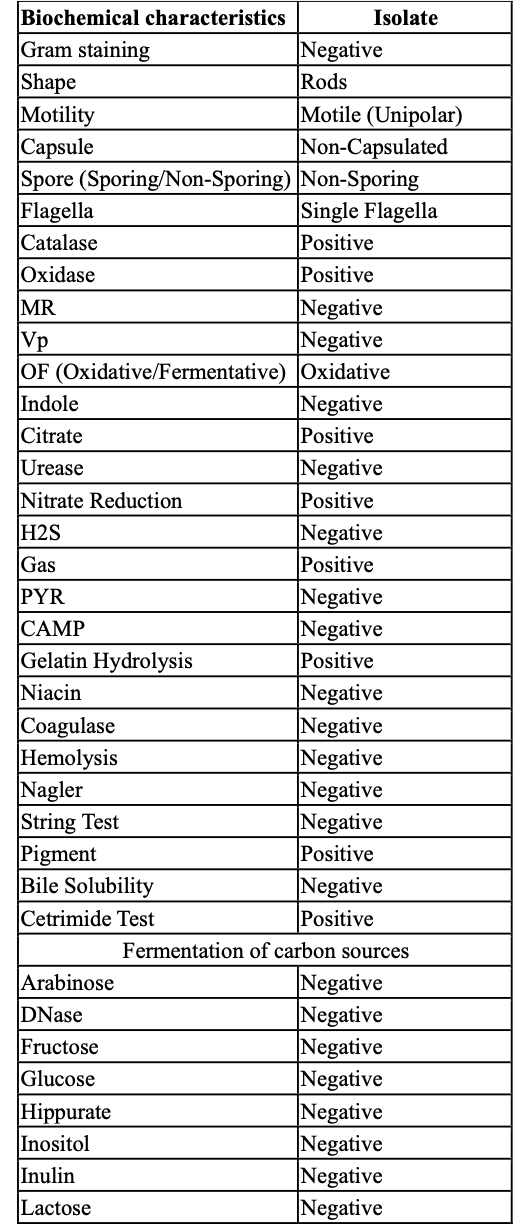
Inhibition Hemolysis (%) = (Control-Test)/Control\*100

The reaction mixture of the compound at various concentrations ranged from 250, 500, 750, and 1000 µg/ml prepared in 0.85% of phosphate-buffered saline (PBS). The enzyme reaction was conducted in a 96-well microplate, with each well receiving 50 µl of beta-hexosaminidase enzyme solution and 50 µl of 1 mM 4-methylumbelliferyl N-acetyl-beta-D-glucosaminide substrate solution. To each well, 50 µl of the respective test compound concentration was added, while control wells received 50 µl of PBS. The microplate was incubated at room temperature (37°C) for 1 hr to improve enzymatic activity. The reaction was stopped by adding 100 µl of 0.2 M glycine (pH 10.4) to each well. Fluorescence intensity, which is directly proportional to beta-hexosaminidase activity, was measured using a microplate reader set at 360 nm and an emission at 450 nm. [(Calzoni et al., 2024)](https://paperpile.com/c/fkvTk7/52iws).

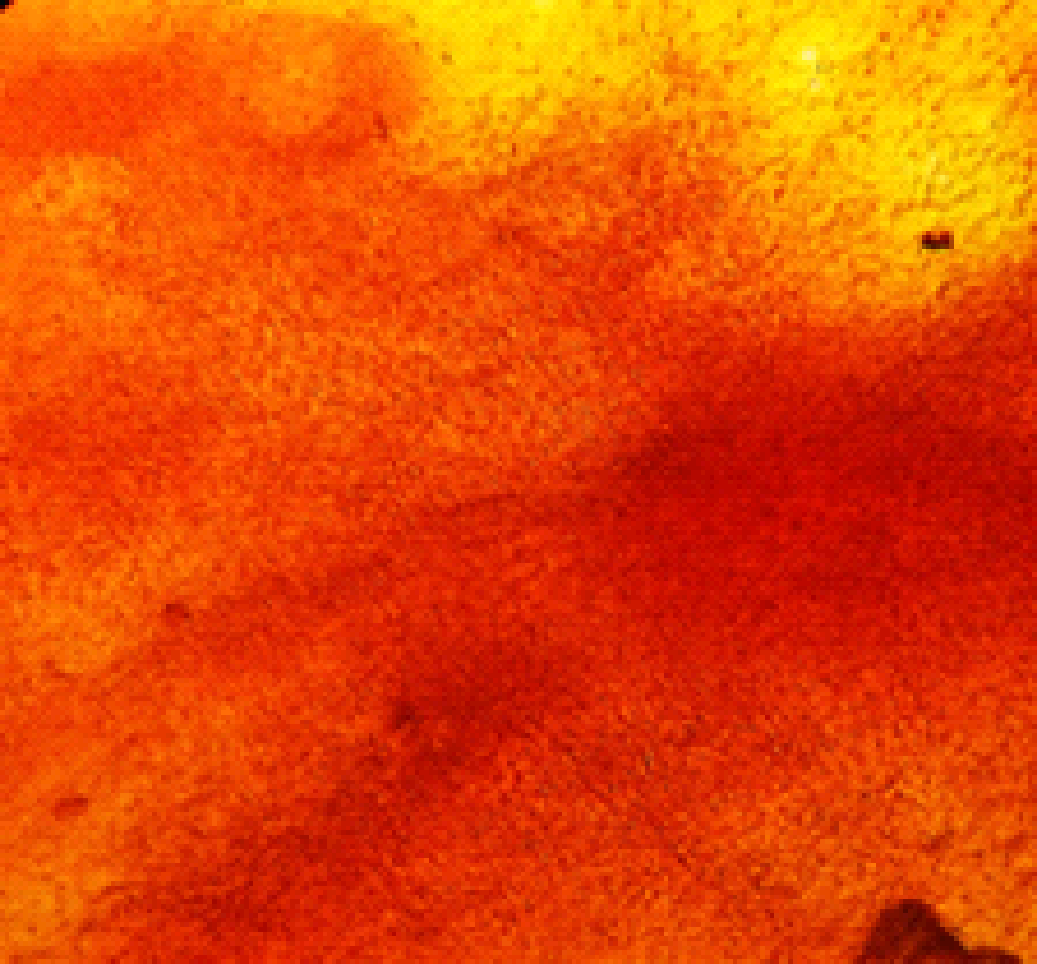
# Results

The biochemical identification of the pathogen reveals it to be a *Pseudomonas species* (Table 1). The Gram stain result is negative, indicating that the bacterium has a thin peptidoglycan layer and does not retain the crystal violet stain, appearing pink under the microscope. Morphologically, the bacterium is rod-shaped and demonstrates motility, suggesting the presence of flagella. The bacterium is negative for indole production, methyl red (MR), and Voges-Proskauer (VP) tests, indicating it does not produce indole, does not perform mixed acid fermentation, and does not produce acetoin, respectively. The triple sugar iron (TSI) test is negative, indicating no fermentation of glucose, lactose, or sucrose, and no hydrogen sulfide production. The organism does not ferment lactose, maltose, sucrose, xylose, or inositol, which is characteristic of non-fermentative metabolism. The citrate test is positive, showing that the bacterium can utilize citrate as a sole carbon source. The bacterium tests positive for oxidase and catalase, confirming the presence of cytochrome c oxidase and the ability to break down hydrogen peroxide into water and oxygen. Urease activity is also positive, indicating the ability to hydrolyze urea to ammonia and carbon dioxide. However, it can hydrolyze starch, as indicated by the positive starch test. Collectively, these biochemical characteristics align with the genus Pseudomonas, known for its Gram-negative, rod-shaped, motile properties, and specific metabolic capabilities.

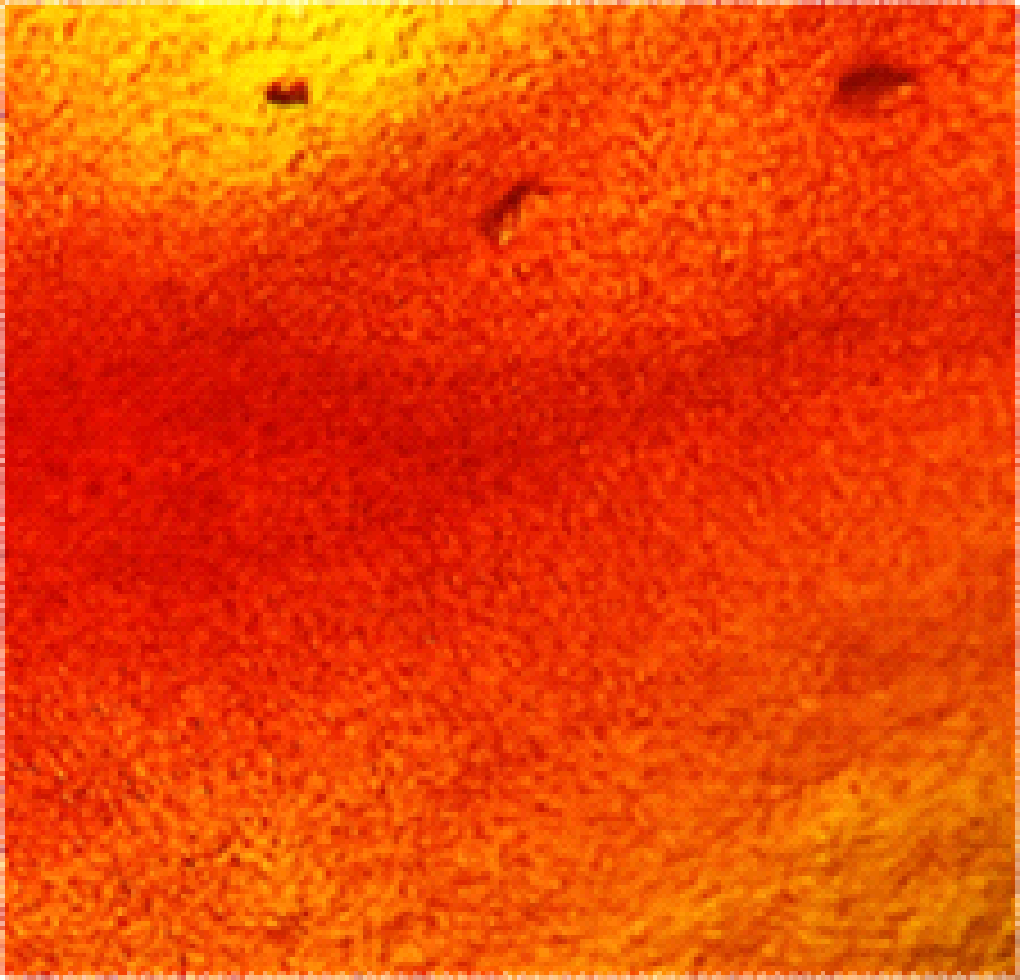
**Table 1.** Biochemical identification of pathogen



The ertapenem treated samples and untreated control (Figure 1) has shown significant insights into the antibiotic's impact on *Pseudomonas aeruginosa* biofilms. The untreated biofilm exhibits a dense and uniform structure with vibrant red and orange hues, indicative of a mature biofilm rich in bacterial cells and extracellular matrix (ECM) (Fig. 1). This intact and robust biofilm structure underscores the strong cell-to-cell adhesion and stability typically associated with *Pseudomonas* biofilms. Ertapenem-treated biofilm shows a marked reduction in bacterial density and diminished color intensity, reflecting a decrease in ECM production and bacterial viability. The treated biofilm's disrupted and less cohesive structure, along with the presence of clear zones and holes, highlights the antibiotic's effectiveness in penetrating and dismantling the biofilm architecture. These observations confirm that Ertapenem significantly impairs biofilm integrity, reducing its viability and structural cohesion. However, the residual presence of biofilm suggests that complete eradication may require higher doses or combination therapies, emphasizing the need for ongoing development of effective treatment strategies against biofilm-associated infections.

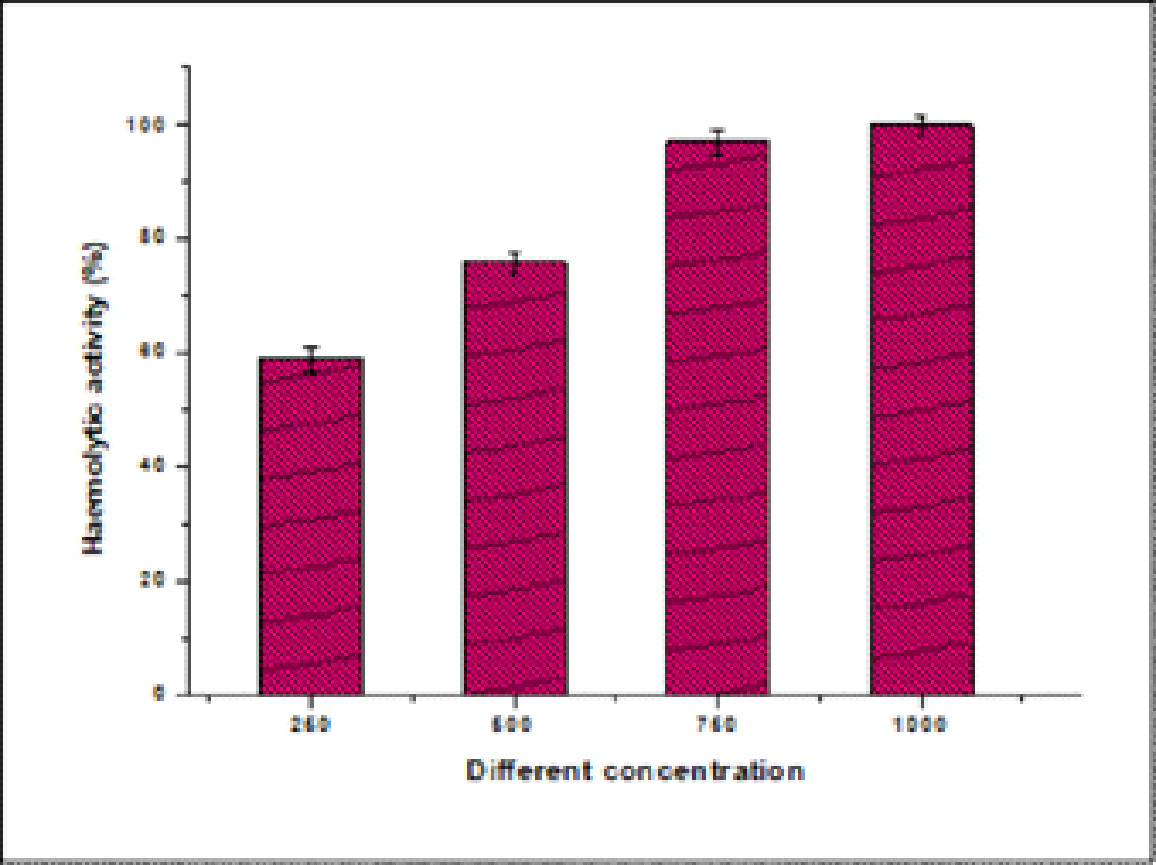


**Figure 1.**Biofilm of *Pseudomonas sp.* indicative of a mature biofilm rich in bacterial cells and extracellular matrix.



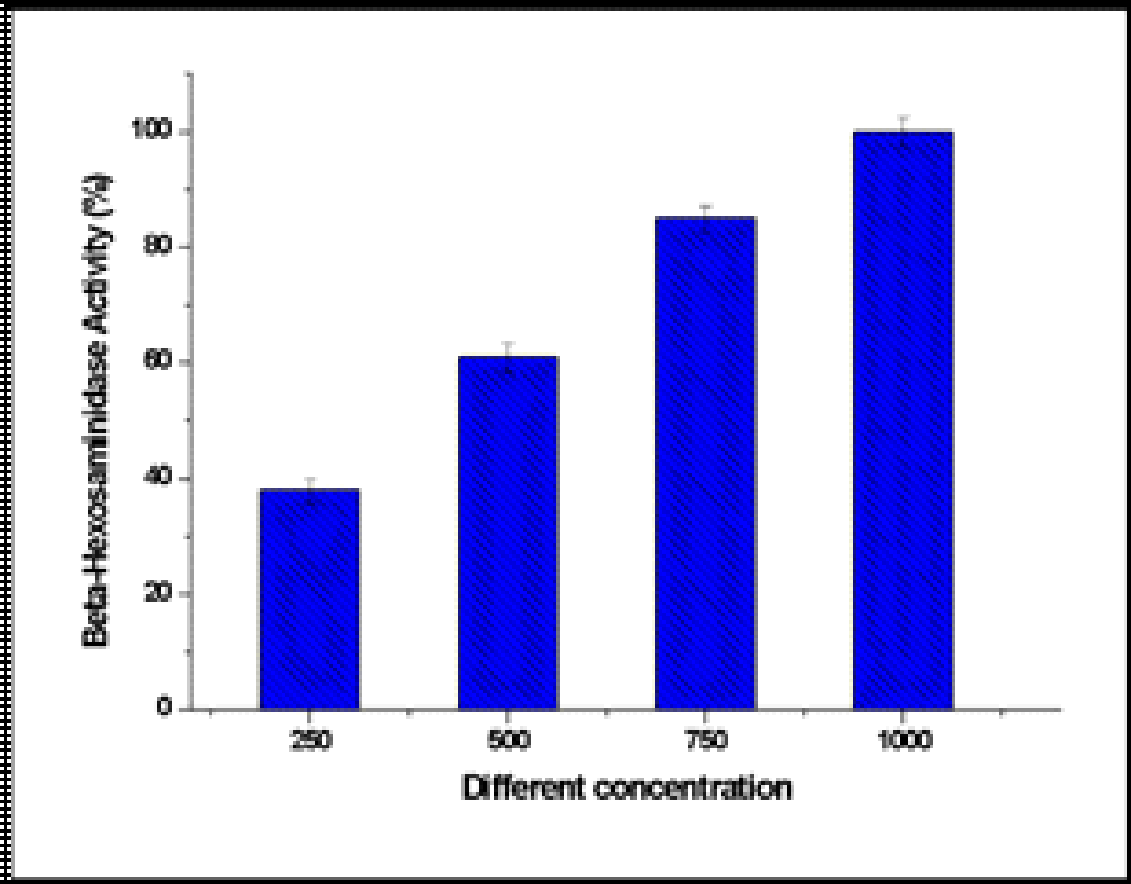
**Figure 2.** Treated with Ertapenem shows reduction in bacterial density and diminished color intensity, reflecting a decrease in ECM production and bacterial viability.

The hemolytic activity of cell lysates at different concentrations was evaluated, and the results are presented as the percentage of hemolysis along with the standard deviation (St.D) (Fig. 3). The concentrations tested were 250 µg/ml, 500 µg/ml, 750 µg/ml, and 1000 µg/ml. The results of the hemolytic activity assay demonstrated a clear dose-dependent increase in hemolysis with increasing concentrations of the cell lysate. At a concentration of 250 µg/ml, the cell lysate caused 59% hemolysis with a standard deviation (St.D) of 2.2, indicating moderate hemolytic activity. When the concentration was increased to 500 µg/ml, hemolytic activity rose significantly to 76% (St.D 1.9), suggesting a substantial increase in the ability of the cell lysate to lyse red blood cells (RBCs). At 750 µg/ml, the hemolytic activity reached 97% (St.D 2.3), indicating near-complete lysis of RBCs. Finally, at the highest concentration of 1000 µg/ml, the cell lysate achieved complete hemolysis (100%) with a St.D of 1.8, reflecting consistent and maximal hemolytic activity(Nikalje et al., 2024). These results highlight the strong and increasing cytotoxic effect of the cell lysate on RBCs as the concentration increases(Chehelgerdi et al., 2023).



**Figure 3.** Hemolytic activity assay demonstrated a clear dose-dependent increase in hemolysis with increasing concentrations of the cell lysate.

Assessment of beta-hexosaminidase activity in the cell lysate shows a dose response to increase enzyme activity with increasing amounts(Fig.4). Beta-hexosaminidase activity was quantified as 38 % with St.D = 2.1 at a concentration of 250 µg/ml. Dilution A has huge increase in enzyme activity was observed, to give 61% (St.D. 2.5), where the concentration is 500 µg/ml Beta-hexosaminidase activity was further increased to 85% (St. D.2.2) at 750 µg/ml, demonstrating high enzyme response of activity. At the highest concentration of 1000 µg/ml, enzyme activity was maximally activated to an extent of 100% as compared with control (St.D = 2.5). This increase in activity may reflect the presence of specific lysate components that activate or upregulate the beta-hexosaminidase enzyme, which is crucial for cellular processes involving glycosaminoglycan degradation and lysosomal function.



**Figure 4** Beta-hexosaminidase activity in the cell lysate shows a dose response to increase enzyme activity with increasing amounts.

# Discussion

Biofilm formation and viability of *Pseudomonas aeruginosa* on glass surface studies against various concentrations of caffeine 40-80μg/mL has shown minimal impact [(Chakraborty et al., 2020)](https://paperpile.com/c/fkvTk7/veYN1). The live cells were stained with acridine orange and the dead cells were stained with ethidium bromide, and the yellow dots in the merged image indicates the dead cells revealed the minimal efficacy of caffeine against either planktonic or biofilm cells of *Pseudomonas aeruginosa*[*(Ramakrishnan et al., 2023; Shenoy & Maiti, 2023; J. S. Sindhu et al., 2023)*](https://paperpile.com/c/fkvTk7/MuIjn+6mHq4+jj5bU). The study reported by [(Topa et al., 2020)](https://paperpile.com/c/fkvTk7/OmKWt) demonstrated that combining 3 mM CAD with COL significantly enhanced the antimicrobial efficacy against biofilm formation by *Pseudomonas aeruginosa*[*(Dharman et al., 2023; S. Sindhu et al., 2023; Sreenivasagan et al., 2023)*](https://paperpile.com/c/fkvTk7/kco94+IsYFj+IEnZl). The minimum inhibitory concentration (MIC) of COL was reduced from 6.8 µM to 1.7 µM in the presence of CAD, as indicated by the checkerboard assay. To further assess the impact of this combination on biofilm inhibition, sub-MIC concentrations of CAD (1.5 µM) and COL (0.9 µM) were used. CAD alone inhibited biofilm formation by 31.3%, while COL and TOB each showed inhibition of thirty five (35%) percent, as determined by the crystal violet assay[(Ajay et al., 2023; Chokkattu et al., 2023; Padarthi et al., 2023)](https://paperpile.com/c/fkvTk7/sxATg+tzSIv+JhiGI). Especially, the combination of CAD with either COL/TOB exhibited a significant biofilm inhibition of 83.9% and 75.2%, respectively, compared to the untreated control. These findings highlight the potential of CAD in combination with COL or TOB as a more effective strategy for combating biofilm-associated infections, suggesting that such combinations may be a promising approach to enhance antimicrobial efficacy against resistant bacterial strains[(Kasabwala et al., 2021; Rajeshkumar & Lakshmi, 2021; Varghese et al., 2023)](https://paperpile.com/c/fkvTk7/SCko7+BCth8+9epTp). This study reveals that Ertapenem alone disrupts biofilm structure but may not fully eradicate the biofilm, while caffeine shows negligible effects on biofilm viability[(Keerthana & Ramesh, 2021; Murugesan, 2021; Tiwari & Jain, 2021)](https://paperpile.com/c/fkvTk7/09JOF+vuf2w+8K8j8)[(Keerthana & Ramesh, 2021; Murugesan, 2021; Subramanian et al., 2021; Tiwari & Jain, 2021)](https://paperpile.com/c/fkvTk7/09JOF+vuf2w+8K8j8+wUzMY). The combination of CAD with COL or TOB provides a significantly enhanced inhibitory effect on biofilm formation. These insights highlight the need for further research into combination strategies to effectively manage resistant *Pseudomonas aeruginosa* infections [(Pranati et al., 2021; Sakthi et al., 2021)](https://paperpile.com/c/fkvTk7/Y9upb+C116z) In, 2024, [(Aguirre-García et al., 2024)](https://paperpile.com/c/fkvTk7/7gFAo) shows that the hemolytic activity of the polyphenolic extract from *Flourensia cernua* reveals that incubation of erythrocytes with the polyphenolic extract at a concentration of 500 µg/ml for 4 hours resulted in a slight toxic effect on the erythrocytes which suggests that the extract exhibits some level of hemolytic activity, though it is not highly toxic under the tested conditions[(G. & Ganapathy, 2022; Kumar & Ramesh, 2021)](https://paperpile.com/c/fkvTk7/VMvJ8+HWnQl)). Study of [(Silva et al., 2020)](https://paperpile.com/c/fkvTk7/rua9i) evaluated the inhibitory effect of RPE on β-hexosaminidase release from mast cells, which is a crucial marker for assessing antiallergic activity. RPE at concentrations of 50 and 100 µg/mL significantly inhibited the release of β-hexosaminidase by 44% and 55%, respectively, demonstrating a potent antiallergic effect. This inhibition was more effective than the mast cell stabilizer ketotifen fumarate (17% inhibition at 100 µg/mL) and suggests that RPE primarily acts by preventing mast cell degranulation rather than directly interfering with the enzyme's function and also underscores the potential of RPE as a therapeutic agent for allergic conditions. Finally, results highlight the dual roles of β-hexosaminidase in both allergic responses and cellular metabolism. While RPE shows promise as an antiallergic agent by inhibiting the release of this enzyme from mast cells and also the activation of β-hexosaminidase in response to cellular components.

# Conclusion

This study provides valuable insights into the resistance mechanisms and biofilm-forming capabilities of ESBL-producing Pseudomonas aeruginosa. The biochemical characterization confirmed the pathogen's identity, consistent with known Pseudomonas metabolic profiles. While Ertapenem showed some efficacy in reducing biofilm formation, its partial success in disrupting biofilm architecture and reducing bacterial density suggests that monotherapy might be inadequate for fully eradicating these resilient strains. The dose-dependent increase in hemolytic activity and beta-hexosaminidase activity observed in this study underscores the cytotoxic potential and complex enzymatic responses associated with the bacterial cell lysate, complicating treatment strategies. These findings highlight the necessity for combination therapies and innovative approaches to overcome the limitations of current treatments. Also, the study underscores the importance of continuous surveillance, stringent infection control measures, and antibiotic stewardship programs to address the growing threat of antimicrobial resistance in clinical settings. Ultimately, this research contributes to a deeper understanding of Pseudomonas aeruginosa resistance patterns, aiding in the development of more effective treatments and strategies to manage infections caused by ESBL-producing strains.

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