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Antimicrobial, Synergistic, and Antibiofilm Activity of Sildenafil Against *Pseudomonas aeruginosa*: Preliminary Studies

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Abstract: The present study tested sildenafil citrate as an example of pharmacological repositioning against the opportunistic pathogen *Pseudomonas aeruginosa*, known for its potent biofilm formation. We evaluated its antimicrobial, synergistic, and antibiofilm effects using broth microdilution, checkerboard assays, and atomic force microscopy techniques. Sildenafil citrate showed antimicrobial activity, effectively inhibiting bacterial growth at minimum inhibitory concentrations ranging from 3.12 to 6.25 mg/mL and minimum bactericidal concentrations between 3.12 and 25 mg/mL. When combined with reference antimicrobial agents—cefepime, imipenem, cilastatin, and polymyxin—sildenafil citrate had a synergistic effect. It also effectively inhibited and eradicated biofilms, reducing total biomass by 87.1% for inhibition and 83.8% for eradication. Atomic force microscopy confirmed the efficacy of sildenafil citrate in destroying and inhibiting biofilms, decreasing the overall amplitude of the biofilm. Consequently, sildenafil citrate appears to be a promising candidate for combination with commercial antimicrobial drugs to prevent and treat *P. aeruginosa* infections.

Keywords: repositioning; sildenafil citrate; *Pseudomonas aeruginosa*; antimicrobial resistance; biofilm

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1. Introduction

Drug repositioning is the process of identifying and investigating new therapeutic indications for existing drugs to treat other diseases. This approach requires less time and fewer developmental steps due to the known profile of the drug, making the investment more cost-effective. A notable example of pharmacological repositioning is sildenafil citrate, a phosphodiesterase inhibitor initially studied for the treatment of angina. This drug is now used in treating erectile dysfunction and idiopathic pulmonary arterial hypertension and has shown therapeutic efficacy in chronic inflammatory diseases [1,2].

Given the indiscriminate use of antimicrobials, treating infections may pose various challenges, often leading to increased costs and high mortality rates. Among these challenges is the enhancement of microbial resistance through processes such as biofilm formation. Biofilms are structures that amplify the pathogenic process [3]. *Pseudomonas aeruginosa*, a Gram-negative, aerobic, and opportunistic bacillus, is known for its ability to form biofilms and cause severe hospital-acquired infections [4].

Phosphodiesterase inhibitors are widely recognized as adjuvants in several infectious or toxigenic processes, including tuberculosis [5], lung damage [6], pulmonary hypertension

caused by endotoxins [7], and COVID-19 [8,9]. Their indirect mechanism leads to cellular protection and consequently improves the patient's clinical conditions. Additionally, evidence suggests that sildenafil may directly affect microorganisms, since phosphodiesterases are enzymes that regulate vital activities in bacteria [10]. Zheng et al. found that a synthetic phosphodiesterase inhibitor inhibits several bacterial virulence factors and swarming motility, closely related to the initial stages of biofilm formation [11]. Thus, the hypothesis is that sildenafil may act as both an adjuvant (as widely reported in the current literature) and a protagonist in treating bacterial infections associated with biofilm.

Therefore, this study aims to evaluate the antimicrobial, antibiofilm, and synergistic effects of sildenafil citrate in combination with commercially available antimicrobials such as cefepime, imipenem, cilastatin, and polymyxin B for the first time.

2. Materials and Methods

2.1. Microorganisms

A *P. aeruginosa* strain (PA01) served as the standard strain for biofilm formation in all assays. In addition, ten clinical isolates (CI) of *P. aeruginosa* were identified using a semi-automated MicroScan Autoscan-4 instrument (Siemens), as listed in Table 1. The strains used in this study were of hospital origin, clinical isolates from various anatomical sites. In our laboratory, they were identified by the following tests/characteristics: colony characteristics (creepy and metallic aspect, green pigmentation), positive for oxidase, negative for lactose, positive for catalase colonies. In triple sugar iron media, they were non-fermenting for glucose. In O/F media (Hugh and Leifson test), they were O+/F−.

Table 1. *P. aeruginosa* clinical isolates, their respective collection locations, and acronyms.

Isolate—Sites	Acronyms
1—Urine	1PAUR
2—Blood	2PAHC
3—Urine	3PAUR
4—Urine	4PAUR
5—Sputum	5PAES
6—Tracheal secretion	6PAST
7—Sputum	7PAES
8—Urine	8PAUR
9—Urine	9PAUR
10—Urine	10PAUR

2.2. Commercial Drugs

Sildenafil citrate, cefepime hydrochloride, imipenem monohydrate, cilastatin sodium, and polymyxin B sulfate were commercially obtained from Sigma-Aldrich, Brazil, and reconstituted according to the manufacturer's instructions. The stock solution for sildenafil citrate was prepared following the Brazilian Pharmacopoeia guidelines, which recommend dissolving in sterile injection water. The drugs were reconstituted in sterile test tubes, diluted to a concentration of 100 mg/mL.

2.3. Antimicrobial Activity

2.3.1. Strain Preparation

P. aeruginosa strains (PA01) and clinical isolates were standardized according to CLSI guidelines [12]. Isolated colonies were cultured for 18–24 h on Mueller–Hinton agar (Himedia). A saline solution suspension (0.85% NaCl) was then prepared, with the density adjusted to a 0.5 McFarland scale (1.5×10^8 CFU/mL) [12].

2.3.2. Minimum Inhibitory and Bactericidal Concentrations

The minimum inhibitory concentration (MIC) was determined using the broth microdilution method in 96-well plates [12,13]. The plates were incubated at 37 °C for 24 h, covered

with aluminum foil, and the assay was performed in triplicate. Microorganism growth was detected with 2,3,5-triphenyl tetrazolium chloride dye. The MIC was identified as the lowest concentration at which no color change occurred. For the minimum bactericidal concentration (MBC), 1 µL from each well was inoculated onto Mueller–Hinton agar plates that showed no microbial growth. After incubating at 37 °C for an additional 24 h, the MBC values were determined as the lowest concentration at which no bacterial growth was observed.

2.4. Antibiofilm Activity

2.4.1. Biofilm Formation

The biofilm-forming capabilities of the standard strain PA01 and clinical isolates were evaluated using 96-well plates. Each well was filled with 100 µL of Mueller–Hinton broth, supplemented with 100 µL of the sildenafil citrate solution and 10 µL of the strain inoculum, adjusted to a 0.5 McFarland scale. The plates were incubated at 37 °C for 24 and 48 h. After incubation, the wells were washed with sterile distilled water and air-dried for 60 min to eliminate weakly adherent cells. The biofilm was stained with 1% crystal violet and fixed with 200 µL of 95% ethanol [12]. Absorbances were measured at 570 nm using a spectrophotometer, with all tests conducted in triplicate. PA01 (without sildenafil) served as the positive control, while a culture medium without inoculum was the negative control.

2.4.2. Inhibition and Destruction of Biofilm Formation

To evaluate the efficacy of sildenafil citrate in both inhibiting and destroying biofilms, treatments were administered using concentrations determined by prior MIC and MBC values. For inhibition studies, 96-well plates were prepared as detailed in Section 2.3.2, incorporating sildenafil citrate at sub-inhibitory levels.

It is critical to note that the inhibition assays employed concentrations below the MIC to assess whether sildenafil can prevent biofilm formation in its early stages through mechanisms that do not involve killing the microorganism. The goal is to explore the potential of sildenafil in preventing microbial biofilm formation. These plates were incubated at 37 °C for 24 h. For biofilm destruction, treatments were applied to pre-formed biofilms after a 24-h incubation to break down the adherent microbial mass. In these destruction assays, the biofilm had already reached a mature phase, making its eradication significantly more challenging.

Therefore, concentrations equal to or greater than the MIC were utilized in this context. Following treatment, these plates were also incubated at 37 °C for 24 h. After incubation, the wells underwent washing and were analyzed using the method outlined in Section 2.4.1 [13].

2.4.3. Atomic Force Microscopy

To investigate the biofilm structure, tests for both inhibition and destruction were performed using 24-well plates. Polyethylene plates (Braskem, São Paulo, Brazil) measuring 5 × 5 mm were placed inside the wells to facilitate biofilm formation and subsequent microscopic analysis. After incubation, as described in Section 2.4.2 [13], the polyethylene plates were fixed in 95% ethanol. The resulting dispersion was then placed on freshly cut mica slices for imaging. A Park NX10 microscope (Park Systems, Suwon, Republic of Korea), equipped with SmartScan software version 1.0.RTM11a, was used to record topographic maps.

Measurements were taken with a Tap-300G probe at a nominal resonance frequency of 300 kHz and a force constant of 40 N/m. Recordings were carried out under ambient conditions at a room temperature of 21 ± 5 °C and a relative humidity of 55 ± 10%, at a scan rate of 0.35 Hz. Image processing was conducted offline using XEI software version 4.3.4Build22.RTM1 [12]. Biofilm samples were analyzed based on their fractal dimension to assess surface roughness and topographical complexity [14].

2.4.4. Fractional Inhibitory Concentration Index

The study included combinations of three commercial antimicrobial drugs (cefepime, imipenem, and polymyxin B), commonly used to treat serious *P. aeruginosa* infections. Initial MIC tests were performed for each drug using the method outlined in Section 2.3.2 [12], with initial concentrations of 200 mg/mL for cefepime, 50 mg/mL for imipenem, and 25 mg/mL for polymyxin B. The fractional inhibitory concentration index (FICI) was calculated by dividing the MIC of each drug in combination by its MIC when used alone.

The combined FICI value determined the interaction between the drugs: synergism (FICI $\leq$ 0.5), indifference (0.5 < FICI $\leq$ 4.0), and antagonism (FICI > 4.0) [15]. Observations of macroscopic turbidity after 24 h indicated the presence or absence of activity. The interpretation of the drug interactions was based on the FICI values and the established classification [16]. The study was performed in three replicates.

2.4.5. Statistics

The results were expressed as mean $\pm$ standard error of the mean (SEM) in Microsoft Excel. The activity was analyzed statistically using one-way analysis of variance (ANOVA) with Systat 11 software (Systat, Richmond, VA, USA). A p-value of less than 0.05 was considered statistically significant. GraphPad Prism 6 software was used for graph creation.

3. Results

3.1. Antimicrobial Activity

The broth microdilution test revealed that sildenafil citrate has antimicrobial activity against both PA01 and clinical isolates (Table 2). MIC values ranged between 3.12 and 6.25 mg/mL, with the clinical isolates 4PAUR, 6AST, and 7PAES showing higher susceptibility. The MBC values varied from 3.12 to 25 mg/mL, with most CIs having an intermediate concentration of 12.5 mg/mL. The lowest concentrations were observed in 5PAES and 7PAES.

Table 2. The MIC and MBC results.

Microorganism	MIC (mg/mL)	MBC (mg/mL)
PA01	3.12	6.25
1PAUR	6.25	12.5
2PAHC	6.25	12.5
3PAUR	6.25	12.5
4PAUR	3.12	25
5PAES	6.25	3.12
6PAST	3.12	12.5
7PAES	3.12	3.12
8PAUR	6.25	12.5
9PAUR	6.25	12.5
10PAUR	6.25	12.5

3.2. Antibiofilm Activity

3.2.1. Biofilm Formation

In evaluating the biofilm-forming capabilities of the CIs, their absorbance levels were compared to those of the standard strain (i.e., PA01). After 24 h of incubation, the CIs 1PAUR, 2PAHC, 3PAUR, and 5PAES exhibited absorbance values significantly lower than those of PA01, categorizing them as non-biofilm forming. However, after 48 h of incubation, the CIs 2PAHC and 3PAUR showed different profiles, with absorbance levels that were equal to or exceeded those of PA01 (Figure 1A,B).

This test confirmed their capacity for biofilm formation. Biofilms were identified at 24 h (A) or 48 h (B) using crystal violet, a dye extensively utilized in biofilm formation assays to quantify total biofilm biomass. The greater the absorbance measured, the more biomass adheres to the microplate, indicating a higher biofilm presence in the well.

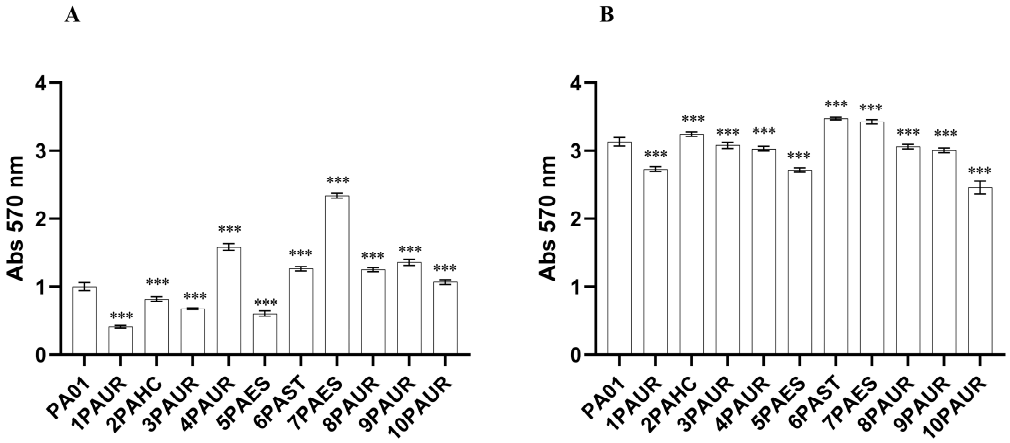

Figure 1. Biofilm formation profile of reference strain (PA01) and clinical isolates after 24 h (**A**) and 48 h (**B**). An analysis of variance followed by Tukey's test was used, considering *p*-values < 0.05 to be statistically significant (***) when compared with the PA01 strain group. The study was performed in three replicates.

3.2.2. Biofilm Inhibition and Destruction

The ability of sildenafil to inhibit biofilm formation and destroy the formed biofilm was recorded (Figures 2 and 3). The percentage values above each bar represent the reduction in total biofilm biomass relative to the positive control for biofilm formation. Sildenafil citrate was found to effectively inhibit biofilm formation at all tested concentrations (3.12–0.78 mg/mL). Notably, Figure 2G shows that at a sub-inhibitory concentration—half of the MIC ($^1/_2$ MIC, 1.56 mg/mL)—sildenafil citrate inhibited PA01 biofilm formation by 33.33%. Similar efficacy was observed in the clinical isolates (Figure 2A–F), with Figure 2C highlighting an inhibition rate of 87.17% at a reduced drug concentration.

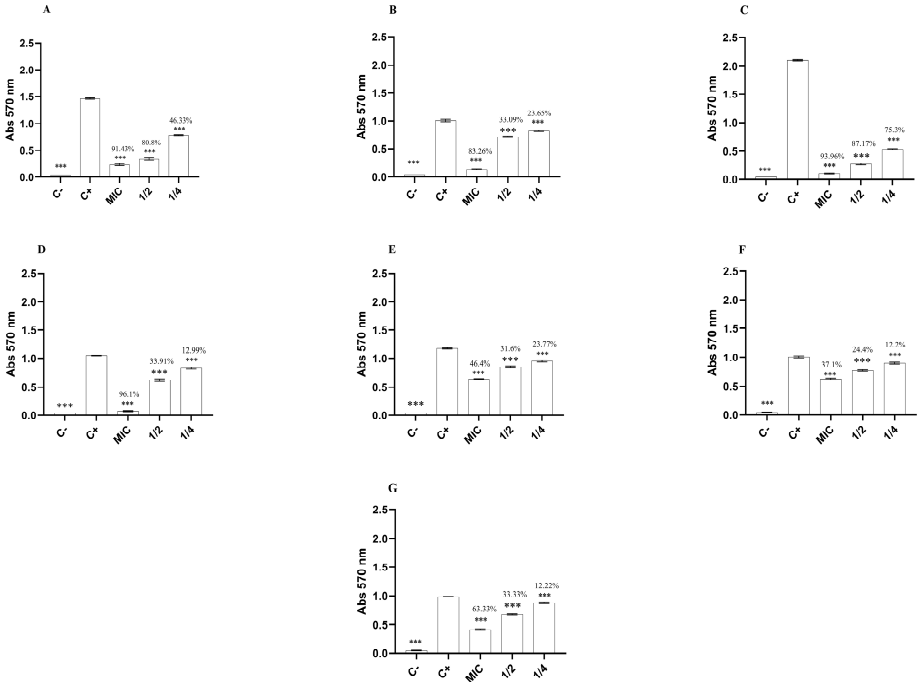

Figure 2. Biofilm inhibition results. (**A**) 4PAUR, (**B**) 6PAST, (**C**) 7PAES, (**D**) 8PAUR, (**E**) 9PAUR, (**F**) 10PAUR, and (**G**) PA01, with C- representing the negative control and C+ the positive control. The percentages above each bar represent the reduction in total biofilm biomass compared to the positive biofilm formation control. Analysis of variance (ANOVA) followed by Tukey's test was conducted, considering $p < 0.05$ as statistically significant (***), specifically when compared with the positive control (C+) group. The study was performed in three replicates.

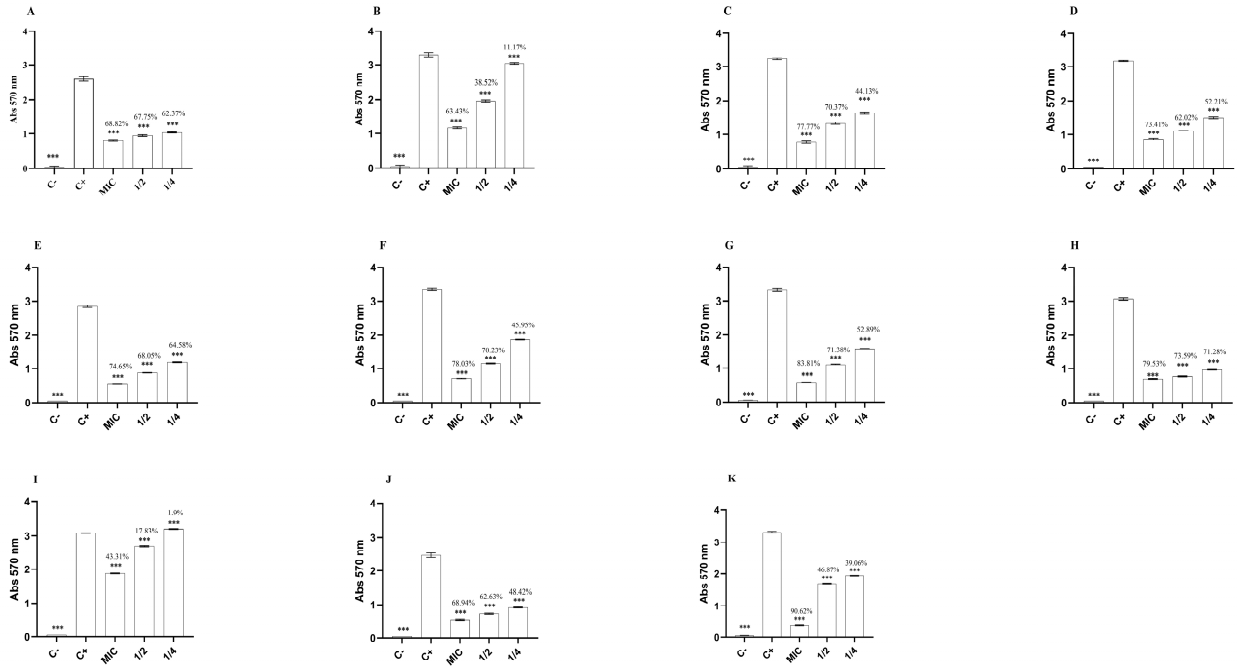

Figure 3. Biofilm destruction results. (**A**) 1PAUR, (**B**) 2PAHC, (**C**) 3PAUR, (**D**) 4PAUR, (**E**) 5PAES, (**F**) 6PAST, (**G**) 7PAES, (**H**) 8PAUR, (**I**) 9PAUR, (**J**) 10PAUR, and (**K**) PA01, with C- representing the negative control and C+ the positive control. The percentage values above each bar show the reduction in total biofilm biomass compared to the control that positively formed biofilms. An analysis of variance (ANOVA) followed by Tukey's test was utilized, considering $p < 0.05$ as statistically significant (***) in comparison with the positive control (C+) group. The study was performed in three replicates.

The results regarding biofilm destruction indicate that sildenafil can disrupt the protective matrix, which enables microorganisms to survive in adverse environments, thus hindering the treatment of biofilm-related infections [8,9]. Figure 3K illustrates that sildenafil citrate eliminated 46.87% of the biofilm at a sub-inhibitory concentration. For the isolates, the percentage of biofilm destruction at the MIC varied between 83.81 and 43.31%. Notably, the sample depicted in Figure 3G exhibited the highest rates of destruction—83.81% at the MIC, 73.38% at $^{1}/_{2}$ MIC, and 52.89% at $^{1}/_{4}$ MIC.

3.2.3. Atomic Force Microscopy

Figures 4 and 5 present 2D and 3D topographical maps generated through atomic force microscopy for MIC-treated high-density polyethylene substrates (Braskem, São Paulo, Brazil) at a concentration of 3.12 mg/mL plus 100 mg/mL sildenafil citrate, focusing on biofilm destruction and inhibition. The negative control showed a smooth surface typical of polyethylene, whereas the positive control sample (using only the PA01 strain) exhibited significant topographical differences compared to the pure polymer.

The formation of biofilms was visible in both 2D and 3D images, marked by the growth of bacterial structures within the polymeric matrices. Figures 4 and 5 demonstrate the compound's success in both inhibiting and destroying biofilms. Evaluations were conducted at three concentrations, MIC, $^{1}/_{2}$ MIC, and $^{1}/_{4}$ MIC, with all concentrations displaying biofilm inhibition and destruction. The most successful outcome was observed at the MIC concentration of 3.12 mg/mL, confirmed by the topographical images showing a surface free of bacterial structures, thus highlighting the efficacy of this treatment in removing the protective bacterial matrix and preventing biofilm formation.

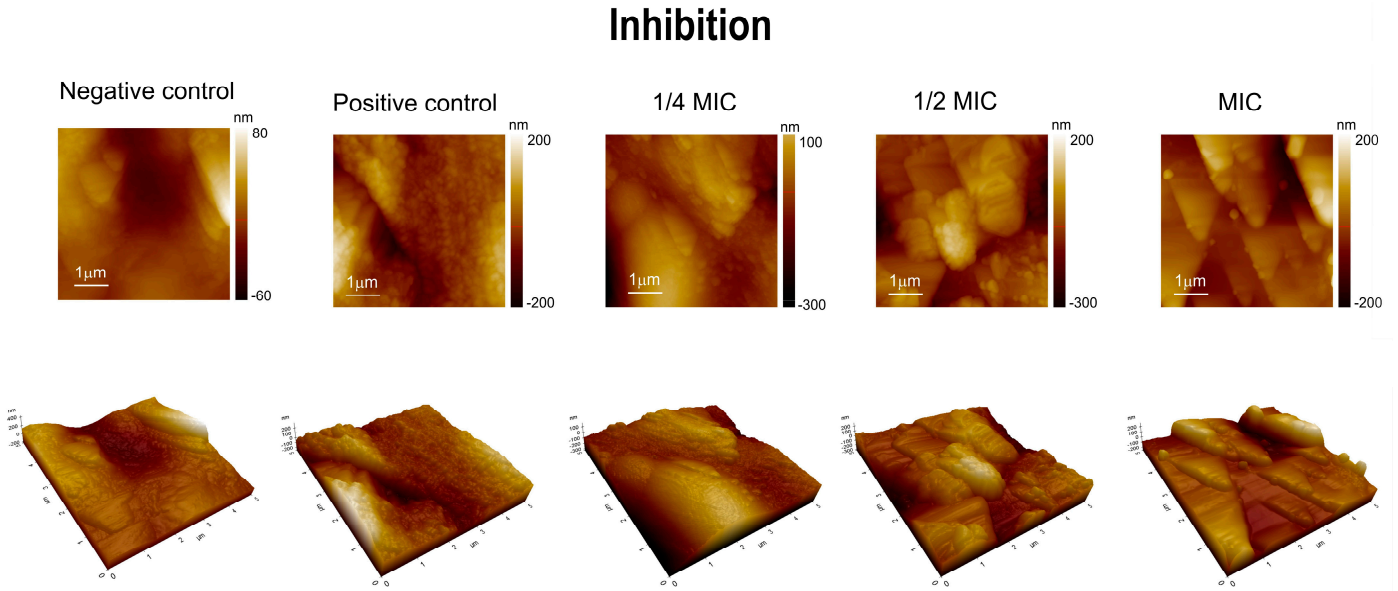

Figure 4. Atomic force microscopy images of *P. aeruginosa* biofilm inhibition by sildenafil citrate.

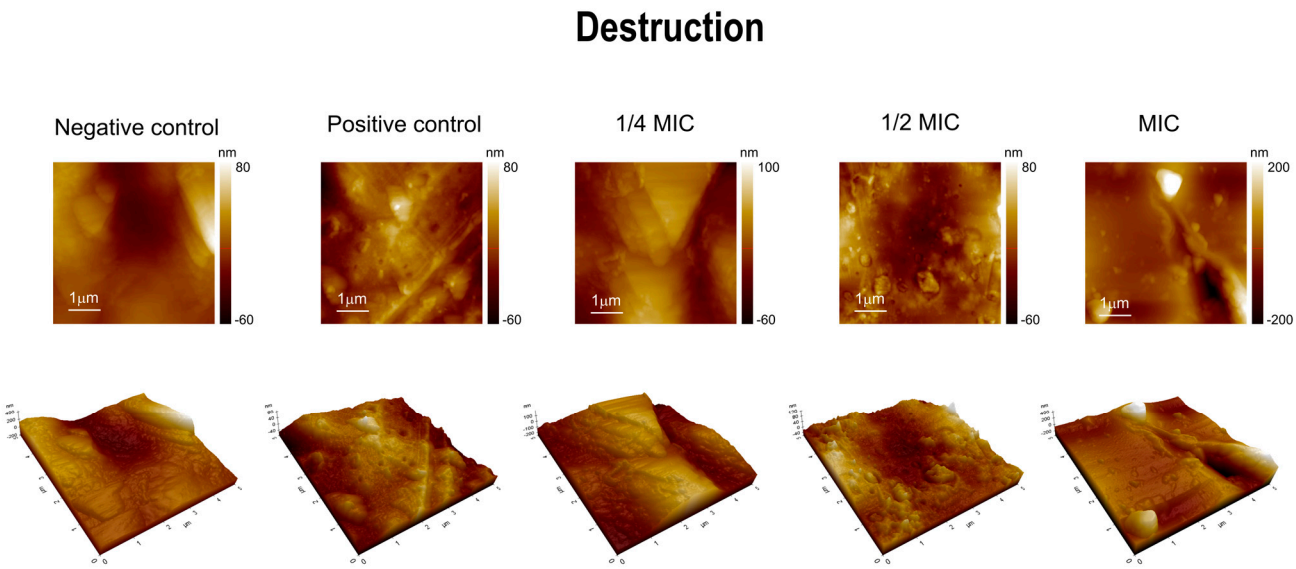

Figure 5. Atomic force microscopy images of *P. aeruginosa* biofilm destruction by sildenafil citrate.

The examination of fractal dimensions revealed that samples with biofilm showed increased topographical complexity. For the positive control, inhibition and destruction values were 2.165 and 2.163, respectively. Better results were seen in the MIC value images, with readings of 2.135 and 2.093, showcasing the drug's effectiveness against the PA01 strain. However, for a comprehensive analysis, a minimum of five images should be reviewed, particularly since the values for $^{1}/_{4}$ and $^{1}/_{2}$ MIC were similar to those of the positive control.

3.2.4. Checkerboard Assay

Antimicrobial concentrations were determined based on the MIC for each drug—cefepime, imipenem, cilastatin, and polymyxin B—against the standard PA01 strain and the isolate 7PAES. The results were evaluated according to the lowest FICI in wells that displayed no turbidity [16,17]. This methodology revealed 100% synergism in antimicrobial interactions, indicating a significant effect of the drug combinations, as shown in Table 3.

Table 3. Evaluation of synergism between sildenafil citrate and polymyxin B, sildenafil citrate and cefepime, and sildenafil citrate and imipenem against 7PAES and PA01.

Antimicrobials	Clinical Isolate (7PAES)	PA01 Strain
Sildenafil/polymyxin B		
Mean FICI	0.063	0.063
Range	0.6–0.7	0.6–0.7
% Synergism	100	100
Sildenafil/cefepime		
Mean FICI	0.093	0.093
Range	0.09–0.1	0.09–0.1
% Synergism	100	100
Sildenafil/imipenem		
Mean FICI	0.187	0.187
Range	0.1–0.2	0.1–0.2
% Synergism	100	100

Synergism: FICI $\leq$ 0.5; indifference: 0.5 < FICI $\leq$ 4.0; antagonism: FICI > 4.0.

4. Discussion

Our findings show that the drug has significant antimicrobial activity and can inhibit and destroy microbial mass effectively, even at low concentrations. Microscopy images support these results, verifying that sildenafil citrate successfully inhibits and disrupts biofilm formation, even at doses below the inhibitory concentration.

It is well-documented that bacterial infections lead to biofilm formation in more than 80% of cases, causing chronic conditions and high morbidity rates, according to the National Institutes of Health [18,19]. *P. aeruginosa* biofilm formation is linked to various chronic human infections, including chronic wound infections, otitis, prostatitis, urinary tract infections, and infections related to medical devices such as intravenous catheters and heart valves. These infections often persist and progress despite treatment, highlighting the importance of biofilm formation as a key virulence factor [6].

Sildenafil citrate works by inhibiting the 5PDE enzyme, which is crucial for converting cGMP to GMP and cAMP to AMP. This inhibition increases cellular cGMP levels, which affects protein kinase G (PKG) activity, influencing ion channel conductance, cellular apoptosis, and glycogenolysis [20,21]. The drug's antimicrobial and antibiofilm effects may relate to this mechanism of action.

Our study also explored the synergistic effects of sildenafil citrate in combination with commonly used antimicrobials using the checkerboard method (FIC index assay). We discovered that when used together, these drugs act synergistically, boosting the drug's effectiveness [22,23]. This finding indicates a promising approach for treating *P. aeruginosa* infections with sildenafil citrate, especially when combined with other antimicrobials. However, further studies are necessary for each strain to determine the impact of specific resistance mechanisms on synergy.

Considering the decrease in newly approved antimicrobials over the past decade and the rise in drug-resistant bacteria, sildenafil citrate represents a promising approach for treating *P. aeruginosa* infections. Although more research is needed, the drug could provide a timely and effective therapeutic option, especially in combination with approved medications.

5. Conclusions

This study revealed for the first time that sildenafil citrate has antimicrobial and antibiofilm efficacy, as well as a synergistic effect with other tested drugs. It showed antimicrobial activity within a range from 3.12 to 6.25 mg/mL and bactericidal activity from 6.25 to 12.5 mg/mL against *P. aeruginosa* (PA01) and ten clinical isolates. The drug also effectively inhibited and destroyed biofilm formation at tested concentrations (3.12–0.78 mg/mL) against *P. aeruginosa* (PA01) and ten clinical isolates. Atomic force microscopy confirmed sildenafil citrate's capability to inhibit and destroy biofilm on polystyrene plates of the standard strain of *P. aeruginosa* (PA01). A synergistic effect was achieved with all associated

drugs, highlighting sildenafil citrate's efficacy. Therefore, sildenafil citrate shows promise in combating *P. aeruginosa*, commonly found in hospitalized patients, especially those in intensive care units, marking a finding of significant clinical importance. The preliminary results presented here are robust and indicate significant antimicrobial and antibiofilm activity. However, further studies should be conducted in order to provide greater safety for the clinical use of sildenafil in infectious processes.

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