

RESEARCH ARTICLE

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Cuminaldehyde in Combination with Ciprofloxacin Shows Antibiofilm Activity against Clinical Strains of *Pseudomonas aeruginosa*: A Study to Explore the Underlying Mechanism

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Abstract

Pseudomonas aeruginosa poses a serious threat in healthcare settings. This bacterium can develop resistance to many antibiotics, rendering even last-resort treatments ineffective. Additionally, it forms protective biofilms that shield it from the immune system, making infection treatment challenging. This study investigated the susceptibility of five clinically isolated strains of the test bacteria to a combination of ciprofloxacin and cuminaldehyde. Cuminaldehyde (a natural phytochemical) and ciprofloxacin (an antibiotic) were separately found to show antimicrobial effect against test organism. However, the combination of selected compounds showed an additive effect in their microbial growth inhibitory activity. The mentioned compounds at their sub-MIC doses subjected to test whether they could show any extent of biofilm inhibition or disintegration property against the clinical strains of *P. aeruginosa*. The chosen concentrations of the compounds demonstrated significant antibiofilm activity against all the tested clinical strains. Additionally, it was observed that the compounds not only accumulated reactive oxygen species (ROS) but also enhanced the cell membrane permeability of the clinical strains. These findings suggest that the combination of ciprofloxacin and cuminaldehyde could explore new directions in fighting *P. aeruginosa*-linked infections.

Keywords: *Pseudomonas aeruginosa*, Ciprofloxacin, Cuminaldehyde, Additive Interaction, Biofilm

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INTRODUCTION

P. aeruginosa, a major threat in clinical settings, is a Gram-negative opportunistic bacterium that could develop biofilms on various abiotic or biotic surfaces.¹ These biofilms are slimy, multicellular communities that defend bacteria from the immune system.² This promotes the rate of chronic infections, such as cystic fibrosis or those associated with implanted medical devices.³ The emergence of antibiotic-resistant biofilms of *P. aeruginosa* presents a noteworthy challenge in treating these chronic infections. Studies suggest a high prevalence of biofilm formation among *P. aeruginosa* isolates. One study reported 83.75% of clinical isolates displayed biofilm-forming ability.⁴ This ability to form biofilms, combined with growing resistance to antibiotics, makes *P. aeruginosa* a major contributor of prolonged untreated infections. Recent studies focus on exploring new treatment strategies to combat these resilient biofilms. One such approach is a combinatorial study investigating the effectiveness of combining natural compounds with antibiotics.^{5,6} In this regard, we had already used ciprofloxacin and cuminaldehyde against *P. aeruginosa* by considering the potential of ciprofloxacin against *P. aeruginosa* (MTCC 424), a non-pathogenic strain.⁷ Ciprofloxacin, a potent fluoroquinolone antibiotic has fairly good activity against the Gram-positive bacteria. However, its efficacy has proved to be more potent in Gram-negative bacteria when compared to other fluoroquinolones.⁸ The recent literature suggests that the clinically isolated strains of *P. aeruginosa* have been found to be more infectious and pathogenic than non-clinical strains.⁹ Clinical-pathogenic strains possess a range of virulence factors, such as toxins and enzymes that allow them to, less virulent strains invade and damage host tissues unlike the non-pathogenic. They also tend to be more adept at forming biofilms exhibiting different physiological patterns than the non-pathogenic strains.¹⁰ Although, the effectiveness of the compounds (cuminaldehyde and ciprofloxacin) has already been reported against the non-clinical strains of *P. aeruginosa*, the potential of the same has yet to be tested on the clinical strains of the same bacteria. Thus, in this study, we have combined ciprofloxacin and

cuminaldehyde against the growth of the clinical strains of *P. aeruginosa*. The study revealed that ciprofloxacin and cuminaldehyde was highly effective and shows promise in inhibiting the microbial growth as well as managing biofilm threats of the clinical strains while applied in combination. Hence, this approach offers a possible solution to combat biofilm-mediated *P. aeruginosa* infections.

MATERIALS AND METHODS

Microbial strains, chemicals and growth media

Five clinical strains of *P. aeruginosa*, obtained from Suraksha Diagnostic Centre, Kolkata, were considered as the organism of interest in the current study. The bacteria was cultivated at 37°C for 24 h in sterile Luria Bertani broth (LB; obtained from Sisco Research Laboratories). Cuminaldehyde, a natural compound (Sigma-Aldrich), and ciprofloxacin, an antibiotic (Sisco Research Laboratories), were selected as test compounds. Cuminaldehyde was dissolved in DMSO (Dimethyl Sulfoxide), while ciprofloxacin (10 mg/mL) was dissolved in MilliQ water and further diluted in LB as per the experimental requirement.

Estimating the minimum inhibitory concentration (MIC)

The minimum amount of any anti-microbials that prevents a microorganism to grow visibly is termed as the MIC value of that antimicrobial.¹¹ Here, MIC of ciprofloxacin and cuminaldehyde was estimated against the clinical strains using a 2020 protocol of Chakraborty *et al.*¹² In this regard, at first, equal numbers (10^6 CFU/mL) of the test organisms were cultured in autoclaved growth media (5 mL) in which various concentrations of the natural compound, cuminaldehyde (ranging from 0 µg/mL to 600 µg/mL) and ciprofloxacin (ranging from 0 µg/mL to 15 µg/mL) were added individually. After incubation at 37°C for a period of 24 h, the extent of microbial growth was determined at 600 nm using a colorimeter.

Determining Fractional-Inhibitory-Concentration Index (FICI)

The checker board assay was conducted to determine FICI to understand the possible

interactions between cuminaldehyde and ciprofloxacin. For this experiment, an equal number (10^6 CFU/mL) of overnight inoculated cultures of all clinical isolates were further cultured into sterile growth media and subsequently treated with varying concentrations of cuminaldehyde (up to 600 μ g/mL) and ciprofloxacin (up to 20 μ g/mL), or left unexposed. After incubation at 37°C for a period of 24 h, microbial growth was assessed, and using the following formula the FICI was calculated:

FICI of cuminaldehyde against clinical strains = MIC (cuminaldehyde) while applied in combination / MIC (cuminaldehyde) while applied alone against clinical strains.

FICI of ciprofloxacin against clinical strains = MIC (ciprofloxacin) while applied in combination / MIC (ciprofloxacin) while applied alone against clinical strains

$FICI = FIC \text{ (cuminaldehyde)} + FIC \text{ (ciprofloxacin)}$

The interaction between the two selected compounds was calculated using the FIC index provided in established documents¹³:

When the value of FIC is ≤ 0.5 , it is synergistic;

When the value of FIC is >0.5 and ≤ 1 , it is additive;

When the value of FIC is >1 and ≤ 4 , it is intermediate;

When the value of FIC is >4 , it is antagonistic

Crystal-Violet (CV) assay

Assessment of bacterial biofilm using CV assay relies on the ability of the basic dye, CV, to bind to negatively charged cells and surface molecules of the extracellular polymeric substance.¹⁴ Thus, the CV staining assay is widely recognized as a conventional method for quantifying microbial biofilm formation on various surfaces.¹⁵ In this study, the degree of biofilm of both untreated and treated test organisms were analyzed using the CV assay, using the protocol adapted from Das *et al.*¹⁶ To proceed with the experiment, 10^6 CFU/mL of the test bacteria were inoculated into autoclaved growth media (5 mL) and subsequently treated with 50 μ g/mL of cuminaldehyde (for all five selected isolates). Ciprofloxacin was either added along with cuminaldehyde (0.3 μ g/mL for

PSA1; 0.1 μ g/mL for PSA2; 0.01 μ g/mL for PSA3 and 1 μ g/mL for PSA4, PSA5) or left unexposed. Following incubation, free-living planktonic cells were removed and the adhered biofilm on the test tubes was CV-stained and kept aside for half an hour. The non-adhered CV solution was discarded post incubation, and the tubes were washed with sterile double-distilled water. Thereafter, 33% acetic acid (glacial) solution (5 mL) was added to the respective tubes to dissolve the stain adhered with the biofilm cells. Finally, OD₆₃₀ was recorded by measuring the intensity of the CV solution using a colorimeter.

Analysis of bacterial biofilm under light microscope

Microscopic images offer detailed visual presentation of biofilm formation of a microorganism under a given condition.¹⁷ Therefore, to analyze the biofilm profiles of test organisms in the absence or presence of selected doses (as mentioned previously) of test compounds, light microscopic image analysis was considered. To perform this analysis, clinical strains of *P. aeruginosa* (10^6 CFU/mL) were inoculated in LB media containing coverslips that supported the formation of biofilm clusters either in the absence or presence of the chosen concentrations of test compounds (ciprofloxacin and/or cuminaldehyde) under same experimental conditions. Following overnight incubation, coverslips from each experimental set were aseptically collected from each experimental set, gently washed with MilliQ water, subsequently stained CV (0.4%) in solution form for half an hour and observed under a light microscope (Olympus CX21i).

Assessment of total biofilm protein

As an indirect measure, quantifying total biofilm protein content offers a reliable method for the relative assessment of biofilm profile.¹⁸ To this end, the Bradford assay was conducted to analyze the total biofilm protein content of all clinically isolated *P. aeruginosa* strains, both in the absence and presence of test compounds, using the protocol outlined by Malik *et al.*⁷ For this assay, cells were either challenged with selected doses of cuminaldehyde and/or ciprofloxacin (as mentioned previously), or left untreated as a control set. After incubation at 37°C for a period

of 24 h, biofilm cells adhered to the tube were considered. NaOH solution (5 mL, 0.3 M) was added in each tube and boiled for half an hour to extract the total protein of the biofilm present on the glass tube surface. Then the supernatant was collected by centrifuging the solution for 10 min at 8000 rpm. Bradford reagent was mixed with the supernatant in a 1:1 ratio and kept aside for 30 min at room temperature (RT). Finally, the OD at 595 nm was recorded to measure the intensity of the color developed by the biofilm protein.

Measurement of extracellular polysaccharides (EPS)

To measure the EPS of all clinical isolates, the phenol-sulfuric acid method, as outlined by Dubois *et al.*¹⁹ was followed. The EPS profile, consisting of primary and major constituents, reflects the degree of biofilm formation on the given surface. For this assay, 10^6 CFU/mL of test organism were cultivated to form biofilm on the glass surface in the presence or absence of the chosen concentrations of cuminaldehyde and/or ciprofloxacin (as mentioned previously). A control set was also prepared where 10^6 CFU/mL of test organism was left untreated. Post incubation, free-living planktonic cells were removed, and the attached biofilm cells on the glass tube were suspended in phosphate-buffered saline (PBS; pH 7.4). This prepared suspension was then subjected to centrifugation at a rate of 6000 rpm for around 10 min. The supernatant obtained from this step was the cell-free EPS and the pellet (re-suspended in 10 mM EDTA) obtained was re-centrifuged (10 min, 6000 rpm) to extract the cell-bound EPS matrix. Then, the supernatant obtained from each of the sets was dissolved in chilled ethanol (1:2.2 ratios) for an hour at 4°C. Then, this suspension was subjected to 6000 rpm centrifugation for 20 min. The pellet obtained from this step was termed as the total EPS content. To measure the degree of EPS extracted in both treated and untreated samples, the extracted pellets were re-suspended in 1 mL of PBS and mixed with phenol and concentrated sulfuric acid in a ratio of 2:1:5 (sample: phenol: sulfuric acid), respectively. The prepared mix solution was then boiled at 100°C for 10 min and cooled for 10 min at RT. Finally, the OD at 490 nm was recorded to measure the amount of EPS in each set.

Analysis of metabolic activity

The degree of metabolically active clinical isolates of the test bacteria was measured by employing FDA hydrolysis assay.²⁰ In this regard, equal numbers of biofilm cells of the clinical strains of *P. aeruginosa* were either untreated or treated with the compounds and following incubation, the cells were collected by scrapping. The cells collected from the previous step were suspended in PBS (5 mL, pH 7.4). Subsequently, 500 µL (5 mg/mL) FDA in solution form was dispensed in each tube for an hour in darkness. All the prepared experimental sets were then subjected to 10,000 rpm centrifugation for a time span of 10 min. Then, fluorescein, collected from the obtained supernatant was recorded at 490 nm.

ROS accumulation estimation

Intracellular ROS accumulation was assessed for all the selected clinical isolates of *P. aeruginosa*. DCFDA method was employed for all the various sample conditions (treated and untreated) by following Das *et al.*²¹ To conduct this experiment, cells were either treated with 50 µg/mL of the natural test compound, cuminaldehyde and/or various concentrations of the antibiotic, ciprofloxacin (0.3 µg/mL for PSA1; 0.1 µg/mL for PSA2; 0.01 µg/mL for PSA3 and 1 µg/mL for PSA4 and PSA5). A separate untreated control set was prepared where only the clinical strains were grown over a similar incubation temperature for 6 h. Then, each set was supplemented with 0.5 µL of DCFDA to achieve a final ratio of 1:2000 (v/v), and incubation at 37°C for half an hour. Finally, the quantity of accumulated ROS produced under each condition was estimated at 488 nm (excitation), and at 535 nm (emission).

Estimation of cell membrane permeability by Ethidium Bromide (EtBr) influx assay

The cell membrane permeability of the clinical strains of the test bacteria was analyzed in response to the test compounds by following the EtBr influx assay.²² For this experiment, overnight-grown cells were centrifuged and the collected cell pellet (1 mL) was suspended in PBS (pH 7.4). The cell pellet was further treated with 50 µg/mL of cuminaldehyde and/or various concentrations of ciprofloxacin (0.3 µg/mL for PSA1; 0.1 µg/mL for PSA2; 0.01 µg/mL for PSA3 and 1 µg/mL for PSA4

and PSA5) at 37°C for 6 h. Following treatment, both the untreated and treated cell suspensions were supplemented with EtBr (0.5 µg/mL) and kept aside without exposure to light for 5 min. Finally, the fluorescence intensity was measured in a fluorometer (excitation at 520 nm, emission at 590 nm).

Analysis of biofilm disintegration

The disintegrating property of the clinically isolated strains of the test bacteria was assessed under the presence of the test compounds. Similar cells were permitted to develop biofilm for a period of 24 h, 37°C. Following incubation, overnight-grown biofilm was either treated with cuminaldehyde or ciprofloxacin or both at similar concentrations stated previously for a span of another 6 h. The degree of residual biofilm denoted the biofilm disintegration percent compared to the control set. Thus, this residual biofilm was subjected to various experiments (CV assay, total biofilm-protein estimation, EPS measurement and FDA assay) already discussed in previous sections.

Analysis of biofilm management on catheter tubes

To analyze the test compounds in managing microbial biofilm formation on catheter surfaces, two sets of experiments were conducted. In the first set, sterile catheter tubes measuring 1 cm in length and 0.3 cm in width were exposed to the clinical strains of the test bacteria under the presence of the selected concentrations of the natural compound, cuminaldehyde and/or the antibiotic, ciprofloxacin. In the second set, a control set was considered without any compound exposure. After similar conditions of incubation time and temperature, catheter tubes from the second set were treated with the chosen concentrations of ciprofloxacin and/or cuminaldehyde for another 6 h. Following treatment, catheter tubes from both sets were aseptically collected, and CV assay, protein estimation, and EPS measurement assay were conducted by following previously established protocol was carried out.

Statistical analysis

To statistically analyze the key observations, ANOVA, One-way analysis of variance was explored (ANOVA). The average recorded value of all the experiment(s) was estimated post a three times repetition. An error bar of each data set represents the standard deviation of each experiment in comparison to the control set. The *p* values less than 0.05 represented with (*), less than 0.01 represented with (**), and less than 0.001 represented with (***) in comparison to the control. Whereas, *p* values beyond 0.005 were denoted as N.S. (statistically no difference).

RESULTS AND DISCUSSION

Antimicrobial activity of ciprofloxacin and cuminaldehyde against *P. aeruginosa*

The increasing challenge of treating infectious diseases caused by the opportunistic pathogen *P. aeruginosa* reinforces the scientific community relying on combinatorial therapies.²³ In this context, in our previous study, cuminaldehyde combined with ciprofloxacin has already shown potential against laboratory strains of *P. aeruginosa* (MTCC 424).⁷ To ensure whether the findings are directly applicable to the challenges encountered in clinical practice,^{24,25} in present study, effort has been accumulated to investigate the applicability of cuminaldehyde along with ciprofloxacin against clinically isolated strains of *P. aeruginosa*. Literature showed that clinical strains of *P. aeruginosa* often exhibited increased virulence through structural, morphological and metabolic adaptation, which is critical for their survival and proliferation in human hosts.²⁶ Moreover, clinical strains could show significant genetic diversity and frequent acquisition of mobile genetic elements, which confer diverse array of virulence factors and resistance to antibiotics commonly found in hospital environments.²⁷ This genetic flexibility allows them to adapt rapidly to selective pressures, enhancing their survival and virulence.²⁷ In contrast, laboratory strains often lack these adaptive mechanisms, making them less capable of interacting with and infecting human hosts. From a biological perspective, understanding host and pathogen interplay is essential for

developing new therapeutic strategies.²⁸ Studies on clinical isolates are likely to provide detailed insights into the drastically diverse forms of *P. aeruginosa* infections.²⁹ Therefore, the present study focused on total five clinical isolates of *P. aeruginosa* (PA1 to PA5). At first, MIC of all the isolates was determined using a broth dilution assay. The observation revealed that the MIC of cuminaldehyde exhibited 600 µg/mL, and that of

ciprofloxacin varied within 0.1-20 µg/mL across all the five isolates of test organism (Table). Furthermore, a checkerboard assay was conducted to understand the interaction between the two compounds. The result showed a reduction in effective concentrations of compounds when used together compared to individual application (Figure 1). Additionally, the FICI of >0.5 indicated a strong additive interaction between ciprofloxacin

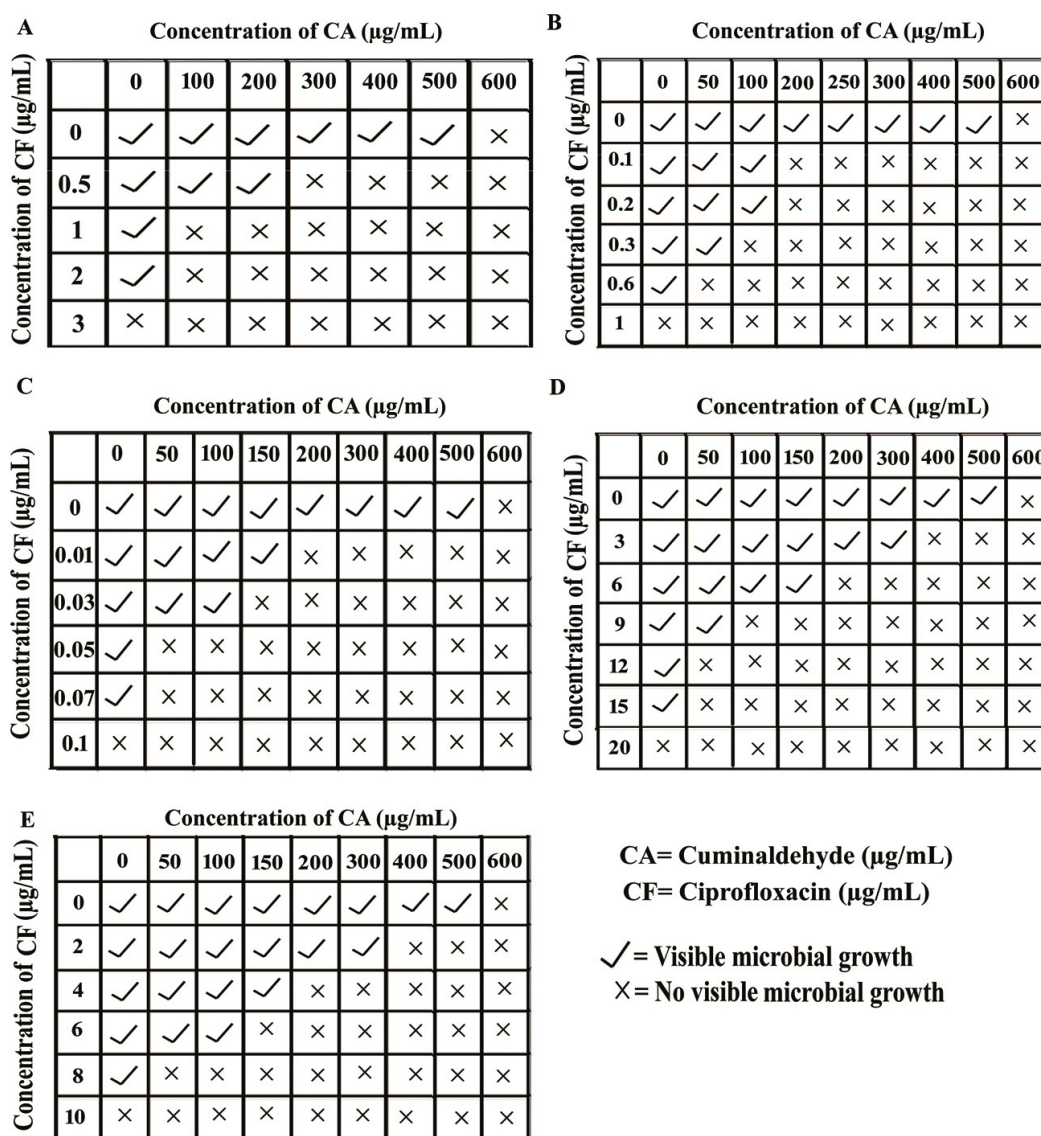
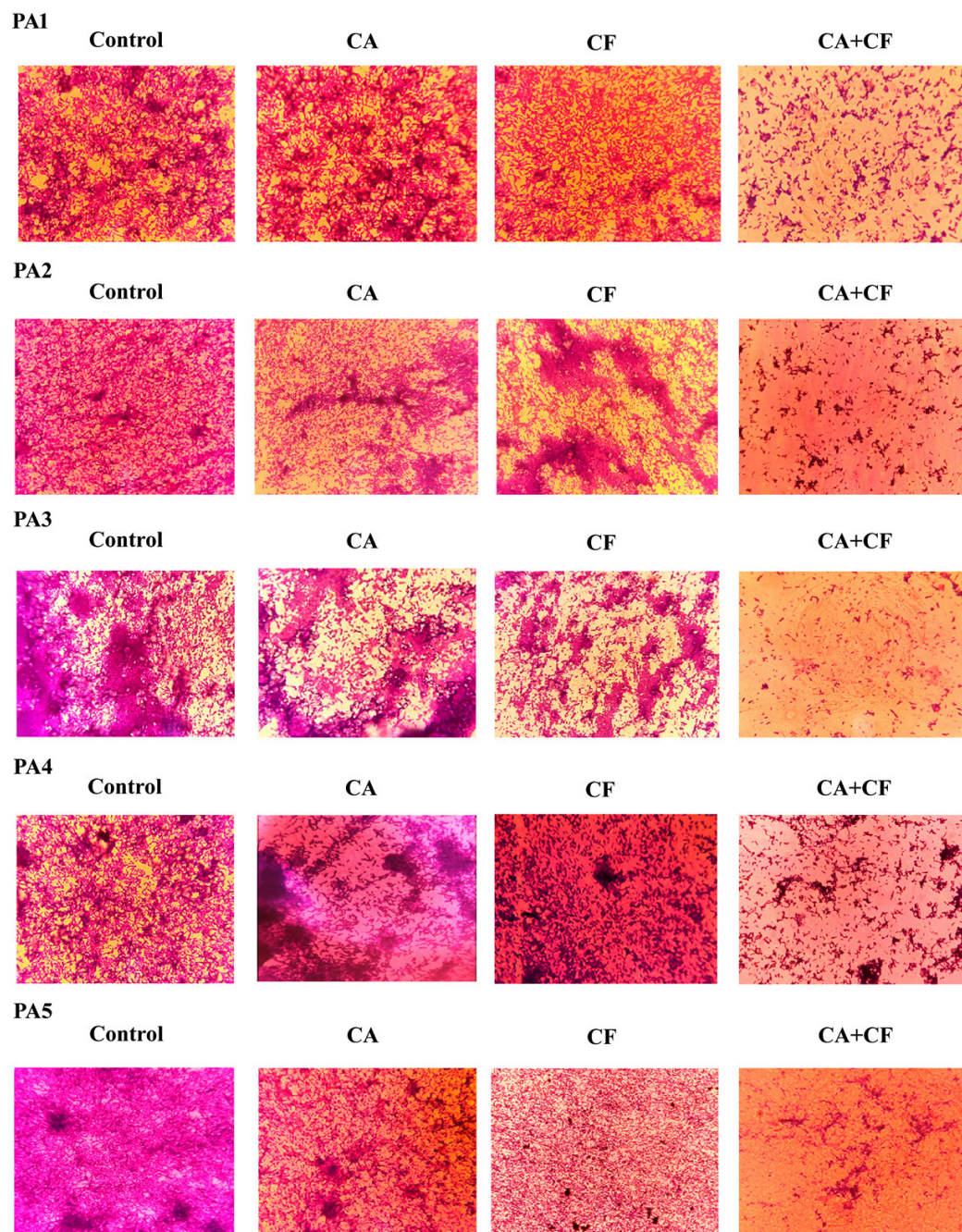


Figure 1. Antimicrobial activity of cuminaldehyde in combination with ciprofloxacin against the clinical strains of *P. aeruginosa*. A. Checkerboard assay of Isolate 1 (PA1). B. Checkerboard assay of Isolate 2 (PA2). C. Checkerboard assay of Isolate 3 (PA3). D. Checkerboard assay of Isolate 4 (PA4). E. Checkerboard assay of Isolate 5 (PA5)



CA= Cuminaldehyde ($\mu\text{g/mL}$) ; CF= Ciprofloxacin ($\mu\text{g/mL}$)

Figure 2. Microscopic image analysis of *P. aeruginosa* treated with cuminaldehyde in combination with ciprofloxacin. Sub-MIC doses of ciprofloxacin and/or cuminaldehyde were added in different tubes and incubated at 37°C for 24 h. Post incubation, coverslips were collected and stained with CV (0.4%) and observed under a light microscope. The figure is a representative image of 20 different spots of three independent experiments. A. Microscopic image of PA1. B. Microscopic image of PA2. C. Microscopic image of PA3. D. Microscopic image of PA4. E. Microscopic image of PA5

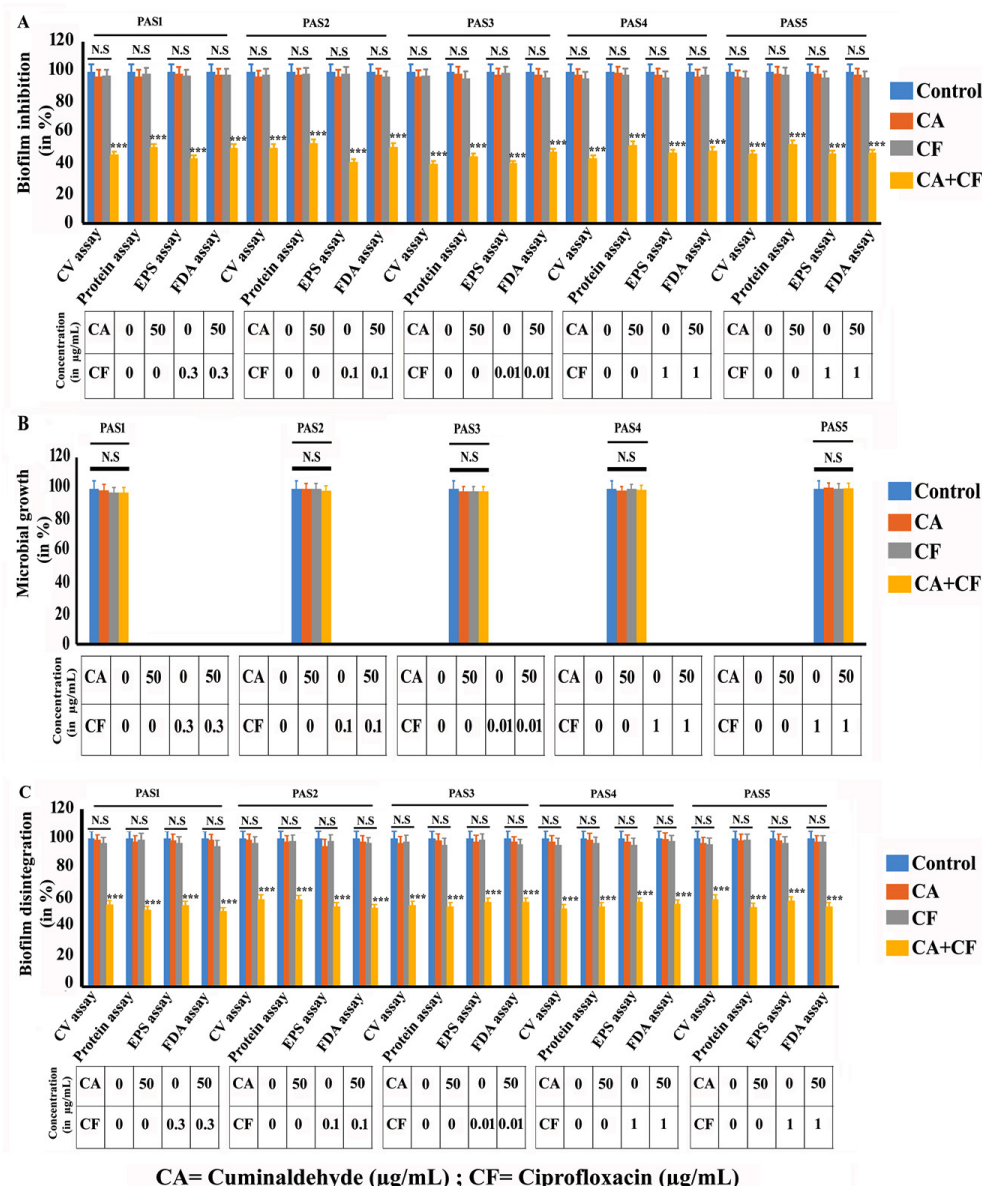


Figure 3. Cuminaldehyde in combination with ciprofloxacin exhibited considerable antibiofilm property against clinical strains of *P. aeruginosa*. A similar number of cells were cultured in sterile LB tube either in presence or in absence of sub-MIC doses of ciprofloxacin and/or cuminaldehyde for 24 h at 37°C. A. Biofilm profile. The extent of biofilm adhered on glass tube was measured by conducting CV assay, EPS measurement assay, total protein estimation assay and FDA assay. B. Antimicrobial profile. To understand the antimicrobial property of the selected sub-MIC doses of test compound upon the clinical strains, the OD of both treated and untreated sets of test samples were measured at 600 nm. C. Biofilm disintegration property. Overnight grown culture of *P. aeruginosa* was either treated with the selected sub-MIC doses of ciprofloxacin and/or cuminaldehyde or left untreated and further incubated for 6 h. Then, the degree of biofilm disintegration was estimated by performing CV assay, total EPS measurement assay, total protein estimation and FDA assay. Each experiment was carried out three times. The standard error of the mean was shown by the error bars. The p values were used to represent the statistical difference between the observations. The p values less than 0.05, 0.01, and 0.001 were marked by *, **, and ***, respectively

and cuminaldehyde against all clinical strains of *P. aeruginosa* (Table). Thus, the present study reports that the compounds either alone or in combination showed considerable antimicrobial activity. However, we reported that the compounds

(ciprofloxacin and cuminaldehyde) in combination could show heightened antimicrobial potential against the clinical strains due to additive interactions.

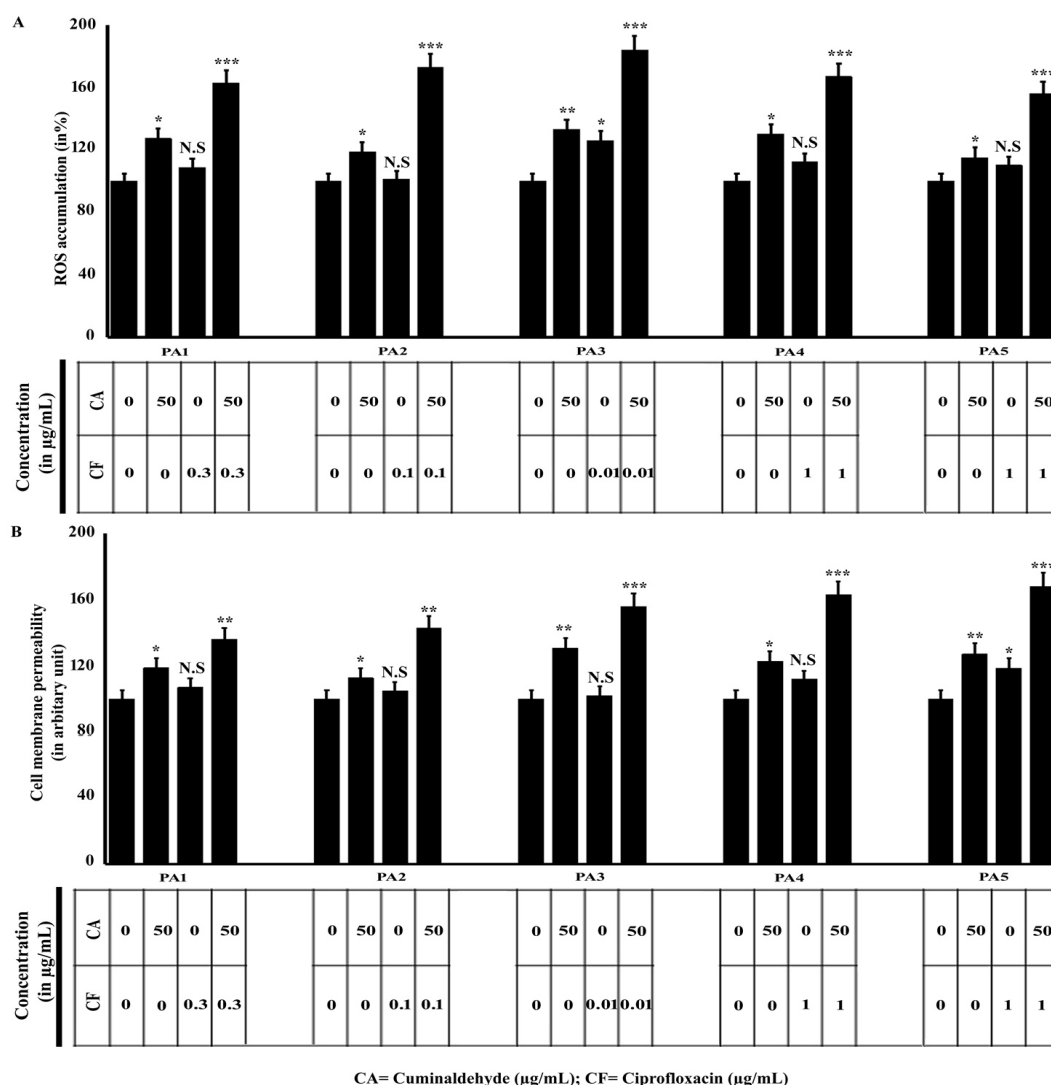
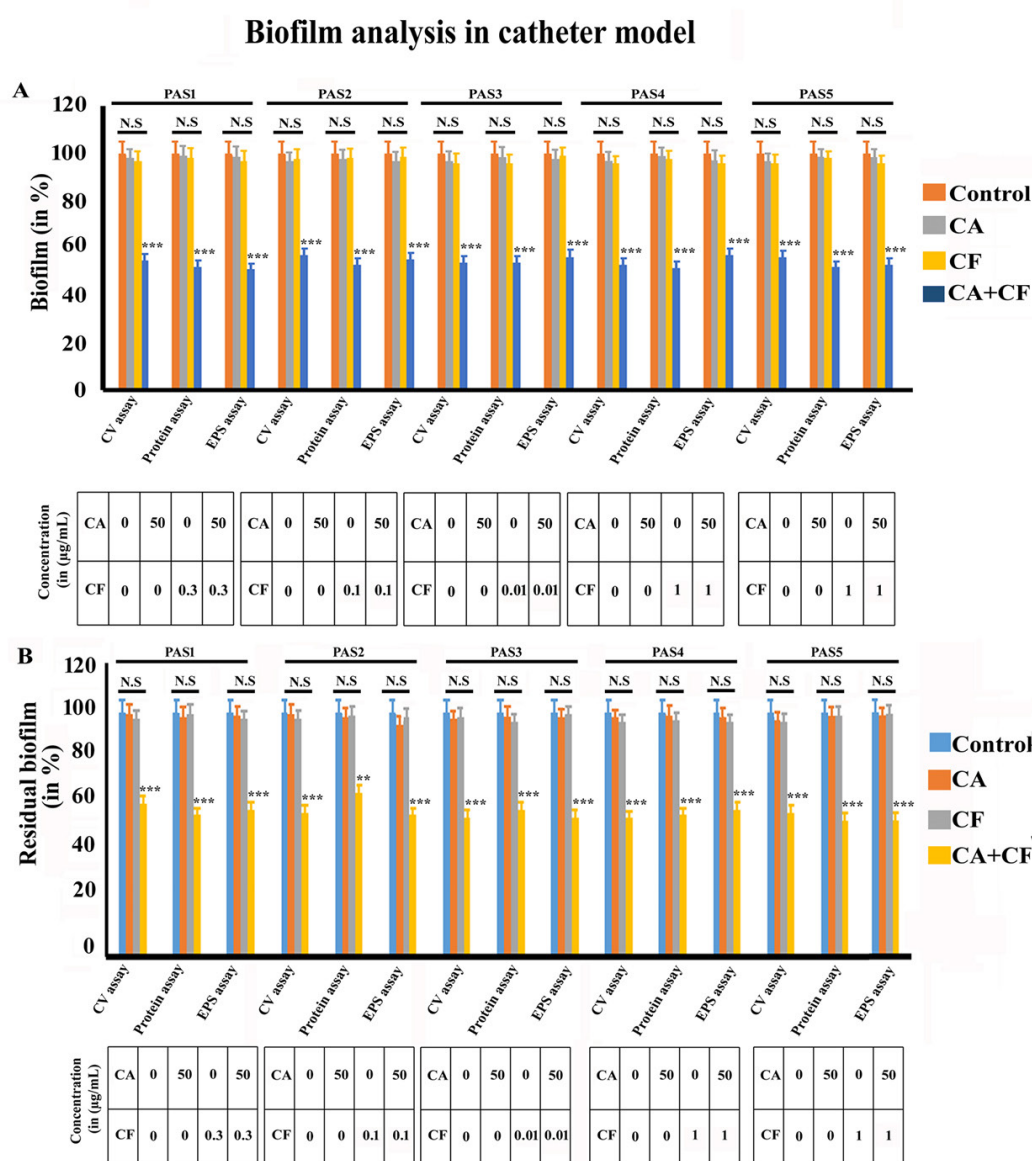


Figure 4. Investigating the mechanisms of cuminaldehyde combined with ciprofloxacin against the clinical strains of *P. aeruginosa*. A. Estimation of intracellular ROS accumulation. The intracellular ROS accumulation in clinical strains of *P. aeruginosa* from both compounds treated and untreated conditions were estimated by following the DCFDA assay. B. Estimation of cell membrane permeability. Accumulation of cell membrane permeability of *P. aeruginosa* cells from both treated and untreated conditions was estimated by EtBr influx assay as mentioned in the materials and method section. Each experiment was repeated three times. The standard error of the mean was shown by the error bars. The p values were used to represent the statistical difference between the observations. The p values less than 0.05, 0.01, and 0.001 were marked by *, **, and ***, respectively and p value greater than 0.05 signified Non-significant (NS)



CA= Cuminaldehyde ($\mu\text{g/mL}$) ; CF= Ciprofloxacin ($\mu\text{g/mL}$)

Figure 5. Ciprofloxacin and cuminaldehyde in combination exhibited considerable reduction in microbial biofilm formation of *P. aeruginosa* on catheter surfaces. A. Biofilm inhibition profile. Equal numbers of *P. aeruginosa* cells were grown on catheter tubes in LB medium for 24 h under various treatment conditions. After the incubation period, catheters were collected aseptically from all experimental sets. The biofilm inhibition profile was then assessed using several assays, including the CV assay, EPS estimation, and protein estimation. B. Biofilm disintegration profile. The extent of disintegration of the existing biofilm on the catheter surface was assessed under the treated and untreated conditions using the CV assay, EPS estimation, and protein estimation. Each experiment was carried out three times. The error bars represented the standard error of the mean. The p values were used to represent the statistical difference between the observations. The p values less than 0.05, 0.01, and 0.001 were marked by *, **, and ***, respectively

Cuminaldehyde and ciprofloxacin showed promising effect in managing biofilm threat of the clinical isolates of *P. aeruginosa*

Combating biofilm-related infections has become a focal point in medical and scientific research, with a particular emphasis on developing new therapeutics targeting biofilm challenges associated with *P. aeruginosa*.³⁰ Previous researches have demonstrated that sub-MIC doses of ciprofloxacin and cuminaldehyde in combination could inhibit microbial biofilm formation of the non-clinical strains of *P. aeruginosa*.⁷ Building upon this foundation, this research work focused in investigating whether the combined use of the selected compounds could inhibit biofilm formation across all the selected clinical strains of *P. aeruginosa*. Light microscopic image assessment, a method capable of visualizing bacterial biofilm morphology, revealed a noticeable reduction in biofilm formation under the combined application of the chosen concentrations of ciprofloxacin and cuminaldehyde in contrast to their individual treatments (Figure 2). To validate the microscopic observations, CV assay (evaluates the degree of biofilm formation on any surface), protein recovery assay (to quantify the protein content recovered from the biofilm adhered to the glass surface), EPS measurement assay (to assess the production of extracellular polysaccharides), and FDA-hydrolysis assay (to analyze the metabolically active biofilm cells adhering to the tube) were conducted.

The results thus cooperatively demonstrated that the combination of the test compounds efficiently impeded microbial biofilm formation by approximately 50% across all five clinical isolates of *P. aeruginosa* (Figure 3A). Moreover, the selected sub-MIC concentrations of the compounds did not exhibit any inhibitory action on the microbial growth of the same microorganisms (Figure 3B). Treating pre-existing microbial biofilm is vital due to its role as a reservoir for persistent infections.³¹ Targeting and disrupting pre-existing biofilm enhance treatment efficacy and reducing recurrent infections.³¹ Therefore, understanding the ability of ciprofloxacin and cuminaldehyde to disintegrate existing biofilm suggest their potential as effective antibiofilm agents, capable of inhibiting as well as disintegrating the pre-existing biofilm, thereby addressing a critical aspect of combating biofilm-related infections.³² To investigate the disintegration property of cuminaldehyde and/or ciprofloxacin against the clinical isolates of test organism, CV-assay, protein recover assay, EPS-assay and FDA-assay were undertaken. The result revealed a significant reduction (approximately 45%) in pre-existing biofilm formation when treated in combination (Figure 3C). Hence, from all these observations, it was quite evident that the selected concentrations of ciprofloxacin and cuminaldehyde in combination could act as a potential antibiofilm agent for the clinical isolates of *P. aeruginosa* cells. The result also suggested

Table. According to the FIC index estimated from the checkerboard assay, cuminaldehyde and ciprofloxacin shows additive interaction against all clinical isolates *P. aeruginosa*. Sub-MIC doses of cuminaldehyde and ciprofloxacin suppressed biofilm development as well as efficiently disintegrated the pre-existing biofilm in all clinical isolate of *P. aeruginosa*

S. No.	Organism name	MIC of Cuminaldehyde (µg/mL)	MIC of Ciprofloxacin (µg/mL)	FICI (FIC index)	Type of Interaction	Amount of biofilm reduction (in comparison to the control group)	Amount of residual biofilm (in comparison to the control group)
01.	PA1	600	3	0.5	Additive	~ 45.23	~ 55.15
02.	PA2	600	1	0.68	Additive	~ 49.52	~ 58.90
03.	PA3	600	0.1	0.58	Additive	~ 39.18	~ 54.89
04.	PA4	600	20	0.68	Additive	~ 42.78	~ 52.70
05.	PA5	600	10	0.88	Additive	~ 45.60	~ 58.90

that the combinations of the compounds at their sub-MIC doses could exhibit antibiofilm effect against the organisms without showing antimicrobial effect.

Exploration of underlying mechanism of the antibiofilm potential of the combination of ciprofloxacin and cuminaldehyde against clinical isolates of *P. aeruginosa*

To investigate the underlying mechanism behind antibiofilm potential of cuminaldehyde combined with ciprofloxacin, the ROS profile of five clinical isolates of *P. aeruginosa* using the DCFDA method was assessed. ROS is a well-documented microbial biofilm inhibitor.⁵ Elevated levels of ROS can trigger oxidative stress leading to damage of cellular components thereby inhibiting biofilm formation.³³ Treatment with ciprofloxacin and cuminaldehyde together resulted in a significant increase in intracellular ROS accumulation compared to untreated cells (Figure 4A). The result also revealed that ROS profile increased by ~60% to ~80% across all the five selected clinical isolates. To confirm this finding, the experiment was repeated with ascorbic acid. Ascorbic acid acts as a ROS scavenger by donating electrons to neutralize ROS.⁷ The findings revealed a notable reduction in ROS levels when exposed to ascorbic acid alongside the test compounds (Data not shown). The finding suggested a marked decrease in biofilm activity correlating with increased ROS generation. Ascorbic acid treatment effectively mitigated ROS levels consequently restored the biofilm profile (Data not shown). These findings suggested that the antibiofilm properties of ciprofloxacin and cuminaldehyde could be attributed to the ROS accumulation. Additionally, cell membrane permeability was measured as it could play an important role in influencing microbial biofilm network. Cuminaldehyde has previously been shown to increase cell membrane permeability in *P. aeruginosa*.⁶ The result demonstrated a significant increase in membrane permeability when both cuminaldehyde and ciprofloxacin were applied together, with enhancements ranging from ~30% to ~70% across all the elected clinical isolates compared to controls (Figure 4B). This suggested that the combined action of these compounds could enhance intracellular ROS accumulation

and increment of cell membrane permeability more effectively than their individual application. According to previous literature, reduced cell membrane permeability typically reinforces biofilm architecture whereas increased membrane permeability has a significantly diminished impact on biofilm structures.³⁴ Taken together, it can be deduced that targeting cell membrane permeability can be an effective strategy for biofilm inhibition. The results demonstrated that a combined application of ciprofloxacin and cuminaldehyde can be considered as a potential cause of biofilm control of clinical strains of *P. aeruginosa*.

Cuminaldehyde in combination with ciprofloxacin could curb biofilm linked threats of clinical isolates of *P. aeruginosa* from catheter model

Catheter-associated biofilm poses significant challenges in healthcare, leading to persistent and recurrent infections.⁵ Addressing these infections poses a major challenge for the healthcare system. Effort was accumulated to assess the effectiveness of our chosen compounds against *in-vitro* catheter-associated biofilm challenges from all five clinical isolates of the test bacteria under investigation. In this regard, various assays including CV-assay, protein recovery assay, and EPS-assay were conducted. The results indicated a substantial reduction in biofilm formation, ranging from 45% to 50%, when the biofilm of the test organism was developed on catheter surface, with or without cuminaldehyde and ciprofloxacin in combination (Figure 5A). Moreover, efficient disintegration of the pre-existing biofilm was assessed in the range from 40% to 45%, across all five selected clinical isolates (Figure 5B). Collectively, our data suggested that selected compounds effectively inhibits and disintegrates pre-existing biofilm on the catheter surface while applied in combination, offering a promising strategy for preventing and treating catheter-associated infections.

CONCLUSION

P. aeruginosa biofilm-related infections are becoming an alarming issue around the world, and as a result, controlling this threat is

an enormous concern for the healthcare sectors. The findings of this study demonstrate that cuminaldehyde in combination with ciprofloxacin can inhibit the development and persistence of *P. aeruginosa* biofilms effectively, thereby offering a promising therapeutic strategy for combating against biofilm-associated infections.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

PT conceived the idea. MM, SD, PP, PC,AD,RR, PT and SS designed the experiments. MM, SD, PP, PC,AD,RR and SS performed the experiments. PT analyzed the data. MM, SD, PP, PC,AD,RR and SS wrote the manuscript. PT edited the manuscript. All authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

Not applicable.

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