Ind. J. Pharm. Edu. Res., 2027; 61(1):10.5530/ijper.20270007.
https://www.ijper.org

Original Article

Targeting Quorum Sensing-Mediated Virulence and Biofilm Formation in *Pseudomonas aeruginosa* Using Ferulic Acid

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ABSTRACT

Background: *Pseudomonas aeruginosa* is a clinically significant opportunistic bacterium that exhibits intrinsic antimicrobial resistance, biofilm formation, and quorum-sensing-regulated virulence. Nowadays, plant-derived compounds are gaining attention as alternative agents for targeting bacterial virulence pathways. **Objectives:** This study aimed to determine the efficacy of Ferulic Acid (FA) against *P. aeruginosa* by antibacterial, anti-biofilm formation and anti-quorum sensing activity. **Materials and Methods:** Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) experiments were used to assess Anti-bacterial activity. Biofilm inhibition and metabolic activity were evaluated by using Crystal violet staining and an XTT reduction assay. Fluorescence microscopy was employed to assess bacterial viability, membrane integrity and intracellular Reactive Oxygen Species (ROS) generation. Quorum-sensing regulator lasR gene expression was analyzed by qPCR. **Results:** FA exhibited a concentration-dependent inhibition of bacterial growth and bactericidal activity at higher concentrations. A significant reduction was observed in biofilm biomass and metabolic activities, including fluorescence analysis, which revealed compromised membrane integrity and reduced cellular viability in treated cells. Elevated intracellular ROS generation suggested oxidative stress-mediated antibacterial activity. The lasR gene, a quorum-sensing regulator, showed downregulation, confirming interference with quorum-sensing-controlled virulence mechanisms. **Conclusion:** The findings demonstrate that FA exerts a multi-targeted antibacterial and anti-virulence effect against *P. aeruginosa*, demonstrating its potential as a naturally occurring phytochemical for the development of an alternative therapeutic approach for targeting resistant and biofilm-associated infections.

Keywords: Biofilm, Ferulic acid, MBC, MIC, *Pseudomonas aeruginosa*, Quorum sensing.

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Received: 12-05-2026;
Revised: 29-06-2026;
Accepted: 07-08-2026.

INTRODUCTION

The rapid proliferation of multidrug-resistant pathogens requires the development of alternative therapeutic approaches that can effectively control the infections without accelerating the development of drug resistance. *P. aeruginosa*is a well-known opportunistic pathogen that can cause severe hospital-acquired infections, particularly in a weakened immune system. It significantly complicates the treatment and increases the risk of recurring and chronic infections, due to its strong biofilm formation and high intrinsic resistance to antibiotics.[1,2]

The quorum sensing system is a density regulatory mechanism that controls the coordinated expression of genes involved in the virulence factor, motility and biofilm formation. The las, rhl, and pqs pathways affect the *P. aeruginosa* Qs network and control the synthesis of virulence factors such as pyocyanin, elastase, proteases, and Rhamnolipids.[3,4] Targeting QS has been a popular anti-virulence strategy due to its lower pathogenicity without immediately preventing bacterial growth, which reduces the pressure for the increase of resistance.[5]

Quorum-sensing targeting has become a promising anti-virulence strategy, especially for the treatment of biofilm-associated and chronic infections. Secondary metabolites derived from plants are gaining attention in recent years, as they are natural quorum-sensing inhibitors of their many biological functions, biocompatibility, and structural diversity. Phenolic compounds, such as phenolic acids, are widely known for their antibacterial, antioxidant, antibiofilm, and modulator of signaling properties. Those substances can affect the QS by interfering with the production of signal molecules, receptor binding or downstream gene regulation.[6,7]

Ferulic Acid (FA) is a phenolic compound and a derivative of hydroxycinamic acid. It is widely distributed in medicinal plants, cereals, fruits and vegetables. According to reports, this FA has important biological actions such as antioxidant,

DOI: 10.5530/ijper.20270007

anti- inflammatory, and antibacterial efficacy. Emerging evidence suggests that FA may suppress the QS regulated phenotypes and affect bacterial communication systems without having a significant bactericidal effect.[8] The potential of FA as a natural antiviral drug is highlighted by this QS-targeted activity.

Although several plant-derived phenolic compounds have been reported to evaluate the antibacterial and anti-biofilm, oxidative stress-inducing quorum-sensing inhibitory effects and ferulic acid remains limited. So, the current work is focused on evaluating the quorum-sensing inhibitory efficacy of Ferulic acid against *P. aeruginosa*, in accordance with the clinical relevance of infections and the increasing interest in plant-derived compound-based anti-virulence approaches. The study was focused on the mechanisms that affect biofilm formation and QS-regulated virulence factors under *in vitro* conditions, without exerting bactericidal pressure. The results of this work may lead to the development of new therapeutic approaches for reducing *P. aeruginosa* pathogenicity and provide scientific evidence for the use of Ferulic acid as a natural quorum-sensing inhibitor.

MATERIALS AND METHODS

Preparation of test chemical and *P. aeruginosa* culture for assays

The strain of *P. aeruginosa* used in this investigation was acquired from King Fahad Hospital (ATCC 27853) and cultured in nutrient broth at 37°C while being stirred at 200 rpm in order to create the inoculum for the *in vitro* study. In culture media, the strain growth was regulated to aturbidity equal to the 0.5% McFarland standard. Ferulic acid was purchased from Sigma.

Minimum Inhibition Concentration (MIC)

In a 96-well microtiter plate, the MIC was calculated by using a two-fold serial dilution method. The minimum standard for the bacterial inoculum was 1% McFarland turbidity. Each well received a twofold diluted inoculum to reach a final volume of 200 µL. The final concentrations of 62.5, 125, 250, 500, and 1000 µg/mL were achieved by preparing Ferulic acid in DMSO (10 mg/mL). The plates were incubated at room temperature overnight. The growth control was provided by the wells that contained only the bacterial suspension. The optical density at 600 nm was measured with an ELISA plate reader to determine the bacterial growth. The following formula was used to determine the percentage of inhibition.

$$\text{Inhibition (\%)} = \frac{(\text{OD control} - \text{OD test})}{\text{OD control}} \times 100$$

Minimum Bactericidal Concentration (MBC)

The Minimum Bactericidal Concentration (MBC) of ferulic acid against *Pseudomonas aeruginosa* was assessed to distinguish bactericidal effects from quorum-sensing-mediated inhibition. The 100 µL of two-fold diluted bacterial inoculum was added to each well of a 96-well microtiter plate to reach the final quantity of 200 µL. The compound was tested at concentrations of 250, 500, and 1000 µg/mL, while wells containing only the bacterial culture served as growth controls. Plates were incubated at room temperature for 24 hr. Following incubation, 20 µL from each well was aseptically plated onto Mueller-Hinton agar without prior agitation of the well contents. After 48 hr of incubation at 37°C, the plates were checked for colony formation. The MBC was defined as the lowest concentration that resulted in no visible colonies or fewer than three colony-forming units, indicating approximately 99-99.5% bacterial killing.

Crystal Violet Assay

Biofilm biomass was measured by using the crystal violet staining assay with modifications based on previously reported methods.[9,10] In short, a 24-well plate was filled with *P. aeruginosa*. To enable the production of mature biofilms, the plates were incubated for 48 hr at 37°C. Following incubation, DMSO was used to gently remove planktonic cells, and the plates were then allowed to air dry. For 20 min, 200 µL of 1% crystal violet was used to dye the adherent biofilms at room temperature. DMSO was used to wash out any remaining discoloration. The absorbance at 600 nm was measured by using a microplate reader.

XTT Assay

The XTT assay was performed on 72-hr old *P. aeruginosa* biofilms. The established biofilms were treated with test samples at varying concentrations (6.25, 12.5, 25, 50, and 100 µg/mL) and incubated at 37°C for 24 hr. Following treatment, 50 µL of reconstituted XTT detection solution was added to both test and control wells. The plate was gently shaken and further incubated at 37°C for 2-5 hr. After incubation, the wells were gently mixed by repeated pipetting to ensure complete solubilization of the Formosan product. The contents were then centrifuged at 6000 rpm for 5 min, and the supernatants were collected. Absorbance was measured at 450 nm using a microplate reader.[11]

The percentage of cellular metabolic activity was calculated using the following formula:

$$\text{\% Cellular Metabolic Activity} = \frac{\text{Mean OD of Sample}}{\text{OD of Control}} \times 100$$

Determination of Bacterial Load Using Fluorescent Di-acetate Staining

Bacterial inoculums of *P. aeruginosa* were prepared and were dispersed to each well in a 96-well plate. After adding 20 µL of inoculums to each well, the plates were incubated at 37°C for 72 hr. A sample was not added to the control well. The plate was again kept in the incubator for 24 hr. After 24 hr, the media was removed, and the cells were washed with sterile PBS to remove excess media and detached cells, and the biofilms were air-dried for 10 min. 10 µL of FDA stain was added to each well and incubated in the dark for 15-20 min. After incubation, the

wells were washed 2 times with sterile PBS, and fluorescence was imaged in a fluorescent microscope.[12]

Estimation of Reactive Oxygen Species (ROS)

P. aeruginosa cultures were prepared and evenly dispensed into a 12-well culture plate. An aliquot of 20 µL bacterial suspensions was added to each well, followed by treatment with the test sample at a concentration of 178.422 µg/mL. Wells lacking FA served as the untreated control. The plates were incubated at 37°C for 24 hr. After incubation, intracellular reactive oxygen species generation was assessed by adding 50 µL of DCFDA to each well and incubating for 30 min. Excess probe was removed by washing with Phosphate-Buffered Saline (PBS). Fluorescence was observed using a fluorescence microscope and quantitative fluorescence intensity was measured using a fluorimeter at an excitation wavelength of 470 nm and emission at 635 nm. The fluorescence intensity was expressed in arbitrary units.[13]

Analysis of mRNA expression by real-time PCR Analysis

Total RNA was isolated from *P. aeruginosa* cultures using a Total RNA Extraction Kit (Invitrogen; Product Code: 10296010) following the manufacturer's guidelines. Complementary DNA (cDNA) was synthesized from 1 µg of purified RNA using a first-strand cDNA synthesis master premix (G-Biosciences; Product Codes: 786-5019S and 786-5020). The resulting cDNA was diluted tenfold with RNase-free distilled water and used as the template for quantitative real-time PCR (qRT-PCR) analysis. Quantitative PCR was carried out using a SYBR Green-based qPCR master mix under optimized amplification conditions. Gene-specific primers targeting the quorum-sensing regulatory gene lasR (Forward: AAGGAAGTGTTGCAGTGGTG; Reverse: GAGCAGTTGCAGATAACCGA) were employed, with 16S rRNA used as the internal reference gene (Forward: CAGGCTTAGATACCCTGGTACTCC; Reverse: CAGGATTAGATACCCTGGTACTCC). Relative gene expression levels were calculated using the comparative Ct normalization method and Agarose gel electrophoresis.

Statistical analysis

All experiments were performed using three independent biological replicates (n=3), and the results are presented as mean±Standard Deviation (SD). Statistical analyses were conducted using GraphPad Prism version 8.1 (GraphPad Software, USA). Comparisons among multiple groups were performed using One-Way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison *post hoc* test. Statistical significance was defined as $p<0.05$. The significance levels are indicated in the figures as follows: $p<0.05$, $p<0.01$ and $p<0.001$.

RESULTS

Minimal Inhibitory Concentration (MIC) analysis

The Minimal Inhibitory Concentration (MIC) analysis of FA against *P. aeruginosa* revealed a clear concentration-dependent antibacterial response. When compared to the control, the FA treatment causes a consistent decrease in bacterial growth with increasing concentration, as seen in Figure 1. These results were statistically significant (***$p<0.001$), indicating that the phenolic compound has strong antibacterial activity against *P. aeruginosa*. Reduced turbidity in wells confirms the visual evaluation of the MIC microtiter plate.

Minimal bactericidal concentration analysis

Colony count analysis on Mueller-Hinton agar plates was used to evaluate the Minimal Bactericidal Concentration (MBC) assay. Figures 2A-2C shows the result of MBC. The strong proliferation of *P. aeruginosa* was confirmed by the control,

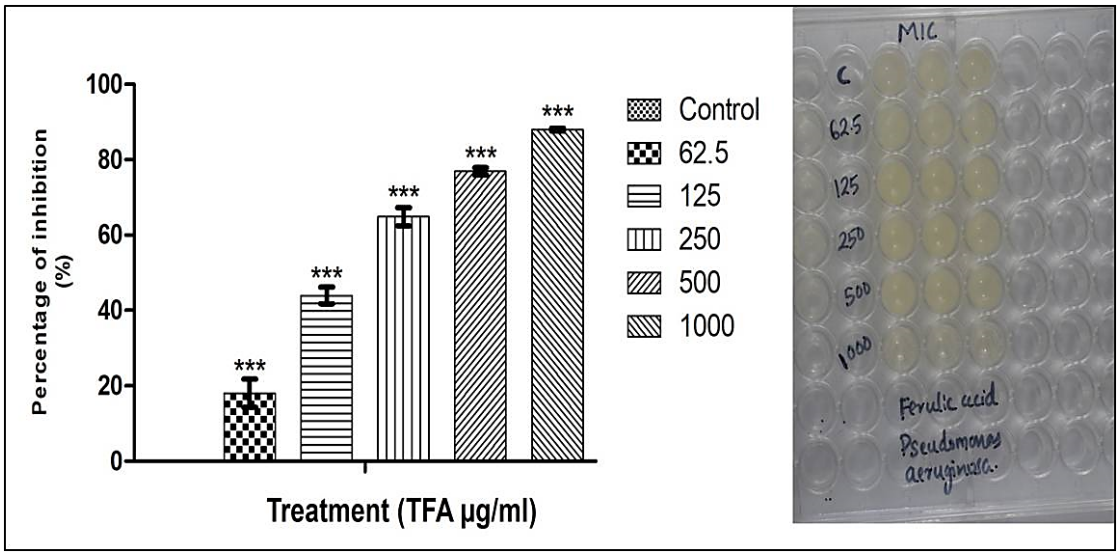

Figure 1: The MIC assay revealed that Ferulic acid inhibited bacterial growth in a concentration-dependent manner (***$p<0.001$), n=3.

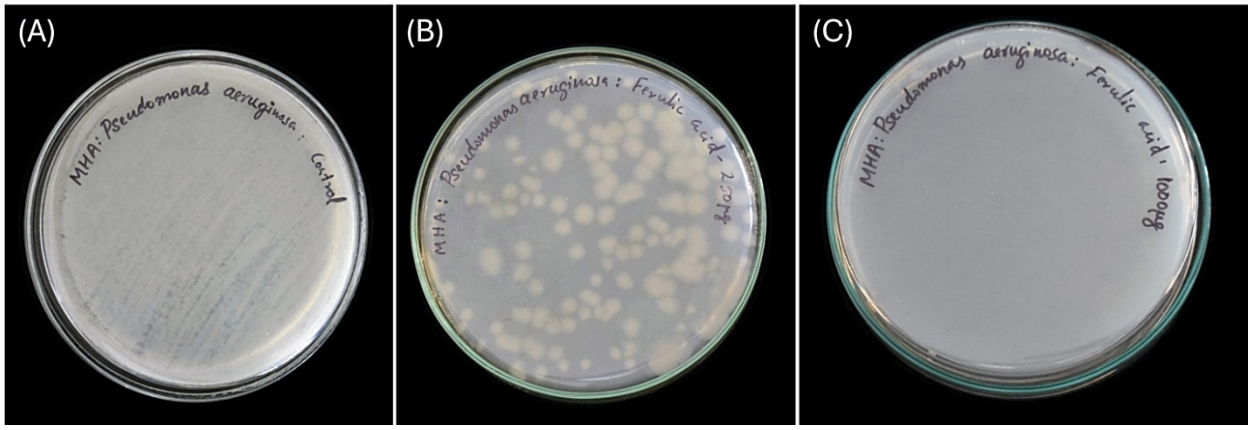

Figure 2: Evaluate the Minimal Bactericidal Concentration (MBC) against *P. aeruginosa* on a Mueller-Hinton agar plate. (A) Control exhibiting confluent growth; (B) FA 250 µg/mL showing decreased colony formation; and (C) FA 1000 µg/mL exhibiting complete inhibition of bacterial growth.

which showed high bacterial growth with 2425±321 colonies. A 250 µg/mL concentration significantly reduced the colonies, showing substantial (168±15) bactericidal activity. A CFU count of 0 demonstrated that FA totally suppressed bacterial growth at a dose of 1000 µg/mL, confirming the function of MBC against *P. aeruginosa*. The above results showed that FA has strong antibacterial properties at higher concentrations.

Crystal violet Assay

Figure 3 depicts the concentration-dependent anti-biofilm and anti-quorum-sensing properties of FA against *P. aeruginosa*. The phenolic compound significantly reduced biofilm biomass, as shown in reduced crystal violet staining, with inhibition progressively increasing with the concentration. At a 250 µg/mL concentration, a decrease in biofilm formation was observed, and these results suggest that FA has a bactericidal effect in addition to interference with quorum-sensing-mediated biofilm formation.

XTT assay

Ferulic acid treatment significantly reduced the metabolic activity of *P. aeruginosa* in a concentration-dependent manner, according to the XTT reduction method (Figure 4). The strong biofilm viability was demonstrated by the control, which was 100% cellular metabolic activity. On the other hand, FA administration at 6.25 µg/mL markedly decreased CMA, indicating early bacterial metabolic function impairment. A dose-responsive inhibitory effect was confirmed by a further decrease in CMA at 12.5 and 25 µg/mL, respectively.

Fluorescence Assay

The fluorescence microscopy study showed that the treated and control groups' bacterial cell viability (Figures 5A-5C). High green fluorescence was seen in the control group, confirming normal membrane integrity and metabolic activity under control circumstances. The treated group 5B, on the other hand, showed a large reduction in viable cells and a significant decrease, indicating membrane damage and bacterial cell death after CFA treatment. The decreased fluorescence intensity is an important indicator of antibacterial and anti-biofilm activity since it shows that the integrity of the bacterial membrane has been effectively damaged. According to the quantitative analysis (Figure 5C) denotes the decreased level in fluorescence intensity of FA treated cells (*p<0.05).

ROS Assay

Fluorescence microscopy showed that the control group slightly produced ROS (Figure 6A), but FA-treated cells showed strong fluorescence (Figure 6B), which indicates a higher intracellular ROS level. FA causes oxidative stress, which may lead to bacterial cell damage and loss of viability according to Quantitative analysis (Figure 6C), which reveals a substantial increase in Fluorescence intensity in FA-treated groups compared to control (*p<0.05).

Real Time PCR

The expression analysis of the quorum-sensing regulator *lasR* in *Pseudomonas aeruginosa* revealed a marked downregulation upon treatment with Ferulic Acid (FA). As shown in Figure 7A, the relative *lasR* gene expression in the FA-treated group was significantly reduced compared to the control and the agarose gel electrophoresis results (Figure 7B) showed a visibly reduced band intensity for *lasR* in the FA-treated cells (lane 2) compared to the control (lane 1), while the 16S rRNA housekeeping gene showed comparable band intensity across both cells, confirming the attenuated quorum-sensing-mediated virulence in *P. aeruginosa*.

DISCUSSION

The ongoing emergence of bacterial strains that are resistant to drugs has raised interest in naturally occurring bioactive compounds that have different antibacterial actions. Particularly, the phenolic acids have gained interest nowadays, because of their capacity to prevent bacterial growth, Quorum Sensing (QS), and interference with the formation of biofilms.[14] Hence,

we have designed the novel study to investigate the anti-bacterial, anti-biofilm and anti-QS qualities of Ferulic Acid (FA) against multi-drug-resistant pathogen *P. aeruginosa*.

P. aeruginosa growth was significantly inhibited in a concentration-dependent manner by the MIC assay, and these results suggest that FA has a strong anti-bacterial property. FA demonstrated improved efficiency of bacterial suppression in lower doses as compared to documents on other phenolic acids, such as ferulic, gallic acid, and hydroxycinamic acid, demonstrating MIC values ranging between 500-1000 µg/mL against gram-negative bacteria.[15] Ferulic acid moderately inhibited growth at 750 µg/mL in the study of Borges *et al.*[14] While FA inhibited the growth and indicated a potentially stronger association with the bacterial membrane.

FA can cause irreversible bacterial mortality at greater concentrations according to the MBC assay, which shows the complete bactericidal efficacy at 1000 µg/mL. This finding is consistent with other phenolic compounds; Gallic acid showed an MBC against *Escherichia coli* in the range of 800-1000 µg/mL concentration.[16] However, the growth suppression ratio of MBC and MIC in this study suggests a different method of action, possibly including membrane rupture and oxidative damage.

One of the main mechanisms for antibiotic resistance and chronic conditions is biofilm formation. The considerable reduction in biofilm biomass observed via crystal violet staining verifies the antibiofilm activity of FA. Plant phenolics have also been shown to suppress biofilm formation.[17-19] FA demonstrated similar biofilm reduction at equivalent doses in the current investigation, suggesting a strong breakdown of biofilm structural integrity.

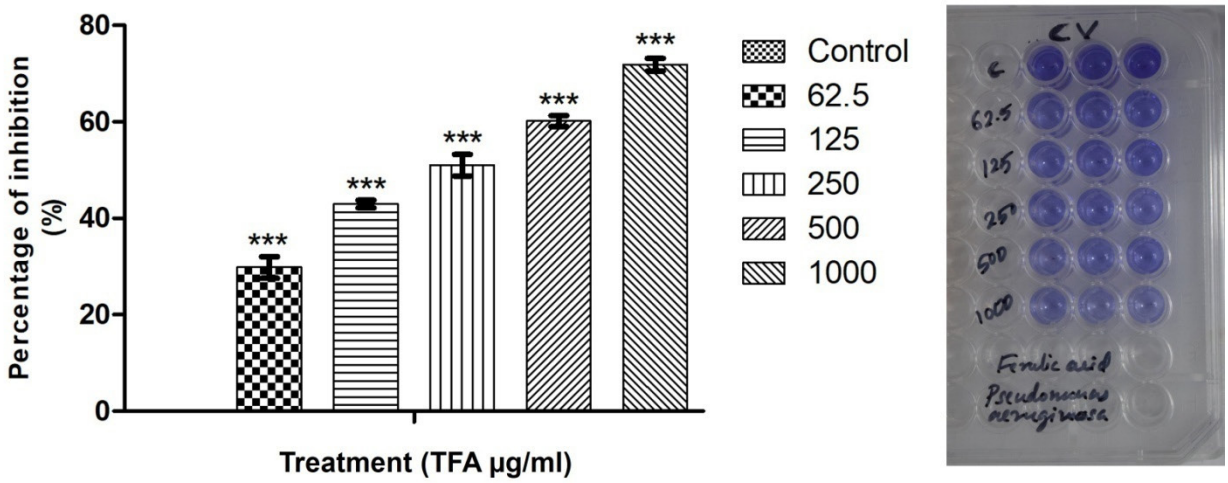

Figure 3: Ferulic acids' anti-biofilm inhibitory effect against *P. aeruginosa* was assessed using the crystal violet assay. The percentage of inhibition after treatment with FA shows a concentration-dependent increase in biofilm suppression when compared to control (***$p<0.001$), $n=3$.

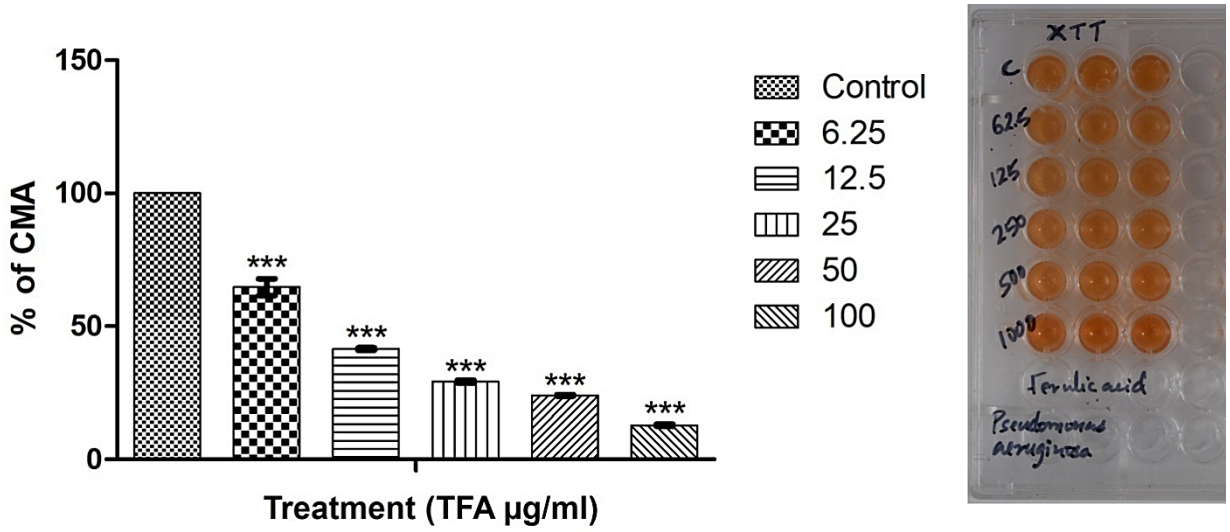

Figure 4: *P. aeruginosa* Cellular Activity (CMA) as determined by XTT assay with respect to FA. The results show that metabolic activity decreases with concentration. ***$p<0.001$ indicates statistical significance when compared to untreated cells, $n=3$.

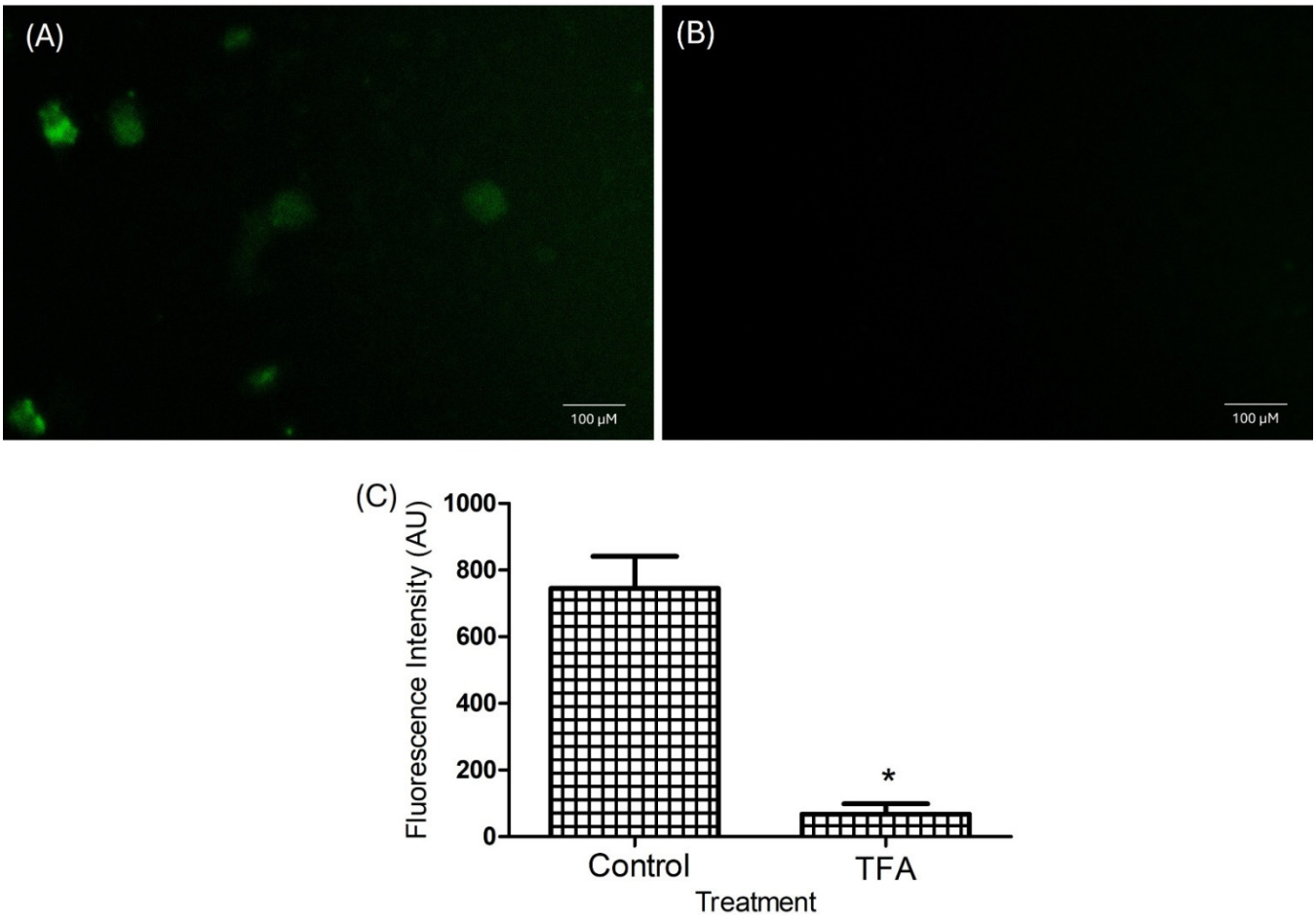

Figure 5: Effect of fluorescence microscopy on FA against *P. aeruginosa.* In (A) the control group exhibited both cellular and biofilm-associated fluorescence. The significantly reduced fluorescence of FA-treated cells in (B). (C) Demonstrates the quantitative analysis of fluorescence intensity (*p<0.05). These validate the inhibitory effect of FA and confirm its disruption of cellular fluorescence, n=3.

Additionally, the XTT assay revealed a significant reduction in metabolic activity in biofilms treated with FA. This result is consistent with studies on tannic acid and catechin, which decrease the survival of *P. aeruginosa* biofilms by inhibiting the formation of extracellular polymeric substances and metabolic pathways.[20] The reduction in metabolic activity at 250 µg/mL concentration implies that FA not only impacts cellular viability but also impairs biofilm physiology, possibly by reducing food nutrients and energy generation.

The results of fluorescence microscopy showed a considerable decrease in viable cells and damaged membrane integrity. Similar membrane disturbances have been documented for thymol and caffeic acid, both of them are interacts with the lipid bilayers to induce intracellular components to cause leakages.[21,22] Strong membrane damage is suggested by the fluorescence reduction seen in FA -treated cells, which is compatible with bactericidal action.

FA-treated cells had more intracellular reactive oxygen species, according to the ROS test. It is well recognized that elevated ROS levels induce oxidative stress, which results in DNA damage, protein oxidation and lipid peroxidation. Phenolic chemicals frequently cause ROS buildup, which greatly helps in bacterial death, according to research by Imlay.[23] These results are consistent with the current findings, suggesting that oxidative stress plays a significant role in the FA mode of action. Jige *et al*. have been shown to have comparable ROS-mediated bactericidal effects in *Staphylococcus aureus*.[24]

The major finding of this study was that the quorum-sensing regulator gene lasR was significantly downregulated. In *P. aeruginosa*, the lasR gene regulates a number of virulence and biofilm-associated genes, such as elastase and pyocyanin production, which are critical for infection establishment and persistence.[25] Quercetin inhibited the generation of virulence factors and biofilm formation by down regulating lasR and associated QS genes.[26] This inhibition of lasR expression is consistent with findings from other phenolic based research. Significantly, phenotypic decreases in biofilm biomass and metabolic activity are strongly correlated with lasR down regulation seen in this investigation supporting the FA anti-virulence impact is mediated via QS inhibition. Additionally,

prior studies have shown that natural QS inhibitors can interfere with gram negative bacteria's communication networks without applying conventional bactericidal pressure, which may decrease the chances that resistance will emerge.[27]

Together, these results show that ferulic acid has a multi-targeted antibacterial and anti-virulence effect that includes membrane disruption, oxidative stress induction, biofilm suppression, and metabolic impairment, inhibition of bacterial growth, bactericidal activity, and quorum-sensing gene modulation. From a pharmacognostic perspective, FA is a promising option for the creation of alternative treatment approaches that target bacterial virulence because of the anti-biofilm and Anti-QS activity.

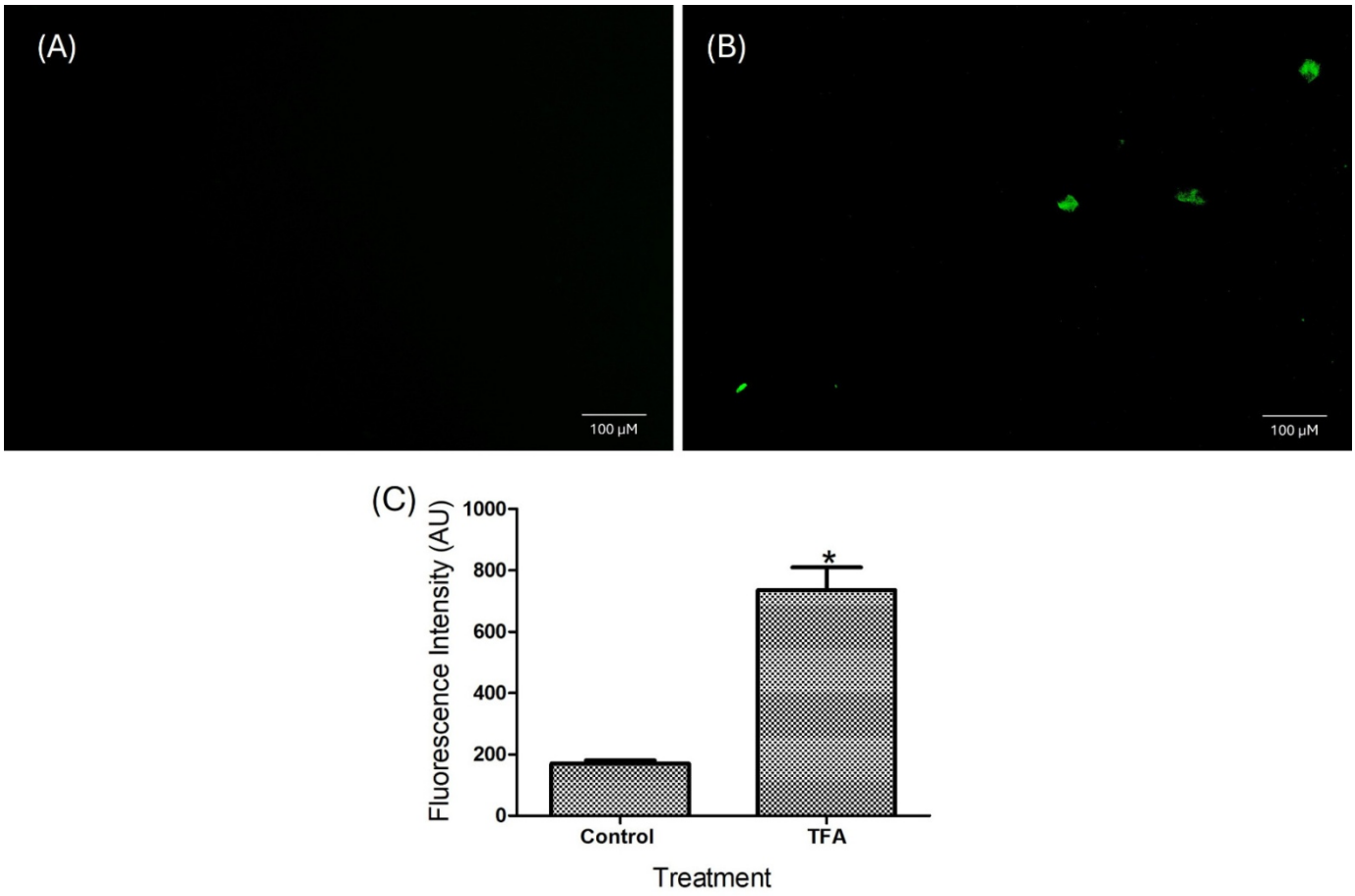

Figure 6: *Pseudomonas aeruginosa* intracellular fluorescence in response to FA. In (A) the fluorescence of control cells is quite low. (B) FA-treated cells exhibiting enhanced green fluorescence, indicating increased intracellular signal. (C) Quantitative analysis of fluorescence intensity demonstrating a significant increase in fluorescence in FA-treated cells compared to control ($p<0.05$), $n=3$.

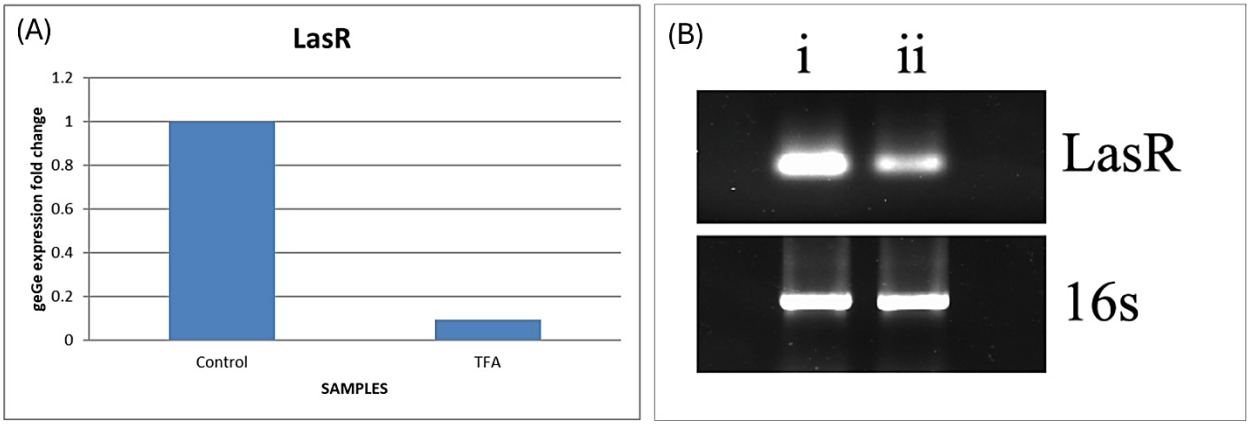

Figure 7: Effect of Ferulic Acid (FA) on lasR expression in *Pseudomonas aeruginosa*. In (A) the relative fold change in lasR gene expression shows significant downregulation in FA-treated cells compared to controls. (B) Agarose gel analysis depicting reduced lasR band intensity in FA-treated cells relative to control and 16S rRNA served as the internal loading control.

CONCLUSION

The current study provides comprehensive evidence that Ferulic acid has a strong antiviral and antibacterial property against *Pseudomonas aeruginosa*. Its antibacterial activity is confirmed by the concentration-dependent inhibition of bacterial growth and the bactericidal effect at higher concentrations. Importantly, Ferulic acid has the potential to decrease the bacterial pathogenicity, highlighted by its capacity to inhibit biofilm formation and metabolic activity at 250 µg/mL inhibitory concentration. Cell damage and viability are triggered by oxidative stress and membrane rupture, according to mechanistic insights obtained from fluorescence and ROS analysis. Additionally, the significant downregulation of the quorum-sensing regulator lasR, which correlates with the suppression in biofilm biomass and cellular metabolic activity, offers molecular evidence that supports FA anti-quorum-sensing activity. All of these results point to a multifunctional mechanism by FA, and it inhibits the growth, biofilm suppression, decreases metabolic activity, induces oxidative stress, and interferes with quorum-sensing activity. FA is a promising natural anti-infective agent that may be used to treat *P. aeruginosa* infections, especially those associated with biofilm-mediated resistance. The present study did not evaluate additional quorum-sensing regulatory genes or downstream virulence factors. Therefore, the precise molecular mechanism underlying quorum-sensing inhibition requires further investigation. Future studies should include comprehensive analysis of the las, rhl, and pqs systems using sub-inhibitory concentrations of ferulic acid. Based on the current results, ferulic acid should be considered a promising candidate for future research rather than an alternative therapeutic agent for biofilm-associated infections.

ACKNOWLEDGEMENT

The author doesn't have any special acknowledgment.

ABBREVIATIONS

ANOVA: Analysis of Variance; **CFU:** Colony Forming Unit; **CMA:** Cellular Metabolic Activity; **cDNA:** Complementary DNA; **Ct:** Cycle Threshold; **DCFDA:** 2',7'-Dichlorofluorescin Diacetate; **DMSO:** Dimethyl Sulfoxide; **DNA:** Deoxyribonucleic Acid; **ELISA:** Enzyme-Linked Immunosorbent Assay; **FDA:** Fluorescein Diacetate; **MIC:** Minimum Inhibitory Concentration; **MBC:** Minimum Bactericidal Concentration; **mRNA:** Messenger RNA; **PBS:** Phosphate Buffered Saline; **PCR:** Polymerase Chain Reaction; **QS:** Quorum Sensing; **qPCR:** Quantitative Polymerase Chain Reaction; **qRT-PCR:** Quantitative Real-Time Polymerase Chain Reaction; **RNA:** Ribonucleic Acid; **ROS:** Reactive Oxygen Species; **rpm:** Revolutions Per Minute; **rRNA:** Ribosomal RNA; **FA:** Ferulic Acid; **XTT:** 2,3-Bis(2-Methoxy-4-Nitro-5-Sulfopheny l)-2H-Tetrazolium-5-Carboxanilide; **OD:** Optical Density; **µg/mL:** Microgram per Milliliter.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

The author doesn't receive any funding for this project.

PATIENT CONSENT

NA.

ETHICAL APPROVAL

NA.

SUMMARY

Ferulic acid demonstrated a potent anti-bacterial action against *P. aeruginosa* and its increase the growth inhibition in a concentration-dependent manner.

By inhibiting the development of biofilms and reducing the CMA, ferulic acid demonstrates strong anti-virulence capability.

FA causes Oxidative stress and membrane damage, which is contributes to bacterial cell death according to the fluorescence microscopy and ROS analysis.

The quorum sensing regulator gene lasR is significantly down regulated and it's indicating that FA disrupts the quorum sensing pathways involved in the biofilm formation and bacterial pathogenicity.

FA is a potential candidate for further research against biofilm associated *P. aeruginosa* infection because it acts effective multiple mechanism including antibacterial, anti-biofilm, oxidative stress and anti-quorum sensing properties.

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Cite this article: Alsaadi KH. Targeting Quorum Sensing-Mediated Virulence and Biofilm Formation in *Pseudomonas aeruginosa* Using Ferulic Acid. Indian J of Pharmaceutical Education and Research. 2027;61(1):10.5530/ijper.20270007.