



Pinostrobin-Functionalized Gold Nanoparticles with Enhanced Antibacterial and Antibiofilm Effects Against *Pseudomonas aeruginosa*

Dini N. Fauzia^{1,2}, Mochamad Z. Fahmi², Titik Taufikurohmah³, Afaf Baktir^{2,*}¹Doctoral Program of Mathematics and Natural Sciences, Faculty of Science and Technology, Universitas Airlangga, Jl. Dr. Ir. H. Soekarno, Mulyorejo, Surabaya 60115, Indonesia²Department of Chemistry, Faculty of Science and Technology, Universitas Airlangga, Jl. Dr. Ir. H. Soekarno, Mulyorejo, Surabaya 60115, Indonesia³Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Negeri Surabaya, Jl. Ketintang, Surabaya 60231, Indonesia

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ABSTRACT

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Pseudomonas aeruginosa is a clinically significant pathogen with strong biofilm-forming capacity, leading to persistent infections and high resistance toward conventional antibiotics. Nanotechnology based therapeutics have emerged as a promising solution to overcome these limitations. In this study, pinostrobin-functionalized gold nanoparticles (Pin-AuNPs) were evaluated for their antibacterial and antibiofilm activities against *P. aeruginosa*. Although gold nanoparticles and flavonoid compounds have individually demonstrated antibacterial activity, their integration as a synergistic antibiofilm nano-system against *P. aeruginosa* remains underexplored. Pin-AuNPs were prepared using a previously validated synthetic protocol. Pinostrobin was immobilized onto the surface of citrate-stabilized AuNPs via non-covalent physisorption, with Tween 20 employed as a non-ionic surfactant to enhance colloidal stability and ensure efficient surface functionalization. Pin-AuNPs exhibited significantly enhanced antibacterial activity compared to free pinostrobin and bare AuNPs, demonstrating the advantage of nanoparticle-based functionalization. Here, we investigated antibacterial potency, biofilm inhibition, biofilm eradication and morphological alterations. Pin-AuNPs exhibited superior antibiofilm efficacy against *P. aeruginosa*, inhibiting biofilm formation by more than 80% and significantly reducing bacterial viability in established biofilms. Notably, Pin-AuNPs achieved a substantially lower MBIC₅₀ value (7.08 µg/mL, R² = 0.95) compared to bare AuNPs (25.79 µg/mL) and free pinostrobin (46.17 µg/mL), indicating markedly improved prevention of biofilm formation. In addition, Pin-AuNPs demonstrated the highest eradication potency against mature biofilms, with an MBEC₅₀ of 10.01 µg/mL, underscoring their potential as an effective nano-antimicrobial strategy for controlling biofilm-associated *P. aeruginosa* infections.

Keywords: Pinostrobin, Gold Nanoparticles Conjugates, Antibiofilm Agents, Antibacterial Activity, *P. aeruginosa*

Introduction

P. aeruginosa is a highly adaptable Gram-negative opportunistic pathogen and a major cause of severe nosocomial infections, particularly in immunocompromised individuals, patients with cystic fibrosis, burn injuries, or ventilator-associated pneumonia.^{1–3} Its exceptional capacity for antimicrobial resistance arises from an array of intrinsic and acquired mechanisms, including multidrug efflux pumps, β -lactamase production, and reduced outer membrane permeability.⁴ A defining feature of *P. aeruginosa* pathogenicity is its ability to form mature biofilms composed of a dense extracellular polymeric substance (EPS) matrix enriched with alginate, proteins, and extracellular DNA. This structured architecture markedly attenuates antibiotic penetration, buffers oxidative stress, stabilizes quorum-sensing (QS) communication, and enhances the persistence of dormant subpopulations.^{4,5}

*Corresponding author. Email: afaf-b@fst.unair.ac.id
Tel: +(62) 82334817019

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Consequently, biofilm-associated cells exhibit up to 100–1,000-fold increased tolerance to antibiotics compared with planktonic cells, posing a major challenge for current antimicrobial therapies.⁶ In recent years, the increasing prevalence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *P. aeruginosa* strains has further exacerbated the clinical challenge, with reduced effectiveness reported even for last-line antibiotics such as colistin, aminoglycosides, and fluoroquinolones. These alarming trends have driven growing interest in alternative anti-infective strategies that target biofilm formation and maintenance rather than bacterial viability alone.^{7,8,9} Consequently, there is an urgent need for therapeutic platforms capable of simultaneously targeting multiple biofilm attributes, including initial adhesion, EPS stabilization, and structural eradication.² Natural products, particularly plant-derived polyphenols, have emerged as promising sources of antivirulence and antibiofilm agents due to their multitarget biological activities and relatively low cytotoxicity profiles.¹⁰ Plants have been used to treat various diseases since ancient times. Numerous novel phytochemicals are currently being screened and reported for their ability to inhibit biofilm formation and eradicate pathogenic bacteria.^{9,11,12} Among these phytochemicals, flavonoids have garnered significant attention for their ability to disrupt QS pathways^{1,13}, suppress virulence factor production, and modulate bacterial membrane integrity.^{12,14} Among them, pinostrobin, a flavanone abundantly found in *Boesenbergia rotunda*, has been reported to exhibit antioxidant^{15,16}, anti-inflammatory¹⁶, antiviral^{17,18}, anticancer^{19–22}, and importantly antibacterial and antibiofilm effects against various pathogens, including *Staphylococcus aureus*, *Escherichia coli*, and *P. aeruginosa*.^{23–26} Notably, pinostrobin has been reported to inhibit bacterial growth and significantly suppress biofilm

formation through interference with quorum-sensing pathways and virulence factor production.^{23,24} Recent studies suggest that flavanones with structural similarity to pinostrobin can interfere with the *Las*, *Rhl*, and *Pqs* QS circuits in *P. aeruginosa*, reducing the synthesis of key virulence determinants such as elastase, pyocyanin, and rhamnolipids. These multitarget interactions position pinostrobin as a compelling candidate for antibiofilm intervention, particularly when coupled with advanced delivery technologies.^{27,28} Despite its broad spectrum of promising biological activities, the clinical and technological application of free pinostrobin remains hindered by its poor water solubility, limited membrane permeability, rapid metabolic degradation, and consequently low systemic bioavailability.²⁹

To overcome these pharmaceutical limitations, loading pinostrobin onto gold nanoparticles represents a highly promising strategy.^{10,30} These developments have also generated new optimism for the discovery of more effective antibacterial and anti-infective agents. Gold nanoparticles are well established nanocarriers with excellent biocompatibility, facile surface functionalization, and intrinsic antibacterial properties mediated by reactive oxygen species (ROS) generation and direct membrane interaction.^{31–33}

Recent advances over the past years have highlighted nanotechnology-based delivery systems as an effective approach to overcome the physicochemical and pharmacokinetic limitations of bioactive phytochemicals. In particular, nanoparticle-based formulations have demonstrated enhanced drug stability, improved cellular uptake, and increased accumulation within bacterial biofilms. Among various nanomaterials, gold nanoparticles (AuNPs) have attracted significant attention due to their excellent biocompatibility, chemical stability, and ease of surface functionalization with bioactive compounds.^{34–37}

Functionalization of AuNPs with flavonoids has been shown to significantly enhance antibacterial and antibiofilm performance by improving solubility, facilitating interaction with bacterial membranes, and enabling deeper penetration into biofilm structures.^{15,38,39} Despite increasing reports on flavonoid nanoparticle conjugates, studies specifically investigating pinostrobin-functionalized gold nanoparticles as antibiofilm agents against *P. aeruginosa* remain limited. Moreover, systematic comparisons between free pinostrobin, bare AuNPs, and pinostrobin-functionalized AuNPs in the context of antibiofilm efficacy are still scarce.

Therefore, the development and evaluation of pinostrobin-functionalized gold nanoparticles represent a relevant and timely strategy to address current limitations in antibiofilm therapy against *P. aeruginosa*. The specific objectives of this study are to assess the antibacterial activity of Pin-AuNPs against *P. aeruginosa*; to evaluate their ability to inhibit and eradicate biofilms using crystal violet-based assays and compare their performance with free pinostrobin and bare AuNPs. We hypothesize that functionalization of pinostrobin onto AuNPs enhances its antibiofilm efficacy through improved physicochemical stability, increased interaction with bacterial cells, and synergistic antibacterial effects.

Materials and Methods

Materials

Pinostrobin was isolated from *Boesenbergia rotunda* rhizomes. The isolated pinostrobin was purified by recrystallization and its purity was confirmed by thin-layer chromatography (TLC) using three different eluent systems. Chloroauric acid trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, $\geq 99.9\%$) was purchased from Sigma-Aldrich. The reference strain *P. aeruginosa* ATCC 9027 was obtained from Balai Besar Laboratorium Kesehatan (BBLK) Surabaya, Indonesia. Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) were purchased from HiMedia Laboratories (Mumbai, India). Sterile blank paper disks (6 mm diameter) were obtained from Macherey-Nagel GmbH & Co. KG (Düren, Germany). Crystal violet, methanol, glacial acetic acid, absolute ethanol, phosphate-buffered saline tablets (PBS, pH 7.4), and meropenem (used as positive control) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and GlpBio (Montclair, CA, USA). Ultrapure water (sterile aqua ad iniectionem) was used throughout the experiments. All other chemicals and reagents were of analytical grade and used as received.

Synthesis and Preparation of Pinostrobin-Functionalized Gold Nanoparticles (AuNP-Pin)

Pinostrobin, isolated from the rhizomes of *B. rotunda*, was subsequently attached to the gold nanoparticle (AuNPs) surfaces via physisorption, with Tween 20 employed as a non-ionic surfactant to enhance colloidal stability and facilitate adsorption. The functionalization was optimized by gradual addition of pinostrobin (dissolved in ethanol) at varying concentrations (0.02–1 % w/v) to 50 mL of the AuNP colloid (1:5 v/v ratio), under strong stirring for 30 min at room temperature. The optimal concentration (0.1 % w/v) was selected based on maximum surface coverage, as determined by UV–Vis spectroscopy (λ_{max} shift from 520 nm to 530 nm). Excess pinostrobin was removed by triple centrifugation ($12,000 \times g$, 15 min, 4 °C) and resuspension in ultrapure water.

The resulting Pin-AuNPs were characterized by UV–Vis spectroscopy, Fourier-transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM), stability (detailed characterization data are presented in Supplementary Material Fig. S1–S4)

Preparation of Bacterial Inoculum

Bacterial Strain Revival and Stock Maintenance

A clinical isolate of multidrug-resistant *P. aeruginosa* ATCC 9027 was maintained on Tryptic Soy Agar (TSA) slants at 4 °C and subcultured every 4 weeks. For each experiment, a single colony from a freshly streaked TSA plate (overnight incubation at 37 °C) was used to ensure consistent physiological state. *P. aeruginosa* was cultured in nutrient broth at 37 °C with shaking until reaching the mid-logarithmic growth phase. Bacterial suspensions were adjusted to approximately 10^6 CFU/mL prior to antibacterial and antibiofilm assays.

Inoculum Preparation

A single colony was suspended in sterile phosphate-buffered saline (PBS, pH 7.4) and adjusted to 0.5 McFarland standard (1×10^8 CFU/mL). This suspension was further diluted 1:100 in TSB supplemented with 1 % (w/v) glucose to yield a working inoculum of approximately 1×10^6 CFU/mL.

Antibacterial Activity

Disk Diffusion Method (Kirby-Bauer)

The antibacterial activity of pinostrobin, AuNPs, and Pin-AuNPs was evaluated using *Disk Diffusion Method (Kirby-Bauer)*. Tryptic Soy Agar (TSA) plates were brought to room temperature before use. Sterile cotton swabs were dipped into the standardized inoculum ($\sim 1 \times 10^8$ CFU/mL), excess fluid was removed by pressing the swab against the inner wall of the tube, and the entire surface of the TSA plate was evenly inoculated by streaking in three directions (60° rotation each time). The plates were allowed to stand for 3–5 min to allow surface drying (pre-diffusion).

Sterile blank disks were placed on the inoculated agar surface using flame-sterilized forceps. Exactly 15 μL of each test solution (Pin, AuNPs, and Pin-AuNPs) and standard antibiotic (Meropenem) was carefully pipetted onto the respective disks. For negative control, 15 μL of the solvent alone was applied. Plates were incubated at 37 °C for 18–24 h in an inverted position.^{12,40}

After incubation, the diameter of inhibition zones (including the 6-mm disk diameter) was measured to the nearest millimeter using a digital caliper under transmitted light from the back of the plate.^{41,42} Measurements were performed in triplicate, and results were expressed as mean $\pm$ standard deviation (SD).

Quality Control and Interpretation

Each batch of tests included *P. aeruginosa* ATCC 9027 as the quality control strain. Zone diameters obtained for the standard antibiotic (meropenem 10 μg /disk) were within the acceptable ranges specified in the latest CLSI M100 guidelines. For the novel compounds (pinostrobin, AuNPs, and Pin-AuNPs) without established interpretive breakpoints, only the mean inhibition zone diameter $\pm$ standard deviation was reported and compared with positive and negative controls.

Inhibition zones were measured to the nearest millimeter using a digital caliper under transmitted light and independently verified by digital image analysis using ImageJ software (v1.54f, National Institutes of

Health, USA). Briefly, photographs of the plates were taken under standardized lighting conditions, converted to 8-bit grayscale, and the scale was set using the known disk diameter (6 mm) as a reference. Zone edges were automatically detected by threshold adjustment, and diameters were calculated from the measured area of the clear zone (including the disk).

Although Mueller-Hinton Agar (MHA) remains the CLSI-recommended medium for standardized disk diffusion susceptibility testing, Tryptic Soy Agar (TSA) was intentionally selected in the present work as an enriched medium to support robust growth and biofilm-related metabolite production of *P. aeruginosa* during preliminary screening of nanoparticle–natural product conjugates. The use of TSA for such comparative studies is well documented and does not affect the validity of relative activity differences between tested compounds.⁴¹

Antibiofilm Activity Assessment

Biofilm Inhibition Assay

Biofilm inhibition and eradication were assessed using the crystal violet (CV) staining method. For biofilm inhibition, bacterial suspensions were incubated in 96-well plates in the presence of test samples and allowed to form biofilms at 37 °C for 24 h. For biofilm eradication, pre-formed biofilms were first established under identical conditions, followed by treatment with the test samples.⁴³ Briefly, 100 µL of standardized inoculum (1×10^6 CFU/mL in TSB + 1 % glucose) was dispensed into each well of a sterile flat-bottom polystyrene 96-well plate. After 90 min of adhesion at 37 °C, non-adherent cells were gently removed by washing twice with 200 µL PBS. Subsequently, 100 µL of two-fold serial dilutions of Pin-AuNPs, bare AuNPs, free pinostrobin, or meropenem (positive control) ranging from 200 to 6.25 µg/mL were added. Wells receiving 100 µL of TSB + 1 % glucose alone served as untreated biofilm growth controls. Plates were incubated statically at 37 °C for 24 h.^{24,34}

After incubation, planktonic cells were discarded, and wells were washed twice with 200 µL PBS. Biofilms were fixed with 100 µL of absolute methanol for 15 min and air-dried. Biofilm biomass was stained with 125 µL of 0.1 % (w/v) crystal violet for 5 min, followed by rinsing with distilled water until no colour was released. Bound dye was solubilized with 100 µL of 30 % (v/v) acetic acid for 10 min, and absorbance was measured at 595 nm using a microplate reader (BioTek ELx808 or equivalent). Each concentration was tested in triplicate within the same plate, and the entire experiment was independently repeated. Percentage biofilm inhibition was calculated as:

$$\% \text{ Inhibition} = \frac{(OD_{595} \text{ control} - OD_{595} \text{ treated})}{OD_{595} \text{ control}} \times 100\% \dots (1)$$

The minimum biofilm inhibitory concentration required to inhibit 50 % of biofilm formation (MBIC₅₀) was determined by non-linear regression (sigmoid DoseResp model).

Biofilm Eradication Assay

Mature biofilms were first established by dispensing 200 µL of standardized inoculum (1×10^6 CFU/mL in TSB + 1 % glucose) into 96-well plates and incubating statically at 37 °C for 24 h. After removal of planktonic cells and two gentle washes with 200 µL PBS, 100 µL of test compounds (200–6.25 µg/mL) were added. Plates were further incubated for 24 h at 37 °C. Biofilm biomass was quantified by crystal violet staining.⁴ Each concentration was tested in triplicate within the same plate, and the entire experiment was independently repeated. Percentage biofilm eradication was calculated as:

$$\% \text{ Eradication} = \frac{(OD_{595} \text{ control} - OD_{595} \text{ treated})}{OD_{595} \text{ control}} \times 100\% \dots (2)$$

The minimum biofilm eradication concentration required to eradicate 50 % of pre-formed biofilm (MBEC₅₀) was determined using the same non-linear regression model.

Scanning Electron Microscopy (SEM) of Biofilm Morphology

Biofilms were grown on sterile glass coverslips (10 × 10 mm) placed in 24-well plates. Each well received 200 µL of standardized inoculum and 200 µL of test compound (or TSB for controls) and was incubated at 37 °C for 24 h. After treatment, planktonic cells were aspirated, and coverslips were gently washed twice with PBS. Biofilms were fixed

with 500 µL of absolute methanol for 30 min at room temperature. ⁴Fixed samples were dehydrated through a graded ethanol series, critical-point dried, sputter-coated with gold, and examined using a field-emission scanning electron microscope (JEOL JSM-7900F) at an accelerating voltage of 5–10 kV.

Statistical Analysis

All experiments were conducted in triplicate (n = 3), and results are presented as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by appropriate post hoc tests to determine significant differences between groups. A p-value of less than 0.05 was considered statistically significant.

Results and Discussion

The emergence of multidrug-resistant *P. aeruginosa* has highlighted the urgent need for new agents that are effective against both planktonic cells and recalcitrant biofilms. *P. aeruginosa* is notorious for its intrinsic and acquired resistance to multiple antibiotic classes, coupled with its ability to form protective biofilms that exacerbate therapeutic challenges in clinical settings.^{5,8,26} In this study, we evaluated the antibacterial efficacy of free pinostrobin, bare gold nanoparticles (AuNPs), and pinostrobin-functionalized gold nanoparticles (Pin-AuNPs) against *P. aeruginosa* ATCC 9027 using standardized disk diffusion and crystal violet biofilm assays. These approaches allowed for direct comparison of planktonic growth inhibition with biofilm formation and eradication, providing comprehensive insights into the therapeutic potential of nanoparticle-mediated flavonoid delivery.

Antibacterial Activity

The antibacterial activity of free pinostrobin, AuNPs, and pinostrobin-conjugated AuNPs was evaluated using the disk diffusion assay, with the results presented in Figure 1D. In the disk diffusion assay, free pinostrobin displayed only weak activity (8.41 ± 0.11 mm), in agreement with earlier studies reporting limited efficacy of flavonoids against Gram-negative pathogens due to poor outer-membrane impermeability and efflux pump activity.¹² Citrate-capped bare AuNPs showed moderate activity (12.32 ± 0.72 mm), attributable to mild ROS generation and surface-charge interactions, as previously described for similarly synthesized gold nanoparticles.^{1,37} The diameter of inhibition zones are summarized in Table 1.

Table 1: Diameter of inhibition zones (mm) obtained by disk diffusion assay on TSA (mean ± SD, n = 5).

Treatment	Zone of inhibition (mm)
Pinostrobin	8.41 ± 0.11
Bare AuNPs	12.32 ± 0.72
Pin-AuNPs	14.69 ± 0.58
Meropenem (K+)	19.77 ± 0.65
Solvent control	No zone

Functionalization of AuNPs with pinostrobin (Pin-AuNPs) significantly improved the inhibition zone to 14.69 ± 0.58 mm ($p < 0.0001$ vs. pinostrobin; $p < 0.01$ vs. bare AuNPs), confirming a true synergistic rather than additive effect. This enhancement is consistent with multiple reports on flavonoid–gold nanoparticle conjugates, where nano-delivery increases local drug concentration, facilitates membrane adhesion/perturbation, and may promote intracellular uptake.^{4,44,45} Although the zones remained smaller than meropenem (19.77 ± 0.65 mm), the relative improvement over the parent compound is substantial and highly reproducible.

One-way ANOVA indicated a highly significant difference among the three treatment groups ($p < 0.0001$). The Pin-AuNPs conjugate exhibited the highest mean activity (14.69 mm), followed by AuNPs (12.32 mm) and pinostrobin alone (8.41 mm). These results clearly demonstrate that strong surface association via physisorption of pinostrobin onto gold nanoparticles substantially enhances its antibacterial performance against *P. aeruginosa* compared to the individual components.^{4,45}

Antibiofilm Activity

The antibiofilm assay was conducted to evaluate the ability of the tested compounds to inhibit biofilm formation or disrupt pre-formed biofilms. In this study, antibiofilm activity was examined through two complementary approaches: biofilm inhibition assay and biofilm eradication assay. The EPS-rich biofilm architecture of *P. aeruginosa* plays a central role in antibiotic tolerance and QS-regulated persistence, providing a critical barrier against conventional antimicrobial agents.

Biofilm Inhibition Assay

The biofilm inhibition assay was designed to evaluate the ability of the test compounds to prevent initial bacterial adhesion, microcolony formation, and subsequent maturation of *P. aeruginosa* biofilms. By exposing freshly inoculated bacterial suspensions to the test agents during the early stages of growth, this assay specifically targets the initiation phase of biofilm development, including adhesin-mediated attachment and extracellular polymeric substance (EPS) secretion.^{34,46} Biofilm biomass was quantified using the standard crystal violet (CV) microtiter plate assay. This method relies on the binding of crystal violet to negatively charged surface molecules and polysaccharides within the biofilm matrix, with the amount of retained dye (measured as absorbance at 595 nm, OD₅₉₅) being directly proportional to the total biofilm mass.⁴⁷ Biofilm inhibition was quantified using the standard crystal violet assay as described in the Methods section.

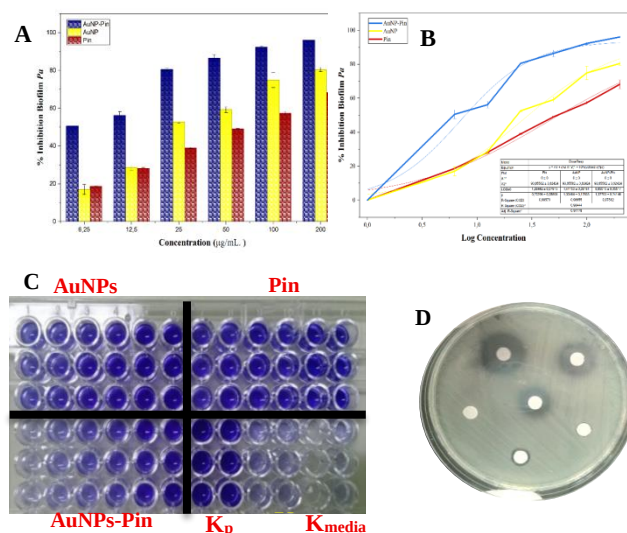


Figure 1: (A) Concentration-dependent inhibition of *P. aeruginosa* biofilm formation by free pinostrobin (Pin), bare gold nanoparticles (AuNP), and pinostrobin-functionalized gold nanoparticles (Pin-AuNP) at concentrations ranging from 6.25 to 200 μg/mL. (B) Determination of the minimum biofilm inhibitory concentration required to inhibit 50 % of biofilm formation (MBIC₅₀) using a *non-linear sigmoid Doseresp fit* model. Values are expressed as mean ± SD (n = 3). Pin-AuNPs exhibited significantly greater inhibitory potency than free pinostrobin and bare AuNPs across the entire concentration range, (C) Visualization of biofilm inhibition assay results against *Pseudomonas aeruginosa* using the crystal violet (CV) staining method on 96-well microtiter plates, (D)

Representative disk diffusion assay on Tryptic Soy Agar showing inhibition zones of *P. aeruginosa* ATCC 9027 after 24 h incubation at 37 °C.

As shown in Figure 1A, a concentration-dependent decrease in OD₅₉₅ was observed for all tested agents over the range 6.25–200 μg/mL. The lowest OD₅₉₅ values, indicative of the thinnest and least dense biofilms, were consistently recorded for pinostrobin-functionalized gold nanoparticles (Pin-AuNPs), followed by bare AuNPs and free pinostrobin. At 200 μg/mL, Pin-AuNPs reduced biofilm biomass by more than 90 %, whereas free pinostrobin achieved only 70 %

inhibition, confirming the marked synergistic enhancement conferred by nanoparticle conjugation. The relationship between test compound concentration and percentage biofilm inhibition, together with the calculated minimum biofilm inhibitory concentration required to inhibit 50 % of biofilm formation (MBIC₅₀), is presented in Figure 1B. The substantially lower MBIC₅₀ of Pin-AuNPs compared with free pinostrobin and bare AuNPs further underscores their superior antibiofilm potency and validates the nanoconjugation strategy as an effective means of potentiating the intrinsic anti-adhesive and anti-maturation properties of pinostrobin against *P. aeruginosa*. Non-linear regression analysis using the *sigmoid Doseresp fit* model yielded the following MBIC₅₀ values: free pinostrobin (Pin), 30.01 μg/mL (R² = 0.99) and Pin-AuNPs, 7.08 μg/mL (R² = 0.95). The approximately eight-fold lower MBIC₅₀ of Pin-AuNPs compared with free pinostrobin unequivocally demonstrates the dramatically enhanced antibiofilm potency achieved through nanoparticle conjugation.

This marked improvement is attributable to multiple synergistic mechanisms. First, pinostrobin itself interferes with *P. aeruginosa* quorum-sensing pathways by reducing extracellular polysaccharide (EPS) secretion and biofilm maturation, mature biofilm.^{40,48} Second, the small hydrodynamic diameter of Pin-AuNPs (14.35 nm) facilitates superior diffusion into the polysaccharide matrix of developing biofilms, enabling direct intracellular delivery of pinostrobin. Third, surface functionalization with pinostrobin significantly enhances the aqueous solubility and local bioavailability of this otherwise hydrophobic flavonoid, further amplifying its anti-adhesive and anti-maturation effects (Figure 2.).

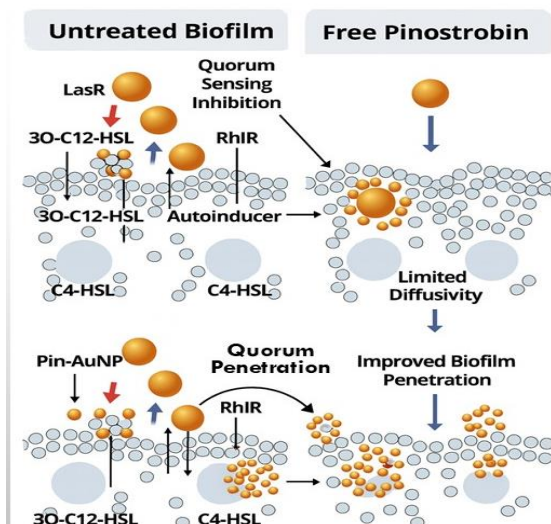


Figure 2: Proposed mechanism of the enhanced antibiofilm activity of pinostrobin-functionalized gold nanoparticles (Pin-AuNPs) against *P. aeruginosa* compared to free pinostrobin

Based on the previous study, pinostrobin displays moderate inhibitory activity against LasR and RhIR receptors; however, its efficacy is severely constrained by poor aqueous solubility, limited diffusion across the EPS-rich matrix, and inadequate intracellular accumulation, resulting in only partial suppression of QS signaling and biofilm maturation.⁴⁸ The pronounced antibiofilm effects observed for Pin-AuNPs are consistent with interference in QS-regulated pathways, as reflected by strong suppression of biofilm biomass, reduced EPS density, and morphological disruption observed by SEM.

These findings align closely with previous reports⁴ on phytosynthesized AuNPs demonstrated that *Tinospora cordifolia* mediated AuNPs (TC-AuNPs) inhibited *P. aeruginosa* biofilm formation in a dose-dependent manner, achieving up to 59.9 % inhibition at 150 μg/mL, with SEM analysis revealing impaired bacterial adhesion and colonization. Similarly, glucosamine-conjugated AuNPs (GlcN-AuNPs) at 0.02 mg/mL synergistically potentiated meropenem, significantly enhancing both antibacterial and antibiofilm efficacy compared with the antibiotic alone.³⁴ Rajkumari et al. (2017) reported that hordenine-stabilized

AuNPs (HD-AuNPs) inhibited *P. aeruginosa* PAO1 biofilm by 73.7–78.4 % and reduced EPS production by 68.5 %, outperforming free hordenine and underscoring the critical role of phytochemical capping in augmenting matrix penetration and interaction.⁹

Compared with previously reported phytosynthesized AuNPs, Pin-AuNPs demonstrated markedly improved antibiofilm potency, achieving >90% inhibition at 200 µg/mL and an MBIC₅₀ of 7.08 µg/mL, substantially lower than values reported for TC-AuNPs, GlcN-AuNPs, and fucoidan-stabilized AuNPs. Gold nanoparticles synthesized biologically and stabilized with fucoidan (F-AuNPs) have been reported to inhibit biofilm formation and eradicate mature biofilms, with an MBIC value of 128 µg/mL.⁴³ Similarly, gold nanoparticles synthesized using *Capsicum annuum* extract (AuNPs-CA) demonstrated a dose-dependent antibiofilm effect against *P. aeruginosa* PAO1. At concentrations of 25, 50, and 100 µg/mL, AuNPs-CA inhibited biofilm formation by 16.96 ± 3.02%, 47.98 ± 1.66%, and 65.03%, respectively. These nanoparticles interfere with quorum sensing, suppress EPS synthesis, disperse biofilm-embedded cells, and enhance the penetration of antibiofilm agents highlighting their multifaceted antimicrobial potential.⁴⁵

Further studies conducted underscore that gold nanoparticles (AuNPs) are effective not only against planktonic bacterial cells but also exhibit substantial potential in preventing biofilm formation and disrupting pre-established biofilms. The antibiofilm mechanisms of AuNPs are multifactorial. Their nanoscale dimensions facilitate deep penetration into the biofilm matrix, enabling interaction with bacterial cells protected by extracellular polymeric substances (EPS). AuNPs also bind to EPS components including polysaccharides, proteins, and extracellular DNA leading to structural disruption and weakening of the protective biofilm architecture. Moreover, AuNPs modulate quorum-sensing regulatory systems, thereby interfering with bacterial adhesion and biofilm maturation processes.⁴

Biofilm Eradication Assay

The mature biofilm eradication assay was performed to assess the ability of the tested compounds to disrupt pre-established *P. aeruginosa* biofilms. Unlike the biofilm inhibition assay, which evaluates the capacity of compounds to prevent early-stage cell adhesion and biofilm formation the eradication assay focuses on the removal or degradation of fully developed, structurally stable biofilms. This model is particularly relevant for simulating chronic infections or biofilm-contaminated surfaces in clinical and environmental settings.⁴⁵ Eradication efficiency was quantified based on reductions in biofilm biomass and observable morphological alterations following treatment. Common mechanistic events involved in biofilm destruction include extracellular polymeric substance (EPS) degradation, increased cell wall permeability, and induction of oxidative stress leading to cell lysis and detachment from the substrate.

Biofilm eradication was then evaluated by exposing the mature biofilms to serial concentrations (6.25–200 µg/mL) of pinostrobin (Pin), gold nanoparticles (AuNP), and the gold nanoparticle–pinostrobin conjugate (AuNP–Pin). The treated plates were incubated for an additional 24 h at 37 °C to enable direct interaction between the compounds and the biofilm matrix. Following treatment, the residual biofilm biomass was quantified using the crystal violet (CV) staining method, following the same post-staining steps described previously. This assay provided a comparative assessment of the ability of Pin, AuNP, and AuNP–Pin to disrupt mature *P. aeruginosa* biofilms and thereby offered mechanistic insights relevant to the treatment of persistent biofilm-associated infections.

As shown in Figure 3B, *non-linear sigmoidal Doseresp fitting* was used to calculate MBEC₅₀ values. The MBEC₅₀ for AuNP–Pin (10.01 µg/mL) was substantially lower than free pinostrobin compound (30.01 µg/mL), confirming the superior efficacy of the conjugate in eradicating mature *P. aeruginosa* biofilms. This finding indicates that AuNP–Pin disrupts biofilm integrity more efficiently, likely by simultaneously compromising cell–cell adhesion and facilitating degradation of the polysaccharide-rich extracellular matrix. The synergistic performance of the conjugate can be attributed to the dual mechanism in which AuNPs enhance the penetration and local concentration of pinostrobin within the dense biofilm layers, while pinostrobin contributes targeted

antibacterial and EPS-inhibitory activity. Collectively, these effects result in substantial structural destabilization and reduction of mature biofilm mass.

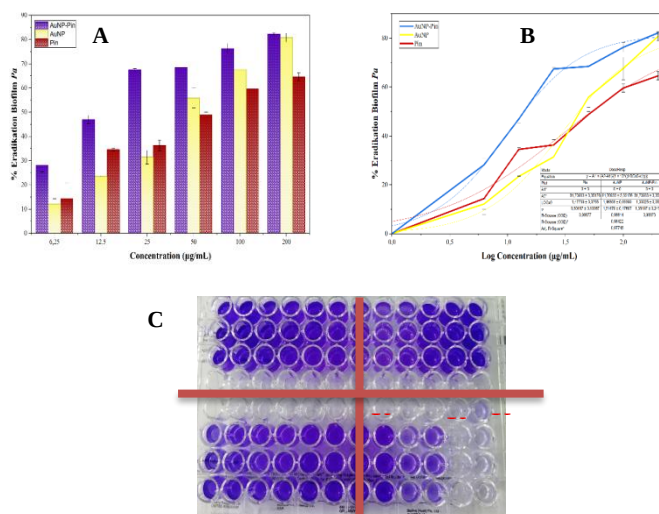


Figure 3: (A) Biofilm eradication curves of *P. aeruginosa* treated with pinostrobin (Pin), gold nanoparticles (AuNP), and gold nanoparticle–pinostrobin conjugates (AuNP–Pin) across concentrations ranging from 6.25 to 200. (B) Determination of MBEC₅₀ values using a *non-linear sigmoidal Doseresp fit* model

Scanning Electron Microscopy (SEM) Observation of Biofilm Morphology

To gain direct visual evidence of the disruptive effect of Pin-AuNPs on *P. aeruginosa* biofilm architecture, scanning electron microscopy (SEM) was employed. This technique allows high-resolution imaging of surface topography and extracellular polymeric substance (EPS) integrity, enabling detailed comparison between untreated mature biofilms and those exposed to Pin-AuNPs. The following protocol was used to prepare and examine the samples.

SEM-based morphological analysis of *P. aeruginosa* biofilms revealed marked structural differences between the untreated control and the samples treated with Pin-AuNPs, as shown in Figure 4. In the untreated control, the substrate surface was uniformly covered by a thick, compact biofilm layer composed of densely aggregated rod-shaped bacterial cells embedded within a highly cohesive polysaccharide-rich matrix. Following treatment with Pin-AuNPs, the biofilm exhibited substantial structural disruption, characterized by the appearance of void areas, reduced matrix density, and fragmentation of the extracellular polymeric substance (EPS). Bacterial cells appeared more dispersed, with evident morphological deformation and a pronounced decline in polysaccharide matrix integrity. The presence of gold nanoparticle aggregates adhering to both the bacterial surface and the EPS further demonstrated the ability of Pin-AuNPs to directly interact with biofilm components, thereby compromising biofilm stability.

These observations indicate that Pin-AuNPs disrupt biofilm integrity through two primary mechanisms: (i) the intrinsic antibacterial activity of pinostrobin, which perturbs membrane stability and interferes with quorum-sensing regulation; and (ii) the capacity of Pin-AuNPs to penetrate and destabilize the polysaccharide matrix via electrostatic interactions and plasmonic effects with organic biofilm components. The combined action of these mechanisms leads to substantial biofilm disintegration and diminishes the ability of *P. aeruginosa* to maintain its protective microenvironment. Collectively, these findings confirm that Pin-AuNPs exert a potent biofilm-disrupting effect through synergistic antibacterial and matrix-degrading activities.

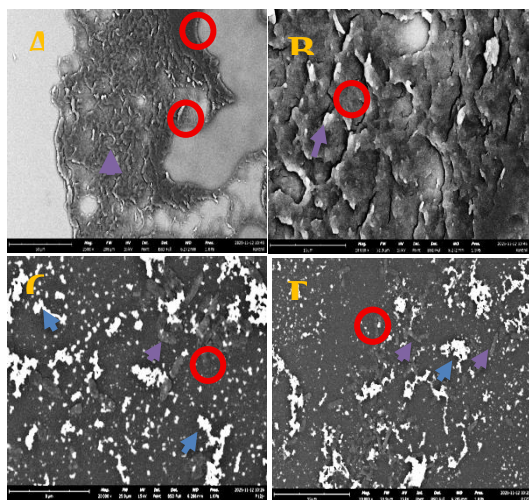


Figure 4: SEM micrographs of *P. aeruginosa* biofilms showing structural alterations following treatment. The untreated control biofilm (A) displays a dense extracellular polymeric substance (EPS) matrix with dispersed bacterial cells. In contrast, biofilms exposed to Pin-AuNPs (C, 20,000×; D, 10,000×) exhibit reduced biomass density along with visible interactions between gold nanoparticle aggregates and both EPS and bacterial cells (red circles: EPS; yellow arrows: *P. aeruginosa* cells; blue arrows: gold nanoparticle aggregates)

Synergistic Enhancement of Quorum Sensing Inhibition by Pinostrobin-Functionalized AuNPs

The superior antibiofilm efficacy of pinostrobin-functionalized gold nanoparticles (Pin-AuNPs) observed in this study aligns with and extends previous findings on the quorum sensing (QS) inhibitory potential of biogenic AuNPs.⁴⁰ While AuNPs synthesized via traditional chemical routes have demonstrated broad-spectrum antimicrobial activity against pathogens such as MRSA and *Salmonella Typhi*^{42,49}, their antibiofilm potency is often limited by poor penetration through the extracellular polymeric substance (EPS) matrix.³⁶

Several studies have demonstrated that gold nanoparticles (AuNPs), particularly those synthesized via intracellular or green routes, possess intrinsic quorum-sensing (QS) inhibitory properties against *Pseudomonas aeruginosa*. These nanoparticles suppress the LasIR–RhIR regulatory network, leading to reduced production of autoinducer molecules (C12-AHL and C4-AHL) and downstream virulence factors, including pyocyanin, elastase, proteases, and alkaline phosphatase. Importantly, AuNPs exhibit this anti-QS effect without significantly affecting bacterial growth, indicating a targeted interference mechanism rather than bactericidal stress. This inhibition of pyocyanin secretion and EPS formation subsequently disrupts microcolony maturation and weakens biofilm structure, consistent with earlier reports on nanostructured materials that attenuate QS-regulated pathogenicity in *P. aeruginosa*.⁴⁰

The findings from previous work strongly support the mechanistic basis observed in our study, where pinostrobin-functionalized AuNPs (Pin-AuNPs) demonstrated superior antibiofilm activity compared to free pinostrobin.

These findings complement reports of mycelium-derived AuNPs reducing pyocyanin production in *P. aeruginosa*⁴⁰ by demonstrating that natural product-nanoparticle hybrids can achieve comparable or superior QS inhibition through physicochemical synergy rather than biological functionalization. The 82.8% biofilm eradication achieved by Pin-AuNPs at 200 µg/mL significantly outperforms both free pinostrobin (64.6%) and bare AuNPs (80.8%), validating this conjugation strategy as a versatile platform for enhancing the therapeutic potential of hydrophobic QS inhibitors.

Thus, Pin-AuNPs represent effective nanotherapeutic platform capable of preventing biofilm establishment and eradicating pre-formed biofilms at concentrations substantially lower than those required for the parent compound or unmodified nanoparticles, offering a promising

new strategy for combating recalcitrant *P. aeruginosa* biofilm-related infections.

Conclusion

This study demonstrates that conjugation of pinostrobin with gold nanoparticles significantly enhances its antibacterial and antibiofilm activities against *Pseudomonas aeruginosa*. The AuNP–pinostrobin conjugates (Pin-AuNPs) consistently outperformed free pinostrobin and bare AuNPs in inhibiting planktonic bacterial growth as well as in preventing and eradicating biofilms, indicating a synergistic effect driven by nanoparticle functionalization. These antibiofilm effects are phenotypically consistent with synergistic nanoscale interactions, including enhanced biofilm penetration, destabilization of the EPS matrix, and disruption of bacterial cell integrity.

These findings highlight the ability of gold nanoparticles to overcome intrinsic limitations of flavonoids against Gram-negative pathogens by enhancing membrane interaction, increasing local drug concentration, and improving biofilm penetration. From a practical standpoint, this nano-enabled approach offers a promising complementary strategy for managing *P. aeruginosa* biofilm-associated infections that are often refractory to conventional treatments.

Overall, Pin-AuNPs represent an effective antibiofilm platform capable of preventing biofilm formation and eradicating mature biofilms at substantially lower concentrations than free pinostrobin or unmodified nanoparticles. Although the present study is limited to in vitro evaluation, it establishes a basis for future in vivo validation and molecular-level investigations to support the development of bioactive compound-based nanotherapeutics.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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