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Exploring the anti-biofilm activity of 2-hydroxy pyridine against clinical isolate of *A. baumannii*

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ABSTARCT
Acinetobacter baumannii (*A. baumannii*) is a major multidrug-resistant (MDR) nosocomial pathogen associated with persistent infections and limited treatment options. In this study, the antimicrobial and antibiofilm potential of 2-Hydroxypyridine (2-HP) was evaluated against a clinical isolate (*A. baumannii* SD-AB-023). Antibacterial activity was assessed using agar well diffusion and broth microdilution assays, while antibiofilm activity at sub-MIC was determined using crystal violet staining, air-liquid interface assays, and light microscopy. The effect on bacterial metabolic activity was evaluated using the Alamar Blue assay. 2-HP exhibited significant antibacterial activity, with a zone of inhibition of 25 mm and a MIC of 20 mg/mL. At sub-MIC concentrations, 2-HP significantly inhibited biofilm formation in a dose-dependent manner, achieving up to 58.02% reduction, and disrupted biofilm architecture as confirmed by microscopic observations. Notably, Alamar Blue assay results demonstrated no significant reduction in metabolic activity at sub-MIC levels, indicating that biofilm inhibition was not due to bactericidal effects. Altogether, these findings suggest that 2-HP possesses both antibacterial and antibiofilm properties and may serve as a promising candidate for the development of alternative therapeutic strategies against MDR *A. baumannii*, although further studies are required to elucidate its mechanism of action and *in-vivo* efficacy.

Keywords: *A baumannii*, 2-hydroxypyridine, antibacterial, antibiofilm

1. INTRODUCTION

Acinetobacter baumannii, an immobile, aerobic Gram-negative *coccobacillus*, has been able to make a striking shift from an organism that is of minor significance from the point of view of its clinical importance to an extremely difficult to treat nosocomial pathogen faced by modern medicine. *A. baumannii* has been transformed within the last few decades from a pathogen that could not be considered as a clinically important agent to one of the most powerful causes of hospital acquired infections owing to its extraordinary ability to become resistant and persistent in healthcare settings, leading to the prevalence of MDR and XDR clones [1]. It is the cause of numerous illnesses that usually affect immunocompromised and critically sick individuals, including pneumonia associated with ventilators, bloodstream infections related to catheter use, urinary tract infections, wound infections, and meningitis. To illustrate the magnitude of this problem, the WHO has classified *A. baumannii* as one of the most important ESKAPE organisms that require new treatment options [2].

The development of Carbapenem-resistant *A. baumannii* (CRAB) as a worldwide clinical dilemma has come to be known as a consequence of the highly adaptable mechanism of resistance. Resistance can be developed by *A. baumannii* through antibiotic inactivation via enzymes, modification of target sites, changes in permeability, and the presence of multidrug efflux pumps [3,4]. The spread of resistance has been facilitated further by various mobile genetic elements, such as resistance islands, plasmids, and insertion sequences, allowing for the rapid propagation of clones in medical environments. In sum, all these factors have led to the diminishing efficacy of last-line antibiotics like carbapenems and colistin. In addition to the problem of antibiotic resistance, one should consider the versatility of pathogens due to the presence of virulence determinants. Among them, it is possible to highlight such a factor as the ability to form biofilms, which is essential and significantly enhances the survival of microorganisms and their antibiotic resistance [5,6]. The complexity of biofilm structure, which consists of polysaccharide, protein, nucleic acid, and lipid matrices, creates a unique environment that helps protect bacteria from antibiotic action and immune system cells; therefore, the amount of the antibiotic required to destroy biofilms is up to 1,000 times higher than the amount required to kill planktonic bacteria. Key genetic factors responsible for biofilm integrity include *bap, ompA, csuE,* and *pgaB*, and the entire biofilm formation process is coordinated via the quorum sensing (QS) system in *A. baumannii. AbaI/AbaR* regulates QS in *A. baumannii*, similar to classic *LuxI/LuxR* system, where abaI produces the enzyme responsible for N-acyl homoserine lactones (AHLs) production and AbaR acts as a receptor [7]. With the overlap between antibiotic resistance, biofilm-induced persistence, and QS-modulated virulence, there is a critical need for the development of alternate therapeutic modalities. In this regard, attempts have included the use of natural products isolated from plants, antimicrobial peptides, synthetic molecules, and nanoparticles. Synthetic heterocycles, especially pyridine analogs, have gained immense popularity due to their versatile biological activities. The pharmacological significance of pyridines in the management of infectious conditions has been reported by several authors, with anti-*A. baumannii* properties of heterocycles being emphasized [8,9]. Among them, hydroxy pyridine analogs have emerged as attractive candidates since the design of QS inhibitors, which would decrease the secretion of virulence factors, can be regarded as an ideal antivirulence approach because of reduced selection pressure imposed on the bacteria, unlike conventional antibiotics [10]. 2-Hydroxypyridine (2-HP) is a heterocyclic compound with notable biological activity, characterized by its tautomeric equilibrium between hydroxyl and pyridine forms, which plays a crucial role in its interaction with biological macromolecules [11,12]. In the present study, 2-HP was investigated as a potential antimicrobial, antibiofilm agent against the multidrug-resistant (MDR) pathogen *A. baumannii*. Accordingly, this research aims to evaluate the multitargeted effects of 2-HP to assess its potential as an effective anti-infective agent against the pathogenicity of *A. baumannii*.

2. METHODOLOGY

2.1 Microbial isolates and maintenance: The *A. baumannii* clinical isolate (SD-AB-023) used in this study was obtained from the SDC Laboratory, Department of Microbiology, Saveetha Dental College and Hospitals, Chennai, India, with the assistance of laboratory personnel. According to Bergey's Manual of Systematic Bacteriology, the VITEK 2 digital identification system verified the isolate (BioMérieux, France) [13,14]. *A. baumannii* maintained on Luria–Bertani (LB) agar 4 °C and subcultured prior to each experiment.

2.2 2-Hydroxypyridine : 2-HP of analytical grade (purity ≥ 98%) was procured from Sigma-Aldrich (St. Louis, MO, USA) and mixed with dimethyl sulfoxide (DMSO) in order to create a stock. This was then diluted in the respective growth media to achieve working concentrations. In order to prevent solvent-mediated confounding effects, the final DMSO content in all experiments was kept at ≤ 1% (v/v).

2.3 Susceptibility profiling and inhibitory concentration: Using the agar well diffusion method on Mueller-Hinton agar (MHA) plates (HiMedia, Mumbai, India), the antimicrobial susceptibility of SD-AB-023 to 2-HP was initially assessed following an established protocol [15]. A sterile cork borer was used to aseptically punch 8 mm diameter wells after standardized bacterial solutions were evenly swabbed onto the surface of MHA plates. After adding 40 µL of 2-HP at 40 mg/mL to each well and incubated for 24 h at 37 °C. Experiment had suitable positive and negative controls. After incubation, the zone of inhibition diameter around each well was measured in millimetres using a vernier calliper [16], MIC was determined by two-fold serial dilutions prepared in LB broth, with a concentration ranging from 40-0.078mg/mL. Following a day of incubation at 37 °C, bacterial growth was detected using 2,3,5-triphenyltetrazolium chloride (TTC) as a colorimetric indicator, and the lowest concentration yielding no visible growth or color change was recorded as the MIC.

2.4 Assessment of biofilm inhibitory potential: Quantitative biofilm inhibition in SD-AB-023 was evaluated using the crystal violet (CV) staining assay in 96-well flat-bottom microtiter plates [17]. Sub-MIC concentrations of 2-HP (10 mg/mL-0.019 mg/mL) were added to wells containing bacterial cultures in LB broth, then static incubation for 48 h at 37 °C. Following incubation, planktonic cells were carefully extracted, and non-adherent cells were removed from the wells by washing them with phosphate-buffered saline (PBS). 0.2% of CV was used to stain the remaining adherent biofilms, and 95% ethanol was then used to dissolve the bound dye. For every strain, absorbance was measured at 525 nm, and the percentage of biofilm inhibition was computed in relation to the untreated control.

Using glass test tubes, the impact of 2-HP (10 mg/mL) on the production of air-liquid interface (ALI) biofilms by SD-AB-023 was evaluated. The distinctive ring-like biofilm that developed at the ALI was stained with CV and gently washed with DH_2O after the cultures were statically incubated at 37 °C for two days. To assess biofilm formation, the stained ring's intensity was visually compared between the treated and untreated control groups.

2.5 Metabolic activity and cell viability assessment: Using the Alamar Blue assay and an established technique [18], the impact of 2-HP on the metabolic activity of SD-AB-023 was assessed. In brief, *A. baumannii* were grown for 24 h at 37 °C in LB broth, both with and without sub-MIC doses of 2-HP (10 mg/mL). Following incubation, the planktonic and biofilm fractions were gathered, processed, and treated with Alamar Blue reagent before being left to incubate in the dark. At 560 nm for excitation and 590 nm for emission, fluorescence was detected.

2.6 Light microscopic examination of surface-attached biofilm: After individually inoculating sterile glass coverslips in plates with 6 wells with SD-AB-023 in LB broth, both with and without sub-MIC of 2-HP, the plates were statically incubated at 37 °C for 24 h. Coverslips were rinsed with PBS, air-dried, and stained with CV. Biofilm architecture of each strain was examined under an Olympus CX23 inverted phase-contrast microscope, and representative micrographs were captured for qualitative comparison.

3. STATISTICAL EVALUATION

The results are presented as mean ± standard deviation (SD), and all studies were conducted in biological triplicates. GraphPad Prism (version 9.0, GraphPad Software, San Diego, CA, USA) was used for statistical analysis. To find significant differences between treatment groups, one-way analysis of variance (ANOVA) and Tukey's post-hoc test were used. For every comparison, a p-value of less than 0.05 was deemed statistically significant.

4. RESULTS

4.1 Antimicrobial susceptibility and minimum inhibitory concentration: The agar well diffusion assay demonstrated that 2-HP exhibited dose-dependent inhibitory activity against SD-AB-023. A distinct zone of inhibition measuring 25 mm was observed around wells loaded with 2-HP at a concentration of 40 mg/mL against the SD-AB-023 isolate. In contrast, the negative control (DMSO ≤ 1%) exhibited no inhibitory zone, while the positive control (PIT 100/10) produced a zone of inhibition measuring 12 mm **(Figure 1)**. The MIC of 2-HP was determined to be 20 mg/mL against SD-AB-023 by broth microdilution, as indicated by the absence of TTC color reduction (**Table 1**). All subsequent assays were conducted at sub-MIC concentrations to assess the antibiofilm potential of the compound.

4.2 Inhibition of biofilm formation: Treatment of SD-AB-023 with sub-MIC concentrations of 2-HP resulted in a significant and concentration-dependent reduction in biofilm biomass, as quantified by the crystal violet staining assay. At 10 mg/mL and 5 mg/mL, biofilm inhibition of 58.02 % and 39.33% was observed in SD-AB-023 compared to the untreated control **(Figure 2A)**.

The capacity of 2-HP to disrupt biofilm development at the air-liquid interface was visually evident in glass test tubes. Untreated cultures of both SD-AB-023 produced robust, clearly visible ring-like structure stained intensely with crystal violet. While, cultures treated with 10 mg/mL of 2-HP exhibited a marked reduction in density and staining intensity **(Figure 2B)**. The inhibitory effect was more pronounced at higher sub-MIC concentrations, corroborating the quantitative biofilm inhibition data.

4.3 Analysis of cell viability: The Alamar Blue assay revealed that sub-MIC of 2-HP did not significantly affect the metabolic activity of SD-AB-023, indicating that bacterial viability was maintained. This confirms that the observed antibiofilm effects were not due to bactericidal action. Fluorescence readings at 590 nm emission showed comparable metabolic activity in SD-AB-023 at 10 mg/mL when compared to untreated controls **(Figure 3)**.

4.4 Microscopic examination of biofilm architecture: Phase-contrast microscopic observation of crystal violet-stained coverslips provided qualitative confirmation of the antibiofilm activity of 2-HP. Untreated coverslips inoculated with SD-AB-023 displayed dense, confluent biofilm layers with extensive intercellular aggregation. In contrast, coverslips exposed to sub-MIC concentrations of 2-HP showed a markedly reduced surface coverage, with sparse and disorganized cellular clusters, indicating disruption of the structural integrity of the biofilm matrix **(Figure 4A:4B)**.

5. DISCUSSION

The global escalation of MDR *A. baumannii* infections represents one of the foremost challenges in contemporary infectious disease management. CRAB has been placed in the critical tier of the 2024 WHO Bacterial Priority Pathogens List, reflecting the urgent need for novel therapeutic strategies beyond conventional antibiotics [19]. The present study demonstrates, for the first time, that 2-HP a bioactive heterocyclic compound exhibits significant multi-targeted antibiofilm activity against a clinical MDR *A. baumannii* isolate (SD-AB-023) at sub-MIC level, encompassing inhibition of biofilm formation.

The agar well diffusion and broth microdilution data confirmed intrinsic antimicrobial activity of 2-HP against *A. baumannii*. Further biofilm assays were deliberately conducted at sub-MIC concentrations. This approach is well-supported in the literature; Halicki et al. (2020) demonstrated that arene carbaldehyde 2-pyridinylhydrazone derivatives inhibited *A. baumannii* biofilm formation at concentrations as low as 12.5 µg/mL [11], and Miao et al. (2023) showed that 3-hydroxypyridin-4(1H)-one derivatives reduced *P. aeruginosa* virulence and antibiotic resistance at sub-bactericidal concentrations [20], both providing structural and pharmacological precedent for the current findings. The concentration-dependent reduction in biofilm biomass quantified by CV assay in SD-AB-023 represents a central finding of this study. Biofilm formation in *A. baumannii* is orchestrated by multiple genetic regulators including *bap, ompA, csuE,* and the *AbaI/AbaR* QS system, and the resulting extracellular matrix confers antibiotic tolerance up to 1000-fold greater than planktonic bacteria [21]. The inhibitory profile observed here is consistent with findings from Raorane et al. (2019), who demonstrated that curcumin and related flavonoids dose-dependently reduced *A. baumannii* biofilm formation by >45–86% at sub-inhibitory concentrations, with *BfmR*-binding affinity correlating with antibiofilm efficacy [22]. The ALI pellicle inhibition data further corroborate these findings; similar dose-dependent pellicle suppression has been reported for resveratrol and natural polyphenols against *A. baumannii* by Migliaccio et al. (2024). The dual-organism inhibitory activity observed in both *A. baumannii* and *C. violaceum* suggests that 2-HP targets conserved biofilm regulatory circuits rather than species-specific pathways [23].The Alamar Blue assay confirmed that at sub-MIC level 2-HP significantly reduced metabolic activity without abolishing bacterial viability, establishing an antibiofilm agent rather than bactericidal mode of action. This mechanistic distinction is clinically important: antibiofilm agents that spare bacterial growth impose reduced selective pressure for resistance development. Selvaraj et al. (2020) similarly reported that myrtenol achieved strong antibiofilm activity against clinical *A. baumannii* strains with no adverse effect on bacterial viability, reinforcing the well-established precedent that QS-targeting antivirulence compounds can uncouple biofilm inhibition from cytotoxicity [24].

Additionally, phase-contrast microscopy confirmed structural disruption of biofilms at the architectural level, the transition from dense, multilayered biofilms in untreated controls to sparse, disorganized clusters in treated samples is a hallmark of antivirulence compound action. This morphological pattern is consistent with microscopic findings reported by Mumtaz et al. (2023) for polyphenolic compounds, and by Migliaccio et al. (2024) for resveratrol, both against *A. baumannii*. The indole derivative 7-hydroxyindole has similarly been shown to produce structurally deficient, dispersed XDRAB biofilms under sub-inhibitory conditions, further corroborating the pattern observed here [25,26].Altogether, this study has several limitations. It was conducted using a single clinical MDR *A. baumannii* isolate; validation across a broader panel of MDR/XDR strains, including ATCC 17978 and ATCC 19606, is needed. Gene expression analysis (qRT-PCR) of key QS and biofilm regulators (*abaI, abaR, bap, ompA, csuE, bfmR*) should be performed to support the proposed mechanism. Molecular docking with targets such as *AbaI, AbaR,* and *BfmR* remains to be explored. Additionally, the findings are limited to *in-vitro* assays; *in-vivo* validation (e.g., *Galleria mellonella* or *Caenorhabditis elegans*) and cytotoxicity assessment in mammalian cells are essential. Finally, potential synergy with last-line antibiotics like colistin and carbapenems warrants investigation

6. CONCLUSION

In conclusion, the present study provides the first evidence of the multitargeted antibiofilm activity of 2-HP against a clinical *A. baumannii* isolate. At sub-MIC level, 2-HP significantly inhibited biofilm formation in a dose-dependent manner, reduced air–liquid interface pellicle formation, and disrupted biofilm architecture without affecting bacterial viability. This indicates that its mode of action is antibiofilm rather than bactericidal. Collectively, these findings highlight 2-HP as a promising scaffold for the development of novel anti-infective strategies targeting biofilm-associated pathogenicity in *A. baumannii*.

However, further investigations are required to validate these observations across a broader panel of MDR and XDR strains. In addition, studies focusing on the gene expression profiling of QS regulators, molecular docking analyses, and *in-vivo* validation will be essential to comprehensively elucidate the therapeutic potential of 2-HP.

CONFLICT OF INTEREST: The authors declare that they have no conflict of interest in relation to the submitted work.
AUTHORS' CONTRIBUTION: Each of the authors mentioned has made significant contributions to the research

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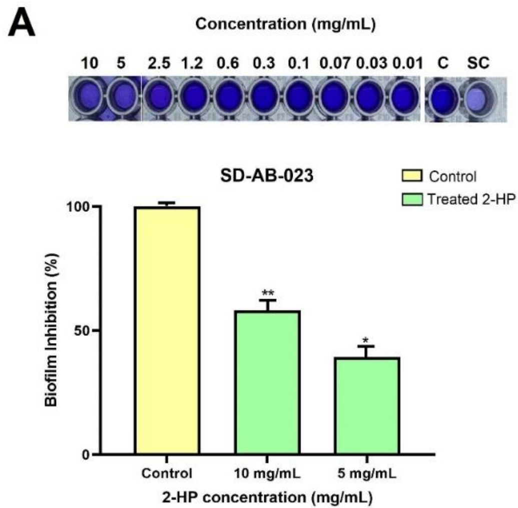
Tables and Figures

Table 1. MIC of 2-HP against *SD-AB-023*.

Concentration of 2-HP (mg/mL)	*A. baumannii* (*SD-AB-023*)
40	−
20	−
10	+
5	+
2.5	+
1.25	+
0.625	+
0.312	+
0.156	+
0.078	+
Control	+

(−) No visible growth; (+) Visible bacterial growth.

Figure 1. Antimicrobial activity of 2-HP against SD-AB-023 by agar well diffusion assay. Wells contain 2-HP at 40 mg/mL (25 mm zone of inhibition), piperacillin/tazobactam positive control (PIT; 100/10 µg/mL, 12 mm zone), and DMSO negative control (no zone).



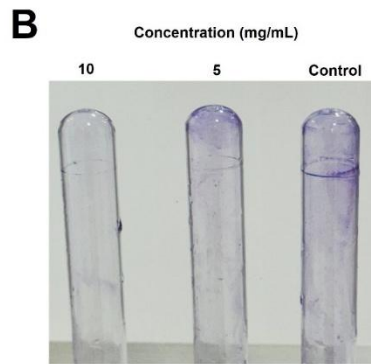


Figure 2. Inhibition of biofilm formation in SD-AB-023 by 2-HP. (A) CV microtiter assay showing concentration-dependent biofilm inhibition of 58.02% and 39.33% at 10 mg/mL and 5 mg/mL of 2-HP, respectively, relative to untreated control. Inset shows representative stained wells across the concentration range. Data represent mean ± SD; * $p < 0.05$, ** $p < 0.01$. **(B)** Visual assessment of air–liquid interface biofilm in glass test tubes stained with CV, demonstrating reduced biofilm ring intensity in 2-HP-treated cultures compared to untreated control.

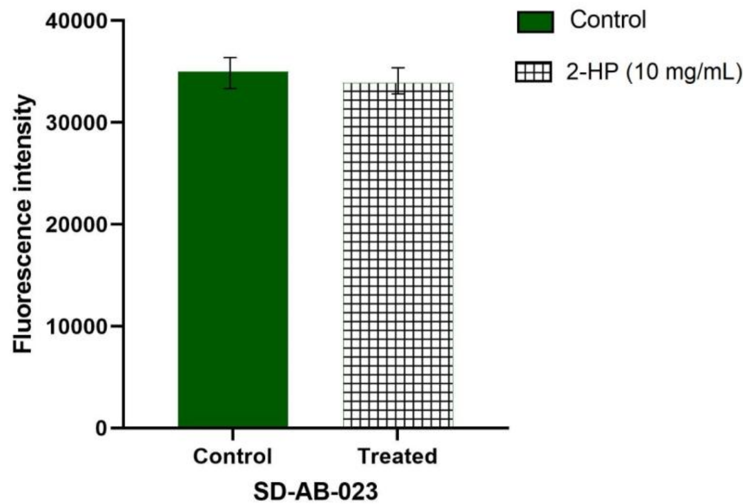


Figure 3. Effect of sub-MIC concentration of 2-HP on metabolic activity of SD-AB-023. Alamar Blue assay showing fluorescence intensity (590 nm emission) of SD-AB-023 treated with 2-HP at 10 mg/mL compared to untreated control. No significant difference in metabolic activity was observed between treated and control groups, confirming that the antibiofilm activity of 2-HP is not attributable to bactericidal effects and that bacterial viability was maintained. Data represent mean ± SD.

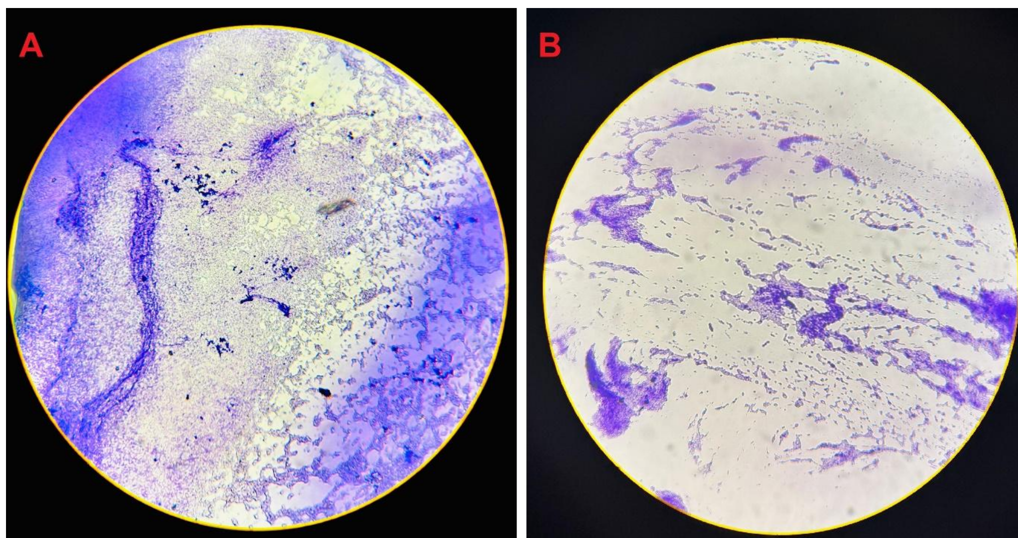


Figure 4. Phase-contrast microscopy of crystal violet-stained biofilms of SD-AB-023. Representative micrographs of biofilms formed on glass coverslips by SD-AB-023 **(A)** untreated control, showing dense, confluent biofilm with extensive intercellular aggregation, and **(B)** Treated with sub-MIC concentration (10 mg/mL) of 2-HP, showing markedly reduced surface coverage with sparse and disorganized cellular clusters, indicative of disrupted biofilm matrix integrity.