EXPLORE THE ANTIBIOFILM ABILITY OF *AMPELOPSIS CANTONIENSIS* EXTRACT AGAINST *STAPHYLOCOCCUS AUREUS* VIA EXPERIMENTAL AND COMPUTATIONAL APPROACHES

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Abstract - Our study aimed to evaluate the anti-biofilm activity of the crude ethanolic extract of *Ampelopsis cantoniensis* against *Staphylococcus aureus* (*S. aureus*) isolated from clinical specimens. Biofilm inhibition was assessed at 6, 16, and 24 h using two concentrations of the extract: 0.4 mg/ml (MIC) and 0.2 mg/ml (½ MIC). At the MIC concentration, the extract achieved a biofilm inhibition rate of 69.91% after 24 h. To explore the bioactive constituents responsible for this effect, virtual screening was conducted, identifying 3',5',5,7-tetrahydroxyflavanone as a promising anti-biofilm compound. Molecular docking analysis revealed that this compound interacts with Sortase A (SrtA) and SarA, two essential regulatory proteins involved in *S. aureus* biofilm formation. These findings suggest that *A. cantoniensis*, particularly its constituent 3′,5′,5,7-tetrahydroxyflavanone, can be potential for the development of novel anti-biofilm agents targeting *S. aureus*.

Key words - *Ampelopsis cantoniensis*; *Staphylococcus aureus*; Anti-biofilm activity; Virtual screening; Sortase A

1. Introduction

Infections remain a pressing global health concern, with *Staphylococcus aureus* being one of the most prevalent pathogens associated with both community- and hospital-acquired infections. This bacterium causes a wide spectrum of diseases, ranging from minor skin infections to severe conditions such as cellulitis, pneumonia, meningitis, and abscesses [1]. A major factor contributing to its antibiotic resistance is the formation of biofilms, which serve as protective barriers that hinder the efficacy of conventional treatments and increase healthcare costs due to the reliance on more potent and effective antimicrobial agents [2].

Biofilms are complex, structured communities of microorganisms embedded in a self-produced matrix of extracellular polymeric substances (EPS). This matrix alters the phenotype of the microbes compared to their planktonic cells. Biofilm formation begins with bacterial adhesion to the surface, facilitated by the expression of various proteins that bind to host extracellular matrix components [3]. The EPS matrix not only organizes the biofilm structure but also enhances water channels that allow nutrients and waste to move efficiently. In *S. aureus*, the biofilm structure confers multiple advantages, including protection from antibiotics, chemicals, ultraviolet light, and environmental stress. Moreover, the EPS impedes drug penetration and supports slow bacterial growth in biofilm slowly, making the cells more tolerant to treatment therapy [4].

Sortase A (SrtA) and staphylococcal accessory regulator A (SarA) proteins play crucial roles in the pathogenesis and biofilm formation of *S. aureus*. SrtA anchors surface proteins to the bacterial cell wall, facilitating adhesion to host tissues and biofilm initiation [5]. SarA is a global regulator that modulates the expression of various genes involved in virulence and biofilm development. It inhibited production of extracellular proteases, thereby stabilizing the biofilm matrix and enhancing bacterial persistence [6]. Together, these proteins enable *S. aureus* to form persistent infections and evade host immune defense.

In response, there is growing interest in natural products as alternative antimicrobial agents. *Ampelopsis cantoniensis* (*A. cantoniensis*) a traditional medicinal plant found abundantly in northern and central mountainous regions of Vietnam, has been reported to possess antibacterial and anti-inflammatory properties. Previous studies have identified bioactive compounds such as myricitrin and dihydromyricetin, which exhibited inhibitory effects against various bacterial strains, including *S. aureus* [7]. Other compounds, like 3′,5′,5,7-tetrahydroxyflavanone and phloretin, have demonstrated anti-inflammatory activity by suppressing nitric oxide production in LPS-induced RAW 264.7 cells [8]. Our previous research showed that extracts of *A. cantoniensis* can suppress *in vitro* growth of *S. aureus* and Methicillin-resistant *S. aureus* after 24 h [9]. Dihydromyricetin (DHM) was identified as a major contributing compound to anti-staphylococcal activity [10].

Although many studies have investigated the biological activities of *A. cantoniensis*, its ability to inhibit biofilm formation in *S. aureus* remains unexplored. Therefore, in this study, we aim to evaluate the effects of *A. cantoniensis* crude ethanolic extract on the biofilm formation of *S. aureus* and elucidate possible mechanisms of action through molecular docking analysis of its bioactive compounds with the SrtA and SarA proteins.

2. Material and Methods

2.1. Plant material and extraction

Ampelopsis cantoniensis samples (including leaves and stems) were collected from the mountainous area of Hoa Bac Commune, Hoa Vang District, Da Nang City, during June-July 2021. The taxonomic identification of these

samples was conducted in our previous study [9].

Ethanolic extract was isolated as previously described. Briefly, the *A. cantoniensis* stems and leaves (100 g) were dried at 50°C using a Memmert UFE 600 drying oven (Buechenbach, Germany), followed by soaking in 1500 mL of 70% ethanol for 72h at 25°C. The liquid extract was then filtered through Whatman No. 4 filter paper, and the filtrate was concentrated using a rotary evaporator (EYELA, Japan). The crude extract was dissolved in DMSO 10% (v/v) to an initial concentration of 100 mg/mL [10].

2.2. Microorganisms

Staphylococcus aureus used in this study was isolated and taxonomically identified in our prior study [9]. The bacterium was cultured and stored at the Laboratory of the Department of Biotechnology, Faculty of Chemical Engineering, The University of Danang - University of Science and Technology.

To determine the biofilm formation of S. aureus strains in an optimized environment.

A pure culture of *S. aureus* strain isolate was cultured overnight in TSB medium supplemented with 1% glucose and 3% NaCl, with shaking at 37°C. The culture was then diluted to 10^6 CFU/ml in TSB medium with 1% glucose and 3% NaCl. A volume of 200 µL of this cell suspension of *S. aureus* was added to each well of a 96-well plate. Following 24h of incubation, the planktonic cells were removed by aspirating the medium, and each well was gently washed with 200 µL of 1× phosphate-buffered saline (PBS) to eliminate non-adherent bacteria. To fix the biofilm, 200 µL of 96% ethanol was added to each well and was left for 30 minutes at room temperature. After discarding the ethanol, the wells were allowed to dry. Next, the biofilm was stained with 200 µL of 0.1% crystal violet (CV) for 10 minutes, and the staining solution was aspirated. Excess stain was removed, and the wells were rinsed twice with 200 µL of 1× PBS. Finally, 200 µL of 96% ethanol was added to solubilize the bound dye, and the absorbance was measured at 595 nm [11].

2.3. Evaluation of the effect of the ethanolic crude extract on the biofilm formation of S. aureus

To evaluate the ability of the extract to inhibit biofilm formation, S. *aureus* were cultured in TSB medium supplemented with 1% glucose and 3% NaCl, with and without the ethanolic extract at different concentrations. At selected time points (6, 16, and 24h), the samples were conducted the CV assay as described above. The cells cultured in medium without the extract served as the control group.

The percentage of biofilm formation was calculated using the following formula:

$$\% \text{ biofilm formation} = (OD_{treated}/OD_{control})*100$$

2.4. Prediction of the mechanism of action of ethanolic extract compounds on biofilm-related proteins (SrtA and SarA) via molecular docking

In this study, we selected five flavonoids from *A. cantoniensis*, previously reported by Nguyen Van Thu *et al.* [8], with molecular structures obtained in SMILES strings from the PubChem database of the National Library of Medicine. The 3D structures were then built from obtained SMILES strings using Chimera. The tool Dock Prep was used to prepare structures for molecular docking: after merging non-polar hydrogens, polar hydrogen was added along with Gasteiger charges. The structures were minimized to obtain stable conformations and then were saved in PDB format.

The membrane-associated proteins SarA (ID: 2FRH) and SrtA (ID: 1T2P) were selected as target proteins, and their 3D structures were retrieved from the Protein Data Bank. The ligand, water molecule, heteroatoms, and co-crystallized solvents were removed. Non-polar hydrogens were merged, and polar hydrogens along with Gasteiger charges were subsequently added. Binding sites of SarA and SrtA protein were identified based on previous studies by Liu *et al*. [12], and Zhuravleva *et al*. [13], respectively. The protein structures were then saved in PDB format. Virtual screening of the selected compounds against these key biofilm-associated proteins was conducted using AutoDock Vina 1.2.5.

To evaluate the binding interactions between the compounds and the target proteins, we compared the binding scores of the protein–ligand complexes with those of the proteins and known inhibitors. The reference compounds used were ZINC990144 [14] and curcumin [15], previously experimentally validated inhibitors of SarA and SrtA, respectively.

2.5. Evaluation of physicochemical properties and toxicity

The physicochemical and toxicological parameters of the bioactive compound isolated from *A. cantoniensis* leaves with the highest potential were analyzed using SwissADME and ProTox-3.0.

2.6. Statistical analysis

The experiments were carried out in triplicate and results are presented as the mean ± standard deviation (SD). GraphPad Prism version 10.4.2 software (GraphPad Software, USA) was used for statistical analyses and data visualization.

3. Results and Discussion

3.1. The formation of biofilms by S. aureus strains under optimal conditions

Several environmental factors, including glucose, osmolarity, ethanol, temperature, and anaerobiosis, have been reported to affect the biofilm-forming ability of *S. aureus* [16]. Glucose enhances biofilm development by promoting the aggregation of bacterial cells during the early stages of biofilm formation. Additionally, sodium chloride (NaCl) has a significant impact, as elevated salt concentrations increase osmolarity, which in turn stimulates biofilm production [17].

In this study, we examined the biofilm formation capability of clinical *S. aureus* isolate using tryptic soy broth (TSB) optimized with 1% glucose and 3% NaCl. As shown in Figure 1, the *S. aureus* clinical isolate produced biofilm at a level approximately 1.6 ± 0.3 times greater than that of the reference strain *S. aureus* ATCC 25923.

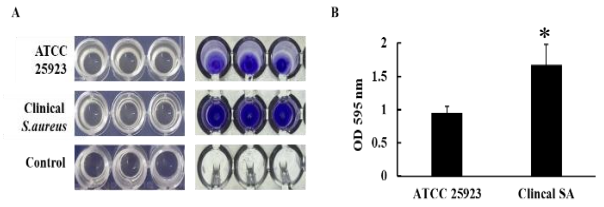


Figure 1. *Evaluating biofilm formation using crystal violet assay*

*(A) Biofilm grown in optimized TSB medium (left) and stained with 0.1% crystal violet (right). (B) Quantification of biofilm formation by measuring absorbance of crystal violet at 595 nm. Data are presented as the means ± SD of two independent experiments. Statistical significance was determined using one-tailed T- test. *p <0.05.*

3.2. Effect of A. cantoniensis ethanolic extract on biofilm formation by S. aureus

Antibacterial activity of *A. cantoniensis* ethanolic extract against S. *aureus* isolate was reported in our previous study [9], where the minimum inhibitory concentration was determined to be 0.4 mg/mL. In this study, we examined the ability of preventing biofilm formation of this extract at both the MIC and half the MIC (½ x MIC). As shown in Figure 2, biofilm development by *S. aureus* was minimal after 6 h of incubation. However, by 16 h (Figure 2B) and 24 h (Figure 2C), treatment with the crude ethanolic extract of *A. cantoniensis* at MIC and ½ x MIC concentrations resulted in a notable reduction in biofilm formation. At the MIC level, the inhibition rate reached 69.91% after 24 h (Figure 2D).

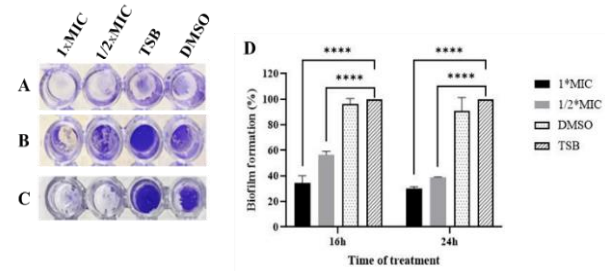


Figure 2. *Biofilm formation of S.aureus isolate in the optimal medium TSB with and without ethanolic extract at different time point*

*(A) – 6h; (B) – 16h; (C) – 24h; (D) – Bar graph represents the biofilm inhibition rate (%) at different time intervals. DMSO was used as vehicle control. Assays were carried out in triplicate and the results were expressed as mean values ± SD. ****p < 0.0001. One-way ANOVA with Dunnet post-hoc test was applied to examine the statistical difference.*

This effect is comparable to previous studies on other plant-based antibiofilm agents. For instance, *Piper betle* ethanolic extract showed 70–85% inhibition of *S. aureus* biofilms at MIC and sub-MIC levels [18], and demonstrated biofilm eradication activity with 60% removal at MIC. Similarly, Opuntia ficus-indica cladode extracts were evaluated for their antibiofilm properties, where at a concentration of 1500 µg/mL, the immature cladode extract inhibited biofilm formation by 85%, while the mature cladode extract achieved 71% inhibition [19]. Notably, the immature cladode extract began to exhibit significant inhibition at 1 mg/mL, whereas no inhibition was observed at 0.5 mg/mL.

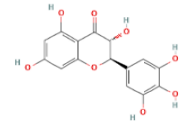
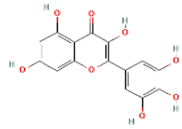
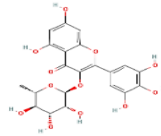
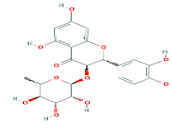
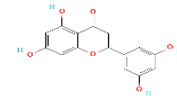
In comparison, *A. cantoniensis* crude extract achieved a similar level of biofilm inhibition (69.91%) at a considerably lower concentration (0.4 mg/mL), highlighting its potent antibiofilm activity. These findings underscore the potential of *A. cantoniensis* as an effective and efficient natural antibiofilm agent, possibly offering advantages in terms of required dosage and extract yield. Moreover, its comparable performance to well-studied plant extracts further supports its development for therapeutic or preventive applications in managing biofilm-associated infections caused by *S. aureus*.

3.3. Prediction of the mechanism of action of potential compounds in total A. cantoniensis extract on biofilm formation using virtual screening

Flavonoids have been reported to be able to prevent biofilm formation by interfering with bacterial adhesion to surfaces and host tissues [20], blocking the bacterial toxin synthesis [21], or inhibiting quorum sensing (QS) [22]. For example, quercetin has been shown to disrupt *S. aureus* biofilm formation by targeting the regulatory protein SarA [23]. Another active flavonoid compound, luteolin (3′,4′,5,7-tetrahydroxyflavone), which is commonly found in fruits and vegetables, has demonstrated anti-biofilm activity and can reduce the pathogenicity of *S. aureus* via the accessory gene regulatory (*agr*) of QS system [24].

In this study, we examined the mechanism of action of five flavonoids identified by Nguyet *et al.* [8] on biofilm formation by molecular docking analysis. Their 2D chemical structures were shown in Table 1.

Table 1. *Major compounds in A. cantoniensis*

No	Compounds	2D Chemical structure
1	Dihydromyricetin	
2	Myricetin	
3	Myricitrin	
4	Astilbin	
5	3’,5’,5,7-tetrahydroxyflavanone	

3.3.1. Molecular docking results of compounds with SrtA protein

S. aureus utilizes the enzyme Sortase A (SrtA) to anchor surface proteins to its cell wall. In this bacterium, surface proteins-known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)-are attached to the cell wall via the action of SrtA. Inhibition of SrtA significantly reduces bacterial virulence, including decreased binding to fibronectin and fibrinogen, as well as diminished biofilm formation. As a result, SrtA is considered a key target for anti-virulence therapies and a promising alternative to broad-spectrum antibiotics. The structure of SrtA has been well-characterized, with several conserved and catalytically essential amino acid residues identified, including Cys184, Arg197, Ile182, Thr180, and Ala118 [13]. Xiaodi Niu *et al*. [15] demonstrated that curcumin, a natural compound, inhibits SrtA enzymatic activity and effectively reduces biofilm formation in *S. aureus*. Given this evidence, curcumin was chosen as the reference compound in our study.

Table 2. *Results of binding affinity between selected compounds and SrtA*

Compounds	Binding Affinity (kcal/mol)
Dihydromyricetin	-7.44
Myricetin	-7.45
Myricitrin	-7.1
Astilbin	-7.04
3',5',5,7-tetrahydroxyflavanone	**-8.03**
Curcumin (reference)	-7.72

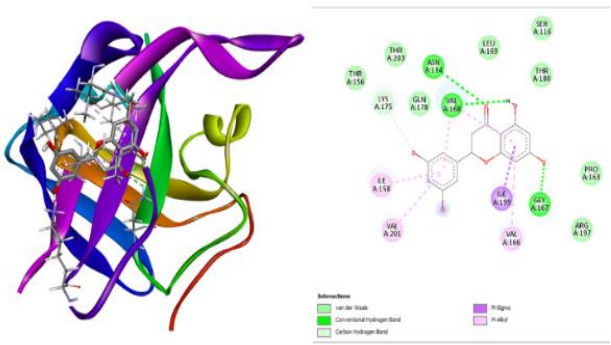


Figure 3. *The 3D (left) and 2D (right) molecular docking pose of 3',5',5,7-tetrahydroxylflavanone with sortase A (ID 1T2P)*

As shown in Table 2, 3',5',5,7-tetrahydroxyflavanone exhibited the highest binding affinity to SrtA (-8.03 kcal/mol). Notably, this interaction was stronger than that of the reference compound, curcumin. 3',5',5,7-tetrahydroxyflavanone bound to SrtA through multiple strong hydrogen bonds (Asn114, Val168, Gly167, and Lys175) and hydrophobic interactions (Ile199, Val166, Ile158, and Val201) (Figure 3). Asn114, located within the β3/β4 loop, plays a role in forming the calcium-binding site, which is crucial for stabilizing the active conformation of the enzyme. Disruption of this site may impair calcium binding, leading to reduced enzymatic activity [20, 21]. Val168, part of the β6/β7 loop, is important for substrate recognition. Mutations at this residue have been shown to significantly decrease SrtA activity [21]. Binding of the compound to these residues suggests a potential mechanism for inhibiting SrtA function, thereby preventing the anchoring of surface proteins essential for bacterial adhesion and biofilm formation.

3.3.2. Molecular docking results of compounds with SarA protein

SarA is a membrane-associated protein that plays a crucial role in biofilm formation in *S. aureus*. The crystal structure of SarA has been determined, revealing several residues essential for its function, including Thr141, Glu145, Asn146, and Asn161 [12]. We chose ZINC990144, a SarA inhibitor capable of reducing biofilm formation in various *S. aureus* strains, as a reference in this study [14].

Among five selected compounds, 3',5',5,7-tetrahydroxyflavanone exhibited the strongest interaction with the SarA protein (Table 3). The interactions between SarA and 3',5',5,7-tetrahydroxyflavanone features pi–pi (Tyr142) and hydrophobic contacts (Leu160 and Ala138) (Figure 4). These residues are part of the SarA dimerization interface. Disruption of this interface by small-molecule binding may inhibit dimerization, thereby impairing SarA's ability to bind DNA and regulate biofilm-related gene expression [12].

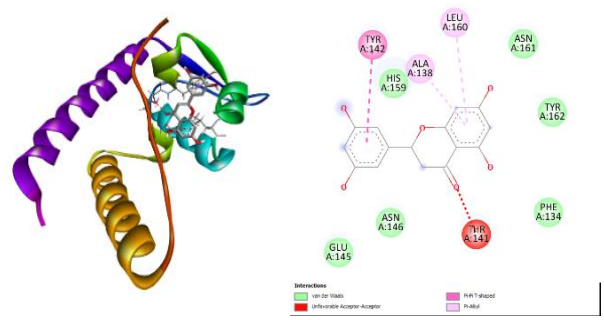


Figure 4. *The 3D (left) and 2D (right) molecular docking pose of 3',5',5,7-tetrahydroxyflavanone with SarA (ID: 2FRH*

Table 3. *Results of compounds having high binding affinity values with SarA*

Compounds	Binding Affinity (kcal/mol)
Dihydromyricetin	-6.34
Myricetin	-6.36
Myricitrin	-6.14
Astilbin	-6.01
3',5',5,7-tetrahydroxyflavanone	**-6.49**
ZINC990144 (reference compound)	-7.83

Although 3',5',5,7-tetrahydroxyflavanone has not been thoroughly studied, it has demonstrated notable anti-inflammatory properties by suppressing nitric oxide (NO) production with an IC_{50} of 18.5 µM. This effect was attributed to a dose-dependent reduction in LPS-induced inducible nitric oxide synthase (iNOS) expression in RAW 264.7 macrophage cells [8]. Additionally, the compound exhibited potential antiviral activity against HIV-1 by inhibiting the RNase H function associated with enzyme reverse transcriptase of the virus [25]. In the present study, we revealed that 3',5',5,7-tetrahydroxyflavanone exhibited promising antibiofilm activity, effectively binding to SarA

and SrtA proteins, thereby interfering with their interaction with substrate surfaces during the early stages of biofilm development. A limitation of our study is the inability to accurately quantify the concentration of 3′,5′,5,7-tetrahydroxyflavanone in the ethanolic extract, and thus we cannot rule out the contributions of other bioactive constituents.

Biofilms are structured microbial communities that are resistant to antibiotics and host immune responses. Pathogenic biofilms can secrete toxins and inflammatory mediators, disrupt the normal wound healing and contribute to chronic inflammation and persistent infection [26]. Interestingly, several flavonoids have been reported to have both anti-biofilm and anti- inflammatory properties. The anti-inflammatory effects of luteolin have been linked to its inhibition of ROS production and activation of antioxidant enzymes; suppression of pro-inflammatory cytokine expression; or stabilization of mast cells [27]. Recently, Hao *et al*. reported that phloretin significantly improves prognostic outcomes and mitigates inflammation in subarachnoid hemorrhage mice model [28].

3.4. Physicochemical properties of 3′,5′,5,7-tetrahydroxyflavanone

3’,5’,5,7-tetrahydroxyflavanone meets all the criteria of Lipinski’s Rule of Five, with a molecular weight of 288.25 g/mol, log P of 1.48, 4 hydrogen bond donors, and 6 hydrogen bond acceptors, indicating strong drug-like properties. It has a total polar surface area (TPSA) of 107.22 Å² and zero rotatable bonds-both favorable for oral bioavailability. The compound is predicted to have high gastrointestinal absorption but is not blood-brain barrier permeant, which may reduce potential CNS side effects.

Table 4. *Physicochemical properties and ADME predictions computed by SwissADME and ProTox-3.0*

Molecular weight (g/mol)		288.25
Molar refractivity		73.59
Log P		1.48
Number of hydrogen bond donors		4
Number of hydrogen bond acceptor(s)		6
Total polar surface area (Å²)		107.22
Water solubility		-3.4
		Soluble
Number of rotatable bond(s)		0
Pharmacokinetics	GI absorption	High
	BBB permeant	No
	P-gp substrate	Yes
	CYP1A2 inhibitor	No
	CYP2C19 inhibitor	No
	CYP2C9 inhibitor	No
	CYP2D6 inhibitor	No
	CYP3A4 inhibitor	Yes
	Log Kp (skin permeation) (cm/s)	-6.46
Druglikeness	Lipinski violation	0
LD50 (mg/kg)		2000
Toxicity		Non-required

It is water-soluble and shows no inhibition of major cytochrome P450 enzymes (CYP1A2, CYP2C19, CYP2C9, CYP2D6), but does inhibit CYP3A4, indicating a potential for metabolic interactions that should be further evaluated. Though it is a P-glycoprotein substrate, this does not preclude its therapeutic potential. Its low dermal permeability (Log Kp −6.46 cm/s) indicates limited transdermal application. With an LD50 of 2000 mg/kg and a “non-required” toxicity classification, the compound demonstrates a favorable safety profile. Overall, these findings support the potential of 5,7,3',5'-Tetrahydroxyflavanone as a promising orally administered drug candidate (Table 4).

4. Conclusion

Our study demonstrated that *A. cantoniensis* ethanolic extract exhibits significant inhibitory activity against the biofilm formation of clinical *S. aureus* isolate after 16 and 24 h. Compound screening and molecular docking analysis identified 3′,5′,5,7-tetrahydroxyflavanone as a key bioactive constituent, exhibiting strong binding affinity to the biofilm-associated proteins SrtA and SarA. These interactions suggest a potential mechanism involving disruption of early biofilm development.

These findings highlight the potential of *A. cantoniensis*, particularly 3′,5′,5,7-tetrahydroxyflavanone, as a promising candidate for anti-biofilm agents for further therapeutic development against *S. aureus* infections. Future studies should focus on isolating and testing the purified compound, evaluating its efficacy against diverse clinical strains of *S. aureus*, and assessing its therapeutic potential in *in vivo* infection models.

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