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Research Article

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Anti-biofilm activity of *Barleria acanthoides* Vahl against Methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

Methicillin-resistant *Staphylococcu aureus* (MRSA) biofilms are notoriously difficult to treat due to their high resistance to antibiotics. This study examines the antibiofilm, antibacterial, and molecular docking potential of *Barleria acanthoides* ethanol extract against MRSA. A total of 18 compounds were identified in GC-MS analysis and the major compounds were beta-asarone (34.09%), Neophytadiene (15.09%) and caryophyllene (9.65%). The extract was very good at killing bacteria; at a minimum inhibitory concentration (MIC) of 0.5 mg/mL, it stopped MRSA biofilm formation by 80.07%. Molecular docking studies revealed the binding affinities of key compounds to regulatory proteins LasR and SarA, which are essential in biofilm formation and quorum sensing. These findings suggest *B. acanthoides* as a promising source for developing new therapeutic agents to combat MRSA biofilm-related infections.

KEYWORDS: MRSA, *Barleria acanthoides*, Molecular docking, Antibiofilm, Quorum sensing inhibition

INTRODUCTION

Staphylococcus aureus, particularly methicillin-resistant *S. aureus* (MRSA), represents a significant global health challenge due to its ability to form resilient biofilms (Arunan *et al.*, 2023). These complex microbial communities, characterized by dense bacterial populations encased in a self-produced extracellular polymeric substance (EPS), contribute substantially to the pathogenesis of various life-threatening infections (Kavanaugh & Horswill, 2016). The biofilm matrix, primarily composed of polysaccharides, proteins, and extracellular DNA, confers remarkable resistance to antimicrobial agents and host immune responses, with antibiotic tolerance up to 1,000-fold higher than planktonic cells.

The formation of *S. aureus* biofilms involves a highly regulated, multi-stage process influenced by environmental factors and governed by intricate regulatory networks, including transcriptional regulators and quorum-sensing systems (Moormeier & Bayles, 2017). Recent research has elucidated key molecular mechanisms underlying biofilm development, revealing potential targets for therapeutic intervention (Paharik & Horswill, 2016). However, the increasing prevalence of antibiotic-resistant strains necessitates the exploration of novel anti-biofilm strategies.

In this context, phytochemicals have emerged as promising candidates for combating bacterial biofilms. The genus Barleria (Acanthaceae) has garnered particular interest due to its rich repertoire of bioactive compounds, including iridoids, flavonoids, and phenolic acids, which exhibit significant antibacterial and anti-biofilm properties (Amoo *et al.*, 2011; Jaiswal *et al.*, 2010). These phytochemicals have been shown to disrupt bacterial cell membranes and interfere with quorum sensing, critical processes in biofilm formation (Aneja *et al.*, 2010).

Barleria acanthoides, a species within this genus, represents an untapped resource for potential anti-biofilm agents. Given the urgent need for novel therapeutic strategies against MRSA biofilms, investigating the antibacterial and anti-biofilm efficacy of *B. acanthoides* is highly warranted. This study aims to evaluate the effects of *B. acanthoides* extracts on *S. aureus* biofilm formation, with a specific focus on Assessing the antibacterial activity against planktonic and biofilm-associated *S. aureus*, investigating the impact on quorum sensing mechanisms and elucidating the molecular basis of

observed anti-biofilm effects through in silico analysis of key regulatory proteins.

By integrating phytochemical analysis, microbiological assays, and molecular docking studies, this research seeks to provide comprehensive insights into the potential of *B. acanthoides* as a source of novel anti-biofilm compounds. The findings may contribute to the development of innovative strategies for managing MRSA infections and addressing the growing threat of antimicrobial resistance in biofilm-associated infections.

MATERIALS AND METHODS

Plant Material and Extract Preparation

Fresh leaves of *Barleria acanthoides* Vahl were collected from Sivanthipatti, Tirunelveli (8.7647° N, 77.8042° E), Tamil Nadu, India, in February 2024. The plant material was authenticated, and a voucher specimen was deposited in the institutional herbarium (voucher number: BA2024-01).

Leaves were washed with distilled water, air-dried at room temperature (25±2 °C) and pulverized using a mechanical grinder. The resulting powder was stored in airtight containers at 4 °C until use. Ethanol extraction was performed using a cold maceration technique (Azwanida, 2015). Briefly, 50 g of dried leaf powder was soaked in 500 mL of analytical grade ethanol (Sigma-Aldrich, USA) for 72 hours at room temperature with intermittent agitation. The mixture was filtered through Whatman No. 1 filter paper, and the filtrate was concentrated under reduced pressure at 45 °C using a rotary evaporator (Buchi R-300, Switzerland). The resulting crude extract was stored at -20 °C in amber glass vials until further analysis.

Phytochemical Profiling

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis: Chemical profiling of the ethanolic extract was performed using a Thermo Scientific Trace GC1310-ISQ system equipped with a TG-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness). The GC oven temperature was programmed as follows: initial temperature 50 °C (2 min hold), ramped to 230 °C at 5 °C/min (2 min hold), then to 290 °C at 30 °C/min (2 min hold). Helium was used as the carrier gas at a constant flow rate of 1 mL/min. Samples (1 µL) were injected in split mode with a 10:1 ratio. Mass spectrometric data were acquired in full scan mode (m/z 40-1000) with an electron ionization energy of 70 eV and ion source temperature of 200 °C. Compound identification was performed by comparing mass spectra with the NIST 11 spectral library.

Antimicrobial Susceptibility Testing

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC): The MIC and MBC of the ethanolic extract against *Staphylococcus aureus* (ATCC 25923) were determined using the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018). Briefly, two-fold serial dilutions of the extract (64 to 0.125 mg/mL) were prepared in Mueller-Hinton broth (MHB) in 96-well microplates. Bacterial inoculum was prepared from fresh culture and adjusted to 5×10^5 CFU/mL. Equal volumes (100 µL) of extract dilutions and bacterial suspension were mixed and incubated at 37 °C for 24 hours. The MIC is defined as the lowest concentration that inhibited visible bacterial growth, as indicated by the absence of turbidity. For MBC determination, aliquots (10 µL) from wells showing no visible growth were sub-cultured on Mueller-Hinton agar plates and incubated at 37 °C for 24 hours. The MBC is defined as the lowest concentration that resulted in no bacterial growth on agar plates. All assays were performed in triplicate with appropriate positive (streptomycin) and negative (sterile MHB) controls.

Anti-quorum Sensing Activity

Pyocyanin Quantification Assay: The effect of *B. acanthoides* extract on quorum sensing was assessed by quantifying pyocyanin production in *Pseudomonas aeruginosa* PAO1, as described by Essar *et al*. (1990) with modifications. *P. aeruginosa* was cultured in Luria-Bertani broth supplemented with sub-inhibitory concentrations of the extract (0.25, 0.5 and 1 mg/mL) and incubated at 37 °C for 24 hours with shaking at 200 rpm. Cultures were centrifuged at 10,000 × g for 10 minutes at 4 °C. Pyocyanin was extracted from 5 mL of supernatant using 3 mL of chloroform, followed by re-extraction with 1 mL of 0.2 M HCl. The absorbance of the resulting pink-red solution was measured at 520 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan). Pyocyanin concentration (µg/mL) was calculated by multiplying the OD520 by 17.072 (Saurav *et al*., 2016).

Anti-biofilm Activity

Crystal Violet Biofilm Assay: The anti-biofilm activity of the extract against methicillin-resistant *S. aureus* (MRSA) was evaluated using a modified tube biofilm assay (Park *et al*., 2022). MRSA (clinical isolate) was cultured overnight in tryptic soy broth (TSB) at 37 °C. The culture was diluted to 1×10^6 CFU/mL in fresh TSB supplemented with varying concentrations of the extract (10, 25 and 50 mg/mL). Aliquots (5 mL) were dispensed into sterile glass tubes and incubated at 37 °C for 24 hours to allow biofilm formation.

Following incubation, the tubes were gently washed twice with phosphate-buffered saline (PBS, pH 7.4) to remove planktonic cells. Adherent biofilms were stained with 0.1% (w/v) crystal violet solution for 30 minutes at room temperature. Excess stain was removed by thorough washing with PBS. The bound crystal violet was solubilized using 5 mL of 33% acetic acid, and the absorbance was measured at 595 nm. The percentage of biofilm inhibition was calculated relative to the untreated control using the formula:

$$\% \text{ Biofilm inhibition} = [(\text{OD control} - \text{OD treatment})/\text{OD control}] \times 100$$

Antibacterial Activity

Agar Well Diffusion Assay: The antibacterial activity of the ethanolic extract was evaluated using the agar well diffusion method as per CLSI guidelines (CLSI, 2018). Test organisms included *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), and *Vibrio cholerae* (clinical isolate). Bacterial suspensions (1.5×10^8 CFU/mL, equivalent to 0.5 McFarland standard) were spread on Mueller-Hinton agar plates. Wells (6 mm diameter) were punched in the agar and filled with 50 µL of extract at concentrations of 10, 50, and 100 mg/mL. Streptomycin (10 µg/well) served as a positive control, while 70% DMSO was used as a negative control. Plates were incubated at 37 °C for 24 hours, after which zones of inhibition were measured. All assays were performed in triplicate.

Molecular Docking Analysis

In silico molecular docking studies were conducted to investigate the potential interactions between major phytoconstituents identified by GC-MS and key bacterial proteins involved in biofilm formation and quorum sensing. The 3D structures of target proteins, including SarA (PDB ID: 2FNP) from *S. aureus*, LasR (PDB ID: 4NG2) from *P. aeruginosa*, and tyrosyl-tRNA synthetase (PDB ID: 1JIJ) from *S. aureus*, were obtained from the Protein Data Bank (Berman *et al.*, 2000). Ligand structures were retrieved from the PubChem database (Kim *et al.*, 2021).

Protein and ligand structures were prepared using AutoDock Tools (Morris *et al.*, 2009). Docking simulations were performed using AutoDockVina (Trott & Olson, 2010) with a grid box encompassing the ligand binding sites of each protein target. The docking results were analysed and visualized using Biovia Discovery Studio.

Statistical Analysis

All experiments were performed in triplicate, and data are presented as mean±standard deviation (SD). Statistical analysis was conducted using SPSS version 25.0 (IBM Corp., Armonk, NY, USA). Differences between groups were analysed using one-way ANOVA followed by Tukey's post-hoc test. A p-value<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Phytochemical Analysis

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the ethanolic extract of *B. acanthoides* revealed a complex phytochemical profile (Figure 1 and Table 1). The major constituents identified were beta-asarone (34.09%), neophytadiene (15.09%), caryophyllene (9.65%), phytol (8.79%), and germacrene D (4.97%). These compounds have been previously reported to possess significant antimicrobial and anti-biofilm properties (Brehm-Stecher & Johnson, 2003; Jiao *et al.*, 2018). Beta-asarone, the most abundant

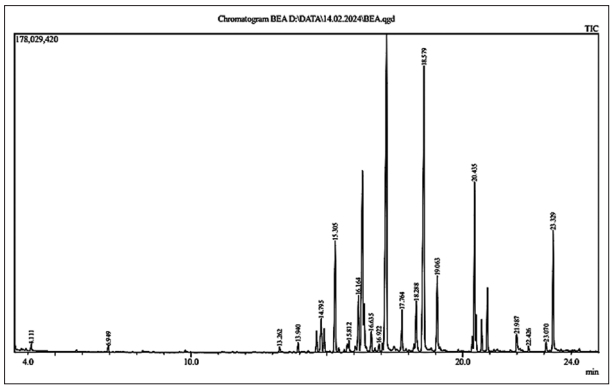

Figure 1: GCMS analysis of *B. acanthoides*

compound, has demonstrated potent antifungal activity (Lee *et al.*, 2004) and may contribute to the extract's antimicrobial effects. Caryophyllene and humulene, both sesquiterpenes, are known to disrupt bacterial cell membranes (Bakkali *et al.*, 2008), while phytol has been shown to enhance the activity of other antibacterial agents through membrane perturbation (Lim *et al.*, 2006). The presence of these bioactive compounds suggests a potential synergistic effect, which could explain the broad-spectrum antimicrobial activity observed in subsequent assays.

Antimicrobial Susceptibility

The ethanolic extract of *B. acanthoides* exhibited notable antimicrobial activity against both Gram-positive and Gram-negative bacteria. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *S. aureus* were determined to be 0.5 mg/mL and 1 mg/mL, respectively. This low MIC value (<1 mg/mL) indicates high antimicrobial potency, as plant extracts with MICs below 1 mg/mL are generally considered to have significant antimicrobial activity (Kuete, 2010). The MBC/MIC ratio of 2:1 suggests that the extract possesses bactericidal rather than bacteriostatic activity against *S. aureus* (Traczewski *et al.*, 2009). This bactericidal nature implies that the extract may target multiple bacterial cellular processes, a characteristic that could potentially mitigate the development of resistance (Kohanski *et al.*, 2010).

Anti-quorum Sensing Activity

The *B. acanthoides* extract demonstrated a concentration-dependent inhibition of pyocyanin production in *P. aeruginosa*. Pyocyanin, a blue-green pigment, is both a virulence factor and a quorum sensing signal molecule in *P. aeruginosa* (Mudaliar & Prasad, 2024). The minimum concentration of the extract that inhibited pyocyanin production was found to be 0.12 mg/mL, indicating potent anti-quorum sensing activity.

This inhibition of pyocyanin production suggests that compounds within the extract may interfere with key regulatory proteins in the quorum sensing pathway, such as LasR or RhlR (Papenfort & Bassler, 2016). The ability to disrupt bacterial

Table 1: GCMS profile of ethanol extract of *B. acanthoides*

R.Time	Compound name	Area %	Mol Formula	Mol weight
4.111	o-Xylene	0.46	C_8H_{10}	106.16
6.949	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	0.46	$C_{10}H_{16}$	136.23
13.262	Safrole	0.45	$C_{10}H_{10}O_2$	162.18
13.940	Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-	0.69	$C_{15}H_{24}$	204.35
14.795	1H-Cyclopenta[1,3]cyclopropane[1,2]benzene, octahydro-7-methyl-3-methylene	7.19	$C_{15}H_{24}$	204.35
15.305	Caryophyllene	9.65	$C_{15}H_{24}$	204.35
15.812	Humulene	1.49	$C_{15}H_{24}$	204.35
16.164	Germacrene D	4.97	$C_{15}H_{24}$	204.35
16.635	(3R,3aR,3bR,4S,7R,7aR)-4-Isopropyl-3,7-dimethyloctahydro-1H-cyclopenta	1.57	$C_{15}H_{26}O$	222.36
16.922	Benzene, 1,2,3-trimethoxy-5-(2-propenyl)-	0.59	$C_{12}H_{16}O_3$	208.25
17.764	Beta -Asarone	34.09	$C_{12}H_{16}O_3$	208.25
18.288	Apiol	4.88	$C_{12}H_{14}O_4$	222.24
19.063	1,7-Octadiyne	6.59	C_8H_{10}	106.16
20.435	Neophytadiene	15.09	$C_{20}H_{38}$	278.5
21.987	1-(Cyclopropylmethyl)-4-(methyloxy) benzene	1.33	$C_{11}H_{14}O$	162.23
22.426	Octadecanal	0.36	$C_{18}H_{36}O$	268.5
23.070	1-Octadecanol, methyl ether	0.62	$C_{19}H_{40}O$	284.5
23.329	Phytol	8.79	$C_{20}H_{40}O$	296.5

communication without directly inhibiting growth represents a promising strategy for anti-virulence therapy, potentially reducing the risk of resistance development (Elfaky, 2024).

Anti-biofilm Activity

The ethanolic extract of *B. acanthoides* demonstrated remarkable anti-biofilm activity against methicillin-resistant *S. aureus* (MRSA), reducing biofilm formation by 80.07% at the highest tested concentration of 50 mg/mL. This potent effect can be attributed to the extract's diverse phytochemical profile, which includes compounds known to interfere with bacterial attachment and biofilm maturation. Beta-asarone, for instance, has been shown to inhibit biofilm formation by disrupting quorum sensing mechanisms (Zhou *et al.*, 2020), while caryophyllene alters cell surface hydrophobicity in *S. aureus* (Subramenium *et al.*, 2015), and phytol inhibits both initial attachment and mature biofilm development (Pejin *et al.*, 2015). The synergistic action of these compounds likely contributes to the extract's efficacy in reducing MRSA biofilm formation.

The presence of flavonoids and phenolic compounds in the *B. acanthoides* extract further enhances its anti-biofilm properties through their antioxidant and antimicrobial activities. These phytochemicals may disrupt the extracellular polymeric substances (EPS) that form the structural scaffold of biofilms, thereby weakening their integrity. The extract's ability to inhibit biofilm formation at such a high percentage suggests its potential as a promising candidate for developing novel anti-biofilm agents against MRSA infections.

Antibacterial Activity

The agar well diffusion assay revealed broad-spectrum antibacterial activity of the *B. acanthoides* extract against both Gram-positive and Gram-negative bacteria (Table 2 and Figure 2). The extract exhibited a clear dose-dependent response, with the highest activity observed at 100 mg/mL concentration. *E. coli* showed the highest susceptibility,

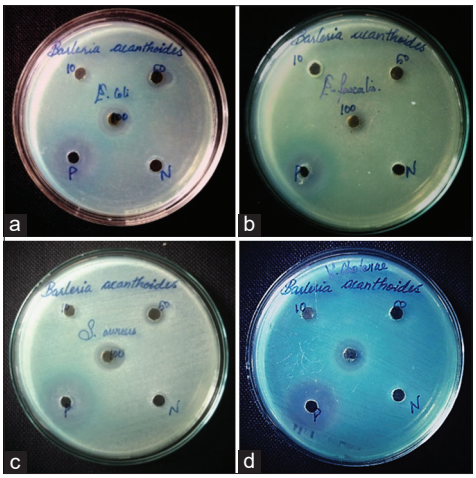

Figure 2: Antibacterial Activity of Ethanolic extract of *B. acanthoides*. a) *E. coli*, b) *E. faecalis*, c) *S. aureus* and d) *V. cholerae* (10, 50, 100 - Extract concentration (mg/mL); P - Positive control; N - Negative Control)

with 87.9% inhibition at 100 mg/mL, followed by *S. aureus* (76.7%), *E. faecalis* (73.1%), and *V. cholerae* (67.4%). Notably, at 100 mg/mL, the extract's efficacy against E. coli (9.5 mm zone of inhibition) nearly matched that of the positive control streptomycin (10.8 mm zone of inhibition).

The broad-spectrum activity of the extract, particularly its efficacy against both Gram-positive (*S. aureus*, *E. faecalis*) and Gram-negative (*E. coli*, *V. cholerae*) bacteria, suggests the presence of multiple bioactive compounds with diverse mechanisms of action. This broad activity spectrum is particularly valuable in the context of polymicrobial infections and could potentially address the challenge of antibiotic resistance in various pathogenic bacteria. The mechanism of action for this compound involved bacterial membrane depolarization, cell membrane damage, and DNA intercalation, leading to impeded DNA replication and disturbed DNA gyrase function (Wang *et al.*, 2024).

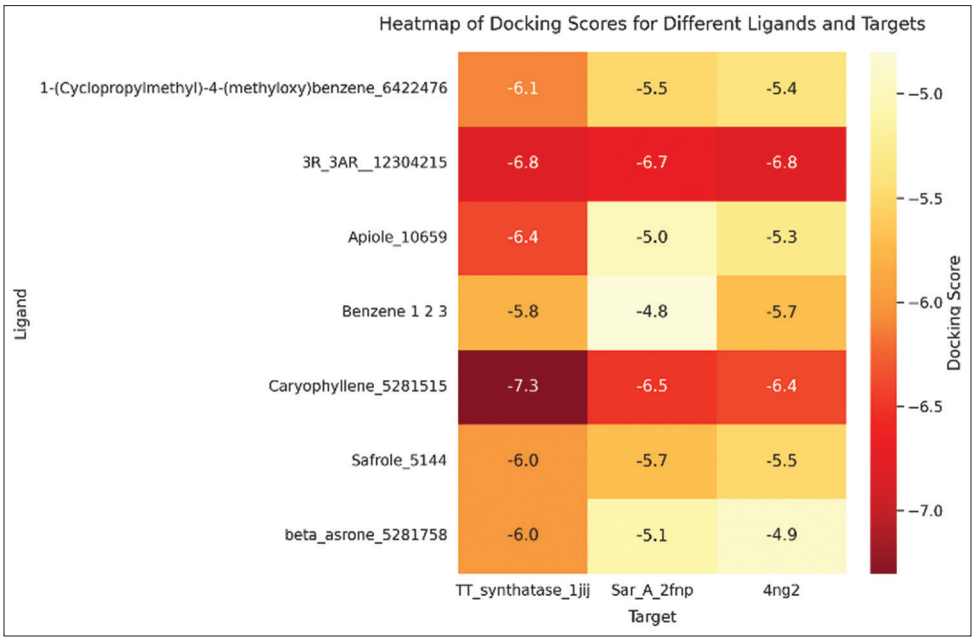

Figure 3: Binding affinity of Ligands with Specific proteins

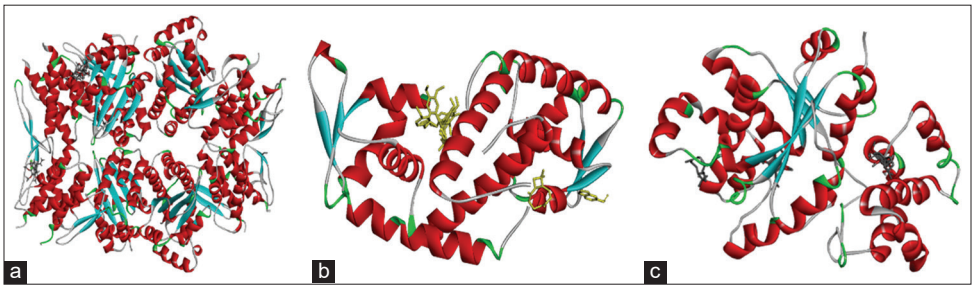

Figure 4: Molecular Docking -Protein Ligand Interaction. a) LasR - 4NG2, b) SarA - 2FNP and c) Tyrosyl-tRNA synthetase - 1JIJ

Table 2: Antibacterial Activity of Ethanolic extract of *B. acanthoides*

Pathogens	Samples	ZoI	% Inhibition	F
Staphylococcus aureus	10	2.66±0.57 a	23.1	66.619048
	50	4.5±1.32 a	39.1	
	100	8.83±0.76 b	76.7	
	P	11.5±0.5 c	-	
	N	0	-	
Escherichia coli	10	1.5±0.5 a	13.8	67.702703
	50	6±1 b	55.5	
	100	9.5±1.32 c	87.9	
	P	10.8±0.28 c	-	
	N	0	-	
Enterococcus faecalis	10	2±0.5 a	14.4	123.956
	50	6.33±0.76 b	45.8	
	100	10.1±0.76 c	73.1	
	P	13.8±1.04 d	-	
	N	0	-	
Vibrio cholerae	10	1.16±0.28 a	8.85	181.818
	50	4.16±0.76 b	31.7	
	100	8.83±0.28 c	67.4	
	P	13.1±1.04 d	-	
	N	0	-	

dF – 3,1; p<0.001.

Molecular Docking Analysis

In silico molecular docking studies provided insights into the potential molecular mechanisms underlying the observed antibacterial and anti-biofilm activities of the *B. acanthoides* extract. The compound 3R_3AR_12304215 (tentatively identified from the GC-MS analysis) showed strong binding affinities to key bacterial proteins: SarA from *S. aureus* (-6.7 kcal/mol) and LasR from *P. aeruginosa* (-6.8 kcal/mol) (Figures 3 and 4).

SarA plays a vital role in regulating biofilm formation in *S. aureus* (Trotonda *et al.*, 2005). It stimulates the ica operon, which is responsible for the production of polysaccharide intercellular adhesin (PIA/PNAG), an essential component of biofilms (Valle *et al.*, 2003; Trotonda *et al.*, 2005). Additionally, SarA controls the expression of other genes associated with biofilm formation, such as bap (Trotonda *et al.*, 2005). In contrast, LasR serves as a critical transcriptional regulator in the quorum sensing system of *P. aeruginosa* (Bottomley *et al.*, 2007). When LasR interacts with the autoinducer 3-oxo-dodecanoyl homoserine lactone (3O-C12-HSL) produced by LasI, it triggers the transcription

of genes encoding virulence-related traits, including elastase, lasI, rhlI, and rhlR (Morales *et al*., 2017). The high binding affinity of 3R_3AR_12304215 to these proteins indicates that it may disrupt their function, potentially explaining the observed anti-biofilm and anti-quorum sensing effects of the extract. Caryophyllene, another major component identified in the extract, also demonstrated affinity for SarA (-6.5 kcal/mol), corroborating its previously reported anti-biofilm properties (Neagu *et al*., 2023). These docking results provide a molecular basis for the broad-spectrum antimicrobial and anti-biofilm activities observed in the experimental assays.

CONCLUSION

In conclusion, this study demonstrates the significant antimicrobial, anti-quorum sensing, and anti-biofilm potential of *Barleria acanthoides* ethanolic extract against clinically relevant pathogens, including antibiotic-resistant strains. The extract's complex phytochemical composition suggests a broad spectrum of biological activities, which contribute to its efficacy in inhibiting bacterial growth and biofilm formation. These findings suggest that *B. acanthoides* may serve as a promising source of novel therapeutic agents for managing biofilm-associated and antibiotic-resistant infections. Further research is necessary to explore its full therapeutic potential and identify the active compounds responsible for these effects.

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