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Investigation of Anti-Biofilm and Quorum Sensing Inhibitory Properties of Isatin-Based Hydrazide Derivatives Against *Klebsiella pneumoniae*

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A B S T R A C T

In this study, previously reported isatin-based hydrazide derivatives (5a–5i) were further evaluated for their anti-biofilm and quorum sensing (QS) inhibitory activities against *Klebsiella pneumoniae* and *Chromobacterium violaceum* CV026, respectively. The biofilm inhibition assay demonstrated that compound 5b, containing a 3-bromo substituent, exhibited the highest biofilm inhibition, reaching 84.19% at a concentration of 100 µg/100 µl. The significant efficacy of 5b is likely due to the enhanced electronic and steric properties conferred by the bromine atom, promoting stronger interactions with bacterial targets. Compound 5i, an unsubstituted benzene derivative, also showed substantial inhibition (86.10%), indicating that both substituted and unsubstituted aromatic rings influence biofilm formation.Further investigation of 5b's anti-quorum sensing potential via a disc diffusion bioassay against *C. violaceum* CV026 revealed a concentration-dependent loss of violacein production, with the largest zone of pigmentation loss (21 mm) at 1 mg/ml. These findings suggest that 5b not only impairs biofilm formation but also disrupts QS signaling pathways. Molecular docking studies provided mechanistic insights into the interactions between the synthesized compounds and bacterial target proteins. The docking analysis revealed that hydrophobic interactions, including pi-pi and pi-alkyl stacking between the indolinone moiety of the isatin-hydrazide scaffold and key protein residues, played a crucial role in the inhibitory activity. Compound 5b exhibited the excellent binding affinity, further supporting its experimental potency.In conclusion, the experimental and computational data identify isatin-based hydrazide derivatives, particularly compound 5b, as promising candidates for the development of novel anti-biofilm and quorum sensing inhibitors, with potential applications in combating multidrug-resistant bacterial infections. These findings provide valuable insights into the structural features essential for antibacterial activity and lay the groundwork for future optimization of these compounds in therapeutic applications.

Keywords: Anti-Biofilm; Synthesis; Quorum Sensing; Isatin-Based Hydrazide Derivatives; Docking study; *Klebsiella pneumoniae*.

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INTRODUCTION

Quorum sensing (QS), the process that involves the endogenously produced signaling molecules controlling gene expression in a dose-dependent way, is one of the key bacterial communication channels (Waters and Bassler, 2005). This communication system enables bacteria to organize simple social networks in which group performances are coordinated using chemical signals called autoinducers (AIs) (Ng and Bassler, 2009). The key molecules involved in intraspecies communication in Gram-negative bacteria are N-acyl homoserine lactones (AHLs); in the case of Gram-positive bacteria, such signaling is performed by oligopeptides (Miller and Bassler, 2001). These molecules attach to specific receptors and stimulate a sequence of gene activation when bacterial density reaches a certain threshold (Ng and Bassler, 2009). Among these gene expressions are those that regulate pathogenicity, antibiotic production, biofilm synthesis, bioluminescence, and motility by swarming (Rutherford and Bassler, 2012). Biofilms, which are heterogeneous and organized clusters of microorganisms surrounded by a protective polymer matrix, are accepted as virulence factors of bacteria and are linked to chronic and persistent infections (Costerton *et al.,* 1999). Biofilms are challenging to eliminate because they are much more resistant to antibiotics, up to a thousand times more than free-swimming bacteria (Stewart and Costerton, 2001). These biofilm-associated infections account for approximately 17 million new cases annually, with over 500,000 deaths worldwide (Donlan and Costerton, 2002). Understanding biofilm formation and developing methods to prevent this process is crucial for tackling bacterial resistance and chronic infections (Fux *et al.,* 2005). Several bacterial models have been used to investigate QS systems and their implications on biofilm formation, including *Agrobacterium tumefaciens*, *Chromobacterium violaceum* CV026, and *Vibrio fischeri* (McClean *et al.,* 1997). Among pathogenic bacteria, *Klebsiella pneumoniae* is feared for its biofilm-forming capability, as well as for causing diseases such as gastroenteritis, lung inflammation, and multiple organ dysfunction (Podschun and Ullmann, 1998). *Cronobacter sakazakii* has been associated with foodborne outbreaks and utilizes both AI-2 transport genes and type-2 QS regulatory molecules in biofilm layer formation (Podschun and Ullmann, 1998). The cross-sensitivity to quinolone and carbapenem is due to signaling involving LuxS-dependent signal molecules, which play a role in biofilm formation in *K. pneumoniae* (Sperandio *et al.,* 2002). Since biofilm formation and bacterial resistance have accelerated over time, there has been a push to discover potential QS inhibitors (Hentzer *et al.,* 2003). A variety of synthetic compounds—including cyclohexanones, macrolides, furanones, fimbrolides, and furanyl hydrazides—have been shown to block quorum sensing and prevent biofilm formation (Geske *et al.,* 2008). However, many of these compounds present issues, such as high toxicity or limited chemical applicability for some therapeutic functions (García-Contreras *et al.,* 2016). Therefore, there is a pressing need to search for and develop more potent and safe QS inhibitors for therapeutic application (García-Contreras *et al.,* 2016). Targeting the QS regulatory system can lead to the development of new-generation antimicrobial compounds to counter bacterial infections, particularly those that form biofilms (Rasmussen and Givskov, 2006). Isatin, a heterocyclic molecule, is incorporated within mammalian tissues and exhibits multiple pharmacological activities (Pandeya *et al.,* 2005). The chemical modification of isatin presents an opportunity to generate new compounds with enhanced pharmacological characteristics (Trost *et al.,* 2001). A wide range of biological activities has been reported for isatin-based compounds, including anticonvulsant, anticancer, antidepressant, antifungal, anti-angiogenic, and anti-HIV effects (Pandeya *et al.,* 2005). The potential of isatin derivatives as biofilm inhibitors has also been investigated, suggesting their suitability for further research in anti-biofilm treatments. In this context, the present study focuses on the biofilm inhibitory properties of nine distinct N'-(1-benzyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene) hydrazide derivatives, previously synthesized and reported by our group (Arif *et al.,* 2024). Altogether, these compounds were tested on their effectiveness to prevent biofilm formation in Klebsiella pneumoniae, a pathogen of clinical importance. This compound was subjected to more experiments to determine if it could eradicate quorum sensing, a key process that contributes to biofilm formation. To further explain its biofilm inhibitory activities, molecular docking studies were performed where only the QS receptor protein LasR ligand-binding domain (LBD) was considered to

predict the compound-receptor binding. This combined experimental and computational approach aims to provide valuable insights into the potential of these compounds as novel antibiofilm agents targeting QS pathways in bacterial pathogens.

MATERIAL AND METHODS

Synthesis and In Silico Characterization of N'-(1-Benzyl-2-Oxo-1,2-Dihydro-3H-Indol-3-ylidene) Hydrazide Derivatives

Nine different N'-(1-benzyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene) hydrazide derivatives were prepared according to published synthetic sequences. These derivatives were also analysed using regular methods including nuclear magnetic resonance (NMR) and mass spectrometry (MS). Additionally, the synthesized compounds received further drug likeness, toxicity and ADME profile analysis using DataWarrior, pkCSM and SwissADME tools. These data were reported in our earlier studies (Arif *et al.,* 2024). The synthesis procedures and the chemical structures of the developed compounds together with the computational predictions of bioavailability and toxicity are described in the earlier publication from our research group.

Media and Compounds Used in Biofilm and QS Assays

Media: LB broth and LB agar were adopted for bacterial cultivation in all biofilm and quorum sensing (QS) experiments, prepared according to standard procedures with concentrations and ingredients from Merck Pakistan (Sezonov *et al.,* 2007). In all experiments, double distilled water was used for accuracy.

Compounds: The synthesized N'-(1-benzyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene) hydrazide derivatives as shown in (Figure1) were dissolved in dimethyl sulfoxide (DMSO) (Merck Pakistan) to prepare stock solutions. Ciprofloxacin was used as the positive control, while DMSO served as the negative control; cefadroxil was obtained from Hilton Pharma, Pakistan.

Crystal violet dye and ethanol were purchased from Sigma Aldrich Pakistan for biofilm quantification (Christensen *et al.,* 1985).

Bacterial Strains and Growth Conditions

The bacterial strains used in this study included Klebsiella pneumoniae (clinical isolate) for biofilm formation assays and Chromobacterium violaceum CV026 for assessing QS inhibition ability (Rasmussen and Givskov 2006).

- *Klebsiella pneumoniae* was cultured in Luria-Bertani (LB) media at 37°C, 200 RPM on an orbital shaker, with a 1% dilution of overnight culture used as inoculum (Singh et al. 2017).
- *Chromobacterium violaceum* CV026 was grown at 27°C on LB agar for QS assays (McClean et al. 1997).

Both strains were obtained from the Pakistan Institute of Medical Sciences (PIMS), Islamabad, and maintained in glycerol stocks at -80°C for long-term preservation.

Determination of Anti-Biofilm Activity

The anti-biofilm activity of the synthesized compounds was assessed using the microtiter plate method, following established protocols (Peeters *et al.,* 2008). Briefly, a 1% dilution from an overnight culture was used to inoculate fresh LB broth containing the test compounds, negative control (DMSO), and positive control (cefadroxil). The plates were incubated at 37.8°C for 48 hours. After incubation, planktonic cells were removed by gentle washing with sterile double-distilled water, and biofilm cells were stained with 0.2% crystal violet for 10 minutes (Stepanović *et al.,* 2007). Excess dye was removed, and the remaining crystal violet was solubilized in 95% ethanol. Biofilm biomass was quantified by measuring absorbance at 595 nm using a microplate reader (EL808; BioTek Instruments, USA). All experiments were performed in triplicate, and results were reported as a percentage reduction in biofilm biomass relative to the control.

A Bioassay for Determining QS Inhibition Activity

The most effective anti-biofilm compound, 5b, was tested for QS inhibition using the disc diffusion method (Choo *et al.,* 2006). N-hexanoyl homoserine lactone (HHL, 1 mg/mL) was added to molten LB agar, which was then poured into Petri dishes and allowed to solidify. An overnight culture of *C. violaceum* CV026 was spread on the agar surface. Sterile discs (6 mm diameter) were impregnated with 20 µL of compound 1b at varying concentrations and placed on the agar plates. The plates were incubated at 27°C for 25 hours, and QS inhibition was indicated by colorless zones around the discs (McClean et al. 1997). The assay was performed in triplicate, and inhibition zones were measured.

Molecular Docking Analysis

Molecular docking was performed to investigate interactions between the synthesized compounds and the LasR receptor (PDB ID: 2UV0), a key protein involved in quorum sensing. The 3D structures of the compounds were prepared using ChemSketch 12.0 and converted to PDB format using Open Babel (O'Boyle *et al.,* 2011). The protein and ligand structures were converted to PDBQT format using AutoDock Tools (Morris *et al.,* 2009), and docking was carried out with AutoDock Vina (Trott and Olson, 2010). Post-docking, interactions were analyzed using Discovery Studio 4.1 and PyMOL. Binding affinities were expressed in kcal/mol, indicating the binding energy and

preferred orientation of each compound within the ligand-binding domain of LasR.

RESULT AND DISCUSSION

Biofilm Inhibition Against *Klebsiella pneumoniae*

The microtiter plate assay was carried out using *Klebsiella pneumoniae* to determine the biofilm inhibition activity of the synthesized N'-(1-benzyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene) hydrazide derivatives. Of all the synthesized compounds, the 3-bromo benzene derivative (5b) demonstrated the highest percentage of biofilm inhibition. At 25 µg / 100 µl, compound 5b decreased the biofilm formation by 79.74% when compared to treatment without any compound, while at 50 µg / 100 µl, the percent inhibition increased to 81.22%, and at 100 µg / 100 µl, inhibition of biofilm formation was 84.19%. These strong activities can perhaps be explained by the presence of the bromine atom in 5b, which seems to improve the interaction with the bacterial targets and therefore plays a significant role in biofilm inhibition (Soto, 2013). (Table 1) Likewise, the unsubstituted benzene derivative (5i) also exhibited good activity (86.10% at 100 µg/100 µl), further confirming the notion that the absence of electronic groups on the benzene ring favors biofilm formation inhibition. Another analog of the compound, the 3,4,5-trihydroxy benzene derivative (5g), also showed good biofilm inhibition, achieving 84.28% inhibition at its maximum concentration, supporting that the high extension of hydroxyl groups offers optimum interaction with bacterial targets. (Figure 2) Compound 5h (2-chloro benzene) also had a concentration-dependent profile, with 100 µg/100 µl exhibiting the highest inhibition of 83.56%. This increase at higher concentrations suggests that the chlorine atom, an electron-withdrawing group, works more effectively in increasing biofilm inhibition. On the other hand, the 3-iodobenzene derivative (5c) and the 4-pyridine derivative (5a) exhibited lower inhibition rates of 83.65% and 82.92%, respectively. The large size of the iodine atom in 5c and the nitrogen atom in the pyridine ring in 5a may have contributed to the reduced overall interaction effectiveness with bacterial biofilm-forming targets. Compound 5d, derived from 2-hydroxy benzene, 5e from 4-fluoro benzene, and 5f from 2-pyridine, exhibited moderate to low activity, with 5f showing concentration-dependent inhibition that increased to 81.92% at 100 µg/100 µl (Borges *et al.,* 2016). The outcome patterns unmistakably indicate that biofilm inhibition depends upon the availability and position of the substituents present on the benzene ring. Increased antibacterial activity is observed in the presence of bromine, hydroxyl groups, or an unsubstituted benzene ring on the aromatic nucleus, while electron-withdrawing groups such as chlorine and fluorine cause a variable degree of inhibition depending on concentration (Donlan, 2002).

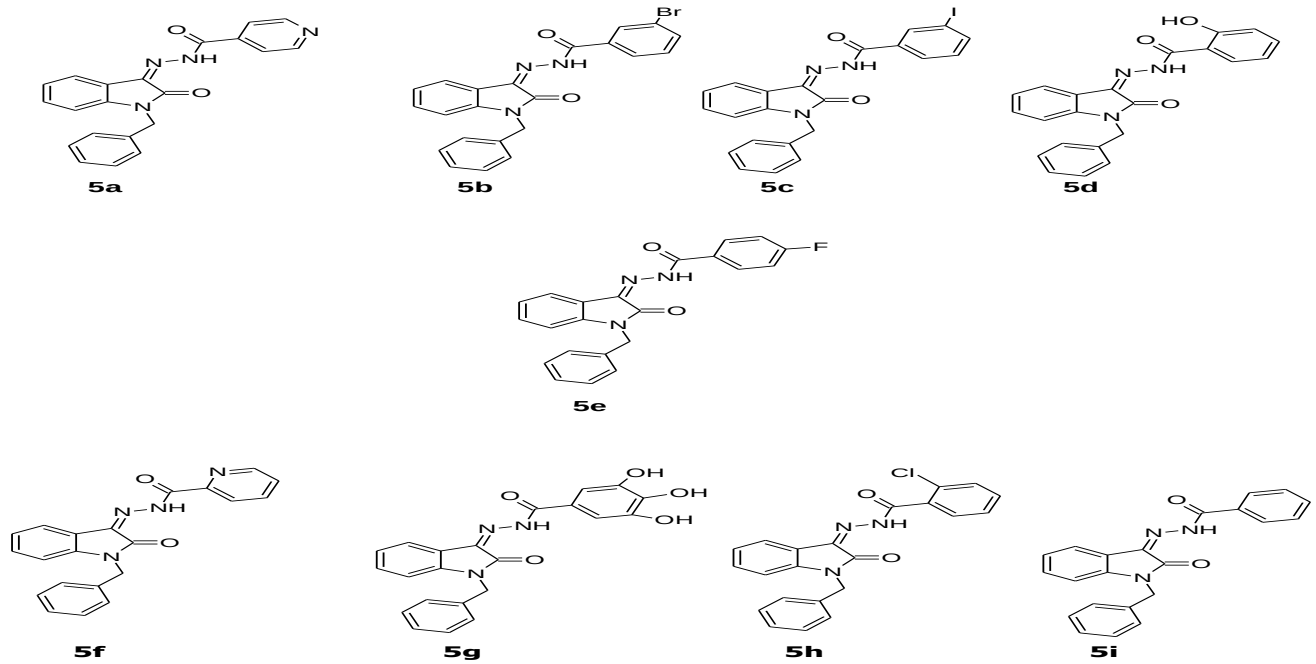

Figure 1. Structures of Isatin Derivatives (5a-5i).

Table 1. Percentage inhibition of Klebsiella pneumoniae biofilm formation by compounds 5(a–i) at different concentrations:

Compounds	*Klebsiella pneumoniae*		
	% inhibition		
	25µg/100µl	50µg/100µl	100µg/100µl
5a	79.90 ± 0.8	80.91 ± 0.9	82.92 ± 1.3
5b	79.74 ± 0.5	81.22 ± 0.8	84.19 ± 1.4
5c	77.49 ± 0.9	79.54 ±1.6	83.65 ± 0.9
5d	53.61 ± 0.6	62.74 ±1.4	81.01 ± 0.8
5e	65.25 ±1.1	70.53 ± 0.9	81.10 ± 0.5
5f	38.27 ±1.4	52.82 ±0.4	81.92 ± 1.3
5g	55.87 ±0.5	65.34 ± 0.7	84.28 ± 0.7
5h	8.70 ± 0.8	30.99 ± 1.3	83.56 ± 0.9
5i	65.05 ±1.3	71.29 ± 0.9	86.10 ± 0. 1.4
+ve control Cefadroxil (5µg/5µl)	78.76 ±0.8	80.45 ± 0.5	83.83 ±0.7
-ve control DMSO	-6.77	-13.89 =0	-22.51 = 0

Quorum Sensing Inhibition (QS)

The QS of the most active biofilm inhibitor, compound 5b, was assessed using the *Chromobacterium violaceum* CV026 strain. This strain synthesizes the purple pigment violacein as a QS-regulated molecule. In the disc diffusion bioassay, the inhibition of violacein production by 5b occurred in a concentration-dependent manner, as demonstrated by the increasing size of the clear zone surrounding the discs loaded with higher concentrations of 5b. A concentration of 0.1 mg/ml showed a 9 mm zone of inhibition, which increased to 19 mm at 0.5 mg/ml and 21 mm at 1.0 mg/ml (Grandclément *et al.*, 2016). The lack of violacein pigmentation indicated that 5b disrupted QS signaling, thus exhibiting anti-QS activity (Rutherford and Bassler 2012) (Table 2).

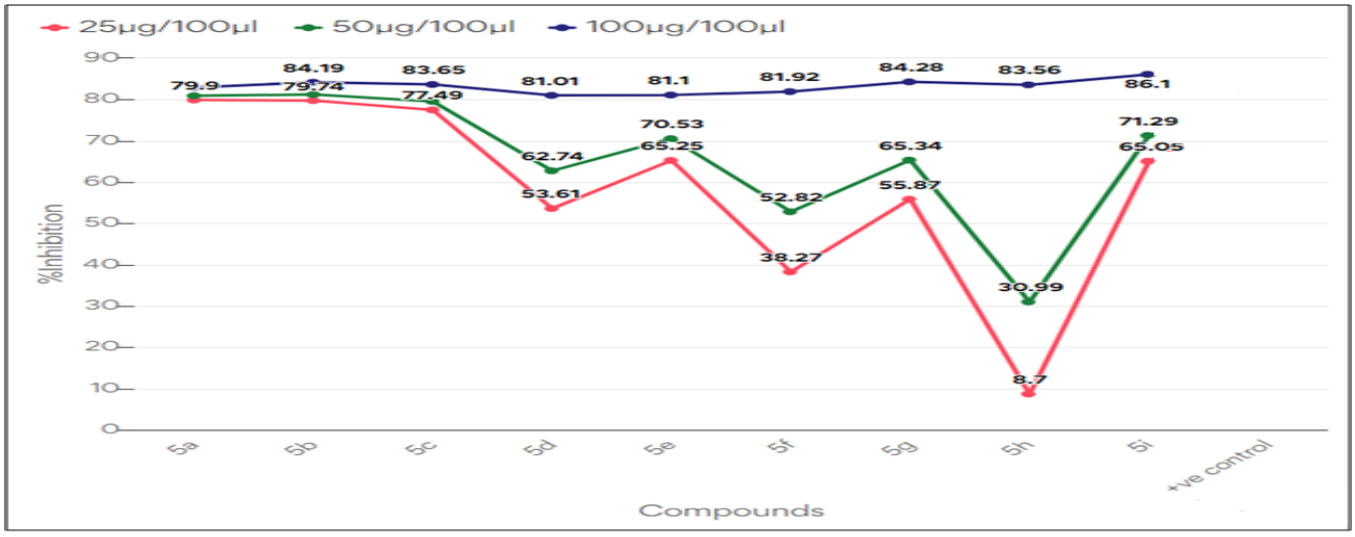

Figure 2. Line Graph depicting the percentage inhibition of *K. pneumoniae* at varying concentration of compound 5(a-i).

Table 2. QS inhibitory activity of compound 5b at different concentrations:

Compound	Concentration (mg/ml)	Zone of pigmentation loss(mm)
	Blank	Nil
5b	0.1	9 ± 0.6
	0.5	19 ± 1.0
	1.0	21 ± 0.9

Molecular Docking

Molecular docking studies were performed to explore the interaction of the synthesized compounds with the QS receptor protein LasR (PDB ID: 2UV0). The docking study showed that all compounds had good binding affinities with the receptor, comparable to the standard ligand (Kim *et al.*, 2015, Szabó *et al.*, 2010). The binding affinity of compound 5b was -11 kcal/mol, indicating strong interactions with key amino acid residues, including TRP301. Specifically, Trp301's hydrogen atom remains connected to the H atom of Leu267 at a distance of 3.39 Å, with NE of Trp301 in van der Waals contact with the O atom of ILE B:326 at 3.49 Å and O atom of ARG B:394 at 3.89 Å (Trott and Olson 2010).

The predominant forces and intermolecular interactions included hydrophobic contacts, such as pi-pi, pi-alkyl, and pi-anion interactions between the indolinone ring and the bacterial QS protein. These results imply that the biofilm inhibition and QS inhibitory properties of the synthesized compounds strongly depend on hydrophobic interactions with target proteins. Substituents that contribute positively to the molecular structure, such as the bromine atom in the 5b position, showed higher binding affinities and improved bioactivity (Rasmussen et al. 2005) (Figure 3-6).

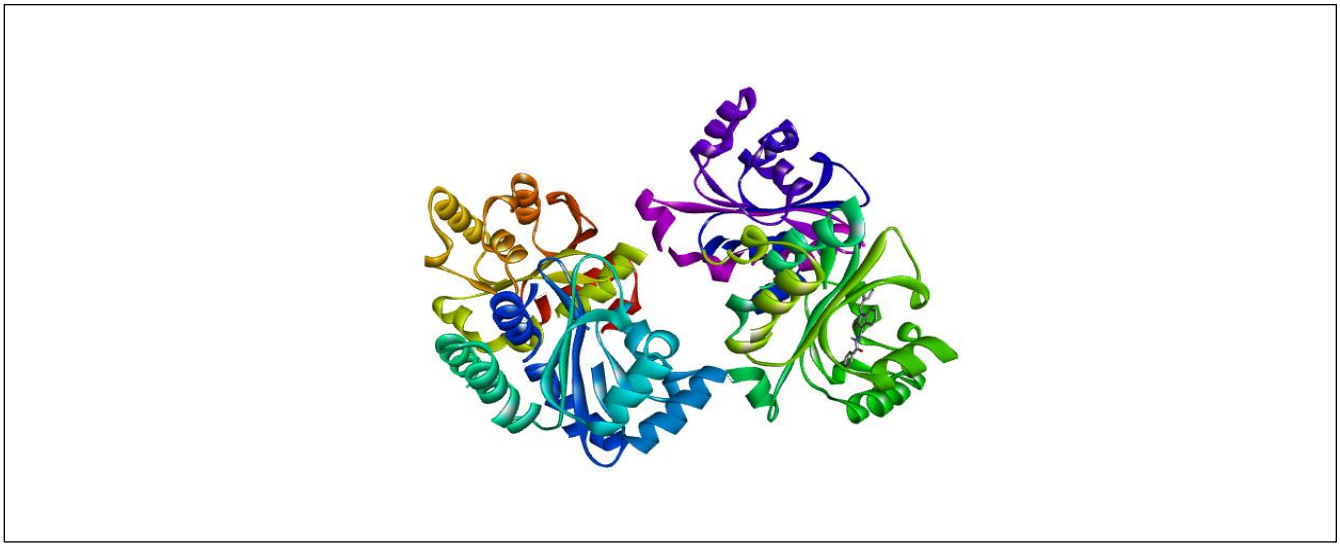

Figure 3. Compound 5h docked with the LasR protein, illustrating the binding pose and orientation of the compound within the active site.

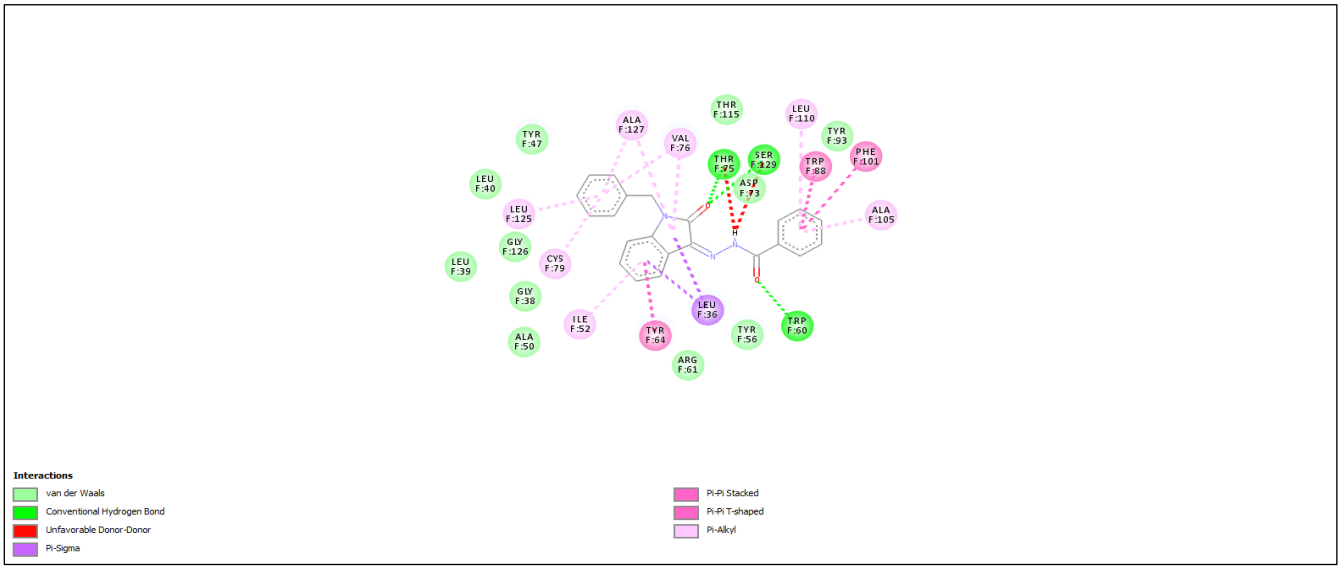

Figure 4. 2D binding interactions of compound 5h with the LasR protein, highlighting key residues involved in the interaction and the nature of the binding interactions.

These findings suggest that enhancing the binding and biological activities of the synthesized indolinone-hydrazide derivatives primarily relies on hydrophobic interactions. Compound 5b demonstrated both moderate biofilm inhibition, restricted QS, and favorable molecular interactions. Therefore, compound 5b was selected as the most promising candidate for further development as an anti-biofilm and anti-QS agent (Nazzaro *et al.,* 2013, Ahmed *et al.,* 2019) (Table 3).

Table 3. Molecular docking results of the synthesized compounds with LasR protein:

Compounds	Binding Affinity (kcal/mol)	Amino acid residue
5a	-12.7	LEU B:320, TRP B:393, LYS B:449, ARG B:394, ILE B:326, PRO B:324, GLU B:353
5b	-11	TRP B:393, ILE B:326, ARG B:394, LEU B:320, PRO B:324
5c	-9.2	LYS A:520, ARG B:548, MET A:427, HIS B:547, ALA A:430, ASP A: 426
5d	-12.2	MET A:427, THR B:460, HIS B:547, ALA B:546, ARG B:548, GLU A:423, LYS A:520
5e	-12.2	LEU B:320, ILE B:326, TRP B:393, LYS B:449, ARG B:394, PRO B:324, GLU B:353
5f	-12.4	LEU B:320, ILE B:326, TRP B:393, GLU B:353, ARG B:394, GLU B:353
5g	-8.00	MET A:427, HIS B:516, HIS B:547, GLU A:423, ARG B:548
5h	-12.6	ARG B:548, HIS B:516, CYS B:381, , HIS B:547, LYS A:520
5i	-12.4	PRO B:324, TRP B:393, ILE B:326, ARG B:394, LEU B:320

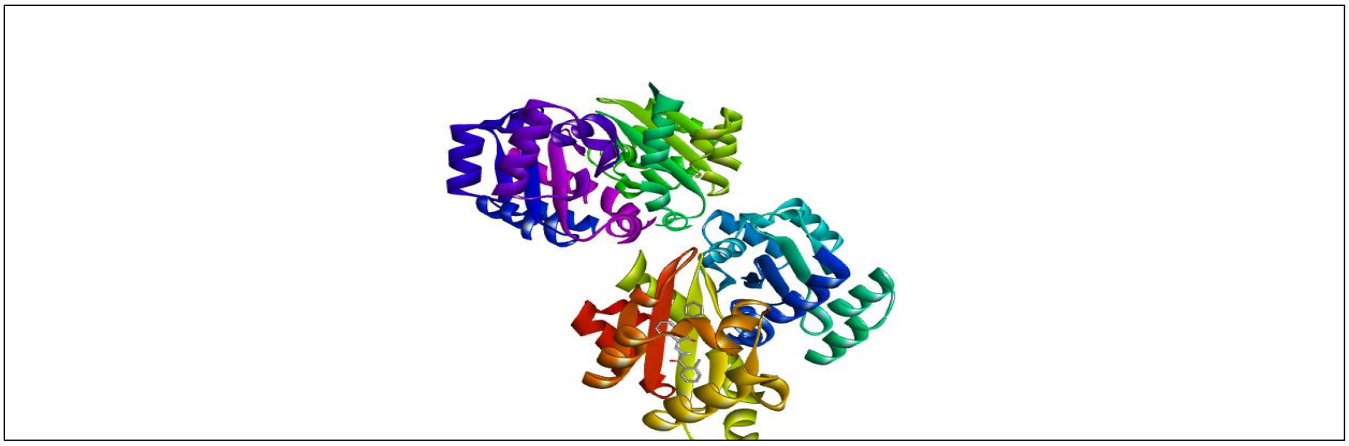

Figure 5. Compound 5i docked with the LasR protein, illustrating the binding pose and orientation of the compound within the active site.

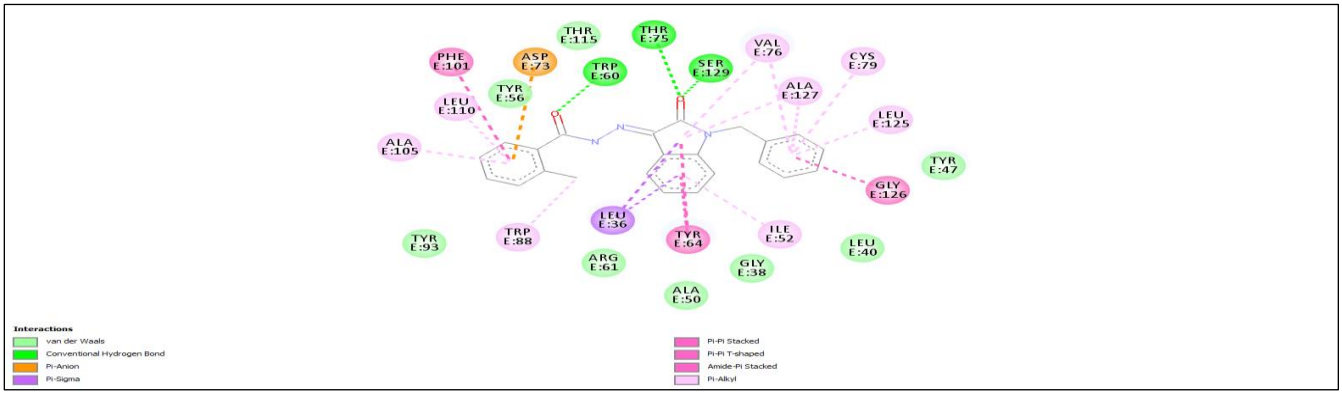

Figure 6. 2D binding interactions of compound 5h with the LasR protein, highlighting key residues involved in the interaction and the nature of the binding interactions.

CONCLUSION

The work effectively proved the presence of considerable biofilm inhibition of the isatin hydrazide derivatives (5a–5i) against the K pneumonia and the highest inhibition (84.19%) from the series was found for the 3-bromo compound 5b at 100 µg/100 µl concentration. Since the bromine substituent was present at the para position of the phenyl ring in compound 5b, it probably improves the compound's affinity to bacterial targets and promotes its potent biofilm inhibition. Compound 5i which is an unsubstituted benzene derivative, also showed high activity (86.10%) at the highest concentration of tested compounds, which underlines the significance of the aromatic ring as part of the chemical structure both substituted and unsubstituted. Tetra-substituted compounds also exhibited significant inhibition: 5g (3,4,5-trihydroxy) and the halo-derivative 5h (2-chloro), and this indicates that the electronic and steric effects of these groups are influential in the biofilm inhibition tendency of these compounds.

The anti QS activity of compound 5b was further established using Chromobacterium violaceum CV026, which exhibits concentration dependent inhibition of violacein formation. This indicates that compound 5b can interfere also with bacterial signaling processes besides biofilm development.

The molecular docking analysis also complemented the experimental data indicating quite reasonable binding energies of the efficient components with the selected bacterial targets. Subsequent docking study showed that the key interactions are hydrophobic one, thus the cations π-π and π-alkyl stacking between the indolinone moiety of the compounds and the bacterial proteins. The binding affinity of compound 5b was highest among all the compounds thus, affirming its experimental effectiveness.

In summary, the current study supports the isatin-based hydrazide derivatives as a novel lead for biofilm and quorum sensing inhibition, and further, compound 5b may be regarded as a lead compound to be developed. Overall, the experimental and computational design gives a qualitative and quantitative insight into the structural components that positively influence the antibacterial activity and hence direction to further improving these molecule types as efficient anti-biofilm working agents.

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Ethical Considerations: This study did not involve any human participants or animal subjects, and therefore, no ethical approval was required.

Consent to Participate: The concept of obtaining consent for participation does not apply to the scope of this study.

Consent for Publication: All the authors have diligently examined and provided their approval for the final version of the manuscript, endorsing its readiness for publication.

Authors' Contributions: All the authors have significantly contributed to the conceptualization and design of the research.

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