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Anti-quorum sensing and anti-biofilm activity of 5-hydroxymethylfurfural against *Pseudomonas aeruginosa* PAO1: Insights from *in vitro*, *in vivo* and *in silico* studies

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ABSTRACT

Pseudomonas aeruginosa is one of the most common pathogens associated with nosocomial infections and a great concern to immunocompromised individuals especially in the cases of cystic fibrosis, AIDS and burn wounds. The pathogenicity of *P. aeruginosa* is largely directed by the quorum sensing (QS) system. Hence, QS may be considered an important therapeutic target to combat *P. aeruginosa* infections. The anti-quorum sensing and anti-biofilm efficacy of aromatic aldehyde, 5-hydroxymethylfurfural (5-HMF) against *P. aeruginosa* PAO1 were assessed. At the sub-inhibitory concentration, 5-HMF suppressed the production of QS-controlled virulence phenotypes and biofilm formation in *P. aeruginosa*. It was also able to significantly enhance the survival rate of *C. elegans* infected with *P. aeruginosa*. The *in silico* studies revealed that 5-HMF could serve as a competitive inhibitor for the auto-inducer molecules as it exhibited a strong affinity for the regulatory proteins of the QS-circuits i.e. LasR and RhlR. In addition, a significant down-regulation in the expression of QS-related genes was observed suggesting the ability of 5-HMF in mitigating the pathogenicity of *P. aeruginosa*.

1. Introduction

Numerous pathogenic microorganisms are known to utilize the cell-density dependent bacterial communication system known as quorum sensing (QS) to regulate numerous virulence phenotypes, contributing to its pathogenicity. In *P. aeruginosa*, the QS system constitutes of two major intertwined circuits, LasI/LasR and RhlI/RhlR. The synthase protein, LasI and RhlR synthesize the autoinducers, 3-oxododecanoyl-L-homoserine lactone (3-oxo-C_{12}-AHL) and N-butanoyl homoserine lactone (C_4-HSL) which binds to their cognate transcriptional regulators, LasR and RhlR respectively. The LasR/3OC_{12}-HSL and RhlR/ C_4-HSL complexes, in turn, regulate the expression of genes related to virulence factor production (Kalia, 2013). QS also amplifies bacterial virulence by stimulating biofilm formation which leads to the development of antibiotic resistance. The biofilm forming ability of *P. aeruginosa* has been implicated for causing severe chronic infections in individuals with cystic fibrosis and burn wounds (Solano et al., 2014). Hence, targeting the QS circuit may serve as one of the most effective ways to counteract the infections caused by *P. aeruginosa*.

Numerous compounds have been extensively studied for their ability to interrupt and/or disrupt the bacterial QS system. Unlike classical antibiotics which target the basic cellular metabolic processes, the quorum sensing inhibitors (QSIs) are thought to hamper the bacterial communication without exerting selective pressure on the bacteria, thereby decreasing the chance for the appearance of MDR strains. It has also been proposed that a potential QSI should be highly specific for the QS regulator and efficiently reduce the QS-regulated gene expression without exerting a bactericidal effect on the pathogen or causing an adverse effect on the host cell (Kalia, 2013). Natural compounds are extensively being investigated for their ability to curtail the bacterial QS circuit and attenuate pathogenicity, without causing a harmful side-effect. 5-hydroxymethylfurfural (5-HMF, $C_3H_6O_3$) is an aromatic aldehyde naturally present in honey, dried fruits, wine, fruit juices, and coffee. Earlier reports have demonstrated the potential use of 5-HMF in the prevention or treatment of sickle-cell anemia and type I allergic reactions. It has also been reported to possess anti-oxidant, anti-

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ischemic, anti-apoptotic, anti-proliferative and hepato-protective properties (Zhao et al., 2013). Keeping in view its inherent pharmacological effects, the anti-QS and anti-biofilm property of 5-HMF was evaluated against P. aeruginosa PAO1. The influence of 5-HMF on the mortality of *C. elegans* infected with PAO1 and expression of QS-regulated genes were also examined. Further, the molecular target of 5-HMF was also identified by *in silico* analysis.

2. Materials and methods

2.1. Materials

Chromobacterium violaceum 2656 and wild type strain of *P. aeruginosa* PAO1 (MTCC 2453) were procured from MTCC, Chandigarh, India. The wild-type strain of *Caenorhabditis elegans* (Bristol N2) were provided by Caenorhabditis Genetics Center, USA. The primers were purchased from Eurofins Genomics Pvt Ltd., Bangalore.

2.2. Detection of anti-quorum sensing activity and violacein inhibition

To detect the anti-quorum sensing activity of 5-HMF, the agar well diffusion method was employed against reporter bacteria, *C. violaceum*. The stock solution of the sample was prepared using dimethyl sulfoxide (0.1% DMSO). Twenty microliters of 0.1% DMSO and 5-HMF (1 µL/20 µL and 2 µL/20 µL) were loaded onto the wells (8 mm diameter) made on LB agar plates, pre-inoculated with *C. violaceum*. The agar plates were observed for the zone of violacein inhibition after 24 h of incubation at 30 °C. Further, *C. violaceum* (1%) was treated with 1.25 µL/mL of 5-HMF for 24 h at 30 °C. The inhibition in violacein production was quantified spectrophotometrically at OD_{585} (Merghni et al., 2018).

2.3. Determination of the minimum inhibitory concentration (MIC)

According to CLSI guidelines (2015), the microbroth dilution method was employed to evaluate the effect of 5-HMF on the growth of PAO1. An overnight culture (1%) of the PAO1 was grown in different concentrations of 5-HMF ranging from 1 µL/mL to 2.5 µL/mL. The MIC and sub-MIC were determined by recording the absorbance (OD_{600}) following 24 h of incubation at 37 °C (Zhou et al., 2018). Further, PAO1 supplemented with 5-HMF (sub-MIC) was incubated at 37 °C with continuous agitation and the absorbance (OD_{600}) was recorded at every 2 h (Husain et al., 2013).

2.4. Effects of 5-HMF on the production of virulence factors in PAO1

P. aeruginosa PAO1 was cultivated with and without the sub-MIC (1.25 µL/mL) of 5-HMF for 24 h and centrifuged. The culture supernatant obtained was filtered using a 0.2 µm filter and employed for the subsequent assays. The supernatant (3 mL) was mixed with chloroform (5 mL) and the organic phase was collected and supplemented with HCl (1 mL, 0.2 M). The absorbance (OD_{520}) was recorded to estimate the pyocyanin concentration (Aybey and Demirkan, 2016). The cell pellet of an overnight culture of *S. aureus* was collected, diluted in 0.02 M Tris − HCl (pH8.5) and boiled for 10 min. The cell suspension (900 µL, OD_{600} = 0.8) was dispensed in 100 µL of the supernatant of PAO1. The LasA protease activity was measured by recording the decrease in OD_{600} (Aybey and Demirkan, 2016). The culture supernatant (150 µL) was mixed with 0.3% azocasein (500 µL) and incubated at 37 °C for 4 h. The LasA protease activity was determined at OD_{400}. The supernatant (100 µL) was supplemented with 900 µL of Elastin Congo Red (ECR) buffer containing 20 mg of ECR and the LasB elastase activity was measured at OD_{495} (Zhou et al., 2018). The supernatant (1 mL) was mixed with 0.5 mL of sodium citrate buffer (0.1 M) supplemented with chitin azure. Following 7 days of incubation at 37 °C, the chitinase activity was determined at OD_{570} (Husain et al., 2013).

2.5. Effects of 5-HMF on the biofilm formation of PAO1

P. aeruginosa PAO1 was inoculated into 200 µL of trypticase soya broth (TSB) and cultivated with and without the sub-MIC (1.25 µL/mL) of 5-HMF at 37 °C for 24 h, without agitation. The biofilm formed was stained with crystal violet (0.2%) and quantified at OD_{570} (Zhou et al., 2018). The culture supernatant of PAO1 treated and untreated with sub-MIC of 5-HMF was extracted using ethyl acetate. The residue obtained was mixed with orcinol reagent (900 µL, 0.19% orcinol) and the rhamnolipid concentration was determined at OD_{421} (Zhang et al., 2018). The culture supernatant of PAO1 (70 µL) was mixed with borate-sulfuric acid reagent (600 µL). Carbazole reagent (20 µL) was added into the samples and the alginate concentration was determined at OD_{530} (Heidari et al., 2017). The absorbance of overnight broth culture of PAO1 treated with and without 5-HMF was recorded at OD_{600}. The bacterial culture (1 mL) was supplemented with toluene (1 mL) and mixed vigorously for 2 min. The absorbance of the aqueous phase was recorded at OD_{600} to determine the cell surface hydrophobicity (CSH). The eDNA (extracellular DNA) from the 5-HMF treated and untreated culture of PAO1 was isolated from the culture supernatant and the concentration was estimated using a NanoDrop spectrophotometer (Chatterjee et al., 2017).

Congo red agar (CRA) composed of 0.8 g/L congo red dye supplemented with and without 5-HMF (1.25 µL/mL) was streaked with PAO1. Following incubation for 48 h at 37 °C, the color of the bacterial colonies were examined for exopolysaccharide (EPS) production (Rajkumari et al., 2018). The EPS produced by the 5-HMF treated and untreated culture of PAO1 was extracted using chilled ethanol (99%) and quantitatively estimated using the phenol-sulfuric acid method at OD_{490} (Chatterjee et al., 2017). Swarm agar plates constituting of 0.5% peptone, 0.2% yeast extract, 1% glucose and 0.5% bactoagar amended with and without 5-HMF ((1.25 µL/mL) were prepared. An overnight culture of PAO1 (2 µL) was spot inoculated in the middle of the semi-solid agar plates and the migration distance was measured after 48 h of incubation (Chatterjee et al., 2017). The biofilm was allowed to form in presence and absence of 5-HMF, rinsed with PBS (pH6.5) and air-dried. PBS (200 mL) supplemented with 50 µL of 2,3,5-triphenyl-tetrazolium chloride solution (TTC, 5 mg/mL) was added in each well and incubated in dark for 6 h (37 °C). The insoluble purple formazan formed was further dissolved in DMSO and the viability of cells was assessed spectrophotometrically at OD_{405} (Sabaeifard et al., 2014).

Coverslips were placed in 24-well microtiter plates containing TSB with PAO1 (1%) and supplemented with and without 5-HMF (1.25 µL/mL). Following incubation at 37 °C for 16 h, the coverslip was gently rinsed twice using PBS, stained using crystal violet (0.2%) and viewed under a light microscope. The biofilm was also stained with 0.01% acridine orange and the 3D-micrograph of the stained biofilm was captured using CLSM (Zhou et al., 2018).

2.6. Effect on the survival of C. elegans

The synchronized *C. elegans* (wild-type) were propagated on a lawn of *E. coli* OP50 grown on Nematode Growth Media (NGM) and incubated at 20 °C. BHI agar incorporated with or without the sub-MIC (1.25 µL/mL) of 5-HMF were seeded with 10 µL of PAO1 and incubated at 37 °C for 24 h. Nematodes (L4 stage) were washed with M9 buffer and 20 nematodes were placed onto the bacterial lawn and incubated at 25 °C. The nematodes were assessed for mortality in every 12 h interval (Sarabhai et al., 2013).

2.7. In silico studies

To identify the molecular targets, 5-HMF was docked to transcriptional regulators of QS circuit, LasR and RhlR. The 3D structure of *P. aeruginosa* regulatory protein, LasR (PDB ID:2UV0) was downloaded from Protein Data Bank while the modeled structure of RhlR was taken

from our data published earlier (Rajkumari et al., 2018). The 3D-crystal structures of 5-HMF and the natural ligands (3-oxo-C_{12}HSL and C_4-HSL) were docked to the binding site of LasR and RhlR using Auto Dock Vina. The number of hydrogen bonds and hydrophobic interactions between the receptor-ligand complexes were determined using LigPlot+.

The best-docked conformations for the ligands were identified based on the docking score and the complexes were subjected to MD simulations using the Gromacs 5.0.4 conjugated with Gromos force field. MM/PBSA analysis was employed to estimate the post-simulation binding free-energy of the protein-ligand complexes and to compute their interaction energies. The MD trajectories were analyzed using the g_mmpbsa tool and Gibb's free energy was estimated (Rajkumari et al., 2018).

2.8. Effects of 5-HMF on the expression of QS genes

The total RNA from 350 µL of the broth culture of PAO1 treated with and without 5-HMF (1.25 µL/mL) was extracted using Trizol reagent (700 µL, Sigma-Aldrich (USA). The cDNA from 0.2 µg of RNA was synthesized using the first strand cDNA Synthesis Kit (Thermo Scientific) and subjected to quantitative real-time polymerase chain reaction (qRT-PCR). The amplification was carried out using SYBR Green Real-time PCR Master Mix (Thermo Scientific) in Roche Light Cycler 480 system with primers listed in Table 1. The housekeeping gene, *proC* was employed as a reference gene for normalizing gene expression, and the differential gene expression of the QS-associated genes was determined using the $2^{-\triangle\triangle Ct}$ method (Zhang et al., 2018).

3. Results

3.1. Detection of anti-quorum sensing activity and violacein inhibition

The appearance of a non-pigmented zone of *C. violaceum* around the well loaded with 5-HMF (2 µL/20 µL) indicated the potential anti-QS activity of 5-HMF (Fig. 1a). In contrast, the well loaded with DMSO was devoid of a zone. At a concentration of 1.25 µL/mL, 5-HMF noticeably decreased the violacein production in *C. violaceum* to 98.12% (Fig. S1).

3.2. Determination of sub-MIC and effect on cell growth

The MIC and sub-MIC of 5-HMF for *P. aeruginosa* PAO1 were determined as 2.5 µL/mL and 1.25 µL/mL, respectively. As depicted in the growth curve (Fig. 1b), the sub-MIC did not cause any inhibitory effect on the growth profile of PAO1 with respect to control. Hence, the sub-MIC was employed to analyze the effect of 5-HMF towards the virulence factors and biofilm formation of PAO1.

3.3. Effects of 5-HMF on the production of virulence factors in PAO1

On treatment with sub-MIC of 5-HMF, a significant suppression in the production of QS-controlled virulence factors in *P. aeruginosa* was observed as evident in Fig. 2a. The production of pyocyanin pigment was inhibited up to 65.34% when grown in the presence of 5-HMF. 5-HMF also reduced the LasA staphylolytic activity by 77.92%. The total protease activity was decreased by 84.49% and a reduction of 36.48% in LasB elastase activity was estimated. The chitinase activity was also decreased by 57.71% on treatment with 5-HMF.

3.4. Effects of 5-HMF on the biofilm formation of PAO1

The sub-MIC of 5-HMF significantly arrest the biofilm development in PAO1, resulting in the biofilm inhibition by 29.80%. A notable inhibition in the production of rhamnolipid and alginate by 31.80% and 55.43% respectively was also observed (Fig. 2b). Furthermore, 5-HMF also decreased the cell surface hydrophobicity and production of eDNA by 16.01% and 17.57% respectively as shown in Fig. 2b.

The colonies of PAO1 developed on CRA plates amended with 5-HMF showed red color colonies indicating restricted EPS production. On the other hand, the untreated control exhibited black colonies (Fig. 3a). A significant arrest in the motility of PAO1 by 30.90% was also observed in the semi-solid agar plates supplemented with sub-MIC of 5-HMF (Fig. 3b). A significant decrease in the EPS production up to 37.16% was found in PAO1 when exposed to the sub-MIC of 5-HMF. However, the 5-HMF-treated PAO1 exhibited only a slight reduction in cell viability by 4.03% as presented in Fig. 3b.

As displayed in Fig. 4a, a poorly developed biofilm of PAO1 with disrupted biofilm architecture was observed on exposure to 5-HMF in the light microscope images. In contrast, the untreated sample exhibited well-developed and compact biofilm growth. Similarly, in the CLSM images, the 5-HMF-treated biofilm exhibited scattered appearance with relatively reduced thickness. On exposure to 5-HMF, the biofilm thickness was decreased from 18 µm (control) to nearly 5 µm (Fig. 4a).

3.5. Effect on the survival of C. elegans

As observed in the Kaplan-Meier survival curves (Fig. 4b), the *C. elegans* exposed to PAO1 were rapidly killed within 60 h of incubation. On treatment with 5-HMF, the PAO1 infected *C. elegans* displayed significant improvement in mortality by 78.33%, indicating the protective effects of 5-HMF towards the killing of *C. elegans* by PAO1. However, the nematodes infected with PAO1 showed the survival of 26.66%.

Table 1
Primers used for qRT-PCR.

Gene	Direction	Sequence (5'-3')	Melting temperature (Tm in °C)	Product size (bp)	Annealing temperature (Ta in °C)
proC (Reference gene)	Forward	CAGGCCGGGCAGTTGCTGTC	68	180	
	Reverse	GGTCAGGCGCGAGGCTGTCT	59		
lasI	Forward	GGCTGGGACGTTAGTGTCAT	60	104	59
	Reverse	AAAACCTGGGCTTCAGGAGT	59		
lasR	Forward	ACGCTCAAGTGGAAAATTGG	56	111	56
	Reverse	TCGTAGTCCTGGCTGTCCTT	61		
rhlI	Forward	AAGGACGTCTTCGCCTACCT	61	130	59
	Reverse	GCAGGCTGGACCAGAATATC	58		
rhlR	Forward	CATCCGATGCTGATGTCCAACC	61	101	51
	Reverse	ATGATGGCGATTTCCCCGGAAC	63		
mvfR	Forward	AACCTGGAAATCGACCTGTG	58	238	59
	Reverse	TGAAATCGTCGAGCAGTACG	57		
lasB	Forward	GACCGAGAATGACAAAGTGGAA	57	80	51
	Reverse	GGTAGGAGACGTTGTAGACCAGTTG	62		
rhlA	Forward	TGGCCGAACATTTCAACGT	58	107	51
	Reverse	GATTTCCACCTCGTCGTCCTT	60		

The data were acquired from three independent experiments and the results are presented as mean ± standard error.

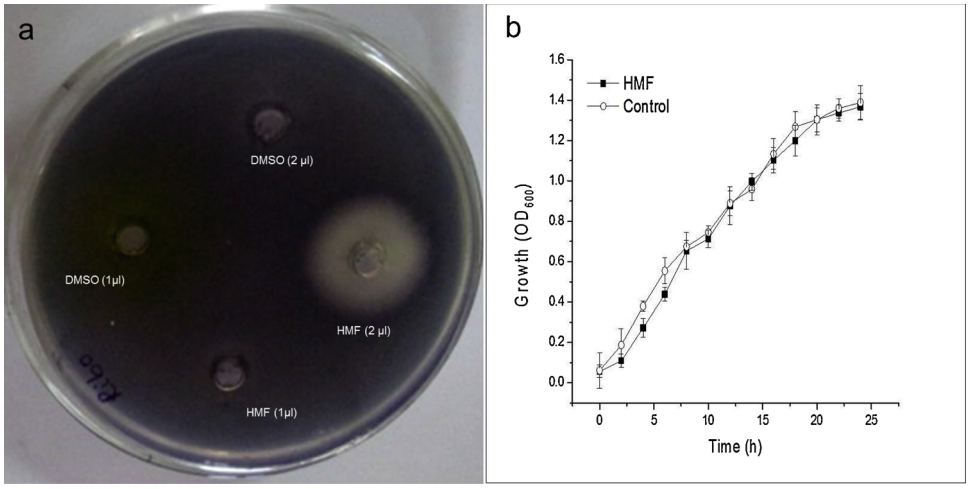

Fig. 1. a) Inhibitory effect of 5-HMF on the violacein production of *C. violaceum* as compared to control (0.1% DMSO) indicated by the zone of inhibition, b) Effect of 5-HMF (sub-MIC) on the growth of PAO1 as compared with untreated control.

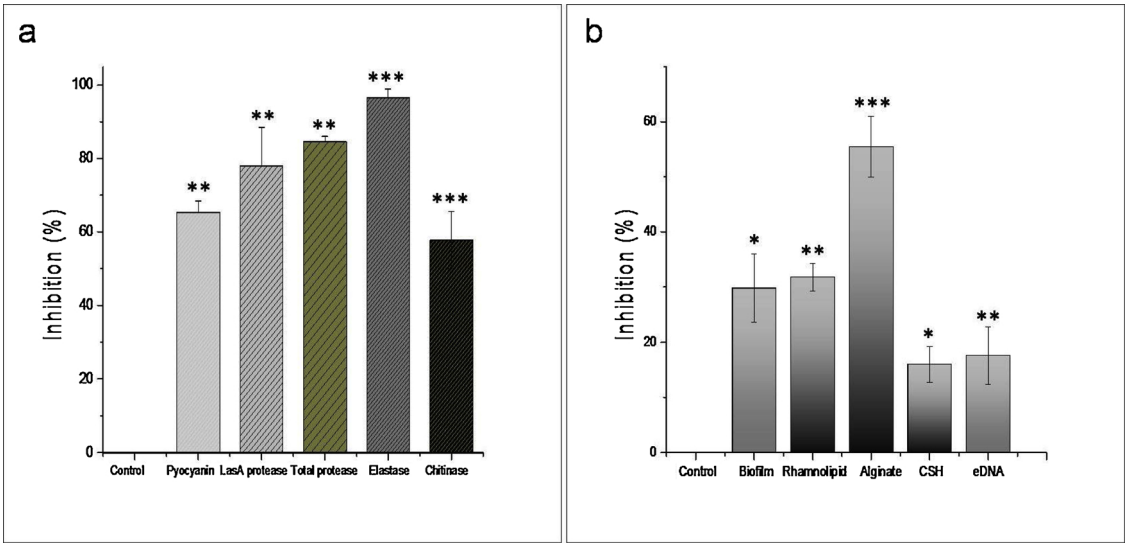

Fig. 2. a) Inhibitory effect of 5-HMF on the production of QS-controlled virulence determinants of PAO1, b) Inhibitory effect of 5-HMF on the biofilm-associated traits of PAO1. The statistical significance (*, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$) relative to untreated control.

3.6. In silico studies

The natural ligands, 3-oxo-C_{12}HSL and C_4HSL showed a strong binding affinity towards their cognate receptors, LasR and RhlR exhibiting a docking score of -8.8 kcal/mol and -5.4 kcal/mol, respectively. As shown in Table 2,3-oxo-C_{12}HSL formed four hydrogen bonds (H-bonds) with Tyr56 (2.8 Å, 3.1 Å), Asp73 (2.6 Å) and Ser129 (2.5 Å) within the catalytic site of LasR. Likewise, RhlR made one H-bond with the residues Ser135 (2.99 Å) in the catalytic site of RhlR (Fig. 5a).

Molecular docking studies showed 5-HMF with a binding affinity of -5.7 kcal/mol for the catalytic site of LasR. As illustrated in Fig. 5a, it establishes one H-bond by the aid of active site residue Ser129 (2.75 Å). It also showed six hydrophobic interactions with residues, Tyr56, Asp73 and Trp88 (Table 2, Fig. 5a). A binding score of -4.7 kcal/mol with two H-bonds with the Ser135 (2.94 Å & 2.93 Å) was exhibited by 5-HMF. The seven hydrophobic interactions were attained with five active site residues, Ala44, Asp81, Ala83, Ile84 and Trp96 (Fig. 5a, Table 2).

The RMSD value of the biomolecular complexes and the natural ligand were found within the range of 2 Å indicating accurate docking. LasR-3-oxo-C_{12}HSL and LasR-HMF complexes showed stability in RMSD after ˜10 ns and maximum RMSD of ˜0.25 nm. In the case of RhlR, 5-HMF showed stability after ˜12 ns with a maximum RMSD of ˜0.6 nm (Fig. 5b). The MD trajectories were used to measure the binding free energy presented in Table 3. LasR-HMF complex exhibited binding free energy of -70.2 kJ/mol while that of RhlR-HMF complex exhibited -58.3 kJ/mol. Both docking and post simulation binding free energy analysis revealed that 5-HMF binds more strongly with LasR as compared to RhlR.

3.7. Effects of 5-HMF on the expression of QS genes

The QS-controlled genes accountable for the expression of virulence factors in PAO1 showed significant down-regulation on treatment sub-MIC of 5-HMF, as shown in Fig. 6. The expression of *lasI* and *lasR* were down-regulated by 92.79% and 90.15% respectively. Similarly, 5-HMF suppressed the relative expression of *rhlI* and *rhlR* in PAO1 by 83.44% and 74.29% respectively. A profound influence on *lasB* and *rhlA* with inhibition in expression by 59.66% and 66.20% were also observed on exposure to 5-HMF. Additionally, the expression of *mvfR* was down-regulated by 99.94% relative to control.

4. Discussion

P. aeruginosa is one of the well-studied pathogen known to cause

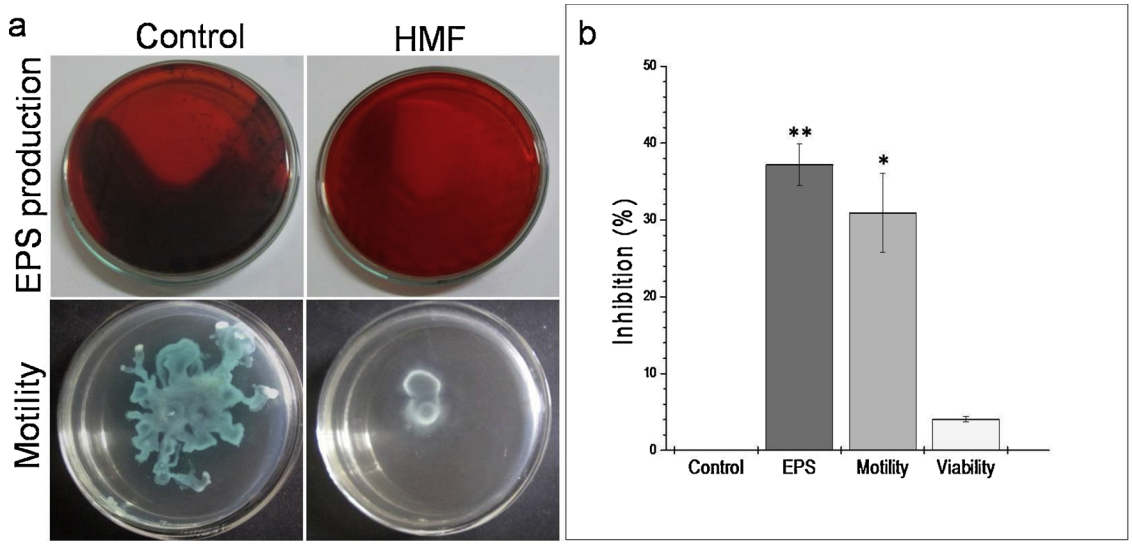

Fig. 3. a) Inhibitory effect of 5-HMF on the EPS production and swarming motility of PAO1 observed on agar plates relative to control, b) Bar diagram showing the effect of 5-HMF on the EPS production, motility and viability of *P. aeruginosa* PAO1. The statistical significance (*, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$) relative to untreated control.

chronic and recurring infections, regulated by the QS circuit (Sharma et al., 2014). The suppression of QS-mediated violacein production in the biosensor organism, *C. violaceum* in the presence of 5-HMF gave the preliminary indication on the anti-QS property of the test compound. In a similar report, Khan et al. (2009) observed a significant arrest in the production of violacein pigment in *C. violaceum* in presence of cinnamon, peppermint and lavender oil (Khan et al., 2009). In a study performed by Packiavathy et al. (2014), curcumin at a concentration of 100 µg/mL exhibited 89% reduction in the production of violacein (Packiavathy et al., 2014). The anti-QS and anti-biofilm potential of 5-HMF against gram-positive pathogens, Streptococcus pyogenes, Streptococcus mutans, Staphylococcus aureus and Staphylococcus epidermidis were recently documented (Vijayakumar and Ramanathan, 2018).

Considering the global situation of the ever-increasing MRD stains, it is therefore, desirable to develop QSIs without bactericidal or bacteriostatic effect towards the test pathogen (Hoiby et al., 2010). According to current findings, the sub-MIC of 5-HMF was determined as 1.25 µL/mL as it was found to be the highest concentration which did not exert bactericidal effect towards PAO1. A similar observation was reported by Packiavathy et al. (2014) wherein at the sub-MIC, curcumin showed no considerable difference in the cell density of *P. aeruginosa* relative to the untreated control (Packiavathy et al., 2014). The pathogenicity in *P. aeruginosa* is governed largely by its secreted virulence attributes which facilitate its survival, colonization, and invasion (Chatterjee et al., 2016). Apart from pyocyanin which produces reactive oxygen species (ROS), the elastase and protease also play a vital part in damaging and colonization of the host tissues (Sarabhai et al., 2013). The production of pyocyanin, proteases, elastase and chitinase were also substantially affected by 5-HMF. Consistent with the present data, Zhou et al. (2013) also documented a notable reduction in the production of pyocyanin pigment in PAO1 up to 56% on treatment with 50µM of eugenol (Zhou et al., 2013). As observed in the present study, a considerable decrease in LasA protease and LasB elastase activity by 39.04% and 37.54% respectively were recorded on the administration of 110 µg/mL of the polyphenolic flavones, vitexin (Das et al., 2016). According to Zhou et al. (2013), the elastase production in PAO1 was decreased up to 32% by 200µM of by eugenol (Zhou et al., 2013). As

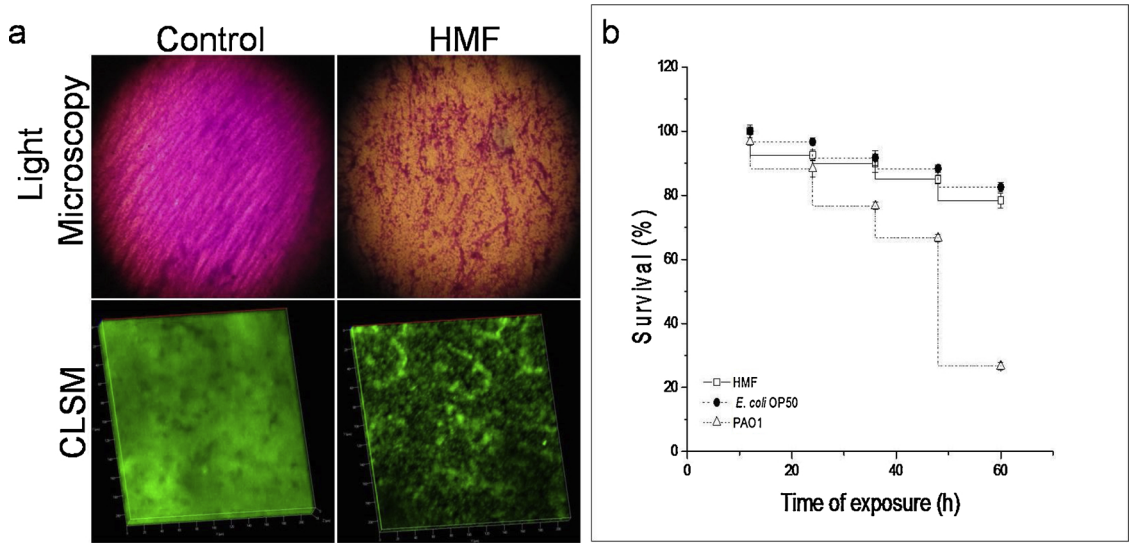

Fig. 4. a) Light and CLSM micrographs of PAO1 biofilm grown in the presence and absence of 5-HMF, b) Kaplan-Meier survival curve showing the effects of 5-HMF on the survival of *C. elegans* infected with PAO1.

Table 2
The binding energies of the ligands with LasR and RhlR tabulated along with their interacting hydrogen.

Ligand	Receptor	Binding affinity (kcal mol^{-1})	Interacting residues (H-bond length in Å)
Natural ligand: 3-oxo-C_{12}HSL	LasR	−8.8	**Tyr 56 (2.8, 3.1), Asp 73 (2.6), Ser 129 (2.5)**
Natural ligand: C_4-HSL	RhlR	−5.4	**Ser 135 (2.99)**
HMF	LasR	−5.7	Tyr 56*, Asp 73*, Thr 75, Tyr 93, Trp 88, Leu 110, **Ser 129* (2.75).**
	RhlR	−4.7	Ala 44*, Gly 46, Asp 81*, Ala 83*, Ile 84*, Trp 96*,Val 133, **Ser 135* (2.94, 2.93)**

* indicates active site residues.

compared to our earlier report (Rajkumari et al., 2017), 5-HMF was found to be a more potent antagonist in the production of pyocyanin, protease, and chitinase in *P. aeruginosa* relative to the positive control, Baicalein. Hence, the results revealed the ability of 5-HMF in attenuating the production of QS-associated virulence factors in PAO1 without affecting its viability.

P. aeruginosa form biofilm on living as well as non-living surfaces like the mucosal lining of lungs, surgical implants, medical devices and contact lenses (Sharma et al., 2014). Inside the biofilm matrix, the bacterial consortium coalesces together by the aid of polymeric matrix constituting of polysaccharides, proteins, and eDNA produced by the pathogen. This matrix provides a protective advantage and often associated with chronic infections such as cystic fibrosis and keratitis (Hoiby et al., 2010). The sub-MIC of 5-HMF effectively inhibited the QS-mediated attributes contributing to the biofilm development in PAO1. The present data also corroborates with the findings of Zhou et al. (2013) wherein at a concentration of 400μM eugenol significantly reduced the biofilm formation in *P. aeruginosa* by 43% (Zhou et al., 2013). The amphipathic glycolipid, rhamnolipid facilitates bacterial motility, increases the CSH and maintains open channels thereby inducing the formation of micro-colonies (Sharma et al., 2014). The polysaccharide, alginate contributes to the adherence, hydration, protection and rigidity of the biofilm matrix (Chatterjee et al., 2016). At sub-MIC, 5-HMF considerably inhibited the production of rhamnolipid and alginate in PAO1 relative to the untreated control. In previous studies by Kim et al. (2015) a decrease in rhamnolipid production by 60% in *P. aeruginosa* was observed on treatment with 100 μM of 6-gingerol (Kim et al., 2015). Kalia et al. (2015) also reported a 54% reduction in the alginate production in presence of 0.2 μL/mL of cinnamon oil (Kalia et al., 2015).

The development of biofilm initiates with the reversible adherence of the planktonic bacteria to either a living or inert surface. This process of attachment is greatly influenced by the CSH of the bacterial cells. Hence, bacteria usually colonize non-polar or hydrophobic surfaces like plastics, relative to the hydrophilic surface like glass (Jamal et al., 2018). Another key component contributing to the initial stages of biofilm development is the eDNA, generated by autolysis of bacteria. It plays a vital part in holding the cells together making them less susceptible to antibiotics and also imparts stability to the biofilm architecture. The reduction in the CSH and eDNA production in PAO1 on treatment with 5-HMF indicated the influence of the test compound on the initiation of biofilm. Likewise, She et al. (2018) also observed inhibition in the production of eDNA on treatment with Meloxicam at the sub-lethal concentration (She et al., 2018). The EPS produced by PAO1 represents the major and most important component of the biofilm. It holds the cells together providing strength and structure to the biofilm matrix (Sharma et al., 2014). In addition, the swarming motility also aids in the initial adherence and colonization of the substratum (Solano et al., 2014). This study evidenced the interference of 5-HMF on the EPS production and motility of PAO1. These present findings were consistent with the earlier observation of Husain et al. (2013) wherein a significant reduction in the EPS production of *P. aeruginosa* by 31% was demonstrated with clove oil, at a concentration of 0.2 (v/v) (Husain et al., 2013).

It is opined that QSIs pose negligible or less selective pressure on the bacteria as they do not directly target their metabolic pathway. Therefore, there exists less possibility for the emergence of resistance towards QSIs unlike conventional antibiotics (Kalia, 2013). The findings of the TTC assay was in a good agreement with the growth curve analysis, suggesting that 5-HMF (sub-MIC) exhibits a profound antibiofilm efficacy without affecting the viability of PAO1. Such findings are may lead to the use of optimal doses of drugs, for safe and efficient treatments. The reduction in the biofilm in presence of 5-HMF was further confirmed using a light microscope and CLSM. The biofilm treated with 5-HMF displayed diffused and thinner biofilm as compared to control.

The susceptibility to various virulence determinants of *P. aeruginosa* makes *C. elegans* a suitable model to investigate the *in vivo* pathogenicity of PAO1. The pyocyanin pigment causes lethal paralysis in *C. elegans*, eventually leading to the death of the nematodes. Besides, the motility of PAO1 also aids in colonization and dissemination of the pathogen in the nematode gut (Luo et al., 2017). Interestingly, 5-HMF treated nematodes infected with PAO1 displayed a profound decrease in mortality compared to untreated control. The investigation revealed that 5-HMF could suppress the QS-regulated pathogenicity in PAO1, leading to significant improvement in the survival of *C. elegans* during *P. aeruginosa* infection. These results were consistent with the reports of Musthafa et al. (2012), wherein the PAO1-preinfected *C. elegans*, on further exposure to phenylacetic acid displayed enhanced survival rate up to 53% in comparison with control (Musthafa et al., 2012). The finding indicated the non-toxic and therapeutic potential of 5-HMF in attenuating the virulence of PAO1 *in vivo*.

The LasI/R and RhlI/R system along with their corresponding autoinducers, 3-oxo-C_{12}-HSL and C_4-HSL respectively, regulates the QS system in *P aeruginosa*. In the LasI/R circuit, the *lasI* gene regulates the production of 3-oxo-C_{12}-HSL, which binds with the cognate receptor, LasI. This interaction subsequently governs numerous virulence attributes like LasB elastase, protease, and LasI itself. Similarly, in the Rhl system, the *rhlI* regulates the synthesis of C_4-AHL, which interacts with RhlR and regulates the expression of *lasA*, *lasB*, pyocyanin and rhamnolipid (Chatterjee et al., 2016). The *in silico* analysis revealed that 5-HMF acted as a strong antagonist for QS-receptors, LasR and RhlR

Table 3
MM/PBSA analysis results: The free energy of binding and their components (kJ mol^{-1}) of LasR and RhlR complexes with 5-HMF after MD simulation.

Ligand	Binding Energy (kJ mol^{-1})	Van der Waal Energy (kJ mol^{-1})	Electrostatic Energy (kJ mol^{-1})	Polar Solvation Energy (kJ mol^{-1})	SASA Energy (kJ mol^{-1})
HMF	LasR				
	−70.19 ± 9.13	−91.21 ± 8.03	−12.10 ± 5.77	42.22 ± 6.71	−9.10 ± 0.56
	RhlR				
	–58.30 ± 8.30	–89.85 ± 6.18	–7.02 ± 3.91	48.05 ± 5.35	–9.48 ± 0.54

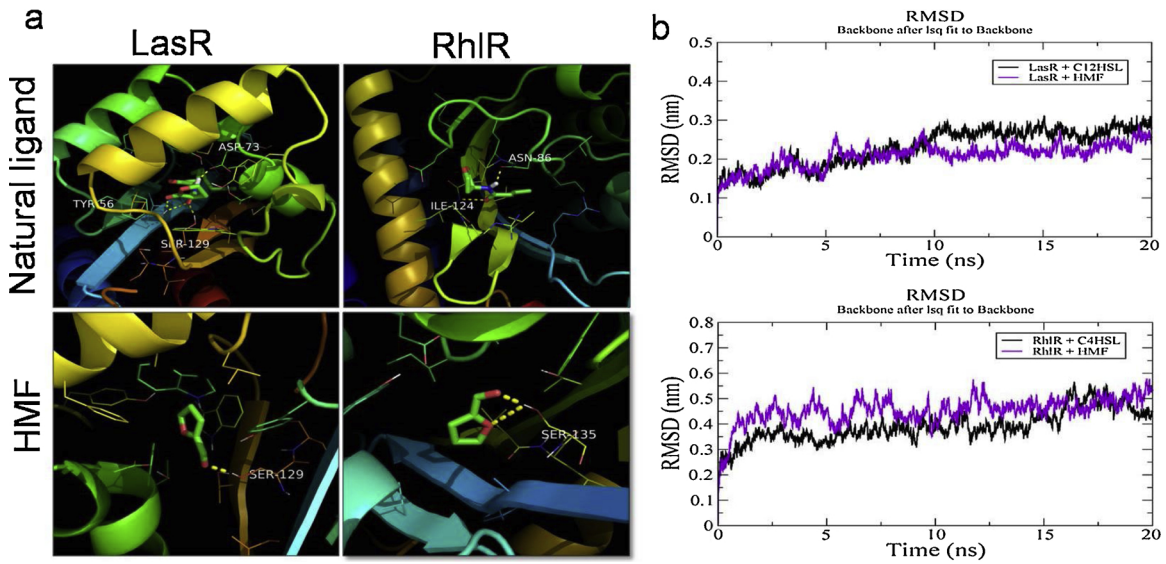

Fig. 5. (a) The top panel shows the interactions of natural ligands, 3-oxo-C_{12}HSL and C_4HSL with LasR and RhlR respectively and the panel below shows the interactions of 5-HMF with LasR and RhlR respectively. The yellows dotted lines represent H-bonds, (b) Comparative RMSD (backbone) with time for natural ligands (3-oxo-C_{12}HSL and C_4HSL) and 5-HMF complexes with LasR and RhlR, respectively.

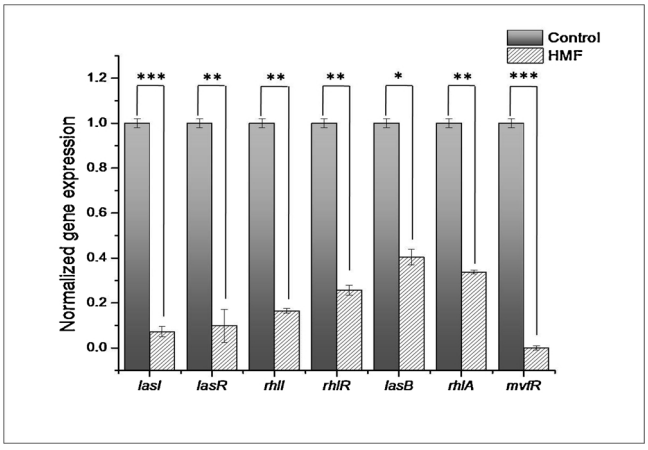

Fig. 6. Inhibitory effect of 5-HMF on the relative expression level of QS-related genes of PAO1 determined using real-time RT-PCR. The housekeeping gene *proC* was used as a reference to normalize the gene expression. The statistical significance (*, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$) relative to untreated control.

relative to their cognate ligands. However, a higher binding affinity of 5-HMF towards LasR was observed in comparison to RhlR. The presence of identical H-bonding and hydrophobic residues in the HMF-receptor complexes to that of the natural ligands suggested the competitive binding of 5-HMF to the receptor binding sites of the QS regulators. In accordance to the present findings, the molecular docking analysis of LasR receptor protein to quercetin showed a rigid binding with the LasR receptor protein with higher docking score when compared with the natural ligand (Gopu et al., 2015).

Further, 5-HMF considerably repressed the expression of auto-inducer synthase (*lasI*, *rhlI*) as well as their receptors (*lasR* and *rhlR*). The gene expression studies also showed the down-regulation of *lasB and rhlA* however, the highest suppression was observed in the transcription of *mvfR* which controls the expression of pyocyanin, rhamnolipid and lectins (Chatterjee et al., 2017). The result obtained further substantiated the *in silico* data wherein it was evidenced that 5-HMF preferentially targeted the LasI/R receptor and down-regulated the *lasI* and *lasR* genes. 5-HMF also downregulated *rhlI, rhlR, lasB* and *rhlA* but comparatively lesser than *lasI* and *lasR*. In the recent findings of She et al. (2018) a significant arrest in the expression of *lasR*, *rhlR* and *mvfR* by 59%, 51% and 88% respectively was attained in presence of Meloxicam (She et al., 2018). The results obtained were also similar to the report of Zhang et al. (2018) wherein the expression of *lasB, lasI*, *lasR, rhlA*, *rhlI* and *rhlR* were downregulated by 58.9%, 16.1%, 24.2%, 31.7%, 23.4% and 36.2%, respectively in presence of 300 μM of equisetin (Zhang et al., 2018). Thus, the binding of 5-HMF to LasR and RhlR receptors and reduced expression of QS-genes might have attenuated the expression of virulence traits resulting in decreased pathogenicity in PAO1.

5. Conclusion

It may be inferred that 5-HMF significantly attenuated the QS-controlled virulence phenotypes and biofilm development in PAO1 without restricting its growth. It also exhibited a competitive inhibition of the QS-regulators and consequently down-regulated the expression of the target genes associated with the QS-circuit. Moreover, 5-HMF significantly improved the survival rate of *C. elegans* infected with PAO1 by attenuating its pathogenicity. Hence, it could serve as a promising tool in the management of infections caused by *P. aeruginosa*.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.05.001.

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