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PARTIAL PURIFICATION AND QUORUM SENSING INHIBITION OF *SYZYGIUM AROMATICUM* ON *PSEUDOMONAS AERUGINOSA* – AN *IN* *VITRO* AND *IN SILICO* APPROACH

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ABSTRACT

Syzygium aromaticum, a popular ethnomedicinal herb, possesses quorum quenching and inhibition of virulence factors of *Pseudomonas aeruginosa*. The objective of the present study was to partially purify and study quorum sensing inhibition of *S. aromaticum* methanolic extract using *in vitro* and *in silico* docking studies. *S. aromaticum* was extracted with methanol using Soxhlet apparatus and was subjected to TLC-bioautography using *Chromobacterium violaceum* as reporter strain. The TLC purified fraction was subjected to biofilm inhibition on *P. aeruginosa* and GC-MS analysis. The identified compounds from GC-MS were docked with LasI and EsaI proteins. TLC-bioautography analysis showed clear zone of inhibition at the bioactive fraction ($R_f = 0.78$) and also exhibited dose dependent (50-200 $\mu\text{g/mL}$) biofilm inhibition on *P. aeruginosa*. GC-MS analysis of active fraction revealed six major compounds viz. 4-hydroxy-3-methoxy-benzaldehyde, 1-phenoxy ethanol, eugenol, chavicol, p-hydroxyacetophenone and 4-azido-phenol that may account for the antibiofilm activity. These compounds were studied for their *in silico* binding patterns with quorum sensing regulated proteins, LasI and EsaI. Among all, chavicol formed three hydrogen bonds with LasI (-6.20 kcal/mol) and three hydrogen bonds with EsaI (-4.36 kcal/mol), indicating best interaction with targeted protein binding sites. The current study demonstrated that the

bioactive fraction has the ability of quorum quenching which is supported by *in silico* analysis and might leads to *in vivo* establishment in future.

Keywords: *Syzygium aromaticum*, *in silico*, TLC-bioautography, LasI, EsaI

INTRODUCTION

In the infectious diseases, at least 65% are linked to the bacterial communities which proliferate by forming biofilms [1]. Bacterial biofilm is the matrix of extracellular polymeric substance which serves a protective layer by reducing antibiotic efficiency and the host immune response. Bacterial behaviour within biofilms is regulated by the phenomenon called quorum sensing (QS). QS, a population density dependent mechanism present in many bacteria, is mediated through small signal molecules called auto inducers that regulate the target gene expression which are responsible for the phenotypes essential to pathogenicity/symbiosis [2]. Among all the QS bacterial systems, *Pseudomonas aeruginosa* is the most intensively studied because of its fatal infections leading to the death of several thousand annually [3]. *P. aeruginosa* has two *N*-acyl-homoserine lactone (AHL) mediated (LasR/I and RhII/R) and one alkylquinolone mediated (2-alkyl-4-(1H)-quinolone) QS systems of which AHL-mediated system was well studied [4, 5]. In AHL mediated QS, LasI produces the N-3-oxododecanyol-L-homoserine lactone (3-oxo-C12 HSL),

activates regulator LasR and modulate the expressions of RhI which encode elastase, a virulence factor. RhII synthesize the N-butanoyl-L-homoserine lactone (C4-HSL), which regulates rhII and phz operon leading to the synthesis of a virulence factor, pyocyanin [6-8]. In this scenario, the QS have become the therapeutic target to attenuate bacterial virulence and thus control infection by degrading QS signals.

Syzygium aromaticum is an aromatic herb, commonly called clove, which grows in hot tropical climate [9]. Clove is used in indigestion, generalized stress, parasitic infestations, cough, toothaches, headache and blood impurities. It also acts as antiviral, antimicrobial, antiseptic and antifungal agent. According to the previous literature, *S. aromaticum* possesses inhibition of AHL short chain and virulence factors in *P. aeruginosa* [10-12].

In our previous study, we demonstrated the efficacy of quorum sensing inhibition (QSI), biofilm inhibition and virulence factors inhibition of *S. aromaticum* [13]. In our continuous research, we aimed to partial purification, antiquorum and GC-MS analysis of *S. aromaticum*. Furthermore, *in silico* docking studies were performed on

GC-MS analysed compounds in order to investigate the specific binding sites of the compounds with LasI and EsaI.

MATERIALS AND METHODS

Collection and extraction of *S. aromaticum*

S. aromaticum (buds) collected from Bengaluru, Karnataka, India, were cleaned, dried and powdered. The powdered sample was extracted with methanol at 1:10 (w/v) concentrations using Soxhlet apparatus. The extract was filtered through Whatman No.1 filter paper and the filtrate was concentrated by rotary evaporator for further use.

Strains and culture condition

Chromobacterium violaceum 12742 was procured from MTCC, Chandigarh and clinically isolated *P. aeruginosa* was collected from Bhagawan Mahaveer Jain Hospital, Bengaluru. The cultures were maintained in the Luria Bertani (LB) medium at regular intervals for further studies.

TLC and bioautographic analysis on *C. violaceum*

Methanolic extract of *S. aromaticum* was subjected to TLC. About 15 µl of extract was applied to the precoated aluminum silica gel 60 F, Merck F 254. Developing solvent system used was toluene: acetone: chloroform (40:35:25). The separated spots were visualized under UV illuminator at 346 nm [14].

For bioautographic analysis, developed TLC plates were dried and placed on the *C. violaceum* spreader agar plate. The plates were incubated for one hour to diffuse the TLC separated fraction into the agar media. After incubation, the TLC plate was removed carefully by aseptic forceps and the agar plate was incubated at 37°C. *C. violaceum* growth appeared as clear zone against purple back ground at bioactive fraction and the R_f value was determined [15].

Effect of bioactive fraction on biofilm formation of *P. aeruginosa*

The effect of *S. aromaticum* bioactive fraction on the biofilm formation was determined by quantifying the biomass through microtitre plate assay [12]. The clinical pathogen was incubated in 96 well plates in the presence (50, 100, 150 and 200 µg/mL) and absence of extracts for 16 h without agitation. The free-floating planktonic cell were removed, washed with sterile water and stained with 0.2% crystal violet solution for 15 min. The excess crystal violet was discarded, wells were filled with 95% ethanol and absorbance was measured at 650 nm using UV-visible spectrophotometer.

Attenuated total reflectance infra-red (ATR-IR) analysis

The bioactive fraction was subjected to ATR-IR (Bruker Alpha ECO-ATR spectrometer) analysis for the functional groups. 10 μ l sample was placed directly on the germanium piece of the IR spectrometer with constant pressure and wave number ranged from 4000 cm^{-1} to 675 cm^{-1} . The characteristic peaks were determined according to the presence of functional groups [16].

GC-MS analysis and identification of compounds

GC-MS analysis of the bioactive fraction was performed with Thermo GC –Trace ultra Ver: 5.0, thermo MS DSQ II and equipped with column DB 5 -MS capillary standard non –polar (35 Mts x 0.25 mm x 0.25 μ m). The analysed patterns were interpreted by the National Institute Standard and Technology (NIST) database [17].

In silicodocking studies

In order to understand the binding sites of GC-MS analysed compounds, docking studies were performed with crystal structures of PDB ID: 1R05 (LasI) and PDB ID: 1KZF (EsaI). Chemspider was employed for drawing, displaying and characterizing the chemical as well as the 3D structures which are required for docking. All the protein molecules were pre-processed computationally by removing water molecules from the crystal structure

and minimised using the MGL toolkit scripts. Theoretically published active sites for each protein molecule were identified from knowledge base. The Docking grid coordinates were plotted around 4Å° radius of active pocket on protein molecule. Protein-compound docking simulation was performed using Auto Dock 4.1[18] and visualization by discovery studio. ADME molecular properties and bioactivity scores of the drug targets were calculated using Molinspiration according to Lipinski's rule for all analysed ligands [19].

Statistical analysis

All experiments were performed in triplicate. Data of each experiment showed the mean $\pm$ SE and the significance of differences among was assessed using one sample t-test at $p \leq 0.05$.

RESULTS AND DISCUSSION

TLC Bioautography and biofilm inhibition

Methanolic extract of *S.aromaticum* was separated on TLC which showed five clear bands under UV illuminator at 346 nm. TLC bioautography of above extract exhibited the clear zone of inhibition with purple background at the bioactive fraction ($R_f = 0.78$) (Figure 1). The earlier studies reported that crude *S.aromaticum* exhibited anti quorum activity against *C. violaceum*, *P. aeruginosa* and *Aeromonas hydrophila* at sub-MIC levels [12]. In the current study,

bioactive fraction showed dose dependent biofilm inhibition on *P. aeruginosa* and maximum inhibition ($72\% \pm 0.35$) was found at $200\mu\text{g/mL}$ (Figure 2).

ATR-IR analysis

In our previous study, ATR-IR analysis of *S.aromaticum* methanolic extract showed the presence of alkanes, alkynes, aldehydes, aromatics, carboxylic acids and halogen groups [13]. In the present study, ATR-IR analysis confirmed the presence of aldehydes and aromatic compounds which showed major peaks at 1709.134 cm^{-1} and 1260.435 cm^{-1} respectively (Figure 3A) indicating that the above functional groups retained in the purified bioactive fraction.

GC-MS analysis

GC-MS analysis of active fraction revealed six major compounds viz. 4-hydroxy-3-methoxy-benzaldehyde, 1-phenoxy ethanol, eugenol, chavicol, p-hydroxyacetophenone and 4-azido-phenol that may account for the antibiofilm activity (Figure 3B; Table 1).

In silico docking

The molecular docking analysis was performed to understand the mechanism of compounds mediated inhibition and predicted their potential binding sites of AHL synthase. LasI is the counterpart of AHL synthase in *P. aeruginosa* which produces 3-oxo-C12-HSL, shares 31% identity (47% homology) of protein

sequence with RhlI. The binding energies of compounds were calculated on LasI (Table 1). Among all, chavicol showed best pose dock with binding energy of -6.20 kcal/mol . The LasI crystal structure showed SAM binding pockets with N-terminal residue e.g. Phe 27, Arg 30 and Trp 33 and with the conserved residue of Phe 105 in acyl-chain binding site. The pose of chavicol was locked deeply into the binding site and formed three hydrogen bonds with LasI amino acids Phe 105, Thr 144 and Val 143 (Figure 4A).

In order to understand the binding properties of compounds to AHL synthase, the second docking was performed with EsaI. The binding energies also revealed that the chavicol showed best binding energy (-4.36 kcal/mol) (Table 1) and formed three hydrogen bonds with Ser 98, Arg100 and Phe101 of EsaI (Figure 4B). The amino acids, Arg100 and Phe101 are in the hydrophobic core of EsaI acyl-chain substrate. Docking pose revealed that chavicol bind to acyl-chain binding site in EsaI.

Earlier study reported that the binding poses of trans-cinnamaldehyde formed one hydrogen bond with Arg 30 of LasI and two hydrogen bonds of EsaI, Arg100 and Phe101 [20]. On the contrary, the present study showed that the chavicol has formed three hydrogen bonds with LasI and three

hydrogen bonds with EsaI, indicating best interaction with targeted protein binding sites. The ADME (Molinspiration-based) of the phytochemical compounds were depicted in table 1. The compounds showed molecular hydrophobicity (miLogP) and topological polar surface areas (TPSA) which reveals the cell permeability and obeyed Lipinski's Rule of Five. For the drug lead properties or designing programs,

the Lipinski's Rule has been used for the sorting of the active ligands. The compounds violating the rules (more than five H-bonds donors, H-bond acceptors, a molecular weight (MW) greater than 500, and log P value greater than five) may show poor absorption or permeation [21] and the compounds in the present study are in the accordance with Lipinski's Rule.

Figure 1: TLC separation and bioautography of *S.aromaticum* extract.

A) TLC separation of *S.aromaticum* extract; B) TLC bioautography of *S.aromaticum* extract on *C. violaceum*

Figure 2: Biofilm inhibition of bioactive fraction of *S.aromaticum*

Figure 3: (A) ATR-IR and (B) GC-MS analysis of bioactive fraction.

Figure 4: Binding and hydrogen bonds formation of 5-phenyldecane with (A) LasI and (B) EsaI

Table 1: Docking scores of GC-MS compounds with active site of *P. aeruginosa* quorum sensing regulators and their ADME analysis

Compound Name	Docking energy(kcal/mol)												
	LasI	EsaI	miLog P	TPSA	N atoms	MW	N violations	N rotb	Ion channel modulators	Kinase inhibitors	Nuclear receptor ligand	Protease inhibitors	Enzyme inhibitors
4-hydroxy-3-methoxy-Benzaldehyde	-5.87	-4.57	1.07	46.53	11	152.15	0	2	-0.54	-1.13	-0.91	-1.65	-0.64
1-phenoxy ethanol	-5.55	-4.03	1.71	29.46	10	138.17	0	2	-0.54	-1.37	-1.12	-0.85	-0.28
Eugenol	-5.98	-4.72	2.10	29.46	12	164.20	0	3	-0.36	-1.14	-0.78	-1.29	-0.41
Chavicol	-6.20	-4.36	2.82	9.23	11	148.21	0	3	-0.51	-1.40	-1.03	-1.39	-0.62
p-Hydroxyacetophenone	-5.98	-4.72	1.36	37.30	10	136.15	0	1	-0.54	-1.50	-1.13	-1.43	-0.70
4-Azido-phenol	-5.84	-3.89	1.17	71.79	10	137.14	0	1	-0.28	-1.04	-1.14	-1.29	-0.54

CONCLUSION

In conclusion, our results demonstrated that the *S.aromaticum* bioactive fraction has quorum quenching ability. The *in silico* analysis supported the *in vitro* study which might lead to *in vivo* establishment in future.

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