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ORIGINAL RESEARCH ARTICLE

Phomopsis tersa as Inhibitor of Quorum Sensing System and Biofilm Forming Ability of *Pseudomonas aeruginosa*

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Abstract Endophytic fungi provide rich reservoir for novel antimicrobial compounds. An endophytic fungus, from *Carica papaya* plant identified as *Phomopsis tersa*, was investigated for attenuating the quorum sensing mediated pathogenicity of *Pseudomonas aeruginosa* PAO1. Crude extract of *P. tersa* was found to reduce the production of redox-active pigments—pyocyanin and pyoverdine in *P. aeruginosa* PAO1 by 92.46% and 71.55%, respectively at sub-MIC concentration of 900 µg/mL. In addition, the crude extract was also able to inhibit the expression of virulence factors involved in biofilm formation: exopolysaccharide (72.21%) and alginate (72.50%). Secretion of cell-lytic enzymes was also found to be reduced: chitinase by 79.73% and elastase by 74.30%. 3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione identified from GC-MS analysis, displayed favorable molecular interactions with *P. aeruginosa* transcriptional regulators, LasR and RhlR with good docking scores of − 6.873 kJ/mol and − 6.257 kJ/mol, respectively. The study thus reveals the potential use of *P. tersa* for discovering drugs against infectious pathogens.

Keywords *Pseudomonas aeruginosa* · Quorum sensing · *Phomopsis tersa* · Biofilm formation · Secondary metabolites

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Introduction

The chronic bacterial infections are major cause of solicitude for healthcare sectors. Biofilm establishing bacteria contributes to around 65% bacterial infection of the world [1]. Bacterial biofilm forming ability and expression of virulence factors are mediated by a unique communication system termed as quorum sensing (QS) [2]. *Pseudomonas aeruginosa* is a nosocomial pathogen causing severe chronic infection in immunocompromised individuals with lung disease, pneumonia and severe burns. QSS, designated as LasI/R and RhlI/R operon, regulates bacterial pathogenicity by controlling the expression of various pathogenic determinants and has positive influence on motility factors and biofilm mediated chronic infections [2–4]. The *P. aeruginosa* QS cascade involves two major signaling molecules, N-3-Oxo-Dodecanoyl-L-Homoserine lactone (C_{12}HSL) and N-Butyryl-L-Homoserine lactone (C_4HSL). These signaling molecules with respect to bacterial density interacts with their cognate receptors and initiate bacterial infection [5].

With increased risk of emergence of antibiotic resistance among infection causing bacteria, conventional antibiotics

have been shown low effectiveness towards infections. The major reason for the antibiotic resistance is the limited permeability of cell membrane which contributes to slow down the penetration of effective dose of the anti-microbial compounds and increases the pathogenicity in the bacterial cell [6]. QS inhibition (QSI) can be achieved via different mechanisms such as inhibition of signaling molecule production, blocking the binding of AHL molecule to the receptors, alteration in the structure of target molecule by enzymes and interrupting the transport facility of the signaling molecule through the membrane channel. Molecules interfering with the QSS have gained importance to develop anti-infective drugs [7–9]. Natural compounds from plants as well as microbial sources are highly important to combat microbial pathogenicity especially drug resistant bacteria [10–12]. Plants are rich source of compounds that can act as QSIs by decreasing the expression level of virulence factor gene cluster without affecting bacterial growth. Microbial secondary metabolites are chemically diverse natural compound that can be utilized as QSIs and anti-biofilm agents.

Microorganisms associated with plants in symbiotic and non-symbiotic relationship generate a wide range of QSI molecules. Microorganisms are also a rich source of bioactive molecules. Endophytic fungi are beneficial microorganism that colonizes different plant parts in symbiotic manner [13]. Endophytic fungal metabolites are biologically active with high antimicrobial, antiviral, antifungal and insecticidal activities. Endophytic fungus, *Alternaria alternata* isolated from *Carica papaya* displayed QSI and altered the biofilm architecture against *P. aeruginosa* PAO1. *A. alternata* also showed inhibition of various pathogenic traits such as pyocyanin pigment, elastase, protease, chitinase and minimized biofilm formation via hindering alginate, EPS production and eDNA secretion [14]. Similarly, a marine fungi also significantly inhibited the biofilm dynamics of *S. aureus*, *Listeria monocytogenes*, *E. coli* and *Salmonella typhi* [15].

In the present work, the QSI activity of *P. tersa* isolated from *C. papaya* leaves was determined. Additionally, the identified bioactive compounds from GC-MS were subjected for their interaction with QS receptors using molecular docking analysis.

Materials and Methods

Bacterial culture, *P. aeruginosa* PAO1 and biosensor strain *Chromobacterium violaceum* (ATCC 12472) were grown overnight and anti-quorum sensing assays were conducted using crude extract of *P. tersa* based on available literature. Isolation, identification and phylogenetic analysis of fungal species were performed. Bioactive metabolites present in the fungal crude extract were determined using Gas chromatography-mass spectrometry (GC-MS) technique [14]. The MIC of *P. tersa* on *P. aeruginosa* was determined as per CLSI guidelines [16]. Anti-quorum sensing activities including inhibition of pigment production i.e. violacein [16], pyocyanin [14] and pyoverdine [17], virulence factors such as hydrogen peroxide [18], secretion of cell lytic enzymes; staphylolytic protease, protease, chitinase [14], elastase [19], biofilm promoting factors; exopolysaccharide [14], cell surface hydrophobicity, alginate [20], bacterial motility [16] and biofilm growth determination [20] using confocal laser scanning microscopic analysis were determined. Molecular docking was used as a tool for analyzing the molecular interaction between identified metabolites and QS receptors, LasR and RhlR [19, 21].

Results and Discussion

Identification of Endophytic Fungi

Twenty-four endo-symbiotic fungal strains were isolated from *C. papaya* leaves. Based on their antimicrobial activities against *P. aeruginosa,* the most active endophytic fungus was selected and identified using ITS region sequencing. BLAST analysis revealed it has maximum similarity with endophytic fungal strain *P. tersa*. The ITS sequencing data and BLAST analysis confirmed the selected strain as *P. tersa* based on 99% similarity among reported strains. The gene sequence was submitted to NCBI GenBank with an accession number KX925281. *P. tersa* was cultivated in large scale to isolate the crude extract for further studies (Fig. 1).

Determination of MIC

The MIC value was determined based on growth inhibition at varying concentrations ranging from 250 to 1500 µg/mL and found to be 1250 µg/mL. All subsequent assays including growth curve analysis were performed at sub MIC value of 900 µg/mL (Fig. 1).

Anti-quorum Sensing Activities

Inhibition of Pigment Production in P. aeruginosa PAO1 and C. violaceum

C. violaceum, a known biomarker strain for determining QSI activity when subjected to 900 µg/mL of *P. tersa* crude extract, resulted in 72.54 ± 4.15% reduction in violacein production (Fig. 2a). *P. aeruginosa* treated with fungal extract showed 92.46 ± 2.34% of reduction in pyocyanin production and 71.55 ± 6.49% reduction in

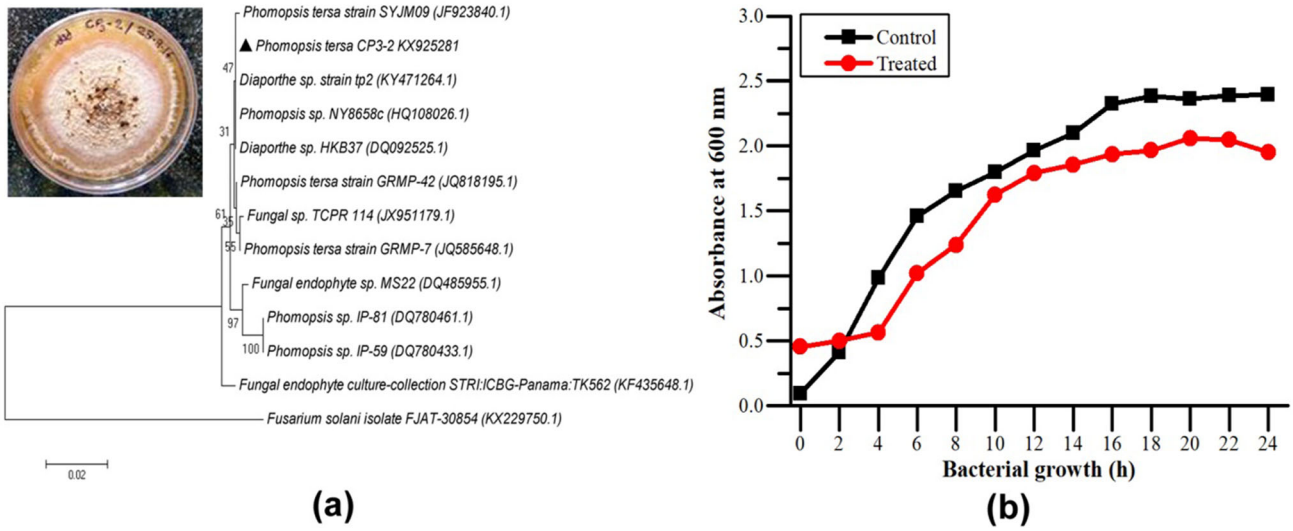

Fig. 1 **a** Morphological and molecular characterization of endophytic fungus, *Phomopsis tersa* CP3 **b** growth pattern of *P. aeruginosa* at sub-MIC concentration 900 µg/mL of fungal extract with control

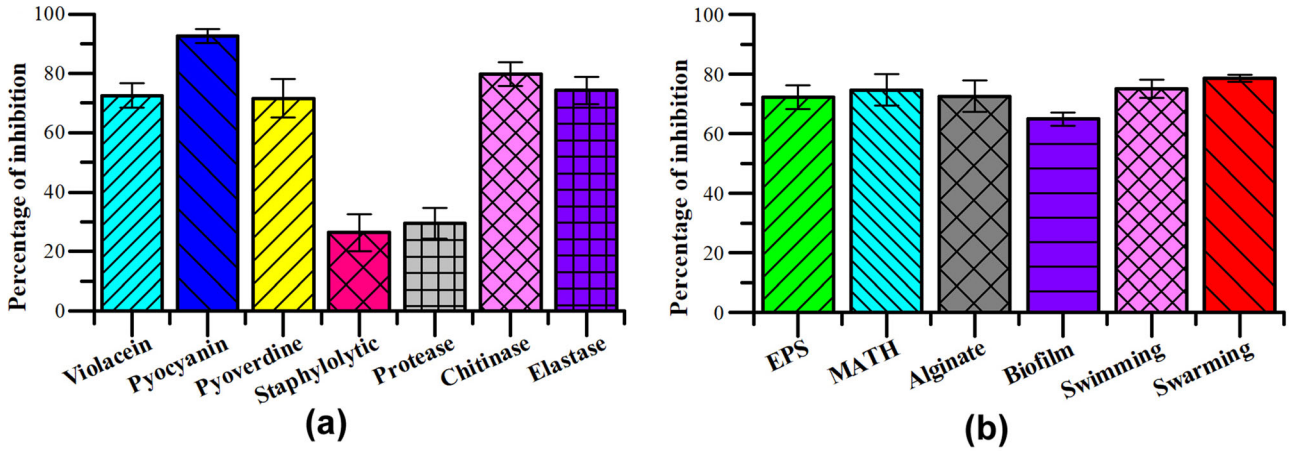

Fig. 2 **a** Inhibitory effect of *P. tersa* crude extract (900 µg/mL) on quorum sensing regulated processes: **a** production of pigments, in *C. violaceum* and *P. aeruginosa* PAO1, lysis of *Staphylococcus aureus* cell, degradation of Azocasein, production of chitinase and elastase. **b** Inhibition of virulent factors: exopolysaccharide (EPS), microbial adhesion to hydrocarbon (MATH), alginate, biofilm formation, swimming and swarming motility

pyoverdine production compared to the control (Fig. 2a). *Streptomyces* sp. TOHO-Y209 and TOHO-O348 secreted bioactive compounds, Piericidin A1, 3′-rhamnopiericidin A1 and piericidin E and showed QSI activity against *C. violaceum* and significantly decreased the production of violacein pigment in a dose dependent manner [22]. The present results were as per the earlier report depicting the potential of *Andrographis paniculata* extract in inhibiting the production of pyocyanin pigment [23].

Inhibition of Hydrogen Cyanide (HCN) Production

P. aeruginosa PAO1 treated with *P. tersa* extract demonstrated noticeable difference in HCN production via color changing properties of picric acid soaked filter paper. The filter paper placed with sample exhibited discoloration of filter paper from pale yellow to orange color. The color change observed in control sample was more compared to the treated sample (Fig. 3a).

Las A Staphylolytic Activity

Staphylolytic enzyme production was significantly altered when treated with sub-MIC of *P. tersa* with an inhibition of 26.41 ± 6.21% (Fig. 2a).

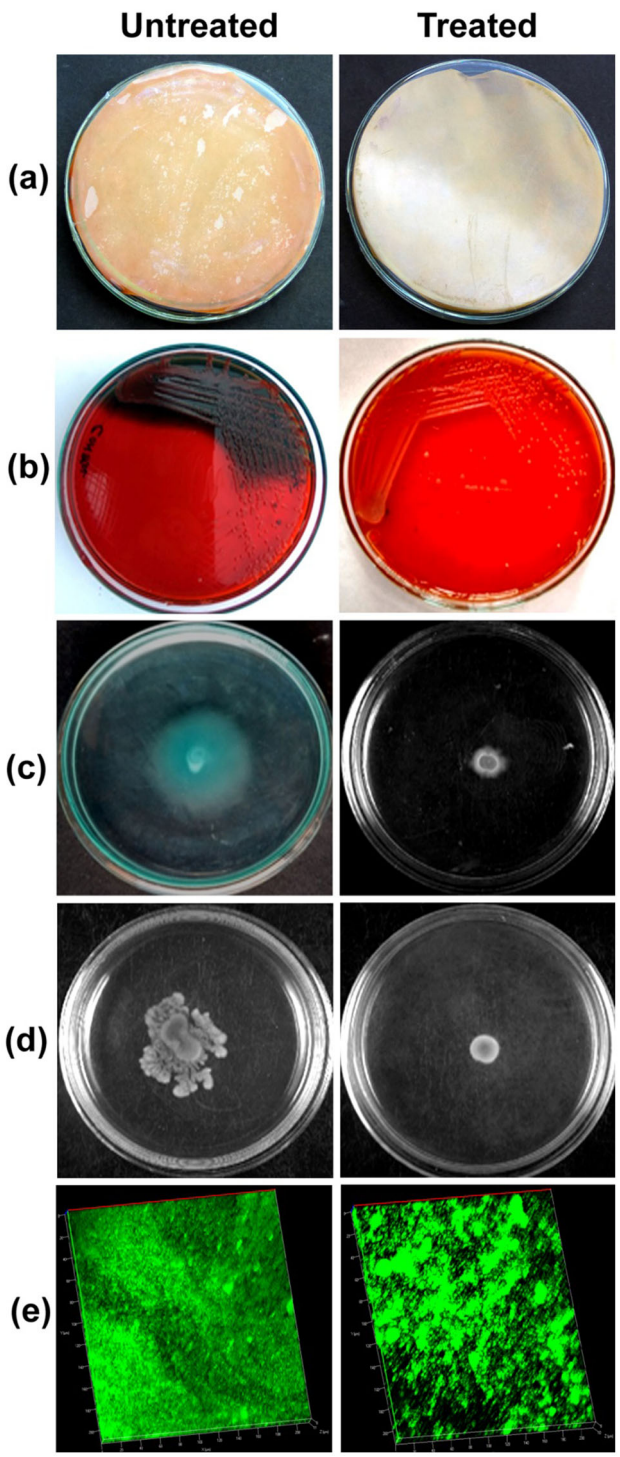

Fig. 3 Inhibitory effect of fungal crude extract, *P. tersa* on quorum sensing mediated characteristics of *P. aeruginosa* PAO1: **a** Hydrogen cyanide (HCN) production, **b** Exopolysaccharide (EPS) production, **c** Swimming, **d** Swarming motility, and **e** Biofilm formation observed using confocal laser scanning microscopy (CLSM)

Las A Protease Activity

Based on the azocasein assay it was observed that QS regulated protease production was inhibited by 29.56 ± 5.24% compared to control (Fig. 2a). Anti-proteolytic activity of *Rhizophora* spp. against the protease produced by PAO1, CI-I and CI-II was recorded with the inhibition of 39%, 93% and 92%, respectively [24].

Las B Chitinase Activity

The production of Las B chitinase enzyme was reduced by 79.73 ± 3.96% compared to the untreated control (Fig. 2a). The methanolic extract of *Mangifera indica* L. at 800 µg/mL concentration showed tremendous reduction in chitinase enzyme production by 55.3% [25].

Las B Elastase Activity

Reduction in elastase production was found to be 74.30 ± 4.55% in *P. tersa* crude extract treated *P. aeruginosa* at sub MIC (900 µg/mL) (Fig. 2a).

Exopolysaccharide (EPS) Inhibition

The EPS producing capacity of *P. aeruginosa* PAO1 was greatly affected when treated with *P. tersa* with an inhibition of 72.21 ± 3.96% compared to the untreated bacterial sample. Fungal crude extract treated agar plate showed pink color colonies with less EPS production compared to the untreated cells (Figs. 2a and 3b).

Microbial Adhesion to Hydrocarbons (MATH)

Cell surface hydrophobicity (CSH) index was measured quantitatively with MATH assay using toluene as substrate. After treatment with fungal crude extract, CSH index of treated *P. aeruginosa* PAO1 was found to be 74.65 ± 5.32% less compared to the control (Fig. 2b). Ability of bacterial cells to attach to hydrophobic solvent after treatment with *Diaporthe phaseolorum* SSP12 crude extract was inhibited in a study by 58.01%, 50.59% and 30.03% at 250, 500 and 750 µg/mL concentrations, respectively [20].

Alginate Inhibition

A significant reduction in alginate production (72.50 ± 5.29%) was observed upon treatment with fungal crude extract when compared with untreated sample (Fig. 2b). *P. cucumerina,* isolated from a Chinese medicinal plant, *Orychophragmus violaceus* reduced alginate production by 52% at a concentration of 1 mg/mL [26].

Swimming and Swarming Motility

The result showed decrease in diameter of bacterial halo zone that confirms the inhibition of swimming and swarming motility upon treatment with *P. tersa* crude extract at sub MIC concentration. The bacterial cell movement from the point of inoculation to petri dish edge was less than the control in both swimming (75.06 ± 2.95%) and swarming assays (78.52 ± 1.20%) (Fig. 3c, d).

Biofilm Inhibition

The cell growth rate after treatment with *P. tersa* crude extract was inhibited by 64.83 ± 2.13% at a sub-MIC concentration of 900 µg/mL compared to the control (Fig. 2b).

Confocal Laser Scanning Microscopy

Microscopic analysis results represent a remarkable decrease in bacterial growth rate and EPS production in biofilm matrix in treated cells. The CLSM results showed highly thick and dense biofilm in untreated sample compared to the treated sample and reduction in bacterial growth was observed in fungal crude extract treated sample (Fig. 3e).

GC-MS Analysis

GC-MS analysis provided the availability of bioactive compounds with diversified chemical structures. A cyclo dipeptide, 3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione was present with retention time (20.815 min) and peak area % (38.86). It has been previously reported to have biological properties such as antibacterial, antifungal and anticancer activities. Presence of several bioactive compounds was observed with different retention times and peak area % such as Sulfurous acid, 2-propyl tridecyl ester, Ethanethioic acid, S-(2-methylbutyl) ester, 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester which could be contributed anti QS activity (Table 1).

Molecular Docking Studies

LasR and RhlR, transcriptional regulators have a vital role in the expression of QSS in *P. aeruginosa.* Docking studies were conducted to use these proteins as potential drug targets to determine the specific interaction and affinity of fungal metabolites. Natural QS signaling molecules in *P. aeruginosa*, C_{12}HSL and C_4HSL were docked against LasR and RhlR receptors, respectively. Molecular docking studies displayed that C_{12}HSL signal molecule exhibited high binding affinity for the receptor protein, LasR with docking score -6.658 kJ/mol. Hydrophobic interaction (Van der Waal force) also contributes to the increased efficacy of signal molecule and cognate receptors binding affinity. Hydrophobic interactions were found to be with amino acids, LEU36, TYR47, ILE52, TYR64, VAL76, PHE101, LEU110, PRO74 (Fig. 4a) and confirmed by formation of hydrogen bond with TYR56, SER129 amino acids. The signal molecule, C_4HSL (Fig. 5a) showed interaction with RhlR receptor protein with the docking score − 5.081 kJ/mol. Fungal bioactive compound, 3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione showed docking score of − 6.873 kcal/mol (Fig. 4b) and -6.257 kcal/mol (Fig. 5b) with LasR and RhlR receptor, respectively. This ligand has two hydrogen bonds with residue TRP60 and SER129 of LasR and additionally possesses hydrophobic interaction with the active site

Table 1 List of identified fungal metabolites in ethyl acetate crude extract of endophytic fungus, *Phomopsis tersa* using GC-MS analysis

S. No.	Fungal metabolites	Molecular formula	Molecular weight (g/mol)	Retention time	Peak area %
1	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	278.348	17.029	39.962
2	Ethanethioic acid, S-(2-methylbutyl) ester	$C_7H_{14}OS$	146.250	17.164	4.992
3	3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_{11}H_{18}N_2O_2$	210.2728	18.170	4.822
				18.215	15.528
4	Heneicosane	$C_{21}H_{44}$	296.583	20.931	3.736
5	Sulfurous acid, 2-propyl tridecyl ester	$C_{16}H_{34}O_3S$	306.505	24.802	3.595
6	Octacosane	$C_{28}H_{58}$	394.772	21.751, 22.551	9.242 7.864
7	Heptacosane	$C_{27}H_{56}$	380.74	23.322	6.412
8	Tetratetracontane	$C_{44}H_{90}$	619.204	24.067	3.846

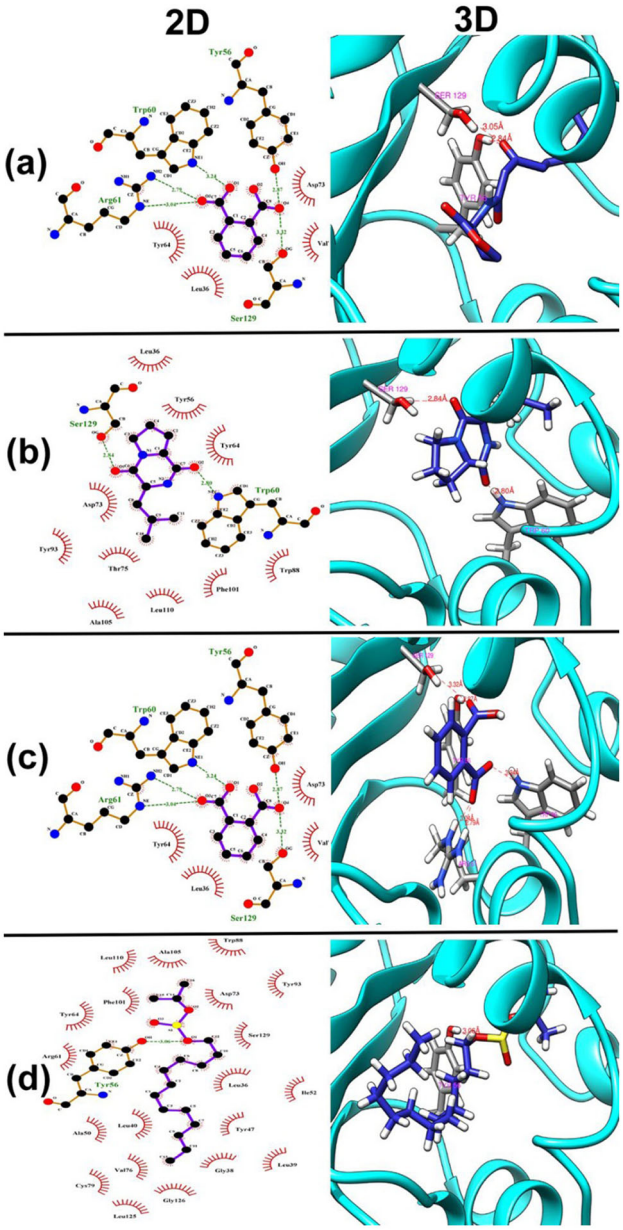

Fig. 4 Molecular docking of ligands with LasR receptor protein from *Pseudomonas aeruginosa* PAO1 showing the key hydrophobic and polar interactions of **a** 3-oxo-C_{12}—HSL **b** 3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione **c** 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester **d** Sulfurous acid, 2-propyl tridecyl ester

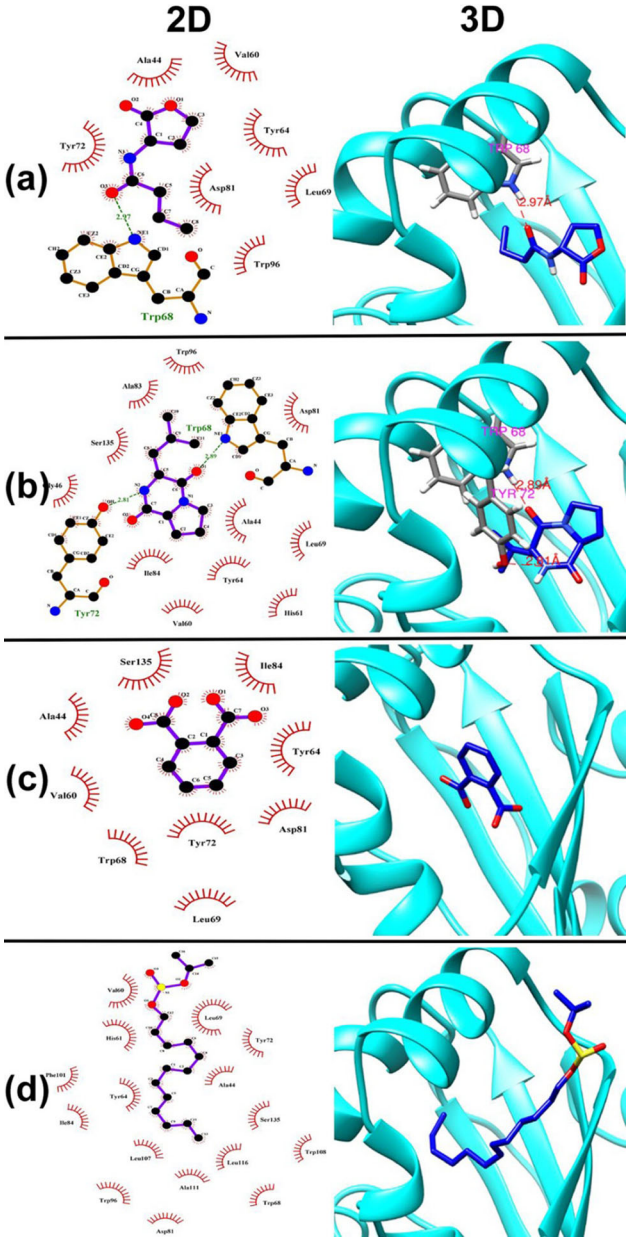

Fig. 5 Molecular docking of ligands with RhlR receptor protein from *P. aeruginosa* PAO1 showing the key hydrophobic and polar interactions of **a** C_4—HSL **b** 3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione **c** 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester **d** Sulfurous acid, 2-propyl tridecyl ester

residues of LasR (LEU36, TYR64, TRP60, TYR56, PRO74, ALA105, TYR93, PHE102, PHE101, TRP88, ALA127, VAL76, LEU110). Fungal compound owned hydrogen bond at TRP68 and TYR72 with non-polar interaction includes ALA83, ALA44, TYR64,TYR72, SER135 and ILE84 that shows high binding efficacy of fungal metabolite towards RhlR receptor protein. Fungal metabolites, 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester (Figs. 4c and 5c), Sulfurous acid, 2-propyl tridecyl ester (Figs. 4d and Fig. 5d) and Ethanethioic acid, S-(2-methylbutyl) ester shares docking score of (− 7.372, − 5.985, − 4.803 kJ/mol) and (− 4.329, − 5.779, − 4.726 kJ/mol), for LasR and RhlR, respectively. Docking studies confirmed the possibility of binding of fungal metabolites at the active binding socket to inhibit the QS cascade in *P. aeruginosa* PAO1 (Table 2). Study conducted to understand the molecular interaction between the bioactive compounds secreted from *A. alternata* and LasR receptor of *P. aeruginosa* PAO1 revealed that sulfurous

Table 2 Molecular docking analysis data represent the electrostatic interaction between signal molecules, N-(3-Oxooctanoyl)-L homoserine and N-butyryl-homoserine lactone with transcriptional regulators, LasR and RhlR of *P. aeruginosa* PAO1. Fungal metabolites displayed propitious interaction involving hydrogen bond formation and hydrophobic interaction with LasR and RhlR receptors

S.No	Compound interacting with Receptor protein	Glide Docking Score	Glide Emodel	Hydrogen Bonds with Residue names	Hydrophobic interaction residues name
LasR receptor					
1	N-(3-Oxooctanoyl)-L homoserine	− 6.658	− 52.888	TYR56, SER129	LEU36, TYR47, ILE52, TYR64, VAL76, PHE101, LEU110, PRO74
2	3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione	− 6.873	− 34.797	TRP60, SER129	LEU36, TYR64, TRP60, TYR56, PRO74, ALA105, TYR93, PHE102, PHE101, TRP88, ALA127, VAL76, LEU110
3	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	− 5.985	− 43.424	TYR56, ARG61, TRP60, SER129, TYR56,	TYR64, ASP73, PHE101, LEU36, ILE52, ALA127, VAL76, LEU110, TRP88, TYR56
4	Sulfurous acid, 2-propyl tridecyl ester	− 7.372	− 67.983	TYR56	LEU40, ASP73, PHE101, ALA105, PHE102, TRP88, TYR93, TYR56, LEU36, ILE52, TYR64, TYR93, TYR56, LEU36, ILE52, TYR64, TYR47, LEU39
5	Ethanethioic acid, S-(2-methylbutyl) ester	− 4.803	− 31.031	TYR56, SER129	LEU36, ASP73, THR75, VAL76, TYR64, TRP60, TRP88, LEU110, TRP88, ALA105, PRO74
RhlR receptor					
1	N-Butyryl homoserine lactone	− 5.081	− 36.629	TRP68,ASP81	ALA44,TYR72,LEU107,LEU116,TYR64
2	3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione	− 6.257	− 15.992	TRP68, TYR72	ALA83, ALA44, TYR64,TYR72, SER135, ILE84
3	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	− 4.329	− 7.548	–	ALA44, VAL60, TYR64, TYR72, ILE84
4	Sulfurous acid, 2-propyl tridecyl ester	− 5.779	− 27.202	–	ALA44, TRP68, LEU107, HIS61, TYR64, LEU69, VAL60, TYR45
5	Ethanethioic acid, S-(2-methylbutyl) ester	− 4.726	− 28.495	TRP68	LEU116, TYR64, LEU69, VAL60, ALA44, TYR64, ASP81, ALA83, TRP96, ALA111

acid-2-propyl tridecyl ester had the highest docking score of − 7.525 kJ/mol with residual interaction involving TYR56 and TRP60. Bioactive compounds, 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester also showed a good docking score of − 6.384 kJ/mol [14]. Our study shows a promising approach to combat *P. aeruginosa* pathogenicity by interfering with the QSS and biofilm formation by the treatment of *P. tersa* crude extract.

In summary, development of antibiotic resistance under the process of selective pressure is one of the major challenges of the present era for the treatment of infectious diseases. The present study explains endophytic fungus *P. tersa* and its efficacy for producing secondary metabolites as QSI molecules against *P. aeruginosa*. The crude extract suppressed QS mediated virulence factors. Bioactive compounds reported in the study can further be utilized as new drug candidates in the fight against chronic infections.

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Compliance with Ethical Standards

Conflict of interest The authors hereby declare no conflict of interest.

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