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ATTENUATION OF QUORUM SENSING ASSOCIATED VIRULENCE FACTORS AND BIOFILM FORMATION IN *PSEUDOMONAS AERUGINOSA* PAO1 BY *PHOMA MULTI-ROSTRATA* PUTY3, A SAPROPHYTIC FUNGUS, ISOLATED FROM *CARICA PAPAYA*

Tanveer Ahmed and V. Venkateswara Sarma *

Department of Biotechnology, School of Life Sciences, Pondicherry University, Kalapet - 605014, Puducherry, India.

Keywords:

Antibiotic resistance, Anti-infective strategy, GCMS, *In-silico* study, Quorum quenching, Secondary metabolites

Correspondence to Author:
V. Venkateswara Sarma

Assistant Professor,
Department of Biotechnology,
School of Life Sciences, Pondicherry University, Kalapet - 605014,
Puducherry, India.

E-mail: sarmavv@yahoo.com

ABSTRACT: *Pseudomonas aeruginosa* is one of the most emerging multidrug-resistant opportunistic pathogens, which poses a threat to immunocompromised patients. Treatment of *P. aeruginosa* in the affected patients has become difficult because of its ability to develop resistant strains against most of the conventional therapeutic antibiotics. Quorum sensing plays a crucial role in the virulence nature of *P. aeruginosa* as it helps the bacteria to produce virulence factors and biofilm formation. In the present study, we studied the quorum quenching potential of fungal secondary metabolites from the well-known fungus *Phoma multirostrata* PUTY3 strain isolated from *Carica papaya*. In this study, we have carried out both *in-vitro* and *in-silico* analyses of fungal secondary metabolites from *P. multrirostrata* against *P. aeruginasa*. *The in-vitro* analysis showed a marked decrease in the production of virulence factors such as pyocyanin, rhamnolipids, protease, elastase, exopolysaccharides, HCN gas production and biofilm formation. In addition to this, a significant inhibition in the motility factors of *P. aeruginosa* such as swimming, swarming, twitching was also observed. *In-silico* studies further validated the results, in which, docking of molecules, present in the GCMS analysis of fungal secondary metabolites, were carried out with the two main quorum sensing regulating proteins, *viz*., LasR and RhlR. These findings indicate a new anti-infective and alternative therapeutic approaches to combat the virulence factors of *Pseudomonas aeruginosa* and explore further the potential of fungal secondary metabolites from other saprophytic fungi colonizing decaying substrata of medicinal plants.

INTRODUCTION: The looming inefficacy of conventional antibiotics against drug-resistant microbial pathogens has become a serious concern worldwide.

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Pseudomonas aeruginosa is one of the examples among drug-resistant opportunistic microbes that is capable of infecting patients suffering from cystic fibrosis, chronic obstructive pulmonary diseases, HIV, and immunocompromised patients.

Furthermore, *P. aeruginosa* is also known for severe burn wound infections, urinary tract infections, bloodstream infections, nosocomial infections [1], and meningitis [2]. *P. aeruginosa* causes 17% of nosocomial pneumonia, 7% urinary tract infections, 8% quotidian cause of surgical-site

infections and 9% infections in general, was found [3]. *P. aeruginosa* releases a variety of extracellular and cell-associated signaling molecules that are predominantly responsible for the progression of the disease by imposing the adhesion, altering the immune response, eluding from phagocytosis and damaging the host tissues [4]. Approximately, 300 genes are responsible for various virulence factors in the pathogenesis of *P. aeruginosa*, which is controlled by acylhomoserine lactone (AHL) [5].

These virulence factors are regulated in a bacterial cell in a density-dependent manner by well-known quorum sensing systems [6]. Among these, the Las system uses N-(3 oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) as the signal transducing molecule, whereas the Rhl system employs N butanoyl-L-homoserine lactone (C4-HSL) [7]. The las system regulates the production of virulence factors such as LasA protease, LasB elastase, alkaline protease, exotoxin A and biofilm formation. Similarly, rhl system regulates the production of pyocyanin, rhamnolipids, LasB elastase and hydrogen cyanide [8, 9, 10]. *P. aeruginosa* also has a species-specific (PQS) signaling system that employs 2-heptyl-3-hydroxy-4-quinolone as the signaling molecule [11] and contributes significantly to quorum sensing and infections. All three systems have a regulatory hierarchy in such a way that las system regulates the rhl and pqs systems [12]. In these quorum-sensing systems, the signaling molecules turn on various virulence determinants by binding to a cognate receptor protein in a threshold amount [13] and bring about a conformational change that in turn exposes DNA binding regions (promoters) upstream of to the virulence genes [14].

During the last few decades, there has been indiscriminate use of antibacterial drugs to treat *P. aeruginosa* related infections that are leading to emergence of multiple drug resistance (MDR) strains, which is a serious public health concern. As such, the development of novel and highly effective anti-infective, targeting the QS systems, along with their virulence factors, is of great importance in the so-called post-antibiotic era [15]. Synthetic quorum sensing inhibitors (QSIs) pose some issues related to their bioavailability and relative toxicity, and hence the focus has now turned towards the development of QSIs using untapped natural resources such as secondary metabolites from plants and other phytoconstituents [16]. Phytoconstituents have been regarded as one of the most potential sources targeting the QS virulence and biofilm formation in *P. aeruginosa* [17]. However, the quorum quenching potential of secondary metabolites derived from the fungi has remained largely unexplored and thus, these secondary metabolites may be useful for the development of novel anti-infective agents. Potential bioactive natural products having antifungal, antiviral, antioxidant, cytotoxic, and antibacterial activities have been isolated from secondary metabolites associated with fungi [18, 19].

Fungi show coexistence with bacteria in some cases but they lack an active immune system. Thus, Fungi rely mainly on chemical defense mechanisms [20]. A series of quorum quenching compounds have been isolated from *Penicillium* sp. such as penicillic acid [21], hydroxyemodin, emodic acid, and(+)-2′S-isorhodoptilometrin [22]. Hence, the use of fungi to control pathogenic bacteria is believed to be a novel renewable approach.

The present study focuses on the quorum quenching potential of *P. multirostrata* PUTY3 extract against quorum sensing activity of *P. aeruginosa* PAO1 virulence factors and biofilm formation. The study is further validated by *in-silico* studies for understanding the possible target of quorum quenching. The present study also explored the new alternative strategy for the development of novel and efficient anti-infective drugs from the unexplored secondary metabolites obtained from fungal sources.

MATERIALS AND METHODS:

Chemicals and Solvents: The chemicals used in the present study, such as azocasein and elastin congo red (ECR), were purchased from Sigma Aldrich, USA. Other chemicals like crystal violet, acridine orange, trichloroacetic acid (TCA), sodium chloride (NaCl), Tris-HCl, and nutrient media such as Tryptone Soy Broth (TSB), Luria Bertani (LB) broth, bacteriological peptone, LB agar were procured from HiMedia Lab. Pvt. Ltd., Mumbai, India, and solvent such as ethyl acetate were purchased from Avra synthesis Pvt. Ltd., Hyderabad, India.

Isolation of Fungal Isolates, Media and Storage: Dead and decomposing leaf litter samples of *Carica papaya* were collected from the main campus of Pondicherry University, Pondicherry, India. A leaf litter samples were processed for isolation of fungal colonies adopting a modified particle filtration technique [23]. Briefly, the leaf litter samples were first cleaned carefully with the help of tap water followed by sterile distilled water and were dried at room temperature. 1g of dried leaves was ground in a blender, diluted with 100 mL sterile distilled water and 500µl from this dilution was poured into the plates using the pour-plate method. The colonies propping up from the PDA plates were sub-cultured to get axenic cultures. All the pure fungal isolates were preserved at -80 °C in 15% glycerol solution in cryovials for further analysis.

Extraction of Fungal Crude Extracts and Sample Preparation: Fungal isolates were aseptically grown in 1000 mL conical flask containing potato dextrose broth (PDB) in static conditions at 25 °C ± 2, by inoculating with freshly growing fungal colonies from potato dextrose agar plate. After a two-week mycelial mat was separated from the broth by using three layers of muslin cloth and broth was centrifuged at 10,000 rpm for 20 min. to avoid any spores and/or very fine fungal mycelial bits. The resulting filtrate was extracted twice with equal volumes of ethyl acetate and kept on a shaker (200 rpm) for 24-48 h for separation of secondary metabolites. Both aqueous and organic phases were separated through glass separatory funnel, and the organic phase of ethyl acetate was concentrated by a rotary vacuum evaporator (Buchi, India) to collect crude extracts [24]. The fungal crude extract was dissolved in DMSO for preliminary screening and further *in-vitro* analysis in required concentrations.

Screening of Fungal Crude Extracts: Fungal isolates were subjected to preliminary screening for their antiquorum sensing potential against widely known quorum sensing and biofilm-forming strains, *viz*., *Chromobacterium violaceum* (MTCC 2656) and *P. aeruginosa* (MTCC 2453), following agar well diffusion method with minor modifications. In brief, 10 mL of molten soft agar (Tryptone 10 g/L, NaCl 5g/L, Agar-agar 6.5 g/L) seeded with 1% overnight culture of *P. aeruginosa* and *C. violaceum* was poured separately onto the fresh LB agar medium and was allowed to solidify. Two wells with a diameter of 8 mm were carefully made, and two different doses of crude fungal extracts (250 and 500 µg/mL) were loaded into each well, and the plates were incubated at 37 °C for 24 h [25].

Identification of Potential Fungal Isolate through Sequencing: Based on the results of initial screening for antiquorum sensing potential of fungal crude extracts, one fungal isolate *P. multirostrata* PUTY3 was chosen for further studies and was selected for molecular identification. The Internal transcribed spacer (ITS) region of the fungal isolate PUTY3 was amplified using specific primers ITS1 (5′-TCCGTAGGTGA ACCTGCGG-3′) and ITS4 (5′- TCCTCCGCTTAT TGATATGC-3′). The amplified PCR products were subjected to gel electrophoresis to check the purity of amplicons, and specific band was purified and sequenced. The sequences obtained were compared for homology using BLASTN (NCBI) database [26]. The ITS sequence was subjected to multiple sequence alignment followed by phylogenetic analysis for molecular identification.

Phylogenetic Analyses: Phylogenetic analysis was performed under maximum-likelihood (ML) and maximum parsimony (MP) criteria. Parsimony analysis was carried with the heuristic search option in PAUP (Phylogenetic Analysis Using Parsimony) v. 4. Maximum likelihood trees were generated using the RAxML-HPC2 on XSEDE (8.2.8) [27] in the CIPRES Science Gateway platform [28] using a GTR+I+G model of evolution. Phylograms were visualized with FigTree v1.4.0 program and reorganized in Microsoft powerpoint (2007) and Adobe Illustrator® CS5 (Version 15.0.0, Adobe®, San Jose, CA).

Determination of Minimum Inhibitory Concentration (MIC): The MIC of *P. multirostrata* extract against *P. aeruginosa* was determined using a broth microdilution method with a bacterial concentration of 1×10^5 CFU/mL in LB broth following the guidelines of Clinical and Laboratory Standards Institute (CLSI) [29].

Violacein Inhibition Activity: In brief, an overnight culture of *C. violaceum* (MTCC 2656)

was inoculated into freshly prepared LB medium supplemented with and without *P. multirostrata* crude extract and incubated at 37 °C for 24 h. After incubation, the cultures were centrifuged at 13,000 rpm, 10 min for the precipitation of insoluble violacein. The pellets were resuspended in DMSO and the suspension was vigorously vortexed followed by recentrifugation at 13,000 rpm for 10 min. The absorbance of the supernatant was measured at 585 nm. The *C. violaceum* culture without PUTY3 extract served as control [29].

Growth Curve Analysis: The effect of sub-MIC concentration of PUTY3 extract on the growth and cell proliferation of *P. aeruginosa* PAO1 was monitored at 600 nm for 24 h at every 1 h interval by using spectrophotometer [30].

Effect of *P. multirostrata* extract on QS Regulated Virulence Factors of *P. aeruginosa*:

Pyocyanin Inhibition Activity: To measure the inhibition of pyocyanin pigment, the bacterium *P. aeruginosa* was inoculated with and without the sub MIC concentration of *P. multirostrata* extract, and after overnight incubation at 37 °C, pyocyanin pigment was extracted from cell-free supernatant using chloroform in the ratio of 3:2 and then re-extracted using 0.2 M HCl to obtain a pink coloured solution. The intensity of the solution was recorded at 520 nm [31].

LasA Protease Activity: Azocasein degrading activity was determined by adding overnight grown culture supernatant of *P. aeruginosa* (with treated or without treated) to 1 mL of 0.3% azocasein in 0.05M Tris–HCl (pH 7.5) and incubated at 37^{o} C for 15 min. Then 10% TCA was added to stop the reaction followed by centrifugation at 10,000 rpm for 5 min to get clear supernatant and absorbance was measured at 440 nm [32].

LasB Elastase Activity: This activity was measured following the method of Das *et al*., (2016). In brief, 100 µL of PUTY3 treated cell-free culture supernatant of *P. aeruginosa* was added to 900 µL of ECR buffer (100 mM Tris, 1 mM CaCl2, pH 7.5) containing 20 mg of Elastin Congo Red and was incubated at 37 °C for 3 h under appropriate shaking conditions. The reaction was stopped by using 1 mL of 0.7M sodium phosphate buffer (pH 6.0) and the tubes were kept in a cold water bath. The insoluble ECR was pelleted by centrifugation at 10,000 rpm for 10 min and the OD was recorded at 495 nm [32].

Motility Inhibition Assay:

Swimming and Swarming Inhibition Assays: The method mentioned by [33] was followed for swimming and swarming motility assays, PUTY3 extract-treated overnight culture of *P. aeruginosa* was point inoculated onto the center of specific swimming agar medium (tryptone 10 g/L, NaCl 5 g/L, Agar-agar 3 g/L). In case of swarming assay, overnight culture of *P. aeruginosa* was point inoculated at the center of swarming agar medium (10 g/L peptone, 5 g/L NaCl, 5 g/L agar and 5 g/L filter-sterilized glucose) having sub-MIC concentration of PUTY3 extract (750 µg/mL). All the plates were incubated at 37 °C for 24h.

Twitching Motility Assay: LB agar plates (10 g/L Bacto agar) with and without treatment with a sub-MIC concentration of PUTY3 extract were prepared for twitching motility assay. Overnight incubated cultures of *P. aeruginosa* were stabbed with a sterile toothpick through the LB agar layer to the bottom of the petridish and then incubated at 37 °C for 48 h. The bacterial adherence to the bottom of the petri plate was examined carefully after removing the agar and staining the adhered cells with crystal violet (0.1%, w/v) solution. The petri plates were then washed gently with tap water to remove un-adhered cells before staining. The diameter of the zone was then measured [34].

Congo Red Agar (CRA) Method (Qualitative Biofilm Production Assay): Briefly, sterilized aqueous solution of Congo red dye (0.8 g/L) was added to pre-cooled sterile medium containing brain heart infusion agar (BHI) (37 g/L), sucrose (50 g/L) and agar (10 g/L) supplemented with PUTY3 extract (750 µg/mL), fresh culture of *P. aeruginosa* was streaked on the CRA plates. The plates were incubated at 37 °C for 48 h and the color of the colonies formed was observed. Plate without *P. multirostrata* PUTY3 extract worked as control [35].

Rhamnolipid Inhibition Assay: Briefly, *P. aeruginosa* was grown in the presence and absence of PUTY3 fungal extract at 37 °C for 18 h in LB medium. Cultures were then centrifuged at 10,000

rpm for 5 min. The supernatant was added with equal portion of ethyl acetate and vortexed rigorously to yield rhamnolipid containing layer. The rhamnolipid containing organic phase was dissolved in 99% chloroform and freshly prepared methylene blue solution (1.4% (w/v), pH 8.6 ± 0.2) in a 10:1 ratio and the resultant was vortexed. The chloroform phase was transferred to a 0.2 N HCl solution and was vortexed again. Finally, OD of the upper acid phase containing the methylene blue complex was measured at 638 nm [36].

EPS Inhibition Assay: The EPS production in *P. aeruginosa* treated with *P. multirostrata* extract was performed as per the Gupta *et al*., (2017) with few modifications. In brief, overnight culture of *P. aeruginosa* was grown with and without PUTY3 extract at 37 °C for 18 h followed by centrifugation at 10,000 rpm, for 15 min and the collected pellets were resuspended in buffer (10 mM KPO_4, pH-7, 5 mM NaCl, 2.5 mM $MgSO_4$) and then recentrifuged at 10,000 rpm for 30 min. The supernatant containing displaced EPS was mixed with three volumes of chilled absolute ethanol and after overnight incubation at 4 °C, the precipitated EPS was added to a mixture of cold phenol (5%) and conc. H_2SO_4 and the absorbance were determined spectrophotometrically at 490 nm [37].

Effect of *P. multirostrata* extract on Alginate Production: Culture of *P. aeruginosa* was grown overnight with and without *P. multirostrata* extract. In brief, 500 µL of 1M NaCl was mixed to 500 µL of overnight bacterial culture and vortexed followed by centrifugation at 10,000 rpm for 20 min. then 500 µL of cetylpyridinium chloride was added in the supernatant, and mixed rigorously and recentrifuged at 10,000 rpm for 10 min. The pellet obtained was resuspended in 500 µL of cold isopropanol for 10 min. This mixture was then again recentrifuged at 10,000 rpm for 10 min, and finally, the obtained pellet was resuspended in 500 µL of 1M NaCl for overnight and alginate was quantified by carbazole assay [38, 39].

LasA Staphylolytic Activity: The LasA staphylolytic protease activity of *P. aeruginosa* was measured by determining its ability to lyse the boiled culture of *Staphylococcus aureus* on treatment with PUTY3 extract. In brief, a 30 mL overnight culture of *S. aureus* was boiled for 10-12 min. and then centrifuged for 10 min at 13,000 rpm, and the resulting pellet was resuspended in 10 mM Na_2PO_4 (pH 4.5). A 100 μL aliquot of *P. aeruginosa* test supernatant with the crude extract was added to 900 μL of the *S. aureus* suspension The OD of the mixture was recorded at every 15 min. interval up to two hours at 600 nm [40]. The percentage of inhibition was calculated as follow,

Staphylolytic activity (%) = Change in OD_{600}/h in control – change in OD_{600}/h in treated sample × 100 / Change in OD_{600}/h in control

Biofilm Inhibition Activity of *P. multirostrata* PUTY3 extract in *P. aeruginosa* (MTP or Microtiter Plate Assay): The biofilm formation activity of *P. aeruginosa* after treated with *P. multirostrata* extract was quantified by following the method of Luciardi *et al*., (2016) with a slight modification. The biofilms formed after 24 h of incubation were stained with an aqueous solution of crystal violet (0.1% w/v) for 20 min followed by washing with water. The biofilms that remained fixed to the polystyrene were gently washed with PBS thrice. The stain bound to biofilm was then dissolved by adding 95% ethanol and the OD was measured at 540 nm [41].

Determination of Cell Surface Hydrophobicity (CSH): For determination of CSH, an overnight culture of *P. aeruginosa* containing with and without *P. multirostrata* extract was grown for 24 h and centrifuged at 12,000 rpm for 2 min. The pellets were washed gently and homogenized in PUM buffer (22.2 g Potassium phosphate trihydrate, 7.26 g Monobasic potassium phosphate, 1.8 g Urea, and 0.2 g Magnesium sulphate heptahydrate/ litre (pH 7.1). 100 µL suspension was taken out to measure the initial cell density (ICD). 400 µL of toluene was added in the rest of suspension and vortexed. The aqueous layer was collected, and the final cell density (FCD) was measured [42]. The percentage of hydrophobicity was calculated as follows:

Hydrophobicity (%) = (ICD-FCD) × 100 / ICD

Inhibition of Hydrogen Cyanide Production: Hydrogen cyanide (HCN) production was observed by the change in the color of the Whatman no. 1 filter paper. Briefly, *P. aeruginosa* PAO1 was streaked on to nutrient agar medium supplemented

with 4.4 g/L of glycine and with and without *P. multirostrata* extract. Filter papers were saturated in a solution (2% sodium carbonate and 0.5% picric acid) and kept in the upper lid of Petri dishes. Plates were sealed with parafilm appropriately to avoid the gas from escaping. Plates were incubated at 28 °C for 4 days. One plate without inoculation of the bacterium was considered as a negative control, while one plate without the fungal extract was considered as a positive control. Production of HCN gas turns yellow filter papers into cream, light brown, dark brown and finally, into reddish-brown color [43].

Microscopic Observation of Adherence of Bacterial Cells on Coverslip: Fresh culture of *P. aeruginosa* was added to the sterilized LB broth, with and without the supplementation of *P. multirostrata* extract, and applied on to sterilized coverslips, was incubated at 37 °C for 16 h. After incubation, the coverslips were washed gently with PBS to remove unattached cells. Then the coverslips with the adhering bacterial cells were stained with a 0.4% crystal violet solution and observed under differential interference contrast (DIC) microscope at 100X magnification [38]. In the case of fluorescence microscopic observation, the bacterial biofilms adhering to the coverslips were stained with 0.01% acridine orange for 15-20 min in dark and allowed to air dry. Adherent cells were fixed by using 2.5% glutaraldehyde. Then the coverslips were observed under a fluorescence microscope at 40X magnification [32].

GC-MS Analysis of *P. multirostrata* Extract: GC-MS analysis of the *P. multirostrata* crude extract was performed to detect the presence of bioactive compounds. The GC-MS analysis was accomplished at a sophisticated instrumentation facility, Vellore Institute of Technology, 632 014, TN, India.

***In-silico* Studies:**

Modeling and Structure Validation: *P. aeruginosa* QS network consists of two main circuits, known as LasI/R and RhlI/R. LasI and RhlI produce N-(3-oxododecanoyl)- homoserine lactone (OdDHL) and N-butyrylhomoserine lactone (BHL), respectively which bind to their corresponding receptors *i.e.* LasR and RhlR and regulate the QS regulated virulence genes. To check the binding affinity of a compound present in the *P. multirostrata* PUTY3 secondary metabolite, *in-silico* studies were carried out. The structure of LasR protein was downloaded from the PDB database (PDB ID: 2uv0, Chain H), because the crystal structure was not available in the PDB database, the amino acid sequence of RhlR protein was retrieved from the UniProt database (ID: P54292.1) and submitted to the online ROBETTA server for the prediction of three-dimensional structure. Finally, the obtained 3D models were confirmed using the online RAMPAGE server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) and the qualities of the predictive models were refined by Verify 3D structure evaluation server (http://nihserver.mbi.ucla.edu/Verify3D/), the best model was preferred for docking studies [44]. The ligand-binding site of both the proteins was predicted *via* Coach Webserver (https://zhanglab.ccmb.med.umich.edu/COACH/). The ligands used for docking studies include the natural ligand, positive control, and ligands from the fungal secondary metabolites for LasR and RhlR. The preparation of ligand, Proteins, and docking studies were performed using PyRx software [45], which comprises AutoDock [46] and AutoDock Vina [47] using the Lamarckian genetic algorithm.

The docking grid for both the receptor proteins (LasR and RhlR), were set around the active site residues of both the individual proteins. The conformations, which showed the strongest binding affinity, were chosen for analysis and LigPlot+ was used for calculating the H-bonding and hydrophobic interactions between the ligand and the protein complex.

Statistical Analysis: All the tests were performed in triplicates. The results were calculated with mean ± standard deviation (SD) values. One-way ANOVA followed by Tukey's test was carried out by using the SPSS software version 20.0, at 5% level of significance. *values significantly differed from 250 µg/mL, because of $P<0.05$.

RESULTS:

Quorum Quenching Activity of *P. multirostrata* crude Extracts: A Total of 46 fungal isolates were isolated from leaf litter samples through a modified particle filtration technique [23]. Among the 46 fungal isolates, PUTY3 crude extract showed

highest anti quorum sensing activity against model organisms *C. violaceum* and *P. aeruginosa* PAO1 with a zone of inhibition of 27 and 20 mm, respectively, at a concentration of 250 µg/ml when compared to other isolates which showed lower values for inhibition zones. Hence, PUTY3 extract was selected for further studies against *P. aeruginosa* PAO1.

Molecular Identification of PUTY3 fungal isolate: The BLAST search analyses of sequences of PUTY3 at NCBI website revealed that our selected fungal isolate (PUTY3) showed a 100% homology with *Phoma multirostrata*. The ITS gene dataset comprised 20 taxa, including our newly sequenced taxon along with *Verticillium nubilum* as the out-group. The trees generated under ML and MP analyses were similar in overall topologies. Phylogeny inferred from ML and MP analyses depicts that our taxon nested with *Phoma multirostrata* with moderate bootstrap support **Fig. 1**. Values greater than 0.60 are given above each node. Our sequence generated was submitted to the NCBI GenBank with the name *Phoma multirostrata* PUTY3 with KX925846 as its accession number.

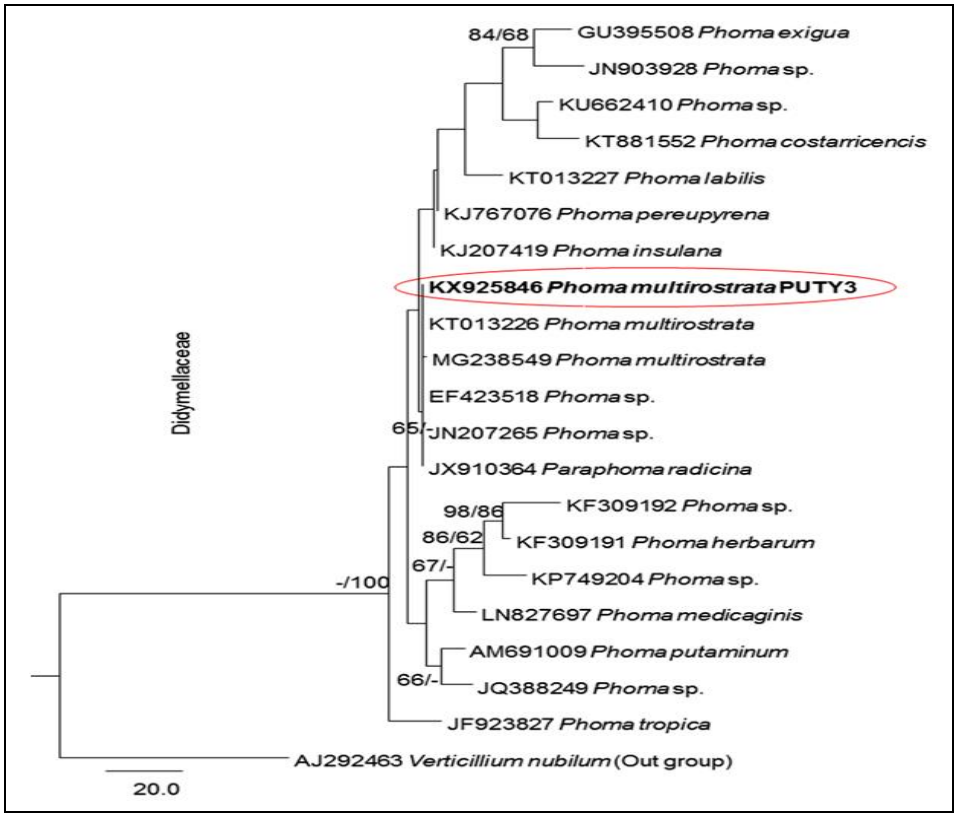

FIG. 1: PAUP TREE CONSTRUCTED BASED ON ANALYSIS OF ITS PARTIAL SEQUENCE DATA. BOOTSTRAP SUPPORT VALUES FOR ML AND MP HIGHER THAN 60% ARE GIVEN ABOVE EACH BRANCH, RESPECTIVELY. THE FUNGAL ISOLATE OF THE PRESENT STUDY IS INDICATED IN BOLD (*PHOMA MULTIROSTRATA* PUTY3). THE TREE IS ROOTED TO *VERTICILLIUM NUBILUM*

Determination of MIC and Sub-MIC: For MIC determination, the effect of different concentrations of *P. multirostrata* PUTY3 extract (from 2000 µg/ml to 15.625 µg/ml), on the growth of *P. aeruginosa* PAO1, was determined. It was found that at the concentration of 1200 µg/ml and beyond that no turbidity was observed. Hence, 1200 µg/ml was used as MIC and three sub-MIC concentrations (250, 500 and 750 µg/ml) were used for further studies.

Violacein Inhibition Assay: *P. multirostrata* PUTY3 extract significantly inhibited the production of violacein by *C. violaceum* as compared to control with 41.63 ± 2.15, 55.54 ± 1.84 and 83.04 ± 2.1% of inhibition at 250, 500 and 750 µg/ml concentrations, respectively **Fig. 2B**.

Growth Curve Analysis: From the growth curve analysis, it was observed that *P. aeruginosa* PAO1 treated with *P. multirostrata* PUTY3 extract

showed a traditional sigmoid growth curve similar to the control but at a slightly slower growth rate as compared to the untreated control as shown in **Fig. 2A**.

Effect of *P. multirostrata* PUTY3 Extract on Quorum Sensing Regulated Virulence in *P. aeruginosa* PAO1:

Pyocyanin Inhibition Assay: The sub-MIC concentrations of *P. multirostrata* PUTY3 extract significantly inhibited the production of pyocyanin as compared to control with 54.56 ± 1.9, 68.8 ± 1.12 and 73.62 ± 1.19% of inhibition at 250, 500 and 750 µg/ml concentrations, respectively **Fig. 2B**.

LasA Staphylolytic Activity: A significant reduction in LasA Staphylolytic activity, as compared to the control, was observed when *P. aeruginosa* PAO1 was treated with sub-MIC levels of *P. multirostrata* PUTY3 extract with inhibition of 42.24 ± 4.59, 59.19 ± 2.98, and 74.85 ± 1.77% at 250, 500 and 750 µg/ml concentrations, respectively **Table 1**.

TABLE 1: EFFECT OF *P. MULTIROSTRATA* EXTRACT (250, 500 AND 750 µg/mL) ON STAPHYLOLYTIC ACTIVITY, BIOFILM FORMING ACTIVITY AND CELL SURFACE HYDROPHOBICITY IN *P. AERUGINOSA*

Concentrations of *P. multirostrata* extracts (µg/mL)	Staphylolytic activity	Biofilm formation assay	Cell surface hydrophobicity (CSH) assay
250	42.24 ± 4.59	41.64 ± 2.08	56.16 ± 3.11*
500	59.19 ± 2.98*	62.79 ± 1.76*	42.63 ± 4.08*
750	74.85 ± 1.77*	74.21 ± 2.28*	34.25 ± 2.14

LasA Protease Activity: The azocasein degrading ability of *P. aeruginosa* PAO1 was significantly reduced on treatment with sub-MIC levels of *P. multirostrata* PUTY3 extract with an inhibition of 35.44 ± 1.74 47.21 ± 3.79 and 65.53 ± 1.91% at 250, 500 and 750 µg/ml concentrations, respectively **Fig. 2B**.

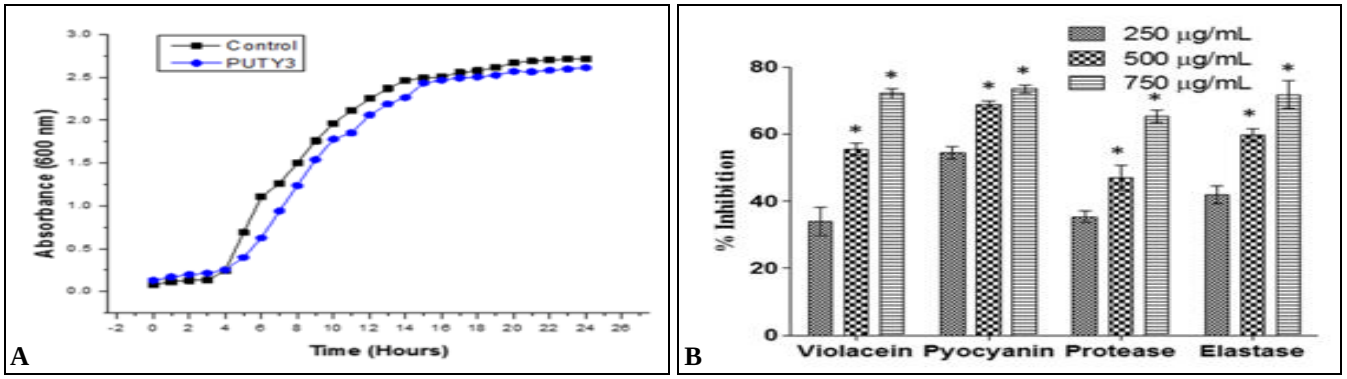

FIG. 2: (A) THE EFFECT OF *P. MULTIROSTRATA* EXTRACT AT A DOSE OF 500 µg/mL ON THE GROWTH OF *P. AERUGINOSA* IN COMPARISON TO CONTROL. (B) EFFECT OF *P. MULTIROSTRATA* EXTRACT AT CONCENTRATIONS OF 250, 500, AND 750 µg/mL ON VIOLACEIN PRODUCTION IN *C. VIOLACEUM* AND PYOCYANIN, PROTEASE, ELASTASE PRODUCTION IN *P. AERUGINOSA*

LasB Elastase Activity: The elastolytic activity of *P. aeruginosa* PAO1 was significantly inhibited on supplementation with sub-MICs of *P. multirostrata* PUTY3 extract. **Fig. 2B** depicted the reduction in elastolytic activity of *P. aeruginosa* PAO1, in a concentration-dependent manner, with inhibition of 42.01 ± 2.63, 59.75 ± 1.83 and 71.95 ± 4.17% at 250, 500 and 750 µg/ml concentrations, respectively.

Anti-motility Activity: From the motility assays, it was observed that *P. multirostrata* PUTY3 extract characteristically inhibited swimming, swarming and twitching motility of *P. aeruginosa* PAO1 with inhibition of 92.55 ± 1.81, 82.68 ± 1.77 and 91.65 ± 1.95% respectively at 750 µg/ml concentrations as shown in **Fig. 3**.

Effect of *P. multirostrata* PUTY3 Extract on Quorum Sensing Regulated Biofilm Formation in *P. aeruginosa* PAO1:

Biofilm Formation Assay (MTP Method): *P. multirostrata* PUTY3 extract significantly inhibited the formation of biofilm in *P. aeruginosa* PAO1. The observed biofilm inhibition was 41.64 ± 2.08, 62.79 ± 1.76 and 74.21 ± 2.28% at concentrations of 250, 500 and 750 µg/ml, respectively **Table 1**.

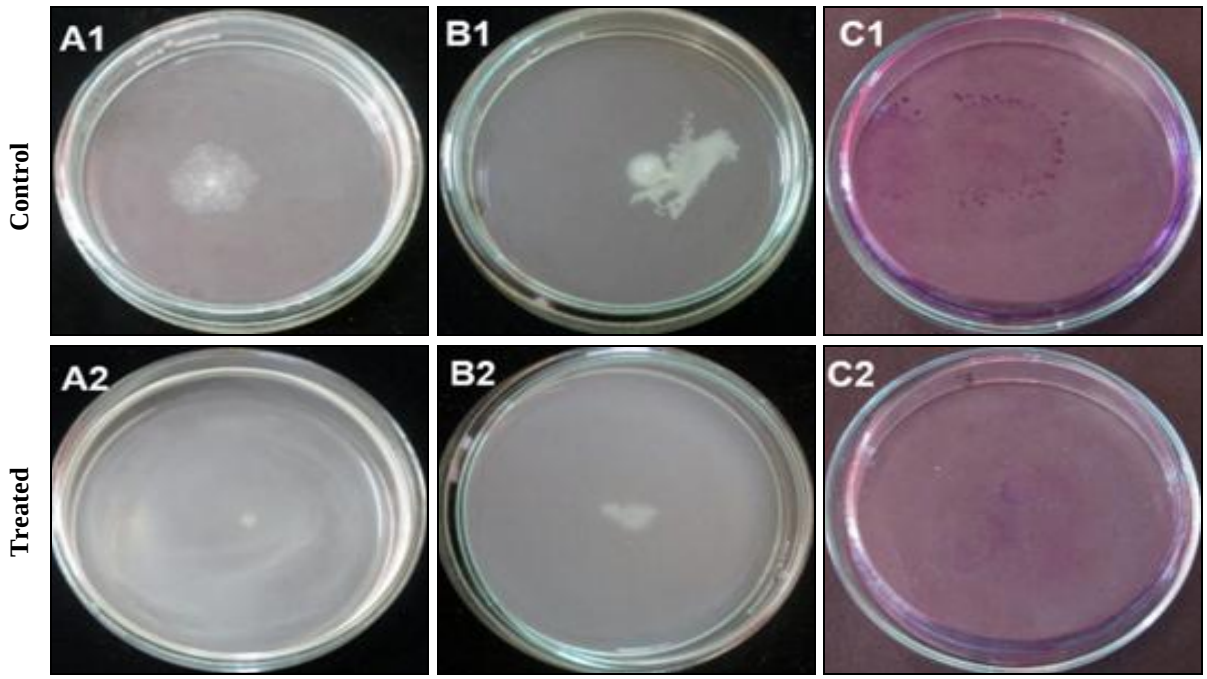

FIG. 3: EFFECT OF *P. MULTIROSTRATA* EXTRACT AT A CONCENTRATION OF 750 µg/mL ON MOTILITIES OF *P. AERUGINOSA*. SWARMING MOTILITY, CONTROL *vs*. TREATED WITH *P. MULTIROSTRATA* EXTRACT (A1, A2), SWIMMING MOTILITY, CONTROL *vs*. TREATED WITH *P. MULTIROSTRATA* EXTRACT (B1, B2), TWITCHING MOTILITY, CONTROL *vs*. TREATED WITH *P. MULTIROSTRATA* EXTRACT (C1, C2).

Biofilm Formation Assay by CRA Method: The results of the biofilm formation assay by CRA method showed that in the presence of *P. multirostrata* PUTY3 extract, the production of EPS was inhibited in *P. aeruginosa* PAO1 as was evident from the formation of whitish colonies when compared to the control that showed dark black crystalline colonies **Fig. 4L**.

EPS Inhibition Assay: The sub-MIC doses of *P. multirostrata* PUTY3 extract showed 39.38 ± 1.26, 46.42 ± 2.39 and 74.41 ± 1.66% of reduction in the EPS production of *P. aeruginosa* PAO1 as compared to the control **Fig. 4K**.

Rhamnolipids Inhibition Assay: When the concentrations of *P. multirostrata* PUTY3 extract were increased from 250 to 750 µg/ml, a concomitant decrease in the production of rhamnolipids with inhibition of 38.11 ± 2.01, 47.14 ± 1.59 and 63.94 ± 2.12% respectively was observed **Fig. 4K**.

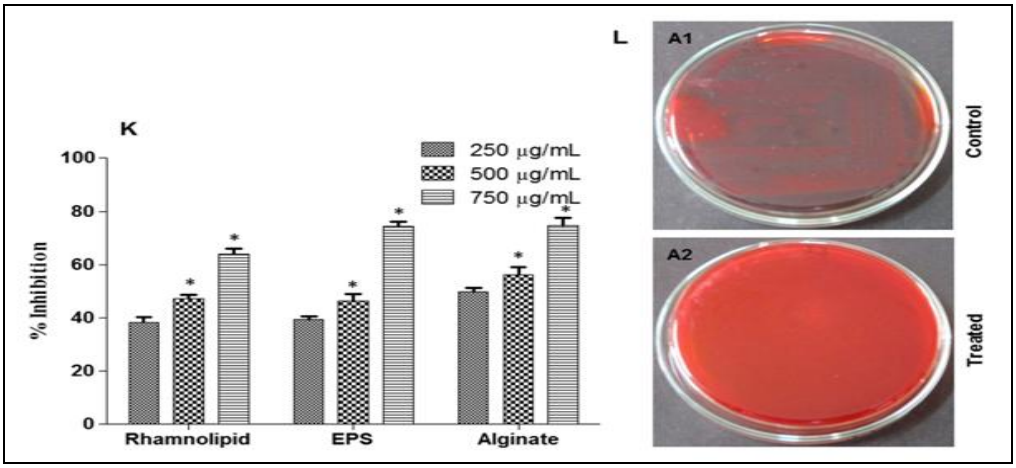

FIG. 4: (K) EFFECT OF *P. MULTIROSTRATA* EXTRACT AT CONCENTRATIONS OF 250, 500, AND 750 µg/mL ON RHAMNOLIPID, EPS AND ALGINATE PRODUCTION IN *P. AERUGINOSA*. (L) EFFECT OF *P. MULTIROSTRATA* EXTRACT AT A CONCENTRATION OF 750 µg/mL ON BIOFILM FORMATION IN *P. AERUGINOSA* BY CONGO RED AGAR (CRA) METHOD, CONTROL (A1) *vs*. TREATED WITH *P. MULTIROSTRATA* EXTRACT (A2)

Cell Surface Hydrophobicity Assay: The treatment with the increasing sub-MIC concentrations of *P. multirostrata* PUTY3 extract resulted in a significant decrease in the cell-to-surface attachment ability of *P. aeruginosa* PAO1. The hydrophobicity percentage of 56.16 ± 3.11, 42.63 ± 4.08 and 34.25 ± 2.14% was observed at 250, 500 and 750 µg/ml concentrations, respectively **Table 1**.

Alginate Production: The sub-MIC doses of *P. multirostrata* PUTY3 extract showed 49.7 ± 1.56, 56.08 ± 2.94 and 74.78 ± 2.88% of reduction in the Alginate production of *P. aeruginosa* PAO1 at 250, 500 and 750 µg/ml concentrations, respectively as compared to the control **Fig. 4K**.

HCN Gas Production: The HCN production is found to be a common trait of *Pseudomonas* (88.89%). A significant HCN reduction was observed as yellow filter paper changed to very light brown color after treatment with *P. multirostrata* PUTY3 extract as compared to the negative (impregnated with the solution but without *P. aeruginosa*) and positive (without PUTY3 extract) controls, which were shown in yellow and dark brown colors, respectively **Fig. 5Z**.

Microscopic Analysis of Bacterial Biofilms: The light microscopic analysis of bacterial biofilm exhibited a significant decrease in the density and coverage of the biofilm formed in bacterial cells grown in the presence of *P. multirostrata* PUTY3 extract as compared to the control. Similarly, fluorescence microscopic analysis of biofilms formed by *P. aeruginosa* PAO1 supplemented with *P. multirostrata* PUTY3 extract showed a marked decrease in the attachment of biofilm-forming cells with scattered appearance as compared to the control with prominent biofilm formation over the glass surface **Fig. 5Y**.

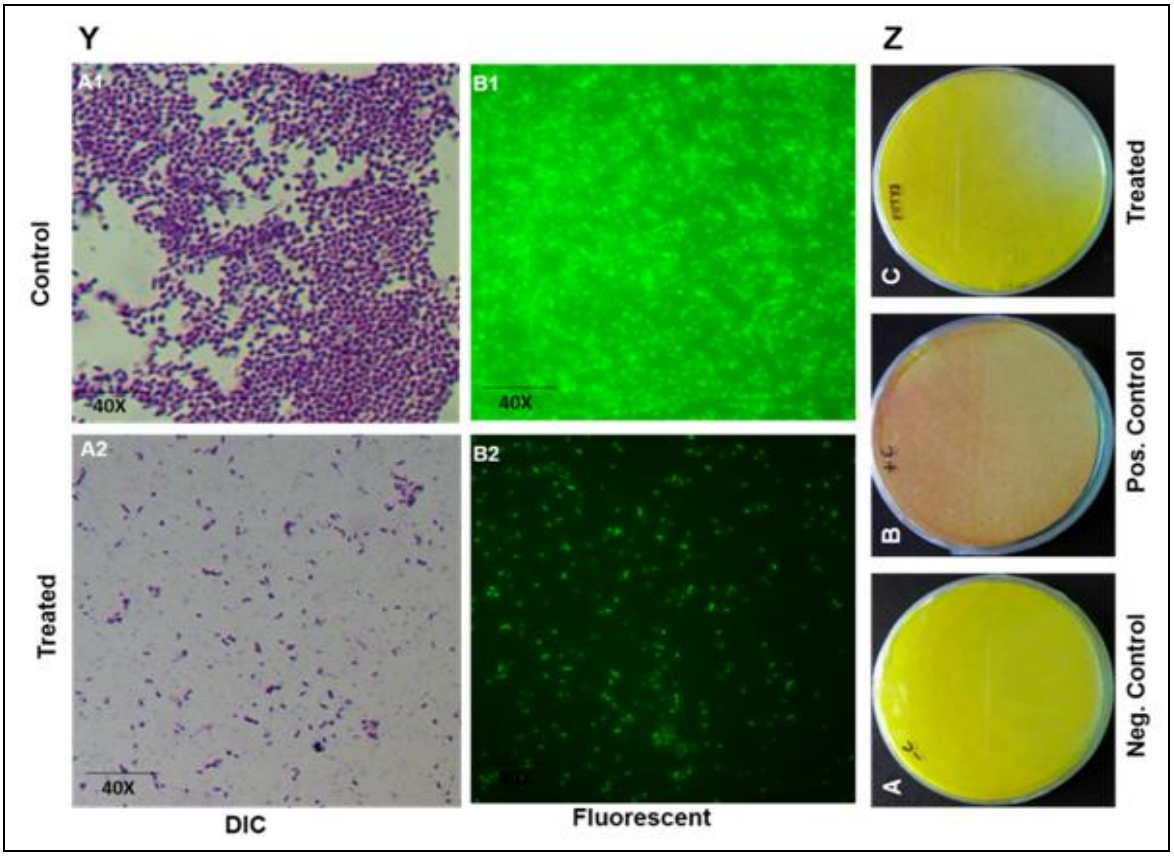

FIG. 5: (Y) MICROSCOPIC OBSERVATION OF EFFECT OF *P. MULTIROSTRATA* EXTRACT AT 750 µg/mL DOSE ON BIOFILM FORMATION BY *P. AERUGINOSA*. DIFFERENTIAL INTERFERENCE CONTRAST *(DIC) MICROSCOPIC* OBSERVATION OF BIOFILM FORMATION BY *P. AERUGINOSA* WHEN TREATED BY *P. MULTIROSTRATA* EXTRACT IN COMPARISON TO CONTROL (A1, A2). FLUORESCENCE MICROSCOPIC OBSERVATION OF BIOFILM FORMATION WHEN TREATED BY *P. MULTIROSTRATA* EXTRACT IN COMPARISON TO CONTROL (B1, B2). (Z)EFFECT OF *P. MULTIROSTRATA* EXTRACT AT A CONCENTRATION OF 750 µg/mL ON HCN PRODUCTION BY *P.AERUGINOSA*. A, B AND C REPRESENT NEGATIVE CONTROL, POSITIVE CONTROL AND *P. MULTIROSTRATA* EXTRACT TREATED PLATES, RESPECTIVELY

GC-MS Analysis: The bioactive compounds present in the fungal crude extract of *P. multirostrata* PUTY3 were identified using the GC-MS analysis. The major bioactive compounds observed from GC-MS analysis in *P. multirostrata* PUTY3 extract were Phenol, 2,4-Bis(1,1-Dimethyl-ethyl)-, 1,2-Benzenedicarboxylic acid, Bis(2-Methylpropyl) Ester, 1,1-Dichloro-2-Methyl-3-(4,4-Diformyl-1,3-Butadien-1-Yl)Cyclopropane, 1-Heptacosanol, 1-Heneicosanol, Hexacosyl Pentafluoropropionate, Hexacosyl Trifluoroacetate, Adamantane-1-Carboxamide, N-(4-Chlorobenzyl)-**.** The molecularweight, molecular formula, retention time, peak area (%) are presented in **Table 2**.

TABLE 2: LIST OF SECONDARY METABOLITES PRESENT IN THE CRUDE EXTRACT OF *P. MULTIROSTRATA* PUTY3 IDENTIFIED BY GC-MS ANALYSIS

S. no.	Bioactive compound	Retention time	Molecular formula	Molecular weight	Peak area (%)
1	2-Butanone, 4-Hydroxy	2.863	$C_4H_8O_2$	88	2.707
2	1-Tetradecene	12.362	$C_{14}H_{28}$	196	5.183
3	Phenol, 2,4-Bis(1,1-Dimethylethyl)-	13.263	$C_{14}H_{22}O$	206	8.965
4	E-15-Heptadecenal	14.153	$C_{17}H_{32}O$	252	10.698
5	1-Heptadecene	16.074	$C_{17}H_{34}$	238	11.532
6	1,2-Benzenedicarboxylic Acid, Bis(2-Methylpropyl) Ester	16.939	$C_{16}H_{22}O_4$	278	12.173
7	1-Heneicosanol	17.935	$C_{21}H_{44}O$	312	10.578
8	1-Nonadecene	19.695	$C_{19}H_{38}$	266	5.028
9	1-Heptacosanol	21.371	$C_{27}H_{56}O$	396	14.451
10	Adamantane-1-Carboxamide, N-(4-Chlorobenzyl)-	22.631	$C_{18}H_{22}ONCl$	303	6.106
11	1,1-Dichloro-2-Methyl-3-(4,4-Diformyl-1,3-Butadien-1-Yl)Cyclopropane	22.757	$C_{10}H_{10}O_2Cl_2$	232	2.569
12	Hexacosyl Trifluoroacetate	22.972	$C_{28}H_{53}O_2F_3$	478	3.149
13	Hexacosyl Pentafluoropropionate	24.557	$C_{30}H_{55}O_2F_5$	542	1.446

Analysis of Docking Study: Binding affinity, residues forming hydrophobic interaction and hydrogen bonds were showed in great detail in **Table 3**. The results obtained from the docking studies revealed that 1,1-Dichloro-2-Methyl-3-(4,4-Diformyl- 1, 3- Butadien- 1- yl) Cyclopropane exhibited the maximum interaction with LasR, as observed by its docking score of -7.49 kcal/mol, with one H-bond (2.86 Å) with Arg-61 of active site residue, while the seven other residues formed hydrophobic and Van der Waal interactions within the catalytic site, which was almost close to its natural autoinducer (3-Oxo-C12-HSL), showed -7.52 and less to positive control, baicalein (a prominent quorum quenching agent), which showed -8.17 docking score. The compound Phenol, 2,4-Bis(1,1-Dimethylethyl) showed lower docking score as compared to the natural autoinducer, which showed -5.34 docking score and hydrophobic and Van der Waal interactions with three active site residues and ten neighboring residues.

TABLE 3: DOCKING CHARACTERISTICS OF THE KNOWN NATURAL CONTROL AND POSITIVE CONTROL LIGANDS AND THE COMPOUNDS SHOWING HIGHER BINDING AFFINITY WITH RECEPTORS LasR AND RhlR OF *PSEUDOMONAS AERUGINOSA* ARE TABULATED ALONG WITH THE INTERACTING RESIDUES (HYDROPHOBIC INTERACTIONS AND HYDROGEN BONDING)

Receptor	S. no.	Ligand	Binding affinity (kcal/mol)	Interacting residues (H-bond length in Å^o)
LasR	1	Natural ligand for LasR (3-Oxo-C12-HSL)	-7.52	L36, L40, Y47, A50, Y56 (2.77), W60 (3.11) R61, Y64, D73 (2.98), T75 (3.22), W88, Y93, F101, L110, L125, A127, V176
	2	Positive ligand for LasR (Baicalein)	-8.17	L36, G38, L39, L40, Y56 (3.19), R61 (2.82), Y64, D73, T75 (2.61), T115 (2.91), L125, G126, A127, S129, V176
	3	Phenol, 2,4-Bis(1,1-Dimethylethyl)-	-5.34	L36, G38, Y47, I52, Y56, R61, Y64, D65, A70, D73, A127, S129, V176
	4	1,1-Dichloro-2-Methyl-3-(4,4-Diformyl-1,3-Butadien-1-yl)Cyclopropane	-7.29	L36, Y56, R61 (2.86), Y64, D73, V76, G126, S129
RhlR	5	Natural ligand for RhlR (C4-	-4.85	V60, Y64, W68 (2.78), L69, Y72, D81, I84,

	HSL)		W96, L116, S135
6	Positive ligand for RhlR (Furanone C30)	-5.13	A44, Y45, H61, Y64, W68 (2.83), L69, Y72, V160
7	1,2-Benzenedicarboxylic Acid, Bis(2-Methylpropyl) Ester	-4.47	A44, Y45, G46, V60, H61, G62, Y64, W68 (3.31), L69, D81, I84, W96, Y72, L116, S135
8	Hexacosyl Pentafluoropropionate	-2.78	R48 (2.87), F53, T54, P56, T58, Q73, G78, A79, L88

In the case of RhlR, 1,2-Benzenedicarboxylic Acid, bis(2-Methylpropyl) ester, showed highest docking score of -4.47 kcal/mol and formed 1 H-bond (2.83Å) with Trp-68, whereas fourteen other neighbouring residues showed hydrophobically and Van der Waal interactions within the active site as compared to natural autoinducer (C4-HSL), which was showed docking score of -4.85 kcal/mol. While Hexacosyl Pentafluoropropionate showed lower docking score of -2.78 with one hydrogen bond with Arg-48 (2.87Å) and eight hydrophobic interactions within the catalytic site **Fig. 6**.

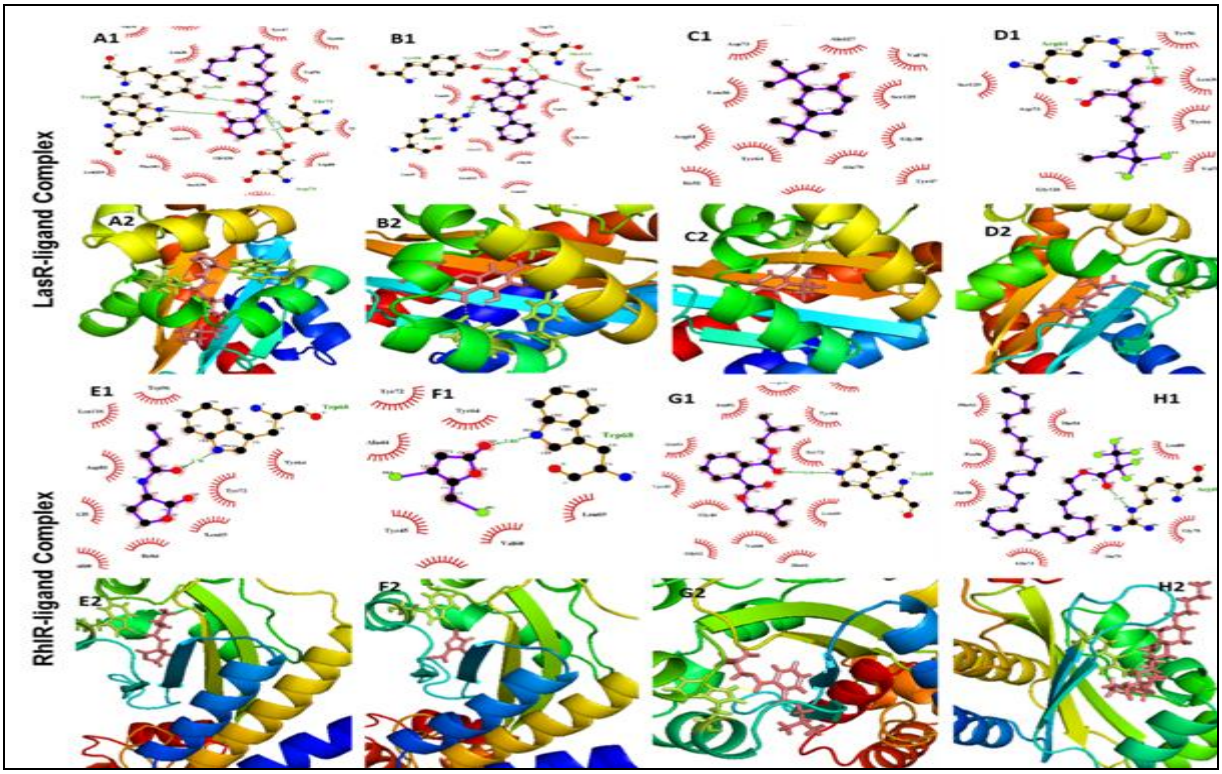

FIG. 6: MOLECULAR DOCKING STUDIES OF PHENOL, 2,4-BIS(1,1-DIMETHYLETHYL)- AND 1,1-DICHLORO-2-METHYL-3-(4,4-DIFORMYL-1,3-BUTADIEN-1-YL)CYCLOPROPANE, BIOACTIVE COMPONENTS OF *P. MULTIROSTRATA* EXTRACT TO INVESTIGATE THE INTERACTION WITH THE RESIDUES OF ACTIVE SITE OF LASR PROTEIN, COMPARED TO NATURAL LIGAND AND POSITIVE CONTROL. (A1) LIGPLOT VIEW OF NATURAL LIGAND (C12-HSL) INTERACTING WITH ACTIVE SITE RESIDUES OF LASR PROTEIN, (A2) 3D VIEW OF NATURAL LIGAND-LASR PROTEIN COMPLEX, (B1) LIGPLOT VIEW OF POSITIVE CONTROL (BAICALEIN) INTERACTING WITH ACTIVE SITE RESIDUES OF LASR, (B2) 3D VIEW OF POSITIVE CONTROL-LASR PROTEIN COMPLEX, (C1) LIGPLOT VIEW OF PHENOL, 2,4-BIS(1,1-DIMETHYLETHYL)- INTERACTING WITH ACTIVE SITE RESIDUES OF LASR, (C2) 3D VIEW OF PHENOL, 2,4-BIS(1,1-DIMETHYLETHYL)- LASR PROTEIN COMPLEX, (D1) LIGPLOT VIEW OF 1,1-DICHLORO-2-METHYL-3-(4,4-DIFORMYL-1,3-BUTADIEN-1-YL)CYCLOPROPANEINTERACTING WITH ACTIVE SITE RESIDUES OF LASR, (D2) 3D VIEW OF 1,1-DICHLORO-2-METHYL-3-(4,4-DIFORMYL-1,3-BUTADIEN-1-YL)CYCLOPROPANE-LASR PROTEIN COMPLEX, WHEREAS MOLECULAR DOCKING STUDIES OF1,2-BENZENEDICARBOXYLIC ACID, BIS(2-METHYLPROPYL) ESTER AND HEXACOSYL PENTAFLUOROPROPIONATE BIOACTIVE COMPONENT OF *P. MULTIROSTRATA* EXTRACT TO INVESTIGATE THE INTERACTION WITH THE RESIDUES OF ACTIVE SITE OF RHLR PROTEIN, AS COMPARED TO NATURAL LIGAND AND POSITIVE CONTROL. (E1) LIGPLOT VIEW OF NATURAL LIGAND (C4-HSL) INTERACTING WITH ACTIVE SITE RESIDUES OF RHLR, (E2) 3D VIEW OF NATURAL LIGAND-RHLR PROTEIN COMPLEX, (F1) LIGPLOT VIEW OF POSITIVE CONTROL (FURANONE C30) INTERACTING WITH ACTIVE SITE RESIDUES OF RHLR, (F2) 3D VIEW OF POSITIVE CONTROL-RHLR PROTEIN COMPLEX, (G1) LIGPLOT VIEW OF 1,2-BENZENEDICARBOXYLIC ACID, BIS(2-METHYLPROPYL) ESTER INTERACTING WITH ACTIVE SITE RESIDUES OF RHLR, (G2) 3D VIEW OF 1,2-BENZENEDICARBOXYLIC ACID, BIS(2-METHYLPROPYL) ESTER-RHLR PROTEIN COMPLEX, (H1) LIGPLOT VIEW OF HEXACOSYL PENTAFLUOROPROPIONATE INTERACTING WITH ACTIVE SITE RESIDUES OF RHLR, (H2) 3D VIEW OF HEXACOSYL PENTAFLUOROPROPIONATE-RHLR PROTEIN COMPLEX

DISCUSSION: It is a well-known fact that filamentous fungi produce SMs into the surrounding vicinity that help in various processes, such as chemical defense against microorganisms [48, 49]. However, the production of these bioactive SMs, must often be triggered by environmental factors, for example, by limiting availability of nutrients, differences in the pH, salinity, temperature and even the competitive pressure by co-culture with other microorganisms [50, 51]. On the basis of morphological and molecular studies, the selected fungal culture PUTY3 was identified as *Phoma multirostrata* (KX925846), which formed a distinct monophyletic clade with other *P. multirostrata* strains. The SMs from the strains belonging to the genus Phoma contribute many bioactive molecules such as topopyrone B as anti-herpesvirus [52], furopyrans a phytotoxic compound [53], pyrenophorol an antifungal macrolide [54], α-pyrones [55], cytotoxic spiciferones [56] and immunomodulatory polyketides [57], protein tyrosine phosphatase 1B (PTP1B) inhibitory biphenyl ether [58] and (10′*S*)-verruculide B [59].

As far as we know, there was no report of anti-quorum sensing activity of *Phoma multirostrata* against the *Pseudomonas aeruginosa* in the literature. Targeting bacteria by conventional antibiotics can be ineffective when the bacteria form biofilms under stress conditions. Hence, present therapies that target these pathogenic bacteria are quite insufficient and infection due to these bacteria lead to serious threats for human beings [60]. Thus, there is a need for alternative treatment strategies that target such kinds of pathogenic bacteria. It was suggested that it is a safer way to leave bacteria vulnerable by targeting their intercellular communications without inducing stress. The strategy of targeting bacterial communications prevents the secretion of bacterial virulence factors and biofilm formation by using the QSIs [61]. Like plant secondary metabolites, molecules present in secondary metabolites of fungi are important sources of targeting the QS process. Thus, based on the preliminary screening, secondary metabolites of *P. multirostrata* were selected for the present study to attenuate the QS process in *P. aeruginosa* and the fungus was identified by morphological and molecular techniques. Violacein, a purple pigment, produced by a modal organism *C. violaceum*, which is an important organism for checking QS activity, has been undertaken in the present study. *P. multirostrata* PUTY3 extract exhibited a dose-dependent reduction in violacein pigment production, a result that is corroborative with a previous report [16].

To develop an anti-infective drug, it is important to target the virulence of the organism rather than killing the organism. It is observed from the growth curve study that *P. aeruginosa* treated with PUTY3 extract showed the sigmoid curve, quite similar to the control, thus suggesting the presence of potential anti-infective activity, as it has not affected the growth of the microorganism. Induction of oxidative stress by a generation of reactive oxygen intermediates and correlation with the severity of disease by Pyocyanin makes *P. aeruginosa*a more virulent pathogen. In this study, *P. multirostrata* extract significantly reduced the production of pyocyanin in a dose-dependent manner with a higher reduction (73%) at 750 µg/ml concentration, which was comparatively higher than the *Plectosphaerella cucumerina* (70%) at 1000 µg/ml concentration [62]. Proteases of *P. aeruginosa* also play an important role as it helps in the bacterial invasion and growth by degrading the host cell protein in the infected tissue. A significant reduction in the protease production was observed when *P. aeruginosa* was treated with increasing concentrations of *P. multirostrata* extracts. The result showed the efficacy of *P. multirostrata* extract in reducing the LasA protease activity, which is accountable for lysis of heat-killed *S. aureus* cells by changing the stability of peptidoglycan. Furthermore, a significant reduction in the azocasein degrading ability of *P. aeruginosa* was also observed when bacteria treated with a sub-MIC concentration of fungal extract suggesting that fungal crude extract attenuating the *P. aeruginosa* colonization efficiency in the host tissues [62].

Similarly, the production of elastase was investigated and it was found that degradation of elastin congo red substrate was reduced by 71.9% which was more than the previous report which showed a 32-35% decrease in the elastase production [63]. LasB elastase is capable of destroying host tissue and inactivate a wide range of immunological components, thus helping in the invasion and colonization of pathogens. Moreover,

it also helps in the swarming motility of bacteria [64]. The motility of the bacteria also plays a crucial role in the formation of biofilm and in pathogenicity. In this study, significant inhibition was observed in the swarming, swimming and twitching activities when *P. aeruginosa* PAO1 was treated with *P. multirostrata* PUTY3 extract (750 µg/ml) in comparison to untreated control [33]. The mucoid nature of *P. aeruginosa* in the lung of cystic fibrosis (CF) patients, with the subsequent production of EPS, plays a crucial role in the development of robust biofilm formation and multi-drug resistant infection. Therefore, a study was carried out against the Rhl mediated EPS, rhamnolipid and alginate production by *P. aeruginosa*.

The results were encouraging as *P. multirostrata* extract was found to decrease the production of EPS, rhamnolipid and alginate significantly in a dose-dependent manner and showed better results when compared to previous reports [65]. The EPS production is confirmed by the Congo red agar assay. In this assay, black colonies are produced by *P. aeruginosa* on Congo red agar plates because of degradation of Congo red by *P. aeruginosa.* The colonies remained red when treated with *P. multirostrata* PUTY3 extract when compared to the control (without fungal extract). HCN, a secondary metabolite of pseudomonads, is generally produced at a late exponential phase or at the start of the stationary phase under microaerophilic conditions because it is QS dependent. HCN gas is used as a biomarker for *P. aeruginosa* in cystic fibrosis patients, and it is a well-known inhibitor of aerobic respiration. A significant reduction in HCN production was observed when the *P. aeruginosa* culture was treated with *P. multirostrata* (PUTY3) extract **Fig. 5Z** as evidenced by the production of light brown color, which indicates a decrease in the HCN gas production, when compared to the reddish-brown color observed in the positive control. Visualization of microscopic images showed a reduction in biofilm formation when treated with *P. multirostrata* extract in comparison to the control, which showed a thick opaque biofilm on the glass coverslips **Fig. 5Y**. These results were further confirmed by MTP assay, where 74.21 ± 1.77% inhibition in the biofilm formation was found at a concentration of 750 µg/ml.

In-vitro results were further validated by *in-silico* analyses. The compound 1,1-Dichloro-2-Methyl-3-(4, 4-Diformyl-1, 3-Butadien-1-yl) Cyclopropane showed the highest -7.29 docking score with LasR receptor protein, which was close to the docking score of its natural ligand3-Oxo-C12-HSL that showed -7.52 docking score, suggesting that the molecule 1,1-Dichloro-2-Methyl-3-(4,4-Diformyl-1,3-Butadien-1-yl)Cyclopropane competes with the natural ligand for a binding with LasR receptor protein. In the present study, for the first time, we report the anti-quorum sensing potential of this molecule against *P. aeruginosa*. This molecule was one of the compounds reported by [66] in the leaf extract of *Homalium zeylanicum* plant, where they found antioxidant and hepatoprotective properties in the leaf extract of this plant against carbon tetrachloride-induced oxidative stress and liver injury in rats.

Similarly, 1,2-Benzenedicarboxylic Acid, Bis(2-Methylpropyl) Ester, showed highest -4.47 docking score with RhlR receptor protein, which was close to its natural ligand C4-HSL that showed a docking score of -4.85, suggesting that the molecule 1,2-Benzenedicarboxylic acid, Bis(2-Methylpropyl) Ester competes with the natural ligand for a binding with the RhlR protein. Saxena *et al*., [67] also reported 1,2-Benzenedicarboxylic Acid, Bis(2-Methylpropyl) Ester molecule in the extract of *Muscodor tigerii*, an endophytic fungus, where they reported antimicrobial activity in the extract of this fungus. Thus, the present study explored the antiquorum sensing activity of *P. multirostrata* and the results suggest that it could be used in the development and production of potent QS inhibitors against multi-drug resistant pathogens that have QS and biofilm-forming abilities.

CONCLUSION: The present study reveals that *P. multirostrata* extract significantly decreases QS regulated virulence factors and biofilm formation in *P. aeruginosa*, thus indicating a vital role played by *P. multirostrata* extract in downregulating the QS regulated genes and significantly alternating the production of virulence factors. Additionally, *P. multirostrata* extract exhibited a significant reduction in the biofilm formation as evidenced by the microscopic observations. The potential of bioactive compounds of *P. multirostrata* extract, identified by GC-MS analysis, was further

confirmed by *in-silico* analysis. The *in-silico* studies have shown that the compound present in the *P. multirostrata* extract *i.e*., 1,1-Dichloro-2-Methyl- 3- (4, 4- Diformyl- 1, 3- Butadien- 1- yl) Cyclopropane, binds to the LasR receptor protein, whereas 1,2 Benzenedicarboxylic acid, Bis(2-Methylpropyl) Ester binds to the RhlR receptor protein, to attenuate the QS of *P. aeruginosa*. Thus, the present study indicates that alternate strategies are available in the form of effective QS inhibitors (QSIs) from unexplored natural resources such as saprophytic leaf litter degrading fungi. Additionally, the present study also throws new possibilities for the application and evolution of secondary metabolites of fungal origin as anti-infective drugs in the post-antibiotic era. Future research endeavors should focus on purification and characterization of the potent compounds, which may further enhance our understanding of fungal secondary metabolites as one of the natural sources in therapeutic use in containing QS pathogens.

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REFERENCES:

1. Muimhneacháin EÓ, Reen FJ, O'Gara F and McGlacken GP: Analogues of *Pseudomonas aeruginosa* signalling molecules to tackle infections. Organic & Biomolecular Chemistry 2018; 16(2): 169-79.
2. Pai S, Bedford L, Ruramayi R, Aliyu SH, Sule J, Maslin D and Enoch DA: *Pseudomonas aeruginosa* meningitis/ ventriculitis in a UK tertiary referral hospital. QJM: An International Journal of Medicine 2015; 109(2): 85-89.
3. Friedrich M: *Pseudomonas aeruginosa* infections. http:// emedicine.medscape.com/article/226748-overview#showall 2015.
4. Moghaddam MM, Khodi S and Mirhosseini A: Quorum Sensing in Bacteria and a Glance on *Pseudomonas aeruginosa*. Clinical Microbiology 2014; 3: 15.
5. Goncalves-de-Albuquerque CF, Silva AR, Burth P, Rocco PR, Castro-Faria MV and Castro-Faria-Neto HC: Possible mechanisms of *Pseudomonas aeruginosa*-associated lung disease. International Journal of Medical Microbiology 2016; 306(1): 20-8.
6. Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH and Quax WJ: The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. Microbiology and Molecular Biology Reviews 2012; 76(1): 46-65.
7. Rasmussen TB and Givskov M: Quorum sensing inhibitors: a bargain of effects. Microbiology 2006; 152(4): 895-04.
8. Pesci EC, Pearson JP, Seed PC and Iglewski BH: Regulation of las and rhl quorum sensing in *Pseudomonas aeruginosa*. Journal of bacteriology 1997; 179(10): 3127-32.
9. Moradali MF, Ghods S and Rehm BH: *Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence. Frontiers in Cellular and Infection Microbiology 2017; 7: 39.
10. Williams P, Winzer K, Chan WC and Camara M: Look who's talking: communication and quorum sensing in the bacterial world? Philosophical Transactions of the Royal Society B: Biological Sciences 2007; 362(1483): 1119-34.
11. Papenfort K and Bassler BL: Quorum sensing signal–response systems in Gram-negative bacteria. Nature Reviews Microbiology 2016; 14(9): 576.
12. Lee J and Zhang L: The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. Protein & cell 2015; 6(1): 26-41.
13. Asfour HZ: Anti-quorum sensing natural compounds. Journal of Microscopy and Ultrastructure 2018; 6(1): 1.
14. Haripriyan J, Omanakuttan A, Menon ND, Vanuopadath M, Nair SS, Corriden R and Kumar GB: Clove Bud Oil Modulates Pathogenicity Phenotypes of the Opportunistic Human Pathogen *Pseudomonas aeruginosa*. Scientific Reports 2018; 8(1): 3437.
15. Chen T, Sheng J, Fu Y, Li M, Wang J and Jia AQ: 1H NMR-based global metabolic studies of *Pseudomonas aeruginosa* upon exposure of the quorum sensing inhibitor resveratrol. Journal of Proteome Research 2017; 16(2): 824-30.
16. Vasavi HS, Sudeep HV, Lingaraju HB and Prasad KS: Bioavailability-enhanced Resveramax™ modulates quorum sensing and inhibits biofilm formation in *Pseudomonas aeruginosa* PAO1. Microbial pathogenesis 2017; 104: 64-71.
17. Rekha PD, Vasavi HS, Vipin C, Saptami K and Arun AB: A medicinal herb *Cassia alata* attenuates quorum sensing in *C. violaceum* and *Pseudomonas aeruginosa*. Letters in Applied Microbiology 2017; 64(3): 231-38.
18. Kandasamy P, Manogaran S, Dhakshinamoorthy M and Kannan KP: Evaluation of antioxidant and antibacterial activities of endophytic fungi isolated from *Bauhinia racemosa* Lam and *Phyllanthus amarus* Schum and Thonn. J Chem Pharm Res 2015; 7(9): 366-79.
19. Zhang SP, Huang R, Li FF, Wei HX, Fang XW, Xie XS and He J: Antiviral anthraquinones and azaphilones produced by an endophytic fungus Nigrospora sp. from *Aconitum carmichaeli*. Fitoterapia 2016; 112: 85-89.
20. Macheleidt J, Mattern DJ, Fischer J, Netzker T, Weber J, Schroeckh V, Valiante V and Brakhage AA: Regulation and role of fungal secondary metabolites. Annual Review of Genetics 2016; 50: 371-92.
21. Wang L, Zou S, Yin S, Liu H, Yu W and Gong Q: Construction of an effective screening system for detection of *Pseudomonas aeruginosa* quorum sensing inhibitors and its application in bioautographic thin-layer chromatography. Biotechnology Letters 2011; 33(7): 1381-87.

22. Figueroa M, Jarmusch AK, Raja HA, El-Elimat T, Kavanaugh JS, Horswill AR and Oberlies NH: Polyhydroxyanthraquinones as quorum sensing inhibitors from the guttates of Penicillium restrictum and their analysis by desorption electrospray ionization mass spectrometry. Journal of Natural Products 2014; 77(6): 1351-58.
23. Bills GF and Polishook JD: Abundance and diversity of microfungi in leaf litter of a lowland rain forest in Costa Rica. Mycologia 1994; 86(2): 187-98.
24. Patil MP, Patil RH and Maheshwari VL: Biological activities and identification of bioactive metabolite from endophytic *Aspergillus flavus* L7 isolated from Aegle marmelos. Current Microbiology 2015; 71(1): 39-48.
25. Oliveira BDÁ, Rodrigues AC, Cardoso BMI, Ramos ALCC, Bertoldi MC, Taylor JG and Pinto UM: Antioxidant, antimicrobial and anti-quorum sensing activities of *Rubus rosaefolius* phenolic extract. Industrial Crops and Products 2016; 84: 59-66.
26. Dolatabad HK, Javan-Nikkhah M and Shier WT: Evaluation of antifungal, phosphate solubilisation, and siderophore and chitinase release activities of endophytic fungi from *Pistacia vera*. Mycological progress 2017; 16(8): 777-90.
27. Stamatakis A, Hoover P and Rougemont J: A rapid bootstrap algorithm for the RAxML web servers. Systematic Biology 2008; 57(5): 758-71.
28. Miller MA, Pfeiffer W and Schwartz T: Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In 2010 gateway computing environments workshop (GCE): 1-8. Ieee.
29. Husain FM, Ahmad I, Al-thubiani AS, Abulreesh HH, AlHazza IM and Aqil F: Leaf extracts of *Mangifera indica* L. Inhibit quorum-sensing–regulated production of virulence factors and biofilm in test bacteria. Frontiers in Microbiology 2017; 8: 727.
30. Sethupathy S, Shanmuganathan B, Kasi PD and Pandian SK: Alpha-bisabolol from brown macroalga *Padina gymnospora* mitigates biofilm formation and quorum sensing controlled virulence factor production in *Serratia marcescens*. Journal of Applied Phycology 2016; 28(3): 1987-96.
31. Luo J, Kong JL, Dong BY, Huang H, Wang K, Wu LH and Chen YQ: Baicalein attenuates the quorum sensing-controlled virulence factors of *Pseudomonas aeruginosa* and relieves the inflammatory response in *P. aeruginosa*-infected macrophages by down regulating the MAPK and NFκB signal-transduction pathways. Drug Design, Development and Therapy 2016; 10: 183.
32. Das MC, Sandhu P, Gupta P, Rudrapaul P, De UC, Tribedi P and Bhattacharjee S: Attenuation of *Pseudomonas aeruginosa* biofilm formation by Vitexin: A combinatorial study with azithromycin and gentamicin. Scientific reports 2016; 6: 23347.
33. Packiavathy IASV, Priya S, Pandian SK and Ravi AV: Inhibition of biofilm development of uropathogens by curcumin–an anti-quorum sensing agent from *Curcuma longa*. Food Chemistry 2014; 148: 453-60.
34. Roudashti S, Zeighami H, Mirshahabi H, Bahari S, Soltani A and Haghi F: Synergistic activity of sub-inhibitory concentrations of curcumin with ceftazidime and ciprofloxacin against *Pseudomonas aeruginosa* quorum sensing related genes and virulence traits. World Journal of Microbiology and Biotechnology 2017; 33(3): 50.
35. Chhibber S, Gondil VS, Sharma S, Kumar M, Wangoo N and Sharma RK: A novel approach for combating *Klebsiella pneumoniae* biofilm using histidine functionalized silver nanoparticles. Front. Microbiol 2017; 8: 1104.
36. Rasamiravaka T, Ngezahayo J, Pottier L, Ribeiro S, Souard F, Hari L and Duez P: Terpenoids from *Platostoma rotundifolium* (Briq.) AJ Paton alter the expression of quorum sensing-related virulence factors and the formation of biofilm in *Pseudomonas aeruginosa* PAO1. International Journal of Molecular Sciences 2017; 18(6): 1270.
37. Gupta P, Sarkar A, Sandhu P, Daware A, Das MC, Akhter Y and Bhattacharjee S: Potentiation of antibiotic against *Pseudomonas aeruginosa* biofilm: a study with plumbagin and gentamicin. Journal of Applied Microbiology 2017; 123(1): 246-61.
38. Kalia M, Yadav VK, Singh PK, Sharma D, Pandey H, Narvi SS and Agarwal V: Effect of cinnamon oil on quorum sensing-controlled virulence factors and biofilm formation in *Pseudomonas aeruginosa*. PLoS one 2015; 10(8): e0135495.
39. Knutson CA and Jeanes A: A new modification of the carbazole analysis: application to heteropolysaccharides. Analytical Biochemistry 1968; 24(3): 470-81.
40. Alasil SM, Omar R, Ismail S and Yusof MY: Inhibition of quorum sensing-controlled virulence factors and biofilm formation in *Pseudomonas aeruginosa* by culture extract from novel bacterial species of Paenibacillus using a rat model of chronic lung infection. International Journal of Bacteriology 2015; 671562.
41. Luciardi MC, Blázquez MA, Cartagena E, Bardón A and Arena ME: Mandarin essential oils inhibit quorum sensing and virulence factors of *Pseudomonas aeruginosa*. LWT-Food Science and Technology 2016; 68: 373-80.
42. García-Lara B, Saucedo-Mora MÁ, Roldán-Sánchez JA, Pérez-Eretza B, Ramasamy M, Lee J and García-Contreras R: Inhibition of quorum-sensing-dependent virulence factors and biofilm formation of clinical and environmental *Pseudomonas aeruginosa* strains by ZnO nanoparticles. Letters in Applied Microbiology 2015; 61(3): 299-05.
43. Ghodsalavi B, Ahmadzadeh M, Soleimani M, Madloo PB and Taghizad-Farid R: Isolation and characterization of rhizobacteria and their effects on root extracts of *Valeriana officinalis*. Australian Journal of Crop Science 2013; 7(3): 338.
44. Lovell SC, Davis IW, Arendall III WB, De Bakker PI, Word JM, Prisant MG and Richardson DC: Structure validation by Cα geometry: ϕ, ψ and Cβ deviation. Proteins: Structure, Function, and Bioinformatics 2003; 50(3): 437-50.
45. Dallakyan Sand Olson AJ: Small-molecule library screening by docking with PyRx. In Chemical Biology2015; 243-250. Humana Press, New York, NY.
46. Morris GM, Huey R and Olson AJ: Using autodock for ligand-receptor docking. Current Protocols in Bioinformatics 2008; 24(1): 8-14.
47. Trott O and Olson AJ: AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. Journal of Computational Chemistry 2010; 31(2): 455-61.
48. Spiteller P: Chemical ecology of fungi. Natural Product Reports 2015; 32(7): 971-93.
49. Künzler M: How fungi defend themselves against microbial competitors and animal predators. PLoS Pathogens 2018; 14(9): e1007184.
50. Cueto M, Jensen PR, Kauffman C, Fenical W, Lobkovsky E and Clardy J: Pestalone, a new antibiotic produced by a marine fungus in response to bacterial challenge. Journal of Natural Products 2001; 64(11): 1444-46.

51. Miao LI, Kwong TF and Qian PY: Effect of culture conditions on mycelial growth, antibacterial activity, and metabolite profiles of the marine-derived fungus Arthrinium cf saccharicola. Applied Microbiology and Biotechnology 2006;72(5): 1063-73
52. Kanai Y, Ishiyama D, Senda H, Iwatani W, Takahashi H, Konno Hand Kanazawa S: (2000). Novel Human Topoisomerase I Inhibitors, Topopyrones A, B, C and D. The Journal of Antibiotics 2000; 53(9): 863-72.
53. Cimmino A, Andolfi A, Zonno MC, Avolio F, Berestetskiy A, Vurro M and Evidente A: Chenopodolans A–C: Phytotoxic furopyrans produced by *Phoma chenopodiicola*, a fungal pathogen of Chenopodium album. Phytochemistry 2013; 96: 208-13.
54. Krohn K, Farooq U, Flörke U, Schulz B, Draeger S, Pescitelli G and Kurtán T: Secondary Metabolites Isolated from an Endophytic Phoma sp.–Absolute Configuration of Tetrahydropyrenophorol Using the Solid-State TDDFT CD Methodology. European Journal of Organic Chemistry 2007; 19: 3206-11.
55. Sang XN, Chen SF, Tang MX, Wang HF, An X, Lu XJ and Chen G: α-Pyrone derivatives with cytotoxic activities, from the endophytic fungus Phoma sp. YN02-P-3. Bioorganic and Medicinal Chemistry Letters 2017; 27(16): 3723-25.
56. Tan XM, Li LY, Sun LY, Sun BD, Niu SB, Wang MH and Xing XK: Spiciferone analogs from an endophytic fungus Phoma betae collected from desert plants in West China. The Journal of Antibiotics 2018: 1.
57. Yi-Zhe S, Kurtan T, Mandi A, Tang H, Chou Y, Soong K, Su L, Sun P, Zhuang CL and Zhang W: Immunomodulatory polyketides from a Phoma-like fungus isolated from a soft coral. Journal of natural products 2017; 80(11): 2930-40.
58. Sumilat DA, Yamazaki H, Kanno SI, Saito R, Watanabe Y and Namikoshi M: Biphenyl ether derivatives with protein tyrosine phosphatase 1B inhibitory activity from the freshwater fungus Phoma sp. The Journal of antibiotics 2017; 70(3): 331.
59. Gubiani JR, Wijeratne EK, Shi T, Araujo AR, Arnold AE, Chapman E and Gunatilaka AL: An epigenetic modifier induces production of (10′ S)-verruculide B, an inhibitor of protein tyrosine phosphatases by Phoma sp. nov. LG0217, a fungal endophyte of Parkinsonia microphylla. Bioorganic and Medicinal Chemistry 2017; 25(6): 1860-66.
60. Koo H, Allan RN, Howlin RP, Stoodley P and Hall-Stoodley L: Targeting microbial biofilms: current and prospective therapeutic strategies. Nature Reviews Microbiology 2017; 15(12): 740.
61. Gökalsın B and Sesal NC: Lichen secondary metabolite evernic acid as potential quorum sensing inhibitor against Pseudomonas aeruginosa. World Journal of Microbiology and Biotechnology 2016; 32(9): 150.
62. Zhou J, Bi S, Chen H, Chen T, Yang R, Li M and Jia AQ: Anti-biofilm and antivirulence activities of metabolites from *Plectosphaerella cucumerina* against *Pseudomonas aeruginosa*. Frontiers in Microbiology 2017; 8: 769.
63. Singh VK, Mishra A and Jha B: Anti-quorum sensing and anti-biofilm activity of *Delftia tsuruhatensis* extract by attenuating the quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa*. Frontiers in Cellular and Infection Microbiology 2017; 7: 337.
64. Wagner S, Sommer R, Hinsberger S, Lu C, Hartmann RW, Empting M and Titz A: Novel strategies for the treatment of Pseudomonas aeruginosa infections. Journal of Medicinal Chemistry 2016; 59(13): 5929-69.
65. Rasamiravaka T, Vandeputte OM, Pottier L, Huet J, Rabemanantsoa C, Kiendrebeogo M and El Jaziri M: (2015). *Pseudomonas aeruginosa* biofilm formation and persistence, along with the production of quorum sensing-dependent virulence factors, are disrupted by a triterpenoid coumarate ester isolated from *Dalbergia trichocarpa*, a tropical legume. PloS one 2015; 10(7): e0132791.
66. Kanhar S and Sahoo AK: Ameliorative effect of *Homalium zeylanicum* against carbon tetrachloride-induced oxidative stress and liver injury in rats. Biomedicine and Pharmacotherapy 2019; 111: 305-14.
67. Saxena S, Meshram V and Kapoor N: *Muscodor tigerii* sp. nov.-Volatile antibiotic producing endophytic fungus from the Northeastern Himalayas. Annals of Microbiology 2015; 65(1): 47-57.

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