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Inhibition of Quorum Sensing Regulated Virulence Factors and Biofilm Formation by *Eucalyptus globulus* against Multidrug-Resistant *Pseudomonas aeruginosa*

Pankaj Kumar Sagar[1], Poonam Sharma[2], Rambir Singh[3]*

[1]Department of Biotechnology, Bundelkhand University, Jhansi (U.P.), India
[2]Department of Zoology, Indira Gandhi National Tribal University (A Central University), Amarkantak, Madhya Pradesh, India
[3]Department of Horticulture, Aromatic and Medicinal Plants, Mizoram University (A Central University), Aizawl, Mizoram, India

***Corresponding Author**
Rambir Singh
Department of Horticulture, Aromatic and Medicinal Plants, Mizoram University (A Central University), Aizawl, Mizoram 796004, India
Tel: +91-947-358-3251
E-mail: rambirsehrawat@gmail.com

Objectives: The quorum-sensing–inhibitory and anti-biofilm activities of the methanol extract of *E. globulus* leaves were determined against clinically isolated multidrug-resistant *Pseudomonas aeruginosa*.

Methods: The preliminary anti-quorum–sensing (AQS) activity of eucalyptus was investigated against a biosensor strain *Chromobacterium violaceum* ATCC 12472 (CV12472) by using the agar well diffusion method. The effect of sub-minimum inhibitory concentrations (sub-MICs) of the methanol extract of eucalyptus on different quorum-sensing–regulated virulence factors, such as swarming motility, pyocyanin pigment, exopolysaccharide (EPS), and biofilm formation, against clinical isolates (CIs 2, 3, and 4) and reference PAO1 of *Pseudomonas aeruginosa* were determined using the swarm diameter (mm)-measurement method, chloroform extraction method, phenol (5%)-sulphuric acid (concentrated) method, and the microtiter plate assay respectively, and the inhibition (%) in formation were calculated.

Results: The preliminary AQS activity (violacein pigment inhibition) of eucalyptus was confirmed against *Chromobacterium violaceum* ATCC 12472 (CV12472). The eucalyptus extract also showed concentration-dependent inhibition (%) of swarming motility, pyocyanin pigment, EPS, and biofilm formation in different CIs and PAO1 of *P. aeruginosa*.

Conclusion: Our results revealed the effectiveness of the *E. globulus* extract for the regulation of quorum-sensing–dependent virulence factors and biofilm formation at a reduced dose (sub-MICs) and suggest that *E. globulus* may be a therapeutic agent for curing and controlling bacterial infection and thereby reducing the possibility of resistance development in pathogenic strains.

Keywords: anti-quorum sensing (AQS) activity, eucalyptus (*Eucalyptus globulus*), exopolysaccharide (EPS), minimum inhibitory concentration (MIC), multidrug-resistant, *Pseudomonas aeruginosa*

INTRODUCTION

The increasing emergence of microbial multi-drug resistance has proven to be an obstacle for the effective treatment of microbial infection worldwide. This severe problem has necessitated the development of new therapeutic agents as well as drug targets. *Pseudomonas aeruginosa* is a Gram-negative, opportunistic human pathogen that can cause respiratory infections, urinary tract infections (UTIs), eye infections, ear infections, infections in bone, skin, and soft tissues, ventilator-associated pneumonia (VAP), and cystic fibrosis [1-3]. The commonly used antibiotics for the treatment of *P. aeruginosa* infection include gentamicin, tobramycin (aminoglycosides); ceftazidime, cefepime (cephalosporins); ciprofloxacin (fluo-

roquinolones); aztreonam (monobactams); and imipenem (carbapenems) [4]. Several Gram-negative bacteria, including *P. aeruginosa*, monitor their population density using signal molecules (auto-inducer) such as *N*-acyl homoserine lactones (AHLs) under a gene regulatory mechanism known as quorum sensing (QS) [5, 6]. In *P. aeruginosa*, las and rhl are two inter-related QS systems that work in a hierarchical manner, and LasI and RhlI are synthetases that produce the auto-inducer signaling molecules *N*-(3-oxododecanoyl)-ʟ-homoserinelactone (OdDHL) and *N*-butanoyl-ʟ-homoserine lactone (BHL), respectively. Another signal named the *Pseudomonas* quinolone signal plays an essential role in the QS system [7-9]. Various virulence factors of *P. aeruginosa*, such as swarming motility and the formation of pyocyanin pigment, exopolysaccharide, LasA protease, and LasB elastase, contribute to the regulation of pathogenesis by the QS mechanism [10, 11]. In comparison with planktonic cells, cells in the biofilm are highly resistant and require greater doses of antibiotics for their removal [12]. Inhibition of QS, also known as quorum quenching (QQ), may be achieved by destruction or modification of QS signaling molecules (AHL), interference or blocking agents of QS, or via formation of antibodies to regulate QS signals [13]. The inhibition of virulence factor production by interference in QS may be a better approach than killing or inhibiting the growth to regulate the pathogenicity and virulence of pathogenic bacteria such as *P. aeruginosa* [14, 15]. Several studies have reported the antibacterial and anti-QS (AQS) activity of plant extracts [16-18]. The doses of antibiotics or natural plant extracts required to inhibit bacterial growth (antibacterial activity) are generally higher than the doses required to interfere with QS and inhibit virulence factor formation.

The use of higher doses of antibiotics for growth inhibition and biofilm removal may lead to serious adverse effects in patients and facilitate the emergence of drug resistance in the microorganism. The common mechanisms of drug resistance in *P. aeruginosa* are efflux pump activity, low outer membrane permeability, formation of antibiotic-inactivating enzymes (intrinsic resistance); mutational alterations or horizontal antibiotic resistant gene transfer (acquired resistance) [19, 20]; and biofilm formation (adaptive resistance) [21]. Thus, in addition to the antibacterial approach, QS inhibition for attenuation of virulence factor formation at reduced doses of plant extracts or antibiotics may be an attractive solution for emergence of microbial drug resistance as well as effective and safe treatment of infections. Over the last several decades, eucalyptus has been used as a medicinal plant for its antibacterial, anti-inflammatory, antifungal, antiviral, anticancer, and antioxidant properties [22]. Essential oils from many *Eucalyptus* species have been previously reported for their antibacterial properties [23-25]. 1,8-Cineole or eucalyptol (63.81%) was reported to be a major constituent of *Eucalyptus globulus* leaves [26], and the anti-biofilm and AQS activity of 1,8-cineole against methicillin-resistant *Staphylococcus aureus* (MRSA) isolates has been reported previously [27]. In a previous study, we had determined the growth-inhibiting activity of the methanol extract of *E. globulus* leaves and their MICs against clinical isolates (CIs) of multidrug-resistant *P. aeruginosa* [17]. As an extension of these findings, the aim of the present study is to reveal the AQS and anti-biofilm efficacy of a crude methanolic extract of *E. globulus* against CIs and a reference strain (PA01) of *P. aeruginosa*.

MATERIALS AND METHODS

1. Collection of plant material and extract preparation

Fresh leaves of *E. globulus* were collected and authenticated by the Council for Research in Ayurvedic Sciences-Regional Ayurveda Research Institute, Jhansi (Uttar Pradesh), India. The maceration method was used for extraction of *E. globulus* leaves. Briefly leaves were air-dried, powdered, and soaked (10 gm) in 100% methanol and subjected to continuous mixing for 3 successive days at room temperature. The crude methanolic extract was filtered through Whatman filter paper no. 1 and subjected to centrifugation at 3,000 rpm for 10 min. The collected supernatant was evaporated and concentrated with a rotary evaporator. The concentrated crude methanol extract of eucalyptus was aseptically preserved at 4℃ in a sterile airtight bottle.

2. Bacterial isolates

All the clinical isolates of *P. aeruginosa* were collected from Department of Microbiology, Sarojini Naidu Medical College, Agra (Uttar Pradesh), India. A biosensor strain *Chromobacterium violaceum* ATCC 12472 (CV12472) and reference strain (PA01) of *P. aeruginosa* were collected from Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh (Uttar Pradesh), India. *P. aeruginosa* was maintained on Luria–Bertani (LB) broth at 37℃ while *C. violaceum* (CV12472) was maintained on nutrient broth at 28℃.

3. Determination of antibacterial activity and MIC

The antibacterial activity of the eucalyptus extract against clinical isolates (CIs) and the reference strain of *P. aeruginosa* was determined using an agar well diffusion method, and the MICs were determined using the broth microdilution method in accordance with the guidelines of Clinical and Laboratory Standard Institute; the protocols for these evaluations were the same as those in our previous study [28].

4. Determination of AQS activity

The AQS activity of the eucalyptus extract was confirmed by showing pigment (violacein) inhibitory activity in CV12472 using the standard agar well diffusion method [29] with slight modification. Briefly, a Mueller–Hinton agar plate with 100 μL of freshly grown and aseptically diluted bacterial suspension with an inoculum size of 2.5×10^6 CFU/mL was prepared. Wells of 6-8 mm diameter were made on the plates by using a sterile cub borer, and the bottom was sealed using soft agar. Next, 50 μL of eucalyptus extract (500 mg/mL) was poured aseptically in the wells. The plates were incubated for 18-20 h at 28℃. DMSO (dimethyl sulfoxide) was used as a negative control. The inhibition zone diameter of pigment inhibition (mm) around the well was measured to determine AQS activity.

5. Inhibition of QS-regulated virulence factors and biofilm formation

1) Assay for swarming motility

The effect on swarming motility was determined using a swarm plate prepared using sterile nutrient broth (8 gm/L) supplemented with 5 gm/L glucose and solidified by bacto agar (0.5%) with sub-MICs of eucalyptus extract (50 μL) [30]. Swarm plates were point-inoculated with freshly grown culture of *P. aeruginosa* and incubated at 37℃ for 18-24 h. The diameter (mm) of the circular turbid zone from the point of bacterial inoculation was measured to determine the effect of eucalyptus on swarming motility. Swarm diameters (mm) of treated and untreated cultures (as control) were noted to calculate the percentage inhibition in swarming motility.

2) Assay for pyocyanin formation

The effect of eucalyptus extract on pyocyanin formation in CIs and the reference strain (PA01) of *P. aeruginosa* was deter-

mined using the standard chloroform extraction method [31]. Freshly prepared bacterial culture (5 mL) was treated with 150 μL of sub-MIC eucalyptus extract and incubated at 37℃ for 18-24 h. A 3-mL volume of chloroform was added to the supernatant of the incubated culture and mixed vigorously. After mixing, the chloroform layer was separated and further mixed with 1 mL of 0.2 M HCL (hydrochloric acid). The solution was centrifuged at 8,000 rpm for 10-12 min at room temperature. The absorbance of the HCL layer (pink to deep red color) was measured at 520 nm. Absorbance of treated and untreated culture (as control) was noted to calculate the percentage inhibition in pyocyanin formation.

3) EPS extraction and estimation

The bacterial isolates were grown in LB broth containing 150 μL of sub-MIC eucalyptus extract at 37℃ for 18-24 h. After incubation, the bacterial culture was centrifuged, and the supernatant was filtered and transferred to fresh sterile tubes. Three volumes of chilled ethanol (100%) were added, and the tube was incubated at 4℃ for 24 h to precipitate extraction and EPS quantification [32]. Quantification of EPS was performed by measuring the sugar concentration with the phenol (5%) - sulfuric acid (concentrated) method [33]. Percentage inhibition of EPS formation was calculated by determining the absorbance of treated and untreated cultures (as control) at 490 nm.
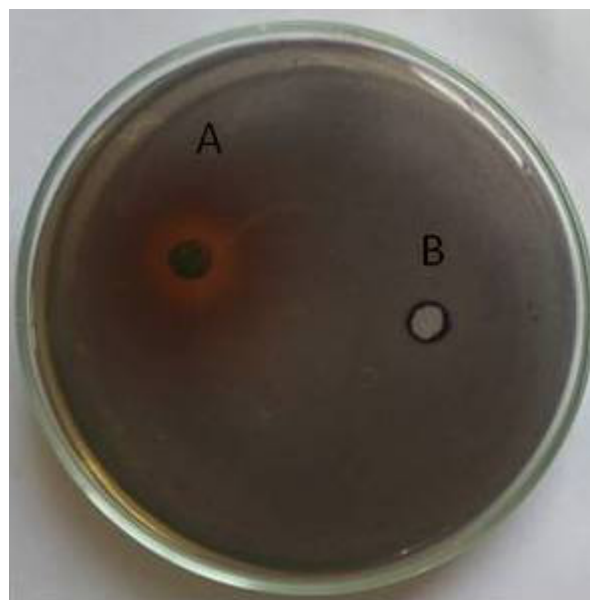

Figure 1. Anti-quorum sensing (violacein pigment inhibition) activity of the methanol extract of eucalyptus against *C. violaceum* CV12472. (A: methanol extract of *Eucalyptus globulus*; B: negative control).

4) Assay for biofilm

The effects of sub-MICs of the eucalyptus extract on biofilm formation were determined by the microtiter plate assay [34], with slight modifications. Briefly, biofilms were developed in the presence of eucalyptus extract (at different sub-MICs) at 37℃ for 18-24 h. The wells were drained properly the next day and washed with sterile PBS (phosphate-buffered saline) three times to remove planktonic cells. For biofilm staining, 150 μL of crystal violet solution (0.1%) was poured into the wells and plate was incubated at room temperature for 5-10 min. After incubation, excess dye was removed by washing with sterile PBS, and the plate was kept at room temperature for air drying. Next, 150 μL of glacial acetic acid (33% v/v) was added to wells to solubilize the dye taken in by the cells in the biofilm. Percentage inhibition in biofilm formation was calculated by determining the absorbance of treated and untreated culture (as control) at 570 nm.

6. Statistical analysis

Experiments were performed in triplicate, and mean ± standard deviation was calculated. The data were statistically analyzed with one-way ANOVA, and the level of significance was expressed as the p value ($p < 0.001$, $p < 0.005$, $p < 0.01$ and $p < 0.05$ were denoted with ****, ***, **, and *, respectively) when inhibition in PA01 was compared to those in clinical isolates. Comparison of inhibition in the reference strain/clinical isolates by sub-MICs was made using Tukey's HSD post-hoc test, and the mean difference was expressed as significant at the 0.05 level. Values sharing a common letter (a, b, c) were not significant (at $p < 0.05$) when comparisons were made between percentage inhibition (%) of strain/clinical isolates by sub-MICs. Statistical analyses were performed in IBM SPSS statistics 20 (Version 20.0. Armonk, NY: IBM Corporation).
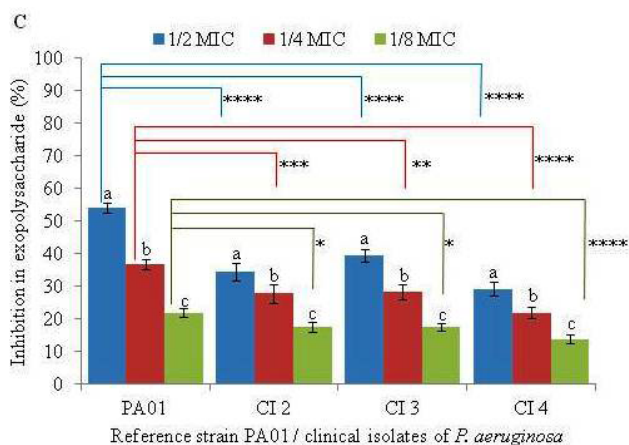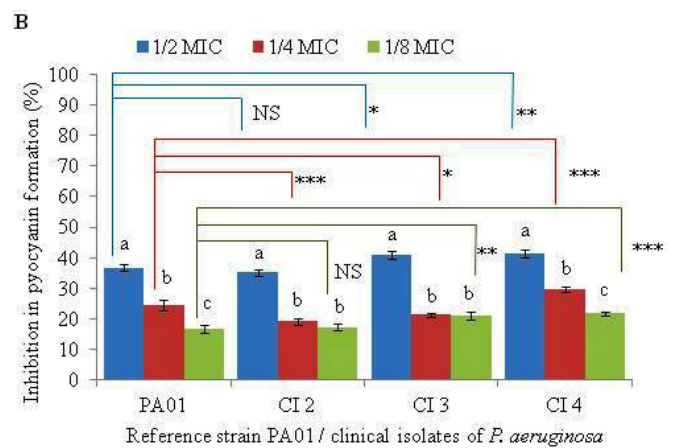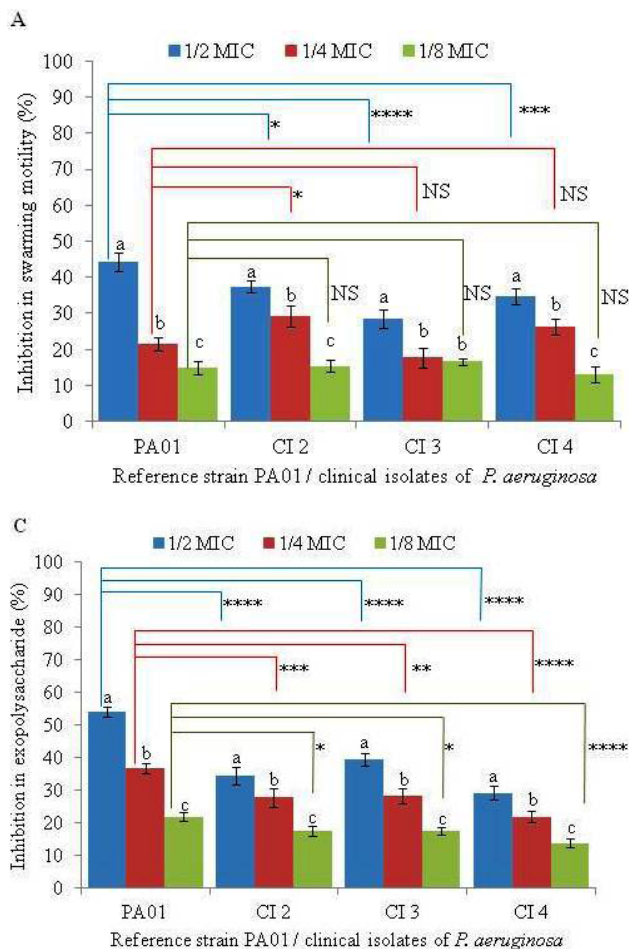
Figure 2. Inhibition of the formation of various virulence factors (A), swarming motility (B), pyocyanin formation (C), and exopolysaccharide (EPS) formation by sub-MICs of eucalyptus extract in the reference PA01 strain and the clinical isolates (CI 2, CI 3, and CI 4) of *P. aeruginosa*, and comparison of the reference strain PA01 to the clinical isolates. Each bar represents the mean value of three independent replicates, and the error bar shows the standard deviation. ****$p < 0.001$, ***$p < 0.005$, **$p < 0.01$, *$p < 0.05$, NS: not significant when the reference PA01 strain was compared to CI 2, 3, and 4. Values sharing a common letter (a, b, c) were not significant (at $p < 0.05$) when comparison was made between inhibitions by sub-MICs with in strain/clinical isolates.

RESULTS

In preliminary evaluation of the QS-inhibitory activity of eucalyptus, the eucalyptus extract showed significant inhibition of violacein pigment formation in CV12472 (Fig. 1). The effects of the eucalyptus extract on the growth of PA01 and CIs 2, 3, and 4 at its sub-MICs (1/2 MIC, 1/4 MIC, 1/8 MIC) were determined using spectroscopic growth curve analysis, and the results showed that the growth of the reference strain/isolates was not affected at sub-MICs (data not shown). A significant ($p < 0.05$) concentration-dependent reduction in swarming motility was observed in PA01 and CIs 2 and 4 at all tested sub-MIC levels (1/2, 1/4, 1/8 MIC), while the reduction in CI 3 was slightly similar at both 1/4 and 1/8 MIC. The reduction in swarming motility in PA01 (approximately 44%) was significantly higher than those in CIs 2, 3, and 4 (approximately 38%, 28%, and 35%, respectively, at 1/2 MIC; Fig. 2). At 1/2 MIC, the maximum and significantly higher inhibition of pyocyanin formation (%) was observed in CI 3 ($p < 0.05$) and CI 4 ($p < 0.01$) than in PA01, while inhibition (%) at 1/4 MIC was significantly higher in PA01 than in CIs 2 and 3 but lower than that in CI 4. At 1/4 MIC and 1/8 MIC, a significant concentration-dependent pyocyanin inhibition (%) was found in PA01 and CI 4 while inhibition was slightly similar in both the CI 2 and CI 3 (Fig. 2). A significant concentration-dependent inhibition

(%) in EPS formation was found in all of the tested CIs and the reference strain (PA01) of *P. aeruginosa* ($p < 0.05$). At all of the tested levels of sub-MICs, inhibition was significantly higher in PA01 than in the other CIs (Fig. 2). The reduction in OD (570 nm) and percentage inhibition (%) of biofilm formation by the eucalyptus extract in the reference strain PA01 and CIs of *P. aeruginosa* are mentioned in Fig. 3. A significant concentration-dependent inhibition in biofilm formation (%) was observed in PA01, CI 2 and CI 3 ($p < 0.05$) at the sub-MICs. For CI 4, the inhibition was slightly similar at 1/2 MIC and 1/4 MIC, but the inhibition at both sub-MIC levels was significantly higher as compared to at 1/8 MIC (Fig. 3).

DISCUSSION

Over the last several decades, plant-based components have been traditionally used as medicines for curing and controlling microbial infections and diseases. Previous research studies have reported the AQS activity of plant materials [35-40]. The methanol extract of eucalyptus leaves was prepared because this solvent allows easy degradation of the cell wall and releases a range of phytoconstituents from the plant cells because of its polar nature [41, 42]. In the preliminary step of research, the growth inhibitory activity and MIC of methanol extract of eucalyptus leaves against the CIs and reference strain (PA01)
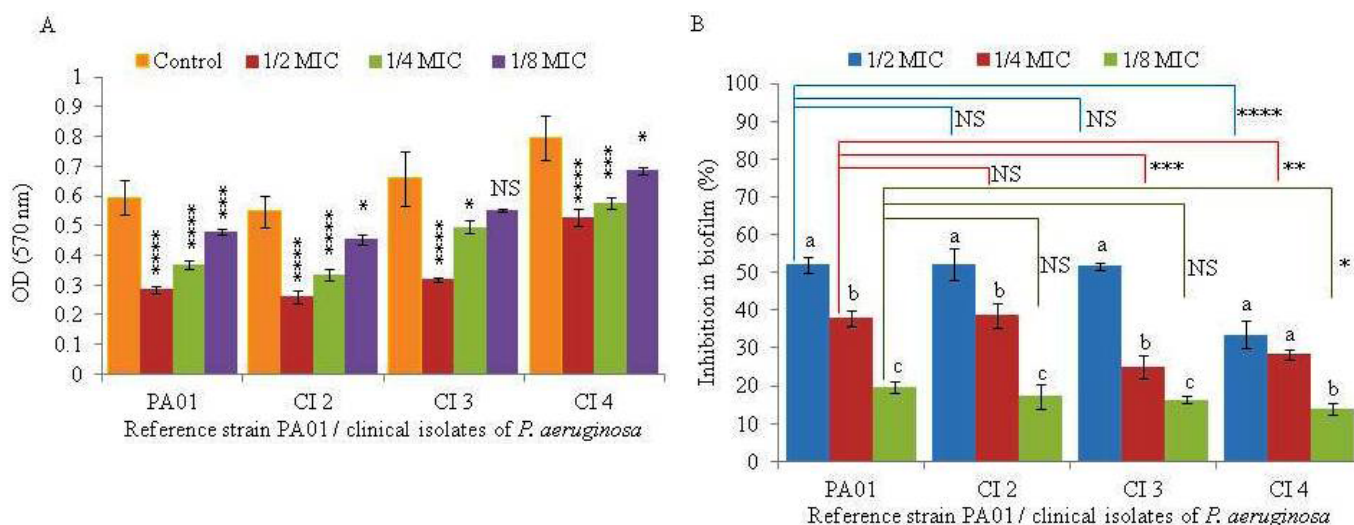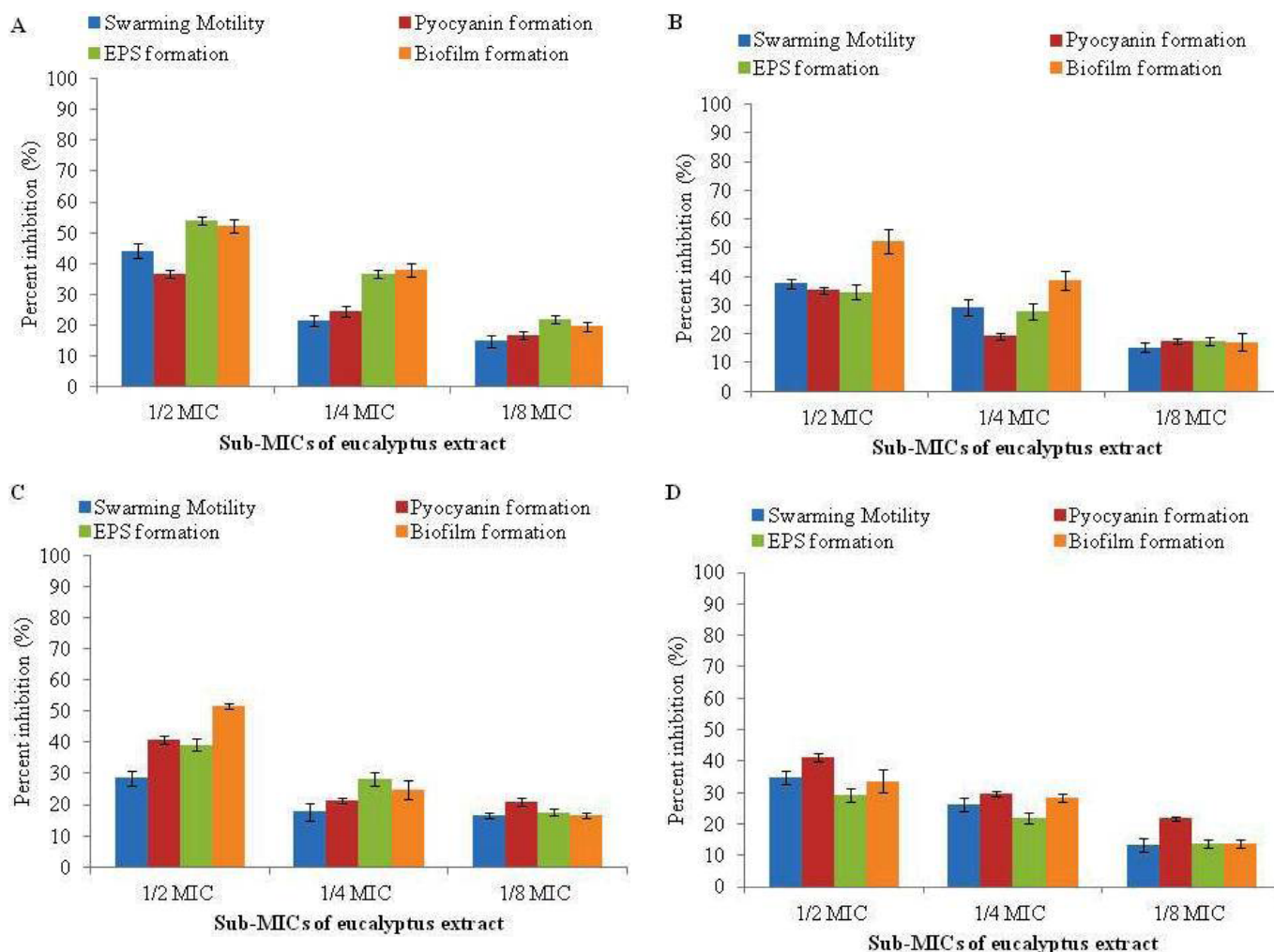

Figure 3. Inhibition of biofilm formation. (A) OD at 570 nm. (B) Percent inhibition (%) by sub-MICs of eucalyptus extract in the reference strain PA01 and clinical isolates (CI 2, CI 3 and CI 4) of *P. aeruginosa*. Each bar represents the mean value of three independent replicates, and the error bar shows the standard deviation. ****$p < 0.001$, ***$p < 0.005$, **$p < 0.01$, *$p < 0.05$, NS: not significant. Values sharing a common letter (a, b, c) were not significant (at $p < 0.05$) when comparison was made between percent inhibitions (%) by sub-MICs within strain/clinical isolates.

of *P. aeruginosa* were determined [17]. As an extension of that study, the present study prepared sub-MICs of the eucalyptus extract (Table 1) and investigated their AQS effects to evaluate whether the virulence factors regulated under QS as well as biofilm formation could be inhibited at the sub-MIC levels. In the present study, effective initial AQS activity of eucalyptus

against CV12472 was observed as violacein pigment inhibition (Fig. 1), indicating the efficacy of the extract to interfere with the QS signaling cascade. Similar inhibition in violacein pigment formation was reported for eucalyptus (*E. globulus* and *E. radiata*) [26], *Mangifera indica* [15], clove [36], *Cuminum cyminum* [39], and *Terminalia bellerica* [40]. In the present

Table 1. MIC and sub-MICs of methanol extract of eucalyptus against clinical isolate (CI) 2, 3, 4 and PA01 of *P. aeruginosa*

Strain/Clinical isolates (CIs)	MIC and sub-MICs (mg/mL) of methanol extract of *Eucalyptus globulus* leaves			
	MIC	1/2 MIC	1/4 MIC	1/8 MIC
PA01	7.81	3.90	1.95	0.97
CI 2	15.62	7.81	3.90	1.95
CI 3	62.5	31.25	15.62	7.81
CI 4	31.25	15.62	7.81	3.90

Figure 4. Inhibition (%) of quorum-sensing–regulated virulence factors and biofilm formation in the (A) reference strain PA01, (B) CI 2, (C) CI 3, and (D) CI 4 of *P. aeruginosa* by sub-MICs of the eucalyptus extract. Each bar represents the mean value of three independent replicates, and the error bar shows the standard deviation.

study, sub-MICs of the eucalyptus extract showed effective concentration-dependent inhibition (%) of QS-regulated virulence factors and biofilm formation in the reference PA01 and different CIs of *P. aeruginosa* (Fig. 4). Pyocyanin pigment is a QS-regulated virulence factor contributing to the pathogenesis of *P. aeruginosa* infections in cystic fibrosis patients [43]. In the present study, effective reduction (%) in pyocyanin pigment formation was observed in CIs and the reference strain PA01. A significant ($p < 0.05$) concentration-dependent reduction in pyocyanin formation was observed in PA01 and CI 4 at all the tested sub-MICs, while the reduction in CI 2 and CI 3 at 1/4 MIC and 1/8 MIC were similar, which exhibited the limit of possible reduction at the selected sub-MICs (Fig. 2). Similar reduction in pyocyanin formation by plants was also previously reported in *P. aeruginosa* [40, 44]. EPS and swarming are the two most important QS-regulated factors closely related to the biofilm. EPS is required for maturation of the biofilm [45], and type IV pilli and flagella-mediated motility, swarming motility [46], is a key factor to keep cells compact and close within the biofilm. Therefore, controlling the swarming and EPS formation through interference in QS signaling may also regulate biofilm formation and maturation. The significant inhibitory effect of eucalyptus extract on biofilm formation (Fig. 3) as well as virulence factors such as swarming and EPS formation was observed in both the CIs and the reference strain (PA01) of *P. aeruginosa* (Fig. 2). The biofilm-forming bacteria are usually resistant to antibiotics due to the formation of EPS as an essential component of biofilms [47].

Thus, reduction in EPS formation may be beneficial to control biofilm formation and the emergence of antibiotic resistance. In the present study, a significant reduction in biofilm formation as well as EPS was observed in CIs and the reference strain PA01 and as a result, the chances of resistance development in *P. aeruginosa* might be decreased at a lower dose (sub-MICs) of the eucalyptus extract. Reduction in biofilm formation using other plant extracts and oils has been previously reported in *P. aeruginosa* [36, 48]. The present study revealed the AQS potential of *E. globulus*, which may contribute to controlling bacterial infection as well reducing the emergence of resistance. Furthermore, molecular investigations are needed to elucidate its actual mechanism of virulence factors and biofilm inhibition.

CONCLUSION

Because of its AQS potential at a low dose, *E. globulus* has therapeutic importance in curing and controlling infections and reducing microbial resistance. Eucalyptus extracts may be an alternative and effective agent to combat infections caused by multi-drug resistant pathogenic bacteria.

CONFLICT OF INTEREST

There are no conflicts of interest.

ORCID

Pankaj Kumar Sagar, https://orcid.org/0000-0002-5335-2398
Poonam Sharma, https://orcid.org/0000-0003-1999-7309
Rambir Singh, https://orcid.org/0000-0002-3895-3005

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