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Article

Essential Oil from *Tagetes minuta* Has Antiquorum Sensing and Antibiofilm Potential against *Pseudomonas aeruginosa* Strain PAO1

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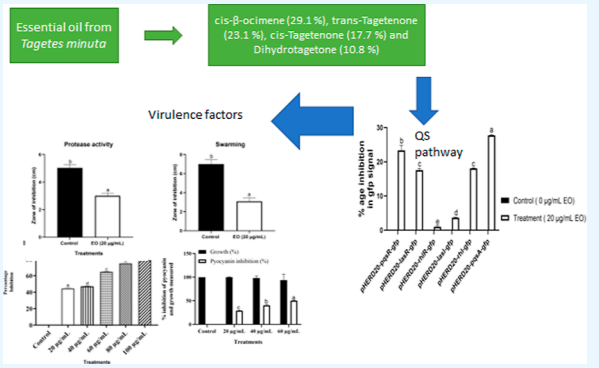
ABSTRACT: Biofilms are complex communities of microorganisms that are enclosed in a matrix that shows increased resistance to antimicrobial and immunological encounters. Mostly, the traditional methods to control biofilm are exhausted; therefore, the aim is to evaluate the potential of essential oil (EO) from *Tagetes minuta* to encounter biofilm and other related virulence factors. The EO of *T. minuta* was extracted through steam-distillation, analyzed on gas chromatography−mass spectrometry, and the biofilm inhibition assays were performed with various concentrations of EO. Mainly the EO from *T. minuta* contains *cis*-*β*-ocimene (29.1%), *trans*-tagetenone (23.1%), and *cis*-tagetenone (17.7%). The virulence factors were monitored while applying different concentrations of EO and it was recorded that the EO from *T. minuta* significantly inhibited the virulence factors linked with quorum sensing (QS), such as pyocyanin production, protease production, and swarming motility. Biofilm formation is one of the most important virulence factors associated with the QS pathway and was inhibited up to 79% in the presence of EO. Antibacterial activity against the PAO1 of EO was not so promising particularly and it has high MIC (325 μg/mL) and MBC (5000 μg/mL). EO is quite efficient to inhibit biofilm in a very small concentration of 20 μg/mL, which confirms that the biofilm inhibition by EO is not by killing bacterial cells but by inhibiting the QS pathway. The study on PAO1 constructs carrying various QS reported genes confirmed that the EO interferes with the QS pathway that ultimately controls various virulence factors caused by PAO1.



1. INTRODUCTION

Biofilms are complex microbial communities surrounded by an extracellular matrix that constitutes proteins, extracellular DNA (eDNA), lipids, and exopolysaccharides.[1] In most biofilms, only 10% of dry mass is composed of cells, whereas 90% is attributed to the extracellular matrix produced by bacteria themselves.[2] The main constituents of the matrix also called extracellular polymeric substance (EPS) are biomolecules such as exopolysaccharides, proteins, and extracellular DNA that form a polar hydrated mixture and are responsible for the overall structure and architecture of the biofilm.[3] Biofilms can be of single microbial species or multiple microbial species and are capable of formation on a variety of natural and artificial surfaces. Although mixed-species biofilms are prevalent in the environment, biofilms comprising single species are found in many diseases and on the surface of medical devices.[4]

Pseudomonas aeruginosa is an opportunistic pathogen found in a wide variety of natural environments and able to thrive in both aerobic as well as anoxic environments.[5] *P. aeruginosa* adapts some unique features especially during infection, biofilm formation under microaerobic conditions is one of them, that make it lethal during infection.[6,7] Owing to its opportunistic nature, it infects mostly immune-compromised individuals such as with severe burns.[8] The considerable metabolic versatility and flexibility of *P. aeruginosa* not only contribute to its ecological success but also render it necessary to understand the mechanisms underlying its pathogenicity and opportunistic nature.[9]

P. aeruginosa uses a regulator process known as quorum sensing to collectively produce a suite of virulence factors, which is a cell−cell communication process.[10] Quorum sensing (QS) starts with the autoinducer, which is the diffusible molecule, initially formed at low concentration and increases with cell density until a threshold concentration is reached. The autoinducer then enters back to bacteria and binds to its specific target protein. This autoinducer−protein complex activates genes that control various virulence factors including

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the biofilm.[11] Therefore, one of the approaches to control biofilm formation is to stop this communication by some natural products and the most potential candidates are plant-based compounds. Medicinal plants have been used from centuries to cure various diseases such as fever, cough, infections, etc.[12] Plants with antimicrobial activity have been used in traditional treatments to cure a variety of diseases and there has been an increased use of these plants since the last few decades.[13]

Medicinal plants have shown the promising attitude to inhibit the formation of biofilms; in a study, the leaf extracts obtained from *Syzygium masukuense*, *Syzygium species* A, and *Eugenia natalitia* efficiently inhibit the biofilm formation as well as have antibacterial activity against various pathogens.[14] Extracts obtained from *Citrus sinensis*, *Laurus nobilis*, *Elettaria cardamomum*, *Allium cepa*, and *Coriandrum sativum* had shown quorum quenching effect and have been able to inhibit production of pyocyanin as well as the formation of biofilm in *P. aeruginosa* PA14.[15] Essential oils (EOs) are natural aromatic oily liquids with complex compositions obtained from various parts of plants by following different methods including expression, fermentation, enfleurage, extraction, and distillation.[16] Due to the complex nature of EOs, they have multiple mechanisms of action through which they control various virulence factors including the formation of biofilms.[17] EO from *Cinnamomum tamala* has antivirulence activity and can be able to inhibit biofilm formation of *P. aeruginosa* separately as well as along with DNase isolated from marine bacterium with a synergistic effect.[18] Similarly, EOs from *Origanum heracleoticum*, *Origanum vulgare*, *Thymus vulgaris*, and *Thymus serpyllum* have antimicrobial as well as antibiofilm activity against *Salmonella enteritidis*.[19] Quorum sensing is the major mechanism through which bacteria control various activities and the virulence factors including biofilm formation are under the control of QS, EOs play a very important role to inhibit bacterial communication by showing their antiquorum sensing activity.[20]

Tagetes minuta L. is one of the widely occurring plants all over the world with several biological activities.[21] The plant *T. minuta* has high medicinal value and grow in various region of Pakistan, especially from an altitude of 1000 to 11,000 ft in the North and Northwest regions. It has allelochemicals and EO which have multidimensional applications such as insecticide, germicide, fungicide, nematicide, etc.[22] It has well-known inflammatory properties and has been used since ancient times as traditional medicine for curing stomach and intestinal discomfort.[23] The plant has enormous medicinal applications used for curing respiratory inflammation, and cough remedies, used in urinary tract infections, it facilitates mucus flow, dislodging congestion, used in skin infections.[21,23,24] The EO extracted from the foliar parts of the plant has, antibacterial, anti-inflammatory, and antioxidative effects.[23,25] The EO obtained from *T. minuta* has significant antibacterial activity against both Gram-positive and Gram-negative bacteria. That shows against *Staphylococcus aureus*, *Mycobacterium smegmatis*, *Streptococcus uberis*, *Listeria ivanovii*, *Vibrio* spp., *Enterobacter cloacae*, and *Escherichia coli*.[26] In another study, the EO was extracted from *T. minuta* grown in Egypt, South Africa, and UK, although have similar constituents but vary in composition, therefore showing a difference in antibacterial activity against various species of bacteria. The oil exhibited antibacterial activity but more effective against Gram-positive bacteria with a minimum inhibitory concentration (MIC) of 6.25 to 25 μg/mL, while for Gram-negative bacteria, the MIC was 25 to 50 μg/mL. It was also observed that the *P. aeruginosa* and *Salmonella typhi* has high MIC of oils.[27] Keeping in view the high medicinal and antimicrobial activity of *T. minuta*, it was hypothesized that the EOextracted from the plant may have potential to inhibit biofilm formation of one of the notorious pathogens *P. aeruginosa* PAO1 by obstructing its QS signaling pathway. Therefore, the current study was focused to discover the antibiofilm potential of EO extracted from *T. minuta* and to explore its mechanism of action.

2. RESULTS

2.1. Chemical Composition of EO. Sixteen compounds were identified in the EO extracted from aerial parts of *T. minuta* (Table 1). The most abundant compounds were *cis*-β-ocimene (29.1%), *trans*-tagetenone (23.1%), *cis*-tagetenone (17.7%), dihydrotagetone (10.8%), *cis*-tagetone (7.1%), and limonene (4.1%) constituting about 92% of EO (Table 1).

Table 1. Composition of EO Extracted from *T. minuta*

compounds	retention time	retention index	% composition
sabinen	12.33	973	0.5
limonene	14.34	1028	4.1
cis-β-ocimene	14.67	1037	29.1
trans-β-ocimene	15.07	1048	0.4
dihydrotagetone	15.25	1053	10.8
α-pinene oxide	16.13	1077	0.5
3,4-dimethyl-2,4,6-octatriene	18.04	1129	1.3
trans-tagetone	18.59	1144	1.2
cis-tagetone	18.92	1153	7.1
cis-tagetenone	21.75	1232	17.7
trans-tagetenone	22.05	1241	23.1
isopiperitenone	23.11	1271	1.7
β-caryophyllene	28.15	1425	0.4
elixene	30.53	1501	0.4
spathulenol	32.94	1583	0.6
caryophyllene oxide	33.12	1589	0.4

2.2. Antibacterial Activity. *T. minuta* EO did not show good antibacterial activity against PAO1 so exhibited quite high MIC and minimum bactericidal concentration (MBC), as shown in Table 2.

2.3. Biofilm Inhibition Assay. The EO of *T. minuta* inhibited biofilm formation when used in various concentrations (20 to 100 μg/mL). The minimum inhibitory concentration for the inhibition of PAO1 biofilm was 20 μg/mL, while the inhibition was significantly increased as the

Table 2. Antibacterial Activity Was Shown by Various Concentrations of EO of *T. minuta*

conc (μg/mL)	CFU growth inhibition (%)	
5000	100 ± 0.0	
2500	94.5 ± 0.8	
1250	54.9 ± 1.1	
625	31.9 ± 1.0	
312.5	0 ± 0.0	
	MIC (μg/mL)	MBC (μg/mL)
T. minuta	312.5	5000
ciprofolxacin	19.5	1250

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concentration of EO increased to 100 μg/mL which is recorded as about 79% but above 100 μg/mL, no significant effect was observed (data not shown), as shown in Figure 1.

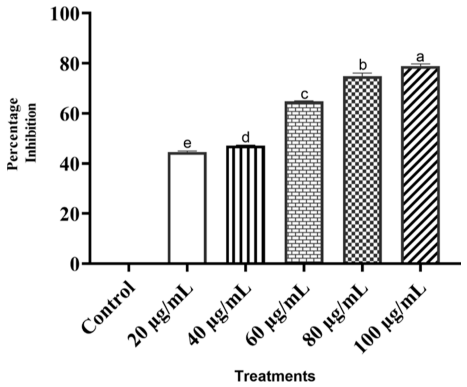

Figure 1. Percentage inhibition of PAO1 biofilm by various concentrations of EO extracted from *T. minuta*; different letters on bars indicate the significant difference ($p< 0.05$) between various treatments (**the letter "a" shows most significant difference as compared to control, then "b", "c", "d", and "e" as compared to control, respectively**).

2.4. Swarming Motility. Swarming is a complex type of motility shown by *P. aeruginosa* and under the control of various genes and considers to be involved in bacterial virulence. Therefore, EO from *T. minuta* was examined to anticipate its effect on the swarming motility of PAO1. It was found that at the concentration of 20 μg/mL of EO, the swarming motility was inhibited as compared to the control carrying no EO but at higher concentrations, the same findings were observed as in the presence of 20 μg/mL EO having no significant difference between each other as shown in Figure 2.

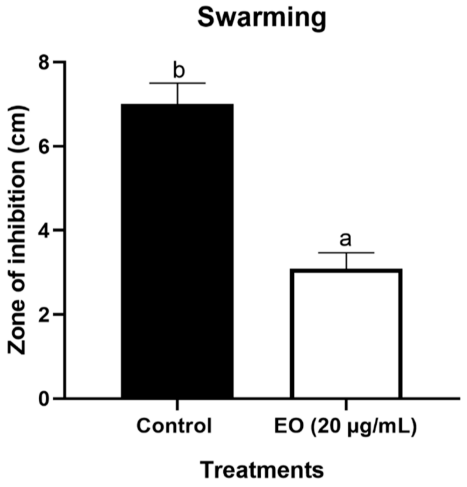

Figure 2. Effect of EO from *T. minuta* on swarming motility of PAO; different letters on bars indicate the significant difference ($p< 0.05$) between various treatments.

2.5. Quantification of Pyocyanin. The production of pyocyanin by PAO1 was significantly reduced in the presence of EO, as shown in Figure 3. It was found that the higher the concentration of EO, the more will be reduction in pyocyanin production and maximum reduction of about 50% was observed when 20 μg/mL of EO was used, as shown in

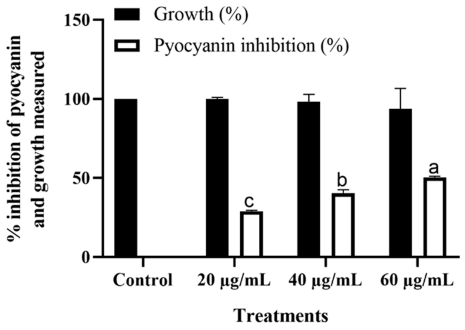

Figure 3. Effect of various concentrations of EO from *T. minuta* on the production of pyocyanin; different letters on bars indicate the significant difference ($p< 0.05$) between various treatments (**the letter "a" shows most significant difference as compared to control, then "b" and "c" as compared to control, respectively**).

Figure 3. However, in the meanwhile, there is no effect on the growth of bacterium, as shown in Figure 3.

2.6. Exoprotease Activity. The EO from *T. minuta* significantly inhibited the activity of proteases, as shown in Figure 4. The production of proteases was done in the

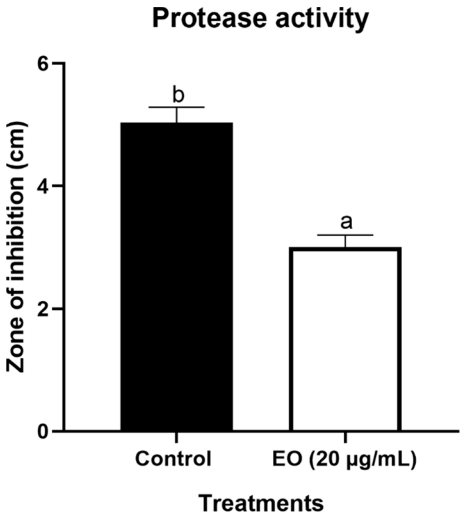

Figure 4. Effect of EO from *T. minuta* on the production of protease by PAO1; different letters on bars indicate the significant difference ($p< 0.05$) between various treatments (**letter "a" indicates that the EO significantly reduces the production of protease as compared to control indicates by letter "b"**).

presence and absence of 20 μg/mL EO and skimmed milk agar plates were employed to measure the clear zone. It was found that the EO has significantly inhibited the production of proteases which was about 42% as compared to the control, as shown in Figure 4.

2.7. Antiquorum Sensing Activity. Quorum sensing inhibition activities of the EO was compared to untreated samples. Results reveal that the EO has the potential to inhibit all the QS pathways, but it is most effective to inhibit pqsA followed by pqsR and lasR, as shown in Figure 5. The maximum inhibition in the gfp signal was recorded for pqsA which was about 27% followed by pqsR that was 23% and the minimum inhibition was for the rhlR pathway which was about 1% as compared to the untreated samples (control), as shown in Figure 5.

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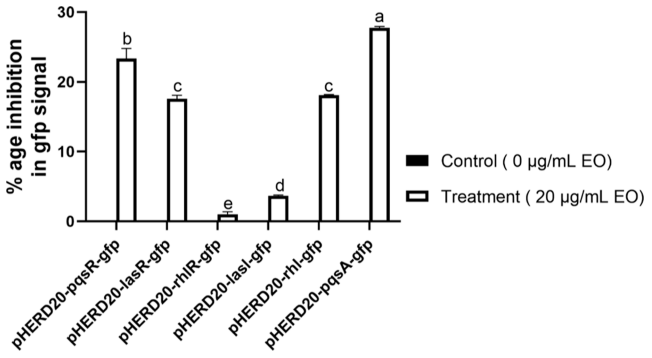

Figure 5. PAO1 harboring QS genes with gfp treated with EO from *T. minuta*; different letters on bars indicate the significant difference ($p < 0.05$) between various treatments (**the letter "a" shows most significant difference as compared to control, then "b", "c", "d", and "e" as compared to control, respectively**).

3. DISCUSSION

Biofilms are a major persistent threat especially during various infections because of an increase in morbidity and mortality.[4] Antibiotics mostly are unable to work against biofilms; therefore, it is required to develop some other strategies to manage biofilm-related infectious diseases. Quorum sensing (QS), which is cell density-dependent regulation of gene expression via extracellular signals, plays a vital role in the regulation of various virulence factors.[28] Therefore, the compounds targeting the QS system will eventually inhibit this cell-to-cell communication by influencing these genes involved in QS.[29] Medicinal plants are the natural resource of such chemicals that can interfere with QS signals and have a direct influence to control virulence caused by various pathogens.[30]

In the present study, the EO of *T. minuta* was used against PAO1 and its activity against various pathogenic factors caused by PAO1 was monitored. The present study exhibited that the main compounds in the EO of *T. minuta* were *cis*-*β*-ocimene, *trans*-tagetenone, *cis*-tagetenone, dihydrotagetone, *cis*-tagetone, and limonene. Several different studies from various parts of the world described the chemical composition of *T. minuta* EO and reported the presence of different major compounds that could be due to several biotic and abiotic factors. For example, a study from Argentina described that the major compounds of *T. minuta* essential contained 66.3% limonene, 11.8% *trans*-*β*-ocimene, 19.7% *trans*-tagetenone, and 2.7% *cis*-tagetenone.[31] Another study from Argentina reported 16.2% *trans*-*β*-ocimene, 10.3% dihydrotagetone, 2.9% *trans*-tagetone, and 62.2% *cis*-tagetone as the main components of *T. minuta* EO.[32] A previous study from Pakistan reported that the main components of *T. minuta* EO were limoene (8.3%), *cis*-*β*-ocimene (12.6%), dihydrotagetone (20.9%), *trans*-tagetone (14.8%), *cis*-tagetone (16.5%), and *cis*-tagetenone (13.1%).[33] The comparison of the present study with several previous studies demonstrated that the *T. minuta* EO contained similar types of compounds but their relative composition was quite different from each other. This could be explained by the fact that climatic conditions, soil type, available nutrients, and harvesting time affect the chemical composition of EO.

The current findings revealed that the EO of *T. minuta* has potential to inhibit the biofilm formation of PAO1 at various concentrations. Though no inhibition was recorded when used in a concentration of 10 μg/mL, but above this concentration until 100 μg/mL, there was a significant decrease in biofilm formation. Previous findings also have same results where the EOs from various plants inhibit the biofilm formation of various pathogens.[19,34] One of the studies on cinnamon oil reported that the oil inhibited biofilm formation along with other QS controlled virulence factors.[35]

The antibiofilm activity of *T. minuta* EO could be attributed due to the presence of its major compounds such as *cis*-*β*-ocimene, *trans*-tagetenone, *cis*-tagetenone, dihydrotagetone, and limonene but the synergetic effect of major and minor compounds could not be neglected. A previous study described that the *T. minuta* EO exhibited moderate antifungal activity against *Aspergillus flavus,* whereas quite good insecticidal activity against stored grain pest insect *Tribolium castaneum*.[36] Qualitatively, the *T. minuta* EO investigated in three different studies were similar; however, the chemical composition of major and minor components was quite different that is why they exhibited different bioactivities.[37] A study from Argentina showed that *T. minuta* EO exhibited medium to good antibiofilm activity against *Bacillus* sp. and *Staphylococcus* sp., respectively;[38] however, the chemical composition of the EO was not reported. Swarming is a complex type of motility influenced by various genes and is involved in the bacterial virulence.[39] Some researchers reported the swarming and biofilm has inverse relation, although if it is, swarming was positively associated with the production of proteases and type 3 exoenzymes.[40] The relation between the swarming and biofilm formation is still unclear, so in a recent study, it was concluded that the swarm cells of *P. aeruginosa* become adapted to UV radiation and enhance the formation of biofilm.[41] In another study, it was discovered that due to exposure to kanamycin the swarming cells of *Bacillus subtilis* activate matrix genes and induces biofilm formation.[42] In the present findings, the EO from *T. minuta* inhibited the formation of biofilm as well as the swarming motility which was quite like previous findings. Previous work on cinnamon EO concludes that the EO not only inhibited the formation of biofilm of *P. aeruginosa* but also restrict swarming motility.[35]

Similarly, pyocyanin is one of the important virulence factors produced by *P. aeruginosa*. Pyocyanin is a nitrogen-containing aromatic tricyclic phenazine compound that oxidizes glutathione and decreases its level in airway epithelial cells which is an important cellular antioxidant, which contributes to the regulation of redox sensitive signaling system.[43] It generates the reactive oxygen species by accepting the electron from NADH or NADPH with subsequent transfer of an electron to the oxygen.[44] EO from *T. minuta* significantly reduced the production of pyocyanin when added in various concentrations but more than 50% reduction was observed when used in higher concentration, i.e., 60 μg/mL. Similar findings were observed when EO from *Ferula assafoetida* L. and *Dorema aucheri* Bioss. were used against *P. aeruginosa* that reduced its pyocyanin production.[45]

In *P. aeruginosa,* number of virulence factors are regulated by QS systems;[46] therefore, the study was designed to observe the effect of EO on various QS systems in *P. aeruginosa*. In *P. aeruginosa* there are three QS systems, the two Las and Rhl systems synthesize and detect *N*-acyl homoserine lactone, while the third one, i.e., *Pseudomonas* quinolone signal (PQS) system, is responsible for 2-alkyl-4(1*H*)-quinolones (AQs) synthesis.[47] The virulence factors such as pyocyanin, elastase, lectin A, rhamnolipids, etc. are under the control of these three systems; therefore, the molecules which obstruct cell−cell

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communication by inhibiting either of these three QS systems will ultimately reduce the virulence caused by *P. aeruginosa*. In the present findings, EO from *T. minuta* significantly inhibited QS signals and mostly the PQS system, i.e., PqsA and PqsR as shown in Figure 5. Thus, the effect of EO on PAO1 virulence including the inhibition of biofilm is because of antiquorum sensing activity of EO from *T. minuta*. The results here of antibacterial activity has also been indicates that the inhibition of biofilm by EO is solely due to obstruction in QS pathway and not by killing effect of bacterial cells as a very high concentration of EO was required to kill *P. aeruginosa* as shown in Table 2. These results were also supported by previous findings where the EO interferes with QS system and control various virulence factors including biofilm formation.[48]

4. CONCLUSIONS

EO from *T. minuta* controlled various virulence factors of *P. aeruginosa* viz. interfering QS pathway and will be a potential candidate for drug discovery to controlled biofilm related infectious diseases.

5. METHODS

5.1. EO Extraction and Chemical Analysis. Aerial parts of *T. minuta* were collected from the Bagnutar area of Abbottabad district, KPK, Pakistan, and were deposited in the herbarium of the COMSATS University Islamabad, Abbottabad campus as a voucher specimen no. CUHA-101. The fresh plant was used to extract the EO by steam-distillation method as described earlier.[33] The plant material was cut into pieces and about 2 kg of the plant material was subjected to steam-distillation in a stainless steel distillatory. The distillate was collected in a separating funnel for 4 h and the EO was recovered from the upper layer in a separating funnel through decantation. The extracted oil was dried over a layer of anhydrous magnesium sulfate and stored at −20 °C until further use.

Chemical profiling of the EO was performed by using the Hewlett-Packard 6890 N gas-chromatograph (GC) coupled with an HP 5973 mass spectrometer (MS) (Agilent Technologies Inc. USA), as described earlier.[36] Briefly, the GC was fitted with a DB-5 capillary column (Agilent Technologies Inc., USA) of 0.25 mm internal diameter, 30 m length, and 0.25 μm stationary phase film thicknesses. The GC injector was set at 235 °C, and the auxiliary tube connecting GC to the MS was set at 240 °C. The temperature of the GC oven was initially set at 40 °C for 2 min and then slowly ramped to 240 °C at a rate of 4 °C/min. The final oven temperature (240 °C) was maintained for 8 min. A constant flow of helium (1 mL/min) was employed as the mobile phase. The MS ion source temperature was set at 180 °C and electron ionization (EI) mass spectra were recorded at 70 eV with a mass scan range of 30 m/z to 400 m/z. The identification and relative composition of separated compounds was carried out by adopting the method described by Azeem et al. (2019).[33]

5.2. Bacterial Strains and Growth Conditions. *P. aeruginosa* PAO was gifted by Professor Luyan Ma, Institute of Microbiology, Chinese Academy of Sciences, China. PAO1 was grown in Luria−Bertani (LB) at 37 °C or in Jensen's chemically defined medium,[49] which was stored at −80 °C in a glycerol stock. The biofilm was cultured by using a freshly prepared seed culture of PAO1 in LB broth having an absorbance value of 0.8 to 1 at 600 nm. Biofilm of *P. aeruginosa* was grown in Jensen's medium at 30 °C in 96 well plates made of polystyrene while incubating for 24 h.[50] All experiments were performed in replicates of three with at least three negative controls with respective to treatments.

5.3. Antibacterial Activity. The broth dilution method was used to check the antibacterial potential of *T. minuta* EO by following the protocol described before with some modification.[51] For this, freshly grown colonies of the bacterium were suspended in 4 mL of sterilized distilled water and the optical density of the suspension was adjusted to 0.5 McFarland standard which was equivalent to 10^8 colony forming units per mL (CFU/mL). This bacterial suspension was further diluted in sterilized water to get the desired concentration of 10^4 CFU/mL. The 10 μL aliquot of bacterial suspension was taken in a 5 mL glass test tube to which 980 μL of nutrient broth (NB) or 980 μL sterilized water, and 10 μL of test sample or control were added. The test tube with water, bacterial suspension, and $DMSO_4$ were worked as a reference to count the number of CFU originally added in any sample or control. Different concentrations of EO were prepared in $DMSO_4$ that was added to bacterial suspension thus the final concentration of the test sample in a test tube was 5000 to 312.5 μg/mL having 2-fold dilutions in each step. After overnight incubation at 37 °C, an aliquot of 10 μL sample mixture from each test, control, or reference test tube was spread on NA Petri plates and incubated at 37 °C for 24 h and the number of bacterial colonies grown on reference or test, or control Petri plates was counted. $DMSO_4$ was used as negative control while ciprofloxacin was used as a positive control, and at least three replicates of each concentration of the test sample were employed.

5.4. Biofilm Inhibition Assay. Crystal violet (CV) biomass assay was employed for the estimation of the inhibition of biofilms. Biofilm inhibition assay was performed with various concentrations of *T. minuta* EO (20−100 μg/mL) and was added to growth media at the time of inoculation. For biofilm inhibition assay, the medium as well as planktonic cells were washed after 24 h of incubation and stained with 0.1% crystal violet.[52] After 20 min, the crystal violet was washed thoroughly, and the biofilm was dissolved in 30% acetic acid and measured the absorbance at 560 nm.

5.5. Swarming Motility. Swarm plates of *P. aeruginosa* PAO1 were prepared containing (1% w/v) glucose, (0.5% w/v) bactoagar, (0.6% w/v) bactopeptone, and (0.2% w/v) yeast extract. The swarming media was seeded with different concentrations of EO of *T. minuta* (20 to 60 μg/mL) and poured immediately into the plates, while the control contain only swarming media. Both the control and the treatment plates were inoculated with 2 μL of *P. aeruginosa* strain PAO1 and incubated for 24 h at 37 °C.[53]

5.6. Pyocyanin Quantification Assay. The pyocyanin was determined in both the treated (containing 20 to 60 μg/mL EO separately) and untreated samples based on spectrophotometric assay where the absorbance was measured at 520 nm in acidic solution.[54] A colony of freshly prepared culture of *P. aeruginosa* PAO1 was inoculated in 10 mL PB (*Pseudomonas* broth, that contains: 20 g Bacto-peptone, 1.4 g $MgCl_2$, and 10 g of K_2SO_4 per liter of distilled water) and incubated for 24 h at 37 °C. The 5 mL culture was used to extract pyocyanin with 3 mL of chloroform and then re-extracted into 1 mL of 0.2 N HCl that gives pink to deep red solution after shaking it firmly. The percentage inhibition of pyocyanin was determined by the following formula.

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$$\%\text{age inhibition of pyocyanin} = \frac{(\text{control} - \text{treament})}{\text{control}} \times 100$$

5.7. Exoprotease Activity. Protease activity was measured by skimmed milk agar assay. The efficacy of EO of *T. minuta* to inhibit protease activity was measured by culturing PAO1 in the presence and absence of 20 μg/mL EO and the clear zone was measured on skimmed milk agar plates. The supernatant was obtained by centrifuging the overnight culture at 10,000 rpm and cell free extract (CFE) obtained was used for protease activity. CFE was loaded in center of skimmed milk agar plates and incubated at 37 °C, and after 24 h of incubation, the clear zone obtained was measured to determine the activity of exoprotease.[35]

5.8. Antiquorum Sensing Activity. The already constructed reporter strains (Table 3) were used for determined the effect of *T. minuta* oil on quorum sensing attribute of *P. aeruginosa* PAO1.[55] The reporter strains were grown in LB carrying 20 μg/mL EO of *T. minuta* as a treatment, while control carries no EO. The overnight culture was diluted to value of 0.25 at OD_{600} for measuring the fluorescence intensities. The growth of each construct were determined simultaneously at OD_{600}. All experiments were conducted in replicates of three.

Table 3. Strains and Plasmid Used in This Study

strain/plasmid	characteristics	references
Pseudomonas aeruginosa		
PAO1	wild type; ATCC 15692	Wei et al., 2012
PAO1/pHERD20-pqsR-gfp	PAO1 containing pqrS-gfp reporter plasmid	Wei et al., 2020[55]
PAO1/pHERD20-lasR-gfp	PAO1 containing lasR-gfp reporter plasmid	Wei et al., 2020[55]
PAO1/pHERD20-rhlR-gfp	PAO1 containing rhlR-gfp reporter plasmid	Wei et al., 2020[55]
PAO1/pHERD20-lasl-gfp	PAO1 containing lasl-gfp reporter plasmid	Wei et al., 2020[55]
PAO1/pHERD20-rhl-gfp	PAO1 containing rhl-gfp reporter plasmid	Wei et al., 2020[55]
PAO1/pHERD20-pqsA-gfp	PAO1 containing pqsA-gfp reporter plasmid	Wei et al., 2020[55]

5.9. Statistical Analysis. All the experiments were conducted in a triplicated manner and the average values were presented. For statistical analysis, SPSS 21 was employed and ANOVA followed by Duncan's test was performed to compare the means of various treatments. Independent "*T*" test was employed where two treatments were compared with each other.

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this article are available in the article.

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Palwasha Khan: investigation, data curation, formal analysis, and methodology; **Amara Waheed**: investigation and data curation; **Muhammad Azeem**: draft writing, analysis, and methodology; **Amna Parveen**: analysis; **Muhammad Arfat Yameen**: draft writing and analysis **Jamshed Iqbal**: analysis; **Muhammad Ali**: resources and analysis; **Shiwei Wang**: analysis and methodology; **Sadaf Qayyum**: writing and analysis; **Awal Noor**: writing, analysis, and funding; and **Tatheer Alam Naqvi**: conceptulization, supervision, draft writing, formal analysis, resources, and project funding.

Notes

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