



Synergism Between α -Terpineol and Terpinen-4-ol Potentiates Antivirulence Response Against *Pseudomonas aeruginosa*

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Abstract Antivirulence strategies have emerged as next-generation therapies that are now becoming refractory to the use of traditional antimicrobial approaches. Considering the global medical burden associated with *Pseudomonas aeruginosa* infections, there is a pressing need to explore therapeutic alternatives. In this direction, the current study was aimed at investigating the combinational effects of α -terpineol (α -T) and terpinen-4-ol (T-4-ol), the principal bioactive components of tea tree oil, against *P. aeruginosa*. The phytochemical combination was examined for synergistic interaction and various biological properties, including antibacterial, quorum quenching (QQ), and antivirulence potential. α -T and T-4-ol displayed synergism and harbored profuse antibacterial properties against *P. aeruginosa*. The phytochemicals inhibited quorum sensing (QS) in biosensor strains of *Agrobacterium tumefaciens* and *Chromobacterium violaceum* by suppressing *lacZ* and diminishing violacein production, respectively. Moreover, α -T and T-4-ol, independently and in combination, extended antivirulence response by significantly reducing hemolysin, pyocyanin, pyochelin, and total protease production in *P. aeruginosa* PAO1 and PA14. Hence, this study suggests that the phytochemical combination of α -T and T-4-ol can be used as a potent antivirulence elixir over antibiotics to combat *P. aeruginosa*.

Keywords α -Terpineol and terpinen-4-ol · Bioactive phytochemicals · *Pseudomonas aeruginosa* · Quorum quenching · Antivirulence therapy

Pseudomonas aeruginosa is an extremely notorious and multidrug-resistant Gram-negative bacterium that has recently been classified as a Priority 1 (critical) pathogen by the World Health Organization [1]. Due to its extensively invasive/toxigenic nature, wide tissue tropism, prevalence of intrinsic/extrinsic drug resistance mechanisms, and production of resilient biofilms and diverse virulence determinants, there is an urgent need to develop alternate intervention strategies against *P. aeruginosa* [2]. Interestingly, pseudomonal virulence is stringently modulated by quorum sensing (QS), a cell density-dependent phenomenon that synchronizes bacterial responses at the community level [3]. The Las, Rhl, and Pqs systems function in a hierarchical manner to intricately regulate QS circuits in *P. aeruginosa*, which in turn modulates the genotypic expression and phenotypic production of virulence factors [4]. Hence, a viable alternative to traditional antimicrobial treatments is the targeted inhibition of QS which can potentially disarm bacterial virulence [5]. Also, antivirulence therapies employing QS inhibitors do not mediate bacterial killing but only silence virulence pathways and are thus known to minimize the development of anthropogenic resistance in bacterial pathogens [2].

In the past two decades, innumerable quorum quenching (QQ) agents harboring antivirulence properties against *P. aeruginosa* have been widely reported [5]. Among these, plant-derived essential oils (EOs) and their bioactive phytochemicals have gained attention from the scientific community due to their natural abundance, convenient accessibility, promising pharmacological attributes, and applications in mainstream medicine [6]. Tea tree oil is one such EO that

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has been thoroughly investigated and widely recognized for its medicinal significance and broad-range antimicrobial properties [7, 8]. Its principal bioactive components, α -terpineol (α -T) and terpinen-4-ol (T-4-ol), have independently been shown to interfere with QS circuits and attenuate virulence in *P. aeruginosa* [9, 10]. Moreover, free- and liposomal-forms of α -T have been shown to rescue *Caenorhabditis elegans* from *P. aeruginosa* infection [11] and biofilm-associated keratitis in mice [12]. Additionally, the combination of α -T and T-4-ol was recently proposed as an effective alternative against ESKAPE pathogens [13]. Although the study recognized the combination to be effective in terms of antimicrobial activity, there was no emphasis laid to explore the possible effects on bacterial virulence. To further extend the encouraging results obtained from our previous research [7–10], the current investigation was undertaken to scrutinize the antibacterial, anti-QS, and anti-virulence prospects of α -T and T-4-ol in combination against *P. aeruginosa*.

Throughout the course of experimentation, standard virulent strains of *P. aeruginosa* (PAO1 and PA14) were employed, while QS inhibition was studied using two different biosensor strains, *Agrobacterium tumefaciens* NTL4 (pZLR4) and *Chromobacterium violaceum* MTCC 2656. Interestingly, *A. tumefaciens* NTL4 has been genetically modified to detect C4-, C8-, C10-, and C12-homoserine lactones (HSLs) via induction of *lacZ* through the *tral* promoter on pZLR4, stimulating the enzymatic degradation of X-Gal and yielding blue-colored colonies [14]. Similarly, *C. violaceum* produces a violet-colored pigment, violacein, which is expressed only upon QS induction via the C6-HSL signal [15]. The present study was initiated by examining the antibacterial potential of α -T and T-4-ol, alone and in combination, against *P. aeruginosa* using a previously reported well-diffusion assay [6]. The minimum inhibitory concentrations (MICs) of the test phytochemicals were determined against *P. aeruginosa* strains following protocols prescribed by the Clinical Laboratory Standards Institute (CLSI) guidelines coupled with a resazurin-based dye reduction test [16, 17]. The standard chequerboard dilution method was then used to evaluate the type of interaction between α -T and T-4-ol in combination [18]. Upon investigating the drug interactions, the QQ potential of test phytoproducts was qualitatively examined using *A. tumefaciens* NTL4 and *C. violaceum* using previously established biosensor assays [14, 19]. The antivirulence prospects of α -T and T-4-ol were subsequently investigated by quantitatively estimating the production of hallmark QS-regulated virulence factors such as pyocyanin, hemolysin, total protease, and pyochelin in drug-treated and -untreated culture supernatants of *P. aeruginosa* [11, 14].

The inhibition zones obtained in well-diffusion assays confirmed the antibacterial properties of α -T and T-4-ol (10 mg/mL) against *P. aeruginosa* PAO1 and PA14

(Fig. 1A, B). Moreover, in combination, the antibacterial effect increased drastically with a notable increment in the diameter of inhibition zones (Table S1). The MIC values of T-4-ol against PAO1 and PA14 were experimentally found to be 1.25 and 2.5 mg/mL, respectively (Fig. 1C). Whereas the MIC for α -T was reported to be 1.25 mg/mL against both the *P. aeruginosa* strains (Fig. 1C), indicating the antibacterial potency of the test phytochemicals [17]. The chequerboard dilution assay revealed a significant reduction in the MIC values for both α -T and T-4-ol, when used in combination against *P. aeruginosa* (Fig. 2). For α -T, the fractional inhibitory concentration (FIC) against PAO1 and PA14 was found to be 0.064 and 0.124, respectively (Table S2). Additionally, the FIC value for T-4-ol was determined as 0.124 against both the *P. aeruginosa* strains. Interestingly, the FIC index (FICI) for α -T and T-4-ol against PAO1 and PA14 was calculated to be 0.188 and 0.248, respectively (Table S2). Considering the FICI for the test phytochemicals was experimentally less than 0.5 [10], a synergistic interaction between α -T and T-4-ol was successfully established.

The QQ potential of α -T and T-4-ol, alone and in combination (at synergistic concentrations), was then probed using biosensor strains. With *A. tumefaciens* NTL4, colorless halos corresponding to anti-QS zones were observed around the wells against a blue-colored background (Fig. 3A). This revealed inhibition of QS via suppression of *lacZ* on pZLR4 by the test phytochemicals [14]. Moreover, α -T and T-4-ol also diminished violacein production in *C. violaceum*, which was distinctly characterized by pigment beaching (colorless bacterial growth) around the phytochemical-loaded wells (Fig. 3B). To our interest, both the phytoproducts exhibited anti-QS activity independently, but when used in combination at synergistic levels, the anti-QS response was notably enhanced [11, 17]. These findings motivated us to investigate the downstream effects of this phytochemical combination on the phenotypic expression of QS-driven hallmark virulence determinants in *P. aeruginosa*. The plant bioactives were able to independently impede the production of all virulence factors. Upon solo treatment with α -T, the production of pyocyanin, hemolysin, total protease, and pyochelin in PAO1 and PA14 significantly lowered by 36.6%, 43.1%, 54.4%, 34.8% and 45.3%, 56.2%, 52.2%, and 37.9%, respectively (Fig. 3D–F). While the inhibition extended by T-4-ol alone was 29.1%, 28.4%, 31.2%, 14.5% and 34.3%, 43.7%, 28.8%, and 25.3%, against PAO1 and PA14, respectively (Fig. 3D–F). Combinational treatment with α -T and T-4-ol further heightened the antivirulence response in PAO1 and PA14, thereby inhibiting the production of virulence factors by 83.5%, 85.4%, 87.3%, 76.2% and 85.7%, 91.4%, 89.2%, and 81.1%, respectively (Fig. 3D–F), thereby indicating that the phytochemical combination of α -T and T-4-ol could effectively revoke the virulent phenotype in *P. aeruginosa* PAO1 and PA14 by abrogating QS circuits [14].

Fig. 1 Antibacterial activity of α -T and T-4-ol (both 10 mg/mL) against **A** *P. aeruginosa* PAO1 and **B** *P. aeruginosa* PA14. **C** Antimicrobial potency of the test phytochemicals in terms of minimum inhibitory concentration (MIC)

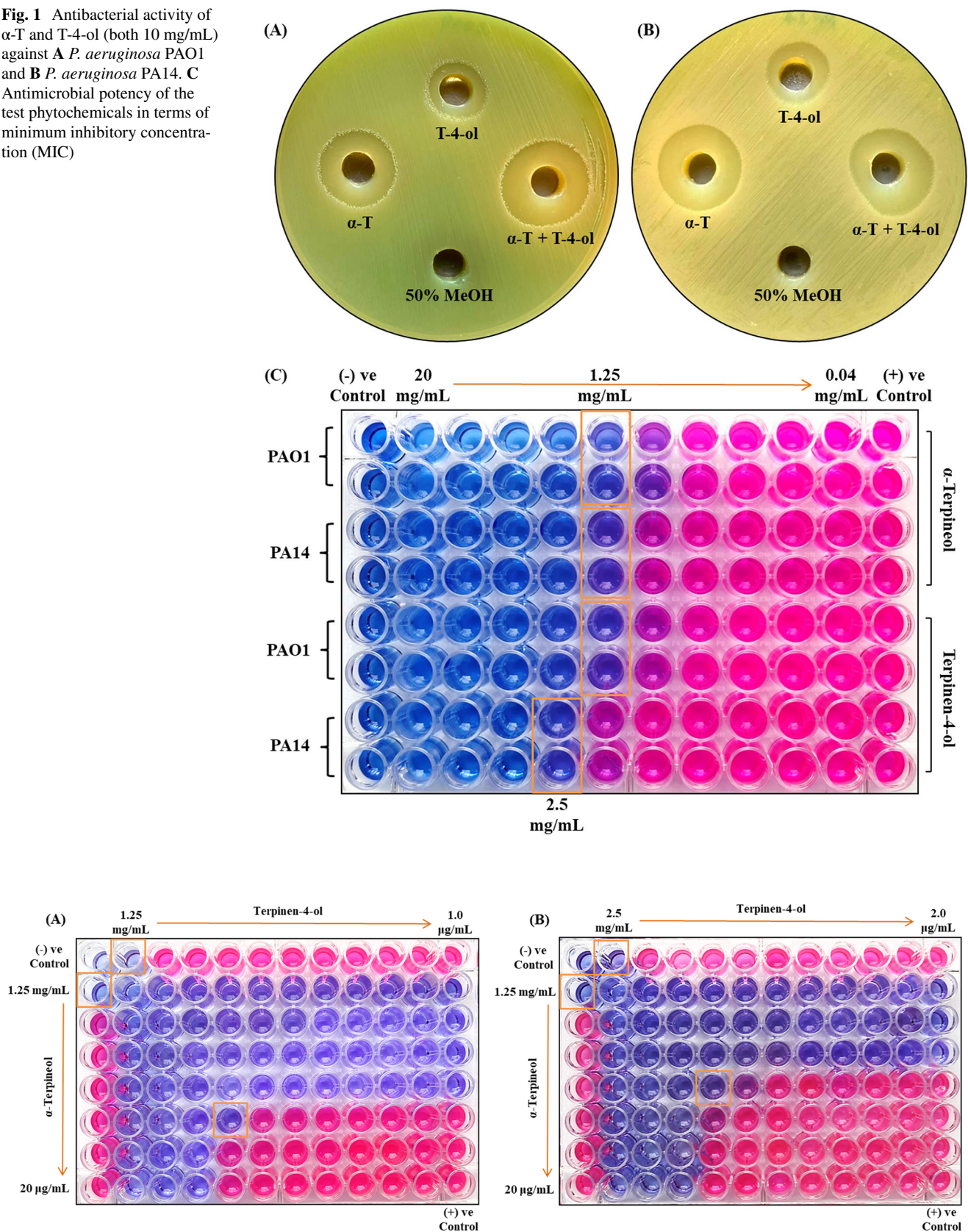


Fig. 2 Checkerboard dilution technique depicting synergistic interactions between α -T and T-4-ol against **A** *P. aeruginosa* PAO1 and **B** *P. aeruginosa* PA14

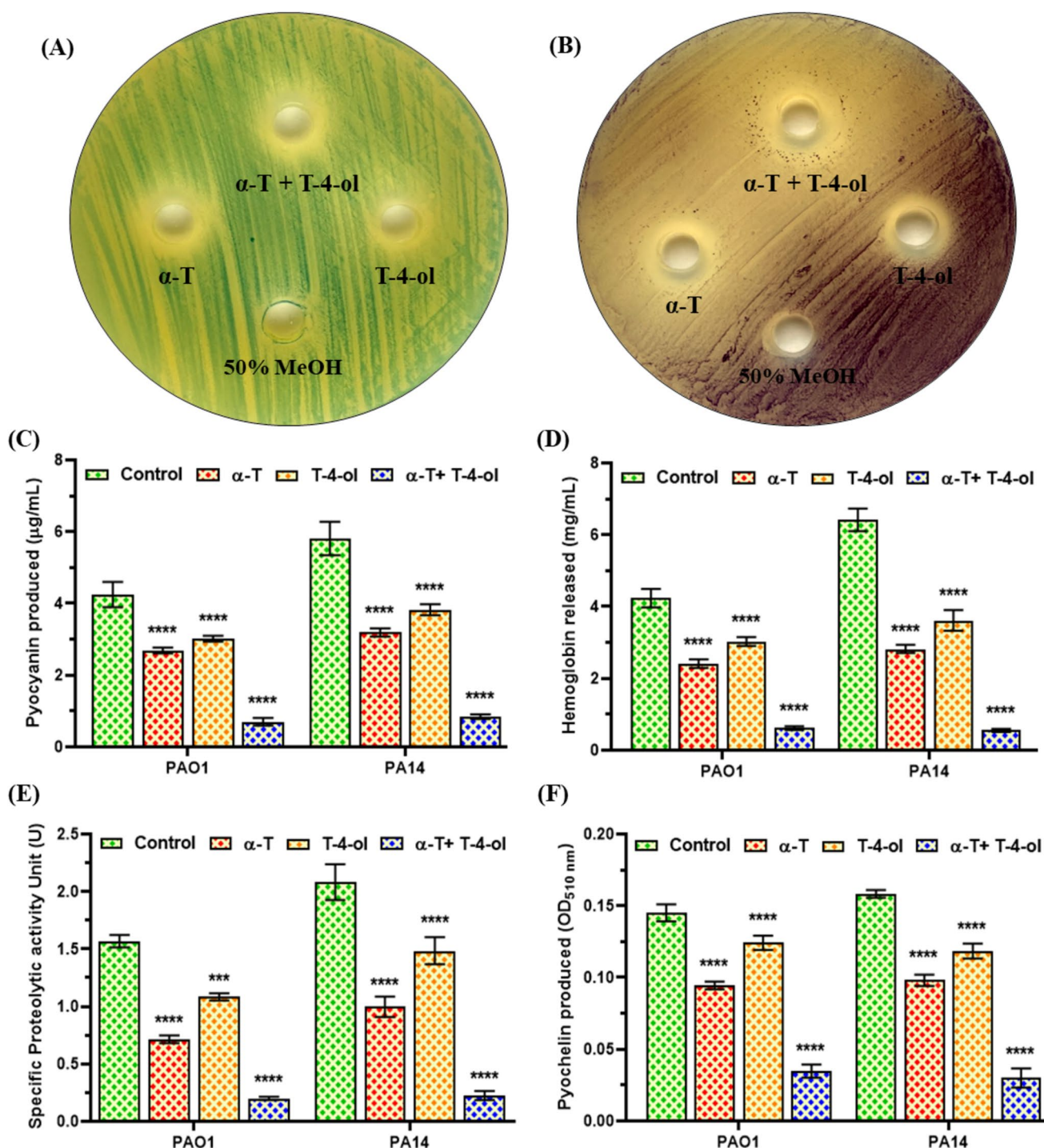


Fig. 3 Anti-QS and antivirulence potential of α-T and T-4-ol against biosensor and standard *P. aeruginosa* strains, respectively. **A** Visualization of anti-QS prospects using *A. tumefaciens* NTL4 and **B** *C. violaceum* MTCC 2656. **C** Effect on pyocyanin, **D** hemolysin, **E** total

bacterial protease, and **F** pyochelin production (statistical analysis has been carried out in reference to the respective untreated controls: *** $p \leq 0.001$, **** $p \leq 0.0001$)

In summary, this investigation confirms the synergistic action of α-T and T-4-ol towards potentiating anti-bacterial activity, anti-QS potential, and antivirulence responses against *P. aeruginosa*. This novel phytochemical

combination was illustrated to interfere with the QS mechanisms in the biosensor strains, thereby silencing QS circuits, and ultimately attenuating bacterial virulence in *P. aeruginosa*. Therefore, as an efficacious alternative to existing

antimicrobial regimens, bioactive phytochemicals displaying remarkable antivirulence properties can be exploited for medical application against *P. aeruginosa*. Coating of α -T-T-4-ol combination onto medical devices like urinary catheters, intravenous catheters, prosthetic implants/stents, and intrauterine devices might help in mitigating pseudomonal infections and resilient biofilms. Moreover, incorporating such antivirulence phytochemicals in skincare products, sanitizers, and topical delivery agents like ointments, wound dressings and bandages can also be beneficial in combating *P. aeruginosa*. Nevertheless, investigations in animal models (in vivo) are needed to translate this bench-based research for the widespread application of α -T-T-4-ol combination in clinical settings.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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