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ORIGINAL PAPER

Growth-phase specific regulation of *cvi*I/R based quorum sensing associated virulence factors in *Chromobacterium violaceum* by linalool, a monoterpenoid

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Abstract

Quorum sensing (QS)-dependent gene regulation in bacteria performs a vital role in synchronization of cell-density-dependent functions. In *Chromobacterium violaceum* QS-dependent *cvi*I/R regulatory genes are activated during the mid- or late-exponential phase of growth. However, sufficient evidence is lacking on the role of QS inhibitors on gene regulation at different phases of growth. Hence, we report the role of linalool, a natural monoterpenoid on QS mediated gene regulation at different stages of growth in *C. violaceum* by performing biosensor, growth kinetic and gene expression studies. In vitro and in vivo studies were performed for establishing role of linalool in reducing the virulence and infection by using HEK-293 T cell lines and *Caenorhabditis elegans* models respectively. *C. violaceum* CV026 with C_6-HSL was used as control. The results showed linalool to be a QS inhibitor with an estimated IC_{50} of 63 µg/mL for violacein inhibition. At this concentration the cell density difference (delta OD_{600}) of 0.14 from the compound was observed indicating the quorum concentration. The expression of *cvi*I/R was initiated at mid-log phase (~ 18 h) and reached the maximum at 36 h in control whereas in treatment it remained significantly downregulated at all time points. The expression of violacein biosynthetic genes *vio*A, *vio*C, *vio*D and *vio*E was also downregulated by linalool. Infection studies with linalool showed higher survival rates in HEK-293T cell lines and *C. elegans* compared to the infection control. Taken together, this study proves linalool to be a QS inhibitor capable of attenuation of QS by controlling the cell density through *cvi*I/R downregulation at the early phase of growth and hence offering scope for its application for controlling infections.

Keywords Quorum sensing · *cvi*I/R regulation · Linalool · *C. violaceum* · In vivo infection

Introduction

Quorum sensing (QS) is a well-studied signalling mechanism in bacteria controlling the expression of group of genes in a cell-density-dependent manner particularly with focus on its role in controlling virulence mechanisms and pathogenicity (Ruer et al., 2015; Jesudhasan et al., 2010). In *Chromobacterium violaceum* QS system consist of *Cvi*I/R, a *Lux*I/R homologue system that is activated in response to the autoinducer, N-hexanoyl-l-homoserine lactone (HSL) (Tarighi and Taheri 2011; Papenfort and Bassler 2016). QS regulates various phenotypes such as violacein production, biofilm formation, chitinolytic activity, cyanide and elastase production among others in *C. violaceum* (Kothari et al., 2017). The QS mechanism is triggered by the bacterial growth in the ecosystem that depends on the various environmental cues, nutritional factors, and presence of other bacterial communities (Whitehead et al. 2001; Mukherjee and Bassler 2019). The QS mediated gene expression is modulated if a threshold concentration is achieved that allows bacteria to coordinate quorum-based community behaviour (FitzGerald et al. 2020). Hence, the threshold density during autoinduction of signals plays a crucial role for the bacterial community expansion including formation of biofilm (Solano et al. 2014; Vikram et al. 2010).

Quorum sensing plays a significant role in pathogenicity and regulate many virulence genes (Sikdar and Elias 2020; Soni et al., 2008). It is of significance in the treatment of infections caused by opportunistic pathogens particularly

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to overcome the antibiotic resistance (Wang et al. 2020). The precise understanding of QS machinery has allowed the identification of inhibitors to attenuate QS controlled virulence. A diversity of inhibitors of QS signal receptors and signal synthases have been discovered, in addition to enzymes that degrade signalling molecules (Ng and Bassler 2009; Wu et al. 2020; Soni et al., 2008). Designing the drugs that interfere with QS to target virulence serves as anti-virulence agents instead of antibacterial agents and, hence it is believed that resistance is less likely to emerge (Rutherford and Bassler, 2012; Kalia 2013). Many natural and synthetic compounds such as flavonoids, furanones and macrolides have been established as QS inhibitors through different experiments (Ouyang et al. 2016; Burr et al. 2016; Vikram et al. 2010).

Linalool (3,7-dimethyl-1,6-octadien-3-ol) is an important constituent of essential oils with the molecular formula being $C_{10}H_{18}O$ and molecular weight of 154.25 g/mol possessing biological activities including antibacterial, antiviral, and anti-inflammatory activities (Pereira et al. 2018). Essential oils like frankincense oil, carrageenin enriched with linalool exhibited anti-inflammatory by targeting the cytokines (Varia et al. 2020; Li et al. 2016), also showed anti-bacterial activity by disrupting the bacterial cell wall and inhibiting enzyme activity (Park et al. 2012; Duarte et al. 2016). Further, a glycol-monoterpinol derived from linalool has shown to inhibit QS phenotypes in *P. aeruginosa* and *C. violaceum*. Essential oils extracted from *Cinnamomum camphora*, *Ocimum basilucum*, *Coriandrum sativum* L., *Salvia sclarea* contain linalool as one of major components and these essential oils have anti-QS activity (Kerekes et al. 2013; Wang et al. 2019; Molina et al. 2020). However, these studies are limited to phenotype level and exploring the transcriptional expression of the genes involved in the QS network is essential for assessing the functioning of the cells under QS inhibitory conditions. Based on this background, we investigated the effect of linalool as a potential candidate to attenuate QS using *C. violaceum* as a model organism. In *C. violaceum* the production of violacein is mediated through the expression of *vio*ABDEC operon regulated by *cvi*I/R system. The *vio*ABDEC operon consists of five enzyme coding genes namely, *vio*A, *vio*B, *vio*C, *vio*D, and *vio*E which are majorly oxidoreductases and mono-oxygenases (Hoshino 2011). As violacein production is a factor of cell density, we investigated the role of linalool by studying the temporal variation in phenotypes and gene expression patterns with respect to the cell density. Further, we also studied the effect of linalool in controlling biofilm and infection using in vitro and in vivo models to establish its potential use.

Methodology

In this study, we used *C. violaceum* ATCC 12472, the wild type of strain that produces violacein pigment and *C. violaceum* CV026, a mini-Tn5 mutant which lacks the AHL synthase gene and produces pigment only in response to exogenous AHL. Luria–Bertani (LB) and Muller Hinton media (HiMedia, India) were used respectively for culturing the bacteria and for the determination of minimum inhibitory concentration (MIC). Linalool was purchased from Sigma Aldrich, USA (CAS number 78-70-6). HEK-293 T cells (Human embryonic kidney 293 cells) were obtained from National Centre for Cell Sciences (NCCS), Pune. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), antibiotic–antimycotic solution, trypsin–EDTA, methyl thiazolyl tetrazolium (MTT), acridine orange and propidium iodide were purchased from HiMedia, India. The nematode *Caenorhabditis elegans* N2 strain used for in vivo infection studies were maintained in nematode growth media at 25 °C. The feed culture *E. coli* OP50 maintained in LB media at 37 °C was used.

Activity of linalool against *C. violaceum* quorum sensing

The anti-QS activity of linalool against *C. violaceum* was performed as described previously (Rekha et al. 2017). Briefly, sterile discs (6 mm) impregnated with different concentrations of linalool were placed on the LB agar plates seeded with overnight of *C. violaceum* ATCC 12472 culture and incubated for 24 h at 32 °C. For the bioassay with mutant *C. violaceum* CV026, LB agar medium was supplemented with C_6-HSL (10 µL of 25 µM). The anti- QS activity was interpreted based on the presence of a zone of colourless but viable cells around the discs and anti-microbial activity was indicated by the presence of transparent zone. The MIC of linalool against *C. violaceum* ATCC 12472 was determined using broth microdilution method according to Clinical and Laboratory Standards Institute guidelines (CLSI 2018; Wayne 2010). For this, linalool stock (10 mg/mL aq.) was diluted in Muller–Hinton broth to obtain the desired final concentration in the range of 0–320 µg/mL in 96-well plates. Initial density of the bacteria was adjusted to OD_{600} of 0.02. Appropriate blanks (without bacteria) and control (without linalool) were used for comparisons. The MIC was interpreted as the lowest concentration of linalool that prevented the visible growth of *C. violaceum* at 24 h incubation.

Estimation of $IC_{50}$and IC_{90} for violacein inhibition

For quantification of violacein and estimation of violacein inhibitory activity (IC_{50}), broth assay was performed at sub-MIC concentrations of linalool as described previously

(Rekha et al. 2017). For this, 10 mL of LB broth containing linalool was seeded with *C. violaceum* to an initial density of 10^5 CFU/mL. The contents were incubated at 32 °C under shaking (120 rpm) for 24 h. Violacein was estimated spectrophotometrically (OD_{585}) after extracting the pigment from cell pellet using water-saturated *n*-butanol. The experiment was performed in triplicates and as three independent sets. The IC_{50} and IC_{90} values were calculated from the graph plotted using percentage violacein inhibition values compared to control.

Biofilm studies

Crystal violet staining method was used to study the effect of linalool on *C. violaceum* biofilm as previously described (Stepanovic et al. 2007). LB broth medium containing linalool was inoculated with *C. violaceum* in 96-well microtiter plates and incubated at 32 °C under static condition. For comparison, *C. violaceum* CV026 supplemented with C_6-HSL was also used. After 24 h, the planktonic cells were decanted, wells were washed with PBS and the adherent biofilm was fixed with methanol (95%) before staining with crystal violet. After solubilising the stain in acetic acid (33%), the biofilm intensity was estimated (OD_{590}) spectrophotometrically.

qRT-PCR studies to understand the gene expression pattern during the different growth phases in response to linalool

Experiments were designed to monitor the regulatory gene expression at different phases of growth under treatment with linalool compared to control conditions. Initially the effect of linalool on the growth kinetics was assessed. The bacteria were grown in LB medium supplemented with linalool (80 µg/mL) and growth was followed at every six hours by recording OD_{600} values. The CFU/mL was calculated by standard serial dilution and plating method. The data obtained were used for generating the growth curve. Appropriate controls were used for comparison.

The expression of regulatory genes *cvi*I and *cvi*R, and the genes involved in the violacein biosynthesis (*vio*A, *vio*C, *vio*D and *vio*E) were quantified by quantitative PCR method (Burt et al. 2014). The cells were harvested from the treatment groups (Group 1: CV-12472 (control); Group 2: CV12472 + Linalool; Group 3: CV026 + C_6-HSl; Group 4: CV026 + C_6-HSL + Linalool) at regular intervals. Total RNA was extracted from the cells using the RNAiso plus Trizol reagent (Catalogue no: 9108/9109 TaKaRa) following the manufacturer's protocol and was quantified using a Nanodrop spectrophotometer. The single-stranded cDNA was prepared using Prime Script 1st strand cDNA synthesis kit (TaKaRa, India) in accordance with manufacturer's protocol and was used as a template for RT-qPCR amplification. PCR was performed using TB Green™ Premix ExTaq TM II master mix (TliRNAase Plus, TaKaRa) using CFX96 qPCR system (Bio Rad). The primers used for *cvi*I, *cvi*R, *vio*A, *vio*C, *vio*D and *vio*E genes and 16S rRNA gene used for reference are given in the supplementary table 1. PCR amplifications were performed using the temperature cycles of 10 min at 95 °C, followed by 40 cycles (10 s at 95 °C and 60 s at 60 °C). The data were calculated using $2^{-\Delta\Delta CT}$ method and expressed as fold change compared to the control sample from three independent experiments.

In vitro and in vivo studies to evaluate the protective effect of linalool against *C. violaceum* infection

Cytotoxicity studies using MTT assay

Prior to conducting the infection studies the cytotoxicity of linalool was assessed in vitro using HEK-293 T cells by Methyl Thiazolyl Tetrazolium (MTT) assay (Mosmann 1983). The cells maintained in DMEM (HiMedia India) supplemented with 10% FBS and antibiotics (Penicillin–streptomycin) in a CO_2 incubator were seeded to 96-well plates at a density of 5000 cells/well. The cells were treated with different concentrations of linalool and incubated for 24 h. Subsequently, the cells were treated with MTT reagent (1 mg/mL), incubated for 4 h and DMSO was added to solubilise the formazan crystals. The absorbance was read as OD_{570} against cell control using a multimode microplate reader (FluoSTAR Omega, BMG Labtech, Germany). The experiment was performed in triplicates and the results are expressed as percentage of viable cells calculated based on OD_{570} values of control and treatment groups.

In vitro infection study on HEK-293 T cell lines

For in vitro infection studies, HEK-293 T cells were seeded in 96-well plates containing DMEM with 10% FBS at a density of 5000 cells/well. For infection 5 µL of bacterial suspension in PBS was added to achieve multiplicity of infection (MOI) of 10. The infected cells were incubated at 37 °C for 6 h in a CO_2 incubator. The experimental groups included infection, treatment (linalool) and infection with treatment (Sharma et al. 2020). For imaging, the cells were fixed with ice-cold methanol, stained with propidium iodide (3 mg/mL) and acridine orange (5 mg/mL) solutions for 30 min protected from light. The cells were observed using a fluorescent cell imager using green and red channel (ZOE, Bio-Rad, USA).

In vivo *C. violaceum* infection using *Caenorhabditis elegans* model

Caenorhabditis elegans was used as an in vivo model system to study *C. violaceum* infection (Durai et al. 2013). Briefly, overnight culture of *C. violaceum* ATCC 12,472 was spread on LB agar plates incorporated with linalool (20–80 µg/mL). After incubating at 37 °C for 24 h, 10 synchronized worms (L4 stage) of the wild-type *C. elegans* N2 strain were transferred onto the bacterial lawn. The plates were further incubated at 20 °C and the nematodes were scored for survival and paralysis every 12 h for 3 consecutive days. The survival analyses were performed using Survival curve and compared with control (GraphPad Prism 8.0).

Statistical analysis

All the experiments were performed in triplicates (n = 3). The data is presented as mean ± standard deviation. Significance of difference between groups was analysed using one-way analysis of variance (ANOVA), and $p < 0.05$ was considered significant unless stated. Bonferroni and Holm post-hoc tests were used for comparison. Student's t-test was used for comparison between two groups. Mantel–Cox test was used to analyse the *C. elegans* infection study data. All the graphs and figures were generated using GraphPad prism 8.02 software and CorelDRAW 2019.

Results

Linalool inhibits quorum sensing-controlled violacein production in *C. violaceum*

The biosensor bioassay using the reporter strain *C. violaceum* CV026 and the wild type of strain *C. violaceum* ATCC 12,472 demonstrated the QS inhibitory potential of linalool (Fig. 1a, b). The diameters of zone of QS inhibition were 65 mm and 87 mm respectively in *C. violaceum* ATCC 12,472 and *C. violaceum* CV026 (supplemented with C_6-HSL). The MIC of linalool against *C. violaceum* ATCC 12,472 was 120 µg/mL estimated based on the broth microdilution method. The QS inhibitory activity of linalool at sub-MIC is shown in Fig. 1c, d. At 40–80 µg/mL complete inhibition (> 90%) of violacein was observed with 10–15% decrease in growth compared to the control. The IC_{50} for growth inhibition and violacein inhibition are 56 and 63 µg/mL.

Linalool suppresses the biofilm formation of *C. violaceum*

The biofilm inhibitory potential of linalool was established by crystal violet staining method. At the tested concentrations, the biofilm was significantly ($p < 0.01$) inhibited in *C. violaceum* ATCC 12,472 (Fig. 2a). *C. violaceum* CV026

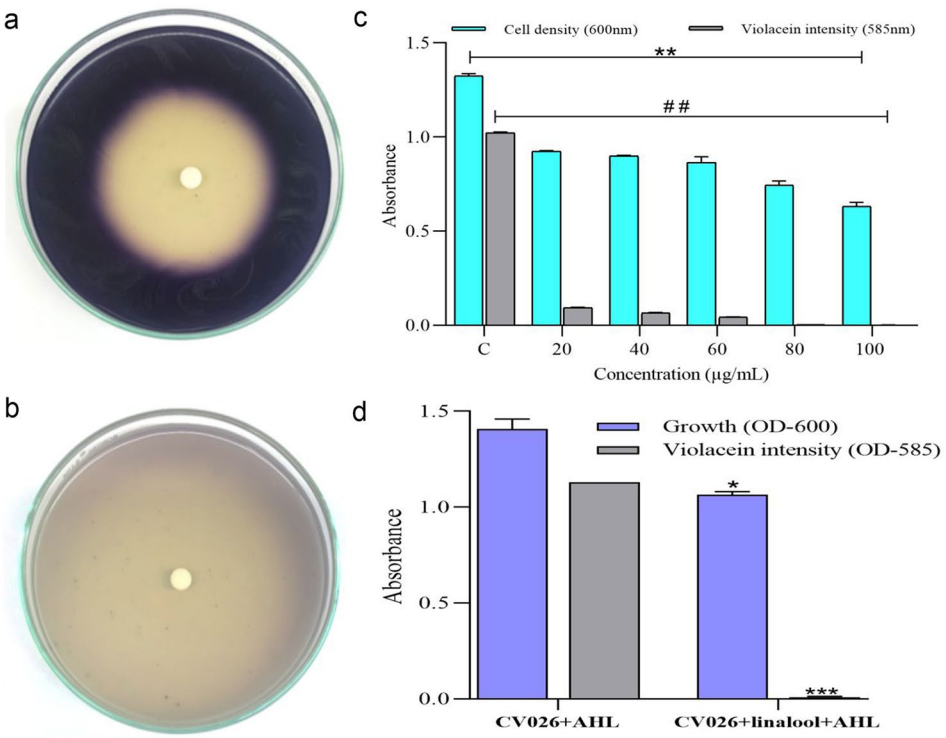

Fig. 1 Quorum sensing controlled violacein production in *C. violaceum* by linalool. Quorum sensing inhibitory activity of linalool showing inhibition of C_6-HSL mediated quorum sensing in *C. violaceum* by biosensor bioassay, against *C. violaceum* ATCC 12472 (**a**), *C. violaceum* CV026 supplemented with C_6-HSL (**b**). Graph (**c**) represents results of *C. violaceum* ATCC 12472 treated with linalool, growth measured as OD-600 and violacein quantified as OD-585. (**d**) *C. violaceum* CV026 treated with linalool in the presence and absence of C_6-HSL Treated group showed statistical difference when compared to respective control ($p < 0.05$)

grown without C_6-HSL could not form profuse biofilm (OD_{585}—0.12), while supplemented with C_6-HSL formed strong biofilm (OD_{585}—1.05). Linalool treatment significantly suppressed the biofilm in the C_6-HSL supplemented CV026 culture indicating the role of QS in controlling the biofilm of *C. violaceum* (Fig. 2b).

Growth phase dependent population density and expression of quorum sensing genes in response to linalool treatment

A series of experiments were conducted to understand the population density for QS activation by following the violacein production in the wild type *C. violaceum* at different time points (0–30 h). The agar plate assay allowed the visualization of colour development corresponding to the cell multiplication at different time intervals (Fig. 3a, b). Based on these time points, the pigment production in the liquid broth medium was followed. The results suggest that between 12 and 18 h of growth, the culture produced the violacein pigment with a corresponding cell density of OD_{600} of > 1.25 (Fig. 3c, d). Beyond this time point there was a significant increase in the pigment production in the control. But in linalool treatment group bacterial growth was maintained at OD_{600} of < 1.25 without inducing the violacein pigment (Fig. 3e, f). We observed that linalool treatment controlled the population below threshold maintaining a uniform difference in the cell density with delta OD_{600} of 0.14 (Fig. S1).

Based on these results, the effect of linalool on the expression of *cvi*I/*cvi*R system was evaluated at different critical time points of growth (12, 18, 24 and 36 h). In the control set, the expression of *cvi*I/*cvi*R was up-regulated with evolution of time corresponding to the pigment intensity. But treatment with linalool significantly reduced ($p < 0.001$) the expression of *cvi*I and *cvi*R genes (Fig. 4a). The mutant *C. violaceum* CV026 grown in presence of C_6-HSL and treatment with linalool also showed similar results (Fig. 4b). In the control set with increase in cell density, up-regulation in the expression of *vio* genes was observed indicative of activated QS phenomena. The expression of violacein biosynthetic genes (*vio*A, *vio*C, *vio*D and *vio*E) in *C. violaceum* were downregulated with linalool treatment. Interestingly, the *vio*A and *vio*E genes were almost completely repressed in linalool treatment group (Fig. 4c–f).

Linalool supresses *C. violaceum* infection in vitro and in vivo

The in vitro and in vivo studies were conducted to evaluate the effect of linalool treatment against *C. violaceum* infection. Prior to the infection studies, the safety of linalool on the cells was tested by MTT assay. Linalool did not exhibit toxicity at the tested concentration range of 10 to 80 µg/mL (Fig. 5a). However, at a concentration of 100 µg/mL, 20% cell death was observed.

The HEK-293 T cells infected with *C. violaceum* in vitro showed significant cell death compared to the control ($p < 0.05$) and linalool treatment was successful in reducing the infectivity significantly (Fig. 5b). The live/dead staining of the cells showed similar morphology in both treatment and control groups, but the infected cells without treatment showed disrupted morphology.

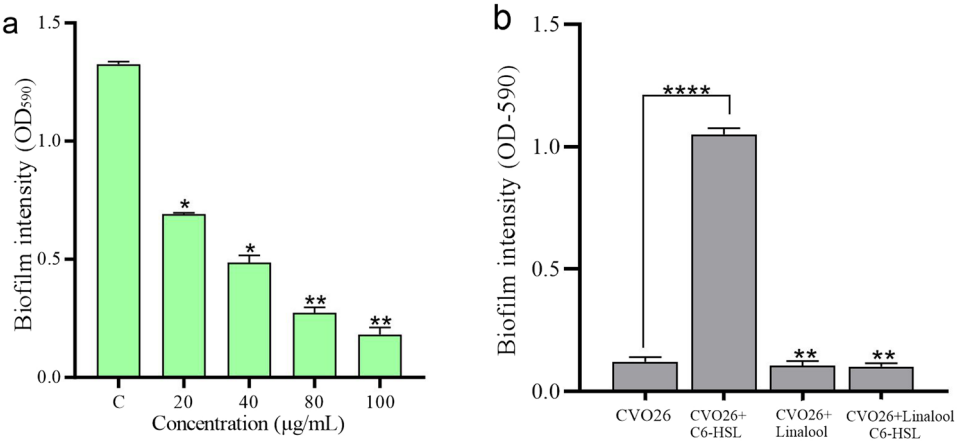

Fig. 2 Linalool targets biofilm inhibition in *C. violaceum.* Graph showing the reduction in biofilm intensity in presence of linalool at different concentrations in *C. violaceum* ATCC 12472 (**a**) and in *C. violaceum* CV026 supplemented with C_6-HSL (**b**). The biofilm intensity was quantified spectrophotometrically (OD_{580}) by crystal violet staining method. Data points represented are mean ± SD and statistical significance was assessed based on *p*-value ($p < 0.05$ was considered statistically significant). Data represented as mean ± SD and statistical significance was assessed based on *p*-value < 0.05

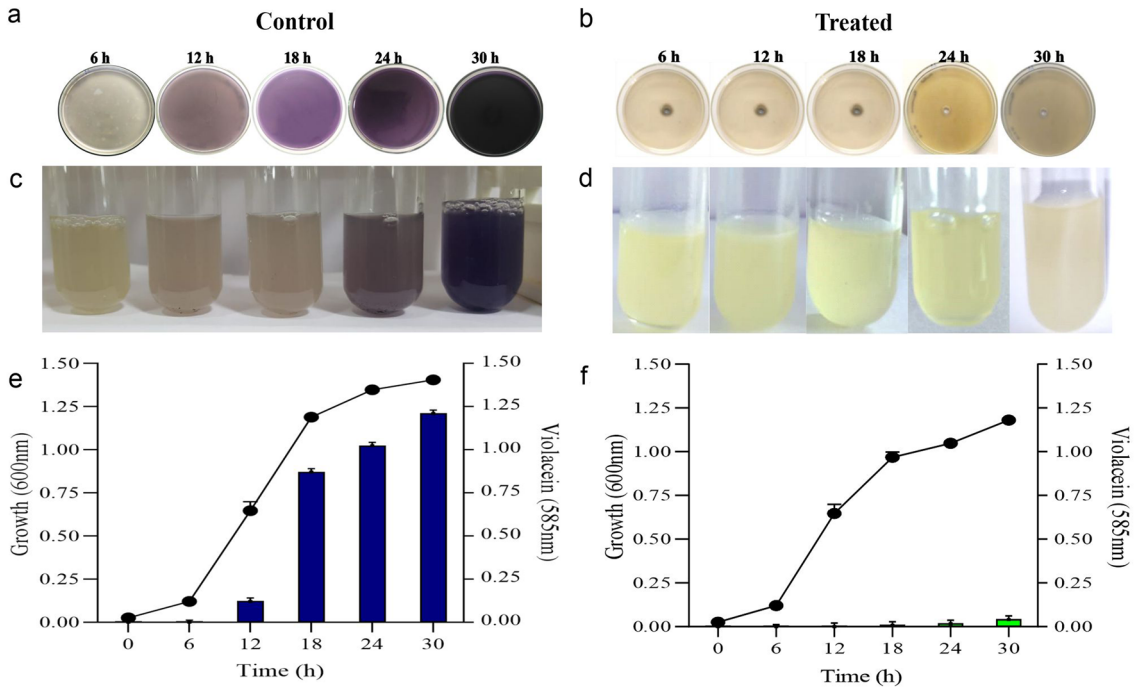

Fig. 3 Growth phase dependent modulation of quorum sensing phenotypes in *C. violaceum.* The agar plate assay exhibiting the violet colour pigment development at every 6 h time interval in control (**a**) and in presence of linalool (**b**) by agar diffusion method. Exponential increase in the pigment production shown in culture tubes representing the growth and violacein pigment (**c** and **d**) development at increasing time intervals and graph representing corresponding cell density and pigment production in control (**e**) and in presence of linalool (**f**). Data points represented are mean ± SD

In vivo *C. elegans* survival assay showed that linalool could reduce the mortality of the nematodes at 40–80 µg/mL concentration. Enhanced survival of the infected nematodes was observed in the linalool treated group. Linalool alone did not exert any significant effect on the *C. elegans* (Fig. 6a). At these concentrations no paralytic activity was observed (Fig. 6b).

Discussion

There is a growing demand for novel antimicrobials and developing new approaches that can target the bacterial virulence. Modulation of QS allows to control the bacterial population at the transcriptional level through suppression of the QS genes (Suresh et al. 2021). In this study, linalool a natural monoterpenoid present in many essential oils having a myriad of biological activities used in this study showed strong QS inhibitory activity against *C. violaceum*. The concentration of linalool required to block the pigment production through QS inhibition was in the range of 60–80 µg/mL, while the MIC was estimated to be approximately 120 µg/mL. A previous study by Lahiri et al. (2021) showed the QS inhibitory potential of linalool extracted from *Ocimum tenuiflorum* plant against *P. aeruginosa* phenotypes at a concentration of 175 µg/mL. A study by Ngenge et al. (2021) showed the anti-QS activity of *Melaleuca alternifolia* at 250 µg/mL against *C. violaceum* and *P. aeruginosa* isolates. Compared to their results the linalool in its pure form used in the study has higher potency for QS inhibition. Using the growth kinetic studies, the threshold population density for the quorum concentration was estimated and we observed at an $OD_{600} > 1.25$, the bacterial culture could produce pigment. Interestingly, linalool treatment controlled the population below this threshold maintaining a uniform difference in the cell density compared to control (delta OD_{600} of 0.14). It is well reported that, when the cell density increases beyond threshold, the temporal patterns of expression of genes in the QS network during growth shows up-regulation of genes in the QS network (Lazdunski et al. 2004). This phenomenon insures the bacterial metabolic activities including virulence factors. Many earlier studies have shown that the QS inhibitors generally do not affect the growth (Vattem et al. 2007). However slight difference in the growth can be attributed to the inhibition of QS controlled growth expansion, without arresting its kinetics. In bacteriostatic mechanism, the growth curve remains at a plateau due to arresting of the bacterial multiplication.

Biofilm is a co-operative group behaviour that involves bacterial population self-embedded in an extracellular

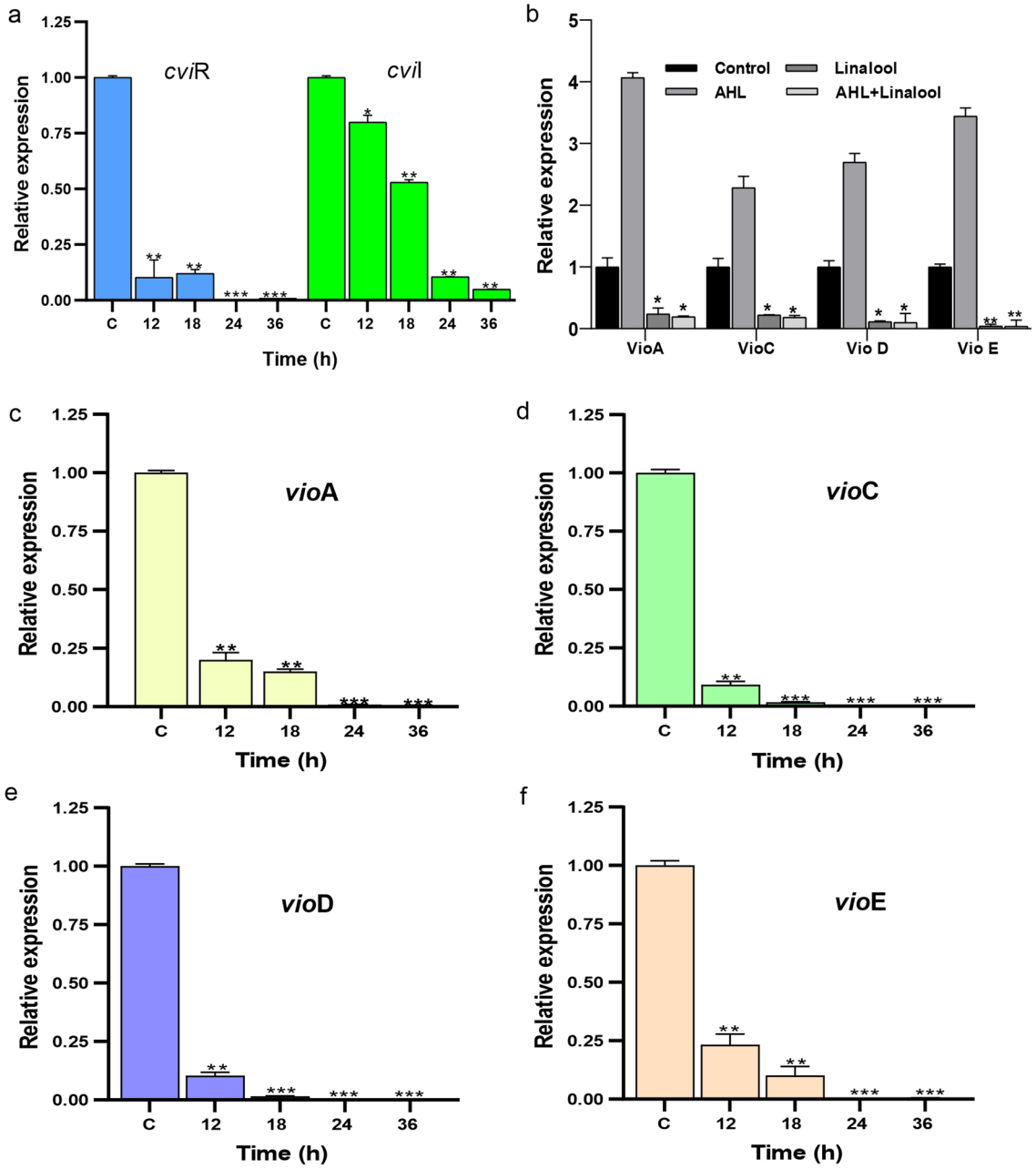

Fig. 4 Linalool effectively targets quorum sensing regulatory and violacein biosynthetic gene expression. RT-PCR data representing growth phase dependent downregulation of regulatory genes *cvi*I and *cvi*R in *C. violaceum* ATCC 12472 in presence of linalool (**a**); violacein biosynthetic genes (*vio*A, *vio*C, *vio*D and *vio*E) *C. violaceum* CVO26 supplemented with AHL (**b**) and *C. violaceum* ATCC 12472 (**c**–**f**) at 6 h time intervals. Data points represented are mean ± SD and statistical significance was assessed based on p-value

polymeric matrix. In response to nutrient availability and environmental stress QS plays a role in switching to the biofilm lifestyle (Solano et al. 2014; Saxena et al., 2019). Hence, QS is considered as a potential target for treating biofilm infections which are generally recalcitrant to antimicrobial agents. The results of biofilm inhibition in *C. violaceum* by linalool in the wild type and mutant supplemented with C_6-HSL were similar confirming the role of linalool in QS modulation. *C. violaceum* biofilm architecture is quite unique and display uniform arrangement of cells that are strongly controlled by QS (Kamaeva et al. 2014). However, studies have shown that compounds like carvacrol, carvone can disrupt this co-ordinated assembly of biofilm architecture (Tapia-Rodriguez et al. 2017; Kanekar et al. 2021). Research on inhibitors of biofilm have vast applications in healthcare sector as biofilms are strongly associated with

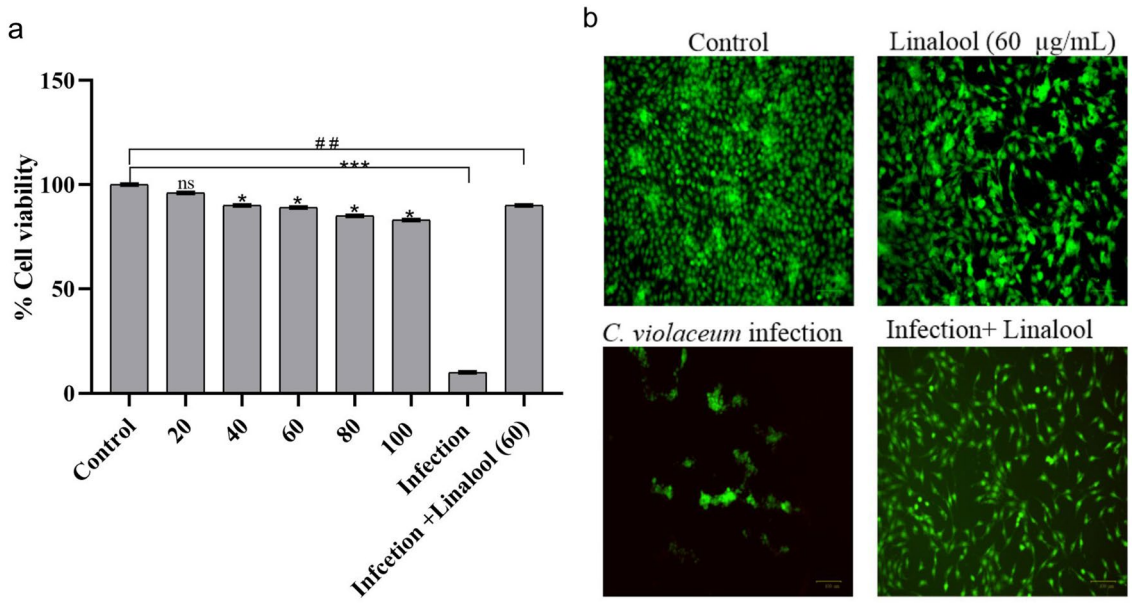

Fig. 5 Effect of linalool on viability of HEK-293T cells infected with *C. violaceum.* **a** Graph representing percentage viability of HEK-293T cells treated with different concentrations of linalool. The results are expressed as mean ± S.D., *p < 0.05 and **p < 0.01, compared to control group. **b** Live dead staining using acridine orange and propidium iodide of HEK-293T cells after 24 h treatment with linalool. Green represents living cells and red the dead cells. Scale bar 100 µm

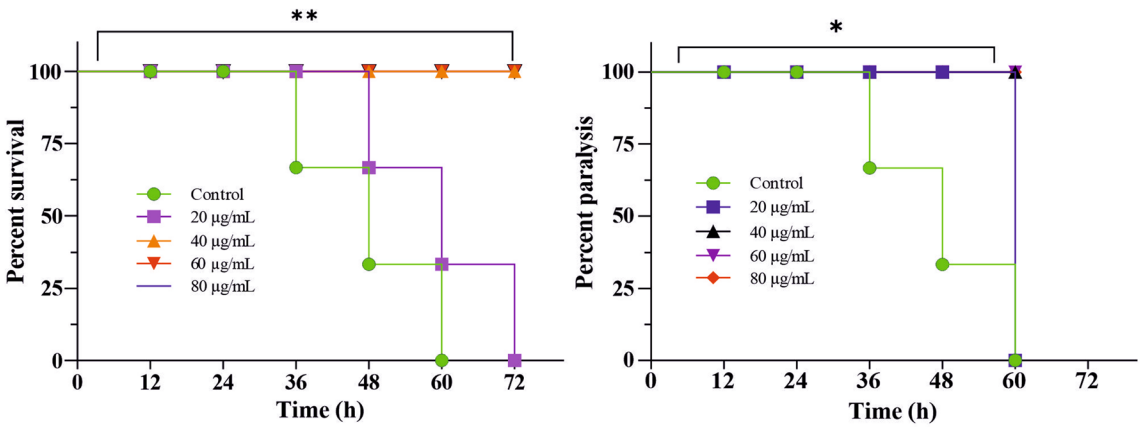

Fig. 6 Linalool enhances the survival of *C. elegans* infected with *C. violaceum.* **a** Survival curve representing the effect of linalool at QS inhibitory concentrations on survival of *C. elegans* infected with *C. violaceum*. **b** Graph representing percentage of paralysis in *C. elegans* infected with *C. violaceum* at 12 h interval. The survival curve comparison done by Mantel–Cox test where ** and * represents p value of 0.0035 and 0.045 respectively

infection transmitted through medical devices (Vipin et al. 2019; Kurmoo et al. 2020).

The QS mediated gene expression system gets activated during the bacterial growth in relation to autoinducer concentration and metabolic status of the cell (Rutherford and Bassler 2012; Venturi 2006). In *C. violaceum*, *Cvi*I/R, the QS regulatory system controls different QS mediated phenotypes depending on the growth conditions (Cámara 2006; Kimyon et al. 2016; Ravichandran et al. 2018). In this study, the expression of *cvi*I and *cvi*R genes were significantly down-regulated in presence of linalool in comparison with control. Similarly, the expression of the violacein biosynthetic genes was also downregulated. The *vio* operon consists of five enzyme coding genes namely, *vio*A, *vio*B, *vio*C, *vio*D, and *vio*E that are transcribed in single direction producing Flavin-dependent L-tryptophan oxidase, 2-imino-3(indol-3-yl) propanoate dimerase, violacein synthase, protodeoxyviolaceinate monooxygenase and

violacein biosynthesis enzyme respectively (Kothari et al., 2017). The experiments using mutant with C_6-HSL and the wild type showed similar results in the gene expression pattern suggesting that linalool has the efficacy to target the AHL synthesis, where the autoinduction feed-forward process is being hindered, demoting the synchronous gene expression in the population. Similar results on the gene expressions were reported in natural extracts constituting terpenoids like carvacrol which downregulated the *cvi*I expression (Burt et al., 2014). In *Pseudomonas aeruginosa* the *cvi*I/R homologue, i.e. *las*I/R and *rhl*I/R has significant role in controlling the virulence factors such as protease, elastase, rhamnolipid and pyocyanin, which are significantly downregulated by treatment with furanones and flavonoids (Ahmed et al., 2019). As per the hypothesis the QS inhibitors can supress the virulence and infection rate.

Further, the safety of the test compound is important for its possible use as a therapeutic agent. The cytotoxicity of linalool assessed using the in vitro model showed no effect on the HEK-293T cells. Similar finding on the safety of linalool have been earlier established with IC_{50} value of 63 µg/mL (Duarte et al., 2016). Animal model studies have indicated the safety of linalool up to 100 mg/kg of body weight per day (Kashiwadani et al., 2021). *C. violaceum* as a pathogen is known to induce cytotoxicity in Hepa 1–6, RAW264.7, HepG2 and HeLa cell lines resulting in activation of the inflammatory cytokines (Miki et al., 2010). Our results of the infection studies demonstrate that linalool could significantly reduce infectivity of *C. violaceum* ATCC 12472 in vitro on HEK-293T cells. This may be due to controlling the virulence of *C. violaceum* as well as arresting the population expansion. Linalool can offer other benefits to cells due to its anti-inflammatory and antioxidant properties that are of significance for protection of cells during infection (Li et al. 2016; Kamatou and Viljoen. 2008). Similarly, the in vivo infection assays also demonstrated an enhanced survival of pre-infected nematode that was treated with linalool. This clearly indicates that linalool reduces the virulence factors during infection by *C. violaceum* directly restoring survival rate of *C. elegans*. Earlier studies using the aqueous extracts of medicinal plants have also shown similar improved survival rate of *C. elegans* infection with *P. aeruginosa* (Alexpandi et al. 2019). Quorum sensing molecules significantly increase when bacteria are under antibiotics exposure, which, in turn, promote the development of antibiotic tolerance and development (Lu et al. 2005; Lu 2004). Thus, quorum sensing inhibitors can also increase the efficacy of the antibiotics in combination therapy by exerting synergistic activity (Zhang and Dong 2004; Vipin et al. 2020). In combination therapy, the anti-QS molecules can alter the biochemical processes that are essential for the population expansion and virulence while, the bacterial survival can be targeted by the action of antibiotics thus offering the scope as adjuvants in antibiotic therapy.

Linalool has been demonstrated to possess QS inhibitory activity both at the phenotypic and genotypic level along with proof for reducing the infection both in vitro and in vivo. The mechanism of action of linalool at the QS expression level in a temporal scale adds to the understanding and provides the precise information at the target gene level. As the QS inhibitor linalool can inhibit the biofilm formation that has implications in healthcare sector particularly in controlling persistent infections indwelling medical devices. Linalool with other biological activities such as anti-inflammatory, antioxidant and anti-microbial that can be further explored in detail for its ability to act in synergy with antibiotics to enhance the antibiotic susceptibility.

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Author contributions KS designed and executed all the experiments, analysed the results, and prepared the manuscript. RPD framed the study, designed all experiments, analysed the data, and prepared the manuscript. Both the authors approved the manuscript for submission.

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Data availability All relevant data are within the paper and its Supporting Information files.

Declarations

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

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