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Essential oils modulate virulence phenotypes in a multidrug-resistant pyomelanogenic *Pseudomonas aeruginosa* clinical isolate

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Pyomelanogenic *P. aeruginosa*, frequently isolated from patients with urinary tract infections and cystic fibrosis, possesses the ability to withstand oxidative stress, contributing to virulence and resulting in persistent infections. Whole genome sequence analysis of U804, a pyomelanogenic, multidrug-resistant, clinical isolate, demonstrates the mechanism underlying pyomelanin overproduction. Seven essential oils (EOs) were screened for pyomelanin inhibition. Garlic, cinnamon and thyme EOs were selected for further studies based on their significant anti-virulent properties, like inhibition of pyomelanin production and biofilm formation. Additionally, downregulation of the expression of virulence genes regulated by quorum sensing (QS) and a decrease in levels of the QS signaling molecule, C12-HSL, were also observed. The EO treatment inhibited the survival of U804 in human blood and increased survival of *C. elegans*, a whole animal model of pathogenesis. EO treatment also resulted in a significant reduction of efflux pump activity, indicative of their effect on antibiotic sensitization. Garlic oil enhanced the permeability of the bacterial membrane, resulting in decreased survival, when combined with sub-MIC concentrations of colistin. This study demonstrates that thyme, cinnamon and garlic EOs can attenuate pyomelanogenic *P. aeruginosa* virulence traits. Additionally, garlic potentiates drug sensitivity, suggesting its promising therapeutic use in combating pyomelanogenic MDR infections.

Keywords *P. aeruginosa*, Pyomelanin, Essential oils, Virulence

Abbreviations

EOs	Essential oils
MDR	Multidrug resistance
eDNA	Extracellular DNA
AMR	Antimicrobial resistance
HGA	Homogentisic acid
NPs	Natural products
LB	Luria Bertani
DMSO	Dimethyl sulphoxide
DCFDA	Dichlorodihydrofluorescein diacetate
qPCR	quantitative real-time PCR
HPLC	High performance liquid chromatography

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P. aeruginosa	*Pseudomonas aeruginosa*
CFU	Colony forming units
C. elegans	*Caenorhabditis elegans*
EtBr	Ethidium bromide
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
MIC	Minimum inhibitory concentration
NPN	N phenyl 1-napthylamine
QS	Quorum sensing
AHL	Acyl homoserine lactone
WGS	Whole genome sequencing
ROS	Reactive oxygen species
LC-MS	Liquid chromatography–mass spectrometry

Pseudomonas aeruginosa, a Gram-negative opportunistic pathogen, can be found in a diverse range of ecological environments. It is associated with both acute and chronic infections, including burn wound infections, pneumonia, urinary tract infections and eye infections such as keratitis[1]. Additionally, *P. aeruginosa* can cause serious and potentially life-threatening invasive infections in persons with compromised immune systems[1]. This nosocomial pathogen is considered a critical public health priority, due to its significant impact on healthcare settings.

P. aeruginosa possesses a large genome with multiple variable regions that contain genes responsible for producing diverse virulence factors. These include secreted proteases, toxins, biofilm formation, motility-related genes, extracellular DNA (eDNA), exopolysaccharide synthesis, rhamnolipid production, and the generation of various pigments[1]. Notably, *P. aeruginosa* is capable of producing four distinct types of pigments, namely pyocyanin, pyoverdine, pyomelanin and pyorubin[2].

Pyomelanin, a reddish-brown pigment, is known to confer a protective effect on *P. aeruginosa*, because of its antioxidant property and is often found in clinical isolates obtained from patients suffering from cystic fibrosis and/or urinary tract infections[3]. Pyomelanin is synthesized via the tyrosine catabolic pathway, either naturally or due to mutations in key enzymes within this pathway[4]. One of the key proteins involved in pyomelanin production is HmgA, which plays a critical role in converting homogentisic acid (HGA) to maleylacetoacetate. When there are point mutations or chromosomal deletions affecting *hmgA*, it leads to the accumulation of HGA. Subsequently, HGA undergoes auto-oxidation and self-polymerization, resulting in the accumulation of pyomelanin, which is then secreted via the HatABCDE transport system[5].

Pyomelanin plays an important role in virulence by providing protection to bacterial cells against oxidative stress induced by H_2O_2. Additionally, it is involved in bacterial persistence during infections[6]. In a mouse model of acute infection, *P. aeruginosa* variants producing pyomelanin, demonstrated increased persistence, compared to the non-pigmented wild-type strains. Studies conducted in a *Caenorhabditis elegans* challenge model, also suggested that pyomelanogenic *P. aeruginosa* strains were more virulent than their non-pigmented counterparts[7].

For the present study, we utilized a hyperpigmented, clinical strain of *P. aeruginosa*, that produced pyomelanin and was isolated from a urine sample. Previous investigations conducted by our laboratory had revealed that this strain exhibited a phage-resistant phenotype[8].

P. aeruginosa possesses a distinctive characteristic of exhibiting elevated levels of both intrinsic and acquired antibiotic resistance, which are attained through various mechanisms[1]. Given the escalating incidence of antimicrobial resistance (AMR), it has become increasingly critical to conduct research on alternative treatment strategies. One such approach involves targeting virulence factors, which hold significant importance in the battle to combat multidrug-resistant (MDR) infections, an ongoing global health crisis[9]. Furthermore, strategies aimed at targeting virulence factors offer the added advantage of enhancing the specificity of therapeutic modalities toward the pathogens, rather than the normal flora. They also focus on the interactions between the host and the pathogen, as opposed to merely targeting the pathogen itself. These advantages could ultimately lead to more favourable clinical outcomes and reduced perturbations to the normal microbial flora[10]. In this context, natural products (NPs), specifically phytochemicals with medicinal properties, have garnered increasing attention, particularly regarding their anti-virulence properties[11].

In the current study, our focus has therefore been on investigating the anti-virulent effect of seven essential oils (EOs), namely, garlic, cinnamon, clove, thyme, davana, palmarosa and fennel on a pyomelanogenic, MDR, clinical isolate of *P. aeruginosa* (U804). The most promising amongst these - garlic, cinnamon and thyme were selected for further studies using in vitro, ex vivo as well as in vivo models.

Materials and methods

Bacterial strains and growth conditions

Pseudomonas aeruginosa strain U804, was isolated from routine screening of urine samples obtained from a 58-year-old female patients at a tertiary care center in South India after informed consent, and following relevant guidelines and regulations, to maintain optimal conditions for the study.

To cultivate U804, we followed a standard procedure, routinely growing it in Luria Bertani (LB) broth at 37 ℃, with continuous shaking at 200 rpm. In our experiments, we treated the strain with essential oils that were dissolved in dimethyl sulfoxide (DMSO).

Essential oils were procured from Plant Lipid Pvt Ltd and Bhumi Naturals, Kerala, India (Suppl. Table. 1).

DNA isolation and whole genome sequencing of *P. aeruginosa* U804

DNA extraction from overnight cultures of U804 was carried out using the DNA isolation kit (Origin Diagnostics and Research, India). Subsequently, a paired-end library was constructed, and sequencing was performed using an Illumina NovaSeq instrument.

Raw reads obtained were quality checked using FastQC and the high-quality reads were assembled using SPAdes within the Galaxy online platform. Quast was used to check the quality of the assembly. Prokka annotation was performed on the Galaxy platform. We also generated a comprehensive genome report for U804 through the BV-BRC platform, using contigs > 200 bp to generate a circular genome map. To assess antibiotic resistance and the presence of virulence-associated genes, we used ABRIcate and screened against databases including ResFinder, NCBI, MegaRes, CARD, ARG-ANNOT, and VFDB. In addition, PlasmidFinder was used to search for sequences potentially associated with plasmids. To identify regions of non-homology, we aligned the contigs to the *P. aeruginosa* PAO1 sequence using ProgressiveMauve.

Effect of tyrosine on pyomelanin production of *P. aeruginosa* U804

The synthesis of pyomelanin is through the tyrosine catabolic pathway. Therefore, to evaluate the impact of tyrosine supplementation on pigment production, specifically pyomelanin synthesis, an overnight *P. aeruginosa* U804 grown in LB broth was first diluted to $OD_{600} = 0.1$. Subsequently, it was subjected to treatment with various concentrations of tyrosine (0.625, 1.25, 2.5, 5 and 10 mM). These treated cultures were then incubated at 37 ℃ with shaking at 200 rpm for a duration of 20 to 24 h. Following incubation, for quantifying pyomelanin, bacterial cultures were centrifuged at 3200 g for 10 min, and 100 µl of the supernatant was transferred to a flat-bottomed 96-well plate. Absorbance at OD_{405} was measured using the BioTek Synergy™ HT Multi-Mode Microplate Reader[12].

Effect of ascorbic acid on pyomelanin production of *P. aeruginosa* U804

Pyomelanin synthesis has previously been shown to be inhibited by ascorbic acid[6].

To investigate the influence of ascorbic acid on pyomelanin production, overnight *P. aeruginosa* U804 grown in LB broth ($OD_{600} = 0.1$) were diluted and treated with various concentrations of ascorbic acid (1.25, 2.5, 5, 10 and 20 mM). These treated cultures were then incubated at 37 ℃ for 24 h with constant agitation at 200 rpm. Following incubation, for quantifying pyomelanin, bacterial cultures were centrifuged at 3200 g for 10 min, and 100 µl of the supernatant was transferred to a flat-bottomed 96-well plate. Absorbance at OD_{405} was measured using the BioTek Synergy™ HT Multi-Mode Microplate Reader[12].

Dose-dependent effect of EOs on pyomelanin production of *P. aeruginosa* U804

Initially EOs were diluted in DMSO and further diluted in LB broth to get a concentration of 0.01, 0.03, 0.06, 0.125, 0.25 and 0.5%. Further *P. aeruginosa* U804 grown in LB broth was treated with different concentrations of seven EOs namely: thyme, cinnamon bark, clove bud, davana, fennel, garlic and palmarosa (0.01, 0.03, 0.06, 0.125, 0.25 and 0.5%) and incubated for 20–24 h at 37 ℃; an untreated U804 culture was maintained as a control. Following incubation, 5 µl of the culture was spot inoculated onto LB agar plates to evaluate the non-inhibitory effect of EOs. Next, the culture was centrifuged at 7500 rpm for 10–15 min at 4 ℃, pyomelanin levels were assessed by the previously described method[12].

Effect of EOs on reactive oxygen species (ROS) production by *P. aeruginosa* U804

An overnight culture of *P. aeruginosa* U804 (1 ml) was subjected to centrifugation. The resulting pellet was washed with phosphate buffered saline (PBS), and then resuspended in 0.5 ml of PBS. Subsequently, the bacterial cells were treated with sub-inhibitory concentrations of EOs and incubated at 37 ℃ for 1 h 30 min. Following this incubation, 5 µM dichlorodihydrofluorescein diacetate (DCFDA) was added to the cell suspension, and it was further incubated for 30 min in the dark. The sample's fluorescence was read at excitation and emission wavelengths of 488 nm and 530 nm, respectively[13].

Effect of EOs on biofilm formation of *P. aeruginosa* U804

Crystal violet assay was utilized to study the impact of varying sub-inhibitory concentrations of EOs (0.01, 0.03, 0.06, 0.125 and 0.25%) on biofilm formation of U804, following a previously established protocol of crystal violet assay[14], with untreated U804 as control.

RNA isolation and cDNA synthesis

RNA extraction was performed using the RNA Extraction Kit (Origin Diagnostics and Research, India). The concentration of the extracted RNA was determined, and cDNA was subsequently synthesized following the manufacturer's instructions (Origin Diagnostics and Research, India).

Gene expression analysis by qPCR

Quantitative PCR (qPCR) was performed by manufacturer's instructions, using the Takara SYBR®Premix ExTaq™ kit, and analyzed using the ΔΔCT method. The primer sequences used as previously reported[14], with *rpsL* serving as the reference gene for normalization. The PCR cycling conditions were adjusted as follows: initial denaturation at 95 °C (10 min) followed by 45 cycles of 95 °C (15 s) and 60 °C (30 s). Melt curve was performed using the following cycle: 61 °C (1 min) and 95 °C (15 s).

Extraction of C12-HSL

Acyl-homoserine lactone (AHL) was extracted as described earlier[11]. Briefly, bacterial cultures were grown for 18 h, and supernatants were collected by centrifugation at 6000 g for 10 min. The lyophilized supernatant

samples were dissolved in an extraction buffer containing acetonitrile and DMSO (4:1 ratio). After centrifugation (12,000 rpm for 15 min), the supernatant was transferred to a fresh Eppendorf tube, and samples were subjected to mass spectrometry analysis as detailed below (or stored at -80 °C until use).

Identification of a quorum sensing molecule (C12-HSL) through LC-MS analysis

The samples were infused into the mass spectrometer (Agilent 6540 Q-TOF LC-MS) through an HPLC system (Agilent 1290 uHPLC) equipped with an ESI source. The samples were resolved using a reversed-phase column (Zorbax Eclipse plus Phenyl Hexyl RRHD C18 column, 2.1 × 50 mm, 1.8 µm) using the mobile phases A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid). The HPLC pumps were set to deliver the solvents at a flow rate of 0.2 ml min^{-1} and a 25 min linear gradient (5 to 95% B) was used to separate the molecules. The MS parameters used for the data acquisition were MS range:100–1000 m/z; MS scan rate: 3 spectra/sec; isolation width MS/MS: medium (~4 amu); the maximum number of precursors: five; sort precursors: by abundance; dry gas temperature: 320 °C; dry gas flow: 6 L/min; nebulizer pressure (psig): 45; capillary voltage: 3000 V; nozzle voltage: 1000 V; sheath gas flow: 10 L/min; sheath gas temp: 350 °C; fragmentor: 180 V; MS ionization mode: positive. The MS/MS data of C12-HSL (theoretical monoisotopic protonated mass, 284.2220 Da) was acquired in positive ionization and targeted MS mode by setting the collision energy at 15 V. The identity of C12-HSL in samples were confirmed through the precursor m/z value, HPLC retention time and the prominent MS/MS fragmentation ions (102.05 and 183.17) observed. Agilent Mass Hunter (Version 7.0) qualitative analysis software was used for the data analysis.

Ethical statement

Blood was collected via venipuncture from healthy volunteers under written informed consent approved by the Ethics Committee of Amrita School of Medicine (ECASM-AIMS-2023-149), Amrita Vishwa Vidyapeetham. All experiments were performed in accordance with relevant guidelines and regulations of Amrita School of Biotechnology, Amrita Vishwa Vidyapeetham, India.

Whole blood survival assay

Whole blood survival assay was performed according to the previously described method[12]. Blood was collected from healthy volunteers in heparinized tubes. Mid-log-phase cultures of *P. aeruginosa* U804 were diluted 1:100 in RPMI 1640 and 20 µl (approximately 3×10^4 CFU) were added to 180 µl of both active and heat-inactivated blood in 0.5 ml tubes, incubated rotating at 37 °C. Aliquots were plated at the indicated time points for CFU determination.

Survival assay of *C. elegans* upon infection with *P. aeruginosa* U804

Survival assay of *C. elegans* infected with *P. aeruginosa* was conducted as described earlier[11]. Briefly, L4 larval stages of *C. elegans* were infected with *P. aeruginosa* U804. Survival of the larvae was observed for 5 days, and percentage survival was calculated.

Effect of EOs on Ethidium Bromide accumulation assay

Late exponential cells of bacterial cultures (1 ml) incubated in LB broth (37 ℃, 200 rpm) were centrifuged at 7000 rpm for 10 min. Pellets were washed twice in PBS and resuspended in 500 µl of PBS (pH 7.4) containing 0.4% glucose. Sub-inhibitory concentrations of EOs were added with 2 µg/ml EtBr and accumulation of EtBr was determined by measuring fluorescence with excitation and emission wavelengths of 544 and 590 nm, respectively. CCCP (100 µM) was used as the positive control[13].

Determination of the minimum inhibitory concentration (MIC)

MIC of colistin and ciprofloxacin were assessed by broth dilution method. Bacterial cultures were adjusted to $OD_{600} = 0.4$ and diluted 1:100 to achieve a bacterial inoculum of 2×10^6 CFU/ml. Bacterial inoculum (180 µl) was treated with 20 µl of 10X antibiotics to achieve final concentrations ranging from 0.125 to 8 µg/ml for colistin and 8–513 µg/ml for ciprofloxacin and incubated overnight at 37 °C. The viability of the bacterial cultures was assessed by resazurin assay. The MIC value was defined as the lowest concentration of antibiotic that prevented the change in color of resazurin from blue to pink. The Minimum Bactericidal Concentration (MBC) was determined by plating 10 µl of the bacterial suspension from the well that did not exhibit a color change[15].

Combinatorial effect of antibiotics with EOs

Checkerboard assays were performed by combining 20 µl of both 10X antibiotic and 10X EO to 160 µl of bacterial inoculum (2×10^6 CFU/ml)[15].

Cell membrane permeability

The effect of EOs on the permeabilization of the outer membrane was analyzed by using N-phenyl-1-napthylamine (NPN) as described earlier by[16].

Statistical analysis

The experiments were repeated at least three times with a minimum of three technical replicates. All data are presented as mean ± standard deviation (SD). Error bars represent variations within the experiment. To analyze the significant difference between the values of control and treated samples, one-way analysis of variance (ANOVA) and Duncan's post hoc test was performed using GraphPad Prism 8.0 (GraphPad Software Inc., CA, USA). The values of $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$ were considered as statistically significant. Asterisks (*) are used to denote levels of significance between treated and untreated samples.

Results

Characterization of *P. aeruginosa* U804 strains

Whole genome sequencing of U804 elucidates mechanisms involved in pyomelanin pigmentation, phage resistance, antibiotic resistance, and virulence traits

U804 is a MDR clinical strain of *P. aeruginosa* isolated from a patient's urine sample. This strain exhibits resistance to a wide range of antibiotics, including aminoglycosides, cephalosporins, monobactams, fluoroquinolones, anti-pseudomonal beta-lactams, and carbapenems. U804 is also resistant to vB_Pae_AM. P2 phage infection[12]. Notably, this strain produces a dark pigment, indicative of pyomelanin. The objective of sequencing the U804 genome was to elucidate the underlying factors contributing to these intriguing phenotypes.

Whole genome sequencing of U804 generated 476 scaffolds, each exceeding 200 bp in length, totaling ~ 6.79 Mb. This genome contains 6293 coding sequences (CDS), 3 rRNA sequences, 4 repeat regions, 68 tRNA sequences, and 1 tmRNA (Fig. 1a). Through BV-BRC annotation, we performed an analysis of the subsystems (Fig. 1b). When aligning the scaffolds to the laboratory reference strain PAO1, we observed a missing 334 Kb region in the clinical isolate, spanning from 2,087,486 bp to 2,421,494 bp in PAO1 (Fig. 1c). This region encompasses *hmgA* (homogentisate 1,2-dioxygenase), which catalyzes the conversion of homogentisate to maleylacetoacetate during tyrosine catabolism, and *galU*, integral to LPS O-antigen biosynthesis. The loss of this region in PAO1 has been linked to pyomelanogenesis and resistance to the AM.P2 phage[12], which accounts for the dark brown pigmentation and phage resistance phenotype in U804.

U804 scaffolds were further analyzed for the presence of antibiotic resistance determinants by using databases such as ResFinder, NCBI Bacterial Antimicrobial Resistance Reference Gene Database, megares, CARD and ARG-ANNOT, with the assistance of ABRicate. This analysis revealed the presence of several genes encoding for antibiotic resistance, including *blaGES-9*, *blaOXA*, *blaPDC*, *blaNDM-1*, *catB7*, *catB*, *bcr1*, *aph(3")-Ib*, *aph(6)-Id*, *fosA*, *aph(3')-IIb*, *arnA*, *basS*, and *crpP*. These genes account for multidrug resistance against tetracyclines, beta-lactams, cephalosporins, carbapenems, monobactams, fluoroquinolones, polymyxins and aminoglycosides (Table S1). Furthermore, U804 carries genes for 11 efflux pump systems (*mexAB-oprM*, *mexCD-oprJ*, *mexEF-oprN*, *mexJK-oprM*, *mexPQ-opmE*, *muxABC-opmB*, *pmpM*, *triABC-opmH*, *mexMN-oprM*, *mexVW-oprM*, *mexGHI-opmD*) along with five associated regulatory elements (*mexR*, *mexL*, *cpxR*, *armR*, *soxR*). This array of efflux pumps and regulators, further enhances resistance to a wide spectrum of antibiotics and even the biocide triclosan.

Upon screening the U804 scaffolds against the virulence factor database (VFDB), we identified 209 virulence genes (Table S2). Many of these genes are associated with flagella, pili, quorum sensing, LPS biosynthesis, type III and IV secretion systems, phenazine biosynthesis, pigment production (pyoverdine), elastase and proteases, pyochelin synthesis, alginate biosynthesis, rhamnolipids and exotoxins. PlasmidFinder did not identify any regions associated with plasmids, suggesting that all resistance and virulence genes are situated on the U804 chromosome. The sequence has been deposited into GenBank NCBI (Accession number CP152087.1).

Dose-dependent effect of tyrosine and ascorbic acid on pyomelanin production of *P. aeruginosa* U804

Different soluble pigments, including pyocyanin, pyoverdine, pyorubin, and pyomelanin, are known to be produced by *P. aeruginosa*[17]. To confirm that the reddish-brown pigment produced by *P. aeruginosa* U804 is indeed pyomelanin, we supplemented the media with tyrosine, the substrate for pyomelanin production, to assess any change in pigmentation phenotype. The supplementation of tyrosine at concentrations ranging from 0.1 mM to 10 mM resulted in a dose-dependent escalation in pyomelanin production. Specifically, with 10 mM tyrosine, there was a 60% increase in pigmentation (Fig. 1d). At 5 mM tyrosine, there was a 46% increase in pigment compared to the control. As the amount of tyrosine supplied to the organism decreased, the pigment formation reduced, corroborating that the pigment produced by U804 was pyomelanin.

To further confirm the presence of pyomelanin, U804 was treated with ascorbic acid, an antioxidant and known inhibitor of pyomelanin synthesis. U804 was treated with different concentrations of ascorbic acid ranging from 1.25 mM to 25 mM, and a dose-dependent inhibition in pyomelanin production was observed up to a 35.6% reduction, at the highest concentration tested (20 mM). Importantly, through a spot assay, we determined that ascorbic acid did not impact the viability of U804. These findings strongly support the conclusion that the reddish-brown pigment produced by U804 is indeed pyomelanin (Fig. 1e).

Dose-dependent effect of EOs on pyomelanin production by U804

To investigate the impact of EOs on pyomelanin production, U804 was treated with seven different EOs, namely: thyme, cinnamon bark, clove bud, davana, fennel, garlic and palmarosa, at concentrations ranging from 0.01 to 0.5% and pyomelanin production was subsequently measured (Fig. 2a). The treatment with thyme, cinnamon or garlic oil resulted in a substantial reduction in pyomelanin production, following a dose-dependent pattern. Treatment with 0.06, 0.125, 0.25, and 0.5% thyme oil demonstrated corresponding reductions of 14.9%, 22.7%, 43% and 78% in pyomelanin production, respectively. Similarly, treatment with 0.06%, 0.125%, 0.25%, and 0.5% cinnamon oil led to reductions of 30%, 34.3%, 67.2% and 75.7% in pyomelanin production, respectively. U804 exposed to 0.06%, 0.125%, 0.25%, and 0.5% garlic oil showed significant decreases in pyomelanin production, with reductions of 25.7%, 32.6%, 41% and 78.7%, respectively.

The present study mainly focuses on the anti-virulent properties of EOs, which target the virulence of bacteria, instead of the antimicrobial activity. Hence, the initial screening of the different EOs targeted inhibition of pyomelanin production, since it is a key virulence factor of *P. aeruginosa*. Of the seven EOs tested for pyomelanin inhibition, the three best EOs, namely, cinnamon, garlic and thyme were selected for further studies.

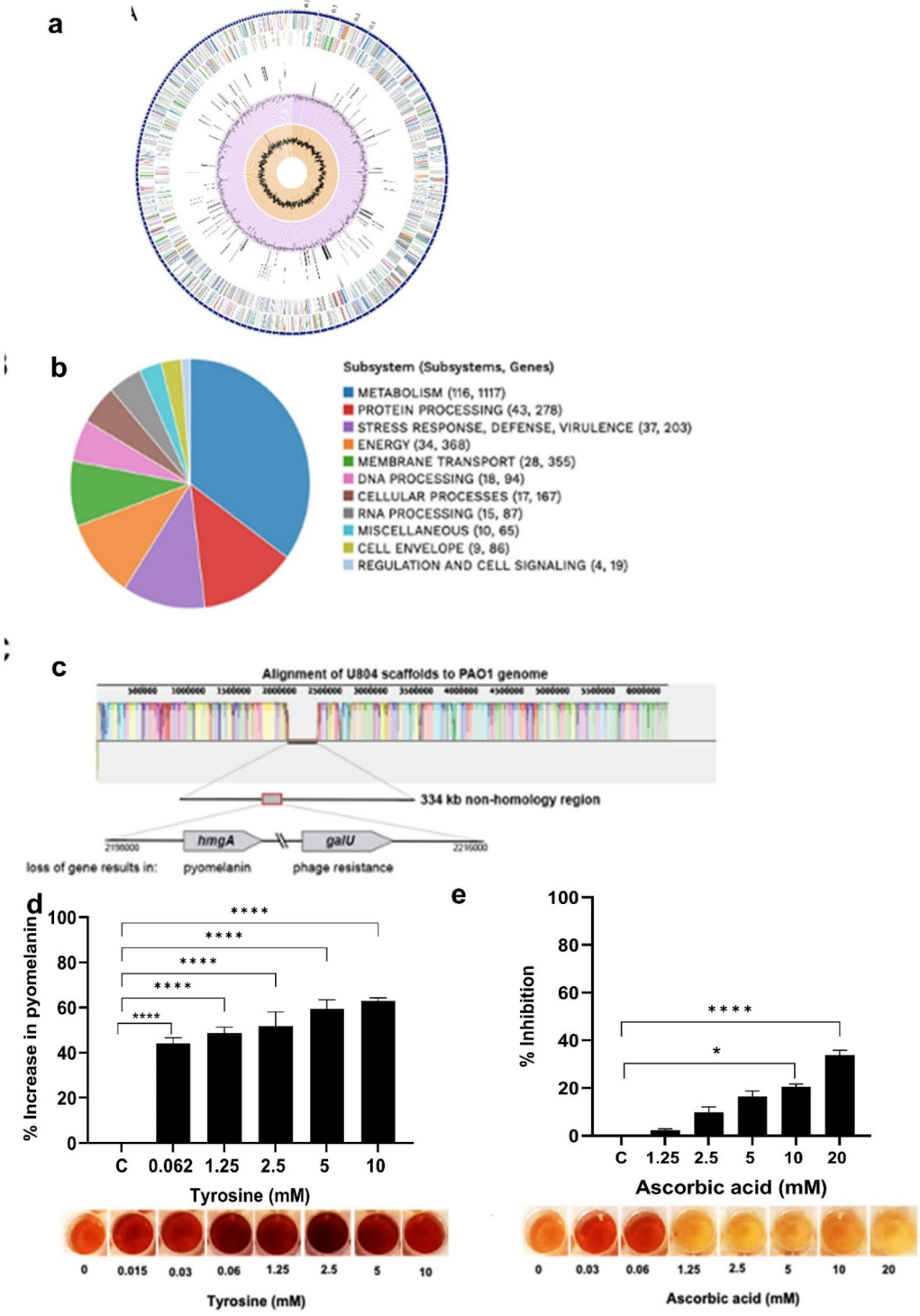

Fig. 1. Characterization of *P. aeruginosa* U804 (**a**) Circular genome map of U804 including the 163 longest contigs. From outer to inner rings: the contigs, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to known virulence factors, GC content and GC skew. The colors of the CDS on the forward and reverse strand indicate the subsystem that these genes belong to (**b**) Subsystem distribution of genes within the U804 genome (**c**) ProgressiveMauve alignment of U804 scaffolds to laboratory reference strain PAO1 highlights a non-homology block spanning 2.09 Mb to 2.42 Mb in PAO1, approximately 334 Kb in length. The missing region includes *hmgA* and *galU* which, when absent, result in pyomelanin production and phage resistance (**d**) Effect of Tyrosine and (**e**) Ascorbic acid on pyomelanin production by *P. aeruginosa* U804. Data are shown as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ compared with untreated by determining One-way ANOVA.

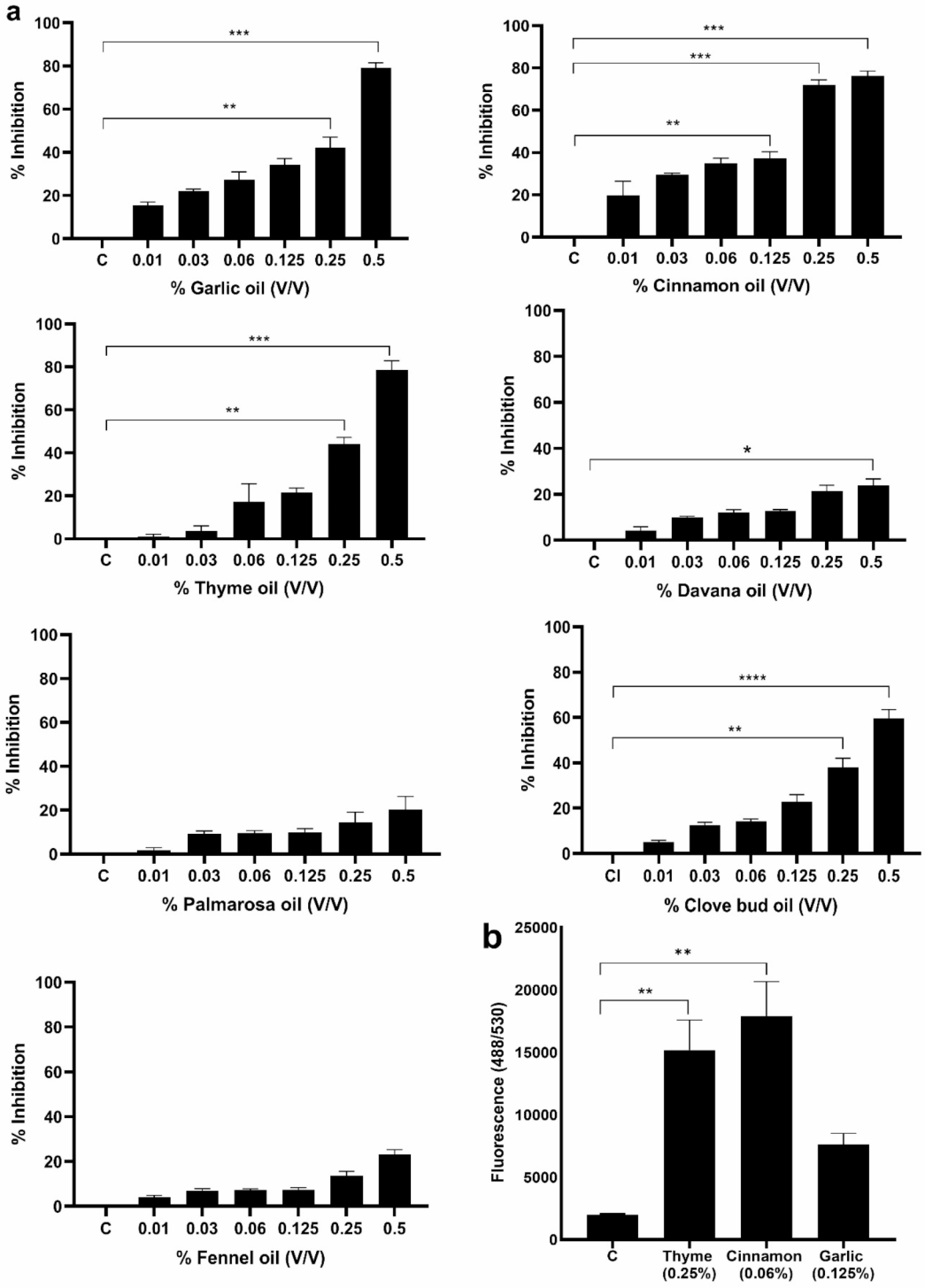

Fig. 2. Effect of EOs on pyomelanin and ROS production. (**a**) Dose-dependent effect of cinnamon, garlic, thyme, clove bud, fennel, davana, and palmarosa EOs on *P. aeruginosa* U804 pyomelanin production (**b**) ROS levels in U804 cultures upon treatment with thyme, cinnamon, and garlic EOs using DCFDA assay. Data are shown as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ compared with untreated by determining One-way ANOVA.

The MIC values for thyme, cinnamon, and garlic oils were determined to be 0.5%, 0.125%, and 0.25%, respectively. The MBC values were 1% for thyme and garlic, and 0.5% for cinnamon. Further studies were performed using sub-MIC concentrations of these EOs.

Treatment of *P. aeruginosa* U804 with EOs increases ROS

Given that thyme, cinnamon and garlic EOs were observed to inhibit the pyomelanin production, which is known to confer protection against ROS, we investigated whether EO treatment could elevate ROS levels in the bacteria. Following treatment of *P. aeruginosa* U804 with sub-inhibitory concentrations of the various EOs, a significant increase in ROS production was observed in cultures treated with thyme and cinnamon EOs, when compared to the control (Fig. 2b). Specifically, treatment with 0.25% thyme or 0.06% cinnamon oil led to 6.6-fold and 7.9-fold increases in ROS production, respectively. However, garlic oil-treated U804 showed only a 0.8-fold increase in ROS production. These observations suggest that compared to garlic, thyme and cinnamon EOs have the potential to significantly increase the levels of free radicals within bacterial cells, inducing oxidative stress that could lead to cell death.

EOs inhibit *P. aeruginosa* U804 biofilm formation in a dose-dependent manner

The significance of *P. aeruginosa* in clinical contexts predominantly reflects its antimicrobial resistance and its ability to form biofilms, which contribute to chronic infections. Biofilms are among the most pathogenic virulent phenotypes, offering protection from environmental stress and enhancing survival rates[18]. *P. aeruginosa* biofilm structure is supported by three polysaccharides: alginate, Psl and Pel[18]. We evaluated the capacity of *P. aeruginosa* U804 to form biofilms following treatment with EOs. As illustrated in Fig. 3a, P. *aeruginosa* U804 showed a dose-dependent reduction in biofilm formation when treated with different concentrations of thyme, cinnamon and garlic oil (ranging from 0.01 to 0.25%) as compared to the untreated control (Fig. 3a-c). Thyme oil and garlic oil at a concentration of 0.25% resulted in a reduction of biofilm formation by 79% and 54%, respectively. However, cinnamon oil exhibited 59% reduction in biofilm formation even at concentrations as low as 0.06%.

EOs modulate quorum sensing gene expression

P. aeruginosa uses three quorum sensing (QS) systems - LasI-LasR, RhlI-RhlR, and PQS-MvfR, which operate in a hierarchical manner and regulate key virulence factors or phenotypes. Expression studies indicate that treatment with EOs leads to the modulation of U804 QS gene expression (Fig. 3d). It was observed that when U804 was treated with 0.25% thyme oil, there was no significant reduction in expression of QS genes (*las*, *rhl* and *pqs*), except for *gacA* and *vfr* genes, where a slight decrease (1.4 and 1.5-fold respectively) was observed. In contrast, treatment with cinnamon oil (0.06%) significantly down-regulated the expression of all three quorum sensing genes - *lasI*, *rhlI* and *pqsA*, as well as *gacA* and *vfr*, with reductions of 1.81-fold, 2.6-fold, 3.0-fold, 2.9-fold, and 1.8-fold, respectively. Treatment with garlic oil (0.125%) also resulted in decreased expression of all QS genes, except for the *lasI* gene. Furthermore, *gacA* expression levels were reduced in U804 treated with cinnamon oil (2.94-fold) and garlic oil.

EOs reduce the AHL signaling molecule C12-HSL

C12-HSL is a major quorum-sensing signaling molecule involved in the Las system. To assess the impact of EOs on AHL signaling molecule levels, *P. aeruginosa* U804 was grown in sub-inhibitory concentrations of EOs, and the supernatant was collected for Mass Spectrometric analysis. The structure and MS/MS spectrum of standard C12-HSL are shown in Fig. 3e. The fragmentation of the protonated parent ion at m/z 284.220 yielded two predominant product ions at m/z 102.05 and 183.17 (Fig. 3e), indicating the presence of homoserine lactone ring and dodecane acyl side chain, respectively, in the structure. When compared to the standard C12-HSL, similar fragmentation patterns were observed in the supernatants of untreated U804 (Fig. 3f) and U804 treated with 0.125% garlic (Fig. 3g), 0.06% cinnamon (Fig. 3h), and 0.25% thyme oil (Fig. 3i). However, in the thyme, cinnamon, and garlic oil-treated samples, the intensity of the product ions representing the presence of C12-HSL, was significantly reduced, compared to untreated U804. These observations demonstrate that the EOs can inhibit the levels of the quorum-sensing molecule C12-HSL.

Effect of EOs on innate immune sensitization of U804

To understand how EOs could modulate *P. aeruginosa* U804's susceptibility to host innate immunity, we pretreated bacteria with sub-inhibitory concentrations of thyme, cinnamon, and garlic oil (0.25%, 0.06% and 0.125% respectively) before exposure to human whole blood. The results revealed that EO pretreatment led to a substantial reduction (1 to 1.5 log fold) in bacterial survival in human whole blood within just 30 min and this reduction was sustained for 2 h (Fig. 4a). Importantly, no significant change in bacterial survival was observed when the blood was heat-inactivated, implying that EO treatment specifically enhanced bacterial susceptibility to active host innate immune components.

EOs enhance the survival of *C. elegans* infected with *P. aeruginosa* U804

C. elegans, a model organism for studying bacterial virulence, was infected with *P. aeruginosa* U804 and treated with sub-inhibitory concentrations of thyme, cinnamon, and garlic oils (0.25%, 0.06% and 0.125% respectively). *C. elegans* infected with untreated U804 and uninfected worms were used as controls, and their survival rates were analyzed. As demonstrated in Fig. 4b, the study revealed a significant increase in the *C. elegans* survival rate (60%, 50%, and 77%) when treated with 0.25% thyme, 0.06% cinnamon, and 0.125% garlic oil respectively, compared to the 22% survival rate of worms infected with untreated *P. aeruginosa* U804. These observations clearly indicate that treatment with EOs enhanced the survival of *C. elegans*, indicative of their anti-infective properties.

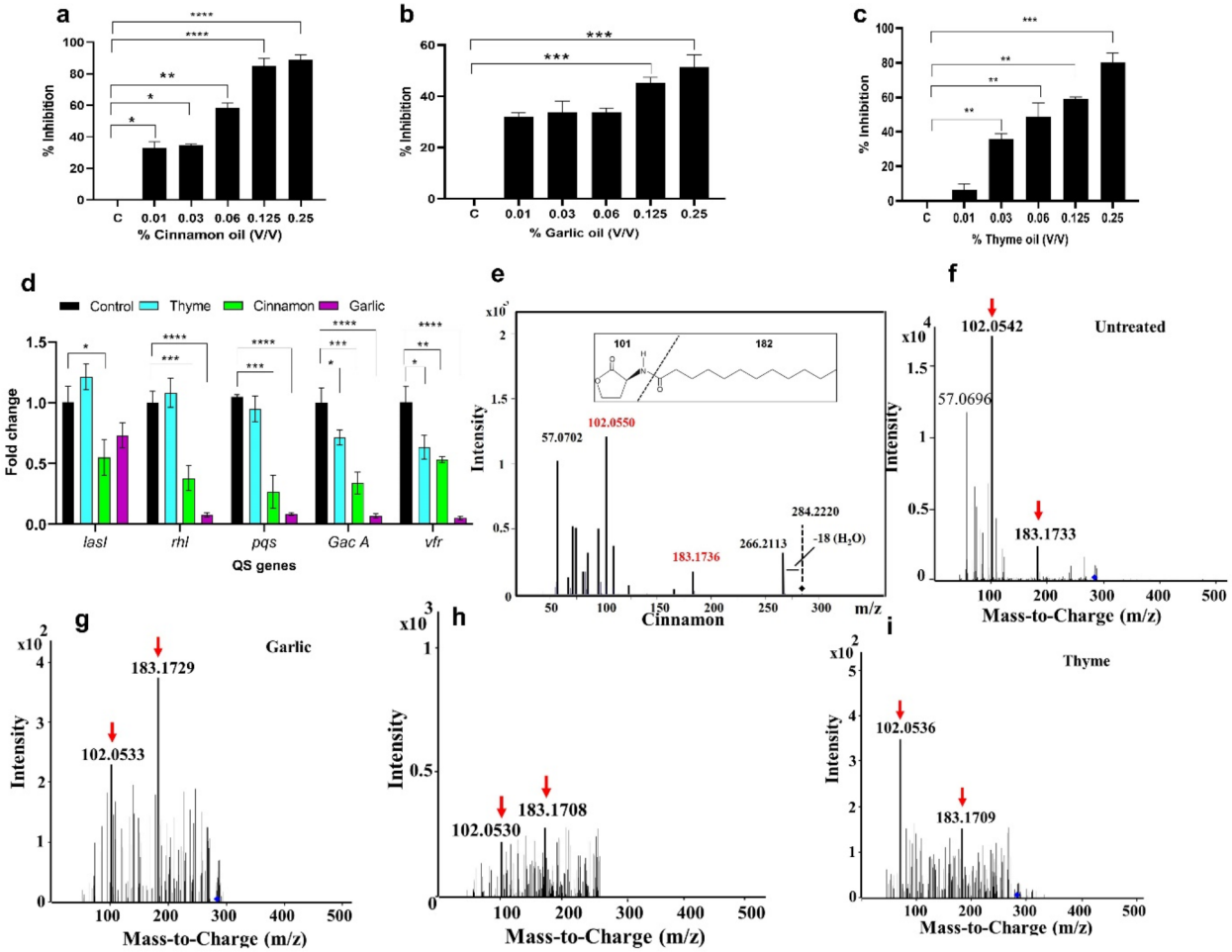

Fig. 3. Effect of EOs on biofilm formation, QS-related gene expression and C12-HSL production of *P. aeruginosa* U 804. Effect of (**a**) cinnamon (**b**) garlic and (**c**) thyme EOs on U804 biofilm formation (**d**) Differential gene expression of U804 quorum sensing genes when treated with EOs (**e**) MS/MS fragmentation pattern for standard C12-HSL; inset shows the structure of C12-HSL; the m/z highlighted in red are the prominent MS/MS product ions for C12-HSL. MS/MS fragmentation pattern for (**f**) untreated (**g**) garlic (**h**) cinnamon and (**i**) thyme treated U804. X-axis represents the m/z values and Y-axis corresponds to the intensity. Data are shown as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ compared with untreated by determining One-way ANOVA.

Effect of EOs on efflux activity of *P. aeruginosa* U804

Increased RND family efflux pump activity is a major contributor to multidrug resistance in *P. aeruginosa*. We assessed the efflux pump activity of MDR *P. aeruginosa* U804 following treatment with sub-MIC concentrations of EOs (0.25% thyme, 0.06% cinnamon, and 0.125% garlic). Treatment with EOs significantly increased the accumulation of ethidium bromide (EtBr) fluorescence in *P. aeruginosa* U804, indicating reduced efflux pump activity (Fig. 5a). Thyme oil showed the most significant increase in fluorescence (5.5-fold), followed by cinnamon bark oil (4.7-fold) and garlic oil (1.6-fold). Substantial accumulation of EtBr inside bacterial cells indicated reduced efflux pump activity. Interestingly, the efflux pump inhibition exhibited by thyme and cinnamon oil was even greater than that of the positive control CCCP, a well-established efflux pump inhibitor (3.5-fold)[19]. These results highlight the potential of thyme oil as an effective agent to inhibit *P. aeruginosa* efflux pump activity, which could be valuable in combinatorial treatment strategies with antibiotics, in order to enhance their effectiveness against MDR strains.

EO increases the outer membrane permeabilization of *P. aeruginosa* U804

Outer membrane permeabilization is an alternative strategy to enhance the susceptibility of gram-negative bacteria to antibacterial compounds. The hydrophobic probe N-phenyl-1-napthylamine (NPN) cannot pass through LPS directly unless the outer membrane is damaged, in which case it passes through and accumulates inside the cytoplasm, resulting in strong fluorescence. MDR *P. aeruginosa* U804 was treated with thyme, cinnamon, and garlic EOs for 1 h, after which 10 µM of NPN was added and the fluorescence measured to assess membrane permeability. Interestingly, only treatment with garlic oil (0.125%) resulted in a significant

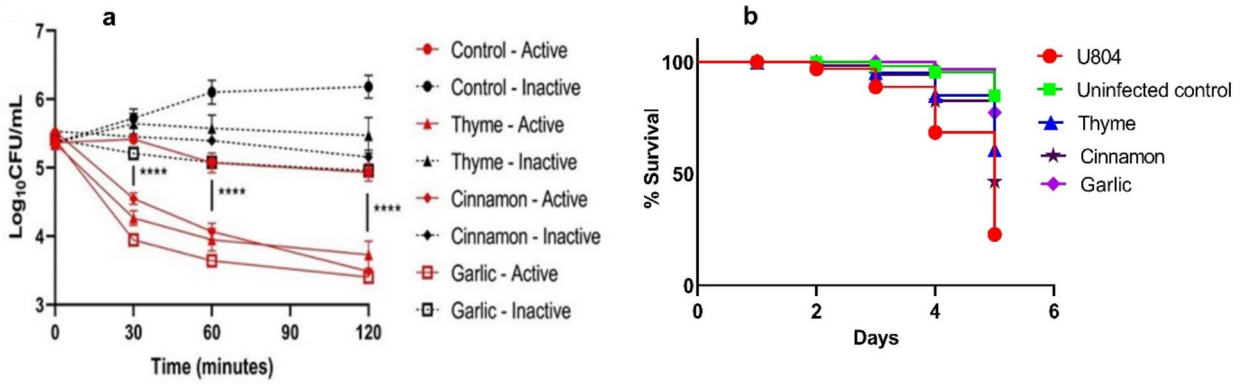

Fig. 4. (**a**) Effect of U804 survival in human whole blood with and without pretreatment with EOs (**b**) survival analysis of *C. elegans* infected with U804 pretreated with EOs.

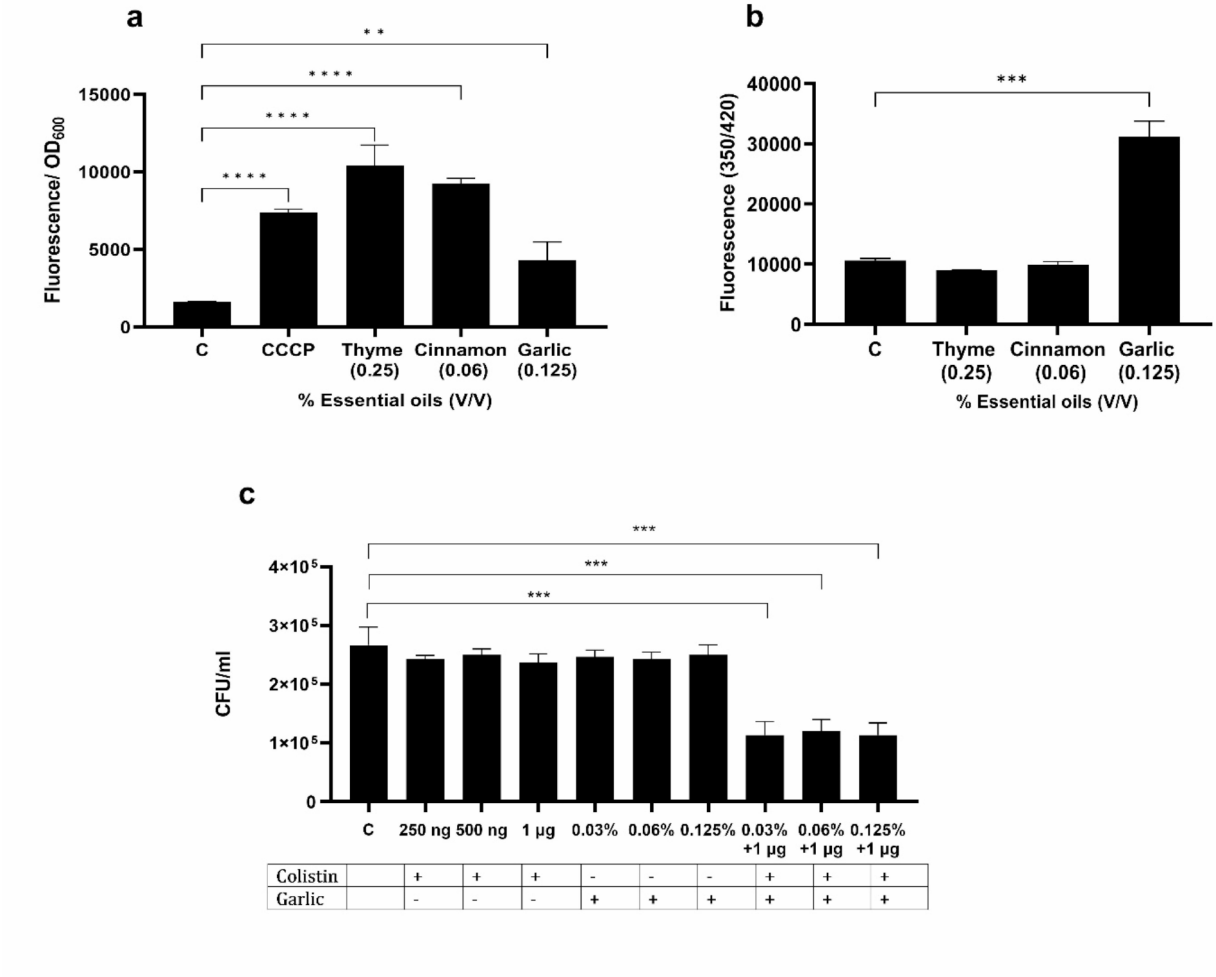

Fig. 5. Effect of essential oils on efflux pump activity, membrane permeability of *P. aeruginosa* U804 (**a**) Ethidium bromide accumulation (**b**) NPN assay for measuring outer membrane permeability in *P. aeruginosa* U804 treated with EOs (Thyme, Cinnamon, Garlic) at sub-MIC concentrations (**c**) Checkerboard assay to determine the combinatorial effect of Garlic oil with Colistin. Data are shown as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ compared with untreated by determining One-way ANOVA.

increase (2-fold) in NPN accumulation, indicating increased membrane permeability. In contrast, sub-MIC concentration of thyme (0.25%) and cinnamon oil (0.06%), did not significantly alter membrane permeability compared to the untreated control (Fig. 5b). These results suggest that treatment with garlic oil disrupted the integrity of the outer membrane of *P. aeruginosa* U804, allowing the hydrophobic substance NPN to pass through. This disruption in the outer membrane could be a potential mechanism for enhancing the susceptibility of the bacteria to antibacterial compounds.

Effect of EO on *P. aeruginosa* U804 antibiotic susceptibility

Given our observations indicating that EOs modulated efflux pump activity and membrane permeability, we sought to investigate whether EOs could enhance the sensitivity of MDR *P. aeruginosa* U804 to antibiotics. We determined the MIC and MBC concentrations for antibiotics namely ciprofloxacin and colistin. U804 exhibited notable resistance to ciprofloxacin, as evidenced by an exceptionally high MIC_{90} (256 µg/ml), while showing susceptibility to colistin (2 µg/ml). However, administration of such high doses of colistin is not optimal and is often avoided due to its potential nephrotoxicity. Consequently, identifying agents that could sensitize U804 to either antibiotic could potentially sensitize the bacteria to antibiotics and enhance current therapeutic modalities. To explore this possibility, we performed a checkerboard assay, using sub-inhibitory concentrations of garlic oil in combination with either ciprofloxacin or colistin. The results revealed a significant reduction in bacterial survival when U804 was treated with a combination of garlic oil and colistin, compared to untreated U804 or U804 treated with garlic oil or colistin alone (Fig. 5c). However, the combination of garlic oil with ciprofloxacin did not alter the MIC of the antibiotic.

Discussion

Pyomelanogenic *P. aeruginosa* presents a significant challenge in health-care settings[20]. The hyperpigmentation observed in certain strains of this bacterium provides adaptive flexibility, particularly during oxidative stress and other environmental challenges[21]. Studies have shown that isogenic hyperpigmented mutants lacking *hmgA* gene, exhibit increased resistance to hydrogen peroxide and contribute to the pathogen's persistence in vivo, as evidenced in a murine lung infection model[12]. The current study underscores the therapeutic potential of EOs in combating pyomelanogenic, MDR, as well as phage-resistant clinical isolate of *P. aeruginosa*.

WGS analysis revealed that the hyperpigmentation phenotype is associated with the absence of the *hmgA* gene, which catalyzes the conversion of homogentisate to maleylacetoacetate during tyrosine catabolism. Genetic linkage of *hmgA* and *galU* often results in coupled pyomelanogenic as well as phage-resistance phenotypes[12].

To confirm that the pigment produced by *P. aeruginosa* U804 was indeed pyomelanin, we have determined the effect of tyrosine and ascorbic acid on the bacterium. Tyrosine, a substrate of pyomelanin, can induce pigment production, while ascorbic acid, an antioxidant, is reported to inhibit the pyomelanin production[6]. Our findings showed an increase in pyomelanin production in the presence of tyrosine, and conversely, a reduction in pigment when ascorbic acid was introduced, supporting the conclusion that the pigment produced by *P. aeruginosa* U804 is indeed pyomelanin. Having established the molecular and phenotypic basis for pyomelanin hyperpigmentation in U804, our focus shifted to understanding the effect of EOs, (specifically thyme, cinnamon, and garlic), on pyomelanin production. ROS are mainly produced during aerobic metabolism, resulting in oxidative stress. Host innate immune cells often employ ROS as a means to kill bacterial pathogens. Pyomelanin, as a known antioxidant, can potentially shield *P. aeruginosa* from ROS-mediated killing[4]. Therefore, the dose-dependent decrease in pyomelanin production induced by EO treatment may compromise this protective effect. Additionally, the significant increase in ROS production observed upon treatment with EOs further enhances this killing effect.

Previous studies from our laboratory have demonstrated the anti-quorum sensing attributes such as anti-virulent, anti-biofilm activity and immune modulatory activity of clove bud oil, on the well characterized *P.aeruginosa* prototype strain PAO1[11,12].

Earlier studies have highlighted the pro-oxidant activity of certain EOs, such as lavender oil, leading to cell membrane damage and eventual bacterial death[22]. Compounds like acetic acid, linalool[23], and pyrrolidines[24] have also demonstrated significant pro-oxidant activity, resulting in elevated ROS production. Moreover, specific components of thyme oil, including p-cymene, 1,8-cineole, and myrcene, have been found to exhibit pro-oxidant activity[25]. Thus, the dual action of EOs, inhibiting pyomelanin production and directly increasing ROS levels, can synergistically enhance pathogen eradication. These observations were further substantiated in an ex vivo model of innate immune sensitization using human blood, where EO treatment led to improved bacterial killing compared to the untreated control.

Efflux pump systems are recognized as important contributors to antibiotic resistance in *P. aeruginosa*[26], and several studies have proposed targeting these pumps as a strategy to reverse bacterial drug resistance. In this context, we investigated whether EOs could influence the efflux pump activity of the MDR *P. aeruginosa* clinical isolate, U804. To assess this, we conducted an influx/efflux assay, using EtBr as a substrate, and our results demonstrated a significant reduction in efflux pump activity. The capacity of EOs to act as efflux pump inhibitors in MDR U804 underscores their potential utility as therapeutic adjuvants. By inhibiting efflux pump activity at subinhibitory concentrations, this approach could contribute to mitigating drug resistance. *P. aeruginosa* is well known to possess many virulence factors that are regulated by various quorum sensing systems. This bacterium harbors three quorum sensing systems - LasI-LasR, RhlI-RhlR, and PQS-MvfR, that function in a hierarchical manner[27]. Given that quorum sensing plays such a pivotal role in regulating bacterial virulence, inhibition of these pathways represents a potential modality to attenuate the pathogenicity of *P. aeruginosa*[27]. Notably, sub-inhibitory concentrations (0.06%) of cinnamon oil were found to significantly reduce the expression of QS genes, including *rhl*, *pqsA*, *gacA* and *vfr*. Garlic oil exhibited similar inhibitory effects on QS gene expression, except for *lasI* that was unchanged. These findings align with prior reports that demonstrated the quorum-sensing

inhibitory potential of cinnamon oil in *P. aeruginosa* PAO1, which likewise resulted in a decreased expression of various virulence factors[28]. The transition of *P. aeruginosa* from planktonic to biofilm lifestyle involves intricate cellular processes, which are modulated by the Gac/Rsm pathway, or signaling by the second messenger cyclic-di-GMP (c-di-GMP)[29]. The reduction in biofilm formation when treated with thyme, cinnamon, and garlic EOs correlates with the reduced expression of GacA, a positive regulator of QS and other associated QS genes.

C12-HSL is a key signaling molecule within the Las QS system of *P. aeruginosa*, which regulates various virulence traits. Our studies revealed a significant reduction in the concentration of C12-HSL, when exposed to sub-MIC concentrations of thyme, cinnamon, and garlic oils. These findings are in line with our gene expression studies, which showed that the *lasI* gene is downregulated by cinnamon and garlic oil, at sub-MIC concentrations. However, thyme oil did not significantly alter the expression of the *lasI*, *rhlI* and *pqs* genes. The observed decrease in virulence phenotypes like biofilm formation, may be attributed to the lowered levels of the LasI signaling molecule, C12-HSL, following treatment with cinnamon or garlic oil. In the case of thyme oil, there was no downregulation of *lasI*, but a reduction in C12-HSL, possibly indicating its interruption after synthesis.

The host innate immune system plays a large role in the early stage of bacterial colonization and the progression of infection. It comprises both cellular and soluble components that act to prevent bacteria from establishing themselves within the host. These include neutrophils, monocyte/macrophages, serum complement, and antimicrobial peptides, all of which are present in humans. However, bacteria have evolved various strategies to evade host innate immunity - for example, *P. aeruginosa* can circumvent the classical, alternative and lectin-based complement to survive within the host[30]. In our study, EO treatment resulted in enhanced clearance of *P. aeruginosa* from human blood, as demonstrated in whole blood killing assays.

C. elegans is widely used as an in vivo model to study host-pathogen interactions. The killing of *C. elegans* by *P. aeruginosa* can occur through various mechanisms[31]. Notably, pyomelanogenic *P. aeruginosa* has demonstrated increased virulence compared to wild-type strains in the *C. elegans* model[32]. Our research, involving the infection of *C. elegans* with pyomelanogenic *P. aeruginosa* treated with EOs, revealed an increased survival rate among the nematodes, highlighting the potential therapeutic value of these natural products.

Conclusion

In conclusion, this study showcases the significant anti-virulent and anti-infective properties of EOs, specifically thyme, garlic and cinnamon. These properties result in enhanced pathogen clearance within human blood in an ex vivo model of innate immune sensitization, as well as increased survival in a whole animal model of pathogenesis. The anti-virulence properties of the EOs tested were assessed by determining their capacity to inhibit pyomelanin and biofilm formation. Cinnamon, thyme and garlic EOs demonstrated comparable results in pyomelanin inhibition. However, cinnamon oil exhibited significantly higher antibiofilm activity, as compared to garlic and thyme. Furthermore, amongst these essential oils, garlic oil exhibited enhanced sensitivity to colistin, compared to thyme and cinnamon. The attenuation of *P. aeruginosa* virulence traits and the EO-mediated potentiation of antibiotics, highlights the plausible therapeutic efficacy of thyme, cinnamon, and particularly garlic EO in combating pyomelanogenic, MDR *P. aeruginosa* infections.

Data availability

The datasets generated and/or analysed during the current study are available in the GenBank NCBI repository (https://www.ncbi.nlm.nih.gov/nuccore/CP152087.1/, Accession number CP152087.1).

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Author contributions

GK: Conceptualisation, project administration, writing- reviewing, editing, JH: Designed the experiments, performed data analysis, and prepared figures and tables, writing original draft CRB: Investigation, Formal analysis, methodology NM: Investigation, Formal analysis, writing-review and editing MV: Investigation, Formal analysis, writing -review and editing, MBH: Investigation, Formal analysis NN: Investigation, Methodology KB: Investigation, Formal analysis, Data curation AK: Conceptualisation, resources BGN: Conceptualization, Funding acquisition, infrastructure support writing-review editing VN: Conceptualization, Writing- review and editing.

Declarations

Competing interests

The authors declare no competing interests.

Ethics approval

The protocols were approved by the ethics committee of Amrita School of Medicine (ECASM-AIMS-2023-149), Amrita Vishwa Vidyapeetham. Blood was collected via venipuncture from healthy volunteers under written informed consent.

Additional information

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