

In vitro experimental evaluation of the antibacterial, antibiotic-synergistic, and anti-motility effects of eugenol against clinical *Pseudomonas aeruginosa* isolates from hospitalized animals

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ABSTRACT

Background and Objectives: *Pseudomonas aeruginosa* is an opportunistic Gram-negative pathogen responsible for severe infections and increasing therapeutic difficulties due to multidrug resistance and virulence traits such as motility and biofilm formation. This study aimed to evaluate the in vitro antibacterial, antibiotic-synergistic, and anti-motility effects of eugenol in combination with cefixime and gentamicin against nine clinical *P. aeruginosa* isolates obtained from hospitalized animals.

Materials and Methods: This preliminary in vitro laboratory investigation was conducted on a convenience sample of nine independent non-duplicate clinical *P. aeruginosa* isolates obtained from wound swabs and urinary tract samples of hospitalized animals at the Small Animal Hospital, Faculty of Veterinary Medicine, University of Tehran. Minimum inhibitory concentrations (MICs) of eugenol alone were determined across all nine isolates using the broth microdilution method in accordance with CLSI guidelines (M07-A11). Synergistic interactions between eugenol and the antibiotics cefixime and gentamicin were evaluated using the checkerboard assay and calculation of the fractional inhibitory concentration index (FICI) on a subset of three selected representative isolates (P3, P6, and P9). Furthermore, the effects of sub-inhibitory concentrations of eugenol ("MIC/2") on bacterial motility were assessed using standardized media containing 0.3%, 0.7%, and 1.5% agar to evaluate swimming, swarming, and twitching motilities, respectively, across this same subset of isolates (P3, P6, and P9) in biological triplicate (n=3).

Results: Antimicrobial analysis across all nine isolates demonstrated intrinsic antibacterial activity of eugenol, with MIC values ranging from 2.5 to 10 µg/mL. Checkerboard synergy testing on the selected representative subset (P3, P6, and P9) revealed synergistic interactions with cefixime ("FICI"=0.5 for isolate P6, P3 and P9) as well as interactions with gentamicin yielding FICI values of 0.256 (for isolates P6 and P9) and 0.384 (for isolate P3), classifying these combinations within synergistic interaction ranges ("FICI"≤0.5). Additionally, sub-inhibitory eugenol treatment of the tested subset significantly reduced bacterial motility compared to untreated controls, decreasing swimming by 44-57%, swarming by 43-46%, and twitching by 39-64% (P<0.05).

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Conclusion: Eugenol demonstrated notable in vitro antibacterial and anti-motility activities against tested clinical *P. aeruginosa* isolates from veterinary sources, alongside favorable FICI interactions with gentamicin and cefixime. Because these findings are strictly restricted to limited in vitro laboratory observations without dosage optimization, toxicity, or in vivo efficacy evaluations, further comprehensive investigations are required before establishing any potential clinical relevance.

Keywords: *Pseudomonas aeruginosa*; Eugenol; Antibacterial activity; Synergistic interactions; Motility assay

INTRODUCTION

Antimicrobial resistance (AMR) represents one of the most serious global health threats, accounting for approximately 1.27 million deaths annually according to recent reports from the World Health Organization. The rapid emergence of resistant pathogens has significantly complicated the management of infectious diseases and reduced the effectiveness of existing antimicrobial therapies (1-3).

Among clinically important resistant bacteria, *Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen frequently associated with severe hospital-acquired infections, including pneumonia, septicemia, urinary tract infections, and wound infections (4). These infections predominantly affect high-risk populations, including immunocompromised individuals and veterinary clinical patients. Intended primarily as a veterinary health investigation, this study addresses clinical isolates recovered from hospitalized animal populations. The remarkable persistence of *P. aeruginosa* is attributed to both intrinsic and acquired resistance mechanisms, including the production of β -lactamases such as AmpC, multidrug efflux pumps (e.g., MexAB-OprM), and reduced outer membrane permeability, all of which limit antibiotic penetration and efficacy (5-7).

In addition to antibiotic resistance, several virulence determinants contribute to the pathogenic success of *P. aeruginosa*. Biofilm formation and diverse motility mechanisms—swimming, swarming, and twitching—play essential roles in colonization, surface attachment, and chronic infection development. Recent epidemiological studies emphasize the urgent need for alternative therapeutic strategies to manage resistant strains. While previous work has examined eugenol's general antimicrobial properties, biofilm disruption, and broad quorum-sensing interference, a critical knowledge gap remains regarding the simultaneous evaluation of antibiotic potentiation alongside multi-phenotype motility suppression across veterinary clinical strains (8). However, evidence remains limited on whether eugenol can

potentiate commonly used antibiotics while simultaneously suppressing distinct motility phenotypes in clinical *P. aeruginosa* isolates recovered from hospitalized animals.

Combination therapy has emerged as a promising approach to overcome antimicrobial resistance by enhancing antibacterial efficacy while potentially reducing required antibiotic dosages and associated toxicity (3). In this context, natural bioactive compounds have gained considerable attention as antibiotic adjuvants (9, 10). Eugenol (4-allyl-2-methoxyphenol), the principal phenolic component of clove oil (*Eugenia aromaticum*), exhibits notable antimicrobial and anti-virulence activities. Previous studies have demonstrated that eugenol can disrupt bacterial membrane integrity, increase permeability to antimicrobial agents, and interfere with regulatory systems involved in motility and virulence factor production. However, these mechanistic descriptions regarding membrane disruption, quorum sensing, flagella, and Type IV pili are presented here as working hypotheses, as direct molecular assays were beyond the scope of this in vitro study (3, 11).

Cefixime, a third-generation cephalosporin that inhibits bacterial cell wall synthesis, was selected to investigate whether eugenol could enhance the activity of a β -lactam agent against the tested *P. aeruginosa* isolates. The inclusion of cefixime and gentamicin also allowed evaluation of eugenol in combination with antibiotics representing distinct antibacterial targets, namely cell-wall synthesis and bacterial protein synthesis, respectively (12).

Meanwhile, gentamicin, an aminoglycoside targeting protein synthesis via binding to the 30S ribosomal subunit, was selected to explore broad adjuvant potentiation against standard susceptibility profiles in veterinary clinical isolates. Increasing resistance has compromised their clinical effectiveness, and earlier investigations have suggested that eugenol may enhance antibiotic activity against Gram-negative bacteria by reducing minimum inhibitory concentrations (MICs), yet evidence regarding its interaction with cefixime remains limited. Furthermore,

although the antibiofilm properties of eugenol have been reported, its influence on bacterial motility—an essential factor in early colonization and infection establishment—warrants comprehensive investigation across veterinary clinical strains (1, 3).

Therefore, this study aimed to evaluate the *in vitro* antibacterial and anti-virulence potential of eugenol against clinical isolates of *P. aeruginosa* obtained from a veterinary hospital setting. Specifically, this study sought to: (1) determine the MICs of eugenol across a panel of nine clinical isolates; (2) assess the synergistic interactions between eugenol and cefixime or gentamicin using the checkerboard assay and fractional inhibitory concentration index (FICI); and (3) investigate the effects of sub-MIC concentrations of eugenol on swimming, swarming, and twitching motility.

MATERIALS AND METHODS

Study design and bacterial isolates. This study was designed as an analytical *in vitro* laboratory investigation to evaluate the antimicrobial activity of eugenol and its synergistic interactions with selected antibiotics against clinical isolates of *P. aeruginosa*. All experiments were conducted in the Microbiology Laboratory, Faculty of Veterinary Medicine, University of Tehran, during 2024. Institutional authorization for utilizing routine diagnostic clinical isolates of hospitalized animals was granted by the Faculty of Veterinary Medicine. This study was conducted in accordance with the reporting standards for *in vitro* laboratory research as outlined by the EQUATOR Network guidelines.

A convenience sample of nine independent, non-duplicate clinical *P. aeruginosa* isolates (designated P1 through P9) was obtained from a total of nine distinct animal hosts, comprising canine (n=6) and feline (n=3) patients. Specifically, the sample set comprised five wound swabs (n=5; canine: 4, feline: 1) and four urinary tract samples (n=4; canine: 2, feline: 2) collected from distinct, non-duplicate hospitalized companion animal patients at the Small Animal Hospital, Faculty of Veterinary Medicine, University of Tehran, between March and September 2024. No epidemiological or clinical metadata beyond sample source and patient species were linked to the isolates, and duplicate isolates from the same clinical source or patient were strictly excluded. The selection of exactly

nine isolates represented a preliminary convenience sampling framework, and the very small, non-random sample size substantially limits generalizability; findings must therefore be interpreted strictly as exploratory *in vitro* observations rather than broad epidemiological evidence (13, 14). The selection of isolates P3, P6, and P9 was performed purposefully based on predefined criteria to represent a spectrum of diverse baseline susceptibility profiles and MIC observed across the sample set. Although all nine clinical isolates originated from veterinary clinical samples consisting of wound and urinary swabs from hospitalized animals, these three particular isolates demonstrated distinct baseline resistance and susceptibility patterns, thereby qualifying them as ideal candidates for detailed downstream checkerboard and motility evaluations. The remaining isolates were intentionally excluded from these specific resource-intensive motility and matrix assays to preserve a focused and exploratory analysis centered on representative phenotypic variations.

Bacterial identification was confirmed using conventional phenotypic characterization methods, including Gram staining, oxidase testing, pigment production assessment, and standard biochemical assays. Due to reliance on standard phenotypic identification, molecular confirmation (such as MALDI-TOF or PCR-based sequencing) was not performed, which represents an acknowledged study limitation. Isolates were stored in cryovials at -80°C in Tryptic Soy Broth (TSB; Merck, Darmstadt, Germany) supplemented with 20% glycerol, subcultured twice on Mueller-Hinton agar prior to assays, and maintained at low passage numbers (passages 2 to 4) to ensure purity and viability. Purity was routinely verified via colony morphology on cefrimide agar and Gram staining. Baseline antimicrobial susceptibility profiles were established for all strains using both disk diffusion and broth microdilution methods. Inoculum standardization and viability were routinely verified for all assays by performing serial ten-fold dilutions and plating quantitative viable counts (CFU/mL) on Mueller-Hinton agar.

Eugenol and antibiotic preparation. The antimicrobial agents evaluated included analytical-grade eugenol ($\geq 99\%$ purity, Sigma-Aldrich, St. Louis, MO, USA; stored in the dark at 4°C, cefixime powder (Sigma-Aldrich, USA), and gentamicin sulfate (Sigma-Aldrich, USA). Due to its hydrophobic nature,

eugenol was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA). A primary stock solution of eugenol (20 mg/mL) was prepared by mixing 200 μ L of eugenol with 40 μ L of DMSO and 1,760 μ L of sterile Tryptic Soy Broth (TSB; Merck, Darmstadt, Germany), yielding a primary stock solution containing 10% DMSO. Eugenol stock solutions were prepared by initial dissolution in a 10% (v/v) aqueous DMSO solution. Through subsequent two-fold serial dilutions into the respective assay media, the final working concentration of DMSO in all experimental wells—including broth microdilution, checkerboard matrix, and motility assays—was strictly maintained at or below 0.5% (v/v). To ensure that the solvent did not influence experimental outcomes, matched vehicle controls containing the maximum corresponding final concentration of DMSO (0.5%) were evaluated in parallel across all assays (including swimming, swarming, and twitching evaluations), confirming no inhibitory effect on bacterial growth or baseline motility. Stock solutions of cefixime (100,000 μ g/mL) and gentamicin (40,000 μ g/mL) were prepared under sterile conditions in appropriate solvents and diluted in sterile broth to obtain required working ranges. Consistent solvent preparation and dilution protocols were applied across all experiments to control for matrix-induced variability in MIC values.

Determination of minimum inhibitory concentration. The MICs of eugenol, cefixime, and gentamicin were determined individually using the broth microdilution method in sterile 96-well microtiter plates according to Clinical and Laboratory Standards Institute guidelines (CLSI M07-A11) (13).

Bacterial inocula were prepared from overnight cultures on Mueller-Hinton agar and suspended in sterile saline to match a 0.5 McFarland turbidity standard (1×10^8 CFU/mL), followed by further dilution in Mueller-Hinton broth (MHB; Merck, Darmstadt, Germany) to achieve a final well inoculation density of approximately 5×10^5 CFU/mL in a total assay volume of 200 μ L. Inoculum standardization was verified by viable plate counts.

Two-fold serial dilutions of eugenol spanned a full range from 10 to 0.078 μ g/mL. Quality control strains (*P. aeruginosa* ATCC 27853) and appropriate controls—including growth controls (bacteria without antimicrobial agent), sterility controls (broth only), and matched solvent controls (1% DMSO)—were included on every plate.

Microplates were incubated aerobically at 37°C for 18–20 h. The MIC was defined as the lowest concentration showing complete visible inhibition of bacterial growth. All MIC assays were performed in biological triplicate ($n=3$ independent experiments performed on separate days), yielding identical replicate values without observable variability across runs. Careful visual and spectrophotometric checks confirmed that eugenol precipitation, turbidity, or optical interference did not occur at the tested concentrations (2.5–10 μ g/mL). The unusually low eugenol MICs observed compared to some historical literatures are methodologically attributable to strict solvent control (maintaining DMSO $\leq 1\%$), fresh high-purity stock preparation, and specific regional strain characteristics.

In parallel, disk diffusion susceptibility testing for gentamicin (10 μ g disks; Oxoid, UK) was performed on Mueller-Hinton agar according to CLSI M100 standards, and associated standard M07 for broth microdilution (34th informational supplement, 2024), with inhibition zone diameters interpreted in millimeters against established CLSI breakpoints for *P. aeruginosa* (≥ 15 mm susceptible, 13–14 mm intermediate, and ≤ 12 mm resistant). Tested parameters included standardized Mueller-Hinton agar and broth media, specific antibiotic disk contents (gentamicin), and standard incubation conditions at 37°C for 18–24 hours, with zone diameters interpreted using established CLSI breakpoints. Because standardized CLSI interpretive breakpoints are not established for cefixime against *P. aeruginosa* due to intrinsic non-susceptibility, cefixime was tested exclusively to evaluate eugenol-mediated synergy and antibiotic potentiation rather than clinical categorization. Cefixime, while typically exhibiting limited intrinsic activity against *P. aeruginosa* due to outer membrane barrier properties and efflux mechanisms, was deliberately included in the checkerboard synergy assays to evaluate whether eugenol could act as an effective membrane-permeabilizing adjuvant to restore or enhance its antibacterial efficacy.

Checkerboard microdilution assay for synergy evaluation. Synergistic interactions between eugenol and cefixime or gentamicin were evaluated using the checkerboard microdilution method on selected representative isolates (P3, P6, and P9) in 96-well microtiter plates.

These three isolates were chosen to represent diverse baseline susceptibility and motility phenotypes

observed across the panel of nine strains; synergy was thus evaluated exclusively in isolates P3, P6, and P9 rather than universally across all nine strains. Two-fold serial dilutions of eugenol were arrayed vertically, and serial dilutions of the antibiotic were arrayed horizontally. Plates were inoculated to achieve a final density of 5×10^5 CFU/mL in 200 μ L total volume and incubated at 37°C for 18–20 h. Checkerboards were performed in biological triplicate ($n=3$ independent experiments). When replicate outcomes showed minor numerical shifts, modal or most conservative FICIs were reported. Full checkerboard matrix distributions for cefixime and gentamicin across isolates P3, P6, and P9 are documented in the supplementary laboratory records. Detailed gentamicin MIC values are presented for these representative isolates to correlate directly with checkerboard interaction matrices.

The interaction was quantified using the FICI, calculated as:

$$FICI = \frac{MIC_A \text{ in combination}}{MIC_A \text{ alone}} + \frac{MIC_B \text{ in combination}}{MIC_B \text{ alone}}$$

Where A represents eugenol and B represents the antibiotic. FICI interpretations were defined strictly by standard pharmacological thresholds: synergistic ($FICI \leq 0.5$), additive ($0.5 < FICI \leq 1$), indifferent ($1 < FICI \leq 4$), and antagonistic ($FICI > 4$). Because FICI categorizes interaction types rather than testing statistical hypotheses, these classifications are presented as pharmacological interaction categories without inferential P-values. Confirmatory time-kill assays were not performed, meaning these combination effects represent isolate-specific *in vitro* findings rather than broadly established synergistic patterns.

Assessment of anti-motility activity. The effect of eugenol on swimming, swarming, and twitching motilities was evaluated exclusively on the selected representative subset of three isolates (P3, P6, and P9) rather than all nine strains. Eugenol was tested at sub-inhibitory concentrations corresponding exactly to one-half of the MIC ($MIC/2$) determined for each specific strain (5 μ g/mL for P3, 2.5 μ g/mL for P6, and 2.5 μ g/mL for P9). Assays were conducted in biological triplicate ($n=3$ independent experiments performed on separate days), with technical duplicates ($n=2$) per run. Luria-Bertani (LB) medium (Merck, Darmstadt, Germany) was prepared with precise agar concentrations: 0.3% (w/v) for swimming, 0.7% (w/v)

for swarming, and 1.5% (w/v) for twitching.

Swimming motility assay. Assayed on 0.3% LB agar plates (20 mL per 90-mm petri dish). Plates were centrally inoculated using a sterile inoculating needle dipped into a standardized bacterial suspension (0.5 McFarland, 3 μ L) and inserted to a depth approximately halfway to the bottom of the agar. Plates were incubated face-up at 37°C for 24 h, and migration zone diameters (including the inoculation point) were measured in millimeters using a digital caliper (15).

Swarming motility assay. Assayed on 0.7% LB agar plates supplemented with 0.5% glucose (15 mL per dish). Plates were surface-dried at room temperature for 30 min prior to inoculation, then surface-inoculated with 3 μ L of standardized bacterial suspension. Plates were incubated at 37°C for 24 h, and the radial migration spread diameter was measured in millimeters (including the inoculation point) (15).

Twitching motility assay. Assayed using a modified sub-surface overlay technique on 1.5% LB agar plates (15 mL per dish) (16). To enhance the precision and reproducibility of the twitching motility assay, we implemented a modified inoculation technique. After placing the bacterial colony (15 mL per dish) at the junction of the agar and the interior wall of the Petri dish, 1000 μ L of the tempered (37°C) LB-agar medium was gently overlaid onto the inoculum. Once solidified, the remaining volume of the medium was poured into the Petri dishes. This procedure secured the colony at the specific interface between the agar and the polystyrene surface, preventing displacement during the pouring of the remaining medium. By ensuring consistent, direct contact between the inoculum and the surface throughout the incubation period, this approach allows for a more stable and accurate measurement of twitching zone diameters. Plates were incubated at 37°C for 48–72 h. After incubation, the agar layer was carefully removed, and the adherent bacterial cells at the plate base were stained with 0.1% crystal violet (Merck, Germany) for 10 min. Excess stain was gently rinsed with distilled water, and the surface was air-dried. Twitching motility was quantified by measuring the diameter of the stained zone at the agar–surface interface (including the inoculation point).

All assays were performed in triplicate to ensure reproducibility. Inocula were independently stan-

standardized for each isolate using spectrophotometric adjustment and viable counts. Colony size and viability differences did not confound readouts. Parallel growth curves (monitoring optical density at 600 nm over 24 h) confirmed that MIC/2 eugenol concentrations did not inhibit baseline planktonic growth rate, verifying that reduced motility was independent of residual growth inhibition. Measurement blinding was not implemented. Furthermore, observed reductions in motility are reported strictly as in vitro anti-motility phenomena and are not equated with demonstrated reductions in in vivo invasion, dissemination, or biofilm formation, as those complex phenotypes were not directly measured.

Statistical analysis. The independent biological replicate, defined as an experiment performed on a separate day, was considered the experimental unit. Technical duplicates were averaged within each experimental run before statistical analysis. MIC assays were performed using three biological replicates across all nine isolates, while checkerboard and motility assays were conducted using three biological replicates for the selected isolates P3, P6, and P9; motility assays additionally included technical duplicates.

Data normality and homogeneity of variance were assessed using the Shapiro–Wilk and Brown–Forsythe tests, respectively. Where appropriate, one-way ANOVA followed by Tukey’s multiple-comparison test was used for comparisons involving multiple groups, while paired two-tailed Student’s *t*-tests were used for direct comparisons between untreated and eugenol-treated samples within the same isolate. Exact *P*-values, means $\pm$ standard deviations, and 95% confidence intervals were reported where applicable. Statistical significance was set at $P < 0.05$. Given the limited number of independent clinical isolates, statistical findings were interpreted as observations within the tested isolates and experimental replicates rather than as evidence of population-level generalizability.

Ethical approval. Although no direct live animal interventions or human participant experiments were performed, institutional permission and ethical clearance for utilizing routine diagnostic veterinary clinical specimens were formally granted by the Small Animal Hospital and the Research Ethics Committee of the Faculty of Veterinary Medicine, University of Tehran.

RESULTS

Antibacterial activity of eugenol against *P. aeruginosa* isolates. The antibacterial activity of eugenol was evaluated by determining the MICs against all nine clinical *P. aeruginosa* isolates ($N=9$). The MIC values ranged from 2.5 to 10 $\mu\text{g/mL}$ among the tested isolates (Fig. 1). Isolates P2 and P5 exhibited the lowest MIC value (2.5 $\mu\text{g/mL}$), indicating the highest susceptibility to eugenol. Four isolates (P1, P7, P8, and P9) showed MIC values of 5 $\mu\text{g/mL}$, whereas isolate P3 demonstrated the highest MIC value (10 $\mu\text{g/mL}$), and isolate P6 showed an MIC of 5 $\mu\text{g/mL}$. No isolates displayed MIC values exceeding 10 $\mu\text{g/mL}$. All MIC determinations were performed in independent biological triplicate ($n=3$), yielding identical replicate values.

Comprehensive isolate susceptibility and interaction summary: to provide complete data transparency across the panel, Table 1 summarizes the isolate ID, clinical source, individual eugenol MICs, gentamicin MICs, and subset-specific checkerboard FICI values for cefixime and gentamicin. Cefixime stand-alone MIC testing yielded a uniform high value of 64 $\mu\text{g/mL}$ across tested representative strains (such as P6), reflecting broad baseline resistance, whereas individual cefixime testing was not exhaustively repeated across all nine isolates due to confirmed uniform high resistance.

Antibiotic susceptibility testing and MIC determination of gentamicin. The antimicrobial susceptibility of *P. aeruginosa* isolates to gentamicin was initially evaluated using the disk diffusion method. The results demonstrated inhibition zone diameters of approximately 20.4 ± 1.2 mm (mean $\pm$ SD, $n=3$), indicating high susceptibility according to CLSI interpretive criteria (susceptibility breakpoint ≥ 15 mm). In addition, the MICs of gentamicin were determined independently using the broth microdilution method in biological triplicate ($n=3$). The obtained MIC values were 0.78 $\mu\text{g/mL}$ (isolate P9) and 1.56 $\mu\text{g/mL}$ (isolates P3 and P6), confirming strong baseline antibacterial activity of gentamicin against the tested subset.

Synergistic interaction between eugenol and cefixime. The checkerboard microdilution assay, performed in biological triplicate ($n=3$), demonstrated a synergistic interaction between eugenol and ce-

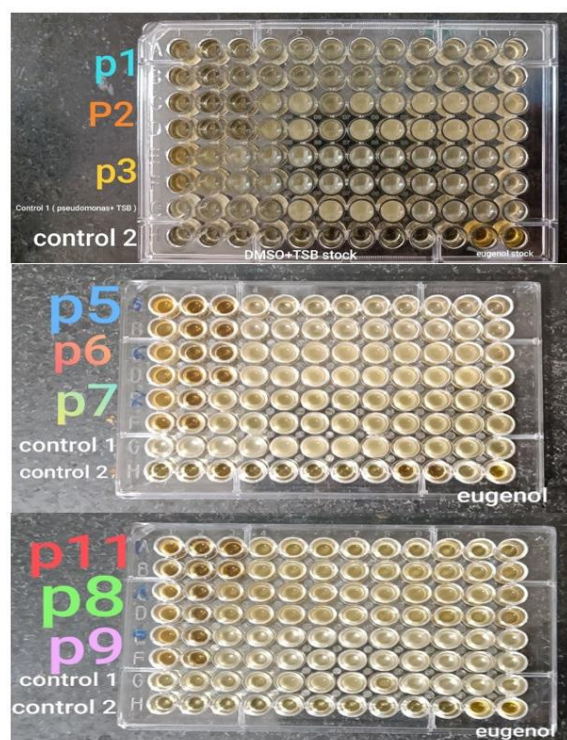


Fig. 1. Minimum inhibitory concentration (MIC) of eugenol against clinical isolates of *Pseudomonas aeruginosa* determined by broth microdilution. Representative 96-well plates showing serial two-fold dilutions of eugenol after 24 h incubation at 37°C. The plates display isolates P1, P2, P3 (top plate), P5, P6, P7 (middle plate), and P11, P8, P9 (bottom plate). The MIC range was 2.5–10 µg/mL. Growth and sterility controls were included in each plate. The displayed 96-well plates represent a single independent experiment out of the three biological replicates performed (n=3).

fixime against the selected representative subset of *P. aeruginosa* isolates (P3, P6, and P9). For isolate P6, the combination markedly reduced the MICs of both agents compared with their individual MIC values. Specifically, the MIC of eugenol decreased from 5 µg/mL to 1.25 µg/mL, while the MIC of cefixime decreased from 64 µg/mL to 16 µg/mL when used in combination. The calculated FICI for isolate P6 was 0.5 (Fig. 2), indicating a synergistic interaction ($FICI \leq 0.5$). Similar synergistic interactions ($FICI \leq 0.5$) were observed for isolates P3 and P9. Because these results were derived exclusively from isolates P3, P6, and P9, they are presented as isolate-specific in vitro findings rather than evidence of a broadly established universal pattern across all nine strains.

Synergistic interaction between eugenol and gentamicin. The checkerboard microdilution assay (n=3 biological replicates) demonstrated a pronounced synergistic interaction between eugenol and gentamicin against the selected representative subset of clinical isolates (P3, P6, and P9). For isolate P6, the MIC of eugenol decreased from 2.5 µg/mL to 0.32 µg/mL when combined with gentamicin, while the MIC of gentamicin was reduced from 1.56 µg/mL to 0.2 µg/mL, resulting in a FICI value of 0.256. Similarly, isolate P9 exhibited identical MIC reductions, yielding an FICI value of 0.256. In isolate P3, the combination produced an FICI value of 0.384 (Fig. 3). Since all calculated FICI values for this subset were ≤ 0.5 , the interaction between euge-

Table 1. Summary of isolate IDs, clinical sources, individual minimum inhibitory concentrations (MICs), and interaction indices (FICI).

Isolate ID	Clinical Source	Eugenol MIC (µg/mL)	Cefixime MIC (µg/mL)	Gentamicin MIC (µg/mL)	FICI (Eugenol + Cefixime)	FICI (Eugenol + Gentamicin)
P1	Canine Wound	5	NT	NT	NT	NT
P2	Canine Wound	2.5	NT	NT	NT	NT
P3	Canine Urine	10	64	1.56	0.5 (Synergistic)	0.384 (Synergistic)
P4	Feline Urine	5	NT	NT	NT	NT
P5	Canine Wound	2.5	NT	NT	NT	NT
P6	Feline Wound	5	64	1.56	0.5 (Synergistic)	0.256 (Synergistic)
P7	Canine Wound	5	NT	NT	NT	NT
P8	Canine Urine	5	NT	NT	NT	NT
P9	Canine Urine	5	64	0.78	0.5 (Synergistic)	0.256 (Synergistic)

Note: NT = Not Tested; checkerboard synergy and combination MIC evaluations were restricted to the selected representative subset of isolates P3, P6, and P9 rather than applied across all nine isolates.



Fig. 2. Synergistic interaction between eugenol and cefixime against *Pseudomonas aeruginosa* clinical isolates (P3, P6, and P9) determined by the checkerboard microdilution assay. Serial two-fold dilutions of eugenol (horizontal axis) and cefixime (vertical axis) were combined in 96-well microplates and incubated for 24 h at 37°C. Representative wells indicating MICs of each agent alone and in combination are highlighted. The combination reduced the MIC of eugenol from 5 to 1.25 µg/mL and cefixime from 64 to 16 µg/mL (isolate P6), yielding a Fractional Inhibitory Concentration Index (FICI) of 0.5, consistent with synergism ($FICI \leq 0.5$). Similar synergistic patterns were observed for isolates P3 and P9. The displayed plate represents an isolate-specific representative experiment out of the three independent biological replicates performed ($n=3$). Representative wells indicating MICs of each agent alone and in combination are highlighted, where the 'Combination' label indicates wells containing the dual mixture of eugenol and cefixime.

nol and gentamicin was classified pharmacologically as synergistic. These findings indicate that eugenol markedly enhances gentamicin activity in these specific isolates.

Inhibitory effects of eugenol on *P. aeruginosa* motility. To clearly distinguish direct antibacterial effects from anti-motility activities, sub-inhibitory



Fig. 3. Synergistic interaction between eugenol and gentamicin against *Pseudomonas aeruginosa* clinical isolates (P3, P6, and P9) determined by checkerboard microdilution assay. Serial two-fold dilutions of eugenol (horizontal axis) and gentamicin (vertical axis) were combined and incubated for 24 h at 37°C. Representative wells indicating MICs of each agent alone and in combination are highlighted. In isolate P6, the MIC of eugenol decreased from 2.5 to 0.32 µg/mL and gentamicin from 1.56 to 0.2 µg/mL, yielding a Fractional Inhibitory Concentration Index (FICI) of 0.256. FICI values for isolates P3 and P9 were 0.384 and 0.256, respectively, confirming strong synergism ($FICI \leq 0.5$). The displayed plate represents an isolate-specific representative experiment out of the three independent biological replicates performed ($n=3$). Representative wells indicating MICs of each agent alone and in combination are highlighted, where the 'Combination' label indicates wells containing the dual mixture of eugenol and gentamicin.

concentrations corresponding exactly to one-half of each strain's MIC (MIC/2; 5 µg/mL for P3, 2.5 µg/mL for P6, and 2.5 µg/mL for P9) were evaluated. Parallel growth curve assays confirmed that bacterial replication rates and turbidity at MIC/2 were indistinguishable from untreated controls, verifying that observed motility reductions were independent of general growth inhibition. Motility inhibition per-

centages were calculated relative to untreated control migration diameters using the formula:

$$\text{Inhibition Rate (\%)} = \left(1 - \frac{\text{Mean Diameter}_{\text{treated}}}{\text{Mean Diameter}_{\text{control}}}\right) \times 100$$

Swimming motility. Assayed across isolates P3, P6, and P9 in biological triplicate (n=3 independent experiments, with technical duplicates averaged per run). Eugenol (MIC/2) significantly reduced swimming migration zone diameters compared with untreated controls (n=3): for isolate P3, mean diameter decreased from 32.4 ± 2.1 mm to 13.9 ± 1.5 mm (57% reduction, paired t-test $P=0.0123$); for isolate P6, diameter decreased from 29.8 ± 1.8 mm to 13.4 ± 1.2 mm (55% reduction, $P=0.013$); for isolate P9, diameter decreased from 35.2 ± 2.4 mm to 19.7 ± 1.6 mm (44% reduction, $P=0.049$). One-way ANOVA across tested strains yielded an overall significance of $P<0.001$ (Fig. 4).

Swarming motility. Assayed across isolates P3, P6, and P9 in biological triplicate (n=3 independent experiments). Eugenol (MIC/2) significantly reduced swarming spread diameters compared with untreated controls (n=3): for isolate P3, mean diameter decreased from 28.6 ± 1.9 mm to 16.0 ± 1.3 mm (44% reduction, $P<0.001$); for isolate P6, diameter decreased from 26.5 ± 1.7 mm to 14.3 ± 1.1 mm (46% reduction, $P=0.013$); for isolate P9, diameter decreased from 30.1 ± 2.2 mm to 17.1 ± 1.4 mm (43% reduction, $P=0.033$). One-way ANOVA yielded an

overall significance of $P<0.013$ (Fig. 5).

Twitching motility. Assayed across isolates P3, P6, and P9 in biological triplicate (n=3 independent experiments). Eugenol ("MIC/2") significantly reduced crystal-violet-stained attachment zone diameters compared with untreated controls (n=3): for isolate P3, mean diameter decreased from 18.5 ± 1.4 mm to 6.7 ± 0.8 mm (64% reduction, $P=0.001$); for isolate P6, diameter decreased from 15.2 ± 1.1 mm to 9.3 ± 0.9 mm (39% reduction, $P<0.001$); for isolate P9, diameter decreased from 16.8 ± 1.3 mm to 8.7 ± 0.7 mm (48% reduction, $P=0.004$). One-way ANOVA yielded an overall significance of $P<0.001$ (Fig. 6). Visual documentation of plate assays is presented in Fig. 7.

DISCUSSION

The present study demonstrated the intrinsic in vitro antibacterial activity of eugenol against clinical isolates of *P. aeruginosa*, with MIC values ranging from 2.5 to 10 $\mu\text{g/mL}$ among the nine examined isolates. When compared with previous literature, these observed MIC values are substantially lower than several earlier reports where average MIC values exceeded 625 $\mu\text{g/mL}$ or reached concentrations over 1000 $\mu\text{g/mL}$ to inhibit *P. aeruginosa* growth (17). Methodologically, these discrepancies are best explained by strict solvent control (maintaining final DMSO concentrations $\leq 1\%$), the use of fresh

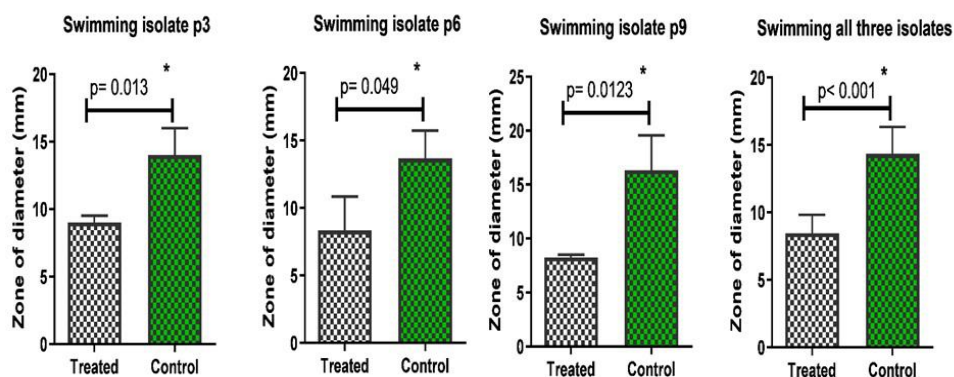


Fig. 4. Inhibitory effect of sub-MIC eugenol on swimming motility of *P. aeruginosa* clinical isolates (P3, P6, and P9). Swimming assays were performed on LB agar containing 0.3% agar and incubated at 37°C for 24 h. Representative plates comparing untreated controls and eugenol-treated groups (MIC/2) are shown. Eugenol significantly reduced swimming motility, with mean inhibition rates of 57% (P3), 55% (P6), and 44% (P9). Statistical analysis confirmed significant differences compared with untreated controls (P3: $P=0.0123$; P6: $P=0.013$; P9: $P=0.049$; one-way ANOVA).

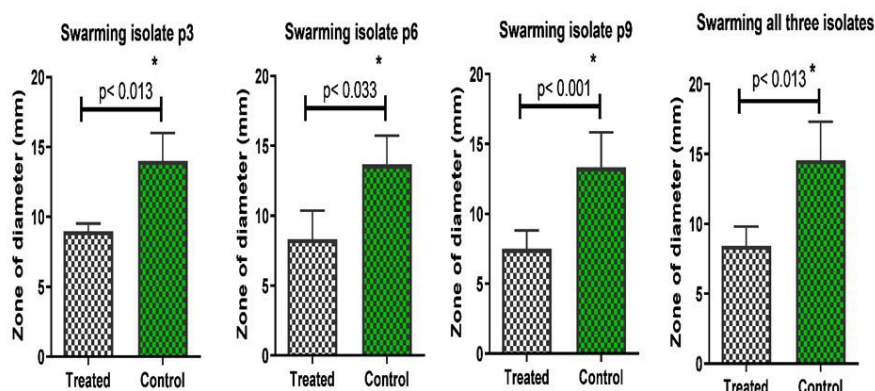


Fig. 5. Effect of sub-MIC eugenol on swarming motility of *P. aeruginosa* clinical isolates (P3, P6, and P9). Swarming assays were performed on LB agar containing 0.7% agar and incubated at 37°C for 24 h. Representative plates comparing untreated controls and eugenol-treated groups (MIC/2) are shown. Eugenol significantly reduced swarming motility, with mean inhibition rates of 44% (P3), 46% (P6), and 43% (P9). Statistical analysis demonstrated significant differences compared with untreated controls (P3: $P < 0.001$; P6: $P = 0.013$; P9: $P = 0.033$; one-way ANOVA).

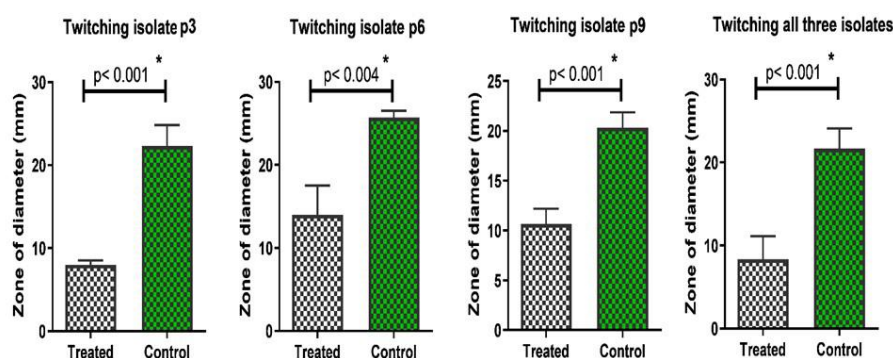


Fig. 6. Inhibitory effect of sub-MIC eugenol on twitching motility of *P. aeruginosa* clinical isolates (P3, P6, and P9). Twitching assays were performed on LB agar containing 1.5% agar and incubated at 37°C for 48-72 h. After incubation, the agar layer was removed and adherent cells were stained with 0.1% crystal violet for visualization. Representative plates comparing untreated controls and eugenol-treated groups (MIC/2) are shown. Eugenol markedly reduced twitching motility, with mean inhibition rates of 64% (P3), 39% (P6), and 48% (P9). Statistical analysis demonstrated highly significant differences compared with untreated controls (P3: $P = 0.001$; P6: $P < 0.001$; P9: $P = 0.004$; one-way ANOVA).

high-purity stock solutions, specific regional strain characteristics, and variations in culture media composition (such as Mueller-Hinton broth versus TSB).

The antimony and microdilution analyses confirmed the susceptibility of the tested veterinary isolates to gentamicin, with disk diffusion inhibition zones averaging approximately 20 mm and low microdilution MICs (0.78 to 1.56 µg/mL) (18). However, the tested isolates were not systematically characterized as multidrug-resistant (MDR); therefore, these findings cannot be extrapolated to broader MDR human clinical infections. All isolates evaluated in this study originated exclusively from companion animal

veterinary patients, limiting the scope of the findings to a preliminary veterinary context (3).

The checkerboard assay demonstrated a clear synergistic interaction between eugenol and cefixime (FICI=0.5 for isolate P6, with comparable outcomes in P3 and P9). An even stronger synergistic interaction was observed for the combination of eugenol and gentamicin, yielding FICI values of 0.256 (isolates P6 and P9) and 0.384 (isolate P3). These interaction indices demonstrate favorable in vitro combination effects for these specific isolate pairs rather than a broadly established universal pattern. Mechanistically, while earlier literature suggests that phenolic

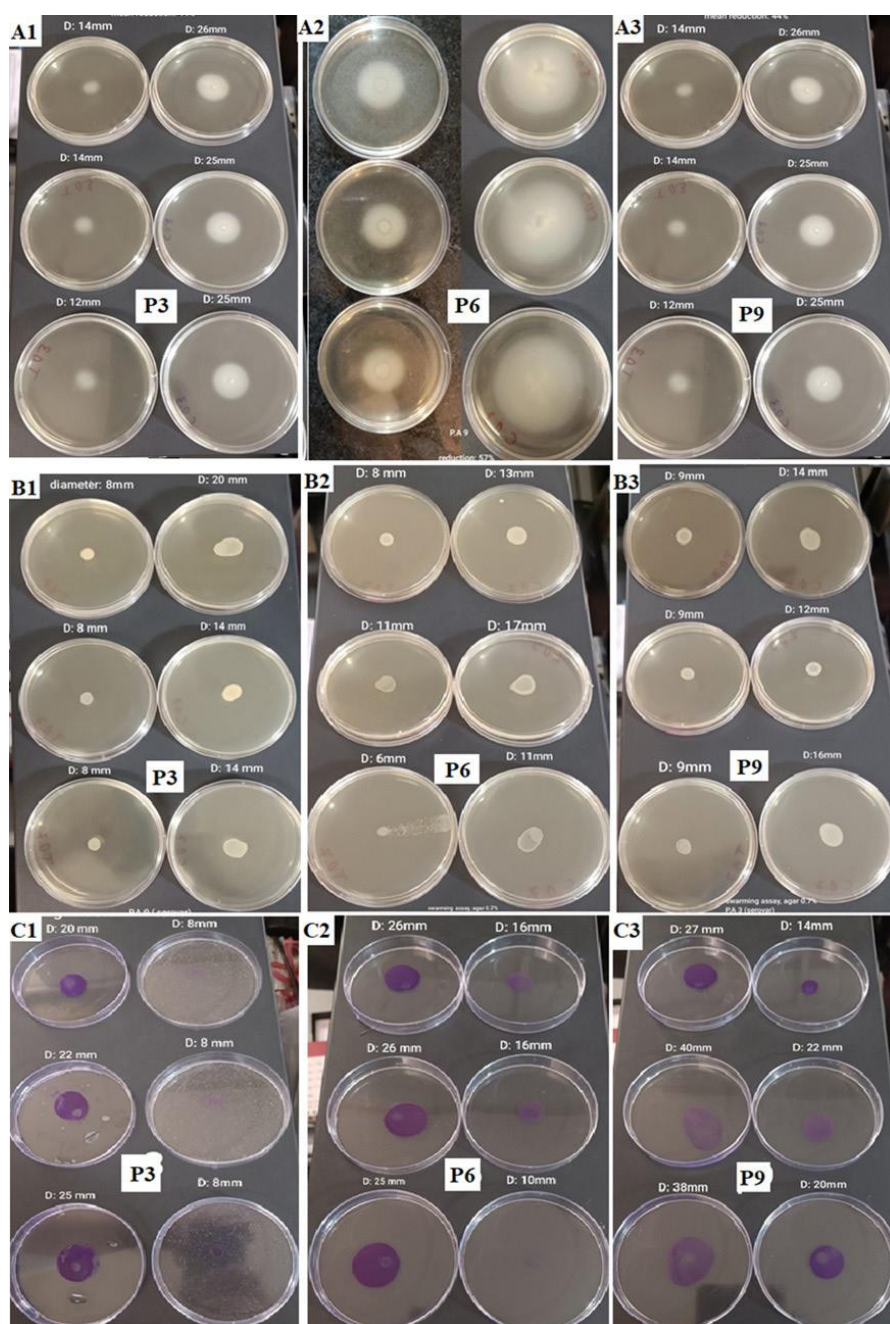


Fig. 7. Inhibition of different motility types of *P. aeruginosa* by sub-MIC eugenol; A1–A3 show images related to swimming motility in three tested isolates (P3, P6 and P9). B1–B3 represent swarming motility, and C1–C3 correspond to twitching motility. The plates on the right were treated with MIC/2 concentration of eugenol, while the plates on the left served as controls. The experiment was performed in triplicate, and the mean diameter of bacterial growth was ultimately used as the analyzed parameter. The displayed plates represent a single independent experiment out of the three biological replicates performed ($n=3$).

compounds like eugenol may alter membrane characteristics or interfere with regulatory pathways, direct membrane permeabilization, quorum-sensing interference, or Type IV pilus disruption were not experimentally measured in this study and must be in-

terpreted strictly as working hypotheses rather than demonstrated mechanisms (3).

The most plausible explanation for this synergy lies in eugenol's membrane-disruptive properties. Eugenol increases bacterial cytoplasmic membrane per-

meability, thereby facilitating enhanced intracellular penetration of cefixime, a β -lactam antibiotic that inhibits cell wall synthesis. Improved antibiotic access to its target site likely accounts for the reduced MIC values observed in combination therapy. Importantly, this finding addresses a previously underexplored therapeutic combination involving eugenol and cefixime against resistant *P. aeruginosa* strains (19). Similar potentiation effects have been described for natural compounds such as cinnamaldehyde, which enhance aminoglycoside efficacy by interfering with quorum sensing (QS) pathways and suppressing virulence factor expression (20).

Beyond antimicrobial synergy, sub-inhibitory eugenol treatment (MIC/2) significantly reduced swimming (44-57%), swarming (43-46%), and twitching (39-64%) motility across the tested isolates ($P < 0.05$). While these reductions in motility are notable in vitro phenotypic observations, motility inhibition does not necessarily imply or demonstrate reduced in vivo invasion, host dissemination, or biofilm formation, as those complex pathogenic phenotypes were not directly evaluated (21).

Furthermore, because no pharmacokinetic, toxicological, longitudinal resistance-monitoring, or in vivo efficacy experiments were performed, claims that eugenol will reduce clinical dosage requirements, minimize toxicity, or prevent resistance emergence must be strictly avoided (22).

Overall, this study provides preliminary in vitro laboratory evidence supporting future investigation rather than direct therapeutic implications. Future research must focus on rigorous molecular target validation, toxicity profiling, and in vivo model evaluations before any clinical relevance can be established. Furthermore, while eugenol holds Generally Recognized as Safe (GRAS) status, its clinical translation is significantly constrained by poor systemic bioavailability and dose-dependent toxicity, rendering topical applications or advanced nano formulations promising strategies for future exploration (23).

CONCLUSION

In conclusion, this in vitro laboratory investigation demonstrated that eugenol exhibits intrinsic antibacterial activity against clinical *P. aeruginosa* isolates obtained from hospitalized animals, alongside favorable FICI interaction profiles when combined with

cefixime and gentamicin. Furthermore, sub-inhibitory eugenol concentrations significantly reduced swimming, swarming, and twitching motilities in the tested representative subset. However, because these findings are strictly restricted to limited in vitro laboratory observations on a small convenience sample of veterinary isolates—without dosage optimization, pharmacokinetic evaluations, toxicity testing, molecular mechanism validation, or in vivo efficacy trials—they do not provide direct therapeutic implications or establish a broadly universal clinical pattern. Future research must focus on rigorous molecular target validation, safety profiling, and in vivo model evaluations before any clinical relevance or adjuvant application can be established.

This study has several limitations. First, the small convenience sample of nine clinical isolates from a single veterinary institution (Small Animal Hospital, University of Tehran) may introduce selection bias and limits the generalizability of the findings. Second, detailed synergy and motility assays were restricted to three representative isolates (P3, P6, and P9). The study also did not include molecular characterization of resistance or virulence determinants, direct assessment of membrane permeability, quorum-sensing or gene-expression analyses, biofilm quantification, time-kill assays, toxicity testing, pharmacokinetic evaluation, or in vivo experiments. In addition, in vitro motility findings cannot establish clinical relevance or predict bacterial invasion or dissemination in vivo. Finally, the present findings do not provide evidence that eugenol can prevent resistance emergence, reduce clinical antibiotic doses, or provide therapeutic benefit. These limitations should be considered when interpreting the preliminary in vitro findings and highlight the need for further studies using larger and more diverse isolate collections and appropriate mechanistic and in vivo models.

REFERENCES

1. Hemaiswarya S, Doble M. Synergistic interaction of eugenol with antibiotics against Gram negative bacteria. *Phytomedicine* 2009; 16: 997-1005.
2. Shaw E, Wuest WM. Virulence attenuating combination therapy: A potential multi-target synergy approach to treat *Pseudomonas aeruginosa* infections in cystic fibrosis patients. *RSC Med Chem* 2020; 11: 358-369.
3. Kong J, Wang Y, Yao Z, Lin Y, Zhang Y, Han Y, et

- al. Eugenol works synergistically with colistin against colistin-resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* isolates by enhancing membrane permeability. *Microbiol Spectr* 2023; 11(5): e0366622.
4. Lashaki SB, Moulavi P, Ashrafi F, Sharifi A, Asadi S. Imipenem/Cilastatin encapsulation in UIO-66-NH₂ carrier as a new strategy for combating imipenem-resistant *Pseudomonas aeruginosa* isolates. *J Glob Antimicrob Resist* 2025; 42: 15-27.
5. Soltani R, Fazeli H, Najafi RB, Jelokhanian A. Evaluation of the synergistic effect of tomatidine with several antibiotics against standard and clinical isolates of *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli*. *Iran J Pharm Res* 2017; 16: 290-296.
6. Vitkauskienė A, Skrodenienė E, Dambrauskienė A, Macas A, Sakalauskas R. *Pseudomonas aeruginosa* bacteremia: resistance to antibiotics, risk factors, and patient mortality. *Medicina (Kaunas)* 2010; 46: 490-495.
7. Aloush V, Navon-Venezia S, Seigman-Igra Y, Cabili S, Carmeli Y. Multidrug-resistant *Pseudomonas aeruginosa*: risk factors and clinical impact. *Antimicrob Agents Chemother* 2006; 50: 43-48.
8. Asmare Z, Reta MA, Gashaw Y, Getachew E, Sisay A, Gashaw M, et al. Antimicrobial resistance profile of *Pseudomonas aeruginosa* clinical isolates from healthcare-associated infections in Ethiopia: A systematic review and meta-analysis. *PLoS One* 2024; 19(8): e0308946.
9. Sharifi A, Mohammadzadeh A, Zahraei Salehi T, Mahmoodi P. Antibacterial, antibiofilm and quorum sensing effects of *Thymus daenensis* and *Satureja hortensis* essential oils against *Staphylococcus aureus* isolates. *J Appl Microbiol* 2018; 124: 379-388.
10. Sharifi A, Mohammadzadeh A, Salehi TZ, Mahmoodi P, Nourian A. *Cuminum cyminum* L. essential oil: A promising antibacterial and antivirulence agent against multidrug-resistant *Staphylococcus aureus*. *Front Microbiol* 2021; 12: 667833.
11. Kothari A, Kherdekar R, Mago V, Uniyal M, Mamgain G, Kalia RB, et al. Age of Antibiotic Resistance in MDR/XDR Clinical Pathogen of *Pseudomonas aeruginosa*. *Pharmaceuticals (Basel)* 2023; 16: 1230.
12. Ulanowska M, Olas B. Biological Properties and Prospects for the Application of Eugenol-A Review. *Int J Mol Sci* 2021; 22: 3671.
13. Lady J, Nurcahyanti ADR, Tjoa E. Synergistic Effect and Time-Kill Evaluation of Eugenol Combined with Cefotaxime Against *Staphylococcus aureus*. *Curr Microbiol* 2023; 80: 244.
14. Eliasi UL, Sebola D, Oguttu JW, Qekwana DN. Antimicrobial resistance patterns of *Pseudomonas aeruginosa* isolated from canine clinical cases at a veterinary academic hospital in South Africa. *J S Afr Vet Assoc* 2020; 91(0): e1-e6.
15. Vakili N, Ashengroph M, Sharifi A, Zorab MM. Eco-friendly synthesis of copper nanoparticles by using *Ralstonia* sp. and their antibacterial, anti-biofilm, and antivirulence activities. *Biochem Biophys Rep* 2025; 42: 101978.
16. Talà L, Fineberg A, Kukura P, Persat A. *Pseudomonas aeruginosa* orchestrates twitching motility by sequential control of type IV pili movements. *Nat Microbiol* 2019; 4: 774-780.
17. Achraf A, Fatima Ezzahra M, Fatima Zahra M, Abdoullah B, Nour-Eddine C, Abdelhakim E, Jamal JE, et al. Antibacterial Potent of Acetylated and Non-Acetylated Clove Bud Essential Oils and Their Main Compounds. *Chem Biodivers* 2023; 20(4): e202201034.
18. Balasubramanian B, Xue J, Luo Y, Upadhyay A. Eugenol nanoemulsion reduces *Listeria monocytogenes* biofilm by modulating motility, quorum sensing, and biofilm architecture. *Front Sustain Food Syst* 2023; 7: 1272373.
19. Attaallah Ibrahim A, Kadhim Mohammed R. Synergistic Antimicrobial Activity of Eugenol in Combination with Fosfomycin to Combat *Escherichia coli* and Potential Effect on Plasmid-Mediated Fosfomycin Resistance Genes. *Chem Biodivers* 2023; 20(12): e202301567.
20. Chadha J, Harjai K, Chhibber S. Repurposing phytochemicals as anti-virulent agents to attenuate quorum sensing-regulated virulence factors and biofilm formation in *Pseudomonas aeruginosa*. *Microb Biotechnol* 2022; 15: 1695-1718.
21. Huber P, Basso P, Reboud E, Attrée I. *Pseudomonas aeruginosa* renews its virulence factors. *Environ Microbiol Rep* 2016; 8: 564-571.
22. Sultan M, Arya R, Kim KK. Roles of Two-Component Systems in *Pseudomonas aeruginosa* Virulence. *Int J Mol Sci* 2021; 22: 12152.
23. Abdelghany SM, Quinn DJ, Ingram RJ, Gilmore BF, Donnelly RF, Taggart CC, et al. Gentamicin-loaded nanoparticles show improved antimicrobial effects towards *Pseudomonas aeruginosa* infection. *Int J Nanomedicine* 2012; 7: 4053-4063.