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Antibacterial and antibiofilm effects of α-pinene on carbapenem-resistant *Klebsiella pneumoniae*: modulation of adhesion-related virulence genes

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Abstract

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is a major cause of healthcare-associated infections with high mortality, largely due to biofilm formation and expression of adhesion-associated genes such as *mrkA*, *mrkD*, and *fimA*. Natural monoterpenes such as α-pinene (PIN) have emerged as potential alternative antimicrobial agents. This study evaluated the antibacterial and antibiofilm effects of PIN against CRKP clinical isolates and investigated its impact on biofilm-related gene expression. CRKP isolates were identified from clinical specimens and screened for *mrkA*, *mrkD*, and *fimA*. The minimum inhibitory concentration (MIC) of PIN was determined using broth microdilution. Time-kill kinetics were assessed at subMIC levels. Antibiofilm activity was evaluated using the crystal violet assay. Gene expression changes following subMIC exposure were analyzed via quantitative real-time PCR (qRT-PCR). Among 89 *K. pneumoniae* isolates, 23 (25.8%) were carbapenem-resistant. MIC values of PIN ranged from 125–250 µg/ml. SubMIC concentrations caused minimal direct bactericidal activity but significantly reduced biofilm biomass ($p < 0.001$). PIN significantly downregulated *mrkD* in most isolates, while *mrkA* and *fimA* showed isolate-dependent reductions ($p < 0.05$). α-Pinene demonstrated significant antibiofilm activity against CRKP, primarily through modulation of fimbrial adhesion gene expression rather than strong bactericidal action. These findings support its potential as an antivirulence adjunctive therapy.

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Introduction

The escalating prevalence of antimicrobial resistance represents one of the most critical challenges in the treatment of infectious diseases worldwide. Among resistant pathogens, carbapenem-resistant enterobacteriaceae-particularly *Klebsiella pneumoniae*-have emerged as major causes of morbidity and mortality in healthcare-associated infections. Infections such as bacteremia and pneumonia caused by carbapenemase-producing *K. pneumoniae* are associated with alarmingly high fatality rates, reported to range between 30% and 70%. Recent surveillance studies further indicate a growing proportion of *K. pneumoniae* isolates exhibiting multidrug-resistant phenotypes (DeLeo *et al*., 2014). The increasing burden of these infections is largely attributed to the organism's remarkable ability to spread within intensive care environments and its rapid acquisition of resistance determinants. Consequently, elevated mortality linked to *K. pneumoniae* infections is often a result of delayed or inappropriate antimicrobial therapy (Struve *et al*., 2008).

One of the key factors contributing to the persistence and severity of *K. pneumoniae* infections is its capacity to form biofilms. Biofilm-associated growth provides a protective niche that enhances bacterial survival, promotes virulence, and limits the effectiveness of antimicrobial agents (Balasubramanian *et al*., 2017). Biofilm development is a dynamic, multistep process encompassing initial attachment, structural maturation, and eventual dispersal. Adhesion to host tissues and abiotic surfaces is mediated by surface-associated virulence factors, particularly fimbrial structures. In *K. pneumoniae*, both type 1 and type 3 fimbriae play crucial roles in colonization and biofilm formation (Podschun and Ullmann, 1998).

Type 1 fimbriae are among the most prominent adhesive organelles expressed by *K. pneumoniae* and are primarily composed of repeating subunits of the *fimA* pilin protein, forming rigid filamentous structures. In parallel, type 3 fimbriae represent another major adhesion system and are predominantly assembled from *mrkA* subunits. These fimbriae are widely produced among *Klebsiella* species and are especially important for binding to extracellular matrix components. The adhesive specificity of type 3 fimbriae is largely mediated by the *mrkD* adhesin, which facilitates interaction with collagen-containing surfaces (Sebghati *et al*., 1998). Experimental evidence has demonstrated that the presence of distinct *mrk* gene clusters enables *K. pneumoniae* to adhere efficiently to extracellular matrix molecules, whereas isolates lacking functional *mrkA* and *mrkD* proteins show markedly reduced binding ability (Ghasemian *et al*., 2019).

In recent years, increasing attention has been directed toward plant-derived compounds as alternative or adjunctive antimicrobial agents. Essential oils obtained from various plant species are rich in pinenes and other bicyclic terpenes (Rivas da Silva *et al*., 2012). Among these compounds, α-pinene (PIN), a monoterpene hydrocarbon with the molecular formula $C_{10}H_{16}$, exists as one of two biologically active isomers. PIN is a common constituent of many essential oils, and multiple studies have explored its antibacterial activity as well as its potential role in modulating antimicrobial resistance (Borges *et al*., 2022). Notably, Griffiths *et al*. (1987) reported that PIN effectively inhibited the growth of several bacterial species, including *Nocardia* spp., *Pseudomonas* spp., and *Pseudomonas fluorescens*, highlighting its promise as a bioactive antimicrobial compound.

Given the reduced efficacy of conventional antibiotics against multidrug-resistant strains, the exploration of natural bioactive molecules has gained renewed attention. Prior studies have demonstrated its inhibitory activity against several bacterial species, suggesting its promise as an alternative or adjunctive therapeutic approach. Despite this, limited data are available regarding the interaction of PIN with virulence-related genes such as *mrkA* and *mrkD* in *K. pneumoniae*, particularly within resistant clinical isolates. The present work focuses on investigating the antibacterial and antibiofilm activities of PIN against carbapenem-resistant *K. pneumoniae* (CRKP) clinical isolates.

Materials and Methods

Bacterial isolation and antibiotic susceptibility

In this descriptive study, 400 clinical specimens were collected from Tehran hospital laboratories and the hospital biobank. Samples were cultured on blood agar plates and incubated at 37°C for 24 hours. Bacterial identification of *K. pneumoniae*

isolates was initially performed using conventional microbiological and biochemical tests, followed by 16S rRNA gene amplification for molecular confirmation. Antimicrobial susceptibility testing was carried out using the disc diffusion method according to CLSI guidelines (CLSI, 2023). *K. pneumoniae* isolates were tested against imipenem, meropenem, and ertapenem. Reference strain of *K. pneumoniae* BAA-1706 served as positive control. Genomic DNA was also extracted using the CinnaPure DNA Kit (CinnaGene, Iran), and DNA purity was verified spectrophotometrically at 260 nm. Detection of biofilm-associated genes was carried out via PCR, targeting *mrkA*, *mrkD*, and *fimA* in CRKP strains.

Antibacterial activity of pin

First PIN was purchased from Merck, Germany Co. The antibacterial activity of PIN against CRKP was determined using the broth microdilution method following CLSI guidelines (Bazargan *et al*., 2024). Serial two-fold dilutions of PIN (0.97–250 µg/ml) were prepared in Mueller–Hinton broth within 96-well microplates. Each well contained 100 µl of PIN solution, 95 µl of broth, and 5 µl of bacterial suspension standardized to 5×10^5 CFU/ml. Plates were incubated at 37°C for 24 hours. The minimum inhibitory concentration (MIC) was defined as the lowest concentration preventing visible growth.

The antibacterial efficacy of PIN against MRSA and CRKP pathogens was assessed over 48 hours using the Time-Kill assay method (Bazargan *et al*., 2024). In summary, 100 µl of samples at sublethal doses (subMIC) were introduced to 96-well microtiter plates that contained 100 µl of each bacterial culture. Following incubation at 37°C, the optical density at OD 600 nm was assessed at 8, 16, 24, 32, 40, and 48 hours using a microplate reader.

Antibiofilm activity of PIN

The antibiofilm effectiveness of PIN against CRKP biofilms was evaluated using a microtiter plate crystal violet (CV) assay (Barakat *et al*., 2014). Biofilm formation was assessed using the 96-well microtiter plate method. Briefly, 180 µl of Mueller–Hinton broth (MHB) and 20 µl of bacterial suspension (OD_{600} = 0.6) were added to each well and incubated at 37°C for 48 hours at 120 rpm to allow biofilm development. Subsequently, PIN at ½ MIC and fresh MHB (negative control) were added, followed by incubation at 37°C for 24 hours. After incubation, wells were gently washed with PBS to remove non-adherent cells and air-dried. Biofilms were fixed with 2% sodium acetate, stained with 0.1% crystal violet for 30 minutes in the dark, and excess stain was removed by washing. The bound dye was solubilized with ethanol, and biofilm biomass was quantified by measuring absorbance at 570 nm.

Analysis of biofilm gene expression

The expression levels of biofilm-associated genes (*mrkA, mrkD*, and *fimA*) in CRKP isolates were evaluated using quantitative real-time PCR (qRT-PCR) with specific primers (Table 1). Following 24 hours exposure to subMIC concentrations of PIN, total RNA was extracted using the RNX-Plus kit (SinaGene, Iran) and reverse-transcribed into cDNA with the YTA cDNA synthesis kit (Yekta Tajhiz, Iran). qRT-PCR reactions (15 µl) were performed on a Corbett 5-Plex HRM system using SYBR Green chemistry, with 16S rRNA as the internal reference gene. Thermal cycling conditions consisted of 95°C for 5 minutes, followed by 40 cycles of 95°C for 20 seconds, 58°C for 40 seconds, and 72°C for 40 seconds, with melt-curve analysis at 53–95°C. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1. List of primer sequences in the study.

Genes	Primer sequences (5' to 3')	Amplification product (bp)
mrkA	TGAACTGGACTGGCGGTAAC	168
	CTTTAGGCTGGGTGGCATCA	
fimA	GCTCTGGCCGATACCGTTAC	120
	AACCTGGCCTAACTGAACGG	
mrkD	CCACCAACTATTCCCTCGAA	226
	TAGCCCTGTTGTTTGCTGGT	
16S rRNA	ATCAGAGCGCGGATCTTTGCCG	155
	ATCAGAGCGCGGATCTTTGCCG	

Statistical analysis

The statistical analysis of this study was conducted utilizing SPSS version 20 and GraphPad Prism V.8 software. The results were evaluated through one-way analysis of variance (One-way ANOVA), and the differential expression of target genes between control and treated samples was determined using Tukey's HSD post-hoc test. The

results were expressed as mean ± standard deviation (SD), with a p value of less than 0.05 deemed significant.

Results

Bacterial identification and antibiotic resistance profile

Among the 400 clinical specimens, 89 isolates (22.25%) were identified as *K. pneumoniae*. Out of the 89 *K. pneumoniae* isolates, 23 strains (25.84%) exhibited simultaneous resistance to imipenem, meropenem, and ertapenem and were therefore classified as carbapenem-resistant *K. pneumoniae* (CRKP). Molecular analysis showed that 18 CRKP isolates (78%) carried the *mrkA* gene, 17 isolates (73.9%) harbored *mrkD*, and 14 isolates (60%) were positive for *fimA*. Based on PCR confirmation, 12 CRKP isolates (52.1%) possessing all three adhesion-related genes were selected for subsequent experiments.

Antibacterial activity of PIN

The antibacterial effect of PIN against the selected CRKP isolates was evaluated using the broth microdilution method. The results demonstrated that PIN inhibited bacterial growth in a concentration-dependent manner. The MIC values of PIN against CRKP strains ranged from 125 to 250/ml, indicating a measurable inhibitory activity of this compound against carbapenem-resistant isolates.

Table 2. MIC value of PIN against CRKP isolates.

Strain No.	α-PIN
1	125
2	125
3	250
6	125
8	250
11	250
13	125
14	125
16	125
17	125
21	250
22	125
Standard	125

Fig. 1 illustrated the time–kill kinetics of CRKP isolates exposed to various subMIC concentrations of PIN. Treatment with subMIC levels of PIN resulted in only a slight reduction in the bacterial load after 72 hours of incubation compared with the untreated control group.

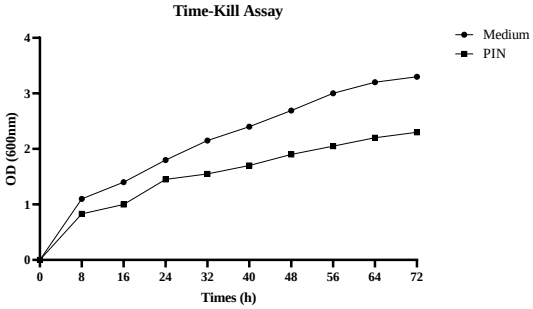


Fig. 1. Time–kill kinetics of CRKP isolates exposed to various subMIC concentrations of PIN compared to medium (untreated group).

Antibiofilm activity of PIN

The antibiofilm activity of PIN against CRKP isolates was evaluated using the crystal violet assay. As shown in Fig. 2, all tested CRKP isolates exhibited strong biofilm-forming ability prior to treatment, with optical density (OD_{570}) values ranging approximately from 0.75 to 0.85. Following exposure to PIN at subMIC concentrations, a significant reduction in biofilm biomass was observed across all CRKP isolates compared to the untreated controls ($p < 0.001$). The OD_{570} values decreased to approximately 0.58–0.68 after PIN treatment, indicating a marked inhibition of biofilm formation.

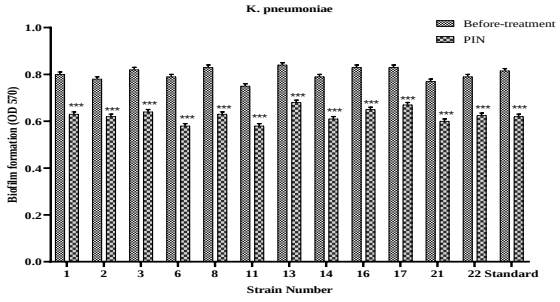


Fig. 2. Antibiofilm study of PIN against CRKP strains using CV test. ***: $p < 0.001$.

mrkA, *mrkD*, and *fimA* genes expression in treated CRKP isolates

To determine whether PIN inhibits the target gene expression, the PIN was administered to CRKP strains. According to Fig. 3A, PIN caused a significant reduction in the expression of *mrkA*

gene in isolates NO. 8, 11, 21, 22, and standard isolate ($p < 0.05$) and did not affect other isolates compared to the control. According to Fig. 3B, treatment with PIN resulted in a significant downregulation of *mrkD* gene expression in isolates No. 2, 6, 8, 11, 13, 17, 21, 22, and standard isolate ($p < 0.05$) compared to the untreated control. In contrast, no statistically significant changes in *mrkD* expression were observed in the remaining isolates following PIN exposure. As shown in Fig. 3C, PIN also significantly reduced the expression of the biofilm-associated *fimA* gene in isolates No. 11, 13, 22, and standard isolate ($p < 0.05$), whereas *fimA* expression in the other CRKP isolates remained unaffected relative to the control group, indicating isolate-dependent responsiveness to PIN.

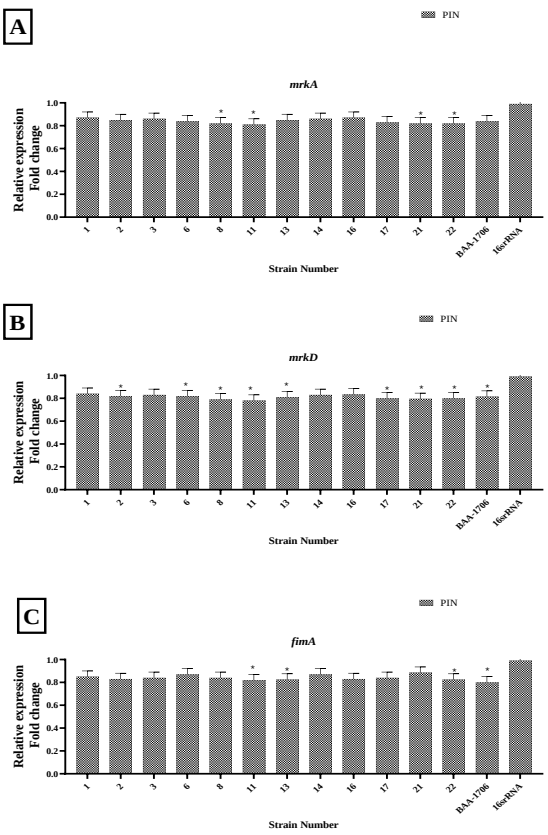


Fig. 3. The evaluation of PIN effects on the expression of (A) *mrkA*, (B) *mrkD*, and (C) *fimA* mRNA in CRKP isolates using real-time PCR. *: $p < 0.05$.

Discussion

The present study demonstrated a considerable burden of carbapenem resistance among clinical isolates of *K. pneumoniae*, with 25.84% of confirmed isolates classified as CRKP. This finding is consistent with global surveillance data indicating rapid dissemination of high-risk carbapenem-resistant clones, particularly ST258 lineages, which have been strongly associated with hospital outbreaks and elevated mortality rates *K. pneumoniae* (DeLeo *et al*., 2014). Mortality associated with CRKP infections, especially in intensive care settings, has been reported to range between 30-70%, largely due to delayed effective therapy and enhanced virulence capacity.

A key virulence determinant of CRKP is its ability to form biofilms. In this study, a high prevalence of adhesion-associated genes (*mrkA* 78%, *mrkD* 73.9%, *fimA* 60%) was observed. Types 1 and 3 fimbriae, encoded by *fimA* and *mrkA/mrkD*, respectively, play central roles in colonization and persistence. Type 3 fimbriae, particularly through the *mrkD* adhesin, mediate binding to collagen and abiotic surfaces, facilitating biofilm establishment on medical devices and host tissues (Podschun and Ullmann, 1998, Sebghati *et al.,* 1998). Likewise, phase-variable expression of type 1 fimbriae contributes to epithelial adhesion and infection dynamics (Struve *et al.,* 2008). Our molecular findings align with previous clinical observations demonstrating a strong correlation between the presence of the *mrk* operon and enhanced biofilm-forming capacity (Struve *et al*., 2008), reinforcing the importance of targeting adhesion mechanisms in CRKP management.

Regarding antibacterial activity, PIN exhibited moderate inhibitory effects with MIC values ranging from 125–250 µg/ml. PIN is a bicyclic monoterpene hydrocarbon commonly present in essential oils and widely investigated for antimicrobial properties (Rivas da Silva *et al*., 2012). Comparable MIC ranges have been reported against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Campylobacter jejuni*, suggesting broad-spectrum but moderate potency (Sá *et al*., 2014, Kovač *et al*., 2015, Bomfim de Barros *et al*., 2023). However, time–kill analysis in our study revealed only mild suppression of bacterial growth at subMIC concentrations over 72 hours, indicating limited bactericidal activity. This observation is consistent with previous reports that monoterpenes typically exert membrane-perturbing and bacteriostatic effects rather than rapid bacteriolysis (Mahizan *et al*., 2019).

Notably, the most prominent effect of PIN in our study was its significant antibiofilm activity.

Crystal violet assays demonstrated marked reduction in biofilm biomass ($p < 0.001$) following subMIC exposure, despite limited planktonic growth inhibition. This finding underscores an antivirulence mechanism distinct from direct bactericidal action (Salinas *et al.,* 2022, Kashi *et al.,* 2024, Wei *et al.,* 2025, Roustaye Gourabi *et al.,* 2026). Gene expression analysis further revealed significant downregulation of *mrkD* in the majority of isolates, partial suppression of *mrkA*, and selective reduction of *fimA*. Since *mrkD* is essential for collagen binding and early biofilm establishment, its downregulation likely compromises stable surface attachment. Experimental disruption of *mrkD* has previously been shown to significantly impair biofilm formation (Rahmati *et al.,* 2022, Karbalaeiheidar and Ashrafi, 2023). Similarly, altered *fimA* expression may interfere with type 1 fimbrial assembly and epithelial adhesion (Cadavid and Echeverri, 2019, Makhrmash *et al.,* 2022, Omer *et al.,* 2022). The isolate-dependent responsiveness observed in our results suggests heterogeneity in regulatory networks among CRKP strains.

The mechanistic basis of PIN activity can be interpreted through multiple complementary pathways. First, its lipophilic structure enables integration into bacterial lipid bilayers, increasing membrane permeability and altering membrane fluidity (Rivas da Silva *et al*., 2012). This membrane perturbation can compromise nutrient transport and energy metabolism. Second, subMIC concentrations appear to modulate transcriptional regulation of fimbrial operons (*mrk* and *fim* clusters), thereby interfering with adhesion and biofilm maturation (Karbalaeiheidar and Ashrafi, 2023). Third, monoterpenes have been associated with induction of oxidative stress via intracellular reactive oxygen species accumulation, contributing to metabolic imbalance. Finally, accumulating evidence suggests that plant-derived terpenes may interfere with quorum-sensing pathways, attenuating coordinated biofilm development and virulence gene expression (Melkina *et al.,* 2021, Karbalaeiheidar and Ashrafi, 2023).

The present study demonstrated that PIN exerts measurable antibacterial activity against CRKP clinical isolates, with MIC values ranging from 125–250 µg/ml. Although its direct bactericidal activity was limited at subMIC concentrations, PIN significantly inhibited biofilm formation and reduced biofilm biomass across tested isolates. Importantly, molecular analysis revealed downregulation of key adhesion-associated genes, particularly *mrkD*, along with partial suppression of *mrkA* and selective reduction of *fimA* expression. These findings indicate that PIN primarily functions as an antivirulence agent, impairing bacterial adhesion and biofilm establishment rather than exerting strong bactericidal effects. Given the critical role of biofilms and fimbrial adhesins in CRKP persistence and therapeutic failure, targeting these pathways represents a promising complementary strategy. PIN may therefore serve as a potential adjunctive compound in combination therapies aimed at controlling multidrug-resistant *K. pneumoniae* infections. Future studies should focus on evaluating synergistic interactions with carbapenems, elucidating quorum-sensing modulation mechanisms, and validating efficacy in *in vivo* infection models.

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Conflict of Interest

The author declares that there is no conflict of interest regarding the publication of this work.

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