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Antibacterial activity of zerumbone and similar compounds in Klebsiella pneumoniae biofilms

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Abstract

Strategies aimed at reducing extracellular polymeric matrix (EPM) components and disrupting biofilm structural integrity have emerged as a major focus of investigation. In this context, the present study was conducted to assess the feasibility of using zerumbone and chemically related compounds for the inactivation of *Klebsiella pneumoniae* biofilms. To this end, compounds structurally related to zerumbone were identified using CAS Finder Software. The cytotoxicity of zerumbone and its structurally related derivatives was evaluated through assays in HeLa cells to assess biocompatibility and safety profiles. In parallel, the minimum bactericidal concentration (MBC) of zerumbone and related compounds was determined against planktonic cultures of *K. pneumoniae*. Furthermore, the antimicrobial efficacy of these compounds against *K. pneumoniae* biofilms was investigated by analyzing viable cell counts and biofilm biomass following compound exposure. The results demonstrated that zerumbone, tropone, purpurogallin and dicinnamalacetone exhibited a half-maximal inhibitory concentration (IC_{50}) of 100 μM in HeLa cells, indicating a moderate cytotoxic effect under the experimental conditions. α-humulene exbibits an IC_{50} of 81 μM in HeLa cells. The IC_{50} of dibenzylideneacetone was 12 μM. Zingerone and 6-gingerol were not cytotoxic in the range of concentrations tested. The results demonstrated that after 24 h of incubation of planktonic cultures with zerumbone, zingerone, 6-gingerol and α-humulene at higher concentrations a reduction was observed in *K. pneumoniae*. These compounds showed moderate activity against *K. pneumoniae* biofilms, with efficacy varying by molecule and concentration of compounds. Zerumbone and α-humulene were the most effective, achieving viability reductions of up to ~ 1.4 and ~ 1.86 log_{10}, respectively, while zingerone and 6-gingerol exhibited reductions of up to ~ 1.14 and ~ 1.47 at 100 μM, respectively. Ciprofloxacin showed no activity against the biofilms. Together, these findings indicate that zerumbone, α-humulene, zingerone, 6-gingerol hold promise as complementary strategies for weakening and inactivating *K. pneumoniae* biofilms.

Introduction

Bacterial infections have become a major global health concern due to the rapid emergence and spread of antimicrobial resistance, which compromises the effectiveness of current antibiotics and increases morbidity, mortality, and healthcare costs. This scenario underscores the urgent need to develop novel antimicrobial strategies capable of overcoming multidrug-resistant pathogens (Ho et al. 2025). *Klebsiella pneumoniae* is a Gram-negative opportunistic pathogen facultatively anaerobic rod-shaped bacterium and a member of the clinically important ESKAPE group, which includes pathogens known for their ability to evade conventional antimicrobial therapies (Chang et al. 2021). Carbapenem-resistant *K. pneumoniae* is classified by the World Health Organization (WHO) as a critical priority pathogen, and severe infections caused by these strains have been associated with mortality rates of up to 70% (Martin et al. 2016).

In humans, *K. pneumoniae* predominantly colonizes the gastrointestinal tract and, to a lesser extent, the nasopharynx, serving as a potential source for dissemination into the bloodstream and other tissues, where it may establish severe infections (Chang et al. 2021). *K. pneumoniae* can colonize the oral cavity,

particularly removable dental prostheses, where it may persist within biofilm communities. Its presence on prosthetic surfaces and its ability to form biofilms support the oral cavity as a potential reservoir for this pathogen (Leoney et al. 2020; Lim et al. 2024). In addition, this bacterium is frequently implicated in hospital-acquired infections, particularly those associated with indwelling medical devices such as urinary and intravascular catheters, prosthetic joints, and vascular grafts (Holt et al. 2015; Zdarska et al. 2026).

The capacity of *K. pneumoniae* to form biofilms on these surfaces contributes significantly to persistent infections and increased resistance to conventional antibiotics (Guerra et al. 2022). Biofilms are structured communities of microbial cells encased in a self-produced extracellular polymeric matrix (EPM), which provides mechanical stability, mediates cell–cell and cell–surface interactions, and reduces susceptibility to antimicrobial treatments (Guerra et al. 2022). Key constituents of the EPM, including exopolysaccharides, extracellular DNA (eDNA), and proteins, play a critical role in maintaining biofilm integrity and protecting the bacterial community from antimicrobial penetration (Wang et al. 2020). The formation of *K. pneumoniae* biofilms, regulated in part by quorum sensing (QS)-mediated cell-to-cell communication, and their increased tolerance to antimicrobial agents pose substantial challenges for infection management in clinical settings. QS contributes to the coordinated regulation of bacterial behaviors associated with biofilm development, persistence, and virulence. Consequently, persistent biofilm-associated infections increase patient morbidity, prolong hospital stays, and elevate healthcare costs (Guerra et al. 2022; Wang et al. 2020). So, strategies aimed at disrupting the extracellular matrix and weakening biofilm structure are of great interest, as they can enhance microbial inactivation and improve therapeutic outcomes. In this context, natural products have emerged as a promising source of bioactive compounds capable of targeting biofilms (Saifi et al. 2024). Among these, zerumbone (2,6,9,9-tetramethyl-2,6,10-cycloundecatrien-1-one,), a cyclic sesquiterpene predominantly found in the essential oils of the rhizomes and leaves of *Zingiber zerumbet* (L.) Smith, has demonstrated remarkable pharmacological properties, including antioxidant, anti-inflammatory, anticancer, and antimicrobial activities (Ibáñez et al. 2023; Dev 1956, 1960). The chemical composition of the essential oil has been investigated and several sesquiterpenes with medicinal significance were identified including humulene, zerumbone (ZER), epoxyhumulene, caryophyllene, epoxycaryophyllene, α-epoxyhumulene, 4-epoxyhumulene, diepoxyhumulene, cardinene, X-(+)-ar-curcumene, dihydro-Ψ-photozerumbone I, dihydro-Ψ-photozerumbone, Ψ-photozerumbone, and humulenol (Dev 1956, 1960).

Previous studies have demonstrated the antimicrobial activity of zerumbone against Gram-negative bacteria, such as *Escherichia coli* and *Helicobacter pylori*, and Gram-positive bacteria, including *Staphylococcus epidermidis* and *Staphylococcus aureus*. In addition to its antibacterial effects, zerumbone has also shown antifungal activity (Sidahmed et al. 2015; Moreira da Silva et al. 2018; Shin and Eom 2019; Abreu-Pereira et al. 2025; Abreu-Pereira et al. 2023). In addition, zerumbone can disrupt the extracellular matrix, reduce microbial viability, and interfere with cell morphology and membrane integrity in biofilms (Fadhel Abbas Albaayit et al. 2022).

Building on the promising antimicrobial activity of ZER, our proposal is to evaluate the potential use of ZER and structurally related compounds against *K. pneumoniae* biofilms. A key advantage of this approach is the validation of an alternative antimicrobial compound against this Gram-negative pathogen, supporting the development of non-antibiotic strategies to combat bacterial infections and antimicrobial resistance. Therefore, this study aimed to investigate the antimicrobial effects of zerumbone and related compounds against *K. pneumoniae* biofilms, addressing an important gap in the literature, as no previous studies have evaluated these compounds for the inactivation of *K. pneumoniae* biofilms.

Materials and methods

Identification of compounds structurally similar to zerumbone with CAS Finder Software

Phytochemical analysis showed that the essential oil of *Zingiber zerumbet* L. Smith (Zingiberaceae) was composed of a complex mixture of various terpenoids. Spectroscopic studies indicated the presence of several medicinally relevant sesquiterpenes, such as humulene, zerumbone, epoxy-humulene, caryophyllene, epoxy-caryophyllene, α-epoxy-humulene, 4-epoxy-humulene, diepoxy-humulene, cardinene, X-(+)-ar-curcumene, dihydro-Ψ-photozerumbone I, dihydro-Ψ-photozerumbone, Ψ-photozerumbone, and humulenol (Dev 1956, 1960). Structural similarity searches were conducted using CAS SciFinder (American Chemical Society), accessed in September of 2025. After accessing the platform, the structure of ZER (Fig. 1) was queried by entering its CAS Registry Number (CAS: 471-05-6) or by using the structure editor to import or draw its chemical structure.

The “Structure Search” tool was then selected, followed by the “Similarity Search” option, which enabled the identification of compounds exhibiting defined structural similarity thresholds relative to zerumbone. The resulting list of candidate molecules was subsequently filtered according to structural features and physicochemical criteria relevant to the study.

Assessment of the cytotoxicity of ZER and structurally related compounds in HeLa cells.

For the determination of the biocompatibility and potential safety for further applications of the compounds, the cytotoxicity was determined in HeLa cells (ATCC® CCL-2™). The cells seeded at a density of 5000 cells per well in 96-well plates, using 100 µL of complete medium per well [(DMEM, high glucose; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C]. The plates were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2 to allow cell adhesion and growth. After incubation, when the cell confluence reached approximately 75%, the medium was removed and replaced with 90 µLof fresh complete medium. Subsequently, 10 µL of each tested solution (10× stock solutions) was added to the wells, resulting in the desired final concentrations (range 100µM until 0.20 µM of each compound). The plate was incubated for 24 h at 37°C. Control groups were included in all experiments. For the negative

control (no expected cytotoxic effect), cells were incubated with complete culture medium only. For the positive control (inducing cytotoxicity), DMSO was used at a final concentration of 50%. The cytotoxicity of ZER and structurally related compounds was evaluated over a concentration range of 0.20 to 100 µM.

Cell viability was evaluated using the ATPlite™ Luminescence Assay System (Revvity, Waltham, MA, USA; Cat. No. 6016941), according to the manufacturer's instructions. Briefly, 50 µL of cell lysis solution was added to each well, avoiding bubble formation and changing pipette tips between rows. The plate was shaken for 5 min at 250 rpm, followed by the addition of 50 µL of substrate solution under the same conditions. After another 5 min of shaking, the plate was incubated for 10 min in the dark, and luminescence was measured [SpectraMax® microplate reader (Molecular Devices, San Jose, CA, USA)] by top reading with an integration time of 1000 ms. All assays were performed in triplicate on three independent occasions. The concentration–response data were analyzed by nonlinear regression using the Hill equation to estimate the half-maximal inhibitory concentration (IC_{50}), defined as the concentration required to reduce cell viability by 50% relative to the untreated control.

Determination of the minimum bactericidal concentration (MBC) of ZER and related compounds against planktonic cultures of K. pneumoniae.

To determine the bactericidal activity of zerumbone and structurally related compounds against planktonic culture of *K. pneumoniae*, the Minimal Bacterial Concentration (MBC) test was performed. This assay evaluates bactericidal effect by exposing a standardized inoculum of *K. pneumoniae* (5×10^5 CFU/mL) to a concentration range of each test compound (100 µM until 0.20 µM) for 24 hours, followed by recovery steps to assess surviving bacteria (Clinical and Laboratory Standards Institute 2024, 2026; EUCAST 2026). The MBC is defined as the lowest concentration of compound that results in bactericidal activity according to the chosen endpoint criteria (e.g., culture-negative or a specified ≥ 3 $\log_{10}$ reduction compared with control), following EUCAST/CLSI guidance (Clinical and Laboratory Standards Institute 2024, 2026; EUCAST 2026). After the exposure period, perform serial dilutions of the recovered cultures (10^{-6}) and plate onto Tryptic soy agar (TSA) [BD Difco™ (Becton, Dickinson and Company, Sparks, MD, USA] medium for colony recovery. Incubate the plates for 24 hours and determine surviving CFU/mL and calculate $\log_{10}$ reductions relative to untreated growth controls. Each condition was evaluated using independent biological replicates and technical replicates.

Evaluation of the antimicrobial activity of ZER and related compounds in K. pneumoniae biofilms.

Following the MBC assay, compounds and concentrations that demonstrated activity against planktonic *K. pneumoniae* cultures were subsequently evaluated against biofilms. This workflow was designed to prioritize compounds with both antimicrobial and antibiofilm potential for further characterization (Santos et al. 2020). Biofilms were established by inoculating 100 µL of the standardized bacteria (turbidity equivalent to the 0.5 McFarland standard, corresponding to approximately 1×10^8 CFU/mL) suspension into the wells of a 96-well microtiter plate, followed by the addition of 100 µL of fresh culture medium (Dan et al. 2023). Plates were incubated for 24 h at 37°C to allow biofilm formation. After

incubation, 100 µL of each test compound in solution (100, 50 and 25 µM) was added to the wells, and the plates were further incubated for 24 h under the same conditions. After the incubation, biofilms were detached, serially diluted (10^{-1} until 10^{-8}), and plated on TSA agar for CFU/mL determination.

Biofilm biomass was also quantified using the crystal violet (CV) staining assay. Biofilms were established and treated as described above (Silva et al. 2021). The medium was carefully removed, and non-adherent cells were eliminated by washing the wells three times with 200 µL of sterile phosphate-buffered saline (PBS). Biofilms were fixed by adding 200 µL of 96% ethanol for 15 min at room temperature. After discarding the ethanol and air-drying the plates, wells were stained with 200 µL of 0.1% (w/v) crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 15 min. Excess dye was removed, and wells were washed three times with Phosphate-Buffered Saline (PBS) until the wash solution was clear, followed by air-drying. To quantify biofilm biomass, 200 µL of 30% (v/v) acetic acid (Sigma-Aldrich, St. Louis, MO, USA) was added to each well to solubilize the bound crystal violet for 15 min. The resulting solution was transferred to a new microplate to avoid interference from residual staining on the wells, and absorbance was measured at 570 nm using a microplate reader (FLUOstar Omega, BMG LABTECH, Ortenberg, Germany). All assays were performed in triplicate on three independent occasions.

Data analysis

Data are presented as mean ± standard deviation (SD). Normality was assessed using the Shapiro–Wilk test, and homogeneity of variances was evaluated using Levene's test. Data were analyzed by analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. Statistical analyses were performed using SPSS software, with the significance level set at 5% ($p < 0.05$).

Results

Identification of compounds structurally similar to zerumbone with CAS Finder Software

The compounds analyzed share structural and/or electronic features that render them comparable to ZER, particularly through the presence of conjugated systems and electrophilic centers capable of similar interactions and reactivity (Fig. 2). Tropone (CAS 539-80-0) and purpurogallin (CAS 569-77-7) exhibit conjugated carbonyl-containing frameworks that confer analogous electronic and electrophilic properties, while dicinnamalacetone (CAS 622-21-9) and dibenzylideneacetone (DBA) (CAS 538-58-9) are characterized by an α,β-unsaturated system essential for Michael-acceptor behavior, mirroring the enone functionality of zerumbone. Zingerone (CAS 122-48-5) and [6]-gingerol (CAS 23513-14-6) combine a partially conjugated carbonyl group with exposed hydrophobic regions, producing a lipophilic–electrophilic profile akin to that of ZER. Finally, α-humulene (CAS 6753-98-6) shares the sesquiterpene scaffold and hydrophobic physicochemical traits, enabling steric and membrane interactions comparable to those of zerumbone.

Assessment of the cytotoxicity of ZER and structurally related compounds through in vitro cytotoxicity assays conducted in HeLa cells to determine their biocompatibility and potential safety for further applications.

The value of IC_{50} represents the concentration required to reduce cell viability by 50%, providing an important reference point for evaluating the compound cytotoxicity and guiding future dose-response investigations (n = 3). Cytotoxicity assays revealed that zerumbone, tropone, purpurogallin and dicinnamalacetone exhibited IC_{50} of 100 μM in HeLa cells, indicating a moderate cytotoxic effect under the experimental conditions. α-humulene exhibits an IC_{50} of 81μM in HeLa cells. The IC_{50} of dibenzylideneacetone was 12μM. Zingerone and 6-gingerol were not cytotoxic in the range of concentrations tested.

Determination of the minimum bactericidal concentration (MBC) of ZER and related compounds against planktonic cultures of K. pneumoniae.

The results demonstrated that after 24 h of incubation of planktonic cultures with different concentrations (100 to 0.2 μM) of the compounds zerumbone, tropone, purpurogallin, dicinnamalacetone, and dibenzylideneacetone, only zerumbone at concentrations of 100 and 50 μM produced an antimicrobial effect, resulting in approximately a 7-$\log_{10}$ reduction (Fig. 3). For the compounds zingerone, 6-gingerol, and α-humulene, the reductions were an average of 2.4 $\log_{10}$ (Fig. 3). Since only the compounds zerumbone, zingerone, 6-gingerol, and α-humulene produced reductions in planktonic cultures, only these compounds were used in the evaluation of *K. pneumoniae* biofilms.

Evaluation of the antimicrobial activity of ZER and related compounds in K. pneumoniae biofilms, focusing on viable colony counts and biofilm biomass after exposure to the compounds.

In *K. pneumoniae* biofilms, only the concentrations of 100 and 50 μM of zerumbone resulted in reductions of approximately 1.5 $\log_{10}$ compared to the control (biofilms only) (Fig. 4). For α-humulene, reductions of 1.86 and 1.72 $\log_{10}$ were observed at concentrations of 100 and 50 μM, respectively. For Zingerone, 100 μM concentration reduces *K. pneumoniae* viability by 1.14 log. The 6-gingerol promoted a reduction of 1.47 $\log_{10}$ at the concentration of 100 μM. Ciprofloxacin did not exhibit antimicrobial activity against the biofilms, as no reduction in viability was observed.

Treatment with zerumbone and structurally related compounds significantly reduced *K. pneumoniae* biofilm biomass in a concentration-dependent manner (Table 1). Among the evaluated compounds, α-humulene promotes biomass reductions of 58%, 55%, and 51% at 100, 50, and 25 μM, respectively. Zerumbone also reduced total biomass by up to 51% at 100 μM. In contrast, zingerone and 6-gingerol displayed comparatively lower biomass reductions of 41% and 34%, respectively in the highest concentration (100 μM).

Table 1
Biomass quantification (crystal violet assay) of *K. pneumoniae* biofilms after the incubation with the compounds

Group	**Absorbance (492nm)**	**Total Biomass (%)**	**Reduction (%)***
K. pneumoniae **(control group)**	0.713	100	-
Zerumbone 100 μM	0.349	49	51
Zerumbone 50 μM	0.386	54	46
Zerumbone 25 μM	0.399	56	44
α-humulene 100 μM	0.301	42	58
α-humulene 50 μM	0.325	45	55
α-humulene 25 μM	0.351	49	51
Zingerone 100 μM	0.421	59	41
Zingerone 50 μM	0.434	61	39
Zingerone 25 μM	0.447	63	37
6-gingerol 100 μM	0.471	66	34
6-gingerol 50 μM	0.487	68	32
6-gingerol 25 μM	0.499	70	30

*The percentage of reduction was calculated compared to the negative control group (biofilm only).

Discussion

Although antibiotics have been highly effective against bacterial infections, the global emergence and spread of multidrug-resistant (MDR) bacteria continue to compromise current antimicrobial therapies, highlighting the urgent need for novel therapeutic strategies with distinct mechanisms of action (Ho et al. 2025). In recent years, strategies aimed at disrupting EPS and weakening the biofilm architecture have garnered increasing attention (Chang et al. 2021). In this context, the present study investigated the potential of ZER and structurally related compounds to inactivate *K. pneumoniae* biofilms. To achieve this objective, the study was conducted through four sequential experimental phases: (I) identification of compounds structurally related to ZER using CAS Finder software; (II) evaluation of the cytotoxicity of ZER and its selected analogues in HeLa cells to establish their biocompatibility; (III) determination of the minimum bactericidal concentration (MBC) against planktonic *K. pneumoniae*; and (IV) investigation of the antimicrobial efficacy of ZER and its analogues against established *K. pneumoniae* biofilms through quantification of viable bacterial cells and total biofilm biomass after treatment.

Identifying compounds with electrophilic and physicochemical comparable features to those of zerumbone may offer more accessible alternatives while preserving the promising biological activities demonstrated by ZER. In this study, several candidate compounds were identified based on shared structural and functional features with ZER. Tropone (CAS 539-80-0) and purpurogallin (CAS 569-77-7) possess conjugated carbonyl-containing frameworks that support comparable electronic reactivity; dicinnamalacetone (CAS 622-21-9) and dibenzylideneacetone (CAS 538-58-9) feature α,β-unsaturated systems associated with Michael-acceptor behavior, a hallmark of ZER's enone group; and zingerone (CAS 122-48-5) along with [6]-gingerol (CAS 23513-14-6) combine partially conjugated carbonyl moieties with hydrophobic domains, generating lipophilic–electrophilic profiles reminiscent of ZER. Additionally, α-humulene (CAS 6753-98-6) retains a sesquiterpene backbone and hydrophobic characteristics that enable steric and membrane-level interactions analogous to those attributed to zerumbone.

The IC_{50} values of 100 μM obtained for zerumbone, tropone, purpurogallin, and dicinnamalacetone are consistent with reports indicating that compounds bearing conjugated carbonyl systems often exert dose-dependent cytotoxicity only at higher micromolar concentrations, supporting their potential applicability within therapeutic windows (Huang et al. 2005). Importantly, ZER exhibited a favorable biocompatibility profile in both monolayer and 3D cell models (Abreu-Pereira et al. 2025; Abreu-Pereira et al. 2026). In NOK-si and HGF monolayers, cell viability was maintained at concentrations ranging from 293 to 1172 μM, whereas a significant reduction was observed at 2344 μM. This concentration-dependent response was also evident in the 3D coculture model comprising NOK-si and HGF cells, in which ZER at 1172 μM remained within biocompatible limits, while 2344 μM reduced cell viability by more than 30% (Abreu-Pereira et al. 2026). Together, these findings suggest that ZER is well tolerated by non-tumoral NOK-si and HGF cells within an appropriate concentration range, whereas higher concentrations may compromise cell viability (Abreu-Pereira et al. 2025; Abreu-Pereira et al. 2026). In contrast, HeLa cells appear to be considerably more sensitive to ZER, with a reported IC_{50} of 100 μM, suggesting a differential cytotoxic response between tumor and non-tumor cell models.

α-humulene, which demonstrated an IC_{50} of 81 μM in HeLa cells, similarly falls within the range previously described for sesquiterpenes with membrane-active properties (Legault et al. 2003; Silva et al. 2007). In contrast, dibenzylideneacetone exhibited a substantially lower IC_{50} (12 μM), reflecting its well-documented potency as a Michael acceptor and its stronger impact on cell viability relative to the other analogs (Dinkova-Kostova et al. 2001). On the other hand, zingerone and 6-gingerol, showed no detectable cytotoxicity within the concentration range assayed, in line with previous studies highlighting their favorable safety profiles (Chakraborty et al. 2012). Collectively, these findings indicate that while several zerumbone-related molecules display dose-dependent cytotoxicity comparable to previously published data, others — particularly zingerone and 6-gingerol — showed no cytotoxicity effect at the concentrations evaluated, positioning them as attractive candidates for further investigation.

The antimicrobial activity observed in planktonic cultures is consistent with a previous study highlighting the selective potency of certain Zingiber-derived compounds (Moreira da Silva et al. 2018). In the present study, zerumbone exhibited a strong bactericidal effect, achieving a ~ 7-log reduction at 100 and 50 μM,

whereas tropone, purpurogallin, dicinnamalacetone, and dibenzylideneacetone showed no meaningful activity under the same conditions. These results align with earlier studies indicating that the electrophilic enone system of zerumbone plays a key role in its antimicrobial efficacy, particularly against Gram-negative pathogens (Moreira da Silva et al. 2018). In addition, zingerone, 6-gingerol, and α-humulene produced moderate reductions (> 2.4 logs), a trend compatible with the literature describing antibacterial effects (Molzahn et al. 2026), often attributed to membrane-active or oxidative stress–mediated mechanisms rather than strong bactericidal action (Elfaky et al. 2024). The fact that only these four compounds—zerumbone, zingerone, 6-gingerol, and α-humulene—displayed detectable activity against planktonic *K. pneumoniae* supports their selection for subsequent biofilm assays and reflects the broader pattern reported in the literature that structural similarity to zerumbone does not necessarily translate into equivalent antimicrobial potency.

In established *K. pneumoniae* biofilms, only the highest concentrations tested of the compounds produced measurable antimicrobial effects, a finding consistent with the well-recognized tolerance of these species to chemical stressors within the biofilm matrix (Van Acker et al. 2014). Zerumbone exhibited modest but detectable antibacterial activity, with 100 and 50 μM, reducing biofilm viability by approximately 1.5 $\log_{10}$, which aligns with previous reports showing that zerumbone can disrupt membrane integrity and inhibit growth but often requires higher concentrations to affect biofilm-embedded cells (Abreu-Pereira et al. 2023). The results observed for α-humulene (1.86 and 1.72 $\log_{10}$ at 100 and 50 μM, respectively), are coherent with studies describing its lipophilic interaction with bacterial membranes and its ability to interfere with cell envelope stability (Adeosun et al. 2022). Zingerone and 6-gingerol showed similar reductions of 1.14 $\log_{10}$ and 1.47 $\log_{10}$ at 100 μM, respectively. Notably, ciprofloxacin failed to reduce viability under these conditions, corroborating studies demonstrating that *K. pneumoniae* biofilms exhibit high tolerance or complete refractoriness to fluoroquinolones due to restricted penetration, efflux pump overexpression, and protective EPS barriers (Emara et al. 2025). Together, these results indicate that while the natural compounds tested displayed only modest antibiofilm activity, they were more effective than ciprofloxacin against mature *K. pneumoniae* biofilms, underscoring their potential value as alternative or adjuvant strategies.

Zerumbone, α-humulene, zingerone and 6-gingerol compounds reduced *K. pneumoniae* biofilm biomass, whereas α-humulene and zerumbone showing the most pronounced effects. Although no studies to date have directly evaluated these two sesquiterpenes against *K. pneumoniae*, their better activity is consistent with evidence obtained in other Gram-negative bacteria (Tian et al. 2020; Jang et al. 2020). α-humulene inhibited biofilm formation and eradicated established *Bacteroides fragilis* biofilms at low concentrations (2–32 μg/mL), accompanied by downregulation of the RND-type efflux pump genes *bmeB1* and *bmeB3* (Jang et al. 2020). Likewise, zerumbone has been described as a disruptor of the extracellular polymeric matrix in *Escherichia coli* biofilms (Abreu-Pereira et al. 2023) and was shown to reduce biofilm formation and motility in *Acinetobacter baumannii* through downregulation of *adeA/adeB/adeC* and *bap* (Kim et al. 2020). This pattern may be partly related to structure–activity differences associated with hydrophobicity (Guimarães et al. 2019). Zerumbone and α-humulene are highly lipophilic sesquiterpenes, which may favor their interaction with bacterial membranes and the

extracellular biofilm matrix, thereby contributing to structural destabilization of both the matrix and lipid bilayer (Burt 2004). In addition, zerumbone has been shown to disrupt the extracellular matrix of *C. albicans* biofilms, as well as mixed *C. albicans–S. mutans* biofilms, by reducing several matrix components, including polysaccharides, proteins, and extracellular DNA (Abreu-Pereira et al. 2025; Abreu-Pereira et al. 2023; Abreu-Pereira et al. 2026; Gorayb Pereira et al. 2025).

In contrast, zingerone and 6-gingerol are more polar phenolic ketones containing a guaiacol-derived aromatic moiety (Wang et al. 2014; Rashpa et al. 2025). Their reported antibiofilm activity has been more closely associated with specific molecular mechanisms, including competitive inhibition of the quorum-sensing receptor LasR and suppression of QS-regulated genes in *Pseudomonas aeruginosa* (Kim et al. 2020), as well as interactions with fimbrial subunits in *Salmonella* (Kharga et al. 2023). Such mechanisms may preferentially interfere with biofilm establishment rather than directly disrupting an already formed extracellular matrix. Therefore, the greater biomass reduction observed with α-humulene and zerumbone in the present study may reflect a more pronounced membrane- and matrix-disruptive activity, whereas the effects of zingerone and 6-gingerol may rely more heavily on specific signaling- or receptor-mediated pathways.

Overall, these findings demonstrate that zerumbone and selected structurally related compounds — particularly α-humulene, zingerone, and [6]-gingerol — reduced the viability and biofilm biomass of *K. pneumoniae*, with α-humulene and zerumbone producing the most pronounced effects. Remarkably, these results were obtained under experimental conditions in which ciprofloxacin, a conventional antibiotic, showed no measurable antimicrobial activity against the same biofilm-associated bacteria. This contrast reinforces the therapeutic relevance of these natural compounds, since it indicates that their antibacterial effect was achieved precisely in a scenario of antibiotic failure, where the biofilm matrix is known to confer tolerance to conventional therapy. Future studies should investigate the combination of these compounds with conventional antibiotics to assess potential synergistic, as well as evaluate their in vivo efficacy and safety, in order to establish their applicability as anti-biofilm therapeutic agents.

Declarations

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Author contributions

CCJ: conceptualization, data curation, formal analysis, investigation, methodology, resources, software, validation, visualization, review and writing; ALGP: data curation, formal analysis, investigation, methodology and software; VD: investigation and resources; ADG: formal analysis, investigation, methodology, software, validation and visualization; FLB: conceptualization, funding acquisition, resources and review and writing; ACP: conceptualization, funding acquisition, supervision, review and writing; JCC: conceptualization, funding acquisition, methodology, software, resources, supervision, review and writing.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest

The authors declare no competing interests.

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Figures

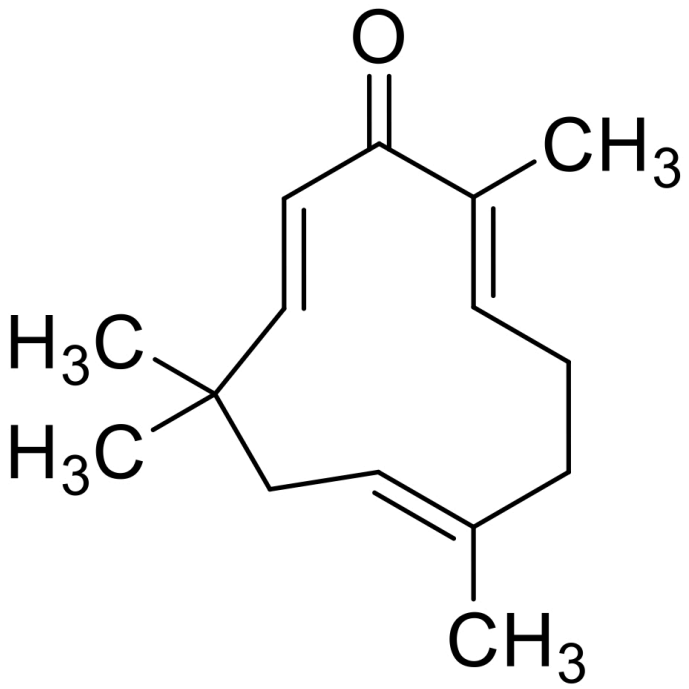

Figure 1

Chemical structure of zerumbone.

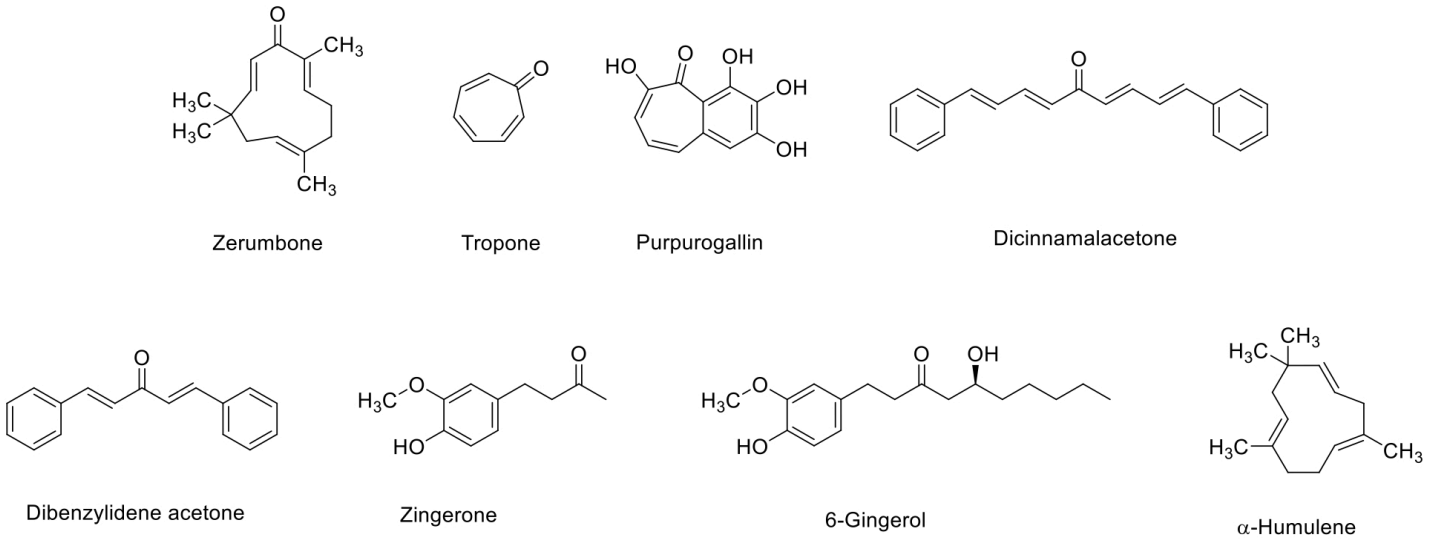

Figure 2

Structural analogs of zerumbone were screened using CAS Finder software.

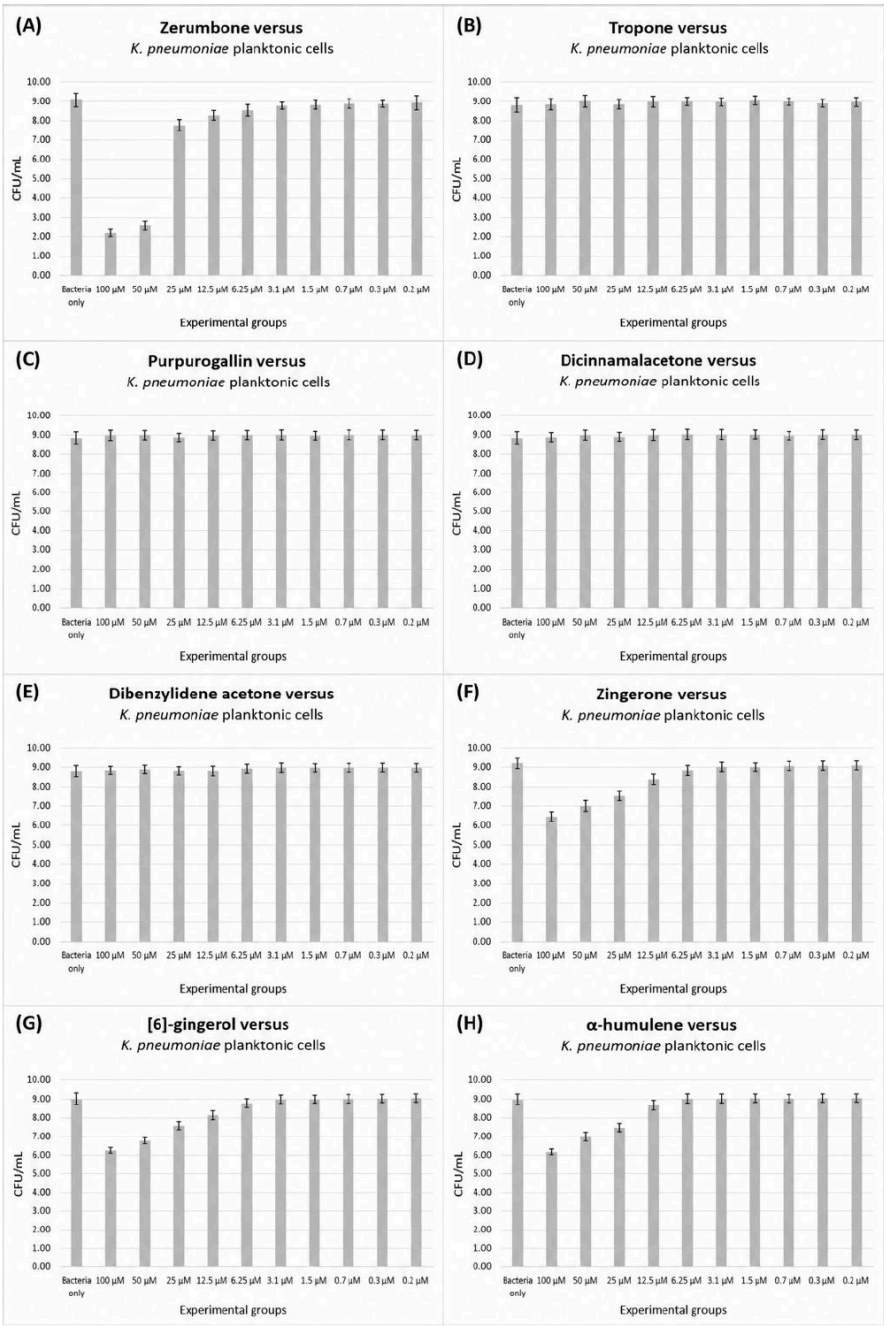

Figure 3

CFU/mL counts of *Klebsiella pneumoniae* planktonic cultures following incubation with a range of concentrations of the tested compounds—zerumbone (A), tropone (B), purpurogallin (C), dicinnamalacetone (D), and dibenzylideneacetone (E) zingerone (F), 6-gingerol (G) and α-humulene (H). The graphs depict dose-dependent effects on bacterial viability, highlighting the distinct antimicrobial responses elicited by each compound (n=3).

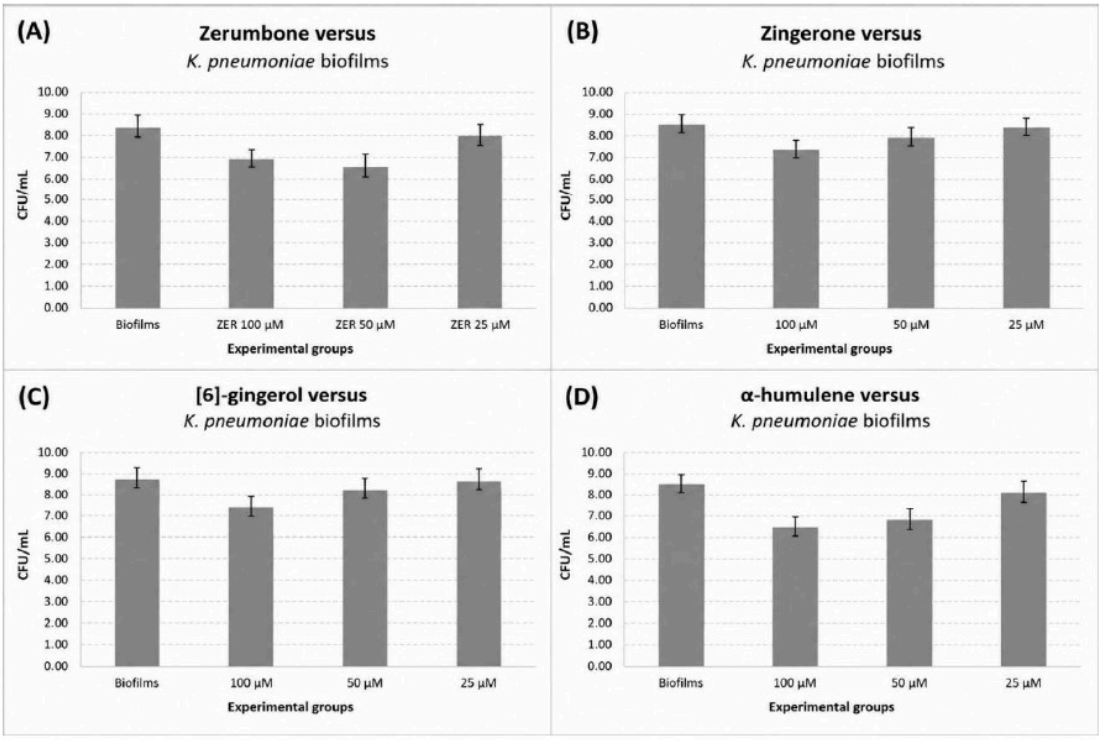

Figure 4

CFU/mL counts of *Klebsiella pneumoniae* biofilms following incubation with a range of concentrations of the tested compounds—zerumbone (A), zingerone (B), 6-gingerol (C) and α-humulene (D) (n=3).

Supplementary Files

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- GraphicalAbstract.png