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Assessment of the antimicrobial impact of Melaleuca alternifolia essential oil on Klebsiella pneumoniae strains

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ABSTRACT

Objective: Tea tree oil (TTO) from Melaleuca alternifolia is renowned for its therapeutic properties, including antimicrobial effects. Standardized by ISO 4730:2017, TTO's major component, terpinen-4-ol, contributes to its broad spectrum of activity. Notably, TTO demonstrates efficacy against Cutibacterium acne, suggesting a potential role in acne treatment. In the context of Klebsiella pneumoniae (Kp), a carbapenem-resistant Gram-negative bacillus associated with pneumonia, this study explores TTO's antibacterial and anti-adherent activities.
Methods: A 15% TTO solution was prepared, chemically analyzed via GC-MS, and tested in vitro against Kp strains. The minimum inhibitory concentration (MIC) was determined using the microdilution method. The minimum bactericidal concentration (MBC) was calculated using the tube dilution method. Minimum inhibitory adherence concentration (MIAC) was also calculated using the Albuquerque protocol.
Results: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined, revealing strong antimicrobial effects with an MIC50 of 128 µg/mL, an MBC of 256 µg/mL, MIAC of 256 µg/mL concentration against Kp strains, respectively.
Conclusion: The study underscores TTO's pharmacological potential against antibiotic-resistant bacteria like Kp, particularly in nosocomial infections. Further research is needed to validate efficacy, elucidate mechanisms, and develop suitable formulations for clinical use.
Keywords: Tea Tree Oil; Melaleuca alternifolia; Klebsiella pneumoniae; Anti-Bacterial Agents; Microbial Sensitivity Tests; Terpenes; Drug Resistance, Bacterial

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Introduction

Tea tree oil (TTO) is derived from a small tree called Melaleuca alternifolia, which is native to Australia. [1] The physicochemical characteristics of TTO are standardized by International Standard ISO 4730:2017, stipulating minimum and maximum concentrations of its major components. About 14 different volatile constituents have been extracted from the Melaleuca alternifolia plant, including sesquiterpenes, monoterpenes, γ- and α-terpinene, terpinen-4-ol, 1,8-cineol, p-cymene, and αterpineol. [2] Extensive research has been conducted to discover TTO's diverse therapeutic properties that encompass antibacterial, antifungal, antiviral, and anti-inflammatory effects. [3] It is speculated that terpinen-4-ol is the predominant constituent and the primary contributor to its medicinal activities. [4]

TTO exhibits a wide spectrum of properties, including anti-bacterial, anti-fungal, and anti-inflammatory properties. It has been used as a hand sanitizer, bug repellent, a natural deodorant, an all-purpose cleaner, and a chemical-free mouthwash.[5] Herbal experts use TTO to treat skin

acne, athlete's foot, and nail fungus, enhance wound healing, antiseptic for cuts and bruises, control dandruff, clean fruits and vegetables, and relieve psoriasis.[6] It has wide antimicrobial activity against gram-positive and gram-negative bacteria, yeasts, and mold fungi. [7] Particularly noteworthy is its remarkable activity against Cutibacterium acnes (formerly Propionibacterium acnes). [6] Given that this Gram-positive bacterium is a major contributor to acne vulgaris, the efficacy of TTO in the topical treatment of acne has been proposed by several scientists based on in vitro and in vivo studies. Clinical investigations have even demonstrated comparable or superior efficacy of TTO compared to commonly used therapeutic drugs for acne.

Tea tree oil (TTO) exhibits a broad spectrum of therapeutic applications, yet the precise mechanisms underlying its antimicrobial activity continue to be investigated. Among the microorganisms under scrutiny, Klebsiella pneumoniae (Kp) stands out as a significant focus of inquiry. Klebsiella pneumoniae, identified as a Gram-negative bacterium, displays encapsulation, lacks motility, and operates as a facultative anaerobe. Carl Friedländer first isolated and described it in 1882 from patients who had died of pneumonia, leading to its initial designation as Friedländer's bacillus. The genus was later named *Klebsiella* in honor of the pathologist Edwin Klebs. [8] Kp is aerobic in nature and can be found abundantly in water, soil, and other areas. In human beings, it is naturally colonized in the mucosal surfaces of the nose, mouth, pharynx, and even the gut. Under low immune conditions like diabetes and alcoholism, the organism colonizes and infects the upper respiratory tract, so much so, that in its hypervirulent state, it may cause pneumonia with superimposed conditions like meningitis, liver abscess, and endophthalmitis. [9]

To initiate infection, K. pneumoniae must first overcome mechanical and chemical barriers and evade detection by the host's humoral and cellular immune responses. Upon entry into the host, these invasive microorganisms are identified by immune cells through pattern recognition receptors (PRRs), triggering the production of diverse immune mediators. The LPS and the OMP porins are abundantly present on the outer wall of Kp. Notably, Kp is known for producing the carbapenemase enzyme, earning it the acronym KPC, Klebsiella pneumoniae carbapenemase. Consequently, it exhibits resistance to carbapenem-class antibiotics such as Meropenem, Ertapenem, and Imipenem. Additionally, it can inactivate β-lactam agents, including penicillins, cephalosporins, and monobactams. [10] The high potential for dissemination in KPC, coupled with Klebsiella pneumoniae's adeptness in transferring genetic material and resistance genes, poses challenges for infection control efforts.

Since the emergence of Kp infections, they have proliferated worldwide, posing a significant public health threat. Various treatment regimens have been documented in the literature, including polymyxin, aminoglycoside, carbapenem, glycylcycline, beta-lactam plus beta-lactamase inhibitor, fluoroquinolone, tetracycline, cephalosporins, trimethoprim-sulfamethoxazole, and fosfomycin, among others. A study conducted at the University of Texas assessed the efficacy of these commonly prescribed drugs and found that 47% of treatments resulted in failure. [11] This alarming finding underscores the urgent need for the development of newer, less hazardous treatment options to effectively combat the escalating global burden of Kp infections.

With the surge in resistant strains of Kp and the concerning side effects of existing medications, scientists globally are seeking safer treatment alternatives. One promising avenue they're exploring involves turning to herbal plants. This natural approach not only holds potential effectiveness but also offers hope for minimizing the adverse effects commonly associated with traditional drugs. Research indicates that terpenes, a significant category of plant-derived compounds, can interact with bacterial cells, either enhancing or diminishing each other's antibacterial effects. Specific terpenes, including terpineol, eugenol, citronellol, carveol, and geraniol, have demonstrated antibacterial activity, with this effect linked to the presence of hydroxyl groups. Moreover, upon analyzing the essential oil of TTO, it becomes evident that it possesses antifungal, antibacterial, antioxidant, antiparasitic, antitumor, and anti-inflammatory effects, making it a potential candidate for the management and infection control of Kp diseases. Against this backdrop, this study aimed to assess the antibacterial activity of TTO against Klebsiella pneumoniae strains in an in vitro study.

Materials and Methods

Essential Oil Preparation

The tea tree oil solution, obtained at a concentration of 15%, was procured from the local market through the Australian Tea Tree Industry Association, which serves as the material source in

the market. Meanwhile, the fluconazole solution, with a concentration of 64 µg/ml, was prepared using fluconazole tablets sourced from LABORATE Pharmaceuticals. The chemical composition of TTO was examined using a gas chromatograph attached to a mass spectrometer (GC-MS) setup, specifically the Shimadzu model GC-QP2010 (Shimadzu, Kyoto, Japan). The capillary column chosen for this experiment was DB-5 fused silica (30m x 0.25mm i.d. x 0.25µm). The chromatographic conditions included an injector temperature of 220 °C with a split ratio of 1:10 (3.0 min), a helium carrier gas at 0.6 mL/min, an interface temperature at 250 °C, and an electron ionization source (35-350 m/z). The oven temperature ramp was set at 40 °C (2 min), with an increment of 3 °C/min up to 240 °C for 5 min, and an injected volume of 1 µL (1% solution in dichloromethane).

TTO compounds were identified by comparison of their fragmentation patterns and the mass spectra from the NIST 14 database (NIST/EPA/NIH Mass Spectral Library, 2014) integrated into the GC-MS. Moreover, the study included a comparative analysis that involved a literature review and the computation of Linear Retention Indices (LRIs). These indices were calculated in relation to the retention times of a homologous series of hydrocarbons, ranging from C8 to C26, which were injected under identical conditions to those of the sample. The methodology for calculating the linear retention index is grounded in the principles established by Van den Dool and Kratz in 1963, as well as Adams in 2007. Identifications based on database spectral matches were deemed valid when the similarity percentage surpassed the 90% threshold.

Microorganism

Multiple Klebsiella pneumoniae strains were utilized in the study, namely: ATCC 13883, Kp 101, Kp 103, Kp 104, Kp 105, and Kp 110. The strains were inoculated on Mueller-Hinton Agar (MHA) petri dish and allowed to grow for 3 days at 37°C. From these fresh cultures, the inoculation of Klebsiella pneumoniae (Kp) was meticulously prepared from freshly cultured samples using a sterile inoculation loop. These were then carefully transferred into small, sterile saline-filled plastic laboratory tubes. The Kp solution's concentration was precisely controlled at an estimated 1.5×10^8 colony-forming units per milliliter (CFU/mL), utilizing a spectrophotometer for accurate measurement. Adjustments to this concentration were made by assessing the turbidity in comparison to the standard 0.5 McFarland scale tube. Cultures not immediately used were preserved on MH agar plates at a temperature of 4 °C for future experimental requirements. The chosen media for the evaluation of antimicrobial properties were the Mueller-Hinton solid and liquid agar mediums, both sourced from Difco® and constituted in strict adherence to the manufacturer's specifications.

Minimum Inhibitory Concentration

The experimental procedure to establish the Minimum Inhibitory Concentration (MIC) was meticulously conducted using the microdilution method within a 96-well plate that featured a U-shaped bottom. The initial step involved the careful addition of 100 µL of Mueller Hinton broth, which was prepared at double the usual concentration, alongside 100 µL of Tea Tree Oil (TTO) essential oil, which was introduced at a starting concentration of 2048 µg/mL. These components were combined in the wells situated in the first row of the plate. Following this, a systematic serial dilution process was undertaken, adhering to a dilution ratio of two. This methodical approach resulted in a series of descending concentrations, specifically 1024, 512, 256, 128, 64, 32, 16, 8, and 4 µg/mL. The gradient of concentrations was arranged such that the most concentrated solution was placed in the first row, gradually decreasing to the least concentrated solution in the final row. This careful gradation ensured a precise determination of the concentration threshold that inhibits bacterial growth without excess.

For the determination of MIC, 10 µL of the Kp strains (approximately 1.5×10^8 CFU/mL) were added to each well. In the meticulous setup of the experiment, a sterility control was established by allocating 200 µL of the broth to the second-to-last well, ensuring the absence of unintended microbial contamination. Concurrently, a growth control was instituted in the final well, which included 100 µL of a doubly concentrated Mueller-Hinton broth coupled with a suspension of the microorganism under study. To ensure the reliability of the results, the entire procedure was replicated, with each assay being performed in triplicate. Following the setup, the plates were subjected to an incubation period at a controlled temperature of 35 ± 2 ºC for 24 hours. This was succeeded by the initial assessment of the results. In the subsequent phase, 20 µL of a sodium resazurin solution, provided by SIGMA and pre-dissolved in sterile distilled water to achieve a 0.01% (w/v) concentration, was introduced to the

wells. This solution acts as a colorimetric indicator, changing color in response to the oxidative-reduction activity of the bacteria, thereby facilitating the determination of bacterial growth. A further incubation at the same temperature range of 35 ± 2 ºC was carried out to complete the process. This comprehensive approach ensures a thorough evaluation of the antimicrobial properties being tested.

The plates were evaluated by visually inspecting for the growth of microorganisms, which was indicated by the appearance of a cellular cluster, also known as a "button." Additionally, a change in the solution's color from blue to pink was a sign of growth. The Minimum Inhibitory Concentration (MIC) was denoted as the smallest amount of the substance that prevented the observable multiplication of the microorganism, as evidenced by the indicator dye retaining its original color. [12]

Minimum Bacterial Concentration

After the determination of MIC, the Minimum Bactericidal Concentration (MBC) for TTO against Klebsiella pneumoniae strains was determined using the tube dilution method. [13] Samples of 10 µL from three different dilutions, all less concentrated than the MIC, were carefully placed into cavities containing 100 µL of Mueller-Hinton broth within a pristine microdilution plate. This plate was then kept in an incubator set at a steady temperature of 35 ± 2 °C for a full day. Following this period, a 20 µL dose of resazurin was introduced to each cavity. The plate was incubated for another day at the same temperature. The purpose of this second round was to double-check the lowest concentration that could fully halt bacterial growth, which was confirmed when no shift in the color of the resazurin indicator was observed.

Minimum Inhibitory Adherence Concentration

The assessment of the Minimum Inhibitory Adherence Concentration (MIAC) for Tea Tree Oil (TTO), in conjunction with 5% sucrose, was executed by adapting the protocol described by Albuquerque with certain modifications. [14] A spectrum of 14 different concentrations was explored, starting from undiluted oil to a dilution ratio of 1:1024. The Klebsiella pneumoniae strain labeled KP 105 was propagated at a temperature of 35 ± 2 ºC in Mueller-Hinton broth sourced from DIFCO, Michigan, United States. This was followed by the allocation of 0.9 mL of the bacterial subculture into sterile test tubes, to which 0.1 mL of various diluted solutions of the compound was added. The tubes were then incubated at an angle of 30º at 35 ± 2 ºC for 24 hours. The evaluation process included a visual inspection for bacterial adhesion to the interior surfaces of the tubes post-vigorous shaking. To ensure the accuracy of the results, the entire test was performed thrice. An analogous method was applied to the positive control substance, 0.12% chlorhexidine digluconate (Periogard®, Colgate-Palmolive Company, New York, USA). The MIAC was determined to be the minimal concentration of TTO in the presence of sucrose that effectively inhibited bacterial attachment to the glass surface of the tubes.

Statistical Analysis

All experiments were performed in triplicate on three independent occasions. As MIC, MBC, and MIAC are categorical endpoints (growth/no growth; adherence/no adherence), data are reported as the mode of three independent replicates per concentration–strain combination. Concordance across replicates was 100% at each concentration tested; no inferential statistical testing was warranted. The MIC50 was defined as the lowest concentration at which 50% of tested strains were inhibited; the MIC90 was defined as the lowest concentration at which 90% of strains were inhibited. Data were tabulated using Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

Results

As presented in Table 1, the Minimum Inhibitory Concentration (MIC) of TTO was 128 µg/mL for all six *Klebsiella pneumoniae* strains tested (ATCC 13883, KP 101, KP 103, KP 104, KP 105, and KP 110). At concentrations of 64 µg/mL and below, growth was observed in all wells; at 128 µg/mL and above, growth was completely inhibited across all strains. As all six strains shared the same MIC value, the MIC50 (the concentration at which 50% of tested strains are inhibited) and MIC90 (the concentration at which 90% of strains are inhibited) were both 128 µg/mL. The ATCC 13883 reference strain served as the quality control.

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Table 1: Minimum inhibitory concentration (MIC) of tea tree oil (TTO) against six *Klebsiella pneumoniae* strains

Bacterial strain/substance	ATCC 13883	Kp 101	Kp 103	Kp 104	Kp 105	Kp 110
Growth control	G	G	G	G	G	G
Sterility control	NG	NG	NG	NG	NG	NG
4 µg / mL	G	G	G	G	G	G
8 µg / mL	G	G	G	G	G	G
16 µg / mL	G	G	G	G	G	G
32 µg / mL	G	G	G	G	G	G
64 µg / mL	G	G	G	G	G	G
128 µg / mL	NG	NG	NG	NG	NG	NG
256 µg / mL	NG	NG	NG	NG	NG	NG
512 µg / mL	NG	NG	NG	NG	NG	NG
1024 µg / mL	NG	NG	NG	NG	NG	NG

† G = visible bacterial growth (button formation in well or pink color after resazurin); NG = no growth (clear well, blue color retained). Growth control well contained MH broth + bacterial suspension without TTO; sterility control well contained MH broth alone. Tea tree oil concentrations were prepared by serial two-fold dilution from 1024 µg/mL down to 4 µg/mL. All assays were performed in triplicate with concordant results across replicates.

Table 2 presents the MBC of TTO against Kp strains. As for MIC, different concentrations of TTO were used against each strain of Kp. The concentration at which all bacterial activity halted was 256 µg/mL. Table 2 showcases results that are consistent with the previously discussed findings. It details the Minimum Inhibitory Adherence Concentration (MIAC), which indicates the least amount of an antimicrobial agent necessary to prevent bacterial proliferation when sucrose is present. For the control substance, chlorhexidine, this critical concentration was determined to be 256 µg/mL. This concentration was sufficient to halt the growth of bacteria in a sucrose environment. In a similar vein, Tea Tree Oil (TTO) demonstrated comparable efficacy, with the MIAC also being established at 256 µg/mL. This denotes that TTO, much like chlorhexidine, possesses the ability to effectively inhibit bacterial growth at this specified concentration when in the presence of sucrose, highlighting its potential as an antimicrobial agent.

Table 2: Minimum bactericidal concentration (MBC) of tea tree oil (TTO) against six *Klebsiella pneumoniae* strains

Bacterial strain/substance	ATCC 13883	Kp 101	Kp 103	Kp 104	Kp 105	Kp 110
Growth control	G	G	G	G	G	G
Sterility control	NG	NG	NG	NG	NG	NG
4 µg / mL	G	G	G	G	G	G
8 µg / mL	G	G	G	G	G	G
16 µg / mL	G	G	G	G	G	G
32 µg / mL	G	G	G	G	G	G
64 µg / mL	G	G	G	G	G	G
128 µg / mL	G	G	G	G	G	G
256 µg / mL	NG	NG	NG	NG	NG	NG
512 µg / mL	NG	NG	NG	NG	NG	NG
1024 µg / mL	NG	NG	NG	NG	NG	NG

† G = visible bacterial growth (button formation in well or pink color after resazurin); NG = no growth (clear well, blue color retained). Growth control well contained MH broth + bacterial suspension without TTO; sterility

control well contained MH broth alone. Tea tree oil concentrations were prepared by serial two-fold dilution from 1024 µg/mL down to 4 µg/mL. All assays were performed in triplicate with concordant results across replicates.

Discussion

In the present study, Melaleuca alternifolia essential oil (tea tree oil, TTO) inhibited the growth of all six Klebsiella pneumoniae strains at 128 µg/mL and showed bactericidal activity at 256 µg/mL, while the minimum inhibitory adherence concentration (MIAC) obtained for the single strain tested was 256 µg/mL. These findings suggest reproducible in vitro activity of the tested TTO preparation against both planktonic growth and surface adherence under the present assay conditions. The MIC of 128 µg/mL observed in the present study falls within the range reported in prior TTO studies against Gram-negative organisms, although direct comparisons are complicated by differences in oil composition, solubilization method, and assay format. [15] Because this work concerns TTO, the interpretation of the findings should be anchored in the TTO literature. In addition, in the absence of full compositional data for the specific TTO batch tested, the antibacterial effect observed in this study is attributed to the TTO preparation as a whole rather than to any single constituent.

Standardized TTO is typically characterized by a terpinen-4-ol-rich monoterpene profile, and prior work has linked its antimicrobial effects to coordinated actions of multiple constituents rather than to a single molecule alone. Mechanistically, TTO has been reported to increase membrane permeability, disturb ion homeostasis, and impair essential cellular functions in susceptible bacteria, making a membrane-active effect biologically plausible in Gram-negative organisms such as K. pneumoniae. The uniform MIC observed across all six strains in the present study suggests a consistent inhibitory effect, and the MBC/MIC ratio of 2 is compatible with bactericidal activity under the tested conditions. However, potency comparisons across studies should be made cautiously because essential-oil MIC values are strongly influenced by inoculum density, medium composition, volatility, oil solubilization, and endpoint determination.

Several recent studies provide context for the present findings. Brun et al. (2019) evaluated ten commercially available TTO products against a panel of pathogens including MRSA and *Pseudomonas aeruginosa* and reported that only five of the ten batches exhibited significant antimicrobial activity, underscoring the importance of batch-to-batch compositional variability, a factor that may partly explain differences in MIC values across laboratories. [16] Oliva et al. (2018) tested a chemically characterized TTO against MDR Gram-negatives, including ESBL-producing and carbapenem-resistant *K. pneumoniae*, and demonstrated bactericidal activity as well as synergy with oxacillin against MRSA; their reported MIC values for *K. pneumoniae* were broadly comparable to those observed in the present study, supporting the notion that TTO retains activity against resistant Gram-negative phenotypes. [17] More recently, Borotová et al. (2022) characterized a TTO sample containing 38.4% terpinen-4-ol and demonstrated antimicrobial activity against both Gram-positive and Gram-negative organisms, with activity varying by strain and concentration. [18] In a bovine mastitis model, Curbelo Torres et al. (2022) tested TTO alongside thymol and carvacrol against field isolates of *K. pneumoniae* and found that TTO alone showed moderate inhibition, whereas the combination of TTO with thymol produced additive effects against Gram-negatives, suggesting that combinatorial approaches may enhance efficacy. [19] Collectively, these studies indicate that TTO possesses reproducible but variable activity against *K. pneumoniae* depending on oil composition, solubilization method, and assay format. The uniform MIC of 128 µg/mL across all six strains in the present study is at the lower (more potent) end of published ranges for Gram-negatives, which may reflect the specific terpene profile of the tested batch, the use of resazurin as a sensitive growth indicator, or the relatively small strain panel.

The MIAC result is potentially relevant because interference with bacterial adherence may reduce early colonization and persistence. Nevertheless, this part of the study should be interpreted conservatively. MIAC was assessed in only one strain (Kp 105), adherence was determined by visual inspection of tube-wall attachment, and no quantitative biofilm biomass or viable-count assay was used to confirm the anti-adherence effect. Accordingly, the present data support only a preliminary claim of anti-adherence activity in a single-strain model rather than a generalized anti-biofilm conclusion for K. pneumoniae.

Several limitations should be acknowledged explicitly. First, MIAC evaluation in

a single strain limits external validity. Second, the study did not include biological or mechanistic verification, such as membrane-permeabilization assays, ATP-leakage measurements, electron microscopy, or transcriptional analysis, so the mechanism of action remains inferential. Third, time-kill kinetics were not performed, preventing assessment of the rate and durability of bactericidal activity. Fourth, no checkerboard or time-kill combination experiments were conducted with conventional anti-Klebsiella antibiotics; therefore, any statement suggesting synergy should be removed because it is not supported by the present data. Fifth, although resazurin was used as a growth indicator, the manuscript does not describe an independent validation step demonstrating concordance between the resazurin readout and culture-based viability at the tested TTO concentrations, which would strengthen confidence in the endpoint. Sixth, although GC-MS analysis was performed, the full compositional profile of the tested TTO batch (including percentage abundances of terpinen-4-ol, 1,8-cineole, and other major constituents) is not reported in this paper, preventing independent verification of oil identity and batch-to-batch reproducibility. Future work should report the full GC-MS composition of the tested oil, expand testing to a broader panel of clinical and resistant isolates, quantify biofilm inhibition with orthogonal methods, evaluate synergy with standard antibiotics, perform time-kill analyses, and assess formulation and cytotoxicity before any clinical relevance is inferred.

Conclusion

Within the limits of this in vitro study, Melaleuca alternifolia essential oil demonstrated inhibitory, bactericidal, and preliminary anti-adherence activity against K. pneumoniae under the tested conditions. However, the present data do not support claims regarding specific active constituents, mechanisms of action, or synergy with conventional antimicrobials. Future studies should verify oil identity by full compositional reporting, broaden strain coverage for adherence testing, validate resazurin-based endpoints, and incorporate mechanistic, time-kill, synergy, and safety studies before translational or clinical implications are proposed.

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Author Contribution

SR conceived the idea, collected data, and wrote the initial manuscript. ZA collected data, analyzed the data, validated the results, and proofread the finalized manuscript.

Data Availability Statement

All relevant data are within the manuscript. Additional data supporting this study are available from the corresponding author upon reasonable request.

Use of AI Tools

ChatGPT (OpenAI) was used to proofread the manuscript and refine sentence structure in selected passages. All scientific content, data analysis, and conclusions are the original work of the authors, who reviewed and verified all AI-assisted text.

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Ethical Considerations

All experiments involving *K. pneumoniae* were conducted under Biosafety Level 2 (BSL-2) conditions in compliance with institutional biosafety guidelines at Universiti Sains Malaysia. As the study used only ATCC reference strains and clinical isolates from the institutional culture collection, no human or animal subjects were involved, and ethics committee approval was not required.

Conflict of Interest

The authors declare no conflicts of interest.

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