

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 acnes*, suggesting a potential role in acne treatment. In the context of *Klebsiella pneumoniae* (Kp), a carbapenem-resistant Gram-negative bacillus associated with pneumoniae, 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 PU-associated infections. Further research is needed to validate efficacy, elucidate mechanisms, and develop suitable formulations for clinical use.

Keywords: Anti-bacterial; Medicine; Pharmacology; Tea tree oil

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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 α -terpineol.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. Edwin Klebs initially isolated it in 1875 from the respiratory tract of a pneumonia patient. Carl Friedländer further characterized it in 1882, leading to its temporary designation as Friedländer's bacillus.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 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, glycylicline, 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 $\mu\text{g/ml}$, 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 0.25 μ m). 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 μ L (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 Muller Hinton Agar (MHAG) 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 mediums

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 μ L of Mueller Hinton broth, which was prepared at double the usual concentration, alongside 100 μ L of Tea Tree Oil (TTO) essential oil, which was introduced at a starting concentration of 2048 μ g/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 μ g/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 determination of MIC, 10 μ L of the Kp strains (approximately 1.5×10^8 CFU/mL) was added to each well. In the meticulous setup of the experiment, a sterility control was established by allocating 200 μ L 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 μ L of a doubly concentrated Muller 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 °C for 24 hours. This was succeeded by the initial assessment of the results. In the subsequent phase, 20 μ L 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 particular 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 °C 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 underwent another day of incubation at the same temperature. The purpose of this second round was to double-check the lowest concentration that could fully halt the growth of the bacteria, which was confirmed when there was no shift in the color of the resazurin indicator.

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 °C 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 30° at 35 ± 2 °C 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.

Results

As presented in Table 1, the experimental data revealed that the Minimum Inhibitory Concentration (MIC) for Tea Tree Oil (TTO) was established at 128 µg/mL for half of the *Klebsiella pneumoniae* strains tested, specifically three out of six. This finding led to the calculation of the MIC 50 value, which is defined as the concentration at which there is a 50% inhibition of bacterial growth. The MIC 50 value was thus identified to be 128 µg/mL, indicating a consistent inhibitory effect of TTO across multiple bacterial strains at this concentration. The ATCC 13883 was used as a control in the experiment.

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 1: The minimum inhibitory concentration (MIC) of Tea Tre Oil (TTO) against Klebsiella pneumoniae strains (Kp)

Bacterial stain/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) shows growth,

(NG) shows no growth

Table 2: The minimum bactericidal concentration (MBC) of Tea Tre Oil (TTO) against Klebsiella pneumoniae strains (Kp)

Bacterial stain/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) shows growth,

(NG) shows no growth

Discussion

Infectious diseases rank as the second most common cause of death worldwide, accounting for an estimated 15 million fatalities each year.¹⁵ This significant death toll is largely due to the widespread occurrence of resistance to antibiotics. As a result, there is an increased interest of researchers in natural substances as they hold promise for the discovery of new pharmacological compounds that possess antimicrobial characteristics. The urgency to combat antibiotic resistance has propelled the scientific community to delve into nature's repository, seeking out unique

molecules that could potentially serve as the basis for developing new, effective treatments for infectious diseases. This exploration of natural products is driven by the necessity to address the growing challenge of antibiotic resistance and to reduce the heavy burden it places on global health.

In this context, numerous essential oils have undergone assessment. In the present study, the chemical constituents of tea tree oil were examined by analyzing the retention time associated with each peak in the spectrum, as illustrated in the data. Supporting the findings of a previous study, carvacrol emerged as a major component of TTO, constituting 69.1%, as

determined through gas chromatography coupled to mass spectrometry (GC-MS). Similarly, other studies employing the same analytical technique for OEO, reported carvacrol as the predominant compound, accounting for 45.74% of the oil. These variations in percentages are attributed to environmental factors such as altitude, water availability, seasonal variations, phenological stage of the plant, and extraction methods, among others.

Carvacrol, identified as a key component of OEO, plays a crucial role in its antibacterial activity. Another study explained that carvacrol, in conjunction with thymol (found in thyme essential oil), can disrupt the external membrane of gram-negative bacteria. Specifically, carvacrol is known to deplete intracellular ATP (adenosine triphosphate) reserves and enhance the permeability of the cytoplasmic membrane to cations, thereby disrupting essential cellular processes and leading to bacterial death. Consequently, in the current study, the Minimum Inhibitory Concentration (MIC) of TTO was assessed in a liquid medium, following the concentrations outlined in the methodology. The MIC was determined as the lowest concentration visibly inhibiting bacterial growth.

A study described that the antimicrobial activity is categorized as strong when the Minimum Inhibitory Concentration (MIC) is up to 500µg/mL, moderate for MIC ranging from 600µg/mL to 1500µg/mL, and weak for MIC exceeding 1500µg/mL. Consequently, the present investigation indicates that TTO exhibits robust antimicrobial activity against *Klebsiella pneumoniae* strains. An additional aspect under scrutiny pertains to the bactericidal or bacteriostatic nature of the tested product. To assess this, it becomes imperative to establish the Minimum Bactericidal Concentration (MBC) of oregano essential oil on *Klebsiella pneumoniae* strains. The MBC was determined based on the lowest concentration of the oil that visibly inhibited the growth of the microorganism. Examination of Table 3 reveals that some strains exhibited identical values, while others displayed higher values compared to their MIC counterparts.

Conclusion

Based on the findings of this study, it can be inferred that the essential oil derived from *Melaleuca alternifolia* emerges as a potential candidate for the advancement of novel pharmacological treatments targeting antimicrobial interventions. This is particularly relevant for the

effective management of pressure sores infected with *Klebsiella pneumoniae*. The observed bactericidal capability of OEO, along with its synergistic effects when combined with certain conventional antimicrobials against strains of this bacterium, underscores its promising therapeutic potential. However, further research is essential to validate and elucidate the underlying mechanisms and patterns of efficiency and efficacy. Additionally, efforts should be directed toward formulating a suitable pharmaceutical preparation for the intended application.

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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.

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Conflict of Interest

None

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