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Original Article

Comprehensive investigation of *Litsea cubeba* antibacterial and antifungal activities across solid, liquid, and vapor phases against key human pathogens

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Abstract

The escalating global incidence of antimicrobial resistance poses a significant public health challenge. In response, exploring alternative antimicrobial agents, particularly derived from plants, becomes crucial to alleviate the selective pressure exerted by conventional antibiotics. The aim of this study was to characterize the composition of essential oil extracted from *Litsea cubeba* fruits and to evaluate its antimicrobial potential, along with its major compound, across solid, liquid, and vapor phases. The antimicrobial activity was assessed against a diverse range of human pathogenic Gram-positive bacteria (n=8), Gram-negative bacteria (n=34), filamentous fungi (n=2), and yeast (n=1). Disk diffusion, broth macrodilution, and vapor-phase diffusion methods were employed. This study found that all phases of *L. cubeba* essential oil and purified limonene exhibited broad-spectrum bactericidal and fungicidal activities (solid-phase: inhibition zone diameter (IZD) 19 mm vs 14 mm; liquid-phase: minimum inhibitory concentration (MIC) 2.0 mg/mL vs 4.0 mg/mL; vapor-phase: IZD 90 mm vs 45 mm), with superior efficacy against filamentous fungi and yeast compared to bacteria (solid-phase: IZD 90 mm vs 17.5 mm; liquid-phase: MIC 2.0 mg/mL vs 0.06 mg/mL; vapor-phase: IZD 90 mm vs 12.5 mm; all *p*-values<0.05). Among bacteria, solid-phase *L. cubeba* essential oil demonstrated increased activity against *Staphylococcus saprophyticus* and *Acinetobacter lwoffii* whereas liquid-phase *L. cubeba* essential oil had optimal activity against *Streptococcus agalactiae* and *Elizabethkingia meningoceptica*. Notably, *Trichophyton rubrum*, *Nannizzia gypsea*, and *Candida albicans* displayed high susceptibility to all phases of *L. cubeba* essential oil. These findings highlight the potential activity of *L. cubeba* essential oil, across its various phases, as a promising alternative antimicrobial agent against medically significant pathogens, providing essential baseline information for further exploration and development of *L. cubeba* essential oil in the pursuit of combating antimicrobial resistance.

Keywords: Antibacterial activity, antifungal activity, *Litsea cubeba*, limonene, essential oil

Introduction

Antimicrobial resistance is a significant global health concern, contributing to an estimated 4.95 million deaths in 2019 [1]. World Health Organization (WHO) has identified ESKAPE pathogens

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Received: October 22, 2024 - Accepted: February 22, 2025 - Published online: March 20, 2025

(*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp.) as critical contributors of antimicrobial resistance and launched a Global Action Plan in 2015 to optimize antimicrobial use [2]. Recent studies have demonstrated that various plant-derived essential oils possess strong antimicrobial activity, exhibiting effectiveness against both susceptible and resistant microorganisms, including colistin-resistant *Escherichia coli* and *Proteus mirabilis*, as well as methicillin-resistant *S. aureus* [3-5].

Litsea cubeba (Lour.) Pers., commonly known as mountain pepper or may chang, is a potential antimicrobial solution [6]. This evergreen tree, belonging to the *Lauraceae* family, is native to high-altitude regions of South and Southeast Asia and has been traditionally used to treat various ailments, including respiratory infections and traumatic injuries [6]. The essential oil of *L. cubeba* demonstrates diverse bioactivities, particularly antimicrobial properties [7-13], which have been evaluated in solid and liquid phases against specific microorganisms with promising results [14-17]. Limonene is a volatile constituent present in a variety of plants in the *Lauraceae* and *Poaceae* families, such as *L. cubeba* and *Cymbopogon citratus*. A previous study demonstrated excellent antifungal activities of vapor-phase *C. citratus* essential oil on some medically significant pathogens (*Candida albicans*, *C. tropicalis*, and *Aspergillus niger*) [18]. The potent antimicrobial activities of limonene, which is a cyclic monoterpene compound, against Gram-positive bacteria, Gram-negative bacteria, and yeasts have also been addressed [19-22]. However, their potential antimicrobial efficacies in the vapor phase remain underexplored.

The aim of this study was to analyze the chemical composition of *L. cubeba* essential oil extracted from fruits and to evaluate its antimicrobial activities, including the effects of its major component (limonene) across solid, liquid, and vapor phases. The study investigated a wide range of microbial strains, including significant human pathogens, and used time-kill assays to determine killing kinetics and bactericidal time points. Given the limited data on the vapor-phase antimicrobial activity of *L. cubeba* essential oil and its components beyond citral, the present study addressed a critical knowledge gap. The findings are anticipated to provide valuable insights into the application of *L. cubeba* essential oil as an alternative antimicrobial agent, presenting innovative strategies to combat the global challenge of antimicrobial resistance.

Methods

Study design and setting

This study employed an applied experimental research design. Plant samples were collected from Mae Hong Son Province, Thailand, in June 2024. Antimicrobial activity was evaluated using disk diffusion, broth microdilution, and vapor-phase diffusion methods. All experiments were conducted at the Faculty of Medical Technology, Pathum Thani, Thailand, in 2024.

Plant material and chemicals

Fresh *L. cubeba* fruits were collected from mountain areas in Mae Hong Son Province, Northern Thailand. The plant sample was identified by personnel from the Department of Botany, Faculty of Science, Chulalongkorn University, Thailand, with a voucher specimen (BCU No. 015829) and deposited in the herbarium of the same department for reference. The fruits of *L. cubeba* (1 kg) were cleaned and cut into small pieces. The sample was suspended in 2.2 liters of distilled water and extracted through hydrodistillation for three hours. During this process, the essential oil was separated and presented on the surface of the water, where it was collected through a pipette connected to the condenser. To eliminate any residual water from the collected oil, anhydrous sodium sulfate (Na_2SO_4) was used as a drying agent. A pale-yellow essential oil with a citrus-like odor was obtained, with a yield of 4.0% and a density of 0.90 g/mL. Limonene, with a purity of 98.0% and a density of 0.84 g/mL, was obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Both the extracted *L. cubeba* essential oil and purified limonene were stored at 4°C before use.

Analysis of *Litsea cubeba* essential oil

The chemical components of *L. cubeba* essential oil were identified using gas chromatography-mass spectrometry (GC-MS) with an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass selective detector (Agilent Technologies, Santa Clara, CA, USA), equipped with a Mega-5MS capillary column. The operating conditions followed those described in a previous study [23]. Briefly, the gas chromatographic conditions were programmed as follows: the injection temperature was set at 230°C; with oven temperature initially set at 60°C for one minute, and then gradually increased at a rate of 3°C/min up to 240°C and held for five minutes. The carrier gas was helium and maintained at a constant flow rate of 1.0 mL/min. The volume of injection was 1 µL of ethanol solution in a split mode (1:20). The mass spectrometry transfer line temperature was maintained at 250°C utilizing electron ionization mode at 70 eV ionization potential. The mass-to-charge (m/z) range was established from 40 to 650 m/z. Compound identification was achieved by matching their mass spectra fragmentation patterns and retention times with the standard reference compounds, and subsequently verifying their mass spectrometry results against the National Institute of Standards and Technology (NIST) 11 Mass Spectral Database (Gaithersburg, MD, USA) for confirmation.

Microorganisms and culture conditions

The microorganisms used in the experiments comprised five American Type Culture Collection (ATCC) bacterial strains, 42 additional bacterial strains, two filamentous fungi strains, and one yeast strain. The ATCC strains included *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603, and *P. aeruginosa* ATCC 27853, serving as controls for antibiotic susceptibility patterns. A full list of microorganisms utilized in the study is presented in **Table 1**. All tested microorganisms were sourced from the stock cultures (maintained at -20°C) at the Faculty of Medical Technology, Rangsit University, Thailand. Bacterial strains were cultured on blood agar plates at 37°C for 18 to 24 hours, while fungal strains were grown on potato dextrose agar plates at 25°C for 2 to 7 days prior to assay.

Antimicrobial activities of solid-phase *Litsea cubeba* essential oil and purified limonene

The antimicrobial activities of *L. cubeba* essential oil and purified limonene in solid-phase media were evaluated using the disk diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [24] (**Figure 1A**).

Antibacterial activities of solid-phase Litsea cubeba essential oil and purified limonene

Bacterial suspension with a concentration of approximately 10^8 CFU/mL was prepared by adjusting turbidity to 0.5 McFarland standards in sterile normal saline, utilizing a DEN-1 densitometer (Biosan, Riga, Latvia). The suspension was then spread onto 90 mm Mueller-Hinton agar plates or Mueller-Hinton agar with 5% sheep blood plates (Clinag, Bangkok, Thailand), depending on the bacterial type. Sterile disks (6 mm in diameter) impregnated with 10 µL (equivalent to 9.0 mg) of *L. cubeba* essential oil or purified limonene were placed on the agar surface [25] and incubated at 37°C for 18–24 hours. The inhibition zone diameter (IZD), defined as the clear zone surrounding the disk, was measured by using a vernier caliper and reported as IZD in millimeters. Antibacterial activity was classified based on criteria from a previous study: no activity for IZD ≤6 mm, weak activity for >6 mm to ≤12 mm, moderate activity for >12 mm to <20 mm, and strong activity for ≥20 mm [26]. Each assay included sterile disk impregnated with 4% dimethyl sulfoxide as a diluent control and 10 µg gentamicin disk (Oxoid, Hampshire, UK) as an antibiotic susceptibility control. The susceptibility pattern for gentamicin was interpreted according to the IZD quality control ranges specified by the CLSI 2020 [24].

Antifungal activities of solid-phase Litsea cubeba essential oil and purified limonene

To assess antifungal activity, a fungal suspension with a concentration of approximately 10^6 CFU/mL was prepared by adjusting turbidity to 2.0 McFarland standards using sterile normal saline. The suspension was spread onto potato dextrose agar plates. Sterile disks impregnated with 10 µL of *L. cubeba* essential oil or purified limonene were placed on the inoculated agar plates. Amphotericin B was used as an antifungal control. The plates were incubated at 25°C for

24 hours for *C. albicans* or for five days for *Trichophyton rubrum* and *Microsporum gypseum*. The IZD was measured by using a vernier caliper, reported as IZD in millimeters, and interpreted according to CLSI 2022 [27,28].

Table 1. List of the microbial strains used in this study

Microorganisms	Strains
Gram-positive bacteria (n=8)	*Staphylococcus aureus*
	S. saprophyticus
	S. epidermidis
	Streptococcus pyogenes
	S. agalactiae
	Enterococcus faecalis
	Listeria monocytogenes
	Corynebacterium diphtheriae
Gram-negative bacteria (n=34)	*Elizabethkingia meningoseptica*
	Pseudomonas aeruginosa
	Vibrio cholerae
	V. vulnificus
	V. parahaemolyticus
	Aeromonas hydrophila
	A. veronii biovar sobria
	Plesiomonas shigelloides
	Escherichia coli
	Klebsiella pneumoniae
	K. aerogenes
	Citrobacter freundii
	Providencia rettgeri
	P. stuartii
	Proteus vulgaris
	P. mirabilis
	Edwardsiella tarda
	Pantoea agglomerans
	Morganella morganii
	Shigella flexneri
	S. dysenteriae
	S. boydii
	S. sonnei
	Salmonella Typhi
	S. Enteritidis
	S. Paratyphi A
	S. arizonae
	Enterobacter cloacae
	Yersinia enterocolitica
	Serratia rubidaea
	S. marcescens
	Acinetobacter lwoffii
	A. baumannii
	Stenotrophomonas maltophilia
Filamentous fungi and yeast (n=3)	*Trichophyton rubrum*
	Microsporum gypseum
	Nannizzia gypsea
	Candida albicans

Antimicrobial activity of vapor-phase *Litsea cubeba* essential oil and purified limonene

The in vitro antimicrobial activities of vapor-phase *L. cubeba* essential oil and purified limonene were assessed using a vapor-phase diffusion assay, as described previously [18] (**Figure 1B**). *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *T. rubrum*, *M. gypseum*, and *C. albicans* were selected for investigation. Microbial suspensions (approximately 10^8 CFU/mL for bacteria and 10^6 CFU/mL for yeast and filamentous fungi) were spread on either Mueller-Hinton agar or potato dextrose agar plates, depending on the microorganism type. A sterile disk impregnated with 10 µL of *L. cubeba* essential oil or purified limonene was placed on the inside of the upper lid of the agar plate, ensuring no direct contact with the agar medium. The distance between the agar surface and the impregnated disk was approximately 2 mm. The lid was then sealed onto the plate with parafilm. The plates were incubated at 37°C for 48 hours (for bacteria) or 25°C for five days

(for yeast and filamentous fungi), and the IZD was measured. To determine the time-killing point of vapor-phase *L. cubeba* essential oil, the incubation time of the impregnated disk was varied from 0.5 to 8 hours. At predetermined time points, the disk was removed, and the plates were further incubated at 37°C for 24 hours (for bacteria), 25°C for 48 hours (for yeast), or 25°C for five days (for filamentous fungi), followed by measurement of the IZD in millimeters.

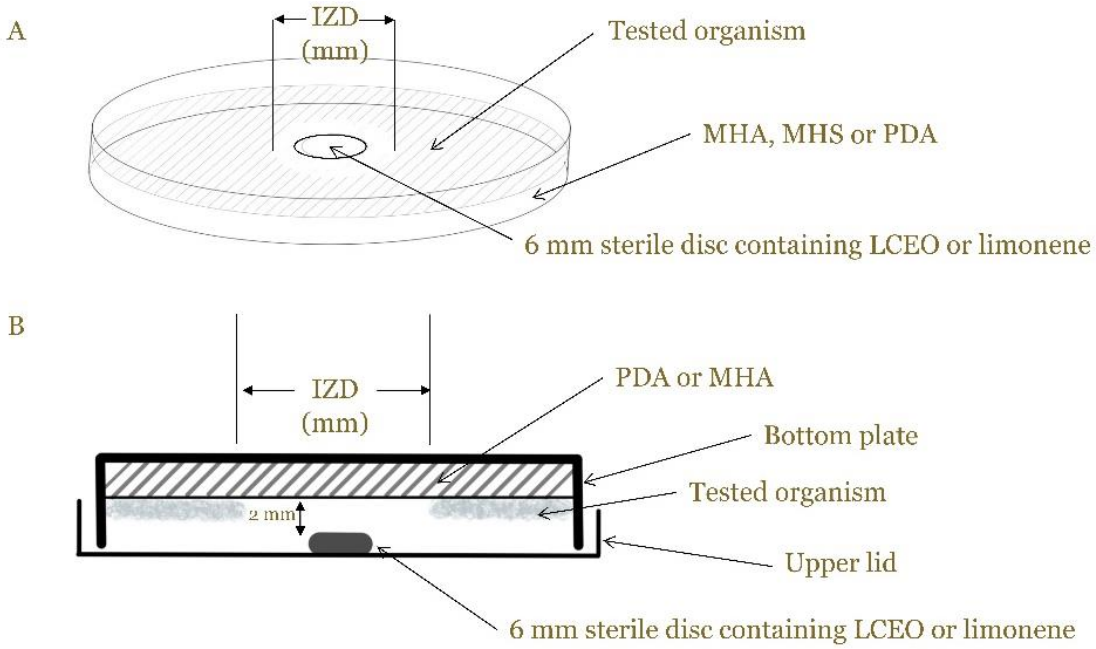


Figure 1. Experimental designs for (A) solid-phase disk diffusion and (B) vapor-phase diffusion assays. IZD: inhibition zone diameter; LCEO: *Litsea cubeba* essential oil; MHA: Mueller-Hinton agar; MHS: Mueller-Hinton agar with 5% sheep blood; PDA: potato dextrose agar.

Antimicrobial activities of liquid-phase *Litsea cubeba* essential oil and purified limonene

Antibacterial activities of liquid-phase Litsea cubeba essential oil and purified limonene

The antibacterial activities of *L. cubeba* essential oil and purified limonene in a liquid-phase medium were assessed using broth macrodilution, as described previously [29]. *L. cubeba* essential oil and purified limonene were dissolved in dimethyl sulfoxide to prepare a stock solution (400 mg/mL). Working solutions were prepared by serial 2-fold dilutions in cationic-adjusted Mueller-Hinton broth (CAMHB) at concentrations ranging from 0.25 mg/mL to 32.0 mg/mL, except for *Streptococcus* species, which were cultured in CAMHB supplemented with 3% lysed horse blood. A bacterial suspension (approximately 10^6 CFU/mL) was added to each concentration, resulting in final concentrations of *L. cubeba* essential oil and purified limonene from 0.125 mg/mL to 16.0 mg/mL. Broth control, bacterial control, and diluent control (4% dimethyl sulfoxide) were included in each experiment. Gentamicin (Himedia, Maharadhtra, India) was used as an antibiotic control, with final concentrations ranging from 0.125 µg/mL to 16.0 µg/mL. The suspensions were incubated at 37°C for 18–24 hours and the bacterial growth was measured by visible turbidity, turbid growth, and clear-no growth. The minimum inhibitory concentration (MIC) was reported as the lowest concentration at which no visible growth was observed. The minimum bactericidal concentration (MBC) was determined by transferring 10 µL of the MIC suspension to Mueller-Hinton agar or Mueller-Hinton agar with sheep blood, followed by incubation at 37°C for 18–24 hours. The MIC index (MBC/MIC ratio) was calculated to classify the antibacterial activity of *L. cubeba* essential oil and purified limonene as bactericidal (when MIC index ≤4), bacteriostatic (when 4< MIC index <32), or tolerant (when MIC index ≥32), following the criteria outlined previously [30].

Antifungal activities of liquid-phase Litsea cubeba essential oil and purified limonene

For antifungal activity testing, *L. cubeba* essential oil and purified limonene were serially diluted in potato dextrose broth at concentrations ranging from 0.03 mg/mL to 2.0 mg/mL. A fungal suspension (approximately 10^3 CFU/mL) was added to each concentration, resulting in final concentrations of *L. cubeba* essential oil and purified limonene from 0.015 mg/mL to 1.0 mg/mL. Amphotericin B (Himedia, Maharashtra, India) was used as an antifungal control, with final concentrations ranging from 0.015 µg/mL to 1.0 µg/mL. The suspensions were incubated at 25°C for 24 hours (for *C. albicans*) or 72 hours (for *T. rubrum* and *M. gypseum*). The MIC was determined as the lowest concentration at which no visible growth was observed. The minimum fungicidal concentration (MFC) was determined by transferring 10 µL of the MIC suspension to a potato dextrose agar plate, followed by incubation at 25°C for 48 hours (for *C. albicans*) or five days (for *T. rubrum* and *M. gypseum*). In each experiment, broth control, fungal control, and diluent control (4% dimethyl sulfoxide) were included. The MFC/MIC ratio was calculated to classify the antifungal activity as fungicidal (when MFC/MIC ratio ≤4) or fungistatic (when MFC/MIC ratio >4), following the criteria outlined by Wiegand *et al*. [31].

Antibacterial kinetic curves of *Litsea cubeba* essential oil

The antibacterial kinetic curve and bactericidal time points of *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 after exposure to *L. cubeba* essential oil were evaluated using a time-killing assay. The bacterial suspension (approximately 5×10^5 CFU/mL) was exposed to *L. cubeba* essential oil at concentrations of 1×MIC and 2×MIC, and incubated at 37°C for 0 to 24 hours. Dimethyl sulfoxide 4% was used as a negative control. Bacterial viability was determined by measuring absorbance at a wavelength of 600 nm using GENESYS 30 visible spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA) at various time points from 0 to 24 hours. The kinetic growth curve was constructed based on bacterial viability at each time point. Additionally, 10 µL of the bacterial suspension exposed to *L. cubeba* essential oil was transferred to Mueller-Hinton agar plates and further incubated for 18 to 24 hours. The bactericidal time point was defined as the specific time at which no bacterial colonies were observed.

Statistical analysis

All experiments were conducted in triplicate. The IZD, MIC, and MBC values were expressed as the median and range. Differences in IZD and MIC values between different compounds were analyzed using the Kruskal-Wallis test and the Mann-Whitney U test. All statistical analyses were performed using SPSS version 21.0 (IBM, Armonk, NY, USA). Statistical significance was considered at a $p<0.05$.

Results

Chemical compositions of *Litsea cubeba* essential oil

The chemical compositions of *Litsea cubeba* essential oil were characterized by GC-MS (**Figure 2**) and 17 compounds were identified in *L. cubeba* essential oil, accounting for 92.34% of the total composition (**Table 2**). The major compounds identified were citral, 1,3,8-p-menthatriene, and *d*-limonene, which comprised 42.53%, 35.18%, and 4.25%, respectively. Citral is an oxygenated monoterpene with a core structure consisting of (2E,6E)-octa-2,6-dienal, substituted with methyl groups at positions 3 and 7. 1,3,8-p-menthatriene is a menthane monoterpenoid, while *d*-limonene is a monoterpene hydrocarbon with core structures of cyclohexa-1,3-diene and cyclohex-1-ene, respectively, each substituted with a methyl group at position 1 and a prop-1-en-2-yl group at position 4 (**Figure 3**).

Antibacterial activities of solid-phase *Litsea cubeba* essential oil and purified limonene

The antibacterial activities of *L. cubeba* essential oil and its primary compound, purified limonene, in a solid-phase medium against reference bacterial strains are summarized in **Table 3**. The antibiotic susceptibility patterns of gentamicin disk (10 µg/disk) against all reference bacterial strains (IZD values 14.0–30.0 mm) were within the acceptable IZD quality control ranges following the CLSI guideline [24]. The activity of solid-phase *L. cubeba* essential oil was

classified into three different groups, including strong, moderate, and weak activities, according to previous study [26]. The results showed that *L. cubeba* essential oil in solid-phase medium had antibacterial activity against all reference bacterial strains (5/5, 100%), with IZD values ranging from 7.0 mm to 29.0 mm. Strong antibacterial activity of *L. cubeba* essential oil in solid-phase medium was observed on *S. aureus* ATCC 25923 and moderate activity was observed on *E. faecalis* ATCC 29212 and *E. coli* ATCC 25922. However, weak activity was observed on *P. aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC 700603. Purified limonene in solid-phase medium showed antibacterial activity against almost all reference bacterial strains (4/5, 80%), with IZD values ranging from 6.0 mm to 19.0 mm. Moderate activity of purified limonene in solid-phase medium was observed against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *K. pneumoniae* ATCC 700603, while weak activity against *E. faecalis* ATCC 29212. However, no inhibitory effect of purified limonene in solid-phase medium on *P. aeruginosa* ATCC 27853. The overall IZD values of *L. cubeba* essential oil in solid-phase medium toward tested reference bacterial strains were comparable to those of purified limonene ($p>0.05$).

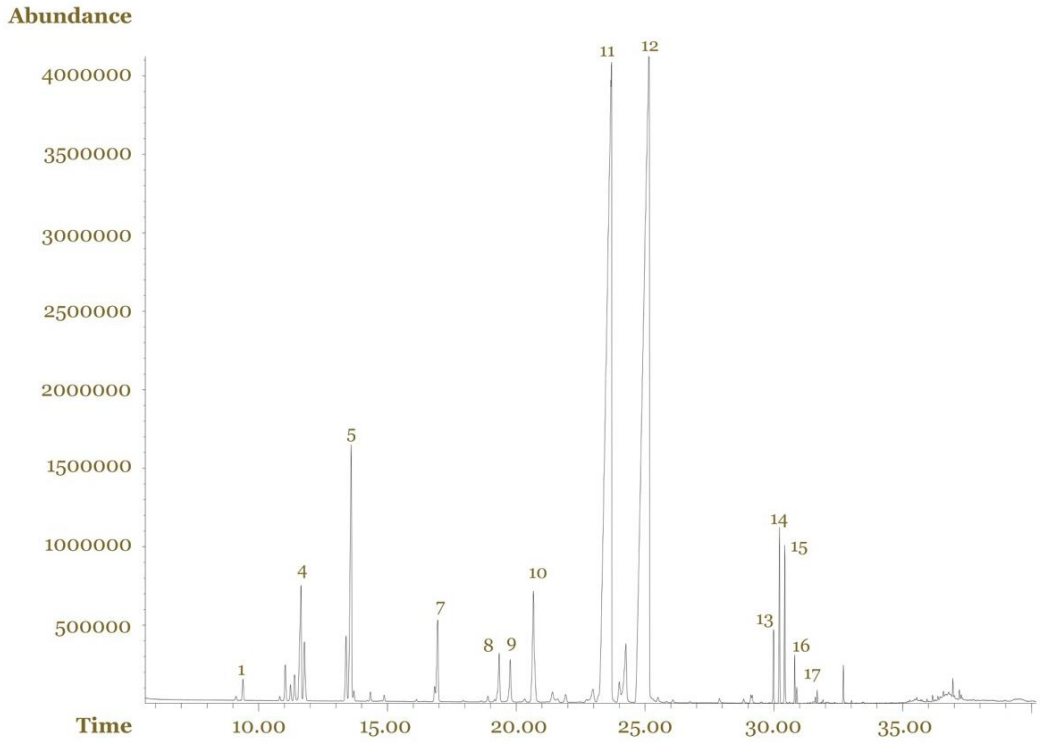


Figure 2. Gas chromatography-mass spectroscopy chromatogram of *Litsea cubeba* essential oil demonstrated three major chemical components, which are composed of *d*-limonene (peak 5), 1,3,8-p-menthatriene (peak 11), and citral (peak 12). The x-axis represents retention time in minute and the y-axis represents abundance of signals in arbitrary unit.

The results of antibacterial activities of *L. cubeba* essential oil and purified limonene in solid-phase medium against 8 strains of Gram-positive bacteria are presented in **Table 4**. The results indicated that antibacterial activity of *L. cubeba* essential oil in solid-phase medium exhibited moderate to strong activity against all tested Gram-positive bacterial strains (n=8/8, 100%), with IZD values ranging from 14.0 mm to 41.0 mm. Strong activity was observed on *S. saprophyticus*, followed by *Listeria monocytogenes*, *S. epidermidis*, *Streptococcus agalactiae*, *Corynebacterium diphtheriae*, *S. pyogenes*, and *S. aureus*, in order of the degree of activity. *S. saprophyticus* is the most susceptible to *L. cubeba* essential oil among Gram-positive bacteria. Meanwhile, moderate activity was observed on *E. faecalis*. Purified limonene in solid-phase medium showed weakly to strongly antibacterial activity against all tested Gram-positive bacterial strains (n=8/8, 100%), with IZD values ranging from 6.0 mm to 30.0 mm. Strong activity was observed on *C. diphtheriae*. Moderate activity was observed on *S. aureus*, *S. epidermidis*, and *S. saprophyticus*, while weak activity was observed on *L. monocytogenes*, *S. agalactiae*, *E. faecalis*, and *S. pyogenes*. The IZD values of *L. cubeba* essential oil in solid-phase

medium against all tested Gram-positive bacterial strains were significantly higher than those of solid-phase purified limonene (p<0.01).

Table 2. Chemical compositions of *Litsea cubeba* essential oil characterized by gas chromatography-mass spectrometry (GC-MS)

Compounds	Molecular formula	Class of compound	Quality	Retention time (min)	Peak area[a] (%)
α-Pinene	$C_{10}H_{16}$	Monoterpene hydrocarbons	94	9.39	0.27±0.005
β-Thujene	$C_{10}H_{16}$	Monoterpene hydrocarbons	81	11.03	0.45±0.006
β-Pinene	$C_{10}H_{16}$	Monoterpene hydrocarbons	95	11.23	0.23±0.002
6-methyl-5-Hepten-2-one	$C_8H_{14}O$	Monoterpenoid ketone	94	11.64	2.04±0.013
d-Limonene	$C_{10}H_{16}$	Monoterpene hydrocarbons	99	13.59	4.25±0.019
β-Ocimene	$C_{10}H_{16}$	Monoterpene hydrocarbons	91	14.34	0.17±0.003
3-Carene	$C_{10}H_{16}$	Monoterpene hydrocarbons	95	16.95	1.22±0.021
Citronellal	$C_{10}H_{18}O$	Monoterpenoid aldehyde	91	19.33	0.83±0.016
cis-p-Mentha-1(7), 8-dien-2-ol	$C_{10}H_{16}O$	Oxygenated monoterpenes	52	19.76	0.78±0.015
2-Methyl-cis-3a,4,7,7a-tetrahydroindan	$C_{10}H_{16}$	Monoterpene hydrocarbons	83	20.66	2.39±0.030
1,3,8-p-Menthatriene	$C_{10}H_{14}$	Menthane monoterpenoids	83	23.69	35.18±0.287
Citral	$C_{10}H_{16}O$	Oxygenated monoterpenes	96	25.16	42.53±0.790
α-Cubebene	$C_{15}H_{24}$	Sesquiterpene hydrocarbons	78	29.16	0.08±0.002
β-Caryophyllene	$C_{15}H_{24}$	Sesquiterpene hydrocarbons	99	30.21	1.44±0.023
β-Farnesene	$C_{15}H_{24}$	Sesquiterpene hydrocarbons	94	30.81	0.32±0.005
α-Caryophyllene	$C_{15}H_{24}$	Sesquiterpene hydrocarbons	98	30.88	0.12±0.002
α-Farnesene	$C_{15}H_{24}$	Sesquiterpene hydrocarbons	83	31.61	0.04±0.001

[a]Data are expressed as mean ± standard deviation from triplicate measurements

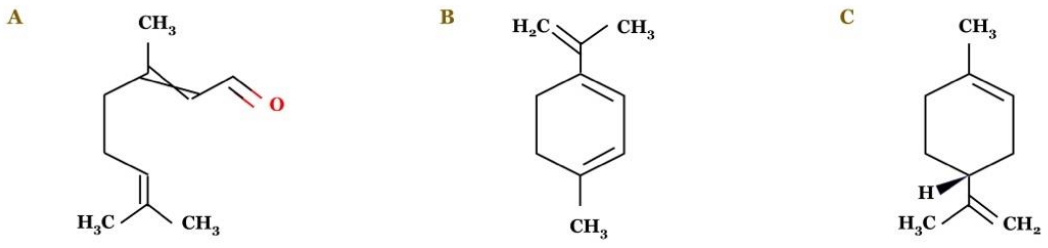


Figure 3. Molecular structures of major compounds in *Litsea cubeba* essential oil; (A) citral, (B) 1,3,8-p-menthatriene, and (C) *d*-limonene.

The antibacterial activities of *L. cubeba* essential oil and purified limonene in solid-phase media against 34 strains of Gram-negative bacteria were also determined (**Table 5**). It showed that *L. cubeba* essential oil in solid-phase medium showed antibacterial activity against all tested Gram-negative bacterial strains (n=34/34, 100%), with IZD values ranging from 7.0 mm to 48.0 mm. Strong antibacterial activity of *L. cubeba* essential oil was observed on 11 strains of Gram-negative bacteria (n=11/34, 32.4%); *A. lwoffii*, *Elizabethkingia meningoseptica*, *Vibrio cholerae*, *Plesiomonas shigelloides*, *Shigella dysenteriae*, *V. parahaemolyticus*, *Aeromonas hydrophila*, *Yersinia enterocolitica*, *V. vulnificus*, *A. veronii* biovar sobria, and *S. flexneri*, in order of the

degree of activity, while moderate activity was observed on 11 strains (n=11/34, 32.4%); *S. boydii*, *Stenotrophomonas maltophilia*, *Edwardsiella tarda*, *P. vulgaris*, *Enterobacter cloacae*, *S. sonnei*, *A. baumannii*, *Providencia stuartii*, *Morganella morganii*, *E. coli*, and *K. aerogenes*. Weak activity was observed on 12 strains of Gram-negative bacteria (n=12/34, 35.2%); *P. mirabilis*, *Serratia rubidaea*, *Salmonella* Typhi, *Citrobacter freundii*, *P. rettgeri*, *S. arizonae*, *K. pneumoniae*, *S.* Enteritidis, *S.* Paratyphi A, *Pantoea agglomerans*, *S. marcescens*, and *P. aeruginosa*. *A. lwoffii* was the most susceptible to *L. cubeba* essential oil among Gram-negative bacteria. In addition, *L. cubeba* essential oil posed an inhibitory effect on gentamicin-resistant *A. baumannii*. Purified limonene in solid-phase medium exhibited antibacterial activity against most tested Gram-negative bacterial strains (n=29/34, 85.3%), with IZD values ranging from 6.0 mm to 22.0 mm. The IZD values of *L. cubeba* essential oil against all tested Gram-negative bacteria were higher than those of purified limonene with significant differences ($p<0.01$). These results demonstrated that the overall antibacterial activities of *L. cubeba* essential oil in solid and liquid-phase media against Gram-negative bacteria were more effective than those of solid and liquid-phase purified limonene.

Antibacterial activities of liquid-phase *Litsea cubeba* essential oil and purified limonene

The antibacterial activity of *L. cubeba* essential oil and its primary compound, purified limonene, in liquid-phase media against reference bacterial strains are presented in **Table 3**. The antibiotic susceptibility patterns of gentamicin in a liquid medium against all reference bacterial strains (MIC values 0.1–16.0 μg/mL) were within the acceptable MIC quality control ranges following the CLSI guideline [24]. Both liquid-phase *L. cubeba* essential oil and purified limonene exhibited antibacterial activity against most reference strains (n=4/5, 80%); *E. faecalis* ATCC 29212, *E. coli* ATCC 25923, *K. pneumoniae* ATCC 700603, and *S. aureus* ATCC 25923 (MIC values: 0.5–8.0 mg/mL vs 2.0–16.0 mg/mL) ($p<0.05$). No inhibitory effect of liquid-phase *L. cubeba* essential oil was observed on *P. aeruginosa* ATCC 27853, with the MIC value higher than 16.0 mg/mL. The MIC indexes of *L. cubeba* essential oil and purified limonene were observed at values lesser than or equal to 4.0 mg/mL against all tested ATCC bacterial strains, except for *P. aeruginosa* ATCC 27853, indicating their bactericidal effects. However, the MIC indexes of *L. cubeba* essential oil and purified limonene against *P. aeruginosa* ATCC 27853 could not be interpreted due to the MIC and MBC values exceeding 16.0 mg/mL.

The results of antibacterial activities of *L. cubeba* essential oil and purified limonene in liquid-phase media against 8 strains of Gram-positive bacteria are presented in **Table 4**. Similarity to solid-phase, it showed that *L. cubeba* essential oil in liquid-phase medium exhibited excellent inhibitory activity against all tested Gram-positive bacterial strains (n=8/8, 100%); *S. agalactiae*, followed by *L. monocytogenes*, *C. diphtheriae*, *S. aureus*, *S. epidermidis*, *S. pyogenes*, *S. saprophyticus*, and *E. faecalis*, in order of the degree of activity, with MIC values ranging from 0.1 mg/mL to 2.0 mg/mL. *S. agalactiae* was the most susceptible to *L. cubeba* essential oil among Gram-positive bacteria. Purified limonene also exhibited inhibitory effect but at different degrees of activity against all tested Gram-positive bacteria (n=8/8, 100%); *S. pyogenes*, *S. agalactiae*, *S. saprophyticus*, *C. diphtheriae*, *S. aureus*, *L. monocytogenes*, *E. faecalis*, and *S. epidermidis*, in order of the degree of activity, with MIC ranging from 0.1 mg/mL to 8.0 mg/mL. The MIC values of *L. cubeba* essential oil against almost all tested Gram-positive bacteria were significantly lower than those of purified limonene, except for only *S. pyogenes* ($p<0.01$). These results demonstrated that the overall antibacterial activities against Gram-positive bacteria of *L. cubeba* essential oil in solid and liquid-phase media were more effective than those of purified limonene. Regarding the MIC indexes, the bactericidal effect of *L. cubeba* essential oil was found on almost all tested Gram-positive bacteria (n=7/8, 87.5%); *S. aureus*, *S. saprophyticus*, *S. epidermidis*, *S. pyogenes*, *E. faecalis*, *L. monocytogenes*, and *C. diphtheriae* (MIC indexes: 1.0–2.0), while the bacteriostatic effect was found only on *S. agalactiae* (MIC index: 5.0).

L. cubeba essential oil in liquid-phase medium exhibited excellent activity on almost all tested Gram-negative bacterial strains (n=33/34, 97.1%), except for *P. aeruginosa*, with MIC values ranging from 0.1 to 8.0 mg/mL (**Table 5**). *E. meningoceptica* was the most susceptible

among Gram-negative bacteria. Meanwhile, purified limonene showed inhibitory effect against most of tested Gram-negative bacterial strains (n=25/34, 73.5%); *V. cholerae*, *V. vulnificus*, *P. shigelloides*, and *A. lwoffii*, followed by *E. meningoseptica*, *V. parahaemolyticus*, *S. flexneri*, *A. hydrophila*, *A. veronii* biovar sobria, *S. sonnei*, *C. freundii*, *E. tarda*, *E. coli*, *S.* Paratyphi A, *S.* Enteritidis, *S. dysenteriae*, *P. agglomerans*, *S. maltophilia*, *S. boydii*, *S. rubidaea*, *Y. enterocolitica*, *P. vulgaris*, *M. morganii*, *K. aerogenes*, and *A. baumannii*, with MIC values ranging from 0.1 mg/mL to higher than 16.0 mg/mL. The MIC values of *L. cubeba* essential oil against almost all tested Gram-positive bacteria were significantly lower than those of purified limonene, except for *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus*, *A. hydrophila*, *A. veronii* biovar sobria, *P. shigelloides*, *C. freundii*, *E. tarda*, *S. sonnei*, and *A. lwoffii* ($p<0.01$). These results demonstrated that the overall antibacterial activities of *L. cubeba* essential oil in liquid-phase medium against Gram-positive and Gram-negative bacteria were more effective than those of purified limonene. Both *L. cubeba* essential oil and purified limonene in liquid-phase media could not inhibit *P. aeruginosa*. Regarding the MIC indexes, the bactericidal effect of *L. cubeba* essential oil was found on almost all tested Gram-negative bacterial strains (n=33/34, 97.1%) (MIC indexes: 1.0–2.0), except for *P. aeruginosa* in which the MIC index could not be interpreted since the MIC and MBC values are higher than 16.0 mg/mL.

Antibacterial activities of vapor-phase *Litsea cubeba* essential oil and purified limonene

The results indicated that vapor-phase *L. cubeba* essential oil and purified limonene exhibited antibacterial activity against *S. aureus* ATCC 25923, and *E. coli* ATCC 25922 after incubation with impregnated disks for 24 hours, as presented in **Figure 4**. Interestingly, a large inhibition zone of vapor-phase *L. cubeba* essential oil was observed on *S. aureus* ATCC 25923 (**Figure 4A**), while a large inhibition zone of vapor-phase purified limonene was observed on *E. coli* ATCC 25922 (**Figure 4D**). No inhibition zone was observed on *S. aureus* ATCC 25923 exposed to purified limonene and *E. coli* ATCC 25923 exposed to *L. cubeba* essential oil (**Figures 4B** and **4C**). These findings demonstrated that both vapor-phase *L. cubeba* and purified limonene contained antibacterial activity in a different manner. A vapor-phase *L. cubeba* essential oil posed a higher activity on Gram-positive bacteria, whereas a vapor-phase purified limonene posed a higher activity on Gram-negative bacteria.

The time point for the inhibitory effect of vapor-phase *L. cubeba* essential oil on the Gram-positive bacterium *S. aureus* ATCC 25923 was further evaluated. It was found that *L. cubeba* essential oil initially inhibited *S. aureus* ATCC 25923 after being exposed to vapor-phase *L. cubeba* essential oil for 5 hours, as presented in **Figure 5**. At this time point, a small inhibition zone had appeared. However, the zone was not clear. The clear zone was observed at 8 hours, with an IZD value of 8.0 mm.

Time-killing kinetic and bactericidal time point

The time-killing kinetic curves and bactericidal time points of *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 after exposure to *L. cubeba* essential oil are presented in **Figure 6**. The viability of *S. aureus* ATCC 25923 in the 4% dimethyl sulfoxide control gradually increased within the first 5 hours and then rapidly increased from 6 to 24 hours (**Figure 6A**). Similarly, the viability of *E. coli* ATCC 25922 in the dimethyl sulfoxide control initially increased within 3 hours, followed by a rapid increase from 4 to 24 hours (**Figure 6B**). These results indicate that dimethyl sulfoxide, used as an oil-dissolving solvent in this study, had no inhibitory effect on the tested bacteria. After exposure to 1×MIC and 2×MIC of *L. cubeba* essential oil, the viabilities of *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were suppressed within 0.5 hours of exposure, showing a horizontal, straight-growth curve with no significant changes over the 24-hour period.

The bactericidal time points were further investigated through sub-cultivation on Mueller-Hinton agar plates. Dimethyl sulfoxide controls of *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 showed bacterial colonies on the agar surfaces at all experimental time points. In contrast, after exposure to *L. cubeba* essential oil at both 1×MIC and 2×MIC, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were completely eradicated, with no observable bacterial colonies present within 0.5 hours of exposure. These findings demonstrated that these concentrations of *L. cubeba* essential oil show rapid bactericidal efficacy.

Table 3. Antibacterial activities of *Litsea cubeba* essential oil and purified limonene in solid and liquid-phase media against American Type Culture Collection bacterial strains by disk diffusion and broth macrodilution assays

Reference bacterial strain (n=5)	Disk diffusion, IZD (mm)				Broth macrodilution									
					Gentamicin (µg/mL)			LCEO (mg/mL)			Purified limonene (mg/mL)			
	Gentamicin	LCEO	Purified limonene	*p*-value[b]	MIC	MBC	MIC index	MIC	MBC	MIC index	MIC	MBC	MIC index	*p*-value[c]
Staphylococcus aureus ATCC 25923	29.0 (29.0–30.0)[a]	28.0 (24.0–29.0) (ST)	18.0 (16.0–19.0) (M)	0.032*	0.1 (0.1–0.1) (NI)	2.0 (2.0–2.0)	20.0 (BS)	4.0 (4.0–8.0)	8.0 (8.0–8.0)	2.0 (BC)	8.0 (8.0–8.0)	8.0 (8.0–8.0)	1.0 (BC)	0.114
Enterococcus faecalis ATCC 29212	22.0 (22.0–22.0) (NI)	18.0 (18.0–18.0) (M)	10.0 (9.0–10.0) (W)	0.034*	16.0 (16.0–16.0)[a]	>16.0	ND	0.5 (0.5–0.5)	1.0 (1.0–1.0)	2.0 (BC)	16.0 (16.0–16.0)	16.0 (16.0–16.0)	1.0 (BC)	0.025*
Escherichia coli ATCC 25922	22.0 (21.0–22.0)[a]	13.0 (12.0–15.0) (M)	13.0 (11.0–13.0) (M)	0.487	1.0 (1.0–2.0)[a]	4.0 (4.0–4.0)	4.0 (BC)	4.0 (4.0–4.0)	4.0 (4.0–4.0)	1.0 (BC)	2.0 (2.0–2.0)	2.0 (2.0–2.0)	1.0 (BC)	0.025*
Pseudomonas aeruginosa ATCC 27853	19.0 (19.0–20.0)[a]	9.0 (7.0–9.0) (W)	6.0 (6.0–6.0) (N)	0.034*	2.0 (1.0–2.0)[a]	4.0 (2.0–4.0)	2.0 (BC)	>16.0	>16.0	ND	>16.0	>16.0	ND	1.000
Klebsiella pneumoniae ATCC 700603	14.0 (14.0–14.0) (NI)	8.0 (8.0–8.0) (W)	17.0 (16.0–18.0) (M)	0.037*	16.0 (8.0–16.0) (NI)	16.0 (8.0–16.0)	1.0 (BC)	4.0 (4.0–4.0)	4.0 (4.0–4.0)	1.0 (BC)	16.0 (16.0–16.0)	16.0 (16.0–16.0)	1.0 (BC)	0.025*
Total	22.0 (14.0–30.0)	13.0 (7.0–29.0)	13.0 (6.0–19.0)	0.648	2.0 (0.1–16.0)	4.0 (2.0–16.0)		4.0 (0.5–8.0)	4.0 (1.0–8.0)		12.0 (2.0–16.0)	12.0 (2.0–16.0)		0.023*

Susceptibility profile against gentamicin was interpreted using disk diffusion and MIC QC ranges by CLSI [24]. The degree of solid-phase activity of *Litsea cubeba* essential oil (LCEO) and purified limonene: no activity (N), weak (W), moderate (M), and strong (ST). The type of antibacterial activity: bactericidal (BC), bacteriostatic (BS), and tolerant (T). IZD: inhibition zone diameter; MBC: minimum bactericidal concentration; MIC: minimum inhibitory concentration; ND: not determined when MIC or MBC values exceed 16.0 µg/mL for gentamicin and 16.0 mg/mL for LCEO and purified limonene; NI: not interpreted due to no IZD or MIC breakpoint by CLSI guidelines

[a]IZD and MIC quality control ranges by CLSI [24]

[b]Comparison in IZD values between LCEO and purified limonene

[c]Comparison in MIC values between LCEO and purified limonene

*Statistically significant at $p<0.05$

Table 4. Antibacterial activities of *Litsea cubeba* essential oil and purified limonene in solid and liquid-phase media against Gram-positive bacteria by disk diffusion and broth macrodilution assays

Gram-positive bacterial strain (n=8)	Disk diffusion, IZD (mm)				Broth macrodilution									
					Gentamicin (µg/mL)			LCEO (mg/mL)			Purified limonene (mg/mL)			
	Gentamicin	LCEO	Purified limonene	*p*-value[a]	MIC	MBC	MIC index	MIC	MBC	MIC index	MIC	MBC	MIC index	*p*-value[b]
Staphylococcus aureus	30.0 (30.0–30.0) (S)	20.0 (16.0–24.0) (ST)	17.0 (12.0–22.0) (M)	0.372	0.3 (0.1–0.3) (S)	1.0 (0.5–1.0)	3.3 (BC)	0.5 (0.5–0.5)	1.0 (1.0–1.0)	2.0 (BC)	2.0 (2.0–4.0)	4.0 (4.0–8.0)	2.0 (BC)	0.034*
S. saprophyticus	36.0 (34.0–38.0) (S)	36.0 (30.0–41.0) (ST)	16.0 (12.0–16.0) (M)	0.028*	0.1 (0.1–0.1) (S)	0.5 (0.5–1.0)	5.0 (BS)	0.5 (0.5–1.0)	1.0 (1.0–1.0)	2.0 (BC)	2.0 (2.0–2.0)	4.0 (4.0–8.0)	2.0 (BC)	0.034*
S. epidermidis	34.0 (32.0–34.0) (S)	30.0 (28.0–40.0) (ST)	16.0 (14.0–16.0) (M)	0.028*	0.1 (0.1–0.1) (S)	0.3 (0.3–0.5)	3.0 (BC)	0.5 (0.5–0.5)	0.5 (0.5–1.0)	1.0 (BC)	8.0 (8.0–8.0)	16.0 (16.0–16.0)	2.0 (BC)	0.025*
Streptococcus pyogenes	28.0 (26.0–30.0) (NI)	26.0 (20.0–30.0) (ST)	7.0 (6.0–8.0) (W)	0.032*	0.3 (0.1–1.0) (NI)	1.0 (0.5–4.0)	3.3 (BC)	0.5 (0.5–0.5)	1.0 (1.0–1.0)	2.0 (BC)	0.3 (0.1–0.3)	1.0 (1.0–1.0)	3.3 (BC)	0.034*

Gram-positive bacterial strain (n=8)	Disk diffusion, IZD (mm)				Broth macrodilution									
					Gentamicin (µg/mL)			LCEO (mg/mL)			Purified limonene (mg/mL)			
	Gentamicin	LCEO	Purified limonene	*p*-value[a]	MIC	MBC	MIC index	MIC	MBC	MIC index	MIC	MBC	MIC index	*p*-value[b]
S. agalactiae	24.0 (23.0–24.0) (NI)	30.0 (27.0–36.0) (ST)	8.0 (8.0–9.0) (W)	0.046*	4.0 (2.0–4.0) (NI)	4.0 (4.0–8.0)	1.0 (BC)	0.1 (0.1–0.1)	0.5 (0.5–0.5)	5.0 (BS)	0.3 (0.1–0.3)	2.0 (2.0–2.0)	6.7 (BS)	0.114
Enterococcus faecalis	19.0 (19.0–20.0) (NI)	18.0 (14.0–20.0) (M)	7.0 (7.0–7.0) (W)	0.019*	16.0 (8.0–16.0) (NI)	>16.0	ND	2.0 (1.0–2.0)	2.0 (2.0–2.0)	1.0 (BC)	8.0 (4.0–8.0)	8.0 (8.0–8.0)	1.0 (BC)	0.043*
Listeria monocytogenes	34.0 (32.0–34.0) (NI)	35.0 (30.0–36.0) (ST)	9.0 (8.0–14.0) (W)	0.032*	0.3 (0.1–0.3) (NI)	0.5 (0.5–0.5)	1.7 (BC)	0.3 (0.3–0.3)	0.5 (0.5–0.5)	1.7 (BC)	4.0 (2.0–4.0)	4.0 (4.0–4.0)	1.0 (BC)	0.034*
Corynebacterium diphtheriae	33.0 (32.0–34.0) (NI)	31.0 (30.0–32.0) (ST)	30.0 (28.0–30.0) (ST)	0.105	0.1 (0.1–0.1) (S)	0.1 (0.1–0.1)	1.0 (BC)	0.3 (0.3–0.3)	0.5 (0.3–0.5)	1.7 (BC)	2.0 (2.0–2.0)	2.0 (2.0–4.0)	1.0 (BC)	0.025*
Total	31.0 (19.0–38.0)	30.0 (14.0–41.0)	12.0 (6.0–30.0)	0.000**	0.2 (0.1–16.0)	0.5 (0.1–8.0)		0.5 (0.1–2.0)	1.0 (0.25–2.0)		2.0 (0.1–8.0)	4.0 (1.0–16.0)		0.001**

Susceptibility profile against gentamicin was interpreted using zone diameter breakpoint: susceptible (S), intermediate (I), and resistant (R) [24]. The degree of solid-phase activity of *Litsea cubeba* essential oil (LCEO) and purified limonene: no activity (N), weak (W), moderate (M), and strong (ST). The type of antibacterial activity: bactericidal (BC), bacteriostatic (BS), and tolerant (T). IZD: inhibition zone diameter; MBC: minimum bactericidal concentration; MIC: minimum inhibitory concentration; ND: not determined when MIC or MBC values exceed 16.0 µg/mL for gentamicin and 16.0 mg/mL for LCEO and purified limonene; NI: not interpreted due to no IZD or MIC breakpoint by CLSI guideline [24]

[a]Comparison in IZD values between LCEO and purified limonene

[b]Comparison in MIC values between LCEO and purified limonene

*Statistically significant at $p<0.05$

**Statistically significant at $p<0.01$

Table 5. Antibacterial activities of *Litsea cubeba* essential oil and purified limonene in solid and liquid-phase media against Gram-negative bacteria by disk diffusion and broth macrodilution assays

Gram-negative bacterial strain (n=34)	Disk diffusion, IZD (mm)				Broth macrodilution									
					Gentamicin (µg/mL)			LCEO (mg/mL)			Purified limonene (mg/mL)			
	Gentamicin	LCEO	Purified limonene	*p*-value[a]	MIC	MBC	MIC index	MIC	MBC	MIC index	MIC	MBC	MIC index	*p*-value[b]
Elizabethkingia meningoseptica	40.0 (36.0–42.0) (NI)	40.0 (40.0–40.0) (ST)	32.0 (32.0–33.0) (ST)	0.034*	>16.0 (R)	>16.0	ND	0.3 (0.1–0.3)	0.3 (0.1–0.3)	1.0 (BC)	0.5 (0.5–0.5)	1.0 (1.0–1.0)	2.0 (BC)	0.034*
Pseudomonas aeruginosa	22.0 (21.0–23.0) (S)	8.0 (8.0–9.0) (W)	6.0 (6.0–6.0) (N)	0.034*	0.5 (0.5–1.0) (S)	0.5 (0.5–2.0)	1.0 (BC)	>16.0	>16.0	ND	>16.0	>16.0	ND	1.000
Vibrio cholerae	26.0 (22.0–26.0) (S)	40.0 (40.0–40.0) (ST)	25.0 (24.0–25.0) (ST)	0.034*	2.0 (1.0–2.0) (S)	2.0 (2.0–2.0)	1.0 (BC)	0.3 (0.3–0.3)	0.5 (0.3–0.5)	1.7 (BC)	0.1 (0.1–0.1)	0.1 (0.1–0.1)	1.0 (BC)	0.025*
V. vulnificus	18.0 (18.0–20.0) (S)	26.0 (21.0–28.0) (ST)	25.0 (24.0–25.0) (ST)	0.507	4.0 (4.0–4.0) (S)	8.0 (8.0–8.0)	2.0 (BC)	4.0 (4.0–4.0)	4.0 (4.0–4.0)	1.0 (BC)	0.1 (0.1–0.1)	0.1 (0.1–0.1)	1.0 (BC)	0.025*
V. parahaemolyticus	19.0 (18.0–22.0) (S)	30.0 (29.0–32.0) (ST)	20.0 (18.0–28.0) (ST)	0.050	4.0 (4.0–4.0) (S)	8.0 (8.0–8.0)	2.0 (BC)	4.0 (4.0–4.0)	4.0 (4.0–8.0)	1.0 (BC)	0.5 (0.5–0.5)	0.5 (0.5–0.5)	1.0 (BC)	0.025*
Aeromonas hydrophila	22.0 (22.0–23.0) (S)	30.0 (28.0–31.0) (ST)	19.0 (14.0–20.0) (M)	0.032*	2.0 (2.0–2.0) (S)	2.0 (2.0–2.0)	1.0 (BC)	4.0 (4.0–4.0)	4.0 (4.0–4.0)	1.0 (BC)	2.0 (1.0–2.0)	8.0 (4.0–8.0)	4.0 (BC)	0.034*
A. veronii biovar sobria	20.0 (19.0–20.0) (S)	22.0 (22.0–23.0) (ST)	18.0 (18.0–20.0) (M)	0.043*	4.0 (4.0–4.0) (S)	8.0 (8.0–8.0)	2.0 (BC)	4.0 (4.0–4.0)	4.0 (4.0–4.0)	1.0 (BC)	2.0 (1.0–2.0)	8.0 (4.0–8.0)	4.0 (BC)	0.034*
Plesiomonas shigelloides	22.0 (16.0–22.0) (S)	40.0 (35.0–44.0) (ST)	36.0 (30.0–38.0) (ST)	0.275	1.0 (1.0–2.0) (S)	8.0 (4.0–8.0)	8.0 (BS)	1.0 (0.5–1.0)	1.0 (0.5–1.0)	1.0 (BC)	0.1 (0.1–0.1)	0.3 (0.1–0.3)	3.0 (BC)	0.034*
Escherichia coli	21.0 (20.0–21.0) (S)	12.0 (12.0–14.0) (M)	14.0 (12.0–15.0) (M)	0.346	1.0 (1.0–1.0) (S)	2.0 (1.0–2.0)	2.0 (BC)	2.0 (2.0–4.0)	2.0 (2.0–4.0)	1.0 (BC)	4.0 (4.0–4.0)	4.0 (4.0–4.0)	1.0 (BC)	0.114

Gram-negative bacterial strain (n=34)	Disk diffusion, IZD (mm)				Broth macrodilution									
					Gentamicin (µg/mL)			LCEO (mg/mL)			Purified limonene (mg/mL)			
	Gentamicin	LCEO	Purified limonene	*p*-value[a]	MIC	MBC	MIC index	MIC	MBC	MIC index	MIC	MBC	MIC index	*p*-value[b]
Klebsiella pneumoniae	22.0 (21.0–24.0) (S)	8.0 (8.0–12.0) (W)	6.0 (6.0–7.0) (W)	0.043*	1.0 (0.5–1.0) (S)	2.0 (1.0–2.0)	2.0 (BC)	8.0 (8.0–8.0)	8.0 (8.0–8.0)	1.0 (BC)	>16.0	>16.0	ND	0.025*
Citrobacter freundii	20.0 (20.0–21.0) (S)	12.0 (8.0–12.0) (W)	14.0 (13.0–20.0) (M)	0.046*	2.0 (1.0–2.0) (S)	2.0 (2.0–2.0)	1.0 (BC)	4.0 (4.0–4.0)	4.0 (4.0–4.0)	1.0 (BC)	2.0 (2.0–2.0)	4.0 (4.0–4.0)	2.0 (BC)	0.025*
Providencia rettgeri	15.0 (15.0–16.0) (S)	10.0 (10.0–11.0) (W)	6.0 (6.0–6.0) (N)	0.034*	>16.0 (R)	>16.0	ND	4.0 (4.0–4.0)	4.0 (4.0–4.0)	1.0 (BC)	>16.0	>16.0	ND	0.025*
P. stuartii	20.0 (19.0–21.0) (S)	14.0 (10.0–15.0) (M)	6.0 (6.0–6.0) (N)	0.037*	16.0 (4.0–16.0) (I)	16.0 (16.0–16.0)	1.0 (BC)	8.0 (8.0–8.0)	8.0 (8.0–8.0)	1.0 (BC)	>16.0	>16.0	ND	0.025*
Proteus vulgaris	23.0 (22.0–24.0) (S)	15.0 (14.0–17.0) (M)	9.0 (6.0–10.0) (W)	0.050	8.0 (4.0–8.0) (S)	8.0 (8.0–8.0)	1.0 (BC)	1.0 (1.0–1.0)	2.0 (1.0–2.0)	2.0 (BC)	16.0 (16.0–16.0)	16.0 (16.0–16.0)	1.0 (BC)	0.025*
P. mirabilis	22.0 (22.0–22.0) (S)	12.0 (12.0–12.0) (W)	6.0 (6.0–6.0) (N)	0.025*	8.0 (4.0–8.0) (S)	8.0 (8.0–8.0)	1.0 (BC)	4.0 (4.0–4.0)	4.0 (4.0–4.0)	1.0 (BC)	>16.0	>16.0	ND	0.025*
Edwardsiella tarda	20.0 (18.0–22.0) (S)	21.0 (10.0–24.0) (M)	8.0 (6.0–9.0) (W)	0.050	4.0 (4.0–8.0) (S)	4.0 (4.0–8.0)	1.0 (BC)	4.0 (4.0–4.0)	8.0 (4.0–8.0)	2.0 (BC)	4.0 (2.0–4.0)	8.0 (8.0–8.0)	2.0 (BC)	0.317
Pantoea agglomerans	21.5 (20.0–22.0) (S)	8.0 (7.0–10.0) (W)	6.0 (6.0–6.0) (N)	0.121	2.0 (0.5–2.0) (S)	2.3 (0.5–4.0)	ND	1.0 (1.0–1.0)	1.0 (1.0–1.0)	1.0 (BC)	8.0 (4.0–8.0)	16.0 (8.0–16.0)	2.0 (BC)	0.034*
Morganella morganii	24.0 (20.0–24.0) (S)	14.0 (11.0–14.0) (M)	10.0 (9.0–10.0) (W)	0.043*	4.0 (2.0–4.0) (S)	4.0 (4.0–4.0)	1.0 (BC)	1.0 (1.0–1.0)	1.0 (1.0–1.0)	1.0 (BC)	16.0 (16.0–16.0)	16.0 (16.0–16.0)	1.0 (BC)	0.025*
Shigella flexneri	18.0 (18.0–22.0) (S)	20.0 (20.0–22.0) (ST)	15.0 (14.0–16.0) (M)	0.046*	4.0 (2.0–4.0) (S)	4.0 (4.0–4.0)	1.0 (BC)	1.0 (1.0–1.0)	2.0 (1.0–2.0)	2.0 (BC)	1.0 (1.0–1.0)	2.0 (1.0–2.0)	2.0 (BC)	1.000
S. dysenteriae	18.0 (18.0–18.0) (S)	30.0 (24.0–38.0) (ST)	21.0 (20.0–23.0) (ST)	0.050*	0.5 (0.5–0.5) (S)	1.0 (0.5–1.0)	2.0 (BC)	1.0 (1.0–1.0)	2.0 (1.0–2.0)	2.0 (BC)	8.0 (2.0–8.0)	16.0 (2.0–16.0)	2.0 (BC)	0.034*
S. boydii	21.0 (20.0–27.0) (S)	20.0 (16.0–22.0) (M)	14.0 (13.0–18.0) (M)	0.127	4.0 (4.0–8.0) (S)	8.0 (8.0–8.0)	1.0 (BC)	1.0 (1.0–1.0)	2.0 (2.0–2.0)	2.0 (BC)	8.0 (8.0–8.0)	16.0 (16.0–16.0)	2.0 (BC)	0.025*
S. sonnei	20.0 (18.0–20.0) (S)	17.0 (10.0–18.0) (M)	17.0 (16.0–17.0) (M)	0.817	4.0 (4.0–4.0) (S)	4.0 (4.0–4.0)	1.0 (BC)	4.0 (2.0–4.0)	4.0 (2.0–4.0)	1.0 (BC)	2.0 (1.0–2.0)	4.0 (2.0–4.0)	2.0 (BC)	0.099
Salmonella Typhi	30.0 (27.0–31.0) (S)	11.0 (10.0–14.0) (W)	14.0 (9.0–14.0) (M)	0.817	0.5 (0.5–0.5) (S)	1.0 (1.0–1.0)	2.0 (BC)	4.0 (4.0–4.0)	4.0 (4.0–4.0)	1.0 (BC)	>16.0	>16.0	ND	0.025*
S. Enteritidis	20.0 (20.0–21.0) (S)	10.0 (8.0–10.0) (W)	15.0 (14.0–15.0) (M)	0.043*	2.0 (1.0–2.0) (S)	8.0 (4.0–8.0)	4.0 (BC)	1.0 (1.0–1.0)	2.0 (2.0–2.0)	2.0 (BC)	4.0 (4.0–8.0)	>16.0	ND	0.034*
S. Paratyphi A	22.0 (22.0–25.0) (S)	7.0 (7.0–10.0) (W)	15.0 (14.0–15.0) (M)	0.043*	1.0 (1.0–1.0) (S)	2.0 (1.0–2.0)	2.0 (BC)	2.0 (2.0–2.0)	4.0 (2.0–4.0)	2.0 (BC)	4.0 (4.0–4.0)	8.0 (8.0–8.0)	2.0 (BC)	0.025*
S. arizonae	21.0 (19.0–22.0) (S)	8.0 (8.0–15.0) (W)	16.0 (15.0–17.0) (M)	0.072	1.0 (1.0–1.0) (S)	2.0 (1.0–4.0)	2.0 (BC)	4.0 (4.0–4.0)	4.0 (4.0–4.0)	1.0 (BC)	>16.0	>16.0	ND	0.025*
Enterobacter cloacae	25.0 (25.0–26.0) (S)	16.0 (14.0–16.0) (M)	7.0 (7.0–8.0) (W)	0.043*	1.0 (0.5–1.0) (S)	2.0 (2.0–2.0)	2.0 (BC)	2.0 (2.0–2.0)	2.0 (2.0–2.0)	1.0 (BC)	>16.0	>16.0	ND	0.025*
K. aerogenes	23.0 (22.0–23.0) (S)	13.0 (10.0–14.0) (M)	8.0 (7.0–8.0) (W)	0.046*	2.0 (2.0–2.0) (S)	2.0 (2.0–2.0)	1.0 (BC)	2.0 (2.0–2.0)	4.0 (4.0–4.0)	2.0 (BC)	16.0 (16.0–16.0)	16.0 (16.0–16.0)	1.0 (BC)	0.034*
Yersinia enterocolitica	26.0 (26.0–27.0) (S)	30.0 (26.0–30.0) (ST)	12.0 (11.0–12.0) (W)	0.043*	2.0 (1.0–4.0) (S)	2.0 (2.0–4.0)	1.0 (BC)	2.0 (2.0–2.0)	2.0 (2.0–2.0)	1.0 (BC)	16.0 (4.0–16.0)	>16.0	ND	0.034*
Serratia rubidaea	26.0 (25.0–27.0) (S)	11.0 (11.0–14.0) (W)	9.0 (9.0–10.0) (W)	0.043*	2.0 (2.0–2.0) (S)	4.0 (4.0–8.0)	2.0 (BC)	2.0 (2.0–2.0)	4.0 (4.0–4.0)	2.0 (BC)	8.0 (8.0–8.0)	16.0 (8.0–16.0)	2.0 (BC)	0.025*
S. marcescens	19.0 (19.0–20.0) (S)	7.0 (7.0–10.0) (W)	6.0 (6.0–6.0) (N)	0.034*	4.0 (2.0–4.0) (S)	4.0 (4.0–4.0)	1.0 (BC)	4.0 (4.0–4.0)	4.0 (4.0–4.0)	1.0 (BC)	>16.0	>16.0	ND	0.025*
Acinetobacter lwoffii	24.0 (24.0–28.0) (S)	44.0 (44.0–48.0) (ST)	22.0 (21.0–22.0) (ST)	0.043*	0.3 (0.3–0.3) (S)	0.3 (0.3–0.3)	1.0 (BC)	4.0 (2.0–4.0)	4.0 (2.0–4.0)	1.0 (BC)	0.1 (0.1–0.1)	1.0 (1.0–1.0)	10.0 (BS)	0.034*

Gram-negative bacterial strain (n=34)	Disk diffusion, IZD (mm)				Broth macrodilution									
					Gentamicin (µg/mL)			LCEO (mg/mL)			Purified limonene (mg/mL)			
	Gentamicin	LCEO	Purified limonene	*p*-value[a]	MIC	MBC	MIC index	MIC	MBC	MIC index	MIC	MBC	MIC index	*p*-value[b]
A. baumannii	10.0 (10.0–11.0) (R)	14.0 (13.0–14.0) (M)	12.0 (10.0–12.0) (W)	0.043*	>16.0 (R)	8.0 (8.0–8.0)	ND	2.0 (2.0–2.0)	4.0 (2.0–4.0)	2.0 (BC)	16.0 (16.0–16.0)	>16.0	ND	0.025*
Stenotrophomonas maltophilia	26.0 (25.0–27.0) (NI)	19.0 (18.0–19.0) (M)	20.0 (20.0–20.0) (ST)	0.034*	2.0 (2.0–2.0) (S)	8.0 (4.0–8.0)	4.0 (BC)	4.0 (2.0–4.0)	4.0 (2.0–4.0)	1.0 (BC)	8.0 (4.0–8.0)	16.0 (16.0–16.0)	2.0 (BC)	0.099
Total	24.5 (10.0–28.0)	14.0 (7.0–48.0)	12.0 (6.0–22.0)	0.004**	2.0 (0.3–4.0)	4.0 (0.3–8.0)		2.0 (0.1–8.0)	4.0 (2.0–4.0)		4.0 (0.1–16.0)	16.0 (1.0–16.0)		0.000**

Susceptibility profile against gentamicin was interpreted using zone diameter breakpoint: susceptible (S), intermediate (I), and resistant (R) [24]. The degree of solid-phase activity of *Litsea cubeba* essential oil (LCEO) and purified limonene: no activity (N), weak (W), moderate (M), and strong (ST). The type of antibacterial activity: bactericidal (BC), bacteriostatic (BS), and tolerant (T). IZD: inhibition zone diameter; MBC: minimum bactericidal concentration; MIC: minimum inhibitory concentration; ND: not determined when MIC or MBC values exceed 16.0 µg/mL for gentamicin and 16.0 mg/mL for LCEO; NI: not interpreted due to no IZD or MIC breakpoint by CLSI guideline [24]

[a]Comparison in IZD values between LCEO and purified limonene

[b]Comparison in MIC values between LCEO and purified limonene

*Statistically significant at $p<0.05$

**Statistically significant at $p<0.01$

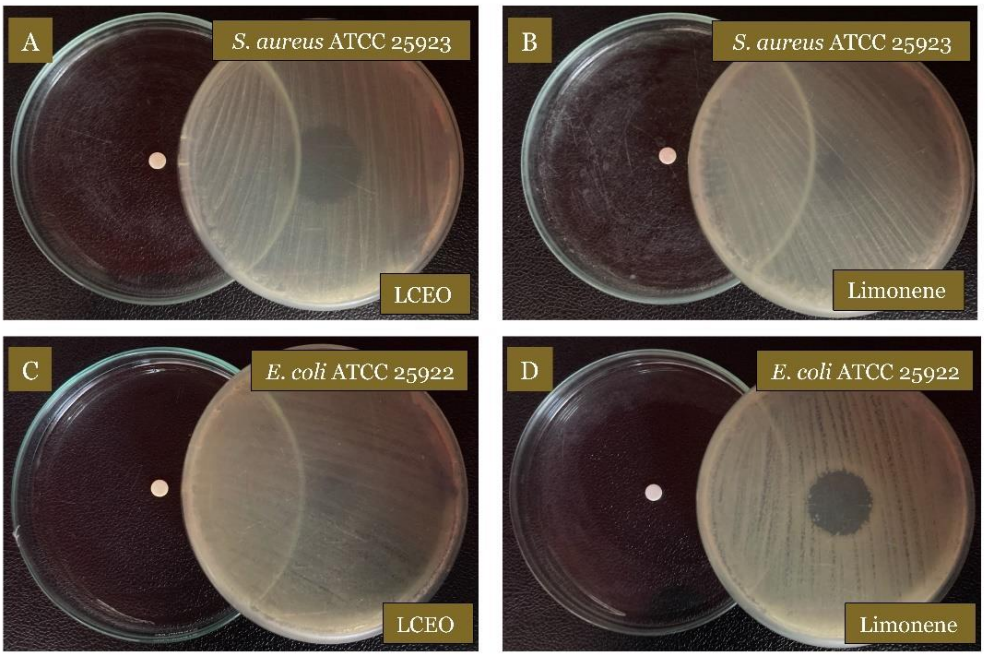


Figure 4. Inhibition zones of vapor-phase (A, C), *Litsea cubeba* essential oil (B, D) and purified limonene against (A, B) *Staphylococcus aureus* ATCC 25923 and (C, D) *Escherichia coli* ATCC 25922.

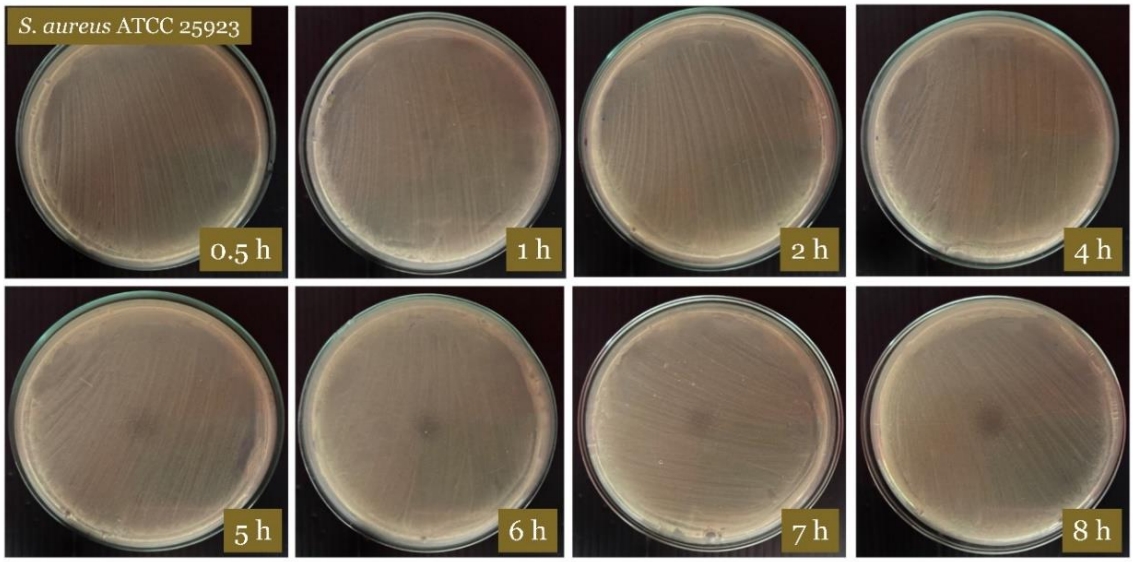


Figure 5. Inhibition zones of vapor-phase *Litsea cubeba* essential oil against *Staphylococcus aureus* ATCC 25923 at several time points of incubation: 0.5, 1, 2, 4, 5, 6, 7, and 8 hours.

Antifungal activities of solid-phase *Litsea cubeba* essential oil and purified limonene

The antifungal activities of *L. cubeba* essential oil and purified limonene in solid-phase medium against two strains of filamentous fungi (*T. rubrum* and *M. gypseum*) and one strain of yeast (*C. albicans*) are presented in **Table 6**. The negative control disk impregnated with 4% dimethyl sulfoxide showed no antifungal activity against the tested microorganisms, with an IZD value of 6.0 mm. In contrast, amphotericin B demonstrated antifungal activity against *T. rubrum*, *M. gypseum*, and *C. albicans*, with IZD ranging from 10.0 mm to 18.0 mm.

When compared to amphotericin B, *L. cubeba* essential oil in solid-phase medium exhibited superior antifungal activity against all tested filamentous fungi and yeast (*T. rubrum*, *M. gypseum*, and *C. albicans*) (n=3/3, 100%), with IZD ranging from 30.0 mm to 90.0 mm. Among

the filamentous fungi, no colonies of *T. rubrum* and *M. gypseum* were observed on potato dextrose agar plates after five days of incubation, indicating complete inhibition. A large inhibition zone was also observed for *C. albicans* (IZD value: 30.0–34.0 mm). These results demonstrate that *T. rubrum* and *M. gypseum* were the most susceptible fungal strains to *L. cubeba* essential oil. Purified limonene also had excellent antifungal activity against all tested filamentous fungi and yeast (n=3/3, 100%), with IZD ranging from 25.0 mm to 67.0 mm. However, the IZD values of *L. cubeba* essential oil in solid-phase medium against all tested fungal strains were significantly higher than those of purified limonene (p<0.05).

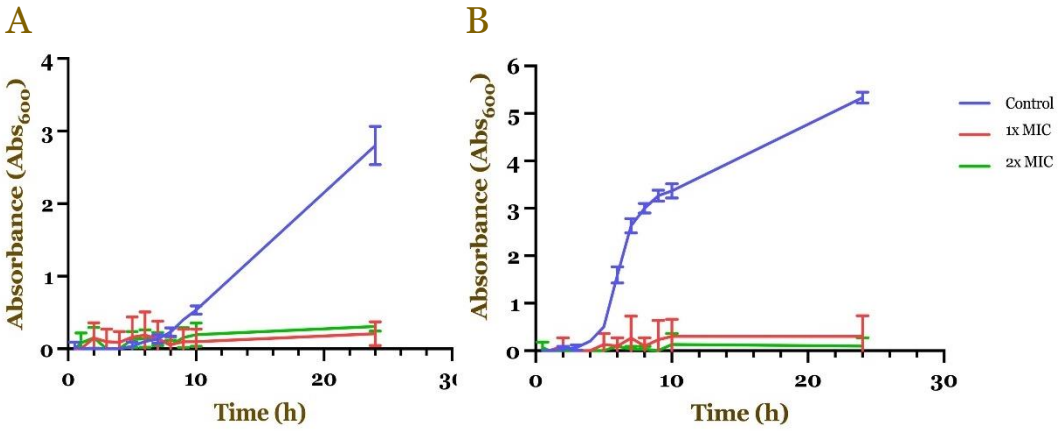


Figure 6. Time-killing kinetic curve and bactericidal time points of (A) *Staphylococcus aureus* ATCC 25923 and (B*) Escherichia coli* ATCC 25922 after exposure to *Litsea cubeba* essential oil (LCEO) at 1×MIC and 2×MIC for 0.5 to 24 hours. Abs_{600}: absorbance at a wavelength of 600 nm; control: 4% dimethyl sulfoxide; MIC: minimum inhibitory concentration.

Antifungal activities of liquid-phase *Litsea cubeba* essential oil and purified limonene

In a liquid-phase medium, *L. cubeba* essential oil demonstrated significant antifungal activity against all tested filamentous fungi and yeast (*T. rubrum*, *M. gypseum*, and *C. albicans*) (n=3/3, 100%) (**Table 6**). The MIC of *L. cubeba* essential oil ranged from 0.03 mg/mL to 0.06 mg/mL, while that of amphotericin B, used as the antifungal control, ranged from 0.06 µg/mL to 1.0 µg/mL. *T. rubrum* and *M. gypseum* were the most susceptible to *L. cubeba* essential oil.

Purified limonene also exhibited inhibitory effects on all tested filamentous fungi and yeast (n=3/3, 100%), with MIC values ranging from 0.06 mg/mL to 1.0 mg/mL. The MIC values of *L. cubeba* essential oil against all tested filamentous fungi and yeast were significantly lower than those of purified limonene (p<0.05). Regarding the MFC to MIC ratio, liquid-phase *L. cubeba* essential oil was considered fungicidal against *T. rubrum* and *M. gypseum* (MFC/MIC ratio: 1.0–2.2) and fungistatic against *C. albicans* (MFC/MIC ratio: 8.3). Interestingly, the IZD and MIC values of *L. cubeba* essential oil against all tested filamentous fungi and yeasts were significantly different from those observed for Gram-positive and Gram-negative bacteria (IZD values: 30.0–90.0 mm vs 14.0–41.0 mm vs 7.0–48.0 mm and MIC values: 0.03–0.06 mg/mL vs 0.1–2.0 mg/mL vs 0.1–8.0 mg/mL) (p<0.01).

Antifungal activities of vapor-phase *Litsea cubeba* essential oil and purified limonene

Both vapor-phase *L. cubeba* essential oil and purified limonene exhibited excellent antifungal activity against all tested filamentous fungi and yeast (*T. rubrum*, *M. gypseum*, and *C. albicans*) (n=3/3, 100%) (**Figures 7**). Vapor-phase *L. cubeba* essential oil completely eradicated *T. rubrum*, *M. gypseum*, and *C. albicans*, as no colonies were observed following exposure (**Figure 7A**, **Figure 7C**, and **Figure 7E**). Additionally, vapor-phase purified limonene efficiently inhibited *T. rubrum*, *M. gypseum*, and *C. albicans*, with large inhibition zones observed after exposure (**Figure 7B**, **Figure 7D**, and **Figure 7F**). The IZD of *L. cubeba* essential oil against all tested filamentous fungi and yeasts were significantly larger than those observed against Gram-positive and Gram-negative bacteria (IZD values: 90.0 mm vs 25.0–28.0 mm vs 0.0 mm)

(p<0.05). These findings indicated that *L. cubeba* essential oil exhibits antimicrobial activity across its solid, liquid, and vapor phases, with the highest activity observed against filamentous fungi and yeast, followed by Gram-positive bacteria, and then Gram-negative bacteria.

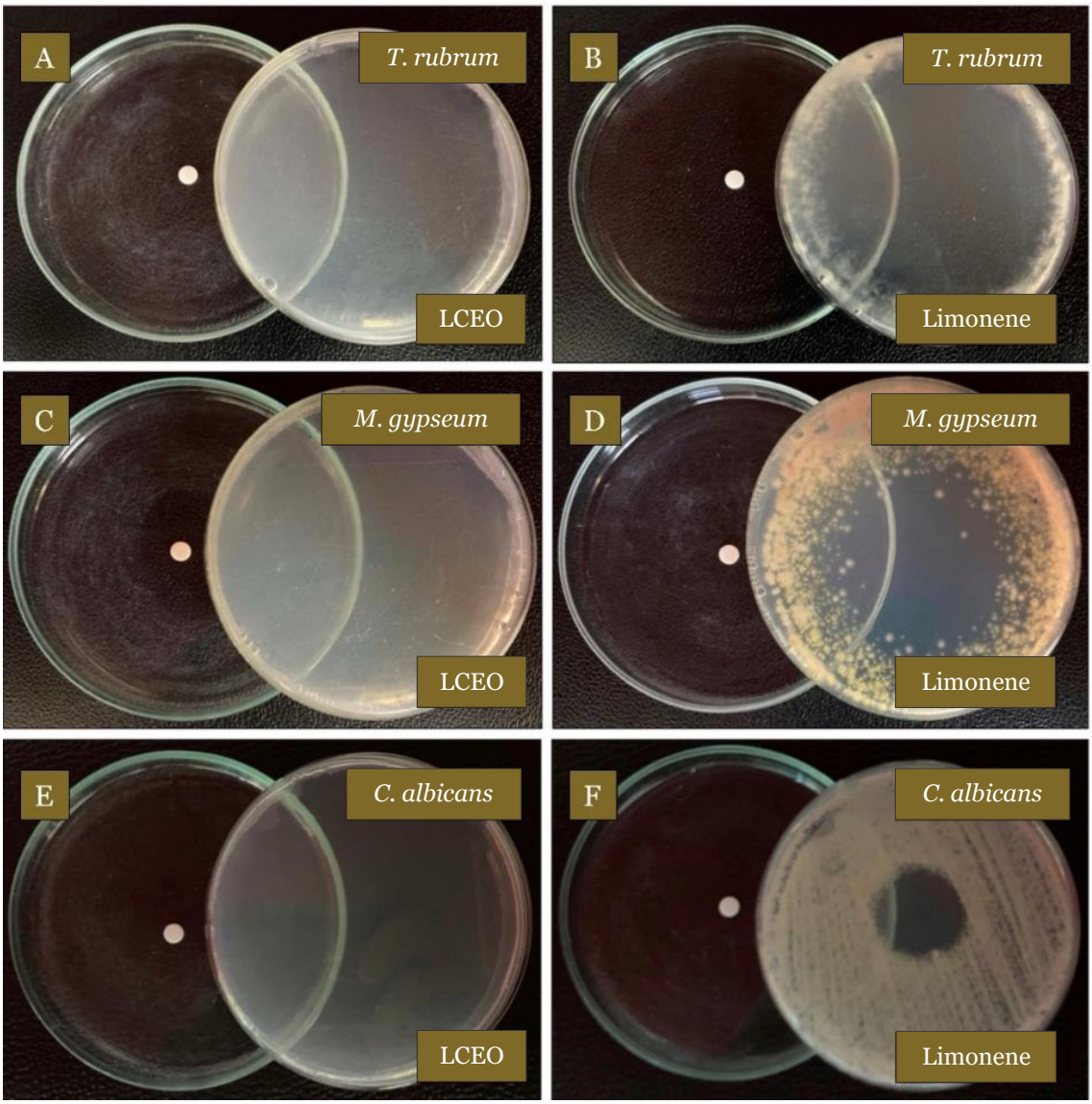


Figure 7. Inhibition zones of (A, C, E) vapor-phase *Litsea cubeba* essential oil; and (B, D, F) vapor-phase limonene toward tested filamentous fungi and yeast: (A, B) *Trichophyton rubrum*; (C, D) *Microsporum gypseum*; and (E, F) *Candida albicans*.

Further evaluation of the antifungal activity of vapor-phase *L. cubeba* essential oil was conducted at specific time points, ranging from 0.5 to 8 hours (**Figure 8**). The results indicated that vapor-phase *L. cubeba* essential oil initially inhibited the growth of *T. rubrum* after one hour of incubation, as evidenced by the appearance of an inhibition zone (IZD value: 11.7±1.5 mm). Complete eradication of *T. rubrum* was observed after 2 hours of incubation (**Figure 8A**). Similarly, vapor-phase *L. cubeba* essential oil initially inhibited the growth of *M. gypseum* after 1 hour of incubation, with an inhibition zone appearing (IZD value: 7.0±1.0 mm), which widened from 1 hour to 4 hours. Complete eradication of *M. gypseum* was observed at 5 hours of incubation (**Figure 8B**). For *C. albicans*, vapor-phase *L. cubeba* essential oil initially inhibited growth after 5 hours of incubation, with the inhibition zone widening in a time-dependent manner from 6 to 8 hours (**Figure 8C**). Partial inhibition of *C. albicans* was still observed after a 1-day incubation period.

Table 6. Antifungal activities of *Litsea cubeba* essential oil and purified limonene in solid and liquid-phase media against filamentous fungi and yeast by disk diffusion and broth macrodilution assays

Fungal and yeast strains (n=3)	Disk diffusion, IZD (mm)				Broth macrodilution									
					AmpB (µg/mL)			LCEO (mg/mL)			Purified limonene (mg/mL)			
	AmpB	LCEO	Purified limonene	*p*-value[a]	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC	*p*-value[b]
Trichophyton rubrum	10.0 (10.0–12.0)	NG	65.0 (63.0–67.0)	0.507	0.25 (0.25–0.25)	0.5 (0.25–0.5)	2.0 (FC)	0.06 (0.03–0.06)	0.13 (0.13–0.13)	2.2 (FC)	0.06 (0.06–0.13)	0.13 (0.13–0.13)	2.2 (FC)	0.197
Microsporum gypseum	10.0 (10.0–16.0)	NG	45.0 (43.0–46.0)	0.507	1.0 (1.0–1.0)	>1.0	ND	0.06 (0.03–0.06)	0.06 (0.03–0.06)	1.0 (FC)	0.06 (0.06–0.13)	0.06 (0.06–0.13)	1.0 (FC)	0.197
Candida albicans	18.0 (18.0–18.0)	34.0 (30.0–34.0)	27.0 (25.0–27.0)	0.043*	0.06 (0.06–0.06)	0.5 (0.5–0.5)	8.3 (FS)	0.06 (0.06–0.06)	0.5 (0.5–0.5)	8.3 (FS)	1.0 (0.5–1.0)	1.0 (1.0–1.0)	1.0 (FC)	0.034*
Total	12.0 (10.0–18.0)	90.0 (30.0–90.0)	45.0 (25.0–67.0)	0.043*	0.25 (0.06–1.0)	0.5 (0.25–0.5)		0.06 (0.03–0.06)	0.13 (0.03–0.5)		0.13 (0.06–1.0)	0.13 (0.06–1.0)		0.019*

FC: fungicidal; FS: fungistatic; MFC: minimum fungicidal concentration; MIC: minimum inhibitory concentration; NG: no growth; ND: not determined when MIC or MFC values exceed 1.0 µg/mL for amphotericin B (AmpB) and 1.0 mg/mL for *Litsea cubeba* essential oil (LCEO)

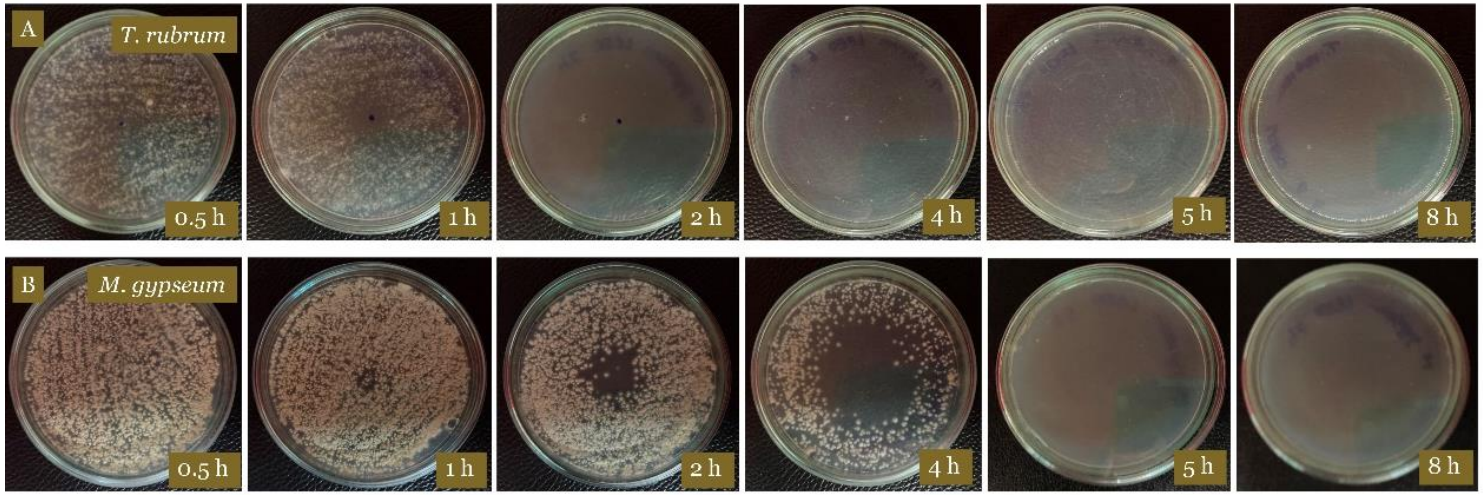
[a]Comparison in IZD values between LCEO and purified limonene

[b]Comparison in MIC values between LCEO and purified limonene

*Statistically significant $p<0.05$

Sreepian *et al*. Narra J 2025; 5 (1): 1685 - http://doi.org/10.52225/narra.v5i1.1685

Filamentous fungi



Yeast

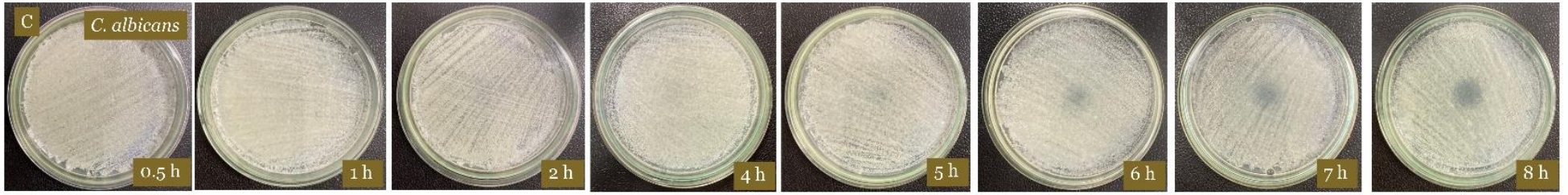


Figure 8. Inhibition zones of vapor-phase *Litsea cubeba* essential oil at several time points of incubation (0.5, 1, 2, 4, 5, 6, 7, and 8 hours) toward tested filamentous fungi and yeast: (A) *Trichophyton rubrum*; (B) *Microsporum gypseum*; and (C) *Candida albicans*.

Discussion

The yield of hydrodistilled *L. cubeba* essential oil in this study was approximately 4.0%, which is consistent with a previous study conducted in Vietnam [32]. Hydrodistilled *L. cubeba* essential oil extracted from the fruits in that study yielded 3% (v/w) with an absolute density of 0.8820 g/mL [32]. The major constituents in *L. cubeba* essential oil identified in this study were citral (42.53%), 1,3,8-p-menthatriene (35.18%), and *d*-limonene (4.25%), relative to the total content. These primary compounds were in agreement with previous studies conducted across several countries in Asia, including China, Taiwan, Vietnam, and Thailand [13,17,33-38]. Specifically, hydrodistilled *L. cubeba* essential oil extracted from fruit in Taiwan contained a total of 23 compounds, with the major components being citral (geranial or α-citral or *trans*-citral 36.16% and neral or β-citral or *cis*-citral 28.29%), and *d*-limonene (22.90%) [33]. Another study from Taiwan demonstrated that hydrodistilled *L. cubeba* essential oil extracted from the fruit contained citral (69.8%), limonene (12.7%), and linalool (1.4%) [34]. However, studies conducted in Vietnam and Thailand reported a higher content of citral (66.1% and 70.95%) and a lower content of limonene (7.0% and 3.59%) [35,36]. These findings suggested that both the oil yield and the chemical composition of *L. cubeba* essential oil may vary depending on the geographical location.

This study aimed to determine the antimicrobial spectrum of hydrodistilled *L. cubeba* essential oil and assess the efficacy of its different phases against various human pathogenic strains. All three phases of *L. cubeba* essential oil and purified limonene had significant antibacterial and antifungal activity against all tested microorganisms, except *P. aeruginosa*. The antimicrobial effects of *L. cubeba* essential oil in solid and liquid-phase media against key medical microorganisms, including Gram-positive and Gram-negative bacteria, filamentous fungi, and yeast, align with findings from several studies [14-17,38]. Hammid and Ahmad reported complete inhibition of *Aspergillus niger* NBRC 4066 and *Saccharomyces cerevisiae* ATCC 9763, as well as strong inhibition of *S. aureus* ATCC 9763 and *Bacillus subtilis* NBRC 3134, but no effect on *P. aeruginosa* NBRC 12689 and *E. coli* NBRC 3301 [14]. A previous study reported antibacterial activity of *L. cubeba* essential oil in solid-phase media against *S. aureus* ATCC 25923, *B. subtilis* ATCC 11774, *E. coli* ATCC 25922, and clinical isolates of *S.* Enteritidis [32]. Songsang *et al*. observed antimicrobial activity against *Streptococcus mutans* ATCC 25175TM and *C. albicans* ATCC 10231TM [16], while Li *et al*. found excellent antifungal activity against *C. albicans* ATCC 0231, with an IZD greater than 50 mm [38]. Gogoi *et al*. reported that *L. cubeba* essential oil combined with solid-phase medium exhibited antibacterial effects against *Bacillus cereus* ATCC 10876 and *S. aureus* ATCC 11632, with a stronger inhibitory effect in liquid-phase medium against *B. subtilis* ATCC 11774, *B. cereus* ATCC 10876, *S. aureus* ATCC 11632, and *S. typhimurium* ATCC 13311 [10]. However, no inhibition was observed against *Aspergillus fumigatus*, *A. niger*, *S. cerevisiae*, and *C. albicans* [10]. This contrasts with the present study, which demonstrated inhibitory effects of all phases of *L. cubeba* essential oil on *T. rubrum*, *M. gypseum*, and *C. albicans*.

A study demonstrated that hydrodistilled *L. cubeba* essential oil exhibits antibacterial activity against *Listeria innocua* ATCC 33090, *B. cereus* ATCC 13061, *B. subtilis* ATCC 11778, *S. aureus* ATCC 25923, methicillin-resistant *S. aureus* ATCC 33591, methicillin-resistant *S. epidermidis* ATCC 35984, *P. vulgaris* ATCC 49132, *P. aeruginosa* ATCC 9027, *A. hydrophila* ATCC 35654, *E. coli* ATCC 25922, *S. typhimurium* ATCC 14028, and *V. parahaemolyticus* ATCC 17802 when combined with a liquid-phase medium, with MIC values ranging from 700 to 5,500 µg/mL, indicating the potential of hydrodistilled *L. cubeba* essential oil in inhibiting bacterial growth [12]. Additionally, another study found that hydrodistilled *L. cubeba* essential oil exhibited promising antibacterial and antifungal activities against *E. coli* and *A. fumigatus* isolated from avian sources [9]. Furthermore, a study reported that hydrodistilled *L. cubeba* essential oil combined with liquid-phase medium demonstrated excellent antibacterial activity against *V. parahaemolyticus* ATCC 17802 and its laboratory isolates, consistent with the findings of the present study, which also observed effectiveness against *Vibrio* species [39].

The proposed antimicrobial mechanism of hydrodistilled *L. cubeba* essential oil is related to its inherent hydrophobic nature, which allows the oil to partition into lipid-rich regions of bacterial cell membranes and mitochondria [40]. This partitioning can disrupt cell structures,

causing the leakage of critical molecules and ions, significantly impairing cell function [40]. Previous study suggested that hydrodistilled *L. cubeba* essential oil may influence bacterial membrane permeability and cell membrane structure, extending its impact beyond the outer membrane to affect the structural integrity of bacterial cells [41]. Additionally, it has been proposed that hydrodistilled *L. cubeba* essential oil may interfere with bacterial cell wall synthesis, further enhancing its antimicrobial activity [41]. In the case of *C. albicans*, a different mechanism has been proposed, involving the inhibition of specific enzyme proteins [38]. The study suggested that *d*-limonene and citral, two major constituents of hydrodistilled *L. cubeba* essential oil, target β-1,3-glucan synthase and secretory aspartate protease in *C. albicans* [38]. These enzymes play critical roles in fungal cell wall synthesis, and their inhibition may contribute to the antifungal activity of hydrodistilled *L. cubeba* essential oil [38]. Overall, these mechanisms highlight the complex and multifaceted antimicrobial actions of hydrodistilled *L. cubeba* essential oil, which involves interactions with both bacterial and fungal cell structures at multiple levels [38,40,41]. The hydrophobic properties of essential oils, such as hydrodistilled *L. cubeba* essential oil [40,41], make them promising candidates for disrupting microbial structures and warrant further research for the development of antimicrobial agents.

The comprehensive findings revealed that hydrodistilled *L. cubeba* essential oil exhibited superior antibacterial activity against Gram-positive bacteria compared to Gram-negative bacteria. This observation is consistent with previous studies, reinforcing its validity and significance [14,15]. This difference in activity can be attributed to the higher membrane complexity of Gram-negative bacteria, which limits the diffusion of hydrophobic compounds [40]. Additionally, hydrodistilled *L. cubeba* essential oil demonstrated higher antimicrobial activity compared to purified limonene, suggesting a synergistic interaction between limonene and other components in hydrodistilled *L. cubeba* essential oil, such as citral [14,17,41].

The antimicrobial activity of citral, a major component of hydrodistilled *L. cubeba* essential oil, has been extensively studied. Previous research indicated that citral exhibited antimicrobial effects against *S. aureus* CECT 239, *E. coli* CECT 516, and *C. albicans* CECT 1394 [42]. However, *P. aeruginosa* CECT 111 demonstrated high resistance to citral [42]. Another study found that citral impacted the growth of *A. flavus* CGMCC 3.4408 [43]. The proposed antibacterial mechanism of citral involves a reduction in intracellular ATP levels and cytoplasmic pH, leading to hyperpolarization of the bacterial cell membrane [44]. Its antifungal mechanism is associated with the inhibition of mycelial biomass synthesis and toxin production [43].

The present study demonstrated that purified limonene exhibited broad-spectrum antibacterial and antifungal activities. Previous studies have reported antimicrobial activity of limonene against *L. monocytogenes* FSCC 178006 [21], *E. coli* MG 1655 and its isolated strains [19] as well as various yeast strains, including *C. albicans* reference strains (ATCC 10231 and ATCC 90028) and clinical isolates, *C. krusei* ATCC 6558, *C. glabrata* ATCC 2001, *C. parapsilosis* ATCC 22019, and *S. cerevisiae* [20,22,45]. The proposed antimicrobial mechanism of limonene involves effects on cell integrity and cell wall structure, the formation of hydroxyl radicals and reactive oxygen species that cause DNA damage, and ultimately cell death [19–21,45,46].

The present study demonstrated that *L. cubeba* essential oil exhibited the most potent antibacterial activities in solid-phase medium against both Gram-positive and Gram-negative bacteria, particularly against *S. saprophyticus* and *A. lwoffii*. In liquid-phase medium, the most effective antibacterial activities of *L. cubeba* essential oil were observed against *S. agalactiae* and *E. meningoseptica*, respectively. *S. saprophyticus* is a commensal bacterium that can cause uncomplicated urinary tract infections, and in some cases, it may lead to acute pyelonephritis, urethritis, epididymitis, and prostatitis [47]. The transmission to humans occurs through the ingestion of contaminated food, followed by colonization in the human intestinal tract [48]. Resistance to ampicillin, ceftriaxone, cephalexin, and ciprofloxacin has been reported [47].

S. agalactiae, or group B streptococcus, is a commensal Gram-positive bacterium that can cause bacteremia, skin and soft tissue infections, as well as septicemia, pneumonia, and meningitis in neonates [49,50]. Transmission typically occurs from mother to newborn during delivery, and resistance to erythromycin and clindamycin has been reported [50]. *A. lwoffii* is a commensal Gram-negative coccobacillus that can cause bacteremia, particularly in association with indwelling catheters [51]. A previous study has shown that *A. lwoffii* exhibits high

susceptibility to gentamicin, amikacin, meropenem, ciprofloxacin, and piperacillin/tazobactam, but low susceptibility to cefuroxime axetil, cefazolin, and cefoxitin [52].

E. meningoseptica is a Gram-negative bacillus that can cause neonatal meningitis, septicemia, bacteremia, endophthalmitis, and necrotizing fasciitis, especially in immunocompromised hosts [53]. Healthcare-associated infections, particularly from contaminated hospital water, are common sources of transmission [54]. *E. meningoseptica* isolates have demonstrated resistance to carbapenems, cephalosporins, and aminoglycosides, as well as relatively low susceptibility to trimethoprim-sulfamethoxazole. Notably, none of the *E. meningoseptica* isolates exhibited susceptibility to vancomycin [54].

The present study found that *L. cubeba* essential oil in solid-phase medium exhibited an inhibitory effect on gentamicin-resistant *A. baumannii*. Previous research has also demonstrated the inhibitory effect of *L. cubeba* essential oil on *A. baumannii* [55]. *A. baumannii* is a Gram-negative coccobacillus that commonly causes infections in both aquatic animals and humans [1]. It is a significant pathogen associated with mortality, particularly in cases of carbapenem-resistant *A. baumannii* and multidrug-resistant *A. baumannii* [1]. Hospital-acquired infections caused by *A. baumannii* are of particular concern, as it is a leading cause of pneumonia, bacteremia, meningitis, urinary tract infections, and wound infections [56]. This pathogen primarily affects immunocompromised patients and those in intensive care units, often presenting as ventilator-associated pneumonia and septicemia [56]. Therefore, further investigation is warranted on a diverse range of *A. baumannii* clinical isolates, including antibiotic-resistant and multidrug-resistant strains, to evaluate the broader antimicrobial potential of *L. cubeba* essential oil.

The present study demonstrated that *L. cubeba* essential oil in both solid and liquid-phase media exhibited excellent antifungal activity against all tested filamentous fungi and yeast, including *T. rubrum*, *M. gypseum*, and *C. albicans*. These results align with a previous report that showed the IZD values of *L. cubeba* essential oil (1.25–5.0% v/v) against *C. albicans* ATCC 10231TM ranged from 8.3 to 14.3 mm, with a MIC value of 11.1 mg/mL [16]. Additionally, the present study provides the first evidence of the antifungal activity of *L. cubeba* essential oil in vapor-phase form. *T. rubrum* is a dermatophyte responsible for tinea pedis and tinea unguium and is known to exhibit resistance to terbinafine in many cases [57]. *M. gypseum*, now classified as *Nannizzia gypsea*, is a geophilic dermatophyte that can cause tinea corporis, tinea capitis, and tinea faciei in both humans and animals, such as cats and dogs [58,59]. *C. albicans*, a common commensal fungus, colonizes the skin, oropharynx, digestive tract, and vaginal tract. In immunocompromised individuals, it can cause superficial mucocutaneous infections and systemic infections [60]. Resistance to azoles, a commonly used class of antifungals, as well as resistance to polyenes, echinocandins, and 5-fluorocytosine, has been reported in *Candida* species [61].

In the present study, no inhibitory effect of *L. cubeba* essential oil in liquid-phase medium was observed, and a weak susceptibility to *L. cubeba* essential oil in solid-phase medium was noted in *P. aeruginosa*, with a high MIC greater than 16.0 mg/mL. This finding is consistent with a previous study [14]. The reduced susceptibility could be attributed to the formation of biofilms, which decreases the interaction between bacterial cells and antimicrobial agents, thereby limiting the effectiveness of *L. cubeba* essential oil [62].

Additionally, vapor-phase purified limonene had antibacterial activity against *E. coli* ATCC 25922, whereas this inhibitory effect was not observed with vapor-phase *L. cubeba* essential oil. A previous study on the antibacterial activity of several vapor-phase plant essential oils against *E. coli* ATCC 25922 reported that the MIC and MBC values of *L. cubeba* essential oil were 1,500 µL/L. However, the highest activity was observed with linaloe wood oil and tea tree oil, both showing MIC and MBC values of 200 µL/L [63]. The antibacterial activity of vapor-phase essential oils may be attributed to several mechanisms, including the degradation of cell walls and cell membranes, changes in membrane protein structure, and alterations in nuclear activity [63].

In this study, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were selected as model strains to evaluate the time-killing assay. The results demonstrated that the rapid bactericidal effects of *L. cubeba* essential oil at 1×MIC and 2×MIC were observed in both *S. aureus* ATCC 25923 and *E.*

coli ATCC 25922 within 0.5 hours after exposure to *L. cubeba* essential oil in liquid-phase medium. A previous study on the time-killing assay reported that *L. cubeba* essential oil (0.5% v/v) had a strong inhibitory effect on the stationary phase of *S. aureus* Newman strain, a broad antibiotic-susceptible clinical isolate, at all experimental time points, including day 3 and day 5 [64]. Previous study found that *L. cubeba* essential oil (0.0625% v/v) prolonged the growth of *E. coli* ATCC 8739 to approximately 12 hours, while *L. cubeba* essential oil (0.125% v/v) completely eradicated the bacteria within 2 hours [41]. Additionally, a separate study observed that the viability of *E. coli* ATCC 25922 gradually diminished and was eventually eradicated after exposure to 1×MIC and 2×MIC of *L. cubeba* essential oil for 7 and 5 hours, respectively [12].

The present findings represent the first report on the antifungal effect of vapor-phase *L. cubeba* essential oil against *T. rubrum*, *M. gypseum*, and *C. albicans*. Vapor-phase *L. cubeba* essential oil rapidly inhibited the growth of these filamentous fungi and yeast within 1 to 5 hours. The filamentous fungi were completely eradicated within 2 to 5 hours following treatment. This study also demonstrated the antifungal activity of vapor-phase purified limonene, a major volatile component in *L. cubeba* essential oil, alongside citral. These findings highlight the potential of vapor-phase *L. cubeba* essential oil as an effective antifungal agent for the treatment of skin infections. With demonstrated potency against a spectrum of human pathogens, *L. cubeba* essential oil presents itself as a versatile and effective antimicrobial agent. The findings suggest the potential to reduce reliance on antibiotics, contributing to the global effort against antibiotic-resistant microorganisms. While paving the way for further research on synergies with antibiotics and safety assessments, the study opens new possibilities for a holistic approach to combat bacterial and fungal infections. *L. cubeba* essential oil, with its diverse applications, emerges as a promising candidate for addressing the pressing challenges of antimicrobial resistance and public health concerns. However, the cytotoxicity of *L. cubeba* essential oil warrants further investigation. An in vitro study reported that *L. cubeba* essential oil at the maximal dose of 30% (v/v) exhibited no cytotoxicity on the human gingival fibroblast cell line [16]. Additionally, an in vivo study on the toxicity of *L. cubeba* essential oil in mice and rats reported that *L. cubeba* essential oil displayed no genetic toxicity and relatively low acute toxicity, with an oral lethal dose (LD50) of 4,000 mg/kg body weight, dermal LD50 of >5,000 mg/kg body weight, and inhalation lethal concentration (LC50) of 12,500 ppm [65].

This study had several limitations. Vapor-phase antimicrobial activity and antibacterial kinetic curves were assessed only in selected strains of common human pathogens, and the precise antimicrobial mechanisms were not investigated. A comprehensive evaluation of vapor-phase antimicrobial activity and kinetic curves against a broader range of microbial strains, along with further investigations on synergistic interactions with standard antibiotics, cytotoxicity in human cells, and mechanisms of action, is necessary to enhance understanding of antimicrobial efficacy and ensure safety before potential application in human subjects.

Conclusion

D-limonene was among major components identified in the *L. cubeba* essential oil. All phases of *L. cubeba* essential oil and purified limonene exhibited remarkable antimicrobial efficacy on a spectrum of human pathogens, with superior efficacy against filamentous fungi and yeast compared to bacteria. The findings suggest the potential to reduce reliance on antibiotics, contributing to the global effort against antibiotic-resistant microorganisms. *L. cubeba* essential oil, with its diverse applications, emerges as a promising candidate for addressing the pressing challenges of antimicrobial resistance and public health concerns.

Ethics approval

The study protocol was approved by the Research Ethics Committee of Rangsit University, Pathum Thani, Thailand (DPE. No. RSUERB2024-019).

Acknowledgments

The authors would like to thank the Faculty of Medical Technology, Rangsit University, Thailand for supporting research facilities. The authors would also like to express our gratitude to Dr. Brian Andrew Vesely for proofreading the manuscript.

Competing interests

All the authors declare that there are no conflicts of interest.

Funding

This work was supported by the Research Institute of Rangsit University, Thailand (Grant number 57/2560).

Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

Declaration of artificial intelligence use

We hereby confirm that no artificial intelligence (AI) tools or methodologies were utilized at any stage of this study, including during data collection, analysis, visualization, or manuscript preparation. All work presented in this study was conducted manually by the authors without the assistance of AI-based tools or systems.

How to cite

Sreepian PM, Popruk S, Rattanasinganchan P, Sreepian A. Comprehensive investigation of *Litsea cubeba* antibacterial and antifungal activities across solid, liquid, and vapor phases against key human pathogens. Narra J 2025; 5 (1): e1685 - http://doi.org/10.52225/narra.v5i1.1685.

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