

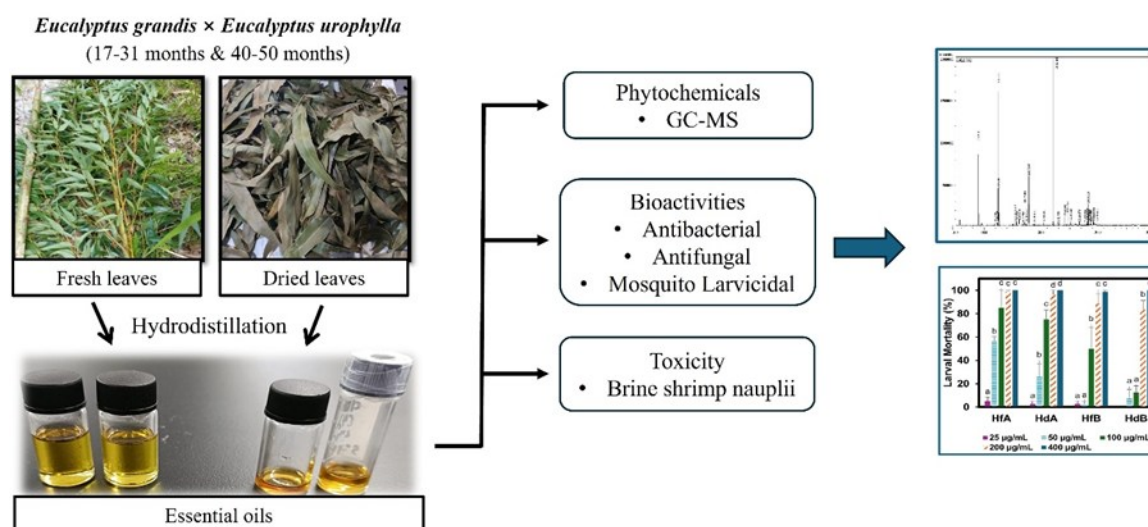
Phytochemical Composition, Biological Activities, and Toxicity of the Leaf Essential Oils Obtained from *Eucalyptus grandis* × *Eucalyptus urophylla* Cultivated in Malaysia

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GRAPHICAL ABSTRACT



Phytochemical Composition, Biological Activities, and Toxicity of the Leaf Essential Oils Obtained from *Eucalyptus grandis* × *Eucalyptus urophylla* Cultivated in Malaysia

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This study aimed to evaluate the phytochemical composition, antimicrobial properties, mosquito larvicidal effects, and brine shrimp toxicity of essential oils obtained using hydrodistillation from the fresh and dried leaves of *Eucalyptus grandis* × *Eucalyptus urophylla* at two age groups. Leaves from trees aged 17 to 31 months old yielded more essential oils than those aged 40 to 50 months. Gas chromatography-mass spectrometric analysis revealed that 1,8-cineol (13.1% to 26.7%) and α -terpinyl acetate (18.3% to 26.1%) were the dominant components across all essential oils. All tested essential oils inhibited Gram-positive bacteria, yeasts, and the dermatophyte *Trichophyton rubrum*, but failed to exhibit activity against most of the tested Gram-negative bacteria and *Aspergillus fumigatus*. The minimum inhibitory concentrations ranged from 0.16 to 2.50 mg/mL for bacteria and 0.04 to 1.25 mg/mL for fungi, highlighting the greater antifungal efficacy of the essential oils. All tested essential oil samples were also active against third instar larvae of *Aedes aegypti* and *Aedes albopictus*, with median lethal concentrations of 52.3 to 134 μ g/mL after 24 h, lower than that of against *Artemia franciscana* nauplii (209 and 222 μ g/mL). Therefore, *Eucalyptus grandis* × *Eucalyptus urophylla* essential oils are potential larvicidal agents for mosquito control with low toxicity to aquatic organisms.

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Keywords: *Eucalyptus grandis* × *Eucalyptus urophylla*; *Aedes aegypti*; *Aedes albopictus*; Antibacterial; Antifungal; Larvicidal

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INTRODUCTION

In Malaysia, afforestation or reforestation through forest plantations has been identified as a crucial strategy to address the declining timber supply resulting from the depletion of natural forest resources. The Forest Plantation Development Program, initiated by the Malaysian Timber Industry Board (MTIB), provides financial incentives to encourage the development of commercial forest plantations of four hectares or more (MTIB 2021). Among the species listed under this program, *Eucalyptus* species stands out as a promising candidate for large-scale planting due to its fast growth, limited space occupancy, high phenotypic plasticity, and adaptability to diverse climates and soil types (Yahya *et al.* 2020). *Eucalyptus* is a genus of evergreen hardwood (angiosperm) species in the family Myrtaceae, which is commonly cultivated in subtropical regions for plantation purposes (Mieres-Castro *et al.* 2021). Globally, the top three *Eucalyptus*-cultivating

countries are Brazil, India, and China. The main planted species in Brazil are *E. camaldulensis*, *E. citriodora*, *E. globulus*, *E. grandis*, *E. saligna*, *E. urophylla*, and their hybrids such as *E. grandis* × *E. camaldulensis*, *E. urophylla* × *E. camaldulensis*, *E. urophylla* × *E. globulus*, and *E. urophylla* × *E. grandis* (Florêncio *et al.* 2022). In India, the commercially important species are *E. camaldulensis*, *E. citriodora*, *E. globulus*, *E. grandis*, and *E. tereticornis* (Shikha *et al.* 2025), while China primarily cultivates *E. camaldulensis*, *E. dunnii*, *E. globulus*, *E. grandis*, *E. maidenii*, *E. saligna*, *E. tereticornis*, *E. urophylla*, as well as *E. urophylla* × *E. grandis* and *E. camaldulensis* × *E. grandis* hybrids (Zhou and Wingfield 2011).

Malaysia is ideal for growing *Eucalyptus*, as its low cold tolerance and high-water requirement are well-suited to the country's hot and humid climate conditions (Zhang and Wang 2021). *Eucalyptus pellita* and *E. grandis* × *E. urophylla* are the commonly cultivated species. However, the hybrid type has gained popularity using clones sourced from Southern China, as its growth outperformed *E. pellita* in terms of tree height and diameter (Yahya *et al.* 2020). This hybrid was developed through controlled pollination, a technique used to select and combine the desirable traits of both parent species. *Eucalyptus grandis* contributes to fast growth and ease of vegetative propagation, while *Eucalyptus urophylla* provides beneficial traits such as enhanced disease resistance, improved adaptability to diverse environmental conditions, and higher wood density (Kullan *et al.* 2012; Van den Berg *et al.* 2015).

Eucalyptus trunks are valued for various applications, including paper pulp, plywood, furniture, poles, and sawn timber (Lu *et al.* 2014), while the remaining parts, including fruits, flowers, and leaves, could be harvested for essential oil extraction. The leaves are particularly noteworthy, as more than 300 *Eucalyptus* species have been reported to contain volatile oils (Mieres-Castro *et al.* 2021). Essential oils are colorless or pale-yellow, aromatic, oily products that are typically soluble in organic solvents and less dense than water (Haro-González *et al.* 2021). According to the International Organization for Standardization (2021), essential oils are natural products derived from botanical sources obtained *via* hydrodistillation, dry distillation, steam distillation, or mechanical compression in the case of citrus fruit, after separation of aqueous phase using physical methods. This definition highlights that essential oils are commonly obtained through hydrodistillation, a method that involves the use of boiling water to release volatile compounds from plant material, which are subsequently condensed and collected. Hydrodistillation is widely used due to its efficiency, simplicity, and the absence of organic solvents (Salehi *et al.* 2019).

The essential oils of *Eucalyptus* species are recognized for their abundance of bioactive compounds, primarily monoterpenes, such as α -pinene, 1,8-cineol (eucalyptol), and limonene, as well as sesquiterpenes, such as β -eudesmol, α -humulene, and globulol (Yip *et al.* 2024). However, their phytochemical composition can vary significantly depending on the extraction techniques, tree age, species, geographical location, and leaf condition (Zhang *et al.* 2010; Achmad *et al.* 2018; Shiferaw *et al.* 2019). Furthermore, existing literature has documented a wide range of biological activities associated with *Eucalyptus* essential oils, including antioxidative, antimicrobial, antiviral, antidiabetic, anti-inflammatory, analgesic, mucolytic, and bronchodilatory effects (Barbosa *et al.* 2016; Salehi *et al.* 2019; Chandorkar *et al.* 2021). For example, Zhou *et al.* (2021) reported minimum inhibitory concentrations of between 0.023 to 0.091 mg/mL for *E. grandis* × *E. urophylla* leaf essential oils, obtained using steam distillation, against human pathogenic bacteria, *i.e.*, *Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*, *Salmonella enterica*

serovar Typhimurium, *Pseudomonas aeruginosa*, and *Escherichia coli*. Moreover, Lucia *et al.* (2008) demonstrated the larvicidal activity of essential oils derived from *Eucalyptus* hybrids: *E. grandis* × *E. camaldulensis* and *E. grandis* × *E. tereticornis*, against *Aedes aegypti*, the yellow fever mosquito.

Given that the biological activities of essential oils derived from *Eucalyptus* species are closely associated with their phytochemical composition, this study aimed to explore the phytochemical composition of essential oils obtained from the leaves of *Eucalyptus grandis* × *Eucalyptus urophylla* cultivated in Malaysia at different tree ages using hydrodistillation. In addition, this study assessed the toxicity of the isolated essential oils and evaluated their antibacterial activity against bacterial pathogens, antifungal efficacy against human pathogenic fungi, and larvicidal potential against *Aedes* mosquitoes, which are the vectors of arboviral diseases such as dengue, chikungunya, and Zika. Through these assessments, this study seeks to explore natural alternatives to synthetic disinfectants and mosquito larvicides.

EXPERIMENTAL

Materials

The iodinitrotetrazolium chloride, itraconazole, along with the standards for alkanes (C7-C40), α -pinene, α -terpineol, borneol, β -caryophyllene, 1,8-cineol, and limonene were purchased from Sigma-Aldrich (St. Louis, USA), whereas the aromadendrene standard was purchased from ChemFaces (Wuhan, China). Chloramphenicol was sourced from Duchefa Biochemie (Haarlem, The Netherlands), 3-morpholinopropanesulfonic acid from Bio Basic (Markham, Canada), 5-fluorocytosine from Acros Organics (Hong Kong, China), temephos from Dr. Ehrenstorfer GmbH (Augsburg, Germany), potassium dichromate from System Chemical (Shah Alam, Malaysia), anhydrous sodium sulphate and acetone (spectroscopy grade) from Merck (Darmstadt, Germany), and methanol and ethanol (analytical grade) from Rank Synergy (Kuala Lumpur, Malaysia). The culture media used included Mueller-Hinton broth and Mueller-Hinton agar (HiMedia, Thane, India), potato dextrose agar (Liofilchem, Roseto degli Abruzzi, Italy), oatmeal agar (Laboratorios Conda, Madrid, Spain), and Roswell Park Memorial Institute (RPMI)-1640 medium (Biowest, Nuaille, France).

Ten bacterial species comprising six reference strains from the American Type Culture Collection (ATCC), *Enterococcus hirae* ATCC[®] 10541[™], *Staphylococcus aureus* ATCC[®] 6538[™], *Pseudomonas aeruginosa* ATCC[®] 15442[™], *Escherichia coli* ATCC[®] 35218[™], *Klebsiella pneumoniae* ATCC[®] 13883[™], and *Bacillus cereus* ATCC[®] 14579[™], as well as four clinical isolates, *Staphylococcus aureus* SA-LWE23#1, *Staphylococcus aureus* SA-LWE23#2, *Klebsiella pneumoniae* KP-LWE23#1, and *Escherichia coli* EC-LWE23#1, were used in this study. Eight fungal species, including six yeasts, namely *Candida tropicalis* ATCC[®] 750[™], *Candida albicans* ATCC[®] 90028[™], *Candida auris* derived from CDC B11903, *Candida parapsilosis* ATCC[®] 22019[™], *Cryptococcus neoformans* ATCC[®] 13690[™], and *Nakaseomyces glabratus* ATCC[®] MYA-2950[™], along with two filamentous fungi, *Trichophyton rubrum* ATCC[®] 28188[™] and *Aspergillus fumigatus* ATCC[®] 204305[™], were also tested in the study.

Preparation of Plant Materials

Fresh leaves from the *Eucalyptus grandis* × *Eucalyptus urophylla* were used for essential oil extraction. The leaf samples were harvested from trees cultivated at two different estates in Gua Musang, Kelantan, Malaysia. Trees aged 17 to 31 months from the first estate (4° 39' 48" N 101° 36' 58" E) were designated as *Eucalyptus* A, while those aged 40 to 50 months from the second estate (4° 48' 192" N 101° 55' 15" E) were designated as *Eucalyptus* B. Leaf vouchers were prepared (code: UTAR/FSC/23/001) and deposited at the Faculty of Science, Universiti Tunku Abdul Rahman, Kampar, Malaysia. The collected leaves were rinsed and divided into two batches. One batch was kept in a 4 °C fridge to maintain freshness, while another batch was dried in an oven (Memmert GmbH, Schwabach, Germany) at 40 °C for 5 to 7 days. The average mass loss (n=3) of the leaves following drying was 32.0% ± 3.46% for *Eucalyptus* A and 59.1% ± 3.59% for *Eucalyptus* B. Both fresh and dried leaf samples were then cut into smaller pieces and blended prior to hydrodistillation.

Hydrodistillation

Hydrodistillation of the plant sample was carried out using a stainless-steel distiller (Laboratory & Scientific Enterprise, Klang, Malaysia) to obtain the essential oils. The distiller consisted of a 10 L distilling pot connected to a condensation tower filled with cold water, as shown in Fig. 1. The cold water was maintained using a chiller (Buchi Labortechnik AG, Flawil, Switzerland). One kg of blended leaf material was placed in the distilling pot and submerged in 4 L of deionized water. The mixture was heated using an induction cooker (Philips, China) operating at 800 W. The pot was equipped with a thermometer for continuous temperature monitoring. The entire extraction was run at 100 °C for 8 h, and the distillate was collected in a glass collecting flask, where the essential oil layer was separated from the hydrosol. The resulting essential oils were collected and dried using anhydrous sodium sulphate, then stored in glass vials at 4 °C prior to analysis. Each extraction was performed in triplicate. The extraction yield (w/w) was calculated based on the fresh leaf weight for fresh leaf essential oils (FLEO) and the dried leaf weight for dried leaf essential oils (DLEO). For clarity, the FLEO samples from *Eucalyptus* A and *Eucalyptus* B were designated as HfA and HfB, respectively, while the corresponding DLEO samples were labeled HdA and HdB, respectively.

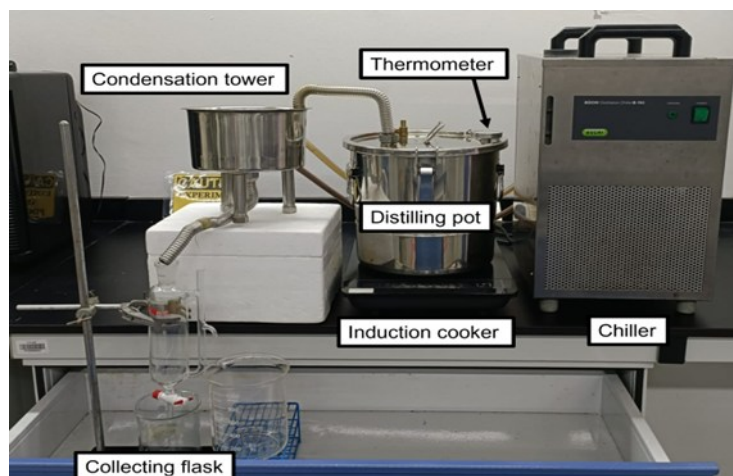


Fig. 1. Instrumental setup for hydrodistillation

Phytochemical Composition

A Shimadzu model, QP2010 Plus (Tokyo, Japan) GC-MS was used to identify the phytochemical composition of essential oil samples. The component separation was performed using a 30.0 m × 0.25 mm × 0.25 µm capillary column of 5% diphenyl-95% dimethyl polysiloxane (SH-I-5Sil MS, Shimadzu, Tokyo, Japan). The temperature of the capillary column was initially set at 50 °C and held for 5 min, then the temperature rose at a rate of 5 °C/min until reaching 200 °C and remained for 5 min, giving a total duration of 40 min for the analysis of each sample. The mobile phase, helium gas, flowed at a linear velocity rate of 36.3 cm/s. The sample was prepared in acetone at 1.0 mg/mL and filtered using a 0.45 µm nylon syringe filter. One µL of sample was injected per run, with a split ratio of 20. The injector port temperature was maintained at 200 °C. For the mass spectrometer settings, both interface temperature and ion source temperature were 200 °C. Electron impact ionization at 70 eV was used. The fragment ions produced by each component were scanned at m/z 35 to 600. The identification of compounds was carried out by matching their mass spectra with those recorded in the NIST 23 Mass Spectral Library (National Institute of Standards and Technology, Gaithersburg, USA). Only components with matching similarity $\geq 86\%$ were reported (Lim *et al.* 2023). Further confirmation of selected identified compounds was accomplished through comparison of their retention times with the authentic standards (aromadendrene, β -caryophyllene, α -terpineol, α -pinene, limonene, borneol, and 1,8-cineol) analyzed under the same settings. The retention indices were determined with respect to C7 to C40 alkanes. The relative percentage of compounds in the total ion chromatogram was calculated using a peak area normalization method. The analysis was performed in duplicate.

Antibacterial Assay

The colorimetric broth microdilution antibacterial assay was adapted from the method of Sit *et al.* (2017) with slight modifications. Bacteria were first cultured on Mueller-Hinton agar prior to the assay. A 10 mg/mL stock solution of essential oil was prepared in an ethanol-water mixture (2:1, v/v) and sterilized using a 0.45 µm syringe filter. This stock solution was subjected to two-fold serial dilutions in a 96-well microtiter plate with Mueller-Hinton broth to obtain final concentrations of 0.02, 0.04, 0.08, 0.16, 0.31, 0.63, 1.25, and 2.50 mg/mL. After that, 50 µL of bacterial suspension (1×10^6 colony-forming unit/mL) was inoculated into each well, bringing the final volume to 100 µL per well. Four controls were included in each assay to validate the results: positive control (chloramphenicol ranging from 1 to 128 µg/mL), medium control (broth), growth control (bacterial inoculum without essential oil), and negative control (essential oil without bacteria). The microtiter plates were incubated at 37 °C for 24 h. After incubation, 20 µL of iodinitrotetrazolium chloride solution at 0.4 mg/mL was added to each well. Bacterial growth was assessed based on the formation of a purple formazan precipitate. The minimum inhibitory concentration (MIC) was recorded as the lowest concentration that inhibited bacterial growth. Subsequently, the minimum bactericidal concentration (MBC), which was the lowest concentration capable of killing 99.9% bacteria, was determined by inoculating 20 µL from wells that showed no bacterial growth onto Mueller-Hinton agar plates. The plates were then incubated at 37 °C for 24 h, and the formation of bacterial colonies was observed to determine the MBC.

Antifungal Assay

The colorimetric broth microdilution method was also used to determine the antifungal properties of the essential oils. All yeasts and *A. fumigatus* were maintained on potato dextrose agar, while *T. rubrum* was cultured on oatmeal agar prior to testing. The 10 mg/mL stock solution of each essential oil, prepared in an ethanol-water mixture and filter-sterilized through a 0.45 µm syringe filter, was two-fold serially diluted in a 96-well microtiter plate to obtain final concentrations ranging from 0.02 to 2.50 mg/mL. The fungal inoculums were prepared according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2008a; 2008b), and 50 µL of each inoculum was added to the wells. Itraconazole and 5-fluorocytosine served as the positive controls for filamentous fungi and yeasts, respectively. Wells containing only RPMI-1640 medium, only essential oil, and only fungal inoculum served as sterility control, negative control, and growth control, respectively. The microtiter plates were incubated at 35 °C for 48 h for *Candida* spp. and *N. glabratus*, 72 h for *C. neoformans* and *A. fumigatus*, and at 30 °C for 5 d for *T. rubrum*. After incubation, 20 µL of iodinitrotetrazolium chloride solution (0.4 mg/mL) was pipetted into each well to determine the MIC. The spread plate technique was performed by swabbing 20 µL from wells that showed no visible purple precipitate on potato dextrose agar. The lowest essential oil concentration that completely inhibited fungal growth on the agar was determined as the minimum fungicidal concentration (MFC).

Mosquito Larvicidal Testing

The mosquito larvicidal properties of the essential oils were evaluated against *Aedes aegypti* and *Aedes albopictus* based on the World Health Organization guidelines (WHO 2005), with slight modifications. The assay began with the hatching of mosquito eggs, sourced from the Vector Control Research Unit (VCRU) of Universiti Sains Malaysia (USM), Penang, Malaysia, in dechlorinated water and maintained until they reached the third instar larval stage. A stock solution of the essential oil, which was prepared at 40 mg/mL in methanol, was then diluted with deionized water to obtain five concentration levels (25, 50, 100, 200, and 400 µg/mL), using round plastic containers. The final methanol concentration in the assay was maintained at ≤1% to avoid methanol-induced larval mortality. For each test, 20 third instar larvae were transferred into plastic containers containing 100 mL of the diluted essential oil solutions, following a 1.0 h holding period. The containers were maintained at room temperature (24 °C) and relative humidity (70%). Larval mortality was recorded at 24 h and 48 h post-treatments. The negative control was 1% methanol, while the positive control was 1000 µg/mL temephos. Larvae were considered dead if they failed to move when gently probed with a pipette tip in the cervical region and were unable to reach the water surface. Dead larvae were examined for morphological abnormalities under a stereo microscope (SMZ-161, Motic Asia, Hong Kong, China). The percentage of larval mortality was calculated as (number of dead larvae in treatment/total number of larvae) × 100.

Brine Shrimp Lethality Testing

The brine shrimp lethality assay was employed to assess the toxicity of the essential oils. *Artemia franciscana* cysts, obtained from Universiti Malaysia Terengganu (UMT), Terengganu, Malaysia, were hatched in artificial seawater under constant aeration at room temperature for 48 h, and the nauplii were then fed every 24 h with PKC-Nutri+® (Tiong *et al.* 2024). A stock solution of essential oil at 10,000 µg/mL was prepared using 10%

ethanol and subsequently diluted using artificial seawater to obtain final concentrations of 1, 10, 100, 500, and 1000 µg/mL in separate plastic containers. Each container had a final volume of 5 mL. Ten brine shrimp nauplii were introduced into each container and incubated at room temperature for 24 h. Artificial seawater containing 1% ethanol served as the negative control, while potassium dichromate prepared at the same concentrations (1, 10, 100, 500, and 1000 µg/mL) served as the positive control. After incubation, the number of surviving nauplii in each container was counted, and the percentage of mortality (%) was calculated as [(number of survivors in control - number of survivors in treatment)/number of survivors in control] × 100.

Statistical Analysis

Antimicrobial assays and toxicity assessment were triplicated, while the mosquito larvicidal assay was performed in four independent experiments. Data from the extraction yields, mosquito larvicidal assays, and brine shrimp lethality assays were presented as means ± standard deviations. Results for antibacterial and antifungal assays were expressed as either mean values or ranges. Microsoft Excel 2019 was used for the calculation of the means and standard deviations, while IBM Statistical Package for the Social Sciences (SPSS) version 27 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The median lethal concentration (LC₅₀) and 95% lethal concentration (LC₉₅) of the essential oils against brine shrimp nauplii and mosquito larvae were determined using Probit analysis. Student's *t*-test was applied to compare the extraction yields between two tree age groups. Two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was applied to analyze brine shrimp mortality rates with essential oil type and concentration as independent factors. Four-way ANOVA followed by Tukey's post-hoc test was employed to assess mosquito larval mortality rates with essential oil type, concentration, exposure time, and mosquito species being independent variables. The significance level of *P* < 0.05 was adopted throughout the study.

RESULTS AND DISCUSSION

Extraction Yield and Phytochemical Composition

After 8 h of hydrodistillation, essential oil yields from fresh *Eucalyptus* hybrid leaves were 0.118% ± 0.012% (w/w) for HfA and 0.026% ± 0.002% (w/w) for HfB, calculated on a fresh weight basis. On the other hand, dried leaves yielded 0.191% ± 0.029% (w/w) for HdA and 0.046% ± 0.004% (w/w) for HdB, based on dry weight. Correspondingly, for 1 kg of plant material, the extraction volumes obtained were 1.225, 1.977, 0.273, and 0.477 mL for HfA, HdA, HfB, and HdB, respectively. Notably, the younger *Eucalyptus* hybrid trees (HfA, HdA) consistently produced significantly higher essential oil yields than the older *Eucalyptus* hybrid trees (HfB, HdB) under both fresh and dried leaf conditions (*P* < 0.05).

Thirty-eight components were successfully identified in the essential oil samples, accounting for 95.6% to 99.7% of the total peak area (Table 1), with corresponding total ion chromatograms shown in Tables S1-S4. 1,8-cineol and α-terpinyl acetate were the dominant components across all samples. 1,8-cineol constituted 22.9% in HfA, 26.7% in HdA, 13.0% in HfB, and 21.9% in HdB, while α-terpinyl acetate accounted for 26.1% in HfA, 19.7% in HdA, 24.6% in HfB, and 18.3% in HdB. In addition to these two major constituents, HfA and HdA also exhibited high abundance of α-pinene (13.9%; 15.7%), α-

terpineol (7.7%; 6.3%), and limonene (5.9%; 8.2%), respectively. In contrast, HfB and HdB displayed more variations in their phytochemical profiles, with HfB being dominated by α -terpineol (9.2%), borneol (6.6%), and globulol (5.1%), while HdB was characterized by high levels of α -terpineol (9.3%), α -pinene (7.8%), and borneol (7.5%).

A comparison of phytochemical profiles between the two age groups revealed that essential oils obtained from younger *Eucalyptus* trees uniquely contained seven chemical constituents, namely aromadendrene (HfA: 1.95%; HdA: 1.79%), epiglobulol (HfA: 0.41%; HdA: 0.53%), isoamyl isobutyrate (HfA: 0.35%), terpinen-4-ol (HfA: 0.63%), bornyl acetate (HfA: 0.50%), isocarveol (HfA: 0.39%), and α -cubebene (HfA: 0.36%). Conversely, five components were found exclusively in essential oils from older *Eucalyptus* trees: butyl isobutyl phthalate (HfB: 1.02%), humulene (HfB: 0.81%), τ -muurolol (HfB: 0.72%), δ -cadinene (HfB: 1.12%; HdB: 0.68%), and caryophyllene oxide (HfB: 1.64%; HdB: 0.93%).

Additionally, some compounds were detected exclusively in either fresh or dried leaf essential oils, regardless of tree age. FLEO contained eight unique compounds that were not present in DLEO: namely, terpinen-4-ol (HfA: 0.63%), isocarveol (HfA: 0.39%), bornyl acetate (HfA: 0.50%), α -cubebene (HfA: 0.36%), isoamyl isobutyrate (HfA: 0.35%), humulene (HfB: 0.81%), τ -muurolol (HfB: 0.72%), and butyl isobutyl phthalate (HfB: 1.02%). Conversely, DLEO exclusively contained fenchene (HdA: 0.51%; HdB: 0.48%), isoterpinolene (HdA: 0.79%; HdB: 0.48%), and cubebin-11-ol (HdA: 0.47%; HdB: 0.71%).

The essential oil yields obtained in the present study were compared to those reported by da Silva *et al.* (2020), who hydrodistilled fresh leaves of *Eucalyptus grandis* $\times$ *Eucalyptus urophylla* cultivated in Brazil for 4 h and achieved an essential oil yield of 1.03% (w/w). In contrast, despite using a longer hydrodistillation period of 8 h, the yields obtained in the present study were much lower, with 0.118% (w/w) for HfA and 0.026% (w/w) for HfB. Essential oil yield in aromatic plants is affected by a complex group of factors, and the observed variations in the yield may be attributed to differences in cultivation practices and environmental conditions such as geographical location, soil type, rainfall, climate, and air temperature (Gilles *et al.* 2010; Malaka *et al.* 2022). Prolonged extraction at high temperatures may cause thermal degradation of essential oils. For example, the yields of *Piper nigrum* (green pepper) essential oil increased as the hydrodistillation time extended from 30 min to 180 min, but they decreased when further prolonged to 300 min (Dao *et al.* 2020).

This study also highlighted the influence of tree age on essential oil yield. Leaves harvested from older trees yielded significantly less essential oil. Essential oils accumulate in the specialized glands during leaf development but may diminish after full leaf expansion *via* evaporation and leakages (Fikremariam *et al.* 2019). In addition, the branches become wider and denser as trees grow, which may reduce sunlight exposure to the leaves, impairing photosynthesis and reducing carbon flux for secondary metabolite production (Fajar *et al.* 2019). These findings align with the research of Shiferaw *et al.* (2019), who reported a decline in essential oil yield from 1.32% to 1.10%, obtained from *Eucalyptus globulus* as the age of the tree increased from 3 years to 8 years.

Table 1. Phytochemical Composition of *Eucalyptus grandis* × *Eucalyptus urophylla* Essential Oils Identified Using NIST 23 Mass Spectral Library

No.	Components	Peak area (%)			
		HfA	HdA	HfB	HdB
1.	α -Pinene*	13.9	15.7	1.36	7.84
2.	Fenchene	-	0.51	-	0.48
3.	Isoamyl isobutyrate	0.35	-	-	-
4.	<i>o</i> -Cymene	0.75	0.79	0.74	1.40
5.	Limonene*	5.92	8.24	3.06	5.53
6.	1,8-Cineol*	22.9	26.7	13.1	21.9
7.	Isoterpinolene	-	0.79	-	0.48
8.	Fenchol	1.57	1.56	2.54	3.03
9.	α -Campholenal	0.64	0.60	0.63	0.71
10.	Sabinol	0.97	0.80	1.43	1.59
11.	Pinocarpone	0.42	0.59	-	0.57
12.	Borneol*	3.58	3.07	6.62	7.46
13.	Terpinen-4-ol	0.63	-	-	-
14.	α -Terpineol*	7.67	6.31	9.22	9.34
15.	Isocarveol	0.39	-	-	-
16.	Bornyl acetate	0.50	-	-	-
17.	α -Terpinyl acetate	26.1	19.7	24.6	18.3
18.	α -Cubebene	0.36	-	-	-
19.	β -Caryophyllene*	1.32	1.34	4.98	3.00
20.	Aromadendrene*	1.95	1.79	-	-
21.	Humulene	-	-	0.81	-
22.	Humulen-(v1)	0.74	0.72	1.23	0.89
23.	Viridiflorene	-	0.38	1.28	0.88
24.	δ -Cadinene	-	-	1.12	0.68
25.	<i>trans</i> -Calamenene	0.53	0.55	1.52	1.13
26.	Epiglobulol	0.41	0.53	-	-
27.	Ledol	-	0.37	0.83	-
28.	Spathulenol	0.78	0.88	4.02	2.56
29.	Caryophyllene oxide	-	-	1.64	0.93
30.	Globulol	3.50	3.69	5.07	2.62
31.	Viridiflorol	0.79	0.77	2.49	1.22
32.	Cubeban-11-ol	-	0.47	-	0.71
33.	Rosifoliol	0.58	0.48	1.49	-
34.	β -Eudesmol	-	0.82	1.61	0.74
35.	1,10-Diepicubenol	0.82	0.87	1.32	0.67
36.	τ -Muurolol	-	-	0.72	-
37.	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl) propan-2-ol	0.74	0.67	1.98	1.02
38.	Butyl isobutyl phthalate	-	-	1.02	-
	Total Identified (%)	98.8	99.7	96.4	95.6
	Monoterpenes (%)	19.8	25.2	4.42	14.3
	Sesquiterpenes (%)	4.37	4.23	9.42	5.45
	Monoterpenoids (%)	65.4	59.3	58.1	62.8
	Sesquiterpenoids (%)	8.15	10.1	22.7	11.6
	Others (%)	1.10	0.79	1.76	1.40

*Identification of components was further confirmed using authentic standards. '-' denotes not detected. HfA: Fresh leaf essential oil from *Eucalyptus* hybrid aged 17 to 31 months; HdA: Dried leaf essential oil from *Eucalyptus* hybrid aged 17 to 31 months; HfB: Fresh leaf essential oil from *Eucalyptus* hybrid aged 40 to 50 months; HdB: Dried leaf essential oil from *Eucalyptus* hybrid aged 40 to 50 months.

Table 2. Phytochemical Composition of Essential Oils Obtained from *Eucalyptus grandis* × *Eucalyptus urophylla* and Its Parental Species via Hydrodistillation

Eucalyptus species	Hydrodistillation condition; duration	Main Constituents	Reference
<i>E. grandis</i> × <i>E. urophylla</i>	Fresh leaves; 8 h (HfA)	α-terpinyl acetate (26.1%), 1,8-cineole (22.9%), α-pinene (13.9%), α-terpineol (7.67%)	Present study
	Dried leaves; 8 h (HdA)	1,8-cineole (26.7%), α-terpinyl acetate (19.7%), α-pinene (15.7%), limonene (8.24%)	
	Fresh leaves; 8 h (HfB)	α-terpinyl acetate (24.6%), 1,8-cineole (13.1%), α-terpineol (9.22%), borneol (6.62%)	
	Dried leaves; 8 h (HdB)	1,8-cineole (21.9%), α-terpinyl acetate (18.3%), α-terpineol (9.34%), α-pinene (7.84%)	
	Fresh leaves; 4 h	1,8-cineole (28.2%, 20.3%), α-terpinyl acetate (14.2%, 17.6%), α-terpineol (10.9%, 11.5%), caryophyllene oxide (7.25%, 10.8%)	Borges <i>et al.</i> 2024
	Fresh leaves; 4 h	1,8-cineole (48.2%), α-pinene (15.6%), α-cubebene (9.86%), α-terpineol (3.26%)	Lu <i>et al.</i> 2014
	Fresh leaves; 4 h	1,8-cineole (41.3%), α-pinene (27.7%), α-terpinyl acetate (7.95%), α-terpineol (6.90%)	da Silva <i>et al.</i> 2020
	Fresh leaves; 2 h	1,8-cineole (26.4%), α-gurjunene (15.4%), o-cymene (15.4%), α-pinene (10.2%)	Gallon <i>et al.</i> 2020
<i>E. grandis</i>	Fresh leaves; 1 h 10 min	α-pinene (52.7%), 1,8-cineole (18.4%), p-cymene (9.70%), α-terpineol (5.67%)	Lucia <i>et al.</i> 2007
	Dried leaves; 6 h	p-cymene (26.8%), α-pinene (15.9%), 1,8-cineole (6.28%), α-terpineol (4.10%)	Tian <i>et al.</i> 2011
	Fresh leaves; 4 h	1,8-cineole (23.6%), α-pinene (21.8%), p-cymene (13.4%), γ-terpinene (9.35%)	Lu <i>et al.</i> 2014
	Fresh leaves; > 3 h	α-pinene (29.6%), p-cymene (19.8%), 1,8-cineole (12.8%), α-terpineol (6.40%)	Sewanu <i>et al.</i> 2015
	Dried leaves; > 3 h	1,8-cineole (47.4%), limonene (13.3%), α-pinene (7.50%), spathulenol (7.10%)	Sewanu <i>et al.</i> 2015
	Fresh leaves; 2 h	α-pinene (17.0%), o-cymene (15.6%), terpineol (8.61%), 1,8-cineole (6.90%)	Gallon <i>et al.</i> 2020
	Dried leaves; 2 h	β-(Z)-ocimene (35.5%), 1,8-cineole (19.3%), α-pinene (9.29%), α-phellandrene (3.84%)	Koursaoui <i>et al.</i> 2023
<i>E. urophylla</i>	Fresh leaves; 7 h	1,8-cineole (57.7%), α-pinene (10.1%), limonene (6.40%), β-ocimene (4.40%), globulol (4.40%)	Cimanga <i>et al.</i> 2002
	Fresh leaves; 6 h	1,8-cineole (58.3%), α-terpinyl acetate (14.9%), α-pinene (6.25%), cis-ocimene (3.55%)	Cheng <i>et al.</i> 2009a
	Fresh leaves; 4 h	1,8-cineole (57.1%), α-cubebene (9.69%), p-cymene (4.22%), α-pinene (3.95%)	Lu <i>et al.</i> 2014
	Fresh leaves (duration not reported)	1,8-cineole (40.8%), γ-terpinene (23.8%), m-cymene (7.43%), terpinyl acetate (6.14%)	Insuan <i>et al.</i> 2021

A comparative summary of essential oil compositions with previous studies is presented in Table 2. Despite the differences in yield, the predominant constituents identified in this study were generally consistent with earlier studies. 1,8-cineole was consistently the major component across all essential oil samples. α -Pinene was also found in considerable abundance in most samples, except in HfB from the current study, as well as the FLEOs reported by Insuan *et al.* (2021) and Borges *et al.* (2024). In addition, the high percentage of α -terpinyl acetate reported by da Silva *et al.* (2020) and Borges *et al.* (2024) in their FLEOs from the same *Eucalyptus* hybrid was similarly observed as one of the dominant constituents in all our samples. In contrast, *p*-cymene, detected in high proportions in some *E. grandis* and *E. urophylla* essential oils, was absent in the current study. These findings suggest that variations in chemical composition may result from differences in species, extraction parameters, and cultivation environments. Moreover, phytochemical analysis revealed that essential oil composition varied according to the plant age and leaf condition, indicated by the presence of unique compounds detected in younger and older *Eucalyptus* hybrids, as well as between fresh and dried leaf samples (Table 1). Disparities in the phytochemical profiles of essential oils between fresh and dried plant samples have been recorded in earlier studies on basil and thyme essential oils (Ghasemi Pirbalouti *et al.* 2013; Rahimmalek and Goli 2013). Such variations may arise due to the degradation or transformation of compounds via oxidation, glycoside hydrolysis, dehydration, and esterification during the drying process, as well as the rupture of plant cells that release the volatile compounds (Díaz-Maroto *et al.* 2004; Sewanu *et al.* 2015).

Bioactivities and Toxicity of Essential Oils

Table 3 summarizes the antibacterial activity of the essential oils against ten tested bacterial species. Generally, Gram-positive bacteria were more susceptible to the essential oils than Gram-negative bacteria. Among the essential oil samples, HdB displayed slightly stronger antibacterial activity against Gram-positive ATCC strains. It inhibited the growth of *Bacillus cereus* and *Staphylococcus aureus* ATCC strains at concentrations between 0.16 to 0.31 mg/mL, and *Enterococcus hirae* at 0.63 mg/mL, representing the lowest MIC values recorded among all tested essential oils. Although all essential oils, except HfA, inhibited the two clinically isolated *S. aureus* strains at 2.50 mg/mL, only HdB exhibited bactericidal activity against *S. aureus* SA-LWE23#1, a methicillin-resistant clinical isolate, at the same concentration. On the other hand, the Gram-negative bacteria were all generally less sensitive. Out of the five Gram-negative bacterial strains tested, only *Klebsiella pneumoniae* ATCC strain showed susceptibility to all four essential oils, with MIC values ranging from 1.25 to 2.50 mg/mL, and with bactericidal effect observed only for HfB at 2.50 mg/mL.

The antibacterial results demonstrated that Gram-positive bacteria were generally more sensitive to the essential oils than Gram-negative bacteria. This difference can be explained by the presence of a lipopolysaccharide outer membrane in Gram-negative bacteria, which serves as a hydrophilic barrier against the penetration of hydrophobic compounds (Simpson *et al.* 2015). In contrast, the more permeable cell wall in Gram-positive bacteria allows hydrophobic compounds in essential oils to penetrate them and interact with the phospholipid bilayer, leading to increased ion permeability, intracellular components leakage, and bacterial enzyme disruption (Silva *et al.* 2011; Barbosa *et al.* 2016). Furthermore, in a related study, Zhou *et al.* (2021) demonstrated stronger antibacterial activity from *E. grandis* $\times$ *E. urophylla* leaf essential oils obtained using steam distillation, which inhibited *Escherichia coli* and *Pseudomonas aeruginosa* at

concentrations of 0.091 mg/mL and 0.023 mg/mL, respectively; unlike the current study, where no inhibition was observed against both bacterial species. Additionally, their reported MIC against *Bacillus cereus* was 0.045 mg/mL, which is substantially lower than the MIC values (0.16-0.63 mg/mL) found in this study (Table 3).

Table 3. Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations of *Eucalyptus grandis* × *Eucalyptus urophylla* Essential Oils Against Bacterial Species

Bacterial Species	MIC Values (mg/mL)					MBC Values (mg/mL)			
	HfA	HdA	HfB	HdB	PC	HfA	HdA	HfB	HdB
Gram-positive Bacteria									
<i>Bacillus cereus</i> ATCC® 14579™	0.63	0.31	0.31	0.16-0.31	2-4	0.63	0.31	0.31	0.31
<i>Enterococcus hirae</i> ATCC® 10541™	1.25-2.50	1.25	1.25-2.50	0.63	32-64	NA	NA	2.50	0.63
<i>Staphylococcus aureus</i> ATCC® 6538™	0.31-0.63	0.31-0.63	0.31-0.63	0.31	1-2	1.25	1.25	1.25	0.63
<i>Staphylococcus aureus</i> SA-LWE23#1	NA	2.50	2.50	2.50	16	-	NA	NA	2.50
<i>Staphylococcus aureus</i> SA-LWE23#2	NA	2.50	2.50	2.50	32-64	-	NA	NA	NA
Gram-negative Bacteria									
<i>Escherichia coli</i> ATCC® 35218™	NA	NA	NA	NA	64-128	-	-	-	-
<i>Escherichia coli</i> EC-LWE23#1	NA	NA	NA	NA	64-128	-	-	-	-
<i>Klebsiella pneumoniae</i> ATCC® 13883™	2.50	2.50	1.25-2.50	1.25-2.50	1-2	NA	NA	2.50	NA
<i>Klebsiella pneumoniae</i> KP-LWE23#1	NA	NA	NA	NA	8	-	-	-	-
<i>Pseudomonas aeruginosa</i> ATCC® 15442™	NA	NA	NA	NA	16-32	-	-	-	-
The data are expressed as mean or range of three consistent replicates. NA denotes no activity. “-” denotes not applicable, as no MIC was determined. PC: Positive control with chloramphenicol and expressed in µg/mL. HfA: Fresh leaf essential oil from <i>Eucalyptus</i> hybrid aged 17 to 31 months; HdA: Dried leaf essential oil from <i>Eucalyptus</i> hybrid aged 17 to 31 months; HfB: Fresh leaf essential oil from <i>Eucalyptus</i> hybrid aged 40 to 50 months; HdB: Dried leaf essential oil from <i>Eucalyptus</i> hybrid aged 40 to 50 months.									

The antifungal activity of essential oil samples was assessed against eight fungal species. As shown in Table 4, the antifungal efficacies of the four essential oils did not differ noticeably, as the variations in MIC and MFC values for each fungal strain did not exceed a two-fold difference. The tested yeasts demonstrated susceptibility to all four essential oils, with MIC and MFC values ranging from 0.16 to 1.25 mg/mL and 0.31 to 2.50 mg/mL, respectively. *Candida tropicalis* was the most susceptible among the *Candida* species, with the lowest MIC values of 0.16 to 0.31 mg/mL recorded for HdA and HfB.

Notably, all four essential oils exhibited fungicidal activity against *Cryptococcus neoformans*, with an MFC of 0.31 mg/mL. Strong antifungal activity was also observed against the ringworm-causing dermatophyte *Trichophyton rubrum*, where the HfB and HdB achieved the lowest MFC value of 0.04 mg/mL, followed by 0.08 mg/mL for HfA and HdA, as their MFC values were <0.10 mg/mL (Saraiva *et al.* 2011). In contrast, *Aspergillus fumigatus* exhibited high resistance to the essential oils, with no inhibitory effects observed for any of the samples. To the best of our knowledge, this is the first study of the essential oils from *E. grandis* × *E. urophylla* leaves using hydrodistillation against human pathogens. Previous antimicrobial studies on essential oils of this hybrid all deployed the steam distillation technique, as summarized in Table 5.

Table 4. Minimum Inhibitory Concentrations and Minimum Fungicidal Concentrations of *Eucalyptus grandis* × *Eucalyptus urophylla* Essential Oils Against Fungal Species

Fungal Species	MIC Values (mg/mL)					MFC Values (mg/mL)			
	HfA	HdA	HfB	HdB	PC	HfA	HdA	HfB	HdB
Yeasts									
<i>Candida albicans</i>	0.63-1.25	0.63	0.63	0.31-0.63	4-8	1.25	0.63	0.63	0.63
<i>Candida auris</i>	1.25	1.25	1.25	1.25	0.25-0.50	2.50	2.50	1.25	1.25
<i>Candida parapsilosis</i>	1.25	1.25	1.25	1.25	2-4	2.50	1.25	2.50	2.50
<i>Candida tropicalis</i>	0.31	0.16-0.31	0.16-0.31	0.63	2-4	0.31	0.31	0.31	0.63
<i>Cryptococcus neoformans</i>	0.31	0.31	0.31	0.31	2-4	0.31	0.31	0.31	0.31
<i>Nakaseomyces glabratus</i>	1.25	0.63	1.25	1.25	0.06-0.13	1.25	2.50	2.50	NA
Filamentous fungi									
<i>Aspergillus fumigatus</i>	NA	NA	NA	NA	8-16	-	-	-	-
<i>Trichophyton rubrum</i>	0.08	0.08	0.04	0.04	16-32	0.08	0.08	0.04	0.04
The data are expressed as mean or range of three consistent replicates. NA denotes no activity. “-” denotes not applicable as no MIC was determined. PC: positive control with 5-fluorocytosine for yeasts and itraconazole for filamentous fungi and expressed in µg/mL. HfA: Fresh leaf essential oil from <i>Eucalyptus</i> hybrid aged 17 to 31 months; HdA: Dried leaf essential oil from <i>Eucalyptus</i> hybrid aged 17 to 31 months; HfB: Fresh leaf essential oil from <i>Eucalyptus</i> hybrid aged 40 to 50 months; HdB: Dried leaf essential oil from <i>Eucalyptus</i> hybrid aged 40 to 50 months.									

This study assessed the larvicidal activity of essential oils obtained from *Eucalyptus* hybrid leaves by exposing third instar larvae of *Ae. aegypti* and *Ae. albopictus* to five concentrations ranging from 25 to 400 µg/mL for 24 h and 48 h. No mortality was observed in the negative control (1% methanol), while temephos (positive control) caused 100% mortality to the larvae of both species within 24 h of treatment. In the assay against *Ae. aegypti* larvae (Fig. 2), essential oils derived from younger *Eucalyptus* hybrid trees (HfA and HdA) consistently induced higher larval mortality after 48 h compared to those obtained from older *Eucalyptus* hybrid trees (HfB and HdB).

Table 5. Reported Bioactivities of Essential Oils Obtained from *Eucalyptus grandis* × *Eucalyptus urophylla* or Its Parental Species

Bioactivity	Eucalyptus species	Extraction and part used	Tested organism	Reported efficacy	Reference
Antibacterial activity	<i>E. grandis</i> × <i>E. urophylla</i>	Steam distillation; leaves	<i>Bacillus cereus</i> , <i>Bacillus subtilis</i> , <i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i> , <i>Salmonella enterica</i> serovar Typhimurium, <i>Staphylococcus aureus</i>	Inhibition zone = 14.3-18.1 mm MIC = 0.023-0.091 mg/mL MBC = 10 mg/mL	Zhou <i>et al.</i> 2021
	<i>E. grandis</i> × <i>E. urophylla</i>	Steam distillation; leaves	<i>Escherichia coli</i> , <i>Staphylococcus aureus</i>	MIC = 0.63%-1.25% MBC = 1.25%	Salvatori <i>et al.</i> 2023
	<i>E. grandis</i> × <i>E. urophylla</i>	Steam distillation; leaves	<i>Streptococcus mutans</i>	Inhibition zone = 23.0 mm MIC = 0.025 mg/mL	Goldbeck <i>et al.</i> 2014
Antifungal activity	<i>E. grandis</i> × <i>E. urophylla</i>	Steam distillation; leaves	<i>Candida albicans</i>	MIC = 0.31% MFC = 0.63%	Salvatori <i>et al.</i> 2023
Mosquito larvicidal activity	<i>E. grandis</i> × <i>E. urophylla</i>	Hydrodistillation; leaves	<i>Aedes aegypti</i>	100% mortality at 100 µg/mL (24 h)	Gallon <i>et al.</i> 2020
	<i>E. grandis</i>	Hydrodistillation; leaves	<i>Aedes aegypti</i>	LC ₅₀ = 32.4 µg/mL (24 h)	Lucia <i>et al.</i> 2007
	<i>E. urophylla</i>	Hydrodistillation; leaves	<i>Aedes aegypti</i>	LC ₅₀ = 95.5 µg/mL (24 h) LC ₉₀ = 166 µg/mL (24 h)	Cheng <i>et al.</i> 2009a
			<i>Aedes albopictus</i>	LC ₅₀ = 286 µg/mL (24 h) LC ₉₀ = >400 µg/mL (24 h)	
MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; MFC: Minimum fungicidal concentration; LC ₅₀ : median lethal concentration; LC ₉₀ : 90% lethal concentration.					

Specifically, HfA and HdA achieved over 60% mortality at the lowest concentration of 25 µg/mL (HfA: 62.8% ± 24.0%; HdA: 69.0% ± 17.4%) and reached complete mortality at 200 and 400 µg/mL after 48 h. In contrast, HfB and HdB exhibited less than 15% mortality (HfB: 12.5% ± 9.57%; HdB: 7.50% ± 6.45%) at the lowest concentration and only achieved complete mortality at the highest concentration. Comparatively, *Ae. albopictus* larvae were more susceptible to the essential oils. HfA, HdA, and HfB induced 100% mortality at 200 and 400 µg/mL within 24 h, while HdB required 48 h to reach the same effect (Fig. 3). Although HdB consistently demonstrated lower larval mortality than the essential oils from younger *Eucalyptus* hybrid trees (HfA and HdA), HfB unexpectedly exhibited higher efficacy at lower concentrations, producing 77.5% ± 11.9% mortality at 50 µg/mL and 95.0% ± 0% at 100 µg/mL after 48 h.

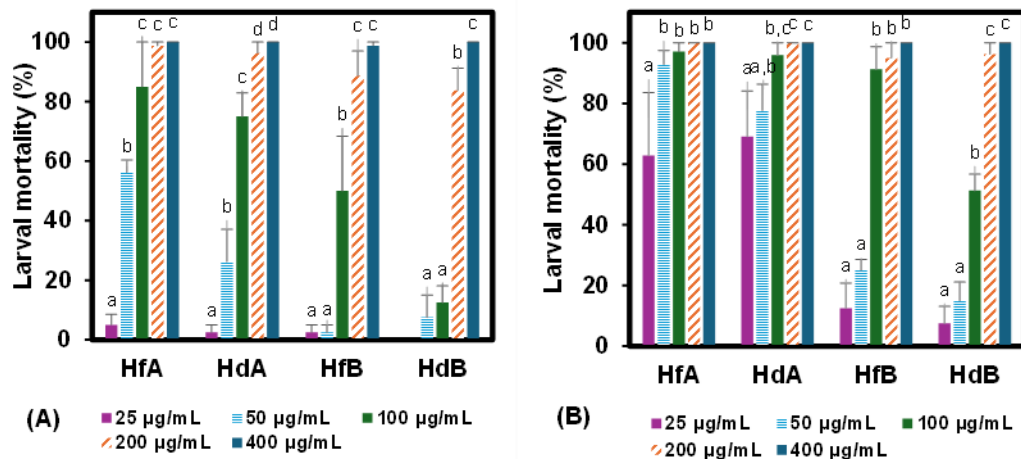


Fig. 2. Larval mortality rate of *Aedes aegypti* at five concentration levels of *Eucalyptus grandis* × *Eucalyptus urophylla* essential oils after 24 h (A) and 48 h (B) post-exposure. Bars with different letters (a, b, c) represent significant differences ($P < 0.05$) between concentrations for each sample. HfA: Fresh leaf essential oil from *Eucalyptus* hybrid aged 17 to 31 months; HdA: Dried leaf essential oil from *Eucalyptus* hybrid aged 17 to 31 months; HfB: Fresh leaf essential oil from *Eucalyptus* hybrid aged 40 to 50 months; HdB: Dried leaf essential oil from *Eucalyptus* hybrid aged 40 to 50 months

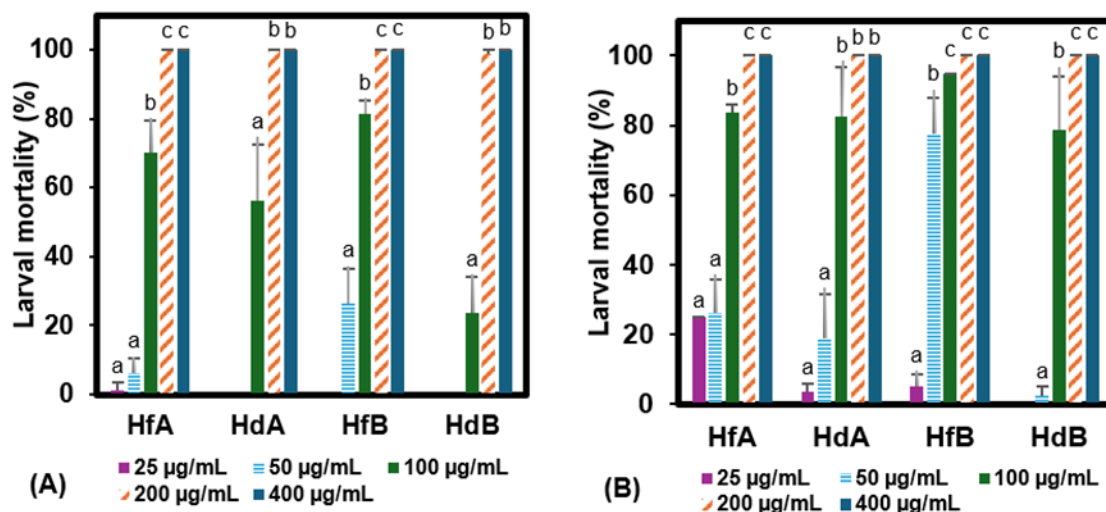


Fig. 3. Larval mortality rate of *Aedes albopictus* at five concentration levels of *Eucalyptus grandis* × *Eucalyptus urophylla* essential oils after 24 h (A) and 48 h (B) post-exposure. Bars with different letters (a, b, c) represent significant differences ($P < 0.05$) between concentrations for each sample. HfA: Fresh leaf essential oil from *Eucalyptus* hybrid aged 17 to 31 months; HdA: Dried leaf essential oil from *Eucalyptus* hybrid aged 17 to 31 months; HfB: Fresh leaf essential oil from *Eucalyptus* hybrid aged 40 to 50 months; HdB: Dried leaf essential oil from *Eucalyptus* hybrid aged 40 to 50 months

Four-way ANOVA analysis demonstrated that larval mortality rates differed significantly ($P < 0.001$) across essential oils, exposure times, mosquito species, and concentration levels. Furthermore, interactions among these variables were also statistically significant ($P < 0.05$), except for the interaction between exposure time and essential oil ($P = 0.057$), as shown in Table 6. Probit regression analysis was performed to determine the median lethal concentration (LC_{50}) and 95% lethal concentration (LC_{95}) of

the essential oils. Among the four samples, HfA exhibited the strongest larvicidal activity against *Ae. aegypti*, consistently demonstrating the lowest LC₅₀ values across both 24 h (52.3 µg/mL) and 48 h (14.3 µg/mL), along with corresponding LC₉₅ values of 130 µg/mL and 71.9 µg/mL, respectively (Table 7). For *Ae. albopictus*, HfB was most effective in killing the larvae, demonstrating the lowest LC₅₀ of 67.3 µg/mL after 24 h and the corresponding LC₉₅ of 131 µg/mL. Its efficacy further increased after 48 h, with the LC₅₀ being reduced to 42.1 µg/mL and the LC₉₅ to 81.2 µg/mL (Table 7). These findings indicate that the larval susceptibility to essential oils was influenced by the mosquito species.

Table 6. Four-way ANOVA Analysis for Mosquito Larvicidal Activity of *Eucalyptus grandis* × *Eucalyptus urophylla* Essential Oils

Dependent Variable: <i>Aedes</i> larval mortality					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	529947.848 ^a	79	6708.201	110.938	< 0.001
Intercept	1221588.899	1	1221588.899	20202.294	< 0.001
Concentration	405356.620	4	101339.155	1675.919	< 0.001
Time	20025.032	1	20025.032	331.168	< 0.001
Essential Oil	20402.315	3	6800.772	112.469	< 0.001
Species	2372.957	1	2372.957	39.243	< 0.001
Concentration * Time	11102.329	4	2775.582	45.902	< 0.001
Concentration * Essential Oil	16339.229	12	1361.602	22.518	< 0.001
Concentration * Species	7390.419	4	1847.605	30.555	< 0.001
Time * Essential Oil	460.329	3	153.443	2.538	0.057
Time * Species	1326.026	1	1326.026	21.929	< 0.001
Essential Oil * Species	14068.449	3	4689.483	77.553	< 0.001
Concentration * Time * Essential Oil	8903.494	12	741.958	12.270	< 0.001
Concentration * Time * Species	1956.616	4	489.154	8.089	< 0.001
Concentration * Essential Oil * Species	14912.610	12	1242.718	20.552	< 0.001
Time * Essential Oil * Species	977.960	3	325.987	5.391	0.001
Concentration * Time * Essential Oil * Species	4353.464	12	362.789	6.000	< 0.001
Error	14512.279	240	60.468		
Total	1766049.026	320			
Corrected Total	544460.127	319			

^aR Squared = 0.973 (Adjusted R Squared = 0.965)

Table 7. Lethal Concentrations and Probit Analysis of *Eucalyptus grandis* × *Eucalyptus urophylla* Essential Oils against Third-Instar Larvae of *Aedes* Mosquitoes

Sample	Time (h)	LC ₅₀ (µg/mL) (LCL-UCL)	LC ₉₅ (µg/mL) (LCL-UCL)	Regression coefficient ± Standard error	Chi-Square, χ ²	P-value
<i>Aedes aegypti</i>						
HfA	24	52.3 (40.4-66.0)	130 (95.4-242)	4.162 ± 0.386	5.426	0.143
	48	14.3 (7.60-19.8)	71.9 (56.3-110)	2.342 ± 0.432	1.950	0.583
HdA	24	70.9 (64.0-78.5)	174 (149-215)	4.210 ± 0.366	0.392	0.942
	48	15.0 (8.25-20.8)	91.8 (70.6-143)	2.090 ± 0.353	4.380	0.223
HfB	24	105 (71.2-156)	261 (172-817)	4.171 ± 0.348	12.062	0.007
	48	59.3 (33.0-100)	165 (97.9-1170)	3.714 ± 0.317	17.162	< 0.001
HdB	24	135 (72.5-283)	297 (180-6130)	4.793 ± 0.423	22.919	< 0.001
	48	84.9 (53.9-137)	240 (145-1090)	3.640 ± 0.297	14.304	0.003
<i>Aedes albopictus</i>						
HfA	24	82.0 (50.3-139)	151 (1040-1350)	6.185 ± 0.624	17.189	< 0.001
	48	53.4 (18.4-119)	170 (88.9-19900)	3.274 ± 0.295	23.837	< 0.001
HdA	24	96.3 (89.3-104)	150 (133-184)	8.559 ± 1.336	0.878	0.831
	48	67.4 (53.6-85.6)	140 (105-261)	5.171 ± 0.481	6.173	0.103
HfB	24	67.3 (61.6-73.4)	131 (115-158)	5.678 ± 0.572	1.611	0.657
	48	42.1 (27.4-60.1)	81.2 (57.8-296)	5.774 ± 0.584	12.719	0.005
HdB	24	120 (111-129)	187 (167-223)	8.493 ± 1.053	0.946	0.814
	48	81.4 (75.4-87.3)	125 (114-145)	8.750 ± 1.035	0.166	0.983

A heterogeneity factor is used in the calculation of confidence limits if P < 0.150. LCL: Lower confidence limit; UCL: Upper confidence limit; HfA: Fresh leaf essential oil from *Eucalyptus* hybrid aged 17 to 31 months; HdA: Dried leaf essential oil from *Eucalyptus* hybrid aged 17 to 31 months; HfB: Fresh leaf essential oil from *Eucalyptus* hybrid aged 40 to 50 months; HdB: Dried leaf essential oil from *Eucalyptus* hybrid aged 40 to 50 months.

Moreover, morphological deformities were observed in the treated larvae compared to the untreated controls (Fig. 4). Larvae of both *Ae. aegypti* and *Ae. albopictus* exposed to the essential oil samples showed notable elongation in the neck region and blackening of the midgut.

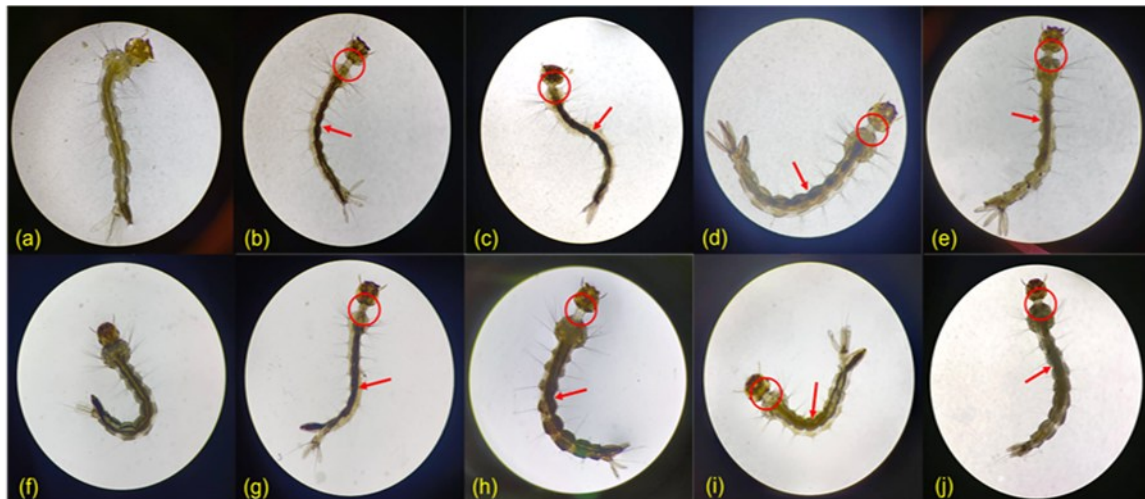


Fig. 4. Elongated necks (circle) and blackened midgut (arrow) were observed in the third instar larvae treated with essential oils of *Eucalyptus grandis* × *Eucalyptus urophylla* at 400 µg/mL (30× magnification under stereo microscope). (a) Control, untreated *Ae. aegypti* larvae; (b) HfA, *Aedes aegypti*; (c) HdA, *Ae. aegypti*; (d) HfB, *Ae. aegypti*; (e) HdB, *Ae. aegypti*; (f) Control, untreated *Ae. albopictus* larvae; (g) HfA, *Ae. albopictus*; (h) HdA, *Ae. albopictus*; (i) HfB, *Ae. albopictus*; (j) HdB, *Ae. albopictus*; HfA: Fresh leaf essential oil from *Eucalyptus* hybrid aged 17 to 31 months; HdA: Dried leaf essential oil from *Eucalyptus* hybrid aged 17 to 31 months; HfB: Fresh leaf essential oil from *Eucalyptus* hybrid aged 40 to 50 months; HdB: Dried leaf essential oil from *Eucalyptus* hybrid aged 40 to 50 months

The mosquito larvicidal activity of *E. grandis* × *E. urophylla* essential oils observed in this study was also compared to those reported in the literature (Table 5). The hybrid essential oils in the study of Gallon *et al.* (2020) showed higher *Ae. aegypti* larval mortality at 100 µg/mL than in the present study. However, the LC₅₀ values in the present study were lower than those described by Cheng *et al.* (2009a) against *Ae. albopictus* after 24 h post-treatment. Moreover, morphological deformities observed in the treated *Aedes* larvae, which revealed elongated necks and blackened midguts, are consistent with previous studies and may reflect underlying physiological damage. For instance, Seye *et al.* (2021) reportedly found intestinal tissue degradation, muscular disruption, and damaged microvilli in *Ae. aegypti* larvae that were treated with *Cymbopogon citratus* (lemongrass) essential oil. Similarly, Soonwera and Phasomkusolsil (2016) reported multiple deformities in *Ae. aegypti* larvae treated with essential oils of *C. citratus* and *Syzygium aromaticum* (clove), including elongated necks, enlarged thorax, and degraded respiratory tracheae and digestive tract, which suggested possible disruptions in hormonal regulation and chitin synthesis during the molting process.

Figure 5 illustrates the concentration-dependent mortality rate of brine shrimp nauplii following 24-h exposure to various concentrations of essential oil samples. A similar mortality trend was observed across all four essential oils, with 100% survival of exposed nauplii at the lower concentrations of 1 and 10 µg/mL. At 100 µg/mL, HfA, HdA, and HfB caused no mortality, whereas HdB resulted in a slight mortality rate of 3.33% ± 5.77%. Complete mortality was observed for all essential oils at the highest concentrations of 500 µg/mL and 1000 µg/mL.

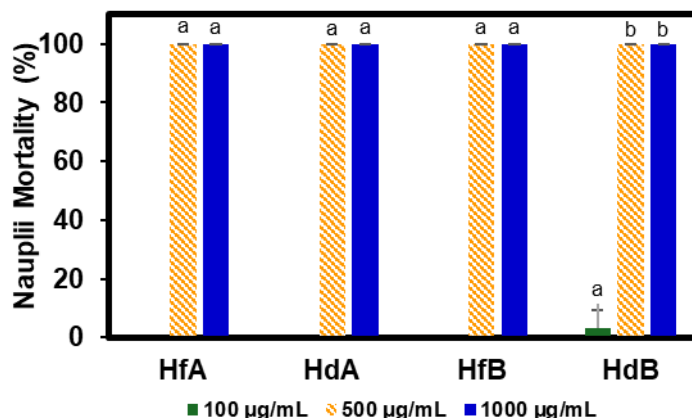


Fig. 5. Mortality rate of *Artemia franciscana* shrimp nauplii at different concentration levels of *Eucalyptus grandis* × *Eucalyptus urophylla* essential oils after 24 h post-exposure. No nauplii mortality was observed at 1 and 10 µg/mL. Bars with different letters (a, b) represent significant differences ($P < 0.05$) between concentrations for each sample. HfA: Fresh leaf essential oil from *Eucalyptus* hybrid aged 17 to 31 months; HdA: Dried leaf essential oil from *Eucalyptus* hybrid aged 17 to 31 months; HfB: Fresh leaf essential oil from *Eucalyptus* hybrid aged 40 to 50 months; HdB: Dried leaf essential oil from *Eucalyptus* hybrid aged 40 to 50 months.

Significant differences in nauplii mortality rates were observed only between concentration levels ($P < 0.001$) in the two-way ANOVA analysis (Table 8), with no significant effects among the essential oil samples ($P = 0.403$) or their interaction ($P = 0.446$). Probit regression analysis revealed LC_{50} and LC_{95} values of 222 and 468 µg/mL, respectively, for all HfA, HdA, and HfB. In contrast, a lower LC_{50} of 209 µg/mL and LC_{95} of 438 µg/mL were determined for HdB (Table 9). The positive control, potassium dichromate, revealed a much lower LC_{50} of 20.2 µg/mL and LC_{95} of 172 µg/mL, indicating that the essential oils possess substantially lower toxicity relative to the control.

Table 8. Two-way ANOVA Analysis for *Artemia franciscana* Nauplii Mortality of *Eucalyptus grandis* × *Eucalyptus urophylla* Essential Oils

Dependent variable: <i>Artemia franciscana</i> nauplii mortality					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	143231.667 ^a	19	7538.509	4523.105	< 0.001
Intercept	96801.667	1	96801.667	58081.000	< 0.001
Concentration	143206.667	4	35801.667	21481.000	< 0.001
Essential Oil	5.000	3	1.667	1.000	0.403
Concentration * Essential Oil	20.000	12	1.667	1.000	0.466
Error	66.667	40	1.667		
Total	240100.000	60			
Corrected Total	143298.333	59			

^aR Squared = 1.000 (Adjusted R Squared = 0.999)

The essential oil's bioactivity is highly related to its phytochemical composition. In this study, the essential oils contained a relatively higher proportion of terpenes and terpenoids, which are known for their bioactivities. Specifically, 1,8-cineol, α -terpinyl acetate, and α -pinene have demonstrated antimicrobial properties against various bacteria and fungi (Ložienė *et al.* 2018; Marei *et al.* 2019; Ivanov *et al.* 2021).

Table 9. Lethal Concentrations and Probit Analysis of *Eucalyptus grandis* × *Eucalyptus urophylla* Essential Oils against *Artemia franciscana* Nauplii

Sample	LC ₅₀ (µg/mL) (LCL-UCL)	LC ₉₅ (µg/mL) (LCL-UCL)	Regression coefficient ± Standard error	Chi- Square, χ ²	P-value
HfA	222 (167-300)	468 (340-785)	5.088 ± 0.901	2.363	0.500
HdA	222 (164-300)	468 (340-785)	5.088 ± 0.901	2.363	0.500
HfB	222 (164-300)	468 (340-785)	5.088 ± 0.901	2.363	0.500
HdB	209 (158-287)	438 (314-775)	5.129 ± 0.911	0.989	0.804
Potassium dichromate	20.2 (12.5-32.0)	172 (93.8-451)	1.765 ± 0.254	2.302	0.512
No heterogeneity factor is used in the calculation of confidence limits as P > 0.150. LCL: Lower confidence limit; UCL: Upper confidence limit; HfA: Fresh leaf essential oil from <i>Eucalyptus</i> hybrid aged 17 to 31 months; HdA: Dried leaf essential oil from <i>Eucalyptus</i> hybrid aged 17 to 31 months; HfB: Fresh leaf essential oil from <i>Eucalyptus</i> hybrid aged 40 to 50 months; HdB: Dried leaf essential oil from <i>Eucalyptus</i> hybrid aged 40 to 50 months					

Moreover, Perumalsamy *et al.* (2009) reported the larvicidal activities of 1,8-cineol, limonene, α -pinene, and α -terpineol against third instar *Ae. aegypti* larvae, with LC₅₀ values ranging from 24.5 to 112 µg/mL.

Cheng *et al.* (2009b) also documented an LC₅₀ of 74.0 µg/mL for α -pinene against *Ae. albopictus* larvae. However, the overall bioactivity of the essential oils was likely attributable to the synergistic or additive interactions between components, rather than from the individual components alone. Badr *et al.* (2021) revealed that pure α -terpinyl acetate exhibited greater antifungal and antibacterial activities on *Candida albicans* (half maximal effective concentration, EC₅₀ = 0.3 mg/mL) and *S. aureus* (MIC = 0.8 mg/mL) than the α -terpinyl acetate-rich lavender essential oil (EC₅₀ = 0.6 mg/mL and MIC = 3.0 mg/mL). Additionally, Mulyaningsih *et al.* (2010) reported synergistic antibacterial effects between aromadendrene and 1,8-cineol in inhibiting multidrug-resistant bacteria, while Sarma *et al.* (2019) found that the binary combinations of 1,8-cineol and α -pinene at a 1:1 ratio demonstrated synergistic larvicidal activity against *Ae. aegypti*, which achieved >90% larval mortality after 24 h. Notably, in the present study, no distinguishable differences in bioactivities were observed between the fresh and dried leaf essential oils or between the younger and older trees. Therefore, constituents such as 1,8-cineole, α -terpinyl acetate, and α -pinene, which were persistently detected in all tested samples, as well as their possible synergistic interactions, are proposed to contribute to the observed bioactivities. Their relatively stable presence across both tree age groups and leaf conditions also indicates that these major constituents are not susceptible to loss during leaf drying or tree maturation.

Importantly, comparison of the LC₅₀ values for brine shrimp lethality and *Aedes* mosquito larvicidal assays revealed relatively lower concentrations were required to achieve larval mortality. Given that the brine shrimp lethality assay is widely used to assess the toxicity of substances towards aquatic organisms, the current findings suggest that essential oils of *E. grandis* × *E. urophylla* are relatively safe for non-target aquatic ecosystems while retaining their potency as natural mosquito larvicide.

CONCLUSIONS

1. Hydrodistillation of fresh and dried leaves from *Eucalyptus grandis* × *Eucalyptus urophylla* at two different tree age groups successfully yielded essential oils, with younger trees consistently producing significantly higher yields.
2. Gas chromatography-mass spectrometry (GC-MS) analysis identified 1,8-cineol and α -terpinyl acetate as the dominant chemical components across all essential oil samples.
3. Although there was no notable variation in bioactivity observed between fresh leaf essential oils (FLEO) and dried leaf essential oils (DLEO), as well as between younger and older *Eucalyptus* hybrid trees, the essential oils exhibited stronger antifungal activity, particularly against the dermatophyte *Trichophyton rubrum*, than antibacterial effects.
4. The essential oils demonstrated concentration-dependent mosquito larvicidal activity against *Aedes aegypti* and *Aedes albopictus*, with effective larval mortality achieved at concentrations lower than those required to produce toxicity in the brine shrimp nauplii, suggesting a favorably safe level for non-target aquatic organisms.
5. The bioactivity of the essential oils could be attributed to the known antimicrobial and larvicidal properties of major components of the essential oils, as well as the possible synergistic interactions between the components.
6. The findings of this study are indicative of the potential of essential oils from *E. grandis* × *E. urophylla* as natural disinfectants and mosquito larvicides, offering a low-risk alternative to synthetic chemicals for combating infectious and vector-borne diseases. This aligns with the Sustainable Development Goal (SDG) 3: Good Health and Well-being. Furthermore, by exploring the valorization of agro-industrial wastes such as *Eucalyptus* hybrid leaves, the study contributes to SDG 12: Responsible Consumption and Production, promoting sustainable applications of natural resources.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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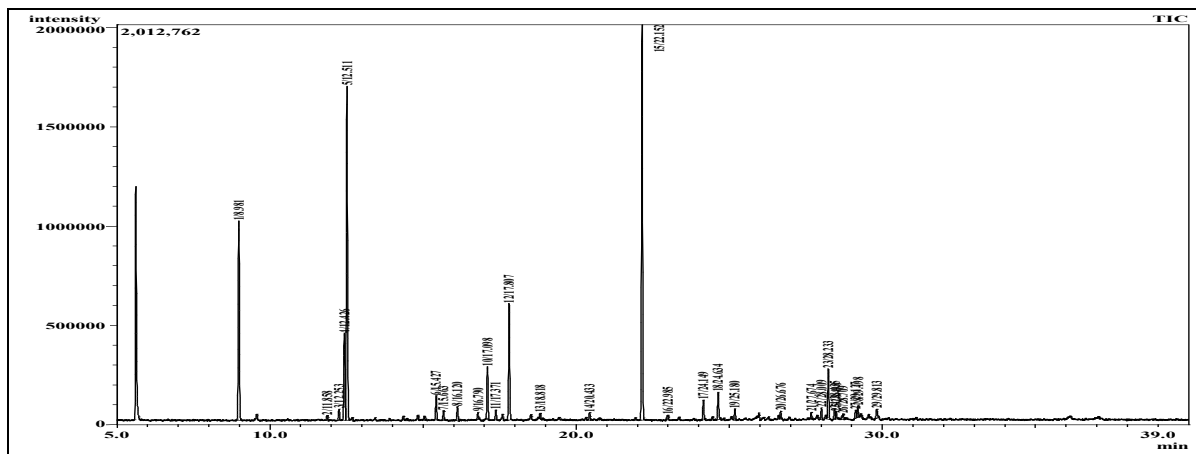
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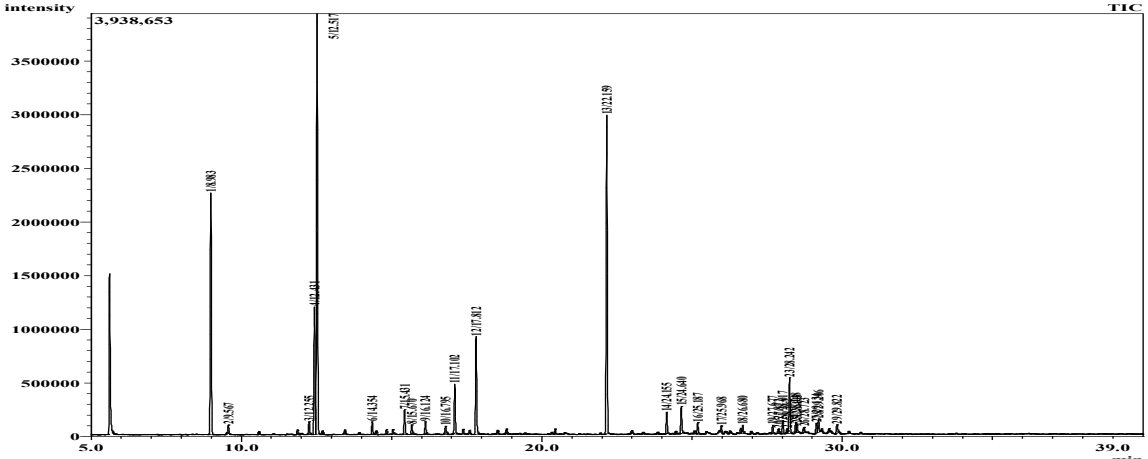
APPENDIX

Table S1. Chemical Composition of the Fresh Leaf Essential Oils Obtained from *Eucalyptus grandis* × *Eucalyptus urophylla* with Tree Ages of 17 to 31 months (HfA) Using Gas Chromatography-Mass Spectrometry



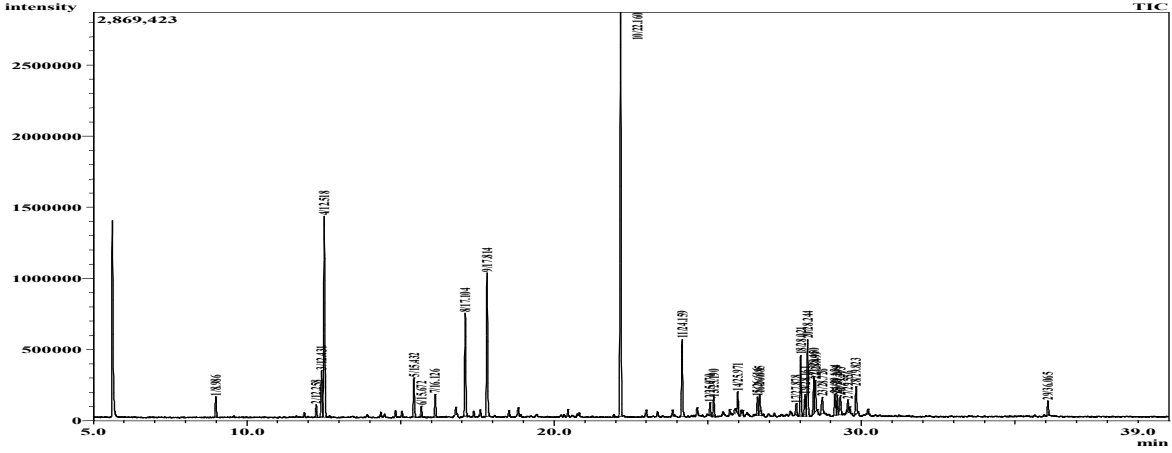
Peak No.	Retention Time (min)	Retention Indices	Peak Area	Peak Area (%)	Component Name	Similarity (%)
1	8.981	932	2586272	13.9	α -Pinene	96
2	11.858	1011	64327	0.35	Isoamyl isobutyrate	94
3	12.253	1023	139608	0.75	<i>o</i> -Cymene	96
4	12.426	1028	1098910	5.92	Limonene	95
5	12.511	1030	4247391	22.9	1,8-Cineol	95
6	15.427	1117	292380	1.57	Fenchol	97
7	15.663	1125	119070	0.64	α -Campholenal	94
8	16.120	1139	179643	0.97	Sabinol	91
9	16.790	1161	77691	0.42	Pinocarvone	93
10	17.098	1170	665338	3.58	Borneol	96
11	17.371	1179	117186	0.63	Terpinen-4-ol	94
12	17.807	1193	1425590	7.67	α -Terpineol	95
13	18.818	1227	71635	0.39	Isocarveol	86
14	20.433	1283	92861	0.50	Bornyl acetate	94
15	22.152	1345	4852792	26.1	α -Terpinyl acetate	95
16	22.985	1376	66917	0.36	α -Cubebene	90
17	24.149	1421	244888	1.32	β -Caryophyllene	96
18	24.634	1440	361502	1.95	Aromadendrene	96
19	25.180	1461	136754	0.74	Humulen-(v1)	92
20	26.676	1522	98795	0.53	<i>trans</i> -Calamenene	94
21	27.674	1563	76195	0.41	Epiglobulol	95
22	28.009	1577	145730	0.78	Spathulenol	95
23	28.233	1587	649767	3.50	Globulol	95
24	28.435	1595	147148	0.79	Viridiflorol	90
25	28.490	1598	86925	0.47	Unknown	-
26	28.709	1607	107945	0.58	Rosifoliol	94
27	29.127	1625	132203	0.71	Unknown	-
28	29.198	1629	151409	0.82	1,10-Diepicubenol	95
29	29.813	1656	137717	0.74	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	90

Table S2. Chemical Composition of the Dried Leaf Essential Oils Obtained from *Eucalyptus grandis* × *Eucalyptus urophylla* with Tree Ages of 17 to 31 months (HdA) Using Gas Chromatography-Mass Spectrometry



Peak No.	Retention Time (min)	Retention Indices	Peak Area	Peak Area (%)	Component Name	Similarity (%)
1	8.983	932	5771020	15.7	α -Pinene	96
2	9.567	948	187421	0.51	Fenchene	88
3	12.255	1023	289337	0.79	<i>o</i> -Cymene	96
4	12.431	1028	3031437	8.24	Limonene	96
5	12.517	1031	9816256	26.7	1,8-Cineol	95
6	14.354	1084	291471	0.79	Isoterpinolene	95
7	15.431	1117	574048	1.56	Fenchol	96
8	15.670	1125	220759	0.60	α -Campholenal	94
9	16.124	1139	293532	0.80	Sabinol	91
10	16.795	1161	218193	0.59	Pinocarpone	94
11	17.102	1170	1130747	3.07	Borneol	96
12	17.812	1193	2320906	6.31	α -Terpineol	95
13	22.159	1346	7259896	19.7	α -Terpinyl acetate	95
14	24.155	1421	492172	1.34	β -Caryophyllene	96
15	24.640	1440	657092	1.79	Aromadendrene	95
16	25.187	1462	264836	0.72	Humulen-(v1)	92
17	25.968	1493	140363	0.38	Viridiflorene	94
18	26.680	1522	203486	0.55	<i>trans</i> -Calamenene	95
19	27.677	1564	196125	0.53	Epiglobulol	95
20	27.872	1572	136090	0.37	Ledol	86
21	28.017	1578	322436	0.88	Spathulenol	95
22	28.162	1584	126094	0.34	Unknown	-
23	28.242	1587	1357506	3.69	Globulol	94
24	28.448	1596	281978	0.77	Viridiflorol	90
25	28.493	1598	172051	0.47	Cubeban-11-ol	90
26	28.723	1608	176877	0.48	Rosifoliol	94
27	29.134	1626	302414	0.82	β -Eudesmol	87
28	29.206	1629	318649	0.87	1,10-Diepicubenol	95
29	29.822	1656	248211	0.67	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	90

Table S3. Chemical Composition of the Fresh Leaf Essential Oils Obtained from *Eucalyptus grandis* × *Eucalyptus urophylla* with Tree Ages of 40 to 50 months (HfB) Using Gas Chromatography-Mass Spectrometry



Peak No.	Retention Time (min)	Retention Indices	Peak Area	Peak Area (%)	Component name	Similarity (%)
1	8.986	932	378096	1.36	α -Pinene	96
2	12.258	1023	205597	0.74	<i>o</i> -Cymene	96
3	12.431	1028	853782	3.06	Limonene	95
4	12.518	1031	3635007	13.1	1,8-Cineol	95
5	15.432	1117	707843	2.54	Fenchol	97
6	15.672	1125	175797	0.63	α -Campholenal	94
7	16.126	1139	399727	1.43	Sabinol	91
8	17.104	1171	1845950	6.62	Borneol	96
9	17.814	1193	2570425	9.22	α -Terpineol	95
10	22.160	1346	6863245	24.6	α -Terpinyl acetate	95
11	24.159	1421	1388972	4.98	β -Caryophyllene	96
12	25.070	1457	225504	0.81	Humulene	95
13	25.190	1462	343082	1.23	Humulen-(v1)	92
14	25.971	1493	356803	1.28	Viridiflorene	93
15	26.616	1519	312672	1.12	δ -Cadinene	91
16	26.685	1522	423319	1.52	<i>trans</i> -Calamenene	95
17	27.878	1572	232415	0.83	Ledol	88
18	28.021	1578	1118746	4.02	Spathulenol	96
19	28.161	1584	455682	1.64	Caryophyllene oxide	89
20	28.244	1587	1412611	5.07	Globulol	94
21	28.450	1596	693380	2.49	Viridiflorol	92
22	28.499	1598	558933	2.01	Unknown	-
23	28.726	1608	414128	1.49	Rosifoliol	95
24	29.134	1626	448438	1.61	β -Eudesmol	86
25	29.209	1629	368323	1.32	1,10-Diepicubenol	95
26	29.313	1634	439230	1.58	Unknown	-
27	29.556	1644	201341	0.72	τ -Muurolol	91
28	29.823	1656	551946	1.98	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	88
29	36.065	1946	282977	1.02	Butyl isobutyl phthalate	96

Table S4. Chemical Composition of the Dried Leaf Essential Oils Obtained from *Eucalyptus grandis* × *Eucalyptus urophylla* with Tree Ages of 40 to 50 months (HdB) Using Gas Chromatography-Mass Spectrometry

Peak No.	Retention Time (min)	Retention Indices	Peak Area	Peak Area (%)	Component Name	Similarity (%)
1	8.989	932	1725627	7.84	α -pinene	96
2	9.573	948	106137	0.48	Fenchene	88
3	12.262	1023	307888	1.40	<i>o</i> -Cymene	96
4	12.435	1028	1216249	5.53	Limonene	95
5	12.522	1031	4813150	21.9	1,8-Cineol	95
6	14.360	1084	105888	0.48	Isoterpinolene	94
7	15.436	1117	666965	3.03	Fenchol	97
8	15.680	1125	155613	0.71	α -Campholenal	94
9	16.130	1139	350374	1.59	Sabinol	91
10	16.798	1161	125954	0.57	Pinocarvone	93
11	17.110	1171	1640863	7.46	Borneol	96
12	17.818	1193	2054584	9.34	α -Terpineol	95
13	18.828	1228	120316	0.55	Unknown	-
14	22.161	1346	4018717	18.3	α -Terpinyl acetate	95
15	24.161	1421	660669	3.00	β -Caryophyllene	96
16	25.195	1462	196541	0.89	Humulen-(v1)	92
17	25.485	1473	643835	2.93	Unknown	-
18	25.975	1493	193968	0.88	Viridiflorene	94
19	26.620	1519	149462	0.68	δ -Cadinene	91
20	26.688	1522	247827	1.13	<i>trans</i> -Calamenene	96
21	28.022	1578	564086	2.56	Spathulenol	96
22	28.164	1584	204986	0.93	Caryophyllene oxide	87
23	28.247	1587	576850	2.62	Globulol	94
24	28.451	1596	267775	1.22	Viridiflorol	89
25	28.503	1598	155260	0.71	Cubeban-11-ol	88
26	29.138	1626	161774	0.74	β -Eudesmol	86
27	29.213	1629	148211	0.67	1,10-Diepicubenol	93
28	29.314	1634	196810	0.89	Unknown	-
29	29.829	1656	224285	1.02	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	89