

## Vector Control, Pest Management, Resistance, Repellents

# Bioinsecticidal potential of *Baeckea frutescens* (Myrtales: Myrtaceae) essential oil: chemical variation, yeast encapsulation, and larvicidal activity against *Aedes aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus* (Diptera: Culicidae)

Hung Huy Nguyen<sup>1,2,✉</sup>, Mau Trinh-Dang<sup>3</sup>, Huy Gia Ngo<sup>1</sup>, Linh Thao Phan<sup>3</sup>, Prabodh Satyal<sup>4</sup>,  
Thuong Thanh Vo<sup>1</sup>, Hoa Van Vo<sup>1</sup>, Thong Quoc Nguyen<sup>5,6</sup>, Anh Quynh Tran-Nguyen<sup>3</sup>,  
Van Thi-Thuy Do<sup>7</sup>, William N. Setzer<sup>4,8</sup>, Phong Hai Nguyen<sup>6</sup>, and Minh Van Vo<sup>\*,3</sup>

<sup>1</sup>Department of Pharmacy, Duy Tan University, Da Nang, Vietnam

<sup>2</sup>Center for Advanced Chemistry, Institute of Research and Development, Duy Tan University, Da Nang, Vietnam

<sup>3</sup>Faculty of Biology, Agriculture & Environmental Science, The University of Da Nang—University of Science and Education, Da Nang City, Vietnam

<sup>4</sup>Aromatic Plant Research Center, Lehi, UT, USA

<sup>5</sup>Ba Ria-Vung Tau Provincial Medical College, Ho Chi Minh City, Vietnam

<sup>6</sup>University of Sciences, Hue University, Hue City, Vietnam

<sup>7</sup>Faculty of Physics and Chemistry, The University of Da Nang—University of Science and Education, Da Nang, Vietnam

<sup>8</sup>Department of Chemistry, University of Alabama in Huntsville, Huntsville, AL, USA

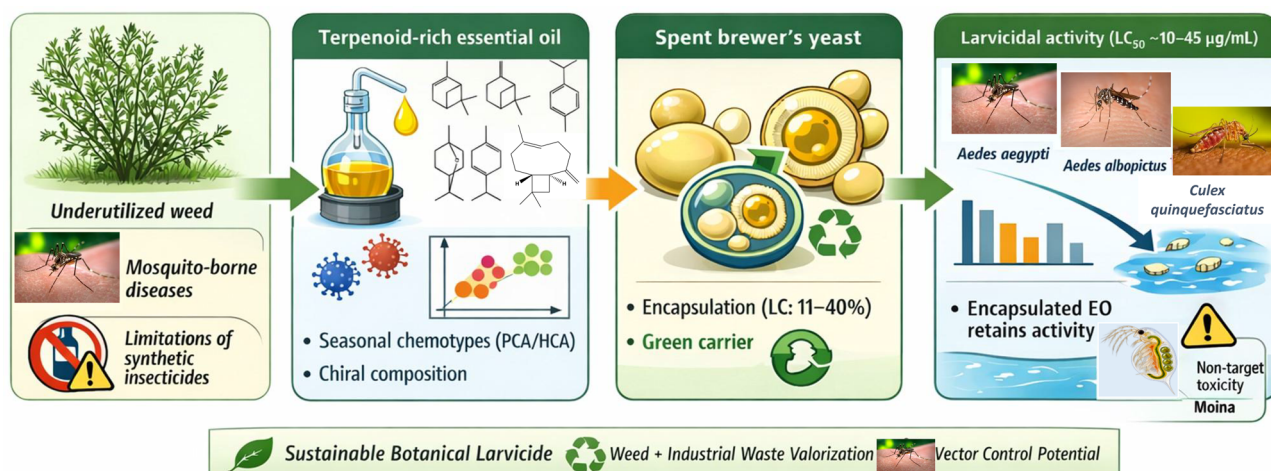
\*Corresponding author. Faculty of Biology, Agriculture & Environmental Science, The University of Da Nang—University of Science and Education, 459 Ton Duc Thang Street, Da Nang 550000, Vietnam (Email: [vominhndn@gmail.com](mailto:vominhndn@gmail.com)).

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Plant essential oils are promising alternatives to synthetic larvicides, but their development is often limited by chemical variability, formulation challenges, and concerns regarding non-target toxicity. *Baeckea frutescens*, a widespread weed species, remains insufficiently characterized in terms of chemotypic diversity, stereochemical composition, and larvicidal selectivity. The essential oils were dominated by mono- and sesquiterpenoids, with *p*-cymene, 1,8-cineole,  $\alpha$ - and  $\beta$ -pinene,  $\alpha$ -humulene, caryophyllene oxide, and humulene epoxide II as major constituents. PCA and HCA revealed distinct chemotypic groups that may reflect temporal variation in essential oil composition. Chiral GC analysis showed pronounced enantiomeric excess for several terpenoids. In WHO-based larvicidal assays, free essential oils exhibited notable activity against *Aedes aegypti* (Linnaeus), *Aedes albopictus* (Skuse), and *Culex quinquefasciatus* Say (Diptera: Culicidae). The corresponding 24-h LC<sub>50</sub> values ranged from 18.97 to 39.33  $\mu$ g/mL, 10.60 to 13.11  $\mu$ g/mL, and 19.79 to 45.62  $\mu$ g/mL, respectively. Yeast encapsulation achieved a loading capacity of approximately 11.1% to 40.71% and retained larvicidal activity in acute assays. This study provides integrated chemotypic and enantiomeric insights into *B. frutescens* essential oil and demonstrates its acute larvicidal activity with preliminary indication of selectivity against multiple mosquito species using WHO-based protocols. The findings support the sustainable utilization of an underutilized weed species and provide a chemically informed basis for the development of sustainable botanical larvicides.

**Keywords:** botanical larvicide, essential oil encapsulation, non-target toxicity, spent brewer's yeast

## Graphical Abstract



## Introduction

The increasing global burden of mosquito-borne diseases has intensified the demand for effective, sustainable, and environmentally responsible vector control strategies (Centers for Disease Control and Prevention 2025, World Health Organization 2025). Conventional synthetic insecticides, although effective, have raised serious concerns related to environmental persistence, non-target toxicity (Punniyakotti et al. 2024), resistance development (World Health Organization 2022), and adverse impacts on ecosystem health. These limitations have stimulated growing interest in bio-based insecticides derived from renewable natural resources, particularly plant essential oils, which are generally biodegradable and possess multiple modes of action against insect vectors (Jyotsna et al. 2024).

However, despite their promising bioactivity, the large-scale application of essential oils in vector control remains constrained by several intrinsic drawbacks, including high volatility, rapid degradation under environmental conditions, and limited persistence in aquatic habitats where mosquito larvae develop (Yammine et al. 2024). Addressing these challenges requires not only the identification of potent botanical sources but also the development of green formulation strategies capable of enhancing stability and prolonging biological efficacy without introducing additional environmental risks.

In this context, the sustainable utilization of underutilized or low-value biomass has emerged as a key pillar of the circular bioeconomy and green chemistry. Transforming abundant, non-food biological resources into functional materials or bioactive agents offers a dual benefit: reducing environmental burdens associated with waste or invasive biomass while simultaneously generating value-added products. Weed species and industrial by-products are particularly attractive in this regard, as their utilization avoids competition with food crops and minimizes land-use pressure.

*Baeckea frutescens* L. (Myrtaceae) is a widely distributed wild shrub occurring across diverse ecological zones, ranging from mountainous regions to coastal areas. Its natural distribution extends from southern China throughout Southeast Asia to Australia (Bean 1997). In many regions, the species is regarded as a weed due to its prolific growth and limited

conventional agricultural value. Despite this status, *B. frutescens* exhibits an unusually high essential oil yield, frequently exceeding 2.23% (w/w) (An et al. 2020), which is considerably higher than that of many commercially exploited aromatic plants. Such a combination of wide availability, minimal cultivation requirements, and high extractable bioactive content positions *B. frutescens* as a promising renewable feedstock for the development of bio-based insecticidal products.

From a sustainability perspective, the exploitation of *B. frutescens* offers several advantages. The plant can be harvested from natural or semi-natural habitats without intensive agro-nomic inputs, thereby reducing the environmental footprint associated with cultivation, fertilization, and irrigation. Moreover, the use of a weed species minimizes ethical and economic concerns related to food security and land competition. These attributes align well with the principles of green chemistry, which emphasize the use of renewable feedstocks, waste minimization, and the design of environmentally benign products.

In parallel with the development of value-added applications for plant biomass, the sustainable management of industrial by-products represents another critical challenge in environmental chemical engineering. The brewing industry generates large quantities of spent brewer's yeast as a fermentation by-product, which is commonly underutilized or disposed of at low economic value (Jaeger et al. 2020). Rich in polysaccharides, proteins, and functional cell wall components, spent yeast biomass possesses physicochemical properties that make it suitable as a natural carrier material for the encapsulation of bioactive compounds (Wei et al. 2024). Importantly, its biological origin, biodegradability, and non-toxic nature make it an attractive alternative to synthetic polymers commonly used in microencapsulation (Lieu et al. 2024).

The integration of waste-derived yeast biomass as a functional carrier for essential oil delivery represents a synergistic strategy that combines plant-based bioactivity with the value-added utilization of industrial by-products. Yeast cell walls provide a porous and protective matrix capable of entrapping volatile compounds, thereby reducing rapid evaporation and degradation while enabling a more sustained release of active constituents. Such green formulation approaches can significantly enhance the

practical applicability of essential oils in environmental and public health interventions, including mosquito vector control (Paramera et al. 2023, Thomas et al. 2026).

Mosquito larvae, particularly those of *Ae. aegypti*, develop in aquatic environments where rapid dilution and loss of active agents often limit the effectiveness of botanical larvicides. A delivery system that prolongs larvicidal activity while minimizing environmental loading is therefore highly desirable. By coupling a high-yield essential oil source such as *B. frutescens* with a waste-derived yeast carrier, it is possible to design a bioinsecticidal system that addresses both efficacy and sustainability considerations.

Accordingly, the present study aims to develop and evaluate a sustainable larvicidal formulation based on the utilization of two underexploited resources: a widely distributed weed species (*B. frutescens*) and spent brewer's yeast generated from industrial fermentation processes. The chemical composition of *B. frutescens* essential oil was characterized using gas chromatography–mass spectrometry and chiral analysis to elucidate its bioactive profile. The essential oil was subsequently encapsulated within yeast biomass, and the resulting formulation was assessed for larvicidal efficacy against *Aedes aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus* (Diptera: Culicidae). By integrating weed biomass utilization, the value-added use of industrial by-products, and green formulation principles, this work seeks to demonstrate a practical pathway toward environmentally responsible mosquito vector control and sustainable bioinsecticide development.

## Materials and Methods

### Plant Material

The aerial parts (stems and leaves) of *B. frutescens* L. (Myrtaceae) were collected from Hoa Khanh Commune, Da Nang City, Vietnam (16°01'13.04" N, 108°05'59.24" E) during different seasons between November 2023 and July 2025. Samples were designated as BF1–BF12, where “BF” denotes *B. frutescens* and the accompanying number identifies each sample. Sample BF1 was prepared by storing a portion of sample BF2 under dry conditions at 25°C for 3 months to evaluate changes in essential oil composition associated with post-harvest drying and storage. The field-collected samples (BF2–BF12) were collected in November 2023 (BF2), March 2024 (BF3), April 2024 (BF4), May 2024 (BF5), June 2024 (BF6), July 2024 (BF7), August 2024 (BF8), November 2024 (BF9), December 2024 (BF10), June 2025 (BF11), and July 2025 (BF12).

The plant material was taxonomically identified by Dr. Pham Van The (Van Lang University, Ho Chi Minh City, Vietnam). Voucher specimens corresponding to each collection period were deposited in the herbarium collection of the Faculty of Biology, Agriculture and Environmental Science, The University of Da Nang—University of Science and Education, Da Nang, Vietnam.

Fresh plant materials were transported to the laboratory immediately after collection and processed for essential oil extraction within 24 h.

### Extraction of Essential Oils

Essential oils were obtained by hydrodistillation using a Clevenger-type apparatus (Witeg Labortechnik, Wertheim,

Germany). For each extraction, 100 g of freshly chopped aerial plant material was immersed in 500 mL of distilled water and hydrodistilled for 5 h. Essential oil yield was determined from three independent extractions and expressed as percentage (w/w) based on fresh plant weight.

The obtained essential oils were separated from the aqueous phase, dried over anhydrous sodium sulfate ( $\text{Na}_2\text{SO}_4$ ), transferred to amber glass vials, and stored at 4°C until further analysis and bioassays (An et al. 2020).

The mean essential oil yields (%) were  $1.12 \pm 0.07$  (BF1),  $1.10 \pm 0.12$  (BF2),  $0.936 \pm 0.09$  (BF3),  $0.897 \pm 0.08$  (BF4),  $0.959 \pm 0.05$  (BF5),  $0.845 \pm 0.06$  (BF6),  $0.793 \pm 0.04$  (BF7),  $0.785 \pm 0.06$  (BF8),  $0.657 \pm 0.04$  (BF9),  $0.687 \pm 0.07$  (BF10),  $1.18 \pm 0.05$  (BF11), and  $1.30 \pm 0.09$  (BF12).

### Gas Chromatographic–Mass Spectral Analysis

Gas chromatography–mass spectrometry (GC–MS) analysis was performed using a Shimadzu GCMS-QP2010 Ultra system (Shimadzu Corporation, Kyoto, Japan) equipped with a ZB-5ms fused silica capillary column (60 m  $\times$  0.25 mm i.d., 0.25  $\mu\text{m}$  film thickness). Helium was used as the carrier gas at a flow rate of 2.0 mL/min. The injector temperature, detector temperature, and interface temperature were maintained at 260°C. The oven temperature was programmed from 50 to 260°C at 2°C/min. Samples were prepared as 1% (w/v) solutions in dichloromethane, and 1.0  $\mu\text{L}$  was injected using a split ratio of 1:24.4. Mass spectra were acquired in electron impact (EI) mode at 70 eV over a mass range of  $m/z$  40 to 400 at a scan rate of 3.0 scans/s. Retention indices (RI) were calculated relative to a homologous series of n-alkanes. Compound identification was based on comparison of RI values and mass spectra with published databases and reference libraries (Pham et al. 2023).

Enantiomeric analysis was carried out using a Shimadzu GCMS-QP2010S system equipped with a Restek B-Dex 325 chiral capillary column. The oven temperature was programmed from 50 to 120°C at 1.5°C/min and then from 120 to 200°C at 2.0°C/min. Samples were prepared as 5% (w/v) solutions in dichloromethane, and 0.1  $\mu\text{L}$  was injected using a split ratio of 1:45. Enantiomeric assignments were made by comparison with authentic reference standards, and enantiomeric percentages were calculated from peak areas without correction factors (Pham et al. 2023).

### Preparation of Pre-Treated Brewing Spent Yeast Cells

Pre-treatment of yeast cells was performed to remove intracellular components and generate hollow structures suitable for essential oil encapsulation. The process consisted of two consecutive steps: autolysis followed by plasmolysis, adapted and modified from previously reported procedures (Herlinger et al. 2023). Unlike many previous studies that employed either autolysis or plasmolysis as a single pre-treatment step, the present study combined both approaches sequentially. This strategy was adopted because spent brewer's yeast contains substantial residual intracellular materials derived from industrial fermentation processes. Autolysis was first applied to promote enzymatic degradation and removal of intracellular constituents, while subsequent plasmolysis further depleted residual cellular contents and increased the internal cavity available for essential oil loading. The combined treatment was therefore expected

to improve encapsulation capacity compared with the use of either method alone.

### Autolysis

Yeast biomass was suspended in distilled water containing 0.5% (w/v) sodium chloride and 0.5% (v/v) ethyl acetate. The pH of the suspension was adjusted to 6.5 using either 1 M NaOH or 1 M HCl. The suspension was incubated at 50 °C with continuous agitation at 200 rpm for 24 h to promote enzymatic self-digestion of intracellular components.

Following incubation, the suspension was centrifuged at 4000 rpm for 10 min, and the supernatant containing the autolysate was discarded. The recovered yeast biomass was subsequently subjected to plasmolysis.

### Plasmolysis

The autolyzed yeast biomass was resuspended in distilled water containing 1.5% (v/v) ethyl acetate. The pH was adjusted to 5.5 using either 1 M NaOH or 1 M HCl. The suspension was incubated at 55 °C with continuous agitation at 200 rpm for 48 h.

After plasmolysis, the suspension was heated at 80 °C for 15 min to terminate biological activity. The treated yeast cells were collected by centrifugation at 4,000 rpm for 10 min, washed three times with distilled water, and stored at 4 °C until use in encapsulation experiments.

## Encapsulation of *Baeckea frutescens* Essential Oil into Spent Yeast Cells

### Water-Assisted Encapsulation

Essential oil (EO) encapsulation was performed using intracellular content-depleted yeast cells as biological carriers. EO, treated yeast cells (Y), and distilled water (W) were mixed at three different ratios: 60:15:25, 30:15:55, and 15:15:70 (EO:Y:W, w/w/w). The mixtures were incubated at 55 °C under continuous agitation at 200 rpm for 72 h to promote EO diffusion into the hollow yeast cell structure. After incubation, the encapsulated products were collected for subsequent determination of loading capacity (Mirza et al. 2020, Soto et al. 2022, 2023). The mixture was washed three times by centrifugation at 4000 rpm for 10 min, then frozen and subsequently lyophilized at −38 °C for 24 h.

### Tween 80-Assisted Encapsulation

In this experiment, 15 g essential oil was emulsified with 40 g Tween 80 4%, 3%, 2%, and 1% solution for 10 min using a magnetic stirrer at 600 rpm at room temperature. Subsequently, 10 g pre-treated yeast cells were slowly added to the emulsion to avoid dust formation. The mixture was incubated at 40 °C under magnetic stirring at 180 rpm for 24 h (Czerniak et al. 2015). The mixture was washed three times by centrifugation at 4,000 rpm for 10 min, then frozen and subsequently lyophilized at −38 °C for 24 h.

### Determination of Loading Capacity and Surface Oil

Total oil loading and surface oil were determined using UV–Vis spectrophotometry. Calibration curves were prepared using BF12 essential oil, the same oil sample used for the encapsulation experiments. BF12 essential oil was dissolved in absolute

ethanol and serially diluted to concentrations of 0.01, 0.02, 0.025, 0.05, 0.10, 0.20, 0.30, 0.40, and 0.60 mg/mL. Absorbance was measured at 265 nm. The calibration curve showed good linearity over the tested concentration range, with the regression equation  $y = 1.201x + 0.1458$  and  $R^2 = 0.9976$ .

For total oil loading, 0.025 g of essential oil-loaded yeast microcapsules was vortexed with 0.5 mL of distilled water for 10 min. Subsequently, 9.5 mL of absolute ethanol was added, and the mixture was vortexed for another 10 min to extract the encapsulated essential oil. The suspension was centrifuged at 4,000 rpm for 10 min, and the clear supernatant was analyzed by UV–Vis spectrophotometry at 265 nm.

For surface oil determination, 0.025 g of essential oil-loaded yeast microcapsules was vortexed with 10 mL of n-hexane for 30 s to selectively remove non-encapsulated oil adsorbed on the particle surface. The suspension was centrifuged at 4000 rpm for 10 min, and the clear supernatant was analyzed at 265 nm.

$$\text{Encapsulated oil loading} = \frac{\text{Total oil (g)} - \text{surface oil (g)}}{\text{Total dry microcapsules (g)}} \times 100$$

Total oil loading and surface oil content were calculated from the BF12 essential oil calibration curve and expressed as percentage of microcapsule dry weight.

### Scanning Electron Microscopy

The morphology of yeast cells before and after essential oil encapsulation was examined using scanning electron microscopy (SEM). Samples of pre-treated yeast cells and essential oil-loaded yeast microcapsules were mounted on aluminum stubs using double-sided conductive carbon tape and sputter-coated with platinum for 20 s.

SEM observations were performed using a JSM-6010LV scanning electron microscope (JEOL, Tokyo, Japan) operated at an accelerating voltage of 10 kV. Images were acquired at different magnifications to evaluate particle morphology, surface characteristics, and structural integrity of the yeast microcapsules. SEM analyses were conducted at the Oil and Gas Laboratory, The University of Da Nang—University of Science and Technology, Da Nang, Vietnam.

### Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) was used to characterize chemical interactions between yeast cells and encapsulated essential oil. Spectra were recorded using an FT/IR-6800 spectrometer (JASCO Corporation, Tokyo, Japan) equipped with an attenuated total reflectance (ATR) accessory.

Samples of pre-treated yeast cells, BF12 essential oil, and essential oil-loaded yeast microcapsules were analyzed over the spectral range of 500 to 4,000  $\text{cm}^{-1}$ . Spectra were collected using 16 scans at a resolution of 4  $\text{cm}^{-1}$ . FTIR analyses were performed at the Faculty of Chemistry, The University of Da Nang—University of Science and Education, Da Nang, Vietnam.

### Fluorescence Microscopy

The localization of essential oil within yeast microcapsules was evaluated using Nile Red fluorescence staining. Nile Red stock

solution (1 mg/mL) was prepared in dimethyl sulfoxide (DMSO) and stored at 4°C in the dark until use.

Yeast microcapsules were suspended in distilled water and stained with Nile Red to obtain a final dye concentration of 5 µg/mL. The suspensions were incubated at 37°C for 30 min in the dark and subsequently washed twice with distilled water to remove excess dye.

Stained samples were mounted on glass slides and examined using a fluorescence microscope equipped with a TRITC/Texas Red filter set. Fluorescence images were acquired using excitation wavelengths of 540 to 560 nm and emission wavelengths of 580 to 620 nm. Empty yeast cells were used as controls for comparison of fluorescence intensity.

### Larvicidal Bioassays

Colonies of *Ae. aegypti* (Linnaeus) and *Ae. albopictus* (Skuse) (Diptera: Culicidae) were maintained continuously at Duy Tan University, Da Nang, Vietnam. Egg rafts of *Cx. quinquefasciatus* Say (Diptera: Culicidae) were collected from domestic wastewater drainage channels in Da Nang City, Vietnam, and subsequently reared under laboratory conditions for bioassays. Mosquito colonies were maintained at 27 ± 2°C under a 12:12 h light photoperiod. Larvae were reared in plastic trays containing dechlorinated water and fed commercial cat food (Me-O Tuna, Perfect Companion Group Co., Ltd., Thailand). Third- and early fourth-instar larvae were used for all larvicidal bioassays.

Larvicidal activity was evaluated following World Health Organization guidelines (WHO 2005) with minor modifications. Test solutions were prepared by dissolving essential oils in ethanol and subsequently diluting with distilled water to obtain final concentrations of 100, 50, 25, 12.5, 6.25, and 3.125 µg/mL. In addition to the essential oils, selected major constituents including 1,8-cineole, α-humulene, β-caryophyllene, caryophyllene oxide, (–)-β-pinene, and (+)-α-pinene were individually evaluated against third- and early fourth-instar *Ae. aegypti* larvae. All reference compounds were purchased from Merck (Darmstadt, Germany). Test solutions were prepared and evaluated using the same concentration range and experimental protocol described above for the essential oils.

Bioassays were conducted in 250-mL glass beakers containing 150 mL of test solution. Groups of 25 third- or early fourth-instar larvae were introduced into each beaker. Four independent replicates were performed for each concentration. For free essential oil assays, ethanol served as the solvent control. The final ethanol concentration in all treatments and solvent controls was maintained at approximately 0.33% (v/v). For yeast-encapsulated essential oil assays, distilled water containing the corresponding amount of treated yeast cells without essential oil served as the negative control. Larval mortality was recorded after 24 and 48 h of exposure. Larvae were considered dead if they failed to respond to gentle probing. Mortality data were corrected using Abbott's formula whenever control mortality ranged between 5% and 20%, in accordance with WHO recommendations. Tests with control mortality exceeding 20% were discarded and repeated (World Health Organization 2005). Permethrin was included as a positive control reference insecticide because of its established larvicidal activity and was used as a laboratory reference to verify larval susceptibility and provide a standardized benchmark for comparison with the essential oils.

### *Moina macrocopa* Toxicity Assay

The non-target aquatic organism *Moina macrocopa* was obtained from the Faculty of Biology, Agriculture and Environmental Science, The University of Da Nang—University of Science and Education, Da Nang, Vietnam. Organisms were maintained in 500-mL glass beakers containing dechlorinated tap water at 25 ± 1°C and fed *Chlorella vulgaris* at a concentration of 4.5 × 10<sup>6</sup> cells/L. Culture water was renewed every 2 to 3 days. A 1% (w/v) stock solution was prepared by dissolving 100 mg of the test substance in 10 mL of dimethyl sulfoxide (DMSO). Working solutions were freshly prepared by diluting the stock solution with dechlorinated tap water to obtain the desired test concentrations, following World Health Organization (2005) guidelines. Acute toxicity of essential oils toward *M. macrocopa* was evaluated over exposure periods of 12, 24, and 48 h. Six concentrations (0, 6.25, 12.5, 25, 50, and 100 µg/mL) were tested, with five replicates per concentration. Each replicate consisted of 10 juvenile individuals aged 6 to 12 h. No food was provided during the exposure period. Mortality was recorded after 12, 24, and 48 h, and LC<sub>50</sub> and LC<sub>90</sub> values were calculated for each exposure period.

### Statistical Analysis

Larvicidal and toxicity data were analyzed using probit regression following Finney (2009) to estimate LC<sub>50</sub> and LC<sub>90</sub> values together with their 95% confidence intervals. Multivariate analyses were performed using the relative abundances (%) of major essential oil constituents (>1% in at least one sample). Prior to analysis, the data were standardized using Z-score transformation. Principal component analysis (PCA) was performed using the correlation matrix, whereas hierarchical cluster analysis (HCA) was conducted using Ward's linkage method and Euclidean distance. PCA and HCA were performed using OriginPro 2024 (OriginLab Corporation, Northampton, MA, USA).

Comparisons among mosquito species were based on LC<sub>50</sub> and LC<sub>90</sub> estimates and their associated confidence intervals. Formal interspecific statistical comparisons were not performed.

## Results and Discussion

### Essential Oil Yield and Chemical Composition

Hydrodistillation of the aerial parts of *B. frutescens* yielded between 0.657 ± 0.04% and 1.30 ± 0.09% (w/w, fresh weight basis) essential oil among the investigated samples. The highest yield was obtained from sample BF12 (1.30 ± 0.09%), followed by BF11 (1.18 ± 0.05%), whereas BF9 produced the lowest yield (0.657 ± 0.04%). Sample BF1, which was prepared by storing BF2 under dry conditions for three months, yielded 1.12 ± 0.07% essential oil, a value comparable to that of the corresponding fresh sample BF2 (1.10 ± 0.12%).

GC–MS analysis revealed that the essential oils were dominated by mono- and sesquiterpenoids, with total identified constituents accounting for 96.8% to 99.9% of the oil composition (Table 1, Table S1). Monoterpene hydrocarbons represented the major chemical class, ranging from 31.8% to 60.2% of the total oil, followed by oxygenated monoterpenoids (13.2% to 26.3%), sesquiterpene hydrocarbons (7.4% to 28.4%), and oxygenated sesquiterpenoids (4.6% to 33.7%). Diterpenoids and benzenoid aromatic compounds were detected only in minor amounts.

**Table 1.** Major components and their relative percentages (%) in BF1–BF12 samples

| RI (calc) | RI (db) | Compound                             | BF1  | BF2  | BF3  | BF4  | BF5  | BF6  | BF7  | BF8  | BF9  | BF10 | BF11 | BF12 |
|-----------|---------|--------------------------------------|------|------|------|------|------|------|------|------|------|------|------|------|
| 933       | 932     | $\alpha$ -Pinene                     | 9.1  | 7.9  | 6.5  | 6.6  | 3.9  | 4.6  | 7.2  | 7.4  | 6.6  | 6.8  | 6.9  | 6.7  |
| 978       | 978     | $\beta$ -Pinene                      | 14.0 | 14.3 | 11.1 | 12.1 | 6.5  | 7.8  | 11.6 | 12.6 | 10.0 | 10.7 | 10.4 | 10.8 |
| 1025      | 1025    | <i>p</i> -Cymene                     | 29.5 | 8.7  | 14.9 | 5.8  | 16.9 | 15.4 | 13.2 | 10.1 | 6.8  | 10.4 | 11.6 | 9.8  |
| 1032      | 1032    | 1,8-Cineole                          | 10.2 | 8.9  | 14.6 | 10.8 | 11.0 | 9.7  | 9.4  | 10.9 | 6.9  | 10.2 | 8.1  | 8.8  |
| 1058      | 1058    | $\gamma$ -Terpinene                  | 1.0  | 4.4  | 1.5  | 6.6  | 2.0  | 2.5  | 2.7  | 4.4  | 19.4 | 26.6 | 2.9  | 5.1  |
| 1418      | 1417    | ( <i>E</i> )- $\beta$ -Caryophyllene | 1.8  | 7.2  | 1.8  | 9.9  | 1.4  | 2.9  | 4.9  | 7.0  | 10.4 | 7.6  | 5.4  | 6.8  |
| 1455      | 1454    | $\alpha$ -Humulene                   | 3.2  | 10.6 | 3.2  | 13.6 | 2.7  | 5.1  | 7.6  | 10.2 | 12.8 | 9.6  | 8.1  | 9.9  |
| 1584      | 1587    | ( <i>E</i> )-Caryophyllene oxide     | 8.0  | 5.4  | 9.5  | 3.5  | 13.2 | 12.9 | 9.6  | 5.4  | 1.2  | 0.9  | 8.2  | 7.1  |
| 1612      | 1613    | Humulene epoxide II                  | 9.2  | 6.0  | 10.3 | 3.7  | 14.4 | 13.6 | 9.6  | 5.8  | 1.5  | 1.1  | 8.9  | 7.7  |
|           |         | Monoterpene hydrocarbons             | 56.4 | 38.0 | 36.5 | 34.1 | 31.8 | 33.0 | 37.7 | 37.5 | 49.1 | 60.2 | 35.8 | 36.9 |
|           |         | Oxygenated monoterpeneoids           | 14.3 | 19.4 | 26.3 | 21.9 | 23.4 | 20.0 | 20.2 | 22.4 | 13.2 | 14.6 | 15.9 | 18.8 |
|           |         | Sesquiterpene hydrocarbons           | 7.4  | 21.4 | 8.0  | 28.3 | 7.5  | 11.2 | 16.3 | 20.9 | 28.4 | 20.1 | 18.3 | 19.7 |
|           |         | Oxygenated sesquiterpenoids          | 20.9 | 20.4 | 26.7 | 14.7 | 33.7 | 32.9 | 24.8 | 18.2 | 8.2  | 4.6  | 28.1 | 22.8 |
|           |         | Diterpenoids                         | 0.0  | 0.0  | 0.0  | 0.1  | 0.0  | 0.0  | 0.0  | 0.0  | tr   | 0.0  | 0.0  | 0.0  |
|           |         | Benzenoid aromatics                  | 0.4  | 0.4  | 0.4  | 0.8  | 0.4  | 0.7  | 0.5  | 0.6  | 0.7  | 0.2  | 1.0  | 1.0  |
|           |         | Total identified                     | 99.4 | 99.6 | 97.8 | 99.9 | 96.8 | 97.7 | 99.4 | 99.6 | 99.7 | 99.7 | 99.1 | 99.2 |

The principal constituents varied among samples but generally included *p*-cymene (5.8% to 29.5%), 1,8-cineole (6.9% to 14.6%),  $\alpha$ -pinene (3.9% to 9.1%),  $\beta$ -pinene (6.5% to 14.3%),  $\alpha$ -humulene (2.7% to 13.6%), caryophyllene oxide (0.9% to 13.2%), and humulene epoxide II (1.1% to 14.4%). Considerable variation was observed in the relative abundance of these compounds among samples collected at different times, indicating substantial chemical heterogeneity within the studied population.

To further investigate patterns of chemical variation among samples, multivariate analyses including principal component analysis (PCA) and hierarchical cluster analysis (HCA) were performed using the major constituents of the essential oils.

### Chemotypic Variation Revealed by PCA and HCA

Principal component analysis (PCA) was performed using the relative abundances of selected major constituents (>1% in at least one sample) to investigate patterns of chemical variation among the 12 *B. frutescens* essential oil samples. The first two principal components accounted for 80.44% of the total variance, with PC1 and PC2 explaining 57.97% and 22.47%, respectively (Fig. 1).

The loading matrix indicated that PC1 (Tables S2 and S3) was positively associated with sesquiterpene hydrocarbons, particularly  $\alpha$ -humulene and (*E*)- $\beta$ -caryophyllene, together with  $\gamma$ -terpinene, whereas negative loadings were observed for oxygenated compounds, including 1,8-cineole, caryophyllene oxide, and humulene epoxide II. PC2 was primarily influenced by the monoterpene hydrocarbons  $\alpha$ -pinene and  $\beta$ -pinene. These variables contributed substantially to the separation of samples observed in the PCA biplot.

To further evaluate relationships among samples, hierarchical cluster analysis (HCA) was performed using Ward's linkage method and Euclidean distance based on Z-score-standardized compositional data (Fig. 2). The dendrogram revealed three major clusters corresponding to distinct chemical profiles.

Cluster I comprised samples BF1, BF3, BF5, and BF6 and was characterized by relatively higher proportions of oxygenated terpenoids, including 1,8-cineole, caryophyllene oxide, and humulene epoxide II. This cluster was clearly separated from the remaining samples in both PCA and HCA analyses.

Cluster II included samples BF2, BF4, BF7, BF8, BF11, and BF12. These samples exhibited more heterogeneous compositions, characterized by mixed contributions of monoterpene hydrocarbons and sesquiterpene hydrocarbons, particularly  $\alpha$ -humulene and (*E*)- $\beta$ -caryophyllene.

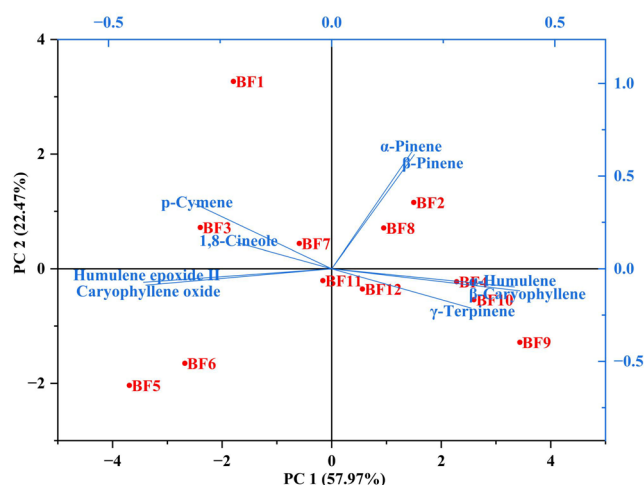
Cluster III consisted of samples BF9 and BF10 and was distinguished by elevated levels of  $\gamma$ -terpinene together with relatively high proportions of sesquiterpene hydrocarbons. These samples formed a distinct group in both multivariate analyses.

Overall, the clustering pattern obtained from HCA was consistent with the distribution of samples observed in the PCA biplot, supporting the presence of marked chemotypic variation among the investigated *B. frutescens* essential oil samples.

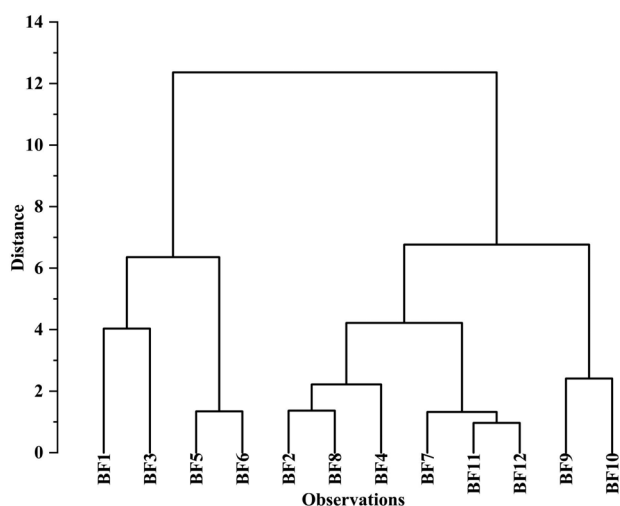
### Enantiomeric Composition

The enantiomeric distribution of selected chiral terpenoids in *B. frutescens* essential oils is presented in Table 2. Several compounds exhibited pronounced enantiomeric predominance with relatively low variation among samples.

$\alpha$ -Thujene was detected exclusively as the (–)-enantiomer in all analyzed samples. Similarly,  $\alpha$ -copaene and (*E*)- $\beta$ -caryophyllene were present almost entirely as a single enantiomeric form, showing complete stereochemical dominance across the investigated samples.  $\beta$ -Pinene also exhibited strong enantiomeric enrichment, with (–)- $\beta$ -pinene accounting for 94.4% to 95.2% of the total  $\beta$ -pinene fraction. In contrast,  $\alpha$ -pinene occurred as an enantiomeric mixture with a moderate predominance of (–)- $\alpha$ -pinene, representing 55.6% to 60.1% of the total  $\alpha$ -pinene content. Camphene displayed a stronger stereochemical bias, with (–)-camphene accounting for 83.5% to 89.0% of the total camphene fraction. Greater variability was observed for limonene and linalool. The proportion of



**Fig. 1.** Principal component analysis (PCA) of *Baeckea frutescens* essential oils. PCA biplot (PC1 vs PC2) constructed from the relative abundances (%) of selected major compounds for 12 essential oil samples (BF1–BF12). PC1 and PC2 explained 57.97% and 22.47% of the total variance, respectively. Blue vectors represent variable loadings, indicating the contribution of major terpenoid constituents to the distribution of samples. PCA was performed on the correlation matrix using Z-score-standardized variables.



**Fig. 2.** Hierarchical cluster analysis (HCA) of *Baeckea frutescens* essential oils. Dendrogram of 12 essential oil samples (BF1–BF12) generated using Ward's method and Euclidean distance based on Z-score-standardized chemical composition data. Three major clusters were identified at a linkage distance corresponding to the main cluster separation. Cluster I (BF1, BF3, BF5, and BF6) was clearly separated from the remaining samples and was associated with higher relative abundances of oxygenated terpenoids, including 1,8-cineole, caryophyllene oxide, and humulene epoxide II. Cluster II (BF2, BF4, BF7, BF8, BF11, and BF12) comprised samples with more heterogeneous compositions, characterized by mixed contributions of monoterpenes and sesquiterpene hydrocarbons such as  $\alpha$ -humulene and  $\beta$ -caryophyllene. Cluster III (BF9 and BF10) formed a distinct group associated with higher levels of monoterpene hydrocarbons, particularly  $\gamma$ -terpinene. The clustering pattern was consistent with the separation observed in the PCA biplot.

(–)-limonene ranged from 54.1% to 71.0%, whereas (+)-limonene accounted for 29.0% to 45.9%. Linalool showed a more balanced distribution, with (+)-linalool ranging from 41.9% to 61.8% and (–)-linalool from 38.2% to 58.1% among

samples. Terpinen-4-ol exhibited a nearly racemic distribution in all analyzed samples, with mean proportions of 49.3% (+)-terpinen-4-ol and 50.7% (–)-terpinen-4-ol. Similarly,  $\alpha$ -terpineol displayed only a slight predominance of the (–)-enantiomer, which accounted for  $57.8 \pm 2.8\%$  of the total  $\alpha$ -terpineol content.

Overall, the investigated essential oils were characterized by a combination of strongly enantiomerically enriched terpenoids, including  $\alpha$ -thujene,  $\beta$ -pinene,  $\alpha$ -copaene, and (E)- $\beta$ -caryophyllene, together with compounds exhibiting more balanced stereochemical distributions such as limonene, linalool, terpinen-4-ol, and  $\alpha$ -terpineol.

### Physicochemical Properties of Yeast Microcapsules

The encapsulation of *B. frutescens* essential oil within yeast cells was further confirmed by the combined analysis of SEM, FTIR, and loading capacity (LC). SEM images revealed that the yeast cells maintained their overall oval to spherical morphology after the encapsulation process, with no significant surface collapse or structural damage, indicating that the cell wall integrity was preserved. This structural stability suggests that the yeast cells can act as an effective encapsulating matrix (Fig. 3).

FTIR analysis provided chemical evidence for the presence of the essential oil within the yeast-based system (Fig. 4). The coexistence of characteristic absorption bands of yeast polysaccharides ( $\sim 1,043 \text{ cm}^{-1}$ ) and essential oil hydrocarbon chains ( $2,922$  and  $2,958 \text{ cm}^{-1}$ ) in the encapsulated sample indicates successful incorporation of the essential oil into the yeast matrix. Changes in the intensity and profile of the C–H stretching bands compared to the individual components further suggest possible interactions between the essential oil and the yeast cell constituents.

Importantly, the relatively high loading capacity (11.1% to 40.71%) provides quantitative confirmation of the encapsulation efficiency, demonstrating that a substantial amount of essential oil was retained within the yeast microcapsules, consistent with effective encapsulation rather than simple surface adsorption (Tables 3 and 4).

Nile Red fluorescence imaging qualitatively corroborated the quantitative loading capacity results. Samples with LC of 34.79% displayed intense and densely distributed fluorescence signals, while samples with LC of 11.1% exhibited weaker fluorescence, consistent with lower amounts of hydrophobic material present (Fig. 5). Fluorescence staining with Nile Red provided direct visual evidence for the intracellular localization of the essential oil within the yeast cells. The stronger intracellular fluorescence observed in samples with higher loading capacity, compared with the weaker signal in samples with lower loading capacity (Fig. 5), supports the retention of essential oil within the yeast matrix. When considered together with SEM observations and loading capacity data, these results collectively demonstrate successful encapsulation and effective retention of the essential oil within the yeast-based microcapsules.

### Larvicidal Activity

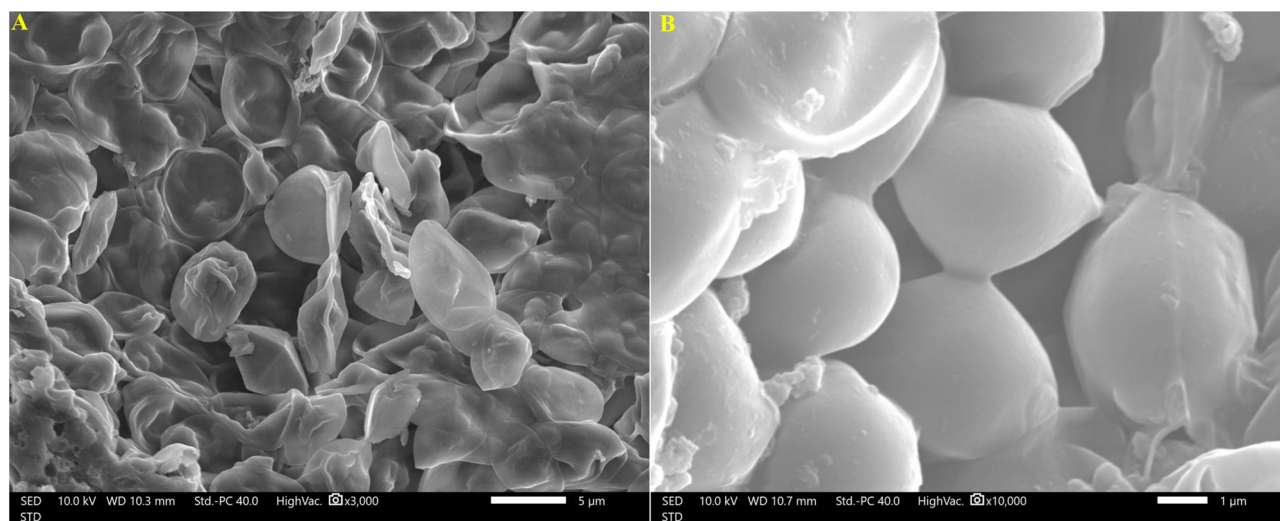
#### Larvicidal Activity Against *Aedes aegypti*

The larvicidal activity of the 12 *B. frutescens* essential oil samples against third- and early fourth-instar *Ae. aegypti* larvae is

**Table 2.** Enantiomeric distribution of chiral terpenoids in *Baeckea frutescens*

| Enantiomer                               | RI <sub>db</sub> | RI <sub>calc</sub> <sup>22</sup> | RI <sub>calc</sub> <sup>25</sup> | BF3   | BF4   | BF5   | BF6   | BF7   | BF8   | BF2   | BF11  | BF12  | Ave±SD    |
|------------------------------------------|------------------|----------------------------------|----------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-----------|
| (+)- $\alpha$ -Thujene                   | 950              | no                               | no                               | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0±0.0   |
| (-)- $\alpha$ -Thujene                   | 951              | 954                              | 956                              | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0±0.0 |
| (-)- $\alpha$ -Pinene                    | 976              | 975                              | 974                              | 55.6  | 56.6  | 56.6  | 57.1  | 56.7  | 60.1  | 58.8  | 57.9  | 59.7  | 57.7±1.4  |
| (+)- $\alpha$ -Pinene                    | 982              | 981                              | 980                              | 44.4  | 43.4  | 43.4  | 42.9  | 43.3  | 39.9  | 41.2  | 42.1  | 40.3  | 42.3±1.4  |
| (-)-Camphene                             | 998              | 1000                             | 1001                             | 83.5  | 86.6  | 84.6  | 84.3  | 86.9  | 86.2  | 89.0  | 87.4  | 84.5  | 85.9±1.6  |
| (+)-Camphene                             | 1005             | 1004                             | 1005                             | 16.5  | 13.4  | 15.4  | 15.7  | 13.1  | 13.8  | 11.0  | 12.6  | 15.5  | 14.1±1.6  |
| (+)- $\beta$ -Pinene                     | 1027             | 1024                             | 1023                             | 4.8   | 4.9   | 5.1   | 5.3   | 5.2   | 5.2   | 5.1   | 5.5   | 5.6   | 5.2±0.2   |
| (-)- $\beta$ -Pinene                     | 1031             | 1027                             | 1025                             | 95.2  | 95.1  | 94.9  | 94.7  | 94.8  | 94.8  | 94.9  | 94.5  | 94.4  | 94.8±0.2  |
| (-)-Limonene                             | 1073             | 1076                             | 1077                             | 69.2  | 66.6  | 63.9  | 63.4  | 67.6  | 69.3  | 71.0  | 54.1  | 54.5  | 64.4±6.4  |
| (+)-Limonene                             | 1081             | 1083                             | 1084                             | 30.8  | 33.4  | 36.1  | 36.6  | 32.4  | 30.7  | 29.0  | 45.9  | 45.5  | 35.6±6.4  |
| (-)-Linalool                             | 1228             | 1228                             | 1228                             | 38.2  | 43.0  | 46.7  | 52.9  | 49.5  | 50.5  | 47.5  | 51.6  | 58.1  | 48.7±4.5  |
| (+)-Linalool                             | 1231             | 1231                             | 1231                             | 61.8  | 57.0  | 53.3  | 47.1  | 50.5  | 49.5  | 52.5  | 48.4  | 41.9  | 51.3±4.5  |
| (+)-Terpinen-4-ol                        | 1297             | 1298                             | 1296                             | 50.3  | 50.6  | 50.2  | 49.3  | 48.9  | 48.2  | 48.9  | 49.6  | 48.0  | 49.3±0.9  |
| (-)-Terpinen-4-ol                        | 1300             | 1301                             | 1299                             | 49.7  | 49.4  | 49.8  | 50.7  | 51.1  | 51.8  | 51.1  | 50.4  | 52.0  | 50.7±0.9  |
| (-)-Borneol                              | 1335             | 1337                             | 1335                             | –     | –     | –     | –     | 100.0 | 100.0 | 100.0 | 91.8  | 85.6  | 95.5±6.6  |
| (+)-Borneol                              | 1340             | no                               | 1342                             | –     | –     | –     | –     | 0.0   | 0.0   | 0.0   | 8.2   | 14.4  | 4.5±6.6   |
| (-)- $\alpha$ -Terpineol                 | 1347             | 1346                             | 1344                             | 55.8  | 57.8  | 53.5  | 54.8  | 58.4  | 60.2  | 60.7  | 58.0  | 61.3  | 57.8±2.8  |
| (+)- $\alpha$ -Terpineol                 | 1356             | 1354                             | 1353                             | 44.2  | 42.2  | 46.5  | 45.2  | 41.6  | 39.8  | 39.3  | 42.0  | 38.7  | 42.2±2.8  |
| (-)- $\alpha$ -Copaene                   | 1381             | 1381                             | 1384                             | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0±0.0 |
| (+)- $\alpha$ -Copaene                   | na               | no                               | no                               | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0±0.0   |
| (-)-( <i>E</i> )- $\beta$ -Caryophyllene | 1461             | 1461                             | 1463                             | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0±0.0 |
| (+)-( <i>E</i> )- $\beta$ -Caryophyllene | na               | no                               | no                               | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0±0.0   |
| (-)- $\delta$ -Cadinene                  | 1563             | no                               | no                               | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0±0.0   |
| (+)- $\delta$ -Cadinene                  | 1576             | 1576                             | 1577                             | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0±0.0 |
| (-)-( <i>E</i> )-Nerolidol               | 1677             | 1677                             | 1677                             | 1.2   | 1.4   | 1.5   | 1.9   | 1.7   | 2.2   | 1.6   | 1.4   | 2.2   | 1.7±0.3   |
| (+)-( <i>E</i> )-Nerolidol               | 1680             | 1680                             | 1679                             | 98.8  | 98.6  | 98.5  | 98.1  | 98.3  | 97.8  | 98.4  | 98.6  | 97.8  | 98.3±0.3  |

na=not available, no=not detected, RI<sub>db</sub>= Retention Index (database), RI<sub>calc</sub>= Calculated Retention Index.



**Fig. 3.** Scanning electron microscopy (SEM) images of yeast cells encapsulating *Baeckea frutescens* essential oil at different magnifications: (A) low magnification showing the overall morphology and aggregation of yeast microcapsules; (B) high magnification revealing the surface structure and shape of individual encapsulated yeast cells.

summarized in Table 5. Considerable variation in toxicity was observed among the essential oil samples. After 24 h of exposure, LC<sub>50</sub> values ranged from 18.97 to 39.33  $\mu$ g/mL, whereas LC<sub>60</sub> values ranged from 26.17 to 49.05  $\mu$ g/mL. Sample BF8 exhibited the highest activity with an LC<sub>50</sub> value of 18.97  $\mu$ g/mL, while BF2 showed the lowest activity with an LC<sub>50</sub> value of 39.33  $\mu$ g/mL. Following 48 h of exposure, LC<sub>50</sub> values ranged from 18.18 to 37.55  $\mu$ g/mL and LC<sub>90</sub> values ranged

from 25.21 to 47.77  $\mu$ g/mL. In most cases, toxicity increased slightly with exposure time, as reflected by lower LC<sub>50</sub> and LC<sub>90</sub> values after 48 h compared with 24 h (Table 5).

#### Larvicidal Activity Against *Aedes albopictus*

The essential oils also exhibited larvicidal activity against *Ae. albopictus* larvae (Table 6). As observed for *Ae. aegypti*, substantial variation in toxicity was detected among samples.

At 24 h,  $LC_{50}$  values ranged from 10.60 to 13.11  $\mu\text{g/mL}$ , whereas  $LC_{90}$  values ranged from 14.57 to 19.9  $\mu\text{g/mL}$ . Sample BF7 was the most active, whereas sample BF9 displayed the lowest activity. After 48 h of exposure,  $LC_{50}$  values ranged from

9.93 to 19.21  $\mu\text{g/mL}$ . A general increase in toxicity with increasing exposure duration was observed for most essential oil samples. Among the 3 mosquito species tested, *Ae. albopictus* generally exhibited the lowest  $LC_{50}$  values.

#### Larvicidal Activity Against *Culex quinquefasciatus*

Larvicidal activity against *Cx. quinquefasciatus* is presented in Table 7. The tested essential oils produced  $LC_{50}$  values ranging from 19.79 to 45.62  $\mu\text{g/mL}$  after 24 h of exposure and from 25.68 to 57.98  $\mu\text{g/mL}$  after 48 h. Among the evaluated samples, BF6 exhibited the lowest  $LC_{50}$  value, whereas BF7 showed the highest  $LC_{50}$  value.

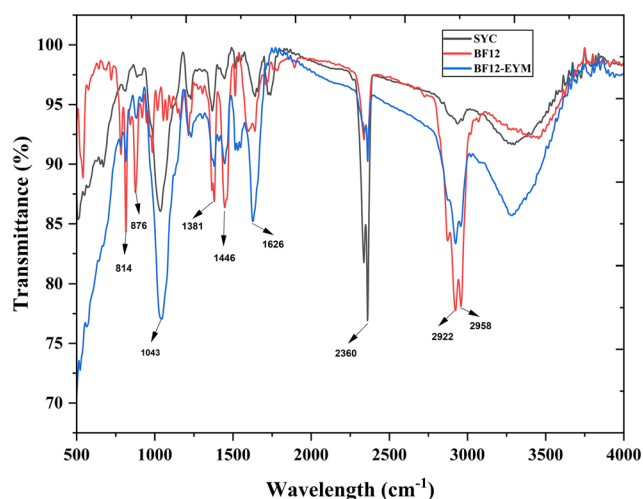
Among the three mosquito species tested, *Ae. albopictus* generally exhibited lower  $LC_{50}$  values than *Ae. aegypti* and *Cx. quinquefasciatus*, suggesting a tendency toward greater susceptibility to *B. frutescens* essential oils. However, formal statistical comparisons among species were not performed.

#### Larvicidal Activity of Encapsulated BF12 Essential Oil

The larvicidal activity of the yeast-encapsulated BF12 formulation (BF12-EYM) against *Ae. aegypti* larvae is presented in Table 5. The encapsulated formulation exhibited  $LC_{50}$  values of 45.71 and 44.41  $\mu\text{g/mL}$  after 24 and 48 h of exposure, respectively. Although the encapsulated formulation remained toxic to mosquito larvae, its  $LC_{50}$  values were higher than those observed for the corresponding free essential oil. This difference is expected because the reported concentrations refer to the total formulation mass rather than the amount of active essential oil released from the microcapsules. The results demonstrate that BF12 essential oil retained larvicidal activity following encapsulation within the yeast microcapsule delivery system.

#### Toxicity to *Moina macrocopa*

The leaf essential oils of *B. frutescens* exhibited acute toxicity toward *M. macrocopa* across all evaluated exposure times (12, 24, and 48 h). At 12 h,  $LC_{50}$  values ranged from 7.81 (6.99 to 8.61) to 20.03 (17.53 to 25.12)  $\mu\text{g/mL}$ , with corresponding  $LC_{90}$  values of 11.30 (10.27 to 12.92) to 31.69 (25.60 to 48.33)  $\mu\text{g/mL}$  (95% confidence intervals). At 24 h,  $LC_{50}$  values remained



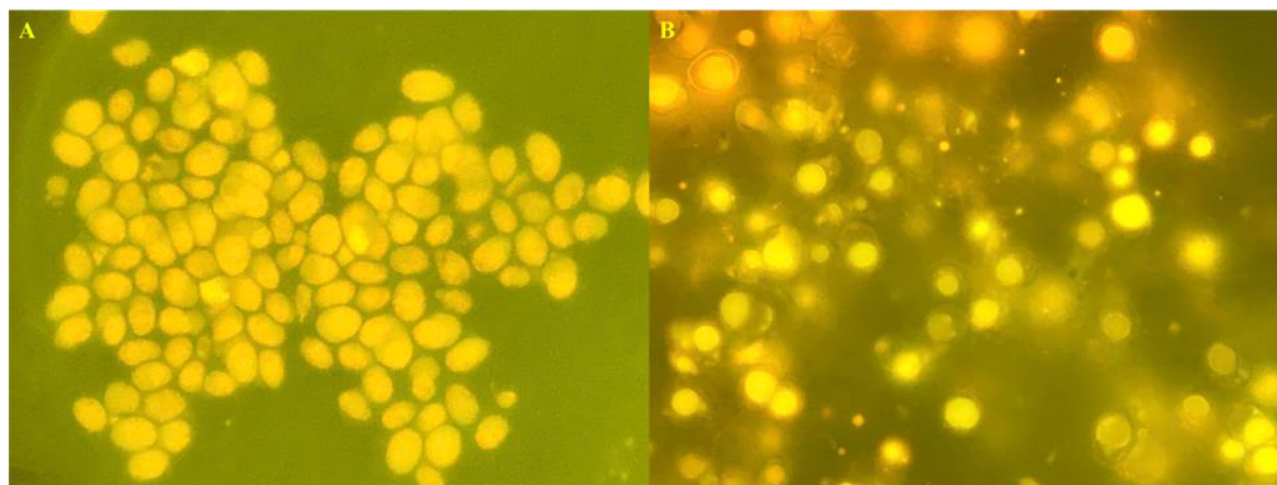
**Fig. 4.** Fourier transform infrared (FTIR) spectra of yeast cells (SYC), *Baeckea frutescens* essential oil (BF12), and yeast cells encapsulating *Baeckea frutescens* essential oil (BF12-EYM).

**Table 3.** Effect of Tween 80 concentration on essential oil loading capacity in yeast cell-based encapsulation

| %               | 1%          | 2%           | 3%           | 4%           |
|-----------------|-------------|--------------|--------------|--------------|
| <b>Tween 80</b> |             |              |              |              |
| LC              | 11.1 ± 0.25 | 29.86 ± 0.54 | 40.71 ± 0.68 | 30.28 ± 0.42 |

**Table 4.** Effect of essential oil–yeast–water ratio on the loading capacity of essential oil in yeast cells

| EO:Y:W | 60:15:25                   | 30:15:55    | 15:15:70     |
|--------|----------------------------|-------------|--------------|
| LC     | 34.79 ± 0.34<br>(BF12-EYM) | 21.4 ± 0.44 | 27.86 ± 0.56 |



**Fig. 5.** Fluorescence microscopy images of Nile Red-stained yeast microcapsules encapsulating essential oil. (A) BF12-EYM microcapsules showing high lipid-associated fluorescence corresponding to a loading capacity (LC) of 34.79%. (B) Microcapsules with a loading capacity of 11.1%, showing weaker and more dispersed fluorescence signals.

**Table 5.** Larvicidal activity of *Baeckea frutescens* leaf essential oils against *Aedes aegypti*

| Material            | LC <sub>50</sub> (95% limits) µg/mL | LC <sub>90</sub> (95% limits) µg/mL | Slope±SE        | χ <sup>2</sup> | df | P    |
|---------------------|-------------------------------------|-------------------------------------|-----------------|----------------|----|------|
| <b>24 h</b>         |                                     |                                     |                 |                |    |      |
| BF1                 | 35.46 (33.30–37.80)                 | 45.28 (42.51–48.89)                 | 0.1304±0.0118   | 0.17           | 3  | 0.98 |
| BF2                 | 39.33 (37.01–41.52)                 | 49.05 (46.65–51.99)                 | 0.1318±0.0122   | 0.02           | 3  | 1.00 |
| BF3                 | 36.18 (33.75–38.92)                 | 43.79 (40.83–47.75)                 | 0.1684±0.0179   | 0.00           | 3  | 1.00 |
| BF4                 | 36.02 (33.84–38.36)                 | 45.50 (42.79–48.99)                 | 0.1352±0.0123   | 0.08           | 3  | 0.99 |
| BF5                 | 38.80 (36.53–41.15)                 | 51.83 (48.86–55.61)                 | 0.0984±0.0083   | 0.78           | 3  | 0.86 |
| BF6                 | 28.19 (26.33–30.87)                 | 37.42 (34.01–43.20)                 | 0.1389±0.0198   | 0.47           | 3  | 0.93 |
| BF7                 | 22.71 (21.50–23.77)                 | 28.31 (26.92–30.43)                 | 0.2286±0.0312   | 0.01           | 3  | 1.00 |
| BF8                 | 18.97 (17.80–20.20)                 | 26.17 (24.54–28.28)                 | 0.1779±0.0153   | 1.83           | 3  | 0.61 |
| BF9                 | 21.26 (19.97–22.31)                 | 26.08 (24.97–27.52)                 | 0.2659±0.0320   | 0.00           | 3  | 1.00 |
| BF10                | 20.79 (19.61–21.98)                 | 27.42 (25.89–29.44)                 | 0.1933±0.0183   | 0.31           | 3  | 0.96 |
| BF11                | 28.57 (26.69–30.96)                 | 38.74 (35.56–43.51)                 | 0.1260±0.0144   | 2.62           | 3  | 0.45 |
| BF12                | 22.19 (20.96–23.24)                 | 27.52 (26.26–29.35)                 | 0.2405±0.0315   | 0.01           | 3  | 1.00 |
| BF12-EYM            | 45.71 (42.46–49.64)                 | 67.57 (61.93–75.41)                 | 0.05862±0.00548 | 5.80           | 3  | 0.12 |
| 1,8-cineole         | >100                                | >100                                | Nd              | Nd             | Nd | Nd   |
| α-Humulene          | 45.25 (41.55–49.29)                 | 88.62 (78.00–105.40)                | 3.78509±0.36291 | 4.80           | 3  | 0.19 |
| β-Caryophyllene     | 106.77 (98.85–115.42)               | 191.41 (169.00–229.12)              | 3.76409±0.40567 | 7.15           | 4  | 0.13 |
| Caryophyllene oxide | 59.03 (53.53–65.52)                 | 123.94 (105.65–154.58)              | 2.96228±0.28519 | 6.33           | 3  | 0.10 |
| (–)-β-Pinene        | 29.46 (27.12–32.04)                 | 51.17 (45.54–59.82)                 | 3.97987±0.37237 | 1.61           | 3  | 0.66 |
| (+)-α-Pinene        | 20.56 (18.67–21.91)                 | 26.04 (24.54–28.19)                 | 9.29745±1.49931 | 0.03           | 3  | 1.00 |
| Permethrin          | 0.00464 (0.00430–0.00503)           | 0.00643 (0.00590–0.00724)           | 715.2±89.6      | 0.545          | 2  | 0.76 |
| <b>48 h</b>         |                                     |                                     |                 |                |    |      |
| BF1                 | 34.64 (32.50–37.07)                 | 44.13 (41.24–48.00)                 | 0.1351±0.0130   | 0.17           | 3  | 0.98 |
| BF2                 | 37.55 (35.33–39.79)                 | 47.77 (45.18–51.01)                 | 0.1254±0.0109   | 0.01           | 3  | 0.99 |
| BF3                 | 35.36 (33.05–38.07)                 | 43.41 (40.40–47.57)                 | 0.1591±0.0170   | 0.01           | 3  | 1.00 |
| BF4                 | 34.92 (32.77–37.28)                 | 45.52 (42.65–49.23)                 | 0.1210±0.0106   | 1.63           | 3  | 0.65 |
| BF5                 | 37.38 (35.08–39.86)                 | 51.80 (48.56–55.95)                 | 0.0889±0.0071   | 0.63           | 3  | 0.89 |
| BF6                 | 27.27 (25.30–29.69)                 | 39.54 (36.19–44.26)                 | 0.1045±0.0102   | 7.73           | 3  | 0.05 |
| BF7                 | 21.17 (19.99–22.36)                 | 27.76 (26.23–29.82)                 | 0.1944±0.0190   | 0.23           | 3  | 0.97 |
| BF8                 | 18.18 (17.04–19.40)                 | 25.21 (23.61–27.27)                 | 0.1824±0.0154   | 0.24           | 3  | 0.97 |
| BF9                 | 19.53 (18.40–20.65)                 | 25.22 (23.89–26.89)                 | 0.2252±0.0199   | 0.16           | 3  | 0.98 |
| BF10                | 20.28 (19.12–21.46)                 | 26.82 (25.32–28.76)                 | 0.1961±0.0179   | 0.37           | 3  | 0.95 |
| BF11                | 28.02 (26.18–30.37)                 | 38.08 (34.94–42.82)                 | 0.1275±0.0148   | 4.00           | 3  | 0.26 |
| BF12                | 21.81 (20.63–22.91)                 | 27.59 (26.24–29.45)                 | 0.2219±0.0250   | 0.03           | 3  | 1.00 |
| BF12-EYM            | 44.41 (41.17–48.29)                 | 66.85 (61.22–74.61)                 | 0.06011±0.00555 | 5.98           | 3  | 0.11 |
| 1,8-cineole         | >100                                | >100                                | Nd              | Nd             | Nd | Nd   |
| α-Humulene          | 36.19 (33.03–39.70)                 | 70.54 (61.88–83.83)                 | 3.29286±0.28933 | 7.29           | 3  | 0.06 |
| β-Caryophyllene     | 95.37 (89.29–101.98)                | 146.24 (136.77–157.97)              | 0.02519±0.00165 | 2.93           | 4  | 0.57 |
| Caryophyllene oxide | 52.02 (47.32–57.41)                 | 105.96 (91.41–129.54)               | 3.08836±0.28893 | 3.27           | 3  | 0.35 |
| (–)-β-Pinene        | 25.34 (23.38–27.47)                 | 43.06 (38.47–50.20)                 | 4.14386±0.40509 | 1.07           | 3  | 0.79 |
| (+)-α-Pinene        | 19.63 (18.02–21.01)                 | 25.29 (23.64–27.52)                 | 8.67047±1.11890 | 0.04           | 3  | 1.00 |

No larval mortality was observed in the ethanol negative control.

low, ranging from 6.89 (5.75 to 7.91) to 12.47 (10.81 to 14.14) µg/mL, with LC<sub>90</sub> values between 11.94 (10.50 to 14.48) and 20.30 (17.95 to 24.36) µg/mL, depending on the essential oil sample. At 48h, the toxicity profile was sustained, with LC<sub>50</sub> values ranging from 6.00 (4.45 to 7.20) to 10.08 (7.35 to 12.17) µg/mL across the samples, and LC<sub>90</sub> values from 11.87 (10.22 to 15.04) to 21.01 (17.84 to 27.59) µg/mL (Table 8).

## Discussion

### Chemical Variation and Enantiomeric Composition

The clustering pattern observed in the present study indicates clear chemotypic variation among the *B. frutescens* essential oil samples. In particular, Group 1 (BF1, BF3, BF5, and BF6) represents a chemically differentiated group characterized by relatively higher contributions of oxygenated terpenoids,

including 1,8-cineole, caryophyllene oxide, and humulene epoxide II. Compared with previously reported profiles that often emphasize variation in monoterpene hydrocarbons, this group highlights an additional axis of chemical variability associated with oxygenated terpenoid constituents.

This pattern suggests an additional dimension of chemical variability in *B. frutescens* essential oils, driven by oxygenated sesquiterpenes and related compounds rather than by monoterpene hydrocarbons alone. The separation of this group was consistently supported by both PCA and HCA, reinforcing the robustness of the observed classification.

Group 2 comprises BF2, BF4, BF7, BF8, BF11, and BF12, forming a central cluster with more heterogeneous chemical profiles. These samples exhibit mixed contributions from both monoterpenes and sesquiterpene hydrocarbons, particularly α-humulene and β-caryophyllene, along with varying levels of γ-terpinene.

**Table 6.** Larvicidal activity of *Baeckea frutescens* leaf essential oils against *Aedes albopictus*

| Material    | LC <sub>50</sub> (95% limits) µg/mL | LC <sub>90</sub> (95% limits) µg/mL | Slope±SE        | χ <sup>2</sup> | df | P     |
|-------------|-------------------------------------|-------------------------------------|-----------------|----------------|----|-------|
| <b>24 h</b> |                                     |                                     |                 |                |    |       |
| BF3         | 12.50 (11.81–13.38)                 | 16.40 (15.12–18.62)                 | 0.32889±0.05006 | 0.00           | 3  | 1.00  |
| BF4         | 11.20 (10.24–12.27)                 | 17.78 (16.06–20.46)                 | 0.19461±0.02438 | 0.64           | 3  | 0.89  |
| BF5         | 12.34 (11.27–13.50)                 | 19.91 (18.14–22.43)                 | 0.16939±0.01785 | 0.35           | 3  | 0.95  |
| BF6         | 12.53 (11.50–13.70)                 | 19.58 (17.78–22.22)                 | 0.18172±0.02019 | 3.51           | 3  | 0.32  |
| BF7         | 10.60 (9.94–11.29)                  | 14.57 (13.59–15.98)                 | 0.32288±0.03623 | 0.00           | 3  | 1.00  |
| BF8         | 12.65 (11.94–13.60)                 | 16.65 (15.30–19.03)                 | 0.32111±0.04980 | 0.00           | 3  | 1.00  |
| BF9         | 13.11 (12.10–14.30)                 | 19.71 (17.96–22.30)                 | 0.19406±0.02135 | 3.17           | 3  | 0.37  |
| BF10        | 12.23 (11.29–13.35)                 | 18.41 (16.70–21.04)                 | 0.20739±0.02517 | 0.77           | 3  | 0.86  |
| Permethrin  | 0.00375 (0.00352–0.00404)           | 0.00493 (0.00454–0.00560)           | 1098±144        | 0.20           | 1  | 0.732 |
| <b>48 h</b> |                                     |                                     |                 |                |    |       |
| BF3         | 11.85 (11.16–12.66)                 | 15.95 (14.76–17.85)                 | 0.31262±0.04139 | 0.00           | 3  | 1.00  |
| BF4         | 9.93 (9.11–10.78)                   | 15.28 (13.93–17.40)                 | 0.23942±0.03082 | 0.02           | 3  | 1.00  |
| BF5         | 11.00 (10.00–12.09)                 | 18.01 (16.25–20.74)                 | 0.18278±0.02267 | 1.05           | 3  | 0.79  |
| BF6         | 11.37 (10.28–12.54)                 | 19.21 (17.34–22.01)                 | 0.16345±0.01900 | 3.88           | 3  | 0.28  |
| BF7         | 9.96 (9.30–10.62)                   | 13.83 (12.92–15.09)                 | 0.33091±0.03509 | 0.00           | 3  | 1.00  |
| BF8         | 12.58 (11.87–13.49)                 | 16.52 (15.21–18.83)                 | 0.32498±0.04995 | 0.00           | 3  | 1.00  |
| BF9         | 12.23 (11.29–13.35)                 | 18.41 (16.70–21.04)                 | 0.20739±0.02517 | 0.77           | 3  | 0.86  |
| BF10        | 11.17 (10.24–12.23)                 | 17.54 (15.85–20.19)                 | 0.20136±0.02555 | 0.43           | 3  | 0.93  |

No larval mortality was observed in the ethanol negative control.

**Table 7.** Larvicidal activity of *Baeckea frutescens* leaf essential oils against *Culex quinquefasciatus*

| Material    | LC <sub>50</sub> (95% limits) µg/mL | LC <sub>90</sub> (95% limits) µg/mL | Slope±SE        | χ <sup>2</sup> | df | P    |
|-------------|-------------------------------------|-------------------------------------|-----------------|----------------|----|------|
| <b>24 h</b> |                                     |                                     |                 |                |    |      |
| BF1         | 23.12 (21.94–24.22)                 | 28.95 (27.44–31.35)                 | 0.21983±0.03110 | 0.01           | 3  | 1.00 |
| BF3         | 42.83 (40.46–45.26)                 | 56.31 (53.14–60.56)                 | 0.09508±0.00930 | 0.28           | 3  | 0.96 |
| BF4         | 35.26 (32.95–37.79)                 | 50.75 (47.33–55.13)                 | 0.08275±0.00648 | 1.21           | 3  | 0.75 |
| BF5         | 38.90 (36.63–41.10)                 | 49.01 (46.53–52.06)                 | 0.12682±0.01140 | 0.05           | 3  | 1.00 |
| BF6         | 19.79 (18.66–20.92)                 | 25.68 (24.32–27.41)                 | 0.21755±0.01943 | 0.19           | 3  | 0.98 |
| BF7         | 45.62 (43.22–47.76)                 | 56.94 (54.10–61.30)                 | 0.11322±0.01558 | 0.01           | 3  | 1.00 |
| BF8         | 39.35 (36.83–42.14)                 | 56.36 (52.50–61.42)                 | 0.07533±0.00625 | 1.03           | 3  | 0.79 |
| BF9         | 27.30 (25.46–29.74)                 | 37.27 (34.01–42.38)                 | 0.12849±0.01581 | 1.07           | 3  | 0.79 |
| BF10        | 33.85 (31.75–36.18)                 | 45.21 (42.28–49.03)                 | 0.11288±0.00979 | 1.33           | 3  | 0.72 |
| BF11        | 45.39 (43.02–47.73)                 | 57.98 (54.83–62.59)                 | 0.10175±0.01222 | 0.05           | 3  | 1.00 |
| BF12        | 33.91 (31.56–36.61)                 | 45.21 (41.36–50.99)                 | 7.63881±0.81709 | 0.08           | 3  | 1.00 |
| Permethrin  | 0.0150 (0.0141–0.0161)              | 0.0197 (0.0181–0.0224)              | 271.69±40.48    | 0.12           | 2  | 0.94 |
| <b>48 h</b> |                                     |                                     |                 |                |    |      |
| BF1         | 21.60 (20.41–22.68)                 | 27.24 (25.93–29.01)                 | 0.22708±0.02516 | 0.03           | 3  | 1.00 |
| BF3         | 38.57 (36.30–40.93)                 | 51.81 (48.81–55.65)                 | 0.09676±0.00812 | 0.98           | 3  | 0.81 |
| BF4         | 24.92 (23.16–27.13)                 | 36.00 (32.87–40.59)                 | 0.11570±0.01238 | 3.70           | 3  | 0.30 |
| BF5         | 35.40 (33.19–37.79)                 | 48.12 (45.09–51.98)                 | 0.10072±0.00808 | 5.09           | 3  | 0.17 |
| BF6         | 18.20 (17.07–19.38)                 | 24.71 (23.20–26.65)                 | 0.19668±0.01652 | 0.01           | 3  | 1.00 |
| BF7         | 39.19 (36.92–41.39)                 | 49.50 (47.02–52.58)                 | 0.12431±0.01122 | 0.05           | 3  | 1.00 |
| BF8         | 29.95 (27.87–32.40)                 | 42.67 (39.37–47.11)                 | 0.10081±0.00889 | 7.51           | 3  | 0.06 |
| BF9         | 21.07 (19.68–22.65)                 | 30.39 (28.10–33.58)                 | 0.13750±0.01345 | 3.82           | 3  | 0.28 |
| BF10        | 30.83 (28.81–33.33)                 | 41.11 (37.91–45.75)                 | 0.12465±0.01337 | 0.17           | 3  | 0.98 |
| BF11        | 42.35 (39.92–44.95)                 | 57.19 (53.69–61.86)                 | 0.08638±0.00800 | 0.88           | 3  | 0.83 |
| BF12        | 33.33 (31.06–35.98)                 | 44.97 (41.06–50.96)                 | 7.33432±0.78838 | 0.115          | 3  | 0.99 |

No larval mortality was observed in the ethanol negative control.

Group 3 (BF9 and BF10) forms a clearly distinct cluster and is characterized by higher proportions of mono-terpene hydrocarbons, particularly γ-terpinene, together with notable contributions from sesquiterpene hydrocarbons. This group is well separated along PC1 in the PCA and represents a chemically distinct profile within the dataset.

Because reports on the enantiomeric distribution of mono-terpenoids in essential oils of *Baeckea* species are still very limited, comparison of the present results with those reported for other genera within the Myrtaceae family represents a reasonable approach to place the enantiomeric data into a broader biological and biosynthetic context. The doctoral thesis by [Cornwell \(1999\)](#) demonstrates that within Myrtaceae, chiral

**Table 8.** Toxicity of *Baeckea frutescens* leaf essential oils against *Moina macrocopa*

| Material    | LC <sub>50</sub> (95% limits) µg/mL | LC <sub>90</sub> (95% limits) µg/mL | Slope±SE      | χ <sup>2</sup> | df | P     |
|-------------|-------------------------------------|-------------------------------------|---------------|----------------|----|-------|
| <b>12 h</b> |                                     |                                     |               |                |    |       |
| BF3         | 20.03 (17.53–25.12)                 | 30.31 (25.19–43.20)                 | 0.1247±0.0285 | 0.02           | 2  | 0.991 |
| BF4         | 15.87 (14.15–18.14)                 | 24.27 (21.20–30.16)                 | 0.1526±0.0269 | 6.10           | 2  | 0.047 |
| BF5         | 7.81 (6.99–8.61)                    | 11.30 (10.27–12.92)                 | 0.3676±0.0536 | 6.21           | 2  | 0.045 |
| BF6         | 14.59 (11.49–19.32)                 | 31.20 (24.17–54.88)                 | 0.0771±0.0220 | 7.26           | 2  | 0.027 |
| BF7         | 12.75 (11.29–16.21)                 | 17.95 (15.03–26.28)                 | 0.2465±0.0625 | 7.80           | 2  | 0.020 |
| BF8         | 14.91 (13.25–16.89)                 | 23.06 (20.31–28.09)                 | 0.1572±0.0262 | 2.30           | 2  | 0.316 |
| BF9         | 19.08 (16.34–24.84)                 | 31.69 (25.60–48.33)                 | 0.1016±0.0248 | 0.73           | 2  | 0.695 |
| BF10        | 18.45 (15.94–23.14)                 | 30.23 (24.86–43.46)                 | 0.1088±0.0245 | 4.44           | 2  | 0.109 |
| <b>24 h</b> |                                     |                                     |               |                |    |       |
| BF3         | 11.36 (9.60–13.00)                  | 19.31 (17.01–23.32)                 | 0.1612±0.0264 | 0.23           | 2  | 0.893 |
| BF4         | 11.77 (10.38–13.14)                 | 17.79 (16.01–20.65)                 | 0.2127±0.0313 | 0.71           | 2  | 0.703 |
| BF5         | 6.89 (5.75–7.91)                    | 11.94 (10.50–14.48)                 | 0.2536±0.0423 | 4.20           | 2  | 0.122 |
| BF6         | 8.77 (7.05–10.20)                   | 15.19 (13.38–18.25)                 | 0.1999±0.0335 | 0.61           | 2  | 0.736 |
| BF7         | 7.85 (6.76–8.99)                    | 13.21 (11.53–16.34)                 | 0.2388±0.0413 | 0.23           | 2  | 0.89  |
| BF8         | 12.47 (10.81–14.14)                 | 20.30 (17.95–24.36)                 | 0.1637±0.0262 | 0.43           | 2  | 0.806 |
| BF9         | 9.28 (7.60–10.73)                   | 15.89 (14.04–18.96)                 | 0.1939±0.0312 | 2.63           | 2  | 0.268 |
| BF10        | 10.10 (8.60–11.50)                  | 16.39 (14.55–19.44)                 | 0.2037±0.0319 | 5.50           | 2  | 0.064 |
| <b>48 h</b> |                                     |                                     |               |                |    |       |
| BF3         | 10.08 (7.35–12.17)                  | 21.01 (17.84–27.59)                 | 0.1172±0.0236 | 0.95           | 2  | 0.62  |
| BF4         | 9.11 (7.05–10.74)                   | 16.98 (14.84–20.72)                 | 0.1628±0.0281 | 0.13           | 2  | 0.938 |
| BF5         | 6.00 (4.45–7.20)                    | 12.21 (10.46–15.65)                 | 0.2066±0.0397 | 2.83           | 2  | 0.243 |
| BF6         | 6.79 (4.20–8.50)                    | 14.26 (12.30–17.72)                 | 0.1715±0.0324 | 2.12           | 2  | 0.347 |
| BF7         | 6.02 (4.58–7.16)                    | 11.87 (10.22–15.04)                 | 0.2189±0.0409 | 0.24           | 2  | 0.887 |
| BF8         | 9.87 (7.47–11.75)                   | 19.48 (16.82–24.50)                 | 0.1333±0.0245 | 3.50           | 2  | 0.174 |
| BF9         | 9.18 (6.11–11.31)                   | 20.25 (17.16–26.69)                 | 0.1157±0.0238 | 0.69           | 2  | 0.709 |
| BF10        | 10.08 (7.77–11.95)                  | 19.59 (16.93–24.55)                 | 0.1219±0.0239 | 4.70           | 2  | 0.127 |

monoterpenoids such as α-pinene, limonene, linalool, terpinen-4-ol, and α-terpineol may exhibit markedly different enantiomeric distributions among genera and species, ranging from strong enantiomeric predominance to near-racemic compositions, reflecting substantial diversity in biosynthetic control across the family (Cornwell 1999).

With respect to terpinen-4-ol, Southwell and Robert (1999) report that natural tea tree oil (*Melaleuca alternifolia* (Maiden & Betche) Cheel) possesses a characteristic and relatively stable enantiomeric ratio, and that deviations in the (+)/(-)-terpinen-4-ol ratio have been used as an important indicator for detecting adulteration or differences in origin (Southwell and Robert 1999). In the present study, the near-racemic distribution of terpinen-4-ol observed in *Baeckea* essential oil suggests that this compound may be formed through multiple parallel biosynthetic pathways, consistent with the range of enantiomeric variability reported among other Myrtaceae species.

Cornwell (1999) further indicates that for monoterpenes such as α-pinene and limonene, enantiomeric ratios can vary considerably among genera within the Myrtaceae, even when the overall chemical composition of the essential oils is relatively similar (Cornwell 1999). The enantiomeric predominance observed for certain monoterpenes in *Baeckea* essential oil therefore falls within the broader range of variation reported for the family and reflects genus- and species-specific stereochemical control by terpene synthases.

Overall, when considered in the context of the available enantiomeric data for the Myrtaceae, the results of the present

study not only contribute new information for the poorly documented genus *Baeckea* but also support the view that enantiomeric distribution of monoterpenoids constitutes a valuable biochemical characteristic for studies of biosynthesis, chemotaxonomy, and authenticity assessment of essential oils within the Myrtaceae family.

Although all samples were collected from the same geographical location, substantial variation in essential oil composition was observed among collection periods. The PCA and HCA analyses revealed the presence of distinct chemotypic groups, suggesting that temporal factors may contribute to chemical variability. Seasonal fluctuations in temperature, rainfall, solar radiation, and plant physiological status are known to influence terpene biosynthesis in aromatic plants. Therefore, the observed differences among BF2–BF12 may partially reflect temporal environmental variation, although dedicated seasonal studies would be required to establish causal relationships.

### Physicochemical Properties of Yeast Microcapsules

The preservation of yeast cell morphology observed by SEM suggests that the encapsulation process did not compromise structural integrity of the cell wall. The simultaneous detection of yeast polysaccharide and essential oil spectral features in FTIR analysis indicates successful incorporation of the essential oil into the yeast matrix rather than simple physical mixing.

The relatively high loading capacity values demonstrate efficient retention of essential oil within the yeast cells. The correlation between LC values and fluorescence intensity further

supports intracellular localization of the oil within lipid-associated domains of the yeast matrix.

Taken together, the morphological evidence from SEM, the chemical fingerprints from FTIR, and the quantitative LC results collectively confirm the successful encapsulation and effective retention of *B. frutescens* essential oil within yeast-based microcapsules. The results in this study are comparable to a previous study by Thomas et al. (2026).

### Larvicidal Activity

Based on PCA-HCA results and their comparison with the larvicidal bioassay data of the major constituents, the relationship between chemotypes and larvicidal activity against *Ae. aegypti* can be discussed at a trend-based level supported by experimental evidence. Group 1 (BF1, BF3, BF5, and BF6) forms a distinct cluster associated with an oxygenated-terpenoid axis, particularly 1,8-cineole and oxide/epoxide-type sesquiterpenes.

However, the oils in this group (Group 1) generally exhibited only moderate larvicidal activity, with  $LC_{50}$  values at 24 h ranging from approximately 27 to 39  $\mu\text{g/mL}$ . This pattern is consistent with single-compound bioassays showing that 1,8-cineole displays weak activity ( $LC_{50} > 100 \mu\text{g/mL}$ ), while the major sesquiterpenes characteristic of this group, such as caryophyllene oxide,  $\alpha$ -humulene, and  $\beta$ -caryophyllene exhibited moderate larvicidal activity, with 24-h  $LC_{50}$  values ranging from 37.91 to 63.18  $\mu\text{g/mL}$ .

In contrast, Group 2 (the central cluster) shows a tendency toward association with sesquiterpene hydrocarbons and/or  $\gamma$ -terpinene and includes most of the samples with lower  $LC_{50}$  values within the BF1–BF12 dataset. This observation is supported by bioassays of major constituents, in which  $\gamma$ -terpinene and *p*-cymene exhibited relatively low  $LC_{50}$  values against *Ae. aegypti* (11.25 and 17.05  $\mu\text{g/mL}$  at 24 h, respectively) (Huang et al. 2019), indicating substantially higher activity than that of many sesquiterpenes tested individually. Moreover, bioassays of mixtures of major constituents demonstrate that larvicidal activity does not follow a linear relationship with the abundance of individual compounds but is strongly dependent on mixing ratios, as illustrated by the very low  $LC_{50}$  value (9.54  $\mu\text{g/mL}$  at 24 h) observed for the caryophyllene oxide/ $\alpha$ -humulene/ $\beta$ -caryophyllene mixture (7/2/1) (Luu-Dam et al. 2023), whereas other combinations resulted in higher  $LC_{50}$  values, suggesting possible synergistic or antagonistic interactions within the essential oil matrix.

The larvicidal activity observed in the present study compares favorably with many plant-derived mosquito larvicides reported in the literature. According to Pavela (2015), essential oils exhibiting  $LC_{50}$  values below 50  $\mu\text{g/mL}$  are generally considered highly active against mosquito larvae (Pavela 2015, Pavela and Benelli 2016). The majority of *B. frutescens* samples evaluated in this study produced  $LC_{50}$  values within this range against *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus*, indicating promising larvicidal potential.

Compared with numerous essential oils previously investigated for mosquito control, the activity of *B. frutescens* essential oil is comparable to that reported for several commercially important aromatic plants. Together with its high essential oil yield, chemical diversity, and successful encapsulation within yeast microcapsules, these results support the potential of *B. frutescens* as a candidate botanical

larvicide. Nevertheless, further studies evaluating residual activity, semi-field performance, formulation stability, and environmental safety will be necessary before practical application can be considered.

Overall, these findings indicate that the larvicidal activity of *B. frutescens* essential oils against *Ae. aegypti* is more likely governed by the overall chemical profile and the balance and interactions among constituent groups, rather than by the dominance of any single major compound, with Group 2 potentially benefiting from the presence of highly active monoterpenes ( $\gamma$ -terpinene and *p*-cymene) and/or favorable compositional ratios.

Yeast-based encapsulation has been widely recognized as an effective strategy to enhance the delivery and performance of essential oils for mosquito larval control. Previous studies reported that yeast-encapsulated essential oils exhibit strong larvicidal activity against *Aedes* mosquitoes, with  $LC_{50}$  values  $< 50 \mu\text{g/mL}$  after 24 h of exposure, and oil loadings of approximately 26% to 30%. (Workman et al. 2020, Kelly et al. 2022, Gomes et al. 2025) In the present study, a higher encapsulation efficiency ( $34.79 \pm 0.34\%$ ) was achieved, indicating an increased essential oil payload per unit mass of yeast carrier. Kaur et al. (2025) reported citronella oil-loaded yeast microcapsules with a high encapsulation efficiency ( $LC = 54.28\%$ ). The formulation exhibited larvicidal activity against third- and fourth-instar *Ae. aegypti* larvae, with  $LC_{50}$  and  $LC_{90}$  values of 1600 and 3400  $\mu\text{g/mL}$ , respectively (Kaur et al. 2025).

Although the formulation-based  $LC_{50}$  of the essential oil-loaded yeast microcapsules (45.71  $\mu\text{g/mL}$  at 24 h) was approximately 2.1-fold higher than that of the free essential oil (22.19  $\mu\text{g/mL}$ ), this apparent difference primarily reflects the inclusion of the biologically inert yeast carrier in the reported formulation concentration rather than a reduction in intrinsic larvicidal potency. Yeast cells serve as protective carrier matrices that physically entrap essential oils without chemically altering their bioactive constituents, thereby preserving their biological activity while improving stability and facilitating delivery (Workman et al. 2020, Kelly et al. 2022, Gomes et al. 2025). Accordingly, when normalized to the actual essential oil loading, the EO-equivalent  $LC_{50}$  of the microcapsules was approximately 15.90  $\mu\text{g/mL}$ , which was comparable to, and slightly lower than, that of the free essential oil, indicating that encapsulation did not compromise the intrinsic larvicidal activity of the essential oil. The absence of corresponding free-oil toxicity data in most previous studies limits direct comparisons between formulations.

### Toxicity to *Moina macrocopa*

When compared with published data for non-target aquatic crustaceans, the toxicity observed against *M. macrocopa* was relatively high, as the  $LC_{50}$  values obtained in the present study were generally lower than many previously reported values for essential oils tested against aquatic invertebrates. Specifically, at 48 h of exposure,  $LC_{50}$  values for *M. macrocopa* ranged from 5.35 to 10.06 mg/L, which are substantially lower than the 48-h  $LC_{50}$  values reported for *Daphnia magna* exposed to *Helichrysum italicum* essential oil (28.71  $\mu\text{g/mL}$ ) and *Thymus mastichina* essential oil (87.84  $\mu\text{g/mL}$ ). However, the same study also documented that certain thyme or white thyme essential oils can exert strong toxicity toward *D. magna*, with 48-h  $LC_{50}$  values as low as 2.5 to 11.79  $\mu\text{g/mL}$

(Afonso et al. 2023), indicating that a pronounced toxicity range within cladocerans has been previously reported. In this context, the  $LC_{50}$  values obtained in the present study are consistent in terms of order of magnitude with published data. From an ecological risk management perspective, the high sensitivity of *Moina*—a key component of aquatic food webs—at low concentrations highlights the need for careful evaluation of non-target risks associated with essential-oil-based formulations intended for mosquito control, particularly under scenarios where release into aquatic environments may occur. Overall, these findings support the use of *M. macrocopa* as a sensitive non-target model organism for ecological risk screening of essential oils and essential-oil-derived formulations, in line with recent meta-analytical evidence indicating high interspecific variability and sensitivity of aquatic invertebrates to essential oils and plant-derived mixtures.

Although *B. frutescens* essential oil exhibited measurable toxicity toward *M. macrocopa*, this finding does not necessarily preclude its practical application. *Moina macrocopa* is a small and sensitive freshwater invertebrate that is widely used as a conservative model for acute aquatic toxicity assessment. In contrast, many previous studies evaluating botanical larvicides have employed larger non-target organisms, such as fish and aquatic insects (Pavela 2015, Pavela and Benelli 2016). Therefore, the present findings should be interpreted as evidence that non-target effects are possible under laboratory exposure conditions and should be carefully considered during product development. Moreover, botanical larvicides are generally intended for localized mosquito breeding habitats, where appropriate application practices and optimized formulation strategies, such as controlled-release encapsulation and habitat-targeted application, may reduce environmental exposure and improve target specificity while minimizing adverse effects on non-target aquatic organisms.

## Conclusions

This study demonstrates that *B. frutescens* is a valuable renewable source of essential oil with larvicidal activity against *Aedes* and *Culex* mosquitoes. Chemical analyses revealed substantial chemical variation and distinct chemotypic groups among samples collected over time, particularly those enriched in oxygenated terpenes, as supported by multivariate analysis. Encapsulation of the essential oil in spent brewer's yeast cells was successfully achieved using a biodegradable, waste-derived carrier, demonstrating a green formulation approach. Laboratory bioassays confirmed larvicidal activity of both free and encapsulated essential oils; however, the study was limited to acute toxicity assessment, and no conclusions regarding prolonged or residual efficacy can be drawn. Overall, this work provides new chemical insights into *B. frutescens* essential oil and establishes a solid basis for future studies on formulation performance and practical application.

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## Author Contributions

Huy Hung Nguyen (Conceptualization [equal], Formal analysis [Equal], Investigation [equal], Methodology [equal], Resources [equal], Supervision [equal], Writing—original draft [equal]), Mau Dang Trinh (Investigation [equal]), Huy Gia Ngo (Investigation [equal]), Linh Thao Phan (Investigation [equal]), Prabodh Satyal (Investigation [equal], Methodology [equal]), Thuong Thanh Vo (Investigation [equal]), Hoa Van Vo (Investigation [equal]), Thong Quoc Nguyen (Investigation [equal]), Anh Quynh Tran-Nguyen (Investigation [equal]), Van Thi-Thuy Do (Investigation [equal]), William N. Setzer (Data curation [equal], Formal analysis [equal], Writing—review & editing [equal]), Phong Hai Nguyen (Investigation [equal]), and Minh Van Vo (Conceptualization [equal], Data curation [equal], Formal analysis [equal], Funding acquisition [equal], Methodology [equal], Project administration [equal], Software [equal], Validation [equal], Visualization [equal], Writing—original draft [equal])

## Supplementary Material

Supplementary material is available at *Journal of Medical Entomology* online.

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## Conflicts of Interest

The authors declare no conflict of interest.

## Data Availability

All data generated or analyzed during this study are included in this published article.

## Ethical Approval

This study was approved by the Duy Tan University Medical–Biological Research Ethics Council, Number DTU/REC2020/NHH02 and DTU/REC2024/NHH15.

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