

**CHEMICAL COMPOSITION OF *OCIMUM BASILICUM*, *SYZIGIUM AROMATICUM*  
AND *EUCALYPTUS GLOBULUS* ESSENTIAL OILS AND THEIR LARVICIDAL  
ACTIVITY ON TWO IMPORTANT MOSQUITOES (DIPTERA: CULICIDAE)**

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**ABSTRACT.** The extensive use of synthetic insecticides has led to insecticide resistance in mosquito vectors such as *Culex pipiens* L. and *Aedes aegypti* L. (Diptera: Culicidae). This study aimed to assess the chemical composition and larvicidal potential of essential oils (EOs) from *Ocimum basilicum*, *Syzygium aromaticum*, and *Eucalyptus globulus*. EOs were extracted via hydro-distillation and analyzed using GC-MS. Major compounds identified were estragole (45.03%) in basil, eugenol (78.70%) in clove, and spathulenol (29.29%) in eucalyptus. Larvicidal activity was evaluated against fourth instar larvae, revealing LC<sub>50</sub> values of 13.13–84.27 ppm for *C. pipiens* and 25.11–168.65 ppm for *A. aegypti*. Basil oil showed the highest efficacy. Enzyme assays on larvae exposed to LC<sub>50</sub> doses indicated increased activity of GSTs,  $\alpha$ -/ $\beta$ -esterases, ALP, and ACP, while AChE and cytochrome P450 activities decreased, suggesting oxidative stress and neurotoxic effects. These results highlight the potential of the tested EOs, particularly *O. basilicum*, as eco-friendly alternatives for mosquito control through both direct toxicity and biochemical disruption.

**KEY WORDS:** GC-MS analysis, Sweet basil, Clove, Tasmanian blue gum, Bioassay, Enzyme activity

## INTRODUCTION

Since ancient times, mosquitoes have caused human suffering, making them the most significant insect pest. *Anopheles*, *Culex*, and *Aedes* mosquitoes are vectors of infections that cause a variety of illnesses, including chikungunya, dengue fever, malaria, filariasis, and Japanese encephalitis [1, 2].

Beyond transmitting diseases, mosquito bites can also trigger allergic reactions in humans, ranging from localized skin irritation to more severe responses such as angioedema [3]. Synthetic pesticides have been widely used to control mosquito populations. These chemicals work by killing adult mosquito vectors, preventing them from biting humans, or by targeting larvae at their breeding sites to disrupt the life cycle [4, 5]. Since mosquitoes develop in water, insecticides find it easy to control them in this habitat, making the larval stage of the insect an appealing target [6, 7]. However, the need for alternative control strategies has grown due to the emergence of resistance to several kinds of synthetic insecticides, including pyrethroids, carbamates, organophosphates, and organochlorides [8]. It is now necessary to find alternate

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methods of controlling vector-borne diseases due to the toxicity of currently available chemical pesticides, their high operational costs, and the ensuing degradation of the environment. In place of synthetic pesticides, plant extracts with the ability to control mosquitoes can be used as repellents to ward against mosquito bites or as insecticides to destroy larvae or adults. Due to their insecticidal qualities, essential oils have drawn particular attention [9, 10]. More recently, these essential oils have been recognized as promising sources of bioactive compounds effective against mosquito larvae [11]. Isoprenoid compounds make up essential oils, mostly monoterpene transporters of scent in aromatic plants, such as different monoterpene components obtained from *Thuja orientalis* [12]. Essential oils' larvicidal and repellent properties have been the subject of a few investigations. Effective pest control measures include *cinnamon umzeylanicum*, *Lavandula angustifolia*, *Ocimum* spp, *Rubdosia melissoides*, *Cuminum cymium*, *Bunium persicum*, and *Piper* spp. [13, 14]. An additional investigation on the repellent properties of essential oils derived from *Ocimum*, *Cymbopogon*, and *Eucalyptus* species demonstrated these oils' potent ability to ward off malaria vectors and other mosquitoes [14].

Furthermore, almost 85% of larvae died after 24 hours of exposure in a study conducted in Nigeria to assess the efficacy of clove EO against *A. aegypti* and *C. quinquefasciatus* [15]. In the meantime, essential oils of clove and lemongrass proved highly successful in killing all larval stages of *Anopheles dries* and *Aedes aegypti* in Thailand. From the Lamiaceae family, basil (*Ocimum basilicum*) is extensively cultivated globally and serves various purposes across continents, including as an insect repellent and as a seasoning for food [16]. The Myrtaceae family of *Eucalyptus* has multiple applications. The compounds derived from these plants are made up of a diverse range of molecules, including sesquiterpenes, monoterpenes, esters, aldehydes, and ketones. Essential oils derived from various *Eucalyptus* species are well known for their antioxidant and antibacterial properties. Among them, the essential oil of *Eucalyptus globulus* has shown potential as a bio-insecticide, owing to its ability to inhibit microbial growth and exert antibiofilm effects during in situ vapor phase exposure [17]. The clove *Syzygium aromaticum* (Family: Myrtaceae) is extensively utilized for its medicinal properties [15]. Because clove buds contain a high concentration of phytochemicals such as monoterpenes, sesquiterpenes, hydrocarbons, and phenolic compounds, their essential oil is well-known for having anti-fungal, antioxidant, antiprotozoal, and antibacterial properties. According to recent findings, clove essential oil exhibits larvicidal activity against *A. aegypti* larvae, with LC<sub>50</sub> values of 92.56 and 62.23 ppm [18].

Additionally, alterations in the levels of defensive enzymes *e.g.*, glutathione-S-transferases (GSTs), acid and alkaline phosphatases (ACP and ALP),  $\alpha$ - and  $\beta$ -esterases ( $\alpha$ -/ $\beta$ -ESTs), and acetylcholinesterase (AChE), and cytochrome P450 are linked to the metabolic detoxification of pesticides. These enzymes assist organisms in converting and getting rid of both endogenous and exogenous substances [18, 19]. Therefore, the main objective of this research was to identify the active ingredients and assess the larvicidal activities of basil, clove, and eucalyptus EOs against *C. pipiens* and *A. aegypti* in a laboratory setting. The secondary aim was to evaluate the impact of EOs of *O. basilicum*, *S. aromaticum*, and *E. globulus* on the biochemical attributes of *C. pipiens* and *A. aegypti* larvae. This was achieved by quantifying alterations in crucial enzymes involved in detoxifying xenobiotic substances. These enzymes include GSTs, AChE,  $\alpha$ -/ $\beta$ -ESTs, ACP, ALP, and cytochrome P450. The findings of this research contribute to a fundamental understanding of plant-based alternatives for vector control and provide valuable insights into the potential application and optimization of such products. This approach presents a promising alternative to synthetic pesticides, particularly in areas with high mosquito populations, while minimizing adverse environmental impacts.

## EXPERIMENTAL

### *Botanical materials and the extraction of EOs*

The leaves of *Ocimum basilicum* (Lamiaceae), *Eucalyptus globulus* (Myrtaceae), and buds of *Syzygium aromaticum* were collected from the Taif region of Saudi Arabia in April 2022, according to permission from the administration of the Department of Biology, Faculty of Science, Taif University. Then, all specimens were transferred to a laboratory and air-dried. Following the British Pharmacopoeia's (1988) recommendation, 100 grams of dried plant buds and leaves were finely chopped and hydro-distilled by a Clevenger apparatus for three hours [20]. After dehydrating over anhydrous sodium sulfate to remove any remaining moisture, the essential oils were sealed in vials and kept at 4 °C until analysis. The dried weight of the plant sample was used to calculate the essential oil yield.

### *GC–MS analysis of essential oils*

A gas chromatography-mass spectrometry apparatus at the Medicinal and Aromatic Plants Research Department, National Research Centre of Egypt, was used to identify components of different essential oils with the following specifications to perform the GC–MS analysis. The instrument used is a THERMO mass spectrometer detector connected to a TRACE GC Ultra Gas Chromatograph (THERMO Scientific Corp., Waltham, MA, USA). A Waltman, Massachusetts, USA, TG-WAX MS column (30 m ± 0.25 mm i.d., 0.25 µm film thickness) was fitted to the GC–MS system. Helium served as the carrier gas during the analyses, operating with a temperature program and a flow rate of 1.0 mL/min, alongside a split ratio of 1:10 as follows: After holding at 240 °C for one minute, the temperature rose 3.0 °C per minute to 60 °C. Both the detector and injector were maintained at 240 °C. A 1 µL diluted mixture sample (1:50 hexane, v/v) was injected. The mass spectrum was generated by employing electron ionization (EI) at 70 electron volts (eV) across a spectral range spanning from m/z 40 to 450. The analytical techniques of mass spectra (genuine chemicals, NSIT, and Wiley spectral library collection libraries) were used to identify compounds.

### *Mosquito rearing*

*C. pipiens* eggs were maintained at the Research Institute of Medical Entomology, Doki, Giza, Egypt. While *A. aegypti* were collected from the artificial freshwater lake located at the Faculty of Agriculture, Cairo University, identification of this strain depended on female morphological characteristics and keys of standard taxonomy according to Rueda [21]. Insects were reared in an insectary at the Economic Entomology and Pesticides Department, Faculty of Agriculture, Cairo University, Egypt. Eggs were placed on white plates containing dechlorinated tap water and maintained under insectary conditions until they developed into fourth instar larvae, which were then collected for subsequent bioassay experiments [22]. The insects were raised in an insectary with carefully regulated temperature ranges of 25–27 °C, 70%–80% relative humidity, and 12 L/12D photoperiod. Dog biscuits that had been finely powdered were fed to the larvae twice daily. Adults emerged from the rearing cages (30 × 30 × 30 cm) when the pupae were moved from the trays to cups filled with dechlorinated tap water [23]. While females were blood-fed to produce eggs, adults raised in the same cages were given constant access to 10% sucrose, including 10% multivitamin syrup. Early-stage *A. aegypti* and *C. pipiens* larvae were available for biochemical tests.

*Mosquito larvicidal assay*

Fourth instar larvae from *C. pipiens* and *A. aegypti* were used to examine the bioactivities of three essential oils: basil, clove, and eucalyptus. Bioassay testing was conducted following the larvicidal test technique guidelines and the WHO standardized procedures [24]. In the manner described below, 25 4<sup>th</sup> larval instar was placed in 500 mL beakers containing water and essential oils of various samples dissolved in dimethyl sulfoxide (DMSO) as 1% stock solution of EOs with five concentrations (100, 75, 50, 25, and 5 ppm). A set of DMSO-based controls was also conducted to compare with every experiment. Following 24 hours and 48 hours of treatment, mortality was recorded. The insect-rearing conditions used before were carried out in each experiment. Four duplicates of each test were performed. When larvae did not rise to the top of the solution or did not react to stimulation, they were deemed dead. The test was considered invalid if any larvae in their fourth instar pupated during the exposure time. Similarly, if over 10% of the larvae in the control group perished, the entire experiment was rerun. Abbott's formula was employed to determine the mortality of the treatment groups if the mortality of the control larvae was less than 10%.

$$\text{Mortality (\%)} = (C - T)/C \times 100$$

C represents the surviving larvae in the control group, while T denotes the surviving larvae in the treated group. Then, mortality data were subjected to Probit analysis using SPSS software [24].

*Chemicals and reagents*

All utilized reagents and chemicals were obtained from local suppliers in Cairo, Egypt, such as Albumin standard and Bio Red protein reagents employed to quantify total protein. Biochemical assays for cytochrome P450,  $\alpha$ -/ $\beta$ -ESTs, and ACP/ALP utilized bovine heart cytochrome c ( $\geq 95\%$  purity),  $\alpha$ -naphthol,  $\beta$ -naphthol, p-nitrophenol, and fast blue B salt dye, respectively. The enzyme analysis substrates included 3,3', 5,5'-tetramethylbenzidine dihydrochloride hydrate powder,  $\alpha$ -naphthyl acetate -,  $\beta$ -naphthyl acetate (98%), 1-chloro-2, 4-dinitrobenzene, L-glutathione reduced, 5,5'-Dithiobis (2-nitrobenzoic acid), acetylthiocholine iodide, and p-nitrophenyl phosphate. The above substrates were dissolved in a solvent: absolute methanol, ethanol, and phosphate buffer. These were used in the biochemical assays of GSTs and AchE, respectively:

*Preparation of whole-body homogenates to be utilized in enzyme determinations*

Four batches of twenty-five freshly hatched 4<sup>th</sup> larval instar of *A. aegypti* and *C. pipiens* were exposed for twenty-four hours to *O. basilicum*, *S. aromaticum*, and *E. globulus* essential oils at the median lethal concentration (LC<sub>50</sub>) of 25.11, 82.83, and 168.65 ppm for *A. aegypti* and 13.13, 37.07, and 84.27 ppm for *C. pipiens*, respectively, as previously estimated in a larvicidal assay. The larvae in a 1% DMSO-distilled water solution comprised the control group. Throughout the trial, the larvae received no nourishment. Bioassays were conducted at  $25 \pm 2$  °C and relative humidity of  $80 \pm 10\%$ , with five replicates. Following a 24-hour exposure to test solutions containing various essential oils, the larvae from both the treated and untreated groups were cleaned using deionized water. After removing all adherent water with tissue paper, the larvae were preserved in 2 mL tubes at -80 °C until required for the enzyme assay. Individual larvae (2–10 per group) from each mosquito species' treated and untreated groups were homogenized separately in the suitable buffer required for each enzyme assay to create whole-body homogenates. Following centrifugation of the whole-body homogenates for 20 min at  $10,000 \times g$

at 4°C, the resulting clear supernatants were promptly utilized to assess the enzyme activity. All enzyme bioassays were conducted in conjunction with negative controls in this investigation.

#### *Determination of total protein*

Larval homogenate total protein was determined by the method outlined in the reference [25] with some minor modifications. The standard curve of bovine serum albumin (0 - 0.5 mg/mL) was used to calculate the protein concentrations in mg/mL.

#### *Determination of GST activity*

GST activity was estimated following a modified procedure based on the method described in the reference [26]. The GST activity was quantified and expressed as  $\mu\text{mol}$  of CDNB conjugated per minute per milligram of protein.

#### *Determination of $\alpha$ -/ $\beta$ -ESTs Activity*

A standard curve of  $\alpha$ - or  $\beta$ -naphthyl acetate was utilized to determine the activities of  $\alpha$ -/ $\beta$ -ESTs [27], with the results expressed as nmol of  $\alpha$ - or  $\beta$ -naphthol released per minute per milligram of protein.

#### *Determination of AChE Activity*

The modified method of [28] was utilized to evaluate the activity of AChE as nmol of the acetylthiocholine hydrolyzed/min/mg protein.

#### *Determination of ACP and ALP activities*

ACP and ALP levels were measured using the method of [28] as nmol of p-nitrophenol released/min/mg protein.

#### *Determination of cytochrome P450 activity*

The Heme-peroxidase method [29] was used to measure cytochrome P450 activity as nanograms of cytochrome c equivalent unit/mg protein.

#### *Statistical analysis*

LC<sub>50</sub> values were determined through Probit analysis. Subsequently, significant differences between each pair of LC<sub>50</sub> values were assessed using relative median potency (RMP) confidence intervals. If the value of 1.0 fell within the 95% confidence interval of RMP, differences between pairs of LC<sub>50</sub> values were considered non-statistically significant. Statistical analyses were conducted using SPSS software, version 20.

## RESULTS AND DISCUSSION

#### *Yields and chemical constituents of the tested EOs*

The resulting yields of EOs were  $1.3 \pm 0.06$ ,  $7.93 \pm 0.14$ , and  $3.27 \pm 0.09$  (w/w) for *O. basilicum*, *S. aromaticum*, and *E. globulus*, respectively. The chemical constituents of the studied volatile oils are indicated in Table 1 and Figure 1, where 27, 7, and 36 compounds were identified in the EOs of *O. basilicum*, *S. aromaticum*, and *E. globulus*, respectively. The obtained results indicated

that the major constituents of *O. basilicum* EO were Estragole (45.03%), Linalool (12.96%), Eucalyptol (11.25%), and Methyl eugenol (5.08%). In *S. aromaticum*, Eugenol (78.70%),  $\beta$ -caryophyllene (12.80%), and Eugenyl acetate (4.82%) were the major components. In comparison, Spathulenol (29.29%), *p*-cymene (10.30%),  $\psi$ -limonene (8.03%), and  $\gamma$ -element (6.63%) were the major components in *E. globulus* EOs.

Table 1. Chemical constituents of the tested EOs of *O. basilicum*, *E. globulus*, and *S. aromaticum*.

| N  | RT    | Compound                                        | Area (%)            |                    |                      |
|----|-------|-------------------------------------------------|---------------------|--------------------|----------------------|
|    |       |                                                 | <i>O. basilicum</i> | <i>E. globulus</i> | <i>S. aromaticum</i> |
| 1  | 4.46  | trans-Thujene                                   |                     | 1.42               |                      |
| 2  | 4.61  | $\alpha$ -pinene                                | 1.92                | 0.61               |                      |
| 3  | 5.32  | (+) Sabinene                                    | 0.51                | 0.69               |                      |
| 4  | 5.73  | Beta-Myrcene                                    | 0.65                | 0.43               |                      |
| 5  | 5.98  | $\alpha$ -Phellandrene                          |                     | 2.49               |                      |
| 6  | 6.36  | P-Cymene                                        |                     | 10.30              |                      |
| 7  | 6.53  | $\gamma$ -Limonene                              |                     | 8.03               |                      |
| 8  | 6.53  | Eucalyptol                                      | 11.25               |                    |                      |
| 9  | 7.03  | $\beta$ -Ocimene                                | 0.65                |                    |                      |
| 10 | 7.26  | $\gamma$ -Terpinene                             | 0.33                | 0.63               |                      |
| 11 | 8.26  | Linalool                                        | 12.96               |                    |                      |
| 12 | 8.70  | Trans-P-menth-2-en-1-ol                         |                     | 0.48               |                      |
| 13 | 8.96  | camphor                                         | 0.52                |                    |                      |
| 14 | 9.13  | Terpinenol                                      |                     | 0.43               |                      |
| 16 | 9.92  | Cryptone                                        |                     | 1.78               |                      |
| 17 | 10.12 | Terpinene-4-ol                                  | 1.11                | 2.10               |                      |
| 18 | 10.61 | Estragole                                       | 45.03               |                    |                      |
| 19 | 11.43 | Benzaldehyde, P-iso propyl                      |                     | 0.50               |                      |
| 20 | 12.35 | Phellandral                                     |                     | 0.38               |                      |
| 21 | 12.90 | Bornyl acetate                                  | 0.09                |                    |                      |
| 22 | 14.55 | Eugenol                                         | 2.45                | 3.11               | 78.70                |
| 23 | 15.06 | Cinnamic acid methyl ester                      | 3.34                |                    |                      |
| 24 | 15.56 | $\alpha$ -Copaene                               |                     |                    | 0.29                 |
| 25 | 15.66 | methyl eugenol                                  | 5.08                |                    |                      |
| 26 | 16.52 | $\beta$ -Caryophyllene                          | 1.62                | 0.70               | 12.80                |
| 27 | 17.02 | Cis -Bergamotene                                | 2.88                |                    |                      |
| 28 | 17.32 | $\alpha$ -Humulene                              | 0.96                |                    | 1.89                 |
| 29 | 17.37 | Naphthalene                                     |                     | 0.46               |                      |
| 30 | 17.49 | Allo-Aromadendrene                              |                     | 1.26               |                      |
| 31 | 17.51 | Beta-Cubebene                                   | 0.52                |                    |                      |
| 32 | 17.95 | Germacrene D                                    | 1.46                |                    |                      |
| 33 | 17.96 | Carbamic acid Bis (1-methyl ethyl)- Ethyl ester |                     | 0.59               |                      |
| 34 | 18.32 | Gamma - Elemene                                 | 0.40                | 6.63               |                      |
| 35 | 18.46 | Eugenyl acetate                                 |                     | 0.22               | 4.82                 |
| 36 | 18.51 | Beta-Elemene                                    | 0.68                | 0.28               |                      |
| 37 | 18.57 | $\beta$ -Vetispiene                             |                     | 0.54               |                      |
| 38 | 18.72 | delta-Cadinene                                  | 1.44                |                    | 0.44                 |
| 39 | 18.97 | $\beta$ -Cadinene                               |                     | 0.30               |                      |
| 40 | 19.48 | $\alpha$ -bisabolene                            | 0.95                |                    |                      |
| 41 | 19.82 | $\gamma$ -Eudesmol                              |                     | 0.55               |                      |
| 42 | 20.11 | Caryophyllene oxide                             |                     |                    | 0.34                 |

|                  |       |                                                  |      |       |       |
|------------------|-------|--------------------------------------------------|------|-------|-------|
| 43               | 20.19 | (-)-Spathulenol                                  |      | 29.29 |       |
| 44               | 20.33 | Epiglobulol                                      | 0.39 | 1.12  |       |
| 45               | 20.47 | Viridiflorol                                     |      | 1.88  |       |
| 46               | 20.70 | Ledol                                            |      | 1.05  |       |
| 47               | 21.42 | Iso- Spathulenol                                 |      | 4.54  |       |
| 48               | 21.47 | Tau-Cadinol                                      | 2.51 |       |       |
| 49               | 21.52 | $\alpha$ -Vetivol                                |      | 1.48  |       |
| 50               | 21.78 | $\alpha$ -Cadinol                                |      | 0.31  |       |
| 51               | 21.91 | Germacre 4(15),5,10<br>(14)trien-1- $\alpha$ -oH |      | 1.69  |       |
| 52               | 22.07 | Ledone oxide(11)                                 |      | 0.65  |       |
| 53               | 23.38 | Ylangenal                                        |      | 6.63  |       |
| 54               | 23.84 | Longipinocarvone                                 |      | 3.04  |       |
| Total compounds  |       |                                                  | 26   | 36    | 7     |
| Total amount (%) |       |                                                  | 99.7 | 96.59 | 99.28 |

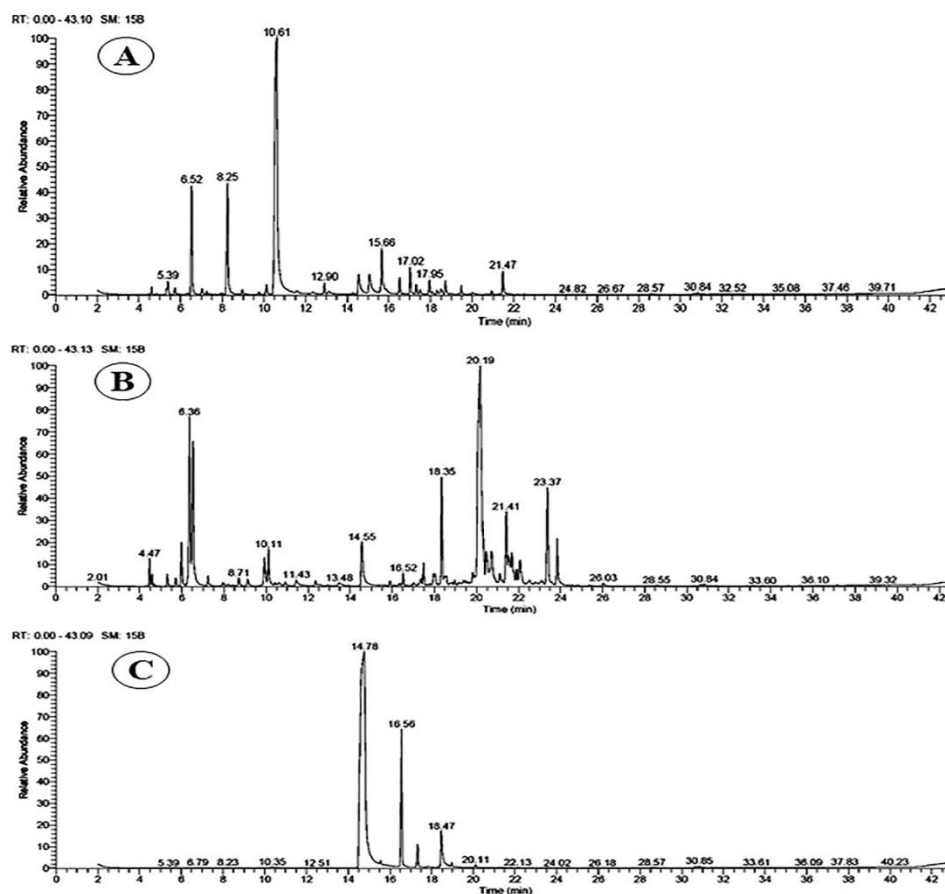


Figure 1. GC-MS analysis of tested EOs of *O. basilicum* (A), *E. globulus* (B), and *S. aromaticum* (C).

*Larvicidal activity of the tested EOs*

The presented data in Figures 2 and 3 indicated the mortality ratios caused by *O. basilicum*, *S. aromaticum*, and *E. globulus* essential oils against the fourth larval instar of *C. pipiens* and *A. aegypti*. The mortality was scored at 24 and 48 hrs. after treatment. Generally, each tested EO had a higher mortality rate on *A. aegypti* than on *C. pipiens*. With probit LC<sub>50</sub> values of the three tested EOs on *C. pipiens*, they indicated that the LC<sub>50</sub> values for *O. basilicum* (13.13 ppm), *S. aromaticum* (37.07 ppm), and *E. globulus* (84.27 ppm) (Table 2). The same results were obtained for *A. aegypti* larvae; the LC<sub>50</sub> values were 25.11, 82.83, and 168.65 ppm for *O. basilicum*, *S. aromaticum*, and *E. globulus*, respectively (Table 2). The comparison between the LC<sub>50</sub> values of each pair of EOs on *C. pipiens* and *A. aegypti* by RMP analyses (Table 3) indicated that all comparisons among each pair of treatments were significantly different, where all these 95% CIs of RMPs did not include 1.0.

Table 2. LC<sub>50</sub> values (ppm) for the tested three plant EOs against larvae of *Culex pipiens* and *Aedes aegypti*.

| Essential oils             | Mosquitos' species   | LC <sub>50</sub><br>(Confidence Interval limits) | Intercept ± SE | Slope ± SE    | χ <sup>2</sup> | P      |
|----------------------------|----------------------|--------------------------------------------------|----------------|---------------|----------------|--------|
| <i>Ocimum basilicum</i>    | <i>Culex pipiens</i> | 13.13 (8.52–17.91)                               | −1.295 ± 0.091 | 1.159 ± 0.059 | 141.23         | <0.001 |
|                            | <i>Aedes aegypti</i> | 25.11 (19.56–31.27)                              | −1.679 ± 0.096 | 1.199 ± 0.060 | 98.16          | <0.001 |
| <i>Syzygium aromaticum</i> | <i>Culex pipiens</i> | 37.07 (29.49–46.65)                              | −1.664 ± 0.097 | 1.061 ± 0.060 | 83.42          | <0.001 |
|                            | <i>Aedes aegypti</i> | 82.83 (65.98–105.29)                             | −1.402 ± 0.098 | 0.673 ± 0.060 | 39.23          | 0.019  |
| <i>Eucalyptus globulus</i> | <i>Culex pipiens</i> | 84.27 (68.46–109.70)                             | −1.963 ± 0.108 | 1.019 ± 0.065 | 49.91          | <0.001 |
|                            | <i>Aedes aegypti</i> | 168.65 (130.56–222.95)                           | −2.121 ± 0.119 | 0.930 ± 0.071 | 43.96          | 0.005  |

Table 3. Relative susceptibilities of *C. pipiens* and *A. aegypti* larvae to the three tested EOs.

| Plant EOs x Insect                       | <i>O. basilicum</i><br>x<br><i>C. pipiens</i> | <i>O. basilicum</i><br>x<br><i>A. aegypti</i> | <i>S. aromaticum</i><br>x<br><i>C. pipiens</i> | <i>S. aromaticum</i><br>x<br><i>A. aegypti</i> | <i>E. globulus</i><br>x<br><i>C. pipiens</i> | <i>E. globulus</i> x<br><i>A. aegypti</i> |
|------------------------------------------|-----------------------------------------------|-----------------------------------------------|------------------------------------------------|------------------------------------------------|----------------------------------------------|-------------------------------------------|
| <i>O. basilicum</i> x <i>C. pipiens</i>  | -                                             |                                               |                                                |                                                |                                              |                                           |
| <i>O. basilicum</i> x <i>A. aegypti</i>  | 0.492                                         |                                               |                                                |                                                |                                              |                                           |
| <i>S. aromaticum</i> x <i>C. pipiens</i> | 0.315                                         | -                                             |                                                |                                                |                                              |                                           |
| <i>S. aromaticum</i> x <i>A. aegypti</i> | -                                             | 0.286                                         | 0.448                                          |                                                |                                              |                                           |
| <i>E. globulus</i> x <i>C. pipiens</i>   | 0.138                                         | -                                             | 0.439                                          | -                                              |                                              |                                           |
| <i>E. globulus</i> x <i>A. aegypti</i>   | -                                             | 0.141                                         | -                                              | 0.491                                          | 0.501                                        | -                                         |

RMP values indicate the comparison of EO in the column versus EO in the row; All values are <1 and indicate more susceptibility and a significant difference (95% CI ≠ 1).

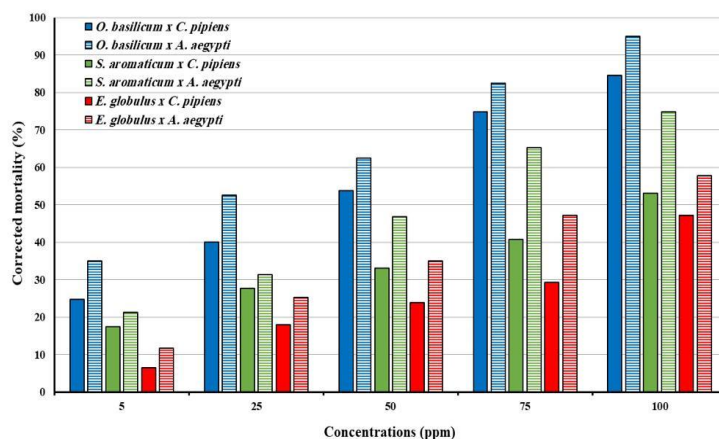


Figure 2. Corrected mortality after 24 hrs. (%) of treatments of the fourth larval instar of *C. pipiens* and *A. aegypti* treated with three essential oils.

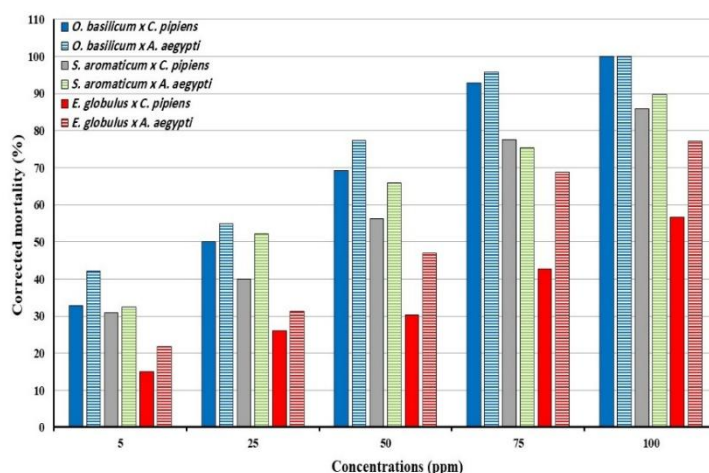


Figure 3. Corrected mortality after 48 hrs. (%) of treatments of the fourth larval instar of *C. pipiens* and *A. aegypti* treated with three essential oils.

#### Effects of EOs on biochemical features of *C. pipiens* and *A. aegypti* larvae.

After 24 hrs of treatments with the LC<sub>50</sub> of all three tested essential oils, it was noticed that cytochrome P450 and AChE significantly decreased in all treatments with all tested EOs compared to those of the control. In these two enzyme analyses, a higher decrease was obtained with EO of *O. basilicum* in both *C. pipiens* and *A. aegypti*, followed by *S. aromaticum* and *E. globulus*. Contrary to what was previously mentioned, in both enzymes, cytochrome P450 and AChE, all five other tested enzymes, i.e., GSTs, ALP,  $\alpha$ - $\beta$ -ESTs, and ACP, significantly increased in all treatments with all tested EOs than in the control. In all these six enzymes, a higher increase was obtained with EO of *O. basilicum* in both *C. pipiens* and *A. aegypti*, followed by *S. aromaticum* and *E. globulus*. These findings showed that the EO of *O. basilicum* had a higher effect on both insects for all analyzed enzymes, whether increased (GSTs,  $\alpha$ - $\beta$ -ESTs, ALP, and

ACP) or decreased (cytochrome P450 and AChE) (Table 4). These results also match the findings of higher mortality rates of this EO on both tested insects.

Table 4. Effects of essential oils on biochemical features of *Culex pipiens* and *Aedes aegypti* larvae.

| Enzyme          |               | Control                                                                                                          |                                 | <i>O. basilicum</i>             |                                 | <i>S. aromaticum</i>            |                                 | <i>E. globulus</i>              |                                 |
|-----------------|---------------|------------------------------------------------------------------------------------------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                 |               | <i>C. pipiens</i>                                                                                                | <i>A. aegypti</i>               | <i>C. pipiens</i>               | <i>A. aegypti</i>               | <i>C. pipiens</i>               | <i>A. aegypti</i>               | <i>C. pipiens</i>               | <i>A. aegypti</i>               |
| cytochrome P450 | Mean $\pm$ SE | 0.068 $\pm$ 0.00 <sup>A</sup>                                                                                    | 0.138 $\pm$ 0.0003 <sup>A</sup> | 0.050 $\pm$ 0.0007 <sup>D</sup> | 0.098 $\pm$ 0.0003 <sup>D</sup> | 0.055 $\pm$ 0.0003 <sup>C</sup> | 0.108 $\pm$ 0.0003 <sup>C</sup> | 0.057 $\pm$ 0.0003 <sup>B</sup> | 0.113 $\pm$ 0.0003 <sup>B</sup> |
|                 | One-way ANOVA | <i>C. pipiens</i> ; $F_{3,8} = 681.2$ , $P = < 0.001$ ; <i>A. aegypti</i> ; $F_{3,8} = 2582.3$ , $P = < 0.001$   |                                 |                                 |                                 |                                 |                                 |                                 |                                 |
| GST             | Mean $\pm$ SE | 0.102 $\pm$ 0.001 <sup>D</sup>                                                                                   | 0.204 $\pm$ 0.002 <sup>C</sup>  | 0.202 $\pm$ 0.001 <sup>A</sup>  | 0.398 $\pm$ 0.002 <sup>A</sup>  | 0.187 $\pm$ 0.001 <sup>B</sup>  | 0.366 $\pm$ 0.003 <sup>B</sup>  | 0.177 $\pm$ 0.001 <sup>C</sup>  | 0.344 $\pm$ 0.013 <sup>B</sup>  |
|                 | One-way ANOVA | <i>C. pipiens</i> ; $F_{3,8} = 1667.0$ , $P = < 0.001$ ; <i>A. aegypti</i> ; $F_{3,8} = 197.8$ , $P = < 0.001$   |                                 |                                 |                                 |                                 |                                 |                                 |                                 |
| $\alpha$ -ESTs  | Mean $\pm$ SE | 92.23 $\pm$ 0.46 <sup>D</sup>                                                                                    | 153.8 $\pm$ 0.53 <sup>D</sup>   | 155.37 $\pm$ 1.12 <sup>A</sup>  | 216.8 $\pm$ 0.92 <sup>A</sup>   | 135.77 $\pm$ 0.26 <sup>B</sup>  | 204.6 $\pm$ 0.41 <sup>B</sup>   | 124.0 $\pm$ 0.35 <sup>C</sup>   | 192.6 $\pm$ 0.67 <sup>C</sup>   |
|                 | One-way ANOVA | <i>C. pipiens</i> ; $F_{3,8} = 1689.5$ , $P = < 0.001$ ; <i>A. aegypti</i> ; $F_{3,8} = 1711.1$ , $P = < 0.001$  |                                 |                                 |                                 |                                 |                                 |                                 |                                 |
| $\beta$ -ESTs   | Mean $\pm$ SE | 89.37 $\pm$ 0.50 <sup>D</sup>                                                                                    | 146.8 $\pm$ 0.36 <sup>D</sup>   | 140.9 $\pm$ 0.36 <sup>A</sup>   | 200.7 $\pm$ 0.23 <sup>A</sup>   | 130.97 $\pm$ 0.38 <sup>B</sup>  | 189.3 $\pm$ 0.56 <sup>B</sup>   | 120.37 $\pm$ 0.72 <sup>C</sup>  | 177.1 $\pm$ 0.50 <sup>C</sup>   |
|                 | One-way ANOVA | <i>C. pipiens</i> ; $F_{3,8} = 1904.4$ , $P = < 0.001$ ; <i>A. aegypti</i> ; $F_{3,8} = 2870.5$ , $P = < 0.001$  |                                 |                                 |                                 |                                 |                                 |                                 |                                 |
| AChE            | Mean $\pm$ SE | 13.93 $\pm$ 0.023 <sup>A</sup>                                                                                   | 16.47 $\pm$ 0.221 <sup>A</sup>  | 8.06 $\pm$ 0.009 <sup>D</sup>   | 11.75 $\pm$ 0.034 <sup>D</sup>  | 9.65 $\pm$ 0.031 <sup>C</sup>   | 12.77 $\pm$ 0.024 <sup>C</sup>  | 10.34 $\pm$ 0.015 <sup>B</sup>  | 14.47 $\pm$ 0.06 <sup>B</sup>   |
|                 | One-way ANOVA | <i>C. pipiens</i> ; $F_{3,8} = 14036.7$ , $P = < 0.001$ ; <i>A. aegypti</i> ; $F_{3,8} = 402.2$ , $P = < 0.001$  |                                 |                                 |                                 |                                 |                                 |                                 |                                 |
| ACP             | Mean $\pm$ SE | 117.9 $\pm$ 0.33 <sup>D</sup>                                                                                    | 134.6 $\pm$ 0.15 <sup>D</sup>   | 157.1 $\pm$ 0.09 <sup>A</sup>   | 173.7 $\pm$ 0.21 <sup>A</sup>   | 148.6 $\pm$ 0.53 <sup>B</sup>   | 165.2 $\pm$ 0.36 <sup>B</sup>   | 140.9 $\pm$ 0.08 <sup>C</sup>   | 160.6 $\pm$ 0.28 <sup>C</sup>   |
|                 | One-way ANOVA | <i>C. pipiens</i> ; $F_{3,8} = 2797.7$ , $P = < 0.001$ ; <i>A. aegypti</i> ; $F_{3,8} = 4177.5$ , $P = < 0.001$  |                                 |                                 |                                 |                                 |                                 |                                 |                                 |
| ALP             | Mean $\pm$ SE | 6.52 $\pm$ 0.02 <sup>D</sup>                                                                                     | 9.57 $\pm$ 0.03 <sup>D</sup>    | 12.97 $\pm$ 0.04 <sup>A</sup>   | 15.98 $\pm$ 0.05 <sup>A</sup>   | 12.23 $\pm$ 0.01 <sup>B</sup>   | 14.75 $\pm$ 0.02 <sup>B</sup>   | 10.61 $\pm$ 0.03 <sup>C</sup>   | 14.06 $\pm$ 0.03 <sup>C</sup>   |
|                 | One-way ANOVA | <i>C. pipiens</i> ; $F_{3,8} = 14080.5$ , $P = < 0.001$ ; <i>A. aegypti</i> ; $F_{3,8} = 7607.1$ , $P = < 0.001$ |                                 |                                 |                                 |                                 |                                 |                                 |                                 |

Means bearing different capital letters in the same row for the same insect are significantly different with  $P < 0.001$ .

The essential oil yields for the three studied plants were 1.3, 3.27, and 7.93 (w/w). In this regard, there was a variation in the central and minor essential oil components for the three studied plant species. Generally, many factors, including time of harvesting, extraction method, maturity stage, place of cultivation, and environmental conditions (temperature, photoperiod, altitude, and hygrometry), and especially altitude, affected both EO yields and chemical components, where altitudes, whether low or high, are considered indirect factors affecting plant metabolism [30]. Our findings indicated that *O. basilicum* EO constitutes the main constituent of estragole (45.03%). Other significant compounds are linalool (12.96%) and eucalyptol (11.25%). Previous reports show that basil leaves exhibited an EO extraction yield of 1.56% and essential oils are

characterized by higher amounts of linalyl acetate and linalool [3]. Our results indicate that *E. globulus* essential oil contains spathulenol (29.29%), p-cymene (10.30%), and  $\psi$ -limonene (8.03%) as significant compounds. Previous studies show that the extraction of *E. globulus* EO yielded 2.64% (w/w). For this EO, 75 compounds were identified, where p-cymene (20.24%), spathulenol (14.10%), and eucalyptol (11.30%) were the major compounds. Moreover, phellandrene,  $\beta$ -thujene, cryptone, elixene, cuminal and phellandral (5.10-2.54%) were the notable compounds identified in this EO [31]. Other investigations indicated that *E. globulus* EO is characterized by higher amounts of L-globulol, aromadendrene, and spathulenol [32].

The obtained results showed that clove buds exhibited an EO extraction yield of 7.93%, and eugenol, eugenol acetate, and  $\beta$ -caryophyllene were the major compounds. This finding agrees with the investigation [33], where clove EO had a higher eugenol, acetate, and  $\beta$ -caryophyllene content.

The larvicidal activity of EOs from different medicinal plants has been ultimately confirmed through their significant components [34], such as terpene compounds that exert toxic effects against insects, especially mosquitoes, by interfering with the metabolic, digestive, respiratory, and behavioral psychological systems of insects [6]. Also, the effect of the EO varied depending on the concentration and plant species, where the mortality increased with EO concentration and exposure time for all EOs on larvae of *C. pipiens* and *A. aegypti* [35]. In the current investigation, the mortality (%) achieved from the EOs increased with the concentration, where the highest mortality was achieved with the highest concentration (100 ppm) of *O. basilicum* (95% and 84.6%), *S. aromaticum* (74.9% and 53%), and *E. globulus* (57.5% and 47.2%) for *A. aegypti* and *C. pipiens*, respectively, after 24 hrs of exposure to different essential oils. These findings follow previous investigations [36] where the effect of EO compounds, linalool (from *O. basilicum*), eugenol (from *S. aromaticum*), and p-cymene (from *E. globulus*) on the larvae of *C. pipiens* and *A. aegypti* was auspicious, as bioinsecticides, especially at higher concentrations. The plant EOs are also eco-friendlier, efficient, and effective in controlling insect pests. Thus, they have been considered an essential natural resource for synthetic pesticides against vector mosquitoes [6]. EO of *O. basilicum* had higher larvicidal activity against larvae of both tested mosquitoes. This may be due to the high content of linalool, eucalyptol, and estragole, which are responsible for larvicidal activity and toxicity of the oil. Additionally, minor compounds such as  $\alpha$ -pinene, germacrene D, and caryophyllene may interfere with oil components and synergize, thus increasing the toxic effect of the oil. Our finding agrees with another study [10], which found that  $\beta$ -linalool in cinnamon EO is strongly related to the toxicity in wild-collected larvae caused by this essential oil. Our findings revealed that the mortality (%) of EO of *S. aromaticum* was found to be higher than that caused by EO of *E. globulus*, which might be associated with the presence of high content of eugenol and  $\beta$ -caryophyllene that has larvicidal activity. This finding agrees with the previous investigation [37], where larvicidal activities of essential oil from *E. valerianifolius* could be associated with the high content of  $\alpha$ -pinene, myrcene, and  $\beta$ -caryophyllene. However, the synergistic effects between EO components may increase the toxic effect. In this respect, an investigation [38] showed that eugenol had high effects for a longer time in successive generations without initiating rapid resistance. Therefore, it could be advocated for managing *C. pipiens* and *A. aegypti*. Also, Limonene,  $\alpha$ -pinene, and caryophyllene oxide were the significant components in EO of *E. hieraciifolius* [39]. Both limonene and  $\alpha$ -pinene have shown suitable larvicidal activities compared with caryophyllene oxide against *A. albopictus* and *A. aegypti*. *Eucalyptus* Sp. EO indicated a weak effect on mortality (%) due to the occurrence of high content from spathulenol (29.29%) and isospathulenol (4.54%) tricyclic sesquiterpene alcohol which had the lowest mortality compared with monoterpenes (limonene and p-cymene) which responsible about the toxicity of the oil where terpene compounds have toxic effects through various ways including the cholinergic system, mitochondrial system. It affects the respiratory system by blocking the larvae' spiracle to stopping their ability to breathe. It also can serve as a regulator for the insect growth through disturbance the endocrinological balance [39].

Additionally, the minor compounds might negatively interfere with compounds and cause negative synergistic effects, which may be essential to decrease the toxic effect of the EO [2]. EO of *Eucalyptus* sp. showed larvicidal activity with LD<sub>90</sub> and LD<sub>50</sub> values of 330.01 ppm and 80.93 ppm, respectively [40].

Detoxification enzymes in insects are mostly shown as the enzymatic protection against unfamiliar compounds and assume significant roles in maintaining their typical physiological function. The detoxifying enzymes respond to insect poisons or mixtures showing insecticidal activity. They incorporate general esterases, glutathione-S-transferase, and phosphatases [41]. Biochemical profiles of *C. pipiens* and *A. aegypti* larvae before treatments with LC<sub>50</sub> from *O. basilicum*, *S. aromaticum*, and *E. globulus* essential oils showed more elevated levels of  $\alpha$ - $\beta$ EST, GSTs, ACP, and ALP in *A. aegypti* than those in *C. pipiens*; this may be explained by the fact that *Aedes* larvae may be a wild strain that has a resistance gene and have previously been exposed to various pesticides and other environmental pollutions, for example, heavy metals which make them to be resistant to multiple insecticides than *C. pipiens*. These findings agreed with previous biochemical investigations, which estimated different insect species in either field populations or laboratory, and demonstrated high levels of GSTs [41],  $\alpha$ - $\beta$ EST [42], and ACP [43] in insecticide-resistant insect pests. The prominent modifications of these enzymes perhaps correspond to the phytochemicals found in the plants [43].

Additionally, the increasing expression of  $\alpha$ / $\beta$  esterases might be required to maintain life support in a distressing climate [44]. Concerning results obtained from the current and previous investigations, the increase in the activity of protective enzymes, such as  $\alpha$ - $\beta$ -ESTs, GSTs, ALP, and ACP, in different EO-treated larvae of *C. pipiens* and *A. aegypti*, showed their role in metabolizing oil compounds. Elevation of these enzymes with different EOs might be credited to their overproduction. It is guessed that some oil components are utilized directly by these detoxifying proteins, while the remainder of the tried oil that binds to receptors could incite more enzyme production [44]. Cytochrome P450 and AChE activities in the treated *C. pipiens* and *A. aegypti* larvae in the current investigation decreased compared to the controls, as obtained after treatment for 24 h with the three tested EOs. The same findings of cytochrome P450 and AChE reduced activity [19] were obtained in many insect species treated with botanicals. Decreasing these enzymes' activity led to the postulation that both enzymes play a minimal or no role in EOs' metabolism. Because of the significant role in different physiological processes, decreasing cytochrome P450 and AChE, resulting from the inhibitory impact of various EOs, probably creates physiological disturbance and possible death of multiple larvae. Esterase is one of the most grounded responses to ecological stimulation. Most insects can utilize the enzyme to decrease the sensitivity of the target sites of insecticides. When the action of acetylcholinesterase is repressed somewhat, it might straightforwardly cause paralysis of insects and death [41]. Of interest reduction of cytochrome P450 activity in strains of *C. pipiens* (16.5%, 19.1%, and 16.2%) and *A. aegypti* (29%, 21.7% and 18%) after treatments with *O. basilicum*, *S. aromaticum* and *E. globulus* essential oils sparked the idea of utilizing these EOs in controlling mosquitoes resistance by combining it with some synthetic pesticides that are degradable by cytochrome P450 as organophosphates and pyrethroids. The combination of *O. basilicum*, *S. aromaticum*, and *E. globulus* essential oils and synthetic pesticides is a targetable prospect for synergist activity, which reduces the dose of pesticides, and increases the toxicological effects [19]. Furthermore, as *O. basilicum*, *S. aromaticum*, and *E. globulus* essential oils are composed of changed and complex dynamic standards with numerous methods of activity through various targets like cytochrome P450 and AChE, a combination of different essential oils and insecticides might contribute to delaying the resistance in insect populations [45, 46]. As a result, this total technique might work on the exhibition of the IPM of various mosquitoes.

## CONCLUSIONS

It can be concluded that the essential oils (EOs) of *O.basilicum*, *S. aromaticum*, and *E. globulus* exhibit potent larvicidal activity against *A. aegypti* and *C. pipiens* larvae. Furthermore, biochemical analyses of larvae exposed to the LC<sub>50</sub> concentrations of these EOs revealed significant alterations in key detoxification enzymes, including  $\alpha$ - and  $\beta$ -esterases (ESTs), glutathione S-transferases (GSTs), alkaline phosphatase (ALP), acid phosphatase (ACP), cytochrome P450, and acetylcholinesterase (AChE). The strong larvicidal efficacy combined with the inhibitory effects on cytochrome P450 and AChE highlight the potential of *O. basilicum*, *S. aromaticum*, and *E. globulus* essential oils as promising alternatives for mosquito control within integrated pest management (IPM) programs.

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