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Larvicidal Activity and Phytochemical Profiling of Sweet Basil (*Ocimum basilicum* L.) Leaf Extract against Asian Tiger Mosquito (*Aedes albopictus*)

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Abstract: Applying larvicides to interrupt a mosquito's life cycle is an important strategy for vector control. This study was conducted to evaluate the larvicidal properties of the hexane extract of sweet basil (*Ocimum basilicum* L.; family Lamiaceae) leaves against the wild strain of Asian tiger mosquito, *Aedes albopictus* (Skuse). Third instar larvae (20 larvae/replicate, n = 3) were exposed to different concentrations of the extract (6.25–200 µg/mL), and the mortality rate was recorded. Probit analysis showed that the median lethal concentration and 95% lethal concentration of the extract were 16.0 (10.9–22.1) and 53.0 (34.6–136.8) µg/mL, respectively, after 24 h exposure. Only the fractions F3, F4, and F5 from the column chromatography displayed high mortality rates of 91.7–100% at 25.0 µg/mL after 24 h exposure. Subsequent column chromatography from the pooled fraction yielded two active subfractions, H-F345-S2 and H-F345-S3, with mortality rates of 100% and 98.3 ± 2.9%, respectively, at 12.5 µg/mL. Gas chromatography–mass spectrometry analysis unveiled that methyl chavicol, 2-(2-butoxyethoxy)ethanol, cedrelanol, methyl eugenol, 2,4,di-tert-butylphenol, and phytol were the major components in both subfractions with some of them being reported as larvicidal compounds. The results suggest that sweet basil has substantial larvicidal activity against *Ae. albopictus* mosquito and is a potential source of naturally derived larvicide.

Keywords: *Aedes albopictus*; gas-chromatography–mass spectrometry; median lethal concentration; 95% lethal concentration; *Ocimum basilicum*; probit analysis

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1. Introduction

The Asian tiger mosquito, *Aedes albopictus* (Skuse), which derives from the subgenus Stegomyia of the family Culicidae, is an invasive species based on its ever-increasing global distribution. For the past few decades, the *Ae. albopictus* has spread from its native range of Southeast Asia to several new regions as they have strong ecological plasticity, which allows them to adapt progressively to anthropogenic influences [1]. Due to its high disease vector potential and its substantial biting activities, *Ae. albopictus* is considered one of the important mosquito species responsible for several pandemic-prone viral diseases. Formerly known as a secondary vector, the *Ae. albopictus* mosquitoes have evolved to be the primary vectors in several parts of the world [2]. They are the known vectors to transmit dengue and chikungunya viruses, and in recent years, they have been reported to be correlated to the Zika virus outbreaks as well [3,4]. The burden of these diseases is typically highest in tropical and subtropical areas, affecting the most impoverished populations [5].

Being a naturally tree-hole mosquito, *Ae. albopictus* are able to establish themselves and survive in non-urbanized and rural localities that lack artificial containers. Although they inhabit densely vegetated rural areas, their ecological flexibility also allows them to colonize a wide range of artificial water containments and also in urban localities. In Malaysia, there were a sizeable amount of evidence which pointed to the *Ae. albopictus* becoming a dominant species over *Ae. aegypti* (Linnaeus) in certain urban areas due to the

higher environmental temperature and precipitation, and adaptability to various breeding habitats [1]. The biting rates of female adult *Ae. albopictus* mosquitoes can be as high as 30 to 45 bites per hour, giving them the name "aggressive daytime biters". They are usually found biting during the early mornings and late afternoons, preferring to bite outdoors rather than indoors. Although they may feed on a variety of hosts, including humans as well as mammals, reptilians, amphibians, and birds, they prefer mammalian blood [6]. Their anthropophilic behavior, frequent biting, and aptitude to create habitats near human dwellings, have led the *Aedes* mosquitoes to be the most effective vectors for arboviruses [5].

In the year 2020, Kinta district of Perak was declared a dengue epidemic area. Out of the 3226 reported dengue cases, Kinta topped the list with 968 cases. Kampar district came in fourth, with 298 reported cases [7]. The constant usage of insecticides has led to the development of resistance among the *Aedes* population in Kampar [8,9].

Rising public health concerns over the mosquito population and the arboviral diseases transmitted by them have intensified the need for adequate mosquito control strategies. The use of synthetic insecticides is usually recommended during outbreaks of any vector-borne diseases in order to reduce the vector population as quickly as possible [10]. Nonetheless, the use of plant-based products that have insecticidal activity has intensified in an effort to combat the development of insecticide resistance in disease-related insect vectors [2]. Until today, various kinds of plant-based products have been identified to possess potential insecticidal activities, with pyrethrum, neem, rotenone, and essential oils being considered as the four major types to control insects [11,12]. In contrast to synthetic insecticides, botanicals and botanical components with potential insecticidal activity have the features of low toxicity to mammals and easy biodegradability, causing less or no harm to beneficial insects, yet still show promising effects that are able to control the targeted insects [13,14].

Sweet basil, *Ocimum basilicum* (Linnaeus), is the most common species in the Lamiaceae family. There are about 50 to 150 members being reported under the *Ocimum* genus based on variation in morphological characteristics such as growing habit, leaf and flower color, size and shape, and aromatic composition [15]. It is the largest among the Lamiaceae family, with many of them considered to be rich sources of essential oils as they have been known to express various biological activities [16]. Other significant representatives of the *Ocimum* genus include *O. americanum* L. (syn. *O. hispidulum* Schum.) (hoary basil), *O. tenuiflorum* L. (syn. *O. sanctum* L.) (holy basil), and *O. gratissimum* L. (syn. *O. suave* Willd.) (clove basil) [17,18].

Ocimum basilicum is incredibly aromatic, and because of its popularity, it is often referred to as the "king of the herbs". Along with its pleasant taste, it is a versatile culinary ingredient, used as a common spice especially in Italian and Southeast Asian cuisines. It is native to Asia, Africa, Central and South America but it has been widely cultivated in many countries, especially in the Mediterranean region. Similar to other aromatic plants grown for fresh herbs, basil is mainly cultivated as a short-lived annual crop, preferably in an organic production system, including hydroponics [19]. It is a green herb that can reach about 90 cm in height, displaying lanceolate leaves, which are glossy and fragrant [20]. The *O. basilicum* has several varieties that differ in the general morphological structure and texture, and in the chemical contents as well [21].

The essential oils from some *Ocimum* spp. have been shown to repel insects and exhibited larvicidal activity against houseflies, blue bottle flies, and mosquitoes [22]. In Cuba, *O. basilicum* is commonly used as insect repellent in greenhouses [23]. In two separate reports, Erler et al. [24] noted the repellency activities of the essential oils extracted from dried foliage of *O. basilicum* against female adult *Culex pipiens*, whereas Adam et al. [21] reported on the repellency properties of *O. basilicum* essential oils against *Anopheles* mosquitoes. Besides having substantial repellency properties, some reports have also noted the larvicidal effects of the *Ocimum* spp. against dengue vectors. Ghosh et al. [25] discovered that the nanoemulsion of essential oils of *O. basilicum* has shown a dose-dependent larvicidal effect against wild strain *Aedes aegypti* larvae. In another study, treatment with granu-

lated formulations of *O. tenuiflorum* led to up to 90% mortality of the *Ae. aegypti* larvae at 6000 ppm [26].

According to Krüger et al. [27], the main components of essential oils distilled from various basil cultivars contain alcohols (linalool), oxides (1,8-cineole), phenols (eugenol, methyl eugenol, methyl isoeugenol, thymol), esters (methyl cinnamate), aldehydes (citral), and camphor. Among all of them, the 1,8-cineole, methyl cinnamate, methyl chavicol, and linalool are the major constituents that are primarily responsible for the distinct aroma of basil plants [28]. The known insecticidal compounds found in the volatile oils of some *Ocimum* spp. are methyl cinnamate, methyl chavicol, and eugenol. Other chemicals which have been reported to have insect repellent activity are thymol, carvacrol, camphor, caryophyllene oxide, cineole, limonene, and myrcene [29]. In spite of that, the chemical components and essential oil levels of *Ocimum* may vary between the different species and cultivars, and under different growing conditions. Various parameters such as genotype, cropping seasons, and geographical properties would also influence the biochemical components of the plants [17].

Increasing number of studies have reported on the toxicity of plant extracts against mosquitoes, indicating that they exhibit potent larvicidal, ovicidal, and repellent activities against these vectors of diseases [30–32]. In this present study, the larvicidal efficacy of the *O. basilicum* leaf extract and its corresponding fractions were investigated against a wild strain of *Ae. albopictus*. The results from the present study highlighted that the hexane extract of the leaves of *O. basilicum* contained many secondary metabolites which were active against the *Ae. albopictus* larvae.

2. Materials and Methods

2.1. Chemicals and Reagents

The following chemicals were purchased from the respective manufacturers: dimethyl sulfoxide (DMSO) from Fisher Scientific Ltd., Loughborough, UK; absolute ethanol, n-hexane (chromatography grade), and silica gel 60 F_{254} from Merck, Darmstadt, Germany; acetone, hexane, and methanol (analytical grade) from Prochem Chemicals Inc., High Point, NC, USA; and temephos from Vector Control Research Unit, Universiti Sains Malaysia, Penang, Malaysia.

2.2. Extraction of Plant Material

The fresh leaves of *O. basilicum* L. were harvested in January 2015 from the Agriculture Park of Universiti Tunku Abdul Rahman, Kampar, Malaysia. The plant species name was authenticated by the ethnobotanist Dr. Hean Chooi Ong, a former professor affiliated with the Faculty of Science, Universiti Malaya, Malaysia. The leaves were cleaned under running tap water to remove soil and dirt, and air-dried under shade for 24 h. The leaves (1.3 kg) were then ground into small pieces using a stainless-steel blender prior to extraction. The blended leaves were soaked in hexane for two days at room temperature and agitated at 120 rpm using an orbital shaker (Orbit LS, Labnet International Inc., Woodbridge, NJ, USA). The maceration was repeated for five cycles. The filtrate was concentrated at 40 °C to dryness using a rotary evaporator (Rotavapor R-200, Buchi Labortechnik AG, Flawil, Switzerland) and the dried extract was stored at −20 °C pending bioassay.

2.3. Mosquito Sampling and Larvae Culturing

Sixty ovitraps (H: 9.0 cm × D: 7.8 cm) were randomly set up outdoors surrounding the West Lake Garden (Kampar, Malaysia) from February 2015 to June 2015 for mosquito oviposition. The garden is a suburban area consisting of moderately populated houses and an exmining lake. The ovitraps were hung on the trees or bamboo plants approximately 20–30 m apart and 1 m above the ground. A hardboard paddle (H: 10.0 cm × L: 2.5 cm × W: 0.3 cm) was inserted diagonally into each ovitrap filled with dechlorinated tap water. The paddles and the water in the ovitrap were replaced every two to three days. The egg-containing paddles were brought back to the laboratory and air-dried for two days before immersing

them in distilled water for hatching. The emerged larvae were fed with ground cat food (Cuties Catz, Perfect Companion (M) Sdn. Bhd., Kuala Lumpur, Malaysia) until they developed into pupae and adults. Temperature and relative humidity in the laboratory were maintained within the range of 22.0 °C to 27.7 °C and 67% to 78%, respectively [33]. The emerged adults were used for species identification [34] using a stereomicroscope (Zoom 2000, Leica Microsystems Inc., Buffalo, NY, USA). Only the third instar larvae of the species *Ae. albopictus* were used for larvicidal bioassay.

2.4. Larvicidal Bioassay

The larvicidal bioassay was performed according to the World Health Organization guidelines [35] with slight modifications. Each extract was dissolved in a dimethyl sulfoxide-ethanol mixture (3:2, *v*/*v*), sonicated for 5 min using an ultrasonic water bath (Elmasonic E 100 H, Elma Schmidbauer GmbH, Singen, Germany), and filtered using 0.45 µm nylon syringe filters to produce a stock solution of 60 mg/mL. Appropriate volumes of the stock solution were pipetted into round plastic containers (H: 6.0 cm × D: 9.2 cm) filled with dechlorinated tap water to produce six different concentrations (6.25, 12.5, 25, 50, 100, and 200 µg/mL) for the assay. The final volume for each container was 150 mL. Later, 20 third instar larvae of *Ae. albopictus* were collected in separate containers and after a one hour holding period they were introduced into each container and the larval mortality was observed and recorded at 24 and 48 h post-treatment. It was ensured that the DMSO-ethanol content in the assay medium was ≤1% to reduce DMSO/ethanol toxicity to the larvae. Food was not given to the larvae during the assay. The larvae were considered dead if they were unable to reach the water surface and did not move when prodded with a needle in the siphon or cervical region. A 1% DMSO-ethanol mixture was used as negative control while temephos at 1 µg/mL was used as positive control. Temephos is a synthetic, organophosphate larvicide widely used in the vector control programs against mosquitoes in Malaysia. The assay was conducted in triplicate. The percentage mortality was calculated. The morphological changes that occurred on the treated larvae were observed under the stereomicroscope and recorded.

2.5. Column Chromatography

Fractionation of the hexane extract was carried out using gravity column chromatography. A glass column (H: 50.0 cm × D: 3.5 cm) was packed with silica gel 60 F_{254}. Two grams of extract was dissolved completely in hexane and introduced into the column. The elution began with 600 mL each of 100% hexane, followed by different compositions of hexane-acetone mixture (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9 *v*/*v*), 100% acetone, and lastly with a different composition of acetone-methanol mixture (8:2, 6:4, and 5:5 *v*/*v*). The collected eluents were dried at 40 °C using the rotary evaporator. A total of 14 dried fractions (F1–F14) were obtained and subjected to the larvicidal bioassay at 12.5 and 25.0 µg/mL, as described in Section 2.4, to identify active fractions. Subsequently, the three most active fractions, F3, F4, and F5 were pooled together (1.1 g) as H-F345 was subjected to another round of gravity column chromatography using different compositions of hexane-acetone mixture (9:1, 8:2, 7.5:2.5, 7:3, 6:5:3.5, 6:4, and 5:5, *v*/*v*). Seven subfractions (H-F345-S1 to H-F345-S7) were collected from the column and together with the pooled fraction H-F345 were evaluated again for larvicidal activity at 12.5 µg/mL.

2.6. Thin-Layer Chromatography

Thin-layer chromatography (TLC) was performed to determine the compound profile in the active fractions F3, F4, and F5. Ten microliters of each fraction (1 mg/mL) was spotted onto a silica gel 60 F_{254} TLC plate (10 cm × 4 cm; Merck KGaA, Darmstadt, Germany) using a microcapillary tube. The TLC plate was developed using hexane-acetone mixture (8:2, *v*/*v*) as a mobile phase. The plate was then viewed at 366 nm using the TLC Visualizer (CAMAG, Muttenz, Switzerland) and the photo was captured using the software winCATS version 1.4.4.6337 (CAMAG, Muttenz, Switzerland).

2.7. Phytochemical Profiling Using Gas Chromatography–Mass Spectrometry (GC-MS)

Profiling of phytochemicals in the most active subfractions, H-F345-S2 and H-F345-S3, was performed using a gas chromatography–mass spectrometry (QP2010 Plus, Shimadzu Corporation, Kyoto, Japan). The components were separated on a BPX-5 capillary column, 30 m × 0.25 mm × 0.25 µm (SGE International Pty Ltd., Ringwood, Victoria, Australia). Helium gas (purity > 99.999%) was used as a carrier gas with a flow rate of 0.65 mL/min and a linear velocity of 29.9 cm/s. The injector port was maintained at 250 °C. The sample was prepared at 1 mg/mL in chromatography grade n-hexane and 1 µL was injected into the column using split mode with a split ratio of 10:1. The column temperature was set at 100 °C for 3 min, then ramped at a rate of 5 °C/min to 300 °C and maintained for 3 min. The total program time for each analysis was 46 min. For the mass spectrometer, the ion source and interface temperatures were maintained at 200 °C and 300 °C, respectively. Electron ionization at 70 eV was used. Each ion peak was scanned from 40 to 600 m/z. The data acquisition was recorded using the GCMS Solution software version 4.45 SP1 (Shimadzu Corporation, Kyoto, Japan). The possible identity of each component was made by comparison of the mass spectrum obtained with the NIST 14 Mass Spectral Library and Search Software (National Institute of Standards & Technology, Gaithersburg, MD, USA).

2.8. Data Analysis

The larval mortality rate was expressed as the mean ± standard deviation of three replicates. The data were subjected to probit analysis to determine median lethal concentration (LC_{50}), 95% lethal concentration (LC_{95}), upper confidence limit, lower confidence limit, and regression coefficient. The percentages of larval mortality were tested for significance using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test as the post hoc test. The analysis was performed using the IBM SPSS Statistics for Windows Version 23.0 (IBM Corp, Armonk, NY, USA). The significance level was set at $\alpha = 0.05$.

3. Results

In this study, the hexane extract of the leaves of *O. basilicum* was evaluated against third instar larvae of *Ae. albopictus* using six concentrations ranging from 6.25 to 200 µg/mL for 24 and 48 h of exposure. As shown in Figure 1, the larval mortality rates increased from 3.3% at 6.25 µg/mL to 100% at 100 and 200 µg/mL after 24 h of exposure. Further exposure for another 24 h resulted in higher larval mortality rates for 12.5 µg/mL (51.7% to 66.7%), 25.0 µg/mL (68.3% to 78.3%), and 50 µg/mL (91.7% to 100%). Statistical comparisons of the larval mortality performed using one-way ANOVA revealed that there were significant differences between groups for both 24 h ($F(6, 14) = [360.205]$, $p = 0.00$) and 48 h of exposures ($F(6, 14) = [277.130]$, $p = 0.00$).

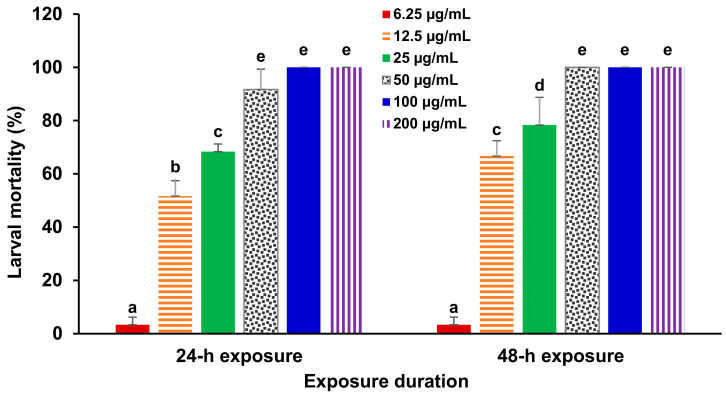

Figure 1. Percentages of mortality of third instar *Ae. albopictus* larvae treated with various concentrations of the hexane extract of *O. basilicum* leaves for 24 and 48 h exposure. The percentages are expressed as mean ± standard deviation of three replicates. Mean values which are statistically not significant ($p > 0.05$) using one-way ANOVA are indicated with the same superscript above the bars.

Probit analysis revealed that the median lethal concentration (LC_{50}) and 95% lethal concentration (LC_{95}) of the extract were 16.0 and 53.0 µg/mL, respectively, after 24 h exposure, and 12.8 and 32.7 µg/mL, respectively, after 48 h exposure (Table 1 and Figure 2). This clearly demonstrated that the larvicidal effect of a plant extract was dependent on the extract concentration and larval exposure period. In terms of morphological changes, the *Ae. albopictus* larva treated with the hexane extract of the leaves of *O. basilicum* showed an extended cephalo-thoracic junction and blackening of the midgut or abdomen compared to the healthy untreated larva (Figure 3).

Table 1. Probit analysis of the larvicidal activity of the hexane extract of *O. basilicum* leaves against third instar *Ae. albopictus* larvae after 24 and 48 h exposure periods.

Extract	24 h Exposure	48 h Exposure
Median lethal concentration (LC_{50}, µg/mL) (LCL-UCL)	15.98 (10.95–22.12)	12.82 (7.64–19.22)
95% lethal concentration (LC_{95}, µg/mL) (LCL-UCL)	53.00 (34.61–136.82)	32.66 (21.11–151.96)
Regression coefficient ± Standard error	3.159 ± 0.312	4.051 ± 0.442
Chi Square, X^2	10.292	17.151
Degree of freedom, df	4	4
p-value	0.036	0.002

LCL denotes lower confidence limit; UCL denotes upper confidence limit.

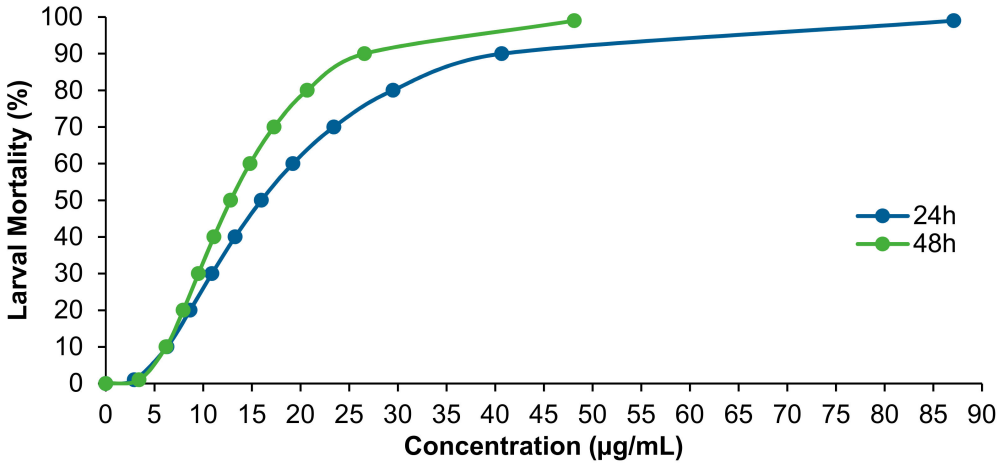

Figure 2. Probit analysis of the larvicidal activity of the hexane extract of *O. basilicum* leaves against third instar *Ae. albopictus* larvae after 24 and 48 h exposure periods.

Fourteen fractions (F1–F14) were obtained from the column chromatography and their larvicidal activity was evaluated at 12.5 and 25.0 µg/mL for 24 and 48 h. As shown in Figure 4, the larvicidal compounds were found to be mainly present in the fractions F3, F4, and F5 as their mortality rates shown were 80.0–100% at 12.5 µg/mL and 91.7–100% at 25.0 µg/mL. The fractions were eluted using the hexane-acetone mixture with the compositions of 8:2, 7:3, and 6:4 *v*/*v*, respectively. Seven fractions (F1, F7, F8, F10, F12, F13, and F14) were devoid of larvicidal activity at both concentrations. The three active fractions were pooled together based on their similar TLC profiles (Figure S1) into a single fraction (H-F345) and subjected to another round of column chromatography. Seven subfractions were obtained and tested for larvicidal activity at 12.5 µg/mL. Out of these, only the subfractions H-F345-S2, H-F345-S3, and H-F345-S4 exhibited larvicidal activity with mortality rates of 100%, 98.3 ± 2.9%, and 75.0 ± 5.0%, respectively, after 24 h of exposure. The latter two subfractions achieved a mortality rate of 100% at 48 h after exposure. These three subfractions were eluted using the hexane-acetone mixture with the compositions of 8:2, 7.5:2.5, and 7:3 *v*/*v*, respectively.

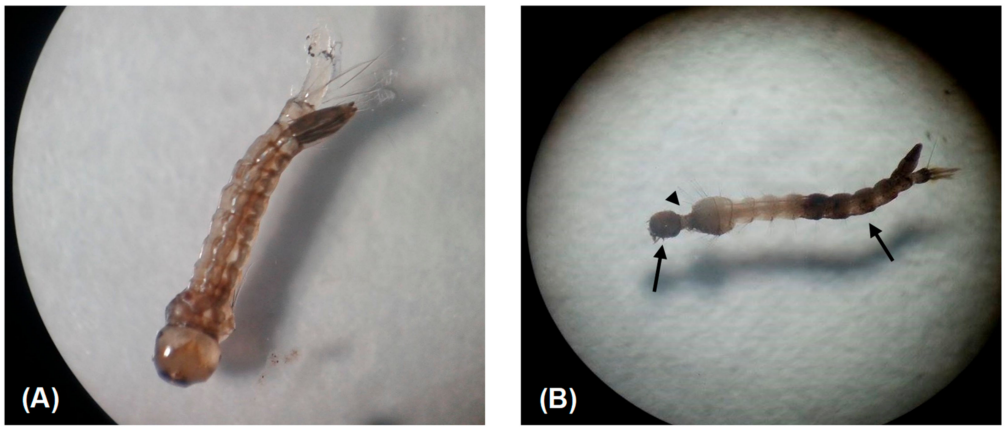

Figure 3. Morphology of third instar larvae of *Ae. albopictus* (40X magnification). (**A**) Normal, untreated larva; (**B**) Larva treated with 200 µg/mL of the hexane extract of *O. basilicum* leaves after 24 h exposure. Deformities such as blackening abdomen (arrow) and extended cephalo-thoracic junction (arrowhead) were noticed on the larva.

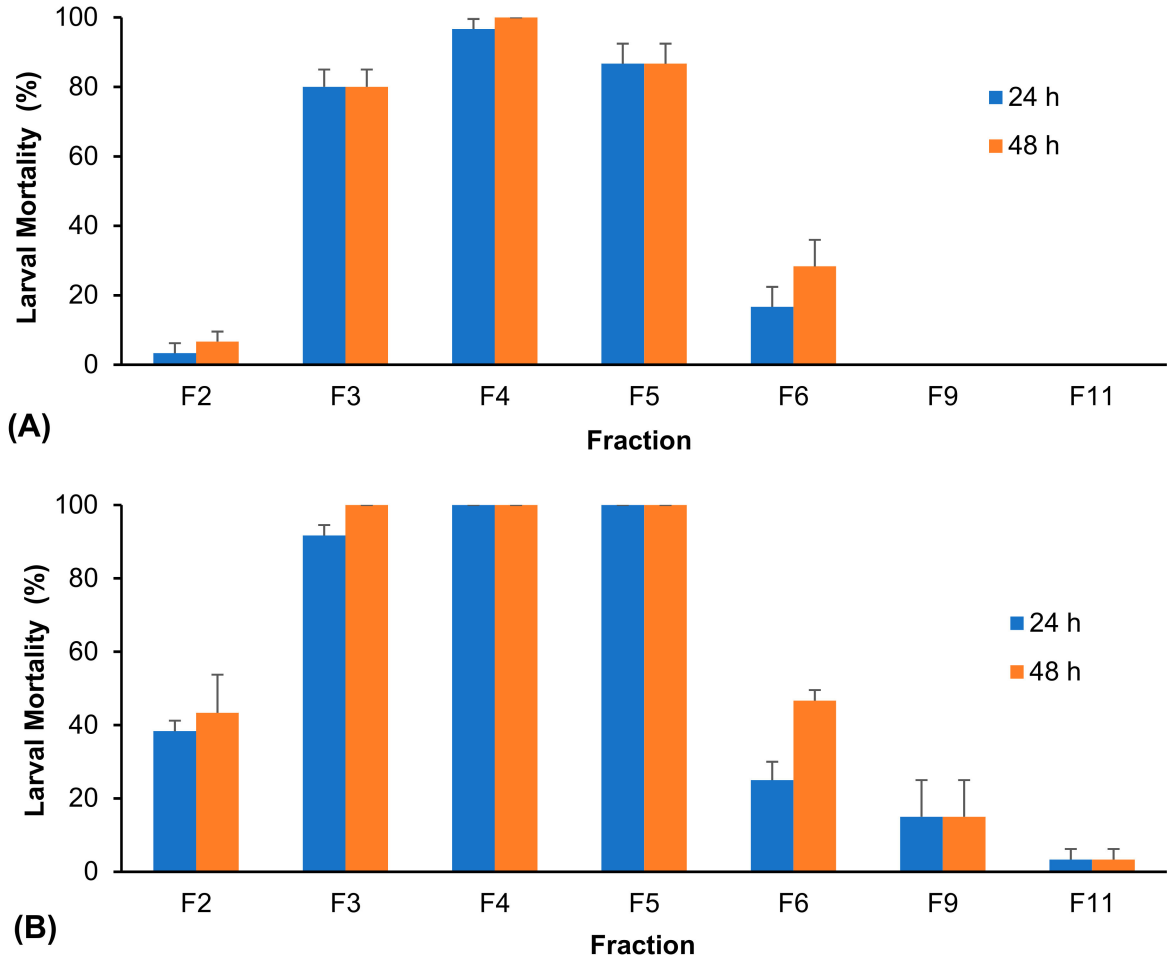

Figure 4. Percentage of mortality of third instar larvae of *Ae. albopictus* treated with fractions of the hexane extract of *O. basilicum* leaves for 24 and 48 h at 12.5 µg/mL (**A**) and 25.0 µg/mL (**B**). The fractions were eluted with different compositions of hexane and/or acetone. The percentage of mortality for each fraction is shown as mean ± standard deviation of triplicate.

In order to gain some insights into the larvicidal compounds, the two most active subfractions (H-F345-S2 and H-F345-S3) were selected for phytochemical profiling using

gas chromatography–mass spectrometry (GC-MS). The subfraction H-F345-S2 contained a higher number of compounds than H-F345-S3. In total, 14 out of 31 peaks detected in the H-F345-S2 were successfully identified and they represented 91.52% of the total peak area or 88.67% of the total peak height of the chromatogram (Table 2). While for the subfraction H-F345-S3, 19 peaks were registered in the chromatogram, and nine of them were successfully identified, accounting for 95.16% of the total peak area or 92.69% of the total peak height (Table 3). The major components present in both subfractions are quite similar, i.e., methyl chavicol, 2-(2-butoxyethoxy)ethanol, cedrelanol, methyl eugenol, 2,4-di-tert-butylphenol, and phytol. The main compound, methyl chavicol constituted 57.67% and 64.34% of the peak area for H-F345-S2 and H-F345-S3, respectively.

Table 2. Phytochemical profiling of the subfraction H-F345-S2 derived from the hexane extract of the leaves of *O. basilicum* using gas chromatography–mass spectrometry.

Peak	Retention Time (min)	Peak Area (%)	Peak Height (%)	Compound	MW	Chemical Formula	Similarity (%)
1	13.811	10.92	8.86	2-(2-Butoxyethoxy)ethanol	162	$C_8H_{18}O_3$	95
2	14.544	57.67	47.12	Methyl chavicol	148	$C_{10}H_{12}O$	93
3	15.918	0.53	0.58	-			
4	16.961	0.18	0.24	-			
5	19.158	0.20	0.28	-			
6	19.316	0.45	0.63	Allyl methallyl ether	112	$C_7H_{12}O$	91
7	19.864	0.40	0.64	beta-Elemene	204	$C_{15}H_{24}$	86
8	19.971	4.35	6.10	Methyl eugenol	178	$C_{11}H_{14}O_2$	92
9	20.922	0.28	0.40	-			
10	21.019	0.49	0.66	-			
11	22.001	0.42	0.62	Humulene	204	$C_{15}H_{24}$	86
12	22.668	2.39	3.16	2,4-Di-tert-butylphenol	206	$C_{14}H_{22}O$	91
13	23.334	1.23	1.81	Germacrene D	204	$C_{15}H_{24}$	88
14	24.443	0.53	0.91	1-Dodecene	168	$C_{12}H_{24}$	90
15	24.851	0.28	0.41	-			
16	25.035	0.25	0.37	-			
17	25.176	0.94	1.37	-			
18	25.408	0.31	0.45	-			
19	25.908	0.31	0.49	-			
20	26.004	1.53	1.89	-			
21	26.589	9.92	13.54	Cedrelanol	222	$C_{15}H_{26}O$	88
22	26.951	0.46	0.63	-			
23	27.140	0.63	0.85	-			
24	27.225	0.55	0.74	-			
25	27.447	0.55	0.44	-			
26	29.068	0.22	0.41	cis-3-Tridecene	182	$C_{13}H_{26}$	88
27	31.949	0.78	1.34	Methyl palmitate	270	$C_{17}H_{34}O_2$	90
28	35.569	0.61	1.01	9,12,15-Octadecatrienal	262	$C_{18}H_{30}O$	88
29	35.669	1.63	2.52	Phytol	296	$C_{20}H_{40}O$	89
30	37.411	0.21	0.34	-			
31	40.552	0.76	1.19	-			

'-' denotes unidentified as the similarity with the NIST 14 Mass Spectral Library is <86%.

Table 3. Phytochemical profiling of the subfraction H-F345-S3 derived from the hexane extract of the leaves of *O. basilicum* using gas chromatography–mass spectrometry.

Peak	Retention Time (min)	Peak Area (%)	Peak Height (%)	Compound	MW	Chemical Formula	Similarity (%)
1	13.816	11.03	9.08	2-(2-Butoxyethoxy)ethanol	162	$C_8H_{18}O_3$	95
2	14.537	64.34	54.66	Methyl chavicol	148	$C_{10}H_{12}O$	94
3	19.867	0.33	0.49	-			
4	19.982	4.13	6.00	Methyl eugenol	178	$C_{11}H_{14}O_2$	91
5	21.038	0.42	0.57	-			
6	22.677	2.00	2.81	2,4-Di-tert-butylphenol	206	$C_{14}H_{22}O$	88
7	23.347	0.96	1.44	Germacrene D	204	$C_{15}H_{24}$	86
8	24.456	0.37	0.67	1-Dodecene	168	$C_{12}H_{24}$	88
9	25.184	0.70	1.12	-			
10	26.010	1.12	1.51	-			
11	26.589	9.30	13.13	Cedrelanol	222	$C_{15}H_{26}O$	88
12	26.958	0.28	0.43	-			
13	27.145	0.43	0.65	-			
14	27.242	0.31	0.48	-			
15	31.952	0.76	1.29	Methyl palmitate	270	$C_{17}H_{34}O_2$	87
16	35.577	0.36	0.66	-			
17	35.674	2.27	3.61	Phytol	296	$C_{20}H_{40}O$	89
18	40.557	0.61	0.95	-			
19	42.595	0.29	0.45	-			

'-' denotes unidentified as the similarity with the NIST 14 Mass Spectral Library is <86%.

4. Discussion

The hexane extract of *O. basilicum* leaves is considered to have strong larvicidal activity as its LC_{50} values were less than 50 µg/mL [36]. Our results are consistent with the findings from other reported studies. The essential oils or extracts from the leaves and/or stems of *O. basilicum* have been shown to possess larvicidal activity against several disease-carrying mosquitoes, including *Culex quinquefasciatus* [37,38], *Cx. pipiens* [39,40], *Culex tritaeniorhynchus*, *Anopheles subpictus* [41], *Anopheles stephensi* [42], *Anopheles culicifacies* [43], and *Ae. aegypti* [44,45]. The leaf essential oils of *O. basilicum* are also active against the larvae of *Ae. albopictus* with LC_{50} values of 11.97–107.7 µg/mL [41,46]. On the other hand, the methanol extract of the aerial part of *O. basilicum* showed a weak larvicidal effect against *Ae. albopictus* with an LC_{50} value of 755.13 µg/mL after 24 h of exposure [43]. Our study using the hexane extract (LC_{50} = 16.0 µg/mL) suggested that the larvicidal compounds from the leaves of *O. basilicum* are likely to be volatile or non-polar compounds.

In this study, the larvae of *Ae. albopictus* treated with the hexane extract showed deformities such as an extended cephalo-thoracic junction and blackening of the mid/hind gut. Similar deformities have also been reported when *Ae. albopictus* larvae were treated with the acetone leaf extract of *Ipomoea cairica* and the ethanol leaf and fruit extracts of *Piper nigrum* [47,48]. This suggests that the mid/hind gut of mosquito larvae is a common target organ for the larvicidal effect of plant extracts. These morphological deformities are likely caused by the interference of hormonal control or interruption of chitin synthesis from the bioactive phytochemicals in the extracts [49].

Analysis using GC-MS showed that the leaves of *O. basilicum* contained many phytochemicals or different classes of secondary metabolites. Methyl chavicol (also known as estragole) and methyl eugenol belong to the phenylpropene group of secondary metabolites. Both phenylpropenes have been reported from the leaves or aerial parts of *O. basilicum* [40,50–52]. Methyl chavicol, isolated from the essential oils of *Clausena anisata*, has been shown to exhibit larvicidal activities against *An. stephensi*, *Ae. aegypti*, and *Cx. quinquefasciatus* with LC_{50} values of 11.01–14.01 µg/mL [53]. Methyl chavicol also exhibited larvicidal activity against *Ae. albopictus* but with a much higher LC_{50} value of 58.7 µg/mL [46]. Similarly, methyl chavicol and methyl eugenol obtained from the root essential oils of *Asarum heterotropoides* possess larvicidal activity against *Ae. aegypti*, *Cx. pipiens*, and *Ochlerotatus togoi* with LC_{50}

values of 46.39–58.52 µg/mL and 53.30–67.02 µg/mL, respectively [54,55]. The LC_{50} values recorded from these studies strongly suggested that methyl chavicol was not the sole active larvicidal compound for *O. basilicum* albeit it is the major component in both active subfractions.

Cedrelanol (tau-cadinol) is a natural oxygenated sesquiterpene that has been documented in some of the *Ocimum* species, such as *O. basilicum*, *O. carnosum*, *O. gratissimum*, and *O. tenuiflorum* [56–58]. It can also be found in the essential oils of aromatic plants such as *Salvia aratocensis*, *Lippia americana*, *Melissa officinalis*, and *Thymus* spp. [59–61]. Several biological activities of cedrelanol have been reported in the literature, these include cytotoxic against human cancer cells, antibacterial, antifungal, antiparasitic, spasmolytic, and immunomodulatory activities [62–65]. Cedrelanol isolated from the bark of *Swartzia polyphylla* killed all the larvae of *Cx. quinquefasciatus* at 300 µg/mL [66]. However, the larvicidal activity of cedrelanol against *Aedes* and other species of mosquitoes remains to be investigated.

2-4-Di-tert-butylphenol is a natural lipophilic phenol and a toxin produced by many living organisms. This toxin has been documented in at least 169 species of bacteria, fungi, diatom, liverwort, pteridophytes, gymnosperms, dicots, monocots, and animals [67]. The GC-MS results of the present study revealed that this toxin is also detected for the first time from a species of *Ocimum* genus, *O. basilicum*. Wang et al. [68] studied the larvicidal effect of 2-4-di-tert-butylphenol and reported LC_{50} values of 1.98–3.90 µg/mL against the larvae of *Cx. pipiens*, *Ae. aegypti*, *Ae. albopictus*, and *Anopheles sinensis*. Our study suggested that the toxin works synergistically with other phytochemicals of the plant in exerting the killing effect on *Ae. albopictus* larvae.

Phytol (3,7,11,15-tetramethylhexadec-2-en-1ol) is a diterpene or isoprenoid alcohol with 20 carbon atoms and a double bond. It is a constituent of plant and phytoplankton chlorophyll. Besides the synthesis of chlorophyll, phytol is involved in the production of tocopherol, phylloquinol, and fatty acid phytyl ester in plants [69]. Hence, it is not surprising to find the presence of phytol in the leaves of many *Ocimum* spp., such as *O. basilicum* [50,70], *O. gratissimum* [71], *O. obovatum* [72], and *O. tenuiflorum* [73]. A comprehensive review of published literature highlighted the diverse biological activities of phytol, including antibacterial, antifungal, antiparasitic, cytotoxic, antimutagenic, antioxidant, anti-inflammatory, insulin-sensitizing, and lipid-lowering activities [74]. Despite these, further study is needed to ascertain whether phytol possesses any larvicidal activities against mosquito larvae.

2-(2-Butoxyethoxy)ethanol or diethylene glycol monobutyl ether is a colorless liquid with a low odor and high boiling point. It is an inert ingredient or a stabilizer in plant protection products, pesticides, or fertilizers, or a common solvent in many washing and cleaning household products, cosmetics, and personal care products [75]. To the authors' knowledge, this compound has not been isolated from plants. Hence, it is unlikely a bioactive product from the secondary metabolism of *O. basilicum*.

In addition to the major components of the two subfractions, some minor components are also believed to play their roles in contributing to the larvicidal activity of *O. basilicum* leaves. Germacrene D, α-humulene (α-caryophyllene), and β-elemene are sesquiterpenes that accounted for 0.40–1.23% of the subfraction H-F345-S2. Germacrene D was also detected (0.96%) in the subfraction H-F345-S3. These sesquiterpenes have also been detected and quantified from the essential oils of *O. basilicum* cultivated in Romania [56], Algeria [39], Greece [46], Serbia [51], Jordan [76], and Saudi Arabia [52]. Germacrene D is able to kill the larvae of *Ae. aegypti*, *An. stephensi*, and *Cx. quinquefasciatus* with LC_{50} values of 16.95–63.6 µg/mL [53,77]. Similarly, α-humulene possesses larvicidal activities against *Ae. aegypti*, *Ae. albopictus*, *An. subpictus*, and *Cx. tritaeniorhynchus* with LC_{50} values of 6.19–90.50 µg/mL [78,79]. β-Elemene is the major isomer of elemene, which is a naturally occurring compound in the herb *Curcuma wenyujin*. It is used as an herbal extract in traditional Chinese medicine for treating various types of cancers owing to its broad-spectrum anticancer properties [80]. The β-elemene isolated from *Syzygium zeylanicum* was found to be active against the larvae of *Ae. albopictus* with an LC_{50} value of 11.15 µg/mL [78].

5. Conclusions

The hexane extract and fractionations obtained from the leaves of *O. basilicum* have demonstrated significant larvicidal effects against wild strain *Ae. albopictus* that are both concentration and time dependent. GC-MS analysis revealed the presence of methyl chavicol, methyl eugenol, cedrelanol, and 2,4-di-tert-butylphenol as the major components in the active subfractions with reported larvicidal activities against disease-carrying mosquitoes, including *Ae. albopictus*. In addition, the larvicidal effects were also contributed by germacrene D, α-humulene, or β-elemene, which were minor components with known larvicidal property. These results are indicative of the use of *O. basilicum* leaves as a potential source of natural mosquitocidal agents in integrated vector management programs. Application of these natural larvicides alongside the reduction of usage of synthetic larvicides may serve to decrease the population of *Aedes* mosquitoes, and eventually the transmission of arboviruses to humans without the decremental effects to the environment. Further investigations using laboratory strains of both dengue vectors are necessary to substantiate the herb's efficacy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8050443/s1, Figure S1: Thin-layer chromatography (TLC) profiles of the hexane fractions of the leaves of *Ocimum basilicum*.

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