

Research article

Combinations of plant essential oils and their major compositions inducing mortality and morphological abnormality of *Aedes aegypti* and *Aedes albopictus*Mayura Soonwera^{*}, Tanapoom Mounghipmalai, Jirapon Aungtikun, Sirawut Sittichok

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HIGHLIGHTS

- Trans-anethole and limonene are strongly insecticidal against two mosquito species.
- *I. verum* EO + trans-anethole was very strong larvicide and pupicide.
- *I. verum* EO + trans-anethole was much more effective than temephos.
- All formulations effected some morphological changes at time of death.
- These long-used natural compounds should be much safer than temephos.

GRAPHICAL ABSTRACT



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ABSTRACT

Extensive uses of synthetic insecticides to control mosquito's populations have induced the insects to develop resistance against them, rendering them ineffective today. Moreover, they cause serious impacts on human health and the ecosystem. Therefore, safe and effective natural alternatives are needed. This study evaluated the larvicidal and pupicidal activities of essential oils (EOs) from *Illicium verum* and *Zanthoxylum limonella* and the major constituents against *Aedes aegypti* and *Aedes albopictus* mosquitoes as well as recorded their morphological aberrations at death. The GC-MS analysis showed that trans-anethole was the major constituent of *I. verum* EO, and limonene was the major constituent of *Z. limonella* EO. Both were more effective against the larvae and pupae of *Ae. aegypti* than those of *Ae. albopictus*. A 2.5% *I. verum* EO + 2.5% trans-anethole combination showed the highest larvicidal and pupicidal effects against *Ae. aegypti* and *Ae. albopictus* with an LT_{50} ranging from 0.2–6.9 h. Between the two tested constituents, trans-anethole exhibited stronger larvicidal and pupicidal activities (LC_{50} ranging 2.4–3.4%) against the two tested mosquito species than d-limonene (LC_{50} ranging 2.5–3.7%). Most importantly, 5% trans-anethole, 5% d-limonene, and 2.5% *I. verum* EO + 2.5% trans-anethole were more effective (LT_{50} ranging 0.1–0.3 h) than 1% (w/w) temephos (LT_{50} ranging 2.9–3.1 h). Morphological aberrations at death observed were such as color pigment and thorax shape abnormalities. To conclude, trans-anethole, d-limonene, and a combination of *I. verum* EO + trans-anethole, are natural compounds that not only are as effective as temephos at the time of this study, but should be also be much safer to human health.

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1. Introduction

Recently, the number of incidents of viral disease contraction in humans, transmitted by *Aedes aegypti* L. and *Aedes albopictus* (Skuse), around the world has increased (Beltrán-Silva et al., 2018; Kraemer et al., 2019). The two mosquito species carry many disease pathogens such as chikungunya (CHIKV), yellow fever (YF), Zika (ZIKV), and dengue (DENV) viruses (Kraemer et al., 2019). Among these diseases, dengue is the most serious disease in Thailand and other Asian countries (Thailand Ministry of Public Health, 2020; WHO, 2020a). The occurrences of dengue cases have increased 30 folds in the past decade (Benelli and Mehlhorn, 2016). A recently updated report by the World Health Organization (WHO, 2020a), on 2 March 2020, estimated that 96 million people will be annually infected with the dengue virus worldwide. In the same vein, in 2019, Asia, as well as North and South America, were affected heavily by CHIKV disease (WHO, 2020b). Regarding ZIKV disease, in 2013, a ZIKV outbreak in French Polynesia caused 73 pregnant women to deliver a baby with a small head (microcephaly) (Benelli and Mehlhorn, 2016).

Conventionally, synthetic chemicals have been used to kill larvae and adult mosquitoes effectively. Unfortunately, by the time of this study, the target insects of these chemicals have developed strong resistance to them, rendering the synthetic chemicals ineffective (Naqqash et al., 2016). This kind of resistance has become a norm for populations of mosquitoes all around the world. Numerous cases of resistance have been reported in Thailand and worldwide (Valle et al., 2019; Chai-phongpachara and Moolrat, 2017; Bisset et al., 2020). To make matters worse, those synthetic chemicals seriously polluted aquatic and other ecosystems and destroyed marine and land animals other than the pest insects (Castillo et al., 2017; Govindarajan et al., 2018). This insect resistance and non-targeted organism safety issues call for a new, safer, and more effective way to control mosquitoes. Meeting this challenge, many current research works have mostly been on developing low-cost, environmentally friendly, and biodegradable insecticides that are safe for non-target animals (Govindaraju et al., 2016; Muthiah et al., 2018).

Essential oils (EOs) are potentially safe and effective insecticidal plant extracts that many researchers have investigated (Benelli and Pavela, 2018; Pavela and Benelli, 2016). Several researchers reported that EOs and their constituents could destroy several mosquito species, such as *Illicium verum* (against *Ae. aegypti*) (Gomes da Rocha Voris et al., 2018); *Myristica fragrans* (against *Ae. aegypti*) (Pavela, 2015a); *Zingiber officinale* (against *Culex quinquefasciatus*) (Soonwera and Phasomkusolsil, 2017; Rabha et al., 2012); *Amomum subulatum* (against *Ae. albopictus* and *Anopheles subpictus*) (Govindarajan et al., 2018), and *Coleus aromaticus* (against *An. stephensi*) (Govindaraju et al., 2016). In addition, several studies reported that some combinations of EOs and EO constituents exhibited a higher degree of toxicity against mosquitoes and other insects than their individual EO and EO constituent components (Pavela, 2015b; Benelli et al., 2017; Ríos et al., 2017). For example, a study by Aungtikun and Soonwera (2021) showed that the KT_{50} against *Ae. aegypti* and *Ae. albopictus* of two combinations of EOs—*Cinnamomum verum* + *Cinnamomum cassia* and *C. cassia* + *Cinnamomum loureiroi*—were shorter, at 1.8–2.4 min, than those of individual *C. verum*, *C. cassia*, and *C. loureiroi* alone, at 5.5–15.0 min.

An effective vector control program for preventing those serious diseases must destroy mosquitoes at all life stages: adult, larva, and pupa (Castillo et al., 2017). Consequently, the primary objective of this study was to find out whether and how strong major constituents of *Illicium verum* and *Zanthoxylum limonella* EOs and two combinations of the EO and their EO constituents were effective against *Ae. aegypti* and *Ae. albopictus*. The secondary objective was to compare their efficacy to that of temphos, a widely used synthetic insecticide. Since the mosquito subjects would be destroyed in efficacy assays regardless of the outcome, their morphological aberrations at time of death were recorded as well, as they were useful data for future research on the mechanisms of action of these substances. These two EOs were selected because *I. verum* EO was

reported to exhibit strong larvicidal and adulticidal activities against *Ae. aegypti* (Gomes da Rocha Voris et al., 2018), and *Z. limonella* EO was reported to exhibit strong larvicidal activity against *An. dirus*, *Ae. aegypti*, and *Ae. albopictus* (Pitasawat et al., 2007; Pavela, 2015a; Rabha et al., 2012) as well as to exhibit ovicidal and oviposition deterrent activities against *Cx. quinquefasciatus* and *Ae. aegypti* (Soonwera and Phasomkusolsil, 2017). The tertiary objective was to observe and record the morphological aberrations at time of death of larvae and pupae of those mosquitoes, which would be valuable for identifying and/or verifying the mechanisms of mosquito-controlling actions of these substances since reported data on aberrations at death from these two EOs at the time of this study was limited.

2. Materials and methods

2.1. Insect rearing

Ae. aegypti and *Ae. albopictus* mosquitoes were reared in the laboratory of Entomology at the Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok, Thailand, under the environmental conditions of 26.5 ± 1.5 °C, $75.0 \pm 1.2\%$ RH. The hatched larvae were fed with fish food pellets (Hipro[®], 38% high protein content). Larvicidal and pupicidal assays were conducted on Stage-4 larvae and pupae.

2.2. Plant essential oils and extraction method

Dried fruits of *I. verum* were purchased from Vejpong Pharmacy (Hock Ann Tung) CO., LTD. 145–149 Jakrawat Rd., Samphanthawong, Bangkok, Thailand, and dried fruits of *Z. limonella* were purchased from an herb store in Den Chai District, Phrae Province, Thailand. They were identified by Jirapon Aungtikun and Cheepchanok Puwanard, the herbal specialists at the KMITL Herbarium. Photos of the samples are shown in Figure 1. Those samples were extracted by a water distillation method (Soonwera and Sittichok, 2020), carried out in the following steps. One kilogram of small fruits was thoroughly washed with distilled water and placed in an extraction column and warmed to 100 °C. The mixture was distilled for 5 h until completion after it started to boil, and the extracted oil was collected in a round-bottomed flask connected to the extraction column. Water was removed from the EO distillate with anhydrous sodium sulfate, and the resulting concentrated EO solution became the stock solution that was diluted and used in all assays. The stock EO was diluted to 1:100 before injection into the GC-MS system. For larvicidal and pupicidal assays, the stock solution was diluted to 0.5%, 1%, 2.5%, and 5% EO solutions in ethyl alcohol, and the two combined formulations were 2.5% *I. verum* EO + 2.5% trans-anethole and 2.5% *Z. limonella* EO + 2.5% d-limonene, from the respective stock solutions. All formulations tested, described in the next-to-last paragraph of the Introduction section, were prepared and stored under laboratory conditions at 26.2 ± 1.5 °C, $75.0 \pm 1.2\%$ RH.

2.3. Gas chromatography-mass spectrometry analysis of EO chemical composition

Chemical analyses of *I. verum* and *Z. limonella* EOs were determined by GC-MS (a service provided by Scientific Instrument Center, KMITL). A GC-MS instrument GC 6890-N (Agilent Technologies Co., Ltd. USA.) was used, with an HP-5MS column (30 m × 0.25 mm i.d.) and a 0.25 μm film thickness. The carrier gas was 99.99% helium. The injector and detector temperatures were set at 270 °C, and the pressure was set at 8.73 psi. The mass range was scanned from 30 to 500 m/z⁻¹. The obtained MS spectrum of every compound was compared with the reference spectra of compounds in the Wiley 7n.l database. Its identity was then confirmed by comparing the retention index (RI) with a homologous series of *n*-alkanes (C₇–C₃₀) in the NIST tandem mass spectral library v 7.1 (NIST 17, 2017). The identified constituents and their corresponding RIs are shown in Table 1.

2.4. Toxicity assay against *Ae. aegypti* and *Ae. albopictus*

The larvicidal and pupicidal assays in this study were the same, but different types of outcomes were observed. This assay followed the WHO-recommended assay (WHO, 2005). It was faithfully used by Phukerd and Soonwera (2013). In the assay, twenty-five 4th instar larvae and pupae of *Ae. aegypti* and *Ae. albopictus* were put in a plastic cup containing each treatment: 1 ml of each essential oil formulation in 99 ml of distilled water. After treatment, the larvae were not fed with any nourishment before the assay. The pupicidal test lasted until every pupa either died or developed into an adult. The decisive sign that the larvae and pupae were dead was that they were not able to move up to the surface of disturbed water or did not exhibit diving reaction characteristics (Chantawee and Soonwera, 2018a). Larval mortality was monitored for 5, 10, 15, 30, 60 min, and 6 h, while pupal mortality was monitored for 5, 10, 15, 30, 60 min, 6 h, 24 h, 48 h, and 72 h (Figures 2 and 3). Lethal times were calculated based on those monitored values. Only the final mortality rates at 6 h for larvae and 72 h for pupae are tabulated in Tables 2 and 3. For each treatment, the assay was run in 10 replicates and conducted under controlled laboratory conditions [28.5±1 °C, 65.5 ± 5% R.H.].

The treatment chemicals, trans-anethole which is a major constituent of *I. verum* EO and d-limonene which is a major constituent of *Z. limonella* EO, were obtained from Sigma-Aldrich (USA) (Figure 1). <https://liquor.or.th/>. The positive control, temephos 1% w/w (Sai GPO-1[®]), was manufactured by Thai Government Pharmaceutical Organization (GPO), 138 Moo 4 Rangsit-Nakhon Nayok Rd., Bueng Sanan, Thanyaburi District, Pathum Thani Province, Thailand. The negative control, 70% v/v ethyl alcohol, was manufactured by Liquor Distillery Organization, Bang Khla District, Chachoengsao Province, Thailand (<https://liquor.or.th/>).

2.5. Expected morphological aberrations at time of death

After treatment, the morphological aberrations at death of larvae, pupae, and adults that did not emerge completely from pupal exoskeleton were observed and categorized under a stereomicroscope (Nikon[®] Type 102) and photographed with a digital camera (Nikon[®] DS-Fi2). The expected morphological aberrations were the nine types of aberrations reported by Chantawee and Soonwera (2018a) and Soonwera and Phasomkusolsil (2016): photos of each aberration are shown in Figure 4:

- Type 1: Normal larvae (NL). This group of pre-pupal-stage larvae retained their normal posture and general appearance at death.
- Type 2: Deformed larvae (DL). This group of larvae died in an abnormal posture. Dorsal splitting of thoracic cuticles could be observed in the dying and dead larvae. The digestive tract of the deformed larvae was darker than normal.
- Type 3: Pre-pupae that did not come completely out of the larval exoskeleton (PP). This group of larvae died before they could emerge successfully from their exoskeleton. Some of them died with their head still enclosed in their exoskeleton.
- Type 4: White pupa (WP). This group of pupae successfully emerged from their exoskeleton, but their cuticles were abnormally white, which made some researchers call them "albinos".
- Type 5: Deformed pupae (DP). This group of pupae died in an abnormal posture. Some died with a deformed head that looked like a miniature elephant head, and thus were named "elephantoids".
- Type 6: Dead normal brown pupae (BP). This group of pupae died in a normal posture, but their exoskeleton was browner than normal.
- Type 7: Adults attached to the pupal case (PA). The tarsi, legs, wings, and abdomen of adult mosquitoes in this group did not completely emerge from their exoskeleton at death.

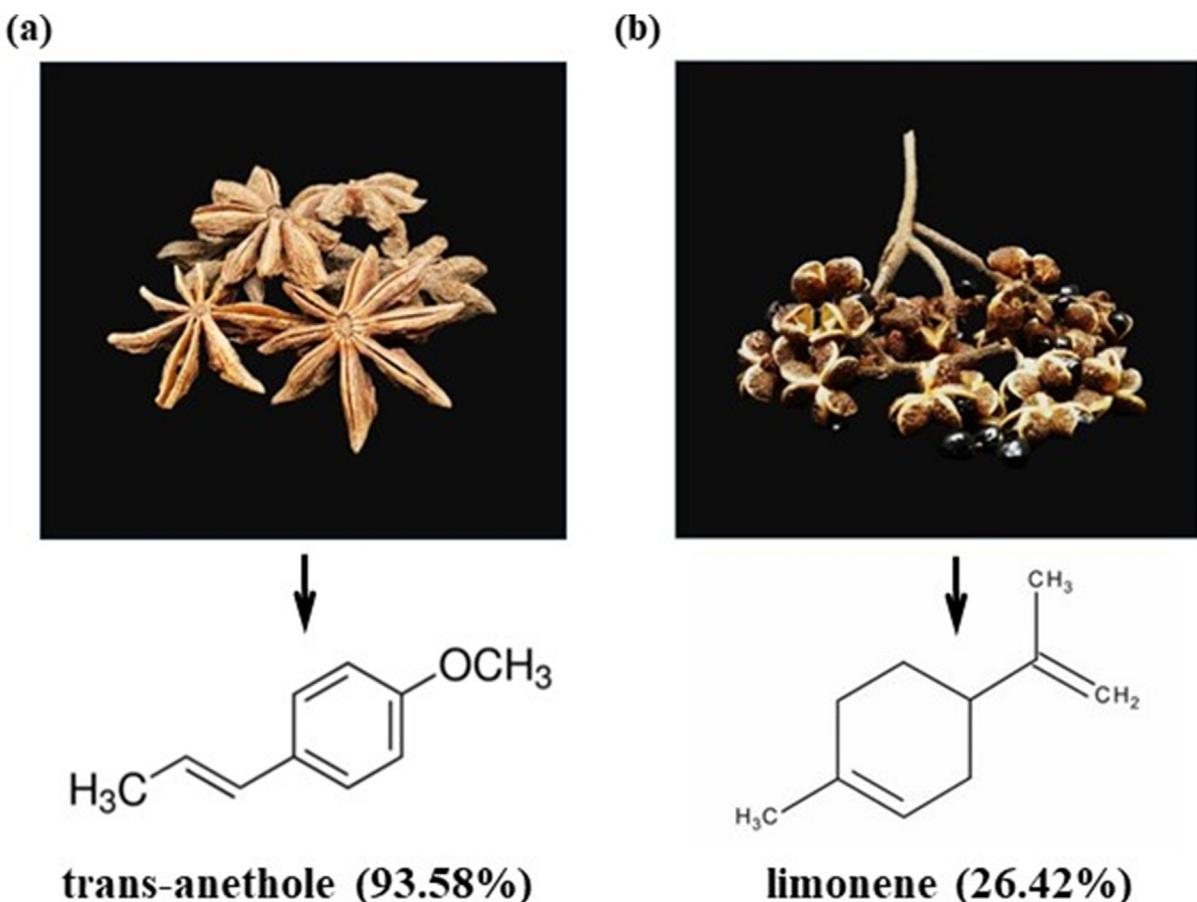


Figure 1. Major chemical constituents of EOs from *I. verum* (a) and *Z. limonella* (b).

- Type 8: Deformed Adults (DA). This group of adult mosquitoes successfully emerged from their exoskeleton, but in many cases, they emerged with morphological defects, e.g., crippled wings, deformed abdomen or legs.
- Type 9: Normal adult (NA): This group of adult mosquitoes emerged completely from their exoskeleton with a normal appearance.

2.6. Statistical analysis

Probit analysis was used to calculate the time that a treatment took to produce 50% and 90% insect mortality (LT_{50} and LT_{90}) and the concentration of the active ingredient in the treatment that produced 50% mortality (LC_{50}) against *Ae. aegypti* and *Ae. albopictus*. Ten replicates of six different groups of larvae were observed at 5, 10, 15, 30, 60 min, and 6 h (6 cups for 6 time points), and ten replicates of nine different groups of pupae were observed at 5, 10, 15, 30, 60 min, 6 h, 24 h, 48 h, and 72 h (9 cups for 9 time points). The end times of the larvae and pupae observation were not specified a priori, rather, they were the times that all larvae and pupae in every cup were observed to metamorphose into the next stage in the mosquito life cycle. In addition, they were the times that we observed in previous research works (Phukerd and Soonwera,

2013; Chantawee and Soonwera, 2018a). Therefore, it can be concluded that every treatment truly affected LT_{50} and LT_{90} strictly in the way that it was designed to do for Probit analysis. Significant differences between the means at $p < 0.05$ were determined by analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) with SPSS Statistical Software Package version 23.0. In this study, the percent of larvicidal activity index (LAI) was used to indicate whether an essential oil treatment destroyed larvae more effectively or less effectively than temephos. The following formula expresses LAI as the ratio of the LT_{50} of the treatment to the LT_{50} of 1% w/w temephos, $LAI = LT_{50}$ of treatment/ LT_{50} of 1% w/w temephos (1)

$LAI < 1.0$ signifies that the treatment was more toxic than 1% w/w temephos and $LAI > 1.0$ signifies that the treatment was less toxic than 1% w/w temephos.

In the design of this study, it was expected that both d-limonene and trans-anethole might provide such high mortality rates and short LT_{50} values at 5% (Aungtikun and Soonwera, 2021) that their efficacy could not be distinguished clearly. Therefore, the slopes of the linear regression lines of mortality rate versus d-limonene and trans-anethole concentration were determined and used as an indicator for distinguishing their relative efficacy at concentrations below 5%.

Table 1. List of chemical constituents of *I. verum* and *Z. limonella* essential oils.

Item	Compound ^a	Peak area %		RI ^b	KI ^c	Mode of identification
		<i>I. verum</i>	<i>Z. limonella</i>			
1	Cyclopentane	-	0.71	721	720	RI, MS
2	α -Thujene	0.09	2.28	923	923	RI, MS
3	α -Pinene	0.11	1.72	933	933	RI, MS
4	Benzene	0.57	7.48	938	938	RI, MS
5	Sabinene	-	4.46	973	973	RI, MS
6	β -Myrcene	-	0.38	983	983	RI, MS
7	α -Phellandrene	-	1.29	992	992	RI, MS
8	α -Terpinene	0.07	6.13	1015	1015	RI, MS
9	3-Carene	0.20	0.29	1017	1017	RI, MS
10	Limonene	1.70	26.42	1018	1018	RI, MS
11	1,8-Cineole	0.35	-	1024	1025	RI, MS
12	trans- β -Ocimene	-	0.30	1043	1043	RI, MS
13	γ -Terpinene	-	6.82	1048	1048	RI, MS
14	Linalyl oxide	0.08	-	1077	1077	RI, MS
15	Terpinolene	0.11	2.20	1084	1084	RI, MS
16	Linalool	-	1.05	1085	1085	RI, MS
17	3-Cyclohexene-1-ol	0.13	-	1096	1097	RI, MS
18	Cyclohexanone	-	0.45	1122	1122	RI, MS
19	Sabina ketone	-	0.70	1157	1158	RI, MS
20	Terpinen-4-ol	-	22.11	1174	1174	RI, MS
21	α -Terpineol	0.36	4.49	1179	1179	RI, MS
22	trans-Carveol	-	2.27	1217	1217	RI, MS
23	Carvone	-	2.38	1242	1242	RI, MS
24	Carvotanacetone	-	0.60	1243	1243	RI, MS
25	Anisaldehyde	1.55	-	1262	1262	RI, MS
26	trans-Anethole	93.58	-	1288	1288	RI, MS
27	Carvacrol	-	1.17	1298	1298	RI, MS
28	Eugenol	0.62	-	1351	1351	RI, MS
29	Acetic acid	-	0.91	1404	1404	RI, MS
30	β -Caryophyllene	-	0.12	1418	1418	RI, MS
31	Anisyl acetone	0.35	-	1462	1462	RI, MS
32	Spathulenol	-	0.29	1575	1575	RI, MS
33	Caryophyllene oxide	-	0.25	1580	1581	RI, MS
Total		99.87	97.27			

RI = Retention index, MS = Mass spectra.

^a Identified constituents are listed in their order of elution.

^b RI are retention indices calculated against C_7 – C_{30} n-alkanes with HP-5MS column.

^c KI = Kovats Retention Index is taken from <https://pubchem.ncbi.nlm.nih.gov>.

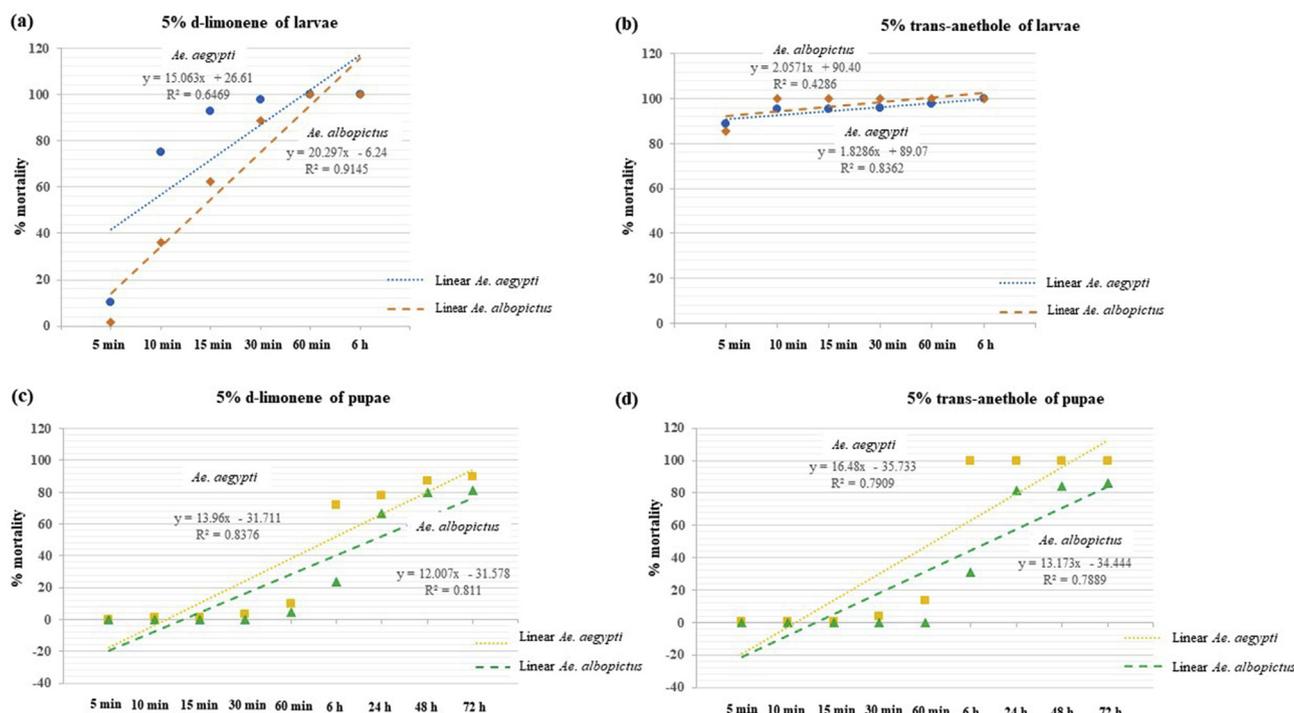


Figure 2. Relationship between mortality rate and exposure period of *Ae. aegypti* and *Ae. albopictus* larvae (a,b) and pupae (c,d) exposed to 5% d-limonene and 5% trans-anethole.

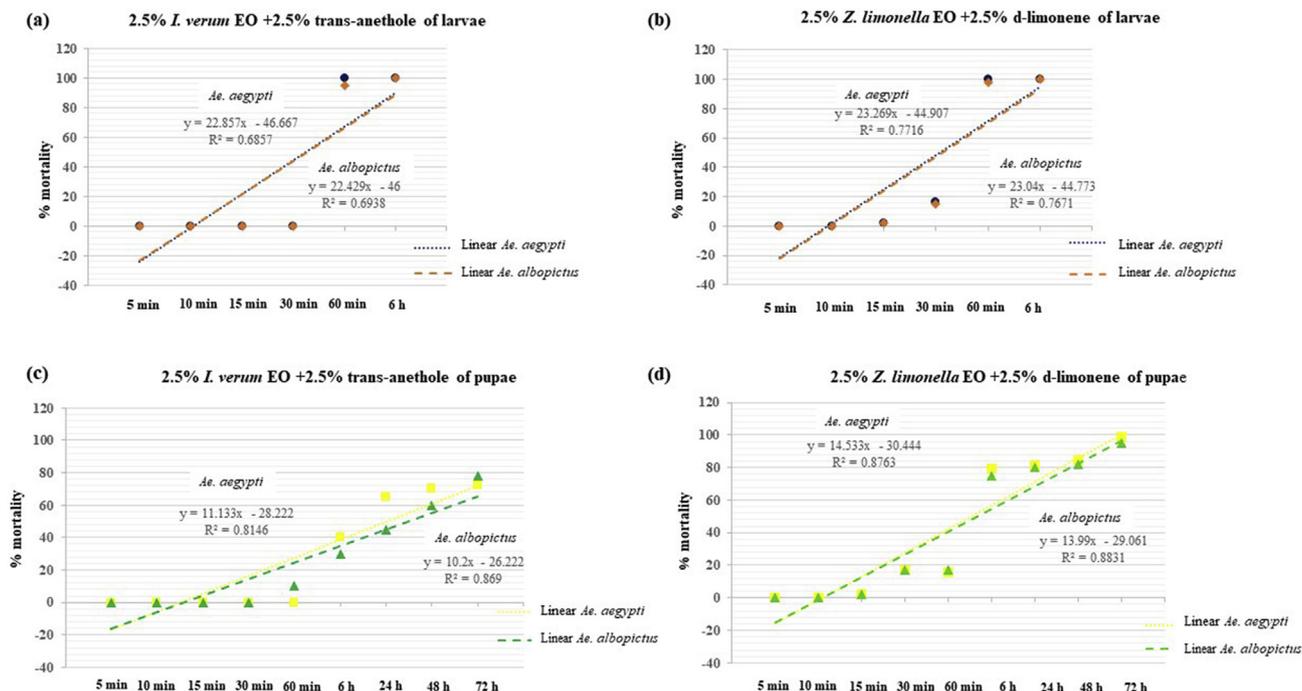


Figure 3. Relationship between mortality rate and exposure period of *Ae. aegypti* and *Ae. albopictus* larvae (a,b) and pupae (c,d) exposed to a combined formulation of EO and EO constituent.

3. Results

3.1. Chemical profile and yield of essential oils

The chemical composition of each tested essential oil was determined by GC-MS. The percentage fractions of each constituent in the total composition as well as their retention index (RI) and Kovats retention

index (KI) are listed in Table 1. A total of 15 constituents of *I. verum* EO were found, accounting for 99.87% of the EO mass. The major constituent was trans-anethole (93.58%). Other constituents that existed at a noteworthy level were limonene (1.70%), anisaldehyde (1.55%), and eugenol (0.62%). For *Z. limonella* EO, a total of 26 constituents were positively identified, accounting for 97.27% of the EO mass. The major constituent was limonene (26.42%). Other constituents that existed at a

Table 2. Mortality rate at 6 h, lethal time for 50% and 90% mortality, and lethal concentration for 50% mortality of every treatment against fourth instar larvae of *Ae. aegypti* and *Ae. albopictus*.

Mosquito Species	Treatment	Conc. (%)	LAI ^a	Mortality (%) ± SD at 6 h	Slope ±SE	LT ₅₀ ^b (LCL-UCL) (h)	LT ₉₀ ^b (LCL-UCL) (h)	χ ^{2c}	R ^{2d}	LC ₅₀ ^e (%) (LCL-UCL)
<i>Ae. aegypti</i>	d-limonene	0.5	4.24	4.8 ± 3.3g*	0.005 ± 0.002	12.3 (9.2–26.6)	16.7 (11.9–39.6)	13.2	0.111	2.9 (1.5–3.9)
		1	4.21	35.2 ± 4.4e	0.003 ± 0.000	12.2 (9.5–17.6)	20.3 (15.6–29.9)	46.6	0.377	
		2.5	0.17	52.6 ± 5.3d	0.012 ± 0.000	0.6 (0.4–1.0)	0.9 (0.5–1.1)	86.5	0.450	
		5	0.07	100a	0.217 ± 0.013	0.2 (-)	0.3 (-)	92.3	0.500	
	trans-anethole	0.5	5.86	8.0 ± 3.3f	0.003 ± 0.001	16.7 (11.4–36.4)	24.5 (16.4–55.3)	51.9	0.015	2.5 (0.8–3.0)
		1	0.72	70.8 ± 7.1cd	0.008 ± 0.000	2.1 (1.6–3.0)	4.9 (2.4–6.3)	338.4	0.537	
		2.5	0.13	97.5 ± 4.1b	0.125 ± 0.006	0.4 (0.2–0.5)	0.7 (0.4–0.9)	88.2	0.752	
		5	0.07	100a	0.114 ± 0.006	0.2 (-)	0.4 (-)	94.6	0.804	
	2.5% <i>I. verum</i> EO + 2.5% trans-anethole	-	0.03	100a	0.095 ± 0.006	0.1 (-)	0.3 (-)	2.6	0.156	
	2.5% <i>Z. limonella</i> EO + 2.5% d-limonene	-	0.07	100a	0.117 ± 0.008	0.2 (0.1–0.2)	0.4 (0.3–0.4)	100.0	0.809	
	1% w/w temephos (positive control)	-	-	79.2 ± 9.5c	0.008 ± 0.000	2.9 (2.1–4.3)	5.6 (4.2–8.6)	590.8	0.246	
	70% v/v ethyl alcohol (negative control)	-	-	0h	ns ^f	ns	ns	ns	ns	
ANOVA Df total; P value				119; P < 0.0001						
<i>Ae. albopictus</i>	d-limonene	0.5	4.83	0.8 ± 0.2f	0.002 ± 0.001	20.1 (-)	29.2 (-)	69.3	0.322	3.1 (1.6–4.3)
		1	6.70	0.8 ± 0.2f	0.004 ± 0.001	14.5 (10.4–28.5)	20.6 (14.4–42.4)	47.7	0.996	
		2.5	0.19	51.4 ± 6.1d	0.035 ± 0.000	0.6 (0.4–1.2)	1.0 (0.8–1.5)	79.3	0.510	
		5	0.10	100a	0.124 ± 0.008	0.3 (0.2–0.3)	0.4 (0.4–0.5)	80.4	0.803	
	trans-anethole	0.5	4.40	1.6 ± 0.3e	0.004 ± 0.002	13.4 (9.7–22.6)	18.4 (13.8–35.6)	30.8	1.000	2.4 (1.5–3.9)
		1	4.43	1.6 ± 1.0e	0.004 ± 0.001	13.3 (9.9–24.5)	18.7 (13.5–36.3)	30.4	0.055	
		2.5	0.16	86.4 ± 6.3c	0.241 ± 0.005	0.5 (0.3–0.6)	0.8 (0.4–1.0)	88.2	0.752	
		5	0.10	100a	0.079 ± 0.005	0.3 (-)	0.6 (-)	94.8	0.632	
	2.5% <i>I. verum</i> EO + 2.5% trans-anethole	-	0.03	100a	0.540 ± 0.040	0.1 (-)	0.1 (-)	2.4	0.825	
	2.5% <i>Z. limonella</i> EO + 2.5% d-limonene	-	0.10	100a	0.106 ± 0.007	0.3 (0.2–0.3)	0.5 (0.4–0.5)	93.3	0.751	
	1% w/w temephos (positive control)	-	-	95.2 ± 8.7b	0.011 ± 0.001	3.1 (2.5–3.6)	4.8 (4.1–6.1)	265.0	0.450	
	70% v/v ethyl alcohol (negative control)	-	-	0g	ns	ns	ns	ns	ns	
ANOVA Df total; P value				119; P < 0.0001						

^a LAI = Larvicidal activity index; LAI <1.0 signifies that the treatment was more toxic than 1% w/w temephos; LAI >1.0 signifies that the treatment was less toxic than 1% w/w temephos.

^b LT₅₀, LT₉₀ = Lethal Time for 50% and 90% mortality with 95% confidence limit; LCL = lower confidence limit; UCL = upper confidence limit.

^c χ² = Chi-square value with α = 0.05.

^d R² = Regression coefficient.

^e LC₅₀ = Lethal Concentration for 50% mortality with 95% confidence limit.

^f ns = not significant.

* The means in each row against each mosquito species that are followed by different letters are significantly different (P < 0.05, by ANOVA and Duncan's Multiple Range Test).

Table 3. Mortality rate at 72 h, lethal time for 50% and 90% mortality, and lethal concentration for 50% mortality of every treatment against the pupae of *Ae. aegypti* and *Ae. albopictus*.

Mosquito Species	Treatment	Conc. (%)	Mortality (%) ± SD at 72 h	Slope ±SE	LT ₅₀ ^a (LCL-UCL) (h)	LT ₉₀ ^a (LCL-UCL) (h)	χ ^{2b}	R ^{2c}	LC ₅₀ ^d (%) (LCL-UCL)
<i>Ae. aegypti</i>	d-limonene	0.5	0f*	-	-	-	-	-	3.7
		1	2.4 ± 1.4d	0.000 ± 0.000	197.8 (131.2–465.0)	197.8 (1297.8–465.0)	106.4	0.074	(2.8–4.7)
		2.5	53.1 ± 3.5c	0.001 ± 0.000	45.3 (40.5–48.7)	55.2 (48.5–58.6)	134.2	0.085	
		5	94.4 ± 5.3b	0.002 ± 0.000	23.8 (16.3–35.5)	59.2 (44.6–90.4)	872.1	0.023	
	trans-anethole	0.5	0.8 ± 0.2e	0.001 ± 0.000	163.2 (119.9–277.2)	247.8 (178.5–433.0)	94.9	0.288	3.3
		1	3.2 ± 1.1d	0.000 ± 0.000	142.5 (103.0–260.2)	218.1 (153.8–413.6)	172.5	0.020	(2.5–3.9)
		2.5	52.6 ± 4.3c	0.002 ± 0.000	47.9 (45.5–52.5)	58.1 (56.5–60.1)	122.5	0.345	
		5	100a	0.005 ± 0.000	6.9 (6.2–7.7)	11.5 (10.4–12.9)	126.6	0.824	
	2.5% <i>I. verum</i> EO + 2.5% trans-anethole	-	100a	0.031 ± 0.003	1.5 (1.4–1.8)	2.2 (2.0–2.6)	39.7	0.360	
	2.5% <i>Z. limonella</i> EO + 2.5% d-limonene	-	100a	0.003 ± 0.003	15.3 (14.2–16.6)	23.8 (21.9–26.0)	94.0	0.823	
	1% w/w temephos (positive control)	-	0f	ns ^e	ns	ns	ns	ns	
	70% v/v ethyl alcohol (negative control)	-	0f	ns	ns	ns	ns	ns	
ANOVA Df total; P value			119; P < 0.0001						
<i>Ae. albopictus</i>	d-limonene	0.5	1.6 ± 1.16e	0.000 ± 0.000	161.0 (120.2–288.7)	224.3 (162.3–422.0)	22.4	1.000	3.7
		1	2.4 ± 1.8d	0.000 ± 0.000	179.8 (126.1–413.7)	249.9 (169.6–604.7)	27.6	0.283	(2.8–4.7)
		2.5	43.2 ± 8.5c	0.001 ± 0.000	53.2 (48.3–59.9)	64.4 (58.1–70.2)	153.5	0.170	
		5	89.6 ± 9.1b	0.002 ± 0.000	28.5 (23.3–35.6)	53.2 (44.3–67.3)	486.0	0.620	
	trans-anethole	0.5	2.4 ± 1.8d	0.000 ± 0.000	207.7 (139.4–508.8)	2967.7 (194.4–752.9)	40.1	0.178	3.4
		1	2.6 ± 2.2d	0.000 ± 0.000	174.5 (-)	232.7 (-)	16.3	0.333	(2.5–3.9)
		2.5	41.7 ± 9.4c	0.001 ± 0.000	57.6 (54.3–60.5)	60.4 (58.2–65.5)	785.1	0.781	
		5	86.4 ± 8.7b	0.002 ± 0.000	28.8 (23.3–36.3)	55.8 (46.2–71.2)	516.1	0.528	
	2.5% <i>I. verum</i> EO + 2.5% trans-anethole	-	100a	0.009 ± 0.001	5.2 (4.8–5.7)	7.6 (7.0–8.5)	98.9	0.979	
	2.5% <i>Z. limonella</i> EO + 2.5% d-limonene	-	100a	0.005 ± 0.000	7.9 (7.3–8.6)	12.4 (11.5–13.6)	86.2	0.903	
	1% w/w temephos (positive control)	-	0f	ns	ns	ns	ns	ns	
	70% v/v ethyl alcohol (negative control)	-	0f	ns	ns	ns	ns	ns	
ANOVA Df total; P value			119; P < 0.0001						

^a LT₅₀, LT₉₀ = Lethal Time for 50% and 90% mortality with 95% confidence limit; LCL = lower confidence limit; UCL = upper confidence limit.

^b χ² = Chi-square value with α = 0.05.

^c R² = Regression coefficient.

^d LC₅₀ = Lethal Concentration for 50% mortality with 95% confidence limit.

^e ns = not significant.

* The means in each row against each mosquito species followed by different letters are significantly different (P < 0.05, by ANOVA and Duncan's Multiple Range Test).



Figure 4. Induced morphological aberrations at time of death of treated *Ae. aegypti* larvae and pupae: (a) normal larvae (NL); (b) deformed larva (DL); (c) larvae died before coming completely out of the larval exoskeleton (PP); (d) pupae came completely out of the larvae exoskeleton, but with a different color than the normal color (WP); (e) dead normal brown pupa (BP); (f) deformed pupa exhibited the appearance of an elephant head (DP); (g) adult mosquitoes died as they were emerging from their exoskeleton (PA); (h) survived adults with morphological defects (DA); and (i) normal adult (NA).

noteworthy level were terpinen-4-ol (22.11%), benzene (7.48%), and γ -terpinene (6.82%).

Regarding yield, the extraction yield of *I. verum* and *Z. limonella* dried fruits EOs by the water distillation method were 9.6% and 4.0% (v/w) of EO per kg of dry weight, respectively. All of the oils were light, pale yellow colors.

3.2. Larvicidal and pupicidal activities

In the following paragraphs, three groups of larvicidal and pupicidal results as three groups: 1) results on the efficacy of each individual EO constituent; 2) results on the efficacy of two combined formulations of EO mixed with EO constituent; and 3) results on the positive and negative control.

The first group assessed the degree of toxicity of individual EO constituents against the larvae and pupae of the two mosquito vectors (*Ae. aegypti* and *Ae. albopictus*), in terms of mortality rate, LT_{50} value, LC_{50} value, and larvicidal activity index (LAI). The observed values of these indicators are tabulated in Table 2 (larvicidal results) and 3 (pupicidal results). The larvae and pupae of *Ae. aegypti* were significantly more susceptible to d-limonene and trans-anethole than the larvae and pupae of *Ae. albopictus* measured by LT_{50} . Both d-limonene and trans-anethole at 5% concentration were significantly more effective against *Ae. aegypti* and *Ae. albopictus* than at lower concentrations. Five percent trans-anethole against the larvae of the two mosquito species was 100% effective after 6 h of exposure, with a short LT_{50} of 0.2–0.3 h as well as a low LAI of 0.07–0.10, as well as 86.4–100% mortality rate against the

pupae after 72 h of exposure, with an LT_{50} ranging from 6.9–28.8 h. Five percent d-limonene caused 100% mortality after 6 h of exposure and provided a short LT_{50} of 0.2–0.3 h as well as a low LAI of 0.07–0.10 against the larvae of both species. Against the pupae, it caused 89.6–94.4% mortality after 72 h of exposure and provided an LT_{50} ranging from 23.8–28.5 h. However, trans-anethole and d-limonene at 0.5–2.5% caused 0.8–97.5% mortality rate (LT_{50} of 0.4–20.1 h) against the larvae and 1.6–53.1% mortality rate (LT_{50} of 45.3–197.8 h) against the pupae of the two mosquito species. More spectacularly, both d-limonene and trans-anethole at 5% were more toxic to the larvae and pupae of both mosquito species than 1% temephos, a common insecticide in the market—they provided an LAI of less than 1.0. Furthermore, both EO constituents, d-limonene and trans-anethole exhibited stronger larvicidal and pupicidal activities against the mosquitoes of both species with no significant difference in terms of LC_{50} .

As stated in the methodology section, linear regression analyses of the larvicidal and pupicidal activities versus concentration provided by trans-anethole against *Ae. aegypti* and *Ae. albopictus* were performed as well as by d-limonene so that the values of the slopes of the regression lines of both compounds could be used to differentiate the relative strength of each compound. All obtained regression lines were positively linear with R^2 close to 1 (Figure 2). The regression line slopes of the larvicidal and pupicidal activities versus the concentration of trans-anethole (below 5%) against *Ae. aegypti* was higher than those against *Ae. albopictus*, signifying that, at a concentration lower than 5%, increasing the concentration of d-limonene and trans-anethole by the same amount would increase the toxicity of trans-anethole more than

that of d-limonene. Therefore, trans-anethole was found more effective than d-limonene, even though their LT_{50} values were not very different.

The second group of results showed the efficacy of two combinations of EO mixed with EO constituent. Both combinations provided the same highest mortality rate (100%) against larvae and pupae, but the combination of 2.5% *I. verum* EO + 2.5% trans-anethole provided a shorter LT_{50} for pupae than 2.5% *Z. limonella* EO + 2.5% d-limonene: the *I. verum* combination provided an LT_{50} of 0.1 h and LAI = 0.03 of larvae and LT_{50} of 1.5–5.2 h of pupae, while the *Z. limonella* combination provided an LT_{50} of 0.2–0.3 h and LAI = 0.07–0.10 of larvae and LT_{50} of 7.9–15.3 h of pupae.

Two combined EO formulations exhibited synergistic effects, manifesting higher toxicity than those of individual EOs to larvae and pupae of both species, with an SI ranging from 0.2–0.6 and an SI status of “synergy”, except 2.5% *Z. limonella* EO + 2.5% d-limonene against the larvae of the two mosquito species with an SI = 1 and “no synergy” status (see Figure 5). Nevertheless, the relative efficacy of the two combined formulations could not be distinguished by the mortality rate and LT_{50} that they provided alone because the mortality rates were the same at 100%, and the LT_{50} against the larvae of both species were also the same. Therefore, it was decided to distinguish their relative efficacy based on the steepness of the

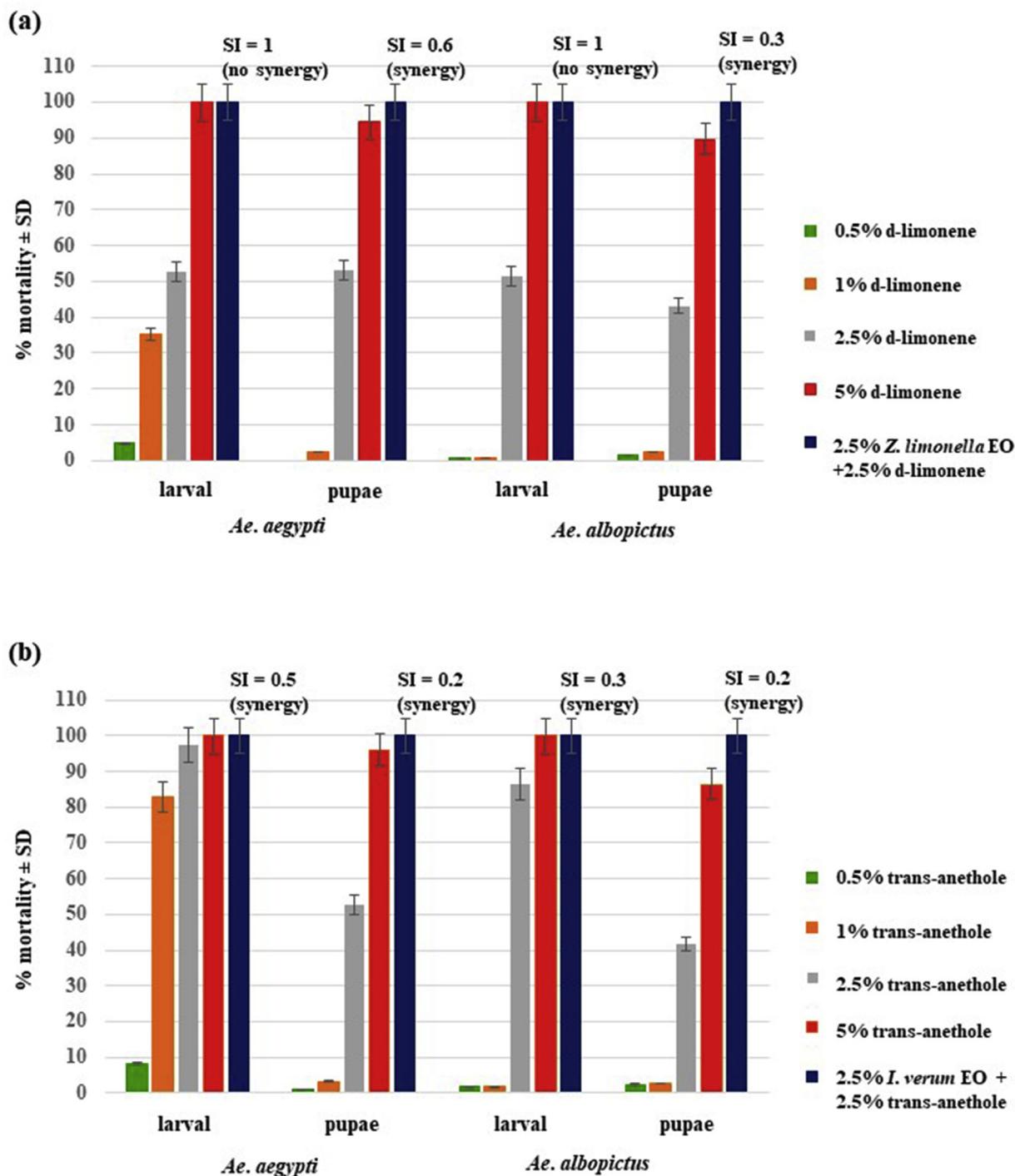


Figure 5. Synergistic effects of combined EO and EO constituent, *Z. limonella* EO + d-limonene (a) and *I. verum* EO + trans-anethole (b) in terms of mortality rate against larvae and pupae of *Ae. aegypti* and *Ae. albopictus*. Note: Synergistic index (SI) = [LT_{50} of combined EO / LT_{50} of individual EO]; SI < 1 indicated a synergistic effect; SI > 1 indicated an antagonistic effect; and SI = 1 indicated a no synergistic effect (Aungtikun et al., 2021).

slope of the linear positive relationship between the mortality rate and exposure period, of which R^2 was very close to 1 for both formulations (see Figure 3). Based on the relative values of these slopes, it can be concluded that the combined formulation of 2.5% *I. verum* EO + 2.5% trans-anethole was more effective than the other combination, and so had a higher potential for developing into a commercial product.

Finally, the third group of results showed the efficacy of the positive and negative controls. The negative control, ethyl alcohol, was not toxic to the larvae and pupae at all. The most significant finding, however, was that the positive control, 1% (w/w) temephos, a widely used synthetic insecticide, took 10 times longer (LT_{50} 2.9–3.1 h) to destroy the mosquitoes than 5% trans-anethole (LT_{50} 0.1–0.3 h) and 5% d-limonene as well as the two combined formulations.

3.3. Morphological aberrations at time of death

After 6 h of exposure to each treatment, the morphological changes at death of larvae, pupae, and adults of *Ae. aegypti* and *Ae. albopictus* were observed. The changes to the larvae were changes in the thorax

pigmentation and shape of head (H) and thorax (TH), abdominal damage (AB) (midgut), as well as damage to respiratory siphon (RS) and anal papillae (AP) surface (Figure 6). It can be seen in Table 4, showing the percentage of dead larvae with each observed morphological aberration to the total number of dead larvae, that d-limonene and trans-anethole at all tested concentrations as well as the two formulations of combined EO mixed with EO constituent all caused certain morphological abnormalities at time of death of most larval-stage mosquitoes. Five percent d-limonene and 5% trans-anethole, as well as 2.5% *I. verum* EO + 2.5% trans-anethole, caused most larvae to die with NL morphology, while 2.5% *Z. limonella* EO + 2.5% d-limonene induced mostly DL morphology. In contrast, 1% (w/w) temephos caused most larvae to die with an NL morphology (Figure 4).

For pupae, the morphological changes at time of death after 72 h of treatment included changes in pigmentation and lyses of cephalothorax cells as well as changes in the shape of the head (H), abdomen (AB), and respiratory trumpets (RT) (Figure 7). Table 5 shows the observed percentage of pupae with each type of morphological aberration at death to the total number of dead pupae. Treated with d-limonene and trans-anethole at 5% concentration as well as each of the two formulations



Figure 6. Morphological changes at the head (H), thorax (TH), abdomen (AB), anal papillae (AP), and respiratory siphon (RS) of treated *Ae. aegypti* larvae induced by control (a–c), d-limonene (d–f), trans-anethole (g–i), and a combined EO and EO constituent formulation (j–l).

Table 4. Percentage of treated fourth-instar larvae of *Ae. aegypti* and *Ae. albopictus* that died with each type of morphological abnormality.

Mosquito Species	Treatment	Conc. (%)	Stage at death (%)								Mortality (%)	Normally-developed adults (NA) (%)
			NL	DL	PP	WP	DP	BP	PA	DA		
<i>Ae. aegypti</i>	d-limonene	0.5	-	-	4	-	-	-	0.8	-	4.8	95.2
		1	18.4	-	1.6	-	-	12	1.6	1.6	35.2	64.8
		2.5	30.6	-	5	-	-	10	3	4	52.6	47.4
		5	96.8	-	-	-	-	3.2	-	-	100	-
	trans-anethole	0.5	3.2	-	2.4	-	-	2.4	-	-	8.0	92.0
		1	84	-	-	-	-	7.2	1.6	-	92.8	7.2
		2.5	95	-	-	-	-	2.5	-	-	97.5	2.5
		5	96	-	-	-	-	4	-	-	100	-
	2.5% <i>I. verum</i> EO +2.5% trans-anethole		-	86	14	-	-	-	-	-	100	-
	2.5% <i>Z. limonella</i> EO +2.5% d-limonene		-	44	46	-	-	10	-	-	100	-
1% w/w temephos (positive control)		-	76	-	3.2	-	-	-	-	79.2	20.8	
70% v/v ethyl alcohol (negative control)		-	-	-	-	-	-	-	-	0	100	
<i>Ae. albopictus</i>	d-limonene	0.5	-	-	-	-	-	0.8	-	-	0.8	99.2
		1	0.8	-	-	-	-	-	-	-	0.8	99.2
		2.5	50	-	1.4	-	-	-	-	-	51.4	48.6
		5	99.2	-	0.8	-	-	-	-	-	100	-
	trans-anethole	0.5	0.8	-	0.8	-	-	-	-	-	1.6	98.4
		1	-	-	-	-	-	1.6	-	-	1.6	98.4
		2.5	85	-	-	-	-	1.4	-	-	86.4	13.6
		5	100	-	-	-	-	-	-	-	100	-
	2.5% <i>I. verum</i> EO +2.5% trans-anethole		-	60	38	-	2	-	-	-	100	-
	2.5% <i>Z. limonella</i> EO +2.5% d-limonene		-	32	54	9	5	-	-	-	100	-
1% w/w temephos (positive control)		-	84.8	-	-	-	4	-	-	88.8	11.2	
70% v/v ethyl alcohol (negative control)		-	-	-	-	-	-	-	-	0	100	

Note: NL = normal larva, DL = deformed larva, PP = pre-pupa (pupa that has not emerged completely out of the larval exoskeleton), WP = white pupa, DP = deformed pupa, BP = dead normal brown pupa, PA = adult still attached to pupal case, DA = deformed Adult, and NA = normal adult.

of combined EO mixed with EO constituent, most pupae died with a BP morphology (Figure 4).

4. Discussion

This section discusses four main groups of results: 1) GC/MS chemical composition analyses; 2) larvicidal and pupicidal efficacy of each individual EO constituent and two combined formulations of EO mixed with EO constituent; 3) types of morphological aberration at time of death of treated larvae and pupae; and 4) comparative efficacy of temephos and EO formulations. In addition, a few further developmental studies of these EOs are needed to develop them into commercial products.

The first main topic of discussion involves GC/MS chemical composition analysis results. To be able to compare the experimental outcomes of this study meaningfully with those of other studies on EO and insect pests, the composition of the extracted EOs in this study must be the same or nearly the same as the composition of the reported EOs in the literature. Therefore, the chemical compositions of all extracted essential oils were determined by GC-MS to ascertain that they were the same as those reported previously (Aungtikun et al., 2021; Wongkattiya et al., 2018). It was observed that the major constituent of *I. verum* EO was trans-anethole (93.58% of the total composition), at nearly the same as that (94.0%) reported in a previous research paper (Aungtikun et al., 2021). On the other hand, a paper by Matos et al. (2020) reported a lower percentage of trans-anethole (88.6%) in the total composition. This discrepancy could be due to many factors, e.g., different geographical areas of the farms where these plants had been grown (Wongkattiya et al., 2018), different soil and fertilizer conditions, and different harvest times (i.e., harvesting the plants at which growth stage and season) (Wongkattiya et al., 2018; Aungtikun and Soonwera, 2021). For *Z. limonella* EO, a total of 26 constituents were positively identified, accounting for 97.14% of the EO mass. The major constituent was

d-limonene, at 26.42%, much lower than 43.6% reported by Charoensup et al. (2016) and 57.9% reported by Wongkattiya et al. (2018). Again, this discrepancy was likely to be from the reasons mentioned above. Incidentally, the yields of the two extracted EOs found in this study were almost identical to those reported in three papers (Aungtikun et al., 2021; Charoensup et al., 2016; Waliwitiya et al., 2009), indicating that these yield figures were valid and reliable.

The second main topic of discussion involves larvicidal and pupicidal efficacy of each individual EO constituent and formulation of EO combined mixed with EO constituent. The first part is about the efficacy of each individual EO constituent. Between the two major EO constituents, d-limonene and trans-anethole at 5% concentration exhibited a significantly higher efficacy against *Ae. aegypti* and *Ae. albopictus* than at lower concentrations, implying that an even higher concentration would provide even higher values for some insecticidal indicators. However, as they were going to be used as insecticidal agents, using the lowest concentration that would achieve the intended mortality rate would be better than using a higher concentration. Therefore, this study was not designed to test d-limonene and trans-anethole at concentrations higher than 5% because they had already been shown to provide a full 100% mortality rate against *Ae. aegypti* at 3% (Dhinakaran et al., 2019). Both d-limonene and trans-anethole have been reported to provide high efficacy against several insect pests. Examples of papers reporting the high efficacy of d-limonene and trans-anethole are such as the following: a paper by Seo et al. (2015) reporting that d-limonene had strong larvicidal activity against *Ae. albopictus*; a paper by Waliwitiya et al. (2009) reporting that trans-anethole had a strong oviposition-deterrent capacity against *Ae. aegypti*; a paper by Pandiyan et al. (2019) indicating that the LC₅₀ of trans-anethole against *Ae. aegypti* larvae were 50.2 mg L⁻¹; and a paper by Aungtikun et al. (2021) reporting a strong insecticidal effect of trans-anethole against *Musca domestica* adults. Supporting by the papers above, the results of this study showed that these two compounds were

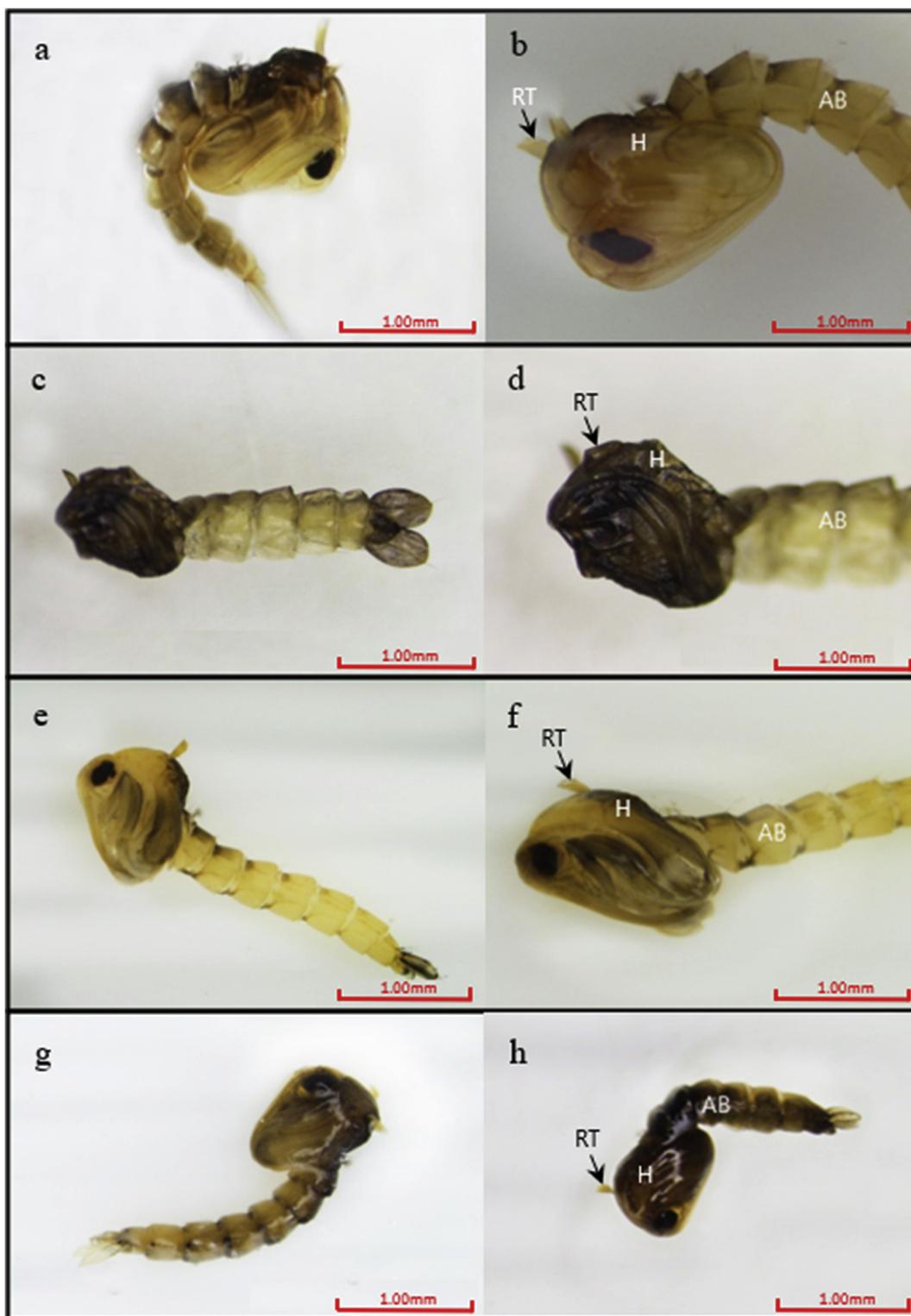


Figure 7. Morphological changes at the head (H), abdomen (AB), and respiratory trumpets (RT) of treated *Ae. aegypti* pupae induced by control group (a–b), d-limonene (c–d), trans-anethole (e–f), and a combined EO and EO constituent formulation (g–h).

highly toxic against the larvae and pupae of *Ae. aegypti* and *Ae. albopictus*. The second part of this paragraph reports the efficacy of formulations of combined EO mixed with EO constituent. The formulation of 2.5% *I. verum* EO + 2.5% trans-anethole exhibited stronger larvicidal and pupicidal activities than 2.5% *Z. limonella* EO + 2.5% d-limonene. Moreover, a strong synergistic effect between *I. verum* EO and trans-anethole seemed to be indicated by their relative LT_{50} values. The value of LT_{50}

against the pupae of *Ae. albopictus* provided by 2.5% *I. verum* EO + 2.5% trans-anethole combination (5.2 h) was quite shorter than that (28.8 h) of trans-anethole alone, seeming to indicate a synergistic effect between *I. verum* EO and trans-anethole. The combined formulation of 2.5% *Z. limonella* EO + 2.5% d-limonene also showed synergistic effect between its components, but weaker than the 2.5% *I. verum* EO + 2.5% trans-anethole formulation. This kind of synergistic effect between EO

Table 5. Percentage of treated pupae of *Ae. aegypti* and *Ae. albopictus* that died with each type of morphological abnormality.

Mosquito Species	Treatment	Conc. (%)	Stage at death (%)				Mortality (%)	Normally-developed adults (NA) (%)
			BP	DP	PA	DA		
<i>Ae. aegypti</i>	d-limonene	0.5	-	-	-	-	-	100
		1	1.6	-	0.8	-	2.4	97.6
		2.5	52	-	1.1	-	53.1	46.9
		5	93.6	-	0.8	-	94.4	5.6
	trans-anethole	0.5	0.8	-	-	-	0.8	99.2
		1	2.4	-	0.8	-	3.2	96.8
		2.5	51	-	1.6	-	52.6	47.4
		5	100	-	-	-	100	-
	2.5% <i>I. verum</i> EO +2.5% trans-anethole	-	82	18	-	-	100	-
	2.5% <i>Z. limonella</i> EO +2.5% d-limonene	-	60	36	4	-	100	-
1% w/w temephos (positive control)	-	-	-	-	-	-	100	
70% v/v ethyl alcohol (negative control)	-	-	-	-	-	-	100	
<i>Ae. albopictus</i>	d-limonene	0.5	-	-	1.6	-	1.6	98.4
		1	2.4	-	-	-	2.4	97.6
		2.5	40.2	-	2	1	43.2	56.8
		5	77.6	-	8	4	89.6	14.4
	trans-anethole	0.5	-	-	2.4	-	2.4	97.6
		1	2.6	-	-	-	2.6	97.4
		2.5	39	-	1.2	1.5	41.7	58.3
		5	72	-	5.6	8.8	86.4	13.6
	2.5% <i>I. verum</i> EO +2.5% trans-anethole	-	86	14	-	-	100	-
	2.5% <i>Z. limonella</i> EO +2.5% d-limonene	-	72	28	-	-	100	-
1% w/w temephos (positive control)	-	-	-	-	-	-	100	
70% v/v ethyl alcohol (negative control)	-	-	-	-	-	-	100	

Note: BP = dead normal brown pupa, DP = deformed pupa, PA = adult still attached to pupal case, DA = deformed Adult, NA = normal adult.

constituents has been reported widely. For example, a synergistic effect between d-limonene and trans-anethole against the larvae of *Cx. quinquefasciatus* was reported in papers by [Pavela \(2015b\)](#) and [Andrade-Ochoa et al. \(2018\)](#). The same synergistic effect against *Ae. aegypti* was reported in a paper by [Dhinakaran et al. \(2019\)](#). The most important conclusion from these experiments was that the two combined formulations in this study were even more potent than 1% (w/w) temephos, a seriously harmful synthetic insecticide that they were intended to replace. In this sense, they were at least as effective as d-limonene and trans-anethole individually as a replacement for temephos.

The third main topic of discussion involves types of morphological aberration of treated larvae and pupae at their time of death. Phytochemicals from several plants could cause morphological abnormalities at time of death of mosquitoes at different developmental stages ([Fujiwara et al., 2017](#); [Senthil-Nathan, 2020](#)). Morphological aberrations can help elucidate the mechanisms of action of various larvicidal and pupicidal agents ([Araújo et al., 2018](#); [Fujiwara et al., 2017](#)). Morphological abnormality in mosquitoes can be the result of their disrupted endocrinological balance as well as damaged muscles by EOs ([Fallatah and Khater, 2010](#)). Moreover, some EOs affected larvae's movement to the water surface to breathe, and some EOs induced neurotoxicity via various mechanisms, disrupting the normal process of morphogenesis and leading to abnormal feeding and development of adult mosquitoes and their flying ability ([Silvério et al., 2020](#)). In this study, the tested treatment formulations caused nine types of morphological aberration at time of death of larvae, pupae, and adults of *Ae. aegypti* and *Ae. albopictus*. The variety of aberration types depended on their concentration: a lower concentration induced a larger variety of morphological aberrations than a higher concentration did. The reason for this result is likely to be that the insects treated by the compound at a lower concentration did not die as quickly as those treated by the same compound at a higher concentration, hence many types of abnormality had more time to develop before they died ([Soonwera and Phasomkusolsil, 2016](#)). However, most

larvae died with an NL morphology, and most pupae died with a BP morphology, indicating that the formulations were very toxic to the mosquitoes and destroyed them so quickly that their body had no time to develop morphological changes ([Soonwera and Phasomkusolsil, 2016](#); [Chantawee and Soonwera, 2018a](#)). Even though most EO treatments caused NL morphology of larvae identical to the morphology that temephos caused, the reasons for this similarity might be different. It is likely that the NL morphology caused by most EO treatments was the result of their high efficacy explained above, while the NL morphology caused by temephos was due to its specific mode of action against larvae ([Naqqash et al., 2016](#)).

Most larvae treated with d-limonene in this study did not survive long. At the time of death, those that died underwent morphological changes in their thorax pigmentation as well as head and thorax shapes and damage to midgut, anal papillae, and respiratory siphon. In addition, the abnormal morphology at time of death of *Ae. aegypti* and *Ae. albopictus* pupae included lysis of cephalothorax cells, cuticle color change, and changes in the shape of the head, midgut, and respiratory trumpets. These findings agree well with those from [Cruz et al. \(2017\)](#). That paper concluded that d-limonene, a monoterpene, exerted its toxic activity via insect cuticle penetration (contact effect). The authors offered an explanation that the lipophilicity of d-limonene and its affinity to the cuticle of insects made the compound easily absorbed through the cuticle, leading to strong toxicity against the insect. These findings on morphological aberration at time of death induced by d-limonene are fully supported by other previous findings in the literature. For example, in this study, d-limonene induced dark pigments all over the chest of *Ae. aegypti* larvae and at the base of its respiratory siphon. It also damaged anal papillae, midgut, and hemolymph, identical to the abnormalities reported by [Rocha et al. \(2015\)](#), [Dhinakaran et al. \(2019\)](#), and [Silvério et al. \(2020\)](#). In addition, d-limonene induced the following changes—a more fragile appearance, limited mobility, wrinkled body surfaces at the head, thorax, and respiratory siphon as well as color change of abdominal

cuticles—identically reported by [Botas et al. \(2017\)](#). Also, these types of morphological changes at time of death were not specific to *Ae. aegypti*: [Soonwera and Phasomkusolsil \(2017\)](#) reported that *Z. limonella* EO, of which d-limonene was the major constituent, induced morphological changes in the anal papillae and thorax pigmentation as well as the thorax shape of *Cx. quinquefasciatus* mosquitoes. Furthermore, the morphological changes at time of death of *Ae. aegypti* pupae were identical to the changes described by [Chantawee and Soonwera \(2018a\)](#) of darkened head, abdomen, and respiratory trumpets cuticles of *Ae. aegypti* pupae induced by *Anethum graueolens* EO, of which d-limonene was the major constituent.

Switching from discussing the results on d-limonene to those on trans-anethole, the morphological changes in the larvae treated with trans-anethole were identical to the changes induced by an EO containing trans-anethole previously reported by [Rocha et al. \(2015\)](#); the changes induced by *An. graueolens* EO reported by [Chantawee and Soonwera \(2018a\)](#); and the changes induced by *C. citratus* and *S. aromaticum* EOs reported by [Soonwera and Phasomkusolsil \(2016\)](#). These identical morphological changes—change in thorax pigmentation, partial lysis of midgut epithelial cells, abnormal thorax cell shape, change in thorax shape, and midgut damage—suggest that the mechanism of toxicity of trans-anethole and those EOs was likely to be the same. It is likely that this mechanism depended strongly on the lipophilicity of trans-anethole, as [Hashem et al. \(2018\)](#) concluded that the lipophilicity of trans-anethole was the key to its easy penetration through the cuticle.

Similar to the morphological changes induced by trans-anethole in the larvae, the changes in the pupae included contraction in the abdominal region, lysis of cephalothorax cell, midgut damage, and change in head shape. These findings are fully supported by findings on morphological changes against *An. dirus* and *Ae. aegypti* pupae exposed to EOs from *C. citratus* and *S. aromaticum* in a study by [Soonwera and Phasomkusolsil \(2016\)](#). In addition, [Chantawee and Soonwera \(2018b\)](#) reported burned and swollen larvae of *M. domestica* housefly as well as swollen pupae with shrunk exoskeleton at the time of death caused by *F. vulgare* EO, of which trans-anethole was the major constituent. The nearly identical aberrations induced by d-limonene, trans-anethole, and the combined formulations, suggested that their modes of action against *Ae. aegypti* and *Ae. albopictus* were the same. This was supported by the internal morphological changes to the larvae and pupae at the time of death induced by these treatments: internal changes to the digestive tract and tracheal systems. The internal changes that were observed in this study—damaged tracheal system, midgut, and anal papillae of *Ae. aegypti* larvae—were similar to those reported by [Fujiwara et al. \(2017\)](#) induced by a combination of EO constituents, methyl cinnamate + linalool at 1:4 ratio.

The fourth main topic of discussion involves the comparative efficacy of temephos and the EO treatments and ends with a few suggestions for further research and development of these EOs.

In the present study, 5% d-limonene, and 5% trans-anethole, 2.5% *I. verum* EO + 2.5% trans-anethole, and 2.5% *Z. limonella* EO + 2.5% d-limonene formulations were found to be more toxic to the larvae and pupae of both mosquito species than 1% (w/w) temephos, a widely-used synthetic insecticide. This anticipated lower efficacy of temephos was most likely due to the developed resistance that today's generation of insect pest populations inherited from the older generations' defensive response to temephos that has been applied too extensively in the past. The resistance ratio of temephos was LC_{50} of 0.008 mg/L and a longer LT_{50} of 34.8 h ([Fatimah et al., 2020](#)). This alarming conclusion was supported by findings from several studies. For example, [Chantawee and Soonwera \(2018a\)](#) showed that, at the time of their study, several EOs provided stronger larvicidal and pupicidal activities against *Ae. aegypti* than temephos. In that study, the larval mortality rate provided by temephos ranged from 79.2–95.2% at 6 h; its LT_{50} ranged from 3.1–5.6 h; and the pupal mortality rate was zero. Others supporting studies are by [Valle et al. \(2019\)](#), [Chaiphongpachara and Moolrat \(2017\)](#), and [Bisset et al. \(2020\)](#) also concluded that *Ae. aegypti* has developed resistance to

temephos. Even more dramatically, [Melo-Santos et al. \(2010\)](#) and [Naqqash et al. \(2016\)](#) reported that the median lethal resistance ratio (RR_{50}) of temephos was up to 200 times higher than when it was introduced 20 years ago. Today's highly elevated insect resistance to temephos was our primary reason to find a more effective alternative to it. The secondary reason was its dangerous neurotoxic side effects on humans. It caused the following human diseases: attention deficit hyperactivity disorder, Alzheimer's disease, and schizophrenia ([Martins Laurentino et al., 2019](#)). Its strong neurotoxicity for both insects and mammals was exerted through a common cholinergic pathway ([Satriawan et al., 2019](#); [Naqqash et al., 2016](#)), a vital excitatory neurotransmission system in insects and mammals ([Thany et al., 2010](#); [Satriawan et al., 2019](#)). A clear piece of evidence of the degree of severity of global temephos poisoning has been reported by [Satriawan et al. \(2019\)](#): cases of temephos poisoning to farmers and environmental monitoring agents were more than 70% of the total pesticide poisoning cases for those occupations. In addition, [Benitez-Trinidad et al. \(2015\)](#) reported damaging genetic effects (damaged lymphocyte DNA) in humans caused by temephos. The US Environmental Protection Agency ([USA EAP, 2001](#)) classified temephos as a contaminant in the marine environment, i.e., their accumulation in soil and water would cause toxicity to aquatic organisms.

In contrast to the harmful temephos, EOs from *Z. limonella* and *I. verum* as well as their main constituents—d-limonene and trans-anethole—have already been used safely since ancient time in Asian countries as food and traditional medicine ([Wongkattiya et al., 2018](#); [Wang et al., 2011](#)). This conclusion was supported by studies by [Benelli and Pavela \(2018\)](#) and [Pavela and Benelli \(2016\)](#). Their experimental results showed that EOs are eco-friendly, non-toxic to humans and mammals, quickly degraded in the environment, and benign to marine animals like fish and plankton. Regarding the possibility of insects developing resistance against EOs, so far, no paper in the literature on plant compounds has reported any indication of emerging insect resistance against any EOs. The reason for this desirable property is likely to be that EOs exert their insect-destroying actions through multiple modes ([Pavela and Benelli, 2016](#)), making it difficult for insects to successfully adapt against all of them. Incidentally, identical multiple modes of action may be the explanation for the nearly identical efficacy of d-limonene and trans-anethole in this study. These multiple action modes were reported by [Dhinakaran et al. \(2019\)](#), i.e., the insecticidal modes of action of d-limonene against *Ae. aegypti* inhibited acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes, leading to disruption of the insect endocrinal system during its molting process, while [Aungtikun et al. \(2021\)](#) and [Shahriari et al. \(2016\)](#) reported the same insecticidal modes of action for trans-anethole against *M. domestica* and *Tribolium castaneum* (Coleoptera: Tenebrionidae). Because of all of the mentioned reasons above, it was not surprising that, in this study, d-limonene from *Z. limonella* and trans-anethole from *I. verum* as well as the two combined formulations of each of these EO constituents with its corresponding EO were more potent in larvicidal and pupicidal activities against *Ae. aegypti* and *Ae. albopictus* than temephos at the time of this study (see [Tables 2 and 3](#)).

To conclude this section, 2.5% *I. verum* EO + 2.5% trans-anethole is recommended as a better mosquito control alternative to temephos because its potency against the two mosquito species was not only 10 times higher than that of temephos at the time of this study, but it also should be much safer to humans and non-targeted animals than temephos since the EOs are long-used natural compounds with no report of strong human toxicity. Moreover, these EOs are from native plant species in Thailand, so they can be readily and economically produced locally ([Pavela, 2015a](#); [Aungtikun et al., 2021](#); [Muthiah et al., 2018](#)). Regarding safety, the European Medicines Agency (EMA—The European Agency for the Evaluation of Medical Products and Veterinary Medicines and Information Technology Unit reported that *I. verum* fruit EO can be used as an expectorant or stomachic in human medicine and as a spice in alcoholic beverages, sweets, and toothpaste. Their recommended daily dose of *I. verum* fruit for humans was 3 g and for essential oil was 0.3 g. The

doses for domestic animals were 20g/200kg for cattle and 15g/200kg for sheep and goats (European Medicines Agency, 2022; Sharafan et al., 2022). Furthermore, an EO solution for effective larvicidal and pupicidal activities in still water in a kitchen, for example, will be 0.25%, 4 times less than the equivalently effective temephos solution of 1%. Therefore, the EO solution should be much less toxic to humans than temephos. Nevertheless, a recognized safety evaluation of this formulation is needed before it can be developed into a commercial insecticide. In the future, toxicity studies of the EOs, major composition, their combinations, and their synergistic effects against non-target aquatic organisms and field evaluation are necessary. These results obtained are useful in the context of a search for natural larvicidal and pupicidal agents.

5. Conclusion

The present study clearly showed that d-limonene and trans-anethole at 5% concentration and a combined formulation of 2.5% *I. verum* EO + 2.5% trans-anethole were very effective larvicidal and pupicidal agents for controlling *Ae. aegypti* and *Ae. albopictus* mosquito vectors and more effective than temephos, a widely used synthetic chemical at the present time. The higher efficacy may be partly due to current mosquito populations becoming highly resistant to temephos. More importantly, the EO alternatives can be assumed to be much safer for humans and more eco-friendly than temephos as their uses in humans and animals have not only been accepted by EMA but they have been used in food and folk medicine in Asia since ancient times. Therefore, they are better alternatives to synthetic insecticides and justify further development into commercial insecticidal products.

Declarations

Author contribution statement

Mayura Soonwera: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Tanapoom Mounghthipmalai; Jirapon Aungtikun: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Sirawut Sittichok: Performed the experiments.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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