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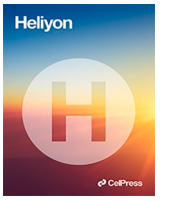

Research article

Adulticidal synergy of two plant essential oils and their major constituents against the housefly *Musca domestica* and bioassay on non-target species

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ABSTRACT

Single and mixture formulations of lemongrass (*Cymbopogon citratus* (DC.) Stapf.) and star anise (*Illicium verum* (J. Presl.)) essential oils (EOs) and their major constituents were assayed for their adulticidal activities against housefly, *Musca domestica* L., and two non-target species, stingless bee (*Tetragonula pegdeni* Schwarz) and guppy (*Poecilia reticulata* Peters). The efficacies of the mixture formulations were compared against those of the single formulations and 1.0% α-cypermethrin, a common synthetic insecticide. GC-MS analysis found that the major constituent of lemongrass EO was geranial (45.23%), and that of star anise EO was *trans*-anethole (93.23%). Almost all mixture formulations were more effective in adulticidal activity against housefly adults than single formulations and 1.0% α-cypermethrin. A mixture of 1.0% lemongrass EO + 1.0% *trans*-anethole exhibited the strongest synergistic insecticidal activity with a 100% mortality rate (KT_{50} of 3.2 min and LT_{50} of 0.07 h). The relative percentage increase in mortality rate over single formulations was between 1.6 and 91.9%. In addition, it was three times more effective than 1.0% α-cypermethrin. To find the mechanism of adulticidal action, scanning electron microscopy (SEM) was done to find morphological aberrations, such as antennal and mouthpart aberrations, after the houseflies were treated with 1.0% lemongrass EO + 1.0% *trans*-anethole. The aberrations included deformed and abnormal shape of arista and flagellum, change in labellum pigmentation, and damage to pseudotracheae. Regarding toxicity against non-target species, all single and mixture formulations were not toxic to the two non-target species, while 1.0% α-cypermethrin was highly toxic. To conclude, a mixture of 1.0% lemongrass EO + 1.0% *trans*-anethole can be an excellent, natural, sustainable housefly adulticidal agent.

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1. Introduction

One serious global insect vector of numerous diseases is the housefly, *Musca domestica* L. This annoyance-causing insect is a source of more than 150 mammalian infections including viruses, bacteria, protozoa, and helminths [1,2]. Housefly adults are a major nuisance that interferes with dairy cow and chicken feeding, which is why they are a critical issue for dairy and poultry farms [2]. Currently, controlling houseflies is difficult due to the target its resistance to common synthetic insecticides (chlorpyrifos, cypermethrin, deltamethrin, and permethrin) [3,4]. For example, the resistance ratios at median lethal doses (LD_{50}) ranged from 30 to 153.7 folds for cypermethrin and from 5 to 16 folds for permethrin [3–5]. In many countries, including Thailand, housefly resistance is increasing [6]. To make matters worse, non-target animals, pollinator insects, beneficial insects, and the ecosystem are adversely affected by synthetic insecticides [7].

Although synthetic insecticides, especially α-cypermethrin, have several side effects, it is still commonly used to control insect pests in public health and agricultural sectors in Thailand [8]. Therefore, it is a proper positive control in the framework of Thailand's pest insect control for the development of brand–new, powerful natural substances that are safe for humans and the environment [8,9]. Many research teams are currently interested in using natural essential oils from plants (EOs) as insecticidal agents [9–11].

EOs and their primary ingredients from *Myristica fragrans*, *Pimpinella anisum*, *Ocimum gratissimum*, *Laurus nobilis*, *Illicium verum*, *Eucalyptus globulus*, *Cuminium cyminum*, *Cymbopogon citratus*, *Cinnamomum osmophleum*, *Cinnamomum verum*, *Cinnamomum cassia*, *Cinnamomum loureiroi*, *Citrus aurantium*, D-limonene, and *trans*-cinnamaldehyde are natural insecticidal substances; all of which induce low resistance in insect pests and are biodegradable [9–16]. Furthermore, EOs from *C. verum*, *C. aurantium, C. citratus*, *C. cyminum*, *E. globulus*, *O. gratissimum*, *P. anisum*, D-limonene, and *trans*-cinnamaldehyde are also safe for non-target species such as honeybee (*Apis mellifera*), moonfish (*Poecilia latipinna*), guppy (*Poecilia reticulata*), manure worm (*Eisenia foetida*), predatory stinkbug (*Podisus nigrispinus*), and Asian lady beetle (*Harmonia axyridis*) [17–19]. EOs have been employed both singly and in combination to manage insect infestation [20,21]. Insect vector control can benefit from the synergy of mixture EO combinations and mixture combinations of EO and their main active ingredient [22–24]. Mixture EO combinations of *E. globulus* + *C. citratus*, *C. loureiroi* + *C. cassia*, *C. verum* + *C. cassia*, and *C. verum* + *C. loureiroi* showed synergistic adulticidal effects against female houseflies and two mosquito vectors, *Aedes aegypti* and *Aedes albopictus* [21–23]. Their mixture combinations have synergistic effects against Dipteran insect vectors [23–25]. The main active ingredients from plant EOs that had an adulticidal effect on houseflies, *Ae. aegypti*, and *Ae. albopictus* mosquitoes were monoterpenes such as *trans*-cinnamaldehyde, citronellal, eugenol, limonene, 1,8-cineole, α-pinene, and *trans*-anethole [22–24]. Mixtures of monoterpene combinations were synergistically effective against housefly and *Ae. aegypti* mosquitoes, such as combinations of *trans*-anethole + α-pinene, α-pinene + geranial, carvone + limonene, 1,8-cineole + eudesmol, and 1,8-cineole + citronellal [21–27]. In addition, several mixture combinations of EO and EO constituents exhibited a higher degree of toxicity against housefly and mosquitoes. Their advantages are shorter knockdown time (KT_{50}), especially a 0.5%:0.5% (w/w) combination of *I. verum* EO + geranial and *M. fragrans* EO + geranial exhibited a highly synergistic insecticidal effect against *M. domestica* [22] and a 2%:1% (w/w) *C. citratus* EO + *trans*-cinnamaldehyde, *C. citratus* EO + geranial, and *C. citratus* EO + alpha-pinene exhibit strong adulticidal activity against *Ae. aegypti* [28]. A substantial adulticidal action on houseflies was also demonstrated by combined formulations of *E. globulus* + geranial and *C. citratus* + 1,8-cineole [29].

Most investigations have found that several formulations combining EO and EO's main active component had a high potential for controlling houseflies in a way that was both environmentally friendly and non-toxic to non-target species [13,24–27]. Therefore, our group was motivated to investigate adult housefly mortality incurred by single formulations and mixture formulations of EOs—lemongrass and star anise—and their active components. At the chosen concentrations, these EO formulations have already been shown to be effective against *M. domestica* and *Ae. aegypti* [20–23]. The chemical components of lemongrass and star anise EOs, the synergistic effect of mixture formulations, and the biosafety of the EO treatments were evaluated against a non-target species, stingless bee (*Tetragonula pegdeni* Schwarz) and guppy (*Poecilia reticulata* Peters), two common non-target organisms, predator, and pollinator, in Thailand and Southeast Asia [13,30]. Once their synergistic adulticidal activity and safety to non-target species have been established, some EO formulations would be a natural, secure, sustainable, and efficient alternative to α-cypermethrin. The most urgently needed application is for reducing the housefly populations and hence house fly-borne diseases in urban, suburban, and livestock areas.

2. Materials and methods

2.1. Collection of plants

All plant material collections and all authors adhered to local, regional, and global regulations. King Mongkut's Institute of Technology Ladkrabang (KMITL) issued an approval, oversight, and authorized the collection of plant materials under permit KDS2021/002.

The two EOs used in this investigation were obtained from fresh stems of lemongrass (*C. citratus*: KMITL-01-08) and dried fruits of star anise (*I. verum*: KMITL-01-09). From August to September 2022, fresh stems of lemongrass were harvested at a natural habitat in Wat Nam Daeng Village (13° 35′ 58″ latitude N/100° 35′ 48″ longitude E) located in the town of Samut Prakan Province, in the central region of Thailand. Dried fruits of star anise were purchased from a Chinese herb vendor in Samut Prakan Province, Thailand. Hataichanok Passara a plant expert at the KMITL herbarium, identified the two plant species at the School of Agricultural Technology (KMITL), Bangkok, Thailand. All voucher specimens (KMITL-01-08 and KMITL-01-09) were deposited at the KMITL Herbarium, School of Agricultural Technology, KMITL for future reference.

2.2. Extraction of two essential oils and their GC-MS analysis

Cleaned and crushed 800 g (in 1600 ml of distilled water) of each plant were hydro-distilled in a Clevenger-type apparatus to extract its EO. The extraction process followed the protocol described by Soonwera and Sittichok [21]. Each EO was then filtered into a 100 ml container with a brown hue. It was then kept at 4 °C until it was used in GC-MS analysis as well as insecticidal experiments.

Each EO was subjected to a mass-spectrometric examination using an Agilent 6890 N gas chromatography-mass spectrometer (GC-MS) at the Scientific Center, KMITL. The used column, the temperature of the column, the temperature of the injector, carrier gas, acquisition mass range, and essential oil constituents' quantification were similar to those described in Aungtikun et al. [22]. Identification of the constituents of essential oils was done by computing retention indices (RD) using a homologous hydrocarbons series (C_8–C_{30} *n*-alkanes) and peak area percentage in the chromatogram. The constituent's identification was completed by comparing the RD and mass spectra with one of three standard literature databases: NIST-MS (National Institute of Standards and Technology) [31], ADAMS [32], or the National Library of Medicine [33]. Every EO was checked three times, and the final observations were presented as mean ± standard deviation.

2.3. Source and purity of reagents

This investigation only employed reagent-grade compounds. Standard homologous hydrocarbons series (C_8–C_{30} *n*-alkanes), geranial, the main ingredient of lemongrass EO (*C. citratus*), which was 96% pure, and *trans*-anethole, the main ingredient of star anise EO (*I. verum*), which was 98% pure, were purchased from Sigma-Aldrich (USA). Alpha-cypermethrin (10% *w/v*) was used as a positive control and ethyl alcohol (95% *v/v*) was used as a negative control. They were supplied by SM Chemical Supplies Company (Bangkok, Thailand).

2.4. Raising housefly (Musca domestica)

Housefly were reared in the laboratory of the Entomological division, School of Agricultural Technology, KMITL, in an air-conditioned room under the conditions of 27.5 ± 3.5 °C, 75.0 ± 2.5 RH, and 12 h photoperiod. A day after their initial feeding, adult females laid eggs on pieces of mackerel fish on sterile coconut husks in a plastic container (17 × 25 × 6 cm^3). The eggs then turned into larvae and pupae. The housefly-rearing protocol has been described by Soonwera and Sittichok [21]. The insecticidal assay was conducted on fresh, 3-day-old adult houseflies.

2.5. Adulticidal assay on housefly

The standard susceptibility test of the World Health Organization (WHO) [34] was used to evaluate the adulticidal efficacy against housefly adults. In a treatment tube that measured 125 mm in length and 44 mm in diameter, ten 3-day-old housefly adults of both sexes were exposed to 2 mL of either single or mixture formulations for an hour. Two milliliters of each formulation, one at a time, were put onto a sheet of Whatman No 1® filter paper with a surface area of 12 cm by 15 cm^2. Housefly adults were subsequently moved to the non-treatment tube and monitored there for 24 h. Each treatment was repeated five times, with 1% (*w/v*) α-cypermethrin serving as the concurrent positive control. To decide whether the adult houseflies had been knocked down or killed, the authors observed the movement of their antennae, head, thorax, wings, legs, and belly. No movement indicated a knockdown or mortality [22]. At 1, 5, 10, 30, and 60 min after exposure, we observed and reported the knockdown rate for each formulation. At 24 h after exposure, we observed and recorded the mortality rate. The knockdown (%K) and mortality (%M) rates of adult houseflies were determined using the following formulae [22; Eqs. (1) and (2)],

$$\text{Knockdown rate } (\%\text{K}) = \text{KD}/\text{TN} \times 100, \tag{1}$$

$$\text{Mortality rate } (\%\text{M}) = \text{MT}/\text{TN} \times 100, \tag{2}$$

where KD is the total number of adult houseflies knocked down; MT is the total number of adult houseflies dead; and TN is the total number of adult houseflies treated.

The knockdown index (KI) was determined using the following formula [22; Eq. (3)],

$$\text{KI} = \text{KT}_{50} \text{ of treatment}/\text{KT}_{50}\text{of}\alpha\text{-cypermethrin}. \tag{3}$$

KI < 1 signifies that the formulation, either single or mixture, was more toxic than α-cypermethrin; and KI > 1 signifies that the formulation was less toxic than α-cypermethrin.

Mortality index (MI) was determined using the following formula [22; Eq. (4)],

$$\text{MI} = \text{LT}_{50} \text{ of treatment}/\text{LT}_{50}\text{of}\alpha\text{-cypermethrin}. \tag{4}$$

MI < 1 signifies that the formulation, either single or mixture, was more toxic than α-cypermethrin; and MI > 1 signifies that the formulation was less toxic than α-cypermethrin.

Synergistic mortality index (SMI) was determined using the following formula [23; Eq. (5)],

$$\text{SMI} = \text{LT}_{50} \text{ of the mixture formulation}/\text{LT}_{50} \text{ of the single formulation}. \tag{5}$$

SMI < 1 suggests that there is a synergistic effect, and SMI > 1 suggests that there is an antagonistic effect.

Increased mortality value (IMV) was determined using the following formula [23; Eq. (6)],

$$\mathrm{IMV} = [\%\mathrm{Mofmixtureformulations\text{-}\%Mofsingleformulations} / \%\mathrm{Mofmixtureformulations}] \times 100. \quad (6)$$

2.6. Safety assays on non-target organisms, stingless bee (Tetragonula pegdeni) and guppy (Poecilia reticulata)

The toxicity of every formulation was evaluated against a non-target pollinator, stingless bee (*T. pegdeni*) using the method of Dorneles et al. [35] and Matos et al. [36]. One hundred adult workers from three natural colonies of stingless bees were gathered from the KMITL organic farm into an insect box (17 × 24 × 5 cm) and transported to our laboratory in 1 h, Jirisuda Sinthusiri an entomologist, completed the stingless bee scientific identification. The stingless bee workers were fed with 40% (*w/v*) glucose and maintained at 28.5 ± 2.0 °C and 79.3 ± 3.0% RH for 2 days before the topical application test. Before the topical application, the stingless bees were anesthetized at −8 °C for 1 min. Then, they were applied 1 μL of each test formulation (at 10,000 ppm concentration) to their mesonotum part. After application, ten stingless bees were transferred into an insect box (7.5 × 10 × 4.5 cm) and fed with 40% (*w/v*) glucose. Five repeats of each treatment were done, along with 1% (*w/v*) α-cypermethrin as a positive control. After 5, 10, 30 min, 1, 12, and 24 h of exposure, mortality and aberrant behaviors, such as reduced feeding and activity, were observed.

The toxicity assay against a non-target aquatic predator guppy (*P. reticulate*) was identical to the method used by Moungthipmalai et al. [13]. The guppy fish species were purchased from an organic farm in Kanchanaburi Province, Thailand. Tanapoom Moungthipmalai, an animal taxonomist, completed the guppy scientific identification. They were individually kept in a plastic bucket (40 × 58 × 30 cm) containing 80 L of water at 36.5 ± 2.0 °C and 60.5 ± 5.0% RH. For testing each formulation, ten adult guppies were put in a plastic bucket (38 × 26 × 20 cm) containing 5 L of water. A treatment was added to it, 10,000 ppm of each formulation. Five repeats of each treatment were done, along with 1% (*w/v*) α-cypermethrin as a positive control. Data on mortality and swimming inefficiency were recorded at 1, 3, 6 h, 1, 2, 3, 4, and 5 days after the exposure.

The mortality rate of non-target species was determined using the following formula [13,36; Eq. (7)],

$$\text{Mortality rate } (\%\mathrm{N}) = \mathrm{NT}/\mathrm{TT} \times 100. \quad (7)$$

where NT is the total number of dead adults of the non-target species, and TT is the total number of treated adults of the non-target species.

Safety index (SI) was determined using the following formula [13; Eq. (8)],

$$\mathrm{SI} = \mathrm{LT}_{50}\mathrm{ofnon\text{-}targetspecies}/\mathrm{LT}_{50}\text{ of target species.} \quad (8)$$

Table 1
Chemical compositions of the two essential oils (EOs) assayed for adulticidal activity in this study.

No.	Component[a]	RD[b]	KR[c]	Peak area (%) ± SD[d]		Identification[e]
				lemongrass EO	star anise EO	
1.	α-Thujene	932	930	–	0.25 ± 0.02	A,B,C
2.	α-Pinene	981	982	3.23 ± 0.11	–	A,B,C
3.	α-Terpinene	1010	1010	0.16 ± 0.02	–	A,B,C
4.	1,8-Cineole	1025	1025	10.83 ± 0.94	0.71 ± 0.04	A,B,C
5.	Limonene	1031	1032	–	1.83 ± 0.08	A,B,C
6.	ϒ-Terpinene	1051	1052	0.17 ± 0.04	–	A,B,C
7.	Linalool	1097	1098	0.82 ± 0.04	–	A,B,C
8.	α-Terpineol	1179	1178	–	0.43 ± 0.01	A,B,C
9.	Neral	1216	1217	24.62 ± 1.23	–	A,B,C
10.	*p*-Anisaldehyde	1247	1247	–	1.51 ± 0.08	A,B,C
11.	Geraniol	1240	1240	6.12 ± 0.98	–	A,B,C
12.	Geranial	1246	1246	45.22 ± 1.12	–	A,B,C
13.	*trans*-Anethole	1283	1283	–	93.24 ± 2.04	A,B,C
14.	Eugenol	1355	1356	–	0.62 ± 0.02	A,B,C
15.	Geranyl acetate	1380	1380	4.11 ± 0.92	–	A,B,C
16.	Caryophyllene oxide	1581	1580	2.12 ± 0.89	–	A,B,C
	Total (%)			97.40 ± 0.52	98.59 ± 0.95	
	Yield (%)			1.19 ± 0.10	3.13 ± 0.08	
	Density (g/ml)			0.96 ± 0.08	0.97 ± 0.09	
	Color			pale yellow	pale yellow	

[a] Major and minor compounds of EOs are listed in order of elution in the HP-5 MS column.
[b] *RD*, Retention index calculated using the retention time in relation to the homologous series of C_8–C_{30} *n*-alkanes.
[c] *KR*, Kovats index is taken from NIST-MS [31] or ADAMS [32] or National Library of Medicine [33].
[d] Average of three runs.
[e] Identification: A, substance matching was done with a readily available analytical standard (Sigma Aldrich); B, mass spectrum matching with those reported in ADAMS [32] or NIST-MS [31] or National Library of Medicine [33]; and C, retention index overlapping with those reported in NIST-MS [31] and National Library of Medicine [33].

SI > 1 signifies that the formulation, either single or mixture, was benign to the non-target species; and SI < 1 signifies that the formulation was toxic to the non-target species.

The Ethics Committee of King Mongkut's Institute of Technology Ladkrabang (number KDS2021/002) had given its approval to each bioassay used in this research.

2.7. Preparation of adult samples for scanning electron microscopy

After 24 h of treatment, the morphology of the head, antennae, and mouthpart of the adult houseflies that underwent a treatment or control was observed under scanning electron microscopy (SEM). Heads of all samples were cut off and placed in 70% ethanol at room temperature. After being washed in 70% ethanol, the samples, the heads, were postfixed in 95% ethanol for 90 min. Then, they were dehydrated in absolute alcohol (100%) three times at 90 min each time. Samples were dried with a CO_2 critical point drier. The dried head samples were then mounted on aluminium stubs with double-sided adhesive tape, coated with gold, and observed with a JSM-6610LV scanning electron microscopy (JEOL Ltd., Tokyo, Japan) at the Scientific and Technological Research Equipment Centre, Chulalongkorn University, Bangkok, Thailand.

2.8. Data analysis

All experiments were of completely randomized design (CRD), and the results were expressed as the mean ± SD. The average knockdown data were subjected to a probit regression analysis to calculate the knockdown time KT_{50}. In the case of mortality data, LT_{50} values were calculated [37]. Using a one-way analysis of variance (ANOVA), the treatment's impact was assessed. If the analysis

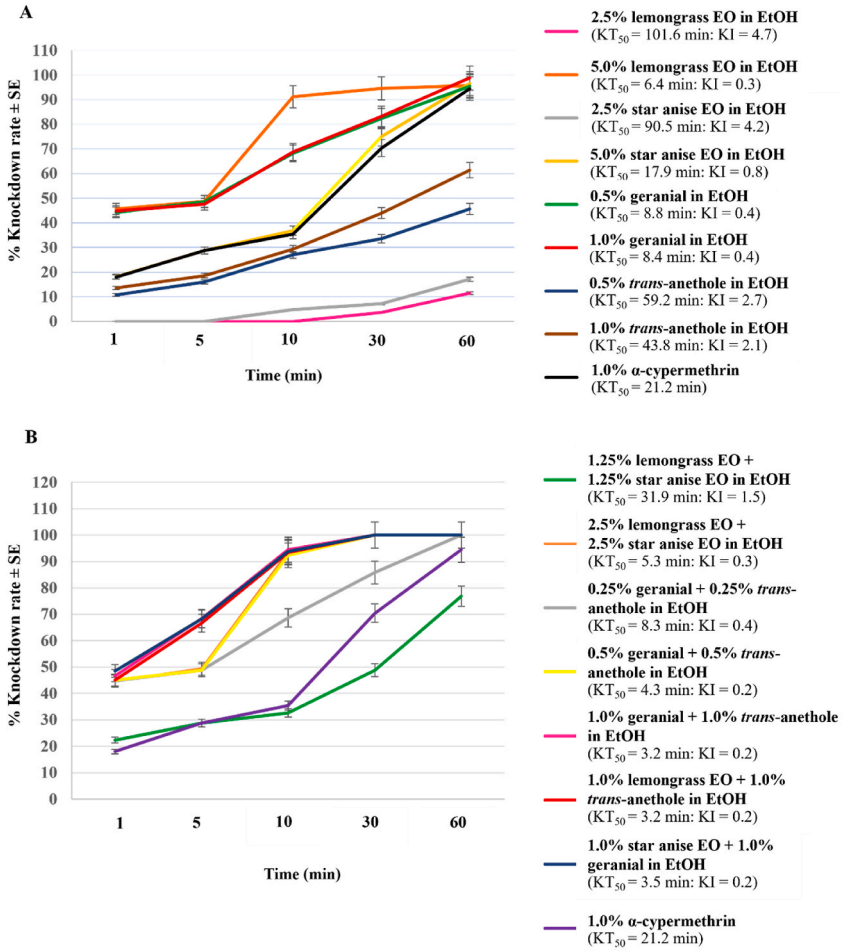

Fig. 1. Knockdown time ±SE against housefly of single EO or EO constituent formulations (A) and mixture formulations (B).
Note: KT_{50} = 50% Knockdown Time; Knockdown index (KI) = [KT_{50} of treatment/KT_{50} of α-cypermethrin] [22]: KI < 1 signifies that the formulation, either single or mixture, was more toxic than α-cypermethrin, and KI > 1 signifies that the formulation was less toxic than α-cypermethrin.

of variance indicated significant differences between the groups ($P < 0.05$), a Tukey's post hoc test ($P < 0.05$) was carried out to ascertain the significant difference between the control and multiple treatment groups. All statistics were calculated with SPSS Statistical Software Package version 25.0.

3. Results

3.1. Yields and chemical profiles of the two EOs

Yields of EOs from the hydro-distillation procedure were identified, and GC-MS analysis of their chemical makeups was conducted. Also identified were some of the EOs' physical characteristics. Table 1 lists each constituent's percentage fraction, as well as its retention index (RD) and Kovats retention index (KR). Pale yellow EOs were produced by lemongrass stems and star anise fruits. Their yields were 1.19 and 3.13%, respectively. Lemongrass EO had a density of 0.96 g/ml, and star anise EO had a density of 0.97 g/ml. The primary components of the lemongrass EO were geranial (45.22%) and neral (24.62%). A total of 10 chemical components were found, accounting for 97.40% of the total composition. The minor constituents were 1,8-cineole (10.83%), geraniol (6.12%), geranyl acetate (4.11%), α-pinene (3.23%), caryophyllene oxide (2.12%), linalool (0.82%), ϒ-terpinene (0.17%), and α-terpinene (0.16%). *Trans*-anethole (93.24%) was the primary constituent of the star anise EO. A total of 7 chemical components were found, accounting for 98.59% of the total composition. The minor constituents were limonene (1.83%), *p*-anisaldehyde (1.51%), 1,8-cineole (0.71%), eugenol (0.62%), α-terpinene (0.43%), and α-thujene (0.25%).

3.2. Effects on housefly of single and mixture formulations

Figs. 1 and 2 showed the evaluation of the knockdown and mortality rates, 50% knockdown time (KT_{50}), 50% lethal time (LT_{50}), knockdown index (KI), and mortality index (MI) of single and mixture formulations against housefly. Housefly was more sensitive to all mixture formulations (KT_{50} value from 3.2 to 31.9 min) (Fig. 1B) than single formulations (KT_{50} value from 6.4 to 101.6 min) (Fig. 1A) at 1 h following exposure. Most single and mixture formulations had a knockdown rate greater than 1.0% (*w/v*) α-cypermethrin; the KI was 0.2. With the lowest KT_{50} of 3.2 min and a KI of 0.2, 1.0% geranial + 1.0% *trans*-anethole and 1.0% lemongrass EO + 1.0% *trans*-anethole had the highest knockdown activity (Fig. 1).

Additionally, housefly was more vulnerable to all mixture formulations 24 h after treatment than it was to single formulations, with mortality rates ranging from 78.5 to 100% and an LT_{50} of 0.09–8.4 h. It was less susceptible to single formulations that offered mortality rates of 21.4–98.4% and an LT_{50} of 0.1–60.9 h. When compared to 1.0% (*w/v*) α-cypermethrin, the adulticidal activity of the mixture formulations was 0.003–0.939 times higher. The strongest adulticidal effect was produced by 1.0% geranial + 1.0% *trans*-anethole and 1.0% lemongrass EO + 1.0% *trans*-anethole, which provided a 100% mortality rate, the lowest LT_{50} of 0.07 h, and an MI of 0.003 (Fig. 2).

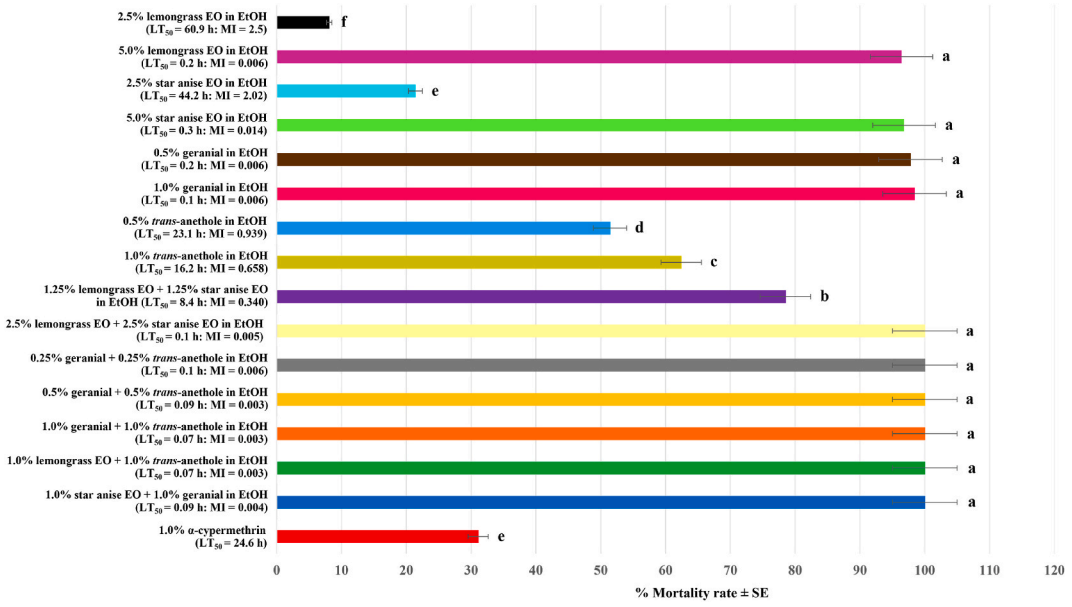

Fig. 2. Mortality rate ±SE against housefly of single EO or EO constituent and mixture formulations.
Note: Mean mortality rates ±SE within a column followed by the same letter do not differ significantly (Tukey's post hoc test $P < 0.05$), LT_{50} = 50% Lethal Time, and Mortality index (MI) = [LT_{50} of treatment/LT_{50} of α-cypermethrin] [22]: MI < 1 signifies that the formulation, either single or mixture, was more toxic than α-cypermethrin, and MI > 1 signifies that the formulation was less toxic than α-cypermethrin.

All mixture formulations demonstrated a synergistic effect, displaying greater toxicity than that of single formulations, with an SMI of 0.001–0.9. Their synergistic effect increased mortality, with IMV increasing from 1.6 to 91.9%. The formulation with the strongest synergistic effect, with an SMI ranging from 0.001 to 0.004, was 1.0% lemongrass EO + 1.0% *trans*-anethole. Its IMV of 37.6–91.9% was the formulation's maximum synergistic impact (Fig. 3).

3.3. Effects on two non-target species, stingless bee and guppy of single and mixture formulations

In this study, the safety index (SI) value of a formulation was defined as LT_{50} of non-target species divided by LT_{50} of houseflies. A value higher than one was considered safe, while a value lower than one was considered toxic. The computed SI of all formulations made from lemongrass and star anise EOs and their major constituent against two non-target species, stingless bee, and guppy, are presented in Fig. 4. All formulations showed low toxicity to two non-target species, with stingless bee (Fig. 4A) and guppy adults (Fig. 4B) having an LT_{50} range of 21.3–353.7 h. All formulations provided an SI of higher than 1 (1.1–4901.4). On the other hand, 1.0% (*w/v*) α-cypermethrin was highly toxic to the adult stingless bee and guppy, with a mortality rate of 100% at 1 h (LT_{50} of 0.08) and 5 days (1.2 h). Its safety index (SI), which ranged from 0.003 to 0.05, was extremely low, signifying that all EO formulations were extremely safe.

4. Discussion

The current study shows that lemongrass and star anise essential oils, gathered from central Thailand, were viable sources of phytochemicals. According to the EO yield data, the extraction of lemongrass and star anise were 1.19 and 3.13% *v/w*, respectively, slightly different from the yields previously reported [21,22]. Several studies reported that the hydro-distillation process produced EO yields of lemongrass (*C. citratus*) that ranged from 0.79 to 1.10% *v/w* [21,22,38], whereas star anise (*I. verum*) reported EO yields ranged from 4.0 to 6.7% *v/w* [22,39]. The GC-MS part of the study revealed that their chemical compositions matched those previously mentioned in the literature [38,39]. Geranial was discovered to be the primary component of lemongrass EO (*C. citratus*), accounting for 45.22% of the total chemical composition, within the previously reported range of 40.7–45.4% [22,38]. *Trans*-anethole was discovered to be the primary component of star anise EO (*I. verum*), accounting for 93.24% of the total chemical composition, within the previously reported range of 88.5–94.0% [22,39,40]. Many important variables, including the quality of the raw plant materials, the management practice of plant cultivations [22], and the extraction procedures [41,42], can be explained by slight variations in the EO yield and the chemical profile of both EOs from the previously published values.

In this study, all mixture formulations showed a pronounced synergistic effect against housefly adults with a high SMI. The formulations 1.0% geranial + 1.0% *trans*-anethole and 1.0% lemongrass EO + 1.0% *trans*-anethole showed the highest level of synergy, improving the mortality rate to more than 90.0%. This result was consistent with the result of another study. That study found that *C. citratus* EO + alpha-pinene (2%:1% (w/w)) exhibits strong adulticidal activity against *Ae. aegypti* [28]. Aungtikun et al. [22] concluded that the mortality rate against *M. domestica* rose to more than 50% when EOs from *I. verum* EO + geranial, *M. fragrans* EO + geranial,

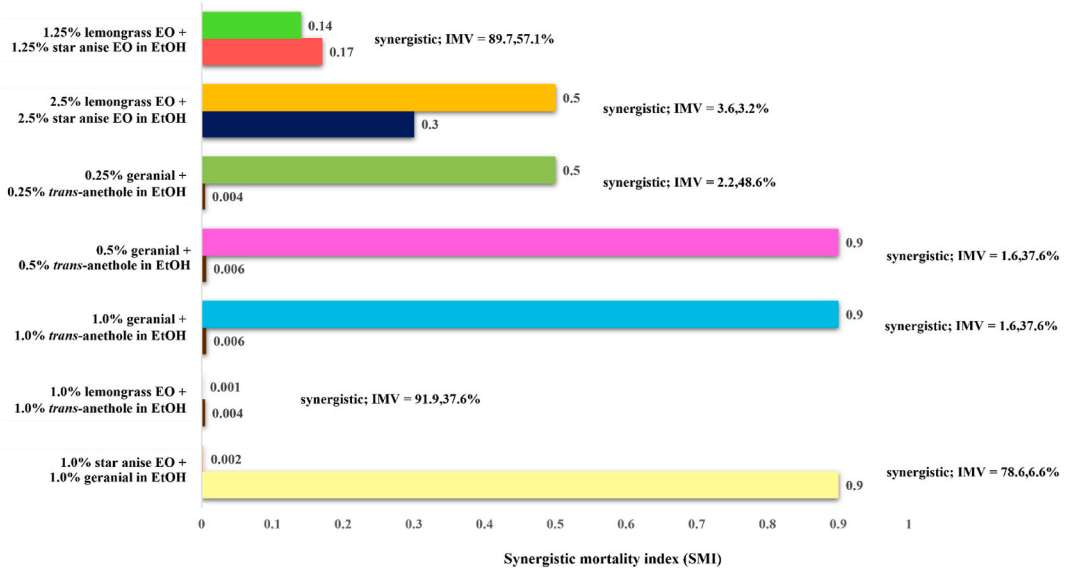

Fig. 3. Synergistic mortality index of mixture formulations against housefly.
Note: Synergistic mortality index (SMI) = [LT_{50} of mixture formulation/LT_{50} of single formulation] [23]: SMI < 1 suggests that there is a synergistic effect, and SMI > 1 suggests that there is an antagonistic effect and Increased mortality value (IMV) = [%M of mixture formulations − %M of single formulations/%M of mixture formulations] × 100 [23].

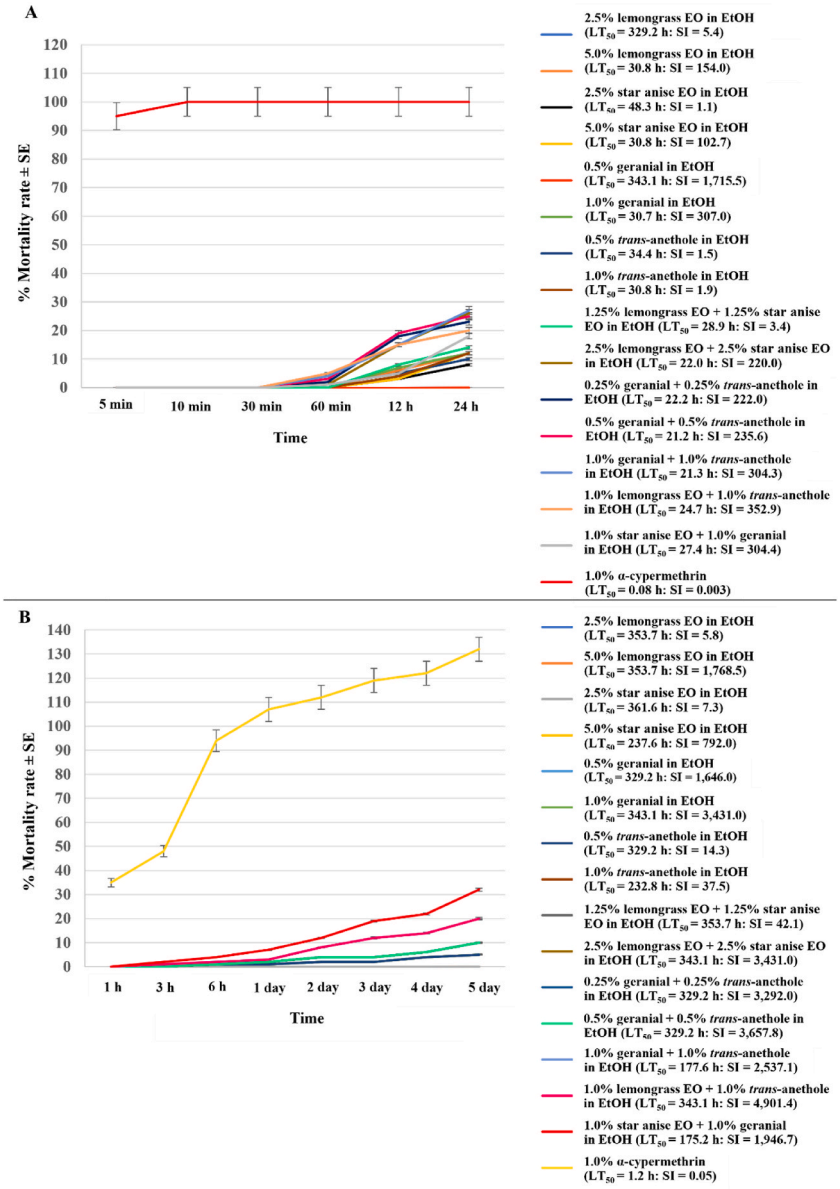

Fig. 4. Mortality rate ±SE of single EO and EO constituent formulations and mixture formulations against two non-target species: stingless bee (A) and guppy (B).
Note: LT_{50} = 50% Lethal Time, Safety index (SI) = [LT_{50} of non-target species/LT_{50} of target species] [13]: SI > 1 signifies that the formulation, either single or mixture, was benign to the non-target species; and SI < 1 signifies that the formulation was toxic to the non-target species.

and α-pinene + geranial at 0.5%:0.5% was combined into a mixture. The EO combination of *C. citratus* EO + *E. globulus* EO (1:1 ratio) had a powerful synergistic impact (100% knockdown and mortality rates) against *M. domestica* females, with an increased mortality rate of more than 90.0% [21]. The monoterpene mixture of 1,8-cineole + geranial (2.5%:2.5%) exhibited synergistic insecticidal effects against *M. domestica* females [21]. In addition, mixtures of monoterpenoid essential oil compounds from *p*-cymene +1, 8-cineole, *p*-cymene + ϒ-terpinene, and ϒ-terpinene +1,8-cineole displayed synergistic toxicity to *M. domestica* adults [43]; citral + limonene, and 1,8-cineole + camphor showed synergistic toxicity against *Tichoplusia ni* [44,45]; and *trans*-anethole + estragole and *trans*-anethole + estragole + linalool showed strong synergistic effects against *Cryptolestes ferrugineus* [46]. Similarly, a mixture of *Rosmarinus officinalis* EO + *E. globulus* EO (10%:10%) showed strong synergistic toxicity against *Cx. pipiens*, *Blattella germanica*, and *M. domestica* [47].

Regarding SEM images, they showed clearly the morphological alterations induced by EO treatments when compared with non-treated adult head (Fig. 5A), non-treated adult antennae (Fig. 5B), and non-treated adult mouthparts (Fig. 6A, B). Fig. 5C, D, E showed morphological damages of external structure in the shape of flagellum and antennae balance as well as deformed maxillary

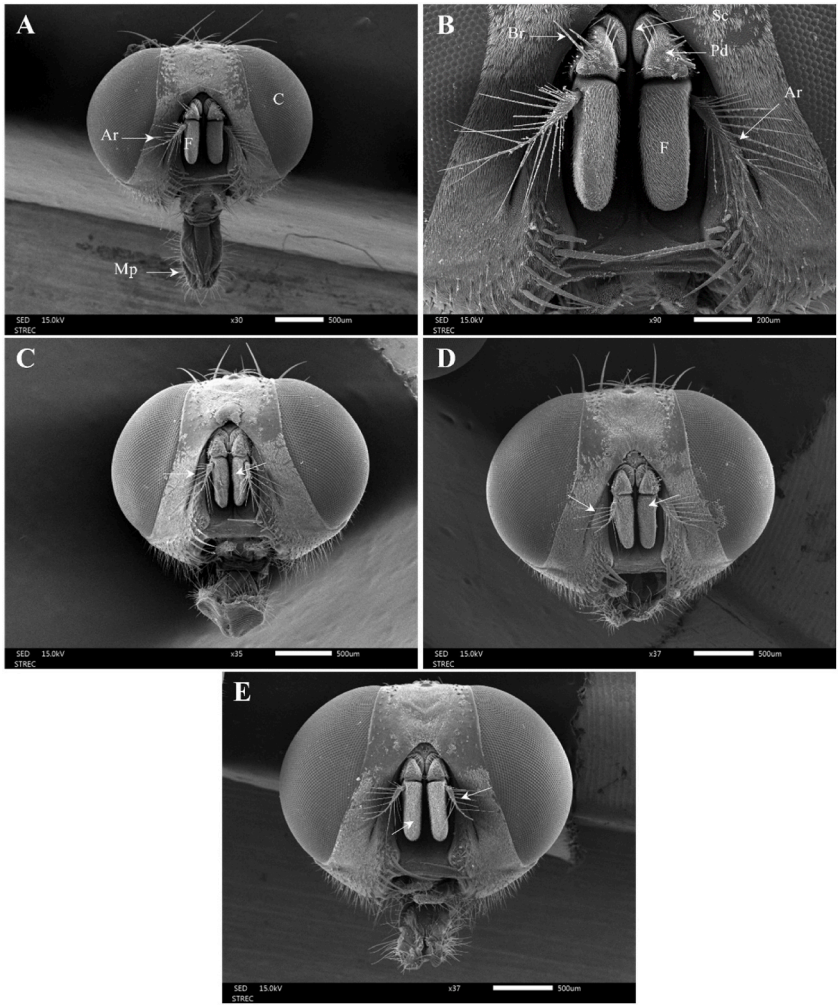

Fig. 5. Scanning electron micrographs of the head of housefly: (A) non-treated adult head, showing full external structure with compound eye (c), arista (Ar), elongated flagellum (F), and maxillary palp (Mp); (B) non-treated adult antennae, showing full external structure with bristle (Br), scape (Sc), and pedicel (Pd); morphological damage to antennae and elongated flagellum after treated with lemongrass EO (C), geranial (D), and a mixture of lemongrass EO + *trans*-anethole (E).

palps, covered with a layer (Fig. 6C, D) that was thought to be an oil layer, that would account for the EOs' insecticidal mechanism.

Regarding the potential for houseflies to develop resistance to these natural agents, it is very unlikely because the mixtures of EOs exert their actions through multiple mechanisms [48]. All the mixture EO formulations examined in this study appeared to have a synergistic mechanism of action that was closely related to the modes of action of their primary ingredients [45,46]. EOs and their major constituents with different mechanisms of action can be more efficient in reducing or preventing resistance rates against insect pests, including housefly [16,45,48]. A mixture formulation, 1.0% lemongrass EO + 1.0% *trans*-anethole, damaged the olfactory sense in the antennae and maxillary palps as well as the neural systems of housefly and other insects [45,47,49]. Lemongrass EO acted toxically on the olfactory sense in the antennae and the integument branes of housefly by inhibiting enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) [22,49]. It was toxic to *M. domestica* larvae causing cell shrinkage, spinous cells to proliferate, pupae to emerge partially, and adults to develop abnormally [50]. *M. domestica*'s nervous system synapses were damaged by star anise EO, geranial, and *trans*-anethole. All caused cell membrane damage, slowed down cellular metabolism, inhibited AChE activity in a neurotoxic manner, and caused acetylcholine to accumulate [49,51]. These effects resulted in hyperactivity, immobility, knockdown, morbidity, and mortality [16,45,50,52,53]. Furthermore, a mixture of monoterpenoids is a more effective AChE inhibitor than a single monoterpenoid. They work together harmoniously [25].

According to the safety data, all EO formulations were safe for the adults of the two non-target species, stingless bee and guppy. Their SI was larger than 1 and their LT_{50} values were high (see Fig. 4A and B). A mixture formulation—1.0% lemongrass EO + 1.0% *trans*-anethole—was outstandingly benign to stingless bee and guppy adults, with a 0.001% mortality rate. In contrast, 1.0% (*w/v*) α-cypermethrin was highly toxic to the two non-target species, with a low SI, and LT_{50}.

EOs and their components are frequently regarded as safe for natural insect adversaries, pollinators, and other invertebrate

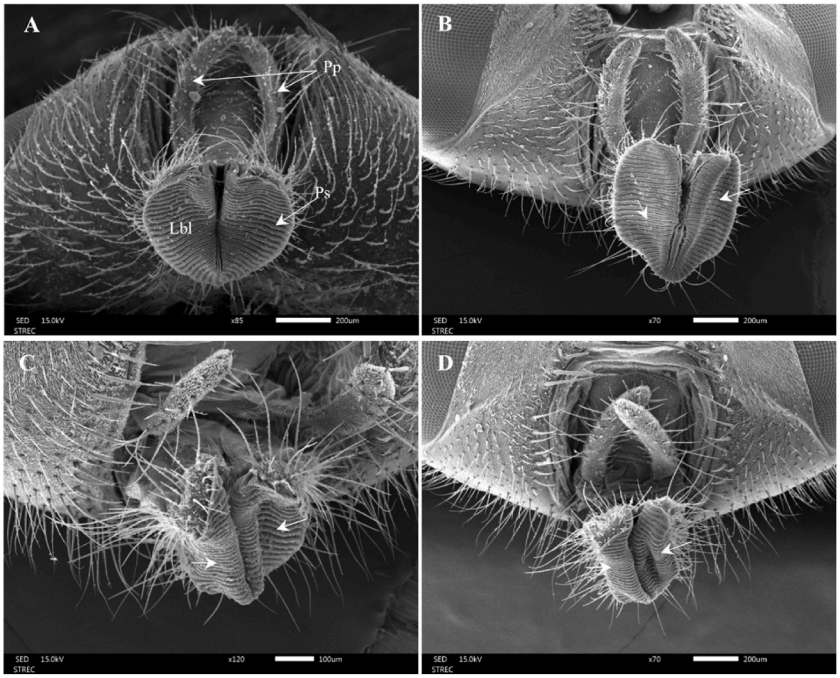

Fig. 6. Scanning electron micrographs of the mouthparts of housefly: (A,B) non-treated, showing full external structure with tips of palpus (Pp), labellum (Lbl), and labellum with pseudotracheae (Ps); morphological damage to mouthparts after treated with lemongrass EO (B), geranial (C), and a mixture of lemongrass EO + *trans*-anethole (D).

organisms [13,14,54,55]. Two studies reported that *C. citratus* EO and geranial were safe to a predator fish, guppy (*P. reticulata*) [13], and a predator bug, *P. nigrispinus* (Heteroptera) [19]. *Trans*-anethole and lemongrass EO did not show any toxic effect against honeybee, *A. mellifera* workers [14,54]. EOs from rosemary pepper and its major compounds, (*E*)-caryophyllene and ρ-cymene were not toxic to stingless bees, *Nannotrigona* aff. *testaceicornis* [56]. In addition, Moungthipmalai et al. [13] supported that a combination of *C. verum* EO + geranial (2:1 ratio) did not show toxicity against two species of predator fish, *P. latipinna* and *P. reticulata*. A combination of EOs from *Cedrus atlatica* + *Corymbia citriodora* + *C. citratus* (1:1:1 ratio) was safe to parasitoid of *Ceratitis capitate* (Diptera) and *Psyttalia concolor* (Hymenoptera) [57]. On the other hand, commonly, synthetic insecticides are regarded as poisonous to pollinators and other naturally occurring insect pest enemies [58]. Cypermethrin is highly toxic to stingless bees, *Meliponula bocandei*, with a low LC_{50} of 0.66–0.76 μg/ml [59]. To make matters worse, cypermethrin contributes significantly to environmental pollution since it can build up in the soil, water, and air. Due to its high accumulation in the environment, it becomes very toxic to fish, pollinator species, and other non-target species [56,59]. In addition, cypermethrin is hazardous to mammals and humans [60]. It is poisonous to the human central nervous system, causing hyperexcitation of neurons, nausea, headaches, incontinence, shortness of breath, convulsion, and even death [60]. In contrast, EOs and their constituents do not accumulate in the environment, making them far safer for non-target species and natural insect pest enemies [11,14]. In this study, all EO formulations had an SI greater than 1, indicating that they were all entirely safe for these non-target species. Furthermore, the primary components of EOs from lemongrass and star anise neither damaged nor altered the histopathology of human cells [61,62]. They have long been used as traditional Asian medicine and food ingredients [38–41].

5. Conclusion

Due to their efficacy and safety, the outstanding mixture formulation—1.0% lemongrass EO + 1.0% *trans*-anethole—should be developed further into a natural adulticidal agent for managing housefly populations in urban and suburban areas and organic farms. Most importantly, the chosen EO and EO constituent—lemongrass EO and *trans*-anethole—are readily available and reasonably priced, which makes them excellent choices for inclusion in an insecticidal formulation. Several tests must be conducted along the way before the formulation can be developed into a commercial product, tests such as post-application humidity and temperature tests. Furthermore, semi-field and field studies on factors impacting the death of target and non-target species (biological stability of temperatures and humidities) as well as non-target effects, and toxicity on human cells should be investigated.

Ethics approval

This study was reviewed and approved by the Ethics Committee of King Mongkut's Institute of Technology Ladkrabang, with the

approval number: KDS2021/002.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Mayura Soonwera: Writing – review & editing, Writing – original draft, Conceptualization. **Tanapoom Moungthipmalai:** Funding acquisition, Formal analysis, Data curation. **Cheepchanok Puwanard:** Methodology, Formal analysis, Data curation. **Sirawut Sittichok:** Writing – original draft, Investigation, Formal analysis. **Jirisuda Sinthusiri:** Writing – original draft, Investigation, Formal analysis. **Hataichanok Passara:** Writing – original draft, Investigation, Formal analysis.

Declaration of competing interest

Tanapoom Moungthipmalai reports financial support was provided by King Mongkut's Institute of Technology Ladkrabang. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

EOs	Essential Oils
EO	Essential Oil
KI	Knockdown Index
MI	Mortality Index
SMI	Synergistic Mortality Index
IMV	Increased Mortality Value
SI	Safety Index; EtOH, Ethyl alcohol

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