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Larvicidal and pupicidal activity of combination of two plant essential oils against *Aedes aegypti*

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Abstract The larvicidal and pupicidal effects of six mixtures of cinnamon (*Cinnamomum verum* J. Presl.) and nutmeg (*Myristica fragrans* Houtt.) essential oils (EOs) and major compositions (geranial, α-pinene and *trans*- cinnamaldehyde) were tested against early fourth instar and pupal stages of *Aedes aegypti*. The combinations (2% cinnamon EO + 1% geranial, 2% cinnamon EO + 1% *trans*-cinnamaldehyde and 2% cinnamon EO + 1% α-pinene) showed high toxicity against early 4th instar larvae, with 100% mortalities at 24 hours and LT_{50} between 1.4 and 2.4 hours. Additionally, 2% nutmeg EO + 1% α-pinene showed >99% mortality at 24 hours with LT_{50} = 4.6 hours. 2% nutmeg EO + 1% geranial and 2% nutmeg EO + 1% α-pinene were highly toxic against pupae, with 100% mortality at 48 hours and LT_{50} values of 5.8 and 7.8 hours. On the other hand, temephos showed LT_{50} for larval and pupal stages at 9.8 and 94.1 hours. It showed that 2% nutmeg EO + 1% α-pinene was able to control immature stages and was more effective than temephos. However nutmeg extracts need to be checked for human and valuable species toxicity.

Keywords: *Aedes aegypti,* Combination essential oils*,* Larvicidal and pupicidal activities, Cinnamon oil, Nutmeg oil

Introduction

Mosquitoes (Culicidae: Diptera) pose a major global menace to both people and animals. Many harmful infections and parasites are spread via them. (Benelli *et al.,* 2016). *Aedes aegypti* (L.) is an important vector of arboviruses: it is responsible for transmitting pathogens that cause various infectious diseases such as dengue, chikungunya and Zika virus (Beltrán-Silva *et al*., 2016; Ahebwa *et al*., 2023). *Ae. aegypt*i females are anthropophilic and prefer humans as hosts, so humans are the main targets of their attacks. *Ae. aegypti* females may spend

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all of their lives in or near the houses and they prefer to lay their eggs in the clean water found in many types of domestic containers inside or near human dwellings (Yu *et al*., 2015; Elumalai *et al*., 2016). All mosquitoes have aquatic immature stages. Therefore, larvicidal and pupicidal activity is a commonstrategy to reduce mosquito populations and prevent disease. Larvicidal activity is very important in vector management because in the growth stage, larvae are the easily killed. Controlling larvae typically requires the long-term administration of organophosphates or other growth regulators, including methoprene and diflubenzuron (Conti *et al*., 2014). Temephos is a registered organophosphate produced commercially that has been extensively used for controlling *Ae. aegypti* larvae (Perumalsamy *et al*., 2009). These days, the effectiveness of synthetic chemical insecticides in managing mosquito vectors is being called into serious question due to the permanent harm they do to the environment and the development of resistance. Plant essential oils (EOs) and their constituents have been proposed as alternative larvicidal and pupicidal treatments for mosquito control in recent years. This is primarily because the bioactive compounds in EOs often have little effects on other creatures and the environment (Perumalsamy *et al*., 2015).

Thailand is a botanically rich country with many kinds of native aromatic plants. These plants (and their oils) are extensively used in traditional Thai medicine. Many plants have been evaluated as controls for mosquito larvae. Moreover, combinations of EO and EO constituents exhibited a higher degree of toxicity against mosquitoes (Soonwera *et al*., 2024). However, the larvicidal and pupicidal activities of combination of *Cinnamomum verum* and *Myristica fragrans* EO and major constituents against *Aedes aegypti* have not been reported. Therefore, this study investigated the insecticidal activity of combinations of cinnamon (*Cinnamomum verum* J. Presl.) and nutmeg (*Myristica fragrans* Houtt.) EO and their major constituents (geranial, α-pinene and *trans*- cinnamaldehyde) against immature *Ae. Aegypti*.

Materials and methods

Ae. aegypti were raised and tested at School of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok, on September 2023 to March 2024, under the environment conditions of 26±2 °C, 75.0±2% RH. Eggs were hatched in plastic 180 mm×270 mm×100 mm boxes, each containing 2.0 L of tap water. Fish food pellets were given to the larvae until they pupated. After being gathered, pupae were placed in an insect cage of 300 mm×300 mm×300 mm, and adult mosquitoes were fed 5% glucose on cotton wool. On day 5, blood meals were given to the female adults following an

artificial membrane feeding method (Phasomkusolsil and Soonwera, 2010). Following 2-3 days of blood meals to prepare the females for spawning, wet filter papers were placed in a cup on the water's surface so they could deposit their eggs there. For 5-7 days, the eggs were kept wet and then put on a pan to hatch. Early fourth instar larvae and pupal stages were used for the experiments. Materials and essential oils used are listed in Table 1, and the used formulations are listed in Table 2.

Table 1. Materials

Name	Source	CAS number	Comments
fish food pellets	OPIMUM®		Thai local company
glucose syrup	Mitr Pohl, Thailand		Sucrose 50%, glucose 25%, fructose 35%
cinnamon oil	Chemipan Corporation Co Ltd, Kanna Yoa, Bangkok		
nutmeg oil	Chemipan Corporation Co Ltd, Kanna Yoa, Bangkok		
geranial	Sigma-Aldrich	CAS 5392-40-5	Technical grade, 96%
α-pinene	Sigma-Aldrich	CAS 80-56-8	Technical grade, 98%
trans-cinnamaldehyde	Sigma-Aldrich	CAS 104-55-2	Technical grade, 98%

Table 2. Formulations

Label	cinnamon oil	nutmeg oil	geranial	α-pinene	*trans*-cinnamaldehyde
F1	2%		1%		
F2	2%			1%	
F3	2%				1%
F4		2%	1%		
F5		2%		1%	
F6		2%			1%

All formulations were mixed with Tween 60 in reverse osmosis water and were kept at room temperature before being testing.

Bioassay

Larvicidal and pupicidal activity were each determined according to a test dipping method by Soonwera and Phasomkusolsil (2016). The experiment was performed using a completely randomized design (CRD), consisting of 6 treatments (T 1-6), each with 5 replications. For each experimental treatment, 1 mL combination essential oil solution was added to 99 mL of distilled water in a 200 mL glass cup. Ten immature stage *Ae. aegypti* (Early fourth instar larvae and pupal stages) were put into each glass cups. All larvae were exposed to combination EOs until pupation, and mortality was observed over 24 h. The

pupae were exposed to combine EOs until some grew into adults and mortality was observed over 48 h. Temephos was used as a positive control and Tween 60 as a negative control. During the experiment, the larvae were not fed, and dead larvae in 24 hours. The dead larvae showed abnormal movement which after 24 h. The pupal were recorded at 48 h and considered dead larvae, if they did not respond to touch by a probe.

The mortality rate of larvae and pupal (%M) was determined as (Soonwera *et al*., 2024):

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

where MT is the total number of larvae or pupal dead, and TN is the total number of larvae or pupal treated.

Mortality index (MI) was determined as (Soonwera *et al*., 2024):

$$\text{MI} = LT_{50} \text{ of treatment} / LT_{50} \text{ of temephos}$$

where MI < 1 signifies that the formulation was more toxic than temephos, and MI > 1 signifies that it was less toxic.

Statistical analysis

The mortality data from each treatment of larvae and pupal were statistically analyzed using one-way ANOVA and means were compared by Duncan's multiple range test (DMRT). The mean mortality data of the five replicates per treatment was used to calculate the LT_{50} values (lethal time for 50% mortality).

Results

The mortality rate, LT_{50} values and mortality index of combination EOs against the early 4th instar larvae of *Ae. aegypti* are presented in Table 3 and Figure 1. Combinations F1, F2 and F3 showed the highest toxicity against the early 4th instar larvae, with 100% mortality at 360 minutes. Moreover, F5 showed 93% at 360 minutes and >99% mortality at 24 hours, with no significant differences in mean mortality rate for these four combinations. Whereas 1% (w/w) temephos showed lower mortality than these combinations, with 90% at 360 minutes and 94% at 24 hours. The best LT_{50} level at 1.4 h was for F1, followed closely by F3 (1.7 h) and F2 (2.4 h) and then F5 (4.6 h), versus temephos (9.8 h). Compared with 1% (w/w) temephos, the mortality indices (MI) of the four combinations were F1 - 0.14, F3 - 0.17, F2- 0.24 and F5 - 0.47.

The mortality rate, LT_{50} and mortality index of combination EOs against the pupal stages are shown in Table 4 and Figure 2. F4 showed the highest

toxicity against the pupae - 100% mortality at 24 hours, with F5 showing 98% at 24 hours but 100% mortality at 48 hours. Both combinations showed no significant differences in mean mortality rate. The LT_{50} for F4 = 5.8 h and F5 = 7.8 h, whereas 1% (w/w) temephos was hardly effective against pupae with mortality 1% at 48 h and LT_{50} = 94 hours. The mortality indices of F4 and F5 were very low at 0.06 and 0.08 versus 1% (w/w) temephos.

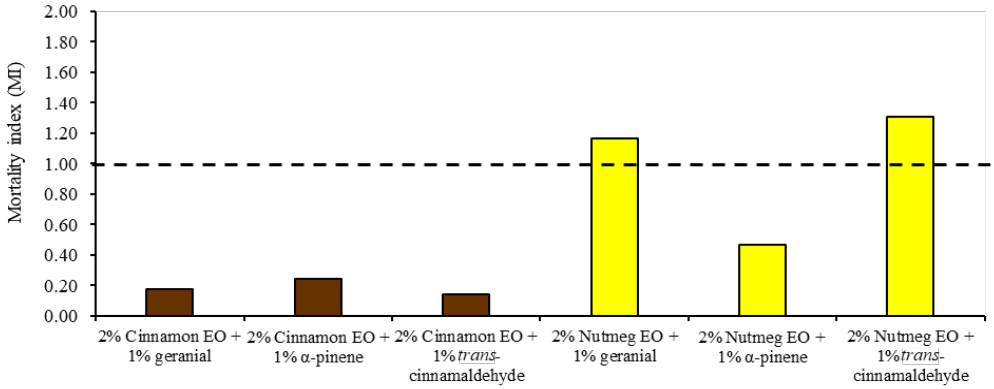

Figure 1. Mortality index (MI) of combination EOs against the early 4th instar larvae of *Ae. Aegypti*

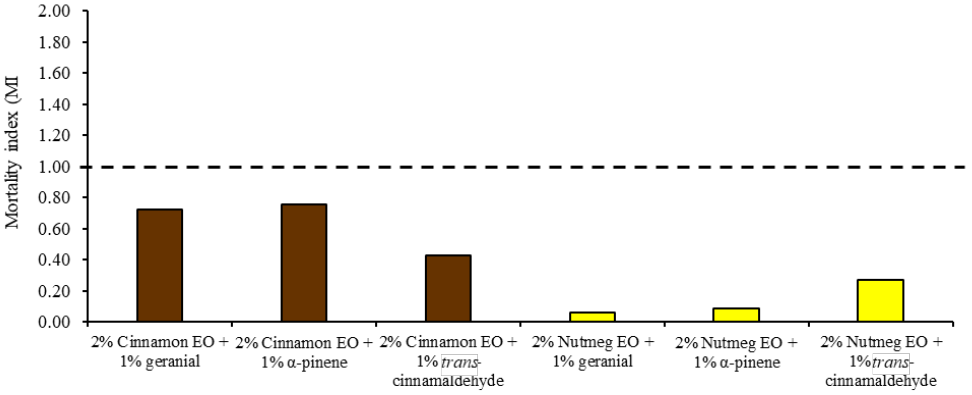

Figure 2. Mortality index (MI) of combination EOs against the pupal stages of *Ae. Aegypti*

Note: MI < 1 signifies that the formulation was more toxic than temephos; and MI > 1 signifies that the formulation was less toxic than temephos.

Table 3. Insecticidal activity of combination EOs against the early 4th instar larvae of *Ae. aegypti*

Combination EOs	% Mortality rate ± SD / Time (min.)									LT_{50}[2/] (h)
	1	5	10	15	30	60	120	360	1440 (24 h)	
F1 = 2% Cinnamon EO + 1% geranial	0±0[1/]	0±0	3.5±3.2^{A}	8.3±2.3^{A}	20.3±5.9^{B}	41.3±9.5^{B}	81.8±11.3^{A}	100±0^{A}	100±0^{A}	1.7 (1.6-1.8)
F2 = 2% Cinnamon EO + 1% α-pinene	0±0	0±0	0±0^{B}	6.8±1.9^{AB}	12.8±10.9^{C}	32.5±13.5^{BC}	59.5±13.1^{BC}	100±0^{A}	100±0^{A}	2.4 (2.2-2.6)
F3 = 2% Cinnamon EO + 1% *trans*-cinnamaldehyde	0±0	0±0	0±0^{B}	6.3±2.0^{AB}	28.3±11.5^{A}	55.3±11.8^{A}	87.3±7.2^{A}	100±0^{A}	100±0^{A}	1.4 (1.3-1.6)
F4 = 2% Nutmeg EO + 1% geranial	0±0	0±0	0±0^{B}	0±0^{C}	2.8±4.6^{D}	16.5±8.0^{D}	34.3±26.7^{D}	46.0±31.4^{B}	94.8±7.4^{B}	11.4 (-)
F5 = 2% Nutmeg EO + 1% α-pinene	0±0	0±0	0±0^{B}	5.3±1.9^{B}	16.3±10.9^{BC}	30±13.1^{C}	56.0±23.3^{C}	93.0±13.0^{A}	99.8±1.0^{A}	4.6 (-)
F6 = 2% Nutmeg EO + 1% *trans*-cinnamaldehyde	0±0	0±0	0±0^{B}	0±0^{C}	0±0^{D}	3.0±1.1^{E}	8.3±4.1^{E}	49.5±22.1^{B}	88.0±11.6^{C}	12.8 (11.5-14.3)
Tween 60 (negative control)	0±0	0±0	0±0^{B}	0±0^{C}	0±0^{D}	0±0^{E}	0±0^{E}	0±0^{C}	0±0^{D}	NA[3/]
1% (w/w) temephos (positive control)	0±0	0±0	0±0^{B}	2.4±1.3^{BC}	11.3±6.9^{C}	35.3±16.2^{BC}	76.3±11.1^{AB}	90.3±8.6^{A}	93.8±8.3^{B}	9.8 (4.8-22.1)
df_{total}, *F*-test	39**	39**	39**	39**	39**	39**	39**	39**	39**	-
C.V. (%)	-	-	110.65	114.99	50.41	28.33	26.73	21.92	4.11	-

[1/] Mean % Mortality in each column marked by different letters were significantly different ($P < 0.05$, by one-way ANOVA and Duncan's multiple range test)
[2/] LT50 = Lethal time for 50% mortality at 95% confidence limit
[3/] NA = Not Available

Table 4. Insecticidal activity of combination EOs against the pupal stages of *Ae. aegypti*

Combination EOs	% Mortality rate ± SD / Time (min.)									LT_{50}[2/](h)
	1	10	15	30	60	120	360	1440 (24 h)	2280 (48 h)	
F1 = 2% Cinnamon EO + 1% geranial	0±0[1/]	0±0	0±0	0±0	4.2±2.0^{BC}	5.2±2.4^{CD}	11.0±5.7^{CD}	14.0±6.9^{D}	22.0±11.4^{C}	68.1 (58.0-83.5)
F2 = 2% Cinnamon EO + 1% α-pinene	0±0	0±0	0±0	0±0	5.2±2.4^{B}	6.0±4.7^{C}	10.1±6.7^{CD}	16.0±5.2^{D}	20.0±6.7^{C}	71.2 (59.8-89.4)
F3 = 2% Cinnamon EO + 1% *trans*-cinnamaldehyde	0±0	0±0	0±0	0±0	8.0±4.2^{A}	11.0±5.7^{B}	14.0±5.2^{BC}	30.0±9.4^{C}	59.0±17.3^{B}	39.9 (36.2-44.4)
F4 = 2% Nutmeg EO + 1% geranial	0±0	0±0	0±0	0±0	13.2±4.7^{A}	17.0±10.6^{A}	49.0±12.9^{A}	100±0^{A}	100±0^{A}	5.8 (5.3-6.4)
F5 = 2% Nutmeg EO + 1% α-pinene	0±0	0±0	0±0	0±0	0±0^{C}	19.5±5.7^{A}	49.0±12.9^{A}	98.0±4.2^{A}	100±0^{A}	7.8 (-)
F6 = 2% Nutmeg EO + 1% *trans*-cinnamaldehyde	0±0	0±0	0±0	0±0	0±0^{C}	4.2±2.0^{DE}	17.1±5.0^{B}	41.0±11.9^{B}	100±0^{A}	25.2 (23.2-27.4)
Tween 60 (negative control)	0±0	0±0	0±0	0±0	0±0^{C}	0±0^{E}	0±0^{D}	0±0^{E}	0±0^{D}	NA[3/]
1% (w/w) temephos (positive control)	0±0	0±0	0±0	0±0	0±0^{C}	0±0^{E}	0±0^{D}	0±0^{E}	1±3.1^{D}	94.1
df_{total}, *F*-test	39**	39**	39**	39**	39**	39**	39**	39**	39**	-
C.V. (%)	-	-	-	-	152.12	64.09	54.99	18.10	16.38	

[1/] Mean % Mortality in each column followed by different letters were significantly different ($P < 0.05$, by one-way ANOVA and Duncan's multiple range test)
[2/] LT50 = Lethal time for 50% mortality at 95% confidence limit
[3/] NA = Not Available

Discussion

The research finding revealed that four combinations (F1, F2, F3 and F5) were effective against the 4th instar larvae and combinations (F4 and F5) were effective against the pupal stage. In contrast, temephos was highly effective only against larvae but had no effect on pupae. Overall, it found F5 had the potential to control both larvae and pupal stages and was more effective than temephos. The combination of nutmeg EO + α-pinene is found to be a new investigation. Previous studies reported to use of nutmeg EO. Gomes da Rocha Voris *et al*. (2018) who reported it was toxic to larvae and adult females at LC_{50} of 28 ug/ml and 18 ug/mg and Abou-Elnaga (2014) who also reported that toxicity to larvae and adults of *Ae. aegypti* and *Culex pipiens*. Similarly, Carolina and Maman (2016) found fruit nutmeg EO was more toxic than leaf nutmeg EO, with LC_{50} values 110 and 134 ug/ml: the active chemical compound in nutmeg oil was believed to cause mosquito larvae death. On the other hand, Sarma *et al*. (2022) showed that isomers of pinene were had good larvicidal properties against 4th instar larvae where α-pinene acted more rapidly than β-pinene, with LC_{50} values being 166 and 349 ppm at 24 hours. Moreover, among other order *Diptera* insects, Aungtikun *et al.* (2021) reported that 5% nutmeg EO was effective against adult *Musca domestica* (house fly) with 90% knockdown rate at 60 minutes (KT_{50} = 28 mins) and 48% mortality at 24 hours (LT_{50} = 54.0 mins). Additionally, combinations of 0.5% nutmeg EO + 0.5% geranial and 0.5% α-pinene + 0.5% geranial showed 100% knockdown at 60 minutes (KT_{50} = 5.9-6.1 mins) and 100% mortality at 24 hours (LT_{50} = 12.0 mins). Cossetin *et al*. (2021) showed that 5% nutmeg EO was very effective against *M. domestica* and *Chysomya albiceps* (blow fly) larvae with 100% and 95% embryo died inside the pupae. In addition, the compounds of nutmeg EO were toxic to adult female german cockroaches, *Blattella germanica* (Jung *et al*., 2007) and nymph and adult whitefly, *Bemisia tabaci* (Wagan *et al*., 2017). These studies demonstrated that nutmeg EO and α-pinene, by themselves, were toxic to immature mosquitoes and several other insect species.

Myristica fragrans Houtt. (Myristicaceae) has been cultivated in many tropical countries. *M. fragrans* (known as Chan-thet in Thailand) is used for food, beverage and cosmetics. The seed of *M. Fragrans* or nutmeg is widely used as a carminative, astringent, hypolipidemic, antithrombotic, antiplatelet aggregation, antifungal, aphrodisiac, anti-flatulence, anti-nausea and anti-dyspepsia agent (Champasuri and Itharat, 2016). Nutmeg oil has various pharmacological properties and active constituents, including α-pinene, elemicin, 4-terpineol, myristicin, eugenol, safrole and linalool. Biological activities of the oil, including antioxidant, analgesic, anti-inflamtory, anticonvulsant, antibacterial, antiparasitic, insecticidal, and anti-carcinogenic activities have been reported.

However, a large intake of nutmeg oil could cause intoxication, shown through effects on the central nervous system and the stomach (Warsito, 2021).

In conclusions, this study indicated that the combination of nutmeg oil + α-pinene had larvicidal and pupicidal effects against *Aedes aegypti* and was more effective than commercial temephos. However, the toxicity of nutmeg oil + α-pinene should be checked for safety with human and other valuable species and general environmental safety. In particular, given the reported toxicities of high concenrations of nutmeg oil, future study should investigate nutmeg oil and α-pinene toxicity against non-target species.

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