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ORIGINAL PAPER

Behavioral responses of *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Culicidae) to four essential oils in Thailand

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Abstract The behavioral effects of four essential oils extracted from orange peel (*Citrus aurantium* L.), cinnamon leaf (*Cinnamomum verum* J. Presl), citronella grass (*Cymbopogon winterianus* Jowitt), and clove flower [*Syzygium aromaticum* (L.) Merrill & Perry] were evaluated against two medically important species of mosquitoes, *Aedes aegypti* (L.) and *Culex quinquefasciatus* Say, using an excito-repellency test system. *Ae. aegypti* was collected from a small village in Kanchanaburi Province and *Culex quinquefasciatus* was captured from an urban area of Bangkok. Mosquitoes from the F1–F3 generations were tested in the excito-repellency test chamber for contact excitation and non-contact spatial repellency. Results showed that both species demonstrated varying levels of behavioral escape responses to different essential oils, showing a clear dose response depending on percent w/v concentration used. Orange oil produced the least response in both mosquito species, while citronella and clove the greatest. In general, *Cx. quinquefasciatus* exhibited much stronger behavioral responses to all four essential oils than *Ae. aegypti*. From this study, we conclude that the essential oils from various botanical sources should continue to be screened for protective properties against mosquitoes and other biting arthropods.

Keywords *Aedes aegypti* · *Culex quinquefasciatus* · Essential oils · Behavioral responses · Excito-repellency test system

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Introduction

Approximately 4,000 known species of mosquitoes have been described throughout the world with some species having wide cosmopolitan distributions in the urban and peri-urban settings in close association with humans, most notably *Aedes* (*Stegomyia*) *aegypti* L. and *Culex quinquefasciatus* Say. *Ae. aegypti*, the primary epidemic vector of dengue viruses, is a predominately urban, day-biting mosquito, often found in and around human dwellings and preferentially feeds on humans, whereas *Cx. quinquefasciatus* is a common urban and rural species with strong night biting patterns and is a major vector of Bancroftian filariasis (*Wuchereria bancrofti*) and several arboviruses in various parts of the world (Sasa 1976). Both mosquito species have been extremely refractory to common control measures and can occur in high-population densities. Despite decades of research, an efficacious and commercially viable multivalent dengue vaccine is not yet available. Therefore, the prevention and control of dengue and many other vector-borne diseases (e.g., malaria and filariasis) remains dependent on various vector control strategies to decrease transmission risk. In some instances this requires the use of chemical insecticides as adulticides for space spray and indoor residual spray applications as well as pyrethroid-impregnated bed nets to control adult mosquito blood-feeding (Roberts and Andre 1994; Reiter and Gubler 1997; Grieco et al. 2007).

A variety of chemical compounds can protect humans from blood-feeding insects by one or more of three identified actions, contact excitation (irritancy), non-contact spatial repellency or physiological knockdown (KD), and toxicity (Grieco et al. 2007). The first two properties are potential outcome behavioral responses of mosquitoes after or before they make physical contact with a chemical (Chareonviriyaphap et al. 1997; Roberts et al. 1997) A lethal or KD response occurs when the mosquitoes receive a toxic dose of an insecticidal chemical (Roberts et al. 2000). Recently, a fourth behavioral action has been described for *N*,*N*-diethyl-*m*-toluamide (DEET), a common synthetic chemical often referred to as having a "repellent" action, in which the chemical actually inhibits an insect's odor activated receptors thereby preventing the location of the host for blood-feeding (Ditzen et al. 2008; Suwannachote et al. 2009). Most studies have focused primarily on the toxic action of chemicals designed to control mosquitoes and disease transmission, whereas relatively few investigations have directed attention to the non-toxic properties, including irritancy and repellency (Grieco et al. 2007; Chareonviriyaphap et al. 1997, 2004). Much of the previous works have focused on synthetic chemicals (Thanispong et al. 2009; Mongkalangoon et al. 2009) that are often non-discriminating in their detrimental effect on non-target organisms and can result in excessive contamination to the surrounding environment. In addition, the development of resistance by mosquitoes to synthetic compounds has resulted in reduced effectiveness of traditional chemical-based prevention and control methods (Thanispong et al. 2008; Chuaycharoensuk et al. 2011). For this reason, the potential for use of native plant derived products as a source of essential oils is an attractive option to replace or supplement synthetic compounds for the control of vectors and prevention of disease transmission.

One of the most common insect "repellent" active ingredients in commercial products is DEET (McCabe et al. 1954; Fradin and Day 2002). This compound has demonstrated to be a broad-spectrum repellent with excellent protection against mosquitoes and other blood-sucking arthropods (Fradin and Day 2002; Yap 1986; Coleman et al. 1993; Walker et al. 1996). However, DEET has been shown to have significant adverse or toxic effects to humans (Brown and Hebert 1997), especially when misapplied and at higher concentrations is a powerful solvent of plastics and other synthetic materials (Odalo et al. 2005). For these reason, there has been an interest on developing insect repellents from natural plant extracts, such as thyme (Park et al. 2005), clove (Bernard 1999; Trongtokit et al. 2004, 2005), celery (Tuetun et al. 2005), citronella (Yang and Ma 2005), and others.

Numerous essential oils extracted from plants have been evaluated for insect repellent activity for protection against mosquitoes and other arthropod pests in Thailand, for example *Ocimum* spp. (Chokechaijaroenporn et al. 1994; Tawatsin et al. 2001) *Nepeta cataria* (Polsomboon et al. 2008b), *Citrus hystrix* (Thavara et al. 2007), *Melaleuca leucadendron*, *Litsea cubeba*, and *Litsea salicifolia* (Noosidum et al. 2008). Recently, over 90 plant species were evaluated as potential repellents or toxicants against blood-feeding invertebrates and pestiferous flies in Lao PDR (De Boer et al. 2010). Although several essential oils have exhibited significant repellent activity against target insects, comparatively little has been done to identify the degree of behavioral responses at varying chemical concentrations or to differentiate the two primary types of behavioral responses (i.e., contact excitation and spatial repellency) to chemical exposure (Polsomboon et al. 2008a; Noosidum et al. 2008). As follow-on to the work conducted by Polsomboon et al. (2008a), we describe the behavioral responses of *Ae. aegypti* and *Cx. quinquefasciatus* based on the two non-toxic properties of four extracted essential oils using an excito-repellency (ER) test system.

Materials and methods

Mosquito populations

Ae. aegypti was collected as larvae and pupae from artificial containers located in Ban Pu Tuey, Ta-Soa Sub-district, Sai-Yok District, Kanchanaburi Province (14°20′N, 98°59′E). Species identification and subsequent colonization was carried out at the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. *Ae. aegypti* mosquitoes were reared following the methods of Kongmee et al. (2004).

Culex quinquefasciatus was captured as larvae and pupae from polluted collections of water near houses in Jatuchak District, Bangkok, Thailand (13°50′50.39″N, 100°34′19.44″E). Species identification and subsequent colonization was conducted at the Department of Entomology, Kasetsart University using morphological keys and standard rearing procedures (Kongmee et al. 2004).

For both species, only F1–F3 generation, 3–5-day-old female mosquitoes were used. Mating status was not examined, but *Ae. aegypti* was assumed mated while status of *Cx. quinquefasciatus* was unknown.

Essential oils

The four essential oils used in this study were purchased in maximum extract concentration form from Thai-China Flavours and Fragrances Industry Co., Ltd. Company (TCFF, Phra Nakhon Si Ayutthaya, Thailand) as follows: orange oil 'A' (CA: *Citrus aurantium* L.), by cold expression of outer peel (product code: MK-20024); cinnamon oil (CV: *Cinnamomum verum* J. Presl. [synonym = *C. zeylanicum* Blume]), by water of steam distillation of leaves and twigs (product code: MK-20086); citronella oil (CW: *Cymbopogon winterianus* Jowitt), by steam distillation of citronella grass (product code: MK-40012); and clove oil (SA: *Syzygium aromaticum* (L.) Merrill & Perry [synonym = *Eugenia caryophyllata* Thunb.]), by water distillation of dried flower (product code: MK-20006). All oils were deemed acceptable for use based on shelf life characteristics and storage criteria (tightly sealed bottles and protection from light) from the manufacturer; however, none were reanalyzed for specific gravity and refractive index after initial testing at point of production.

Treated papers

Three different concentrations of each oil product as serial dilutions 2.5, 5.0, and 10.0 % (w/v) were individually impregnated onto filtered test papers (14.7 × 17.3 cm^2) for use in the ER test system, following World Health Organization general procedures for treating papers with insecticidal compounds (World Health Organization 1998). Three serial concentrations were prepared by dilution of individual stock oil in absolute ethanol only. All papers were treated at the rate of 2.8 ml of test solution per 254.3 cm^2 surface area and allowed to air dry. Control paper was treated separately with absolute ethanol only. All papers were allowed to air dry for at least 12-h before use in the ER tests and were used only once per test series.

Excito-repellency test system

An ER test system was used as previously described (Polsomboon et al. 2008a; Noosidum et al. 2008; Chareonviriyaphap et al. 2002). Four test chambers were used per test trial. One pair of chambers served as contact and control, the other pair as non-contact and control configurations. The ER test apparatus consists of a screened inner chamber that is used to prevent mosquitoes coming in direct contact with chemical-treated surfaces and thus serving as a non-contact (repellency) chamber (Fig. 1). Alternatively, contact test designs had treated papers attached inside the chamber so that the exposed surfaces were available for mosquitoes to land on. All females were deprived of sucrose solution and provided only water 12 h before testing.

To begin the test, 15, 3–5-day-old, non-blooded female mosquitoes were introduced per chamber using a mouth aspirator and allowed a 3-min ″resting″ (adjustment) period, after which the escape funnel was opened to begin the observation period. A receiving cage (21.5 × 24.5 × 21 cm^3 paper carton) was connected to the exit portal for collecting all escaped mosquitoes. The number of escaping mosquitoes from each ER chamber was recorded at 1-min intervals for a full test period of 30 min. Immediately following test completion, the number of dead or KD (moribund) specimens either remaining inside the chamber and those that had escaped into the receiving cage were recorded for each of the 4 chambers. All moribund and live specimens that had successfully escaped or remained inside the test chamber were transferred to clean holding cups, labeled and provided with a 10 % sucrose solution for sustenance over a 24-h holding and observation period, at which time mortality was recorded. All test pairs were replicated four times.

Data analysis

A Kaplan–Meier survival analysis method was used to analyze and interpret the behavioral response data (Roberts et al. 1997; Kleinbaum 1995). Mosquitoes that escaped from the chamber were categorized as "dead" and those remaining inside the chamber as "survivors" (Chareonviriyaphap et al. 1997). Survival analysis was used to estimate probability of mosquito escape over time and comparison of differences in escape response between difference test populations, essential oils, and percent concentrations. The use of survival curves minimized loss of valuable information by estimating mosquito escape probability over all time points. The log-rank method (Mantel and Haenzel 1959) was used to compare patterns of escape response

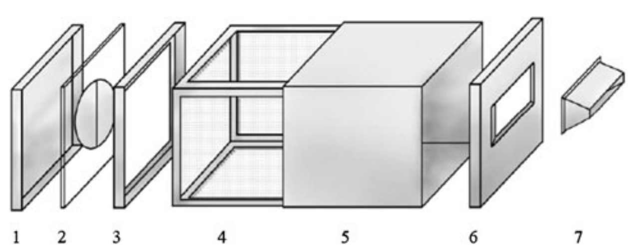

Fig. 1 Breakdown diagram of ER test system used in behavioral response studies (based on Chareonviriyaphap et al. 2002). Rear door cover (1), Plexiglas with rubber-sealed door (2), Plexiglas holding frame (3), screened inner chamber (4), outer chamber (5), front panel (6), and exit portal (7). Receiving cage not shown

within and between different groups by time (Chareonviriyaphap et al. 2004). The discriminating level of statistical significance for all tests was set at 95 % ($p < 0.05$). Mean percent mortality was calculated per test chamber. For those tests in which the control (untreated) mortality exceeded 5 %, a correction factor (Abbott's formula) was applied to the data (Abbott 1925).

Results

ER responses of *Ae. aegypti* and *Cx. quinquefasciatus*, exposed to 2.5, 5.0, and 10.0 % w/v essential oils of orange (CA), cinnamon (CV), citronella (CW), and clove (SA) were evaluated for contact irritancy and non-contact repellency responses using an ER test system. Post-exposure mortality of *Ae. aegypti* and *Cx. quinquefasciatus* after 24-h post-exposure holding period are documented in Tables 1 and 2, respectively. Knockdown data of escape and non-escape specimens were also recorded at the end of the 30 min test. In general, higher mortality was recorded from non-escape mosquitoes compared to mosquitoes successfully exiting the chambers and from exposure to higher concentrations vice lower. In all paired tests, very few mosquitoes (0–5 %) escaped from untreated control chambers and both mortality and KD were very low (data not shown).

The escape response of both species varied significantly, depending upon oil and chemical concentration used. In general, a stronger escape response was observed when both species were tested with clove and citronella compared to orange and cinnamon extracts. A greater escape response was observed in the contact trials compared to the spatial non-contact trial designs. Specifically, a greater overall escape response was recorded for *Cx. quinquefasciatus* compared to *Ae. aegypti*.

For *Ae. aegypti* contact trials, the highest escape responses were seen with 2.5 % clove (36.9 %), the lowest response with exposure to 10 % cinnamon (8.9 %). In non-contact trials, 2.5 % clove and 5 % citronella produced the strongest escape responses (38.8 and 39.0 %, respectively) and 5 % cinnamon gave the lowest escape response (3.3 %) at 30 min. No mortality was observed in escape specimens from contact and non-contact for all three concentrations of orange oil and comparatively low-percent mortality were seen in the non-escape mosquitoes, ranging from 0 to 2 % only. For clove oil, a higher percent mortality was observed in both escape and non-escape *Ae. aegypti* at the concentration of 10 % (Table 1).

Thirty min KD was recorded for all escape and non-escape mosquitoes. For *Ae. aegypti*, KD was very low (0–2 %) for escape and non-escape specimens exposed to orange oil regardless of concentrations. Cinnamon at 10 % concentration produced the greatest percent KD of escaped and non-escaped specimens from both contact and non-contact trials (Table 1). Citronella and clove oil at 10 % gave a significantly higher percent KD as compared to the two lower concentrations.

For *Cx. quinquefasciatus*, the highest escape response in contact and non-contact trials was observed with 10 % citronella (87.7 and 83.0 %, respectively). The lowest percentage escape response in contact trials and non-contact trials were observed with 5 % orange oil (1.7 and 0 %, respectively). No mortality was observed from those escaping from contact and non-contact chambers for the four essential oils, except for a single exception with 10 % orange oil in the contact trials. Comparatively low-percent mortality was seen from all non-escape tests regardless of oil and concentration (ranging 0–7.1 %) (Table 2). Percent KD presented a much different picture. For orange oil, escape and non-escape KD at 1 h it was 0–4.7 %. However, 10 % cinnamon oil resulted in a high-percent KD of non-escape mosquitoes in both contact (82.6 %) and non-contact experiments (96.8 %) (Table 2). In addition, 10 % clove also resulted in relatively high-percent KD in contact (78.6 %) and non-contact (70.6 %) tests. In general, greater than 75 % of KD specimens recovered within 24 h post-exposure holding period, except non-escape mosquitoes from non-contact chamber treated with 5 % citronella oil and contact chamber treated with 10 % orange oil.

The escape patterns based on time (minutes) required for test populations to escape was defined proportionally as 25 % (ET25), 50 % (ET50), and 75 % (ET75) of the exposed females exiting the chamber over the 30 min period (Table 3). Overall, *Ae. aegypti* demonstrated a lower overall escape response than that of *Cx. quinquefasciatus*. In contact trials, *Ae. aegypti* had the greatest ET25 escape values with 2.5 and 5 % citronella (6 and 1 min, respectively), whereas 10 % citronella had a contact ET25 of 30 min. In non-contact trials, 5 % citronella also produced the most rapid escape response at 1 min ET25. In all cases, both ET50 and ET75 values of the four essential oils could not be estimated due to low number of escapees during the 30 min testing period. For *Cx. quinquefasciatus*, ET25 values at 5 and 10 % concentrations of cinnamon, citronella, and clove in contact and non-contact trials were comparatively rapid, ranging between 1 and 5 min for escape. Orange oil produced the slowest escape response even at the highest concentration (contact ET25 at 18 min). *Culex quinquefasciatus* demonstrated a much greater overall escape percentage compared to *Ae. aegypti*. ET50 values were seen for 5 and 10 % cinnamon, and ET50 and ET75 escape times (ET) for citronella and clove were clearly evident (Table 3).

Multiple log-rank comparisons were performed in paired contact, non-contact, and control trials for the four

Table 1 Escape response, KD at 30 min and mortality after 24 h holding period for *Ae. aegypti* following contact and non-contact with three serial concentrations of 4 essential oils in ER tests

Oil	Test	Dose (% w/v)	*N*	Mean # Esc ± SD	Esc (%)*	% KD (30 min)		% Mort (24 h)	
						Esc	Not esc	Esc	Not esc
CA	C	2.5	59	2.25 ± 1.0	12.3	0	0	0	2.0
		5.0	60	4.25 ± 2.1	25.1	0	0	0	0
		10.0	59	2.0 ± 1.6	12.1	0	2.0	0	2.0
	NC	2.5	60	0.75 ± 0.5	3.4	0	0	0	0
		5.0	60	1.75 ± 0.5	7.0	0	0	0	0
		10.0	57	1.0 ± 1.2	7.0	0	0	0	0
CV	C	2.5	59	5.0 ± 2.4	25.9	0	0	0	2.3
		5.0	59	3.0 ± 1.6	9.4	0	83.0	8.3	2.1
		10.0	59	2.0 ± 1.2	8.9	37.5	100.0	0	5.9
	NC	2.5	60	4.75 ± 1.5	8.5	0	0	0	0
		5.0	60	2.5 ± 1.7	3.3	40.0	84.0	30.0	4.0
		10.0	60	3.52 ± 2.2	21.7	30.8	97.9	0	2.1
CW	C	2.5	59	5.25 ± 1.5	30.4	0	0	0	2.6
		5.0	60	5.25 ± 1.5	29.1	0	5.1	0	0
		10.0	57	3.5 ± 1.7	24.6	14.3	58.1	7.1	2.3
	NC	2.5	59	1.5 ± 1.3	10.2	0	0	0	0
		5.0	60	6.0 ± 1.4	39.0	0	0	0	0
		10.0	58	3.0 ± 2.2	20.7	0	73.9	25.0	8.7
SA	C	2.5	60	7.0 ± 0.8	36.9	0	40.6	3.6	0
		5.0	58	4.25 ± 0.5	22.6	0	12.2	0	2.4
		10.0	54	2.75 ± 1.0	17.6	9.1	97.7	0	46.5
	NC	2.5	58	6.25 ± 2.1	38.8	0	32.0	0	6.1
		5.0	59	3.25 ± 1.7	19.3	0	32.6	0	0
		10.0	57	3.25 ± 1.0	22.8	7.7	97.7	23.1	29.6

C contact, *NC* noncontact, *N* combined sample size; *KD* knockdown, *Esc* escaped mosquitoes, *Not esc* non-escaped mosquitoes, *CA Citrus aurantium* (orange oil), *CV Cinnamomum verum* (cinnamon oil), *CW Cymbopogon winterianus* (citronella oil), *SA Syzygium aromaticum* (clove oil)

* Adjusted rate based on paired control response

essential oils by concentration. No statistical differences in pattern of escape were observed in comparisons of contact versus non-contact ($p > 0.05$), except for *Ae. aegypti* at 2.5 % citronella and 5 % orange and for *Cx. quinquefasciatus* at 2.5 % cinnamon oil (Table 4). For all concentrations, *Ae. aegypti* showed no significant differences ($p > 0.05$) in escape response between orange and citronella in contact trials, and orange and cinnamon in non-contact trials, and similarly with *Cx. quinquefasciatus* for cinnamon and clove extracts in both contact and non-contact tests. No significant differences ($p > 0.05$) in escape patterns were observed for *Cx. quinquefasciatus* comparing concentrations at 5 and 10 % for cinnamon, citronella and clove in both contact and non-contact trials. Comparisons between both contact and paired control and non-contact and pair control showed significant differences ($p < 0.05$) for all pairings, except for *Ae. aegypti* in contact and paired control at 5 and 10 % cinnamon and in non-contact and paired control at 2.5 and 5 % orange and 5 % cinnamon oil. No significant differences were seen with *Cx. quinquefasciatus* in both contact and non-contact versus pair controls at 2.5 and 5 % orange oil concentrations.

Using survival analysis, the proportion of mosquitoes remaining in exposure chambers treated with three different concentrations of essential oils in the contact and non-contact test designs during a 30-min exposure period are graphically presented in Figs. 2 and 3. Overall, the escape rate by 1 min intervals was greater in *Cx. quinquefasciatus* than *Ae. aegypti* regardless of test concentration or type of oil used. Orange oil produced the weakest escape response *Ae. aegypti* and *Cx. quinquefasciatus* in both contact (Fig. 2a) and non-contact trials (Fig. 3a), regardless of concentration. Much stronger escape patterns were seen in *Cx. quinquefasciatus* compared to *Ae. aegypti* for all

Table 2 Escape response, KD at 30 min and mortality after 24 h holding period for *Culex quinquefasciatus* following contact and non-contact with three serial concentrations of four essential oils in ER tests

Oil	Test	Dose (% w/v)	*N*	Mean # Esc ± SD	Esc (%)*	% KD (30 min)		% Mort (24 h)	
						Esc	Not esc	Esc	Not esc
CA	C	2.5	58	0.25 ± 0.5	1.7	0	0	0	0
		5.0	60	0.5 ± 1.0	1.7	0	0	0	0
		10.0	58	3.75 ± 3.3	24.6	0	4.7	13.3	4.7
	NC	2.5	60	0.5 ± 0.6	1.7	0	0	0	0
		5.0	60	0 ± 0	0	0	0	0	0
		10.0	60	2.5 ± 2.1	16.7	0	0	0	0
CV	C	2.5	59	9.0 ± 2.9	59.7	0	0	0	0
		5.0	60	10.0 ± 1.8	65.5	0	20.0	0	5.0
		10.0	58	8.75 ± 4.1	60.3	0	82.6	0	0
	NC	2.5	58	6.5 ± 1.7	44.8	0	0	0	0
		5.0	59	7.25 ± 1.7	49.2	0	6.7	0	0
		10.0	60	7.25 ± 3.3	47.5	0	96.8	0	0
CW	C	2.5	59	5.75 ± 3.5	36.9	0	0	0	0
		5.0	60	11.25 ± 2.9	75.0	2.2	6.7	0	0
		10.0	60	13.25 ± 1.3	87.7	0	0	0	0
	NC	2.5	60	5.0 ± 2.9	28.6	0	0	0	0
		5.0	60	11.0 ± 2.8	73.3	0	6.3	0	6.3
		10.0	60	12.5 ± 1.0	83.0	0	30.0	0	0
SA	C	2.5	59	11.25 ± 1.9	76.3	0	0	0	0
		5.0	60	11.75 ± 1.3	76.8	2.1	53.9	0	0
		10.0	60	11.5 ± 2.6	75.4	0	78.6	0	7.1
	NC	2.5	59	8.75 ± 3.9	57.9	0	8.3	0	0
		5.0	58	10.0 ± 1.2	69.0	5.0	44.5	0	0
		10.0	60	10.75 ± 1.7	70.7	0	70.6	0	5.9

C contact, *NC* noncontact, *N* combined sample size; *KD* knockdown, *Esc* escaped mosquitoes, *Not esc* non-escaped mosquitoes, *CA Citrus aurantium* (orange oil), *CV Cinnamomum verum* (cinnamon oil), *CW Cymbopogon winterianus* (citronella oil), *SA Syzygium aromaticum* (clove oil)

* Adjusted rate based on paired control response

concentrations of cinnamon in both contact (Fig. 2b) and non-contact trial configurations (Fig. 3b). For citronella, the escape response was stronger for *Cx. quinquefasciatus* compared to *Ae. aegypti* for all three concentrations in both contact (Fig. 2c) and non-contact (Fig. 3c) tests as was the case with clove oil in contact (Fig. 2d) and non-contact trials (Fig. 3d).

Discussion

The two primary objectives of the study were to (1) evaluate four essential oil extracts for avoidance response in two different species of mosquitoes, and (2) demonstrate that the ER test system can be a useful tool to conduct preliminary screening of different compounds to determine acceptable candidates that could proceed to more advance evaluation. Although this method of evaluation presents limitations for extrapolating the findings to actual applications for protection, ER testing does provide an approach for quickly assessing a compound's potential qualities without the use of live hosts and associated ethical considerations.

All four essential oils showed excito-repellent actions against *Ae. aegypti* and *Cx. quinquefasciatus*. Higher mortality (toxicity) was observed in both escaped and non-escaped *Ae. aegypti* (maximum 46.5 % in contact with 10 % clove oil) as compared to *Cx. quinquefasciatus* (maximum 13.3 %) after a 24-h holding period. Greater mortality was associated with higher concentrations of oils. Citronella and clove were significantly more effective in both excitation and repelling *Ae. aegypti* compared to orange and cinnamon extracts. However, 5 % orange was shown to have more marked contact irritancy properties

Table 3 Estimated ET in minutes for 25 % (ET_{25}), 50 % (ET_{50}), and 75 % (ET_{75}) of *Ae. aegypti* and *Cx. quinquefasciatus* populations to escape from the exposure chambers of ER tests

Oil	% w/v	*Ae. aegypti*			*Cx. quinquefasciatus*		
		ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}
CA	2.5						
	CT	–	–	–	–	–	–
	NT	–	–	–	–	–	–
	5.0						
	CT	27	–	–	–	–	–
	NT	–	–	–	–	–	–
	10.0						
	CT	–	–	–	18	–	–
	NT	–	–	–		–	–
CV	2.5						
	CT	13	–	–	18	–	–
	NT	14	–	–	–	–	–
	5.0						
	CT	–	–	–	1	12	–
	NT	–	–	–	3	–	–
	10.0						
	CT	–	–	–	3	7	–
	NT	–	–	–	3	–	–
CW	2.5						
	CT	6	–	–	2		–
	NT	–	–	–	12	–	–
	5.0						
	CT	1	–	–	1	3	28
	NT	1	–	–	1	2	–
	10.0						
	CT	30	–	–	1	4	15
	NT	–	–	–	1	2	19
SA	2.5						
	CT	3	–	–	1	11	26
	NT	8	–	–	1	12	–
	5.0						
	CT	16	–	–	4	8	28
	NT	–	–	–	5	11	–
	10.0						
	CT	–	–	–	1	3	18
	NT	–	–	–	2	9	–

CA Citrus aurantium (orange oil), *CV Cinnamomum verum* (cinnamon oil), *CW Cymbopogon winterianus* (citronella oil), *SA Syzygium aromaticum* (clove oil), *CT* contact, *NT* non-contact

with *Ae. aegypti* in comparison to non-contact and paired controls, suggesting that excitation may be the only major action of orange oil. Similarly, citronella and clove at the lowest concentration were more likely to perform as contact irritants against *Ae. aegypti* than greater concentrations suggesting that non-contact repellency is the predominant escape response as concentration increases. Moreover, the *Aedes aegypti* that successfully escaped the treated chambers of either chemical were far more likely to survive than those remaining inside the contact and non-contact chambers indicating that the lowest dose of citronella and clove were not toxic. Lastly, higher numbers of *Ae. aegypti* were KD as concentrations increased; however, over 52 % recovered during 24-h post-exposure indicating a limited toxic action of these two active oils.

For *Cx. quinquefasciatus*, the lowest concentration of clove produced the strongest escape response in both contact (76.3 %) and non-contact trials (57.9 %), followed by cinnamon and citronella. As no statistical differences were seen among the three concentrations of clove and cinnamon in both contact and non-contact configurations, it appears the lowest concentrations tested are also the most

Table 4 Multiple log-rank comparisons in paired contact, non-contact, and control trials for the four essential oils by concentration

Oils	Dose (% w/v)	Comparisons	*Ae. aegypti* *p* value*	*Cx. quinquefasciatus* *p* value*
CA	2.5	NC vs. NT	0.3080	0.5703
		CC vs. CT	0.0246	0.3132
		NT vs. CT	0.0649	0.5804
	5.0	NC vs. NT	0.1883	NA
		CC vs. CT	0.0007	0.5670
		NT vs. CT	0.0285	0.1556
	10.0	NC vs. NT	0.0392	0.0011
		CC vs. CT	0.0160	0.0002
		NT vs. CT	0.2488	0.2026
CV	2.5	NC vs. NT	<0.0001	<0.0001
		CC vs. CT	<0.0001	<0.0001
		NT vs. CT	0.8358	0.0093
	5.0	NC vs. NT	0.6594	<0.0001
		CC vs. CT	0.2416	<0.0001
		NT vs. CT	0.6041	0.0912
	10.0	NC vs. NT	0.0002	<0.0001
		CC vs. CT	0.1014	<0.0001
		NT vs. CT	0.2458	0.2423
CW	2.5	NC vs. NT	0.0123	0.0003
		CC vs. CT	<0.0001	<0.0001
		NT vs. CT	0.0013	0.4295
	5.0	NC vs. NT	<0.0001	<0.0001
		CC vs. CT	0.0003	<0.0001
		NT vs. CT	0.5625	0.9741
	10.0	NC vs. NT	0.0002	<0.0001
		CC vs. CT	<0.0001	<0.0001
		NT vs. CT	0.6848	0.4972
SA	2.5	NC vs. NT	<0.0001	<0.0001
		CC vs. CT	0.0003	<0.0001
		NT vs. CT	0.5788	0.2067
	5.0	NC vs. NT	0.0023	<0.0001
		CC vs. CT	0.0042	<0.0001
		NT vs. CT	0.3491	0.2188
	10.0	NC vs. NT	<0.0001	<0.0001
		CC vs. CT	0.0039	<0.0001
		NT vs. CT	0.7914	0.1553

CA Citrus aurantium (orange oil), *CV Cinnamomum verum* (cinnamon oil), *CW Cymbopogon winterianus* (citronella oil), *SA Syzygium aromaticum* (clove oil), *CT* contact treatment, *NT* non-contact treatment, *CC* contact control, *NC* non-contact control, *NA* insufficient numbers of mosquitoes escaping to conduct analysis

* The discriminating level of statistical significance for all tests was set at 95 % ($p < 0.05$)

appropriate for protecting against this species. With the same two oils, the 30-min KD among mosquitoes that did not escape in both contact and non-contact tests showed a clear dose response in relation to increased serial concentrations, yet the majority of escape mosquitoes made a full apparent recovery within the 24-h holding period indicating relatively poor toxicity of clove and citronella to *Cx. quinquefasciatus*.

One of the most significant components of a mosquito abatement program is an understanding of how mosquito vectors behave in response to chemical control measures. These responses can be characterized by three primary actions; excitation ("irritancy"), repellency, and toxicity (Grieco et al. 2007). The combination of the first two behavioral actions is sometimes referred to as "ER," whereas toxicity implies insecticidal action in the form of lethality or reduced survival. Behavioral actions of mosquitoes are part of an innate response repertoire to active ingredients having "irritant" and/or "repellent" properties causing movement away from a point source; whereas, toxicity is a purely physiological response to direct contact with a poison. Numerous attempts have been made to more

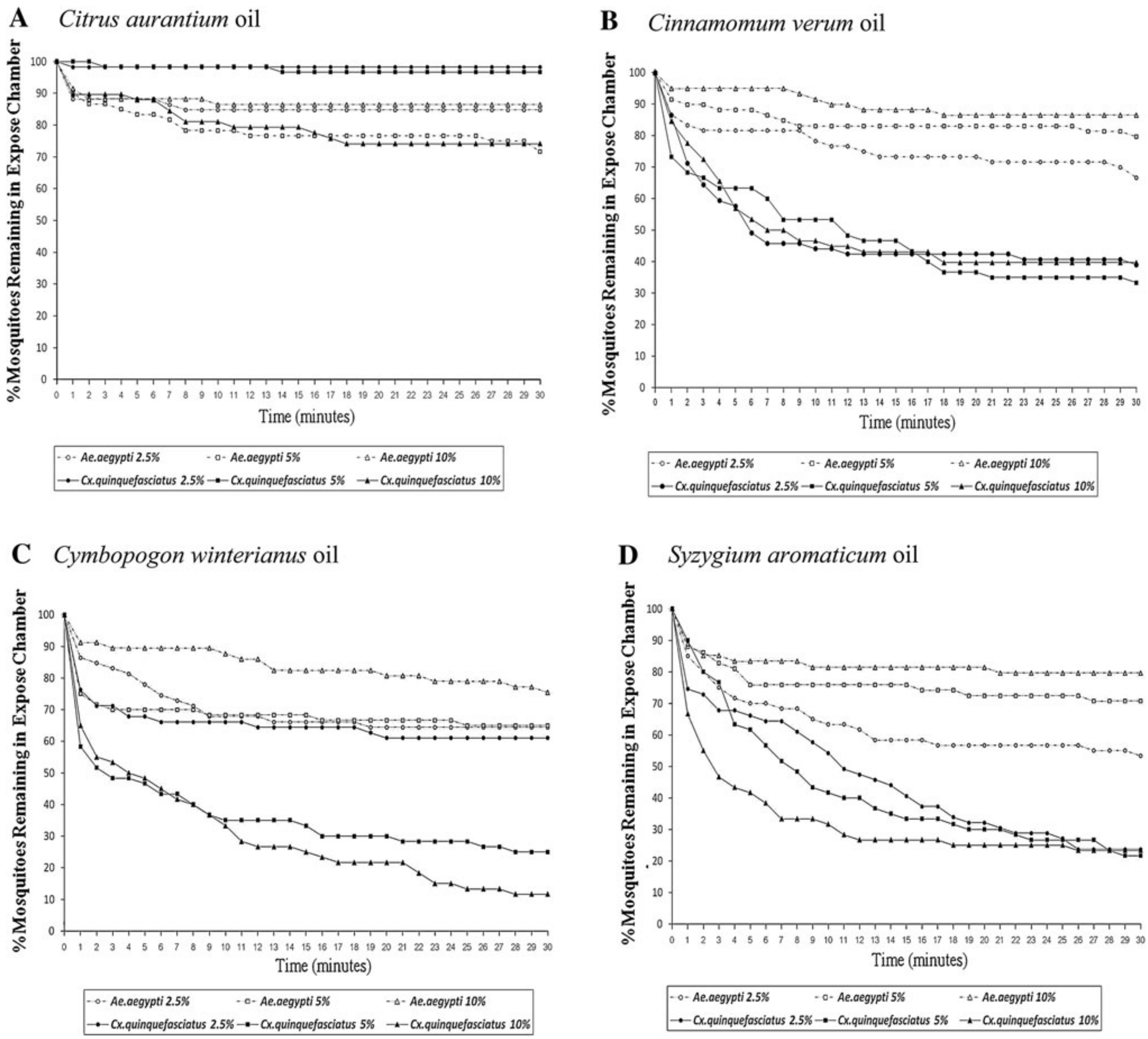

Fig. 2 Escape probability (rate) of female *Ae. aegypti* and *Culex quinquefasciatus* exposed in contact excitation trials using three serial percent concentrations (w/v) of orange (*Citrus aurantium*) oil (**a**), cinnamon (*Cinnamomum verum*) oil (**b**), citronella (*Cymbopogon winterianus*) oil (**c**), and clove (*Syzygium aromaticum*) oil (**d**)

accurately measure the behavioral responses of mosquitoes to test chemicals, as documented in the designs of various ER test systems (Chareonviriyaphap et al. 1997; Roberts et al. 1984; Rutledge et al. 1999; Sungvornyothin et al. 2001; Polsomboon et al. 2008a). In 2005, a modular, high-throughput screening system was developed to observe all three primary actions of test chemicals depending on the assay configuration (Grieco et al. 2005). Although the modular system is smaller in size compared to the previous ER test chambers thereby minimizing the amount of chemical required and the number of test specimens needed per test, this system is more applicable for use in the laboratory setting rather than in the field and more useful for mass screening of different chemical libraries for mode of actions.

In this study, we choose a well-documented ER test system for observing the behavioral responses of two field collected mosquito species, *Ae. aegypti* and *Cx. quinquefasciatus*, to four essential oil extracts produced by a commercial company in Thailand. The value of many essential oils, some traditionally used to ″repel″ or otherwise prevent mosquitoes from biting, has been underestimated as possible alternatives to the most common synthetic and other natural active ingredients currently on the market (Walker et al. 1996; Rutledge et al. 1983; Debboun et al. 2007). Many studies in Thailand and

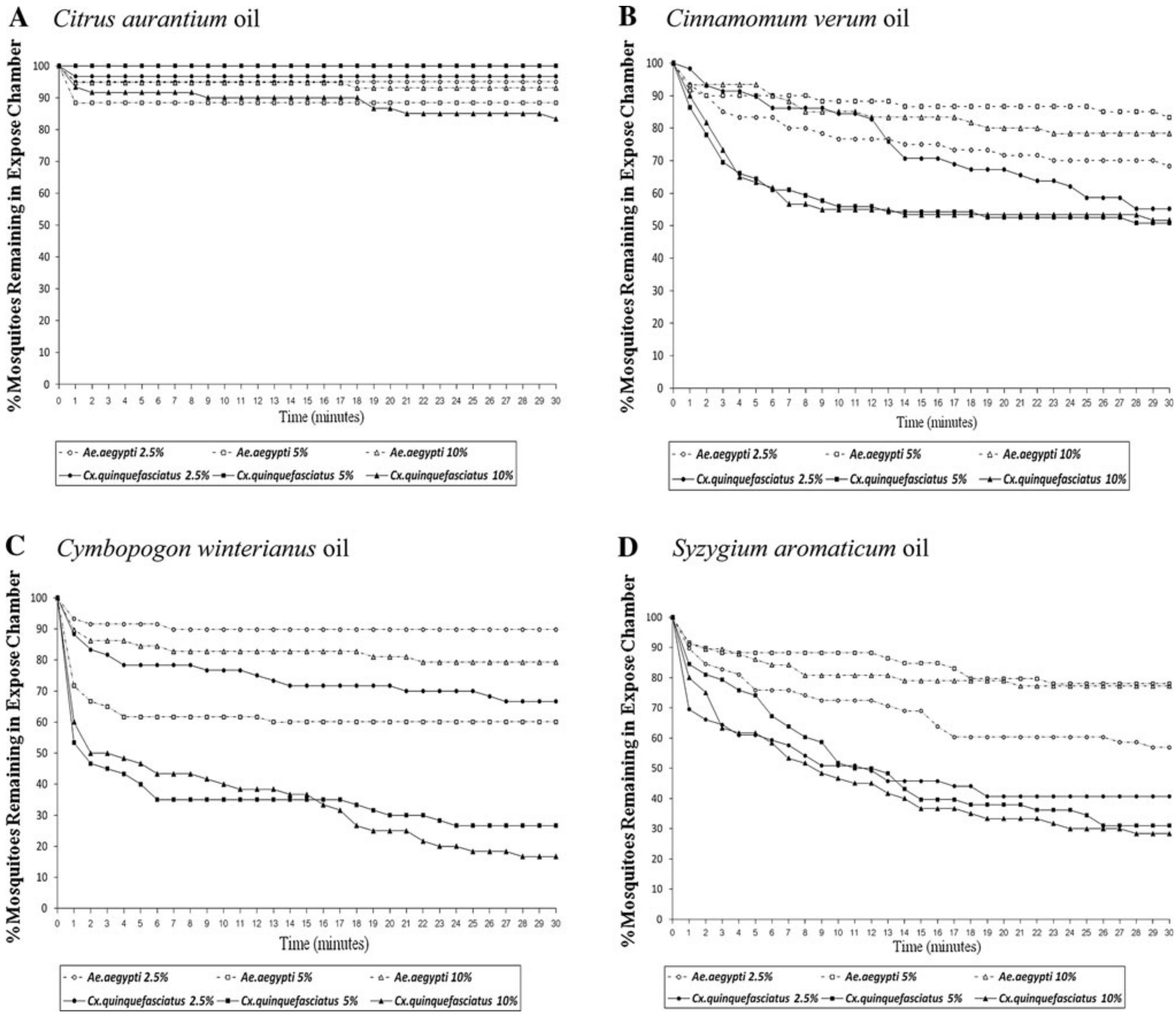

Fig. 3 Escape probability (rate) of female *Ae. aegypti* and *Culex quinquefasciatus* exposed in non-contact repellency trials using three serial percent concentrations (w/v) of orange (*Citrus aurantium*) oil (**a**), cinnamon (*Cinnamomum verum*) oil (**b**), citronella(*Cymbopogon winterianus*) oil (**c**), and clove (*Syzygium aromaticum*) oil (**d**)

elsewhere have shown that various plant species contain select phytochemicals that can perform as insect repellents against mosquitoes and other arthropods (Odalo et al. 2005; Bernard 1999; Trongtokit et al. 2004, 2005; Yang and Ma 2005; Chokechaijaroenporn et al. 1994; Tawatsin et al. 2001; Noosidum et al. 2008; De Boer et al. 2010; Suwonkerd and Tantrarongroj 1994; Tawatsin et al. 2006; Adewoyin et al. 2006; Zhu et al. 2006; Gillij et al. 2008; Kumar et al. 2011). Surprisingly, comparatively few studies have actually demonstrated or identified the precise chemical actions being elicited on mosquitoes and have only focused on the outcome (bite protection) (Polsomboon et al. 2008a; Noosidum et al. 2008).

This study was not designed to measure protection response time of oils on mosquito behavior. However, both citronella and clove oil demonstrated the strongest excitation and repellency actions on both *Ae. aegypti* and *Cx. quinquefasciatus* mosquitoes based on comparatively rapid ET. For citronella, a common ingredient in commercial repellent products, this study represents the first observation that shows the marked behavioral effects of this oil on mosquitoes. Clove extract is also capable of eliciting a strong excitation and repellency response in the two species, followed by cinnamon oil. Clove and cinnamon deserve more in depth investigation on their protective properties using other testing methods (e.g., human exposure trials) as possible additives in commercial product formulations.

An understanding of the basic behavioral responses of mosquitoes to natural and synthetic compounds is a more complete measure of their overall effect and potential for reducing human-vector contact and disease transmission. In this study, two types of behavioral response, contact excitation and spatial repellency, were evaluated with two mosquito species against four essential oils. These responses were quantitatively evaluated and demonstrated that cinnamon, citronella, and clove oils were more effective in eliciting escape responses in mosquitoes than orange oil. This study demonstrated how essential oils can significantly impact mosquito behavior with the possibility they might be incorporated into topical skin repellent formulations or used on other innovative vector control tools. In addition, this study demonstrated varying avoidance responses based on different concentrations that may also prove useful as starting points for determining the lowest effective concentrations and potential synergistic and antagonistic effects against various mosquito species. Lastly, the ER test system appears suitable for use in the preliminary screening of chemicals for behavioral avoidance characteristics by mosquitoes in the absence of host bait. Chemicals that demonstrate significant avoidance response in the test system can be advanced as potential candidates for advanced testing using animal models or human hosts.

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