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SCIENTIFIC NOTE

COMPARATIVE BEHAVIORAL RESPONSES OF Β-CARYOPHYLLENE AGAINST *ANOPHELES* MOSQUITO SPECIES, POTENTIAL VECTORS OF MALARIA IN THAILAND

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ABSTRACT. Insecticide-based mosquito control poses risks of environmental harm and resistance development. As a safer alternative, β-caryophyllene, a nontoxic compound derived from plants, was evaluated as a safer alternative for its excito-repellency against four *Anopheles* mosquito species. Using an excito-repellency assay, *An. minimus* showed the highest escape rates (100%) at 2.5% (contact) and 5% (noncontact) concentrations. *An. harrisoni* also exhibited strong responses at 7.5%, with 94.34% (contact) and 72.73% (noncontact) escape. In contrast, *An. epiroticus* showed minimal response (12–25.42%). Significant differences between contact and noncontact trials were noted only in *An. minimus* and *An. harrisoni*. No knockdown or mortality occurred. Kaplan-Meier analysis revealed faster escape in contact conditions, especially for *An. minimus*. Log-rank tests confirmed dose-dependent repellency effects. These findings suggest β-caryophyllene has promising potential as a natural spatial repellent against malaria vectors.

KEY WORDS *Anopheles*, β-caryophyllene, behavioral response, excito-repellency

Malaria remains a major global health concern, disproportionately affecting impoverished populations and straining national economies (WHO 2023). Thailand has committed to eliminating malaria by 2030 through its National Malaria Elimination Strategy, which incorporates the 1-3-7 surveillance system (Sudathip et al. 2022). Despite this progress, resurgence of cases, particularly along Thailand's western border, poses a significant challenge, with 15,367 cases reported in 2024 (MOPH 2023). Malaria transmission is transmitted by *Plasmodium*-infected *Anopheles* mosquitoes. In Thailand, primary vectors include *Anopheles dirus* Peyton and Harrison in forested highlands and *Anopheles minimus* Theobald in village-forest fringes, whereas *Anopheles harrisoni* Harbach and Manguin is considered as a secondary vector in the west, and *Anopheles epiroticus* Linton and Harbach is a potential vector along the coastal areas (Sinka et al. 2011, Tainchum et al. 2015, Tananchai et al. 2019). Current vector control efforts rely heavily on chemical insecticides, notably pyrethroids and organophosphates (Chareonviriyaphap et al. 2013). However, their long-term application raises concerns regarding environmental safety, cost, and the emergence of insecticide resistance (Chaumeau et al. 2017). Consequently, there is a growing interest in sustainable and environmentally friendly alternatives. Among these, plant-derived compounds offer promising solutions (Tisgratog et al. 2016). β-caryophyllene, a naturally occurring bicyclic sesquiterpene found in essential oils of clove, basil, hops, and rosemary, is recognized as safe by the U.S. Food and Drug Administration (FDA) and exhibits low mammalian toxicity alongside diverse therapeutic properties (Oliveira et al. 2017). Its volatile, aromatic nature lends itself to potential application as an insect repellent. Studies have reported repellent efficacy of β-caryophyllene and its oxide derivative against *Aedes aegypti* (L.), *Ae. albopictus* (Skuse), and *An. dirus* (Gillij et al. 2008; Nerio et al. 2010). Nevertheless, data on its behavioral effects against major *Anopheles* vectors in Thailand remain limited.

This study evaluated the contact irritancy and noncontact repellency of β-caryophyllene against four *Anopheles* species using an excito-repellency assay. Laboratory strains included *An. dirus*, *An. minimus*, and *An. epiroticus*, all insecticide-susceptible. A field population of *An. harrisoni* was collected as adults from Kanchanaburi Province and reared under semi-field conditions. Mosquito colonies were maintained at Kasetsart University under controlled insectary conditions (25 ± 2 °C, 80 ± 10% RH, 12:12 h L:D). Larvae of *An. epiroticus* were reared in artificial brackish water, whereas adults were sustained on 10% sucrose and human blood via membrane feeding (Sukkanon et al. 2020). *Anopheles dirus* required artificial insemination

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(Yang et al. 1963), *An. minimus* and *An. epiroticus* were self-mating. For testing, 3–5-day-old sugar-starved, non-blood-fed females were used. Pure β-caryophyllene was diluted in absolute ethanol to prepare 2.5%, 5%, and 7.5% (v/v) solutions. Whatman No. 1 filter papers (14.7 × 17.5 cm) were treated with 2.8 ml of each solution and air-dried for at least 1 h (Sukkanon et al. 2020). Each paper was used only once. Experiments were conducted using an excito-repellency assay system composed of four stainless steel chambers with escape windows (Choomsang et al. 2018). In noncontact (repellency) trials, treated papers were placed behind mesh screens; for contact (irritancy) trials, papers were laid directly on the mesh. Fifteen mosquitoes were introduced per chamber (60 total per test) after a 3-min acclimation period. Escaping mosquitoes were recorded at 1-min intervals for 30 min. Post-exposure mortality was assessed at 24 h. All experiments were replicated 4 times during daylight hours.

Data were corrected using Abbott's formula (Abbott 1925). Contact-specific irritancy was isolated using a modification of the Henderson-Tilton method (Sukkanon et al. 2020). Escape responses were analyzed using Kaplan–Meier survival analysis, with escape time (ET) for 25%, 50%, and 75% of mosquitoes (ET25, ET50, ET75) compared, using log-rank tests (Mantel and Haenszel 1959, Roberts et al. 1997). Statistical analyses were performed using SAS software, with $P < 0.05$ considered significant.

β-caryophyllene significantly enhanced mosquito escape behavior in both contact and noncontact trials, with no knockdown or mortality observed after 24 h (Table 1). In noncontact trials, *An. minimus* demonstrated 100% escape at 5%, whereas *An. harrisoni* reached 72.73% at 7.5%. *An. epiroticus* had the lowest escape rates (12.07–25.42%). In contact trials, *An. minimus* again exhibited 100% escape at 2.5%, with *An. harrisoni* reaching 94.34% at 7.5%. In contrast, *An. dirus* and *An. epiroticus* had modest responses at 7.5% (64.29% and 20.69%, respectively; Table 2). Escape responses between contact and noncontact trials were largely comparable, except for *An. minimus* at 2.5% and *An. harrisoni* at 7.5%, which showed stronger responses in contact trials. After adjusting for spatial repellency, most contact irritancy values showed no significant difference, though numerical escape rates were often higher. *Anopheles minimus* consistently had the fastest escape times (ET75 within 2–6 minutes), whereas *An. harrisoni* had ET50 values of 14–21 min in noncontact trials and 2–4 min in contact trials. *Anopheles dirus* showed an ET50 of 11 minutes at 7.5% in noncontact trials (Table 2). Escape times for *An. epiroticus* were not calculable because of insufficient responses. Log-rank analysis indicated significant differences in escape rates for *An. minimus* across concentrations in both test types (Table 1). *Anopheles harrisoni* exhibited dose-dependent responses in contact trials. *Anopheles dirus* showed significant differences only at 7.5%, whereas *An. epiroticus* displayed no significant variation.

Table 1. Percentage escape responses and log-rank statistics for four mosquito species exposed to β-caryophyllene.

Conc. (%)	ER assay	Percentage escape ± SD (log-rank test)			
		Anopheles dirus	*An. epiroticus*	*An. minimus*	*An. harrisoni*
2.5	Contact	23.21 ± 1.03^{a} (0.3567*, < 0.0001**)	14.03 ± 0.47^{a} (0.6297*, 0.4621**)	100.00 ± 0.00^{a} (0.0551*, 0.0086**)	42.86 ± 1.87^{a} (0.0016*, < 0.0001**)
	Noncontact	19.29 ± 0.86^{a} (0.319*, < 0.0001**)	12.07 ± 0.47^{a} (0.633*, 0.1175**)	87.72 ± 1.43^{b} (0.0469*, 0.0424**)	52.82 ± 2.17^{a} (0.8535*, 0.2921**)
5	Contact	31.07 ± 0.86^{a} (0.3567*, 0.0006***)	17.55 ± 0.47^{a} (0.6297*, 0.8075***)	98.24 ± 0.25^{a} (0.0551*, 0.2681***)	72.22 ± 2.46^{a} (0.0016*, 0.0056***)
	Noncontact	26.79 ± 0.85^{a} (0.319*, 0.0007***)	14.03 ± 0.85^{a} (0.633*, 0.2679***)	100.00 ± 0.00^{b} (0.0469*, 0.8958***)	63.64 ± 0.91^{a} (0.8535*, 0.3052***)
7.5	Contact	64.29 ± 0.70^{a} (< 0.0001**, 0.0006***)	20.69 ± 0.64^{a} (0.4621**, 0.8075***)	93.10 ± 1.00^{a} (0.0086**, 0.2681***)	94.34 ± 0.75^{a} (< 0.0001**, 0.0056***)
	Noncontact	59.32 ± 0.81^{a} (< 0.0001**, 0.0007***)	25.42 ± 0.40^{a} (0.1175**, 0.2679***)	98.24 ± 0.25^{a} (0.0424**, 0.8958***)	72.73 ± 0.85^{b} (0.2921**, 0.3052***)

Escape percentages were adjusted using Abbott's formula to account for control responses. Values are presented as means ± standard deviations (SD), and different superscript letters within the same concentration row denote statistically significant differences between contact and noncontact assays, as determined by log-rank tests ($P < 0.05$).
Asterisks indicate statistically significant differences at $P < 0.05$: * denotes comparisons between 2.5% and 5.0%, ** between 2.5% and 7.5%, and *** between 5.0% and 7.5% concentrations.

Table 2. Time (min) for 25% (ET_{25}), 50% (ET_{50}), and 75% (ET_{75}) of adult female mosquitoes to escape from treated excito-repellency chambers containing various concentrations of β-caryophyllene.

Mosquito species	Conc. (%)	Contact			Noncontact		
		ET_{25}	ET_{50}	ET_{75}	ET_{25}	ET_{50}	ET_{75}
Anopheles dirus	2.5	24	N/A	N/A	N/A	N/A	N/A
	5	17	N/A	N/A	20	N/A	N/A
	7.5	1	11	N/A	1	23	N/A
An. epiroticus	2.5	N/A	N/A	N/A	N/A	N/A	N/A
	5	N/A	N/A	N/A	N/A	N/A	N/A
	7.5	N/A	N/A	N/A	28	N/A	N/A
An. minimus	2.5	< 1	< 1	< 2	< 1	< 2	6
	5	< 1	< 1	< 2	< 1	< 2	5
	7.5	< 1	< 1	< 2	< 1	< 2	2
An. harrisoni	2.5	< 1	N/A	N/A	< 1	14	N/A
	5	< 1	4	30	4	21	N/A
	7.5	< 1	2	14	2	18	30

Use 'N/A' to indicate results that cannot be measured.

This study demonstrated that β-caryophyllene effectively induces both contact irritant and spatial repellent responses in several *Anopheles* species, particularly *An. minimus* and *An. harrisoni*. These findings align with earlier reports on β-caryophyllene oxide's efficacy against *An. minimus* (Nararak et al. 2019) and its synergistic effects when combined with essential oils (Nararak et al. 2023). The comparatively lower responses observed in *An. dirus* and *An. epiroticus* suggest species-specific sensitivities and possibly underlying genetic factors. The strong repellent responses of field-collected *An. harrisoni* support the practical applicability of β-caryophyllene in outdoor settings. This is particularly relevant in Southeast Asia, where many malaria vectors exhibit exophilic and exophagic behaviors, limiting the effectiveness of indoor-based interventions (Saeung et al. 2024). Formulations based on β-caryophyllene could offer a safe, plant-based alternative for personal protection and outdoor vector control, contributing to reduced insecticide reliance and resistance development (Deng et al. 2023). β-caryophyllene shows promise as a natural excito-repellent agent against important *Anopheles* vectors in Thailand, especially *An. minimus* and *An. harrisoni*. Its ability to trigger rapid escape responses without inducing mortality underscores its potential as a nonlethal control tool. Further field evaluations are warranted to validate these laboratory findings and explore formulation strategies for effective outdoor use.

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