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*Acta Tropica*

**Excito-repellency and biological safety of  $\beta$ -caryophyllene oxide against *Aedes albopictus* and *Anopheles dirus* (Diptera: Culicidae)**

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## Highlights

- $\beta$ -caryophyllene oxide had stronger repellent and irritant effect than DEET
- *Ae. albopictus* was more sensitive to  $\beta$ -caryophyllene oxide than *An. dirus* while, knockdown responses (37%) were observed in *An. dirus* exposed to 1%  $\beta$ -caryophyllene oxide in the contact trial.
- $\beta$ -caryophyllene oxide did not show any phototoxic activity.
- None of the tested  $\beta$ -caryophyllene oxide induced a significant increase of micronucleated cells with or without metabolic activation.
- $\beta$ -Caryophyllene oxide could be considered as a safe repellent, effective against mosquitoes.

## Abstract

The activity of  $\beta$ -caryophyllene oxide as either a contact or non-contact repellent was evaluated against two laboratory strains (*Aedes albopictus* and *Anopheles dirus*) using an excito-repellency test system. *N, N*-Diethyl-3-methylbenzamide (DEET) was used as a standard reference baseline for comparative purposes.  $\beta$ -Caryophyllene oxide and DEET were tested at concentrations of 0.1, 0.25, 0.5 and 1.0% (v/v). In addition, the phototoxic and genotoxic effects of  $\beta$ -caryophyllene oxide were investigated on Balb/c 3T3 mouse fibroblasts (3T3-L1) and Chinese hamster ovary cell line (CHO-K1). The results demonstrated that the higher concentrations of test compounds (0.5 and 1.0%) produced greater behavioral responses. *Ae. albopictus* was more sensitive to  $\beta$ -caryophyllene oxide than *An. dirus*. Moderate avoidance response rates (25-56% escape) of *Ae. albopictus* at 0.5% and 1.0%  $\beta$ -caryophyllene oxide were observed in contact and non-contact trials compared with low response rates from *An. dirus* (26-31% escape). DEET at  $\leq 1\%$  displayed lower irritancy and repellency (1-38%) than  $\beta$ -caryophyllene oxide when tested against the two mosquito species. Knockdown responses (37%) were only observed in *An. dirus* exposed to 1%  $\beta$ -caryophyllene oxide in the contact trial.  $\beta$ -Caryophyllene oxide did not show any phototoxic potential (PIF= 0.38) nor was there any significant genotoxic response as indicated by no increase in micro-nucleated cells with or without metabolic activation.  $\beta$ -Caryophyllene oxide could be considered as a safe repellent, effective against mosquitoes.

**Keywords:** Excito-repellency test system,  $\beta$ -Caryophyllene oxide, Phototoxic, Genotoxic, Mosquitoes

## 1. Introduction

Mosquito-borne diseases represent a key hazard for millions of people worldwide. Mosquitoes serve as vectors of pathogens for devastating human scourge, including malaria, filariasis, yellow fever, dengue, West Nile virus and chikungunya (Benelli and Mehlhorn, 2018). Malaria and dengue are the two most common mosquito-borne diseases that cause high morbidity and mortality (Wiwanitkit, 2011). Millions of humans in undeveloped and developing countries are now at risk from these diseases. To a lower extent, developed countries are too at risk of dengue outbreaks due to the invasion of *Aedes albopictus* into temperate regions and population movement from dengue-endemic areas. In 2017, an estimated 219 million cases of malaria occurred worldwide, with an estimated 435,000 deaths from malaria globally (WHO, 2018). *Anopheles dirus* is the one of the most important malaria vector in Southeast Asia (Tainchum et al., 2015). This species inhabits forest and forest fringes and exhibits mostly exophagic behavior (Baimai et al., 1984; 2019; Tananchai et al., 2012). In contrast, dengue and chikungunya are arboviral infections transmitted by two species of *Aedes* mosquitoes—*Ae. aegypti* L. and *Ae. albopictus* (Skuse). The latter, known as the Asian tiger mosquito, is native from Southeast Asian countries (Smith, 1956). The distribution of *Ae. albopictus* has been extended recently by invasion into more northerly latitudes as well as into higher altitudes such as the United States of America and Southern Europe (Chouin-Carneiro et al., 2016; Fidy et al., 2016).

Personal protection using insect repellents is considered as one of the most efficient measures which has been widely used to reduce the outdoor transmission of mosquito-borne diseases (Debboun and Strickman, 2013). Commercial mosquito repellents containing *N, N*-Diethyl-3-methylbenzamide (DEET) have been used by people worldwide. Research has suggested that DEET is not safe when applied to children's skin or if improperly used

25 (Briassoulis et al., 2001). Several side effects have been reported from DEET including rash,  
26 skin irritation and eye irritation (Amichai et al., 1994; Patel et al., 2012).

27 In addition, mosquitoes have developed resistance to all main groups of insecticides  
28 (organochlorines, organophosphates, carbamates). For example, the commonly used  
29 pyrethroids are applied to control malaria mosquitoes but have become increasingly less  
30 effective due to the development of physiological resistance in mosquito populations  
31 (Chareonviriyaphap et al., 2000; 2013). Consequently, alternative plant-based repellents have  
32 been used to protect people, particularly children, during outdoor activities. In addition, such  
33 alternative repellents cause no harm to various nontarget organisms (with additional benefits  
34 as a potential source of bioactive chemicals, fragrances and flavoring agents) and are  
35 recommended as an alternative source of materials for insect control (Isman, 2002; Yang et al.,  
36 2005).

37  $\beta$ -Caryophyllene oxide is generally found in essential oils of various plant species such  
38 as *Artemisia anomala* S. Moore, *Salvia miltiorrhiza* Bunge, *Chloroxylon swietenia* DC.,  
39 *Psidium guajava* L., *Origanum vulgare* L., *Cinnamomum* spp., *Syzygium aromaticum* (L.)  
40 Merr. & L.M. Perry, *Piper nigrum* L. and *Serjania yucatanensis* Standl. (Garneau et al., 2013;  
41 Gertsch et al., 2008; Jun et al., 2011; Liang et al., 2009; Polanco-Hernández et al., 2012; Shell,  
42 1997; Telang et al., 2003; Zhao et al., 2013) Furthermore,  $\beta$ -caryophyllene oxide has been  
43 reported to repel mosquitoes. Suleiman et al. (2014) reported that leaves of *Artabotrys*  
44 *hexapetalus* (L. f.) Bhandari contained large amounts of  $\beta$ -caryophyllene oxide with high  
45 mosquito repellent activity against *Anopheles gambiae* s.s. in Africa. A more recent study  
46 reported that *Aedes aegypti* and *Anopheles minimus* exhibited high avoidance response rates at  
47 0.5% and 1% concentrations of  $\beta$ -caryophyllene oxide compared to DEET (Nararak et al.,  
48 2019), showing the high repellent potential of this molecule.

49 In recent years, the food and cosmetic industries and both national and international  
50 health and food safety authorities have extensively debated the safety of novel plants and plant-  
51 derived ingredients for their use in foods. These discussions consistently produced a consensus  
52 that adequate specifications of plant identity and composition are key issues in the safety  
53 assessment of plant-derived ingredients (Antignac et al., 2011). Although  $\beta$ -caryophyllene  
54 oxide is beneficial as a mosquito repellent, this compound may produce toxic and adverse  
55 effects on humans or animals or both. Consequently, potential cytotoxic effects of hazardous  
56 substances must be assessed prior to product development. Little scientific data is available on  
57 the possible adverse effects or the biological safety of  $\beta$ -caryophyllene oxide in experimental  
58 administration to animals. What has been reported is that based on a biomembrane model,  $\beta$ -  
59 caryophyllene oxide could pass through the cell membrane without inducing genotoxic effects  
60 at the gene or chromosomal level (Di Sotto et al., 2013).

61 In the current study, following the repellent study already done on *Aedes aegypti* and  
62 *An. minimus* (Nararak et al., 2019), we investigated the active excito-repellent properties of  $\beta$ -  
63 caryophyllene oxide against *Ae. albopictus* and *An. dirus*, using an excito-repellency (ER) test  
64 system. The excito repellency (ER) test system is the gold standard to quantitatively determine  
65 the two distinct forms of behavioral responses in mosquito population in response to test  
66 chemicals (Sathantriphop et al., 2006). A test chamber system and protocol that can easily  
67 differentiate these two types of behavioral responses has been described (Chareonviriyaphap  
68 et al., 1997; Roberts et al., 1997). Moreover, in the current study, we evaluated the safety of  
69  $\beta$ -caryophyllene oxide using an *in vitro* phototoxicity test and an *in vitro* micronucleus assay.

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## 2. Materials and Methods

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### 2.1 Mosquitoes used

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### 2.2 Test compounds

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### 2.3 Filter paper treatment

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Samples of *Aedes albopictus* were originally captured in 1996 in Chanthaburi province, eastern Thailand by staff from the Ministry of Public Health, Thailand. Representatives of this population have been maintained in the entomological laboratory at Kasetsart University for since 2013 and females only were used in the current study. Samples of *Anopheles dirus* were based on individuals originally collected in 1981 in Khao Mai Kaeo sub-district, Bang Lamung district, Chonburi province, eastern Thailand. These two mosquito species were reared in separate insectaries of the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. All larvae and adults were held under laboratory-controlled conditions of  $25\pm 5^\circ\text{C}$  and  $80\pm 10\%$  relative humidity with a 12:12 (L:D) photoperiod. Larvae were fed with fish food twice daily. Adults were reared in a screened cage and provided with 10% sugar solution as food. Three-to-five day-old female mosquitoes were starved for 24 h before testing.

DEET (*N,N*-Diethyl-3-methylbenzamide) with 97% purity obtained from the Sigma-Aldrich Company Ltd. (Lot No: MKBH0428V) was used as the gold standard insect repellent.  $\beta$ -Caryophyllene oxide was purchased from the Acros Organics Company Ltd. (95% purity, Lot No: A0356135).

$\beta$ -Caryophyllene oxide and DEET were diluted with absolute ethanol to provide respective concentrations of 0.1, 0.25, 0.5 and 1.0% (v/v). The 2.8 mL test solution was spread evenly over the fine surface of filter paper ( $14.7 \times 17.5$  cm) using a 5 mL serological pipette and pipette controller. Similar treated papers for four replicates were prepared for each concentration and the control papers were treated with absolute ethanol only. All treated papers



95 were air-dried in a horizontal position at room temperature for 1 h prior to starting the test. All  
96 solutions and treated papers were freshly prepared on the day required.

#### 97 **2.4 Excito-repellency test system**

98 Experiments were conducted to compare the behavioral responses of mosquitoes using  
99 an excito-repellency test system described by (Chareonviriyaphap et al., 2002). The test system  
100 consisted of two pairs each for contact and noncontact exposure chambers. For a pair of contact  
101 chambers, the treated papers were placed on the inside of a screened inner chamber so that  
102 female mosquitoes could rest directly on the treated papers. Alternatively, a pair of noncontact  
103 trials had treated papers outside the screened inner chamber, which prevented the female  
104 mosquitoes from resting directly on the treated papers. Fifteen healthy, unfed female  
105 mosquitoes were introduced into four chambers simultaneously using a mouth aspirator and  
106 allowed 3 min to adjust to the chambers. Subsequently, the escape funnels were opened to  
107 begin the observation period. The number of mosquitoes escaping from the chamber into the  
108 receiving cage was recorded at 1 min intervals for a total of 30 min. After the 30 min exposure  
109 period, the numbers of knockdown and dead mosquitoes were recorded separately. All  
110 mosquitoes that either escaped to the receiving box or remained inside the chamber were kept  
111 in clean plastic cups and provided with cotton pads soaked with 10% sugar solution for 24 h.  
112 The numbers of both knockdown and mortality were respectively recorded after 30 min  
113 exposure and after 24 h.

114 The mean percentage of escaped mosquitoes was calculated per test chamber. Abbott's  
115 formula was applied to adjust escape responses based on paired control escape responses  
116 (Abbott, 1987). A log-rank test was used to analyze paired comparisons of the escape patterns  
117 based on the compound, species and concentrations (Mantel and Haenszel, 1959). Statistical  
118 significance was accepted for all tests at  $P < 0.05$ .

119

## 120           **2.5 Safety evaluation procedures for $\beta$ -caryophyllene oxide**

121           In accordance with OECD (2004) for the identification of efficacy of  $\beta$ -caryophyllene  
122 oxide, the safety evaluation showed no prohibited or restricted components. For safety reasons,  
123 the *in vitro* 3T3 NRU phototoxicity test (OECD N°432) was first conducted to identify the  
124 toxicity of the test substance induced by the excited chemicals after exposure to light. The 3T3  
125 NRU phototoxicity test is based on the comparison of a chemical when tested in the presence  
126 and in the absence of exposure to a non-cytotoxic dose of simulated solar light. Cytotoxicity  
127 is expressed as a concentration-dependent reduction of the uptake of the vital dye Neutral Red  
128 when measured 24 h after chemical treatment and irradiation.

129           Balb/c 3T3 mouse fibroblasts (3T3-L1) (ATCC, USA, ATCC® CL-173™, N°  
130 P6110401, Lot. 09I006), low passage number (<50), maintained into DMEM (Dulbecco's  
131 Minimum Essential Medium, PAN BIOTECH. lot 1874561) supplemented with penicillin 100  
132 IU/mL, streptomycin 100  $\mu$ g/mL (PAN BIOTECH, Lot 945514) and 10% inactivated calf  
133 serum (PAN BIOTECH, Lot P56314), were seeded into two 96-well tissue culture plates (0.1  
134 mL per well) at concentration of  $1 \times 10^5$  cells/mL, and incubated at 37°C (5% CO<sub>2</sub>) for 24 h  
135 until semi-confluent. At the end of the incubation period, the culture medium was decanted  
136 and replaced by 100  $\mu$ L of Hank's balanced salt solution (HBSS) containing the appropriate  
137 concentrations of the test substance (eight different concentrations). Subsequently, cells were  
138 incubated at 37°C (5% CO<sub>2</sub>) in the dark for 60 min. From the two plates prepared for each  
139 series of test substance concentrations and the controls, one was randomly selected for the  
140 determination of cytotoxicity without irradiation (-Irr), and the other for the determination of  
141 photo-cytotoxicity with irradiation (+Irr). The irradiation procedure was performed using a  
142 solar simulator Suntest CPS+ apparatus (Atlas Material Testing Technology BV, Mousy le  
143 Neuf, France) equipped with a xenon arc lamp (1,100 W), a special glass filter restricting  
144 transmission of light below 290 nm and a near IR-blocking filter. The irradiance was fixed at

145 750 W/m<sup>2</sup> throughout the experiments and the combined light dose was 5 J/cm<sup>2</sup>/min based on  
146 UVA irradiation (0.41 J/cm<sup>2</sup>) or visible irradiation (4.06 J/cm<sup>2</sup>). The test solution was removed,  
147 cells were rinsed twice with 150 µL HBSS and incubated for 18-22h in 0.1 mL of culture  
148 medium at 37°C (5% CO<sub>2</sub>). Cells were washed, placed into Neutral Red medium (50 µg/mL  
149 Neutral Red in complete medium) and incubated for 3 h at 37°C, 5% CO<sub>2</sub>. Then the medium  
150 was removed and cells were washed three times with 0.2 mL of HBSS to remove any excess  
151 dye. The Neutral Red medium was removed and destaining solution (50% ethanol, 1% acetic  
152 acid, 49% distilled water; 50 µL per well) was added into each well. Then, the plates were  
153 shaken for 15-20 min at room temperature in the dark. All the test samples and controls were  
154 run in triplicate in three independent experiments. A positive control (chlorpromazine  
155 (SIGMA), final concentrations 1-100 µg/mL without irradiation and 0.01 to 1 µg/mL with  
156 irradiation) and a negative control (HBSS) were included in each set of experiments. Cell  
157 viability (increase Neutral Red uptake) was measured using a fluorescence-luminescence  
158 reader (Infinite M200 Pro, TECAN). The optical density (OD) of each well was read at 540  
159 nm. The results obtained for wells treated with the test material were compared to those of  
160 untreated control wells (HBSS, 100% viability) and converted to percentage values. The  
161 concentrations were calculated for the test material causing a 50% release of the preloaded  
162 Neutral Red without irradiation (IC<sub>50</sub> -Irr) and with irradiation (IC<sub>50</sub> +Irr) compared to the  
163 control culture using the Phototox Version 2.0 software (Federal Institute for Risk Assessment,  
164 [zebet@bfr.bund.d](mailto:zebet@bfr.bund.d)). The mean OD value of blank wells (containing only Neutral Red desorbed  
165 solution) was subtracted from the mean OD value of three treated wells (dilutions of the test  
166 material, positive control or HBSS). The percentages of cell viability were calculated as:

167

$$168 \quad \text{Viability (\%)} = \frac{\text{Mean OD of test wells} - \text{mean OD of blanks}}{\text{Mean OD of negative control} - \text{mean OD of blanks}}$$

169

170 The photo-irritation-factor (PIF) was calculated using the following formula:

171

$$172 \quad \text{PIF} = \frac{\text{IC50} (- \text{Irr})}{\text{IC50} (+ \text{Irr})}$$

173

174 Based on validation studies, a test substance with a PIF < 2 predicts no phototoxicity,  
175 a PIF between 2 and 5 predicts a probable phototoxicity and a PIF > 5 predicts phototoxicity.

176 Then, the *in vitro* micronucleus assay (MNvit) was used to detect the long-term toxicity  
177 of the chemical. Micronucleus assay is a mutagenicity assay which is based on the detection  
178 of micronuclei (MNC) in the cytoplasm of interphase cells and allows the detection of the  
179 cytogenetic activity of clastogenic or aneugenic compounds or both in cell culture (Johnson et  
180 al., 2010). The micronucleus assay was performed on a Chinese hamster ovary cell line (CHO-  
181 K1; ATCC, USA) maintained in McCoy's 5A medium supplemented with 1 mM glutamine,  
182 100 unit/mL and 10 µg/mL of a mixture of penicillin and streptomycin, respectively and 10%  
183 of inactivated calf serum. The CHO-K1 cells were transferred into Labteck wells at a  
184 concentration of 100,000 cells/mL, and incubated for 24 h at 37°C in CO<sub>2</sub> (5%). When the test  
185 was performed without metabolic activation, the test substance was added into cell cultures at  
186 the concentrations previously defined. A negative control containing culture medium, a solvent  
187 control containing 1% DMSO and a positive control containing 0.6 µg/mL of mitomycin C  
188 were added.

189 When the assay was performed in the presence of metabolic activation, S9 mix  
190 metabolizing mixture was added to cell cultures at a concentration of 10%. The metabolic  
191 activation system (S9) was a 9,000 g centrifuged supernatant of a liver homogenate (S9) and  
192 was prepared from male Sprague-Dawley rats treated with a single injection of Aroclor 1254  
193 (500 mg/kg body weight). The protein concentration in the S9 homogenate was 26 mg/ mL as  
194 determined by the method of Lowry. S9 mix contained 10% S9, 5 mM G6P, 4 mM NADP, 33

195 mM KCl, and 8 mM MgCl<sub>2</sub> diluted in saline phosphate buffer. Then, the test substance was  
196 added to the cell cultures at the concentrations previously defined. A negative control  
197 containing culture medium, a solvent control containing 1% DMSO and a positive control  
198 containing 5 µg/mL of benzo[a]pyrene were added.

199 After 3 h of incubation at 37 °C in CO<sub>2</sub> (5%), the culture medium was removed, the  
200 cells were rinsed with phosphate buffered saline (PBS), and then returned to culture in McCoy's  
201 5A medium containing 3 µg/mL of cytochalasin B. After incubation for 21 h at 37°C, the cells  
202 were rinsed with PBS, fixed with methanol and stained with 10% Giemsa for 20 min.

203 The results were analyzed under a microscope at ×1,000 magnification. The  
204 antiproliferative activity of test substances was estimated by counting the number of  
205 binucleated cells relative to the number of mononucleated cells for 500 cells for each dose (250  
206 cells counted per well). The cytokinesis blocked proliferative index (CBPI) was calculated  
207 using the following formula:

208

$$209 \quad \text{CBPI} = \frac{2 \times (\text{BI} + \text{MONO})}{500}$$

210

211 where, BI is the number of binucleated cells and MONO is the number of mononucleated cells

212 The cytostasis index (CI%) is the percentage of cell replication inhibition and was  
213 calculated using the following formula:

214

$$215 \quad \text{CI}\% = 100 - \left( \frac{100 \times (\text{CBPI}_{\text{test material}} - 1)}{\text{CBPI}_{\text{solvent control}} - 1} \right)$$

216

217 After this step, only the doses inducing a decrease of less than 55±5% of CI% compared  
218 to the negative control were considered for counting micronuclei. The rates of micronuclei

219 were evaluated for the presence of independent nuclear core entities in 1,000 binucleated cells  
220 per well, which corresponded to 2,000 cells examined by test substance dose. Micronuclei  
221 were identified as small nuclei well-differentiated from the cell nucleus, stained in the same  
222 manner and having a diameter less than one-third of that of the cell nucleus. The micronuclei  
223 rates obtained for different doses of test substances were compared to the negative control using  
224 a  $\chi^2$  test. The assay was considered positive if a dose-response relationship was obtained  
225 between the rate of micronuclei and the doses tested, where at least one of these doses induced  
226 a statistically significant increase ( $P < 0.05$ ) in the number of micronucleated cells compared  
227 to the negative control.

228

### 3. Results

229           Excito-repellency responses of *Ae. albopictus* and *An. dirus* exposed to 0.1, 0.25, 0.5,  
230 1.0% v/v of DEET and  $\beta$ -caryophyllene oxide were evaluated for contact irritancy and non-  
231 contact repellency responses using an excito-repellency system. DEET was used as a standard  
232 repellent for comparison purposes. The results showed that *Ae. albopictus* (3.51-56.36%) had  
233 a much higher escape response to  $\beta$ -caryophyllene oxide than *An. dirus* (0-32.73%) in both the  
234 contact and non-contact trials (Table 1). A higher escape response percentage was seen from  
235 the contact chamber than from the noncontact chamber and control, regardless of test  
236 compounds and concentrations.

237           The escape responses of *Ae. albopictus* to  $\beta$ -caryophyllene oxide were characterized in  
238 contact and noncontact exposure chambers by comparison to DEET (Table 1).  $\beta$ -  
239 Caryophyllene oxide at 0.5 and 1.0% elicited stronger escape responses than DEET in the  
240 contact and noncontact treatments. No knockdown and mortality were observed from  
241 treatments and control chambers at all concentrations. The greater escape response percentages  
242 of *Ae. albopictus* were observed at 0.5 and 1.0% in the contact trial with  $\beta$ -caryophyllene oxide  
243 (46.43 and 56.36%, respectively) and DEET (38.98 and 38.18% respectively). For *An. dirus*,  
244 the stronger escape responses were found at 0.5 and 1.0%, in both contact (26.32 and 32.73%  
245 respectively) and non-contact trial (31.03 and 31.67%, respectively) as shown in Table 1. A  
246 knockdown response (37%) was observed in *An. dirus* exposed to 1%  $\beta$ -caryophyllene oxide  
247 in the contact trial.

248           The escape patterns from the chambers using survival curves at 1 min intervals in the  
249 contact and noncontact designs with paired controls under different concentrations of  $\beta$ -  
250 caryophyllene oxide and DEET against *Ae. albopictus* and *An. dirus* are shown in Figures 1-4.  
251 The rates represent probabilities for escaping from a chamber with a particular compound and  
252 concentration. Overall,  $\beta$ -caryophyllene oxide exhibited a strong escape response in both

253 contact and noncontact trials at all concentrations. Delayed escape responses were observed  
254 for 0.1 and 0.25% with all compounds in the contact and noncontact trials (Figures 1-2). With  
255 *Ae. albopictus*, strong contact escape patterns were evident for 0.5 and 1.0%  $\beta$ -caryophyllene  
256 oxide (Figures 3-4).

257 The multiple log-rank comparisons of *Ae. albopictus* and *An. dirus* in the paired contact  
258 and noncontact treatment trials for  $\beta$ -caryophyllene oxide and DEET are shown in Table 2.  
259 Interestingly, there were no significant differences in escape patterns for contact *versus*  
260 noncontact trials, except for 0.5% and 1.0% with *Ae. albopictus* (Table 2).

261 The statistical comparison of the escape responses for mosquitoes exposed to  $\beta$ -  
262 caryophyllene oxide compared to DEET, at different concentrations, are presented in Table 3.  
263 In both the contact and noncontact trials using *Ae. albopictus*, the escape responses were  
264 significantly higher with  $\beta$ -caryophyllene oxide than DEET at 1.0% concentration. For *An.*  
265 *dirus*, the escape responses were significantly more important with  $\beta$ -caryophyllene oxide than  
266 DEET in the noncontact trials at 0.5 and 1%. The statistical comparisons between the two  
267 species exposed to  $\beta$ -caryophyllene oxide and DEET in either the contact or noncontact trials  
268 are shown in Table 4. For  $\beta$ -caryophyllene oxide, *Ae. albopictus* showed significantly higher  
269 escape responses compared to *An. dirus* in the contact trials at 0.5% and 1.0% ( $P = 0.0184$  and  
270  $0.0024$ , respectively), while significance differences in the escape responses were found at  
271 0.5% in both contact and noncontact trials using DEET.

272 The cytotoxic potential of  $\beta$ -caryophyllene oxide was evaluated in murine fibroblasts  
273 (3 T3) incubated with the compound (0.1-10 mg/mL). The compound exhibited negligible  
274 cytotoxicity ( $IC_{50} = 13.23 \pm 1.37 \mu\text{g/mL}$ ) as shown in Table 5. PIF values were used to classify  
275 the phototoxicity potential (Table 5). The phototoxicity assay considered dark ( $IC_{50} =$   
276  $13.23 \pm 1.37 \mu\text{g/mL}$ ) and irradiated ( $IC_{50} = 34.79 \pm 5.49 \mu\text{g/mL}$ ) conditions. According to the



277 analyses,  $\beta$ -caryophyllene oxide did not exhibit phototoxic potential (PIF = 0.368) for the dose  
278 levels tested.

279 Genotoxicity was assayed starting from the highest concentration at which neither  
280 necrosis nor cytotoxic or cytostatic effects was observed. When tested on the Chinese hamster  
281 ovary (CHO)-K1 cell line,  $\beta$ -caryophyllene oxide did not produce any cytotoxic effects up to  
282 a concentration of 5  $\mu\text{g}/\text{mL}$ . At concentrations of 0.5, 1.0 and 5.0  $\mu\text{g}/\text{mL}$ ,  $\beta$ -caryophyllene  
283 oxide did not increase the MNC (micronucleated cells) frequency with respect to the control  
284 (Table 6). No concentration of  $\beta$ -caryophyllene oxide induced an increase in MNC with or  
285 without metabolic activation. These results indicated that the compound was not derived from  
286 clastogenic/aneugenic activity and did not produce clastogenic/aneugenic metabolites (Table  
287 6).

288

#### 4. Discussion

289           Approximately 3,500 species of mosquitoes have been formally described, with some  
290 species having ecological distributions in many urban, peri-urban, or forest settings in close  
291 association with humans, and these species include *Aedes albopictus* and *Anopheles dirus*. *Ae.*  
292 *albopictus*, an important vector of dengue viruses in many urban and semi-forest communities,  
293 is a predominately day-biting mosquito, often found in and around human dwellings and  
294 preferentially feeds on humans (Chareonviriyaphap et al., 2003; Gomes et al., 2003), whereas  
295 *An. dirus* occurs in forest and forest-fringe habitats with a preference for night feeding and it  
296 is a major vector of malaria in several Asian countries (Suwonkerd et al., 2013; Tainchum et  
297 al., 2015). These two mosquito species have been found highly refractory to common control  
298 tools due to their mostly exophagic behavior and are regarded as excellent vectors even though  
299 they are present in low population densities. Therefore, the prevention and control of most  
300 vector-borne diseases remains dependent on various vector control strategies to decrease the  
301 transmission risk, which is a major challenge for outdoor-biting mosquitoes such as these 2  
302 species.

303           Among the available vector control tools, chemical control remains the most used and  
304 effective in combatting mosquito vectors. A number of chemical compounds can protect  
305 humans from blood-feeding pests by one or more of three identified actions: contact irritancy,  
306 non-contact spatial repellency, or toxicity (Grieco et al., 2007). The first two properties are  
307 potential outcome behavioral responses of mosquitoes after or before they make tarsi contact  
308 with treated surfaces (Chareonviriyaphap et al., 1997; Roberts et al., 1997). Most studies have  
309 focused primarily on the insecticidal action of chemicals to control mosquitoes, whereas few  
310 investigations have played attention to the non-toxic properties, including irritancy and  
311 repellency (Chareonviriyaphap et al., 2013; Chareonviriyaphap et al., 2004;  
312 Chareonviriyaphap et al., 1997; Grieco et al., 2007). Moreover, much of the previous work

313 has focused on potent synthetic compounds (Mongkalangoon et al., 2009; Thanispong et al.,  
314 2009). For years, the development of physiological resistance by mosquitoes to synthetic  
315 compounds has resulted in the reduced effectiveness of chemical-based prevention and control  
316 methods (Chareonviriyaphap et al., 2013). For this reason, the potential use of plant extracts  
317 or products derived from native plants as a source of essential oils for use as natural repellents  
318 is an attractive option to replace or supplement synthetic compounds for the control of vectors  
319 and the prevention of disease transmission (Desgrouas et al., 2016; Maia and Moore, 2011)

320 Several plant-based essential oils have been evaluated for mosquito repellent activity  
321 as protection against mosquitoes and other arthropod pests in Thailand. These have included  
322 *Ocimum americanum* L. (Hairy basil), *Cymbopogon nardus* (L.) Rendle (Citronella), *Vetiveria*  
323 *zizanioides* (L.) Nash (Vetiver), *Citrus hystrix* DC. (Kaffir lime), *Cinnamomum verum* J. Presl  
324 (Cinnamon leaf), *Syzygium aromaticum* (L.) Merr. & L.M. Perry (Clove flower), and *Zingiber*  
325 *officinale* Roscoe (Ginger) (Boonyuan et al., 2014; Nararak et al., 2016; Nararak et al., 2017;  
326 Suwansirisilp et al., 2013). These essential oils have shown great promise as insect repellents  
327 and have been effective against several species of mosquitoes due to the presence of a variety  
328 of bioactive constituents that interfere with insect behavior and growth (Tisgratog et al., 2016;  
329 Tisgratog et al., 2018). The plant products have been effective as insect repellents or  
330 insecticidal agents and one of the potential repellent compounds is  $\beta$ -caryophyllene oxide  
331 (Nararak et al., 2019).

332  $\beta$ -Caryophyllene oxide is a bicyclic sesquiterpene generated from the oxidation of  $\beta$ -  
333 caryophyllene and is found in a large number of plants worldwide (Fidyt et al., 2016).  $\beta$ -  
334 Caryophyllene oxide appears to be common among the essential oils that exhibit mosquito  
335 repellent ability (Jaenson et al., 2006; Trongtokit et al., 2005). *Artabotrys hexapetalus* (L.f.)  
336 Bhandari oil, obtained from leave parts, contains  $\beta$ -caryophyllene oxide as one of its major  
337 constituents and displays strong repellent activity against females of *An. gambiae* (Suleiman et

338 al., 2014). Strong repellency against *An. gambiae* was also reported from a combination of  
339 linalool,  $\beta$ -caryophyllene oxide,  $\gamma$ -terpinene, and 1-methylpyrrole (45:39:8:8), and essential oil  
340 of *Croton pseudopulchellus* Pax (Odaló et al., 2005). Furthermore, (-)-caryophyllene oxide  
341 and (-)-limonene are the major chemical constituents found in essential oil extracted from the  
342 leaves of *Perilla frutescens* (L.) Britton, and provide good biting-deterrent activity (Tabanca et  
343 al., 2015).

344  $\beta$ -Caryophyllene oxide has been approved as a food additive by the Food and Drug  
345 Administration (FDA) and the European Food Safety Authority (EFSA) (Fidyt et al., 2016).  
346 The two most common plant-associated repellent compounds are p-menthane-3,8-diol (derived  
347 from the Australian lemon-scented gum tree) and picaridin (a synthetic derivative of pepper);  
348 these have been tested for toxicity (U.S. EPA Biopesticide Registration Documents 011550  
349 and 7505C) (EPA, 2019; Zhu et al., 2009). Phototoxic potential is assessed by comparing the  
350 differences in  $IC_{50}$  between negative control plates (not exposed to UVA) and test plates  
351 (exposed to UVA) (Roesler et al., 2010). The phototoxicity results obtained using the *in vitro*  
352 method are crucial because topical repellent formulations are also used during the day to protect  
353 against day-biting mosquitoes such as *Aedes* species, and this involves exposure to the sun and  
354 artificial light. The current study investigated the cytotoxicity and phototoxicity of  $\beta$ -  
355 caryophyllene oxide and showed that the compound was neither cytotoxic, nor phototoxic  
356 (PIF=0.38). The potential genotoxic effects from  $\beta$ -caryophyllene oxide have also been  
357 evaluated in mammalian cells. Likewise, the current study showed that  $\beta$ -caryophyllene oxide  
358 did not induce genotoxicity at the chromosomal level, as observed in the micronucleus assay.  
359 Di Sotto et al. (2013) reported that  $\beta$ -caryophyllene oxide was tested for mutagenic effects  
360 using the Ames test and micronucleus assay. Their results showed that the flavoring agent was  
361 devoid of mutagenic effects, both at the gene level (frameshift or based-substitution mutations)  
362 and on chromosomes (clastogenicity and aneuploidogenicity), suggesting that  $\beta$ -caryophyllene

363 oxide is also safe when used as a flavoring/fragrance ingredient. Then, this finding highlights  
364 the fact this component could be safe for human topical applications.

365 Nararak et al. (2016, 2017) reported that essential oils of citronella, vetiver, hairy basil,  
366 and kaffir lime had good irritant and repellent effects on mosquito vectors compared to DEET.  
367 These studies suggested that plant-based substances have good potential efficacy to be  
368 alternative insect repellents. In the current study, mosquitoes displayed varying levels of  
369 behavioral escape responses to the compounds, indicating a clear dose response with  
370 concentration. The study showed that at higher concentrations (0.5 and 1.0%),  $\beta$ -caryophyllene  
371 oxide had significantly greater repellent and irritant effects compared to DEET. Moreover, *Ae.*  
372 *albopictus* exhibited much stronger escape responses against  $\beta$ -caryophyllene oxide than *An.*  
373 *dirus* for both contact and non-contact assays. Knockdown (37.83%) was also found only in  
374 non-escape *An. dirus* mosquitoes in the contact trial at 1.0% concentration of  $\beta$ -caryophyllene  
375 oxide, whereas no knockdown and mortality at 24 h post exposure were observed for *Ae.*  
376 *albopictus*. The results in contact and non-contact trials indicated that escape responses of  
377 mosquitoes to the  $\beta$ -caryophyllene oxide were significantly greater than with DEET, similar to  
378 the previous study by Nararak et al. (2019) in which  $\beta$ -caryophyllene oxide was tested at  
379 concentrations of 0.1, 0.25, 0.5 and 1.0% (v/v) against *Ae. aegypti* and *An. minimus* and the  
380 results were compared to DEET at the same concentrations. The results showed that DEET  
381 displayed lower irritancy and repellent responses than  $\beta$ -caryophyllene oxide and *An. minimus*  
382 exhibited higher avoidance response rates (86–96% escape) at 0.5% and 1.0% concentrations  
383 in contact and non-contact trials compared with *Ae. aegypti* (22–59% escape). When  
384 comparing the results obtained with the four mosquito species tested with  $\beta$ -caryophyllene  
385 oxide, *An. minimus* presented the highest sensitivity to both types of escape responses (contact  
386 irritancy and noncontact-spatial repellency) at 0.5-1% (v/v), followed by *Ae. aegypti*, *Ae.*  
387 *albopictus*, and *An. dirus*. Comparatively, DEET was less efficient than  $\beta$ -caryophyllene oxide

388 at 0.5-1% (v/v) for all 4 species as they presented lower escape responses in both trials, contact  
389 irritancy and noncontact-spatial repellency. Based on these results,  $\beta$ -caryophyllene oxide had  
390 stronger repellent and irritant effects than DEET at the same concentrations, suggesting the  
391 former has high potential for further development as an alternative active ingredient in  
392 mosquito repellent formulations, safe for humans and the environment. Such efficient repellent  
393 products are in need. In a study done in Vietnam, forest-goers were in favor of using repellent  
394 products to avoid mosquito-biting pressure (Ohrt et al., 2018). The use of repellent is a  
395 complementary vector control method to standard ones such as bednets, long-lasting  
396 insecticidal nets (LLINs), indoor residual spraying (IRS), which has the advantage of repelling  
397 outdoor-biting mosquitoes, then reducing residual malaria transmission risk (Durnez and  
398 Coosemans, 2013). Then, appropriate formulations using  $\beta$ -caryophyllene oxide await  
399 development for its use as repellent for a safe protection against mosquito bites.

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420

421 **Conflict of interest**

422 The authors declare no conflict of interest.

423

424 **Author Contributions**

425 TC conceived and designed the experiments. JN, CS and CG performed the  
426 experiments. JN, CS and CG analyzed the data. JN, CS and CG wrote the manuscript. SM,  
427 VML, EO and TC consulted and edited the manuscript. All authors read and approved the  
428 manuscript.

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- 439 Abbott, W.S., 1987. A method of computing the effectiveness of an insecticide. 1925. J. Am.  
440 Mosq. Control Assoc. 3, 302-303.
- 441 Amichai, B., Lazarov, A., Halevy, S., 1994. Contact dermatitis from diethyltoluamide.  
442 Dermatitis 30, 188-189.
- 443 Antignac, E., Nohynek, G.J., Re, T., Clouzeau, J., Toutain, H., 2011. Safety of botanical  
444 ingredients in personal care products/cosmetics. Food Chem. Toxicol. 49, 324-341.
- 445 Baimai, V., Green, C., Andre, R., Harrison, B., Peyton, E., 1984. Cytogenetic studies of some  
446 species complexes of *Anopheles* in Thailand and Southeast Asia. Southeast Asian J.  
447 Trop. Med. Public Health 15, 536-546.
- 448 Benelli, G., Mehlhorn, H., 2018. Mosquito-borne Diseases: Implications for Public Health.  
449 Springer.
- 450 Boonyuan, W., Grieco, J.P., Bangs, M.J., Prabaripai, A., Tantakom, S., Chareonviriyaphap,  
451 T., 2014. Excito-repellency of essential oils against an *Aedes aegypti* (L.) field  
452 population in Thailand. J. Vector Ecol. 39, 112-122.
- 453 Briassoulis, G., Narlioglou, M., Hatzis, T., 2001. Toxic encephalopathy associated with use  
454 of DEET insect repellents: a case analysis of its toxicity in children. Hum. Exp.  
455 Toxicol. 20, 8-14.
- 456 Chareonviriyaphap, T., Akratanakul, P., Nettanomsak, S., Huntamai, S., 2003. Larval habitats  
457 and distribution patterns of *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse), in  
458 Thailand. Southeast Asian J. Trop. Med. Public. Health 34, 529-535.
- 459 Chareonviriyaphap, T., Bangs, M.J., Ratanatham, S., 2000. Status of malaria in Thailand.  
460 Southeast Asian J. Trop. Med. Public Health 31, 225-237.



461 Chareonviriyaphap, T., Bangs, M.J., Suwonkerd, W., Kongmee, M., Corbel, V., Ngoen-Klan,  
462 R., 2013. Review of insecticide resistance and behavioral avoidance of vectors of  
463 human diseases in Thailand. *Parasites & vectors* 6, 280.

464 Chareonviriyaphap, T., Prabaripai, A., Bangs, M.J., 2004. Excito-repellency of deltamethrin  
465 on the malaria vectors, *Anopheles minimus*, *Anopheles dirus*, *Anopheles*  
466 *swadiwongporni*, and *Anopheles maculatus*, in Thailand. *J. Am. Mosq. Control*  
467 *Assoc.* 20, 45-54.

468 Chareonviriyaphap, T., Prabaripai, A., Sungvornyothin, S., 2002. An improved excito-  
469 repellency test chamber for mosquito behavioral tests. *J. Vector Ecol.* 27, 250-252.

470 Chareonviriyaphap, T., Roberts, D., Andre, R.G., Harlan, H., Manguin, S., Bangs, M., 1997.  
471 Pesticide avoidance behavior in *Anopheles albimanus*, a malaria vector in the  
472 Americas. *J. Am. Mosq. Control Assoc.* 13, 171-183.

473 Chouin-Carneiro, T., Vega-Rua, A., Vazeille, M., Yebakima, A., Girod, R., Goindin, D.,  
474 Dupont-Rouzeyrol, M., Lourenço-de-Oliveira, R., Failloux, A.-B., 2016. Differential  
475 susceptibilities of *Aedes aegypti* and *Aedes albopictus* from the Americas to Zika  
476 virus. *PLoS Negl. Trop. Dis.* 10, e0004543.

477 Debboun, M., Strickman, D., 2013. Insect repellents and associated personal protection for a  
478 reduction in human disease. *Bull. Entomol. Res.* 27, 1-9.

479 Desgrouas, C., Nararak, J., Tisgratog, R., Mahiou-Leddé, V., Bory, S., Ollivier, E., Manguin,  
480 S., Chareonviriyaphap, T., 2016. Comparative excito-repellency of three Cambodian  
481 plant-derived extracts against two mosquito vector species, *Aedes aegypti* and  
482 *Anopheles minimus*. *J. Am. Mosq. Control Assoc.* 32, 185-194.

483 Di Sotto, A., Maffei, F., Hrelia, P., Castelli, F., Sarpietro, M.G., Mazzanti, G., 2013.  
484 Genotoxicity assessment of  $\beta$ -caryophyllene oxide. *Regul. Toxicol. Pharmacol.* 66,  
485 264-268.

486 Durnez, L., Coosemans, M., 2013. Residual transmission of malaria: An Old issue for new  
487 approaches. In *Anopheles mosquitoes-New insights into malaria vectors*. Manguin S.  
488 Ed., IntechOpen, London, UK, 671-704.

489 EPA, 2019. Skin-Applied Repellent Ingredients. [https://www.epa.gov/insect-repellents/skin-](https://www.epa.gov/insect-repellents/skin-applied-repellent-ingredients)  
490 [applied-repellent-ingredients](https://www.epa.gov/insect-repellents/skin-applied-repellent-ingredients).

491 Fidyk, K., Fiedorowicz, A., Strzadala, L., Szumny, A., 2016.  $\beta$ -caryophyllene and  
492  $\beta$ -caryophyllene oxide—natural compounds of anticancer and analgesic properties.  
493 *Cancer Med.* 5, 3007-3017.

494 Garneau, F.-X., Collin, G.J., Jean, F.-I., Gagnon, H., Arze, J.B.L., 2013. Essential oils from  
495 Bolivia. XII. Asteraceae: *Ophryosporus piquerioides* (DC) Benth. ex Baker. *J. Essent.*  
496 *Oil Res.* 25, 388-394.

497 Gertsch, J., Leonti, M., Raduner, S., Racz, I., Chen, J.-Z., Xie, X.-Q., Altmann, K.-H.,  
498 Karsak, M., Zimmer, A., 2008. Beta-caryophyllene is a dietary cannabinoid. *Proc.*  
499 *Natl. Acad. Sci.* 105, 9099-9104.

500 Gomes, A., Silva, N., Marques, G., Brito, M., 2003. Host-feeding patterns of potential human  
501 disease vectors in the Paraíba Valley region, State of São Paulo, Brazil. *J. Vector*  
502 *Ecol.* 28, 74-78.

503 Grieco, J.P., Achee, N.L., Chareonviriyaphap, T., Suwonkerd, W., Chauhan, K., Sardelis,  
504 M.R., Roberts, D.R., 2007. A new classification system for the actions of IRS  
505 chemicals traditionally used for malaria control. *PLoS One.* 2, e716.

506 Isman, M.B., 2002. Plant essential oils as green pesticides for pest and disease management.  
507 *J. Am. Chem. Soc.* 223, U668-U669.

508 Jaenson, T.G., Pålsson, K., Borg-Karlson, A.-K., 2006. Evaluation of extracts and oils of  
509 mosquito (Diptera: Culicidae) repellent plants from Sweden and Guinea-Bissau. *J.*  
510 *Med. Entomol.* 43, 113-119.

511 Johnson, G.E., Jenkins, G.J., Thomas, A.D., Doak, S.H., 2010. Vinblastine and  
512 diethylstilboestrol tested in the in vitro mammalian cell micronucleus test (MNvit) at  
513 Swansea University UK in support of OECD draft Test Guideline 487. *Mutat Res*  
514 *Genet Toxicol Environ Mutagen* 702, 189-192.

515 Jun, N.J., Mosaddik, A., Moon, J.Y., Ki-Chang, J., Dong-Sun, L., Ahn, K.S., Cho, S.K.,  
516 2011. Cytotoxic Activity of [beta]-Caryophyllene Oxide Isolated from Jeju Guava  
517 (*Psidium cattleianum* Sabine) Leaf. *Rec. Nat. Prod.* 5, 242.

518 Liang, Q., Liang, Z.-S., Wang, J.-R., Xu, W.-H., 2009. Essential oil composition of *Salvia*  
519 *miltiorrhiza* flower. *Food Chem. Toxicol.* 113, 592-594.

520 Maia, M.F., Moore, S.J., 2011. Plant-based insect repellents: a review of their efficacy,  
521 development and testing. *Malar. J.* 10 (Suppl 1), S11.

522 Mantel, N., Haenszel, W., 1959. Statistical aspects of the analysis of data from retrospective  
523 studies of disease. *J. Natl. Cancer Inst.* 22, 719-748.

524 Mongkalagoon, P., Grieco, J.P., Achee, N.L., Suwonkerd, W., Chareonviriyaphap, T., 2009.  
525 Irritability and repellency of synthetic pyrethroids on an *Aedes aegypti* population  
526 from Thailand. *J. Vector Ecol.* 34, 217-224.

527 Nararak, J., Sathantriphop, S., Chauhan, K., Tantakom, S., Eiden, A.L., Chareonviriyaphap,  
528 T., 2016. Avoidance Behavior to Essential Oils by *Anopheles minimus*, a Malaria  
529 Vector in Thailand. *J. Am. Mosq. Control Assoc.* 32, 34-43.

530 Nararak, J., Sathantriphop, S., Kongmee, M., Bangs, M.J., Chareonviriyaphap, T., 2017.  
531 Excito-Repellency of Citrus hystrix DC Leaf and Peel Essential Oils Against *Aedes*  
532 *aegypti* and *Anopheles minimus* (Diptera: Culicidae), Vectors of Human Pathogens. *J.*  
533 *Med. Entomol.* 54, 178-186.

534 Nararak, J., Sathantriphop, S., Kongmee, M., Mahiou-Leddet, V., Ollivier, E., Manguin, S.,  
535 Chareonviriyaphap, T., 2019. Excito-repellent activity of  $\beta$ -caryophyllene oxide  
536 against *Aedes aegypti* and *Anopheles minimus*. *Acta Trop.* 197, XX-XX

537 Odalo, J.O., Omolo, M.O., Malebo, H., Angira, J., Njeru, P.M., Ndiege, I.O., Hassanali, A.,  
538 2005. Repellency of essential oils of some plants from the Kenyan coast against  
539 *Anopheles gambiae*. *Acta Trop.* 95, 210-218.

540 OECD, 2004. OECD Guidelines for the testing of chemical, No. 432: In vitro 3T3 NRU  
541 phototoxicity test. . Paris, France: Organization for economic cooperation and  
542 development. 4.

543 Ohrt, C., Ngo, T.D., Nguyen, T.Q., 2018. Preparing for the Next Global Threat: A Call for  
544 Targeted, Immediate Decisive Action in Southeast Asia to Prevent the Next Pandemic  
545 in Africa. In: Manquin, S. (Ed.), *Towards Malaria Elimination: A Leap Forward*.  
546 IntechOpen.

547 Patel, E., Gupta, A., Oswal, R., 2012. A review on: mosquito repellent methods. *Int. J.*  
548 *Pharm. Chem. Biol. Sci.* 2, 310-317.

549 Polanco-Hernández, G., Escalante-Erosa, F., García-Sosa, K., Chan-Bacab, M.J., Sagua-  
550 Franco, H., González, J., Osorio-Rodríguez, L., Peña-Rodríguez, L.M., 2012.  
551 Metabolites from the leaf extract of *Serjania yucatanensis* with trypanocidal activity  
552 against *Trypanosoma cruzi*. *Parasitol. Res.* 111, 451-455.

553 Roberts, D.R., Chareonviriyaphap, T., Harlan, H.H., Hshieh, P., 1997. Methods of testing and  
554 analyzing excito-repellency responses of malaria vectors to insecticides. *J. Am. Mosq.*  
555 *Control Assoc.* 13, 13-17.

556 Roesler, R., Lorencini, M., Pastore, G., 2010. Brazilian cerrado antioxidant sources:  
557 cytotoxicity and phototoxicity in vitro. *Food Sci. Technol.* 30, 814-821.

558 Sathantriphop, S., Ketavan, C., Prabaripai, A., Visetson, S., Bangs, M.J., Akkratanakul, P.,  
559 Chareonviriyaphap, T., 2006. Susceptibility and avoidance behavior by *Culex*  
560 *quinquefasciatus* Say to three classes of residual insecticides. J. Vector Ecol. 31, 266-  
561 274.

562 Shell, E.R., 1997. Atlantic Monthly. Resurgence of a deadly diseases, 45-60.

563 Smith, C., 1956. The history of dengue in tropical Asia and its probable relationship to the  
564 mosquito *Aedes aegypti*. Am. J. Trop. Med. Hyg. 59, 243-251.

565 Suleiman, R.A., Mgani, Q.A., Nyandoro, S.S., 2014. Chemical compositions and mosquito  
566 repellency of essential oils from *Artabotrys hexapetalus* and *Artabotrys rupestris*. Int.  
567 J. Biol. Chem. Sci. 8, 2804-2812.

568 Suwansirisilp, K., Visetson, S., Prabaripai, A., Tanasinchayakul, S., Grieco, J.P., Bangs, M.J.,  
569 Chareonviriyaphap, T., 2013. Behavioral responses of *Aedes aegypti* and *Culex*  
570 *quinquefasciatus* (Diptera: Culicidae) to four essential oils in Thailand. J. Pest Sci. 86,  
571 309-320.

572 Suwonkerd, W., Ritthison, W., Ngo, C.T., Tainchum, K., Bangs, M.J., Chareonviriyaphap,  
573 T., 2013. Vector biology and malaria transmission in Southeast Asia, *Anopheles*  
574 mosquitoes-New insights into malaria vectors. IntechOpen.

575 Tabanca, N., Demirci, B., Ali, A., Ali, Z., Blythe, E.K., Khan, I.A., 2015. Essential oils of  
576 green and red *Perilla frutescens* as potential sources of compounds for mosquito  
577 management. Ind. Crop. Prod. 65, 36-44.

578 Tainchum, K., Kongmee, M., Manguin, S., Bangs, M.J., Chareonviriyaphap, T., 2015.  
579 *Anopheles* species diversity and distribution of the malaria vectors of Thailand.  
580 Trends Parasitol. 31, 109-119.

581 Tananchai, C., Pattanakul, M., Nararak, J., Sinou, V., Manguin, S., Chareonviriyaphap, T.,  
582 2019. Diversity and biting patterns of *Anopheles* species in a malaria endemic area,  
583 Umphang Valley, Tak Province, western Thailand. *Acta Trop.* 190, 183-192.

584 Tananchai, C., Tisgratog, R., Juntarajumnong, W., Grieco, J.P., Manguin, S., Prabaripai, A.,  
585 Chareonviriyaphap, T., 2012. Species diversity and biting activity of *Anopheles dirus*  
586 and *Anopheles baimaii* (Diptera: Culicidae) in a malaria prone area of western  
587 Thailand. *Parasites & vectors* 5, 211.

588 Telang, T., Awasthy, S., Oswal, V., 2003. Qualitative improvement of the essential oil of  
589 *Chloroxylon swietenia* (Roxb. corom). *Indian Perfum.* 47, 79-82.

590 Thanispong, K., Achee, N.L., Bangs, M.J., Grieco, J.P., Suwonkerd, W., Prabaripai, A.,  
591 Chareonviriyaphap, T., 2009. Irritancy and repellency behavioral responses of three  
592 strains of *Aedes aegypti* exposed to DDT and alpha-cypermethrin. *J. Med. Entomol.*  
593 46, 1407-1414.

594 Tisgratog, R., Sanguanpong, U., Grieco, J.P., Ngoen-Kluan, R., Chareonviriyaphap, T., 2016.  
595 Plants traditionally used as mosquito repellents and the implication for their use in  
596 vector control. *Acta Trop.* 157, 136-144.

597 Tisgratog, R., Sukkanon, C., Grieco, J.P., Sanguanpong, U., Chauhan, K.R., Coats, J.R.,  
598 Chareonviriyaphap, T., 2018. Evaluation of the Constituents of Vetiver Oil Against  
599 *Anopheles minimus* (Diptera: Culicidae), a Malaria Vector in Thailand. *J. Med.*  
600 *Entomol.* 55, 193-199.

601 Trongtokit, Y., Rongsriyam, Y., Komalamisra, N., Apiwathnasorn, C., 2005. Comparative  
602 repellency of 38 essential oils against mosquito bites. *Phytother. Res.* 19, 303-309.

603 WHO, 2018. *World malaria report 2018*. World Health Organization, Geneva, Switzerland,  
604 p. 165.

605 Wiwanitkit, V., 2011. Concurrent malaria and dengue infection: a brief summary and  
606 comment. *Asian Pac. J. Trop. Biomed.* 1, 326-327.

607 Yang, Y.C., Lee, H.S., Lee, S.H., Clark, J.M., Ahn, Y.J., 2005. Ovicidal and adulticidal  
608 activities of *Cinnamomum zeylanicum* bark essential oil compounds and related  
609 compounds against *Pediculus humanus capitis* (Anoplura : Pediculicidae). *Int. J.*  
610 *Parasitol.* 35, 1595-1600.

611 Zhao, J., Zheng, X., Newman, R.A., Zhong, Y., Liu, Z., Nan, P., 2013. Chemical composition  
612 and bioactivity of the essential oil of *Artemisia anomala* from China. *J. Essen. Oil*  
613 *Res.* 25, 520-525.

614 Zhu, J., Zeng, X.P., Berkebile, D., Du, H.J., Tong, Y., Qian, K., 2009. Efficacy and safety of  
615 catnip (*Nepeta cataria*) as a novel filth fly repellent. *Med. Vet. Entomol.* 23, 209-216.

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618 Table 1. Percentage escape of *Aedes albopictus* and *Anopheles dirus* exposed to serial doses of  $\beta$ -caryophyllene oxide and DEET in contact and  
 619 non-contact chambers.

Compound	Test	Dose (%)	<i>Aedes albopictus</i>				<i>Anopheles dirus</i>			
			Treatment		Control		Treatment		Control	
			N	% Esp <sup>(a)</sup>	N	% Esp	N	% Esp <sup>(a)</sup>	N	% Esp
$\beta$ -Caryophyllene oxide	C	0.1	60	3.51	60	5.00	60	0	60	8.33
		0.25	60	3.85	58	10.34	60	0	58	10.34
		0.5	60	46.43	61	8.20	62	26.32	61	6.56
		1.0	60	56.36	60	8.33	62	32.73	60	8.33
	NC	0.1	60	1.75	60	5.00	60	3.51	60	5
		0.25	60	1.79	60	6.67	60	1.79	60	6.67
		0.5	60	31.03	61	4.92	60	31.03	61	4.92
		1.0	60	25.45	60	8.33	60	31.67	60	0
DEET	C	0.1	60	0	60	8.33	60	3.57	60	5.08
		0.25	60	5.26	60	5	61	3.51	60	5.00
		0.5	60	38.98	60	1.67	60	16.98	59	11.67
		1.0	60	38.18	61	9.84	60	26.42	60	13.11
	NC	0.1	60	6.7	60	0	60	3.57	60	5.08
		0.25	60	0	58	6.9	60	1.79	58	6.90
		0.5	60	8.93	60	6.7	59	8.93	60	6.67
		1.0	60	10	60	0	60	1.82	60	8.33

620 C=contact; NC=non-contact; Esp= escaped mosquitoes; NE= Non escape mosquitoes; <sup>a</sup> Escape rates adjusted with paired controls using

621 Abbott's formula.



622 Table 2. Comparisons of mosquito escape responses between contact and non-contact  
 623 chambers for *Ae. albopictus* and *An. dirus* exposed to  $\beta$ -caryophyllene oxide and DEET.

Compound	Dose (%)	P-value	
		<i>Ae. albopictus</i>	<i>An. dirus</i>
$\beta$ -Caryophyllene oxide	0.1	0.7430	0.7274
	0.25	0.1510	0.7348
	0.5	0.0394*	0.7644
	1.0	0.0009*	0.5754
DEET	0.1	0.9902	0.9898
	0.25	0.4858	0.7063
	0.5	0.3908	0.1368
	1.0	<0.0001*	0.0011*

624 \* Indicates significant difference ( $P < 0.05$ ) between contact and non-contact.

625 Table 3. Comparisons of irritant and repellent actions between  $\beta$ -caryophyllene oxide and  
 626 DEET against *Ae. albopictus* and *An. dirus* in contact and non-contact chambers.

Compound	Dose (%)	P-value	
		Contact	Non-contact
<i>Ae. albopictus</i>	0.1	0.7473	0.9828
	0.25	0.2850	0.7496
	0.5	0.1272	0.1994
	1.0	0.0142*	0.0030*
<i>An. dirus</i>	0.1	0.9529	0.7945
	0.25	0.7748	0.7496
	0.5	0.5365	0.0193*
	1.0	0.7112	0.0030*

627 \* Indicates significant difference ( $P < 0.05$ ) between  $\beta$ -caryophyllene oxide and DEET.

628 Table 4. Comparisons of escape responses between *Ae. albopictus* and *An. dirus* in contact  
 629 and non-contact chambers treated with  $\beta$ -caryophyllene oxide and DEET.

Compound	Dose (%)	P-value	
		Contact	Non-contact
$\beta$ -Caryophyllene oxide	0.1	0.7374	0.7334
	0.25	0.2681	1.0000
	0.5	0.0184*	1.0000
	1.0	0.0024*	1.0000
DEET	0.1	0.9606	0.9606
	0.25	0.7495	1.0000
	0.5	0.0995*	0.0013*
	1.0	0.3584	1.0000

630 \* Indicates significant difference ( $P < 0.05$ ) between *Ae. albopictus* and *An. dirus*.

631 Table 5. *In vitro* cytotoxic and phototoxic activity of  $\beta$ -caryophyllene oxide against mouse  
632 normal fibroblast (BALB/c 3T3) cell lines.

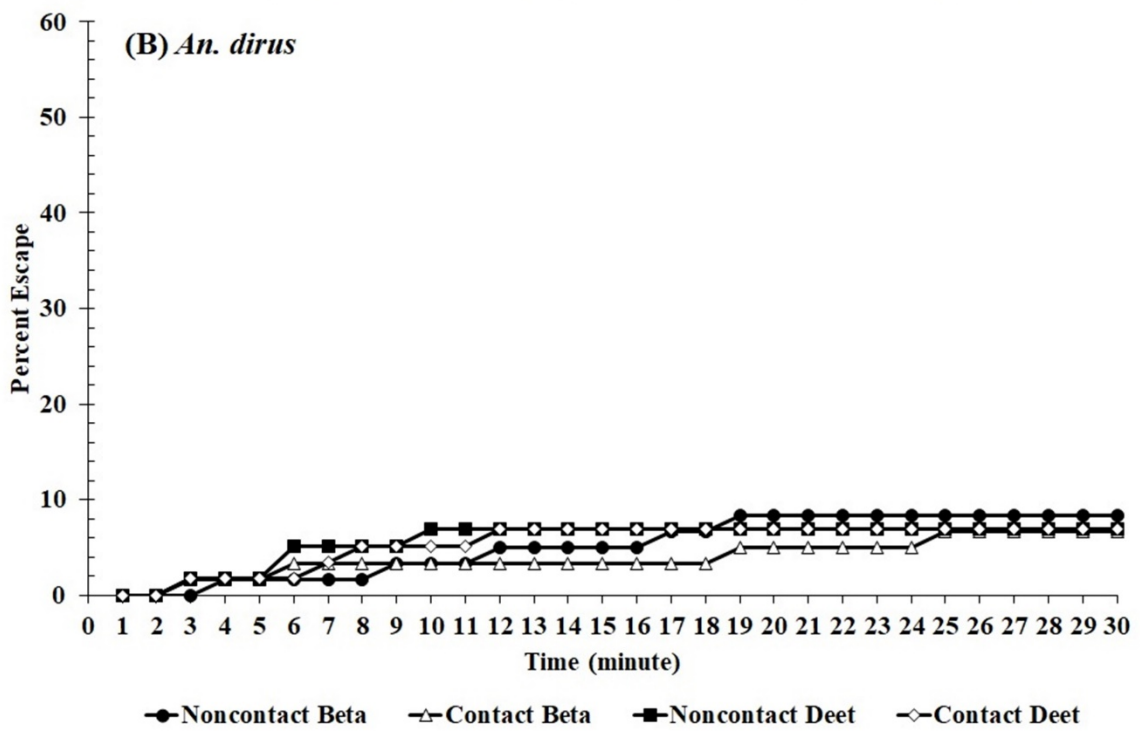
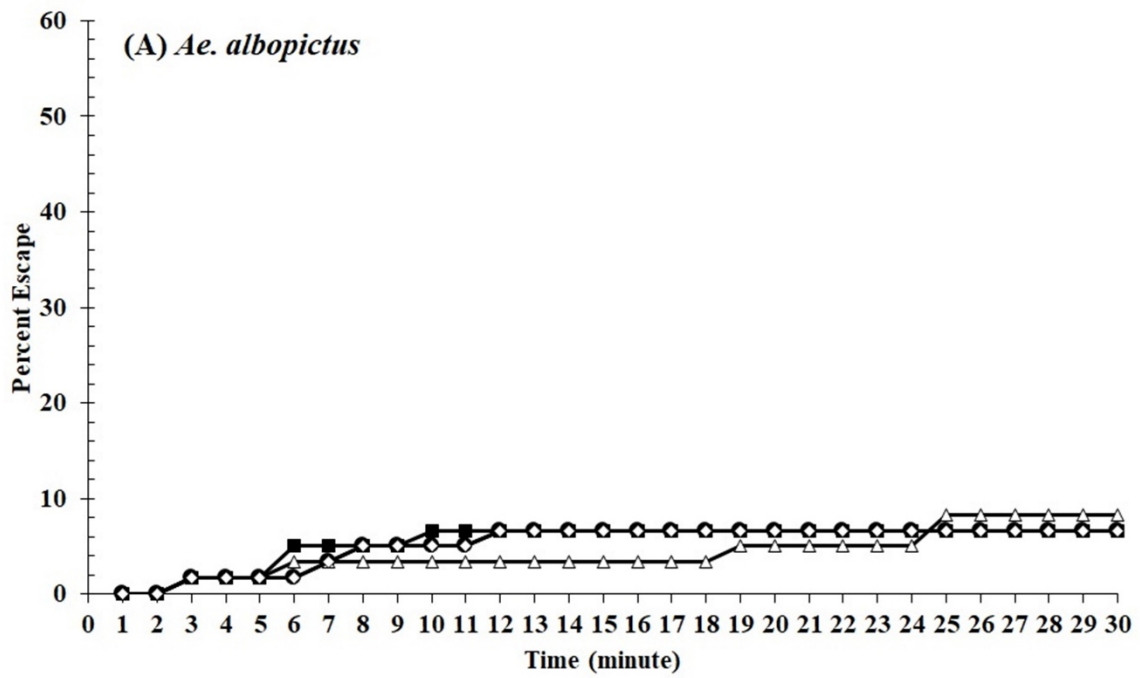
Compound	IC <sub>50</sub> without irradiation	IC <sub>50</sub> with irradiation	PIF	Phototoxicity
$\beta$ -Caryophyllene oxide	13.23 $\pm$ 1.37	34.79 $\pm$ 5.49	0.38	Non-phototoxic
Chlorpromazine	48.9 $\pm$ 3.26	1.05 $\pm$ 0.29	54.71	Phototoxic

633 Results are expressed as mean  $\pm$  SD

634 Table 6. *In vitro* genotoxicity activity of  $\beta$ -caryophyllene oxide on CHO-K1 cells.

Compound (% or $\mu\text{g.mL}^{-1}$ )	Assay performed without S9 mix			Assay performed with S9 mix			
	Proliferative Index (%)	MNC (per 1,000)	<i>P</i>	Proliferative Index (%)	MNC (per 1,000)	<i>P</i>	
Negative control	100	10.5 $\pm$ 0.7	-	100	10.5 $\pm$ 2.1	-	
Positive control <sup>§</sup>	98.2	31.5 $\pm$ 2.1	<0.001	97.6	24.0 $\pm$ 1.4	<0.001	
Solvent control	98.6	9.5 $\pm$ 0.7	NS <sup>a</sup>	98.4	10.0 $\pm$ 1.4	NS	
$\beta$ -caryophyllene oxide	0.5	99.7	10.5 $\pm$ 2.1	NS	98.8	9.5 $\pm$ 0.7	NS
	1	98.4	11.0 $\pm$ 1.4	NS	97.4	13.0 $\pm$ 2.8	NS
	5	65.1	9.5 $\pm$ 2.1	NS	73.2	11.0 $\pm$ 1.4	NS
	10	TOX	-	-	TOX	-	-

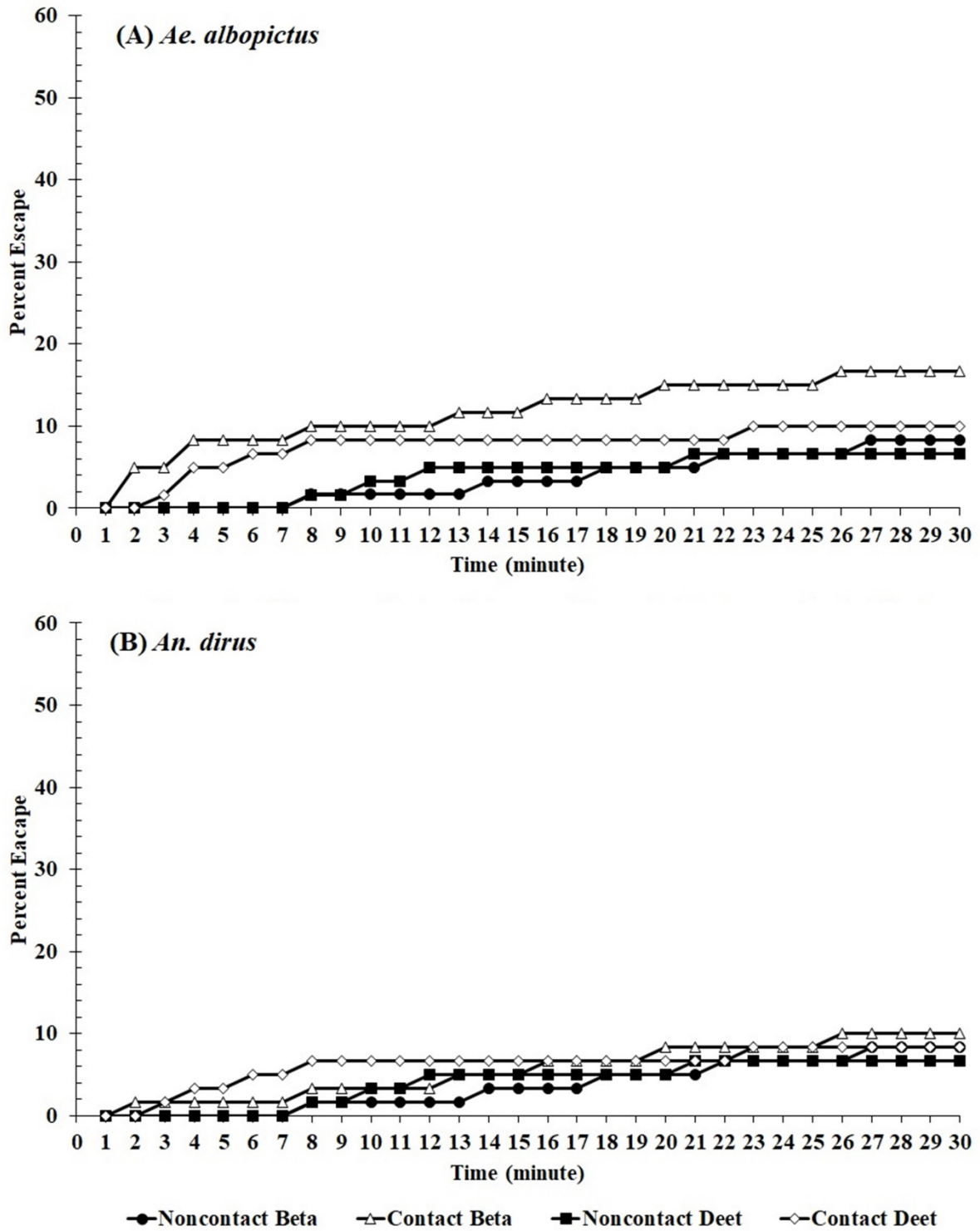
635 <sup>§</sup>Positive controls: mitomycin C (0.05  $\mu\text{g.mL}^{-1}$ ) without S9 mix and benzo-[a]-pyrene (5  
636  $\mu\text{g.mL}^{-1}$ ) with S9 mix; MNC: Micronucleated cells per 1,000; P: Probability of the comparison  
637 between the negative control and the tested dose using the Chi-squared test; TOX: Toxic.  
638 <sup>a</sup>NS: non-significant activity; Results are expressed as mean  $\pm$  SD



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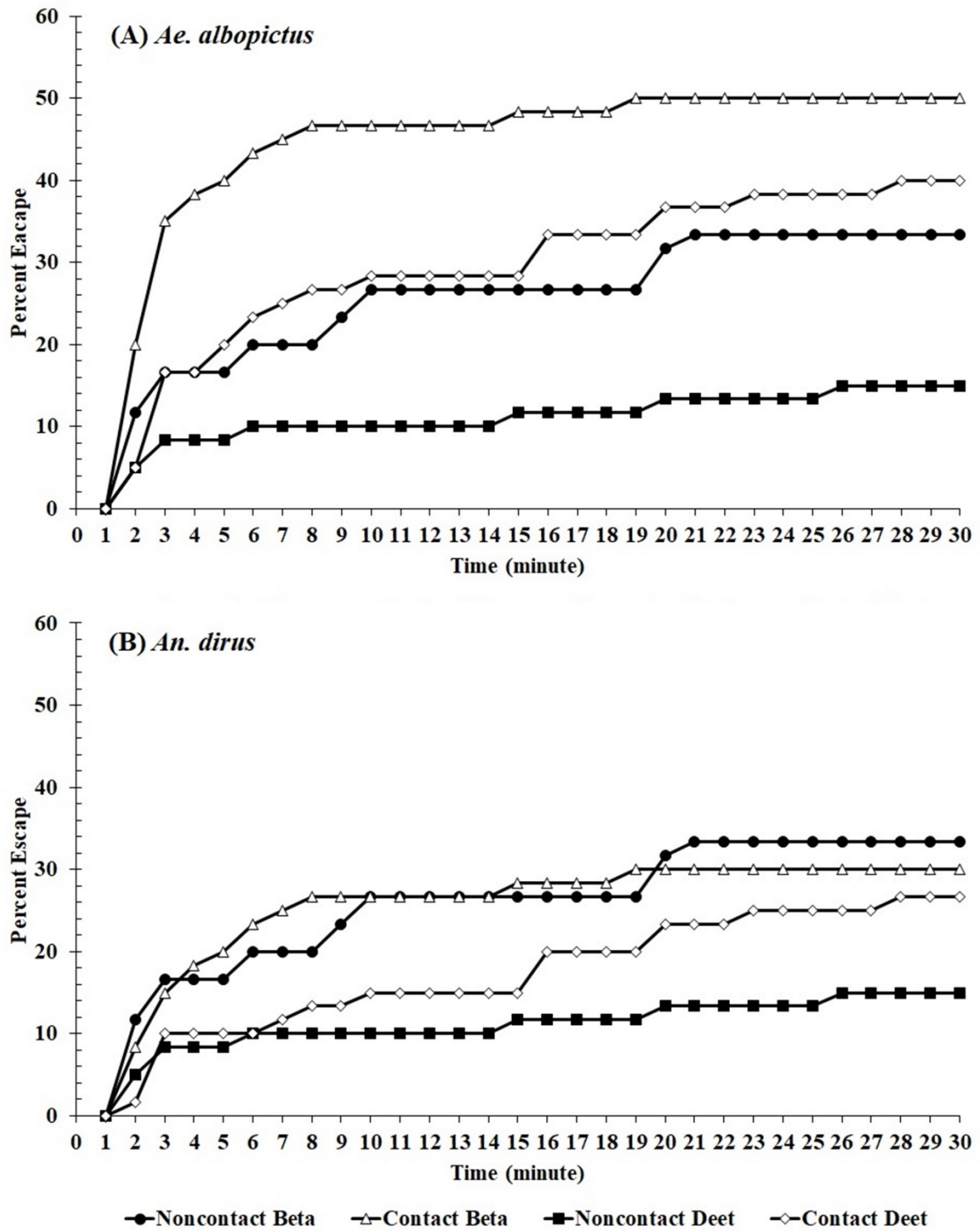
640 **Fig. 1** Patterns of escape percentage from ER chambers for contact and noncontact assay  
 641 designs during 30 min exposure to  $\beta$ -caryophyllene oxide and DEET at 0.1%: (A) *Ae.*  
 642 *albopictus*, (B) *An. dirus*.

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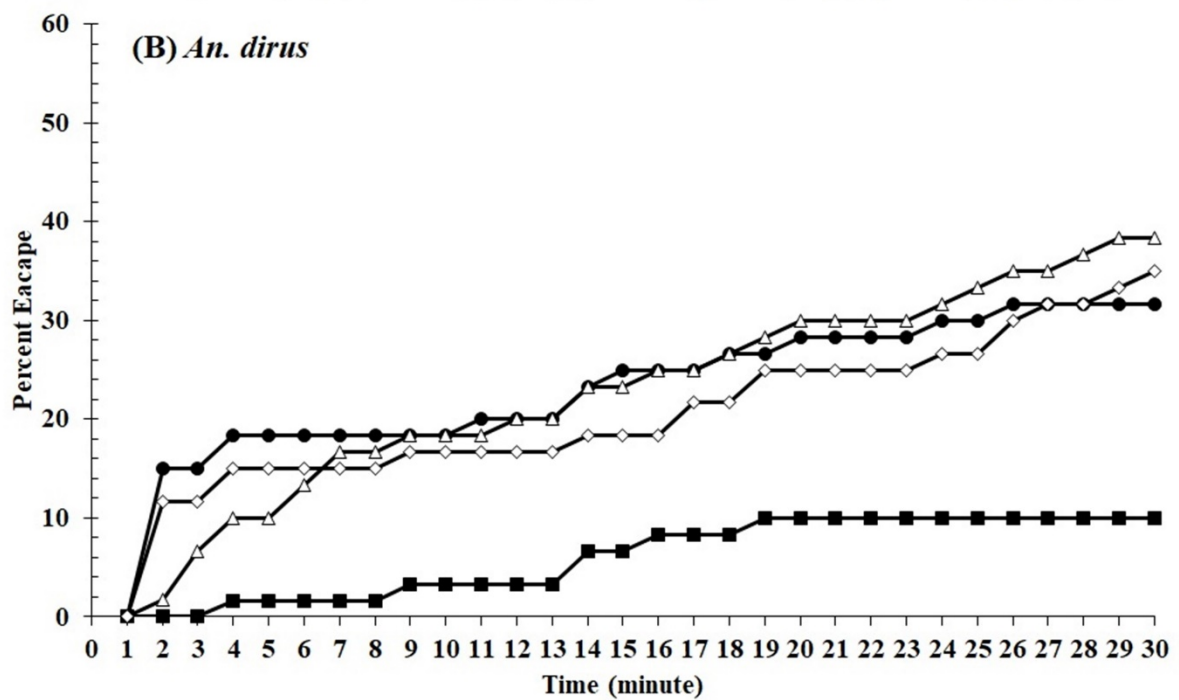
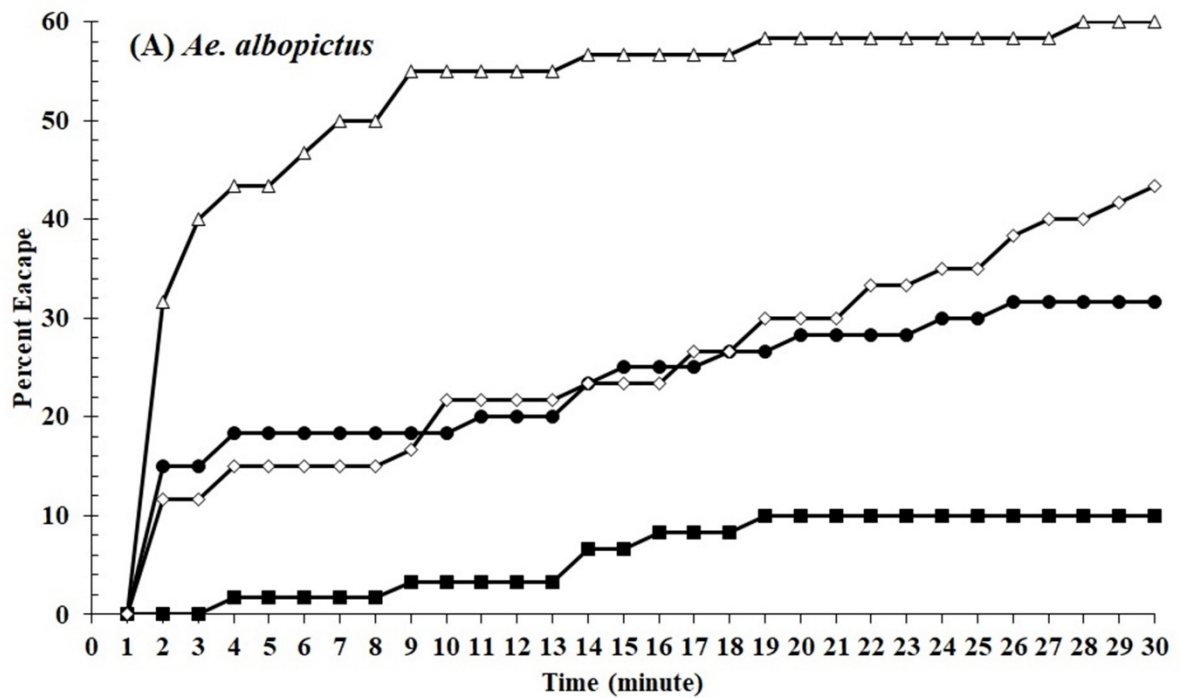
645 **Fig. 2** Patterns of escape percentage from ER chambers for contact and noncontact assay  
 646 designs during 30 min exposure to  $\beta$ -caryophyllene oxide and DEET at 0.25%: (A) *Ae.*  
 647 *albopictus*, (B) *An. dirus*



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649 **Fig. 3** Patterns of escape percentage from ER chambers under contact and noncontact assay  
 650 designs during 30 min exposure to  $\beta$ -caryophyllene oxide and DEET at 0.5%: (A) *Ae.*  
 651 *albopictus*, (B) *An. dirus*.





● Noncontact Beta    ▲ Contact Beta    ■ Noncontact Deet    ◇ Contact Deet

652

653 **Fig. 4** Patterns of escape percentage from ER chambers under contact and noncontact assay

654 designs during 30 min exposure to  $\beta$ -caryophyllene oxide and DEET at 1%: (A) *Ae. albopictus*,

655 (B) *An. dirus*.

656