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Highlights

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

Acta Tropica

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

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Abstract

The activity of β -caryophyllene oxide as either a contact or noncontact 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. β -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 β -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. *Aedes albopictus* was more sensitive to β -caryophyllene oxide than *An. dirus*. Moderate avoidance response rates (25-56% escape) of *Ae. albopictus* at 0.5% and 1.0% β -caryophyllene oxide were observed in contact and noncontact trials compared with low response rates from *An. dirus* (26-31% escape). DEET at $\leq 1\%$ displayed lower irritancy and repellency (1-38%) than β -caryophyllene oxide when tested against the two mosquito species. Knockdown responses (37%) were only observed in *An. dirus* exposed to 1% β -caryophyllene oxide in the contact trial. β -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. β -Caryophyllene oxide could be considered as a safe repellent, effective against mosquitoes.

Keywords: Excito-repellency test system, β -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 the least developed and developing countries are mainly at risk from these diseases. To a lower extent, now developed countries are too at risk of dengue outbreaks due to the invasion of *Aedes albopictus* (Skuse) into temperate regions and population movement from dengue-endemic areas (Vairo et al., 2018). In 2017, an estimated 219 million cases of malaria occurred worldwide, with an estimated 435,000 deaths from malaria globally (WHO, 2018). *Anopheles dirus* Peyton & Harrison is 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; Tananchai et al., 2012; 2019). In contrast, dengue and chikungunya are arboviral infections transmitted by two species of *Aedes* mosquitoes—*Ae. aegypti* L. and *Ae. albopictus*. 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 Europe (Chouin-Carneiro et al., 2016; Martinet et al., 2019). These two mosquito species have been found highly refractory to common control tools due to their highly exophagic behavior and are regarded as great potential vectors even though they are present in low population densities. Therefore, the prevention and control of most vector-borne diseases remain dependent on various vector control strategies to decrease the transmission risk, which is a major challenge for outdoor-biting mosquitoes such as these two *Aedes* species.

25 Among the available vector control tools, chemical methods remain the most used and
26 effective in combatting mosquito vectors. A number of chemical compounds can protect
27 humans from blood-feeding pests by one or more of three identified actions: contact irritancy,
28 noncontact spatial repellency, or toxicity (Grieco et al., 2007). The first two properties are
29 potential outcome behavioral responses of mosquitoes after or before they make tarsi contact
30 with treated surfaces (Chareonviriyaphap et al., 1997; Roberts et al., 1997). Most studies have
31 focused primarily on the insecticidal action of chemicals to control mosquitoes, whereas few
32 investigations have paid attention to the non-toxic properties, including irritancy and
33 repellency (Chareonviriyaphap et al., 1997; 2013; 2004; Grieco et al., 2007). Moreover, much
34 of the previous work has focused on potent synthetic compounds (Mongkalangoon et al., 2009;
35 Thanispong et al., 2009). The extensive use of these synthetic compounds has raised key
36 concerns over the selection pressure induced by insecticides on resistance gene mechanism
37 (Chareonviriyaphap et al. 2003). For example, the commonly used pyrethroids are applied to
38 control malaria mosquitoes but have become increasingly less effective due to the development
39 of physiological resistance in mosquito populations (Chareonviriyaphap et al., 2000; 2013).
40 Consequently, alternative plant-based repellents have been used to protect people, particularly
41 children, during outdoor activities. In addition, such alternative repellents cause no harm to
42 various nontarget organisms (with additional benefits as a potential source of bioactive
43 chemicals, fragrances and flavoring agents) and are recommended as an alternative source of
44 materials for insect control (Isman, 2002; Yang et al., 2005).

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

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

52 The compound tested in this study is β -caryophyllene oxide, generally found in
53 essential oils of various plant species such as *Artemisia anomala* S. Moore, *Salvia miltiorrhiza*
54 Bunge, *Chloroxylon swietenia* DC., *Psidium guajava* L., *Origanum vulgare* L., *Cinnamomum*
55 spp., *Syzygium aromaticum* (L.) Merr. & L.M. Perry, *Piper nigrum* L. and *Serjania*
56 *yucatanensis* Standl. (Garneau et al., 2013; Gertsch et al., 2008; Jun et al., 2011; Liang et al.,
57 2009; Polanco-Hernández et al., 2012; Shell, 1997; Telang et al., 2003; Zhao et al., 2013). The
58 interest of β -caryophyllene oxide is that it has been reported to repel mosquitoes. Suleiman et
59 al. (2014) reported that leaves of *Artabotrys hexapetalus* (L. f.) Bhandari contained large
60 amounts of β -caryophyllene oxide with high mosquito repellent activity against *Anopheles*
61 *gambiae* s.s. in Africa. A more recent study reported that *Aedes aegypti* and *Anopheles minimus*
62 exhibited high avoidance response rates at 0.5% and 1% concentrations of β -caryophyllene
63 oxide compared to DEET (Nararak et al., 2019), showing the high repellent potential of this
64 molecule.

65 In recent years, the food and cosmetic industries and both national and international
66 health and food safety authorities have extensively debated the safety of novel plants and plant-
67 derived ingredients for their use in foods. These discussions consistently produced a consensus
68 that adequate specifications of plant identity and composition are key issues in the safety
69 assessment of plant-derived ingredients (Antignac et al., 2011). Although β -caryophyllene
70 oxide is beneficial as a mosquito repellent, this compound may produce toxic and adverse
71 effects on humans or animals or both. Consequently, potential cytotoxic effects of hazardous
72 substances must be assessed prior to product development. Little scientific data is available on
73 the possible adverse effects or the biological safety of β -caryophyllene oxide in experimental
74 administration to animals. What has been reported is that based on a biomembrane model, β -

75 caryophyllene oxide could pass through the cell membrane without inducing genotoxic effects
76 at the gene or chromosomal level (Di Sotto et al., 2013).

77 In the current study, following the repellent study already done on *Ae. aegypti* and *An.*
78 *minimus* (Nararak et al., 2019), we investigated the active excito-repellent properties of β -
79 caryophyllene oxide against *Ae. albopictus* and *An. dirus*, using an excito-repellency (ER) test
80 system. The excito repellency (ER) test system is the gold standard to quantitatively determine
81 the two distinct forms of behavioral responses in mosquito population in response to test
82 chemicals (Sathantriphop et al., 2006). A test chamber system and protocol that can easily
83 differentiate these two types of behavioral responses has been described (Chareonviriyaphap
84 et al., 1997; Roberts et al., 1997). Moreover, in the current study, we evaluated the safety of
85 β -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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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.

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2.2 Test compounds

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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. β -Caryophyllene oxide was purchased from the Acros Organics Company Ltd. (95% purity, Lot No: A0356135).

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2.3 Filter paper treatment

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β -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 (14.7×17.5 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

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

113 **2.4 Excito-repellency test system**

114 Experiments were conducted to compare the behavioral responses of mosquitoes using
115 an excito-repellency test system described by Chareonviriyaphap et al. (2002). The test system
116 consisted of two pairs each for contact and noncontact exposure chambers. For a pair of contact
117 chambers, the treated papers were placed on the inside of a screened inner chamber so that
118 female mosquitoes could rest directly on the treated papers. Alternatively, a pair of noncontact
119 trials had treated papers outside the screened inner chamber, which prevented the female
120 mosquitoes from resting directly on the treated papers. After starvation for 24h, fifteen healthy,
121 non-bloodfed female mosquitoes were introduced into four chambers simultaneously using a
122 mouth aspirator and allowed 3 min to adjust to the chambers. Subsequently, the escape funnels
123 were opened to begin the observation period. The number of mosquitoes escaping from the
124 chamber into the receiving cage was recorded at 1 min intervals for a total of 30 min. After
125 the 30 min exposure period, the numbers of knockdown and dead mosquitoes were recorded
126 separately. All mosquitoes that either escaped to the receiving box or remained inside the
127 chamber were kept in clean plastic cups and provided with cotton pads soaked with 10% sugar
128 solution for 24h. The numbers of both knockdown and mortality were respectively recorded
129 after 30 min exposure and after 24h.

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

135

136 **2.5 Safety evaluation procedures for β -caryophyllene oxide**

137 In accordance with OECD (2004) guideline for the identification of efficacy of β -
138 caryophyllene oxide, the safety evaluation showed no prohibited or restricted components. For
139 safety reasons, the *in vitro* 3T3 NRU phototoxicity test (OECD N°432) was first conducted to
140 identify the toxicity of the test substance induced by chemicals after exposure to light. The
141 3T3 NRU phototoxicity test is based on the comparison of a chemical when tested in the
142 presence and in the absence of a non-cytotoxic dose of simulated solar light. Cytotoxicity is
143 expressed as a concentration-dependent reduction of the uptake of the vital dye Neutral Red
144 when measured 24 h after chemical treatment and irradiation.

145 Balb/c 3T3 mouse fibroblasts (3T3-L1) (ATCC, USA, ATCC® CL-173™, N°
146 P6110401, Lot. 09I006), low passage number (<50), maintained into DMEM (Dulbecco's
147 Minimum Essential Medium, PAN BIOTECH. lot 1874561) supplemented with penicillin 100
148 IU/mL, streptomycin 100 μ g/mL (PAN BIOTECH, Lot 945514) and 10% inactivated calf
149 serum (PAN BIOTECH, Lot P56314), were seeded into two 96-well tissue culture plates (0.1
150 mL per well) at concentration of 1×10^5 cells/mL, and incubated at 37°C (5% CO₂) for 24 h
151 until semi-confluent. At the end of the incubation period, the culture medium was decanted
152 and replaced by 100 μ L of Hank's balanced salt solution (HBSS) containing the appropriate
153 concentrations of the test substance (0, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, and 0.5 μ M).
154 Subsequently, cells were incubated at 37°C (5% CO₂) in the dark for 60 min. From the two
155 plates prepared for each series of test substance concentrations and the controls, one was
156 randomly selected for the determination of cytotoxicity without irradiation (-Irr), and the other
157 for the determination of photo-cytotoxicity with irradiation (+Irr). The irradiation procedure
158 was performed using a solar simulator Suntest CPS+ apparatus (Atlas Material Testing
159 Technology BV, Mousy le Neuf, France) equipped with a xenon arc lamp (1,100 W), a special
160 glass filter restricting transmission of light below 290 nm and a near IR-blocking filter. For

161 the +Irr, the irradiance was fixed at 750 W/m² throughout the experiments and the combined
162 light dose was 5 J/cm²/min based on UVA irradiation (0.41 J/cm²) or visible irradiation (4.06
163 J/cm²). The test solution was removed, cells were rinsed twice with 150 µL HBSS and
164 incubated for 18-22h in 0.1 mL of culture medium at 37°C (5% CO₂). Cells were washed,
165 placed into Neutral Red medium (50 µg/mL Neutral Red in complete medium) and incubated
166 for 3 h at 37°C, 5% CO₂. Then the medium was removed and cells were washed three times
167 with 0.2 mL of HBSS to remove any excess dye. The Neutral Red medium was removed and
168 destaining solution (50% ethanol, 1% acetic acid, 49% distilled water; 50 µL per well) was
169 added into each well. Then, the plates were shaken for 15-20 min at room temperature in the
170 dark. All the test samples and controls were run in triplicate in three independent experiments.
171 A positive control (chlorpromazine (SIGMA), final concentrations 1-100 µg/mL without
172 irradiation and 0.01 to 1 µg/mL with irradiation) and a negative control (HBSS) were included
173 in each set of experiments. Cell viability (increase Neutral Red uptake) was measured using a
174 fluorescence-luminescence reader (Infinite M200 Pro, TECAN). The optical density (OD) of
175 each well was read at 540 nm. The results obtained for wells treated with the test material were
176 compared to those of untreated control wells (HBSS, 100% viability) and converted to
177 percentage values. The concentrations were calculated for the test material causing a 50%
178 release of the preloaded Neutral Red without irradiation (IC₅₀ -Irr) and with irradiation (IC₅₀
179 +Irr) compared to the control culture using the Phototox Version 2.0 software (Federal Institute
180 for Risk Assessment, zebet@bfr.bund.d). The mean OD value of blank wells (containing only
181 Neutral Red desorbed solution) was subtracted from the mean OD value of three treated wells
182 (dilutions of the test material, positive control or HBSS). The percentages of cell viability were
183 calculated as:

184

185

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

186

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

188

$$189 \quad \text{PIF} = \frac{\text{IC50} (- \text{Irr})}{\text{IC50} (+ \text{Irr})}$$

190

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

193 Then, the *in vitro* micronucleus assay (MNvit) was used to detect the long-term toxicity
194 of each treatment chemical. The micronucleus assay is a mutagenicity assay which is based on
195 the detection of micronuclei (MNC) in the cytoplasm of interphase cells and allows the
196 detection of the cytogenetic activity of clastogenic and/or aneugenic compounds in the cell
197 culture (Johnson et al., 2010). The micronucleus assay was performed on a Chinese hamster
198 ovary cell line (CHO-K1; ATCC, USA) maintained in McCoy's 5A medium supplemented
199 with 1 mM glutamine, 100 unit/mL and 10 µg/mL of a mixture of penicillin and streptomycin,
200 respectively, and 10% of inactivated calf serum. The CHO-K1 cells were transferred into
201 Labteck wells at a concentration of 100,000 cells/mL, and incubated for 24 h at 37°C in CO₂
202 (5%). The test without metabolic activation was performed where, the test substance was
203 added into cell cultures at the concentrations previously defined. A negative control containing
204 culture medium, a solvent control containing 1% DMSO and a positive control containing 0.6
205 µg/mL of mitomycin C were also run.

206 When the assay was performed in the presence of metabolic activation, S9 mix
207 metabolizing mixture was added to cell cultures at a concentration of 10%. The metabolic
208 activation system (S9) was a 9,000 g centrifuged supernatant of a liver homogenate (S9) and
209 was prepared from male Sprague-Dawley rats treated with a single injection of Aroclor 1254
210 (500 mg/kg body weight). The protein concentration in the S9 homogenate was 26 mg/ mL as

211 determined by the method of Lowry. The S9 mix contained 10% S9, 5 mM G6P, 4 mM NADP,
212 33 mM KCl, and 8 mM MgCl₂ diluted in saline phosphate buffer. Then, the test substance was
213 added to the cell cultures at the concentrations previously defined. A negative control
214 containing culture medium, a solvent control containing 1% DMSO and a positive control
215 containing 5 µg/mL of benzo[a]pyrene were added.

216 After 3 h of incubation at 37 °C in CO₂ (5%) the culture medium of both with (S9) and
217 without (-S9) metabolic activation assays were removed, the cells were rinsed with phosphate
218 buffered saline (PBS), and then returned to culture in McCoy's 5A medium containing 3 µg/mL
219 of cytochalasin B. After incubation for 21 h at 37°C, the cells were rinsed with PBS, fixed
220 with methanol and stained with 10% Giemsa for 20 min.

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

226

$$227 \quad \text{CBPI} = \frac{2 \times (\text{BI} + \text{MONO})}{500}$$

228

229 BI is the number of binucleated cells and MONO is the number of mononucleated cells

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

232

$$233 \quad \text{CI}\% = 100 - \left(\frac{100 \times (\text{CBPI}_{\text{test material}} - 1)}{\text{CBPI}_{\text{solvent control}} - 1} \right)$$

234

235 After this step, only the doses inducing a decrease of less than 55 ± 5 CI% compared to
236 the negative control were considered for counting micronuclei. The rates of micronuclei were
237 evaluated for the presence of independent nuclear core entities in 1,000 binucleated cells per
238 well, which corresponded to 2,000 cells examined per test substance dose. Micronuclei were
239 stained in the same manner and identified as small nuclei well-differentiated from the cell
240 nucleus, having a diameter less than one-third of that of the cell nucleus. The micronuclei rates
241 obtained for different doses of test substances were compared to the negative control using a
242 χ^2 test. The assay was considered positive if a dose-response relationship was obtained
243 between the rate of micronuclei and the doses tested, where at least one of these doses induced
244 a statistically significant increase ($P < 0.05$) in the number of micronucleated cells compared
245 to the negative control.

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3. Results

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Excito-repellency responses of *Ae. albopictus* and *An. dirus* exposed to 0.1, 0.25, 0.5, 1.0% v/v of DEET and β -caryophyllene oxide were evaluated for contact irritancy and noncontact repellency responses using an excito-repellency system. DEET was used as a standard repellent for comparison purposes. The results showed that *Ae. albopictus* (3.51-56.36%) had a much higher escape response to β -caryophyllene oxide than *An. dirus* (0-32.73%) in both the contact and noncontact trials (Table 1).

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The escape responses of *Ae. albopictus* to β -caryophyllene oxide were characterized in contact and noncontact exposure chambers by comparison to DEET (Table 1). β -Caryophyllene oxide at 0.5 and 1.0% elicited stronger escape responses than DEET in the contact and noncontact treatments. No knockdown and mortality were observed from treatments and control chambers at all concentrations. The greater escape response percentages of *Ae. albopictus* were observed at 0.5 and 1.0% in the contact trial with β -caryophyllene oxide (46.43 and 56.36%, respectively) and DEET (38.98 and 38.18% respectively). In noncontact trial, however, the low percentage responses were 31.03 and 25.45% for β -caryophyllene oxide and were 8.93 and 10% for DEET at 0.5 and 1.0%, respectively. For *An. dirus*, the stronger escape responses were found at 0.5 and 1.0%, in both contact (26.32 and 32.73% respectively) and noncontact trial (31.03 and 31.67%, respectively) as shown in Table 1. A knockdown response (37%) was observed in *An. dirus* exposed to 1% β -caryophyllene oxide in the contact trial.

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

271 contact and noncontact trials at all concentrations. Delayed escape responses were observed
272 for 0.1 and 0.25% with all compounds in the contact and noncontact trials (Figures 1-2). With
273 *Ae. albopictus*, strong contact escape patterns were evident for 0.5 and 1.0% β -caryophyllene
274 oxide (Figures 3-4).

275 The multiple log-rank comparisons of *Ae. albopictus* and *An. dirus* in the paired contact
276 and noncontact treatment trials for β -caryophyllene oxide and DEET are shown in Table 2.
277 Interestingly, there were no significant differences in escape patterns for all contact *versus*
278 noncontact trials, except at the concentrations of 0.5% and 1.0%, which were statistically
279 significant in escape patterns for contact *versus* noncontact trials for *Ae. albopictus* (Table 2).

280 The statistical comparison of the escape responses for mosquitoes exposed to β -
281 caryophyllene oxide compared to DEET, at different concentrations, are presented in Table 3.
282 In both the contact and noncontact trials using *Ae. albopictus*, the escape responses were
283 significantly higher with β -caryophyllene oxide than DEET at 1.0% concentration. For *An.*
284 *dirus*, the escape responses were significantly higher with β -caryophyllene oxide than DEET
285 in the noncontact trials at 0.5 and 1%. The statistical comparisons between the two species
286 exposed to β -caryophyllene oxide and DEET in either the contact or noncontact trials are
287 shown in Table 4. For β -caryophyllene oxide, *Ae. albopictus* showed significantly higher
288 escape responses compared to *An. dirus* in the contact trials at 0.5% and 1.0% ($P = 0.0184$ and
289 0.0024, respectively), while significance differences in the escape responses were found at
290 0.5% in both contact and noncontact trials using DEET.

291 Overall, a higher percent escape reaction was observed when *Ae. albopictus* and *An.*
292 *dirus* were tested on β -caryophyllene oxide when compared to DEET at 0.5-1%. A greater
293 escape response was seen from the contact chamber than that from the noncontact chamber and
294 control, regardless of test compounds and concentrations.

295 The cytotoxic potential of β -caryophyllene oxide was evaluated in murine fibroblasts
296 (3 T3) incubated with the compound (0.1-10 mg/mL). The compound exhibited negligible
297 cytotoxicity ($IC_{50} = 13.23 \pm 1.37 \mu\text{g/mL}$) as shown in Table 5. PIF values were used to classify
298 the phototoxicity potential (Table 5). The phototoxicity assay considered dark ($IC_{50} =$
299 $13.23 \pm 1.37 \mu\text{g/mL}$) and irradiated ($IC_{50} = 34.79 \pm 5.49 \mu\text{g/mL}$) conditions. According to the
300 analyses, β -caryophyllene oxide did not exhibit phototoxic potential (PIF = 0.368) for the dose
301 levels tested.

302 Genotoxicity was assayed starting from the highest concentration at which neither
303 necrosis nor cytotoxic or cytostatic effects was observed. When tested on the Chinese hamster
304 ovary (CHO)-K1 cell line, β -caryophyllene oxide did not produce any cytotoxic effects up to
305 a concentration of 5 $\mu\text{g/mL}$. At concentrations of 0.5, 1.0 and 5.0 $\mu\text{g/mL}$, β -caryophyllene
306 oxide did not increase the MNC (micronucleated cells) frequency with respect to the control
307 (Table 6). No concentration of β -caryophyllene oxide induced an increase in MNC with or
308 without metabolic activation. These results indicated that the compound was not derived from
309 clastogenic/aneugenic activity and did not produce clastogenic/aneugenic metabolites (Table
310 6).

311

4. Discussion

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

323 Nararak et al. (2016; 2017) reported that essential oils of citronella, vetiver, hairy basil,
324 and kaffir lime had good irritant and repellent effects on mosquito vectors compared to DEET.
325 These studies suggested that plant-based substances have good potential efficacy to be
326 alternative insect repellents. In the current study, mosquitoes displayed varying levels of
327 behavioral escape responses to the β -caryophyllene oxide, indicating a clear dose response with
328 different concentrations. The study showed that at higher concentrations (0.5 and 1.0%), β -
329 caryophyllene oxide had significantly greater repellent and irritant effects compared to DEET.
330 Moreover, *Ae. albopictus* exhibited much stronger escape responses against β -caryophyllene
331 oxide than *An. dirus* for both contact and noncontact assays. Knockdown (37.83%) was also
332 found only in non-escape *An. dirus* mosquitoes in the contact trial at 1.0% concentration of β -
333 caryophyllene oxide, whereas no knockdown and mortality at 24 h post-exposure were
334 observed for *Ae. albopictus*. The results in contact and noncontact trials indicated that escape
335 responses of mosquitoes to the β -caryophyllene oxide were significantly greater than with

336 DEET, similar to the previous study by Nararak et al. (2019) in which β -caryophyllene oxide
337 was tested at concentrations of 0.1, 0.25, 0.5 and 1.0% (v/v) against *Ae. aegypti* and *An.*
338 *minimus* and the results were compared to DEET at the same concentrations. The results
339 showed that DEET displayed lower irritancy and repellent responses than β -caryophyllene
340 oxide and *An. minimus* exhibited higher avoidance response rates (86–96% escape) at 0.5%
341 and 1.0% concentrations in contact and noncontact trials compared with *Ae. aegypti* (22–59%
342 escape). When comparing the results obtained with the four mosquito species tested with β -
343 caryophyllene oxide, *An. minimus* presented the highest sensitivity to both types of escape
344 responses (contact irritancy and noncontact-spatial repellency) at 0.5-1% (v/v), followed by
345 *Ae. aegypti*, *Ae. albopictus*, and *An. dirus*. Comparatively, DEET was less efficient than β -
346 caryophyllene oxide at 0.5-1% (v/v) for all 4 species as they presented lower escape responses
347 in both trials, contact irritancy and noncontact-spatial repellency.

348 β -Caryophyllene oxide has been approved as a food additive by the Food and Drug
349 Administration (FDA) and the European Food Safety Authority (EFSA) (Fidyt et al., 2016).
350 The two most common plant-associated repellent compounds are p-menthane-3,8-diol (derived
351 from the Australian lemon-scented gum tree) and picaridin (a synthetic derivative of pepper);
352 these have been tested for toxicity (U.S. EPA Biopesticide Registration Documents 011550
353 and 7505C) (EPA, 2019; Zhu et al., 2009). Phototoxic potential is assessed by comparing the
354 differences in IC_{50} between negative control plates (not exposed to UVA) and test plates
355 (exposed to UVA) (Roesler et al., 2010). The phototoxicity results obtained using the *in vitro*
356 method are crucial because topical repellent formulations are mainly used during the day to
357 protect against day-biting mosquitoes such as *Aedes* species, and this involves exposure to the
358 sun and artificial light. The current study investigated the cytotoxicity and phototoxicity of β -
359 caryophyllene oxide and showed that the compound was neither cytotoxic, nor phototoxic
360 (PIF=0.38). The potential genotoxic effects from β -caryophyllene oxide have also been

361 evaluated in mammalian cells. Likewise, the current study showed that β -caryophyllene oxide
362 did not induce genotoxicity at the chromosomal level, as observed in the micronucleus assay.
363 Di Sotto et al. (2013) reported that β -caryophyllene oxide was tested for mutagenic effects
364 using the Ames test and micronucleus assay. Their results showed that the flavoring agent was
365 devoid of mutagenic effects, both at the gene level (frameshift or based-substitution mutations)
366 and on chromosomes (clastogenicity and aneuploidogenicity), suggesting that β -caryophyllene
367 oxide is also safe when used as a flavoring/fragrance ingredient. Then, this finding highlights
368 the fact this component will be safe for human topical applications.

369 β -Caryophyllene oxide is a bicyclic sesquiterpene generated from the oxidation of β -
370 caryophyllene and is found in a large number of plants worldwide (Fidyt et al., 2016). β -
371 Caryophyllene oxide appears to be common among the essential oils that exhibit mosquito
372 repellent ability (Jaenson et al., 2006; Trongtokit et al., 2005). *Artabotrys hexapetalus* (L.f.)
373 Bhandari oil, obtained from leave parts, contains β -caryophyllene oxide as one of its major
374 constituents and displays strong repellent activity against females of *An. gambiae* (Suleiman et
375 al., 2014). Strong repellency against *An. gambiae* was also reported from a combination of
376 linalool, β -caryophyllene oxide, γ -terpinene, and 1-methylpyrrole (45:39:8:8), and essential oil
377 of *Croton pseudopulchellus* Pax (Odalo et al., 2005). Moreover, (-)-caryophyllene oxide and
378 (-)-limonene are the major chemical constituents found in essential oil extracted from the leaves
379 of *Perilla frutescens* (L.) Britton, and provide good biting-deterrent activity (Tabanca et al.,
380 2015). One study examining the repellent effect on the olfactory system of *Cimex lectularius*
381 (Bed Bug) antennal sensilla neurons showed that (-)-caryophyllene oxide produces a strong
382 neuronal response on *Da* sensilla (82 spikes/s) (Liu et al., 2014). Li et al. (2019) discovered
383 that the essential oils from *Sauaaurea amara* (L.) DC. and *Sigesbeckia pubescens* Makino were
384 analyzed for their chemical composition by Gas Chromatography-Mass Spectrometry
385 (GC-MS) and their repellent activities against adults of the red flour beetle, *Tribolium*

386 *castaneum* Herbst, and the booklouse, *Liposcelis bostrychophila* Badonnel. Results of GC-MS
387 analysis indicated that both essential oils were characterized by high content of caryophyllene
388 oxide (Synonyms: β -caryophyllene oxide) and exerted beneficial repellent effects on *T.*
389 *castaneum* and *L. bostrychophila* at 2 and 4 h post-exposure, respectively. These works
390 confirmed the potent repellent efficacy of β -caryophyllene oxide for controlling insects and
391 suggested their potential to be developed into botanical repellents.

392 Based on these results, β -caryophyllene oxide had stronger repellent and irritant effects
393 than DEET on four vector species at the same concentrations, suggesting the former has high
394 potential for further development as an alternative active ingredient in mosquito repellent
395 formulations, safe for humans and the environment. Such efficient repellent products are in
396 need. In a study done in Vietnam, forest-goers were in favor of using repellent products to
397 avoid mosquito-biting pressure (Ohrt et al., 2018). The use of repellent is a complementary
398 vector control method to standard ones such as bednets, long-lasting insecticidal nets (LLINs),
399 indoor residual spraying (IRS), which has the advantage of repelling outdoor-biting
400 mosquitoes, then reducing residual malaria transmission risk (Durnez and Coosemans, 2013).
401 Then, appropriate formulations using β -caryophyllene oxide await development for its use as
402 repellent for a safe protection against mosquito bites.

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410

411 **Conflict of interest**

412 The authors declare no conflict of interest.

413

414 **Author Contributions**

415 TC conceived and designed the experiments. JN, CS and CG performed the
416 experiments. JN, CS and CG analyzed the data. JN, CS and CG wrote the manuscript. SM,
417 VML, EO and TC consulted and edited the manuscript. All authors read and approved the
418 manuscript.

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601 Table 1. Percentage escape of *Aedes albopictus* and *Anopheles dirus* exposed to serial doses of β -caryophyllene oxide and DEET in contact and
 602 noncontact chambers.

Compound	Test	Dose (%)	<i>Aedes albopictus</i>								<i>Anopheles dirus</i>							
			Treatment		Control		% Knockdown after 30 min exposure				Treatment		Control		% Knockdown after 30 min exposure			
							Treatment		Control						Treatment		Control	
			N	% Esp ^(a)	N	% Esp	Esp	NE	Esp	NE	N	% Esp ^(a)	N	% Esp	Esp	NE	Esp	NE
β -Caryophyllene oxide	C	0.1	60	3.51	60	5.00	0	0	0	0	60	0	60	8.33	0	0	0	0
		0.25	60	3.85	58	10.34	0	0	0	0	60	0	58	10.34	0	0	0	0
		0.5	60	46.43	61	8.20	0	0	0	0	62	26.32	61	6.56	0	0	0	0
		1.0	60	56.36	60	8.33	0	0	0	0	62	32.73	60	8.33	0	37	0	0
	NC	0.1	60	1.75	60	5.00	0	0	0	0	60	3.51	60	5	0	0	0	0
		0.25	60	1.79	60	6.67	0	0	0	0	60	1.79	60	6.67	0	0	0	0
		0.5	60	31.03	61	4.92	0	0	0	0	60	31.03	61	4.92	0	0	0	0
		1.0	60	25.45	60	8.33	0	0	0	0	60	31.67	60	0	0	0	0	0
DEET	C	0.1	60	0	60	8.33	0	0	0	0	60	3.57	60	5.08	0	0	0	0
		0.25	60	5.26	60	5	0	0	0	0	61	3.51	60	5.00	0	0	0	0
		0.5	60	38.98	60	1.67	0	0	0	0	60	16.98	59	11.67	0	0	0	0
		1.0	60	38.18	61	9.84	0	0	0	0	60	26.42	60	13.11	0	0	0	0
	NC	0.1	60	6.7	60	0	0	0	0	0	60	3.57	60	5.08	0	0	0	0
		0.25	60	0	58	6.9	0	0	0	0	60	1.79	58	6.90	0	0	0	0
		0.5	60	8.93	60	6.7	0	0	0	0	59	8.93	60	6.67	0	0	0	0
		1.0	60	10	60	0	0	0	0	0	60	1.82	60	8.33	0	0	0	0

603 C=contact; NC=noncontact; Esp= escaped mosquitoes; NE= Non escape mosquitoes; ^a Escape rates adjusted with paired controls using Abbott's
 604 formula.

605 Table 2. Comparisons of mosquito escape responses between contact and noncontact
 606 chambers for *Ae. albopictus* and *An. dirus* exposed to β -caryophyllene oxide and DEET.

Compound	Dose (%)	<i>P</i> -value	
		<i>Ae. albopictus</i>	<i>An. dirus</i>
β -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*

607 * Indicates significant difference ($P < 0.05$) between contact and noncontact.

608 Table 3. Comparisons of irritant and repellent actions between β -caryophyllene oxide and
 609 DEET against *Ae. albopictus* and *An. dirus* in contact and noncontact chambers.

Compound	Dose (%)	<i>P</i> -value	
		Contact	Noncontact
<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*

610 * Indicates significant difference ($P < 0.05$) between β -caryophyllene oxide and DEET.

611 Table 4. Comparisons of escape responses between *Ae. albopictus* and *An. dirus* in contact
 612 and noncontact chambers treated with β -caryophyllene oxide and DEET.

Compound	Dose (%)	<i>P</i> -value	
		Contact	Noncontact
β -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

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

614 Table 5. *In vitro* cytotoxic and phototoxic activity of β -caryophyllene oxide against mouse
615 normal fibroblast (BALB/c 3T3) cell lines.

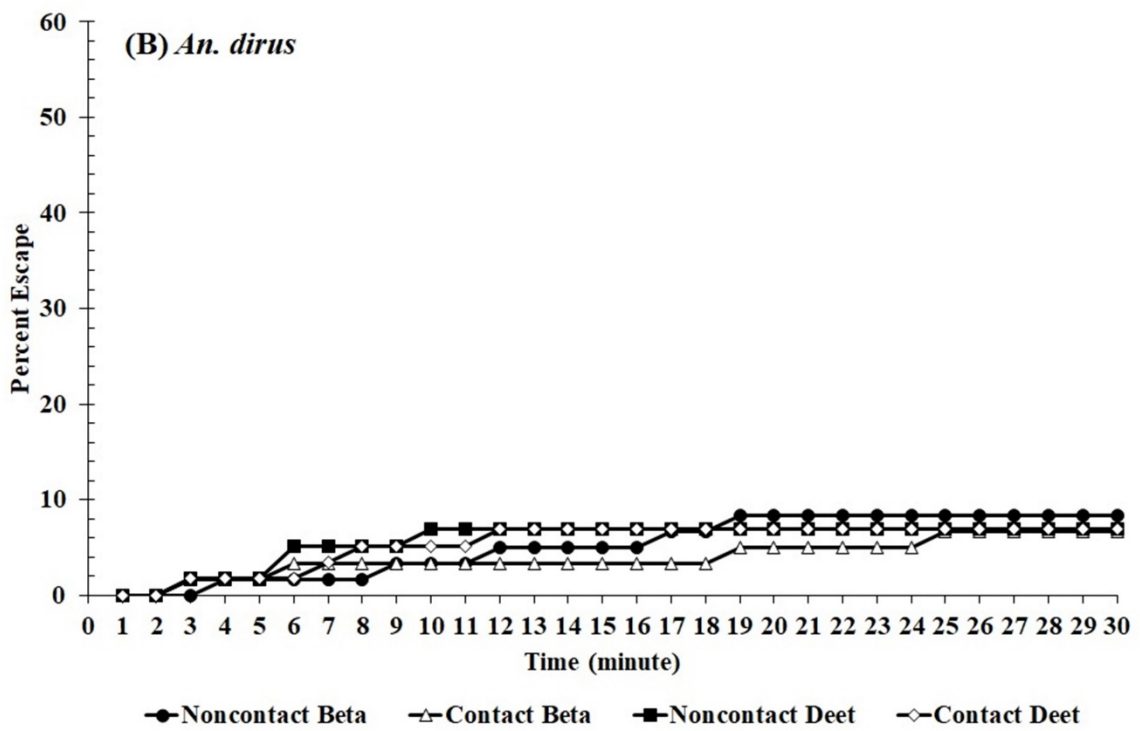
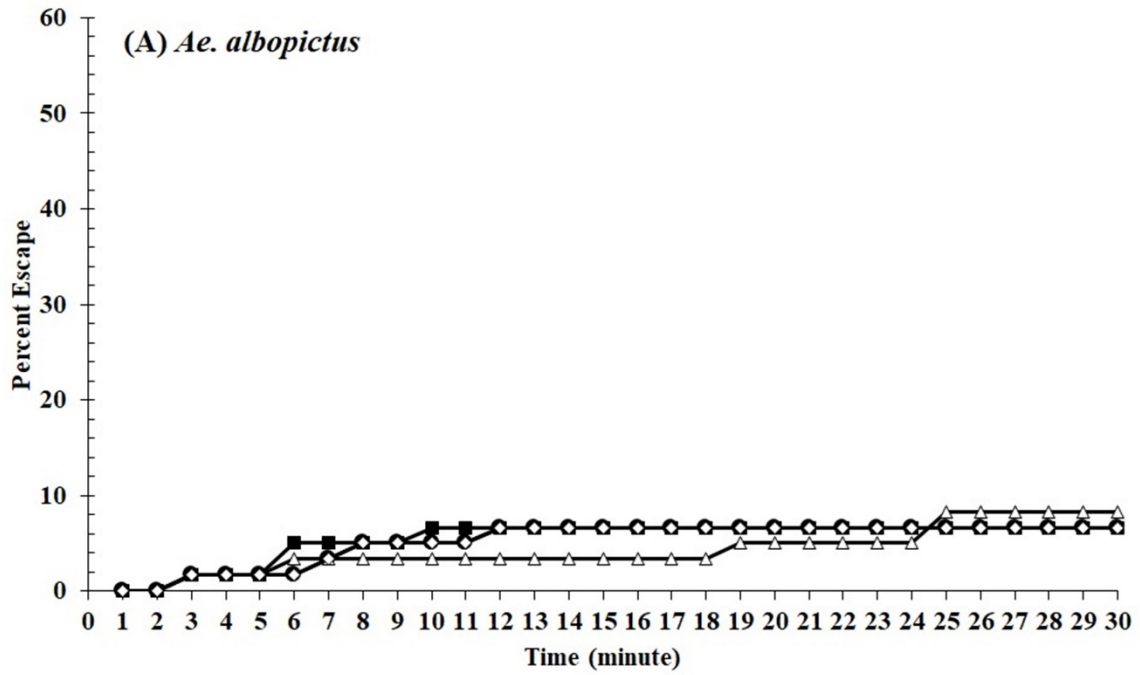
Compound	IC ₅₀ without irradiation	IC ₅₀ with irradiation	PIF	Phototoxicity
β -Caryophyllene oxide	13.23 \pm 1.37	34.79 \pm 5.49	0.38	Non-phototoxic
Chlorpromazine	48.9 \pm 3.26	1.05 \pm 0.29	54.71	Phototoxic

616 Results are expressed as mean \pm SD

617 Table 6. *In vitro* genotoxicity activity of β -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 [§]	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 ^a	98.4	10.0 \pm 1.4	NS	
β -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	-	-

618 [§]Positive controls: mitomycin C (0.05 $\mu\text{g.mL}^{-1}$) without S9 mix and benzo-[a]-pyrene (5
619 $\mu\text{g.mL}^{-1}$) with S9 mix; MNC: Micronucleated cells per 1,000; P: Probability of the comparison
620 between the negative control and the tested dose using the Chi-squared test; TOX: Toxic.
621 ^aNS: non-significant activity; Results are expressed as mean \pm SD



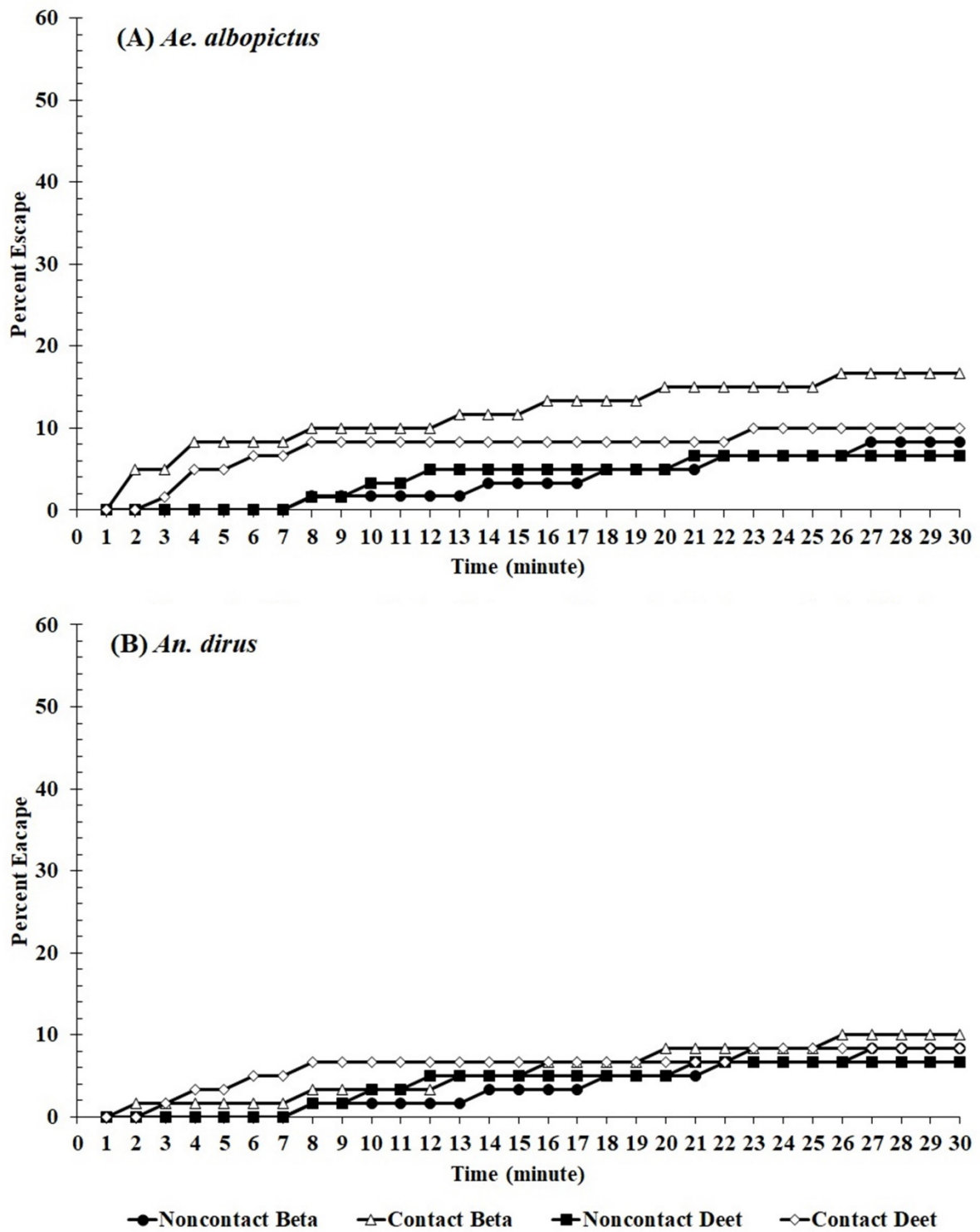
622

623 **Fig. 1** Patterns of escape percentage from ER chambers for contact and noncontact assay

624 designs during 30 min exposure to β -caryophyllene oxide and DEET at 0.1%: (A) *Ae.*

625 *albopictus*, (B) *An. dirus*.

626

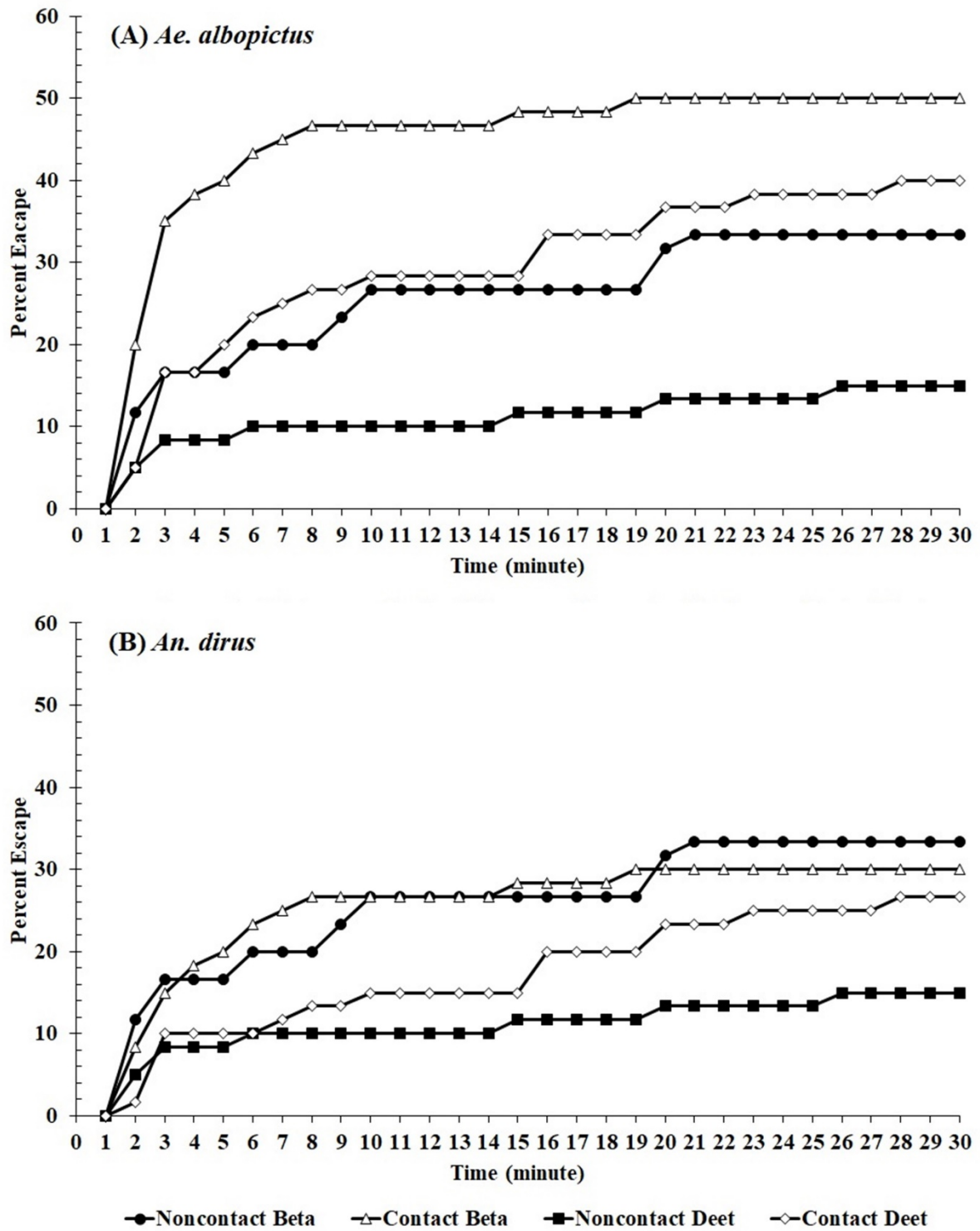


627

628 **Fig. 2** Patterns of escape percentage from ER chambers for contact and noncontact assay

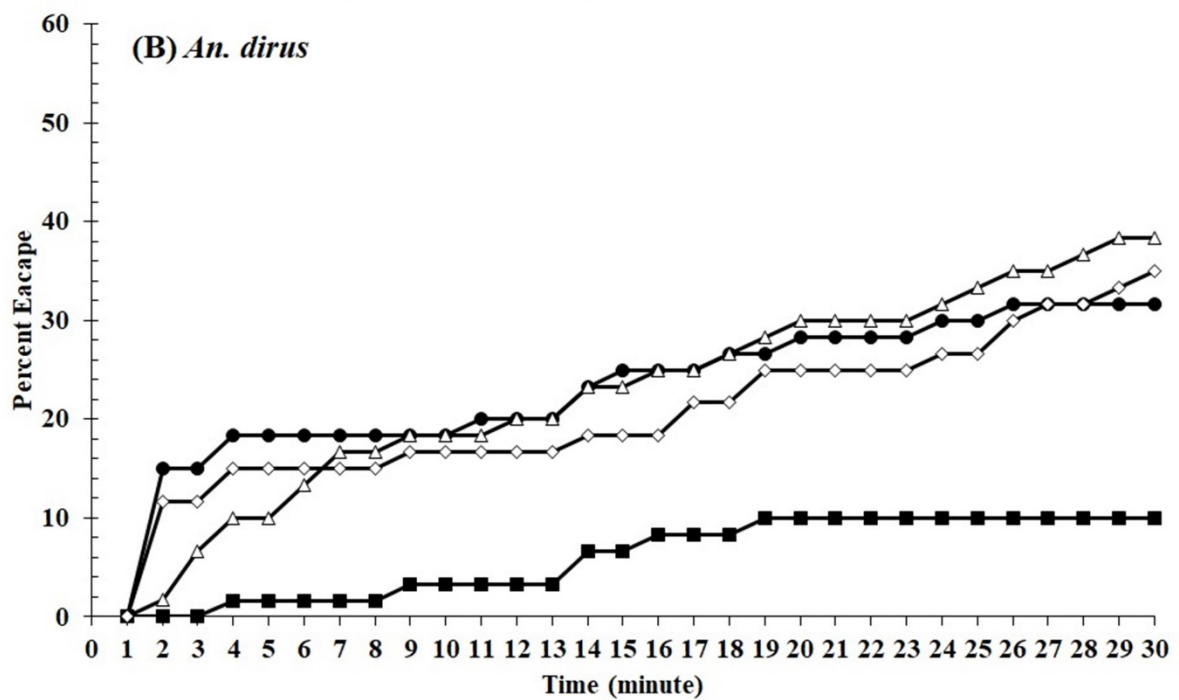
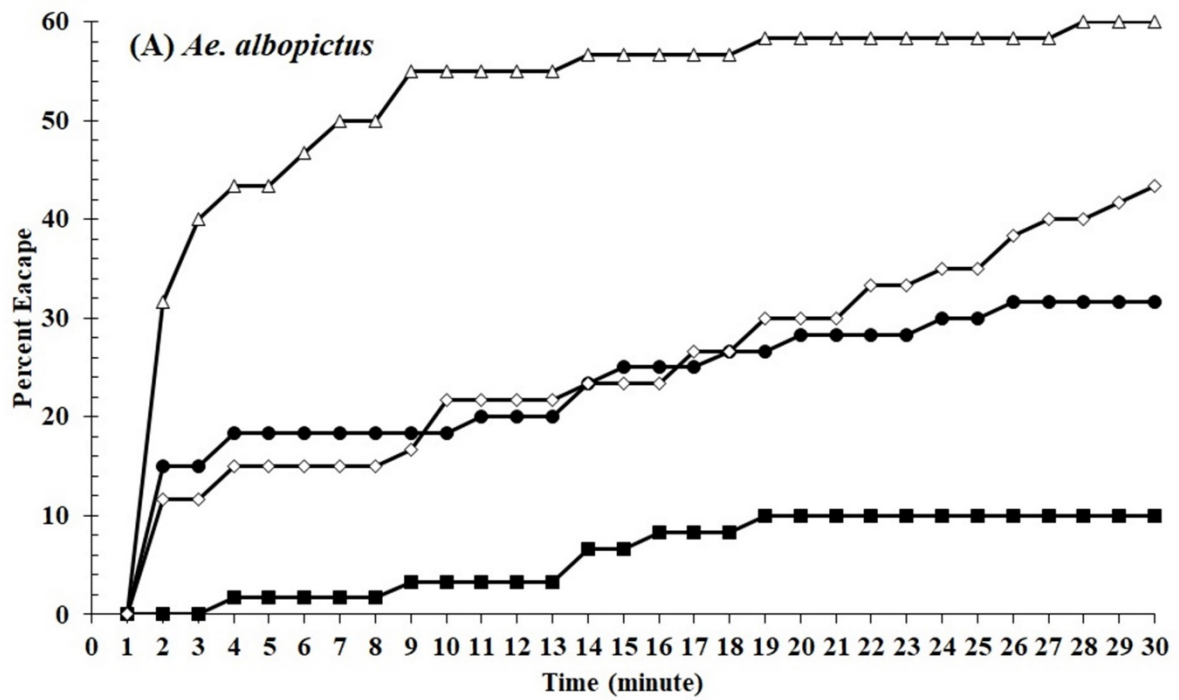
629 designs during 30 min exposure to β -caryophyllene oxide and DEET at 0.25%: (A) *Ae.*

630 *albopictus*, (B) *An. dirus*



631

632 **Fig. 3** Patterns of escape percentage from ER chambers under contact and noncontact assay
 633 designs during 30 min exposure to β -caryophyllene oxide and DEET at 0.5%: (A) *Ae.*
 634 *albopictus*, (B) *An. dirus*.



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

635

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

637 designs during 30 min exposure to β -caryophyllene oxide and DEET at 1%: (A) *Ae. albopictus*,

638 (B) *An. dirus*.

639

Conflict of Interest and Authorship Conformation

Conflict of interest

The authors declare no conflict of interest.

Author Contributions

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

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Author Contributions

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