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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.

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.

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

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

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

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

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

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

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

2. Materials and Methods

2.1 Mosquitoes used

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.

2.2 Test compounds

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).

2.3 Filter paper treatment

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

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

2.4 Excito-repellency test system

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

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

2.5 Safety evaluation procedures for β -caryophyllene oxide

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

$$CI\% = 100 - \left(\frac{100 \times (CBPI_{test\ material} - 1)}{CBPI_{solvent\ control} - 1} \right)$$

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.

3. Results

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).

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.

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

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

Based on these results, β -caryophyllene oxide had stronger repellent and irritant effects than DEET on four vector species at the same concentrations, suggesting the former has high potential for further development as an alternative active ingredient in mosquito repellent formulations, safe for humans and the environment. Such efficient repellent products are in need. In a study done in Vietnam, forest-goers were in favor of using repellent products to avoid mosquito-biting pressure (Ohrt et al., 2018). The use of repellent is a complementary vector control method to standard ones such as bednets, long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS), which has the advantage of repelling outdoor-biting mosquitoes, then reducing residual malaria transmission risk (Durnez and Coosemans, 2013). Then, appropriate formulations using β -caryophyllene oxide await development for its use as 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 (%)	P-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 ± 0.7	-	100	10.5 ± 2.1	-
Positive control [§]		98.2	31.5 ± 2.1	<0.001	97.6	24.0 ± 1.4	<0.001
Solvent control		98.6	9.5 ± 0.7	NS ^a	98.4	10.0 ± 1.4	NS
β -caryophyllene oxide	0.5	99.7	10.5 ± 2.1	NS	98.8	9.5 ± 0.7	NS
	1	98.4	11.0 ± 1.4	NS	97.4	13.0 ± 2.8	NS
	5	65.1	9.5 ± 2.1	NS	73.2	11.0 ± 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

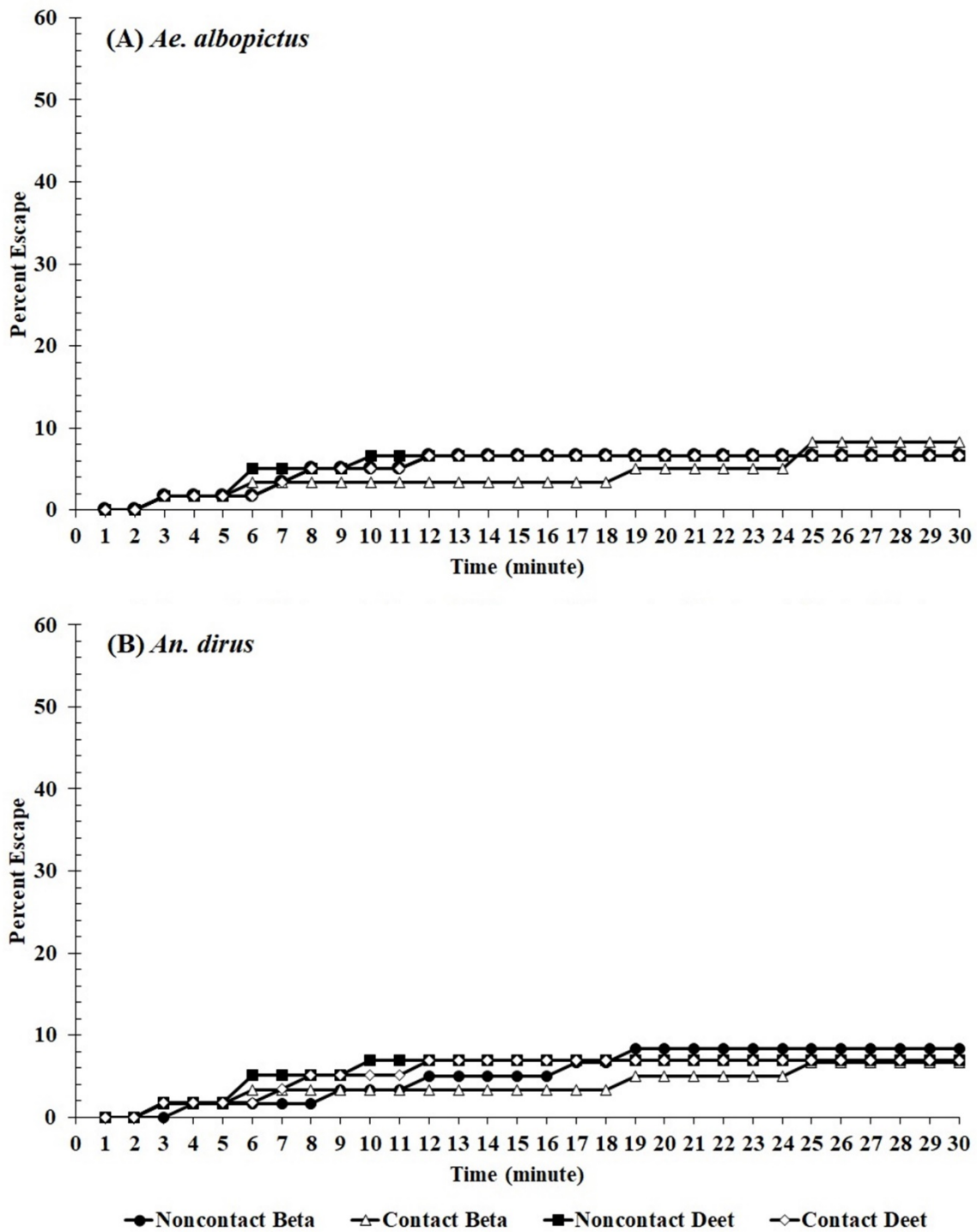


Fig. 1 Patterns of escape percentage from ER chambers for contact and noncontact assay designs during 30 min exposure to β -caryophyllene oxide and DEET at 0.1%: (A) *Ae. albopictus*, (B) *An. dirus*.

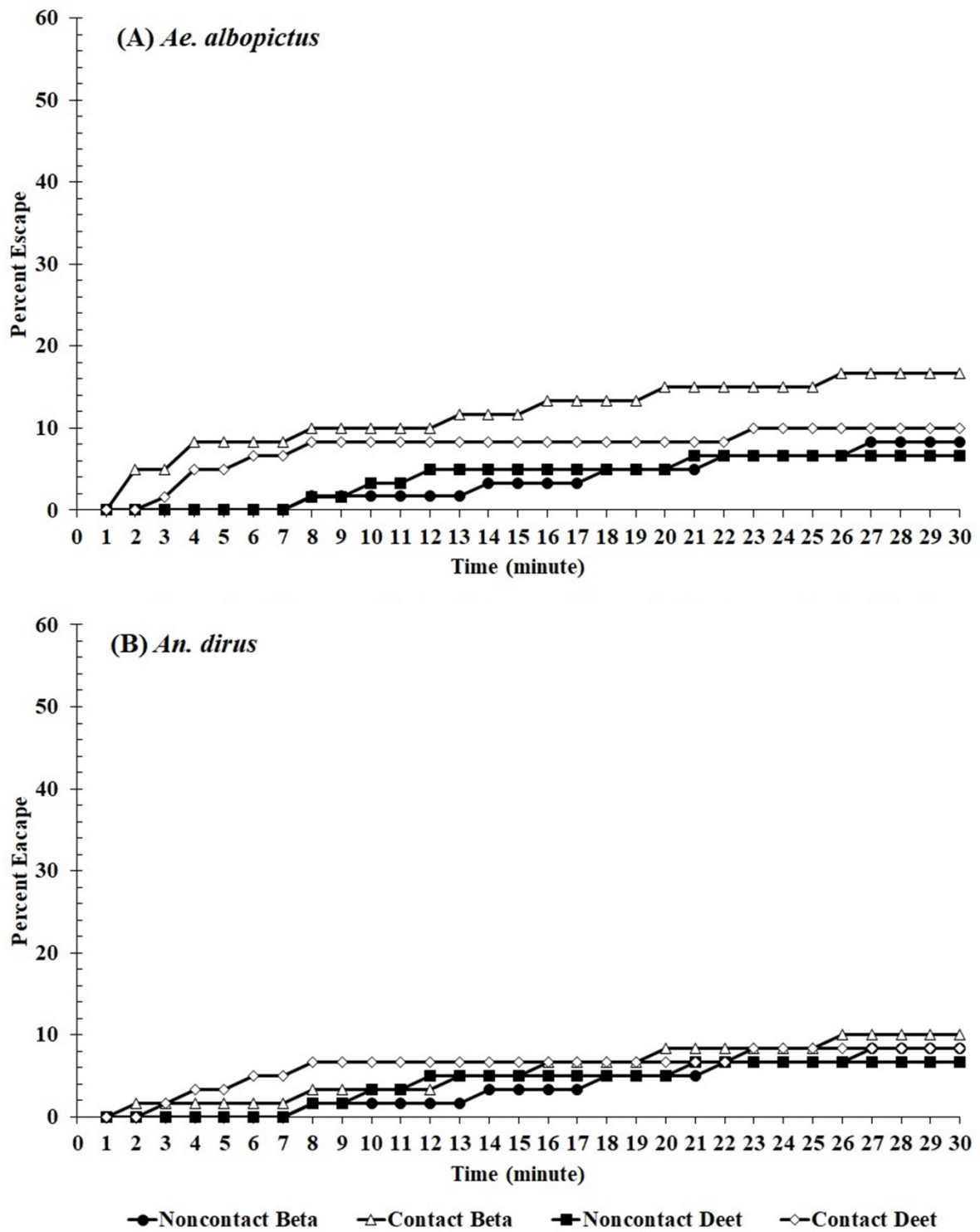


Fig. 2 Patterns of escape percentage from ER chambers for contact and noncontact assay designs during 30 min exposure to β -caryophyllene oxide and DEET at 0.25%: (A) *Ae. albopictus*, (B) *An. dirus*

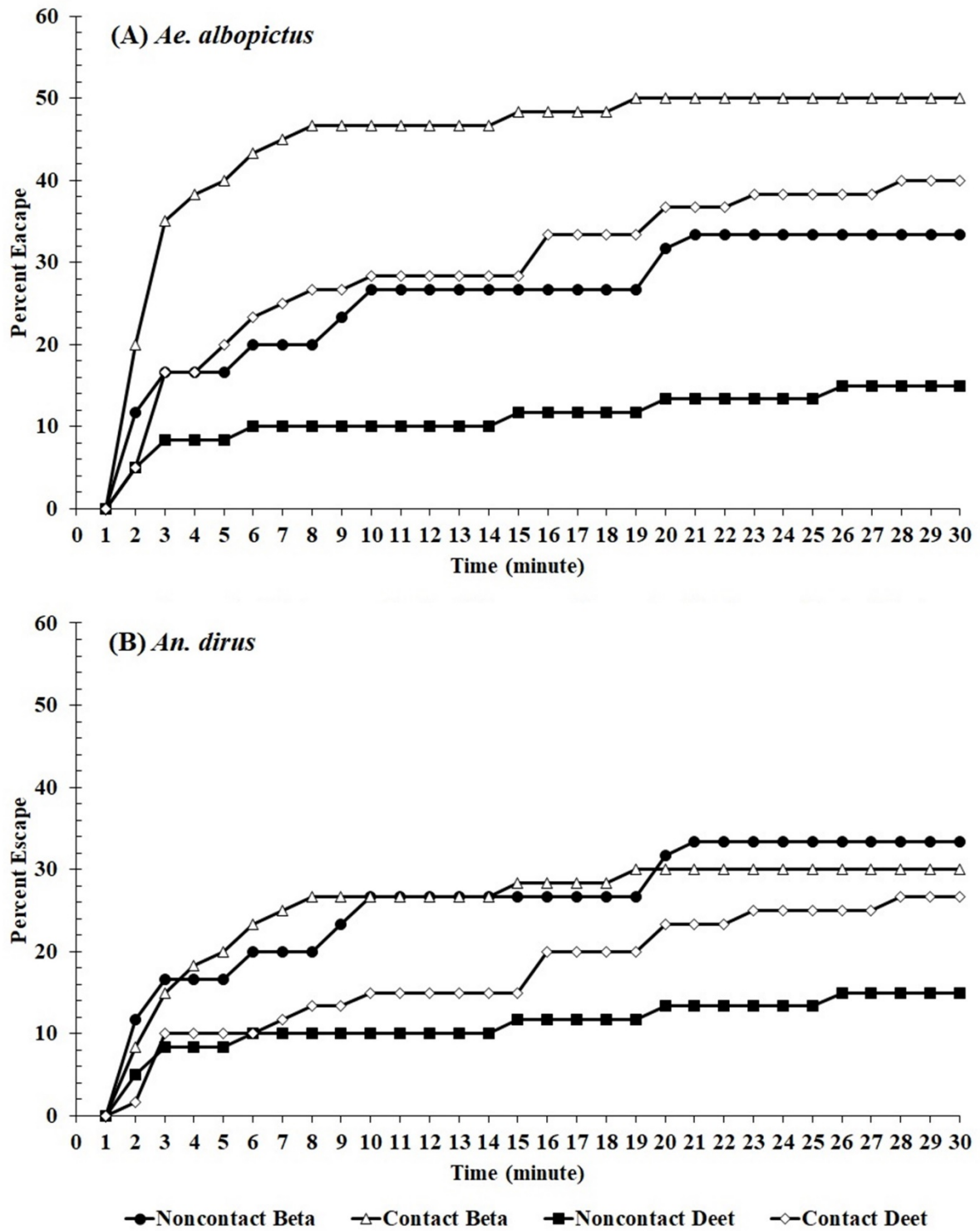


Fig. 3 Patterns of escape percentage from ER chambers under contact and noncontact assay designs during 30 min exposure to β -caryophyllene oxide and DEET at 0.5%: (A) *Ae. albopictus*, (B) *An. dirus*.

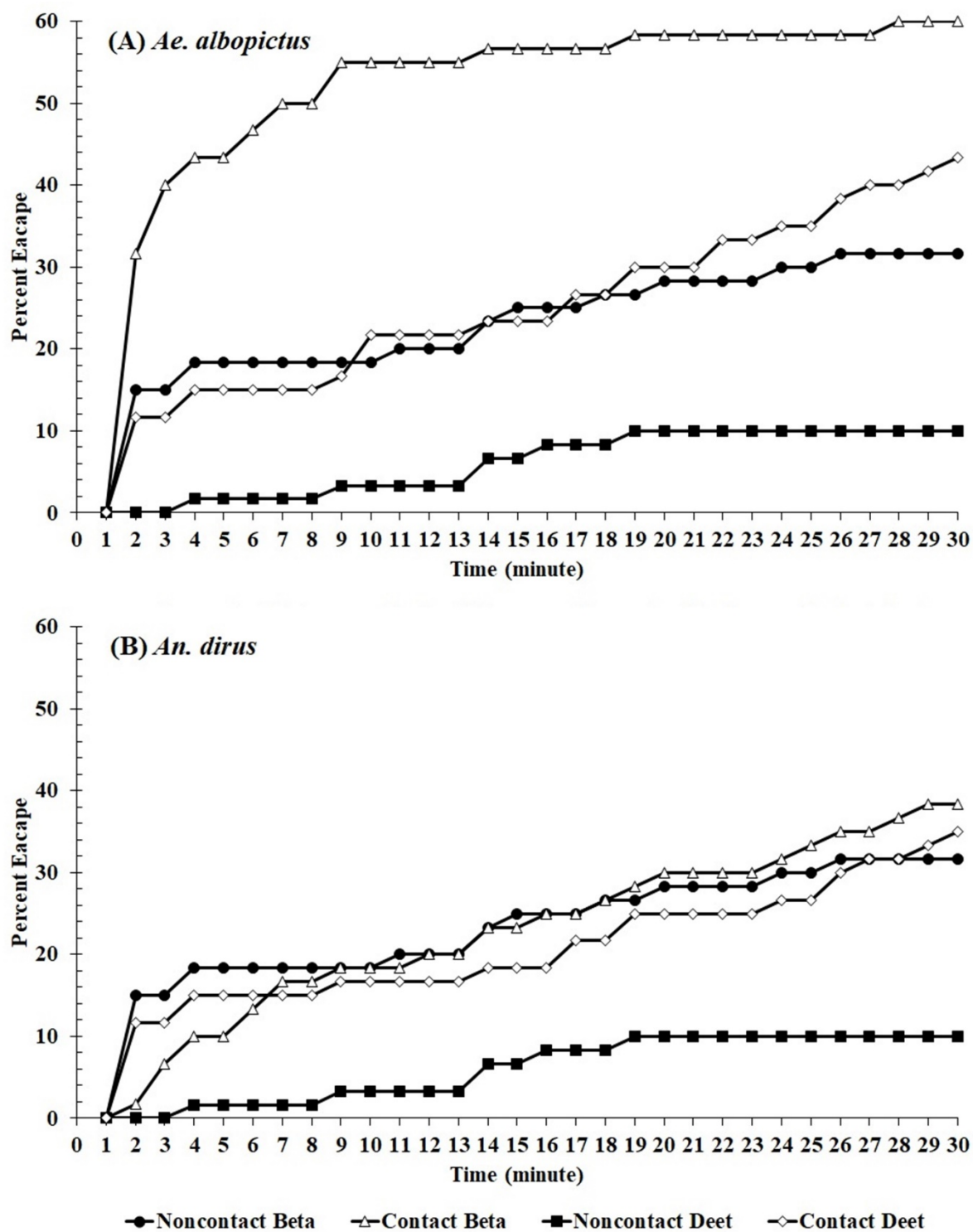


Fig. 4 Patterns of escape percentage from ER chambers under contact and noncontact assay designs during 30 min exposure to β -caryophyllene oxide and DEET at 1%: (A) *Ae. albopictus*, (B) *An. dirus*.

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.