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Behavioral avoidance and biological safety of vetiver oil and its constituents against *Aedes aegypti* (L.), *Aedes albopictus* (Skuse) and *Culex quinquefasciatus* Say

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ABSTRACT

Numerous plant-based repellents are widely used for personal protection against host-seeking mosquitoes. *Vitiveria zizanioides* (L.) Nash essential oil and its constituents have demonstrated various mosquito repellent activities. In this study, three chemical actions of vetiver oil and five constituents (terpinen-4-ol, α -terpineol, valencene, vetiverol and vetivone) were characterized against *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus* by using the high-throughput screening assay system (HITSS). Significant contact escape responses in *Ae. aegypti* and *Ae. albopictus* to all test compounds at concentrations between 2.5 and 5% were observed. Spatial repellency responses were also observed in some tested mosquito populations depending upon concentrations. The most significant toxic response on mosquitoes was found at the highest concentration, except for vetivone which had no toxic effect on *Ae. aegypti* and *Ae. albopictus*. Results on phototoxic and genotoxic hazard revealed that vetiver oil and their constituents showed no phototoxic potential or any significant genotoxic response. In conclusion, vetiver oil and two constituents, valencene and vetiverol, are potentials as active ingredients for mosquito repellency and present no toxicity.

1. Introduction

Mosquitoes are the most detrimental insect in terms of public health concerns. Some blood-feeding female mosquitoes play a role in the transmission of a large number of pathogens responsible of vector-borne diseases, such as malaria, filariasis, dengue, Japanese encephalitis, to cite a few, causing an estimated 700,000 deaths annually (WHO, 2020). *Aedes aegypti* (L.) is the principal vector that carries arboviruses responsible for dengue, chikungunya, Zika and other arboviruses (Sukhralia et al., 2019). Moreover, *Aedes albopictus* (Skuse), the Asian tiger mosquito, originated from Southeast Asia, has the potential to transmit 26 arboviruses including those cited above for *Ae. aegypti* and is considered to be the fastest and most invasive mosquito species in the world as it is now well established on every continent (Kamal et al., 2018; Paupy et al., 2009; Pereira-dos-Santos et al., 2020). *Culex quinquefascia-*

tus Say represents a major nuisance as a night-biting mosquito in semi-urban and urban areas and is a potential vector of Japanese encephalitis virus (JEV) in Thailand (Phumee et al., 2019). This species can transmit both arboviruses responsible for several encephalitis and parasite of the Bancroftian lymphatic filariasis in urban areas, where this species is widely distributed (Manguin et al., 2010; Tawatsin et al., 2019).

Synthetic insecticides are the first baseline strategies to control mosquitoes at the adult stage (Buxton et al., 2020). However, repeated usage of insecticides for vector management, as well as for agriculture, has resulted in their lesser efficacy and higher resistance rates and, more importantly, greater environmental risks and potential human health loss due to this indiscriminate usage of synthetic chemicals (Yogarajalakshmi et al., 2020). The phyto-compounds present in the natural based insecticides have proved their effective properties as observed by several scientists (Amala et al., 2021; Senthil-Nathan, 2020;

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Tisgratog et al., 2016). Topical insect repellents protect users from mosquito bites as people go out for their daily activities and therefore offer a potential tool against outdoor-biting mosquitoes (Wilson et al., 2014). Widely used repellents are based on synthetic compounds. DEET (*N, N*-Diethyl-meta-toluamide, also called diethyltoluamide) is the most common active ingredient used in repellent products and is available to the public market in various forms such as lotions, gels, creams, aerosols sprays, sticks, and impregnated towelettes (Diaz, 2016). The concentrations of DEET in marketed products is high, varying between 5 to 100% (Corbel et al., 2009). DEET is a broad-spectrum repellent against mosquitoes, biting flies, chiggers, fleas, and ticks (Fradin, 2019). However, DEET plays a limited role in disease control in endemic areas because of its high cost, unpleasant odor, and inconvenience of a continuous application on the exposed skin at high doses, without mentioning the fact that DEET melt plastic materials (Deletre et al., 2016; Leal, 2014). Therefore, varieties of plant-based products are of upmost interest for their effect to repel mosquitoes and other arthropod pests in many laboratories interested in developing new, efficient and more environmental-friendly repellents. Several essential oils have been reported to exhibit significant repellent activity against target insects, especially mosquitoes including citronella or lemon grass (*Cymbopogon citratus*), catnip (*Nepeta cataria*), clove (*Syzygium aromaticum*), cinnamon (*Cinnamomum verum*), ginger (*Zingiber officinale*), kaffir lime (*Citrus hystrix*), hairy basil (*Ocimum americanum*), Chinese yellow ginger *Cassumunar* ginger (*Zingiber cassumunar*), sweet basil (*Ocimum basilicum*), vetiver (*Vitiveria zizanioides*), and ylang-ylang (Boonyuan et al., 2014; Nararak et al., 2016; Polsomboon et al., 2008; Sathantriphop et al., 2014; Sukkanon et al., 2022; Suwansirisilp et al., 2013; Tisgratog et al., 2016). These essential oils are composed of a complex mixture blend of constituents among which some exhibit excellent mosquito repellent activity.

Vetiver (*Vitiveria zizanioides* (L.) Nash, Poaceae) essential oil consists of a complex mixture of more than 200 compounds, a major portion of oil consisting of sesquiterpenoids, hydrocarbons and their oxygenated derivatives, but also phenols and nitrogen compounds. Vetiver oil has been reported to repel several insects such as termites, mosquitoes, weevils, beetles (Nararak et al., 2016; Sujatha, 2010; Zhu et al., 2001). St. Pfau and Plattner (1939) identified in vetiver oil α - and β -vetivones comprising the major constituents. Möllenbeck et al. (1997) found that α -terpineol and terpinen-4-ol are minor components in vetiver. Six other compounds in vetiver oil have demonstrated some repellent properties against arthropods and these include α -vetivone, β -vetivone, khusimone, zizanal, epizizanal and (+)-(1S, 10R)-1, 10-dimethyl bicyclo [4,4,0]-dec-6-en-3-one (Jain et al., 1982). Tisgratog et al. (2018) reported that vetiverol, valencene, terpinen-4-ol and isolongifolene, which are constituents of vetiver essential oil, exhibited repellency and irritancy actions against *Anopheles minimus* at a concentration <5 %. A recent study showed that vetiver oil displayed a strong repellent activity (78%) against house flies (*Musca domestica*) and exhibited 100% contact toxicity to larval and adult house flies (Khater and Geden, 2019).

Relatively few studies have measured the types of responses of mosquitoes to chemicals (Carrasco et al., 2019; Grieco et al., 2005). The excito-repellency system (ER) developed by Chareonviriyaphap et al. (2002) and later modified (Tanasinchayakul et al., 2006) was used to characterize both types of mosquito behavioral responses, contact irritancy and spatial repellency, against test compounds. However, the ER test system requires a large amount of chemical on a treated paper surfaces due to the size of the interior surface of each test system. In 2005, a suite of the high-throughput screening system (HITSS) was developed and used to quantitatively describe the responses of mosquitoes to different actions of chemicals, which are contact irritancy, spatial repellency, and toxicity to mosquitoes (Grieco et al., 2005). The HITSS was subsequently applied to determine the chemical behavioral actions on several mosquito species against selected synthetic compounds (Dusfour et al., 2009; Thanispong et al., 2010). Relatively few studies

have used the HITSS to characterize the chemical actions of natural plant-based repellents (Sathantriphop et al., 2015; Tisgratog et al., 2018). In this study, we used the HITSS to investigate three chemical actions: irritancy, spatial repellency and toxicity of vetiver oil and its constituents against *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*. In addition, the toxic safety of vetiver oil and vetiver constituents was determined using an *in vitro* phototoxicity test and an *in vitro* micronucleus assay.

2. Materials and Methods

2.1. Mosquito test populations

Laboratory strains of *Ae. aegypti* (USDA strain), *Ae. albopictus* (KU strain) and *Cx. quinquefasciatus* (NIH strain) were used in this study. *Aedes aegypti* eggs were obtained from the U.S. Department of Agriculture, Gainesville, FL, USA. The colony has been continuously maintained under laboratory-controlled conditions for over 50 years and is completely susceptible to all insecticides (Juntarajumnong et al., 2012). *Aedes albopictus* population was originally captured in 1996 in Chanthaburi Province, eastern Thailand by the staff from the Ministry of Public Health, Thailand. Representatives of this population have been maintained in the entomological laboratory at Kasetsart University (KU) since 2013. *Culex quinquefasciatus* was obtained from the National Institute of Health (NIH), Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand, in 2015. This colony has been continuously maintained by the NIH for nearly 40 years.

All three species were reared separately in the insectary of the Department of Entomology, Faculty of Agriculture, Kasetsart University under the conditions of $25\pm 5^\circ\text{C}$ and $80\pm 10\%$ relative humidity with a 12:12 light:dark photoperiod (Boonyuan et al., 2017). Pupae were collected daily and placed in small cups until adult emergence in wire-mesh cages ($30 \times 30 \times 30$ cm). Adults were provided 10% sucrose solution *ad libitum*. Human blood was provided using an artificial membrane feeding system. Female mosquitoes with the age of three to five days old were starved for 24 hrs before testing.

2.2. Chemical analysis

Vetiver oil and five pure compounds were tested with the high throughput assay system (HITSS) to characterize the repellent, irritant and toxic activity of each mosquito species. The repellents include:

- 1 Vetiver root essential oil purchased from Thai-China Flavors and Fragrances Industry Co., Ltd. Company (TCFF, Phra Nakhon Si Ayutthaya Province, Thailand). The main components of this batch are vetiveryl acetate, vetiverol, vitivone, terpenes, these are provided by Thai - China Flavours and Fragrances Industry (TCFF) Co., Ltd. (TCFF, 2013).
- 2 Valencene obtained from Sigma-Aldrich Company 3050 Spruce, St. Louis, MO 63103, USA.
- 3 Terpinen-4-ol provided by Professor Dr. Joel R. Coats from the Department of Entomology, Iowa State University, Ames, Iowa 50011-3140, USA.
- 4 Vetiverol, vetivone (mixture of α , β) and α -terpineol: these three constituents were supplied from Dr. Kamlesh R. Chauhan, Invasive Insects Biocontrol & Behavior Laboratory, USDA-ARS, BARC-West Bldg. 007, room 303, 10300 Baltimore Avenue, Beltsville, MD 20705, USA.

The constituent solutions were dissolved in absolute ethanol (Merck, Darmstadt, Germany) and diluted to obtain the concentrations of 1.0, 2.5, and 5.0% (w/v).

2.3. Net impregnations

Vetiver oil and vetiver constituents were diluted with absolute ethanol into solutions of 1% (0.2 mg in 19.8 ml solution), 2.5% (0.5

mg in 19.5 ml solution) and 5% (1 mg in 19 ml solution) of active ingredient. Nylon netting was cut into $11 \times 25 \text{ cm}^2$. A volume of 1.5 mL solution was applied to a strip using a 1,000 μL micropipette. The treated nets were air-dried for 15 min before being attached to the test cylinder. Control nets were treated with ethanol only.

2.4. High-throughput screening system (HITSS)

A complete set of high-throughput screening system (HITSS) consists of three test assays including toxicity, contact irritancy, and spatial repellency. Toxicity is indicative of knockdown or death after the mosquito makes tarsal contact with the test chemical. Contact irritancy stimulates directed movement away from the chemical source after the mosquito makes physical contact. Spatial repellency stimulates directed movement away from the chemical source without any physical contact of the mosquito with the treated surface (Grieco et al., 2005). Standard operating protocols followed that described previously (Achee et al., 2009; Grieco et al., 2007). Ten female mosquitoes were used in the contact irritancy assay, this number was increased in both spatial repellency and toxicity assays (20 females) based on baseline experiments that were conducted to determine the sample size required for statistical power in the smallest number of replicates with the least difficulty in manual observation (Achee et al., 2009). The sample size for toxicity assays followed guidelines established for insecticide resistance testing (Achee et al., 2009; WHO, 1998).

2.4.1. Contact Irritancy Assay (CIA)

In the contact irritancy assay (CIA), a test metal cylinder lined with a treated net was fixed to a darkened control clear cylinder using a butterfly valve placed in the open position. Ten unfed female mosquitoes were released into the metallic cylinder with the treated net. After a 30 sec acclimation duration, the butterfly valve was opened and the distribution of the mosquitoes between the two compartments was recorded after 10 min. Individuals remaining in the clear cylinder at the end of the test were recorded as escaped mosquitoes, after which the butterfly valve was off. The number of mosquitoes that escaped into the clear cylinder, those still present in the metallic cylinder, and knockdown mosquitoes within both cylinders were counted (Thanispong et al., 2010). Six replicates were conducted for each test and concentration.

2.4.2. Spatial Repellency Assay (SRA)

The spatial repellency assay (SRA) configuration contains three chambers, two metallic cylinders connected to a clear central cylinder using butterfly valves. Twenty unfed female mosquitoes were introduced into the central clear cylinder and allowed to rest for 30 sec, then the butterfly valves were opened for 10 min to allow free movement of mosquitoes in either direction of both ends of the test chamber. After a 10-min exposure time, the butterfly valves were closed and the number of mosquitoes in each chamber was counted. Spatial repellency is determined by considering the number of mosquitoes that have moved into the untreated, control chamber (away from the treated surface) relative to the total number of mosquitoes that have moved in either direction. Nine replicates for each compound and concentration were conducted (Achee et al., 2009; Grieco et al., 2005).

2.4.3. Toxicity Assay (TOX)

The toxicity assay (TOX) comprises a metallic cylinder only (control and treatment) fixed with an end cap. The treated net was fixed inside the cylinder of each treatment and matched control assay. Twenty starved female mosquitoes were transferred into test cylinders and exposed for 1 hr. Then, the number of knocked down mosquitoes was recorded and all (knocked down and those still mobile) were moved into clean plastic cups. These mosquitoes were provided with a cotton ball soaked in 10% sucrose water and maintained in the insectary. Their mortality was monitored and recorded after 24 hrs. Six replicates were performed for each treatment combination including a control test, and each compound at each concentration.

2.4.4. Data analysis

Contact irritancy data were analyzed using the Wilcoxon 2-sample test (SAS Institute, 1999) to calculate the difference in the number of escaped mosquitoes in the treated and untreated control cylinders. The spatial activity index (SAI) value was calculated for each chemical using the following equation:

$$\text{SAI} = \frac{(\text{Nc} - \text{Nt})}{(\text{Nc} + \text{Nt})}$$

where Nc is the number of mosquitoes in the control chamber and Nt is the number of mosquitoes in the treated chamber. The SAI varies from -1 to 1, with 0 indicating no attractant or repellent response. When a SAI value is < zero, this indicates a greater proportion of mosquitoes that moved into the treatment chamber than the control chamber, resulting in an attractant response. When a SAI value is > zero, this indicates a greater proportion of mosquitoes moving into the control chamber (away from the treatment end of the assay device), suggesting a repellent response (Kamal et al., 2018). Spatial repellency data were analyzed by a nonparametric signed-rank test (SAS Institute, 1999) to calculate whether the mean spatial activity index for each treatment was significantly different from zero. Toxicity data, percentage of knockdown and mortality at 24 hrs were corrected using Abbott's formula and transformed using the arcsine square root of the data for analysis of variance (ANOVA). The knockdown and mortality of the treatment at each concentration were compared using Tukey's honestly significant difference test at $P=0.05$.

2.5. Safety evaluation procedures for Vetiver oil and pure components

2.5.1. The 3T3 NRU phototoxicity test

The *in vitro* 3T3 NRU phototoxicity of vetiver oil and vetiver components were measured to evaluate the relative reduction in viability of cells exposed to them in the presence of light versus absence of light. The 3T3 NRU phototoxicity test (OECD N°432) is based on the comparison of a chemical when tested in the presence and in the absence of exposure to a non-cytotoxic dose of simulated solar light (OECD, 2004). Cytotoxicity is expressed as a concentration-dependent reduction of the uptake of the vital dye Neutral Red when measured 24 hrs after chemical treatment and irradiation. Briefly, mouse fibroblasts Balb/c 3T3 cell line (ATCC, USA, ATCC® CL-173™) was grown in DMEM (Dulbecco's Minimum Essential Medium) and supplemented with penicillin 100 IU/mL, streptomycin 100 $\mu\text{g}/\text{mL}$, and 10% inactivated calf serum. 3T3 murine fibroblasts were seeded into two 96-well tissue culture plates, at the concentration of 1×10^5 cells/mL, and incubated at 37°C (5% CO_2) for 24 hrs until semi-confluent. The culture medium was decanted and replaced by 100 μL of HBSS containing the appropriate concentrations of the test substances, then cells were incubated at 37°C (5% CO_2) in the dark for 60 min. From the two plates prepared for each series of test substance concentrations and the controls, one was selected, generally at random, for the determination of cytotoxicity without irradiation (-Irr), and the other for the determination of photocytotoxicity with irradiation (+Irr).

Irradiation procedure was performed with a solar simulator Suntest CPS+ (Atlas Material Testing Technology BV, Moussy le Neuf, France) apparatus equipped with a xenon arc lamp (1100 W), a special glass filter restricting transmission of light below 290 nm and a near IR-blocking filter. The irradiance was fixed at 750 W/m^2 throughout the experiments and the combined light dose was 5 J/cm^2 for one-minute UVA/visible irradiation (0.41 J/cm^2 of UVA and 4.06 J/cm^2 of visible light).

The test solution was removed, cells were rinsed twice with 150 μL HBSS and incubated for 18-22 hrs culture medium at 37°C (5% CO_2). Cells were washed, placed into Neutral Red medium (50 $\mu\text{g}/\text{mL}$ Neutral Red in complete medium) and incubated for 3 hrs at 37°C, 5% CO_2 . Then, the Neutral Red medium was removed and the destaining solution (50% ethanol, 1% acetic acid, 49% distilled water; 50 μL per well) was added into the wells. Then, the plates were shaken for 15-20 min at

room temperature in the dark. All test samples and controls were run in triplicates in three independent experiments.

Cell viability was measured by a fluorescence-luminescence reader Infinite M200 Pro (TECAN). The optical density (OD) of each well was read at 540 nm. 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. Neutral Red desorbed solution serves as blank. The percentages of cell viability (Nararak et al., 2020) 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 results were expressed as a percentage of untreated control cell and concentration dependent curves in the presence and absence of light. The photo-irritation-factor (PIF) was calculated with concentration (obtained by the software Phototox Version 2.0) of the test material causing a 50% release of the preloaded Neutral Red without irradiation (IC₅₀ -Irr) and with irradiation (IC₅₀ +Irr) as compared to the control culture using the following formula (Kim et al., 2020).

$$\text{PIF} = \frac{\text{IC}_{50} (-\text{Irr})}{\text{IC}_{50} (+\text{Irr})}$$

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

2.5.2. In vitro micronucleus assay (MNvit)

The *in vitro* micronucleus assay (MNvit) was used to detect the long-term toxicity of the chemical. The micronucleus assay MNvit is a mutagenicity assay based on the detection of micronuclei (MNC) in the cytoplasm of interphase cells and allows detecting the cytogenetic activity of clastogenic and/or aneugenic compounds in cell culture (Johnson et al., 2010). The micronucleus assay was performed on a Chinese Hamster Ovary cell line CHO-K1 (ATCC, USA). The CHO-K1 cells, suspended in Mac Coy's 5A medium, were transferred into LabTek wells at a concentration of 100,000 cells/ml, and incubated for 24 hrs at 37°C in 5% CO₂. The test was performed in the presence (+) or absence (-) of the S9 metabolic activation system, and cultures were done in duplicate. When the test was performed without metabolic activation, the test substances were added into cell cultures at doses of 0.5-10 µg/ml. A negative control containing culture medium, a solvent control containing 1% ethanol and a positive control containing 0.06 µg/ml of mitomycin C were added. 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%. Then, the test substances were added to the cell cultures at doses previously defined. A negative control containing culture medium, a solvent control containing 1% ethanol, and a positive control containing 5 µg/ml of benzo-a-pyrene were added. After 3 hrs of incubation at 37°C in CO₂ (5%), the culture medium was removed, 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 a 21-hrs incubation period at 37°C, cells were rinsed with phosphate buffered saline (PBS), fixed with methanol and stained with 10% Giemsa for 20 min. The proliferation index (cytokinesis blocked proliferative index CBPI) was calculated. 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:

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

where BI is the number of binucleated cells and MONO is the number of mononucleated cells (Cardoso Trento et al., 2019). The cytostasis index (CI%) is the percentage of cell replication inhibition and was calculated

using the following formula (Bhavsar and Chandel, 2019).

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

Statistical differences between negative controls and treated samples were determined using the χ^2 test. The assay was considered positive if a dose-response relationship was obtained between the rate of micronuclei and the doses tested, where at least one of these doses induced a statistically significant increase ($P < 0.05$) in the number of micronucleated cells compared to the negative control.

2.6. Data availability statement

Raw data of the contact irritancy assay (CIA), spatial repellency assay (SRA) and toxicity assay (TOX) are available in three excel files in the supplementary materials. In addition, two tables on the raw data of the cytotoxicity, phototoxicity and genotoxicity have been added as supplementary materials. Codes are provided for each file.

3. Results

Contact irritant, spatial repellent and toxic actions of six compounds including vetiver essential oil and five pure compounds (valencene, terpinen-4-ol, vetiverol, α -terpineol, and vetivone) were performed using the HITSS against *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus*. Responses of female mosquitoes in the contact irritancy, spatial repellency and toxicity assays to three different concentrations (1, 2.5, and 5%) of each component or vetiver oil are shown in Tables 1-5 and Figures 1-3.

3.1. Contact irritancy

Behavioral escape responses of *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus* varied drastically depending upon the concentration (1, 2.5, and 5%) and tested species. For *Ae. aegypti*, the greater percent escape responses were observed in the treatment group compared to the control, except at 1% vetiver oil (19.35±9.37), terpinen-4-ol (8.88±5.13), α -terpineol (5.60±7.99), vetiverol (9.12±3.42), and vetivone (0) ($P > 0.05$) (Table 1). For *Ae. albopictus*, there was no significant difference in treatment group versus control group at 1% vetiver oil (6.64±10.49), terpinen-4-ol (8.51±4.78), valencene (17.65±7.13), vetivone (5.83±6.07), and 2.5% vetivone (6.85±3.37) ($P > 0.05$) (Table 2). In contrast, *Cx. quinquefasciatus* females escaped significantly more ($P > 0.05$) in treatment compared to control tests, except at 1% α -terpineol (13.88±8.00), and 1% and 2.5% terpinen-4-ol (0 and 12.27±6.29, respectively) (Table 3).

The strongest contact irritancy action (>87%) was observed from vetiver oil at 5% when exposed to *Ae. aegypti* and *Cx. quinquefasciatus*, as well as valencene at 2.5% and 5% for the latter species (Tables 1, 3). *Aedes albopictus* exhibited a strong contact irritancy action at 84% and 83% respectively with valencene and vetiverol at 5% (Table 2). The rank corrected percent escaping was found higher when tested with vetiver oil (50.29-92.06%), valencene (66.11-98.33%), and vetiverol (52.54-82.82%) against *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* at 2.5 and 5% respectively compared to terpinen-4-ol, α -terpineol and vetivone (Tables 1-3).

3.2. Spatial repellency

Aedes aegypti was repelled by 2.5-5% vetiver oil, terpinen-4-ol and valencene as indicated by the positive value of SAI (Figure 1A). In contrast, *Ae. albopictus* was repelled at 1-5% vetiver oil and 2.5-5% terpinen-4-ol and valencene (Figure 1B). No significant differences in spatial repellency were found from terpineol, vetiverol, and vetivone in *Ae. aegypti* and *Ae. albopictus* compared to control ($P > 0.05$). Significant differences ($P < 0.05$) in responses due to repellency were found between all

Table 1
Escape response of *Aedes aegypti* in the contact irritancy assay to vetiver oil and pure compounds.

Repellent	Concentration (%)	Number escaping (mean±SE)		Percent escaping (mean±SE)	P-value
		Treated	Control		
Vetiver oil	1	3.00± 0.63	1.16±0.40	19.35±9.37	0.0866*
	2.5	6.10±0.70	1.83±0.30	52.21±9.37	0.0022
	5	9.10±0.40	2.16±0.30	91.53±5.41	0.0022
Terpinen-4-ol	1	1.83±0.40	1.00±0.23	8.88±5.13	0.0758*
	2.5	1.50±0.34	0.33±0.21	12.03±3.04	0.0476
	5	5.00±0.68	1.16±0.47	42.42±8.55	0.043
α -Terpineol	1	1.50±0.34	0.83±0.40	5.60±7.99	0.3723*
	2.5	2.16±0.40	0.66±0.21	16.11±3.75	0.0108
	5	4.88±1.16	0.33±0.21	47.22±11.54	0.0065
Valencene	1	2.83±0.30	0.50±0.34	23.52±6.06	0.0065
	2.5	6.83±0.47	0.50±0.34	66.15±5.53	0.0022
	5	6.83±0.70	0.83±0.40	66.11±7.75	0.0022
Vetiverol	1	1.83±0.40	1.00±0.36	9.12±3.42	0.2532*
	2.5	5.66±0.71	0.83±0.30	52.54±8.03	0.0022
	5	6.16±0.94	1.00±0.42	57.08±9.77	0.0022
Vetivone	1	0.83±0.30	1.00±0.0	0.0±0.0	0.4545*
	2.5	1.33±0.33	0.16±0.16	11.48±4.90	0.0411
	5	6.16±0.94	1.00±0.44	59.17±9.14	0.0022

* P-value < 0.05 indicates a significant difference between the number escaping in treatment chamber and control chamber.

Table 2
Escape response of *Aedes albopictus* in the contact irritancy assay to vetiver oil and pure compounds

Repellent	Concentration (%)	Number escaping (mean±SE)		Percent escaping (mean±SE)	P-value
		Treated	Control		
Vetiver oil	1	2.50±0.71	1.33±0.21	6.64±10.49	0.177*
	2.5	6.00±0.51	1.50±0.21	50.29±7.08	0.002
	5	6.33±0.33	1.33±0.21	65.34±6.53	0.002
Terpinen-4-ol	1	1.83±0.30	1.00±0.36	8.51±4.78	0.205*
	2.5	6.83±0.87	1.83±0.30	59.78±12.33	0.002
	5	4.50±0.92	0.33±0.21	42.77±10.37	0.006
α -Terpineol	1	3.00±0.51	1.00±0.36	21.99±5.20	0.028
	2.5	5.50±0.42	1.83±0.30	43.81±7.09	0.002
	5	6.83±0.33	1.33±0.21	68.14±5.27	0.002
Valencene	1	3.33±0.33	1.83±0.21	17.65±7.13	0.205*
	2.5	7.33±0.55	2.16±0.30	66.45±6.64	0.002
	5	8.50±0.56	0.50±0.34	84.02±5.87	0.002
Vetiverol	1	7.60±0.21	0.50±0.22	77.22±1.51	0.002
	2.5	8.16±0.60	0.83±0.54	75.47±9.23	0.002
	5	8.50±0.56	0.83±0.30	82.82±6.68	0.002
Vetivone	1	1.33±0.21	0.66±0.42	5.83±6.07	0.316*
	2.5	1.16±0.40	0.50±0.34	6.85±3.37	0.372*
	5	5.83±0.79	0.83±0.54	51.83±6.07	0.004

* P-value < 0.05 indicates a significant difference between the number escaping in treatment chamber and control chamber.

Table 3
Escape response of *Culex quinquefasciatus* in the contact irritancy assay to vetiver oil and pure compounds

Repellent	Concentration (%)	Number escaping (mean ± SE)		Percent escaping (mean ± SE)	P-value
		Treated	Control		
Vetiver oil	1	5.83±0.47	1.33±0.33	51.66±6.00	0.002
	2.5	7.66±0.42	1.33±0.33	72.17±5.69	0.002
	5	9.33±0.33	1.83±0.30	92.06±3.84	0.002
Terpinen-4-ol	1	1.00±0.36	1.00±0.36	0.0±0.0	1.000*
	2.5	6.00±0.36	1.33±0.55	12.27±6.29	0.138*
	5	5.66±0.76	1.00±0.36	50.55±9.66	0.002
α -Terpineol	1	3.33±0.49	2.16±0.30	13.88±8.00	0.138*
	2.5	2.66±0.42	1.00±0.36	17.03±7.53	0.002
	5	6.33±0.42	0.66±0.33	59.95±5.68	0.032
Valencene	1	7.00±0.51	1.50±0.42	62.77±8.32	0.002
	2.5	9.00±0.36	2.16±0.30	87.36±4.35	0.002
	5	9.83±0.17	0.66±0.21	98.33±1.66	0.002
Vetiverol	1	4.33±0.21	1.00±0.36	36.52±3.40	0.002
	2.5	7.16±0.30	1.00±0.36	68.05±4.18	0.002
	5	7.16±0.30	1.00±0.36	69.44±3.69	0.002
Vetivone	1	3.16±0.40	1.66±0.21	17.59±5.76	0.032
	2.5	6.83±0.47	1.00±0.36	64.30±6.07	0.002
	5	7.50±0.61	0.16±0.16	74.25±6.58	0.002

* P-value < 0.05 indicates a significant difference between the number escaping in treatment chamber and control chamber.

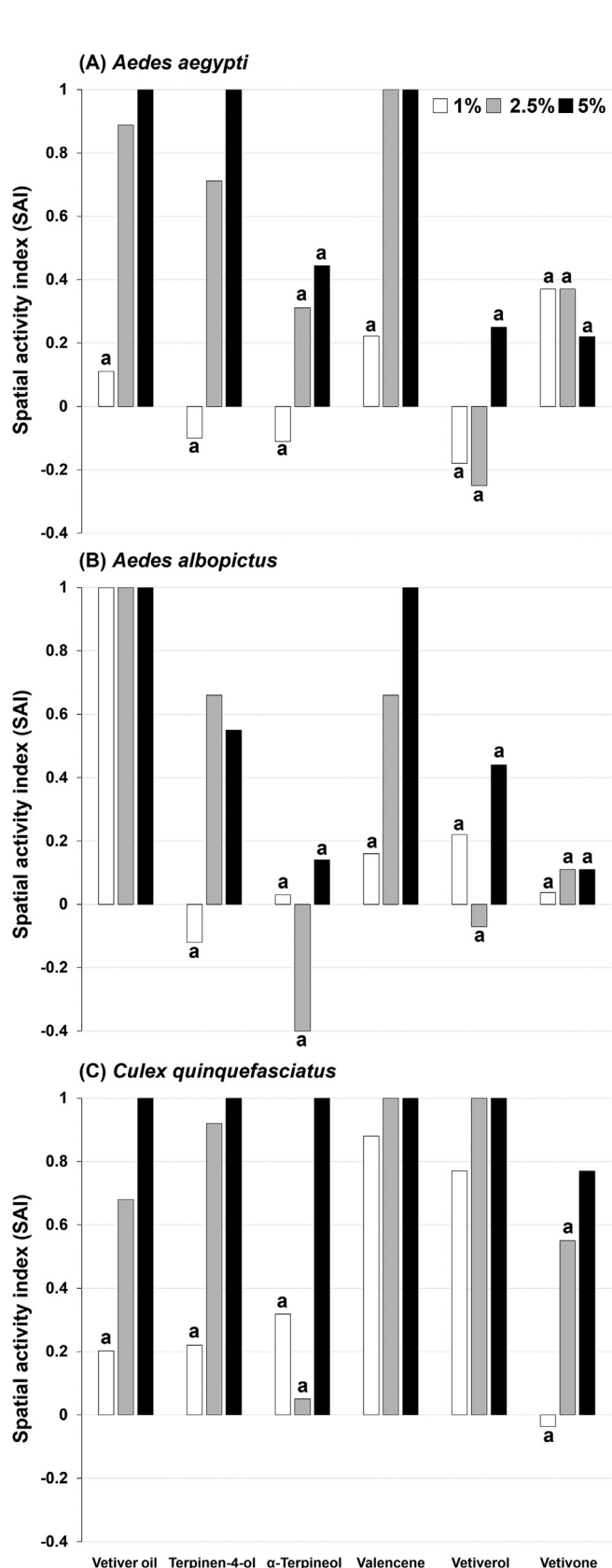


Figure 1. Spatial repellent responses of *Aedes aegypti* (A), *Aedes albopictus* (B), and *Culex quinquefasciatus* (C) exposed to vetiver oil and its constituents at 1%, 2.5% and 5% concentrations. a, denotes statistically not significant (signed rank test, $P > 0.05$) repellent response compared with matched controls.

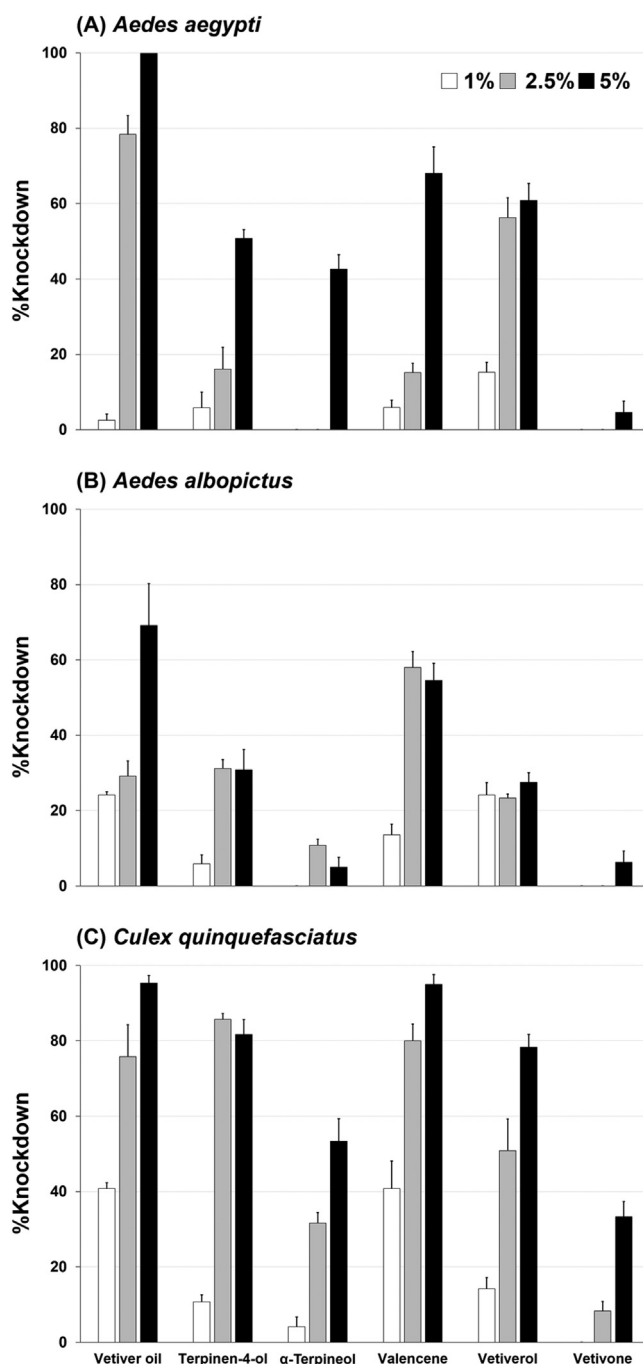


Figure 2. Percentage 1-hour Knock-down rates (TOX) with standard error (SE) in *Aedes aegypti* (A), *Aedes albopictus* (B), and *Culex quinquefasciatus* (C) laboratory strains exposed to three concentrations of vetiver oil, terpinen-4-ol, α -terpineol, valencene, vetiverol and vetivone.

treatment and control pairs for valencene and vetiverol against *Cx. quinquefasciatus* (Figure 1C). Overall, vetiver oil and valencene showed the most promising spatial repellency against *Aedes* mosquitoes, whereas *Cx. quinquefasciatus* was repelled by all test compounds (Figure 1A-C).

3.3. Toxicity assay

Positive relationship between knockdown and mortality rates and concentrations of test compound was observed for all three mosquito species (Figures 2-3). Vetiver oil and two constituents (valencene and vetiverol) produced high knockdown and mortality effects at 2.5-5% on

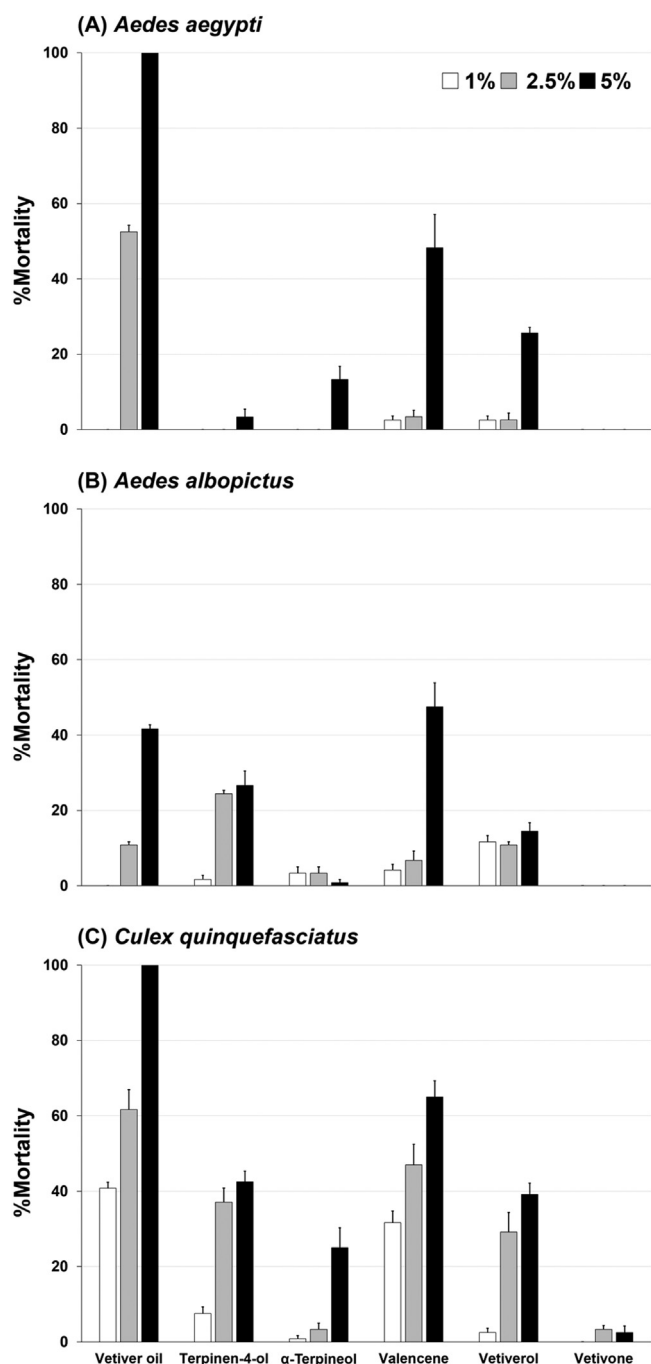


Figure 3. Percentage 24-hour mortality rates (TOX) with standard error (SE) in *Aedes aegypti* (A), *Aedes albopictus* (B), and *Culex quinquefasciatus* (C) laboratory strains exposed to three concentrations of vetiver oil, terpinen-4-ol, -terpineol, valencene, vetiverol and vetivone.

Ae. aegypti, *Ae. albopictus*, and *Cx. quinquefasciatus*. Specifically, vetiver oil showed the highest knockdown and mortality rates at 5%. Low toxicity, knockdown, or mortality was detected with vetivone in *Ae. aegypti* and *Ae. albopictus* (Figures 2-3).

3.4. The 3T3 NRU phototoxicity test

The cytotoxic potential of vetiver oil and pure compounds were evaluated in murine fibroblast (3T3) and tested in the presence and absence of exposure to a non-cytotoxic dose of simulated solar light. Results of phototoxicity were reported in Table 4. PIF values were used to cate-

gorize the phototoxicity potential. A total of six tested samples including vetiver oil, terpinen-4-ol, α -terpineol, valencene, vetiverol, and vetivone, were investigated by the phototoxicity assay and all samples showed no sign of phototoxicity as indicated by the low PIF values as compared to the positive sample (chlorpromazine) (Table 4).

3.5. In vitro micronucleus assay (MNvit)

Genotoxicity was assayed starting from the highest concentration at which neither necrosis nor cytotoxic or cytostatic effect was observed. When tested on the Chinese hamster ovary (CHO)-K1 cell line, vetiver oil and pure compounds did not produce any cytotoxic effects up to a concentration of 5 μ g/mL. Percent micronucleated binucleated cells performed with and without S9 mix is presented in Table 5. Vetiver oil and all five compounds had no significant effect on the number of micronuclei induced at the four concentrations tested in the study when compared to the negative control. All the positive controls with mitomycin C or benzo-[a]-pyrene without or with S9 mix respectively, significantly enhanced the number of micronuclei (Table 5). These results indicated that vetiver oil and all constituents were not derived from clastogenic/aneugenic activity and did not produce clastogenic/aneugenic metabolites. Therefore, they are safe to be used as topical repellent.

4. Discussion

Insecticide resistance is recognized as a severe danger to vector control programs by many parties, including industry, the WHO, regulatory organizations, and the general public, and is seen as a problem that requires urgent attention (Pavela, 2015). Researchers have been focusing on finding alternate ways and chemicals to reduce mosquito vectors due to the fast development of chemical resistance in mosquito populations and the high costs of synthetic pesticides (Chellappandian et al., 2018). Vetiver essential oil has shown a promising repellent property against structural arthropod pests, e.g., termites, due to its long residual activity, as well as blood-sucking insects, e.g., mosquitoes and flies, due to its high repellent activity from its unique smell (Nuchuchua et al., 2009; Tisgratog et al., 2016). In this study, we investigated for the first time three chemical actions, comprising spatial repellency, contact irritancy, and toxicity of vetiver oil and five of its main constituents using the HITSS against three mosquito species, including *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus*, under laboratory-controlled conditions.

The constituents in vetiver oil, including α - and β -vetivone, khusimone, zizanal, and epizizanal, were found to be repellent to insects (Jain et al., 1982). Zhu et al. (2001) also reported that vetiver oil was found to be the most effective repellent with a major bioactive ingredient α - and β -vetivone. In this study, GC-MS analysis revealed that β -vetivone, khusimol, and α -vetivone are the main components of vetiver oil, with nootkatone as one of the minor components (Boonyuan et al., 2022). However, TCF (2013) reported that the major components of vetiver were vitiveryl acetate, vetiverol, vitivone, and terpenes.

Different bioactive compound produces distinguish behavioral responses and actions against a certain mosquito species. In 2018, vetiver oil and four bioactive constituents, valencene, terpinen-4-ol, isolongifolene, and vetiverol, were investigated against *An. minimus*, a major malaria vector species in Southeast Asia, including Thailand, using the HITSS (Tisgratog et al., 2018). The authors demonstrated that these four constituents of vetiver exhibited both behavioral and insecticidal actions depending upon the test concentrations and type of exposures. Similarly, in our study, vetiver oil and five constituents also showed variable behavioral responses, depending upon the type of assays including CIA, SRA and TOX.

Each mosquito species responded differently in escape patterns to the various test compounds. Barnard (1999) explained the differences in responses of mosquito species by their preference of food sources. *Aedes aegypti* and *Ae. albopictus* are mainly anthropophilic species with high biting activities in laboratory bioassays. *Culex quinquefasciatus* is mainly

Table 4
In vitro cytotoxic and phototoxic activity of vetiver components against mouse normal fibroblast (BALB/c 3T3) cell lines.

Compound	Without irradiation			With irradiation			PIF	Phototoxicity
	IC ₅₀ (µg.mL ⁻¹)	IC ₉₀ (µg.mL ⁻¹)	Slope	IC ₅₀ (µg.mL ⁻¹)	IC ₉₀ (µg.mL ⁻¹)	Slope		
Vetiver oil	5.29 ± 0.98	73.19 ± 1.65	12.45	13.74 ± 2.01	36.53 ± 3.37	4.44	0.38	Non-phototoxic
Terpinen-4-ol	>100	>100	ND	>100	>100	ND	N/A	Non-phototoxic
α -Terpineol	>100	>100	ND	>100	>100	ND	N/A	Non-phototoxic
Valencene	>100	>100	ND	>100	>100	ND	N/A	Non-phototoxic
Vetiverol	96.09 ± 6.22	>100	0.62	87.98 ± 8.26	>100	0.89	1.09	Non-phototoxic
Vetivone	>100	>100	ND	>100	>100	ND	N/A	Non-phototoxic
Chlorpromazine	48.9 ± 3.26	>100	0.99	1.05 ± 0.29	12.65	65.23	54.71	Phototoxic

Results are expressed as mean ± SD,
 IC₅₀ = Concentration inducing a 50% decrease of cell viability, IC₉₀ = Concentration inducing a 90% decrease of cell viability
 Slope = Cell viability decrease (%) observed at 1µg.mL⁻¹, as assessed by not linear regression analysis with Table Curve 2.0 software
 N/A = showed no sign of phototoxicity as indicated by the low to no PIF values as compared
 ND = non-Determined activity

Table 5
In vitro genotoxicity activity of vetiver oil and pure compounds on CHO-K1 cells.

Compound(% or µg.mL ⁻¹)	Assay performed without S9 mix			Assay performed with S9 mix		
	Proliferative Index (%)	MNC(per 1,000)	P	Proliferative Index (%)	MNC(per 1,000)	P
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
Vetiver oil	0.1	98.6	NS	99.4	10.5±1.4	NS
	0.5	96.8	NS	97.5	9.5±0.7	NS
	1	81.2	NS	85.6	11.5±1.4	NS
	5	TOX	-	TOX	-	NS
	5	99.8	NS	99.9	10.5±2.1	NS
Terpinen-4-ol	5	99.8	NS	99.9	10.5±2.1	NS
	10	99.1	NS	98.9	10.5±0.7	NS
	50	89.5	NS	86.4	12.0±2.8	NS
	100	82.4	NS	78.6	10.5±0.7	NS
	100	TOX	-	TOX	-	NS
α -Terpineol	5	99.3	NS	99.4	8.5 ± 0.7	NS
	10	93.5	NS	95.3	9.0±1.4	NS
	50	78.4	NS	80.2	13.0±1.4	NS
	100	TOX	-	TOX	-	NS
	100	TOX	-	TOX	-	NS
Valencene	5	100	NS	98.7	12.5±0.7	NS
	10	99.6	NS	99.4	12.5±0.7	NS
	50	94.5	NS	89.1	12.0±2.8	NS
	100	89.5	NS	86.6	12.5±0.7	NS
	100	TOX	-	TOX	-	NS
Vetiverol	5	98.7	NS	99.2	9.0±1.4	NS
	10	88.4	NS	97.8	12.5±2.1	NS
	50	81.2	NS	74.1	11.5±2.1	NS
	100	TOX	-	TOX	-	NS
	100	TOX	-	TOX	-	NS
Vetivone	5	98.4	NS	97.4	10.0±1.4	NS
	10	92.2	NS	91.3	12.5±0.7	NS
	50	87.3	NS	87.3	12.0±2.8	NS
	100	78.5	NS	79.3	12.5±0.7	NS
	100	TOX	-	TOX	-	NS

[§] Positive controls: mitomycin C (0.05 µg.mL⁻¹) without S9 mix and benzo-[a]-pyrene (5 µg.mL⁻¹) with S9 mix; MNC: Micronucleated cells per 1,000; P: Probability of the comparison between the negative control and the tested dose using the χ^2 test; TOX: Toxic.

^a NS: non-significant activity; Results are expressed as mean ± SD

an ornithophilic species, which has only reduced appetite in laboratory trials. Therefore, using the plant-based insect repellent compounds against mosquitoes need thorough laboratory test evaluation on factors that may influence mosquito behavior, e.g., chemical compositions, test concentrations of the essential oils and mosquito species (Rehman et al., 2014).

In the present study, vetiver oil and all five pure compounds tested had strong spatial repellency, contact irritancy, and toxicity against *Cx. quinquefasciatus*. One study examining the repellent effect on the olfactory system of *Culex* mosquito antennal sensilla neurons showed that terpene-derived chemical repellents produce stronger behavioral avoidance than non-terpene-derived chemicals such as dimethyl phthalate (Liu et al., 2013). In addition, our results showed a strong knockdown and mortality response by *Cx. quinquefasciatus* when exposed to valencene and vetiverol, two sesquiterpenes, at 5%. These findings were

similar to a study conducted by Tisgratog et al. (2018) in which valencene and vetiverol at 5% killed *An. minimus* up to 62% and 71%, respectively.

Plant essential oils frequently exhibit stronger insecticidal activity in comparison to each of their individual constituents (Tak et al., 2016). Our results showed that vetiver oil at 2.5 and 5% had a strong contact irritancy against all three species tested. Moreover, *Cx. quinquefasciatus* exhibited much stronger irritancy escape responses against vetiver oil and its constituents than *Ae. aegypti* and *Ae. albopictus*. In contrast, terpinen-4-ol and vetivone displayed the least contact irritancy on *Ae. aegypti* and *Ae. albopictus*. For spatial repellency, at 2.5 and 5% concentrations, vetiver, valencene, and vetiverol produced the highest SAI against all three mosquito species. In a previous study, Nararak et al. (2016) observed a great excito-repellency activity of vetiver oil against *An. minimus* using an excito-repellency test system. Ve-

tiver oil at 1-5% showed strong irritant effects with >80% escape, while repellent effects of vetiver oil was observed at 5% concentration with 83.9% escape. Sathantriphop et al. (2015) also tested CIA and SRA actions of six repellent essential oils and pure compounds (citronella, hairy basil, catnip, vetiver, DEET, and picaridin) against *Ae. aegypti* and *An. minimus* using HITSS. The results showed that vetiver oil had the greatest repellency effect against *An. minimus* (0.5-2%), which was less pronounced against *Ae. aegypti* at the same concentration. Moreover, our results showed that vetiver oil and their component presented strong repellency against *Ae. aegypti* from 2.5-5%. Guo et al. (2019) reported the biological activity of valencene at 0.3%, which exhibited strong repellency actions against *Tribolium castaneum* (Herbst) adults (red flour beetles). Appropriate formulations using synergistic and additive interactions among constituents of these oils await development to increase the effectiveness and persistence of vetiver repellent activity.

The greatest responses of TOX were seen from the vetiver oil followed by valencene, vetiverol, and terpinene-4-ol. Similar study by Afshar et al. (2017) reported the toxicity of *Helosciadium nodiflorum* (L.) Koch (Apiaceae) essential oil and its main constituents against the cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera). The result showed that the complete oil was the most potent and showed the lower LD₅₀ values (LD₅₀ = 101.6-128.4 µg/larva) than other components, which were limonene (LD₅₀ = 427.3 µg/larva), (Z)-β-ocimene (LD₅₀ = 771.9 µg/larva), terpinolene (LD₅₀ = 699.7 µg/larva), β-pinene (LD₅₀ = 614.9 µg/larva), and ecoTrol™, containing rosemary essential oil (LD₅₀ = 29.5 µg/larva) (Afshar et al., 2017). It is therefore possible that the toxicity showed in the present study is the result of synergistic action of various constituents of vetiver oil. Further experiments of synergistic phenomena would provide an insight into the characterization of vetiver oil and its constituents biological properties.

Natural plant-based repellents are one of the best alternatives to chemical repellents (Asadollahi et al., 2019). Many topical repellents contain essential oils, e.g., citronella, catnip, eucalyptus, etc (Da Silva and Ricci-Júnior, 2020). However, some essential oils or terpenes are known to irritate the skin and mucous membranes, and prolonged exposure to them had caused contact dermatitis (Türkmenoğlu and Özmen, 2021). The shortage of toxicological studies of natural products is also an argument against their use, due to concerns for potential mutagenic or genotoxic effects (Pavela and Benelli, 2016). The phototoxicity results obtained using the *in vitro* method are important because topical repellent formulations are also used during day-time to protect against day-biting mosquitoes such as *Aedes* species, and this involves exposure to the sun or artificial light. It is imperative to conduct extensive research to ensure the activity or effect on human users. The current study showed that the tested compounds were neither cytotoxic nor phototoxic. Likewise, this study also showed that all tested repellents did not induce genotoxicity at the chromosomal level, as observed in the micronucleus assay. Sinha et al. (2014) demonstrated that the vetiver oil induced cytotoxicity and genotoxicity at higher concentrations (400-800 µg/mL). Based on the results, the vetiver oil is considered safe for human topical use at low concentrations (25-400 µg/mL). Acute toxicity determination indicated that vetiver oil has LD₅₀ values of 2985.38 mg/kg, which is practically less toxic at oral doses in rat (Tripathi et al., 2006).

Topical repellents offer much promise as potential tools for prevention against indoor and outdoor biting vectors. This study produced the first findings on the contact irritancy, spatial repellency, and toxicity of vetiver compounds against three main arbovirus vectors. This research showed for the first time that vetiver oil, three sesquiterpenes (valencene, vetiverol, and vetivone), and two monoterpenic alcohols (terpinen-4-ol and α-terpineol) could be used as plant-based repellents or green insecticides. Topical repellents could offer adequate personal protection, especially against outdoor-biting mosquitoes that have very little ways to be controlled. Then, they could be potentially used as a complement to other vector control methods under an integrated vector control management strategy. Repellent products obtained from these

studies should be tested in the field to further support the effectiveness of the test compounds in natural settings because human host and environmental factors could potentially affect a repellent activity.

Author Contributions

SM and TC conceived and designed the experiments. JN, CS and CDG performed the experiments. JN, KT and CDG analyzed the data. JN, CS, US and CDG wrote the manuscript. SM, VML, US, EO and TC revised and edited the manuscript. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2022.100044.

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