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Keywords
Vetiver oil, contact irritancy, spatial repellency, Anopheles minimus, natural repellents

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Research Article

Evaluation of the Constituents of Vetiver Oil Against *Anopheles minimus* (Diptera: Culicidae), a Malaria Vector in Thailand

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Abstract

The development of resistance by mosquitoes to current synthetic compounds has resulted in reduced effectiveness of prevention and control methods worldwide. An alternative nonchemical based control tools are needed to be evaluated particularly plant-derived essential oils. Several components of vetiver oil have been documented as insect repellents. However, detailed knowledge of those components action against insect remains unknown. In this study, behavioral response of *Anopheles minimus* to four constituents of vetiver oil (valencene, terpinen-4-ol, isolongifolene, vetiverol) was evaluated by using the high-throughput screening assay system. Vetiverol and isolongifolene exhibited strong contact irritancy action at 1.0% (80.2% escaping) and 5.0% (81.7% escaping) concentration, respectively, while moderate action was found in both valencene and terpinen-4-ol at 5.0% (57.6% escaping). Only at 1.0% (0.7 spatial activity index [SAI]) and 5.0% (1.0 SAI) of valencene and 0.5% (0.7 SAI) of isolongifolene showed spatial repellency activity. High mortality (58.9–98.2%) was recorded in all concentration of vetiverol and isolongifolene. Meanwhile, valencene exhibited high mortality only at 5.0%, terpinen-4-ol showed very low toxic action (0–4.3%) in all concentration. These proved that valencene in vetiver oil is the promising constituent that can be developed as an alternative mosquito control mean in efforts to prevent disease transmission.

Key words: Vetiver oil, contact irritancy, spatial repellency, *Anopheles minimus*, natural repellents

Malaria is one of the most common infectious diseases and a worldwide public health problem, including the country of Thailand (Manguin et al. 2008, Sriwichai et al. 2016). The greatest number of cases of malaria continues to occur in provinces that share a border with Myanmar, Cambodia, and Laos, especially in forested and forest fringe areas of these provinces (CDC 2013, Sriwichai et al. 2016). The number of confirmed malaria cases reported in these regions of Thailand demonstrated a decrease from 2.9 million to 1.6 million cases between the years 2000 to 2014. Thailand projects to achieve an additional 50% decrease in case incidence by 2015 (WHO 2016). In 2014, a reported 37,921 confirmed cases and 38 reported deaths occurred in nationwide (WHO 2016) and 5,933 known malaria cases and four deaths in 2015 (Bureau of Epidemiology 2016). In 2015, the proportion between *Plasmodium falciparum* and *P. vivax* was 1:1 (*P. falciparum* 50% [up to 75% in some areas] and *P. vivax* 50% [up to 60% in some areas]) with *Plasmodium vivax* being higher on the Thai side of the border (CDC 2016). *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi* have been found in small portions (CDC 2013).

Anopheles minimus is the primary malaria vector in Thailand (Rattanarithikul et al. 2006, Manguin et al. 2008, Saeung 2012). Indoor residual spraying, impregnated mosquito nets, and alternative vector control strategies such as fogging, the use of mosquito repellents, and bioenvironmental control have all been used in the current mosquito control programs in Thailand.

The use of synthetic insecticides for mosquito control has negatively impacted biological control efforts as the result of their effects on nontarget organisms and natural predators (Gill and Garg 2014). It has also resulted in the development of insecticide resistance as well as having undesirable effects on the environment and human health (Thomas et al. 2004, Gokulakrishnan et al. 2013). Safer and more environmental friendly alternatives, such as plant essential oils,

need to be evaluated for their use in mosquito control (Sukumar 1991, Mulla and Su 1999).

The mosquito repellent and antibiting properties of many plant essential oils have been known for a considerable amount of time (Curtis 1992, Tawatsin et al. 2001). Those oils most commonly reported as insect repellents include citronella, clove, makaen and patchouli (pim sane bai), cedar, verbena, geranium, lavender, pine, cinnamon, rosemary, basil, thyme, garlic, peppermint, and vetiver (Sharma et al. 1993, Zhu et al. 2001, Trongtokit et al. 2005). However, these oils are composed of a complex blend of constituents which may or may not elicit behavior-modifying activity. Therefore, it is critical that a thorough evaluation and understanding of the constituents of these essential oils be researched.

The excitorepellency test system and the arm-in-cage test have both been used to characterize the behavioral responses and protection time in mosquitoes to chemicals and essential oil (Tawatsin et al. 2001, Sathantriphop et al. 2014, Nararak et al. 2016). Vetiver (*Vetiveria zizanioides*) oil was evaluated as an insect repellent against several species of mosquitoes (Tawatsin et al. 2001, Sathantriphop et al. 2014). The components of vetiver oil have also been found to have strong repelling actions against a variety of insect species (Zhu et al. 2001). More than 300 compounds have been identified in vetiver oil with α- and β-vetivones comprising the major constituents (St Pfau and Plattner 1939). Six other compounds in vetiver oil have demonstrated some repellent properties against arthropods and these include α-vetivone, β-vetivone, khusimone, zizanal, epizizanal and (+)-(1*S*, 10*R*)-1, 10-dimethyl bicyclo [4,4,0]-dec-6-en-3-one (Jain et al. 1982).

The high-throughput screening assay system (HITSS) was developed by Achee et al. (2009) and Grieco et al. (2005). The system was used in this research to characterize the contact irritant, spatial repellent, and mortality actions of four constituents of vetiver oil at four concentrations. Although several published articles described repelling action of some components of vetiver oil to some insects, none observed these four components.

Contact irritancy means the insects make physical contact with chemically-treated surfaces, whereas spatial repellency results from the insects make avoidance from a chemical substance detected from a distance without making physical contact (Roberts et al. 1997). The toxicity means the number of mortality after exposure. The evaluation of the dose response relationship with these compounds is vital to understanding how best to use the constituent against biting insects. The goal of this study is finding the important information for evaluation and development of repellent compounds.

Materials and Methods

Mosquito

Anopheles minimus (laboratory) has been maintained in the Department of Entomology, Kasetsart University for >15 y. The colony originated from the Malaria Division, Department of Communicable Disease Control (CDC), Ministry of Public Health, Nonthaburi, Thailand, and was established in 1993. Female mosquitoes were provided a human blood via an artificial membrane feeding system on the fifth–seventh day post emergence. Resulting eggs were harvested and hatched in white plastic pans (BioQuip Products, 2321 Gladwick Street Rancho Dominguez, CA 90220). Larvae were reared in pans under identical physical and nutritional conditions throughout the study period. Four- to 6-day-old female mosquitoes were used for all tests. All adult mosquito test cohorts were denied blood and were maintained on a 10% sugar solution for nutritional energy. The sugar solution was removed, and mosquitoes were starved for at least 12 h prior to testing.

Repellents

A series of components from vetiver oil were isolated using gas chromatography/mass spectrometry. Of these components, four were selected for testing using the HITSS to characterize the irritant, repellent, and toxic actions of each constituent against *An. minimus*. The components included valencene, terpinen-4-ol, isolongifolene, and vetiverol. Valencene and terpinen-4-ol were provided by Professor Dr. Joel R. Coats Iowa State University. Isolongifolene used in this study was perchased from Sigma-Aldrich Company, 3050 Spruce Street, St. Louis, MO and vetiverol was supplied from Dr. Kamlesh R. Chauhan, USDA, MD. Four concentrations (0.5, 1.0, 2.5, and 5.0%) of each component were investigated in this study. There are four components chosen for this study, including valencene, terpinen-4-ol, isolongifolene, and vetiverol. Several published articles describe the repelling action of vetiver oil to some insect species; however, none of these studies were conducted using the components of the raw oil. For this reason, the components of vetiver oil were identified using gas chromatography/mass spectrometry and a selection of the resulting constituents was made based on their commercial availability. One component (isolongifolene) was acquired from Sigma-Aldrich and three components (valencene, terpinen-4-ol, and vetiverol) were obtained from a collaborating laboratory at the USDA, Beltsville, MD and Iowa State University, IA.

Impregnated Netting

Reagent grade valencene, terpinen-4-ol, isolongifolene, and vetiverol were dissolved in absolute ethanol. This solution was then applied evenly by micropipette across the surface of 11 × 25 cm pieces of nylon organdy netting (No. I10N, G-Street Fabrics, Bethesda, MD) and allowed to air-dry a minimum of 1 h prior to use.

Study Parameters

The assays were tested within 1–7 h of treating the nettings, and testing was carried out between the hours of 9:00 a.m. and 5.00 p.m. For each component tested, the response to the lowest treatment concentration was evaluated first, followed by the next highest concentration. For all test days, the laboratory temperature averaged 24°C (range 23–26°C) and the relative humidity averaged 75% (range 70–80). Assay cleaning involved washing all parts of the system that were in direct contact with treated materials with acetone. All other components of the assay were washed in a detergent solution (Liqui-Nox, Aloconox, Inc., New York). Before reuse, both acetone- and detergent-washed components were allowed to air-dry overnight.

System Testing

Contact irritant, spatial repellent, and toxic behaviors were evaluated using a HITSS previously described by Grieco et al. (2005) and recently adopted by the WHO as a standard procedure for in vitro efficacy testing of spatial repellents.

Contact Irritancy Assay

A clear cylinder and the treatment cylinder were connected with a linking section so that the narrow end of the funnel pointed toward the clear cylinder. The linking section's butterfly valve was turned to the closed position. An end cap was then placed on the open end of the clear cylinder, and opaque-felt cloth was wrapped around the clear cylinder and placed over the viewing port on the end cap

to prevent light from enducing any type of phototactic response. A metal drum, with treatment netting affixed to it, was inserted into the metal cylinder and an end cap was affixed to the completed chamber. The viewing port of the end cap was also covered with opaque-felt cloth. Ten mosquitoes were transferred into the treatment end of the assembly and, after 30 s, the butterfly valves were placed in the open position. After 10 min, the valve was again closed, and counts were immediately made of the number of mosquitoes in the clear ends of the assay (number escaping) along with the numbers of mosquitoes that appeared to be knocked down. For all trials, a second assay was simultaneously run to serve as a control in which the treatment was an ethanol-treated net. The numbers of mosquitoes escaping from the control chamber were used to correct for the number of mosquitoes escaping the treatment chamber. Six replicates were done at each treatment concentration (Grieco et al. 2005, Achee et al. 2009).

Spatial Repellency Assay

The spatial repellency assay (SRA) dual-choice chamber system, which allows the observation of directional mosquito movement in response to a single chemical stimulus outside the context of host cues, consists of a clear Plexiglass central unit connected at opposite ends to one treatment chamber housing repellent-treated netting and one control chamber housing a net treated with acetone only.

Briefly, cohorts of 20 mosquitoes were introduced into the central HITSS chamber and, after a 30-s acclimation period, butterfly valves situated at both ends of the central chamber were opened simultaneously to allow free movement of mosquitoes in either direction into either end chamber. After 10-min exposure period, the butterfly valves were closed and the numbers of mosquitoes in each chamber were counted. Spatial repellency is measured 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 (Grieco et al. 2005, Achee et al. 2009).

Toxicity Assay

The assembly configuration for this assay was similar to the contact irritancy assay (CIA), minus the clear cylinder and its end cap. After preparing a chamber to include the appropriate treatment netting and assembling the test unit, 20 mosquitoes were transferred into the chamber, and the test unit was set in the cradle. After 1 h, the number of knocked down mosquitoes were recorded and all (knocked down and those still mobile) were transferred to holding cartons. These mosquitoes were provided a 10% sucrose-soaked cotton ball and returned to the insectary. Their mortality was recorded after 24 h. As with the CIA and for all trials, an accompanying assay in which the treatment was ethanol-treated netting served as a control. The ratio of treatment to control assays was either 1:1 or 1:2. Six replicates were done at each treatment concentration (Grieco et al. 2005, Achee et al. 2009).

Data Analysis

The CIA and SRA data were analyzed, as previously described (Grieco et al. 2005, Achee et al. 2009). The Wilcoxon two-sample test was used to analyze and interpret the CIA (SAS Institute 1999). One-way analysis of variance and Student–Newman–Keuls multiple mean comparison tests were conducted to compare corrected percentage of escape (i.e., magnitude of response) among test populations. A spatial activity index (SAI) based on the oviposition activity index of Kramer and Mulla (1979) was used to interpret the SRA (SAS Institute 1999). The SAI value was calculated for each chemical using the following equation: SAI=(Nc – Nt)/(Nc + Nt), where Nc is the number of females in the control chamber and Nt is the number of females in the treated chamber. The SAI varies from –1 to 1, with 0 indicating no attractant or repellent response. An SAI value of –1 indicates that a greater proportion of mosquitoes moved into the treatment chamber than the control chamber, indicating an attractant response. A SAI value of 1 indicates a greater proportion of mosquitoes moved into the control chamber (away from the treatment end of the assay device), indicating a repellent response. A weighted SAI was also performed to factor into account the percentage of mosquitoes responding within a particular SRA (SAS Institute 1999). This was used to interpret variations in magnitude of response among test populations.

Results

Contact Irritancy

Anopheles minimus showed a significant contact irritancy response to most of the constituents of vetiver oil (Table 1). Statistically significant differences in the percent of escaping mosquitoes were found at all concentrations when exposed to isolongifolene and vetiverol and was also seen for valencene and terpinen-4-ol at a 5% concentration between treatment and control. The corrected percent escaping ranged between 0.00 and 81.7% for all four constituents. The lowest corrected percent escaping was seen in terpinen-4-ol at a 0.5% concentration whereas the highest occurred for isolongifolene at a 5.0% concentration. Moderate behavioral responses were observed from valencene at 1.0 and 2.5%, with the percent escaping equal to 11.7 and 13.3%, respectively.

Spatial Repellency

Anopheles minimus was repelled at 1.0 and 5.0% of valencene and 0.5% of isolongifolene. No significant spatial repellent activity was documented for terpinen-4-ol or vetiverol (Table 2). Other constituents tested also showed no repellent activity in *Anopheles* mosquitoes.

Toxicity

Two constituents (isolongifolene and vetiverol) of vetiver oil resulted in high knockdown and mortality at all four concentrations tested whereas terpinen-4-ol resulted in very low levels of both knockdown and mortality. The percentage of mosquito mortality in relation to both valencene and terpinen-4-ol exposure was associated with increased concentration. The lowest concentration (0.5%) of vetiverol resulted in the highest mortality (98.2%) when compared to other concentrations tested. At 5% of each constituent, isolongifolene resulted in a greater knockdown and mortality (87.2%) as compared with vetiverol (71.0%), valencene (62.0%), and terpinen-4-ol (4.3%), respectively. No knockdown and mortality were investigated from valencene at 0.5% and terpinen-4-ol at 0.5 and 1.0% (Table 3).

Dose Response Curve for CIA

The strong contact irritancy action was observed from isolongifolene at 5% (81.7) and 2.5% (72.8), vetiverol at 1.0% (80.2) and 0.5% (73.5) when exposed to *An. minimus* (laboratory strain). The corrected percent escaping was found higher when tested with isolongofolene and vetiverol whereas terpinen-4-ol and valencene at 0.5–2.5% gave lower percentage responses (0–13.3) except at 5.0% of each component (54.3 and 57.6, respectively) (Fig. 1).

Table 1. Responses of female *Anopheles minimus* in the contact irritancy assay to four different concentrations of each constituent of vetiver oil (valencene, terpinen-4-ol, isolongifolene, vetiverol)

Repellent	Concentration (%)	Number of trials (no. mosquitoes)	Number escaping (mean ± SE)		Corrected percent escaping (mean ± SE)	*P*
			Treated	Control		
Valencene	0.5	6(61)	0.3 ± 0.2	0.0 ± 0.0	1.7 ± 3.1	0.4545
	1.0	6(60)	1.2 ± 0.7	0.0 ± 0.0	11.7 ± 6.5	0.1818
	2.5	6(60)	1.3 ± 0.5	0.0 ± 0.0	13.3 ± 4.9	0.0606
	5.0	6(60)	5.5 ± 0.9	0.2 ± 0.2	54.3 ± 9.2	0.0022*
Terpinen-4-ol	0.5	6(60)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.0000
	1.0	6(60)	0.3 ± 0.2	0.0 ± 0.0	3.3 ± 2.1	0.4545
	2.5	6(60)	0.3 ± 0.3	0.0 ± 0.0	3.3 ± 3.3	1.0000
	5.0	6(60)	5.8 ± 0.5	0.2 ± 0.2	57.6 ± 4.8	0.0022*
Isolongifolene	0.5	6(60)	7.2 ± 0.6	0.3 ± 0.2	70.0 ± 6.0	0.0022*
	1.0	6(58)	5.5 ± 0.9	0.3 ± 0.2	54.8 ± 9.1	0.0022*
	2.5	6(60)	7.2 ± 0.7	0.5 ± 0.3	72.8 ± 6.5	0.0022*
	5.0	6(61)	8.3 ± 0.5	0.5 ± 0.3	81.7 ± 4.9	0.0022*
Vetiverol	0.5	6(58)	7.0 ± 0.7	0.3 ± 0.2	73.5 ± 8.0	0.0022*
	1.0	6(60)	7.7 ± 0.9	0.3 ± 0.2	80.2 ± 5.4	0.0022*
	2.5	6(58)	6.8 ± 0.3	0.2 ± 0.2	70.2 ± 5.7	0.0022*
	5.0	6(62)	6.8 ± 1.2	0.2 ± 0.2	67.0 ± 11.6	0.0022*

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

Table 2. Responses of female *Anopheles minimus* in the spatial repellency assay to four different concentrations of each constituent of vetiver oil (valencene, terpinen-4-ol, isolongifolene, vetiverol)

Repellent	Concentration (%)	Number of trials (no. mosquitoes)	Mean percent responding (SE)	Mean SAI (SE)	*P*
Valencene	0.5	9 (181)	0.0 (0.0)	0.0 (0.0)	1.0000
	1.0	9 (176)	5.1 (1.7)	0.7 (0.2)	0.0313*
	2.5	9 (177)	5.2 (2.3)	0.6 (0.2)	0.0625
	5.0	9 (189)	14.1 (2.3)	1.0 (0.0)	0.0039*
Terpinen-4-ol	0.5	9 (180)	19.9 (3.5)	-0.1 (0.3)	0.8047
	1.0	9 (175)	10.8 (2.1)	-0.1 (0.3)	0.8438
	2.5	9 (177)	11.8 (1.6)	0.6 (0.2)	0.1250
	5.0	9 (179)	6.8 (2.7)	0.3 (0.2)	0.3750
Isolongifolene	0.5	9 (175)	7.3 (1.7)	0.7 (0.2)	0.0156*
	1.0	9 (174)	1.8 (1.3)	0.2 (0.1)	0.5000
	2.5	9 (176)	3.9 (2.2)	0.4 (0.2)	0.1250
	5.0	9 (182)	4.5 (1.6)	0.4 (0.2)	0.1250
Vetiverol	0.5	9 (180)	5.6 (1.7)	0.6 (0.2)	0.0625
	1.0	9 (175)	5.6 (1.9)	0.3 (0.2)	0.2500
	2.5	9 (177)	2.3 (0.9)	-0.2 (0.2)	0.6250
	5.0	9 (175)	5.1 (1.9)	-0.1 (0.2)	1.0000

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

Dose Response Curve for SRA

The highest mean percent escaping was found from terpinen-4-ol at 0.5% (19.9), valencene at 5.0% (14.1), terpinen-4-ol at 2.5% (11.8) and 1.0% (10.8), respectively. The results showed lowest escape responding from valencene at 0.5% (0), isolongofolene at 1.0% (1.8), vetiverol at 2.5% (2.3), and isolongifolene at 5.0% (4.5) (Fig. 2).

Discussion

Vector control involves using proven methods to eradicate or control disease-carrying insects with the ultimate goal of eliminating the transmission and spread of vector-borne disease. Chemical control that results in a toxic action remains the most effective method of mosquito control as either larvicides applied to aquatic habitats or adulticides applied as space sprays, indoor residual sprays, or insecticide-treated materials (Suwansirisilp et al. 2012, Chareonviriyaphap et al. 2013). In Thailand, at least four classes of chemical have been labeled for use in vector control: organochlorines, organophosphates, carbamates, and pyrethroids (Chareonviriyaphap et al. 2013). However, many mosquito populations have developed resistance to all four chemical classes with the highest percentage being resistant to the synthetic pyrethroids (Chareonviriyaphap et al. 2013). For this reason, this study was conducted to find alternative classes of chemistry with differing modes of action that could be employed for the control of biting insects. The primary focus, being on the components of vetiver oil to include valencene, terpinen-4-ol, isolongifolene, and vetiverol.

Table 3. Percentage of knockdown and 24-h mortality of female *Anopheles minimus* after exposure to four different concentrations of each constituent of vetiver oil (valencene, terpinen-4-ol, isolongifolene, vetiverol)

Repellent	Concentration (%)	Number of trials (no. of mosquitoes)	% knockdown (SE)	% mortality (SE)
Valencene	0.5	6 (117)	0 (0)	0 (0)
	1.0	6 (116)	0 (0)	2.59 (0.55)
	2.5	6 (119)	12.60 (0.84)	25.21 (2.10)
	5.0	6 (116)	96.55 (2.16)	62.07 (3.95)
Terpinen-4-ol	0.5	6 (117)	0 (0)	0 (0)
	1.0	6 (119)	0 (0)	0 (0)
	2.5	6 (118)	0 (0)	1.69 (0.52)
	5.0	6 (115)	1.74 (0)	4.35 (1.17)
Isolongifolene	0.5	6 (117)	23.93 (2.66)	58.97 (4.46)
	1.0	6 (120)	61.67 (1.03)	87.50 (3.27)
	2.5	6 (113)	84.07 (3.31)	96.46 (1.83)
	5.0	6 (125)	83.20 (1.37)	87.20 (2.04)
Vetiverol	0.5	6 (115)	85.22 (1.51)	98.26 (0.75)
	1.0	6 (117)	89.74 (1.87)	63.25 (3.83)
	2.5	6 (118)	88.98 (1.87)	71.86 (2.28)
	5.0	6 (114)	87.72 (2.66)	71.05 (2.59)

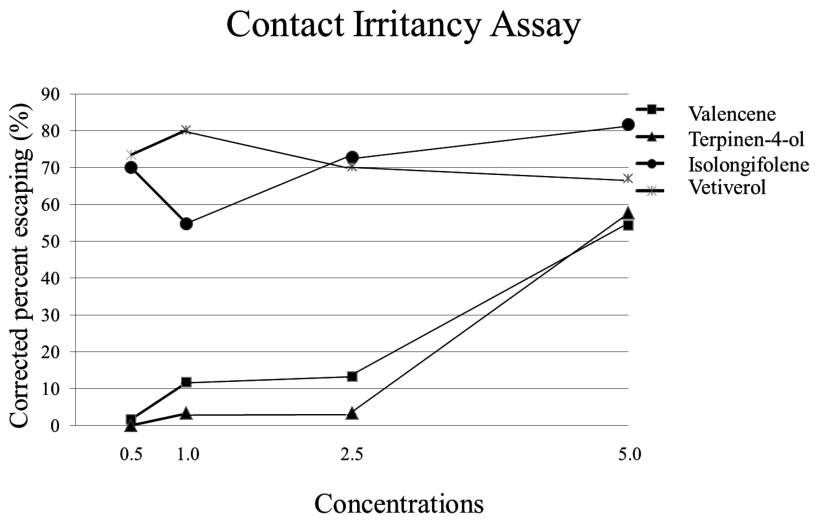

Fig. 1. Dose response curve from contact irritancy assay of *Anopheles minimus* exposed to four components of vetiver oil (valencene, terpinen-4-ol, isolongifolene, vetiverol).

Our results showed a strong behavioral response by *An. minimus* when exposed to valencene at 5.0%, with 54.3% of the mosquitoes escaping from the CIA and 14.1% being repelled from the SRA. The percent mortality was found to be 62.0%. These findings were similar to a study conducted by Zhu et al. (2003) that reported valencene exhibited a weak repellent activity in termites whereas nootkatone (synthesized from the sesquiterpene hydrocarbon valencene) exhibited strong repellent and toxic effects in both termites and ticks (Dietrich et al. 2006, Zhu et al. 2010). These findings are encouraging given that both valencene and its constituents are currently being systhesized as food additives (Beekwilder et al. 2014).

The mean spatial repellent response (6.8–19.9%) from terpinen-4-ol was relatively high as compared with other constituents of vetiver oil. As such, it represents the compound with the greatest potential to serve as a topical repellent from those compounds tested in the study. Isman (2000) reported on the biological activity of this chemical when it was determined that it possessed both contact and fumigant toxic actions against the bean weevil as well as repellent actions in the Brown tick (*Rhipicephalus appendiculatus*) and Maize weevil (*Sitophilus zeamais* [Coleoptera: Curculionidae]) (Ndungu et al. 1995, Jaenson et al. 2005). The high vapor pressure of this compound makes it an ideal candidate for use as a repellent as is its ability to affect insects in multiple orders.

The primary action of isolongifolene and vetiverol was found to be as contact irritants and toxicants with little or no spatial repellent activity indicated. Isolongifolene has long been used as a natural insecticide and pesticide. The current findings differ slightly with previous work that demonstrated that isolongifolene exhibits insect repelling activities (Zhang et al. 2011). More specifically, isolongifolene has been used as a repellent against *Aedes aegypti* (Diptera: Culicidae) and *Anopheles stephensi* with the results demonstrating this compound to be more effective than standard repellent (DEET) in laboratory bioassays (Zhang et al. 2009). The reasons for the differences are the result of test modality. Zhang et al (2009) utilized the K&D assay system which measures antibiting but cannot discriminate between contact irritancy and spatial repellency, both of which can result in an antibiting response. It is very much possible that the contact irritancy that was documented in our study was the ultimate driver for the repellency (antibiting) documented in the previous study.

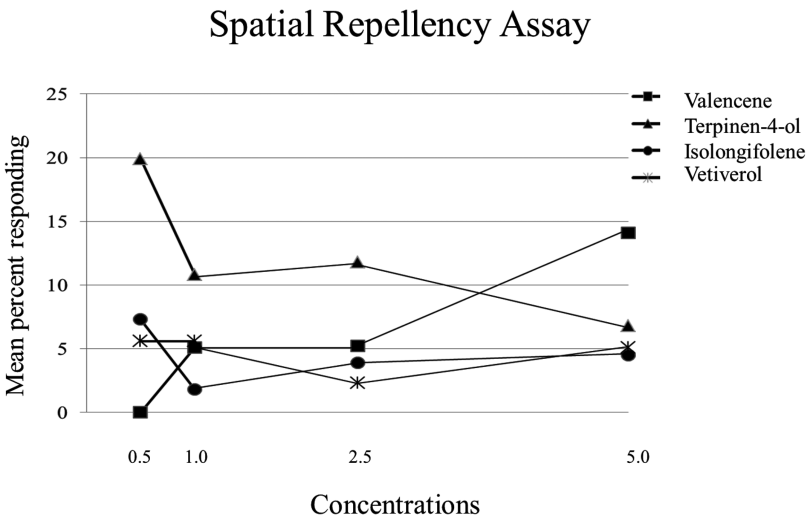

Fig. 2. Dose response curve from spatial repellency assay of *Anopheles minimus* exposed to four components of vetiver oil (valencene, terpinen-4-ol, isolongifolene, vetiverol).

Given that insecticide resistance is quickly reducing our arsenal of tools available for mosquito control, it is imperative that new chemicals and novel modes of action are explored to combat this growing problem. This study has identified several of the components of vetiver oil that could be used in the absence of these commonly used insecticides. These compounds are already commercially available making them suitable candidates for immediate use. It is important to note that, although these chemicals show promise as irritants and toxicants against *An. minimus*, more work is needed to determine their effect against a range of insect vectors. It is also important to note that this study did not address the issue of duration. Future evaluations should be conducted to determine the effect of these chemicals over time. In many regards, the true impact that these chemicals could have directly related to the duration of protection that they could impart to the user.

This study demonstrates that there are a number of natural products that could be used to protect humans from the bite of blood-feeding insects. These chemicals function in a range of ways to modify insect behavior to inhibit the biting response. The detailed knowledge of how and at what doses behavioral responses and toxicity occur in mosquitoes is critical to any screening program aimed at discovering novel active ingredients to be used for vector control. Such research must be conducted as we strive to reach malaria elimination and continue our battle against the re-immergence vector-borne diseases such as Dengue and Zika.

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