

Ovicidal and Larvicidal Effects of Garlic and Asafoetida Essential Oils Against West Nile Virus Vectors

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Subject Editor: Nannan Liu

Received 15 February 2018; Editorial decision 26 March 2018

Abstract

We examined the chemical composition of garlic and asafoetida essential oils and their individual and combined toxicity against larvae of *Culex pipiens* Linnaeus and *Culex restuans* Theobald (Diptera: Culicidae). The effect of the two essential oils on egg hatch was also examined. Ten and 12 compounds, respectively, were identified in garlic and asafoetida essential oils. Allyl disulfide (49.13%) and diallyl trisulfide (31.08%) were the most abundant compounds in garlic essential oil accounting for 80.2% of the total oil. In contrast, (E)-sec-butyl propenyl disulfide (30.03%), (Z)-sec-butyl propenyl disulfide (24.32%), and disulfide, methyl 1-(methylthio)propyl (21.87%) were the most abundant compounds in asafoetida essential oil. Allyl disulfide accounted for 7.38% of the total oil in asafoetida essential oil and was one of only three compounds found in both oils. For both mosquito species, garlic essential oil was more toxic than asafoetida essential oil with *Cx. restuans* (LC₅₀: garlic = 2.7 ppm; asafoetida = 10.1 ppm) being more sensitive than *Cx. pipiens* (LC₅₀: garlic = 7.5 ppm; asafoetida = 13.5 ppm). When combined, the two essential oils had antagonistic effects. The majority of *Culex* egg rafts exposed to garlic (73.1%) or asafoetida (55.8%) essential oils failed to hatch and larvae of the few that did hatch mostly died as first instars. Allyl disulfide exhibited strong ovicidal and larvicidal activity suggesting its important contribution to the overall toxicity of the two essential oils. Thus, garlic and asafoetida essential oils are potent mosquito ovicides and larvicides but if used jointly, they could undermine vector control programs.

Key words: asafoetida, *Culex pipiens*, *Culex restuans*, essential oil, garlic

Mosquitoes transmit a variety of parasitic (e.g., malaria and lymphatic filariasis) and viral (e.g., dengue, Zika, and yellow fever) diseases that pose serious public health challenge worldwide. One of the key strategies for controlling these diseases involves the use of synthetic insecticides targeting both larval and adult stages of the primary vectors. Although this strategy has helped avert thousands of deaths related to mosquito-borne diseases, the rapid emergence of mosquito resistance to these chemicals and the ecological and public health risks posed by these chemicals has created an immediate need for alternative vector control products (Brogdon and McAllister 1998, Corbel et al. 2007, Aktar et al. 2009, Bisset et al. 2011, Chareonviriyaphap et al. 2013).

In many parts of the world, botanical insecticides were commonly used to control insects of agricultural and public health significance from ancient times (Isman 2006) until the practice was pushed into the periphery in the mid-1930s and 1950s when the major classes of synthetic insecticides were discovered. However, it is now being promoted as an attractive alternative to synthetic

insecticides. Among plant-derived compounds, essential oils have shown great potential for vector control because they are relatively easy to obtain, have low mammalian toxicity, degrade quickly in soil and water (Isman 2000), and elicit a variety of biological activities in mosquitoes ranging from ovicidal, larvicidal, and adulticidal effects to oviposition deterrent, feeding deterrent, and repellent effects (Chauhan et al. 2005; Amer and Mehlhorn 2006a,b; Chaiyasit et al. 2006; Moore et al. 2006; Tawatsin et al. 2006; Champakaew et al. 2007; Maia and Moore 2011; Warikoo et al. 2011). Unfortunately, the commercial market for these oils has lagged behind due to a myriad of factors, among them their inferior performance relative to synthetic insecticides and availability of other products with low environmental impact such as *Bacillus thuringiensis* var *israelensis* and *Lysinibacillus sphaericus*. These microbe-based products have a proven efficacy in mosquito control but in the long term, mosquitoes can develop resistance to them (Mulla et al. 2003, Stalinski et al. 2014). In this context, the discovery and development of new vector control products remain critical.

Although several hundreds of essential oils are commercially available, a substantial number of them have not been examined for their biological activity against mosquitoes. In addition, most studies focus on biological activity of individual oils. However, a growing number of studies have shown that certain essential oils can interact synergistically against the target insect populations including mosquitoes (Pavela 2008, 2015, Zibae and Khorram 2015, Benelli et al. 2017, Muturi et al. 2017). These studies suggest that in-depth understanding of the combined toxicity of essential oils may propel discovery of essential oils that may work in synergy against mosquitoes and could thus be developed for application in integrated vector management.

The purpose of this study was to evaluate the individual and combined effects of garlic (*Allium sativum* L.) and asafoetida (*Ferula asafoetida* L.) essential oils against larvae of *Culex pipiens* and *Culex restuans*, the primary vectors of West Nile virus (WNV) in northeastern United States. The ovicidal potential of the two essential oils against the two mosquito species was also assessed. Both mosquito species occur in large numbers in urban ecosystems where they colonize and develop in thousands of storm water catch basins that are intended for storm water management (Harbison et al. 2017). Although the larvicidal activity of garlic essential oil against mosquitoes has been documented before, it remains unknown how this oil may interact with other essential oils. In addition, the biological activity of asafoetida essential oil against mosquitoes remains poorly understood. The results of our study show that essential oils of these plants show promise for development as ovicides and larvicides.

Materials and Methods

Acute Toxicity of Essential Oils to *Cx. pipiens* and *Cx. restuans* Larvae

Bioassays were conducted using field-collected samples of *Cx. pipiens* and *Cx. restuans* from Peoria, IL. *Culex* egg rafts were collected using ovitraps baited with grass infusion prepared by fermenting approximately 500 g of fresh grass clippings in 160 liters of tap water for 5 d (Reiter 1986). Egg rafts from multiple locations were hatched individually in 300 ml of deionized (DI) water held in

tripour beakers and supplemented with 0.1 g of Tetramin fish food (Spectrum Brands Inc., Blacksburg, VA). The containers were maintained at 26°C, 70% relative humidity, and 10:14 light: dark cycle. At first instar stage, a single larva from each container was identified either as *Cx. pipiens* or *Cx. restuans* based on the presence of a clear scale anterior to the sclerotized egg-breaker which is lacking in *Cx. pipiens* but present in *Cx. restuans* (Crabtree et al. 1995). The species identity was further confirmed at the third instar stage based on the arrangement and morphology of setae on the siphon. For each species, late third instar larvae from all locations were pooled for use in bioassays.

Twenty late third instar larvae of either *Cx. pipiens* or *Cx. restuans* were added into 120 ml of DI water held in 400-ml tripour beakers. Treatments included garlic essential oil, asafoetida essential oil purchased from New Directions Aromatics (Mississauga, Ontario Canada), a combination of the two essential oils, and allyl disulfide ($\geq 80\%$, Sigma-Aldrich, St. Louis, MO), which was one of the major constituents of garlic essential oil and a minor constituent of asafoetida essential oil (Table 1). The two essential oils were extracted through steam distillation of bulbs (garlic) and stem and roots (asafoetida) originating from India. Each treatment was tested at 10 concentrations ranging from 2 to 20 ppm. A control group received 20 μ l of absolute ethanol without oil treatment. The stock solution for each essential oil (100,000 ppm) was prepared by mixing 900 μ l of absolute ethanol with 100 μ l of the target essential oil. The stock solution for oil combination treatment was prepared by mixing 50 μ l garlic essential oil with 50 μ l of asafoetida essential oil and 900 μ l of absolute ethanol. The experiment was replicated three times for a total of 246 containers. The containers were held at room temperature at 10:14 light: dark cycle and the total number of larvae surviving 24 h post-treatment were counted and recorded.

Ovicidal Activity of Essential Oils

The hatch rate of larvae from *Culex* egg rafts was examined following exposure to allyl disulfide, garlic oil, or asafoetida oil at concentrations that resulted in 50% larval mortality. These concentrations were 5, 12, and 11 ppm, respectively, for garlic, asafoetida, and allyl disulfide. These values were derived by summing

Table 1. Chemical composition of garlic and asafoetida essential oils

Compound	Retention time	Garlic (%)	Asafoetida (%)
Allyl disulphide	5.24	49.13	7.38
1-Allyl-2-isopropyl disulfane	5.44	0.52	0.00
Allyl methyl trisulfide	6.07	4.67	0.00
4-Methyl-1,2,3-trithiolane	6.26	0.98	1.50
n-Propyl sec-butyl disulfide	6.44	0.00	2.92
(Z)-sec-Butyl propenyl disulfide	6.51	0.00	24.32
(E)-sec-Butyl propenyl disulfide	6.58	0.00	30.03
Diallyl trisulfide	8.34	31.08	4.55
(E)-1-(Prop-1-en-1-yl)-3-propyltrisulfane	8.71	0.00	0.15
Cyclohexasiloxane, dodecamethyl-	8.78	0.00	2.37
5-Methyl-1,2,3,4-tetrathiane	9.16	0.49	0.00
Ally methyl disulfide	9.41	1.18	0.00
1-(1-(Methylthio)propyl)-2-propyl disulfane	9.95	0.00	1.59
Disulfide, methyl 1-(methylthio)propyl	10.04	0.00	21.87
Cycloheptasiloxane, tetradecamethyl-	10.97	0.00	2.01
Diallyl tetrasulfide	11.40	11.01	0.00
Cyclooctasiloxane, hexadecamethyl-	12.94	0.00	1.30
2-Methoxymethyl-2-methyl-[1,3]dithiane 1-oxide	14.06	0.48	0.00
1-Allyl-3-(2-(allylthio)propyl)trisulfane	14.46	0.46	0.00

the LC_{50} for both mosquito species and dividing the resulting value by two (i.e., mean LC_{50} for the two mosquito species). The stock solutions (100,000 ppm) were prepared as described above and 200 ml of 5, 12, and 11 ppm solutions of garlic oil, asafoetida oil, and allyl disulfide were prepared in 500-ml glass bottles. The glass bottles were vigorously shaken to homogenize the content and 3 ml of respective treatment were dispensed in 12-well cell culture plates. A single *Culex* egg raft was transferred onto each well and held at room temperature at 10:14 light: dark cycle. A control treatment received 24 μ l of ethanol (equivalent to the highest volume of oil used) instead of oil. Fifty-two egg rafts were evaluated for each treatment. The eggs were monitored every day for 7 days and the number of eggs that hatched in each treatment counted and recorded.

Chemical Analysis of Essential Oils

Ten microliters of either oil was diluted in 4.990 ml of hexane and vortexed. Ten microliters of this solution were again mixed with 990 μ l of hexane and vortexed: 1 μ l samples of this mixture were analyzed by a Thermo Scientific ISQ QD mass selective detector interfaced with a Thermo Scientific Trace 1300 gas chromatography (Thermo Scientific, Waltham, MA). Inlet temperatures were maintained at 200°C and samples were injected in splitless mode. A 30-m DB-5 capillary column (0.25 mm I.D., 0.25 μ m film thickness; Agilent Technologies, Santa Clara, CA) was employed. Helium, the carrier gas, was set at constant pressure (6 psi) and injector temperature was set at 280°C. Oven temperature was increased from 50 to 280°C at 10°C/min. The NIST mass spectral library on the MS data system was used for identification.

Statistical Analyses

Probit analysis conducted using SAS 9.4 statistical package was used to calculate LC_{50} and LC_{95} values for different oil treatments. To examine the effect of oil mixtures, we used cotoxicity coefficient (CTC) to determine whether the interaction was additive, synergistic or additive (Sun and Johnson 1960). This was computed as follows with garlic serving as the standard:

Toxicity index (TI) of garlic (G) = 100,

$$TI \text{ of asafoetida (A)} = \frac{LC_{50} \text{ of G}}{LC_{50} \text{ of A}} \times 100$$

$$\text{Actual TI of mixture (AG)} = \frac{LC_{50} \text{ of G}}{LC_{50} \text{ of AG}} \times 100$$

$$\text{Theoretical TI of AG} = TI \text{ of G} \times \% \text{ of G in AG} + TI \text{ of A} \times \% \text{ of A in AG}$$

$$CTC = \frac{\text{Actual TI of AG}}{\text{Theoretical TI of AG}} \times 100$$

A CTC of a mixture near 100 indicates probability of additive effects while CTC value lower than or greater than 100 indicates antagonism and synergism, respectively (Sun and Johnson 1960, Islam et al. 2010).

Results

Chemical Composition of Garlic and Asafoetida Essential Oils

Gas chromatography-mass spectrometry (GC-MS) analysis revealed the presence of 10 compounds in garlic essential oil and 12 compounds in asafoetida essential oil (Table 1). The five most abundant compounds obtained in garlic essential oil were allyl disulfide (49.13%), diallyl trisulfide (31.08%), diallyl tetrasulfide (11.01%), allyl methyl trisulfide (4.67%), and allyl methyl disulfide (1.18%). For asafoetida essential oil, the five most abundant compounds were (E)-sec-butyl propenyl disulfide (30.03%), (Z)-sec-butyl propenyl disulfide (24.32%), disulfide, methyl 1-(methylthio)propyl (21.87%), allyl disulfide (7.38%), and diallyl trisulfide (4.55%). Only three compounds, allyl disulfide, diallyl trisulfide, and 4-Methyl-1,2,3-trithiolane, occurred in both oils.

Ovicidal and Larvicidal Activity of Garlic and Asafoetida Essential Oils

Out of 52 *Culex* egg rafts that were exposed to garlic essential oil, 38 (73.1%) did not hatch while the remaining 14 (26.9%) hatched but the larvae died as first instars (Table 2). For the 52 egg rafts that were exposed to asafoetida essential oil, 29 (55.8%) did not hatch, 19 (36.5%) hatched but the larvae died as first instars, and 4 (7.7%) hatched and the larvae survived (Table 2). Allyl disulfide had similar effects suggesting it was one of the principle compounds responsible for inhibiting egg hatch. Of the 52 egg rafts that were exposed to allyl disulfide, 50 (96.2%) did not hatch and 2 (3.8%) hatched but the larvae died as first instars.

Garlic essential oil was more toxic than asafoetida essential oil with *Cx. restuans* larvae being more sensitive to both oils compared to *Cx. pipiens* larvae (Table 3; Fig. 1). The LC_{50} values for garlic and asafoetida essential oils against *Cx. pipiens* larvae were 7.5 and 13.5 ppm, respectively, while the LC_{50} values for *Cx. restuans* larvae were 2.7 ppm for garlic essential oil and 10.1 ppm for asafoetida essential oil (Table 3). Allyl disulfide was also highly toxic to larvae of both mosquito species (LC_{50} : *Cx. pipiens* = 12.5 ppm, *Cx. restuans* = 9.4 ppm), suggesting its important contribution to overall toxicity of both oils. The cotoxicity coefficient values were 80.3% for *Cx. pipiens* and 46.9% for *Cx. Restuans*, suggesting that the two oils interacted antagonistically.

Discussion

We used field populations of two WNV vectors, *Cx. pipiens* and *Cx. restuans*, to examine the ovicidal potential of *E. asafoetida* and *A. sativum* (garlic) essential oils as well as their individual and

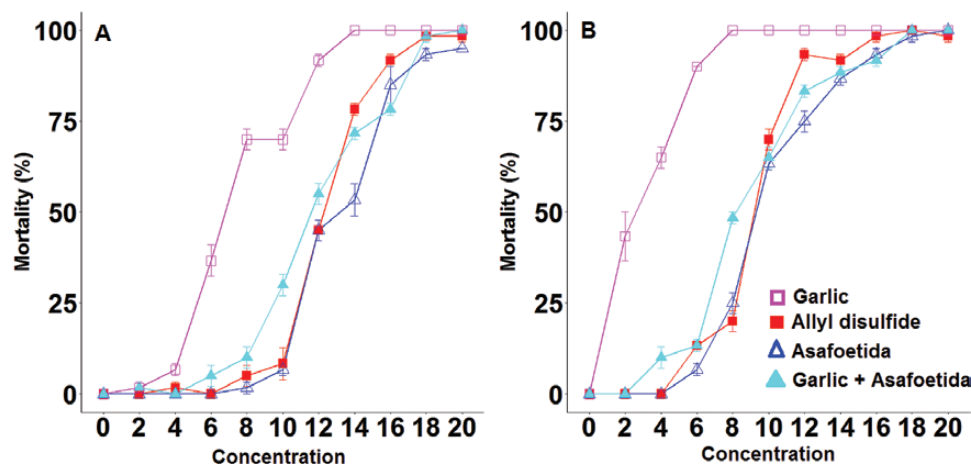
Table 2. Effect of garlic essential oil, asafoetida essential oil, and one of their components (allyl disulfide) on hatch rate of *Culex* eggs

Treatment	Concentration (ppm)	Did not hatch (%)	Hatched and died (%)	Hatched and survived (%)
Control		0 (0.0)	0 (0.0)	52 (100.0)
Garlic	5	38 (73.1)	14 (26.9)	0 (0.0)
Asafoetida	12	29 (55.8)	19 (36.5)	4 (7.7)
Allyl disulfide	11	50 (96.2)	2 (3.8)	0 (0.0)

Table 3. LC₅₀ and LC₉₅ values for garlic essential oil, asafoetida essential oil, their combination, and one of their components (allyl disulfide) against *Cx. pipiens* and *Cx. restuans* larvae

Species	Oil type	LC ₅₀ (95 % CI)	LC ₉₅ (95% CI)	Equation
<i>Cx. pipiens</i>	Allyl disulfide	12.5 (12.0–12.9)	16.9 (16.2–17.8)	$y = 0.37x - 4.66$
	Asafoetida	13.5 (13.0–13.9)	18.4 (17.7–19.4)	$y = 0.33x - 4.50$
	Garlic	7.5 (7.0–8.0)	12.3 (11.5–13.2)	$y = 0.34x - 2.59$
	A+ G	12.0 (11.5–12.5)	17.9 (17.0–18.9)	$y = 0.28x - 3.36$
<i>Cx. restuans</i>	Allyl disulfide	9.4 (8.9–9.8)	14.0 (13.3–14.9)	$y = 0.36x - 3.36$
	Asafoetida	10.1 (9.6–10.6)	15.4 (14.6–16.3)	$y = 0.31x - 3.19$
	Garlic	2.7 (2.0–3.2)	6.7 (6.0–7.9)	$y = 0.41x - 1.09$
	A+ G	9.1 (8.5–9.6)	15.1 (14.3–16.2)	$y = 0.27x - 2.44$

Values in parenthesis are 95% confidence limits.

**Fig. 1.** Mean (\pm SE) mortality of late third instar larvae of (A) *Cx. pipiens* and (B) *Cx. restuans* in different concentrations of garlic essential oil, asafoetida essential oil, their combination, and one of their components (allyl disulfide).

combined toxicity against larvae of the two mosquito species. We also characterized the chemical composition of the two essential oils and examined the ovicidal and larvicidal activity of allyl disulfide, one of the major and minor components of garlic and asafoetida essential oils, respectively. Both essential oils were quite effective at suppressing egg hatch and killing the larvae, demonstrating their potential application as mosquito ovicides and larvicides. Although garlic extracts and/or essential oil have been shown to possess ovicidal and larvicidal activities against a wide range of insects including mosquitoes (Park and Shin 2005, Kimbaris et al. 2009, Zhao et al. 2013, Sangha et al. 2017), this is the first report of the biological activity of asafoetida and a mixture of the two essential oils against mosquitoes.

The LC₅₀ values for individual oils and their combinations against larvae of the two mosquito species ranged from 2.7 ppm for garlic to 13.5 ppm for asafoetida. These values are much lower than those of many essential oils that have been examined for larvicidal activity against mosquitoes (Amer and Mehlhorn 2006a, Koliopoulos et al. 2010, Dias and Moraes 2014, Benelli et al. 2017). Garlic was the more toxic of the two essential oils and *Cx. restuans* larvae were more susceptible to both oils compared to *Cx. pipiens*. Differential sensitivity of mosquito species to essential oils is a common observation (Amer and Mehlhorn 2006a, Fayemiwo et al. 2014, Govindarajan et al. 2016). The high toxicity of garlic essential oil against mosquito larvae is well documented (Amonkar and Reeves 1970, Kimbaris et al. 2009). In fact, previous studies have shown that garlic essential oil can be an effective pesticide against mosquitoes when microencapsulated and used in an attractive toxic

sugar baits system (Junnala et al. 2015). Asafoetida is used as a flavoring agent and as a traditional medicine for many diseases in many parts of the world (Mahendra and Bisht 2012, Divya et al. 2014, Amalraj and Gopi 2017). Asafoetida leaf extracts are also effective against a variety of cotton pests (Noonari et al. 2016) and moderately (acetone extracts LC₅₀ = 65.02 ppm; petroleum ether extracts LC₅₀ = 62.61 ppm) toxic to *Aedes aegypti* larvae (Harve and Kamath 2004). Thus, our results demonstrate that asafoetida essential oil is more potent to mosquito larvae than acetone and petroleum ether leaf extracts of the same plant and could be developed as a novel mosquito larvicide.

Previous studies have shown that allyl disulfide and diallyl trisulfide are the primary constituents responsible for the larvicidal activity of garlic essential oil with the former being more toxic than the later (Kimbaris et al. 2009). The two compounds were the most abundant constituents in our garlic essential oil accounting for 80.2% (allyl disulfide = 49.13%; diallyl trisulfide = 31.08%) of the total oil. These values are much higher than the 7.2% (allyl disulfide) and 16.3% (diallyl trisulfide) reported by Kimbaris et al. (2009) and may explain why our LC₅₀s were much lower than those reported in their study. Allyl disulfide (7.38%) and diallyl trisulfide (4.55%) occurred as minor components in asafoetida essential oil, and were two of only three constituents that occurred in both essential oils. Thus, despite their low abundance, both compounds are likely among the minor components influencing the toxicity of asafoetida essential oil. Further studies are needed to determine the contribution of the major constituents towards the overall toxicity of asafoetida essential oil and how

these constituents may interact with allyl disulfide and other minor constituents.

Some essential oils contain bioactive compounds that may interact synergistically or antagonistically against mosquitoes when combined (Intirach et al. 2012, Pavela 2015, Muturi et al. 2017). The discovery of essential oil combinations that interact synergistically may provide critical leads for the development of cheap, effective, and safe alternatives to synthetic insecticides. Nevertheless, we found that garlic and asafetida essential oils interacted antagonistically against *Cx. pipiens* and *Cx. restuans* and would thus be more effective when used individually than in combination. These findings indicate that combining two highly toxic essential oils may not necessarily yield optimal results, and knowledge-guided decision-making processes should be used to select essential oil combinations that do not undermine vector control programs (Muturi et al. 2017).

When exposure to oil concentrations that killed 50% of the larvae (LC_{50}), the majority of eggs rafts from both garlic (73.1%) and asafetida (55.8%) essential oil treatments did not hatch. In addition, larvae from 19 of the 23 egg rafts that hatched in asafetida essential oil treatments and all the 14 egg rafts that hatched in garlic essential oil treatments died as first instars. Similar bioassays using allyl disulfide revealed its significant role towards inhibition of egg hatch: 96.2% of eggs exposed to this compound failed to hatch and larvae from the two egg rafts that hatched died as first instars. Diallyl trisulfide which was also a major component of garlic essential oil is also known to suppress egg hatch in some beetle species and may also have contributed to egg hatch inhibition by garlic essential oil (Huang et al. 2000). Because allyl disulfide (7.38%) and diallyl trisulfide (4.55%) were minor components of asafetida essential oil, our results suggest that either only small amounts of allyl disulfide and diallyl trisulfide are required to inhibit egg hatch or that other components of asafetida essential oil also contribute to egg hatch inhibition. Further studies are needed to determine the role of various components of asafetida essential oil towards inhibition of egg hatch. The ovicidal properties of garlic essential oils have been demonstrated in diamondback moth *Plutella xylostella* (L.) (Sangha et al. 2017) and garlic extracts are also known to exhibit ovicidal effects against red spider mite, *Oligonychus coffeae* (Nietner) (Roobakkumar et al. 2010) and mosquitoes (Jarial 2001). In contrast, this is the first report of ovicidal effects of asafetida essential oil against mosquitoes. We did not examine the mechanism(s) underlying the ovicidal properties of the two essential oils but previous studies suggest that garlic extracts inhibit egg hatch by interfering with the ability of mosquito embryos to break the egg shells (Jarial 2001).

In conclusion, we investigated the individual and combined toxicity of garlic and asafetida essential oils. We found that both essential oils possess ovicidal and larvicidal properties, suggesting that the application of these oils could be integrated with other vector control methods. However, the two essential oils had antagonist effects against mosquito larvae when combined, suggesting their potential to undermine vector control programs if used jointly. Additional studies are needed to determine the role of all components in the overall toxicity of each essential oil. This knowledge may facilitate discovery of components that work in synergy which could provide leads for development of cheap, effective, and eco-friendly botanicals for mosquito control.

Acknowledgments

We thank Kevin Foley for his technical support. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the

author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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