Research Article

Received: 16 May 2008 Revised: 23 August 2008 Accepted: 24 August 2008 Published online in Wiley Interscience: 11 December 2008

(www.interscience.wiley.com) DOI 10.1002/ps.1675

Larvicidal and oviposition-altering activity of monoterpenoids, *trans*-anethole and rosemary oil to the yellow fever mosquito *Aedes aegypti* (Diptera: Culicidae)†

Ranil Waliwitiya,* Christopher J Kennedy and Carl A Lowenberger

Abstract

BACKGROUND: *Aedes aegypti* L. is the major vector of dengue fever and dengue hemorrhagic fever. In an effort to find effective tools for control programs to reduce mosquito populations, the authors assessed the acute toxicities of 14 monoterpenoids, *trans*-anethole and the essential oil of rosemary against different larval stages of *Ae. aegypti*. The potential for piperonyl butoxide (PBO) to act as a synergist for these compounds to increase larvicidal activity was also examined, and the oviposition response of gravid *Ae. aegypti* females to substrates containing these compounds was evaluated in behavioral bioassays.

RESULTS: Pulegone, thymol, eugenol, *trans*-anethole, rosemary oil and citronellal showed high larvicidal activity against all larval stages of *Ae. aegypti* (LC_{50} values 10.3–40.8 mg L^{-1}). The addition of PBO significantly increased the larvicidal activity of all test compounds (3–250-fold). Eugenol, citronellal, thymol, pulegone, rosemary oil and cymene showed oviposition deterrent and/or repellent activities, while the presence of borneol, camphor and β-pinene increased the number of eggs laid in test containers.

CONCLUSIONS: This study quantified the lethal and sublethal effects of several phytochemical compounds against all larval stages of *Aedes aegypti*, providing information that ultimately may have potential in mosquito control programs through acute toxicity and/or the ability to alter reproductive behaviors.

Keywords: *Aedes aegypti*; acute toxicity; essential oils; larvicides; monoterpenoids; oviposition; piperonyl butoxide

1 INTRODUCTION

Mosquitoes are the vectors of important human pathogens, including those responsible for causing malaria, dengue, filariasis and yellow fever.[1] Malaria, with 300–500 million new cases annually and approximately 2.5 million annual deaths,[2] is one of the most devastating diseases affecting humans. *Aedes aegypti* (L.) is the main vector of dengue viruses which cause more human mortality and morbidity than any other arthropod-transmitted viral disease, and rank second only to malaria among mosquito-transmitted infections.[1] An estimated 2.5 billion people are at risk, and over 100 million new cases of dengue occur worldwide each year.[3]

Vector control using insecticides has been the primary means of reducing dengue virus transmission,[4] but wide-scale applications of synthetic pesticides can lead to environmental contamination and adverse effects on non-target species, including humans.[5,6] In addition, *Ae. aegypti* has developed resistance to organochlorine, organophosphate, carbamate and pyrethroid insecticides in many regions of the world,[7–11] which has hindered control efforts.

Plant-based chemicals, or phytochemicals, have been used for many years to control insect pests on agricultural crops.[12] Their insecticidal, fungicidal, bactericidal, antiviral, antifeedant or insect growth retardant properties[13–15] often are the result of synergistic interactions among different biologically active constituents such as terpenoids, alkaloids and phenolics.[16] Phytochemicals degrade rapidly, are unlikely to persist in soil and leach into groundwater,[17] often have a reduced impact on non-target populations and are important components of integrated pest management systems used by organic farmers.[17] Many of the active components of phytochemicals are termed essential oils, which refers to the steam-distillable fraction of plant tissues that often are responsible for their characteristic scent or odor.[17] Essential oils such as citronella have become popular alternative compounds to *N*,*N*-diethyl-*m*-toluamide (DEET) for personal protection against mosquitoes and other biting flies.[17,18]

While essential oils have been demonstrated to have antifeedant,[19] growth inhibitor[20] or repellent and toxic effects against various insects,[21–24] their modes of action are not well

* *Correspondence to: Ranil Waliwitiya, Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby, BC V5A 1S6, Canada. E-mail: rwaliwit@sfu.ca*

† *This article was published online on December 11, 2008. The spelling of trans-anithole has since been corrected to trans-anethole, and tolumide to toluamide, throughout the article. This notice is included in the online and print versions to indicate that both have been corrected [January 14, 2009].*

Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby, BC V5A 1S6, Canada

characterized.[25] Most insects treated with essential oils display characteristic neurotoxic symptoms including agitation, hyperactivity, paralysis and quick knockdown.[17,26–28] Neem-based products have been used to suppress blood feeding, reduce oviposition and inhibit larval growth in *Culex tarsalis* Coquilett, *Culex quinquefasciatus* Say[29] and *Ae. aegypti*.[30] Marigold extract (an essential oil) is lethal to larvae and adults of *Ae. aegypti* and *Anopheles stephensi* Liston,[31] while some plant essential oils demonstrate oviposition deterrent activities in mosquitoes.[32]

In order to enhance the toxicity of specific compounds to target organisms, some commercial insecticides contain synergists. Piperonyl butoxide (PBO) has been used widely as a synergist to enhance the efficacy of natural pyrethrins and synthetic pyrethroids.[33,34] PBO is a well-known inhibitor of microsomal monooxygenases which are involved in the metabolism and detoxification of many insecticides,[35–37] and thus its use limits the ability of insects to biotransform and detoxify these compounds.

In this study, the authors investigated the toxicity of 14 structurally different monoterpenoids, *trans*-anethole and one complex essential oil (rosemary oil from *Rosemarinus officinalis* L., family: Lamiaceae) on first through fourth larval instars of *Ae. aegypti* with the following specific objectives: (1) to quantify the acute toxicities of these compounds to *Ae. aegypti* larvae; (2) to evaluate the possible synergistic effects of PBO on the larvicidal activity of selected compounds; (3) to evaluate selected compounds for their ability to modify the ovipositional activity of *Ae. aegypti*.

2 MATERIALS AND METHODS

2.1 Insects

Aedes aegypti larvae and adults were raised and maintained as described previously[2] at 27 °C and 80–85% relative humidity under a 14 : 10 h light : dark cycle. Adults were provided with a 10% sucrose solution *ad libitum*. Larvae were raised at densities of 100 larvae L^{-1} distilled water and fed with ground Nutrafin Basix fish food (Rolf C Hagen Inc., Montreal, QC). All bioassays were conducted in a walk-in environmental chamber with these environmental conditions.

2.2 Chemicals

1,8-Cineole (95% purity, source *Eucalyptus globules* Labill.), linalool, pulegone and *trans*-anethole (>95% purity) were obtained from Ecosafe Natural Products (Victoria, BC). Eugenol, *p*-cymene, bornyl acetate, camphor (>98% purity), α-pinene, β-pinene, α-terpineol, citronellal, thymol, camphene, rosemary oil (>95% purity) and PBO (90% purity) were purchased from Sigma Aldrich (St Louis, MO). Stock solutions of the chemicals were made using aqueous acetone (10% v : v) as the solvent and stored in dark bottles. From the stock solutions, 5, 10, 20, 50, 100, 200 and 500 mg L^{-1} treatment solutions were prepared using distilled water. In order to increase the solubility of *trans*-anethole, rosemary oil and the monoterpenoids, and to keep them from binding to test containers, 5 µL of Tween 20 (Uniqema, New Castle, DE) were added to 100 mL of each treatment solution as a solubilizing agent. Control treatments contained distilled water, acetone and Tween 20 in the same concentrations as the test solutions. In the synergist studies, 10 mg L^{-1} of piperonyl butoxide (PBO) was added to treatment solutions after preliminary experiments had indicated that this was the minimum sublethal concentration of PBO that could be used with Tween 20. Control treatments for PBO plus phytochemical solution contained distilled water, acetone and PBO in the same concentrations as the test solutions.

2.3 Larval bioassays

All bioassays were carried out in the environmental chamber in which the mosquitoes were raised. In each bioassay, ten *Ae. aegypti* larvae from each larval instar stage were placed in 140 mL plastic cups (Sunfresh Ltd, ON) containing 50 mL of prepared treatment solution (as described in Section 2.2) and 0.1 g of ground Nutrafin Basix fish food. Cups were covered with mosquito mesh, and larvae were monitored for mortality at 24, 48, 72 and 96 h. Larvae were considered dead if they were immobile and unresponsive to touch with a probe, and dead larvae were removed. In experiments with PBO, mortality was determined for up to 48 h of exposure because, in the previous experiment, all mortality occurred at time points <48 h. All bioassays were replicated 3 times.

2.4 Ovipositional activity bioassay

All oviposition assays were carried out in the environmental chamber under standard rearing conditions. The ovipositional response of adult mosquitoes to essential oils was examined using a binary choice design described previously.[38] Based on preliminary studies, a 20 mg L^{-1} concentration of each test chemical was used in all ovipositional assays. Solutions were prepared as described for acute toxicity bioassays. Two 6 cm diameter pyrex petri dishes containing either 30 mL of treatment solution or 30 mL of control solution (distilled water with acetone and Tween 20) were placed in a cage (45 × 23 × 15 cm) containing ten 3–4-day-old female and five male *Ae. aegypti*. Mosquitoes were blood fed by keeping the primary author's right hand on the mesh of the rearing cup for 5 min prior to release into the cage. Subsequently they were blood fed by keeping the primary author's right hand inside the cage for 5 min every 2 days. Cumulative egg numbers were recorded in treatment and control dishes on days 3 and 5. Differential oviposition was measured in duplicate cages for each treatment solution, and each experiment was replicated a minimum of 3 times.

2.5 Analysis

Larvicidal bioassay data from three experiments were pooled and analyzed by standard probit analysis.[39] LC_{50} values (concentrations that caused mortality in 50% of a sample population) were determined at 48 h because no further mortality occurred after this time point. LC_{50} values were considered to be significantly different ($P \leq 0.05$) from each other if the confidence intervals did not overlap. Abbott's formula[40] was used to correct the mortality values. The synergism ratio was calculated as

$$(LC_{50} \text{ without PBO})/(LC_{50} \text{ with PBO})$$

In oviposition assays, the numbers of eggs laid were counted on days 3 and 5. For each day, and for each cage, the total numbers were converted to proportions of eggs laid $cage^{-1}$ day^{-1}, transformed by arcsine square root transformation and compared using a paired *t*-test as described previously.[41] Differences in oviposition were considered significant at $P \leq 0.05$. This analysis has been used previously to determine if an individual solution repels/deters or attracts oviposition by gravid females as compared with a control solution. The oviposition activity index (OAI) for each solution was also calculated to generate a global comparison among the test solutions, as described previously:[42]

$$OAI = [(T - C)/T + C]$$

where T is the number of eggs collected from the treated dish and C is the number of mosquito eggs collected from the control dish.

3 RESULTS

3.1 Larval bioassays

The susceptibilities of *Ae. aegypti* larvae to 14 different monoterpenoids, *trans*-anethole and one complex essential oil (rosemary oil) were examined. The range of concentrations used in these bioassays was sufficient to calculate LC_{50} values and their 95% confidence intervals (CIs) for 13 out of 16 (13/16), 5/16, 5/16 and 5/16 of the compounds for larval instars 1 to 4 respectively. For those compounds where LC_{50} values could be calculated, all test compounds showed increasing mortality with increasing concentration against all four larval instar stages. The acute toxicities of selected compounds with and without PBO to all larval instars of *Ae. aegypti* are presented in Tables 1 and 2. The least toxic compounds, resulting in no mortality even at the highest concentration, were borneol acetate, camphor, cineol, linalool and myrcene.

Rosemary oil (R. oil) was highly toxic to the first-instar larval stage (L1), but was not toxic at the highest concentration tested for L2–L4 stages. Pulegone, *trans*-anethole, thymol, eugenol and citronellal were consistently the most toxic compounds to all larval instars; however, these compounds possessed different levels of toxicity for different stages. The LC_{50} values of these five compounds against first- to fourth-instar larvae of *Ae. aegypti* increased with larval instars, as demonstrated in Tables 1 and 2. Linear regression analysis of LC_{50} values of these five compounds shows 3.5-, 4.8-, 5.4-, 6.4- and 13.4-fold reductions in toxicity against L4 as compared with L1 instar larvae for thymol, *trans*-anethole, pulegone, citronellal and eugenol respectively.

Exposure to PBO alone did not result in mortality of any larval stage of *Ae. aegypti*, but the addition of PBO to the test solutions significantly increased the larvicidal activity of all chemicals against *Ae. aegypti* larvae (Tables 1 and 2). The synergism ratios varied from 3 to 250 for all the test chemicals. Interestingly, the range of LC_{50} values for all 14 different monoterpenoids, *trans*-anethole and rosemary oil against all instar stages was very narrow compared with the very wide range of LC_{50} values in the absence of PBO. For example, the range of values for non-PBO-treated first instars was from 10.3 to >500 mg L^{-1} and from 2 to 5.2 mg L^{-1} for first instars with the addition of PBO. LC_{50} values for second-, third- and fourth-instar larvae ranged from 19.6 to >500, from 39.6 to >500 and from 48.7 to >500 mg L^{-1} respectively for non-PBO-treated larvae, and from 2 to 10.7, from 3.9 to 18.5 and from 8.6 to 99.5 mg L^{-1} respectively for PBO-treated larvae.

3.2 Oviposition assays

Differential oviposition was measured among the various treatment solutions and their controls (Table 3). This bioassay does not make it possible to distinguish between a repellent or deterrent activity, so these responses were combined under the term 'deterrent'. There were no significant differences in the numbers of eggs laid on distilled water or on distilled water containing Tween 20 and acetone for day 3 ($P = 0.6412$) or day 5 ($P = 0.1344$). Solutions containing terpineol or α-pinene did not receive significantly different numbers of eggs to the control solution, suggesting that they are neither deterrent nor attractive to gravid females. Solutions containing β-pinene, borneol acetate, borneol or camphor received more eggs than the controls, while those containing cineol, citronellal, eugenol, linalool, *p*-cymene, pulegone, rosemary oil, *trans*-anethole or thymol received significantly fewer eggs than did their control solutions.

The oviposition activity index allows for a global comparison of the attractant or repellent nature of different solutions (Fig. 1). The pattern of OAI is maintained for individual solutions on days 3 and 5, except for terpineol, which had a negative OAI on day 3 but a positive OAI on day 5 (Fig. 1). However, there was no significant difference in the numbers of eggs laid on terpineol or its control on day 3 ($P = 0.939$) and day 5 ($P = 0.857$). In order of strength of activity, as measured by OAI, eugenol, citronellal, thymol, cineol, pulegone, rosemary oil, *p*-cymene, linalool and *trans*-anethole deterred oviposition, whereas β-pinene, borneol, camphor and borneol acetate acted as oviposition attractants.

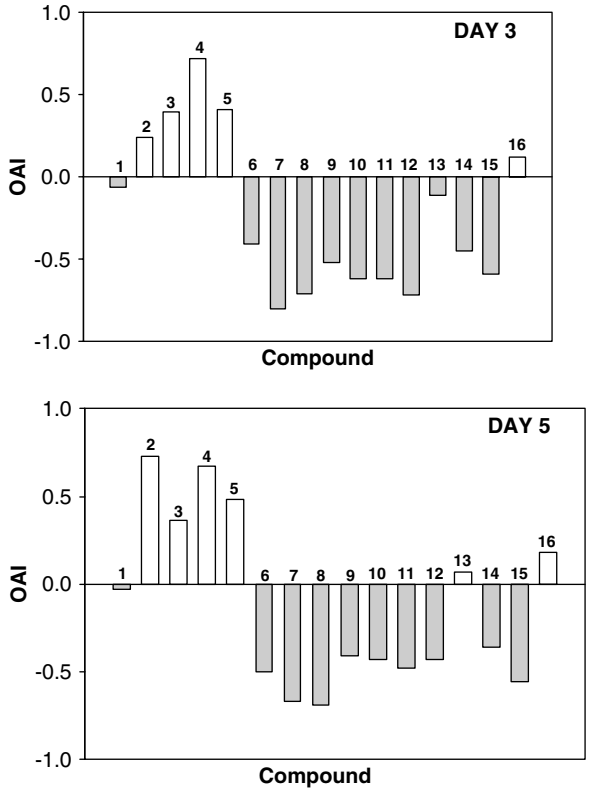

Figure 1. Oviposition activity index values for tested compounds on days 3 and 5 (for names of compounds, see Table 3).

4 DISCUSSION

4.1 Larval bioassays

Mosquito larvae are attractive targets for pesticide management programs because they are limited to discrete aquatic habitats. In this study, the authors evaluated the toxicities of phytochemicals and an essential oil (rosemary oil) that contains some of the individual compounds tested (α-pinene, 1,8-cineole, camphor, β-pinene and borneol) to compare the relative toxicities of phytochemicals against mosquito larvae. All of the chemicals tested in this study have some larvicidal activity at the tested concentrations against first-instar larvae. Certain compounds, including camphor, linalool and bornyl acetate, had LC_{50} values >500 mg L^{-1} and therefore are less likely to be useful in mosquito control programs. The present results confirm the reports of others that the monoterpenoids thymol and 1,8-cineol are acutely toxic to fourth-instar larvae of *Ae. aegypti*.[43] The present LC_{50} estimations differ slightly from other reports, which probably reflects differences in methodologies and analyses.[43–46] It is difficult to compare directly the effects of compounds used in different studies because the relative composition of major

Table 1. Larvicidal activity of selected monoterpenoids, *trans*-anethole and rosemary oil with and without PBO to first- and second-instar larvae of *Aedes aegypti* exposed for 24 h. Lethal concentration (LC) values are expressed in mg L^{-1}. All values are means of $n = 3$ experiments

	First-instar larvae without PBO				First-instar larvae with PBO					Second-instar larvae without PBO				Second-instar larvae with PBO				
Chemical	LC_{50}	95% CL	Slope	χ^2 value	LC_{50}	95% CL	Slope	χ^2 value	SR[a]	LC_{50}	95% CL	Slope	χ^2 value	LC_{50}	95% CL	Slope	χ^2 value	SR[a]
α-Pinene	82.3	77–122	1.7	11.07	2.8	2–3	5.91	12.9	29	>500	–	–	–	3.3	3–4	2.1	6.1	151
β-Pinene	96.2	71–133	1.6	11.04	2.5	2–3	4.9	12.6	39	>500	–	–	–	3	1–5	1.2	7.6	167
Borneol acetate	>500	–	–	–	3	2–4	2.5	1.3	167	>500	–	–	–	6.3	4–9	1.5	3.2	79
Borneol	183.1	113–374	0.96	3.2	2.1	1–4	1.7	2.1	87	>500	–	–	–	2	1–4	0.94	9.4	250
Camphor	>500	341–835	0.83	1.9	2.1	1–3	1.5	3.1	238	>500	–	–	–	3.3	1–6	0.86	2.6	151
Cineol	>500	–	–	–	2.1	1–4	1.3	2.1	238	>500	–	–	–	4.5	2–8	0.96	6.1	111
Citronellal	40.7	30–56	1.5	6.9	2.9	3–4	6.2	12.5	14	118	51–188	1.2	14	3.2	3–4	5.4	1.6	37
Eugenol	24.5	20–30	2.8	2.9	2.3	4–6	1.4	19.8	11	32.6	24–43	1.7	7.3	4	5–9	1.4	19.7	8
Linalool	>500	–	–	–	2	2–3	3.7	12.6	250	>500	–	–	–	7.2	1–9	1.4	8.5	69
Myrcene	>500	–	–	–	–	–	–	–	–	>500	–	–	–	–	–	–	–	–
p-Cymene	226.2	137–490	0.9	1.8	5.2	4–7	2.2	3.9	44	>500	–	–	–	10.7	8–14	1.5	1.7	47
Pulegone	10.3	9–12	1.1	1.1	3.2	2–4	1.6	2	3	19.6	16–25	2.7	5.4	4.1	3–6	1.7	1.8	4.8
R. oil	40.8	18–92	2	23.6	2.4	2–3	5.7	12.6	17	>500	–	–	–	7.2	4–12	0.9	1.3	69
trans-Anethole	13	9–18	1.7	8.5	2.6	1–4	1.8	2.3	5	51.9	38–72	1.4	11	3	1–5	1.3	3.3	17
Terpineol	83.9	63–115	1.6	5.3	3.2	3–4	9.2	12.5	26	>500	–	–	–	4	3–5	6.5	7.7	125
Thymol	17.3	14–22	2.5	3.2	2.7	2–3	7.1	12.9	6	23.7	19–30	3.5	2.4	3.1	1–7	2.1	31	8

[a] SR = synergism ratio.

Table 2. Larvicidal activity of selected monoterpenoids, *trans*-anethole and rosemary oil with and without PBO to third- and fourth-instar larvae of *Aedes aegypti* exposed for 24 h. Lethal concentration (LC) values are expressed in mg L^{-1}. All values are means of $n = 3$ experiments

	Third-instar larvae without PBO				Third-instar larvae With PBO					Fourth-instar larvae without PBO				Fourth-instar larvae with PBO				
Chemical	LC_{50}	95% CL	Slope	χ^2 value	LC_{50}	95% CL	Slope	χ^2 value	SR[a]	LC_{50}	95% CL	Slope	χ^2 value	LC_{50}	95% CL	Slope	χ^2 value	SR[a]
α-Pinene	>500	–	–	–	4.6	3–6	1.8	8.4	109	>500	–	–	–	20.7	15–29	1.4	9.7	24
β-Pinene	>500	–	–	–	5.2	3–8	1.3	11.7	96	>500	–	–	–	30.7	22–43	1.4	3.8	16
B. acetate	>500	–	–	–	12	8–17	1.2	1.4	42	>500	–	–	–	39.3	28–56	1.3	1.6	13
Borneol	>500	–	–	–	7	4–11	0.93	7.9	71	>500	–	–	–	24.6	15–35	1.2	3.2	20
Camphor	>500	–	–	–	6	2–11	0.75	4.5	83	>500	–	–	–	71.8	46–124	0.9	1.9	7
Cineol	>500	–	–	–	11.9	7–18	0.96	4.4	42	>500	–	–	–	96.2	63–162	1	1.8	5
Citronellal	174.3	134–249	4.7	8.2	7.2	6–9	2.6	2.1	24	262.9	232–356	0.5	1.6	15.6	12–20	1.9	9.4	17
Eugenol	82.2	38–223	1.5	16	8.8	7–13	1.6	11.9	9	142.9	101–217	1.4	6.8	52.3	16–36	1.2	5	3
Linalool	>500	–	–	–	18.5	6–12	0.98	7.2	27	>500	–	–	–	99.5	37–77	1.1	2.1	5
Myrcene	>500	–	–	–	–	–	–	–	–	>500	–	–	–	–	–	–	–	–
p-Cymene	>500	–	–	–	9.8	7–13	1.7	2.4	51	>500	–	–	–	23.2	16–33	1.3	5.9	22
Pulegone	39.6	17–86	1.5	14.8	5	4–7	1.9	5	8	48.7	18–79	1.6	13.8	15.1	10–22	1.2	3.7	3
R.Oil	>500	–	–	–	10.7	6–16	0.98	3	47	>500	–	–	–	41.1	29–59	1.2	1.2	12
trans-Anethole	67.1	29–193	1.1	11.7	5.9	2–7	1.1	4.1	11	88.5	75–177	1.2	10.5	25.3	19–34	1.5	8.3	4
Terpineol	>500	–	–	–	3.9	2–7	2.9	54.9	128	>500	–	–	–	8.5	3–18	1.2	16	59
Thymol	27.3	22–34	2.5	3.6	4.2	4–8	1.9	10.8	7	53.5	32–90	2.5	12.6	19.8	11–35	1.5	13.6	3

[a] SR = synergism ratio.

components in different mixtures may affect the toxicity of the mixture.[43–50]

As such, although two different oils of Brazilian crotons containing α-pinene and β-pinene as major constituents had LC_{50} values of 102 mg L^{-1} and 104 mg L^{-1}, respectively, against third-instar larvae of *Ae. aegypti*,[46] it is difficult to compare these data with the present studies using pure α-pinene or β-pinene, or rosemary oil which contains α-pinene or β-pinene along with other compounds. The present data agree with other studies that have evaluated different plant essential oils for toxicity to *Ae. aegypti* larvae,[49] although there are some differences in absolute values. The reported LC_{50} values of essential oils can vary greatly, depending on their chemical composition, which depends upon the plant species, the plant part extracted, maturity and the extraction method. Essential oils contain many different compounds, which may interact additively, synergistically and even antagonistically, increasing, decreasing or resulting in no change in the larvicidal activity of test oils compared with the purified major active ingredient. Studies on the modes of action or the synergistic interactions of the major constituents of essential oils might help to explain why these combinations are more toxic to larvae.

Several phytochemicals have been evaluated against larvae and adults of different mosquito species that occupy very different ecological niches.[43–50] Different species appear to be more susceptible or tolerant to specific compounds. A comparison of the limited available data suggests that *Ae. aegypti* may be more tolerant to the toxic effects of natural and synthetic pesticides than other mosquito species, although more research into this question needs to be done to show this conclusively.

The toxicity of most compounds tested varied with the larval instar (Table 1). In the cases where toxicity values could be determined, the toxicity of chemicals decreased significantly with increasing larval stage, a trend that has been observed previously.[43–50] A number of factors could attribute to this, including the following:

1. Larger instars present a smaller surface area to volume ratio, and at the same water concentration would absorb less chemical than smaller instars.
2. Alterations in cuticle thickness and composition with increasing size may reduce the permeability of chemicals in larger instars.
3. Detoxification potential is higher in more developed insect larvae, possibly resulting in increased biotransformation of absorbed chemicals.
4. It is possible that, in conjunction with enhanced detoxification ability, increased elimination potential [through various mechanisms such as higher basal expression of xenobiotic efflux pumps (e.g. *p*-glycoprotein)] may be higher in larger instars.

PBO is a well-known inhibitor of microsomal monooxygenases (cytochrome P_{450} inhibitor), which are involved in the metabolism and detoxification of a very large number of insecticides.[37] Several studies have demonstrated the synergistic effects of PBO with many synthetic insecticides against *Ae. aegypti* larvae and adults.[37] The present study, however, is the first to report its synergistic effects with natural phytochemicals. The increases in acute toxicity of individual phytochemicals by the addition of PBO to the test solutions were not directly proportional to the non-PBO-treated values. The toxicity values of all compounds with PBO against first-instar larvae were in the same range ($<$10 mg L^{-1}), in spite of non-PBO values ranging from 10 to 500 mg L^{-1}. The toxicity of α-pinene to first- and fourth-instar larvae was increased at least 24-fold (the LC_{50} without PBO could only be estimated as $>$500 mg L^{-1}), and the toxicity of linalool to first-instar larvae of *Ae. aegypti* was increased 250-fold by the addition of PBO (Tables 1 and 2). Similarly, the addition of PBO to the test solution containing borneol acetate, which was non-toxic to first-instar larvae without PBO, resulted in a synergism ratio of 167, and rendered borneol lethal at a low concentration. The synergism ratios of all compounds are presented in Tables 1 and 2.

The present experiments demonstrate that high mosquito larvae mortality levels can be achieved with relatively low concentrations of some natural plant compounds, and that this lethality can be enhanced further by formulations including PBO. Traditionally, the use of plant-based products has required higher volumes and concentrations of compounds than conventional products. The present data suggest that these volumes can be reduced significantly by using synergists such as PBO. Equally important is that some of the more 'non-toxic' compounds become lethal when combined with PBO, therefore increasing the list of potential natural pesticides. Alternative synergists could be evaluated to determine which ones combine optimally with individual compounds to enhance toxicity, and to reduce the quantity of chemical used, particularly if such compounds are to be used effectively in pest and disease control programs.

Compounds used commonly in mosquito control programs include synthetic pesticides, insect growth regulators and chitin inhibitors. Data on the interactions of synergists such as PBO with methoprene (juvenile hormone homolog), dimilin (a chitin inhibitor) and conventional insecticides might produce combinations that reduce the volumes required to achieve control. Whereas many mosquito species have developed resistance to conventional insecticides, combinations of insecticides, phytochemicals, growth inhibitors or juvenile hormone inhibitors with synergists might provide better control with lower doses and lower costs. Such approaches might render phytochemicals more efficient in practical applications.

4.2 Oviposition assay

The choice of an oviposition site by gravid mosquito females is a principal factor that determines species proliferation, population densities and dispersion in different geographical areas.[32] *Aedes aegypti* breeds in domestic and peridomestic water containers, follows visual and olfactory cues to find appropriate oviposition sites and then uses both physical and chemical factors of the waters to discriminate between suitable sites.[50] Oviposition repellents cause mosquitoes to move away from the source,[50–53] whereas, in the presence of oviposition deterrents, females move towards and land upon a site, assess site quality, but lay few or no eggs before flying away.[51–53] While attractance could be demonstrated, the present experimental design did not allow for discrimination between oviposition repellence and deterrence of the test compounds. It was demonstrated that gravid females laid significantly more eggs on waters that contained β-pinene, borneol acetate, borneol or camphor than their controls and significantly fewer eggs on waters that contained cineol, citronellal, eugenol, linalool, *p*-cymene, pulegone, rosemary oil, *trans*-anethole or thymol compared with their controls (Table 3). Only terpineol and α-pinene did not induce a statistically significant differential oviposition. The oviposition activity index (OAI) represents a global view of the relative preference of a substrate by gravid females. The OAI can be overly influenced

Table 3. Analysis of the proportions of eggs laid in treatment or control waters in binary choice bioassays

Compound number	Compound[a]	Day 3 Total number of eggs	Day 3 Control (%)	Day 3 Treatment (%)	Day 3 *P*-value[b]	Day 5 Total number of eggs	Day 5 Control (%)	Day 5 Treatment (%)	Day 5 *P*-value[b]
1	α-Pinene	545	53	47	0.804	1247	54	46	0.29
2	β-Pinene*	550	18	82	0.014	1098	20	80	0.008
3	B. acetate*	626	31	69	0.045	1114	33	67	0.0001
4	Borneol*	665	15	85	0.017	1338	24	76	0.017
5	Camphor*	508	30	70	0.03	950	31	69	0.034
6	Cineol**	557	70	30	0.033	1058	66	34	0.004
7	Citronellal**	308	88	12	0.035	997	83	17	0.009
8	Eugenol**	410	84	16	0.002	816	83	17	0.001
9	Linalool**	326	75	25	0.004	660	69	31	0.001
10	*p*-Cymene**	389	73	27	0.003	733	71	29	0.002
11	Pulegone**	449	80	20	0.005	877	76	24	0.0004
12	R. oil**	872	82	18	0.018	623	74	26	0.0003
13	Terpineol	1391	67	33	0.939	981	48	52	0.857
14	*trans*-Anethole**	438	72	28	0.001	759	71	29	0.001
15	Thymol**	412	79	21	0.004	651	80	20	0.007
16	Control	799	64	36	0.641	757	43	57	0.134

[a] * Solutions that were preferred by gravid females;
** solutions that repelled gravid females.
[b] If $P < 0.05$, there was a significant difference in egg numbers between the treatment petri dish and the control petri dish for the compound.

by single events in which there is significantly more oviposition in one cage, as it calculates a value based on total egg counts. Compounds with negative OAI values act as repellents/deterrents, while positive values act as attractants (Fig. 1). Thymol, pulegone, citronellal and eugenol showed strong repellent/deterrent activity, whereas β-pinene, borneol acetate, borneol and camphor acted as strong oviposition attractants (Fig. 1). Rosemary oil, the only essential oil evaluated in this experiment, had a strongly negative OAI. It is important to note that a single concentration was tested, and it is possible that different concentrations may alter the OAI.

The specific activity of compounds also may change with time. The repellent/deterrent activity of pulegone, one of the most lethal compounds tested, decreased from day 3 to day 5, whereas the oviposition attractant activity of camphor increased from day 3 to day 5. The other compounds showed consistent oviposition-modifying activity over the 5 day period.

Repellents/deterrents and attractants could be utilized in mosquito control programs by manipulating the attractiveness of existing oviposition sites. Ideally, attractant compounds could be provided in oviposition traps for use in monitoring programs, while repellent/deterrent compounds could be used to reduce oviposition in specific habitats. Because *Ae. aegypti* predictably lays eggs in peridomestic dark containers, it may be possible to develop ovitraps containing compounds that attract gravid females but kill emerging larvae, and whose effectiveness might be enhanced by the addition of a synergist such as PBO.

The present study demonstrates the potential for using natural phytochemicals as larvicides and for altering reproductive behaviors in *Ae. aegypti*. The synergistic effects of PBO with these compounds was also demonstrated, a fact which could be exploited in developing more effective strategies to prevent and control mosquitoes. Furthermore, most of the compounds tested, particularly those that showed high larval toxicity and oviposition repellency (thymol, eugenol, rosemary oil, pulegone) are listed in the US Food and Drug Administration (FDA) 'generally recognized as safe' (GRAS) list,[54] indicating their safe use on food products.[17] The positive attributes of these compounds, such as relative safety to non-target aquatic invertebrates, long-term oviposition deterrency and larval control, warrant further research into their potential development as compounds for the control of mosquitoes.

ACKNOWLEDGEMENTS

The authors thank Dr Russell Nicholson for editorial assistance and Kendra Foster for providing *Ae. aegypti* eggs. This study was supported by an NSERC award to RW and by an NSERC award (261940), a CIHR award (69558), the Canada Research Chair Program and an MFSHR scholar award to CL.

REFERENCES

1 Paul A, Laura CH and Scott JG, Evaluation of novel insecticides for control of dengue vector *Aedes aegypti* (Diptera: Culicidae). *J Med Entomol* **43**:55–60 (2006).
2 Lowenberger CA, Kamal S, Chiles J, Paskewitz S, Bulet P, Hoffmann JA, *et al*, Mosquito–*Plasmodium* interactions in response to immune activation of the vector. *Exp Parasitol* **91**:59–69 (1999).
3 Gubler DJ, The global emergence/resurgence of arboviral diseases as public health problems. *Arch Med Res* **33**:330–342 (2002).
4 Rodriguez MM, Bisset J, Fernandez MD, Lauzan L and Soca A, Detection of insecticide resistance in *Aedes aegypti* (Diptera: Culicidae) from Cuba and Venezuela. *J Med Entomol* **38**:623–628 (2001).
5 Chaton PF, Ravanel P, Tissut M and Meyran JC, Toxicity and bioaccumulation of fipronyl in the nontarget arthropodan fauna associated with subalpine mosquito breeding sites. *Ecotoxicol Environ Sat* **52**:8–12 (2002).
6 Schulze TLRA, Jordan RW, Hung AJ, Krivenko J, Schulze JJ and Jordan TM, Effects of an application of granular carbaryl on nontarget forest floor arthropods. *J Econ Entomol* **94**:123–128 (2001).

7 Resistance of vectors and reservoirs of disease to pesticides. *WHO Tech Rep Ser* **737**:(1986).
8 Georghiou GP, Wirth M, Tran H, Saume F and Knudsen AB, Potential for organophosphate resistance in *Aedes aegypti* in the Caribbean area and neighboring countries. *J Med Entomol* **24**:290–294 (1997).
9 Rawlins SC, Spatial distribution of insecticide resistance in Caribbean populations of *Aedes aegypti* and its significance. *Pan Am J Public Health* **4**:243–251 (1998).
10 Rawlins SC and Ragoonansingh R, Comparative organophosphorus insecticide susceptibility in Caribbean populations of *Aedes aegypti* and *Toxorynchites moctezuma*. *J Am Mosq Control Assoc* **6**:315–317 (1999).
11 Rawlins SC and Wan JOH, Resistance in some Caribbean populations of *Aedes aegypti* to several insecticides. *J Am Mosq Control Assoc* **11**:59–65 (1995).
12 Lee S, Tsao T, Peterson C and Coats JR, Insecticidal activity of monoterpenoids to western corn rootworm (Coleoptera: Chrysomelidae), two-spotted spider mite (Acari: Tetranychidae), and house fly (Diptera: Muscidae). *J Econ Entomol* **90**:883–892 (1997).
13 Benner JP, Pesticidal compounds from higher plants. *Pestic Sci* **39**:95–102 (1993).
14 Singh D, Siddiqui MS and Sharma S, Reproduction retardant and fumigant properties in essential oils against rice weevil (Coleoptera: Curculionidae) in stored wheat. *J Econ Entomol* **82**:727–733 (1989).
15 Wilson CL, Solar JM, El Ghaouth A and Wisniewski ME, Rapid evaluation of plant extracts and essential oils for antifungal activity against *Botrytis cinerea*. *Plant Dis* **81**:204–210 (1997).
16 Panella NAMC, Dolan JJ, Karchessy Y, Xiong J, Peralta-Cruz M, Khasawneh JA, *et al*, Use of novel compounds for pest control: insecticidal and acaricidal activity of essential oil components from heartwood of Alaska yellow cedar. *J Med Entomol* **42**:352–358 (2005).
17 Isman MB, Pesticides based on plant essential oils. *Pestic Outlook* **10**:68–72 (1999).
18 Karr LL and Coats JR, Insecticidal properties of d-limonene. *J Pestic Sci* **13**:287–290 (1988).
19 Hough-Goldstein JA, Antifeedent effects of common herbs on the Colorado potato beetle (Coleoptera: Chrysomelidae). *Environ Entomol* **19**:234–238 (1990).
20 Sharma RN and Saxena KN, Orientation and developmental inhibition in the housefly by certain terpenoids. *J Med Entomol* **11**:617–621 (1974).
21 Gengaihi E, Amer SE and Mohamed SAA, Biological activity of thyme oil and thymol against *Tetranychus urticae* Koch. *Anz Schäd Pflanz Umwelt* **69**:157–159 (1996).
22 Isman MB, Wan AJ and Passreiter CM, Insecticidal activity of essential oils to the tobacco cutworm, *Spodoptera litura*. *Fitoterapia* **72**:65–68 (2001).
23 Masatoshi H and Hiroaki K, Repellency of rosemary oil and its components against the onion aphid, *Neotoxopteraf ormosana* (Takahashi) (Homoptera, Aphididae). *App Entomol Zool* **32**:303–310 (1997).
24 Masatoshi H, Repellency of rosemary oil against *Myzus persicae* in a laboratory and in a screenhouse. *J Chem Ecol* **9**:1425–1432 (1998).
25 Enan E, Insecticidal activity of essential oils: octopaminergic sites of action. *Comp Biochem Physiol* **130**:325–337 (2001).
26 Brattsten LB, Cytochrome P-450 involvement in the interactions between plant terpenes and insect herbivores, in *Plant Resistance to Insects*. *ACS Symp Ser* No. 208, ed. by Hedin PA. American Chemical Society, Washington, DC, pp. 173–195 (1983).
27 Grundy DL and Still CC, Inhibition of acetylcholinesterases by pulegone-1,2-epoxide. *Pestic Biochem Physiol* **23**:383–388 (1985).
28 Coats JR, Karr LL and Drews CD, Toxicity and neurotoxic effects of monoterpenoids in insects and earthworms, in *Naturally Occurring Pest Bioregulators*, *ACS Symp Ser* No. 449, ed. by Hedin PA. American Chemical Society, Washington, DC, pp. 305–316 (1991).
29 Su T and Mulla MS, Ovicidal activity of neem products (azadirachtin) against *Culex tarsalis* and *Culex quinquefaciatus* (Diptera: Culicidae). *J Am Mosq Control Assoc* **14**:204–209 (1998).
30 Boschitz C and Grunewald J, The effect of neem Azal on *Aedes aegypti* (Diptera: Culicidae). *J Appl Parasitol* **35**:251–256 (1994).
31 Perich MJ, Wells C, Bertsch W and Tredway KE, Toxicity of extracts from three *Tagetes* against adults and larvae of yellow fever mosquito and *Anopheles stephensi* (Diptera: Culicidae). *J Med Entomol* **31**:833–837 (1994).
32 Tawatsin A, Asavadachanukorn P, Thavara U, Wongsinkongman P, Bansidhi J, Boonruad T, *et al*, Repellency of essential oils extracted from plants in Thailand against four mosquito vectors (Diptera: Culicidae) and oviposition deterrent effects against *Aedes aegypti* (Diptera: Culicidae). *Southeast Asian J Trop Med Pub Health* **37**:915–931 (2006).
33 Casida JE, Mixed-function oxidases involvement in the biochemistry of insecticide synergist. *J Agric Food Chem* **18**:753–772 (1970).
34 Jao LT and Casida JE, Insect pyrethroid hydrolyzing esterases. *Pestic Biochem Physiol* **4**:465–472 (1974).
35 Feyereisen R, Insect P450 enzymes. *Annu Rev Entomol* **44**:507–533 (1999).
36 Hemingway J and Ranson H, Insecticide resistance in insect vectors of human disease. *Annu Rev Entomol* **45**:371–391 (2000).
37 Kumar S, Thomas A, Sahgal A, Verma A, Samuel T and Pillai MKK, Effect of the synergist, piperonyl butoxide, on the development of deltamethrin resistance in yellow fever mosquito, *Aedes aegypti* L. (Diptera: Culicidae). *Arch Insect Biochem Physiol* **50**:1–8 (2002).
38 Lowenberger CA and Rau ME, Selective oviposition by *Aedes aegypti* (Diptera Culicidae) in response to a larval parasite, *Plagiorchis elegans* (Trematoda: Plagiorchiidae). *Environ Entomol* **23**:1269–1276 (1994).
39 Finney DJ, *Probit Analysis*, 3rd edition. Cambridge University Press, London, UK (1964).
40 Abbott WS, A method of computing the effectiveness of an insecticide. *J Econ Entomol* **18**:265–267 (1925).
41 Lowenberger CA and Rau ME, Selective oviposition by *Aedes aegypti* (Diptera: Culicidae) in response to a larval parasite, *Plagiorchis elegans* (Trematoda: Plagiorchiidae). *Environ Entomol* **23**:1269–1276.
42 Kramer WL and Mulla MS, Oviposition attractants and repellants of mosquitoes: oviposition responses of Culex mosquitoes to organic infusions. *Environ Entomol* **8**:1111–1117 (1979).
43 Silva WJ, Doria GAA, Maia RT, Nunes RS, Carvalho GA, Blank AF, *et al*, Effects of essential oils on *Aedes aegypti* larvae: alternatives to environmentally safe insecticides. *Biotech* **99**:3251–3255 (2008).
44 Cheng SS, Liu JY, Tsai KH, Chen WJ and Chang ST, Chemical composition and mosquito larvicidal activity of essential oils from leaves of different *Cinnamomum osmophloeum* provenances. *J Agric Food Chem* **52**:4395–4400 (2004).
45 Chantraine JM, Laurent D, Ballivian C, Saavedra G, Ibanez R and Vilaseca LA, Insecticidal activity of essential oils on *Aedes aegypti* larvae. *Phytother Res* **12**:350–354 (1998).
46 Morais SM, Calvacanti ESB, Bertini LM, Oliveira CL, Rodrigues JRB and Cardoso JHL, Larvicidal activity of essential oils of Brazilian Croton species against *Aedes aegypti* L. *J Am Mosq Control Assoc* **22**:161–164 (2006).
47 Sosan MB, Adewoyin FB and Adewunmi CO, Larvicidal properties of three indigenous plant oils on the mosquito *Aedes aegypti*. *Nigerian J Nat Prod Med* **5**:30–33 (2001).
48 Cavalcanti ESB, Morais SM, Lima MA and Santana EWP, Larvicidal activity of essential oils from Brazilian plants against *Aedes aegypti*. *Mem Inst Oswaldo Cruz* **99**:541–544 (2004).
49 Amer A and Mehlhorn H, Larvicidal effects of various essential oils against *Aedes, Anopheles*, and *Culex* larvae. *Parasitol Res* **99**:466–472 (2006).
50 Clements AN, Egg laying, in *The Biology of Mosquitoes, Vol. 2*. CABI Publishing, Wallingford, UK, p. 559 (1999).
51 Xue RD, Barnard DR and Ali R, Laboratory evaluation of 18 repellent compounds as oviposition deterrents of *Aedes albopictus* and as larvicides of *Aedes aegypti, Anopheles quadrimaculatus*, and *Culex quinquefasciatus*. *J Am Mosq Control Assoc* **11**:72–76 (2003).
52 Bentley MD and Day JF, Chemical ecology and behavioral aspects of mosquito oviposition. *Annu Rev Entomol* **34**:401–21 (1989).
53 Clements AN, Sensory reception and behaviour, in *The Biology of Mosquitoes, Vol. 2*. CABI Publishing, Wallingford, UK (1999).
54 Waliwitiya R, Isman MB, Vernon RS and Isman A, Insecticidal activity of selected monoterpenoids and rosemary oil to *Agriotes obscurus* (Coleoptera: Elateridae). *J Econ Entomol* **98**:1560–1565 (2005).

