# Alternative control of *Aedes aegypti* resistant to pyrethroids: lethal and sublethal effects of monoterpenes bioinsecticides

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Running title: **Bioinsecticides to control *Aedes aegypti* resistant to pyrethroids**

## ABSTRACT

**BACKGROUND:** The mosquito *Aedes aegypti* is intensely controlled because it is vector of viruses that cause innumerous diseases, especially in tropical regions. Due to the indiscriminate use of insecticides, populations from different regions have been resistant to pyrethroids. Here,

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we analyzed the lethal and sublethal effects of essential oil of *Aristolochia trilobata* and its major compounds on *A. aegypti* from susceptible and pyrethroid resistant populations.

**RESULTS:** Our results showed that the toxicity and behavioral changes to different compounds are dependent of the stage of the insect life cycle. The monoterpene ρ-cymene caused high mortality in both larvae and adult females of *A. aegypti*, including those from the pyrethroid resistant population. The monoterpenes limonene and linalool caused a sublethal effect in the larvae triggering changes in the swimming pattern.

**CONCLUSIONS:** This study highlights the potential of the essential oil of *A. trilobata*, ρ-cymene and limonene to the control of *A. aegypti* and reveals the importance of analyzing the sublethal effects for the population dynamics of the *A. aegypti* mosquito.

**Keywords**: *Aristolochia trilobata*; sublethal effect; plant essential oils; ρ-cymene; toxicity.

## 1 INTRODUCTION

Insects are the main vectors of pathogens in the tropical regions, causing serious diseases that result in high mortality and economic costs.[1,2] The mosquito *Aedes aegypti* (Diptera: Culicidae) is considered the most important vector of viral diseases for humans, including the dengue fever, urban yellow fever, Zika and Chicungunya.[3–6] In the last decades, the control of *A. aegypti* has been performed basically by insecticides,[7,8] mainly pyrethroids,[9] which nowadays represent the class of insecticide most used *per* area.[10] However, *A. aegypti* populations in several regions of the world have been shown resistance to this group of insecticides, limiting their control.[11–13]

Studies have reported the occurrence of costs associated with resistance, with consequent reduction in the fitness of the individuals in environment free of selection factors or when exposed to other types of bioactive molecules.[14,15] In this sense, an alternative to the control of resistant populations of *A. aegypti* is the use of complex mixtures of bioactive compounds that show different mechanisms and sites of action from insecticides, as is the case of essential oils (EOs) of plants.[16–20] In fact, some studies have been shown that populations of *A. aegypti*, including those resistant, can be controlled by these products.[21–23] Among the effective bioinsecticides, the EO from *Aristolochia trilobata* (Aristolochiaceae) stands out for its lethal and/or sublethal effects investigated in fungi,[24] bacteria[25] and pest insects.[26] The compounds present in the EO of this plant species have synergistic and rapid actions on cutter ants *Atta sexdens* and *Acromyrmex balzani*, as well as sublethal effects in the locomotion behavior of these insects.[26] However, modification in the motor coordination in rats was not verified, which may indicate an apparent absence of toxicity in humans.[27] In addition, fundamental steps to obtain the botanical insecticide from *A. trilobata* have already been elucidated, including chemical analyzes about the spatial geometry and the isolation of its major compound.[28] Thus, the EO of *A. trilobata* may emerge as a promising source of molecules for the development of alternative insecticides.

In addition to the efficiency of bioinsecticides, the use of such compounds may also have other advantageous, such as selectivity to non-target organisms, low residual power and longer demand for resistance selection due to the complexity of compounds.[16,29] The prospection of EOs from plants as bioinsecticides is increasing, and the procedures such as nanoencapsulation can optimize the bioactivity of the compounds as well as make it physically more stable.[30] Currently, a range of EOs from plants are used in botanical insecticides products available on the market and there is a high prospect that these products will occupy much of the commercial production of insecticides, due to environmental safety, the demand for selective

toxic efficiency, and the recent discussions by the European Community (European Food Safety Authority) about the use of botanical insecticides and the definition of these products as Low Risk Active Substances (SABRs).[30] However, the sublethal effects of bioinsecticides in the different stages of the life cycle of *A. aegypti* populations resistant to pyrethroid are still poorly understood.

The selection of resistance to insecticides may interfere with the characteristics of the life history of organisms, triggering ecological trade-offs.[31] Thus, it is important that the selection of new molecules to the control of resistant insects be not only based on the lethal effects, but also on the analysis of sublethal effects in different stages of the life cycle of the individuals. Such effects can trigger behavioral changes that result in changes in the fitness of these individuals when subjected to different biotic and abiotic pressures in natural situations.

Here, we evaluated the lethal and sublethal effects of EO of *A. trilobata* and its major compounds on resistant and susceptible populations of *A. aegypti*. Larval and female adult mortality, as well as the larval swimming behavior, were analyzed in individuals treated with bioinsecticides and the pyrethroid deltamethrin. The comprehension of toxicity levels and the sublethal effects of bioinsecticides, especially in resistant populations, can provide important information that allows a more efficient control of this vector.

## 2 MATERIALS AND METHODS

### 2.1 *A. aegypti* populations

Populations of *A. aegypti* used in the bioassays (*N=2*) were obtained from Federal University of Viçosa (Departamento de Biologia Geral, Viçosa, Minas Gerais, Brazil). The populations were the PPCampos – population susceptible to pyrethroids (municipality of Campos dos Goytacazes, state of Rio de Janeiro, Brazil) – and F2 Oiapoque – population resistant to pyrethroids (municipality of Rio de Janeiro, state of Rio de Janeiro, Brazil).

Populations were reared following the procedure described in Marriel *et al.* (2016).[32] The larvae of *A. aegypti* populations were kept in plastic trails (6 L) with 5 L of water (chlorine free) and they were fed with animal ration. The adults were conditioned separately by populations in plastic cages (20 cm x 18 cm ϕ) covered with organza. Sucrose solution in a cotton was offered daily for feeding. Insects were kept under controlled temperature, humidity and photoperiod conditions (25 ± 2ºC, 60 ± 2% and 12:12 L).

### 2.2 Obtaining bioinsecticides and pyrethroid

The EO was extracted from stems of *A. trilobata* plants collected in a mangrove area, in the municipality of Pirambu, Sergipe, Brazil (10º40'42" S, 36º52'25" W). The mean annual temperature and rainfall in the region are 26 °C and 1,650 mm, respectively, with a rainy season from March to August.[33] The sampled material was oven dried at 60±1 °C for four days.[34] Then, the EO was obtained by hydrodistillation in a Clevenger-type apparatus[35] and it was separated from the aqueous phase and stored in an amber vial at a temperature of -4 °C.

The analysis of EO components was performed by GC/MS/FID (GCMSQP2010 Ultra, Shimadzu Corporation, Kyoto, Japan) equipped with an automatic injector AOC-20i (Shimadzu). The separations were performed on a Rtx®-5MS Restek (5%-diphenyl-95%-dimethylpolysiloxane) fused-silica capillary column with 30 m x 0.25 mm internal diameter, 0.25 mm film thickness, in a constant flow of Helium 5.0 with the rate of 1.0 ml $min^{-1}$. The injection temperature was 280 °C, and 1.0 µL (10 mg $ml^{-1}$) of the sample was injected in a split ratio of 1:30. The oven temperature program started with 50 °C (isotherm for 1.5 min) with an increase of 4 °C $min^{-1}$ to 200 °C, then at 10 °C $min^{-1}$ to 300 °C, remaining for 5 min.

For GC/MS, molecules were ionized by ionization of electrons with an energy of 70 eV. The fragments were analyzed by a quadrupole system programmed to filter fragments/ions with m/z in the order of 40 to 500 Da and detected by an electron multiplier. The ionization method for the GC/FID was performed by the flame coming from hydrogen gases 5.0 (30 ml $min^{-1}$) and synthetic air (300 ml $min^{-1}$). The species sampled and the electric current generated were amplified and processed. The data were processed in the GC Postrun Analysis (Labsolutions-Shimadzu).

The identification of compounds was performed based on the retention index present in the literature.[36] It was used the Van den Dool e Kratz (1963)[37] equation in relation to a homologous series of *n*-alkanes (*n*C9- *n*C18). It was also used three libraries from the equipment (WILEY8, NIST107 e NIST21), which allow the comparison of spectra data with those in the libraries by a similarity index of 80%.

Compounds of EO of *A. trilobata* were considered as major compounds when present proportion higher than 6% of total chemical composition: ρ-cymene, limonene, linalool and sulcatyl acetate (see Fig. 1A). Chemical standards of these compounds were purchased from Sigma-Aldrich® (Steinheim, Germany), except for sulcatyl acetate, which was synthetized by chemical reduction of 6-methyl-5-hepten-2-one (sulcatone, 500 mg, 3.97 mmol, Sigma-Aldrich®) (see Fig. 1B).[26]

The deltamethrin pyrethroid (K-othrine 25 CE, 25 g i.a./L, emulsifiable concentrate, Bayer S.A.®, Estrada da Boa Esperança, Belford Roxo, Rio de Janeiro, Brazil) was obtained commercially.

**2.3 Bioassays**

Bioassays of toxicity were performed, at first, to determine the concentration and dose-mortality curves to larvae and adults of susceptible population using: i) the EO of *A. trilobata*; and its major compounds: ii) ρ-cymene, iii) limonene, iv) linalool, v) sulcatyl acetate; and vi) deltamethrin pyrethroid. The control was done using surfactant or solvent. The $LC_{90}$ (larvae) and $LD_{90}$ (adult females) obtained from the susceptible population were used to evaluated the toxicity of these compounds on pyrethroid resistant population. The lethal time was estimated for both populations, considering only the compounds that showed toxicity for larvae and adult females of the pyrethroid resistant population. Subsequently, behavioral assays were performed to evaluate the sublethal effects of all treatments on both susceptible and pyrethroid resistant populations.

In all bioassays with larvae of *A. aegypti*, the treatments EO of *A. trilobata* and its major compounds were diluted in distilled water with the surfactant dimethylsulfoxide at 0.2% (ppm). In the bioassays with adult females, dilutions of these treatments were done with acetone (μg/mg of insect). Preliminary tests showed that the surfactant dimethylsulfoxide does not alter the swimming behavior of larvae (3rd instar – L3) and that acetone does not affect the survivorship of *A. aegypti* adult females.

All bioassays were performed as recommended by World Health Organization.[11,38,39]

*2.3.1 Toxicity*

The toxicity bioassays to obtain the concentration and dose-mortality curves were performed with larvae (L3) and adult females of the pyrethroid susceptible population.

Larvae were exposed to treatments by contact and ingestion in aqueous solution. Each experimental unit consisted of 10 *A. aegypti* larvae in 100 ml of treatment solution conditioned in closed plastic pot (140 ml). It was conducted eight replicates *per* treatment *x* population (susceptible and pyrethroid resistant).

For adults, individuals were exposed to treatments by contact. For this, each experimental unit consisted of 10 *A. aegypti* females treated conditioned in a plastic pot (3 cm ϕ). Treatments were applied (0.5 μL) in the thoracic region of the mosquitoes with microsyringe

of 10 µL (Hamilton, Reno, NV, USA). Females were previously sedated with $CO_2$ and handled on a table with dry ice at 4 °C. It was conducted four replications *per* treatment *x* population.

Posteriorly, bioassays with the same method were performed for larvae and adult females of the pyrethroid resistant population, except for sulcatyl acetate which did not kill 90% of individuals from the susceptible population (see Table 2).

In all bioassays, mortality was evaluated after 48 hours of exposure of insects to treatments.

*2.3.2 Lethal time*

Bioassays were performed evaluating the mortality of insects from both susceptible and pyrethroid resistant populations to determine survival curves and lethal times (LTs). It was used only treatments that showed toxicity for larvae and adult females of the pyrethroid resistant population: $LC_{90}$ and $LD_{90}$ to deltamethrin and to ρ-cymene (see Tables 2 and 3).

All bioassays were performed according to the method described in the toxicity bioassays. It was conducted 10 replicates for each treatments *x* population. The mortality of larvae was evaluated each 10 min in the first hour, 30 min from 1 h, 120 min from 30 h to 54 h, 360 min from 54 h to 78 h and 720 min from 78 h until the end of the bioassay when the pupae emerged in adults. The mortality of adult females was evaluated each 5 min in the first hour, 10 min from 1 h to 2 h, 15 min from 2 h to 3 h, 30 min from 3 h to 6 h, 60 min from 6 h to 12 h, 120 min from 12 h to 24 h, 240 min from 24 to 48 h, 360 min from 48 h to 72 h and 720 min from 72 h until the end of bioassay when the mortality of control reached 20%.

*2.3.3 Swimming behavior of larvae*

Bioassays were performed to verify the effect of treatments and the exposure time on the larval swimming behavior of both susceptible and pyrethroid resistant populations, considering: *(i)* displacement, *(ii)* swimming speed, *(iii)* the sinuosity of the path travelled (meander), *(iv)* the change of orientation (angular velocity) and *(v)* the activity (time of rest or moving).

Bioassays were performed in Petri dish (9 cm ϕ x 1.5 cm) with 20 ml of solution with treatments diluted in the $LC_{20}$ determined to larvae of the susceptible population. In each Petri dish, a single larva was conditioned and it was video-recorded for 10 min using a video camera (Panasonic SD5 Superdynamic - modelWV-CP504 – Spacecom lens 1/3" 3-8 mm), coupled to a computer. Video recorded was performed immediately and 48 h after exposure of larvae to treatments. The analyzes of behaviors were performed using Ethovision XT software (version

8.5; Noldus Integration System, Sterling, VA) and Studio 9 (Pinnacle Systems, mountain View, CA). It was conducted 25 replicates *per* population in each treatment and exposure time.

### 2.4 Statistical analyses

Probit analyzes were performed to obtain the concentration- and dose- mortality curves for larvae and adult females of *A. aegypti* from susceptible and pyrethroid resistant populations. Analyzes were conducted in the SAS software (PROC PROBIT; SAS, 2011).[40] The LCs and LDs estimated by these curves were compared using the criteria of non-overlapping of the confidence intervals ($IC_{95}$) with the origin of the interval.

The values of mortality of larvae and adult females from pyrethroid resistant population were submitted to Analysis of Variance (ANOVA) and compared by Tukey test at the 5% of probability level, using SAS software (PROC GLM, com Tukey; SAS, 2011).[40]

For each combination of treatment *x* population *x* development stage survival curves were obtained using Kaplan-Meier estimators in the SigmaPlot 11.0 program. By means of these curves, it was possible to estimate the times necessary to cause mortality of 50% of the individuals of each population ($LT_{50}$). Survival curves were compared by pair by Holm-Sidak method, in the SigmaPlot 11.0.

In the behavioral bioassays of larvae swimming, firstly it was analyzed the behavior of individuals from susceptible and pyrethroid resistant populations in the absence of treatments (control) in the two exposure times with Multivariate Analysis of Variance (PROC GLM, with MANONVA; SAS) to verify possible intrinsic differences between populations. Then, data of behavior of larvae swimming exposed to control and treatment were also analyzed with Multivariate Analysis of Variance (PROC GLM, with MANONVA; SAS).

Univariate Analysis of Variance (ANOVA) (for each population and time of exposure) were performed individually for the following parameters: displacement, swimming velocity, meander, angular velocity and activity (rest/moving time of larvae) in function of treatments. Afterwards, data were analyzed by Dunnett test to compare means of the control with the means of the treatments (PROC GLM, com Dunnett; SAS).

## 3 RESULTS

### 3.1 Chemical characterization of EO of *A. trilobata*

The EO from *A. trilobata* shows an average yield (oil mass/plant mass) of 3%. A total of 25 compounds were identified and quantified in the EO of *A. trilobata*, which represented 98.72% of the total composition. The compounds in greater proportions were the monoterpenes: sulcatyl

acetate (25.64%), limonene (24.80%), ρ-cymene (10.41%) and linalool (9.51%). The other constituents represented less than 5.3% of the composition of EO of *A. trilobata* (Table 1).

### 3.2 Bioassays

#### *3.2.1 Toxicity*

For larvae of the *A. aegypti* susceptible population, the required concentration of the compounds to kill 50% of the individuals varied from 3.3 x $10^{-5}$ to 238.80 ppm (Table 2). The insecticide deltamethrin was more toxic to larvae than the EO of *A. trilobata* and its major compounds. Among the bioinsecticides, the highest toxicity was observed for limonene, followed by ρ-cymene, EO of *A. trilobata* and linalool. The compound sulcatyl acetate did not cause mortality in individuals even when applied at the maximum dose tested (600 ppm). This pattern was the same for the $LC_{90}$ (Table 2).

For adult females of the susceptible population, the insecticide deltamethrin was also the more toxic compound, presenting $LD_{50}$ of 3.2 x $10^{-8}$ μg $mg^{-1}$. Among bioinsecticides, the $LD_{50}$ varied from 12.25 to 32.81 μg $mg^{-1}$, presenting a different toxicity pattern from that observed for larvae: the EO of *A. trilobata* was the compound more toxic, followed by linalool, sulcatyl acetate and ρ-cymene (Table 3).

The mortality of larvae and adult females of the pyrethroid resistant population treated with deltamethrin ($LC_{90}$ and $LD_{90}$, respectively) did not differ from control, as expected. The same result was observed for the linalool compound (Fig. 2). However, larvae and adult females of the pyrethroid resistant population presented high mortality (≈ 100%) when treated with the compound ρ-cymene. Similar mortality was observed for adult females treated with limonene. On the other hand, the EO of *A. trilobata* and the sulcatyl acetate resulted in mortality about 50-60% of adult females but had no effect on larval mortality (Fig. 2).

#### *3.2.2 Lethal time*

The survivorship of *A. aegypti* susceptible population exposed to deltamethrin and ρ-cymene was significantly reduced over the time (larvae/pupae: Log-rank test: $\chi 2=260.97$, df=2, $P<0.001$ and adult females: Log-rank test $\chi 2=223.50$, df=2, $P<0.001$) (Fig. 3A and 3C). Larvae in this population showed a quick reduction in survival when exposed to deltamethrin ($LT_{50}$= 0.24h) and ρ-cymene ($LT_{50}$= 0.55h), differing significantly from the control. In less than 10 h, all individuals died (Fig. 3A). The adult females of this same population had their survival reduced more quickly by ρ-cymene ($LT_{50}$= 0.08h) than by deltamethrin ($LT_{50}$= 57.9h) and

control. All adult females exposed to ρ-cymene die instantly. However, 34% of adult females exposed to deltamethrin survived until the end of the bioassays (Fig. 3C).

Likewise, the survivorship of *A. aegypti* from pyrethroid resistant population exposed to deltamethrin and ρ-cymene was significantly reduced over the time (larvae/pupae: Log-rank test: $\chi 2$=394.35, df=2, $P$<0.001 and adult females: Log-rank test: $\chi 2$=127.29; df=2, $P$<0.001) (Fig. 3B and 3D). In this population, as expected, the lethal time to kill 50% of individuals (larvae-pupae and adult females) did not differ between deltamethrin and control. However, ρ-cymene showed a rapid potential to reduce the survival of the population, with a lethal time of 9.5 and 0.08 h for larvae and adult female, respectively (Fig. 3B and 3D). All adult females exposed to ρ-cymene die instantly (Fig. 3D) and only 4% of the larvae/pupae exposed to this compound survived until the end of the bioassays (Fig. 3B).

### 3.3 Larval swimming behavior

In the control, the swimming behavior of larvae from susceptible and pyrethroid resistant populations differed when evaluated immediately (Wilks' lambda=0.532, $F$=7.73; df num/den=5/44, $P$<0.001) or after 48 h of swimming (Wilks' lambda 0.758, $F$=19.27, df num/den=5/44, $P$<0.001).

Similarly, treatments modified the swimming behavior of larvae from susceptible (Wilks' lambda=0.232, $F$=20.38, df num/den=25/1056, $P$<0.001) and pyrethroid resistant population (Wilks' lambda=0.510, $F$= 8.41, df num/den=25/1056, $P$<0.001).

#### *3.3.1 Displacement and swimming speed*

There were significant differences in the displacement and swimming speed of larvae from susceptible ($F_{5,288}$=26.92, $P$<0.001 and $F_{5,288}$=26.92, $P$<0.001) and pyrethroid resistant ($F_{5,288}$=15.35, $P$<0.001 and $F_{5,288}$=19.30, $P$<0.001) exposed to treatments. The most representative trails of the displacement of the two populations exposed to treatments are shown in Fig. 4.

In general, when there was a significant effect of the treatments, and they caused a reduction in the displacement and the swimming speed of the larvae compared with control (Fig. 5). The displacement of individuals from the susceptible population (at both exposure times) was significantly reduced when exposed to all treatments, except for deltamethrin that did not differ from the control (Fig. 5A). On the other hand, the larvae from pyrethroid resistant population had their displacement affected by different compounds depending on the time of exposure. Immediately after contact, deltamethrin, ρ-cymene and limonene reduced the

displacement of individuals. However, after 48 h of exposure, this effect was observed only for ρ-cymene, limonene and linalool (Fig. 5B).

The swimming speed (Fig. 5C and D) followed a pattern similar to that described above. For the susceptible population, the exception was the absence of effect of ρ-cymene immediately after contact and the absence of effect of limonene after 48 h of exposure. The deltamethrin increased the swimming speed immediately after contact (Fig. 5C). For the pyrethroid resistant population, the only exception was the absence of change in the speed of larvae after 48 h of exposure in the ρ-cymene treatment (Fig. 5D).

*3.3.2 Meander and angular velocity of larvae*

There were significant differences in the number of meanders and the angular velocity of larvae from susceptible ($F_{5,288}$=4.16, $P$<0.001 and $F_{5,288}$=5.08, $P$<0.001) and pyrethroid resistant population ($F_{5,288}$=5.24, $P$<0.001 and $F_{5,288}$=5.08, $P$<0.001) exposed to treatments.

When there was a significant effect of the treatments, in general, these caused an increase in the disorientation (increase in a number of meanders and the angular velocity) of the larvae during swimming when compared to the control (Fig. 6). Larvae of the susceptible population performed more meanders and greater angular velocity of the swim when they were immediately exposed to linalool (Fig. 6A and 6C). After 48h of exposure, the EO of *A. trilobata* also increased the angular velocity of the larvae of this population (Fig. 6C).

The pyrethroid resistant population presented more meanders and angular velocity immediately when exposed to ρ-cymene and limonene (Fig. 6 B and 6D). After 48 h of exposure, the meanders were also increased when exposed to linalool (Fig. 6B).

*3.3.3 Activity of larvae*

There were significant differences in the time that larvae from the susceptible ($F_{5,288}$=8.07, $P$<0.001) and pyrethroid resistant ($F_{5,288}$=10.41, $P$<0.001) populations remained immobile when exposed to treatments.

Larvae from susceptible population treated with linalool, at both exposure times, remained immobile for a longer time than those in the control (Fig. 7A and 7B).

On the other hand, larvae from pyrethroid resistant population when immediately treated with deltamethrin, ρ-cymeno and limonene remained a greater proportion of the time immobile than larvae in the control (Fig. 7C). After 48 h of exposure, larvae from this population remained more time immobile when treated with deltamethrin, limonene and linalool compared with larvae from control (Fig. 7D).

## 4 DISCUSSION

In the last years, the botanical insecticides based in the EOs from plants have been considered an environmental and health safe product, including absence of deleterious effects on non-target organisms (*e.g.* bees, wasps, fish and human) and decreasing problems of selection of pest resistance. So, these products can be a safe solution for decrease the environmental problems caused by the indiscriminate use of insecticides.[30] The selection of *A. aegypti* resistance to insecticides, mainly in the tropical regions, has been a major obstacle to the control of this important vector of diseases. Our results highlight the high potential of monoterpenes, ρ-cymene and limonene, found in the EO of *A. trilobata* on larvae and adult females from a population highly resistant to pyrethroids (deltamethrin and permethrin).[41] Additionally, our results also indicate that other monoterpenes trigger sublethal effects by changing the behavioral patterns that can reduce the fitness of these individuals in natural situations.

The lethal effects of the different compounds found in the EO of *A. trilobata* were dependent on the life cycle of *A. aegypti* individuals, which is related to morphological and physiological characteristics of each development stage. As expected, the pyrethroid resistant population, when exposed to deltamethrin did not present significantly different mortality from that observed in the control. In general, pyrethroids provoke a prolonged opening of the sodium channels,[42] inducing a strong excitatory action on the nervous system of insects. However, the pyrethroids resistant populations of *A. aegypti* may have metabolic resistance (*e.g.* cytochrome P450 enzymes,[13] esterases[43] and glutathione S-transferases[44]) or mutations at target sites of insecticides binding (*i.e.* knockdown mutation - *Kdr*), not being intoxicated with deltamethrin. Larvae from the pyrethroid resistant population used in the present study have resistance associated with metabolic detoxification through cytochrome P450 enzymes, esterase and glutathione *S*-transferases and the adults are insensitive to the synergists of these enzymes, showing the high influence of the V410L mutation on the *Kdr* phenomenon.[41] Interestingly, our results show that the monoterpenes ρ-cymene and limonene present high toxicity for individuals of the pyrethroid resistant population. Two different mechanisms can be related to this result: *(i)* low metabolization of the ρ-cymene by the larvae or *(ii)* different sites of action of ρ-cymene and limonene from those of deltamethrin in the female adults.

The compound linalool did not cause toxicity to the pyrethroid resistant population. This result could be associated with cross-resistance or some physio-chemical properties of linalool

(*e.g.* presence of hydroxyl group), which may decrease the lipophilicity of the molecules and, consequently, its penetration into the cuticle of insect reducing the larvicide activity.[45–47]

The monoterpene ρ-cymene acted very quickly provoking a high mortality in individuals of susceptible and pyrethroid resistant populations. It is important to highlight that, unlike deltamethrin, which provokes lethal effect only in adult individuals, ρ-cymene showed to be efficient also for larvae. These results show the potential of the use of this compound for the control of *A. aegypti* mosquito. Our results corroborate with previous studies that have been shown that EOs from plants present an efficient insecticidal action on several pests.[48–50] However, few studies have been evaluated the potential insecticidal and sublethal effects of these compounds on populations of resistant insects, including *A. aegypti*. In the best of our knowledge, the present work is the first study to demonstrate the bioactivity of monoterpenes present in EOs against *A. aegypti* population resistant to deltamethrin as well as its sublethal effects in the swimming behavior of larvae from pyrethroid resistant population.

The occurrence of resistance in populations of insects provides to individuals an adaptive advantage to the pressure of insecticides. However, such resistance can be accompanied also by a high cost in the fitness of individuals.[31] Thus, resistance is accompanied by trade-offs which involve resource allocation between adaptive advantage *vs.* associated costs, affecting physiological, reproductive and/or behavioral process of individuals from resistant populations.[51] Specifically for *A. aegypti*, the presence of resistance has been reported to interfere within the vector capacity, biological (*e.g.* development, reproduction and survival), physiological and also behavioral parameters.[31,52,53] In the present study, individuals of *A. aegypti* from susceptible and the pyrethroid-resistant populations show naturally distinct developmental and behavioral parameters. Individuals from pyrethroid resistant population, in addition to having a shorter life cycle, also had a greater locomotion activity than individuals from the susceptible population. A reduction in the life cycle of individuals (L3-pupa) in the pyrethroid resistant population observed in the present study has also been reported in other studies.[53,54] Such reduction in the development time is an important point: in a long-term, a quick growth rate of individuals from pyrethroid-resistant populations may occur. In addition, the increased locomotion activity of individuals from pyrethroid resistant populations could increase their ability to escape from predators, increasing their likelihood of survival and reproduction. All these changes in the development and behavior increase the concern about vector activity of these mosquito.[31] However, here we showed that under effect of bioinsecticide, individuals from susceptible and pyrethroid-resistant populations suffered sublethal effects with a reduction of displacement and swimming speed, and a greater

disorientation in the swimming. These behaviors may indicate a possible perception and toxicity of compounds to the individuals tirar since the reduction of the locomotor activity is considered a strategy widely used by insects to minimize the effects of toxicity by insecticides.[55] Although it is known that deltamethrin provokes a reduction in the locomotor activity of individuals,[32] this effect was not observed in individuals from the susceptible population. In fact, there was an increase in the swimming speed of individuals from the susceptible population when exposed to deltamethrin, which could be related to the attempt to escape.

The characteristics of life history, as well as the morphology, physiology, and behavior, may represent adaptations to defend against predators. Among these characteristics is the behavior of reduction the swimming activity of mosquito larvae, which can avoid the detection by predators,[56] and also interfere in the feeding capacity of the larvae.[57] Thus, a reduction in the displacement and swimming speed of larvae from susceptible and pyrethroid resistant populations treated with monoterpenes of EOs could, at first, make them less perceptible to predators in natural situations. However, once detected by predators, its escape ability may be compromised since they have smaller displacement capacity and higher disorientation of swimming. Sublethal doses of monoterpenes from the EO of *Thymus vulgaris* was already reported to act as potential repellents against females of mosquitoes (*Culex pipiens pallens*).[58] Thus, sublethal effects may interact with external factors in the environment determining the survival and viability of mosquito populations.

## 5 CONCLUSIONS

Our results demonstrate that the EO of *A. trilobata* and its major compounds are a promising alternative to be used to control larvae and adult of *A. aegypti*. The EO of *A. trilobata* and its major compounds have a potential for the development of new insecticides' molecules for control of *A. aegypti* from both pyrethroid resistant and susceptible populations. In addition, this study contributes also to increase the comprehension of the effect of pesticides on the juvenile phases of *A. aegypti* and its possible consequences in the fitness of individuals in natural populations.

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**Table 1.** Composition of the essential oil of *Aristolochia trilobata* characterized by GC/MS/FID.

| Compounds | Retention Time (min) | Retention Index[a] | Peak Area (%)[b] |
|---|---|---|---|
| Tricyclene | 8.985 | 929 | 1.88±0.02 |
| Camphene | 9.469 | 944 | 3.24±0.012 |
| β-pinene | 10.353 | 973 | 1.05±0.02 |
| Myrcene | 10.681 | 984 | 0.68±0.03 |
| 6-Methyl-5-hepten-2-ol | 10.728 | 985 | 0.64±0.08 |
| *ρ*-cymene | 11.859 | 1020 | 10.41±0.03 |
| Limonene | 12.021 | 1025 | 24.80±0.26 |
| *(Z)*-β-ocimene | 12.546 | 1041 | 5.27±0.42 |
| Linalool | 14.257 | 1092 | 9.51±0.05 |
| Sulcatyl acetate | 15.220 | 1122 | 25.64±0.45 |
| Borneol | 16.568 | 1165 | 0.64±0.05 |
| α-terpineol | 17.328 | 1189 | 0.28±0.02 |
| Bornyl acetate | 20.222 | 1283 | 0.79±0.007 |
| β-elemene | 23.345 | 1391 | 0.30±0.012 |
| *(E)*-caryophyllene | 24.260 | 1424 | 1.15±0.03 |
| Aromadendrene | 24.633 | 1438 | 0.27±0.01 |
| Allo-aromadendrene | 25.415 | 1467 | 0.27±0.00 |
| Germacrene D | 25.922 | 1485 | 0.70±0.007 |
| Bicyclogermacrene | 26.343 | 1501 | 1.52±0.02 |
| δ-cadinene | 26.933 | 1524 | 0.40±0.06 |
| Spathulenol | 28.508 | 1586 | 3.04±0.10 |
| Globulol | 28.695 | 1594 | 4.29±0.13 |
| Viridiflorol | 28.903 | 1602 | 0.49±0.03 |
| Isospathulenol | 29.971 | 1646 | 0.94±0.05 |
| α-Cadinol | 30.348 | 1662 | 0.55±0.05 |
| Monoterpenes (%) | | | 84.83 |
| Sesquiterpenes (%) | | | 13.89 |
| Total (%) | | | 98.72 |

[a] Retention index calculated using the Van den Dool & Kratz (1963) equation relative to a homologous series of *n*-alkanes (*n*C9- *n*C18).

[b] Values (± SE) for the content of compounds by averaging three different determinations obtained by GC/MS/FID.

**Table 2.** Toxicity of deltamethrin insecticide, the essential oil of *Aristolochia trilobata* and its major compounds (ρ-cymene, limonene, linalool and sulcatyl acetate) on larvae (L3) of *Aedes aegypti* from susceptible population after 48 hours of exposure.

| Treatment | *N* | $LC_{50}$ ($CI_{95}$) (ppm) | $LC_{90}$ ($CI_{95}$) (ppm) | Slope | $\chi 2$ | *P* |
|---|---|---|---|---|---|---|
| Deltamethrin | 1116 | $3.3x10^{-5}$ ($3.2x10^{-5}$-$3.5x10^{-5}$) | $5.3x10^{-5}$ ($4.8x10^{-5}$-$6.3x10^{-5}$) | 6.25 | 0.01 | 0.99 |
| Essential oil | 1360 | 97.38 (95.51-99.78) | 113.99 (109.28-121.67) | 18.7 | 3.31 | 0.18 |
| ρ-cymene | 2229 | 97.89 (88.98-104.58) | 167.54 (154.47-189.80) | 5.48 | 0.93 | 0.81 |
| Limonene | 2559 | 74.35 (72.00-76.61) | 104.34 (99.33-111.30) | 8.69 | 3.88 | 0.57 |
| Linalool | 2708 | 238.80 (233.21-244.09) | 295.34 (285.97-307.94) | 11.1 | 0.78 | 0.85 |
| Sulcatyl acetate | 1440 | >600 | -† | - | - | - |

† It was not possible to determine the concentration curve due the low toxicity of compound.

**Table 3.** Toxicity of deltamethrin insecticide, the essential oil of *Aristolochia trilobata* and its major compounds (ρ-cymene, limonene, linalool and sulcatyl acetate) on adult females of *Aedes aegypti* from susceptible populations after 48 hours of exposure.

| Treatment | *N* | $LD_{50}$ ($CI_{95}$) (μg $mg^{-1}$) | $LD_{90}$ ($CI_{95}$) (μg $mg^{-1}$) | Slope | $\chi 2$ | *P* |
|---|---|---|---|---|---|---|
| Deltamethrin | 441 | $3.2x10^{-8}$ ($2.2x10^{-8}$-$4.8x10^{-8}$) | $2.4x10^{-6}$ ($1.1x10^{-6}$-$6.6x10^{-6}$) | 0.68 | 1.85 | 0.76 |
| Essential oil | 471 | 12.25 (11.70-12.82) | 18.49 (17.19-20.39) | 7.16 | 3.36 | 0.18 |
| ρ-cymene | 471 | 32.81 (29.08-36.95) | 93.46 (78.20-117.80) | 2.81 | 1.79 | 0.58 |
| Limonene | 506 | 26.11 (24.54-27.66) | 45.81 (42.16-50.98) | 5.24 | 4.45 | 0.21 |
| Linalool | 542 | 16.38 (13.84-19.74) | 87.71 (57.23-187.02) | 1.75 | 0.72 | 0.70 |
| Sulcatyl acetate | 773 | 25.23 (23.73-26.74) | 40.21 (37.07-44.73) | 6.32 | 2.32 | 0.31 |

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**Figure 1.** (A) Structural formula of major compounds found in the essential oil of *Aristolochia trilobata*: (1) ρ-cymene, (2) limonene, (3) linalool and (4) sulcatyl acetate. (B) Scheme of chemical synthesis of sulcatyl acetate. (1) sulcatone, (2) sulcatol and (3) sulcatyl acetate.

**Figure 2.** Mortality (%) (±S.E.) of larvae (L3) and adult females of *Aedes aegypti* from pyrethroid resistant population exposed to $LC_{90}$ (of larvae (L3) from susceptible population) and $LD_{90}$ (of adult females from susceptible population) of deltamethrin insecticide, essential oil of *Aristolochia trilobata* and its major compounds (ρ-cymene, limonene, linalool and sulcatyl acetate) after 48 hours of exposure. Bars with same lowercase letter (comparison of larvae mortality) and uppercase letter (comparison of adult female mortality) indicates no significant difference with Tuckey test ($P<0.05$). ***** The compound sulcatyl acetate was not tested on larvae of *Aedes aegypti* from pyrethroid resistant population, once it was not possible to determine the $LC_{90}$ to larvae from susceptible population.

**Figure 3.** Survival curves and lethal time required to kill 50% ($LT_{50}$) of *Aedes aegypti* larvae/pupae from susceptible (A) and pyrethroid resistant (B) populations and adult females from susceptible (C) and pyrethroid resistant (D) populations exposed to ρ-cymene and deltamethrin insecticide. It was used the $LC_{90}$ to larvae/pupae and $LD_{90}$ to adult females.

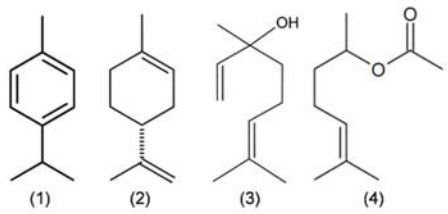
**Figure 4.** Most representative trails of displacement of *Aedes aegypti* larvae (L3) from susceptible and pyrethroid resistant populations exposed immediately and after 48 hours to $LCs_{20}$ of deltamethrin insecticide, essential oil of *Aristolochia trilobata* and its major compounds (ρ-cymene, limonene and linanool).

**Figure 5.** Displacement (±S.E.) (m) (A and B) and swimming speed (±S.E.) (pixels $s^{-1}$ x $10^{-2}$) (B and C) of *Aedes aegypti* larvae (L3) from susceptible and pyrethroid resistant populations, exposed immediately and after 48 hours to $LCs_{20}$ of deltamethrin insecticide, the essential oil of *A. trilobata* and its major compounds (ρ-cymene, limonene and linalool). * indicates significant differences compared with control with Dunnett test ($P < 0.05$).

**Figure 6.** Meander (±S.E.) (deg $mm^{-1}$) (A and B) and angular velocity (±S.E.) (deg $s^{-1}$) (C and D) of *Aedes aegypti* larvae (L3) from susceptible and pyrethroid resistant populations, exposed immediately and after 48 hours to $LCs_{20}$ of deltamethrin insecticide, the essential oil of *A. trilobata* and its major compounds (ρ-cymene, limonene and linalool). * indicates significant differences compared with control with Dunnett test ($P<0.05$).

**Figure 7.** Activity (proportion of time rest or moving) of *Aedes aegypti* larvae (L3) from susceptible (A and B) and pyrethroid resistant (C and D) populations, exposed immediately and after 48 hours to $LCs_{20}$ of deltamethrin insecticide, the essential oil of *A. trilobata* and its major compounds (ρ-cymene, limonene and linalool). * indicates significant differences compared with control with Dunnett test ($P<0.05$).

A




B