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# Evaluation of larvicidal activity and ecotoxicity of linalool, methyl cinnamate and methyl cinnamate/linalool in combination against *Aedes aegypti*

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ABSTRACT

The frequent use of synthetic pesticides to control *Aedes aegypti* population can lead to environmental and/or human contamination and the emergence of resistant insects. Linalool and methyl cinnamate are presented as an alternative to the synthetic pesticides, since they can exhibit larvicidal, repellent and/or insecticidal activity and are considered safe for use. The aim of this study was to evaluate the larvicidal activity of methyl cinnamate, linalool and methyl cinnamate/linalool in combination (MC-L) (1:4 ratio, respectively) against *Aedes aegypti*. The *in vitro* preliminary toxicity through brine shrimp lethality assay and hemolytic activity, and the phytotoxic potential were also investigated to assess the safety of their use as larvicide. Methyl cinnamate showed significant larvicidal activity when compared to linalool ($LC_{50}$ values of 35.4 μg/mL and 275.2 μg/mL, respectively) and to MC-L ($LC_{50}$ 138.0 μg/mL). Larvae morphological changes subjected to the specified treatments were observed, as the flooding of tracheal system and midgut damage, hindering the larval development and survival. Preliminary *in vitro* toxicity through brine shrimp showed the high bioactivity of the substances (methyl cinnamate $LC_{50}$ 35.5 μg/mL; linalool $LC_{50}$ 96.1 μg/mL) and the mixture (MC-L $LC_{50}$ 57.7 μg/mL). The results showed that, despite the higher larvicidal activity of methyl cinnamate, the use of MC-L as a larvicide seems to be more appropriate due to its significant larvicidal activity and low toxicity.

## 1. Introduction

*Aedes aegypti* Linnaeus (Diptera: Culicidae) is the main vector of dengue, Chikungunya and Zika virus, which are responsible for significant human morbidity and mortality in many countries (Brasil, 2015; World Health Organization (WHO), 2016). These mosquitoes are common in tropical and subtropical areas, where environmental and climatic conditions of temperature and humidity allow them to proliferate (Jansen and Beebe, 2010; Silva et al., 2014a, 2014b; Brasil, 2015).

Virus transmission by *Ae. aegypti* can be reduced or prevented with environmental management and by the use of synthetic insecticides belonging to the group of organophosphates and pyrethroids, such as Temephos (Lima et al., 2006; Silva et al., 2014a, 2014b) to minimize the spread of the mosquitoes and human contact (World Health Organization (WHO), 2009). However, frequent use of these insecticides can result in phytotoxicity, human poisoning and emerging of resistant insects (Lima et al., 2006; Pandey et al., 2013; Kumar et al., 2014). In this context, researchers have worked to develop alternative strategies to control *Ae. aegypti* proliferation, such as the use of phytolarvicides composed of plant essential oils or their compounds (Nour et al., 2009; Grzybowski et al., 2013; Kabir et al., 2013; Lima et al., 2014).

Linalool and methyl cinnamate are used in pharmaceutical, cosmetic and food industries (Letizia et al., 2003; Bathia et al., 2007) and some authors have reported their repellent, insecticidal and larvicidal activities (Peterson et al., 2000; Nour et al., 2009; Dekker et al., 2011; Kwon et al., 2011).

Recent researches have demonstrated the bioactivity of different substances used in combination, aiming to verify the existence of

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synergistic or additive effect between them (Azeredo and Soares, 2013; Grzybowski et al., 2013; Mitsopoulou et al., 2014). Annies et al. (2012) evaluated the repellent activity of linalool and methyl cinnamate, and reported that when the substances were used in combination, it was observed a higher repellent activity than when the substances were used separated.

Considering the repellent activity of linalool and methyl cinnamate used in combination and the lack of studies about their larvicidal activity, this study aimed to evaluate the larvicidal activity of methyl cinnamate, linalool, and methyl cinnamate/linalool in combination (MC-L) (ratio 1:4) against *Aedes aegypti* L. (Diptera: Culicidae) and the morphological changes of the larvae exposed to these treatments. Toxicological assays were also assessed, since the use of MC-L as larvicide involves exposing it to the environment, which may result in toxicity to plants, aquatic species and mammals, due to direct contact during application or the presence of its residues in water.

## 2. Material and methods

### 2.1. Reagents

Methyl cinnamate and the racemic mixture of linalool were obtained from Sigma-Aldrich. Marine salt was purchased from Qingdao. Span® 60 (Croda), Methanol (Vetec), Tween® 80 (Galena), NaCl (Vetec), $KH_2PO_4$ (Vetec) and $Na_2HPO_4$ (Vetec) were used in analytical grade. Water was purified by reverse osmosis (Gehaka) before use.

### 2.2. Brine shrimp lethality assay

The assay was performed according to Meyer et al. (1982) with modifications. Cysts of *Artemia salina* L. (200 mg) were incubated in 400 mL artificial sea water (38 g marine salts dissolved in 1000 mL purified water). The system was kept under constant stirring and aeration at 30 °C for 48 h for hatching. After hatching, 10 nauplii of *A. salina* L. were transferred to tubes containing different concentrations of the substances for evaluation, which were solubilized in artificial sea water with 0.5% Tween® 80 (m/v) added. Quinidine sulfate was used as a positive control at the same concentrations as the samples, and artificial sea water containing 0.5% Tween® 80 (m/v) was used as a negative control. After 24 h, the live and dead nauplii were counted.

### 2.3. Hemolytic activity

The assay was conducted according to Banerjee et al. (2008). Sheep erythrocytes were obtained commercially (Newprov) and washed with phosphate buffered saline (PBS) (150 mM NaCl, 0.58 mM $KH_2PO_4$ and 3.4 mM $Na_2HPO_4$, pH 7.4). After washing, a 2% (v/v) suspension of red blood cells was prepared in PBS.

Standard solutions (1 mg/mL) were prepared using samples solubilized in 100 µL of methanol and PBS, which were subsequently diluted to concentrations of 10 µg/mL, 50 µg/mL, 100 µg/mL and 1000 µg/mL in PBS. A 100% hemolysis sample was prepared with distilled water, and the negative control consisted of PBS solution containing methanol.

For each sample concentration, 200 µL of a 2% suspension of red blood cells and 200 µL of the sample solution were placed in Eppendorf tubes. The samples were homogenized and incubated at 37 °C for 3 h. Following the incubation, the tubes were centrifuged for 5 min at 3000 rpm and the supernatant transferred to 96-well microplates. The samples absorbance was measured in a Multiskan FC spectrophotometer (Thermo Scientific) at 540 nm. The hemolysis percentage was calculated as follows:

$$\%Hemolysis = \frac{Abs_t - Abs_n}{Abs_w - Abs_n} \times 100$$

Where $Abs_t$ is the absorbance of test sample, $Abs_n$ the absorbance of control (saline) and $Abs_w$ the absorbance of control (distilled water).

### 2.4. Phytotoxicity assay

The phytotoxicity assay was determined according to Silva et al. (2014b), with modifications. The samples were emulsified with Tween® 80 in a 1:1 (m/v) proportion and diluted with purified water in concentrations of 0.001%, 0.01%, 0.1% and 1.0% (m/v). A 1% (m/v) solution of Tween® 80 was used as a negative control. For the germination assays, 50 seeds of *Lactuca sativa* cv. Grand Rapids were displaced in sterile Petri dishes (θ 90 mm) containing a sheet of Whatman No. 1 filter paper moistened with 5 mL of purified water. After the seeding, 3 mL of the solution of each sample concentration was placed on two filter papers attached (by double-sided adhesive tape) in the inner side of the cover of the dish, avoiding direct contact with the seeds. The dishes were wrapped with Parafilm® and incubated in BOD incubator (Novatecnica) at 25 °C.

Each day the germinated seeds were counted; the criterion for germination was a protruding radicle at least 2.0 mm long. The Speed of Germination Index (SGI) was calculated according to Hoffmann et al. (2007) and the Percentage of Germination (%G) according to Rosado et al. (2009).

The same procedure was adopted to check the growth of seedlings, and the hypocotyl and radicle lengths were evaluated after 7 days of incubation. The assay was performed in quadruplicate.

### 2.5. Toxicity against Aedes aegypti

The larvicidal activity against the third-stage larvae of *Aedes aegypti* was evaluated according to the World Health Organization (World Health Organization (WHO), 2005) and Silva et al. (2008). Eggs of *Aedes aegypti*, Rockefeller strain, provided by the Fundação Oswaldo Cruz, Rio de Janeiro, Brazil, were placed in a plastic beaker containing 500 mL of chlorine-free water. The beaker was placed in a BOD incubator at 27 °C and 80% relative humidity. The larvae were fed with fish food from hatching to the third stage.

For each concentration tested, 100 mL of the sample and 20 third-stage larvae of *Aedes aegypti* were placed in a plastic container of 120 mL capacity. The samples were placed in a BOD incubator, temperature 27 °C, relative humidity 80% and photoperiod of 12 h. After 24 h, the number of living and dead larvae was determined. Each test was conducted in quadruplicate, with a control consisting of 100 mL chlorine-free water containing 0.5% (m/v) Tween® 80.

### 2.6. Morphological analysis

The larvae of *Aedes aegypti* were evaluated by comparing photos (taken with a Nikon Coolpix AW110) of the control group and treated groups. For structural studies, an Olympus CX41 microscope (100x magnification) was used.

### 2.7. Statistical analysis

The Probit method (Finney, 1971) was used to determine the $LC_{50}$ and $LC_{90}$ values, as well as the corresponding 95% confidence intervals and chi square values for the assays with *Artemia salina* L. and *Aedes aegypti*, using the SPSS Statistical Software Package version 23.0.

The data obtained in the evaluation of phytotoxicity were subjected to analysis of variance, and the means were compared by the Scott-Knott test ($p < 0.05$), using the software Sisvar (Ferreira, 2011).