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Dissuasive and biocidal activity of *Salvia officinalis* (Lamiaceae) with induction of malformations in *Aedes aegypti* (Diptera: Culicidae)

Actividad disuasiva y biocida de *Salvia officinalis* con inducción de malformaciones en *Aedes aegypti* (Diptera: Culicidae)

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Abstract: Different studies have evaluated the biocidal activity of essential oils (EO) in mosquito larvae of medical importance. However, limited research focused on analyzing the EO effect at all the stages of the life cycle of *Aedes aegypti* has been done. This study evaluates the biological activity of the EO *Salvia officinalis* against *Ae. aegypti*. The ovicidal activity was evaluated at 1, 5, 37 and 50 mg.L^{-1} concentrations in eggs from 0-12 h and eggs from 0-72 h. Larvicidal, pupicidal and adulticidal activities were assessed at exploratory concentrations (EC) and multiple concentrations. We employed an EC of 1,000 mg.L^{-1} for repellent activity and used an exposure time of 0-2 min and 2-15 min on the forearms of volunteers. The deterrent action was estimated at EC of 5, 50 and 200 mg.L^{-1}. The EO caused malformations in embryos as well as larvae alteration. The highest larvicidal activity was at 63 and 76 mg.L^{-1} (27 ± 13.4 and 37 ± 18.6 %) with 24 h exposure. The greatest pupicidal mortality was at 310 and 390 mg.L^{-1} (89 ± 1.53 and 100 ± 0 %) with 48 h exposure. Adulticidal mortality at 300 mg.L^{-1} was 57.5 ± 0 % and the percentage of repellency was 42 ± 4.7 %. The dissuasive action at 200 mg.L^{-1} was 97 ± 4.81 %, with an oviposition activity rate of -0.94. *S. officinalis* showed a biocidal effect on embryos and mortality of pupae and adults, indicating that its potential use in control programs should be focused on these stages of development.

Keywords: Botanical insecticide, oviposition dissuasive activity, essential oil, ovicidal activity, 1-8 cineol.

Resumen: Diferentes estudios han evaluado la actividad biocida de aceites esenciales (AEs) en larvas de mosquitos de importancia médica. Sin embargo, son limitadas las investigaciones que analizan los efectos de los AEs en todos los estadíos del ciclo de vida de *Aedes aegypti.* Este estudio evalúa la actividad biológica del AE de *Salvia officinalis* frente a *Ae. aegypti.* Se evaluó la actividad ovicida a concentraciones de 1, 5, 37 y 50 mg.L^{-1} en huevos de 0-12 h y huevos de 0-72 h. La actividad larvicida, pupicida y adulticida fue evaluada a concentraciones exploratorias (CE) y múltiples. Para la actividad repelente se empleó una CE de 1.000 mg.L^{-1} en intervalos de 0-2 min y de 2-15 min de exposición en antebrazos de voluntarios. La actividad disuasiva de oviposición se estimó a CE de 5, 50 y 200 mg.L^{-1}. El AE causó malformaciones en embriones y alteración de las larvas. La mayor actividad larvicida fue a 63 y 76 mg.L^{-1} (27 ± 13,4 y 37 ± 18,6 %) a 24 h. La mayor mortalidad pupicida fue a 310 y 390 mg.L^{-1} (89 ± 1,53 and 100 ± 0 %) a las 48 h. La mortalidad adulticida a 300 mg.L^{-1} fue de 57,5 ± 0 % y el porcentaje de repelencia fue de 42 ± 4,7 %. La acción disuasiva a 200 mg.L^{-1} fue de 97 ± 4,81 %, con un índice de actividad de ovipostura de -0,94. S. *officinalis* mostró un efecto biocida en embriones y mortalidad de pupas y adultos, lo que revela que tiene un uso potencial en programas de control focalizados en estos estadios de desarrollo.

Palabras clave: Insecticida botánico, actividad disuasiva de oviposición, aceite esencial, actividad ovicida, 1-8 cineol.

Introduction

Aedes aegypti (Linnaeus, 1762) (Diptera: Culicidae) is the main vector of pathologies of major impact in urban and peri-urban areas of tropical and subtropical zones, namely Dengue, Chikungunya and Zika virus (Rodríguez-Morales 2015a, 2015b). This mosquito is distributed under 2,200 masl although in Colombia, it is reported

at altitudes of up to 2,302 masl, (Ruiz-López *et al*. 2016). *Ae. aegypti* is present in 80 % of the national territory, where a large part of the population lives and is susceptible to the three pathologies mentioned above (Singh *et al*. 2012; Soonwera and Phasomkusolsil 2014; Castrillón *et al.* 2015).

The efforts to stop the circulation of diseases transmitted by *Ae. aegypti* are focused on vector control through the use of chemical insecticides and the elimination of their breeding sites (Licastro *et al.* 2010; Rodríguez 2002). For juvenile phases, temephos and growth regulators are used, and for the control of adults, pyrethroid insecticides are applied (Álvarez González *et al.* 2014; Ardila-Roldán *et al.* 2013; Maestre-Serrano and Gómez-Camargo 2013). However, the vector has not shown a population decline due to the inappropriate application of insecticides. This has led to an increase in the number of cases of diseases caused by arboviruses in the neotropical regions (Castrillón *et al.* 2015; Rodríguez-Morales 2015a, 2015b). Additionally, the indiscriminate use of chemicals induces environmental contamination and generates resistance to the active principles in populations of *Ae. aegypti* (Leyva *et al.* 2012; Maheswaran and Ignacimuthu 2012; Bhatt *et al.* 2013).

As an alternative to insecticides of a synthetic origin (organophosphates-OP and pyrethroids-PI), the use of essential oils (EOs) and extracts obtained from plants have been proposed. These botanical products are synthesized naturally by the secondary metabolism of plants when exposed to sudden changes in temperature or as a chemical defense mechanism against the harmful action of pathogens and phytophagous insects (Ávalos and Pérez-Urria 2009; Koul *et al.* 2008; Leyva *et al.* 2012; Arango 2013). The EOs offer multiple applications in vector control programs that are not found in traditional insecticides. For example, biocidal action can be achieved throughout the insect´s life cycle (egg, larva, pupa and adult), as well as benefits such as repellent and dissuasive action (Maheswaran and Ignacimuthu 2012; Zoubiri and Baaliouamer 2011).

Different studies have evaluated the biocidal activity of EOs in mosquito larvae of medical importance (Aciole *et al.* 2011; El-Gendy and Shaalan 2012; Elango *et al.* 2012; Gokulakrishnan *et al.* 2013; Leyva *et al.* 2012; Mathivanan *et al.* 2010; Ponnusamy *et al.* 2010; Suman *et al.* 2013). However, the majority of researches have not focused on analyzing the effects of EO at all of *Ae. aegypti* life cycle stages and as such have not detected other significant biocidal effects. In this sense, the present study sought to evaluate whether *Salvia officinalis* L. (Lamiales: Lamiaceae) EO has ovicidal, larvicidal, pupicidal and adulticidal activity together with repellent, attractive and dissuasive action in *Ae. aegypti*.

Materials and methods

Abbreviations. EO, Essential oil; LC, Lethal concentration; DMSO, Dimethyl Sulfoxide; OAI, oviposition activity index; MWHD, Microwave-assisted hydrodistillation; LD, Lethal doses; KT, knockdown time; RO, olfactory receptor; EDO, odour degrading enzymes.

Essential oil extraction. *S. officinalis* EO was provided by the "Centro Nacional de Investigación para la Agroindustrialización de Plantas Aromáticas y Medicinales Tropicales – CENIVAM" of the Universidad Industrial de Santander. The oil extraction methodology, as well as its chemical characterization, was carried out in accordance with the methodology described by Ríos *et al.* (2017).

***Aedes aegypti* colony.** Bioassays were performed with a colony of *Ae. aegypti,* Rockefeller strain, maintained in an insectary at 25 ± 5 °C, with a humidity of 70 ± 5 % and photoperiod (12:12). Male adults were fed on a permanent basis with a sugar solution of 10 % honey (75 % carbohydrates). Females were blood-fed on an albino Wistar rat (WI IOPS AF/Han strain) provided by Universidad Industrial de Santander bioterium. This research was approved by the Ethics Committee (CEINCI) (Minutes No. 11, 8 May 2015).

To obtain the larvae in the experiments, eggs were submerged in mineral water obtained through osmosis (Pure Pro EC106M-P; Pure Pro Water Corporation, Bogotá, Colombia) plus TetraMin Tropical Flakes® fish concentrate (Manufactured by Tetra GMBH Henenteich, Melle, Germany). After emergence, the larvae were kept in plastic trays and fed every three days until they reached the appropriate instar for each assay.

The ovicidal activity. We used the Rezende *et al.* (2008) and Suman *et al.* (2013) methodology to establish the ovicidal activity. We separated 20 gravid females of *Ae. aegypti* in a security cage with dimensions of 40 x 40 x 40 cm. Inside were six plastic cups with 50 µL of EO dissolved in dimethyl sulfoxide (DMSO) at different concentrations plus a glass as a control treatment (49 mL of water + 1 mL of 0.5 % DMSO). Initially, we used a screening selection with exploratory concentrations (5, 50 and 200 $mg.L^{-1}$). Then, we evaluated multiple concentrations of 1, 5, 37 and 50 $mg.L^{-1}$. The inside of each glass was coated with Whatman® #1 filter paper cut in half and folded into a cone shape, in order to collect and count the eggs. The oviposition lasted for eight consecutive days, changing the filter paper daily. Three replicates were used by concentration and the experiment was repeated on three different days.

When the females oviposited, in order to obtain embryonated eggs and non-embryonated eggs, the time of oviposition was considered: from 0 to 12 h for non-embryonated eggs; and more than 72 h embryonated eggs. Between 50 and 100 eggs per glass were taken at random. In each case, the eggs were transferred to individual containers according to the evaluated concentration, where they were subsequently counted and examined under a stereoscope in order to verify their integrity. The hatching percentage of the eggs was evaluated up to 120 h after oviposition, submerging the eggs obtained for each concentration in mineral water. The first instar larvae that emerged were counted under a microscope, and eggs that did not hatch after seven days were considered unviable. The hatching value of the eggs was estimated as the percentage of eggs that went on to the larval stage in a normal manner.

To determine whether the EO caused alteration in embryonic development and hatching of *Ae. aegypti*, a random sample of non-embryonated and embryonated eggs from glass treated at different concentrations (1, 5, 37 and 50 $mg.L^{-1}$) was selected. The eggs selected from each concentration and exposure time were immersed in a solution of 80 % sodium hypochlorite for 10 min until the eggshell lost its pigmentation and became transparent. These eggs were examined under a microscope to detect morphological abnormalities caused by exposure to the EO (malformed embryos, malformations in the head and abdomen and merged corporal tagmas). The morphological changes were recorded to calculate the percentage of abnormalities in relation to the embryo's maturation times.

Larvicidal activity. Larvae at the final L_3 and initial L_4 stages were used. These were transferred individually to 200 mL plastic cups with 99 mL of mineral water. 1 mL of the EO of *S. officinalis* was added to each plastic cup for a final volume of 100 mL where the concentration-response bioassays were performed. Firstly, we conducted exploratory concentration of 30, 300, and 1,000 mg.L^{-1} (Vera *et al.* 2014). Subsequently, multiple concentrations were evaluated (30, 47, 51, 63 and 76 mg.L^{-1}) in order to establish mortalities between 2 and 98 %. In each case, the negative control treatment was DMSO at 0.5 % and positive control with the insecticide propoxur (1.50 mg.L^{-1}) were used in this assay. Quadruplicate tests (40 larvae) were performed for each concentration evaluated by repeating the experiment three times on different days until a total of 120 larvae were used by each treatment. The larvae were not fed throughout the treatment. Mortality at 24 and 48 h was recorded in each assay. The results of mortality and survival were subjected to Probit analysis (Wiegand 1972).

Pupicidal activity. Twenty pupae, between 12 and 24 h of age, were removed with a Pasteur pipette and placed in four plastic cups containing 48 mL of mineral water and 2 mL of EO of *S. officinalis* (final volume: 50 mL). The selective screening was carried out with exploratory concentrations of 250, 310, and 390 mg.L^{-1} of EO. Pupae in the control group were placed in water with 0.5 % dimethyl sulfoxide (DMSO) as this was the solvent used in the EO, with no toxicity to *Ae. aegypti*. Pupicidal activity was determined by the percentage of dead pupae at 24 and 48 h; the pupae that showed no movement under external stimuli were considered dead. The replicas and experimental design followed the same criteria of larvicidal activity.

Adulticidal activity. The adulticidal activity of EO was assessed, using the CDC bottle bioassay method (Brogdon and McAllister 1998). The interiors of Wheaton 250 mL glass bottles were treated with 1 mL of EO of *S. officinalis* at exploratory concentrations of 30, 300 y 1,000 mg.L^{-1}. In the present study, the use of acetone (1 mL) as a negative control is based on the fact that this substance is used as a solvent for EOs and is highly volatile. This characteristic is extremely important, since when applying the treatments, it is necessary that the solvent evaporates, avoiding its interference in the results obtained (Debboun *et al*. 2007, World Health Organization 2009).

In each bottle, 10 individuals (males and females) were introduced, and mortality was recorded every 15 min for three hours. Triplicate tests (30 adults) were performed for each concentration evaluated by repeating the experiment on three times on different days. The mortality percentage was calculated after 2 min of exposure, followed by every 15 min thereafter for up to 2 h, and a final recorded at 24 h of exposure.

Repellent activity. Twenty females of *Ae. aegypti* between 5 and 10 days emerged, copulated, fed only of carbohydrates and were maintained nulliparous. They were placed in rectangular acrylic chambers (18 cm x 5 cm x 4 cm) (Castillo *et al.* 2017). Each device had an opening of 29 mm in diameter at the lower end so that only a section of the forearm of the volunteer is exposed to mosquitoes and treatment. These tests followed the methodology of the American Society for Testing and Materials (ASTM) and ASTM-E951-94 for repellency bioassays (Debboun *et al*. 2007).

Volunteers were used after signing the informed consent approved by the "Comite de Ética en Investigación Científica" (CEINCI), Minutes No. 3/2013. A 4 cm^2 area was initially marked on the forearm with removable ink for application of the treatments. The right arm was used to evaluate 1 mL of *S. officinalis* EO, at a single concentration of 1,000 mg.L^{-1} and the left arm was used for the control treatment (1 mL of acetone at 99 %). After the application of the treatments, we waited 20 min (drying time) for the ubication of the acrylic chamber in each arm with the females of *Ae. aegypti*. During the test, the number of bites and landings were recorded at 0 min and 2 min, and subsequently, in the interval of 2 to 15 min. DEET was used as a positive control. Four replicates of each experiment were performed on different days (40 adults for treatment), which always consisted of four different volunteers for each test, with a maximum exposure time of 1 hour. In all the experiments, the protection percentage was estimated according to the formula proposed by Phasomkusolsil and Soonwera (2011) in time intervals of 0-2 min, from 3 to 15 min and 15 min onwards, as follows:

$$\% \, Protection = \frac{Nc - Nt}{Nc}$$

Nc = Number of bites received by control arm; Nt = Number of bites received by the treated arm.

Additionally, the percentage of bites was calculated according to the authors Tawatsin *et al.* (2001) and Thavara *et al.* (2001):

$$\text{Biting } \% = \frac{B}{60} \, x \, 100$$

Where: B = Total numbers of bites by the end of the test.

Oviposition dissuasive activity. Ten females that were nulliparous, gravid and with a visibly swollen abdomen due to the blood supply were placed in four aluminium cages of 70 x 70 x 70 cm. Four 30 mL plastic cups were introduced in each of the cages, each with 14.25 mL mineral water with 75 µL of *S. officinalis* EO (final volume: 15 mL). We used a screening selection with exploratory concentrations (5, 50 and 200 mg.L^{-1}). The control treatment was 14.25 mL of water + 75 µL of 0.5 % DMSO. They were subsequently marked and covered with Whatman® #1 filter paper cut in half manually and folded into a cone shape, to allow the oviposition of *Ae. aegypti* females.

The dissuasive oviposition activity was evaluated for 6 days. Every 24 h the plastic cups were rotating inside the cages counterclockwise and maintaining the same concentrations mentioned above. Each experiment was performed by triplicate and repeated for four different days, counting the number of eggs laid per day, over 6 days. The experiment was evaluated by the Oviposition Activity Index (OAI) between + 1 to - 1 as follows:

$$\text{Oviposition Activity Index (OAI)} = \frac{Nt - Ns}{Nt + Ns}$$

Nt = Total number of eggs in the treatment; Ns = Total number of eggs in the control.

Values greater than or equal to + 0.3 can be considered as attractants, and less than or equal to - 0.3, are considered as repellents (Kramer and Mulla 1979).

Additionally, we calculated the Inhibition Effect (IE) of the different treatments on the oviposition:

$$\text{Inhibition Effect (IE)} = \frac{Nc - Nt}{Nc} \times 100$$

Nc = Total number of eggs in the control; Nt = Total number of eggs in the treatment (El-Gendy and Shaalan 2012; Phasomkusolsil and Soonwera 2012).

Statistical analyses. The data was tabulated and descriptive statistics, normality test of Kolmogorov-Smirnorv and, Shapiro Wilk were employed. When normal distribution was found, ANOVA and Tukey or Newman-Keuls test were applied. When distribution was not normal, non-parametric tests were employed (Kruskal-Wallis test), and when it was significant, multiple comparison tests were used. According to the experiment, the Spearman correlation test was applied with $P \leq 0.05$ considered as statistically significant. The results were analyzed with Statistics V11.

Results

Chemical composition of essential oil. The EO of *S. officinalis* showed a yield of 0.4 %. The main components of the EO were the monoterpenes 1,8-cineol (26.6 %), α-tujone (18.1 %), the sesquiterpene trans-β-caryophyllene (7.3 %) and the hydrocarbon α-humulene (5.4 %) (Ríos *et al.* 2017).

Ovicidal activity. We obtained 580 non-embryonated eggs (0-12 h) and 1,054 embryonated eggs (0-72 h). When comparing hatching percentages in eggs from 0-12 hours, a decrease in hatching percentage is observed as the concentration of EO increases, going from 74.7 % for 1 mg.L^{-1} to 0 % for 50 mg.L^{-1} (Fig. 1). In the case of eggs from 0-72 hours, an increase in hatching percentage is observed at 5 mg.L^{-1} (97.6 %), 37 mg.L^{-1} (100 %) and 50 mg.L^{-1} (77.6 %) when compared to the other concentrations evaluated. Statistically significant differences were found between the treatments of eggs from 0-12 hours to 37 mg.L^{-1} and 50 mg.L^{-1}, and the control group (ANOVA test $P \leq 0.05$). No statistically significant differences were found between the treatments of the 0-72 hour eggs and the control group [Kruskal-Wallis test: H (4, N = 10) = 7.228125, P = 0.1243]. However, when observing the hatching percentages obtained, it seems that the AE can reduce the hatching percentage of eggs with a few hours of exposure, even at low concentrations (Fig. 1).

In relation to embryonic development, eggs from 0-12 h showed damage in the cephalic capsule. At the highest concentration (50 mg.L^{-1}) 3.4 % of eggs presented malformations. The formation of structures, such as eyes and mouthparts, were not observed. At concentrations of 37 and 50 mg.L^{-1}, the thorax was not adequately formed, and the embryo was completely deformed or nonexistent (Fig. 2). There was a positive correlation between the increase in the concentration of the EO and malformations ($R = 0.66$, $P \leq 0.05$).

Regarding eggs from 0-72 h, the percentage of malformations was lower than in eggs of 1-12 h, in 20.7 % at a concentration of 37 mg.L^{-1}. The Spearman correlation coefficient showed a negative relationship ($R = 0.265$, $P \leq 0.05$) suggesting that malformations in embryos from 0-72 h do not have a relationship directly proportional to the increase in EO concentration. The malformations caused by the EO were present in the head (non-existent mouthparts, large cephalic capsule), abnormal formation of the thorax and abdomen (scarcely differentiated, incomplete and with malformations at the end of the last segments) (Fig. 2).

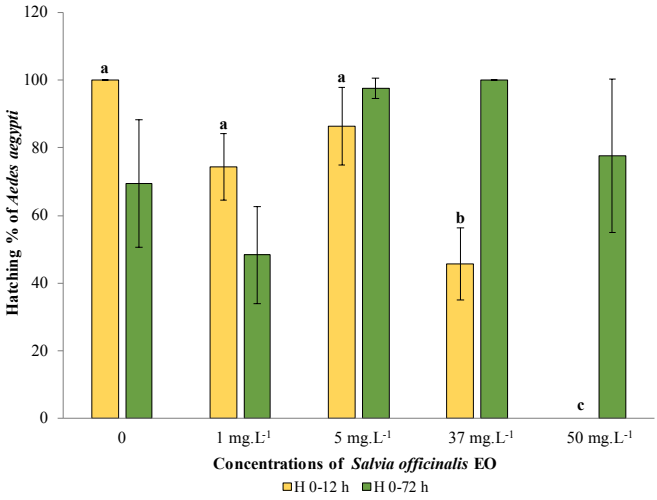

Figure 1. Egg hatching percentage of *Aedes aegypti* from 0-12 h and 0-72 h (± SD), in different concentrations of *Salvia officinalis* EO (1, 5, 37 and 50 mg.L^{-1}). [a,b,c] Different letters indicate significant statistical differences between concentrations between eggs from 0-12 h *versus* the control group (ANOVA test $P \leq 0.05$).

The abnormal hatching of the eggs was significantly higher in eggs from 0-12 h at 50 mg.L^{-1} when compared to the control group (ANOVA test $P \leq 0.05$), where the percentage of abnormalities in the hatching was 36.6 %. Regarding eggs from 0-72 h, significant statistical differences were found between the concentration of 37 mg.L^{-1} and the rest, except at 50 mg.L^{-1} (ANOVA test $P \leq 0.05$). The highest percentage of abnormal hatching was recorded at 37 mg.L^{-1} at 3.7 %. When comparing hatching for each concentration, statistically significant differences were found at 50 mg.L^{-1} between eggs from 0-12 h and from 0-72 h (NK test P = 0.0034) (Fig. 3).

In relation to the larvae obtained from the eggs in each of the treatments and concentrations evaluated, a higher percentage of abnormalities was observed between 37 and 5 mg.L^{-1} for eggs from 0-12 h (15.6 %). No significant statistical differences were found in eggs from 0-12 h [KW test H (4, N = 10) = 5.951613; P = 0.2028]. In eggs from 0-72 h, a higher percentage of abnormalities was observed at 5 mg.L^{-1} (7.5 %) and 50 mg.L^{-1} (3.4 %). No significant statistical differences were found in eggs from 0-72 h [KW test H (4, N = 10) = 5.834483; P = 0.2119] when compared to the control group. No statistically significant differences were found when comparing larval abnormalities from 0 -12 h and from 0-72 h at each concentration; 1 mg.L^{-1} (KW Test P = 0.5353), 5 mg.L^{-1} (Tukey Test $P \geq 0.05$), 37 mg.L^{-1} [KW test H (2, N = 6) = 4, 80; P = 0.090], and 50 mg.L^{-1} [KW test H (2, N = 6) = 1.30; P = 0.522] (Fig. 4). Regarding the abnormalities observed, morphological damages were detected, mainly represented by the decrease in the amount of chitin in the head of the larvae and in the head-thorax junction. In addition, it was observed that the larvae showed slow or abnormal movements, and in some cases, death.

Larvicidal activity. The highest mortality value was observed at 76 mg.L^{-1} at 24 h (37 ± 18.6) and 48 h (37 ± 19.2). No significant differences were found between the treatments evaluated at 24 h and 48 h [KW test: H (5, N = 18) F = 8.072327 P = 0.1523; KW test: H (5, N = 18) F = 7.970630 P = 0.1579]. The lethal concentrations of *S. officinalis* EO at 24 h were LC_{50} = 76.43 mg.L^{-1} (71.84 - 83.79), LC_{95} = 123.92

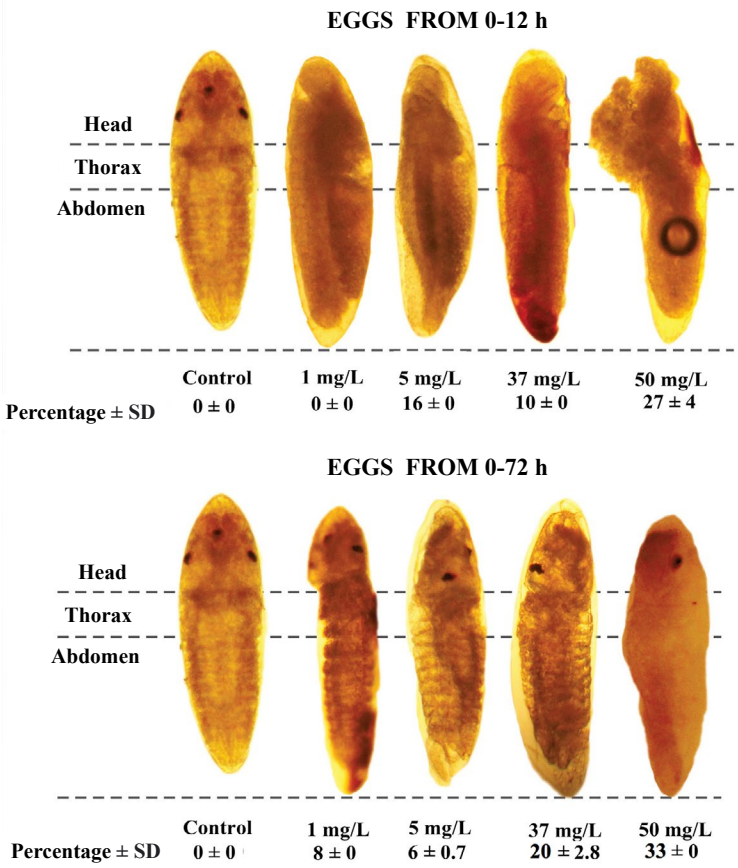

Figure 2. Types of malformations presented in the embryos of *Aedes aegypti* for each *Salvia officinalis* EO concentrations in eggs and percentages of malformations from 0-12 h: 1 mg.L^{-1}: malformed embryos, indistinguishable cephalic capsules; 5 mg.L^{-1}: malformations in the head, segments of the abdomen with malformations; 37 mg.L^{-1}: malformed and fused with the thorax; 50 mg.L^{-1}: weak chorion, the embryos are not clearly distinguishable. Eggs without distinguishable embryo, not viable to go on to the larval stage. Eggs from 0-72 h: 1 mg.L^{-1}: malformations in the head, an abnormal increase in egg size, thorax and abdomen with malformations; 5 mg.L^{-1}: malformations in the head, or cephalic capsule indistinguishable, thorax of small size; 37 mg.L^{-1}: embryos with scarcely distinguishable thorax, large head and abdominal segments with malformations; 50 mg.L^{-1}: indistinguishable sections of the embryo, malformations in the head and abdominal segments.

mg.L^{-1} (106.98 - 136.75). At 48 h the lethal concentrations were LC_{50} = 123.92 mg.L^{-1} (106.98 - 136.75), LC_{95} = 198.20 mg.L^{-1} (149.17 - 322.5) (Table 1).

Table 1. Larvicidal activity (in mortality percent ± SD) of the essential oil of *Salvia officinalis* against *Aedes aegypti* at 24 and 48 h. LC_{50} is the lethal concentration causing mortality of 50 % of organisms exposed to the treatment. LC_{95} is the lethal concentration causing mortality of 95 % of organisms exposed to the treatment. The confidence interval is given in parentheses. The statistical analysis was well adjusted to the Probit model (Finney 1949).

Concentration (mg. L^{-1})	Mortality (% ± SD)	
	24 h ± SD	48 h ± SD
0	0	0
30	3 ± 2.3	5 ± 1.7
47	6 ± 3.3	16 ± 6.8
63	27 ± 13.4	34 ± 14.8
76	37 ± 18.6	37 ± 19.2
Temephos	100 ± 0	100 ± 0
LC_{50}	76.4 (71.8 - 83.8)	123.9 (106.9 - 136.7)
LC_{95}	123.9 (106.9 - 136.7)	198.2 (149.2 - 322.5)
X^2	5.46	1.37

SD: Standard deviation; LC: Lethal Concentration; X^2: Chi squared.

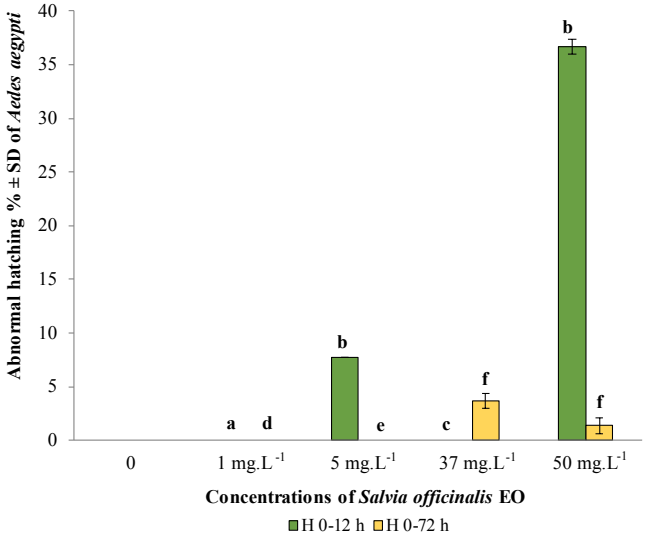

Figure 3. Percentage of eggs of *Aedes aegypti* with abnormal hatching obtained from 0-12 h and 0-72 h, in different concentrations of *Salvia officinalis* EO (1, 5, 37 and 50 mg.L^{-1}). Different letters indicate significant statistical differences. [a, b, c] between the eggs from 0-12 h versus the control group (Tukey test P ≤ 0.05), [d, e, f] between eggs from 0-72 h versus the control group (Tukey test P ≤ 0.05). [a, f] and statistically significant differences at 50 mg.L^{-1} between eggs from 0-12 h and from 0-72 h (NK test P = 0.0034).

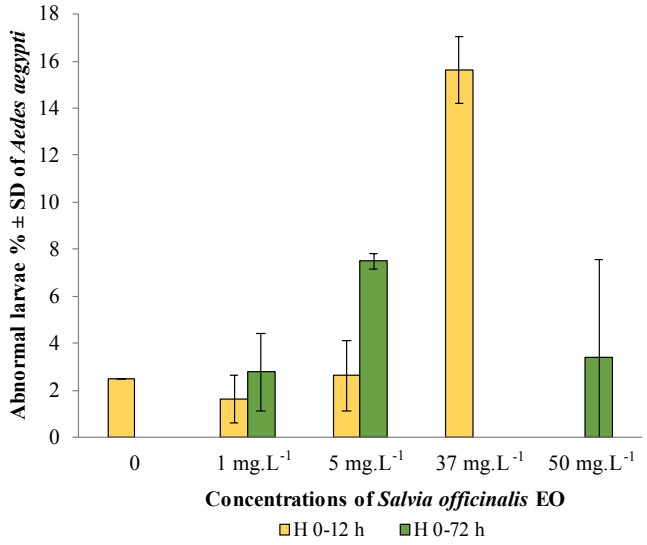

Figure 4. Percentage of abnormal larvae hatching of *Aedes aegypti* observed in eggs from 0-12 h and 0-72 h obtained at different concentrations of *Salvia officinalis* EO (1, 5, 37 and 50 mg.L^{-1}).

Pupicidal activity. The highest pupicidal action was found in the concentration of 390 mg.L^{-1}, at 24 h and 48 h of exposure (69 ± 3.1 and 100) (Table 2). It was observed that the pupicidal action increased significantly from 24 to 48 h of EO exposure. Statistically significant differences were observed with respect to the negative control at 24 h [KW test: H (3, N = 12) F = 7.533333 P = 0.0567], and 48 h [KW test: H (3, N = 12) F = 8.569604 P = 0.0356].

Adulticidal activity. It was observed that *Ae. aegypti* mortality increased significantly with higher concentrations of the EO and greater exposure time [60 min KW test H (3, N = 16) = 9.548033; P = 0.022; 120 min KW test H (3, N = 16) = 9.118421; P = 0.028; 24 h KW test H (3, N = 16) = 11.72727; P = 0.0084]. The highest percentage of mortality was reached at concentrations of 30 and 1,000 mg.L^{-1} (100 %) at 24 h of exposure. From 0 to 2 min of exposure, no effect was observed in the exposed individuals (Table 3).

Mosquito repellency activity. The highest percentage of protection was observed at the time interval from 0 to 2 min (67 %), with a bite percentage of 3.3 %. No statistically significant differences were found between the time intervals and the negative and positive controls (DEET) (ANOVA P = 0.667563). However, significant statistical differences were found between the time intervals (NK test P = 0.041) (Table 4).

Table 2. Pupicidal activity (in mortality percent ± SD) of the essential oil of *Salvia officinalis* against *Aedes aegypti* at 24 and 48 h. * Statistically significant differences between negative control and mortality percent.

Concentration (mg. L^{-1})	Mortality (% ± SD)	
	24 h ± SD	48 ± SD
0	0	0
250	16 ± 1.5	73 ± 3.5 *
310	44 ± 5.8	89 ± 1.5 *
390	69 ± 3.1	100 *

SD: Standard deviation of mortality percent at 24 and 48 h.

Table 3. Adulticidal activity (in mortality percent ± SD) of the essential oil of *Salvia officinalis* against *Aedes aegypti* at 0-2 min, 60 min, 120 min and 24 h. [a,b,c] Equal letters indicate significant statistical differences between control and mortality percentage observed during test intervals.

Concentration (mg.L^{-1})	Mortality (% ± SD)			
	0-2 min	60 min	120 min	24 h
0	0	0	0	0
30	0	55 ± 3^{a}	62 ± 2^{b}	100^{c}
300	0	40 ± 2.4^{a}	50 ± 3^{b}	100^{c}
1,000	0	65 ± 2.6^{a}	70 ± 2.2^{b}	100^{c}

Oviposition deterrent activity. In relation to the deterrence of oviposition, the three concentrations evaluated (5, 50 and 200 mg.L^{-1}) of *S. officinalis* showed significant inhibition percentages > 80 % [KW test H (3, N = 32) = 10, 39201; P = 0.015]. The concentration of 200 mg.L^{-1} presented the highest value of oviposition inhibition, directly related to the value obtained in the Oviposition Activity Index (-0.94). Accordingly, female *Ae. aegypti* avoid laying their eggs in all the treatments evaluated (Table 5).

Discussion

According to Abou-Elnaga (2014), five chemotypes of the essential oil of *S. officinalis* have been characterized according to major components (1. Camphor > α-tujone > 1,8-cineol > β-tujone, 2. Camphor > α-tujone > β-tujone > 1,8-cineol; 3. β-tujone > camphor > 1,8-cineol > α-tujone; 4. 8-cineol > camphor > α-tujone > β-tujone; and 5. α-tujone > camphor > β-tujone > 1,8-cineol). 1,8-cineole constitutes the major component (26.6 %) of the essential oil of *S. officinalis* used in this study, corresponding to chemotype 4. This variation of chemotypes is explained by the inter and intra-specific factors of the genotype, plant cultivation conditions (climate and geographical location), the vegetative organ used for its extraction, time of year in which it was collected and extraction conditions (Bernotiené *et al.* 2007; Santana *et al.* 2012).

The chemical characterization described coincides with that reported by Castañeda *et al.* (2007), who also performed extraction by microwave-assisted hydrodistillation (MWHD) of plants obtained from the same department (Santander, municipality of Sucre) with the major compounds 1,8-cineol (26.6 %) and α- tujona (18.3 %).

Table 4. Protection percentage (% ± SD) and bite percentage of the essential oil of *Salvia officinalis* against *Aedes aegypti* evaluated during test intervals 0-2, 0-15 min. [a,b] Different letters indicate significant statistical differences between time intervals of protection percentage (Newman-Keuls Test P = 0.041).

Time	Protection percentage (% ± SD)	Control ± SD	DEET ± SD	% Bite
2 min	67^{a} ± 1	6 ± 2	0 ± 2	3.3
15 min	26^{b} ± 4.7	19 ± 3.5	0 ± 0	23.3

SD: Standard deviation of protection percentage in negative control and positive control with DEET.

Table 5. Oviposition inhibition activity of the essential oil of *Salvia officinalis* against *Aedes aegypti*. OAI: Oviposition activity index (negative values indicate a greater number of eggs in the controls, positive values indicate a greater number of eggs in the treatments). IE: Inhibition effect (% ± SD). * Significant statistical differences between control and Inhibition effect (IE) [KW test H (3, N = 32) = 10, 39201; P = 0.015].

Concentration ($mg.L^{-1}$)	IE % (% ± SD)	OAI
0	0 ± 103.2	0
5	92 ± 12.7 *	- 0.853
50	91 ± 13 *	- 0.831
200	97 ± 4.8 *	- 0.941

Bioassays of ovicidal activity. According to the larvae hatching results, it was observed that *Ae. aegypti* were susceptible to biocidal action by penetration of *S. officinalis* EO. This indicates that, although the embryos are protected by a hard shell (Phasomkusolsil and Soonwera 2011), they were permeable to EO. The exposure of eggs from 0-12 h with EO was more effective in decreasing hatching percentage. Accordingly, it can be inferred that exposure time, the concentration used, and embryo age directly affects ovicidal activity (Valarmathy *et al.* 2011). In relation to the 0-72 eggs where the embryo is already formed, apart from causing morphological damage to the embryo, larvae hatching is also affected. This explains the percentage of emerged larvae that presented abnormalities with the concentrations used.

Arivoli and Tennyson (2011) mention that some substances of vegetable origin can induce deformities and degenerative effects during the development of mosquito eggs, as observed in embryos exposed to EO. According to Valarmathy *et al.* (2011), longer exposure time to the toxic agent increases the entry capacity of compounds through the shell and affects embryogenesis. In this study, high percentages of abnormalities were observed in eggs from 0-12 h, which are related to the passage of the compounds of *S. officinalis* through the egg chorion. In general, the chorion is more resistant to drying and the action of external compounds up to two hours after oviposition. During this period, structural modifications occur in its constituent proteins, which lead to its insolubilization (Devillers *et al.* 2014; Rezende *et al.* 2008). It is precisely in this time that the components of the *S. officinalis* EO can penetrate the egg and exert its ovicidal action, or cause malformations. After these two hours, the entry of the essential oil into the egg occurs in smaller quantities through the small pores in the surface of the shell.

With respect to the hatching patterns, the 0-72 h eggs presented significant percentages of larvae malformations. These are directly related to embryo affectation, or damage in the structural conformation of the shell, which would prevent normal eclosion. In relation to the larvae, the type of damage that predominated was the little cephalic chitinization, observed mostly in the eggs of this same period. Even when the larva changed state, this type of damage continued until the pupal stage. Thus, the exposure time of the EO components causes the possible inhibition of chitin synthesis and generates a weaker integument (Arivoli and Tennyson 2011). Accordingly, any type of damage caused at the embryonic stage will affect the following stages of insect development. In this regard, Arivoli and Tennyson (2011) mention that the metamorphic abnormalities caused in early stages of development, also cause abnormalities in pupae and adults, which adds up as an important effect that justifies the utility of EO in mosquito control.

Although high inhibition of hatching could be expected in eggs from 0-72 h, it is important to note that during this time, the embryo was fully developed; therefore, inhibition was not significant. Accordingly, the effect of the essential oil possibly caused physiological alterations of the hormonal type. This phenomenon is similar to that observed with pyriproxyfen (juvenile hormone analogue) and azadirachtin (ecdysone agonist or molting hormone) (Suman *et al.* 2013). Here the effects are observable in the later development of larval stages, represented in scarce cephalic chitinization, malformations at the digestive tract level and abnormal behaviour, which was consistent with this study.

Larvicidal activity. *S. officinalis* presents larvicidal action against mosquitoes and the LC_{50} and LC_{90} presented in the study are lower than those reported by Pavela (2008). Pavela (2008) studied the larvicidal activity of 56 species of plants from the Euro-Asian region, including *S. officinalis*, with a mortality of 84.6 % at an LD_{50} of 159 $mg.L^{-1}$ and an LD_{90} > 500 $mg.L^{-1}$ in *Culex quinquefasciatus* larvae. According to this result, Pavela did not consider the plant *S. officinali*s as an important larvicidal agent. The same was indicated by Arivoli and Tennyson (2011) who mentioned that some species of *Salvia* have moderate larvicidal activity, with LC_{50} values between 100 and 250 $mg.L^{-1}$. However, more recent studies than those mentioned, show that this plant is considered an important agent for the production of insecticidal molecules. For instance, Ali *et al.* (2015) presented different species of *Salvia* with larvicidal activity similar to the results of our study for larvae of *Ae. aegypti*, with reports of LC_{50} of 56.9 $mg.L^{-1}$ and LC_{90} of 92.4 $mg.L^{-1}$; and Abou-Elnaga (2014) with LC_{50} of 25.1 $mg.L^{-1}$ and LC_{90} of 120.5 $mg.L^{-1}$ at 24 h of exposure, and an LC_{50} of 18.4 $mg.L^{-1}$ and LC_{90} of 106, 8 $mg.L^{-1}$ at 48 h. The lethal concentrations obtained by these authors are lower than those reported in our study. This might be explained by the major compounds reported by these authors, which where are different from those detected in this study. For example, viridiflorol (20.4 %) and α-tujone (25.8 %) in Ali *et al.* (2015), and miristicin (20.58 %), sabinene (15.37 %) and terpinen-4-ol (14.92 %) in Abou-Elnaga (2014).

The content of monoterpenoids in the essential oil of *S. officinalis* (thujone, camphor and 1,8-cineol) has a strong larvicidal effect against other mosquito species, such as *Anopheles* and *Culex* (Abou-Elnaga 2014). The major component of *S. officinalis* in this work, 1,8-cineol, has been reported with insecticidal action against various insect species, even being extracted from other plant species (Leyva *et al.* 2009). Kishore *et al.* (2011) mention that 1,8-cineole isolated from leaves of *Hyptis martiusii* Benth (Lamiaceae) showed a larvicidal effect on *Ae. aegypti* at concentrations of 25 $mg.mL^{-1}$ (10 % mortality), 50 $mg.mL^{-1}$ (53 % mortality) and 100 $mg.mL^{-1}$ (100 % mortality). Lucia *et al.* (2007), evaluated the larvicidal activity of *Eucaliptus grandis* W. Hill ex Maid (Myrtaceae) and its major components, and report that 1,8-cineol showed low larvicidal activity, with an LC_{50} of 57.2 $mg.L^{-1}$.

Pupicidal activity. The pupae of *Ae. aegypti* have strong cuticle sclerotization as the hatching time of the adult individual approaches, going from a thin, smooth and colorless layer, to a darker and thicker cuticle before emergence (Popa *et al.* 2011).

This degree of cuticle sclerotization contributes to a lower susceptibility of this stage to the action of external agents (Ramar *et al.* 2013), whether from synthetic or natural origin.

Since the pupa stage is more resistant, there are few studies that report the mortality of essential oils in mosquitoes, and the high concentrations used to achieve 100 % mortality. No studies were found in the literature involving the effect of *S. officinalis* on *Ae. aegypti*. However, when examining the concentrations used in studies evaluating the action of different plant species, it is evident that the concentrations used are between 160 and 1,000 $mg.L^{-1}$ (Gleiser *et al.* 2007; Ramar *et al.* 2013) and between 100 and 1,000 µL / mL (Sivagnaname and Kalyanasundaram 2004; Souza *et al.* 2011; Candido *et al.* 2013; Phasomkusolsil and Soonwera 2013).

The concentrations used in this paper to observe the pupicidal effect showed mortality percentages higher than 50 % at 48 h of exposure in concentrations that did not exceed 400 $mg.L^{-1}$. Since the pupae used for the bioassay were less than 24 h of emergence, that is, they had a less sclerotic cuticle, it is possible that the observed mortality is related to the action by contact with the components of the AE present in the liquid medium. Since the cuticle of insects is made up of different regions that differ markedly with respect to their chemical composition and properties, some studies consider that there is an ideal polarity value for the entry of xenobiotics in the organism (Lucia *et al.* 2013).

Adulticidal activity. The observed adulticidal effect is possibly due to the presence of monoterpenes in the EA. Studies indicate that they are the cause of a neurotoxic effect similar to that of organophosphorus compounds and carbamates in the inhibition of the enzyme acetylcholinesterase (Lucia *et al.* 2013). In this study, the major compounds, 1,8-cineol and α-tujone, play an important adulticidal role, due to the fact that both have neurotoxic effect in mosquitoes, reflected in a knockdown effect (Lucia *et al.* 2007; Lucia *et al.* 2009a). Being volatile monoterpenes, these compounds enter the body through the tracheae and tracheoles, entering in circulation through these ducts until they reach the hemolymph, where they are absorbed by proteins and spread throughout the body of the insect (Lucia *et al.* 2013). Although there are few studies evaluating the adulticidal effect of *S. officinalis*, the action times reported in this study coincide with those published by Lucia *et al.* (2009b), who obtained a fumigant effect of 1,8-cineol with a knockdown time (KT_{50min}) between 4.95-5.73, attributable to a neurotoxic effect of this compound. Subsequently, Lucia *et al.* (2011) evaluated the fumigant action of 15 plants of the genus *Eucalyptus* and found a KT_{50} between 10.65-0.076 min for species with a high content of 1,8-cineol. The minor adulticidal activity obtained with 300 $mg.L^{-1}$ is possibly related to the volatility of the compounds of the EO. Lucia *et al.* (2009b) reported that species with a high content of 1,8-cineole in their essential oils have a greater fumigant activity against *Ae. aegypti,* and this activity is related with the vapor pressure of this compound.

Repellency activity. The first line of defense against mosquito bites is the application of repellents. For this reason, to understand that there are substances as EOs that have this effect on mosquitoes can be a powerful tool to prevent its bites (Nerio *et al.* 2010; Jaramillo Ramirez *et al.* 2012). The protection and repellency percentages obtained from 0 to 2 min indicate that *S. officinalis* EO constitutes a repellent and a deterrent element (Phasomkusolsil and Soonwera 2011) against bites in this period, with a subsequent decrease in its effectiveness in the period from 2 to 15 min. This effect may be due to the fact that repellents work by creating a vapor barrier that prevents the insect from coming into contact with the surface of the skin (Nerio *et al.* 2010), but this same vapor phase of the EA makes them effective only during a relatively short period of time due to the high volatility and the type of components involved (Leyva *et al.* 2012; Phasomkusolsil and Soonwera 2011).

Some authors suggest that monoterpenes such as α-pinene, 1,8-cineol, eugenol, limonene, terpinolene, citronellol, citronellal, camphor and thymol present insecticidal activity in adult mosquitoes as natural repellents (Leyva *et al.* 2009; Nerio *et al.* 2010; Abou-Elnaga 2014). Individual compounds of the oil, such as 1,8-cineol, are detected by a specific olfactory receptor (RO) of the antenna of the mosquitoes (located in the sensilla's), which, in combination with odor degrading enzymes (EDO) recognizes the stimulus (Jaramillo Ramirez *et al.* 2012). If the EO compound acts by inhibiting the activity or production of the EDO enzymes, the specific olfactory receptor (RO) continues to function even though there would be no enzymes to break it down, which would cause confusion and disorientation in the insects, making them unable to locate their target (Jaramillo Ramirez *et al.* 2012).

However, this effect cannot only be attributed to these components, since bioactivity is associated with the joint presence of monoterpenes and sesquiterpenes in the EO (Nerio *et al.* 2010; Leyva *et al.* 2012) as is the case with *S. officinalis*. Therefore, in the case of the *S. officinalis* EO, a formulation should be considered to help stabilize their volatile components and improve protection times.

Deterrent activity. The high percentages of effectiveness in the inhibition of oviposition indicate that the females are sensitive to the smell of EO. The egg oviposition restriction action occurs because the sensory system of the insects is able to detect the volatile compounds of *S. officinalis* EO, which, when dissipated in the oviposition medium, produce secondary metabolites that act together or independently to avoid laying eggs (Navarro-Silva *et al.* 2009; El-Gendy and Shaalan 2012).

Davis and Bowen (1994) mention that mosquitoes use physical and chemical signals to detect potential oviposition sites through sensory receptors in the antennae, which respond to specific stimuli depending on the stage of development. Gravid females will be more likely to look for suitable oviposition sites, with adequate color and optical density of the water, texture, moisture and temperature of the oviposition substrate (Guha *et al.* 2012).

Only a few studies are available on the deterrent potential of EO from different plants. It has been reported that flavonoid compounds and formulations based on neem reduce oviposition and affect *Ae. aegypti* fertility (Guha *et al.* 2012). However, the EO of *S. officinalis* has monoterpenes and sesquiterpenes, which due to their volatility can cause an evasive action on the part of the females to these compounds, acting as semiochemicals (Navarro-Silva *et al.* 2009). These data support the values obtained at the three concentrations for the oviposition activity index (OAI), indicating high deterrent action potential against *Ae. aegypti.*

In conclusion, *S. officinalis* EOs presented a biocidal effect in all stages of development of the *Ae. aegypti* mos-

quito. However, this effect was more pronounced in the egg, pupa and adult stages (mortality and detergent activity). The results of the present study helped to understand the form like the *S. officinalis* EO has an insecticide effect over the different developmental stages of *Ae. aegypti*. According to this, the information contributes presented here can be useful to propose the components of EO of *S. officinalis* as possible replacement candidates for conventional chemical substances like temephos.

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Author contribution

The first author, Ruth M. Castillo-Morales, performed the experiments, analyzed the information, and wrote the paper. The second author, Jonny E. Duque, worked on designing experiments, analyses, and writing the paper.