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Interactions of *Schinus terebinthifolius* (Anacardiaceae) essential oil against *Aedes aegypti* (Diptera: Culicidae) larvae

Interações do óleo essencial de *Schinus terebinthifolius* (Anacardiaceae) contra larvas de *Aedes aegypti* (Diptera: Culicidae)

Interacciones del aceite esencial de *Schinus terebinthifolius* (Anacardiaceae) contra larvas de *Aedes aegypti* (Diptera: Culicidae)

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Abstract
Essential oils arouse the interest of research for insect control. *Schinus terebinthifolius* is described in the literature for being bioactive against *Aedes aegypti* larvae. However, studies are scarce to fully assess the larvicidal potential of this species. This study aimed to evaluate the chemical composition, bioactivity, time of death and bioavailability of the essential oil from different parts of *S. terebinthifolius* obtained from the Brazilian cerrado on *Ae. aegypti* larvae. For this, plants grown in the city of Goiânia-GO were used and the elucidation of the chemical composition of essential oils was carried out by means of gas chromatography coupled with mass spectrometry. *Ae. aegypti* larvae were used in the bioassays to assess larvicidal activity, determine the time of death and bioavailability of the essential oil in solution. In addition, the interference of essential oil in the activity of the enzyme acetylcholinesterase was also investigated. Based on the results obtained, it was observed that the most promising essential oil for the development of larvicidal formulations is that of fruits, based on having higher yield, greater bioactivity, time of death similar to synthetic insecticides. An inhibitory interaction of acetylcholinesterase was also observed. However, the essential oil had low bioavailability, so it is necessary to develop formulations to increase its bioactivity period.
Keywords: Bioavailability; Vector control; Time of death.

Resumo

Óleos essenciais despertam o interesse das pesquisas para controle de insetos. *Schinus terebinthifolius* é descrita na literatura por ser bioativa contra larvas de *Aedes aegypti*. No entanto, os estudos são escassos para a avaliar a fundo o potencial larvicida dessa espécie. Este estudo teve como objetivo avaliar a composição química, bioatividade, tempo de morte e biodisponibilidade do óleo essencial de diferentes partes de *S. terebinthifolius* obtidas do cerrado brasileiro sobre larvas de *Ae. aegypti*. Para isso, utilizou-se plantas cultivadas na cidade de Goiânia-GO e a elucidação da composição química dos óleos essenciais foi realizada por meio de cromatografia a gás acoplada a espectrometria de massas. Nos bioensaios foram empregadas larvas de terceiro estádio de *Ae. aegypti* para avaliação da atividade larvicida, determinação do tempo de morte e da biodisponibilidade do óleo essencial em solução. Além disso, a interferência do óleo essencial na atividade da enzima acetilcolinesterase também foi investigada. Mediante os resultados obtidos, observou-se que o óleo essencial mais promissor para o desenvolvimento de formulações larvicidas é o dos frutos, baseando-se no maior rendimento, maior bioatividade e tempo de morte semelhante a inseticidas sintéticos. Observou-se também interação inibitória da acetilcolinesterase. No entanto, o óleo essencial de *S. terebinthifolius* possuiu baixa biodisponibilidade, assim, faz-se necessário o desenvolvimento de formulações para aumentar o período de bioatividade do mesmo.

Palavras-chave: Biodisponibilidade; Controle de vetores; Tempo de morte.

Resumen

Los aceites esenciales despiertan el interés de la investigación para el control de insectos. *Schinus terebinthifolius* se describe en la literatura por ser bioactivo contra las larvas de *Aedes aegypti*. Sin embargo, los estudios son escasos para evaluar completamente el potencial larvicida de esta especie. Este estudio tuvo como objetivo evaluar la composición química, bioactividad, tiempo de muerte y biodisponibilidad del aceite esencial de diferentes partes de *S. terebinthifolius* obtenido del cerrado brasileño sobre las larvas de tercer estadio de de *Ae. aegypti*. Para ello se utilizaron plantas cultivadas en la ciudad de Goiânia-GO y se determinó la composición química de los aceites esenciales mediante cromatografía de gases acoplada a espectrometría de masas. Se utilizaron larvas de *Ae. aegypti* en los bioensayos para evaluar la actividad larvicida, determinar el tiempo de muerte y la biodisponibilidad del aceite esencial en solución. Además, también se investigó la interferencia del aceite esencial en la actividad de la enzima acetilcolinesterasa. Con base en los resultados obtenidos, se observó que el aceite esencial más prometedor para el desarrollo de formulaciones larvicidas es el de los frutos, por poseer mayor rendimiento, mayor bioactividad, tiempo de muerte similar a los insecticidas sintéticos. También se observó una interacción inhibidora de la acetilcolinesterasa. Sin embargo, el aceite esencial tenía baja biodisponibilidad, por lo que es necesario desarrollar formulaciones para aumentar su período de bioactividad.

Palabras clave: Biodisponibilidad; Control de vectores; Tiempo de muerte.

1. Introduction

Essential oils (OE) are compounds derived from the secondary metabolism of plants, which are characterized for being volatile and hydrophobic liquids with various applications. This is because EOs are composed of complex mixtures formed mainly by monoterpenes and sesquiterpenes (Bakkali *et al*., 2008). These compounds are widely used by the pharmaceutical and food industries mainly due to their antimicrobial, antioxidant, and organoleptic properties (Bhavaniramya *et al*., 2019; Mishra *et al*., 2020; Goudjil *et al*., 2020; He *et al*., 2020). They are promising products in the research and development of bioinsecticides because they interact with the nervous system of insects due to anticholinesterase activity, inhibition, or antagonism of gamma-aminobutyric receptors (Braga *et al*., 2007) or acting on the digestive system (Camaroti *et al*., 2018). Therefore, they play an important part in the control of agricultural pests (Deb *et al*., 2020), urban vectors (Bouabida *et al*., 2020; Zeghib *et al*., 2020), and insecticide formulations, since they present low toxicity and are alternatives to the use of synthetic insecticides (Sharma *et al*., 2020; Amado *et al*., 2020).

The Anacardiaceae family has approximately 81 genera subdivided into 800 species, present mainly in tropical and subtropical regions (Pell, 2011). It is a family with plants known for food consumption, but many of these species have insecticidal potential against mosquitoes vectors of diseases such as *Anacardium occidentale* L. (Vani *et al*., 2018; Kala *et al*., 2019) and *Spondias mombin* L. (Famuyiwa *et al*., 2020; Ajaegbu *et al*., 2016), known as cashew and yellow mombin, respectively. In addition to these, the genus *Schinus* has species widely distributed throughout the Brazilian territory, among

them, *Schinus molle* L., which is investigated in the control of urban disease vectors such as *Culex pipiens* (Diptera: Culicidae) for example (Zahran *et al*., 2017).

Schinus terebinthifolius, popularly known as rose pepper, "aroeira", red "aroeira", beach "aroeira", among other denominations, is widely described in the literature due to its diverse bioactivity, among them studies aimed at the control of agricultural pests and vectors of urban pathogens. That is a promising species against *Anticarsia gemmatalis* (Lepidoptera: Noctuidae), also known as velvetbean caterpillar, which affects various crops such as sugar cane, cotton and cauliflower (Vicenço *et al*., 2020); *Plutella xylostella* (Lepidoptera: Plutella), popularly known as diamondback moth, responsible for affecting rice, potato, and cotton crops among others (Silva, 2019); *Bemisia tabaci* and *Trialeurodes ricini* (Hemiptera: Aleyrodidae) known as sweet potato whitefly and castor bean whitefly respectively (Hussein, 2017). 13 in addition, this plant is widely studied in the fight against mosquitoes vectors such as *Cx. pipiens*, vector of encephalitis, avian malaria, and filarial virus (Zahran *et al*., 2017) and *Aedes aegypti*, vector of dengue, chikungunya and zika virus (de Campos Bortolucci *et al*., 2019; Procopio *et al*., 2015). Thus, it is a promising species against agricultural pests causing numerous damages in the productive sector, and urban vectors, carriers of high incidence and high prevalence pathologies in tropical and subtropical regions.

Considering that *S. terebinthifolius* is a plant known for its bioactivity against several vectors of urban diseases, among them the *Ae. aegypti*, and there are no studies in the literature that investigate the comparative larvicidal activity between leaves, fruits and seeds, it is necessary to analyze these EOs to determine the plant part with the most promising biological activity. Thus, this paper aims to analyze the chemical composition and larvicidal activity of the EOs, as well as determine the time of death, and the bioavailability of the EO in solution. Its interference with the activity of the enzyme acetylcholinesterase was also investigated. These studies aim to support the development of natural formulations for vector control.

2. Methodology

2.1 Plant material and obtaining the essential oil

The branches, leaves, fruits and seeds were collected in the municipality of Goiânia, State of Goiás, Brazil. A voucher specimen was stored in the herbarium of the conservation unit at the Federal University of Goiás for identification purposes under number 66.444. The samples of the leaves and fruits were separated, and the seeds were peeled manually. They were then desiccated in a forced air convection oven at 40ºC for three days. Afterwards, they were crushed in Ika® A11 processor and immediately after processing underwent hydrodistillation in Clevenger apparatus for two hours. The resulting essential oil was desiccated with sodium sulfate anhydrous and stored in an amber vial under refrigeration at -22°C. The yield of the extractive process was calculated from the ratio between the mass of oil obtained and the crushed sample (Farmacopéia Brasileira, 2019).

2.2 Essential oil chemical composition determination

An aliquot of the EO obtained underwent gas chromatographic analysis, coupled to mass spectrometry (GC-MS) in a Shimadzu GC-QP2010A apparatus, with silica capillary column DB-5 (30m × 0.25mm × 0.25m with 5%-Phenyl-methylpolysiloxane). Heating ramp programmed in the following scheme: starting with 60-240°C at 3°C/min, then 280°C at 10°C/min, ending with 10 min at 280°C. Carrier gas was helium with a flow of 1 mL/min. Injection port has been set to 225°C. Mass spectrometer operating with interface temperature of 240°C; electron ionization at 70 eV with scanning mass range of 40-350 m/z and sampling rate of 1 scan/s. Chemical components of essential oil were identified by comparing the mass spectra

and retention indices with those reported in the literature for the most common components of essential oils (Adams, 2007). The retention indices were calculated by co-injecting a mixture of hydrocarbons, C_9-C_{28}, and using the Van Den Dool & Kratz equation (Dool; Kratz, 1963; Adams, 2007).

2.3 Bioassays

Bioassays with larvae of *Ae. aegypti* were performed at the Laboratory of Insect Biology and Physiology (IPTSP/UFG), under controlled conditions of climate control. In the biological chamber, the insects develop and are tested at a temperature of 28°C ± 1°C, relative humidity of 85% ± 5% and 12-hour photophase of light-dark. The larvae used in the tests grew in basins with water supplied by the public supply network and fed with cat food. Tests were carried out in polystyrene containers with a capacity of 50mL.

2.3.1 Assessment of larvicidal activity

To assess the larvicidal potential of fruit, leaves, seeds, and branches, aliquots of *S. terebinthifolius* EO were dispersed with surfactant Tween 80 (v/v) to produce an aqueous solution at 100µg/mL. A total of 20 third-stage larvae of *Ae. aegypti* were exposed to 20mL of test solution in serial dilutions of 20-100µg/mL. A solution of water + surfactant was used as negative control and temephos (Abate® Basf Chemical Group) at 0.012µg/mL as the positive control, according to the methodology proposed by WHO (WHO, 2005). Mortality was quantified after 24-hour exposure to treatments and confirmed by lack of response to mechanical stimulus and stiffening of the cephalic capsule. Three repetitions were performed for each assay. Subsequent tests for larvicidal activity were performed only with the EO considered more promising according to the lower lethal concentration (LC) and higher yield in the extractive process. Lethal time (Lt) determination to determine the mean time required for larval mortality, the assay proposed by Aguiar *et al*., (2015) was conducted, with some adaptations. In this sampling, 20 larvae of *Ae. aegypti* in the third stage were exposed to the EO solution of fruits of *S. terebinthifolius* at LC_{90}. After exposure, the larvae were monitored and classified as living, lethargic or dead every 40 minutes. For classification, the larvae were observed in stereomicroscope (Leica M50) augmented 20-fold. Death was confirmed with the absence of contraction in the respiratory and circulatory muscles. The evaluation was performed in triplicate.

2.3.2 Assessment of persistence time and bioavailability of essential oil (Bt)

At first the test was performed to verify the residual effect of the EO of the fruits of *S. terebinthifolius*. In this test 20 third-stage larvae of *Ae. aegypti* were exposed to 50mL EO solution in LC_{90} for 24 hours. After counting mortality events, new larvae were exposed to the solution, without any renewal of the solution, and mortality events were quantified after 24 hours of exposure. The replacement of larvae every 24 hours should occur until the nullity of the solution effect (Romano *et al*., 2018; Menezes *et al*., 2019). However, the solution showed no residual effect. Given this condition, an exposure scheme was developed to verify the bioavailability time of the EO in the test solution. Considering the volatility of essential oils, the experiment to determine the bioavailability time (Bt) was drawn from the correlation between the preparation time of the solution and the occurrence of larval mortality. For this 140mL of EO solution of *S. terebinthifolius* was prepared in LC_{90} and immediately distributed in seven containers, containing 20mL of solution each. The exposure of the larvae in the solution obeyed a temporal scheme so that the first container received 20 larvae of *Ae. aegypti* in third instar immediately after preparation. The second container received the larvae after 40 minutes of filling with the solution. The exposure of the larvae to the test solution obeyed this interval of 40 minutes, so that at the last exposure the larvae found a ready solution at 280 minutes. Larval mortality was observed after 24 hours of exposure. Tests were performed in triplicate.

2.3.3 Enzymatic activity on acetylcholinesterase

Enzyme activity assay on acetylcholinesterase was performed according to the methodology proposed by Sugumar *et al*., (2014), with minor modifications. To observe the interference of EO of fruits of *S. terebinthifolius* on acetylcholinesterase activity, 50 fourth-stage larvae were exposed to the EO test solution in increasing solutions from 50 to 400ppm. Larvae treated with EO were macerated with 500µL PBS buffer pH 7.2 and Triton X-100 at 10% to produce the body homogenized. The homogenized was centrifuged 12,000rpm, at 4°C for 10min. Enzymatic activity was measured by the Ellman's reaction (1961). Homogenized prepared with larvae exposed to temephos at 0.012ppm was used as a positive control and the negative control was prepared with larvae exposed to water solution and surfactant only. For the reaction, 50µL of homogenized body was incubated in 125µL of PBS buffer and 50µL of 10mM of 5,5′-Dithiobis(2-nitrobenzoic acid) and 50µL of 12.5mM of acetylcholine iodide as a substrate, for 5 min. The absorbance of the reaction was measured at 405nm.

2.4 Statistical analysis

The lethal concentration (LC) responsible for 50 and 90% of mortality was estimated by nonlinear regression (Probit) (α=0.05). The percentage of enzyme inhibition was calculated with the absorbance obtained in the enzyme assay using the formula I = (A0 – At) A0*100, where I equals the percentage of enzyme inhibition, A0 corresponds to the absorbance of the negative control and the absorbance of the test solution (Owokotomo et al., 2015). The inhibition rate was used to predict the concentration required for minimum enzyme inhibition (MI) of 50 and 90%, also calculated by nonlinear regression. All statistical analyses were performed using Statistica 12.0 software (StatSoft, 2013).

3. Results and Discussion

3.1 Essential oil chemical composition determination

The chemical composition of the EOs of branches, leaves, fruits and seeds is shown in Table 01. Results show that the components found in fruits and seeds are formed predominantly by monoterpenes. Experiments carried out by Barbosa *et al*., (2007) and Cavalcanti *et al*., (2015) evaluated the extraction kinetics of the volatile components of *S. terebinthifolius* and found the composition of the EO of the fruits formed predominantly by monoterpenes during the first hours of extraction, just as in this study. The 17 monoterpenes are described in the literature for their satisfactory larvicidal activity, being the main compounds of interest evaluated in this study (Kweka *et al*., 2016).

After the extraction of the EOs, the yield of the extractive process was determined to verify which EO is most viable for large-scale use in the production of larvicides. The results obtained in this study are shown in Table 1. Thus, it was possible to verify that the yield of fruits and seeds are higher than that of leaves and branches, which have low yield that disfavors their use in the development of formulations. The seeds, despite having higher yields, have an additional step (peeling) in the acquisition process, which would require a higher production cost, whether with equipment or labor. Therefore, when considering the yield factor alone, it can be said that the essential oil extracted from the total fruit is the most viable.

Table 1: Chemical composition and yield of the extraction process of essential oil from branches, leaves, fruits and seeds of *Schinus terebinthifolius* (Anacardiaceae) evaluated by the process of gas chromatography coupled with mass spectrometry.

IK	Substance	Branches	Leaves	Fruits	Seeds
921	Tryciclene	-	1,76	-	-
939	α-Pinene	1,53	-	2,95	5,84
940	4-Methylpentanoic acid	0,97	-	-	-
974	β-Pinene	0,16	-	0,21	-
988	Myrcene	1,57	0,56	6,32	4,32
1002	α-Phellandrene	0,25	6,92	15,25	21,82
1008	δ-3-Carene	-	16,13	13,86	50,11
1014	α-Terpinene	-	-	0,44	-
1020	ρ-Cimene	-	0,49	1,11	-
1025	β-Phellandrene	-	2,71	5,58	12,24
1029	Limonene	3,54	-	-	-
1032	Z- β-Ocimene	-	-	1,81	-
1088	Terpinolene	-	0,6	-	-
1338	δ-Elemene	-	-	3,96	-
1375	α-Ylangene	18,83	-	-	-
1388	β-Cubebene	0,43	-	-	-
1390	β-Elemene	-	2,67	1,76	-
1408	Z-Caryophyllene	-	11,62	-	-
1419	β-Caryophyllene	10,31	1,36	9,38	-
1419	β-Ylangene	-	1,31	-	-
1436	ɣ-Elemene	3,41	-	1,05	-
1451	α-Himachalene	2,28	-	-	-
1452	α-Humulene	1,69	1,23	0,65	-
1456	E-β-Farnesene	1,94	-	-	-
1461	Cis-cadina-1(6),4-diene	-	11,17	-	-
1465	Cis-muurola-4(14),5-diene	-	1,39	0,62	-
1475	ɣ-Gurjunene	-	1,32	-	-
1482	ɣ-Himachalene	3,48	-	-	-
1484	Germacrene D	12,64	-	14,46	5,67
1495	ɣ-Amorphene	-	-	0,51	-
1498	β-Alaskene	4,39	-	-	-
1500	Biciclogermacrene		10,69	-	-
1500	α-Muurolene	2,01	0,98	-	-
1505	β-Bisabolene	1,95	-	-	-
1511	δ-Amorphene	-	-	1,23	
1513	ɣ-Cadinene	1,05	1,2	-	-
1515	Z-ɣ-Bisabolene	1,01	-	-	-
1523	δ-Cadinene	9,34	3,28	-	-
1546	Selina-3,7(11)-diene	0,74	-	-	-
1559	Germacrene B	4,04	-	0,65	-
1563	E-Nerolidol	1,85	-	-	-
1600	Rosifoliol	-	2,48	-	-
1628	1-epi-Cubenol	0,75	-	-	-
1642	epi-α-Muurolol (torreyol)	1,82	-	-	-
1646	α-Muurolol	0,85	-	-	-
1654	α-Cadinol	2,59	-	-	-
1680	Elemol acetato	-	-	0,99	-
Oxigenated monoterpenes		-	-	-	-
Monoterpenes		8,74	29,17		100
Oxigenated sesquiterpenes		7,87	-		-
Sesquiterpenes		81,30	70,83		-
Total (%)		97,91	100	100	100
Yeld (%)		0,098	0,22	5,89	7,75

Source: Authors.

The chromatographic analysis of the essential oil of the seeds revealed the presence of six components, the monoterpenes δ-3-Carene (50.1%), α-Phellandrene (21.8%), and β-Phellandrene (12.2%) being the majority. Studies involving seeds have not yet been reported in the literature, however, the analyzed compounds are also present in other parts of the

vegetable. Results of the chemical composition of the branches indicated the presence of forty components, the following being the majority: α-Ylangene (17.39%) and ɣ-Muurolene (11.68%). Ennigrou *et al*., (2018) reported the presence of these compounds in the branches of samples collected in Tunisia. However, the majority of compounds reported in the literature were α-Phellandrene (36.18%), α-Pinene (14.85%), and limonene (8.79%), which were also described in this study in different proportions.

Analysis of the chemical composition of the fruits detected twenty-five compounds, the majority of which were α-Phellandrene (15.25%), germacrene D (14.46%) and δ-3-Carene (13.86%). In Dannenberg's (*et al*., 2019) studies the following major compounds were found: β-Myrcene (41%), β-Cubebene (12%), limonene (9%), and α-Pinene (8%) in samples obtained in Capão Leão, Rio Grande do Sul (Brazil). In the analysis of Hussein *et al*., (2017) were detected: α-Pinene (36.9%), α-Phellandrene (32.8%), limonene (11.9%), and α-Terpineol (6.0%). Cavalcanti (2015) found the majority components α-Pinene (44.9%), β-Pinene (15.1%), and germacrene D (17.6%) in samples collected of *S. terebinthifolius* obtained in Rio de Janeiro (Brazil). Whereas Bendaoud *et al*., (2010) analyzed samples also obtained in Tunisia, and the results found were α-Phellandrene (34.4%), β-Phellandrene (10.6%), ɣ-Cadinene (18.0%), and α-Pinene (6.5%). Thus, the compounds described in this study are in accordance with those described in the literature for the fruit samples.

Analysis of the leaves detected the presence of thirty components, majority compounds being δ-3-Carene (16.13%), Z-Caryophyllene (11.62%), and cis-Cadina-1(6),4-diene (11.17%). Cavalcanti *et al*., (2015) found the majority β-Caryophyllene (35.2%), α-Pinene (28.1%) and germacrene D (15.5%) in samples collected in Rio de Janeiro (Brazil). Uliana (2016) found the compounds δ-3-Carene (68.8%), β-Caryophyllene (8.22%), Myrcene (6.8%) and α-Pinene (4.0%) as the majority in samples collected in Vitória, Espírito Santo (Brazil). The study of Ennigrou *et al*., (2018) also found the following compounds: α-Phellandrene (33.1%), α-Pinene (15.2%), limonene (6.6%), and β-Phellandrene (4.8%). The compounds found in this study have been previously described in samples of *S. terebinthifolius* and show that despite the chemical variation that naturally occurs according to edaphic and climatic factors, the samples of *S. terebinthifolius* tend to present α and β-Phellandrene, and β-Caryophyllene between their majority in the leaves.

3.2 Assessment of larvicidal activity and enzymatic activity on acetylcholinesterase

The evaluation of the larvicidal activity of the leaf, fruit, seed, and branch samples is shown in Table 2. The mean lethal concentration (LC) is the lowest concentration necessary for a population exposed to a given substance, in a pre-established period, to die (Minho *et al*., 2017). When analyzing the samples, it is possible to see that the seeds and fruits have greater lethality against larvae of *Ae. aegypti*. However, for large-scale production of bioinsecticide, the additional steps in obtaining the essential oil from seeds can drive up costs. Therefore, the fruits are considered as the most promising sample for the evaluation of larvicidal potential. In studies by Bortolucci *et al*., (2019), the larvicidal activities of the essential oil of fruits of *S. terebinthifolius* collected in Juranda, Paraná (Brazil) were determined in third-stage larvae of *Ae. aegypti* and found a LC_{50} of 374mg.L^{-1}. Variations in the composition of essential oil influence the larvicidal activity and are explained due to differences in seasonality, temperature, water availability, light incidence, among other factors that alter both the presence and concentration of certain metabolites in the plant (Gobbo-Neto & Lopes, 2007).

Table 2: Lethal concentrations obtained in larvicidal bioassays with essential oil from leaves, fruits, seeds, and branches of *Schinus terebinthifolius* on third-stage larvae of *Aedes aegypti*.

EO sample	LC_{50} (µg/mL)	LC_{90} (µg/mL)
Branches	72.8 (69.7–75.9)	107.3 (104.1–110.45)
Leaves	65.20 (61.8–68.6)	96.00 (90.3–101.7)
Fruits	34.89 (34.0–35.7)	50.13 (48.7–51.7)
Seeds	33.8 (32.1–35.6)	55.40 (52.7–58.0)

Source: Authors.

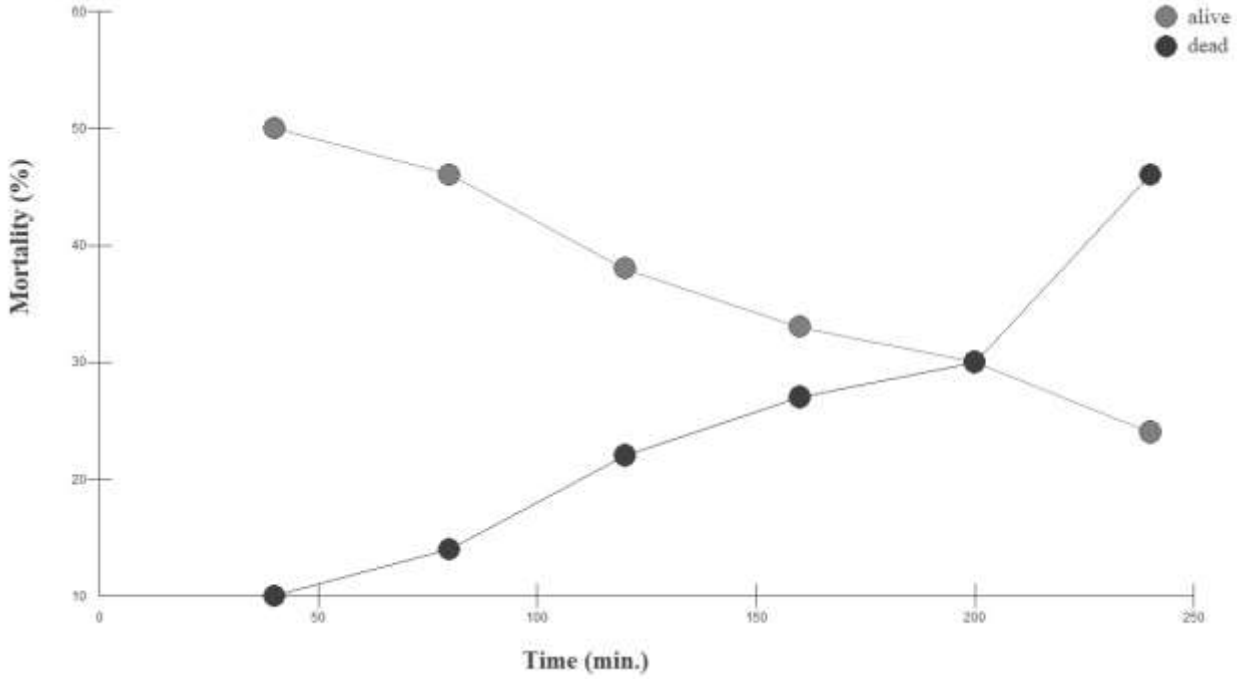
S. terebinthifolius is described in the literature for its larvicidal activity against several vectors of tropical diseases and crop pests due to the presence of compounds of great relevance in various parts of the vegetable. The mechanism of action of these compounds suggests that they can act at different active sites. In this study, an enzyme extracted from fourth-stage larvae was used and the results of enzymatic activity on acetylcholinesterase indicated inhibition in MIC_{50} of 436.95µg/mL and MIC_{90} of 1,204.37µg/mL for fruit EO. The use of enzymes extracted from larvae of *Ae. aegypti* allows matching the analysis conditions closer to reality, showing the real activity of the EO. In an enzyme kinetics experiment using purified enzyme in studies by Bortolucci *et al*., (2019), it was found that the EO of *S. terebinthifolius* fruits acts through the mechanism of acetylcholinesterase inhibition in *Ae. aegypti*. Thus, we can infer that one of the probable mechanisms that are involved in the larvicidal activity of the EO of *S. terebinthifolius* fruits is the inhibition of enzymatic activity on acetylcholinesterase. However, due to the high concentration required for the inhibition of 90% of enzyme activity, it can be assumed that enzyme activity is not the only path for activation of lethality.

In addition to enzymatic activity on acetylcholinesterase, other probable mechanisms of action are described in the literature in distinct species of vector and agricultural pests. Studies involving Essential Oil of *S. terebinthifolius* showed promising larvicidal activity against fourth-stage larvae of *Cx. pipiens* which suggests the probable mechanism of death is enzymatic activity (Zahran *et al*., 2017). Bilobol, an isolated alkylresorcinol, obtained from the leaves presented results of LC_{50} 7.67mg.L^{-1}, considered a potent candidate for natural larvicide (Schulte *et al*., 2021). Plant extracts produced from leaves of *S. terebinthifolius* collected in Recife, Pernambuco (Brazil) were tested in fourth-stage larvae of *Ae. aegypti*, thus observing a larvicidal effect caused by damage to their midgut (Procópio *et al*., 2015). In addition, studies using samples also collected in Recife, Pernambuco (Brazil) demonstrated interference in intestinal enzymes of *Sitophilos zeamais* (Coleoptera: Curculionidae), a major pest of corn and other stored grains (Camaroti *et al*., 2018).

3.3 Determination of lethal time (Lt) and assessment of persistence and bioavailability (Bt) of essential oil

To assess the time of death of larvae after exposure to the EO of the fruits, the time in which there was 50% mortality (LT_{50}) was determined in 183.31 minutes and 90% mortality (LT_{90}) in 349.83 minutes using the lethal concentration of 90% (LC_{90}). From Figure 1 it is possible to analyze that the percentage of live and lethargic larvae reduces over time, as well as an increase in the percentage of mortality.

Figure 1: Determination of time of death of third-stage larvae of *Aedes aegypti* after exposure to essential oil of *Schinus terebinthifolius* fruits. Higher mortality values are observed after three hours of exposure, showing the rapid effect of essential oil on larvae.



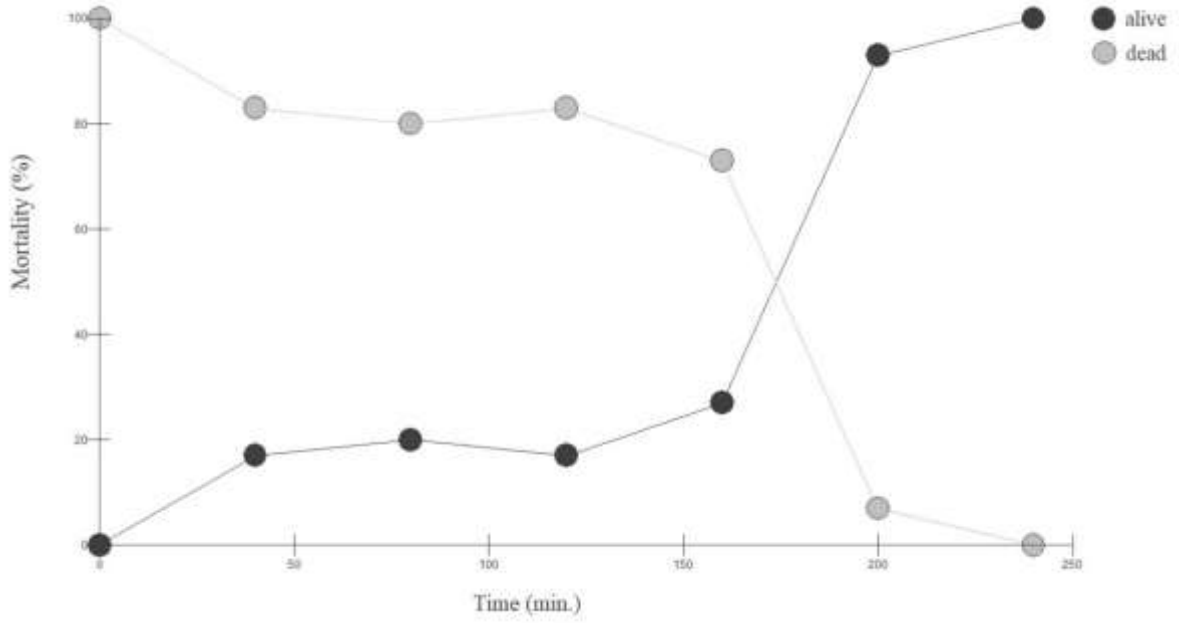
Source: Authors.

In the literature, there are no scientific studies that analyze the time of death of larvae of *Ae. aegypti* using EO of *S. terebinthifolius.* These data are fundamental to understand how long a potential larvicide produced from the EO of the fruits of Pink Pepper will take to reach the mortality of the exposed population. In Lethal time studies involving EO of *Siparuna guianensis* Aubl (Siparunaceae) in third-stage larvae of *Ae. aegypti*, a LT_{50} of 12.11 min was obtained using samples collected in Gurupi, Tocantins, Brazil (Aguiar *et al*., 2015). Studies with EO of *Plectranthus amboinicus* (Lour.) Spreng. (Lamiaceae) collected in Taiwan found a LT_{50} of 61.16 minutes in larvae of *Ae. aegypti* at 100mg.L^{-1} (Huang *et al*., 2019).

Synthetic insecticides are widely used in the control of urban vectors; however, they have as a disadvantage their high toxicity to the environment. Assays with *temephos* have shown that the insecticide can reach LT_{50} and LT_{90} in 2,088.6 and 3,432 minutes respectively (Fatimah *et al*., 2020). Studies using DDT (dichlorodiphenyltrichloroethane), a currently banned insecticide due to high environmental toxicity, showed LT_{50} and LT_{90} in 177.73 and 493.54 minutes (Nazni *et al*., 2009). Thus, *S. terebinthifolius* has a smaller lethal time than the *temephos*, the neurotoxic insecticide indicated by WHO as standard for testing and for field use in places where the mosquito population is not yet resistant. Its lethal time is similar to that of DDT; however, it has the advantage of a low toxicity to the environment and may be a possible candidate for the replacement of synthetic larvicides.

The determination of the bioavailability of the EO was performed using LC_{90}. Since EOs are volatile substances, this parameter is essential to verify their bioactivity period. Therefore, it was possible to calculate that Bt_{90} was nine minutes and Bt_{50} approximately 134 minutes as shown in Figure 2.

Figure 2: Assessment of the bioavailability of essential oil from *Schinus terebinthifolius* fruits. The lethal effect of the essential oil reduces with increasing exposure time of the prepared solution to the environment.



Source: Authors.

The bioassay showed that with time there was a reduction in the bioactivity of the EO, probably caused by its volatilization to the environment. Consequently, the development of formulations that increase the permanence of EO in an aqueous medium is fundamental for the use of *S. terebinthifolius* with larvicidal purposes. This was the first study that sought to evaluate the bioavailability time of essential oils guided by the larval mortality event.

4. Conclusion

Thus, it is possible to determine that the most promising essential oil of *S. terebinthifolius* is that of the fruits since it has a satisfactory yield and potent larvicidal activity against larvae of *Ae. aegypti*. However, the result of the bioavailability test allowed us to observe that this oil remains bioavailable for a short time in an aqueous solution, which suggests that it is necessary to develop dispersion systems that favor the permanence of the oil dispensed in the liquid medium for a longer time, thus avoiding the loss of volatile compounds to the environment. Therefore, it is a plant with the potential for the development of low environmental impact and effective larvicidal products against *Ae. aegypti*.

Furthermore, the bioavailability assessment methodology guided by larval mortality was an efficient and relatively low-cost way to measure the persistence time of essential oils in solution, working as an indicator for cases where residual effect tests would not generate accurate information.

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