

## Larvicidal activity of cloves as an alternative to chemical control in mosquito vectors in the Amazonia under laboratory conditions

Atividade larvívica do cravo-da-índia como alternativa ao controle químico em mosquitos vetores na Amazônia em condições de laboratório

Actividad larvívica del clavo de olor como alternativa al control químico en mosquitos vectores de la Amazonia en condiciones de laboratorí

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### Abstract

Mosquito-borne diseases continue to be the main causes of death in many tropical countries. Among the infectious diseases transmitted by mosquitoes, malaria and dengue fever remain a major public health concern. Chemical control is the most widely used method for controlling and preventing mosquito-borne diseases. However, there are reports in the literature about resistance in populations due to the continuous use of chemical insecticides. In this sense, products of plant origin, such as plant extracts, essential oils and plant derivatives, have emerged as a promising alternative. In this study, bioassays were carried out to evaluate the activity of the aqueous and methanolic extracts of cloves (*Syzigium aromaticum*) in laboratory conditions against *Anopheles darlingi* and *Aedes aegypti* larvae. The bioassays carried out with *An. darlingi* showed CL<sub>50</sub> values of 6.41 µg/mL for the methanolic extract and CL<sub>50</sub> values of 5.65 µg/mL for the aqueous extract. Considering the bioassays with *Ae. aegypti* larvae (aqueous extract), the CL<sub>50</sub> value was 78.81 µg/mL and the methanolic extract showed a CL<sub>50</sub> value of 131.10 µg/mL. The larvicidal activity of the methanolic extract (CL<sub>50</sub> 131.10 µg/mL) against *Ae. aegypti* larvae was much lower than that obtained with *An. darlingi*. Comparing the two mosquito species, *An. darlingi* was more susceptible to the aqueous and methanolic extracts. However, when comparing the activity of the extracts against *An. darlingi*, it was found that the aqueous and methanolic extracts were active. With regard to *Ae. aegypti* larvae, they were more susceptible to the aqueous extract.

**Keywords:** Cloves; *Syzigium aromaticum*; Insecticidal activity; Malaria; Dengue fever.

### Resumo

As doenças transmitidas por mosquitos continuam a ser as principais causas de morte em muitos países tropicais. Entre as doenças infecciosas transmitidas por mosquitos, a malária e a dengue permanecem como grande preocupação na saúde pública. O controle químico é o método mais utilizado no controle e prevenção de doenças transmitidas por mosquitos. Porém, há relatos nas literaturas sobre a resistência em populações pelo uso contínuo de inseticidas químicos. Nesse sentido os produtos de origem vegetal, como extratos vegetais, óleos essenciais e derivados de plantas, surgem como uma alternativa promissora. No presente trabalho foram realizados bioensaios para se avaliar a atividade do extrato aquoso e metanólico do cravo-da-índia (*Syzigium aromaticum*) em condições de laboratório com larvas de *Anopheles darlingi* e *Aedes aegypti*. Os bioensaios realizados com *An. darlingi*, observou-se valores de CL<sub>50</sub> igual a 6.41 µg/mL para o extrato metanólico, e CL<sub>50</sub> igual a 5,65 µg/mL para o extrato aquoso. Considerando os bioensaios com larvas de *A. aegypti* (extrato aquoso), o valor de CL<sub>50</sub> foi de 78,81 µg/mL e extrato metanólico apresentou valor de CL<sub>50</sub> igual à 131,10 µg/mL. Atividade larvívica do extrato metanólico (CL<sub>50</sub> 131.10 µg/mL) em larvas de *Ae. aegypti* foi bastante inferior ao obtido com *An. darlingi*. Comparando-se as duas espécies de mosquito,

observou-se maior susceptibilidade de *An. darlingi* ao extrato aquoso e metanólico. Contudo, ao comparar-se a atividade dos extratos sobre *An. darlingi*, verificou-se que o extrato aquoso e metanólico se mostraram ativos. Em relação às larvas de *A. aegypti*, mostraram-se mais susceptíveis ao extrato aquoso.

**Palavras-chave:** Cravo-da-índia; *Syzigium aromaticum*; Atividade inseticida; Malária; Dengue.

### Resumen

Las enfermedades transmitidas por mosquitos siguen siendo las principales causas de muerte en muchos países tropicales. Entre las enfermedades infecciosas transmitidas por mosquitos, la malaria y el dengue siguen siendo un importante problema de salud pública. El control químico es el método más utilizado para controlar y prevenir las enfermedades transmitidas por mosquitos. Sin embargo, existen informes en la literatura sobre la resistencia en las poblaciones debido al uso continuado de insecticidas químicos. En este sentido, los productos de origen vegetal, como extractos de plantas, aceites esenciales y derivados de plantas, han surgido como una alternativa prometedora. En este estudio se realizaron bioensayos para evaluar la actividad de los extractos acuosos y metanólicos de clavo (*Syzigium aromaticum*) en condiciones de laboratorio sobre larvas de *Anopheles darlingi* y *Aedes aegypti*. Los bioensayos realizados con *An. darlingi* mostraron valores  $CL_{50}$  de 6,41  $\mu\text{g/mL}$  para el extracto metanólico y valores  $CL_{50}$  de 5,65  $\mu\text{g/mL}$  para el extracto acuoso. Considerando los bioensayos con larvas de *Ae. aegypti* (extracto acuoso), el valor  $CL_{50}$  fue de 78,81  $\mu\text{g/mL}$  y el extracto metanólico mostró un valor  $CL_{50}$  de 131,10  $\mu\text{g/mL}$ . La actividad larvicida del extracto metanólico ( $CL_{50}$  131,10  $\mu\text{g/mL}$ ) sobre larvas de *Ae. aegypti* fue muy inferior a la obtenida con *An. darlingi*. Comparando las dos especies de mosquitos, *An. darlingi* fue más susceptible a los extractos acuoso y metanólico. Sin embargo, al comparar la actividad de los extractos sobre *An. darlingi*, los extractos acuosos y metanólicos resultaron ser activos. En cuanto a las larvas de *Ae. aegypti*, fueron más susceptibles al extracto acuoso.

**Palabras clave:** Clavo; *Syzigium aromaticum*; Actividad insecticida; Paludismo; Dengue.

## 1. Introduction

Mosquitoes are the main vectors responsible for disease transmission within medical entomology, and the Culicidae family is the one that has attracted the most public health attention. This is due to the fact that this family is involved in the transmission of multiple infections to humans and domestic animals (Forattini, 2002). Several species belonging to the genera *Aedes*, *Anopheles* and *Culex* are the vectors of various pathogens of various diseases such as dengue, Zika, chikungunya, yellow fever, malaria, encephalitis, filariasis, etc. (Borah et al., 2012). Some diseases transmitted by these vectors have become naturally transmitted in urban or peri-urban areas, due to the emergence or re-emergence of their vectors in these areas, classic examples being dengue, visceral leishmaniasis and malaria (Tauli, 2006). Malaria and dengue are more prevalent in tropical and subtropical countries, where the hot and humid climate favors the proliferation of *Anopheles* and *Aedes* mosquitoes (WHO, 2020).

Dengue is an infectious disease transmitted by a mosquito of the genus *Aedes*. The main vector is the *Aedes aegypti* (Forattini, 2002). The disease presents itself in more severe forms such as dengue hemorrhagic fever, dengue shock syndrome, or with unusual manifestations such as central nervous system involvement, according to the World Health Organization (WHO, 2023), around two-fifths of the world's population is at risk of contracting dengue. In Brazil, dengue, Zika and chikungunya are responsible for annual epidemic cycles. In 2023, more than 1.6 billion dengue infections were recorded in Brazil, accounting for 65% of all infections in South America. In addition, there were more than 49,000 infections caused by chikungunya, while there were 2,700 notifications of Zika (Brasil, 2023).

The rural and peri-urban areas of the nine states that make up the Brazilian legal Amazonia are drastically affected by malaria, an endemic disease in the Amazonia region, which in 2021 caused the infection of 145,000 people. Malaria is an acute or chronic infectious disease caused by protozoan parasites of the genus *Plasmodium*. It is transmitted by mosquitoes of the genus *Anopheles*, the main vector of which in the Amazonia is *Anopheles darlingi*. Malaria is a public health problem in more than 100 countries and around 3 million people die each year, 90% of them in Africa. It affects more than 500 million people every year (WHO, 2023). The Ministry of Health faces difficulties in managing diseases caused by mosquito vectors, because the vector of malaria, *An. darlingi*, and the vector of dengue, Zika and chikungunya, *Ae. aegypti*, have a rapid capacity for adaptation.

Another difficulty is anthropogenic and environmental changes, which alter the rainfall regime, increasing the availability of natural breeding sites for *An. darlingi* and artificial ones for *Ae. aegypti*, increasing the population density of these mosquitoes and, consequently, the transmission of dengue throughout Brazil and malaria specifically in the Amazonia region. Associated with these difficulties are also social factors, such as inefficient basic sanitation, problems with medical care, especially in locations far from urban centers, and biological factors, such as the resistance that *An. darlingi* and *Ae. aegypti* mosquitoes have to various synthetic insecticides, which are still used as the main method of control, making it difficult to control the vectors efficiently (Borah et al., 2012).

In this sense, preventive measures to combat mosquitoes should not be neglected, given that malaria and dengue fever are of great importance to public health. Vector control remains the most widely used method for preventing the transmission of mosquito-borne diseases. Among the control methods is the use of chemical insecticides, such as pyrethroids, carbamates and organophosphates. This is one of the most widely used approaches to controlling mosquito populations, as it reduces the number of cases of the diseases they transmit (Adhikari et al., 2022). These synthetic insecticides, although highly effective for the target species, are promoting the development of resistance through constant use, hampering efforts to achieve worldwide control (Nascimento & Melnyk 2016; Adhikari et al., 2022). In addition, these insecticides have a high toxicity to humans and other non-target organisms, as well as a high potential for contaminating soil and water (Mossa et al., 2018).

Therefore, in order to overcome these problems, it is necessary to look for alternative methods of mosquito control that are environmentally safe. In this context, insecticides of botanical origin have emerged as a cheap and environmentally friendly alternative to synthetic insecticides, due to their low toxicity to human health and the environment and their ability to interfere with the development of the vector, keeping it at low population levels (Benelli, 2015; Pavela & Benelli 2016). This study investigated the larvicidal activity of the species *Syzigium aromaticum* (Myrtaceae), popularly known as cloves. The great importance of cloves is the presence of eugenol, a monoterpene, the main constituent of the essential oil (Chaieb et al., 2007; Adhikari, 2022). Products obtained from cloves have been shown to be effective on a wide variety of pests, including mosquitoes (Obeng-Ofori et al., 1997; Huang et al., 2019).

In order to assess the toxicity of the methanolic and aqueous extracts of *S. aromaticum*, a study was carried out to determine the lethal concentration (LC<sub>50</sub>) in *An. darlingi*, the malaria vector, and *Ae. aegypti*, the dengue, Zika and chikungunya vector, under laboratory conditions.

## 2. Methodology

### Obtaining clove flower buds

The extracts used in this study were taken from flower buds purchased at the Adolpho Lisboa Municipal Market in Manaus, Amazonia.

### Preparing the aqueous extract

To obtain the aqueous extract (AE), 60g of clove flower buds were added to 300 mL of distilled water. The mixture was then ground in a domestic blender for approximately two minutes and filtered through a fabric strainer. The resulting solution was stored in glass recipients.

### Preparation of the methanolic extract

320g of clove flower buds were used, the ground plant material was extracted with methanol in a soxhlet apparatus (solid-liquid extraction) for 3 times (3 × 6h), adding new solvent after each extraction period, for a total of 18h. The extracts were combined and evaporated. Subsequently, the methanolic extract (ME) was stored in a glass bottle in a refrigerator at 4 °C.

### **Rearing the larvae used in the bioassays**

The larvae used in the bioassays were reared in the insectary of the INPA Malaria and Dengue Vector Laboratory, at a temperature of  $26 \pm 2$  °C, relative humidity of over 85% and 12-hour photophase (Scarpassa & Tadei, 1990).

#### ***Aedes aegypti* larvae**

The eggs were obtained from colonies maintained at the INPA Malaria and Dengue Vector Insectarium, where the winged mosquitoes mated and oviposited in 50 mL plastic cups. These cups contained 20 mL of water and the sides were lined with a strip of filter paper, measuring 3 cm high by 22 cm long. The strips of filter paper containing adhered eggs were dehydrated and stored in the insectary. To obtain new larvae, these filter paper strips were transferred to enameled containers containing artesian well water.

In the containers, the larvae were reared until the third stage of larval development and fed a mixture of fish meal and liver powder (8:1) diluted in 1000 mL of distilled water. The food was added to these containers every two days, when the water in the containers was changed. This procedure was repeated until the larvae reached the third stage. The larvae that reached the pupal stage were transferred to a plastic cup and placed in the cages to allow the adult mosquitoes to emerge. In the cages, the males were fed a 10% sucrose solution soaked in absorbent cotton. In addition to the solution, the females received a blood meal (twice a week), in which case a hamster (*Mesocricetus auratus*) was used.

#### ***Anopheles darlingi* larvae**

Similarly, the bioassays with *An. darlingi* were carried out using third-stage larvae obtained from periodic collections of adult female mosquitoes in the field, given the difficulty of maintaining colonies of this species in an insectary. Collections were made in the municipality of Coari and Cacao Pirera (Amazonia). The *Anopheles* females were captured before being fed blood and fed *Gallus gallus* blood in the laboratory. The engorged females were placed to lay eggs in individual covered cups lined with damp filter paper to prevent the eggs from drying out. After oviposition, on average three to five days, the spawn was transferred to containers with distilled water and liquid food. The *Anopheles* species were identified using the identification key of Consoli & Lourenço-de-Oliveira (1994), separating only the *An. darlingi* species.

### **Bioassays**

The bioassays followed the protocol of the World Health Organization (WHO, 2005) and the criteria of Dulmage et al., (1990). The bioassays were conducted at the Malaria and Dengue Vector Laboratory at INPA, at an ambient temperature of  $26 \pm 2$  °C and relative humidity of over 85%. A stock solution was prepared for each extract. For this, the aqueous (20 mg) and methanolic (20.2 mg) extracts were solubilized in 600 and 603  $\mu$ L of dimethylsulfoxide solvent (DMSO, Dinâmica®), respectively. Initially, preliminary tests were carried out to test the toxicity of cloves against *Anopheles darlingi* larvae. Bioassays were carried out with four 50 mL beakers, each containing 20 mL of distilled water, eleven third-stage *An. darlingi* larvae and the concentrations of the methanolic extract. There were no replicates in this test, as it was a selective test. After applying the extract, readings were carried out after 24, 48 and 72 hours.

In the dose bioassays with AE and ME against *Ae. aegypti* larvae, replicates of five plastic cups with a capacity of 180 mL were prepared for each concentration, containing: 100 mL of distilled water, 1 mL of liquid food, 20 third-stage larvae and the concentration of the extracts (500, 250, 125, 60 and 30  $\mu$ g/mL). The bioassays with AE and ME against *An. darlingi* larvae were set up in the same way as described above, however, differing only in the volume of 50 mL for the disposable cup. In both tests, after the application of ME and AE, the live and dead larvae were counted at intervals of 24, 48 and 72 hours. In each bioassay, a control replicate was prepared, without the addition of the plant extract, only with DMSO. The control served

as a basis for measuring the natural mortality of the larvae which, according to the criteria established by Dulmage et al., (1990), should not exceed 10%. The bioassays were carried out on three alternate days and the confidence interval was 95%.

### Evaluation of the median lethal concentration (LC<sub>50</sub>)

Based on the larval mortality data obtained in the bioassays, the Median Lethal Concentration - LC<sub>50</sub> was determined, considering a significance level of 95%, calculated using the POLO-PC program (LeOra Software Berkeley, CA). Probit analysis, using dose-response linear regression, was carried out according to Finney (1981) and Haddad (1998).

## 3. Results

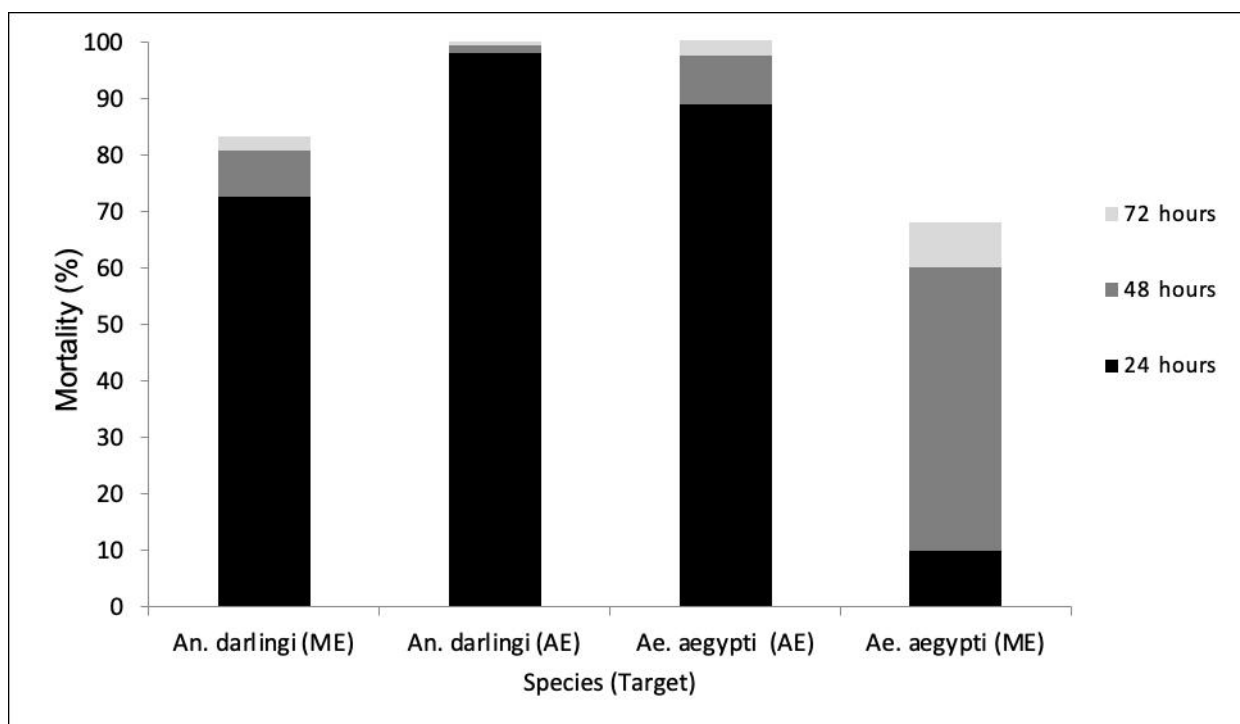
### Selective Bioassays

The selective tests with ME against *An. darlingi* larvae caused 73% mortality at a concentration of 500 µg/mL in 24 hours, with the 72-hour reading showing total larval mortality. At a concentration of 250 µg/mL, 82% of the larvae were killed in 24 hours, with equivalent mortality values being maintained throughout the three readings. At the next concentration of 125 µg/mL, the highest larval mortality was 36% at 72 hours.

### Dose bioassays

The larvicidal activity of AE and ME on the target species is shown in Figure 1. The highest percentage of mortality of *An. darlingi* larvae occurred in the first 24 hours, with 98% mortality for AE. ME showed 72% larval mortality within 24 hours. For the *Ae. aegypti* the highest rate was at 24 hours with 89% of larvae dead in the AE, while at 72 hours mortality reached a percentage of 3%, and for the ME larval mortality was 10%, 50% and 8% at 24, 48 and 72 hours, respectively.

**Figure 1** - Graphical representation of mortality of *Anopheles darlingi* and *Aedes aegypti* larvae in bioassays with methanolic and aqueous extracts at 24, 48 and 72 hours of contact per target species.



ME– Methanolic extract; AE - Aqueous extract. Source: Authors (2023).

Considering the total number of dead larvae observed in the bioassays, it was found that in those carried out with EA and EM, the greatest impact of mortality occurred in the first 24 hours, reaching percentages of over 90% in the bioassays carried out with *An. darlingi* larvae.

#### Median Lethal Concentration – LC<sub>50</sub>

The results of the calculation of the Median Lethal Concentration (LC<sub>50</sub>) obtained for the bioassays with the ME and AE of cloves are shown in Table 1. Initially, considering the bioassays carried out with *An. darlingi*, LC<sub>50</sub> values equal to 6.41 µg/mL were observed for ME and LC<sub>50</sub> equal to 5.65 µg/mL for AE. Considering the bioassays with *Ae. aegypti* larvae (AE), the LC<sub>50</sub> value was 78.81 µg/mL and ME showed a LC<sub>50</sub> of 131.10 µg/mL.

**Table 1** - Median Lethal Concentration values (LC<sub>50</sub> µg/mL) obtained in bioassays with plant extracts on *Anopheles darlingi* and *Aedes aegypti* larvae after 24 hours.

Target species	Extracts	n	24h (CI 95%) µg/mL CL <sub>50</sub>
<i>Anopheles darlingi</i>	Methanolic	600	6.41 (5.66 – 7.30)
	Aqueous	600	5.65 (2.32 – 9.87)
<i>Aedes aegypti</i>	Methanolic	3.300	131.10 (95.63 – 286.15)
	Aqueous	3.300	78.81 (60.05 – 89.44)

n = Number of larvae tested; CI = Confidence Interval. Source: Author (2023).

Comparing the two mosquito species, *An. darlingi* was more susceptible to AE and ME from cloves. However, when comparing the activity of the extracts against *An. darlingi*, it was found that AE was more effective than ME. With regard to *Ae. aegypti* larvae, they were more susceptible to AE.

## 4. Discussion

A similar study was carried out by Rodrigues et al., (2019) with the methanolic extract of clove flower buds against *Ae. aegypti* and *Ae. albopictus* larvae, finding a LC<sub>50</sub> value of 138.10 ppm/mL for *Ae. aegypti* larvae. Thongwat et al., (2017) tested the aqueous extract of *Dracaena loureiri* with *Ae. aegypti* larvae and obtained a LC<sub>50</sub> value of 1,067.53 ppm/mL, which is 12 times higher than the value presented in this study. Ali et al., (2017) evaluated the methanolic extract of the flower of *Juglans regia* against *Ae. aegypti* larvae and obtained a LC<sub>50</sub> value of 77.87 ppm/mL, corroborating the results presented in this study with a LC<sub>50</sub> value of 78.81 µg/mL. Kamaraj et al., (2018) tested the methanolic extract of *Lycopodium clavatum* against *Ae. aegypti* larvae and obtained a LC<sub>50</sub> value of 388.66 ppm/mL, which is twice as high as the LC<sub>50</sub> obtained, which was 78.81 µg/mL. Carneiro et al., (2021) demonstrated the larvicidal activity of the methanolic extract of *Eugenia astringens* leaves against *Ae. aegypti*, obtaining a LC<sub>50</sub> value of 23.58 ppm/mL, five times lower than that presented in this study (LC<sub>50</sub> 131.10 µg/mL). Studies carried out by Yadav et al., (2019) with the aqueous extract of *Piper longum* leaves against *Ae. aegypti* showed a LC<sub>50</sub> equal to 1,685.6 µg/mL, this value differs from the value presented in the present work (LC<sub>50</sub> of 78.81 µg/mL), showing that the aqueous extract of *Syzigium aromaticum* is more active.

Regarding the results with *An. darlingi* larvae, Benelli et al., (2018) evaluated the oil extracted from *Syzygium lanceolatum* leaves against *An. stephensi* and *An. subpictus* larvae, finding LC<sub>50</sub> values equal to 51.20 ppm/mL and LC<sub>50</sub> 61.34 ppm/mL, when compared with the LC<sub>50</sub> values of the aqueous extract (5.65 µg/mL) and the methanolic extract (6.41 µg/mL) against *An. darlingi* larvae in the present work, we can conclude that it was less efficient. Yadav et al., (2019) evaluated the



aqueous and methanolic extract of *Piper longum* leaves against *An. stephensi* larvae, showing LC<sub>50</sub> values equal to 2,465.33 µg/mL and LC<sub>50</sub> 1,508.41 µg/mL, respectively. These results differ from those presented in the present study, as the methanolic extract (LC<sub>50</sub> 6.41 µg/mL) and the aqueous extract (LC<sub>50</sub> 5.65 µg/mL) proved to be active against *An. darlingi* larvae.

The results of the bioassays using *An. darlingi* larvae showed that the extracts were highly active, with a LC<sub>50</sub> value of < 50 µg/mL. The activity of the extracts was classified as efficient. As for the results with *Ae. aegypti* larvae, the methanolic extract was considered efficient, and the aqueous extract was moderately active. The criteria used to classify botanical substances as insecticides of plant origin have yet to be determined, but their activity is defined through tests comparing LC<sub>50</sub> values with those of synthetic insecticides. In this work we used the criteria of Komalamisra et al., (2005), who classify botanical insecticides as active (LC<sub>50</sub> < 50 µg/mL), moderately active (LC<sub>50</sub>< 100 µg/mL), efficient (LC<sub>50</sub> < 750 µg/mL) and inactive (LC<sub>50</sub>> 750 µg/mL).

## 5. Conclusion

The results of this study demonstrate the larvicidal effect of clove extracts against *An. darlingi* and *A. aegypti*. The methanolic extract showed toxicity to *Ae. aegypti* larvae, but the aqueous extract was the most effective for controlling the larvae of this mosquito. In relation to *An. darlingi*, it was also found that the extracts showed larvicidal activity, and it was evident that the methanolic and aqueous extracts were active against *An. darlingi* larvae, showing that they are promising. Thus, based on the results obtained in this study, the extracts from the flower buds of the clove tree are an alternative for product development studies with insecticidal action against both mosquitoes.

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