



## Larvicidal effect of hydroethanolic extract from the leaves of *Acmella oleracea* L. R. K. Jansen in *Aedes aegypti* and *Culex quinquefasciatus*

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### ABSTRACT

Mosquitoes, such as *Aedes aegypti* and *Culex quinquefasciatus*, are important vectors of diseases, such as dengue fever, chikungunya fever, Zika virus, and filariasis, and these diseases are public health problems. The present study was carried out to evaluate the larvicidal activity of the hydroethanolic extract from leaves of *Acmella oleracea* leaves against 3rd instar larvae of the *Ae. aegypti* dengue vector and the *Cx. quinquefasciatus* filariasis vector. The hydroethanolic extract caused significant mortality in *Ae. aegypti* and *Cx. quinquefasciatus*. After 24 h of exposure to the extract, it was possible to establish the LC<sub>50</sub> values for the extract: 11.41 ppm for *Ae. aegypti* and LC<sub>50</sub> 32.40 ppm for *Cx. quinquefasciatus*. The hydroethanolic extract from leaves of *A. oleracea* showed very low ecotoxicity suggesting that it can be used without causing environmental damage. This is the first study that shows the use of hydroethanolic extract from leaves of *A. oleracea* as an alternative to synthetic larvicides to eliminate larvae of *Ae. aegypti* and *Cx. quinquefasciatus* in an easy, cheap and safe way.

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### 1. Introduction

Mosquito-borne diseases are still of significant concern, considering the high mortality and morbidity of these diseases among underdeveloped and developed countries (Carvalho and Moreira, 2017). In Brazil 925 people died due to diseases transmitted by *Aedes aegypti*, such as dengue, chikungunya and, more recently, Zika virus, in 2016 (Brazil, 2017); *Ae. aegypti* is the main vector of these diseases.

*Culex quinquefasciatus* is the main vector of *Wuchereria bancrofti*, an agent responsible for lymphatic filariasis, which is an important cause of acute and chronic morbidity (WHO, 1997; Brazil, 2011) and has recently been reported to have the potential for expressive transmission of the Zika virus through *Culex pipiens quinquefasciatus* (Guo et al., 2016). The control of *Cx. quinquefasciatus* is based on breeding prevention measures and the elimination of breeding places through improvements in basic sanitation or the use of larvicides (WHO, 1997).

It is therefore clear that the best way to prevent these diseases is to control their vectors through the use insecticides and larvicides; however, the uncontrolled and rampant use of these products, directly or indirectly, makes resistant populations of mosquitoes and many of

these substances used are considered harmful to the environment (Nkya et al., 2013; Belinato and Valle, 2015).

The search for alternatives to synthetic insecticides stimulates the development of new technologies. In the Amazon, due to its rich biodiversity, oils, extracts, or active constituents from certain plants are being exploited for their uses as bioactive products (Guissoni et al., 2013).

Among several species, *Acmella oleracea* (L.) R. K. Jansen, popularly known as jambu, stands out. It belongs to the Asteraceae family, a small herbaceous plant with creeping and branching stems (Cardoso and Garcia, 1997; Barbosa et al., 2016). Jambu leaves and stalks are used in local cuisine in the Amazon (Cardoso and Garcia, 1997).

Jambu is rich in bioactive isobutylamides; the major molecule this species is the alkaloid (2E,6Z,8E)-N-Isobutyl-2,6,8-decatrienamide, known as spilanthol (Gilberto and Favoreto, 2010). The presence of this substance and its derivatives gives the plant potential in the pharmaceutical, food, and health industries, with its best-studied property being its anesthetic activity (Pandey and Agrawal, 2009). Interestingly, it also has insecticidal activity via spilanthol against *Periplaneta americana* L. (Kadir et al., 1989), *Plutella xylostella* (Sharma et al., 2012), and *Tuta absoluta* (Moreno et al., 2012).

The objective of this study was to evaluate the larvicidal activity of the *A. oleracea* hydroethanolic extract (of the leaves) against *Ae. aegypti* and *Cx. quinquefasciatus* due to easy access and procurement of jambu in

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a tropical region, as such Brazil. It also evaluated ecotoxicity by the fungus *Trichoderma* ssp. and antioxidant activity of the *A. oleracea* hydroethanolic extract.

## 2. Material and methods

### 2.1. Plant and larvae

The leaves of the jambu were collected in March 2017 in the Fazendinha District (S 02°30.40/W 5106'37.5), Macapá-AP. The species was identified by Professor Rosângela Sarquis and deposited in the Herbarium IAN of Embrapa Amazônia Oriental under numbering: 196011.

For the larvicidal test, we used third instar larvae of *Ae. aegypti* Rockefeller and *Cx. quinquefasciatus* Macapá strain from the Arthropoda Laboratory of the Federal University of Amapá. The assay was conducted under controlled conditions, with temperatures between  $25 \pm 2$  °C, relative humidity of  $75 \pm 5\%$ , and a photoperiod of 12 h (Fig. 1).

### 2.2. Preparation of the hydroethanolic extract of *A. oleracea*

The leaves of *A. oleracea* were dried at room temperature for 10 days, triturated, and stored. Subsequently, 74 g of crushed leaves was weighed and placed under maceration for 10 days using ethyl alcohol (70%) (1.5 L) as the solvent, and then the solution was filtered and excess solvent was subjected to rotary evaporation under reduced pressure and thereafter lyophilized.

### 2.3. Gas chromatography–mass spectrometry (GC–MS)

We evaluated the samples using a gas chromatograph (GCMS-QP 2010) equipped with an auto-sampler injection system (AOC-20i, Shimadzu). The following settings were used: electron impact detection

(Shimadzu MS2010 Plus), electronic impact of 70 eV, and fragments detected from 50 to 400 Da. Separations were performed on a fused silica capillary column (RTX-5MS with i.d. = 0.25 mm, length = 30 m, and film thickness = 0.25  $\mu$ m) in a stream of helium (1.03 mL/min). The sample was solubilized in dichloromethane (2  $\mu$ g/mL) and 1.0  $\mu$ L of the solution was subjected to the following experimental conditions: injector temperature, 210 °C; detector temperature, 250 °C; carrier gas, helium; flow rate, 3.0 mL/min; and split injection with a split ratio of 1/10. The column temperature was programmed from 80 °C, with an increase of 6 °C/min, to 250 °C, ending with a 5-min isothermal step at this temperature; the total analysis time was 35.33 min.

### 2.4. Larvicidal activity

The extract were dissolved in dimethylsulfoxide (DMSO) at different concentrations (15, 12.5, 10, 7.5, 5, and 2.5 ppm) for *Ae. aegypti* and at (40, 30, 20, 10, and 5 ppm) for *Cx. quinquefasciatus*. Five replicates were carried out with ten larvae each. Negative controls contained distilled water containing the same amount of DMSO (1%) present in the respective test sample. The larval mortality rate was determined after 24 h of incubation. Larvae were considered dead when they did not respond to stimuli or did not rise to the solution surface, in contrast to those observed in the control. The bioassay experiments were conducted according to the WHO standard (2005).

### 2.5. Morphological study of larvae

After treatment, larvae were fixed in formalin (10%) and the external morphology was analyzed under light microscopy (Output DC 6 V/20 W) and photographed with a digital camera (MDCE-SC USB 2.0) with ScopelImage 9.0 software.

### 2.6. Antioxidant activity

The antioxidant test was performed using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). The procedure consisted of preparing a stock solution of DPPH in ethanol according to the methodology of Melagraki et al. (2009), with modifications, and the final solutions had a concentration of 0.05 mmol DPPH and 16, 31, 63, 125, 190, and 250  $\mu$ g/mL of extract.

The mixture was stirred at 450 rpm for 30 min and kept in an environment without light at room temperature. The analysis was performed using a spectrophotometer (UV–VIS Shimadzu) with each sample containing 1.0 mL of the extract at the concentration to be tested and 1.0 mL of ethanol (Borges and Castle, 2015; Malki et al., 2017). The experiment was carried out in triplicate.

The antioxidant activity index (AAI) was calculated according to the method of Scherer and Godoy (2009).

### 2.7. Isolation of the filamentous fungus *Trichoderma* ssp

The fungus used in this study was obtained from the Brazil nut (*Bertholletia excelsa*). To obtain the isolates, the malt extract medium (2%) was treated with the antibiotic chloramphenicol. Isolates from Brazil nut urchins were obtained by surface scraping of the structures of the microorganisms. After the isolation procedure, the petri dishes containing the isolate were transferred to a BOD greenhouse incubator with an adjusted photoperiod of 12 h and temperature of  $31 \pm 1$  °C. After 7 days of incubation under the conditions described above, the colony and morphological structure (conidiophores and conidia) of the isolate were evaluated for the identification of *Trichoderma* at the genus level, based on the morphological keys of the sections and species developed by Gams and Bissett (1998).



Fig. 1. Image of the leaves of *A. oleracea* collected for later drying and production of the extract.

## 2.8. Activity of *Trichoderma ssp*

The fungi were grown in solid BDA medium, where 20 g of agar and 20 g of dextrose were weighed and added to distilled water and potato broth. The pH of the medium was corrected to 7.0 with the aid of NaOH (0.1 M) and HCl (0.1 M). After autoclaving, 32.40 ppm of the solution of the jambu extract, was impregnated in the culture medium; DMSO (1%) was used as a control. The test was performed in triplicate. Growth was observed at 24, 48, 72, and 96 h. Inhibition of mycelial growth (PIC) was calculated by Equation: (%) PIC =  $\frac{RGC - RGT}{RGC} \times 100$ , where RGC = radial growth of control (cm) and RGT = radial growth of treatment (cm) (Vale et al., 2011).

## 2.9. Statistical analysis

Lethal concentrations (LC<sub>50</sub> and LC<sub>90</sub>) were determined after 24 h of incubation and calculated using Probit analysis with StatGraphics Centurion XV software, version 15.2.11. If the control mortality of the treated groups was between 5 and 20%, the analysis was corrected according to the WHO (2005) formula: mortality (%) =  $\frac{X - Y}{X} \times 100$ , where X = percentage survival in the untreated control and Y = percentage survival in the treated sample.

## 3. Results

### 3.1. Gas chromatography–mass spectrometry (GC–MS)

The presence of four major substances was observed by gas chromatography (Fig. 2). Among the compounds found in the extract, the majority was spilanthalol (1), followed by linoleic acid (3), and palmitic acid (2), respectively in the forms of ethyl ester and octadecanamide (4).

### 3.2. Larvicidal activity of *A. oleracea* extract

The larvicidal bioassays for *Ae. aegypti* showed 58% mortality in 24 h at a concentration of 15 ppm and 18% at the lowest concentration (2.5 ppm) (Fig. 3). This demonstrates a promising result, considering that the concentrations are low.

In the tests for *Cx. quinquefasciatus*, no mortality was observed for the concentration of 15 ppm, in this way the concentration was increased to 40 ppm where it was possible to observe the mortality 54% after the period of 24 h in exposure to the extract and of 12% for the (5 ppm) in the same period.

The LC<sub>50</sub> and LC<sub>90</sub> values for *Ae. aegypti* in 24 h were 11.41 ppm and 23.23 ppm, respectively (Table 1), with a *p* value <0.05 (0.0124). The LC<sub>50</sub> and LC<sub>90</sub> values for *Cx. quinquefasciatus* were 32.40 ppm and 68.24 ppm, respectively, with a *p* value <0.05 (0.0148), thus demonstrating that the higher the concentration, the greater the mortality and efficiency of the extract of *A. oleracea* for larval control.

### 3.3. Analysis of larval morphology after 24 h

In the obtained optical microscope images, the larvae of *Ae. aegypti* (Fig. 3A–C) have normal morphological segments (head (H), thorax (TH), and abdomen (AB)) and no changes were observed in their cuticle, respiratory tract (S), or anal papillae. However, the larvae treated with 15 ppm of extract showed, after 24 h (Fig. 4d–f), a discoloration that started from the thorax and continued to the end of the abdomen.

When observing the larvae of *Cx. quinquefasciatus* (Fig. 5a–f), it is possible to verify that both the larvae in the control and in the treatment did not present alterations in the external structure, making it possible to observe the division of the segments, the presence of the bristles, and the siphon and papilla without apparent changes.

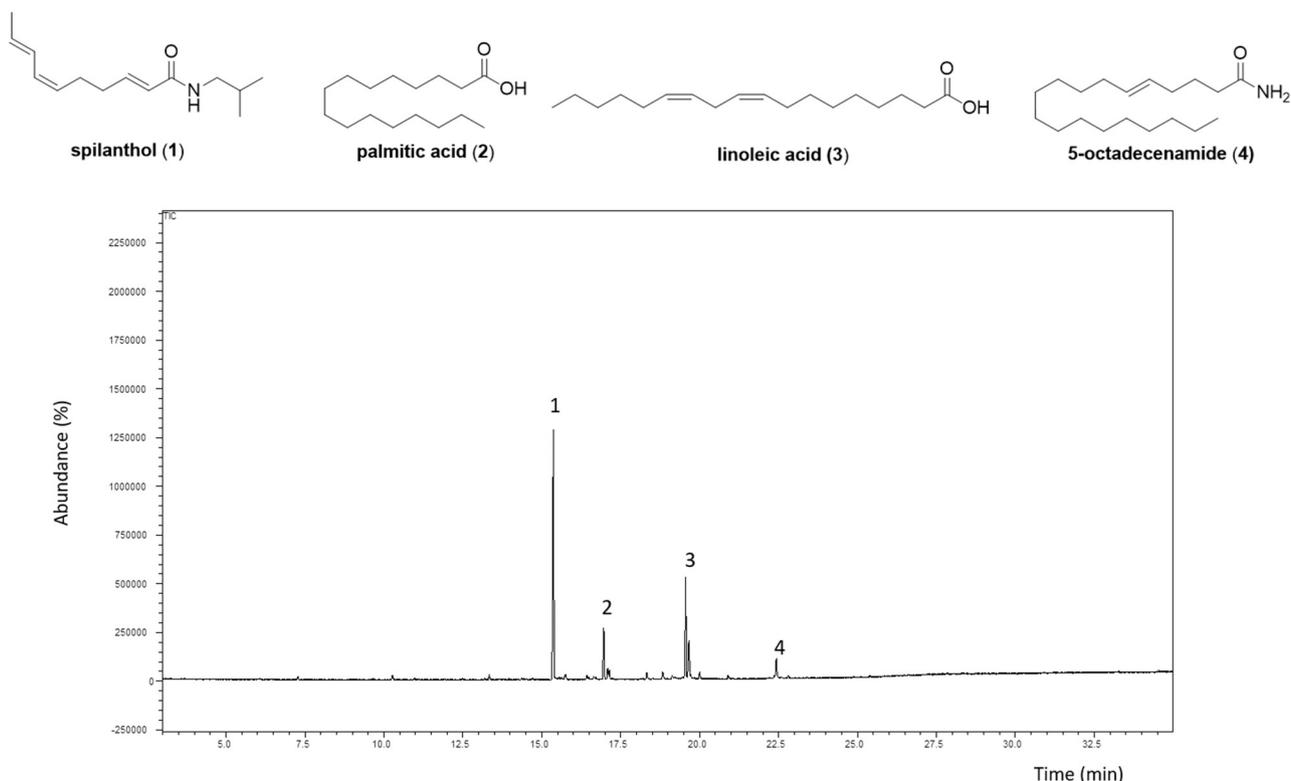


Fig. 2. Major compounds of the hydroethanolic extract from leaves of the *A. oleracea*.

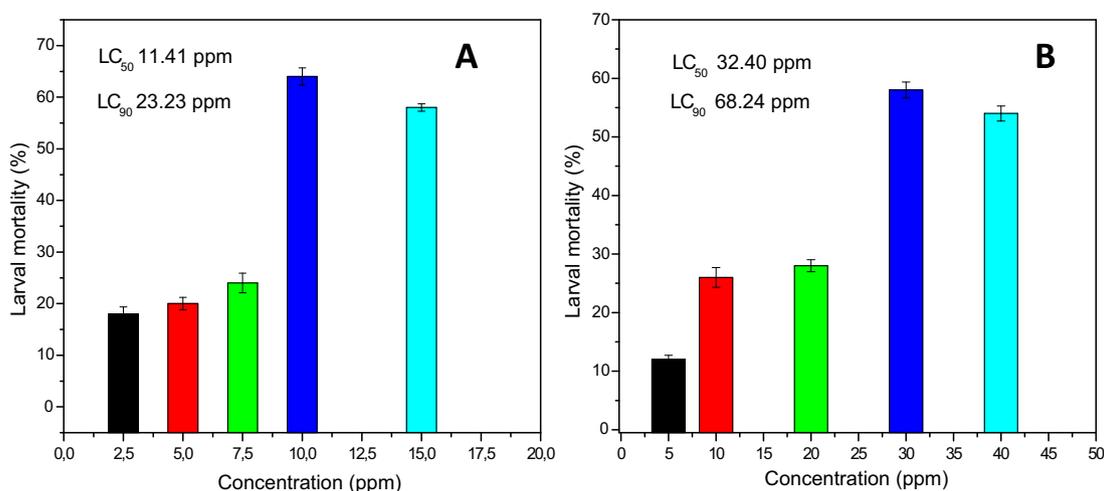


Fig. 3. Larvicidal activity of *Acemella oleracea* hydroethanolic extract in *Aedes aegypti* (A) and *Culex quinquefasciatus* (B) larvae at 24 h.

#### 3.4. Antioxidant activity

The hydroethanolic extract showed low antioxidant activity, based on the AAI: 0.15 (AAI <0.5); this amount could inhibit 90% of the DPPH (0.5 mmol/mL) and presented an IC<sub>50</sub> value of 130.0 µg/mL.

#### 3.5. Activity fungus *Trichoderma* spp

The study showed low values for %PIC 1.78. This result indicates that the hydroethanolic extract from leaves of *A. oleracea* did not induce significant toxic responses on test-microorganisms, fungus *Trichoderma* spp., in 96 h for concentration of 32.40 ppm.

### 4. Discussion

The compound (2*E*,6*Z*,8*E*)-*N*-Isobutyl-2,6,8-decatrienamamide (spilanthol) was identified as the major substance present in the hydroethanolic extract of jambu leaves. The identification of spilanthol by electron impact (70 eV) showed the appearance of two characteristic fragmentation signals, resulting from hemolytic C—C bond cleavage, at *m/z* = 81 (100%) and *m/z* = 141 (72%) (Hiserodt et al., 2004).

In this study, the hydroethanolic extract of *A. oleracea* showed an LC<sub>50</sub> of 11.41 ppm after 24 h for *Ae. aegypti*, unlike the results described by Simas et al. (2013), who studied the crude ethanolic extract of the leaves of *A. oleracea* and observed an LC<sub>50</sub> value of 251 ppm overall and an LC<sub>50</sub> value of 145 ppm in the hexane partition. Notably, the chemical composition of the metabolites can be influenced by the development site, seasonality, age, temperature, water stress, ultraviolet radiation, mechanical factors, and pathogen attack of the product (Gobbo-Neto and Lopes, 2007).

The hydroethanolic extract of the leaves of *A. oleracea* collected in the district of Fazendinha — Macapá was effective for killing the larvae of *Ae. aegypti*, required only a low dose to be effective, was easy to

prepare, and was cheap. It is noteworthy that the presence of the 2*E*-type unsaturated bonds present in alkanolamines is associated with insect toxicity (Jacobson, 1954).

In contrast, other studies reported insecticidal activity from *Clausena anisata* extract with an LC<sub>50</sub> value of 59.65 ppm in 24 h (Mukandiwa et al., 2015), *Xanthium strumarium* seed extract with an LD<sub>50</sub> of 531.07 ppm against *Aedes caspius* and 502.32 ppm for *Cx. pipiens* larvae (Mekhlafi et al., 2017), and methanolic extract from the leaves of *Crataeva magna* with LC<sub>50</sub> values of 121.69, 132.09, and 147.27 ppm for *Anopheles stephensi*, *Ae. aegypti*, and *Cx. quinquefasciatus*, respectively, at 24 h of exposure (Veni et al., 2016).

The insecticidal activity of spilanthol isolated from the *Spilanthes acmella* extract for *P. americana* L. showed high activity against the adults of this species, with an LD<sub>50</sub> value of 2.46 ppm; electrophysiological experiments suggested that spilanthol interferes in the nervous system (Kadir et al., 1989). A study of the *S. acmella* flower head extract also showed activity against the 2nd instar larvae of *P. xylostella*, presenting LC<sub>50</sub> values of 1.49, 5.14, and 5.04 ppm for spilanthol, the hexanic extract, and the methanolic extract, respectively (Sharma et al., 2012).

In the study by Pandey et al. (2007), the hexanic extract of the flowers of *S. acmella* L. var. *oleraceae* Clarke showed variable mortality for the larvae of three species of vectors: *A. stephensi*, *Anopheles culicifacies*, and *Cx. quinquefasciatus*, with LC<sub>50</sub> values of 4.57 ppm, 87 ppm, and 3.11 ppm, respectively.

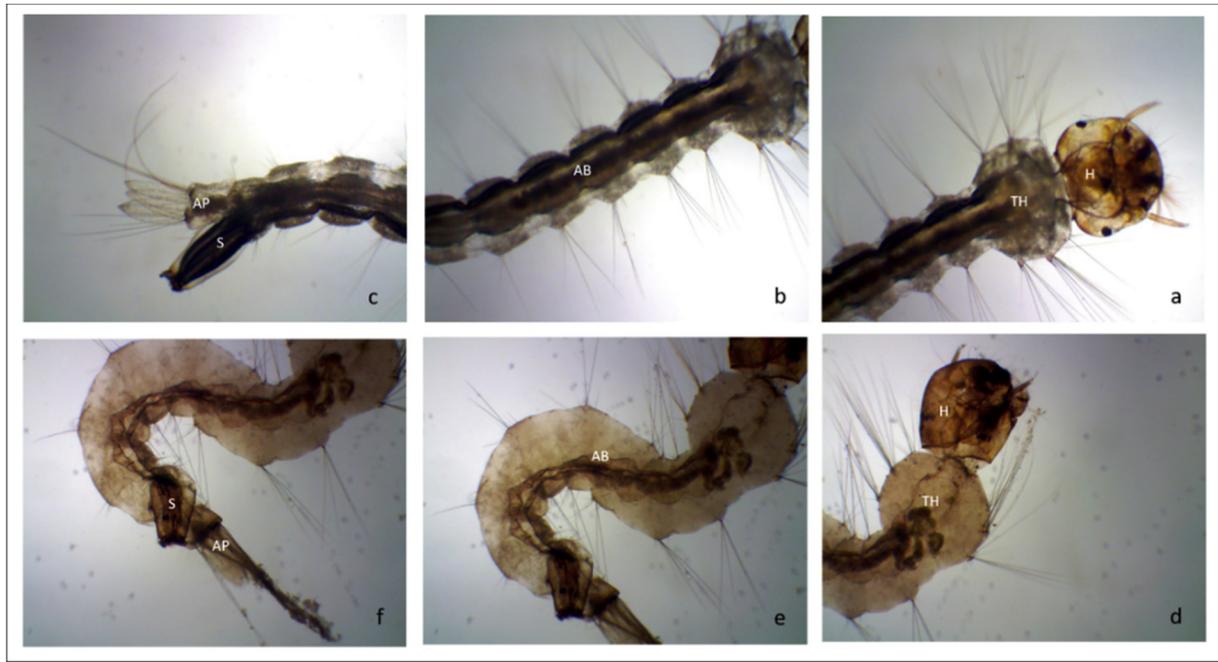
In the tests carried out in this work, the LC<sub>50</sub> value for *Cx. quinquefasciatus* was 32.40 ppm; however, the solvent used and the part of the plant used to determine this activity were different in both works, and therefore the larvicidal actions of the extracts are more difficult to compare. It is also worth mentioning that ethanol was used as the solvent in this work and not hexane; ethanol is less toxic to the environment than hexane, relatively easy to access, less volatile, and, consequently, safer to handle.

Soonwera and Phasomkusolsil (2016), when studying the effect of the oils of *Cymbopogon citratus* and *Syzygium aromaticum* on the morphology of *Ae. aegypti* and *Anopheles dirus*, observed morphological alterations in the larvae in comparison to the control, where they presented deformations in the neck and stretching, as well as loss of siphon; in this work, it was also possible to observe changes in *Ae. aegypti*, where there was a loss in the clarity of the segmentation when compared to the control and a discoloration in the cuticle, suggesting that substances present in the extract may interact with chitin. However, Valotto et al. (2010), did not identify external morphological alterations in the larvae but instead observed the expulsion of the peritrophic matrix to the external environment, containing all of the food, as a means of eliminating the larvicidal substance.

**Table 1**  
Larvicidal activity (LC<sub>50</sub> and LC<sub>90</sub>) for *Aedes aegypti* and *Culex quinquefasciatus* in 24.

<i>A. oleracea</i>	<i>Aedes aegypti</i>	<i>Culex quinquefasciatus</i>
	24 h	24 h
LC <sub>50</sub> <sup>a</sup>	11.41 ppm	32.40 ppm
C.I.	7.98 ± 26.27 ppm	22 ± 84.67 ppm
LC <sub>90</sub> <sup>a</sup>	23.23 ppm	68.24 ppm
C.I.	16.11 ± 86.44 ppm	46.15 ± 284 ppm

<sup>a</sup> LC50 and LC90 in ppm. C.I. = confidence interval.

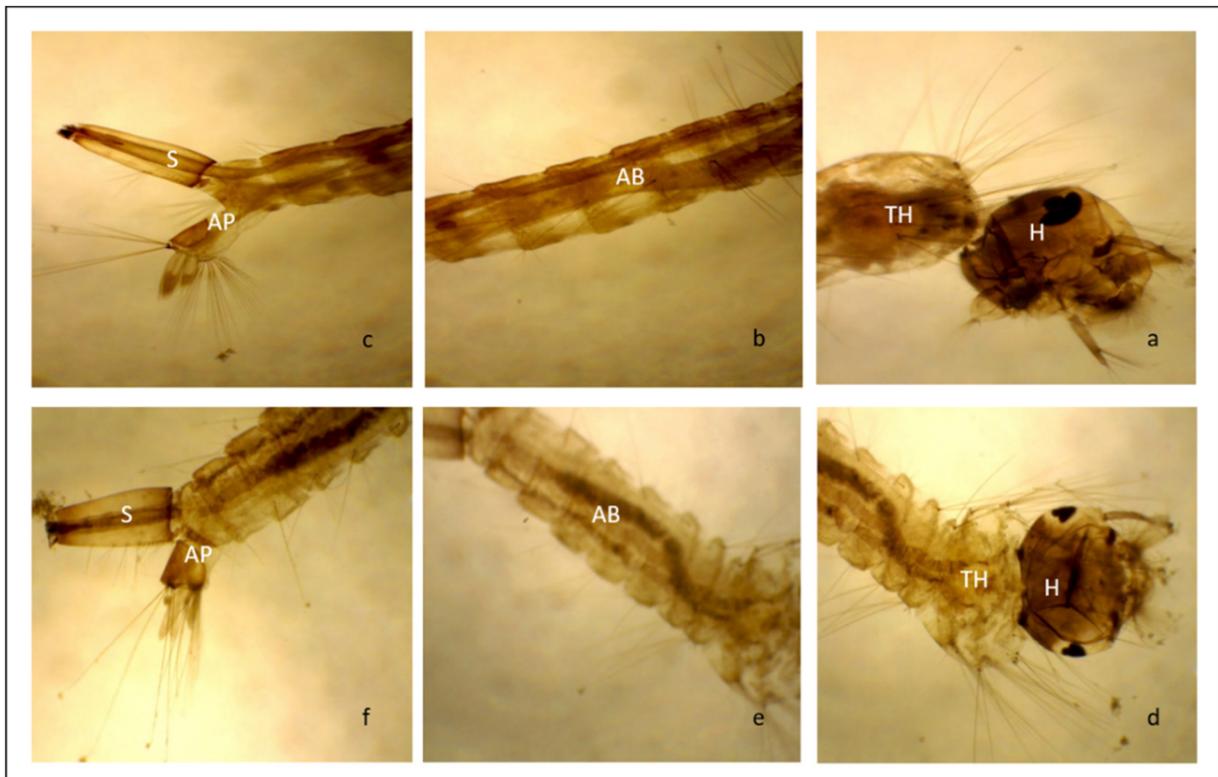


**Fig. 4.** Optical microscope images of *Aedes aegypti* larvae. Control (a–c) not showing changes), head (H), thorax (TH), abdomen (AB), respiratory siphon (S) and papilla anal (AP). Treatment with extract at 15 ppm after 24 h exposure (d–f).

Morphological studies in the larvae of *Cx. quinquefasciatus* also demonstrated alterations caused by a nanoemulsion of *Pterodon emarginatus* in the abdomen, thorax, and anal papillae (Oliveira et al., 2017); however, this effect was not observed in our results, suggesting that the mortality of *Cx. quinquefasciatus* is not related to external damage of the integument. Studies have shown that secondary metabolites with

insecticidal effects may act in different ways, such as inhibiting feeding, regulating growth, or acting on the neuroendocrine system and interfering with tegument exchange and/or metamorphosis (Menezes, 2005; Maciel et al., 2010).

The low antioxidant activity of the *A. oleracea* hydroethanolic extract in this study may be related to fertilizers, the extraction method, the



**Fig. 5.** Optical microscope images of *Culex quinquefasciatus* larvae. It was submitted to *Acnella oleracea* extract at 40 ppm (d–f), head (H), thorax (TH), abdomen (AB), respiratory siphon (S) and anal papilla (AP). Control (a–c) with no changes.

time of collection, and the solvent used. In a previous study, conventional fertilizers helped increase the antioxidant activity and vitamin C concentrations, when compared to organic fertilizers, of *A. oleracea* (Borges et al., 2015). The extraction method is also an important factor because it can interfere with the amount of bioactive compounds extracted; for example, the supercritical extraction by CO<sub>2</sub> that occurs in low temperatures can cause less damage due to the properties of the compounds, when compared to hydrodistillation (Uquiche and Garcés, 2016). This result was also observed by Dias et al. (2012), who obtained a better extraction of non-polar compounds with significant antioxidant capacity, which was associated with the amount of alkylamides present in the extract of *A. oleracea*. In this way, we suppose that the extraction method, or the time of collection of the jambu leaves, may have interfered in the quantification of the compounds that present this activity, considering that studies on the total antioxidant capacity (TAC) of the extract of *A. oleracea* previously showed antioxidant values of  $5.29 \pm 0.85$ ,  $3.42 \pm 0.59$ , and  $1.42 \pm 0.40$  mg/TE/DM/g for leaves, flowers, and stems, respectively (Abeyisiri et al., 2013).

Endophytic fungi that live in association with plants without inducing any visible symptoms of pathogenicity (Dastogeer et al., 2017) may associate with the roots and produce interesting metabolites with applications in agriculture, industry, and pharmaceuticals (Souza et al., 2004).

The study indicates too that hydroethanolic extract from leaves of jambu is an optimal source of nitrogen or carbon for culture of fungus *Trichoderma* ssp. under laboratory incubation condition (pH 7 and  $31 \pm 1$  °C), however field studies would be required to confirm whether the degradation of the jambu extract would be the same, enhanced or reduced in the real situation.

The non-toxicity of the hydroethanolic extract from leaves of jambu for the fungus *Trichoderma* ssp. is promising since it is an endophytic and cosmopolitan fungus, can be present in the soil, and presents as a bioprotector, promoting growth and relieving the biotic and abiotic stress of plants (Mastouri et al., 2010). *Trichoderma harzianum* are also aids in the growth of plants in saline environments, which have higher water content and better photosynthetic performance (Yasmeen and Siddiqui, 2017). Therefore, it is suggested that the hydroethanolic extract from sheets of *A. oleracea* has no toxicity and can be used without damage to the environment.

The hydroethanolic extract of *A. oleracea*, against the larvae of the *Ae. aegypti* and *Cx. quinquefasciatus*, showed a significant result when compared to the adopted literature, mainly because it is a compound of the chemical derivatives and not only of an isolated product.

We must emphasize, however, that the chemical specificity of the species used is directly influenced by the seasonality, leading to the biochemical changes of the main metabolites, both constituent and extractable, as well as the extraction method.

## 5. Conclusions

It is concluded that the hydroethanolic extract from leaves of jambu was more toxic to *Ae. aegypti* larvae (LC<sub>50</sub> 11.41 ppm); consequently, higher selectivity was suggested in the studied concentrations when compared to the effects on *Cx. quinquefasciatus* larvae (LC<sub>50</sub> 32.40 ppm). This is the first study that shows the use of hydroethanolic extract from leaves of *A. oleracea* as an alternative to synthetic larvicides to eliminate larvae of *Ae. aegypti* and *Cx. quinquefasciatus* in an easy, cheap and safe way.

It was observed that the hydroethanolic extract from leaves of jambu has no toxicity and can be used without causing environmental damage.

## Conflict of interest statement

We declare that we have no conflict of interest.

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