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Larvicide potential of genus *Croton* L. (Euphorbiaceae) in biological control of *Aedes aegypti* L. (Diptera: Culicidae)

Vitor Emanuel Sousa da Silva[1*], André Ricardo Ferreira da Silva Rocha[1], Victor Mateus Pinheiro Fernandes[1], Hayla Nunes da Conceição[1] and Eliana Campelo Lago[1,2]

[1]Departamento de Ciências da Saúde, Universidade Estadual do Maranhão, Rua do Morro do Alecrim, 65604-380, Caxias, Maranhão, Brazil. [2]Departamento de Enfermagem, Centro Universitário UniFacid Wyden, Teresina, Piauí, Brazil. *Author for correspondence. E-mail: gaarakasekaque@gmail.com

ABSTRACT. The reemerging diseases caused by *Aedes aegypti* are one of the main public health problems in the world. The control of mosquitoes using larvicidal compounds from products of plant origin is an excellent alternative. This study aims to evaluate the larvicidal potential of fractions in hexane, chloroform, ethyl acetate and hydromethanol from the ethanolic leaf extract of two species of the genus *Croton* L. (Euphorbiaceae) against larval forms of *A. aegypti*, as an alternative tool to control this vector. Dry leaves of *Croton betaceus* Baill. and *Croton lundianus* (Didr.) Müll. Arg. were used for biological tests. The compounds were extracted with ethanol (99.8%). The ethanolic extracts of the leaves were suspended in a methanol / water solution and were successively subjected to the liquid-liquid division process with solvents of different polarities: hexane, chloroform and ethyl acetate, giving rise to the four fractions. Larvicidal tests were performed with the ethanol extract and fractions resulting from the partition. In the study, the crude extract and the fractions showed larvicidal potential, being hexane fraction the one with greatest activity. Mortality in *C. betaceus* fractions was up to 40%. *Croton lundianus* presented mortality of up to 93.33% of the larvae submitted to the test. Data analysis showed larvicidal activity in the crude extract and fractions. The hexane fraction was more effective, especially in *C. lundianus*.

Keywords: plant extracts; public health; reemerging diseases.

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Introduction

In 2018, the World Health Organization [WHO], (2018) considered vector-borne diseases - dengue, filariasis, yellow fever, malaria, leishmaniasis and Chagas disease - as responsible for around 17% of infectious pathologies worldwide, causing up to 700.000 deaths per year (WHO, 2018). Thus, reemerging diseases are one of the main global public health problems, mainly in tropical regions, with Brazil being highly affected (Bhatt et al., 2013; Guirado & Bicudo, 2016).

Dengue, an infectious disease caused by arboviruses, has *Aedes aegypti* as its main vector, and it is constantly expanding. Since its resurgence in Brazil, the main control strategies adopted are educational activities and home inspection in search of breeding sites, besides the application of organophosphate and pyrethroid insecticides in epidemic situations (Freitas, Santos, & Wakimoto, 2019).

The focus of the majority of control programs is to decrease the population density of the vector, using techniques that aim to eliminate or control mosquito-breeding sites (Brasil, 2018). In 2012, WHO launched the global strategy for prevention and control of the vector, aiming at more ecologically appropriate means (World Health Organization [WHO], 2012).In this context, plants are an alternative, having important volatile compounds with larvicidal bioactivity (Costa et al., 2005), such as terpenes and phenylpropanoids, which are synthesized by some plant species and have insecticidal properties.

Croton L. is the second most diverse genus in the Euphorbiaceae family, comprising about 1300 species in tropical and subtropical regions (Santos et al., 2014). In Brazil, it has approximately 356 species, found mainly in the northeast region (Secco et al., 2012). Species of this genus are a viable alternative for prospection of new larvicidal bioproducts, since they are widely distributed and very abundant, thus having great availability and low cost (Osanloo, Sedaghat, Dehkordi, & Amani, 2019).

This study arose from the need to develop alternative products for *A. aegypti* control, since the increase in larvae resistance to commercial products affects control programs; therefore, the chemical prospecting of

plants with larvicidal properties is a viable option. We aim to evaluate the larvicidal potential of crude ethanolic extract and fractions in hexane, chloroform, ethyl acetate and hydromethanol from leaves of two species of *Croton* L. (Euphorbiaceae) against larval forms of *Aedes aegypti*. We believe it could be used as an alternative product in the control of *A. aegypti*.

Material and methods

Selection and obtaining of plant material

Fresh leaves of *Croton betaceus* Baill (3.5 kg/-4.885973, -43.410064) and *Croton lundianus* (Didr.) Müll. Arg (5.5 kg/-4.913059, -43.429960) were collected in the Cerrado region of Caxias, Maranhão, Brazil (Figure 1) during the morning shift. They were used for the bioassays and had their exsiccates deposited at *Herbário Afrânio Gomes Fernandes (HAF)*, under voucher HAF-04642 and HAF-04644, respectively. The procedures were submitted to *Sistema Nacional de Gerenciamento do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen) under A8C212C.

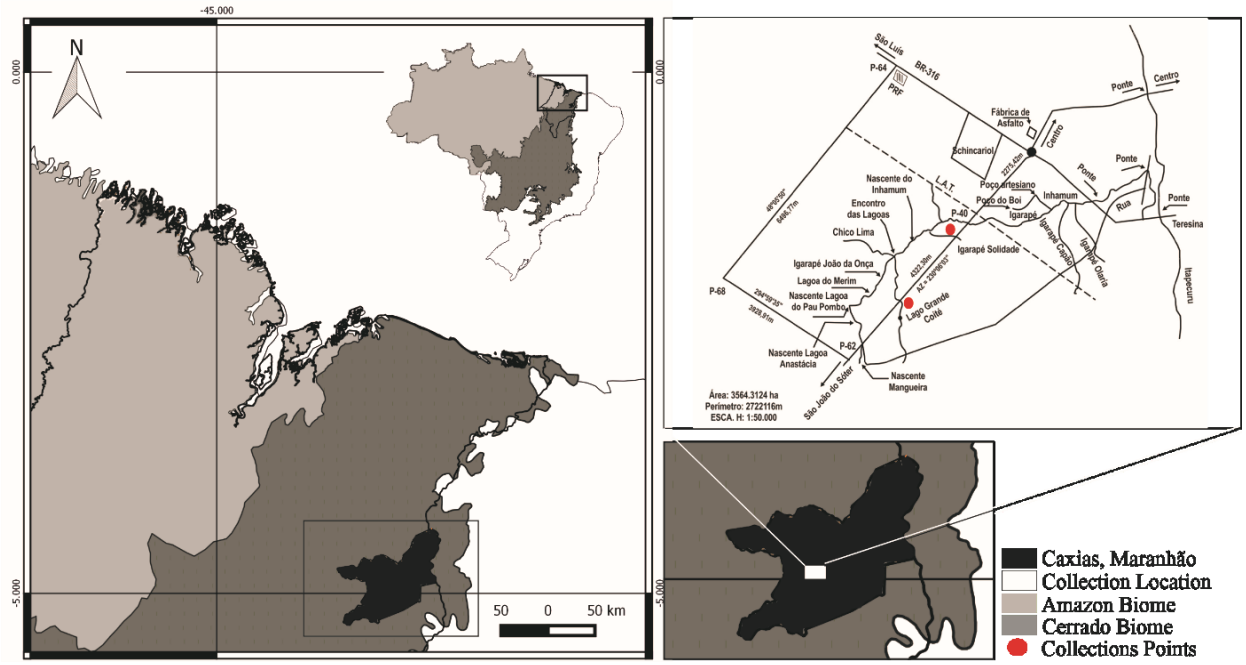

Figure 1. Geographic location of the place where plant material was obtained. The red dots indicate the geographical coordinates of *Croton betaceus* and *C. lundianus* used in this study.

Source: The authors, 2020.

Compound extraction and fractionation

After drying at 26°C ± 1, in the dark, the leaves of both *Croton* species were ground to powder. The extract was obtained using ethanol (absolute ethyl alcohol PA ACS, C_2H_6O/99.8%, Dinâmica) as solvent, in a glass extractor (26°C ± 1). The solvent was removed on a rotary evaporator (Fisatom 801) at 180 rpm/45 minutes at 38°C ± 1, coupled to a vacuum pump (Prismatec 132) and lyophilized later (Micro Modulyo Edwards). The ethanolic extracts were suspended in a methanol (neutral hydrated alcohol PA, CH_3OH, Dinâmica) and deionized water mixture (2:3), and successively submitted to the liquid-liquid division process with solvents of different polarities: hexane (PA C_6H_{14}, Dinâmica), chloroform (PA $CHCl_3$, Dinâmica) and ethyl acetate (PA$C_4H_8O_2$, Dinâmica).

After removing the last fractionation solvent (ethyl acetate), we considered the methanol/water residue to be also a fraction. Then, all were evaporated and lyophilized separately, resulting in four fractions: hexane (HX), chloroform (CH), ethyl acetate (AC) and hydromethanolic (HD) plus the crude fraction (CE).According to the manufacturer, the solvents used are removed from the solution by evaporation, obtaining a maximum of 0.001 to 0.003% residue. Thus, they did not interfere in toxicity analyzes. The total yield (%) is given by the weight of the crude extract (g) after evaporation and lyophilization divided by the weight of dry leaves (g). The process followed the model by Ûchoa et al. (2016).

$$\left(\text{Total Yiel (\%)} = \frac{\text{Fraction (g)}}{\text{Dry leaves (g)}}\right) \text{x } 100$$

Larvicidal bioassay

To assess the larvicidal effect, tests were performed following World Health Organization's methodology (WHO). Adult mosquitoes (male and female) were kept in entomological cages at 26ºC ± 2, with a 10% sucrose solution for feeding. The spawning was induced from blood meal to females, on moistened filter paper. The eggs were placed in a container with water for hatching. The larvae, in stage L3, used in the experiments were kept at a constant temperature of 26°C ± 1 and fed with fish food.

The larvae were put in containers containing different concentrations of *Croton* spp. fractions (10µL mL^{-1}, 20µL mL^{-1} and 40µL mL^{-1}). In order to obtain the concentrations needed, fractions were diluted in distilled water, using a vortex shaker (K40-1020, Kasvi) for total homogenization. The tests were performed with triplicates of 10 viable larvae per container. Temephos ($C_{16}H_{20}O_6P_2S_3$) and distilled water were used as the control test. Lethality was evaluated at 24 and 48 hours of exposure of the larvae to the extracts or fractions, considering dead those that did not respond to mechanical stimulus. The results are demonstrated by the number of dead larvae in each concentration (µL mL^{-1}) in different fractions.

Statistical analysis

Larvicidal toxicity data was expressed by mean ± standard deviation of triplicate values from independent experiments, for each concentration of the fractions. The values were submitted to Analysis of Variance (ANOVA) and the means were compared by Tukey's test at 5% ($p < 0.05$) using BioEstat 5.0 software (Ayres, Ayres Junior, Ayres, & Santos, 2007).

Results and discussion

In *Croton betaceus* there was 3.39% total yield of the extract in relation to dry leaves (460.86 g), with a predominance of 58.18% of hexane fraction (CBHX). A lower total yield was obtained in *C. lundianus*, being 1.03% for 1974.11 g of dry leaves. These total yield values are equivalent to those described in the literature for the genre, in which solvents order in the partition process and the solvent used in the extraction influences the yield percentage of each fraction (Oliveira et al., 2016).

Larvicidal potential

The crude ethanolic extract and the fractions of *Croton betaceus* showed larvicidal activity with different mortality rates. The bioassays made with the crude extract (CBCE) from this species showed larvicidal potential of 3.33%, in 24 hours, at the highest concentrations (20 and 40 µL).In 48 hour analysis, larval mortality increased in both concentrations, being 10% at 20 µL and increasing significantly to 16.66% at 40 µL(Table 1).

Table 1. Percentage of mortality of *Aedes aegypti* larvae exposed to different concentrations of fractions of ethanolic extract, obtained from the leaves of *Croton betaceus* (CB)

Fraction	Mortality (%)					
	24 hours			48 hours		
	10 *	20	40	10	20	40
CB CE	0.00±0[a]	3.33±0.4	3.33±0.4	0.00±0[a]	10.00±0.8	16.66±0.4[b]
CB HX	10.00±1.4	10.00±0.8	16.00±0.4	16.00±1.6	20.00±1.4	40.00±0.8
CB CH	0.00±0[a]	0.00±0[a]	10.00±0.8	10.00±0.8	16.66±0.4[b]	16.66±0.4[b]
CB AC	0.00±0	0.00±0	6.66±0.4	0.00±0[a]	0.00±0	10.00±0
CB HD	0.00±0[a]	6.66±0.4	10.00±0	3.33±0.4	13.33±0.4	16.66±0.4[b]
PC	90.00±0[c]	90.00±0[c]	90.00±0[c]	96.66±0[c]	96.66±0[c]	96.66±0[c]
NC	0.00±0[a]	0.00±0[a]	0.00±0[a]	00.00±0[a]	0.00±0[a]	0.00±0[a]

Data is expressed as mean ± standard deviation of number of dead larvae. The superscripts (a, b and c) represent data with significant similarity ($p < 0.05$) to each other by Tukey's test. *Relative concentration of extract/fraction, given in µL mL^{-1}. CE: Crude extract; HX: Hexane fraction; CH: Chlorophoric fraction; AC: Ethyl acetate fraction; HD: Hydromethanolic fraction. PC: Positive control NC: Negative control. Source: Research data, 2019.

According to Silva, Gualberto, Carvalho, and Fries (2014), the extract obtained from *Croton linearifolius* stem has 65% activity in 24 hours, at a concentration of 13.3 mg mL^{-1}. The mortality rates reported by these authors are higher than those observed in this study, however, the concentrations used are also higher than those from *C. betaceus*, indicating the possibility of increased mortality in high concentrations.

CBCE data showed significant activity, especially at 48 hours, which contradicts the literature data for *Croton* spp. crude extracts. Silva et al. (2014) consider these extracts incapable of causing mortality at low concentrations, as in *C. linearifolius*. However, mortality in *C. betaceus*is similar to the effect observed in *Guarea guidonia, Arrabidaea florida, Renealmia alpinia,* widely studied species and considered alternatives in *A. aegypti* biocontrol (Silva et al., 2014).

In studies with *C. linear*, Amado et al., (2020) determined that low concentrations are necessary for high mortality in mosquito larvae, and this effect comes mainly from the compounds present in leaves of these species. *Croton*s pecies are promising in studies of biological control of larval phases of *A. aegypti*.

In tests with the hexane fraction of *C. betaceus* (CBHX), it was observed that, in 24 hours, 40 µL caused mortality of 16% while other volumes (10 and 20 µL) lead to 10%. The results of CBHX in 48 hours demonstrated an increase in mortality, where 10 µL had a potential of 16% and at 40 µL, the activity was 40%. This difference in mortality between the different fractions corroborates Silva et al. (2014), who observed an increase in the activity of hexane fraction in relation to the crude fraction in *C. linearifolius*. It is notable that CBHX has a significantly greater activity when compared to crude fractions.

All other fractions of *C. betaceus* showed lower mortality than CBCE and CBHX, which tend to be more chemically complex due to the fractionation process. The chlorophoric fraction of *C. betaceus* (CBCH) showed a larvicidal potential of 10% at its highest concentration (40 µL) in 24 hours; in 48 hours, the lowest concentrations (10 and 20 µL) showed mortality of 10 and 16% at 40 µL, respectively. The ethyl acetate fraction (CBAC) showed larvicidal activity at 40 µL in 24 and 48 hours with mortality of 6.66 and 10%, respectively.

In *C. betaceus* hydromethanolic fraction (CBHD) bioassays, larvicidal action observed was 6 % and 10 % at 20 and 40 µL, respectively, in 24 hours; in 48 hours, mortality increased to 13.33 and 16.66%, which is similar to the rates presented by CBCH and CBAC. According to Barbosa et al. (2013) who analyzed extracts and residual fractions, *Magonia pubescens, Lantana camara, Palicourea marcgravii, Siparuna guianensis* and *Ocotea velloziana* did not affect insecticidal mortality.

Croton betaceus, both crude extract and fractions, demonstrated larvicidal activity. The hexane fraction is more expressive, possibly due to the concentration of present compounds. Other fractions potential may be greater with increasing concentration in tests, since studies with *Croton* spp. use higher concentrations.

In bioassays with *C. lundianus* crude extract (CLCE), it was observed larvicidal activity of 6.66 % at 40 µL (24 hours). In 48 hours, all concentrations showed mortality, with 3.33% at 10 and 20 µL and 30% at 40 µL (Table 2).

Table 2. Mortality percentage of *Aedes aegypti* larvae exposed to different concentrations of fractions of the ethanolic extract, obtained from *Croton lundianus* (CL) leaves.

Fraction	Mortality (%)					
	24 hours			48 hours		
	10*	20	40	10	20	40
CL CE	0.00±0^{a}	0.00±0^{a}	6.66±0.4^{a}	3.33±0.4^{a}	3.33±0.4^{a}	30.00±0.8^{b}
CL HX	13.33±0.4^{a}	13.33±0.9	30.00±0	43.33±0.9^{b}	46.66±0.9^{b}	93.33±0.9^{c}
CL CH	13.33±0.4^{a}	36.66±0.4	36.66±0.9	66.66±0.4	73.33±1.2^{b}	76.66±0.9^{b}
CL AC	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	6.66±0.4^{a}
CL HD	3.33±0.4^{a}	6.66±0.4^{a}	10.00±0.8	6.66±0.4^{a}	13.33±0.4	26.66±0.4^{b}
PC	90.00±0.8^{c}	90.00±0.8^{c}	90.00±0.8^{c}	96.66±0.4^{c}	96.66±0.4^{c}	96.66±0.4^{c}
NC	0.00±0^{a}	0.00±0^{a}	0.00±0^{a}	00.00±0^{a}	0.00±0^{a}	0.00±0^{a}

Data is expressed as mean ± standard deviation of number of dead larvae. The superscripts (a, b and c) represent data with significant similarity ($p < 0.05$) to each other by Tukey's test. *Extract relative concentration / fraction, given in µL mL^{-1}. CE: Crude extract; HX: Hexane fraction; CH: Chlorophoric fraction; AC: Ethyl acetate fraction; HD: Hydromethanolic fraction. PC: Positive control; NC: Negative control. Source: Research data, 2019.

When comparing fraction activities of *Croton betaceus* and *C. lundianus*, it is evident that CLCE larvicidal activity is superior to CBCE. This may be due to the fact that *C. lundianus* has more compounds with larvicidal capacity and/or these were present in the fraction. It is also suggested that the small amount of antagonistic substances could contribute to the decrease of potential in *C. betaceus*.

In tests with *C. lundianus* hexane fraction (CLHX), larvicidal activity was observed at all concentrations in 24 hours, with 40 µL showing 30% mortality while other concentrations (10 and 20 µL) showed 13.33%. In 48 hours, 10 µL concentration displayed 43.33% mortality, while at 20 µL it was 46.66%. At 40 µl there was 93.33% larvicidal action, which is significantly similar to the positive control.

The CLHX data reinforces the hypothesis that among ethanolic fractions, hexanes have greater larvicidal activity and, therefore, may be better candidates for larvae control. The larvicidal potential of complex

extracts is usually attributed to a synergistic action between the compounds (You et al., 2014), which may occur in CLHX. These data demonstrate the strong potential of this fraction in biocontrol of larval forms of *A. aegypti*.

Mortality evidenced in CLCE is higher than found by Barbosa et al. (2014), who observed 20.75% in 24 hours for bark extracts of *Ximenina americana* and 26.25% for root extracts of *Mimosa hostilis*; according to the author, these findings have great relevance for the study of biological control of the mosquito. These data also corroborate with Falkowski et al., (2020) who classifies the *Croton* genus as a potential alternative as natural larvicide.

In 24 hours, *C. lundianus* chloroform fraction (CLCH) showed 36.66% activity at the highest concentrations (20 and 40 µL) and 13.33% at the lowest (10 µL).In 48 hour analysis, 10 µL concentration showed 66.66% activity; 20 µL showed 73.33%; and the highest concentration (40 µL) presented 76.66% mortality. Ribeiro et al. (2020) showed the toxic effects of *Croton rudolphianus* at 0.75 µL mL^{-1}, indicating that *Croton* species may have high action potential, even at low concentrations.

Bioassays with the ethyl acetate fraction (CLAC) showed absence of larvicidal activity at all concentrations in 24 hours; in 48 hours, only 40 µL expressed 6.66% of larvicidal activity. According to Silva et al. (2014) the ethyl acetate fraction of *Croton linearifolius* extract showed larvicidal activity at 13.3 mg ml^{-1}, causing mortality of 20.84% of the larvae in 24 hours. These results suggest that at high concentrations it may present toxicity.

Santos, Bandeira, Lemos, and Santiago (2017) studied compounds from *C. nepetaefolius* leaves that demonstrated larvicidal activity of 77.6 mg mL^{-1}. The authors associate this effect to the presence of secondary metabolites already described as active against *A. aegypti.* As in *C. nepetaefolius*, the leaves of *C. lundianus* can be used as a natural larvicide being effective, biodegradable and non-toxic to the environment.

In bioassays with hydromethanolic fraction of *Croton lundianus* ethanolic extract (CLHD), it was found that within 24 hours there was larvicidal action at all concentrations. At 10 µL, it presented 3.33% mortality; at 20 µL it was 6.66% and at 40 µL, 10%. In 48 hours, there was an increase in the potential of all concentrations: at 10 µL there was mortality of 6.66%, while at 20 µL it was 13.33% and at 40 µL, 26.66 %.

Gomes et al. (2009) evaluated the insecticidal activity of essential oil *Zingiber officinale Roscoe* against *A. aegypti* larvae, where it showed activity of 10% in the concentration 20 µg mL^{-1}. This percentage is lower than the showed by fractions of *C. betaceus* and *C. lundianus*. This shows that these species can be useful in the development of bioproducts for *A. aegypti* control, improving public health sector especially for poor communities.

Considering the need for strategies for *A. aegypti* control, an applicable measure is the use of methods based on plant origin bioactive compounds with larvicidal activity, and *Croton* species are viable alternatives. The results presented using the hexane fraction of both *Croton* species proved to be very effective, especially *C. lundianus*, which showed the highest mortality rate.

Conclusion

In view of the data presented in this study, it is possible to evidence larvicidal activity both in crude extract and fractions originated from the two plant species, *Croton betaceus* and *C. lundianus*. CBHX and CLHX fractions expressed higher larvicidal activity than the others, being 40 and 93.33%, respectively. *C. lundianus* showed even greater larvicidal activity in all fractions, when compared to *C. betaceus*. In addition to CLHX, in *C. lundianus*, the CLCH fraction stands out with a mortality rate of 76.66% at the highest concentration (40µL mL^{-1}).

The ethanolic extract from the studied species, *Croton betaceus* and *C. lundianus* are very promising in biocontrol of *Aedes aegypti* larvae. However, there is still a need for studies on phytochemical properties of extracts and fractions of *Croton* species, especially regarding the stability of these compounds, which could contribute a lot to the development of bioproducts.

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