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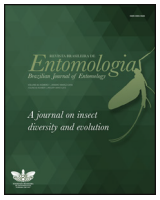

Isolation of 4-nerolidylcatechol from leaves of *Piper peltatum* L., and evaluation of larvicidal activity in mosquito vectors, with emphasis on *Aedes aegypti* (Diptera: Culicidae)

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ABSTRACT

Synthetic chemical insecticides are widely used in population control of pests. *Aedes aegypti* is the primary vector of dengue, Zika, chikungunya and yellow fever to humans, and has proven resistance to chemical insecticides. As an alternative vector control method, the ethanolic extract of the leaves of *Piper peltatum* L. (Piperaceae) showed larvicidal activity against *Ae. aegypti*. Despite the wide medicinal use of this plant, the biological activity of its isolated constituents remains unexplored. In this sense, we isolated, identified and evaluated the larvicidal activity of 4-nerolidylcatechol (4-NC) from *P. peltatum* against *Ae. aegypti*, *Culex quinquefasciatus* and *Anopheles darlingi*, focusing on the larvicidal, adulticidal and genotoxic potential of 4-NC on *Ae. aegypti*. Larvae were captured in the city of Manaus, Amazonas state, Brazil. 4-NC was isolated from the extract of the leaves of *P. peltatum* via silica gel chromatography. This was identified using nuclear magnetic resonance spectroscopy and tested in *Artemia franciscana* (6.25, 12.5, 25, 50, 100 and 200 µg/mL). In the toxicity bioassay, *Ae. aegypti* larvae were exposed to 30, 50, 70, 90, and 110 µg/mL of 4-NC, while *Cx. quinquefasciatus* and *An. darlingi* were exposed to 6.25, 12.5, 25, 50 and 100 µg/mL. *Ae. aegypti* larvae were also subjected to 40 and 60 µg/mL of 4-NC (genotoxicity bioassay), and adult females to 62.5 to 1,000 µg/mL (adulticidal bioassay). The results of the 4-NC toxicity assays showed that there was 100% mortality in larvae of *Ar. franciscana* at the concentration of 200 µg/mL, with an LC_{50} of 8.0 µg/mL. In the larvae of *Ae. aegypti,* mortality was 100%, with an LC_{50} of 62 µg/mL. In larvae of *Cx. quinquefasciatus*, 97% mortality occurred, with an LC_{50} of 52.3 µg/mL, and in *An. darlingi* larvae there was an 83% mortality rate, with an LC_{50} of 55.8 µg/mL. In adults of *Ae. aegypti*, however, there was no adulticidal activity. In the larvae of *Ae. aegypti*, the genotoxic effect of 4-NC (40 and 60 µg/mL) showed significant frequency ($p < 0.05$) of cellular abnormalities (micronuclei, budding and nuclear bridges) of interphasic nuclei of neuroblasts and oocytes in relation to the negative control. This result may be associated with a decrease in oviposition of females, which was observed in two generations. We can confirm that 4-NC has larvicidal activity against *Ae. aegypti*, *Cx. quinquefasciatus* and *An. darlingi*. Although it does not present adulticidal activity in *Ae. aegypti*, it reduced the oviposition of females. Therefore, 4-NC seems to be a strong candidate for the development of an alternative method for the control of these mosquitoes in the immature phase.

Introduction

Mosquitoes of the genera *Aedes* Meigen, 1818, *Anopheles* Meigen, 1818 *and Culex* Linnaeus, 1758, belong to the family Culicidae, and are distributed in two subfamilies – Anopheline and Culicinae. They are dipterans of epidemiological importance because some species transmit pathogens such as *Plasmodium* species, arboviruses, and microfilariae (Gaffigan et al., 2015; WHO, 2021).

Aedes (*Stegomyia*) *aegypti* (Linnaeus 1762) is the main vector of the four serotypes of dengue virus (DENV-1, -2, -3 and -4), chikungunya (CHIKV), yellow fever (YFV), and Zika (ZIKV) (Adler and Moncada-Álvarez, 2016). In Brazil, in 2021, 502,983 probable cases of dengue were reported, in addition to chikungunya and Zika, with 93,043 and 6,020 cases, respectively (Brasil, 2021a).

Malaria is an infectious disease caused by parasites of the genus *Plasmodium* that affected, in 2019, more than 241 million people worldwide (WHO, 2021). The mosquito *Anopheles* (*Nyssorhynchus*)

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darlingi Root, 1926 is considered the primary vector of malaria in South America (Tadei et al., 1988). In 2020, Brazil reported a total of 145,188 cases of malaria (Brasil, 2021b). Of this total, more than 99% occurred in the Brazilian Amazon region, and the main etiological agent was *Plasmodium vivax* (Brasil, 2021b). In this region, *P. vivax* infection predominates (83.7%) (Costa et al., 2012; Pimenta et al., 2015), while infections by *Plasmodium falciparum* (13.05%) and *Plasmodium malariae* (0.037%) are less common (Pimenta et al., 2015).

Culex quinquefasciatus Say (1823) is an anthropophilic mosquito that is distributed in several regions of the planet and presents epidemiological importance as a vector of several pathogens, such as *Wuchereria bancrofti* (Lai et al., 2000), West Nile virus, St. Louis, the Western Equine encephalitis virus (2012) and the Oropouche virus (Vasconcelos et al., 2011). The principal population control strategy of *Ae. aegypti, An. darlingi* and *Cx. quinquefasciatus* continues to be based on neurotoxic synthetic chemical insecticides from the group of organophosphates and pyrethroids (WHO, 2022). Although this type of intervention is crucial in endemic areas, continued use has contributed to the emergence and spread of insecticide resistance, and also affects non-target species and pollutes the environment (Valle et al., 2019, WHO, 2022). In this sense, health agencies need to discover new alternatives so as to reduce dependence on interventions that are based on synthetic chemical insecticides.

Plants are a source of promising secondary metabolites and have ovicidal, larvicidal, adulticidal and repellent activities against proven mosquito vectors (Ríos et al., 2017; Demirak & Canpolat, 2022). Essential oils (EOs) from *Acacia nilotica* and *Eucalyptus globulus*, for example, cause high mortality in larvae of *Anopheles stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* (Vivekanandhan et al., 2018a, 2020), and the extract of *Acanthospermum hispidum* showed larvicidal, pupicidal and adulticidal activity against these species (Vivekanandhan et al., 2018b). The EOs from *Citrus aurantium* and *Cymbopogon citratus* showed ovicidal activity against *Ae. aegypti* and *Aedes albopictus* (Moungthipmalai et al., 2023), and oil from *Piper cordoncillo* was toxic to *Ae. aegypti* larvae (Alonso-Hernández et al., 2023). The extract of *Naringi crenulata* was also toxic to *Cx. quinquefasciatus* (Pratheeba et al., 2019), and the combination of the nanoemulsion with essential oil from *Eucalyptus grandis* and *Corymbia citriodora* was highly toxic to larvae of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* (Vivekanandhan et al., 2023).

Essential oils from plants of the genus *Piper*, family Piperaceae, have been extensively investigated for their insecticidal effect against mosquito vectors (França et al., 2021; Oliveira et al., 2022; Morais et al., 2023). *Piper peltatum* L., commonly known as cordoncillo, is a shrub that is easily found in edges of forests in the Amazon region (Guimarães et al., 2020). Although its methanolic extract has potent action against *Ae. aegypti* larvae (Mongelli et al., 2002), the effects of its chemical constituents against the species *Anopheles* and *Culex* were not found in the literature. Such activity of this substance may be related to 4-nerolidylcatechol (4-NC; $C_{21}H_{30}O_2$), which is one of the main components of *P. peltatum* (Pinto et al., 2006). It is a sesquiterpene that is isolated from the parts of this plant (roots and leaves) and makes up > 5% of the dry weight of its roots (Pinto et al., 2006). This substance has a number of proven biological activities, among them, antimalarial (Pinto et al., 2009; Bagatela et al., 2013), antitumor (Alves-Fernandes et al., 2020) and antioxidant (Lima et al., 2013).

Considering the importance of using *Artemia salina* Leach, 1812, *or Artemia franciscana* Kellogg, 1906, some studies correlate toxicity for *Ar. salina* as a biomarker with anticancer, antifungal and antimicrobial activities (Meyer et al., 1982). Both organisms are small in size, and easy to handle and acquire, thus allowing for rapid bioassays.

The micronucleus (MN) test has been used to detect clastogenic and aneugenic agents (Fenech et al., 2021). It has also been used in larvae of *Ae. aegypti* and *Ae. albopictus*, as the main genotoxic marker of natural and semisynthetic substances, such as dillapiole and its derivatives, demonstrating DNA damage (budding, polynucleated cells and interphasic and anaphasic bridges), according to Rafael et al. (2008) and Domingos et al. (2014). Studies with dillapiole and its semisynthetic derivatives from *P. aduncum,* with ovicidal, larvicidal, adulticidal and genotoxic effects, have generated promising results for the effective control of *Ae. aegypti* and *Ae. albopictus* (Rafael et al., 2008; Pinto et al., 2012; Domingos et al., 2014; Meireles et al., 2016; Santos et al., 2020; Silva et al., 2021).

Due to the absence of data on the toxic effect of 4-NC in mosquitoes, this research evaluated, for the first time, the toxic potential of this substance in *Ae. aegypti* (as a larvicide and adulticide) and in *Cx. quinquefasciatus* and *An. darlingi* (as a larvicide), using the microcrustacean *Ar. franciscana* as a marker of toxicity. In *Ae. aegypti,* the genotoxic aspects of 4-NC were also studied.

Materials and methods

Obtaining 4-nerolidylcatechol (4-NC), catechol and nerolidol

The isolation of the 4-nerolidylcatechol was made from the extract of the roots of *P. peltatum*, which was cultivated at Embrapa Amazônia Ocidental, Manaus, Amazonas state, Brazil (Pinto et al., 2006). The extraction was done from 150 g of dry roots subjected to ultrasound extraction (Ultrasonic Cleaner), using the mixture of $CHCl_3$:EtOH (1:1). The isolation of 4-NC was carried out from the extract by chromatography on silica gel (70-230 *mesh*, Merck), with the solvent mixture $CHCl_3$:EtOH (9: 1). The isolated 4-NC was identified by nuclear magnetic resonance spectrometry of hydrogen (1H), carbon (13C) and DEPT 135 (Varian, INOVA 500, CDCl3). Catechol and nerolidol were obtained from Sigma-Aldrich® and used in this study to compare their larvicidal activity in *Ar. franciscana*.

Capture of mosquitoes

The capture of *Ae. aegypti*, and *Cx. quinquefasciatus* occurred in the Aleixo neighborhood (03º 05´ 29, 1´´ S, 59º 59´ 40, 7´´ W) and *An. darlingi* occurred in the Puraquequara lake (03º 06´ 18,89´´ S, 59º 84´ 36.92´´ W), in the municipality of Manaus, Amazonas state, Brazil. This activity was carried out with the authorization of the Chico Mendes Institute for Biodiversity Conservation (ICMBio), and Biodiversity Information and Authorization System (SISBIO), Brazil (permanent license number 32941, issued May 21st, 2012 to Dr. Míriam Silva Rafael, INPA, Manaus, Amazonas state), for the collection of zoological material.

The specimens were transported to the insectarium at the Department of Society, Environment and Health, of the National Institute for Amazonian Research (COSAS/INPA), and were identified with the taxonomic identification keys of Forattini (2002) and Consoli and Lourenço-de-Oliveira (1994). The feeding of the larvae was with fishfood (TetraMin Tropical Flakes®) and they were kept at 26± 2 ºC and 70± 5%, relative humidity. After the emergence of adult individuals, the feeding of mosquitoes was with glucose solution (5%) and the females with hamster blood (*Mesocrisetus auratus*), according to authorization No. 020/2017 from the Ethics Committee on the Use of Animals (CEUA) of the Central Vivarium at INPA.

Cytotoxicity test using Artemia franciscana

Cysts of *Ar. franciscana* were purchased from a commercial pet shop in Manaus, Amazonas, Brazil. The cysts were hatched in artificial

seawater (2 gL^{-1} $NaHCO_3$ + 8 gL^{-1} NaCl) at room temperature (25 ºC) for 48 h, under constant illumination.

Third-instar larvae were exposed to 4-NC at concentrations of 6.25, 12.5, 25, 50, 100 and 200 µg/mL. The test took place in 24-well ELISA microplates, with the addition of 20 µL dimethylsufoxide (DMSO) for solubilization of each concentration of the substance, plus 1 mL of saline solution (35 g/L) for the 10 *Ar. franciscana,* in each replicate. For the negative control (NC), the addition of 20 µL of DMSO in saline solution occurred in 10 larvae. The bioassay was performed in triplicate and had its activity evaluated after 24 h of exposure in the absence of light.

The acquisition of the *Ar. franciscana* samples (1 vial of 10 g) was from PRODAC (aquarium products) International S.r.l, 35013, Cittadella PD Italy.

Larvicidal test of mosquitoes

A total of 1,300 *Ae. aegypti, Cx. quinquefasciatus* and *An. darlingi larvae* (3rd and 4th instar) were used in the 24-hour bioassay. These were distributed in five replicates, with ten individuals for each treatment (n = 5) and controls (n = 2), per species. Larvae of *Ae. aegypti* (n = 250) were exposed to concentrations 30, 50, 70, 90 and 110 µg/mL of 4-NC. *Cx. quinquefasciatus* (n = 250) and *An. darlingi* (n = 250) were submitted to concentrations of 6.25, 12.5, 25, 50 and 100 µg/mL. Each species tested had a negative control (NC; n = 50) in water and 0.02% DMSO (n = 150) and a positive control (PC; n = 50), which was temephos at 40 µg/mL (n = 150).

Aedes aegypti *adulticide test*

About 12 h before the adulticide test, also known as the biological bottle test (WHO 2006), with adaptations, 315 females *Ae. aegypti* were fed with blood. The solubilization process of the 4-NC was in acetone (w/v) in concentrations of 62.5 to 1,000 µg/mL. The NC was with acetone and the PC was with pyrethroid insecticide type II-alfacipermethrin. Then, Schott glass bottles (1 mL/vial) impregnated with different concentrations of 4-NC, NC with acetone and PC with alfacipermethrin received 15 previously bloodfed females each with blood, in triplicate. Readings took place every 15 min until 90 min had passed.

Aedes aegypti *genotoxicity bioassay*

First generation (G_1) third instar larvae of *Ae. aegypti* (n=600) were exposed to 4-NC at concentrations of 40 and 60 µg/mL (in triplicate/50 larvae each) as well as to the NC and the PC, for 4 h. Of these, 120 specimens had the brain ganglia (somatic cells) extracted for the cytological preparations, and the rest continued their development until adulthood. Then, the preparation of ovariole slides (gametic cells) of adult females (n=120) was performed, and the other samples were used to obtain G_2, according to the steps previously used in G_1.

Cytological preparations of Aedes aegypti

Cytological preparations of *Ae. aegypti* were performed according to Imai et al. (1988). For each concentration, brain ganglia of the G_1 and G_2 larvae (n=60) were hypotenized in buffer (0.8% sodium citrate in 0.005% colchicine) and incubated at 37 ºC, for 1 hour. Then, fixators I (ethanol: acetic acid: H_20); II (ethanol: acetic acid) and III (glacial acetic acid) were added to the cell material, and dried at room temperature (RT). Giemsa solution in phosphate buffer 8%, pH 6.8, was used to stain the slides for 20 minutes. The manufacture of ovariole slides (n=30) of G_1 followed the same procedures used for the neuroblasts.

Analysis of slides and photographs

In each slide (n=10) 1,000 nuclei of each concentration of 4-NC and NC of G_1 and G_2 of neuroblasts, and of G_1 of oocytes were counted, totaling 90,000 cells. The abnormalities abnormal found in the cells were counted using a mechanical DigiTimer blood cell counter[ADAmTM-CelT (SATRA Technology Centre, Telford Way, Kettering, Northamptonshire, UK) and the microphotographs were obtained using an AxioCam MRcA camera under an Axioplan Zeiss light microscope (100× immersion objective with 1×, 1.25×, and 1.6× optovar; Carl Zeiss MicroImaging, Inc., Thornwood, NY, U.S.A.).

Oviposition of Aedes aegypti

The G_1 and G_2 generations of the adult female *Ae. aegypti* permitted the analysis in triplicate of the average oviposition of the individuals submitted to the concentrations of 40 and 60 µg/mL of 4-NC and the NC. The separation of adult females (n=10) aged between 3 and 5 days was in 50 mL paraffin cups, using screens, filter paper and 30 mL of drinking water.

Statistical analysis

Probit analysis determined the LC_{50} and LC_{90} and the percentage of mortality from the larvicidal test with *Ar. franciscana*, larvae and adult females of *Ae. aegypti*, and larvae of *Cx. quinquefasciatus* and *An. darlingi*. Using ANOVA ($p<0.05$), followed by the Tukey test ($p<0.05$), we observed the frequency of genotoxic damage of 4-NC in the neuroblasts and oocytes of *Ae. aegypti*. The mean oviposition of females between treatments and control, as well as between generations (G_1 and G_2) of this mosquito was significant.

Results

The isolation of 4-NC gave a yield of 8.6 g (44.1% of the total extracted) and a total yield of 5.7% based on the dried and ground root (m/m), and presented the molecular chemical structure $C_{21}H_{30}O_2$ (Fig. 1).

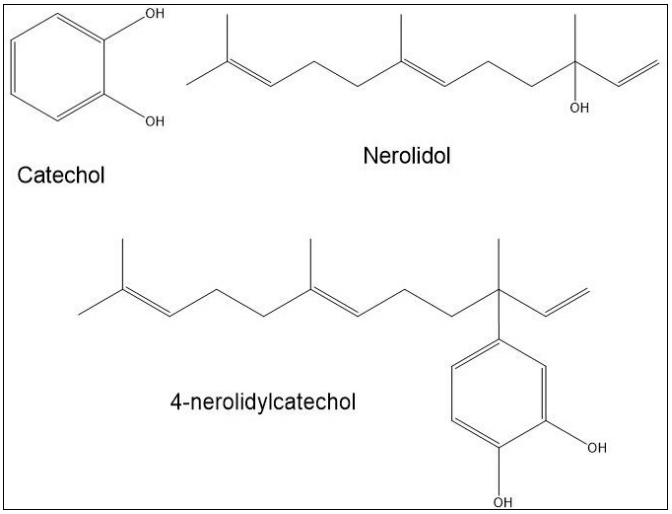

Figure 1 Molecular chemical structure of the substances 4-nerolidylcatechol (4-NC), isolated of *Piper peltata*, and catechol and nerolidol were obtained from Sigma-Aldrich®, used in larvicidal and cytotoxicity bioassays.

4-NC was identified using ^{1}H and ^{13}C NMR spectroscopy, and presented the following chemical displacements: NMR ^{1}H ($CDCl_3$; 500 MHz): 5.09 dd (*J*=10.8; 1.5 HZ); 5.02 dd (*J*= 17.4; 1.5 Hz); 5.99 dd (*J*= 17.4; 10.8 Hz); 1.95 m; 1.74 m; 5.07 m; 2.04 m; 1.81 m; 5.11 m; 6.75 dd (*J*= 8.4; 2.1 Hz); 6.80 d (*J*= 8.1 Hz); 6.85 d (*J*=2.0 Hz); 1.52 s; 1.60 s; 1.68 s; 1.33 s. NMR ^{13}C ($CDCl_3$; 125 MHz): 140.9; 143.1; 114.9; 141.3; 119.1; 111.5; 147.0; 43.8; 41.1; 23.1; 124.3; 134.9; 39.7; 26.7; 124.5; 131.2; 25.7; 17.6; 15.9; 24.9. Calculated molecular weight: 314.4617 g/Mol. Density of 0.993 ± 0.06 g/cm^3.

In *Ar. franciscana*, the substances 4-NC, catechol and nerolidol caused 100% mortality at the concentration of 200 µg/mL, and the LC_{50} of the 4-NC was 8.0 µg/mL; catechol had an LC_{50} of 23 µg/mL and nerolidol had an LC_{50} of 9.4 µg/mL (Table 1).

In larvae of *Ae. aegypti*, after 24 h, the 4-NC had caused 100% mortality at the concentration of 110 µg/mL and presented an LC_{50} of 62 µg/mL and an LC_{90} of 95 µg/mL. In the tests with the larvae of *Cx. quinquefasciatus,* the observed mortality was 97% at the concentration of 100 µg/mL, with an LC_{50} of 52.3 µg/mL and an LC_{90} of 99.2 µg/mL. In the larvae of *An. darlingi,* there was a mortality rate of 83% at the concentration of 100 µg/mL with an LC_{50} of 55.8 µg/mL and an LC_{90} of 139.3 µg/mL. For the catechol and nerolidol standards, 100% mortality was only achieved in *Cx. quinquefasciatus* with an LC_{50} of 209.5 and an LC_{90} of 265.6 µg/mL for catechol, and an LC_{50} 62.9 and an LC_{90} 125.7 µg/mL for nerolidol (Table 1).

The tested concentrations of 4-NC did not cause mortality *Ae. aegypti* in adulthood during the evaluation time of the test (90 min). This was perhaps due to this substance not being volatile and since it presents a high molecular weight.

The genotoxicity of substance 4-NC in neuroblasts and oocytes of *Ae. aegypti*, at the concentrations of 40 and 60 µg/mL showed the presence of nuclear and chromosomal alterations, such as micronucleus (MN), nuclear sprouts (NS), chromosomal breaks (CB), among other malformations, as shown in Fig. 2.

The frequency of nuclear and chromosomal damage in interphasic nuclei of neuroblasts of *Ae. aegypti* exposed to 4-NC (40 and 60 µg/mL) for 4 hours was significantly higher ($p < 0.05$) when compared to the NC, but not between their treatments. The frequency of damage in individuals exposed to the substance also showed a significant increase in G_2 when compared with G_1 (Fig. 3A). In interphasic nuclei of oocytes of first-generation females exposed to 4-NC (40 and 60 µg/mL), there was a significant difference ($p<0.05$) in the number of nuclear and chromosomal abnormalities in relation to the NC, but not between treatments (Fig. 3b).

Table 2 shows the decrease in the amount of eggs laid by female *Ae. aegypti* treated with 4-NC compared to untreated mosquitoes (control). The largest reduction in the number of eggs was with 40 µg/mL, in which the average number of eggs was 70.0 ± 5.1 in G_1, decreasing to 51.8 ± 6.0 in G_2; while in the control the average was 90.0 ± 4.6 and 82.0 ± 2.7 in G_1 and G_2, respectively.

Table 1
Lethal concentrations (LC_{50} and LC_{90}) of 4-nerolidylcatechol, catechol and nerolidol in *Artemia franciscana*, and 4-nerolidylcatechol in larvae of *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles darlingi*, after exposure for 24 hours.

Organism	Lethal concentration	4-NC	Catechol	Nerolidol	
*Artemia franciscana**	(%)	100	100	100	
	LC_{50} (µg/mL)	8.0 ± 1.0	23.0 ± 1.0	9.4 ± 1.0	
		(7.8 – 9.6)	(19.6 – 27.1)	(8.4 – 10.6)	
Aedes aegypti	(%)	100	0	13	100
	LC_{50} (µg/mL)	62 ± 0.8	-	-	33.8 ± 1.0
		(52.5 – 72.1)			(32.4 – 47.5)
	LC_{90} (µg/mL)	95 ± 2.0	-	-	103.2 ± 1.0
		(81.1 – 133.2)			
Culex quinquefasciatus	(%)	97	100	100	100
	LC_{50} (µg/mL)	52.3 ± 1.0	209.5 ± 2.7	62.9 ± 0.6	-
		(46.1 – 59.3)	(190.3 – 222.5)	(51.5 – 69.1)	
	LC_{90} (µg/mL)	99.2 ± 1.1	265.6 ± 2.0	125.7 ± 1.1	
		(79.6 – 173.3)	(247.7 – 304.7)	(97.3 – 240.7)	
Anopheles darlingi	(%)	83	-	-	100
	LC_{50} (µg/mL)	55.8 ± 1.0	-	-	-
		(46.6 – 66.9)			
	LC_{90} (µg/mL)	139.3 ± 1.0	-	-	-
		(85.8 – 197.5)			

*C= 200 µg/mL

Table 2
Mean and standard deviation of oviposition of adult females of *Aedes aegypti* survivors of the genotoxicity bioassay at concentrations 40 and 60 µg/mL of 4-neroldycatechol (4-NC), and negative control, for two generations (G_1 and G_2).

Compound	Concentration (µg/mL)	Generation	
		G_1	G_2
Negative control	0	90.0 ± 4.6	82.0 ± 2.7
4-NC	40	70.0 ± 5.1	51.8 ± 6.0
	60	56.0 ± 3.2	41.5 ± 8.1

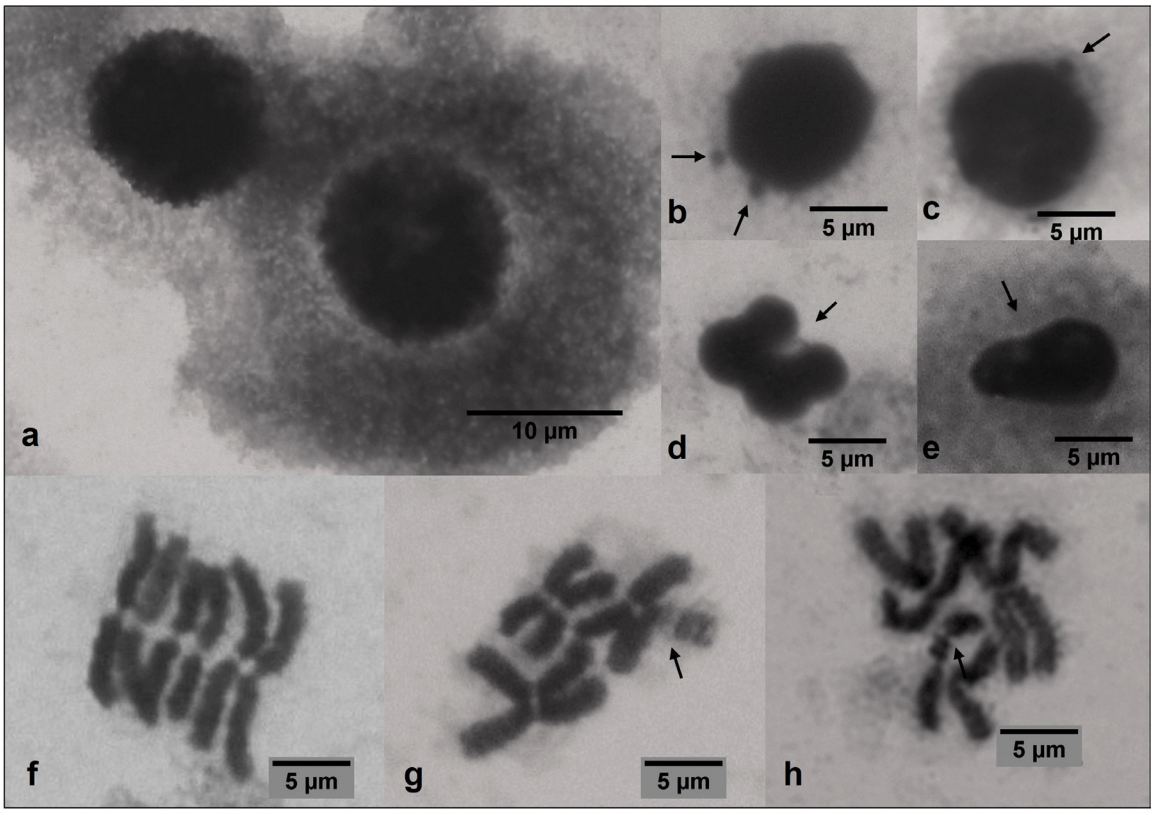

Figure 2 Microphotographs of abnormalities in interphasic and metaphasic nuclei of neuroblasts and oocytes of *Aedes aegypti*, stained with Giemsa (pH 5.8) and lacto-acetic orcein (2%). Arrows indicate: a – normal interphasic nuclei of neuroblasts of the NC group, G_1; b and c – micronuclei in interphasic nuclei of neuroblasts of G_2 (40 and 60 µg/mL), respectively; d – budding and telophasic bridging nucleus of neuroblasts (60 µg/mL, G_2); e – budding in interphasic nucleus of oocytes (40 µg/mL, G_1); f – normal metaphasic chromosomes (NC, G_1); g and h – chromosomal metaphases of neuroblasts showing achromatic secondary constriction (60 µg/mL, G_2) . Magnification: 1600×. Scale bar: 5 and 10 µm.

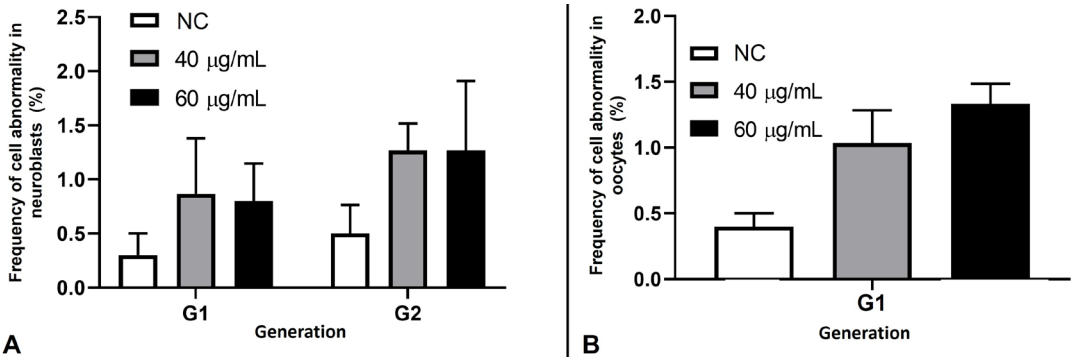

Figure 3 Frequency of anomalies in interphase nuclei. a – damage to *Aedes aegypti* neuroblasts exposed to 4-NC for two generations. b – data on *Ae. aegypti* oocytes exposed to 4-NC in G_1.

Discussion

In this study, the 4-NC used in the highest concentrations was cytotoxic in *Ar. franciscana*, and caused 100% mortality of the exposed individuals. These data corroborate those of Mongelli et al. (1999) who observed toxicity of the methanolic extract of *P. peltata* leaves (LC_{50} 89 µg/mL) against *Artemia salina*.

In mosquitoes, the larvicidal activity of isolated natural substances is poorly reported in the literature. In the present study, the high larval mortality recorded in *Ae. aegypti* corroborates the work of Mongelli et al. (2002). 4-NC demonstrated high lethality in larvae of *Ae. aegypti* (LC_{50} of 9.1 µg/mL and LC_{50} of 38.7 µg/mL), respectively, and it is present in the methanolic extract of the leaves of *P. peltata* and in the chloroform/ethanolic extract of the roots of that plant, respectively in these studies. Other studies with extracts, essential oils and their derivatives have reported ovicidal, larvicidal, and adulticidal activities in *Ae. aegypti* (Rafael et al., 2008; Pinto et al., 2012; Ríos et al., 2017; França et al., 2021; Oliveira et al., 2022; Morais et al., 2023).

Rafael et al. (2008) used dillapiole and observed larval mortality of 3rd instar *Ae. aegypti* at concentrations of 200 and 400 µg/mL (53% and 67%), respectively, after 72 hours. After 24 hours, derivatives of dillapiole ethyl ether (80 µg/mL) and *n*-butyl ether (30 µg/mL) killed

83.75 and 100% of the *Ae. aegypti* larvae, respectively (Domingos et al., 2014). The dillapiole methyl ether (DME), at a concentration of 140 µg/mL, made 97% of the eggs unviable and caused 99% larval mortality (Silva et al., 2019). Nerolidol, at concentrations of 100 and 200 µg/mL, also caused 100% mortality of the larvae (Meireles et al., 2021). Despite the high mortality caused by 4-NC in *Ae. aegypti* larvae, there was no adulticidal activity, probably because it is not a very volatile substance and has a high molecular weight. Studies are lacking to document this toxic activity in mosquitoes; however, Pinto et al. (2012) tested dillapiole, isodillapiole and butyl, ethyl and propyl ethers derived from dillapiole isolated from *Piper aduncum* at a concentration of 100 µg/mL in *Ae. aegypti*, and recorded 100% mortality in adults.

On the other hand, genotoxicity studies in *Ae. aegypti* neuroblasts recorded frequent budding and micronuclei in interphasic nuclei in larvae exposed to 4-NC for 4 h, which was significant ($p < 0.05$) in relation to the NC, and chromosomal breaks in metaphases. It is likely that the action of genotoxic agents on DNA occurs during de-espiralization, a moment of greater vulnerability of the molecule (Rafael et al., 2008; Fenech et al., 2021). This can lead to the formation of micronuclei and damage to the mitotic spindle mechanism involved in chromosome separation (Albertini et al., 2000), while breaks in chromosomal arms, also observed in *Ae. aegypti,* are associated with the regions of secondary constrictions on chromosome 3 of this mosquito (Rafael et al., 2008). Studies by Valadares et al. (2007) evaluated the mutagenicity and antimutagenicity of 4-NC in bone marrow cells of mice, and noted that this substance has no mutagenic effect on these cells and that there was a protective effect against genotoxicity. Barros et al. (2005) evaluated the mutagenicity of the ethanolic extract of 4-NC in mice and observed the absence of mutagenic activity. On the other hand, Alves-Fernandes et al. (2020) noted low genotoxicity of 4-NC in *in vitro* test in human cells. Dillapiole and its derivatives, even at low concentrations, showed toxic effects against eggs, larvae and adults of *Ae. aegypti* and *Ae. albopictus* (Pinto et al., 2012), and was able to alter genes that are important to the development of individuals (Meireles et al., 2016; Silva et al., 2021).

In this study, in adult females previously treated in the larval phase with 4-NC, there was a slight decline in fertility. This result is consistent with the data in *Ae. aegypti* (Domingos et al., 2014) and *Ae. albopictus* treated with dillapiole ethyl ether and dillapiole *n*-butyl ether (Meireles et al., 2016). These authors associated the reduced oviposition rate of adult females with DNA damage, as observed in *Ae. aegypti* after treatment with E-isodillapiole (Santos et al., 2020) and dillapiole methyl ether, and that even the smallest concentrations can cause damage to the genome of individuals and interfere with their function (Silva et al., 2021).

The data reported, in addition to the findings of the present study, reinforce the importance of toxicity analyses of candidate substances for effective vector control of *Ae. aegypti*, *Cx. quinquefasciatus* and *An. darlingi,* and genotoxicity in *Ae. aegypti*, because of the resistance of these mosquitoes to synthetic insecticides. In this sense, dillapiole *n*-butyl ether was cytotoxic for *Ae. aegypti* and *Ae. albopictus* (Domingos et al., 2014; Meireles et al., 2016). This substance, with $LD_{25, 50}$ and $_{80}$, however, was promising in hepatic, renal and cardiac tissues of Balb/C mice, since it was not cytotoxic at the lowest concentration (Viana Cruz et al., 2020) in relation to those previously tested in *Ae. aegypti* and *Ae. albopictus*. This suggests future testing of the toxicity and genotoxicity of 4-NC against immature forms of *Ae. aegypti* in field environments, and other non-target insects and mammals, for clarification of its larvicidal potential.

Conclusions

The toxicity of the substance 4-NC showed high mortality rates in eggs and of 3rd instar *Ae. aegypti* larvae, in *Cx. quinquefasciatus* and *An. darlingi* larvae and in *Ar. franciscana*, but was absent in adult subjects of *Ae. aegypti.* Genotoxicity, in the *Ae. aegypti* bioassay with 4-NC showed nuclear and chromosomal damage and reduced oviposition of adult females. These data suggest the need for future research on the application and effects of 4-NC as an alternative and effective tool in the control of *Ae. aegypti* in the field, and its application in other organisms.

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Conflicts of interest

The authors declare that there have no conflicts of interest.

Author contribution statement

SRLN: Methodology (Supporting), Acquisition of data, Analysis and interpretation of data, Writing-original draft. ACSP: Methodology (Supporting), Acquisition of data, Contributed with analyzed data. SFM: Supervision, Acquisition of data, helped in the writing-original draft & editing the main manuscript. JSS: Methodology (Supporting), Contributed with writing-original draft. FCMC: Contributed with a material, to carry out the Methodology study, Validation. MSR: Conception and design of the study, Funding acquisition, Writing-review & editing of the main manuscript, Supervision, and final approval of the version for publication.

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