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Full Length Research Paper

Larvicidal activity of clove (*Eugenia caryophyllata*) extracts and eugenol against *Aedes aegypti* and *Anopheles darlingi*

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Malaria and dengue are the cause of a heavy public health burden in the Amazon region. The present work evaluated the larvicidal activity of aqueous and methanolic extracts from clove, *Eugenia caryophyllata* Thunberg (Myrtaceae), and a chemical component are found in cloves, eugenol, against malaria and dengue mosquito vectors. Bioassays were carried out with these extracts and eugenol on *Anopheles darlingi* Root, 1926 and *Aedes aegypti* Linnaeus, 1762 (Diptera, Culicidae) third instar larvae. The median lethal concentration values obtained with aqueous extract against *A. aegypti* (LC_{50} = 6.4 mg/mL) were higher than those observed against *A. darlingi* (LC_{50} = 99 mg/mL). Eugenol exhibited an LC_{50} value of 3.6 mg/mL against *A. aegypti* larvae. These findings show eugenol´s potential as a larvicide against malaria and dengue vectors.

Key words: Myrtaceae, mosquito, dengue vector, malaria vector, plant extracts.

INTRODUCTION

Mosquitoes are major vectors responsible for the transmission of diseases. The Culicidae family attracts the greatest attention within public health care programs. This is because of the action of the species belonging to the genera *Aedes*, *Anopheles* and *Culex*. These species are the vectors of several pathogens that cause diseases, such as, malaria, encephalitis, dengue, yellow fever and filariasis (Borah et al., 2012). Some diseases transmitted by these vectors came to be naturally transmitted in urban or adjacent areas, thanks to the emergence or re-emergence of their vectors within those areas. Dengue and malaria are the classical examples (Tauil, 2006). Malaria and dengue are more commonly found in tropical countries where the hot humid climate favors the proliferation of many different species of *Anopheles* and *Aedes* mosquitoes.

Dengue is an infectious disease transmitted by mosquitoes of the genus *Aedes*. In Brazil and other countries *Aedes aegypti* Linnaeus, 1762 is a major vector of the virus that causes dengue fever. The most severe forms of this disease are: Hemorrhagic dengue fever, dengue shock syndrome, or uncommon manifestations like affected central nervous system. According to the World Health Organization, about two fifths of world's population is at risk of infection by dengue (WHO, 2011). Malaria is an acute or chronic infectious disease caused by protozoan parasites of the genus *Plasmodium*. It is transmitted by mosquitoes of the *Anopheles* genus. *Anopheles darlingi* Root, 1926 is the main malaria vector in the Amazon region. Malaria is a public health burden in over 100 countries. It causes the deaths of nearly one million people with 90% of these deaths occurring in Africa (WHO, 2011).

There is no vaccine for malaria or dengue at present.

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The best vaccine candidates to date do not provide immunity to a significant number of those vaccinated. Thus, vector control becomes indispensible for preventing malaria, dengue and other vector-borne diseases (Borah et al., 2012). Various pesticides have been employed against mosquito vectors in an effort to control their populations. Even though they are highly efficacious against target species, pesticides are losing efficacy due to their steady use and the development of pesticide resistance of mosquitoes and other insect vectors.

There is a great need to investigate new insecticidal substances. Ideally, these substances would be able to interfere with vector development and keep vectors under control, that is, at very low population levels. Cloves are the unblossomed flower buds of *Eugenia caryophyllata* (synonyms *Syzygium aromaticum* L., *Eugenia aromatica* L., *Caryophyllus aromaticus* L., Myrtaceae family). Clove essential oil (EO) has been widely investigated on account of its popularity, availability and high (80 to 90%) eugenol content (Chaieb et al., 2007; Machado et al., 2011). The *in vitro* activity of *E. caryophyllata* extract has been demonstrated against pathogenic bacteria (Fu et al., 2007), viruses (Kurokawa et al., 1995; Hussein et al., 2000) and fungi (Ranasinghe et al., 2002). Eugenol is medicinally used as a stimulant against digestive ailments and diarrhea (Chaieb et al., 2007). Various biological activities of eugenol have been reported, such as, fungicidal (Gayoso et al., 2005; Braga et al., 2007), antiviral, antibacterial (Shokeen et al., 2008), antitumor, anesthetic and antioxidant (Sadeghian et al., 2008; Hemaiswarya and Doble, 2009) and insecticidal (Chaieb et al., 2007; Pohlit et al., 2011a, b). This work investigated the larvicidal activity of water and methanol extracts of *E. caryophyllata* and commercial eugenol against *A. darlingi* and *A. aegypti* under laboratory conditions.

MATERIALS AND METHODS

Preparation of plant extracts

Extracts utilized in this study were extracted from *E. caryophyllata* (clove) flower buds, acquired at Adolpho Lisboa municipal market, in the city of Manaus, Amazonas State, Brazil. Clove flower buds (60 g) were added to 300 mL of distilled water. Next, the mixture was ground in a blender and filtered. The main solution was composed by 200 g/mL. Doses to be tested were obtained from this solution. Clove flower buds (320 g) were extracted three times with methanol in a soxhlet apparatus (boiling point range 60 to 80°C) for 6 h (3 × 6 h). The extracts were filtered and concentrated under reduced pressure. The residue was stored at 4ºC. 6 g of crude extract were first dissolved in 60 mL of water (stock solution). The doses tested were prepared from the initial solution of the 100 g/mL.

Eugenol

The concentration of eugenol (Sigma-Aldrich) utilized in these bioassays were determined by preliminary tests that were performed on a solution prepared by dissolving 5 µL of eugenol in 60 mL of water in the procedure described below. In the concentration-larvicidal activity tests carried out for larvae of *A. aegypti* with eugenol, the following concentrations were used: 0.5, 1, 2, 3 and 4 ppm.

Rearing of mosquitoes

A. aegypti larvae

Breeding of *A. aegypti* was performed at the INPA Malaria and Dengue Vectors Laboratory Insectarium at 26 ± 2°C, relative humidity >85% and using a 12 h photophase (Scarpassa and Tadei, 1990). For maintenance in screened cages, male mosquitoes were fed on a cotton ball soaked with a 10% sugar solution. Female mosquitoes were fed sugar solution and were also provided with a blood meal furnished by hamsters (*Mesocricetus auratus*) twice a week. Eggs were obtained from colonies kept in the insectarium where winged mosquitoes mated and layed their eggs in 50 mL volume plastic cups. These cups contained 20 mL of water and the upper perimeter of each cup was coated with a 3 × 22 cm strip of filter paper. The filter paper strips with adhered eggs were dehydrated and stored in the insectarium. To obtain larvae, filter paper strips containing eggs were transferred to enameled containers containing water where larvae hatched after one days and reached the third instar larval stage after a total of four days.

Anopheles spp. larvae

Larvae were obtained through periodic collections of female mosquitoes in the field due to the difficulty of maintaining colonies of this species in the insectarium. Collections were performed in the townships of Coari and Cacau Pirera, Amazonas State, Brazil. Anopheline females were captured before their blood meal and then fed in the laboratory on chicken (*Gallus gallus*) blood. Engorged females were placed to lay eggs on screened, individual cups lined with wetted filter paper so as to prevent egg desiccation. Following oviposition, which lasted for three to five days, the deposited eggs were transferred to enameled containers containing distilled water and liquid food. Anopheline species were identified according to the Consoli and Lourenço-de-Oliveira (1994) identification key.

Larvicidal bioassays

During preliminary screening the larvae of *A. albitarsis* and *A. nuneztovari* were collected from the insect-rearing cage. Six grams of crude extract were first dissolved in 60 mL of water (stock solution). From this stock solution, 100 ppm was prepared with water. The larvicidal activity was assessed using the procedure described by WHO (1996) with some modifications according to criteria established by Dulmage et al. (1990). For bioassays, batches of 11 larvae were placed in plastic cups containing 10 mL of water and concentrations of 10, 5 or 2.5 ppm of the extract. The control was set up with tap water. Controls and experiments were performed in triplicate at each of the concentrations evaluated. The numbers of dead larvae were counted after 24 h of exposure. The experimental samples in which 100% mortality of larvae occurred were selected for dose-response bioassays.

Dose - response bioassay

Based on the preliminary screening findings, clove methanol and

Table 1. Percent mortality per target species on water extract, methanol extract and eugenol after 24, 48 and 72 h of exposure.

Reading (h)	n	*A. darlingi* (methanol)	n	*A. darlingi* (aqueous)	n	*A. aegypti* (aqueous)	n	*A. aegypti* (eugenol)
	600		900		3300		900	
24		435 (72)		734 (97)		1305 (88)		16 (16)
48		50 (8)		10 (1)		125 (8)		76 (76)
72		15 (2)		6 (0,8)		40 (2)		8 (8)
Total number of dead larvae		500		750		1470		100

( ) = percentage values; n = Number of tested individuals.

Table 2. Lethal concentration LC_{50} of the methanol extract, water extract and eugenol against *A. darlingi* and *A. aegypti* larvae after 24 h.

Reading (h)	Target species	Extract	n	LC_{50} ($IC_{0,05}$)
24	*A. darlingi*	methanolic	600	0.53 (0.47 – 0.59) mg/mL
		aqueous	900	99 (NE) g/mL
	A. aegypti	aqueous	3300	6.4 (5.7 – 7.3) mg/mL
		eugenol	900	3.6 (2.3 - 24.1) g/mL

NE = Not estimated at 95%; n = number of tested individuals, and CI = confidence interval.

aqueous extracts were subjected to dose-response bioassay for larvicidal activity against *A. darlingi* and *A. aegypti* larvae. From the stock solution of each extract, different concentrations ranging from 0.5 to 40 ppm were prepared for studies of concentration-larvicidal activity.

In the tests carried out with aqueous extract against *A. aegypti* and *A. darlingi* larvae, the concentrations used were 0.5, 1, 2, 3, 4, 10,000, 20,000, 30,000 and 40,000 ppm and 0.5, 1, 2, 3 and 4 ppm, respectively. For methanolic extract against *A. darlingi* larvae the concentrations tested were 0.5, 1, 2, 3 and 4 ppm. In each bioassay we prepared a control blank with tap water (no extract or eugenol). The numbers of dead larvae were counted after 24, 48 and 72 h of exposure, and the percentage of mortality from the average of five replicates was used.

The average larval mortality data was subjected to probit analysis for calculating LC_{50}, by considering the level of significance of 95%, calculated through the POLO-PC software (LeOra Software Berkeley, CA), according to Finney (1971) and Haddad (1998). Results with $P<0.05$ were considered to be statistically significant.

RESULTS

Bioassays

Preliminary screening is a good means of evaluating the larvicidal activity of plants commonly used for this purpose. All extracts showed larvicidal effects after 24 h.

Preliminary larvicidal assays

The preliminary larvicidal activity on the target mosquito species are presented in Table 1. The highest mortality rate of *A. darlingi* larvae took place in 24 h for the aqueous extract. The methanol extract presented 72% mortality in 24 h. For *A. aegypti* larvae, the highest mortality rates occurred within 24 h in the aqueous extract. Eugenol exhibited the highest mortality rate on *A. aegypti* larvae at 48 h. It must be pointed out that bioassays using eugenol were carried out only with *A. aegypti* which was less sensitive to the aqueous extract. Given the number of dead larvae observed in the bioassays for aqueous and methanol extracts, the greatest mortality occurred in the first 24 h reaching over 90% for *A. darlingi* larvae.

The LC_{50} obtained for extracts and eugenol is presented in Table 2. The bioassays carried out with clove methanol and aqueous extracts on *A. darlingi* exhibited LC_{50} values of 530 and 99 ppm, respectively. Bioassays with the aqueous extract against *A. aegypti* larvae exhibited an LC_{50} value of 6.4 mg/mL. For the tests performed with eugenol, the LC_{50} value was 3.6 mg/mL.

DISCUSSION

Control of the species targeted in this study has been facing great threats due to the emergence of resistance to conventional synthetic insecticides. This situation warrants counter measures such as the development of new insecticides to achieve higher efficiency in ongoing vector control programs. The utilization of plant extracts is a promising method for controlling immature forms of mosquitoes. This is evident for eugenol (a major component of the essential oils of basil, cinnamon, cloves and other plants) and clove extracts that caused mortality

in *A. aegypti* and *A. darlingi* larvae.

A similar study conducted by Bagavan and Rahuman (2011) reported that the hexane, ethyl acetate and methanolic extracts were effective against the *Anopheles vagus* and *Culex vishnui* fourth instar larvae. *E. caryophyllata* flower bud hexane extract presented the highest mortality values in *A. vagus* larvae (LC_{50} = 85.9 mg/mL). On the other hand, the high mortality of larvae brought about by eugenol in the present study (after 48 h), diverges from that found by Costa et al. (2005) in bioassays carried out with *A. aegypti* and *C. quinquefasciatus* larvae. In the Costa et al. study, total larvae mortality at time intervals of only 10 min (at concentrations of 1,000, 500 and 250 ppm), 30 min (100 ppm) and 24 h (50, 25 and 10 ppm) were reported. However, it is worth mentioning that the concentrations utilized in the present study: 0.5, 1, 2, 3 and 4 ppm were quite lower than the ones employed by Costa et al. (2005) which could account for the longer time it took to reach total mortality.

The results of the present study agree in general with Shaalan et al. (2005) and a recent review (Pohlit et al., 2011b) showed that *Aedes* spp. larvae tend to be more robust and less sensitive to insecticides and botanical extracts than *Culex* spp. larvae. Yet, *Anopheles* spp. larvae susceptibility to larvicidal agents may vary depending on the species or environmental conditions. Therefore, *A. aegypti* is the most commonly used mosquito species for evaluating the insecticidal activity of plant extracts and substances on account of its lower sensitivity to insecticides and ease of colonization in the laboratory.

Generally, the clove extracts killed larvae within the first 24 h pointing to its importance in the search for safe control methods that will provide fast results such as the reduction of disease-vector mosquito larvae in periods of outbreaks and epidemics.

Conclusions

The results reported in this study merit further investigations on the larvicidal and repellent properties in these natural product extracts. The LC_{50} values for *A. aegypti* (LC_{50} = 6.3 mg/mL) obtained herein were higher (63 fold), those obtained for larvae of *A. darlingi* a clear indication that the former species is less sensitive than the latter to these extracts. Tests with eugenol revealed the highest larvicidal activity (the lowest LC_{50} value 3.56 mg/mL) of all and against the relatively hardy larvae of *A. aegypti*.

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