

ORIGINAL ARTICLE

**TOXICITY OF HEXANIC AND METHANOLIC FRACTIONS
FROM *Copaifera reticulata* DUCKE (LEGUMINOSAE –
CAESALPINOIDEA) AGAINST *Aedes aegypti* LINNAEUS
(DIPTERA – CULICIDAE), IN FIELD ASSAYS**

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ABSTRACT

The three most common artificial containers found in natural environmental conditions, are discarded tires, plastic and glass objects. These were tested to verify the effectiveness of hexanic (CRH) and methanolic (CRM) fractions of the oil-resin, from *Copaifera reticulata* (Ducke) (Leguminosae-Caesalpinoideae) against 3rd instar larvae of *Aedes aegypti* (Linnaeus) (Diptera - Culicidae). Larvicidal activity was noted in all the containers, varying according to type. The glass and plastic containers interfered less in the larvicidal activity than the tires. The most promising fraction was CRH1, which presented 18.2 of LC₉₀. Glass containers and tires presented LC₉₀ of 18.8 and 42.8 ppm respectively.

KEY WORDS: Dengue; yellow fever; chikungunya; natural products.

RESUMO

Toxicidade de frações hexânicas e metanólicas de *Copaifera reticulata* Ducke (Leguminosae – Caesalpinoidea) sobre *Aedes aegypti* Linnaeus (Diptera – Culicidae), em ensaios de campo

Testes foram realizados no meio urbano, em condições ambientais, nos três criadouros artificiais mais comuns (pneu, plástico e vidro) para verificar a eficácia das frações hexânicas (CRH) e metanólicas (CRM) do óleo-resina de *Copaifera reticulata* (Ducke) (Leguminosae-Caesalpinoideae) sobre larvas de 3^o estágio de *Aedes aegypti* (Linnaeus) (Diptera: Culicidae). A atividade larvicida foi verificada em todos os criadouros, mas apresentou diferenças de acordo com o tipo de recipiente. Os criadouros de vidro e plástico interferiram menos na atividade larvicida do que o pneu. A fração mais promissora foi a CRH1, que apresentou CL90 igual a 18,2 ppm, 18,8 ppm e 42,8 ppm, respectivamente, para os recipientes de plástico, vidro e pneu.

DESCRITORES: Dengue; febre amarela; chikungunya; produtos naturais.

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INTRODUCTION

The prevalence of dengue and the exposure of 2.5 billion people to its main transmitter *Aedes aegypti* (WHO, 2009) in intertropical countries, has led to a relentless search for new alternatives to control this vector, particularly as this mosquito is also a vector for chikungunya (Albuquerque et al. 2012; MS 2015) and yellow fever (Mascheretti et al. 2013), currently presenting epidemics in Brazil. In addition to chemical products, certain bacteria, fungi and plants have been considered in the control of *Ae. aegypti* (Veiga Jr 2003; Carvalho et al. 2011; Guissoni et al. 2013; Lima et al. 2013; Dias et al. 2014). There is no vaccine for dengue and chikungunya, and the development of such a vaccine is not simple since it must immunize against the five types of virus to be effective (Yauch & Shresta, 1992; Amaku et al. 2012; Lang 2012).

Copaifera reticulata (Ducke) is a medicinal plant presenting various pharmacological indications, such as antioxidant activity (Desmarchelier et al. 2001), and as an immunomodulator (Santiago et al. 2015), as well as activity against *Leishmania amazonensis* parasite (Santos et al. 2008), antinociceptive activity (Gomes et al. 2007), as an insecticide or larvicide (Silva et al. 2003b; Mendonça et al. 2005; Silva et al. 2007; Geris et al. 2008) and also antimicrobial activities (Ziech et al. 2013). This plant is found in Tropical America, throughout the Brazilian Amazon, mainly in the states of Pará and Amazonas. Compounds from this plant may be alternatives in the control of these mosquitoes (Geris et al. 2012). Other than the mentioned activities, the oil-resin and compounds showed toxicity against *Ae. aegypti* and *Culex quinquefasciatus* (Silva et al. 2003b; Silva et al. 2007; Geris et al. 2008; Geris et al. 2012).

Chemical insecticides, used in mosquito control, have shown degrees of resistance and/or tolerance and also interference in the health of the population and in the environment (Cunha et al. 2005; Beserra et al. 2007; Braga & Valle 2007; Gambarra et al. 2013). The larvicidal agent currently available for sale, temephos, has been used for more than three decades in the control of *Ae. aegypti* around the world (Beserra et al. 2007; Gambarra et al. 2013) causing various levels of resistance, which have already been detected in several countries.

Studies and the need to improve mosquito control by means of the discovery of new drugs, led to testing the larvicidal activity of *C. reticulata* against *Ae. aegypti* in the main artificial containers where the mosquito completes its life cycle in urban areas. Studies were also carried out regarding the activity of the oil-resin in ultra-low volume against adult mosquitoes.

MATERIAL AND METHODS

Plant material

The oil-resin was collected in the municipality of Jacundá, PA, Brazil. The collection was performed by making an incision in the trunk of the tree with a borer at a height of 70cm. The hole was then sealed with clay to prevent infestation by fungi or termites. The oil-resin was stored in an amber colored flask and taken to the laboratory, where it was filtered using a thin nylon cloth, weighed and stored in other similar flasks.

Extraction and separation

A 200g sample of the oil-resin, from *C. reticulata*, underwent three liquid-liquid partitions, with n-hexane methanol (1:1). After the evaporation of each solvent, using a rotational evaporator under vacuum, 170.12g of hexane extract (CRH) and 18.63g of methanolic extract (CRM) were obtained.

The extracts were chromatographed on silica gel CC (70-230 mesh) using n-hexane, methanol and dichloromethane for the mobile phase. Eight fractions from each extract were obtained, which were analyzed for their larvicidal activity. Of these, three hexanic fractions (CRH₁, CRH₄ and CRH₅), and three methanolic fractions (CRM₃, CRM₄ and CRM₅) were tested in the field.

The fractions from the hexanic extract were named CRH₁ to CRH₅, and the fractions from the methanolic extract were named CRM₁ to CRM₅. The active fractions were rechromatographed on a silica flash CC (230-400 mesh), eluted with n-hexane, ethyl acetate and methanol gradient for the mobile phase. The fractions were analyzed by thin layer chromatography (TLC) using a sulphuric acid solution of vanillin as revealing reagent. Fractions with similar TLC patterns (r.f.) were added for subsequent bioassays.

Bioassays

The larvae were obtained from a cyclical breeding unit of *Ae. aegypti*, at the Biology and Insects Physiology Laboratory, IPTSP/UFG, placed in a heated chamber at $28 \pm 1^\circ\text{C}$, $80 \pm 5\%$ relative humidity for a 12h photophase.

The tests were performed using 3rd instar larvae, as they proved more tolerant than the other stages (Silva et al. 2003a). The fractions were first weighed and dissolved in dimethylsulphoxide (DMSO), to prepare a mother solution at 300ppm. The quantity of solvent used to prepare this solution was previously determined by tolerance tests of the larvae to the solvent. Tolerance ended at the proportion of 0.8mL of DMSO in 24.2mL of water (Silva et al. 2007).

From the mother solution, a series of dilutions were prepared in order to obtain lower concentrations. The bioassays were conducted using 200 ml plastic cups and glasses and in a bicycle tire, in which 33 mL of each of the solutions and twenty larvae were placed. All these assays were performed in four replicates, in the urban area of Goiânia. All experiments were followed by a control, containing the same number of larvae and the same volume of DMSO in distilled water contrasted with 1ppm temephos, the same dosage used in larvicidal campaigns.

The mortality readings were performed 24h after the exposure of the larvae to the solutions. Death was verified by total lack of movement, even when exposed to a stimulus, and by body and cephalic capsule darkening (Silva et al. 2007).

The field tests were performed outside homes, located in Vila Morais, Goiânia, Goiás, using three types of containers: tires, glass and plastic. During the experiments, the average temperature was $25.9\pm 0.8^{\circ}\text{C}$ and relative humidity $64.7\pm 3.7\%$ with approximately 12 hours of photophase. Each container (tire, plastic and glass) received 33mL of the oil-resin solution, in concentrations of 60, 80, 85 and 90 ppm and twenty larvae of *Ae. aegypti*. The mortality readings were performed after 24 hours.

The hexanic fractions (CRH₁ and CRH₄) were tested at 300, 250, 200, 170, 150, 100 and 80 ppm concentrations. The methanolic fractions (CRM₃ and CRM₄) at 250, 210, 180, 150 and 100 ppm concentrations, in the three types of containers.

All tests were followed by a positive control containing water and DMSO 0.6% concentration and one negative control containing 1 ppm of temephos as applied in the larvicidal campaigns.

Ultra-low volume (ULV) for adults

The oil-resin was dissolved in DMSO and applied in ultra-low volume (ULV), via costal pump, in the dosage of 100 mL/min (WHO 1992; Silva et al. 1996; Lima et al. 2013). The bioassays were performed at 500, 1000, 2000, 3000, 5000, 10000, 15000 and 20000 ppm concentrations, replicates (n=4). Cages were used (Veiga Jr 2003), with 20 fed females. All tests were followed by a control with 40% concentration DMSO.

Ethics

This study was reviewed and approved by the Research Ethics Committee of Federal University of Goiás (UFG). The residents of the homes where the field tests were performed signed a Statement of Free and Informed Consent (SFIC), as demanded by the committee.

Statistical analysis

The data obtained regarding mortality x concentration (ppm) were analyzed by the Statistical Analysis System (SAS) program, in Probit graphics, to determine the lethal concentration (LC₅₀ and ₉₀) and its confidence interval (IC).

RESULTS

The chemical analysis of the fractions that presented larvicidal activity against 3rd instar larvae of *Ae. aegypti* evidenced that the major components were monoterpenes and sesquiterpenes for hexanic fractions, and diterpenes for methanolic fractions. The fractions were approximately five times more powerful than the oil-resin. The larvicidal activity of diterpenes, monoterpenes and sesquiterpenes were similar or presented slight differences from one container to another.

The larvicidal activity of the oil-resin from *C. reticulata* was detected in all containers tested, with concentrations varying according to the type, all with 3rd instar larvae of *Ae. aegypti*. The lowest efficiency of the oil-resin was found in the tires and the highest in the plastic cups.

The oil-resin was also tested on adult *Ae. Aegypti*, to check its activity by applying an ultra low volume. The doses were increased, starting at 500ppm through to 20000ppm.

The hexanic fractions, CRH₄ and CRH₅ (sesquiterpenes) and methanolic fraction, CRM₅ (diterpenes) were harmless to the larvae in the tire experiments. Here, the activity in all tests was significantly lower than in the other containers. The other hexanic and methanolic fractions, presenting larvicidal activity and their lethal concentrations and confidence intervals, are seen in Tables 1, 2 and 3.

Table 1. Larvicidal activity of *in natura* oil-resin from *Copaifera reticulata*, against 3rd instar larvae of *Aedes aegypti*, in field bioassays, in different containers, after 24 h.

Container	CL ₅₀ ⁽¹⁾ (IC ⁽²⁾ 95%) ppm ⁽³⁾	CL ₉₀ ⁽⁴⁾ (IC 95%) ppm
Tire	147.2 (137.9 – 158.4)	268.1 (235.6 – 322.2)
Plastic	80.6 (75.4 – 85.9)	143.2 (129.7 – 163.7)
Glass	89.4 (86.6 – 92.2)	110.1 (104.7 – 119.1)

⁽¹⁾CL₅₀ – lethal concentration for 50% of the larvae, ⁽²⁾IC – confidence interval, ⁽³⁾ppm – parts-per-million, ⁽⁴⁾CL₉₀ – lethal concentration for 90% of the larvae. There was no mortality in DMSO control. There was total mortality in the temephos control.

Table 2. Larvicidal activity of hexanic fractions (CRH₁, CRH₄ and CRH₅) from *Copaifera reticulata*, against 3rd instar larvae of *Aedes aegypti*, in field bioassays, in different containers, after 24 h.

	Container	CL ₅₀ ⁽¹⁾ (IC ⁽²⁾ 95%) ppm ⁽³⁾	CL ₉₀ ⁽⁴⁾ (IC 95%) ppm
Fraction CRH ₁	Tire	21.8 (20.8 – 23.4)	42.8 (36.7 – 55.5)
Monoterpenes	Plastic	12.5 (11.8 – 13.1)	18.2 (17.3 – 19.3)
	Glass	12.4 (11.6 – 13.1)	18.6 (17.5 – 20.1)
Fraction CRH ₄	Tire	-	-
Sesquiterpenes	Plastic	18.4 (17.7 – 19.2)	26.3 (24.7 – 27.8)
	Glass	17.3 (16.5 – 18.2)	28.5 (26.4 – 31.3)
Fraction CRH ₅	Tire	-	-
Sesquiterpenes	Plastic	21.1 (20.3 – 22.8)	29.4 (27.5 – 32.3)
	Glass	18.1 (16.9 – 19.2)	28.6 (26.7 – 31.2)

⁽¹⁾CL₅₀ – lethal concentration for 50% of the larvae, ⁽²⁾IC – confidence interval, ⁽³⁾ppm – parts-per-million, ⁽⁴⁾CL₉₀ – lethal concentration for 90% of the larvae. There was no mortality in DMSO control. There was total mortality in the temephos control.

Table 3. Larvicidal activity of the methanolic fractions (CRM₃, CRM₄ and CRM₅) from *Copaifera reticulata*, against 3rd instar larvae of *Aedes aegypti*, in field bioassays, in different containers, after 24 h.

	Container	CL ₅₀ ⁽¹⁾ (IC ⁽²⁾ 95%) ppm ⁽³⁾	CL ₉₀ ⁽⁴⁾ (IC 95%) ppm
Fraction CRM ₃	Tire	43.7 (32.6–77.8)	70.6(63.3–85.9)
Diterpenes	Plastic	16.5 (15.2 – 17.9)	36.1 (30.2 – 47.6)
	Glass	18.8 (17.5 – 19.8)	31.4 (28.0 – 38.4)
Fraction CRM ₄	Tire	38.2 (30.4 – 99.4)	61.6 (41.3 – 67.9)
Diterpenes	Plastic	16.7 (15.3 – 17.7)	32.7 (27.4 – 42.3)
	Glass	21.2 (19.2 – 22.6)	37.8 (32.1 – 51.1)
Fraction CRM ₅	Tire	-	-
Diterpenes	Plastic	63.6 (38.7 – 75.6)	76.3 (67.6 – 105.6)
	Glass	33.7 (25.4 – 72.7)	81.6 (74.6 – 94.3)

⁽¹⁾CL₅₀ – lethal concentration for 50% of the larvae, ⁽²⁾IC – confidence interval, ⁽³⁾ppm – parts-per-million, ⁽⁴⁾CL₉₀ – lethal concentration for 90% of the larvae. There was no mortality in DMSO control. There was total mortality in the temephos control.

DISCUSSION

Terpenes were the major chemical compounds found in the oil-resin from *C. reticulata*. These chemical compounds come from various vegetable substances and biosynthetic derivatives of the condensing units of isoprene, obtained from mevalonic acid. According to the number of isoprene units and carbon atoms, the chemical class terpene is divided into monoterpenes, sesquiterpenes, diterpenes, triterpenes, tetraterpenes and polisoprenes. The literature on this subject, has related activity of these substances only in reducing reproduction, appetite suppression, repulsion and larval mortality (Silva et al. 2007; Geris et al. 2008; Geris et al. 2012). In this work the terpenes that presented toxicity against *Ae. aegypti* were monoterpenes, sesquiterpenes and diterpenes. The monoterpenes were the best larvicids as they were the most active and proved toxic in all containers. The sesquiterpenes (CRH₄ and CRH₅) and diterpenes CRM₅ interacted with the tire container, proving harmless to the larvae of *Ae. aegypti*. These data confirm the reference results (Silva et al. 2003a; Veiga Jr 2003; Silva et al. 2007; Geris et al. 2012).

The oil-resin from *C. reticulata* presented larvicidal activity on *Ae. aegypti* in all containers tested, being more efficient in the plastic (LC₅₀ = 89.4ppm) and less efficient in the tire (LC₅₀ = 147.2ppm). These data are similar to those of Silva et al. (2003b), tested in *C. quinquefasciatus* larvae.

Larvicidal activity of many plants against *Ae. aegypti* has been reported and their extracts have produced various lethal concentrations, although smaller when compared to *C. reticulata*, but none have yet been tested under field conditions, only in the laboratory (Silva et al. 2003b; Silva et al. 2007; Geris et al. 2012; Mendonça et al. 2005). These differences in LC₅₀ are, possibly due to the fact that they are different plants with specific active ingredients and also in view of the different environments in which the tests were performed.

In this experiment the oil-resin presented lower larvicidal activity in all containers when comparing its hexanic and methanolic fractions.

These fractions showed varied larvicidal activity against third-instar larvae of *Ae. aegypti* in the field, depending on the container. In all tests the activity in the tire varied from very low to no activity (CRH₄ and CRH₅). Activity was detected in the plastic and glass containers. The CRH₁, CRM₃ and CRM₄ fractions were active in the tire, with CRH₁ presenting the highest activity. This observation is in agreement with the findings in the tire, where it is assumed that an interaction occurs between these vegetable oil products and the rubber, inactivating them or even, reducing their larvicidal action.

The hexanic and methanolic fractions in this work presented similar lethal concentrations in the plastic and glass containers, suggesting similar chemical products. These results were similar to those obtained by Silva et al. (2003b) and Geris et al. (2012).

All the hexanic fractions showed higher activity than the methanolic fractions in glass and plastic containers. According to Veiga Jr (2003) and Geris et al. (2012) the hexanic fractions have large quantities of monoterpenes and sesquiterpenes, while the methanolic fractions, CRM₄ and CRM₅ are the richest in diterpenes, demonstrating that the presence of both components increases the larvicidal activity under laboratory conditions, data supported by observations by Veiga Jr (2003) and Geris et al. (2012). It is therefore assumed that the hexanolic fractions have larger quantities of these components than the methanolic fractions.

When comparing the methanolic fractions, only the CRM₅ fraction was not active in the tire, in spite of its high dosage. The CRM₃ fraction presented the best larvicidal result in the glass container, and the CRM₄ fraction in the plastic and tire containers. However, the CRM₅ fraction presented reduced larvicidal activity in the plastic and glass containers in a high dosage and presented no activity in the tire. These findings suggest the reduction of the monoterpene and sesquiterpene components as observed by Veiga Jr (2003).

None of the concentrations, tested in an ultra low volume, caused death to adult *Ae. aegypti*.

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