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Larvicidal activity of oil-resin fractions from the Brazilian medicinal plant *Copaifera reticulata* Ducke (Leguminosae-Caesalpinoideae) against *Aedes aegypti* (Diptera, Culicidae)

Atividade larvicida das frações do óleo-resina da planta medicinal brasileira *Copaifera reticulata* Ducke (Leguminosae-Caesalpinoideae) sobre o *Aedes aegypti* (Diptera, Culicidae)

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ABSTRACT

Oil-resin fractions from Copaifera reticulata *Ducke (Leguminosae-Caesalpinoideae) were evaluated for larvicidal activity on third larval instars of* Aedes aegypti*, in searching for alternative control methods for this mosquito. The bioactive fractions were chemically monitored by thin-layer chromatography, ^{1}H and ^{13}C nuclear magnetic resonance and mass spectrometry. Bioassays were performed using five repetitions, at a temperature of 28 ± 1°C, relative humidity of 80 ± 5% and light and dark cycles of 12h. Mortality was indicated by darkening of the cephalic capsule after 24h of exposure of the larvae to the solutions. The most active fractions were CRM_{1-4} (sesquiterpenes) and CRM_{5-7} (labdane diterpenes), which showed LC_{50} values of 0.2 and 0.8ppm, respectively.*

Key-words: Copaifera reticulata. Aedes aegypti. *Terpenoids. Larvicidal activity.*

RESUMO

A atividade larvicida das frações do óleo-resina de Copaifera reticulata *Ducke (Leguminosae-Caesalpinoideae) foi avaliada em larvas de 3º estádio de* Aedes aegypti*, na busca de alternativas para o controle desse mosquito. As frações bioativas foram monitoradas quimicamente através de cromatografia de camada delgada, analisada por ressonância magnética nuclear de hidrogênio (^{1}H e ^{13}C) e espectrometria de massas. Os bioensaios foram realizados à temperatura de 28±1°C, 80±5% de umidade relativa e fotofase de 12h, com cinco repetições. A mortalidade foi determinada através do escurecimento da cápsula cefálica, após 24h de exposição das larvas às soluções. As frações mais ativas foram CRM_{1-4} (sesquiterpenos) e CRM_{5-7} (diterpeno labdano), que mostraram os valores de CL_{50} de 0,2 e 0,8ppm, respectivamente.*

Palavras-chaves: Copaifera reticulata. Aedes aegypti. ***Terpenoides. Atividade larvicida.***

Aedes aegypti (Lin), a mosquito that is disseminated around the world, has medical importance because it is a vector for dengue in Asia and for dengue and yellow fever in Africa and the Americas[16]. According to the World Health Organization[27], around 2.5 billion people are exposed to dengue transmission risk, in the urban environment of the cosmotropical region. This problem could increase through adaptation of the mosquito to polluted water[19], which could make control a big challenge in the near future.

There is still no vaccine available for dengue prevention. Dengue control has been limited to combating the vector using synthetic and biological insecticides. However, due to continuous use, the vector has become resistant to some chemical products[2 3 9 11 12]. This factor, allied to the growth of environmental

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concerns about the ecological damage produced by synthetic insecticides, has motivated scientific research towards finding active products of botanical origin, and a number of species have been investigated with this purpose[5 6 7 13 14 15 17 20 21 23].

Our own screening of plant extracts for their larvicidal activity has revealed that *Magonia pubescens* St Hil (Sapindaceae) and *Copaifera reticulata* (Leguminosae) were the most active extracts[20 22]. *Copaifera reticulata* is a plant found in the tropical region of Latin America, with widespread distribution in the Brazilian Amazon forest, particularly in the States of Pará and Amazonas[8]. It is also known as *copaibeira* and *pau-d'oleo*, and is one of the principal sources of copaiba oil-resin, which is widely exported from Brazil. Local populations in this region have been using this oil-resin as a primary therapeutic source for several ethno-pharmacological indications[1 4 8 26].

A study on *Copaifera reticulata* would therefore be expected to have great potential for finding bioactive compounds, since its extract has been shown to present good larvicidal activity. The present paper describes the activity and chemical composition of fractions derived from *Copaifera reticulata* against third-instar *Aedes aegypti* larvae.

MATERIALS AND METHODS

Plant material. Crude oil from *Copaifera reticulata* was obtained as an exudate from direct perforation of its trunk, in Jacundá, Pará, Brazil (October 2001). The trunk was perforated at 70cm from the ground. The oil-resin exudate was collected, filtered through nylon cloth, and stored in amber flasks until use. The borehole in the plant was sealed with clay.

Extraction and separation. An oil sample (186.5g) was subjected to liquid-liquid partition with *n*-hexane and methanol (1:1), thus resulting in two extracts: hexane soluble (162.3g) and methanol soluble (22.8g). These extracts were subsequently chromatographed on silica gel CC (70-230 mesh), using *n*-hexane, dichloromethane and methanol, by means of the gradient polarity elution technique. Fractions from the hexane extract were named CRH_1 to CRH_8, and those from the methanol extract were named CRM_1 to CRM_8. The active fractions were re-chromatographed on a silica flash CC (230-400 mesh), using an *n*-hexane, ethyl acetate and methanol gradient. The fractions were analyzed by thin layer chromatography (TLC) using sulfuric acid solution of vanillin as the revealing reagent. Fractions with similar TLC patterns (retention factor) were combined for subsequent bioassays.

Analyses of bioactive subfractions. The bioactive subfractions were monitored by TLC and analyzed by ^{1}H nuclear magnetic resonance (^{1}H NMR) in a Bruker ARX-200 spectrometer, using deuterated chloroform ($CDCl_3$) as the solvent and tetramethylsilane (TMS) as the internal standard. The non-polar fractions (from the hexane extract) were analyzed by gas chromatography coupled to mass spectrometry (GC-MS), using electron impact.

Bioassays. For the larvicidal activity assays, third-instar larvae of *Aedes aegypti* were used. These were obtained from a cyclic colony that has been maintained for more than 10 years at the Institute of Tropical Pathology and Public Health, Federal University of Goiás, at a temperature of 28 ± 1°C, relative humidity of 80 ± 5% and light and dark cycles of 12h[18]. Each fraction containing test compounds was weighed, dissolved previously in dimethylsulphoxide (DMSO), and the volume was made up with distilled water to obtain a stock solution (500ppm). The non-lethal concentration of DMSO that was used to prepare the solutions was determined previously, in experiments with *Aedes aegypti* larvae, to be 16.2% (v/v). From the stock solution, a series of dilutions was prepared in order to obtain the desired concentrations. The bioassays were carried out as five replicates in a climate-controlled environment similar to that of the colony. Twenty third-instar larvae of *Aedes aegypti* were placed in a 25ml test solution. Control assays were conducted using the same number of larvae in a DMSO-distilled water solution. The larva mortality after 24h was recorded. Total absence of larval movement as well as dark body color and cephalic capsule were used as indicative of death. The lethal concentrations and their respective confidence intervals were calculated by data interpolation, by means of Probit analyses using the Statistical Analyses System (SAEG).

RESULTS AND DISCUSSION

Liquid-liquid partition of *Copaifera reticulata* oil-resin (186.5g) resulted in 162.3g of hexane extract and 22.8g of methanol extract, which both showed larvicidal activity against *Aedes aegypti*. The bioassay-guided fractionation of these extracts produced eight fractions for each one. Two hexane (CRH_1, CRH_5) and two methanol (CRM_1, CRM_5) fractions showed the highest toxicity against *Aedes aegypti* larvae. The lethal concentrations (LC), with their respective confidence intervals, are shown in Table 1. There was no mortality in the control group.

The results showed that the two fractions CRH_1 (LC_{50} 2.3ppm) and CRM_1 (LC_{50} 0.8ppm) are more active than the oil-resin (LC_{50} 8.9ppm) (Table 1). The two fractions CRH_5 (LC_{50} 13.9ppm) and CRM_5 (LC_{50} 10.5ppm) were slightly less active. Examination of these fractions by TLC and ^{1}H NMR showed their terpenoid nature and GC/MS analysis (NIST library) verified that they

Table 1 - Chemical constituents of the fractions obtained from* Copaifera reticulata *and their activity against third-instar larvae of* Aedes aegypti, *after 24h of exposure.

Fractions	Chemical class	LC_{50} (95% CI) ppm	LC_{90} (95% CI) ppm
Oil-resin		8.9 (6.9 – 10.8)	59.4 (41.9 – 102.2)
CRH_1	monoterpenes	2.3 (0.9 – 3.4)	8.8 (7.3 – 11.5)
CRH_4	sesquiterpenes	22.6 (22.5 – 26.4)	43.9 (40.2 – 49.1)
CRH_5	sesquiterpenes	13.9 (11.2 – 15.3)	40.9 (34.5 – 52.4)
CRM_1	mono-, di-, sesquiterpenes	0.8 (0.01 – 2.5)	8.1 (7.9 – 11.9)
CRM_3	diterpenes	17.3 (15.3 – 19.1)	61.1(50.4 – 80.3)
CRM_4	diterpenes	15.5 (11.8 – 18.6)	93.1 (67.2 – 163.0)
CRM_5	diterpenes	10.5 (9.1 – 11.7)	21.4 (18.6 – 26.5)

95% CI - confidence interval at 95% probability

LC_{50} - lethal concentration required to kill 50% of the population exposed

LC_{90} - lethal concentration required to kill 90% of the population exposed

ppm - parts per million

consisted of monoterpenes and sesquiterpenes. CRH_1, which is rich in monoterpenoids that are mainly from the pinane and para-menthane groups, was four times more active than the oil-resin. CRM_1, which was 11 times more active than the oil-resin, consisted mainly of sesquiterpenoids from the caryophyllene, bisabolane, cadinane and copaene groups. It was refractioned to give, among other subfractions, CRM_{1-4} with an LC_{50} of 0.2ppm and an NMR spectrum that confirmed the presence of sesquiterpenoids (Table 2). CRM_5 was rather less active than the oil-resin and, on further fractionation, gave the subfraction CRM_{5-7} with an LC_{50} of 0.8ppm, such that the TLC and NMR (^1H and ^{13}C) data showed the presence of clerodane and labdane diterpenoids (Table 2).

Table 2 - Chemical constituents of the subfractions CRM_1 and CRM_5 from* Copaifera reticulata *and their activity against third-instar larvae of* Aedes aegypti*, after 24h of exposure.

Fractions	Chemical class	LC_{50} (95% CI) ppm	LC_{90} (95% CI) ppm
CRM_{1-2}	monoterpenes	27.5 (12.1 – 41.9)	> 100
CRM_{1-3}	monoterpenes	3.9 (2.1 – 5.5)	37.8 (26.6 – 69.4)
CRM_{1-4}	sesquiterpenes	0.2 (0.1 – 1.2)	12.2 (6.1 – 22.2)
CRM_{1-5}	sesquiterpenes	7.5 (4.9 – 15.7)	65.1 (59.4 – 85.2)
CRM_{1-6}	sesquiterpenes	>100	>100
CRM_{1-7}	sesquiterpenes	> 100	>100
CRM_{1-8}	labdane diterpenes	> 100	> 100
CRM_{1-9}	labdane diterpenes	1.3 (0.2 – 2.5)	>100
CRM_{5-7}	labdane diterpenes	0.8 (0.1 – 1.9)	8.2 (6.5 – 11.3)
CRM_{5-8}	labdane diterpenes	7.9 (7.1 – 8.4)	12.5 (11.3 – 15.1)
CRM_{5-9}	furane labdane diterpenes	32.3 (30.4 – 34.2)	56.2 (51.8 – 62.1)
CRM_{5-10}	furane labdane diterpenes	10.6 (9.7 – 11.5)	21.6 (19.4 – 25.2)
CRM_{5-11}	clerodane diterpenes	6.2 (5.7 – 6.8)	11.1 (9.9 – 13.0)
CRM_{5-14}	clerodane diterpenes	7.3 (5.1 – 9.4)	68.5 (48.4 – 117.2)

95% CI - confidence interval at 95% probability

LC_{50} – Lethal concentration required to kill 50% of the population exposed

LC_{90} - Lethal concentration required to kill 90% of the population exposed

ppm – parts per million

Summarizing these results, CRH_1 consisted mainly of monoterpenoids, CRM_{1-4} consisted mainly of sesquiterpenoids and CRM_{5-7} consisted of diterpenoids. These were, respectively, 4.44 and 13 times more active than the oil-resin.

Terpenoids are often reported as candidate insecticides that could be an effective alternative for insect control, with a lower impact on human health, household animals and the environment[25]. The terpenoids reported from *Copaifera reticulata* oil-resin were much more active than the terpenoids reported in the literature. For example, the sesquiterpenes *E*-nerolidol, farnesol and nerolidol (the first of these isolated from *Myroxylon balsamum* and the other two obtained from commercial sources) showed LC_{50} values of 6.0, 13.0 and 17.0ppm, respectively[23]. The monoterpene isolated from oil produced by *Tagetes minuta* was active at concentrations above 40ppm[10], and the diterpene isolated from *Melantheria albinervia* showed an LC_{100} of 62.5ppm[24]. Furthermore, a triterpene isolated from *Azadirachta indica* showed an LC_{50} of 21ppm[17]. All of these concentrations were much higher than those observed for fractions of *C. reticulata* oil-resin.

The results described in the present paper suggest that some oil-resin fractions (CRM_{1-4} and CRM_{5-7}) of *Copaifera reticulata* are comparatively more active against *Aedes aegypti* larvae. These results encourage the search for new natural larvicides. However, further investigations on larvicidal mechanisms of action and the effects of subfractions on non-target organisms and the environment in general, as well as formulations for improving larvicidal potency and stability, are needed for them to be used in practice as naturally occurring mosquito larval control agents.

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