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Copaifera multijuga ethanolic extracts, oil-resin, and its derivatives display larvicidal activity against *Anopheles darlingi* and *Aedes aegypti* (Diptera: Culicidae)

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Abstract: *Copaifera* spp. is a common tree species found in the tropical region of Latin America, popularly known as copaiba or pau-d'alho. Oil-resin from different *Copaifera* species and its components present several biological activities such as antimicrobial, anti-inflammatory, antioxidant and insecticidal, including larvicidal activity against mosquitoes. Thus, bark and leaf ethanolic extracts, oil-resin, essential oil and alepterolic acid from *Copaifera multijuga* Hayne, Fabaceae, were tested as larvicides against the main malaria vector in the north of Brazil, Anopheles darlingi and also *Aedes aegypti*, the dengue vector. *A. darlingi* larval mortality was significantly higher than *A. aegypti* for most tested compounds. Bark and leaf extracts resulted in lower Lethal Concentrations (LC50) values for *A. darlingi*, 3 and 13 ppm, respectively, while the essential oil provided the lowest LC50 value for *A. aegypti*, 18 ppm. Despite of that, the lowest LC values were from the alepterolic acid for both species, *i.e*. 0.9 and 0.7 ppm for *A. darlingi* and *A. aegypti*, respectively.

Introduction

Mosquitoes (Diptera: Culicidae) are important vectors of several human diseases including malaria and dengue. *Anopheles darlingi* is the principal vector of malaria in Rondonia and other Amazonian states; it is captured in high densities and frequency in anthropized environments (Gil et al., 2003). The dengue vector, *Aedes aegypti*, is a domestic and highly endophilous mosquito species (Consoli & Lorenço-de-Oliveira, 1994). Among many variables, the use of insecticides in vector control plays an important role in preventing transmission of these diseases. However, since chemical control often results in insect resistance to synthetic insecticides (Hemingway & Ranson, 2000), identifying new molecules with insecticidal properties remains a priority in the biotechnology field.

Copaifera spp. (commonly know as copaiba or pau-d'alho) is a tree species found throughout the tropics of Latin America that exudes oil-resin from the trunks (Leandro et al., 2012). Although many *Copaifera* species exists in the Amazon region, *Copaifera multijuga* Hayne, Fabaceae, provides a considerable amount of oil-resin commercialized (≈5%) (Lawrence, 1980), that peaks during the rainy season, especially ones growing in clay soils (Alencar, 1982). Up to 60% of the oil-resin composition comprises diterpene acids and volatile substances, such as sesquiterpene hydrocarbons. Plant nutrition, insect herbivory and light conditions greatly influences the concentration and composition of copaiba's oil-resin (Veiga Jr. et al., 2005; Langenheim, 1990).

The medicinal properties of *Copaifera* oil-resin have been described since the sixteenth century (see Veiga Jr et al., 2005 for a review). Oil-resin and other substances derived from different *Copaifera* species displayed antimicrobial, anti-inflammatory, antioxidant, and insecticidal activities (Leandro et al., 2012). Regarding insecticides, previous studies reported that oil-resin effectively killed mosquito larvae (Prophiro et al., 2012; Silva et al., 2003). Therefore, we evaluated the larvicidal activity of copaiba extracts, oil-resin, essential oil and

alepterolic acid from *C. multijuga* against the malaria and dengue vectors, *A. darlingi* and *A. aegypti.*

Material and Methods

Processing the plant material

Oil-resin, bark, and leaves from *Copaifera multijuga* Hayne, Fabaceae, were collected in March 2008 from a tree located in Candeias do Jamari, Rondonia State, Brazil. Taxonomists from the Instituto Nacional de Pesquisas da Amazônia performed plant identification and the voucher specimen was deposited in the herbarium with identification number 223.353.

Dried and grounded *C. multijuga* trunk bark (1.6 kg) and leaves (1.9 kg) were extracted with 99% ethanol (2.0 L and 2.5 L, respectively) at room temperature over the course of five days. The solvent was evaporated under reduced pressure, leaving 36.0 g of bark ethanolic extract and 29.6 g of leaves ethanolic extract.

Extracting and analyzing the essential oil

Ten milliliters of oil-resin was distillated in a 500 mL round-bottom flask fitted with a 40 cm distillation column and a serpentine condenser coupled with an oil separator. The distillation process took 4 h and yielded 4 mL of essential oil.

The essential oil was analyzed by GC/MS (determination of Kovats Indices) with a HP-5971 apparatus, using a 25 m x 0.25 mm fused silica capillary SP-2100 column, helium as carrier gas 1 mL/min flow rate, and temperature programmed from 50-250 ºC at 4 °C/min. Compound identification was initially done using a MS library search with Kovats indices as a pre-selection routine (Alencar et al. 1984; 1990) followed by visual confirmation to the standard MS obtained from literature (Adams, 2007).

Isolating and characterizing the components from the remaining oil-resin

After extracting the volatile compounds from the oil-resin, the remaining oil was separated from the aqueous phase using a separation funnel. This fraction was dried using anhydrous sodium sulphate and submitted to silica gel column chromatography eluted with *n*-hexane and chloroform in increasing polarity. The resulting white amorphous solid was subjected to IR, RMN ^{1}H and ^{13}C spectroscopic analysis.

Collecting mosquitoes and breeding larvae

Adult *A. darlingi* mosquitoes were captured using a modified BG sentinel trap in peri-urban regions of Porto Velho, Rondonia. *A. aegypti* eggs were obtained from the Laboratório de Ecologia Química de Insetos Vetores, UFMG, Brazil and colonized under laboratory conditions (25-28 °C, 12 h photoperiod and 80% humidity). Adult mosquitoes fed on blood from rabbits for 15 min to stimulate egg production and then were transferred to plastic cups covered with fine netting, where they fed on 20% sucrose. Three days after a meal, oviposition was induced in female mosquitoes by removing one wing from *A. darlingi* or introducing beakers containing distilled water to *A. aegypti*. The resulting larvae were fed with TetraMin Tropical Flakes fish food *ad libitum* until the 3rd or 4th instar stage.

Larvicidal bioassays

Larvicidal tests were performed in two sets. Set 1 was a dose response test with nine concentrations for extracts and oils (ppm: 5, 10, 25, 50, 125, 250, 500, 750, 1000) in order to determine the concentrations for the lethal concentration (LC50 and LC90) assay (Set 2) using five different concentrations and four replicates (WHO, 2005). Set 2 tests were repeated three times. Larval mortality was recorded from 24 to 48 h for LC calculation and interrupted after 96 h. Plant extracts or oils were diluted in DMSO and pipetted under the water surface of plastic cups (150 mL) containing 100 mL of distillated water and larvae (10 for Set 1 or 25 for Set 2) were introduced in the cups 30 min after pipetting. Lethal concentrations (LC) were calculated using Probit analysis and Weibull distribution (Minitab, Minitab Inc).

Statistical analysis

The effects of extract oils and isolated substance concentration on larval mortality were analyzed by TwoWay Anova or by non-parametric analysis, *i.e.,* Kruskal-Wallis or Rank sum test (SigmaStat 2.0, 1992-1997) when data transformation for normalization failed.

Results

The essential oil extracted from the oil-resin was comprised of sequiterpene hydrocarbons with β-caryophyllene (57.1%) and α-humulene (10.2%) making up the principal compounds (Table 1).

The IR, RMN ^{1}H and ^{13}C spectroscopic analysis of the white amorphous solid isolated from the remaining oil-resin (after essential oil extraction) was identified as alepterolic acid or 3-hydroxy-copalic acid. Comparison of the RMN ^{1}H e ^{13}C data with published data (Geris et al., 2008) confirmed the proposed structure.

The mortality rate of *A. darlingi* and *A. aegypti* larvae was significantly affected by different concentrations of extracts, oils, and alepterolic acid,

Table 1. Chemical composition of the essential oil extracted from the *Copaifera multijuga* oil-resin.

Components	(IK)	%	Components	(IK)	%
δ-elemene	1338	1.2	nerolidol	1504	0.3
α-cubebene	1551	1.3	δ-amorfene	1512	0.9
β-elemene	1399	1.1	cubenol	1515	0.5
β-caryophyllene	1419	57.1	β-sesquifelandrene	1523	9.9
β-copaene	1432	1.0	elemol	1550	0.7
α-amorphene	1433	1.1	germacrene B	1561	1.7
aromadendendre	1436	0.8	caryophyllene oxide	1583	0.6
α-humulene	1455	10.2	10-*epi*-γ-eudesmol	1624	1.0
γ-gurjunene	1477	1.3	β-eudesmol	1650	1.0
(*E*)-*9-epi*-caryophyllene	1478	1.5	α-cadinol	1650	0.3
γ-muurolene	1480	1.7	β-selinene	1490	0.9
germacrene D	1583	0.4	bicyclogermacrene	1500	1.5
Total identified					98.0

often showing, an increasing number of deaths from 24 to 96 h. The larval mortality of *A. aegypti* and *A. darlingi* in most assays increased up to 48 and 72 h, accordingly (Figure 2 and 3). However, *A. aegypti* larval mortality was significantly lower than *A. darlingi*'s when fed with bark (T=5.68; $p<0.001$) or leaf extract (T=8700.0; $p<0.001$), or oil-resin (T=7323.5; $p=0.003$), but not for essential oil (T=6593.0; $p=0.603$) even at high concentrations (Figure 1 and 2, respectively).

The *C. multijuga* bark extract affected of *A. aegypti* and *A. darlingi* larval mortality significantly in most of the extract concentrations tested (F=294,81; $p<0,001$ and F=29.36; $p<0.001$, respectively). At 200 ppm, bark extracts killed 86% of *A. aegypti* larvae after 48 h, while 83% of the *A. darlingi* larvae were dead within the same period at 125 ppm. A. darlingi larvae mortality reached 93% after 96 h (Figure 1A and 2A).

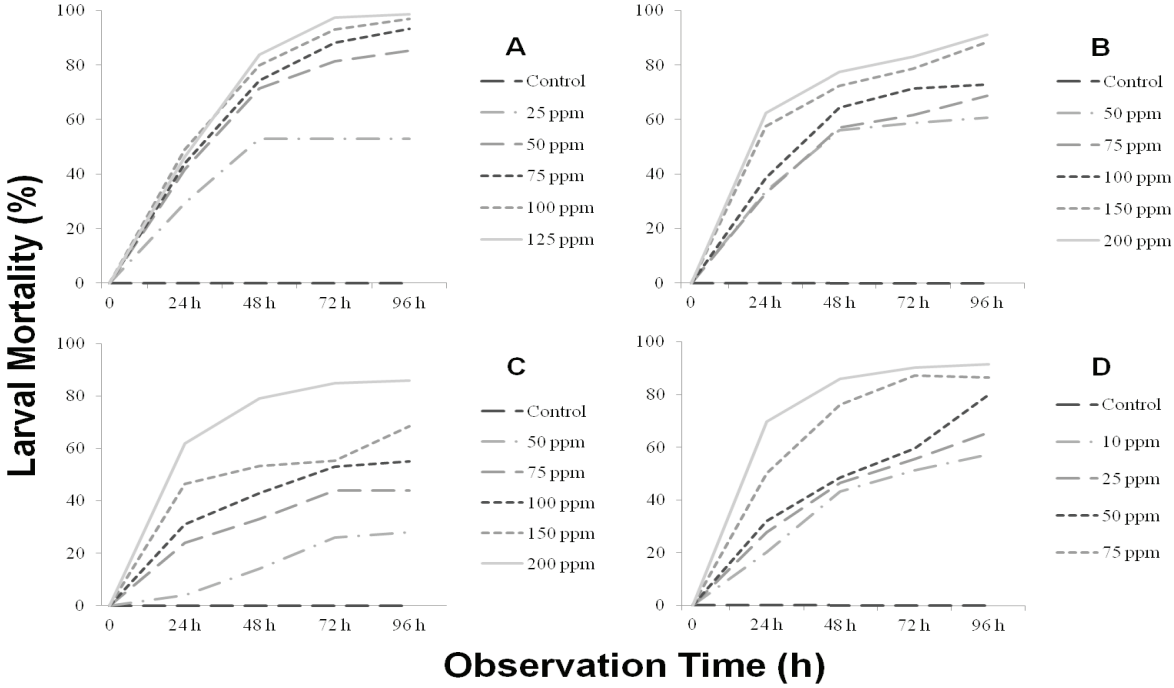

Figure 1. Larvicidal activity of *Copaifera multijuga*: ethanolic extract of bark (A), leaf (B), essential oil (C), oil-resin (D) against 3rd-4th instar larvae of *Anopheles darlingi* (Diptera: Culicidae).

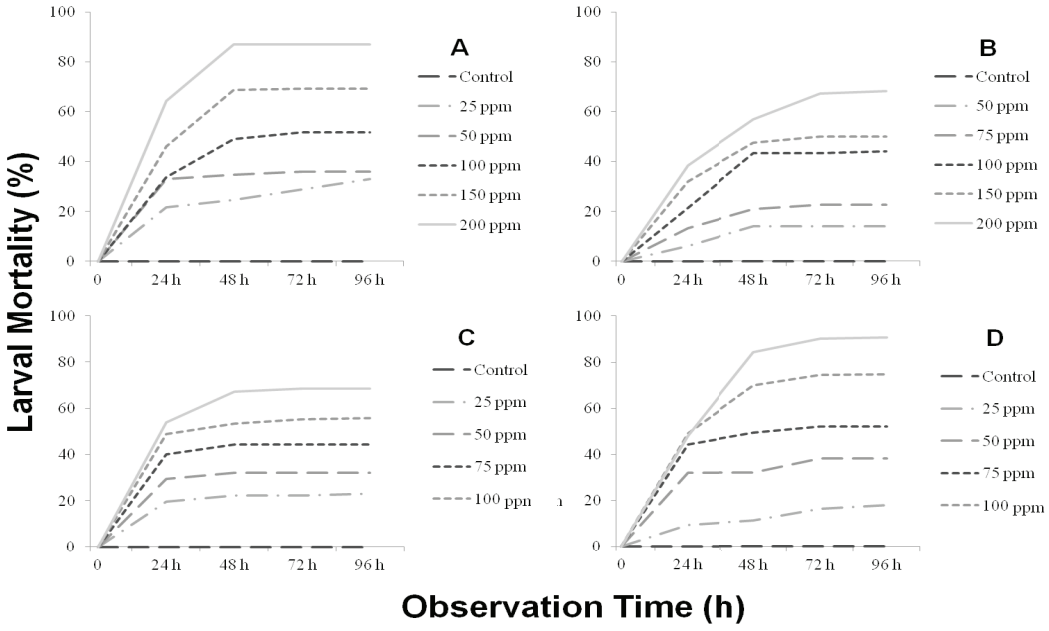

Figure 2. Larvicidal activity of *Copaifera multijuga*: ethanolic extract of bark (A), leaf (B), essential oil (C), oil-resin (D) against 3rd-4th instar larvae of *Aedes aegypti* (Diptera: Culicidae)

Larvicidal leaf extract caused larval mortality of both species in a similar temporal pattern described above, but with weaker effects compared to bark extract. For example, larval mortality at 200 ppm after 48 h reached 57% for *A. aegypti* (F=102.2; p<0.001) and 72% for *A. darlingi* (F=13.17; p<0.001) (Figure 1B and 2B).

Oil-resin from *C. multijuga* was tested at different concentrations for *A. aegypti* and *A. darlingi*. Oil-resin caused *A. darlingi* larval mortality only at higher concentrations (100-75 ppm) (F=30,1; p<0,001). For all other concentrations tested, *A. aegypti* larval mortality differed significantly (F=282.2; p<0.001). Although the larval mortality caused by the oil-resin was generally significantly higher for *A. darlingi* than *A. aegypti* (T=7325.5; p=0.003), the larval mortality of *A. aegypti* reached 90% after 96 h at 200 ppm, while for *A. darlingi,* 90% larval mortality was reached with 100 ppm (Figure 1D).

When mosquito larvae were exposed to the *C. multijuga* essential oil, the mortality rate did not differ between mosquito species. The number of deaths increased significantly after 24 h (F=6.84; p<0.001 - *A. aegypti* and F=5.52; p=0.002 - *A. darlingi*) and with increasing concentrations (F=175.34; p<0.001- *A. aegypti* and F=22.26; p<0.001 - *A. darlingi*) (Figure 1C and 2C).

The larvicidal activity of alepterolic acid did not differ significantly between species (T=6113.5; p=0.26) or change over the course of time (H=0.67; p=0.87 - *A. aegypti* and H=0.12; p=0.98 - *A. darlingi*) (*i.e.*, highest mortality was reached after 24 h and did not increase afterwards) (Figure 3). However, larvae mortality increased significantly (H=77.3; p<0.001 - *A. aegypti* and H=77.5; p<0.001 - *A. darlingi*) with the concentration range tested, surpassing 50% for the lowest concentration evaluated at 1 ppm (Figure 3).

Bark and leaf extracts resulted in lower LC50 values for *A. darlingi* while the essential oil provided the lowest LC50 value for *A. aegypti*. Despite of that, the lowest LC values were from the alepterolic acid for both species (Table 2).

Table 2. Lethal Concentrations in part per million of bark, leaf extract, oil-resin, essential oil and alepterolic acid from *Copaifera multijuga* for *Anopheles darlingi* and *Aedes aegypti* (Diptera: Culicidae) larvae.

Copaifera multijuga	Target	LC50	LC90
Bark		3	157
Leaf		13	297
Essential oil	*Anopheles darlingi*	128	231
Oil-resin		31	121
Alepterolic acid		0,9	8,8
Bark		81	224
Leaf		166	293
Essential oil	*Aedes aegypti*	18	201
Oil-resin		93	248
Alepterolic acid		0,7	7,0

LC50 and LC90: Concentrations needed to kill 50% and 90%, respectively, of the larvae in the bioassays

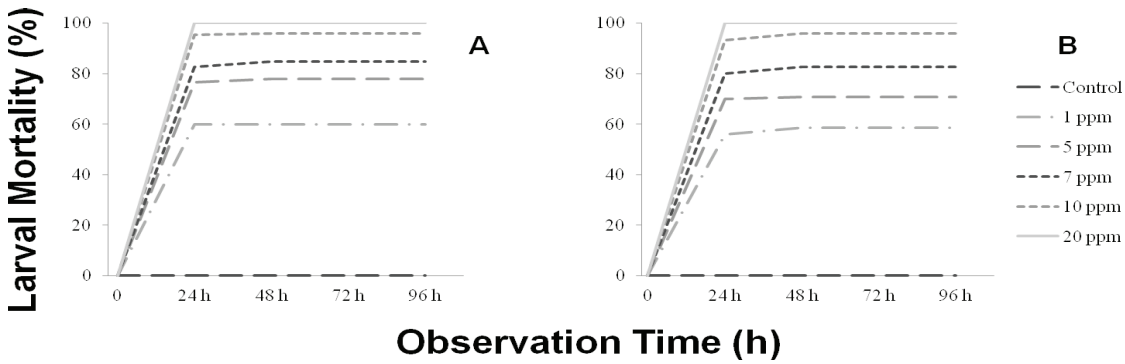

Figure 3. Larvicidal activity of alepterolic acid isolated from *Copaifera multijuga* against 3rd-4th instar larvae of *Anopheles darlingi* (A) and *Aedes aegypti* (B) (Diptera: Culicidae).

Discussion

All the *C. multijuga* extracts tested affected larval mortality of the malaria vector *A. darlingi* and the dengue vector *A. aegypti*. However, the degree of larvicidal activity depended on concentration and duration of the assays.

The present data indicate that *Copaifera* ethanolic extracts from different organs have significant larvicidal activity against the mosquito species tested. Ribeiro et al. (2009) reported that fruit peel and leaf extracts of *C. langsdorffii* lead to greater reduction in food consumption and larvae weight of *Spodoptera frugiperda* compared to fruit pulp and seed extracts. The fungitoxicity of *C. multijuga* essential oil was higher than the oil-resin. Furthermore, the response of different fungus species to this species was variable (Deus et al., 2011). Such variation in biological activity probably arises from differences in qualitative and quantitative active chemical compounds from *Copaifera*. For example, Chen et al. (2009) showed that the concentration of individual sesquiterpenes of *C. officinalis* isolated from various plant organs (leaves, stems and roots) were different.

Thus, comparing biological activity using *Copaifera* extracts or mixtures was not a straightforward process even when using the same species due to different biological aspects, such as the chemical composition of extracts and compound mixtures and species variation. For example, Silva et al. (2007) and Kanis et al. (2011) evaluated the larvicidal activity of the oil-resin of *C. reticulata* on *A. aegypti* and acquired very different lethal concentration (LC) values.

Oliveira et al. (2006) studied the oil-resin production and composition of *C. duckei, C. marttii*, and *C. reticulata* in Moju, Para, Brazil for several months in 2003 and 2004 and related differences in oil-resin production and composition, even between individuals of the same species.

A. darlingi larvae were more susceptible to *C. multijuga* extracts compared to *A. aegypti* larvae. However, susceptibility to larvicides in Anophelinae and Culicinae species did not follow a pattern. Joseph et al. (2004) demonstrated that *Culex quinquefasciatus* larvae acquired a higher LC50 than *A. gambiae* when exposed to petroleum extracts of *Neotatenia mitus*. Other studies showed that *A. stephensis* larval mortality to pure oil of *Dalbergia sissoo* was much lower than *A. aegypti* and *C. quinquefasciatus* (Ansari et al., 2000). Bond et al. (2004) related a similar larvicidal activity of spinosad, a neurotoxin mixture produced by an actinomycete, against *A. aegypti* and *A. albimanus*.

The calculated LC for *A. aegypti* was generally greater than *A. darlingi*, except for when essential oil and isolated substance was applied. Bark extract had a LC50 ten times lower than oil-resin for *A. darlingi*, but most biological assays and chemical characterization comprise *Copaifera* oil-resin only. Silva et al. (2001; 2003) tested the susceptibility of *A. aegypti* and *C. quinquefasciatus* larvae to the oil-resin extracted from *C. langsdorffii* and *C. reticulata*, respectively, and observed a gradual decrease in susceptibility as the larvae progressed from 1st to 4th instar. The estimated LC50 ranged from 0.4 (1st instar) to 80 ppm (4th instar); Prophiro et al. (2012) related a LC50 of 47 and LC90 of 91 ppm for wild *A. aegypti* using *Copaifera* spp. oil.

Larvicidal activity of isolated substances or subfractions of *Copaifera* spp oil-resin components resulted in a much lower LC for *A. aegypti*. For example, the LC50 of methanolic subfractions of *C. reticulata* for mono, sesquiterpenes, and labdane diterpenes were 3.9, 7.5 and 0.8 ppm, respectively (Silva et al., 2007). The labdane diterpene 3-β-acetoxylabdan-8 (17)-13-dien-15-oic acid isolated from *C. reticulata* with LC50 0.8 ppm (Geris et al., 2008) caused several ultra-structural alterations in the *A. aegypti* midgut and killed the larvae after 22 h of exposure (Valotto et al., 2011). Geris et al. (2008) also evaluated the larvicidal effect of the alepterolic acid against *A. aegypti* but received LC

values 100 times higher compared to the present work. This was not surprising since resistance to insectides (*e.g*., temephos) and detoxifying enzymes, such as GST and alfa esterases activity, may vary greatly within *A. aegypti* populations that are 10 km apart (Lima et al., 2011).

Finally, *C. multijuga* larvicidal activity against *A. darlingi*, the main malaria vector in Northern Brazil, is presented here for the first time. Chemical characterization of bark and leaf extract derived from *C. multijuga* and larvicidal evaluation of the isolated compounds against *A. darlingi* or the principal essential oil compounds against *A. aegypti* may provide insight into potential larvicidal compounds with a lower lethal concentration as demonstrated with alepterolic acid. The present data also show that *A. darlingi* are more susceptibility to *Copaifera* extracts compared to the dengue vector *A. aegypti*.

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Authors' contributions

FTTT contributed in the larvicidal experiments, analysis of the data and drafted the paper. AAP contributed in the phytochemical analysis. VAF supervised the phytochemical analysis and critical reading of the manuscript. RGS contributed to critical reading of the manuscript. AAS designed the study, supervised the laboratory work, drafted the paper and contributed with data analysis. All the authors have read the final manuscript and approved the submission.

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