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Evaluation of larvicidal activity of the methanolic extracts of *Piper alatabaccum* branches and *P. tuberculatum* leaves and compounds isolated against *Anopheles darlingi*

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Abstract: *Piper* is a notable genus among Piperaceae due to their secondary metabolites such as lignans, amides, esters and long chain fatty acids used as anti-herbivore defenses with comparable effects of pyrethroids, that holds a promise in insect control, including malaria vectors such as *Anopheles darlingi*, the main vector in the North of Brazil. Methanolic extracts of *Piper tuberculatum* Jacq., Piperaceae, and *P. alatabaccum* Trel. & Yunck., Piperaceae, and some isolated compounds, *i.e*, 3,4,5-trimetoxy-dihydrocinamic acid, dihydropiplartine; piplartine, piplartine-dihydropiplartine and 5,5',7-trimetoxy-3',4'-metilenodioxiflavone were tested as larvicides against *A. darling*i. The Lethal Concentrations (LC50 and LC90) of methanolic extracts were 194 and 333 ppm for *P. tuberculatum* and 235 and 401 ppm for *P. alatabacum*, respectively. Isolated compounds had lower LC values, *e.g.* the LC50 and LC90 of the piplartine-dihidropiplartine isolated from both plant species was 40 and 79 ppm, respectively.

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Introduction

Anopheles darlingi, among several anopheline species, is the main malaria vector in Rondonia and other Amazonian States. This is species is found in high densities and frequency in human anthropized environments (Gil et al., 2003). In this regard, the any biological study including this species is important for malaria control.

Insect resistance to synthetic insecticides proved to be a serious obstacle in the control of medical and agricultural important insects (Hemingway & Ranson, 2000), and, therefore, has stimulated researches aiming to the development of new chemicals (Mueller-Beilschmidt, 1990).

Several plants produce secondary metabolites that inhibit insect development (Chariandy et al., 1999), while others are repellents (Mohan & Fields, 2002). Thus, the use of plant metabolites is an interesting perspective to control insects, both adults and larvae.

The Piperaceae is a vast family of plants, which has been extensively used for medicinal purposes (Chauret et al., 1996). Within the Piperaceae family, the genus *Piper* has over 700 species, distributed throughout the tropical and subtropical regions of the world. Its phytochemistry has been object of extensive reviews (Sengupta & Ray, 1987; Parmar et al., 1997). With regard to the ethnopharmacological information, while the pungent and aromatic fruits of some species of *Piper* are used as spices, most of them find wide application in traditional systems of medicine (Sengupta & Ray, 1987; Parmar et al., 1997), as insecticides (Chauret et al., 1996; Park et al., 2002; Yang et al., 2002), antivirals (Lohézic-le et al., 2002) antimicrobials (Costantin et al., 2001) and particularly antifungals (Lopez et al., 2001). These biological properties have been attributed to the presence of lignans and/or amides, such as alkyl or olefinic isobutylamides, flavonoids, kawa-lactones, butenolides and cyclohexane epoxides, among

others (Parmar et al., 1997). In this study, we evaluated the larvicidal activity of the methanolic extracts of *P. alatabaccum* branches and *P. tuberculatum* leaves and compounds isolated against *Anopheles darlingi* (Diptera: Culicidae).

Materials and Methods

Plant material

The leaves of *Piper alatabaccum* Trel. & Yunck., Piperaceae, and *P. tuberculatum* Jacq., Piperaceae, were harvested in August 2009 Porto Velho, Rondonia State, Brazil. A voucher specimen has been deposited at the Herbarium of Instituto Nacional de Pesquisa da Amazonia, under the number 211720 for *P. alatabaccum* and 211724 for *P. tuberculatum.*

Isolation and identification of compounds

The air-dried leaves of *P. alatabaccum* (450 g) were extracted with 95% EtOH (3 x 1 L). The extract was concentrated, defatted with *n*-hexane, and partitioned with EtOAc. The EtOAc layer was concentrated and chromatographed on silica gel column (200-300 mesh, 90 g), eluting with *n*-hexane and subsequently with *n*-hexane-EtOAc in mixtures of increasing polarity (95:5-30:70) 52 fractions (80 mL each) were collected, from these fractions, was isolated 35.5 and 42.0 mg the two white solids called PAFET-1 and PAFET-2, respectively. The structures of PAFET-1 and PAFET-2 were deduced as a mixture of piplartine and dihydropiplartine and 5,5',7-trimetoxi-3',4'-metilenodioxiflavone , respectively (Facundo et al., 2005). The leaves of *P. tuberculatum* (1.2 kg) was extracted with 95% EtOH (3 x 3 L) and the extract was dried by a rotary evaporator under reduced pressure for to give 45 g EtOH extract. Part of this extract (36 mg) was submitted to column chromatography over 280 g of silica gel and eluted with a gradient of *n*-hexane, *n*-hexane:EtOAc, EtOAc:MeOH with increasing polarity and methanol. Fractions 8-15 (547.26 mg, extracted with *n*-hexane:EtOAc 20:80) were rechromatographed as in previous cases, and eluted with *n*-hexane, *n*-hexane:EtOAc gradient and EtOAc, and given 64.5 mg of dihydropiplartine , 33.1 mg of piplartine and 99.8 mg of 3,4,5-trimetoxi-dihydrocinamic acid (Facundo et al., 2008).

Mosquito collection and breeding

Adult mosquitoes were captured using a modified BG-Sentinel trap (Gama, data not published) in different localities of Porto Velho-RO, *i.e*., Vila Candelária (63° 55' 01''W 08° 47'17''S), Bate-Estaca (63° 55' 48'' W 08° 47' 55''S) e Santo Antônio (08° 48' 21.3''W 63° 56' 37''S). Females were blood fed on rabbits in the laboratory and oviposition was induced by wing removal after 72 h. Hatched larvae were kept under laboratory conditions (28 ºC, 80% RU and 12 h photoperiod) and fed with grinded fish food (TetraMin Tropical Flakes) up to 3° and 4° instar.

Larvicidal bioassays

Larvicidal tests were set in two phases, *i.e*. phase 1: a dose response test with three replicates and nine concentrations (ppm: 5, 10, 25, 50, 125, 250, 500, 750, 1000 extracts and ppm: 1, 5, 10, 20, 40, 60, 80, 100 isolated compounds) to estimate the concentrations for the Lethal Concentration (LC50 and LC90) assay (phase 2) using five different concentrations and four replicates. Phase 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 compounds were diluted in DMSO and pippeted under the water surface of plastic cups (150 mL) containing 100 mL of distillated water and larvae (10 for phase 1 or 25 for phase 2) were introduced in the cups 30 min after pippeting. Lethal Concentrations (LC) were calculated using Probit analysis and Weibull distribution (Minitab, Minitab Inc). The effects of extract and compound concentration on larval mortality were analyzed by TwoWay Anova (SigmaStat 2.0, 1992-1997) (WHO 2005).

Results

Larval mortality due to the exposure to extracts and isolated substances varied significantly with concentration and time ($p<0.05$), but no interactions were found ($p>0.05$) between them.

Piper alatabaccum Trel. & Yunck., Piperaceae, and *P. tuberculatum* Jacq., Piperaceae, methanolic extracts killed a mean up to 94% of the *A. darlingi* larvae in the highest concentrations related to lower concentrations tested (450 and 400 ppm, respectively ($F=22.4$; $p<0.001$ and $F=32.4$; $p<0.001$) (Figure 1A and Figure 2A). Larvae mortality differed significantly only between the 24 h observation and the other periods evaluated (*i.e.* 48, 72 and 96 h) for *P. tuberculatum* ($F=4,1$; $p=0.009$), but not among different periodic observations for *P. alatabaccum* ($F=2.3$; $p=0.082$) (Figure 2A).

Isolated substances from both plant species were also evaluated as larvicides to *A. darlingi.* Generally, the larvicidal effect of the isolated substances tested almost doubled from 24 h observation to the 48 h observation and larval mortality increased significantly ($p<0.0001$) until 96 h observation (Figure 1 and 2). Dihydropiplartine (Figure 1D), Piplartine (Figure 1B) and 3,4,5

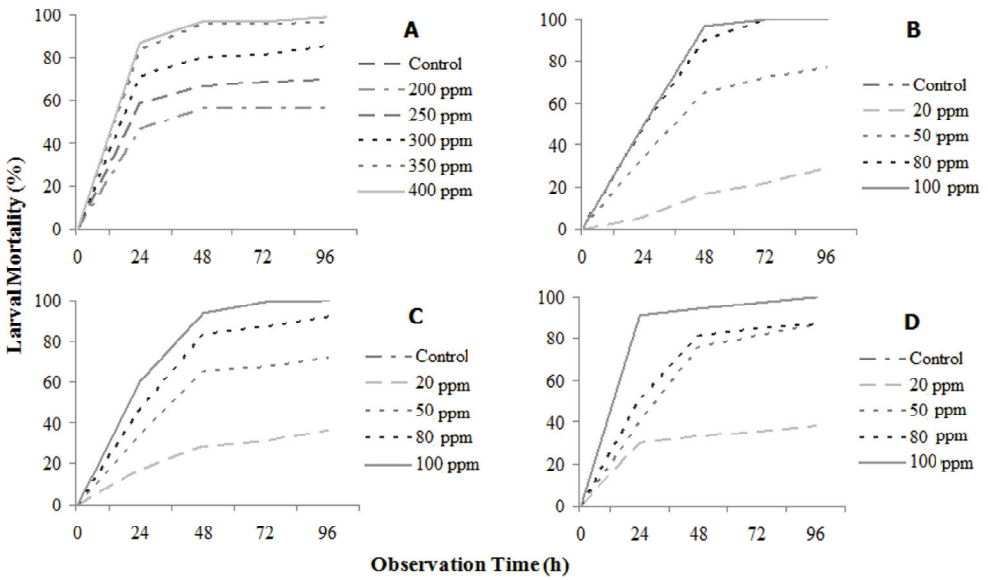

Figure 1. Larvicidal activity of *Piper tuberculatum*: Methanolic extract of leaves (A), Piplartine (B), 3,4,5-trimetoxy-dihydrocinnamic acid (C), dihydropiplartine (D) against 3º-4º instar larvae of *Anopheles darlingi* (Diptera: Culicidae).

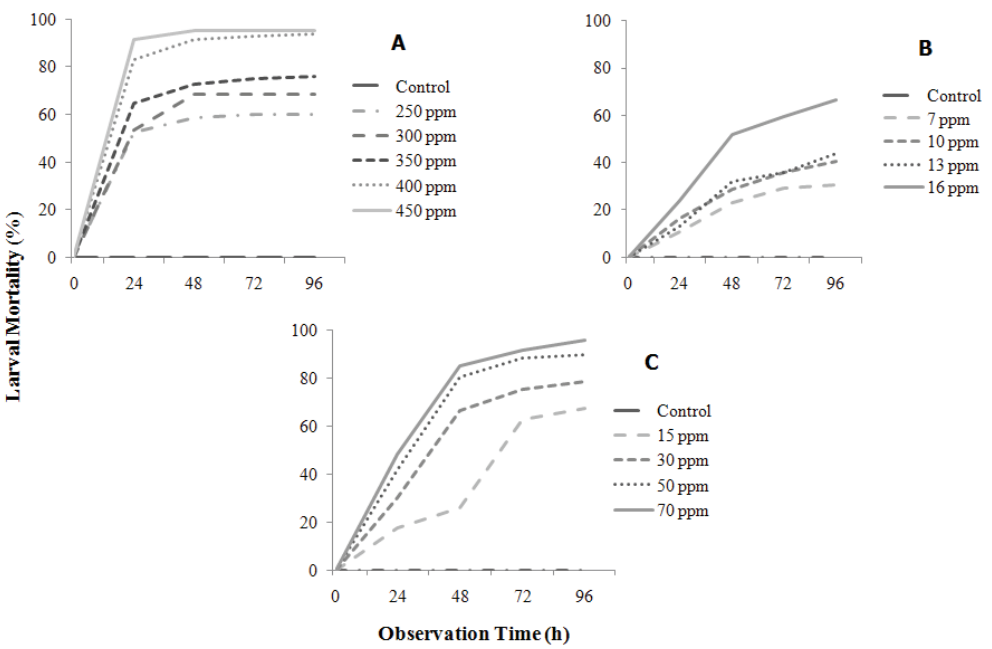

Figure 2. Larvicidal activity of *Piper alatabaccum*: Methanolic extract of branches (A), PAREM (piplartine-dihidropiplartine) (B) and 5,5',7-trimetoxy -3',4'-metilenodioxiflavone) (C) against 3°-4° instar larvae of *Anopheles darlingi* (Diptera: Culicidae).

trimetoxidihydrocinamic acid (Figure 1C) killed 85% of the larvae in the highest concentration tested (*i.e.*, 100 ppm) related to lower concentrations (F=133.2; $p<0.0001$; F=115.1, $p<0.0001$; F=179.4; $p<0.0001$, respectively). Other substances tested, 5,5',7-trimetoxi-3',4'-metilenodioxiflavone and PAREM killed a mean of 80% and 50% of the A. darlingi larvae in the highest concentrations tested (*i.e.*, 70 and 16 ppm, respectively), a significantly higher mortality than the lower concentrations tested (F=45.4; $p<0.0001$ and F=35.4; $p<0.0001$, respectively).

The Lethal Concentrations LC50 and LC90 for *P. alatabaccum* and *P. tuberculatum* were 235-401 and 194-333, respectively. The Piplartine-Dihydropiplartine (PAREM) had the lowest LC50 and LC90 values of the larvicidal assays (Table 1).

Table 1. Lethal Concentration (LC) in PPM of the extracts and isolated substances from *Piper tuberculatum* e *Piper alatabaccum* against de 3°-4° instar larvae of *Anopheles darlingi* (Diptera: Culicidae).

Extract/Substances	LC50	LC90
Piper tuberculatum (leaves)	194	333
Piplartine	40	79
Dihydropiplartine	29	95
3,4,5-trimetoxy-dihydrocinnamic acid	35	92
Piper alatabaccum (braches)	235	401
Piplartine-Dihydropiplartine (PAREM)	17	46
5,5',7-trimetoxy-3',4'-metilenodioxiflavone	24	72

LC50 and LC90: lethal concentrations necessary to kill 50% e 90%, respectively, of the larvae during assays; ppm: part per million.

Discussion

Most recognized insecticidal compounds from Piperaceae were isolated from *P. nigrum, P. guineense* and *P. tuberculatum* with several modes of action that includes contact toxicity, synergism, repellent, and antifeedant properties (see Scott et al., 2008 for a review).

Larvicidal activity of *Piper* sp. extracts or isolated substances greatly varies according to the mosquito species studied. Several studies focused on the larvicidal potential on culicine species, mostly *Aedes aegypti* using essential oils (*e.g*. Morais et al., 2007), but also *Culex* (*e.g*. Chansang et al., 2005) and fewer studies included anophelines (*e.g.* Cruz et al., 2011) or anophelines only (*e.g*. Matasyoh et al., 2011). The larval mortality caused by the crude extracts tested against the main malaria vector in Brazil, *i.e. A. darlingi,* in present work are similar or slightly lower than some crude extracts from other Piperaceae species tested against the dengue vector, *Aedes aegypti*. During laboratory tests with the aqueous extract of *Piper nigrum* the FNS (2001) related a larval mortality of 80% after 64 h. Pohlit et al. (2004) performed a screening for larvicidal effects of several Amazon plants against *A. aegypti,* including some Piperaceae. The 500 ppm of *P. tuberculatu*m extract of leaves, stalk and fruits killed all the larvae within 24 h.

But, Lethal Concentrations (LC) are also affected by extractions from different plant parts, *e.g.*, Regasini et al. (2009) related, in general, that crude extracts of *P. tuberculatum* obtained from leaves exhibited stronger antifungal activity than those from green fruits and branches.

Isolated substances from *P. tuberculatum* and *P. alatabaccum* had up to ten times lower LC for *A. darlingi* than crude extracts. Essential oil extracts or isolated compounds of some *Piper* species against larvae of *A. aegypti* or *A. gambiae* had much lower LC than crude aerial extracts. Morais et al. (2007) related an LC50 54 and 36 ppm of the essential oils of *P. hostmanianum* and *P. pernocronatum* on *A. aegypti* and Matasyoh et al. (2011) found that *P. capense* essential oil had an LC50 34,9 and LC90 85 ppm on *A. gambiae.* Despite of that, essential oils of *P. jacquemontianum* and *P. variabili* had no activity on *A. albimanus* and *A. aegypti* even at 1000 ppm (Cruz et al., 2011).

The 3,4,5-trimetoxi-dihydrocimanic acid activity against the larvae of *A. darlingi* was similar to the amides tested. Narasimhan et al. (2004) related that several derivatives of cinnamic acid presented antibacterial and antifungal similar or even higher than the standard control salicylic acid. Moreover, the author argued that the cinnamic acid moiety is necessary for the activity studied. On the other hand, Norton & Dowd (1996) tested the steryl cinnamic acid derivative fraction from corn bran at 100 ppm on the corn earworm (*Helicoverpa zea*) and the driedfruit beetle (*Carpophilus hemipterus*) and related no toxic activity on both insect species.

The flavone, 5,5',7-trimetoxy-3',4'-metilenodioxiflavone, isolated from *P. alatabaccum* was tested as a larvicide on *A. darlingi* and had the lowest LC value from isolated substances. Campos et al. (2005) related that orientin, a flavone isolated from *P. solmsianum*, was active against several filamentous fungi (dermatophytes) and Hoa et al. (2003) found that meliternatin, a flavone isolated from *Melicope subunifoliata,* was highly toxic to *A. aegypti* larvae.

Among the substances tested in the present work, the amide piplartine was, previously, evaluated for different purposes and presented promising results including anti-cancer (Costa-Lotufo et al., 2010); schistosomicidal (Moraes et al., 2011), and insecticidal (herbivores) activity (Dyer et al., 2003). Interestingly, the lowest LC values found resulted from a mixture of piplartine-dihydropiplartine. Dyer et al. (2003) also argued that the mixture of different amides from *P. cenocladum* had synergistic action on the toxic and deterrent effect against several herbivore species. Yang et al. (2002) related that pipernonaline isolated from *P. longum* has LC similar to a commercial mosquito larvicide, pirimiphos-methyl.

In a review, Scott et al. (2008) argued that piperamides, which act as neurotoxins with distinct mechanisms of pyrethroids, either singly or in combination, could replace contact insecticides with neurotoxic activity such as carbanates, organophosphates or pyrethoids to which insect resistance has developed. Thus, the investigation of the insecticidal potential of different *Piper* species on important vectors such as *A. darlingi* may provide new sources of molecules for vector control.

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