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Major Article

Larvicidal effects of endophytic and basidiomycete fungus extracts on *Aedes* and *Anopheles* larvae (Diptera, Culicidae)

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ABSTRACT

Introduction: *In vitro* bioassays were performed to access the larvicidal activity of crude extracts from the endophytic fungus *Pestalotiopsis virgulata* (Melanconiales, Amphisphaeriaceae) and the saprophytic fungus *Pycnoporus sanguineus* (Basidiomycetes, Polyporaceae) against the mosquitoes *Aedes aegypti* and *Anopheles nuneztovari.* **Methods**: The extracts were tested at concentrations of 100, 200, 300, 400 and 500ppm. Ethyl acetate mycelia (EAM) extracts and liquid culture media (LCM) from *Pe. virgulata* and *Py. sanguineus* were tested against third instar larvae of *Ae. aegypti* and *An. nuneztovari*. **Results**: The larvicidal activity of the EAM extracts from *Pe. virgulata* against *Ae. aegypti* had an LC_{50}=101.8ppm, and the extract from the basidiomycete fungus *Py. sanguineus* had an LC_{50}=156.8ppm against the *Ae. aegypti* larvae. The *Pe. virgulata* extract had an LC_{50}=16.3ppm against the *An. nuneztovari* larvae, and the *Py. sanguineus* extract had an LC_{50}=87.2ppm against these larvae. **Conclusions**: These results highlight the larvicidal effect of EAM extracts from the endophyte *Pe. virgulata* against the two larval mosquitoes tested. Thus, *Pe. virgulata* and *Py. sanguineus* have the potential for the production of bioactive substances against larvae of these two tropical disease vectors, with *An. nuneztovari* being more susceptible to these extracts.

Keywords: Endophytic fungi. Saprophytic fungi. Larvicidal. Tropical diseases.

INTRODUCTION

Dengue and malaria are tropical diseases of major concern for worldwide public health, particularly in underdeveloped countries. The global expansion of *Aedes aegypti* L. (1762) and the spread of dengue virus are currently major public health problems[1]. Demographic and social factors contribute to the epidemic levels of this disease[2] in urban areas, as the simultaneous circulation of all four dengue serotypes was described for the first time in the City of Manaus, State of Amazonas, Brazil, in 2011[3]. Additionally, malaria, which is caused by protozoans of the genus *Plasmodium*, is transmitted by mosquito vectors of the genus *Anopheles* and is important in rural and natural areas. In the Brazilian Amazon, *Anopheles nuneztovari* Gabaldón (1940) populations (cytotype A) are predominantly zoophilic and are not implicated as important malaria vectors[4]. However, in Colombia and Venezuela, populations of the same species are highly anthropophilic and are the primary malaria vectors[5,6]. Malaria interferes with child growth and affects the productivity of laborers, resulting in obstacles to socioeconomic development in affected countries[7]. The search for practical uses for natural compounds is one of the oldest activities of civilization. Control of mosquito larvae could represent an effective tool for integrated vector management to reduce malaria transmission[8,9]. Thus, the extracellular secondary metabolites produced by entomopathogenic fungi have been a focus of interest for insect pathologists[9]. Metabolites from fungal genera, such as *Metarhizium*[10], *Trichophyton*[11], *Chrysosporium*[12] and *Lagenidium*[13], as well as some actinomycetes[14] and several basidiomycetes[15], have shown potential insecticidal activity.

In this study, we utilized the Amazonian fungus *Pycnoporus sanguineus* (L.), Murrill 1904 (Basidiomycetes, Polyporaceae), to determine whether it contains metabolites with larvicidal activity. *Py. sanguineus* is a slow-growing saprophytic fungus, and it has long been used in popular medicine by indigenous tribes of Africa and the Americas to treat a number of illnesses[16-18]. In particular, cinnabarin, a substance produced

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by *Py. sanguineus,* has been shown to have antibacterial[19], cytotoxic and antiviral activities[20]. The genus *Pestalotiopsis* has also received attention because members of this genus have been shown to produce secondary metabolites with potential antimicrobial activity[21,22]. Most recent *Pestalotiopsis* studies have been based on endophytic isolates[15,23,24]. The aims of the present study were to examine products of the Amazon biodiversity and to identify fungi with potential larvicidal activity to control mosquito-vectored diseases.

METHODS

Mosquito culture and management

Aedes aegypti Linnaeus 1762: eggs were obtained from colonies at the Laboratório de Malária e Dengue of Instituto Nacional de Pesquisas da Amazônia – INPA, and the mosquitos were kept in cages in the insectary. Mosquitoes were maintained for oviposition in the insectary at 26 ± 2ºC with a photoperiod of 12:12 (L/D) and 80 - 90% relative humidity[25].

Anopheles nuneztovari Gabaldón 1940: collections of species from the genus *Anopheles* was conducted in the Manaus east area (Amazonas State, Brazil) near Farm Natan (03º04'10"S, 59º51'40"W). Catches were performed in cattle pens, and only feeding females were selected. Samples were obtained between the hours of 18 to 21h by manual capture. Mosquitoes were maintained for oviposition in the insectary, according to the criteria of Scarpassa and Tadei[26]. Females and eggs were identified according to a dichotomous key for the Culicidae family, generated by Faran[27].

Fungus collections and extractions

Plant origin and endophytic fungi: crude extracts of mycelium and metabolic medium were donated by the Laboratório de Produtos Bioativos de Origem Microbiana (LPBOM/UFAM). The endophytic fungi were isolated from *Murraya paniculata* (L.) Jack, Rutaceae, an exotic plant collected in São Paulo State, Brazil. And the endophytic fungus *Pestalotiopsis virgulata* was isolated from *Gustavia* cf. *elíptica* (S.A.), the Mori fruit, which is a member of the Lecythidaceae family from the Brazilian Amazon, which was collected from Fazenda Experimental da Agronomia (UFAM) in October 2008 (02º38'58"S, 60º03'09"W) and was preserved at LPBOM/UFAM, using the methodology described by Castelanni[28]. Crude extracts were used to perform the screening for larvicidal activity (data not shown).

Reactivation and culture of Pestalotiopsis virgulata: after preliminary test results to verify *Ae. aegypti* larva mortality (data not shown), the fungal stock and experimental cultures were grown at 26±2°C in stationary conditions in Erlenmeyer flasks, containing 300mL of PDA (potato dextrose agar) growth medium with glucose (10g/L) as a carbon source and L-asparagine (2.5g/L) as a nitrogen source. The stock culture was inoculated with a section from the agar culture, and the stock culture was grown until the mycelia had totally covered the surface. The 47 experimental flasks of liquid culture were inoculated with floating mycelium fragments (~4mm in diameter, aseptically cut from the stock culture) and were grown for 14 days.

Secondary metabolite extractions from Pe. virgulata: After 28 days, the mycelia from the liquid culture medium (LCM) of *Pe. virgulata* were isolated by filtration through Whatman no. 4 Chr filter paper. Mycelia were added to ethanol for macerating, and after 24h, the resulting mixture was filtered. This process was repeated twice. The mycelia extracts obtained were then concentrated in a rotary evaporator at reduced pressure. After observing the boiling point of each solvent, the extracts were weighed to obtain yield. The LCM was extracted in solvents with different polarity: ethyl acetate (EtOAc) and water (aqueous extract).

Secondary metabolite extractions from Pycnoporus sanguineus: Pycnoporus sanguineus specimens were collected in Reserva Forestal Adolpho Ducke (02º58'30"S, 59º56'01"W) of Instituto Nacional de Pesquisas da Amazônia - INPA (Manaus-AM, Brazil). The criteria described by Smânia et al.[29] were used for cultivating and obtaining crude extracts from *Py. sanguineus,* with some modifications. The above protocol was used to perform secondary metabolite extractions from *Py. sanguineus* extracts.

Larvicidal selective bioassay

These experiments were performed following the results of the first stage and were aimed to calculate the LC_{50} of selected extracts against the third instar larvae of the *Ae. aegypti* mosquito species. The selective bioassay results were used as a biological model for subsequent application of the extracts at the bioassay dose, to obtain the lethal concentrations (LC_{50} and LC_{90}) for larvae of *Ae. aegypti* and *An. nuneztovari*. The trials were performed at two concentrations (250 and 500ppm). Two selected extracts were targeted in these trials (data not shown), and these extracts showed larval mortality of more than 50% after 24h of exposure. The experiment was repeated three times. The percentage mortality was calculated using the mortality formula, and corrections were made when necessary using Abbott's[30] formula.

Larvicidal bioassay dose

The larvicidal bioassay was performed according to the recommendations of the WHO[31], with minor modifications. The following criteria were considered: the mortality in the control group should not exceed 10%, the confidence limit was set at 95%, and the bioassays were repeated on three alternate days. The bioassay dose experiments were performed using two selective bioassay extracts: GaFr3 2.3 Mycelium EtOAc (*Pe. virgulata*) and Pyc Mycelium EtOAc (*Py. sanguineus*) (data not shown). The larvicidal bioassay dose experiments were performed using five concentrations: 100, 200, 300, 400 and 500ppm. The negative control received only DMSO (dimethylsulfoxide) at the same concentrations, and the mortality rate of the control was not to exceed 10%.

Statistical analysis

The lethal concentrations (LC_{50} and LC_{90}) were determined by a probit analysis using POLO-PC®[32] LeOra Software, Berkeley, CA, USA. The mortality rate was corrected using Abbott's[30] formula.

RESULTS

Selective bioassay evaluation

The percentage mortality of *Ae. aegypti* following treatment with different concentrations of extracts from endophytic fungi and basidiomycetes fungi was analyzed in a selective bioassay evaluation (data not shown). The ethyl acetate mycelia (EAM) extract from *Py. sanguineus* was very effective against the third instar larvae of *Ae. aegypti*, promoting mortality of 67% of the larvae at a concentration of 250ppm and up to 98% of the larvae at a concentration of 500ppm. The EAM extract of the *Pe. virgulata* endophyte caused 81% mortality of the larvae at 250ppm and up to 100% of the larvae at 500ppm, thus showing greater effectiveness than the extract from the basidiomycete.

Dose evaluation to obtain the lethal concentrations (LC_{50} - LC_{90})

Aedes aegypti. As shown in **Table 1,** three of six fungal extracts that were assessed to determine their lethal concentrations against *Ae. aegypti* larvae showed toxicity values in which LC_{50} < 500ppm. The results calculated using the Polo-PC® program indicate that the data fit the probit model. The ethyl acetate mycelia extracts from *Pe. virgulata* and *Py. sanguineus* showed better mortality results against *Ae. aegypti* third instar larvae than the LCM extracts (**Table 1**, **Figures 1** and **2)**.

Figure 1 shows the regression lines for the larval mortality induced by 24h of treatment with the mycelia or LCM EtOAc extracts from *Pycnoporus* and *Pestalotiopsis*. We first compared the results from the *Pestalotiopsis* extracts **(Figure 1A)**. In this comparison between the LCM EtOAc-treated *Ae. aegypti* larvae and the EAM-treated *Ae. aegypti* larvae, the value of X^2 was significant. This finding led us to reject the hypothesis that the larvicidal effects of the two extracts would be equal **(Table 2)**. Therefore, the effective doses were qualitatively equal but quantitatively different. The relative potency of the larvicidal effects of the *Pestalotiopsis* LCM EtOAc extract (0.128) were less than those of the *Pestalotiopsis* EAM extract (7.769) **(Table 2)**.

We next compared the results of the *Pycnoporus* extracts against *Ae. aegypti* larvae. **(Figure 1B)**. In the comparison

TABLE 1 - Bioassay dose results of *Aedes aegypti* and *Anopheles nuneztovari* (third instar) larvae following 24h of treatment with different tested extracts.

Larvae species	Extract codes	LC_{50} ppm (95% CI)	LC_{90} ppm (95% CI)	χ^2 (*df* = 3)	Regression equation
Ae. aegypti	Pest LCM Aqueous Fr.	n.s.	n.s.	1.04	2.25x - 2.11
Ae. aegypti	Pest Mycelium Aqueous Fr.	n.s.	n.s.	1.35	1.42x - 0.51
Ae. aegypti	Pest LCM EtOAc	787.5 (623.8 - 1199.1)	2879.1 (1699.3 - 7911.1)	1.56	2.27x - 1.59
Ae. aegypti	Pyc LCM EtOAc	401.3 (330.0 - 532.2)	3448.8 (1811.7 - 12162.0)	0.65	1.37x + 1.43
Ae. aegypti	Pest Mycelium EtOAc	101.8 (54.7 - 138.2)	379.0 (290.3 - 619.7)	4.12	2.24x + 0.50
Ae. aegypti	Pyc Mycelium EtOAc	156.8 (55.5 - 228.0)	665.4 (405.6 - 4877.6)	10.01	2.03x + 0.53
An. nuneztovari	Pest LCM EtOAc	585.7 (466.1 - 862.7)	3777.5 (1976.3 - 13756.0)	1.46	1.58x + 0.62
An. nuneztovari	Pest LCM Aqueous Fr.	685.7 (545.1 - 1025.1)	3360.1 (1868.8 - 10498.0)	1.06	1.85x - 0.26
An. nuneztovari	Pest Mycelium EtOAc	16.3 (0.75 - 38.5)	74.4 (24.7 - 108.1)	1.69	1.94x + 2.65
An. nuneztovari	Pest Mycelium Aqueous Fr.	389.3 (339.1 - 462.9)	2289.3 (1510.7 - 4455.8)	0.92	1.66x + 0.69
An. nuneztovari	Pyc Mycelium	87.2 (16.5 - 137.9)	343.7 (237.0 - 947.1)	8.16	2.15x + 0.83

LC_{50}: median lethal concentration; CI: confidence interval; LC_{90} 90%: lethal concentration; n.s: not significant; *df:* degree of freedom. *Ae:Aedes; An: Anopheles.* Pest: *Pestalotiopsis;* LCM: Liquid culture medium; EtOAc: ethyl acetate; Pyc: *Pycnoporus.*

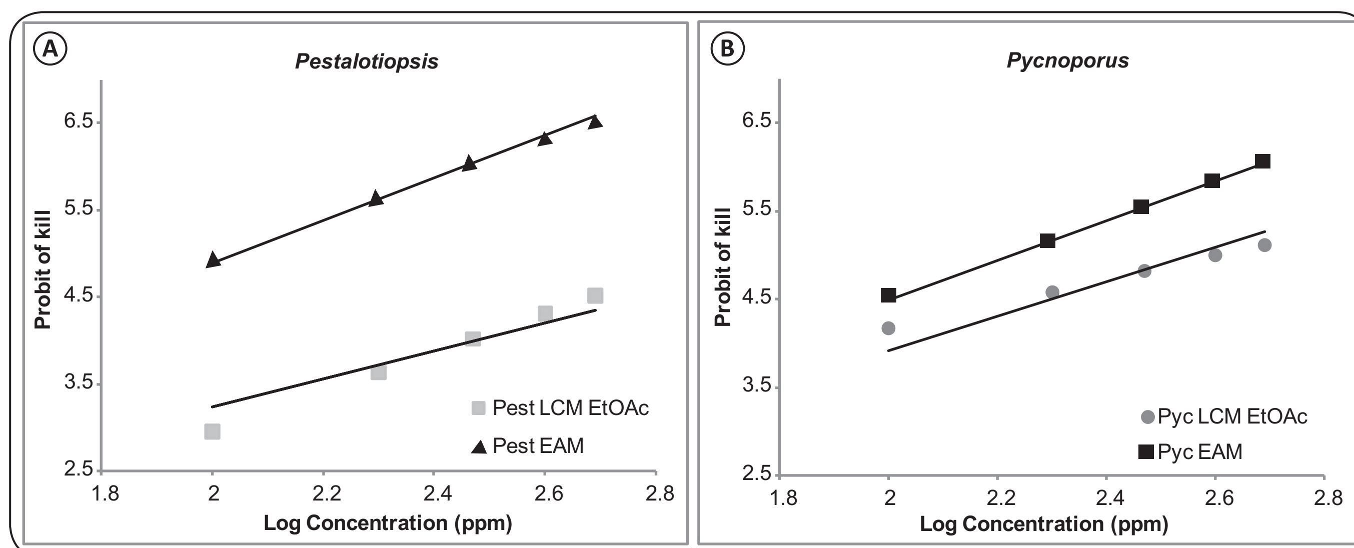

FIGURE 1 - Graphical representations of the probit values of mortality against the third instar larvae of *Aedes aegypti* following 24h of treatments with: A: ethyl acetate mycelia (EAM) extract and liquid culture medium (LCM) from *Pestalotiopsis virgulata;* and B: ethyl acetate mycelia (EAM) extract and liquid culture medium (LCM) from *Pycnoporus sanguineus*. Pyc: *Pycnoporus;* EtOAc: ethyl acetate.

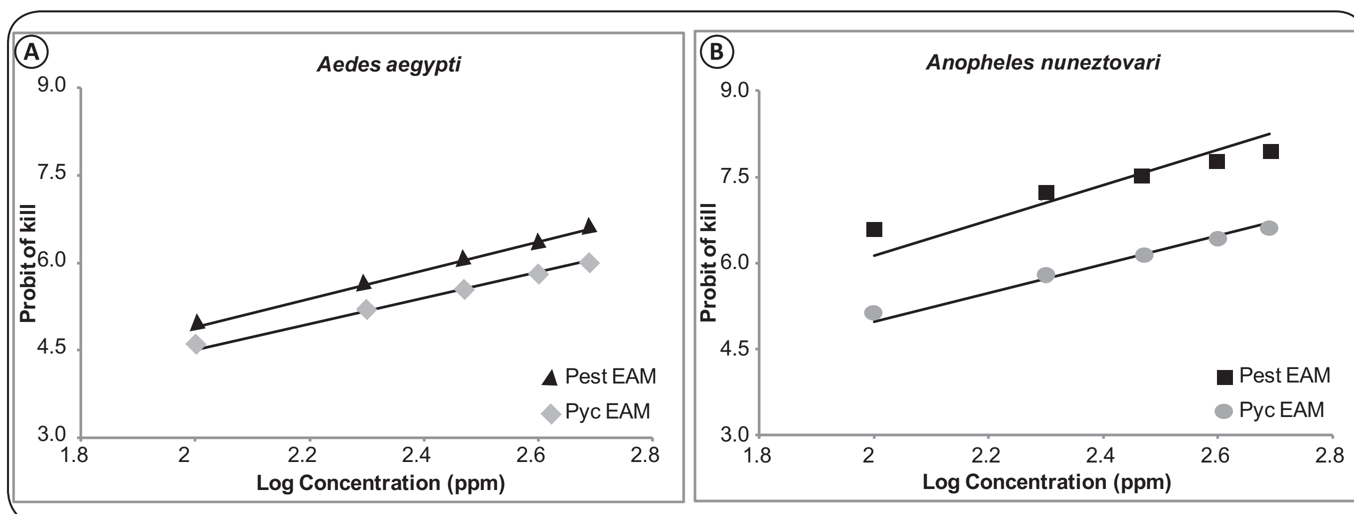

FIGURE 2 - Graphical representations of the probit values following 24h of treatment with ethyl acetate mycelia (EAM) extract from *Pestalotiopsis virgulata* and *Pycnoporus sanguineus* against third instar larvae of *Aedes aegypti* (A) and *Anopheles nuneztovari* (B). Pest: *Pestalotiopsis;* Pyc: *Pycnoporus.*

between the mortality induced by the *Pycnoporus* EAM extract and that induced by the *Pycnoporus* LCM EtOAc extract, the hypothesis that the extracts would result in equal rates of mortality was rejected, while the hypothesis that the slopes of the mortality curves would be parallel was not rejected. These data suggest that the effective doses of these fungal extracts were qualitatively equal but quantitatively different. The greatest larvicidal effects were observed for the mycelia extract from *P. sanguineus*, which also had the highest relative potency **(Table 2)**. Comparing the data from the mycelia fungus extracts from the two fungal species, we observed that the greatest potential larvicidal effects against *Ae. aegypti* were obtained for the genus *Pestalotiopsis*. We obtained an LC_{50} value of 101.8ppm for the *Pestalotiopsis* EAM and 156.8ppm for *Pycnoporus* EAM **(Table 1** and **Figure 2A)**. As shown in **Table 2**, the relative potency (RP) values were also calculated, and the *Pestalotiopsis* EAM (1.634) was shown to be more effective than the *Pycnoporus* EAM (0.612).

Anopheles nuneztovari

As shown in **Table 1,** three of five fungal extracts that were assessed to determine the lethal concentration against *An. nuneztovari* larvae showed toxicity values in which LC_{50} < 500ppm. The results presented using the Polo-PC® program indicated that the data fit the probit model. As shown in **Table 2,** the relative potency (RP) values were calculated, and the *Pestalotiopsis* EAM extract (RP = 36.221) was more effective than the *Pestalotiopsis* Mycelium Aqueous Fr. extract (RP = 0.027). In addition, the *Pestalotiopsis* EAM extract (PR = 47.981) was more effective than the *Pestalotiopsis* LCM

TABLE 2 - The tests for equal efficacy and parallel regression lines comparing the bioassays using *Pestalotiopsis virgulata* and *Pycnoporus sanguineus* extracts to treat against *Aedes aegypti* and *Anopheles nuneztovari* (third instar) larvae for 24h. The hypothesis for equal efficacy or parallel regression lines and values showing χ^2, p-value, slope ± SE and RP.

Larvae species	Extracts Compared	Hypothesis of Equal Efficacy	χ^2 (df = 2)	P	Hypothesis of Parallel Regression Lines	χ^2 (df = 1)	P	Slope ± SE	RP
Ae. aegypti	Pest LCM EtOAc x	rejected	541.8	< 0.001	not rejected	0.005	0.943	2.27 ± 0.37	0.128
	Pest EAM							2.24 ± 0.25	7.769
Ae. aegypti	Pyc LCM EtOAc x	rejected	88.41	< 0.001	rejected	4.223	0.040	1.37 ± 0.23	0.387
	Pyc EAM							2.03 ± 0.23	2.585
Ae. aegypti	Pyc EAM x	rejected	30.34	< 0.001	not rejected	0.371	0.542	2.03 ± 0.23	0.612
	Pest EAM							2.24 ± 0.25	1.634
An. nuneztovari	Pest Mycelium Aqueous Fr. x	rejected	600.4	< 0.001	not rejected	0.277	0.599	1.66 ± 0.24	0.027
	Pest EAM							1.56 ± 0.46	36.221
An. nuneztovari	Pest LCM Aqueous Fr. x	rejected	6.47	0.039	not rejected	0.478	0.489	1.85 ± 0.31	0.758
	Pest LCM EtOAc							1.58 ± 0.27	1.318
An. nuneztovari	Pest LCM EtOAc	rejected	679.7	< 0.001	not rejected	0.012	0.973	1.58 ± 0.27	0.019
	Pest EAM							1.56 ± 0.46	47.981
An. nuneztovari	Pyc EAM x	rejected	117.0	< 0.001	not rejected	0.033	0.856	2.15 ± 0.26	0.209
	Pest EAM							2.02 ± 0.65	4.770

LCM: Liquid culture medium; EAM: Ethyl acetate mycelia; EtOAc: ethyl acetate; *df*: degree of freedom; Pest: *Pestalotiopsis;* Pyc: *Pycnoporus;* χ^2: chi-square; SE: standard error; RP: relative potency; *Ae:Aedes; An: Anopheles.*

TABLE 3 - The ranking of the effective activity ranging LC_{50}.

	Aedes aegypti		*Anopheles nuneztovari*	
Extracts	LC_{50} - (ppm) (95% CI)	Effect	LC_{50} - (ppm) (95% CI)	Effect
Pest. LCM Aqueous Fr.	ns	nontoxic	689.1 (471.7 - 1737.2)	nontoxic
Pest Mycelium Aqueous Fr.	ns	nontoxic	397.9 (265.0 - 1202.6)	weak
Pest LCM EtOAc	805.4 (553.7 - 2766.5)	nontoxic	585.7 (466.1 - 862.7)	nontoxic
Pyc LCM EtOAc	335.1 (272.1 - 441.2)	weak	na	na
Pyc Mycelium EtOAc	153.6 (52.6 - 223.8)	moderate	87.2 (16.5 - 137.9)	moderate
Pest Mycelium EtOAc	101.8 (54.7 - 138.2)	moderate	11.9 (0.27 - 33.4)	high

LCM: Liquid culture medium; EtOAc: ethyl acetate; LC_{50}: median lethal concentration; CI: confidence interval; ns: not significant; na: not available; Pest: *Pestalotiopsis;* Pyc: *Pycnoporus.*

EtOAc extract (PR = 0.019). However, the *Pestalotiopsis* LCM EtOAc (PR = 1.318) extract was more effective than the *Pestalotiopsis* LCM Aqueous Fr. (PR = 0.758). The *Pestalotiopsis* LCM EtOAc was compared with the *Pestalotiopsis* LCM Aqueous Fr., and statistical analysis led us to reject the hypothesis that the larvicidal effects of the two extracts would be equal, while the hypothesis that the slopes of the mortality curves would be parallel was not rejected. These data indicate that the effective doses of these extracts were qualitatively equal but quantitatively different.

The larvicidal activity of the *Pestalotiopsis* EAM extract against *An. nuneztovari* larvae was compared with the larvicidal effects of the *Pycnoporus* EAM extract against the same larval species, and the LC_{50} values were observed to be 16.3 and 87.2ppm, respectively, **(Table 1** and **Figure 2B)** while the LC_{90} values were observed to be 74.4 and 343.7ppm. **Table 2** shows that the *Pestalotiopsis* EAM extract (PR = 4.770) was more effective than the *Pycnoporus* EAM extract (PR = 0.209). Statistical analysis led us to reject the hypothesis that the larvicidal effects of the two extracts would be equal, while the hypothesis that the slopes of the mortality curves would be parallel was not rejected. These data indicate that the effective doses were qualitatively equal but quantitatively different. Probit results indicated that the hypothesis that the larvicidal effects of the two extracts would be equal could be rejected and that the regression lines were parallel and were thus not significantly different.

Classification following the criteria of Komalamisra et al.[33], with minor modifications, classifies plant larvicidal activities as nontoxic when the LC_{50} is greater than 750ppm, weakly effective when the LC_{50} ranges from 200 to 750ppm, moderate when the LC_{50} is 50 - 100ppm and high when the LC_{50} is less than 50ppm. Thus, the activity of the ethyl acetate extract from *Pe. virgulata* against *Ae. aegypti* was considered moderate, while the ethanol extract was considered effective, and the aqueous (alkaline hydroethanolic) extracts showed without/low activity. Among the extracts evaluated against *Ae. aegypti* larvae **(Table 3)**, three of the fractions were nontoxic, one showed weak activity, and the other two fractions exhibited moderate toxicity. Among the extracts evaluated against *An. nuneztovari* larvae **(Table 3)**, two of the fractions were nontoxic, one had weak activity, one displayed moderate activity, and one displayed high activity. Against *Ae. aegypti* larvae, the EAM extracts from *Pe. virgulata* and *Py. sanguineus* showed weak toxicity, while the aqueous extracts were, for the most part, nontoxic. In contrast, against *An. nuneztovari* larvae, the EAM extracts from *Pe. virgulata* and *Py. sanguineus* had moderate to high toxicity, while the aqueous extracts showed without/low activity.

DISCUSSION

General

There is growing interest in the use of natural insecticides to reduce the use of synthetic pesticides and avoid environmental damage. The use of larvicidal compounds involves the application of chemicals to habitats to kill pre-adult mosquitoes. This practice can reduce overall pesticide use in a control program, by reducing or eliminating the need for ground or aerial chemical applications to kill adult mosquitoes[34]. The efficiency in killing larval instars of important vector species and the lack of effects on non-target organisms, as well as the biological stability of extracellular metabolites, make this practice a promising alternative to mycelium- and conidial-based larvicides[9]. These products could be considered fungal-based natural larvicides for vector control. Biological control of immature forms of *Anopheles nuneztovari* and *Anopheles darlingi*, which are species that are endemic to the Brazilian Amazon, under both laboratory and field conditions, has been well-documented in the literature[35,36].

The purpose of a general screen for bioactivity is to isolate as many potentially active constituents from a species as possible. This goal is achieved using two solvents: water, the most polar solvent, with a polarity index (PI) of 10.2; and an intermediary solvent, such as ethyl acetate (PI = 4.4)[37]. In this study, the separated fractions were assessed for their ability to control two major species of mosquito larvae (*Ae. aegypti* and *An. nuneztovari*). This experiment validated this approach and revealed the efficacies of the extracellular metabolites from *Py. sanguineus* and *Pe. virgulata* that were extracted with ethyl acetate.

Liquid culture medium

The insecticidal activity assessment of an aqueous extract allows for rapid and easy exploration of many species and compounds that are effective for the control of mosquito larvae[38]. In the first stage of this study, the aims were to evaluate ethyl acetate extracts from the mycelia and LCM and to evaluate the aqueous fractions of the mycelia and LCM. Thus, the larvicidal activities of the secondary metabolites and extracellular metabolites in the LCM from *Pe. virgulata* and *Py. sanguineus* were not confirmed, as our results showed weak/nontoxic activity. As shown in **Table 3**, we verified that, against *Ae. aegypti* larvae, only the LCM extract from *Py. sanguineus* showed weak toxicity (LC_{50} > 200ppm) at 24h. Unfortunately, it was not possible to perform a bioassay using the *Pycnoporus* LCM against *An. nuneztovari* larvae, due to insufficient amounts of the extract.

Liquid culture medium extracts from *Pe. virgulata* showed an LC_{50} > 500ppm in every assay; therefore, these extracts had weak/nontoxic activity against both vector species tested **(Tables 1** and **3)**. However, this fungal species was relevant in the initial screening, as the aqueous extract caused larva mortality at high extract concentrations[38]. This relevance was also evident using aqueous extracts from LCM, which did not have significant larvicidal effects against any of the tested larvae.

The results observed showed that ethyl acetate extracts from the studied fungus mycelia presented better larvicidal activity and that the ethyl acetate extract from the LCM did not induce satisfactory results. Fungi secrete secondary metabolites into their external environment to perform several activities, including obtaining food and providing defense against microorganisms[12,22]. These results suggest that the studied fungi did not produce metabolites with larvicidal activity when grown in liquid culture medium. It is also possible

that these metabolites were not produced by these fungi due to other factors, such as the lack of the necessity to produce such compounds in the absence of competition from microbes in the LCM or the lack of some other compound that would induce a fungal response. Finally, it might not be possible to obtain these secondary metabolites from LCM using the methodology applied in this work. Vyas et al.[13] used a different extract protocol to obtain second metabolites, and they demonstrated that *Lagenidium giganteum* metabolites, when filtered through Whatman filter paper applied directly to third instar larvae of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*, had LC_{50} and LC_{90} values against *An. stephensi* of 7.21 and 24.29ppm, respectively and against *Cx. quinquefasciatus* of 4.09 and 12.12ppm. However, the *Ae. aegypti* larval instars and all non-target organisms were not found to be susceptible. Mohanty and Prakash[11] demonstrated that filtrate extracellular metabolites from *Trichophyton ajelloi* were efficacious against larvae of the mosquito species *Cx. quinquefasciatus* and *An. stephensi*. Further results of Mohanty and Prakash[12] indicated that an LC_{50} of secondary metabolites against third instar *An. stephensi* of SDB-2.33mg/250mL, CB-1.28mg/250mL and against *Cx. quinquefasciatus* of SDB-1.08mg/250mL, CB-0.65mg/250mL was obtained. The purposes of these filtration processes were to decrease impurities in the filtrates and to enhance efficacy. This finding might suggest that further bioassays should utilize the successful techniques used by these authors[11-13], which might enable investigators to select more efficiently the active components present in the cellular mycelium of *Pe. virgulata* and *Py. sanguineus.*

However, *An. nuneztovari* larvae were susceptible to the aqueous extract fraction from the endophytic fungus *Pe. virgulata* **(**LC_{50} 389.3 ppm; **Table 1)**. Prakash et al.[39] utilized filtered metabolites of *Fusarium oxysporum* as test materials for larvicidal activity against *Cx. quinquefasciatus* and *An. stephensi* larvae, while the mycelium mass was discarded.

Ethyl acetate mycelium extract

The ethyl acetate mycelium **(**EAM) extracts from the entophytic fungus *Pe. virgulata* and from the basidiomycete fungus *Py. sanguineus* demonstrated larvicidal activity against both mosquito species tested. The EAM extract from *Py. sanguineus* (less polar), containing at least two major components (cinnabarin and a mixture of other phenoxazin-3-one substituted components), had higher larvicidal activity and presented greater potentially lethal results than the aqueous extracts (more polar). This finding indicates that the active larvicidal components of mycelia extracts of *Pe. virgulata* and *Py. sanguineus* must be low-polarity constituents. This observation agrees with the results of Aivazi and Vijay[40], who examined the ethyl acetate extract from oak gall (LC_{50} 116.92ppm). The aqueous extract in this study (more polar) had less activity against the two mosquito species.

The highest larval mortality was induced by the EAM extract from *Pe. virgulata* and *Py. sanguineus*. The results of the LC_{50} calculation of these two extracts demonstrated that these extracts were more effective against *Ae. aegypti,* with the EAM extract of *Pestalotiopsis* leading to an LC_{50} of 101.8ppm **(Table 1)** and the EAM extract of *Pycnoporus* leading to an LC_{50} of 156.8pm. Against *An. nuneztovari,* the EAM extract of *Pestalotiopsis* led to an LC_{50} of 16.3 ppm **(Table 1),** and the EAM extract of *Pycnoporus* led to an LC_{50} of 87.2ppm. Therefore, different sensitivities of mosquito species were observed in response to these extracts. Similar to these results, Bagavan et al.[41] showed that the ethyl acetate extract from the *Achyranthes aspera* leaf had effective larvicidal activity against *Ae. aegypti* and *Cx. quinquefasciatus* of 18.2 and 27.2 ppm, respectively, due to the presence of saponin. The xanthone sterigmatocystin, isolated from the endophytic fungus *Podospora* sp. and the plant *Laggera alata* (Asteraceae), was reported[42] to have a high level of potency against third instar larvae of *Anopheles gambiae,* with LC_{50} and LC_{90} values of 13.3 and 73.5ppm, respectively.

As shown in **Table 1**, many different reactions to mycelia extract appeared between the two tested mosquito species. The LC_{50} values of the mycelia extract were very high against *Ae. aegypti* cases, compared to *An. nuneztovari*, verifying the increased susceptibility of *An. nuneztovari* compared to *Ae. aegypti* larvae. According to Amer and Hehlhorn[34], these variations are not abnormal. In their results, the LC_{50} values of many oils were very high against *An. stephensi,* compared to the two other mosquito species tested, and except for few cases, *Ae. aegypti* was more sensitive than *Cx. quinquefasciatus*. Prakash et al.[39] showed that the extracellular metabolites from *F. oxysporum* were less effective against *An. stephensi* but were highly effective against *Cx. quinquefasciatus* larvae. This finding might have been due to the size of *Culex,* which has more surface area.

With the primary information available, studies can be undertaken to standardize extracts and to identify and isolate active components. Our results clearly show that *Py. sanguineus* extract, which contains cinnabarin, demonstrates high larval mortality. From the results, we can conclude that the two mosquito species' larvae were susceptible to the compounds in fungus extracts. Such findings could be useful in promoting research aimed at the development of new mosquito control agents, based on bioactive chemical compounds from indigenous fungus sources as an alternative to chemical larvicides, to suppress malaria vector mosquito populations.

The evaluation of the role of fungus extracts in the larvicidal bioassay against *Aedes aegypti* and *Anopheles nuneztovari* has demonstrated promising larvicidal activity. Studies investigating new substances are important to advancing the availability of control alternatives against malaria. Therefore, we conclude that the ethyl acetate mycelia extracts from *Pe. virgulata* and *Py. sanguineus* were more effective than ethyl acetate aqueous extracts and liquid culture extracts. The mycelia extract from *Pe. virgulata* was more effective than the ethyl acetate mycelia extract from *Py. sanguineus* against *Ae. aegypti,* as well as against *An. nuneztovari* larvae. *An. nuneztovari* larvae were shown to be more susceptible to mycelia extracts from both fungi, in comparison to *Ae. aegypti* larvae.

The results reported here provide the possibility for further efficient investigation of the larvicidal properties of natural product extracts. The isolation and purification of endophytic fungus crude extracts are in progress.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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