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Pathogenicity of Metabolites of Nematophagous Fungus *Paecilomyces Lilacinus* Against the Larvae of *Anopheles Stephensi* And *Culex Quinquefasciatus*

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Abstract
The main aim of the present investigation was to elucidate the pathogenic effect of secondary metabolites nematophagous fungus *Paecilomyces lilacinus* (Order: Monilialis) against all larval instars of malaria vectors, *Anopheles stephensi* and filaria vectors, *Culex quinquefasciatus*. However, the efficacy of these metabolites has so far not been tested as mosquito larvicide and this is the first report on it. *P. lilacinus* was previously isolated from adult housefly cadaver and grown on PDA medium, thereafter was cultured on PDB at 25 ∓ 1 C° and 70 ∓ 5% R.H for fifteen days, the extracellular metabolites were filtered by using whatman no.1 filter paper. The efficacies were conducted at five different concentrations (25, 50, 75, 100 and 150) ppm. and the mortalities of all larval instars for both mosquito vectors were assessed after 24, 48 and 72 hrs. and LC_{50} values were determined by probit analysis. The results showed that mortality percentages of all tested instar were directly proportional for both metabolites concentration, exposure time and inversely to larval age. Early instars of *An. stephensi* and *Cx. quinquefasciatus* were highly vulnerable to metabolites and mortality exceeded 90% after 72 hrs., therefor the metabolites of *P. lilacinus* were found better equipped against early instars for both species. The LC50 values were 24.5, 28.72, 32.78 and 40.96 ppm. for the four larval instars of *An. stephensi* respectively while in case of *Cx. quinquefasciatus* these values were 37.65, 42.88, 47.36 and 53.10 ppm. after 72 hrs. Overall, the results of susceptibility had shown the degree of tolerance to metabolite was in order $1^{st} > 2^{nd} > 3^{rd} > 4^{th}$ instar, and the metabolites induced high mortalities in all instars of *An. stephensi* comparable to *Cx. quinquefasciatus*. Eventually, the larvicidal potential of *P. lilacinus* metabolites may be altributed to secretion of bioinsecticidal molecules, like paecilotoxins and dipliconic acid, these can be fast acting metabolites for controlling larvae of malaria and filaria vectors.

Keywords: *P. lilacinus*; *An. stephensi*; *Cx. quinquefasciatus*; paecilotoxins; dipliconic acid.

Introduction

Globally, there are millions of humanbeings suffering from mosquito borne diseases such as malaria, filaria and viral encephalites. Malaria is caused by different species of *Plasmodium*, this obligatory protozoan parasite can´t develop and reproduce unless it passes through the haemocoel of certain species of *An. stephensi* mosquito. These vectors are *An. stephensi* in the middle and south of Iraq, while *An. sacharovi* in the north region (Abul-hab, 1979). On the other hand, filaria commonly known as elephantiasis is caused by *Wuchereria* or *Brugia*, these pathogenic nematodes are transmitted by different species of *Culex*, mainly *Cx. quinquefasciatus*. This species is also responsible for transmitting viral pathogens like, Chikungunga, West Nile Virus, Raft Valley Fever and Japanese encephalites.

Combat of these diseases was achieved by targeting the causative pathogens or controlling vectors, which has become the main dependant strategy, since it is easier than controlling pathogens. Chemical control has been one of most widely used traditional methods and still the main approach to reduce incidence of epidemic diseases, since it is inexpensive and causing prompt mortality.

However, problems associated with application chemical insecticides, they have adverse impacts on environment involving resources pollution, targeting beneficial animals and creating insecticides resistance in principal vectors (Hemingway and Ranson, 2000). These limitations are a serious drawback and highlighted the need to explore alternative approach that either integrate or replaced the conventional insecticides. Microbial control is considered a fundamental part of recently launched vectors control program and has so far shown promising results. One of the most promising tools are fungi and their derivatives.

Different fungal species were tested and found potentially mosquitocidal such as *Leptolegina* and *Laginidium* (Oomycota), *Coelomomyces* (Chytridiomycota), *Entomopathora* (Zygomycota) (Scholte *et al.*, 2004).

However, *Hyphomycetes* (Deutromycota) includes many

other fungi that kill larvae or adult's mosquito, like *Metarhizium*, *Beauveria* and *Culicinomyces*. *Paecilomyces* (Order: Monilialis) includes thirty – one species, among them many species are known pathogenic for Arthropoda, such as *P. fumoroseus*, *P. farinosus*, *P. amoeneroseus*, *P. javanicus*, *P. tenuipes* and *P. lilacinus*, which is a common soil hyphomycetes (Family: Moniliaceae).

Virtually, it is nematophagous against eggs and cysts of root – knote nematodes (Khan *et al.*, 2004).

However, this fungal species was isolated from different insects like, Cimicids, Weevils, Bugs, Whiteflies and Triatomin bugs (Marti *et al.*, 2006), moreover, it is isolated from house fly adults in Iraq (Burhan and Annon, 2020).

Little information's and rare reports on evaluating the pathogenicity of culture filtrate of this fungus against larvae of *An. stephensi* and *Cx. quinquefasciatus*.

In Iraq, as far as the author aware the larvicidal activity of *P. lilacinus* against Malaria and Filaria vectors has not been determined and the present results is pioneered and can be suggested as alternative tool for mosquito control.

Material and Methods

Insects Culture

1- Cx. quinquefasciatus

Laboratory colony was established since April, 2018 following the rearing method of (Gerberge *et al.*, 1994).

The culture was maintained at 28 ∓ 2 C°, 75% R.H and 12 D: 12 L. photoperiod. Larvae were removed and placed in jars (1000ml capacity) at a density of 100 larvae per jar tilled with dechlorinated water and supplemented with 100mg yeast powder. Larvae were procured for bio efficacy according to their larval age (first to fourth) and were put in discrete jars.

2- An. stephensi

Engorged adults' females were collected by aspirator and kept in a cage containing a number of pans as deposition sites. WHO (2006) procedure was followed. To colonies a permanent culture, the latter was maintained at lab. conditions as previously mentioned for *Cx. quinquefasciatus*. Newly laid eggs were transferred to a new jars (1000ml) containing distilled water and supplied with 100gm yeast powder.

Newly hatched larvae were reared for requisite instar (L_1 to L_4) and were put separately in discrete jars for Larvicidal assay.

Source of fungal strain

P. lilacinus isolates was obtained from Lab. of Mycology, Coll. of Science and maintained in culture on potato dextrose agar (PDA) slopes in universal tube. Subculture were grown at 25 C°.

Preparation of broth and isolation of extracellular metabolites

P. lilacinus was cultured on potato dextrose broth (PDB) according to (Soni and Prakash, 2012) as follows: five 250ml conical flasks, each containing 100ml of (PDB) [Infusion of potatoes, 200gm, dextrose 20gm, deionized water 1000ml) were autoclaved.

Thereafter, each conical flask was inoculated with 0.5cm. section from the PDA culture using sterilized corkborrer, these conical flasks were incubated at 25C° for 15 days.

After this incubation period, the extracellular metabolites were isolated by filtering the broth through whatman No.1 filter paper from many times.

Larvicidal activity

To test the larvicidal activity of extracellular metabolites of *P. lilacinus* against all instars of *An. stephensi* and *Cx. quinquefasciatus*.

The standard method (WHO, 2005) was followed. Five test concentrations of extracellular metabolites in 100ml of distilled water were prepared (i.e.: 25, 50, 75, 100 and 150) ppm in 250ml beakers.

Twenty larvae of each instar and for each mosquito species were separately exposed to 100ml of selective test conc. Each bioassay replicated for three times and the control treatment was run to estimate the natural mortality in which only distilled water was used. The mortalities were counted after 24, 48 and 72 hrs. post treatment.

The larvae in all bioassay experiment were fed on yeast powder.

Statistical Analysis

The data were managed according to completely randomized design (CRD). Least significant differences (L.S.D) with 95% confidence limit were assessed to evaluate the statistical differences between treatments.

Mortality rates were corrected according to Abbott formula (Abbott, 1925).

Efficacy of extracellular metabolites against larvae of both mosquito species assessed by probit analysis (Finney, 1971).

Results and Discussion

The lethal effects of extracellular metabolites of *P. lilacinus* was evaluated against the four instars larvae of *An. stephensi* and *Cx. quinquefasciatus* at five different test concentration (25, 50, 75, 100 and 150) ppm after 24, 48 and 72 hrs. The mortality percentages of all instars of both mosquito species were directly proportional to both metabolites' concentration and exposure period, and inversely related with larval age.

Table (1) elucidated that mortality percentage of first instar of *An. stephensi* was increased from 16.7% to 75% post treatment with (25 - 150) ppm after 24 hrs., while the percentage of mortality was elevated to (50 – 93.3%) after 72 hrs. of treatment. Whilst, the subsequent instars more tolerant to the culture filtrate, For example:

The mortality percentages were recorded as 90%, 81.66%, and 73.33% for second, third and fourth instar respectively when treated with higher concentration of extracellular metabolite after 72 hrs. Similarly, the mortality percentages in case of larval instars of *Cx. quinquefasciatus* were apparently affected when exposed to different concentrations of secondary metabolites of *P. lilacinus* and showed highest mortality at maximum concentration for all instar larvae after 24, 48 and 72 hrs.

For instance, the mortality percentage attained 83.3%, 75%, 73.66% and 71.6% for first, second, third and fourth instar after a period of 72 hrs. post treatment with 150 ppm of culture filtrate of *P. lilacinus* (Table 1).

Table 1. Mortality percentages of larval instars of *An. stephensi* and *Cx. quinquefasciatus* at different concentration of extracellular metabolites of *P.lilacinus*.

Instar	Conc. ppm	% Mortality after					
		An. stepensi			*Cx. quinquefasciatus*		
		24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.
First	25	16.66	33.33	50.00	11.66	25	40.00
	50	30.00	58.33	66.66	25.00	50	56.66

	75	36.66	61.66	75.00	30.00	53.33	60.00
	100	50.00	70	86.66	43.33	61.66	75
	150	75.00	65.00	93.33	65.0	73.33	83.33
	Control	0.00	0.00	0.00	0.00	0.00	0.00
Second	25	13.33	28.33	43.33	11.66	20.00	33.33
	50	25.00	51.66	60.00	25.00	45.00	50.00
	75	31.66	56.66	66.66	26.00	46.66	60.00
	100	45.00	63.33	78.33	36.00	53.33	66.66
	150	68.33	76.66	90.00	56.66	66.66	75.00
	Control	0.00	0.00	0.00	0.00	0.00	0.00
Third	25	10.00	23.33	35.00	6.66	18.33	30.00
	50	20	45.00	51.66	18.33	38.33	46.66
	75	23.3	48.33	58.33	20.00	41.66	53.33
	100	35.00	55.00	70.00	30.00	46.66	63.33
	150	56.66	68.33	81.66	50.00	61.66	71.66
	Control	0.00	0.00	0.00	0.00	0.00	0.00
Fourth	25	5.00	16.66	26.66	3.33	13.33	23.33
	50	15.00	36.66	43.33	11.66	33.33	40.00
	75	20.00	41.66	50.00	13.33	35.00	46.66
	100	25.00	45.00	61.66	18.33	40.00	56.66
	150	35.00	58.33	73.33	28.33	53.33	73.33
	Control	0.00	0.00	0.00	0.00	0.00	0.00

Value of L.S.D (0.05) for: Conc.=1.6; Time=1.3; Instar=1.6; Species=1.14

Moreover, relevant results were evidenced that extracellular metabolites of fungus *P. lilacinus* were induced higher mortality in all instars larvae of *An. stephensi* comparable to that of *Cx. quinquefasciatus* (Fig 1).

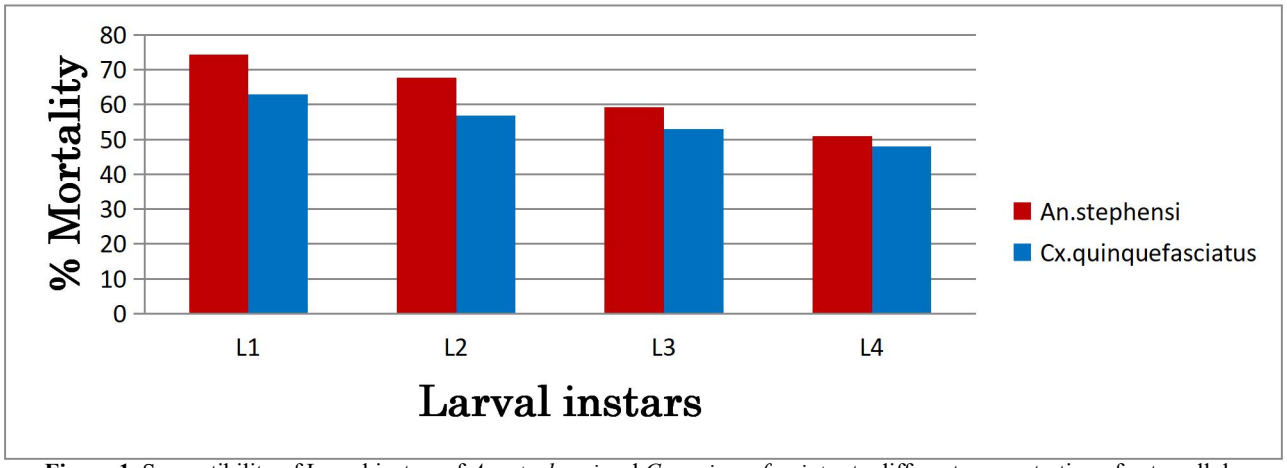

Figure 1. Susceptibility of Larval instars of *An. stephensi* and *Cx. quinquefasciatus* to different concentration of extracellular metabolites of *P.lilacinus*.

Additionally, when data on efficacy were subjected to probit analysis, the lethal concentration that kill 50% of individuals (LC_{50}) and probit regression equation were estimated (Table 2 and 3).

In case of *An. stephensi*, Table (2) indicated that the LC_{50} values for first, second, third and fourth instar after 24 hrs. were 32.9, 35.65, 39.33 and 44.97 ppm while after 48 hrs., the values of LC_{50} for mentioned instars respectively were 29.82, 31.43, 36.25 and 42.38 ppm.

Lastly, after 72 hrs. LC50 were 24.50, 28.72, 32.78 and 40.96 ppm for first, second, third and fourth instars respectively. Whereas in case of *Cx. quinquefasciatus* Table (3) summarized that LC50 values for first instars were 45.90, 40.72 and 37.65 ppm for second instars were 48.66, 46.23 and 42.88 ppm, for third instar were 52.76, 49.95 and 47.36 ppm, for fourth instar 60.22, 56.46 and 53.10 ppm after 24.48 and 72 hrs.

Overall, the results of susceptibility of larval instars for both malaria and filaria vectors had shown the degree of tolerance to whatman filtered metabolites was in order first > second > third > fourth instar.

Table 2. Probit equation and larvicidal activity of extracellular metabolites of *P. lilacinus* against *An. stephensi* after 24, 48 and 72 hrs

1st instar			2nd instar			3rd instar			4th instar		
24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	
32.98	29.82	24.50	35.65	31.43	28.72	39.33	36.25	32.78	44.97	42.38	
15.70–51.25	22.6 – 48.28	18.63–40.91	17.27–55.91	16.30–51.81	20.97–68.6	22.16–61.8	20.63–59.68	21.47–51.52	26.28–48.17	30.09–50.82	
12.47	9.48	4.67	4.68	8.95	16.04	5.61	1.86	15.78	3.54	6.12	

0.052	0.007	0.00	0.009	0.016	0.00	0.024	0.013	0.004	0.013	0.031
Y=−0.56 + 0.54X	Y=−0.73 + 0.76X	Y=−1.41 + 3.32X	Y=−1.11 + 0.78X	Y=−1.18 + 0.66X	Y=−0.49 + 1.13X	Y=−1.15 + 0.60X	Y=−0.81 + 0.71X	Y=−0.46 + 0.83X	Y=−0.46 + 0.83X	Y=−0.95 + 0.63X

Table 3. Probit equation and larvicidal activity of extracellular metabolites of *P. lilacinus* against *Cx. quinquefasciatus* after 24, 48 and 72 hrs.

1st instar			2nd instar			3rd instar			4th instar	
24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.
45.90	40.72	37.65	48.66	46.23	42.88	52.76	49.95	47.36	60.22	56.46
28.27–60.23	21.89–51.66	18.29–41.72	25.72–56.7	23.95–51.56	25.5–58.41	22.33–61.8	20.72–55.9	19.92–49.63	30.22–61.85	30.19–58.25
10.47	7.09	6.22	6.68	10.95	14.04	8.90	10.86	12.78	6.12	3.54
0.018	0.007	0.033	0.020	0.011	0.070	0.021	0.014	0.010	0.013	0.111
Y=−0.78 + 0.74X	Y=−0.69 + 1.86X	Y=−0.90 + 0.71X	Y=−0.87 + 0.82X	Y=−0.92 + 0.78X	Y=−1.05 + 0.82X	Y=−0.85 + 0.64X	Y=−0.50 + 0.80X	Y=−0.70 + 0.90X	Y=−0.56 + 0.81X	Y=−0.82 + 0.68X

P. lilacinus is one of soil – borne fungi and infects principally cyst and eggs of different nematodes, mosquito are not listed as natural host for *P. lilacinus* (Scholte *et al.*, 2004) it his fungus been tested against some agricultural and medical insects, such as rice plant hopper *Nilaparvata lugens* (Homoptera: Delphacidae) (Rombatch *et al.*, 1986).

Larvae and adults of *Musca domestica* (Mwamburil *et al.*, 2011; Burhan and Annon, 2020) and *Tetranychus kanazawi* (Sanjaja *et al.*, 2016). Nevertheless, the efficacy of filtrated secondary metabolites of this nematophagous fungus has so for not been tested as mosquito larvicide and this is the first report on it.

Many researches have shown the efficacy of secondary metabolites of different fungal species against different mosquito vectors.

Prakash *et al.*, (2010) have tested the pathogenicity of *Fusarium oxysporum* against the larvae of *An. stephensi* and *Cx. quinquefasciatus*, they observed that extracellular metabolites of mentioned fungus were less effective on larvae of *An. stephensi* but highly effective on *Cx. quinquefasciatus*, and they added that the first and fourth instars of malaria vector were more sensitive, whereas third and fourth instars of filaria vector were more sensitive on the contrary of our results.

The role *Metarhizium anisopliae* metabolites for controlling *An. stephensi* and *Cx. quinquefasciatus* have been assessed, these metabolites were found more effective on larvae of *An. stephensi* (Almuhana, 2011) this finding seemed to be consistent with the present results. Almashkoor (2014) affirmed that secondary metabolites of *Chrysoporium keratinophilum* were most effective on first instars of *Cx. quinquefasciatus* and caused more than 90% mortality, these results seemed in accord with efficiency of *P. lilacinus.*

Therefore, the extracellular metabolites of *P. lilacinus* was found to be better equipped against the early instars of *An. stephensi* and *Cx. quinquefasciatus*.

Furthermore,Vyas *et al.* (2015) have demonstrated that larvicidal activity of *Metarhizium anisopliae* against *Aedes aegyptii* and *Cx. quinquefasciatus* and found that lethal concentration have shown the degree of susceptibility for all larval instars of *Culex* was in order of $1^{st} > 2^{nd} > 4^{th} > 3^{rd}$ and for *Aedes* in order of $1^{st} > 4^{th} > 2^{nd} > 3^{rd}$, and showed that *Culex* larvae were more sensitive than *Aedes* larvae, while the present results proved that secondary metabolites of *P. lilacinus* have shown that susceptibility of larval instars for both *An. stephensi* and *Cx. quinquefasciatus* in order of $1^{st} > 2^{nd} > 3^{rd} > 4^{th}$.

Moreover, Alganimi and Al-hasnawi (2017) have tested the larvicidal activity of *Penicillium marneffei* against *Cx. quinquefasciatus* and concluded that first instars were very most sensitive while the fourth instars were tolerant.

Later, Aljumaily and Al-hasnawi (2017) have determined the pathogenic effects of *Asperigillus* parasitcus on *Cx. quinquefasciatus* larvae, they proved that first larval instars were Vulnerable to the secondary metabolites and exhibited higher mortality than subsequent instars. *P. lilacinus* like the rest of fungal species, created during its developmental stages a variety of metabolic products that were secreted into its ambient, these vital molecules were played a substantial role in perpetuating fungal persistence and environmental sustainability and enhanced fungus to obtain food and enabled with standing harmful circumstances and undesirable enemies (resistance), also these biological constituents determined the fungus virulence, like proteolytic enzymes and different toxins which may act in synergetic action.

P. lilacinus has produced diverse of enzymes like Lipaze, Chitnase, Protease and array of toric substances such as paecilotoxins that includes leucinostation A and B in addition to secretetion of Diplicomic acid, this can be fast – acting metabolite for controlling larvae of mosquito (Barhan and Annon, 2020).

Overall, the larvicidal activity of secondary metabolites of *P. lilacinus* may be due to the presence of these major bio insecticides molecules.

The outcome of present communication is that secondary metabolites of *P. lilacinus* can be promising larvicidal potential against early larval instars of malaria and filaria vectors which afford, easy, cheap and ecofriendly attitude.

Further efforts on characterization of biologically active molecules or compounds one necessitated, besides application in field. This may be modest recommendation for mosquito vector control.

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