Original Article

Larvicidal Effects of Metabolites Extracted from *Nocardia* and *Streptomyces* Species against the Forth Larval Stage of *Anopheles stephensi* (Diptera: Culicidae)

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Abstract

Background: Larvicidal agents can be produced using microbial resources, which are environmentally friendly, biodegradable, and economical. The study's goal was to evaluate the larvicidal activity of metabolites isolated from *Nocardia* (*N. fluminea*, *N. soli* and *N. pseudobrasiliensis*) and *Streptomyces* (*S. alboflavus*) bacterial species against *Anopheles stephensi*.

Methods: Four metabolites isolated from *Nocardia* and *Streptomyces* strains were exanimated for larvicidal activity. The experiments were performed for 24, 48, and 72 hours. 300, 350, 400, 450, 500, 550, and 600 µl of *Actinobacteria* metabolites were added to 100 cc of dechlorinated water. Fourth-stage larvae were placed in dechlorinated water as a control. LC_{50} and LC_{90} were calculated using toxicity data and analyzed.

Results: All metabolites had a statistically significant influence on mosquito larvae ($P< 0.05$). At 24, 48, and 72 hours, the LC_{50} for N2 (*N. fluminea*) was 417, 386, and 370 ppm, respectively, and the LC_{90} was 650, 595, and 561 ppm. Moreover, LC_{50} for N4 (*N. soli*) was 389, 376, and 347 and LC_{90} were 591, 565, and 533 and LC_{50} for N5 (*N. pseudobrasiliensis*) was 390, 357, and 341 ppm and LC_{90} were 589, 532 ppm. In addition, LC_{50} for S921 (*S. alboflavus*) was 484, 416, and 382 ppm, and LC_{90} was 701, 612, and 574 ppm.

Conclusion: The four bacterial metabolites tested in our study were found to have a notable effect on the mortality rate of *Anopheles stephensi* larvae, indicating their potential as natural larvicides. This is an effective technique for controlling *Anopheles stephensi* that has no detrimental environmental impact.

Keywords: *Anopheles stephensi*; Larvicidal; *Nocardia*; *Streptomyces*

Introduction

Anopheles stephensi is a mosquito species that is considered a potent disease vector, as it can transmit several diseases, including malaria, lymphatic filariasis, dengue fever, chikungunya, and Zika virus, among others (1–3). Malaria threatens the lives of over a million people every year and infects an estimated 300 million people worldwide annually (5). Djibouti (2012), Ethiopia (2016), Sri Lanka (2017), and most recently, the Republic of Sudan (2017) have all reported the presence of *A. stephensi* (1). Each year, more than 700,000 people die due to vector-borne diseases, which account for more than 17 percent of all infectious diseases (6).

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In Iran, several mosquito-borne diseases can be found, including six arboviral (avian pox, bovine ephemeral, Rift Valley, Sindbis, and West Nile fever), two bacterial (anthrax and tularemia), four helminthic (*Deraiophoronema evansi* infection, dirofilariasis, wuchereriosis, and seteriasis), and two protozoal (bird malaria and human malaria) diseases (7–10).

Controlling mosquitoes is critical to preventing the spread of these diseases, but the widespread use of chemical insecticides has made mosquitoes more resistant to them (11). Efforts to control mosquitoes are primarily based on the use of natural or synthetic insecticides, but their effectiveness has been declining due to the development of resistance (12, 13). As a result, researchers are exploring alternative methods, such as the use of microbial resources to produce larvicidal agents, which are eco-friendly, biodegradable, and cost-effective (14). Microbial metabolite compounds have been observed to be very toxic to mosquitoes while having fewer adverse effects on non-specific targets (15, 16).

Actinobacteria are a group of Gram-positive bacteria responsible for producing about half of the discovered secondary metabolites with significant medicinal and commercial value (17). Several antibiotics, including streptomycin, erythromycin, and chloramphenicol, have been derived from *Actinobacteria* (18–20). Additionally, extremophilic *Actinobacteria* constitutes a valuable antibiotic source due to their rapid growth rate and propensity for their mycelium to break down and lyse quickly (21). These bacteria decompose resistant substances in soil and produce antibiotics such as aminoglycosides, erythromycin, and chloramphenicol (22, 23).

Members of the genus *Actinomycetes*, which are a type of *Actinobacteria*, produce insecticidal active compounds used to develop biocontrol of the housefly (*Musca domestica*) (24). Following the use of *Actinomycetes* insecticide, the mortality of larval and pupal stages has been reported to reach up to 90% (25). The purpose of this study was to determine the larvicidal activity of metabolites isolated from the *Nocardia* (*N. fluminea*, *N. soli* and *N. pseudobrasiliensis*) and *Streptomyces* (*S. alboflavus*) species against *An. stephensi*.

Materials and Methods

Preparation of the larvicidal solution

The larvicidal activities of four metabolites were evaluated, extracted from *Nocardia* strains N2 ($C_{40}H_{64}O_{12}$) (data unpublished), N4 ($C_{21}H_{28}N_7O_{14}$) (26), and N5 ($C_{18}H_{36}O_2$) (27), as well as *Streptomyces* strain S921 ($C_{32}H_{32}O_{14}$) (28). These metabolites were achieved using the following methods:

From randomly selected districts in Tehran (the North of Tehran, Darabad Park; the South of Tehran, Razi Park; the East of Tehran, Red Hisar Park, and the West of Tehran, Chitgar Park.), soil samples were collected at depths of 3 to 5 cm below the surface. The soil samples were stored in sterile zip-lock bags under 4 °C to maintain their sterility and to prevent them from becoming contaminated. A soil sample weighing five grams was suspended in 45 ml of physiological saline (0.9% NaCl) to prepare serial dilutions at 10-fold intervals (from 10^{-3} to 10^{-5} CFU/ml). Separate dilutions were cultured on actinomycete isolation agar (AIA) and potato dextrose agar (PDA) medium (Merck KGaA, Darmstadt, Germany). The plates were incubated at 28 °C for a period of 1–4 weeks. In order to isolate Actinobacteria strains, cream to white colonies were cultured on nutrient agar, sabouraud dextrose agar, and blood agar media (Merck, Germany), followed by paraffin baiting (29).

For the culture of the Actinobacteria isolates, 250 ml of brain heart infusion broth medium (BHI, Merck, Germany) was used. The culture was maintained at 30 °C in a shaker incubator (150 rpm) for five days. Following this, 10% of the medium was transferred to another flask containing 250 ml of Yeast Extract-Malt Extract (YEME) liquid medium. The flask was then incubated at 30 °C for seven days at 200

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rpm. The liquid medium was centrifuged for 4 minutes at 4 °C at 12000 rpm after fermentation. In a ratio of 1:1, the supernatant was mixed with ethyl acetate and kept at room temperature for one hour. Ethyl acetate was separated from the organic phase after two layers were formed. In a rotary vacuum evaporator, the organic phase containing the anti-larval metabolites was collected and concentrated. In order to further purify the sample, silica gel column chromatography was used (255 cm, Silica gel 60, Merck). HPLC semi-preparative columns were used to purify fractions showing anti-larval activity. The metabolite structure was assessed using mass spectrometry (MS) (30).

Mass rearing of *Anopheles* mosquitoes

Anopheles stephensi mosquitoes that had already been inoculated in the Insectarium of Biology and Vector Control of Diseases Department of the School of Public Health at Tehran University of Medical Sciences (SPH-TUMS) were employed. Adult mosquitos were housed at 28±2 °C, 75%±2 humidity, and a 10–12 h light cycle (dark 10 and light 12). Artificial lighting was used to illuminate the room (fluorescent lamp). Five percent sugar water was employed to feed the adults, and guinea pigs were anesthetized and used as blood sources for direct feeding. Chlorinated water was combined with fish meal including protein and nutritional supplements (micronutrients) to enable mass rearing of the larvae.

Larvicidal Assay of *Actinobacteria* metabolites

The larvae of *An. stephensi* were obtained from the Insectarium of the Department of Biology and Vector Control of Diseases, SPH-TUMS. The effects of metabolites extracted from four isolates of Actinobacteria (*N. fluminea*, *N. soli*, *N. pseudobrasiliensis*, and *S. alboflavus*) on fourth-stage mosquito larvae were evaluated using a modified version of the approach from the World Health Organization (WHO) in 2005 (31).

A combination of chromatographic techniques and bioassays was used to determine which fraction had insecticidal properties. After the extraction process, all the given chromatographical peaks were carefully collected and each fraction was tested for anti-insect activity using bioassays. The fractions showing anti-larval activity were selected for further purification and analysis using silica gel column chromatography and HPLC semi-preparative columns. The metabolite structure of the most active fraction was determined using mass spectrometry (MS). Therefore, the determination of which fraction had insecticidal properties was based on the results of the bioassays, and the fractions that showed anti-larval activity were selected for further analysis.

A total of 756 larvae were used (seven larvae per replication due to limited larvae). The experiments were performed for 24, 48, and 72 h at room temperature (28±2 °C). Dechlorinate tap water was used to make 100 mL of stock solution. Each of Actinobacteria metabolites in various volumes (300, 350, 400, 450, 500, 550, and 600 µl) was added to a series of containers containing 100 cc of dechlorinated water. The extracted metabolite concentration was considered as 100%, and the mentioned volumes were used from the metabolite stock. As a control, 21 larvae of the fourth stage were placed in a glass of dechlorinated water. Each larva was placed in its own glass. The number of dead larvae was counted at 24, 48, and 72 h after exposure, and the mortality rate was calculated using an average of three replications.

Data analysis

SPSS statistical software version 18 was used for statistical analysis (IBM, Armonk, NY, USA). The Abbott 's formula was used to correct mortality control (32). Toxicity data was used to calculate LC_{50} (Lethal concentration 50) and LC_{90} (Lethal concentration 90), which were then evaluated using appropriate analysis (33). In order to determine LC_{50} and LC_{90} values, probit analysis is used as a statistical method. For all statistical analyses in this study, $P \leq 0.05$ are considered significant.

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Results

The results of anti-larval metabolite activities are presented in Tables 1–2. All metabolites had a statistically significant influence on mosquito larvae ($P< 0.05$) (Table 1). The lowest LC_{50} was observed in metabolite N5 at 72 h (341 ppm) and the highest LC_{50} was observed at metabolite S921 at 24 h (484 ppm). The lowest LC_{90} was observed in metabolite N5 at 72 h (484 ppm) and the highest LC_{90} was observed at metabolite S921 at 24 h (701 ppm) (Table 2).

Table 1. Probit model based on time and treatment group

Group	Time		Estimate	Std. Error	Z	P	95% CI	
							Lower	Upper
N2	24	concentration	0.01	0.00	6.79	<0.001	0.00	0.01
		Intercept	-2.29	0.35	-6.54	<0.001	-2.65	-1.94
	48	concentration	0.01	0.00	7.30	<0.001	0.00	0.01
		Intercept	-2.38	0.36	-6.67	<0.001	-2.73	-2.02
	72	concentration	0.01	0.00	7.61	<0.001	0.00	0.01
		Intercept	-2.48	0.37	-6.77	<0.001	-2.84	-2.11
N4	24	concentration	0.01	0.00	7.36	<0.001	0.00	0.01
		Intercept	-2.47	0.37	-6.77	<0.001	-2.84	-2.11
	48	concentration	0.01	0.00	7.46	<0.001	0.01	0.01
		Intercept	-2.56	0.38	-6.76	<0.001	-2.94	-2.18
	72	concentration	0.01	0.00	7.67	<0.001	0.01	0.01
		Intercept	-2.39	0.36	-6.59	<0.001	-2.75	-2.02
N5	24	concentration	0.01	0.00	7.38	<0.001	0.00	0.01
		Intercept	-2.51	0.37	-6.79	<0.001	-2.88	-2.14
	48	concentration	0.01	0.00	7.78	<0.001	0.01	0.01
		Intercept	-2.61	0.38	-6.83	<0.001	-2.99	-2.23
	72	concentration	0.01	0.00	7.81	<0.001	0.01	0.01
		Intercept	-3.07	0.45	-6.86	<0.001	-3.52	-2.62
S921	24	concentration	0.01	0.00	6.39	<0.001	0.00	0.01
		Intercept	-2.86	0.42	-6.81	<0.001	-3.28	-2.44
	48	concentration	0.01	0.00	7.26	<0.001	0.00	0.01
		Intercept	-2.72	0.39	-6.96	<0.001	-3.11	-2.33
	72	concentration	0.01	0.00	7.55	<0.001	0.00	0.01
		Intercept	-2.55	0.37	-6.85	<0.001	-2.92	-2.17

*CI: Confidence Interval; N refers to *Nocardia* and S to *Streptomyces*; August 2022, Tehran Iran

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Table 2. LC_{50} and LC_{90} values of *Actinobacteria* metabolites based on time

Group	Time	LC%	Estimate	95% CI	
				Lower	Upper
N2	24	50	417	379	455
N2	24	90	650	588	754
N2	48	50	386	350	420
N2	48	90	595	544	675
N2	72	50	370	335	402
N2	72	90	561	517	629
N4	24	50	389	354	422
N4	24	90	591	542	668
N4	48	50	376	342	407
N4	48	90	565	520	634
N4	72	50	347	310	378
N4	72	90	533	491	595
N5	24	50	390	355	422
N5	24	90	589	540	665
N5	48	50	357	323	387
N5	48	90	532	493	591
N5	72	50	341	310	368
N5	72	90	484	451	531
S921	24	50	484	449	529
S921	24	90	701	632	825
S921	48	50	416	382	449
S921	48	90	612	562	693
S921	72	50	382	347	413
S921	72	90	574	528	644

*CI: Confidence Interval; N refers to *Nocardia* and S to *Streptomyces*; August 2022, Tehran Iran

Discussion

Insecticides, including pyrethroids, organophosphates, carbamates, and organochlorines, are commonly used in malaria prevention programs to kill or repel mosquitoes that carry the malaria parasite. However, the overuse of these insecticides has led to the development of resistance in mosquito populations, which can be caused by mechanisms such as the intensification of carboxyl-cholinesterase activity, resulting in resistance to multiple classes of insecticides (34). Insecticides such as benzoylurea and the bacterium *Bacillus thuringiensis* Israelensis, which acts as a larvicide, have a limited impact on mosquito populations (35, 36). Changes in the natural or human-made environment can significantly impact vector biology, leading to the disruption of original habitats, an increase in vector populations, and the spread of vector-borne diseases. These changes can limit the effectiveness of mosquito control measures and make it more difficult to manage disease transmission (37).

Actinomycetes have recently been identified as a valuable source of effective and efficient bioactive compounds with industrial potential (38). Mosquito populations can be managed us-

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ing biological control. A study conducted in India reported that various forms of biolarvicidal agents exhibit potent LC_{50} and LC_{90} values against numerous mosquito-borne disease vectors (39). Previous research has demonstrated the high effectiveness of the metabolite produced by the fungus in reducing mosquito populations, with particularly notable mortality rates observed in *Culex* sp. and *Anopheles* sp. (40, 41). *Actinobacteria* are Gram-positive bacteria that produce a variety of physiologically active secondary metabolites in addition to antibiotics. Mosquito larvae can be poisoned by these metabolites (42). As a result, metabolites of *Actinobacteria* are one of the most important biological control agents for insects.

In the current study, all four investigated metabolites exhibited statistically significant anti-larval activity. Our investigation found that Actinobacterial metabolites have high potential as a complement to existing larval control measures, as they showed significant toxicity against mosquito larvae. These findings are consistent with the results of previous studies, such as the research conducted by Singh and Prakash, who demonstrated the potential of fungal metabolites as a novel strategy for filariasis and dengue control (43). Additionally, significant effects of *Lagenidium giganteum* metabolites against *Cx. quinquefasciatus* and *Ae. aegypti* have been reported (44). A study conducted in India demonstrated that extracellular secondary metabolites produced by 35 different Actinobacterial isolates were shown to have high larvicidal activity against *Culex* and *Anopheles* mosquitoes (45). The larvicidal activity of *Streptomyces* isolates against *Anopheles* larvae was previously reported (46). A study synthesized silver nanoparticles using the filtration of GRD cells of *Streptomyces* species and demonstrated larvicidal activity against *Aedes* and *Culex* larvae. They examined the larvicidal effect of Actinobacterial extracts on *Culex* larvae and found that 1000ppm concentrations of *Streptomyces* KA13-3 and *Streptomyces* KA25-A isolates killed 100% and 90% of mosquito larvae, respectively (47). The current study's findings were agreed with the results of Tanvir et al. who isolated 21 *Actinomycetes* from *Asteraceae* plants and screened their potential to kill *Cx. quinquefasciatus* larvae in the fourth stage of development (48). A study conducted in India isolated 30 *Actinomycetes* from sediments of maritime soil and showed that *Streptomyces* isolates were highly effective against *An. stephensi* (49). Many studies have discovered that insect mortality may be caused by the release of bioactive compounds by *Actinobacteria* against *Culex* mosquito species (50). The effects of fungal and actinomycete metabolites on *Cx. quinquefasciatus*, *An. stephensi*, and *Ae. aegypti* egg, larval, and adult stages have been studied. The metabolites of three *Actinomycetes* had anti-egg activity, 35 *Actinomycetes* had larvicidal activity, and no activity against adults was discovered, according to Vijayan and Balaraman. With LC_{50} values of 1 to 3 microliters per milliliter, two *Streptomyces* species and one *Paecilomyces* species were very active (45).

According to our investigation, the LC_{50} and LC_{90} values of 341 (for *Nocardia pseudobrasiliensis*) and 484 ppm (for *Streptomyces alboflavus*), respectively, were less potent. Few studies on the larvicidal activity of pure chemicals against *An. stephensi* have been reported. According a study in Tanzania, after 24 h at a concentration of 237 ppm, a himachalane sesquiterpenoid isolated from *Hugonia busseana* had moderate efficacy against *Anopheles* larvae (51). The ethanol extract of *Leucas aspera* showed an LC_{50} of 9.70–12.73 ppm against *An. stephensi* (52). *Streptomyces alboflavus* extract was tested on *An. stephensi* larvae, as demonstrated by Balakrishnan et al. (LC_{50} 1.3±0.09 and LC_{90} 3.13±0.21) (53). Comparing the metabolites extracted in the study to the ones given above, they were generally more effective.

In the present study, the lowest LC_{50} in metabolite N5 was observed in 72 h and the highest LC_{50} in metabolite S921 in 24 hours. Also, the lowest LC_{90} was observed in metabolite N5 at 72 h and the highest LC_{90} was observed

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in metabolite S921 at 24 hours. Karthik et al. isolated the extract of *S. gedanensis* and tested it against the larvae of *Cx. gelidus* and *Cx. tritaeniorhynchus* tested (54). Their results showed promising activity with LC_{50} values of 108.08 ppm and 609.15 ppm. Our study agrees with their findings that *Streptomyces* metabolites have larvicidal activity against mosquito larvae. However, there were differences in the potency of the metabolites between the two studies.

Conclusion

The current investigation elucidates the diversity and distribution of metabolites in distinct actinomycete strains, as well as the antilarval capability of selected isolates. Actinobacterial metabolites were found to be effective against *An. stephensi*. As a result, it could be exploited as an alternate source of anti-mosquito larvae. More research is needed to identify active compounds that may be employed in a broad range for mosquito control, as well as to determine the pathways of action of these compounds. The importance of larvicidal *Actinomycetes* as a useful resource for the identification of novel insecticidal compounds was investigated in this study.

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Ethical considerations

This study was performed based on Tehran University of Medical Sciences (TUMS) Ethical Committee Guideline.

Conflict of interest statement

The authors declare that they have no competing interests.

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