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Biological control of *Phlebotomus papatasi* larvae by using entomopathogenic nematodes and its symbiotic bacterial toxins

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Abstract. The sand fly *Phlebotomus papatasi* is an important disease-bearing vector. Five entomopathogenic nematodes (EPNs) – *Steinernema carpocapsae* DD136, *Steinernema* sp. (SII), *S. carpocapsae* all, *S. abbasi*, and *Heterorhabditis bacteriophora* HP88 – were applied as biocontrol agents against the late third instar larvae of *P. papatasi*. In addition, the effect of toxin complexes (TCs) of *Xenorhabdus nematophila* and *Photorhabdus luminescens laumondii* bacteria was evaluated. Results revealed that *S. carpocapsae* DD136 was the most virulent species followed by *Steinernema* sp. (SII) and *S. carpocapsae* all where LC_{50} were 472, 565, 962 IJs/ml, respectively. Also, the crude TCs were slightly more active and toxic than their fractionated protein. Histopathological examination of infected larvae with *H. bacteriophora* HP88 showed negative effect on their midgut cells. In conclusion, EPNs with their symbiotic bacteria are more effective as biocontrol agents than the crude or fractionated TCs against sand fly larvae.

INTRODUCTION

Sand flies (Diptera: Psychodidae, Phlebotominae) have medical and veterinary considerations as they are the main vector of several diseases, especially protozoal disease (e.g., *Leishmania*, causing both visceral "VL" and cutaneous "CL" leishmaniasis) (Pessoa *et al*., 2007). Costa *et al*. (2007) reported that stray dogs are the main cause of transmitting the disease to human through their contact with wild reservoirs. In endemic areas, entomological and serological surveys in dog populations revealed a high prevalence of VL in dogs as well as an abundance and dominating presence of vector, increasing the hazards of disease transmission to man (Monteiro *et al*., 2005).

In Egypt, an epidemic of infantile VL occurred in EL Agamy, Alexandria governorate in 1982 and extended along the northern west coast to the borders of Libya. *Phlebotomus langeroni* is the proven vector of VL (El Sawaf *et al*., 2012). *Leishmania tropica* was recently identified as the pathogen responsible for anthroponotic CL cases in North Sinai (Shehata *et al*., 2009).

Chemical pesticides achieved convenient results in breading sites of sand fly. However, some problems arise from using this method, such as exceeding the maximum limits of pesticides residues, environmental pollution, human and animal toxicity as well as development of insect resistance to the chemical pesticide (Guglielmone *et al*., 2001). Because of these problems, scientists gave a great attention to find out an alter-

native approach to control sand fly. Biological control involves the introduction of a natural living antagonist (a predator, parasite or a pathogen) to lower pest populations to acceptable, non-harmful level (Hajek & Eilenberg, 2018). Biological control is considered a promising method, which can help to maintain environmental balance. Biological control can target the intermediate host or vector of *Leishmania* (i.e., sand fly) through either predatory enemies or infection and interfering with its biology (Ebrahim, 2015). Some species of entomopathogenic bacteria were experimentally applied to control sand fly larvae. De Barjac *et al.* (1981) demonstrated the role of *Bacillus thuringiensis var israelensis* in the control of larvae of *Phlebotomus papatasi* and *Lutzomyia longpalpalis*. Also, Robert *et al*. (1997) successfully used *Bacillus sphaericus* in the control of *P. martini* in Kenya. Moreover, some plants such as *Solanum jasminoides*, *Ricinus communis*, *Bougainvillea glabra*, and *Capparis spinosa* were found to be toxic to *P. papatasi* (Schlein *et al*., 2001).

Entomopathogenic nematodes (EPNs) that belong to *Steinernema* and *Heterorhabditis* species are obligatory parasites to a wide range of insect pests and parasites. They represent unique and excellent models for biological control agents. Their infective juveniles (IJs) invade the host with the help of their symbiotic bacteria *Xenorhabdus* or *Photorhabdus* and kill the host within days (Jawish *et al*., 2015). Multiplication of *Xenorhabdus* and *Photorhabdus* bacteria result in production of proteins known as toxin complexes (TCs). These TCs represent novel insecticides against different pests. The TC is composed of three different proteins: XptA2, XptB1, and XptC1, representing products from class A, B, and C toxin complex genes, respectively (Ffrench-Constant *et al*., 2007; Eleftherianos *et al*., 2010).

Some studies have addressed the efficacy of EPNs against members of the order Diptera, including *Cephalopina titillator*, *Gasterophilus intestinalis*, *Parasarcophaga dux* (Derbala *et al*., 1997; Zayed *et al*., 1997; El-Sadawy *et al*., 1997), *Lucilia sericata*, *Calliphora vicina*, *Musca domestica* and *Stomoxys calcitrans* (Mahmoud *et al*., 2007; Leal *et al*., 2017), *Culex quinquefasciatus* (Zohdy *et al*., 2013), *Aedes aegypti* (Peschiutta *et al*., 2014; Cardoso *et al*., 2015), and *Musca domestica* (Archana *et al*., 2017). However, there is only one study that evaluated the effect of EPNs on *P. papatasi* and *P. sergenti* (El Sawaf *et al*., 2011).

In this laboratory study, we evaluated the use of five species/strains of EPNs (*Steinernema carpocapsae* DD136 Wiser, *Steinernema* sp. SII, *S. carpocapsae* all, *S. abbasi Ab*, and *Heterorhabditis bacteriophora* HP88) and the effect of crude and fractionated TCs of EPNs symbiotic bacteria (*Xenorhabdus nematophila* and *Photorhabdus luminescens laumondii*) as biocontrol agents against the larvae of sand fly *P. papatasi*. Also, we investigated the effect of EPNs on the sand fly midgut cells.

MATERIALS AND METHODS

Ethical approval

The experiments were conducted in compliance with the requirements and recommendations of the International Animal Ethics Committee and the current Egyptian law and regulations for the protection of experimental animals to minimize the harmful effect and improve feeding and housing conditions. The study obtained approval (No. 19124) from the Ethical Committee of the National Research Centre, Giza, Egypt.

Rearing of *Phlebotomus papatasi*

Colonies were maintained in an insectary at the Research and Training Center on Vectors of Diseases at Ain Shams University, Cairo, Egypt. This species was reared at the laboratory for many generations according to the technique described by Modi & Tesh (1983), using a group of four hamsters of 6-month average age and 200 g average weight for feeding adult fly.

Entomopathogenic nematode

The present study was conducted at the laboratory of Parasitology and Animal Diseases Department, Veterinary Division, National Research Center, Giza, Egypt. *Steinernema carpocapsae* DD136, *Steinernema* sp. SII., *S. carpocapsae* all, *S. abbasi* Ab, and *H. bacteriophora* HP88 were applied to the late third instar larvae of sand fly. Nematodes were propagated on sixth instar larvae of the great wax moth *Galleria mellonella* according to the method described by Dutky *et al.* (1988).

Larvicidal activity of EPNs

Larvicidal activity of EPNs against Late third larvae of *P. papatasi* was conducted in plastic cups (5 cm high and 2.5 cm in diameter at the top) with 2 cm height in perforated bottom lined plaster of Paris. The plastic cups were supplied with 0.1 g larval food. According to a pilot study, every fold of nematode concentration (39, 78, 156, 312, 625, 1 250, 2 500, 5 000 IJs/cup) was suspended in 1 ml tap water added to the cups and left for an hour. Ten larvae of sand fly were then placed in each cup. The check replicates were treated with tap water only. Cups were covered with muslin cloth and tightly banded with rubber. The cups were moistened with water as needed. The experiment was replicated for five times. All cups were incubated in darkness at 25°C. Dissecting microscope (MAC-10) was used for mortality observation. Mortality was recorded every day for 6 days.

Isolation of symbiotic bacteria

Two symbiotic bacteria, *X. nematophila* and *P. luminescens laumondii*, were isolated from *S. carpocapsae* DD136 and *H. bacteriophora* HP88, respectively. Bacterial isolation was conducted according to Thomas and Poinar (1979). About 20 sterilized infective juveniles (IJs) of *S. carpocapsae* DD136; and *H. bacteriophora* HP88; were infected of the greater wax moth larvae *(Galler*ia mellonella). After 24h, a leg of surface sterilized *G. mellonella* larvae was removed and the released haemolymph was streaked on Nutrient-Agar (NA) plates containing 8% nutrient broth (LAB) and 1,2% agar. Bromothymol Blue (BTB) 0.025g and 0.04g Triphenyltetrazolium Chloride (TTC) were added to NA plates as an indicator. Plates were incubated at 25°C for 3 days. Then, the characteristic of bacterial colonies developed. These were repeatedly subculture until a pure culture was obtained. The potential *Photorhabdus* sp. and *Xenorhabdus* sp. were identified by adsorption of blue dye (Akhurst, 1986). Pure cultures were inoculated to nutrient broth slants. Incubated at 25°C for 3 days. Stored at 6°C and sub-culture at least once a month to maintain viability. The culture was incubated on rotatory shaker at 150 rpm at 30°C for 24 h.

Bacterial cultures and growth conditions

Bacterial cultures and growth conditions were conducted according to the method described by Bowen & Ensign (1998).

Cultures of *X. nematophila* and *P. luminescens* strains were grown into four 1-liter flasks each one containing 500 ml of 2% Proteose Peptone no. 3 (PP3) broth supplemented with 0.5% polyoxyethylene sorbitan mono stearate (Tween 60; Sigma Chemical Co., St. Louis, Mo.). The cultures were incubated for 72 h at 28°C on a rotary shaker at 150 rpm.

Separation of toxin complexes of symbiotic bacteria

Fast protein liquid chromatography (FPLC): The TC proteins of *X. nematophila* and *P. luminescens* were separated by ÄKTA Avant 150 system (Germany) according to the technique stated by Sheets *et al.* (2011), with some modification. Cell pellets of *X. nematophila* bacteria and *P. luminescens* were extracted from two liters of bacterial culture. The protein pellets were suspended in 50 mM Tris-HCl pH 8.0 (Bio Shop), 100 mM NaCl (Modern Lab), 1 mM DTT, 10% glycerol, lysozyme (0.6 mg/ml) (BioShop) and bacterial protease inhibitor cocktail (Sigma, St. Louis). Cells were smashed using small amount of glass beads and sonicated for 3 min. The cells were then centrifuged

at 48 000 xg for 60 min. at 4°C, and the supernatant was collected and dialyzed against 25 mM Tris-HCl, pH 8.0 overnight. The protein was then loaded onto a Q Sepharose XL (1.6 x 10 cm) anion exchange column. Bound proteins were eluted using a linear 0 to 1M NaCl gradient in 10 column volumes. The high molecular weight TCs eluted in the early fractions and were concentrated by dialysis bags (Avg. flat width 35 mm) USA, for R&D use only. Polyethylene glycol powder MW 20 000 (PEG-Sigma Chemical Co.) was added to the dialysis bag to absorb water and precipitate the toxins.

Native-PAGE Electrophoresis: Protein components were separated using vertical electrophoresis apparatus (SCIE-PLAS, Cambridge, UK) according to the method of Laemmli, (1970). Total protein of crude and fractionated bacterial TCs was determined according to Lowry *et al.* (1951).

Larvicidal activity of the bacterial toxins (*X. nematophila* and *P. luminescens*)

Bacterial toxicity of the crude and fractionated TCs of *X. nematophila* and *P. luminescens* was studied on second instar larvae of *P. papatasi*. The experiment was conducted in plastic cups as previously mentioned. The plastic cups containing 0.1 g larval food were mixed with 1 ml of TC protein at a concentration of 3 mg/ml, and then 10 larvae of sand fly were added to each cup. Tap water was mixed with larval food to the check cups. The cups were covered with muslin and tightly banded and were moistened with water as needed. Five plastic cups were set up for each treatment dose and incubated in the dark at 25°C. Mortality rate was recorded using a dissecting microscope (MAC-10) every day to observe the adult emergence. Adults were observed for mating and female egg laying. Female egg count was recorded in all toxic experiments.

Histopathological examination

Infected third instar larvae of *P. papatasi* with 5 000 IJs of *H. bacteriophora* HP88 were prepared for histopathological examination according to the method of Stiles (1934). Larvae, 48 hours post infection, were fixed in a mixture of formaldehyde-glutareldehyde pH 7.4 at room temperature for 4 hour and then rinsed twice in 0.1 M phosphate buffer (15 min each). Specimens were fixed in 1.0% buffered osmic acid for 30 min at 4 C and then washed twice in phosphate buffer for 30 min each. Specimens were then dehydrated in normal butyle alcohol (butanol). The material was then embedded in tissue preparation embedding media (melting point 55-57°C, Fisher Scientific Co.) and prepared for sectioning. Longitudinal and transvers sections were cut at the thickness of 6 U using a microtome and were mounted on glass slides with a drop of dist. Water at 37°C. They were deparaffinized in toluene, hydrated in aggraded ethanol / distilled water series, and stained with (haematoxylin and eosin). The sections were then dehydrated in absolute ethanol and toluene and then mounted in pro-Texx mounting medium. Good sections were examined for general orientation on a light microscope (OLYMPUS CX41).

Statistical Analysis

All data were subjected to statistical analysis using SPSS version 20 software. The mean and standard error (mean ± SE) were calculated. Differences between treated groups were tested for significance using a one-way analysis of variance followed by Duncan's multiple range test. Differences were considered significant at $P<0.05$ (Snedecor and Cochran, 1989).

RESULTS

Virulence of entomopathogenic nematodes to third instar larvae of sand flies

Mortality of the late third instar larvae of *P. papatasi* that were exposed to the five species/ strains of EPNs in this study increased by increasing the concentration. At the highest concentration (5 000 IJs), *S. carpocapsae* DD136 and *Steinernema* sp. (SII) achieved the highest mean mortality rate at three days' post exposure (94%), followed by *S.*

carpocapsae all (84%), while *H. bacteriophora* HP88 and *S. abbasi* Ab achieved the lowest mean mortality rates (50% and 42%, respectively). The Egyptian isolate *Steinernema* sp. (SII) was slow in low concentrations but its larvicidal effect dramatically increased as the concentration increased with the mortality rate reaching 100% at a concentration of 5 000 IJs/ml at 6 days' post exposure (Table 1). Evaluation of LC_{50} and LC_{90} revealed that *S. carpocapsae* DD136 was the most virulent one (LC_{50}= 472 and LC_{90}= 4179 IJs/ml), while *H. bacteriophora* (HP88) and *S. abbasi* were the lowest virulent ones (LC_{50}= 5377, 7389; $LC_{90=}$ 151631, 75646 IJs/ml, respectively) (Table 2). The control group completed its life cycle normally.

Fast protein liquid chromatography (FPLC)

Chromatographic resolution of *X. nematophila* and *P. luminescens* TCs was studied. Fractionation of *X. nematophila* crude protein revealed a four-peak chromatogram (Figure 1), whereas the fractionation of crude protein of *P. luminescens* TCs revealed a three-peak chromatogram (Figure 2). The elution buffer (50 mM Tris-HCl) appeared as an upward sloping line.

Structural characterization of bacterial crude toxins and fractionated toxins by NATIVE–PAGE

Electrophoretic profiles of the toxins were characterized by NATIVE–PAGE. They showed the bands at both high and low M.W. ranges. The results revealed that there were variations in the number of protein bands as shown in Figure 3. The protein marker consists of 11 bands (ranging from 10 to 300 kDa). *Xenorhabdus* crude protein had six bands (216.1, 72.0, 67.3, 58.3, 56.3, and 34.4 kDa). *Xenorhabdus* fractionated protein revealed 12 bands (194.7, 103.6, 79.6, 76.3, 71.9, 66.6, 57.4, 55.6, 33.6, 30.0, and 25.7 kDa). *Photorhabdus* crude protein had nine bands (213.8, 162.6, 128.1, 96.3, 94.6, 77.9, 69.1, 65.2, and 47.8 kDa). *Photorhabdus* fractionated protein revealed 13 bands (194.7, 157.0, 117.3, 95.1, 93.1, 76.9, 68.3, 64.4, 55.0, 46.0, 37.9, 35.3, and 31.9 kDa).

Virulence of symbiotic bacterial toxins to second instar larvae of sand flies

The crude protein of *X. nematophila* and *P. luminescens* TCs showed a higher mean rate of larval mortality compared with its fractionated protein of the same bacterial species (36% vs 32% and 34% vs 22%, respectively; Table 3). The morality rate of the treated sand fly larvae was continuously recorded for 25 days till the end of their life cycle. Results showed that larval mortality rate increased with increasing the time of exposure to bacterial toxins.

Histopathological examination of *P. papatasi* larval midgut

Histopathological study of the control larvae midgut showed normal cells with clear nucleus, continuous basement membrane, regular columnar cells, normal peritrophic matrix, and normal lumen. Midgut cells of infected larvae had degenerated nuclei. Examination revealed also damage to the epithelial lining columnar cells, large gaps between the cells, interrupted peritrophic matrix, vacuoles in fat cells, discontinuous basement membrane and leakage of contents of the lumen leading to its narrowing (Figure 4).

Table 1. Mortality rates (Mean±SE) of *P. papatasi* at 3 days and 6 days' post exposure to Entomopathogenic nematodes under laboratory conditions

Nematode concentration (IJs)	Mortality percentage (Mean ± SE)									
	After 3 days					After 6 days				
	DD136	SII	ALL	Ab	HP88	DD136	SII	All	Ab	HP88
39	6±2a	2±2a	0±0a	0±0a	0±0a	6±2a	2±2a	2±2a	0±0a	0±0a
78	14±2ab	10±3ab	4±2ab	0±0a	4±2ab	14±2ab	10±3ab	8±4ab	0±0a	4±2ab
156	22±4b	16±5bc	14±2b	0±0a	12±4ab	22±4b	16±5bc	18±4b	4±2ab	12±4ab
312	44±8c	22±4c	28±4c	4±2ab	14±7b	48±7c	24±2c	34±5c	10±3bc	14±7b
625	60±3d	60±6d	44±5d	10±3bc	16±4b	62±4d	62±6d	48±4d	14±4cd	16±4b
1250	66±4d	70±5d	58±4e	14±2c	30±5c	68±4d	74±5e	62±5e	22±4d	30±5c
2500	80±5e	94±2e	66±7e	28±4d	38±5cd	84±5e	94±2f	70±6e	46±4e	40±6c
5000	94±2f	94±4e	84±2f	42±4e	50±6d	96±3f	100±0f	88±4f	56±4f	54±5d
F value	57.298	91.241	63.995	37.931	15.341	68.220	110.730	54.918	46.300	17.472
P value	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

a, b, c, ……. letters within the same column denote significance according to Duncan test; *Significant at P<0.05 (F-test); R= 5; N = 10; Σ_n = 50; EPNs = entomopathogenic nematodes; DD136 = *S. carpocapsae* DD136; SII = *Steinernema* sp.; all = *S. carpocapsae* all; Ab = *S. abbasi*; HP88 = *H. bacteriophora*HP88; IJs = Infective Juveniles; Mortality rate of the control = 0.0.

Table 2. LC_{50} and LC_{90} of different entomopathogenic nematodes (IJs) to *P. papatasi*

strains	LC_{50}	Lower limit	Upper limit	LC_{90}	Lower limit	Upper limit
DD136	472	398	561	4179	3124	5981
SII	565	492	650	3076	2494	3941
all	962	805	1160	8366	5938	13014
Ab	7389	5087	13437	75646	33086	312878
HP88	5377	3585	9604	151631	58829	651696

DD136 = *S. carpocapsae* DD136; SII = *Steinernema* sp.; all = *Steinernema carpocapsae* all; Ab = *S. abbasi*; HP88 = *H. bacteriophora* HP88; IJs = Infective Juveniles.

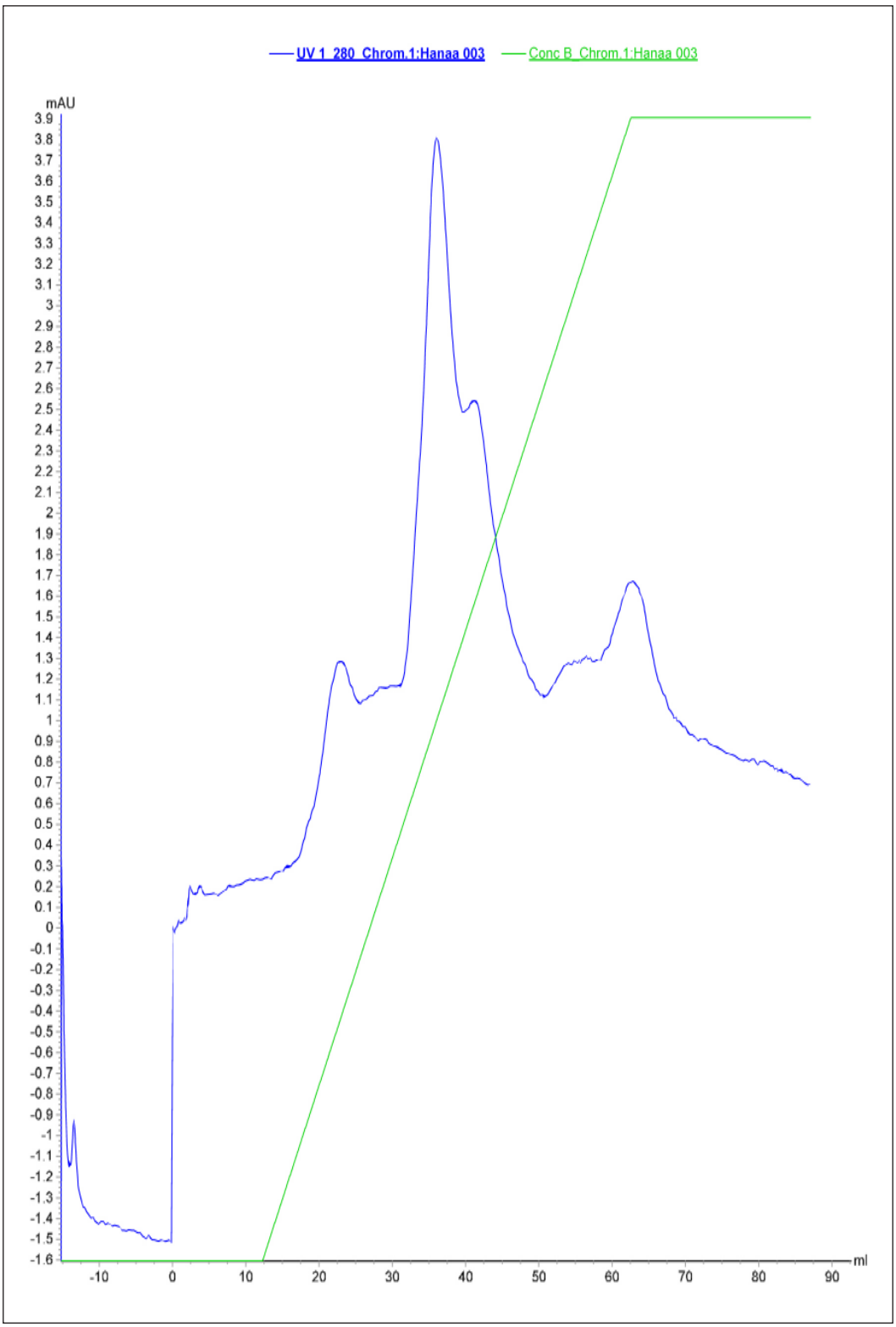

Figure 1. Chromatographic resolution of *Xenorhabdus* toxin complex.

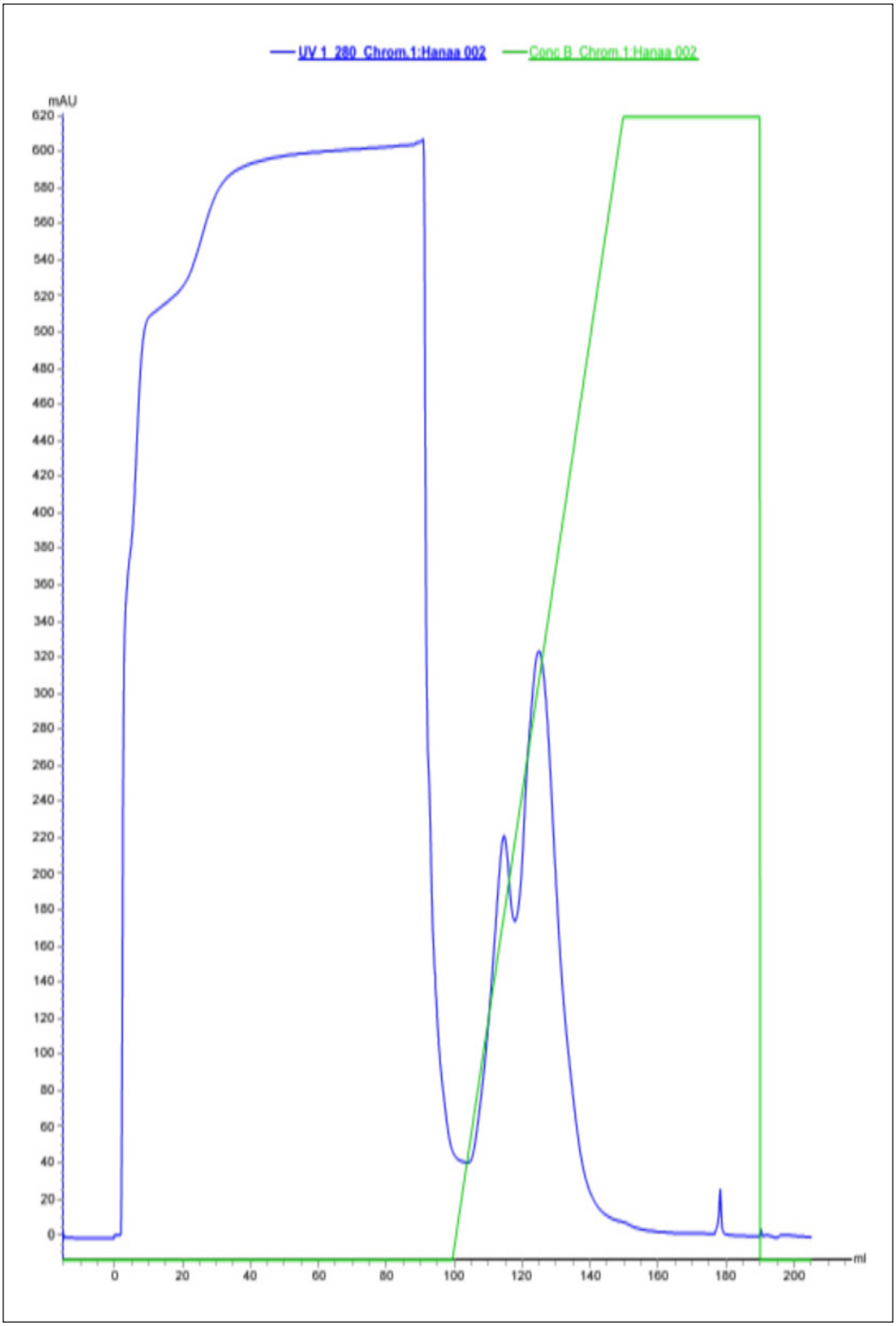

Figure 2. Chromatographic resolution of *Photorhabdus* toxin complex.

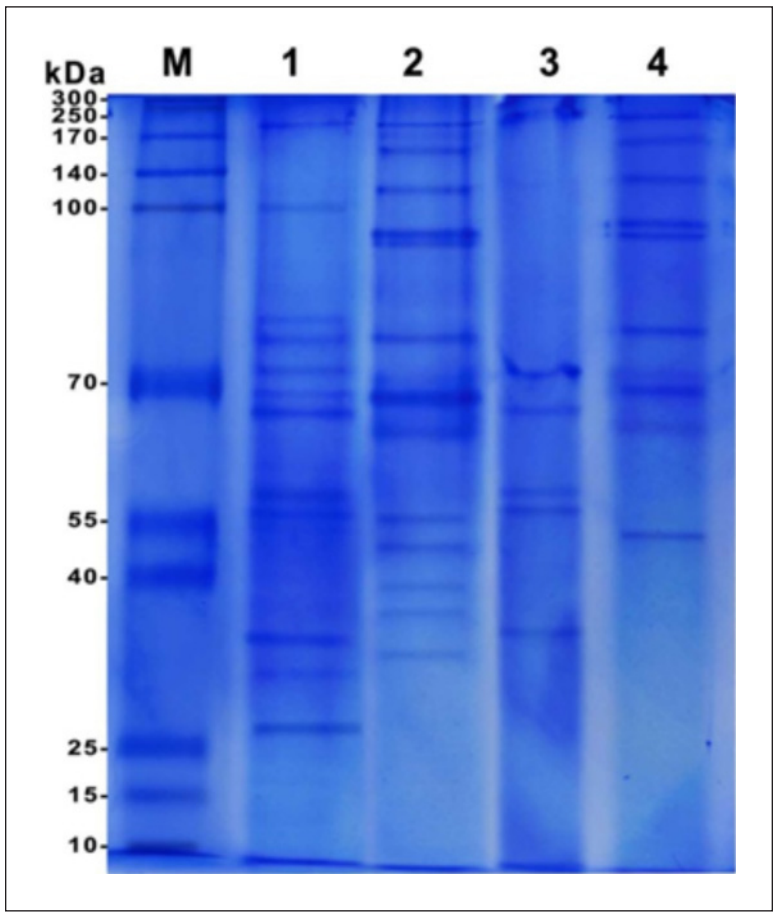

Figure 3. Characterization of bacterial crude protein and fractionated protein by NATIVE -PAGE: Lane M: Prosieve Quadwwr protein Marker. Lane 1: *Xenorhabdus nematophila* "fractionated". Lane 2: *Photorhabdus luminescens* "fractionated". Lane 3: *X. nematophila* "crude". Lane 4: *P. luminescens* "crude".

Table 3. Mortality rates (Mean±SE) of *P. papatasi* at 4, 7, 11 and 25 days' post exposure to *Xenorhabdus nematophila* and *Photorhabdus luminescens* toxin complexes

Strains	Days				F	P
	4	7	11	25		
Xenorhabdus "fractionated"	6 ± 4a	8 ± 6a	16 ± 4a	32 ± 4b	6.983	0.003*
Photorhabdus "fractionated"	4 ± 2a	6 ± 2a	10 ± 3a	22 ± 6b	4.643	0.016*
Xenorhabdus "crude"	6 ± 2a	10 ± 3a	16 ± 5a	36 ± 4b	12.23	0.001*
Photorhabdus "crude"	4 ± 2a	6 ± 4a	12 ± 4a	34 ± 2b	18.032	0.001*
F	0.363	0.371	0.355	1.807		
P	NS	NS	NS	NS		

NS = non-significant; *significant at $p<0.05$.
N= 10 R= 5 Σ= 50.

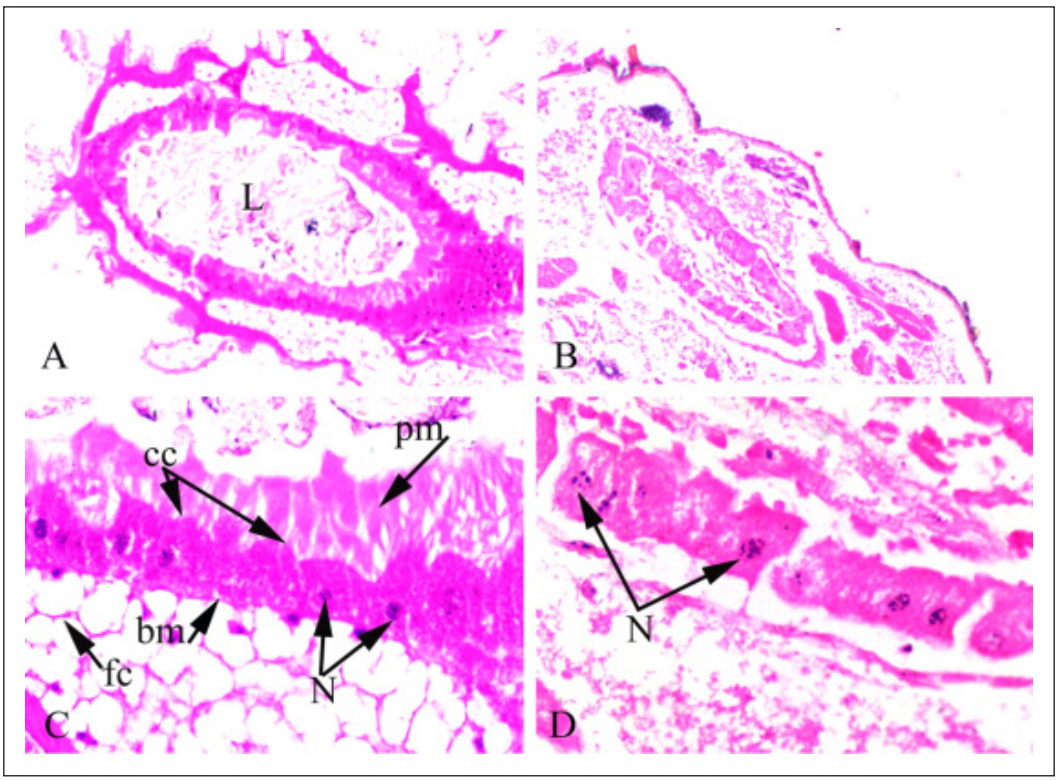

Figure 4. Effect of entomopathogenic nematodes (*H. bacteriophora* HP88) on sand fly (*P. papatasi*) 48h post exposure at a dose of 5 000 IJs/ml. LS of midgut of 3rd instar larvae of *P. papatasi* (x 10). A: control, B: infected. TS of midgut of 3rd instar larvae of *P. papatasi* (x 40) C: control, D: infected. N: nucleus, cc: columnar cells, pm: peritrophic matrix, fc: fat cells, bm: basement membrane and L: lumen.

DISCUSSION

This work focused on investigating the potential use of EPNs and its symbiotic bacterial TCs as biocontrol agents against sand fly larvae. Mortality rates and LC_{50} of the five species of EPNs were recorded. *S. carpocapsae* DD136 and *Steinernema* sp. SII species were the most virulent ones against sand fly larvae, while *H. bacteriophora* HP88 and *S. abbasi* exerted very weak effect on those larvae.

In a relevant study by El Sawaf *et al.* (2011). Their findings were partially consistent with ours. They reported almost the same results with *S. carpocapsae* DD136. However, they found that third instar larvae of sand fly exhibited high resistance to *Steinernema* sp. SII and moderate resistance to *Heterorhabditis bacteriophora* HP88 and *S. abbasi* Ab nematode species. Our results showed that the larvae were very susceptible to SII species and very resistant to HP88 and Ab nematode species. This controversy may be attributed to the nematode self-behavior.

In addition, previous laboratory studies were carried out on some mosquito species. Cardoso *et al.* (2015) recorded with mortality of the third and fourth instar larvae of *Aedes aegypti*, which were exposed to IJs of *H. indica*. Also, Zohdy *et al.* (2013) found that *H. bacteriophora* was significantly more virulent than *S. carpocapsae* against *Culex quinquefsciatus*. Moreover, Peschiutta *et al.* (2014) observed that *H. bacteriophora* achieved 84% mortality of *Aedes aegypti* at the high concentration. Leal *et al.* (2017) evaluated *H. bacteriophora* HP88 virulence to *Stomoxys calcitrans* larvae; mortality

rate reached more than 90% 4 days' post infection. Virulence differences among nematode species/strains may be attributed to the differences in attractiveness of fly maggots to the IJs or to the tested nematode behavior (Abdel-Razik, 2006).

X. nematophila and *P. luminescens* are characterized by their symbiotic relationships with some entomopathogenic nematodes, and both bacteria are rarely found isolated from their nematode host (Hinchliffe *et al.*, 2010). *Photorhabdus* and *Xenorhabdus* species multiply within the insect hemocoel and produce toxic substances and bacterial proteinases causing septicemia, histological lesions, and killing of the host insect (Sheets *et al.*, 2011; Owuama, 2001).

In the present study, crude and fractionated *X. nematophila* and *P. luminescens* bacterial TCs that were used against second instar larvae of *P. papatasi* were not promising as mortality rate was not higher than that achieved by nematode infection.

That finding agrees with the results of El Sawaf *et al.* (2014) who tested the activity of *P. luminescens akhurstii* HRM1 bacterial toxin against *P. papatasi* larvae. They reported mortality rates of 60%, 32% and 2% three days' post treatment at concentrations of 11.39, 5.67, and 2.85 mg/ml toxin protein, respectively. However, they found significant negative effects due to toxin treatment including decrease in egg number and hatchability. As far as we know, this controversy may be due to differences in toxin origin and concentration. Bowen & Ensign (2001) recorded the presence of intracellular protein inclusions that were produced by the entomopathogenic bacterium *Photorhabdus luminescens*. This protein when injected or fed to *Galleria mellonella* larvae was not toxic. Another study was carried out by Khandelwal & Banerjee-Bhatnagar (2003) who recorded a low level of oral toxicity of outer membrane toxin of *X. nematophilus* to *Helicoverpa armigera* neonatal larvae.

On the other hand, some previous studies reported results that differed from ours. Sheets *et al.* (2011) found that the insecticidal TC proteins from *X. nematophilus* were highly active against neonate larvae of *Helicoverpa zea* and *Heliothis virescens*. Wang *et al.* (2012) reported that *X. nematophila* HB310 showed high oral virulence to larvae of diamondback moth *Plutella xylostella*. Da Silva *et al.* (2013) reported that *P. luminescens* killed 73% of the fed and 83% of the unfed *Aedes aegypti* larvae. Vani & Lalithambika (2014) recorded maximum mortality (93.32%) against the malarial vectors *anopheles gambiae* treated with outer membrane vesicles protein of *Xenorhabdus* sp. Ahmed *et al.* (2017) declared that extracted TCs from the two *Photorhabdus* types (HRM1 and HS1) showed promising larvicidal activities against *Culex pipiens*. Vitta *et al.* (2018) investigated the high oral toxicity of *Xenorhabdus* and *Photorhabdus* bacteria against *Aedes* spp. and El-Sadawy *et al.* (2018) demonstrated that both *X. indica* and *P. luminescens laumondii* HP88 exhibited significant larvicidal toxicity for *Culex pipiens*. These previous controversies may be attributed to the difference in toxin preparation and the used host.

In spite of the importance of histopathological examination of the EPNs symbiotic bacterial toxin efficiency, only few studies investigated this point. In this study, *H. bacteriophora* Hp88 nematode with the help of its symbiotic bacteria *P. luminescens* had severe effect on the midgut cells of *P. papatasi* larvae. These effects are comparable to those caused by other gut-active toxins such as δ-endotoxins from *B. thuringiensis* (Endo & Nishiitsutsuji-Uwo, 1980) and the *P. luminescens* toxin complex (TCa) (Blackburn *et al.*, 1998). Released toxin resulted in decrease in midgut intercellular adhesion. The same effect had been recorded for midgut cells of *Aedes aegypti* larvae treated with *B. thuringiensis* toxins (Charles & De-Barjac, 1983). Also, distortion of the pretrophic matrix lining of the midgut cells was observed. All of these tissues were noted to be damaged in *Heliothis armigera* larvae when treated with txp40 toxin protein from *Xenorhabdus* and *Photorhabdus* bacteria (Brown *et al.*, 2006). The results therefore corroborated that EPNs are potent pathogens and useful as biological control agents of sand fly larvae.

CONCLUSION

Entomopathogenic nematodes and their symbiotic bacteria together are more effective than the isolated bacterial toxin in case of sand fly *P. papatasi* larval control. This difference could be attributed to the nematode acting as an injectable tool for the bacterial toxin, achieving high mortality rate. However, further studies should be conducted to elucidate the exact mechanism of the effect of ENPs and their symbiotic bacteria on sand fly larvae.

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