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Original Article

Antibacterial Activities of Extracellular Metabolites of Symbiotic Bacteria, *Xenorhabdus* and *Photorhabdus* Isolated from Entomopathogenic Nematodes

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Xenorhabdus and *Photorhabdus* are members of the family *Enterobacteriaceae*, and are engaged in a mutualistic association with the entomopathogenic nematodes, *Steinernema* and *Heterorhabditis*, respectively. In the present study, the organic fraction of ethyl acetate bacterial crude extracts of the symbiotic bacteria, *Xenorhabdus stockiae* and *Photorhabdus luminescens* isolated from entomopathogenic nematodes, were characterized by High Performance Liquid Chromatography (HPLC) and Fourier Transform Infrared Spectroscopic (FTIR) methods, and evaluated for their antibacterial activity on selected pathogenic bacteria, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi* and *Klebsiella pneumonia* by agar well plate method. When compared to *X. stockiae*, *P. luminescens* showed higher antibacterial activity at maximum concentration on *K. pneumonia* and *S. aureus*, respectively. FTIR analysis of the crude ethyl acetate extract of *X. stockiae* showed the corresponding peaks values of 2373.33 and 544.93 cm^{-1} whereas *P. luminescens* showed the presence of peaks at 2128.00 and 552.80 cm^{-1}, respectively indicating the functional group of P-H and isocyanides stretching. The present study suggests that the antibacterial activity might be due to the effective compounds from the symbiotic bacteria of entomopathogenic nematodes, and that they could be used as bioinsecticides in the future.

Keywords: *Steinernema*, *Heterorhabditis*, *Xenorhabdus*, *Photorhabdus*, antimicrobial activity

Entomopathogenic nematodes (EPN) are soil-inhabiting lethal insect pathogenic organisms that belong to the phylum *Nematoda*, commonly known as roundworms. Their body is soft and non-segmented. They may be obligate or facultative parasites of insects (1). *Heterorhabditidae* and *Steinernematidae* are two nematode families that have been used as natural insecticides (2). The infective juvenile stage (IJ) is the only free living stage of EPNs and this active stage of the nematode is able to invade an insect. The IJ is a free-living stage which occurs in the soil, and without feeding. During the juvenile stage, insects penetrate their host via the spiracles, mouth, anus, or sometimes through intersegmental membranes of the cuticle, before entering into the hemocoel (3).

Both *Heterorhabditis* and *Steinernema* are mutualistically associated with bacteria of the

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genera *Photorhabdus* and *Xenorhabdus*, respectively (4). During the juvenile stage, insects release their symbiotic bacteria from their intestines into the hemocoel. Therefore, the bacteria multiply and cause the death of their host within 24 to 48 h. Even then, the nematode's feeding continue on the host tissue, which allows insect maturation through four juvenile stages, and reproduction. Secondary metabolites production is common to *Xenorhabdus spp*. and *Photorhabdus spp*. when cultured *in vitro*, and several secondary metabolites, such as stilbene derivatives, anthraquinone derivatives, genistein and a phenol derivative exhibiting insecticidal, antioxidant, antibiotic, and even anticancer and antiulcer activities have been identified. Some of these bioactive molecules are effective against drug resistant bacteria as well as a number of cancer cell lines while presenting no toxicity toward healthy cells (5). Recently, researchers have focused on the chemical compounds produced by these symbiotic bacteria which can affect the behavior of scavengers (6-9).

In the present study, the ethyl acetate crude extracts of *Xenorhabdus stockiae* and *Photorhabdus luminescens* were screened for their antibacterial activity. Furthermore, some metabolites were characterized from the active fraction using thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and were examined for their antibacterial effects.

Materials and methods

Insect culture

The greater wax moth *Galleria mellonella* eggs were obtained from Department of Biotechnology, Bharathidasan University, Tiruchirappalli, Tamil nadu, India (N 10.6779° and E 78.7445°) and were kept in rearing plastic boxes with artificial diet, and the insects were maintained in aerated plastic containers (22.5 x 17 x 10 cm) at 25 ± 2 ˚C Late instar stage of *G. mellonella* larvae were collected and used for nematode rearing.

Nematode cultures

Established cultures of *Steinernema siamkayai* (KPR-4) and *Heterorhabditis indica* (KPR-8) were obtained from Department of Biotechnology, Periyar University (N 11.7184°, E 78.0773°), India, and were maintained in the greater wax moth larvae *Galleria mellonella*. The insect larvae were infected with individual nematode species. After three days, the dead larvae were collected and transferred into a white trap (10) to confirm the infection and to collect the infective juveniles. After 10 days the collected infective juveniles were checked for their pathogenicity against *Galleria* larvae.

Bacterial cultures

The bacterial strains *X. stockiae* (isolated from *S. siamkayai* (KPR-4)) and *P. luminescens* (*H. indica* (KPR-8)) were used in the experiments. The bacterial strains were obtained from the haemolymph of *G. mellonella* infected with the respective nematode species. Last instar *G. mellonella* were placed on the top of a Whatmann no.1 filter paper in a petri dish. After 24 h larvae were removed, rinsed in sterile distilled water, and then sterilized with 70 % ethanol, and left to dry in a laminar air flow cabinet. Haemolymph was collected with a sterile loop and streaked on nutrient agar plates containing 0.004% 2, 3, 5–triphenyl tetrazolium chloride and 0.025% bromothymol blue (NBTA). The bacterial colonies were grown for 48 h at 28 ˚C. Single colonies showing morphological differences (color, shape, and size) were subcultured to fresh NBTA plates. Physiological characters of symbiotic bacteria were examined. To distinguish primary and secondary forms of symbiotic bacteria, NBTA test based on absorption of bromothymol blue was conducted (11). Blue colonies (absorption of dyes) or green colonies were selected and analyzed for the main genotypic characteristics.

Crude compounds extraction

Extraction of metabolites was conducted from the symbiotic bacteria *X. stockiae* and *P. luminescens* which were isolated from their nematode symbionts, *S. siamkayai* and *H. indica* respectively. Bacterial colonies were established on

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NBTA agar plates. Soluble organic metabolites were then extracted from the bacterial cultures. Briefly, bacterial cultures were scaled up for metabolite isolation through liquid culture in TSY (Tryptic Soy Broth + 0.5 % yeast extract). A loopful of bacteria was added to 50 ml of fresh TSY in a 300 ml Erlenmeyer flask and kept incubated under static conditions of 130 rpm for 24 h at 25 ˚C. The bacterial cultures (*X. stockiae* and *P. luminescens*) were then transferred into 900 ml TSY in 2 L flasks and placed on a rotary shaker at 25 ˚C for 72 h. The culture media were then centrifuged (10,000 × g, 20 min, 4 °C). Then 500 ml of supernatant was taken and extracted with an equal volume of ethyl acetate at thrice. The ethyl acetate layers were used to separate the organic fraction combined, which was then air dried, and concentrated using a rotary flash evaporator at 30 ºC. The dry residue was weighed and reconstituted in 6 ml methanol and stored at -20 ºC for further studies.

Thin layer chromatography

Thin layer chromatography (TLC) was performed on a silica gel plate (TLC Aluminium Sheets Silica Gel 60 F254, 0.2 mm, 20×20 cm, SDFCL) for compounds isolation. The solvent system (mobile phase) of hexane: ethyl acetate (9:1, 8:2, 7:3 v/v) and chloroform: methanol (6:4) was prepared and poured in a TLC chamber. The movement of the analyte was expressed by its retention factor (R_f). Different bands were observed and corresponding R_f values were calculated.

High performance liquid chromatographic (HPLC) analysis

HPLC (Shimadzu LC solution 20 AD, Japan) analysis was performed for both the bacterial crude ethyl acetate extracts and their fractionation. The obtained fractions were analyzed by HPLC at specific wavelength (254 nm) and individual R_T values of peaks were recorded.

Fourier transform infrared spectroscopic (FT-IR) analysis

FT-IR (Perkin-Elmer Spectrum 2000) analysis was carried out for both bacterial crude ethyl acetate extracts. FT-IR spectra were recorded in the absorption range between 4000 and 400 cm^{-1} at room resolution 4 cm^{-1} with scans using Thermo Nicolet FT-IR Nexus spectrometer coupled with TGS (Tri-glycine sulphate) detector by the KBr pellet technique. The spectrum was recorded using attenuated total reflectance (ART) technique bench measurement.

Antibacterial activity assessment

The following four bacteria, *Klebsiella pneumoniae* MTCC 8911, *Escherichia coli* MTCC 2622, *Staphylococcus aureus* MTCC 902 and *Salmonella typhi* MTCC 3224 were purchased from Microbial type culture collection and gene bank (MTCC), Chandigarh, India and maintained on nutrient agar (NA) slants. Antibacterial activities of the crude ethyl acetate extracts were measured using agar well diffusion assay according to Perez et al. against the test organism *B. subtilis*, *E. coli*, *S. aureus* and *P. aeruginosa* (12). Five wells (6 mm diameter well) were made using cork borer. Different amounts (50, 100, 150 and 200 µl) of ethyl acetate crude extract dissolved in dimethyl sulfoxide (DMSO), and chloramphenicol (5 µl) as positive control, were added in respective wells. The zone of inhibition was measured in millimeter.

Results

The phenotypic characterization of symbiotic bacteria *X. stockiae* and *P. luminescens* is represented in Table 1. The NBTA plates of the bacterial culture *Xenorhabdus stockiae* and *Photorhabdus luminescens* are shown in Figure 1 (a, b).

The bacterial suspension was prepared in 15 L of TSY broth. The cell free culture filtrate of 72 h showed maximum antibacterial activity, and was therefore separated into aqueous and organic fractions (Figure 1 c). Organic fractions were conc-entrated and were used for antibacterial assay.

Thin layer chromatographic (TLC) analysis

The TLC was performed to separate the biom-

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Table 1. Phenotypic characteristics of isolated symbiotic bacteria

Characteristics	*X. stockiae*	*P. luminescens*
Colony color on NBTA agar	Blue	Greenish Yellow
Bioluminescence	-	+
Insect pathogenicity	+	+
Growth on 28 °C	+	+
Growth on 37 °C	w	w
Absorption of bromothymol blue	+	+
Catalase	-	+

-: negative; +: positive; w: weak positive

olecules using the ethyl acetate extract of *X. stockiae* and *P. luminescens*. The band separation was accomplished in the solvent system of hexane/ethyl acetate (9:1 ratio) for *X. stockiae* and chloroform/methanol (6:4 ratio) for *P. luminescens*. The clear band separation was observed from the solvent system of 9:1 and 6:4 ratio, which were observed under ultraviolet illumination using potassium permanganate ($KMnO_4$) (Figure 1 d and e). The R_f values of the separated fractions were

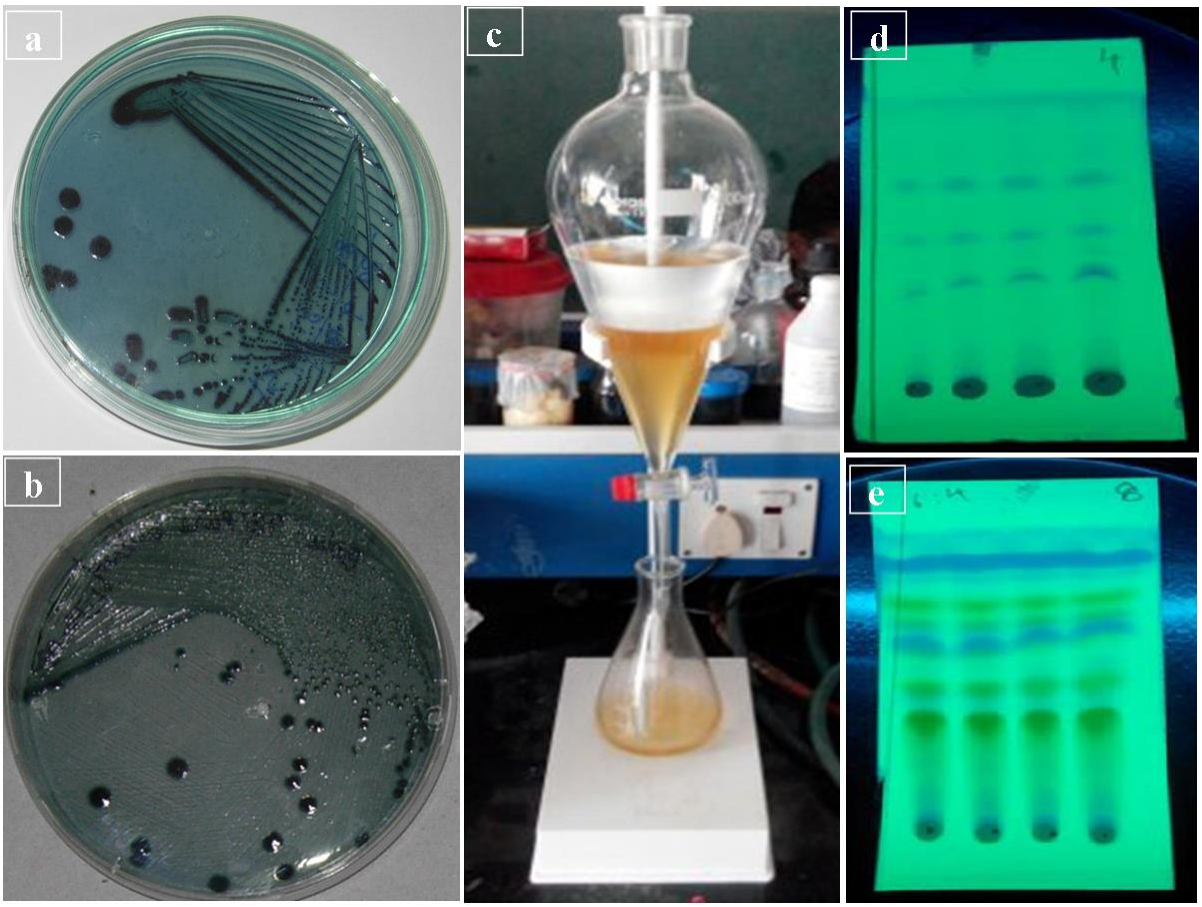


Figure 1. Chemical analysis of bacterial cultures. a: *X. stockiae* culture showing blue color colonies; b: *P. luminicense* culture showing greenish yellow colonies on NBTA agar plates; c: separation of crude compounds using separating funnel; d: TLC profile analysis of *X. stockiae;* e: TLC profile analysis of *P. luminescens*.

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calculated. Fraction 1: R_f – 3/5.5= 0.5454 cm. Fraction 2: R_f – 3.9/5.5=0.7090 cm. Fraction 3; R_f– 4.5/5.5= 0.8181 cm. Fraction 4: R_f – 5/5.5= 0.9090 cm. Fraction 5: R_f – 5.3/5.5= 0.9636 cm. Based on the R_f values, the fractions were found to be phenolic group of compounds.

High performance liquid chromatographic (HPLC) analysis

HPLC analysis was achieved using polar solvents methanol: water (50:50) as the mobile phase. The wavelength of 254 nm was found to be optimal for the highest sensitivity. The analysis of crude extract has revealed 1 peak in *X. stockiae* and 3 peaks in *P. luminescens*. The R_f values with respective areas (%) covered by the individual peaks are represented in Figure 2.

Fourier transform infrared spectroscopic (FT-IR) analysis

The FTIR was used to identify the functional group of the active components based on peak value of the infrared radiation. The results revealed the presence of various functional groups in ethyl acetate crude extracts (Figure 3).

FT-IR predicts the molecular configuration of different functional groups present in the symbiotic bacterial extracts. The FTIR spectrum of ethyl acetate extract of *X. stockiae* showed bands with

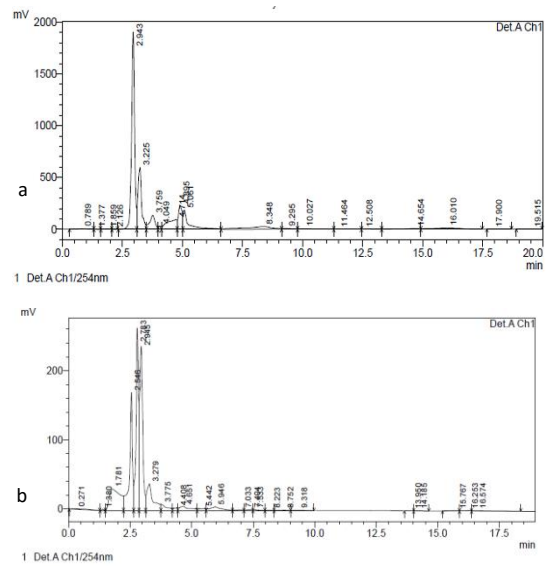


Figure 2. HPLC analysis of ethyl acetate bacterial crude extracts. a: *X. stockiae*; b: *P. luminescens.*

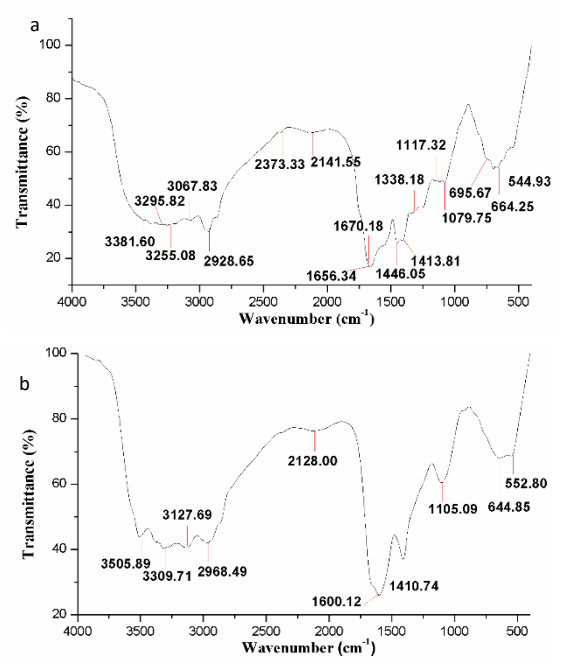


Figure 3. FTIR analysis of ethyl acetate bacterial crude extracts. a: *X. stockiae*; b: *P. luminescens.*

their respective stretches and corresponding functional groups at 3381.60 (O-H stretch; carboxylic groups), 3295.82 (C=H stretch; alkene group), 3255.08 (C-H stretch; alkene groups), 3067.83 (C=H stretch; alkene group), 2928.08 (CH stretch; alkene group), 2373.33 (P-H stretch), 2141.55 (isocyanides stretch; miscellaneous), 1670.18 (C=H stretch; alkene group), 1656.34 (C-H stretch; alkene group), 1446.05 (S=O sulfate ester stretch), 1413.81 (Ar C-C stretch; C-C in ring), 1338.18 (NH stretch; amines), 1117.32 (P-H phosphine stretch; miscellaneous), 1079.75 (NH Stretch; amines), 695.67 (C=C Stretch; alkenes), 664.25 (C=C stretch; alkynes) and 544.63 cm^{-1} (C-H wag (-CH2X) stretch; alkyl halides). Whereas the FTIR spectrum of ethyl acetate extract of *P. luminescens* showed bands at 3505.89 (NH Stretch (free);Amides), 3309.71 (C≡C stretch; alkynes), 3127.69 (C≡C stretch; alkynes group), 2968.49 (C=C stretch; trans RCH=CHR), 2128.00 (isocyanides stretch; miscellaneous), 1600.12 (C-O stretch; carboxylic acid), 1410.74 (S=O Sulfate ester stretch; miscellaneous), 1105.09 (C-H wag (-CH2X) stretch; alkyl halides), 644.85 (C=C stretch; alkynes) and 552.80 cm^{-1} (C-H wag (-CH2X)

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stretch; alkyl halides). In addition the recorded, FTIR peaks indicated the presence of alcohols, alkynes, aromatics, carboxylic acids and alkyl halides.

Antibacterial activity

The antibacterial activity of the ethyl acetate extract of *X. stockiae* and *P. luminescens* was evaluated using different concentrations on four selected bacterial pathogens. Among the bacteria tested, *Staphylococcus aureus* and *Salmonella typhi* were found to be very sensitive to all the tested extract. Similarly, *K. pneumonia* had the least susceptibility and *S. aureus* had the highest sensitivity to symbiotic bacteria of the entomopathogenic nematode. Entomopathogenic bacterial (EPB) antibiotics were used to monitor activity during isolation and identification of EPB bioactive compounds by well plate method (Figure 4).

All the concentrations of *X. stockiae* extract were effective against *S. aureus*. Both extracts of *X. stockiae* and *P. luminescens* did not produce any inhibition zone in all studied concentrations, against *K. pneumonia* and *E. coli* (Table 2 and 3). When compared to *X. stockiae*, *P. luminescens* showed antimicrobial activity, at maximum amount of 150 and 200 µl with the inhibition zone of 11 and 15 mm, respectively against *K. pneumonia* and *S. aureus*. However, no inhibition zone was observed against *E. coli* strain. In the positive control, the maximum zone of inhibition (28 mm) was observed for *S. typhi* and the minimum was observed for *K. pneumonia* strain (18 mm). The extract which did not produce any zone of inhibition was mentioned as 6 mm (diameter of well).

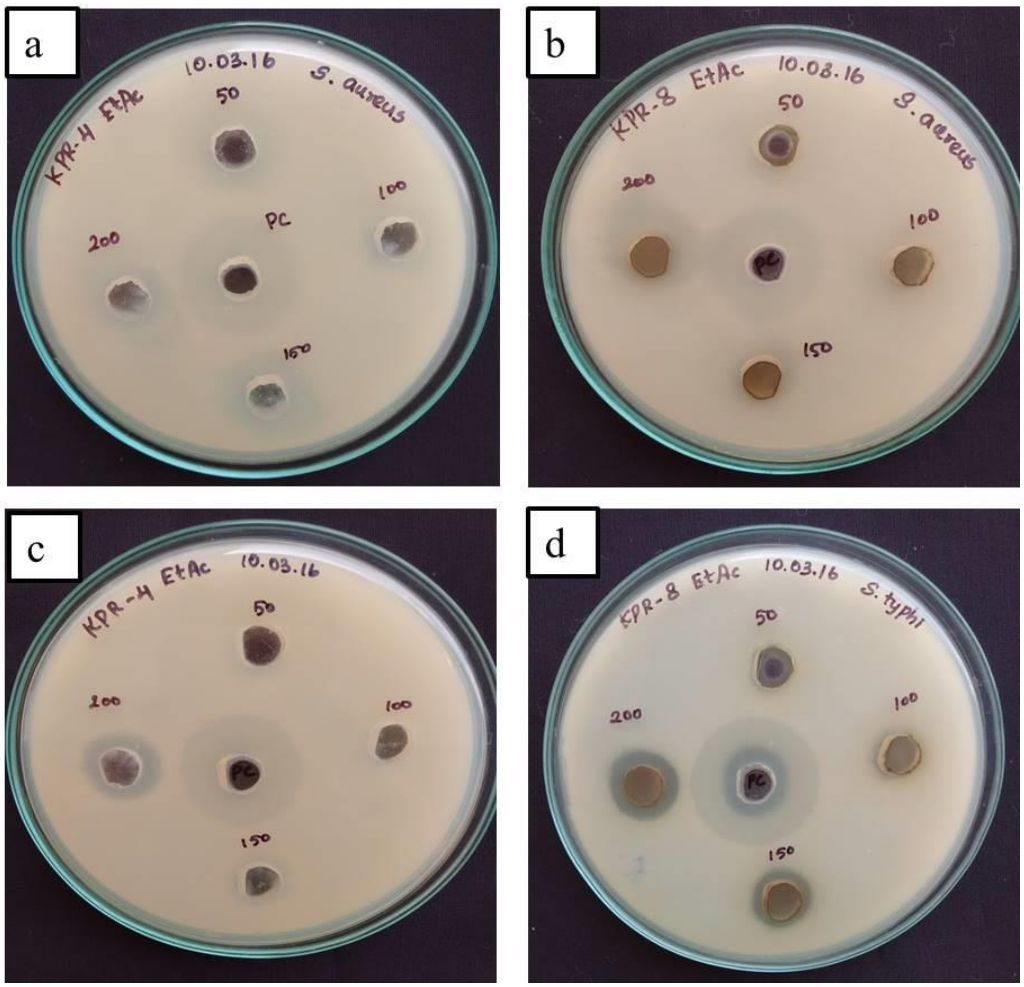


Figure 4. Antibacterial activity of organic fraction of ethyl acetate bacterial crude extracts. a: *X. stockiae* ETAC against *S. aureus*; b: *P. luminescens* against *S. aureus*; c: *X. stockiae* against *S. typhi*; d: *P. luminescens* against *S. typhi*.

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Table 2. Antibacterial activity of crude extract of *X. stockiae*

Conecentration (µl)/disc	Zone of inhibition*			
	S. aureus	*K. pneumonia*	*E. coli*	*S. typhi*
50	11±1	5.67±0.58	6.33±0.58	6.33±0.58
100	15±1	6±0	7.33±0.58	17±1
150	21±1	6.67±0.58	5.66±0.58	22±2
200	22.66±1.54	7±1	17±1	23.67±1.53
Positive	24±1	16.67±1.53	21.67±1.53	25±2

*Values are expressed as mean±standard deviation.

Table 2. Antibacterial activity of crude extract of *X. stockiae*

Conecentration µl/disc	Zone of inhibition*			
	S. aureus	*K. pneumonia*	*E. coli*	*S. typhi*
50	6.33±0.58	6.33±0.58	6±0	6.33±0.58
100	7.67±0.58	7.33±0.58	6.33±0.58	14.67±2.52
150	16±1	12.67±2.08	6.67±0.58	16±1
200	18±1	16.67±1.53	7±1	17.33±2.08
Positive	23.33±1.53	26±1	22.67±1.53	26.67±1.53

*Values are expressed as mean±standard deviation.

Discussion

Synthetic insecticides are progressively replaced by biological insecticides, as they show various side effects. The majority of the biocontrol agents need days or weeks to kill insects/pathogens, but nematodes and bacterial complexes kill insect hosts within 24 to 48 h. The increased interest towards application of the biocontrol method is due to absence of adequate tools allowing an effective, and environmentally acceptable control of the soil-inhabiting insect pests (13). In general, EPNs act as vector by transmitting the symbiotic bacteria to insect host, which cause septicemia within a few days of infection. Regarding the high cost of production in comparison with synthetic pesticides, EPNs were introduced primarily against soil pests in high value crops. The presently obtained results strengthen the hypothesis of a strong specificity in the symbiotic interactions between EPN and bacteria. These symbiotic bacteria are closely related to the *Enterobacteriaceae* family, and present many common properties with their enteric neighbors. The phylogeny of these organisms is relatively definite in the sense that they are clearly placed in the gamma group of *Proteobacteria*.

Purification of the active protein complex from the symbiotic bacteria such as *Xenorhabdus* and *Photorhabdus* bacteria, by various chromatographic steps including HPLC revealed the presence of four distinct proteins called "Toxin complexes" (14). San-Blas et al. studied the *Xenorhabdus* and *Photorhabdus* bacteria by FTIR spectrum and found a unique spectrum characterized by several functional groups (15). The spectra were different at the region below 1400 cm^{-1} which corresponds to the stretching vibrations of phosphate and carbohydrates. Böszörményi et al. studied the crude extract of the symbiotic *Xenorhabdus* bacteria on TLC with butanol: acetic acid: water ratio of 4:1:1, and found that the R_f value was 0.49 (16). In the prospected area, each nematode species was associated with a distinct bacterial isolate belonging

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to different *Xenorhabdus* or *Photorhabdus* species or subspecies. Our results support the suggestion of a strong specificity in the symbiotic interactions between EPN and bacteria.

To maintain their monoxenic condition, symbiotic bacteria are able to produce both wide and narrow spectral bacteriocins. The monoxenic state leads to lethal septicemia of the target host, which is necessary for growth and survival of the associated nematodes in the cadaver (17). Webster et al. reported the synthesis and secretion of various types of antibiotics against bacteria and fungi within the bacterial symbionts culture (5). Antibacterial compounds present in the extract of *Xenorhabdus* and *Photorhabdus* bacteria cultures were shown to be active against many medically and agriculturally challenging Gram-positive and Gram-negative bacteria (18).

Bacteriocins production is useful for symbiosis because the natural symbiont can out-compete closely related bacteria (19). Xenorhabdicin is a bacteriocin isolated from *X. nematophila*, and is active against *Xenorhabdus* spp., *P. luminescens*, and *Proteus* sp. (20). Our results suggest that the entomopathogenecity of *Steinernema* and *Heterorhabditis* is mainly attributable to extracellular toxic factors associated with their symbiotic bacteria *Xenorhabdus* and *Photorhabdus*. Relatively, these bacteria may represent possible sources of novel compounds with effective antibacterial activity which may be used as bio insecticides. Overall, this study extends the knowledge on metabolites of EPNs and is potentially useful to develop new commercial strains for biological control of insects.

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Conflict of interest

The authors declared no conflict of interest.

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