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Antiprotozoal activity of different *Xenorhabdus* and *Photorhabdus* bacterial secondary metabolites and identification of bioactive compounds using the easyPACId approach

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Natural products have been proven to be important starting points for the development of new drugs. Bacteria in the genera *Photorhabdus* and *Xenorhabdus* produce antimicrobial compounds as secondary metabolites to compete with other organisms. Our study is the first comprehensive study screening the anti-protozoal activity of supernatants containing secondary metabolites produced by 5 *Photorhabdus* and 22 *Xenorhabdus* species against human parasitic protozoa, *Acanthamoeba castellanii*, *Entamoeba histolytica*, *Trichomonas vaginalis*, *Leishmania tropica* and *Trypanosoma cruzi*, and the identification of novel bioactive antiprotozoal compounds using the easyPACId approach (easy Promoter Activated Compound Identification) method. Though not in all species, both bacterial genera produce antiprotozoal compounds effective on human pathogenic protozoa. The promoter exchange mutants revealed that antiprotozoal bioactive compounds produced by *Xenorhabdus* bacteria were fabclavines, xenocoumacins, xenorhabdins and PAX peptides. Among the bacteria assessed, only *P. namnaoensis* appears to have acquired amoebicidal property which is effective on *E. histolytica* trophozoites. These discovered antiprotozoal compounds might serve as starting points for the development of alternative and novel pharmaceutical agents against human parasitic protozoa in the future.

Infectious diseases are caused by the invasion and continued presence of pathogenic microorganisms such as viruses, bacteria, fungi, protozoa, nematodes, etc. in a host`s body organ, tissue, or cells. Protozoa in particular, such as *Acanthamoeba castellanii* (*A. castellanii*), *Entamoeba histolytica* (*E. histolytica*), *Trichomonas vaginalis* (*T. vaginalis*), *Leishmania tropica* (*L. tropica*) and *Trypanosoma* spp., are eukaryotic single-celled organisms that are the leading cause of numerous untold deaths and devastating chronic diseases worldwide, especially in underdeveloped and developing countries of sub-Saharan Africa, Asia and South America[1–3]. They are transmitted directly or indirectly through contact, air contaminated food or water, or by vectors from infected humans and animals to healthy others[1]. Poverty, inadequate sanitation and unhygienic living conditions, malnutrition, suitable climatic factors, ineffective anti-parasitic drugs, inept vector control interventions, insecticide resistance

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are some of the factors that contribute to the persistence and incidence of such parasitic infectious diseases in various parts of the world[4–6].

Since protozoan parasites are eukaryotic organisms that share functional homology with mammalian cells, currently available drugs for the treatments of parasitic diseases are generally toxic to human cells and have adverse side effects[7,8]. Owing to these undesired effects and considering the development of resistant strains of parasites against pharmaceutical products, new drugs with different modes of action on target parasites and minimal toxicity to host cells are urgently required[9,10].

Natural products (or secondary metabolites) have been proven to be an important starting point for the development of new drugs. Screening natural products provide the chance of discovering new molecules with unique structure, high activity, and selectivity[11]. The most important natural product sources in nature are fungi[12,13], plants[14] and bacteria[11,15,16]. Various fungi and bacteria produce antimicrobial compounds as secondary metabolites to compete with other organisms. One of the sources of novel bioactive therapeutics against parasites are insect pathogenic *Photorhabdus* and *Xenorhabdus* bacteria. These bacteria encode several putative biosynthetic pathways for natural product biosynthesis[17–19] of which several of them are conserved since they fulfill important ecological functions in their ecological niche[20]. *Photorhabdus* and *Xenorhabdus* bacteria are associated with entomopathogenic nematodes which are obligate and lethal insect parasitic organisms[21,22]. When these nematodes penetrate an insect host, they release their mutualistic bacteria into the insect hemolymph and within 48 h the insect host is killed because of bacterial toxins and enzymes[23,24]. Furthermore, to protect the nematode-infected cadaver from opportunistic microorganisms (e.g., bacteria, fungi, protozoa, and viruses) both *Xenorhabdus* and *Photorhabdus* bacteria produce a variety of natural products that have antimicrobial activities[19,25,26]. Although several studies have reported the antibacterial[27–30], antifungal[29–35], and insecticidal[36–38] activities, only very few studies have investigated the antiprotozoal effect of the secondary metabolites produced by these bacteria[39,40]. Currently, more than 40 different species of *Photorhabdus* and *Xenorhabdus* bacteria have been identified[23,41] that produce different sets of natural products[42]. The aim of our study was to investigate natural products produced by five *Photorhabdus* and 22 *Xenorhabdus* species against human parasitic protozoa, *A. castellanii, E. histolytica, T. vaginalis, L. tropica,* and *Trypanosoma cruzi* (*T. cruzi*), and the identification of novel bioactive antiprotozoal compounds by using the easyPACId (easy Promoter Activated Compound Identification) approach[43].

Material and methods

Bacterial sources and preparation of cell-free supernatants. The cell-free supernatants of 22 *Xenorhabdus* and 5 *Photorhabdus* species were tested against human parasitic protozoa (Table 1). All bacteria strains were obtained from the Bode lab and were kept at − 80 °C as stock culture until use.

A loopfull of bacteria taken from stock culture was inoculated to Luria Bertani (LB) (Merck) agar medium and incubated at 30 °C for 24 h. A single colony was picked and inoculated to 10 ml sterilized Tryptic Soy Broth (TSB) medium (Merck) and cultivated at 30 °C for 24 h to be used as overnight culture. Subsequently, 1 ml from overnight culture was transferred to 50 ml sterilized TSB medium and incubated at 30 °C and 150 rpm for 120 h (it is known that these bacteria produce the most secondary metabolite after 120 h)[30,44]. To obtain cell-free supernatant, the bacterial broth was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was collected carefully and filtered through a 0.22 µm Millipore filter (ISOLAB)[45]. An aliquot of the filtrated suspension was streaked onto NBTA agar to verify the absence of bacterial cells[46]. The supernatants were poured into the 50 ml sterile centrifuge tubes (Corning, NY) and kept at − 20 °C for up to 2 weeks prior to use[47,48].

In vitro cultures of parasitic protozoons. Axenic cultures of *A. castellanii* trophozoites (ATCC 30010) were maintained in liquid PYG (protease peptone—yeast extract—glucose) medium supplemented with penicillin G (500 U/ml) and streptomycin (50 µg/ml)[49] (Pérez-Serrano et al. 2000). The cultures were refreshed weekly in 25 ml cell culture flasks (Sigma) and incubated at 30 °C, until use[50,51]. Cells from the culture medium were harvested by centrifugation at 2000 rpm for five minutes and washed three times with Phosphate-Buffered Saline (PBS). *Acanthamoeba castellanii* trophozoites adhering to flasks were collected by placing the flasks on ice for 30 min with gentle agitation[52,53].

Entamoeba histolytica (ATCC 30459) strain was kindly provided by Dr. Charles Graham Clark from the London School of Hygiene and Tropical Medicine. *Entamoeba histolytica* trophozoites were cultured axenically in LYI medium (880.0 ml LYI Broth, 20.0 ml Vitamin Mixture, 100.0 ml Heat Inactivated Adult Bovine Serum) supplemented with penicillin G (500 U/ml) and streptomycin (50 µg/ml)[54]. The cultures were routinely maintained by subculturing into screw capped test tube containing 7 mL of LYI medium[55,56].

Trichomonas vaginalis (ATCC 30001) trophozoites were grown in Diamond's trypticase yeast-extract maltose (TYM) medium (0.5 mg of L-cysteine HCl, 0.1 g of ascorbic acid, 0.4 g of K_2HPO_4, 0.4 g of KH_2PO_4, 10 g of trypticase, 2.5 g of maltose and 10 g of yeast extract in one ml of distilled water, pH:6) supplemented with 100 IU/ml streptomycin, 100 IU/ml penicillin and 10% heat-inactivated Fetal Bovine Serum (FBS). *T. vaginalis* subcultures were cultured regulaly to maintain viability and for use in the assays[57].

Leishmania tropica (ATCC 50129) promastigotes were routinely cultured at 27 °C in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated FBS (Cegrogen, Stadtallendorf-Germany). The culture was sustained in 25 ml flasks and stationary phase of promastigotes were obtained[58].

Trypanosoma cruzi (CBU-TC01) trypomastigotes were obtained from the parasite biobank of Manisa Celal Bayar University School of Medicine Department of Parasitology Manisa, Turkey. The trypomastigotes were incubated at 27 °C in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 200 U penicillin/ml, and 0.2 mg streptomycin/ml. Subcultures were maintained in 25 ml flasks until use in the experiments[59,60].

	Bacteria species
1	*Xenorhabdus nematophila* ATCC 19061
2	*X. bovienii* SS-2004
3	*X. vietnamensis* DSM 22392
4	*X. cabanillasii* JM26-1
5	*X. szentirmaii* DSM 16338
6	*X. stockiae* DSM 17904
7	*X. ehlersii* DSM 16337
8	*X. koppenhoferi* DSM 18168
9	*X. indica* DSM 17382
10	*X. maulenoii* DSM 17908
11	*X. poinarii* G6
12	*X. griffiniae* DSM 17911
13	*X. ishibashi* DSM 22670
14	*X. doucetiae* DSM 17909
15	*X. innexi* DSM 16336
16	*X. japonica* DSM 16522
17	*X. khoisanae*
18	*X. beddingii* DSM 4764
19	*X. budapestensis* DSM 16342
20	*X. miraniensis* DSM 17902
21	*X. hominickii* DSM 179903
22	*X. kozodoii* DSM 17907
23	*Photorhabdus kayaii* DSM 15194
24	*P. namnaoensis* PB 45.5
25	*P. laumondii* TTO1
26	*P. akhurstii* DSM 15138
27	*P. thracensis* DSM 15199

Table 1. Bacterial species used in antiprotozoal activity tests.

In vitro antiprotozoal activity of bacterial secondary metabolites. Except for *E. histolytica*, the microdilution method was used to assess the antiprotozoal activity of the bacterial supernatants against *A. castellanii*, *T. vaginalis*, *L. tropica* and *T. cruzi*. The four parasites were seeded in 96-well Microtiter plates (Greiner, Germany) and the supernatants were applied at serial concentrations ranging from 10% to 1.25%. Briefly, Trophozoites of *A. castellanii* and *T. vaginalis* were adjusted to 5×10^4 and 2×10^6 cells/mL, respectively. The density of *L. tropica* promastigotes and trypomastigotes of *T. cruzi* were adjusted to 1×10^6 cells/mL[61,62]. Plates with the isolates were incubated at 30 °C for 24 h, 37 °C for 48 h and 27 °C for 72 h for *A. castellanii, T. vaginalis*, *L. tropica,* and *T. cruzi* respectively. Screw capped test tubes were used for *E. histolytic*a instead of the plates used for other parasites. *Entamoeba histolytica* trophozoites (200 µl of 3×10^5 cells/mL) were inoculated into the tubes containing 1.8 ml of fresh axenic LYI medium with the bacterial supernatants at final concentrations of 10%, 5%, 2.5%, and 1.25%. The tubes were incubated at 37 °C for 48 h[56].

Two methods were used to determine the antiprotozoal effects of the bacterial supernatants in vitro. To assess the anti-leishmanial activity was performed by using the XTT (sodium 3,39- [1- (phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) cell proliferation kit (Roche Molecular Biochemicals, Mannheim, Germany) as previously described[63].

Cell viability assay test was used for *A. castellanii*, *T. vaginalis*, *E. histolytica* and *T. cruzi*. The assay was evaluated by adding 0.1% trypan blue stain (TB) [the number of live (unstained) and dead (stained)] using a hemocytometer[64,65]. The parasite mortality (in %) for each bacterial supernatants sample was caluclated according to the formula: % Mortality of parasites = (Control Negative-Test sample) × 100%/Control negative. Only 100% inhibition of the parasite was considered when no motile parasite was observed.

Two negative and one positive control group were included in each experiment. Bacterial culture medium (TSB) and parasite medium was used as a negative control. Metronidazole (Specia Rhone Poulenc Rorer, Paris, France) for *T. vaginalis* and *E. histolytica*, Chlorhexidine (Sigma, Spain) for *A. castellanii*, N-methyl meglumine (Glucantime™, Rhone Poulenc, France) for *L. tropica* and Benzimidazole (Sigma, Spain) for *T. cruzi* were used as positive controls. Each assay was performed at least three times in triplicate.

Identification of antiprotozoal compounds using the easyPACId method. *Generating promoter exchange mutant strains.* The easyPACId approach method recently developed by Bode et al.[43] was used to identify the antiprotozoal compounds in *Xenorhabdus* spp. bacteria. Briefly, Δ*hfq* mutants of each bacterial species (*X. budapestensis, X. cabanillasii, X. doucetiae, X. hominickii, X. nematophila, X. stockiae* and *X. szentirmaii*)

were first generated and then the native promoter regions of selected natural product biosynthetic gene clusters of these bacteria were exchanged with the chemically inducible promoter P_{BAD} (addition of L-arabinose) via integration of the pCEP-KM plasmid[43,66]. This allows the selective production of a desired single natural product compound class and enables direct bioactivity analysis of the corresponding supernatant instead of time-consuming isolation of single compound(s) from analytically complex wild type extracts. The generation of the described *Xenorhabdus spp.* Δ*hfq* as well as *Xenorhabdus spp.* Δ*hfq* pCEP-KM-xy mutants listed in Table 3 (xy describes the locus of the first biosynthetic gene cluster) is described in detail by Bode et al.[43].

Obtaining cell-free supernatants from different *Xenorhabdus spp.* Δ*hfq* promoter exchange mutants. A single *Xenorhabdus spp.* Δ*hfq* pCEP-KM-xy mutant colony, cultivated on LB agar supplemented with a 50 µg/mL final concentration of kanamycin at 30 °C for 48 h, was transferred into LB medium (10 mL) also supplemented with a 50 µg/mL final concentration of kanamycin and incubated at 150 rpm and 30 °C. Then, this overnight culture was inoculated into a fresh 20 mL LB with the final optical density (OD_{600}) adjusted to 0.1. After an hour incubation at 30 °C, these cultures were induced with 0.2% L-arabinose and incubated again for 72 h at 150 rpm and 30 °C[43,67]. Cultures of non-induced mutants contained no L-arabinose. The cell-free supernatants were obtained by centrifugation at 10.000 rpm for 20 min in 50 ml Falcon tubes at 4 °C and filteration through a 0.22-µm Millipore filter (Thermo scientific, NY) to ensure total removal of bacterial cells[34]. The cell free supernatants were stored at − 20 °C and used within 2 weeks[48].

Testing the antiprotozoal activity of cell-free supernatants of mutant strains. Antiprotozoal activity of 5-day-old cell-free supernatants of wild type strains, as well as induced (with arabinose) and non-induced (without arabinose) promoter exchange mutant strains were tested in microdilution bioassay as previously described in the in vitro antiprotozoal activity tests section.

Anti-protozoal activity of bioactive extracts obtained from hfq mutants. As a last step, extracts containing identified anti-protozoal compounds were tested again on the parasite species at different concentrations ranged from 10 to 0.078% (v/v). The same experimental method used in antiprotozoal activity tests was carried out here.

Anti-protozoal bioactive compound extraction was performed by culturing induced *X. nematophila* Δ*hfq*_pCEP_ kan_XNC1_1711 for xenocoumacin production and *X. doucetiae* Δ*hfq*_P_{BAD}_ PAX_km for PAX peptide production in LB (6L) with 2% XAD® resin at 30 °C for 3 days. Afterwards the resin was exhaustively extracted with methanol (3 × 2 L) at 24 ± 1 °C and concentrated under reduced pressure to give a crude extract enriched by the desired natural compound class. The extracts were then dissolved in DMSO and prepared as a stock solution with distilled water. Fabclavine was obtained by concentrating the supernatant of the induced *X. cabanillasii* Δ*hfq*_128-129 culture 10-fold using an evaporator.

Statistical analysis. Differences in antiprotozoal activity of the supernatants were compared with one-way ANOVA and the means separated using Tukey's test. P values < 0.05 were considered as significant[68]. The results are reported as mean ± SD for all values.

Ethics approval. This article does not contain any studies with human participants or animals performed by any of the authors.

Results

In vitro antiprotozoal activity tests. *Acanthamoeba castellanii.* The in vitro activity assays against the trophozoite of *A. castellanii* showed that 12 of 22 *Xenorhabdus* species exhibited effective antiprotozoal activity whereas, none *Photorhabdus* strain showed any activity (Table 2). At 10% supernatant concentration, *A. castellanii* cell mortality ranged between 78 and 100% depending on *Xenorhabdus* species. Relative to the negative control, 12 of 22 tested *Xenorhabdus* supernatants caused significant *A. castellanii* mortality ($F = 1828.80$; df = 28, 232; $P < 0.0001$). Chlorhexidine used as positive control showed 100% mortality and no statistical difference was observed between chlorhexidine and *X. budapestensis, X. cabanillasii, X. doucetiae* and *X. innexii* supernatants. At 5% concentration of bacterial supernatants, the highest level of mortality (> 95%) was exhibited by *X. budapestensis, X. cabanillasii, X. innexii* and chlorhexidine (which were not statistically different from each other). All *Xenorhabdus* species with antiprotozoal activity presented statistically significant mortality compared to the negative controls ($F = 1357.38$; df = 28, 232; $P < 0.0001$). In the following concentration (2.5%), the supernatants of *X. budapestensis, X. cabanillasii* and *X. innexii* exhibited more than 90% mortality on *A. castellanii* trophozoites and no significant difference was observed between this group and chlorhexidine (Table 2). *Xenorhabdus miraniensis* and *X. nematophila* supernatants caused the lowest mortality (55%) on the trophozoites. Despite this, there was a significant difference between all effective 12 *Xenorhabdus* supernatant treatments and negative controls ($F = 653.63$; df = 28, 232; $P < 0.0001$). At the lowest concentration of tested bacterial supernatants (1.25%), *X. budapestensis* and *X. innexii* species showed equal mortality with chlorhexidine. Following these species, supernatants of *X. cabanillasii*, *X. doucetiae*, *X. hominickii, X. stockiae,* and *X. szentirmaii* were more effective compared with the other treatments. Even at highly diluted concentrations of the supernatants compared to negative controls, significant mortalities were obtained ($F = 550.64$; df = 28, 232; $P < 0.0001$) (Table 2).

Trichomonas vaginalis. It was noted that 10 of the 22 tested *Xenorhabdus* supernatants were significantly lethal against *T. vaginalis* when compared with negative controls. Of these, *X. cabanillasii, X. doucetiae, X. hominickii,*

antiprotozoal activity (%)

0-20	21-40	41-60	61-80	81-100

Low High

	A. castellanii				*T. vaginalis*				*L. tropica*				*T. cruzi*				*E. histolytica*			
	Concentrations of bacterial supernatants (%)																			
Bacteria species	10	5	2.5	1.25	10	5	2.5	1.25	10	5	2.5	1.25	10	5	2.5	1.25	10	5	2.5	1.25
X. beddingii	f	g	f	h	f	e	f	f	f	e	g	e	e	e	d	f				
X. bovienii	f	g	f	h	f	e	f	f	f	e	g	e	e	e	d	f				
X. budapestensis	100^{a}	99^{a}	97^{a}	95^{a}	f	e	f	f	100^{a}	100^{a}	98^{a}	97^{a}	89^{b}	82^{c}	80^{c}	77^{e}				
X. cabanillasii	97^{ab}	96^{a}	93^{ab}	89^{b}	94^{cd}	76^{d}	50^{e}	49^{cd}	100^{a}	100^{a}	96^{a}	93^{ab}	100^{a}	100^{a}	98^{a}	97^{a}				
X. doucetiae	98^{ab}	91^{bc}	89^{bc}	86^{b}	93^{cde}	83^{c}	56^{e}	42^{cd}	57^{e}	45^{d}	41^{ef}	31^{d}	58^{d}	31^{d}						
X. ehlersii	f	g	f	h	f	e	f	f	f	e	g	e	e	e	d	f				
X. griffinae	f	g	f	h	f	e	f	f	53^{e}	51^{d}	33^{f}	e	e	e	d	f				
X. hominickii	93^{bc}	93^{bc}	87^{bc}	78^{cd}	91^{de}	83^{c}	71^{d}	55^{c}	100^{a}	100^{a}	97^{a}	96^{a}	91^{b}	43^{d}	11^{d}	f				
X. indica	89^{cd}	84^{d}	75^{d}	70^{d}	85^{e}	82^{c}	59^{e}	46^{cd}	100^{a}	100^{a}	100^{a}	97^{a}	95^{ab}	94^{b}	92^{b}	90^{b}				
X. innexi	97^{ab}	97^{a}	96^{a}	95^{a}	f	e	f	f	100^{a}	100^{a}	98^{a}	96^{a}	88^{b}	81^{c}	82^{c}	78^{e}				
X. ishibashi	f	g	f	h	f	e	f	f	f	e	g	e	e	e	d	f				
X. japonica	f	g	f	h	f	e	f	f	f	e	g	e	e	e	d	f				
X. koppenhoeferii	f	g	f	h	f	e	f	f	f	e	g	e	e	e	d	f				
X. kozodoi	f	g	f	h	98^{ab}	88^{bc}	83^{c}	49^{cd}	f	e	g	e	e	e	d	f				
X. mauleonii	82^{e}	77^{e}	70^{d}	62^{e}	96^{bc}	93^{b}	85^{c}	38^{de}	78^{d}	73^{bc}	64^{d}	49^{c}	e	e	d	f				
X. miraniensis	78^{e}	59^{f}	55^{e}	36^{f}	97^{bc}	93^{b}	92^{ab}	87^{b}	96^{b}	98^{a}	97^{a}	96^{a}	100^{a}	100^{a}	100^{a}	98^{a}				
X. nematophila	90^{cd}	83^{d}	55^{e}	21^{g}	88^{bcd}	81^{c}	49^{e}	44^{cd}	82^{d}	73^{bc}	50^{de}	36^{d}	97^{ab}	93^{b}	90^{b}	85^{d}				
X. poinarii	f	g	f	h	f	e	f	f	f	e	g	e	e	e	d	f				
X. romanii	f	g	f	h	f	e	f	f	f	e	g	e	e	e	d	f				
X. stockiae	93^{bc}	92^{bc}	88^{bc}	78^{cd}	86^{e}	57^{f}	52^{e}	33^{e}	100^{a}	98^{a}	95^{ab}	94^{ab}	68^{c}	38^{d}	12^{d}	f				
X. szentirmaii	93^{bc}	89^{cd}	81^{cd}	77^{cd}	f	e	f	f	90^{c}	85^{b}	31^{f}	30^{d}	e	e	d	f				
X. vietnamensis	83^{e}	75^{e}	69^{d}	62^{e}	93^{bcd}	84^{c}	82^{c}	82^{b}	97^{b}	95^{ab}	93^{ab}	92^{b}	51^{d}	44^{d}	14^{d}	f				
P. akhurstii	f	g	f	h	f	e	f	f	f	e	g	e	e	e	d	f				
P. kayaii	f	g	f	h	f	e	f	f	f	e	g	e	e	e	d	f				
P. laumondii	f	g	f	h	f	e	f	f	f	e	g	e	e	e	d	f				
P. namnaoensis	f	g	f	h	f	e	f	f	f	e	g	e	e	e	d	f	53	12		
P. thracensis	f	g	f	h	f	e	f	f	f	e	g	e	e	e	d	f				
Negative control-1	f	g	f	h	f	e	f	f	f	e	g	e	e	e	d	f				
Negative control-2	f	g	f	h	f	e	f	f	f	e	g	e	e	e	d	f				
Positive control	100^{a}	100^{a}	98^{a}	95^{a}	100^{a}	97^{a}	95^{a}	90^{a}	100^{a}	100^{a}	96^{a}	96^{a}	100^{a}	100^{a}	95^{a}	94^{a}	100	98	95	91

Table 2. Antiprotozoal activity of cell-free culture supernatants of *Xenorhabdus* and *Photorhabdus* spp. against different human parasitic protozoa species. Bioactivities are shown for none (white) to highest activity (dark red) in the different assays. Negative control-1: Bacterial culture medium (TSB), Negative control-2: Parasite growth medium. Positive controls: Metronidazole for T. *vaginalis* and E. histolytica, Chlorhexidine for *A. castellanii*, N-methyl meglumine for *L. tropica* and Benzimidazole for *T. cruzi*. Columns with a common superscript letter do not differ significantly at $P = 0.05$).

antiprotozoal activity (%)

0-20	21-40	41-60	61-80	81-100

Low — High

Bacteria species	Mutant name	Compound Name	*A. castellanii*				*T. vaginalis*				*L. tropica*				*T. cruzi*			
			Supernatant concentrations (%)															
			10	5	2.5	1.25	10	5	2.5	1.25	10	5	2.5	1.25	10	5	2.5	1.25
X. szentirmaii	**DSM 16338**	**Wild type**	93	89	81	77					90	85	31	30				
	Δhfq_ pCEP_KM_0346	GameXPeptide																
	Δhfq_pCEP_KM_0377	Pax-short																
	Δhfq_pCEP_KM_1979	Szentirazin																
	Δhfq_pCEP_KM_3397	Rhabdopeptide																
	Δhfq_pCEP_KM_3460	Szentiamid																
	Δhfq_pCEP_KM_3680	Xenobactin																
	Δhfq_pCEP_KM_3942	Rhabduscin																
	Δhfq_pCEP_KM_5118	Pyrrolizixenamide																
	Δhfq_pCEP_KM_fclC	**Fabclavine +**	100	100	100	92					96	86	58	44				
		Fabclavine -																
	Δhfq_pCEP_KM_xfsA	Xenofuranone																
X. nematophila	**ATCC 19061**	**Wild type**	90	83	55	21	88	81	49	44	82	73	50	36	97	93	90	85
	Δhfq_pCEP_kan_XNC1_2022	Xenotetrapeptide																
	Δhfq_pCEP_kan_XNC1_1711	**Xenocoumacin +**	98	81	58	34	100	100	98	94	100	94	78	62	52	33		
		Xenocoumacin -																
	Δhfq_P_{BAD}_XNC1_xndA	Xenortide																
	Δhfq_pCEP_kan_XNC1_2783	PAX																
	Δhfq_P_{BAD}_XNC1_2228	Rhabdopeptide																
	Δhfq_P_{BAD}_XNC1_2713	Xenematide																
	ΔPPTase_P_{BAD}_XNC1_isnA	Rhabduscin																
	Δhfq_ΔisnAB_P_{BAD}_XNC1_2300	Xenortide																
X. doucetiae	**DSM 17909**	**Wild type**	98	91	89	86	93	83	56	42	57	45	41	31	58	31		
	ΔPPTase_P_{BAD}_isnA	Rhabduscin																
	Δhfq _P_{BAD}_xcnA_km	**Xenocoumacin +**	87	82	53	27	98	96	82	75	86	82	68	54	79	55		
		Xenocoumacin -																
	Δhfq _P_{BAD}_xrdA_km	**Xenorhabdin +**	64	62	52	44	91	83	72	54	35	22						
		Xenorhabdin -																
	Δhfq _P_{BAD}_xabA_km	Xenoamicin																
	Δhfq _P_{BAD}_PAX_km	**PAX +**	98	94	68	33	75	68	32									
		PAX -																
	Δhfq _P_{BAD}_gxpS_km	GameXPeptide																
	Δhfq _P_{BAD}prtA	Protegomycin																
	Δhfq _P_{BAD}_DC_km	Acylamide																
X. cabanillasii	**JM26-1**	**Wild type**	97	96	93	89	94	76	50	49	100	100	96	93	100	100	98	97
	Δhfq_128-129	**Fabclavine +**	100	100	100	100	100	100	100	100	100	100	100	100	100	100	97	85
		Fabclavine -																
X. hominickii	**DSM 179903**	**Wild type**	93	93	87	78	91	83	71	55	100	100	97	96	91	43	11	
	Δhfq_130-131	**Fabclavine +**	100	100	96	91	100	100	95	84	100	100	100	92	100	100	75	58
		Fabclavine -																
X. budapestensis	**DSM 16342**	**Wild type**	100	99	97	95					100	100	98	97	89	82	80	77
	Δhfq_pCEP_fclC	**Fabclavine +**	100	100	97	92					100	100	100	98	100	100	94	88
		Fabclavine -																
X. stockiae	**DSM 17904**	**Wild type**	93	92	88	78	86	57	52	33	100	98	95	94	68	38	12	
	Δhfq_pCEP_fclC	**Fabclavine +**	100	100	98	90	100	100	99	96	100	100	100	98	76	56	24	
		Fabclavine -																

Table 3. Activity of promoter exchange mutants and their respective natural products against the *Acanthamoeba castellanii, Trichomonas vaginalis, Leishmania tropica* and *Trypanasoma cruzi.* Activity of all easyPACId strains (delta hfq mutants) was determined after induction of the PBAD Promoter with L-arabinose. Bioactivities are shown for none (white) to highest activity (dark red) in the different assays. In order to confirm the active compounds, these are also shown non-induced (−) and induced (+).

X. kozodoii, X. mauleonii, X. miraniensis and *X. vietnamensis* species displayed more than 90% mortality against *T. vaginalis* at 10% supernatant concentration ($F = 334.60$; $df = 29, 240$; $P < 0.0001$) (Table 2). At 5% concentration, though the positive control metronidazole caused statistically significant mortality compared to the supernatant treatments, all the other effective bacterial supernatants exhibited mortality ranging between 57 and 93% ($F = 288.74$; $df = 29, 240$; $P < 0.0001$). Among the effective bacterial supernatants, *X. miraniensis, X. mauleonii, X. kozodoii* and caused 92, 85 and 83% *T. vaginalis* mortality, respectively, at 2.5% concentration ($F = 288.74$; $df = 29, 240$; $P < 0.0001$). There was a significant difference between the effective treatments and controls ($F = 237.78$; $df = 29, 240$; $P < 0.0001$). *Xenorhabdus miraniensis* and *X. vietnamensis* still exhibited more than 80% mortality, when the supernatants were diluted to the lowest concentration (1.25%) ($F = 255.82$; $df = 29, 240$; $P < 0.0001$). However, none of the *Photorhabdus* species caused *T. vaginalis* mortality at any concentration (Table 2).

Leishmania tropica. *Xenorhabdus budapestensis, X. cabanillasii, X. hominickii, X. indica, X. innexii* and *X. stockiae* supernatants caused 100% mortality at the highest tested concentration (10%) against the promastigote form of *L. tropica*. No differences occurred between this treatment group and positive control ($P > 0.05$). Following those bacteria species *X. vietnamensis, X. miraniensis,* and *X. szentirmaii* supernatants exhibited 97, 96 and 90% mortality, respectively. The other effective bacterial supernatants presented mortalities that ranged between 53 and 82%. There was a significant difference between all effective treatment groups and negative control ($F = 880{,}33$; df = 29, 240; $P < 0.0001$) (Table 2).

Similarly, at 5% concentration, *X. budapestensis, X. cabanillasii, X. hominickii, X. indica, X. innexii, X. miraniensis, X. stockiae* and Stibogluconate caused 98–100% *Leishmania* mortalities. No significant difference was observed among these groups. Although *X. doucetiae* presented the least *Leishmania* mortality (40%), there were statistically significant differences between all effective treatments and negative controls ($F = 232.16$; df = 29, 240; $P < 0.0001$) (Table 2). At 2.5% supernatant concentration, *X. budapestensis, X. cabanillasii, X. hominickii, X. indica, X. innexii, X. miraniensis, X. stockiae* and *X. vietnamensis* showed the highest efficacy (93–100%), whereas *X. griffinae* and *X. szentirmaii* supernatants exhibited the lowest mortalities (33 and 31%, respectively). However, there was a significant difference between all effective treatments and negative control groups ($F = 425.10$; df = 29, 240; $P < 0.0001$) (Table 2).

Bacterial supernatant of *X. budapestensis, X. cabanillasii, X. hominickii, X. indica, X. innexii, X. miraniensis* and *X. stockiae* were still as effective as N-methyl meglumine even at 1.25% concentration. There was a significant difference between the effective bacterial supernatants and negative controls ($F = 393.67$; df = 29, 240; $P < 0.0001$) (Table 2).

Trypanosoma cruzi. At 10% concentration, 10 of 27 bacterial supernatants showed antiprotozoal activity against *T. cruzi* trypomastigotes. Among the bacterial species, *X. cabanillasii* and *X. miraniensis* exhibited 100% mortality followed by *X. nematophila, X. indica, X. hominickii, X. budapestensis* and *X. innexi* supernatants (88–97%). There was a statistically significant difference between effective bacterial supernatants and negative controls ($F = 288.53$; df = 29, 240; $P < 0.0001$) (Table 2).

When the bacterial supernatants were diluted to 5% concentration, *X. cabanillasii* and *X. miraniensis* still exhibited 100% mortality. *Xenorhabdus indica, X. nematophila, X. budapestensis* and *X. innexi* supernatans displayed between 94 and 81% mortality. The effect of *X. hominickii* dropped drastically from 91 to 43%. Significant differences were observed between negative controls and the effective supernatants ($F = 178.60$; df = 29, 240; $P < 0.0001$). At 2.5% concentration of the supernatants, *X. miraniensis* and *X. cabanillasii* had the highest mortality (98–100%) and there was no significant difference between this bacterial species and Benzimidazole (positive control). Among the other bacterial supernatans, *X. budapestensis, X. indica, X. innexi* and *X. nematophila* also caused relatively high mortality which ranged between 80 and 92%. However, *X. doucetiae, X. hominicki, X. stockiae,* and *X. vietnamiensis* lost their antiprotozoal effects at this concentration. A significant statistical difference was observed between the six effective bacterial supernatants and negative controls ($F = 589.60$; df = 29, 240; $P < 0.0001$) (Table 2).

At the lowest tested concentration (1.25%), *X. cabanillasii* and *X. miraniensis* maintained their high activity (97 and 98% mortality, respectively). *Xenorhabdus indica* followed this group with a mortality of 90%. There was a statistical difference between negative control groups and six bacterial supernatants ($F = 929.48$; df = 29, 240; $P < 0.0001$) (Table 2).

Entamoeba histolytica. Unlike *A. castellanii, E. histolytica,* a different amebic parasite, was resistant to the secondary metabolites of *Xenorhabdus* and *Photorhabdus* bacteria. Only *P. namnaoensis* species showed significant mortality compared to the negative controls ($F = 1.02$; df = 28,232; $P < 0.0001$) (Table 2). Positive control (metronidazole) and *P. namnoensis* supernatant caused 100% and 53% cell mortality on *E. histolytica* trophozoites, respectively. However, the other 26 of 27 tested species exhibited only between 0 and 6% mortality at 10% concentration.

Identification of antiprotozoal compounds. The promoter exchange mutants in Δ*hfq* background revealed that antiprotozoal bioactive compounds produced by *Xenorhabdus* bacteria were fabclavines, xenocoumacins, xenorhabdins and PAX peptides (Table 3, Fig. 1). The supernatants obtained from induced mutants showed very high mortality against the parasite cells, non-induced mutants of the same compounds exhibited no activity (Table 3).

Fabclavines produced by *X. cabanillasii, X. hominickii* and *X. stockiae* species had antiprotozal activity against *A. castellanaii, T. vaginalis L. tropica* and *T. cruzi* parasites. Fabclavines produced by *X. budapestensis* was not effective against *T. vaginalis*. *Xenorhabdus szentirmaii* also produces fabclavines being only effective against *A. castellanii* and *L. tropica* with no antiprotozoal activity against *T. vaginalis* and *T. cruzi*.

Xenocoumacins produced by *X. nematophila* species was the bioactive antiprotozoal compound against all tested pathogens. In contrast to other species, *X. doucetiae* species produce more than one antiprotozoal compound. Δhfq_P_{BAD}_PAX_km of *X. doucetiae* producing PAX peptides exhibited antiprotozoal effect on *A. castellanii* and *T. vaginalis*, but *L. tropica* was killed by xenocoumacins and xenorhabdins. *Xenorhabdus doucetiae* Δhfq_P_{BAD}_xcnA_km showed antiprotozoal activity only with xenocoumacins against *T. cruzi* (Table 3). The active compound in *P. namnaoensis* which was the only species that caused mortality on *E. histolytica* trophozoites was not identified due to the lack of promoter exchange mutants of this species.

Anti-protozoal activity of bioactive extracts obtained from hfq mutants. Supernatants containing xenocoumacins, fabclavines and PAX peptides showed variable activity depending on parasite species and

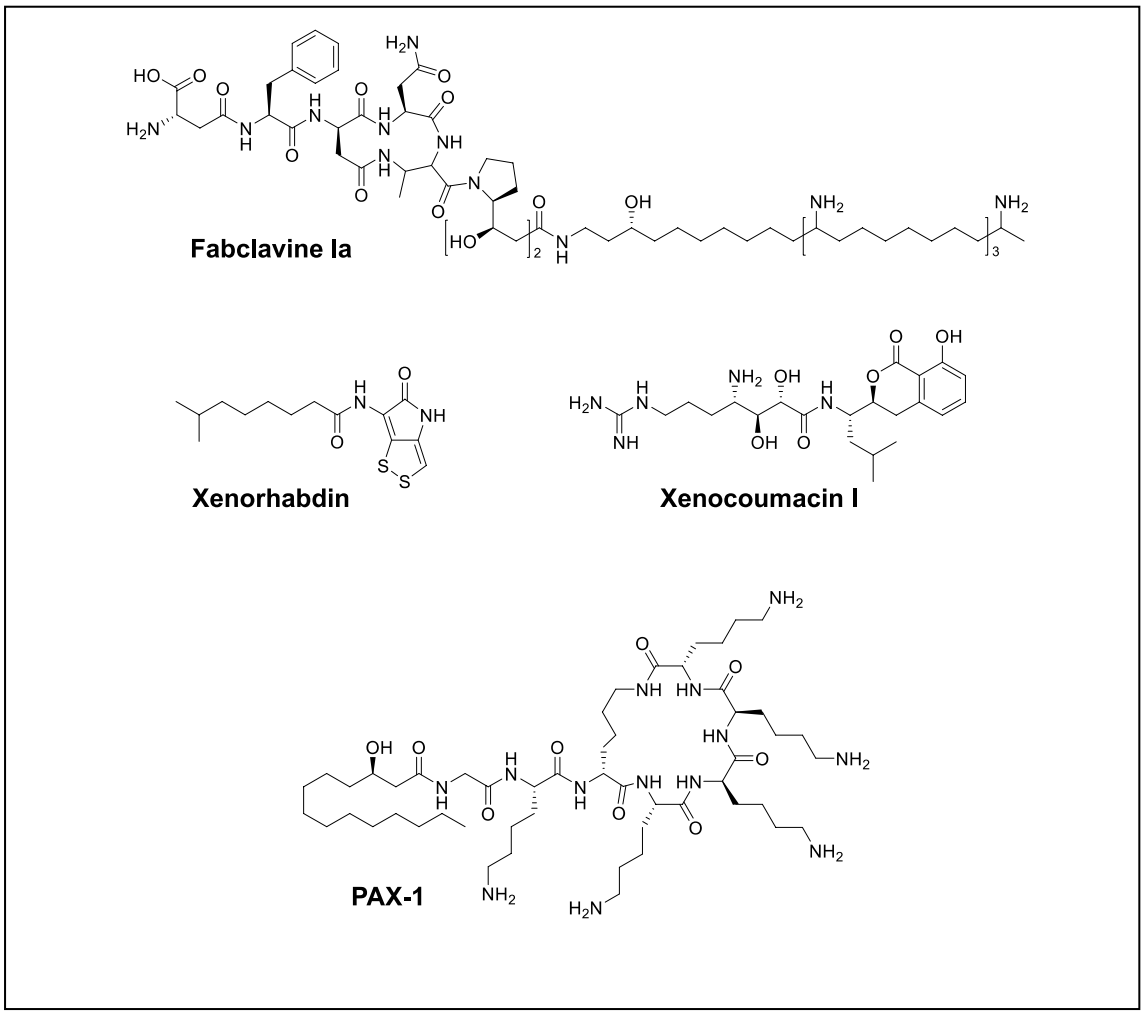

Figure 1. Chemical structure of antiprotozoal natural products from *Xenorhabdus* species.

concentrations; no mortality was observed in the control (Fig. 2). Overall fabclavine molecules were highly effective on all tested parasite species even at very low concentrations.

Discussion

Our data revealed that *Xenorhabdus* and *Photorhabdus* produce antiprotozoal compounds effective on human pathogenic protozoa. However, not all *Xenorhabdus* or *Photorhabdus* species showed this activity. Except for *E. histolytica*, only some of *Xenorhabdus* species exhibited antiprotozoal activity. It was reported that *Xenorhabdus* bacteria produce broad-spectrum compounds with various activity against several organisms such as bacteria, fungi, insects, nematodes, mites, protozoa etc. to protect and bioconvert the host cadaver[69–72]. With the easy-PACId approach we were able to assign the described activities on respective natural products from *Xenorhabdus*.

The bioactivity of fabclavines could be confirmed for *X. budapestensis, X. cabanillasii, X. hominickii, X. stockiae* and *X. szentirmaii* mutants. Biochemically fabclavines are peptide/polyketide hybrids connected to a polyamine moiety generated by a fatty acid/polyketide synthase with similarity to enzymes producing polyunsaturated fatty acids (PUFAs)[67,73,74]. Fabclavines 1a and 1b exhibit various bioactivities against different bacterial, fungal and protozoal organisms[73] and due to such broad-sprectrum activity, fabclavines might serve as protective agents against saprophytic food competitors/microorganisms that attack insect cadavers; this enables *Xenorhabdus/Steinernema* to maintain a monoculture in the infected insect[73]. Fabclavines are structurally very similar to zeamines identified in *Serratia plymuthica* so might similarly permeabilize artificial bacterial and eucaryotic model membranes[75,76]. A structurally yet-undentified fabclavine derivative from *X. innexi* (Xlt) induces membrane degradation at low concentrations in selected mosquito cell lines which led to apoptosis[77]. Production of fabclavine is widespread in *Xenorhabdus* strains whereas, except for *Photorhabdus asymbiotica,* other *Photorhabdus* species do not produce fabclavines[42]. This can explain partially why none of our tested *Photorhabdus* species showed antiprotozoal activity. However, *X. bovienii* is a producer of only the polyamine part of fabclavine[74] and it did not exhibit any activity. There are 32 different types of fabclavine with important variations among their activity[67].

Xenorhabdus nematophila and *X. doucetiae* species do not produce fabclavine[42] but they are effective species on tested parasites except for *E. histolytica*. According to promoter exchange data, it became obvious that *X. nematophila* and *X. doucetiae* perform this task with different compounds. Xenocoumacins are produced using

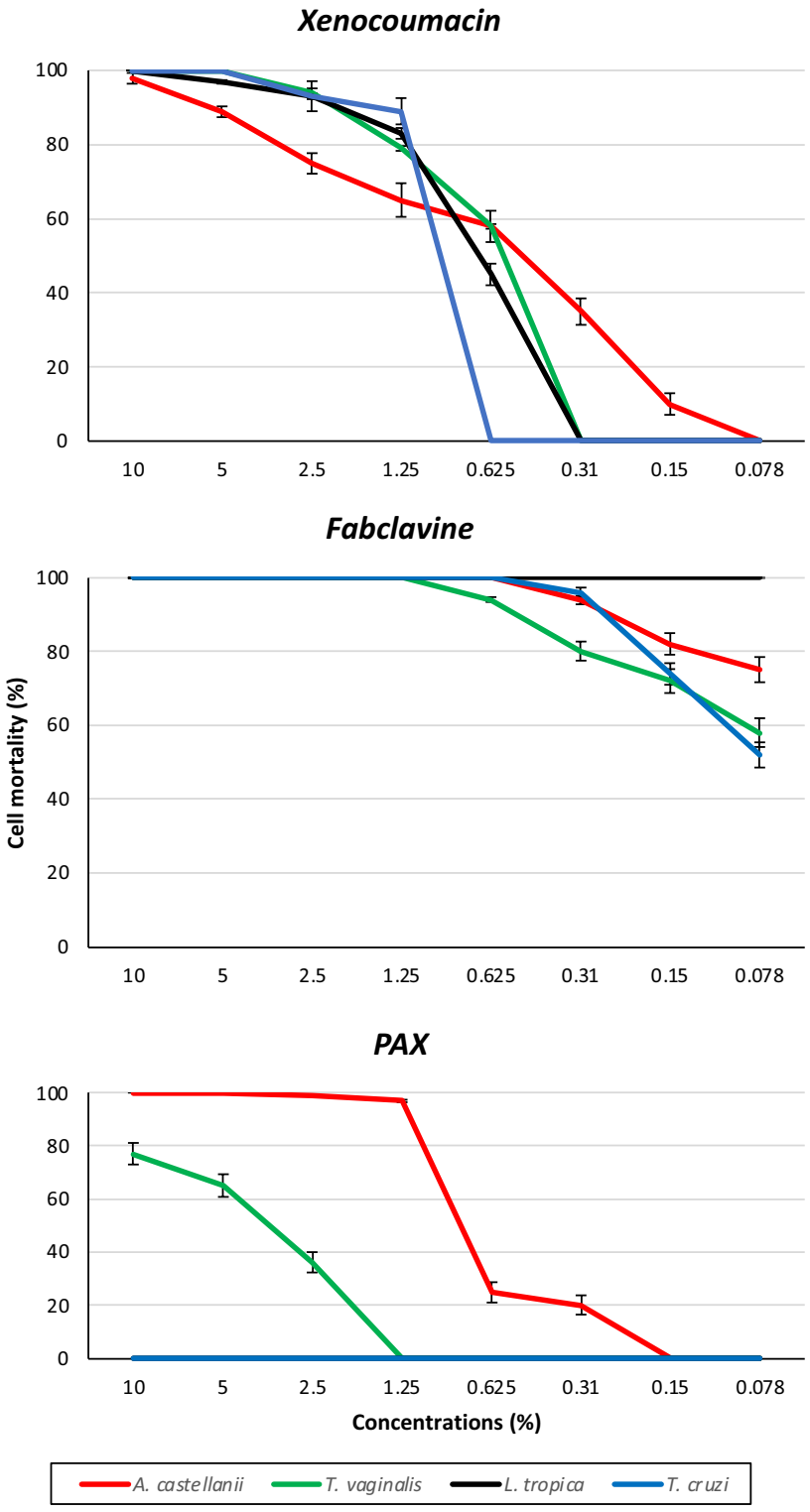

Figure 2. Antiprotozoal activity of supernatants containing bioactive compounds. Xenocoumacins, fabclavines and PAX peptides were obtained from *Xenorhabdus nematophila, Xenorhabdus cabanillasii* and *Xenorhabdus doucetiae,* respectively.

a hybrid nonribosomal peptide synthetase (NRPS) multienzyme (XcnA-N) and polyketide synthase (PKS)[78–80]. When tested for biological activity against *T. b. rhodesiense*, *T. cruzi*, *L. donovani* and *Plasmodium falciparum,* good activities were observed against *T. b. rhodesiense* and *P. falciparum*[80]. Possibly, xenocoumacins inhibit protein biosynthesis in these organisms[80,81]. However, xenocoumacins are not widely distributed in *Xenorhabdus* spp. as one would expect. Among 25 *Xenorhabdus* and *Photorhabdus* strains, xenocoumacins or the corresponding

biosynthetic gene cluster were only be identified from seven *Xenorhabdus* subspecies (*X. nematophila, X. indica, X. miraniensis, X. stockiae, X. kozodoii, X. mauleonii* and *X. doucetiae*)[42]

According to our data, we have determined that xenorhabdins and PAX peptides produced by *X. doucetiae* are other effective secondary metabolites. Xenorhabdins are dithiolopyrrolone compounds[82] and it is reported that they have antibacterial, antifungal, and insecticidal activities[83–85]. Their suggested mode of action is the inhibition of RNA synthesis affecting translation as similar to xenocoumacins[86,87]. PAX peptides are lysine-rich cyclolipopeptides. Gualtieri et al.[88] first described five PAX peptides from *X. nematophila* and then additional eight PAX peptides were identified, and their structures elucidated by Fuchs et al.[73]. Three NRPS genes (*paxABC*) are responsible for the biosynthesis of the PAX compounds. These peptides have antifungal and antibacterial activity. They exhibited strong anti-fungal activity against the opportunistic human pathogen *Fusarium oxysporum* as well as several plant pathogenic fungi[88].

Interestingly, among the tested 27 *Xenorhabdus* and *Photorhabdus* strains only *P. namnoensis* appears to have acquired amoebicidal property which is effective on *E. histolytica* trophozoites. The bioactive compound responsible for this activity and its mode of action needs to be identified in the future.

The determined bioactive compounds may offer new opportunities for treating important parasitic diseases or be useful as lead compounds in the development of new antiprotozoal agents. For this purpose, new bioactive compounds should have no or very low cytotoxicity on human cells. Bode et al.[43] tested the efficacy of bioactive compounds isolated from *Xenorhabdus* and *Photorhabdus* bacteria on the human microvascular endothelial cell (EC) line (CDC.EU.HMEC-1). Fabclavine, PAX peptide, xenocoumacin and xenorhabdin had no or low impact on the metabolic activity, apoptosis and cell cycle G2-block. However, xenocoumacin and xenorhabdin exhibited toxic effects on cell proliferation.

In conclusion, this is the first extensive study screening the anti-protozoal activity of *Xenorhabdus* and *Photorhabdus* secondary metabolites against important human parasites *A. castellanii, E. histolytica, T. vaginalis, L. tropica* and *T. cruzi* and using the easyPACId technique to identify new potential antiprotozoal compounds. Future studies should investigate in detail the mode of action of these promising antiprotozoal compounds. Also, after a close structural investigation of these NPs, novel and safer pharmaceutical drugs can be potentially designed and synthesized.

Data availability

All data generated from this study are included in this article.

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Author contributions

S.G.H, S.H, H.E, S.E and H.B.B. designed the research. S.G.H. and E.T. carried out the research. E.B. and S.W. generated promoter exchanged mutant strains. H.C., M.T., D.U., D.B., and I.Y. assisted with the experiments. S.H.G., S.H., C.H., E.B. and H.B.B. wrote the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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