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ORIGINAL PAPER

# Nematicidal activity of fervenulin isolated from a nematicidal actinomycete, *Streptomyces* sp. CMU-MH021, on *Meloidogyne incognita*

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**Abstract** An isolate of the actinomycete, *Streptomyces* sp. CMU-MH021 produced secondary metabolites that inhibited egg hatch and increased juvenile mortality of the root-knot nematode *Meloidogyne incognita* in vitro. 16S rDNA gene sequencing showed that the isolate sequence was 99% identical to *Streptomyces roseoverticillatus*. The culture filtrates form different culture media were tested for nematocidal activity. The maximal activity against *M. incognita* was obtained by using modified basal (MB) medium. The nematicidal assay-directed fractionation of the culture broth delivered fervenulin (**1**) and isocoumarin (**2**). Fervenulin, a low molecular weight compound, shows a broad range of biological activities. However, nematicidal activity of fervenulin was not previously reported. The nematicidal activity of fervenulin (**1**) was assessed using the broth microdilution technique. The lowest minimum inhibitory concentrations (MICs) of the compound against egg hatch of *M. incognita* was 30 μg/ml and juvenile mortality of *M. incognita* increasing was observed at 120 μg/ml. Moreover, at the concentration of 250 μg/ml fervenulin (**1**) showed killing effect on second-stage nematode juveniles of *M. incognita* up to 100% after incubation for 96 h. Isocoumarin (**2**), another bioactive compound produced by *Streptomyces* sp. CMU-MH021, showed weak nematicidal activity with *M. incognita*.

**Keywords** *Streptomyces* sp. CMU-MH021 · Fervenulin · Nematicidal activity · Root-knot nematode

**Abbreviations**

| | |
|---|---|
| RKNs | Root-knot nematodes |
| J2 | Second-stage nematode juveniles |

## Introduction

Plant-parasitic nematodes such as root-knot nematodes (RKNs) or *Meloidogyne* spp. are microscopic obligate biotrophic pathogens that feed on plant roots. They cause severe damage to a wide variety of crops and lead to significant yield losses of approximately 78 billion dollar worldwide annually (Barker 1998; Caillaud et al. 2008; Sun et al. 2006; Verdejo-Lucas 1999). They are found in all temperate and tropical areas (Caillaud et al. 2008; Trudgill and Block 2001). It has been reported that RKNs spread out in agricultural areas in north, northeastern and central regions of Thailand (Cliff and Hirschmann 1984; Handoo et al. 2005; Ruanpanun et al. 2010).

Biological control is an interesting option to control plant-parasitic nematodes instead of the chemical control

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because it is eco-friendly and has no effect on human health (Dong and Zhang 2006; Sun et al. 2006). Microorganisms are the main tool for managing these pathogens (Dicklow et al. 1993; Saxena 2004).

Actinomycetes are Gram-positive bacteria and have been reported for their nematicidal activity (Sun et al. 2006). *Streptomyces* spp. are the major group of actinomycetes which show activity against plant-parasitic nematodes by producing nematicidal metabolites (Dicklow et al. 1993; Mishra et al. 1987; Samac and Kindel 2001; Sun et al. 2006). According to El-Nagdi and Youssef (2004), abamectin, a member of the avermectin group obtained from the fermentation products of *Streptomyces avermitilis*, showed nematicidal activity by the seed soaking treatment of faba beans infested with *M. incognita*. Moreover, it was found that the mortality of *M. incognita* and *Rotylenchulus reniformis* reached 100 and 97% respectively, after 24 h exposure to 21.5 μg abamecin/ml (Faske and Starr 2006). Furthermore, nematicidal actinomycetes have been implicated in antagonistic ways on other plant pathogens, on fungal diseases (Chung et al. 2005; Liu et al. 2004; Prabavathy et al. 2006; Yu et al. 2008) and bacterial diseases (El-Shanshoury 1994; Liu et al. 1995; Ndonde and Semu 2000). The present study describes the bioassay-guided isolation of a compound responsible for in vitro inhibition of *M. incognita* hatch and juvenile mortality by the culture broth, crude extract and pure compounds [fervenulin (**1**) and 6,8-dihydroxy-3-methylisocoumarin (**2**)] produced by a nematicidal actinomycete, *Streptomyces* sp. CMU-MH021 (Ruanpanun et al. 2010). Products or pure compounds from this isolate seem to be a new alternative biological agent to control root-knot nematodes in the future.

## Materials and methods

### Nematicidal actinomycete isolate

*Streptomyces* sp. CMU-MH021, a nematicidal actinomycete, was isolated from plant-parasitic nematode infested rhizosphere soils in Thailand (Ruanpanun et al. 2010). A stock culture of the strain was maintained on Hickey-Tresner (HT) slant agar and kept in 20% (v/v) glycerol suspension at −20°C.

### DNA extraction, amplification and sequencing of 16S rDNA

The *Streptomyces* sp. CMU-MH021 was grown for 4 days at 28°C with agitation in 250 ml Erlenmeyer flasks containing 50 ml of YM broth. Biomass was harvested by centrifugation at 11,000 rev/min and washed twice with sterile distilled water. About 250 mg of mycelia was used for DNA extraction by using Wizard® genomic DNA purification kit (Promega, WI).

The 16S rDNA was amplified by using the PCR method with *Taq* DNA polymerase and primers 27f (5′ AGA GTT TGA TCM TGG CTC AG 3′) and 1525r (5′ AAG GAG GTG WTC CAR 3′; Khamna et al. 2009). The condition for thermal cycling were as following: denaturation of target DNA at 95°C for 5 min followed by 30 cycles at 95°C for 1 min, primer annealing at 55°C for 1 min and primer extension at 72°C for 1 min. At the end of the cycling, the reaction mixture was kept at 72°C for 10 min and cooled to 4°C. Negative controls (no added DNA) were included in all reaction sets. PCR amplifications were detected by 1% (w/v) agarose gel electrophoresis and visualized by ultraviolet (UV) fluorescence after stained with ethidium bromide. The PCR products were purified by using a QIA Quick Gel Extraction kit (Qiagen, Valencia, CA) and directly sequenced by Macrogen Inc., (Seoul, South Korea). The sequencing primers including primer 27f, primer MG3f (5′ CTA CGG GRS GCA GCA G 3′) and primer MG5f (5′ AAA CTC AAA GGA ATT GAC GG 3′) were used for this purpose (Nimnoi et al. 2010). The obtained sequence was compared using BLAST program for similarity with the reference species of bacteria contained in GenBank, which is available at http://www.ncbi-nlm-nih.gov/.

### Media optimization and production

Five different media including Bennett's broth (beef extract 0.1%, glucose 1%, NZ amine type A 0.2%, yeast extract 0.4%; pH 7.3) (Gerber 1973), Emerson's broth (soluble starch 1.5%, yeast extract 0.4%, $MgSO_4$ 0.5%, $Na_2HPO_4$ 0.1%; pH 6.8) (Weiss 1975), F-4 (glycerol 4.0% soybean meal 2.5%, yeast extract 0.5%, $CaCO_3$ 0.2%, NaCl 0.05%) (Cheeptham et al. 1999), Basal medium (soluble starch 3.3%, defatted peanut powder 1.7%, NaCl 0.2%, $(NH_4)_2SO_4$ 0.23%) and MB medium (soluble starch 5.3%, soybean meal 1.0%, $(NH_4)_2SO_4$ 0.62%, NaCl 0.58%) (Yu et al. 2008) were preliminary used to determine the optimal nutrient for the growth and production of bioactive compounds.

### Inoculum preparation and liquid culture

The strain was sub-cultured on starch-casein agar (SCA) plates for 5 days at 28°C. Three small pieces (5 mm diam.) of the agar culture were then used to inoculate into one-L Erlenmeyer flasks each containing 300 ml of MB medium composed of starch 5.33%, soybean meal 0.94%, $(NH_4)_2SO_4$ 0.62%, NaCl 0.58% and pH was adjusted to 7.0

(Liu et al. 2004; Yu et al. 2008). All flasks were incubated on a rotator shaker at 130 rev/min at 28°C for 7 days.

### Preparation of crude extract

The culture broth was mixed with Celite (~1 kg) and filtrated under pressure. The mycelium was extracted three times with ethyl acetate and twice with acetone. The liquid phase was adsorbed on an Amberlite XAD-16 column and the organic compounds were eluted with methanol. The methanol was removed by evaporation *i. vac*., the aqueous residue was then extracted with ethyl acetate. The organic phase was also evaporated to dryness. The resulting crude extract (5 g) having nematicidal activity was then subjected to separation on silica gel and Sephadex LH-20.

### Purification of active compound

Crude extract (5 g) of *Streptomyces* sp. CMU-MH021 was chromatographed on silica gel (column 50 × 4 cm) with a stepwise $CH_2Cl_2$/MeOH gradient of increasing polarity. Fractions were monitored by thin-layer chromatography (TLC, silica gel, $CH_2Cl_2$/MeOH 19:1, spraying with anisaldehyde/sulfuric acid reagent and warming). Similar fractions were combined and tested with eggs and second-stage nematode juveniles (J2) of *M. incognita*.

The active fraction (1 g) was further purified twice on Sephadex LH-20 (columns 40 × 3 cm, MeOH and then 60 × 1 cm, $CH_2Cl_2$/MeOH 1:1, 0.5 ml/min) (Fig. 1). The pure compounds were analyzed by $^{1}H$ and $^{13}C$ nuclear magnetic resonance spectroscopy (NMR) and ESI mass spectrometry.

### Nematicidal activity

Culture broth, crude extract, 12 fractions, and pure compounds (fervenulin and 6,8-dihydroxy-3-methylisocoumarin from fraction 5) from the nematicidal actinomycete isolate, *Streptomyces* sp. CMU-MH021 were tested on eggs and second-stage nematode juveniles (J2) of *M. incognita*. The samples were dissolved in dimethyl sulfoxide (DMSO) before adding water (final concentration: 0.5% DMSO) while the culture broth was tested directly. The concentration of samples was adjusted to 250 µg/ml. Active samples were further tested at 120, 60 and 30 µg/ml (Nitao et al. 2002).

*Meloidogyne incognita* was cultured on chili (*Capsicum frutescens* L.) in the green house for 45 days, and its eggs were collected from the roots following the procedures described by Sun et al. (2006) with some modifications. Eggs were surface-disinfected with 1% sodium hypochlorite (NaOCl) and washed three times with sterile distilled water to remove residual NaOCl. They were incubated at room temperature (25 ± 3°C) for 48 h for the newly hatched J2. A 50 µl J2 suspension (150–200 J2/50 µl) was transferred into each well of a 24-well tissue culture plate containing 1 ml of sample solution. As negative control, 0.5% DMSO in water was used. The plate was incubated at room temperature (25 ± 3°C) for 96 h and daily examined for dead J2. Three replicates were done for each sample. Living and dead juveniles were counted under the microscope; mortality was calculated according to the formula: juvenile mortality = 100 × dead juveniles/total juveniles (Sun et al. 2006). The immobile, malformed, or motionless juveniles when probed with a fine needle were considered to be dead.

### Hatching inhibition analysis

All chemical substances were prepared as previously described. Eggs from root-knot nematode cultured in a green house on chili were surface-disinfected with 1% sodium hypochlorite (NaOCl) and washed three times with sterile distilled water to remove residual NaOCl. A 50 µl egg suspension (150–200 eggs/50 µl) was pipetted into each well of a 24-well tissue culture plate containing 1 ml of test solution. The plate was incubated at room temperature (25 ± 3°C) for 7 days and daily examined for egg hatch rate. Three replicates were done for each sample. Percentage of egg hatch was determined by counting all eggs and juveniles under the microscope and calculated according to the formula: percentage of egg hatch = 100 × juveniles/(eggs + juveniles) (Sun et al. 2006).

### Toxicity assay

The nematode effective compound fervenulin (**1**) was tested for brine shrimp (*Artemia salina*) toxicity. Fervenulin (**1**) was dissolved in DMSO at a concentration of 1 mg/ml for this experiment (Jumpathong et al. 2010; McLaughlin et al. 1998).

*Artemia salina* eggs were suspended in artificial seawater and kept in a separation funnel under aeration for 3 days for hatching. A 990 µl larvae suspension (~50 larvae) was pipetted into each well of a 24-well tissue culture plate. Ten µl of the DMSO solution was added into each well, DMSO was added as control. The plate was incubated at room temperature (25 ± 3°C) for 24 h. The mortality of *A. salina* was determined by counting living and dead larvae and calculated according to % mortality = 100 × dead larvae/total number of larvae.

### Statistical analysis

All experimental data were analyzed by the statistic package SPSS (program version 16.0 (2007), SPSS Inc.,

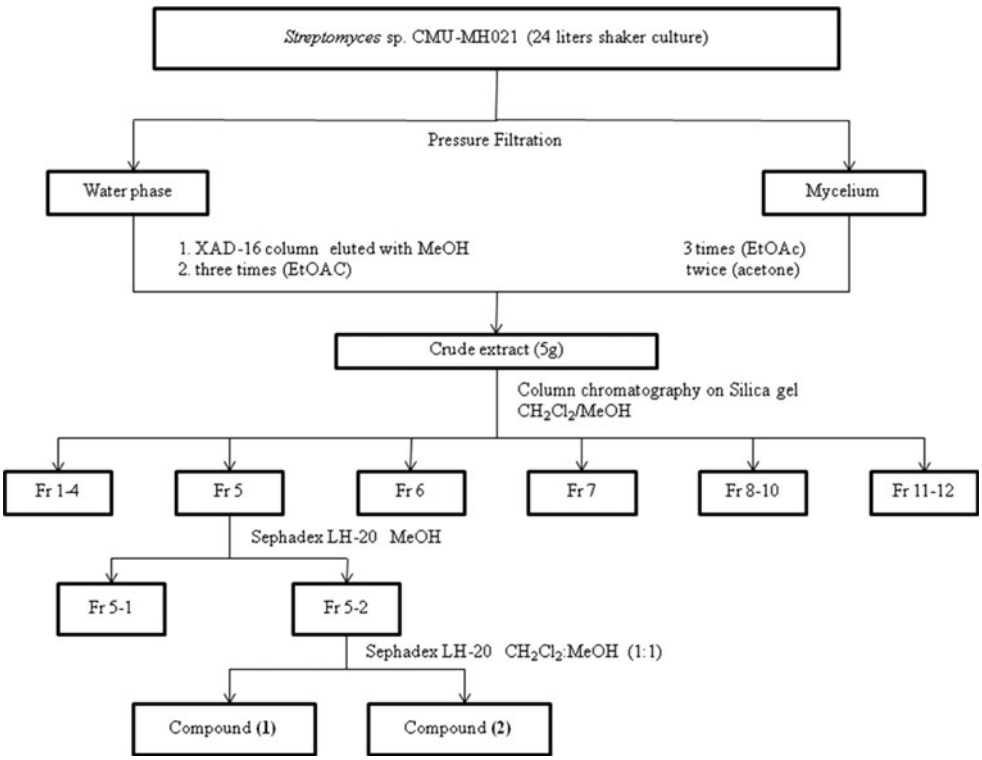


**Fig. 1** Work-up schema for the purification of active molecule of the nematicidal actinomycete, *Streptomyces* sp. CMU-MH021

Chicago, IL). The data were subjected to variance analysis (ANOVA) using Duncan's test ($P < 0.05$).

## Results

### Nematicidal Actinomycete, *Streptomyces* sp. CMU-MH021

A nematicidal actinomycete strain CMU-MH021 was previously isolated from plant-parasitic nematode infested rhizosphere soil at Mae Hae village, Chiang Mai province, Thailand. The strain exhibited nematicidal activity against *M. incognita* and was chemotaxonomically classified as *Streptomyces* sp. (Ruanpanun et al. 2010). The 16S rDNA gene sequence (GenBank, accession number HM101167) of this strain was similar to *Streptomyces roseoverticillatus* (99% identity).

### Media optimization

Different phenotypes of *Streptomyces* sp. CMU-MH021 were observed on different media including Bennett's broth, Emerson's broth, F-4 medium, Basal medium and MB medium. The isolate grew on all media, and culture broths and crude extracts showed significant effects ($P < 0.05$) on egg hatch and J2 mortality when compared with the control. The culture broth and crude extract from fermentation in MB medium showed the highest inhibition of egg hatch and the highest toxicity for J2 of *M. incognita*. The culture filtrate decreased percentage of egg hatch to 10.39 ± 1.3% after 7 days and increased percentage of J2 mortality to 100% after 96 h; the crude extract decreased percentage of egg hatch to 32.25 ± 7.3% and also increased percentage of J2 mortality to 100% (Table 1). Thus MB medium was selected to culture the isolate for this study.

### Nematicidal and hatching inhibition assays

Culture broth (MB medium) and crude extract (250 µg/ml) from *Streptomyces* sp. CMU-MH021 significantly reduced root-knot nematode hatch and increased J2 mortality in vitro. The crude extract was separated by fractionation on silica gel. Twelve fractions were tested on eggs and J2 of *M. incognita*. Fraction 5 decreased percentage of egg hatch to 14.6 ± 4.0% after incubation for 7 days and increased percentage of J2 mortality to 69.2 ± 2.5% after incubation for 96 h, in contrast to the control with 85.8 ± 4.6 and 3.9 ± 0.9%, respectively (Fig. 2). From this fraction, two pure compounds were isolated by column chromatography on Sephadex LH-20. The first compound **1** appeared on thin-layer chromatography as a yellow, UV absorbing spot, whose color did not change with anisaldehyde/sulfuric acid; it yielded a yellow crystalline solid. EI MS indicated a molecular ion at *m/z* 193, which was confirmed by (+)-ESI MS with *pseudo*molecular ions at 194 ($[M + H]^+$) and 216 ($[M + Na]^+$). ESI HRMS afforded the formula

**Table 1** Nematicidal activities of crude extract at 250 μg/ml (A) and culture broth (B) from *Streptomyces* sp. CMU-MH021

| Sample | Egg hatch rate (%) (7 days)[a] | Juvenile mortality rate (%) | | | |
|---|---|---|---|---|---|
| | | 24 h* | 48 h* | 72 h* | 96 h[a] |
| Bennett's broth (A) | 60.83 ± 4.8def** | 15 | 35 | 40 | 67.63 ± 2.4c |
| Bennett's broth (B) | 69.13 ± 8.3fg | 20 | 35 | 40 | 69.00 ± 6.5c |
| Emerson's broth (A) | 64.06 ± 7.7ef | 10 | 30 | 50 | 59.53 ± 3.2d |
| Emerson's broth (B) | 49.19 ± 13.0cd | 25 | 30 | 45 | 71.13 ± 5.4bc |
| AHU-5 (A) | 53.62 ± 12.6cde | 20 | 30 | 50 | 57.47 ± 4.7d |
| AHU-5 (B) | 71.66 ± 5.8fg | 30 | 35 | 45 | 66.27 ± 6.1c |
| Basal medium (A) | 30.43 ± 5.0b | 20 | 35 | 45 | 57.93 ± 2.0d |
| Basal medium (B) | 43.12 ± 3.8bc | 20 | 30 | 50 | 77.17 ± 3.9b |
| Modified basal medium (A) | 10.39 ± 1.3a | 30 | 40 | 50 | 100a |
| Modified basal medium (B) | 32.25 ± 7.3b | 40 | 60 | 100 | 100a |
| Control ($H_20$) | 86.46 ± 7.7h | 0 | 0 | 0 | 3.93 ± 0.8e |
| Control (1% DMSO) | 82.69 ± 9.9gh | 0 | 0 | 0 | 5.57 ± 0.8e |

[a] Average ± standard error from three replicates

* Observed mortality (%)

** Value with the same letter are not significantly different ($P < 0.05$) according to Duncan's test

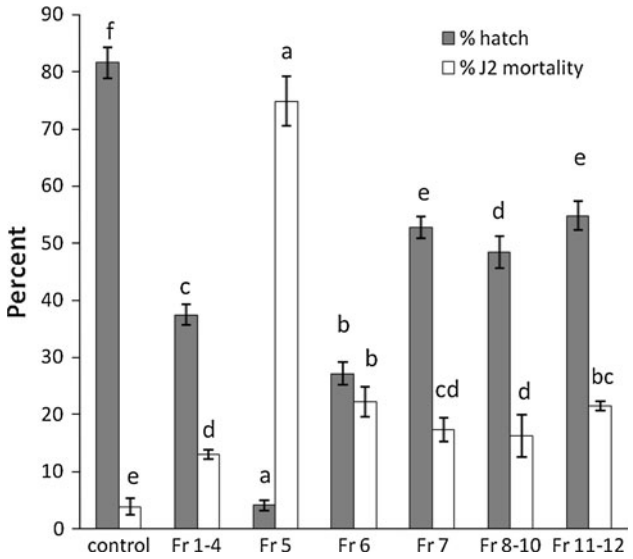


**Fig. 2** The in vitro egg hatch after 7 days and juvenile mortality after 96 h of *Meloidogyne incognita* in respond to solutions of compounds in medium pressure liquid chromatography fraction of *Streptomyces* sp. CMU-MH021 at a concentration of 250 μg/ml. The representation's *bars* are standard deviations. The *same letter* above *bars* within a graph indicates no significant difference according to the Duncan's test at $P < 0.05$

$C_7H_7N_5O_2$ (exp. 216.04921, calcd. 216.04920 for $[M + Na]^+$). The $^1H$ NMR spectrum of this compound ($CDCl_3$, 600 MHz) showed singlets at $\delta$ 9.80 (1H), 3.88 (3H), and 3.54 (3H), the $^{13}C$ NMR spectrum (MeOD, 125 MHz) gave signals at $\delta$ 161.6, 154.6, 152.9, 151.2, 133.9, 29.8, and 29.4. A search in AntiBase (Laatsch 2010) with these data delivered fervenulin (**1**, Fig. 3); the

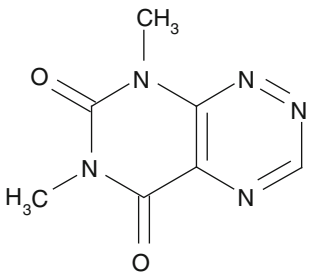


**Fig. 3** Chemical structure of fervenulin (6,8-Dimethylpyrimido(5,4-e)-1,2,4-triazine-5,7(6H,8H)-dione)

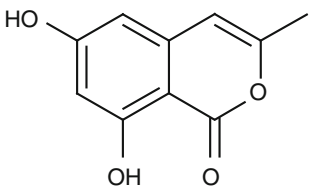


**Fig. 4** Chemical structure of isocoumarin (6,8-Dihydroxy-3-methylisocoumarin)

isomeric toxoflavin was clearly excluded by the NMR data (Esipov et al. 1973).

The second compound **2** appeared on thin-layer chromatography as a UV absorbing colorless spot, which turned yellow with anisaldehyde/sulfuric acid. A search with the NMR values [$^1H$ (DMSO-*d6*, 600 MHz): $\delta$ 6.42, 6.27, 6.24 (3 s, 1H each), 2.19 (s, 3H); $^{13}C$ (MeOD, 125 MHz): $\delta$ 167.7, 167.3, 164.7, 155.4, 141.3, 105.4, 103.4, 102.4, 99.4, 19.2] and the (+)-ESI MS data (*m/z* 215 for $[M + Na]^+$) in

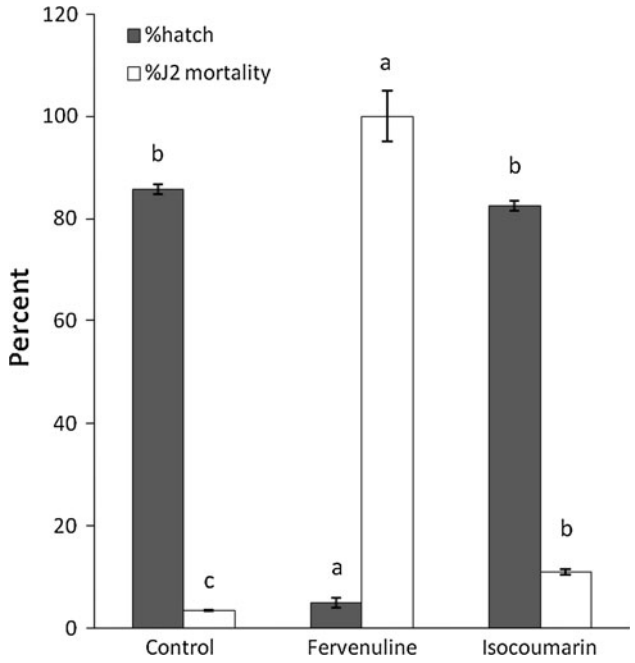


**Fig. 5** The in vitro egg hatch after 7 days and juvenile mortality after 96 h of *Meloidogyne incognita* in respond to fervenulin and isocoumarin, the compound from fraction 5 of *Streptomyces* sp. CMU-MH021 at a concentration of 250 μg/ml. The representation's *bars* are standard deviations. The *same letter* above *bars* within a graph indicates no significant difference according to the Duncan's test at $P < 0.05$

AntiBase (Laatsch 2010) delivered 6,8-dihydroxy-3-methylisocoumarin (**2**, Fig. 4) (Ayer et al. 1986).

Fervenulin (**1**) decreased percentage of egg hatch 5.0 ± 2.0% after incubation for 7 days and increased percentage of J2 mortality to 100.0 ± 0.0% after incubation for 96 h at concentration of 250 μg/ml (Figs. 5, 6), which significantly ($P < 0.05$) differed from the control. A significant effect on hatch compared with the control was also found after 7 days at concentrations of 120, 60 and 30 μg/ml (Fig. 7a). Significant effects were found on J2 mortality as well after 96 h at a concentration of 120 μg/ml, but not at 60 or 30 μg/ml (Fig. 7b). The isocoumarin **2** showed weak effect on J2 mortality but no effect on egg hatching inhibition on *M. incognita* at a concentration of 250 μg/ml.

### Toxicity assay

The nematicidal compound, fervenulin (**1**), showed no toxic effect to brine shrimps (*Artemia salina*) at a concentration of 10 μg/ml after incubation for 24 h.

## Discussion

Natural products from actinomycetes, especially *Streptomyces* spp., are a very promising source of new chemicals

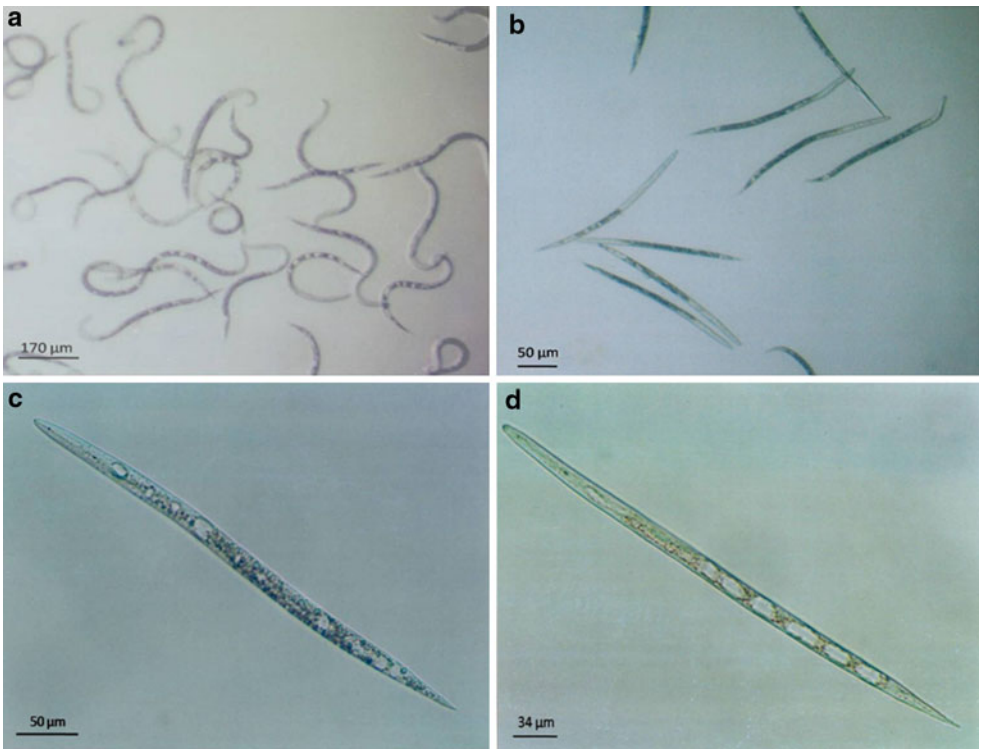


**Fig. 6** *Meloidogyne incognita* 2nd-stage juvenile after treatment with fervenulin isolated from *Streptomyces* sp. CMU-MH021 **a** Non-treated; **b** Treated with fervenulin for 96 h; **c** Dead J2 of *M. incognita* caused by fervenulin; **d** Dead J2 of *M. incognita* caused by lacking nutrient

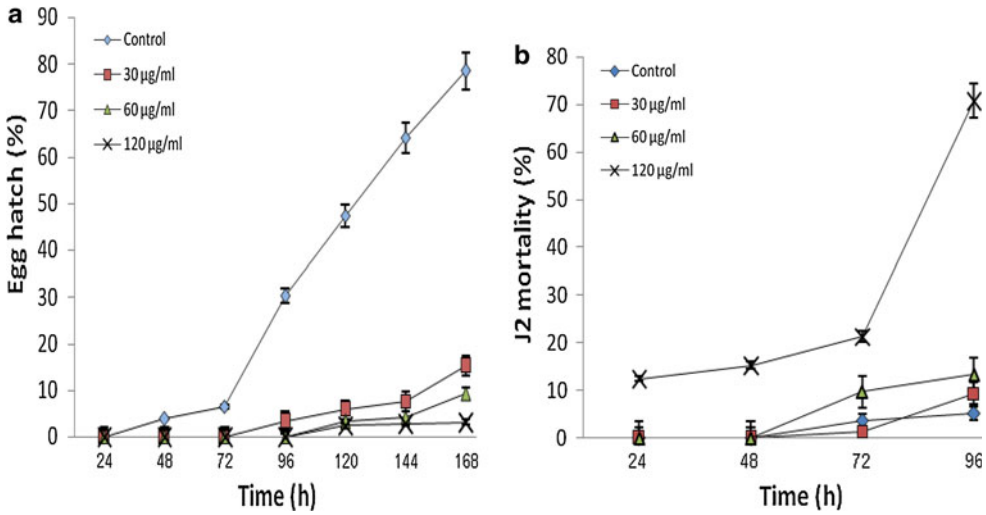


**Fig. 7** Percentage of egg hatch (**a**) and J2 mortality (**b**) of *M. incognita* after 7 days and 96 h, respectively in vitro in three concentration of fervenulin isolated from *Strptomyces* sp. CMU-MH021 (mean ± SE, $n = 3$; 150–200 eggs or J2 per replication)

to manage plant-parasitic nematodes (Chubachi et al. 1999; Dong and Zhang 2006; El-Nagdi and Youssef 2004; McGovern et al. 2002; Samac and Kindel 2001;). Fervenulin (**1**), known also as planomycin, is a yellow crystalline solid, which was previously isolated from *S. fervens* and S. *rubrireticuli* (Liao et al. 1965). Fervenulin (**1**) has a broad range of biological activities, e.g., in vitro antibacterial, antifungal, antiparasitic, and antitumor properties (DeBoer et al. 1960). In this study, it was found that it decreased percentage of egg hatch and increased percentage of J2 mortality of *M. incognita* significantly when compared with the control.

The second compound from *Streptomyces* sp. CMU-MH021 was identified as 6,8-dihydroxy-3-methylisocoumarin (**2**), previously isolated from the fungus *Ceratocystis minor* (Kumagai et al. 1990), from *Streptomyces* sp. GT061089 (Tang et al. 2000) and *Streptomyces* sp. TN97 (Mehdi et al. 2006). This compound was reported to strongly inhibit horse radish peroxidase, to show antitumor activity (McGraw and Hemingway 1977), antiviral (Tang et al. 2000), antimicrobial properties (Mehdi et al. 2006); it had weak nematocidal activity (Fig. 5) in our experiments, although it significantly increase J2 mortality.

Although the two compounds from *Streptomyces* sp. CMU-MH021 have already been described from other microorganism and have had their biological activities studied, this is the first report demonstrating that fervenulin (**1**) has nematicidal activity and inhibits egg hatch and J2 mortality of *M. incognita*. In contrast, the antimicrobial isocoumarin **2** was less active against nematodes in our tests.

The producing strain *Streptomyces* sp. CMU-MH021 was identified by 16S rDNA analysis as belonging to *Streptomyces roseoverticillatus* (99% identity). MB medium was best suited for strain growth and antagonistic substance production. Scale-up and modification for this strain should be further developed to control root-knot nematode in actual agricultural application.

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