

# Nematotoxicity of drupacine and a *Cephalotaxus* alkaloid preparation against the plant-parasitic nematodes *Meloidogyne incognita* and *Bursaphelenchus xylophilus*

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## Abstract

**BACKGROUND:** Species of *Cephalotaxus* (the plum yews) produce nematotoxic compounds of unknown identity. Consequently, bioassay-guided fractionation was employed to identify the compound(s) in *Cephalotaxus fortunei* twigs and leaves with activity against plant-parasitic nematodes.

**RESULTS:** A crude alkaloid extract, particularly drupacine, was responsible for much of the nematotoxicity. The ED<sub>50</sub> of drupacine for *Bursaphelenchus xylophilus* was 27.1 µg mL<sup>-1</sup>, and for *Meloidogyne incognita* it was 76.3 µg mL<sup>-1</sup>. Immersion of *M. incognita* eggs in 1.0 mg mL<sup>-1</sup> crude alkaloid extract (the highest tested concentration) reduced hatch by 36%; immersion of second-stage juveniles (J2) resulted in 72–98% immobility. Crude alkaloid extract and drupacine suppressed protease activity in extracts of the microbivorous nematode *Panagrellus redivivus* by 50% and 80%, respectively. Application of 0.02–0.5 mg mL<sup>-1</sup> crude alkaloid extract to soil with *M. incognita* inoculum did not significantly reduce pepper plant shoot length or weight, compared with nematode-inoculated, water-treated controls, but the number of eggs and J2 per root system respectively decreased by 69% and 73% at 0.5 mg mL<sup>-1</sup>.

**CONCLUSION:** Drupacine and a crude alkaloid extract suppress nematode hatch, activity of mixed life stages, and population numbers on plant roots. This is the first demonstration of nematotoxicity of crude *Cephalotaxus* alkaloids and drupacine.

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**Keywords:** alkaloid; *Bursaphelenchus*; *Meloidogyne*; natural product; nematicide; nematode; *Panagrellus*; phytochemical; pinewood nematode; root-knot nematode

## 1 INTRODUCTION

Plant-parasitic nematodes are responsible for annual crop losses of ca. ten billion dollars in the United States and 125 billion worldwide.<sup>1</sup> Because many conventional chemical nematicides have been de-registered for use due to safety and environmental concerns, an urgent need exists for novel, safe and effective nematicides. Consequently, natural products derived from plants, fungi, bacteria and other organisms are being investigated as potential biobased nematicides.

The search for phytochemicals antagonistic toward nematodes has been pursued in many plant species,<sup>2</sup> but few of the studied plants belong to the small family Cephalotaxaceae. Species of *Cephalotaxus* (the plum yews) are known to produce compounds that can be used as medicines; for example, traditional Chinese medicine has employed compounds from *C. sinensis* for various human ailments. Substances isolated from *Cephalotaxus* extracts include alkaloids, biflavonoids, flavonoids, diterpenes and lactones.<sup>3,4</sup> Alkaloids and their esters isolated from *Cephalotaxus* possess anticancer activity.<sup>5,6</sup> A patent application has been submitted on synthesis and use of structurally related non-natural *Cephalotaxus* esters against hematological and solid tumors.<sup>7</sup>

Crude extracts from *Cephalotaxus drupacea* were nematicidal,<sup>8</sup> and examination of a broad spectrum of Chinese plants for nematotoxicity revealed that crude extracts of *C. fortunei* (the Chinese plum yew) were quite active against the pinewood nematode *Bursaphelenchus xylophilus*, the rice root nematode *Hirschmanniella oryzae*, and the root-knot nematode *Meloidogyne arenaria*.<sup>9</sup> Moreover, crude extracts from *C. fortunei* twigs retarded embryogenesis and larval development of *M. arenaria*,<sup>10</sup> and a

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dried, pulverized powder prepared from *C. fortunei* twigs provided disease control when incorporated into field or greenhouse soils.<sup>11</sup> Because no attempts have been made to fractionate any of these crude extracts or powders, the nature of the compounds responsible for this nematode antagonism remains unknown. The purpose of the present study was to isolate and identify the compounds within *C. fortunei* responsible for its activity against nematodes, with an emphasis on the economically important root-knot nematode *M. incognita*, which infects and damages many crop plants. Additionally, the compounds were evaluated for possible mode of action and effects on root-knot nematode hatch and reproduction.

## 2 MATERIALS AND METHODS

### 2.1 Extraction

Young *Cephalotaxus fortunei* twigs with attached leaves were collected in Hubei Province, China, air-dried, and ground to a powder sufficiently fine to pass through a 40-mesh sieve in an electric mill (FY 130, Tianjin Taisite, China). The powder (4.15 kg) was soaked three times in 95% ethanol (31.8 L, 5–7 days each time) at room temperature. The extracts were pooled and concentrated under reduced pressure in a rotary evaporator; the residue was dissolved in 2.0% HCl (1200 mL) and filtered. The filtrate was extracted with chloroform (3 × 350 mL). The chloroform phase was removed and saved, and the aqueous phase was adjusted to pH 9.0 and re-extracted with chloroform (4 × 300 mL). The combined chloroform solutions were concentrated to obtain the crude alkaloid extract (20.7 g).

### 2.2 Isolation and identification of drupacine

A portion of the crude alkaloid extract (8.8 g) was subjected to column (4.4 × 47.5 cm) chromatography over silica gel 60 (299 g, 100–200 mesh; Qingdao Marine Chemical Ltd., Qingdao, China) and eluted with CHCl<sub>3</sub>–MeOH mixtures of increasing polarity [100:0 (3750 mL), 98:2 (1200 mL), 95:5 (1350 mL), and 90:10 (150 mL)] to afford 43 fractions of ca. 150 mL. The fractions were analyzed by TLC (Merck Kieselgel 60 GF254 HPTLC plates, developed with CHCl<sub>3</sub>–MeOH (9:1), visualized with Dragendorff reagent), and seven pooled fractions with similar compositions, as indicated by color and location of TLC spots, were prepared from the 43 smaller fractions. The combined fractions were tested in the *B. xylophilus* bioassay described below. The most active combined fraction produced a single spot on TLC and was concentrated and dried under reduced pressure to yield drupacine (3240 mg, Fig. 1): a white powder; positive ESI-MS: *m/z* 685 [2M + Na]<sup>+</sup>, 663 [2M + H]<sup>+</sup>, 354 [M + Na]<sup>+</sup>, 332 [M + H]<sup>+</sup>; <sup>1</sup>H NMR δ: 3.99 (1H, t, *J* = 8.8 Hz, H-1), 2.62 and 1.44 (each 1H, d, *J* = 14.0, H<sub>2</sub>-3), 1.74 (2H, m, H<sub>2</sub>-4), 2.13 and 2.00 (each 1H, m, H<sub>2</sub>-5), 3.02 and 2.38 (each 1H, m, H<sub>2</sub>-6), 3.06 (1H, dd, *J* = 13.2, 4.8 Hz, H-8a), 2.95 (1H, dd, *J* = 13.2, 0.8 Hz, H-8b), 4.84 (1H, br d, *J* = 4.0 Hz, H-9), 6.61 (1H, s, H-10), 5.91 (2H, br d, *J* = 11.2 Hz, H<sub>2</sub>-12), 6.62 (1H, s, H-14), 3.42 (1H, d, *J* = 8.8 Hz, H-15), 3.45 (3H, s, 2-Me); <sup>13</sup>C NMR δ: 73.4 (CH, C-1), 108.5 (C, C-2), 35.6 (CH<sub>2</sub>, C-3), 65.1 (C, C-3a), 43.3 (CH<sub>2</sub>, C-4), 22.2 (CH<sub>2</sub>, C-5), 51.9 (CH<sub>2</sub>, C-6), 56.5 (CH<sub>2</sub>, C-8), 78.1 (CH, C-9), 129.9 (C, C-9a), 107.6 (CH, C-10), 146.5 (C, C-10a), 101.1 (CH<sub>2</sub>, C-12), 147.7 (C, C-13a), 111.8 (CH, C-14), 130.9 (C, C-14a), 59.5 (CH, C-15), 53.8 (CH<sub>3</sub>, 2-Me). The data were in accord with those previously reported.<sup>12,13</sup>

<sup>1</sup>H NMR (400 MHz), and <sup>13</sup>C NMR (100 MHz) spectra were recorded on a Bruker DRX-400 instrument with TMS as reference. ESI-MS data were collected on a MDS SCIEX API2000 LC/MS/MS instrument.

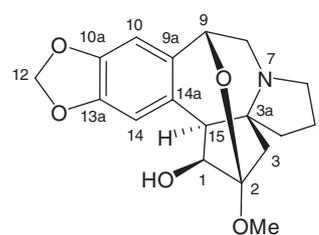


Figure 1. Structure of drupacine.

### 2.3 Nematode cultures and assays in China with drupacine

*Bursaphelenchus xylophilus* was collected from diseased pine trees in Guangdong Province and cultured for 6 months on *Pestalotia* sp. (supplied by Dr Zhou Huijun, Nematology Laboratory, SCAU) in 6-cm-diameter plates containing potato dextrose agar (PDA). For bioassays, *Pestalotia* sp. was cultured on PDA for 5 days in an incubator (25 °C). A cotton cube (5 mm edge) was then placed in the middle of each plate. A stock solution of drupacine was prepared by dissolving it in acetone, then diluting with water to 1.0 mg mL<sup>-1</sup> in 2.0 % acetone. The drupacine used in all experiments was the material contained in the most active combined column chromatographic fraction subsequently analyzed by MS and NMR as described in the section 'Isolation and identification of drupacine'. Each cotton cube received 0.5 mL of the tested drupacine solutions (1.0, 0.5, 0.1, 0.01 and 0.001 mg mL<sup>-1</sup>) or a 2.0 % acetone control, followed by 0.1 mL of *B. xylophilus* (10 000 mixed stage nematodes mL<sup>-1</sup> water).<sup>14</sup> Assay plates were placed in an incubator (25 °C) for 5 days. Nematodes + PDA were then moved to Baermann funnels and the nematodes were collected and counted after 48 h. The experiment was conducted once and each treatment was replicated four times.

*Meloidogyne incognita* was cultured on tomato (*Solanum lycopersicum* L.) in a greenhouse; the egg masses were collected, washed with water, and placed in an incubator (25 °C) for 3 days to obtain second-stage juveniles (J2). Assays were conducted in 3-cm-diameter culture plates. Drupacine stock solution and dilutions were prepared as above. Each well then received 0.5 mL of stock or diluted drupacine solution, followed by 0.1 mL of J2 in water (30–50 nematodes). Final drupacine concentrations were 1.0, 0.5, 0.1, 0.05 and 0.01 mg mL<sup>-1</sup>, with 2.0% acetone in water as the control. The plates were incubated at 25 °C for 54 h, the drupacine solutions were removed and replaced with water, and 24 h later active and inactive nematodes, based on response to a needle probe, were counted. Treated nematodes were defined as inactive if they did not respond to a needle probe or exhibit any movement under direct observation. Inactive nematodes were defined as dead if no movement was observed after transfer to a water rinse. The experiment was conducted once and each treatment was replicated four times. Statistical analyses for assays with both species of nematodes were conducted with Duncan multiple comparisons, Statistical Product and Service Solution (SPSS); *P* = 0.05. Assays in China were not repeated due to low amounts of the test compounds available for this purpose.

### 2.4 Nematode cultures for assays in the United States with crude alkaloid extract

Cultures of *M. incognita*, originally isolated from Maryland soil, were grown on pepper (*Capsicum annuum* L.) 'PA-136' in greenhouse pots and used for all experiments done in the USA. For some experiments, surface-sterilized eggs and J2 were collected as described.<sup>15</sup> Briefly, egg masses were collected from plant roots

and rinsed three times with sterile distilled water. The egg masses were agitated for 3 1/2 min in 0.6% sodium hypochlorite, and the surface-sterilized eggs were removed, stored overnight at 7 °C and used the following day for assays. Additional sterilized eggs were placed on a Spectra/Mesh Nylon Filter (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) with 30-µm-diameter openings in an autoclaved storage dish, and J2 that passed through the filter within 72 h were collected and used immediately for assays. In other experiments, eggs were released from egg masses by agitation in water without sodium hypochlorite, or were selected manually.

## 2.5 Microwell assays with hypochlorite-treated eggs

Microwell assays with crude alkaloid extract and *M. incognita* were set up in 24-well polystyrene plates, similar to described procedures.<sup>15</sup> An aqueous stock solution of crude alkaloid extract (1 mg mL<sup>-1</sup>) was prepared in ethanol; 0.05 g alkaloid + 0.2 mL 95% ethanol was dissolved in 50 mL water and then filter-sterilized. Treatments were: (1) 1.0 mg mL<sup>-1</sup> crude alkaloid extract prepared from the stock solution; (2) 0.1 mg mL<sup>-1</sup> crude alkaloid extract; (3) 0.01 mg mL<sup>-1</sup> crude alkaloid extract; (4) 0.38% ethanol control (0.2 mL of 95% ethanol in 50 mL water); (5) 0.038% ethanol control; (6) 0.0038% ethanol control; and (7) water control. The pH range for all treatment solutions was 7.2 to 7.9. Approximately 100 surface-sterilized *M. incognita* eggs or 50 J2 in 0.1 mL water, and 0.9 mL extract or control solution, were placed into each well. A plastic adhesive sheet was placed on each plate and the plates were incubated at 26 °C. Each treatment was replicated five times in each of two trials. The J2 that hatched from eggs were counted after 3, 5 and 7 days, and active and inactive J2 were counted after 1, 2 and 3 days. Data were analyzed with SPSS Statistics 17.0 software (Statistical Product and Service Solution, Duncan multiple comparisons,  $P = 0.05$ ).

## 2.6 Behavioral and hatching assays with non-hypochlorite treated eggs

For J2 behavior and hatch assays using eggs not exposed to sodium hypochlorite, stock solutions of *C. fortunei* crude alkaloids were prepared in 0.1% ethanol. Dilutions (prepared as needed) and controls were all 0.1% ethanol. *Meloidogyne incognita* egg masses were harvested from pepper and eggs were released by shaking in water. Eggs were held in hatch chambers prepared as described,<sup>16</sup> comprising a 30 µm nylon mesh (Spectrum Laboratories) supported by a 500 µm Netwell insert (Corning Costar, Corning, NY, USA). Eggs in mesh assemblies were suspended in wells of a 24-well Netwell plate (Corning), 1.0 mL tap water per well, and incubated at 27 °C. After 3 days, hatched J2 were collected by pipette in a minimum volume of water and transferred to a fresh 24-well plate (Corning) at a final volume of 1.0 mL per well of alkaloid solution (1.0, 0.5, 0.1, 0.02 mg mL<sup>-1</sup> final concentration) or ethanol control. The plate was incubated 3 days at 27 °C, and J2 were then observed with an inverted microscope at × 40 and × 100 to assess movement.<sup>17</sup> J2 were classified as inactive if no body or stylet movement was detected during a 5 s observation. For each treatment, 100–120 individual J2 were observed and the percent inactive was calculated as  $[N_{\text{inactiveJ2}} / (N_{\text{activeJ2}} + N_{\text{inactiveJ2}}) \times 100]$ . Each treatment was replicated four times and results expressed as the mean % inactive ( $N = 4$ ), with means compared by one-way ANOVA.

In hatching assays with non-hypochlorite treated eggs, eggs from *M. incognita* egg masses obtained from pepper roots were

manually picked under a dissecting microscope (× 15 to × 40) and gently washed in tap water. Groups of eggs were placed in wells of a 48-well assay plate (Corning; 7–11 eggs/well); each well contained 500 µL of 0.5 mg mL<sup>-1</sup> alkaloid mixture or ethanol control. Plates were incubated at 27 °C. One day after plate setup (Day 1) all wells were examined at × 40 for egg and J2 counts. Accuracy in quantifying responses was attained by observing each egg and J2 individually in each treatment group over the course of each experiment, for total observations >1000 per experiment. Wells were monitored periodically for 2 weeks, and total cumulative percent hatch in each egg group on Day14 was calculated as  $[(J2_{\text{Day14}} - J2_{\text{Day1}}) / (J2_{\text{Day14}} + \text{Eggs}_{\text{Day14}}) \times 100]$ .  $N =$  four replicates per treatment.

## 2.7 Protease inhibition assays

The microbivorous nematode *Panagrellus redivivus* was cultured sterilely in aqueous medium containing yeast extract, soy peptone, hemoglobin and dextrose;<sup>18</sup> nematode extractions and protease reactions were performed as described.<sup>19</sup> Briefly, mixed-stage cultures of *P. redivivus* were rinsed with distilled/deionized (D/D) water to remove culture medium and frozen at –20 °C as whole nematode pellets. Freshly hatched *M. incognita* J2 were collected from Baermann funnels, centrifuged at low speed to pellet the J2 and remove water, and frozen as J2 pellets. Nematodes were extracted in sterilized D/D water (typically 100 000 nematodes in 6.0 mL water) using a Bead Beater (BioSpec Products, Bartlesville, OK, USA). Extracts were centrifuged at 40 000 ×  $g$ , 20 min, 5 °C, and the supernatants dried as aliquots with vacuum centrifugation. Total protein was estimated using microBCA (Pierce Chemical, Rockford, IL, USA).

Reactions were performed at 27 °C in 384-well assay plates (flat bottom, non-treated, Corning) in 100 mM TRIS, pH 7.8 assay buffer, 25 µL reaction volume. Extract aliquots were dissolved directly into assay buffer. KSAYMRFa, a highly expressed member of the FLP family of neuropeptides ubiquitous among nematodes of all life histories,<sup>20,21</sup> was used as substrate in modified form. Cleavage of QXL520-KSAYMRF-K(5-FAM)a resulted in a fluorescent signal monitored (Ex = 490 nm, Em = 520 nm) with a SpectraMAX EM fluorescent plate reader (Molecular Devices, Sunnyvale, CA, USA). Reactions contained 0.08–0.12 µg µL<sup>-1</sup> extract protein and were initiated by the addition of substrate (1.0 µM final concentration). For the inhibitor assays, crude alkaloids or drupacine were initially dissolved in 95% ethanol and diluted in D/D water to 0.5% ethanol. Alkaloids were added to reaction mixtures 15 min prior to the addition of substrate. Final concentration of crude alkaloid extract or drupacine was 0.3 mg mL<sup>-1</sup> and ethanol was 0.1% in all reactions. Reactions were monitored for 60 min and digestion rates were quantified using SoftMax Pro instrument control and data collection software (Molecular Devices). Increase in relative fluorescence units (RFU) over time was recorded and reduced to  $V_{\text{max}} s^{-1}$ . Each reaction type (control, inhibitors) was run a minimum of four separate times, data were converted to  $V_{\text{max}} \text{min}^{-1} / \text{total reaction } \mu\text{g}^{-1}$ , and data means were compared by the  $t$ -test.

## 2.8 Greenhouse tests for suppression of *M. incognita* on pepper

In a greenhouse study, pepper seeds (*Capsicum annuum* 'PA-136') were planted in Premier Pro-mix<sup>®</sup> starter mix (Premier Pro-mix<sup>®</sup>; Premier Horticulture Inc., Quakertown, PA, USA). Roots of 5-week-old seedlings were dipped in water to remove potting mix, and

the seedlings were then transplanted into 10 cm diameter pots (one seedling per pot) that each contained 400 g dry greenhouse soil mixture (16:9 sand:compost steamed prior to use). Following transplant, the seedlings were watered to 70% water holding capacity (48 mL water or treatment solution per pot). Eight treatments were used, with 8 pots per treatment in each of two trials. Treatments were: (1) water control, no *M. incognita*; (2) water control, + *M. incognita*; (3) 0.1% ethanol (0.048 mL ethanol per pot), + *M. incognita*; (4) 0.02% ethanol (0.0096 mL ethanol per pot), + *M. incognita*; (5) 0.004% ethanol (0.00192 mL ethanol per pot), + *M. incognita*; (6) 0.5 mg alkaloid mL<sup>-1</sup> 0.1% ethanol (24 mg alkaloid per pot), + *M. incognita*; (7) 0.1 mg alkaloid mL<sup>-1</sup> 0.02% ethanol (4.8 mg per pot), + *M. incognita*; and (8) 0.02 mg alkaloid mL<sup>-1</sup> 0.004% ethanol (0.96 mg per pot), + *M. incognita*. On the day of transplant, 5000 nematodes in 1 mL water were added to holes made in the soil near the plant roots in each pot receiving nematodes. The greenhouse was maintained at 21–26 °C, with natural and supplemental lighting combined for a 15-h day length. Pots were arranged in a randomized complete block design. Seedlings were harvested eight weeks after transplant. Eggs and J2 were extracted from roots and counted,<sup>22</sup> and shoot lengths, shoot fresh and dry weights, and root fresh weights were determined.

## 2.9 Statistical analysis

Except where stated above, all statistical analyses were conducted with SPSS, one-way ANOVA with Duncan post-hoc multiple comparisons,  $P = 0.05$ . For the greenhouse study, one-way ANOVA and Tukey's multiple comparison were used; once the mean total eggs/pot was determined for each treatment, data from the two plants with egg counts furthest from the mean for each treatment were not included in the final analysis. ED<sub>50</sub>s were determined via regression analysis.

## 3 RESULTS

### 3.1 Assays in China with drupacine

Assays for effects of drupacine on *B. xylophilus* indicated that the highest tested concentrations, 1.0 and 0.5 mg mL<sup>-1</sup>, resulted in nearly 100% immobility in mixed life stages of the nematode (Table 1). The ED<sub>50</sub> of drupacine (calculated on the basis of drupacine concentration applied to the cotton cube) was 27.1 µg mL<sup>-1</sup>, and the ED<sub>95</sub> was 254 µg mL<sup>-1</sup>. In initial tests with drupacine on *M. incognita* in China, effects of the higher drupacine concentrations were similar to effects on *B. xylophilus* (Table 1). At 1.0 mg mL<sup>-1</sup>, *M. incognita* J2 immobility was 100%, and immobility exceeded 90% with 0.5 mg mL<sup>-1</sup> drupacine. The ED<sub>50</sub> of drupacine for *M. incognita* was calculated as 76.3 µg mL<sup>-1</sup>, and the ED<sub>95</sub> of drupacine for *M. incognita* was 301.1 µg mL<sup>-1</sup>.

### 3.2 Assays in the United States with crude alkaloid extract

When eggs of the US population of *M. incognita* were immersed in the treatment solutions, percent hatch [(number of J2 that hatched/total eggs × 100)] in the ethanol controls was the same as that recorded in water controls for each day (Table 2). Hatch was reduced in all three tested concentrations of crude alkaloids but tended to be lowest in the 0.01 mg mL<sup>-1</sup> treatment and in the 1.0 mg mL<sup>-1</sup> treatment. By day 7, there was a 35.9% decrease in hatch in the highest concentration of crude alkaloid extract compared to hatch in the corresponding ethanol control, and a 30.0% decrease in the lowest alkaloid concentration.

**Table 1.** Nematicidal activity of drupacine (isolated from *Cephalotaxus fortunei*, the Chinese plum yew) against mixed stages of the pinewood nematode (*Bursaphelenchus xylophilus*) and second-stage juveniles (J2) of the root-knot nematode *Meloidogyne incognita* (Chinese populations)

Concentration (mg mL <sup>-1</sup> )*	<i>Bursaphelenchus xylophilus</i> immobility (%)†	<i>Meloidogyne incognita</i> immobility (%)‡
1.0	99.63 ± 0.01 <sup>a</sup>	100.00 ± 0.00 <sup>a</sup>
0.5	98.86 ± 0.15 <sup>a</sup>	94.36 ± 0.47 <sup>b</sup>
0.1	82.09 ± 1.36 <sup>b</sup>	51.82 ± 0.44 <sup>c</sup>
0.05	Not tested	47.23 ± 0.13 <sup>d</sup>
0.01	24.86 ± 1.79 <sup>c</sup>	15.07 ± 0.91 <sup>e</sup>
0.001	8.28 ± 2.98 <sup>d</sup>	—
Control	—	15.04 ± 1.42 <sup>e</sup>

Data are expressed as the means ± standard errors of four replicates at each concentration. Within a column, values followed by the same letter are not significantly different ( $P < 0.05$ ) by Duncan multiple comparisons.

\* The 1.0 mg mL<sup>-1</sup> drupacine was in 2% acetone, and the other concentrations were made from this stock.

† Counts made after the nematodes were exposed to drupacine treatments for 5 days in *Pestalotia* sp. cultures. Data are expressed relative to the number of nematodes obtained in control assays after extraction with Baermann funnels.

‡ Counts made after nematodes were immersed for 54 h in drupacine treatments and then in a water rinse for 24 h. Data are not adjusted for controls.

Crude alkaloid extract was more toxic to J2 (Table 3) than to eggs. After 1 and 2 days of immersion in the solutions, the greatest percentages of inactive J2 were observed in the two highest concentrations of crude alkaloid extract. By 3 days, the highest crude alkaloid extract concentration resulted in the greatest number of inactive J2. When J2 had been immersed in alkaloid solutions for 3 days and then rinsed in water for 24 h, almost 98% of J2 were dead in the highest concentration. Approximately 71% of the J2 were inactive in 0.1 mg mL<sup>-1</sup> crude alkaloid extract. However, the % of inactive J2 did not differ among the water and ethanol controls, and the lowest concentration of crude alkaloid extract. The percentages of inactive J2 recorded in this assay were similar to those recorded with the Chinese population in drupacine (Table 1).

In J2 activity and stylet movement assays using *M. incognita* J2 from eggs not treated with hypochlorite, *C. fortunei* crude alkaloid extract significantly reduced J2 activity after a 3-day exposure *in vitro* (Table 4). Mean J2 activity decreased nearly four-fold at 0.02 mg mL<sup>-1</sup> alkaloid, and more than 11-fold at 0.1 mg mL<sup>-1</sup> and greater (Table 4) relative to the control level. Alkaloid concentrations greater than 0.1 mg mL<sup>-1</sup> did not significantly increase inactivity, although inhibition at 0.5 mg mL<sup>-1</sup> approached 74%. The 0.5 mg mL<sup>-1</sup> dose was then used to assess effects on hatching. At Day 14 of exposure, total cumulative percent hatch from alkaloid-exposed eggs (11.31 ± 4.08;  $N = 4$ ) was reduced 67% ( $P < 0.05$ ) from the control level (34.48 ± 5.46;  $N = 4$ ). Hatch did not increase significantly in either group after Day 14.

### 3.3 Protease inhibition

Protease activity in *P. redivivus* extracts was strongly suppressed by both crude alkaloid extract and drupacine (Table 5). Drupacine inhibition (80%) was significantly greater ( $P < 0.05$ ) than inhibition

**Table 2.** Inhibition of *Meloidogyne incognita* (Maryland population) egg hatch by crude alkaloid extract from *Cephalotaxus fortunei*, the Chinese plum yew

Treatment (alkaloid extract) or control	Hatch (%)		
	3 days	5 days	7 days
1.0 mg mL <sup>-1</sup>	42.6 ± 3.0 <sup>c</sup>	54.4 ± 4.1 <sup>bc</sup>	57.4 ± 4.9 <sup>b</sup>
0.38% ethanol (control)	67.4 ± 1.3 <sup>a</sup>	84.8 ± 1.9 <sup>a</sup>	89.6 ± 1.2 <sup>a</sup>
0.1 mg mL <sup>-1</sup>	53.4 ± 4.3 <sup>b</sup>	59.4 ± 3.4 <sup>b</sup>	64.8 ± 3.0 <sup>b</sup>
0.038% ethanol (control)	71.2 ± 2.0 <sup>a</sup>	89.4 ± 4.3 <sup>a</sup>	96.0 ± 4.0 <sup>a</sup>
0.01 mg mL <sup>-1</sup>	33.8 ± 1.5 <sup>c</sup>	45.8 ± 1.2 <sup>c</sup>	63.8 ± 3.1 <sup>b</sup>
0.0038% ethanol (control)	72.4 ± 4.4 <sup>a</sup>	91.8 ± 5.0 <sup>a</sup>	91.2 ± 4.3 <sup>a</sup>
Water (control)	68.2 ± 5.3 <sup>a</sup>	86.4 ± 4.6 <sup>a</sup>	88.8 ± 5.7 <sup>a</sup>

Data are expressed as the means ± standard errors of five replicates at each concentration. Within a column, values followed by the same letter are not significantly different ( $P < 0.05$ ) by Duncan multiple comparisons.

**Table 3.** Effects of crude alkaloid extract from *Cephalotaxus fortunei* on mobility and mortality of second-stage juveniles (J2) of *Meloidogyne incognita* (Maryland population) after 1, 2 and 3 days in treatments, and after a 24 h water rinse

Treatment (alkaloid extract) or control	Inactive second-stage juveniles (%)			Mortality (%), Water rinse 24 h
	1 day	2 days	3 days	
1.0 mg mL <sup>-1</sup>	23.5 ± 2.6 <sup>ab</sup>	89.7 ± 1.4 <sup>a</sup>	98.2 ± 0.8 <sup>a</sup>	97.9 ± 1.0 <sup>a</sup>
0.38% ethanol (control)	15.3 ± 2.7 <sup>cd</sup>	23.1 ± 2.8 <sup>b</sup>	22.2 ± 2.1 <sup>d</sup>	18.0 ± 3.0 <sup>c</sup>
0.1 mg mL <sup>-1</sup>	28.6 ± 2.8 <sup>a</sup>	84.7 ± 1.1 <sup>a</sup>	82.8 ± 1.1 <sup>b</sup>	71.4 ± 3.7 <sup>b</sup>
0.038% ethanol (control)	8.0 ± 1.2 <sup>e</sup>	11.2 ± 1.9 <sup>c</sup>	13.5 ± 1.6 <sup>e</sup>	13.1 ± 1.2 <sup>c</sup>
0.01 mg mL <sup>-1</sup>	18.3 ± 1.7 <sup>bc</sup>	27.3 ± 4.0 <sup>b</sup>	36.3 ± 3.8 <sup>c</sup>	17.0 ± 4.0 <sup>c</sup>
0.0038% ethanol	10.6 ± 0.9 <sup>de</sup>	14.9 ± 1.4 <sup>c</sup>	15.8 ± 0.8 <sup>e</sup>	14.1 ± 2.0 <sup>c</sup>
Water	12.8 ± 1.9 <sup>cde</sup>	15.9 ± 1.5 <sup>c</sup>	16.3 ± 1.2 <sup>e</sup>	12.8 ± 1.2 <sup>c</sup>

Data are expressed as the means ± standard errors of five replicates at each concentration. Within a column, values followed by the same letter are not significantly different ( $P < 0.05$ ) by Duncan multiple comparisons. The second-stage juveniles were from eggs treated with sodium hypochlorite.

**Table 4.** Effect of *Cephalotaxus fortunei* crude alkaloid extract on movement of *Meloidogyne incognita* second-stage juveniles (J2) (Maryland population) after 3 days immersion *in vitro*

Alkaloid concentration (mg mL <sup>-1</sup> )	Inactive (%)
1.0	72.4 ± 1.3 <sup>a</sup>
0.5	75.6 ± 0.7 <sup>a</sup>
0.1	72.2 ± 1.2 <sup>a</sup>
0.02	23.4 ± 1.8 <sup>b</sup>
Control	6.1 ± 0.9 <sup>c</sup>

Data are expressed as means ± standard errors of four replicates. Means followed by different letters are significantly different (one-way ANOVA;  $P < 0.05$ ). Second-stage juveniles were from eggs that had not been treated with sodium hypochlorite.

by crude alkaloid extract (50%). While protease activity in *M. incognita* was lower than in *P. redivivus* (Table 5), consistent with previous observations,<sup>19</sup> it was also significantly (7%;  $P < 0.05$ ) inhibited by drupacine.

### 3.4 Greenhouse tests for suppression of *M. incognita* on pepper

In greenhouse experiments, general plant health, determined by above-ground (shoot) and root measurements, was marginally

influenced by only the highest alkaloid dose used (Table 6). Shoot lengths and shoot fresh weights were not affected by any treatment. However, mean shoot dry weight of the 0.5 mg mL<sup>-1</sup> alkaloid group was 14.0% lower ( $P < 0.05$ ) than that of the water–nematode controls. Mean root fresh weight of the 0.5 mg mL<sup>-1</sup> alkaloid group was less ( $P < 0.05$ ) than all other treatments (19.2% lower than the water–nematode control group).

Nematode reproduction was affected by alkaloid treatment. The mean number of eggs collected from the 0.5 mg mL<sup>-1</sup> alkaloid group was less ( $P < 0.05$ ) than all other treatments, and was more than 69% lower than in water–nematode controls. The mean number of J2 collected from the 0.5 mg mL<sup>-1</sup> alkaloid treatment group was lower than in all other treatments ( $P < 0.05$ ) and was 72.6% lower than in the water–nematode controls. Numbers of root galls were similar among all treatments. Root galls were counted up to 100; all treatments had 100 root galls, except the highest concentration of crude alkaloid extract, which had 98.

## 4 DISCUSSION

Crude alkaloid extract from young *C. fortunei* twigs with attached leaves was nematotoxic to two species of plant-parasitic nematodes, and the alkaloid drupacine was the most active isolated component. When drupacine was tested against Chinese populations of *B. xylophilus* and *M. incognita*, a concentration of 1.0 mg mL<sup>-1</sup> resulted in ca. 100% immobility of vermiform stages from both species. A somewhat similar result occurred when J2

**Table 5.** Effect of *Cephalotaxus fortunei* crude alkaloid extract drupacine on proteolytic activities in the microbivorous nematode *Panagrellus redivivus* and the root-knot nematode *Meloidogyne incognita*

Species	Control	Activity ( $V_{\max} \text{ min}^{-1} \mu\text{g}^{-1}$ )	
		Crude alkaloid extract	Drupacine
<i>P. redivivus</i>	14.86 ± 1.13 <sup>a</sup>	7.40 ± 0.76 <sup>b</sup>	2.90 ± 0.34 <sup>c</sup>
<i>M. incognita</i>	4.37 ± 0.14 <sup>a</sup>	ND	4.06 ± 0.03 <sup>b</sup>

Data are expressed as the mean ± standard error of four to nine separate reactions. Means within species followed by different letters are significantly different ( $P < 0.05$ ; two-tailed, unpaired *t*-test). All alkaloid and drupacine treatments were at 0.3 mg mL<sup>-1</sup>. ND, not detected.

**Table 6.** Effects of *Cephalotaxus fortunei* crude alkaloid on pepper (*Capsicum annum*) plant health and on reproduction of *Meloidogyne incognita* (Maryland population)

Treatment	Shoot length (cm)	Shoot fresh wt (g)	Shoot dry wt (g)	Root fresh wt (g)	Number of eggs on roots	Number of second-stage juveniles on roots
0.5 mg mL <sup>-1</sup>	21.36 ± 0.82 <sup>a</sup>	4.80 ± 0.31 <sup>a</sup>	0.86 ± 0.05 <sup>a</sup>	3.15 ± 0.17 <sup>a</sup>	168 400 ± 23 800 <sup>a</sup>	8 550 ± 2 520 <sup>a</sup>
0.1 mg mL <sup>-1</sup>	23.32 ± 0.75 <sup>a</sup>	5.31 ± 0.26 <sup>a</sup>	0.96 ± 0.05 <sup>ab</sup>	3.97 ± 0.17 <sup>b</sup>	421 500 ± 42 100 <sup>b</sup>	18 660 ± 2 140 <sup>b</sup>
0.02 mg mL <sup>-1</sup>	21.44 ± 0.59 <sup>a</sup>	4.80 ± 0.20 <sup>a</sup>	0.93 ± 0.04 <sup>ab</sup>	4.01 ± 0.13 <sup>b</sup>	526 900 ± 43 300 <sup>b</sup>	31 530 ± 2 780 <sup>b</sup>
0.1% ethanol	21.74 ± 0.67 <sup>a</sup>	4.80 ± 0.19 <sup>a</sup>	0.94 ± 0.04 <sup>ab</sup>	3.94 ± 0.18 <sup>b</sup>	490 100 ± 39 500 <sup>b</sup>	19 820 ± 2 360 <sup>b</sup>
0.02% ethanol	22.93 ± 0.80 <sup>a</sup>	4.94 ± 0.32 <sup>a</sup>	0.97 ± 0.06 <sup>ab</sup>	3.88 ± 0.18 <sup>b</sup>	410 400 ± 28 300 <sup>b</sup>	21 880 ± 2 380 <sup>b</sup>
0.004% ethanol	23.37 ± 0.92 <sup>a</sup>	5.08 ± 0.31 <sup>a</sup>	1.01 ± 0.07 <sup>ab</sup>	3.82 ± 0.12 <sup>b</sup>	572 000 ± 35 900 <sup>b</sup>	21 810 ± 2 910 <sup>b</sup>
Water + RKN	22.54 ± 0.91 <sup>a</sup>	5.01 ± 0.36 <sup>a</sup>	1.00 ± 0.07 <sup>ab</sup>	3.90 ± 0.14 <sup>b</sup>	547 600 ± 53 300 <sup>b</sup>	31 140 ± 3 600 <sup>b</sup>
Water, no RKN	21.50 ± 0.53 <sup>a</sup>	5.31 ± 0.23 <sup>a</sup>	1.16 ± 0.06 <sup>bc</sup>	4.41 ± 0.21 <sup>b</sup>	—	—

The experiment was conducted twice with eight pots per treatment in each trial ( $n = 15-16$  for each treatment); data were obtained 8 weeks after inoculation with nematodes, log<sub>10</sub> transformed, and tested for normality. Relationships among means within each column were analysed with one-way ANOVA and Tukey's multiple comparison. Means followed by different letters are significantly different ( $P < 0.05$ ). RKN, root-knot nematode.

from a US population of *M. incognita* were immersed in crude alkaloid extract: 1.0 mg mL<sup>-1</sup> resulted in ca. 72–98% J2 inactivity/mortality (in J2 from eggs that had not been surface-sterilized versus surface-sterilized in sodium hypochlorite, respectively). Observed differences in assay results could be due to several factors, including use of drupacine vs. crude alkaloid extract, acetone vs. ethanol as a solvent, or use of Chinese and US nematode populations. Despite quantitative differences, the results indicate that the crude alkaloid and drupacine are nematotoxic.

Assays with *M. incognita* eggs indicated that J2 hatch was less affected by the highest concentration of crude alkaloid extract than was J2 viability. In 1.0 mg mL<sup>-1</sup> crude alkaloid extract, hatch relative to controls was reduced by ca. 35%, far less than the 72–98% reduction in J2 activity. In both 0.1 and 0.01 mg mL<sup>-1</sup> crude alkaloid extract, relative hatch was reduced by ca. 33%, compared with ca. 70% and 3.3% decreases in J2 viability at 0.1 and 0.01 mg mL<sup>-1</sup>, respectively. The results indicate that, at the higher crude alkaloid extract concentrations, suppressive effects on nematode populations would be primarily due to nematotoxic effects on J2. However, reductions in hatch indicate that eggs may also be a significant target in suppression of reproduction. Effects on the embryo were not examined, but behavioral effects of crude alkaloid extract on infective J2 suggest that a similar response by unhatched J2 may disrupt normal behaviors necessary for the nematode to leave the egg. Such effects have been observed before in plant-parasitic nematodes exposed to behavior modifying chemicals.<sup>23</sup>

Crude alkaloid extract from *C. fortunei* applied to greenhouse soil suppressed *M. incognita* populations on plant roots when

applied at 0.5 mg mL<sup>-1</sup>, with 65.6% decrease in egg numbers and 56.9% decrease in J2 counts compared with the ethanol control. This was the same concentration of crude alkaloid extract that reduced J2 activity by approximately 75% in laboratory assays. Lower tested concentrations of the crude alkaloid extract were not effective in suppressing nematode populations in the greenhouse study. Although there was no difference in shoot length or weights between the 0.5 mg mL<sup>-1</sup> crude alkaloid treatment and the water + *M. incognita* treatment, there was a decrease in root weight. A smaller greenhouse trial indicated that 0.5 mg mL<sup>-1</sup> of crude alkaloid extract increased fresh shoot weight and decreased numbers of *M. incognita* eggs by 93.5% on pepper roots (unpublished). Further studies are needed to determine if a concentration between 0.1 and 0.5 mg mL<sup>-1</sup> can suppress nematode numbers but not affect plant root vigor.

Alkaloids encompass a very broad spectrum of structurally and biosynthetically diverse compounds with equally diverse modes of action, when known, in other organisms. The nematotoxicity of alkaloids has been known since the discovery several decades ago that three tetracyclic alkaloids were nematotoxic to two unidentified species of free-living nematodes.<sup>24</sup> Because of their ease of culture and observation, free-living nematodes have been the subject of many other demonstrations of nematode antagonism by alkaloids from natural sources, e.g. steroidal alkaloids vs. *P. redivivus*,<sup>25</sup> various isoquinolones vs. an unidentified diplogastrid,<sup>26</sup> and carbazole alkaloids, two quinolones and nicotine vs. *C. elegans*.<sup>27–30</sup> The inhibition of protease activity in *P. redivivus* preparations suggests a mode of action not previously

reported. Identification of the proteases affected is a necessary next step towards understanding the interactions of *Cephalotaxus* alkaloids, and drupacine in particular, with nematode metabolism.

Mammalian parasites have also been successful targets of naturally occurring alkaloids, e.g. quinoline alkaloids vs. *Haemonchus contortus*,<sup>31</sup> the oxindole alkaloid paraherquamide vs. *H. contortus* and *Trichostrongylus colubriformis*,<sup>32</sup> the aromatic pentacyclic alkaloid plakinidine vs. *Nippostrongylus brasiliensis*,<sup>33</sup> brominated alkaloids vs. *H. contortus*,<sup>34</sup> a quinolone vs. *Teladorsagia circumcincta*,<sup>28</sup> and isoquinoline alkaloids vs. two species of *Strongyloides*.<sup>35</sup> Similarly, activity of alkaloids against phytoparasitic nematodes has been described, e.g. ergot and loline alkaloids vs. *Pratylenchus scribneri*,<sup>36</sup> serpentine vs. *M. incognita*,<sup>37</sup> peniprequinolone vs. *Pratylenchus penetrans*,<sup>38</sup> quinolizidine alkaloids vs. *B. xylophilus*,<sup>39–41</sup> a piperidine vs. *Radopholus similis*,<sup>42</sup> and pyrrolizidine alkaloids vs. *M. incognita*, *P. penetrans* and *Heterodera schachtii*.<sup>43</sup>

One mode of action of drupacine in nematodes may involve protease inhibition. The significant but slight inhibition of *M. incognita* protease activity by drupacine, compared with the more marked inhibition in *P. redivivus*, may be attributed in part to the different physiological conditions of the two enzyme sources.<sup>19</sup> *Panagrellus redivivus* extractions were made using mixed developmental stages, whereas the *M. incognita* preparation represented J2 only. A wider variety of proteases might be expected in the mixed stage preparations than in those of the more homogeneous J2 population. While proteases in the *P. redivivus* preparation may be generally more sensitive to drupacine than those in *M. incognita*, it is possible that drupacine has multiple protease targets. The identification of specific proteases in *M. incognita* that are inhibited by drupacine, and the physiological processes involved, can help in revealing the metabolic effects of alkaloids in plant-parasitic nematodes and in designing control strategies incorporating these phytochemicals.

None of the compounds evaluated by others for toxicity or other antagonistic properties against nematodes are structurally related to the cephalotaxine alkaloids (tetracyclic spirobenzazepines) we examined. Our research has provided the first demonstration of nematotoxicity of crude *Cephalotaxus* alkaloids and the purified component drupacine. Moreover, crude alkaloid extract effectively suppressed root-knot nematode numbers on plant roots. Although the mode of action of drupacine and other cephalotaxine alkaloids against nematodes is not known, we are the first to demonstrate in any nematode that a plant alkaloid inhibits proteolytic activity. Additional research on the mode of action of these compounds and their spectrum of activity is warranted.

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