SUPPLEMENTARY MATERIAL

Two nematicidal furocoumarins from *Ficus carica* L. leaves and their physiological effects on pine wood nematode (*Bursaphelenchus xylophilus*)

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Abstract: The ethanol extract of the *Ficus carica* L. leaves was tested to show strong nematicidal activity against pine wood nematode (PWN), *Bursaphelenchus xylophilus*, causing 90.93% corrected mortality within 72 h at 1.0 mg/mL. From the ethyl acetate soluble fraction of the *Ficus carica* L. leaves extract, the main nematicidal constituents were obtained by bioassay-guided isolation and identified as linear furocoumarins bergapten (**1**) and psoralen (**2**) by mass and NMR spectral data analysis. Bergapten and psoralen had significant nematicidal activity against PWN with the LC₅₀ values of 97.08 µg/mL and 115.03 µg/mL within 72 h, respectively. The two furocoumarins could inhibit the activities of amylase, cellulase and acetylcholinesterase (AChE) from pine wood nematode. The morphologies of PWNs changed much after they were treated by bergapten and psoralen. The physiological effects of bergapten and psoralene on pine wood nematode might provide helpful clues to elucidate their nematicidal mechanisms.

Keywords: *Ficus carica* L.; nematicidal activity; *Bursaphelenchus xylophilus*; bergapten; psoralene

1. Experimental

1.1. Materials and instruments

Ficus carica L. leaves were picked from campus of Qingdao University. The pine wood nematodes (PWN), *B. xylophilus* were isolated from chips of infected pine

wood collected in Nanjing, Jiangsu Province, China by Baermann funnel techniques (Viglierchio and Schmit 1983). The pine wood nematodes were reared on a lawn of *Botrytis cinerea* cultured on potato dextrose agar medium (PDA) in the dark at 26 °C for about 7 days and collected. Aqueous suspension of the nematodes (about 2,000 nematodes per mL) was prepared by appropriate dilution for use. EIMS was measured on an Agilent 5973 mass spectrometer. NMR spectra were recorded on Bruker Avance 400 instrument in CDCl₃ with TMS as internal standard. Morphology of PWNs were observed with a Hitachi S-4800 scanning electron microscope (SEM) at 20 kV.

1.2. Isolation and identification of nematicidal compounds from Ficus carica L. leaves

Fresh *Ficus carica* L. leaves (200 g) were cut into pieces and extracted with ethanol (2 L×2) by the aid of intermittent ultrasonic oscillation for 24 h at room temperature. The filtrate was concentrated to dryness (12.84 g) under reduced pressure. The ethanol extract was partitioned three times between ethyl acetate and water. The combined ethyl acetate fractions were concentrated to dryness (6.1 g), and the water-soluble fraction was freeze-dried (6.5 g). The two fractions were tested for their nematicidal activity against PWN. The much more active ethyl acetate soluble fraction was chromatographed on a silica gel column (500g, 200-300 mesh), and successively eluted with a stepwise gradient of petroleum ether-ethyl acetate-methanol to obtain eight fractions, F1~F8. F3 and F4 were tested to be significantly active through nematicidal assays against PWN and subjected to further purification. F3 (126 mg) was purified on silica gel column (200-400 mesh, 100 g) eluted by petroleum ether /ethyl acetate (4:1, v/v), and then recrystallized in petroleum ether /ethyl acetate (4:1, v/v), yielding compound **2** (67 mg). F4 (208 mg) was further separated through silica gel column (200-400 mesh, 100 g) eluted by petroleum ether /ethyl acetate (from 6 : 1, 5:1 to 4:1, v/v) to give two subfractions. The subfractions were purified by preparative HPLC (Waters 600, Symmetry PrepTM C18, 7μm, 19×300 mm) using MeOH-H₂O (60:40, v/v) as mobile phase at a flow of 3 mL/min to yield compound **2** (83 mg) and **1** (46 mg).

Compound **1**. White microcrystalline powder (ethyl acetate). MS(m/z):216[M·]⁺. ¹H NMR (CDCl₃) : δ4.27 (3H, s, OCH₃), 6.27 (1H, d, J=9.8 Hz, H-3), 7.03 (1H, d, J=2.4 Hz, H-3'), 7.13 (1H, s, H-8), 7.60 (1H, d, J=2.4 Hz, H-2'), 8.15 (1H, d, J=9.8 Hz, H-4). ¹³C NMR (CDCl₃): δ60.01 (C-OCH₃), 93.90 (C-8), 105.09 (C-3'), 106.43 (C-10), 112.58 (C-3), 112.72 (C-6), 139.39 (C-4), 144.88 (C-2'), 149.50 (C-5), 152.73 (C-9), 158.31 (C-7), 161.4 (C-2). The NMR data were in accordance with reported data of bergapten (Kislev et al. 2006; Yu et al. 2010), so the compound was determined to be bergapten.

Compound **2**. Colorless to faint yellow needle (ether /ethyl acetate). MS(m/z):

186[M-]⁺. ¹H NMR(CDCl₃) : δ6.38 (1H, d, J=9.6 Hz, H-3), 6.84 (1H, d, J=2.4 Hz, H-3'), 7.48 (1H, s, H-8), 7.68 (1H, s, H-5), 7.70 (1H, d, J=2.4 Hz, H-2'), 7.80 (1H, d, J=9.6 Hz, H-4). ¹³C NMR (CDCl₃): δ99.92 (C-8), 106.39 (C-3'), 114.70 (C-3), 115.43 (C-10), 119.84 (C-5), 124.88 (C-6), 144.08 (C-4), 146.92 (C-2'), 152.06 (C-9), 156.43 (C-7), 161.04 (C-2). By comparing the NMR data with the relative literature (Chawla et al. 2012), the compound was deduced as psoralen.

1.3. Nematicidal assay

The *Ficus carica* L. leaves extracts, column fractions and purified compounds were dissolved with dimethylsulphoxide (DMSO) at a concentration of 20 mg/mL. Their test solutions were prepared by serial dilution with distilled water containing 0.5% Triton X-100. Test solution (50 µL) and aqueous suspension of nematodes (50 µL) were introduced into the wells of 24-well plates. Each treatment was replicated four times, with the same concentration of DMSO in distilled water containing 0.5% Triton X-100 as a negative control and carbofuran as a positive control. Dead and active nematodes in each well were recorded after incubation at 26 °C for 24 h, 48 h, 72 h respectively. Nematodes were observed under stereo microscope and considered dead if their bodies were straight and did not move even by physical stimuli with a fine needle.

The mortality in the assays was corrected by eliminating that in the control according to the Schneider–Orelli formula (Puntener 1981):

Corrected mortality % = (Mortality % in treatment – Mortality% in control) / (100-Mortality % in control)

1.4. Assays of bergapten and psoralen on endogenous enzymes from PWN

1.4.1. Preparation of enzyme solutions.

Healthy pine wood nematodes were quickly mixed with physiological saline at 1:5 (w/v), and then homogenated in ice bath. The homogenates were centrifuged at 4°C, 12 000×g for 30 min and the supernatants were collected as enzyme solutions used for assays. Protein concentrations of enzyme solutions were determined according to Bradford's method with bovine serum albumin as the standard (Bradford et al. 1976).

1.4.2. Enzyme treatment by bergapten and psoralen.

The compounds were dissolved in DMSO at a concentration of 20 mg/mL, and 25, 20, 15, 10, 5 µL compound solutions were mixed with appropriate amount of enzyme solution to obtain treated enzyme solutions with 500, 400, 300, 200, 100 µg/ mL compound, respectively. The mixed solutions were preincubated at 37°C for 15 min.

1.4.3. Effects of bergapten and psoralen on enzymes in PWN and IC₅₀.

The activities of amylase were tested by amylase test kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manuals in the kit. One unit of amylase was defined as the amount of enzyme that hydrolyzed 10 mg starch in 30 min. The activities of cellulase were tested by cellulase test kit (Beijing Solarbio Science &

Technology Co., Ltd, China). One unit of cellulase was defined as the amount of enzyme that catalyzed substrates to obtain 1 µg glucose per minute. The activities of acetylcholineesterase (AChE) was tested by AChE test kits (Nanjing Jiancheng Bioengineering Institute, China). One unit of AChE was defined as the amount of enzyme that hydrolyzed 1µmol acetylcholine in 6 min under assay conditions. Each experiment was performed in four replications. Inhibitory effects of the compounds on enzymes were expressed as the percentage of enzyme inhibition in the above assays and calculated as $(1-B/A) \times 100$, where A is the activity of the enzyme without the test compounds and B is the activity of the enzyme treated by the compounds.

1.5. Observation of PWN by SEM.

Approximate 5000 pine wood nematodes were treated with 500 µg/ml bergapten and psoralen dissolved in 5% DMSO containing 0.5% Triton X-100 at 26°C for 48 h, and nematodes in controlled group were treated with 5% DMSO containing 0.5% TritonX-100 at the same condition. The PWNs treated by nematicidal compounds, bergapten and psoralen, and in controlled group were washed with PBS for three times (each for 15 min), then they were re-fixed with 1.0% osmic acid for 1 h. PWNs were then washed with PBS for three times (each for 15 min), followed by dehydrated in an ethanol series (30%, 50%, 70% , 80%, 90%), two changes of 100% ethanol, ethanol-tertiary butyl alcohol (1:1,v/v) and two changes of 100% tertiary butyl alcohol for 10 min at each concentration. After PWNs were incubated at 100% tertiary butyl alcohol at -18°C and vacuum freeze dried, they were sputter-coated with 1-2nm platinum, observed with a Hitachi S-4800 SEM at 20 kV.

1.6. Statistical analysis.

The median lethal concentrations (LC₅₀) against PWN and the median inhibitory concentrations (IC₅₀) on enzymes were obtained according to probit analysis. All of the data processings were based on SPSS version 17.0 software.

Table S1. Nematicidal activities of column chromatography fractions from the ethyl acetate extract of the *Ficus carica* L. leaves

Fraction (500 µg/mL)	Corrected mortality (% , mean±SD)		
	24 h	48 h	72 h
1	0.53±0.40f	1.82±0.25e	2.54±0.41f
2	1.17±0.31f	2.96±0.50e	4.83±0.30e
3	33.76±0.96b	51.83±2.63b	72.97±1.10b

4	42.41±0.62a	71.70±0.85a	90.10±0.53a
5	7.00±0.26d	14.43±0.65cd	28.60±0.90c
6	4.40±0.56e	12.67±0.75d	26.40±0.70d
7	0.47±0.15f	2.60±0.30e	4.60±0.46e
8	9.40±0.46c	16.27±0.61c	29.23±0.31c

Notes: The data were means of 4 replicates. Means followed by the same letter in each column are not significantly different at $P < 0.05$ according to the least significant difference test.

Table S2. IC₅₀ values of bergapten and psoralen on amylase, cellulase and AchE

Compound	Inhibition on amylase (µg/mL)		Inhibition on cellulase (µg/mL)		Inhibition on AchE (µg/mL)	
	IC ₅₀	95% CL ^a	IC ₅₀	95% CL ^a	IC ₅₀	95% CL ^a
Bergapten	372.45	353.27- 394.56	450.40	424.88- 480.92	493.11	449.87- 550.60
Psoralen	1072.82	921.39- 1300.20	458.92	432.41- 490.80	564.59	516.51- 627.88
Positive control ^b	1124.49	1053.89- 1211.56	4944.37	4859.22- 5039.84	29.03	26.27- 31.72

^a CL stands for confidence limit.

^b positive control on amylase is acarbose; positive control on cellulase is copper sulfate-ammonia complex; positive control on AchE is galanthamine hydrobromide.

Figure S1. Chemical structures of bergapten (1) and psoralen (2) isolated from *Ficus carica* L. leaves.

Figure S2. Observation by scanning electron microscope on morphologies of pine wood nematodes treated by bergapten and psoralen. A. Nematodes in control; B. Nematodes treated with bergapten; C. Nematodes treated with psoralen.

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