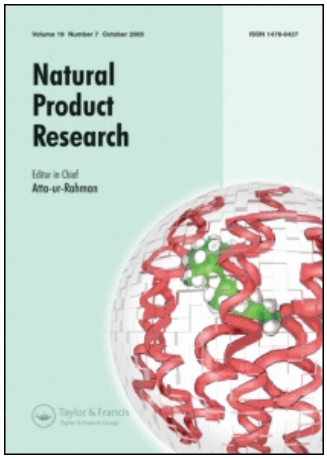

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Nematicidal coumarins from *Heracleum candicans* Wall.

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The root extract of *Heracleum candicans* Wall. exhibited antagonistic activities against nematodes *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle and *Panagrellus redivivus* (Linn.) Goodey. Through bioassay-guided fractionations, three coumarins were obtained from the extract of *H. candicans* and determined to be 8-geranyloxypsoralen (**1**), imperatorin (**2**), and heraclenin (**3**) based on spectra data. All three compounds possessed nematicidal activities against the two tested nematodes. The median lethal concentrations (LC_{50}) of compounds **1–3** at 72 h were 188.3, 161.7, and 114.7 mg L^{-1} respectively against *B. xylophilus* and were 117.5, 179.0, and 148.7 mg L^{-1} respectively against *P. redivivus*. This is the first report about species in the Umbelliferae family that possesses nematicidal activity.

Keywords: *Heracleum candicans* Wall.; Nematode; Coumarin; Nematicidal activity

1. Introduction

Plant parasitic nematodes have caused serious damages to agriculture and forestry. It has been estimated that plant parasitic nematodes cause as much as $100 billion worth of losses to the global crops and trees annually [1]. The majority of nematode damages to the pine trees are caused by the pine wood nematode *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle [2], typically results in rapid wilting and death of the host trees. Traditional treatments using chemically synthesized nematicides have generated significant environmental problems and the development of resistance among nematodes to chemical nematicides has emerged [3]. Therefore, it is necessary to search for more environmentally friendly, low side effects, and more selective and effective nematicides [4,5]. Plants provide a large source of nematicidal chemicals [6–9]. We describe here the isolation and structure elucidation of three coumarins from the root extract of *Heracleum candicans* Wall. (figure 1). We provide evidence that all three compounds have nematicidal activities against *B. xylophilus* and *Panagrellus redivivus* (Linn.) Goodey.

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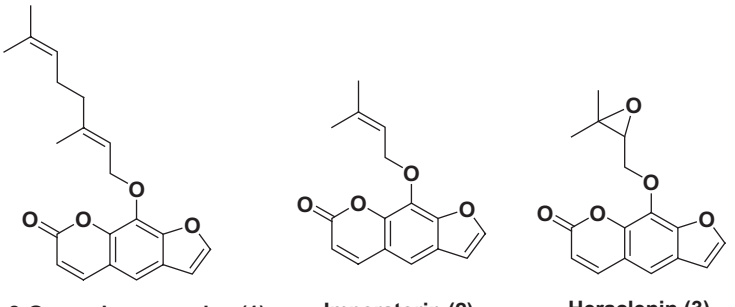

Figure 1. Structure of compounds **1**–**3**.

2. Results and discussion

2.1. *Structure determination of compounds 1–3*

Compound **1** ($C_{21}H_{22}O_4$): amorphous colourless solid; $[\alpha]_D^{19} = +25.0$ (*c* 0.4, $CHCl_3$); m.p. 51–53°C; IR (KBr, cm^{-1}): 2922; 2856; 1728; 1640; 1594; 1476; 1125; and 1094; ESI/MS *m/z* 339 $[M+H]^+$; ^1H NMR (500 MHz, $CDCl_3$): δ 7.77 (H-4; d; $J = 9.6$ Hz); 7.69 (H-2′; d; $J = 2.1$ Hz); 7.36 (H-5; s); 6.81 (H-3′; d; $J = 2.1$ Hz); 6.37 (H-3; d; $J = 9.6$ Hz); 5.61 (H-2″; t; $J = 7.3$ Hz); 5.04 (H-1″ and H-6″; m); 2.03 (H-4″ and H-5″; d; $J = 3.0$ Hz); 1.69 (Me-3″; s); 1.63 (H-8″; s); 1.56 (Me-7″; s); ^{13}C NMR (125 MHz, $CDCl_3$): δ 160.5 (C-2); 114.7 (C-3); 144.3 (C-3); 113.3 (C-5); 125.9 (C-6); 148.8 (C-7); 131.7 (C-8); 143.1 (C-9); 116.5 (C-10); 146.6 (C-2′); 106.7 (C-3′); 70.1 (C-1″); 119.6 (C-2″); 144.0 (C-3″); 16.6 (Me-3″); 39.6 (C-4″); 26.4 (C-5″); 123.8 (C-6″); 131.7 (C-7″); 17.7 (Me-7″); 25.6 (C-8″). Compared to the data reported previously [10,11], compound **1** was determined to be 8-geranyloxypsoralen.

Compound **2** ($C_{16}H_{14}O_4$): amorphous colourless solid; $[\alpha]_D^{19} = +30.0$ (*c* 0.8, $CHCl_3$); m.p. 100–103°C; IR (KBr, cm^{-1}): 1728; 1632; 1594; 1476; and 1160; ESI-MS *m/z* 271 $[M+H]^+$; ^1H NMR (500 MHz, $CDCl_3$): δ 7.77 (H-4; d; $J = 9.3$ Hz); 7.69 (H-2′; d; $J = 2.4$ Hz); 7.36 (H-5; s); 6.81 (H-3′; d; $J = 2.4$ Hz); 6.38 (H-3; d; $J = 9.3$ Hz); 5.61 (H-2″; t; $J = 7.3$ Hz); 5.01 (H-1″; d; $J = 7.2$ Hz); 1.72 (H-3″; s); 1.74 (H-4″; s); ^{13}C NMR (125 MHz, $CDCl_3$): δ 160.5 (C-2); 114.8 (C-3); 144.3 (C-3); 113.2 (C-5); 125.9 (C-6); 148.7 (C-7); 131.8 (C-8); 143.9 (C-9); 116.6 (C-10); 146.6 (C-2′); 106.7 (C-3′); 70.2 (C-1″); 119.8 (C-2″); 139.8 (C-3″); 25.8 (C-4″); 18.1 (C-5″). Compound **2** was identified as imperatorin based on reference [12].

Compound **3** ($C_{16}H_{14}O_5$): amorphous colourless solid; $[\alpha]_D^{19} = +53.8$ (*c* 0.8, $CHCl_3$); m.p. 102–107°C; IR (KBr, cm^{-1}): 1728; 1640; 1594; and 1138; ESI-MS *m/z* 287 $[M+H]^+$; 309 $[M+Na]^+$; ^1H NMR (500 MHz, $CDCl_3$): δ 7.79 (H-4; d, $J = 9.6$ Hz), 7.71 (H-2′; d; $J = 2.4$ Hz); 7.36 (H-5; s); 6.81 (H-3′; d; $J = 2.4$ Hz); 6.37 (H-3; d; $J = 9.3$ Hz); 4.60 (H-1″; t (dd); $J = 5.4$ Hz); 3.34 (H-2″; t; $J = 5.5$ Hz); 1.35 (H-4″; s); 1.29 (H-5″; s); ^{13}C NMR (125 MHz, $CDCl_3$): δ 160.3 (C-2); 114.9 (C-3); 144.3 (C-3); 113.9 (C-5); 126.0 (C-6); 148.4 (C-7); 131.6 (C-8); 143.7 (C-9); 116.6 (C-10); 146.8 (C-2′); 106.8 (C-3′); 72.8 (C-1″); 61.4 (C-2″); 58.2 (C-3″); 24.6 (C-4″); 18.9 (C-5″). Based on data reported in reference [13], we determined that compound **3** was identical to heraclenin.

2.2. *Nematicidal activity of plant extract*

The root extract of *H. candicans* showed obvious activities against *P. redivivus* and *B. xylophilus*. When the concentration of the extract was at 5 mg mL^{-1}, the mortalities of *P. redivivus* and *B. xylophilus* were 57.8 and 85.8% at 48 h and 92.6 and 100% at 72 h, respectively. The plant *H. candicans* is commonly used for medical purposes in China [14]. However, this is the first report that species in Umbelliferae possess nematicidal activity. It is possible that other plants in this family could be sources of nematicidal substances. Additional screening is needed to test this hypothesis.

2.3. *Nematicidal activity of compounds 1–3*

Compounds **1**–**3** caused significant mortalities of *P. redivivus* and *B. xylophilus* at the concentrations of 400 and 200 mg L^{-1} (table 1). The median lethal concentrations (LC_{50}) of the compounds **1–3** against *B. xylophilus* and *P. redivivus* at 72 h were 188.3 and 117.5 mg L^{-1}, 161.7 and 179.0 mg L^{-1}, 114.7 and 148.7 mg L^{-1}, respectively. The test nematocide standard avermectin showed no effect against *P. redivivus* even at the concentration of 200 mg L^{-1}. However, it caused a high mortality on *B. xylophilus*, with LC_{50} value 0.156 mg L^{-1} at 48 h.

Coumarins are known to have a variety of biological activities. For example, they can affect the morphology of roots of maize seedings [15] and they have been used as anticoagulants [16]. In addition, compound imperatorin has been reported to inhibit HIV-1 replication [17] and to kill larvae of the cotton leafworm *Spodoptera littoralis* [18]. However, the nematicidal activities of compounds 8-geranyloxypsoralen, imperatorin, and heraclenin have not been reported. It should be mentioned that the three compounds are obtained, based on bioassay-guided fractionations from *H. candicans*. These results suggest that more nematicidal compounds might be obtained from plants using nematicidal assay model.

Table 1. Effect of compounds **1**–**3** on the mortality (%) of the two tested nematodes.

Compounds	Concentrations (mg L^{-1})	Mortality (%, *Bursaphelenchus xylophilus*/*Panagrellus redivivus*)			
		12 h	24 h	48 h	72 h
1	400	16.7/13.9	27.3/34.7	50.7/56.9	69.0/82.7
	200	8.0/9.3	19.7/28.7	32.6/45.8	48.0/66.7
	100	5.3/4.3	18.0/12.5	21.4/27.1	36.5/49.0
	50	2.3/3.7	9.8/9.7	14.7/15.1	21.7/22.5
2	400	16.0/9.6	27.4/23.6	48.6/41.9	73.3/66.7
	200	11.8/6.4	29.4/20.1	41.6/32.6	54.7/50.4
	100	8.2/5.4	19.0/14.6	28.7/21.9	35.7/36.2
	50	3.7/4.5	9.3/11.6	17.1/19.6	23.5/29.8
3	400	25.3/22.8	44.9/47.6	67.1/64.1	87.3/80.5
	200	15.4/11.3	29.0/29.5	42.7/43.2	59.2/56.7
	100	8.7/5.4	14.2/11.7	29.5/21.5	44.3/36.0
	50	3.2/2.1	8.2/4.9	15.2/10.7	28.6/20.3
Avermectin*	1	31.3	98.1	100	100
	0.5	25.7	83.7	97.1	100
	0.25	18.3	54.2	69.1	75.0
	0.125	10.1	31.7	42.7	56.4
Control (5% ethanol)		1.1/2.6	2.2/4.3	5.1/6.8	6.9/8.9
Distilled water control		0.8/1.3	1.5/2.6	3.1/5.4	5.5/7.2

*Avermectin showed no effect on *P. redivivus* at these concentrations, the data is the mortality of *B. xylophilus*.

3. Experimental

3.1. *Culture of nematodes*

The pine wood nematode *B. xylophilus* and the saprophytic nematode *P. redivivus* were cultured in the laboratory based on a protocol described previously [19]. The cultured nematodes were extracted from medium using Baermann funnel method for bioassay [20].

3.2. *Preparation and testing for nematicidal effect of plant extracts*

The plant *H. candicans* was collected from Kunming Institute of Botany, P.R. China. The methods of preparing plant extracts and testing for nematicidal activity followed the protocols described previously [21]. The nematodes were considered to be dead when they failed to respond to physical stimuli with a fine needle [22,23]. The number of dead/total (live + dead) nematodes was recorded after 12 h, 24 h, 48 h, and 72 h. Five percent ethanol and distilled water were used as controls. The experiments for nematicidal activity were repeated twice.

3.3. *Spectroscopic measurements and chromatography*

NMR experiments were carried out on a Bruker DRX-500 spectrometer. Mass spectra were recorded on a Finnigan LCQ-Advantage spectrometer mass spectrometer. The melting points were measured without correction on a Kofler-microscope (Reichert). Infrared (IR) spectra were measured on a Paragon 1000 pc spectrometer. Optical rotations were measured on a Jasco DIP-370 digital polarimeter. Column chromatography was carried out on silica gel (200–300 mesh; Qingdao Marine Chemical Factory, Qingdao, P.R. China.) and Sephadex LH-20 (Pharmacia). Thin-layer chromatography (TLC) was performed on silica gel (Si gel G; Qingdao Marine Chemical Factory, Qingdao, China.).

3.4. *Isolation of coumarins from* H. candicans *roots*

The dried roots (30.0 g) of *H. candicans* were extracted with 80% ethanol to produce the crude residue. The crude residue was dissolved in water and then extracted using ethyl acetate (EA). The bioassay result showed that the EA fraction contained a nematicidal activity. In further experiments, bioassay was used to isolate the specific nematicidal compounds for each step. The EA fraction (1.455 g) was subjected to a silica gel column (30 g, 200–300 mesh) eluting with petroleum ether and ethyl acetate (20+1, 9+1, 8+2 and 2 + 1 by volume), followed with acetone to yield fractions A_1–A_9. Fraction A_2 was further fractionated on a Sephadex LH-20 column eluted with acetone to produce compound **1** (17 mg). The fraction A_4 (0.295 g) was purified on a Sephadex LH-20 column eluted with acetone to give fractions A_{41}–A_{46}. Fraction A_{44} (0.166 g) was further purified on a Sephadex LH-20 column eluted with acetone to give compound **2** (61 mg). Fraction A_6 (0.518 g) was isolated on a Sephadex LH-20 column eluted with acetone to give fractions A_{61}–A_{69}. The active fraction A_{66} was subjected to a silica gel column (3 g, 200–300 mesh) eluted with petroleum ether and ethyl acetate

(2 + 1 by volume), followed with acetone to give fractions A_{661}–A_{668}. Fraction A_{666} was further purified on a Sephadex LH-20 column eluted with acetone to obtain compound **3** (14 mg).

3.5. *Assay of the nematicidal activity of compounds*

The method for assaying nematicidal activity of compounds followed that described previously [21]. As a standard, avermectin (Lynhi Fine Chemical Co. Ltd, Shijiazhuang, China) was used. Each treatment was replicated three times, with the same concentration of ethanol as a control. Mean percentage mortality was calculated based on the three replicates.

To quantify the nematicidal effects of these three compounds against nematode, LC_{50} values were calculated using Microsoft Excel (version 2003 software, USA). Regression analyses were also conducted using the linear regression model implemented in Excel. In the regression analysis, nematode mortalities were transformed into probit value, and concentrations (C) of compounds were transformed using $\log_{10}(C)$ before analysis.

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