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Nematotoxic coumarins from *Angelica pubescens* Maxim. *f. biserrata* Shan *et* Yuan roots and their physiological effects on *Bursaphelenchus xylophilus*

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Abstract

The ethanol extracts from the roots of *Angelica pubescens* Maxim. *f. biserrata* Shan *et* Yuan was toxic against the pine wood nematode *Bursaphelenchus xylophilus*. The ethyl acetate-soluble fraction derived from this extract increased its potency with a mortality of 95.25% in 72 hr at 1.0 mg/mL. Four nematotoxic coumarins were obtained from the ethyl acetate extract by bioassay-guided isolation. These were identified as osthole **1**, columbianadin **2**, bergapten **3** and xanthotoxin **4** by mass and nuclear magnetic resonance spectral data analysis. The LC_{50} values against *B. xylophilus* in 72 hr were 489.17, 406.74, 430.08, and 435.66 μM, respectively. These compounds also altered the smooth morphology of the *B. xylophilus* exoskeleton to a rough and pitted appearance as visualized by electron microscopy. The coumarins **1-4** possessed significant acetylcholinesterase inhibitory activities but had negligible effects on amylase and cellulase. This research provides additional clues to the nematotoxic mechanism of coumarins against the pine wood nematode *B. xylophilus*. This work will assist in the development of coumarin nematicides with enhanced activity using molecular modifications of the core coumarin structure.

Key words

Roots of *Angelica pubescens* Maxim. *f. biserrata* Shan *et* Yuan, Coumarins, Nematotoxic, Pine wood nematode.

Pine wilt disease is a highly destructive epidemic disease of pine trees that can rapidly spread from infected to healthy trees. The disease affects a variety of pine species in Asia, Europe, America, and Africa and has become a severe worldwide threat to forest resources (Mota et al., 1999; Abelleira et al., 2011; Fonseca et al., 2012). The pine wood nematode (PWN), *Bursaphelenchus xylophilus,* is causal pathogen of pine wilt and its control is crucial for management of the disease (Mamiya, 1983; Wingfield et al., 1986; Mota et al., 1999; Cheng et al., 2017). However, insect resistance, environmental pollution, and off-target lethality of synthetic nematicides used for disease treatment indicate that alternative and ecofriendly nematicides are needed (Zhao, 2008; Andres et al., 2010; Seo et al., 2014; Cheng et al., 2017).

Phytonematicides as natural products are considered popular candidates. Many plants have been reported to provide potential nematotoxic constituents including essential oils, alkaloids, and other constituents (Matsuda et al., 1989, 1991; Zhao, 1999; Choi et al., 2006, 2007, 2008; Kong et al., 2006, 2007; Andrrk et al., 2007; Park et al., 2007; Kim et al., 2008; Wang et al., 2008; Faria et al., 2010, 2013; Zhang et al., 2011; Wang et al., 2012; Ntalli and Caboni, 2012; Cui et al., 2014; Guo et al., 2016, 2017). The plant *Angelica pubescens* Maxim. *f. biserrata* Shan *et* Yuan is distributed in the Hubei, Sichuan, and Shaanxi provinces of

China. Its roots (Duhuo) are used in traditional Chinese herbal medicine for the treatment of arthralgia, rheumatic arthritis, headaches, and limb contracture (Zhang et al., 2008; Zaugg et al., 2011). In this study, we explored the nematotoxic constituents of Duhuo and their effects on the pine wood nematode.

Materials and methods

The roots of *A. pubescens* were purchased from the Qingdao Jianlian Pharmacy, Qingdao, China, in March 2016. Nematodes were isolated from chips of infected pine wood collected in Nanjing, Jiangsu Province, China using the Baermann funnel technique (Viglierchio and Schmit, 1983). The isolated nematodes were cultured on a lawn of *Botrytis cinerea* grown on potato dextrose agar (PDA) in the dark at 26°C for 7 d. An aqueous suspension of 2,000/mL was used for testing. Freeze-dried processing of the samples was carried out in Freezone lyophilizer (Labconco, Kansas City, MO, USA). Silica gel for column chromatography (200-300 mesh) was purchased from Qingdao Marine Chemical, Qingdao, China. Thin-layer chromatography (TLC) was performed on silica gel plates containing fluorescent indicator F-254 (Yantai Jiangyou Silica Gel Development, Yantai, China) and observed under UV light at 254 nm or by iodine fumigation. Electron-impact mass spectra (EIMS) were measured on a Bruker maXis UHR-TOF mass spectrometer. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance-500 spectrometer operating at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR in chloroform-*d* ($CDCl_3$) with tetramethylsilane (TMS) as internal standard. Nematode morphology was observed using a Hitachi S-4800 scanning electron microscope (SEM) and a Hitachi H-7650 transmission electron microscope (TEM).

Isolation of nematotoxic compounds

Duhuo (50 g) was mashed and extracted with ethanol (1 L×2) using intermittent ultrasonic oscillation for 48 hr at room temperature. The ethanol extract was concentrated under vacuum to a brown paste (6.3 g) and then partitioned between ethyl acetate and distilled water three times. The ethyl acetate-soluble fraction was concentrated to dryness (2.5 g), and the aqueous fraction was freeze-dried into a powder (3.6 g). The ethyl acetate extract was fractionated by silica gel column chromatography for active components. The column was eluted with a stepwise gradient of hexane: ethyl acetate at 15:1, 10:1; 9:1; 8:1; 6:1; 5:1; 4:1, 2:1, 1:1, 1:3, 1:4, 1:6, 1:8, 1:10, and 0:1 (v/v). We obtained nine fractions as judged by TLC. Fractions 5 and 6 possessed high nematotoxic activity against pine wood nematodes and were further purified. Fraction 5 was chromatographed on a silica gel column eluted with hexane: ethyl acetate (8:1, v/v) to yield two active compounds, osthole (73 mg) and columbianadin (11 mg). Fraction 6 was subjected to silica gel column chromatography eluted with hexane: ethyl acetate (6:1, v/v) and two active compounds bergapten (7 mg) and xanthotoxin (3 mg) were obtained.

Chemical structure elucidation

7-methoxy-8-(3-methylbut-2-enyl)-2-chromenone (Osthole, 1). White needle-like crystals, EIMS m/z: 244 [M]+. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.26 (1 H, d, J = 9.5 Hz, H-3), 7.63 (1 H, d, J = 9.5 Hz, H-4), 7.29 (1 H, d, J = 8.6, H-5), 6.85(1 H, d, J = 8.6, H-6), 3.94 (3 H, s, OCH3), 5.24 (1 H, m, H-2'), 3.56 (2 H, d, J = 7.2 Hz, H-1'), 1.84 (3 H, s, H-4'), 1.67 (3 H, s, H-5'). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 160.30 (C-2), 113.08 (C-3), 143.68 (C-4), 126.18 (C-5), 107.40 (C-6), 161.32 (C-7), 107.40 (C-8), 152.93 (C-9), 118.14 (C-10), 21.97 (C-1'), 121.1(C-2'), 132.64 (C-3'), 25.76 (C-4'), 17.93 (C-5'), 56.08 (OCH3). The EIMS and NMR data conformed to those of osthole reported previously (Fujioka et al., 1999; Zhang et al., 2008).

(S)-8-[1-[(Z)-2-Methyl-2-butenoyloxy]-1-methylethyl]-8,9-dihydro-2H-furo(2,3-h)-1-benzop yran-2-one (Columbianadin, 2). White crystals, EIMS m/z: 328[M]+. ^{1}H NMR (CDCl3, 500 MHz): δ 6.23 (1 H, d, J = 9.6 Hz, H-3), 7.65 (1 H, d, J= 9.6 Hz, H-4), 7.28 (1 H, d, J = 8.3 Hz, H-5), 6.76 (1 H, d, J = 8.3 Hz, H-6), 5.14 (1 H, m, H-2'), 3.40 (2 H, m, H-3'), 1.66 (3 H, m, H-5'), 1.60 (3 H, m, H-6'), 5.99 (1 H, m, H-8'), 1.69 (3 H, dd, J = 1.7 Hz, 7.3 Hz, H-10), 1.90 (3 H, dd, J = 1.3 Hz, 7.2 Hz, H-11'). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 161.00 (C-2), 112.23 (C-3), 143.95 (C-4), 128.82 (C-5), 106.66 (C-6), 164.05 (C-7), 112.23 (C-8), 151.34 (C-9), 113.57 (C-10), 89.30 (C-2'), 27.67 (C-3'), 82.07(C-4'), 21.25 (C-5'), 22.36 (C-6'), 167.12 (C-7'), 128.74 (C-8'), 137.53 (C-9'), 15.59 (C-10'), 20.5 (C-11'). The EIMS and NMR data coincided with previous reports (Zhang et al., 2008).

4-Methoxy-7H-furo(3,2-g)-1-benzopyran-7-one (Bergapten, 3). White crystals, EIMS m/z: 216 [M]+. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 4.27 (s, 3, OCH3), 6.27 (1 H, d, J = 9.8 Hz, H-3), 8.15 (1 H, d, J = 9.8 Hz, H-4), 7.60 (1 H, d, J = 2.4 Hz, H-2'), 7.03 (1 H, d, J = 2.4 Hz, H-3'), 7.13 (1 H, s, H-8). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 60.01 (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 EIMS and NMR data coincided with those reported for bergapten (Kislev et al., 2006; Yu et al., 2010).

9-Methoxy-7H-furo(3,2-g)chromen-7-one (Xanthotoxin, 4). White needle-like crystals, EIMS m/z: 216[M]+. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.39 (1 H, d, J = 9.7 Hz, H-3), 7.78 (1 H, d, J = 9.7 Hz, H-4), 7.37 (1 H, s, H-5), 4.32 (3 H, s, OCH3), 6.84 (1 H, d, J = 2.2 Hz, H-2'), 7.71 (1 H, d, J = 2.2 Hz, H-3'). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 160.41 (C-2), 114.84 (C-3), 144.27 (C-4), 112.91 (C-5), 126.16 (C-6), 147.81 (C-7), 132.92 (C-8), 143.13 (C-9), 116.58 (C-10),c 61.35 (OCH3), 146.65 (C-2'), 106.74 (C-3'). The above data were consistent with previous reports of xanthotoxin (Fujioka et al., 1999; Zhang et al., 2008).

Chemical structures of compounds 1-4 are shown in Figure 1.

Nematotoxic assay

The extracts of Duhuo and the column fractions 1-9 were dissolved in 5% dimethylsulphoxide (DMSO) aqueous solution containing 0.5% Triton X-100 at a concentration of 1 mg/mL and used as test solutions. Compounds 1-4 were dissolved with DMSO at 20 mg/mL and then diluted with 0.5% Triton X-100 aqueous solution to 200-1,000 μM for testing in for nematotoxic assays. Each test solution (50 [xL) and aqueous suspension of nematodes (50 μL) were introduced into the wells of 96-well plates. In each well, the concentration of nematodes was about one nematode per μL solution. Nematodes were mixtures of juveniles and adults with males, females, and juveniles at a ratio of approximately 1:1:2. Each treatment was replicated four times using the same solvent as negative controls and aloperine as a positive control. Dead and active nematodes in each well were observed under a stereo microscope and their numbers were recorded after incubation at 26°C for 24, 48, and 72 hr.

Nematode mortality was corrected using the Schneider–Orelli formula (Puntener, 1981; Ntalli et al., 2010): Corrected mortality (%) = ((Mortality % in treatment – Mortality% in negative control)/(100% – Mortality % in negative control)) × 100.

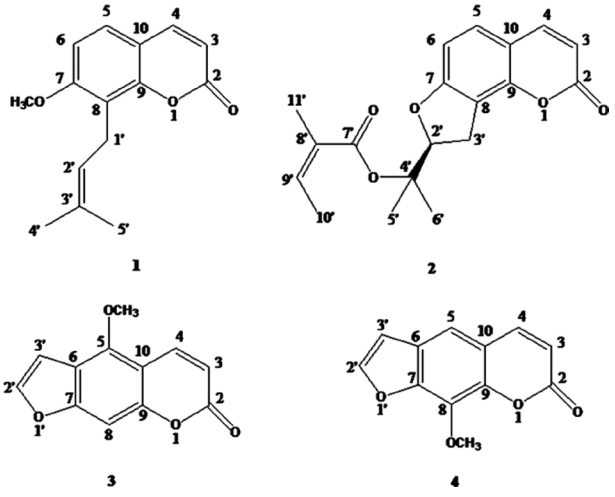

Figure 1: Chemical structures of nematicidal compounds **1–4** isolated from Roots of *Angelica pubescens* Maxim. *f. biserrata* Shan et Yuan (Duhuo).

SEM and TEM

Approximately 5,000 pine wood nematodes were treated with compounds 1-4 at 500 μM in 5% DMSO containing 0.5% Triton X-100 at 26°C for 48 hr. Controls were treated with 5% DMSO containing 0.5% TritonX-100 under the same conditions. Nematodes were washed with 0.1 M phosphate buffer saline (PBS, pH 7.4) three times and then fixed with 2.5% glutaraldehyde overnight at 4°C and then with 1.0% osmium tetroxide for 1 hr after washing with PBS three times each for 15 min. The fixed nematodes were dehydrated in an ethanol series (30, 50, 70, 80, and 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 each treatment. The nematodes were then incubated with 100% tertiary butyl alcohol at –18°C and vacuum freeze-dried. They were sputter-coated with 1 to 2 nm platinum for SEM.

The fixed nematodes were also dehydrated in an acetone series (30, 50, 70, 80, and 90%) and three changes of 100% acetone (each treatment for 10 min). The dehydrated nematodes were embedded in acetone-Spurr resin (Polysciences, US) at 3:1 (v/v) for 4 hr, 1:1 for 6 hr, 1:3 for 12 hr, and in Spurr resin for 24 hr. They were then sectioned for TEM.

In vitro assays of compounds 1-4

Healthy pine wood nematodes were mixed with physiological saline at 1:5 (w/v) and then homogenized on ice. 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 were determined using the Bradford method with bovine serum albumin as the standard (Bradford, 1976). Different amounts of each tested compound were mixed with 5 mL enzyme solution to obtain the treated enzyme solutions containing 2.0, 1.5, 1.0, 0.75, and 0.5 mM of the test compound. The enzyme solutions treated with galanthamine hydrobromide (0.20-0.05 mM), acarbose (2.0-0.5 mM), and copper sulfate-ammonia complexion (50-5 mM) to measure

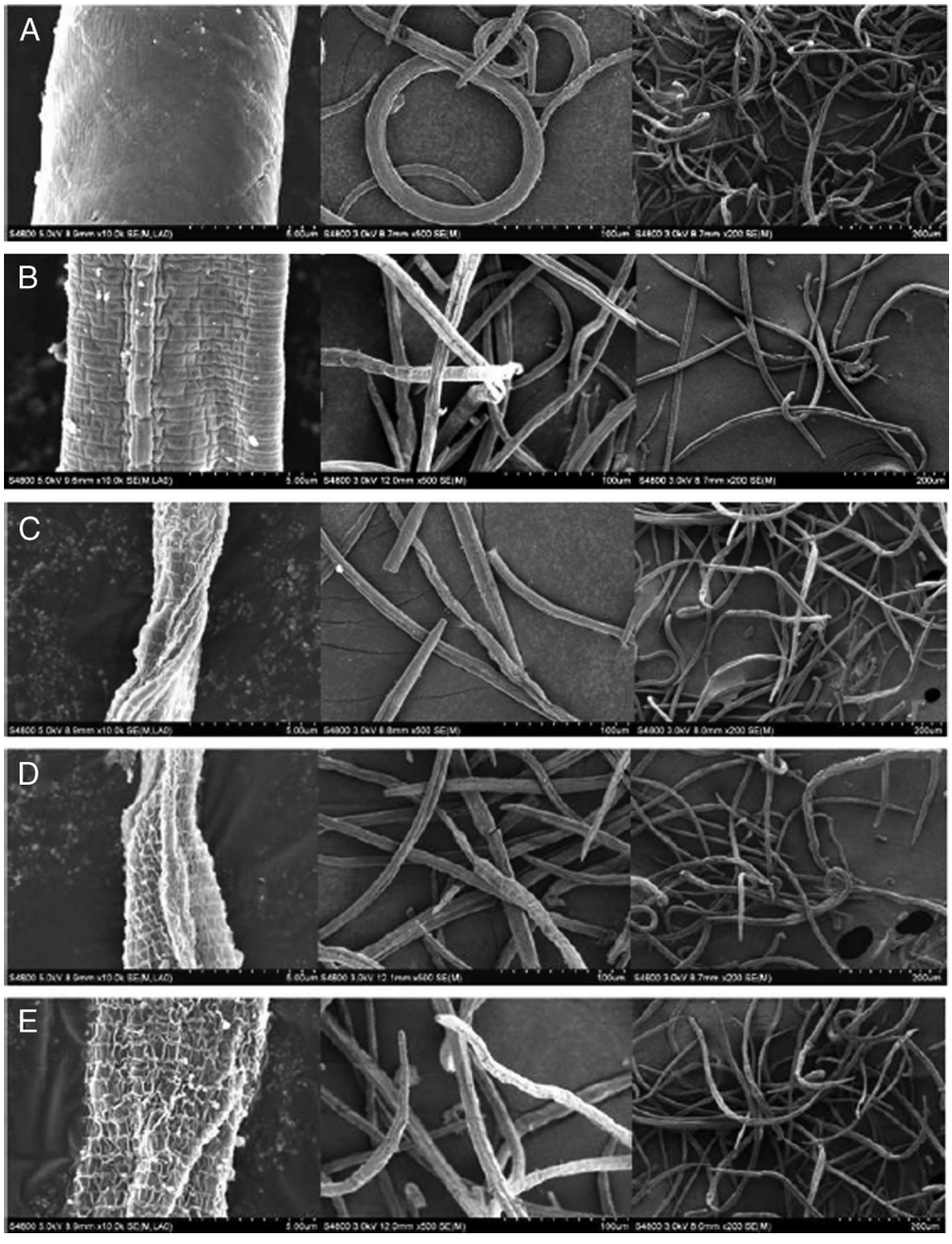

Figure 2: Micrographs of pine wood nematodes treated with compounds 1-4 under SEM. (A) Nematodes in control; (B-E) Nematodes treated with osthole 1, columbianadin 2, bergapten 3, xanthotoxin 4, respectively. Minimum scales in A-E are 5, 100, 200 µm, successively.

inhibitory effects on acetylcholinesterase (AchE), amylase, and cellulase, respectively. The enzyme solution without any treatment served as the negative control. All of the treated enzyme solutions were preincubated at 37°C for 15 min for enzyme activity assays. Each treatment was replicated three times.

AchE and amylase activities were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, China). One unit of AchE was defined as the amount of enzyme that hydrolyzed 1.0 xmol acetylcholine in 6 min under assay conditions. One unit of amylase was defined as the amount of enzyme that hydrolyzed 10 mg starch in 30 min. Cellulase activity was also measured using a commercial kit (Beijing Solarbio, China). One unit of cellulase was defined as the amount of enzyme that catalyzed cellulose degradation in the reaction system to obtain 1.0 × g glucose per minute. Each experiment was performed

in triplicate. Inhibitory effects were expressed as the percentage of enzyme inhibition in the above assays and calculated as (1–B/A) × 100: where A is the activity of the enzyme in negative control and B is the activity of the treated enzyme.

In vivo assays of compounds 1-4

Approximately 100,000 pine wood nematodes were introduced into 5 mL solutions of compounds 1-4 at 500 μM in 5% DMSO containing 0.5% Triton X-100 and cultured in the dark at 26°C. Nematodes cultured in 5% DMSO containing 0.5% Triton X-100 at the same condition were used as the negative control. Each treatment was replicated three times. The same amount of nematode suspension was removed from each treatment at 12, 24, 36, and 48 hr and were then homogenized as per above in physiological saline. The AchE, amylase, and cellulase activities were tested as described above.

Statistical analysis

Analysis of variance (ANOVA) was performed on the data of corrected mortality and the means were compared and separated by Duncan's multiple-range test at the a = 0.05 level. The median lethal concentrations (LC50) and the median inhibitory concentrations (IC50) on enzymes were obtained according to Probit analysis. All of the data were analyzed with SPSS version 17.0 software.

Results

Nematotoxic activity

The Duhuo ethanol extract had a relatively strong nematotoxic activity with a corrected mortality of 85.83% in 72 hr at 1.0 mg/mL. The ethyl acetate-soluble fraction derived from the ethanol extract was more active with a corrected mortality of 95.25% compared with 36.55% for the aqueous fraction in 72 hr at 1.0 mg/mL (Table 1). The four compounds osthole 1, columbianadin 2, bergapten 3, and xanthotoxin 4 from the most active column chromatography fractions (5 and 6) all showed nematotoxic activity (Table 2).

Effects of compounds 1-4 on nematode morphology

Treatment of nematodes with compounds 1-4 resulted in conversion of the nematode smooth exoskel-

Table 1. Nematotoxic activities of the root extracts of *Angelica pubescens* Maxim. *f. biserrata* Shan et Yuan against the Pine Wood nematode *B. xylophilus*.

	Corrected mortality[a] (%, Mean ± SD)		
Sample (1.0 mg/mL)	24 hr	48 hr	72 hr
Ethanol extract	32.50 ± 1.95 c	57.47 ± 1.56 c	85.83 ± 1.60 b
Ethyl acetate fraction	46.93 ± 2.10 c	80.06 ± 1.01 b	95.25 ± 1.16 ab
Aqueous fraction	16.59 ± 0.45 f	27.26 ± 0.99 g	36.55 ± 1.33 e
Fr.1	3.28 ± 0.71 i	10.74 ± 1.11 i	15.48 ± 1.25 h
Fr.2	4.73 ± 1.13 hi	12.78 ± 0.71 i	17.48 ± 1.50 h
Fr.3	10.74 ± 1.27 g	15.54 ± 1.45 h	23.19 ± 1.05 f
Fr.4	20.53 ± 0.66 e	35.68 ± 1.46 e	40.89 ± 0.40 d
Fr.5	56.97 ± 0.94 a	83.78 ± 1.48 a	96.37 ± 1.24 a
Fr.6	49.89 ± 1.19b	76.41 ± 1.22 c	93.58 ± 1.35 b
Fr.7	21.27 ± 1.35 e	33.14 ± 0.39 f	40.76 ± 0.66 d
Fr.8	10.69 ± 1.41 g	17.36 ± 1.60 h	20.27 ± 1.20 g
Fr.9	6.01 ± 80.76 h	11.52 ± 1.49 i	16.74 ± 1.53 h

[a]The data were means of four replicates. Means followed by the same letter in each column were not significantly different (a = 0.05) according to Duncan's multiple-range test.

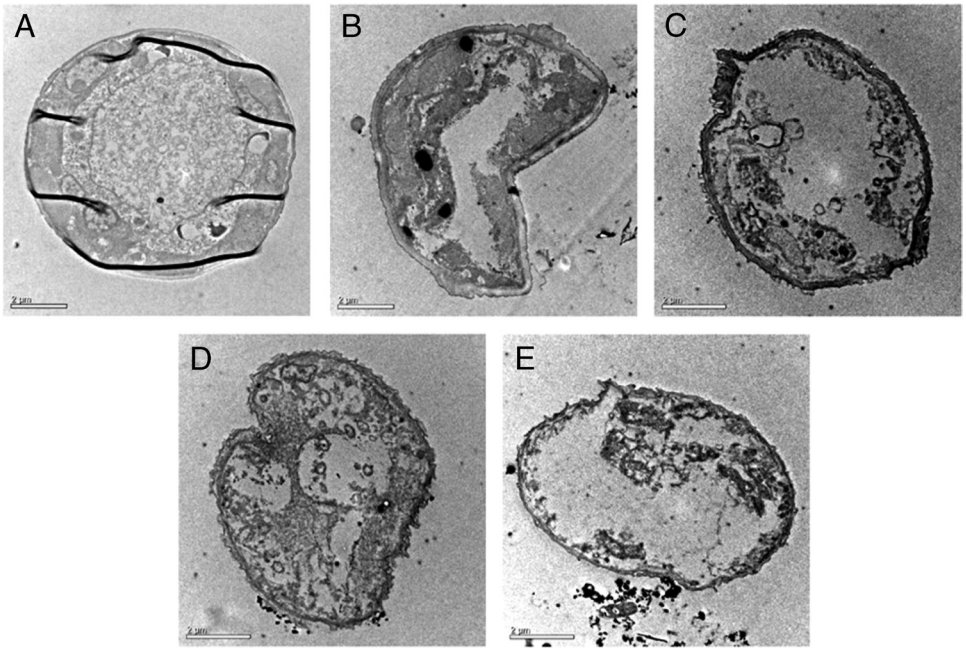

Figure 3: Observation by TEM on pine wood nematodes treated with compounds **1-4**. (A) Nematodes in control; (B-E) Nematodes treated with osthole **1**, columbianadin **2**, bergapten **3**, xanthotoxin **4**, respectively. Bars represent 2 μm.

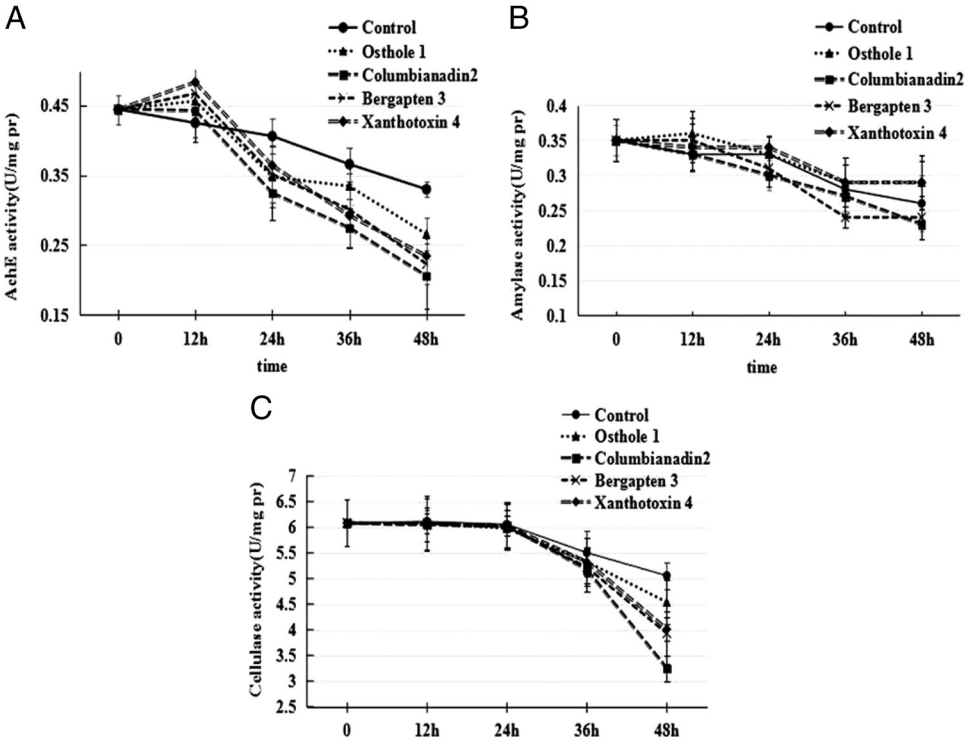

Figure 4: The effects of nematicidal compounds on PWN enzymes *in vivo*. Effects on AchE (A) amylase (B) and cellulase (C) of PWN, respectively.

Table 2. Nematotoxic activities against *B. xylophilus* of compounds 1-4 isolated from the root extracts of *Angelica pubescens* Maxim. *f. biserrata* Shan et Yuan.

Compound (200–1,000 µm)	LC_{50} (72 hr, µM)	95% CL[a] (µM)	χ^2
Osthole 1	489.17	459.09–527.01	1.25
Columbianadin 2	406.74	387.61–422.06	2.14
Bergapten 3	430.08	401.51–458.02	1.13
Xanthotoxin 4	435.66	410.47–459.20	2.02
Aloperine[b]	369.76	350.07–388.93	2.85

[a]CL, confidence limit; bPositive control.

etons to rough and significantly shriveled especially with compounds 2-4. In addition, the SEM fixation procedure of the treated nematodes resulted in structural damage (Fig. 2). TEM micrographs also showed that the insect cuticles were rough with abnormal cavities compared with controls (Fig. 3).

Inhibition of enzyme activities

The four nematotoxic compounds were all able to inhibit AchE activity in vitro. Columbianadin 2 showed greatest inhibitory activity with an IC_{50} value of 1.62 mM (Table 3). Nematodes treated in vivo had

Table 3. IC_{50} values of compounds 1-4 on in vitro enzyme activities of *B. xylophilus*.

Enzyme	Compound	IC_{50} (µM)	95% CL[a] (µM)	χ^2
AchE	Osthole **1**	3.24	3.01–3.49	2.35
	Columbianadin **2**	1.62	1.42–1.85	1.01
	Bergapten **3**	2.28	2.08–2.55	2.93
	Xanthotoxin **4**	2.40	2.16–2.71	1.86
	Galanthamine Hydrobromide[b]	0.079	0.071–0.086	3.10
Amylase	Osthole **1**	4.89	4.15–5.51	2.12
	Columbianadin **2**	1.35	1.12–1.59	1.82
	Bergapten **3**	1.72	1.64–1.83	0.40
	Xanthotoxin **4**	3.25	2.79–3.65	3.51
	Acarbose[b]	1.74	1.63–1.88	1.17
Cellulase	Osthole **1**	6.13	5.85–6.42	3.01
	Columbianadin **2**	4.25	3.94–4.49	1.68
	Bergapten **3**	2.09	1.97–2.23	2.21
	Xanthotoxin **4**	2.35	2.01–2.65	4.01
	Copper Sulfate–Ammonia Complexion[b]	20.10	19.75–20.49	1.82

[a]CL, confidence limit; bPositive control.

greater activity the longer they were exposed to the chemicals. All four compounds showed weak activation at 12 hr that subsequently changed to inhibition over time (Fig. 4A). This early AchE activation was most likely was a nematode stress response.

The four nematotoxic compounds also significantly inhibited the nematode amylase and cellulase activities in vitro (Table 3). However, these effects occurred in vivo at 48 hr for amylase and 36 hr for cellulase (Fig. 4B,C). The reduction in cellulase may have been the result of decreased survival in addition to the direct inhibitory effect on cellulase.

Discussion

We identified and purified four nematotoxic coumarins from Duhuo. Our previous research had identified two nematotoxic phytochemicals, from *Ficus carica* L. leaves that were also coumarins (Guo et al., 2016). Coumarins lethal to nematodes have also been previously identified from other laboratories (Takaishi et al., 2008; Wang et al., 2008; Caboni et al., 2015; Pan et al., 2016). Together these findings implicate the 2H-1-benzopyran-2-one core moiety of coumarins as the key structure contributing to nematotoxicity.

Our studies about effects of compounds 1-4 on morphology and pine wood nematode enzymes were focused on identifying possible targets for the compounds. Interestingly the compounds physically altered the microstructures of the nematode body. Inhibition of insect cholinesterase is the primary target for screening natural pesticides. AchE hydrolyzes acetylcholine to acetic acid and choline and terminates synaptic transmission. Inhibition of AchE leads to accumulation of acetylcholine in the synaptic cleft, impedes neurotransmission, and results in insect mortality. There are numerous phytochemicals that show significant inhibitory activity against the AchE of *B. xylophilus* (Kang et al., 2013a, 2013b). In addition, amylase and cellulase are vital digestive enzymes for nematodes (Yan and Yang, 1997). Our four nematotoxic compounds 1-4 showed significant effects on AchE activity both in vitro and in vivo. However, they did not affect amylase and had an uncertain effect on cellulase in vivo.

Therefore, the most likely target of compounds 1-4 that contributed to nematode lethality was AchE inhibition.

We also found that columbianadin 2 had the greatest nematotoxic activity and the strongest effect on AchE among the four compounds (Tables 2,3; Fig. 4A). The α, β-unsaturated carbonyl in the dihydrofuran side chain is the most likely inhibitory functional group. Conjugated aldehydes inhibit AchE due to their high affinity with an active site cysteine (Kang et al., 2013a). We speculate this was the case for columbianadin 2.

This report is the first study of the nematicidal activity of *Angelica pubescens* Maxim. *f. biserrata* Shan *et* Yuan roots (Duhuo) against the pine wood nematode, *B. xylophilus.* Our findings provide additional clues for the development of coumarin phytonematicides.

Acknowledgments

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References

Abelleira, A., Picoaga, A., Mansilla, J. P., and Aguin, O. 2011. Detection of *Bursaphelenchus xylophilus,* causal agent of pine wilt disease on Pinus pinaster in northwestern Spain. Plant Disease. 95:776.

Andres, M. F., Gonzalez-Coloma, A., Sanz, J., Burillo, J., and Sainz, P. 2010. Nematicidal activity of essential oils: a review. Bioresource Technology 101:372–8.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248–54.

Caboni, P., Saba, M., Oplos, C., Aissani, N., Maxia, A., Menkissoglu-Spiroudi, U., Casu, L., and Ntalli, N. 2015. Nematicidal activity of furanocoumarins from parsley against Meloidogyne spp. Pest Management Science 71:1099–105.

Cheng, L., Xu, S., Xu, C., Lu, H., Zhang, Z., Zhang, D., Mu, W., and Liu, F. 2017. Effects of trans-2-hexenal on reproduction, growth and behaviour and efficacy against the pinewood nematode, *Bursaphelenchus xylophilus*. Pest Management Science 73:888–95.

Choi, I. H., Park, J. Y., Shin, S. C., and Park, I. K. 2006. Nematotoxic activity of medicinal plant extracts and two cinnamates isolated from Kaempferia galanga L. (Proh Hom) against the pine wood nematode, *Bursaphelenchus xylophilus*. Nematology 8:359–65.

Choi, I. H., Shin, S. C., and Park, I. K. 2007, Nematicidal activity of onion *(Allium cepa)* oil and its components against the pine wood nematode *(Bursaphelenchus xylophilus)*. Nematology 9:231–5.

Choi, N. H., Kwon, H. R., Son, S. W., Choi, Y. H., Jang, K. S., Lee, S. O., Choi, J. E., Ngoc, L. H., and Kim, J. C. 2008. Nematotoxic activity of malabaricones isolated from *Myristica Malabarica* fruit rinds against *Bursaphelenchus xylophilus*. Nematology 10:801–7.

Cui, H., Jin, H., Lium, Q., Yan, Z., Ding, L., and Qin, B. 2014. Nematotoxic metabolites from roots of Stellera chamaejasme against *Bursaphelenchus xylophilus*

and *Bursaphelenchus mucronatus*. Pest Management Science 70:827–35.

Faria, J., Barbosa, P., Mota, M., Sanches, J., and Figueiredo, A. C. 2010. Nematicidal activity of essential oil-bearing plants, from Portuguese flora, against the pinewood nematode Bursaphelenchus xylophilus. Journal of Nematology 42:8–16.

Faria, J., Barbosa, P., Bennett, R. N., Mota, M., and Figueiredo, A. C. 2013. Bioactivity against *Bursaphelenchus xylophilus*: nematotoxics from essential oils, essential oils fractions and decoction waters. Phytochemistry 94:220–8.

Fonseca, L., Cardoso, J. M. S., Lopes, A., Pestana, M., Abreu, F., Nunes, N., Mota, M., and Abrantes, I. 2012. The pinewood nematode, *Bursaphelenchus xylophilus,* in Madeira Island. Helminthologia 49:96–103.

Fujioka, T., Furumi, K., Fujii, H., Okabe, H., Mihashi, K., Nakano, Y., Matsunaga, H., Katano, M., and Mori, M. 1999. Antiproliferative constituents from umbelliferae plants. V: a new furanocoumarin and falcarindiol euranocoumarin ethers from the root of *Angelica japonica*. Chemical and Pharmaceutical Bulletin 47:96–100.

Guo, Q. Q., Du, G. C., He, H. W., Xu, H. K., Guo, D. S., and Li, R. G. 2016. Two nematotoxic furocoumarins from Ficus carica L. leaves and their physiological effects on pine wood nematode (*Bursaphelenchus xylophilus*). Natural Product Research 30:1969–73.

Guo, Q. Q., Du, G. C., Qi, H. T., Zhang, Y. N., Yue, T. Q., Wang, J. C., and Li, R. G. 2017. A Nematicidal tannin from *Punica granatum* L. rind and its physiological effect on pine wood nematode (*Bursaphelenchus xylophilus*) pesticide biochemistry. Physiology 135:64–8.

Kang, J. S., Moon, Y. S., Lee, S. H., and Park, I. K. 2013a. Inhibition of acetylcholinesterase and glutathione S-transferase of the pinewood nematode (*Bursaphelenchus xylophilus*) by aliphatic compounds. Pesticide Biochemistry Physiology 105:184–8.

Kang, J. S., Kim, E., Lee, S. H., and Park, I. K. 2013b. Inhibition of acetylcholinesterases of the pinewood nematode, *Bursaphelenchus xylophilus,* by phytochemicals from plant essential oils. Pesticide Biochemistry Physiology 105:50–6.

Kim, J., Seo, S. M., Lee, S. G., Shin, S. C., and Park, I. K. 2008. Nematicidal activity of plant essential oils and components from coriander (Coriandrum sativum), oriental sweet gum (Liquidambar orientalis), and valerian (Valeriana wallichii) essential oils against pine wood nematode (*Bursaphelenchus xylophilus*). Journal of Agriculture and Food Chemistry 56:7316–20.

Kislev, M. E., Hartmann, A., and Bar-yosef, O. 2006. Early domesticated fig in the Jordan valley. Science 312:1372–4.

Kong, J. O., Lee, S. M., Moon, Y. S., Lee, S. G., and Ahn, Y. J. 2006. Nematicidal activity of plant essential oils against *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae). Journal of Asia-Pacific Entomology 9:173–8.

Kong, J. O., Lee, S. M., Moon, Y. S., Lee, S. G., and Ahn, Y. J. 2007. Nematotoxic activity of Cassia and Cinnamon oil compounds and related compounds toward *Bursaphelenchus xylophilus* (Nematoda: Parasitaphelenchidae). Journal of Nematology 39:31–6.

Mamiya, Y. 1983. Pathology of the Pine Wilt Disease caused by *Bursaphelenchus xylophilus*. Annual Review of Phytopathology 21:201–20.

Matsuda, K., Kimura, M., Komai, K., and Hamada, M. 1989. Nematicidal activities of (-)-N-methylcytisine and (-)-anagyrine from Sophora flavescens against pine wood nematodes. Agricultural Biology and Chemistry 53:2287–8.

Matsuda, K., Yarnada, K., Kimura, M., and Hamada, M. 1991. Nematicidal activity of matrine and its derivatives against pine wood nematodes. Journal of Agriculture and Food Chemistry 39:189–91.

Mota, M. M., Braasch, H., Bravo, M. A., Penas, A. C., Burgemeister, W., Metge, K., and Sousa, E. 1999. First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. Nematology 1:727–34.

Ntalli, N., and Caboni, P. 2012. Botanical nematicides: a review. Journal of Agriculture and Food Chemistry 60:9929–40.

Ntalli, N., Vargiu, S., Menkissoglu-Spiroudi, U., and Caboni, P. 2010. Nematicidal carboxylic acids and aldehydes from *Melia azedarach* fruits. Journal of Agriculture and Food Chemistry 58:11390–4.

Pan, L., Li, X. Z., Sun, D. A., Hui, J., Guo, H. R., and Qin, B. 2016. Design and synthesis of novel coumarin analogs and their nematicidal activity against five phytonematodes. Chinese Chemical Letters 27:375–9.

Park, I. K., Kim, J., Lee, S. G., and Shin, S. C. 2007. Nematicidal activity of plant essential oils and components from Ajowan *(Trachyspermum ammi),* Allspice (*Pimenta dioica*) and Litsea (*Litsea cubeba*) essential oils against Pine Wood Nematode (*Bursaphelenchus Xylophilus*). Journal of Nematology 39:275–9.

Puntener, W. 1981, Manual for field trials in plant protection 2nd ed., Ciba-Geigy Limited, Basle, p. 205.

Seo, S. M., Kim, J., Koh, S. H., Ahn, Y. J. and Park, I. K. 2014). Nematicidal activity of natural ester compounds and their analogues against pine wood nematode, *Bursaphelenchus xylophilus*. Journal of Agriculture and Food Chemistry 62:9103–8.

Takaishi, K., Izumi, M., Baba, N., Kawazu, K., and Nakajima, S. 2008. Synthesis and biological evaluation

of alkoxycoumarins as novel nematicidal constituents. Bioorganic & Medicinal Chemistry 18:5614–7.

Viglierchio, D. R., and Schmit, R. V. 1983. On the methodology of nematode extraction from field samples: Baermann funnel modifications. Journal of Nematology 15:438–44.

Wang, J., Pan, X., Han, Y., Guo, D., Guo, Q., and Li, R. 2012. Rosmarinic acid from eelgrass shows nematicidal and antibacterial activities against pine wood nematode and its carrying bacteria. Marine Drugs 10:2729–40.

Wang, X. B., Li, G. H., Li, L., Zheng, L. J., Huang, R., and Zhang, K. Q. 2008. Nematicidal coumarins from Heracleum candicans. Wall Natural Product Researc 22:666–71.

Wingfield, M. J., Bedker, P. J., and Blanchette, R. A. 1986. Pathogenicity of *Bursaphelenchus xylophilus* on Pines in Minnesota and Wisconsin. Journal of Nematology 18:44–9.

Yan, D., and Yang, B. 1997. The enzymes in the secretions of pine wood nematode (*Bursaphelenchus xylophilus*). Forest Research 10:265–9.

Yu, H. W., Li, B. G., Chen, X. Z., Li, C. S., and Zhang, G. L. 2010. Chemical study on Evodia vestita. Chinese Journal of Applied & Environmental Biology 16:72–5.

Zaugg, J., Eickmeier, E., Rueda, D. C., Hering, S., and Hamburger, M. 2011. HPLC-based activity profiling of *Angelica pubescens* roots for new positive GABA: a receptor modulators in Xenopus oocytes. Fitoterapia 82:434–40.

Zhang, C. Y., Zhang, B. G., and Yang, X. W. 2008. Studies on the chemical constituents of the root of *Angelica pubescens f. biserrata.* pharm. Pharmaceutical Journal of Chinese People's Liberation Army 23:241–5.

Zhang, H. M., Wang, G. L., Bai, C. Q., Li, P., Liu, P., Liu, Z. M., Liu, Q. Z., Wang, Y. Y., Liu, Z. L., Du, S. S., and Deng, Z. W. 2011. A new eudesmane sesquiterpene glucoside from *Liriope muscari* fibrous roots. Molecules 16:9017–24.

Zhao, B. G. 1999. Nematicidal activity of quinolizidine alkaloids and the functional group pairs in their molecular structure. Journal of Chemical Ecology 25:2205–14.

Zhao, B. G. 2008. Pine wilt disease in China. In Zhao, B. G., Futai, K., Sutherland, J. R. and Takeuchi, Y. (Eds), Pine wilt disease, Springer, Tokyo, Japan: 18–25.