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Screening and isolation of antinematodal metabolites against *Bursaphelenchus xylophilus* produced by fungi

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Abstract - Sixty crude cultural filtrates of thirty fresh water fungi individually grown in both Potato Dextrose broth and GPC broth were assayed *in vitro* for antinematodal activity against *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle using immersion test. Filtrates from *Camposporium quercicola* YMF1.01300*, Periconia digitata* YMF1.00948, *Caryospora callicarpa* YMF1.01026 grown in both PDB and GPC broth, and the cultural filtrate of *Melanospora zamiae* YMF1.00948 grown in PDB were found to be pathogenic to the tested nematodes. The degree of activity varied with the fungal species, length of exposure time, and media composition. From a nematicidal cultural extract of *Caryospora callicarpa* YMF1.01026, four known naphthalenones were isolated and identified as 4,8-dihydroxy-3,4-dihydronaphthalen-1(2*H*)-one (**1**), 4,6-dihydroxy - 3,4-dihydronaphthalen- 1(2*H*)-one (**2**), 4,6,8-trihydroxy-3,4-dihydronaphthalen-1(2*H*)-one) (**3**), 3,4,6,8-tetrahydroxy-3,4-dihydronaphthalen-1(2*H*)-one (*cis*-4-hydroxyscytalone) (**4**) by NMR and MS analysis. All four metabolites showed noticeable biological activity against *B. xylophilus* nematode. This is the first published report of these compounds affecting plant-parasitic nematodes.

Key words: antinematodal acitivity, *Bursaphelenchus xylophilus*, *Caryospora callicarpa*, naphthalenone.

INTRODUCTION

Bursaphelenchus xylophilus (Steiner et Buhrer 1934) is both a plant-parasitic and fungal-feeding nematode that caused multimillion dollar loses to pine forests, especially in some Asian countries (Mamiya, 1984; Sutherland and Webster, 1993). Control of this disease depends primarily on fumigation of disease-infected trees, aerial application of synthetic pesticides against the vector for the pine wood nematode, *Monochamus alternatus*, or killing the pine wood nematode by injection of tree trunks with nematicides, such as mesulfenfos, morantel tartrate and levamisol hydrochloride, etc (Takai *et al*., 2000). However, increasing awareness of environmental and human health concerns associated with chemical nematicides and removal of several efficacious products from the world market in recent years provide impetus for a search of environmentally compatible products for nematode management. Finding safer alternatives to chemical nematicides is especially urgent for turf and ornamentals due to their proximity to people, pets, and permanent dwellings. One way of searching for such antinematodal compounds is to screen naturally occurring compounds in fungi.

Fungi have already appeared to be a source of effective pesticidal compounds and may come to be regarded as an inexhaustible source of harmless pesticides having low plant and human toxicity and being easily biodegradable (Siddiqui and Mahmood, 1996). Many fungi have been reported to possess antinematodal activities against nematodes such as *Heterodera glycines* (Ichinohe), *Meloidogyne incognita* (Kofoid et White)*, Meloidogyne javanica* (Treub)*, Caenorhabditis elegans, Panagrellus redivivus* (Goodey), *Rotylenchulus reniformis* (Linford et Oliveira) etc. (Desai *et al*., 1972; Alam *et al*., 1997; Sing *et al*., 1983; Khan and Hussain, 1989; Khan and Kgan, 1992; Chattopadhyay and De, 1995; Pathak and Kumar, 1995; Sankaranarayanan *et al*., 1997). Many fungal metabolites with antinematodal activity have also been isolated and identified as alkaloids, peptides, terpenes, fatty acids etc. (Anke and Sterner, 1997; Chitwood, 2002; Li *et al*., 2007). However, among these, only very few reports are related to antinematodal fungi and metabolites against pine wood nematodes (Kawazu *et al*., 1993; Huang *et al*., 2004).

Therefore, a screening of antinematodal substances from fungi against *B. xylophilus* was carried out by the authors in recent years (Dong *et al*., 2004, 2006a). Our prior research has indicated that the fresh water-derived fungi have strong ability to produce antinematodal substances against *B. xylophilus,* and many novel nematicidal metabolites have already been found in these fungi (Dong *et al*., 2004, 2005a, 2005b, 2006b, 2007). The present paper deals with the results of further antinematodal screening and involved the isolation of four secondary metabolites from a nematicidal cultural extract of *Caryospora callicarpa* YMF1.01026*.* Based on spectroscopic analysis, these metabolites were identified as four known naphthalenones, 4,8-dihydroxy-3,4-dihydronaphthalen-1(2*H*)-one (**1**), 4,6-dihydroxy-3,4-dihydronaphthalen-1(2*H*)-one (**2**), 4,6,8-trihydroxy-3,4-dihydronaphthalen-1(2*H*)-one) (**3**), 3,4,6,8-tetrahydroxy-3,4-dihydronaphthalen-1(2*H*)-one (*cis*-4-hydroxyscytalone) (**4**) (See Fig. 1). The *in vitro* antinematodal activities of these metabolites were evaluated against *B. xylophilus*. This is the first published report of these compounds affecting plant-parasitic nematodes.

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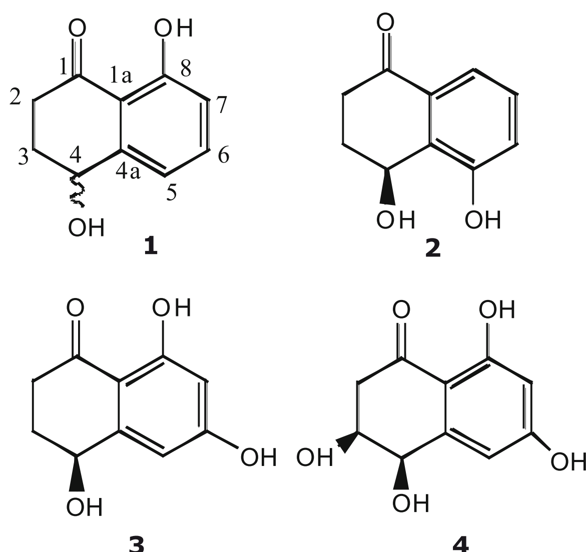

FIG. 1 - Structure of compounds **1**-**4** of *Caryospora callicarpa* YMF1.01026.

MATERIALS AND METHODS

General experimental instruments and materials. Infrared (IR) spectra were obtained in KBr pellets with a Bio-Rad FTS-135 spectrophotometer (Bio-Rad, Richmond, CA, USA). UV spectra were taken on a Shimadzu double-beam 210A spectrophotometer (Shimadzu, Kyoto, Japan). Optical rotations were measured with a Horiba SEPA-300 polarimeter (Horiba, Tokyo, Japan). MS was performed on an Autospec-3000 spectrometer (VG, Manchester, England). The nuclear magnetic resonance (NMR) spectra were recorded on DRX-500 NMR (Bruker, Karlsruhe, Germany) spectrometers, with TMS as an internal standard and coupling constants were represented in Hertz. Silica gel (200-300 mesh) for column chromatography was produced by Qingdao Marine Chemical Factory, Qingdao, China. Sephadex LH-20 for column chromatography was purchased from Pharmacia. Thin layer chromatography (TLC) was performed on silica gel (Si gel G; Qingdao Marine Chemical Factory), and the spots were visualised under 5% vitriol ethanol solution. Solvents were of the industrial purity and distilled prior to use.

Fungi, media, and culture conditions. A total of 30 fungal strains were initially isolated from a submerged woody substrate collected from a freshwater habitat in Yunnan Province, China, and have been deposited in the Laboratory for Conservation and Utilization of Bio-resources, Yunnan University, Yunnan Province, the People's Republic of China (Table 1). All cultures were maintained on Potato Dextrose agar slants at 4 °C and transferred once every 6 months.

All 30 strains were cultured in Potato Dextrose broth (PDB: potato 200 g, glucose 20 g, and water 1000 mL) or GPC broth (glucose 20 g, peptone 2.5 g, K_2HPO_4 2 g, KCl 1 g, $MgSO_4{\cdot}7H_2O$ 0.1 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, VB_1 6 mg, VB_6 2 mg, VB_2 2 mg, Ca $(C_9H_{16}NO_5)_2$ 3 mg, water 1000 mL, pH 7.0). Erlenmeyer flasks (250 mL) containing 70 mL of liquid medium were each inoculated with a 1 cm^2 block from a 2-week-old fungal colony growing on potato dextrose agar. These flasks were cultured on a rotary shaker (200 rpm) at 25 °C. After 24 and 48 h of incubation on different media, the cultures were firstly filtered through sterilised Whatman no. 2 filter paper which removes mycelium and secondly through 0.22 µm Millipore filter avoiding spore contamination in the culture filtrate obtained. This procedure remained identical in all the experiments. Each of the filtrates thus collected were served as stock solutions for screening antinematodal activity against *B. xylophilus*.

The fungal strain *Caryospora callicarpa* YMF1.01026 was selected for further scale-up fermentation grown in shake cultures on GPC medium in 200-mL batches in 500-mL flasks for 15 days at 25 °C, extraction with EtOAc, fractionation and structural elucidation of its bioactive secondary metabolites.

Extraction and isolation of secondary metabolites of *Caryospora callicarpa* YMF1.01026. A total of 10 L of cultural filtrate of *Caryospora callicarpa* YMF1.01026 grown in GPC broth was filtered, first through muslin (10 µm) and then a pad of Celite (an inert support) on a filter funnel. The filtrate was extracted with EtOAc three times and dried over anhydrous sodium sulphate, filtered and the solvent removed under reduced pressure. The residue (600 mg), possessing strong antinematodal activity against *B. xylophilus in vitro* screening, was chromatographed on a Sephadex LH-20 gel column using CH_3OH as eluent solution and four fractions were collected in order of elution by TLC (Silica gel G, 0.25-mm film thickness). Fraction 1 (210 mg) was further separated through a silica gel column [1.5 g Silica gel G (200~300 mesh), 0.7 cm i.d. x 15 cm; $CHCl_3$/CH_3OH 9.95:0.05 to 9.50:0.50], yielding pure compound **3** (4.5 mg) and compound **4** (3.5 mg) as yellow powders, respectively. Fraction 3 (150 mg) was subjected to a silica gel column [1.0 g Silica gel G (200~300 mesh), 0.7 cm i.d. x 10 cm; $CHCl_3$/EtOAc 7.0:3.0], yielding compound **2** (6 mg) as a red powder. Fraction 4 (100 mg) was eluted with $CHCl_3$/CH_3OH 9.7:0.3 on a silica gel column [40 g Silica gel G (200~300 mesh), 0.5 cm i.d. x 8 cm; $CHCl_3$/CH_3OH 9.7:0.3], furnishing compound **1** (3.5 mg) as a colourless powder.

Culture and preparation of nematodes. The test nematodes *B. xylophilus* were cultured and prepared as described in the literature (Mackeen *et al*., 1997).

Assay of antinematodal activity.

Antinematodal activity of fungal filtrates. The antinematodal bioassay for the activity of fungal filtrates was conducted as described in the literature (Dong *et al*., 2004, 2006a).

Antinematodal activity of compounds. The antinematodal bioassay for the activity of compounds **1**-**4** was conducted as described in the literature (Anke *et al*., 1995). Compounds **1**-**4** (3 mg each) were weighed separately and dissolved in DMSO (0.35 mL) and the volume was made up to 5 mL by 0.5 emulsified water (5 mL of Tween 80 in 1 L of distilled water) to get a stock solution of 600 ppm. Test solutions of 400, 300, 200, 100 ppm were prepared by serial dilutions of the stock solutions with 0.5 emulsified water. The antinematodal bioassay of compounds **1**-**4** was conducted in Corning polystyrene 96-well plates as described in the literature (Anke *et al*., 1995). The nematodes were added to 2 mL of physiological saline in a scintillation vial. This was diluted until the nematode counts were about 300 nematodes in a 0.5 mL aliquot. A solution (0.5 mL) containing nematodes was delivered to each of three wells per treatment. To 0.5 mL of this nematode suspension in Petri plates, an equal volume of the test solutions was added separately to obtain the desired test concentrations of 200, 150, 100, 50 ppm, respectively. Control solution was 0.5% DMSO in emulsified water. The treated and control plates were covered with parafilm, and kept in a humid chamber. Dead and

TABLE 1 - Effects after different exposure periods of cultural filtrates from aquatic fungi, grown on two culture media, on the mortality of *Bursaphelenchus xylophilus in vitro* (mean of 4 replicates)

Fungi	Per cent mortality after different exposure periods			
	PDB		GPC	
	24 h	48 h	24 h	48 h
Camposporium quercilola YMF1.01300	47.22	75.16	53.60	74.16
Caryospora callicarpa YMF1.01026	53.18	72.46	73.51	90.23
Caryospora minima YMF1.02118	21.71	37.26	18.70	25.93
Dactylella intermedia YMF1.00589	20.41	34.69	17.25	30.09
Dactylella leptospora YMF1.01832	7.68	15.97	8.54	13.98
Dactylella oxyspora YMF1.01000	4.95	13.66	12.11	17.93
Dictyosporium beptasporum YMF1.01216	10.83	26.78	9.27	19.96
Dictyosporium cocophilum YMF1.01123	15.38	23.47	11.45	20.31
Dactyosporium heptasporum YMF1.01231	13.64	30.57	25.35	34.21
Massarina bipolaris YMF1.01191	25.48	32.08	17.29	28.44
Massarina fronsisubmersa YMF1.01028	8.97	15.73	13.62	19.16
Massarina peerallyi YMF1.01144	10.87	29.64	12.91	22.04
Melanospora zamiae YMF1.00965	48.61	68.34	37.11	47.82
Monacrosporium longiphorum YMF1.01402	11.74	19.49	9.71	17.06
Monacrosporium microscaphoides YMF1.01453	3.33	10.31	4.76	13.74
Monacrosporium psychrophilum YMF1.01819	15.16	34.56	21.45	23.92
Monacrosporium schlerophyum YMF1.01401	10.60	22.81	17.04	28.37
Ophioceras commune YMF1.00982	15.61	30.42	16.38	27.83
Ophioceras dodichostomum YMF1.00988	14.76	23.24	19.51	33.72
Periconia digitata YMF1.00948	59.32	82.94	44.31	70.56
Periconia minutissma YMF1.00955	25.60	31.93	18.47	29.61
Phaeoisaria clematidis YMF1.01165	11.06	24.34	8.67	19.22
Pseudohalonectria lignicola YMF1.01213	7.71	12.39	13.69	20.31
Pseudohalonectria fuxianii YMF1.01207	15.43	27.86	16.67	25.32
Savorylla lignicola YMF1.01094	13.40	21.89	15.26	24.43
Saccardoella minuta YMF1.00961	6.53	10.08	9.83	15.17
Tiarosporella paludosa YMF1.01051	21.76	41.33	17.81	27.62
Torula graminis YMF1.001053	14.75	28.16	10.41	19.63
Torula herbarum YMF1.01021	15.06	25.71	17.52	30.67
Xylaria migricans YMF1.01010	5.63	10.11	10.85	22.38
Xylomycetes chlamydosporis YMF1.00956	17.38	30.74	20.41	33.05
Control (broth alone)	0.00	1.21	0.00	1.44
Control (water alone)	0.00	2.33	0.00	2.11

active nematodes were counted after incubation at 25 °C for 12, 24 and 36 h. Nematodes were defined as dead if their body was straight and they did not move, even after mechanical prodding, and the toxicity was estimated based on the percentage of dead nematodes. Each treatment was replicated three times and the mean percentage mortality was calculated.

Statistical analysis. To describe the nematicidal effects of the compounds against *B. xylophilus,* the median lethal concentrations (LC_{50}) were calculated according to probit analysis (Sporleder *et al*., 2005). Regression analyses were also carried out by SPSS for linear model. Data on proof mortality of nematodes were transformed into probit values, and the concentrations (*C*) of compounds were also changed to $\log_{10}$ (*C*) before analysis.

To evaluate the antinematodal activities of the compounds under different exposure times of 12, 24 and 36 h, and different concentrations of 200, 150, 100 and 50 mg L^{-1}, data were subjected to independent sample *F*-test using ANALYZE (SPSS/ version 11.0 software; SPSS, Chicago, IL, USA). Data on proof mortality were changed to sin1/2 (M) before analysis.

RESULTS AND DISCUSSION

Screening of fresh water fungi for antinematodal metabolites

Results of the antinematodal activity of the crude fungal filtrates against *B. xylophilus* are shown in Table 1. As can be see from the Table 1, the antinematodal activity differs significantly among the 30 fungi selected by length of exposure time and media composition. Out of 30 fungal strains only the filtrates of *Camposporium quercicola* YMF1.01300*, Periconia digitata* YMF1.00948, *Caryospora callicarpa* YMF1.01026 grown in both PDB and GPC broth, and the cultural filtrate of *Melanospora zamiae* YMF1.00948 grown in PDB were found to immobilise over 50% of nematodes following 48 h exposure, which accounted for 11.67% of all culture filtrates. During the 48 h exposure, a maximum mortality of 90.23% of individuals was recorded using isolates of *Caryospora callicarpa* YMF1.01026 grown in GPC broth, followed by *Periconia digitata* YMF1.00948 (82.94%) and *Camposporium quercicola* YMF1.01300 (75.16%), both grown in PDB broth. It was observed that the antinematodal activity of fungi enhanced with the exposure time (Table 1). After 24 h exposure the mortality of these fungi grown in PDB broth varied from 3.33 to 59.32%, and the maximum mortality of 59.32% was recorded in *Periconia digitata* YMF1.00948 isolate while the minimum mortality of 3.33% in *Monacrosporium microscaphoides* YMF1.01453 isolate. After 48 h exposure, the mortality of *Periconia digitata* YMF1.00948 was up to 82.94% and that of *Monacrosporium microscaphoides* YMF1.01453 was also up to 10.31%. There was an increase in mortality after 48 h exposure and significantly differed from control. Additionally, it was also observed that the nematicidal activity of fungi were dependent from the nutrient component of the culture medium selected. With PDB medium, the maximum mortality of 82.94% was recorded in *Periconia digitata* YMF1.00948 isolate, followed by *Camposporium querci-*

TABLE 2 - The effects of metabolites **1**-**4** of *Caryospora callicarpa* YMF1.01026 on the proof mortality of *Bursaphelenchus xylophilus* using *in vitro* tests

Compound	Exposure time (h)	Mortality (%)		LC_{50} (mg L^{-1})
		200 mg L^{-1}	150 mg L^{-1}	
	12	18.9	16.2	540.2
1	24	38.5	30.4	436.6
	36	52.7	47.6	209.7
	12	16.7	13.4	1169.8
2	24	36.7	31.2	461.3
	36	51.6	45.8	229.6
	12	16.9	14.5	1011.6
3	24	37.2	28.6	522.5
	36	52.1	45.8	220.3
	12	17.1	14.3	854.9
4	24	37.4	29.8	468.4
	36	53.1	46.7	206.1

cola YMF1.01300 (75.16%), *Caryospora callicarpa* YMF1.01026 (72.46) and *Melanospora zamiae* YMF1.00948 (68.34%) after 48 h exposure. When these fungi were grown in GPC broth, the mortality of *Periconia digitata* YMF1.00948 against *B. xylophilus* decreased from 82.94 to 70.56% while that of *Caryospora callicarpa* YMF1.01026 increased from 72.46 to 90.23% after 48 h exposure.

This is the first report on the nematicidal activity of *Camposporium quercicola* YMF1.01300, *Periconia digitata* YMF1.00948, *Melanospora zamiae* YMF1.00948. Previously, we have already reported the antinematodal activity of *Caryospora callicarpa* YMF1.01026 against *B. xylophilus* and identified three nematicidal resorcylic macrolides, caryospomycins A~C, from a bioactive cultural extracts of *Caryospora callicarpa* YMF1.01026 grown on wheat (Dong *et al*., 2007). However, the antinematodal substances produced by *Caryospora callicarpa* YMF1.01026 grown on GPC broth obviously was not due to the caryospomycins, as these substances appeared blue or black spots on TLC when visualised by spraying with 5% (v/v) H_2SO_4 in ethanol, not purple spots what would be expected with caryospomycins. Thus, we have been especially interested in the active components of *Caryospora callicarpa* YMF1.01026 grown on GPC broth.

Identification structures of compounds 1-4 of *Caryospora callicarpa* YMF1.01026

Compound **1** was isolated as a colourless powder (CH_3OH): [α]±0° (CH_3OH; *c*0.44); FABMS *m/z* (rel. int) 363 $[M+2 glycerol+1]^+$ (75), 271 $[M+glycerol+1]^+$ (15), 179 $[M +1]^+$ (100); ^1H NMR (CD_3COCD_3, 500MHz, δ) 12.33 (1H, s, OH-8), 7.42 (1H, t, *J* = 7.95 Hz, H-6), 7.00 (1H, d, *J* = 7.45 Hz, H-5), 6.71 (1H, d, *J* = 8.20 Hz, H-7), 4.78 and 4.61 (1H, m, H-4), 2.78 (1H, dt, H-2β, *J* = 18.2, 9.9, 4.8 Hz), 2.62 (1H, m, H-2α), 2.20 (1H, m, H-3β), 2.00 (1H, m, H-3α); ^{13}C NMR (CD_3COCD_3, 500MHz, δ) 206.0 (s, C-1), 163.7 (s, C-8), 149.5 (s, C-4a), 137.9 (s, C-6), 118.7 (d, C-5), 117.5 (d, C-7), 116.0 (s, C-1a), 68.2 (d, C-4), 36.3 (t, C-2), 33.0 (t, C-3). These data agree with those reported previously (Morita and Aoki, 1974; Liu *et al*., 2007).

Compound **2** was isolated as a red powder (CH_3OH): [α]-29.51° (CH_3OH; *c* 0.38); EIMS *m/z* (rel. int) 178 $[M]^+$ (8), 160 (100), 132 (25), 131 (78), 121 (7), 103 (18), 77 (13); ^1H NMR ($CDCl_3$, 400MHz, δ) 7.59 (1H, d, H-8, *J* = 7.75 Hz), 7.32 (1H, t, H-7, *J* = 7.85 Hz), 7.12 (1H, d, H-6, *J* = 7.75 Hz), 5.37 (1H, w, H-4, *J* = 10.1 Hz), 2.84 (1H, dt, H-2β, *J* = 18.2, 9.9, 4.8 Hz), 2.61 (1H, dt, H-2α, *J* = 18.0, 9.7, 4.7 Hz), 2.56 (1H, m, H-3β), 2.23 (1H, m H-3α); ^{13}C NMR ($CDCl_3$, 400MHz, δ) 196.5 (s, C-1), 156.0 (s, C-5), 132.3 (s, C-1a), 129.5 (d, C-7), 128.5 (s, C-4a), 122.4 (d, C-8), 119.2 (d, C-6), 68.5 (d, C-4), 36.0 (t, C-2), 32.4 (t, C-3). These data agree with those reported previously (Suzuki *et al*., 1968).

Compound **3** was isolated as a yellow powder (CH_3OH): [α]-53.78° (CH_3OH; *c*0.44); EIMS *m/z* (rel. int) 194 $[M]^+$ (100), 176 (4), 167 (15), 166 (38), 149 (46), 138 (55), 137 (100), 69 (6); ^1H NMR (CD_3OD, 400MHz, δ) 12.87 (1H, s, O**H**-8), 6.54 (1H, s, H-5), 6.17 (1H, s, H-7), 4.73 (1H, w, H-4, *J* = 10.1 Hz), 2.80 (1H, dt, H-2β, *J* = 18.2, 9.9, 4.8 Hz), 2.63 (1H, m H-2α), 2.26 (1H, m, H-3β), 2.04 (1H, m H-3α); ^{13}C NMR (CD_3OD, 400MHz, δ) 203.7 (s, C-1), 166.8 (s, C-6), 166.7 (s, C-8), 151.0 (s, C-4a), 110.2 (s, C-1a), 107.4 (d, C-5), 102.5 (d, C-7), 68.5 (d, C-4), 32.7 (t, C-3), 35.7 (t, C-2). These data agree with those reported previously (Iwasaki *et al*., 1973; Venkatasubbaiah and Chilton, 1992).

Compound **4** was isolated as a yellow powder (CH_3OH): [α]-62.37° (CH_3OH; *c*0.41); EIMS *m/z* (rel. int) 210 $[M]^+$ (56), 192 (14), 166 (29), 147 (43), 138 (50), 137 (100), 73 (25); ^1H NMR (CD_3COCD_3, 500MHz, δ) 12.75 (1H, s, O**H**-8), 6.71 (1H, s, H-5), 6.22 (1H, s, H-7), 4.55 (1H, d, *J* = 7.75Hz, H-4), 4.02 (1H, m, H-3), 2.91 (1H, m, H-2β), 2.67 (1H, m, H-2α); ^{13}C NMR (CD_3COCD_3, 500MHz, δ) 201.7 (s, C-1), 166,5 (2 × C, s, C-6 and C-8), 149.2 (s, C-4a), 110.8 (s, C-1a), 108.0 (d, C-5), 102.5 (d, C-7), 74.2 (d, C-4), 72.0 (d, C-3), 44.9 (t, C-2); COSY ^1H-^1H (CD_3COCD_3, 500MHz, δ) 6.71/4.55, 6.71/6.22, 4.55/4.02, 4.02/2.91, 4.02/2.67, 2.91/2.67; NOESY ^1H-^1H (CD_3COCD_3, 500MHz, δ) 6.71/4.55, 4.55/4.02, 4.55/2.91, 4.02/2.67, 4.02/2.91, 2.91/2.67. These data agree with those reported previously (Iwasaki *et al*., 1973; Gremaud and Tabacchi, 1996).

Antinematodal activity of metabolites 1-4 of *Caryospora callicarpa* YMF1.01026

The antinematodal activities of metabolites **1-4** against *B. xylophilus* were assayed as described above and the nematicidal activity varied according to metabolites, exposure time, and dose. As shown in Table 2, the order of activity of naphthalenes **1**-**4** was 3,4,6,8-tetrahydroxy-3,4-dihydronaphthalen-1(2*H*-one (**4**) > 4,8-dihydroxy-3,4-dihydronaphthalen-1(2*H*)-one (**1**) > 4,6,8-trihydroxy-3,4-dihydronaphthalen-1(2*H*)-one) (**3**) > 4,5-dihydroxy- 3,4-dihydronaphthalen-1(2*H*)-one (**2**). Although their performance was still poor, it was interesting to

note that compound **4** substituted with 3-OH was more active than the other compounds, and compounds **2** and **3**, both containing 6-OH, showed a tendency to reduce activity. Likewise, we observed that the nematicidal activity of metabolites **1**-**4** enhanced significantly with the length of exposure times at the same concentration and they showed higher antinematodal activity against *B. xylophilus* in evaluations at 36 h than at 12 and 24 h exposure. The fact suggested that the mode of action of these substances was systemic, rather than contact poisons or anti-feedants (Ferris and Zheng, 1999). Additionally, we also observed that probit value of proof mortality showed a linear increase with increasing $\log_{10}$ (C) and the LC_{50} values of metabolites **1**-**4** against *B. xylophilus* were 209.7, 229.6, 220.3, and 206.1 mg L^{-1}, at 36 h exposure, respectively.

The suppressive effects of some fungal phytochemical metabolites on nematode populations have been well documented in several pathosystems (Anke and Sterner, 1997). These compounds could be developed for use as nematicides themselves, or could serve as model compounds for the development of environmentally friendly synthetic derivatives. The present study has already found four nematicidal metabolites from the culture filtrates of *Caryospora callicarpa* YMF1.01026 grown in GPC broth and they were 4,8-dihydroxy-3,4-dihydronaphthalen-1(2*H*)-one (**1**), 4,5-dihydroxy-3,4-dihydronaphthalen-1(2*H*)-one (**2**), 4,6,8-trihydroxy-3,4-dihydronaphthalen-1(2*H*)-one) (**3**), 3,4,6,8-tetrahydroxy-3,4-dihydronaphthalen-1(2*H*)-one (**4**), which were completely different from our previous obtained nematicidal metabolites found in the same fungus (Dong *et al*., 2007). Much of this inconsistency has been ascribed to differences in selected cultural mediums and cultural conditions. This indicated the production of bioactive metabolites was greatly influenced by the culture media and culture conditions. That is in agreement with some other papers according to which the synthesis of active metabolites in fermentative cultures by fungi is directly related to the growth and bioactivity of fungi depending on the nutrient components of the culture media selected (Smith and Moss, 1985; Cayrol *et al*., 1989; Anke *et al*., 1995; Chen *et al*., 2000; Siddiqui and Shaukat *et al*., 2002). Hence it appears that the media necessary for bioactive metabolites production vary from one fungus to the other. Consequently when the ability of fungi to produce any bioactive metabolites is studied, it is absolutely necessary to test several parameters. Also to establish the identity of bioactive metabolites in the near future, it is absolutely necessary to define a well-known synthetic media, which gives optimal results. Likewise, the bioactive properties of the fungus were greatly influenced by the culture conditions. Thus, modification of fermentation parameters has already been proved to be a valuable tool to increase the number of novel bioactive metabolites (Anke *et al*., 1995). The knowledge of the biosynthetic pathways, the understanding of their regulation, and the ecological functions of fungal secondary metabolism may greatly help make use of this rich natural resource.

Structurally, compounds **1**-**4** belong to the known naphthalenone antibiotics and are biosynthetically related. Previously, they have been isolated mainly from plant pathogenic fungi, *Cytospora eucalypticola* (Kokubun *et al*., 2003), *Ceratocystis fimbriata* (Gremaud and Tabacchi, 1996), *Discula* sp. (Venkatasubbaiah and Chilton, 1991), *Sclerotinia sclerotiorum* (Suzuki *et al*., 1968; Morita and Aoki, 1974), *Pyrucularia oryzae* (Iwasaki *et al*., 1973), *Tubakia dryina* (Venkatasubbaiah and Chilton, 1992), and from *Juglans* plant materials (Talapatra *et al*., 1988; Fufimoto *et al*., 1998; Ayer *et al*., 2000; Li *et al*., 2003; Koichi *et al*., 2005; Liu *et al*., 2007) etc., and showed phytotoxic activity (Findlay and Kwan, 1973; Morita and Aoki, 1974; Venkatasubbaiah and Chilton, 1991, 1992) and interesting plant hormone type activity (Suzuki *et al*., 1968). But, to the best of our knowledge, the present study is the first to show nematicidal activities of naphthalenones. To obtain additional information on the structural factors needed for the potent activity, further studies are required to examine the capacities of this fungus to biosynthetic these metabolites and to obtain enough quantities of them and their analogues for evaluating nematicidal and other biological activities.

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