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Nematicidal Activity and Chemical Component of *Poria cocos*

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***Poria cocos*, a famous traditional Chinese medicine, was found to have nematicidal activity in experiments searching for nematicidal fungi. The experiment showed it could kill 94.9% of the saprophytic nematode, *Panagrellus redivivue*, 92.6% of the root-knot nematode, *Meloidogyne arenaria*, and 93.5% of the pine nematode, *Bursaphelenchus xylophilus*, on PDA plate within 12 hours. According to the nematicidal activity, three new compounds, 2, 4, 6-triacetylenic octane diacid, 2, 4, 5, 6-tetrahydroxyhexanoic acid and 3, 4-dihydroxy-2-keto-*n*-butyl 2,4,5,6-tetrahydroxyhexanate, were isolated from submerged cultures of *Poria cocos*. Of these, 2, 4, 6-triacetylenic octane diacid could kill 83.9% *Meloidogyne arenaria* and 73.4% *Panagrellus redivivus* at 500 ppm within 12 hours. Here, it is reported for the first time that *Poria cocos* has nematicidal activity.**

Key words: *Poria cocos*, nematicidal activity, nematicidal fungi, structural analysis

Plant parasite nematodes have inflicted serious damage on agricultural crops and plants. Biocontrol has drawn great interest from researchers in the prevention of nematodes due to the environmental pollution problems induced by chemical insecticides. Nematophagous fungi, which are infectious to nematodes, have become a major source for the control of nematodes (Tunlid *et al.*, 1999). Among the nematophagous fungi, some are able to kill nematodes with their mycelium and others by their metabolites. Due to the effects of biotic and abiotic factors of soil toward the mycelium of microbes it is more feasible to search for metabolites from microbes for the control of nematode than to use the mycelium. Some nematicidal metabolites, isolated from higher fungi, have been reported (Anke and Sterner, 1997).

Poria cocos, named Fuling in China, is a very old and widely used herb, especially in Chinese medicine. It is traditionally used as a tonic to benefit the internal organs, and is considered highly nourishing. The main chemical constituents of *Poria cocos* include: polysaccharide (β-pachyman), several organic acids, such as tumulosic acid, eubricoic acid, pinicolic acid and pachymic acid, proteins, fats, lecithin, sterols and gum, etc. (Chang and Ding, 2002). In the experiments screening and searching for nematicidal metabolites, *Poria cocos* exhibited strong activity toward three tested nematodes, so the active components in submerged cultures of *Poria cocos* were isolated according to the nematicidal activity, and the nematicidal activity of the isolated compounds tested.

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Materials and Methods

General experimental instruments and materials

The infrared (IR) spectra were measured on a Perkin-Elmer-577 spectrophotometer (USA) and the ultraviolet (UV) spectra on a Shimadzu double-beam 210A spectrophotometer (Japan). The nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX-500 spectrometer (500 MHz, 300 K). Mass spectrometry (MS) was performed on a VG Auto Spec-3000 spectrometer, and Finnigan Trace DSQ. Thin-Layer Chromatography (TLC) was performed on plates precoated with Si gel (Qingdao Marine Chemical Ltd., People's Republic of China). The reverse-phase (RP) C_{18} silica gel for the column chromatography was obtained from Merck (USA) and the Sephadex LH-20 from GE Wealthcare Bioscience (USA). All solvents were distilled before use, and the NMR solvent was deuterated CD_3OD.

Culture and fermentation of P. cocos

The strain of *P. cocos* was purchased from Sanming Institute of Mycology, Fujian Province, People's Republic of China, and stored in the Laboratory for Conservation and Utilization of Bio-resources, Yunnan University, Yunnan Province, People's Republic of China. The strain was stored on PDA medium (potato 200 g, sucrose 20 g, agar 18 g, and water 1000 mL) and fermented with fluid Sab-

ouraud's medium (peptone 10 g, glucose 40 g and water 1000 ml) at 28°C, with shaking at 120 rpm, for 7 days.

Culture of nematodes

Panagrellus redivivus: The saprophytic nematode was cultured on oatmeal medium (oatmeal: 20 g, water 80 ml) at 25°C for 7 days, and then refrigerated prior to use.

Bursaphelenchus xylophilus: *Botrytis cinerea* was cultured on a PDA plate at 25°C, and when the fungus was fully grown the plate was inoculated with the pine nematode, and then cultured until the fungal mycelia had been completely consumed.

The two cultured nematodes were separated from the culture medium using the Baerman funnel technique (Gray, 1984), and an aqueous suspension of the nematode prepared for use as a working stock.

Meloidogyne arenaria: The root-knot nematode was cultured on tomatoes under greenhouse conditions, and second stage juveniles extracted and stored, according to the methods of Kerry (Kerry and Bourne, 2002).

Assay of the nematicidal activity: The assay method was based on those described in the literature (Thorn and Barron, 1984; Barron and Thorn, 1987; Kwok *et al.*, 1992).

The nematicidal activity of fungal mycelium

P. cocos was inoculated onto the PDA plate and the nematodes were added when the mycelium had overgrown the plate, then the numbers of active and inactive nematodes counted under a dissection scope at different times (30 min, and 4, 12 and 24 h). Nematodes were considered dead if they showed no response to physical stimuli, and the toxicity was estimated based on the percentage of dead nematodes. A plate containing just medium was used as a control, and each treatment replicated three times, and the proofreading death rate (%) then calculated.

The nematicidal activities of each compound: Each compound was diluted to different concentrations (1000, 500, 100 and 50 ppm) with sterile water to assay for the nematicidal activity. Dilution was performed in an Eppendorf tube, with sterile water used as a control. The numbers of active and inactive nematodes were counted at different times, consistent with the method described earlier.

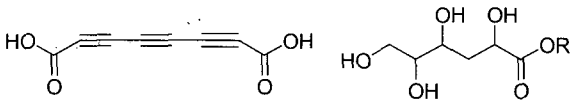
Extraction and isolation

The liquid of the fermentation broth (2 L) of *P. cocos* was removed under vacuum and extracted with methanol exhaustively, the solvent was then removed and 15g of residue was obtained. The residues were subjected to chromatography on a MPLC RP-18 (125 g, 40-63 μm) column, eluted with distilled water (20 ml/min) and the eluents were combined according to the nematicidal activity. The active fraction was again subjected to chromatography on a Sephadex LH-20 (120 g) column, using methanol as the eluent, and the eluents were combined according to the nematicidal activity and TLC ($CHCl_3$: MeOH = 3:2) results obtained during the process; three compounds, including **1** (28 mg), **2** (8 mg) and **3** (5 mg), were obtained.

Results and Discussion

Identification of structures of compounds

Compound **1** was obtained as colorless crystals, $[\alpha]_D^{26}$ 0 (*c*, 0.5, MeOH), and the molecular formula determined to be $C_8H_2O_4$ (*m/z*160.9870 [M - H], calcd. 160.9874) by the negative HRESIMS. The UV spectra showed an absorbance (MeOH) at λ_{max} (log ε) 202 (3.023). The IR (KBr) spectra of **1** revealed the presence of carbonyl stretching



1

2 R=H

3 R=3',4'-dihydroxy-2'-keto-n-butyl

Fig. 1. The structures of compounds **1, 2** and **3**.

(1754 cm^{-1}, s), hydroxyl stretching (3424 cm^{-1}), CO (1274 cm^{-1}) and an alkyne bond (1986 cm^{-1}).

The ^{1}H-NMR of Compound **1** had no proton signal. The ^{13}C-NMR data showed 8 signals, including 2 carbonyl (δ 161.0 and 160.4) and six quaternary carbons (δ 79.3, 78.7, 67.7, 67.2, 64.0 and 63.7) belonging to the carbon of an alkyne type compound. The six carbon chemical shifts were changed to high field, as the alkyne bonds combined to the conjugated system; in the same manner, the two carbonyl chemical shifts were also changed. According to Allan *et al.* (1980), the carbonyl was conjugated with alkenyl, and the carbonyl chemical shift changed to high field (δ 164.0). From the IR spectra, the three alkyne bonds were a conjugated system, which was also conjugated with the carbonyl; therefore, the absorption of the carbonyl changed to low field (1754 cm^{-1}, s), and the absorptions of the alkyne bonds then changed (1986 cm^{-1}). Therefore, compound **1** was determined to be 2, 4, 6-triacetylenic octane diacid (Fig. 1).

Compound **2** was obtained as a colorless powder, $[\alpha]_D^{26}$ 0 (*c*, 0.5, MeOH), FAB-MS *m/z*: 179 [M - H]. From the

Table 1. The NMR data of compound 2 in CD_3OD

Position	^{13}C	^{1}H	HMBC
1	179.0	/	/
2	69.2	4.56, dt, 2.2, 8.6	33.0(w), 179.0
3	33.0	2.56, m 2.16, m	69.2, 179.0 69.2, 73.0, 77.8
4	77.8	4.48, m	63.6
5	73.0	3.83, dd, 5.3, 5.3	33.0, 63.6, 77.8,
6	63.6	3.57, d, 5.1	73.0, 77.8

NMR (Table 1) and MS data, the molecular formula of compound **2** was determined to be $C_6H_{12}O_6$. The ^{13}C-NMR showed compound **2** to have six carbon signals, including two methylenes (δ 33.0, δ 63.6), three methenyl (δ 69.2, δ 77.8, δ 73.0) and one carbonyl. Their chemical shifts showed that one methylene and the three methenyls were connected to an oxygen atom.

The HMBC experiments showed the ^1H-^{13}C long-range correlations between the methenyl proton at δ 4.56 (H-2) and the carbons at δ 179.0 (C-1) and δ 33.0 (C-3), between the methylene protons at δ 2.56 (H-3a) and the carbons at δ 69.2 (C-2) and δ 179.0 (C-1), between the methylene protons at δ 2.16 (H-3b) and the carbons at δ 69.2 (C-2), δ 77.8 (C-4) and δ 73.0 (C-5), between the methenyl proton at δ 4.48 (H-4) and the carbon at δ 63.6 (C-6), the methenyl proton at δ 3.83 (H-5) and the carbons at δ 69.2 (C-2), δ 77.8 (C-4) and δ 63.6 (C-6) and between the methylene protons at δ 3.57 (H-6) and the carbons at δ 73.0 (C-5) and δ 77.8 (C-4). The ^1H-^1H COSY experiments showed, correlations between the H-2, H-3a and H-3b, between the H-3a, H-3b and H-2, between the H-4, H-3a and H-3b, between the H-5, H-4 and H-6 and between the H-6 and H-5 protons. Therefore, the structure of compound **2** was determined to be 2, 4, 5, 6-tetrhydroxyhexanoic acid (Fig. 1).

Compound **3** was obtained as a colorless powder, $[\alpha]_D^{26}$ 0 (*c*, 0.5, MeOH), FAB-MS *m/z*: 281 [M - H]. From the NMR (Table 2) and MS data, the molecular formula of compound **3** was determined to be $C_{10}H_{18}O_9$. The ^{13}C-NMR data showed compound **3** to have ten signals, including three methylenes, four methenyl and two quaternary carbons. The ^{13}C-NMR spectra indicated the presence of two carbonyls (δ 179.0 and δ 213); therefore, **3** was considered as an acid or ester type compound. The HMBC experiments showed ^1H-^{13}C long-range correlations between the methenyl proton at δ 4.56 (H-2) and the carbons at δ 179.0 (C-1) and δ 33.0 (C-3), the methylene protons at δ 2.56 (H-3a) and the carbons at δ 69.2 (C-2) and δ 179.0 (C-1), the methylene protons at δ 2.16 (H-3b) and the carbons at δ 69.2 (C-2), δ 77.8 (C-4) and δ 73.0 (C-5), the methenyl proton at δ 4.48 (H-4) and the carbon at δ 63.6 (C-6), the methenyl proton at δ 3.83 (H-5) and the carbons at δ 69.2 (C-2), δ 77.8 (C-4) and δ 63.6 (C-6) and between the methylene protons at δ 3.57 (H-6) and the carbons at δ 73.0 (C-5) and δ 77.8 (C-4). The ^1H-^1H COSY experiments showed correlations between the H-2, H-3a and H-3b, the H-3a, H-3b and H-2, the H-3b, H-3a and H-2, the H-4, H-3a and H-3b, the H-5, H-4 and H-6 and between the H-6 and H-5 protons. Furthermore, the HMBC experiments showed ^1H-^{13}C long-range correlations between the methenyl proton at δ 4.56 (H-2) and the carbons at δ 67.6 (C-1) and δ 213.0 (C-2), the methylene protons at δ 4.48 (H-1) and the carbon at δ 213.0 (C-2), the methenyl proton at δ 77.8 (H-3) and the carbons at δ 213.0 (C-2) and δ 64.8 (C-4) and between the methylene protons at δ 3.78 (H-4) and the carbons at δ 213.0 (C-2) and δ 77.8 (C-3). Therefore, the structure of **3** was determined to be 3,4-dihydroxy-2-keto-*n*-butyl 2, 4, 5, 6-tetrahydroxyhexanate (Fig. 1).

Table 2. The NMR data of compound 3 in CD_3OD

Position	^{13}C	^1H	HMBC
1	179.0	/	/
2	69.2	4.56,dt, 2.2, 8.6	33.0(w), 67.6(w), 179.0, 213(w)
3	33.0	2.56, m 2.16, m	69.2, 179.0 69.2, 73.0, 77.8
4	77.8	4.48, m	63.6
5	73.0	3.83, dd, 5.3, 5.3	33.0, 63.6, 77.8,
6	63.6	3.57, d, 5.1	73.0, 77.8
1	67.6	4.48, m	213.0
2	213.0	/	/
3	77.8	4.23, t, 4.2	64.8, 213
4	64.8	3.78, m	77.8, 213

Table 3. The proofreading death rate (%) of the mycelium of *P. cocos* toward the nematodes on the PDA plate

Incubation time (h)	*P. redivivus*	*M. arenaria*	*B. xylophilus*
0.5	16.0	11.2	12.8
4	70.4	67.3	60.4
12	94.9	92.6	93.5
24	94.9	92.6	96.4

The nematicidal activity

The mycelium of *P. cocos* was able to kill 94.9% of the *P. redivivue*, 92.6% of the *M. arenaria* and 93.5% of the *B. xylophilus* on the PDA plate within 12 h (Table 3). Compound **1** was also able to kill 83.9% of the *M.*

Table 4. The proofreading death rates (%) of compound 1 toward *P. redivivus* and *M. arenaria*

Incubation time (h) \ Concentration (ppm)	*P. redivivus*				*M. arenaria*			
	1000	500	100	50	1000	500	100	50
4	38.3	35.0	13.5	6.4	50.5	48.6	16.7	14.8
8	67.7	68.3	19.9	6.5	90.8	81.0	18.9	24.3
12	84.2	73.4	27.5	24.5	92.0	83.9	25.3	23.3
24	90.4	86.4	46.4	36.8	92.0	90.9	27.9	26.0

arenaria and 73.4% of the *P. redivivus* at 500 ppm within 12 h (Table 4), but compound **1** exhibited no nematicidal activity against *B. xylophilus* at 1000 ppm. Compounds **2** and **3** showed no obvious activity to the three nematodes at 1000 ppm.

This is the first report on the nematicidal activity of *Poria cocos*. Three new compounds were also isolated according to their nematicidal activities. Our results implied that searching for new compounds by screening with different models was an efficient sourcing method. The presence of alkyne carbons may play an important role in the nematicidal activity of **1**, as many natural acetylene compounds are known to be nematicidal (Kimura *et al.*, 1981; Mori *et al.*, 1982). In the primary screening, the mycelium of *Poria cocos* on the plate showed strong nematicidal activity toward the three nematodes, but only one of the three isolated compounds had activity toward the nematodes *Panagrellus redivivus* and *Meloidogyne arenaria*, but no nematicidal activity toward *Bursaphelenchus xylophilus*; therefore, maybe different active compounds exist against the different types of nematode. Moreover, the active compound showed weaker activity than the mycelium, implying that some other active compound had still not been isolated, and maybe a synergistic action existed in the crude extracts. The results indicated that the nematicidal components and nematicidal effects of *Poria cocos* were complex, and will require further study.

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