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ORIGINAL PAPER

Anthelmintic activity of the crude extracts, fractions, and osthole from *Radix angelicae pubescentis* against *Dactylogyrus intermedius* in goldfish (*Carassius auratus*) in vivo

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Abstract A bioassay-guided fractionation was performed to evaluate the anthelmintic activity of the crude extract fractions and osthole from *Radix angelicae pubescentis* against *Dactylogyrus intermedius* in goldfish (*Carassius auratus*) in vivo. Among four extracts (petroleum ether, ethyl acetate, acetone, and ethanol), only ethanol extract exhibited the best anthelmintic efficacy with 100% mortality of *Dactylogyrus* and no death of fish at the optimal anthelminthic concentration of 120 mg/L. Therefore, ethanol extract was subjected to column chromatography to obtain sixteen fractions. The activity was found in fraction F with 100% mortality of *Dactylogyrus* and no toxicity to fish at dose of 2.0 mg/L. A white crystal was isolated from fraction F and identified as osthole which exhibited the optimal activity with 100% mortality of *Dactylogyrus* at 1.6 mg/L had and no toxicity to fish at dose up to 6.2 mg/L. This is the first report on the isolation and identification of anthelmintic active compound from *R. angelicae pubescentis* against *D. intermedius* in goldfish (*C. auratus*) in vivo.

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Introduction

Dactylogyrus spp. is common ectoparasite living on the gills in freshwater fish (Woo 2006). This genus includes numerous species, while the pathologic significance is very dependent on the species and intensity of infection (Alvarez-Pellitero 2004). *Dactylogyrus intermedius* is one of the important veterinary ectoparasites in Asia, Central Europe, Middle East, and North America (Paperna 1964). *D. intermedius* may cause gill inflammation, excessive mucous secretions, accelerated respiration (Reed et al. 2009), and mixed infections with other parasites and secondary bacterial infections (Woo et al. 2002). This parasite always leads to the loss of appetite, productivity, and high mortalities. In recent years, the infestation of *D. intermedius* in freshwater fish has increased (Topić et al. 2001; Kritsky and Heckmann 2002; Tóro et al. 2003; Bagge et al. 2004; İsmail and Selda 2007; Shamsi et al. 2009) and caused serious economic damage. Despite the availability of chemotherapy for *Dactylogyrus*, many side effects of chemical parasiticides have been found, such as resistance, environmental contamination, and toxicity (Goven et al. 1980; Marshall 1999). It has prompted a search for new alternatives including medical plants.

Many medical plants have been studied to control fish parasites, such as *Pinus elliottii* (Tóro et al. 2003), *Mucuna pruriens*, *Carica papaya* (Ekanem et al. 2004a), *Piper guineense* (Ekanem et al. 2004b), the green tea (Suzuki et

al. 2006), and *Terminalia catappa* L.(Chansue 2007). However, there is little information about the use of medical plant for *D. intermedius*, including *Fructus cnidii* (Wang, et al. 2008), *Fructus arctii* (Wang et al. 2009), *Macleaya microcarpa* (Wang et al. 2010), *Radix angelicae pubescentis*, *Fructus bruceae*, *Caulis spatholobi*, *Semen aesculi*, and *Semen pharbitidis* (Liu et al. 2010). Our previous research also demonstrated that the *R. angelicae pubescentis* exhibited the best anthelmintic activity among 28 Chinese medicinal plants against *D. intermedius* in goldfish (*Carassius auratus*) in vivo. However, to the best of our knowledge, there is no report on the isolation and identification of anthelmintic activity compounds from *R. angelicae pubescentis*. In this paper, the anthelmintic activities of crude extracts, fractions, and active compounds from *R. angelicae pubescentis* against *D. intermedius* were studied.

Materials and methods

Plant material

R. angelicae pubescentis with the production batch number 071102 was supplied by Bozhou City Yonggang Chinese Herbal Pieces Co. Ltd(Bozhou, P.R. China). The dried plant material was crushed and reduced to fine powder by using a strainer (50–60 mesh).

Selection of extraction solvent

Four dried and powdered samples of *R. angelicae pubescentis*, each weighing 50.0 g, were extracted with petroleum ether, ethyl acetate, acetone, and ethanol for 2 h and three replicates, respectively. Portions of each extract were evaporated to dryness under reduced pressure in a rotary evaporator. The dried extracts were dissolved with ethanol or water at a concentration of 0.5 g/mL. All extracts were assayed for anthelmintic activity in vivo. Water and ethanol was used as control group.

Isolation and identification of active compounds

Results revealed activity in the ethanol extract. A large-scale of plant sample (8 kg) were then extracted with ethanol in 75° C water bath for 2 h, this process was performed in three replicates. The extract was evaporated to dryness under reduced pressure in a rotary evaporator to yield the ethanol extract (1,890.8 g). The ethanol extract (120 g) was fractionated by column chromatography (120×10 cm) over silica gel G (2,300 g, 100–200 mesh) eluting gradiently with petroleum ether/ethyl acetate (40:1, 30:1, 20:1, 10:1, 5:1, 2:1, 1:1, 0:1, *v*/*v*), ethyl acetate/methanol (100:1, 50:1, 20:1, 10:1, 5:1, 3:1, 2:1 , 1:1, 0:1, *v*/*v*), and water affording 340 fractions (300 mL each). Thin layer chromatography (TLC) analysis was used for monitoring the eluents, and fractions with similar chromatograms were combined into sixteen fractions as follows: Fr.A (1–14), Fr.B (15–30), Fr.C (31–42), Fr.D (43–49), Fr.E (50–70), Fr.F (71–75), Fr.G (76–95), Fr.H (96–108), Fr.I (109–124), Fr.J (125–147), Fr.K (148–231), Fr.L (232–252), Fr.M (253–270), Fr.N (271–302), Fr.O (303–327), Fr.P (328–340). Each fraction was tested for its anthelmintic activity in vivo. Among these fractions, only the Fr.F showed the most anthelmintic efficacy and became a crystal when it was concentrated.

The structure of this crystal was identified by melting point measure, TLC, high performance liquid chromatography (HPLC), and spectroscopic techniques including ultraviolet (UV) spectroscopy, infrared (IR) spectroscopy, electron ionization mass spectrometry (EI-MS), and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy.

Establishment of model of infected fish

Healthy goldfish (*C. auratus*), weighing 5.0±0.5 g, were obtained from Chengdu Ornamental Fish Farm. Healthy fish were acclimatized under laboratory conditions for 7 days. The diseased fish infested with *D. intermedius* were supplied by the Laboratory of Aquatic Animal Diseases in Sichuan Agricultural University. The animal model infected with *D. intermedius* were established according to Wang's method (Wang et al. 2008). The healthy goldfish were co-habitated with the ones infected with *D. intermedius* for 7 days. Five fish were randomly selected and checked for the presence and intensity of parasites living on the gills in fish under a stereomicroscope. Fish with 100% infection rate and a moderate infestation (about 30 parasites on gills per fish) were used for all in vivo anthelmintic assay.

In vivo anthelmintic activity assay

The experiments were performed in enamel basin with 8 L capacity. Each basin contained 5 L aerated ground water and ten previously infected goldfish. The water temperature was constant at 25±1°C and the water pH ranged from 7.0 to 7.5. The crude extracts (petroleum ether, ethyl acetate, acetone, and ethanol extract), fractions of ethanol extract, and the crystal compound were respectively added in enamel basin at a different series of concentrations. The blank control group with no extract was used under the same experimental conditions. All treatment and control groups were conducted in triplicate. After 48 h, all goldfish were biopsied, and the lamella branchialis were placed on glass slides for counting the number of surviving parasites under a stereomicroscope.

The effective concentration, the mortality of *Dactylogyrus*, and the mortality of fish were used to evaluate the

anthelmintic efficacy of each treatment. The optimal anthelminthic concentration was the concentration which led to the highest mortality of *Dactylogyrus* with no intoxication of fish. The drug concentration which resulted in less than 20% mortality of *Dactylogyrus* was considered ineffective concentration. No parasite or dead *Dactylogyrus* on gills represented 100% mortality of *Dactylogyrus*. The mortality of *Dactylogyrus* of each treatment was calculated according to the following formula (Wang et al. 2010):

$$\mathrm{MD}(\%) = \frac{B - T}{B} \times 100\% \quad (1)$$

where MD is the mortality of *Dactylogyrus*, B is the average number of surviving *D. intermedius* in the blank control, and T is that in the treatment. The mortality of fish was also calculated by the follow equation:

$$\mathrm{MF}(\%) = \frac{B - E}{B} \times 100\% \quad (2)$$

where MF is the mortality of fish, B is the average number of surviving fish in the beginning of test, and E is that at the end of test.

Results

Selection of extraction solvent

Anthelmintic efficacy and toxicity of four extracts from *R. angelicae pubescentis* are shown in Table 1. The results revealed that the ethanol extract displayed the best anthelmintic efficacy with 100% mortality of *Dactylogyrus* and no death of fish at the optimal anthelminthic concentration of 120 mg/L. Followed by the petroleum ether extract and the acetone extract and the maximum mortality of *Dactylogyrus* were 48.3% (200 mg/L) and 72.8% (140 mg/L), respectively. The ethyl acetate extract exhibited the weakest activity with the maximum mortality of *Dactylogyrus* of 64.8% (800 mg/L). Therefore, the ethanol extract was chosen for the extraction of *R. angelicae pubescentis*.

In vivo anthelmintic efficacy of fractions

Sixteen fractions obtained from the ethanol extract of *R. angelicae pubescentis* by column chromatography were tested for anthelmintic activity against *D. intermedius* in vivo. The anthelmintic efficacy and toxicity of fractions are shown in Table 2. The average number of surviving *D. intermedius* in the blank control was 36.0 per fish. The fraction F exhibited the optimal anthelmintic activity with 100% mortality of *Dactylogyrus* and no toxicity to fish at dose of 2.0 mg/L. This followed by the fraction K with 79.7% mortality of *Dactylogyrus* at 5.0 mg/L and showed to be toxic to fish with 33.3% mortality of fish at 20.0 mg/L. The other fractions showed minor efficacy to *D. intermedius*. The active compound was contained in the fraction F which yielded a white needle crystal (in ethanol).

Table 1 Anthelmintic efficacy and toxicity of four extracts after 48 h

Samples	Concentration (mg/L)	Surviving number of *Dactylogyrus*	Mortality of *Dactylogyrus* (%)	Mortality of fish (%)
Petroleum ether extract	220	–	–	100
	200	12.2	48.3	76.6
	160	14.6	38.1	73.3
	100	17.5	25.8	43.3
	80	20.3	13.9	26.6
Ethyl acetate extract	1,000	–	–	100
	800	8.3	64.8	100
	500	13.1	44.4	73.3
	300	18.8	20.3	50.0
	200	21.4	9.3	26.6
	150	22.0	6.7	0
Acetone extract	200	–	–	100
	140	6.4	72.8	80.0
	100	7.0	70.3	56.6
	90	9.3	60.5	53.3
	80	12.7	46.1	26.6
	70	16.9	28.3	20.0
	50	19.2	18.6	16.6
Ethanol extract	200	–	–	100
	180	0	100	86.6
	150	0	100	60.0
	130	0	100	33.3
	120	0	100	0

"–" indicates that the number of *Dactylogyrus* was not counted when all fish are dead at 48 h

In vivo anthelmintic efficacy of active compound

The anthelmintic efficacy and toxicity of active compound are shown in Table 3. The optimal anthelminthic concentration of this compound was 1.6 mg/L with no surviving *D. intermedius* which retained at concentrations ranging from 1.6 to 6.2 mg/L. However, the average number of surviving parasite in the blank control was 33.3 per fish, and fish death occurred when the concentration reached 6.4 mg/L with 23% mortality of fish.

Identification of active compound

The active compound was obtained as white needle crystal (in ethanol) with the melting point of 83–84°C. It was only

Table 2 Anthelmintic efficacy and toxicity of 16 fractions after 48 h

Samples	Concentration (mg/L)	Surviving number of *Dactylogyrus*	Mortality of *Dactylogyrus* (%)	Mortality of fish (%)
A	30.0	26.3	26.9	0
	25.0	28.6	20.5	0
	20.0	31.0	13.8	0
	15.0	32.5	9.7	0
B	15.0	19.4	46.1	50.0
	10.0	22.1	38.6	36.6
	5.0	27.6	23.3	0
C	25.0	–	–	100
	20.0	20.0	44.4	63.3
	15.0	24.2	32.7	30.0
	10.0	26.7	25.8	0
D	10.0	19.3	46.3	53.3
	5.0	25.0	30.5	36.6
	4.0	29.8	17.2	0
E	25.0	21.9	39.1	30.0
	20.0	27.1	24.7	0
	10.0	31.5	12.5	0
F	3.0	0	100	4.6
	2.0	0	100	0
	1.0	11.3	68.6	0
G	20.0	25.4	29.4	20.0
	10.0	29.0	19.4	0
H	20.0	31.1	13.6	0
	10.0	34.3	4.7	0
I	20.0	29.4	18.3	0
	10.0	31.7	11.9	0
J	20.0	23.0	36.1	0
	10.0	28.2	21.6	0
K	20.0	5.4	85.0	33.3
	15.0	7.3	79.7	0
	10.0	12.7	64.7	0
L	20.0	29.3	18.6	0
	10.0	32.5	9.7	0
M	20.0	25.0	30.5	0
	10.0	28.5	20.8	0
N	20.0	22.3	38.0	0
	10.0	27.9	22.5	0
O	20.0	26.2	27.2	0
	10.0	31.0	13.8	0
P	20.0	21.3	40.8	0
	10.0	25.8	28.3	0

"–" indicates that the number of *Dactylogyrus* was not counted when all fish are dead at 48 h

Table 3 Anthelmintic efficacy and toxicity of active compound after 48 h

Concentration (mg/L)	Surviving number of *Dactylogyrus*	Mortality of *Dactylogyrus* (%)	Mortality of fish (%)
1.2	6.0	81.9	0
1.4	4.3	87.0	0
1.6	0	100	0
1.8	0	100	0
2.0	0	100	0
2.2	0	100	0
2.4	0	100	0
2.6	0	100	0
2.8	0	100	0
3.0	0	100	0
3.2	0	100	0
3.4	0	100	0
3.6	0	100	0
3.8	0	100	0
4.0	0	100	0
6.0	0	100	0
6.2	0	100	0
6.4	0	100	23.3

one point on TLC with different development system including petroleum, petroleum/acetic ether, petroleum/acetic ether/chloroform, petroleum/chloroform, chloroform, chloroform/acetone, chloroform/methanol, and methanol. The HPLC (the chromatographic column was SinoChrom ODSBP C18, 200×4.6 mm) property of this compound displayed a single peak. UV (MeOH) ν/nm, 322, 257. IR (KBr) ν/cm^{-1}, 3,040.1, 2,961.0, 2,913.2, 1,719.8, 1,605.4, 1,486.4, 1,178.3, 1,027.8, 829.2. EI-MS (70 eV) *m*/*z*, (M+) 244.5. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ ppm, 1.65 (3H, *s*), 1.80 (3H, *s*), 3.51 (2H, *d*), 3.92 (3H, *s*), 5.19 (1H, *m*), 6.20 (1H, *d*), 6.87 (1H, *d*), 7.32 (1H, *d*), 7.72 (1H, *d*). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ ppm, 17.9 (C-13), 22.3 (C-14), 25.7 (C-10), 56.2 (C-15), 107.9 (C-4), 113.2 (C-7), 114.3 (C-9), 118.13 (C-2), 121.9 (C-11), 127.0 (C-1), 133.1(C-12), 145.0 (C-8), 153.4 (C-3), 160.3 (C-6), 161.8 (C-3). The data were in agreement with previous reports(Wei et al. 2004; Wang et al. 2008; Sajjadi et al. 2009), and the structure of this compound was identified as osthol with the molecular formula of $C_{15}H_{16}O_3$ (Fig. 1).

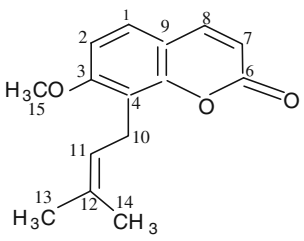

Fig. 1 The structure of active compound isolated from ethanol extract of *Radix angelicae pubescentis*

Discussion

Dactylogyrids are common monogenean parasites with more than 900 described species (Knipes and Janovy 2009), and they may cause a serious pathogen in the aquaculture of many freshwater fish (Ogawa 2002). A variety of chemical treatments including trichlorfon (Prost and Studnicka 1966), formalin (Willomitzer 1980), mebendazole (Goven and Amend 1982), praziquantel (Schmahl and Mehlhorn 1985), and toltrazuril (Schmahl and Mehlhorn 1988) are available to control *Dactylogyrus* infection. In recent investigations done by Schmahl and Mehlhorn (1988), toltrazuril has been reported to be effective against *Dactylogyrus* species at 10 mg/L, resulting in irreversible lesions in the tegument of parasites with vacuolization and disintegration. The similar efficacy and mechanism were previously reported on praziquantel treatment (Schmahl and Mehlhorn 1985; Schmahl and Taraschewski 1987). However, some of chemotherapy such as formalin, praziquantel, and salt-water baths should not be considered absolute cures (Aquatic Unit Technician 2009). The reports of the drug resistance, drug residue, environmental contamination, and toxicity to host have discouraged the use of some anthelmintic chemicals such as trichlorfon and formalin (Goven et al. 1980; Schmahl 1991; Diggles et al. 1993). Despite the availability of chemotherapy, many side effects of chemical anthelmintic have prompted a search for new alternatives. Recently, the plant-based products have been extensively studied to control *Dactylogyrus* infection for their lower side effects as compared with the chemicals. In our previous work, *R. angelicae pubescentis* was observed to be more efficient than the other 28 medicinal plants against *D. intermedius* in vivo. Earlier authors also reported that the methanolic extract of the root of *R. angelicae pubescentis* exhibited potential anthelmintic active to *D. intermedius* with median effective concentration (EC_{50}) value of 57.45 mg/L (Liu et al. 2010).

In the present study, four different solvents (petroleum ether, ethyl acetate, acetone, and ethanol) with an increasing polarity were used to screen the anthelmintic active site of the *R. angelicae pubescentis*. Among the four solvent extracts, the ethanol extract presented the most activity while the other three extracts showed less activity (Table 1). It indicated that the active compound was mainly solved in the polar solvent. The similar report was described by Liu et al. (2010) who found that ethyl acetate extract presented less activity than methanol extract. In order to isolate the active constituents of this plant, a bioassay-guided fractionation was used to obtain osthol from fraction F. The results showed that osthol exhibited the optimal activity with 100% mortality of *Dactylogyrus* at 1.6 mg/L and has no toxicity to fish at dose up to 6.2 mg/L (Table 3). The 100% lethal concentration is similar to mebendazole against *D. intermedius* (Wang et al. 2008). Osthol as a parasiticide against *D. intermedius* with EC_{50} value of 0.807 mg/L was also isolated from *F. cnidii* (Wang et al. 2008). It was confirmed that *D. intermedius* was fully sensitive to osthol.

Osthol is an important coumarin compound for its extensive medical activities including anti-inflammatory (Liu et al. 2005), antitumor (Chou et al. 2007), prevention of atherosclerosis (Ogawa et al. 2007), anti-aging (Hsieh et al. 2004), and antiproliferative (Guh et al. 1996) properties. Osthol also has been used to control crop insects (Xia et al. 2009; Ya et al. 2006) and pathogenic bacterium (Li et al. 2009; Huang et al. 2005). However, there have been few reports related to its antiparasitic activity. In our study, osthol exhibited potential anthelmintic activity against *D. intermedius* with less toxicity to fish, which indicated that this compound may be used in the control of *D. intermedius*. Although osthol has been isolated from many plants such as *F. cnidii* (Wei et al. 2004), *Prangos asperula* Boiss. (Sajjadi et al. 2009), *Angelica pubescens* (Ko et al. 1989), and *R. angelicae pubescentis* (Guo et al. 2006), this is the first report that osthol as a parasiticide against *D. intermedius* is isolated from *R. angelicae pubescentis*.

The data demonstrate that osthol may be effective and safe to control *D. intermedius* in goldfish (*C. auratus*); the mechanism of the anthelmintic activity of osthol and its efficacy to other species of *Dactylogyrus* should be further carried out.

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