

In vitro and *in vivo* assessment of the effect of antiprotozoal compounds isolated from *Psoralea corylifolia* against *Ichthyophthirius multifiliis* in fish



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ABSTRACT

Ichthyophthirius multifiliis, an external fish parasite, often causes significant economic damage to the aquaculture industry. Since the use of malachite green was banned, the search of alternative substance to control *I. multifiliis* infections becomes stringent. In present study, *in vitro* and *in vivo* anti-ich efficacies of isopsoralen and psoralidin, two active compounds isolated from methanol extract of *Psoralea corylifolia* by bioassay-guided fractionation based on the efficacy of anti-ich encysted tomonts, were evaluated. *In vitro* antiprotozoal efficacy of psoralidin is much better than that of isopsoralen. Psoralidin can kill all theronts at concentrations of 0.8 mg/L or more during 4 h exposure; and terminate reproduction of *I. multifiliis* post 6 h exposure of protomonts to 0.9 mg/L and encysted tomonts to 1.2 mg/L. *In vivo* trials showed that 5 h exposure of infected fish to 2.5 mg/L of psoralidin significantly reduced the number of theronts released from tomonts. Furthermore, we observed that a part of protomonts, collected from infected fish post treatment, presented characteristic morphological changes of apoptosis after staining with Annexin V-EGFP/propidium iodide, indicating the possible mechanism of psoralidin against *I. multifiliis* trophont *in situ*. On the basis of these results, psoralidin can be used as a potential lead compound for the development of commercial drug against *I. multifiliis*.

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1. Introduction

Ichthyophthirius multifiliis (also called “ich”) is a holotrichous protozoan parasite of freshwater fish in both temperate and tropical regions throughout the world, and causes an extensive economic loss to the aquaculture industry, including the ornamental fish trade (Matthews, 2005). This parasite has direct, temperature-dependent life cycle, which consists of free-living stage and feeding stage (Matthews, 2005; Picón-Camacho et al., 2012a; Shinn et al., 2012). A free-swimming theront penetrates into the host’s epidermis to feed on mucus and tissue, and differentiates into a trophont. After a period of growth and development, it leaves the host actively and

becomes a protomont. The protomont settles on an appropriate substrate in water to transform into a tomont with a cyst wall. The encysted tomont undergoes binary division, producing several hundreds to thousand tomites, which subsequently turn into infective theronts. The theronts are then released from the cyst, and find a host to begin a new life cycle.

In the past years, malachite green was considered as the most effective parasiticide against both the free-living and parasitic stage of *I. multifiliis* (Wahli et al., 1993; Tieman and Goodwin, 2001; Buchmann et al., 2003; Picón-Camacho et al., 2012a). However, the use of malachite green has been banned since its genotoxic carcinogenesis was reported (Alderman, 1985). In addition, a large number of studies demonstrated that other chemicals including copper sulfate (Ling et al., 1993; Schlenk et al., 1998), sodium chloride (Selosse and Rowland, 1990), potassium permanganate (Straus and Griffin, 2002) could kill the free-living stages of *I. multifiliis*, but these agents had limited efficacy against the trophont *in situ* (Matthews, 2005). Therefore, there is a pressing need to develop an alternative parasiticide, which not only exerts a markedly detrimental effect on all stages of *I. multifiliis*, but also is safe for human health and environment.

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Recently, the application of extracts or active compounds from medicinal plant to control *I. multifiliis* has attracted increasing attention because of demonstrable efficacy and low environmental hazard. Plant extracts, such as aqueous extract of *Capsicum frutescens* (Ling et al., 2012) and acetone extract of *Morus alba* (Fu et al., 2014a), and compounds isolated from plant extracts, such as pentagalloylucose (Zhang et al., 2013), cynaratoside-C (Fu et al., 2014b), dihydrosanguinarine and dihydrochelerythrine (Yao et al., 2011), demonstrated notably antiprotozoal activity against *I. multifiliis*. However, few reports on anti-trophont efficacy of these extracts or compounds and potential action mechanism are available. In our previous study, *P. corylifolia* methanol extract showed high antiprotozoal activity against *I. multifiliis* in free-living stage and feeding stage (Ling et al., 2013). The purpose of this study was to: (1) isolate and identify anti-ich compounds from *P. corylifolia* methanol extract using an *in vitro* bioactivity-guided method; (2) evaluate the efficacy of isolated compound(s) against all stages of *I. multifiliis*; (3) investigate possible mechanism of the most active compound against the trophont *in situ*.

2. Materials and methods

2.1. Fish and parasite

Goldfish (*Carassius auratus*), weighing 4.38 ± 0.95 g, were obtained from a fish farm located in Anziying township (Nanyang, Henan province, China, $112^{\circ}15'30''\text{N}$; $32^{\circ}56'9''\text{W}$). The fish, referred as “naïve fish”, were kept in several 200-L glass aquariums equipped with aquarium filters and air stones (water temperature $19.5\text{--}22.0$ °C, pH 7.1 ± 0.3 , dissolved oxygen $5.0\text{--}7.0$ mg/L). They were fed once at 1% body weight daily with commercial fish pellet feed.

A local strain of *I. multifiliis* was isolated from goldfish obtained from Zhuque ornamental fish market (Xi'an, Shaanxi, China) and maintained by serial transmission on goldfish (Ling et al., 2010). The parasitized fish and naïve fish were held in a static 200-L glass aquarium under the same conditions as described above to develop *I. multifiliis* infection. *I. multifiliis* protomonts was collected using a method described by Clayton and Price (1988) and Shinn et al. (2012). The heavily infected fish were placed into several beakers with filtered aquarium water for 30 min (100 mL/fish). Mature parasites actively exited from the fish by body movements, and then rinsed three times using distilled water in order to discard fish mucus. The protomonts were incubated at $21.5\text{--}22.5$ °C until they reached either the encysted tomont (*i.e.* after ~6 h) or theront stage

(*i.e.* after 20–24 h). After theronts were released, the theront concentration was determined according to a method described by Schlenk et al. (1998) and Straus and Griffin (2001). Briefly, ten 2- μL droplets of the theront suspension were pipetted onto a glass slide and counted under a microscope ($\times 40$ magnification). Theront concentration was represented as the mean count in ten droplets per milliliter.

2.2. Preparation of *P. corylifolia* methanol extract

The methanol extract of *P. corylifolia* was prepared according to Ling et al. (2013). The dried fruits of *P. corylifolia* were purchased from Xi'an Wanshou Chinese Medicinal Herbs Market, and voucher reference (accession no P.2013.12.5.1) was deposited in the College of Animal Science and Technology, Northwest A&F University, China. The plant materials were reduced to fine powder using an electrical pulverizer. The powder (5 kg) was extracted with 2.5 L methanol in 60 °C water bath under reflux for 1 h, and the process was repeated three times. The extract was subsequently filtered and concentrated under reduced pressure in a vacuum rotary evaporator until the solvent was completely evaporated to get some solidified crude extracts (936 g).

2.3. Bioassay-guide isolation and identification of active compounds

The active compounds were isolated through an *in vitro* antiprotozoal efficacy assay (see section 2.4) based on anti-ich encysted tomonts effect of fractions from *P. corylifolia* extract. The fractions with strong antiprotozoal activity were further isolated until the target compound was obtained. The isolation procedure was shown in Fig. 1. Part of the extract (91.0 g) was completely dissolved in 200 mL methanol, subjected to a silica gel column chromatography (silica gel: 197 g, 100–200 mesh) and continuously eluted with petroleum ether, petroleum ether/ethyl acetate (1:1, v/v), ethyl acetate and methanol to obtain a total of 85 eluents (500 mL each). Based on TLC (Thin Layer Chromatograph) analysis (Wang et al., 2009), these fractions were pooled in four new fractions as follows: Fr. A (1–26), Fr. B (27–55), Fr. C (56–71) and Fr. D (72–85). Two fractions (Fr. A and Fr. B) showed significant antiprotozoal efficacy and were further isolated respectively. Fr. A (15.7 g) was fractionated on a silica gel column (silica gel: 80 g, 100–200 mesh) and uninterruptedly eluted with petroleum ether/ethyl acetate (100:1–0:1, v/v) to get 9 pooled fractions (Fr. A1: 1–26, Fr. A2: 27–65, Fr. A3: 66–89, Fr. A4: 90–104, Fr. A5: 105–402, Fr. A6:

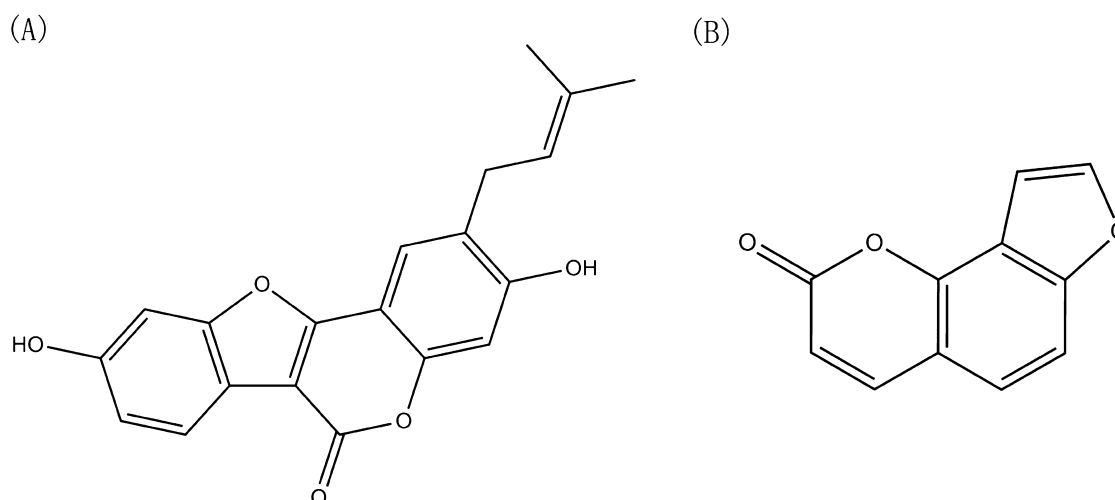


Fig. 1. Chemical structures of isopsoralen (A) and psoralidin (B).

Table 1
Effects of fractions from methanol extract of *P. corylifolia* on reproduction of *I. multifiliis* encysted tomons.^a

Fraction (Fr.)	Antiprotozoal efficacy		
	2.5 mg/L	1.0 mg/L	0
Fr. A	+	–	–
Fr. B	–	+	–
Fr. C	–	–	–
Fr. D	–	–	–
Fr. A1	–	–	–
Fr. A2	–	–	–
Fr. A3	–	–	–
Fr. A4	+	–	–
Fr. A5	–	–	–
Fr. A6	–	–	–
Fr. A7	–	–	–
Fr. A8	–	–	–
Fr. A9	–	–	–
Fr. B1	–	–	–
Fr. B2	–	–	–
Fr. B3	+	+	–
Fr. B4	–	–	–
Fr. B5	–	–	–
Fr. B6	–	–	–
Fr. B7	–	–	–
Fr. B8	–	–	–
Fr. B9	–	–	–

^a Protomonts were allowed to attach 6 h, and then exposed to fractions for 6 h. The number of theronts was counted in each well at 10 h post exposure. +: no theronts produced by encysted tomons; –: the fraction at the listed concentration could not prevent tomons from producing theronts.

403–438, Fr. A7: 439–610, Fr. A8: 611–676 and Fr. A9: 677–685, 40 mL each eluent). Fr. A4, with remarkable antiprotozoal activity, was purified by recrystallization to provide colorless crystal (compound 1). Similar to the isolation procedure of Fr. A, Fr. B (18.7 g) was chromatographed on the same silica gel column (silica gel: 41 g) and continuously eluted with petroleum ether/ethyl acetate/methanol (10:1:0, 1:1:0, 1:3:0, 0:1:0 and 0:0:1, v/v/v) to obtain 9 pooled fractions (Fr. B1: 1–7, Fr. B2: 8–12, Fr. B3: 13–20, Fr. B4: 21–26, Fr. B5: 27–36, Fr. B6: 37–40, Fr. B7: 41–52, Fr. B8: 53–58 and Fr. B9: 59–62, 500 mL each eluent). Among these fractions, Fr. B3 showed the highest activity against encysted tomons, and yellowish crystal (compound 2) was obtained by recrystallization from Fr. B3. Chemical structures of the active compounds were identified by electron ionization mass spectrometry (EI-MS), nuclear magnetic resonance hydrogen spectrum (¹H NMR) and nuclear magnetic resonance carbon spectrum (¹³C NMR). The voucher specimens of these two compounds were deposited in the College of Animal Science and Technology (accession nos. C.2014.3.5.1-2), Northwest A&F University, China.

Table 3
Encystment and reproduction of *I. multifiliis* post 6 h exposure of protomonts (*N* = 50) to different concentrations of isopsoralen and psoralidin.^a

Concentration (mg/L)	Isopsoralen		Psoralidin	
	MNET	MNRT (×1000)	MNET	MNRT (×1000)
0 (negative control)	45.7 ± 1.2 a	12.9 ± 0.6 a	46.0 ± 2.0 a	13.1 ± 1.5 a
0.05 (malachite green)	24.3 ± 2.5 c	1.2 ± 0.3 e	22.3 ± 4.2 b	1.2 ± 0.3 c
0.1 (malachite green)	0	0	0	0
0.3	45.0 ± 2.6 a	13.8 ± 1.0 a	40.3 ± 3.5 a	10.4 ± 1.7 b
0.6	41.0 ± 2.6 a	12.2 ± 1.5 a	25.3 ± 4.7 b	3.6 ± 1.2 c
0.9	35.3 ± 3.1 b	8.7 ± 1.1 b	0	0
1.2	25.7 ± 4.2 c	5.6 ± 1.0 c	0	0
1.5	17.0 ± 3.6 c	3.5 ± 1.1 d	0	0
1.8	0	0	0	0

^a The number of encysted tomons was recorded after 6 h exposure of protomonts to isopsoralen and psoralidin for 6 h, and reproduction (number of released theronts per encysted tomons) was determined post incubation without any test chemicals for 16 h. MNET = mean number of encysted tomons for each treatment; MNRT = mean number of released theronts for each treatment. Each value was expressed as mean ± S.D. of three replicates, and within a column, the values followed by different lower case letters were significantly different (*P* < 0.05).

Table 2
The mean mortality of theronts (*N* = 300) in different concentrations of isopsoralen and psoralidin isolated from *P. corylifolia* and malachite green (positive controls) at 4 h after exposure.

Concentration (mg/L)	Mortality (%)	
	Isopsoralen	Psoralidin
0 (negative control)	0	0
0.01 (malachite green)	0	0
0.025 (malachite green)	65.7	68.5
0.05 (malachite green)	100	100
0.2	*	*
0.4	*	61.7
0.6	*	94.1
0.8	*	100
1.0	53.3	100
1.2	65.4	100
1.4	86.1	100
1.6	100	100

* More than 50% of theronts in each well were still live after 4 h exposure.

2.4. Efficacy assay of fractions against *I. multifiliis* encysted tomons

The solidified extracts of each fraction were obtained through evaporation, and dissolved in 0.02% DMSO (dimethyl sulfoxide) to make a stock solution. Thirty protomonts were distributed to each well of 24-well tissue culture plate, and allowed to settle for 6 h. Then, stock solution of each fraction was added to each well in triplicate to make final concentrations of 2.5, 1.0 and 0 (control), respectively. The numbers of released theronts in each well were counted by microscope examination (×40 magnification) after a total of 22 h of incubation. The fraction was considered as one with strong antiprotozoal activity when this fraction led to all encysted tomons failed to produce theronts (Table 1).

2.5. Bioactivity of compounds 1 and 2 against free-living stages of *I. multifiliis*

Three experiments were performed to evaluate bioactivity of compounds 1 and 2 against free-living stages of *I. multifiliis* (theront, protomont and tomont). In experiment 1 (Table 2), approximately 300 theronts were placed into each well of a 96-well microtiter plate and exposed to concentrations of compounds 1 and 2 at 1.6, 1.4, 1.2, 1.0, 0.8, 0.6, 0.4 and 0.2 mg/L, respectively. A negative control (0 mg/L) and a positive control (0.01, 0.025 and 0.05 mg/L of malachite green) were set up. After 4 h incubation (21.0–22.0 °C), mortality of theronts was determined by the absence of motility and abnormal morphology. The second experiment (Table 3) was carried out to determine the effect of 6 h exposure of protomonts to compound 1 or 2 on encystment and reproduction of *I. multifiliis*. Fifty

Table 4

Effect of 6 h exposure of encysted tomites to isopsoralen and psoralidin against reproduction of *I. multifiliis*.^a

Concentration (mg/L)	Isopsoralen		Psoralidin	
	NET	NRT (×1000)	NET	NRT (×1000)
0 (negative control)	47.7 ± 1.5	13.4 ± 1.1 ab	46.7 ± 0.6	13.1 ± 0.7 a
0.05 (malachite green)	45.3 ± 2.5	3.9 ± 0.5 d	38.7 ± 1.5	3.8 ± 0.9 b
0.1 (malachite green)	46.7 ± 2.5	0	45.0 ± 3.5	0
0.9	42.0 ± 3.5	14.5 ± 1.5 a	42.3 ± 3.5	4.7 ± 0.7 b
1.2	39.7 ± 2.1	11.4 ± 1.5 b	45.3 ± 3.1	0
1.5	48.0 ± 1.0	8.9 ± 1.2 c	46.3 ± 4.6	0
1.8	44.7 ± 3.1	4.7 ± 1.6 d	41.7 ± 3.5	0
2.1	43.3 ± 3.5	2.6 ± 1.0 d	44.3 ± 2.1	0

^a Reproduction (number of released theronts per encysted tomites) was determined after 6 h exposure of encysted tomites to isopsoralen and psoralidin.

NET = number of encysted tomites per well for each treatment; NRT = number of released theronts per well for each treatment. Each value was expressed as mean ± S.D. of three replicates; within a column, the values followed by different lower case letters were significantly different ($P < 0.05$).

protomonts in 0.5 mL of filtered aquarium water were distributed to each well of 24-well tissue culture plate. Then, 0.5 mL of compound 1 and 2 stock solutions was added into each well to yield final concentrations of 1.8, 1.5, 1.2, 0.9, 0.6 and 0.3 mg/L, respectively. A negative control (0 mg/L) was set up, and malachite green solutions at 0.05 and 0.1 mg/L were used as positive controls. At 6 h after exposure, the solution in each well was replaced with fresh filtered aquarium water, and then the number of encysted parasites in each well was counted. After a total of 22 h incubation, the number of released theronts in each well was recorded using the method described above and reproduction was represented as number of released theronts per encysted tomites. For the third experiment (Table 4), a 0.5 mL of filtered aquarium water with 50 protomonts was added into each well of 24-well tissue culture plate, and the parasites were allowed to attach for 6 h in order to produce the cyst wall. The encysted tomites were counted and subsequently exposed to compound 1 and 2 solutions at final concentrations of 2.1, 1.8, 1.5, 1.2, 0.9 and 0 (negative control), and malachite green solutions at 0.05 and 0.1 mg/L (positive controls), respectively. Reproduction was determined as described above at 16 h post exposure. The three experiments were replicated 3 times respectively.

2.6. Effect of more active compound 2 against trophont in situ

An *in vivo* trial was performed to examine the effect of compound 2 against the trophont *in situ* according to Ling et al. (2011). Thirty-six heavily infected fish were split into 4 groups (9 fish per group) and exposed to compound 2 at concentrations of 2.5 and 1.25 mg/L and malachite green at 0.5 and 0.25 mg/L in opaque breakers, respectively. The protomonts were collected from the fish after a total of 1, 3 and 5 h exposure, respectively, and distributed to each well ($N = 50$) of 24-well tissue culture plate as described above. Fifty protomonts collected from untreated fish were used as a control well. The number of released theronts in each well was determined at a total of 22 h incubation. The trial was performed in triplicate.

An Annexin V-EGFP/propidium iodide apoptosis detection kit (Bioxbox, Nanjing) was used to determine early and late apoptotic changes of protomonts after treatment. Several heavily infected fish were exposed to compound 2 at concentrations of 2.5 mg/L and malachite green at 0.5 mg/L in opaque beakers, respectively; and then dozens of protomonts were collected from the fish at 3 and 5 h-exposure, respectively. After 4 h, the parasites were stained with 5 μ L Annexin V-EGFP and 5 μ L propidium iodide for 10 min in the dark according to the manufacturer's instructions. The stained para-

Table 5

The number of released *I. multifiliis* theronts when protomonts were collected from infested goldfish exposed to psoralidin isolated from *P. corylifolia* for 1, 3 or 5h, respectively.^a

Chemicals	Concentration (mg/L)	Mean number of released theronts (×1000)		
		1 h-exposure	3 h-exposure	5 h-exposure
Control	0	13.4 ± 1.0 a	12.4 ± 1.2 a	14.8 ± 2.0 a
Malachite green	0.25	12.3 ± 0.8 a	8.1 ± 1.5 c	4.2 ± 1.4 c
	0.5	7.6 ± 1.1 b	0	0
Psoralidin	1.25	14.4 ± 1.7 a	11.3 ± 1.1 ab	9.6 ± 1.5 b
	2.5	12.4 ± 1.0 a	8.9 ± 1.7 bc	4.5 ± 1.3 c

^a Each value was expressed as mean (±S.D.) of three replicates, and within a column, the values followed by different lower case letters were significantly different ($P < 0.05$).

sites were then dispensed onto slides and photographed under an Olympus fluorescence microscope (BX53+ DP2-BSW).

2.7. Acute toxicity of compound 2 to goldfish

An aqueous static 96-h bioassay was performed to determine the acute toxicity of compound 2 to goldfish (DeLorenzo et al., 2006; Ling et al., 2010). Goldfish were placed into several 20 L aquariums (10 fish/aquarium), and exposed to compound 2 at concentrations of 2.5, 4, 5.5, 7 and 8.5 mg/L for 96 h. The test included a negative control with no compound 2 and DMSO and a positive control with 0.02% DMSO. The aquarium water was not renewed and water quality parameters (dissolved oxygen, pH, and temperature) were measured in all aquariums during the bioassay. Mortality observations were taken from each aquarium every day. All fish were not fed during the exposure.

2.8. Statistical analysis

All data in the study were processed by the software PASW (Predictive Analytics Software) Statistic v. 18.0. The number of encysted tomites and reproduction were compared with S–N–K (Student–Newman–Keuls Test) procedure for multiple comparisons ($\alpha = 0.05$). The lethal concentration for 50 percent of fish (LC_{50}) with 95% confidence intervals (CI) was calculated using the PROBIT procedure.

3. Results

3.1. Isolation and identification of antiprotozoal compounds

A series of fractions were obtained from *P. corylifolia* methanol extract based on bioassay-guide isolation, and the effects on reproduction of *I. multifiliis* encysted tomites are shown in Table 1. The Fr.A4 and Fr.B3 demonstrated the antiprotozoal activity, and through further purification, two active compounds were acquired and identified as isopsoralen and psoralidin (Fig. 1) on the basis of comparison between reported and obtained data (Table S1; Lee et al., 2009; Xiao et al., 2010).

3.2. Bioactivity of isopsoralen and psoralidin against free-living stages of *I. multifiliis*

The results of isopsoralen and psoralidin against *I. multifiliis* theronts are listed in Table 3, which showed a significant dose–response relationship, with higher doses causing greater mortality. At 4 h after exposure, psoralidin at concentrations of 0.8 mg/L or more resulted in 100% mortality, while the minimum concentration of isopsoralen causing the same mortality was 1.6 mg/L; however, 0.05 mg/L of malachite green can kill all theronts in each well.

The efficacy of isopsoralen and psoralidin against *I. multifiliis* protomonts and tomites are shown in Tables 4 and 5, respective-

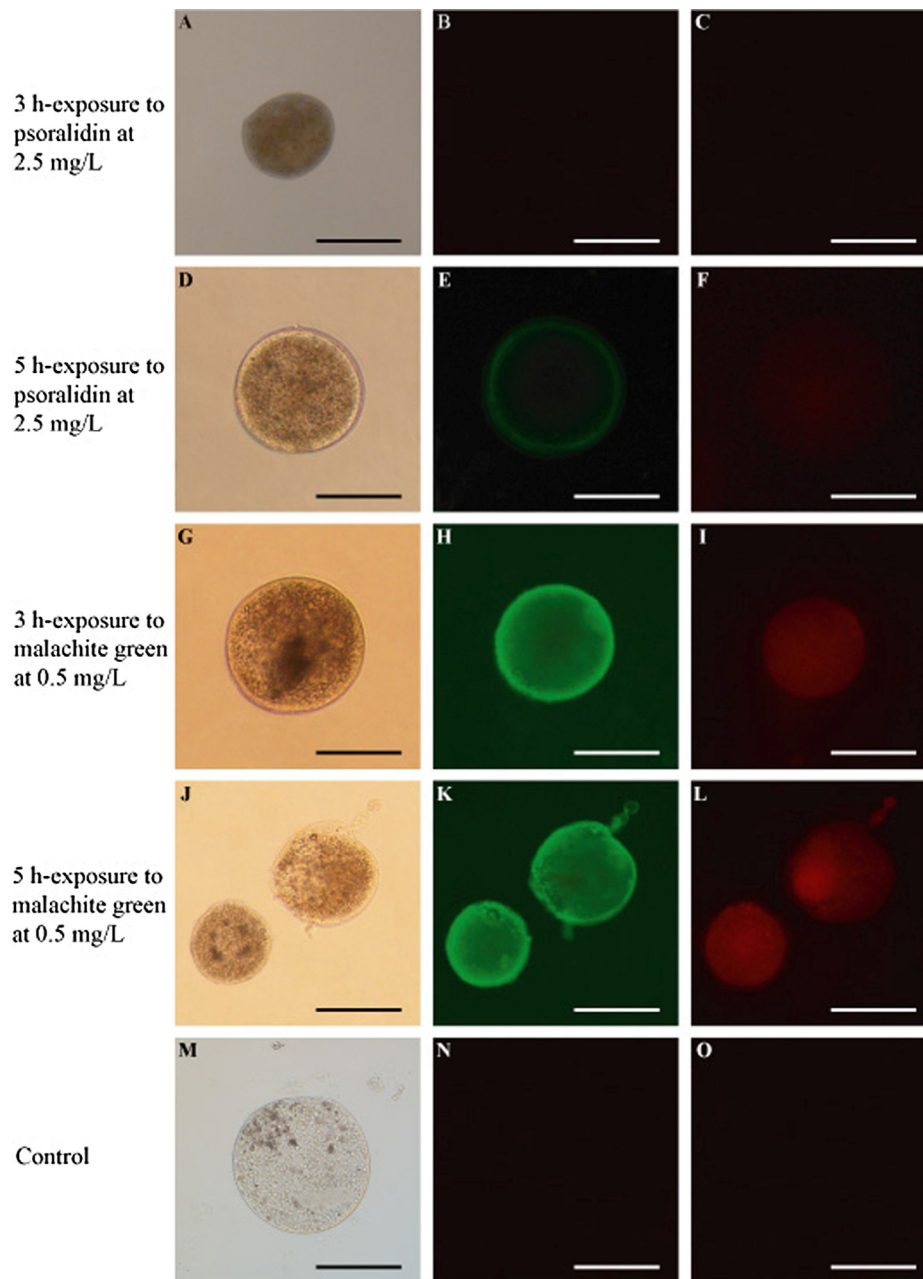


Fig. 2. *I. multifiliis* protomonts stained with Annexin V-EGFP/PI when collected from infected fish exposed to 2.5 mg/L of psoralidin and 0.5 mg/L of malachite green for 3 and 5 h, respectively. The samples are analyzed for bright field (left column), green fluorescence (EGFP, center column) and red fluorescence (PI, right column) under fluorescence microscope (scale bar = 100 μ m).

ly. Table 4 demonstrated that increasing concentrations of exposure to isopsoralen and psoralidin were associated with decreasing number of encysted tomonts, but no significant difference was noted in reproduction between protomonts exposed to 1.5 mg/L or less of isopsoralen and those exposed to 0.6 mg/L of psoralidin or less. It was observed that high concentration of isopsoralen (>1.8 mg/L), psoralidin (>0.9 mg/L) and malachite green (>0.1 mg/L) prevented all protomonts from producing the cyst, and then no theronts were released. As shown in Table 5, the efficacy of psoralidin against reproduction of tomonts is better than isopsoralen. Psoralidin at a concentration of 0.9 mg/L significantly reduced the reproduction of *I. multifiliis*, while isopsoralen with concentrations ranging from 0.9 to 1.8 mg/L did not influence the reproduction. Moreover, 6 h exposure to 1.2 mg/L of psoralidin resulted in no theronts released from the tomonts.

3.3. Effect of psoralidin against trophont *in situ* (Table 5)

The high dose and long duration of exposure of infected goldfish to psoralidin or malachite significantly reduced the number of theronts released from tomont (Table 5). Approximately 4500 theronts were produced by 50 protomonts collected from the infected fish exposed to 2.5 mg/L of psoralidin for 5 h, comparable with 14,800 theronts in the control; however, no theront was released from the protomonts after 3 h exposure of the infected fish to malachite green at a concentration of 0.5 mg/L.

Fig. 2 illustrates the effect of psoralidin against trophont *in situ*. Following staining with Annexin V-EGFP/PI, a part of protomonts collected from infected fish exposed to 2.5 mg/L of psoralidin for 5 h presented characteristic morphological changes of apoptosis, in which the protomonts with a cell membrane stained by green color

Table 6Acute toxicity of psoralidin to goldfish (mean \pm S.D.) ($n = 3$).

Concentration of psoralidin (mg/L)	No. of fish/aquarium	No. dead				Survival (%)
		24 h	48 h	72 h	96 h	
0 (control)	10	0	0	0	0	100
0 (0.02% DMSO)	10	0	0	0	0	100
2.5	10	0+0+0	0+0+0	0+0+0	0+0+0	100
4	10	0+0+0	1+0+2	0+0+0	0+0+0	90 \pm 10
5.5	10	2+2+1	3+2+2	0+0+1	0+0+0	56.7 \pm 5.8
7	10	6+6+4	2+3+4	1+0+0	0+0+0	13.3 \pm 5.8
8.5	10	9+10+8	1+0+2	0+0+0	0+0+0	0

(Annexin V-EGFP) and chromatin fragmentation stained red color (PI) were observed (Fig. 2D–F); on the contrary, 3 h exposure to psoralidin at the same concentration did not induce apoptosis. It is noteworthy that apoptosis was induced in all protomonts after the infected fish were treated by malachite green (0.5 mg/L) (Fig. 2G–L), and even the lysis of some protomont was induced by long duration of exposure.

3.4. Acute toxicity of psoralidin to goldfish

The acute toxicity bioassay demonstrated that LC₅₀ value for psoralidin in goldfish was 5.6 mg/L (95% CI of 5.3–6 mg/L). No visible effect was observed in the treatment with psoralidin at a concentration of 2.5 mg/L, and more than 7 mg/L of psoralidin resulted in the agitated movement and an increased respiration frequency of most fish within 24 h or before death (Table 6).

4. Discussion

In recent years, increasing studies were conducted to investigate the effects of medicinal plants against *I. multifiliis*, because medicinal plants containing many active compounds are important sources of new parasiticide. The dried fruit of *P. corylifolia*, one of the most popular traditional Chinese medicines, showed significant *in vitro* and *in vivo* antiprotozoal activity against *I. multifiliis* (Ling et al., 2013); however, it remains unclear which ingredient(s) of the medicinal plant is(are) responsible for the activity. In this study, a bioactivity-guided fractionation, a proven method for the discovery of active compounds from extracts of medicinal plants (Wang et al., 2008; Fu et al., 2014b), was applied to isolate anti-ich compounds from the methanol extract of *P. corylifolia*. Two active compounds were finally obtained, and then identified as isopsoralen and psoralidin by NMR and ESI-MS.

The theront stage of *I. multifiliis* is infective to the fish host. Therefore killing theronts will prevent the invasion of fish, and then it is essential in evaluating anti-ich activity by *in vitro* anti-theront trials. This study showed that psoralidin at 0.8 mg/L and isopsoralen at 1.6 mg/L killed all theronts within 4 h (Table 3). Anti-theronts efficacy of psoralidin was better than isopsoralen and those compounds isolated in the previous studies. A concentration of 2.5 mg/L of pentagalloylglucose from *Galla chinensis* could kill all theronts during 4 h of exposure (Zhang et al., 2013), and chelerythrine from *Toddalia asiatica* at 1.2 mg/L could be 100% effective against theronts (Shan et al., 2014). Recently, Yao et al. (2015) and Liang et al. (2015) respectively reported that nystatin from extracellular products of *Streptomyces griseus* at a concentration 6.0 mg/L and two flavonoids from root bark of *Morus alba* at more than 2 mg/L caused 100% mortality of theronts after 4 h exposure. Thus, in comparison to the previous reports, psoralidin is more effective in preventing theronts from infecting fish.

Protomont and tomont are the key free-living stages in the life cycle of *I. multifiliis*. The mature trophont transforms to the protomont, which actively leaves the host tissues and swim briefly

before settling on an appropriate substrate, and then the settled protomont encysts and undergoes binary division, producing hundreds to thousands of infective theronts (Matthews, 2005; Picón-Camacho et al., 2012b). Therefore, it is important to kill the parasites at the reproductive stage in order to prevent infestation of theronts by termination of the reproduction of *I. multifiliis* (Zhang et al., 2013). The present study demonstrated that 6 h exposure of protomonts to 0.9 mg/L of psoralidin could kill all protomonts, but for encysted tomonts, the same treatment did not cause 100% mortality, and a higher concentration of psoralidin (1.2 mg/L) could prevent all encysted tomonts from producing infective theronts, indicating that a protomont without a cyst wall is more susceptible to chemicals than encysted tomonts (Tables 4 and 5). Similar results have also been reported in previous studies (Ling et al., 2011, 2013). Combined with the published data and our findings, we draw a conclusion that the encysted tomont is most resistant to chemicals in free-living stages of *I. multifiliis*. As a result, bioassay-guide isolation based on anti-theronts efficacy is likely to produce false positive results, in which a concentration of compound can kill all theronts but not all encysted tomonts. In this study, *in vitro* antiprotozoal activity of psoralen, the main component of crude extract of *P. corylifolia*, was investigated, and results showed that a concentration of 4.0 mg/L of psoralen did not influence the reproduction of encysted tomonts, although the same concentration caused 100% mortality of theronts (unpublished data). Therefore, we suggested that isolation based on anti-ich encysted tomonts is an effective way to reduce false positive results.

At present, a large number of studies showed that many compounds were used to control the infections of *I. multifiliis* through the treatment aimed at interrupting the life cycle by killing the free-living stages of the parasite. However, little report has referred to the effect of bath administration of the compounds against the trophont *in situ*, which is another key stage of *I. multifiliis* life cycle. Some studies confirmed that growth and development of trophonts had an important influence on the encystment and further reproduction within the aquatic environment (Hines and Spira, 1973; Ewing and Kocan, 1986; Matthews, 2005). This study, therefore, was designed to assess anti-trophont efficacy of psoralidin, and results demonstrated that 5 h exposure of infected fish to psoralidin at 2.5 mg/L induced partially apoptosis of protomonts, and then significantly reduced the number of released theronts (Table 6 and Fig. 2). We also found that anti-trophont efficacy of malachite green is clearly much better than that of psoralidin, with preventing all tomonts from producing theronts post 3 h exposure to 0.5 mg/L of malachite green. To our best knowledge, this is the first report evaluating the effect of malachite green against *I. multifiliis* trophont *in situ*. However, the exact mechanism for inducing apoptosis of trophont needs to be further studied.

In summary, two compounds, psoralidin and isopsoralen, were isolated from the methanol extract of *P. corylifolia* by bioassay-guided fractionation based on activity of anti-ich encysted tomonts. Furthermore, *in vitro* and *in vivo* efficacies of these two compounds against the free-living and parasitic stages of *I. multifiliis* were

determined, and we found that psoralidin not only could effectively kill theronts, protomonts and encysted tomonts, but also had a detrimental effect on *I. multifiliis* trophont *in situ*, indicating that psoralidin can be chosen as a potential lead compound for the development of commercial drug against *I. multifiliis*.

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Conflict of interest

The authors declared that they have no conflicts of interest to this work.

Appendix Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.ijpddr.2015.04.001.

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