

Parasiticidal effects of *Morus alba* root bark extracts against *Ichthyophthirius multifiliis* infecting grass carp

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ABSTRACT: *Ichthyophthirius multifiliis* (Ich), an important fish parasite, can cause significant losses in aquaculture. To find efficacious drugs to control Ich, the root bark of white mulberry *Morus alba* was evaluated for its antiprotozoal activity. Bark was powdered and extracted with 1 of 5 organic solvents: petroleum ether, chloroform, ethyl acetate, acetone, or methanol. The extracts were concentrated, dissolved in 0.1% (v/v) DMSO, and used for anti-Ich trials. Acetone and ethyl acetate extracts significantly reduced the survival of Ich tomonts and theronts. *In vitro*, acetone extract at 25 mg l⁻¹ killed all non-encysted tomonts, at 50 mg l⁻¹ eradicated all encysted tomonts, and at 8 mg l⁻¹ caused mortality of all theronts. Ethyl acetate extract at 50 mg l⁻¹ eliminated all non-encysted tomonts, at 100 mg l⁻¹ killed all encysted tomonts and terminated tomtom reproduction, and at 8 mg l⁻¹ killed all theronts. Low concentrations (2 and 4 mg l⁻¹) of acetone and ethyl acetate extracts could not kill all theronts after 4 h exposure, but a significant decrease in theront infectivity was observed following 30 min of pretreatment with the extracts. The 96 h LC₅₀ values of acetone and ethyl acetate extracts to grass carp were 79.46 and 361.05 mg l⁻¹, i.e. much higher than effective doses for killing Ich theronts (8 mg l⁻¹ for both extracts) and non-encysted tomonts (12.5 and 25 mg l⁻¹, respectively). Thus *M. alba* extract may be a potential new, safe, and efficacious drug to control Ich.

KEY WORDS: Antiparasitic effect · White mulberry · Ich · Lethal dose · Acetone · Ethyl acetate

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INTRODUCTION

Ichthyophthirius multifiliis ('Ich') is widely distributed and is among the most virulent ciliated parasites (Schmahl et al. 1992, Kawano & Hirazawa 2012). The parasite's life cycle is divided into 3 distinct stages: parasitic trophont, reproductive tomtom, and infective theront. Trophonts parasitize fish, then leave the fish and enter the water to become non-encysted tomonts after they have matured. The tomonts ad-

here to plants, gravel, or other substrates, secrete a cyst wall, and become encysted tomonts, which reproduce and release numerous infective theronts (Matthews 2005, Dickerson 2012). The theronts invade the skin and gills of fish, causing mortality.

Infection with *I. multifiliis* causes significant losses in aquaculture (Martins et al. 2011). Control of this highly pathogenic ectoparasite is difficult after the parasite penetrates into the skin and gills of fish. The free-living stages of the parasite, viz. theronts and

tomonts, are much easier to kill (Straus & Meinelt 2009, Sudová et al. 2010, Picón-Camacho et al. 2012b, Shinn et al. 2012). Therefore, it is necessary to develop safe and efficacious parasiticides to eradicate free-living *I. multifiliis* to disrupt the parasite's life cycle, and further prevent the spread of the disease, especially following the ban of malachite green to treat food fish by governments in many countries (Picón-Camacho et al. 2012a,b). Studies have evaluated the antiparasitic efficacy against *I. multifiliis* of chemicals such as copper sulfate (Ling et al. 1993, Schlenk et al. 1998, Straus 2008), sodium chloride (Miron et al. 2003, Garcia et al. 2007), chloramine-T (Cross & Hursey 1973), potassium permanganate (Straus & Griffin 2002), potassium ferrate (Ling et al. 2010, 2011), peracetic acid (Straus & Meinelt 2009, Sudová et al. 2010), and chlorophyllin (Wohllebe et al. 2012). However, none of these therapeutants is both effective and safe for treating *I. multifiliis*, especially trophonts in fish tissues (Tieman & Goodwin 2001). Bronopol shows significant ability to treat Ich, but it is still weak when compared to malachite green (Buchmann et al. 2003, Picón-Camacho et al. 2012a,b, Shinn et al. 2012).

Recently, medicinal plant extracts have received attention as alternative chemotherapeutants to control *I. multifiliis*. These studies demonstrated that crude extracts of velvet bean *Mucuna pruriens* and papaya *Carica papaya* (Ekanem et al. 2004), garlic *Allium sativum* (Buchmann et al. 2003), magnolia *Magnolia officinalis* and sophora root *Sophora alopecuroides* (Yi et al. 2012), and chili pepper *Cap-sicum frutescens* (Ling et al. 2012) were able to kill *I. multifiliis* effectively. The Pharmacopoeia of the People's Republic of China has compiled a list of 1146 species used in traditional Chinese medicine (National Pharmacopoeia Committee 2010). White mulberry *Morus alba*, which is one of these medicinal plants, is adapted to a wide range of climatic, topographical, and soil conditions. *M. alba* can be found from temperate to subtropical regions of the northern hemisphere to the tropics of the southern hemisphere (Ercisli & Orhan 2007). Almost all parts of *M. alba*, including the leaves, roots, and fruits, have been used in traditional Chinese medicine (Bae & Suh 2007, Katsube et al. 2009, Hsu et al. 2012). The root bark of *M. alba*, called 'Sang-Bai-Pi' in China, has been used for its anti-inflammatory, hypoglycemic, and antibacterial activity (Park et al. 2003, Dat et al. 2010), and has been highlighted in various scientific investigations to explore its medicinal functions (Lin et al. 2009). However, the anti-Ich activity of *M. alba* root bark has not been reported. The purpose

of this study was to investigate the parasiticidal effect of *M. alba* extracts against *I. multifiliis* and the toxicity of the extracts to grass carp.

MATERIALS AND METHODS

Fish

Healthy grass carp *Ctenopharyngodon idella* weighing 13.8 ± 0.5 g (mean \pm SD) were obtained from a commercial fish farm at Huadu, Guangzhou City, Guangdong Province. The fish were maintained in 100 l opaque tanks filled with aerated tap water at a temperature of $23.0 \pm 0.3^\circ\text{C}$, and one-quarter of the water in every tank was exchanged with dechlorinated fresh water each day. The experimental fish were fed daily with a commercial diet (Guangdong Haid Group) at 1% of fish weight.

Parasite

I. multifiliis was isolated from goldfish obtained from the ornamental fish market of Guangzhou, China. Five naïve grass carp were cohabited with an infected goldfish in a 30 l opaque tank for 7 d to develop *I. multifiliis* infection. Fish heavily infected with mature trophonts were anesthetized with 150 mg l^{-1} tricaine methanesulfonate (MS-222, Sigma), and the skin was gently scraped to dislodge the trophonts. The isolated trophonts were rinsed several times with dechlorinated water to remove fish mucus.

Preparation of *Morus alba* extracts

M. alba was purchased from the Chinese medicinal market at Guangzhou. It was kept in an oven at 55°C until completely dried. The dried plant materials were then powdered by a pulverizer with a 50 mesh strainer. Five powder samples (200 g each) of *M. alba* were extracted 4 l of either petroleum ether, chloroform, ethyl acetate, acetone, or methanol. The extractions were repeated 3 times for 8 h each time. Each extract was subsequently filtered and concentrated under reduced pressure in a vacuum rotary evaporator. Final amounts of petroleum ether, chloroform, ethyl acetate, acetone, and methanol extracts were 10, 8.7, 21.6, 32.6, and 41 g, respectively. Fifty ml of each solvent used in the 5 extracts were also concentrated under reduced pressure in a vacuum rotary evaporator until most of the solvent had evap-

orated, and then 50 ml dechlorinated freshwater containing 1% (v/v) dimethylsulfoxide (DMSO) were added to the evaporator to dissolve the residual solvent. These solutions were used as controls in anti-tomont or anti-theront trials. The same DMSO (0.1%) was added to each of the 5 extracts to increase water solubility of the extracts in this experiment.

Effect of *Morus alba* extracts on *Ichthyophthirius multifiliis*

Non-encysted tomonts. In the anti-tomont experiment, non-encysted tomonts came from trophonts which were obtained as previously described. Five 100 μl non-encysted tomont suspensions were counted under a microscope using a Sedgewick-Rafter cell, and the average non-encysted tomont number per 100 μl was calculated as the total non-encysted tomont number ml^{-1} . Approximately 100 non-encysted tomonts in 300 μl of dechlorinated freshwater were placed into each well of a 24-well tissue culture plate. Extract solution (300 μl) was added to each well in triplicate ($n = 3$) to make final concentrations of 0 (control), 12.5, 25, 50, 100, and 200 mg l^{-1} . Live and dead tomonts were identified based on their movement; tomonts were considered dead if no motion of the parasite was observed. Tomonts were enumerated under a microscope at 4 \times magnification at 1, 2, 3, and 4 h post treatment.

Tomont reproduction. For the tomont reproduction experiment, 300 μl of solution with 20 non-encysted tomonts were distributed into each well of 24-well plates. The non-encysted tomonts were incubated for 6 h until encysted. The parasites were then exposed to the extracts at 6 concentrations: 0 (control), 12.5, 25, 50, 100, and 200 mg l^{-1} , and maintained at $23 \pm 0.3^\circ\text{C}$ for 12 h. Survival or dead encysted tomonts were determined in every well 12 h post treatment; meanwhile, the theronts in each well were enumerated under a microscope (10 \times) with 5 droplets (10 μl each) from each well using a Sedgewick-Rafter counting cell, and the effect of extracts on *I. multifiliis* tomont reproduction was evaluated based on the number of theronts released per encysted tomont.

Theronts. For the anti-theront experiment, the non-encysted tomonts were placed into 12 cm Petri dishes with 50 ml dechlorinated fresh water and incubated at $23 \pm 0.3^\circ\text{C}$ for 18 h. After theronts were released, 5 droplets (10 μl) of theronts were counted under a microscope (10 \times) using a Sedgewick-Rafter cell after the theronts were killed with 1 μl formaldehyde solution (1%). Theront concentration was calculated as

the number of theronts μl^{-1} . A 100 μl solution with approximately 200 theronts was put into each well of 96-well microtiter plates. The theronts were exposed to the extracts at concentrations of 0 (control), 2, 4, 6, 8, 16, 32, and 64 mg l^{-1} in triplicate for each concentration. Status of the theronts (alive or dead) in each well was assessed every 5 min under a microscope, and the lethal exposure duration (min) was recorded.

Effect of low extract concentrations on theront infectivity. The infectivity of theronts was assessed by exposing theronts to low concentrations of acetone or ethyl acetate extracts for 30 min, and then challenging grass carp using the pretreated theronts. Ten ml of water containing approximately 80 000 theronts (enumerated and calculated per milliliter described as above) were added to 1 of 27 beakers (80 ml). A 10 ml extract solution or dechlorinated water (control) was added to each beaker to make final concentrations of 0 (control), 1, 2, 4, and 8 mg l^{-1} with which to treat theronts for 30 min. Aquaria ($n = 27$, 15 l each) containing 4 l of filtered water and 10 grass carp were used for the infection trial at $23 \pm 0.3^\circ\text{C}$. The theronts pretreated in the extract solution in each beaker were poured into 1 of the 27 aquaria at a concentration of ~ 8000 theronts fish^{-1} . After the grass carp were exposed to the pretreated theronts for 2 h, 6 l of filtered water were added to each aquarium. Water (10 l) was fully changed with dechlorinated water every day. On the fifth day following exposure of the grass carp to the theronts, the prevalence (number of infected fish/total number of fish) and infection intensity (total number of white spots on skin and gills/number of infected fish) were determined in each aquarium after the grass carps were anesthetized with 150 mg l^{-1} MS-222 (Sigma).

Acute toxicity test for grass carp

Based on the preliminary trial results, final concentrations of 0 (control), 12.5, 25, 50, 100, and 200 mg l^{-1} were used for acetone extract, and 0 (control), 50, 100, 200, 400, and 800 mg l^{-1} for ethyl acetate extract. Ten grass carp were treated in each of 33 tanks (15 l) with 10 l solutions of acetone or ethyl acetate extracts at $23 \pm 0.3^\circ\text{C}$ for 96 h. Three replicates were used for each treatment. Air was provided to each tank with an air stone. Tank water was replaced daily with fresh extract solution at the same concentration. Dead fish were recorded and removed daily during the 96 h trial. The median lethal concentration (LC_{50}) of the extracts to naive fish was determined at 24, 48, 72, and 96 h.

Table 1. *Ichthyophthirius multifiliis*. Mortality of non-encysted tomonts exposed *in vitro* to different concentrations of methanol, acetone, chloroform, ethyl acetate, or petroleum ether extracts of *Morus alba*. Values are means \pm SD of 3 replicates

Extract	Exposure time (h)	Mortality (%) at concentration (mg l ⁻¹)					
		0	12.5	25	50	100	200
Methanol	1	0	1.0 \pm 1.0	16.7 \pm 2.1	33.3 \pm 2.5	64.0 \pm 3.0	100.0 \pm 0.0
	2	0	8.7 \pm 1.5	36.7 \pm 3.5	53.3 \pm 3.2	100.0 \pm 0.0	100.0 \pm 0.0
	3	2.3 \pm 1.5	10 \pm 2.0	50.3 \pm 2.5	64.7 \pm 3.8	100.0 \pm 0.0	100.0 \pm 0.0
	4	3.6 \pm 2.5	10.7 \pm 1.5	52.0 \pm 3.5	76.3 \pm 4.7	100.0 \pm 0.0	100.0 \pm 0.0
Acetone	1	0	33.0 \pm 2.0	59.0 \pm 1.0	88.7 \pm 4.5	100.0 \pm 0.0	100.0 \pm 0.0
	2	0	45 \pm 5.3	72.6 \pm 2.1	97.3 \pm 3.1	100.0 \pm 0.0	100.0 \pm 0.0
	3	2.3 \pm 1.5	56.7 \pm 3.2	92.0 \pm 3.0	100.0 \pm 0.0	100.0 \pm 0.0	100.0 \pm 0.0
	4	3.6 \pm 2.5	66.7 \pm 3.5	100.0 \pm 0.0	100.0 \pm 0.0	100.0 \pm 0.0	100.0 \pm 0.0
Chloroform	1	1.3 \pm 1.5	0.6 \pm 1.2	18.7 \pm 0.6	46 \pm 5.0	78.3 \pm 3.1	100.0 \pm 0.0
	2	4.3 \pm 0.5	1.0 \pm 1.0	34.7 \pm 5.0	53.3 \pm 3.8	100.0 \pm 0.0	100.0 \pm 0.0
	3	7.3 \pm 2.5	4.7 \pm 1.5	40.3 \pm 3.2	60.0 \pm 2.0	100.0 \pm 0.0	100.0 \pm 0.0
	4	8.6 \pm 3.2	10.7 \pm 2.1	43.0 \pm 3.8	63.3 \pm 3.5	100.0 \pm 0.0	100.0 \pm 0.0
Ethyl acetate	1	1.3 \pm 1.5	27.3 \pm 2.5	75.3 \pm 4.5	84.3 \pm 2.1	99.3 \pm 0.6	100.0 \pm 0.0
	2	4.3 \pm 0.5	33.7 \pm 3.8	84.0 \pm 3.7	93.7 \pm 1.2	100.0 \pm 0.0	100.0 \pm 0.0
	3	7.3 \pm 2.5	41.3 \pm 1.5	85.0 \pm 2.6	100.0 \pm 0.0	100.0 \pm 0.0	100.0 \pm 0.0
	4	8.6 \pm 3.2	51.7 \pm 6.5	90.3 \pm 1.5	100.0 \pm 0.0	100.0 \pm 0.0	100.0 \pm 0.0
Petroleum ether	1	1.3 \pm 1.5	0.7 \pm 1.1	7.0 \pm 3.6	40.7 \pm 2.1	79.0 \pm 2.6	100.0 \pm 0.0
	2	4.3 \pm 0.5	3.7 \pm 3.1	14.0 \pm 1.7	44.3 \pm 2.5	100.0 \pm 0.0	100.0 \pm 0.0
	3	7.3 \pm 2.5	5.0 \pm 2.0	17.7 \pm 3.1	48.3 \pm 2.1	100.0 \pm 0.0	100.0 \pm 0.0
	4	8.6 \pm 3.2	19 \pm 4.6	23.3 \pm 4.0	54.3 \pm 4.1	100.0 \pm 0.0	100.0 \pm 0.0

Statistical analysis

Data were analyzed using SPSS 16.0 software. The infectivity of pretreated theronts exposed to acetone or ethyl acetate extracts was determined using a Student-Newman-Keul's test. A probit procedure was used to determine LC₅₀ and median effective concentration (EC₅₀) with 95% confidence intervals (CI). Probabilities of 0.05 or less were considered statistically significant.

RESULTS

Effects of *Morus alba* extracts on *Ichthyophthirius multifiliis*

Non-encysted tomonts. All non-encysted tomonts were killed after 1 h of exposure at a concentration of 200 mg l⁻¹ of methanol extract, acetone extract, chloroform extract, ethyl acetate extract, and petroleum ether extracts (Table 1). Non-encysted tomonts showed 100% mortality after a 4 h exposure to acetone extract at 25 mg l⁻¹ and a 3 h exposure to ethyl acetate extract at 50 mg l⁻¹. A concentration of 50 mg l⁻¹ methanol, chloroform, and petroleum ether extract caused 76.3, 63.3, and 54.3% mortality of non-encysted tomonts, respectively. The lowest and highest 4 h EC₅₀ were caused by acetone extract (10 mg

l⁻¹) and petroleum ether extract (37.56 mg l⁻¹), respectively (Table 2).

Tomont reproduction. All encysted tomonts were killed by 50 mg l⁻¹ acetone or ethyl acetate extracts, 100 mg l⁻¹ methanol or chloroform extracts, or 200 mg l⁻¹ petroleum ether extract after a 12 h exposure. No tomont reproduction was noted, and no theronts were released after encysted tomont exposure to the extracts at those concentrations (Table 3). Theronts were released from encysted tomonts when exposed to *M. alba* extracts at concentrations below 50 mg l⁻¹. The encysted tomonts released more theronts when exposed to lower extract concentrations (Table 3).

Theronts. The 5 *M. alba* extracts killed 100% of *I. multifiliis* theronts at a dose of 64 mg l⁻¹ within

Table 2. *Ichthyophthirius multifiliis*. Median effective concentration (EC₅₀, mg l⁻¹) and 95% confidence intervals (CI) of methanol, acetone, chloroform, ethyl acetate, and petroleum ether extracts of *Morus alba* against non-encysted tomonts after 4 h exposure. EC₅₀ and 95% CI were estimated by a probit procedure

Extract	EC ₅₀ (95% CI)
Methanol	31.78 (25.00, 40.71)
Acetone	10.00 (9.30, 10.70)
Chloroform	36.84 (29.74, 46.20)
Ethyl acetate	12.45 (11.51, 13.39)
Petroleum ether	37.56 (32.95, 43.09)

10 min, but could not kill all theronts at 4 mg l⁻¹ by the end of the 4 h exposure (Table 4). During the 4 h *in vitro* trial, either acetone or ethyl acetate extract killed all theronts at a concentration of 8 mg l⁻¹ within about 40 min. At the same concentration, the methanol, chloroform, and petroleum extracts killed only a few theronts (Table 4).

Effect of low extract concentrations on theront infectivity. Theront infectivity was significantly decreased after theronts were pretreated by exposure to *M. alba* extracts for 30 min (Table 5). When grass carp were exposed to the theronts pretreated with either acetone extract or ethyl acetate extract at concentrations of 1, 2, 4, and 8 mg l⁻¹, the infection intensity decreased with increased extract concentrations from 0 (control) to 8 mg l⁻¹. The infection rates ranged from 100 to 40% for acetone extract, and from 100 to 60% for ethyl acetate extract. The average infective intensity was 27 for acetone extract at 4 mg l⁻¹, and 22 for ethyl acetate extract at 2 mg l⁻¹, compared to 590 in control fish (0 mg l⁻¹), which was significantly higher than that of grass carp exposed to theronts pretreated with various concentrations of acetone extract or ethyl acetate extract ($p < 0.05$).

Acute toxicity test for grass carp

In toxicity tests of *M. alba* extracts to grass carp, no fish died in the control group (Table 6). All treated fish died in acetone extract at 200 mg l⁻¹ within 48 h and in ethyl acetate extract at 800 mg l⁻¹ within 24 h. No mortality was

observed in fish in acetone extract at 12.5 mg l⁻¹ and ethyl acetate extract at 50 mg l⁻¹ within 96 h. Mortality usually occurred 24 to 72 h post treatment. The 96 h LC₅₀ values were 79.46 mg l⁻¹ for acetone extract and 361.05 mg l⁻¹ for ethyl acetate extract (Table 7).

DISCUSSION

Results of previous studies evaluating plant extracts for their anti-Ich efficacy and toxicity to fish have suggested that crude extracts from some plants

Table 3. *Ichthyophthirius multifiliis*. Reproduction of tomonts after exposure to *Morus alba* extracts. The non-encysted tomonts were allowed to attach for 6 h and were then exposed to different concentrations of 5 *M. alba* extracts for 12 h. Theronts were counted in every well and values are expressed as mean \pm SD of 3 replicated wells. Values with different superscripts in the same row are significantly different ($p < 0.05$)

Extract	Theronts released per tomont at concentration (mg l ⁻¹)					
	0	12.5	25	50	100	200
Methanol	115 \pm 13 ^a	101 \pm 3 ^b	71 \pm 1 ^c	29 \pm 4 ^d	0 ^e	0 ^e
Acetone	115 \pm 13 ^a	60 \pm 2 ^b	19 \pm 2 ^c	0 ^d	0 ^d	0 ^d
Chloroform	115 \pm 13 ^a	69 \pm 5 ^b	24 \pm 5 ^c	12 \pm 2 ^d	0 ^e	0 ^e
Ethyl acetate	115 \pm 13 ^a	55 \pm 6 ^b	11 \pm 3 ^c	0 ^d	0 ^d	0 ^d
Petroleum ether	115 \pm 13 ^a	91 \pm 8 ^a	86 \pm 6 ^b	52 \pm 9 ^c	13 \pm 1 ^d	0 ^e

Table 4. *Ichthyophthirius multifiliis*. Mean duration (min) to mortality of theronts treated with *Morus alba* extracts at various concentrations during a 4 h exposure. Values are expressed as mean \pm SD from 3 replicates. (+) Live theronts were found in the test wells when the trial was terminated at 4 h

Extract	Time to mortality at concentration (mg l ⁻¹)						
	0	2	4	8	16	32	64
Methanol	+	+	+	+	100.0 \pm 8.7	42.7 \pm 2.5	8.7 \pm 1.2
Acetone	+	+	+	38.7 \pm 1.4	24.3 \pm 2.3	9.7 \pm 1.5	3.7 \pm 1.5
Chloroform	+	+	+	+	43.0 \pm 2.6	25.7 \pm 5.9	6.3 \pm 0.6
Ethyl acetate	+	+	+	36.0 \pm 3.6	10.7 \pm 0.6	5.0 \pm 0.0	2.3 \pm 0.6
Petroleum ether	+	+	+	+	34.0 \pm 2.5	9.3 \pm 1.5	5.0 \pm 1.0

Table 5. *Ichthyophthirius multifiliis*. Infectivity of theronts after 30 min exposure to acetone and ethyl acetate extracts of *Morus alba*. Values are expressed as mean \pm SD from 3 replicates. Within a column, values with different superscripts are significantly different ($p < 0.05$). Infection rate (%) = (infected fish / total fish) \times 100. Infection intensity = total number of white spots on skin and gills / number of infected fish

Concentration (mg l ⁻¹)	Acetone extract				Ethyl acetate extract			
	Total fish	Infected fish	Infection rate (%)	Infection intensity	Total fish	Infected fish	Infection rate (%)	Infection intensity
0	30	30	100 \pm 0	590 \pm 90 ^a	30	30	100 \pm 0	590 \pm 90 ^a
1	30	30	100 \pm 0	195 \pm 149 ^b	30	30	100 \pm 0	33 \pm 8 ^b
2	30	30	100 \pm 0	176 \pm 112 ^b	30	30	100 \pm 0	22 \pm 5 ^b
4	30	24	80 \pm 17	27 \pm 25 ^c	30	30	100 \pm 0	17 \pm 9 ^b
8	30	12	40 \pm 10	14 \pm 4 ^c	30	18	60 \pm 10	16 \pm 7 ^b

Table 6. *Ctenopharyngodon idella*. Acute toxicity of acetone and ethyl acetate extracts of *Morus alba* to grass carp. Each tank contained 10 grass carp, and each treatment group contained triplicate tanks. (–) No fish exposed to this concentration

Concentration (mg l ⁻¹)	Acetone extract					Ethyl acetate extract				
	No. dead fish at timepoint				Mortality (%)	No. of dead fish at timepoint				Mortality (%)
	24 h	48 h	72 h	96 h		24 h	48 h	72 h	96 h	
0	0	0	0	0	0	0	0	0	0	0
12.5	0	0	0	0	0	–	–	–	–	–
25	0	0	1	0	3	–	–	–	–	–
50	4	2	2	0	27	0	0	0	0	0
100	12	6	3	0	70	0	0	1	0	3
200	23	7	0	0	100	3	1	0	0	13
400	–	–	–	–	–	10	4	4	0	60
800	–	–	–	–	–	30	0	0	0	100

Table 7. *Ctenopharyngodon idella*. Median lethal concentrations (LC₅₀, mg l⁻¹) and 95% confidence intervals of grass carp exposed to acetone or ethyl acetate extracts of *Morus alba*. LC₅₀ and 95% confidence intervals (CI) were estimated by a probit procedure

Time (h)	LC ₅₀ (95% CI)	
	Acetone extract	Ethyl acetate extract
24	138.46 (120.38, 161.82)	443.88 (386.68, 522.99)
48	88.51 (77.81, 103.34)	398.80 (349.91, 471.33)
72	79.46 (69.53, 92.57)	362.72 (321.16, 419.88)
96	79.46 (69.53, 92.57)	361.05 (316.96, 422.90)

have compounds with significant effects against *I. multifiliis* and are potential resources for production of anti-Ich drugs (Buchmann et al. 2003, Ekanem et al. 2004, Chu et al. 2010, Yao et al. 2010, Ling et al. 2012, Yi et al. 2012, Zhang et al. 2013). Crude plant extracts are usually less costly than purified plant compounds, so it may be possible to use efficacious and safe crude extracts against *I. multifiliis* for controlling ichthyophthiriasis in aquaculture. We used 5 organic solvents (methanol, acetone, chloroform, ethyl acetate, and petroleum ether) to create 5 different extracts of the medicinal plant *M. alba*. Among these 5 extracts, acetone and ethyl acetate extracts exhibited the best efficacy for killing *I. multifiliis*.

Tests were conducted *in vitro* to evaluate the activity of methanol, acetone, chloroform, ethyl acetate, and petroleum ether extracts of *M. alba* against *I. multifiliis* tomonts and theronts. Our results showed that theronts are more susceptible to the extracts than tomonts. Similar results were also reported in previous studies; for example, garlic extract killed theronts at 62.5 mg l⁻¹ within 15 h and encysted tomonts at 117 mg l⁻¹ within 24 h (Buchmann et al. 2003). Pentagalloylglucose caused 100% mortality of theronts at 2.5 mg l⁻¹ and 87.7% mortality of non-encysted tomonts at 40 mg l⁻¹ in a 4 h exposure (Zhang et al. 2013).

When non-encysted tomonts are attached, they become encysted tomonts with a cyst wall. In this study, encysted tomonts were treated for 12 h, whereas non-encysted tomonts were treated for 4 h. Our results indicated that the encysted tomonts were significantly more resistant to extracts compared to the non-encysted tomonts with no cyst walls. A similar phenomenon was reported by Ling et al. (2011): in their study, both non-encysted and encysted tomonts were exposed to potassium ferrate(VI) at a concentration of 2.4 mg l⁻¹. Their results revealed that 2.4 mg l⁻¹ potassium ferrate(VI) significantly reduced the survival of non-encysted tomonts, but had no influence on encysted tomonts, perhaps due to the resistance of the cyst wall against treatment. In addition, both acetone and ethyl acetate extracts exhibited better effects against *I. multifiliis* than the other 3 extracts. Based on our results, further experiments were designed to evaluate acetone and ethyl acetate extracts.

Although acetone or ethyl acetate extracts at concentrations of 2 and 4 mg l⁻¹ could not kill all theronts within 4 h, the 30 min pretreatment of theronts significantly decreased their infectivity at 2 or 4 mg l⁻¹. Similar phenomena were found in previous studies (Shinn et al. 2012, Zhang et al. 2013). Pretreatment with the plant compound pentagalloylglucose at 2 mg l⁻¹ for 1 h did not kill all theronts, but significantly reduced the prevalence and intensity of infectivity (Zhang et al. 2013). The chemical bronopol at a concentration of 1 mg l⁻¹ could not kill all theronts during a 12 h exposure, but significantly reduced infestation by theronts in rainbow trout *Oncorhynchus mykiss* (Shinn et al. 2012). Long exposure duration to low doses of bronopol has also resulted in the reduction of trophont density in rainbow trout (Picón-Camacho et al. 2012b). This suggests that these therapeutants, including the 2 *M. alba* extracts at low concentrations, could significantly reduce infectivity of theronts and partially protect fish from *I. multifiliis*.

infestation. In addition, acetone or ethyl acetate extracts at 8 mg l^{-1} could kill all theronts within a 40 min exposure, but could not kill all theronts when the exposure was 30 min or less. Grass carp exposed to theronts pretreated at 8 mg l^{-1} for 30 min had mean infection intensity of 14 for acetone extract and 16 for ethyl acetate extract. This suggests that treatment time is an important factor for killing all theronts.

A preliminary trial was conducted to find the lowest lethal concentration for all treated fish (200 mg l^{-1} for acetone extract, 800 mg l^{-1} for ethyl acetate extract) and the highest survival concentration for every fish (25 mg l^{-1} for acetone extract, 50 mg l^{-1} for ethyl acetate extract) during a 96 h trial. After the trial, acetone extract of *M. alba* showed higher toxicity to grass carp than ethyl acetate extract. This may be due to different compounds in the 2 kinds of extracts. Some compounds in medicinal plants might have no effect on parasites but are toxic to fish (Lu et al. 2012). For the acetone extract, the 96 h LC_{50} to grass carp was 79.46 mg l^{-1} , approximately 8 times that of the 4 h EC_{50} (10.00 mg l^{-1}) to non-encysted tomonts. For ethyl acetate extract, the 96 h LC_{50} to grass carp was 361.05 mg l^{-1} , 29 times that of the 4 h EC_{50} (12.45 mg l^{-1}) to non-encysted tomonts. The 96 h LC_{50} values of the 2 extracts were also much higher than the LC_{50} values of other plant compounds, such as dihydrosanguinarine (13.3 mg l^{-1}) and dihydrochelerythrine (18.2 mg l^{-1}) to barbel chub *Squaliobarbus curriculus* (Yao et al. 2011), pentagalloylglucose (151.3 mg l^{-1}) to channel catfish *Ictalurus punctatus* (Zhang et al. 2013), and chemicals used for controlling ichthyophthiriasis, such as potassium ferrate(VI) (42.51 mg l^{-1} ; Ling et al. 2010), potassium permanganate (17.6 mg l^{-1} ; Tucker 1987), copper sulfate (6.89 mg l^{-1} ; Straus 2006), and malachite green (0.05 mg l^{-1} ; Bills et al. 1977). Our results suggest that toxicity of the 2 extracts (acetone and ethyl acetate) to grass carp is low and that they are safe for grass carp in treating ichthyophthiriasis.

In conclusion, our results showed that acetone and ethyl acetate extracts of *M. alba* root bark could kill *I. multifiliis* non-encysted tomonts and theronts, could stop tomont reproduction within the treatment period and could reduce infectivity of theronts at low concentrations. The 96 h LC_{50} values of acetone and ethyl acetate extracts to grass carp were much higher than the 4 h EC_{50} values to *I. multifiliis* non-encysted tomonts, indicating that these 2 *M. alba* extracts are safe for use in fish. The concentration of 8 mg l^{-1} for the 2 extracts is considered as a reference dose to protect fish from *I. multifiliis* infection. Further studies are needed to evaluate the effect of these 2 *M. alba* extracts to control ichthyophthiriasis in fish farms.

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