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ORIGINAL PAPER

# Evaluation of medicated feeds with antiparasitical and immune-enhanced Chinese herbal medicines against *Ichthyophthirius multifiliis* in grass carp (*Ctenopharyngodon idellus*)

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**Abstract** Since malachite green was banned for using in food fish due to its carcinogenic and teratogenic effects on human, the search of alternative drug to treat *Ichthyophthirius multifiliis* becomes urgent. This study aimed to (1) evaluate the ethanol extracts of medicinal plants *Cynanchum atratum*, *Zingiber officinale*, *Cynanchum paniculatum*, immunostimulant (A), and immunostimulant (B) for their efficacy against *I. multifiliis*, and (2) determine effects of medicated feeds with *C. atratum*, *Z. officinale*, *C. paniculatum*, and immunostimulant (A) to treat *I. multifiliis* in grass carp. The results in this study showed that the minimum concentrations of *C. atratum*, *Z. officinale*, and *C. paniculatum* extracts for killing all theronts were 16, 8, and 16 mg/L, respectively. In vivo experiments, fish fed with medicated feeds of *C. atratum* for 10 days, or *Z. officinale* for 3 days, or combination of three plants for 10 days resulted in a significant reduction in the *I. multifiliis* infective intensity on grass carp after theronts exposure. Grass carp fed with medicated feeds of immunostimulant (A) for 21 days showed no infection and 100 % of survival 15 days post theronts exposure. Therefore, immunostimulant (A) is a promising feed supplement to treated *I. multifiliis* with good antiparasitic efficacy.

**Keywords** *Ichthyophthirius multifiliis* · *Cynanchum atratum* · *Zingiber officinale* · *Cynanchum paniculatum* · Immunostimulants · Medicated feed

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## Introduction

*Ichthyophthirius multifiliis* (Ich) is a widespread ciliated ectoparasite that penetrates into the skin, gill, and fin of fish to cause ichthyophthiriasis, referred to 'white spot' disease (Buchmann et al. 2001; Farley and Heckmann 1980), and results in heavy economic loss in the aquaculture industry (Matthews 2005; Valtonen and Keranen 1981). The life cycle of *I. multifiliis* comprises a parasitic trophont, a reproductive tomont, and an infective theront. The mature trophonts leave host into water and become nonencysted tomonts. The nonencysted tomonts adhere to substrates and transform into encysted tomonts. An encysted tomont produce numerous theronts. Then, released theronts infect fish and start another life cycle (Dickerson and Findly 2014; Xu et al. 2012). Eliminating any stage of *I. multifiliis* can terminate the life cycle of the parasite and prevent ichthyophthiriasis.

In past decade, several chemical therapeutants were used to treat ichthyophthiriasis by bath treatment, such as formalin and sodium percarbonate (Forwood et al. 2014), copper sulfate (Ling et al. 1993; Schlenk et al. 1998; Straus et al. 2009), potassium permanganate (Straus and Griffin 2002), and malachite green. However, the effective drugs, such as malachite green, have been banned for use in food fish due to its carcinogenic and teratogenic effects on human (Alderman 1985; Srivastava et al. 2004; Tojo Rodriguez and Santamarina

Fernández 2001). Treatment of ichthyophthiriasis by bath is difficult because the trophonts are protected by fish skin and mucus, and the encysted tomonts were protected by a cyst wall (Meinelt et al. 2009; Schumacher et al. 2011). Therefore, it is necessary to find an effective and environmentally friendly new drug and its administration routes to treat ichthyophthiriasis.

In the previous studies, compounds of Chinese medicinal plants were evaluated against *I. multifiliis* in vitro or in vivo with promising results, such as, sanguinarine from *Macleaya cordata* (Yao et al. 2010), dihydrosanguinarine and dihydrochelerythrine from *Macleaya microcarpa* (Yao et al. 2011), chelerythrine and chloroxylonine from *Toddalia asiatica* (Shan et al. 2014), pentagalloylglucose from *Galla chinenisis* (Zhang et al. 2013), kuwanons G and O from *Morus alba* (Liang et al. 2015), cynatratoside-C from *Cynanchum atratum* (Fu et al. 2014), gracillin and zingibernsis from *Costus speciosus* (Zheng et al. 2015), and isopsoralen and psoralidin from *Psoralea corylifolia* (Song et al. 2015). The plant-derived compounds and extracts are secondary metabolites and can be easily degraded, and safe for humans and environment. Thus, these compounds may be used as alternative therapeutants to treat Ich.

Medicinal plants could also be used as immunostimulants that enhanced fish immunity by boosting non-specific immune response. Several plants have been reported to enhance fish immunity to prevent disease in the aquaculture, including *Allium sativum* (Sahu et al. 2007), *Rheum officinale* (Xie et al. 2008), *Astragalus radix* and *Ganoderma lucidum* (Yin et al. 2009), *Withania somnifera* (Sharma et al. 2010), *Achyranthes aspera* (Sheikhzadeh et al. 2012), *Ficus benghalensis* and *Leucaena leucocephala* (Verma et al. 2013), *Psidium guajava* (Giri et al. 2015), and banana peels (Rattanavichai et al. 2015). A high efficacy of immunostimulant against *I. multifiliis* and *Aeromonas hydrophila* has been reported (Li et al. 2012).

Medicated feed will be beneficial for treating ichthyophthiriasis because it directly affects the parasites on the host, is not affected by water parameters such as PH, conductivity, organic load, and results in less environmental contamination (Abdel-Hafez et al. 2014). Currently, there is limited study using traditional Chinese medicine plants to make medicated feed for controlling ichthyophthiriasis. This study aimed to (1) evaluate the ethanol extracts of *C. atratum*, *Zingiber officinale*, *Cynanchum paniculatum*, immunostimulant (A) (Table 1), and immunostimulant (B) (Table 1) for their efficacy against *I. multifiliis*, and (2) determine effects of medicated feeds with *C. atratum*, *Z. officinale*, *C. paniculatum*, and immunostimulant (A) to treat *I. multifiliis* in grass carp.

**Table 1** Proportion of ingredients of immunostimulant (A) and immunostimulant (B)

| Ingredients | Proportion of ingredients | |
|---|---|---|
| | Immunostimulant (A) | Immunostimulant (B) |
| *Astragalus membranaceus* | 2 | 1 |
| *Lonicera japonica* | 1 | 1 |
| *Allium sativum* | 1 | 1 |
| *Crataegus pinnatifida* | 1 | 1 |
| *Aloe barbadensis* | 1 | 1 |
| *Atractylodes macrocephala* | 1 | 1 |
| *Isatidis radix* | 1 | 1 |
| *Angelica sinensis* | 1 | 1 |
| *Dendranthema morifolium* | 0.5 | 1 |
| *Morus alba* | 0.5 | 1 |

## Material and methods

### Fish and parasite

Healthy grass carp (total length = 13.7 ± 1.1 cm, body weight = 29.2 ± 6.9 g) was used on trials from a commercial fish farm at Huadu, Guangzhou City, Guangdong Province. All fish were acclimated in several 100 L of aquaria equipped with an aerator and a water suction pump for 2 weeks before trials and fed two times daily (0930 and 1730 hours) with diets at 1 % fish weight. The water temperature was controlled at 23 ± 0.3 °C. Dissolved oxygen was maintained at 5.0 mg/L or higher. *I. multifiliis* was isolated from a goldfish purchased from an ornamental fish market at Guangzhou, China. The infected goldfish were maintained with six grass carp in a 30-L aquarium with 20 L water for 10 days to permit infection of grass carps by *I. multifiliis*. The parasites used to evaluate anti-Ich activity were collected and cultured as previously reported (Zhang et al. 2013). Theronts number were calculated in five droplets (10 μL) of 1 % formalin-fixed theronts suspensions on a glass slide under a microscope (×10) (Liang et al. 2015). All fish were handled and treated according to the protocol approved by the Animal Experimentation Ethics Committee of Jinan University.

### Collection of medicinal plants and preparation of ethanol extracts

*C. atratum*, *Z. officinale*, *C. paniculatum*, *Astragalus membranaceus*, *Lonicera japonica*, *Allium sativum*, *Crataegus pinnatifida*, *Aloe barbadensis*, *Atractylodes macrocephala*, *Isatidis radix*, *Angelica sinensis*, *Dendranthema morifolium*, and *Morus alba* were obtained from the traditional Chinese medicine plant market at Guangzhou, China, and kept in an oven at 55 °C until completely dried before usage. Then, the *C. atratum*, *Z. officinale*, *C. paniculatum*, immunostimulant (A), and

immunostimulant (B) weighting 20 g were powered by a pulverizer with a 50-mesh strainer, respectively. The powder samples were extracted with 500 mL 95 % ethanol at the room temperature for 24 h. The ethanol extracts were then evaporated at 50 °C in a vacuum rotary evaporator. The dried extracts were stored at 4 °C in a refrigerator prior to the evaluation of anti-Ich activity.

### Bioassay of plant ethanol extracts against *I. multifiliis* theronts

Ethanol extracts of *C. atratum*, *Z. officinale*, and *C. paniculatum* weighting 3.84 mg were dissolved in 1.5 mL dechlorinated water containing 1 % (*v*/*v*) dimethyl sulfoxide (DMSO) to obtain a 2560 mg/L stock solution. Ethanol extracts of immunostimulant (A) and immunostimulant (B) weighting 24.58 mg were dissolved in 1.5 mL dechlorinated water containing 1 % (*v*/*v*) DMSO to obtain a 16,384 mg/L stock solution. The stock solutions were diluted with dechlorinated water in a 96-well plate to make required concentration. Each concentration was 100 μL with three replicates. Then, 100 μL water with about 600 theronts was added into each well to yield final concentrations of 128, 64, 32, 16, 8, 4, 2, 1, 0.5, and 0 mg/L for *C. atratum*, *Z. officinale*, and *C. paniculatum*, and 4096, 2048, 1024, 512, 256, 128, 64, and 0 mg/L for immunostimulant (A) and immunostimulant (B), respectively. The concentration of 0 mg/L was used as negative control. Theronts were considered dead when they were no motion and deformation. The lethal exposure duration and mortality were recorded during 4-h exposure. All procedures on the bioassay were performed at 23 ± 0.3 °C.

### Bioassay of plant ethanol extracts against *I. multifiliis* tomonts

The stock solutions of extracts were prepared as described above and diluted with dechlorinated water in a 24-well tissue culture plate to make required concentrations. There were three replicated wells with 200 μL extract dilution in each concentration. Then, 200 μL water with about 50 nonencysted tomonts was added into each well to make final concentrations of 128, 64, 32, 16, 8, 4, 2, 1, 0.5, and 0 mg/L for *C. atratum*, *Z. officinale*, and *C. paniculatum*, and 16,384, 8192, 4096, 2048, 1024, 512, 256, and 0 mg/L for immunostimulant (A) and immunostimulant (B). The lethal duration for all nonencysted tomonts was monitored at different time point during 5-h exposure. After 6-h and 22-h exposure to each test solution, the live encysted tomonts and released theronts were counted under a microscope (×10). Theronts numbers in each well were determined as described above.

To evaluate effect of extracts on encysted tomonts, approximately 50 nonencysted tomonts with 200 μL water were placed into each well of a 24-well plate to develop for 6 h. After nonencysted tomonts transformed into encysted tomonts, the live encysted tomonts in every well was counted under a microscope (×10). Subsequently, the encysted tomonts were exposed to extracts of *C. atratum*, *Z. officinale*, and *C. paniculatum* at concentrations of 128, 64, 32, 16, 8, 4, 2, 1, 0.5, and 0 (control) mg/L in triplicate. The encysted tomonts were exposed to extracts of immunostimulant (A) and immunostimulant (B) at concentrations of 8192, 4096, 2048, 1024, 512, 256, and 0 (control) mg/L in triplicate. After 16-h exposure, theronts numbers in each well were determined under a microscope (×10) as described above. All procedures on the bioassay were performed at 23 ± 0.3 °C.

### In vivo experiment

#### *Production of medicated feeds*

*C. atratum*, *Z. officinale*, and *C. paniculatum* were purchased and dried as described above. Commercial granule feed were obtained from Guangzhou Haid Group Co., Ltd. The plants and feed were powered by a pulverizer with an 80-mesh strainer, respectively. The powered plants and feed were mixed as follow: (1) 40 g *C. atratum* and 1 kg feed; (2) 40 g *Z. officinale* and 1 kg feed; (3) 40 g *C. paniculatum* and 1 kg feed; (4) 40 g mixture of *C. atratum*, *Z. officinale*, and *C. paniculatum* (*m*/*m*/*m* = 1:1:1) and 1 kg feed; (5) 40 g immunostimulant (A) and 1 kg feed; (6) 1.04 kg feed (control). The six medicated feeds were prepared in the Sun Yat-sen University and stored at 4 °C in a refrigerator before usage.

#### *Experiments*

Trials were performed to evaluate the efficacy of *C. atratum*, *Z. officinale*, and *C. paniculatum* against theronts in grass carp using the published method by Schumacher et al. (2011). Four groups of grass carp were fed with medicated feed for (1) 3 days, (2) 7 days, (3) 10 days, and (4) with commercial feed for 10 days (control). There were three replicated 30 L aquaria with 10 fish, and 20 L static water in each group. Fish in each group were fed with feed twice daily at 0930 and 1730 hours in an amount of 1 % body weight. Water in each tank was completely replaced with fresh water 2 h post feeding. Theronts were added to each tank at 20,000 theronts/fish to challenge fish at the end of feeding treatment. During infection period, all dead fish were removed and fish mortality was recorded daily. Fifty percent water in each tank was replaced daily with fresh water for 5 days. After grass carp were infected by theronts for 5 days, all live fish were anesthetized by 150 mg/L tricaine methanesulfonate (MS-222, Sigma). The number of infected fish was recorded and the number of

trophonts on the body surface of each fish (including gill, fin, and skin) was counted under a microscope (×10). The infective intensity, infective incidence, and survival rate were determined for each treatment group. Infective intensity was defined as the total number of trophonts divide by the number of remaining live infected fish. During the experiment, water temperature was kept at 23 ± 0.3 °C.

To evaluate the effect of medicated feed with immunostimulant (A) against theronts in grass carp, two groups of grass carp were fed using the published method by Verma et al. (2013) as follows: (1) with medicated feed for 21 days; (2) with commercial feed for 21 days (control). There were triplicated 30 L aquaria with 20 fish, and 20 L static water in each group. Fish in each group were fed twice daily at 0930 and 1730 hours in an amount of 1 % body weight. Water in each tank was completely replaced with fresh water 2 h post feeding. Each tank was added 100,000 theronts (5000 theronts/fish) to challenge fish 22 days post feeding medicated feed. During infection period, all dead fish were removed and fish mortality was recorded daily. Fifty percent water in each tank was replaced daily with fresh water for 15 days. After grass carp were infected by theronts for 8 and 15 days, all live fish were anesthetized by 150 mg/L MS-222. The number of trophonts on the body surface of each fish (including fin and skin) were counted under a microscope (×10). Infective incidence, infective intensity, and survival rate were determined for each treatment group as described above.

## Statistical analysis

All data were expressed as mean ± SD (standard deviation). Statistical analysis was conducted with Student-Newman-Keul's test using a statistical analysis software package (SPSS 17.0). $EC_{50}$ was calculated by probit analysis with 95 % confidence intervals (CI). $p$ values <0.05 were considered of significant difference.

## Results

### In vitro bioactivity of plant ethanol extracts on *I. multifiliis*

#### *Theronts*

The anti-theront efficacy of ethanol extracts were positively correlated with the test concentrations (Table 2). The lethal duration of all theronts was significantly shorter in high concentration of each ethanol extract, respectively. All theronts were killed by *C. atratum* extract at the concentration of 16 mg/L within 124.0 min, by *Z. officinale* extract at 8 mg/L within 131.0 min, by *C. paniculatum* extract at 16 mg/L within 148.7 min, by immunostimulant (A) extract at 1024 mg/L within 131.7 min, and by immunostimulant (B) extract at 1024 mg/L within 143.0 min. Mortalities of theronts ranged from 100 % at 16 mg/L, 34.9 % at 2 mg/L to 0 % at 0 mg/L after 4-h exposure to *C. atratum* extract. After 4-h exposure to *Z. officinale* extract, mortalities of theronts were 100 and 37.0 % by exposure to 8 and 2 mg/L, respectively. Mortalities of theronts ranged from 100 % at 16 mg/L, to 29.0 % at 2 mg/L after 4-h exposure to *C. paniculatum* extract. After 4-h exposure to immunostimulant (A) extract, mortalities of theronts were 100 and 57.9 % by exposure to 1024 and 512 mg/L, respectively. Mortalities of theronts were 100 % at 1024 mg/L, and 51.4 % at 512 mg/L after 4-h exposure to immunostimulant (B) extract. The 4-h $EC_{50}$ of theronts were 4.87, 2.53, 6.45, 407.32, and 419.65 mg/L after exposure to *C. atratum*, *Z. officinale*, *C. paniculatum*, immunostimulant (A), and immunostimulant (B) extracts, respectively (Table 6).

#### *Nonencysted tomonts*

The efficacy of ethanol extracts against nonencysted tomonts increased significantly with an increase in the extract concentration (Table 3). The minimum doses of the extracts for killing all nonencysted tomonts were 8, 32, 32, 4096, and 4096 mg/L, of *C. atratum*, *Z. officinale*, *C. paniculatum*, immunostimulant (A), and immunostimulant (B), respectively. *C. atratum*, *Z. officinale*, and *C. paniculatum* extracts demonstrated 78.3, 35.6, and 45.5 % mortalities of nonencysted tomonts after 5-h exposure at the concentration of 4 mg/L. Immunostimulant (A) and immunostimulant (B) extracts caused 58.3 and 56.5 % nonencysted tomonts death after 5-h exposure at a concentration of 2048 mg/L.

Mean numbers of infective theronts released from each live encysted tomonts decreased significantly ($p < 0.05$) from 210.3 in control to 50.7, 104.7, and 153.8 after 22-h exposure to *C. atratum*, *Z. officinale*, and *C. paniculatum* extracts at the concentration of 2 mg/L, respectively (Table 4). Similarly, mean numbers of infective theronts released from each live encysted tomonts decreased significantly ($p < 0.05$) from 210.3 in control to 84.8 and 89.1 after 22-h exposure to immunostimulant (A) and immunostimulant (B) extracts at the concentration of 2048 mg/L, respectively (Table 4).

#### *Encysted tomonts*

The effects of extracts on encysted tomonts were shown in Table 5. All encysted tomonts were killed by *C. atratum* extract at 4 mg/L, by *Z. officinale* or *C. paniculatum* extract at 16 mg/L, and by immunostimulant (A) or immunostimulant (B) extract at 4096 mg/L. Infective theronts were released from each live encysted tomonts when exposed to *C. atratum* extract at the concentration of 2 mg/L or lower and to *Z. officinale* or *C. paniculatum* extract at the

**Table 2** Lethal duration (min) and mortality of plant ethanol extracts against *I. multifiliis* theronts in 4-h exposure. Values are expressed as mean ± standard deviation (SD) for three replicates. Values with different letters in the same column are significant differences ($p < 0.05$). MDDT, mean duration until death of all theronts (maximum observation time 4 h). MT, mortality of *I. multifiliis* theronts at 4 h. CA, *C.atratum*. ZO, *Z. officinale*. CP, *C. paniculatum*. IA, immunostimulant (A). IB, immunostimulant (B). ND, Not detectable in the tested concentration. (−) Ethanol extracts at the listed concentrations did not kill all theronts after 4-h exposure

| Concentration (mg/L) | MDDT (min, mean ± SD) | | | | | MT (%, mean ± SD) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CA | ZO | CP | IA | IB | CA | ZO | CP | IA | IB |
| 0 (control) | – | – | – | – | – | 0.0 ± 0.0a | 0.0 ± 0.0a | 0.0 ± 0.0a | 0.0 ± 0.0a | 0.0 ± 0.0a |
| 0.5 | – | – | – | ND | ND | 7.6 ± 3.1b | 16.3 ± 4.1b | 1.3 ± 0.6a | ND | ND |
| 1 | – | – | – | ND | ND | 31.6 ± 1.7c | 28.3 ± 3.2c | 5.4 ± 1.7a | ND | ND |
| 2 | – | – | – | ND | ND | 34.9 ± 1.7c | 37.0 ± 5.6d | 29.0 ± 6.6b | ND | ND |
| 4 | – | – | – | ND | ND | 48.3 ± 2.3d | 76.9 ± 2.3e | 30.5 ± 3.5b | ND | ND |
| 8 | – | 131.0 ± 3.6d | – | ND | ND | 68.7 ± 4.4e | 100.0 ± 0.0f | 57.1 ± 5.6c | ND | ND |
| 16 | 124.0 ± 3.6c | 57.7 ± 4.0c | 148.7 ± 3.5d | ND | ND | 100.0 ± 0.0f | 100.0 ± 0.0f | 100.0 ± 0.0d | ND | ND |
| 32 | 30.3 ± 3.1b | 20.3 ± 1.5b | 64.3 ± 2.5c | ND | ND | 100.0 ± 0.0f | 100.0 ± 0.0f | 100.0 ± 0.0d | ND | ND |
| 64 | 13.3 ± 4.2a | 12.0 ± 2.0a | 44.3 ± 3.1b | – | – | 100.0 ± 0.0f | 100.0 ± 0.0f | 100.0 ± 0.0d | 10.5 ± 3.1b | 11.6 ± 1.8b |
| 128 | 7.0 ± 2.6a | 5.7 ± 1.2 a | 19.0 ± 1.7 a | – | – | 100.0 ± 0.0f | 100.0 ± 0.0f | 100.0 ± 0.0d | 23.3 ± 5.2c | 18.7 ± 5.0b |
| 256 | ND | ND | ND | – | – | ND | ND | ND | 31.5 ± 6.6d | 38.6 ± 4.7c |
| 512 | ND | ND | ND | – | – | ND | ND | ND | 57.9 ± 2.6e | 51.4 ± 7.6d |
| 1024 | ND | ND | ND | 131.7 ± 21.4b | 143.0 ± 13.5b | ND | ND | ND | 100.0 ± 0.0f | 100.0 ± 0.0e |
| 2048 | ND | ND | ND | 14.7 ± 2.1a | 17.7 ± 3.1a | ND | ND | ND | 100.0 ± 0.0f | 100.0 ± 0.0e |
| 4096 | ND | ND | ND | 8. 3 ± 1.5a | 9.3 ± 2.1a | ND | ND | ND | 100.0 ± 0.0f | 100.0 ± 0.0e |

**Table 3** Lethal duration (min) and mortality of plant ethanol extracts against *I. multifiliis* nonencysted tomonts in 5-h exposure. Values are expressed as mean ± SD for three replicates. Values with different letters in the same column are significant differences ($p < 0.05$). MDDNT, mean duration until death of all nonencysted tomonts (maximum observation time 5 h). MNT, mortality of *I. multifiliis* nonencysted tomonts at 5 h. ND, not detectable in the tested concentration. (−) Ethanol extracts at the listed concentrations did not kill all nonencysted tomonts after 5-h exposure

| Concentration (mg/L) | MDDNT (min, mean ± SD) | | | | | MNT (%, mean ± SD) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CA | ZO | CP | IA | IB | CA | ZO | CP | IA | IB |
| 0 (control) | – | – | – | – | – | 0.0 ± 0.0a | 0.0 ± 0.0a | 0.0 ± 0.0a | 0.0 ± 0.0a | 0.0 ± 0.0a |
| 0.5 | – | ND | ND | ND | ND | 4.3 ± 1.5b | ND | ND | ND | ND |
| 1 | – | – | – | ND | ND | 18.3 ± 3.1c | 11.9 ± 2.3b | 14.7 ± 5.9b | ND | ND |
| 2 | – | – | – | ND | ND | 30.7 ± 2.5d | 20.0 ± 3.3c | 32.1 ± 7.8c | ND | ND |
| 4 | – | – | – | ND | ND | 78.3 ± 1.4e | 35.6 ± 2.9d | 45.5 ± 4.0d | ND | ND |
| 8 | 196.7 ± 31.4d | – | – | ND | ND | 100.0 ± 0.0f | 44.4 ± 2.2e | 58.3 ± 9.1e | ND | ND |
| 16 | 127.7 ± 2.5c | – | – | ND | ND | 100.0 ± 0.0f | 70.7 ± 3.5f | 81.4 ± 4.0f | ND | ND |
| 32 | 88.3 ± 8.5b | 148.3 ± 32.5b | 143.3 ± 40.4b | ND | ND | 100.0 ± 0.0f | 100.0 ± 0.0g | 100.0 ± 0.0g | ND | ND |
| 64 | 54.0 ± 10.1a | 46.0 ± 3.6a | 88.7 ± 7.8a | ND | ND | 100.0 ± 0.0f | 100.0 ± 0.0g | 100.0 ± 0.0g | ND | ND |
| 128 | 32.0 ± 6.6a | 26.7 ± 5.7a | 55.0 ± 5.0a | ND | ND | 100.0 ± 0.0f | 100.0 ± 0.0g | 100.0 ± 0.0g | ND | ND |
| 256 | ND | ND | ND | – | – | ND | ND | ND | 14.0 ± 4.9b | 9.8 ± 1.8b |
| 512 | ND | ND | ND | – | – | ND | ND | ND | 20.9 ± 2.4c | 17.3 ± 5.8c |
| 1024 | ND | ND | ND | – | – | ND | ND | ND | 30.4 ± 2.5d | 25.5 ± 5.2d |
| 2048 | ND | ND | ND | – | – | ND | ND | ND | 58.3 ± 5.3e | 56.5 ± 7.5e |
| 4096 | ND | ND | ND | 155.0 ± 9.2c | 158.7 ± 11.5c | ND | ND | ND | 100.0 ± 0.0f | 100.0 ± 0.0f |
| 8192 | ND | ND | ND | 61.7 ± 5.9b | 65.0 ± 10.5b | ND | ND | ND | 100.0 ± 0.0f | 100.0 ± 0.0f |
| 16,384 | ND | ND | ND | 27.0 ± 5.6a | 31.7 ± 5.9a | ND | ND | ND | 100.0 ± 0.0f | 100.0 ± 0.0f |

**Table 4** Effect of plant ethanol extracts on reproduction of *I. multifiliis* nonencysted tomonts. Values are expressed as mean ± SD for three replicates. Values with different letters in the same column are significant differences ($p < 0.05$). MNET, mean number of encysted tomonts transformed from nonencysted tomonts at 6 h. MNTRLET, mean number of theronts released from each live encysted tomont at 22-h exposure. ND, not detectable in the tested concentration

| Concentration (mg/L) | MNET (mean ± SD) | | | | | MNTRLET (mean ± SD) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CA | ZO | CP | IA | IB | CA | ZO | CP | IA | IB |
| 0 (control) | 54 ± 2.6 | 54 ± 2.6 | 54 ± 2.6 | 54 ± 2.6a | 54 ± 2.6a | 210.3 ± 8.5a | 210.3 ± 8.5a | 210.3 ± 8.5a | 210.3 ± 8.5a | 210.3 ± 8.5a |
| 0.5 | 53 ± 2.0 | ND | ND | ND | ND | 170.1 ± 5.9b | ND | ND | ND | ND |
| 1 | 41 ± 14.0 | 50 ± 5.5 | 53 ± 4.4 | ND | ND | 158.6 ± 3.1b | 180.0 ± 12.8b | 191.6 ± 6.9a | ND | ND |
| 2 | 25 ± 3.5 | 41 ± 2.5 | 43 ± 8.2 | ND | ND | 50.7 ± 15.0c | 104.7 ± 10.1c | 153.8 ± 27.1b | ND | ND |
| 4 | 12 ± 2.5 | 32 ± 2.0 | 42 ± 10.4 | ND | ND | 0.0 ± 0.0d | 71.9 ± 5.7d | 112.1 ± 3.7c | ND | ND |
| 8 | 0.0 ± 0.0 | 24 ± 3.2 | 28 ± 3.0 | ND | ND | 0.0 ± 0.0d | 21.0 ± 9.5e | 34.1 ± 4.8d | ND | ND |
| 16 | 0.0 ± 0.0 | 10 ± 4.6 | 14 ± 3.5 | ND | ND | 0.0 ± 0.0d | 0.0 ± 0.0f | 0.0 ± 0.0e | ND | ND |
| 32 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | ND | ND | 0.0 ± 0.0d | 0.0 ± 0.0f | 0.0 ± 0.0e | ND | ND |
| 64 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | ND | ND | 0.0 ± 0.0d | 0.0 ± 0.0f | 0.0 ± 0.0e | ND | ND |
| 128 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | ND | ND | 0.0 ± 0.0d | 0.0 ± 0.0f | 0.0 ± 0.0e | ND | ND |
| 256 | ND | ND | ND | 52 ± 2.1 | 49 ± 1.0 | ND | ND | ND | 199.3 ± 8.9ab | 198.9 ± 4.9a |
| 512 | ND | ND | ND | 45 ± 1.5 | 47 ± 2.1 | ND | ND | ND | 177.3 ± 10.6b | 180.2 ± 6.4b |
| 1024 | ND | ND | ND | 37 ± 1.7 | 41 ± 3.6 | ND | ND | ND | 130.7 ± 13.7c | 141.5 ± 7.2c |
| 2048 | ND | ND | ND | 22 ± 4.2 | 29 ± 4.4 | ND | ND | ND | 84.8 ± 21.5d | 89.1 ± 13.1d |
| 4096 | ND | ND | ND | 0.0 ± 0.0 | 0.0 ± 0.0 | ND | ND | ND | 0.0 ± 0.0e | 0.0 ± 0.0e |
| 8192 | ND | ND | ND | 0.0 ± 0.0 | 0.0 ± 0.0 | ND | ND | ND | 0.0 ± 0.0e | 0.0 ± 0.0e |
| 16,384 | ND | ND | ND | 0.0 ± 0.0 | 0.0 ± 0.0 | ND | ND | ND | 0.0 ± 0.0e | 0.0 ± 0.0e |

concentration of 8 mg/L or lower. Theronts were released from live encysted tomonts after exposure to immunostimulant (A) or immunostimulant (B) ethanol extracts at 2048 mg/L or lower (Table 6).

*Effects of medicated feeds on I. multifiliis in grass carp*

When fish were fed using granule feeds with 4 % *C. paniculatum*, the infective incidence and survival rate were

**Table 5** Effect of plant ethanol extracts on reproduction of *I. multifiliis* encysted tomonts. Values are expressed as mean ± SD for three replicates. Values with different letters in the same column are significant differences ($p < 0.05$). MNET, mean number of encysted tomonts transformed from nonencysted tomonts at 6 h. MNTRLET, mean number of theronts released from each live encysted tomonts at 22-h exposure. ND, not detectable in the tested concentration

| Concentration (mg/L) | MNET (mean ± SD) | | | | | MNTRLET (mean ± SD) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CA | ZO | CP | IA | IB | CA | ZO | CP | IA | IB |
| 0 (control) | 45 ± 4.2 | 45 ± 4.2 | 45 ± 4.2 | 45 ± 4.2 | 45 ± 4.2 | 206.7 ± 2.1a | 206.7 ± 2.1a | 206.7 ± 2.1a | 206.7 ± 2.1a | 206.7 ± 2.1a |
| 0.5 | 42 ± 2.6 | 44 ± 3.0 | 42 ± 3.0 | ND | ND | 171.7 ± 6.0b | 170.5 ± 7.4b | 181.4 ± 7.5b | ND | ND |
| 1 | 39 ± 5.1 | 45 ± 5.0 | 46 ± 1.5 | ND | ND | 156.3 ± 9.5c | 155.2 ± 7.0c | 171.8 ± 4.5bc | ND | ND |
| 2 | 36 ± 7.4 | 40 ± 3.0 | 43 ± 6.0 | ND | ND | 45.7 ± 1.8d | 143.2 ± 2.8d | 161.4 ± 5.4c | ND | ND |
| 4 | 40 ± 2.0 | 38 ± 2.6 | 40 ± 4.0 | ND | ND | 0.0 ± 0.0e | 106.6 ± 3.9e | 126.2 ± 22.7d | ND | ND |
| 8 | 42 ± 4.4 | 41 ± 5.9 | 43 ± 5.1 | ND | ND | 0.0 ± 0.0e | 62.9 ± 4.0f | 38.8 ± 9.3e | ND | ND |
| 16 | 43 ± 4.0 | 40 ± 5.5 | 38 ± 7.5 | ND | ND | 0.0 ± 0.0e | 0.0 ± 0.0g | 0.0 ± 0.0f | ND | ND |
| 32 | 37 ± 4.0 | 41 ± 5.6 | 44 ± 5.5 | ND | ND | 0.0 ± 0.0e | 0.0 ± 0.0g | 0.0 ± 0.0f | ND | ND |
| 64 | 40 ± 4.5 | 37 ± 5.5 | 41 ± 7.1 | ND | ND | 0.0 ± 0.0e | 0.0 ± 0.0g | 0.0 ± 0.0f | ND | ND |
| 128 | 44 ± 10.5 | 42 ± 5.9 | 44 ± 6.0 | ND | ND | 0.0 ± 0.0e | 0.0 ± 0.0g | 0.0 ± 0.0f | ND | ND |
| 256 | ND | ND | ND | 49 ± 6.1 | 52 ± 3.8 | ND | ND | ND | 197.8 ± 8.9a | 210.3 ± 6.5a |
| 512 | ND | ND | ND | 49 ± 4.4 | 49 ± 1.5 | ND | ND | ND | 176.9 ± 6.2b | 176.0 ± 5.4b |
| 1024 | ND | ND | ND | 46 ± 7.8 | 45 ± 1.5 | ND | ND | ND | 143.8 ± 9.7c | 159.0 ± 11.9c |
| 2048 | ND | ND | ND | 42 ± 5.6 | 43 ± 7.5 | ND | ND | ND | 90.8 ± 9.9d | 103.5 ± 6.7d |
| 4096 | ND | ND | ND | 43 ± 2.1 | 39 ± 3.2 | ND | ND | ND | 0.0 ± 0.0e | 0.0 ± 0.0e |
| 8192 | ND | ND | ND | 37 ± 5.6 | 47 ± 4.6 | ND | ND | ND | 0.0 ± 0.0e | 0.0 ± 0.0e |

100 % in both the control and treatment groups (Table 7). The difference was not significant on the infective intensity between control group and treatment group.

The infective intensity was significantly lower in fish fed with feed at 4 % *C. atratum* for 10 days (55.0) than that in control (233.8) (Table 7). However, the infective intensity showed no significant difference compared with that in control fish fed with medicated feed for only 3 or 7 days. The infective incidence and survival rate were 100 % in both the control and treatment groups.

*Z. officinale* was antiparasitic against theronts infection. The infective intensity of fish fed with feed at 4 % *Z. officinale* was significant lower than that of the control fish (Table 7). The infective incidence and survival rates were 100 % in both the treatment and control groups.

The survival rates were all 100 % and the infective intensity were 312.7, 259.0, and 195.7 after fish were fed using feed with 4 % *C. atratum*, *Z. officinale*, and *C. paniculatum* for 3, 7, and 10 days, respectively (Table 7). After the trial, fish were fed with medicated granule feed for 10 days, the infective intensity was significantly lower than that of the control group. In the control group, the survival rate and infective intensity were 90 % and 286.0, respectively.

The grass carp fed with 4 % immunostimulant (A) feed had infective intensity of 1.3 and survival rate of 100 % after 8 days exposure to theronts (Table 8). In the treatment group, the infective intensity decreased to 0 at 15 days post theront exposure. The infective incidence of treatment group was significantly lower than that of the control. In the control group, the survival rate was 0 8 days post theront exposure.

## Discussion

The dried roots of *C. atratum*, *Z. officinale*, and *C. paniculatum* are popular traditional Chinese medicines, and have been widely used for thousands of years and officially listed in the Chinese Pharmacopoeia. *C. atratum* is a perennial herb native to East Asia and its roots have been used for treating hectic fever, acute urinary infection, and abscesses in China (Bai et al. 2005, 2008). Pharmacological studies on this medical plant have demonstrated anti-tobacco mosaic virus (TMV), anti-inflammatory, anti-Amnesic, and acetylcholinesterase-inhibitory activities (Bai et al. 2005, 2008; Lee et al. 2003a, 2005; Yan et al. 2014). Biologically active compounds in *C. atratum* include cynatratoside-A, cynatratoside-B, cynatratoside-D, cynatratoside-E, and cynanosides A-J.

*Z. officinale* has been proven to contain essential oil, zingiberone, zingiberene, zingerone, [6]-gingerol, and [6]-shogaol (Lin et al. 2014). The previous studies have indicated that *Z. officinale* has anti-cancer, antinociceptive, anti-inflammatory, anti-hyperglycemic, anti-hyperlipidemic, antioxidant, and anti-emetic properties (Abdel-Azeem et al. 2013; Jeena et al. 2013, 2014). In addition, a high efficacy of extract of *Z. officinale* roots against *Hymenolepis nana* and *Streptococcus mutans* has been reported (Hasan et al. 2015; Lin et al. 2014).

In the *Asclepiadaceae* family, *C. paniculatum* is mainly distributed in East Asia. This plant has shown many biological activities such as anti-aggregatory, anti-inflammatory, antimicrobial, antioxidant, and cytotoxic activities (Kim et al. 2013a, b; Lee et al. 2003b). Acetophenone derivatives containing hydroxyl group, methoxy group, and homosyringaldehyde were isolated and identified from *C. paniculatum* roots (Kim et al. 2013b; Weon et al. 2012). The acaricidal activities of acetophenone and its derivatives against *Dermatophagoides* spp. and *Tyrophagus putrescentiae* have been confirmed using an impregnated fumigant bioassay (Kim et al. 2013b). Homosyringaldehyde showed significant neuroprotective against glutamate-induced neurotoxicity in hippocampal HT22 cell line in a previous research (Weon et al. 2012).

To the best of our knowledge, this study is the first report to show anti-Ich efficacy of the ethanol extracts of *C. atratum*, *Z. officinale*, and *C. paniculatum*. The bioassay results indicated that the minimum doses of ethanol extracts for killing all theronts were 16, 8, and 16 mg/L of *C. atratum*, *Z. officinale*, and *C. paniculatum*, respectively. In comparison to the reported data in previous studies, the anti-Ich efficacy of *C. atratum*, *Z. officinale*, and *C. paniculatum* ethanol extracts were better than garlic (*Allium sativum*), *Magnolia officinalis*, *Sophora alopecuroides* extracts, and some other chemicals. Garlic extract killed all theronts at 62.5 mg/L within 15 h (Buchmann et al. 2003). *M. officinalis* and *S. alopecuroides* methanol

**Table 6** Efficacy of plant ethanol extracts on *I. multifiliis* theronts or nonencysted tomonts. $EC_{50}$, median effective concentration. 95 % CI, 95 % confidence interval

| Plant ethanol extract | Anti-theronts efficacy at 4 h | | Anti-nonencysted tomonts efficacy at 5 h | |
|---|---|---|---|---|
| | $EC_{50}$ (mg/L) | 95 % CI (mg/L) | $EC_{50}$ (mg/L) | 95 % CI (mg/L) |
| CA | 4.87 | 3.04-8.69 | 2.75 | 2.52-3.01 |
| ZO | 2.53 | 2.11-3.09 | 9.96 | 7.74-13.28 |
| CP | 6.45 | 5.08-8.70 | 7.48 | 5.16-10.93 |
| IA | 407.32 | 332.48-514.91 | 1631.30 | 1244.99-2262.46 |
| IB | 419.65 | 332.30-551.87 | 1726.24 | 1387.89-2244.53 |

**Table 7** Effects of medicated feeds with *C. atratum*, *Z. officinale*, and *C. paniculatum* on infective intensity, infective incidence, and survival rate in grass carp after exposure to *I. multifiliis*. There were triplicate tanks with 10 fish each for each treatment. Values are expressed as mean ± SD. Values with different letters in the same column are significant differences ($p < 0.05$). Infective intensity = number of trophonts on grass carp/infected fish number. Infective incidence (%) = (number of infected fish / fish number) × 100. Survival rate (%) = (number of remaining live fish / fish number) × 100. DGCFMF, days for which the grass carp were fed with medicated feed. DGCET, days after grass carp exposed to theronts

| Treatment | DGCFMF (day) | DGCET (day) | Infective intensity | Infective incidence (%) | Survival rate (%) |
|---|---|---|---|---|---|
| Control | 10 | 5 | 248.9 ± 96.2a | 100.0 ± 0.0a | 100.0 ± 0.0a |
| CP | 3 | | 241.3 ± 44.5a | 100.0 ± 0.0a | 100.0 ± 0.0a |
| | 7 | | 213.3 ± 15.3a | 100.0 ± 0.0a | 100.0 ± 0.0a |
| | 10 | | 202.7 ± 24.0a | 100.0 ± 0.0a | 100.0 ± 0.0a |
| Control | 10 | 5 | 233.8 ± 13.9a | 100.0 ± 0.0a | 100.0 ± 0.0a |
| CA | 3 | | 174.0 ± 15.1a | 100.0 ± 0.0a | 100.0 ± 0.0a |
| | 7 | | 179.8 ± 40.3a | 100.0 ± 0.0a | 100.0 ± 0.0a |
| | 10 | | 55.0 ± 8.3b | 100.0 ± 0.0a | 100.0 ± 0.0a |
| Control | 10 | 5 | 236.8 ± 68.6a | 100.0 ± 0.0a | 100.0 ± 0.0a |
| ZO | 3 | | 114.6 ± 15.0b | 100.0 ± 0.0a | 100.0 ± 0.0a |
| | 7 | | 70.1 ± 8.2b | 100.0 ± 0.0a | 100.0 ± 0.0a |
| | 10 | | 92.3 ± 22.8b | 100.0 ± 0.0a | 100.0 ± 0.0a |
| Control | 10 | 5 | 286.0 ± 48.3a | 100.0 ± 0.0a | 90.0 ± 10.0a |
| CSP | 3 | | 312.7 ± 21.5a | 100.0 ± 0.0a | 100.0 ± 0.0a |
| | 7 | | 259.0 ± 61.0ab | 100.0 ± 0.0a | 100.0 ± 0.0a |
| | 10 | | 195.7 ± 12.5b | 100.0 ± 0.0a | 100.0 ± 0.0a |

extracts killed all theronts at 10 mg/L within 3 or 4 h, respectively (Yi et al. 2012). Potassium ferrate killed all theronts at 24 mg/L within 30 min (Ling et al. 2010).

This study indicated that the effective dose of *Z. officinale* extract to tomonts was 16 mg/L. The toxicity of *Z. officinale* extract to tomonts was much lower than that to theronts. The same phenomenon was also reported in previous studies (Buchmann et al. 2003; Yi et al. 2012). Tomonts are protected by a cyst wall and show better resistibility to *Z. officinale* extract than theront. However, *C. atratum* extract caused 100 % mortality of *I. multifiliis* encyst tomonts at 4 mg/L and demonstrated to be toxic to *I. multifiliis* encysted tomonts at low concentration. A higher concentration of *C. atratum* extract was needed to induce acute toxicity on theronts. Moreover, the minimum doses of *C. paniculatum* for killing all theronts and tomonts were 16 mg/L.

Medicated feed are not affected by water quality and easy to use in aquaculture (Abdel-Hafez et al. 2014). In several previous studies, medicated feed was demonstrated effective against ichthyophthiriasis or other diseases. Medicated feeds with acetylsalicylic acid, doxycycline, erythromycin, imidocarb dipropionate, sulphadiazine, toltrazuril, and quinine have been shown effective against *I. multifiliis* (Abdel-Hafez et al. 2014; Schumacher et al. 2011). Sulfonamides, dihydrofolate reductase inhibitors, and their combinations have been used in feed to control *Cryptocaryon irritans*

**Table 8** Effects of medicated feeds with immunostimulant (A) on infective intensity, infective incidence, and survival rate in grass carp after exposure to *I. multifiliis*. There were triplicate tanks with 10 fish for each treatment. Values are expressed as mean ± SD. Values with different letters in the same column are significant differences ($p < 0.05$). Infective intensity = number of trophonts on grass carp/infected fish number. Infective incidence (%) = (number of infected fish / fish number) × 100. Survival rate (%) = (number of remaining live fish / fish number) × 100. DGCFMF, days for which the grass carp were fed with medicated feed. DGCET, days after grass carp exposed to theronts. (−), all fish were dead

| Treatment | DGCFMF (day) | DGCET (day) | Infective intensity | Infective incidence (%) | Survival rate (%) |
|---|---|---|---|---|---|
| Control | 21 | 8 | – | 100.0 ± 0.0a | 0.0 ± 0.0a |
| IA | | | 1.3 ± 1.5 | 5.0 ± 5.0b | 100.0 ± 0.0b |
| Control | 21 | 15 | – | – | – |
| IA | | | 0.0 ± 0.0 | 0.0 ± 0.0 | 100.0 ± 0.0 |

infection in *Pagrus major* and *I. multifiliis* infection in *Carassius auratus* (Kawano and Hirazawa 2012). Medicated feed with nicarbazin at the concentration of at least 2.5 g/kg has been proven effective against *Kudoa thyrsites* in Atlantic salmon (Jones et al. 2012). This study was first reported to demonstrate medicated feeds with traditional Chinese plants to prevent grass carp from *I. multifiliis* infection.

In vitro experiments, *C. atratum*, *Z. officinale*, and *C. paniculatum* were effective against *I. multifiliis*. In vivo experiment, there was no significant reduction of trophont numbers on grass carp after 10 days medicated feed with 4 % *C. paniculatum*, and then challenged with theronts. Grass carp fed medicated feeds of *C. atratum* for 10 days, or *Z. officinale* for 3 days, or combination of three plant extracts for 10 days resulted in a significant reduction on the *I. multifiliis* infective intensity, but the parasites in fish were not completely killed. A similar result was reported by Schumacher et al. (2011). This result was attributed to that the substance may not be absorbed from the intestinal tract of grass carp and the concentration of anti-Ich compound was low when reaching to trophonts within fish tissues.

In vitro experiments, both immunostimulant (A) and immunostimulant (B) can kill all theronts at the concentration of 1024 mg/L or higher. The median effective concentration ($EC_{50}$) of immunostimulant (A) for killing theronts was 407.32 mg/L, which was approximately 80, 160, and 60 times the $EC_{50}$ of *C. atratum*, *Z. officinale*, and *C. paniculatum* to theronts, respectively. In vivo experiment, the grass carp were not infected by the parasites and showed a significantly higher survival than that of control after 21 days of medicated feed with 4 % immunostimulant (A) and then challenged with theronts. The infective intensity was lower in fish fed wih immunostimulant (A) than the infective intensity of fish fed medicated feeds with *C. atratum*, *Z. officinale*, or *C. paniculatum*. The ingredients of immunostimulant (A) include *Astragalus membranaceus*, *Lonicera japonica*, *Allium sativum*, *Crataegus pinnatifida*, *Aloe barbadensis*, *Atractylodes macrocephala*, *Isatidis radix*, *Angelica sinensis*, *D. morifolium*, and *Morus alba*. Among them, *Astragalus membranaceus*, *Lonicera japonica*, *Allium sativum*, and *Angelica sinensis* were able to enhance phagocytic activity of macrophage and lysozyme, and complement activities (Ardó et al. 2008; Jian and Wu 2004; Sahu et al. 2007). Base on the result in this study, immunostimulant (A) may be considered to enhance the non-specific response of grass carp against *I. multifiliis* infection.

Medicated feeds with the antiparasitic plants such as *C. atratum*, *Z. officinale*, or *C. paniculatum* for 10 days were not effective to treat *I. multifiliis* in grass carp. However, the dietary supplementation with 4 % immunostimulant (A) for 21 days can be effective to prevent grass carp from Ich infection.

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