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Cynatratoside-C efficacy against theronts of *Ichthyophthirius multifiliis*, and toxicity tests on grass carp and mammal blood cells

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ABSTRACT: Infection by *Ichthyophthirius multifiliis*, a ciliated protozoan parasite, results in high fish mortality and causes severe economic losses in aquaculture. To find new, efficient anti-*I. multifiliis* agents, cynatratoside-C was isolated from *Cynanchum atratum* by bioassay-guided fractionation in a previous study. The present study investigated the anti-theront activity, determined the toxicity of cynatratoside-C to grass carp *Ctenopharyngodon idellus* and mammalian blood cells, and evaluated the protection of cynatratoside-C against *I. multifiliis* theront infection in grass carp. Results showed that all theronts were killed by 0.25 mg l^{-1} of cynatratoside-C in 186.7 ± 5.8 min. Cynatratoside-C at 0.25 mg l^{-1} was effective in treating infected grass carp and protecting naive fish from *I. multifiliis* infestation. The 96 h median lethal concentration (LC_{50}) of cynatratoside-C to grass carp and 4 h median effective concentration (EC_{50}) of cynatratoside-C to theront were 46.8 and 0.088 mg l^{-1}, respectively. In addition, the hemolysis assay demonstrated that cynatratoside-C had no cytotoxicity to rabbit red blood cells. Therefore, cynatratoside-C could be a safe and effective potential parasiticide for controlling *I. multifiliis*.

KEY WORDS: Cynatratoside-C · *Cynanchum atratum* · *Ctenopharyngodon idellus* · Antiparasitic activity · Toxicity · *Ichthyophthirius multifiliis*

INTRODUCTION

Ichthyophthirius multifiliis (Ich), belonging to the Ophryoglenidae, is a freshwater fish ciliate parasite, with a life cycle including an infective theront, a parasitic trophont, and a reproductive tomont (Buchmann et al. 2001, Dickerson & Findly 2014). Theronts and tomonts live in water, and trophonts parasitize fish skin and gills (Matthews 2005, Zhang et al. 2013). The parasite causes 'white spot' disease which results in high fish mortality and heavy economic losses in aquaculture (Shinn et al. 2012, Xu & Klesius 2013)

Chemical treatment is commonly used to treat fish white spot disease. However, in aquaculture, a lack of safe and effective chemical therapeutants exists (Tieman & Goodwin 2001). Hence, it is necessary to discover new effective, safe, and degradable compounds as potential anti-*I. multifiliis* drugs. Several studies have demonstrated that plant products have effective and safe anti-*I. multifiliis* properties (Buchmann et al. 2003, Yi et al. 2012, Zhang et al. 2013). In

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the past decade, 88 plant extracts were reported to be used to treat *I. multifiliis in vitro* or *in vivo*, showing various antiparasitic effects (Buchmann et al. 2003, Ekanem et al. 2004, Chu et al. 2010, Yao et al. 2010, 2011, Gholipour-Kanani et al. 2012, Ling et al. 2012, 2013, Yi et al. 2012, Zhang et al. 2013, Fu et al. 2014a,b, Shan et al. 2014, Zheng et al. 2015). Fourteen anti-*I. multifiliis* compounds have been isolated and purified from 9 plants since 2010 by using bioassay-guided fractionation (Yao et al. 2010, 2011, Zhang et al. 2013, Fu et al. 2014a, Liang et al. 2014, 2015, Shan et al. 2014, Song et al. 2015, Zheng et al. 2015). The plant compounds are environmentally friendly, as they are easily biodegradable (Reverter et al. 2014). Therefore, these plant products could be used in aquaculture as promising anti-*I. multifiliis* agents.

A class of such plant products are C-21 steroidal glycosides. These compounds contain a pregnane skeleton and are the major constituents of Asclepiadaceae which exhibit various bioactivities (Ni & Ye 2010). Their structures have been classified into polyhydroxypregnane-type glycosides or seco-pregnane-type glycosides (Bai et al. 2009). Cynatratoside-C was first isolated from *Cynanchum atratum* as one of the seco-pregnane-type glycosides (Zhang et al. 1985). Previous work demonstrated that cynatratoside-C at 0.25 mg l^{-1} caused 100% mortality of tomonts *in vitro* after a 5 h exposure, at 0.06 mg l^{-1} inhibited the development of theronts from tomonts, and at 2 mg l^{-1} cured the infected grass carp within 48 h (Fu et al. 2014a). However, to date, no information on anti-theront activity or on the toxicity of cynatrasoside-C for fish is available. These data are needed to determine whether cynatrasoside-C could be a promising parasiticide. Thus, the objective of this study was to test cynatratoside-C for its anti-theront activity, and its potential to prevent or treat 'white spot' disease. Further, its toxicity to grass carp and mammal blood cells was investigated.

MATERIALS AND METHODS

Fish and parasite

Grass carp *Ctenopharyngodon idellus* (Cyprinidae), with a mean (±SD) length of 13.1 ± 1.7 cm, were obtained from a commercial fish farm at Huadu, Guangzhou City, Guangdong Province. All fish were kept in 100 l opaque tanks. The water temperature was maintained at 23.0 ± 0.3°C, and the fish were fed daily with granule feed (Haid, Guangzhou, China) at 1% of fish weight. *Ichthyophthirius multifiliis* was originally isolated from goldfish obtained from the ornamental fish market of Guangzhou and maintained by serial transmission on grass carp as previously described (Fu et al. 2014b). Mature trophonts were collected according to Zhang et al. (2013). Isolated trophonts were placed in petri dishes with dechlorinated water and incubated at 23.0 ± 0.3°C for 22 h. After theronts were released, the average number of theronts per 100 µl of water was determined with the aid of a microscope (4×) according to Zhang et al. (2013).

Preparation of cynatratoside-C

The roots of *Cynanchum atratum* (200 kg) were purchased from the Chinese medicinal market at Guangzhou, China, and powdered by a pulverizer with 50 mesh strainer. Then the dry root powder was extracted with 95% ethanol in an extracting and concentrating tank (Tianzhong, Wenzhou, China) at Guangzhou University of Chinese Medicine. Purification and analytic methods followed those described by Fu et al. (2014a). Briefly, column chromatography was performed using 200 to 300 mesh and 100 to 200 mesh silica gels (Qingdao Marine Chemical). Preparative high-performance liquid chromatography (HPLC) was conducted with an Agilent 1100 series HPLC system (Agilent) equipped with a XB-C18 column (5 µm, 21.2 mm × 250 mm; Welch). Another Agilent 1100 series instrument (Agilent) equipped with a TC-C18 column (5 µm, 4.6 mm × 250 mm; Agilent) was used to analyze the purity of active compounds. Finally, cynatratoside-C (11.3 g) was isolated and purified from the ethanol extract (40 kg).

In vitro bioactivity of cynatratoside-C on *I. multifiliis* theronts

A stock solution of cynatratoside-C was prepared by dissolving 5 mg of the substance in 2.5 ml of dechlorinated water containing DMSO (1%, v/v), resulting in a final concentration of 2000 mg l^{-1}. The 6.4 µl stock solution was added to 93.6 µl dechlorinated water to make a 128 mg l^{-1} cynatratoside-C solution. The 128 mg l^{-1} cynatratoside-C solution was serially diluted with 100 µl dechlorinated water in a 96-well plate to concentrations of 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03, 0.015, and 0 (negative control) mg l^{-1}. There were 3 replicates for each concen-

tration. Then, 100 µl of theront solution containing about 200 theronts was added to each well of a 96-well plate; the final concentrations of cynatratoside-C were 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03, 0.015, 0.0075, and 0 mg l^{-1}, respectively. Malachite green at concentrations of 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03, 0.015, and 0.0075 mg l^{-1} was used as a positive control. The duration until death of all theronts was monitored continually for 4 h. The numbers of live theronts in each well were counted under a microscope (4×) for 4 h, according to Zhang et al. (2013).

To monitor pathological changes in theronts after exposure to cynatratoside-C, approximately 200 theronts in 100 µl of dechlorinated freshwater were placed into 1 well of a 96-well plate, and 100 µl of 16 mg l^{-1} cynatratoside-C solution was added into the well to make a final concentration of 8 mg l^{-1}. The treated theronts were observed and photographed under a Nikon ECLIPSE Ti-S inverted microscope (Nikon) at 40× magnification.

Evaluation of cynatratoside-C in protection of grass carp against Ich

Cynatratoside-C weighing 240 mg was dissolved in 24 ml of dechlorinated freshwater containing 1% (v/v) dimethyl sulfoxide (DMSO) to make 10 000 mg l^{-1} (Stock Solution 1). Then 15, 7.5, 1.5, and 0 ml of Stock Solution 1 were diluted with 15, 22.5, 28.5, and 30 ml of dechlorinated freshwater, respectively, to give final concentrations of the 4 diluted solutions (Stock Solutions 2) of 5000, 2500, 500, and 0 (control) mg l^{-1} in 30 ml. The Stock Solutions 2 were stored at 4°C in a refrigerator prior to use.

A total of 120 naive grass carp were randomly divided into 12 tanks (15 l) with 10 fish and 10 l of static dechlorinated water per tank. Sixty fish lightly infected with Ich (38 ± 10 white spots on body surface per fish) were marked by clipping the caudal fin. Then, 5 infected fish were randomly selected, transferred to each tank, and cohabitated with 10 naive fish. Then, 1 ml of cynatratoside-C Stock Solutions 2 was added to 1 of 12 tanks to make final concentrations of 0.5, 0.25, 0.05, and 0 (control) mg l^{-1}, respectively. There were 3 replicates for each concentration. Water in each tank was completely replaced daily for 30 d with fresh water, and cynatratoside-C Stock Solutions 2 were added to tanks to make designated (final) concentrations daily for the first 10 d. Dead fish were counted, recorded, and removed twice a day during the trial. On Days 15 and 30 following the initial day of exposure to cynatratoside-C liquids, all the grass carp were anesthetized with 150 mg l^{-1} MS-222, then the infection incidence and intensity (as defined by Bush et al. 1997), as well as the survival of infected and naive fish, were determined for each concentration.

Acute toxicity of cynatratoside-C to grass carp

In a preliminary acute toxicity trial, cynatratoside-C at 100, 55, and 10 mg l^{-1} was used to determine the concentrations resulting in 0 or 100% fish mortality. Cynatratoside-C at 100 and 10 mg l^{-1} caused 100 and 0% fish mortality, respectively. Based on initial results, further acute toxicity trials were conducted. Ten fish were distributed to 1 of 15 tanks containing 10 l of water. Cynatratoside-C stock solutions were added to make concentrations of 0 (control), 20, 40, 55, and 70 mg l^{-1} in triplicate tanks for each concentration. Water in the tanks was completely replaced daily with fresh cynatratoside-C solution at the designated concentration. Dead fish were counted, recorded, and removed twice a day during the 96 h trial. The median lethal concentration (LC_{50}) of cynatratosid-C to grass carp was determined at 24, 48, 72, and 96 h.

Hemolysis assay

Rabbit blood (4 ml) from the auricular vessels of a healthy New Zealand white rabbit was centrifuged at 685 × *g* for 5 min. The plasma was removed and red blood cells (RBCs) were washed 5 times with 0.9% sodium chloride solution. Then, 0.5 ml of RBCs was suspended in 49.5 ml of 0.9% saline solution to make a 1% blood cell solution (v/v). The cynatratoside-C was added to the 0.9% saline solution (6 ml) to make final concentrations of 200, 1000, and 2000 mg l^{-1}, and then incubated at 37°C for 30 min. A mixture of 1.5 ml RBC suspension and 1.5 ml cynatratoside-C solution were prepared in a 5 ml centrifuge tube, and incubated at 37°C for 60 min. There were 3 replications in each group. After incubation, the mixtures were centrifuged at 685 × *g* for 5 min. The supernatant was collected and measured for optical density at 575 nm using an UV-2450 UV-visible spectrophotometer (Shimadzu). Distilled water was used as the positive control, and 0.9% saline solution was used as the negative control. The hemolysis rate (HR) was calculated according to the equation: $100 \times (A_t - A_n)/(A_p - A_n)$, where A_t, A_n, and A_p were the optical

density of the supernatant fraction of the mixtures in contact with the test, negative, and positive control, respectively.

Statistical analysis

Statistical analyses were performed with Student-Newman-Keul's test using 1-way ANOVA in a statistical analysis system software package (SPSS 19.0) with p-values <0.05 considered significant. All experimental data were expressed as means (±SD). A probit procedure was used to determine median effective and lethal concentrations (EC_{50} and LC_{50}) with 95% confidence intervals.

RESULTS

In vitro bioactivity of cynatratoside-C on *Ichthyophthirius multifiliis* theronts

Theront mortality showed a positive response to the concentrations of cynatratoside-C (Table 1). Mortalities of theronts ranged from 100% at 0.25 mg l^{-1} and 59.5% at 0.125 mg l^{-1} to 0% at 0 mg l^{-1} 4 h after exposure to cynatratoside-C. Mean durations until death of all theronts were significantly shorter in high concentrations of cynatratoside-C and ranged from 3 min at 32 mg l^{-1} to 186.7 min at 0.25 mg l^{-1}. In the malachite green treatment group (positive control), the mean duration until death of all theronts was 123 min at 0.06 mg l^{-1} and 0.5 min at 8 mg l^{-1}. The 4 h EC_{50} of cynatratoside-C to theronts was 0.088 mg l^{-1} (see Table 4), and the 4 h EC_{50} of malachite green to theronts was 0.021 mg l^{-1}. After exposure to cynatratoside-C at 8 mg l^{-1} for 7 min, most theronts lost coordination, ceased swimming forwards, and became spherical in shape. Subsequently, the plasma membranes of the theronts broke, and the cytoplasm spilled out (Fig. 1).

Table 1. Effect of cynatratoside-C on survival of *Ichthyophthirius multifiliis* theronts. Values are means ± SD of 3 replicates. Values with different letters in the same column are significantly different ($p < 0.05$). MDDT: mean duration until death of all theronts (maximum observation time 4 h); MT: mortality of *I. multifiliis* theronts after 4 h; NC: negative control; PC: positive control; ND: not detectable at the tested concentrations; –: cynatratoside-C at the listed concentrations did not kill 100% theronts after 4 h exposure

Concentration (mg l^{-1})	MDDT (min)		MT (%)	
	Cynatratoside-C	Malachite green (PC)	Cynatratoside-C	Malachite green (PC)
0 (NC)	–	–	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
0.0075	–	–	0.0 ± 0.0^{a}	3.7 ± 3.3^{a}
0.015	–	–	3.1 ± 2.8^{a}	27.8 ± 6.4^{b}
0.03	–	–	19.3 ± 12.9^{b}	85.0 ± 4.0^{c}
0.06	–	122.3 ± 8.7^{a}	32.2 ± 13.3^{c}	100.0 ± 0.0^{d}
0.125	–	75.3 ± 4.6^{b}	51.9 ± 4.8^{d}	100.0 ± 0.0^{d}
0.25	186.7 ± 5.8^{a}	37.3 ± 3.8^{c}	100.0 ± 0.0^{e}	100.0 ± 0.0^{d}
0.5	83.3 ± 11.5^{b}	18.0 ± 1.7^{d}	100.0 ± 0.0^{e}	100.0 ± 0.0^{d}
1	43.3 ± 2.9^{c}	5.3 ± 0.6^{e}	100.0 ± 0.0^{e}	100.0 ± 0.0^{d}
2	12.3 ± 2.1^{d}	1.0 ± 0.0^{f}	100.0 ± 0.0^{e}	100.0 ± 0.0^{d}
4	7.7 ± 0.6^{de}	0.5 ± 0.0^{f}	100.0 ± 0.0^{e}	100.0 ± 0.0^{d}
8	6.0 ± 0.0^{de}	0.5 ± 0.0^{f}	100.0 ± 0.0^{e}	100.0 ± 0.0^{d}
16	4.0 ± 0.0^{e}	ND	100.0 ± 0.0^{e}	ND
32	3.0 ± 0.0^{e}	ND	100.0 ± 0.0^{e}	ND

Evaluation of cynatratoside-C in protection of grass carp against Ich

The infection incidence and intensity of Ich decreased significantly with the increase of cynatratoside-C concentrations for both infected and naive grass carp (Table 2). The infected and naive fish treated with cynatratoside-C at 0.25 mg l^{-1} showed mean infection intensities of 0.7 ± 1.2 and 0.8 ± 1.4 parasites per fish at Day 15, respectively. The infection intensity decreased to 0 for infected and naive fish at Day 30. The fish treated with cynatratoside-C at 1 mg l^{-1} demonstrated 0 infection incidence and intensity. All infected and naive fish treated with cynatratoside-C at 0 or 0.05 mg l^{-1} were heavily infected by Ich and dead between Days 15 and 30. All fish survived at concentrations of 0.25 and 1 mg l^{-1}.

Acute toxicity

The grass carp showed different mortalities when treated with various concentrations of cynatratoside-C, and fish mortality increased with increasing dose and exposure time (Table 3). All fish treated with 100 or 70 mg l^{-1} cynatratoside-C died within 24 and 48 h, respectively. The fish in tanks with 55 or 40 mg l^{-1} cynatratoside-C showed mortalities of 73 ± 6 and 30 ± 10%, respectively, on the third day. No fish was found dead at a concentration of 55 or 40 mg l^{-1} cynatratoside-C on Day 4 or at a concentration of 20 or 0 mg l^{-1} cynatratoside-C during the 96 h exposure. Both the 72 h

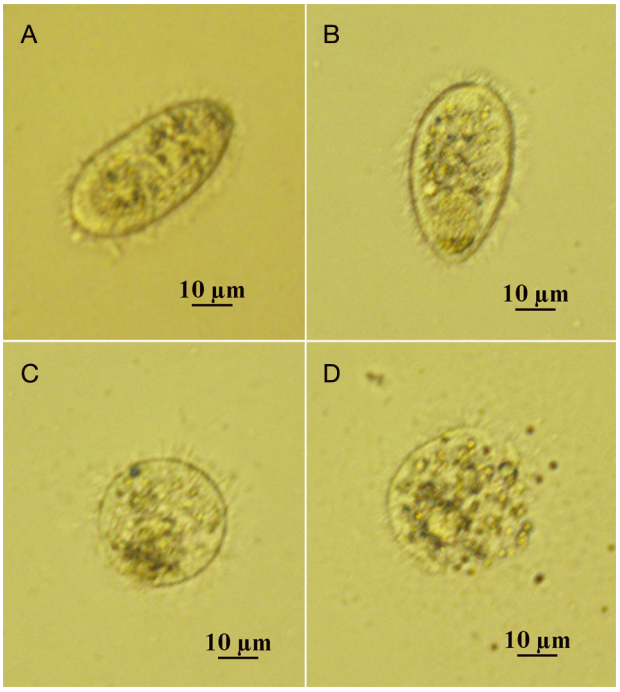

Fig. 1. *Ichthyophthirius multifiliis* theront morphology. (A) Theront untreated with cynatratoside-C. (B,C) Theront exposed to 8 mg l^{-1} cyantratoside-C for 7 min, the theront lost co-ordination, ceased swimming forwards, and became spherical in shape. (D) Subsequently, the plasma membrane broke, and cytoplasm spilled out of the theront

and 96 h LC_{50} values were 46.8 mg l^{-1}, with a 95% confidence interval of 43.0 to 50.3 mg l^{-1} (Table 4).

Hemolysis assay

Results of the hemolysis test are given in Table 5. The optical density in the negative and positive control groups were 0 and 0.598, respectively. Cynatratoside-C at the concentrations of 1000, 500, and 100 mg l^{-1} induced 5, 1.8, and 1.3% RBC hemolysis, respectively, and demonstrated weak cytotoxicity to RBCs.

DISCUSSION

Cynatratoside-C, a main anti-*Ichthyophthirius multifiliis* compound in *Cynanchum atratum*, is one of the C-21 steroidal glycosides and has a carbohydrate chain at the C-3 position in the 13,14:14,15-diseco-pregnane-type skeleton. It was postulated that the type of carbohydrate chain might influence the anti-parasitic effect (Fu et al. 2014b). The anti-tomont and anti-trophont properties of cynatratoside-C have been reported previously (Fu et al. 2014b). The present work is the first report on an anti-theront property, the potential to prevent or treat white spot disease, and on the toxicity of cynatratoside-C to grass carp and mammal blood cells. The theront is the infective stage within the life cycle of *I. multifiliis* and freely swims in water. One reproductive tomont can produce several hundred to a thousand infective theronts in about 22 h at 23°C (Xu et al. 2012, Dickerson & Findly 2014). Killing Ich theronts is an important way to protect fish from infection (Zhang et al. 2013). This study evaluated the parasiticidal effects of cynatratoside-C on theronts, and the results showed that cynatratoside-C significantly reduced theront survival even at low concentrations. Within a 4 h *in vitro* exposure time, cynatratoside-C killed all Ich theronts at a lower concentration (0.25 mg l^{-1}) than many other compounds, e.g. acetone and ethyl acetate extracts of *Morus alba* root bark, which had the same effect within 4 h, but required a much

Table 2. Effect of a 10 d cynatratoside-C treatment on *Ichthyophthirius multifiliis* infection incidence, infection intensity, and trial grass carp survival at Days 15 and 30. Values are means ± SD of 3 replicate tanks per treatment. Values with different letters in the same column are significantly different ($p < 0.05$). IGC: infected grass carp; NGC: naive grass carp; –: all fish dead; infection incidence (%): (infected fish/total fish) × 100; infection intensity: number of white spots on fish/number of infected fish

Day	Concentration (mg l^{-1})	Grass carp/tank		Infection incidence (%)		Infection intensity		Survival rate (%)	
		IGC	NGC	IGC	NGC	IGC	NGC	IGC	NGC
15	0 (control)	5	10	100 ± 0^a	100 ± 0^a	75.2 ± 48^a	52.7 ± 28.0^a	73.3 ± 23.1^a	100 ± 0^a
	0.05	5	10	100 ± 0^a	100 ± 0^a	56.3 ± 21.2^a	38.3 ± 19.0^a	80 ± 20.0^a	100 ± 0^a
	0.25	5	10	20 ± 34.6^b	13.3 ± 23.1^b	0.7 ± 1.2^b	0.8 ± 1.4^b	100 ± 0^a	100 ± 0^a
	1	5	10	0 ± 0^b	0 ± 0^b	0 ± 0^b	0 ± 0^b	100 ± 0^a	100 ± 0^a
30	0 (control)	5	10	–	–	–	–	0 ± 0	0 ± 0
	0.05	5	10	–	–	–	–	0 ± 0	0 ± 0
	0.25	5	10	0 ± 0	0 ± 0	0 ± 0	0 ± 0	100 ± 0	100 ± 0
	1	5	10	0 ± 0	0 ± 0	0 ± 0	0 ± 0	100 ± 0	100 ± 0

Table 3. Acute toxicity of cynatratoside-C to grass carp according to concentration and exposure time. Values of mortality are means ± SD of 3 replicate tanks per treatment (10 fish per tank)

Concentration (mg l^{-1})	No. of dead grass carp after 24 h	48 h	72 h	96 h	Mortality (%)
100	30	0	0	0	100 ± 0
70	0	30	0	0	100 ± 0
55	0	0	22	0	73 ± 6
40	0	0	9	0	30 ± 10
20	0	0	0	0	0 ± 0
0	0	0	0	0	0 ± 0

Table 4. Median lethal concentrations (LC_{50}) of cynatratoside-C to grass carp, and median effective concentration (EC_{50}) of cynatratoside-C to theronts

Anti-theront efficacy at 4 h EC_{50} (mg l^{-1})		— Acute toxicity to fish — Time	LC_{50} (mg l^{-1})	
Median	95% CI	(h)	Median	95% CI
0.088	0.037–0.181	24	83.8	77.9–91.0
		48	62.0	59.2–64.9
		72	46.8	43.0–50.3
		96	46.8	43.0–50.3

Table 5. Percent hemolysis of rabbit red blood cells after 30 min incubation with different concentrations of cynatratoside-C. Values are means ± SD of 3 replicates. Within a column, values with different letters are significantly different ($p < 0.05$). A_t, A_n, and A_p are the optical density (OD) of the supernatant fraction of the mixtures in contact with the test, negative, and positive control, respectively. Hemolysis rate (HR, %) = $100 \times (A_t - A_n)/(A_p - A_n)$

Concentration (mg l^{-1})	OD	HR (%)
1000	0.030 ± 0.001	5.0 ± 0.1[a]
500	0.010 ± 0.002	1.8 ± 0.3[b]
100	0.008 ± 0.002	1.3 ± 0.3[c]
Negative control	0 ± 0	0 ± 0[d]
Positive control	0.598 ± 0.001	100 ± 0.1[e]

higher concentration of 8 mg l^{-1} (Fu et al. 2014b). The plant-derived compound pentagalloylglucose from *Galla chinensis* killed all theronts at a concentration of 2.5 mg l^{-1} in 233.9 min (Zhang et al. 2013). Chemical compounds, such as potassium ferrate (VI), at a concentration of 4.8 mg l^{-1}, resulted in 100% mortality of theronts in 4 h (Ling et al. 2010). An N-butanol extract of *Streptomyces griseus* SDX-4 was effective against theronts with EC_{50} values of 0.86 mg l^{-1} (Yao et al. 2014). To assess the efficacy of cynatratoside-C against *I. multifiliis* theronts, malachite green, an effective drug for killing *I. multifiliis* (Srivastava et al. 2004), was used as a positive control in this study. Results demonstrated that malachite green at a concentration of 0.06 mg l^{-1} resulted in 100% mortality of theronts within 122 min of exposure. Although the effect of cynatratoside-C against theronts was not as efficient as that of malachite green, cynatratoside-C is still a more promising alternative parasiticide to control *I. multifiliis* in comparison with the other compounds mentioned above.

Morphological alteration was pronounced after theronts were exposed to cynatratoside-C. When treated with cynatratoside-C, theronts gradually lost their forward swimming ability and became spherical in shape. The membrane of the dead theront finally broke open. This phenomenon is similar to that of the membrane of a theront damaged by pentagalloylglucose (Zhang et al. 2013). The mechanism of cynatratoside-C for killing theronts is similar to that for killing tomonts (Fu et al. 2014a). The loss of membrane integrity and cytoplasm spilling out of the theront were observed microscopically.

Published reports indicated that theronts are more sensitive to drugs than tomonts and trophonts (Buchmann et al. 2003, Ling et al. 2012, Zhang et al. 2013). The dose of *Magnolia officinalis* methanol extract needed to kill tomonts was more than twice that for killing theronts (Yi et al. 2012). Malachite green at a concentration of 0.125 mg l^{-1} showed 100% mortality of tomonts by 4 h (Fu et al. 2014a), while malachite green at a concentration of 0.06 mg l^{-1} resulted in 100% mortality of theronts after 4 h of exposure. The EC_{50} values of cinnamaldehyde from *Cinnamomum cassia* on tomonts and theronts were 13.9 and 1.8 mg l^{-1}, respectively (Liang et al. 2014). The mean duration until death of all tomonts exposed to 32 mg l^{-1} for cynatratoside-C was 14.7 min (Fu et al. 2014a), but death occurred in only 3 min for all theronts. At a concentration of 0.25 mg l^{-1} tomonts survived for 270 min (Fu et al. 2014a), while theronts were already dead after 186.7 min. Therefore, theronts are more susceptible to cynatratoside-C than tomonts.

Trophonts of *I. multifiliis* parasitize skin or gills of fish and are covered by a host epidermis layer. The host layer makes trophonts more difficult to kill than other life stages. Therefore, controlling *I. multifiliis* at the parasitic stage needs a higher concentration of drugs. Cynatratoside-C at a concentration of 2 mg l^{-1} killed all trophonts completely and cured the infected fish within 48 h (Fu et al. 2014a). To investigate the efficacy of low concentrations of cynatratoside-C against Ich infestation in fish, lightly infected and naive grass carp were co-habitated in the same tanks and exposed to different concentrations of cynatratoside-C. The results demonstrated that cyna-

tratoside-C at a concentration of 0.25 mg l^{-1} was effective in protecting both infected and naive fish from Ich infection.

Although there were some individual infected fish in the 0.25 mg l^{-1} cynatratoside-C treatment groups at 15 d, only 3 infected and 4 naive grass carp were infected by Ich, and the mean infection intensities were 0.7 for the infected fish and 0.8 for the naive grass carp, respectively. The adaptive immune response was evidenced as early as 7 to 10 d after theronts initially entered the fish (Dickerson & Findly 2014). The increasing immunity with time would protect fish from light theront infection after termination of the usage of cynatratoside-C to kill Ich from Day 10 on. Thus, all grass carp treated with 0.25 mg l^{-1} of cynatratoside-C could survive and recover from the *I. multifiliis* infection on Day 30, due to the development of an immune response in fish against Ich.

Acute toxicity and a hemolysis assay were conducted to investigate the toxicity of cynatratoside-C to fish and mammals, respectively. The 96 h LC_{50} of cynatratoside-C on grass carp was 46.8 mg l^{-1}, which was approximately 564 times the 5 h EC_{50} for tomonts (0.083 mg l^{-1}) (Fu et al. 2014b) and 532 times the 4 h EC_{50} for theronts (0.088 mg l^{-1}). The 96 h LC_{50} value of cynatratoside-C was also much higher than the LC_{50} values of other plant and chemical 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) and potassium ferrate (VI) to goldfish (42.51 mg l^{-1}) (Ling et al. 2010). The results suggested that the toxicity of cynatratoside-C to grass carp was low and the compound was safe for the control of white spot disease. The hemolysis assay served as an indicator to assess the cytotoxicity of compounds to mammal blood cells. Rabbit RBCs were used as a model to investigate the cytotoxicity of cynatratoside-C and determine whether cynatratoside-C might have potential negative impacts on humans or not. RBCs were incubated with 4 different cynatratoside-C concentrations within the range of 100 to 1000 mg l^{-1} for 30 min. Results showed cynatratoside-C at a concentration of 1000 mg l^{-1} induced only 5% RBC hemolysis. Hemolysis <10% indicates good hemocompatibility and non-toxicity with respect to erythrocyte membranes (Sathler et al. 2014). The results of this study demonstrated that cynatratoside-C shows no cytotoxicity toward RBCs.

In conclusion, cynatratoside-C from *C. atratum* at 0.25 mg l^{-1} significantly eradicated theronts and protected naive fish from Ich infestation. Cynatratoside-C was safe for grass carp and rabbit RBCs at levels effective against parasites. Further studies will produce the compound by chemical synthesis and evaluate its effectiveness for controlling white spot disease under aquaculture conditions.

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