Antiparasitic efficacy of Gracillin and Zingibernsis newsaponin from *Costus speciosus* (Koen ex. Retz) Sm. against *Ichthyophthirius multifiliis*

WEI ZHENG[1]*, CHUN-MEI YAN[1], YA-BIN ZHANG[1], ZE-HONG LI[2], ZHONGQIANG LI[1], XIU-YING LI[1], ZHANG-WEI WANG[1], XIULAN WANG[1], WEI-QIANG CHEN[1] *and* XIN-HAI YU[2]

[1] *Jilin Province Fisheries Research Institute, 1195 Kunshan Road, Changchun, Jilin, China*
[2] *Jilin Agriculture University, 2888 Xincheng Road, Changchun, Jilin, China*

(*Received 23 May 2014; revised 18 July 2014; accepted 21 July 2014; first published online 20 August 2014*)

SUMMARY

The present study aims to evaluate the antiparasitic activity of active components from *Costus speciosus* against *Ichthyophthirius multifiliis*. Bioassay-guided fractionation was employed to identify active compounds from *C. speciosus* yielding 2 bioactive compounds: Gracillin and Zingibernsis newsaponin. *In-vitro* assays revealed that Gracillin and Zingibernsis newsaponin could be 100% effective against *I. multifiliis* at concentrations of 0·8 and 4·5 mg L^{-1}, with median effective concentration (EC_{50}) values of 0·53 and 3·2 mg L^{-1}, respectively. All protomonts and encysted tomonts were killed when the concentrations of Gracillin and Zingibernsis newsaponin were 1·0 and 5·0 mg L^{-1}. *In-vivo* experiments demonstrated that fish treated with Gracillin and Zingibernsis newsaponin at concentrations of 1·0 and 5·0 mg L^{-1} carried significantly fewer parasites than the control ($P<0·05$). Mortality of fish did not occur in the treatment group (Zingibernsis newsaponin at 5·0 mg L^{-1}) during the trial, although 100% of untreated fish died. Acute toxicities (LD_{50}) of Gracillin and Zingibernsis newsaponin for *grass carp* were 1·64 and 20·7 mg L^{-1}, respectively. These results provided evidence that the 2 compounds can be selected as lead compounds for the development of new drugs against *I. multifiliis*.

Key words: *Ichthyophthirius multifiliis*, Gracillin, Zingibernsis newsaponin, antiparasitic.

INTRODUCTION

Ichthyophthirius multifiliis (Ich) is a holotrichous protozoan parasite that invades the skin and gills of freshwater fish and thereby causing the so-called white spot disease, which can cause high aquaculture mortalities. It is one of the most common and persistent diseases of freshwater fish (Traxler *et al.* 1998).

Ichthyophthiriosis-infected ornamental fish can be treated effectively with a wide spectrum of agents, especially malachite green. Fish for human consumption also had been treated with malachite green until the early 1990s. It was highly effective, inexpensive and potent against many different parasites (Callinan and Rowland 1995). However, because of its carcinogenic potential, it is no longer permitted to treat fish for human consumption with malachite green in China and many other countries. Numerous studies have been conducted to find alternatives to malachite green (Lahnsteiner and Weismann 2007; Wohllebe *et al.* 2012; Ghada *et al.* 2014), but till now without great success. Therefore, the urgent need for the development of affordable, effective and safe alternative agents to combat the disease cannot be overemphasized.

Recently, the utilization of traditional plants for the control of fish parasites has attracted increasing attention. The studies have suggested that some plants, including traditional Chinese medicinal plants, possess effective parasiticide properties. These compounds undergo degeneration in fish and water and show no harmful effects on human health or the environment (Chu *et al.* 2010). Yao *et al.* (2010, 2011) found that sanguinarine, dihydrosanguinarine and dihydrochelerythrine from *Macleaya cordata* were found to be effective against *I. multifillis*. Two compounds, Chelerythrine and Chloroxylonine, from *Toddalia asiatica* have been reported to have antiparasitic efficacy against *I. multifillis* (Shan *et al.* 2014). Use of antiparasitic compounds extracted from plants could be a new approach to treat ichthyophthiriasis. In our previous study, we found that the methanolic extract of *C. speciosus* (Koen ex. Retz) Sm. was highly active against *I. multifillis* which leads us to consider whether it is capable of controlling ichthyophthiriasis. The principal objective of this study was to assess the antiparasitic properties of *C. speciosus* and isolate active constituents responsible for its properties. Additionally, the acute toxicity of active compounds against grass carp was evaluated.

* Corresponding author: Jilin Province Fisheries Research Institute, 1195 Kunshan Road, Changchun, Jilin, China. E-mail: yaojiamm131@163.com

Parasitology (2015), **142**, 473–479.
doi:10.1017/S0031182014001358

MATERIALS AND METHODS

Parasites and hosts

Goldfish (*Carassius auratus*) were obtained from a local Ornamental fish market in Changchun. Prior to the experiments, the skin surface and gills of 10 randomly sampled fish were examined under a microscope to confirm that they were not infected with parasites. Heavy *I. multifiliis*-infected grass carp (*Ctenopharyngodon idella*), weighing ~22 g each, were obtained from a local juvenile producer in Changchun and were maintained in a 1500-L tank with aerated groundwater. The water temperature was 23 ± 1 °C (controlled by automatic aquarium heater) and the pH was 7·2. Aeration was supplied by airstones and the dissolved oxygen level was 5·0 mg L^{-1} or higher. A light:dark period of 12:12 h was maintained at the experimental facility.

Ichthyophthirius multifiliis was collected using a method described by Clayton and Price (1988). Several heavily infected grass carp were placed into 5000 mL of filtered aquarium water for 30 min. Mature trophonts were allowed to dislodge from the host by body movements of the fish whilst in close proximity. Isolated trophonts were randomly divided into 2 batches, 1 was used to assay the activity of the compounds for killing the tomonts, and the other placed in Petri dishes with dechlorinated water filtered via a 0·22 μm filter and incubated for 24 h at 23 °C. After the theronts were released from cysts in water, five 20 μl theront suspension were counted under a microscope using a Neubauer hemocytometer. Theront concentration was calculated as the number of theronts per millilitre before being utilized to assess the antiparasitic activity of the compound extracted from *C. speciosus*.

Plant material

Costus speciosus were collected from Zhejiang Province, China, in March 2013. The rhizome was cut into small pieces, shade dried, and ground coarsely.

Isolation and identification of antiparasitic components from Costus speciosus

The antiparasitic component was isolated via an *in-vitro* bioactivity method based on anti-Ich (theronts) effect of fractions from *C. speciosus*. Only the fractions with strong anti-Ich activity were further purified until the target component was obtained.

Air-dried and powdered rhizomes of *C. speciosus* (6·0 kg) were exhaustively extracted with 50 L methanol at room temperature by percolation, giving 1347·6 g dry extract. This extract was subjected to column chromatograph and successively eluted with chloroform/water/methanol (65:10:35) gradients to afford 548 fractions (300 mL in each fraction). Fractions were monitored using thin-layer chromatography (TLC) and fractions showing similar TLC chromatograms were combined into 4 fractions (Fr. A: 1–98 fractions, Fr. B, 99–244 fractions; Fr. C, 245–370 fractions; and Fr. D, 371–548 fractions). These 4 fractions were submitted to anti-Ich (theronts) test and Fr. D was the most active. Fr. D was then applied to reverse-phase high-performance liquid chromatography, repetition of the chromatographic separations and recrystallization led to the isolation of the 2 active compounds. The structures of these compounds were elucidated by comparing spectroscopic data with those reported for Gracillin (Chen and Yin 1995; Kang *et al.* 2005), Zingibernsis newsaponin (Jain, 1987).

In-vitro *antiparasitic activity of extracts and active compounds from* Costus speciosus *against* I. multifiliis *theronts*

The tests were conducted to access a comprehensive antiparasitic activity of fractions and active compounds against *I. multifiliis* theronts. The crude extracts, fractions and the pure compound were dissolved in 1 mL DMSO, respectively, and made up to 50 mL with distilled water which were used for the preparation of different concentrations of test solutions. Tests were conducted in each well of a 24-well tissue culture plate, the theronts were placed into plates at a final concentration of about 100 theronts per well and exposed to different concentrations of test samples. Microscopic examination (×40 magnifications) was used to determine the antiparasitic activity of each well at various intervals up to 4 h after exposure. The trial was repeated 3 times.

Antiparasitic activities of Gracillin and Zingibernsis newsaponin against I. multifiliis *protomonts and encysted tomonts*

For the protomonts trial, 20 protomonts were placed into each well of a 24-well tissue culture plate. After carefully discarding the water in each well with a pipette, 1 mL Gracillin at concentrations of 0·2, 0·4, 0·6, 0·8 and 1·0 mg L^{-1}, 1 mL Zingibernsis newsaponin at concentrations of 1·0, 2·0, 3·0, 4·0 and 5·0 mg L^{-1} were added to each well (each concentration was repeated 3 times), respectively. A negative control was included using aerated groundwater containing the same amount of DMSO as the maximum concentration test group. The solutions were replaced by filtered aquarium water with no active compounds after 6-h exposure. Then the plates were incubated for 20 h at 23·5 ± 0·5 °C. After counting dead protomonts (the parasites with the absence of internal cell motility or abnormal cell division and the ones that cannot produce theronts were considered dead), theronts in each well were enumerated as

https://doi.org/10.1017/S0031182014001358 Published online by Cambridge University Press

described above. The mortality and reproduction of tomonts were determined for each well. The tomont reproduction was expressed as the number of theronts released by each tomont, calculated by total theronts/live tomonts.

For the encysted tomont trial, the mature trophonts were collected and distributed as above. Until the parasites had produced a cyst coat, the water in each well was removed carefully using a pipette, and 1 mL solution with Gracillin (0·2, 0·4, 0·6, 0·8 and 1·0 mg L^{-1}), Zingibernsis newsaponin at concentrations of 1·0, 2·0, 3·0, 4·0, and 5·0 mg L^{-1} were added to each well. After 6-h exposure, the solutions were changed by filtered aquarium water with no sample test. The next process was the same as the protomonts trial.

In-vivo *efficacy of Gracillin and Zingibernsis newsaponin from* C. speciosus *in the protection of goldfish against Ich*

An experiment was adapted from the method of Zhang (2013) to determinate effective concentration of the 2 active compounds used to protect goldfish against Ich. Tests were conducted in 50-L glass tanks, each containing 35 L of the test solution. The water pH ranged from 7·2 to 7·4. All tests were performed at 25 °C.

Ten naïve goldfish and 10 grass carp infected with Ich (38·7–12·3 whitespots on body surface per fish) were randomly added to each glass tank and exposed to different concentrations of Gracillin (0·2, 0·4, 0·6, 0·8 and 1·0 mg L^{-1}) and Zingibernsis newsaponin (3·0, 3·5, 4·0, 4·5 and 5·0 mg L^{-1}), triplicated tanks were used in each concentration. The Gracillin and Zingibernsis newsaponin solution in each tank was replaced daily for 10 days with a fresh solution at the same concentration. All the fish were then kept in aerated groundwater (with no Gracillin and Zingibernsis newsaponin) for another 5 days. Fish were fed with commercial food pellets every other day. The deaths of fish were recorded when the opercula movement and tail beat stopped and the fish no longer responded to mechanical stimulus. The observed dead fish were removed from the water in time. The fish mortality was recorded daily during the trial. The prevalence, intensity and survival of infected and naïve goldfish were determined for each concentration 15 days post-treatment as described above.

Acute toxicity of Gracillin and Zingibernsis newsaponin to grass carp

Acute toxicity tests (LD_{50}) of active compounds were performed for grass carp using a standard laboratory protocol (Li *et al*. 2013). The tests were conducted in 50-L glass tanks, each containing 35 L of test solution and 10 healthy grass carp. Dilutions were prepared from the stock solution as the following concentrations: 0·9, 1·2, 1·5, 1·8, 2·1, 2·4 and 2·7 mg L^{-1} for Gracillin, 15, 18, 21, 24, 27, 30 and 33 mg L^{-1} for Zingibernsis newsaponin. The tests were conducted in triplicate, as well as controls (under the same test conditions with no chemicals). The fish were carefully observed for any signs of distress indicative of toxic insult such as increased respiration frequency and erratic behaviour. Under the circumstances, the experiments were stopped and fish were transferred to freshwater. Mortality response was noted after 48 h of exposure, during which no food was offered to the fish.

Data analysis

All data in this study were performed using the SPSS 16.0 probit procedure, Tomont survival, tomont reproduction and infection intensity were compared with the Student–Newman–Keuls test procedure for multiple comparisons ($\alpha = 0{\cdot}05$).

RESULTS

In-vitro *antiparasitic efficacy of fractions and active compounds from* C. speciosus *against* I. multifiliis *theronts*

The *in-vitro* antiparasitic efficacy of 4 fractions from the methanol extract were depicted in Fig. 1,which showed that Fr. D had a 100% antiparasitic activity effective against *I. multifiliis* at concentrations of 15·0 mg L^{-1}, after 4 h of exposure. Fr. C was 87·5% effective against *I. multifiliis* at a concentration of 35·0 mg L^{-1} after 4 h of exposure. Fr. A and Fr. B had low efficacy at 40·0 mg $L^{-1}$with an efficacy of 55·0 and 35·0%, respectively. Fr. D was considered to be the fraction that contained active compounds.

The treatment with Gracillin and Zingibernsis newsaponin leads to a significant dose-dependent decrease in the number of *I. multifiliis* theronts compared with the controls. *In-vitro* tests exhibited that Gracillin and Zingibernsis newsaponin could be 100% effective against *I. multifiliis* at concentrations of 0·8 and 4·5 mg L^{-1}, with median effective concentration (EC_{50}) values of 0·53 and 3·2 mg L^{-1}, respectively (Fig. 2).

In-vitro *antiprasatic activity of Gracillin and Zingibernsis newsaponin against* I. multifiliis *protomonts and encysted tomonts*

The effects of Gracillin and Zingibernsis newsaponin on *I. multifiliis* protomonts and encysted tomonts are shown in Tables 1 and 2, respectively. The results showed that the 2 active compounds were effective against *I. multifiliis* protomonts and encysted tomonts, while encysted tomonts were less susceptible to the 2 compounds than protomonts. Exposure of *I. multifiliis* protomonts to 0·8 mg Gracillin caused 100% mortality, while Gracillin at the same

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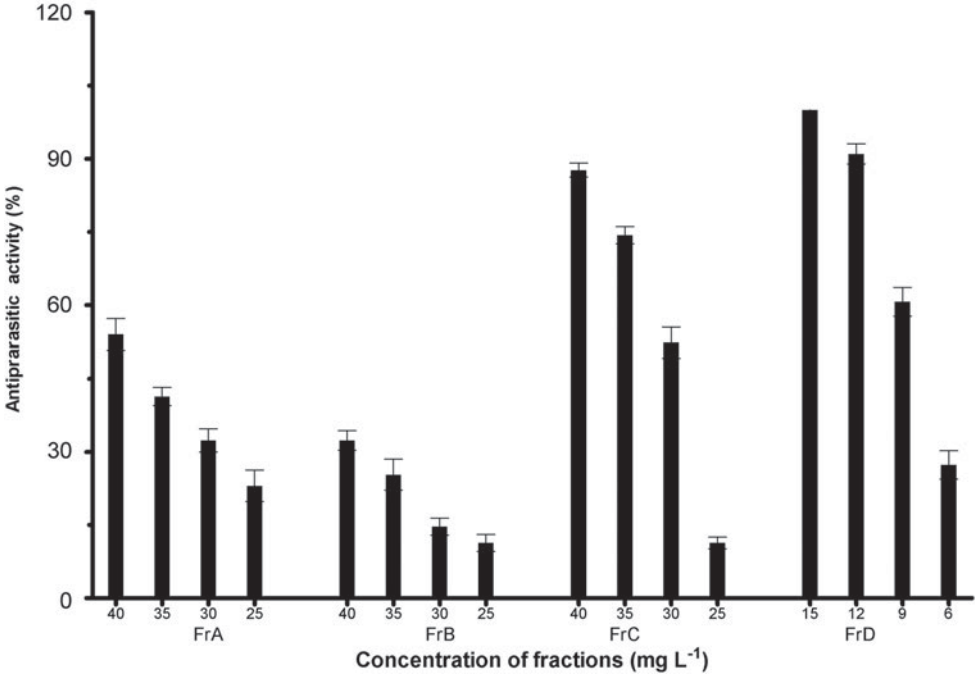

Fig. 1. Antiparasitic efficacy of the 4 fractions against *I. multifiliis* theronts after 4 h exposure. Note: Antiparasitic efficacy (%) = (mean surviving number of theronts of the blank control group − mean surviving number of theronts of treatment group)/mean surviving number of theronts of the blank control group ×100%.

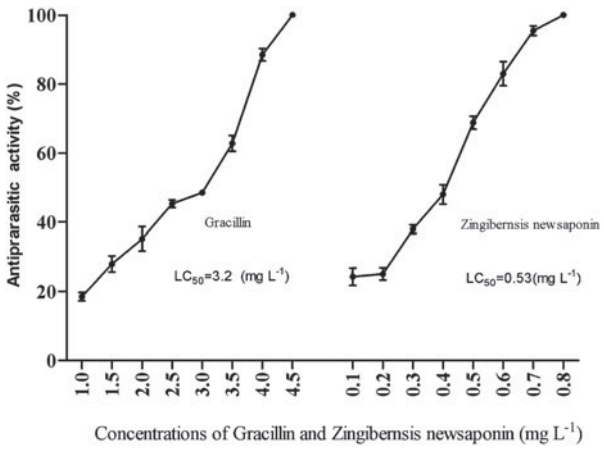

Fig. 2. Antiparasitic efficacy of Gracillin and Zingibernsis newsaponin against *I. multifiliis* theronts after 4 h exposure. Note: Antiparasitic activity (%) = (mean surviving number of theronts of the blank control group–mean surviving number of theronts of treatment group)/mean surviving number of theronts of the blank control group ×100%.

concentration killed barely 92·5% of encysted tomonts until all surviving parasites in the controls reached the theront stage. All tomonts were killed when the concentrations of Gracillin reached 1·0 mg L^{-1}. As for Zingibernsis newsaponin, at a concentration of 5·0 mg L^{-1} could kill 100% of protomonts and encysted tomonts. Additionally, the reproduction of tomonts was remarkably reduced after the protomonts or tomonts were exposed to Gracillin and Zingibernsis newsaponin for 6 h.

In-vivo *efficacy of Gracillin and Zingibernsis newsaponin against Ich*

Table 3 shows the results of an *in-vivo* study on the efficacy of Gracillin against Ich. The increase in fish survival was positively correlated with Gracillin concentration. A concentration of 1·0 mg L^{-1} of Gracillin demonstrated 90·0% survival to infected grass carp and 93·3% survival to naïve goldfish at the end of the trial. The fish in this treated group also showed the lowest infectivity incidence and intensity. Exposure of infected grass carp and naïve goldfish to 0·8 mg L^{-1} of Gracillin resulted in 66·7 and 73·3% survival, respectively. All fish died from Ich infection when treated with 0·2 mg L^{-1} Gracillin or untreated as control.

The survivals of infected and naïve fish treated with Zingibernsis newsaponin are shown in Table 4. As revealed, mortality in the treated group was markedly decreased compared with that in the control group. Infected grass carp and naïve goldfish treated with 5·0 mg L^{-1} Zingibernsis newsaponin led to 93·3 and 100·0% survival on day 15th, respectively. A 90·0% survival to infected grass carp and 93·3% survival to naïve goldfish were demonstrated when the concentration was 4·5 mg L^{-1}. The treatment with Zingibernsis newsaponin lead to a significant dose-dependent decrease in the number of *I. multifiliis* on the body surface of treated fish compared with the controls. When the concentrations of Zingibernsis newsaponin were 5·0 mg L^{-1}, the number of parasites was reduced by 16·7 and 26·7% for infected grass scarp and naïve goldfish,

https://doi.org/10.1017/S0031182014001358 Published online by Cambridge University Press

Table 1. *Ichthyophthirius multifiliis* tomont survival and reproduction after 6-h exposure of protomonts to Gracillin and Zingibernsis newsaponin

	Gracillin			Zingibernsis newsaponin	
Concentrations	Tomont survival (%)	Reproduction	Concentrations	Tomont survival (%)	Reproduction
0 (control)	96·7 ± 1·3a	456·7 ± 47·7a	0 (control)	96·7 ± 1·3a	411·3 ± 38·3a
0·2	92·3 ± 2·7a	437·3 ± 35·3a	1·0	80·0 ± 4·3b	378·3 ± 33·7b
0·4	58·7 ± 1·1b	301·3 ± 33·3b	2·0	70·0 ± 5·7c	321·7 ± 35·7c
0·6	21·3 ± 1·7c	286·3 ± 43·3c	3·0	53·3 ± 3·7d	267·3 ± 22·3d
0·8	0·0 ± 0·0	0·0 ± 0·0	4·0	16·6 ± 2·3e	211·7 ± 37·3e
1·0	0·0 ± 0·0	0·0 ± 0·0	5·0	0·0 ± 0·0	0·0 ± 0·0

The reproduction was represented as the number of theronts released by each live tomont. Each value is expressed as mean ± standard deviation of 3 replicates, and within a column, values followed by the different letters are significantly different ($P < 0·05$).

Table 2. *Ichthyophthirius multifiliis* tomont survival and reproduction after 6-h exposure of tomonts to Gracillin and Zingibernsis newsaponin

	Gracillin			Zingibernsis newsaponin	
Concentrations	Tomont survival (%)	Reproduction	Concentrations	Tomont survival (%)	Reproduction
0	96·7 ± 1·3a	489·3 ± 36·7a	0	96·7 ± 1·3a	489·3 ± 36·7a
0·2	92·3 ± 2·7a	464·3 ± 45·3a	1·0	83·3 ± 11·7b	433·3 ± 46·4b
0·4	58·7 ± 1·1b	367·1 ± 38·9b	2·0	73·3 ± 13·3c	378·7 ± 22·9c
0·6	21·3 ± 1·7c	245·3 ± 23·4c	3·0	53·3 ± 7·7d	320·3 ± 29·1d
0·8	7·5 ± 0·7d	116·4 ± 35·6d	4·0	26·7 ± 3·3e	241·7 ± 22·2e
1·0	0·0 ± 0·0	0·0 ± 0·0	5·0	0·0 ± 0·0	0·0 ± 0·0

The reproduction was represented as the number of theronts released by each live tomont. Each value is expressed as mean ± standard deviation of 3 replicates, and within a column, values followed by the different letters are significantly different ($P < 0·05$).

Table 3. *In-vivo* efficacy of Gracillin against Ich at 15 days

	Infective incidence (%)		Infective level		Survival rate (%)	
Concentration (mg/L)	Infected	Naïve	Infected	Naïve	Infected	Naïve
0 (control)	–	–	–	–	0·0 ± 0·0	0·0 ± 0·0
0·2	–	–	–	–	0·0 ± 0·0	0·0 ± 0·0
0·4	100·0 ± 0·0	93·3 ± 6·7	123·3 ± 16·7	90·7 ± 12·0	10·0 ± 3·3	10·0 ± 3·3
0·6	96·7 ± 3·3	86·7 ± 6·7	70·8 ± 13·3	66·2 ± 13·3	30·0 ± 6·7	33·3 ± 3·3
0·8	43·3 ± 10·0	46·7 ± 10·0	58·2 ± 16·7	32·6 ± 5·8	66·7 ± 10·0	73·3 ± 6·7
1·0	26·7 ± 13·3	23·3 ± 6·7	21·5 ± 6·7	8·9 ± 3·3	90·0 ± 3·3	93·3 ± 6·7

'–' denotes that fish died and no fish was present at 15 days. Infective incidence (%) = (number of infected goldfish (carp)/number of total goldfish (carp)) × 100. Infective level = number of white spots on goldfish (carp) body/number of infected goldfish (carp) body. Values marked by different letters within the same column are significantly different ($P < 0·05$).

respectively. Mortality of 100% fish was observed in the control group, whereas mortality of fish did not occur in the 5·0 mg L^{-1} treatments to goldfish.

Acute toxicity of Gracillin and Zingibernsis newsaponin

In the acute toxicity test for Gracillin for grass carp, most of the fish exhibited a slow reaction to external stimulation when the concentration exceeded 1·2 mg L^{-1}. At 1·8 mg L^{-1}, grass carp showed exophthalmia and signs of disturbed balance after about 10 h exposure. Mortality occurred when the concentration reached 1·2 mg L^{-1}, the linear equation $y = -12·26 + 25·12\times$ was derived from the regression analysis of probit mortality of *grass carp* in test solution bioassay. The calculated LD_{50} at 48 h was 1·64 mg L^{-1} with 95% fiducial limits of 1·52–1·71 mg L^{-1}. For Zingibernsis newsaponin, the linear equation $y = -23·67 + 9·16\times$ was derived

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Table 4. *In-vivo* efficacy of Zingibernsis newsaponin against Ich at 15 days

Concentration (mg/L)	Infective incidence (%)		Infective level		Survival rate (%)	
	Infected	Naïve	Infected	Naïve	Infected	Naïve
0 (control)	–	–	–	–	0·0 ± 0·0	0·0 ± 0·0
3·0	100·0 ± 0·0	93·3 ± 6·7	72·8 ± 8·8	65·3 ± 6·5	20·0 ± 3·3	20·0 ± 10·0
3·5	93·3 ± 10·0	80·0 ± 6·7	53·5 ± 6·2	45·7 ± 5·1	43·3 ± 10·0	43·3 ± 6·7
4·0	73·3 ± 3·3	66·7 ± 6·7	36·1 ± 4·7	28·8 ± 9·3	66·7 ± 10·0	70·0 ± 6·7
4·5	36·7 ± 6·7	30·0 ± 3·3	24·6 ± 6·7	22·3 ± 7·3	80·0 ± 6·7	86·7 ± 3·3
5·0	26·7 ± 3·3	16·7 ± 6·7	12·5 ± 4·5	48·9 ± 3·0	93·3 ± 6·7	100 ± 0·0

'–' denotes that fish died and no fish was present at 15 days. Infective incidence (%) = (number of infected goldfish (carp)/number of total goldfish (carp)) × 100. Infective level = number of white spots on goldfish (carp) body/number of infected goldfish (carp) body. Values marked by different letters within the same column are significantly different ($P < 0·05$).

from the regression analysis of probit mortality with an LD_{50} of 20·7 mg L^{-1} and 95% confidence interval of 19·5–22·3 mg L^{-1}. No fish mortality occurred in the control groups during the experiments.

DISCUSSION

Plants, microorganisms and marine organisms are potential sources of new drugs since they contain a countless quantity of natural products with a great variety of structures and pharmacological activities (Newman *et al*. 2003). These compounds undergo degeneration in fish and water and show no harmful effects on human health or the environment (Chu *et al*. 2010). Use of antiparasitic compounds extracted from plants could be a new approach to treat ichthyophthiriasis (Yao *et al*. 2010, 2011; Zhang *et al*. 2013). *Costus speciosus* (Koen.) Sm., spiral or wild ginger, belongs to the family Costaceae. There were many reports about the anticholinesterase (Battacharya *et al*. 1972), antidiabetic and antilipidaemic (Eliza *et al*. 2009) activity of the crude extracts and active compounds of *C. speciosus*. However, there were no reports about the investigation of *C. speciosus* in aquaculture. In this study, bioactivity-guided isolation was applied on methanol extract of *C. speciosus* rhizomes to provide 2 active compounds, Gracillin and Zingibernsis newsaponin. To our best knowledge, Zingibernsis newsaponin was isolated from *C. speciosus* for the first time. This study is the first report of the antiparasitic efficacy of Zingibernsis newsaponin assessed by both *in-vitro* and *in-vivo* experiments in fish. This result extended the general knowledge about the antiparasitic activity of the Zingibernsis newsaponin and the plants' application to control fish parasites.

Gracillin and Zingibernsis newsaponin, naturally occurring in a variety of plants belonging to the family Costus, are representative steroidal saponins. Different pharmacological activities of saponin have been reported previously and relative mechanism of these activities has been demonstrated. Cheung *et al*. (2005) and Lee *et al*. (2005) found that saponins performed most antitumour activity and echibited a similar pathway on antitumour effect. They elicited apoptosis through mitochondrial dysfunction and cell cycle arrest. Wang *et al*. (2006) reported that the steroidal saponins show potent antiproliferative activities against most cell lines from leukaemia to solid tumours. Proteomic analysis revealed that it induced apoptosis via the mitochondrial and some other pathway. Sparg *et al*. (2004) found that saponins could change the integrity and potential of cell membrane resulting in a strong antiproliferative action on all the stages of development of the parasite *Leishmania infantum*. Both Gracillin and Zingibernsis newsaponin belong to steroidal saponin. Thus, it is postulated that the antiparasitic mechanisms of action of Gracillin and Zingibernsis newsaponin might also be attributed to these factors; however, the exact mechanism of action regarding their antiparasitic efficacy remains to be further investigated.

Fish treated with Gracillin and Zingibernsis newsaponin at concentrations of 1·0 and 5·0 mg L^{-1} carried significantly fewer parasites than the control. Although the parasites were not completely eliminated in the *in vivo*, a substantial reduction of *I. multifilliis* burden, as shown in Tables 3 and 4, led to the recovery of the fish. If an effective therapy leads to the survival of fish exposed to a first infection with *I. multifiliis*, the acquired protective host immunity can develop (Clark and Dickerson, 1997). This could be seen as a better alternative for aquaculture and the environment than the use of some chemicals.

The *in-vitro* experiments have advantages in avoiding animal experiments and in allowing rapid assessment of the efficiency of the tested chemicals against *I. multifiliis*, so it may become the model of the new anti-*I. multifiliis* drug findings (Yao *et al*. 2011). Theronts are the infective stage in the life cycle of *I. multifilliis*. Therefore it is important to kill theronts in order to control ichthyophthiriasis. In recent studies, the theront mortality (Ling *et al*. 2010, 2012; Yi *et al*. 2012) and survival (Shinn *et al*. 2012) have been used as parameters for the evaluation of parasiticide efficacy at various time intervals. In the present study, the strategy of *in-vitro* anti-theront trial guided isolation monitoring the chromatographic

https://doi.org/10.1017/S0031182014001358 Published online by Cambridge University Press

separation was used. By bioactivity-guided isolation, 2 active compounds were isolated from *C. speciosus*. *In-vivo* data showed that the 2 compounds were most active against *I. multifilliis* (Tables 3 and 4). The EC_{50} of 2 active compounds were also higher than some plant compounds, such as dihydrosanguinarine (13·3 mg L^{-1}) and dihydrochelerythrine (mg L^{-1}) (Yao *et al*. 2011) and other chemicals used for controlling ichthyophthiriasis, such as potassium permanganate (mg L^{-1}). Thus, the 2 compounds could effectively eradicate theronts in water.

The results of the *in-vivo* test showed that Zingibernsis newsaponin has strong antiparasitic efficacy against *I. multifilliis* when the concentration reached 5·0 mg L^{-1}, and it was 4-fold more efficient than pentagalloyl glucose (Zhang *et al*. 2013). The 48 h LD_{50} value of Zingibernsis newsaponin was 20·7 mg L^{-1} which is 4 times more than the effective one. These findings ensure the safety or the use of Zingibernsis newsaponin in the control of *I. multifilliis* infection, and suggest that it has great potential for the development of a new parasiticide. In the case of Gracillin, the effective concentration was nearly 2 times higher than the corresponding toxic dose, indicating that Gracillin is of high risk, which may limit its application in the control of *I. multifilliis*.

In conclusion, the results obtained in the present study provided a scientific basis for use of the Gracillin and Zingibernsis newsaponin from *C. speciosus* for the treatment of *I. multifilliis*. The active compounds isolated in this work, especially Zingibernsis newsaponin, could be useful for the development of new antiparasitic agents. However, whether further studies are required for field evaluations in the practical system remained to be further elucidated. Furthermore, the cost of isolation of the 2 compounds from *C. speciosus* is high. To the best of our knowledge there are few reports on the synthesis of Gracillin and Zingibernsis newsaponin; so more research is required to produce cheap and large quantities of the 2 compounds to meet the needs of practical use in ornamental or farm-raised fish.

ACKNOWLEDGEMENT

The research was supported by the Jilin Province Science and Technology Development Plan Item (grant no. 201205069).

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