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Research paper

Efficacy and antiparasitic mechanism of 10-gingerol isolated from ginger *Zingiber officinale* against *Ichthyophthirius multifiliis* in grass carp

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ABSTRACT

Ichthyophthirius multifiliis is a ciliate parasite of freshwater fish with a global distribution and results in severe economic losses in aquaculture. The present study aimed to investigate the efficacy and antiparasitic mechanism of active compounds isolated from *Zingiber officinale* against *I. multifiliis*. Three compounds were isolated from the *Z. officinale* extract and identified as 10-gingerol, 6-dehydroshogaol, and 6-dehydro-10-gingerol. 10-gingerol demonstrated the greatest antiparasitic efficacy *in vitro*. 10-gingerol resulted in 100% mortalities of theronts, nonencysted tomonts, and encysted tomonts at concentrations of 2, 8, and 16 mg/L, respectively. 10-gingerol significantly reduced theronts infectivity ($p < 0.05$) at a concentration of 1 mg/L, and it was effective in treating infected grass carp and protecting naïve fish from *I. multifiliis* infestation at a concentration of 4 mg/L. The antiparasitic mechanism might be attributed to the increase of intracellular osmotic pressure, accumulation of free radicals, and membrane damage of *I. multifiliis* post 10-gingerol treatment. The study demonstrated that 10-gingerol had the potential as a therapeutic agent against *I. multifiliis*.

1. Introduction

Ichthyophthiriasis is one of the most important fish diseases and results in significant losses in aquaculture industry (Buchmann et al., 2001; Matthews, 2005). This disease is commonly named "white spot disease" and is caused by *Ichthyophthirius multifiliis*, a ciliated parasite with a global distribution (Dickerson and Findly, 2014; Xu et al., 2016). The life cycle of *I. multifiliis* consists of an infective theront, a parasitic trophont, and a reproductive tomont (Jørgensen, 2017; Zhang et al., 2013). The tomont stage includes nonencysted tomonts and encysted tomonts (Fu et al., 2014a).

Chemotherapeutics used to control *I. multifiliis* cause food safety concerns and have potential mammalian and environmental toxicities (Reverter et al., 2014). For example, malachite green has been banned for use in food fish by many countries due to its carcinogenic and teratogenic effects on humans (Shinn et al., 2012). Vaccination has been regarded as a potential prevention against ichthyophthiriasis in aquaculture (Jørgensen, 2017; Xu et al., 2009). However, immobilizing antibodies are serotype-specific, and only protect fish against *I. multifiliis* with the same serotype of i-antigens (Wang and Dickerson, 2002). Currently vaccines against *I. multifiliis* is not yet available commercially (Dickerson and Findly, 2014). An alternate approach to control *I. multifiliis* is to use natural products (Fu et al., 2014a; Liu et al., 2017; Zhang et al., 2013). In the past decade, there is an increasing interest in using plant extracts to treat *I. multifiliis* and more plant products have been found to have anti-*I. multifiliis* properties (Fu et al., 2015; Liang et al., 2015). Most plant products are environmentally friendly since they are easily biodegradable (Reverter et al., 2014). Therefore, plant products could be used in aquaculture as anti-*I. multifiliis* agents.

Zingiber officinale, commonly called ginger, is a plant in the Zingiberaceae family and has a long history of medicinal usage (Schoenknecht et al., 2016). Ginger contains several phytochemicals among which non-volatile resins and volatile essential oils are the most important groups (Pang et al., 2017). The major non-volatile pungent constituents of oleoresin are polyphenolic compounds that are responsible for their unique pharmacological effects (Wang et al., 2011).

In a preliminary screening study of herbs, ginger was noted for its

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anti-*I. multifiliis* activity. Three compounds were subsequently isolated from the ginger extract by bioassay-guided fractionation. Among them, 10-gingerol was the most effective compound. There is no information available on 10-gingerol against *I. multifiliis* in fish. Thus, this study was conducted to investigate the efficacy and antiparasitic mechanism of 10-gingerol *in vitro* and *in vivo* against *I. multifiliis*.

2. Materials and methods

2.1. Fish and collection of parasites

Grass carp (*Ctenopharyngodon idellus*) with 13.1 ± 1.7 cm (mean ± standard deviation) in length were obtained from a commercial fish farm at Huadu, Guangzhou City, Guangdong Province. Fish were kept in 100-L opaque tanks and fed daily with granule feed (Haid, Guangzhou, China) at 1% of fish weight. Water temperature was adjusted to 23.0 ± 0.3 °C in the laboratory with an air conditioner. *I. multifiliis* was originally isolated from goldfish from the ornamental fish market at Guangzhou and maintained by serial transmission on grass carp as previously described (Fu et al., 2014a). Trophonts and theronts were collected according to Zhang et al. (2013). Use of grass carp was approved by the Animal Experimentation Ethics Committee of Jinan University.

2.2. Isolation and identification of bioactive compound

Active compounds in ginger with anti-*I. multifiliis* efficacy were isolated, purified, and identified by bioassay-guided fractionation. Ginger oleoresin extracted by supercritical CO_2 extraction was purchased from Chulin Bio-Technique Co. Ltd, Guangzhou, China. Two kg of ginger oleoresin was suspended in 2 L of water and partitioned with petroleum ether (5 × 2 L) and ethyl acetate (5 × 2 L). The active ethyl acetate extract (1.2 kg) was fractionated on a silica gel column and continuously eluted using a stepwise gradient of petroleum ether/ethyl acetate (1:0, 1:1, and 0:1) and ethyl acetate/methanol (1:1 and 0:1) to obtain fractions A–E. The most active fraction B (590 g) was fractionated on a silica gel column and eluted with petroleum ether/ethyl acetate (1:0, 4:1, 1:1, and 0:1) and methanol to get five fractions BA-BE. The active fraction BB (350 g) was further isolated and eluted with petroleum ether/ethyl acetate (1:0, 10:1, 4:1, and 1:1) and methanol to obtain five fractions BB1-BB5. The active fraction BB2 (95 g) was separated by preparative high performance liquid chromatography (PHPLC) equipped with a XB-C18 column (5 μm, 21.2 mm × 250 mm, Welch, MD) to collect fractions BB2-1, BB2-2, BB2-3, BB2-4, and BB2-5. The mobile phase used for PHPLC was methanol/H_2O (80:20, v/v) with a flow rate of 10 mL/min, and ultraviolet detection was at 210 nm. Fraction BB2-5 was designated as compound 1 (24.5 g, t_R = 29 min) with a purity > 99%. Fraction BB2-4 (1.7 g) was further purified by PHPLC to get compound 2 (0.3 g, t_R = 30.6 min) and compound 3 (0.2 g, t_R = 32.5 min). The mobile phase used for PHPLC was methanol/H_2O (75:25, v/v) for fraction BB2-4 purification. The active compounds were identified by ESI-MS, ^{1}H NMR, and ^{13}C NMR.

Compound 1. ^{13}C NMR (125 MHz, MeOD) *δ*: 13.06 (C-14), 22.34 (C-13), 25.24 (C-12), 28.96 (C-11), 29.06 (C-10), 29.3 (C-9), 29.31 (C-8), 29.33 (C-7), 31.67 (C-6), 37.01 (C-1), 45.01 (C-2), 49.91 (C-4), 54.96 (3′-OCH_3), 67.52 (C-5), 111.72 (C-1′), 114.74 (C-6′), 120.3 (C-5′), 132.65 (C-2′), 144.34 (C-3′), 147.47 (C-4′), 210.65 (C=O). ^{1}H NMR (500 MHz, MeOD) *δ*: 0.92 (3H, t, *J*=7.0 Hz, H-10), 1.29–1.37 (14H, m, H-6 to H-13), 2.54 (2H, qd, *J*=15.8 Hz and 6.3 Hz, H-4), 2.80 (4H, d, *J*=10.3 Hz, H-1, H-2), 3.84 (3H, s, -OCH_3), 4.01 (1H, m, H-5), 6.63 (1H, dd, *J*=8.0Hz and 2.0 Hz, H-6′), 6.7 (1H, m, H-2′), 6.79 (1H, d, *J*=1.9Hz, H-5′).

Compound 2. ^{13}C NMR (125 MHz, MeOD) *δ*: 190.47 (C=O), 148.48 (C-2), 148.05 (C-4), 147.47 (C-4′), 144.53 (C-3′), 130.03 (C-2′), 128.64 (C-1), 124.42 (C-5), 123.4 (C-5′), 114.77 (C-6′), 110.63 (C-1′), 54.96 (3′-OCH_3), 32.33 (C-6), 31.26 (C-7), 27.69 (C-8), 22.32 (C-9), 14.89 (C-10). ^{1}H NMR (500 MHz, MeOD) *δ*: 7.66 (1H, d, *J* = 15.9 Hz, H-1), 7.29 (1H, d, *J* = 1.9 Hz, H-6′), 7.18 (1H dd, *J* = 8.2, 1.9 Hz, H-2′), 7.08 (1H, dt, *J* = 15.6, 7.0 Hz, H-5), 7.02 (1H, d, *J* = 3.0 Hz, H-5′), 6.86 (1H, d, *J* = 1.5 Hz, H-2), 6.52 (1H, t, *J* = 1.5 Hz, H-4), 5.77 (1H, d, *J* = 10.2 Hz, 4′-OH), 3.93 (3H, s, 3′-OMe), 2.34 (2H, dd, *J* = 7.1, 1.3 Hz, H-6), 1.49 (2H, d, *J* = 2.8 Hz, H-7), 1.37 (4H, d, *J* = 3.5 Hz, H-8, H-9), 0.98 - 0.96 (3H, m, H-10).

Compound 3. ^{13}C NMR (125 MHz, MeOD) *δ*: 14.34 (C-14), 22.9 (C-13), 25.67 (C-12), 29.51 (C-11), 29.53 (C-10), 29.76 (C-9), 29.8 (C-8), 36.68 (C-1), 45.66 (C-2), 49.57 (C-4), 56.09 (3′-OCH3), 67.86 (C-5), 111.18 (C-1′), 114.57 (C-6′), 120.95 (C-5′), 131.81 (C-7), 132.11 (C-6), 132.86 (C-2′), 144.18 (C-3′), 146.63 (C-4′), 211.69 (C=O). ^{1}H NMR (500 MHz, MeOD) *δ*:0.93 (3H, d, *J*=6.7, H-10), 1.30–1.35 (6H, m, H-8-H-13), 2.56 (3H, m, H-4), 2.8 (4H, m, H-1, H-2), 3.85 (3H, d, *J*=2.5, OCH_3), 4.04 (1H, ddd, H-5), 5.38 (2H, m, H-6, H-7), 6.71 (1H, dd, *J*=8.0, 3.4, H-6′), 6.79 (1H, d, *J*=1.9, H-2′), 6.82 (1H, dd, *J*=8.6, 2.3).

2.3. In vitro bioactivity of compounds on I. multifiliis

For each compound 5 mg was dissolved in 2.5 mL of dechlorinated freshwater containing 1% (v/v) dimethyl sulfoxide (DMSO) to make a 2000 mg/L solution 1. The 25.6 μL solution 1 was added to 174.4 μL dechlorinated water to make a 256 mg/L stock solution.

The stock solution of each compound was serially diluted with dechlorinated water to concentrations of 128, 64, 32, 16, 8, 4, and 0 mg/L with 200 μL in volume per well in a 24-well plate. Next, 200 μL of water containing about 100 nonencysted tomonts was added to each well and the final concentrations of each compound were 64, 32, 16, 8, 4, 2, and 0 mg/L, respectively. There were triplicates for each concentration. Nonencysted tomonts were monitored continually and the numbers of live nonencysted tomonts in each well were counted under a microscope (40×) for 4 h according to Zhang et al. (2013).

The stock solution of 10-gingerol was serially diluted with dechlorinated water to concentrations of 64, 32, 16, 8, 4, 2, 1, and 0 (negative control) mg/L with 100 μL per well in a 96-well plate. Then, 100 μL of water containing about 200 theronts was added to each well (in triplicate) and the final concentrations of 10-gingerol were 32, 16, 8, 4, 2, 1, 0.5, and 0 mg/L. All theronts were monitored continually and the numbers of live theronts in each well were counted under a microscope (40×) for 4 h according to Zhang et al. (2013).

A 200 μL of water containing about 30 nonencysted tomonts was added to each well of a 24-well plate. The nonencysted tomonts were incubated for 6 h to become encysted tomonts, and the encysted tomonts were enumerated in every well. Subsequently, the encysted tomonts were exposed to 10-gingerol at concentrations of 64, 32, 16, 8, 4, 2, and 0 mg/L, respectively. There were 5 replicates for each concentration. The numbers of live encysted tomonts were counted at 4 h in each well under a microscope (40×) and then maintained at 23 ± 0.3 °C for 12 h. After 16 h exposure, theronts in each well were counted as described by Fu et al. (2014a).

2.4. Effect of low 10-gingerol concentrations on theront infectivity

Thirty mL of water containing approximately 200,000 theronts was added to each of 15 beakers (100 mL). Then 30 mL 10-gingerol solution was added to each beaker to make final concentrations of 1, 0.5, 0.25, 0.125, and 0 (control) mg/L to treat theronts for 2 h. There were 3 replicates for each concentration.

One hundred and fifty healthy grass carp were distributed to fifteen 20-L aquaria (10 fish per aquarium) containing 5 L static dechlorinated water. The theronts pretreated with the 10-gingerol solution in each beaker were poured into one of the 15 aquaria. After the grass carp were exposed to the pretreated theronts for 2 h, 5 L of filtered water was added into each aquarium. The temperature was maintained at 23 ± 0.3 °C by an air conditioner. On the fifth day following the exposure to theronts, all fish were anesthetized with 150 mg/L tricaine

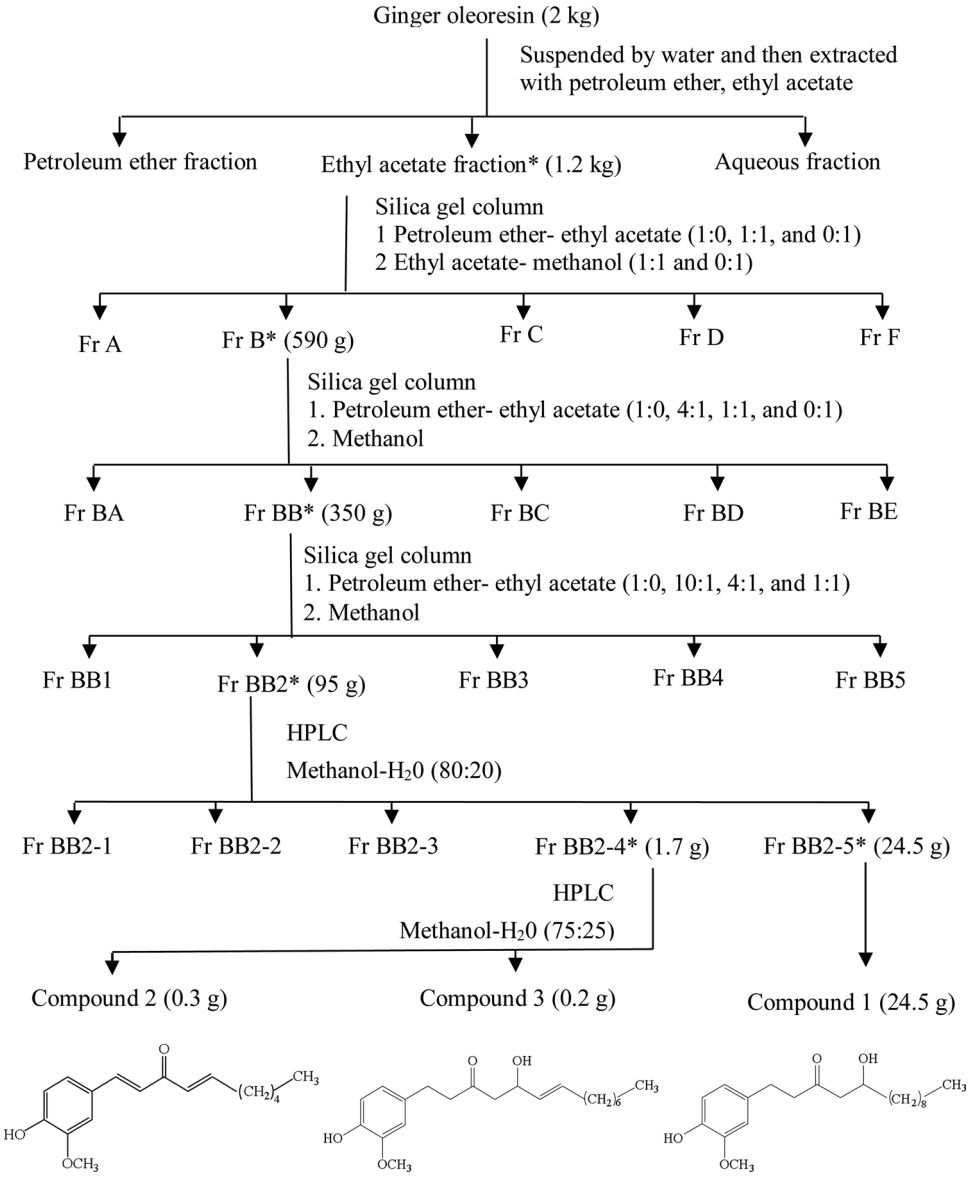

Fig. 1. Bioassay-guided fractionation for identification of parasiticidal compounds and the structure of active compounds. Ginger oleoresin was resuspended in water and extracted with petroleum ether or ethyl acetate. The ethyl acetate extract demonstrated the highest activity against *Ichthyophthirius multifiliis*. The ethyl acetate extract was further isolated by silica gel column, followed by preparative high performance liquid chromatography (PHPLC). The purified compounds were identified by ESI-MS, ^{1}H NMR, and ^{13}C NMR. *: Best antiparasitic activity from the same row; Fr, Fraction.

methanesulfonate (MS-222, Sigma). The survival rates of infected and naïve fish were determined for each concentration. Numbers of white spots on fish skin were counted, and the infection incidence and mean infection intensity were determined as defined by Bush et al. (1997).

2.5. Evaluation of 10-gingerol in protection of grass carp against I. multifiliis

Sixty fish lightly infected with *I. multifiliis* (40 ± 9 white spots on body surface per fish) were marked by clipping the caudal fin and randomly distributed into twelve 20-L aquaria (5 fish/aquarium) with 15 L water. Then, 10 naïve grass carp were added to each aquarium, and cohabitated with the infected fish. The 10-gingerol stock solution was added into each aquarium to make final concentrations of 4, 2, 1, and 0 (control) mg/L, respectively, with 3 replicates for each concentration. Water in each tank was replaced with fresh water and 10-gingerol stock solution was added to each tank to make designated concentrations daily for the first 10 d. Dead fish were counted, recorded, and removed once per day during the trial. On day 15 following the initial exposure to 10-gingerol, all fish were anesthetized with 150 mg/L MS-222 to determine the infection incidence, mean infection intensity, and survival rate for both infected and naïve fish.

2.6. Enzyme activities

Theronts at a concentration of 1×10^4 cell/mL were treated with 2 mg/L of 10-gingerol solution. Nonencysted tomonts at a concentration of 100 cell/mL were treated with 8 mg/L of 10-gingerol solution. After the theronts and nonencysted tomonts were treated with 10-gingerol for 0.5, 1, and 1.5 h, the mixtures were centrifuged at 239 × g for 10 min, and the supernatant was discarded. Phosphate buffer solution (PBS) was added into each centrifuge tube to suspend the cells, and the suspensions were frozen by liquid nitrogen three times to lyse cells. The cell suspensions were centrifuged at 10,956 × *g* for 20 min, and the supernatants were collected. The activities of lactic dehydrogenase (LDH), superoxide dismutase (SOD), glutathione S-transferase (GST),

Ca^{2+}-Mg^{2+}-ATPase, Na^{+}-K^{+}-ATPase, T-ATPase, as well as the content of malondialdehyde (MDA) were determined with assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the kit's protocols (Cao et al., 2016; Li et al., 2017).

2.7. Morphology change of nonencysted tomonts

One hundred nonencysted tomonts with 200 μL water were placed into a well of 24-well plate. Then 200 μL 10-gingerol solution at the concentration of 16 mg/L was added into the well to make the final concentration of 8 mg/L solution. The treated nonencysted tomonts were observed and photographed under a Nikon ECLIPSE Ti.S inverted microscope.

2.8. Transmission electron microscope

Theronts were exposed to 2 mg/L 10-gingerol for 0.5 h and nonencysted tomonts were exposed to 8 mg/L 10-gingerol for 1.5 h. After cells were centrifuged at 239 × g for 10 min, the supernatant was discarded. The cells were prefixed in a 2.5% glutaraldehyde solution for 2 h at 4 °C, and fixation solution was removed following centrifugation. The cells were again fixed by 2.5% glutaraldehyde solution for 2 h. The cells were then fixed in 1% osmium tetroxide for 2 h and washed three times for 10 min with pre-cooled PBS. Next, the samples were dehydrated with a graded acetone series, embedded in Epon812, dried for 48 h, and cut with an ultramicrotome. Ultrathin sections were stained with uranyl acetate and lead citrate for 30 min, respectively. The samples were then examined and analyzed in a transmission electron microscope (Philips TECNAI10, Holland).

2.9. Statistical analysis

All experimental data were expressed as the mean ± SD (standard deviation). Assumptions of normality and homogeneity of variances were confirmed with Shapiro-Wilk test and Levene's test, respectively, using a statistical analysis system software (SPSS 19.0). Student-Newman-Keul's (SNK) test was used for multiple comparisons when ANOVA showed significant differences. Results were statistically significant when $p < 0.05$.

3. Results

3.1. Isolation and identification of bioactive compound

A bioassay-directed fractionation was performed to trace the active components (Fig. 1). Three compounds were isolated and identified by spectroscopic methods. Compound 1 (24.5 g) was identified as 10-gingerol with a molecular weight of 350.49 and a molecular formula of $C_{21}H_{34}O_4$. Compound 2 and compound 3 were identified as 6-dehydroshogaol (0.3 g, molecular weight: 274, formula: $C_{17}H_{22}O_3$) and 6-dehydro-10-gingerol (0.2 g, molecular weight: 348, formula: $C_{21}H_{32}O_4$), respectively.

3.2. In vitro bioactivity of active compounds on I. multifiliis theronts and tomonts

A wide range of concentrations of 10-gingerol, 6-dehydroshogaol, and 6-dehydro-10-gingerol (from 2 to 32 mg/L) were evaluated for their antiparasitic efficacy using *in vitro* anti-nonencysted tomonts assay. 10-gingerol, 6-dehydroshogaol, and 6-dehydro-10-gingerol showed an interdependence between concentration and antiparasitic effects on nonencysted tomonts (Fig. 2A). The minimum lethal concentration of 10-gingerol was 8 mg/L with a mean death duration of 141.3 ± 4.1 min for all nonencysted tomonts. The minimum lethal concentrations of 6-dehydroshogaol and 6-dehydro-10-gingerol were both 16 mg/L, with mean death duration of 120.5 ± 4.7 and 132.3 ± 5.1 min, respectively, for all nonencysted tomonts. The 4 h-EC_{50} of 10-gingerol, 6-dehydroshogaol, and 6-dehydro-10-gingerol to nonencysted tomonts was 3.7, 5.5, and 5.7 mg/L, respectively.

Since 10-gingerol exhibited the maximal activity against *I. multifiliis* in initial screening, its antiparasitic activity was more thoroughly evaluated on *I. multifiliis* theronts (Fig. 2B) and encysted tomonts (Fig. 2C). Theronts and encysted tomonts showed clear dose-response to 10-gingerol. The mean death duration ranged from 3.1 ± 0.5 min at a concentration of 16 mg/L to 131.2 ± 4.5 min at a concentration of 2 mg/L to all theronts. The minimum lethal concentration of 10-gingerol against encysted tomonts was 16 mg/L. The number of theronts released from encysted tomonts sharply decreased from 844.5 ± 24.3 in the negative control to 67.2 ± 3.1 in the 8 mg/L treated group 16 h post exposures. The EC_{50} of 10-gingerol to theronts and encysted tomonts were 0.7 and 4.9 mg/L, respectively.

The infectivity of the live theronts was decreased significantly ($p < 0.05$) after pretreated with 10-gingerol for 2 h. The mean intensity ranged from 2.3 ± 0.5 at a concentration of 1 mg/L to 595.3 ± 25.2 at a concentration of 0 mg/L (Fig. 2D). The treatment at a concentration of 1 mg/L 10-gingerol did not kill all theronts, but significantly reduced the infection incidence and mean intensity.

3.3. Evaluation of 10-gingerol in protection of grass carp against I. multifiliis infection

Since 10-gingerol effectively killed *I. multifiliis* theronts and tomonts, and significantly decreased theronts infectivity at low concentrations *in vitro* experiments, the further study was performed to investigate the protective effects of 10-gingerol on grass carp against *I. multifiliis* infection (Table 1). After 10-days of treatment, 10-gingerol at a concentration of 4 mg/L cured the infected grass carp and protected the naïve fish from *I. multifiliis* infection. 10-gingerol at a concentration of 2 or 1 mg/L significantly ($p < 0.05$) decreased the infection incidence and mean infection intensity, which increased the survival of infected and naïve fish ($p < 0.05$).

3.4. Enzyme activities

In the experiment, theronts and nonencysted tomonts were treated with 10-gingerol at 2 mg/L and 8 mg/L, respectively. The Na^{+}-K^{+}-ATPase (Fig. 3A), Ca^{2+}-Mg^{2+}-ATPase (Fig. 3B), T-ATPase (Fig. 3C), and LDH (Fig. 3D) activities of theronts and nonencysted tomonts were significantly lower in the 10-gingerol treated groups than those in control groups, and decreased notably with the increment of treatment time ($p < 0.05$). The activities were increased significantly for the SOD (Fig. 4B) and GST (Fig. 4C) at 0.5 and 1 h post treatment, then decreased sharply at 1.5 h in nonencysted tomonts ($p < 0.05$). Similarly, SOD (Fig. 4B) and GST (Fig. 4C) activities in theronts were increased significantly at 0.5 h but decreased sharply at 1 and 1.5 h ($p < 0.05$). MDA content (Fig. 4A) showed an opposite trend compared with other enzymes and increased significantly with the treatment time increment ($p < 0.05$). The MDA content was especially higher in theronts treated with 10-gingerol than that in control groups.

3.5. Morphology change of nonencysted tomonts

When exposed to 10-gingerol at 8 mg/L for 60 min, nonencysted tomonts showed a colloidal protoplasm (Fig. 5B). Plasma membrane was ruptured, and the cytoplasm spilled out of the nonencysted tomont at 90 min after exposure (Fig. 5C). With the increment of exposure time for 96 min, the plasma membrane was disintegrated (Fig. 5D).

3.6. Ultrastructural changes of theronts and nonencysted tomonts

When tomonts were treated with 10-gingerol at 8 mg/L for 1.5 h, the cell showed cytoplasmic vacuolation (Fig. 6A), damaged cilia and

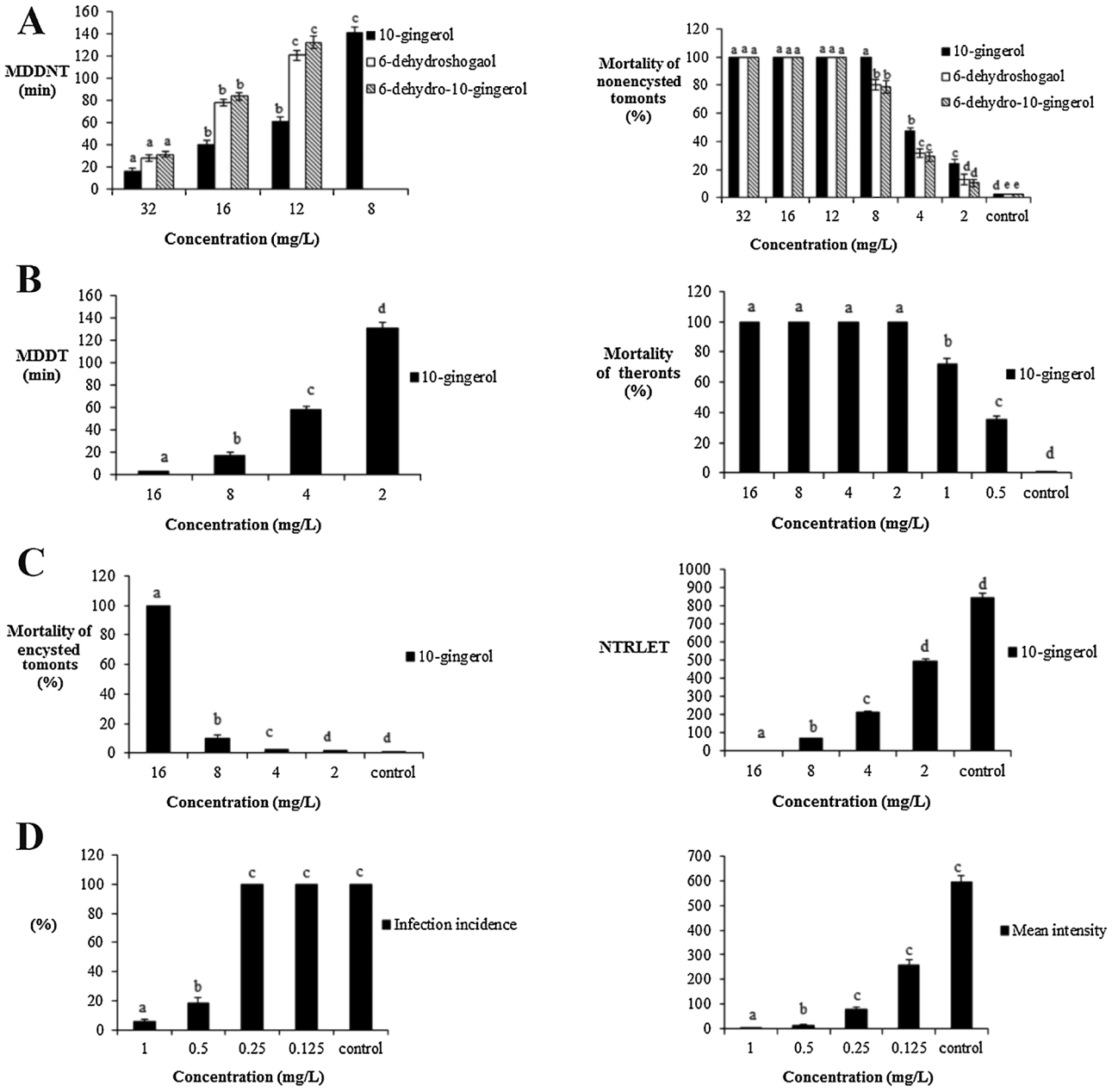

Fig. 2. *In vitro* antiparasitic efficacy of active compounds against *Ichthyophthirius multifiliis*. Values are means ± SD (standard deviation). Values with different letters in the group treated by the same compound are significantly different ($p < 0.05$). (A) Nonencysted tomont (n = 3). MDDNT: mean death duration of all nonencysted tomonts (maximum observation time 4 h). (B) Theronts (n = 3). MDDT: mean death duration of all theronts (maximum observation time 4 h). (C) Encysted tomonts (n = 5). Nonencysted tomonts were allowed to attach for 6 h to become encysted tomonts, exposed to 10-gingerol for 16 h, and theronts were counted in every well. NTRLET: number of theronts released from each live encysted tomont. (D) Infection incidence and mean intensity of theronts pretreated for 2 h with various concentrations of 10-gingerol on grass carp on day 5 (n = 3). Infection incidence (%): (infected fish/total fish) × 100; Mean intensity: number of white spots on fish/number of infected fish.

Table 1
Effect of a 10-day treatment using 10-gingerol on infection incidence and mean intensity of *Ichthyophthirius multifiliis*, and survival rate of grass carp during 15 d trial. 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: naïve grass carp; infection incidence (%): (infected fish/total fish) × 100; mean intensity: number of white spots on fish/number of infected fish.

Concentration (mg/L)	Grass carp/tank		Infection incidence(%)		Mean intensity		Survival rate (%)	
	IGC	NGC	IGC	NGC	IGC	NGC	IGC	NGC
0	5	10	100 ± 0^{a}	100 ± 0^{a}	75.2 ± 48.0^{a}	52.7 ± 28.0^{a}	73.3 ± 23.1^{a}	100 ± 0^{a}
1	5	10	100 ± 0^{a}	100 ± 0^{a}	56.3 ± 21.2^{b}	38.3 ± 19.0^{b}	80 ± 20^{a}	100 ± 0^{a}
2	5	10	20 ± 4.6^{b}	13.3 ± 3.1^{b}	12.7 ± 1.2^{c}	6.8 ± 1.4^{c}	100 ± 0^{b}	100 ± 0^{a}
4	5	10	0 ± 0^{c}	0 ± 0^{c}	0 ± 0^{d}	0 ± 0^{c}	100 ± 0^{b}	100 ± 0^{a}

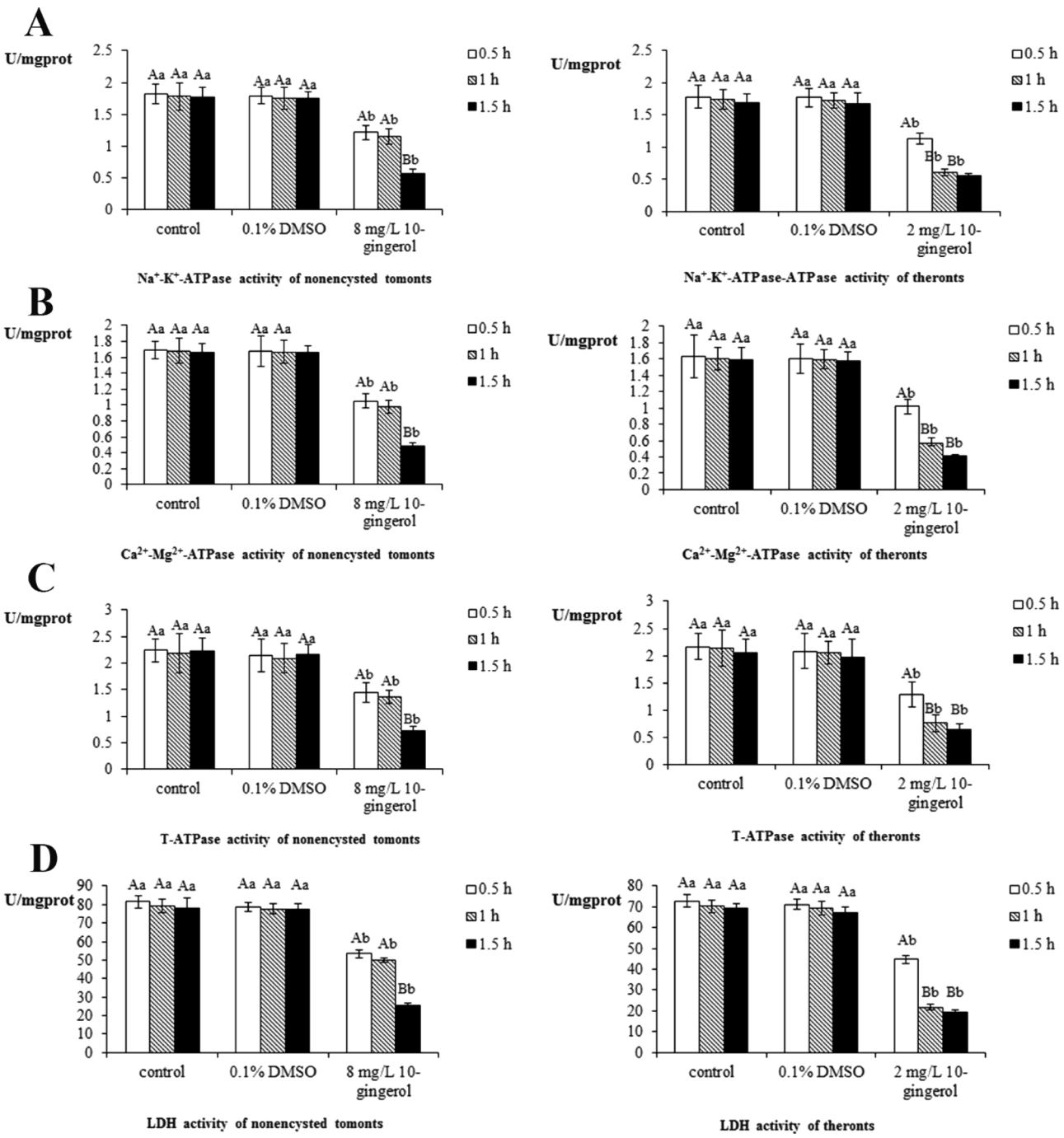

Fig. 3. Effects of 10-gingerol on ATPases and lactic dehydrogenase (LDH) activities of *Ichthyophthirius multifiliis* theronts and nonencysted tomonts. Values are means ± SD of 3 replicates. Within the same group, values with different capitals are significantly different ($p < 0.05$). Within the same time point, values with different lowercase letters are significantly different ($p < 0.05$). (A) Na^+-K^+-ATPase activity. (B) Ca^{2+}-Mg^{2+}-ATPase activity. (C) T-ATPase activity. (D) LDH activity.

kinetosomes (Fig. 6B). Cell membrane breakage (Fig. 6C) and mitochondria atrophia (Fig. 6D) were observed in the treated tomonts. In addition, a number of the organelle fragments were present in the cytoplasm of treated tomonts (Fig. 6D).

After exposure to 10-gingerol, theronts showed similar lesions with those of tomonts (Fig. 7). The chromatin of macronucleus and micronucleus were condensed, and the nuclear membrane was damaged (Fig. 7A). The cilia fragments and damaged kinetosomes were distributed around the cell membrane (Fig. 7B). The endoplasmic reticula were broken into fragments (Fig. 7C) and the mitochondria were atrophy (Fig. 7D).

4. Discussion

It is widely accepted that some plant products have the ability to protect fish from *I. multifiliis* infection (Ling et al., 2012; Liu et al., 2017; Zhang et al., 2013). Most of these studies concentrated on the antiparasitic effects of plant extracts and compounds against *I. multifiliis*, such as acetone extract of *Morus alba* root bark (Fu et al., 2014b), pentagalloylglucose (Zhang et al., 2013), cynatratoside-C (Fu et al., 2014a), cinnamaldehyde (Liu et al., 2017), sanguinarine (Yao et al., 2010), dihydrosanguinarine (Yao et al., 2011), and dihydrochelerythrine (Yao et al., 2011). The present study focused on the active

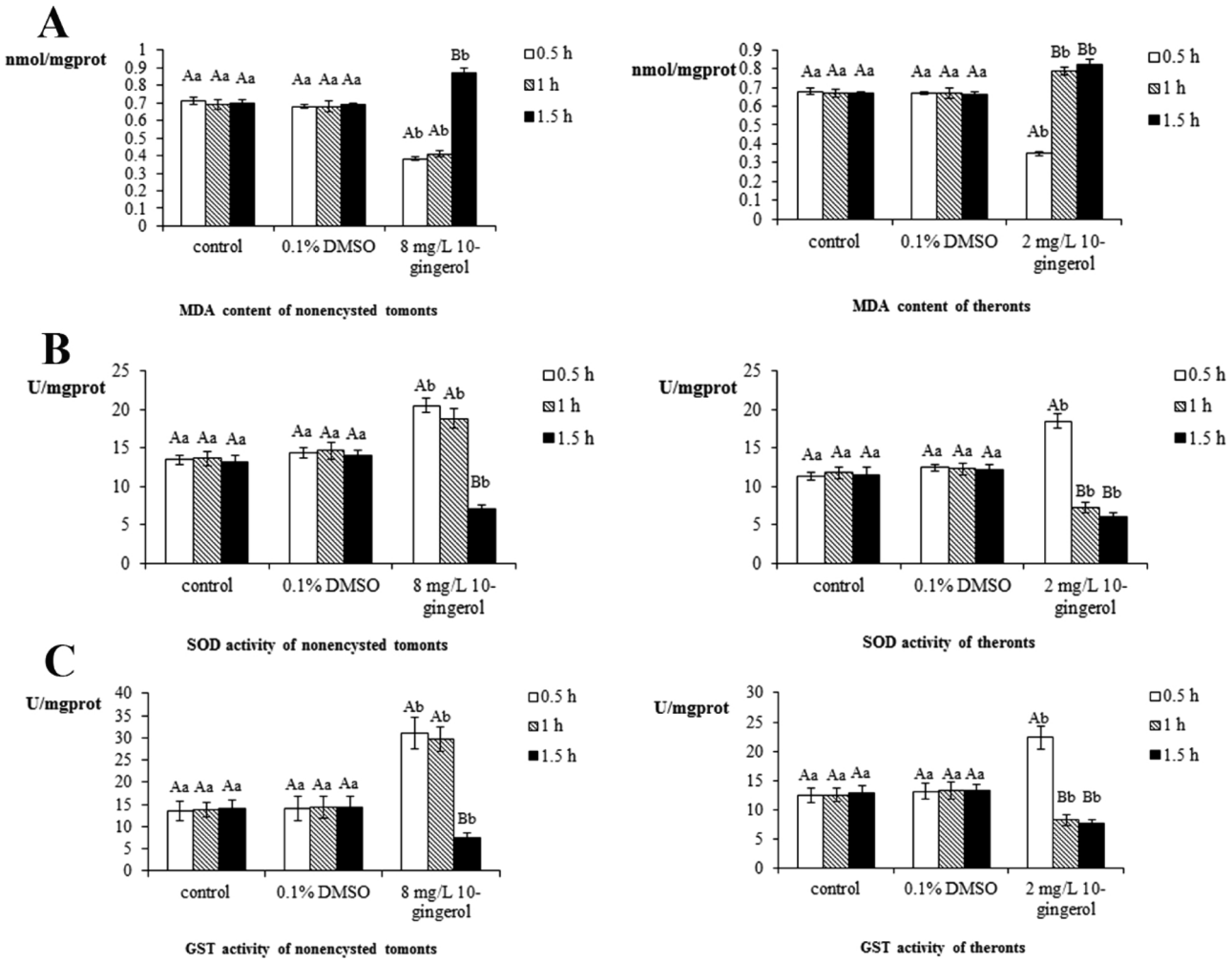

Fig. 4. Effects of 10-gingerol on the malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, and glutathione S-transferase (GST) activity of *Ichthyophthirius multifiliis* theronts and nonencysted tomonts. Values are means ± SD of 3 replicates. Within the same group, values with different capitals are significantly different ($p < 0.05$). Within the same time point, values with different lowercase letters are significantly different ($p < 0.05$). (A) Content of MDA. (B) SOD activity. (C) GST activity.

compounds of ginger screened in previous work.

Ginger oleoresin is a dark golden brown viscous oil extracted from ginger by supercritical carbon dioxide. It contains gingerols and shogaols, which are responsible for the typical ginger pungency (Panjvini et al., 2016). The major constituents of oleoresin have been identified as 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol (Ryu and Chung, 2015). These compounds have diverse biological activities, such as antioxidant, anti-inflammatory, and anticarcinogenic properties (Chen et al., 2013; Joo et al., 2016). In this study, three compounds were isolated from ginger oleoresin with bioassay-guided fractionation, and identified as 10-gingerol, 6-dehydroshogaol, and 6-dehydro-10-gingerol with NMR and ESI-MS. This is the first report on anti-*I. multifiliis* property for these three compounds.

The anti-nonencysted tomonts results demonstrated that 10-gingerol was the most active compound among the three compounds. The 10-gingerol resulted in 100% mortality of nonencysted tomonts at 8 mg/L, which was 2 times lower in concentration than that of 6-dehydroshogaol or 6-dehydro-10-gingerol. In addition, the weight of 10-gingerol, 6-dehydroshogaol, and 6-dehydro-10-gingerol was 24.5, 0.3, and 0.2 g, respectively. The content of 10-gingerol in ginger was higher than other compounds. Thus, 10-gingerol was the main ingredient and appeared to have been the principal compound in ginger responsible for killing *I. multifiliis*. Therefore, the further study was focused on the antiparasitic efficacy and mechanism of 10-gingreol against *I. multifiliis*.

10-gingerol at concentrations of 2, 8, and 16 mg/L eliminated all theronts, nonencysted tomonts, and encysted tomonts, respectively. This indicated that killing the tomonts and trophonts of *I. multifiliis* needed a higher concentration of 10-gingerol. In the life cycle of *I. multifiliis*, encysted tomonts are covered by a thick cyst wall in water and trophonts are covered by a host epidermis layer plus a thick layer of mucus. The protective barriers of encysted tomonts and trophonts make them more difficult to be killed than theronts and nonencysted tomonts. The protective phenomenon has been demonstrated by previous studies (Fu et al., 2014b; Zhang et al., 2013). For example, pentagalloylglucose from *Galla chinensis* killed all theronts at a concentration of 2.5 mg/L, but it required concentration of 40 mg/L to kill all tomonts (Zhang et al., 2013); Acetone extract of *M. alba* at concentrations of 8, 25, and 50 mg/L resulted in 100% mortality of theronts, nonencysted tomonts, and encysted tomonts, respectively (Fu et al., 2014b); Cynatratoside-C killed all theronts at a concentration of 0.25 mg/L, but it needed a concentration of 2 mg/L to eliminate all trophonts (Fu et al., 2015).

A 10-days of treatment of low concentrations of 10-gingerol against *I. multifiliis* infestation in fish was conducted. The results demonstrated that 10-gingerol at a concentration of 4 mg/L cured the infected grass carp and protected the healthy fish from *I. multifiliis* infestation. 10-gingerol at a concentration of 2 mg/L significantly decreased the infection incidence and mean infection intensity, then improved the survival of grass carp. In another experiment to investigate low

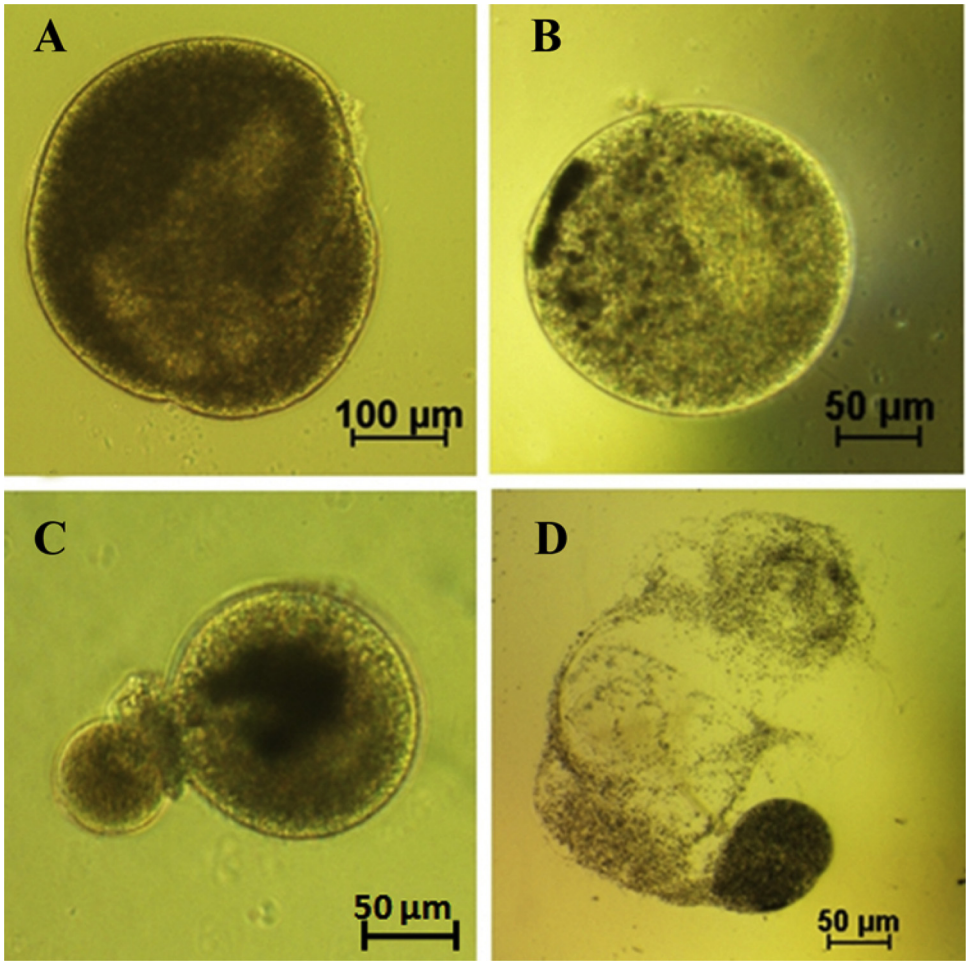

Fig. 5. Morphology of nonencysted tomonts treated with 10-gingerol at 8 mg/L. (A) Untreated nonencysted tomont. Scale bar = 100 μm. (B) The nonencysted tomont showing a colloidal protoplasm. Scale bar = 50 μm. (C) The nonencysted tomont showed raptured cell membrane and spilled out cytoplasm. Scale bar = 50 μm. (D) The plasma membrane completely disintegrated. Scale bar = 50 μm.

concentrations of 10-gingerol on the theronts infectivity, pretreatment of theronts significantly decreased their infectivity at concentrations of 1 or 0.5 mg/L for 2 h. After the parasite infected fish, trophonts remain on the fish for approximately 7 days at 20 °C. Mature trophonts leave the host into the water, become tomonts, and reproduce hundreds to thousands of infective theronts (Dickerson and Findly, 2014). Since theronts are more sensitive to drugs than tomonts and trophonts. Therefore, killing the parasite at the theront stage is an important way to prevent *I. multifiliis* infestation and reduce the dosage of drugs (Zhang et al., 2013). Similar phenomena were found in previous studies (Fu et al., 2014b; Shinn et al., 2012; Zhang et al., 2013). The chemical bronopol at a concentration of 1 mg/L did not kill all theronts during a period of 12 h exposure, but significantly reduced the infectivity in rainbow trout *Oncorhynchus mykiss* (Shinn et al., 2012). Those results demonstrated that low concentrations of 10-gingerol could prevent the infestation of *I. multifiliis* by killing the parasite or decrease the infectivity of theronts.

The activities of lactate dehydrogenase (LDH) and adenosine triphosphatases (ATPases) were decreased significantly after nonencysted tomonts and theronts treated with 10-gingerol for 0.5 h. LDH is the last enzyme in the glycolytic pathway in converting pyruvate to lactate and simultaneously in the conversion of NADH to NAD^+ (Cameron et al., 2004). ATPases are a class of enzymes that catalyze the decomposition of ATP into ADP and a free phosphate ion. LDH and ATPases are the key enzymes of energy metabolism. Inhibition of LDH and ATPases decreased energy metabolism and production of ATP, which cause *I. multifiliis* stopping motion and leading to cell death.

The activities of Na^+-K^+-ATPase and Ca^+-Mg^+-ATPase in *I. multifiliis* were inhibited significantly following the treatment with 10-gingerol. ATPases are a group of membrane-bound enzymes that mediate the active transport of ions across the plasma membranes, and thus maintain the membrane potential and osmotic equilibrium of the cell (Cao et al., 2016). Na^+-K^+-ATPase is the enzyme responsible for recovering the polarization of the cell membranes by efflux of Na^+ and influx of K^+ (Khadrawy et al., 2017). The inhibition of Na^+-K^+-ATPase and Ca^+-Mg^+-ATPase activity lead to the Na^+ and Ca^{2+} overload (Yan et al., 2017; Yu and Gao, 2017). The increasing of Na^+ resulted in the increasing of intracellular osmotic pressure and cellular edema (Yang et al., 1992). The Ca^{2+} overload will lead to the deposition of Ca^{2+} in mitochondrial in the form of calcium phosphate, which is harmful to the mitochondrial function (Yu and Gao, 2017). In addition, Ca^{2+} could also lead to the generation of intracellular free radicals and the damage of cytomembrane structure (Yu and Gao, 2017). Therefore, the membranes and mitochondria of theronts and tomonts were damaged with the treatment of 10-gingerol.

When *I. multifiliis* were treated with 10-gingerol, the superoxide dismutase (SOD) and glutathione S-transferase (GST) activities increased significantly at 0.5 h. At this stage, *I. multifiliis* improved the antioxidant ability to defense the oxidative damage. With the increment of exposure time, the SOD and GST activities decreased constantly, and were significantly lower than those in control group. SOD is of the first-line defense in the antioxidant enzymes, catalyzing O_2^- anion dismutation into O_2 and H_2O_2 (Shen et al., 2016). GST is the important phase II detoxification enzyme, playing important role in protecting

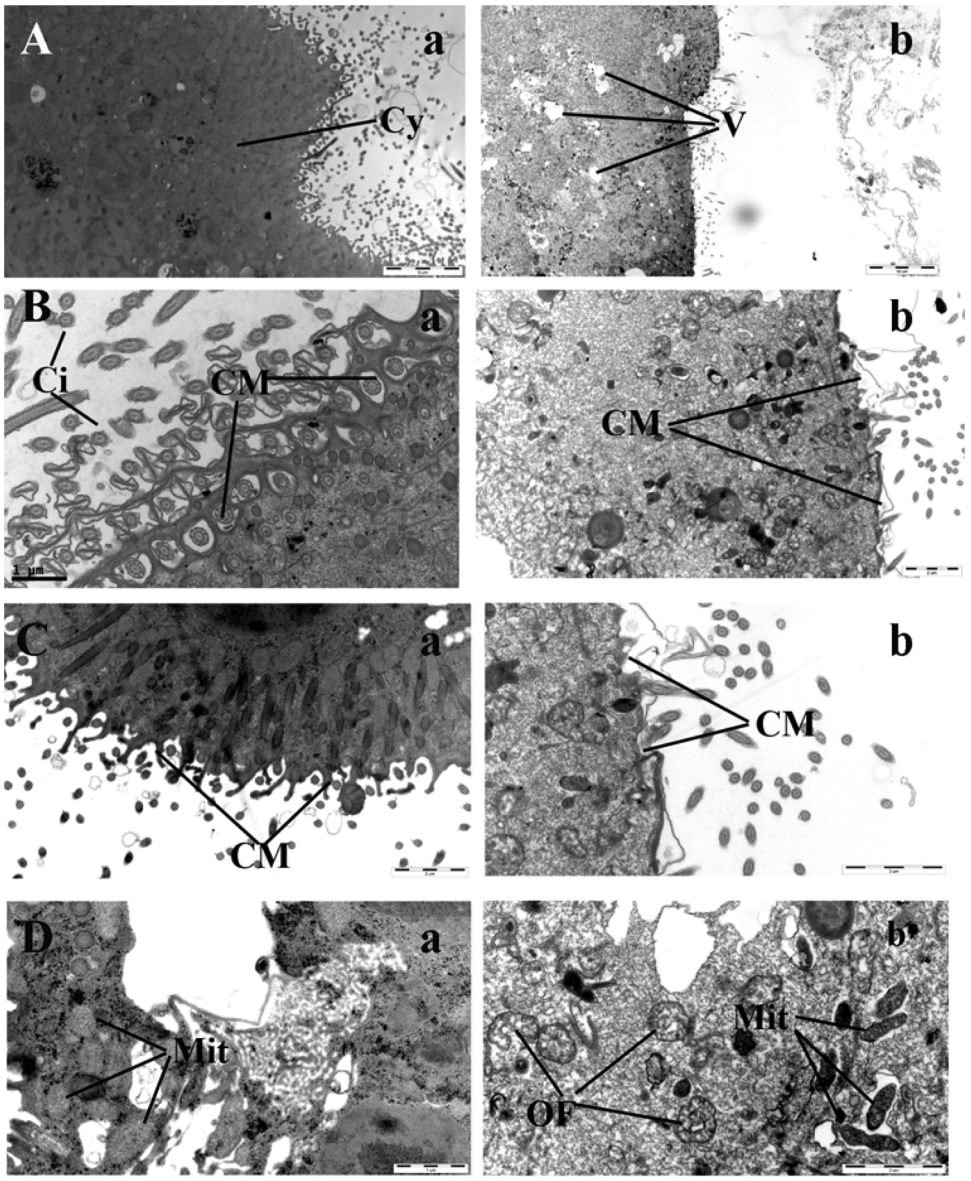

Fig. 6. Ultrastructural changes of *Ichthyophthirius multifiliis* nonencysted tomonts treated with 10-gingerol at 8 mg/L. (A) a: normal cytoplasm (Cy) in control group. Scale bar = 5 μm. b: Nonencysted tomont showed cytoplasmic vacuolization in treatment group. V: vacuole; a: control group (CG); b: treatment group (TG). Scale bar = 10 μm. (B) Ci: cilium. CM: cell membrane. The protrusion and holes on the cell membrane disappeared. a: CG; b: TG. Scale bar of a = 1 μm. Scale bar of b = 2 μm. (C) CM: cell membrane. B: breakage. OF: organelle fragment. Cell membrane breakage and organelle fragments were found in the treatment group. a: CG; b: TG. Scale bar of a and b = 2 μm. (D) Mit: mitochondria. OF: organelle fragment. Mitochondria were atrophied in the treatment group. a: CG; b: TG. Scale bar of a = 1 μm. Scale bar of b = 2 μm.

tissues against the oxidative damage (Zheng et al., 2016). While the content of malondialdehyde (MDA), a biomarker of lipid peroxidation, increased significantly at 1 and 1.5 h. This indicated that normal balance between the producing and scavenging of intracellular free radical was broken (Tsikas, 2017). Free radicals excessively accumulated in cells could further damage structure of *I. multifiliis*.

TEM analysis results backed up the above observation. *I. multifiliis* treated with 10-gingerol resulted in the damage of organelles and cytomembrane, such as cell membrane breakage, mitochondria atrophia, endoplasmic reticulum and Golgi apparatus lysis, chromatin condensation, as well as nuclear membrane fracture. Morphological changes of mitochondria with disrupted internal membrane cristae indicated cell injury resulting from oxidative damage (Matei and Trombetta, 2016). Changes of mitochondria may also directly contribute to the apoptotic process. In previous study, reactive oxygen species produced by mitochondria in artemisinin-treated cells could induce endoplasmic reticulum stress and DNA damage, as well as affect calcium metabolism (Crespo-Ortiz and Wei, 2012; O'Neill et al., 2015). Therefore, the anti-*I. multifiliis* mechanism of 10-gingerol was postulated as follows: 10-gingerol decreased the ATPases activities and resulted in Na^+ and Ca^{2+} overload. The Na^+ and Ca^{2+} accumulated excessively and resulted in the increasing of intracellular osmotic pressure and accumulation of free radicals. The increment of oxidative stress induced morphological changes of mitochondria, which accelerated the damage of cytomembrane and organelles, and resulted in *I. multifiliis* apoptosis.

5. Conclusion

The anti-*I. multifiliis* active compounds 6-dehydroshogaol, 6-dehydro-10-gingerol, and 10-gingerol were isolated from ginger oleoresin. Among three compounds, 10-gingerol showed the highest efficacy against *I. multifiliis*. The current study demonstrated that 10-gingerol killed the theronts and encysted tomonts effectively *in vitro*, and could decrease the infectivity of theronts at low concentrations. 10-gingerol at a concentration of 4 mg/L protected fish from *I. multifiliis* infection. The antiparasitic mechanism study indicated that 10-gingerol resulted in the increase of intracellular osmotic pressure and accumulation of free radicals, which damaged the membrane system of *I. multifiliis*. Thus, 10-gingerol is a potential alternative parasiticide to prevent and control *I. multifiliis*.

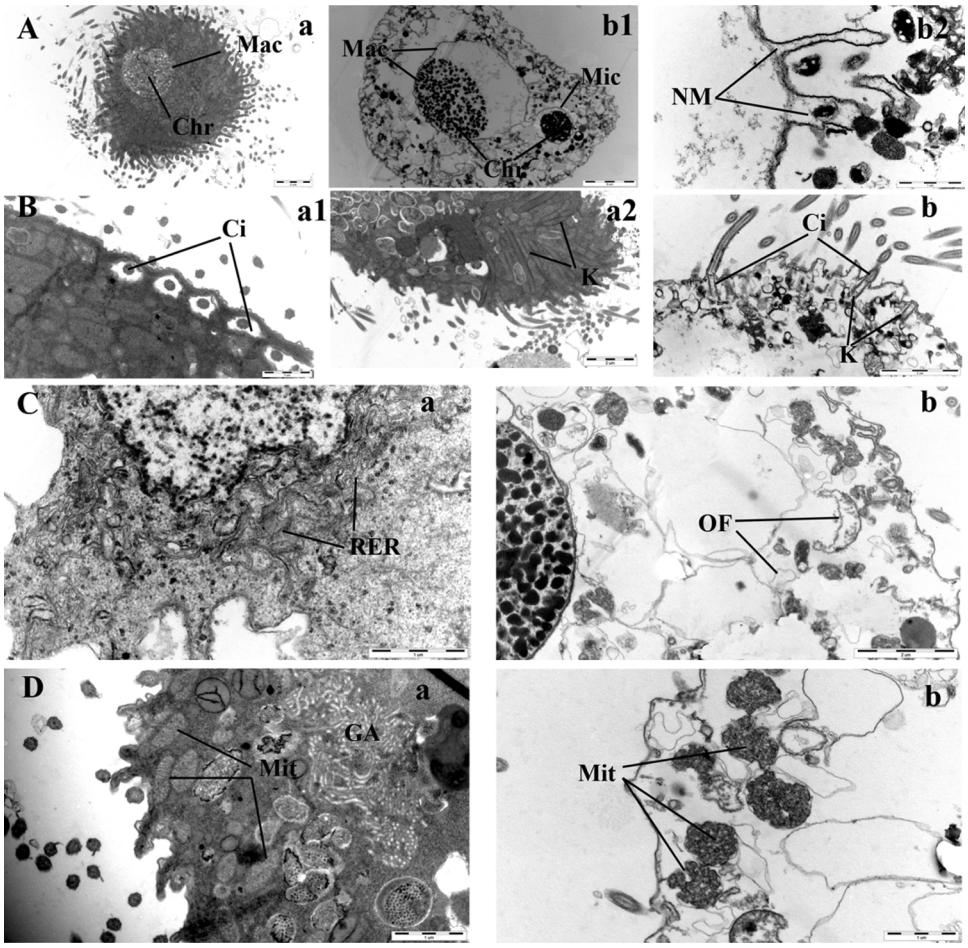

Fig. 7. Ultrastructural changes of *Ichthyophthirius multifiliis* theronts treated with 10-gingerol at 2 mg/L. (A) Mac: macronucleus. Mic: micronucleus. Chr: chromatin. NM: nuclear membrane. The chromatin of macronucleus and micronucleus was condensed, and the nuclear membrane were damaged in the treated theronts. a: control group (CG); b: treatment group (TG). Scale bars of a, b1, and b2 are 2 μm, 5 μm, and 1 μm, respectively. (B) Ci: cilium. K: kinetosome. b: Cilia and kinetosomes were damaged. a: CG; b: TG. Scale bars of a1, a2, and b are 1 μm, 2 μm, and 2 μm, respectively. (C) RER: rough endoplasmic reticulum. OF: organelle fragment. b: Organelle fragments were found in the treatment group. a: CG; b: TG. Scale bar of a = 1 μm. Scale bar of b = 2 μm. (D) Mit: mitochondria. GA: Golgi apparatus. OF: organelle fragment. b: Mitochondria were atrophied after theronts treated with 10-gingerol at a concentration of 2 mg/L. a: CG; b: TG. Scale bar for a and b is 1 μm.

Conflict of interest

The authors have no competing interests to declare.

Acknowledgments

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