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Antiparasitic efficacy and safety assessment of magnolol against *Ichthyophthirius multifiliis* in goldfish

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ABSTRACT

Ichthyophthirius multifiliis causes high mortality rate of freshwater fish. It is necessary to find the effective alternatives that may both be affordable and environmentally acceptable since malachite green is banned to use. In this work, magnolol exhibited great anti-parasitic activity against all stages of *I. multifiliis*. Magnolol at concentrations of 0.6 mg/L or more resulted in 100% mortality of *I. multifiliis* theronts within 4 h exposure; and terminated reproduction of *I. multifiliis* post 6 h exposure of protomonts and encysted tomonts to 0.8 and 1.0 mg/L, respectively. Moreover, some of tomonts presented morphological characteristic changes and a larger part of tomonts were unable to reproduce. Magnolol possibly had a directly effect on the cell membrane, and more importantly, it could permeate into an encysted tomont across the cyst wall and then impact on the tomont proliferation. *In vivo* trials showed that 5 h exposure of infected fish to 1.5 mg/L of magnolol significantly reduced the number of theronts released from tomonts. In addition to determination of anti-*Ichthyophthirius* activity, we also systematically evaluated the safety of magnolol against fish. First of all, the results of acute toxicity experiment showed that the 96 h median lethal concentration (LC_{50}) of magnolol to goldfish was 6.02 mg/L. Furthermore, gene expression (*cyp1a*, *hsp70*, *gst* and *sod*) and enzyme activity (GSH, SOD and T-AOC) in liver, spleen and kidney could recover to the same level as the control group up to 48 h, though magnolol induced goldfish stress response during 6–24 h exposure. More than that, magnolol can be easily synthesized with low cost, and further embellished and modified for higher bioactivity and lower toxicity because of its simple and symmetry chemical structure. Therefore, magnolol has the potential to be a safe and effective therapeutant, and it also can be used as a probable leading compound for the development of commercial drug to control ichthyophthiriasis in aquaculture.

1. Introduction

Ichthyophthirius multifiliis, which can cause white spot disease, is a globally-distributed pathogenic parasitic ciliate due to low host specificity and wide distribution. *I. multifiliis* infection causes severe ichthyophthiriasis and mass economic losses for the aquaculture industry (Wahli and Meier, 1987). *I. multifiliis* has a temperature-dependent life cycle, which divides into four key stages: the infective theront, the parasitic trophont, the free-swimming protomont and the reproductive encysted tomonts (Matthews, 2005). Theronts penetrate into epidermis of host and differentiate into the trophonts, which resides within the surface epithelium of gills, fins and other portions of body. The free-swimming protomonts derived from the mature trophonts leave the host actively, and then settle on the substrate to transform into encysted tomonts. The encysted tomonts undergo a series of binary fission and product a large number of tomites which develop into infective theronts.

Up to now, malachite green is considered as the most effective parasiticide against both the free-living and parasitic stage of *I. multifiliis* (Buchmann et al., 2003; Picón-Camacho et al., 2012). However, the use of malachite green has been banned for food fish because of its carcinogenic and teratogenic effects on humans and adverse effects on the aquatic environment (Alderman, 2010). Other chemotherapeutants, such as formalin (Rintamäki-Kinnunen et al., 2005a, 2005b; Rowland et al., 2010), chloramine-T (Rintamäki-Kinnunen et al., 2005a), copper sulfate (Ling et al., 1993; Schlenk et al., 1998), potassium permanganate (Straus and Griffin, 2002; Buchmann et al., 2003) and hydrogen peroxide (Rach et al., 2000; Lahnsteiner and Weismann, 2007), lack efficacy for eliminating *I. multifiliis* trophont *in situ* (Matthews, 2005) or have potential harmful impacts on human health and environment (Tieman and Goodwin, 2001). Therefore, recently, more and more attentions were attracted to the application of medicinal plants to control *I. multifiliis*, because of demonstrated efficacy and low environmental hazard (Chu et al., 2010). Amounts of medicinal plant extracts were

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evaluated for anti-*I. multifiliis* efficacy, for instance, ethanol extract of *Macleaya cordata* (Yao et al., 2010), aqueous extract of *Capsicum frutescens* (Ling et al., 2012) and acetone extract of *Morus alba* (Fu et al., 2014a). Additionally, the natural compounds such as kuwanons G and O from *Morus alba* (Liang et al., 2015), gracillin and zingibernsis from *Costus speciosus* (Zheng et al., 2015), isopsoralen and psoralidin from *Psoralea corylifolia* (Song et al., 2015), had great effect on free-living stage of *I. multifiliis*. However, large-scale uses of these natural products were actually difficult to apply in aquaculture due to their lower yield or high cost. Thus, it is necessary to develop an efficient, cheap and easily obtained natural compound, which would provide reference for the further development of eco-friendly anti-*I. multifiliis* drug.

In our previous study, the methanol extracts of *Magnolia officinalis* displayed high antiprotozoal activity against *I. multifiliis* (Yi et al., 2012). The magnolol (5,50-diallyl-2,20-dihydroxybiphenyl) is the principal active component of magnolia bark extract, and shows a variety of biological properties including anti-oxidativity, antitumor activity, anti-depressant activity, anti-inflammatory activity, neuroprotective activity, anti-diabetic activity, antiviral activity, and antimicrobial activity (Chen et al., 2011). However, there has been no report on anti-*I. multifiliis* activity of this natural compound. Importantly, magnolol can be synthesized with high quality and cost effective according to an easy work-up and purification methodology (Agharahimi and Lebel, 1995; Reddy et al., 2013). Given that this natural compound has high antiprotozoal activity, then it will become a promising alternative for controlling ichthyophthiriasis in aquaculture industry.

In addition to antiprotozoal activity, safety evaluation of natural compounds is also important for their application. However, a large number of previous studies have much focused on the evaluation of antiparasitic activity and often neglected the safety assessment to the host. For further the safety assessments, biomarker is considered as one of promising tools to determine the severity of toxicants (Weeks, 1995; Voelker et al., 2007). Generally, the stress response related genes (*e.g.* hsp70, cyp1a, *etc.*) or enzymes (*e.g.* GSH, SOD, *etc.*) are selected to evaluate the toxicity of some drugs on fish (Zhu et al., 2013; Liu et al., 2015). In this study, acute toxicity experiments in combination with analysis of stress response biomarkers are essential to determine whether magnolol can safely apply in aquaculture. The primary goals of this study were to (1) investigate the efficacy of magnolol against all stages of *I. multifiliis*; (2) systematically evaluate the safety of magnolol against goldfish.

2. Materials and methods

2.1. Fish, parasite and natural compounds

Goldfish (*Carassius auratus*), weight 10.4 ± 1.3 g, were purchased from a commercial aquafarm at Xi'an, Shaanxi, China. All fish were acclimated for 2 weeks in 200 L of aquaria equipped with an aerator and a water suction pump (water temperature 22.0 ± 1.5 °C, pH 7.2 ± 0.3, with dissolved oxygen 8.0 ± 1.0 mg/L). They were fed once at 1% body weight daily with commercial fish pellet feed.

A local strain of *I. multifiliis* was isolated from goldfish obtained from Zhuque ornamental fish market at Xi'an, China. The parasite was maintained by continuous serial passages through goldfish as Ling et al. (2009) described. The parasitized fish and healthy goldfish were held in a static 50-L glass aquarium under the same conditions as described above in order to develop *I. multifiliis* infection. The parasites were collected as previously described (Ling et al., 2013; Song et al., 2015). The heavily infected fish were placed into several beakers with filtered aquarium water (100 mL/fish). Mature parasites actively exited from the fish by gentle mechanical stimulation, and then were rinsed three times using distilled water in order to discard fish mucus. The collected protomonts could develop into the encysted tomonts after 6 h or product theronts after 22–24 h incubation at 22.0 ± 0.5 °C. Theront numbers were enumerated in ten 2-μL of 1% formalin-fixed theront suspension onto a glass slide (× 40 magnification) and the mean count in ten droplets was expressed as the final number of theronts (Schlenk et al., 1998; Straus and Griffin, 2001).

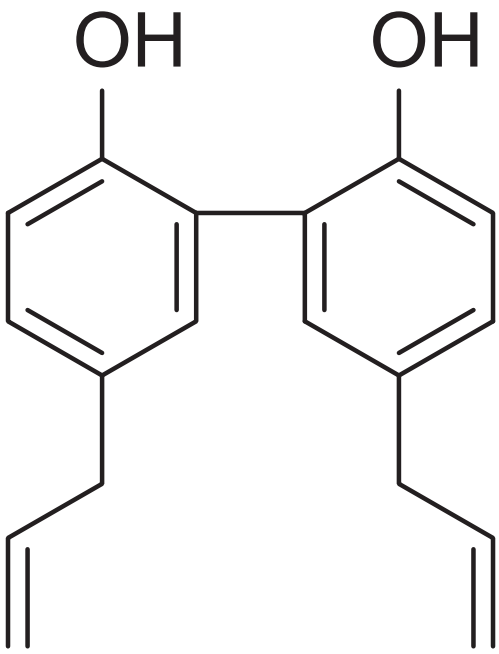

Fig. 1. The chemical structure of magnolol.

Magnolol was purchased from Sigma-Aldrich (St. Louis, MO, USA), and the chemical structure of magnolol was shown in Fig. 1.

2.2. Effect of magnolol on free-living stages of I. multifiliis

Three experiments were performed to evaluate bioactivity of magnolol against free-living stages of *I. multifiliis* (theront, protomont and encysted tomont). In this study, 0.02% (v/v) of DMSO was used to increase the water solubility of compounds. As confirmed by a previous study, *I. multifiliis* was not influenced by this concentration of DMSO (Ekanem et al., 2004).

In experiment 1, approximately 300 theronts were dispensed to each well of a 96-well microtiter plate and exposed to final concentrations of magnolol at 0.8, 0.7, 0.6, 0.5, 0.4, 0.3 and 0.2 mg/L in a final volume of 100 μL per well, respectively. A negative control (0 mg/L) and a positive control (0.01, 0.025 and 0.05 mg/L of malachite green) were set up. After 4 h incubation, mortality of theronts was determined based on the number of theronts with absence of motility and abnormal morphology. The experiment was conducted at 22 ± 0.5 °C and replicated three times using separate populations of theronts for each concentration.

The second experiment was carried out to determine the effect of magnolol on encystment and reproduction of protomonts after 6 h exposure. Thirty protomonts in 500 μL of filtered aquarium water were distributed to each well (N = 3) of 24-well tissue culture plate. Then, 500 μL of the tested solution were added into each well to yield final concentrations of 1.2, 1.0, 0.8, 0.6, 0.4 and 0.2 mg/L, respectively. A negative control (0 mg/L) was set up, and malachite green solutions at 0.05 and 0.1 mg/L were used as positive controls. At 6 h after exposure, the solution was replaced with fresh filtered aquarium water in each well, and then the number of encysted parasites in each well was counted. After a total of 22 h incubation at 22 ± 0.5 °C, the number of theronts released from tomonts in each well was recorded using the method described above, and reproduction was represented as number of released theronts per encysted tomont. The experiment was replicated 3 times.

For the third experiment, protomonts were collected and distributed