Veterinary Parasitology 236 (2017) 128–136

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Antiparasitic efficacy of curcumin from *Curcuma longa* against *Ichthyophthirius multifiliis* in grass carp

Yan-Meng Liu[a], Qi-Zhong Zhang[a,*], De-Hai Xu[b], Yao-Wu Fu[a], De-Jie Lin[a], Sheng-Yu Zhou[a], Jian-Pei Li[a]

[a] *Key Laboratory of Aquatic Eutrophication and Red Tide Prevention of Guangdong Higher Education Institutes, Engineering Research Center of Tropical and Subtropical Aquatic Ecological Engineering Ministry of Education, Institute of Hydrobiology, Jinan University, West 601 Huangpu Avenue, Tianhe District, Guangzhou 510632, People's Republic of China*

[b] *United States Department of Agriculture, Agricultural Research Service, Aquatic Animal Health Research Unit, 990 Wire Road, Auburn, AL 36832-4352, USA*

ARTICLE INFO

Article history:
Received 28 July 2016
Received in revised form 13 February 2017
Accepted 14 February 2017

Keywords:
Ichthyophthirius multifiliis
Curcumin
Curcuma longa
Antiparasitic efficacy
Acute toxicity

ABSTRACT

Ichthyophthirius multifiliis is a ciliated parasite that elicits great economic losses in aquaculture. In the present study, a polyphenol compound, curcumin, was obtained from the rhizome of *Curcuma longa* by bioassay-guided isolation based on the efficacy of anti-*I. multifiliis* theronts. Anti-*I. multifiliis* efficacy of curcumin was evaluated *in vitro* and *in vivo*. Curcumin resulted in 100% mortality of *I. multifiliis* theronts at a concentration of 1 mg/L within 21.7 ± 1.2 min and killed all tomonts at 8 mg/L within 31.0 ± 1.0 min. Curcumin at 4 mg/L for 16 h exposure can completely terminate the reproduction of tomonts. The pre-treatment with curcumin at concentrations of 0.5, 0.25, and 0.125 mg/L for 2 h significantly reduced the infectivity of *I. multifiliis* theronts. Curcumin at 4 mg/L completely cured the infected grass carp and protected naive fish from *I. multifiliis* infection after 10 days exposure. The 4 h median effective concentration (EC_{50}) of curcumin to *I. multifiliis* theronts and the 5 h EC_{50} of curcumin to *I. multifiliis* tomonts were 0.303 mg/L and 2.891 mg/L, respectively. The 96 h median lethal concentration (LC_{50}) of curcumin to grass carp was 56.8 mg/L, which was approximately 187.4 times EC_{50} of curcumin to theronts and 19.6 times EC_{50} of curcumin to tomonts. The results demonstrated that curcumin has the potential to be a safe and effective therapeutant for controlling ichthyophthiriasis in aquaculture.

1. Introduction

Ichthyophthirius multifiliis, a holotrichous obligate parasite, elicits "white spot disease" of fish, and commonly leads to great economic losses in aquarium and farm-raised fishes (Dickerson and Clark, 1996; Buchmann et al., 2001). The life cycle of *I. multifiliis* includes four stages: the trophont, the tomont, the cyst, and the theront. When mature trophonts leave the fish and transform into tomonts, tomonts attach to substrates where they encyst, and then become cysts. The cysts undergo a rapid division with the production of several hundred to thousand tomites, and tomites differentiate into infective theronts. The free-swimming theronts penetrate into the epithelia of fish and become parasitic trophonts again (Dickerson and Clark, 1998; Matthews, 2005; Dickerson and Findly, 2014).

In the past decades, malachite green was used as an effective compound to control ichthyophthiriasis, but it was never registered as a veterinary drug for use in food fish in many countries because of its potential properties of carcinogenicity, mutagenicity and teratogenicity (Srivastava et al., 2004; Sudova et al., 2007). Studies have been conducted to evaluate anti-*I. multifiliis* efficacy of chemotherapeutants including formalin (Rowland et al., 2008), chloramine-T (Rintamaki-Kinnunen et al., 2005), copper sulfate (Ling et al., 1993; Schlenk et al., 1998), potassium permanganate (Straus and Griffin, 2002), and peracetic acid (Meinelt et al., 2009; Straus and Meinelt, 2009; Sudova et al., 2009). The majority of tested products have a certain efficacy against the free-living stages of the parasite (tomonts and theronts) but very limited against the parasitic stage (trophonts) and reproductive stage (cysts), and some are not currently licensed as medicines for this purpose and can not be used on food fish (Picon-Camacho et al., 2012). Thus, it is necessary to develop an alternative therapeutant to control *I. multifiliis*.

Traditional Chinese herbal medicinal plants have been used to control diseases of human beings and livestock for a long time

* Corresponding author.
E-mail addresses: zhangqzdr@126.com, zqz666@sina.com (Q.-Z. Zhang).

http://dx.doi.org/10.1016/j.vetpar.2017.02.011
0304-4017/

(Acharya and Acharya, 2010; Tan and Vanitha, 2004). Recently, more and more medicinal plant extracts were investigated for their anti-*I. multifiliis* efficacy, and some of them were found to have a significant efficacy of treating *I. multifiliis* (Yao et al., 2010, 2011; Zhang et al., 2013; Fu et al., 2014b; Liang et al., 2014; Shan et al., 2014; Liang et al., 2015; Song et al., 2015; Zheng et al., 2015). These compounds could be degraded in fish and environment, and showed low toxicity on human health (Chu et al., 2010). The rhizome of turmeric, *Curcuma longa* (Zingiberaceae), commonly called "jianghuang" in Chinese, has been widely used in food for its flavor and color. It is also commonly used as a medicine to treat menstrual difficulties, hemorrhage, hematuria, jaundice, and colic in traditional Chinese medicine (Labban, 2014). The constituents from *C. longa* have been demonstrated to be numerous pharmacological activities, including anti-inflammatory, anti-bacteria, anti-tumor, anti-HIV, and antioxidant (Araujo and Leon, 2001). However currently there is no report on the isolation of components with anti-*I. multifiliis* activity from this medical plant. In this study, an active compound from ethanol extract of turmeric rhizome was isolated by bioassay-guided fractionation, and then the compound was evaluated for the anti-*I. multifiliis* efficacy and acute toxicity to grass carp.

2. Materials and methods

2.1. Fish and parasite

The naive grass carp (*Ctenopharyngodon idellus*) (22.0 ± 1.6 g in weight and 14.1 ± 1.7 cm in total length) were purchased from a fish farm in Huadu District, Guangzhou City, Guangdong Province, China. All fish were kept in several 100-L glass aquaria equipped with re-circulated aerated tap water (water temperature 23 ± 0.6 °C, pH 7.1 ± 0.2, and dissolved oxygen 5.0–7.0 mg/L). The fish were fed daily at 1% body weight with granule feed (Haid, Guangzhou, China). A light/dark period of 12:12 h was provided. Experimental use of fish was approved by the Animal Experimentation Ethics Committee of Jinan University.

Goldfish (*Carassius auratus*) infected with *I. multifiliis* were obtained from the aquatic retail trade market at Guangzhou, China. *I. multifiliis* was cultured in the laboratory by serial transmission between infected goldfish and some naive grass carp. *I. multifiliis* trophonts were prepared for this experiment as described by Zhang et al. (2013). Briefly, trophonts were gently scraped from the skins and mucus of several infected grass carp to petri dishes with distilled water, and filtered through a 425-μm filter. Isolated trophonts were randomly divided into three groups. The trophonts were used to assess the efficacy of the active compound against the tomonts in group 1, and firstly incubated for 6 h at 23 ± 0.4 °C, then to test the efficacy of the active compound against the cysts in group 2, and to produce theronts after incubation for 22 h at 23 ± 0.4 °C to assay anti-theront efficacy of the active compound in group 3. Five 10 μL theront suspensions were counted on a glass slide under a CX21 light microscope (Olympus Inc., Tokyo, Japan) and theront concentration was calculated as the number of theronts per milliliter.

2.2. Medicinal plant preparation

The rhizomes of *C. longa* (5 kg) were purchased from the Chinese medicinal market at Guangzhou, China. The clean medicinal material was pulverized in an electronic grinder with 50 mesh strainer prior to use.

2.3. Bioassay-guided isolation and identification of the active compound

Five kilograms of dried *C. longa* powder were extracted with 95% ethanol (6 × 25 L) overnight at room temperature. The extract solution was filtered and concentrated in a RE-2000A rotary evaporator (Shanghai Yarong Biochemical Instrument Plant, Shanghai, China) at 70 °C until the ethanol was entirely evaporated to obtain crude extract (1.2 kg). The ethanol extract was suspended in 2 L distilled water, and extracted successively with petroleum ether (3 × 2 L), ethyl acetate (3 × 2 L), and *n*-butyl alcohol (3 × 2 L).

A bioassay-guided isolation was performed to get target compound based on anti-*I. multifiliis* theronts efficacy (Fig. 1). The ethyl acetate extract (560 g) showed the strongest anti-*I. multifiliis* activity, and was subjected to further column chromatography. The ethyl acetate extract was dissolved in 500 mL methanol, mixed with 560 g 200–300 mesh silica gel and dried. The column chromatography was conducted on the dried mixture in a silica gel column packed with 2000 g 100–200 mesh silica gel, and successively eluted with petroleum ether-ethyl acetate gradients (10:0, 6:1, 2:1, 1:4, 0:10, v/v) and ethyl acetate-methanol gradients (1:1, 0:1, v/v). The eluent was divided into seven fractions (Fr. A–G). The fraction C showed the highest anti-*I. multifiliis* efficacy. The fraction C (230 g) was mixed with equal amount of 200–300 mesh silica gel and further isolated on a silica gel column containing 2300 g 100–200 mesh silica gel. The mixture was continuously eluted with petroleum ether-ethyl acetate gradients (6:1, 4:1, 2:1, 1:1, 1:4, v/v) and ethyl acetate-methanol gradients (1:0, 1:1, 0:1, v/v) to obtain seven fractions (Fr. C_1–C_7) on the basis of similar high performance liquid chromatography (HPLC) chromatograms. The fraction C_2 (75 g) showed the highest anti-*I. multifiliis* activity. The fraction C_2 was applied to further chromatographic separation using a XB-C18 preparative HPLC column (5 μm, 21.2 mm × 250 mm, Welch, MD) followed by a column with rinsing and equilibrating procedure as follows: methanol/H_2O (70:30, v/v) mobile phase, 210 nm wavelength of ultraviolet detection, and a flow rate of 10 mL/min at 30 °C column temperature. Three subfractions (Fr. $C_{2\text{-}1}$, Fr. $C_{2\text{-}2}$ and Fr. $C_{2\text{-}3}$) were yielded. The fraction C_{2-2} showed the strongest anti-*I. multifiliis* efficacy, and then was analyzed on an Agilent 1100 series instruments (Agilent, Santa Clara, CA) equipped with a TC-C18 column (5 μm, 4.6 mm × 250 mm, Agilent, CA). An active compound (50 g, retention time = 10 min) with high purity was obtained (Fig. 2). Chemical structure of the active compound was identified by physico-chemical characteristics, electrospray ionization mass spectrometry (ESI–MS), nuclear magnetic resonance hydrogen spectrum (^{1}H NMR), and nuclear magnetic resonance carbon spectrum (^{13}C NMR).

2.4. In vitro *tests*

2.4.1. Preparation of stock solution

The active compound (3 mg) isolated from *C. longa* was dissolved in 20 μL DMSO and adjusted to 2560 mg/L as stock solution with distilled water. The stock solution was stored at a −20 °C freezer for *in vitro* anti-*I. multifiliis* efficacy tests. The 150 μL stock solution was added to 1350 μL distilled water in a 1.5 mL centrifuge tube to make a 256 mg/L solution. Twofold serial dilutions were made in several 1.5 mL centrifuge tubes to yield concentrations of 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0 (control, 0.1% DMSO), and 0 mg/L (control, distilled water).

2.4.2. Antiparasitic efficacy of the active compound against *I. multifiliis* theronts

Anti-*I. multifiliis* theronts tests were conducted in a 96-well tissue culture plate filled with 100 μL water with 500 theronts and 100 μL sample solutions in each well to get final concentrations of