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Short Communication

Isolation of active compounds from methanol extracts of *Toddalia asiatica* against *Ichthyophthirius multifiliis* in goldfish (*Carassius auratus*)

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ABSTRACT

The parasitic ciliate *Ichthyophthirius multifiliis* infests all species of freshwater fish and can cause severe economic losses in fish breeding. The present study aims to evaluate the antiparasitic activity of the active components from *Toddalia asiatica* against *I. multifiliis*. Bioassay-guided fractionation and isolation of compounds with antiparasitic activity were performed on the methanol extract of *T. asiatica* yielding two bioactive compounds: chelerythrine and chloroxylonine identified by comparing spectral data (NMR and ESI-MS) with literature values. Results from *in vitro* antiparasitic assays revealed that chelerythrine and chloroxylonine could be 100% effective against *I. multifiliis* at the concentration of 1.2 mg L^{-1} and 3.5 mg L^{-1}, with the median effective concentration (EC_{50}) values of 0.55 mg L^{-1} and 1.90 mg L^{-1} respectively. *In vivo* experiments demonstrated that fish treated with chelerythrine and chloroxylonine at the concentrations of 1.8 and 8.0 mg L^{-1} carried significantly fewer parasites than the control ($P < 0.05$). The acute toxicity (LC_{50}) of chelerythrine for goldfish was 3.3 mg L^{-1}.

1. Introduction

The ciliate *Ichthyophthirius multifiliis* Fouquet, 1876, commonly called "ich", is the main parasitic threat to freshwater fish in most climatic zones (Buchmann et al., 2001). It can cause sever morbidity and high mortality in most species of freshwater fish worldwide and can result in heavy economic losses for aquaculture (Traxler et al., 1998).

The most effective treatment of *I. multifiliis* has been achieved by using malachite green and mixtures of malachite green and formalin (Dickerson and Dawe, 1995; Buchmann et al., 2003). However, the use of malachite green for the treatment of disease has been no longer permitted by some government agencies, such as the Food and Drug Administration of the USA, due to its potential carcinogenic and teratogenic properties (Srivastava et al., 2004). Since the ban, other chemicals, including formalin (Rowland et al., 2009), copper sulfate (Straus et al., 2009), permanganate (Straus and Griffin, 2002), hydrogen peroxide (Lahnsteiner and Weismann, 2007) have been evaluated for chemotherapy of ichthyophthiriasis. However, the threats of anthelmintic resistance, risk of residue, environmental contamination, and toxicity to host caused by the frequently use of these drugs have led to the need of other alternative control methods.

Toddalia asiatica (Rutaceae) is a medicinal plant commonly known as Orange climber (Eng.) (Orwa et al., 2008). It is widely distributed in Southeast Asia, South Africa and tropical Africa and is widely used as a folk medicine in

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China. The leaves are used to treat lung and skin diseases, rheumatism (Karunai Raj et al., 2012; Duraipandiyan and Ignacimuthu, 2009), stomach ache, to relieve pain in the bowel and as tooth power (Rajkumar et al., 2010). The root and its bark have been traditionally used to treat malaria and cough (Duraipandiyan and Ignacimuthu, 2009). *T. asiatica* is also used to treat nasal and bronchial pains and snake bite (Kokwaro, 1993). However, there is no information about the isolation of components with antiparasitic activity from this medicinal material. The observation that methanol extract of *T. asiatica* exhibits potent antiparasitic activity against *I. multifiliis*, prompted us to perform a detailed chemical evaluation, leading to the isolation of active compounds. Here we describe the isolation, structural elucidation, and antiparasitic activity of the active compounds from *T. asiatica*.

2. Materials and methods

2.1. Plant material

Leaves of *T. asiatica* were collected in Guizhou province, China, in October 2011. They were cleaned and air dried for a week at 35–40 °C and pulverized in electric grinder. The powdered plant samples were stored at −20 °C until further use.

2.2. Parasites and hosts

Heavy *I. multifiliis*-infected grass carp (*Ctenopharyngodon idella*), weighing approximately 12 g each, were obtained from a local juvenile producer in Changchun and were maintained in a 1500-L tank with aerated groundwater at 25 °C.

2.2.1. Source of uninfected fishes

Goldfish (*Carassius auratus*), weighing approximately 6 g each, were obtained from a local Ornamental fish market in Changchun and were kept in 1500-L tanks. Prior to experiments, the skin surface and gills of ten randomly sampled fish from each species were examined under a microscope to confirm that they were not infected with gill or skin parasites.

2.2.2. Infection procedure

I. multifiliis was collected using a method described by Clayton and Price (1988). Several heavily infected grass carp were placed into 1000 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. The cysts thus obtained were incubated at 23.5 ± 0.5 °C for 18–20 h, and theronts were allowed to emerge naturally. Uninfected goldfish were exposed to approximately 5000 theronts per liter for 3 h and randomly redistributed into the aquaria (Ekanem et al., 2004). After 2 weeks of infection, ten fish were randomly selected, killed by spinal severance and examined for the prevalence and intensity of parasite under a light microscope (Olympus BX31, Tokyo, Japan) at 10 × 10 magnification prior to the experiment. The infection rate was 100% and the mean number of parasites on the gills and skin surface was approximately 50 per fish.

2.3. In vitro tests

In vitro tests were undertaken for the crude extracts, fractions isolated from methanol extract and the pure compounds (crystals). Tests were conducted in 24-well tissue culture plate, filled with 2 mL aerated groundwater each containing the test samples. A total of 20 mature trophonts was distributed to each well of a 24-well tissue culture plate and exposed (at 25 °C) to concentrations of crude extracts, fractions and pure compounds. The behavior of the trophonts was observed under a microscope (100×) every hour. Immobilized and lysed trophonts cells were considered dead. The mortality of trophonts in groups with different test sample concentrations was evaluated. A negative control was included using aerated groundwater containing the same amount of DMSO as the maximum concentration test group. All the tests were performed in duplicate. Median effective concentrations (EC_{50}) were calculated.

2.4. Activity-guided isolation of active compounds

2.4.1. Selection of extraction solvent

Five dried and powdered samples of *T. asiatica*, each weighing 50.0 g, were extracted with petroleum ether, chloroform, ethyl acetate, methanol, and water for 2 h and three replicates, respectively. Portions of each extract were evaporated to dryness under reduced pressure in a rotary evaporator. The dried extracts were subjected to *in vitro* antiparasitic efficacy test. The bioassay showed that the methanol extract was the highest in antiparasitic efficacy among all extracts. So, it was then subject to further separation.

2.4.2. Extraction and isolation procedure

Air-dried and powdered herbs of *T. asiatica* (6.0 kg) were exhaustively extracted with 50 L methanol at room temperature by percolation, giving 754 g dry extract. This extract was subjected to column chromatograph and successively eluted with a mixture of chloroform–ethyl acetate/methanol to afford 298 eluents (250 mL each eluent). Eluents were monitored using thin-layer chromatography (TLC) and fractions showing similar TLC chromatograms were combined into five fractions (Fr. A: 1–51 fractions, Fr. B: 52–94 fractions, Fr. C: 95–188 fractions, Fr. D: 189–234 fractions, Fr. E: 235–298 fractions). These five fractions were submitted to *in vitro* test (Table 1), Fr. C showed the most active and was then applied to a silica gel column using a mixture of chloroform-ethyl acetate as an eluent system to give four subfractions (Sfr. C_1: 1–14 fractions, Sfr. C_2: 14–58 fractions, Sfr. C_3: 59–80 fractions, Sfr. C_4: 81–118 fractions, 150 mL each fraction). The most active subfractions (*in vitro* assay) Sfr. C_2 and Sfr. C_4 were selected to separate further (Table 1).

Sfr. C_2 and Sfr. C_4 was applied to reversed-phase high performance liquid chromatography (RP-HPLC) with following chromatographic conditions: XBridge OBD C_{18} (5 μm; 30 mm × 150 mm; Waters, USA) column,