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Antiparasitic efficacy of dihydrosanguinarine and dihydrochelerythrine from *Macleaya microcarpa* against *Ichthyophthirius multifiliis* in richadsin (*Squaliobarbus curriculus*)

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ABSTRACT

Ichthyophthirius multifiliis is a holotrichous protozoan that invades the gills and skin surfaces of fish and can cause morbidity and high mortality in most species of freshwater fish worldwide. The present study was undertaken to investigate the antiparasitic activity of crude extracts and pure compounds from the leaves of *Macleaya microcarpa*. The chloroform extract showed a promising antiparasitic activity against *I. multifiliis*. Based on these finding, the chloroform extract was fractionated on silica gel column chromatography in a bioactivity-guided isolation affording two compounds showing potent activity. The structures of the two compounds were elucidated as dihydrosanguinarine and dihydrochelerythrine by hydrogen and carbon-13 nuclear magnetic resonance spectrum and electron ionization mass spectrometry. The *in vivo* tests revealed that dihydrosanguinarine and dihydrochelerythrine were effective against *I. multifiliis* with median effective concentration (EC_{50}) values of 5.18 and 9.43 mg/l, respectively. The acute toxicities (LC_{50}) of dihydrosanguinarine and dihydrochelerythrine for richadsin were 13.3 and 18.2 mg/l, respectively. The overall results provided important information for the potential application of dihydrosanguinarine and dihydrochelerythrine in the therapy of serious infection caused by *I. multifiliis*.

1. Introduction

The ciliate *Ichthyophthirius multifiiis* is one of the most pathogenic parasites of fish, and it constitutes a major disease problem in aquaculture (Bisharyan et al., 2003; Dickerson and Dawe, 1995; McCallum, 1985, 1986). The morbidity rate due to this disease may reach up to 100%, causing great economic losses in aquaculture and ornamental fish breeding (Osman et al., 2009).

To cope with the parasitism and deleterious consequences, various parasiticides have been used in aquaculture. Formerly, ichthyophthiriasis was treated effectively with malachite green, a compound that has been banned on fish farms due to its confirmed carcinogenicity in most countries (Alderman, 1985; Wahli et al., 1993). Since the ban, formalin (Stoskopf, 1993; Rowland et al., 2009), copper sulfate (Straus et al., 2009; Rowland et al., 2009), potassium permanganate (Straus and Griffin, 2001; Buchmann et al., 2003), hydrogen peroxide (Lahnsteiner and Weismann, 2007), sodium percarbonate (Heinecke and Buchmann, 2009) have been used with varying levels of success. Strategic treatments using sodium percarbonate in combination with continuous filtration of the pond water

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are suggested to provide a powerful and more environmentally friendly method to minimize and control infections with *I. multifiliis*. In terms of current strategies for controlling ichthyophthiriasis in aquaculture, chemical agents aimed at interrupting the life cycle by killing the free-living stages of the parasite play the major role, although in some situations water management and vaccine can also be effective (Matthews, 2005). However, improved methods of disease management are still highly needed to comply with increasing demands for safer and environmentally sustainable production methods in freshwater aquaculture.

Recently, there have been increased research activities into the utilization of traditional plant-based medicines to control *I. multifiliis* infection. Plant extracts from garlic (Buchmann et al., 2003), *Mucuna pruriens* and *Carica papaya* (Ekanem et al., 2004) have been assessed, showing some potential for killing the free-swimming stages of *I. multifiliis*. However, most of the compounds responsible for such activities have not been isolated and their structures determined so as to evaluate their potential for the development of novel antiparasitic drugs.

In our previous study, we investigated the anti-*I. multifiliis* efficacy of *Macleaya cordata*, a congeneric of *Macleaya microcarpa*, and an active compound, sanguinarine, was isolated by using the strategy of bioactivity-guided isolation (Yao et al., 2010). It lead us to consider if *M. microcarpa*, as another specie of this genus, widely distributed in northwest and southwest of China, is also capable of controlling the parasite.

The principal objective of this study was to assess the antiparasitic properties of *M. microcarpa* and isolate active constituents responsible for the activity using *in vitro* antiparasitic assay associated with bioassay-guided fractionation. Additionally, the acute toxicity of the active compounds against richadsin (*Squaliobarbus curriculus*) of was evaluated.

2. Materials and methods

2.1. Fish

Richadsin (mean length: 6.3 ± 0.3 cm), naturally infected with *I. multifiliis*, were obtained from aquatic fry farm of Zhejiang Institute Freshwater Fisheries in China and maintained in a 1 m^3 tank at $24 \pm 1\,^{\circ}C$ (controlled by automatic aquarium heater) with aeration for 7 days. On the seventh day, ten richadsin were randomly sampled, killed by spinal severance, and eight gill filaments of each fish were biopsied to determine the *I. multifiliis* infestation level and intensity under a light microscope (Olympus BX51, Tokyo, Japan) at 10×4 magnification before they were used for the assays.

For acute toxicity tests, parasite-free richadsin were obtained from commercial fish farm and maintained in a 1 m^3 tank supplied with filtered groundwater under the same conditions as parasitized fish. On arrival, the absence of the parasites was carefully checked by examining ten fish randomly selected.

2.2. Bioactivity-guide isolation

The chromatographic separation was monitored by the strategy of bioactivity-guided isolation (*in vitro* tests guided), only the extracts or fractions showed strong activity (100% effective concentration <50 mg/l) against *I. multifiliis* were subjected to further separation and purification.

2.2.1. Preparation of extracts

Dry and powdered leaves of *M. microcarpa* (10 kg) were extracted with 95% ethanol (25 l × 3 times) at room temperature for 24 h. The extract was evaporated to dryness under reduced pressure in a rotary evaporator to yield the ethanol extract (EE, 1.36 kg). Part of the ethanol extract was reserved for activity assays whilst the rest of the extract was suspended in water and extracted with *n*-butanol. The yield of *n*-butanol extract (BE) and aqueous extract (AE) was 654.5 g and 126.6 g, respectively. The *n*-butanol extract was redissolved in chloroform/water (1:1, v/v) mixture, the pH value of the mixture was adjusted to 10–11 with 1% NaOH (m:v), then, extracted with chloroform for three times to give 256.0 g chloroform extract (CE) and 152.3 g remaining aqueous extract (RAE).

The bioassay showed that chloroform extract was the highest in antiparasitic efficacy among all extracts. So, it was then subject to further separation.

2.2.2. Fractionation and isolation of pure compounds

The chloroform extract (245.0 g) was subjected to open column chromatography on normal phase silica gel and eluted with a solvent mixture of petroleum ether/ethyl acetate (2:1, v/v) and finally eluted with methanol affording 6 major fractions (Fr. A: 1–115 fractions, Fr. B: 116–298 fractions, Fr. C: 299–365 fractions, Fr. D: 366–478 fractions, Fr. E: 479–535 fractions, Fr. F: 536–656 fractions). These six fractions were submitted to *in vitro* test, and Fr. B was the most active. Fr. B (22.5 g) was subjected to column chromatograph and successively eluted with petroleum ether/ethyl acetate gradients. Repetition of the chromatographic separations and recrystallization led to the isolation of two active compounds: Compound 1 (221.0 mg) and Compound 2 (144.0 mg).

2.3. Identification of active compounds

Based on the physico-chemical properties and electron ionization mass spectrometry, nuclear magnetic resonance hydrogen spectrum (^{1}H NMR) and nuclear magnetic resonance carbon spectrum (^{13}C NMR), the chemical structures of active compounds were identified.

2.4. In vitro tests

The crude extracts, fractions and the pure compounds isolated from *M. microcarpa* were dissolved in 10 ml of dimethyl sulfoxide (DMSO) to get 1.0 g/ml (sample/solvent) of stocking solutions which were used for the preparations of the desired concentrations for *in vitro* tests and *in vivo* test.