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Short communication

Isolation of bioactive components from *Chelidonium majus* L. with activity against *Trichodina* sp.

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ABSTRACT

Trichodinids are the most common ciliates parasite present on the skin of pond-reared fish and can cause severe economic losses in aquaculture and ornamental fish breeding. The present study aims to evaluate the antiparasitic activity of the active components from *Chelidonium majus* L. against *Trichodina* sp. Bioassay-guided fractionation and isolation of the compounds with antiparasitic activity were performed on the ethanolic extract of *C. majus* yielding three bioactive alkaloids namely: chelidonine (1), chelerythrine (2) and sanguinarine (3). Results from *in vivo* antiparasitic assays revealed that these compounds when isolated could be 100% effective for the elimination of *Trichodina* sp. at the concentrations of 1.0, 0.8, and 0.7 mg L^{-1}, with the median effective concentration (EC_{50}) values of 0.6, 0.33, and 0.32 mg L^{-1}, respectively. Furthermore, the promising chelidonine, chelerythrine and sanguinarine were subjected to acute toxicity tests for the evaluation of their safety to the host (*Parabramis pekinensis*). It was found that the 48 h median lethal concentration (48 h-LC_{50}) values determined by the acute toxicity tests on *P. pekinensis* were 2.5, 3.8 and 1.5 mg L^{-1} respectively. These results provided evidence that the isolated compounds, especially chelerythrine, can be exploited as novel antiparasitic agents for the control of *Trichodina* sp.

1. Introduction

The continuing growth of fish aquaculture worldwide has been accompanied by the increasing importance of parasites as agents of disease. Trichodinids, which are common ectoparasites living on the gills of fish, present the largest group of metazoan fish parasites and are of major importance in the fish pathology (Lyholt and Buchmann, 1995). They can deteriorate the normal health condition of fish and lead to fish mortality by causing gill infestations and inhibiting oxygen exchange across gill lamella. Therefore, the control of this parasite has become an urgent need in fish farming.

Formaldehyde has traditionally been used for the control of trichodinids infection but appears to be inefficient in recent years (Madsen et al., 2000a). Other chemicals, including malachite green, potassium permanganate, acriflavin, bithionol have been evaluated for chemotherapy of trichodinids (Madsen et al., 2000a). However, the frequent use of these chemical drugs has resulted in serious drawbacks such as development of parasites resistance, environmental contamination, drug residue and pressure to host, which has emerged the need of alternative strategy (Goven et al., 1980; Klinger and Floyd, 2002).

Recently, there have been increased research activities into the utilization of traditional plant-based medicines to control parasitic infections in human and animals (Orhan et al., 2006; Wang et al., 2010). Generally, the plant-based products are more non-resistible for frequent use as compared with the chemical drugs. Moreover, the novel products might be potential sources of new antiparasitic drug. Madsen et al. (2000b) reported that raw and squeezed garlic (*Allium sativum*) at 200 mg L^{-1} had potential to treat trichodiniasis in eel. El-Deen (2010) found that the green tea extract (GTE) were active against *Trichodina* sp. An alkaloid sanguinarine from *Macleaya cordata* has been reported to be active against *I.multifillis* (Yao et al., 2010).

Chelidonium majus L. (Papaveraceae), which is widely distributed in Europe and Asia, is a plant of great interest for its wide use in various diseases in European countries and in Chinese herbal medicines (Pavao and Pinto, 1995; Colombo and Bosisio, 1996). The principal objective of this study was to assess the antiparasitic properties of *C. majus* and isolate the active constituents responsible for the activity using *in vivo* antiparasitic assay associated with bioassay-guided silica gel column chromatography isolation. Additionally, the acute toxicities of active compounds from *C. majus* were evaluated.

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2. Materials and methods

2.1. Parasites and hosts

Parabramis pekinensis (mean length ± SD: 5.8 ± 0.4 cm), naturally infected with *Trichodina* sp., were obtained from aquatic fry farm of Zhejiang Institute Freshwater Fisheries in China and maintained in a 1-m^3 tank with an oxygen content higher than 85% saturation at 23 ± 1 °C. Fish were acclimatized under laboratory conditions for 7 days. On the seventh day, 10 fish were randomly selected, killed by spinal severance and examined for the prevalence and intensity of parasite under a light microscope (Olympus BX51, Tokyo, Japan) at 10 × 4 magnification prior to the experiment.

For acute toxicity tests, parasite-free *P. pekinensis* 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 10 fish randomly selected.

2.2. Plant material

C. majus L. were collected in Zhejiang province, China, in March 2010 and identified by Prof. X.L. He in Northwest A&F University, China. A voucher specimen has been deposited in the Herbarium of College of Life Science of the university. 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.

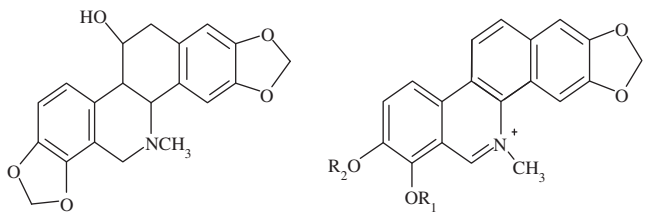
2.3. Activity-guided isolation of active compounds

2.3.1. Selection of extraction solvent

Five powdered samples, each weighing 50.0 g, were respectively extracted under reflux with different solvent (petroleum ether, chloroform, ethyl acetate, ethanol, and water) of increasing polarity at 65 °C for 4 h. The process was performed in three replicates. Each extract was subsequently filtered, and the five filtrates were evaporated under reduced pressure in a vacuum rotary evaporator for the complete removal of solvents. The dried extracts were subjected to *in vivo* antiparasitic efficacy test. The bioassay showed that the ethanol extract was the highest in antiparasitic efficacy among all extracts. So, it was then subject to further separation.

2.3.2. Extraction and isolation procedure

Air-dried and powdered herbs of *C. majus* (3.2 kg) were exhaustively extracted with 50 L ethanol at room temperature by percolation, giving 405 g dry extract. The ethanol extract was subjected to column chromatography on a silica gel and sequentially eluted with petroleum ether, ethyl acetate and methanol with increasing polarity. Repetition of the chromatographic separations and recrystallization led to the isolation of three known compounds (1–3) whose structures are presented in Fig. 1. The structures of these compounds were elucidated by comparing spectroscopic data with those reported as chelidonine (1) (Pandey et al., 1977; Swinehart and Stermitz, 1980; Zhang et al., 1995), chelerythrine (2) (Radek et al., 1999; Pavlina et al., 2002), and sanguinarine (3) (Perez Gutierrez et al., 2002).



Chelidonine (1)

Chelerythrine(2) R_1-R_2=CH_2
Sanguinarine (3) R_1=R_2=CH_3

Fig. 1. Chemical structures of compounds isolated from *C. majus*.

2.4. In vivo antiparasitic efficacy test

The crude extracts and the pure compounds isolated from *C. majus* were dissolved in 1 mL of dimethyl sulfoxide (DMSO) to get 0.5 g mL^{-1} (sample/solvent) of stocking solutions which were used for the preparations of the desired concentrations for *in vivo* antiparasitic efficacy assay. The highest concentration of DMSO in the treatment was less than 1% (Initial tests with 1% DMSO showed no antiparasitic activity).

In vivo tests were conducted in 40 × 30 × 20 cm glass tanks, each containing 10 L of the test solution, and 10 parasitized fish (*P. pekinensis* were placed on glass slides, and the presence of parasites on the paddle of the fish were determined microscopically, heavy infected fish were transferred into the glass tank immediately, fish infected with little parasites were returned to the tanks.). The test samples were assayed at a different series of concentrations based on initial tests. Negative control groups with no chemical were set up under the same experimental conditions, while formalin was used as the positive control. All treatments and control groups were conducted with three replicates.

After 48 h treatment, *P. pekinensis* in all treatments and control groups were killed by a spinal severance for biopsy. The total number of trichodinids in the collected gills and paddle from each fish was counted. The antiparasitic efficacy of tested samples was determined by comparison of the number of parasites in the treatments with those in the negative control groups, and calculated using the following equation described in our previous work (Yao et al., 2010).

$$E = (C-T) \times 100 / C \quad (1)$$

where E is the antiparasitic efficacy, C is the mean number of *Trichodina* sp. in the negative control, and T is the treatment groups.

2.5. Acute toxicity

Acute toxicity of chelidonine, chelerythrine and sanguinarine were carried out according to the method of Wang et al. (2010). The tests were conducted in plastic pot of 5.0 L capacity, each containing 2.0 L of test solution, and 10 healthy *P. pekinensis*. The water temperature was 15–18 °C, the pH ranged from 7.0 to 7.5 and the dissolved oxygen was approximately 6.5–7.8 mg L^{-1}. Dilutions were prepared from the stock solutions as the following concentrations: 2.0, 2.3, 2.6, 2.9, 3.1, 3.4, 3.7 mg L^{-1} for chelidonine, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 5.5 mg L^{-1} for chelerythrine, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, and 2.2 mg L^{-1} for sanguinarine. The tests were conducted in triplicate, as well as controls (under the same test conditions with no chemicals). The deaths of fish were recorded when the opercula movement and tail beat stopped and the fish no longer responded to mechanical stimulus. Any observed dead fish was removed from the medium in time to avoid deterioration of the water quality. Mortality of fish was recorded throughout the experiments, during which no food was offered to the fish. Under the circumstances, the experiments were stopped and fish were transferred to freshwater. Fish mortalities in the treatment and control groups were registered after 48 h of exposure.

2.6. Data analysis

The homogeneity of the replicates of the samples was checked by the Mann–Whitney *U* test. The LC_{50} and their 95% confidence intervals of test samples were calculated by log concentration–probit equation using the SPSS 16.0 probit procedure.