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In vivo assessment of anthelmintic efficacy of ginkgolic acids (C13:0, C15:1) on removal of *Pseudodactylogyrus* in European eel

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ABSTRACT

Pseudodactylogyrus is a significant monogenean parasite of the gills of aquacultured European eels, and can cause severe gill pathology. In this study, effects of the crude extracts, fractions and compounds of exopleura of *Ginkgo biloba* against *Pseudodactylogyrus* were investigated under in vivo conditions by bio-assay guided isolation method. Four solvents (petroleum ether, ethyl acetate, n-butanol and water) were applied for the extraction of exopleura of *G. biloba*. Among them, only the petroleum ether extract showed strong activity and therefore, subjected to further separation and purification using various chromatographic techniques. Two compounds showing potent activity were identified by comparing spectral data (IR, NMR, and EI-MS) with literature values to be ginkgolic acid C13:0 and C15:1. They were found to be 100% effective at the concentration of 2.5 mg l^{-1} and 6.0 mg l^{-1}, with ED_{50} values of 0.72 mg l^{-1} and 2.88 mg l^{-1}, respectively. In the 5-days safety test, ginkgolic acid C13:0 and C15:1 were shown to be safe for healthy juvenile eels when the concentration were up to 10.0 and 18.0 mg l^{-1}, respectively. The two compounds exhibited potential results and can be explored as plant-derived antiparasitic for the control of *Pseudodactylogyrus*.

1. Introduction

European eel (*Anguilla anguilla*), as an important aquaculture species, plays an important role in Chinese aquatic products in recent years. However, parasite infections have become a major hurdle for further development of eel culture, one parasitic problem recently observed is the infestation with *Pseudodactylogyrus* (Buchmann et al., 1987). The eggs of *Pseudodactylogyrus* hatch on the gills of eel and become oncomiracidia, causing impaired respiration problems (Kennedy, 2007). Moreover, the infection makes it easier to be attacked by other deleterious organisms, including fungus, bacteria and virus. All these pathogens can cause serious problems to host, such as loss of appetite and reduction of growth performance. The *Pseudodactylogyrus* on fish gill can be killed, to some extent, by using some chemicals such as the mebendazole, azamethiphos, potassium permanganate and methylene blue (Buchmann and Bjerregaard, 1990; Pretti et al., 2002; Umeda et al., 2006). However, the frequently use of these drugs have caused the development of drug-resistance parasites and environmental pollution.

Nowadays, the traditional plant-based substances for the control of some fish parasites have been studied and some effective substances have been found. Hirazawa et al. (2000) described the anthelmintic effects of four natural agents, caprylic acid, orange oil, peppermint oil and cinnamon oil against the monogenean *Heterobothrium okamotoi* in the tiger puffer. And further study found that the caprylic acid were particularly effective on this parasite (Hirazawa et al., 2001). It was also reported that the crude extracts of *Pinus elliottii* (Tóro et al., 2003), *Mucuna pruriens* (Fabaceae), *Carica papaya* (Caricaceae) (Ekanem et al., 2004), and the green tea (Suzuki et al., 2006) were helpful for the control of some fish parasitic worms.

Ginkgo biloba, which belongs to the family Ginkgoaceae, is considered to be a living fossil due to its survival over millions of years. Its longevity might be due to its unusual resistance against the insects, bacteria, viruses, fungi, and other pathogens (Major, 1967). The extract from *G. biloba* leaves was found to possess the activities against many pathogens including *Pneumocystis carinii* (Atzori et al., 1993), *Toxoplasma gondii* (Chen et al., 2008) and *Oncomelania hupensis* (Yang et al., 2008). However, the use of *G. biloba* extract for the treatment of *Pseudodactylogyrus* in eels has rarely been reported. Therefore, an attempt has been made under the present work to evaluate the potential anthelmintic efficacy of crude extract, fractions and compounds from the exopleura of *G. biloba*. The safety tests of active compounds to eels were also discussed.

2. Materials and methods

2.1. Infected European eel preparation

One-year-old European eels (n = 40, 15 ± 3 g), naturally infected with adult *Pseudodactylogyrus* were collected from Zhisheng eel farm

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(Fuqing city, Fujian province, China) and maintained in glass aquarium containing 180-l groundwater at 25 ± 1 °C (controlled by automatic aquarium heater) with aeration for 3 days. On the third day, 10 eels were randomly selected, killed by a spinal severance, and loaded on the surgical tray. Every lamella branchialis was separated and put into 6-wells cell culture cluster (Corning Incorporated, New York, USA) containing 1.0 ml RPMI-1640 medium with 20% fetal bovine serum at 25 °C for 10 h in order to collect parasite eggs. The *Pseudodactylogyrus* eggs (n = 300) in lamella branchialis were collected using capillary glass tube under the stereomicroscope and subsequently placed into a 1-l glass tank containing filtered groundwater at 25 ± 1 °C. After 3 days incubation, oncomiracidia were hatched from eggs. Five parasite-free eels were then added to the tank to perform the experimental infestation. Ten days later, the larvae developed to adult, the 5 eels were co-habitated with 10 parasite-free eels in 180-l glass aquarium to get infected ones. The co-habitation was performed for 7-10 days and the ratio of the infected eels to the parasite-free ones was 1:2. The sufficient infected eels used for the in vivo tests were prepared following the method described above. During the experiment (May to June, 2006 and May to June, 2007), about 3500 infected eels were obtained. Ten eels were randomly selected, killed and checked for the presence of parasite under a lightmicroscope (Olympus BX41, Tokyo, Japan) at 10 × 4 magnification prior to the experiment. Eels were chosen for the test when the infection rate is 100% and the mean number of the parasite on gills of each eel was 90-110.

For the experimental infestation, parasite-free eels (14 ± 3 g) were also obtained from Zhisheng eel farm (Fuqing city, Fujian province, China) and maintained under the same conditions as parasitized eel. The absence of *Pseudodactylogyrus* was checked on arrival.

2.2. Extraction, isolation of active compounds

2.2.1. Selection of extraction solvent

Four powdered samples, each weighing 20.0 g, were respectively extracted under reflux with petroleum ether (200 ml), ethyl acetate (200 ml), n-butanol (200 ml), and water (200 ml) for 2 hours and this process was repeated 3 times. The filtrates were then concentrated under vacuum on reduced pressure to give four residues. The four residues were then dissolved in 40 ml dimethyl sulfoxide (DMSO) (for petroleum ether extract), ethanol (for ethyl acetate, n-butanol extracts) and distilled water (for water extract) to prepare the stock solutions of 0.5 g ml^{-1} which were used for the further anthelmintic efficacy tests. And the control groups containing no plant extract were performed under the same experimental conditions as the test groups. To discard the possible effects of ethanol and DMSO on parasites, other controls containing the corresponding percentage of ethanol and DMSO were also included. Among them, only the petroleum ether extract showed strong anthelmintic activity when compared with other extracts.

2.2.2. Isolation of active compounds

Air-dried and powdered exopleura (20.0 kg) of *G. biloba* were extracted with ethanol for 2 hours and repeated for three times and then filtered through a Buchner funnel and Whatman No. 1filter paper (Whatman, Maidstone, England). The ethanol filtrates were collected and concentrated under reduced pressure by a rotary evaporator at 50 °C to dryness producing 6640.0 g of ethanol extract. This extract was then portioned with petroleum ether based on the result of Section 2.2.1, yielding petroleum ether fraction (3276.0 g). Part of the petroleum ether fraction (100.0 g) was subjected to column chromatography (120 × 10 cm) on a silica gel (2000.0 g, 100-200 mesh) and eluted with an increasingly polar gradient of chloroform: methanol (1:0, 100:1, 50:1, 25:1, 12.5:1, 5:1, 2:1, 1:1, 0:1, v/v) affording 165 fractions (200 ml each). TLC analysis was performed on silica gel using the same solvent system as the mobile phase, compounds were visualized under UV light (254 nm and 365 nm) or by spraying the plates with ethanol-sulphuric acid reagent (Wagner et al., 1984). these fractions were then pooled in five new fractions as follows: Fr A (1–15), Fr B (16–62), Fr C (63–105), Fr D (106–135), Fr E (136–165). Among them, the Fr B showed higher activity than the other fractions. The Fr B was selected for further purification.

Fr B (25.0 g) was subjected to column chromatography (120 × 10 cm) on a column of silica gel (750.0 g, 300-400 mesh) and eluted with petroleum ether: ethyl acetate gradient (1:0, 200:1, 100:1, 99:1, 98:2, 97:3, 95:5, 90:10, 80:20, 2:1, 0:1, v/v). 126 fractions of 100 ml each were collected: Sfr B1 (1–10), Sfr B2 (11–40), Sfr B3 (41–80), Sfr B4 (80–126). The results of anthelmintic efficacy assay indicated that Sfr B2 exhibited the highest activity and subjected to further isolation. However, by the TLC analysis on silica gel for Sfr B2, only one spot was observed, while HPLC analysis showed five mainly peaks and all the five peaks were confirmed to be ginkgolic acids by compared with standard ginkgolic acids. That is to say, the fraction was hardly to be isolated by silica gel chromatography. Therefore, reversed-phase high performance liquid chromatography (RP-HPLC) was used for the further isolation, with the chromatographic conditions as follows: Octadecyl Silane-A C18 (5 μm 120Å 10 mm × 250 mm) column, MeOH - 4% HAc (93.5:6.5, v/v) mobile phase, 310 nm wavelength, 5.0 ml min^{-1} flow rate, 35 °C column temperature and 500 μl capacity. The sufficient compounds for the tests were obtained by repeatedly using RP-HPLC method.

2.3. In vivo anthelmintic efficacy test

The treatments were conducted in 20-l aerated glass aquaria, each containing 10 l test solution, and 20 infected eels at 25 °C. The water pH ranged from 7.0 to 7.5. During the isolation process, the stock solutions of all the combined fractions and pure compounds were dissolved in ethanol and prepared at the concentration of 100 and 10 mg ml^{-1}, respectively. The desired concentrations of the fractions and compounds were prepared by adding respective stock solutions. Control groups containing no drugs were set up under the same experimental conditions as the test groups. The treatments of fractions during the isolation process were performed once, while the compounds and control groups were repeated for 3 times.

After 48 h, all the surviving eels in the treatment and control groups were killed by a spinal severance for biopsy. The lamella branchialis were placed on glass slides and the numbers of parasites on the gills were counted under a light microscope to determine the

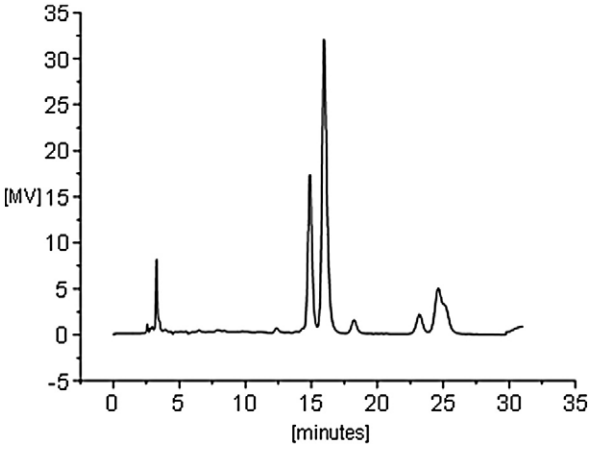

Fig. 1. HPLC fingerprints of the isolated ginkgolic acids. Column condition: Hyper ODS-2 C_{18} (250 mm × 10 mm); mobile phase: MeOH-4%HAC (93.5:6.5, v/v); detection wavelength: 310 nm; flow rate: 1.0 ml/min^{-1}; column temperature: 35 °C; capacity: 5 μl.