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Efficacy of algal *Ecklonia cava* extract against viral hemorrhagic septicemia virus (VHSV)

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ABSTRACT

The inhibition efficacy of an extract from *Ecklonia cava* (*E. cava*) was studied to determine whether the extract and compounds exhibited inhibitory activity against VHSV in the fathead minnow (FHM) cell line and following oral administration to the olive flounder. Based on its low toxicity and effective concentration, the *E. cava* extract (Ext) and compounds (eckol and phlorofucofuroeckol A) were selected for further analysis. In the plaque reduction assay, simultaneous co-exposure of VHSV to Ext, eckol and phlorofucofuroeckol A showed a higher level of inhibition than the pre- and post-exposure groups. The antiviral activity in the FHM cell line was time-dependent and increased with the exposure time with the virus and Ext or the compounds. In the *in vivo* experiments, different Ext concentrations were orally administered to the olive flounder. In trial I, the relative percent survival (RPS) following oral administration of 500 and 50 μg/g/day of Ext was 31.25% and 12.50%, respectively. In trial II, the RPS for 1000, 500 and 50 μg/g/day of Ext was 31.57%, 0% and 0%, respectively. In trial III, the RPS after 1 and 2 weeks (1000 μg/g/day) of exposure to Ext was 26.31% and 31.57%, respectively. Oral administration of Ext (1000 μg/g/day) significantly induced inflammatory cytokine responses (IL-1β, IL-6 and IFN-γ) at 1 and 2 days post-oral administration (dpa). Additionally, IFN-α/β (7–12 dpa), ISG15 (2, 7 and 10 dpa) and Mx (7–12 dpa) were significantly activated in the olive flounder. In conclusion, we demonstrated an inhibitory ability of the *E. cava* extract and compounds against VHSV in the FHM cell line. Moreover, oral administration of the *E. cava* extract to the olive flounder enhanced antiviral immune responses and the efficacy of protection against VHSV, resulting in an anti-viral status in the olive flounder.

1. Introduction

Viral hemorrhagic septicemia virus (VHSV) is a negative-sense, single-stranded RNA virus belonging to genus *Novirhabdovirus* in family *Rhabdoviridae*. In Korea, VHSV infection was first reported in 2001 in juvenile olive flounder (*Paralichthys olivaceus*) from the eastern sea at a low water temperatures (8–15 °C) [1]. Since the first outbreak, high mortality due to VHSV infection has occurred annually in olive flounder. Several preventive measures against VHSV have been reported in olive flounder via the injection route [2–7]; however, VHSV continues to cause mass mortality, especially in olive flounder juveniles. Moreover, injection measures are difficult to apply under field condition in olive flounder due to side effects to the small size of the fish [8]. Hence, the development of preventive measures against VHSV by oral administration is required to support the juvenile olive flounder aquaculture industry.

Seaweeds are a widely available source of biomass, with more than 2 million tons either harvested from the oceans or cultured annually. Among these seaweeds, brown seaweeds are composed of fucoxanthin pigment, polysaccharides and a rich supply of total polyphenolic compounds [9] that possess many bioactive properties [10,11]. For example, phlorotannins from *Ecklonia cava* (*E. cava*) have neuraminidase inhibitory activity against influenza virus [12] and have shown 50% inhibitory concentrations (IC_{50}) ranging from 10.8 to 22.5 μM against porcine epidemic diarrhea virus (PEDV) in Vero cells [13]. Moreover, this compound inhibited the reverse transcription [14] and replication of HIV-1 in CEM-SS and H9 cells [15].

Recent studies have reported the development of safe disease control measures via oral administration of an algal mixture of *Hizikia fusiformis* and *Ecklonia cava* against *Edwardsiella tarda* in olive flounder [16]. Moreover, the *Eclipta alba* leaf enhanced resistance for *Aeromonas hydrophila* in Mozambique tilapia (*Oreochromis mossambicus*) [17], and

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herbal biomedicines administered to grouper juveniles (*Epinephelus tauvina*) to protect against *Vibrio harveyi* infection showed anti-bacterial and immunostimulant activities [18]. *In vitro* antiviral activity of the red alga *Polysiphonia morrowii* and marine brown agla *Eisenia bicyclis* against fish pathogenic infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV) have been reported [19,20]. Moreover, extracts from marine microalgae exhibited inhibitory activity against VHSV replication *in vitro* [21]. However, to the best of our knowledge, no reports have investigated the *in vivo* efficacy of natural sources from the aquatic environment against VHSV.

Hence, this study investigated the inhibitory ability of the extract and compounds from *E. cava* against VHSV in the fathead minnow (FHM) cell line *in vitro*. Moreover, to find successful disease control measures against VHSV in olive flounder, the present study investigated the efficacy of oral administration of the *E. cava* extract to olive flounder and then analyzed the relative percent survival of the fish. Finally, immune-modulatory responses were assessed after oral administration of *E. cava* to olive flounder.

2. Materials and methods

2.1. Virus culture

VHSV was propagated in the FHM cell line with DMEM (Dulbecco's minimum essential medium) (Gibco, USA) containing 10% fetal bovine serum (FBS), 50 IU/ml of penicillin and 50 μg/ml of streptomycin (Gibco, USA). VHSV was harvested from the FHM cell line after the development of cytopathic effects (CPEs) and stored at −80 °C. The VHSV titer was determined by the Reed-Muench method using the FHM cell line to prepare a final titration of $10^{8.8}$ $TCID_{50}$/ml.

2.2. Extraction and isolation of E. cava

Dried powder of *E. cava* (2.0 kg) was extracted with ethanol (20 L) for one week at room temperature. The ethanol extract was concentrated on a rotary evaporator, and the dried extract (157 g) was suspended in H_2O and partitioned with *n*-hexane (25.4 g) and ethyl acetate (60.5 g). We subjected the ethyl acetate layer of the ethanol extract of *E. cava* to a succession of chromatographic procedures, including silica gel, Sephadex LH-20, and octadecyl-functionalized silica gel to yield six compounds as previously described [22]. Through analysis of spectroscopic data and comparison with previous studies, the isolated six compounds were identified as the known species phloroglucinol, eckol, dioxinodehydroeckol, 7-phloroeckol, phlorofucofuroeckol A and dieckol [22].

2.3. Inhibitory activity of E. cava against the FHM cell line

2.3.1. Dissolution of the extract and compounds

The *E. cava* extract was called Ext. Six compounds (phloroglucinol, eckol, dioxinodehydroeckol, 7-phloroeckol, phlorofucofuroeckol A and dieckol) were dissolved in dimethyl sulfoxide (DMSO) (Daejung, Korea) to obtain final concentrations of 100 μg/μl. The dissolved extract and compounds were serially diluted in serum-free DMEM with 50 IU/ml of penicillin and 50 μg/ml of streptomycin.

2.3.2. Toxicity to the FHM cell line

FHM cells cultured in DMEM with 10% FBS, 50 IU/ml of penicillin and 50 μg/ml of streptomycin were seeded into 96-well plates (SPL Life Sciences, Korea). Different concentrations of the extract and compounds (150, 100, 50 and 10 μg/ml) were added to assess their toxicity in the FHM cells in duplicate. After treatment with the extract and compounds, the cells were incubated at 15 °C and monitored by microscopy for one week to assess toxicity.

2.3.3. Determination of the effective concentration 50 (EC_{50})

Extract or compounds were mixed with VHSV at the final concentration of 100, 50 and 10 μg/ml for the algal product and $10^{2.8}TCID_{50}$/ml for the VHSV. The concentration showing 100% inhibition (no CPE) at a 10 μg/ml was selected to determine the EC_{50} values. The final stock concentration of the extract and compounds was 50 μg/μl. The stock was subsequently diluted using serum-free DMEM to obtain a final concentration of 1 ng/μl. The experiment was conducted twice in a 96-well plate in quadruplicate (4 wells in 96wells) for each extract and compound concentration. Predetermined concentrations of the selected extract and compounds were mixed with VHSV ($10^{2.8}$ $TCID_{50}$/ml) and diluted to 100 μl using DMEM. The mixture was incubated at 15 °C for 1 h prior to addition to FHM cells seeded into a 96-well plate. Post-treatment, the plate was kept in a 15 °C incubator and observed daily by microscopy to assess CPE for two weeks. The EC_{50} of the extract and compounds was determined by following the method and equation proposed by Sebaugh [23].

2.3.4. Plaque reduction assay (Co-, pre- and post-exposure antiviral activity)

FHM cells (2×10^6 cells/ml) were seeded into 24-well plates and cultured at 20 °C for 24 h to allow the cells to obtain 80–90% confluence. The plates were shifted to 15 °C for 1 h before starting the experiment. After incubation, the co-, pre- and post-exposure experiments were performed as described below.

In the co-exposure experiment, each concentration of the *E. cava* extract (Ext: 10, 5 and 1 μg/ml) and the compounds (eckol and phlorofucofuroeckol A: 1, 2 and 4 μg/ml) was mixed with VSHV in 500 μl ($10^{5.1}$ $TCID_{50}$/ml) and incubated at 15 °C for 1 h. After 1 h, the culture media were aspirated, and the mixtures were added to each well and exposed for 1 h.

In the pre-exposure experiment, 1 ml of fresh DMEM containing each concentration of the *E. cava* extract and compounds was added to each well. After 1 h, media containing the *E. cava* extract and compounds were removed, and the cells were washed with serum-free DMEM. Fresh DMEM (with 10% FBS) containing VHSV ($10^{4.8}$ $TCID_{50}$/ml) was added to each well and exposed for 1 h.

In the post-exposure experiment, 1 ml of fresh DMEM (with 10% FBS) containing VHSV ($10^{4.8}$ $TCID_{50}$/ml) was added to each well. After 1 h, the medium containing VHSV was removed, and the cells were washed with serum-free DMEM. Fresh DMEM containing each concentration of the *E. cava* extract and compounds was added to each well and exposed for 1 h.

To count the plaque numbers, the following procedure was followed for all three exposure groups. The exposure media were removed, and the cells were washed with serum-free DMEM. Fresh medium (1.6% methyl cellulose in DMEM with 5% FBS) was overlaid onto each well, and the plates were placed in 15 °C incubator. Upon obtaining clear visibility of plaques formed by VHSV, the cells were fixed with 10% formalin and stained with Giemsa stain. The plaque numbers were counted, and the pfu/ml was determined by multiplying the plaque numbers by the dilution factor. The percent inhibition was calculated as follows.

VHSV inhibition (%) = {(pfu/ml in positive control – pfu/ml in treatment) ÷ pfu/ml in positive control} × 100.

2.4. Antiviral activity against VHSV by oral administration of the E. cava extract

2.4.1. Efficacy of the E. cava extract against VHSV infection (Trial I)

Olive flounder (10.6 ± 0.9 g, 10.4 ± 0.6 cm) were divided randomly into four groups (20 fish per group) and used for evaluation of the survival rate at 2 different *E. cava* extract concentrations (500 and 50 μg/g (body weight)/day). The extract was dissolved in ethanol (Sigma, USA) to obtain a final concentration of 500 μg/g/day (this group was called Ext 500); this extract was serially diluted in ethanol to