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Synthesis and antiviral activity of a new arctigenin derivative against IHNV *in vitro* and *in vivo*

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ABSTRACT

Viral diseases in aquaculture were challenging because there are few preventative measures and/or treatments. Our previous study indicated that imidazole arctigenin derivatives possessed antiviral activities against infectious hematopoietic necrosis virus (IHNV). Based on the structure-activity relationship in that study, a new imidazole arctigenin derivative, 4-(8-(2-ethylimidazole)octyloxy)-arctigenin (EOA), was designed, synthesized and its anti-IHNV activity was evaluated. By comparing inhibitory concentration at half-maximal activity (IC_{50}), we found that EOA (IC_{50} = 0.56 mg/L) possessed a higher antiviral activity than those imidazole arctigenin derivatives in our previous study. Besides, EOA could significantly decrease cytopathic effect (CPE) and viral titer induced by IHNV in epithelioma papulosum cyprinid (EPC) cells. In addition, EOA significantly inhibited apoptosis induced by IHNV in EPC cells. Further data verified that EOA inhibited IHNV replication in rainbow trout, with reducing 32.0% mortality of IHNV-infected fish. The results suggested that EOA was more stable with a prolonged inhibitory half-life in the early stage of virus infection (1–4 days). Consistent with above results, EOA repressed IHNV glycoprotein gene expression in virus sensitive tissues (kidney and spleen) in the early stage of virus infection. Moreover, histopathological evaluation showed that tissues from the spleen and kidney of fish infected with IHNV exhibited pathological changes. But there were no lesions in any of the tissues from the control group and EOA-treaten group. In accordance with the histopathological assay, EOA could elicited anti-inflammation response in non-viral infected rainbow trout by down-regulating the expression of cytokine genes (*IL-8*, *IL-12p40*, and *TNF-α*). Altogether, EOA was expected to be a therapeutic agent against IHNV infection in the field of aquaculture.

1. Introduction

As a negatively single stranded RNA virus, infectious hematopoietic necrosis virus (IHNV) belongs to the genus *Novirhabdovirus* in the family Rhabdoviridae. Ever since first detected in the northwestern region of the United States Atlantic, IHNV has widely occurred in North America [1,2] as well as Europe, Asia, and Russia [3–6]. Additionally, outbreaks of IHNV infection have been well known for causing mass mortality worldwide in farm-reared freshwater fish, and marine fish [6–9]. Therefore, infectious hematopoietic necrosis (IHN) has been listed as a notifiable animal disease by the Office International des Epizooties (OIE) and recognized as one of the Class II viral disease by animal epidemic prevention law in China.

Traditionally, most researches of prevention on IHNV have been centered on developing vaccines, such as DNA vaccines [10], attenuated vaccines [11,12], inactivated virus vaccines [13] and oral vaccines [14,15]. However, vaccines are mainly designed to protect against diseases through manipulation of the immune response before the infection process is established [16]. Remarkably, the vaccine can be high labor and production costs and has efficient vector constraints and the handling stress, which makes it impractical for large numbers of susceptible fish [17,18]. Therefore, researchers have turned their attention to antiviral drugs. Notably, two bromophenols isolated from *Polysiphonia morrowii* and three flavonoids isolated from *Rhus verniciflua* significantly inhibited the replication of IHNV *in vitro* [19,20]. Nevertheless, there are no drugs could be used in salmonid aquaculture to treat the IHNV infection.

In aquaculture, the applications of the natural products/derivatives and chemical agents on virus infection have been generally explored [21,22]. For instance, honokiol and moroxydine hydrochloride exhibited anti-grass carp reovirus (GCRV) effects for increasing cell viability [23,24]. Saikosaponin D and coumarin derivatives showed the

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admirable activity against spring viraemia of carp virus (SVCV) [25–27]. In the previous studies, natural product arctigenin was measured the half maximal inhibitory concentration (IC_{50}) of 0.29 mg/L against SVCV [28]. In addition, arctigenin derivatives were synthesized and prosessed anti-SVCV and anti-IHNV activity in epithelioma papulosum cyprinid (EPC) cells [29,30]. Meanwhile, the results suggested that imidazole arctigenin derivatives with eight carbon atoms length of linker were effective to the treatment of IHNV infection in EPC cells.

To obtain a compound with higher anti-IHNV activity, a new arctigenin derivative, 4-(8-(2-Ethylimidazole) octyloxy)-arctigenin (EOA), was designed, synthetized and identified based on structureactivity relationship in this study. As expected, by real-time quantitative PCR (RT-qPCR), we found that EOA showed higher anti-IHNV activity in EPC cells compared with our previously reported imidazole arctigenin derivatives [30]. Cytopathic effect (CPE) reduction assays and titer assay were used to confirm the anti-IHNV activity of EOA in EPC cells. It was further validated in secondary assays, including microtubule structure, nucleus damage observation, and apoptosis test. Furthermore, the anti-IHNV activity of EOA in rainbow trout was evaluated by RTqPCR and survival rate assay. In order to explore how EOA protected rainbow trout from IHNV infection, the effects of EOA on inflammation response were also investigated.

2. Materials and methods

2.1. Cell lines, virus and rainbow trout husbandry

The EPC cell line was kindly provided by Prof. Ling-Bing Zeng (Yangtze River Fisheries Research Institute, Wuhan, Hubei, China). Cells were maintained at 25 °C in 5% CO_2 atmosphere in Medium 199 (Hyclone, USA) cell culture containing 10% fetal bovine serum (FBS) (ZETA LIFE, USA), streptomycin 100 μg/mL and penicillin 100 U/mL. The IHNV (strain Sn-1203, isolated from infected rainbow trout in China, kindly provided by Prof. Tong-yan Lu, Heilongjiang River Fishery Research Institute Chinese Academy of Fishery Sciences, (Harbin China) was propagated in EPC cells at 15 °C [31].

Juvenile rainbow trout (n = 1200) with the average length and weight of 4.50 ± 0.15 cm and 0.70 ± 0.07 g were purchased from administration of shaanxi province stone river reservoir irrigation and maintained in three 500 L aquarium with a flowthrough system of carbon filtered tap water under laboratory conditions for 4 weeks prior to the beginning of experiments. (temperature 15.0 ± 0.5 °C, pH 7.4 ± 0.1, and dissolved oxygen 9.0 ± 1.0 mg/L).

2.2. Synthetization of arctigenin derivative 4-(8-(2-Ethylimidazole) octyloxy)-arctigenin (EOA)

General ^1H and ^{13}C NMR spectra were measured with a Bruker AM500 spectrometer at 500 and 126 MHz. The chemical shifts are expressed in parts per million (δ value) downfield from tetramethylsilane, using tetramethylsilane (TMS) (δ = 0) and/or residual solvents such as dimethyl sulfoxide (DMSO) (δ = 2.50) as an internal standard. High resolution ESI-MS data were recorded on an AB SCIEX ESI-LC-MS/MS mass spectrometer (TripleTOF5600 +, AB SCIEX, USA). Throughout this study, silica gel H (200–300 mesh; Qingdao Marine Chemical Factory, China) was used for the column chromatography. For TLC plates, Silica gel (GF_{254}) (Qingdao Marine Chemical Factory, China) were used for thin layer chromatographic (TLC) analysis, and all of thespots and bands were detected by UV irradiation (254, 365 nm). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Organic solvents were purchased from Sinopharm chemical reagent Co., Ltd and purified by distillation and moisture was excluded from the glass apparatus using $CaCl_2$ drying tubes.

The synthetic route of EOA is shown in Fig. 1 To a stirred solution of arctigenin (372.0 mg, 1.0 mmol) in 20 mL acetone were added anhydrous K_2CO_3 (110.0 mg, 0.8 mmol) at room temperature. After stirring at room temperature for 30 min, 1,8- dibromooctane (408.0 mg, 1.5 mmol) was added to the reaction mixture, and whole was refluxed at 70 °C for 24 h. The precipitate was filtered off and washed with acetone (4 × 30.0 mL). The solvent was evaporated under reduced pressure, and the residue was treated with water (50.0 mL) and extracted with dichloromethane (4 × 30.0 mL). The organic layer were combined, dried with anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude product was purified via silica gel column chromatography with mixed petroleum ether and ethyl acetate (4:3, v/v) as eluent, and resulted in a white solid, the intermediate compound 4-(8-Bromooctyloxy)-arctigenin. Further, 4-(8-Bromooctyloxy)-arctigenin (562.0 mg, 1.0 mmol) in 30.0 mL acetonitrile were added anhydrous K_2CO_3 (275.0 mg, 2.0 mmol) and 2-ethylimidazole (288.0 mg, 3.0 mmol) at 25 °C. Then the mixture was stirred at 25 °C for 24 h. The reaction mixture was filtered, and the filtrate was evaporated under reduced pressure. The residue was treated with water (50.0 mL) and extracted with dichloromethane (4 × 30.0 mL). The organic layer was combined, dried with anhydrous Na_2SO_4, and concentrated under reduced pressure. The obtained residue was purified by silica gel chromatography with chloroform/methanol (5:1) as eluent to give compound EOA as solid.

Stock solutions were prepared at a concentration of 5×10^4 mg/L in DMSO and stored at −20 °C during the experiment. Due to the structural changes affecting the solubility of EOA, the medium containing compound EOA was sonicated for approximately 10 min to ensure that the compounds were fully dissolved and mixed before treating EPC cells.

2.3. Toxicity of EOA on EPC cells

Briefly, approximately 90% confluent cells in 96-well plates were exposed to EOA at six concentrations (0.625, 1.25, 2.5, 5, 10 and 20 mg/L). After incubation for 72 h, the cell viability was examined with cell counting kit-8 assay (CCK-8, Beyotime, China) according to the manufacturer's protocol. The highest safe concentration of EOA in which 80% of the cells survived, was chosen for further antiviral assay.

2.4. Antiviral activity of EOA in vitro

To detect IHNV by RT-qPCR, EPC cells were cultured in 12-well plates to a monolayer and infected with IHNV (1×10^3 50% tissue culture infective dose ($TCID_{50}$)) for 2 h at 15 °C. Subsequently, the media was removed, cells were washed for three times and further incubated in 5% FBS M199 containing EOA (logarithmic doses, 0.2–2 mg/L) as treatments for 72 h. Afterwards, supernatants were removed and EPC cells were washed three times with 0.1 M phosphate buffer (PBS). Then, RT-qPCR which was explained in section 2.9 were carried out to detect IHNV.

2.5. CPE and virus titration reduction assays

Virus multiplication and titration assays were performed as described in a previous study [28]. EPC cells were cultured in 96-well plates (1×10^4 cells/well) for 24 h. Then, the medium was replaced with 100 μL cell maintenance medium containing 1×10^3 $TCID_{50}$ IHNV. After 2 h of infection, the medium was replaced again with maintenance medium containing 2 mg/L EOA. Each sample was directly observed and photographed under an inverted microscope.

2.6. Fluorescence observation for nucleus damages

Cells were incubated with 1×10^3 $TCID_{50}$ IHNV and EOA (2 mg/L) -virus mixture for 72 h at 15 °C. Then samples were collected and washed with 0.1 M PBS three times. Subsequently, cells were dyed with 1 mg/L DAPI (2-(4-Amidinophenyl)-6-indolecarbamidine

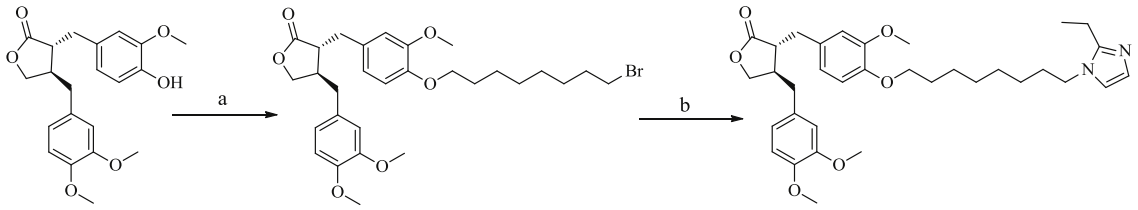

Fig. 1. Synthetic route of EOA. Reagents and conditions: (a) 1,8- dibromooctane, K_2CO_3, dry acetone, reflux, 20–24 h; (b) 2-ethylimidazole, K_2CO_3, CH_3CN, r.t., 20–24 h.

Table 1
Sequences of primer pairs used for the analysis of gene expression by real-time PCR.

Genes		Primer sequences (from 5′ to 3′)
IHNV glycoprotein (G)	Forward	GCACAAAGGCTCCATCTATC
	Reverse	TGTACTGGGCGACGTATT
β-actin (E)	Forward	GCTATGTGGCTCTTGACTTCGA
	Reverse	CCGTCAGGCAGCTCATAGCT
β-actin (H)	Forward	ATGGAAGGTGAAATCGCC
	Reverse	TGCCAGATCTTCTCCATG
IL-8	Forward	CACAGACAGAGAAGGAAGGAAAG
	Reverse	TGCTCATCTTGGGGTTACAGA
TNF-α	Forward	CAAGAGTTTGAACCTTGTTCAA
	Reverse	GCTGCTGCCGCACATAGAC
IL-12p40	Forward	GAACCCAGACGACGATGATT
	Reverse	GTTCAAACTCCAACCCTCCA

dihydrochloride) and 5 mg/mL DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) for 20 min (Beyotime, China). The cover glass grown cells was mounted on a glass slide and fluorescence was observed with an upright fluorescence microscopy (LeicaDM5000, Germany).

2.7. Cellular apoptosis assay

EPC cells were cultured in six-well plates and grown to a monolayer. 24 h later, cells were treated with M199 containing 5% FBS and IHNV ± EOA (2 mg/L) for 72 h incubation. Cell apoptosis was measured by flow cytometry-based Annexin V/propidium iodide (PI) staining Kit (Beyotime, China) according to the manufacturer's protocol. Of which, Annexin V and PI were used to give cell membrane and cell nucleus a staining separately to distinguish cells in the different periods of apoptosis. After staining, fluorescence intensity was measured by flow cytometry using a FACSCalibur (Becton Dickinson, USA).

2.8. Antiviral activity of EOA in vivo

A total of 150 Juvenile rainbow trout were reared in three aquarium containing 100 L UV-sterilized water, and rearing temperatures of each aquaria were kept at 15 ± 0.5 °C. The rainbow trout were divided into a M199 control group and two IHNV infection groups (IHNV/M199 and IHNV/EOA injection) and executed with the following treatments: (1) For the control group, each rainbow trout was injected intraperitoneally with 15 μL M199 and reared for 14 d; (2) Based on pretest, IHNV (10^3 $TCID_{50}$) was mixed with M199 or EOA (50 mg/L) in equal volume, then each rainbow trout was injected intraperitoneally with 15 μL mixture in the infection groups and reared for 14 d. The fish were fed three times daily with commercial dry feed pellets (administration of shaanxi province stone river reservoir irrigation, Shaanxi, China). The rainbow trout were monitored over 14 d period for survival rate. Under the same operational procedure, a total of 200 Juvenile rainbow trout were intraperitoneally injected with mixture (M199, EOA/M199, IHNV/M199 or IHNV/EOA, 50 rainbow trout per treatments), and six samples from each treatment were collected in control (M199), EOA/M199, IHNV/M199 and IHNV/EOA groups on the 1st, 4th and 7th days, respectively. Then the kidney and spleen were collected for RT-qPCR detection, anti-inflammatory response assay and histopathology analysis. To evaluate the histopathology induced by the IHNV, the spleen and kidney tissues were biopsied and processed for histological examination at 4th day. Transverse sections approximately 5 mm thick were excised from the organs, fixed in 4% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin.

2.9. RNA isolation, cDNA synthesis, and qPCR assays

Total mRNA from each sample was extracted using Trizol (TaKaRa, Japan) according to the manufacturer's protocols. RNA was reverse transcribed using HiScript Q Select RT SuperMix for quantitative polymerase chain reacti (qPCR) (+gDNA wiper) (TaKaRa, Japan). Quantitative PCR was performed with CFX96 Real-Time PCR Detection System (Bio-Rad, USA) using AceQ® qPCR SYBR® Green Master Mix (TaKaRa, Japan) with the following parameters: 95 °C for 30 s and then 40 cycles at 95 °C denaturation for 5 s, followed by at 60 °C annealing for 40 s. The sequences of primer pairs are listed in Table 1 [32–34]. Relative mRNA expression was calculated using $2^{-\triangle\triangle Ct}$ method [35]. The *β-actin* (E) and *β-actin* (H) were used to normalize the data *in vitro* and *in vivo*, respectively.

2.10. Statistical analysis

Drug response curves were represented by a logistic sigmoidal function with a maximal effect level (Amax) and a Hill coefficient represented the sigmoidal transition, which was performed with Origin 8.1. The data were analyzed by probit analysis which was used for calculating the half maximal inhibitory concentration (IC_{50}) and 20% cytotoxic concentration (CC_{20}) of the compound at the 95% confidence interval by using the SPSS 18.0 for Windows (SPSS Inc. an IBM Company). Values were expressed as the mean ± standard deviation (SD) or the mean ± standard error (SEM) and statistical analysis was performed with SPSS 18.0 software (SPSS Inc., USA), using one-way ANOVA after normalization to determine significance. *P* values less than 0.05 were considered statistically significant, **, $p < 0.01$; *, $p < 0.05$.

Key resources table

Resource	Source	Identifier
Antibodies		
ß-actin		
CellLine		
EPC cells		
Chemical		
1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate		
1,8- dibromooctane		
2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride		
2-ethylimidazole		
acetone		

acetonitrile
arctigenin
CaCl2
CCK-8
CO2
DAPI
dichloromethane
DiI
DMSO
ethyl acetate
formalin
K_2CO_3
methanol
octyloxy
propidium iodide
streptomycin

3. Results

3.1. Synthetization of EOA

4-(8-Bromooctyloxy)-arctigenin (compound **2**), which was crucial to the synthesis of EOA (compound **3**), was synthesized from arctigenin (compound **1**) by reacting with corresponding α, ω-dibromoalkanes in anhydrous acetone at reflux condition. Compound EOA was synthesized in 40% yield by treatment of compound **2** with corresponding amines and anhydrous potassium carbonate in acetonitrile at room temperature. The structure of EOA was confirmed by HRMS, ^{1}H, and ^{13}C NMR (Fig. 1).

The spectroscopic data of EOA: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.91 (d, J = 1.2 Hz, 1H), 6.80 (d, J = 1.3 Hz, 1H), 6.75 (t, J = 8.0 Hz, 2H), 6.68 (d, J = 1.9 Hz, 1H), 6.63 (dd, J = 8.1, 1.9 Hz, 1H), 6.54 (dd, J = 8.1, 1.9 Hz, 1H), 6.48 (d, J = 1.9 Hz, 1H), 4.09 (dd, J = 9.1, 7.1 Hz, 1H), 3.95 (t, J = 6.8 Hz, 2H), 3.87 (s, 1H), 3.85 (s, 1H), 3.83 (s, 3H), 3.82 (s, 1H), 3.80 (s, 3H), 3.80 (s, 3H), 2.97–2.81 (m, 4H), 2.65–2.48 (m, 4H), 1.83–1.69 (m, 4H), 1.42 (dd, J = 13.7, 6.2 Hz, 2H), 1.32 (td, J = 7.5, 4.2 Hz, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 178.82, 149.60, 149.16, 149.08, 148.00, 147.60, 130.60, 130.39, 126.78, 121.53, 120.70, 120.49, 119.04, 113.04, 112.04, 111.52, 71.33, 69.09, 56.09, 56.03, 55.97, 46.66, 45.84, 41.23, 38.25, 34.61, 30.98, 29.32, 29.28, 29.19, 26.70, 26.01, 20.14, 12.29. HRMS: exact mass calculated for $[M+H]^+$ ($C_{34}H_{47}N_2O_6$) requires m/z 578.3356, found m/z 579.3407.

3.2. Antiviral activity of EOA against IHNV infection in EPC cells

CCK-8 data on cell viability determined that EOA had little cytotoxic effect on EPC cells under the examined doses (Fig. 2B). To determine the antiviral activity of EOA on IHNV replication in EPC cells, the expression of IHNV glycoprotein (*G*) was analyzed by RT-qPCR in a six-point dose of EOA. The results in Fig. 2A showed that the 72 h IC_{50} of EOA on *G* was 1.15 mg/L. In addition, EOA at 2 mg/L had a strong antiviral activity on IHNV with a maximum inhibitory rate more than 90%. The dropped expression of *G* in the present study collectively represented that IHNV replication was inhibited by EOA *in vitro*. In accordance with the protein expression, CPE of IHNV-infected cells in presence of EOA with 2 mg/L was significantly decreased at 72 h (Fig. 2C). As shown in Fig. 2D, significant inhibition of IHNV was shown in EOA-treated EPC cells in the measurement of the viral titer. IHNV titers were $10^{4.91}$ (48 h post infection (p.i.)), $10^{6.52}$ (72 h p.i.), and $10^{7.70}$ (96 h p.i.) $TCID_{50}$/0.1 mL; whereas IHNV titers were $10^{3.48}$ (48 h p.i.), $10^{5.14}$ (72 h p.i.), and $10^{6.43}$ (96 h p.i.) $TCID_{50}$/0.1 mL in the EOA-treated group. The results above indicated that EOA could significantly inhibit IHNV replication in EPC cells.

3.3. Effects of compound EOA on IHNV-induced apoptosis

Based on the observation that typical apoptotic features including cellular morphology disappeared, nuclear fragmentation and cytoplasmic degradation in viral-infected cells (Fig. 3), we found that the apoptotic features weakened in drug-treated cells, which included the quantity of apoptosis body reduced sharply and the nucleus remained a normal spindle shape. Remarkably, flow cytometry showed that EOA could decrease IHNV-induced apoptosis in EPC cells after 72 h (Fig. 4). The results in Fig. 4 showed that apoptosis in drug treatments reduced with 22.7% compared with IHNV group for compound EOA. The treatment with EOA blocked the occurrence of apoptosis in IHNV-infected cells to some extent.

3.4. Antiviral activity of EOA against IHNV infection in rainbow trout

Before evaluating the anti-IHNV activity of EOA, we determined the highest safe inject concentration of EOA was 50 mg/L. To evaluate the *in vivo* antiviral activity of EOA in rainbow trout, we first evaluated the survival rate after treatment with EOA. The results showed that EOA treatment increased the survival rate of infected rainbow trout by 32.0% (Fig. 5A). Fig. 5A showed that rainbow trout died significantly within 3 and 5 days post infection (dpi) and the cumulative morality of IHNV infected fish reached up to 88.0% at 9 dpi. After EOA treatment, the cumulative morality of rainbow trout reduced to 56.0%. To further confirm the antiviral activity of EOA *in vivo*, the expression of *G* gene in kidney and spleen were evaluated by RT-qPCR. As expected, the expression of *G* gene was significantly inhibited by EOA treatment at 1 and 4 dpi (Fig. 5B and C). At 7 dpi, the expression of *G* gene was slightly inhibited (data not significant) by EOA treatment. Overall, these results suggested that EOA has an antiviral effect on IHNV in rainbow trout.

3.5. EOA efficiently ameliorated inflammation in rainbow trout

Histopathological analysis was performed to compare the lesions associated with IHNV in different host tissues. Evaluation of paraffin-embedded biopsied tissues from rainbow trout infected IHNV using light microscopy showed most of the spleen and kidney tissue had hemorrhaged. Moreover, the spleen and kidney tissue showed bleeding, and erythrocytes were observed in abundance. However, in contrast to infection by IHNV, there were no lesions in any of the tissues from rainbow trout from the control group and EOA-treaten group. (Fig. 6A). In accordance with histopathological assay, the similar results can also be seen in the measurement of inflammatory response. The results in Fig. 6B suggested that the expressions of *IL-8*, *IL-12p40*, and *TNF-α* genes were all down-regulated in rainbow trout after treated for 1 d. In kidney, the expressions of *IL-8*, *IL-12p40*, and *TNF-α* genes were down-regulated for 5.59-, 22.22-, and 37.98-fold, respectively. Besides, treatment with EOA for 1 d down-regulated the expressions of *IL-8*, *IL-12p40*, and *TNF-α* genes more strongly in spleen (142.85-, 6.02-, and 60.07-fold, respectively). These data implied that EOA ameliorated inflammation response in rainboe trout. After injection of EOA for 4 d, the expressions of inflammation-related genes were not significantly altered.

4. Discussion

As a phenylpropanoid and dibenzylbutyrolactone lignan present in medical plants, such as those used in traditional Chinese herbal medicine, including *Arctium lappa L.*, arctigenin exhibits antitumor and antiviral activities [36–39]. In our previous study, 23 arctigenin derivatives were synthesized and they possessed anti-IHNV activity in EPC cells [30]. Based on structure-activity relationship, EOA with a length of eight carbon atoms linker was synthesized to improve the drug potency against IHNV infection. Due to the higher antiviral activity of EOA against IHNV in EPC cells, we further explored whether EOA is also effective to IHNV *in vivo*. Our results suggested that rainbow trout died in a high speed within 4 and 5 dpi. This indicated that IHNV replicated quickly in rainbow trout within this period. Gladly, EOA was also highly effective to IHNV infection in rainbow trout, the survival

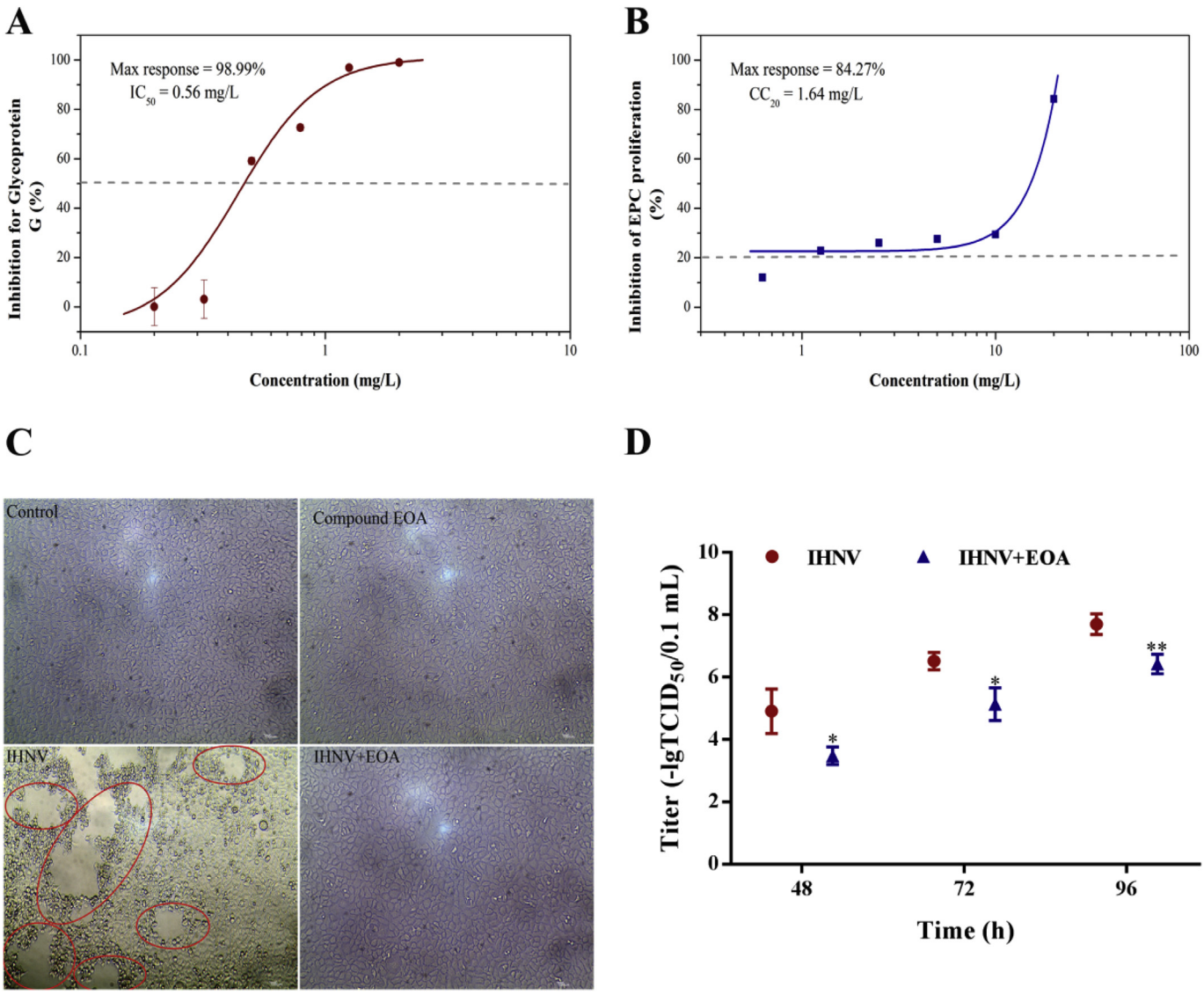

Fig. 2. Antiviral activity of EOA against IHNV in EPC cells. (A) Confirmatory six-point dose-response curves for EOA in EPC cells. The percent inhibition of the compound in the IHNV assay is shown in red. Data were shown as mean ± SEM. (B) Confirmatory six-point dose-response curves for EOA in EPC cells. The percent cytotoxicity of the compound on the host cell is shown in blue. Data were shown as mean ± SEM. (C) Morphologically protective effect of EOA against IHNV in EPC cells. CPE were shown in red ovals. (D) EOA reduced the titers of IHNV in EPC cells. Data were shown as mean ± SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

rate of infected rainbow trout increased after EOA treatment. The data of RT-qPCR indicated that EOA mainly played an antiviral role to IHNV in the first four days. These results suggested that EOA played its antiviral effect at the peak of viral replication and then protected rainbow trout from virus-induced death. Hence, EOA has been considered with antiviral activity against IHNV *in vivo* and can be an alternative anti-IHNV agent in aquaculture.

Synthesis is a common methods for the development of drugs. There are many commecial drugs which were producted by synthesization. Aspirin, one of the most widely used drug, was synthesized by the salicylic acid. It has been reported many activities, such as antiplatelet, anticoagulant, and anti-inflammation [40–42]. Another important commecial drug, diazepam, was also a synthetic drug which can be used as anticonvulsants [43]. In aquaculture, two coumarin derivatives has been synthesized and could be used for treat SVCV infection in zebrafish [27,44]. In addition, the synthetic coumarin derivatives showed anti-parasites activity in goldfish [45]. More importantly, our previous study demonstrated that imidazole arctigenin derivatives possessed an anthelmintic effect on *Dactylogyrus intermedius* in goldfish [46]. Based on these researches, we chosed the synthesis to be the method of development of anti-IHNV drugs. And the method proved to be effective.

Inflammation is a highly regulated defensive process characterized by the release of cytokines, chemokines and growth factors with transmigration of inflammatory cells, such as neutrophils, monocytes and lymphocytes, from the blood to the affected tissue [47]. IHNV infection is associated with excess expression of cytokine genes and induces inflammation in rainbow trout [48,49]. In accordance with previous studies, our study demonstrated IHNV infection could induce inflammation. And EOA could efficiently ameliorated inflammation induced by IHNV in rainbow trout. Moreover, we examined expression of the cytokine genes from different categories, namely *IL-8, IL-12p40,* and *TNF-α*. *IL-8* is often used as markers related with an activated inflammatory response [50]. *IL-12p40* is a regulator of cell-mediated immune responses and provides immune defense against parasites, viruses and intracellular bacteria through stimulating the production of IFN-g from Th1 and NK cells [51]. *TNF-α* is a member of the β-jellyroll family of cytokines, that also includes lymphotoxin, CD40 ligand, CD30

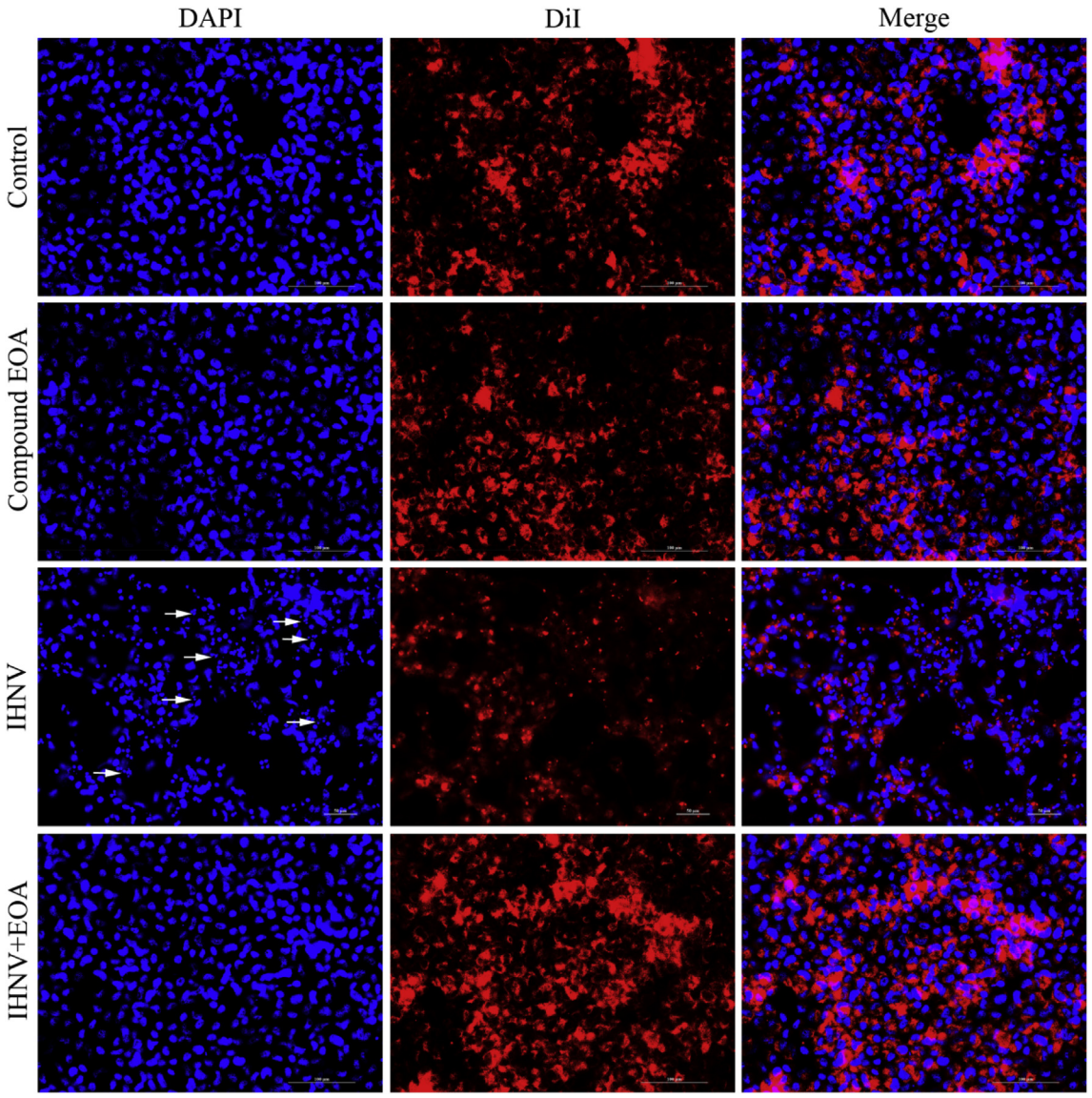

Fig. 3. Fluorescence microscopy images of nucleus damage in EPC cells. EPC cells in 12-well plate were treated in the presence or absence of compound EOA with 1×10^3 $TCID_{50}$ IHNV infection. The cells were dyed after 72 h post infection and apoptosis body was detected as arrows indicating.

ligand, and CD29 ligand [52]. On the other hand, they are the earliest expressed pro-inflammatory cytokine in living organisms and released from different cells to regulate inflammation [52]. The results showed that EOA could significantly inhibit the expression of pro-inflammatory cytokine. In addition, there are some researchers reported that arctigenin had the anti-inflammation activity [53,54]. Therefore, it should be noted that EOA might be a anti-inflammation agent and an immunosuppressive agent.

Interestingly, several immunosuppressive agents have also been confirmed to possess antiviral activities. For instance, dexamethasone, a classic immunosuppressive agent, has been reported with antiviral activity against Human immunodeficiency virus and Parainfluenza virus [55,56]. Rapamycin, the third-generation immunosuppressive agent, was found with antiviral activity against rift valley fever virus and so on [57,58]. In addition, these immunosuppressive agents usually possess anti-inflammatory activity and can improve pathological features [56,59,60]. Here, we speculated that the suppressive effect of EOA on specific cellular immunity might induce the anti-inflammatory activity and contribute to its protective effect on IHNV-infected rainbow throut, which needs further studies.

To better understand the mechanism by which EOA inhibited IHNV replication, we investigated whether EOA can interfere with IHNV adsorption to EPC cells. Hence, the viral binding assay was conducted (Fig. S1A). The results in Fig. S1B indicated that EOA did not affect viral adsorption. Notably, previous study found that IHNV infection could induce autophagy in EPC cells and that activation of autophagy inhibited both IHNV intracellular viral replication and extracellular viral yields [31]. Rapamycin, the autophagy inducer, was found with antiviral activity against IHNV [31]. Moreover, rapamycin also showed inhibitory effect on *IL-8*, *IL-12p40*, and *TNF-α* [61–63]. Given the above, we speculate that the antiviral mechanism of EOA might be the same as that of rapamycin. Nevertheless, the hypothesis that EOA might show the anti-IHNV effect by inducing autophagy needs more studies in future.

In summary, a new arctigenin derivative, EOA, was designed, synthesized and its anti-IHNV activity was evaluated. Our study provided the evidence that EOA played a positive role in anti-IHNV effect in EPC cells and rainbow trout. EOA is regarded as a new compound with a high antiviral activity against IHNV and is expected to be a therapeutic agent against IHNV infection in aquaculture.

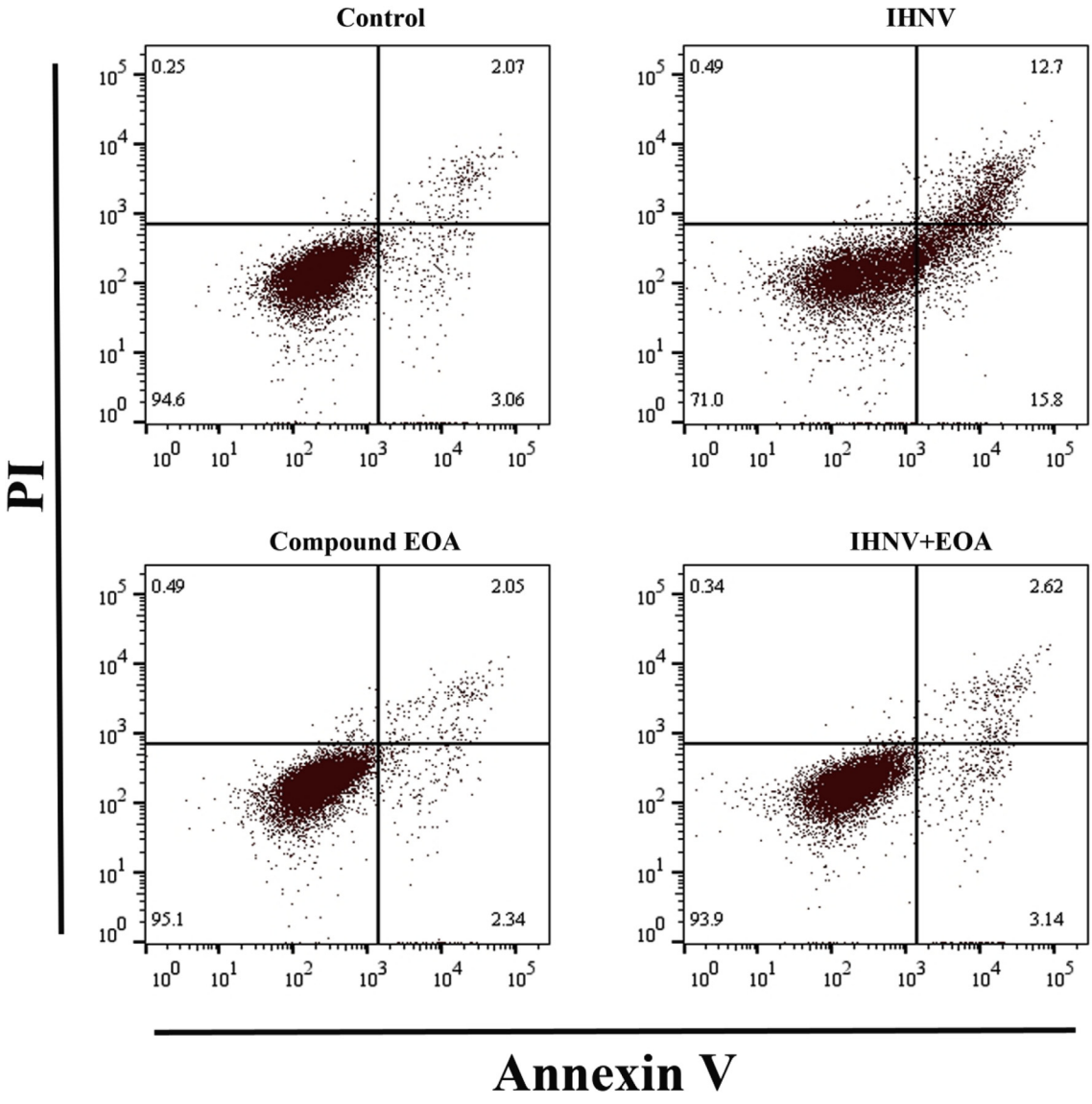

Fig. 4. Compound EOA inhibited apoptosis induced by IHNV in EPC cells. The percentage of viable, early apoptosis and late apoptosis/necrosis cells was assessed by Annexin V/PI staining at 72 h post-infection. Flow cytometric analysis of apoptosis used Annexin V/PI staining of EPC cells.

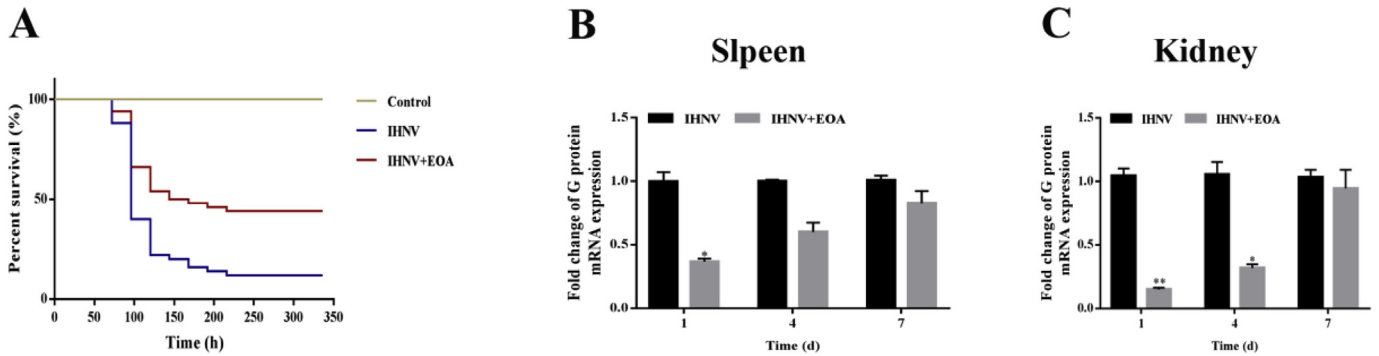

Fig. 5. Antiviral activity of EOA against IHNV in rainbow trout. (A) Cumulative survivorship curves of fish intraperitoneally injected with IHNV and EOA. (B) Expression of IHNV *G* in spleen after injection. Data were shown as mean ± SEM. (C) Expression of IHNV *G* in kidney after injection. Data were shown as mean ± SEM.

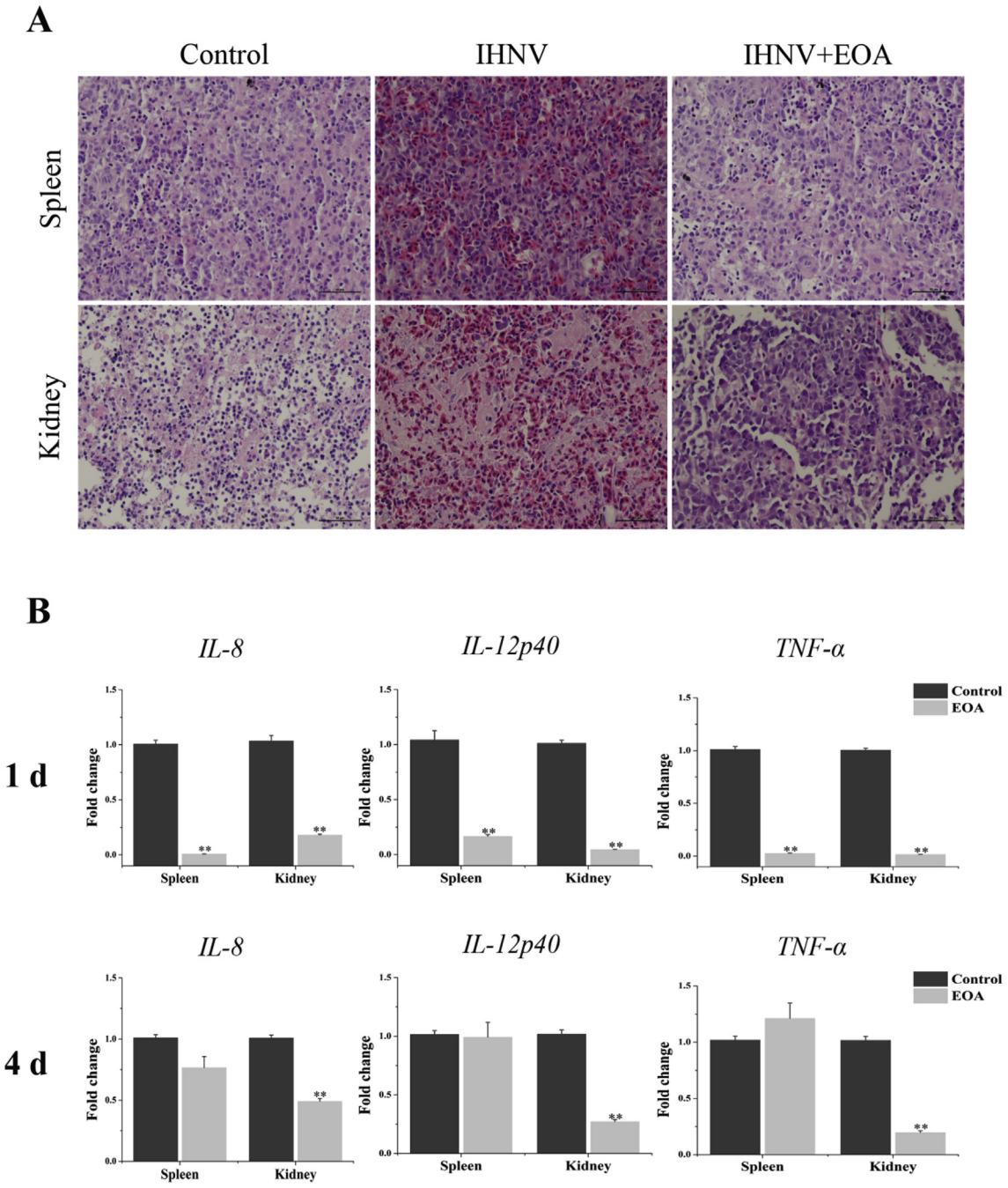

Fig. 6. EOA efficiently ameliorated inflammation in rainbow trout. (A) Histological analysis of rainbow trout intraperitoneally injected with IHNV and EOA. (B) Expression of *IL-8*, *IL-12p40*, and *TNF-α* in spleen and kidney after injection with M199 or EOA. Data were shown as mean ± SEM.

Conflicts of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.006.

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