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Ursolic acid from *Prunella vulgaris* L. efficiently inhibits IHNV infection *in vitro* and *in vivo*

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ABSTRACT

Infectious hematopoietic necrosis virus (IHNV) is a fish viral pathogen that causes severe disease and huge economic losses in the salmonid aquaculture industry. However, anti-IHNV drugs currently are scarce. For the purpose of seeking out anti-IHNV drugs, the anti-IHNV activities of 32 medicinal plants were investigated by using epithelioma papulosum cyprini (EPC) cells. Among these plants, *Prunella vulgaris* L. (PVL) showed the strongest inhibition on IHNV replication with an inhibitory percentage of 99.3% at the concentration 100 mg/L. Further studies demonstrated that ursolic acid (UA), a major constituent of PVL, also showed a highly effective anti-IHNV activity. The half-maximal inhibitory concentration (IC_{50}) at 72 h of UA on IHNV was 8.0 μM. Besides, UA could significantly decrease cytopathic effect (CPE) and the viral titer induced by IHNV in EPC cells. More importantly, UA also showed a strong anti-IHNV activity *in vivo*, as indicated by increasing the survival rate of rainbow trout and inhibiting viral gene expression. Intraperitoneal injection of UA increased the relative percentage of survival of rainbow trout by 18.9% and inhibited IHNV glycoprotein mRNA expression by > 90.0% in the spleen at the 1st-day post-infection. Altogether, UA was expected to be a therapeutic agent against IHNV infection in aquaculture.

1. Introduction

As the causative agent of infectious hematopoietic necrosis (IHN), infectious hematopoietic necrosis virus (IHNV) is presently one of the most serious pathogens harming the salmonid industry (Ammayappan et al., 2010). Since IHNV was discovered in western North America in Washington and Oregon in the 1950s (Rucker et al., 1953), it has rapidly spread to Europe and Asia (Bovo et al., 1987; Enzmann et al., 1992; Park et al., 1993; Rudakova et al., 2007; Sano et al., 1977; Vardić et al., 2007; Winton, 1991). In addition, outbreaks of IHNV usually cause symptoms of hematopoietic necrosis in a variety of salmon and trout, with a fatality rate of 80% (Ahmadivand et al., 2017). Therefore, it is imperative to develop an effective antiviral strategy to combat highly lethal IHNV outbreaks.

Traditionally, most researches of prevention on IHNV have been centered on developing vaccines, such as inactivated virus vaccines (Anderson et al., 2008), attenuated vaccines (Ristow et al., 2000), DNA vaccines (Anderson et al., 2008; Xu et al., 2017), and oral vaccines (Zhao et al., 2017a). These are mainly administered by intraperitoneal injection or intramuscular injection. In addition, vaccines are mainly designed to protect against diseases through manipulation of the immune response before the infection process is established (Gotesman et al., 2015). In this line of combat against IHNV investigators have focused on the discovery of anti-IHNV drugs. Previous studies report that chemical drugs ribavirin, benzimidazole, and guanine show the antiviral activity against IHNV (Amend, 1976; Hasobe and Saneyoshi, 1985; Hu et al., 2019d). Benzyloxycarbonyl-phenylalanyl-alanyl-fluoromethyl ketone, a protease inhibitor, also exhibit a potential anti-IHNV activity (Roscow et al., 2018). However, the use of these chemical drugs have significant risks, such as environmental pollution, host toxicity, and so on (Bundschuh et al., 2016; Chapman et al., 1999; Sommadossi and Carlisle, 1987).

Generally, the natural products are more environmentally for frequent use as compared to the chemical drugs. Moreover, the applications of medicinal plants and their active constituents on IHNV infection have been generally explored. For instance, two bromophenols isolated from *Polysiphonia morrowii* and three flavonoids isolated from *Rhus verniciflua* significantly inhibit the replication of IHNV *in vitro* (Kim et al., 2011; Kang et al., 2012). Moreover, lentinan from *Lentinus edodes* mycelia shows the anti-IHNV activity in EPC cells (Ren et al.,

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2018). Notably, previous studies report that several natural products and their derivatives show good anti-IHNV potential (Hu et al., 2019a, b; Hu et al., 2019c). Hence, it is feasible to find anti-IHNV agents from medicinal plants.

In this study, the anti-IHNV activities of 32 medicinal plants were checked *in vitro*. Data showed that *Prunella vulgaris* L. (PVL) had the strongest anti-IHNV activity among the 32 plant crude extracts. Additionally, as a major constituent of PVL, ursolic acid (UA) was found with strong antiviral activity against IHNV which was assessed *in vitro* and *in vivo*. To test the anti-IHNV activity *in vitro*, real time quantitative PCR (RT-qPCR), titer test, and cytopathic effects (CPE) reduction assay were carried out. Moreover, the antiviral activity of UA against IHNV in rainbow trout was evaluated by RT-qPCR and survival rate assay. Our results demonstrated the potential application of UA as anti-IHNV agent in aquaculture.

2. Materials and methods

2.1. Cell lines, virus and rainbow trout husbandry

The epithelioma papulosum cyprini (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 strain Sn-1203 IHNV was 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), and propagated in EPC cells as-previously described (Zhao, et al., 2017b).

Juvenile rainbow trout (n = 2000) were obtained from the administration of Shaanxi province stone river reservoir irrigation, with the total length (cm) and body weight (g) of 4.50 ± 0.15 and 0.70 ± 0.07. Fish were maintained in four 500 L aquarium with a flowthrough system of carbon filtered tap water under laboratory conditions for 4 weeks prior to the beginning of experiments. Water-quality readings were taken daily to monitor the following parameters: temperature 15.0 ± 0.5 °C, pH 7.4 ± 0.1, and dissolved oxygen 9.0 ± 1.0 mg/L. Photoperiod was maintained on a constant 16:8 h (light: dark) cycle. The fish were fed three times daily with either commercial granular food (Hanye, Beijing, China) at a daily rate of 0.1% body weight.

2.2. Medicinal plants and UA

Medicinal plants were extracted according to a previous study (Chen et al., 2017). Briefly, the dry powder (50.0 g) of plants was respectively extracted with methanol (500 mL × 3 times) for 4 h. Then the extracts were filtered and evaporated under reduced pressure in a vacuum rotary evaporator (R-201, Shanghai Shenshen) to get solidified crude extracts. The extracts of different plants were stored at 4 °C until used. UA was purchased from Nanjing spring & autumn biological engineering Co., Ltd.

2.3. Cytotoxicity assays of 32 medicinal plants and UA

For cytotoxicity assay, EPC cells with a density of 1×10^4 per well were seeded into 96-well plates containing 100 μL growth medium and incubated for reaching approximately 80 ~ 90% confluence. Subsequently, the cells were exposed to cell maintenance medium (medium 199 supplemented with 5% FBS) with or without drugs [32 plant crude extracts (100 mg/L) or UA (8.7 ~ 87.0 μM)] and incubated for 72 h. Cells treated without medicinal plants or UA were used as the controls. After incubation, the viability of cells was examined with cell counting kit-8 assay (CCK-8, Beyotime, China) according to the manufacturer's protocol. The viability was determined by measuring the optical density at 450 nm using a microplate reader (M200, Tecan, Mannedorf, Switzerland). Cell viability = [(experimental group - blank group)/(control group - blank group)] × 100%. EPC cells treated without medicinal plants or UA were used as the controls, while those without cells were used as a blank group. According to the formula to calculate the cell viability, 80% of the cell survival rate was determined as the highest safe concentration of the extracts and was chosen for further antiviral assay.

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

2.4. Anti-IHNV activity assay in EPC cells

Firstly, 100 mg/L was chosen for the screening assay based on testing cytotoxicity in EPC cells. To detect IHNV by 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 with or without drugs (32 plant crude extracts (100 mg/L) or UA (5.5 ~ 17.4 μM)) as treatments for 72 h. Afterward, 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 2.7 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 (Liu et al., 2015). The viral titer was evaluated using a $TCID_{50}$ assay. Virus was serially diluted 10-fold in M199. Then EPC cells were inoculated with the diluted virus. After 2 h of infection, the medium was replaced again with maintenance medium with or without drugs [PVL (100 mg/L) or UA (6.6, 13.2, and 19.8 μM)]. Each sample was directly observed and photographed under an inverted microscope at 48, 72, and 96 h. Viral titers were determined by the Reed–Muench method at the indicated times.

To determine the efficacy of PVL and UA against CPE in EPC cells, the cells were cultured in 12-well plates (1×10^5 cells/well) for 24 h. Then, the medium was replaced with 1.5 mL cell maintenance medium containing 1×10^3 $TCID_{50}$ IHNV. After 2 h of infection, the medium was replaced again with maintenance medium with or without drugs (PVL (100 mg/L) or UA (20.0 μM)). Each sample was directly observed and photographed under an inverted microscope at 72 h.

2.6. Antiviral activity of UA in vivo

To determine the toxicity of UA to rainbow trout, a total of 420 juvenile rainbow trout were reared in 21 aquariums containing 50 L UV-sterilized water, and rearing temperatures of each aquarium was kept at 15 ± 0.5 °C. The rainbow trout were divided into seven groups (60 rainbow trout per treatments, 20 rainbow trout per aquarium) 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) For the UA-treated group, each rainbow trout was injected intraperitoneally with 15 μL mixture (2.5, 5.0, 10.0, 20.0, 40.0, and 80.0 mg/L UA with M199) and reared for 14 d.

To determine the efficacy of UA against IHN disease, a total of 450

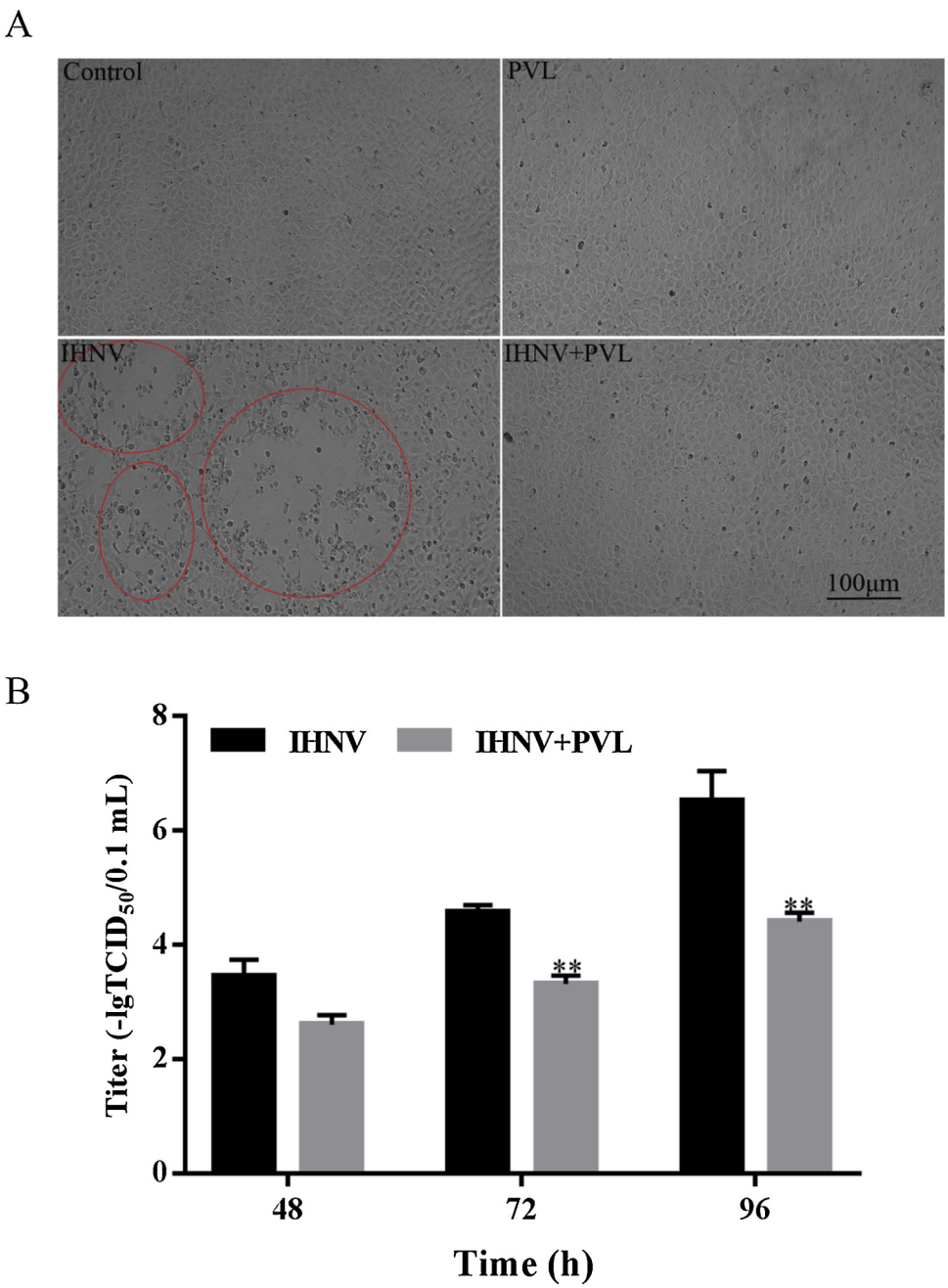

Fig. 1. Antiviral activity of PVL against IHNV in EPC cells. (A) Morphologically protective effect of PVL against IHNV in EPC cells. EPC cells cultured in 12-well plates were exposed to 1×10^3 $TCID_{50}$ IHNV for 2 h and then the medium with IHNV was removed and cells were incubated in fresh medium containing PVL (100 mg/L) for 72 h. (B) PVL reduced the titers of IHNV in EPC cells. EPC cells cultured in 96-well plates were exposed to 1×10^3 $TCID_{50}$ IHNV for 2 h and then the medium with IHNV was removed and cells were incubated in fresh medium containing PVL (100 mg/L) for 96 h. Data were shown as mean ± SD of three replicate samples of three independent experiments. Significance between control and PVL-treated groups are indicated by $^{**}P < 0.01$, $^{*}P < 0.05$.

juvenile rainbow trout were reared in nine aquariums containing 100 L UV-sterilized water, and rearing temperatures of each aquarium was kept at 15 ± 0.5 °C. The rainbow trout were divided into three groups (M199, IHNV/M199 or IHNV/UA, 150 rainbow trout per treatments, 50 rainbow trout per aquarium) 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 pre-test, IHNV (1×10^3 $TCID_{50}$) was mixed with M199 or UA (20 mg/L), then each rainbow trout was injected intraperitoneally with 15 μL mixture in the infection groups and reared for 14 d. To determine the efficacy of UA against IHNV replication *in vivo*, a total of 450 Juvenile rainbow trout were intraperitoneally injected with mixture (M199, IHNV/M199 or IHNV/UA, 150 rainbow trout per treatments, 50 rainbow trout per aquarium), and three samples from each aquarium were collected on the 1 st, 4th and 7th days, respectively. Then the spleen was collected for RT-qPCR detection.

2.7. RNA isolation, cDNA synthesis, and qPCR assays

The EPC cells with a total of 1×10^7 were taken from in 12-well plates and immediately frozen in liquid nitrogen for subsequent RNA isolation. Total RNA was extracted by the Trizol reagent (Takala, Japan). Under the NanoDrop spectrophotometer (ND-1000, Nano-Drop Technologies Inc., Wilmington, DE), nucleic acid concentrations were measured at 260 nm. The purity of the extracted total RNA was determined. by A260/A280 ratio, in which ratios of the absorbance at 260 and 280 nm ranged from 1.8 to 2.0. To further ensuring RNA purity, DNA contamination was removed by treating with DNase I (Takara, Japan) following the manufacturer's instruction. The purified RNA was reverse transcribed using HiScript Q Select RT SuperMix for qPCR (+gDNA wiper) (Vazyme, China), and 500 ng/μL of RNA was used per reaction in cDNA generation. 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

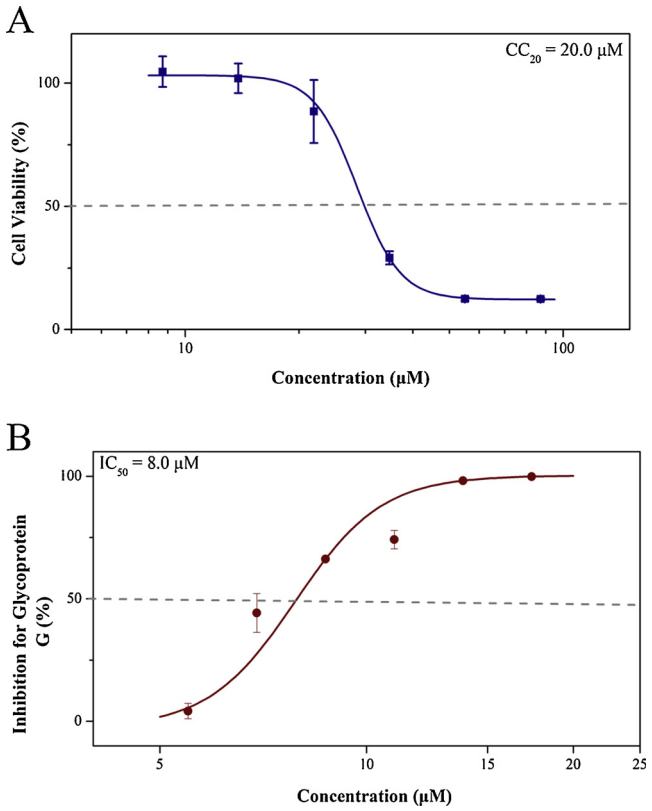

Fig. 2. The cytotoxicity and anti-IHNV activity of UA in EPC cells. (A) The toxicity of UA on EPC cells. EPC cells cultured in 96-well plates were exposed to UA at six concentrations (87.0, 55.0, 34.7, 21.9, 13.8, and 8.7 μM) for 72 h. After incubation periods, cell viability was tested by CCK-8 assay. Data were shown as mean ± SEM of three replicate samples of three independent experiments. (B) The antiviral activity of UA against IHNV in EPC cells. EPC cells cultured in 12-well plates were exposed to 1×10^3 $TCID_{50}$ IHNV for 2 h and then the medium with IHNV was removed and cells were incubated in fresh medium containing UA (5.5, 6.9, 8.7, 11.0, 13.8, and 17.4 μM) for 72 h. The relative RNA levels of glycoprotein genes were calculated based on the $2^{-\triangle\triangle Ct}$ method and relative to the control. Data were shown as mean ± SEM of three replicate samples of three independent experiments.

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 (Bilen et al., 2016; Hu et al., 2019c; Shao et al., 2016). Relative mRNA expression was calculated using $2^{-\triangle\triangle Ct}$ method with the formula (Livak and Schmittgen, 2001), $F = 2^{-\triangle\triangle Ct}$, $\triangle\triangle Ct = (C_{t,\ target\ gene} - C_{t,\ reference\ gene}) - (C_{t,\ target\ gene} - C_{t,\ reference\ gene})_{control}$. *β-actin* (E) and *β-actin* (H) were used to normalize the data in EPC cells and in rainbow trout, respectively.

2.8. Statistical analysis

Drug response curves (Fig. 2) 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). Data (Fig. 1B, Fig. 2, Fig. 3B, Fig. 4A, and C) were expressed as the mean ± SD or the mean ± standard error (SEM). Statistical analysis was performed with SPSS 18.0 software (SPSS Inc., USA), using one-way ANOVA after logistic normalization to determine significance. Data in Fig. 4B were shown as 95% confidence level (CL) and statistical analysis was performed with GraphPad Prism 6 (GraphPad Software, USA), using the Log-rank (Mantel-Cox) test to determine significance. *P* values less than 0.05 were considered statistically significant, **, $P < 0.01$, *, $P < 0.05$.

3. Results

3.1. Antiviral activity of 32 plant extract

Thirty-two kinds of medicinal plants were used in this study. In the initial test, the cell viability of EPC cells was greater than 80% in 32 crude extracts at 100 mg/L (Table 2). Medicinal plant extracts were considered safe if the cell viability was greater than 80%. Subsequently, the cells were treated with all plant extracts with the 100 mg/L in infection experiment. The antiviral efficacies of selected plant extracts were evaluated at 72 h, and the results were shown in Table 2. Medicinal plant extracts were considered active if the expression of IHNV G was lower than 50%. Among the screened plants, *Uncaria rhynchophylla* (Miq.) Jacks., *Notopterygium incisum* Ting ex H. T. Chang, *Ligusticum chuanxiong* Hort., and *Psoralea corylifolia* L., were identified to possess > 50% IHNV inhibition activity, and PVL showed the highest antiviral activity against IHNV (99.34 ± 0.04%).

To further confirm the antiviral activity of PVL against IHNV, we evaluated the related indexes, including CPE and the titer of IHNV after treatment with PVL. As shown in Fig. 1A, CPE of IHNV-infected cells was decreased significantly at 72 h in the presence of PVL. In accordance with CPE assay, significant inhibition of IHNV was shown in PVL-treated EPC cells in the measurement of the viral titer (Fig. 1B). IHNV titers were $10^{3.45}$ (48 h post-infection (p.i.)), $10^{4.57}$ (72 h p.i.) and $10^{6.52}$ (96 h p.i.) $TCID_{50}$ /0.1 mL in the control group; whereas IHNV titers were $10^{2.60}$ (48 h p.i.), $10^{3.31}$ (72 h p.i.) and $10^{4.41}$ (96 h p.i.) $TCID_{50}$/0.1 mL in PVL-treated group. The results above indicated that PVL could significantly inhibit IHNV replication in EPC cells.

3.2. Cytotoxicity and anti-IHNV activity of UA in EPC cells

UA, the major constituent of PVL, had been reported with diverse biological activities (Chen et al., 2015; Kong et al., 2013; Oloyede et al., 2017). Hence, we further evaluated whether UA is the major antiviral constituents of PVL. In EPC cells, the safe concentration of UA was calculated as 20.0 μM (Fig. 2A). As expected, by examining the antiviral activity of UA, we found that UA was highly effective to IHNV. As shown in Fig. 2B, UA had a concentration-dependent inhibition on the expressions of IHNV G. The IC_{50} of UA on IHNV G was 8.0 μM at 72 h p.i.

In accordance with gene expressions, CPE of IHNV-infected cells in the presence of 20.0 μM UA was decreased significantly at 72 h (Fig. 3A). In addition, we further evaluated the titer of IHNV after UA treatment. As shown in Fig. 3B, UA had a concentration-dependent inhibition on IHNV replication in the measurement of the viral titer. The results indicated that IHNV titers were $10^{5.09}$ (48 h p.i.), $10^{6.20}$ (72 h p.i.), and $10^{8.12}$ (96 h p.i.) $TCID_{50}$/0.1 mL in the control group. By contrast, UA treatment significantly reduced the viral titer in a concentration-dependent manner. After the IHNV-infected EPC cells were treated with 6.6, 13.2, and 19.8 μM of UA, IHNV titers were $10^{4.93}$ (48 h p.i.), $10^{5.65}$ (72 h p.i.), $10^{6.66}$ (96 h p.i.), $10^{2.98}$ (48 h p.i.), $10^{3.51}$ (72 h p.i.), $10^{4.75}$ (96 h p.i.), and $10^{2.22}$ (48 h p.i.), $10^{2.80}$ (72 h p.i.), $10^{3.89}$ (96 h p.i.) $TCID_{50}$/0.1 mL, respectively.

3.3. UA improved survival rate and inhibit IHNV replication in rainbow trout

To further confirm the highest safe inject concentration of UA *in vivo*, we evaluated the toxicity of UA to rainbow trout with serial concentrations. Fig. 4A showed that all of the rainbow trout were survived after treatment with 20 mg/L UA, while the mortality rate exceeded 50% at 40 mg/L. Therefore, 20 mg/L was selected as the highest

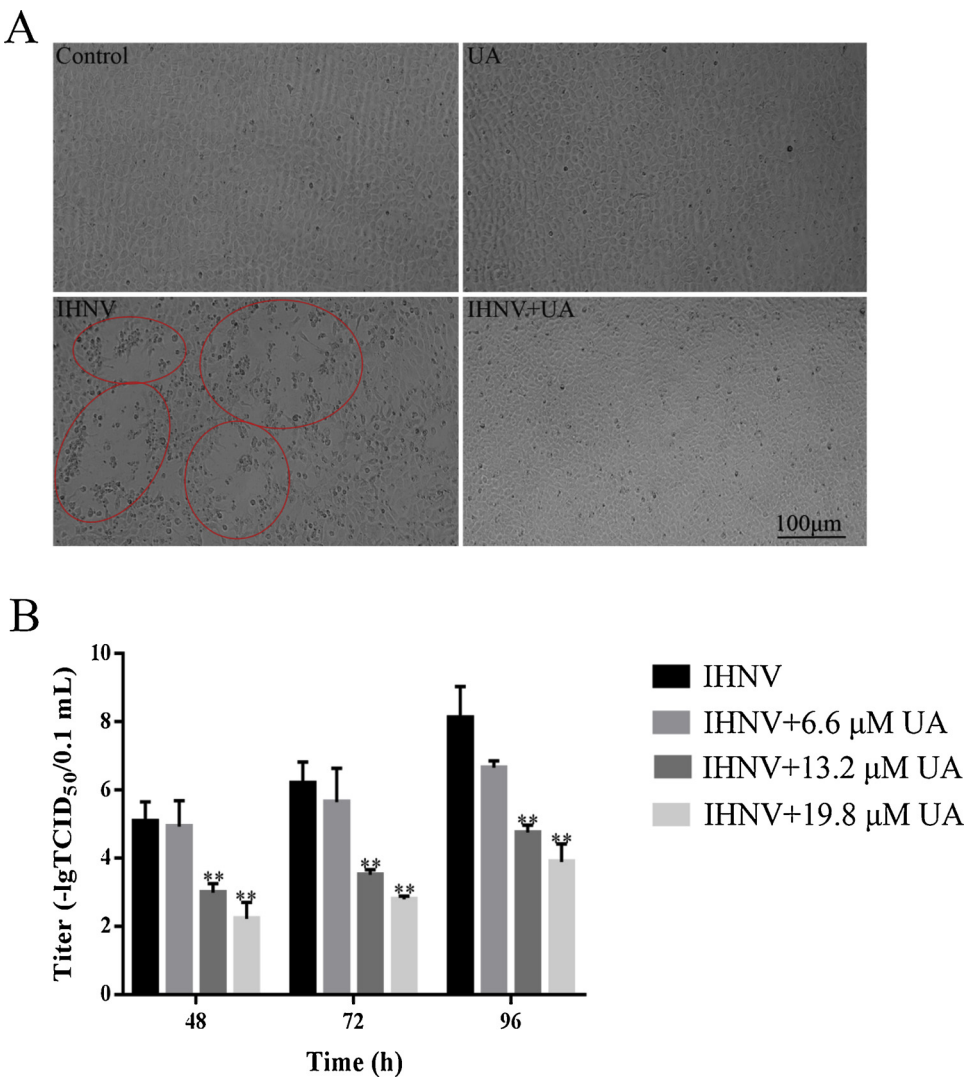

Fig. 3. Antiviral activity of UA against IHNV in EPC cells. (A) Morphologically protective effect of UA against IHNV in EPC cells. EPC cells cultured in 12-well plates were exposed to 1×10^3 $TCID_{50}$ IHNV for 2 h and then the medium with IHNV was removed and cells were incubated in fresh medium containing UA (20.0 μM) for 72 h. (B) UA reduced the titers of IHNV in EPC cells. EPC cells cultured in 96-well plates were exposed to 1×10^3 $TCID_{50}$ IHNV for 2 h and then the medium with IHNV was removed and cells were incubated in fresh medium containing UA (6.6, 13.2, and 19.8 μM) for 96 h. Data were shown as mean ± SD of three replicate samples of three independent experiments. Significance between control and UA-treated groups are indicated by $^{**}P < 0.01$, $^{*}P < 0.05$.

safe inject concentration. To evaluate the antiviral activity of UA in rainbow trout, we first evaluated the survival rate after treatment with UA. As shown in Fig. 4B, UA treatment increased the survival rate of infected rainbow trout by 18.7%. Rainbow trout died significantly within 3 and 6 days post-infection (dpi) and the cumulative mortality of IHNV infected fish reached up to 78.7% at 10 dpi. After UA treatment, the cumulative mortality of rainbow trout reduced to 60.0%. To further confirm the antiviral activity of UA *in vivo*, the expression level of the G in the spleen was evaluated by RT-qPCR. As expected, the expression of the G was significantly inhibited following UA treatment at 1 and 4 dpi (Fig. 4C). At 7 dpi, the expression of the G was slightly inhibited (data not significant) by UA treatment. Overall, these results suggested that UA has an antiviral effect on IHNV in rainbow trout.

4. Discussion

Among the screened plants extracts in this study, PVL exerted the highest inhibition activity against IHNV. PVL is a perennial plant commonly found in China and Europe, which was proved to be rich in phenolic acid, flavonoids, and triterpenes (Jiang et al., 2008; Zhu, 2000). There was little information about the antiviral effect of phenolic acid. In addition, kaempferol and quercetin, the two representative flavonoids in PVL, had no anti-IHNV activity according to our initial study. Notably, UA as one of the most representative triterpenes possesses considerable pharmacological effects including hepatoprotective (Jin et al., 2012), immunomodulatory (Saaby et al., 2011), anti-inflammatory (Ali et al., 2007; Zhang et al., 2013), and so on. Recently, UA has been attracted rising attention for its multi-functional antiviral activities (Kong et al., 2013; Wu et al., 2011). These results presumed that UA might be the potential active ingredient in the anti-IHNV effect. Under this assumption, the anti-IHNV activity of UA was investigated. Excitingly, our study confirmed that UA significantly inhibited IHNV replication in EPC cells.

Interestingly, the results of survival rate assay suggested that the antiviral effect of UA begins to decline gradually after a period of protection. Meanwhile, some studies have reported similar results that drugs only worked in the early stages of injection (Liu et al., 2019; Shen et al., 2018, 2019). In addition, the study on the transcription rate of the gene encoding IHNV G documents an effect of UA on virus replication over the first 4 days after infection, which is abolished by the 7th day. This limited effect might be responsible for the only slightly increased survival to IHNV infection in rainbow trout treated with UA. Given the above, we speculate that the decreased anti-IHNV activity may be due to the drug metabolism in rainbow trout. The drug is hydrolyzed or metabolized by the fish over time. Nevertheless, these speculations need to be studied in the future.

Although some studies have reported the regulation of UA on NF-kB (Jang et al., 2009; Lu et al., 2011), a large number of articles have reported that UA might be an immunosuppressive agent (Checker et al., 2012; Li et al., 2012; Sun et al., 2005), which inhibits pro-inflammatory

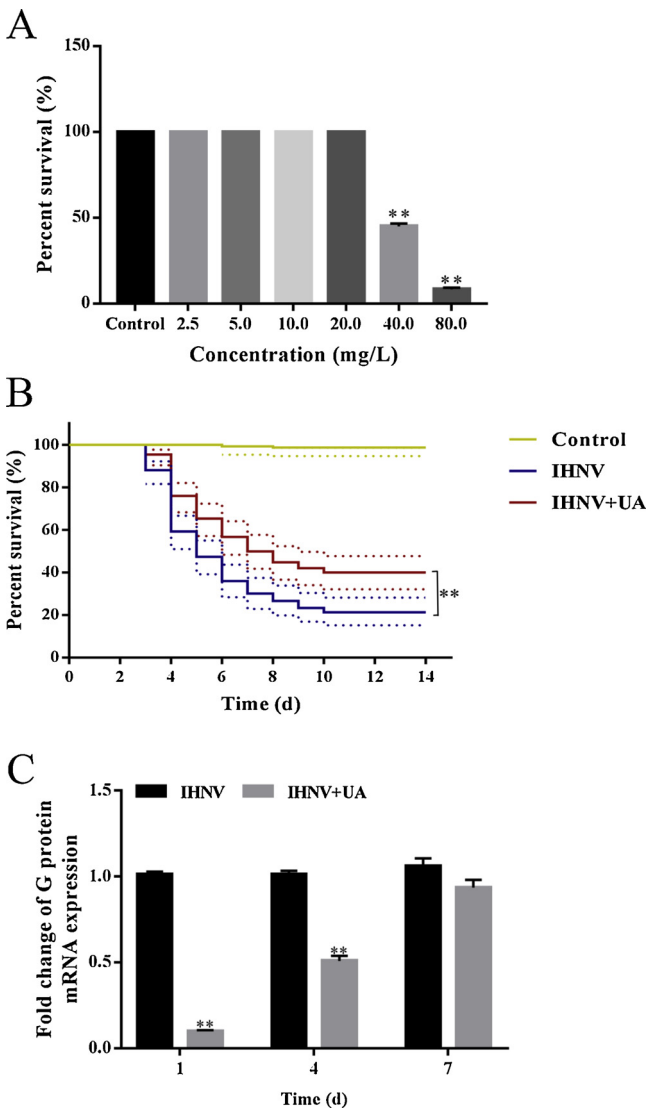

Fig. 4. Antiviral activity of UA against IHNV in rainbow trout. (A) The toxicity of UA on rainbow trout. Data were shown as mean ± SEM of three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$. (B) Cumulative survivorship curves of fish intraperitoneally injected with IHNV and UA (20 mg/L). Data were shown as 95% confidence level (CL) of three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$. (C) Expression of IHNV glycoprotein genes in the spleen after injection. The relative RNA levels of glycoprotein genes were calculated based on the $2^{-\triangle\triangle Ct}$ method and relative to the control. Data were shown as mean ± SEM of three replicate samples of three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$.

factors such as *IL-8*, *IL-12*, *TNF-α*, and so on (Chun et al., 2014; Ikeda et al., 2008; Takada et al., 2010). Interestingly, several immunosuppressive agents have also been confirmed to possess antiviral activities. For instance, rapamycin, the third-generation immunosuppressive agent, was found with antiviral activity against rift valley fever virus, herpes simplex virus, and so on (Bell et al., 2017; Canivet et al., 2015; Ko et al., 2017). Notably, rapamycin also showed inhibitory effect on IHNV, *IL-8*, *IL-12*, and *TNF-α* (Bertagnolli et al., 1994; Charreau et al., 2000; Wang et al., 2014; Zhao et al., 2017b). Meanwhile, rapamycin was the autophagy inducer. It's reported that rapamycin showed the anti-IHNV activity by inducing autophagy in EPC cells. Therefore, we speculate that the antiviral mechanism of UA might be the same as that of rapamycin, which needs further studies.

In summary, the natural product UA from PVL possessed a high antiviral activity against IHNV both *in vitro* and *in vivo*. More

Table 2
List of all medicinal plants used in the study, their cell viability and effects on the expression of IHNV glycoprotein.

Medicinal plants	Cell Viability (%)[a]	Expression of IHNV glycoprotein(%)[a]	Medicinal plants	Cell Viability (%)[a]	Expression of IHNV glycoprotein(%)[a]
Fallopia multiflora Thunb.	90.34 ± 2.13	67.52 ± 0.73	*Curcuma phaeocaulis* Valeton.	86.52 ± 3.45	75.83 ± 6.23
Ligustrum lucidum Ait.	89.14 ± 3.22	467.23 ± 11.91	*Paeonia suffruticosa* Andr.	87.34 ± 4.12	561.77 ± 20.87
Adenophora stricta Miq.	94.36 ± 6.84	483.21 ± 10.09	*Plantago asiatica* L.	89.18 ± 11.58	129.68 ± 3.03
Ligusticum Sinense Oliv.	84.42 ± 3.16	271.21 ± 11.42	*Angelica dahurica* (Fisch. ex Hoffm.) Benth. et Hook.f.	82.31 ± 2.34	103.79 ± 1.69
Peucedanum praeruptorum Dunn.	91.57 ± 8.76	122.34 ± 6.86	*Notopterygium incisum* Ting ex H. T. Chang.	99.12 ± 1.12	31.39 ± 1.38
Gastrodia elata Bl.	96.77 ± 4.75	186.48 ± 12.68	*Acorus calamus* L.	84.37 ± 6.44	113.26 ± 4.00
Glycyrrhiza uralensis Fisch.	86.40 ± 0.44	27.78 ± 1.70	*Spatholobus suberectus* Dunn.	84.34 ± 3.76	44.06 ± 1.71
Gentiana macrophylla Pall.	81.80 ± 0.62	85.65 ± 4.95	*Ligusticum chuanxiong* Hort.	88.68 ± 1.97	43.32 ± 1.61
Pinellia ternata (Thunb.) Breit.	98.34 ± 9.27	19.23 ± 0.75	*Homalomena occulta* (Lour) Schott.	87.51 ± 1.36	80.21 ± 2.31
Citrus aurantium L.	96.52 ± 6.59	120.46 ± 3.30	*Stellaria dichotoma* L. var. lanceolata Bge.	90.45 ± 3.11	95.35 ± 5.71
Asparagus cochinchinensis (Lour.) Mevr.	97.50 ± 3.19	384.54 ± 23.76	*Prunella vulgaris* L.	86.45 ± 2.74	0.66 ± 0.04
Atractylodes macrocephala Koidz.	91.23 ± 2.23	417.31 ± 27.67	*Acanthopanax gracilistylus* W.W.Smith.	92.78 ± 6.14	33.00 ± 1.54
Cynomorium songaricum Rupr.	83.20 ± 0.48	78.90 ± 7.68	*Psoralea corylifolia* L.	88.27 ± 5.89	43.67 ± 0.97
Rehmannia glutinosa Libosch.	85.85 ± 2.63	137.06 ± 4.87	*Platycodon grandiflorus* (Jacq.) A. DC.	96.13 ± 3.18	360.63 ± 13.13
Uncaria rhynchophylla (Miq.) Jacks.	94.54 ± 3.22	44.56 ± 1.17	*Paeonia lactiflora* Pall.	95.14 ± 4.28	86.59 ± 2.59
Bletilla striata (Thunb.) Reichb.f.	92.52 ± 2.53	54.62 ± 2.40	*Cinnamomum cassia* Presl.	86.34 ± 6.92	73.83 ± 1.90

[a] The concentrations of all medicinal plants were 100 mg/L. Each value represents mean ± SEM.

importantly, UA treatment could reduce the mortality rate of rainbow trout. Results so far indicated that UA is a new compound with high anti-IHNV activity and expected to be a therapeutic agent against IHNV infection in aquaculture.

Declaration of Competing Interest

The authors declare no competing financial interest.

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