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Antiviral Activities of Flavonoids Isolated from the Bark of *Rhus verniciflua* Stokes against Fish Pathogenic Viruses *In Vitro*

So Young Kang*, Ji-Young Kang, and Myung-Joo Oh

Department of Aqualife Medicine, Chonnam National University, Yeosu 550-749, Republic of Korea

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An 80% methanolic extract of *Rhus verniciflua* Stokes bark showed significant anti-viral activity against fish pathogenic infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) in a cell-based assay measuring virus-induced cytopathic effect (CPE). Activity-guided fractionation and isolation for the 80% methanolic extract of *R. verniciflua* yielded the most active ethyl acetate fraction, and methyl gallate (1) and four flavonoids: fustin (2), fisetin (3), butin (4) and sulfuretin (5). Among them, fisetin (3) exhibited high antiviral activities against both IHNV and VHSV showing EC_{50} values of 27.1 and 33.3 μM with selective indices ($SI = CC_{50}/EC_{50}$) more than 15, respectively. Fustin (2) and sulfuretin (5) displayed significant antiviral activities showing EC_{50} values of 91.2–197.3 μM against IHNV and VHSV. In addition, the anti-viral activity of fisetin against IHNV and VHSV occurred up to 5 hr post-infection and was not associated with direct virucidal effects in a timed addition study using a plaque reduction assay. These results suggested that the bark of *R. verniciflua* and isolated flavonoids have significant anti-viral activity against IHNV and VHSV, and also have potential to be used as anti-viral therapeutics against fish viral diseases.

Keywords: *Rhus verniciflua*, anti-viral activity, infectious hematopoietic necrosis virus, viral hemorrhagic septicemia virus, flavonoids

Introduction

Aquaculture is now a major global industry with total annual production exceeding 50 million tonnes and an estimated value of almost US$ 80 billion. With an average annual growth of 6.9% from 1970–2007, it has been the fastest growing animal food-producing sector and will soon overtake capture fisheries as the major source of seafood (Walker and Winton, 2010). However, like any other intensive farming activity, the threat of viral diseases within an intensive aquaculture system is considered serious given the economic repercussions. To date, a number of viral diseases have been reported within the fish aquaculture industry (Biering *et al.*, 2005). Infectious hematopoietic necrosis (IHN) is one of the most important viral diseases in aquaculture facilities for salmonid fishes because outbreaks of IHN result in losses approaching 100%, depending on the species and size of the fish, the virus strain and environmental conditions (Wolf, 1988). Viral hemorrhagic septicemia (VHS) is also one of the most important viral diseases affecting the European farmed finfish species, causing estimated losses of 40 million pounds per year in 1991 (Hill, 1992).

Because of the high mortality of these viral diseases in farmed finfish, and the lack of specific treatments or practical prevention methods, such as vaccines, these diseases are notifiable to the Office International des Epizooties (OIE). Infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) are the causative agents of IHN and VHS, respectively. These viruses are bullet-shaped, enveloped negative sense single-stranded RNA viruses belonging to the genus *Novirhabdovirus* in the family Rhabdoviridae, whose genomes, similar to other rhadoviruses, contain the following five structural genes: nucleocapsid protein (N), polymerase-associated phosphoprotein (P), matrix protein (M), surface glycoprotein (G), and virus polymerase (L) genes, as well as one nonstructural NV gene, which codes for NV protein that is expressed in infected cells but is not present in the virion particles. (Gomez-Casado *et al.*, 2011). At present, uses of vaccines for preventing these fish viral diseases are limited and their efficacy is not complete (Gomez-Casado *et al.*, 2011). Furthermore, since there are no effective anti-viral agents to prevent and treat these fish viral diseases, the establishment of effective anti-viral agents against fish viral diseases is urgently needed.

Many screening efforts have been made to find anti-viral agents from natural sources. Plants have the ability to synthesize a wide array of compounds and have long been used as remedies, and many are now being collected and examined in an attempt to identify possible sources of antivirals (Naithani *et al.*, 2008). To obtain safe and effective natural anti-viral agents against fish pathogenic viruses, we have screened various sources of natural products and found that the 80% methanolic extract of *Rhus verniciflua* Stokes barks possessed significant anti-IHNV and anti-VHSV activities. *R. verniciflua* Stokes (Anacardiaceae) is a native plant to East Asian countries such as Korea, China and Japan, and is used as a traditional herbal medicine (Kim, 2009). *R. verniciflua* extract has been found to have various biological activities including antioxidant activity

*For correspondence. E-mail: sykang1@chonnam.ac.kr; Tel.: +82-61-659-7176; Fax: +82-61-659-7176

(Kim *et al.*, 2002, 2010a), antimicrobial activity (Kim *et al.*, 2010a), anti-mutagenic activity (Park *et al.*, 2004), anti-arthritic effects (Choi *et al.*, 2003), anti-obesity effects (Jeon *et al.*, 2003), anti-platelet effects (Jeon *et al.*, 2006), and anti-cancer effects (Lee *et al.*, 2009). Previously, we also found that the 80% methanolic extract of *R. verniciflua* barks and its fractions have significant anti-microbial activities against gram negative fish pathogenic bacteria *Edwardsiella tarda* and *Vibrio anguillarum* (Kang, 2005). However, little information regarding anti-viral activity of *R. verniciflua* extract has been available to date (Nam *et al.*, 1996), and no studies have been conducted to evaluate the anti-viral activity of fractions or compounds isolated from *R. verniciflua* against fish pathogenic viruses.

In the present study, we report the isolation and identification of active compounds attributed for its anti-viral activities from the extract of *R. verniciflua,* and their anti-viral activities against IHNV and VHSV.

Materials and Methods

General experimental procedure

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were run on a Bruker UltraShield 400 instrument (Bruker, Germany) at 400 MHz and 100 MHz, respectively, with tetramethylsilane as internal standard. Electron impact mass sprectrometry spectra were obtained on a VG Trio II spectrometer. Column chromatography was performed using YMC-gel ODS-A (12nm, S-150 µm, YMC Co., Japan) and Sephadex LH-20 (GE Healthcare Bio-Sci, Sweden). Medium Pressure Liquid Chromatography (MPLC) was run on a Combi Flash graduate (Isco, USA) using RediSep reversed phase C-18 columns (Isco). Analytical thin layer chromatography was performed on precoated Merck F_{254} silica gel plates and visualized by spraying with anisaldehyde-H_2SO_4. Other chemicals were of analytical grade.

Plant material

The bark of *Rhus verniciflua* Stokes was purchased from a commercial supplier in the Kyung-dong market in Seoul, Korea in 2006. A voucher specimen has been deposited in our laboratory at Chonnam National University.

Extraction, fractionation, and isolation

Dried and chopped bark of *R. verniciflua* (6.6 kg) was extracted with 80% methanol in water in an untrasonic apparatus. The extracted solution was evaporated *in vacuo* and the 80% methanolic extract (97.6 g) was obtained. The extract was suspended in water and then partitioned successively with *n*-hexane, chloroform ($CHCl_3$), ethylacetate (EtOAc), and water-saturated *n*-butanol (*n*-BuOH). Each fraction (fr.) was evaporated to dryness *in vacuo* and yielded 15.7 g (*n*-hexane fr.), 7.8 g ($CHCl_3$ fr.), 25.0 g (EtOAc fr.), 21.6 g (*n*-BuOH fr.), and 27.3 g (H_2O fr.). Octadecylsilane (ODS) column chromatography of the EtOAc fr. (25.0 g) with a mixture of H_2O-methanol (MeOH) (95:5 to 0:100) as gradient eluent afforded eight fractions (Fr. I–VIII). Fr. II (2.190 g) was subjected to Sephadex LH-20 column chromatography two subsequent times with 100% MeOH, yielding compound **1** (430 mg). Fr. III (3.550 g) was eluted on by Sephadex LH-20 column chromatography with a mixture of MeOH and H_2O (95:5) to obtain seven subfractions (III-1–7). Subfraction III-5 (570 mg) was eluted by Sephadex LH-20 column chromatography with 100% MeOH and then on MPLC (H_2O:acetonitrile, 90:10; 43 g RediSep ODS column) to finally yield compound **2** (130 mg). Fr. V (4.240 g) was subjected to Sephadex LH-20 column chromatography with 100% MeOH to obtain five subfractions (V-1–5). Recrystallization for the subfraction V-1 yielded compound **3** (286 mg). Compound **4** (90 mg) was obtained from MPLC with a mixture of H_2O-MeOH (90:10 to 0:100) for the subfraction V-2 (400 mg). Fr. VI (5.290 g) was eluted on Sephadex LH-20 column chromatography with 100% MeOH to yield nine subfractions (VI-1–9). The subfraction VI-5 (310 mg) was subjected to Sephadex LH-20 column chromatography with 100% MeOH and subsequently to MPLC (H_2O:MeOH=90:10) with an ODS column and afforded compound **5** (85 mg).

Five compounds (**1**–**5**; Fig. 1) were identified by the direct comparison of their physicochemical and spectroscopic data with those previously reported (Kashiwada *et al.*, 1986; Park *et al.*, 2000; Prachayasittikul *et al.*, 2008).

Methyl gallate (**1**): amorphous colorless powder; $C_8H_8O_5$; MS: 184. ^{1}H-NMR (400 MHz, methanol-d_4): δ 7.03 (2H, s, H-2, H-6), 3.78 (3H, s, -OCH_3).

Fustin (**2**): white needles; $C_{15}H_{12}O_6$; MS: 288. ^{1}H-NMR (400 MHz, dimethyl sulfoxide (DMSO)-d_6): δ 7.63 (1H, *d*, *J* = 8.7 Hz, H-5), 6.89 (1H, *d*, *J* = 1.7 Hz, H-2′), 6.75 (1H, *dd*, *J* = 7.0 and 1.8 Hz, H-6′), 6.74 (1H, *d*, *J* = 7.0 Hz, H-5′),

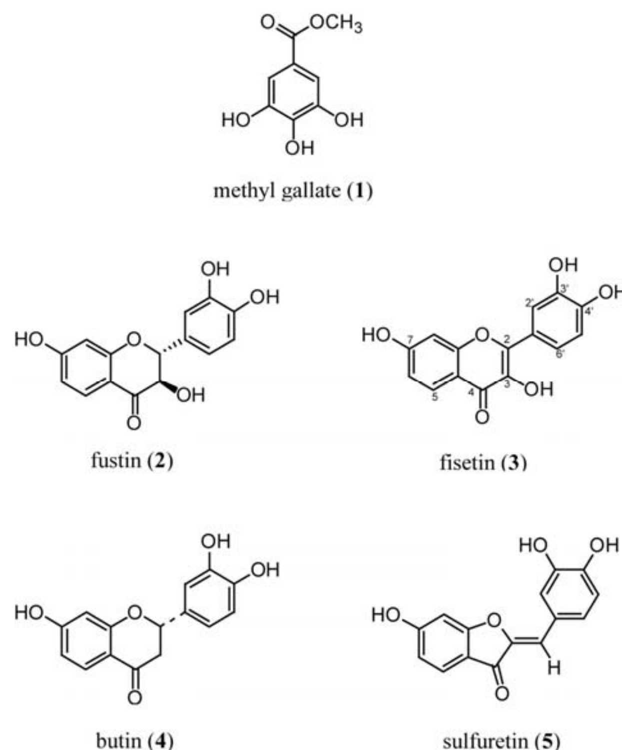

Fig. 1. **Chemical structures of compounds isolated from the bark of *R. verniciflua*.**

6.52 (1H, *dd*, *J* = 8.6 and 2.2 Hz, H-6), 6.28 (1H, *d*, *J* = 2.1 Hz, H-8), 4.96 (1H, *d*, *J* = 11.3 Hz, H-2), 4.4 (1H, *d*, *J* = 11.3 Hz, H-3). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 184.9 (C-4), 157.7 (C-9), 155.6 (C-7), 137.6 (C-4′), 136.8 (C-3′), 120.6 (C-5), 120.6 (C-1′), 111.4 (C-6′), 106.6 (C-5′), 106.4 (C-2′), 103.9 (C-8), 102.7 (C-6), 94.2 (C-10), 76.1 (C-2), 65.1 (C-3).

Fisetin (**3**): yellowish needles; $C_{15}H_{10}O_6$; MS: 286. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.06 (s, 3-OH), 7.93 (1H, *d*, *J* = 9.3 Hz, H-5), 7.69 (1H, *d*, *J* = 2.1 Hz, H-2′), 7.55 (1H, *dd*, *J* = 8.5 and 2.1 Hz, H-6′), 6.92 (3H, *m*, H-5′, H-6, H-8). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 171.9 (C-4), 162.2 (C-7), 156.2 (C-9), 147.2 (C-4′), 145.0 (C-3′), 145.0 (C-2), 137.1 (C-3), 126.4 (C-5), 122.5 (C-1′), 119.6 (C-6′), 115.5 (C-2′), 114.9 (C-6), 114.6 (C-5′), 114.2 (C-10), 101.8 (C-8).

Butin (**4**): yellowish solid; $C_{15}H_{12}O_5$; MS: 272. ^{1}H-NMR (400 MHz, methanol- d_4): δ 7.72 (1H, *d*, *J* = 8.7 Hz, H-5), 6.93 (1H, *d*, *J* = 1.7 Hz, H-2′), 6.79 (1H, *d*, *J* = 8.2 Hz, H-5′), 6.78 (1H, *dd*, *J* = 8.2 and 1.8 Hz, H-6′), 6.49 (1H, *dd*, *J* = 8.7 and 2.3 Hz, H-6), 6.35 (1H, *d*, *J* = 2.2 Hz, H-8), 5.31 (1H, *dd*, *J* = 12.9 and 2.9 Hz, H-2), 3.09 (1H, *dd*, *J* = 16.9 and 12.9 Hz, H-3a), 2.69 (1H, *dd*, *J* = 16.9 and 3.0 Hz, H-3b). ^{13}C-NMR (100 MHz, methanol- d_4): δ 193.5 (C-4), 166.8 (C-7), 165.5 (C-9), 146.8 (C-3′), 146.5 (C-4′), 132.1 (C-1′), 129.8 (C-5), 119.2 (C-6′), 116.3 (C-5′), 115.0 (C-10), 114.7 (C-2′), 111.7 (C-6), 103.8 (C-8), 81.1 (C-2), 45 (C-3).

Sulfuretin (**5**): orange-yellowish prisms; $C_{15}H_{10}O_5$; MS: 270. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.57 (1H, *d*, *J* = 8.4 Hz, H-5), 7.49 (1H, *d*, *J* = 1.9 Hz, H-5′), 7.26 (1H, *dd*, *J* = 8.3 and 1.9 Hz, H-6′), 6.88 (1H, *d*, *J* = 8.2 Hz, H-2′), 6.69 (1H, *d*, *J* = 1.7 Hz, H-8), 6.66 (1H, *dd*, *J* = 8.5 and 1.7 Hz, H-6), 6.6 (1H, s, H-2). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 182.3 (C-4), 170.8 (C-9), 169.5 (C-7), 149.8 (C-4′), 147.7 (C-3), 147.3 (C-3′), 127.1 (C-6′), 126.0 (C-5), 125.1 (C-1′), 119.4 (C-5′), 117.8 (C-2′), 115.8 (C-10), 113.3 (C-6), 112.6 (C-2), 100.1 (C-8).

Viruses and cell cultures

Two fish pathogenic viruses, infectious hematopoietic virus (IHNV, RtPy91) and viral hemorrhagic septicemia virus (VHSV, Wando2005) were used in the present study. IHNV (RtPy91) and VHSV (Wando2005) were propagated in plastic flasks infecting flounder spleen (FSP) cells and Chinook salmon embryo (CHSE-214) cells at a multiplicity of infection of 0.01 plaque forming unit (PFU)/cell, respectively. After 1 week, when an extensive cytopathic effect (CPE) had occurred, the flasks were shaken to detach the cells. To release the virus, the infected cells were frozen and thawed twice, and stored in aliquots at -80°C. As described previously (Kang *et al.*, 2008; Kim *et al.*, 2011), the FSP cells and CHSE-214 cells were used as the host cells for IHNV and VHSV, respectively, and were cultured using Dulbeco's Modified Essential Medium (Invitrogen, USA), which was supplemented with fetal bovine serum (Invitrogen) at 2% for virus production and 10% for routine cell culture. For routine cell propagation, the cells were incubated at 20°C under normal atmospheric conditions.

Cytotoxicity assay

The cytotoxicity was evaluated by a neutral red uptake assay as described by Thompson (1998) with slight modification (Kim *et al.*, 2011). FSP cells or CHSE-214 cells were seeded onto a 96-well plate with DMEM (without phenol red) at 10^5 cells/well. The next day, medium was removed and the 96-well plates were replaced with medium containing the serially 2-fold diluted test samples (five concentrations) and the cells were further incubated for 48 h at 20°C. The medium was removed and the plates were replaced with 200 μl of medium containing 50 μg/ml neutral red dye (Sigma-Aldrich, USA) and then incubated for 2 h at 20°C. The dye solution was removed and the plates were washed with phosphate buffered saline (PBS) and then added with a solution containing 1% acetic acid in 50% ethanol to extract the dye. The plates were incubated for 10 min in a dark place. The absorbance of colored solution was measured at 540–690 nm with a SpectraMax 340 microplate reader (Molecular Devices, USA) and the growth rate as an index of cytotoxicity was calculated by dividing the test cell's absorbance by the absorbance of the corresponding control cells. The 50% cytotoxic concentration (CC_{50}) was estimated from graphic plots. The maximal non-cytotoxic concentration (MNCC) was determined as the maximal concentration of samples that did not show toxic effect detected by microscopic monitoring. The antiviral activities of samples were tested at less than MNCC.

Anti-viral activity assay

Cytopathic effect (CPE) reduction assay (Kujumgiev *et al.*, 1999) was adopted to evaluate the anti-viral activities of test samples. As described previously (Kim *et al.*, 2011), test samples were first mixed with equal volume of the virus suspension (at a final dose of 100 $TCID_{50}$) and incubated for 1 h at 15°C. The mixture was overlaid to quadruplicate confluent FSP or CHSE-214 cell (10^5 cells/well) monolayers in 96-well plate and the plate was incubated at 15°C for 5–6 days. The virus-induced CPE of the tests was scored by light microscopy using a DIAPHOT 300 (Nikon, Japan) in comparison with the parallel virus control and cell control. The concentration that reduced 50% of CPE with respect to that of virus control was estimated from the plots of the data and was defined as 50% effect concentration (EC_{50}). Ribavirin (Sigma-Aldrich) was used as a positive control drug in the anti-viral study.

Time course study of IHNV and VHSV anti-viral activity of fisetin

The inhibitory effects of fisetin on the replication of IHNV and VHSV following timed addition were examined by a plaque reduction assay (Kamei and Aoki, 2007; Kim *et al.*, 2011). Briefly, monolayer of cells was grown on 24-well plate (1×10^6 cells/well). Duplicate confluent cells were pretreated with fisetin (50 μM) at 15°C for 1 h, washed with PBS and then inoculated with 200 μl virus suspensions containing approximate 80–100 pfu at 15°C for 1 h. Following virus adsorption, the cells were washed twice with PBS and with 1 ml of 0.8% methylcellulose/DMEM with 2% FBS (methylcellulose medium) was overlaid in each well. After incubation for 7 days at 15°C, the infected cells were then fixed with 10% formalin and stained with crystal violet. The

Table 1. **Antiviral activities of methanolic extract and its fractions of *R. verniciflua* bark against IHNV and VHSV**

Fractions	IHNV				VHSV			
	CC_{50}[a]	MNCC[b]	EC_{50}[c]	SI[d]	CC_{50}	MNCC	EC_{50}	SI
80% MeOH extract	150.2±6.1	10.0	10.0±1.0	15.0	180.0±8.0	10.0	9.4±1.0	19.1
n-Hexane	5.3±0.5	1.3	1.3±0.1	4.1	6.0±0.5	1.3	0.9±0.5	6.7
$CHCl_3$	55.8±3.7	2.5	NE[f]	-	57.0±4.0	2.5	1.8±0.8	31.7
EtOAc	73.2±0.2	5.0	5.0±0.1	14.6	79.0±0.5	5.0	3.6±1.5	21.9
n-BuOH	>500.0[e]	50.0	NE[f]	-	>500.0[e]	50.0	37.5±17.7	>13.3
H_2O	>500.0[e]	100.0	NE[f]	-	>500.0[e]	100.0	23.4±18.0	>21.4

Results are presented as the mean values obtained from three independent experiments±standard deviation (S.D.)
[a] Concentration required to reduce cell growth by 50% (μg/ml)
[b] Maximal non-cytotoxic concentration (μg/ml)
[c] Concentration required to inhibit virus-induced cytopathic effect (CPE) by 50% (μg/ml)
[d] Selective index=CC_{50}/EC_{50}
[e] Due to sample's solubility, the higher concentrations could not be tested.
[f] Not effective

numbers of plaques were then counted. The wells overlaid with methylcellulose medium without test sample were used as the cell control. To investigate if fisetin had a direct virucidal effect, IHNV and VHSV were preincubated with an equal volume of fisetin (50 μM) at 15°C for 1 h and then the mixture was inoculated to duplicate confluent cells for 1 h at 15°C. Following virus adsorption, the cells were washed twice with PBS and 1 ml of methylcellulose medium was overlaid in each well. The plate was incubated at 15°C for 7 days and then the plaques were enumerated as mentioned above. Separately, for the timed addition of fisetin, duplicate confluent cells were inoculated with same titer of virus mentioned above at 15°C for 1 h, washed twice with PBS. Then, methylcellulose medium containing fisetin (50 μM) was overlaid on cells immediately after, 4 h after and 22 h after. The plate was incubated at 15°C for 7 days and then the number of plaques was counted as mentioned above.

Statistical analysis

Data were evaluated for statistical significance using one-way ANOVA. P values <0.05 were considered statistically significant.

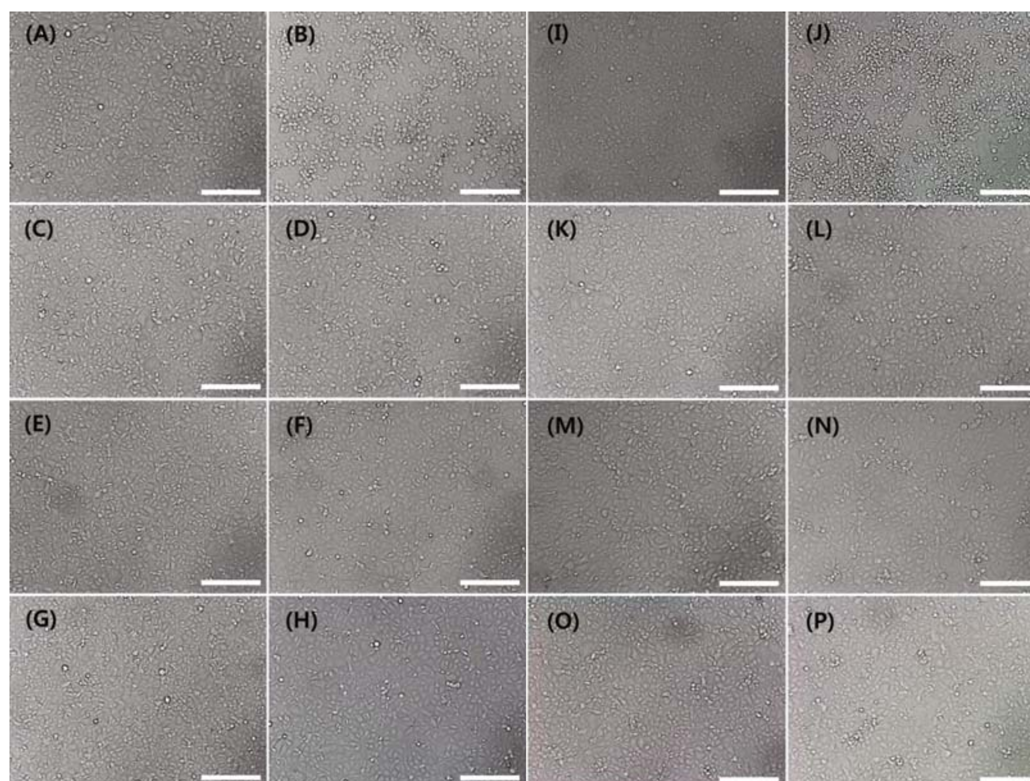

Fig. 2. **Anti-viral effects of 80% methanolic extract of *R. verniciflua* and isolated compounds.** (A and I) Normal FSP and CHSE-214 cells, respectively; (B and J) IHNV and VHSV-infected cells; (C and K) *R. verniciflua* 80% methanolic extract of 10 μg/ml – treated; (D and L) 5 μg/ml EtOAc fraction-treated; (E and M) 200 μM fustin-treated; (F and M) 50 μM fisetin-treated; (G and O) 200 μM sulfuretin-treated; (H and P) 1 μM ribavirin-treated. Scale bar: 200 μm.

Table 2. Antiviral activities of compounds isolated from *R. verniciflua* against IHNV and VHSV

Compounds	IHNV				VHSV			
	CC_{50}[a]	MNCC[b]	EC_{50}[c]	SI[d]	CC_{50}	MNCC	EC_{50}	SI
Methyl gallate (**1**)	370.6±30.1	20.0	NE[g]	-	450.1±20.0	25.0	NE[g]	-
Fustin (**2**)	>1000.0[e] (6%)[f]	200.0	91.2±15.3	>11.0	>1000.0[e] (10%)[f]	200.0	142.0±30.1	>7.0
Fisetin (**3**)	>500.0[e] (43%)[f]	50.0	27.1±2.9	>18.5	>500.0[e] (18%)[f]	100.0	33.3±14.4	>15.0
Butin (**4**)	>500.0[e] (19%)[f]	25.0	NE[g]	-	>500.0[e] (17%)[f]	25.0	NE[g]	-
Sulfuretin (**5**)	>1000.0[e] (23%)[f]	200.0	178.2±37.8	>5.6	>1000.0[e] (14%)[f]	200.0	197.3±3.8	>5.1
Ribavirin	248.0±20.0	1.3	1.0±0.2	248.0	290.0±30.0	1.3	1.2±0.1	241.7

Results are presented as the mean values obtained from three independent experiments±standard deviation (S.D.)
[a] Concentration required to reduce cell growth by 50% (μM)
[b] Maximal non-cytotoxic concentration (μM)
[c] Concentration required to inhibit virus-induced CPE by 50% (μM)
[d] Selective index=CC_{50}/EC_{50}
[e] Due to sample's solubility, the higher concentrations could not be tested.
[f] Average cytotoxicity (%) at the indicated concentrations
[g] Not effective

Results

Anti-viral activity of *R. verniciflua* bark 80% methanolic extract and its fractions against IHNV and VHSV

To obtain the most active fraction from 80% methanolic extract of *R. verniciflua* bark, antiviral activities of its five fractions, n-hexane, $CHCl_3$, EtOAc, n-BuOH, and H_2O fractions against IHNV and VHSV were evaluated using a CPE reduction assay. As shown in Table 1, the 80% methanolic extract of R. verniciflua bark showed significant anti-IHNV and anti-VHSV activities showing EC_{50} values (EC_{50}s) of about 10 μg/ml with selective indices (SIs) more than 15. Among the five fractions, the EtOAc fraction exhibited significant anti-viral activity showing EC_{50}s of 5.0 and 3.6 μg/ml against both IHNV and VHSV, respectively, with similar or increased SIs compared to those of the mother extract, the 80% methanolic extract. As shown in Fig. 2, IHNV and VHSV-infected virus control cells (B and J) showed severe characteristic CPE showing rounded, grape-like cells, while in 10 μg/ml 80% methanolic extract-treated (C and K) and 5 μg/ml EtOAc fraction-treated cells (D and L), CPE was significantly reduced. While the n-hexane fraction showed lower EC_{50}s for both viruses than the EtOAc fraction, its higher cytotoxicity rendered it less effective on anti-viral activity, showing lower SIs than those of the EtOAc fraction. The $CHCl_3$, n-BuOH, and H_2O fractions effectively inhibited virus-induced CPE only in VHSV-infected cells.

Isolation of compounds from the active EtOAc fraction and their anti-viral activity against IHNV and VHSV

The EtOAc fraction, which showed significant anti-viral activities against both IHNV and VHSV, was repeatedly subjected to various column chromatography using ODS and Sephadex LH-20. The procedure yielded five compounds (**1**–**5**, Fig. 1): methyl gallate (**1**) and four flavonoids. The flavonoids were fustin (**2**, a dihydroflavonol), fisetin (**3**, a flavonol), butin (**4**, a flavanone), and sulfuretin (**5**, an aurone) by the direct comparison of their physicochemical and spectroscopic data with those previously reported (Kashiwada *et al.*, 1986; Park *et al.*, 2000; Prachayasittikul *et al.*, 2008).

Compounds **1**–**5** were investigated for their anti-viral activities against IHNV and VHSV. As shown in Table 2, fisetin (**3**) showed relatively high anti-viral activities against both IHNV and VHSV among the compounds tested, showing EC_{50}s of about 30 μM with SIs more than 15. Fustin (**2**) and sulfuretin (**5**) were also effective on anti-viral acvitity against two viruses having EC_{50}s of 90–200 μM with SIs exceeding 5. Ribavirin, which was used as a positive control, showed favourable effects on antiviral activity against IHNV and VHSV with SIs more than 240. As shown in Fig. 2, fustin (200 μM), fisetin (50 μM), and sulfuretin (200 μM) significantly reduced CPE induced by IHNV and VHSV infection by more than 70–80%, with their effects being comparable to that of ribavirin (1 μM) (E–H and M–P). In contrast, methyl gallate (**1**) and butin (**4**) did not show significant anti-viral activity against IHNV and VHSV.

Time course study of anti-viral activities of fisetin against IHNV and VHSV

To investigate the mechanisms of antiviral activity of fisetin, which displayed higher efficacy than other flavonoids, a

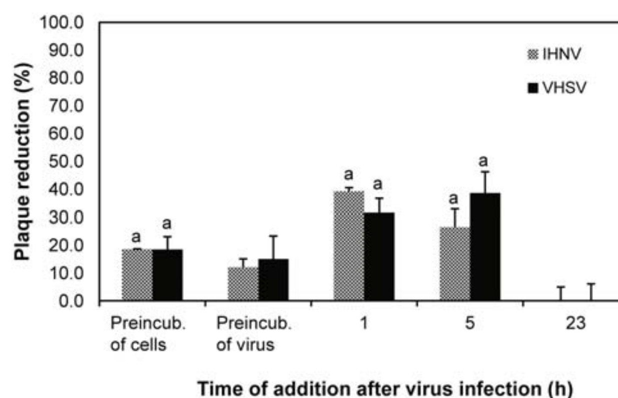

Fig. 3. Time course study of the anti-viral activity of fisetin against IHNV and VHSV. Fisetin (50 μM) was added at the indicated times after viral infection. Preincubation of cells: Cells were pretreated with fisetin (50 μM) at 15°C for 1 h, washed and then inoculated with virus for 1 h. Preincubation of virus: Fisetin (50 μM) was preincubated with virus at 15°C for 1 h and then the mixture was inoculated to cells for 1 h. At 7 days post-infection, virus titer was measured by plaque reduction assay.
[a] significance vs. each virus-infected control group: $P<0.05$

timed addition study of fisetin was performed. As shown in Fig. 3, pre-incubation of viruses with fisetin (50 μM) did not significantly reduce plaque formation, indicating that the anti-viral effects of fisetin were not related with a direct virucidal effect on IHNV and VHSV. The pre-incubation of cells with fisetin (50 μM) 1 h before virus inoculation decreased the plaque formation about 20% for IHNV and VHSV ($P<0.05$). Furthermore, the addition of fisetin up to 5 h post-infection significantly reduced plaque formation by about 30–40% of virus control ($P<0.05$), but not after 23 h following virus inoculation.

Discussion

Recent studies have shown that phytochemicals have great potential to provide efficient antiviral agents against pathogenic viruses for humans. However, few studies have attempted to search for anti-viral agents against fish viruses (Kamei and Aoki, 2007; Naithani *et al.*, 2008; Modak *et al.*, 2010; Kim *et al.*, 2011). In the present study, the 80% methanolic extract of *R. verniciflua* bark and its EtOAc fraction showed efficient anti-viral activities against the fish pathogenic viruses IHNV and VHSV. Although the *n*-hexane fraction displayed the lowest EC_{50} values against two viruses among five fractions from the 80% methanolic extract, its cytotoxicity against FSP and CHSE-214 cells rendered the fraction relatively low in performance, resulting in lower SIs than those of EtOAc fraction. The other three fractions, $CHCl_3$, *n*-BuOH, and H_2O, exerted their anti-viral activities only against VHSV, suggesting that these fractions have the potential to provide compounds for anti-VHSV activity. Although *R. verniciflua* extract has been reported to have various biological activities (Kim *et al.*, 2002, 2010; Choi *et al.*, 2003; Jeon *et al.*, 2003, 2006; Park *et al.*, 2004; Kang, 2005; Lee *et al.*, 2009), its anti-viral activity was reported in only one study (Nam *et al.*, 1996). The latter study reported that the EtOAc fraction of *R. verniciflua* stem inhibited hepatitis B virus DNA replication in HepG2-derived 2.2.15 cells without providing information on the exact compounds responsible for anti-viral activity. Among the five compounds isolated from the active EtOAc fraction in the present study, four were identified as flavonoids (**2**–**5**), except for methyl gallate (**1**). In our study, fustin, fisetin and sulfuretin showed effective anti-viral activities against IHNV and VHSV, while butin did not. Concerning flavonoid structure, fisetin is classified as a flavonol characterized by the presence of OH group on C_3 (Fig. 1) of flavone, fustin as a dihydroflavonol, butin as a flavanone and sulfuretin as an aurone. Based on the structures of the fisetin, fustin and butin flavonoids and their anti-viral activities, we can suggest that OH group on C_3 may play an important role in antiviral activity. This suggestion is supported by previous finding of Inouye *et al.* (1989) that the 3-OH group of flavone is one of the important moieties in inhibiting reverse transcriptase of avian myeloblastosis virus. Flavonoids have been reported to enhance the anti-bacterial, anti-viral or anti-cancer activities of compounds including naringenin, acycloguanosine and tamoxifen. In case of fisetin, it was reported that rutin enhanced its anti-bacterial activities against *Bacillus cereus* and *Salmonella enteritidis* (Arima *et al.*, 2002). This suggests that the three anti-viral flavonoids studied presently in the present study may have some possibility to show different results according to their ratio of combination. This data will be helpful for development of antiviral agents using active fraction such as EtOAc fraction of *R. verniciflua*. In compounds' cytotoxicity assay, only the CC_{50} value of methyl gallate was able to be calculated, while those of the other four flavonoids could not be determined due to their low cytotoxicities in the range of soluble concentrations. As shown in Table 2, four flavonoids were found to have low cytotoxicities showing <50% at their maximal soluble concentrations (500–1,000 μM). Especially, fustin displayed only 6% and 10% cytotoxicity in FSP and CHSE-214 cells, respectively, at 1,000 μM. This suggests that although fustin showed 3–4-fold higher EC_{50}s for IHNV and VHSV than fisetin, its anti-viral efficiency could be same or better than that of fisetin by virtue of its very low cytotoxicities.

Until now, several lines of evidences have shown that fisetin has a wide anti-viral spectrum against various viruses, regardless of whether they are DNA and RNA viruses. Inouye *et al.* (1989) and Chu *et al.* (1992) reported the potent fisetin-mediated inhibition of reverse transcriptases from avian myeloblastosis virus and moloney murine leukemia virus. In addition, fisetin has anti-viral activities against DNA viruses such as herpes simplex virus type 1 (HSV-1) and avian herpes virus, and RNA viruses influenza A virus and human immunodeficiency virus (HIV) with various SIs from 1–40, but the mechanisms of the anti-viral activity was not reported (Konig and Dustmann, 1985; Olivero-Verbel and Pacheco-Londono, 2002; Lyu *et al.*, 2005; Kim *et al.*, 2010b). The presently-reported anti-viral activity of fisetin against fish pathogenic viruses is novel. In contrast to fisetin, there have been few reports on the anti-viral activity of fustin (Malhotra *et al.*, 1996). In the latter study, sulfuretin also showed significant inhibitory activity against tomato ringspot virus. Anti-viral activities of sulfuretin were also reported by Liu *et al.* (2008) and Mercader and Pomilio (2010). Both groups described that sulfuretin showed significant inhibitory activities on neuraminidases from influenza viruses while in cell level, it did not work.

Apoptosis, or programmed cell death, is an important physiological process for host defense against viral infection, and it occurs at the early stage of viral infection, thus limiting viral propagation. To overcome host resistance, many viruses carry anti-apoptotic factors to inhibit apoptosis. However, in some cases, viruses may trigger apoptosis at the late stage of viral replication to facilitate viral release and spread (Ammayappan and Vakharia, 2011). Like many members of the Rhabdoviridae family, IHNV and VHSV induce apoptosis in host cells. Chiou *et al.* (2000) reported that IHNV matrix protein inhibits host-directed gene expression and induces apoptosis in fish cells. Recently, Ammayappan and Vakharia (2011) reported that the NV protein of *Novirhabdovirus* suppresses apoptosis at the early stage of VHSV infection in fish cells showing that NV-deficient and NV-knockout mutant VHSVs induced apoptosis earlier in EPC cells than the wild-type recombinant VHSV by DNA laddering and CPE presented in cells. Several lines

of evidence indicate that flavonoids from *R. verniciflua* including fisetin, fustin and sulfuretin cause apoptotic effects on different cancer cell lines (Jang *et al*., 2005; Ying *et al*., 2012). These results suggest that fisetin, fustin, and sulfuretin may have presently exerted their anti-viral activity by inducing apoptosis of cells. Recently, Yuan *et al*. (2012) reported that flavonols including fisetin significantly inhibited the binding of an oncoprotein E6 of DNA virus human papillomaviruses (HPVs) to FADD and caspase 8. Since the E6 protein has among its functions the ability to prevent apoptosis of infected cells through its binding to FADD and caspase 8, this means that fisetin can exert antiviral activity through proapoptotic effects.

This suggestion was supported by our time course study data of fisetin. Fisetin did not have a significant direct antiviral effect, and incubation of cells with fisetin prior to viral infection inhibited viral replication. Furthermore, the addition of fisetin up to 5 h post-infection significantly reduced plaque formation, but not after 23 h following virus inoculation. These results suggest that the anti-viral mechanism of fisetin may involve the early stages of viral replication. In addition, lower efficacies compared to that of 50 μM fisetin shown in the CPE reduction assay may be due to higher cell number (10^6/well vs. 10^5/well) and higher virus titer (about 300-fold) in the plaque reduction assay. Ammayappan and Vakharia (2011) also showed that in wild-type recombinant VHSV-infected cells, DNA fragmentation was detectable between 20–24 h and completed between 32 and 36 h post-infection. This result explains why fisetin did not show anti-viral activity in cells treated at 23 h following virus inoculation. However, since there are few reports on anti-apoptotic activities of these flavonoids (Lee *et al*., 2002; Park *et al*., 2007) and induction of caspases 3, 8, and 9 by VHSV infection in fish cells were also observed in the study of Ammayappan and Vakharia (2011), we cannot exclude the possibility for flavonoids to exert antiviral activity through their anti-apoptotic activity.

In a previous study (Kang, 2005), we reported that methyl gallate was the major antibacterial compound of the EtOAc fraction of *R. verniciflua* for fish pathogenic Gram-negative bacteria. However, methyl gallate did not show anti-viral activities against the RNA viruses IHNV and VHSV in the present study. A similar phenomenon was found by Kane *et al*. (1988), who reported that methyl gallate inhibited plaque formation of HSV-1 or type 2, but was ineffective against RNA viruses, e.g., vesicular stomatitis virus (VSV) and influenza virus.

In the present study, a synthetic nucleoside, ribavirin, which exhibits anti-viral activity against a broad range of both DNA and RNA viruses *in vitro* as an inhibitor of inosine monophosphate dehydrogenase, was used as a positive control (Jason and Cameron, 2006). In our experimental condition, ribavirin exhibited favourable effects on anti-viral activity in FSP cells infected with IHNV and in CHSE-214 cells infected with VHSV showing SIs more than 240. These results agree with previous data obtained in IPNV-infected RTG-2 cells and in IPNV-infected CHSE-214 cells (Hudson *et al*., 1988; Jashes *et al*., 1996; Kim *et al*., 2011). Although ribavirin has shown very effective anti-vial activities against various fish pathogenic viruses, there are disadvantages related to food safety in the use of antiviral agents for fishes as food sourse. Therefore, flavonoids including fisetin, fustin, and sulfuretin abundant in various food sources could be alternatives for antiviral agents for aquacultures.

At the present time, the application of vaccine-based immunization strategies is very limited, and the use of chemicals is restricted due to their potential harmful impact on the environment and human health. Therefore, although further studies are needed on *in vivo* efficacy, toxicity and the action-mechanisms involved in the antiviral activity, the isolated flavonoids, the fractions or the extract from *R. verniciflua* could be candidates in the development of anti-viral agents and health-promoting feed for fish.

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