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In Vitro Antiviral Activity of Red Alga, *Polysiphonia morrowii* Extract and Its Bromophenols Against Fish Pathogenic Infectious Hematopoietic Necrosis Virus and Infectious Pancreatic Necrosis Virus

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Our previous investigation revealed that 80% methanolic extract of the red alga *Polysiphonia morrowii* has significant antiviral activities against fish pathogenic viruses, infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV). The present study was conducted to identify compounds attributed for its antiviral activities and investigate their antiviral activities against IHNV and IPNV. Activity-guided fractionation for 80% methanolic extract of *Polysiphonia morrowii* using a cell-based assay measuring virus-induced cytopathic effect (CPE) on cells yielded a 90% methanolic fraction, which showed the highest antiviral activity against both viruses among fractions yielded from the extract. From the fraction, two bromophenols were isolated and identified as 3-bromo-4,5-dihydroxybenzyl methyl ether (1) and 3-bromo-4,5-dihydroxybenzaldehyde (2) based on spectroscopic analyses. For both compounds, the concentrations to inhibit 50% of flounder spleen cell (FSP cell) proliferation (CC_{50}) and each viral replication (EC_{50}) were measured. In the pretreatment test, 3-bromo-4,5-dihydroxybenzyl methyl ether (1) and 3-bromo-4,5-dihydroxybenzaldehyde (2) exhibited significant antiviral activities showing selective index values (SI = CC_{50}/EC_{50}) of 20 to 42 against both IHNV and IPNV. In direct virucidal test, 3-bromo-4,5-dihydroxybenzyl methyl ether (1) showed significant antiviral activites against both viruses while 3-bromo-4,5-dihydroxybenzaldehyde (2) was significantly effective against only IHNV. Although antiviral efficacies of both compounds against IHNV and IPNV were lower than those of ribavirin used as a positive control, our findings suggested that the red alga *Polysiphonia morrowii* and isolated two bromophenols may have potential as a therapeutic agent against fish viral diseases.

***Keywords*:** *Polysiphonia morrowii*, antiviral, fish pathogenic viruses, 3-bromo-4,5-dihydroxybenzylmethylether, 3-bromo-4,5-dihydroxybenzaldehyde

The aquaculture industry is an important component of the global high-yield animal protein resources and contributes significantly to the economic base of many countries around the world. But like any other intensive farming activity the threat of viral diseases within an intensive aquaculture system are considered serious given the economic repercussions. To date, a number of diseases have been reported within the fish aquaculture industry (Biering *et al*., 2005). Infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV) are important pathogens of salmonid fish causing serious losses to the rainbow trout- and salmon-farming industries. Outbreaks of infectious hematopoietic necrosis (IHN) result in losses approaching 100%, depending on the species and size of the fish, the virus strain, and environmental conditions (Wolf, 1988). IPNV has been isolated from epizootics in cultured salmonids, and from a variety of aquatic animals in freshwater and seawater environments around the world (Suzuki *et al*., 1998; Reno, 1999; Suzuki and Nojima, 2000; Nishizawa *et al*., 2005). Its acute infections associated with salmonids occur in 1- to 4-month-old fish and may cause mortalities up to 90%, while susceptibility does decreases with increasing age (Samuelsen *et al*., 2006). At the present time, vaccines are used as a preventive measure against these viral diseases; however, their efficacy is not complete. Furthermore, since there are no effective antiviral agents to prevent and treat fish viral diseases, the establishment of effective antiviral agents against fish viral diseases is urgently needed. Marine algae are historically an exceptionally rich source of pharmacologically active metabolites, with antineoplastic, antimicrobial, and antiviral effects (Faulkner, 2000). In the course of our search for antiviral substances against fish pathogenic viruses from marine algae, we found that the 80% methanolic extract of the red alga *Polysiphonia morrowii* Harvey possessed potent anti-IHNV and anti-IPNV activity (Kang *et al*., 2008). *P. morrowii* belongs to the family of Rhodomelaceae and has been known to be distributed in Japan, China, and Korea (Kim *et al.*, 1994). However, little information regarding the biological or phytochemical characteristics of *P. morrowii* has been available to date (Kurihara *et al.*, 1999; Kang *et al*., 2005; Je *et al.*, 2009), and no studies have been conducted to evaluate antiviral activity of fractions or compounds isolated from *P. morrowii* against fish pathogenic viruses.

In this study, we report the isolation and identification of active compounds attributed for its antiviral activities from the extract of *P. morrowii,* and their antiviral activities against

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IHNV and IPNV.

Materials and Methods

General experimental procedure

^{1}H and ^{13}C NMR spectra were run on a BRUKER ULTRASHIELD 400 spectrometer at 400 MHz and 100 MHz, respectively, with TMS as internal standard. EIMS spectra were obtained on a VG Trio II spectrometer. Column chromatographies were performed using YMC-gel ODS-A (YMC Co., Japan) and Sephadex LH-20 (GE Healthcare Bio-Sci, Sweden). Analytical TLC was performed on pre-coated Merck F_{254} silica gel plates and visualized by spraying with anisaldehyde-H_2SO_4.

Viruses and cell cultures

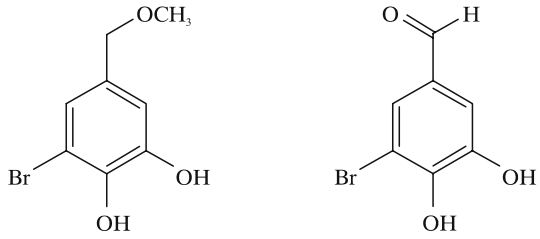
Two fish pathogenic viruses, IHNV (RtPy91) and IPNV (VR299), were used in the present study. The viruses were propagated in plastic flasks infecting flounder spleen (FSP) cells at a multiplicity of infection of 0.01 plaque forming unit (PFU)/cell. After 1 week, when an extensive cytopathic effect (CPE) 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), the FSP was used as the host cells for IHNV and IPNV and cultured using Dulbeco's Modified Essential Medium (Invitrogen Co, USA), which was supplemented with fetal bovine serum (Invitrogen Co) at 2% for virus production and 10% for routine cell culture. For routine cell propagation, the FSP cells were incubated at 20°C under normal atmospheric conditions.

Algal materials

P. morrowii was collected from Yeosu coast in the Republic of Korea in April 2007 and authenticated by Prof. J.-A. Shin (Division of Fisheries and Ocean Sciences, Chonnam National University, Korea). The voucher specimen has been deposited in our laboratory at Chonnam National University.

Extraction and antiviral activity-guided isolation

After cleaning the surface of the thalli to remove visible epiphytes and dirt, *P. morrowii* was freeze-dried and then ground to be used for extraction. We described the detailed processes for the extraction of the alga sample in a previous report (Kang *et al*., 2008). Briefly, 133 g of freeze-dried alga was extracted with three liter of 80% (v/v) methanol (in water, 80% MeOH) at 80°C 8 times, each of which took 1 h for a total of 10 h. The extracted solution was evaporated *in vacuo*. The 80% MeOH extract of *P. morrowii* (33.54 g) was suspended in deionized distilled water and then partitioned with CH_2Cl_2. The CH_2Cl_2 fraction (6.98 g) was evaporated *in vacuo* to be dissolved in 90% (v/v) methanol (in water, 90% MeOH) and then be partitioned with n-hexane (3.95 g). The 90% MeOH-soluble fraction (3.03 g), which showed the most active antiviral acvitities against IHNV and IPNV, was subjected to reversed phase ODS gel column chromatography (200 g) eluted with a stepwise gradient of H_2O-MeOH (9:1 to 1:9) to give 9 fractions (Fraction 1~Fraction 9). Fraction 2 (325 mg) was further chromatographed over Sephadex LH-20 eluting with a 100% MeOH to yield six subfractions. Second subfraction (Fraction 2-2, 180 mg) was further purified by Sephadex LH-20 eluting with MeOH-Acetone (1:9), respectively, to yield compound 1 (24.9 mg) and compound 2 (46.9 mg). The chemical structures of compounds **1** and **2** were elucidated as 3-bromo-4,5-dihydroxybenzyl methyl ether (**1**) and 3-bromo-4,5-dihydroxybenzaldehyde (**2**) by spectroscopic analyses and comparison with the published data (Kurihara *et al*., 1999; Han *et al*., 2005) (Fig. 1).



3-bromo-4,5-dihydroxybenzyl methyl ether (**1**)

3-bromo-4,5-dihydroxybenzaldehyde (**2**)

Fig. 1. Chemical structures of compounds **1** and **2** isolated from *P. morrowii*.

Cytotoxicity assay

The cytotoxicity was evaluated by neutral red uptake assay as described by Thompson (1998) with slight modification. FSP 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 media containing the serially 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, USA) and then incubated for 2 h at 20°C. The dye solution was removed and the plates were washed with PBS and then added with a solution containing 1% acetic acid in 50% ethanol to extract the dye and the plates were incubated for 10 min in a dark place. The absorbance of colored solution was measured at 540-690 nm with a microplate reader (SpectraMax 340, 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 antiviral activities of 80% methanolic extract of *P. morrowii*, its fractions and isolated compounds test were tested at non-cytotoxic concentrations.

CPE reduction assay

CPE (cytopathic effect) reduction assay (Kujumgiev *et al*., 1999) was adopted to evaluate the antiviral activities of 80% methanolic extract of *P. morrowii* and its fractions. In brief, for pretreatment test, quadruplicate confluent FSP cell monolayers (10^5 cells/well) in 96 well plate were overlaid with equal volume of 2× test samples and then incubated at 15°C for 24 h. The medium was removed from the plates and then the cell monolayers were infected with diluted virus suspension containing100 $TCID_{50}$ (50% tissue culture infective dose) of the virus stock. To act as the virus control and cell control, the virus suspension and maintenance medium without samples were added, respectively. The plate was incubated at 15°C for 5-6 days. The virus-induced CPE of the tests was scored under light microscopy in comparison with the parallel virus control and cell control. The concentration that reduced 50% of CPE in respect to that of virus control was estimated from the plots of the data and was defined as 50% effect concentration (EC_{50}). For direct virucidal test, test samples were first mixed with equal volume of the virus suspension (at final dose, 100 $TCID_{50}$) and incubated for 1 h at 15°C. The mixture was overlaid to quadruplicate confluent FSP cell monolayers in 96 well

Table 1. Antiviral activities of *P. morrowii* methanolic extract and its fractions against IHNV and IPNV

Test materials	IHNV					IPNV			
		Pretreatment test		Direct virucidal test		Pretreatment test		Direct virucidal test	
	CC_{50}[a]	EC_{50}[b]	SI[c]	EC_{50}	SI	EC_{50}	SI	EC_{50}	SI
80% methanolic extract	>1250[d]	68.0±8.4	>18.4	10.0±5.9	>125	>100[e]	–	100.0±6.2	>12.5
n-Hexane fraction	121±2	9.0±1.7	13.4	14.0±1.0	8.6	25.0±6.7	4.8	8.0±3.0	15.1
90% MeOH fraction	1267±113	14.0±0.4	90.5	5.0±0.7	253.4	21.0±14.7	60.3	6.0±5.7	211.2
H_2O fraction	>625[d]	>100[e]	–	>100[e]	–	>100[e]	–	>100[e]	–

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

plate and the plate was incubated at 15°C for 5-6 days and then CPE was scored as mentioned above.

Plaque reduction assay

The inhibitory effects of compounds isolated from 90% MeOH fraction of *P. morrowii* on the replication of IHNV and IPNV in FSP cells were studied by the plaque reduction assay, which was performed according to the method of Kamei and Aoki (2007) with slight modifications. Briefly, monolayer of FSP cells was grown on 24 well plate (2×10^6 cells/well). For pretreatment test, triplicate confluent FSP cell monolayers in 24 well plate were overlaid with equal volume of 2× test samples and then incubated at 15°C for 24 h. The medium was removed from the plates and the cells were washed with fresh medium, and then infected with the virus suspension of approximate 80-100 plaque-forming units (PFU)/well and incubated for 1 h at 15°C. Following virus adsorption, the cells were washed twice with fresh medium and 1 ml of 0.8% methylcellulose/DMEM with 2% FBS 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 numbers of plaques were then counted. The wells overlaid with methylcellulose medium without test sample were used as the cell control. The percentage of inhibition of plaque formation was calculated as follows: [(mean number of plaques in virus control)-(mean number of plaques in sample)]×100/(mean number of plaques in virus control). The values of EC_{50}, which was the concentration of test sample required to inhibit up to 50% of virus growth as compared with the virus control group, were estimated from the graphic plots of the data. Ribavirin (Sigma) was used as a positive control drug in the antiviral study. For direct virucidal test, test samples were first mixed with equal volume of the virus suspension and incubated for 1 h at 15°C. Each mixed virus suspension was overlaid to triplicate confluent FSP cell monolayers in 24 well plate and the plate was incubated for 1 h at 15°C. Following virus adsorption, the cells were washed twice with fresh medium and 1 ml of 0.8% methylcellulose/DMEM with 2% FBS was overlaid in each well. The plate was incubated at 15°C for 7 days and then the number of plaques was counted as mentioned above.

Results and Discussion

Antiviral activity of *P. morrowii*'s 80% methanolic extract and its fractions against IHNV and IPNV

To obtain the most active fraction from 80% methanolic extract of *P. morrowii*, antiviral activities of its three fractions, n-hexane, 90% MeOH and H_2O fractions against IHNV and IPNV were evaluated using CPE reduction assay. As shown in Table 1, the 80% methanolic extract of *P. morrowii* showed significant anti-IHNV activities in pretreatment and direct virucidal test, while showing relatively lower antiviral activities against IPNV. Among the three fractions, 90% MeOH fraction exhibited the most active antiviral activities against IHNV and IPNV, showing significantly increased SI values of 60 to 250 compared to those of the mother extract (the 80% methanolic extract). It is noteworthy that the 90% MeOH fraction showed significant antiviral activity when it was pretreated before viral infection as well as directly inhibiting viral infectivity or adsorption to cells. These results suggest that the compounds of 90% MeOH fraction may interact with or persist locally on the FSP cell surfaces. A similar profile of antiviral activity has been found with the rabbit antimicrobial peptide, a-defensin 1 (NP-1), which protected cells *in vitro* from infection by herpes simplex virus type2 (HSV-2) (Sinha *et al*., 2003). *n*-Hexane fraction also effectively inhibited virus-induced CPE in IHNV-infected and IPNV-infected cells showing EC_{50} values ranging from 8 to 25 μg/ml. However, its cytotoxicity for FSP cells was over ten-fold of that of the 90% MeOH fraction and its SI values of antiviral activities against two viruses turned out to be much lower than those of the 90% MeOH fraction.

Antiviral activity of two bromophenols against IHNV and IPNV

For bromophenols 3-bromo-4,5-dihydroxybenzyl methyl ether (1) and 3-bromo-4,5-dihydroxybenzaldehyde (2) isolated from the active fraction, the 90% MeOH fraction of *P. Morrowii*, their antiviral activities against IHNV and IPNV were evaluated through the plaque reduction assay. As shown in Table 2, 3-bromo-4,5-dihydroxybenzyl methyl ether (1) showed comparable EC_{50} values of 19.0 to 27.0 μM against IHNV and IPNV irrespective of treatment condition. In contrast, 3-bromo-4,5-dihydroxybenzaldehyde (2) exhibited comparable antiviral activites against IHNV in both treatment while it did not show antiviral activity against IPNV in the direct virucidal activity test. These results suggest that although the two bromophenols are very similar in their chemical structures, they may exert on IHNV and IPNV through different mechanisms. Here, it was observed that compared to high SI values (60.3 to 253.4) of the 90% MeOH fraction against IHNV and IPNV, SI values (15.7 to 48.7) of two bromophenols against both viruses became so much lower than those. However, considering

Table 2. Antiviral activities of isolated compounds against IHNV and IPNV

Test compounds	IHNV					IPNV			
		Pretreatment test		Direct virucidal test		Pretreatment test		Direct virucidal test	
	CC_{50}[a]	EC_{50}[b]	SI[c]	EC_{50}	SI	EC_{50}	SI	EC_{50}	SI
3-Bromo-4,5-dihydroxybenzyl methyl ether (**1**)	926±153	27.0±6.3	34.3	19.0±4.1	48.7	22.0±0.6	42.1	26.0±8.5	35.6
3-Bromo-4,5-dihydroxybenzaldehyde (**2**)	1175±154	45.0±9.1	26.1	75.0±14.2	15.7	57.0±10.6	20.6	>100[d]	–
Ribavirin	475±11	2.0±0.4	237.5	2.6±1.5	182.7	4.0±0.4	118.8	>50[d]	–

Results are presented as the mean values obtained from three independent experiments±standard deviation (SD)
[a] Concentration required to reduce cell growth by 50% (μM)
[b] Concentration required to inhibit virus-induced CPE by 50% (μM)
[c] Selective index=CC_{50}/EC_{50}
[d] due to sample's cytotoxicity, the higher concentrations could not be tested.

different experimental conditions of the CPE reduction assay which was used for the assessment of antiviral activities of the extract and fractions of *P. morrowii* and the plaque reduction assay for measuring isolated compounds' antiviral activities, it is not thought to be reasonable to directly compare SI values from two different assays. In the CPE reduction assay of the present study, 96 well plate-seeded cultures were used and each well's cell number was 1×10^5/well. They were infected with virus suspension containing 100 $TCID_{50.}$ Whereas, in plaque reduction assay, 24 well plate-seeded cultures with 2×10^6 cells /well were used. And the virus suspension used in this experiment yielded 80-100 PFU/well, which meant that the viral load used in the plaque reduction assay was almost 300 times higher than that used in CPE reduction assay of the present study. Thus, it can be suggested that 300 times higher viral load and 20 times higher cell numbers in the plaque reduction assay than those in the CPE reduction assay required the higher efficient concentrations and then the increases in EC_{50} values caused the decreases of SI values in isolated compounds' antiviral activities. The similar phenomena can be found in other reports. For example, in Hsuan *et al*. (2009), indirubin (an active compound from *Isatis indigotica*) at 100 μg/ml showed the inhibitory activity of 80% against pseudorabies virus-induced CPE in 96 well cultured porcine kidney cells which had 2×10^4 cells/well and were infected with virus suspension containing 50 $TCID_{50}$ in CPE inhibition assay, whereas in plaque reduction assay in which 24 well-plate seeded cultures with 1.6×10^5 cells/well were infected with the virus suspension of 100 PFU/well, indirubin at the same concentration showed the inhibitory activity of only 20%. We also found that two bromophenols showed 2 times to 6 times lower EC_{50} values against IHNV and IPNV in the CPE reduction assay than in the plaque reduction assay (our unpublished data). These results suggest that two bromophenols isolated are among active compounds of the 90% MeOH fraction of *P. morrowii* although we cannot rule out that there can be more active compounds. Until now, only a handful number of studies have addressed biological activities of 3-bromo-4,5-dihydroxybenzyl methyl ether (1) and 3-bromo-4,5-dihydroxybenzaldehyde (2) (e.g. Shoeib *et al*., 2004; Han *et al*., 2005; Li *et al*., 2008). According to the literature, 3-bromo-4,5-dihydroxybenzyl methyl ether (1) shows moderate cytotoxicity in some cancer cell-lines while 3-bromo-4,5-dihydroxybenzaldehyde (2) exhibits cytotoxicity in some cancer cell-lines as well as antioxidative activities such as DPPH radical scavenging. In contrast, the literature is abundant of studies that report biological activities of bromophenols such as antioxidative, antibacterial, anti-inflammatory, feeding-deterrent, and antimutagenic activities (e.g. Wall *et al*., 1989; Wiemer *et al*., 1991; Kurata *et al*., 1997; Xu *et al.,* 2003; Li *et al.,* 2008). They are also known to inhibit a variety of enzymes including phospholipase A_2, 15-lipoxygenase, inosine monophosphate dehydrogenase, guanosine monophosphate and α-glucosidase (Wiemer *et al*., 1991; Chen *et al.*, 1994; Fu *et al*., 1995; Kurihara *et al*., 1999). As for antiviral activity, the literature appears to be scarce. Park *et al*. (2005) reported that 2,3,6-tribromo-4,5 dihydroxybenzyl methyl ether (TDB) isolated from *Symphyocladia latiuscula,* belonging to the family of Rhodomelaceae, exhibited antiviral activity against herpes simplex virus (HSV-1) *in vitro* and in mice.

In the present study, a synthetic nucleoside, ribavirin, which is known to exhibit antiviral activity against a broad range of both DNA and RNA viruses *in vitro* as an inhibitor of inosine monophosphate (IMP) dehydrogenase, was used as a positive control (also see Jason and Cameron, 2006). In our experimental condition, ribavirin exhibited favourable effects on antiviral activity in FSP cells infected with IHNV and IPNV with SI values of about 200 to 350. 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). Among literature cited above, Chen *et al.* (1994) and Fu *et al*. (1995) reported that some polybrominated bromophels significantly inhibited IMP dehydrogenase. Considering the broad antiviral activities *in vitro* of ribavirin as an inhibitor of IMP dehydrogenase, it is suggested that this activity could be an antiviral action-mechanisms of the two isolated bromophenols against IHNV and IPNV, although further studies on mechanismm of action of isolated two bromophenols are needed.

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. Therefore, although further studies are needed on *in vivo* toxicity, and the action-mechanisms involved in the antiviral activity, the isolated bromophenols, or extracts from *P. morrowii* could be candidates in the development of antiviral agents and health-promoting feed for fish.

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