Antiviral Activity of Seaweed Extracts against Feline Calicivirus

**Kyoung-Lan Kim[1], Dae-Sung Lee[2], Mi-Sun Park[1], Sung-Hwan Eom[3],
Keun-Sik Lim[3], Jong-Soon Kim[4], Dong-Ho Lee[5], Chang-Keun Kang[2],
Young-Mog Kim[3] and Myung-Suk Lee[1]***

[1]*Department of Microbiology, Pukyong National University, Busan 608-737, Korea*
[2]*POSTECH Ocean Science and Technology Institute, Pohang University of Science and
Technology (POSTECH), Pohang 790-784, Korea*
[3]*Department of Food Science & Technology, Pukyong National University,
Busan 608-737, Korea*
[4]*NGETECH Inc., Busan 618-818, Korea*
[5]*Children's Dietary Life Safety Division, Korea Food and Drug Administration,
Seoul 122-704, Korea*

Norovirus, which causes gastroenteritis in humans, is an important food-borne pathogen worldwide. In an effort to discover an antiviral substance against norovirus, extracts from several seaweeds were evaluated for antiviral activity against feline calicivirus (FCV), which was used as a surrogate. The methanolic extract of *Undaria pinnatifida* exhibited the most significant antiviral activity and virucidal efficacy against FCV. The concentrations of the extract that reduced viral replication by 50% (EC_{50}) and resulted in the death of 50% of the host cells (CC_{50}) were 0.05 mg/mL and 1.02 mg/mL, respectively. The selectivity index, calculated from the ratio of the CC_{50} and EC_{50} was 20.4. No FCV infection of host cells occurred following a 1-h incubation in the presence of 12.50 mg/mL *U. pinnatifida* extract, indicating that the virus was completely inactivated by the extract treatment. The results obtained in this study will contribute to the development of a natural antiviral substance that will prevent food-borne disease caused by norovirus.

Key words: Antiviral activity, Feline calicivirus, Food-borne pathogens, Norovirus, Seaweed

Introduction

Norovirus, previously known as "Norwalk-like virus" or "small round-structured virus," comprises a diverse group of non-enveloped RNA viruses of the family Caliciviridae (Widdowson et al., 2005). Norovirus causes epidemic gastroenteritis in humans and is considered one of the most important food-borne pathogens in the world (Lopman et al., 2004; Bull et al., 2006); norovirus may be responsible for more than 80% of nonbacterial gastroenteritis reported to the Centers for Disease Control and Prevention (Fankhauser et al., 2002; Widdowson et al., 2005). Norovirus transmission occurs through the feces or vomit of infected patients and person-to-person contact; ingestion of contaminated water, or raw or minimally cooked foods such as seafood, fruits, and vegetables; and via airborne droplets. In addition, norovirus has a high attack rate owing to a very low infective dose; 10-100 viral particles are needed to produce an active infection (Fankhauser et al., 2002; Blackburn et al., 2004; Lopman et al., 2004; Bull et al., 2006). Norovirus has seasonal characteristics and is prevalent in the winter months, but its recent occurrence in the summer has caused public health concerns (Lopman et al., 2004). Since norovirus cannot be grown in tissue culture, disinfection efficacy and inactivation studies have been performed using closely related viruses such as the feline calicivirus (FCV) (Bidawid et al., 2003). As a result, norovirus is known to survive freezing, temperatures as high as 60°C, and low pH, and to be highly resistant to common disinfectants such as 70% ethanol and 1,000–5,000 ppm chlorine (Steinmann, 2004). Chemical disinfectants used to control the virus are corrosive and irritants; as consumer awareness of food safety issues increases, natural disinfectants must be de-

*Corresponding author: leems@pknu.ac.kr

veloped to replace these chemical compounds.

Terrestrial microorganism-based discovery programs have been used over several decades to identify drugs from natural sources. Recently, attempts have been made to isolate novel bioactive metabolites from marine resources. Seaweeds exhibit potent antitumor (Faulkner, 2002; Topcu et al., 2003; Blunt et al., 2006), antiviral (Santos et al., 1999; Mazumder et al., 2002; Barbosa et al., 2004), antifungal (Kubanek et al., 2003), antimicrobial (Fenical and Paul, 1984; Mayer et al., 1993; Deslandes et al., 2000), and other pharmaceutical activities (Kang et al., 2005; Moo-Puca et al., 2008). In this study, we screened seaweed extracts for antiviral activities against norovirus using FCV as a surrogate.

Materials and Methods

Sample preparation

Seaweeds used in this study were purchased from a commercial market located in Gijang, Busan, Korea, in March 2009 (Table 1). Each sample was dried at 40°C for 2 days and finely powdered. Each powdered sample (100 g) was extracted twice with 1 L of 70% methanol at 80°C for 3 h, and filtered. The combined filtrate was concentrated by rotary evaporation at 40°C. Concentrated extracts were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/mL and used for further study.

Table 1. Natural seaweeds used in this study

Scientific name	Commercial name	Materials
Ecklonia cava	Kajime	stem
Ecklonia stolonifera	gom pi	stem
Laminaria japonica	sea tangle	stem
Undaria pinnatifida	sea mustard	stem

Cells and FCV

Crandall-Reese feline kidney cells (CrFK cells, ATCC CCL-94) and FCV (ATCC VR-782), a surrogate for norovirus, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). FCV was proliferated in CrFK cells and used to test antiviral and virucidal activities. CrFK cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO/BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO/BRL) and 100 U/mL penicillin-100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in 75 cm^2 tissue culture flasks and incubated at 37°C in 5% CO_2. Cell monolayers at 80-90% confluence were subcultured every 2 days (Fig. 1).

FCV was propagated in monolayers of CrFK cells to prepare a virus pool; CrFK cells were incubated at 37°C in 5% CO_2 for 120 min to allow for FCV adsorption, and 10 mL DMEM supplemented with 2% FBS was added to promote virus propagation. Virus-induced cytopathic effects (CPEs) were seen in nearly 90% of the CrFK monolayers following incubation at 37°C in 5% CO_2 for 16-24 h (Fig. 1). The virus was harvested by freeze-thawing three times followed by the removal of cell debris via centrifugation (10 min, 1,800×g, 4°C). The FCV suspension was stored at -80°C in 0.5 mL aliquots until use.

MTT assay

CrFK cells were seeded in 96 well plates at 1.0×10^4 cells/well and cultured for 48 h until confluence. They were treated with various concentrations of seaweed extracts and incubated for 24 h before cell viability assays were performed; cells treated with an equal volume of maintenance medium were controls. Cell cytotoxicity was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in which mitochondrial succinate dehydrogenase activity converts the tetrazolium salt (MTT) to the dark blue formazan. The culture medium was aspirated and 100 µL of MTT solution (2 mg/mL) was added to each well and incubated for 4 h. Following aspiration, the dark blue formazans were dissolved in DMSO (Kanto Chemical Co., Tokyo, Japan) and the absorbance of each well was measured at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The median cellular cytotoxicity concentration (CC_{50}) was the concentration of the extracts that resulted in the death of 50% of the CrFK cells.

Plaque reduction assay

The antiviral activity of seaweed extracts against FCV was determined by the plaque reduction assay. CrFK cells were cultured in 6-well plates at 4.0×10^5 cells/mL as described above. Growth medium was aspirated from each well, a FCV suspension with 200 plaque forming units (PFU) per well was inoculated into the CrFK cells, and the inoculated plates were incubated for 120 min at 37°C in 5% CO_2. After infection by the virus, 4 mL of overlay medium, which consisted of equal volumes of 1.5% agarose and 2×DMEM containing serial dilutions of the seaweed extracts, was added to each well. The plates were left at room temperature to ensure overlay solidification. Once completely cooled, plates were
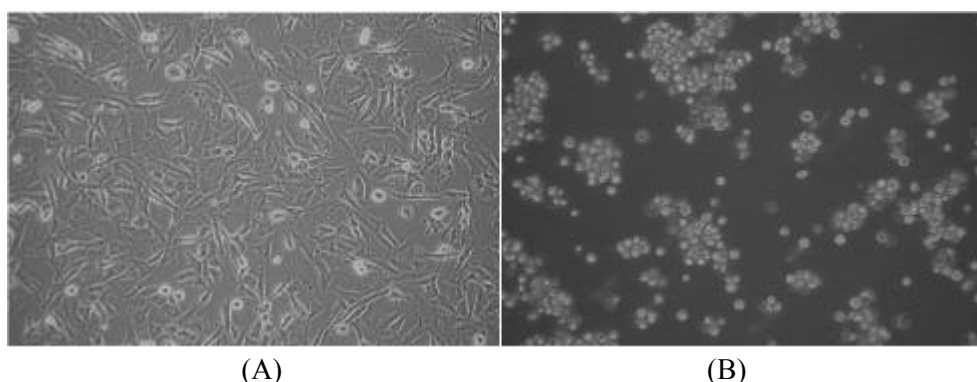
Fig. 1. Inverted microscopic photograph of normal Crandell-Reese Feiline kidney (CrFK) CCL-94 cells (A) and CrFK cells infected by feline calicivirus (B).

incubated at 37°C for 24-48 h in 5% CO_2. The monolayers were fixed for a minimum of 4 h with 3.7% formaldehyde. Overlay plugs were removed with a gentle stream of cold running tap water aimed at the edge of the well. The fixed cell monolayers were stained with 0.1% crystal violet solution for 20 min. Plaques were counted to determine the virus titers. Antiviral effective concentration was expressed as an EC_{50}, defined as the concentration of the sample required to inhibit virus-induced CPEs by 50% (Yoon et al., 2000; Bidawid et al., 2003).

Virucidal assay

The virus titer was estimated by the Reed-Muench method (Mahy and Kangro, 1996; Kampf et al., 2005); the virus infectivity titer was expressed as a $TCID_{50}$, defined as the dose at which 50% of the tissue culture was infected. The virucidal activities of the seaweed extracts against FCV were determined using serial dilutions to determine the TCID50. Briefly, 500 μL of FCV suspension (10^5 PFU/mL) was exposed to 500 μL of serially diluted seaweed extracts at room temperature for different times (3, 5, 24 h), after which 100 μL of mixture was diluted into 900 μL of maintenance medium. The diluted mixture was added to a monolayer of CrFK cells in a 96-well plate, incubated at 37°C for 3-4 days in 5% CO_2, and stained with crystal violet solution. The cultures were monitored for CPEs, and $TCID_{50}$ titers were determined with the endpoint dilution method. Untreated controls were virus suspension with maintenance medium instead of extract.

Results and Discussion

Cytotoxicity test

Cytotoxicity of each seaweed extract to CrFK cells was determined by the MTT assay. Serially diluted seaweed extracts (diluted six times by the two fold serial dilution method; maximum concentration 2.5 mg/mL) were added to wells containing CrFK cells. The amount of formazan crystal in the presence or absence of extract gave a measure of the cyto-toxicity of each extract. The seaweed extracts used in this study showed different cytotoxicities to CrFK cells (Table 2). The *Ecklonia stolonifera* extract was the least cytotoxic (CC_{50}=2.43 mg/mL) and the *Laminaria japonica* extract was the most cytotoxic (CC_{50}=0.49 mg/mL). The *Ecklonia cava* and *Undaria pinnatifida* extracts had relatively low cytotoxicities (CC_{50}=1.82 and 1.02 mg/mL, respectively).

Antiviral activity of seaweed extracts

Antiviral activities of the seaweed extracts against FCV were measured by the plaque reduction assay and expressed as EC_{50} values, defined as the concentrations of the extracts that inhibited 50% of virus replication. The plaque formation caused by FCV in CrFK cells decreased as the concentrations of the seaweed extracts increased (Fig. 2). The extract of *U. pinnatifida* showed the most antiviral activity; the EC_{50} value at which the number of plaques decreased by 50% compared to the control was 0.05 mg/mL. Selectivity index (SI) for *U. pinnatifida*, cal-culated from the CC_{50} and EC_{50} values, was 20.4 (Table 2). This suggests that the *U. pinnatifida* extract could be a suitable antiviral agent for use against FCV. The extract of L. japonica also strongly inhibited plaque formation by FCV at low concentrations (0.17 mg/mL). However, the SI value of the *L. japonica* extract was the lowest of the four seaweed extracts as it was highly cytotoxic to the host cells. In this system, the *L. japonica* extract was not a suitable antiviral agent against FCV. The extracts of *E. cava* and *E. stolonifera* also exhibited low SI values although they were higher than that of the *L. japonica*

Table 2. Antiviral activity of methanolic extracts from seaweeds against feline calicivirus by plaque reduction assay

Seaweeds	CC_{50}[a] (mg/mL)	EC_{50}[b] (mg/mL)	SI[c]
Ecklonia cava	1.82	0.39	4.67
Ecklonia stolonifera	2.43	0.60	4.05
Laminaria japonica	0.49	0.17	2.88
Undaria pinnatifida	1.02	0.05	20.4

[a]50% cytotoxic concentration (CC_{50}) is the concentration of the 50% cytotoxic effect.
[b]50% effective concentration (EC_{50}) is the concentration of the sample required to reduce plaque formation of virus by 50%.
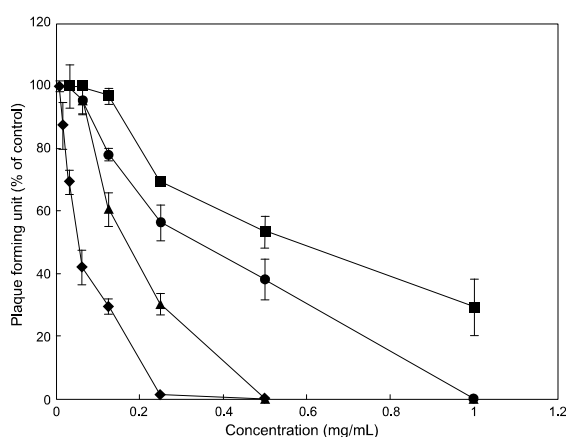[c]Selecticity Index (SI) = CC_{50}/EC_{50}.

Fig. 2. Antiviral activity of seaweed extracts against feline calicivirus (FCV). FCV was infected into Crandall-Reese Feline kidney cells and incubated in the presence or absence of various concentration of seaweed extracts. Antiviral activity was expressed as a comparative inhibitory ratio to inhibit virus-induced cytopathic effect, plaque, compared with control cells. This experiment was performed as triplicate. Each value is the result of mean±S.E. ●, *Ecklonia cava* extract; ■, *E. stolonifera* extract; ▲, *Laminaria japonica* extract; ◆, *Undaria pinnatifida* extract.

extract.

Virucidal efficacy

In CrFK cells infected with FCV, CPEs occur (Fig. 1); these may be reduced by an antiviral substance. We measured the virucidal effects of the seaweed extracts against FCV by mixing serially diluted extracts with a FCV suspended solution for different times and infecting CrFK cells with the reaction mixture. Virucidal activities of the seaweed ex-tracts against FCV were expressed as log $TCID_{50}/mL$ values and were dose- and time-dependent (Table 3).

Table 3. Virucidal activity of methanolic extracts from seaweeds against feline calicivirus by $TCID_{50}$ reduction assay

Medical seaweeds	Concentration (mg/mL)	log $TCID_{50}/mL$			
		0 time	1 hr	3 hr	5 hr
Ecklonia cava	12.50	5.00	4.17	2.40	neg[*]
	06.25	5.00	4.83	3.00	2.17
	03.13	5.00	4.83	3.67	2.50
	01.56	5.00	5.00	4.17	3.83
Ecklonia stolonifera	12.50	5.00	4.83	4.27	3.27
	06.25	5.00	4.67	4.33	3.50
	03.13	5.00	5.00	4.27	3.67
	01.56	5.00	5.00	4.83	4.67
Laminaria japonica	12.50	5.00	2.00	neg	neg
	06.25	5.00	3.00	neg	neg
	03.13	5.00	3.17	2.17	neg
	01.56	5.00	4.83	4.83	4.17
Undaria pinnatifida	12.50	5.00	neg	neg	neg
	06.25	5.00	3.00	neg	neg
	03.13	5.00	3.00	neg	neg
	01.56	5.00	3.67	2.27	neg
Control	-	5.00	5.00	5.00	5.00

TCID50, 50% tissue culture infectious dose; *neg, feline calicivirus are inactivated.

U. pinnatifida extract exhibited the strongest virucidal efficacy against FCV and seemed to inhibit the infectivity of FCV; no viral infection of CrFK cells was observed after 1, 3, and 5 h in the presence of 12.50, 6.25, 3.13, and 1.56 mg/mL of *U. pinnatifida* extract. *L. japonica* extract also exhibited strong virucidal efficacy against FCV. FCV was completely inactivated when incubated with 6.25 mg/mL and 12.50 mg/mL *L. japonica* for over 3 h, and with 3.13 mg/mL for over 5 h (Table 3); FCV was not completely inactivated at 1.56 mg/mL *L. japonica* extract. Virucidal efficacy of the *E. cava* extract against FCV was limited compared to those of *U. pinnatifida* and *L. japonica* even though the extract exhibited some virucidal activity. *E. stolonifera* extract did not completely inactivate FCV under our experimental conditions, even though a 2-log decrease in the viral $TCID_{50}/mL$ value occurred after treatment for 5 h at 12.50 mg/mL.

An antiviral effect is assumed when a 4-log decrease occurs in the viral $TCID_{50}/mL$ value (Bellamy, 1995). Extracts of *L. japonica* and *U. pinnatifida* caused a 5-log decrease in viral $TCID_{50}/mL$ values at ≥ 3.13 mg/mL, indicating that these extracts are effective agents that can be used to inhibit infection of host cells by FCV. A comparison of the SI values of *U. pinnatifida* (20.4) and *L. japonica* (2.88) suggest that the *U. pinnatifida* extract

is more useful than that of *L. japonica* for controlling infection of host cells by FCV (Table 2). The virucidal efficacies of *E. cava* and *E. stolonifera* extracts were negligible.

We propose that natural antiviral substances can be used in place of chemical compounds for the effective control of norovirus. We investigated the antiviral activities of seaweeds against FCV as a surrogate for norovirus, and found that extracts of *U. pinnatifida* and *L. japonica* exhibited outstanding antiviral effects. Fucoidan is a polysaccharide derived from *U. pinnatifida* and *L. japonica*, which possesses anti-tumor, antiviral, and anticoagulant activities, and is effective against viruses like the herpes simplex virus and the human immunodeficiency virus (Hudson et al., 1998; Kang et al., 2005); future research will confirm if fucoidan also inactivates FCV. Our investigations characterizing the in vitro inactivation of FCV by natural compounds will contribute to the development of a natural antiviral substance for the prevention of food-borne diseases caused by noro-virus.

Acknowledgments

References

Barbosa JP, Pereira RC, Abrantes JL, Dos Santos CC, Rebello MA, Frugulhetti IC and Teixeira VL. 2004. In vitro antiviral diterpenes from the Brazilian brown alga *Dictyota pfaffii*. Planta Med 70, 856-860.

Bellamy K. 1995. A review of the test methods used to establish virucidal activity. J Hosp Infect 30, 389-396.

Bidawid S, Malik N, Adegbunrin O, Sattar SA and Farber JM. 2003. A feline kidney cell line-based plaque assay for feline calicivirus, a surrogate for Norwalk virus. J Virol Methods 107, 163-167.

Blackburn BG, Craun GF, Yoder JS, Hill V, Calderon RL, Chen N, Lee SH, Levy DA and Beach MJ. 2004. Surveillance for waterborne-disease outbreaks associated with drinking water-United States. 2001-2002. MMWR Surveillance Summaries 53, 23-45.

Blunt JW, Copp BR, Munro MHG, Northcote PT and Prinsep MR. 2006. Marine natural products. Nat Prod Rep 23, 26-78.

Bull RA, Tu ETV, McIver CJ, Rawlinson WD and White PA. 2006. Emergence of a new norovirus genotype II. 4 variant associated with global outbreaks of gas-troenteritis. J Clin Microbiol 44, 327-333.

Deslandes E, Pondaven P, Auperin T, Roussakis C, Guezennec J, Stiger V and Payri C. 2000. Preliminary study of the in vitro anti-proliferative effect of a hydroethanolic extract from the sub-tropical seaweed *Turbinaria ornata* (Turner) J. Agardh on a human non-small-cell bronchopulmonary carcinoma line (NSCLC-N6). J Appl Phycol 12, 257-262.

Fankhauser RL, Monroe SS, Noel JS, Humphrey CD, Bresee JS, Parashar UD, Ando T and Glass RI. 2002. Epidemiologic and molecular trends of "Norwalk-like viruses" associated with outbreaks of gastroenteritis in the Unites States. J Infect Dis 186, 1-7.

Fenical W and Paul VJ. 1984. Antimicrobial and cytotoxic terpenoids from tropical green algae of the family *Udoteaceae*. Hydrobiologia 116/117, 135-140.

Hudson JB, Lee MK, DeWreede RE and Hong YK. 1998. Antiviral compounds in extracts of Korean seaweeds: Evidence for multiple activities. J Appl Phycol 10, 419-505.

Kampf G, Grotheer D and Steinmann J. 2005. Efficacy of three ethanol based hand rubs against feline calici-virus, a surrogate virus for norovirus. J Hosp Infect 60, 144-149.

Kang SY, Oh MJ and Shin JA. 2005. Antimicrobial activities of Korean marine algae against fish pathogenic bacteria. J Fish Pathol 18, 147-156.

Kubanek J, Jensen PR, Keifer PA, Sullards MC, Collins DO and Fenical W. 2003. Seaweed resistance to microbial attack: a targeted chemical defense against marine fungi. Proc Natl Acad Sci USA 100, 6916-6921.

Lopman B, Vennema H, Kohli E, Pothier P, Sanchez A, Negredo A, Buesa J, Schreier E, Reacher M, Brown D, Gray J, Iturriza M, Gallimore C, Bottiger B, Hedlund KO, Torven M, von Bonsdorff CH, Maunula L, Poljsak-Prijatelj M, Zimsek J, Reuter G, Szucs G, Melegh B, Svennson L, van Duijnhoven Y and Koopmans M. 2004. Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant. Lancet 363, 682-688.

Mahy BWJ and Kangro HO. 1996. Virology Method Manual. Academic Press, London, UK, 41-43.

Mayer AMS, Paul VJ, Fenical W, Norris JN, De Carvalho MS and Jacobs RS. 1993. Phospholipase A2 inhibitors from marine algae. Hydrobiologia 260/261, 521-529.

Mazumder S, Ghosal PK, Pujol CA, Carlucci MJ, Damonte EB and Ray B. 2002. Isolation, chemical investigation and antiviral activity of polysaccharides from *Gracilaria corticata* (Gracilariaceae, Rhodo-phyta). Int J Biol Macromol 31, 87-95.

Moo-Puca R, Robledoa D and Freile-Pelegrina Y. 2008. Evaluation of selected tropical seaweeds for in vitro

anti-trichomonal activity. J Ethnopharmacol 120, 92-97.

Santos MGM, Lagrota MHC, Miranda MMFS, Yoneshingue-Valentin Y and Wigg MD. 1999. A screening for the antiviral effect of extracts from Brazilian marine algae against acyclovir resistant Herpes Simplex virus type 1. Bot Mar 42, 227-230.

Steinmann J. 2004. Surrogate viruses for testing virucidal efficacy of chemical disinfectants. J Hosp Infect 56, 49-54.

Topcu G, Anydoqmus Z, Imre S, Goren AC, Pezzuto JM, Clement JA and Kingston DG. 2003. Brominated sesquiterpenes from the red alga *Laurencia obtusa*, J Nat Prod 66, 1505-1508.

Widdowson MA, Sulka A, Bulens SN, Beard SR and Chaves SS. 2005. Norovirus and foodborne disease, United States. 1991-2000. Emerg Infect Dis 11, 95–102.

Yoon JC, Cho JJ, Yoo SM and Ha YM. 2000. Antiviral activity of ascorbic acid against herpes simplex virus. J Korean Soc Microbiol 35, 1-8.