

Phaeophyta Extracts Exhibit Antiviral Activity against Feline Calicivirus

Yuri Choi^{1,2}, Eunjung Kim², Sunyoung Moon³, Jong-Duck Choi^{4,5}, Myung-Suk Lee¹ and Young-Mog Kim^{3*}

¹Department of Microbiology, Pukyong National University, Busan 608-737, Korea

²LG Electronics, Changwon, 641-711, Korea

³Department of Food Science & Technology, Pukyong National University, Busan 608-737, Korea

⁴Department of Seafood Science and Technology, Gyeongsang National University, Tongyeong 650-160, Korea

⁵Institute of Marine Industry, Gyeongsang National University, Tongyeong 650-160, Korea

Abstract

The objective of this study was to evaluate the antiviral activity of Phaeophyta extracts against feline calicivirus (FCV), used as a norovirus surrogate. A bioassay-guided cytotoxicity and virus infectivity assay revealed that methanolic extracts of Phaeophyta possessed significant antiviral activity against FCV. Among them, *Eisenia bicyclis* extract exhibited the highest antiviral activity against FCV. The 50% effective concentration of the extract (EC₅₀) inhibiting FCV viral replication by 50% was 80 µg/mL. The extract also showed the highest selectivity index, calculated from the ratio of the median cellular cytotoxicity concentration (CC₅₀) and EC₅₀, indicating antiviral efficacy against FCV. In addition, significant interruption of FCV infection was observed by pretreatment of host Crandall-Reese feline kidney cells with the *E. bicyclis* extract (200 µg/mL) prior to virus infection, in a dose-dependent manner.

Key words: Antiviral activity, Feline calicivirus, Norovirus surrogate, Phaeophyta

Introduction

In recent decades, norovirus has been reported to cause mass food poisoning (Food Safety and Infection Service, 2009). According to the US Centers for Disease Control and Prevention, ~54 million people develop illness induced by norovirus annually in the United States, and 96% of viral gastroenteritis occurs secondary to the virus (Cheek et al., 2002; Koopmans and Duizer, 2004). In Korea, the first epidemic of food poisoning caused by norovirus occurred in 1999, and norovirus was designated a contagious disease in 2006 (Jee et al., 1999; Ministry of Health and Welfare, 2006). Norovirus can infect the human body through drinking water or food, and is then released into the environment via the vomit or feces. Next, it is transmitted by the ingestion of groundwater and

food contaminated with norovirus through the fecal-oral route (Fretz et al., 2005).

Most recent studies of norovirus utilized a norovirus surrogate because norovirus is not cultivatable in laboratory conditions, and appropriate experimental protocols have not been established (Duizer et al., 2004). In this study, a feline calicivirus (FCV) was used as a cultivatable norovirus surrogate (Kim et al., 2009, 2010). Norovirus reportedly shows resistance to several sanitizers (Liu et al., 2010; Park et al., 2010; Centers for Disease Control and Prevention, 2011). Moreover, chemical sanitizers can induce various side effects, such as fever and itching, in humans. Therefore, the development of effective and eco-friendly disinfectants against norovirus is necessary

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*Corresponding Author

E-mail: ymkim@pknu.ac.kr

(Malik et al., 2006). Some disinfectant substances originating from natural sources are effective against bacteria and viruses. However, no effective disinfectant against norovirus has been reported. We have focused on Phaeophyta, a potential antimicrobial additive and functional ingredient (Cho et al., 1990; Smit, 2004; Kim et al., 2010). In this study, we reported the antiviral activity of Phaeophyta extracts against norovirus using FCV as a surrogate.

Materials and Methods

Sample preparation

Phaeophyta samples were obtained from a commercial market located in Busan, Korea- in September 2012. Each sample was dried at 40°C for 2 days and then finely powdered. Each powder (100 g) was exhaustively extracted with aqueous 90% methanol (v/v) at 70°C for 3 h. The methanolic extract was filtered and concentrated by rotary evaporation at 40°C. The concentrated extract was dissolved in dimethyl sulfoxide to a concentration of 10 mg/mL.

Cells and virus

Crandall-Reese feline kidney cells (CRFK cells, ATCC CCL-94) and FCV (ATCC VR-782) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). CRFK cells were grown in Dulbecco's modified Eagle's medium (Gibco/BRL, Green Island, NY, USA) as described by Kim et al. (2010). FCV was propagated in CRFK cells and harvested by freeze-thawing. The virus suspension was stored at -80°C until use.

Cytotoxicity assay

The cytotoxicity of Phaeophyta extracts was determined by quantifying the viability of CRFK cells using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described by Kim et al. (2010). The median cellular cytotoxicity concentration (CC₅₀) that resulted in the death of 50% of CRFK cells was determined by MTT assay.

Antiviral assay

The antiviral activity of Phaeophyta extracts against FCV was evaluated in terms of the tissue culture infectivity dose 50 (TCID₅₀), as described by Kim et al. (2010). The antiviral effective concentration was expressed as the EC₅₀ value, defined as the concentration of the sample required to inhibit virus-induced cytopathogenic effects (CPEs) by 50% (Bidawid et al., 2003). In brief, the FCV suspension (log 5 TCID₅₀ per mL) was treated with identical volumes of serially diluted extracts at room temperature for 24 h. The mixtures were added to a

monolayer of CRFK cells in a 96-well plate. After incubating at 37°C for 2 to 3 days, the cells were stained with crystal violet solution and monitored for CPEs. Untreated controls were suspended with maintenance medium instead of extract. The selectivity index (SI) was calculated as the ratio of CC₅₀ to EC₅₀ for each compound. The SI indicates the efficacy of antiviral activity. A higher SI means greater antiviral activity with low cellular toxicity, as well as higher commercialization potential (Oh et al., 2013).

Pretreatment effect of Phaeophyta extracts prior to FCV infection

CRFK cells were treated with Phaeophyta extracts and incubated for 24 h at 37°C prior to virus infection. The effect of Phaeophyta extract on FCV infection was then evaluated in terms of the TCID₅₀ values, as described above.

Results and Discussion

Antiviral activity of Phaeophyta extracts against FCV

Our previous study suggested that some seaweeds exhibit antiviral activity against FCV (Kim et al., 2010). In this study, we evaluated the antiviral activity of Phaeophyta methanolic extracts (Table 1). The cytotoxicity of the methanolic extracts against host CRFK cells was first determined by MTT assay and reported as the CC₅₀ value. As shown in Table 1, *Ecklonia cava* showed the lowest cytotoxicity (620 µg/mL) among the six Phaeophyta species tested. *Ecklonia stolonifera*, *Eisenia bicyclis*, *Undaria pinnatifida*, and *Laminaria japonica* also showed relatively low cytotoxicity levels against CRFK cells, ranging from 300 to 450 µg/mL. The methanolic extract of *Sargassum fulvellum* exhibited the highest cytotoxicity. The cytotoxicities of the *E. cava*, *E. stolonifera*, *L. japonica*, and

Table 1. Antiviral activity of Phaeophyta methanolic extracts against feline calicivirus

Phaeophyta	CC ₅₀ [*] (µg)	EC ₅₀ (µg)	SI
<i>Ecklonia cava</i>	620	210	2.95
<i>Ecklonia stolonifera</i>	450	340	1.32
<i>Laminaria japonica</i>	300	1,000	0.3
<i>Eisenia bicyclis</i>	410	80	5.13
<i>Undaria pinnatifida</i>	388	> 1,000	NA ^d
<i>Sargassum fulvellum</i>	80	100	0.8

NA, not applicable.

^{*}50% cytotoxic concentration (CC₅₀) is the concentration of the 50% cytotoxic effect,

[†]50% effective concentration (EC₅₀) is the concentration of the sample required to reduce plaque formation of virus by 50%,

[‡]Selectivity index (SI) = CC₅₀/EC₅₀.

U. pinnatifida extracts were slightly lower than those reported previously (Kim et al., 2010). This difference may be due to harvesting during a different season or the different origin of the raw materials (Arunkumar and Sivakumar, 2012). Interestingly, the cytotoxicity of Phaeophyta extracts against CRFK cells was higher than that of territorial plant extracts, including those of *Allium scorodorpasum*, *Capsicum annuum*, *Hovenia dulcis*, and *Citrus aurantium* (Kim et al., 2009; Oh et al., 2013), suggesting that the Phaeophyta extracts exert a more negative effect on cell adherence and growth compared with extracts of medicinal herbs and spices.

The antiviral activities of Phaeophyta extracts against FCV were measured by TCID₅₀ assay, and expressed as EC₅₀ values. The *E. bicyclis* extract showed the lowest EC₅₀ value (80 µg/mL), indicating that this extract exhibits the highest anti-FCV activity, followed by *S. fulvellum*, *E. cava*, *E. stolonifera*, and *L. japonica* (Table 1). The antiviral activity of Phaeophyta extracts was then evaluated to determine the SI. The *E. bicyclis* extract showed the highest SI (5.13), suggesting marked antiviral activity and low cytotoxicity, followed by *E. cava*, *E. stolonifera*, *S. fulvellum*, and *L. japonica*. However, these results are not consistent with our previous finding that the *U. pinnatifida* extract exhibited the highest SI (Kim et al., 2010). This discrepancy may be a result of differences in the raw materials used in terms of harvest season and location, as described above (Kulanthayesu and Sadaiyappa, 2012). In addition, the SI of Phaeophyta extracts were similar to those of territorial plant extracts (with the exception of *Camelia sinensis*), ranging from 0.53 to 6.29 (Oh et al., 2013).

Protective effect of Phaeophyta extracts on FCV infection

To evaluate the protective effect of Phaeophyta extracts on FCV infection, CRFK cells were treated with various concentrations of methanolic extracts prior to virus infection. As shown in Fig. 1, FCV infectivity was completely interrupted by treatment with 200 µg/mL *E. bicyclis* and *E. stolonifera* extracts. *E. cava* extract also exhibited a strong protective effect at 500 µg/mL. However, no significant protective effect against FCV infection resulted from treatment with *L. japonica*, *S. fulvellum*, or *U. pinnatifida*. Therefore, we concluded that *Eisenia bicyclis* may inhibit norovirus infection.

Several reports of protection against viral infection have been published. Cho et al. (2013) reported a protective effect of red ginseng extract against vaginal herpes simplex virus infection, which was mediated by promotion of host defence systems. In addition, Carlucci et al. (2004) reported a protective effect of carrageenans against genital herpes simplex virus infection, which was mediated by interruption of viral replication. Although the precise mechanism of the protective effect against FCV infection has not yet been defined, our results suggested that *E. bicyclis* extract inhibits viral penetration or replication, as has been reported previously (Carlucci

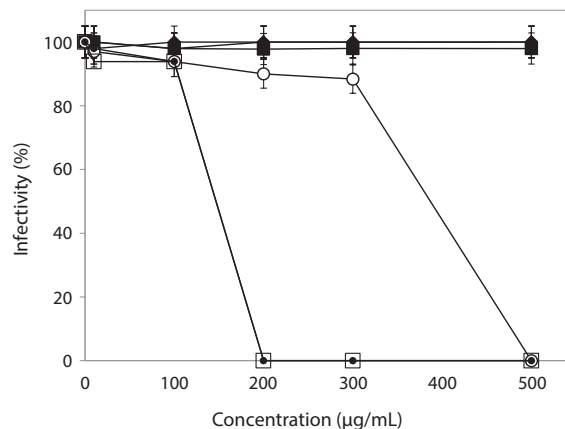


Fig. 1. Protective effect of Phaeophyta extracts on feline calicivirus (FCV) infection. CRFK cells were treated with Phaeophyta extracts for 24 h prior to FCV infection. Antiviral activity was determined by TCID₅₀ assay and the infectivity ratio was compared to control. ○, *Ecklonia cava*; ●, *Ecklonia stolonifera*; □, *Eisenia bicyclis*; ■, *Laminaria japonica*; ◇, *Sargassum fulvellum*; ◆, *Undaria pinnatifida*.

et al., 2004; Cho et al., 2013). To address this issue, further studies should elucidate the mechanism underlying the protective effect of *E. bicyclis* against FCV infection.

To our knowledge, this is the first report of the protective effect of Phaeophyta extract against infection with a norovirus surrogate. Our results will contribute to the development of a food contact sanitizer effective against norovirus because Phaeophyta extracts can be used as a food additive.

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