In vitro antiviral activity of dieckol and phlorofucofuroeckol-A isolated from edible brown alga *Eisenia bicyclis* against murine norovirus

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This research was conducted to develop effective and safe marine-derived antiviral compounds against norovirus. The ethyl acetate (EtOAc)-extract from *Eisenia bicyclis* exhibited strong antiviral activity against murine norovirus (MNV) as a norovirus surrogate. Among the phlorotannins from *E. bicyclis*, dieckol (DE) and phlorofucofuroeckol-A (PFF) were known to possess the strongest antibacterial activity. In this study, DE and PFF were evaluated for antiviral activity against MNV. DE and PFF exhibited strong anti-MNV activity with 50% effective concentration (EC₅₀) of 0.9 μM. However, PFF exhibited more effective antiviral activity against MNV with higher selective index (668.87) than that of DE (550.60), due to its lower cell toxicity against RAW 264.7. This is the first report on the anti-MNV activity of phlorotannins from seaweed. The results obtained in this study suggest that the phlorotannins could be used as a potential source of natural antiviral agents.

**Key Words:** antiviral activity; *Eisenia bicyclis*; murine norovirus; phlorofucofuroeckol-A; phlorotannins

**INTRODUCTION**

Norovirus, a genus in the family Caliciviridae, is a group of non-enveloped viruses that have a single-stranded, positive sense RNA genome (Atmar 2010). Norovirus infection can usually be caused by contaminated water or food, and it spreads via human contact with infected materials through the fecal-oral route (Choi et al. 2014). This infection has been recognized as a leading cause of epidemics with the symptoms of vomiting, diarrhea, mild fever, abdominal cramping, and nausea in the community (La Rosa et al. 2013). According to the Centers for Disease Control and Prevention, norovirus is the leading cause of outbreaks of viral gastroenteritis in the United States. It is estimated that norovirus is responsible for over 21 million illnesses and 45,000 hospitalizations annually. The potential for widespread and severe outbreaks of norovirus highlights the need for effective antiviral therapies.

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Control and Prevention (2011), over 70% of water-related gastroenteritis patients and over 50% of patients due to food-consumption resulted from norovirus infection in USA. Likewise, norovirus causative issues have been a great concern over the world during the recent years.

Norovirus has the characteristics such as low infectious dose, prolonged shedding period, strong stability, great diversity, and frequent genome mutations (Lee et al. 2014b). Norovirus could be effectively reduced by using disinfectants such as alcohols and povidone iodines (Belliot et al. 2008). However, chemical sanitizers can cause various side effects in humans, such as fever and itching (Choi et al. 2014). Recently, attempts have been made to find sustainable solutions from medicinal plants and marine organisms (Balunas and Kinghorn 2005, Jain et al. 2008, Choi et al. 2014). Among marine organisms, chitosan and chitosan oligosaccharides were reported to be able to effectively reduce the infectivity of human enteric viral surrogates (feline calicivirus [FCV] F-9) (Su et al. 2009, Davis et al. 2012). However, there are limitations in the progress of this study, due to inefficient cell culture amplification process for norovirus (Guix et al. 2007, Lay et al. 2010). Recent studies revealed that the structure and genetic relatedness of murine norovirus (MNV) to human norovirus makes this virus a promising and relevant surrogate for studying the environmental survivability of human norovirus (Cannon et al. 2006, Zhang et al. 2012).

In this study, we investigated the possibility of using the marine alga *Eisenia bicyclis* extract and its ingredients as an alternative antiviral agent against MNV. The brown algae have also been reported to exhibit several antimicrobial activities against pathogenic bacteria and FCV (Eom et al. 2013a, Choi et al. 2014, Lee et al. 2014a). Since inadequate scientific research findings are available on the antiviral activity from marine organisms against MNV, the present study with MNV as a norovirus surrogate may have great contribution to the development of effective antiviral agents to control human norovirus.

**MATERIALS AND METHODS**

In the late September of 2013, *E. bicyclis* was purchased from Ulleung Trading Co. (Ulleung, Korea). A voucher specimen was refrigerated at -80°C. Dried *E. bicyclis* was triturated into powder with electronic grinder (HMF-1000A; Hanil Electronics, Seoul, Korea). The dried *E. bicyclis* powder (1.0 kg) was extracted with methanol (MeOH; 10 L × 3) at 68°C for 3 h (3 times), and the solvent was evaporated using rotary evaporator (Eyela Co., Tokyo, Japan) under vacuum at 45°C. The combined crude MeOH extract (164.3 g) was suspended in 10% MeOH (1.0 L), and then it was fractionated in turn with n-hexane (Hexane; 1.0 L × 3), dichloromethane (DCM; 1.0 L × 3), ethyl acetate (EtOAc; 1.0 L × 3), and n-butanol (BuOH; 1.0 L × 3) solution, to yield Hexane-soluble extract (42.3 g), DCM-soluble extract (2.5 g), EtOAc-soluble extract (23.8 g), BuOH-soluble extract (26.5 g), and H2O-soluble extract (69.1 g).

Phlorofucofuroeckol-A (PFF) Fig. 1A and dieckol (DE) Fig. 1B were isolated from the EtOAc-soluble extract of *E. bicyclis* by using Sephadex LH-20 and RP-18 open column chromatography, as a part of previous research, along with several other phloroglucinol derivatives (Eom et al. 2013b) (Fig. 1).

RAW 264.7 cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, Grand Island, NY, USA) with 1% penicillin (100 U mL⁻¹; Gibco BRL)-streptomycin (100 μg mL⁻¹; Gibco BRL) and heat inactivated 10% fetal bovine serum (Gibco BRL).
RESULTS AND DISCUSSION

In recent years, norovirus has gained a lot of attention concerning the management of food safety, since 50% of all outbreaks related to food-poising were caused by norovirus across the nations (Centers for Disease Control and Prevention 2011). Although various biological activities of marine organism have been reported, there are still many unknown potentials that could be utilized. We previously reported that *E. bicyclis* methanolic extract exhibit antiviral activity against FCV (Choi et al. 2014). However, as described above, FCV may not be the most suitable surrogate for evaluating the efficacy of disinfectants against human norovirus (Cannon et al. 2006, Zhang et al. 2012). Therefore, the antiviral activity of *E. bicyclis* extracts against MNV as a norovirus surrogate was evaluated. Cytotoxicity of *E. bicyclis* extracts was evaluated by determining CC<sub>50</sub> value using RAW 264.7 cells. It has been proposed the following cutoff points to determine a cytotoxicity of plant extracts toward normal cells: 1) significant or strong cytotoxicity: <100 μg mL<sup>-1</sup>; 2) moderate cytotoxicity: 100 μg mL<sup>-1</sup> to <300 μg mL<sup>-1</sup>; 3) low cytotoxicity: 300 μg mL<sup>-1</sup> to <1,000 μg mL<sup>-1</sup>; 4) no cytotoxicity: >1,000 μg mL<sup>-1</sup> (Kuete and Efferth 2015). Thus, the MeOH extract and its solvent-soluble extracts showed no severe cytotoxicity towards RAW 264.7 cells with the CC<sub>50</sub> values of 322.48 to 2,146.42 μg mL<sup>-1</sup> (Table 1). The cytotoxicity of *E. bicyclis* methanolic extract against RAW 264.7 cells was higher than that of our previous report using CrFK cells (CC<sub>50</sub> = 410) (Choi et al. 2014). This result could be due to the difference in toxic susceptibility of host cells to the methanolic extract. Antiviral activity of *E. bicyclis* extracts, inhibiting viral infection of host cells, was evaluated by measuring EC<sub>50</sub> value as described in MATERIALS AND METHODS. *E. bicyclis* methanolic extract showed an antiviral activity against MNV in RAW 264.7 cells with EC<sub>50</sub> value of 42.34 μg mL<sup>-1</sup> (Table 1). It has been reported that *E. bicyclis* methanolic extract exhibit EC<sub>50</sub> value of 80 μg mL<sup>-1</sup> against FCV (Choi et al. 2014). Then, the antiviral activity of the methanolic extract was evaluated by SI analysis, as described above. It is desirable to have a high SI giving maximum antiviral activity, with minimal cell toxicity as well as higher possibility of application (Oh et al. 2013). The SI value of *E. bicyclis* methanolic extract against MNV is 22.27, while SI value of the same extract against FCV is 5.13 (Choi et al. 2014). Thus, *E. bicyclis* methanolic extract exhibits higher inhibiting effectiveness against MNV infection in host cells, compared to that of FCV. In addition, the SI value is similar to that of green tea (*Camellia sinensis*) methanolic extract with SI value of 18.57 against FCV.
and PFF inhibited the infection of MNV in RAW 264.7 cells with an EC\textsubscript{50} value of 0.90 μM. Consequently, the calculated SI values of DE and PFF were 550.60 and 668.87, respectively, which is suggesting that PFF can more effectively inhibit MNV infection in host cells compared to that of DE (Table 2). Compared to epigallocatechin gallate (a catechin from green tea) with SI value of 26.67 against FCV (Oh et al. 2013), the present study showed SI values of DE and PFF against MNV were about 20 and 25 times higher, respectively, than that of epigallocatechin gallate. This finding clearly highlights the existence of potential antiviral compounds against MNV in edible brown alga E. bicyclis.

Recent studies have revealed the antiviral effect of plant-derived compounds against MNV (Li et al. 2013). However, to our knowledge, there has been no previous report on marine-derived polyphenols exhibiting antiviral activity against MNV. Choi et al. (2014) reported that E. bicyclis methanolic extract possess an anti-norovirus activity which inhibits FCV infection in CrFK cells with EC\textsubscript{50} value at the concentration of 80 μg, but MNV belongs to the human norovirus unlike FCV. In addition, MNV is demonstrated as enteric pathogens of mice and humans, respectively, whereas FCV infects via nasal, oral, and con-

<table>
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<tr>
<th>Solvent-soluble extracts</th>
<th>CC\textsubscript{50} (μg mL\textsuperscript{-1})\textsuperscript{a}</th>
<th>EC\textsubscript{50} (μg mL\textsuperscript{-1})\textsuperscript{b}</th>
<th>SI\textsuperscript{c}</th>
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<tbody>
<tr>
<td>MeOH\textsuperscript{d}</td>
<td>936.67 ± 55.08c</td>
<td>42.34 ± 3.45c</td>
<td>22.27 ± 3.09c</td>
</tr>
<tr>
<td>Hexane</td>
<td>1,103.33 ± 106.04b</td>
<td>302.71 ± 20.01a</td>
<td>3.64 ± 0.11d</td>
</tr>
<tr>
<td>DCM</td>
<td>667.67 ± 54.54d</td>
<td>179.49 ± 13.78b</td>
<td>3.72 ± 0.02d</td>
</tr>
<tr>
<td>EtOAc</td>
<td>504.64 ± 38.13e</td>
<td>3.99 ± 0.28d</td>
<td>127.17 ± 18.72a</td>
</tr>
<tr>
<td>BuOH</td>
<td>322.48 ± 22.18f</td>
<td>20.03 ± 1.31d</td>
<td>16.20 ± 2.21cd</td>
</tr>
<tr>
<td>H\textsubscript{2}O</td>
<td>2,146.42 ± 155.49a</td>
<td>44.40 ± 3.32c</td>
<td>48.35 ± 0.45b</td>
</tr>
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\textsuperscript{a}CC\textsubscript{50}: mean (50%) value of cytotoxic concentration.
\textsuperscript{b}EC\textsubscript{50}: mean (50%) value of effective concentration.
\textsuperscript{c}SI: selective index, CC\textsubscript{50} / EC\textsubscript{50} - 1.

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<th>Compounds</th>
<th>CC\textsubscript{50} (μM)\textsuperscript{a}</th>
<th>EC\textsubscript{50} (μM)\textsuperscript{b}</th>
<th>SI\textsuperscript{c}</th>
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<tr>
<td>Dieckol</td>
<td>495.71 ± 36.96</td>
<td>0.90 ± 0.06</td>
<td>550.60 ± 6.09</td>
</tr>
<tr>
<td>Phlorofucofuroeckol-A</td>
<td>579.02 ± 22.57</td>
<td>0.90 ± 0.07</td>
<td>668.87 ± 73.06</td>
</tr>
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</table>

\textsuperscript{a}CC\textsubscript{50}: mean (50%) value of cytotoxic concentration.
\textsuperscript{b}EC\textsubscript{50}: mean (50%) value of effective concentration.
\textsuperscript{c}SI: selectivity index, CC\textsubscript{50} / EC\textsubscript{50} - 1.
junctival routes (Radford et al. 2007, Howell and D’Souza 2013). MNV has been increasingly used as a surrogate for human norovirus in virucidal efficacy evaluations (Howell and D’Souza 2013). Our results provide evidence that the phlorotannins (DE and PFF) could provide a way to develop effective marine-derived antiviral agents against human norovirus. However, further antiviral experiments should be performed to delineate how phlorotannins deactivate MNV and the mechanism of action. Generally, although it is not fully understood, the antiviral action mechanism of natural compounds against human norovirus or its surrogates is usually supposed to be the prevention of the viral attachment to host cells (Howell and D’Souza 2013).

CONCLUSION

The EtOAc-soluble extract from *E. bicyclis* exhibited the strongest antiviral activity against MNV among five solvent-soluble extracts. The antiviral activity of EtOAc-soluble extract against MNV may also be correlated with their marine-derived polyphenolic components. Therefore, previously isolated phlorotannins, such as DE and PFF, were evaluated for antiviral activity against MNV. DE and PFF demonstrated strong anti-MNV activity with low cytotoxicity against RAW 264.7 cells. The results of the present investigation are expected to contribute to the development of an alternative phytotherapeutic agent against MNV, as natural disinfectant for the improvement in food safety.

ACKNOWLEDGEMENTS

This research was a part of the project titled ‘Development of manufactured goods and oyster, Crassostrea gigas free from norovirus’, funded by the Ministry of Oceans and Fisheries, Korea. Graduate students were supported by the special fund of Pukyong National University donated by the SKS Trading Co. in Lynnwood, WA, USA in memory of the late Mr. Young Hwan Kang, who had inspiration and a deep concern for fishery science.

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