



Research article

Antiviral activity of ginsenosides against coxsackievirus B3, enterovirus 71, and human rhinovirus 3



Jae-Hyoung Song^{1,☆}, Hwa-Jung Choi^{2,☆}, Hyuk-Hwan Song³, Eun-Hye Hong¹, Bo-Ra Lee¹, Sei-Ryang Oh³, Kwangman Choi⁴, Sang-Gu Yeo⁵, Yong-Pyo Lee⁵, Sungchan Cho^{4,*}, Hyun-Jeong Ko^{1,**}

¹ College of Pharmacy, Kangwon National University, Chuncheon, Korea

² Department of Beauty Science, Kwangju Women's University, Gwangju, Korea

³ Natural Medicine Research Center, Korea Research Institute Bioscience and Biotechnology, Chungcheongbuk-do, Korea

⁴ Targeted Medicine Research Center, Korea Research Institute Bioscience and Biotechnology, Chungcheongbuk-do, Korea

⁵ Division of Vaccine Research, Center for Infectious Diseases, National Institute of Health, Korea Centers for Diseases Control and Prevention, Chungcheongbuk-do, Korea

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ABSTRACT

Background: Ginsenosides are the major components responsible for the biochemical and pharmacological actions of ginseng, and have been shown to have various biological activities. In this study, we investigated the antiviral activities of seven ginsenosides [protopanaxatriol (PT) type: Re, Rf, and Rg2; protopanaxadiol (PD) type: Rb1, Rb2, Rc, and Rd] against coxsackievirus B3 (CVB3), enterovirus 71 (EV71), and human rhinovirus 3 (HRV3).

Methods: Assays of antiviral activity and cytotoxicity were evaluated by the sulforhodamine B method using the cytopathic effect (CPE) reduction assay.

Results: The antiviral assays demonstrated that, of the seven ginsenosides, the PT-type ginsenosides (Re, Rf, and Rg2) possess significant antiviral activities against CVB3 and HRV3 at a concentration of 100 µg/mL. Among the PT-type ginsenosides, only ginsenoside Rg2 showed significant anti-EV71 activity with no cytotoxicity to cells at 100 µg/mL. The PD-type ginsenosides (Rb1, Rb2, Rc, and Rd), by contrast, did not show any significant antiviral activity against CVB3, EV71, and HRV3, and exhibited cytotoxic effects to virus-infected cells. Notably, the antiviral efficacies of PT-type ginsenosides were comparable to those of ribavirin, a commonly used antiviral drug.

Conclusion: Collectively, our findings suggest that the ginsenosides Re, Rf, and Rg2 have the potential to be effective in the treatment of CVB3, EV71, and HRV3 infection.

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1. Introduction

The Picornaviridae are currently divided into nine genera, three of which (*Hepatoviruses*, *Rhinoviruses*, and *Enteroviruses*) are causative agents of human diseases [1]. *Enteroviruses* such as coxsackievirus, poliovirus, and echovirus are small, nonenveloped

viruses possessing a single-stranded RNA genome in positive orientation that acts directly as mRNA in infected cells. *Enteroviruses* are of high clinical relevance with coxsackievirus B3 (CVB3), which can cause heart-muscle infection, being an important member. In addition, Enterovirus 71 (EV71) is a causative agent of hand, foot, and mouth disease and herpangina that

* Corresponding author. Targeted Medicine Research Center, Korea Research Institute Bioscience and Biotechnology, 30-Yeongudanji-ro, Ochang-eup, Cheongwon-gun, Chungcheongbuk-do 363-883, Korea.

** Corresponding author. College of Pharmacy, Kangwon National University, Hyoja 2-dong, Chuncheon 200-701, Korea.

E-mail addresses: sungchan@kribb.re.kr (S. Cho), hjko@kangwon.ac.kr (H.-J. Ko).

☆ These two authors contributed equally to this work.

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can also cause severe neurological disease including brainstem encephalitis and poliomyelitis-like paralysis [2–5]. Human rhinovirus (HRV) represents one of the most important etiological agents of the common cold [6]. Although HRV-induced upper respiratory illness is usually mild and self-limiting, there is increasing evidence linking HRV infection to more serious medical complications including asthma exacerbation [7].

To date, no effective antiviral therapies have been approved for either the prevention or treatment of diseases caused by viruses classified within the Picornaviridae family, including CVB3, EV71, and HRV [8]. In this regard, many trials have been conducted to find antiviral components from plants. Such trials have specifically targeted plants with intrinsic defense mechanisms in the form of secondary metabolites against a broad range of viral infections, in contrast to adaptive immunity induced in mammals. Indeed, medicinal plants are gaining popularity as suitable alternative sources of antiviral agents because of their multiple targets, minor side effects, low potentials to cause resistance, and low costs [9–13]. Although several hundreds of plants with the potential to contain novel antiviral agents have been studied, a number of potentially useful medicinal plants still need to be evaluated and exploited for therapeutic applications against the genetically and functionally diverse virus families. Of these potential agents, we have focused on ginsenosides, which are some of the major components of the ginseng plant, *Panax ginseng* Meyer. The root of *P. ginseng* (Araliaceae) is the most well-known medicinal plant in the Asian region and is frequently used in traditional medicine [14]. Ginsenosides are triterpenoid glycosides containing dammarane [15], and are generally divided into two groups: the protopanaxadiol (PD) and protopanaxatriol (PT) ginsenoside groups. The sugar moieties in the PD group including Rb1, Rb2, Rc, Rd, Rg3, and Rh3 are attached at the 3-position of dammarane-type triterpenes, whereas the sugar moieties in the PT group including Re, Rf, Rg1, Rg2, and Rh1 are attached at the 6-position of dammarane-type triterpenes [16]. As the major components in ginseng, ginsenosides have various biological activities such as anticancer [17], antiaging [18,19], and antitumor activities [20]. Moreover, the antiviral activities of ginseng against influenza virus [15], norovirus [21], and HBV [22] have recently been reported. Although a variety of pharmacological activities associated with seven ginsenosides (PT group: Re, Rf, and Rg2; PD group: Rb1, Rb2, Rc, and Rd) have been demonstrated, antiviral activities especially against CVB3, EV71 and HRV3, which are representative viruses of the picornaviridae and have drawn a great attention in terms of potential therapeutics, have not been reported. Therefore, in the current study, we investigated the antiviral activities of seven ginsenosides against CVB3, EV71, and HRV3.

2. Materials and methods

2.1. Viruses, cell lines, and reagents

CVB3, EV71, and HRV3 were supplied by Korea Research Institute Bioscience and Biotechnology, Ochang-eup, South Korea. A human cervix epithelial cell line (HeLa, CCL-2) and African green monkey kidney cells (Vero, CCL-81) were purchased from the American Type Culture Collection (Manassas, VA, USA). HeLa and Vero cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum and 0.01% antibiotic–antimycotic solution. Antibiotic–antimycotic solution, trypsin–EDTA, fetal bovine serum and minimal essential medium were supplied by Gibco BRL (Grand Island, NY, USA). Tissue culture plates were purchased from Falcon (BD Biosciences, Franklin Lakes, NJ, USA). Ribavirin and sulforhodamine B (SRB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The seven ginsenosides were obtained

from Dr. Bae I (Elohim, Co., Daejeon, South Korea). Stock solutions (100 mg/mL) of the antiviral compounds were dissolved in dimethyl sulfoxide (DMSO) and were subsequently diluted in the culture medium. The final DMSO concentration in the culture medium did not exceed 0.1%, which was found to have no visible toxic effect on the cells. As a negative control, 0.1% DMSO was also added to all no-drug control samples.

2.2. SRB assays of antiviral activity

Assays of antiviral activity and cytotoxicity were evaluated by the SRB method using cytopathic effect (CPE) reduction recently reported [23]. Briefly, 1 day prior to infection, Vero cells were seeded onto a 96-well culture plate at a concentration of 2×10^4 cells/well. The following day, the culture medium was removed and cells were washed with phosphate-buffered saline (PBS). The infectivity of each virus was determined by the SRB method monitoring CPE, allowing for the percentage of cell viability to be determined. Based on the mammalian cell viability determined for each virus, 0.09 mL of diluted virus suspension of CVB3 or EV71 containing CCID₅₀ (50% cell culture infective dose) of virus stock was added to mammalian cells. This dose was selected to produce the appropriate CPEs 48 hours after infection. For compound treatments, 0.01 mL of the medium containing the selected concentration of compound was added to the cells. The antiviral activity of each test material was determined using a 10-fold diluted concentration range of 0.1–100 µg/mL. Four wells were used as virus controls (virus-infected, nondrug-treated cells), whereas four wells were used as cell controls (noninfected, nondrug-treated cells). Culture plates were incubated at 37°C in 5% CO₂ for 48 h. After washing once with PBS, 100 µL of cold (–20°C) 70% (v/v) acetone was added to each well and left for 30 min at –20°C. The acetone was removed from cells, after which 96-well plates were left to dry in oven at 60°C for 30 min. Then, 100 µL of 0.4% (w/v) SRB in 1% acetic acid (v/v) was added to each well and incubated at room temperature for 30 min. Unbound SRB was removed by washing the plates five times with 1% acetic acid (v/v), and the plates were then left to dry in an oven. After drying for 1 day, cell morphology was assessed under a microscope at 4 × 10 magnification (AXIOVERT10; Zeiss, Göttingen, Deutschland) and images were acquired. Fixed SRB in wells was solubilized with 100 µL of unbuffered Tris-base solution (10 mM), and plates were incubated at room temperature for 30 min. Absorbance in each well was read at 540 nm using a VERSAmix microplate reader (Molecular Devices, Palo Alto, CA, USA) and a reference absorbance of 620 nm. The antiviral activity of each test compound in CVB3- or EV71-infected cells was calculated as a percentage of the corresponding untreated control.

2.3. Cell Titer-Glo assays of antiviral activity

The antiviral activity of seven ginsenosides against HRV3 was determined using a Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega, Madison, Wisconsin, USA). The Cell Titer-Glo Reagent induces cell lysis and the generation of luminescence proportional to the amount of ATP present in cells. The resulting luminescence intensity is measured using a luminometer (Molecular Devices) according to the manufacturer's instructions. Briefly, HeLa cells were seeded onto a 96-well culture plate, after which 0.09 mL of diluted HRV3 suspension containing CCID₅₀ of the virus stock, and 0.01 mL culture medium supplemented with 20 mM MgCl₂ and the appropriate concentration of ginsenosides, was added to the cells. The antiviral activity of each test material was determined using a concentration series of 0.1 µg/mL, 1 µg/mL, 10 µg/mL, and 100 µg/mL. Culture plates were incubated at 37°C in 5% CO₂. After 48 h,

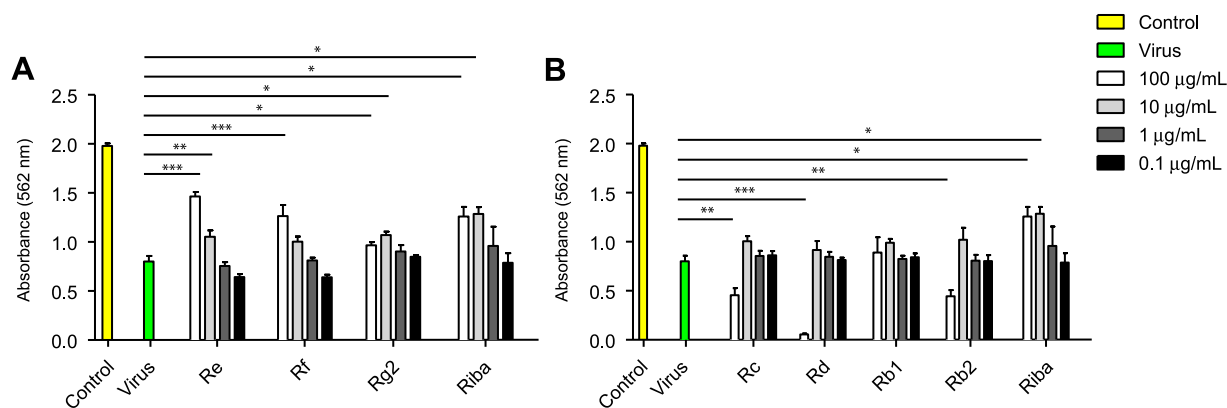


Fig. 1. Antiviral activity of ginsenosides against CVB3 in Vero cells. Vero cells were infected with EV71, after which they were treated with the indicated concentrations (0.1–100 µg/mL) of ginsenosides for 48 h. Ribavirin was used as a positive control for antiviral activity. Antiviral activity was investigated using a CPE reduction assay. Data are presented as mean \pm SD from three independent experiments each carried out in triplicate. CPE, cytopathic effect; CVB3, coxsackievirus B3; EV71, enterovirus 71.

100 µL of Cell Titer-Glo reagent was added to each well, and the plate was incubated at room temperature for 10 min. The resulting luminescence was measured and the percentage cell viability was calculated as described for the antiviral activity assays. Cell morphology was assessed as described for the SRB assay.

2.4. Cytotoxicity

To measure cytotoxicity, cells were seeded onto a 96-well culture plate at a density of 2×10^4 cells/well. The following day, the culture medium containing serially diluted compounds was added to the cells and incubated for 48 h, after which the culture medium was removed and cells were washed with PBS. The next step was conducted as described above for the antiviral activity assay. To calculate the CC_{50} values, the data were expressed as percentages relative to controls, and CC_{50} values were obtained from the resulting dose–response curves.

2.5. Statistical analyses

Differences across more than three groups were analyzed using one-way analysis of variance (Graphpad PRISM, version 5.01, San Diego, CA, USA). All results were expressed as mean \pm standard deviation. Significant differences in direct comparisons were determined using a Tukey's *post hoc* test. Differences with $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered statistically significant.

3. Results

3.1. Antiviral activity of ginsenosides against CVB3

The antiviral activities of ginsenosides against CVB3 were assessed using the SRB method, which monitors the alteration of CPE induced by virus infection. As a positive control, ribavirin, a commonly used antiviral drug, was included. Of the seven ginsenosides tested, ginsenosides Re, Rf, and Rg2, which are classified as PT-type ginsenosides, significantly inhibited CVB3-induced CPE, and increased the cell viability of Vero cells (Fig. 1). CVB3 infection induced approximately 60% cell death in Vero cells (40% of cell viability), and the treatment of cells with 100 µg/mL of Re, Rf, and Rg2 increased the cell viability to 75%, 60%, and 50%, respectively. Furthermore, 10 µg/mL of ginsenosides Re and Rg2 also significantly reduced the CPE of CVB3 infection in Vero cells, albeit a weaker protective effect than that of ribavirin at the same concentration. By contrast, the PD-type ginsenosides Rb1, Rb2, Rc, and Rd did not

exhibit any antiviral activity against CVB3, and 100 µg/mL of Rd, Rc, and Rb2 even significantly increased CVB3 infection-induced cytotoxicity (Fig. 1).

In Vero cells treated with ribavirin after CVB3 infection, the drug exhibited significant antiviral activity at 100 µg/mL and 10 µg/mL (Fig. 1), and the maximal efficacy of ribavirin was comparable to those of PT-type ginsenosides. Ribavirin itself was slightly toxic to Vero cells (cell viability of approximately 81% at 100 µg/mL), whereas none of the seven ginsenosides alone was toxic to Vero cells at the same concentration (Table 1). Collectively, these results suggest that ginsenosides Re, Rf, and Rg2 have significant antiviral activity against CVB3 without inducing cytotoxicity in Vero cells.

3.2. Antiviral activity of ginsenosides against EV71 infection

Together with coxsackievirus A16, EV71 is one of the two major causative agents of hand, foot, and mouth disease, and thus we sought to investigate whether ginsenosides have antiviral activity against EV71 infection in Vero cells. Most ginsenosides assessed using the SRB method did not have significant antiviral activity against EV71, and only ginsenoside Rg slightly inhibited EV71 infection-induced cytotoxicity (Fig. 2). Infection with EV71 induced substantial cell death in Vero cells, resulting in approximately 25% cell viability. The antiviral effect of Rg2 (10 µg/mL and 100 µg/mL) in EV71-infected cells improved cell viability by 40%. The antiviral effect of Rg2 was shown to be dose-dependent, and the maximal antiviral efficacy of the compound is comparable to that of

Table 1
Cytotoxicity of Ginsenosides in Vero Cells

Ginsenosides		Concentration (µg/mL)		
		1	10	100
PT type	Re	109 \pm 1.02	109 \pm 2.53	106.9 \pm 1.03
	Rf	117 \pm 1.92	121 \pm 1.63	105 \pm 3.41
	Rg2	102 \pm 1.15	109 \pm 1.50	106 \pm 2.35
	Ribavirin	101 \pm 2.90	105 \pm 0.13	80.7 \pm 1.22
PD type	Rb1	110 \pm 0.62	117 \pm 2.16	106 \pm 0.40
	Rb2	110 \pm 4.49	106 \pm 1.04	107 \pm 1.08
	Rc	104 \pm 1.04	111 \pm 1.02	106 \pm 1.01
	Rd	101 \pm 1.41	101 \pm 2.35	104 \pm 1.72
	Ribavirin	101 \pm 1.86	105 \pm 1.22	80.7 \pm 0.13

Results are presented as the mean percentage values obtained from three independent experiments carried out in triplicate \pm SD
PD, protopanaxadiol; PT, protopanaxatriol

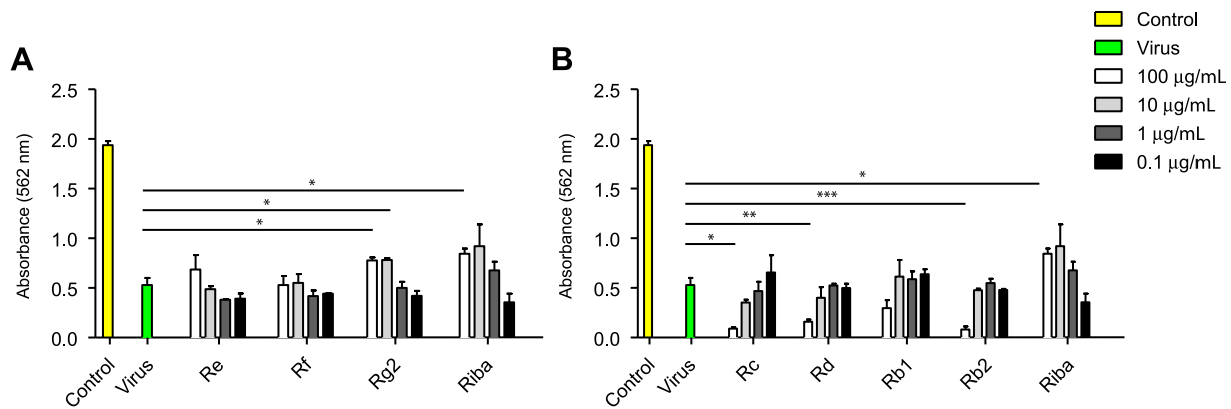


Fig. 2. Antiviral activity of ginsenosides against EV71 in Vero cells. Vero cells were infected with EV71, after which they were treated with the indicated concentrations (0.1–100 µg/mL) of ginsenosides for 48 h. Ribavirin was used as a positive control for antiviral activity. Antiviral activity was assessed using a CPE reduction assay. Data are presented as mean \pm SD from three independent experiments each carried out in triplicate. CPE, cytopathic effect; EV71, enterovirus 71.

ribavirin. By contrast, other ginsenosides tested did not have significant antiviral activity against EV71 infection (Fig. 2).

3.3. Antiviral activity of ginsenosides against HRV3 infection

To assess the antiviral activity of ginsenosides against HRV, HeLa cells were infected with HRV3 and treated with each of the seven ginsenosides of interest at the indicated concentrations. HRV3 infection itself induced cell death in HeLa cells and resulted in 50% cell viability (Fig. 3). Similar to the antiviral effect against CVB3, two PT-type ginsenosides (Rf and Rg2) significantly increased cell viability to 80% (Fig. 3) as shown using the luminescent cell viability assay described in the “Materials and methods” section. The ginsenoside Re, however, had little protective effect in HRV3-infected HeLa cells. Furthermore, none of the PD-type ginsenosides (Rd, Rc, Rb1, and Rb2) had a protective on cell viability, but instead the compounds (100 µg/mL) significantly increased HRV3 infection-induced cell death in HeLa cells (Fig. 3), despite not inducing cytotoxicity in uninfected HeLa cells (Table 1). Collectively, these results suggest that the PT-type ginsenosides Rf and Rg2 have antiviral activity against HRV3.

3.4. Effect of ginsenosides on CVB3- and HRV3-induced cytotoxicity

In order to examine the potential morphological alteration of Vero cells by ginsenosides, cells were treated with the compounds for 48 h and assessed by microscopy. In the absence of infection with CVB3, cells treated with DMSO or 100 µg/mL ginsenosides showed no obvious signs of cytotoxicity, exhibiting the typical spread-out shape associated with the normal morphology of Vero cells (Fig. 4). Infection of Vero cells with CVB3 resulted in a severe CPE, whereas CVB3-infected Vero cells treated with ginsenosides Re, Rf, and Rg2, exhibited noticeably reduced CPE compared with untreated CVB3-infected cells. Treatment of CVB3-infected Vero cells with ribavirin significantly reduced CPE. These results indicate that the CPE of CVB3 infection is prevented by ginsenosides Re, Rf, and Rg2.

The viability of HeLa cells following HRV3 infection was also monitored. In the absence of HRV3 infection, the treatment of HeLa cells with ginsenosides for 48 h altered neither the viability nor the morphology of the cells compared with vehicle-treated cells (Fig. 5). HRV3 infection reduced the viability of cells, and as assessed using the SRB assay, ribavirin was found to significantly inhibit HRV3 infection-induced cell death. Likewise, ginsenosides

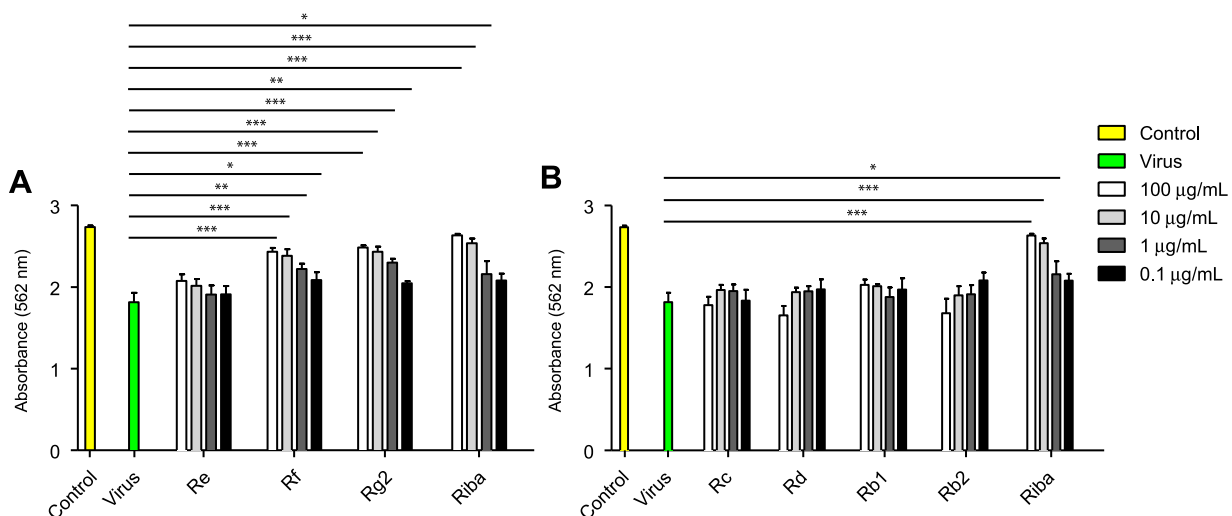


Fig. 3. Antiviral activity of ginsenosides against HRV3 in HeLa cells. HeLa cells were infected with HRV3, after which they were treated with the indicated concentrations of ginsenosides for 48 h. Antiviral activity was assessed using a Cell Titer-Glo Luminescent cell viability assay. Data are presented as mean \pm SD from three independent experiments each carried out in triplicate. HRV3, human rhinovirus 3.

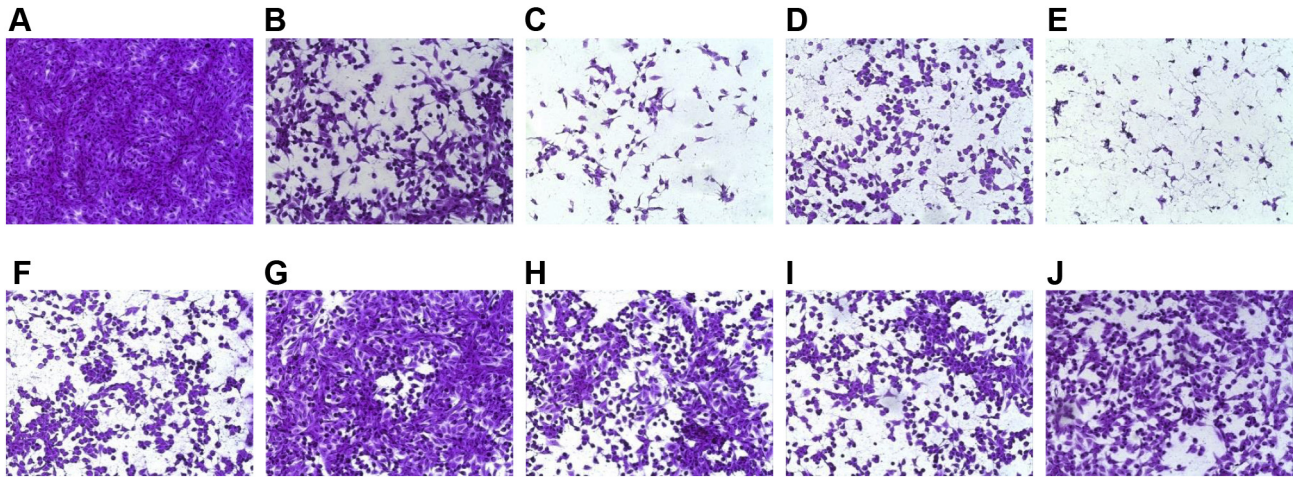


Fig. 4. Morphological assessment of coxsackievirus B3 (CVB3)-infected Vero cells following ginsenoside treatment. CVB3-infected and uninfected Vero cells were treated with either ribavirin or 100 $\mu\text{g}/\text{mL}$ ginsenosides. After staining of viable cells with sulforhodamine B (SRB), cell morphology was assessed by microscopy. (A) Untreated, uninfected cells; (B–I) uninfected cells treated with ginsenosides (B) Rb1, (C) Rb2, (D) Rd, (E) Rc, (F) Re, (G) Rf, and (H) Rg2; and with ribavirin; (J) untreated CVB3-infected cells; (K–R) CVB3-infected cells treated with ginsenosides (K) Rb1, (L) Rb2, (M) Rd, (N) Rc, (O) Re, (P) Rf, (Q) Rg2; and with (R) ribavirin.

Re, Rf, and Rg2 reduced HRV3 infection-induced cell death, whereas ginsenosides Rd, Rc, and Rb2 induced severe cytotoxicity in HeLa cells infected with HRV3. The CPE of HRV3 infection is thus prevented by treatment with ginsenosides Re, Rf, and Rg2.

4. Discussion

P. ginseng is a traditional medicine that has been used in Korea and China for more than 5000 years [24]. Steaming and fermentation of skinned ginseng resulted in red ginseng having a somewhat different chemical composition compared with the original ginseng. Many saponins including ginsenosides found in ginseng and red ginseng have been shown to have various beneficial effects including adjuvant properties and antiviral activity. Some ginsenosides elicited adjuvant effects when used in combination with several vaccines including influenza and porcine parvovirus vaccines [15,25]. Ginsenosides Rg1, Rg2, Rg3, Rb1, and Re, in particular, exhibit potent adjuvant activity, and ginsenoside Re increased the immune response against an inactivated H3N2 influenza vaccine [15]. Furthermore, the oral

administration of ginsenoside Rb2 prior to infection of mice with hemagglutinating virus of Japan protected the infected mice from severe acute lung infection. This effect was shown to be due to antiviral activity of Rb2 as well as an enhancement of mucosal immunity by the compound [26]. Interestingly, a recent study showed that ginsenosides Rg1 and Rb1, as well as red ginseng extract, exhibited antiviral activity against hepatitis A virus, which is classified in the Picornaviridae family together with *Enteroviruses* [27]. However, there have been no previous reports on the antiviral activity of ginsenosides against other viruses included in Picornaviridae. In the current study, we show that ginsenosides Re, Rf, and Rg2 have significant antiviral activity against CVB3 and HRV3 infection, and thus, considering their potential adjuvant activity, these compounds may be effective in eliminating CVB3 and HRV3 in infected hosts.

It is believed that CVB3 is an etiological agent causing myocarditis and dilated cardiomyopathy, and outbreaks of CVB3 infection occur worldwide annually [28]. Currently, there are no effective therapeutic agents against CVB3, and only ribavirin has been shown to have weak antiviral activity against CVB3 infection

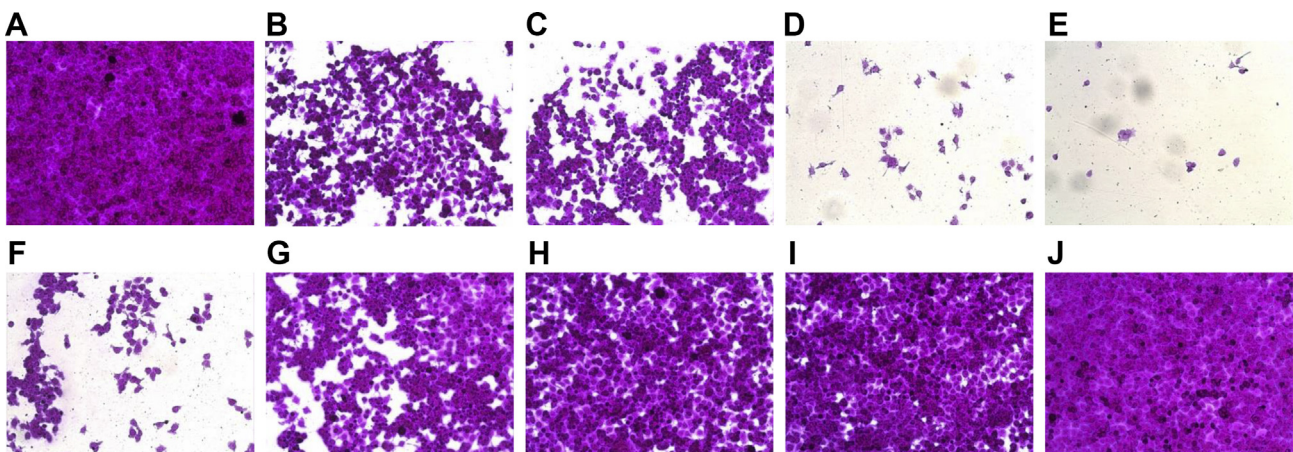


Fig. 5. Morphological assessment of human rhinovirus 3 (HRV3)-infected HeLa cells following ginsenosides treatment. HRV3-infected and uninfected HeLa cells were treated with either ribavirin or 100 $\mu\text{g}/\text{mL}$ ginsenoside. After staining of viable cells with sulforhodamine B (SRB), cell morphology was assessed by microscopy. (A) Untreated, uninfected cells; (B–I) uninfected cells treated with ginsenosides (B) Rb1, (C) Rb2, (D) Rd, (E) Rc, (F) Re, (G) Rf, (H) Rg2; and with (I) ribavirin; (J) untreated HRV3-infected cells; (K–R) HRV3-infected cells treated with ginsenosides (K) Rb1, (L) Rb2, (M) Rd, (N) Rc, (O) Re, (P) Rf, (Q) Rg2; and with (R) ribavirin.

[29–31]. Similarly, no therapeutics are available for the treatment of HRV infection, and most associated treatments function only to reduce the symptoms of the infection. Because of the challenges associated with the development of appropriate vaccines as a means of controlling rhinovirus infection, mainly due to the genetic variability of rhinoviruses, most research efforts toward combating rhinovirus infection have been focused on the development of effective antiviral drugs. A great variety of compounds and compound classes have been shown to exhibit antirhinovirus activity *in vitro*, but few have been found to be effective at the clinical level. The antiviral activities of whole extracts produced from *Uncaria tomentosa*, *Guettarda platypoda* [32], rhizome of *Tamus communis* [33], *Calendula arvensis* [34], root of *Allium sativum* [35], *Zingiber officinale* [36], and *Eleutherococcus senticosus* [37] have been reported; however, antiviral activities of ginsenosides and even of ginseng against HRV have not yet been reported.

Pleconaril is an orally administrable small-molecule inhibitor of human picornavirus replication. The compound is known to be integrated into a hydrophobic pocket within the major coat protein of viruses including human Picornaviridae, and to inhibit the correct functioning of this protein. Consequently, pleconaril inhibits the attachment of some viruses to their cellular receptors and blocks the viral uncoating process [38,39]. Recently, however, the US Food and Drug Administration did not approve pleconaril for clinical use owing to concerns of emergence of viral resistance and side effects in patients [40]. Ribavirin has also been used in attempts to treat various DNA and RNA virus infections, although acquired resistance to the drug has been demonstrated in various virus populations and in some patients [29]. The development of antiviral drugs targeting viruses classified in the Picornaviridae family is therefore urgently required. In the current study, the antiviral activities of ginsenosides against CVB3, EV71, and HRV3 have been evaluated and compared with the currently used antiviral drug ribavirin, which exhibits some antiviral activity. The results of our study demonstrating the antiviral activities of ginsenosides suggest that the compounds may provide a therapeutic option for the treatment of CVB3, EV71, and HRV3 infection; furthermore, the compounds could potentially be effective against Picornaviridae viruses in general.

Strong anti-CVB3 and anti-HRV3 activity was demonstrated for PT-type ginsenosides (Re, Rf, and Rg2), and ginsenoside Rg2 of the PT type showed anti-EV71 activity, despite its relatively weak activity. By contrast, PD-type ginsenosides (Rb1, Rb2, Rc, and Rd) did not show any antiviral activity against CVB3, EV71 and HRV3, and even increased the cytotoxicity induced by virus infection. Taken together, these results indicate that the antiviral activities of ginsenosides against CVB3, EV71, and HRV3 appear to be selectively dependent on the type of ginsenosides. Ginsenoside is divided into PD saponin and PT saponin by its chemical structure. The other study group investigated and compared the antiobesity activity of PD-type and PT-type saponins in rats fed a high fat diet. In conclusion, PD- and PT-type saponins have been shown to exert antiobesity effects in the rats fed with a high fat diet by reducing their body weight, their food consumption, and their fat storage. However, PD-type saponins have more potent antiobesity properties than PT-type saponins [41]. We think our data also demonstrate that antiviral activities are related to the chemical structures. Therefore, further studies are required to explore the detailed antiviral mechanisms of ginsenosides of the PT type as well as to assess *in vivo* antiviral activity.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgments

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