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ORIGINAL PAPER

Inhibition of Murine Norovirus and Feline Calicivirus by Edible Herbal Extracts

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Abstract Human noroviruses (HuNoVs) cause foodborne and waterborne viral gastroenteritis worldwide. Because HuNoV culture systems have not been developed thus far, no available medicines or vaccines preventing infection with HuNoVs exist. Some herbal extracts were considered as phytomedicines because of their bioactive components. In this study, the inhibitory effects of 29 edible herbal extracts against the norovirus surrogates murine norovirus (MNV) and feline calicivirus (FCV) were examined. FCV was significantly inhibited to 86.89 ± 2.01 and 48.71 ± 7.38% by 100 μg/mL of *Camellia sinensis* and *Ficus carica*, respectively. Similarly, ribavirin at a concentration of 100 μM significantly reduced the titer of FCV by 77.69 ± 10.40%. *Pleuropterus multiflorus* (20 μg/mL) showed antiviral activity of 53.33 ± 5.77, and 50.00 ± 16.67% inhibition was observed after treatment with 20 μg/mL of *Alnus japonica*. MNV was inhibited with ribavirin by 59.22 ± 16.28% at a concentration of 100 μM. Interestingly, MNV was significantly inhibited with 150 μg/mL *Inonotus obliquus* and 50 μg/mL *Crataegus pinnatifida* by 91.67 ± 5.05 and 57.66 ± 3.36%, respectively. Treatment with 20 μg/mL *Coriandrum sativum* slightly reduced MNV by 45.24 ± 4.12%. The seven herbal extracts of *C. sinensis*, *F. carica*, *P. multiflorus*, *A. japonica*, *I. obliquus*, *C. pinnatifida*, and *C. sativum* may have the potential to control noroviruses without cytotoxicity.

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Keywords Norovirus · Murine norovirus (MNV) · Feline calicivirus (FCV) · Inhibition · Herbal extract

Introduction

To date, approximately 20000 plant and herb species have been traditionally used as medicinal agents for therapeutic purposes (Singh 2011). In 1000 B.C., 600 plants with medical uses were included in books of Ayurveda of India, and herbs were used in Chinese and Oriental medicine about 3000 years ago (Singh 2011). Extracts of leaves, flowers, roots, stems or barks, seeds, and even whole plants were widely used with food additives or medicinal products in Asian countries (Bent and Ko 2004; Cai et al. 2004; Negi 2012). Phytochemicals of herbal extracts include terpenes, steroids, alkaloids, and flavonoids, which are secondary metabolites of plants (Singh 2011). These components structurally interact with the cell membrane, proteins, and DNA bases, and have single or synergistic effects (Wink 2015).

There are many previous studies on herbal extracts with various beneficial effects such as antimicrobial, antioxidant, antiinflammatory, anticancer, and antidiabetic activity (Atta and Alkofahi 1998; Cai et al. 2004; Dorman and Deans 2000; Patel et al. 2012). In particular, various herbal extracts such as *Glycyrrhiza uralensis*, *Ardisia japonica*, *Paeonia lactiflora*, *Ganoderma lucidum*, *Curcuma longa*, and *Ficus carica* were shown to have antiviral activity against rotavirus, human immunodeficiency virus (HIV), respiratory syncytial virus, herpes simplex virus (HSV), influenza A virus, and adenovirus (ADV), respectively (Adianti et al. 2014; Dao et al. 2012; Eo et al. 1999; Lazreg Aref et al. 2011; Lin et al. 2013; Piacente et al. 1996).

Human noroviruses (HuNoVs) are positive-sense and single-stranded RNA viruses belonging to the *Caliciviridae*

family. They mainly spread through person-to-person transmission or via the fecal–oral route and cause foodborne and waterborne viral gastroenteritis worldwide (Green et al. 2000; Thornton et al. 2004). Recently, a HuNoV culture system using B cells was introduced, but there are limitations that need to be overcome, such as the use of unfiltered stool as a viral source and the inverse correlation between viral inoculum level and infection efficiency (Jones et al. 2015). Evaluation of the survival of HuNoVs cannot be commonly tested with B cell lines and is still conducted using norovirus surrogates such as murine norovirus (MNV) and feline calicivirus (FCV). For these reasons, antiviral drugs or vaccines for the HuNoVs infection have not been developed yet.

Recently, the antiviral activity of natural compounds for controlling or inactivating foodborne viruses was reviewed (Li et al. 2013). Treatment with polyphenols, proanthocyanidins, saponins, polysaccharides, organic acids, and milk constituents inhibited MNV, FCV, rotavirus, coxsackievirus, and hepatitis A virus (HAV). In that review, bioactive substances that can control foodborne viruses at various stages of viral infection were discussed (Li et al. 2013). Pre-treatment with Korean red ginseng extract and ginsenosides reduced MNV and FCV titers in RAW264.7 and Crandell Reese Feline Kidney (CRFK) cells (Lee et al. 2011). However, herbal extracts were not extensively studied against norovirus or its surrogates (Table 1). Therefore, the aim of this study was to investigate the inhibitory effects of 29 Korean native plant extracts used as edible herbs against the norovirus surrogates MNV and FCV.

Materials and Methods

Samples

Methanol extracts of 15 herbs (*Artemisia annua*, *Ginkgo biloba*, *Allium thunbergii*, *Agrimonia pilosa*, *Coriandrum sativum*, *Vitis vinifera*, *Pleuropterus multiflorus*,

Table 1 Antiviral herbal plants and their target viruses described in previous publications

Target viruses	Viral genome	Species of plant	References
Human immunodeficiency virus	*Single-stranded RNA*	*Camellia sinensis*	Nakane and Ono. (1990)
		Crataegus pinnatifida	Min et al. (1999)
		Coriandrum sativum	Asres et al. (2001)
		Coriolus versicolor	Collins and Ng (1997)
		Eucommia ulmoides	Lv et al. (2008)
		Citrus aurantium	Behbahani (2014)
Influenza virus	*Single-stranded RNA*	*Curcuma longa*	Dao et al. (2012)
		Alnus japonica	Tung et al. (2010)
		Allium fistulosum	Lee et al. (2012)
		Agrimonia pilosa	Shin et al. (2010)
		Ginkgo biloba	Haruyama and Nagata (2013)
		Cordyceps militaris	Ohta et al. (2007)
		Portulaca oleracea	Dong et al. (2010)
		Allium sativum	Weber et al. (1992)
Hepatitis C virus	*Single-stranded RNA*	*Glycyrrhiza uralensis*	Adianti et al. (2014)
		Inonotus obliquus	Shibnev et al. (2011)
Human respiratory syncytial virus	*Single-stranded RNA*	*Paeonia lactiflora*	Lin et al. (2013)
Echovirus	*Single-stranded RNA*	*Ficus carica*	Lazreg Aref et al. (2011)
Coxsackievirus	*Single-stranded RNA*	*Cornus officinalis*	Song et al. (2015)
Rotavirus	*11 Sigmented double-helix RNA*	*Glycyrrhiza uralensis*	Alfajaro et al. (2012)
Herpes simplex virus	*Double-stranded DNA*	*Vitis vinifera*	Orhan et al. (2009)
		Ganoderma lucidum	Eo et al. (1999)
		Portulaca oleracea	Dong et al. (2010)
		Lentinus edodes	Sarkar et al. (1993)
Human cytomegalovirus	*Double-stranded DNA*	*Artemisia annua*	Efferth et al. (2002)
Adenovirus	*Double-stranded DNA*	*Ficus carica*	Lazreg Aref et al. (2011)
Hepatitis B virus	*Circular double-stranded DNA*	*Sophora flavescens*	Ding et al. (2006)
		Schisandra chinensis	Xue et al. (2015)

Eleutherococcus senticosus, *Allium sativum*, *Sophora flavescens*, *Allium fistulosum*, *Cornus officinalis*, *Paeonia lactiflora*, *Alnus japonica*, and *Eucommia ulmoides)* were obtained from the Korea Plant Extract Bank at the Korean Research Institute of Bioscience & Biotechnology (KRIBB; Cheongwon, Korea). Ethanol extracts of 14 herbs (*Zizania latifolia, Portulaca oleracea*, *Schisandra chinensis*, *Glycyrrhiza uralensis*, *Curcuma longa*, *Coriolus versicolor*, *Inonotus obliquus*, *Lentinus edodes*, *Ficus carica*, *Citrus aurantium*, *Ganoderma lucidum*, *Cordyceps militaris*, *Camellia sinensis*, and *Crataegus pinnatifida*) were purchased from KOC Biotech (Daejeon, Korea) (Table 2). All extracts were received in the form of dried powder and dissolved in dimethyl sulfoxide as instructed by providers. Initial concentrations of methanol and ethanol herbal extracts were 20 and 100 mg/mL, respectively. Final concentrations of each extract were adjusted with sterile distilled water. Ribavirin as a positive control was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in sterile distilled water. All herbal extracts and ribavirin were sequentially filtered using syringe filters with pore sizes of 5, 1.2, 0.8, 0.45, and 0.20 μm

Cells and Viruses

Murine norovirus 1 (MNV-1) was kindly provided by Dr. Skip Virgin from the University of Washington. FCV strain F9, RAW264.7 cells, and Crandell Reese Feline Kidney (CRFK) cells were purchased from the American type culture collection (ATCC, Manassas, VA, USA).

Cell Viability Assay

Cell viability was measured using CCK-8 (Cell Counting Kit, Sigma) (Yang et al. 2008). Briefly, 100 μL of cell suspension (5000 cells/well) was dispensed into a 96-well plate and incubated for 24 h at 37 °C in a 5% CO_2 incubator. The cells of each well were treated with 50, 100, and 150 μg/mL of 14 herbal extracts (*Z. latifolia*, *P. oleracea*, *S. chinensis*, *G. uralensis*, *C. longa*, *C. versicolor*, *I. obliquus*, *L. edodes*, *F. carica*, *C. aurantium*, *G. lucidum*, *C. militaris*, *C. sinensis*, and *C. pinnatifida*) and 10, 20, and 30 μg/mL of 15 herbal extracts (*A. annua, G. biloba, A. thunbergii, A. pilosa, C. sativum, V. vinifera, P. multiflorus*, *E. senticosus*, *A. sativum*, *S. flavescens*, *A. fistulosum*, *C. officinalis*, *P. lactiflora*, *A. japonica*, *and E. ulmoides*), and incubated for 24 h. Then 10 μL of CCK-8 was added to each plate, and the plates were incubated for 1 h at 37 °C in a 5% CO_2 incubator. The OD (optical density) values were measured at 450 nm using a microplate reader. Cytotoxicity (%) was calculated as follows:

Table 2 Species and locations of herbal extracts used in this study

Family	Genus and species	Locations
Rosaceae	*Agrimonia pilosa*	Whole plant
Umbelliferae	*Coriandrum sativum*	Whole plant
Polygonaceae	*Pleuropterus multiflorus*	Whole plant
Liliaceae	*Allium sativum*	Whole plant
Liliaceae	*Allium fistulosum*	Whole plant
Liliaceae	*Allium thunbergii*	Whole plant
Ranunculaceae	*Paeonia lactiflora*	Whole plant
Compositae	*Artemisia annua*	Leaf
Ginkgoaceae	*Ginkgo biloba*	Leaf
Betulaceae	*Alnus japonica*	Leaf
Araliaceae	*Eleutherococcus senticosus*	Leaf and stem
Vitaceae	*Vitis vinifera*	Fruit
Cornaceae	*Cornus officinalis*	Fruit
Eucommiaceae	*Eucommia ulmoides*	Stem bark
Leguminosae	*Sophora flavescens*	Root
Polyporaceae	*Coriolus versicolor*	Fruit
Hymenochaetaceae	*Inonotus obliquus*	Fruit
Marasmiaceae	*Lentinus edodes*	Fruit
Ganodermataceae	*Ganoderma lucidum*	Fruit
Cordycipitaceae	*Cordyceps militaris*	Fruit
Schisandraceae	*Schisandra chinensis*	Fruit
Moraceae	*Ficus carica*	Fruit
Rosaceae	*Crataegus pinnatifida*	Fruit
Theaceae	*Camellia sinensis*	Leaf
Poaceae	*Zizania latifolia*	Leaf
Portulacaceae	*Portulaca oleracea*	Leaf
Fabaceae	*Glycyrrhiza uralensis*	Leaf
Zingiberaceae	*Curcuma longa*	Leaf
Rutaceae	*Citrus aurantium*	Bark

$$\text{Cell viability } (\%) = (OD_{\text{test}} - OD_{\text{blank}}) / (OD_{\text{control}} - OD_{\text{blank}}) \times 100,$$

where OD_{test} is absorbance of plant extracts or ribavirin in cells with CCK-8, OD_{blank} is absorbance of medium and CCK-8 without cells present, and OD_{control} is absorbance of solvent blanks (sterile distilled water or dimethyl sulfoxide) in cells with CCK-8.

Antioxidant Capacity Assay

A Trolox equivalent antioxidant capacity assay was carried out using ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation solution as previously described (Payet et al. 2005; Re et al. 1999). A working solution of ABTS radical cation was prepared with 7 mM ABTS and 2.45 mM potassium persulfate. Trolox standard curve was constructed with 1.5 mM Trolox stock solution

diluted with pH 7.4 PBS. After the addition of 20 μL of sample or Trolox standard to each well of the 96-well microplate, 200 μL of ABTS radical cation working solution was mixed into each well of the 96-well microplate. Microplate was incubated for 5 min at 25 °C, and absorbance at 734 nm was measured with a plate reader. The antioxidant capacity was calculated as follows:

$$\text{Inhibition } (\%) = 100 - 100(A_S/A_0),$$

where A_S is absorbance of plant extracts or ribavirin or Trolox and A_0 is absorbance of solvent blanks (sterile distilled water or dimethyl sulfoxide or PBS).

Inhibition of MNV and FCV

MNV-1 and FCV titrations were carried out as previously described (Lee et al. 2011; Su and D'Souza 2013). By investigating the effects of 29 herbal extracts on pre-, co-, and post-treatment cells, the antiviral activity of the extracts in virus-infected cells was measured.

The pre-treatment effect experiment was conducted after treatment of confluent CRFK and RAW264.7 cells with herbal extracts in 24-well plates (Corning) for 24 h at 37 °C in a 5% CO_2 incubator. Then ~7 $\log_{10}$ PFU/mL FCV and MNV serially diluted tenfold with Dulbecco's modified Eagle's medium (DMEM) were inoculated into confluent CRFK and RAW264.7 cells, respectively, for 2 h at 37 °C in a 5% CO_2 incubator. They were overlaid with DMEM containing 0.75% agarose (Sigma, Milwaukee, WI, USA), 5% fetal bovine serum (FBS), and 1% penicillin–streptomycin (Hyclone Laboratories, Logan, UT, USA). The CRFK and RAW264.7 cells were maintained for 24 and 48 h, respectively, at 37 °C in a 5% CO_2 incubator. Plaques were counted after staining with neutral red solution.

The co-treatment effect was identified as follows. Confluent CRFK and RAW264.7 cells in 24-well plates were inoculated with ~7 $\log_{10}$ PFU/mL FCV and MNV serially diluted tenfold with DMEM, respectively, and mixed with herbal extracts for 2 h at 37 °C in a 5% CO_2 incubator. All plates were overlaid with DMEM containing 0.75% agarose, 5% fetal bovine serum, and 1% penicillin–streptomycin. The CRFK and RAW264.7 cells were maintained for 24 and 48 h, respectively, at 37 °C in a 5% CO_2 incubator. Plaques were counted after staining with neutral red solution.

In the post-treatment experiment, the CRFK and RAW264.7 cells were inoculated with ~7 $\log_{10}$ PFU/mL FCV and MNV diluted tenfold with DMEM, respectively, and incubated for 18–24 h at 37 °C in a 5% CO_2 incubator. Then the cell media were removed from wells and washed with PBS (pH 7.2). The CRFK and RAW264.7 cells were treated with herbal extracts and overlaid with DMEM containing 0.75% agarose, 2% fetal bovine serum, and 1% penicillin–streptomycin. Each plate was maintained for 24 h and 48 h, respectively, at 37 °C in a 5% CO_2 incubator. Plaques were counted after staining with neutral red solution.

Statistical Analysis

All experiments in this study were performed in triplicate. Virus titers, antioxidant capacity assay, and cytotoxicity assay data were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple range test using Statistical Analysis System software (SAS 9.1 version; Cary, NC, USA). Antioxidant activity was statistically compared with antiviral effect. Differences were considered significant when P-values were less than 0.05.

Results

Cell Viability (%)

The viability of CRFK and RAW264.7 cells is shown in Tables 3 and 4. Ribavirin of 50 and 100 μM was used as antiviral drug to control norovirus in previous study (Chang and George 2007). The CRFK cell viability of ribavirin was 69.23 ± 4.49 and 54.00 ± 5.23% in concentrations of 50 and 100 μM, respectively ($P < 0.01$). The viability of CRFK cells significantly decreased in an herbal extract concentration-dependent manner, except for cells treated with *A. thunbergii*, *V. vinifera*, *G. uralensis*, *I. obliquus*, *F. carica*, and *G. lucidum* ($P < 0.01$). Treatment with 15 of the herbal extracts (*A. annua*, *G. biloba*, *A. thunbergii*, *A. pilosa*, *C. sativum*, *V. vinifera*, *P. multiflorus*, *E. senticosus*, *A. sativum*, *S. flavescens*, *A. fistulosum*, *C. officinalis*, *P. lactiflora*, *A. japonica*, and *E. ulmoides*) resulted in a cell viability above 80%, excluding *A. thunbergii* and *S. flavescens* in concentrations below 20 μg/mL. Treatment with another 14 of the herbal extracts (*Z. latifolia*, *P. oleracea*, *S. chinensis*, *G. uralensis*, *C. longa*, *C. versicolor*, *I. obliquus*, *L. edodes*, *F. carica*, *C. aurantium*, *G. lucidum*, *C. militaris*, *C. sinensis*, *and C. pinnatifida*) resulted in a cell viability above 80%, except for *S. chinensis* in concentrations of less than 100 μg/mL.

The viability of RAW264.7 cells significantly decreased in an herbal extract concentration-dependent manner, except for *G. uralensis*, *C. longa*, and *L. edodes* ($P < 0.01$). When RAW264.7 cells were treated with ribavirin, the viability was 49.08 ± 4.18 and 39.83 ± 4.54% in concentrations of 50 and 100 μM, respectively ($P < 0.01$). Treatment with 15 of the herbal extracts (*A. annua*, *G. biloba*, *A. thunbergii*, *A. pilosa*, *C. sativum*, *V. vinifera*, *P. multiflorus*, *E. senticosus*, *A.*

Table 3 Viability (%) of CRFK cells pre-treated with each herbal extract for 24 h

Methanolic extracts	Concentration (μg/mL)			Ethanolic extracts	Concentration (μg/mL)		
	10	20	30		50	100	150
A. annua	86.10 ± 3.65[a]	81.31 ± 1.89[a]	62.23 ± 2.06[b]	*Z. latifolia*	94.48 ± 5.17[a]	80.06 ± 9.10[b]	56.13 ± 5.14[c]
G. biloba	92.58 ± 1.63[a]	75.69 ± 4.03[b]	59.71 ± 6.04[c]	*P. oleracea*	95.51 ± 2.85[a]	85.60 ± 3.54[b]	60.19 ± 1.61[c]
A. thunbergii	102.97 ± 8.52[a]	90.79 ± 5.87[a,b]	82.57 ± 4.94[b]	*S. chinensis*	100.68 ± 3.06[a]	75.24 ± 6.05[b]	57.47 ± 1.57[c]
A. pilosa	94.00 ± 2.92[a]	80.86 ± 6.39[b]	66.35 ± 6.49[c]	*G. uralensis*	91.73 ± 2.94[a]	85.48 ± 3.79[a,b]	79.85 ± 2.57[b]
C. sativum	91.49 ± 2.91[a]	83.72 ± 2.62[b]	79.41 ± 4.82[b]	*C. longa*	89.77 ± 5.36[a]	80.22 ± 3.78[b]	70.44 ± 5.06[b]
V. vinifera	101.91 ± 5.46[a]	96.71 ± 6.06[a]	91.32 ± 3.54[a]	*C. versicolor*	101.81 ± 4.96[a]	95.69 ± 8.42[a]	77.85 ± 3.72[b]
P. multiflorus	103.97 ± 6.83[a]	97.61 ± 5.27[a]	91.47 ± 2.35[b]	*I. obliquus*	101.20 ± 3.05[a]	94.40 ± 7.47[a]	90.77 ± 3.83[a]
E. senticosus	97.08 ± 2.01[a]	85.39 ± 4.83[b]	69.35 ± 2.55[c]	*L. edodes*	102.69 ± 6.19[a]	93.28 ± 3.92[b]	82.49 ± 2.24[c]
A. sativum	99.60 ± 6.76[a]	90.72 ± 0.64[a]	79.86 ± 7.04[b]	*F. carica*	99.81 ± 2.81[a]	99.03 ± 7.92[a,b]	89.53 ± 1.50[b]
S. flavescens	100.30 ± 2.90[a]	77.42 ± 4.77[b]	69.79 ± 1.58[c]	*C. aurantium*	102.80 ± 5.05[a]	97.23 ± 8.04[b]	92.19 ± 4.10[b]
A. fistulosum	107.22 ± 4.91[a]	89.08 ± 3.69[b]	78.65 ± 1.89[c]	*G. lucidum*	98.49 ± 2.09[a]	94.44 ± 0.61[a]	95.08 ± 2.96[a]
C. officinalis	101.49 ± 5.60[a]	92.53 ± 7.04[b]	62.65 ± 3.29[c]	*C. militaris*	101.01 ± 3.10[a]	96.30 ± 5.39[b]	98.13 ± 5.53[b]
P. lactiflora	105.28 ± 1.99[a]	91.93 ± 2.59[b]	81.76 ± 4.10[c]	*C. sinensis*	92.86 ± 4.36[a]	83.77 ± 2.52[b]	54.64 ± 3.39[c]
A. japonica	102.98 ± 5.28[a]	90.08 ± 1.06[b]	74.95 ± 1.90[c]	*C. pinnatifida*	93.44 ± 4.34[a]	83.94 ± 1.62[b]	72.98 ± 4.05[c]
E. ulmoides	99.35 ± 1.30[a]	90.59 ± 3.01[b]	88.94 ± 2.47[b]	Ribavirin	69.23 ± 4.49[a]*	54.00 ± 5.23[b]*	

Asterisk indicates viability (%) of CRFK cells when the concentration of ribavirin is 50 and 100 μM

[a,b,c] Letters indicate statistical difference between concentrations ($P < 0.05$)

Table 4 Viability (%) of RAW264.7 cells pre-treated with each herbal extract for 24 h

Methanolic extracts	Concentration (μg/mL)			Ethanolic extracts	Concentration (μg/mL)		
	10	20	30		50	100	150
A. annua	103.49 ± 9.81[a]	95.53 ± 3.93[b]	89.41 ± 4.08[c]	*Z. latifolia*	106.18 ± 4.55[a]	89.49 ± 4.73[b]	79.05 ± 5.02[c]
G. biloba	97.90 ± 2.44[a]	81.01 ± 2.99[b]	50.11 ± 4.20[c]	*P. oleracea*	101.88 ± 3.89[a]	93.28 ± 3.18[b]	85.10 ± 3.82[c]
A. thunbergii	101.98 ± 3.38[a]	92.85 ± 7.11[b]	83.37 ± 4.17[c]	*S. chinensis*	100.01 ± 4.93[a]	92.92 ± 4.21[b]	83.46 ± 3.02[c]
A. pilosa	103.10 ± 4.40[a]	92.93 ± 2.97[b]	70.40 ± 5.42[c]	*G. uralensis*	99.98 ± 6.15[a]	92.48 ± 3.15[a,b]	86.37 ± 4.03[b]
C. sativum	113.73 ± 5.25[a]	91.31 ± 4.95[b]	82.79 ± 4.74[c]	*C. longa*	91.97 ± 5.52[a]	85.22 ± 9.59[a,b]	73.16 ± 1.55[b]
V. vinifera	107.19 ± 2.55[a]	90.00 ± 6.62[b]	86.37 ± 4.12[b]	*C. versicolor*	103.26 ± 6.18[a]	94.79 ± 6.05[b]	86.70 ± 3.44[c]
P. multiflorus	100.98 ± 6.37[a]	93.22 ± 4.78[b]	84.22 ± 5.06[c]	*I. obliquus*	100.07 ± 2.92[a]	92.11 ± 3.12[b]	87.31 ± 6.79[b]
E. senticosus	104.55 ± 3.40[a]	93.89 ± 3.10[b]	86.99 ± 2.83[c]	*L. edodes*	100.37 ± 6.73[a]	95.49 ± 3.92[a,b]	82.00 ± 0.83[b]
A. sativum	103.09 ± 7.20[a]	95.32 ± 6.75[b]	84.09 ± 4.46[c]	*F. carica*	104.57 ± 7.07[a]	96.45 ± 4.63[b]	91.08 ± 4.44[b]
S. flavescens	97.70 ± 1.46[a]	83.14 ± 3.93[b]	77.48 ± 1.65[b,c]	*C. aurantium*	101.73 ± 7.04[a]	90.38 ± 3.61[b]	87.50 ± 2.69[b]
A. fistulosum	105.52 ± 3.62[a]	88.29 ± 3.51[b]	79.60 ± 4.82[c]	*G. lucidum*	99.84 ± 1.91[a]	94.01 ± 4.17[a]	82.30 ± 4.72[b]
C. officinalis	91.59 ± 4.05[a]	80.17 ± 6.25[b]	71.02 ± 3.03[c]	*C. militaris*	96.70 ± 2.80[a]	95.74 ± 6.47[a]	79.91 ± 5.05[b]
P. lactiflora	98.19 ± 2.40[a]	88.08 ± 3.50[b]	70.87 ± 2.79[c]	*C. sinensis*	104.29 ± 2.87[a]	95.19 ± 4.31[b]	89.61 ± 4.63[bc]
A. japonica	97.86 ± 5.20[a]	84.88 ± 6.80[b]	72.96 ± 2.93[c]	*C. pinnatifida*	107.04 ± 5.03[a]	93.75 ± 2.96[b]	84.94 ± 5.05[c]
E. ulmoides	97.31 ± 0.31[a]	94.15 ± 6.19[a]	79.74 ± 3.51[b]	Ribavirin	49.08 ± 4.18[a]*	39.83 ± 4.54[b]*	

Asterisk indicates viability (%) of RAW264.7 cells when the concentration of ribavirin is 50 and 100 μM

[a,b,c] Letters indicate statistical difference between concentrations ($P < 0.05$)

sativum, *S. flavescens*, *A. fistulosum*, *C. officinalis*, *P. lactiflora*, *A. japonica*, *and E. ulmoides*) resulted in a cell viability above 80% at concentrations below 20 μg/mL, and treatment with 14 of the herbal extracts (*Z. latifolia, P. oleracea, S. chinensis, G. uralensis, C. longa, C. versicolor, I. obliquus*, *L. edodes*, *F. carica*, *C. aurantium*, *G. lucidum*, *C. militaris*, *C. sinensis*, *and C. pinnatifida*) resulted in a cell viability above 80% at concentrations below 100 μg/mL.

Through a cell viability assay of CRFK and RAW264.7 cells, the concentrations of herbal extracts were determined as 10 and 20 μg/mL for 15 of the herbal extracts (*A. annua*,

Table 5 Antioxidant activity (%) of herbal extracts determined by Trolox equivalent antioxidant assay

Methanolic extracts	Concentration (μg/mL)			Ethanolic extracts	Concentration (μg/mL)		
	10	20	30		50	100	150
A. annua	7.80 ± 1.63[a]	10.99 ± 2.26[a,b]	12.31 ± 1.59[b]	*Z. latifolia*	4.29 ± 1.14[a]	6.19 ± 3.52[a,b]	9.77 ± 1.19[b]
G. biloba	9.03 ± 1.43[a]	13.16 ± 1.15[b]	18.36 ± 2.70[c]	*P. oleracea*	3.57 ± 1.36[a]	7.44 ± 5.33[a,b]	14.41 ± 3.73[b]
A. thunbergii	5.89 ± 1.55[a]	8.99 ± 0.68[b]	12.75 ± 1.65[c]	*S. chinensis*	1.88 ± 1.21[a]	2.29 ± 4.39[a]	4.50 ± 1.88[a]
A. pilosa	3.79 ± 1.80[a]	6.09 ± 1.47[a,b]	9.46 ± 2.04[b]	*G. uralensis*	9.87 ± 2.09[a]	14.77 ± 6.12[a,b]	21.07 ± 3.08[b]
C. sativum	1.80 ± 0.69[a]	1.85 ± 0.84[a]	2.27 ± 1.38[a]	*C. longa*	4.03 ± 1.56[a]	4.12 ± 4.21[a]	9.04 ± 1.99[a]
V. vinifera	5.28 ± 1.35[a]	5.25 ± 1.90[a]	6.58 ± 1.80[a]	*C. versicolor*	3.61 ± 0.84[a]	4.13 ± 3.50[a]	10.03 ± 2.95[b]
P. multiflorus	3.50 ± 0.45[a]	5.27 ± 1.84[a]	9.92 ± 3.29[b]	*I. obliquus*	10.83 ± 1.99[a]	17.04 ± 1.49[b]	25.59 ± 3.27[c]
E. senticosus	4.15 ± 2.88[a]	3.81 ± 3.10[a]	6.10 ± 3.86[a]	*L. edodes*	1.74 ± 1.01[a]	1.90 ± 1.65[a]	2.10 ± 1.14[a]
A. sativum	1.95 ± 1.00[a]	1.99 ± 2.78[a]	2.48 ± 1.09[a]	*F. carica*	1.77 ± 0.86[a]	2.77 ± 0.80[a]	4.23 ± 0.81[a]
S. flavescens	15.18 ± 2.00[a]	20.25 ± 2.77[a]	22.21 ± 11.40[a]	*C. aurantium*	2.43 ± 1.88[a]	2.51 ± 3.34[a]	4.68 ± 1.07[a]
A. fistulosum	5.40 ± 4.92[a]	5.79 ± 3.46[a]	6.19 ± 3.53[a]	*G. lucidum*	2.23 ± 0.59[a]	2.27 ± 3.43[a]	4.76 ± 1.20[a]
C. officinalis	40.08 ± 3.08[a]	70.90 ± 1.98[b]	91.10 ± 2.26[c]	*C. militaris*	2.14 ± 0.71[a]	3.00 ± 0.64[a]	4.28 ± 0.37[a]
P. lactiflora	18.28 ± 1.22[a]	38.11 ± 0.59[b]	60.41 ± 3.02[c]	*C. sinensis*	36.17 ± 4.60[a]	60.08 ± 2.68[b]	76.14 ± 2.04[c]
A. japonica	8.54 ± 2.19[a]	9.33 ± 3.25[a]	11.59 ± 0.56[a]	*C. pinnatifida*	0.52 ± 0.92[a]	1.39 ± 1.11[a]	2.00 ± 1.54[a]
E. ulmoides	3.35 ± 0.59[a]	4.44 ± 1.69[a,b]	7.24 ± 2.38[b]				

[a,b,c] Letters indicate statistical difference between concentrations ($P < 0.05$)

G. biloba, *A. thunbergii*, *A. pilosa*, *C. sativum*, *V. vinifera*, *P. multiflorus*, *E. senticosus*, *A. sativum*, *S. flavescens*, *A. fistulosum*, *C. officinalis*, *P. lactiflora*, *A. japonica*, *and E. ulmoides*) and 50 and 100 μg/mL for the other 14 herbal extracts (*Z. latifolia*, *P. oleracea*, *S. chinensis*, *G. uralensis*, *C. longa*, *C. versicolor*, *I. obliquus*, *L. edodes*, *F. carica*, *C. aurantium*, *G. lucidum*, *C. militaris*, *C. sinensis*, *and C. pinnatifida*).

Antioxidant Activity (%)

Among the 29 herbal extracts, the antioxidant activity significantly increased in a concentration-dependent manner following the treatment with extracts of *G. biloba*, *A. thunbergii*, *C. officinalis*, *P. lactiflora*, *I. obliquus*, and *C. sinensis* ($P < 0.01$) (Table 5).

Inhibitory Activity of Herbal Extracts Against FCV and MNV

While co-treatment and post-treatment effects of herbal extracts were not observed against MNV or FCV, pre-treatment with herbal extracts for 24 h reduced the plague formation of MNV and FCV (Table 6 and 7) ($P < 0.01$). Significant reductions of FCV (86.89 ± 2.01 and 48.71 ± 7.38%) were observed after treatment with 100 μg/mL of *C. sinensis* and *F. carica*, respectively ($P < 0.01$). *P. multiflorus* at 20 μg/mL showed antiviral activity of 53.33 ± 5.77% ($P < 0.05$), and 50.00 ± 16.67% inhibition was observed after treatment with 20 μg/mL *A. japonica.* Ribavirin at a concentration of 100 μM significantly inhibited the titer of FCV by 77.69 ± 10.40% ($P < 0.01$). MNV was significantly inhibited by 91.67 ± 5.05% after treatment with 150 μg/mL *I. obliquus* (data not shown). The titer of MNV significantly decreased to 57.66 ± 3.36% after treatment with 50 μg/mL *C. pinnatifida* ($P < 0.01$). Slight inhibition of MNV (45.24 ± 4.12%) was observed after treatment with 20 μg/mL *C. sativum.* Ribavirin at a concentration of 100 μM significantly reduced the titer of MNV by 59.22 ± 16.28% ($P < 0.01$).

Discussion

This study demonstrated that pre- rather than co- or post-treatment with herbal extracts significantly reduced the norovirus surrogates. Based on previous publications, the antiviral mechanisms of bioactive substances or drugs are related to the inhibition of genome replication, protein synthesis, or viral enzymes, or are mediated by anti-adhesive effects, direct virucidal action, or immune enhancement (Jassim and Naji 2003; Lee et al. 2014). In addition, cellular enzymes, cell membranes, and nucleic acids of the host cell were important target molecules of the natural antiviral compounds (Wink 2015). Su and D'Souza (2013) reported that the effect of pre-treatment with antiviral herbal extracts was mediated by their interference with the ability of the virus to bind to the cell receptor. Furthermore, immune enhancement and formation of an antiviral environment were suggested as other mechanisms. When host

Table 6 Inhibition (%) of FCV in CRFK cells pre-treated with herbal extracts for 24 h

Methanolic extracts	Concentration (μg/mL)		Ethanolic extracts	Concentration (μg/mL)	
	10	20		50	100
A. annua	41.95 ± 14.92[a]	26.89 ± 14.53[a]	*Z. latifolia*	29.97 ± 2.91[a]	36.21 ± 11.64[a]
G. biloba	29.74 ± 6.23	ND	*P. oleracea*	33.95 ± 6.00[a]	34.41 ± 12.89[a]
A. thunbergii	28.34 ± 8.65[a]	36.51 ± 9.97[a]	*S. chinensis*	44.44 ± 11.11	ND
A. pilosa	17.23 ± 16.69[a]	26.89 ± 14.53[a]	*G. uralensis*	22.00 ± 9.81[a]	5.34 ± 5.85[a]
C. sativum	6.12 ± 10.59[a]	21.13 ± 25.88[a]	*C. longa*	5.16 ± 5.60[a]	13.23 ± 17.35[a]
V. vinifera	23.34 ± 8.65[a]	18.55 ± 21.56[a]	*C. versicolor*	39.34 ± 5.61[a]	19.31 ± 6.63[b]
P. multiflorus	11.11 ± 19.25[a]	53.33 ± 5.77[b]	*I. obliquus*	39.06 ± 14.51[a]	40.95 ± 7.41[a]
E. senticosus	−8.33 ± 14.43[a]	24.44 ± 21.43[a]	*L. edodes*	2.90 ± 5.03[a]	13.36 ± 13.01[a]
A. sativum	16.67 ± 14.43[a]	11.11 ± 19.25[a]	*F. carica*	7.67 ± 7.20[a]	48.71 ± 7.38[b]
S. flavescens	−9.70 ± 16.80	ND	*C. aurantium*	15.33 ± 21.56[a]	39.59 ± 1.13[a]
A. fistulosum	19.44 ± 17.35[a]	13.33 ± 23.09[a]	*G. lucidum*	6.34 ± 19.83[a]	9.38 ± 6.21[a]
C. officinalis	27.78 ± 5.89[a]	28.89 ± 7.70[a]	*C. militaris*	1.58 ± 11.99[a]	13.36 ± 5.15[a]
P. lactiflora	36.11 ± 12.73[a]	35.56 ± 3.85[a]	*C. sinensis*	55.29 ± 9.27[a]	86.89 ± 2.01[b]
A. japonica	27.78 ± 4.81[a]	50.00 ± 16.67[a]	*C. pinnatifida*	−7.94 ± 7.28[a]	17.35 ± 10.47[b]
E. ulmoides	8.33 ± 14.43[a]	16.67 ± 14.43[a]	Ribavirin	52.51 ± 11.68[a]*	77.69 ± 10.40[b]*
NC	−6.32 ± 8.94[a]	−0.69 ± 9.62[a]	NC	1.18 ± 5.24[a]	−6.72 ± 12.27[a]

Asterisk indicates inhibition of MNV when the concentration of ribavirin is 50 and 100 μM

FCV feline calicivirus, *CRFK* Crandell Reese feline kidney cells, *ND* not done, *NC* negative control

[a,b] Letters indicate statistical difference between concentrations ($P < 0.05$)

Table 7 Inhibition (%) of MNV in RAW264.7 cells pre-treated with herbal extracts for 24 h

Methanolic extracts	Concentration (μg/mL)		Ethanolic extracts	Concentration (μg/mL)	
	10	20		50	100
A. annua	14.36 ± 34.10[a]	5.28 ± 13.44[a]	*Z. latifolia*	3.33 ± 5.77[a]	27.78 ± 25.46[a]
G. biloba	−1.62 ± 22.55[a]	−5.56 ± 9.62[a]	*P. oleracea*	21.11 ± 18.36[a]	30.56 ± 4.81[a]
A. thunbergii	21.67 ± 2.89[a]	19.84 ± 7.65[a]	*S. chinensis*	16.67 ± 5.77[a]	18.89 ± 20.09[a]
A. pilosa	18.10 ± 3.30[a]	19.05 ± 8.25[a]	*G. uralensis*	13.33 ± 5.78[a]	17.78 ± 16.78[a]
C. sativum	38.33 ± 12.58[a]	45.24 ± 4.12[a]	*C. longa*	10.00 ± 10.00[a]	9.44 ± 10.05[a]
V. vinifera	21.67 ± 2.89[a]	36.51 ± 5.50[a]	*C. versicolor*	11.03 ± 11.57[a]	38.33 ± 2.89[a]
P. multiflorus	−2.78 ± 20.97[a]	27.38 ± 2.06[a]	*I. obliquus*	18.33 ± 7.64[a]	36.15 ± 5.38[b]
E. senticosus	19.44 ± 17.35[a]	25.56 ± 12.62[a]	*L. edodes*	12.50 ± 14.43[a]	35.00 ± 13.23[a]
A. sativum	1.19 ± 26.81[a]	5.56 ± 9.62[a]	*F. carica*	20.56 ± 4.19[a]	32.78 ± 2.55[b]
S. flavescens	17.86 ± 6.19[a]	16.19 ± 3.30[a]	*C. aurantium*	10.26 ± 11.75[a]	26.03 ± 7.92[a]
A. fistulosum	3.57 ± 19.88[a]	22.22 ± 9.62[a]	*G. lucidum*	2.56 ± 4.44[a]	26.67 ± 5.77[b]
C. officinalis	28.97 ± 5.89[a]	23.33 ± 8.82[a]	*C. militaris*	20.00 ± 17.32[a]	33.33 ± 15.28[a]
P. lactiflora	13.10 ± 12.54[a]	6.67 ± 11.55[a]	*C. sinensis*	14.23 ± 18.39[a]	38.33 ± 2.89[a]
A. japonica	8.33 ± 14.43[a]	9.52 ± 8.25[a]	*C. pinnatifida*	57.66 ± 3.36[a]	12.86 ± 7.91[b]
E. ulmoides	21.67 ± 2.89[a]	39.68 ± 5.50[b]	Ribavirin	21.60 ± 17.51[a]*	59.22 ± 16.28[b]*
NC	−2.78 ± 8.33[a]	−0.09 ± 9.50[a]	NC	2.22 ± 6.67[a]	0.37 ± 8.68[a]

Asterisk indicates inhibition of MNV when the concentration of ribavirin is 50 and 100 μM

MNV murine norovirus, *NC* negative control

[a,b] Letters indicate statistical difference between concentrations ($P < 0.05$)

cells were pre-treated with red ginseng extracts or ginsenosides, interferons and interferon-stimulated genes were induced, which reduced the FCV titer (Lee et al. 2014). Since pre-treatment with herbal extracts induced anti-noroviral activity in this study, the regulation of antiviral cytokines and immune enhancement needs to be investigated in future studies.

In the review of extracts and natural compounds derived from foods and plants, co-treatment with numerous samples was reported to inactivate foodborne viruses (Li et al. 2013). FCV and MNV were significantly inhibited by co-treatment with myricetin and epicatechin in citrus fruits or carvacrol which is a component of oregano essential oil (Sánchez et al. 2015; Su and D'Souza 2013). The antiviral mechanism of co-treatment with natural compounds could mediate a direct virucidal effect by degradation of the viral capsid and nucleic acids or an anti-adhesive effect by interference with the binding of the virus and its receptor on host cells (Arthur and Gibson 2015; Su and D'Souza 2013). Since co-treatment with herbal extracts was not effective in reducing the MNV or FCV titer, the possibility that anti-noroviral herbal extracts could damage the capsid protein, nucleic acids, or viral receptor is low.

The post-treatment effect of antiviral herbal extracts is attributable to their inhibition of viral replication Su and D'Souza (2013). Epigallocatechin gallate (EGCG) and epicatechin gallate (ECG), which have catechin structures with an additional phenyl ring with a hydroxy substitution and a galloyl group, inhibited HIV reverse transcriptase. Structural analysis demonstrated that treatment with EGCG and ECG inhibited HIV reverse transcriptase by competing with the primer template (Nakane and Ono 1990; Tillekeratne et al. 2001). Unlike the HIV, norovirus has RNA-dependent RNA polymerase (RdRp) instead of reverse transcriptase. Since HG23 cells were established to measure the RdRp activity of the human norovirus (Chang and George 2007), the interaction of RdRp and anti-noroviral herbal extracts should be examined with this assay system in further studies.

According to previous studies, extraction solvent could influence the antiviral activity. The hexanic and hexane–ethyl acetate extracts of *F. carica* clearly inhibited the herpes simplex virus, echovirus, and adenovirus more potently than the methanolic, ethyl acetate, and chloroform extracts (Lazreg Aref et al. 2011). Fractions of *C. sativum* extracted with petroleum ether inhibited HIV-1 and HIV-2 more markedly than those extracted with dichloromethane, acetone, and methanol (Asres et al. 2001). Since all herbal extracts used in this study were extracted with ethanol or methanol, their antiviral activity may differ depending on the solvent used.

We identified an anti-noroviral effect in cells pre-treated with *I. obliquus*, *C. pinnatifida*, *C. sativum*, *C. sinensis*, *F. carica*, *P. multiflorus*, and *A. japonica* without cytotoxicity in this study. Similarly, Korean red ginseng extract and its ginsenosides reduced FCV and upregulated antiviral cytokines in host cells (Lee et al. 2014). In China, 68 herbal extracts are traditionally consumed as food uses and herbal medicines to enhance the immune system and adjust metabolic disorders (Liu et al. 2008). They have medicinal effects such as antiviral, anticancer, antiinflammatory, and antibiotic activities (Liu et al. 2008). Therefore, the anti-noroviral herbal extracts used in this study could be considered functional foods with physiological benefits because host cells pre-treated with these extracts showed antiviral effects.

Additionally, foods or food components such as fruit juices, herb extracts, and flavonoids have been reported as potential antiviral candidates against noroviruses or foodborne viruses (D'Souza 2014; Li et al. 2013). Since co-treatment with these substances was shown to reduce foodborne viruses by direct inhibitory mechanism, they have found applications as food additives or natural disinfectants (Perumalla and Hettiarachchy 2011; Sánchez et al. 2015; Su and D'Souza 2013). Grape seed extract at 0.25–1 mg/mL reduced HAV, MNV, and FCV titers on produce such as lettuce and jalapeno peppers (Su and D'Souza 2013). In addition, 0.5 or 1% carvacrol was applied to lettuce to reduce MNV and FCV titers (Sánchez et al. 2015). However, this study identified the anti-noroviral effect by the pre-treatment with the herbal extracts at low concentrations of 10–100 μg/mL. Considering that previous studies used 10–100 times higher concentration to control norovirus surrogates by the co-treatment with natural substances (Sánchez et al. 2015; Su and D'Souza 2013), it is needed to determine whether the co-treatment with high concentrations of herbal extracts can inhibit norovirus effectively.

To our knowledge, this is the first study to investigate the inhibitory activity of 29 Korean herbal extracts against the norovirus surrogates, MNV and FCV. Despite the limitation that pre-treatment with *C. sinensis*, *F. carica, P. multiflorus*, *A. japonica*, *I. obliquus, C. pinnatifida*, and *C. sativum* only inhibited MNV and FCV, this study provided evidence that these extracts are promising natural antiviral candidates for controlling noroviruses. Therefore, their antiviral activity should be re-evaluated in further studies using reliably developed human norovirus culture systems.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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