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Research Paper

Inactivation of Norovirus by Lemongrass Essential Oil Using a Norovirus Surrogate System

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ABSTRACT

This study investigated the effect of lemongrass essential oil (LGEO) on the infectivity and viral replication of norovirus. Murine norovirus 1 (MNV-1), a surrogate of human norovirus, was preincubated with LGEO and then used to infect RAW 264.7 cells in a plaque reduction assay. LGEO exhibited a significant reduction in MNV-1 plaque formation in both time- and dose-dependent manners. The quantification of viral genome by quantitative real-time PCR showed similar results in line with those of the plaque reduction assay. It was revealed that citral, a single compound in LGEO, showed dramatic reduction in MNV-1 infectivity (–73.09% when using a treatment of 0.02%, v/v). The inhibitory activity of LGEO on viral replication was further investigated in HG23 cells that harbored a human norovirus replicon. LGEO treatment significantly reduced viral replication in HG23 cells, which suggests that LGEO may have dual inhibitory activities that inactivate viral coat proteins required for viral infection and suppress norovirus genome replication in host cells. In animal experiments, oral administration of murine norovirus preincubated with LGEO significantly suppressed virus infectivity in vivo. Collectively, these results suggest that LGEO, in particular the LGEO component citral, inactivates the norovirus and its subsequent replication in host cells. Thus, LGEO shows promise as a method of inhibiting norovirus within the food industry.

Key words: Antinorovirus activity; Lemongrass essential oil; Murine norovirus; Norovirus; Plaque reduction assay

Food industries and consumers consider foodborne pathogens a major concern in food supply systems. Although authorized synthetic food preservatives are widely used in many countries, investigation of effective antimicrobial natural sources is critical because consumers perceive that natural substances are safer than synthetic chemicals; thus, the use of natural substances to control microbial food safety has been a new trend *(41)*. Among foodborne pathogens, norovirus (NoV) has been identified as a major cause of acute nonbacterial gastroenteritis outbreaks in the United States *(16, 29)*. NoV is responsible for approximately 50% of total foodborne outbreaks in many countries, including the United States and several European countries *(15, 33, 47)*. Moreover, NoVs have been reported as the top five highest-ranking pathogens with respect to the total cost of foodborne illness in the United States *(48)*, suggesting the importance of NoV prevention.

NoVs are nonenveloped, single-stranded, and positive-sense RNA viruses that are highly stable and resistant to environmental degradation *(9, 17, 53)* and are transmitted mainly through the oral route with the ingestion of contaminated water and food and physical contact, but airborne transmission, and vomit from an infected person, can spread viruses as well *(21)*. Acute infection of NoV causes diarrhea, fever, and projectile vomiting *(31, 39)*, and the symptoms can cause hospitalization or death, especially in children, the elderly, and immunocompromised patients *(32)*. However, there is currently no effective NoV vaccine owing to a high mutation frequency of viral coat proteins and no specific and effective drug treatment to prevent human NoV infection *(43)*. Thus, prevention is critical in the control of NoV prevalence. Accordingly, it is important to obtain an effective NoV inactivation strategy that is safe and environmentally friendly. For this purpose, the use of effective natural substances has been suggested as an appropriate approach.

Cymbopogon citratus, commonly known as lemongrass, is recognized for several biological activities and has been widely used in various countries as common tea, medicinal supplement, and insect repellent *(13, 38)*. Lemongrass improves inflammatory symptoms, including fever, infection, stomachaches, headaches, and rheumatic

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pain *(1, 11, 13, 28, 46)*. Reports have also suggested the antioxidative and anticancer activities of lemongrass as well *(5, 6, 12–14, 22, 40, 44)*. Several others also indicated that lemongrass essential oil (LGEO) shows potent antibacterial activity on several microorganisms, including *E. coli, Sterptococus pyonenes,* and *Pseudomonas fluorescence (10, 23, 25, 26, 37, 50, 56, 58)*. In this study, we investigated anti-NoV activity of LGEO both in vitro and in vivo. In vitro study was performed to examine the inhibitory effects of LGEO on NoV infectivity by plaque reduction assay with murine norovirus 1 (MNV-1), a surrogate of human NoV. Alternatively, suppression of NoV replication by LGEO was investigated on HG23 cells harboring a human NoV replicon *(8)*. An in vivo mouse experiment further examined the collective anti-NoV effect of LGEO.

MATERIALS AND METHODS

Materials, viruses, and RAW 264.7 cell culture. LGEO and major single compounds, including citral (Table 1), examined in this study were purchased from Sigma-Aldrich (St. Louis, MO). LGEO and single compounds were dissolved in dimethyl sulfoxide (DMSO; Bio Basic Inc., Markham, Ontario, Canada) to 20% (v/v) and stored at room temperature. We used two types of MNV-1 strains, MNV-1.CW1 (MNV-1) for in vitro plaque assay *(3, 17, 19, 20, 36, 45, 53)* and MNV-1.CR6 for in vivo chronic infection experiments *(55)*. Those strains of MNV-1 were first isolated from laboratory mice by Dr. Herbert Virgin and colleagues *(54)* and have been widely used as a surrogate system for hardly culturable human NoV. The MNV-1.CW1 strain is widely used and suitable for inactivation assay owing to its fast replication rate in the in vitro system *(27, 30, 49)*; this strain cannot induce persistent infection in wild-type mice. Therefore, the MNV-1.CR6 strain was used for the mouse study. MNV-1.CR.6 has been shown to cause persistent infection in mice *(4, 34, 35)*. Both the MNV-1.CW1 and MNV-1.CR6 strains were kindly provided by Dr. Virgin (Washington University, St. Louis, MO).

The RAW 264.7 cells were purchased from the Korean Cell Line Bank (Seoul, Republic of Korea) and cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Pittsburgh, PA) containing 10% fetal bovine serum (Hyclone), 10 mM nonessential amino acids, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 10 mM sodium bicarbonate, and 1% gentamicin (all from GIBCO, Grand Island, NY). The RAW 264.7 cells were maintained at 37°C in a 5% CO_2 humidified incubator.

Cell viability test. The cytotoxicity was assessed by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay. This is a colorimetric assay for the measurement of mitochondrial activity of live cells. Mitochondrial reductase reduces MTT to its insoluble formazan, which has a purple color, and the production of formazan is quantified by spectrophotometry. LGEO and citral dissolved in DMSO were diluted by volume percentage in DMEM media to concentrations of 0.001, 0.002, 0.004, and 0.005 and 0.0003125, 0.000625, 0.00125, 0.0025, and 0.005% (v/v), respectively. The RAW 264.7 cells were treated and incubated with these concentrations for 24 h before media removal. Cells were incubated with 200 μL of DMEM, with a 10% MTT solution for 3 h at 37°C. After removal of the MTT solution, DMSO (200 μL) was added to the cells to extract formazan, and the absorbance of DMSO with formazan was measured at 570 nm by using a microplate reader (Bio-Rad, Hercules, CA).

TABLE 1. *Composition analysis of LGEO by gas chromatography–mass spectrophotometry*

Compound	Relative mass % in LGEO
Ocimene	33.91
α-Terpinolene	17.42
Citral	9.48
d-Limonene	4.18
1,4-Cineole	1.73
Geraniol	0.18

Preparation of MNV and plaque reduction assay. We used MNV-1.CW6 (MNV-1) for plaque reduction assay because MNV-1 is a human NoV surrogate commonly used in plaque reduction assay. To obtain a large stock of MNV-1 (5 log PFU/mL), a monolayer of RAW 264.7 cells (2×10^6 cells per well) in a 175-cm^2 cell culture dish (SPL Life Science, Gyeonggi-do, Republic of Korea) was infected with MNV-1 at a multiplicity of infection of 0.01 in a volume of 5 mL of DMEM *(18, 52)*. The cells were incubated with the virus at 37°C in an atmosphere of 5% CO_2, with occasional agitation every 15 min. The viral inoculum was removed, the cells were washed with serum-free DMEM, complete medium was added to the cell culture dish, and then the cells were incubated at 37°C in 5% CO_2 for another 48 h until approximately 90% viral-induced cytopathic effects were observed. The virus infected cells were subjected to three consecutive freeze-thaw cycles, and the virus was purified, as previously described *(59)*. The supernatants were concentrated at 5,000 × *g* for 20 min by using an Amicon Ultra-15 filter (Millipore, Bedford, MA), with a molecular weight cutoff of 10 kDa. The MNV suspension was stored at –80°C in 110-μL aliquots until use.

The anti–MNV-1 activity of LGEO (0.4%, v/v) and single compounds (0.2%, v/v) were evaluated by plaque reduction assay. The RAW 264.7 cells were seeded into six-well culture plates at a density of 2×10^6 cells per well and incubated overnight at 37°C in an atmosphere of 5% CO_2, and the MNV-1 at 10^5 PFU/mL in DMEM was pretreated with LGEO or single compounds in DMSO for 72 h at 4°C. The RAW 264.7 cells incubated with MNV-1, pretreated with DMSO, were controls. To the incubated RAW 264.7 cells with MNV-1, viral suspensions were serially diluted 10-fold with serum-free DMEM, and 500 μL of the suspension was added to confluent RAW 264.7 cell monolayers in six-well culture plates. The plates were incubated at 37°C for 1 h, with agitation every 15 min. After MNV-1 infection, a viral inoculum was removed, and cells were overlaid with 3 mL of 1% methylcellulose (Sigma-Aldrich) in normal growth media. After 2 days of infection, the cells were fixed and stained with 2% crystal violet (Merck Millipore, Darmstadt, Germany) in 20% ethanol. These experimental conditions examined the anti-NoV effect of LGEO during the food processing condition that is commonly associated with NoV outbreak. For testing the effect of incubation temperature and time, the MNV-1 (10^5 PFU/mL) in DMEM was pretreated with the LGEO (0.4%, v/v) for a shorter time period of 0 to 60 min at 4, 25, and 37°C, and plaque formation was measured as described previously. These experiments were aimed to examine anti-NoV effects of LGEO for food application in ordinary environmental conditions. Controls in the plaque reduction assay were a group of RAW cells incubated with MNV-1, preincubated with DMSO, at a given temperature for a specified time

TABLE 2. *Primer sequences of specific genes used for qPCR*

Gene	Primer (5′–3′)	Accession no.	Sequence
Glyceraldehyde 3-phosphate dehydrogenase	Forward	NM_001289726	ACCTTTGGCATTGTGGAAGG
	Reverse		ACACATTGGGGGTAGGAACA
MNV	Forward	DQ285629	ACGCCACTCCGCACAAA
	Reverse		GCGGCCAGAGACCACAAA
β-Actin	Forward	NM_001101	GGCATCCACGAAACTACCTT
	Reverse		AGCACTGTGTTGGCGTACAG
Human NoV GI (ORF1-ORF2 conjunction)[a]	Forward	M87661	CGYTGGATGCGNTTYCATGA
	Reverse		CTTAGACGCCATCATCATTYAC

[a] GI = genogroup I; ORF, open reading frame.

Plaques were counted, and DMSO-treated specimens at a given temperature used as an untreated control. Antiviral activity was expressed as percent relative plaque formation (%) and inhibitory concentrations of the control group. The 50% inhibitory concentration (IC_{50}) value was calculated by using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA).

Measurement of infected MNV-1 gene in RAW 264.7 cells by qPCR. To confirm the results from the plaque reduction assay, we measured the MNV-1 RNA by quantitative real-time PCR (qPCR). MNV-1 in 5 log PFU/mL at multiplicity of infection of 0.1 *(18, 52)* was preincubated with LGEO (0.4%, v/v) for 72 h at 4°C. Then, MNV-1 was incubated with RAW 264.7 cells for 1 h at 37°C, the inoculum was removed, and RAW 264.7 cells were washed with 1 mL of PBS. Next, the MNV-1–infected RAW 264.7 cells were lysed by freezing and thawing three times, and the cell lysates were collected to isolate total RNA by using RNAiso Plus (Takara, Kusatsu, Shiga, Japan). The cDNAs were synthesized from 0.5 μg of total RNA by using oligo dT primer and ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan), and qPCR was performed in a 20-μL reaction volume by using Thunderbird SYBR qPCR Mix (Toyobo) containing 2 μL of cDNA, 10 μL of SYBR Master Mix, 600 nM of each primer (forward and reverse), and 0.4 μL of ROX dye. Forward and reverse PCR primers for MNV are shown in Table 2. The PCR amplification was performed by using an iQ5 Real-Time PCR Detection System (Bio-Rad) under the following conditions: initial denaturation at 95°C for 3 min, followed by 50 cycles of amplification with denaturation at 95°C for 20 s, and annealing and extension at 60°C for 20 s and 72°C for 1 min. The data were calculated using iQ5 Optical System Software, Version 2 (Bio-Rad). The glyceraldehyde 3-phosphate dehydrogenase was used as a reference gene to normalize the MNV-1 gene expression (e.g., the infected MNV-1 to the host cell was normalized by glyceraldehyde 3-phosphate dehydrogenase expression of the RAW 264.7 cells).

Preparation of MNV-1.CR6, in vivo viral infection, and analysis of viral genomes in the mouse stool MNV-1.CW1 (MNV-1) strain is a human NoV surrogate commonly used in plaque reduction assay. However, this strain shows a low and transient infection rate in mice. Therefore, we used the MNV-1.CR6 strain in the mouse experiment, instead of using MNV-1, which is known to show a persistent enteric infection in mice *(55)*. The MNV-1.CR6 strain was propagated in RAW 264.7 cells grown in DMEM supplemented with 10% fetal bovine serum, 10 mM sodium bicarbonate, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, and 50 μg/μL gentamicin at 37°C in a humidified atmosphere of 5% CO_2. Virus stocks were generated, and titers of virus stocks were determined by plaque assay on RAW 264.7 cells.

The C57BL/6 mice were purchased from Orient Bio Inc. (Seongnam, Republic of Korea) and housed at the animal facility of the Seoul National University Hospital Biomedical Research Institute under specific-pathogen-free conditions according to university guidelines. Animal protocols were approved by the Seoul National University Animal Studies Committee (Protocol SNUIACUC-2013-139). Six- to eight-week-old female mice were infected with 10^6 PFU of MNV-1.CR6, which had been incubated with LGEO (0.5%, v/v) in DMEM or with DMEM only (vehicle control) for 72 h at 4°C. LGEO-treated virus or vehicle control virus was administered to mice by the oral route in a volume of 200 μL. Starting on day 1 postinfection, stool samples were harvested (days 1, 2, 3, 5, and 7 postinfection) and stored at −80°C before quantification of viral RNA by qRT-PCR.

The quantification of viral RNA from mouse stool was conducted following previously reported methods *(4)*. The RNA from stool was extracted with a Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA). For the quantification of MNV-1.CR6 RNA, TaqMan assays were performed as described previously. Briefly, extracted viral RNA template were reverse transcribed at 48°C for 30 min and denatured initially at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, and 60°C for 1 min. The forward (5′-CAC GCC ACC GAT CTG TTC TG-3′) and reverse (5′-GCG CTG CGC CAT CAC TC-3′) primers and a probe (5′-6FAM-CGC TTT GGA ACA ATG-MGBNFQ-3′) were used as previously described *(5)*. A standard curve was generated by using 10-fold serial dilutions (10^7 genomic copies to 1 genomic copy) of the plasmid containing a cDNA clone of MNV-1 sequence. The quantified viral genomes from stool samples were expressed as genome copies per fecal pellet after normalization by fecal pellet count.

GC-MS analysis. The major single compounds were analyzed in LGEO by gas chromatography–mass spectrophotometry (GC-MS) analysis by using a 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) and a Quattro GC with ion trap tandem mass spectrometer (Micromass, Waters, Milford, MA) equipped with an AT-1701 capillary column (length, 50 m; inside diameter, 0.25 mm; film thickness, 0.2 mm; Alltech, Lexington, KY) as previously described *(6)*. The mass spectrometer settings were optimized to provide the best resolution at 69, 219, 502, and 614 *m*/*z* using perfluorotributylamine. Mass measurements in the range of 33 to 350 *m*/*z* were obtained at 240°C by using an electron ionization–positive ion source in SCAN mode. Total ion chromatograms were analyzed by using MassLynx 4.0 software (Micromass), and compounds were positively identified with the aid of the Wiley Mass Spectral Database (included in the software). The detection limit of compounds was calculated as the method detection limit by

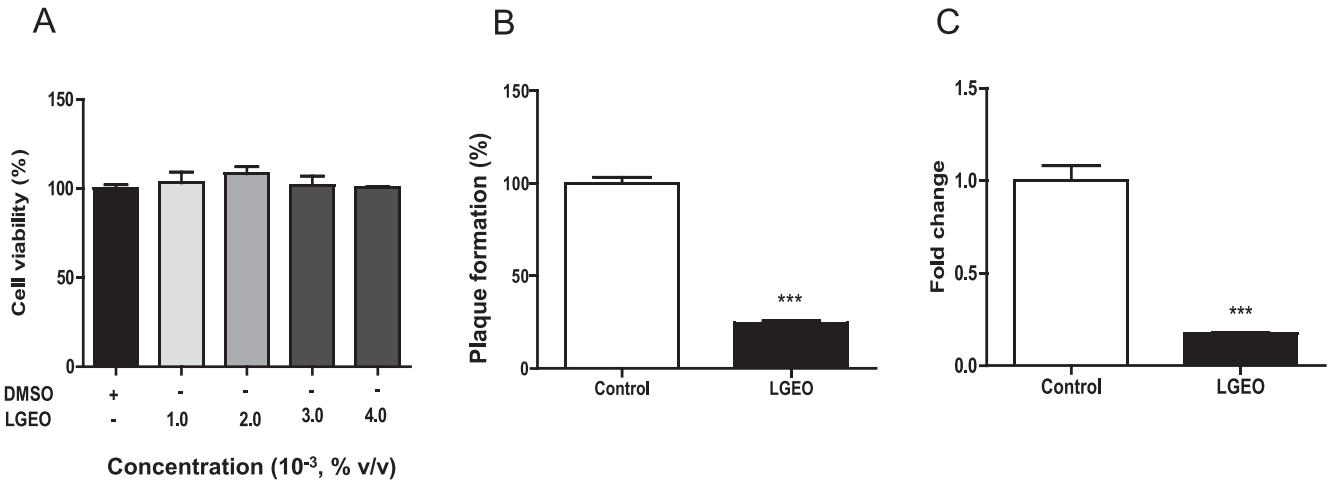

FIGURE 1. *LGEO suppresses MNV-1 infectivity. (A) Cell viability was determined by the MTT assay. (B) Plaque reduction assay. The plaque number in the DMSO-treated sample was set as 100% and used to determine the relative plaque number (percent) in LGEO-treated (0.4%, v/v) samples. (C) Quantification of the MNV genome in RAW 264.7 cells. The number of MNV-1 genome copies was quantified by qPCR, and relative expression levels are presented as fold change compared with gene expression levels in DMSO-treated controls. Values represent the mean ± SEM (*n = 3*). *** P < 0.001 versus control.*

following the relevant U.S. Environmental Protection Agency guidelines *(57)*. The method detection limit values of measured volatile compounds in LGEO were all <100 ppb expressed in the measured mass quantities and were translated into air concentrations by using ideal gas equation assuming a 1-L sample volume. The ideal gas equation is $PV = nRT$, where pressure (P) = 1 atm, sample volume (V) = 1 L, mole (n) = mass (g)/MW (g/mole), $R = 0.082057$ atm·L·mole^{-1}·K^{-1}, and temperature (T) = 298.15 K.

Effects of LGEO on human NoV replication using HG23 cells. The HG23 cells, Huh-7 cells stably expressing a human NoV replicon, were kindly provided by Dr. Kim Y. Green at the Department of Health and Human Services, National Institute of Health (Bethesda, MD) and maintained in DMEM (GIBCO) with 10% heat-inactivated fetal bovine serum (GIBCO), 1% penicillin-streptomycin (Hyclone), and 1 mg/mL G418 (Sigma-Aldrich) at 37°C in a humidified atmosphere of 5% CO_2. During the evaluation of the antiviral activity, HG23 cells were incubated in the same medium without G418. HG23 cells were seeded at a density of 8×10^4 cells per well in a 24-well plate and treated with DMSO, ribavirin (100 μM), and LGEO (0.005%, v/v) for 72 h. Total RNA was isolated using RNAiso PLUS (Takara, Japan). The cDNAs were synthesized by using oligo dT primers, and qPCR was performed in a 20-μL reaction volume by using Thunderbird SYBR qPCR Mix (Toyobo) containing 2 μL of cDNA, 10 μL of SYBR master mix, 600 nM of each primer (forward and reverse), and 0.4 μL of ROX dye. Forward and reverse PCR primers are shown in Table 2. PCR amplification was performed by using an iQ5 Real-Time PCR Detection System (Bio-Rad) under the following conditions: 50°C for 2 min, followed by 45 cycles at 95°C for 10 s, 55°C for 15 s, and then 72°C for 20 s, and a melting curve analysis was performed. The results were analyzed by using the Rotor-Gene real-time analysis software 6.0 (Qiagen, Hilden, Germany). The level of NoV gene expression was normalized to that of β-actin expression of HG23 cells.

Statistical analysis. All experimental data were expressed as the mean ± standard error of the mean (SEM). Data were analyzed by using a Student's *t* test in two-group comparisons and one-way analysis of variance (ANOVA) followed by Tukey's test in a multiple group comparison. An asterisk denotes a significant difference between groups in the *t* test and among groups in one-way ANOVA, followed by Tukey's test ($P < 0.05$). All measurements were performed at least in triplicate. Analyses were performed by using GraphPad Prism 5 software (GraphPad Software).

RESULTS

Effect of LGEO on cell viability and MNV-1 infectivity. Prior to the experiment of plaque reduction assay, we first evaluated the effect of LGEO on cell viability of RAW 264.7 cells by using a MTT assay. Results showed that LGEO did not affect cell viability on RAW 264.7 cells at all tested concentrations compared with the controls (Fig. 1A). Then, antiviral activities of LGEO against MNV-1 were investigated by using a plaque reduction assay and qPCR analysis after previrus treatment with LGEO. This approach examined the collective ability of LGEO to inhibit MNV-1 attachment to host cells or prevent MNV-1 entry into host cells or both *(36)*. Preinactivation of MNV-1 by LGEO was conducted at 4°C for 72 h. As mentioned previously, these experimental conditions examined the anti-NoV effect of LGEO during food processing and storage conditions that are commonly associated with NoV outbreaks. For food processing and storing environments, the risk factor of NoV contamination is the survival of NoV particles at a refrigerating temperature. This preincubation condition mimics such an environment in food processing and storage; thus, we aimed to investigate the effectiveness of LGEO at long-term and low-temperature conditions. The results revealed that MNV-1 plaque formation was reduced 75.5% when MNV-1 was pretreated with LGEO (Fig. 1B). This reduced viral infectivity following LGEO pretreatment was further examined by qPCR analysis. The qPCR results showed that MNV-1 RNA in the RAW 264.7 cells were also dramatically reduced (Fig. 1C). These results suggest that

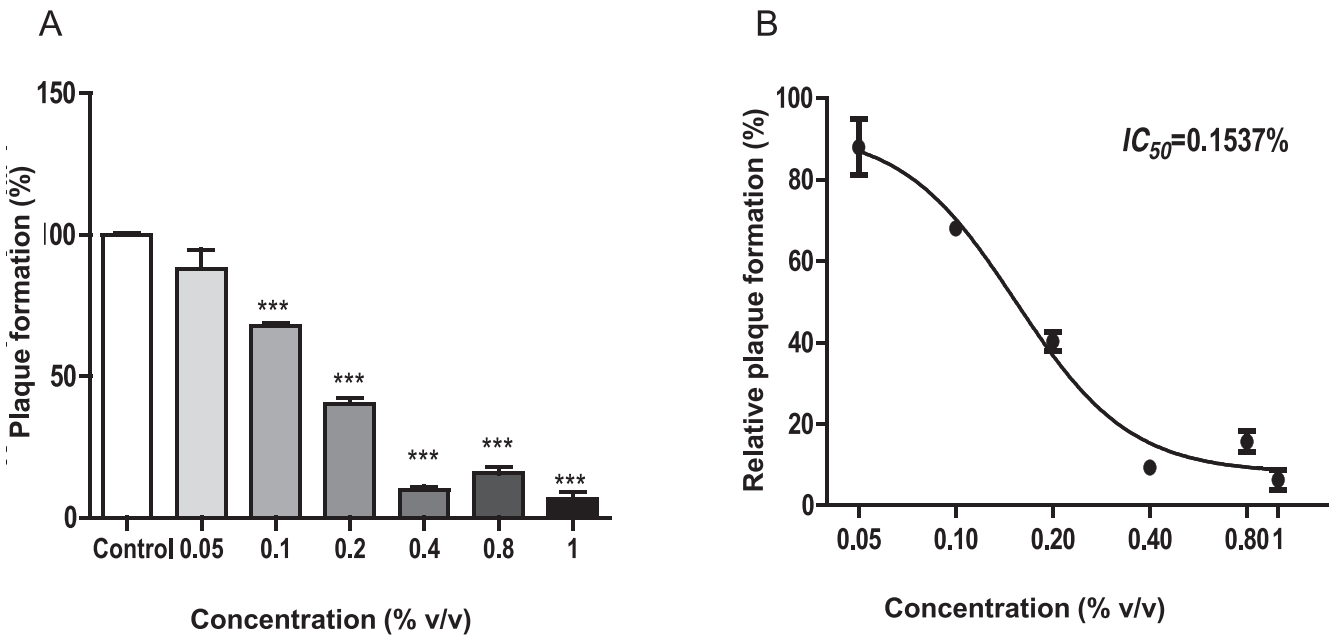

FIGURE 2. *LGEO inhibits MNV-1 infectivity in a dose-dependent manner. (A) Effects of LGEO on plaque formation (percent). A plaque reduction assay was performed as described in the "Materials and Methods" section by using increasing concentrations of LGEO. The virus was preincubated with different concentrations of LGEO at 4°C for 72 h. (B) The IC_{50} value of LGEO for MNV-1 infectivity. Plaque formations (percent) were calculated as relative plaque formation by using DMSO as a control. Values represent the mean ± SEM* (n = 3). ***** $P < 0.001$ *versus control.*

LGEO inhibits MNV-1 attachment or adsorption to RAW 264.7 host cells.

The anti-NoV activity of LGEO is dose dependent. To assess anti-MNV-1 effect of LGEO further, we performed plaque reduction assay at increasing concentrations of LGEO (0, 0.05, 0.1, 0.2, 0.4, 0.8, and 1%, v/v) at 4°C for 72 h and calculated the IC_{50} value of LGEO for MNV-1. The results revealed that anti-NoV activity of LGEO was dose dependent (Fig. 2A and 2B), and the calculated IC_{50} value of LGEO was 0.1537% (v/v; Fig. 2B). These results suggest that pretreatment with LGEO significantly inhibits MNV-1 infection to the host cells in a dose-dependent manner.

Effect of incubation time and temperature of LGEO on MNV-1 infectivity. Subsequently, MNV-1 was pretreated with LGEO (0.4%, v/v) for various amounts of time and at different temperatures and MNV-1 activity. In these experiments, the virus was pretreated with the LGEO (0.4%, v/v) for a shorter time period of 0 to 60 min at 4, 25, and 37°C. These experiments were aimed to examine anti-NoV effects of LGEO for food application in daily life, as mentioned previously. The MNV-1 infectivity at 4°C was marginally affected by LGEO when incubated for up to 60 min (Fig. 3A and 3B), although incubation for 72 h showed dramatic effects (Fig. 1B and 1C). However, the MNV-1 infectivity was inversely correlated with incubation time with LGEO at 25°C; MNV-1 infectivity was decreased rapidly within 5 min (Fig. 3C and 3D). Total MNV-1 plaque formation was 75.1% when MNV-1 was pretreated with LGEO for 5 min compared with plaque formations of DMSO-treated controls at 25°C. At 37°C, MNV-1 infectivity was dramatically decreased in both LGEO-treated samples and controls, but LGEO treatment further reduced MNV-1 plaque formation in a time-dependent manner compared with controls (Fig. 3E and 3F). These results suggest that LGEO inhibits MNV-1 activity in a time-dependent manner, and the pretreatment of the virus with LGEO for 5 min at 25°C could markedly reduce viral infectivity.

LGEO suppresses NoV replication. The effects of LGEO on the replication of human NoV were investigated by using HG23 cells harboring the human NoV replicon. HG23 cells were treated with LGEO (0.005%, v/v) for 72 h at 37°C prior to qPCR analysis. The HG23 cells showed no toxic effects as a result of LGEO treatment at 0.005% (v/v, data not shown). The results showed that LGEO reduced replication of the viral RNA replicon by 58.4% compared with the control (Fig. 4). This result suggests that LGEO could also suppress human NoV genome replication in host cells, in addition to inhibiting viral attachment or entry into host cells, as shown by the results of the pretreatment experiments. Thus, LGEO may exert dual anti-NoV activities.

Citral is an active anti-NoV compound in LGEO. A major single compound of LGEO was analyzed with GC-MS. The results identified 34 compounds (data not shown). Among them, ocimene, α-terpinolene, citral, *d*-limonene, 1,4-cineole, and geraniol were commercially available major components of LGEO; thus, we performed plaque assay with the selected six compounds. The anti-MNV activities of

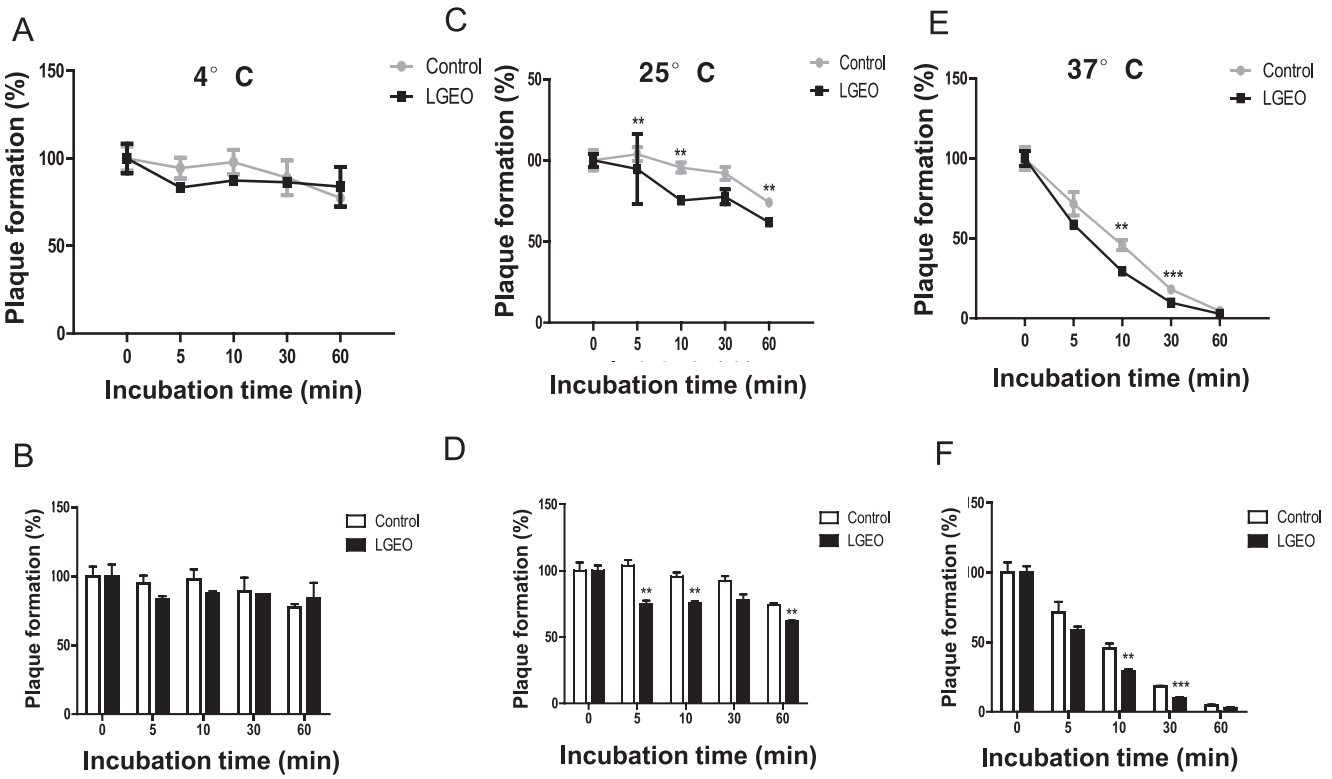

FIGURE 3. *Effects of incubation time and temperature of LGEO pretreatment (0.4%, v/v) on MNV-1 plaque formation. The effects of LGEO incubations at 4°C (A and B), 25°C (C and D), and 37°C (E and F). MNV-1 plaque formations (percent) were assessed by using RAW 264.7 cells at different incubation times (0, 5, 10, 30, and 60 min). MNV-1 infectivity was calculated as a percentage of plaque formation. Plaque formations (percent) were calculated as relative plaque formation by using DMSO as a control. Values represent the mean ± SEM* (n = 3). ** P < *0.01 versus control;* *** P < *0.001 versus control.*

these six single compounds (2%, v/v) were examined by plaque reduction assay. The results showed that citral was the most effective at reducing MNV infectivity among the tested compounds (Fig. 5A). Ocimene, α-terpinolene, *d*-limonene, 1,4-cineole, and geraniol did not have any significant effect on MNV infectivity. Next, we further analyzed potential cytotoxicity of citral using MTT assay with RAW 264.7 cells (Fig. 5B). Results showed that citral may be a key bioactive compounds within LGEO that exhibits anti-MNV activity.

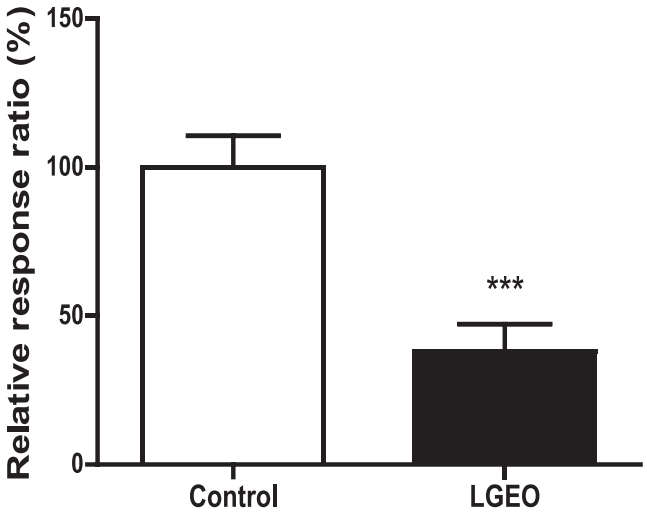

FIGURE 4. *LGEO inhibits the replication of the human NoV replicon in HG23 cells. Human NoV replicon–bearing HG23 cells were incubated with LGEO (0.005%, v/v) for 72 h, and total RNA was prepared for qPCR to detect the NoV genome. Cells treated with DMSO were used as a control. The relative response ratio (percent) was calculated by using the control expression levels as a reference. Values represent the mean ± SEM* (n = 3). *** P < *0.001 versus control.*

Oral administration of LGEO-pretreated MNV-1 shows reduced viral RNA shedding from mice stools. Next, we performed animal experiments to confirm preincubation of LGEO-reduced viral RNA replication and shedding in vivo. Preinactivation of the MNV-1.CR6 strain by LGEO was conducted at 4°C for 72 h, which is the food processing and storage condition commonly associated with NoV outbreak. Thus, we investigated anti–MNV-1 effects of LGEO shown in the plaque reduction assay (Fig. 1B and1C) that could be still exerted in the in vivo model (Fig. 6A). MNV viral RNA was detected starting on day 3 postinfection in both LGEO-treated (2.15 ± 0.12 log copies per fecal pellet) and vehicle control groups (2.21 ± 0.12 log copies per fecal pellet). On day 5 postinfection, the viral load in the stool increased to 3.23 ± 0.09 log copies per fecal pellet in the control group, while that in the LGEO-treated mice did not (compared with levels on day 5). On day 7 postinfection, the inhibitory effect of LGEO on MNV infection was still maintained, but the viral load within the control group was 3.20 ± 0.21 log copies per fecal pellet (Fig. 6B and 6C). These results showed that the inactivated NoV particles by

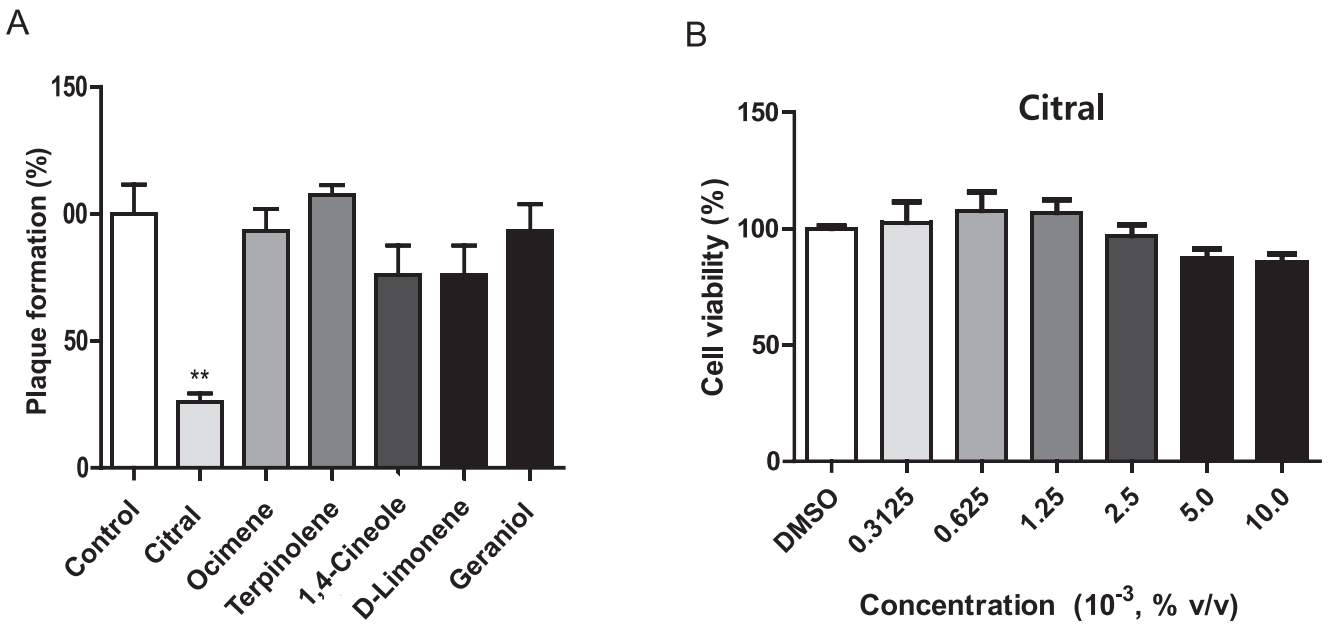

FIGURE 5. *Effects of citral on MNV plaque formation. (A) Citral and other major single compounds in LGEO (0.2%, v/v) were preincubated with MNV at 4°C for 72 h prior to infection of RAW 264.7 cells. Plaque formations (percent) were calculated as relative plaque formation by using DMSO as the control. (B) Cell viability was determined by the MTT assay. Values represent the mean ± SEM (*n = 3). ** P < 0.01 versus control.

preincubation with LGEO were not able to replicate and decreased viral shedding in the in vivo condition.

DISCUSSION

This study investigated the effects of LGEO on the inactivation of NoV, which is a major cause of gastroenteritis outbreaks worldwide *(16, 29)*. Because of the lack of vaccines or a specific cure against NoV, inactivation of NoV is important for the prevention of future infection. Accordingly, researchers have investigated the anti-NoV activity of natural substances, which has recently been reviewed by our group *(45)*. Polyphenols and flavonoids in grape seed, pomegranate, mulberry, black raspberry, cranberry, red ginseng, and persimmon and nonpolyphenols

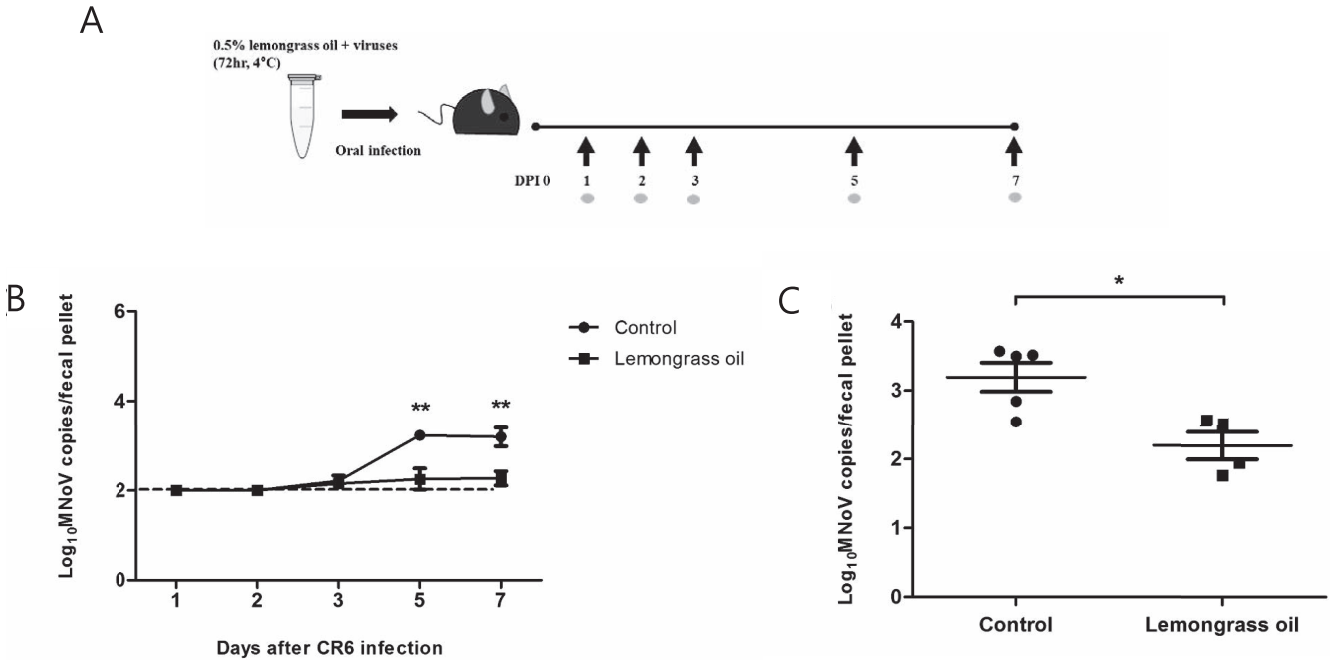

FIGURE 6. *The anti-NoV effects of LGEO in vivo. (A) Schematic flow of antiviral assay (in vivo). (B) Viral loads during the experimental period. Time course changes in viral loads after MNV-1.CR6 infection were analyzed on 1, 2, 3, 5, and 7 days postinfection by two-way ANOVA (*n = 5 mice, P < 0.05). (C) Quantification of MNV in mouse stool. MNV genome copies were quantified in fecal pellets on day 7 postinfection (using a 10^6 PFU dose) and analyzed by the Mann-Whitney test (P < 0.05). Inhibition of MNV-1.CR6 infection after pretreatment with lemongrass oil. * P < 0.05 versus control; ** P < 0.01 versus control.

such as chitosan and citrate have shown anti-NoV activity. In addition, herbal essential oils, mixture of volatile aromatic phytochemicals, have long been used by practitioners of ethnopharmacology, and a previous study revealed that oregano (*Origanum vulgare*) essential oil reduced MNV-1 titers in a dose-dependent manner when the viruses were pretreated with the oils before infection was initiated.

In an effort to investigate natural substances with anti-NoV activity, we initially performed anti-NoV plaque assays by using 148 selected natural substances, from a public database and ancient medical documents in Korea, which were suggested to have antimicrobial and antidiarrheal activities. We identified that LGEO revealed the highest and most consistent activity in the repeated activity assays (unpublished data); thus, we performed more experiments to examine anti-NoV activity both in vitro and in vivo.

Lemongrass has been traditionally consumed in the diet and is a common substance used in alternative medicine. In addition, it has various biological activities, and its antimicrobial and anti-MNV effects in vitro have been recently reported. This study confirmed the anti-NoV activity of LGEO in vitro and also demonstrated this activity in vivo. A previous study reported that LGEO reduced MNV-1 activity when used at concentrations of 2 to 4% (v/v) *(20)*. However, these levels of LGEO led to severe cellular toxicity and thus cannot be used for food applications. In this study, we showed that LGEO and its active compound, citral, have anti-NoV activity at noncytotoxic concentrations in RAW 264.7 and HG23 cells. Using a plaque reduction assay, both LGEO and citral suppressed viral infection of NoV. In particular, the effect of LGEO was dose dependent with an IC_{50} of 0.1537% (v/v) at 4°C for 72 h preincubation. Preincubation of LGEO with virus at room temperature (25°C) for 5 min was also effective. Treatment of the virus with LGEO prior to infection has previously been used to assess the ability of natural substances to inhibit the binding of viruses to cells by blocking cell receptors or directly destroying virus capsid protein *(36)*. These results demonstrate that LGEO prevents NoV infection at the point of attachment or following internalization into cells. LGEO may cause degradation of the viral capsid, and the anti-NoV compounds present in LGEO may act directly on the exposed RNA.

We used MNV-1 as a surrogate of human NoV. As is known, human NoV investigation has been hindered, in part, because human Nov is difficult to culture in vitro. Thus, in the investigation of human NoV, MNV, specifically the MNV-1 subtype has been widely used as a surrogate model system *(2, 60)*. MNV-1 has genetic, biochemical, and morphological similarities to human NoV *(7)*. The capsid structure, size (30 to 40 nm), replication cycle, shape, and genomic organization of MNV are very similar to those of human NoV *(24, 51, 60)*; thus, it has been used commonly in NoV research.

We also demonstrated that LGEO suppresses human NoV replication in HG23 cells in addition to inhibiting NoV viral infectivity. Thus, LGEO has dual inhibitory effects on NoV. LGEO suppressed the replication of the human NoV replicon in HG23 cells up to 58% compared with that in control samples, which implies that LGEO suppresses viral infection by directly interacting with NoV viral particles and reduces viral replication by acting on the host cells. Together, these suggest the potential application of LGEO on food or for the purpose of personal hygiene. HG23 cells were created by transfecting RNA transcripts containing the nearly complete human NoV genome, of which the VP1 gene of human NoV is replaced with a neomycin-resistant gene *(8)*; thus, the HG23 cell has been widely used in the investigation of human NoV replication mechanisms, such as the inhibition of the replicative enzymes and nonstructural proteins *(42)*. Our results showed that LGEO significantly suppressed human NoV replication and, thus, have a dual anti-NoV activity: inactivation of NoV infection and suppression of infected NoV replication in the host mammalian cells. We do not yet understand the mechanism of NoV replication by LGEO. Our preliminary results using HG23 cells showed that LGEO did not directly affect NoV replication machinery in HG23 cells depleting the guanosine nucleotide (data not shown). It has been reported that reduced cholesterol biosynthesis in host cells could inhibit human NoV replication; however, our results also showed that LGEO did not affect key gene expressions in cholesterol metabolism in HG23 cells (data not shown). LGEO may alter cytokine expression, such as interferon-γ, to suppress human NoV replication. We will investigate the possibility in the future.

The inhibitory effects of LGEO on MNV were further confirmed in an established persistent MNV-infected mouse model. The results showed that LGEO pretreatment at 4°C for 72 h significantly suppressed MNV infection, and the effect was sustained until day 7. Thus, pretreatment of virus with LGEO was effective at reducing MNV in vivo potentially by the dual inhibitory mechanisms of LGEO. The effect of LGEO pretreatment on NoV infectivity was higher at 25 and 37°C rather than at 4°C (Fig. 3). NoV particles are widely known to be unstable at an ambient or higher temperature, which is why the NoV outbreak is prevalent in winter not in summer. The untreated NoV particles can be naturally inactivated at those temperatures for hours (Fig. 3C and 3E); therefore, we chose the 4°C as the inactivation temperature for the in vivo experiment. Refrigerating temperature (4°C) is the targeted temperature in food processing and real NoV-related outbreak problems. We showed that LGEO can inactivate MNV after 72 h of exposure at 4°C (Fig. 1B), and we confirmed this effect of LGEO is maintained in the in vivo state. To our knowledge, this is the first report that showed the in vitro inactivation at low temperature is effective in real infection in the in vivo model.

Collectively, our results indicate that LGEO has anti-MNV activity both in vitro and in vivo. Citral was found to be an active compound within LGEO. LGEO may have a dual inhibitory effect by directly interacting with MNV viral particles and indirectly acting on the host cell to suppress viral replication. LGEO could potentially be used as a preventive food additive against NoV infection.

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