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Mechanisms of Antiviral Action of Plant Antimicrobials against Murine Norovirus

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Numerous plant compounds have antibacterial or antiviral properties; however, limited research has been conducted with nonenveloped viruses. The efficacies of allspice oil, lemongrass oil, and citral were evaluated against the nonenveloped murine norovirus (MNV), a human norovirus surrogate. The antiviral mechanisms of action were also examined using an RNase I protection assay, a host cell binding assay, and transmission electron microscopy. All three antimicrobials produced significant reductions ($P \leq 0.05$) in viral infectivity within 6 h of exposure (0.90 $\log_{10}$ to 1.88 $\log_{10}$). After 24 h, the reductions were 2.74, 3.00, and 3.41 $\log_{10}$ for lemongrass oil, citral, and allspice oil, respectively. The antiviral effect of allspice oil was both time and concentration dependent; the effects of lemongrass oil and citral were time dependent. Based on the RNase I assay, allspice oil appeared to act directly upon the viral capsid and RNA. The capsids enlarged from ≤35 nm to up to 75 nm following treatment. MNV adsorption to host cells was not significantly affected. Alternatively, the capsid remained intact following exposure to lemongrass oil and citral, which appeared to coat the capsid, causing nonspecific and nonproductive binding to host cells that did not lead to successful infection. Such contrasting effects between allspice oil and both lemongrass oil and citral suggest that though different plant compounds may yield similar reductions in virus infectivity, the mechanisms of inactivation may be highly varied and specific to the antimicrobial. This study demonstrates the antiviral properties of allspice oil, lemongrass oil, and citral against MNV and thus indicates their potential as natural food and surface sanitizers to control noroviruses.

Human noroviruses (NoV) cause illness in an estimated 19 to 21 million people in the United States each year, resulting in 56,000 to 71,000 hospitalizations and 570 to 800 deaths (1). In addition, NoV is responsible for 73% to 95% of epidemic nonbacterial gastroenteritis cases worldwide (2). NoV is the leading cause of food-borne illness in the United States (3), responsible for more than 58% of all food-borne cases with known etiologies (4) and 49% of all food-borne outbreaks of gastroenteritis (1).

NoV outbreaks have occurred in various settings, including hospitals, assisted-living communities, military barracks (5), cruise ships, schools, restaurants, and family dinners (1). The transmission of NoV may occur via a variety of routes, such as through contaminated food, water, or fomites (inanimate surfaces) (6). In a study of NoV outbreaks, 607 of 680 (89%) were linked to person-to-person transmission (7) that often involved poor hand hygiene as well as surface-to-surface transmission (8). In addition, successive NoV outbreaks on cruise ships have strongly implicated environmental contamination (8). Viruses that cause symptoms such as vomiting and diarrhea, particularly with sudden onset such as with NoV, are likely to contaminate the environment, contributing to their transmission via fomites.

In general, viruses without envelopes, such as NoV (a calicivirus), are resistant to various environmental conditions and antimicrobials (8, 9). The protein capsid of human NoV is resistant to both lipophilic disinfectants (e.g., quaternary ammonium compounds) and solvents (10, 11), and the virus may survive for weeks to months on surfaces at ambient temperatures (12). Under dry conditions at room temperature, feline calicivirus may persist for up to 28 days (13). Rabbit hemorrhagic disease virus (also a calicivirus) survives for at least 105 days under similar conditions (14). Such stability in the environment contributes to the role of fomites in the transmission of NoV.

There is currently no practical method for culturing NoV (15, 16, 17); therefore, several related viruses, including murine norovirus (MNV) (18), feline calicivirus (FCV) (19, 20, 21), Tulane virus (a simian calicivirus) (22, 23), and porcine sapovirus (a swine calicivirus) (24, 25), have been used in laboratory studies as surrogates for NoV. MNV is the most widely accepted surrogate, as it is the most closely related virus (the only culturable virus within the genus *Norovirus*) and is similar in its size, capsid structure, genomic organization, and replication cycle to NoV (18, 26, 27). Like NoV, MNV is resistant to a wide pH range and to inactivation by heat, organic solvents, and antimicrobials (28, 29). The use of MNV as a NoV surrogate has also been supported by previous studies conducted with various disinfectants using multiple NoV surrogates (28, 29).

Plant essential oils are complex mixtures of volatile and lipophilic secondary metabolites that are primarily responsible for a plant's fragrant properties (30). Numerous plant essential oils and extracts may be found in the average household kitchen; such common usage has led to many being generally recognized as safe (GRAS) for human exposure and/or consumption (31, 32, 33, 34). Many plant essential oils, extracts, and their individual components have been shown to possess antibacterial properties in previous studies (30, 34, 35, 36, 37, 38, 39, 40, 41). Some have also

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been shown to have antifungal (30, 42, 43, 44, 45) and antiviral (30, 46, 47, 48, 49, 50, 51, 52, 53, 54) properties.

The majority of such antiviral research has been directed toward clinically relevant enveloped viruses (30, 39, 47, 51, 54, 55, 56, 57, 58, 59, 60, 61, 62), such as herpes simplex virus 1 (HSV-1) (48, 52, 53) and influenza A virus (46, 49, 50), with only limited work performed using nonenveloped viruses such as NoV. A few studies have compared the antiviral efficacies of plant antimicrobials with both enveloped and nonenveloped viruses. The observed antiviral effect has usually been greater for enveloped viruses. For example, in a study by Siddiqui et al. (63), oregano oil and clove oil were effective against enveloped viruses (HSV-1 and Newcastle disease virus) but not against nonenveloped viruses (poliovirus type 1 [PV1] and adenovirus type 3). In another study, hydroxytyrosol, a phenolic compound extracted from olive tree leaves, was effective against several strains of enveloped influenza A virus and Newcastle disease virus but was not effective against either of the nonenveloped viruses bovine rotavirus group A and fowl adenovirus (50).

In recent years, a few researchers have focused their efforts on examining the efficacy of plant antimicrobials against nonenveloped viral pathogens or their surrogates. Tait et al. (64) found homoisoflavonoids to be effective against several enteroviruses, including coxsackieviruses (B1, B3, B4, and A9) and echovirus 30, but not against PV1. In a study by Cermelli et al. (65), eucalyptus essential oil was found to be ineffective against adenovirus. A few studies have been conducted with juices and extracts (or their active components) against NoV surrogates (MNV and FCV) and the bacteriophages MS2 and ϕX174 (also sometimes used as enteric virus surrogates). For instance, grape seed extract (66, 67), cranberry juice (68, 69), cranberry proanthocyanidins (PAC) (68), pomegranate juice (70), and pomegranate phenolic extracts (PPE) (70) were evaluated in several studies. MNV was typically more resistant than the other NoV surrogates for all of these plant antimicrobials. Treatment with grape seed extract reduced NoV (strain GII.4) specific binding to Caco-2 cells by >1.0 $\log_{10}$ genomic copies/ml (67) and hepatitis A virus (HAV) cell culture infectivity by up to 2.89 $\log_{10}$ PFU/ml (66) in separate studies. Elizaquível et al. (71) studied the antiviral efficacies of oregano, clove, and zataria oils against FCV and MNV at 4°C and 37°C. Concentrations of 2% oregano oil, 1% clove oil, and 0.1% zataria oil were not as effective against MNV at 4°C as at 37°C. The oils were not effective against FCV at 4°C (≤0.25-$\log_{10}$ reduction) but were effective at 37°C (3.8-, 3.8-, and 4.5-$\log_{10}$ reductions in FCV for oregano, clove, and zataria oils, respectively). In a recent study, carvacrol (found in oregano oil) was found to be effective against MNV, with reductions in cell culture infectivity approaching 4 $\log_{10}$ within 1 h of exposure (72).

In the current study, we employed MNV (strain S7-PP3) as a surrogate for human NoV in laboratory experiments to determine the antiviral efficacies of allspice and lemongrass oils and also of citral, one of the main active components in lemongrass oil. Attempts were also undertaken to elucidate the mechanism(s) of antiviral action of these plant compounds.

MATERIALS AND METHODS

Viruses and cells. MNV (strain S7-PP3), obtained from Yukinobu Tohya (Department of Veterinary Medicine, Nihon University, Kanagawa, Japan), was propagated on RAW 264.7 (ATCC TIB-71) cell line monolayers with Dulbecco's modified Eagle medium (DMEM; Mediatech Inc., Manassas, VA) containing 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT), 10 mM HEPES buffer (Mediatech Inc.), 0.113% sodium bicarbonate (Fisher Scientific, Fair Lawn, NJ), and 1.0% antibiotic-antimycotic (Mediatech Inc.). The cells were incubated at 37°C with 5% CO_2 as described previously (16). MNV was concentrated and purified using polyethylene glycol precipitation and Vertrel XF extraction to promote monodispersion of the virus and the removal of lipids (73, 74). The virus stocks were stored at −80°C until used in experiments.

Viral titrations were performed using the Reed-Muench method (75) to determine the viral dilution in which 50% of the wells containing cells were visibly affected (the 50% tissue culture infective dose [$TCID_{50}$]). Serial 10-fold dilutions of the virus sample were assayed in 96-well tissue culture plates (Nunclon, Roskilde, Denmark) containing RAW 264.7 cell monolayers and 50 μl of DMEM containing 10% FBS (DMEM-FBS), with incubation at 37°C with 5% CO_2 as described above. A volume of 50 μl of each dilution was used to inoculate eight replicate wells to ensure adequate precision of the assay. Each well was checked every day for 5 days for cytopathic effects (CPE). The highest dilution in which >50% of the wells exhibited CPE was used to determine the virus $TCID_{50}$/ml. The use of such a method for MNV has been widely reported in the literature (25, 71, 72, 76, 77, 78, 79).

Poliovirus type 1 (PV1; strain LSc-2ab) was obtained from the Department of Virology and Epidemiology at the Baylor College of Medicine (Houston, TX) and was included as a process control in several experiments. PV1 was propagated on monolayers of BGM cells (Buffalo green monkey kidney cells) obtained from D. Dahling (Environmental Protection Agency, Cincinnati, OH) with minimal essential medium (MEM; Irvine Scientific, Santa Ana, CA) containing 5% calf serum (HyClone Laboratories) with an incubation temperature of 37°C and an atmosphere of 5% CO_2. PV1 was purified in the same manner as described previously for MNV, and the titer was determined using 10-fold serial dilutions in plaque-forming assays on BGM cell monolayers as described by Bidawid et al. (80).

Antimicrobial preparation. Lemongrass and allspice oils were obtained from Lhasa Karnak Herbal Co. (Berkeley, CA). No information was available regarding the concentration of citral in this specific lemongrass oil product; however, citral may account for up to 85% of the composition of lemongrass oil (81). Purified citral (mixture of *cis* and *trans*, >96%) was purchased from Sigma-Aldrich (St. Louis, MO). The antimicrobials were diluted to the specific concentrations used in the experiments with sterile phosphate-buffered saline (PBS; pH 7.4; Sigma-Aldrich). Since alcohol could potentially enhance the antimicrobial effect of a solution, it was not used in these experiments to suspend these viscous antimicrobials. Though the oils did not dissolve completely in the PBS, the results from these experiments were found to be consistent and repeatable.

Antimicrobial efficacy experiments. Allspice oil, lemongrass oil, and citral were evaluated in separate experiments at concentrations of 2.0% and 4.0% (vol/vol). The experiments were performed at room temperature (~24°C) in triplicate in PBS (1-ml volume in 5-ml polystyrene tubes) (Becton, Dickinson and Company, Franklin Lakes, NJ). MNV was added separately to each of the tubes (to a final concentration of ~10^6 to 10^7 $TCID_{50}$/ml), and the tubes were placed on an orbital shaker (model G33; New Brunswick Scientific, Edison, NJ) with agitation at 300 rpm. Control tubes (no antimicrobials added) containing MNV in PBS were also included in each experiment. The control tubes were sampled immediately (0 h) by removing 100 μl from each and placing this volume in 900 μl of DMEM-FBS. At time intervals of 0.5, 6, and 24 h of exposure, 100-μl samples were removed from each tube and diluted in 900 μl of DMEM-FBS to neutralize the antimicrobials. All samples were placed at −80°C until subsequent assays were performed using the $TCID_{50}$ cell culture method (as described previously) to determine the infectious virus titer.

Antimicrobial neutralization and cytotoxicity experiments. Neutralization tests were performed with both the 2.0% and 4.0% concentrations of lemongrass oil, citral, and allspice oil in which a PBS solution

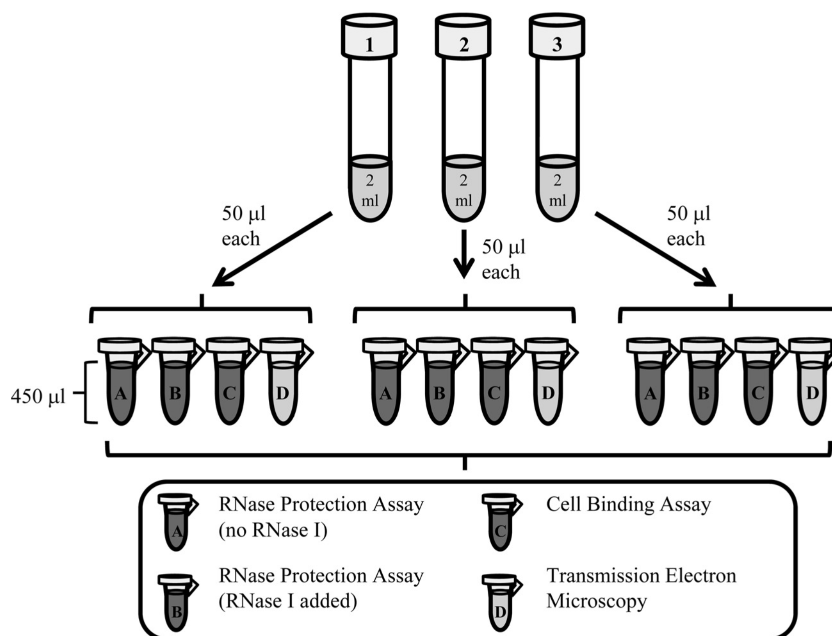

FIG 1 Sampling strategy for mechanism of action experiment. At each time exposure, four 50-μl volumes were removed from each of the replicate test tubes (1, 2, and 3) and placed into separate microcentrifuge tubes containing 450 μl of either Dulbecco's modified Eagle medium with 10% fetal bovine serum (tubes A, B, and C) or phosphate-buffered saline (tube D). These four tubes were used in subsequent experiments or assays (described in box) to determine the antiviral mechanism(s) of action.

containing the desired concentration of the antimicrobial was placed into either DMEM-FBS or PBS (1 ml into 9 ml). The solution was mixed thoroughly, and then approximately 1×10^7 $TCID_{50}$ of MNV was added. The solution was mixed again and then allowed to sit for 5 min at room temperature. Tenfold serial dilutions of the solutions were assayed on RAW 264.7 cells as described previously. If the antimicrobials were completely neutralized in the DMEM-FBS or PBS solution, it was expected that there would be no reduction in MNV numbers in comparison to the controls with either PBS or DMEM-FBS alone.

In a separate experiment, 10-fold serial dilutions of the 2.0% and 4.0% concentrations of the lemongrass oil, citral, and allspice oil were added to RAW 264.7 cells which were then examined daily for 6 days for any signs of cell toxicity.

Mechanism-of-action experiments. To determine the mechanism(s) of antiviral action, another set of tests was performed. Each antimicrobial was added to three replicate test tubes containing approximately 1.0×10^6 $TCID_{50}$/ml of MNV in 2 ml of PBS and a 4.0% (vol/vol) concentration of the plant antimicrobial (i.e., allspice oil, lemongrass oil, or citral). These were tested as described previously. After each exposure period (0.5, 6, or 24 h), volumes of 50 μl were removed from each tube (including the control tubes) and placed separately into four microcentrifuge tubes (replicates), three containing 450 μl of DMEM-FBS and one that contained 450 μl of PBS (Fig. 1). Two of the tubes containing DMEM-FBS (Fig. 1, tubes A and B) were used immediately in an RNase I protection experiment to assess the virus capsid integrity. The other two tubes were stored at −80°C until used in a cell binding experiment (Fig. 1, tube C with DMEM-FBS) to assess the ability of the treated MNV to bind to host RAW 264.7 cells and for transmission electron microscopy (TEM) imaging (Fig. 1, tube D with PBS) to directly observe any physical changes to the MNV particles following antimicrobial treatments. These experiments are described in detail below.

(i) RNase I protection experiment. To one of these tubes (Fig. 1, tube B), 1.0 μl of RNase I (100 U; Ambion Inc., Austin, TX) was added. This tube and another (Fig. 1, tube A without RNase I) were then placed in a 37°C incubator for 30 min. RNase I should degrade any viral RNA that is exposed to the environment (e.g., if the RNA is no longer protected by an intact capsid). The tube without RNase I was included as a control. Following the 30-min incubation, the samples were immediately placed at −80°C to halt the enzymatic activity and held at this temperature for storage.

Immediately prior to the nucleic acid extraction step, the samples were thawed and 200 μl of each was added (separately) to 600 μl of the ZR viral RNA buffer of the ZR viral RNA kits (Zymo Research, Irvine, CA). This freeze-thaw step should denature the RNase I enzyme. The ZR viral RNA buffer should also denature the enzyme, and β-mercaptoethanol (BME) was added to each sample to inhibit RNases (as per the manufacturer's instructions). A volume of 2 μl of purified PV1 (~2.0×10^6 genome copies total) was added to each of the samples as a process control (explained in detail below). The viral RNA (from both MNV and PV1) was purified from each sample using the ZR viral RNA kits according to the manufacturer's protocol, with the exception that the final volume was adjusted to 20 μl.

The reverse transcription (RT) step was performed using high-capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA) in the following manner. Three microliters of extracted virus RNA was added to 3 μl of RT mixture containing 0.6 μl of 10× reverse transcription buffer, 0.24 μl of 25× deoxynucleoside triphosphate (dNTPs), 0.6 μl of 10× random hexamers, 15 U of MultiScribe reverse transcriptase, and 6 U of RNase inhibitor (Applied Biosystems). This RT reaction mixture was incubated at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min to inactivate the enzyme.

A TaqMan-based quantitative PCR (qPCR) assay which targets a 129-nucleotide sequence in the open reading frame 1 (ORF1)-ORF2 junction region of MNV was performed in a 25-μl reaction volume containing 2.5 μl of cDNA from the RT reaction, 12.5 μl of LightCycler 480 Probes Master (Roche Diagnostics, Mannheim, Germany), 400 nM (each) concentrations of the primers MNV-S (5′-CCGCAGGAACGCTCAGCAG-3′) and MNV-AS (5′-GGYTGAATGGGGACGGCCTG-3′), and 300 nM TaqMan MGB probe MNV-TP (5′-FAM-ATGAGTGATGGCGCA-MGB-NFQ-3′) as described previously (76). The qPCR amplification was

TABLE 1 Controls included in the cell binding assay for murine norovirus (MNV) treated with either lemongrass oil, citral, or allspice oil

Control(s)	Description	Purpose
Positive control	RAW 264.7 cells seeded with ~4.1×10^5 copies of MNV from the 0-min control samples (no antimicrobials present)	To determine the normal amt of cell binding by MNV
Negative control 1	RAW 264.7 cells without virus	To ensure that the nucleic acid from the RAW 264.7 cells is not amplified by the MNV or the poliovirus 1 RT-qPCR
Negative control 2	Wells without cells seeded with ~4.1×10^5 copies of MNV from the control samples from 0 min	To determine if MNV is able to bind nonspecifically to the plastic of the 24-well plates
Naked RNA control 1	RAW 264.7 cells seeded with ~3.5×10^6 copies of MNV-RNA extracted from the control samples at 0 min	To determine if the naked MNV RNA is able to bind directly to the RAW 264.7 cells
Naked RNA control 2	Wells without cells seeded with ~3.5×10^6 copies of MNV RNA extracted from the control samples at 0 min	To determine if the naked MNV RNA is able to bind directly to the plastic of the 24-well plates
No-cell controls 1–3	Wells without cells seeded with MNV treated with 4.0% lemongrass oil (control 1), citral (control 2), or allspice oil (control 3) from 24 h	To determine if the treated MNV particles are able to bind nonspecifically to the plastic of the 24-well plates

performed using a LightCycler 480 Real-Time PCR Instrument II (Roche Diagnostics) with the following conditions: denaturation at 95°C for 15 min to activate the DNA polymerase, followed by 50 cycles of amplification with denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Tenfold serial dilutions (from 10^2 to 10^8 copies per PCR tube) of the standard plasmid DNA containing an insert of approximately 500 nucleotides including the ORF1-ORF2 junction region of the MNV-S7 PP3 strain were used to generate a standard curve for the quantification of MNV cDNA copy numbers (76). The average copy number of two PCR tubes was used in these calculations. The amplification data were analyzed using the LightCycler 480 software (version 1.5; Roche Diagnostics).

A subsequent separate qPCR assay was performed for the PV1 process control (an enterovirus) using the cDNA from the RT as the template. The qPCR was performed as described previously for MNV, but with primers and probes specific to the enteroviruses (forward primer EV1F [400 nM], 5′-CCCTGAATGCGGCTAAT-3′; reverse primer EV1R [400 nM], 5′-TGTCACCATAAGCAGCCA-3′; and EV probe [120 nM], 5′-FAM [6-carboxyfluorescein]-ACGGACACCCAAAGTAGTCGGTTC-BHQ [Black Hole Quencher]-1-3′) and with the following conditions: denaturation at 95°C for 10 min, followed by 50 cycles with denaturation at 94°C for 15 s and annealing and extension at 60°C for 1 min. This assay results in a 143-bp product (82). Since 2 μl of the PV1 process control was added prior to nucleic acid extraction to each of the samples, the PV1 concentration (copy number) that was determined by qPCR in the control samples (which did not contain any extraction-, RT-, and/or qPCR-inhibiting substances) was used to determine if there was any inhibition in the samples with antimicrobials (leading to reduced MNV amplification and an underestimate of the viral copy number).

(ii) Cell binding experiment. A cell binding assay was performed to assess the ability of the MNV treated with the plant antimicrobials to bind to host cells. RAW 264.7 cell monolayers were prepared in 24-well plates, and the growth medium was carefully aspirated from each well. The cells were rinsed with Tris-buffered saline (TBS; 2.53 g/liter of Trizma base, 6.54 g/liter of NaCl, 0.3 g/liter of KCl, 0.046 g/liter of Na_2HPO_4 [anhydrous], 4 liters of ultrapure H_2O), and then the TBS was removed. Following this, 100 μl from the third replicate tube from each sample (Fig. 1, tube C) was added to two wells and the plate was incubated at 4°C for 1 h to prevent the virus from entering the cells (62), with gentle agitation every 15 min to ensure that the cells remained covered to prevent them from drying. In addition to the MNV treated with allspice oil, lemongrass oil, or citral, numerous controls were also included in duplicate wells (Table 1).

After the 1-h incubation period, the cells were washed three times with TBS (with careful aspiration as before) to remove any unbound virus particles or virus RNA, and then an additional 198 μl of PBS was added to each well. This was followed first by the addition of 600 μl of the extraction buffer of the ZR viral RNA kit (Zymo Research) and finally by addition of 2 μl of the PV1 process control (~2.0×10^8 total genome copies). This entire 800-μl volume was then extracted using the ZR viral RNA kits as described previously. The extracts were used as the template in the MNV and PV1 RT-qPCR assays as described previously. The 1-h incubation period at 4°C does not allow for viral replication or the development of any CPE in the cells. This therefore was used to determine the number of viruses that were able to bind to the cells (while discounting those that did not bind), but it did not measure successful infection of the cells or assess virus viability.

(iii) TEM imaging. To directly observe any structural changes to the MNV particles following treatment with the plant antimicrobials, TEM imaging was performed. A drop (5 to 10 μl) of each sample from the 24-h exposure from the mechanism of action experiments (Fig. 1, tube D with PBS) was applied to separate glow discharge carbon-coated EM grids. The grids were then stained with 2% aqueous uranyl acetate for 3 min, dried, and examined using an FEI CM12S TEM (FEI Electronics Instruments, Co., Hillsboro, OR) operated at 80 kV. The images were captured using an AMT 420 camera (Advanced Microscopy Techniques, Woburn, MA). The 24-h exposure was chosen since it was likely to produce the greatest antiviral effects which could then be observed under TEM to better understand the mechanisms of action.

Statistical analyses. For the cell culture infectivity (antimicrobial efficacy) assays, the data were reported as the logarithmic reduction in infectivity using the formula $-\log_{10}(N_t/N_0)$, where N_0 was the concentration of MNV particles measured via cell culture infectivity at time zero and N_t was the infectious particle concentration at time t. A two-tailed Student t test was used to compare the reductions in cell culture infectivity observed with the controls or with the antimicrobial treatments. The reductions observed for the antimicrobial treatments at each time exposure were compared to the reduction in the controls, if any. Differences were considered statistically significant if the t test resulted in a P value of ≤ 0.05. Differences between the reductions in cell culture infectivity observed between the two concentrations of each antimicrobial were also evaluated for statistical significance.

In order to allow for statistical comparisons between the reductions in cell culture infectivity observed with different plant antimicrobials in separate experiments, the average reduction in each experiment for the controls (after 24 h) was subtracted from the reductions reported for each sample exposed to an antimicrobial (for all time exposure intervals) in order to normalize the reductions. These normalized data were used only for the Student t tests; the values reported in the tables are thus the actual, nonnormalized results.

For the RNase I protection assays, the data were reported as the $\log_{10}$ reduction [$-\log_{10}(N_t/N_0)$] in the amplifiable virus copy number as determined by RT-qPCR in comparison to the untreated controls. A two-tailed Student t test was used to compare any differences between the

TABLE 2 Antimicrobial efficacy of lemongrass oil[a]

Time (h)	Log_{10} reduction (mean ± SD) Control	2.0% (vol/vol) lemongrass oil	4.0% (vol/vol) lemongrass oil
0.5	0.46 ± 0.4	0.38 ± 0.3	0.59 ± 0.1
6	0.17 ± 0.3	0.74[b,c] ± 0.0	0.90[b,c] ± 0.0
24	0.01 ± 0.0	2.19[b,c] ± 0.1	2.74[b,c] ± 0.2

[a] Results shown are the log_{10} reductions in cell culture infectivity of murine norovirus (initial titer, 6.64×10^6 $TCID_{50}$/ml) after various time exposures to lemongrass oil at two concentrations. The experiment was conducted in triplicate.
[b] Reduction was statistically significant ($P \leq 0.05$) in comparison to the control (with no antimicrobials) at the same time exposure.
[c] Reductions were significantly different ($P \leq 0.05$) between 2.0% and 4.0% lemongrass oil.

TABLE 4 Antimicrobial efficacy of allspice oil[a]

Time (h)	Log_{10} reduction (mean ± SD) Control	2.0% (vol/vol) allspice	4.0% (vol/vol) allspice
0.5	0.22 ± 0.2	0.22[c] ± 0.3	1.39[b,c] ± 0.4
6	0.28 ± 0.3	0.67[c] ± 0.2	1.83[b,c] ± 0.1
24	0.13 ± 0.2	2.97[b] ± 0.3	3.41[b] ± 0.4

[a] Results shown are the log_{10} reductions in cell culture infectivity of murine norovirus (initial titer, 4.21×10^6 $TCID_{50}$/ml) after various time exposures to allspice oil at two concentrations. The experiment was conducted in triplicate.
[b] Reduction was statistically significant ($P \leq 0.05$) in comparison to the control (with no antimicrobials) at the same time exposure.
[c] Reductions were significantly different ($P \leq 0.05$) between 2.0% and 4.0% allspice oil.

controls and the antimicrobial treatments. The reduction (if any) in the viral copy number in each sample was compared to the corresponding control samples (i.e., with or without RNase I). Also, comparisons were performed between the samples that had been treated with antimicrobials with or without subsequent RNase I digestion. Differences were considered statistically significant if the results of the Student *t* test indicated that the *P* value was ≤0.05.

RESULTS

Antimicrobial neutralization and cytotoxicity experiments. No reductions in MNV were observed in the neutralization tests with any of the plant antimicrobials in comparison to the DMEM-FBS or PBS controls (with no antimicrobials). Therefore, this method was confirmed to completely neutralize the antimicrobials at these concentrations. This dilution method was used for all subsequent assays.

Cell toxicity was observed in the RAW 264.7 cells with allspice oil, lemongrass oil, and citral in the 10^{-1} dilution wells. These 10^{-1} wells were therefore not included in the determination of the viral $TCID_{50}$/ml in the subsequent cell culture assays. The 10^{-2} wells were the lowest dilution which could be accurately read. This effectively increased the limit of detection of these assays 10-fold (to 6.3×10^2 $TCID_{50}$/ml).

Antimicrobial efficacy experiments. The antiviral efficacy of each plant antimicrobial was determined by comparison to the reductions (if any) in the cell culture infectivity of MNV observed in the controls (with no antimicrobials) at the same time interval. The results for lemongrass oil and its active component citral are shown in Tables 2 and 3, respectively. Concentrations of 2.0% and 4.0% were used for both antimicrobials. Both concentrations of lemongrass oil and citral produced significant reductions in MNV cell culture infectivity within 6 h of exposure in comparison to those of the controls ($P \leq 0.05$). The reductions observed with 4.0% lemongrass oil were significantly greater than those with the 2.0% concentration following 6 and 24 h of exposure ($P = 0.005$ and $P = 0.01$, respectively). No significant differences ($P > 0.05$) were found between the two citral concentrations for any of the exposure times. Both the lemongrass oil and the citral required 24 h to reach at least a 2.0-log_{10} reduction (range of 2.19 log_{10} to 3.00 log_{10}). There were no statistically significant differences ($P > 0.05$) between the reductions observed between lemongrass oil and citral for either concentration or for any of the exposure times.

TABLE 3 Antimicrobial efficacy of citral[a]

Time (h)	Log_{10} reduction (mean ± SD) Control	2.0% (vol/vol) citral	4.0% (vol/vol) citral
0.5	0.15 ± 0.3	0.67 ± 0.3	0.70 ± 0.4
6	0.05 ± 0.1	1.40[b] ± 0.5	1.88[b] ± 1.1
24	0.23 ± 0.3	2.40[b] ± 0.5	3.00[b] ± 0.3

[a] Results shown are the log_{10} reductions in cell culture infectivity of murine norovirus (initial titer, 1.79×10^6 $TCID_{50}$/ml) after various time exposures to citral at two concentrations. The experiment was conducted in triplicate.
[b] Reduction was statistically significant ($P \leq 0.05$) in comparison to the control (with no antimicrobials) at the same time exposure.

The antiviral efficacies of 2.0% and 4.0% allspice oil are shown in Table 4. The 4.0% concentration produced a significant reduction ($P = 0.014$ in comparison to the control) within 30 min of exposure, whereas the reduction observed with the 2.0% concentration was not significant within 30 min or 6 h of exposure ($P = 1.0$ and $P = 0.12$, respectively) but became significant after 24 h of exposure ($P = 0.001$). The reductions observed for the two concentrations were significantly different from each other after 30 min ($P = 0.01$) and 6 h ($P = 0.0005$) of exposure, but the reductions after 24 h of exposure (2.97 log_{10} and 3.41 log_{10} for the 2.0% and 4.0% allspice oil concentrations, respectively) were not ($P = 0.21$). Similar to the case with lemongrass oil and citral, reductions greater than 2.0 log_{10} were not observed until 24 h of exposure, though the 4.0% allspice oil yielded a reduction near to this within 6 h of exposure (1.83 log_{10}).

The reductions observed with 2.0% allspice oil and 2.0% lemongrass oil differed significantly ($P = 0.03$) after 24 h of exposure (2.97 log_{10} versus 2.19 log_{10}, respectively). The reductions observed with 4.0% allspice oil were greater than those with 4.0% lemongrass oil for all time exposures; the reductions were significantly greater after 30 min and 6 h ($P = 0.005$ and $P = 0.001$, respectively) but not after 24 h ($P = 0.09$). Therefore, allspice oil may have greater and faster-acting antiviral efficacy than lemongrass oil. No significant differences were found between citral and allspice oil at either concentration or any of the exposure times ($P > 0.05$), suggesting that these two antimicrobials have comparable antiviral efficacies against MNV.

RNase I protection experiment. An RNase I protection experiment was performed in order to assess if the MNV capsid was degraded by allspice oil, lemongrass oil, or citral. Two of the four replicate tubes from each sample (Fig. 1, tubes A and B) were used. One of these tubes (tube B) was treated with RNase I to digest any exposed viral RNA; both tubes (with and without RNase I digestion) were then subjected to RT-qPCR to determine the degrada-

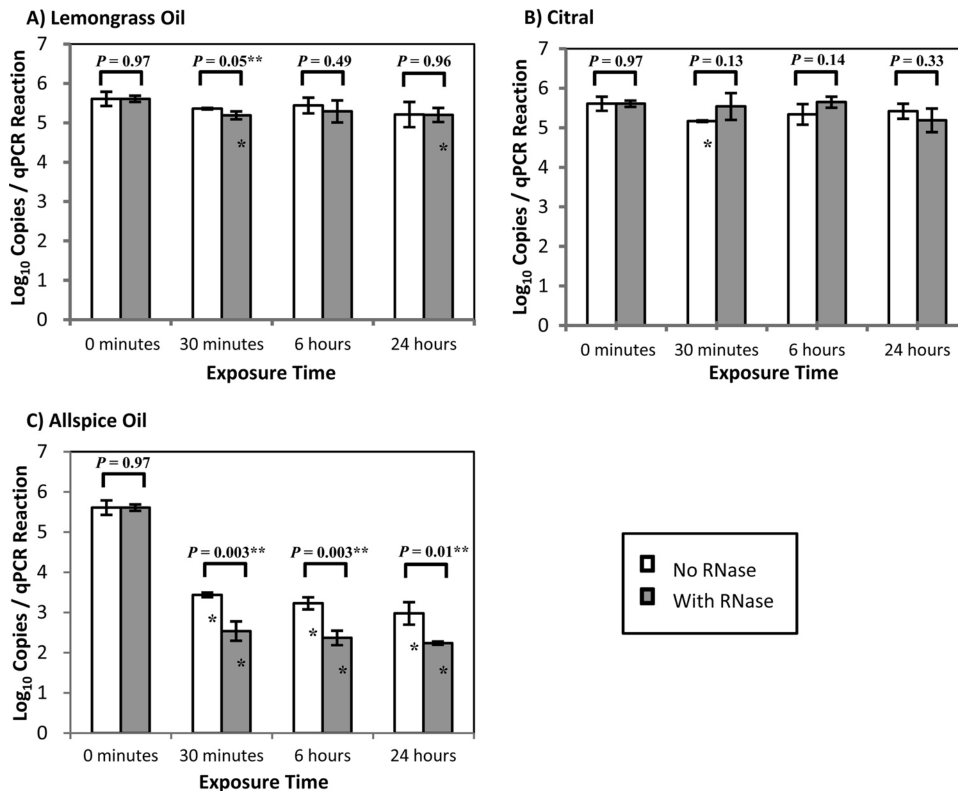

FIG 2 Results of the RNase I protection assay for MNV after exposure to a concentration of 4.0% of lemongrass oil (A), citral (B), or allspice oil (C). The $\log_{10}$ genome copy numbers of MNV RNA recovered were determined by RT-qPCR after exposure to each antimicrobial (for 30 min, 6 h, or 24 h) followed by RNase I digestion. The *P* values for the Student *t* tests comparing the $\log_{10}$ virus copy numbers recovered are also presented. Values with statistical significance ($P \leq 0.05$) for differences between the antimicrobial-treated samples and their corresponding untreated control (with or without RNase I digestion) are indicated with an asterisk; values with statistical significance ($P \leq 0.05$) for differences between the antimicrobial-treated samples either with or without RNase I digestion are indicated with double asterisks.

tion of the viral capsid as well as the viral RNA by comparing the amounts of amplifiable viral RNA in the digested and undigested samples to the corresponding controls (no antimicrobial treatment; with and without RNase I digestion).

The RT-qPCR results ($\log_{10}$ reduction in viral copy number) for the RNA protection experiment with all three antimicrobials (at a 4.0% concentration) are shown in Fig. 2. No reductions were observed in the controls (with no exposure to antimicrobials) after 24 h, regardless of whether they had been digested with RNase I (data not shown). For both lemongrass oil and citral, small reductions in virus RNA copy numbers ($\leq$0.44-$\log_{10}$ reductions with and without RNase I digestion) were observed (Fig. 2A and B). A few of these reductions were significant (indicated by single asterisks) in comparison to the corresponding (i.e., with or without RNase I digestion) no-antimicrobial control: the 30-min and 24-h exposures to lemongrass oil with RNase ($P = 0.005$ and $P = 0.02$, respectively) and the 30-min exposure to citral without RNase I digestion ($P = 0.01$). None of the reductions differed significantly between the samples treated with lemongrass oil or citral with and without RNase I digestion, with the exception of lemongrass oil after 30 min of exposure ($P = 0.05$; indicated by double asterisks).

All of the reductions observed for the samples treated with allspice oil were highly significant ($P \leq 0.0001$) in comparison to the no-antimicrobial controls, with more than 3- and 2-$\log_{10}$ reductions with and without RNase I digestion, respectively (single asterisk in Fig. 2C). Following treatment with allspice oil, the $\log_{10}$ reductions were greater in the samples that were subsequently digested with RNase I; these differences were statistically significant ($P \leq 0.05$; double asterisks in Fig. 2C) for all exposure times (i.e., 30 min, 6 h, and 24 h).

No inhibition was observed in the RNA extraction, the RT, or the qPCR steps with the PV1 process control for any of the samples from the RNase I protection experiment (data not shown).

Cell binding experiment. The results for the cell binding experiment to determine if the antimicrobials inhibited the ability of MNV to bind to host cells are shown in Table 5. Under the experimental conditions, the positive control (MNV with no antimicrobials) was able to bind to the RAW 264.7 cells; nevertheless, although each control well was originally inoculated with approximately 5.6 $\log_{10}$ MNV genome copies, only 2.7 $\log_{10}$ remained bound to the cells at the end of the assay. The multiple wash steps may have removed much of the MNV (or possibly cells with bound MNV) from the wells.

MNV did not bind nonspecifically to the cell culture plates either in the control (negative control 2 in Table 1; not detected by RT-qPCR; data not shown) or the 4.0% allspice oil-treated samples (no-cell control 3 in Table 1); however, MNV treated with 4.0% lemongrass oil (no-cell control 1 in Table 1) or 4.0% citral (no-cell control 2 in Table 1) was able to bind to the cell culture

TABLE 5 Results for the assay of binding of MNV to RAW 264.7 cell monolayers after various time exposures to 4.0% (vol/vol) lemongrass oil, citral, or allspice oil

Sample	Log_{10} MNV genome copy no./cell culture well (avg ± SD) after indicated antimicrobial exposure time (h)[a]			
	0	0.5	6	24
MNV with lemongrass oil, with cells	2.7 ± 0.3	1.8 ± 1.3	3.0 ± 0.2	3.1 ± 0.1
MNV with lemongrass oil, no cells[b]	—	—	—	2.6 ± 0.4
MNV with citral, with cells	2.7 ± 0.3	1.9 ± 1.4	2.9 ± 0.2	2.4 ± 1.2
MNV with citral, no cells[c]	—	—	—	2.5 ± 0.2
MNV with allspice oil, with cells	2.7 ± 0.3	2.8 ± 0.3	2.8 ± 0.1	2.2 ± 1.0
MNV with allspice oil, no cells[d]	—	—	—	ND

[a] Determined by RT-qPCR. —, not tested. ND, not detected by RT-qPCR.
[b] No-cell control 1 in Table 1.
[c] No-cell control 2 in Table 1.
[d] No-cell control 3 in Table 1.

plates (2.6 log_{10} and 2.5 log_{10} virus genome copies, respectively). The MNV RNA did not bind to the RAW 264.7 cells or the cell culture plate (naked RNA controls 1 and 2 in Table 1; not detected by RT-qPCR; data not shown). Finally, the extracted RNA from the RAW 264.7 cells did not result in nonspecific amplification in the RT-qPCR assays (negative control 1 in Table 1; data not shown).

The numbers of virus particles that were able to bind to the RAW 264.7 cells following treatment with 4.0% lemongrass oil and citral were initially (after 30 min of exposure) lower than the number of untreated viruses binding to cells in the control samples. Interestingly, the number of bound virus particles increased with longer exposures to both antimicrobials, particularly for the lemongrass oil treatment. This is directly opposite the cell culture infectivity (as shown in Table 2 and 3), which decreased over time.

In contrast, the number of 4.0% allspice oil-treated virus particles that were able to bind to the cells was initially comparable to that of the untreated control, 2.8 log_{10} genome copies per well after 30 min and 6 h of exposure, but fell to 2.2 log_{10} genome copies after 24 h of exposure.

No inhibition was observed with the extraction, RT, or qPCR steps for the PV1 process control for any of the samples from the cell binding experiment (data not shown). The values for the virus genome copy numbers determined by the RT-qPCR were considered accurate. Nevertheless, the number of bound viruses was likely overestimated to some degree due to the nonspecific direct binding of the lemongrass- and citral-treated virus particles to the plates (see no-cell control data in Table 5).

TEM imaging. TEM imaging was used to directly observe MNV particles following treatment with the plant antimicrobials to determine if there were any structural changes to the virus particles. The TEM images for the untreated MNV, the MNV after exposure to 4.0% lemongrass oil and 4.0% citral for 24 h, and the MNV after exposure to 4.0% allspice oil for 30 min are shown in Fig. 3. The MNV following a 24-h exposure to allspice oil was included in the original TEM imaging, yet very few particles (one or two per entire grid) could be found. In addition, there was also a larger amount of debris present, making it more difficult to identify MNV on the grids (images not shown). Therefore, the 30-min exposure, with presumably less damage to the virus particles from the effects of the antimicrobial, was examined.

Untreated MNV particles range from approximately 20 nm to 35 nm in diameter and have an icosahedral symmetry (appear spherical in most images) (Fig. 3A). The MNV particles exposed to allspice oil appeared to be slightly larger (~25 to 75 nm) but still morphologically similar to the untreated MNV control and seemingly intact (Fig. 3B). Following exposure to allspice oil for 24 h, the few virus particles that were observed were similar in size and appearance (~60 to 70 nm) (images not shown).

The MNV treated with lemongrass oil and its active component, citral, were greatly expanded in size. The virus particles following treatment with lemongrass oil ranged in size from approximately 100 to 500 nm, with an average size of ~300 nm (Fig. 3C). The MNV particles treated with citral were even larger, with an average size of ~600 nm (range of ~350 to 750 nm) (Fig. 3D). The MNV particles treated with these two antimicrobials appeared to be intact. In addition, there was an appreciable amount of increased texture to the surfaces of the treated virus particles in the TEM images. This appeared to be a buildup of small round components on the surface of the virus capsid rather than clumping of virus particles together, since the size of the textured particles was too small and the particles were too numerous to be MNV. No such large particles were observed in control grids which included only the antimicrobial in PBS and no virus particles.

DISCUSSION

In previous experiments, allspice oil, lemongrass oil, and citral have all been shown to have antimicrobial efficacy against *Escherichia coli*, with significant reductions of >4 log_{10} observed within 5 min of exposure (D. H. Gilling and K. R. Bright, unpublished data). In other studies, lemongrass oil has exhibited antibacterial activity against *Escherichia coli*, *Salmonella enterica*, *Serratia marcescens*, and *Staphylococcus aureus* (42, 83, 84, 85). Citral is a major component of lemongrass oil that is a natural mixture of two isomeric acyclic monoterpene aldehydes, geranial and neral. It has also been demonstrated to have antibacterial activity (83). Lemongrass oil has also been shown to possess antifungal activity against yeasts (42), molds, and dermatophytes (43), as well as antiviral activity against HSV-1 (47). Antiviral activity of citral against HSV-1 and yellow fever virus has also been demonstrated (51, 54). Allspice oil is used in bakery products and has antimicrobial, antioxidant, and medicinal properties (86). Allspice oil has been found to possess antibacterial efficacy against *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* (87).

In the current study, allspice oil, lemongrass oil, and citral were examined for their antiviral efficacies against MNV. All three produced significant reductions within 6 h of exposure; 4.0% allspice oil produced a significant reduction (in comparison to the untreated control with no antimicrobials) within 30 min of exposure. Despite this, reductions greater than 2 log_{10} were not observed until after 24 h of exposure with all three antimicrobials. The antimicrobial effect of allspice oil appeared to be both time and concentration dependent (i.e., greater reductions were observed with increasing exposure time or allspice oil concentration), whereas the most relevant factor in the antimicrobial efficacy of lemongrass oil and citral seemed to be the duration of exposure. The greatest reductions in cell infectivity were observed

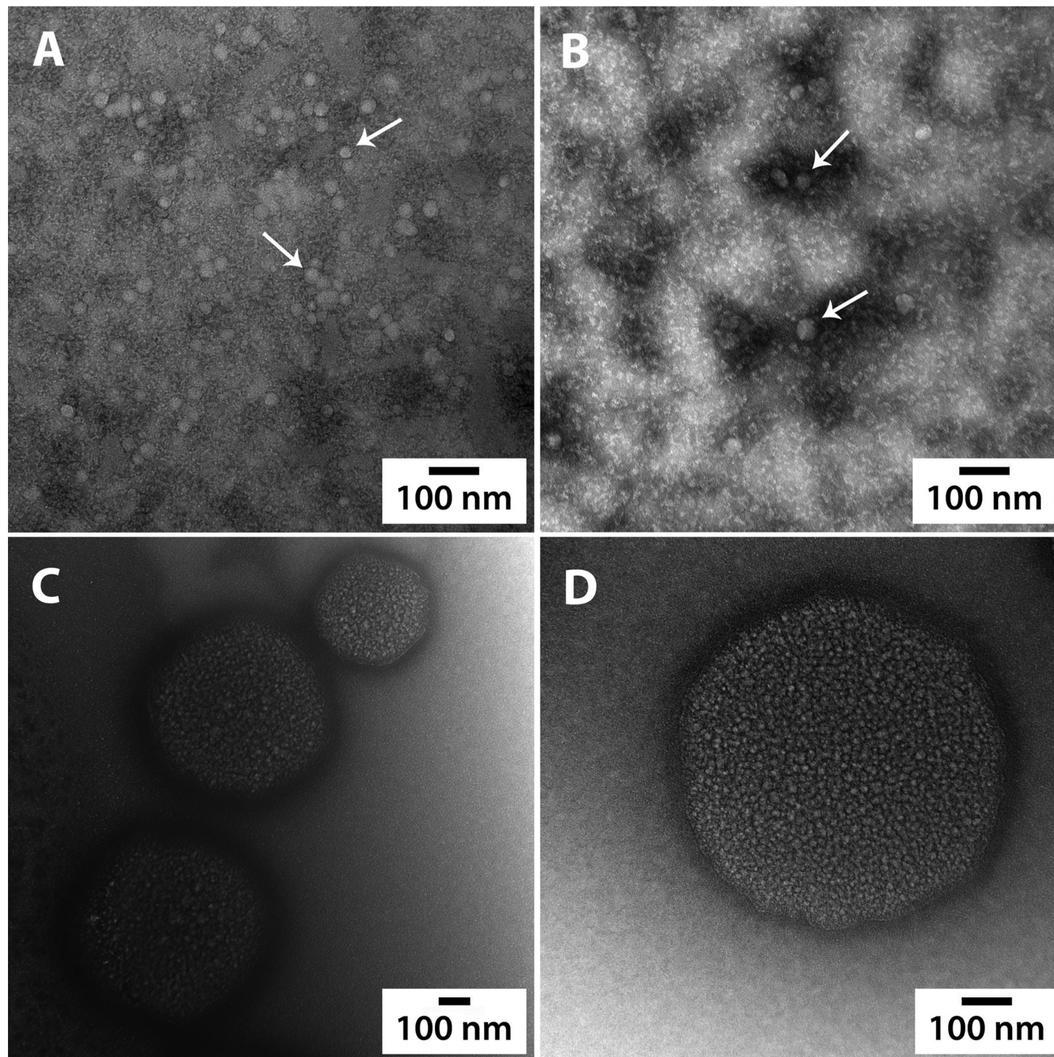

FIG 3 Transmission electron microscope images of MNV. (A) Untreated (no antimicrobials) MNV control (examples of MNV particles are indicated by arrows); (B) MNV following exposure to 4.0% allspice oil for 30 min (examples of MNV particles are indicated by arrows); (C) MNV following exposure to 4.0% lemongrass oil for 24 h; (D) MNV following exposure to 4.0% citral for 24 h.

for allspice oil, followed by citral and then by lemongrass oil. Not surprisingly, citral had greater antimicrobial efficacy than lemongrass oil. Essential oils are mixtures of numerous compounds; the active antimicrobial ingredient often accounts for more than 50% of the total chemical composition of the oil. Lemongrass oil contains multiple components, including citral (57.5%), citral diethylacetal (24.7%), limonene (6.4%), citral acetate (2.1%), myrcene (1.2%), and methyl heptenone (1.2%) (88); nonetheless, citral may account for up to 85% of the composition of lemongrass oil (81).

It is often difficult to distinguish between virus inactivation and the simple prevention of virus adsorption to host cells. To date, little is understood regarding the antiviral mechanisms of action for most plant antimicrobials. As the majority of this research has been conducted with clinical treatments in mind against medically relevant enveloped viruses, the focus has been on either the inhibition of viral adsorption to host cells or examination of the effectiveness of plant antimicrobials against intracellular viruses. Several studies have found that various plant antimicrobials seem to act directly on enveloped viruses (e.g., HSV-1 and HSV-2), since they do not appear to prevent adsorption of the viruses to host cells (39, 46, 47, 52, 89). Wen et al. (90) found that two phytocompounds, betulinic acid and savinin, appeared to inhibit postbinding entry of severe acute respiratory syndrome (SARS) coronavirus into cells. Evidence from TEM imaging indicates that some plant antimicrobials may act directly upon the virus envelope (50, 53, 63).

Often, prior exposure of the enveloped viruses to the antimicrobial may prevent cell infection (e.g., HSV-1, HSV-2, and dengue viruses), yet the antimicrobials are ineffective against the viruses once they are located within cells (39, 47, 52, 60). Alternatively, in a few studies, the plant antimicrobial has been found to be somewhat effective against the intracellular state of enveloped viruses such as HSV-1, bovine herpesvirus type 2, human immunodeficiency virus type 1, influenza A virus, influenza B virus, and human respiratory syncytial virus (62, 91, 92, 93, 94); however, this effect was usually only observed within a short period following viral uptake into the cells (62, 91, 92). Some re-

searchers have found that the plant antimicrobial inhibited viral uncoating by interfering with endosome-lysosome fusion or the acidification of the intralysosomal compartment (62, 91, 93). Such varying effects suggest that different plant antimicrobials may exhibit distinct mechanisms of antiviral action against enveloped viruses.

Though some of the antiviral mechanisms of action of plant antimicrobials may be shared between enveloped and nonenveloped viruses, others may be unrelated. To some extent, antimicrobials that inactivate small enteric RNA viruses such as the picornaviruses (e.g., poliovirus and hepatitis A virus), astroviruses, and caliciviruses (e.g., NoV and MNV) all act on the virus capsid (95). The capsid in such nonenveloped viruses serves to protect the integrity of the viral nucleic acid and to initiate infection by adsorbing to the host cell (95). The viral RNA may be unaffected even though the virus is no longer infectious (95). Studies in recent years have attempted to elucidate the mechanisms of action of plant antimicrobials against nonenveloped viruses. As with enveloped viruses, plant antimicrobials often are effective against nonenveloped viruses when used prior to infection but have limited efficacy against intracellular viruses (96, 97). In a study by Cermelli et al. (65), eucalyptus essential oil was not effective against intracellular adenovirus and only minimally effective against intracellular mumps virus.

Several antimicrobials appear to directly modify the virus capsid. For instance, in a study by Su et al. (69), feline calicivirus treated with cranberry juice and proanthocyanidins appeared to be damaged under TEM. Lipson et al. (98) also observed anomalous rotavirus SA-11 virus-like particles following treatment with cranberry juice. NoV GII.4 virus-like particles treated with grape seed extract exhibited clumping, particle inflation (to twice the original size), and deformation under TEM (67). MNV treated with oregano oil and its primary active component, carvacrol, were greatly expanded in size under TEM (up to 2× for oregano oil and >20× for carvacrol), with visible capsid disintegration in the carvacrol-treated samples (72).

In the current study, multiple experiments or assays were performed in an attempt to determine the mechanism(s) of antiviral efficacy for the three plant antimicrobials tested. These included (i) a cell culture infectivity assay, (ii) an RNase I protection experiment, (iii) a host cell binding experiment, and (iv) TEM imaging. Each experiment or assay provides a particular piece of information that, when evaluated in conjunction with information provided by the others, creates a more complete picture of the antiviral mechanism(s) that lead to a reduction in MNV infectivity.

The $\log_{10}$ reductions for allspice oil were significantly greater ($P \leq 0.05$) in the samples that had been treated with RNase I (Fig. 2C). This suggests that there was at least some degradation of the MNV capsid. This was supported by the observation that the controls that had not been exposed to any antimicrobials were unaffected by digestion with RNase I. The viral RNA was protected from RNase I digestion in these controls, and therefore, the capsid was still intact (99, 100). The reductions observed for the allspice oil treatment followed by RNase I digestion were typically substantially higher than the reductions observed in the cell culture infectivity assays (reductions of 3.1, 3.3, and 3.4 $\log_{10}$ versus 1.4, 1.8, and 3.4 $\log_{10}$, respectively, for the 30-min, 6-h, and 24-h exposure times). This may suggest that there was at least some degradation of the viral capsid in the samples that had been exposed to allspice for shorter durations (i.e., 30 min and 6 h) that was not sufficient to render the particle noninfectious but was enough to allow for the entry of the RNase I enzyme into the virus particle. In addition, the specific binding of virus particles to host cells is unchanged at these earlier exposures to the antimicrobial, suggesting that viral adsorption is not affected until the latter stages of capsid degradation. After 30 min of exposure, there were fewer virus particles observed under TEM than in the untreated control samples; by 24 h of exposure, the number of virus particles had been reduced even further so that it was difficult to find recognizable virus particles, and there was notably more debris observed on the grids. This is possibly indicative of the virus capsid being degraded, particularly with increasing durations of exposure to allspice oil.

Significant reductions (from 2.2 $\log_{10}$ to 2.6 $\log_{10}$) were also observed in the viral RNA following treatment with allspice oil in samples that had not been digested with RNase I (Fig. 2C). This suggests that the viral RNA was also significantly degraded by the antimicrobial itself. These were similar to the reductions that were observed for the cell culture infectivity assays. It therefore appears that the primary mechanism of action for allspice oil against MNV is likely capsid degradation, with subsequent degradation of the viral RNA as well. Such degradation of the virus capsid would also explain why the antiviral efficacy appears to be both concentration and time dependent. Higher concentrations would act to degrade the capsid at a higher rate, and greater effects would be observed with longer exposures to the antimicrobial.

In contrast, the results for lemongrass oil and citral (with one exception) indicated no significant differences between the treated virus particles either with or without RNase I digestion. This suggests either that these virus capsids are still intact enough to protect the viral RNA from RNase I digestion or that they are being shielded from the enzyme by some other means (e.g., the antimicrobial coating the capsid surface). Even following 24 h of exposure to the 4.0% lemongrass oil and citral, which resulted in reductions in the MNV cell culture infectivity of $\geq$2.74 $\log_{10}$, only slight reductions in the virus genome copy numbers were observed with or without RNase I digestion ($\leq$0.43 $\log_{10}$; determined by RT-qPCR). This indicates that although the virus particles may no longer be infectious, their nucleic acid is still intact; therefore, there is a different reason for this loss in MNV infectivity. For example, the antimicrobial might bind to the virus capsid and block the epitopes required for specific adsorption of the virus to host cells. Alternatively, this could cause the virus particles to agglomerate or cause a conformational change in the capsid proteins. All of these types of effects could prevent specific virus adsorption (95). A slow buildup of the antimicrobial on the surface of the capsid over time could lead to greater reductions in cell culture infectivity with increasing duration of exposure to the antimicrobial. The TEM imaging and the cell binding assay results support this scenario. It appears that the lemongrass oil and citral bind directly to the virus capsid. This coating leads to the MNV binding nonspecifically to host cells and to the plastic of the cell culture plates and also possibly prevents the specific adsorption of the virus to host cell receptors that would lead to successful infection. The MNV particles appear to be greatly enlarged following treatment with lemongrass oil and citral, possibly due to such a buildup of the antimicrobial coating on the surfaces. This may also explain why the antiviral effect appears more dependent on time than on the concentration of the lemongrass oil or citral.

In previous studies, allspice oil, lemongrass oil, and citral have

all been demonstrated to have antibacterial activities (42, 83, 84, 85, 87). This antimicrobial efficacy appears to be broad spectrum, as they also inactivated MNV in the current study. Allspice oil appears to cause the viral capsid to lose its integrity, ultimately leading to exposure of the viral genome. In addition, the allspice oil subsequently acts directly upon the viral RNA. With shorter durations of exposure to the antimicrobial, the virus is able to adsorb specifically to host cells; however, it may or may not be able to cause successful infection depending upon the integrity of the viral genome. As the viruses appear to be substantially degraded over time (as evidenced by the reduction in particles observed under TEM with increasing durations of exposure to the antimicrobial and the drop in cell binding after 24 h of exposure), the antiviral effect is likely irreversible and thus true virus inactivation. On the other hand, lemongrass and citral leave the virus capsid and genome intact. These antimicrobials appear to exert their antiviral effect by coating the capsid and thereby preventing specific adsorption of the virus to host cells. There is an increase in cell binding following treatment with lemongrass oil and citral; however, this appears to be nonspecific and therefore nonproductive binding of the virus to the host cell that does not lead to infection. It is unclear whether this leads to permanent virus inactivation, yet the significant expansion in the virus particles is likely irreversible.

In conclusion, the present study provides new information regarding the antiviral properties and mechanisms of action of allspice oil, lemongrass oil, and citral (one of the main active components of lemongrass oil) against MNV, a nonenveloped virus. While the treatment of viruses with different plant-based antimicrobials may result in similar overall reductions in infectivity, the mechanisms of inactivation can be highly varied and specific to the plant compound used. Our results demonstrate that these plant essential oils inactivate MNV, a human NoV surrogate. These antimicrobials could potentially be used as natural surface and food sanitizers to control NoV and possibly other nonenveloped enteric viruses. Even though they do not immediately exhibit antiviral efficacy, they are generally recognized as safe (GRAS) and thus could be left on surfaces or foods for long periods to provide an additional residual antiviral effect.

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