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## ORIGINAL ARTICLE

# Antiviral efficacy and mechanisms of action of oregano essential oil and its primary component carvacrol against murine norovirus

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**Keywords**

plant antimicrobials, human norovirus, sanitizer, mechanism of action, nonenveloped viruses.

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**Abstract**

**Aims:** To investigate the antiviral efficacy of oregano oil and its primary active component, carvacrol, against the nonenveloped murine norovirus (MNV), a human norovirus surrogate.

**Methods and Results:** Along with an observed loss in cell culture infectivity, the antiviral mechanisms of action were determined in side-by-side experiments including a cell-binding assay, an RNase I protection assay and transmission electron microscopy (TEM). Both antimicrobials produced statistically significant reductions ( $P \leq 0.05$ ) in virus infectivity within 15 min of exposure (c.  $1.0\text{-log}_{10}$ ). Despite this, the MNV infectivity remained stable with increasing time exposure to oregano oil ( $1.07\text{-log}_{10}$  after 24 h), while carvacrol was far more effective, producing up to  $3.87\text{-log}_{10}$  reductions within 1 h. Based on the RNase I protection assay, both antimicrobials appeared to act directly upon the virus capsid and subsequently the RNA. Under TEM, the capsids enlarged from  $\leq 35$  nm in diameter to up to 75 nm following treatment with oregano oil and up to 800 nm with carvacrol; with greater expansion, capsid disintegration could be observed. Virus adsorption to host cells did not appear to be affected by either antimicrobial.

**Conclusions:** Our results demonstrate that carvacrol is effective in inactivating MNV within 1 h of exposure by acting directly on the viral capsid and subsequently the RNA.

**Significance and Impact of the Study:** This study provides novel findings on the antiviral properties of oregano oil and carvacrol against MNV and demonstrates the potential of carvacrol as a natural food and surface (fomite) sanitizer to control human norovirus.

**Introduction**

Worldwide, human noroviruses (NoVs) are responsible for more than 267 million cases of gastroenteritis (Donaldson *et al.* 2008), including an estimated 21 million in the United States each year (<http://www.cdc.gov/norovirus/trends-outbreaks.html>. Accessed on February 4, 2014). NoVs are the causative agents of 50% of all gastroenteritis cases and more than 85% of all nonbacterial cases (Fankhauser *et al.* 2002; Lopman *et al.* 2003; Patel *et al.* 2009).

Outbreaks of NoV illness have occurred in numerous venues including assisted living communities, hospitals, military barracks (Donaldson *et al.* 2008), restaurants,

cruise ships, schools, summer camps and during domestic gatherings (<http://www.cdc.gov/norovirus/trends-outbreaks.html>. Accessed on February 4, 2014). Once NoV is introduced into these types of closed settings, person-to-person transmission plays an important role in its spread (Lopman *et al.* 2003); however, the transmission of NoV may occur via a variety of routes. The virus may be transmitted through contaminated food or water, and fomites (inanimate surfaces) also play a significant role in its spread (Carling *et al.* 2009). Often what is perceived as person-to-person transmission is more likely spread from person-to-fomite-to-person (Gerba and Pepper 2009).

Nonenveloped viruses such as NoV (a calicivirus) are generally quite resistant to environmental conditions and the action of antimicrobials (Watanabe *et al.* 1989; Barker *et al.* 2001). NoV has a protein capsid that is very resistant to lipophilic disinfectants (e.g. quaternary ammonium compounds) and solvents (e.g. alcohol) (Said *et al.* 2008; <http://www.infectioncontrolday.com/articles/2012/02/understanding-the-physiology-of-healthcare-pathogens-for-environmental-disinfection.aspx>. Accessed on February 4, 2014). The virus is also able to survive on many environmental surfaces for weeks to months at ambient temperatures and can be transmitted sequentially to up to seven different surfaces (Barker *et al.* 2004). Such environmental stability of NoV contributes to the role of fomites as a significant route of transmission.

Despite repeated attempts, there currently is no practical method for culturing NoV *in vitro* (Duiser *et al.* 2004; Malik *et al.* 2005; Lay *et al.* 2010). Therefore, several culturable viruses within the family *Caliciviridae* such as feline calicivirus (Slomka and Appleton 1998; Clay *et al.* 2006; Bright *et al.* 2009; Elizaquível *et al.* 2013), murine norovirus (MNV) (Wobus *et al.* 2006), Tulane virus (a monkey calicivirus) (Farkas *et al.* 2008; Wei *et al.* 2008) and porcine sapovirus (a pig calicivirus) (Esseili *et al.* 2012; Wang *et al.* 2012) have been used as NoV surrogates in laboratory studies. MNV is currently the most widely accepted surrogate as it is genetically the closest to human NoV among the potential NoV surrogates (the only one within the genus *Norovirus*); the MNV size, capsid structure, genomic organization and replication cycle are very similar to NoV (Karst *et al.* 2003; Sosnovtsev *et al.* 2006). MNV is also resistant to a wide range of pHs, organic solvents, thermal inactivation and antimicrobials (Cannon *et al.* 2006; Bae and Schwab 2008).

Plants produce antimicrobial compounds in areas such as in the roots, leaves, bark and stem (Burt 2004). Plant essential oils are complex mixtures of lipophilic and volatile secondary metabolites isolated from plants such as monoterpenes, sesquiterpenes and/or phenylpropanoids. They are primarily responsible for a plant's fragrant and biological properties (Reichling *et al.* 2009). A variety of plant essential oils can be found in the average kitchen cabinet. Their long-standing usage has led to many being generally regarded as safe (GRAS) for human exposure/consumption (Dillon 1999; Ress *et al.* 2003; Adams *et al.* 2004; Knowles *et al.* 2005). Numerous essential oils and their components have significant antibacterial properties (Didry *et al.* 1994; Friedman *et al.* 2002; Knowles *et al.* 2005; Peñalver *et al.* 2005; Callaway *et al.* 2008; Ravishankar *et al.* 2009, 2010; Reichling *et al.* 2009). In addition, many have also been shown to have some antifungal and antiviral activities (Hammer *et al.* 2002; Carson *et al.* 2006; Pinto *et al.* 2006; Reichling *et al.* 2009), although

the research with respect to viruses has primarily been limited to enveloped viruses (Reichling *et al.* 2009) with very limited work performed with nonenveloped viruses.

Recently, some studies have been conducted with MNV and other nonenveloped NoV surrogates (feline calicivirus, MS2 and  $\Phi$ X-174 bacteriophages) using juices and extracts (not essential oils) from a single plant. Examples are studies with grape seed extract (Su and D'Souza 2011; Li *et al.* 2012), cranberry juice and cranberry proanthocyanidins (Su *et al.* 2010a,b), and pomegranate juice and pomegranate polyphenols (Su *et al.* 2011). MNV tended to be more resistant than the other NoV surrogates and thus provided a more conservative estimation of the inactivation of NoV; therefore, MNV appears to be the most appropriate surrogate for NoV in these types of antimicrobial studies.

In this study, we employed MNV (strain S7-PP3) as a surrogate for human NoV in laboratory experiments to determine the antiviral efficacy of oregano oil, an essential oil, and its primary active component, carvacrol. The active antimicrobial ingredient of essential oils is often the dominant component, accounting for greater than 50% of the chemical composition. For instance, the carvacrol content of oregano oil may be as high as 85%, depending on its geographical origin (Ravishankar *et al.* 2009). In addition to the assessment of antiviral activity, an attempt was undertaken to elucidate the mechanism(s) of antiviral action of these compounds.

## Materials and methods

### Viruses and cells

MNV (strain S7-PP3) was obtained from Dr. Yukinobu Tohya from the Department of Veterinary Medicine, Nihon University (Kanagawa, Japan). The virus 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% foetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), 0.113% sodium bicarbonate (Fisher Scientific, Fair Lawn, NJ), 10 mmol l<sup>-1</sup> HEPES buffer (Mediatech Inc.) and 1.0% antibiotic/antimycotic (Mediatech Inc.) at an incubation temperature of 37°C with 5% CO<sub>2</sub> as described previously (Wobus *et al.* 2004). MNV propagated on RAW 264-7 cells was concentrated and purified using the methods described by Black *et al.* (2009). This included a Vertrel XF extraction to promote monodispersion of the virus and the removal of lipids (Black *et al.* 2009). The virus stocks were stored at -80°C until use.

Viral titrations were performed using the Reed-Muench method (Payment and Trudel 1993) to determine the tissue culture infectious dose that affected 50% of the wells

(TCID<sub>50</sub>). In short, serial 10-fold dilutions of the virus sample were assayed in 96-well tissue culture plates (Nunc, Roskilde, Denmark) containing monolayers of RAW 264.7 cells and 50 µl of DMEM containing 10% FBS (DMEM-FBS) with incubation at 37°C with 5% CO<sub>2</sub> as before. Eight wells were inoculated with 50 µl of each dilution. This number of wells was used to ensure adequate precision of the assay. Each well was checked every day for 5 days for viral cytopathogenic effects (CPE). The greatest dilution in which 50% or higher of the wells were positive was used to determine the virus TCID<sub>50</sub> ml<sup>-1</sup>. The use of the TCID<sub>50</sub> method for murine norovirus has been widely reported in the literature (Kitajima *et al.* 2010; Sánchez *et al.* 2011; Kim *et al.* 2012; Wang *et al.* 2012; Elizaquível *et al.* 2013; Toffan *et al.* 2014).

Poliovirus type 1 (PV1; strain LSc-2ab; included in several experiments as a process control) was obtained from the Department of Virology and Epidemiology at the Baylor College of Medicine (Houston, TX). PV1 was propagated on BGM cell line monolayers (Buffalo green monkey kidney; obtained from D. Dahling at the United States Environmental Protection Agency, Cincinnati, OH) with minimal essential medium (MEM; modified with Earle's salts; Irvine Scientific, Santa Ana, CA) containing 5% calf serum (HyClone Laboratories) at an incubation temperature of 37°C with 5% CO<sub>2</sub>. PV1 was purified as described previously for MNV and quantified using 10-fold serial dilutions in plaque-forming assays as described by Bidawid *et al.* (2003) on BGM cell monolayers.

#### Antimicrobial preparation

Oregano oil was obtained from Lhasa Karnak Herbal Co. (Berkeley, CA). No information was available regarding the concentration of carvacrol in this specific product. Purified carvacrol (>98%) was purchased from Sigma-Aldrich (St. Louis, MO). The antimicrobials were diluted using sterile phosphate-buffered saline (PBS; pH7.4; Sigma-Aldrich) to specific test concentrations. Although alcohol is sometimes used to dissolve such viscous antimicrobials, its use could potentially enhance the antimicrobial effect of the solution, and thus, alcohol was not used in these experiments. The oils dissolved mostly, though not completely, in the PBS; nevertheless, the results from these experiments were found to be consistent and repeatable.

#### Antimicrobial efficacy experiments

Oregano oil and its primary active ingredient, carvacrol, were evaluated in separate experiments. Screening trials were performed to identify the appropriate working concentrations for each antimicrobial with regard to its

efficacy against MNV. Initially, they were evaluated at 0.1 and 1.0% (v/v) (data not shown). These concentrations were then increased or decreased as needed based on the experimental results. The final experiments were performed with the following antimicrobial concentrations (v/v): 4.0% oregano oil; 0.25 and 0.5% carvacrol.

The experiments were performed in triplicate at room temperature (24°C) in 1-ml volumes of PBS in 5-ml polystyrene tubes (Becton Dickinson and Company, Franklin Lakes, NJ). Purified stocks of MNV were added separately to each of the tubes (to a final concentration of  $c. 1.0 \times 10^7$  TCID<sub>50</sub> ml<sup>-1</sup>), and the tubes were placed on an orbital shaker (Model G33; New Brunswick Scientific, Edison, NJ) at 300 rev min<sup>-1</sup>. Control tubes containing MNV in PBS but no added antimicrobials were also included in each experiment. The controls were sampled immediately ( $t = 0$  h) by removing 100 µl from each control tube and placing this volume in 900 µl of DMEM-FBS. At predetermined time intervals (0.25, 0.5, 1, 3, 6 and 24 h of exposure), 100 µl was removed from each tube and diluted in 900 µl of DMEM-FBS to neutralize the antimicrobials. All samples were stored at -80°C and subsequently assayed using the TCID<sub>50</sub> method (as described previously) to determine the infectious virus titre.

#### Antimicrobial neutralization and cytotoxicity experiments

A neutralization test was performed with the 4.0% oregano oil and the 0.25 and 0.5% carvacrol concentrations in which a PBS solution containing the desired concentration of the antimicrobial was placed into either PBS or DMEM-FBS at a ratio of 1 : 10 (1 ml into 9 ml). The solution was vortexed, and then, approximately  $1 \times 10^7$  TCID<sub>50</sub> of MNV was added. The solution was mixed again and then allowed to stand for 5 min at room temperature. Following this, 10-fold serial dilutions of the solution were assayed on RAW 264.7 cell monolayers in a TCID<sub>50</sub> assay as described previously. If the antimicrobials are completely neutralized, it is expected that there will be no reduction in MNV numbers in comparison with controls with either PBS or DMEM-FBS (alone with no antimicrobial).

In a separate experiment, 10-fold serial dilutions of the 4.0% oregano oil and the 0.25 and 0.5% carvacrol concentrations were added to RAW 264.7 cell monolayers. The cells were then examined daily for 6 days to look for cell toxicity.

#### Mechanism of action experiments

To determine the mechanisms of antiviral action, each of the antimicrobials was added to three separate test tubes

(replicates) containing approximately  $1.0 \times 10^6$  TCID<sub>50</sub> ml<sup>-1</sup> of MNV in 2 ml of PBS and either 4.0% oregano oil or 0.5% carvacrol (v/v). These were tested as described previously with the exception that samples were only collected after 0.5, 6 and 24 h of exposure. After each exposure period, four 50- $\mu$ l volumes were removed from each tube (including the three control tubes) and placed into separate Eppendorf tubes: three which contained 450  $\mu$ l of DMEM-FBS and one which contained 450  $\mu$ l of PBS (see Fig. 1). Two of the tubes containing DMEM-FBS were used immediately in the RNase I protection experiment to assess the capsid integrity. The other two tubes were stored at  $-80^\circ\text{C}$  until used subsequently in the cell-binding experiment (tube with DMEM-FBS) to assess the ability of the treated MNV to bind to host cells and for transmission electron microscopy (TEM) imaging (tube with PBS) to directly observe any structural changes to the MNV particles following antimicrobial treatments.

#### RNase I protection experiment

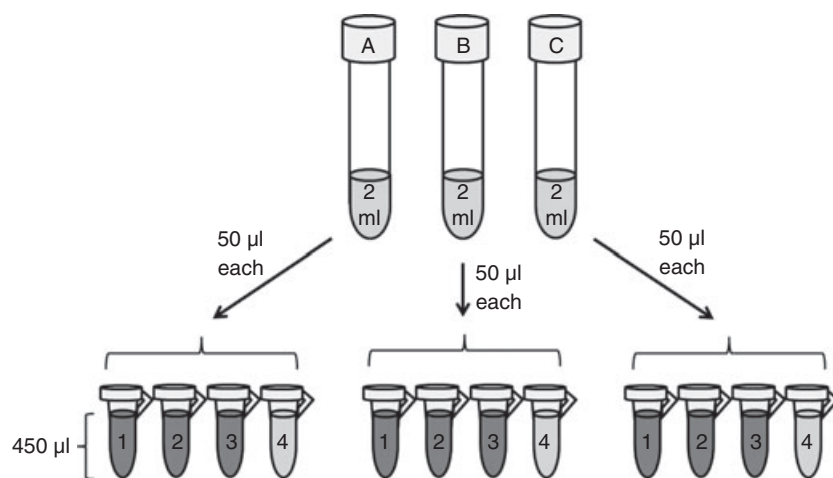
A volume of 1  $\mu$ l of RNase I (100 units; Ambion Inc., Austin, TX) was added to one of the aforementioned tubes (Fig. 1, tube 2). This tube and another (Fig. 1, tube 1—without RNase I) were then incubated at  $37^\circ\text{C}$  for 30 min. RNase I should degrade any viral RNA that is no longer protected by the viral capsid. The replicate tube without RNase I was included as a control. Following this incubation period, all of the samples were immediately placed in a  $-80^\circ\text{C}$  freezer for storage prior to nucleic acid extraction and molecular assay.

The samples were thawed immediately prior to the nucleic acid extraction step in which 200  $\mu$ l of each sample was added (separately) to 600  $\mu$ l of the ZR viral RNA buffer of a ZR Viral RNA Kit (Zymo Research, Irvine, CA). The RNase I enzyme is presumably denatured in

this buffer.  $\beta$ -Mercaptoethanol (BME) was also added to each sample (as per the manufacturer's instructions) to inhibit any RNases. Additionally, 2  $\mu$ l of purified PV1 stock (*c.*  $2.0 \times 10^6$  genome copy numbers total) was added to each of the samples prior to the nucleic acid extraction as a process control (explained in detail later). The viral RNA was extracted from each sample using this kit according to the manufacturer's protocol with the exception that the final elution volume was adjusted to 20  $\mu$ l rather than 10  $\mu$ l.

The reverse transcription (RT) was performed using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA). Briefly, 3  $\mu$ l of extracted virus RNA was added to 3  $\mu$ l of RT mixture containing 0.6  $\mu$ l of  $10 \times$  reverse transcription buffer, 0.24  $\mu$ l of  $25 \times$  dNTPs, 0.6  $\mu$ l of  $10 \times$  random hexamers, 15 units of MultiScribe<sup>TM</sup> reverse transcriptase and 6 units of RNase inhibitor (Applied Biosystems). The RT reaction mixture was incubated at  $25^\circ\text{C}$  for 10 min,  $37^\circ\text{C}$  for 120 min and  $85^\circ\text{C}$  for 5 min to inactivate the enzyme.

A TaqMan-based real-time quantitative polymerase chain reaction (qPCR) assay for MNV, which targets 129 nucleotides in the open reading frame 1 (ORF1)—ORF2 junction region, was performed in a 25- $\mu$ l reaction volume containing 2.5  $\mu$ l of cDNA from the RT reaction, 12.5  $\mu$ l of LightCycler<sup>®</sup> 480 Probes Master (Roche Diagnostics, Mannheim, Germany), 400 nmol l<sup>-1</sup> each of the primers MNV-S (5'-CCGCAGGAACGCTCAGCAG-3') and MNV-AS (5'-GGYTGAATGGGGACGGCCTG-3'), and 300 nmol l<sup>-1</sup> of the TaqMan MGB probe MNV-TP (5'-FAM-ATGAGTGATGGCGCA-MGB-NFQ-3') as described previously (Kitajima *et al.* 2010). The PCR amplification was performed with a LightCycler<sup>®</sup> 480 Real-Time PCR Instrument II (Roche Diagnostics) under the following conditions: initial denaturation at  $95^\circ\text{C}$  for 15 min to activate the DNA polymerase, followed by 50 cycles of



**Figure 1** Sampling strategy for mechanism of action experiment. At each time exposure, four 50- $\mu$ l volumes were removed from each of the replicate test tubes (A, B and C) and placed into separate Eppendorf tubes containing 450  $\mu$ l of either Dulbecco's modified Eagle medium with 10% foetal bovine serum (tubes 1, 2 and 3) or phosphate-buffered saline (tube 4). These four tubes were used in subsequent experiments or assays to determine the antiviral mechanism(s) of action: (1) RNase Protection Assay (no RNase I); (2) RNase Protection Assay (RNase I added); (3) Cell-Binding Assay and (4) Transmission Electron Microscopy.

amplification with denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. The amplification data were collected and analysed with the LightCycler® 480 software (version 1.5; Roche Diagnostics). Tenfold serial dilutions ( $1.0 \times 10^2 - 1.0 \times 10^8$  copies per PCR tube) of the standard plasmid DNA containing an insert of approximately 500 nucleotides encoding the ORF1–ORF2 junction region of the MNV-S7 PP3 strain (Kitajima *et al.* 2010) were used for the quantification of MNV-cDNA copy numbers. The average copy number of two PCR tubes was used for subsequent calculations.

To determine whether the presence of the antimicrobials in the samples caused inhibition of either the RT or the qPCRs, a subsequent separate qPCR assay was performed using the cDNA from the RT as template. The qPCR was performed as described previously for MNV, but with primers/probes specific to the enteroviruses [forward primer EV1F ( $400 \text{ nmol l}^{-1}$ ), 5'-CCCTG AATGCGGCTAAT-3'; reverse primer EV1R ( $400 \text{ nmol l}^{-1}$ ), 5'-TGTCACCA-TAAGCAGCCA-3'; EV probe ( $120 \text{ nmol l}^{-1}$ ), 5'-FAM-ACGACACCCAAAGTAGTCGGTTC-BHQ-1-3'] and with the following conditions: initial denaturation at 95°C for 10 min, followed by 50 cycles of amplification with denaturation at 94°C for 15 s, and annealing and extension at 60°C for 1 min. This assay resulted in a 143-bp product (Gregory *et al.* 2006).

As  $2 \mu\text{l}$  of the PV1 (an enterovirus) process control from the same stock was added to each of the samples prior to nucleic acid extraction, the copy number determined by qPCR to be present in the control samples (which did not contain any extraction-, RT-, and/or qPCR-inhibiting substances) was therefore used to determine whether there was any inhibition in the other samples (leading to reduced amplification and an underestimate of the viral copy number).

#### Cell-binding experiment

The media was removed from RAW 264.7 cell monolayers in a 24-well plate, and then, the cells were rinsed with  $0.025 \text{ mol l}^{-1}$  Tris-buffered saline (TBS;  $0.32 \text{ l}$  of the following solution in  $3.68 \text{ l}$  of ultrapure  $\text{H}_2\text{O}$ :  $31.6 \text{ g l}^{-1}$  Trizma base,  $81.8 \text{ g l}^{-1}$  NaCl,  $3.73 \text{ g l}^{-1}$  KCl and  $0.57 \text{ g l}^{-1}$   $\text{Na}_2\text{HPO}_4$ -anhydrous). Following this,  $100 \mu\text{l}$  from the third replicate tube from each sample (Fig. 1, tube 3) that had previously been treated with oregano oil or carvacrol (from the mechanism of action experiment) was added to duplicate wells, and the plate was incubated for 1 h at 4°C to prevent the virus from entering the cells (Garozzo *et al.* 2011) with gentle agitation every 15 min to prevent drying of the cell monolayers. In addition to the MNV previously treated with either oregano oil or carvacrol, numerous controls were also included in duplicate wells (see Table 1).

After the 1-h incubation period, the cells were washed three times with TBS to remove any unbound virus particles or RNA and then an additional  $198 \mu\text{l}$  of PBS was added to each well, followed by  $600 \mu\text{l}$  of the extraction buffer of the ZR Viral RNA Kit (Zymo Research) and then  $2 \mu\text{l}$  of the PV1 process control (*c.*  $2.0 \times 10^8$  total genome copy numbers). This entire  $800 \mu\text{l}$  volume was then extracted using the ZR Viral RNA Kit as described previously. The RNA extracts were used as template in the RT-qPCR assays for MNV and PV1 as described previously. As the goal of this assay is to assess the effect of the antimicrobials on viral binding to the cells and not their effect on virus viability, the short, 1-h incubation period specifically does not allow for viral replication or the development of any CPE in the cells. This therefore provides a measure of the number of viruses that bind to the cells (while discounting those that do not), but is not meant to measure successful infection of the cells.

#### Transmission electron microscopy (TEM) imaging

Samples from the fourth tube (with PBS) from the 24-h exposure from the mechanism of action experiment (Fig. 1, tube 4) were used to visualize the viruses using TEM. The 24-h exposure was chosen as it was likely to produce the greatest effects which could be observed under TEM to better understand the antimicrobial mechanisms of action. A drop of each sample ( $5-10 \mu\text{l}$ ) was applied to separate glow discharge carbon-coated EM grids and stained with 2% aqueous uranyl acetate for 3 min. The stained grids were then dried and examined using an FEI CM12S TEM (FEI Electronics Instruments, Co., Hillsboro, OR) operated at 80 kV, and images were captured using an AMT 420 camera (Advanced Microscopy Techniques, Woburn, MA).

#### Statistical analyses

For the antimicrobial efficacy (cell culture infectivity) assays, the data were reported as the logarithmic reduction using the formula  $-\log_{10} (N_t N_0^{-1})$ , where  $N_0$  was the concentration of MNV measured by cell culture infectivity ( $\text{TCID}_{50}$ ) at time = 0 and  $N_t$  was the infectious concentration at time =  $t$ . A Student's  $t$ -test was used to determine whether there were significant differences between the controls and the antimicrobial treatments (the reduction at each time exposure was compared to the reduction in the controls, if any). Differences were considered statistically significant if the resultant  $P$  value was  $\leq 0.05$ . Differences between the reductions observed between the two concentrations of carvacrol were also evaluated for statistical significance in the same manner.

To allow for direct statistical comparisons between separate experiments using different antimicrobials, the

**Table 1** Controls included in the cell-binding assay for murine norovirus (MNV) treated with either 4.0% oregano oil or 0.5% carvacrol

| Control              | Description   | Purpose   |
|----------------------|---|---|
| Positive control     | RAW 264.7 cells seeded with c. 5.61 log <sub>10</sub> copy numbers of MNV from the <i>t</i> = 0 min control samples (no antimicrobials present) | To determine the amount of cell binding by intact 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 c. 5.61 log <sub>10</sub> copy numbers of MNV from the control samples from <i>t</i> = 0 min                    | To determine whether MNV is able to bind nonspecifically to the plastic of the 24-well plates                         |
| Naked RNA control #1 | RAW 264.7 cells seeded with c. 6.54 log <sub>10</sub> copy numbers of MNV-RNA extracted from the control samples at <i>t</i> = 0 min            | To determine whether the naked MNV-RNA is able to bind directly to the RAW 264.7 cells                                |
| Naked RNA control #2 | Wells without cells seeded with c. 6.54 log <sub>10</sub> copy numbers of MNV-RNA extracted from the control samples at <i>t</i> = 0 min        | To determine if the naked MNV-RNA is able to bind directly to the plastic of the 24-well plates                       |
| No-cell control #1   | Wells without cells seeded with MNV treated with 4.0% oregano oil from <i>t</i> = 24 h  | To determine whether the treated MNV particles are able to bind non-specifically to the plastic of the 24-well plates |
| No-cell control #2   | Wells without cells seeded with MNV treated with 0.5% carvacrol from <i>t</i> = 24 h  | To determine whether the treated MNV particles are able to bind nonspecifically to the plastic of the 24-well plates  |

average reduction observed for the controls after 24 h in each experiment was subtracted from the reductions reported for each treated sample (for all time exposure intervals) to normalize the reductions. These normalized data were only used for the statistical tests (Student's *t*-tests) and are not reported in the tables.

For the RNase I protection assays, the data were reported as the log<sub>10</sub> of the viral genome copy number recovered per PCR in comparison with the untreated controls. A Student's *t*-test was used to determine whether there were significant differences between the controls and the antimicrobial treatments. The reduction in the viral copy number in each sample was compared to the corresponding control samples (with or without RNase I). Also, comparisons between the samples with and without RNase I were performed for each of the antimicrobial treatments. Differences were considered statistically significant if the resultant *P* value was ≤0.05.

## Results

### Antimicrobial neutralization and cytotoxicity experiments

No reductions in MNV numbers were observed in the neutralization tests with any of the antimicrobial concentrations in comparison with the controls in DMEM-FBS alone or PBS alone with no antimicrobial. Therefore, complete neutralization by this dilution method was confirmed and this method was used for all subsequent assays.

Cell toxicity was observed in the RAW 264.7 cells with both oregano oil and carvacrol for the 10<sup>-1</sup> dilution. These wells were therefore not included in the determination of the viral TCID<sub>50</sub> ml<sup>-1</sup> in the subsequent cell culture infectivity assays. This had the effect of increasing the virus limit of detection of these assays by 10-fold.

### Antimicrobial efficacy experiments

The antiviral efficacy of each antimicrobial was determined by comparison with the reductions (if any) observed in the controls (with no antimicrobials) at the same time interval. The antiviral effect of 4.0% oregano oil is shown in Table 2. This concentration resulted in a statistically significant reduction in MNV of 0.95-log<sub>10</sub> within 0.25 h (15 min) of exposure (*P* = 0.002); however, the observed reductions did not significantly increase from this level even at much longer exposures (≥6 h). Concentrations greater than 4.0% oregano oil are not reasonably soluble and therefore were not included in this study.

Carvacrol was examined at concentrations of 0.25% and 0.5% (Table 3). Both concentrations produced statistically significant log<sub>10</sub> reductions within 15 min (and every time interval thereafter) in comparison with the controls (*P* = 0.003). Both high (0.5%) and low (0.25%) concentrations were effective, with ≥3.27-log<sub>10</sub> reductions after 1 and 3 h, respectively. The higher concentration consistently outperformed its lower counterpart, although this difference was only significant (*P* = 0.032) at the 1-h exposure time (3.87-log<sub>10</sub> vs 1.95-log<sub>10</sub>). Carvacrol was

**Table 2** Antimicrobial efficacy of oregano oil. Results shown are the  $\log_{10}$  reductions (mean  $\pm$  SD) in cell culture infectivity of murine norovirus\* after various time exposures to oregano oil at a concentration of 4.0% (v/v)

| Time (h) | Control Reduction $\pm$ SD | 4.0% Oregano oil Reduction $\pm$ SD |
|----------|----------------------------|-------------------------------------|
| 0.25     | 0.00 $\pm$ 0.00            | 0.95† $\pm$ 0.21                    |
| 0.5      | 0.66 $\pm$ 0.50            | 0.83 $\pm$ 0.00                     |
| 1        | 0.22 $\pm$ 0.38            | 0.98† $\pm$ 0.17                    |
| 3        | 0.00 $\pm$ 0.00            | 0.91† $\pm$ 0.23                    |
| 6        | 0.00 $\pm$ 0.00            | 1.10† $\pm$ 0.12                    |
| 24       | 0.22 $\pm$ 0.38            | 1.07† $\pm$ 0.31                    |

\*Titre was  $2.9 \times 10^7$  TCID<sub>50</sub> ml<sup>-1</sup>. The experiment was conducted in triplicate.

†Reduction was statistically significant ( $P \leq 0.05$ ) in comparison with the control (with no antimicrobial) at the same time exposure.

more effective than oregano oil (which was used at 8 or 16 times the concentration for carvacrol) at all exposure time intervals. These differences were statistically significant within either 1 h ( $P = 0.015$  for 0.5% carvacrol vs 4.0% oregano oil at 1 h of exposure) or within 3 h ( $P = 0.006$  for 0.25% carvacrol vs 4.0% oregano oil at 3 h of exposure) and at each time exposure thereafter ( $P \leq 0.05$ ).

### RNase I protection experiment

To assess whether the capsid is degraded by the plant antimicrobials, two (of four) replicate tubes from each sample (Fig. 1, tubes 1 and 2) were used in an RNase I

**Table 3** Antimicrobial efficacy of carvacrol. Results shown are the  $\log_{10}$  reductions (mean  $\pm$  SD) in cell culture infectivity of murine norovirus\* after various time exposures to carvacrol at two concentrations (v/v)

| Time (h) | Control Reduction $\pm$ SD | 0.25% Carvacrol Reduction $\pm$ SD | 0.5% Carvacrol Reduction $\pm$ SD |
|----------|----------------------------|------------------------------------|-----------------------------------|
| 0.25     | 0.05 $\pm$ 0.08            | 1.03† $\pm$ 0.19                   | 1.28† $\pm$ 0.32                  |
| 0.5      | 0.07 $\pm$ 0.10            | 1.27† $\pm$ 0.40                   | 1.77† $\pm$ 0.78                  |
| 1        | 0.50 $\pm$ 0.38            | 1.95†‡ $\pm$ 0.83                  | 3.87†‡ $\pm$ 0.61                 |
| 3        | 0.26 $\pm$ 0.34            | 3.27† $\pm$ 0.55                   | >4.15† $\pm$ 0.71                 |
| 6        | 0.21 $\pm$ 0.24            | 4.19† $\pm$ 0.27                   | 4.54† $\pm$ 0.05                  |
| 24       | 0.21 $\pm$ 0.37            | 4.45† $\pm$ 0.27                   | >4.52† $\pm$ 0.16                 |

>At least one of the triplicate samples was below the limit of detection of the assay (i.e.  $<6.3 \times 10^2$  TCID<sub>50</sub> ml<sup>-1</sup>).

\*Titre was  $2.8 \times 10^7$  TCID<sub>50</sub> ml<sup>-1</sup>. The experiment was conducted in triplicate.

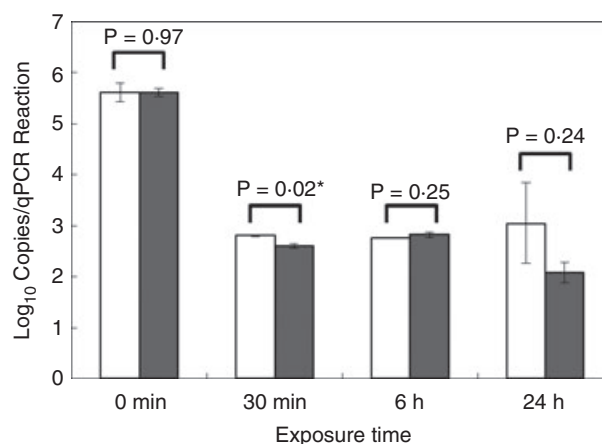
†Reduction was statistically significant ( $P \leq 0.05$ ) in comparison with the control (with no antimicrobial) at the same time exposure.

‡Reductions were significantly different ( $P \leq 0.05$ ) between 0.25% and 0.5% carvacrol.

protection experiment; one tube was treated with RNase I to digest the viral RNA exposed to the outside environment. Both tubes (with and without RNase treatment) were then used as template for RT-qPCR to quantify the amount of viral RNA that was protected by the capsid by comparing the amount of intact viral RNA in the digested and undigested samples. These were also compared to two controls (no antimicrobial treatment, with and without RNase I digestion) to determine the reductions from the initial amount of viral RNA present. The RT-qPCR results ( $\log_{10}$  viral copy number recovered) for the RNase I protection experiment are shown in Figs 2 and 3. No reductions were observed in the controls after 24 h, regardless of whether or not they had been treated with RNase I.

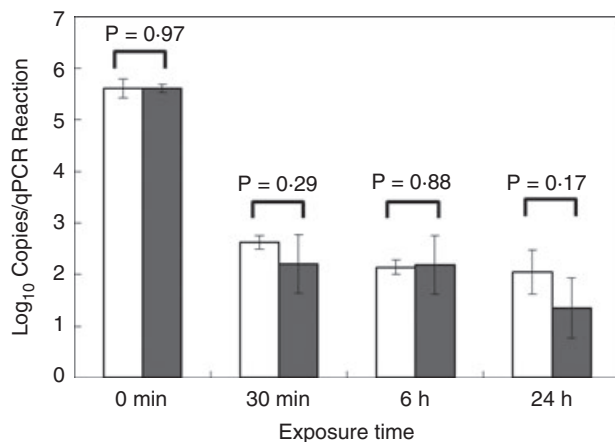
For oregano oil (Fig. 2) and carvacrol (Fig. 3), the  $\log_{10}$  reductions were usually greater in the samples that had been treated with RNase I; however, with the exception of oregano oil after a 0.5-h (30-min) exposure ( $P = 0.02$ ), this difference was not statistically significant ( $P > 0.05$ ). Nevertheless, the reductions in viral RNA amplification observed both with and without RNase I were significant ( $P \leq 0.05$ ) in comparison with the untreated controls (with and without RNase I) at all exposure times (30 min, 6 h and 24 h) for both oregano oil and carvacrol.

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



**Figure 2** Results of the RNase I protection assay for murine norovirus (MNV) after exposure to 4.0% oregano oil. The  $\log_{10}$  genome copy numbers of MNV-RNA recovered was determined by RT-qPCR after exposure to oregano oil (for 30 min, 6 h or 24 h) followed by RNase I digestion.  $P$  values for the Student's  $t$ -tests comparing the  $\log_{10}$  virus copy numbers recovered with and without RNase I digestion are also presented; values with statistical significance ( $P \leq 0.05$ ) are indicated with an asterisk (\*). (□) No RNase and (■) with RNase.





**Figure 3** Results of the RNase I protection assay for murine norovirus (MNV) after exposure to 0.5% carvacrol. The log<sub>10</sub> genome copy numbers of MNV-RNA recovered was determined by RT-qPCR after exposure to carvacrol (for 30 min, 6 h or 24 h) followed by RNase I digestion. *P* values for the Student's *t*-tests comparing the log<sub>10</sub> virus copy numbers recovered with and without RNase I digestion are also presented. (□) No RNase and (■) with RNase.

### Cell-binding experiment

To determine whether the antimicrobials inhibited the ability of MNV to subsequently bind to host cells, a cell-binding assay was performed. The results for this experiment are shown in Table 4. The positive control (MNV with no antimicrobials) was able to bind to the RAW 264.7 cells under the experimental conditions; however, it appears that the multiple wash steps removed much of the virus (or possibly cells with bound virus) from the wells. Although each well was inoculated with approximately 5.6 log<sub>10</sub> MNV genome copy numbers, only 2.7 log<sub>10</sub> remained bound to the cells at the end of the assay. Nevertheless, it was apparent that there was a reduction in the ability of the MNV that had been previously exposed to carvacrol to bind to the RAW 264.7 cells, with

**Table 4** Results\* for the cell-binding assay of murine norovirus (MNV) to RAW 264.7 cell monolayers after various time exposures to 4.0% oregano oil and 0.5% carvacrol

| Sample                       | Antimicrobial exposure time (h) |           |           |           |
|------------------------------|---------------------------------|-----------|-----------|-----------|
|                              | 0                               | 0.5       | 6         | 24        |
| MNV w/oregano oil—with cells | 2.7§ ± 0.3                      | 2.5 ± 1.2 | 3.1 ± 0.3 | 2.4 ± 1.2 |
| MNV w/oregano oil—no cells†  | —                               | —         | —         | ND        |
| MNV w/carcacrol —with cells  | 2.7§ ± 0.3                      | ND        | ND        | ND        |
| MNV w/carcacrol —no cells‡   | —                               | —         | —         | ND        |

—, not tested; ND, not detected by RT-qPCR.

\*Expressed as the average log<sub>10</sub> MNV genome copy number per cell culture well (±SD) as determined by RT-qPCR.

†No-cell control #1 in Table 1.

‡No-cell control #2 in Table 1.

§Positive control (untreated MNV) described in Table 1.

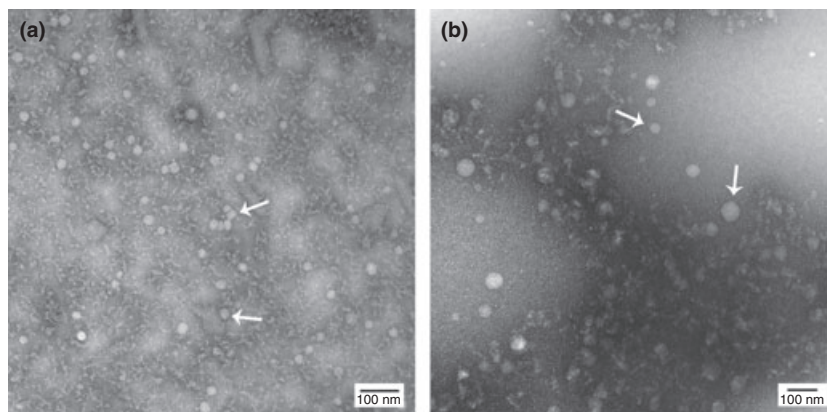
no virus detected by RT-qPCR even after only a 30-min exposure. The amount of oregano oil-treated MNV bound to cells was more variable, but still comparable to the untreated control.

MNV did not bind nonspecifically to the cell culture plates either in the control (not detected by RT-qPCR, data not shown) or the antimicrobial-treated samples (Table 4, oregano oil/carcacrol, no cells). In addition, MNV-RNA was not able to bind to the RAW 264.7 cells or the cell culture plate (not detected by RT-qPCR, data not shown). Finally, the extracted RNA from the RAW 264.7 host cells did not result in nonspecific amplification in the RT-qPCR assays (not detected by RT-qPCR, data not shown).

No extraction, RT or qPCR inhibition was observed with the PV1 process control for any of the cell-binding assay samples (data not shown); therefore, the values determined by the RT-qPCR assay were considered accurate.

### Transmission electron microscopy (TEM) imaging

The structural changes in the MNV particles following the antimicrobial treatments were directly observed through TEM imaging. The TEM images for the untreated MNV and the MNV after exposure to oregano oil for 24 h are shown in Fig. 4. The MNV after exposure to carvacrol for 24 h is shown in Fig. 5. Untreated MNV particles range in size from approximately 20 nm to 35 nm in diameter and have an icosahedral symmetry (appear spherical in most images) (Fig. 4a). The particles exposed to the antimicrobials appeared to expand in size; nevertheless, although the virus particles exposed to oregano oil were larger (*c.* 40–75 nm) than the untreated virus particles, they appeared intact and otherwise normal in morphology (Fig. 4b). In contrast, in samples of MNV exposed to carvacrol, there were virus particles ranging from 100-nm particles that appeared to be intact to



**Figure 4** Transmission electron microscope image of a) untreated murine norovirus (MNV) and b) murine norovirus (MNV), following exposure to 4.0% oregano oil for 24 h. Examples of MNV particles are indicated by arrows.

900-nm particles that were completely broken apart into capsid components (Fig. 5).

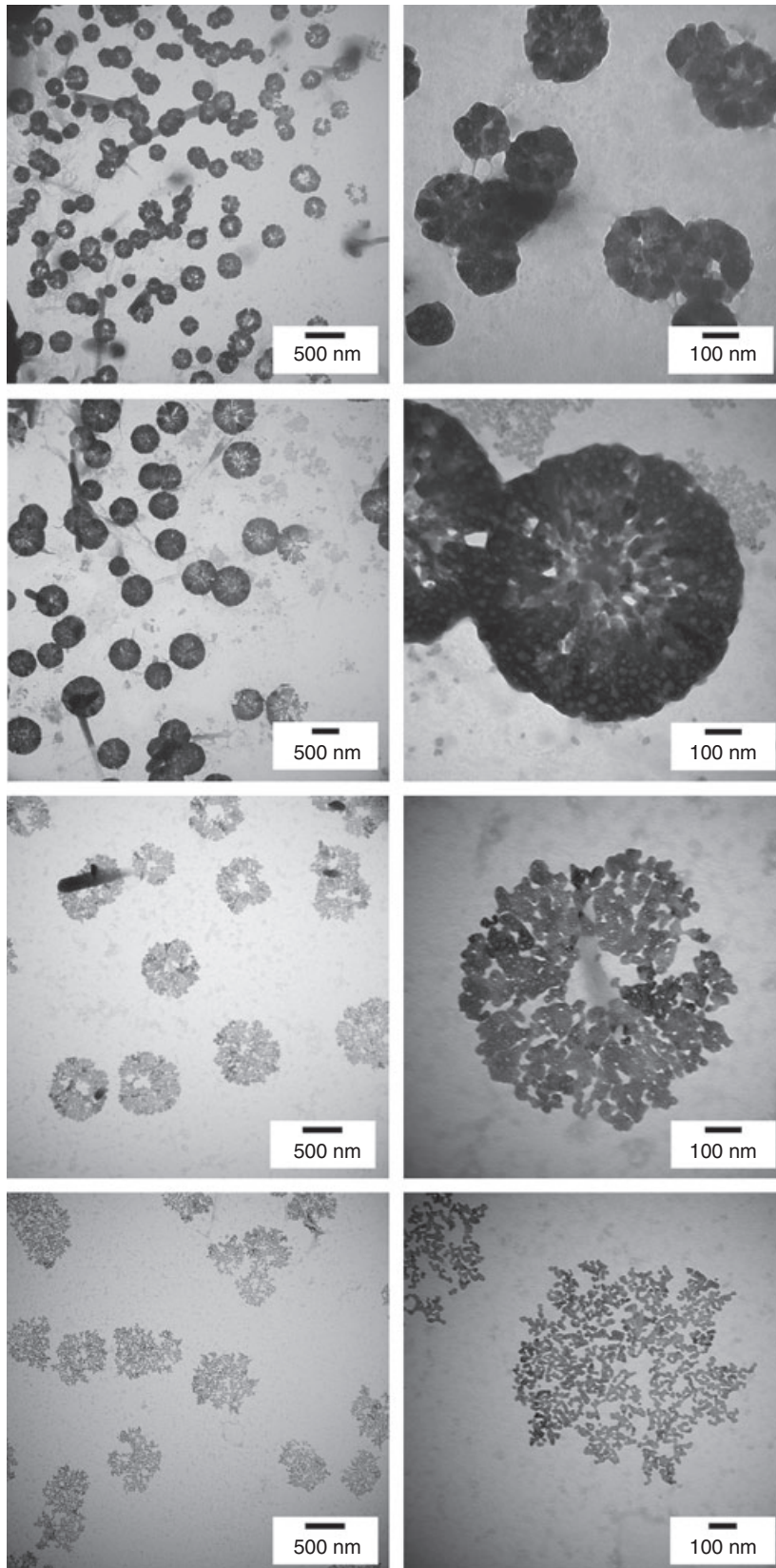
## Discussion

While the antimicrobial properties of plant based essential oils have been examined in detail against bacteria (Didry *et al.* 1994; Friedman *et al.* 2002; Knowles *et al.* 2005; Peñalver *et al.* 2005; Callaway *et al.* 2008; Ravishankar *et al.* 2009, 2010; Reichling *et al.* 2009), they have only recently been examined for their antiviral properties. The majority of this work has been directed towards enveloped viruses of clinical importance (Reichling *et al.* 2005, 2009; Koch *et al.* 2008; Loizzo *et al.* 2008; Meneses *et al.* 2009; Garcia *et al.* 2010; Jackwood *et al.* 2010; Ocazonez *et al.* 2010; Wu *et al.* 2010; Garozzo *et al.* 2011). Limited research has looked at the efficacy of essential oils against nonenveloped viruses. Cermelli *et al.* (2008) found no significant effect from eucalyptus essential oil against adenovirus, a nonenveloped virus. In another study, homoisoflavonoids were found to be effective against several of the enteroviruses (also nonenveloped viruses) including Coxsackieviruses B1, B3, B4, A9 and echovirus 30, but not PV1 (Tait *et al.* 2006). Búfalo *et al.* (2009) found that essential oils and extracts from Brazilian propolis produced some inhibition of PV1 infectivity. Elizacuível *et al.* (2013) found significant reductions of both MNV and feline calicivirus (both non-enveloped human norovirus surrogates) with essential oils from oregano, clove and zataria; however, the reductions appeared to be temperature dependent. MNV was more resistant than feline calicivirus to all three essential oils. Pilau *et al.* (2011) found that carvacrol, but not the oil from Mexican oregano was effective against human rotavirus. Both were effective against several enveloped viruses (HSV-1, human respiratory syncytial virus, bovine herpesvirus 2, bovine viral diarrhoea virus). Conversely,

Garozzo *et al.* (2009) determined tea tree essential oil to be ineffective against PV1, echovirus 9, Coxsackievirus B1 and adenovirus 2. It thus appears that the effectiveness of plant essential oils (and their active ingredients) varies greatly with the antimicrobial, the type of virus and the exposure temperature.

In the present study, oregano oil and its primary active component, carvacrol, were evaluated for their antiviral activity against MNV as a surrogate for NoV for their use in potential applications such as surface disinfection (including use in clinical settings) or food sanitization. As such, a discussion of quantitative  $\log_{10}$  reductions following various periods of exposure is relevant. Oregano oil and carvacrol both significantly reduced MNV titres within 15 min of exposure ( $0.95\text{-}\log_{10}$  and  $\geq 1.03\text{-}\log_{10}$ , respectively). Despite this, the infectivity of MNV treated with oregano oil remained stable over time ( $1.07\text{-}\log_{10}$  reduction in infectivity within 24 h of exposure), whereas carvacrol was far more effective, producing  $\geq 3.87\text{-}\log_{10}$  reductions in MNV within as little as 1 h of exposure. In the study by Elizacuível *et al.* (2013), a 2.0% concentration of oregano oil was found to reduce MNV and feline calicivirus by  $1.62\text{-}$  and  $3.75\text{-}\log_{10}$  TCID<sub>50</sub>/ml, respectively, at an incubation temperature of 37°C; no significant reductions were observed at 4°C. This suggests that the antiviral effect is temperature dependent and possibly explains why the reductions observed with 4.0% oregano oil in the present study were lower, as the experiment was conducted at room temperature (24°C).

It is difficult to determine whether such reductions in virus infectivity are due to actual damage to the virus particles or a simple inhibition of virus adsorption to the host cells. To date, the antiviral mechanisms of action for plant essential oils have not been adequately evaluated. As the majority of this work has been conducted with enveloped viruses and with clinical treatments in mind, the focus has been on the inhibition of viral adsorption to host cells by



**Figure 5** Transmission electron microscope images of murine norovirus (MNV), following exposure to 0.5% carvacrol for 24 h. All images are from the same sample and are included as examples of the varying degree of MNV particle disintegration. The photographs on the right are a magnification of the corresponding images on the left.

essential oils or on examining their effectiveness against intracellular viruses. For instance, several studies determined that pretreatment of herpes simplex viruses (HSV-1 and HSV-2, enveloped viruses) with various essential oils (manuka oil, star anise oil, hyssop oil, thyme oil and ginger oil) inhibited cell infectivity, but pretreatment of the host cell did not (Reichling *et al.* 2005; Koch *et al.* 2008; Astani *et al.* 2011). The authors concluded that the mechanism of action of these essential oils was direct binding to the virus and likely inhibition of virus adsorption to host cells. In contrast, two studies with influenza A virus, an enveloped virus, determined that essential oils (tea tree oil or a blend of wild orange, clove, cinnamon, eucalyptus and rosemary oils) did not prevent adsorption of the virus to host cells (Wu *et al.* 2010; Garozzo *et al.* 2011). Lai *et al.* 2012 reported disruption of the envelope of HSV-1 (as visualized via TEM) by carvacrol and thymol. Another study reported the disintegration of the envelopes of HSV-1 and Newcastle disease virus after treatment with oregano and clove oils using TEM imaging (Siddiqui *et al.* 1996); however, aside from these reports, others have not attempted to differentiate between binding of the antimicrobial to the viral envelope and degradation of the envelope.

A few recent studies have attempted to elucidate the mechanism of action of plant antimicrobials against non-enveloped viruses. The capsid in nonenveloped viruses serves to protect the integrity of the viral RNA and to initiate infection by adsorbing to the host cell (Cliver 2009). In a review by Cliver (2009), it was found that antimicrobials and other treatments that inactivate small enteric viruses such as the picornaviruses (e.g. poliovirus), caliciviruses (such as NoV and MNV), astroviruses and hepatitis viruses all act on the capsid to some extent; in many cases, the viral RNA is unaffected although the virus is no longer infectious. Su *et al.* (2010b) found structural changes via TEM in the capsid of feline calicivirus after exposure to cranberry juice and cranberry proanthocyanidins. Li *et al.* (2012) observed that NoV particles treated with grape seed extract also appeared deformed under TEM. Such information is not quantitative and is therefore still somewhat limited.

In the present study, multiple experiments/assays were performed in an attempt to determine the reason(s) for the observed reductions in virus infectivity. These included (i) a cell-binding assay, (ii) an RNase I protection assay and (iii) TEM imaging. Each assay provides a small amount of information that, when evaluated together with a cell culture infectivity assay, presumably create a more complete picture of the mechanism(s) of antiviral action.

The reductions observed in cell culture infectivity for carvacrol increased with greater durations of exposure to the antimicrobial (e.g. from  $1.28\text{-log}_{10}$  after 15 min to

$>4.52\text{-log}_{10}$  after 24 h of exposure to the 0.5% concentration), whereas the reductions observed in the viral RNA were initially greater and increased only minimally with time (e.g.  $2.99\text{-log}_{10}$  after 30 min to  $3.56\text{-log}_{10}$  after 24 for samples with no RNase I digestion). This suggests that although the capsid is at least partially degraded by carvacrol after a brief exposure, the virus may still be infectious. Therefore, there may be a second mechanism which contributes to the loss in infectivity in cell culture. For instance, the antimicrobial might bind to the capsid or block epitopes required for virus adsorption to the host cells, or cause a conformational change in the capsid. Any of these scenarios could prevent virus adsorption (Cliver 2009).

The results for the cell-binding assay for the carvacrol-treated MNV were inconclusive. There was no cell binding observed after only 30 min of exposure to the antimicrobial; however, as there was a several  $\log_{10}$  reduction observed in the untreated MNV controls, the detection limit of the assay may have been too high to observe a low level of cell binding. Also, the results of the RNase I protection assay indicate that many of the virus particles are no longer intact following a 30-min exposure to carvacrol, which may also contribute to the lack of cell binding. In contrast, the reductions in cell culture infectivity and cell binding for oregano oil-treated MNV appeared to be stable with increasing exposure times. This suggests that the antiviral effect occurs rapidly following exposure and then the antimicrobial (or perhaps its active ingredient) is depleted.

In the RNase I protection assay, the controls that had not been exposed to any antimicrobials were unaffected by the treatment with RNase I. The virus capsid was still intact in these controls, and therefore, the viral RNA was protected from RNase I digestion (Nuanalsuwan and Cliver 2002). In contrast, for both antimicrobials, there was an immediate reduction in the viral RNA even after the 30-min exposure without RNase I treatment. This indicates that there must be some degradation of the virus capsid and it also suggests that the antimicrobials act in some part directly upon the exposed RNA. The observed  $\log_{10}$  reductions were in general greater in the samples that had been treated with RNase I (Figs 2 and 3). Although, for the most part, this difference was not statistically significant, this may suggest that there was at least some additional degradation of the viral capsid that was not sufficient to expose the RNA to degradation by the antimicrobial, but was enough to allow entry of the RNase I enzyme into the virus particle. This is seemingly supported by the results for the TEM of the carvacrol-treated MNV in which various degrees of capsid disintegration can be found within the same sample. All of the carvacrol-treated virus particles were greatly expanded in

size, the largest being those that were visibly losing capsid integrity. Although the MNV particles appeared intact after the treatment with oregano oil, they were also larger than normal (up to *c.* 75 nm vs 35 nm for the untreated control) and thus were also possibly losing capsid integrity.

Oregano oil and its primary active component, carvacrol, have both been found to have antimicrobial efficacy against numerous pathogenic bacterial species in previous studies (Ultee *et al.* 2002; Knowles *et al.* 2005; Cox and Markham 2007; Ravishankar *et al.* 2009, 2010; García-García *et al.* 2011; Mild *et al.* 2011). Based on the results of the present study, this antimicrobial efficacy is also broad-spectrum, as they were also fairly effective against MNV, a nonenveloped virus. This was especially true for carvacrol, which exhibited reductions nearing 4-log<sub>10</sub> within 1 h of exposure. Both compounds appear to cause the viral capsid to lose its integrity, rather than merely inhibiting infectivity by binding to the capsid and preventing adsorption of the virus to host cells. This indicates that this is likely irreversible and thus true virus inactivation. The antibacterial mechanisms of action are likely quite different due to the greater complexity of bacterial cell wall structures and components; nonetheless, there is some evidence that carvacrol acts directly upon bacterial cell walls/membranes as well (Ultee *et al.* 2002; Cox and Markham 2007; García-García *et al.* 2011).

In conclusion, the present study provides novel findings on the antiviral properties and mechanisms of action of oregano oil and its active component, carvacrol, against MNV, a nonenveloped virus. Our results demonstrate that carvacrol is effective in inactivating MNV, a human NoV surrogate. Highly purified carvacrol is commercially available (e.g. the >98% solution purchased from Sigma-Aldrich used in the present study) and is effective at concentrations of ≤0.5%. As carvacrol is a generally regarded as safe (GRAS) plant antimicrobial, it may be used in applications in which other antimicrobials might not be preferred (e.g. on foods or food contact surfaces, or used in place of corrosive compounds on surfaces). Thus, carvacrol could potentially be used as a natural food and surface (fomite) sanitizer to control NoV.

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### Conflict of interest

No conflict of interest declared.

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