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The effect of carvacrol on enteric viruses

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ABSTRACT

Carvacrol, a monoterpenic phenol, is said to have extensive antimicrobial activity in a wide range of food spoilage or pathogenic fungi, yeast and bacteria. The aim of this study was to assess its antiviral activity on norovirus surrogates, feline calicivirus (FCV), murine norovirus (MNV), and hepatitis A virus (HAV), as well as its potential in food applications. Initially, different concentrations of carvacrol (0.25, 0.5, 1%) were individually mixed with each virus at titers of ca. 6–7 log $TCID_{50}$/ml and incubated 2 h at 37 °C. Carvacrol at 0.5% completely inactivated the two norovirus surrogates, whereas 1% concentration was required to achieve ca. 1 log reduction of HAV. In lettuce wash water, carvacrol efficacy on MNV was dependent on the chemical oxygen demand (COD), with no effect over 300 ppm. A 4 log reduction in FCV infectivity was observed when 0.5% carvacrol was used to sanitize lettuce wash water, regardless of COD. Carvacrol was also evaluated as a natural disinfectant of produce, showing 1% carvacrol reduced inoculated NoV surrogates titers in lettuce by 1 log after 30 min contact. These results represent a step forward in improving food safety by using carvacrol as an alternative natural additive to reduce viral contamination in the fresh vegetable industry.

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1. Introduction

There is increasing awareness of the importance of foodborne diseases caused by enteric viruses; furthermore, several international organizations have detected an upward trend in their incidence. Epidemiological evidence indicates that enteric viruses, in particular human norovirus (NoV) and hepatitis A virus (HAV), are the leading causes of foodborne illnesses in developed countries mainly associated with the consumption of shellfish, soft fruits and leafy greens (Anonymous, 2013; EFSA, 2014a). In the EU, foodborne viruses were identified as the most frequently detected causative agent of foodborne outbreaks in vegetables in 2012, accounting for 25.6% of the reported cases (EFSA, 2014a).

The transmission of enteric viruses associated with the consumption of leafy greens is regularly reported after contamination by virus-infected food handlers during harvesting, packaging, or food preparation or by polluted irrigation water. Therefore, it is important to find natural antiviral biopreservatives which are safe, environment friendly, and preferably inexpensive for use in the food industry.

The growing demand for the use of natural additives has produced a substantial increase in the number of studies based on natural extracts such as essential oils or their main compounds in the last decade. They are categorized as Generally Recognised as Safe (GRAS), and are therefore potential alternatives to chemical additives. Furthermore, the antibacterial, antifungal, insecticidal, antitoxigenic and antiparasitic activities of these compounds have been extensively reported (Burt, 2004). In contrast, reports on the antiviral effects of essential oils or their compounds are somewhat limited (Table 1).

Carvacrol, a monoterpenic phenol, has emerged due to its wide spectrum activity extended to food spoilage or pathogenic fungi, yeast and bacteria (Nostro and Papalia, 2012). Carvacrol is the primary component of oregano essential oil and has been identified as a natural economical food preservative (Lu and Wu, 2010; Obaidat and Frank, 2009) with potential for incorporation in food packaging (Guarda et al., 2011). It has recently been reported that carvacrol could effectively reduce the infectivity of murine norovirus (MNV) (Gilling et al., 2014a), a human norovirus surrogate, and rotavirus (Pilau et al., 2011). However, the effectiveness of carvacrol against other foodborne viruses, as well as its efficacy in food applications has yet to be explored.

In this study, the effect of carvacrol on the infectivity of HAV and two norovirus surrogates, MNV and feline calicivirus (FCV) has been assessed. We have also studied the efficacy of carvacrol in reducing viral loads in fresh-cut lettuce wash water and on a produce model.

2. Material and methods

2.1. Virus strains and cell lines

The cytopathogenic MNV-1 strain (kindly provided by Prof. H. W. Virgin, Washington University School of Medicine, USA), the F9 strain of FCV (ATCC VR-782) and the HM-175 strain of HAV (ATCC VR-1402)

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Table 1
Efficacy of essential oils and their compounds on different enteric viruses.

	Tested virus	Experimental setup (time, temperature)	Inactivation (log reduction)	References
Essential oils				
Allspice (4%)	MNV	24 h, 24 °C	3.41	Gilling et al. (2014b)
Clove (1%)	FCV, MNV	2 h, 37 °C	3.75; 0.67	Elizaquível et al. (2013)
Hyssop (0.2%)	AdV-2, MNV	2 and 24 h 4, 25 and 37 °C	No effect	Kovac et al. (2012)
Lemongrass (4%)	MNV	24 h, 24 °C	2.74	Gilling et al. (2014b)
Marjoram (0.2%)	AdV-2, MNV	2 and 24 h 4, 25 and 37 °C	No effect	Kovac et al. (2012)
Mexican oregano	RV		No effect	Pilau et al. (2011)
Oregano (2%)	FCV, MNV	2 h, 37 °C	3.75; 1.62	Elizaquível et al. (2013)
Oregano (4%)	MNV	6 h, 24 °C	1.10	Gilling et al. (2014a)
Tea tree (0.025%)	PV 1, Echo 9, Coxsackie B1, AdV-2	1 h, 37 °C	No effect	Garozzo et al. (2009)
Zataria (0.1%)	FCV, MNV	2 h, 37 °C	4.51; 0.25	Elizaquível et al. (2013)
Main compounds				
Carvacrol (0.5%)	MNV	3 h, 24 °C	3.87	Gilling et al. (2014a)
Citral (4%)	MNV	24 h, 24 °C	3.00	Gilling et al. (2014b)

were propagated and assayed in RAW 264.7 (kindly provided by Prof. H. W. Virgin), CRFK (ATCC CCL-94) and FRhK-4 cells (kindly provided by Prof. Albert Bosch, University of Barcelona), respectively. Semi-purified stocks were subsequently produced from the same cells by centrifugation of infected cell lysates at 660 ×g for 30 min. Infectious viruses were enumerated by determining the 50% tissue culture infectious dose ($TCID_{50}$) with eight wells per dilution and 20 μl of inoculum per well.

2.2. Cytotoxicity determination of carvacrol on cell lines

Carvacrol (≥98% purity; Sigma Aldrich) at various concentrations were added to individual wells of confluent RAW 264.7, CRFK and FRhK-4 cells in 96-well plates and incubated 2 h under 5% CO_2. Thereafter cells were added with 150 μl of supplemented with 2% of fecal calf serum (FCS) and incubated further for 2 to 15 days. Cytotoxicity effects were determined by visual inspection under the optical microscope.

2.3. Antiviral effects of carvacrol

Carvacrol diluted in 50% ethanol was added to virus suspensions in DMEM with 2% FCS (ca. 6–7 log $TCID_{50}$/ml) and further incubated at 37 °C (unless indicated) in a shaker 2 h. Then, infectious viruses were enumerated by cell culture assays as described above. Each treatment was done in triplicate. Positive controls were virus suspensions added with ethanol in amounts corresponding to the highest quantity present.

For exposure time experiments, carvacrol at 0.5% was added to MNV and FCV suspensions at ca. 6–7 log $TCID_{50}$/ml. Samples were immediately removed ($t = 0$) and at different time intervals (30, 60 and 120 min), and ten-fold diluted in DMEM with 2% FCS to neutralize the carvacrol action. Then, infectious viruses were enumerated by cell culture assays as described above. Experiments were performed in triplicates. Antiviral activity of carvacrol was estimated by comparing the number of infectious viruses on suspensions without carvacrol and on the carvacrol-treated virus suspensions.

2.4. Antiviral activity of carvacrol in fresh-cut lettuce wash water

The process wash water with high chemical oxygen demand (COD) was prepared as described by López-Gálvez et al., 2011. Fresh-cut lettuce wash water was diluted with tap water to obtain a batch with 500 mg/l COD. The process wash water were further diluted with tap water to obtain COD values of ca. 200, 300, and 400 mg/l. Lettuce wash water with different COD values were inoculated with MNV and FCV at ca. 10^6 $TCID_{50}$/ml, and carvacrol was added to the inoculated water to obtain concentrations of 0.5%. Triplicate samples were incubated at 37 °C during 2 h with gentle agitation (150 rpm) in a water bath. Treated and non-treated samples were 10-fold serial diluted, and cell culture assays were performed the same day.

2.5. Produce wash test using lettuce

Determination of the virucidal activity of carvacrol wash was performed by adapting the procedure described by Su and D' Souza, 2013. Briefly, locally purchased fresh lettuce was cut in pieces of 3 × 3 cm and sterilized with UV light in a safety cabinet under laminar flow for 15 min prior to inoculation of the test viruses. A suspension of FCV or MNV diluted in PBS buffer (at 10^6 or 10^4 $TCID_{50}$for both viruses) was seeded by distributing 30 μl over 3 spots onto the lettuce surface. Inoculated samples were air dried in a laminar flow hood for 45 min. Thereafter, 0.15 ml of water or a carvacrol solution at 0.5 or 1% was added for 30 min to inoculated lettuce samples. After treatments, viruses were eluted from the lettuce surface by pipetting with 0.85 ml DMEM containing 2% FCS, and samples were 10-fold serial diluted, and cell culture assays were performed the same day. Each treatment was done in triplicate.

2.6. Statistical analysis

The significance of differences among the mean numbers of viruses determined after the various treatments was determined by Student's t test with a significance level of $P < 0.05$ (Microsoft Office Excel; Microsoft, Redmond, WA, USA).

3. Results

3.1. Determination of cytotoxicity of carvacrol on cell lines

Carvacrol was found to be cytotoxic at concentrations that exceeded 1%, for the three cell lines. Thus, this value was the maximum concentration of carvacrol added to determine the antiviral effects of carvacrol against MNV, FCV and HAV.

3.2. Effect of carvacrol on the infectivity of norovirus surrogates and HAV

Incubation of MNV and FCV with carvacrol at concentrations of 0.25, 0.5 and 1% for 2 h at 37 °C decreased the titer of the two norovirus surrogates (Table 2). Carvacrol at 0.5 and 1% reduced FCV and MNV titers to undetectable levels, while carvacrol at 0.25% reduced FCV by 3.4 log

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