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# Effect of (−)-epigallocatechin gallate at different pH conditions on enteric viruses

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ABSTRACT

Epigallocatechin gallate (EGCG), a flavonoid from green tea, is said to have extensive antimicrobial activity in a wide range of food spoilage or pathogenic fungi, yeast and bacteria. In this work, the antiviral activity of EGCG was assessed against hepatitis A virus (HAV) and murine norovirus (MNV), a human norovirus surrogate, at different temperatures, contact times and pH conditions by cell-culture methods. EGCG was effective in reducing the titers of HAV and MNV in a dose-dependent manner at neutral pH and 25 and 37 °C, while no effect was reported at 4 °C. HAV and MNV infectivity was completely removed after overnight treatment with EGCG at 2.5 mg/mL at 37 °C. Furthermore, results also revealed that EGCG was very effective inactivating MNV and HAV at neutral and alkaline pH but was ineffective at pH 5.5. Results from cell-culture assays and viability RT-qPCR assays indicated that EGCG did not dramatically affect viral capsid, which instead may suffer subtle alterations of proteins. Moreover, HPLC/MS analysis of catechin solutions at different pHs indicated that antiviral activity was most likely due to catechin derivatives rather than EGCG itself, given the evolution of these compounds at the various pH conditions tested. These findings suggest that green tea catechins appear to be a suitable natural option for foodborne viral reduction.



## 1. Introduction

Nowadays, foodborne viral outbreaks are a growing concern for food safety authorities. Indeed, enteric viruses, in particular human noroviruses, which cause gastroenteritis, are the leading causes of foodborne illnesses in industrialized countries (Control & Prevention, 2013; EFSA, 2015). Moreover, hepatitis A virus (HAV) is considered as a re-emerging pathogen and is responsible for about half the total number of human hepatitis infections diagnosed worldwide (Sprenger, 2014). Norovirus and HAV can be transmitted directly from person-to-person, but also indirectly via virus-contaminated food (mainly associated with shellfish, soft fruits, leafy greens, and ready-to-eat meals), water, and surfaces.

Due to their low infectious dose (10–100 viral particles) (Teunis et al., 2008; Yezli & Otter, 2011) and to their stability and resistance to inactivation processes, the development of alternative methods for the viral decontamination of food has been recently promoted by public authorities (WHO, 2013). Amongst them, promising results have been reported for many natural compounds tested as antivirals *in vitro*, but when they were evaluated in food model systems or food applications, the viral decay was somewhat limited (Bozkurt, D'Souza, & Davidson, 2015; D'Souza, 2014; C. Sánchez, Aznar, & Sánchez, 2015). Many factors could be responsible for such decrease in efficacy such as the interaction of the active compound or the virus with food matrices, the pH, the water activity, etc.

From the commercially available natural extracts, green tea extract (GTE) has demonstrated inhibitory properties against foodborne bacteria (Perumalla & Hettiarachchy, 2011) and more recently against norovirus surrogates as well (Ueda et al., 2013). Chemically, GTE mainly contains catechins, a group of flavonoids with antioxidant properties (Yilmaz, 2006). Specifically, epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), gallocatechin (GC), catechin (C) and epigallocatechin gallate (EGCG) have been found to be the main catechins present in GTE (Kajiya

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et al., 2004). These bioactive compounds possess many health benefits (Singh, Shankar, & Srivastava, 2011), including protective effects against infections (Steinmann, Buer, Pietschmann, & Steinmann, 2013), cardiovascular and neurodegenerative diseases (Fu, Zhen, Yuskavage, & Liu, 2011), inflammation and arthritis (Singh, Akhtar, & Haqqi, 2010) and cancer (Larsen & Dashwood, 2010). EGCG and ECG are the most abundant compounds in GTE and they have showed strong antimicrobial (Shimamura, Zhao, & Hu, 2007; Veluri, Weir, Bais, Stermitz, & Vivanco, 2004) and antiviral (Dhiman, 2011; Savi, Barardi, & Simões, 2006; Xiao, Yang, Shi, Liu, & Chen, 2008) activities, even when encapsulated within chitosan electrosprayed microcapsules (Gómez-Mascaraque, Soler, & Lopez-Rubio, 2016) or applied as hand sanitizer formulations (Zhang, Yang, Yang, Wu, & Wu, 2016). Due to their autooxidation and varying degree of polymerization, catechins show diverse structural features (Li, Taylor, Ferruzzi, & Mauer, 2012), which result in different binding modes and inhibitory effects. Evidences clearly showed a pH dependent effect on the antiviral activity of EGCG. For example, it was reported that EGCG at neutral pH inactivates herpes simplex virus (HSV), an enveloped virus, but it was ineffective below pH 7.4. In contrast, when EGCG was oxidatively coupled to form dimers with one or two gallate moieties, the antiviral activity at acid pH was substantially increased (Isaacs et al., 2011).

Most studies aiming to determine the antiviral activity of natural compounds have been performed by artificially adding a known amount of the selected compound to a given viral suspension, determining the reduction in the infectious titer after subjecting the treated sample to designated conditions, and applying statistical procedures to determine the significance of virus decay. Obviously, this implies the use of virus strains that may be propagated in cell cultures and enumerated through infectivity, thus greatly restricting the range of viruses to be used in these studies. This is extremely relevant for human norovirus, since only very recently, a human norovirus culture system using enteroids cells has been developed (Ettayebi et al., 2016), but there are limitations that need to be overcome before this assay can be routinely used. Until then, evaluation of the efficacy of natural compounds on human norovirus is still performed using norovirus surrogates such as feline calicivirus (FCV), murine norovirus (MNV), and Tulane virus (TV). Moreover, a novel approach to assess human norovirus and HAV infectivity by combining intercalant dyes and RT-qPCR (Moreno, Aznar, & Sánchez, 2015; Randazzo, López-Gálvez, Allende, Aznar, & Sánchez, 2016; Sánchez, Elizaquível, & Aznar, 2012) has been recently developed with the potential to be used for inactivation studies.

In the present work, the antiviral activity of EGCG was assessed against enteric viruses at different temperatures, contact times and pH conditions by cell-culture methods. Furthermore cell-culture results were compared to results obtained by viability RT-qPCR. Moreover, HPLC analysis of the catechin solutions at different pHs were performed to correlate the antiviral effect of EGCG and its derivatives formed at the various tested conditions and, thus, be able to explain the different antiviral activity displayed.

## 2. Material and methods

### 2.1. Viral strains, cell lines and infections

HAV, HM-175/18f strain (ATCC VR-1402), was propagated and assayed in FRhK-4 cells (kindly provided by Prof. A. Bosch, University of Barcelona, Spain). Murine norovirus, MNV-1 strain, was propagated and assayed in RAW 264.7 cells (kindly provided by Prof. H. W. Virgin, Washington University School of Medicine, USA). 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 using the Spearman-Karber method (Pintó, Diez, & Bosch, 1994).

Faecal suspension of human norovirus genogroup II genotype 4 (GII.4 variant Den Haag 2006b) was kindly provided by Dr. J. Buesa, University of Valencia, Spain. Norovirus stool sample was suspended (10%, wt/vol) in phosphate-buffered saline (PBS) containing 2 M $NaNO_3$ (Panreac, Spain), 1% beef extract (Conda, Spain), and 0.1% Triton X-100 (Fisher Scientific, USA) (pH 7.2) and pelleted at 1000 × g for 5 min. The supernatant was stored at −80 °C in aliquots.

### 2.2. Effect of EGCG on HAV and MNV suspensions

EGCG from green tea (Sigma-Aldrich, CAS number 989-51- 5, Spain) was dissolved in PBS (pH 7.2) to obtain concentrations of 0.25 and 2.5 mg/mL. Each EGCG solution was mixed with an equal volume of HAV and MNV suspensions (ca. 6–7 log $TCID_{50}$/mL), followed by incubation at 4, 25 and 37 °C in a shaker for 2 or 16 h (overnight incubation). Ten-fold dilutions of EGCG-treated and untreated virus suspensions were inoculated into confluent FRhK-4 and RAW monolayers in 96-well plates. 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 PBS only. The decay of HAV and MNV titers was calculated as $\log_{10}(N_x/N_0)$, where $N_0$ is the infectious virus titer for untreated samples and $N_x$ is the infectious virus titer for EGCG-treated samples.

### 2.3. Pre-treatment with EGCG prior to virus infection

Ninety six-well cell culture plates were seeded with RAW 264.7 or FRhk-4 cells. After 24 h or 72 h, RAW 264.7 or FRhk-4 cells, respectively, cell media was removed and washed two times with PBS pH 7.2. Cell lines were treated for 1 h with 0.1 and 1 mg/mL (0.2 and 2 mM, respectively) of EGCG in PBS pH 7.2. Then, EGCG was removed from the 96-well plate and the cells were washed twice with PBS. Untreated and EGCG-treated monolayers were then infected to ten-fold dilutions of MNV and HAV. Infectious viruses and effectiveness of the treatments were calculated as described above.

### 2.4. Effect of pH on the antiviral activity of EGCG

In order to elucidate the effect of pH on the antiviral activity of EGCG, virus suspensions were ten-fold diluted in PBS at different pHs and incubated with a EGCG solution at 0.25 and 2.5 mg/mL prepared in PBS at different pHs (5.5, 6.5, 7.2, 8.0 and 8.5). Samples were further incubated at 37 °C in a water-bath shaker at 150 rpm for 2 h. Infectious viruses and effectiveness of the treatments were calculated as described above.

### 2.5. Efficacy of EGCG on human norovirus and HAV using viability RT-qPCR

Human norovirus and HAV suspensions were overnight incubated with 2.5 mg/mL of EGCG at 37 °C in a shaker. Positive controls were virus suspensions added with PBS only. To assess virus infectivity, a viability-RT-qPCR procedure recently developed was applied (Moreno et al., 2015; Randazzo et al., 2016). Briefly, one-hundred microliters of EGCG-treated virus and un-treated virus were added to PMAxx 50 μM (Biotium, USA) and 0.5% Triton X-100