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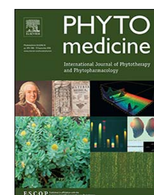

# Inhibitory mechanism of five natural flavonoids against murine norovirus

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ABSTRACT

*Background:* Human noroviruses (HuNoV), which are responsible for acute gastroenteritis, are becoming a serious public health concern worldwide. Since no effective antiviral drug or vaccine for HuNoV has been developed yet, some natural extracts and their active components have been investigated for their ability to inhibit noroviruses. However, their exact antiviral mechanisms have not been investigated.
*Purpose:* This study was performed to investigate the expression of interferon (IFN)-α, IFN-λ, tumor necrosis factor-α (TNF-α), Mx, and zinc finger CCCH type antiviral protein 1 (ZAP), 2′-5′ oligo (A) synthetase (OAS), and inducible nitric oxide synthase (iNOS) in RAW 264.7 cells pre-treated with fisetin, daidzein, quercetin, epigallocatechin gallate (EGCG), and epicatechin gallate (ECG) that have anti-noroviral activity.
*Study design:* Based on the antiviral activity of the five flavonoids, recently reported by our group, the expression of antiviral factors such as IFN-α, IFN-λ, TNF-α, IL-1β, IL-6, Mx, ZAP, OAS, and iNOS was investigated in RAW 264.7 cells pre-treated with these flavonoids.
*Methods:* Anti-noroviral effect was determined by performing a plaque assay on cells treated with the flavonoid. RAW 264.7 cells were treated with fisetin, daidzein, quercetin, EGCG, and ECG. Then, mRNA of IFN-α, IFN-λ, TNF-α, IL-1β, IL-6, Mx, ZAP, OAS, and iNOS were measured by real-time RT-PCR. IFN-α, TNF-α, IL-1β, and IL-6 proteins were measured by ELISA.
*Results:* Pre-treatment with fisetin (50 μM), fisetin (100 μM), EGCG (100 μM), quercetin (100 μM), daidzein (50 μM), and ECG (150 μM) significantly reduced MNoV by 50.00 ± 7.14 to 60.67 ± 9.26%. The mRNA levels of IFN-α, IFN-λ, TNF-α, Mx, and ZAP were upregulated in RAW 264.7 cells pre-treated with fisetin, quercetin, and daidzein, but not in those pre-treated with EGCG or ECG. Regarding protein levels, IFN-α was significantly induced in cells pre-treated with fisetin, quercetin, and daidzein, whereas TNF-α was significantly induced only in cells pre-treated with daidzein.
*Conclusion:* Pre-treatment of RAW 264.7 cells with the five flavonoids inhibited MNoV by upregulating the expression of antiviral cytokines (IFN-α, IFN-λ, and TNF-α) and interferon-stimulating genes (Mx and ZAP).

## Introduction

Human noroviruses (HuNoV), which are responsible for acute gastroenteritis, are becoming a serious public health concern worldwide (Karst et al., 2014). HuNoV is a non-enveloped positive-sense RNA virus that belongs to the family *Caliciviridae* (Karst et al., 2014; Naczk and Shahidi, 2006). Several attempts have been made to propagate HuNoV using *in vitro* cell culture techniques and animal models; however, HuNoV culture systems could not be successfully established (Karst et al., 2014; Duizer et al., 2004). Recently, it was reported that HuNoV could be replicated in a B-cell culture model; however, this method has limitations such as the viral source, e.g., stools, and inverse correlation between virus inocula and infectivity (Jones et al., 2015). Since murine norovirus (MNoV) shares several biological and molecular characteristics with HuNoV, it can be used instead of HuNoV to study norovirus replication and pathogenesis (Wobus et al., 2006).

Previous studies have used natural extracts and their active components to control various viruses (Chiang et al., 2003; Su and D'Souza, 2011; Seo et al., 2016). For example, Su and D'Souza (2011) showed that treatment with grape seed extract inhibited MNoV, feline calcivirus (FCV), and hepatitis A virus (HAV) (Su and D'Souza, 2011). Similarly Chiang et al. (2003) showed that treatment with quercetin

*Abbreviations:* **D50**, daidzein (50 μM); **ECG**, epicatechin gallate; **EGCG**, epigallocatechin gallate; **ELISA**, enzyme linked immunosorbent assay; **F50**, fisetin (50 μM); **F100**, fisetin (100 μM); **FCV**, feline calicivirus; **HCV**, hepatitis C virus; **HEV**, hepatitis E virus; **HuNoV**, human norovirus; **HSV**, herpes simplex virus; **IFN-α**, interferon-α; **IFN-λ**, interferon-λ; **IL-1**, Interleukin-1; **IL-6**, Interleukin-6; **iNOS**, inducible nitric oxide synthase; **MNoV**, murine norovirus; **NO**, nitric oxide; **OAS**, 2′-5′-oligo (A) synthetase; **Q100**, quercetin (100 μM); **R50**, ribavirin (50 μM); **RT-PCR**, reverse transcription polymerase chain reaction; **TNF-α**, tumor necrosis factor-α; **ZAP**, zinc finger CCCH type antiviral protein 1

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inhibited herpes simplex virus (HSV) and adenovirus (Chiang et al., 2003). We reported the comparative activities of 10 antiviral flavonoids including epigallocatechin gallate (EGCG), epicatechin gallate (ECG), quercetin, daidzein, fisetin, baicalein, kaempferol, biochanin A, theaflavin digallate, and theaflavin against MNoV and FCV (Seo et al., 2016). The pre-treatment of RAW 264.7 cells or Crandell-Reese feline kidney cells with fisetin, daidzein, quercetin, EGCG, and ECG showed a potent antiviral activity against both MNoV and FCV (Seo et al., 2016). However, anti-noroviral mechanisms of fisetin, daidzein, quercetin, EGCG, and ECG have not been investigated.

Virus-infected cells express several cytokines like type I, type II, and type III interferons (IFNs) that can block viral replication to limit the spread of the viral infection (Mossman, 2011). Type I IFNs such as IFN-α, IFN-β, and IFN-ω stimulate the transcription of many interferon-stimulated genes (ISGs) like zinc finger CCCH type antiviral protein 1 (ZAP), Mx, and 2′-5′ oligo (A) synthetase (OAS), leading to degradation of the viral RNA genome (Mossman, 2011). IFN-β and IFN-γ blocked the translation of intermediate RNA of MNoV in macrophages and dendritic cells (Changotra et al., 2009). The co-treatment of HG23 cells, transfected with Norwalk virus replicon plasmid, with IFN-α and ribavirin inhibited the RNA-dependent RNA-polymerase (RdRp) activity of HuNoV (Chang and George, 2007). Recently, the antiviral mechanism of Korean red ginseng (KRG) extract and ginsenosides Rb1 and Rg1 against FCV has been shown to be mediated by the induction of IFN-α, IFN-β, IFN-ω, Mx, and ZAP (Lee et al., 2014).

Since the pretreatment of cells with fisetin, daidzein, quercetin, EGCG, and ECG inhibited MNoV, we hypothesized that these flavonoids induce cytokines that create an antiviral environment, thus suppressing viral replication. Therefore, we investigated the expression of antiviral cytokines in RAW 264.7 cells pre-treated with fisetin, daidzein, quercetin, EGCG, and ECG.

## Materials and methods

### *Reagents, virus, and cells*

EGCG, ECG, quercetin, daidzein, fisetin, and ribavirin were purchased from Sigma-Aldrich (St. Louis, MO, USA) (Fig. 1). Ribavirin, EGCG, and ECG were dissolved in sterile distilled water, while quercetin, daidzein, and fisetin were dissolved in dimethylsulfoxide (DMSO). All flavonoids and ribavirin were filtered through 0.20-μm syringe filters.

MNoV-1 was kindly provided by Professor Herbert W. Virgin from the University of Washington, and was cultivated by the standard protocol (Wobus et al., 2004). RAW 264.7 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Ribavirin was used as a standard anti-noroviral drug at concentrations of 50–200 μM (Chang and George, 2007). Therefore, we used 50 μM of ribavirin as a positive control in our study. Concentrations of the flavonoids were determined by our previous study as follows: fisetin, 50 and 100 μM; EGCG, 100 μM; quercetin, 100 μM; daidzein, 50 μM; and ECG, 150 μM (Seo et al., 2016).

### *The inhibitory effect of flavonoids on MNoV activity*

RAW 264.7 cells were seeded at a density of $1 \times 10^5$ cells/well in 12-well plates in a 5% $CO_2$ incubator at 37 °C. The RAW 264.7 cell monolayer was treated with water, DMSO, fisetin (50 μM), fisetin (100 μM), EGCG (100 μM), quercetin (100 μM), daidzein (50 μM), ECG (150 μM), and ribavirin (50 μM), and incubated in a 5% $CO_2$ incubator at 37 °C for 24 h. Then, plaque assay of MNoV-1 was performed as described in the previous study with slight modification (Su and D'Souza, 2013). The titer of the MNoV-1 stock was 7 $\log_{10}$ plaque forming units (PFU)/ml. Plaques were stained with neutral red solution and counted.

### *Experimental design*

RAW 264.7 cells were seeded at a density of $1 \times 10^6$ cells/well in 6-well plates and cultured for 24 h at 37 °C in a 5% $CO_2$ incubator. Cells were treated with fisetin (50 μM), fisetin (100 μM), EGCG (100 μM), quercetin (100 μM), daidzein (50 μM), ECG (150 μM), and ribavirin (50 μM) for 24 h then with 2 $\log_{10}$ PFU/ml MNoV-1 for 48 h. Broadly, cells were divided into two experimental groups, one group infected, and the other group uninfected with MNoV. Uninfected cells were classified as follows: NC, negative control cells untreated with any flavonoid; R50, cells treated with 50 μM ribavirin; D50, cells treated with 50 μM of daidzein; F50, cells treated with 50 μM of fisetin; F100, cells treated with 100 μM of fisetin; Q100, cells treated with 100 μM of quercetin; ECG150, cells treated with 150 μM of ECG; and EGCG100, cells treated with 100 μM of EGCG. Similarly, cells infected with MNoV were classified as follows: V, infected untreated cells; RV50, infected cells treated with 50 μM ribavirin; DV50, infected cells treated with 50 μM daidzein; FV50, infected cells treated with 50 μM fisetin; FV100, infected cells treated with 100 μM fisetin; QV100, infected cells treated with 100 μM quercetin; ECGV150, infected cells treated with 150 μM ECG; and EGCGV100, infected cells treated with 100 μM EGCG. Cells from each experimental group were washed with sterile phosphate-buffered saline, and total RNA extraction was performed directly on each cell using the NucleoSpin RNA Plus kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Contamination with genomic DNA was eliminated by the NucleoSpin RNA Plus kit.

### *Real-time reverse transcription polymerase chain reaction (Real-time RT-PCR)*

For reverse transcription, a mixture was prepared with 1X PCR buffer, 10 mM dNTP, 25 μM oligo d(T), 10 U of RNase inhibitor, 25 U of MuLV reverse transcriptase, and 100 ng/μl total RNA. The mixture was incubated at 42 °C for 15 min, and the enzymes were inactivated at 99 °C for 5 min. To evaluate the relative mRNA expression of antiviral cytokines, quantitative real-time RT-PCR was performed. To detect the mRNA of IFN-α, IFN-λ, IL-1β, IL-6, TNF-α, OAS, Mx, ZAPS, and iNOS in RAW 264.7 cells, the reaction mixture consisted of 12.5 μl of 2X premix Ex Taq (TAKARA), 5 pM of each the forward primer, reverse primer, and probe (Table 1), 100 ng of cDNA, and distilled water to a final volume of 25 μl. Amplification was performed as follows: initial denaturation for 30 s at 95 °C, 40 cycles of 95 °C for 15 s, and 60 °C for 45 s. Primers and probe sequences are shown in Table 1.

### *Enzyme linked immunosorbent assay (ELISA)*

The cell supernatants were used to detect the expression of IFN-α, TNF-α, IL-1β, and IL-6 in RAW 264.7 cells pre-treated with antiviral flavonoids. TNF-α, IL-1β, and IL-6 were analyzed by MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel Kit using Luminex 200 System based on flow cytometry (Merck-millipore, Billerica, MA, USA), while IFN-α was measured by VeriKine Mouse Interferon ELISA Kit (PBL assay science, Piscataway, NJ, USA). Absorbance was measured at 450 nm using an Epoch Spectrophotometer (Biotek, Wakefield, MA, USA), and a standard curve was created for the quantitative analysis.

### *Statistical analysis*

Each experiment was conducted three times independently; each treatment group had three replicates per experiment. Data were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple range test using the Statistical Analysis System software (SAS 9.1 version; Cary, NC, USA). Differences were considered significant when *P* values were less than 0.05.

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