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Comparison of the antiviral activity of flavonoids against murine norovirus and feline calicivirus

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ABSTRACT

Antiviral effect of 10 flavonoids against feline calicivirus (FCV) and murine norovirus (MNV) was investigated on Crandell-Reese feline kidney (CRFK) cells and RAW 264.7 cells. Each cells infected with FCV or MNV was treated with different flavonoids at concentrations between 50 and 300 μM. A 200 μM dose of kaempferol and daidzein significantly reduced the FCV titer by 69.76 ± 8.71 and 63.47 ± 9.82%, respectively. A 300 μM dose of quercetin and fisetin reduced the titer of FCV titer by 56.70 ± 11.39 and 41.84 ± 11.35%, respectively. The titer of MNV was reduced by 50.47 ± 6.91 and 51.21 ± 13.38% by a 50 μM dose of fisetin or a 150 μM dose of epicatechin gallate, respectively. A 50 μM dose of daidzein and 100 μM of quercetin reduced the MNV titer by 46.32 ± 8.70 and 45.78 ± 9.81%, respectively. The pre-treatment of quercetin, fisetin, daidzein, and epicatechin gallate demonstrated the anti-noroviral activity against MNV and FCV.

1. Introduction

Flavonoids are commonly found in vegetables, fruits, nuts, spices, and medicinal herbs. Blueberries, blackberries, cranberries, and red beans are known to have a particularly high flavonoid content, and more than 4000 flavonoids are known to date (Di Carlo, Mascolo, Izzo, & Capasso, 1999; de Groot & Rauen, 1998; Nijveldt et al., 2001). Different flavonoid compounds have different substituents on the A and B rings (Supplementary Fig.1); according to the oxidation level and substitution of the C ring, flavonoids are categorized into six classes: flavones, flavonols, flavanols, flavanones, and anthocyanins (Heim, Tagliaferro, & Bobilya, 2002; Hollman & Katan, 1999). Flavonoids are well-known to have anti-inflammatory, antimicrobial, anticancer, antiallergic, antidiabetic, antidiarrhoeal, antioxidant, and antihepatotoxic activities (Abdullahi, Agho, Amos, Gamaniel, & Wambebe, 2001; Banskota et al., 2001; Chinnam et al., 2010; Ferrandiz & Alcaraz, 1991; Kawai et al., 2007; Kuo, Liu, & Chao, 2004; Yang et al., 2014). As many flavonoid compounds have been shown to have beneficial effects on health, it is very important that flavonoids with potential effects be studied for possible therapeutic use (Di Carlo et al., 1999).

Several flavonoids have been shown to have an antiviral effect against RNA viruses such as influenza A virus, human immunodeficiency virus (HIV), rotavirus, poliovirus, coxsackievirus, dengue fever virus, and Japanese encephalitis virus (Johari, Kianmehr, Mustafa, Abubakar, & Zandi, 2012; Li et al., 2000; Mukoyama et al., 1991; Sithisarn, Michaelis, Schubert-Zsilavecz, & Cinatl, 2013; Zandi et al., 2011). Recently, the antiviral effect against influenza A and B of catechins, proanthocyanidins, theaflavins, flavonols, and one structurally modified derivative of epigallocatechin was reported along with their structural analysis (Yang et al., 2014).

Noroviruses belong to the family *Caliciviridae* and are non-enveloped, positive-sense RNA viruses (Bull et al., 2005; Glass et al., 2000). Human norovirus (HNoV) is a serious public health concern worldwide (Glass et al., 2000). *In vitro* culture systems and animal models of HNoV have not been established although 3-dimensional cell culture systems and human volunteer studies have attempted to propagate HNoV (Richards, 2012). Therefore, no antiviral drug or effective vaccine has been developed against HNoV. Ribavirin has been used as an antiviral drug to inhibit the

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replication of HNoV; however, its clinical use has been limited by its toxicity (Chang & George, 2007; Chen et al., 2011). In recent publications, the anti-noroviral activities of grape seed, pomegranate, mulberry, black raspberry, cranberry, green tea, persimmon, and red ginseng were measured against norovirus surrogates because of the lack of cultivation techniques for HNoV (Lee, Seo, Kang, Oh, & Choi, 2014; Li, Baert, & Uyttendaele, 2013; Ryu et al., 2015; Song, Lee, & Seong, 2005; Su & D'Souza, 2013). Polyphenols and proanthocyanins have been identified as the active components responsible for the antiviral effect of several herbal extracts, but the antiviral activity of flavonoid compounds has not been examined extensively. In order to compare the inhibitory effect of flavonoids against norovirus surrogates, we selected candidate flavonoids by reviewing previous publications on flavonoids with antiviral effects against human RNA viruses. Therefore, this study investigated the antiviral activities of epigallocatechin gallate (EGCG), epicatechin gallate (ECG), quercetin, daidzein, fisetin, baicalein, kaempferol, biochanin A, theaflavin digallate, and theaflavin against the known norovirus surrogates—feline calicivirus (FCV) and murine norovirus (MNV).

2. Materials and methods

2.1. Reagents

Epigallocatechin gallate (EGCG), epicatechin gallate (ECG), quercetin, daidzein, fisetin, baicalein, kaempferol, biochanin A, theaflavin digallate, theaflavin, and ribavirin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ribavirin, EGCG, and ECG were dissolved in sterile distilled water. Quercetin, daidzein, fisetin, baicalein, kaempferol, biochanin A, theaflavin digallate, and theaflavin were dissolved in dimethylsulfoxide. All flavonoids were sequentially filtered through 5-, 1.2-, 0.8-, 0.45-, and 0.20-μm syringe filters.

2.2. Cells and viruses

MNV-1 was kindly provided by Professor Skip Virgin from the University of Washington. FCV strain F9, RAW 264.7 cells, and Crandell-Reese Feline Kidney (CRFK) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained following the guideline of ATCC. MNV-1 and FCV titrations were carried out as described previously with slight modifications (Lee et al., 2011; Lee et al., 2014; Su & D'Souza, 2013).

2.3. Cell viability assay

Cell viability was measured using the CCK-8 kit (Cell Counting Kit, Sigma) (Yang, Lopina, DiPersio, & Schmidt, 2008). Briefly, cell suspension (100 μL; 5000 cells/well) was added to the wells of a 96-well plate and incubated for 24 h at 37 °C in a 5% CO_2 incubator. The cells were treated with ribavirin and flavonoids at concentrations of 50, 100, 150, 200, and 300 μM and incubated for 6, 12, and 24 h. CCK-8 (10 μL) was added to each well and cells were incubated for 2 h at 37 °C in a 5% CO_2 incubator. The OD (optical density) was measured at 450 nm using a microplate reader. Cytotoxicity (%) was calculated as follows:

$$\text{Cell viability } (\%) = (OD_{test} - OD_{blank})/$$

where OD_{test} is the absorbance of flavonoids or ribavirin in CCK-8–treated cells; $OD_{control}$ is the absorbance of medium and CCK-8 alone (no cells); OD_{blank} is the absorbance of solvent blanks (sterile distilled water or dimethylsulfoxide).

2.4. Antiviral activity

The effect of pre-treatment, co-treatment, and post-treatment with 10 flavonoids and the known antiviral drug ribavirin were examined in this study. The concentrations of each flavonoid used to treat CRFK and RAW 264.7 cells were determined based on the cytotoxicity assay, and a range was used that caused less than 50% toxicity. To investigate the effect of pre-treatment, confluent CRFK and RAW 264.7 cells in 24-well plates were incubated for 6, 12, and 24 h at 37 °C in a 5% CO_2 incubator. CRFK and RAW 264.7 cells were treated with 50, 100, 200, and 300 μM or 50, 100, 150, and 200 μM concentrations of each flavonoid, respectively. After pre-treatment with flavonoid, samples of approximately 7 $\log_{10}$ plaque-forming units (PFU)/mL of FCV and MNV were serially diluted 10-fold with Dulbecco's modified Eagle's medium (DMEM) and inoculated onto confluent CRFK or RAW 264.7 cells, respectively, for 2 h at 37 °C in a 5% CO_2 incubator. DMEM containing 0.75% agarose (Sigma, Milwaukee, WI, USA), 5% fetal bovine serum (FBS), and 1% penicillin-streptomycin (Hyclone Laboratories, Logan, UT, USA) was then added to all wells. CRFK and RAW 264.7 cells were incubated for 24 h and 48 h, respectively, at 37 °C in a 5% CO_2 incubator. Plaques were counted after staining with neutral red solution.

To investigate the effect of co-treatment with flavonoids, serially 10-fold diluted FCV and MNV samples were mixed with flavonoids or ribavirin of final concentrations of 50, 100, 200, and 300 μM and 50, 100, 150, and 200 μM, respectively. The FCV and MNV mixtures were then added to confluent CRFK and RAW 264.7 cells, respectively, in 24-well plates and incubated for 2 h at 37 °C in a 5% CO_2 incubator. DMEM containing 0.75% agarose, 5% FBS, and 1% penicillin-streptomycin was then added to all wells. CRFK and RAW 264.7 cells were incubated for 24 h and 48 h, respectively, at 37 °C in a 5% CO_2 incubator. Plaques were counted after staining with neutral red solution.

To investigate the effect of post-treatment, CRFK and RAW 264.7 cells were inoculated with 7 $\log_{10}$ PFU/mL of FCV and MNV, respectively, and incubated for 18–24 h at 37 °C in a 5% CO_2 incubator. After removing cell culture media, each well was washed with sterile PBS (pH 7.2) and treated with flavonoids or 50 μM ribavirin. DMEM containing 0.75% agarose, 5% FBS, and 1% penicillin-streptomycin was then added to all wells and CRFK and RAW 264.7 cells were incubated for 6 h and 24 h, respectively, at 37 °C in a 5% CO_2 incubator. Plaques were counted after staining with neutral red solution.

2.5. Antioxidant capacity assay

A Trolox equivalent antioxidant capacity assay was carried out using ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation solution as previously described (Payet, Shum, Cheong Sing, & Smadja, 2005). ABTS radical cation working solution was prepared with 7 mM ABTS and 2.45 mM potassium persulfate. A Trolox standard curve was created using 1.5 mM Trolox stock solution diluted with PBS, pH 7.4. After sample or Trolox standard (20 μL) was added to each well of the 96-well microplate, ABTS radical cation working solution (200 μL) was added to each well of the 96-well microplate. They were incubated for 5 min at 25 °C and then the absorbance was measured at 734 nm on plate reader. The antioxidant capacity was calculated as follows:

$$\text{Antioxidant capacity } (\%) = 100 - 100 \times (A_S/A_0)$$

where A_S is absorbance of flavonoids or ribavirin or Trolox; A_0 is absorbance of solvent blanks (sterile distilled water, dimethylsulfoxide or PBS).