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# 5 Fisetin and rutin as 3C protease inhibitors of enterovirus A71

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27 **Running title: Fisetin and rutin inhibit EV-A71**

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5 **Abstract**

6 Enterovirus A71 (EV-A71) causes severe complications: encephalitis,
7 pulmonary edema, and death. No effective drug has been approved for clinical use.
8 This study investigated the antiviral effects of flavonoids against EV-A71. An *in vitro*
9 inhibitor screening assay using recombinant EV-A71 3C protease (3Cpro)
10 demonstrated fisetin and rutin inhibiting 3Cpro enzymatic activity in a
11 dose-dependent manner. Cell-based fluorescence resonance energy transfer (FRET)
12 assay with an EV-A71 3Cpro cleavage motif probe also confirmed that fisetin and
13 rutin inhibited the replication of EV-A71 in cells. Virus replication assay indicated
14 that fisetin and rutin reduced significantly the EV-A71-induced cytopathic effect and
15 viral plaque titers in RD cells culture. The $IC_{50}$ values of plaque reduction against
16 EV-A71 were 85 μM for fisetin and 110 μM for rutin. Therapeutic indices
17 (CC50/IC50 of plaque reduction assays) of fisetin and rutin exceeded 10. The study
18 suggests that fisetin and rutin inhibit the replication of EV-A71.

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21 **Keywords:** enterovirus A71, 3C protease, flavonoids, fisetin, rutin

## 1. Introduction

Enterovirus A71 (EV-A71), an important pathogen in the *Picornaviridae* family, causes hand-foot-mouth disease (HFMD), herpangina, aseptic meningitis, or severe neurological complications like encephalitis and poliomyelitis-like paralysis (Singh et al., 2002). EV-A71-induced brainstem encephalitis has a poor prognosis and a high fatality rate (Mizuta et al., 2009). Several EV-A71 outbreaks were reported in Western Pacific countries, in association with severe neurological disease (Mizuta et al., 2009; Lin et al., 2006; Yang et al., 2009; Yang et al., 2009). For example, EV-A71 outbreaks in Taiwan caused 78 deaths in 1998 (Lin et al., 2003; Huang et al., 1999; Ho et al., 1999; Chang et al., 1999), 25 deaths in 2000, and 26 deaths in 2001 (Lin et al., 2006). Prevention and/or treatment of EV-A71 infection are very important, but the FDA-approved drugs are not available. Developing effective agents against EV-A71 infection could contribute to public health.

The enterovirus genome consists of a 7.4 kb single-stranded positive-sense RNA genome (Pallansch and Roos, 2007) encoding a single large polyprotein that is cleaved by enteroviral 2A and 3C proteases. After protein synthesis, viral polyproteins cleave into capsid proteins (VP1-VP4), and nonstructural proteins (2A, 2C, 3A-3D). Inhibition of 2A protease (2Apro), 3C protease (3Cpro) or 3D RNA polymerase could block enterovirus replication significantly, indicating potential molecular targets for

1 development of antiviral agents (Chen et al., 2008; Pawlotsky et al., 2007). Moreover,
2 3Cpro is required for the additional cleavage events within viral protein precursors
3 that produce factors critical to protein processing and RNA replication. 3Cpro has also
4 been showed to cleave several important host proteins including poly(A)-binding
5 protein (PABP), cleavage stimulation factor 64 (CstF-64), TIR-domain-containing
6 adapter-inducing interferon-β (TRIF), and interferon regulatory factor 9 (IRF9)
7 (Rivera et al., 2008; Weng et al., 2009; Hung et al., 2011; Lei et al., 2011). EV-A71
8 3Cpro affects mammalian cell Pol I-III transcription and innate immune defense.
9 Comparison of published amino acid sequences from rhinoviruses and enteroviruses
10 shows considerable variability in the 3Cpro-coding region but strict conservation of
11 catalytic triad residues. The viral 3Cpro structure resembles that of well-defined
12 serine protease chymotrypsin (Kuo et al., 2008). Conservation of critical 3Cpro amino
13 acid residues offers to design potent and broad-spectrum anti-enterovirus agents.
14 Antiviral compounds that inhibit the 3Cpro activity have emerged by mimicking of
15 3Cpro substrates (peptide inhibitors) (Dragovich et al., 1998; Patick et al., 1999;
16 Matthews et al., 1999). An inhibitor of rhinoviruses 3Cpro, rupintrivir (formerly
17 AG7088) shows potency *in vitro* against many enteroviruses (Binford et al., 2005).
18 Screening 3Cpro inhibitors highlights function-based approaches to develop rapidly
19 antiviral agents against enteroviral infections.

Flavonoids occur in vegetables, *Citrus* herbs in particular (Benavente et al., 2008). Researches show their biological properties, including multiple anticancer, cardiovascular antimicrobial and anti-inflammatory activities (Manthey et al., 2001; Orhan et al., 2009). They also exhibit a broad-spectrum antiviral activity, efficiently inhibiting replication of human rhinovirus, Sabin type 2 poliovirus, hepatitis A virus, coxsackievirus B4 and echovirus 6 (Conti et al., 1992; Conti et al., 1990; Genovese et al., 1995; Salvati et al., 2004). However, their antiviral mechanisms are still unclear. Lack of scientific evidence showing the molecular pathways of their action diminishes their clinical utility.

This study investigated the inhibitory effects of flavonoids on the 3Cpro activity and EV-A71 replication in RD cells. *E. coli*-expressed EV-A71 3Cpro was purified for *in vitro* 3Cpro activity assays; fluorescence resonance energy transfer (FRET) probe with a 3Cpro cleavage motif was used for testing the substrate specificity of EV-A71 3Cpro in cells. Among flavonoids, fisetin and rutin showed significantly inhibitory effects on 3Cpro activity and EV-A71 replication.

## 2. Materials and methods

### *2.1. Viruses and cells*

EV-A71 isolate CMUH01 was obtained from the Clinical Virology Laboratory,

China Medical University Hospital in Taiwan. RD cells (ATCC accession no. CCL-136) were grown in Dulbecco's Modified Eagle's Medium (DMEM; HyClone Laboratories, Logan, Utah, USA) with 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel). HeLa-G3CwtR HeLa cells transfected with the plasmid expressing the recombinant fusion protein (green fluorescent protein-a 3Cpro cleavage motif-red fluorescent protein) as a FRET probe were cultured in DMEM with 10% FBS and 20 μg/ml zeocin. All media were supplemented with 100 U/mL of penicillin and streptomycin, and 2mM $_{L}$-glutamine.

*2.2. Chemicals*

Kaempferol, 5-methoxyflavone, myricetin and rutin were purchased from Sigma (St. Louis, MO, USA), chrysin and fisetin from Extrasynthese (Genay, France).

*2.3. Construction, expression and purification of EV-A71 3C protease*

A full-length gene encoding EV-A71 3Cpro was amplified by PCR with cDNA of EV-A71 isolate CMUH01. Forward primer 5'–GCGCGGATCCGGGCCCAGCTTAGACTT-3' and reverse primer 5'-GCGCCTCGAGTTGCTCGCTGGCAAAATA -3' were used, containing BamHI and XhoI restriction enzyme sites (underlined), respectively. The PCR products were

digested with BamHI and XhoI, and then cloned into pET24a (Merck KGaA, Darmstadt, Germany). Plasmid containing EV-A71 3Cpro gene was subsequently transformed into *E. coli* Origami B (DE3) (Merck KGaA, Darmstadt, Germany). For expression of recombinant 3Cpro fused with a C-terminal His tag as described in a recent report (Lu et al., 2011), a 20 ml overnight culture of a single colony was used to inoculate 2 L of fresh LB medium containing 25μg/ml kanamycin. Cells were grown to an A600 of 0.6 and then induced with 1mM IPTG. After a 5-h incubation at 37 ℃, cells were harvested by centrifugation at 7000g for 15 min. Cell pellets obtained from 2 L cell culture were suspended in 80 mL lysis buffer containing 25 mM Tris-HCl, pH 7.5, and 150 mM NaCl. French-press instrument (AIM-AMINCO spectronic Instruments, NY, USA) disrupted cells at 12,000 psi. After the centrifugation, the supernatant of the cell lysates was loaded onto a 20-mL Ni-NTA column equilibrated with 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 5 mM imidazole. The column was washed with 5 mM imidazole followed by a 30 mM imidazole-containing buffer. His-tagged 3Cpro was eluted with 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 300 mM imidazole. Purified His-tagged 3Cpro recombinant protein was dialyzed against a 2× 2 L buffer containing 12 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.1 mM EDTA, 7.5 mM β-mercaptoethanol, and 1 mM DTT. The concentration of purified 3Cpro protein was determined using the Bio-Rad Protein

Assay Kit (catalog number 500-0001).

*2.4. Protease activity assay*

Horseradish peroxidase (HRP) type VI-A (Sigma, Saint Louis, Missouri, USA) containing a 3Cpro cleavage motif (Gln-Gly pairs at the residues 295-296) was used as the EV-A71 3Cpro substrate for establishing an *in vitro* enzymatic assay. To examine the cleavage activity of recombinant 3Cpro protein, the 3Cpro at concentrations of 0, 0.005, 0.01, 0.05, and 0.1 μg/ml was incubated with HRP (0.2 μg/ml) for 2h at 37℃, and then the remaining HRP activity was determined using a chromogenic substrate ABTS [2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid)]/$H_2O_2$ and the optical intensity of the developed color was measured at 405 nm.

For screening EV-A71 3Cpro inhibitors, fisetin, chrysin, myricetin, kaempferol, rutin, puerin, and 5-methoxyflavone were screened for anti-EV-A71 3Cpro activity. Each flavonoid (200 μM concentration), EV-A71 3Cpro and HRP were incubated for 2h at 37℃in 96-well plates. The remaining activity of HRP in each reaction was determined with a chromogenic substrate ABTS/$H_2O_2$ and the intensity of the developed color was measured at 405 nm. Percentage inhibition of EV-A71 3Cpro activity was calculated as $(OD405_{HRP+drug+3C\ protease} – OD450_{HRP+3C\ protease}) / (OD450_{HRP\ only} – OD450_{HRP+3C\ protease}) \times 100\%$. To determine the $IC_{50}$ values, fisetin and rutin at

1 concentrations of 0, 10, 100, 250, 750 and 1000 μM were incubated with 3Cpro and
2 HRP for 2h at 37℃ in 96-well plates *in vitro*. The inhibitory percentage was
3 determined as described above. Concentration for inhibiting the 3Cpro enzymatic
4 activity by 50% ($IC_{50}$) was then determined, using the ID50 program developed by
5 John L. Spouge (National Center for Biotechnology Information) (Spouge, 1992).
6
7 *2.5. Cell viability assay*
8 For the cell viability assay, RD cells were cultured overnight on 96-well plates.
9 DMEM medium containing fisetin or rutin at concentrations of 0, 1, 10, 100 and 1000
10 μM was added and incubated for another 48 hours, followed by WST-1 assay. Cell
11 proliferation reagent WST-1 (Roche, Basel, Switzerland) was used for
12 spectrophotometric quantification of cell proliferation, viability, and chemosensitivity
13 in accordance with manufacturer's directions. After a 1-h incubation of a WST-1
14 reagent (10μL per well) at 37℃, the optical density was measured at 450 nm was
15 measured using a 96-well plate reader. Cell survival rates were calculated as the
16 optical density ratio at 450 nm of treated to untreated cells. Quadruplicate wells were
17 analyzed for each concentration; data represent mean ± SD of three independent
18 experiments. Cytotoxic concentration showing 50% toxic effect ($CC_{50}$) was
19 determined using the ID50 program.

1

2 *2.6. Fluorescence resonance energy transfer (FRET) assay*

3 Approximately 80-90% confluent monolayers of the HeLa-G3CwtR cells

4 expressing the FRET probe (described above) were infected with EV-A71 at a

5 multiplicity of infection (MOI) of 0.25, 0.5, or 1. After 90-min adsorption, inoculum

6 was aspirated and 500 µl of medium (Dulbecco's modified Eagle's medium containing

7 2% FBS) alone or medium with fisetin or rutin at 200 µM concentration was added to

8 each well. Cells were harvested 48 h post-infection, and lysates were transferred to

9 96-well plates for fluorescence. The fluorescent intensity of the FRET probes was

10 measured by a fluorescent-plate reader with an excitation wavelength at 390/20 nm

11 (for $GFP^2$ at 510/10 nm) and an emission wavelength at 590/14 nm (for DsRed2), in

12 which DsRed2 was excited by the emission of $GFP^2$ at 510/10 nm. Data presented are

13 mean values of experiments in triplicate.

14

15 *2.7. Cytopathic reduction (CPE) assay*

16 RD cells were cultured in DMEM medium containing 2% FBS and infected with

17 EV-A71 at MOI of 0.5 in the absence and presence of fisetin or rutin at 200 µM

18 concentration. After 48-h incubation at 37℃, EV-A71-induced cytopathic effect was

19 observed and photographed.

1

2 *2.8. Plaque reduction assay*

3 Confluent layers (> 90%) of RD cells in 6-well plates were inoculated with
4 EV-A71 (100 pfu per well) in the absence and presence of fisetin or rutin at
5 concentrations of 10, 100 and 1000 μM. After 1-h incubation at 37°C, confluent layers
6 of RD cells were covered with 3 mL of the overlay medium containing 1.5% agar and
7 incubated in a $CO_2$ incubator for 2 days at 37°C. Finally, cell layers were fixed with
8 formaldehyde and stained with 0.1% Crystal violet, as described previously (Chen et
9 al., 2009). Concentration for reducing the number of plaques by 50% ($IC_{50}$) was then
10 determined, using ID50 program.

11

12 *2.9. Virucidal Activity Assays*

13 EV-A71 ($10^4$ pfu) was mixed with test compounds (200μM) or medium, and
14 then incubated for 60 min at room temperature. Residual infectivity of 1000-fold
15 dilutions of each virus/compound mixture was determined using the plaque assay as
16 described above.

17

18 **3. Results**

19 *3.1. Preparation of recombinant 3C protease*

1 For preparing recombinant 3Cpro protein, the cDNA fragment of EV-A71 3Cpro
2 gene was cloned into the pET24a expression vector (Supplemental Figs. 1A-1B) and
3 in-frame fused with a C-terminal hexa-His-tag. Recombinant 3Cpro protein was
4 expressed in the transfected *E. coli* cells after IPTG induction and purified by
5 immobilized-metal affinity chromatography (IMAC) (Supplemental Fig. 2A).
6 Western blot analysis of purified recombinant 3Cpro protein with anti-His-tag
7 antibody revealed an immunoreactive band near 21-kDa as expected molecular weight
8 (Supplemental Fig. 2B).
9
10 *3.2. Inhibiting* in vitro *cleavage of 3C protease by fisetin and rutin*
11 To evaluate recombinant 3Cpro activity, HRP type VI-A containing a 3Cpro
12 cleavage motif (the Gln-Gly pair) as a 3Cpro substrate was used for *in vitro* enzymatic
13 assays. The enzymatic activity of purified 3Cpro protein at the desired concentration
14 was proportional to the amount of the cleaved HRP, being inversely proportional to
15 the remaining HRP activity in the reaction mixture. The optical density of the color
16 developed by the remaining HRP with its chromogenic substrate exhibited a reverse
17 dose-dependent manner as 3Cpro concentration rose (Table 1). This study established
18 a HRP-based enzymatic assay for initially screening potent EV-A71 3Cpro inhibitors
19 according to substrate specificity, and the anti-3Cpro assay method is capable of

1 accurately screening potent inhibitors.

2 Flavonoids show a wide range of anti-enterovirus activity, efficiently inhibiting 3 human rhinovirus, Sabin type 2 poliovirus, hepatitis A virus, coxsackievirus B4 and 4 echovirus 6 infections (Conti et al., 1992; Conti et al., 1990; Genovese et al.,1995; 5 Salvati et al., 2004). This study tested flavonoids including chrysin, fisetin, 6 kaempferol, myricetin, rutin, puerin, and 5-methoxyflavone for *in vitro* anti-EV-A71 7 3Cpro activity in 96-well plates (Table 2). Data show fisetin and rutin at 200 μM 8 concentration decreasing EV-A71 activity by over 30%. We selected fisetin and rutin 9 (0, 10, 100, 250, 750 and 1000 μM concentrations) for more detailed examination of 10 3Cpro inhibition study. Figure 1 proves both fisetin and rutin exhibited the 3Cpro 11 inhibition activity in a dose-dependent manner. Results highlighted dose-dependently 12 inhibitory effects of fisetin and rutin on *in vitro* cleavage activity of recombinant 13 3Cpro using HRP-based enzymatic assays.

14

15 *3.3. Inhibition of EV-A71 replication* in vitro *by fisetin and rutin*

16 Both fisetin and rutin (less toxic compounds) showed $CC_{50}$ values above 1000 17 μM (data not shown). HeLa-G3CwtR cells continuously expressing a FRET probe 18 (GFP2-3C cleavage motif-DsRed2 fusion protein) were used to test the substrate 19 specificity and the enzymatic activity of EV-A71 3Cpro in cells, as described in prior

1 study (Tsai et al., 2009). This cell-based FRET assay was also used to test inhibitory
2 ability of fisetin and rutin on the EV-A71 3Cpro activity and viral replication in cells
3 (Fig. 2). To test the proteolytic efficiency of the FRET probe by EV-A71,
4 HeLa-G3CwtR cells were infected with EV-A71 at MOIs of 0.25, 0.5, and 1. Cells
5 were harvested 48 hours post infection, and then added into a fluorescent-plate.
6 Relative fluorescent intensity of the FRET probe was performed using excitation
7 wavelength at 390/20 nm (for $GFP^2$) and emission wavelength at 590/14 nm (for
8 DsRed2) by a fluorescent-plate reader. Compared with mock control, infected cells
9 exhibited declining the emission intensity as well as increasing the cleaved FRET
10 probes in a virus titer-dependent manner (data not shown), indicating that the
11 emission intensity of the cell-based FRET assay inversely correlates with EV-A71
12 3Cpro amount and virus multiplication.

13 To evaluate the inhibition of fisetin and rutin on the 3Cpro activity and EV-A71
14 replication in cells, HeLa-G3CwtR cells were infected with EV-A71 at a MOI of 1 in
15 the absence and presence of fisetin and rutin. After 90 min of virus adsorption,
16 inoculum was aspirated and 500 µl of DMEM medium with or without fisetin or rutin
17 at various concentrations added to each well (Fig. 2). Cells were harvested 2 days post
18 infection and the emission intensity of the FRET probe in each lysate was measured
19 by fluorescent-plate reader with an excitation wavelength at 390/20 nm and an

emission wavelength at 590/14 nm (for DsRed2). Relative fluorescent emission intensity at 590/14 nm revealed that fisetin and rutin exhibited an inhibitory effect on *in vitro* replication of EV-A71 in a dose-dependent manner. The $IC_{50}$ values of both flavonoids against EV-A71 *in vitro* using the cell-based FRET assays were similar: 142.8 μM for fisetin and 83 μM for rutin (Table 3).

### *3.4. Inhibitory Effect of fisetin and rutin on EV-A71 replication*

To evaluate antiviral effect of fisetin and rutin on EV-A71 replication, this study analyzed their effects by *in vitro* virucidal activity, cytopathic effect (CPE) reduction and viral plaque reduction assays (Fig. 3). The results demonstrated that fisetin and rutin at 200 μM had no virucidal activity on EV-A71 ($10^4$ pfu) infectivity, but significantly reduced EV-A71-induced cytopathic effect (data not shown). In addition, fisetin and rutin definitely inhibited EV-A71 plaque formation in a dose-dependent manner (Fig. 3): $IC_{50}$ values of 84.48 μM and 109.63 μM, respectively (Table 3).

## 4. Discussion

This study characterized two *in vitro* enzymatic assays of EV-A71 3Cpro with the 3Cpro cleavage substrates HRP and recombinant FRET probe (Figs. 1 and 2), indicating the 3Cpro enzymatic assays as the rapid methods for anti-EV-A71 drug

1 discovery. Among flavonoid compounds, fisetin and rutin significantly inhibited in
2 enzymatic activity of recombinant 3Cpro proteins and viral 3Cpro in EV-A71-infected
3 cell while also blocking EV-A71 replication in cytopathic effect (CPE) reduction and
4 viral plaque reduction assays (Fig. 3, Table 3). Taken together, the results suggest
5 fisetin and rutin acting as novel inhibitors of EV-A71 3Cpro, exhibiting moderately
6 potent anti-EV-A71 activities.

7 Soluble recombinant EV-A71 3Cpro was expressed in *E. coli*, purified with
8 Ni-NTA column, and its proteolysis activity characterized by HRP containing the
9 3Cpro cleavage peptide sequence (Supplemental Fig 2 and Fig. 1). This *in vitro*
10 proteolysis assay for EV-A71 3Cpro activity proved useful in rapid screening of
11 3Cpro inhibitors; it may be non-specific and non-competitive. In cell-based FRET
12 assay, the FRET probe with 3C cleavage motif was cleaved by EV-A71 3Cpro, but not
13 phylogenetically distant herpes simplex virus (Tsai et al., 2009); the specificity of
14 EV-A71 3Cpro has been demonstrated with rupintrivir (an irreversible 3Cpro inhibitor)
15 (Binford et al., 2007), mutational analysis of FRET probe at 3Cpro cleavage motif and
16 Western blotting (Tsai et al., 2009). Hence, 3Cpro inhibitors identified by this assay
17 were confirmed by cell-based FRET assay of viral 3Cpro activity. Among flavonoids,
18 fisetin and rutin decreased EV-A71 activity by 30%, inhibiting 3Cpro activity in both
19 *in vitro* and cell-based assays. These belong to the flavonoid family of naturally

1 occurring polyphenolic compounds with anti-cancer, cardiovascular anti-microbial
2 and anti-inflammatory activities (Manthey et al., 2001; Benaventw et al., 2008; Orhan
3 et al., 2009). They show a broad spectrum of antiviral activity, efficiently inhibiting
4 human rhinovirus, Sabin Type 2 poliovirus, hepatitis A virus, coxsackievirus B4 and
5 echovirus 6 (Conti et al., 1992; Conti et al., 1990; Genovese et al., 1995; Salvati et al.,
6 2004). Fisetin exhibited antiviral activity against anti-herpes simplex virus Type 1
7 (HSV-1) and anti-moloney murine leukemia virus (Lyu et al., 2005; Chu et al., 1992).
8 Rutin showed anti-HCV, anti-HIV-1, anti-HSV-1, anti-EMC effects (Orhan et al.,
9 2009; Panasiak et al., 1989; Tao et al., 2007; Zuo et al., 2005), yet not in poliovirus
10 infection (Castrillo et al., 1986). These prior studies prove that fisetin and rutin with
11 similar chemical structures of flavonoids have diverse antiviral activities. Besides
12 inhibition of 3Cpro, fisetin and rutin may be involved in other mechanisms inhibiting
13 EV-A71 replication.

14 The $IC_{50}$ values determined by three different assays were diverse. Amounts of
15 fisetin and rutin required to inhibit purified EV-A71 3Cpro activity *in vitro* were more
16 than those required in the cell-based system and in blocking EV-A71 replication. This
17 may arise from use of horseradish peroxides (HRP) containing Gln-Gly pairs as
18 substrates for assay and ABTS/H2O2 as coupling assay to measure protease activity.
19 Since flavonoids are known as anti-oxidants due to redox structural feature, inference

1 of flavonoids with peroxidase reaction yields higher $IC_{50}$ values of *in vitro* 3Cpro
2 activity by peroxidase-based assay. Antiviral compounds targeting 3Cpro activity
3 have been developed based on mimicking of 3Cpro substrates (peptide inhibitor)
4 (Dragovich et al., 1998; Patick et al., 1999). Other studies searched for inhibitors
5 bound to 3Cpro structure (Patick et al., 1999; Matthews et al., 1999). Further study of
6 the affinity of fisetin and rutin for 3Cpro is warranted to ascertain whether these
7 flavonoids affect other viral or cellular functions crucial for EV-A71 replication.
8 In sum, this study used EV-A71 3Cpro as a significant target for antiviral drug
9 discovery to demonstrate that fisetin and rutin inhibited 3Cpro activity *in vitro* and
10 cell-based assays and blocked EV-A71 replication, suggesting these flavonoids as
11 moderately potent anti-EV-A71 agents. Our approach could be crucial to fast,
12 cost-effective development of anti-EV-A71 agents.
13
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18
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1 ## Table and Figure Legends

2 **Table 1. Characterization of EV-A71 3Cpro activity with the substrate**

3 **horseradish peroxidase.**

4 **Table 2. Screening of EV-A71 3Cpro inhibitors from flavonoid derivatives.**

5 **Table 3. Antiviral activities of fisetin and rutin against of EV-A71 by two**

6 **different methods.**

7 **Fig. 1. Dose-dependent effects of fisetin and rutin on inhibition of *in vitro* EV-A71**

8 **3Cpro activity.** A horseradish peroxidase (HRP) containing Gln-Gly pairs

9 corresponding to the cleavage site by 3Cpro was designed as substrate for 3Cpro

10 enzyme. Rutin and fisetin at concentrations of 0, 10, 100, 250, 750 and 1000 μM

11 were incubated with 3C protease (0.1 μM) and HRP (0.2 μg/ml) for 2h at 37℃in

12 96-well plates in vitro. Mixtures were then developed with ABTS/$H_2O_2$ and

13 measured at $OD_{405}$. Percentage inhibition of EV-A71 3Cpro activity was

14 calculated as ($OD405_{HRP+drug+3C\ protease}$ – $OD450_{HRP+3C\ protease}$) / ($OD450_{HRP}$

15 $_{only}$ –$OD450_{HRP+3C\ protease}$) x 100%.

16 **Fig. 2. Inhibitory effects of fisetin and rutin on intracellular EV-A71 3Cpro**

17 **activity with a FRET probe.** HeLa-G3CwtR cells expressed a FRET probe with

18 an in-frame fusion product of GFP[2], the 3Cpro cleavage linker, and DsRed2.

19 HeLa-G3CwtR cells were infected with EV-A71 at MOI= 1 for 90-min of

20 adsorption; the inoculum was aspirated and 500 μl of Dulbecco's modified Eagle's

1 medium containing 2% FBS with rutin or fisetin at various concentrations—0 μM,
2 1 μM, 10 μM, 100 μM, 200 μM and 400 μM—was added to each well. After 48-h
3 incubation, cells were harvested and subjected to measurement by a
4 fluorescent-plate reader. Relative intensity of fluorescent emission at 590/14 nm
5 (for DsRed2) was detected using an excitation wavelength at 390/20 nm (for
6 GFP[2]).

7 **Fig. 3. EV-A71 plaque reduction by rutin and fisetin.** RD cells were infected with
8 EV-A71 at a titer of 100 pfu per well of cells with of medium alone or medium
9 containing fisetin or rutin at concentrations of 10, 100 and 1000 μM. After 1 h
10 incubation, each cell monolayer was covered with 3 mL of agar overlay medium.
11 At the end of 2-day incubation, the cells were fixed with formaldehyde and stained
12 with 0.1% Crystal Violet. The number of plaques in each well was counted.