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Apigenin inhibits enterovirus 71 replication through suppressing viral IRES activity and modulating cellular JNK pathway

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ABSTRACT

Enterovirus 71 (EV71) is a member of genus *Enterovirus* in *Picornaviridae* family, which is one of the major causative agents for hand, foot and mouth disease (HFMD), and sometimes associated with severe central nervous system diseases in children. Currently there are no effective therapeutic medicines or vaccines for the disease. In this report, we found that apigenin and luteolin, two flavones that differ only in the number of hydroxyl groups could inhibit EV71-mediated cytopathogenic effect (CPE) and EV71 replication with low cytotoxicity. Both molecules also showed inhibitory effect on the viral polyprotein expression. They prevented EV71-induced cell apoptosis, intracellular reactive oxygen species (ROS) generation and cytokines up-regulation. Time-of-drug addition study demonstrated that apigenin and luteolin acted after viral entry. We examined the effect of apigenin and luteolin on $2A^{pro}$ and $3C^{pro}$ activity, two viral proteases responsible for viral polyprotein processing, and found that they showed less inhibitory activity on $2A^{pro}$ or $3C^{pro}$. Further studies demonstrated that apigenin, but not luteolin could interfere with viral IRES activity. Also, apigenin inhibited EV71-induced c-Jun N-terminal kinase (JNK) activation which is critical for viral replication, in contrast to luteolin that did not. This study demonstrated that apigenin may inhibit EV71 replication through suppressing viral IRES activity and modulating cellular JNK pathway. It also provided evidence that one hydroxyl group difference in the B ring between apigenin and luteolin resulted in the distinct antiviral mechanisms. This study will provide the basis for better drug development and further identification of potential drug targets.

1. Introduction

Enteroviruses are members of the *Picornaviridae* family, which has more than 70 serotypes (Bruu, 2003). Enterovirus 71 (EV71), consisting of a single-stranded, positive-sense RNA of approximately 7500 nt (Bruu, 2003), is an etiological agent for a number of severe central nervous system (CNS) sicknesses such as aseptic meningitis, encephalitis, monoplegia and acute flaccid paralysis (McMinn, 2002). It is also associated with hand, foot and mouth disease (HFMD), herpangina and pulmonary edema (Solomon et al., 2010; Van Tu et al., 2007; Zhang et al., 2010). EV71 is usually a high risk agent for infants or young children under 6 years of age and the infection can result in deleterious clinical consequences and even death (Yi et al., 2011). This virus was originally isolated and identified from the feces of an infant suffering from encephalitis in 1969 in California, USA (Schmidt et al., 1974). After that, outbreaks of EV-71 infection have periodically occurred worldwide, particularly in the Asia-Pacific region. Unfortunately, no vaccines or specific antiviral agents are currently available for the prevention or the treatment of EV71 infection.

EV71 genome contains only one open reading frame (ORF) and encodes a single polyprotein as NH_2-VP4-VP2-VP3-VP1-2A-2B-2C-3A-3B-3C-3D-COOH driven by the internal ribosome entry site (IRES) element contained in its 5′-untranslated region (UTR). This polyprotein is then cleaved proteolytically by its proteases, $2A^{pro}$ and $3C^{pro}$ to form the mature structural (VP1, VP2, VP3 and VP4) or non-structural (2A, 2B, 2C, 3A, 3B 3C and 3D) proteins (Bruu, 2003). Various stages of the EV71 life cycle are considered as potential targets for antiviral agents, such as viral proteases, 3D polymerase, and IRES, etc. (Chen et al., 2008). And efforts were also made using traditional Chinese herbs and herbal products as an alternative medicine and therapy. These herbs are usually used for the treatment of inflammatory or infective diseases.

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Flavonoids are a large group of low molecular weight polyphenolic compounds and exhibit multiple functions, such as anti-cancer, antimicrobial and anti-inflammatory properties (Manthey et al., 2001; Orhan et al., 2010). Apigenin is a flavone compound found in parsley, artichoke, basil, celery and other plants while luteolin can be found in *Terminalia chebula* and also in celery, parsley, and thyme perilla. These two flavones differ structurally only by one hydroxyl group in the B-ring (molecular structure shown in Fig. 1A). Apigenin and luteolin were previously reported as anti-inflammatory, anti-cancer, anti-oxidant and anti-allergic agents (Kawai et al., 2007; Kim et al., 2004; Ren et al., 2003; Seelinger et al., 2008a,b; Ueda et al., 2002). There are also many reports on their potentials as antiviral agents against hepatitis C virus (HCV), herpes simplex virus type I (HSV-1), influenza virus and human immunodeficiency virus type I (HIV-1) (Amoros et al., 1992; Lee et al., 2003; Liu et al., 2008, 2012). In this study, we demonstrated that both apigenin and luteolin inhibited EV71 replication and showed that these two compounds blocked viral polyprotein expression and genomic RNA synthesis. The further study implied that the possible mechanism of action of apigenin was related to its inhibitory effects on viral IRES translational activity and cellular JNK activation, but not luteolin. The current study suggests that the study of the relationship of antiviral activity and structures of apigenin and luteolin can provide information for drug development and also can be as the leading compounds for antiviral drugs.

2. Materials and methods

2.1. Reagents, cell lines, plasmids and viruses

Apigenin, luteolin, apiin, cynaroside, quercetin, kaempferol and chrysin were obtained from National Institutes for Food and Drug

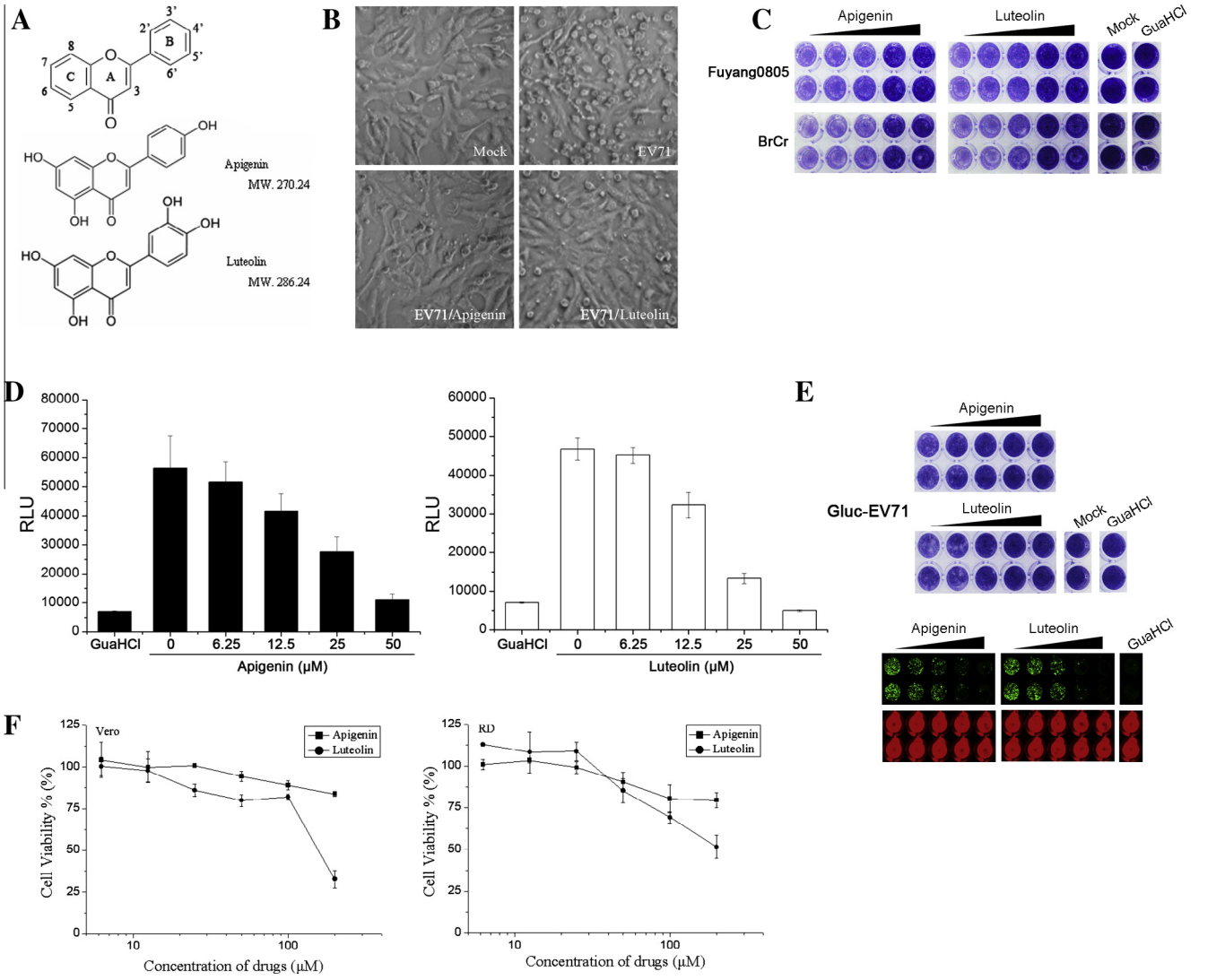

Fig. 1. Apigenin and luteolin inhibited EV71 replication. (A) The ring skeleton of flavonoid and the molecular structures of apigenin and luteolin. (B) Apigenin and luteolin inhibited EV71-induced CPE. Vero cells were mock-infected or infected with EV71 Fuyang0805 strain (moi = 0.2) in the presence or absence of apigenin and luteolin (25 μM). Images were obtained 24 h p.i. under an inverted microscope. (C) Vero cells cultured in 96-well plate were infected with EV71 Fuyang0805 or BrCr strain (moi = 0.2) in the presence of apigenin or luteolin. Cells were stained with crystal violet 72 h p.i. And GuaHCl was set as a positive control. (D) Vero cells were infected with GLuc-EV71 (moi = 0.2) in the presence of apigenin or luteolin. After 4 h, the cultural medium was discarded and 200 μl flesh medium was dispensed into each well. Culture supernatants were collected 24 h p.i., and *gaussia* luciferase activity was determined as described. (E) Thirty microliters of the supernatants from (D) were dispensed onto Vero cell confluent monolayers cultured in a 96-well plate. In-cell western was carried out to evaluate the inhibition of VP2 expression 24 h p.i. And the crystal violet assay was performed as described 72 h p.i. (F) The cytotoxicity of apigenin and luteolin in Vero and RD cells. Vero cells were treated with serial concentrations of apigenin and luteolin. The cell viability was determined by CCK-8 colorimetric assay after 24 h. All experiments were performed three times. The representative results were shown. Data are means of triplicate determinations with standard error.

Control in China (Beijing, China). SB203580, SP600125, 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and Phorbol-12-myristate-13-acetate (PMA) were purchased from Beyotime Ltd. (Haimen, Jiangsu, China). IRDye 680 goat-anti-rabbit and IRDye 800 goat-anti-mouse were obtained from Li-COR (Lincoln, NE, USA). Anti-EV71 antibody was purchased from Millipore (Billerica, MA, USA). Antibodies specific for JNK2, GAPDH and β-catenin were purchased from Santa Cruz (Santa Cruz, CA, USA). Antibodies specific for caspase-3 and p-JNK1/2 were from Cell Signaling Technology (Beverly, MA, USA). Antibody specific for human eIF4G was obtained from Millipore (Billerica, MA, USA). Recombinant human TNF-α was obtained from PeproTech (Rocky Hill, NJ, USA).

pG5luc vector was obtained from Promega (Madison, WI, USA). pCMV-Luc was constructed by Dr. Ying Chu in our lab. pEGFP-N3-2A was constructed by cloning $2A^{pro}$ coding sequence of EV71 Fuyang0805 strain into pEGFP-N3 vector (Clontech, Palo Alto, CA, USA). pEGFP-N3-2Amut contained a single point mutation (C110S). pRHF-EV71 and pRHF-EV71-AS (Lin et al., 2009) was kindly provided from Dr. Shin-Ru Shih, Chang Gung University, Taiwan. pBAK8-MTEGFP-M3-3C-VP16 and pBAK8-MTEGFP-M3-3Cmut-VP16 (Lee et al., 2008) were gifted by John T.A. Hsu, National Health Research Institutes, Taiwan.

Rhabdomyosarcoma (RD) cell was purchased from Cell Bank of Chinese Academy of Sciences (CAS) (Shanghai, China). Vero and HEK293T cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). EV71 Fuyang0805 strain and BrCr strain were kindly gifted from Dr. Bin Wu, Jiangsu Provincial Centers of Disease Control, and Erguang Li, Nanjing University, respectively and propagated on RD cells. In detail, confluent RD cells maintained in DMEM containing 2% FBS were inoculated with the viruses at a multiplicity of infectivity (moi) of 0.2. The virus stocks were collected from the supernatants of infected RD cells 2 days postinfection (p.i.) and titrated in Vero cells with plaque assay. GLuc-EV71 that carried a *gaussia* luciferase reporter gene in EV71 genome was from Dr. Bo Zhang, Wuhan Institute of Virology, CAS (Wuhan, China) and propagated on Vero cells. To measure the *gaussia* luciferase activity, 50 μl cultural supernatants were collected and the luciferase activity was determined using BioLux *gaussia* luciferase assay kit (New England Biolabs, Beverly, MA, USA).

2.2. In vitro antiviral assay

The *in vitro* antiviral activity was determined via crystal violet staining assay. Vero cells cultured in 96-well plate were infected with EV71 (moi = 0.2) in the presence of serial concentrations of apigenin or luteolin. GuaHCl (2 mM) was set as a positive control. Cell monolayers were fixed with 4% paraformaldehyde and then staining with crystal violet 72 h p.i. After 3 times wash, the plate was air-dried and the images were captured using a camera.

2.3. In vitro cytotoxicity assay

The *in vitro* cytotoxicity was determined using a commercial CCK-8 kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, 2×10^4 Vero or RD cells per well were dispersed into 96-well plates and cultured for 24 h at 37 °C in an atmosphere of 5% CO_2 in air before compounds were added in triplicate. After 24 h, 10 μl CCK-8 assay solution was added into each well, and the plate was incubated for additional 2 h at the same condition. The absorbance at 450 nm was measured, and cell viability was plotted as the percent viable cells of the mock-treated control cells.

2.4. Annexin-V-FITC/PI staining and microscope

Vero cells cultured in 24-well plate were washed twice with cold phosphate-buffered saline (PBS, pH7.4). Subsequently, Binding Buffer (eBioscience, San Diego, CA, USA) was dispensed into each well and subsequently added 5 μl of annexin-V-FITC and PI work solution (eBioscience). The plate was incubate at room temperature (RT) in the dark for 10 min, and then washed with Binding Buffer. Images were acquired using Olympus XDS-1B fluorescence microscope immediately (Tokyo, Japan).

2.5. Cytokine array

Cytokines concentrations in culture medium were measured using the Proteome Profiler human cytokine array Panel A array kit (R&D systems, Minneapolis, MN, USA) according to manufacturer's protocol. IRDye 800CW streptavidin (Li-COR) were used to detect protein expression level and the data were captured by scanning the membrane under Li-COR Odyssey Infrared Imager (Li-COR).

2.6. Western blot and In-cell Western

Cells were lysed using RIPA lysis buffer (Santa Cruz) on ice for 30 min and then centrifuged at 12,000×*g* for 10 min at 4 °C. Total protein concentrations in the supernatants were determined using BCA protein assay kit (Pierce, Rockford, IL, USA). After separated using SDS–PAGE, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked using Odyssey Blocking buffer (Li-COR) and then incubated with primary antibodies for 2 h at room temperature (RT). After 5 washes with PBS-0.1% Tween-20 (PBS-T buffer), the membranes were incubated in IRDye IgG (1:10,000) for 1 h at RT and visualized under Li-COR Odyssey Infrared Imager (Li-COR).

In-cell Western was performed in 96-well plate. The cells cultured in a 96-well plate were fixed with 4% paraformaldehyde for 20 min at RT and permeabilized by 5 washes in PBS-0.1% Triton-X 100 with 5 min for each wash. Cell monolayers were blocked for 90 min in blocking buffer (4% non-fat dry milk) and then incubated with primary antibodies diluted in blocking buffer (1:200) for 2 h at RT. After washing with PBS-T buffer, the cell layers were stained with IRDye IgG (1:1500) for 1 h, rinsed and scanned in Odyssey Infrared Imager. Relative protein expression level was normalized against β-catenin.

2.7. RNA extraction and realtime PCR

Total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer's protocol. Complementary DNA was reverse-transcribed using ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan). Realtime PCR was performed in triplicate on ABI Prism 7300 Sequence Detection System using the SYBR Green PCR Master Mix (Life Technologies). Messenger RNA (mRNA) transcription levels were standardized against housekeeping gene GAPDH. The sequences of primer pairs were listed: EV71 VP1 (Forward: GGAGATAGGGTGGCAGATG; Reverse: CCA ATTTCAGCGGCTTGGAG) and GAPDH (Forward: TGCACCA CCAACTGCTTAGC; Reverse: GGCATGGACTGTGGTCATGAG).

2.8. Time-of-drug addition assay

The time-of-drug addition assay was determined via measuring viral protein or genomic RNA copies level. Vero cells cultured in 96-well plate or 24-well plate were infected with EV71 (moi = 1). Apigenin or luteolin were added at 50 μM as a final concentration

at indicated time point. Viral protein level was determined in 96-well plate via In-cell Western 12 h p.i. as described.

2.9. Intracellular ROS detection

Vero cells were mock-infected or infected with EV71 (moi = 0.2) in the presence or absence of the compounds for 24 h. Cells were washed with PBS, and then exposed to DCFH-DA diluted in DMEM medium (10 μM). After 20 min incubation, cells were rinsed 3 times and trypsinized. Intracellular ROS level was measured by flow cytometry (FACSCalibur, BD Biosciences, San Jose, USA) and analyzed by FlowJo software (Treestar Software, San Carlos, CA, USA).

2.10. Ex vivo $2A^{pro}$ and $3C^{pro}$ activity assay

EV71 $2A^{pro}$ and $3C^{pro}$ activity assay was described by Hung et al. (2010), but modified. For determining *in vivo* $2A^{pro}$ activity, pEGFP-N3-2A or pEGFP-N3-2Amut (negative control) and a firefly luciferase expression vector, pCMV-Luc were co-transfected into RD or Vero cells cultured in a 96-well plate using Lipofectamine 2000 transfection reagent (Life Technologies). *In vivo* $3C^{pro}$ activity assay was based on a mammalian cell-based reverse two-hybrid system. Vero cells cultured in 96-well plate were co-transfected with pG5luc (the vector contains 5×GAL4 binding sites upstream of a minimal TATA box) and pBAK8-MTEGFP-M3-3C-VP16 or pBAK8-MTEGFP-M3-3Cmut-VP16 (negative control). Serial concentrations of compounds were added 24 h post-transfection. Cells were lysed after 24 h and the relative luminescence unit (RLU) was determined using Bright-Glo luciferase assay system (Promega).

2.11. Bi-cistronic expression assay

pRHF-EV71, which containing EV71-5′-UTR region between firefly and *Renilla* luciferase genes, was used to determine EV71 IRES activity (Lin et al., 2009). The plasmid was transfected into Vero, RD cells or HEK293T cells. After 24 h, serial concentrations of the compounds were added into each well. Cell lysates were collected and analyzed to determined firefly luciferase (FLuc) and *Renilla* luciferase (RLuc) activity using dual-luciferase reporter assay system (Promega), following the manufacturer's instructions 24 h post-treatment.

2.12. Statistics

Statistical analysis was performed using two-tailed student *t*-test. Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$.

3. Results

3.1. Apigenin and luteolin inhibited EV71 replication

We screened a natural product library containing 502 compounds for their anti-EV71 activity in Vero cells, and the results showed that apigenin and its analog luteolin, differed only by one hydroxyl group in the B-ring (molecular structures shown in Fig. 1A), were the two most effective agents to prevent EV71-induced cytopathogenic effect (CPE). To investigate the anti-EV71 effect of apigenin and luteolin, Vero cells were pretreated with 25 μM compound followed by infection with EV71 Fuyang0805 strain (moi = 0.2). The result showed that both apigenin and luteolin inhibited EV71-induced CPE as shown in Fig. 1B. The similar result was obtained on RD cells (data not shown). We also carried out crystal violet staining assay on Vero cells to quantify their inhibitory effects on the virus-induced CPE. In this experiment, we also employed another strain, BrCr, which was an American laboratory-adapted strain. Consistently, both apigenin and luteolin suppressed both Fuyang0805- and BrCr-induced CPE in a dose-dependent manner (Fig. 1C). GLuc-EV71, an EV71 clone containing a *gaussia* luciferase reporter gene, was employed to confirm their antiviral effect, and the results also showed that apigenin and luteolin could inhibit viral IRES-driven *gaussia* luciferase expression in a dose-dependent manner (Fig. 1D). For better understanding whether apigenin and luteolin could inhibit the infectious virions formation, the supernatants from Fig. 1D were collected and dispensed onto Vero cell monolayer. As shown in Fig. 1E, these two flavones exhibited inhibitory effect on EV71 infectious particles formation. We also evaluated the inhibition of infectious virions formation via In-cell Western, and the results were consistent with that in crystal violet assay (Fig. 1E). To rule out the direct cytotoxicity of the two compounds to EV71 indicator cells, we showed that apigenin and luteolin had low cytotoxicities to both Vero and RD cells (Fig. 1F). The 50% cytotoxicity concentrations (CC_{50}) of apigenin to Vero and RD cells were greater than 200 μM, which were significantly higher than the highest concentration used in viral inhibitory assay. Although luteolin was more cytotoxic than apigenin, the CC_{50} values of luteolin to Vero and RD cells were 157 μM and >200 μM, respectively. Together, we concluded that the anti-EV71 activity of either apigenin or luteolin was not due to its direct cytotoxicity.

3.2. Apigenin and luteolin inhibited EV71 viral protein expression and genomic RNA synthesis

Due to their inhibitory effect on EV71-induced CPE, the effect of apigenin and luteolin on viral polyproteins expression or genomic RNA synthesis was also investigated. Vero cells were pretreated with apigenin or luteolin and then infected with EV71 for 24 h. EV71 polyprotein expression level was determined by In-cell Western, and the results showed that apigenin and luteolin inhibited EV71 Fuyang0805 strain and BrCr strain VP2 expression in a dose-dependent manner (Fig. 2A and B). Similar results were also observed in RD cells, suggesting that the antiviral activity of apigenin and luteolin was not a cell-specific phenomenon (data not shown). Genomic RNA synthesis was determined by realtime-PCR amplifying a specific fragment within VP1 region. As shown in Fig. 2C, apigenin and luteolin inhibited EV71 viral RNA synthesis in a dose-dependent manner in Vero cells. We also evaluated RNA synthesis in RD cells, and the similar results were obtained (data not shown). Thus it was demonstrated that these two flavones inhibited EV71 replication through suppressing viral protein expression and genomic RNA synthesis.

3.3. The antiviral effect of the glycoside derivatives of apigenin and luteolin

In view of the close structural similarity between apigenin and luteolin, we intended to further investigate the structural properties needed for the antiviral activity by analyzing their glycoside derivatives. Apiin, a glycoside derivative of apigenin, and cynaroside, a glycoside derivative of luteolin (molecular structures shown in Fig. 3A), were investigated for their anti-EV71 activities. As shown in Fig. 3B, cynaroside inhibited EV71 replication in a dose-dependent manner, but apiin did not. We concluded that 7-apioglucoside substituent in C-ring of apigenin might also influence its anti-EV71 activity.

3.4. Apigenin and luteolin inhibited EV71-induced cell apoptosis and ROS generation

Several reports showed that EV71 could induce apoptosis in many types of cell lines *in vitro*, such as neuroblastoma cells, colo-

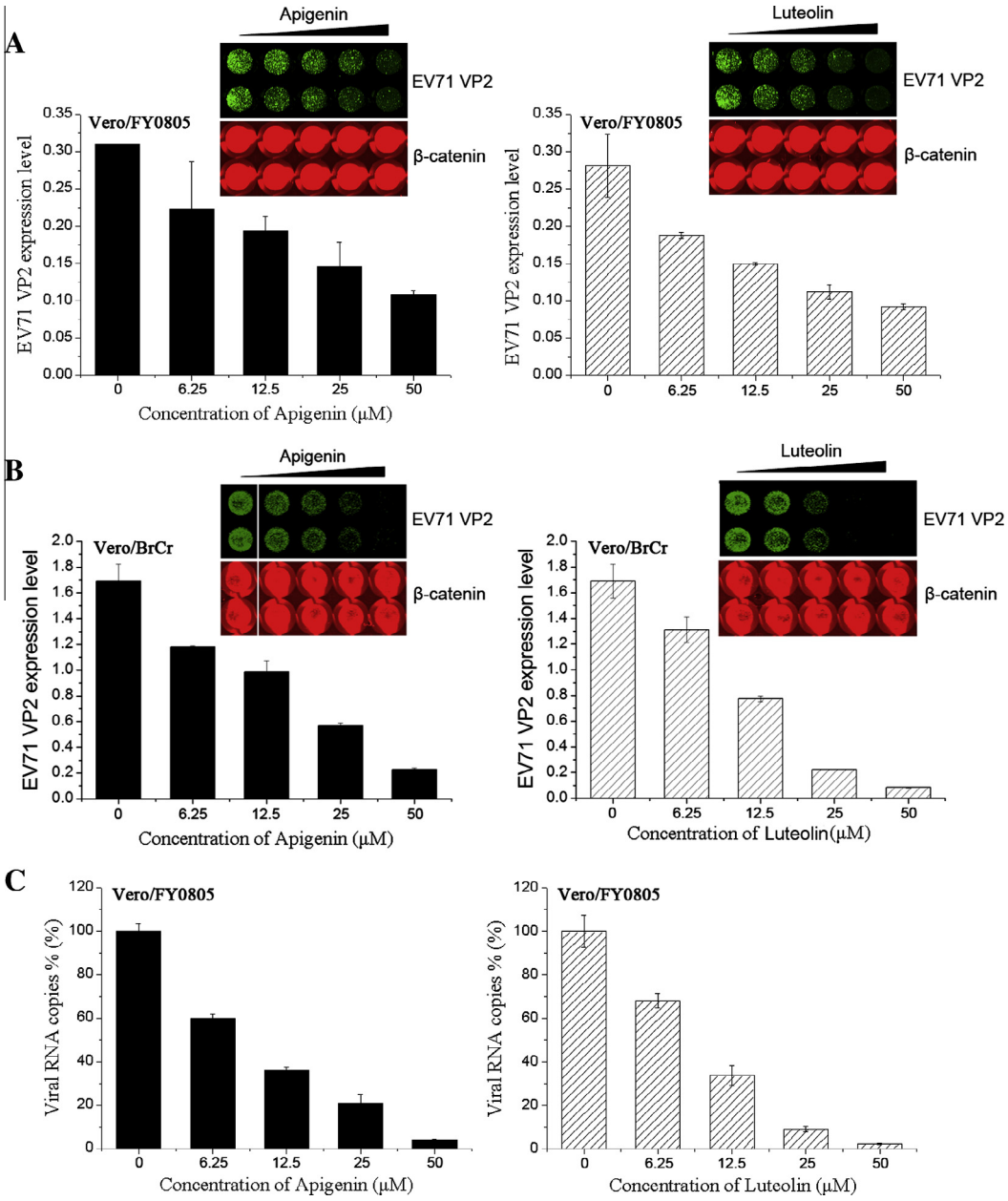

Fig. 2. Apigenin and luteolin inhibited EV71 viral protein expression and genomic RNA synthesis. (A)–(B) Apigenin and luteolin inhibited EV71 VP2 expression. Vero cells were infected with EV71 Fuyang0805 (A) or BrCr (B) (moi = 0.2) prior to treated with apigenin or luteolin. EV71 VP2 expression level was determined via In-cell Western and normalized by β-catenin 24 h p.i. (C) Viral genomic RNA copy numbers in Vero cells were quantified via realtime PCR. All experiments were performed three times. And the representative results were shown. Data are means of triplicate determinations with standard error.

rectal adenocarcinoma and rhabdomyosarcoma (Chi et al., 2013; Wang et al., 2004; Xi et al., 2013). We investigated whether apigenin and luteolin were able to attenuate EV71-induced cell apoptosis by analyzing the cells infected with EV71 in the presence or absence of either apigenin or luteolin. The apoptotic cells were stained with annexin-V-FITC (green) and the dead cells or cells at the late-stage apoptosis were labeled by PI (red). EV71 infection increased the number of both apoptotic and dead cells (Fig. 4A), which was inhibited by either apigenin or luteolin, indicating that these compounds inhibited virus-induced cell apoptosis or death. Caspase-3 is known as a critical executioner of cell apoptosis (Fernandes-Alnemri et al., 1994). Therefore, we examined caspase-3 cleavage after EV71 infection and showed that EV71 infection led to caspase-3 cleavage, which was in parallel to VP2 expression (Fig. 4B), and both apigenin and luteolin inhibited the EV71-induced caspase-3 cleavage level in a dose-dependent manner (Fig. 4C).

Oxidative stress is known to be a determinant of a host's susceptibility to pathogens and EV71 infection resulted in oxidative stress, which might cause damage for tissues. In order to investigate the impact of apigenin and luteolin on EV71-mediated ROS generation, we measured the intracellular ROS level using DCFH-DA probe. As shown in Fig. 4D, EV71 infection increased the proportion of ROS positive cells (from 7.83% to 28.80%) and the treatment of the cells with either apigenin or luteolin suppressed the ROS generation, bringing down the ROS producing cells to the basal level, demonstrating that both flavones exhibited potent activity in downregulating ROS generation. Not only could they down-regu-

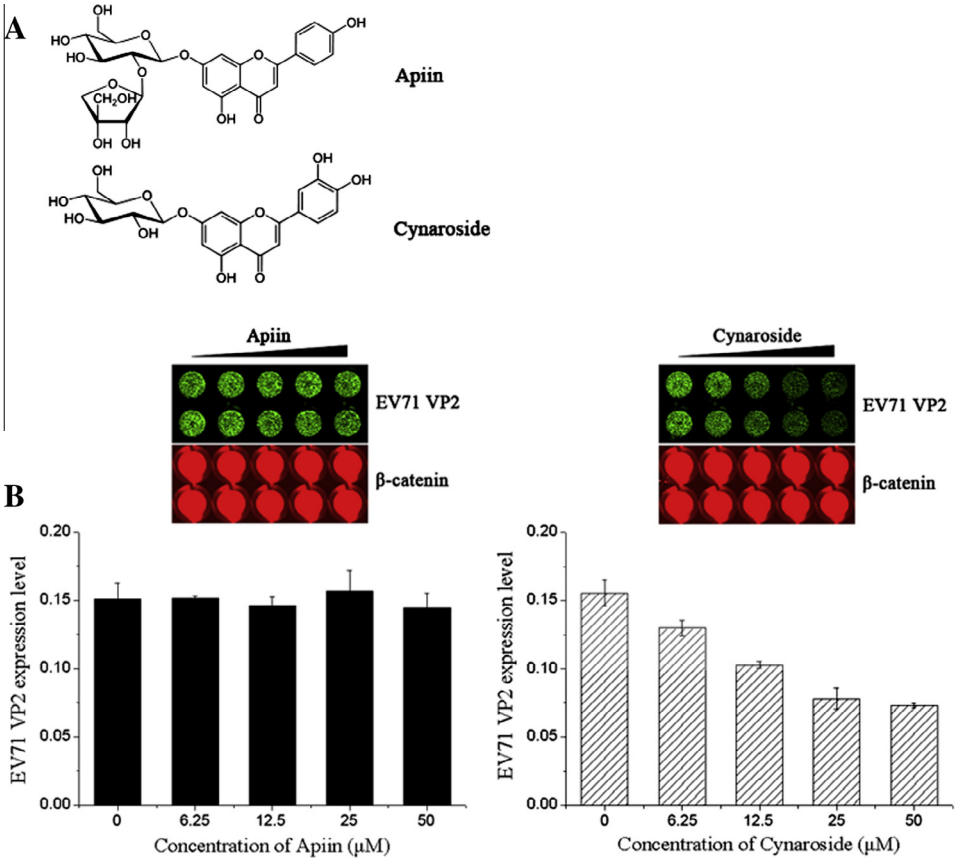

Fig. 3. The antiviral effect of the glycoside derivatives of apigenin and luteolin. (A) Molecular structures of apiin and cynaroside. (B) Cynaroside, but not apiin, inhibited EV71 replication. Vero cells were infected with EV71 (moi = 0.2) prior to treated with apiin or cynaroside. EV71 VP2 expression level was determined as described 24 h p.i.

late EV71-induced ROS generation, but also attenuated hydrogen peroxide-induced ROS production (Fig. 4E), demonstrating that these two flavones exhibited its anti-oxidant activity.

3.5. Apigenin and luteolin reduced EV71-induced cytokine expression

A significant increase of cytokines, known as a cytokine storm, was associated with EV71 patients with encephalitis and pulmonary edema, the leading causes of death (Wang et al., 2003, 2008). In the current study, we investigated whether these two flavones might impact on the virus-induced cytokine expression using a cytokine microarray. As shown in Fig. 5, among 36 cytokines, EV71 infection significantly up-regulated the expressions of G-CSF, GM-CSF, IL-1ra, IL-2, IL-6 and CCL5 in RD cells and the treatment of the cells with either apigenin or luteolin suppressed the expression of most of these cytokines except GM-CSF and IL-2 to basal levels, implying that both apigenin and luteolin have anti-inflammatory effect on EV71-induced cytokine expression, consistent with an early report that apigenin and luteolin have anti-inflammatory activity (Kim et al., 2004).

3.6. Apigenin and luteolin acted at an early stage during infection, likely after viral entry

To determine the antiviral mechanisms of apigenin and luteolin, we performed a time-of-drug addition assay to determine whether these two compounds acted at viral entry or post-entry steps. Vero cells infected with EV71 were exposed to either compounds at indicated time points and EV71 polyprotein level was determined 12 h p.i. via In-cell Western. As shown in Fig. 6, these two flavones protected Vero cells from EV71 infection during −0.5 to 4 h p.i., but EV71 began to escape from their inhibitory effects after 4 h. A time-lapse detection of EV71 VP2 demonstrated that the polyprotein expression and processing mainly occurred after 8 h. Therefore, we concluded that both apigenin and luteolin likely acted at an early stage of infection but after viral entry.

3.7. The effect of apigenin and luteolin on EV71 $2A^{pro}$ and $3C^{pro}$

$2A^{pro}$ and $3C^{pro}$ auto-cleavage activities are critical for EV71 infection and also potential targets for antiviral drug development. Whether apigenin or luteolin could interfere with viral $2A^{pro}$ and $3C^{pro}$ activity was then investigated. RD cells were co-transfected with pCMV-Luc, harboring firefly luciferase, and EV71 2A expression vector, or EV712 2A mutant (C110S) (2Am) as a negative control. $2A^{pro}$ has been shown to cleave the host translation proteins, eIF4G (Kuo et al., 2002). If $2A^{pro}$ retained its protease activity after drug treatment, eIF4G would be cleaved and the cellular cap-dependent translation would be shut off. Thus, the expression of firefly luciferase would be inhibited and luminance intensity should decrease, and vice versa. We observed that the addition of either apigenin or luteolin showed less effect on the luminance intensity, implying that these two compounds have no inhibitory effect on EV71 $2A^{pro}$ activity in RD cells (Fig. 7A). We also considered that whether these two flavones could inhibit EV71-mediated eIF4G cleavage in RD cells via Western blot. As shown in Fig. 7B, EV71 infection caused cellular eIF4G cleavage 6 h p.i., and apigenin and luteolin did not shown any significant effect, which confirmed that these two compounds have less inhibitory effect on $2A^{pro}$ activity.

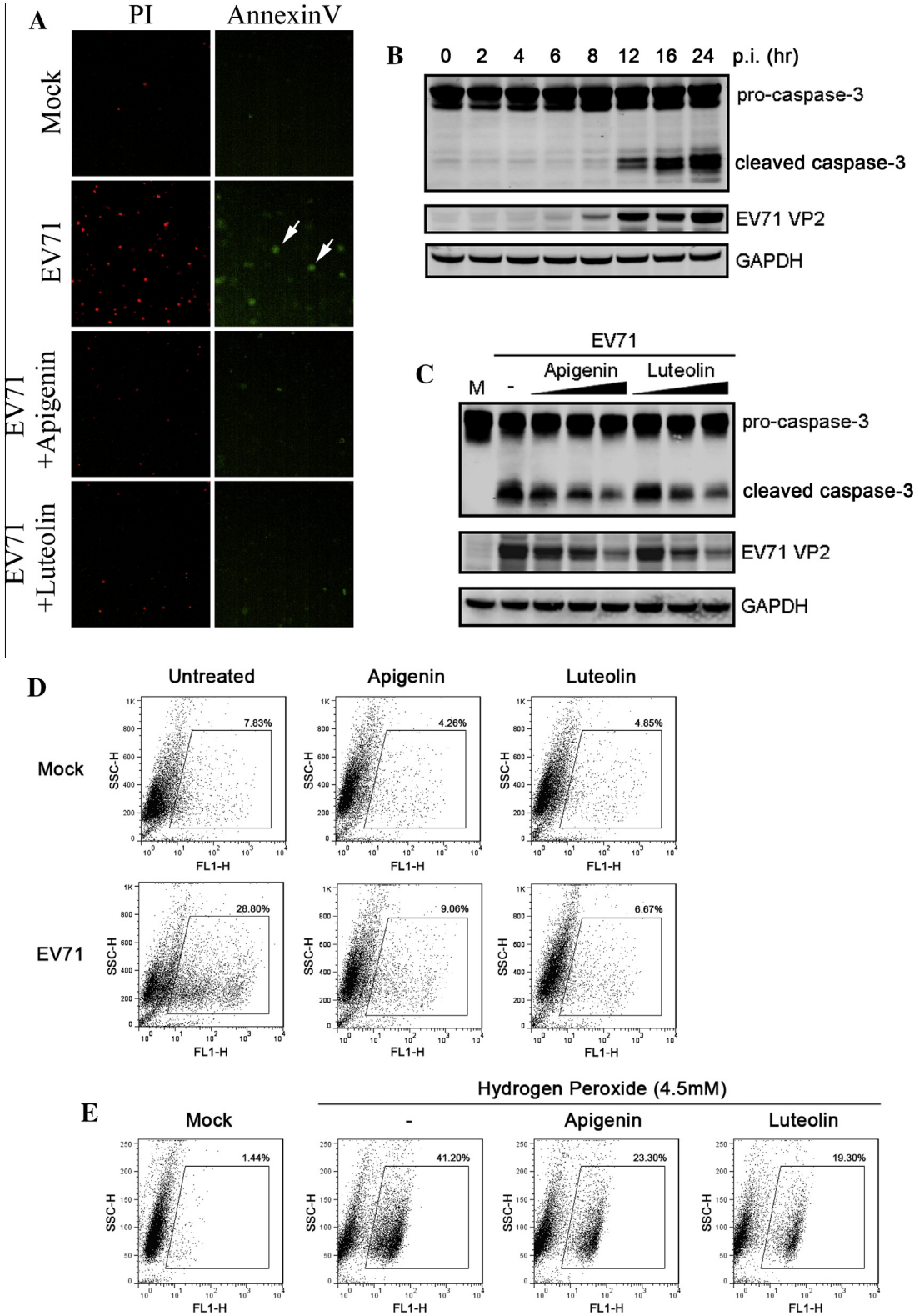

Fig. 4. Apigenin and luteolin inhibited EV71-induced cell apoptosis and ROS generation. (A) Apigenin and luteolin inhibited EV71-induced cell apoptosis and death. Vero cells were mock-infected or infected with EV71 (moi = 0.2) in the presence or absence of apigenin or luteolin (25 μM). Cells were stained with annexin-V-FITC/PI 24 h p.i., and images were obtained under a fluorescence microscope. (B) EV71 infection caused caspase-3 activation. Vero cells were infected with EV71 (moi = 0.2) for indicated hours. (C) Apigenin and luteolin suppressed EV71-induced caspase-3 cleavage. Vero cells were mock-infected or infected with EV71 (moi = 0.2) in the presence or absence of serial concentrations of apigenin or luteolin (6.25, 12.5, 25 μM). Caspase-3 and VP2 were visualized via Western blot 24 h p.i. (D) Apigenin and luteolin downregulated EV71-mediated ROS generation. Vero cells were treated as described. Cells were collected 24 h p.i. and ROS generation was determined via a flow cytometry. (E) Apigenin and luteolin attenuated H_2O_2-induced ROS generation. Vero cells were mock-treated or treated with apigenin or luteolin (25 μM) for 30 min, and then exposed to H_2O_2 (4.5 mM) for another 30 min. ROS generation was measured as described. All experiments were performed three times and the representative results were shown. Data are means of triplicate determinations with standard error.

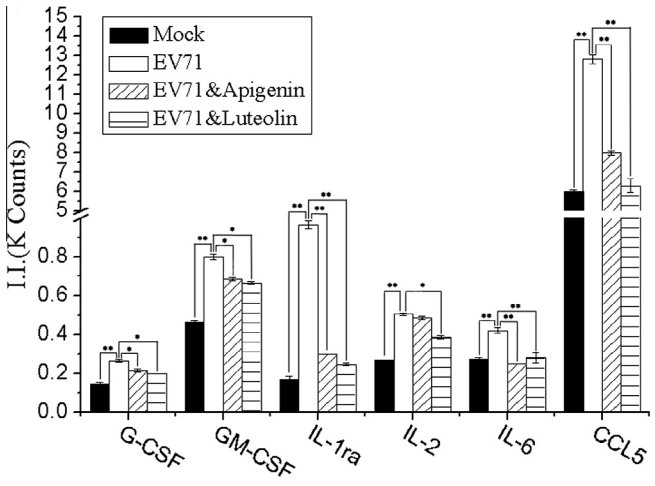

Fig. 5. Apigenin and luteolin decreased EV71-induced cytokines expression. RD cells were mock-infected or infected with EV71 (moi = 0.2) in the presence or absence of apigenin or luteolin (25 μM). After 24 h, culture supernatants were collected. Cytokines expression levels were determined as described.

To examine whether either apigenin or luteolin inhibited the EV71 $3C^{pro}$ activity, a mammalian cell-based reverse two-hybrid system for the analysis of $3C^{pro}$ auto-cleavage activity was employed as described (Lee et al., 2008). The rationale of the analytical system was also illustrated in Fig. 7B. As a result, both apigenin and luteolin showed weak inhibitory effect on $3C^{pro}$ activity (Fig. 7C). Together, neither apigenin nor luteolin could inhibit viral $2A^{pro}$ and $3C^{pro}$ activity at the antiviral concentrations, suggesting that other antiviral mechanisms contributed to their activity.

3.8. Apigenin inhibited EV71 IRES-driven translational activity

One characteristic property of EV71 infection is the shut-down of cap-dependent translation and turn-on of IRES-dependent translation. EV71 polyprotein translation depends on its IRES in 5′-UTR of its genome. Therefore, we investigated whether these two compounds could suppress viral IRES-mediated translation using a bicistronic expression system containing an EV71 IRES sequence as described (Lin et al., 2009). The expression of RLuc was under the control of CMV promoter, and the IRES region was responsible for FLuc translation and expression (Fig. 8A). The efficiencies of the IRES-driven translation initiation are reflected by the ratio of FLuc and RLuc (FLuc/RLuc). As shown in Fig. 8B, apigenin inhibited IRES-mediated translation in both Vero and RD cells in a dose-dependent manner. However, luteolin showed no significant inhibitory activity until the highest concentration (50 μM) was used. The similar results were observed in HEK293T cells (data not shown), suggesting that the differential activity of these two compounds was not cell-type specific.

We also examined the effects of the glycoside derivatives of apigenin and luteolin, cynaroside and apiin, on IRES activity. Cynaroside did not suppress EV71-IRES activity such as luteolin, and apiin showed marginal effect on IRES activity (Fig. 8C), implying that the distinct IRES inhibitory effect between apigenin and apiin might be associated with their differential antiviral activities.

To explore the relationship between hydroxyl groups on B-ring of flavones and IRES inhibitory activity, the structural homologs of apigenin and luteolin were employed. As shown in Fig. 8D, kaempferol, chrysin and apigenin could be able to inhibit EV71-IRES activity, but other compounds showed no activity. We concluded that 3′- and 4′-substituent groups affected the IRES inhibitory activity of these flavones.

3.9. EV71 infection induced cellular JNK activation, which was inhibited by apigenin

We determined that EV71 stimulated cellular p38 MAP kinase and JNK phosphorylation. As shown in Fig. 9A, the phosphorylation level of p38 MAP kinase and JNK increased in Vero cells as the infection proceeded. Therefore, we investigated whether these two kinases played critical roles in viral replication. We employed the p38 MAP kinase and JNK specific inhibitors, SB203580 and SP600125, respectively to determine their antiviral activities. As shown in Fig. 9B, both inhibitors exhibited anti-EV71 properties. The same experiment was also carried out in RD cells, and the results were consistent with that in Vero cells (data not shown). The observation suggested that p38 MAP kinase and JNK pathways might be prerequisite for EV71 infection.

The effects of apigenin and luteolin on EV71-induced p38 MAP kinase and JNK activation were further investigated. As shown in Fig. 9C, The EV71-induced JNK phosphorylation was potently inhibited by apigenin but only weakly by luteolin. However, these two flavones showed minimal inhibitory effect on p38 MAP kinase activation. Their inhibition on JNK phosphorylation induced by other stimulators was also determined. Both TNF-α and PMA induced JNK phosphorylation in Vero cells, and apigenin inhibited TNF-α- and PMA-induced JNK phosphorylation while luteolin elevated the TNF-α-induced JNK phosphorylation but showed no effect on PMA-induced JNK phosphorylation, implying that these

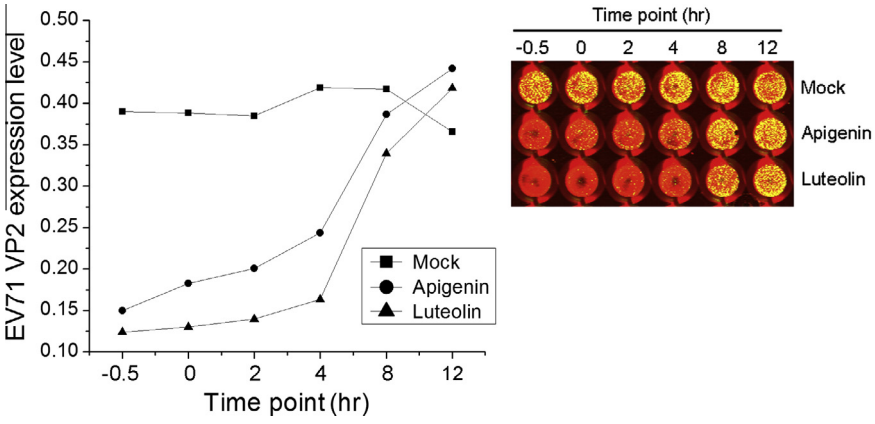

Fig. 6. Apigenin and luteolin acted at early stage infection, but after viral entry. Vero cells were infected with EV71 (moi = 1) and exposed to apigenin or luteolin (50 μM) at indicated time points. After 12 h, EV71 VP2 expression was determined via In-cell Western. The experiment was performed three times and the representative results were shown. Data are means of triplicate determinations with standard error.

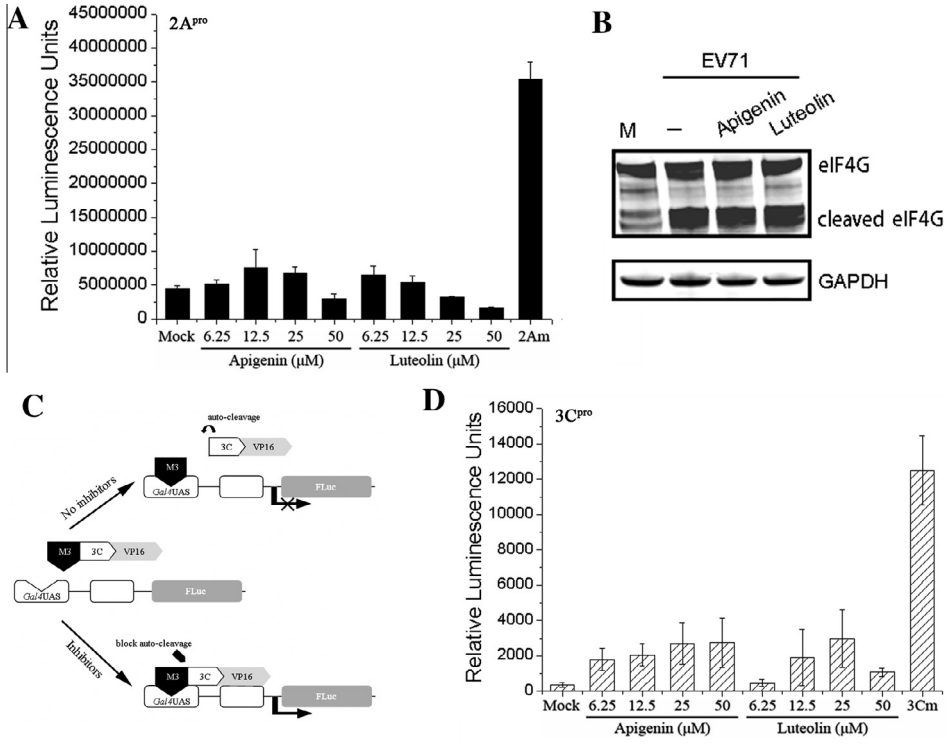

Fig. 7. Apigenin and luteolin showed less inhibitory effect on EV71 $2A^{pro}$ and $3C^{pro}$ activity. (A) Neither Apigenin nor luteolin inhibited EV71 $2A^{pro}$ activity in RD cells. (B) Apigenin and luteolin did not inhibit EV71 $2A^{pro}$-induced eIF4G cleavage. RD cells were mork-treated or treated with apigenin or luteolin (25 μM) for 30 min, and then infected with EV71 (moi = 0.2) for 6 h. eIF4G was detected via Western blot. (C) The rationale of the mammalian cell-based reverse two-hybrid system for functional analysis of $3C^{pro}$. (D) apigenin and luteolin shown weak inhibitory effect on $3C^{pro}$ activity. All experiments were performed three times and the representative results were shown. Data are means of triplicate determinations with standard error.

two flavones act with distinct mechanisms in modulating JNK pathway. Thus we concluded that the effect of apigenin on JNK pathway might contribute to its anti-EV71 activity.

4. Discussion

EV71, the etiologic agent for hand, foot and mouth disease, has caused several epidemics in the Asia-Pacific region and is an important challenge to the public health. Currently, there are no effective vaccines or antiviral medicines against this virus though efforts are being undertaken to develop such a medicine. In the current report, we screened a natural product library and identified two flavones, apigenin and luteolin that could effectively suppress EV71 polyprotein expression and genomic RNA synthesis, and inhibit viral replication *in vitro* with low cytotoxic effect. We found the discrepancies in inhibitory activities as measured by VP2 and viral RNA copies (Fig. 2), and it may be attributable to the aspecific staining of VP2 antibody. These two flavones have similar structures and have been reported to have anti-inflammatory, anti-cancer, anti-oxidant and anti-allergic activities (Kawai et al., 2007; Kim et al., 2004; Ren et al., 2003; Seelinger et al., 2008a,b; Ueda et al., 2002). They were also reported as antiviral agents against several viruses (Amoros et al., 1992; Lee et al., 2003; Liu et al., 2008, 2012).

EV71 infection induces cytopathogenic effects on the host cells, leading to eventual cell death. EV71 has been reported to induce apoptosis in variety of cell lines *in vitro* (Chi et al., 2013; Wang et al., 2004; Xi et al., 2013). Shih et al. demonstrated that EV71 viral protein synthesis was required for its induction of cell apoptosis (Shih et al., 2008). Apoptosis induced by certain viruses is also an effective strategy to release viral particles. Coxsackievirus B3, another member of *Enterovirus* genus, caused caspase-3 cleavage, which played a vital role in viral progeny release (Carthy et al., 1998, 2003). Therefore, it was concluded that enterovirus replication is a necessary and sufficient condition for virus-induced apoptosis. We demonstrated that EV71 infection of Vero cells induced caspase-3 cleavage, and apigenin and luteolin could attenuate this effect (Fig. 4). We postulated that these two flavones inhibit caspase-3 activation and subsequent cell apoptosis through suppressing viral protein expression, which resulted in the reduction of progeny virus release. Oxidative stresses can cause disruptions in normal mechanisms of cellular signaling or reactive oxygen species can be used beneficially by the immune system to attack and kill pathogens and thus influence a host's susceptibility to infections. Apigenin and luteolin were reported as the potent intrinsic scavengers of ROS (Lien et al., 1999; Romanova et al., 2001; Saija et al., 1995). In the current study, we demonstrated that apigenin and luteolin decreased the proportion of the cells with high intracellular ROS level caused by viral infection or H_2O_2 treatment, implying that apigenin and luteolin were able to protect cells from ROS-induced damage. It was also demonstrated that both apigenin and luteolin could attenuate EV71-induced cytokines expression. The elevated expressions of these cytokines are the hallmark of the clinical manifestations in EV71 infections, particularly in patients with encephalitis and pulmonary edema. Our observations suggested that apigenin and luteolin could be potential anti-inflammatory agents in antiviral treatments.

The time-of-drug addition analysis of viral protein or RNA expression demonstrated that apigenin and luteolin might exert their antiviral activity at an early stage of viral infection, likely after viral entry (Fig. 6). Viral $2A^{pro}$ and $3C^{pro}$ are the key proteases for processing the EV71 precursor polyprotein, which plays important roles in viral maturation and infection (Kuo and Shih, 2013). $2A^{pro}$

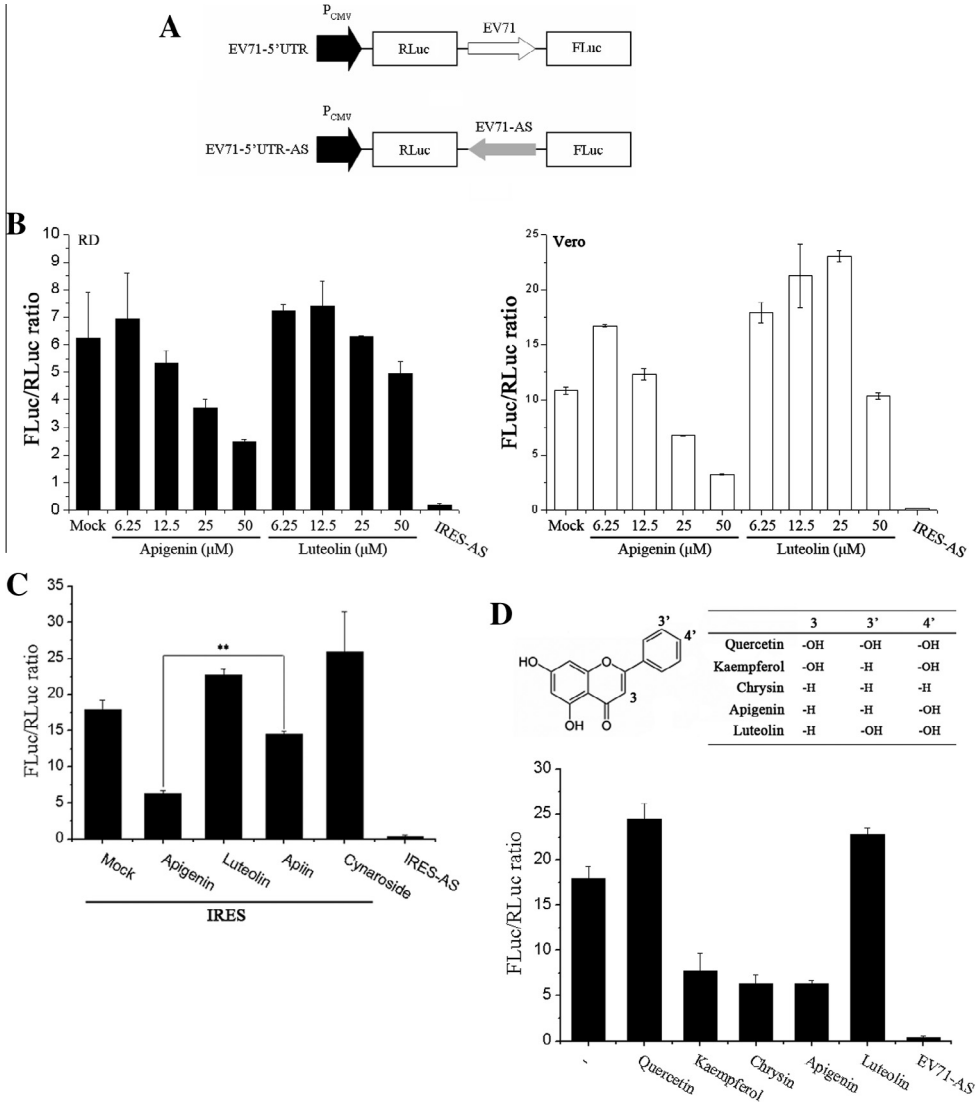

Fig. 8. Apigenin inhibited EV71 IRES-driven translational activity. (A) Diagram of the EV71 bicistronic detection system. (B) Apigenin, but not luteolin inhibited EV71 IRES-driven translation activity. Vero cells were treated as described in Section 2. (C) Apiin lost IRES-inhibitory effect compared with apigenin. (D) The effect of the homologs of apigenin and luteolin on IRES activity. Vero cells were treated as described, and the compounds used in this experiment were at the concentration of 50 μM. IRES-driven translation activity was represented as FLuc/RLuc. These experiments were performed three times and the representative results were shown. Data are means of triplicate determinations with standard error.

cleaves eIF4G, a component of cap binding complex, resulting in a significant decrease of cellular cap-dependent translation (Kuo et al., 2002). The shutoff of cap-dependent and activation of IRES-dependent translation is a characteristic mechanism utilized by members of *Picornaviridae* family. For further mechanistic studies, we employed two cell-based systems to investigate whether the flavones have inhibitory activities on EV71 $2A^{pro}$ or $3C^{pro}$ activity and showed that apigenin and luteolin inhibited less effect on $2A^{pro}$ or $3C^{pro}$ cleavage activity in RD cells (Fig. 7). However we found that $2A^{pro}$-mediated inhibition of luciferase expression was attenuated by apigenin and luteolin in Vero. It was concluded that this phenomenon may be cell-specific. These results suggested that other mechanisms may account for the antiviral activities of both agents.

EV71 5′UTR contains an IRES element that directs initiation of translation in a cap-independent manner (Thompson and Sarnow, 2003). EV71 IRES belongs to type I IRESs such as poliovirus IRES (Barton et al., 2001). We showed that apigenin inhibited EV71-IRES activity, while in contrast luteolin did not. Tsai et al. previously reported that kaempferol and chrysin, two analogs of apigenin, showed anti-IRES activity (Tsai et al., 2011). Although the exact targets of apigenin for anti-IRES activity were unknown, based on the analysis using derivatives of apigenin or luteolin, we speculated that the number and position of hydroxyl groups in the B-ring in flavones may be critical for their anti-IRES activity. The precise structure–activity relationship will be investigated further.

We previously found that EV71 infection resulted in p38 MAP kinase and JNK phosphorylation in Vero cells, which might be associated with viral protein accumulation (Fig. 9A). This phenomenon was also observed in RD cells (data not shown). Hirasawa et al. (2003) reported that encephalomyocarditis virus, another member of the *Picornaviridae* family activated JNK and p38 MAP kinase pathway, and p38 inhibitors could attenuate viral replication. Si et al. (2005) showed that Coxsackievirus B3 infection increased the phosphorylation of JNK and p38, and inhibition of p38 resulted in significant reduction of viral progeny release. In this study, we demonstrated that both p38 MAP kinase and JNK activation might be critical for EV71 replication (Fig. 9B). However, both of the two

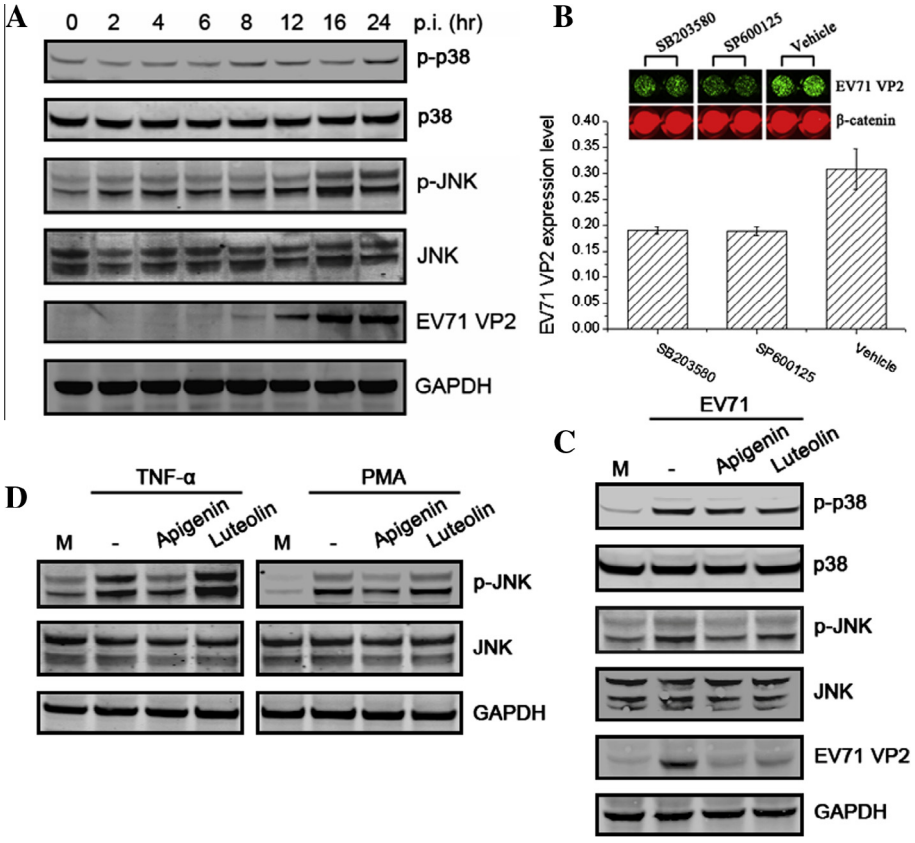

Fig. 9. EV71 infection induced cellular JNK activation, which was inhibited by apigenin. (A) EV71 induced p38 MAP kinase and JNK activation. Vero cells were infected with EV71 (moi = 0.2), and cells were collected at indicated time points. p38 MAP kinase, JNK and their phosphorylated forms and EV71 VP2 were determined via Western blot. (B) The p38 MAP kinase and JNK specific inhibitors, SB203580 and SP600125, respectively, inhibited EV71 replication and polyprotein expression. Vero cells were infected with EV71 (moi = 0.2) prior to treated with SB203580 (20 μM) or SP600125 (20 μM). EV71 VP2 expression level was determined via In-cell Western and normalized by β-catenin 24 h p.i. (C) The effect of apigenin or luteolin on EV71-induced p38 MAP kinase and JNK activation. Vero cells were mock-treated or treated with apigenin or luteolin (25 μM), and then exposed to EV71 (moi = 0.2). Cells infected with EV71 were collected 24 h p.i., and p38 MAP kinase, JNK and their phosphorylated forms and EV71 VP2 were determined via Western blot. (D) Apigenin, but not luteolin could attenuate TNF-α- and PMA-induced JNK phosphorylation. Vero cells were mock-treated or treated with apigenin or luteolin (25 μM), and then exposed to TNF-α (10 ng/ml) or PMA (100 ng/ml). JNK and p-JNK were determined 20 min post-treatment.

flavones exhibited less inhibitory effect on virus-induced p38 MAP kinase activation, and apigenin but not luteolin could inhibit JNK activation induced by various stimulators. We concluded that the inhibitory effect of apigenin on JNK pathway was relatively specific and the exact target might be the upstream of JNK pathway. The relationship between molecular structure and JNK inhibition activation should be further discussed.

A reasonable assumption is that these flavones target a number of kinases or viral proteins, and only a small difference will result in distinct biological properties. Our observations also suggest that the mechanisms of action by apigenin and luteolin may be quite distinct. Further research will be needed to illustrate the relationship between the molecular structures and bioactivity, and it may be benefit for antiviral drug design and development.

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References

Amoros, M., Simõs, C., Girre, L., Sauvager, F., Cormier, M., 1992. Synergistic effect of flavones and flavonols against herpes simplex virus type 1 in cell culture. Comparison with the antiviral activity of propolis. J. Nat. Prod. 55, 1732–1740.

Barton, D.J., O'Donnell, B.J., Flanegan, J.B., 2001. 5′ cloverleaf in poliovirus RNA is a cis-acting replication element required for negative-strand synthesis. EMBO J. 20, 1439–1448.

Bruu, A.L., 2003. Enteroviruses: Polioviruses, Coxsackieviruses, Echoviruses and Newer Enteroviruses. A Practical Guide to Clinical Virology, second ed. pp. 44–45.

Carthy, C.M., Granville, D.J., Watson, K.A., Anderson, D.R., Wilson, J.E., Yang, D., Hunt, D.W., McManus, B.M., 1998. Caspase activation and specific cleavage of substrates after coxsackievirus B3-induced cytopathic effect in HeLa cells. J. Virol. 72, 7669–7675.

Carthy, C.M., Yanagawa, B., Luo, H., Granville, D.J., Yang, D., Cheung, P., Cheung, C., Esfandiarei, M., Rudin, C.M., Thompson, C.B., 2003. Bcl-2 and Bcl-xL overexpression inhibits cytochrome *c* release, activation of multiple caspases, and virus release following coxsackievirus B3 infection. Virology 313, 147–157.

Chen, T.-C., Weng, K.-F., Chang, S.-C., Lin, J.-Y., Huang, P.-N., Shih, S.-R., 2008. Development of antiviral agents for enteroviruses. J. Antimicrob. Chemother. 62, 1169–1173.

Chi, C., Sun, Q., Wang, S., Zhang, Z., Li, X., Cardona, C.J., Jin, Y., Xing, Z., 2013. Robust antiviral responses to enterovirus 71 infection in human intestinal epithelial cells. Virus Res. 176, 53–60.

Fernandes-Alnemri, T., Litwack, G., Alnemri, E.S., 1994. CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. J. Biol. Chem. 269, 30761–30764.

Hirasawa, K., Kim, A., Han, H.-S., Han, J., Jun, H.-S., Yoon, J.-W., 2003. Effect of p38 mitogen-activated protein kinase on the replication of encephalomyocarditis virus. J. Virol. 77, 5649–5656.
Hung, H.-C., Chen, T.-C., Fang, M.-Y., Yen, K.-J., Shih, S.-R., Hsu, J.T.-A., Tseng, C.-P., 2010. Inhibition of enterovirus 71 replication and the viral 3D polymerase by aurintricarboxylic acid. J. Antimicrob. Chemother. 65, 676–683.
Kawai, M., Hirano, T., Higa, S., Arimitsu, J., Maruta, M., Kuwahara, Y., Ohkawara, T., Hagihara, K., Yamadori, T., Shima, Y., 2007. Flavonoids and related compounds as anti-allergic substances. Allergol. Int. 56, 113–123.
Kim, H.P., Son, K.H., Chang, H.W., Kang, S.S., 2004. Anti-inflammatory plant flavonoids and cellular action mechanisms. J. Pharmacol. Sci. 96, 229–245.
Kuo, R.-L., Kung, S.-H., Hsu, Y.-Y., Liu, W.-T., 2002. Infection with enterovirus 71 or expression of its 2A protease induces apoptotic cell death. J. Gen. Virol. 83, 1367–1376.
Kuo, R.-L., Shih, S.-R., 2013. Strategies to develop antivirals against enterovirus 71. Virol. J. 10, 28.
Lee, J.-C., Shih, S.-R., Chang, T.-Y., Tseng, H.-Y., Shih, Y.-F., Yen, K.-J., Chen, W.-C., Shie, J.-J., Fang, J.-M., Liang, P.-H., 2008. A mammalian cell-based reverse two-hybrid system for functional analysis of 3C viral protease of human enterovirus 71. Anal. Biochem. 375, 115–123.
Lee, J.S., Kim, H.J., Lee, Y.S., 2003. A new anti-HIV flavonoid glucuronide from *Chrysanthemum morifolium*. Planta Med. 69, 859–861.
Lien, E.J., Ren, S., Bui, H.-H., Wang, R., 1999. Quantitative structure–activity relationship analysis of phenolic antioxidants. Free Radic. Biol. Med. 26, 285–294.
Lin, J.-Y., Li, M.-L., Shih, S.-R., 2009. Far upstream element binding protein 2 interacts with enterovirus 71 internal ribosomal entry site and negatively regulates viral translation. Nucleic Acids Res. 37, 47–59.
Liu, A.-L., Liu, B., Qin, H.-L., Lee, S.M.Y., Wang, Y.-T., Du, G.-H., 2008. Anti-influenza virus activities of flavonoids from the medicinal plant *Elsholtzia rugulosa*. Planta Med. 74, 847–851.
Liu, M.-M., Zhou, L., He, P.-L., Zhang, Y.-N., Zhou, J.-Y., Shen, Q., Chen, X.-W., Zuo, J.-P., Li, W., Ye, D.-Y., 2012. Discovery of flavonoid derivatives as anti-HCV agents via pharmacophore search combining molecular docking strategy. Eur. J. Med. Chem. 52, 33–43.
Manthey, J.A., Guthrie, N., Grohmann, K., 2001. Biological properties of citrus flavonoids pertaining to cancer and inflammation. Curr. Med. Chem. 8, 135–153.
McMinn, P.C., 2002. An overview of the evolution of enterovirus 71 and its clinical and public health significance. FEMS Microbiol. Rev. 26, 91–107.
Orhan, D.D., Özçelik, B., Özgen, S., Ergun, F., 2010. Antibacterial, antifungal, and antiviral activities of some flavonoids. Microbiol. Res. 165, 496–504.
Ren, W., Qiao, Z., Wang, H., Zhu, L., Zhang, L., 2003. Flavonoids: promising anticancer agents. Med. Res. Rev. 23, 519–534.
Romanova, D., Vachalkova, A., Cipak, L., Ovesna, Z., Rauko, P., 2001. Study of antioxidant effect of apigenin, luteolin and quercetin by DNA protective method. Neoplasma 48, 104.
Saija, A., Scalese, M., Lanza, M., Marzullo, D., Bonina, F., Castelli, F., 1995. Flavonoids as antioxidant agents: importance of their interaction with biomembranes. Free Radic. Biol. Med. 19, 481–486.
Schmidt, N.J., Lennette, E.H., Ho, H.H., 1974. An apparently new enterovirus isolated from patients with disease of the central nervous system. J. Infect. Dis. 129, 304–309.
Seelinger, G., Merfort, I., Schempp, C.M., 2008a. Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. Planta Med. 74, 1667–1677.
Seelinger, G., Merfort, I., Wölfle, U., Schempp, C.M., 2008b. Anti-carcinogenic effects of the flavonoid luteolin. Molecules 13, 2628–2651.
Shih, S.-R., Weng, K.-F., Stollar, V., Li, M.-L., 2008. Viral protein synthesis is required for enterovirus 71 to induce apoptosis in human glioblastoma cells. J. Neurovirol. 14, 53–61.
Si, X., Luo, H., Morgan, A., Zhang, J., Wong, J., Yuan, J., Esfandiarei, M., Gao, G., Cheung, C., McManus, B.M., 2005. Stress-activated protein kinases are involved in coxsackievirus B3 viral progeny release. J. Virol. 79, 13875–13881.
Solomon, T., Lewthwaite, P., Perera, D., Cardosa, M.J., McMinn, P., Ooi, M.H., 2010. Virology, epidemiology, pathogenesis, and control of enterovirus 71. Lancet Infect. Dis. 10, 778–790.
Thompson, S.R., Sarnow, P., 2003. Enterovirus 71 contains a type I IRES element that functions when eukaryotic initiation factor eIF4G is cleaved. Virology 315, 259–266.
Tsai, F.-J., Lin, C.-W., Lai, C.-C., Lan, Y.-C., Lai, C.-H., Hung, C.-H., Hsueh, K.-C., Lin, T.-H., Chang, H.C., Wan, L., 2011. Kaempferol inhibits enterovirus 71 replication and internal ribosome entry site (IRES) activity through FUBP and HNRP proteins. Food Chem. 128, 312–322.
Ueda, H., Yamazaki, C., Yamazaki, M., 2002. Luteolin as an anti-inflammatory and anti-allergic constituent of *Perilla frutescens*. Biol. Pharm. Bull. 25, 1197–1202.
Van Tu, P., Thao, N.T.T., Perera, D., Truong, K.H., Tien, N.T.K., Thuong, T.C., How, O.M., Cardosa, M.J., McMinn, P.C., 2007. Epidemiologic and virologic investigation of hand, foot, and mouth disease, southern Vietnam, 2005. Emerg. Infect. Dis. 13, 1733.
Wang, S.-M., Lei, H.-Y., Huang, K.-J., Wu, J.-M., Wang, J.-R., Yu, C.-K., Su, I.-J., Liu, C.-C., 2003. Pathogenesis of enterovirus 71 brainstem encephalitis in pediatric patients: roles of cytokines and cellular immune activation in patients with pulmonary edema. J. Infect. Dis. 188, 564–570.
Wang, S.-M., Lei, H.-Y., Yu, C.-K., Wang, J.-R., Su, I.-J., Liu, C.-C., 2008. Acute chemokine response in the blood and cerebrospinal fluid of children with enterovirus 71-associated brainstem encephalitis. J. Infect. Dis. 198, 1002–1006.
Wang, Y.-F., Chou, C.-T., Lei, H.-Y., Liu, C.-C., Wang, S.-M., Yan, J.-J., Su, I.-J., Wang, J.-R., Yeh, T.-M., Chen, S.-H., 2004. A mouse-adapted enterovirus 71 strain causes neurological disease in mice after oral infection. J. Virol. 78, 7916–7924.
Xi, X., Zhang, X., Wang, B., Wang, T., Wang, J., Huang, H., Wang, J., Jin, Q., Zhao, Z., 2013. The interplays between autophagy and apoptosis induced by enterovirus 71. PloS One 8, e56966.
Yi, L., Lu, J., Kung, H.-F., He, M.-L., 2011. The virology and developments toward control of human enterovirus 71. Crit. Rev. Microbiol. 37, 313–327.
Zhang, Y., Zhu, Z., Yang, W., Ren, J., Tan, X., Wang, Y., Mao, N., Xu, S., Zhu, S., Cui, A., 2010. An emerging recombinant human enterovirus 71 responsible for the 2008 outbreak of hand foot and mouth disease in Fuyang city of China. Virol. J. 7, 94.