Original Article

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Inhibitory effects of kaempferol, quercetin and luteolin on the replication of human parainfluenza virus type 2 *in vitro*

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SUMMARY The eight flavonoids, apigenin, chrysin, hesperidin, kaempferol, myricetin, quercetin, rutin and luteolin were tested for the inhibition of human parainfluenza virus type 2 (hPIV-2) replication. Three flavonoids out of the eight, kaempferol, quercetin and luteolin inhibited hPIV-2 replication. Kaempferol reduced the virus release (below 1/10,000), partly inhibited genome and mRNA syntheses, but protein synthesis was observed. It partly inhibited virus entry into the cells and virus spreading, and also partly disrupted microtubules and actin microfilaments, indicating that the virus release inhibition was partly caused by the disruption of cytoskeleton. Quercetine reduced the virus release (below 1/10,000), partly inhibited genome, mRNA and protein syntheses. It partly inhibited virus entry and spreading, and also partly destroyed microtubules and microfilaments. Luteolin reduced the virus release (below 1/100,000), largely inhibited genome, mRNA and protein syntheses. It inhibited virus entry and spreading. It disrupted microtubules and microfilaments. These results indicated that luteolin has the most inhibitory effect on hPIV-2 relication. In conclusion, the three flavonoids inhibited virus replication by the inhibition of genome, mRNA and protein syntheses, and in addition to those, by the disruption of cytoskeleton *in vitro*.

Keywords Virus replication, flavonoid, recombinant green fluorescence protein-expressing hPIV-2 without matrix protein

1. Introduction

Human parainfluenza virus type 2 (hPIV-2) is one of the major human respiratory tract pathogens of infants and children. hPIV-2 is a member of the genus *Rubulavirus* in the family *Paramyxoviridae*, and it possesses a single-stranded, non-segmented, negative stranded RNA genome of 15,654 nucleotides (*1*). hPIV-2 has 7 structural proteins, NP, V, phospho (P), matrix (M), F, HN and large (L) proteins. The gene order of hPIV-2 is 3'-(leader)-NP-V/P-M-F-HN-L-(trailer)-5'. All genes of hPIV-2 were sequenced by our group (*2-7*). Monoclonal antibodies (mAbs) were made, and antigenic diversity of clinical isolates was investigated by Tsurudome (*8*). The infectious hPIV-2 from cDNA clone was constructed by Kawano, and it was shown that its growth property was the same as that of control natural hPIV-2 (*9*).

In the present investigation, eight flavonoids which have inhibitory effect on major viruses (*10*) were tested for hPIV-2 growth, and it was found that three flavonoids, kaempferol, quercetin and luteolin, out of the eight had dose-dependent inhibitory effect on hPIV-2. The three had no or sufficiently low cytotoxicity at the concentration used in the present investigation (*11-15*). To investigate the effects of the flavonoids on viral genome synthesis, virus RNA was prepared and analyzed by PCR and real-time PCR. To elucidate the effects of the three flavonoids on mRNA synthesis, cDNA was synthesized using oligo(dT) primer and PCR was carried out. Virus protein expression was observed by indirect immunofluorescence study using mAbs against NP, F and HN proteins of hPIV-2 (*8*). The inhibitory effects of the three flavonoids on cell-to-cell spreading of hPIV-2 were analyzed using a recombinant green fluorescence protein-expressing hPIV-2 without matrix protein (rhPIV-2ΔMGFP) (*9,16,17*). The number of viruses released from infected cells was determined. Cytoskeleton was reported to have an important role in paramyxovirus replication. Actin microfilaments are important in the hPIV-3 life cycle, specifically at the level of viral transport and replication (*18*). Tubulin also acts as a positive transcription factor for *in vitro* RNA synthesis by Sendai virus (*19*). The effects of the three flavonoids on actin microfilaments and microtubules were analyzed using rhodamine phalloidin and anti-tubulin α mAb, respectively.

2. Materials and Methods

2.1. Flavonoids

Eight flavonoids, apigenin ($C_{15}H_{10}O_5$: molecular weight (MW) 270.24), chrysin ($C_{15}H_{10}O_4$: MW 254.24), hesperidin ($C_{28}H_{34}O_{15}$: MW 610.56), kaempferol ($C_{15}H_{10}O_6$: MW 286.24), myricetin ($C_{15}H_{10}O_8$: MW 318.24), quercetin dihydrate ($C_{15}H_{10}O_7 \cdot 2H_2O$: formula weight 338.27), rutin ($C_{27}H_{30}O_{16}$: MW 610.52) and luteolin ($C_{15}H_{10}O_6$: MW 286.24) were purchased from Fuji Film Wako Pure Chemical (Osaka, Japan).

Apigenin was extracted from the flowers or leaves of various plants, for example parsley and chamomile. Chrysin is a naturally occurring flavone chemically extracted from the blue passion flower (*Passiflora caerulea*). Hesperidin is contained in the envelope of citrus fruits. Kaempferol mainly exists in raspberries, capers, brussels sprouts, black beans and grapes. Myricetin is a naturally-occurring flavonoid found in many grapes, berries, fruits, vegetables, herbs, as well as other plants. Quercetin is in red grape wine, leaves of radish and fennel. Luteolin is in leaves of basil, parsley and spinach.

They were dissolved in an appropriate solvent or vehicle at a concentration of 10 mg/mL, and added to the cell culture. Apigenin was dissolved in methanol, chrysin in dimethyl sulfoxide (DMSO), hesperidin in phosphate buffered saline (PBS) with 1/10 volume of 1 mol/L NaOH added. Kaempferol, myricetin, quercetin dihydrate, rutin and luteolin were dissolved in ethanol. The flavonoid solutions were stored in aliquots of 50 µL at −80°C until use, and not reused. 5 µL/mL methanol, DMSO or ethanol in culture medium was not toxic to the cells examined by cell culture microscope.

2.2. Virus and recombinant virus

The virus and the recombinant virus were approved by the relevant biosafety committees of Suzuka University of Medical Science and Mie University. hPIV-2 (Toshiba strain) was used. rhPIV-2ΔMGFP was constructed according to the method described previously (*9,16,17*), and it was shown that it did not produce infectious virus particles without addition of M protein gene *in trans* (data not shown). The virus titer was determined using Vero cells and the titer was about 1×10^5 $TCID_{50}$/mL.

2.3. Cell line and cultivation of cells

$LLCMK_2$ cells (rhesus monkey kidney cell line) were cultured in a flat-bottomed 24-well plate in 1 mL culture medium. Minimum essential medium α (MEMα: Fuji Film Wako Pure Chemical), supplemented with 2% fetal calf serum (FCS) and 0.1 mg/mL kanamycin, was used. The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. After three days, when the cells became confluent (5×10^5 cells), the medium was changed to MEMα with 0.5% FCS and 0.1 mg/mL kanamycin. The flavonoid solution was added to the cells, and the cells were infected with hPIV-2 (3×10^2 $TCID_{50}$).

2.4. Cytopathogenic assay

Cell fusion was observed at four days post infection under a cell culture microscope.

2.5. RNA preparation, cDNA synthesis, real-time PCR and PCR

RNA was extracted from the cells (2×10^6 cells) cultured in a flat-bottomed 6-well plate using TRIZOL reagent (Invitrogen, CA, USA) according to the manufacturer's method. cDNA was synthesized with 1 µg RNA using Reverse Tra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) and NP gene specific primer (nucleotide number 1661-1679: 5'-CAACATTCAATGAATCAGT-3'). Real-time PCR was performed on the ABI PRISM 7700 Sequence Detection System (Life Technologies, Tokyo, Japan) using TaqMan Probe (1932-1956: 5'-FAM-AAGCACCGGATTTCTAACCCGTCCG-TAMRA-3'), forward primer (1851-1875: 5'-ACACACTCATCCAG ACAAATCAAAC-3'), and reverse primer (1958-1980: 5'-TGTGGAGGTTATCTGATCACGAA-3').

cDNA was synthesized with 1 µg RNA using forward primers for NP (nucleotide number 1,081-1,100: 5'-CATGGCCAAGTACATGGCTC-3'), F (5,821-5,840: 5'-CCCTATCCCTGAATCACAAT-3') and HN (7,741-7,760: 5'-ATTTCCTGTATATGGTGGTC-3') and superscript II reverse transcriptase (Invitrogen), and PCR was carried out with forward primers for NP (nucleotide number 1,081-1,100), F (5,821-5,840) and HN (7,741-7,760), and reverse primers for NP (1,466-1,489: 5'-CC TCCGAGTATCGATTGGATTGAA-3'), F (6,661-6,681: 5'-TGTCACGAGACGTTACGGACA-3') and HN (8,481-8,500: 5'-GAACTCCCCTAAAAGAGATG-3') genes and Ex Taq (Takara Bio, Kusatsu, Japan).

2.6. Detection of messenger RNA (mRNA)

cDNA was synthesized with 1 μg RNA using oligo(dT) primer and superscript II reverse transcriptase (Invitrogen), and PCR was carried out with forward primers for NP (nucleotide number 1,081-1,100: 5'-CATGGCCAAGTACATGGCTC-3'), F (5,821-5,840: 5'-CCCTATCCCTGAATCACAAT-3') and HN (7,741-7,760: 5'-ATTTCCTGTATATGGTGGTC-3') genes of hPIV-2, and reverse primers for NP (1,466-1,489: 5'-CC TCCGAGTATCGATTGGATTGAA-3'), F (6,661-6,681): 5'-TGTCACGAGACGTTACGGACA-3') and HN (8,481-8,500: 5'-GAACTCCCCTAAAAGAGATG-3') genes and Ex Taq (Takara Bio).

2.7. Immunofluorescence study

To detect virus proteins in the infected cells, the cells were fixed with 3.7% formaldehyde solution in PBS at room temperature for 15 min. The cells were further incubated with 0.05% Tween-20 in PBS at room temperature for 15 min to detect NP protein that exists mainly in the cytoplasm, or 3 min to detect F and HN proteins that are both in the cytoplasm and in the cell membrane, washed with PBS, and incubated with a mouse mAb against NP, F or HN protein of hPIV-2 at room temperature for 30 min. After washing with PBS, the cells were incubated with Alexa 488 conjugated secondary antibody anti-mouse IgGs (Invitrogen) at room temperature for 30 min, and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Actin was detected using rhodamine phalloidin (Invitrogen) and microtubules were observed using anti-tubulin α mAb against sea urchin tubulin α (clone B-5-1-2, Sigma-Aldrich, St Louis, MO, USA) at four days of cultivation. The cells were fixed with 3.7% formaldehyde solution in PBS at 37°C for 15 min, washed with PBS, and further incubated with 0.05% tween 20 in PBS at 37°C for 3 min to detect actin and for 15 min to detect microtubules.

2.8. Cell-to-cell spreading of hPIV-2

The flavonoid was added to the cells, and immediately after the addition, the cells were infected with rhPIV-2ΔM-GFP (1×10^4 $TCID_{50}$), and cultured for four days. They were then fixed with 1.2% formaldehyde solution in PBS at room temperature for 15 min and observed under a fluorescence microscope.

3. Results

3.1. Inhibitory effects of the three flavonoids

Different doses of the flavonoids (1 μg to 50 μg) were added to the 1 mL cell culture medium, and immediately after the addition the cells were infected with hPIV-2 (3×10^2 $TCID_{50}$), and cultured for four days. The cell fusion was observed under cell culture microscope at four days post infection. The three exhibited dose-dependent inhibitory effects. Kaempferol, quercetin dihydrate and luteolin almost completely inhibited hPIV-2 induced cell fusion, at 25 μg/mL, 20 μg /mL (17.9 μg /mL as quercetin) and 20 μg/mL, respectively.

3.2. Effect of the flavonoids on the release of hPIV-2

The supernatants of the cells were harvested at four days post the flavonoid addition and virus infection. The harvested supernatants were diluted, infected to the cells, and the virus titer was determined by the observation of cell fusion at four days post addition of the supernatants. Figure 1 shows the titer of the virus of the supernatants, indicating that the three flavonoids inhibited the virus release dose-dependently. Both kaempferol (25 μg/mL) and quercetin dihydrate (20 μg/mL) inhibited the release of the virus into the medium (below 1/10,000). Luteolin (20 μg/mL) almost completely inhibited the virus release (below 1/100,000). The three flavonoids of the concentration mentioned above were used in the following experiments.

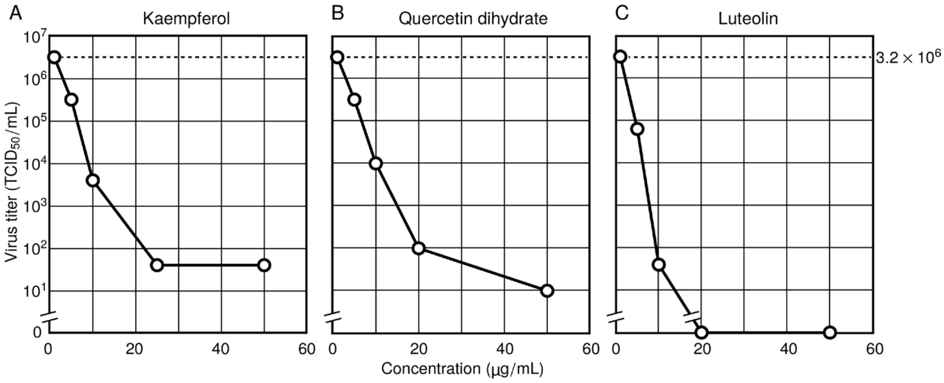

Figure 1. Dose-dependent virus release inhibition into culture medium by the three flavonoids. Both kaempferol (25 μg/mL) (**A**) and quercetin dihydrate (20 μg/mL) (**B**) inhibited the virus release into the medium (below 1/10,000). Luteolin (20 μg/mL) (**C**) almost completely inhibited the virus release (below 1/100,000).

Table 1. Effect of the three flavonoids on viral genome RNA synthesis analyzed by quantitative real-time PCR

	No drug	Kaempferol	Quercetin	Luteolin
Number of virus genome copies	1,219,753	79,435	47,291	3,757
Relative amount of virus genome RNA	1	0.065	0.039	0.003

The flavonoids were added to the cell culture, which was then infected with hPIV-2 and cultured for four days. RNA was extracted and viral genome RNA was analyzed by real-time PCR. The value of the virus infected cells was shown as 1. Kaempferol: 0.065, quercetin: 0.039 and luteolin: 0.003. Luteolin has a good effective inhibition ability to hPIV-2.

3.3. Effects of the flavonoids on viral genome RNA and mRNA syntheses

RNA was prepared from the flavonoid-treated infected cells using TRIZOL reagent according to the manufacturer's method at four days post infection, and viral genome RNA was analyzed by both real-time PCR and PCR. Viral mRNA was also analyzed by PCR.

Real-time PCR shows that kaempferol, quercetin and luteolin almost completely inhibited viral genome RNA syntheses. The inhibitory effect of luteolin was the most outstanding (Table 1).

Quite similar results were obtained by PCR (Figure 2). In control cells, no bands were seen. In virus-infected cells NP, F and HN bands were clearly detected. HN has two bands, because the primers might bind to similar nucleotide sequences. In kaempferol-treated infected cells, both NP gene and F gene bands were slightly detected, but the HN gene band was very faint. In quercetin-treated infected cells, NP and F genes were visible. In luteolin-treated infected cells, NP, F and HN gene syntheses were almost completely inhibited. These results are similar to those of real-time PCR.

Figure 3 shows the three flavonoids also inhibited viral mRNA syntheses. NP, HN or F mRNA was not detected in control cells, but all three mRNAs were clearly seen in virus infected cells. In kaempferol or quercetin-treated infected cells, NP, F and HN mRNA were slightly detected. However, in luteolin treated-infected cells, no visible band of NP, F or HN of mRNA was detected. The result of gene syntheses and mRNA syntheses were in good accordance. These results indicated that the three flavonoids had inhibitory effects on both viral genome and mRNA syntheses.

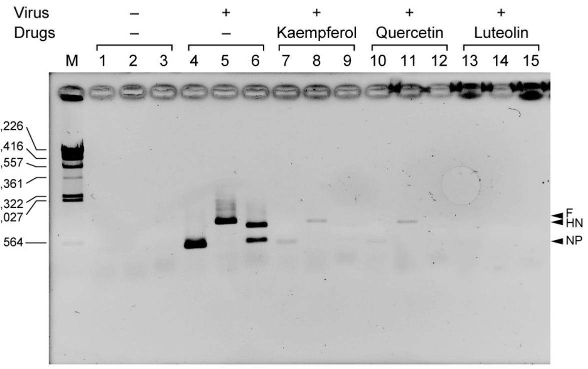

Figure 2. Effect of the flavonoids on viral genome RNA synthesis analyzed by PCR. NP, F and HN genes were detected using specific primers. In virus infected cells, NP, F and HN genes were clearly detected. In kaempferol-treated cells, NP and F genes were faintly detected. Quercetin inhibited HN gene synthesis. Luteolin almost completely inhibited the three gene syntheses.

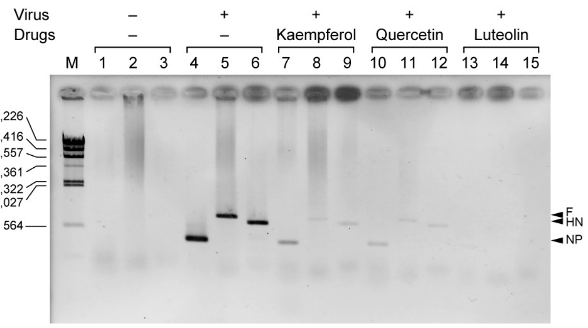

Figure 3. Effect of the flavonoids on viral mRNA synthesis analyzed by PCR. RNA was extracted from the cells and cDNA was synthesized by oligo(dT) primer. NP, F and HN mRNA were detected using specific primers. Both kaempferol and quercetin slightly inhibited NP, F and HN mRNA syntheses. Luteolin almost completely inhibited NP, F and HN mRNA syntheses.

3.4. Effects on protein syntheses

Indirect immunofluorescence study was carried out using mAbs against NP, HN and F proteins to examine the effects of the three flavonoids on viral protein syntheses at four days post infection (Figure 4). In non-infected cells, NP, HN or F protein was not detected (data not shown). Figures 4A, 4B and 4C show the NP, F and HN protein expression in hPIV-2 infected cells, respectively. In hPIV-2 infected cells, NP, F and HN proteins were observed in almost all the cells: NP protein was observed in many big strong fluorescent dots mainly in the cytoplasm, while F and HN proteins were seen in small dots in the cytoplasm and on the cell surface. Kaempferol itself has auto-fluorescence (Figures 4D, 4E and 4F). It only slightly inhibited the protein syntheses: there were many big fluorescence dots of NP protein. A large number of cells had many small fluorescent dots of F and HN proteins of infected cells cultured with kaempferol (Figures 4D, 4E and 4F, respectively). Quercetin largely inhibited NP, F and HN protein syntheses. (Figures 4G, 4H and 4I). In luteolin-treated cells, only a few positive cells were found (Figures 4J, 4K and 4L; NP, F and HN, respectively), indicating that luteolin inhibited almost completely the synthesis of viral proteins.

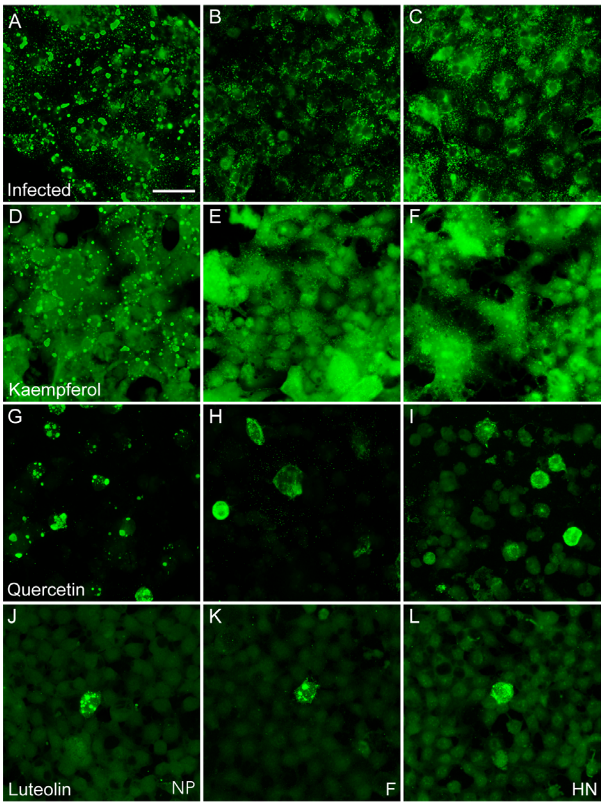

Figure 4. Effect of the three flavonoids on viral protein synthesis. NP (**A**), F (**B**) and HN (**C**) are virus infected positive controls. Kaempferol only partly inhibited NP (**D**), F (**E**) and HN (**F**) protein syntheses: there are many positive spots. Kaempferol itself has auto green fluorescence, indicating that kaempferol penetrated into the cells and stayed there. Quercetin largely inhibited NP (**G**), F (**H**) and HN (**I**) protein syntheses. Luteolin inhibited NP (**J**), F (**K**) and HN (**L**) protein syntheses: there are few positive cells. Bar: 50 µm.

3.5. Effects on the multinucleated giant cell formation

The flavonoids were added to the cells, and immediately after that they were infected with rhPIV-2ΔM-GFP (1 × 10^4 $TCID_{50}$) and cultured for four days. The cells were fixed with 1% paraformaldehyde and observed under the fluorescence microscope. Figure 5A is a positive control. There are many multinucleated giant cells with strong fluorescence. Kaempferol (Figure 5B) and Quercetin (Figure 5C) largely inhibited the giant cell formation: there were a small number of fused cells, but the size is smaller than that of the positive control. In luteolin-treated cells (Figure 5D), no fluorescent cells were found, indicating that luteolin almost completely inhibited the infection of hPIV-2 to the neighboring cells, and as a result multinucleated giant cell formation was not observed. The multinucleated giant cell formation, the number of released virus from the cells, genome RNA syntheses and protein syntheses were in good accordance.

3.6. Effects on actin microfilaments

The three flavonoids were added to the cell culture without virus infection and cultured for four days. F-actin was stained with rhodamine phalloidin. Figure 6A is the positive control: bundles of actin microfilaments were clearly seen. Kaempferol partly disrupted actin microfilaments (Figure 6B), but quercetin (Figure 6C) and luteolin (Figure 6D) caused severe damage in actin microfilaments. These results showed the damage to actin microfilaments caused some inhibitory effects on the release of virus from the cells to culture medium.

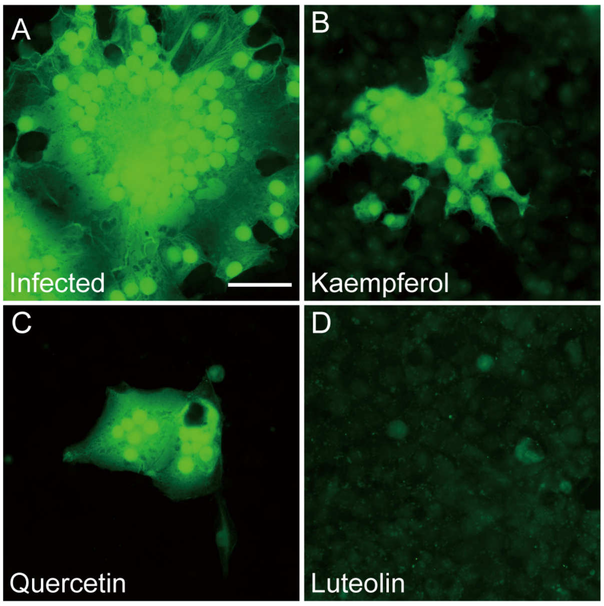

Figure 5. Effect of the flavonoids on multinucleated giant cell formation. The cells were added with the flavonoids and infected with rhPIV-2ΔM-GFP. Immunofluorescence study was carried out at four days of post infection. In positive control cells, many multinucleated giant cells with strong fluorescence were observed (**A**). Kaempferol (**B**), quercetin (**C**) largely inhibited giant cell formation: the fused cell size is small. Luteolin inhibit cell fusion. There were no fused fluorescent cells. Bar: 50 µm.

3.7. Effects on microtubules

The cells were added with the flavonoids without virus infection, and cultured for four days. Microtubules were stained with anti-tubulin α mAb. Figure 7A is the positive control: microtubule networks were seen in the cytoplasm. Kaempferol (Figure 7B), quercetin (Figure 7C), and luteolin (Figure 7D) partly disrupted microtubules. Microtubules are also important for virus replication, so one of the causes of virus replication inhibition had some relation with the disruption of microtubules.

4. Discussion

Three flavonoids, kaempferol, quercetin and luteolin were tested for hPIV-2 replication *in vitro*. In the present investigation, the effects of the three flavonoids on genome RNA synthesis, viral mRNA synthesis, protein expression, multi-nucleated giant cell formation and

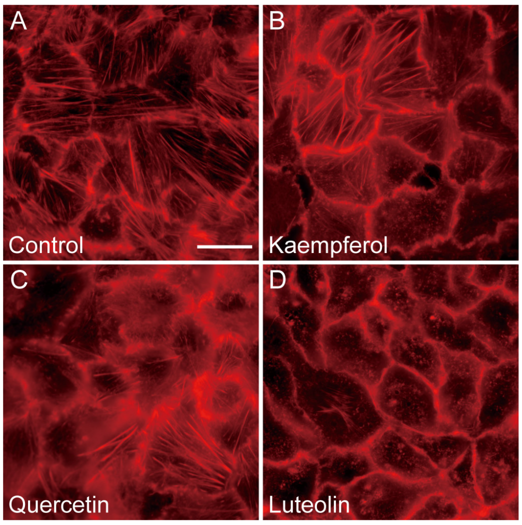

Figure 6. Effect of the flavonoids on actin microfilaments. The flavonoids were added to the cell culture without virus infection, and cultured for four days. The cells were stained with rhodamine phalloidin. In non-treated cells (**A**), actin microfilaments were clearly seen. Kaempferol (**B**) partly destroyed the filaments. Quercetin (**C**) and luteolin (**D**) caused damage the filaments. Bar: 50 µm.

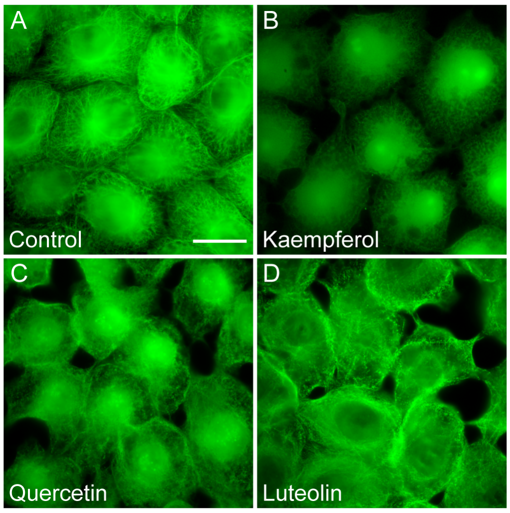

Figure 7. Effect of the flavonoids on microtubules. The flavonoids were added to the cell culture without virus infection, and cultured for four days. The cells were stained with anti-tubulin α mAb against sea urchin tubulin α. In non-treated cells (**A**), microtubules were clearly seen in the cytoplasm. Kaempferol (**B**) and quercetin (**C**) slightly destroyed microtubules. Luteolin (**D**) destroyed microtubules. Bar: 50 µm.

cytoskeleton (actin microfilaments and microtubules) were analyzed.

The three had inhibitory effects on hPIV-2 replication. Kaempferol, quercetin and luteolin reduced the release of the virus from the cells, and they had inhibitory effects on viral genome and mRNA syntheses. They inhibited largely the protein syntheses and multi-nucleated giant cell formation. They caused slight damage to actin microfilaments and microtubules, indicating that the inhibition of virus release was in part caused by the cytoskeletal damage.

Kaempferol has inhibitory activity against human cytomegalovirus (*20*). Kaempferol acts on the influenza virus neuraminidase and inhibits H1N1 and H9N2 virus (*21*). hPIV-2 also has hemagglutinin-neuraminidase (HN) protein, so kaempferol might act on HN protein, and inhibit release of hPIV-2 from the cells. It was also shown that kaempferol and kaempferol-7-*O*-glucoside (100 µg/mL) have strong inhibitory effect on human immunodeficiency virus 1 (HIV-1) reverse transcriptase (*22*). Kaempferol-3-*O*-glucoside binds to HIV-1 reverse transcriptase (*23*). Kaempferol exhibited potent inhibitory activity against feline calicivirus (*24*).

Anti-viral effect of quercetin on viruses were extensively investigated by many researchers. It has dose-dependent inhibitory effects on herpes simplex virus 1 (HSV-1) and HSV-2 in cell culture (*25,26*). Quercetin blocks binding and penetration of HSV-1 and -2 into host cells (*25*). It also inhibits H1N1, H5N2, H7N3 and H9N2 influenza virus *in vivo* (*27*). It may be an inhibitor of neuraminidase of type H1N1 and H7N9 influenza virus (*28-30*), and interacts with hemagglutinin of influenza virus (*31*), resulting in cell fusion between virus and host cells. Quercetin has anti-rhinovirus activity by inhibiting endocytosis, genome transcription and protein synthesis (*32*). In addition, it has inhibitory activity for many other viruses, such as cytomegalovirus (*12*), canine distemper virus (*33,34*), porcine diarrhea virus (*35,36*), dengue virus serotype 2 (*37*), *etc*.

Luteolin was also reported to have antiviral activity *in vitro*. For example, it has antiviral activity against HIV-1 (*38*), Epstein-Barr virus (*39*), severe acute respiratory syndrome-related coronavirus (SARS-CoV) (*40,41*), and Japanese encephalitis virus (*13*). It also has inhibitory activity for influenza A virus by interfering with the coat protein I complex expression (*14*).

Many investigators have shown the inhibitory effects of flavonoids on a wide range of viruses, for example, genistin inhibits adenovirus, arenavirus, HSV-1, HSV-2, human herpesvirus-8, rotavirus and respiratory syncytial virus (*43*), quercetin inhibits adenovirus (*10*), arenavirus (*43*) and coronavirus (*44*), luteolin inhibits coronavirus (*45*), kaempferol inhibits HSV-1 (*10*), myricetin inhibits Moloney murine leukemia virus and SARS-CoV (*10*), chrycin inhibits HSV-1 and coxsackie B virus (*10*), morin inhibits canine distemper virus and Moloney murine leukemia virus (*10*), *etc*.

The antiviral mechanisms of flavonoids are viral binding inhibition, inhibition of viral genome, mRNA and protein syntheses. In the present investigation, the inhibitory mechanisms are similar among the three flavonoids. The three flavonoids had inhibitory effects on viral genome RNA, mRNA and protein syntheses. They also inhibited multinucleated giant cell formation in size and number, indicating that they might inhibit virus entry and/or cell-to-cell spreading. In addition, they caused damage in actin microfilaments and microtubules, resulting in the inhibition of virus release from the infected cells. These results are based on the *in vitro* study. Some researchers reported *in vivo* effects of quercetin (*27,32,36*). The next aim is to elucidate *in vivo* effects of the three flavonoids.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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