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ARTICLE

# Antiviral activities of hydroxylated flavones on feline foamy viral proliferation

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**Abstract** Many hydroxylated flavones are reported to have antibacterial, anticancer, or antiviral activities. In this study, eleven hydroxylated flavones including 3,3′,4′,5,7-pentahydroxyflavone (quercetin), three polymethoxyflavones, two polyethoxyflavones, two polypropoxyflavones, one butoxyflavone, and two benzoxyflavones were tested for antiviral activity using feline foamy virus. Most of the compounds tested showed that they did not have significant cytotoxic effect on the crandell-Ress feline kidney cells. However, four compounds, including quercetin, 5,3′-dihydroxy-3,7,4′-trimethoxyflavone, 5-hydroxy-3,7,3′,4′-tetramethoxyflavone, and 3,5,7,3′,4′-pentahydroxyflavone, showed a strong inhibitory effect on feline foamy virus (FFV) proliferation, by reducing viral production to 7–24% of that in the un-treated control when they were added to the cells at a final concentration of 10 μM. These results were supported by western blot detecting viral protein in the infected cell lysate. In further analysis, quercetin was observed to have a direct inhibitory effect on reverse transcriptase and integrase in vitro, which can explain the mechanism by which quercetin inhibits FFV proliferation. These preliminary results suggest that hydroxylated flavones such as quercetin, 5,3′-dihydroxy-3,7,4′-trimethoxyflavone, 5-hydroxy-3,7,3′,4′-tetramethoxyflavone, and 3,5,7,3′,4′-pentahydroxyflavone have strong antiretroviral activities.

**Keywords** Antiviral activity · Foamy virus · Hydroxylated flavones

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## Introduction

Flavones are the most abundant phytochemicals found in plant extracts. Currently, more than 4000 different flavones are estimated to be present in nature [1]. Their basic chemical structure is composed of diphenylpropanes with two aromatic rings connected with a pyran ring. One of the best-studied hydroxylated flavonoids is 3,3′,4′,5,7-pentahydroxyflavone (quercetin). It has been demonstrated to have anticancer activity in various cancer cell types [2]. At present, quercetin derivatives containing methoxy, ethoxy, propoxy, butoxy, or benzoxy groups on their aromatic rings have been reported to have diverse pharmacological activities.

Polymethoxyflavones (PMFs) are flavonoid compounds containing multiple methoxy groups. PMFs are the major phytocompounds in the rhizome extracts of *Kaempferia parviflora,* from which at least 11–13 PMFs have been isolated [3]. Many investigators have shown that PMFs isolated from *K. parviflora* have a variety of biological and pharmacological activities including antimicrobial, anticancer, and antiviral properties [4–6]. In addition, PMFs isolated from other plant sources were reported to have diverse anticancer activities in in vitro studies. 5-Hydroxy-6,7,3′,4′,5′-pentamethoxyflavone isolated from the African medicinal plant *Lantana ukambensis* was reported to have an anticancer effect on human cell lines [7]. Methoxyflavones obtained from citrus peel showed potent anticancer activities in human prostate xenograft tumors [8]. Several PMFs have also been suggested to suppress lipid accumulation. Hydroxylated PMFs suppressed adipogenesis and obesity in mice fed a high-fat diet [9]. 5-Hydroxy-3,6,7,8,3́,4́-hexamethoxyflavone isolated from orange peel was shown to have a suppressive effect on adipogenesis in

3T3-L1 cells [10]. 5-Demethylnobiletin and 5-acetoxy-6,7,3́,4́,-pentamethoxyflavone inhibited lipid accumulation by activating the LKB1–AMPK pathway in 3T3-L1 preadipocytes and C57BL/6 mice fed a high-fat diet [11]. Recently 5,3́-dihydroxy-3,7,4́-triethoxyflavone was reported to induce apoptosis in human cancer cell line [12].

Feline foamy virus (FFV) is a member of *Spumaretrovirinae,* a subfamily in the *Retroviridae* family of viruses. FFV replicates well in feline kidney cells, like a common retrovirus, by inserting viral DNA into cellular DNA [13]. Recently, it is shown to be able to infect several human cell lines, including neural cell types. Therefore, it is one of the representative retroviruses on which antiviral agents can be screened in in vivo and in vitro systems.

Many hydroxylated flavones are reported to have antiviral activities against various viruses. Quercetin, 5,6,7-trihydroxyflavone (baicalein), 3,3′,4′,5,6,7-hexahydroxyflavone (quercetagetin), and 3,3′,4′,5,5′,7-hexahydroxyflavone (myricetin) were reported to be potent inhibitors of reverse transcriptase and integrase of human immunodeficiency virus [14, 15]. However, the antiviral effects of PMFs and polyethoxyflavone have not been reported. Very recently, PMF found in the supercritical fluid extract of Guangchenpi was reported to possess antiviral activity against respiratory syncytial virus [16]. In order to evaluate antiretroviral activities of hydroxylated flavones in the present study, eleven chemicals including quercetin, three polymethoxyflavones, two polyethoxyflavones, two polypropoxyflavones, one butoxyflavone, and two benzoxyflavones were analyzed using FFV.

## Materials and methods

### Reagents and cell culture

The flavonoids used for the study are listed in Table 1. Quercetin, 3′-azido-3′-deoxythymidine (AZT), AZT-triphosphate (AZT-TP), and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other flavonoid chemicals were obtained from Professor Han Jaehong (Chung-Ang University, Republic of Korea) who had isolated them from *K. parviflora* and synthesized chemically [17, 18]. Flavonoids were dissolved in 50% dimethylsulfoxide. AZT and AZT-triphosphate (AZT-TP) were dissolved in distilled water. The Crandell-Rees feline kidney (CRFK) cell line (Korean Cell Line Bank, Seoul, Korea) was maintained in Dulbecco's modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin.

**Table 1** Chemicals used in the study and their abbreviation

| Mark | Chemical names |
|---|---|
| QUE | 3,3′,4′,5,7-pentahydroxyflavone (quercetin) |
| TMF | 5,3′-dihydroxy-3,7,4′-trimethoxyflavone |
| TeM | 5-hydroxy-3,7,3′,4′-tetramethoxyflavone |
| PMF | 3,5,7,3′,4′-pentamethoxyflavone |
| TeE | 5-hydroxy-3,7,3′,4′-tetraethoxyflavone |
| PEF | 3,5,7,3′,4′-pentaethoxyflavone |
| TPE | 5,3′-dihydroxy-3,7,4′-tripropoxyflavone |
| TEP | 5-hydroxy-3,7,3′,4′-tetrapropoxyflavone |
| TBF | 5,3′-dihydroxy-3,7,4′-tributoxyfalvone |
| TBn | 5,3′-dihydroxy-3,7,4′-tribenzoxyfalvone |
| TeB | 5-hydroxy-3,7,3′,4′-tetrabenzoxyflavone |

### Viral production and FeFAB assay for viral titer determination

FFV was produced in CRFK cells transfected with pCF7 DNA (FFV molecular clone; kind gift from Dr. Martin Löchelt, Heidelberg, Germany) and then used for infection in the inhibitor experiments. Viral titer was evaluated by FeFAB assy. The FeFAB cell line (kind gift from Dr. Martin Löchelt, Heidelberg, Germany) derived from the CRFK cell line containing FFV LTR-β-galactosidase reporter cassettes was maintained in the presence of 100 μg/ml G418 (Invitrogen, Carlsbad, CA, USA). Approximately $2 \times 10^5$ FeFAB cells were infected with serially diluted FFV in 48-well culture plates. At 48 h after infection, cells were fixed with fixative solution of 0.2% glutaraldehyde and 1% formaldehyde in PBS. The fixed FeFAB cells were incubated for 4 h with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining solution, and the number of blue cells was counted on an inverted microscope.

### Cytotoxicity test

The in vitro cytotoxic effects of flavonoids on the cultured cells were determined by MTT colorimetric dye reduction method as described previously [19]. Briefly, about $4 \times 10^4$ cells/well were grown on 96-well tissue culture plates in the presence of chemicals at 100 μM as a final concentration. After 48 h, 50 μl of 0.1 mg/mL MTT was added and incubated for 4 h. After discarding the solution, cells were digested by DMSO and incubated at room temperature for 10 min. The absorbance was read at 595 nm using a microplate reader. Three independent experiments were performed for each replicate at different time points.

### Antiviral effect test

Antiviral activities were studied in FFV-infected CRFK cells in the presence of 1, 10, and 100 μM chemicals. Briefly, the CRFK cells cultured in a 48-well plate were infected with FFV of 1 multiplicity of infection (MOI) and then incubated with the chemicals at the indicated concentrations for 48 h. At this time, AZT was added (final concentration 1 μM) as a positive control for inhibition of viral growth. After 48-h incubation, the culture supernatants were collected and the viral titers were evaluated by FeFAB assay.

### In vitro reverse transcriptase assay

Anti-reverse transcriptase (RT) activities were investigated using Moloney murine leukemia virus (MoMLV) RT. The reactions were composed of 50 μL containing 40 unit of MoMLV RT (Invitrogen), 5 μg of poly(dA)/$(dT)_{12–18}$ (Midland Scientific, Omaha, NE, USA), and 1 μL of $^{3}$H-TTP (TRK354, 1 mCi/mL, 37 MBq, GE Healthcare UK Ltd, Little Chalfont, UK) in the presence of hydroxylated flavones at the indicated concentrations [20]. The reactions were incubated for 1 h at 37 °C, and all the solution in the reaction then absorbed onto DE81 filters (Whatman, GE Healthcare, Piscataway, NJ, USA). After washing three times with 4X SSC (0.6 M sodium chloride, 60 mM sodium citrate) and two times with ethanol, the dried filters were immersed in liquid scintillation cocktail (AquaLight Beta, Hidex, Finland) and LSC was used to measure radioactivity.

### In vitro FFV integrase reaction

FFV integrase was purified according to our previous reports [21, 22]. For the integrase reaction, DNA substrate was first prepared by using the two oligonucleotides: FFVU5/20S, 5′-CAGGTATAGGCCACGACAGT-3′; FFVU5/20A, 5′-ACTGTCGTGGCCTATACCTG-3′. The substrates were prepared by labeling the 5′-end of sense direction oligonucleotides (FFVU5/20S) with [γ-$^{32}$P]-ATP and T4 polynucleotide kinase, and then by annealing the 5′-end-labeled FFVU5/20S with its complementary oligonucleotide FFVU5/20A. In the reaction, 0.1 pmol labeled DNA substrate was incubated with 3 pmol integrase for 60 min at 37 °C in 10 μL of reaction buffer containing 20 mM HEPES (pH 7.5), 5 mM $MnCl_2$, 30 mM NaCl, 10 mM DTT, 0.01 m M EDTA, 1 mM CHAPS, and 0.05% Nonidet P40. The reaction was stopped by adding 10 μl of stop solution (95% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue, and 20 mM EDTA) and heated at 95 °C for 3 min before electrophoresis on 15% polyacrylamide gels with 7 M urea in Tris–taurine–EDTA buffer. After gel drying, the products were quantified with a Cyclone Molecular Dynamic Phospho-Imager (PerkinElmer, Waltham, MA, USA).

### Western blot analysis

No-infected and infected CRFK cells were lysed in RIPA buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% SDS, and 0.5% sodium deoxycholate) as previously described [23], and protein concentration was measured using the Bradford assay. An equal amount of collected proteins was loaded onto 12.5% SDS–PAGE and separated at 120 V for 1 h 30 min. Proteins were then transferred to Immobilon-P membranes (Millipore) at 40 V for 4 h. The membranes were blocked for 2 h at room temperature with blocking buffer (5% nonfat dry milk, 0.1% Tween 20 in PBS). The blotted membranes were probed with rabbit polyclonal antibody against FFV-Gag protein (1:50 dilution, Novopro, China) in PBS overnight. After three washes with PBS/0.1% Tween 20, the membranes were incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase secondary antibody (1:10,000 dilution, Sigma-Aldrich) in blocking buffer for 1 h at room temperature. The membranes were washed three times with PBS/0.1% Tween 20 and developed using chemiluminescence detection kit (Bionote, Seoul, Korea). As an internal control, actin was probed with mouse monoclonal antibody against actin (1:1000 dilution, Santa Cruz Biotechnology) and then with anti-mouse IgG (1:10,000 dilution, Sigma-Aldrich).

### Statistical analysis

All data shown in the figures are expressed as mean ± SEM. Data were obtained at least from three independent experiments. Statistical significance was analyzed with a two-tailed Student's $t$ test. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

## Results

### Chemicals and their cytotoxic effect

Most of the chemicals investigated for antiviral activities were isolated from *K. parviflora* [17], and their chemical names are listed in Table 1. First, we investigated the cytotoxicity of the chemicals on CRFK cells. Cells were incubated with chemicals at a final concentration of 100 μM for 48 h. Growth inhibition was measured by the MTT assay. Compared to un-treated control cells, the chemical-treated cells showed 50–138% of growth of the control cells (Fig. 1). Therefore, it was concluded that most

of the compounds did not have significant cytotoxic effect on the CRFK cells.

### Inhibitory effects on FFV proliferation

Next, in order to observe any antiviral activities, the CRFK cells cultured in the 48-well plate were infected with FFV of 1 MOI and then incubated with the chemicals at a concentration of 100 μM for 48 h. At this time, 1 μM AZT was added to infected cells as a positive control for inhibition of viral growth. After 48-h incubation, the culture supernatants were collected, and the viral titers were evaluated using the FeFAB assay (Fig. 2A). As a positive control for inhibition, the culture supernatant collected from the infected cells treated with 1 μM AZT showed $(3.25 \pm 0.94) \times 10^2$ blue cells, whereas the un-treated control showed $(5.24 \pm 0.71) \times 10^5$ blue cells. It confirmed that AZT is a strong inhibitor for retroviral growth. In addition our data indicated that some hydroxylated flavones, including quercetin, 5,3′-dihydroxy-3,7,4′-trimethoxyflavone (TMF), 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (TeM), and 3,5,7,3′,4′-pentahydroxyflavone (PEF), have a strong inhibitory effect on FFV growth. Treatment with QUE, TMF, TeM, and PEF resulted in $(2.40 \pm 0.77) \times 10^3$, $(8.67 \pm 0.43) \times 10^3$, $(4.00 \pm 0.15) \times 10^4$, and $(1.20 \pm 0.19) \times 10^4$ blue cells per ml of culture supernatants, which are about 0.4–7.6% of the un-treated control cells, respectively (Fig. 2A). Microscopic observations in the FeFAB assay demonstrated that the culture supernatants harvested from the infected and inhibitor-treated cells contained infectious FFV particles, which induce expression of the β-galactosidase gene in the indicator cells (Fig. 2B). Microscopic pictures of the infected cells showed difference in the blue cell numbers depending on the chemicals used. Therefore, the four compounds quercetin, TMF, TeM, and PEF that showed relatively strong inhibition of viral growth at a concentration of 100 μM were selected for further experiments.

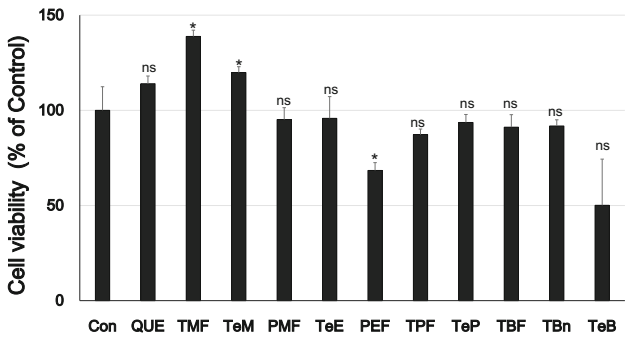


**Fig. 1** Effects of hydroxylated flavones on the cell growth. Approximately $4 \times 10^4$ CRFK cells/well were grown on 96-well plates in the presence of chemicals at a final concentration of 100 μM. After 48 h, the cell growth was detected by MTT assay. All data shown in the figure are expressed as mean ± SEM. Statistical significance was analyzed with a two-tailed Student's *t* test. $^{ns}P > 0.05$, $^{*}P < 0.05$

In further experiments, we collected culture supernatants from the FFV-infected cells treated with 1 and 10 μM of the four chemicals (Fig. 3A). Treatment with quercetin, TMF, TeM, and PEF at 10 μM yielded $(7.67 \pm 1.22) \times 10^4$, $(4.06 \pm 1.32) \times 10^4$, $(1.27 \pm 0.21) \times 10^5$, and $(7.01 \pm 1.33) \times 10^4$ blue cells, respectively, which were about 7.7–24.2% of the un-treated control. In addition, treatment with quercetin, TMF, TeM, and PEF at 1 μM yielded $(3.04 \pm 0.22) \times 10^5$, $(1.21 \pm 0.38) \times 10^5$, $(1.82 \pm 0.39) \times 10^5$, and $(2.30 \pm 0.62) \times 10^5$ blue cells, respectively, which were about 23.1–58.0% of the un-treated control (Fig. 3A). In order to investigate whether the level of viral production is correlated with the production of the viral structural protein Gag in the cytoplasm of the infected cells, we prepared cell lysate from the FFV-infected cells treated with the inhibitors and analyzed the amounts of FFV-Gag protein in the cell lysates by western blotting (Fig. 3B). The levels of FFV-Gag protein detected in the cell lysates prepared from the virus-infected cells treated with quercetin, TMF, TeM, and PEF are 5–20% of that of control cells.

### In vitro enzymatic assays

In general, viral enzymes are essential for viral replication. Therefore, they are frequent targets for antiviral chemotherapy. Reverse transcriptase (RT) is a key retroviral enzyme that is one of the major targets in developing antiretroviral agents. By performing an in vitro RT reaction in the presence of the chemicals, we have directly attempted to investigate whether the hydroxylated flavones have anti-RT activity. As a positive control, addition of 1 μM AZT-TP to the reaction decreased $^3$H-TTP incorporation to 51.0 ± 1.2% of that of the control reaction where any inhibitor is not added (Fig. 4A). Addition of 1, 10, and 100 μM quercetin resulted in 79.3 ± 4.4, 26.5 ± 5.2, and 15.3 ± 1.7% reduction in $^3$H-TTP incorporation compared to that of the control reaction. However, the other 3 chemicals did not inhibit RT activities in vitro.

Integrase is another essential enzyme for the retroviral life cycle. Several inhibitors for retroviral integrase have been reported [24]. Previously, we had first reported the isolation and characterization of FFV integrase [21, 22]. As our laboratory has already established the retroviral integrase reaction, the above hydroxylated flavones were tested to determine whether they have anti-integrase activities in vitro. In the positive reaction of integrase activity (marked '+'; it has only integrase without inhibitor), 72.2% of the 20mer oligonucleotide substrates were converted to the 18mer oligonucleotide products (Fig. 4B).

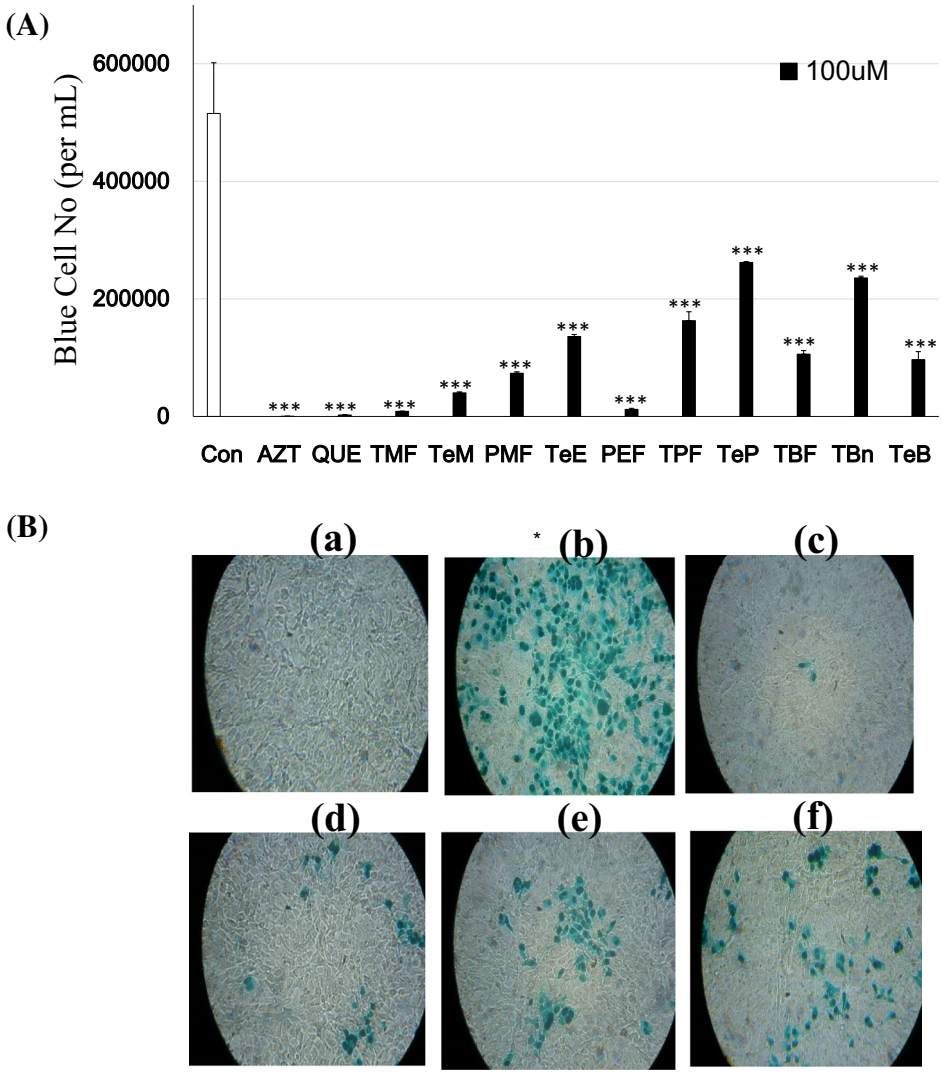


**Fig. 2** Suppression of FFV proliferation byhydroxylated flavones. (**A**) Reduced viral production in the presence of hydroxylated flavones. The CRFK cells infected with FFV of 1 MOI were incubated with 100 μM of the flavones for 48 h. For the positive control of inhibition, 1 μM AZT was used. The culture supernatants were collected, and viral titers were monitored by FeFAB assay. Data are presented as the mean ± SD for triplicate samples. Con: control for FFV-infected CRFK cells without drug treatment. (**B**) Microscopic observation of FeFAB assy. 1000-fold dilution of the supernatants collected in (**A**) was used to infect FeFAB cells. At 48 h post-infection, cells were treated with X-gal solution. *a* no infection, *b* Con, *c* QUE, *d* TeM, *e* PMF, *f* TeE. Statistical significance was analyzed with a two-tailed Student's *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

Under the same condition, addition of quercetin at 1, 10, and 100 μM caused 57.7 ± 4.8, 22.7 ± 3.6, and 9.8 ± 2.6% in the conversion ratio of the substrate to the product, respectively. The IC50 for quercetin is approximately 2.9 ± 0.9 μM. These results indicate that quercetin inhibits integrase activity dose dependently, whereas the other 3 flavones had no direct inhibitory effect on integrase activity.

## Discussion

Hydroxylated flavones are the most abundant plant phenolic compounds. They have been suggested to have diverse biological and pharmacological activities on various cells and experimental animals. Recently, their derivatives containing methoxy or ethoxy groups as substitutes have been extensively investigated for potential use as anticancer and antiviral agents. In this study, we compared the antiviral activities of eleven hydroxylated flavones using a model of retroviral infection. In cell growth inhibition experiments using 100 μM as a final concentration, none of the compounds significantly inhibited CRFK cell growth (Fig. 1). In viral growth inhibition experiments, however, four hydroxylated flavones including OUE, TMF, TeM, and PEF showed strong repression of viral production in the FFV-infected cells when they were added at concentrations of 10 and 100 μM (Figs. 2, 3). This reduction in viral production in these cells was confirmed with reduced production of the viral Gag protein detected by western blot analysis of the infected cell lysates (Fig. 3). Previously, polyhydroxylated flavones such as quercetin, baicalein, quercetagetin, and myricetin were suggested to have antiretroviral activities, since they were potent inhibitors of reverse transcriptase and integrase in human immunodeficiency virus [14, 15]. However, the antiviral effects of polymethoxyflavone and polyethoxyflavones have not yet been reported. In this study, it is

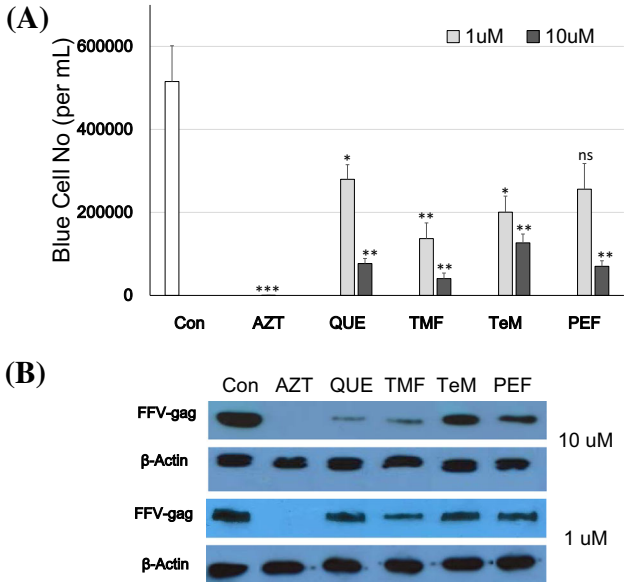


**Fig. 3** Inhibition of FFV growth by hydroxylated flavones. (**A**) The flavones marked above were added to the FFV-infected CRFK cells at final concentrations of 1 and 10 μM. At 48 h post-infection, culture supernatants were collected and analyzed for viral titer. For the positive control of inhibition, cells were treated with 1 μM AZT. (**B**) Western blot analysis of FFV-infected cell lysates. The virus-infected and flavone-treated cells described in (**A**) were lysed at 48 h post-infection. FFV-Gag protein was detected by polyclonal anti-FFV-Gag antisera. *β*-actin was also detected in cell lysates as a loading control. All data shown in the figures are expressed as mean ± SEM. Statistical significance was analyzed with a two-tailed Student's *t* test. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$

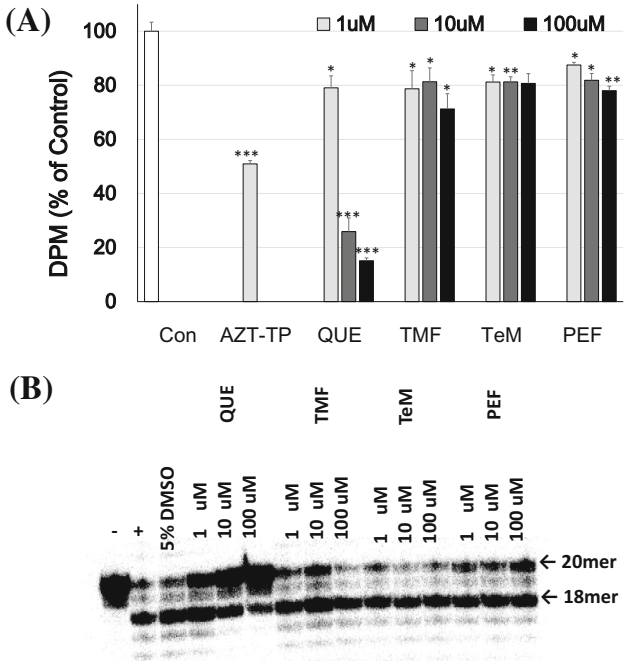


**Fig. 4** Effect of hydroxylated flavones on activities of retroviral enzymes. (**A**) Analysis of RT activities in the presence of hydroxylated flavones. MoMLV RT activities in vitro were investigated in the presence of hydroxylated flavones at 1 and 10 μM. (**B**) Analysis of FFV integrase activities in vitro in the presence of hydroxylated flavones. The biochemically purified FFV integrase was incubated with $^{32}$P-labeled duplex oligonucleotides for 90 min in the presence of hydroxylated flavones of 1 and 10 μM. Then the reactions were analyzed in a 15% denaturing PAGE and then visualized with a Molecular Dynamic Phospho-Imager. Statistical significance was analyzed with a two-tailed Student's *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

suggested that these hydroxylated flavones containing a few methoxy or ethoxy groups have antiretroviral activities. Among the hydroxylated flavones tested here, the three flavones TMF, TeM, and PEF have a methoxy or ethoxy group in their structure. They showed antiviral activities by suppressing viral growth in the infected cells. To our knowledge, it is the first report that hydroxylated flavones containing methoxy or ethoxy group have antiretroviral activities. These data taken together indicate that the hydroxylated flavones have a great advantage over the non-hydroxylated flavones regarding bioavailability. It also appears that the hydroxylated flavones containing methoxy and ethoxy groups may be more pharmacologically diverse than only hydroxylated flavones, although more studies in this area are needed.

To summarize, we have studied the inhibitory effect of eleven hydroxylated flavones on FFV proliferation. Among eleven compounds, four compounds quercetin, TMF, TeM, and PEF showed a strong inhibitory effect on FFV proliferation, by reducing viral production to 7–24% of untreated control. In addition, these results were supported by western blot detecting viral protein in the infected cell lysates. In further analysis using the RT and integrase assays, quercetin was observed to have a direct inhibitory effect on RT and integrase activity in vitro, which can explain the mechanism by which quercetin inhibits FFV proliferation. These preliminary results suggest that the hydroxylated flavones containing methoxy or ethoxy group such as TMF, TeM, and PEF have strong antiretroviral activities. In addition, we are currently preparing to screen for more effective inhibitors of retroviral proliferation among their derivatives.

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