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Antiherpetic Activities of Flavonoids against Herpes Simplex Virus Type 1 (HSV-1) and Type 2 (HSV-2) *In Vitro*

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Flavonoids, a group of low molecular weight phenylbenzopyrones, have various pharmacological properties including antioxidant, anticancer, bactericidal, and anti-inflammatory. We carried out anti-herpetic assays on 18 flavonoids in five classes and a virus-induced cytopathic effect (CPE) inhibitory assay, plaque reduction assay, and yield reduction assay were performed. When flavonoids were applied at various concentrations to Vero cells infected by HSV-1 and 2, most of the flavonoids showed inhibitory effects on virus-induced CPE. Among the flavonoids, EC, ECG (flavanols), genistein (isoflavone), naringenin (flavanone), and quercetin (flavonol) showed a high level of CPE inhibitory activity. The antiviral activity of flavonoids were also examined by a plaque reduction assay. EC, ECG, galangin, and kaempferol showed a strong antiviral activity, and catechin, EGC, EGCG, naringenin, chrysin, baicalin, fisetin, myricetin, quercetin, and genistein showed moderate inhibitory effects against HSV-1. In these experiments, flavanols and flavonols appeared to be more active than flavones. Furthermore, treatment of Vero cells with ECG and galangin (which previously showed strong antiviral activities) before virus adsorption led to a slight enhancement of inhibition as determined by a yield reduction assay, indicating that an intracellular effect may also be involved.

Key words: Flavonoids, Herpes simplex virus (HSV), Cytopathic effect (CPE)-inhibitory assay, Selectivity index (SI), Plaque reduction assay, Yield reduction assay, Vero cells

INTRODUCTION

Flavonoids are plant pigments that are synthesized from phenylalanine, generally display marvelous colors known from flower petals and are members of a class of natural compounds that recently has been the subject of considerable scientific and therapeutic interest. They are found in seeds, citrus fruits, olive oil, tea, and red wine and are commonly consumed with the human diet (Harborne, 1994; Middleton Jr. *et al.*, 2001) approximately 1 g flavonoids/day. The flavonoids are ubiquitous to green plant cells and detailed evidence of the role of flavonoids in gene regulation and growth metabolism is known (Havsteen, 2002). In addition, various pharmacological roles including antioxidant, bactericidal, anti-malarial, anti-inflammatory, and anticancer effects have been demonstrated. Many of the pharmacological properties of these compounds have been related to their ability to inhibit enzymes involved in cell activation, such as phosphodiesterases, kinases, topoisomerases, and other regulatory enzymes (Manthey *et al.*, 2001). Although flavonoids have been studied extensively, the application of flavonoids in treating human diseases is still uncommon because of their high effective concentration and poor absorption after oral intake (Shen *et al.*, 2002).

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are common pathogens that cause localized skin infections of the mucosal epithelia of the genitals, the oral cavity, the pharynx, the oesophagus and the eye, depending upon the type involved. Moreover, HSV infections may also cause severe problems to infected individuals due to the following virus properties. Firstly, the virus establishes latent infections that can be periodically reactivated. And secondly, under certain circumstances, the virus can produce serious infections of the central nervous system including acute necrotizing encephalitis and meningitis; the viruses may also produce fatal infections in patients with immune deficiencies (Whitley, 2001). Thirdly, the immediate-early genes of HSV-1 can stimulated the activation of genes belonging to different

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viruses such as human immunodeficiency virus (Ostrove *et al.*, 1987), varicella-zoster virus (Felser *et al.*, 1988) or human papillomavirus type 18 (Gius and Laimins, 1989). Additionally, HSV infections were reported to be recognized as risk factor for human immunodeficiency virus (HIV) infection (Hook *et al.*, 1992). HSV-2 is also known as oncogenic virus which has the ability to convert cells into tumor cells (Lapucci *et al.*, 1993).

Many *in vitro* and *in vivo* studies have been published on the antiviral activity of flavonoids. Recent reviews (Selway, 1986; Vlietinck *et al.*, 1986; Hudson, 1990) have emphasized the great variety of viruses tested and also the diversity of methods used which demonstrated different effects: direct inactivation or anti-replicative effects. Regarding the action of flavonoids against HSV, quercetin, procyanidin, and pelargonidin were found to be virucidal (Mucsi *et al.*, 1977) whereas luteolin was inactive. The direct inactivation of HSV by catechin and hesperitin has also been verified (Kaul *et al.*, 1985).

Acute and recurrent HSV infections remain a most important problem. The search for selective antiviral agents has been vigorous in recent years but the need for new antiviral therapies still exists since many of the problems relating to the treatment of HSV infections remain unresolved, such as generation of viral resistance and conflicting efficacy in recurrent infection and in immunocompromised patients (Serkedjieva and Ivancheva, 1999). We carried out anti-herpetic assays on 18 flavonoids in five classes. A convenient virus-induced cytopathic effect (CPE) inhibition assay, plaque reduction assay, and yield reduction assay were performed to evaluate *in vitro* anti-herpetic activity of various flavonoids. The present paper describes the results of these evaluations against *Herpes simplex* virus type 1 and type 2.

MATERIALS AND METHODS

Reagents

Flavanol; (+/-) Catechin hydrate, epigallocatechin (EGC), epigallocatechin gallate (EGCG), epicatechin (EC), epicatechin gallate (ECG), flavanone; naringenin, naringin, flavone; apigenin, luteolin, chrysin, rutin, isoflavone; genistein, and flavonol; baicalin, fisetin, myricetin, kaempferol, quercetin, galangin were purchased from Sigma (Poole, UK) (Fig. 1, Table I). Agar, formaldehyde solution, crystal violet, acycloguanosine, piodium iodide, and ribonuclease A from bovine pancreas were obtained from Aldrich Chemical Co. (St. Louis, MO, U.S.A.). Flavonoids

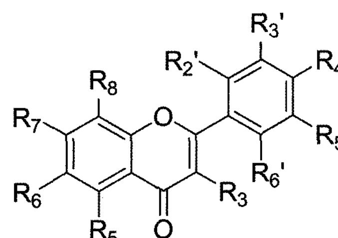

Fig. 1. Structure of the flavonoid

Table I. Classification, structure, and substitution patterns of flavonoids

Compound	R_3	R_5	R_6	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$
(+/-) Catechin hydrate	OH	OH	H	OH	H	H	OH	OH	OH
EC	OH	OH	H	OH	H	H	OH	OH	H
ECG	OH	OH	H	OH	H	OH	OH	H	H
EGC	OH	OH	H	OH	H	H	OH	OH	H
EGCG	OH	OH	H	OH	H	H	OH	OH	OH
Naringenin	H	OH	H	OH	H	H	H	OH	H
Naringin	H	OH	H	Rhamnoglucoside	H	H	H	OH	H
Apigenin	H	OH	H	OH	H	H	H	OH	H
Chrysin	H	OH	H	OH	H	H	H	H	H
Luteolin	H	OH	H	OH	H	H	OH	OH	H
Rutin	manno/gluco-pyranosyl	OH	H	OH	H	H	H	OH	OH
Baicalin	H	OH	OH	OH	H	H	H	H	H
Fisetin	OH	H	H	H	H	H	OH	OH	H
Galangin	OH	OH	H	OH	H	H	H	H	H
Kaempferol	OH	OH	H	OH	H	H	H	OH	H
Myricetin	OH	OH	H	OH	H	H	OH	OH	OH
Quercetin	OH	OH	H	OH	H	H	OH	OH	H
Genistein	H	OH	H	OH	H	H	H	OH	H

were dissolved in Dimethylsulfoxide (DMSO, DUCHEFA Biochemie, Netherlands) on the day of experiment and diluted with phosphate-buffered saline (PBS, Sigma, Poole, UK) into appropriate concentrations. Fetal bovine serum (FBS), penicillin-streptomycin, RPMI 1640, modified Eagle's medium (MEM) without phenol red were from Gibco BRL (Grand Island, NY, U.S.A.). Vero (African green monkey kidney cell line) cells were obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea) and the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). *Herpes simplex* virus type 1 (HSV-1 KOS strain) and type 2 (HSV-2 G strain) were obtained from the Department of Biological Sciences, Kon-Kuk University, Korea.

Cell culture

Vero cells were maintained in RPMI 1640 in 75 cm^2 plastic flasks (Falcon-Becton Dickinson Labwares, Franklin Lakes, NJ, U.S.A.) and were supplemented with 10% (v/v) heat-inactivated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at a temperature of 37°C in a 5% CO_2 humidified incubator (Sanyo, Japan).

Cell density determination

Cell numbers and viability were assessed by trypan blue (Sigma, Poole, UK) dye exclusion. The dye exclusion test is based on the concept that viable cells do not take up trypan blue dye, whereas dead cells are permeable to this dye. Twenty microliters of trypan blue solution was mixed with 20 μL of cell suspension in a microtube to obtain a final density about $0.3\text{-}2\times10^6$ cells/mL, and was loaded onto a hemocytometer. The cells that excluded the dye were counted in the standard manner within 1-5 min after mixing of the dye and cell suspension.

Virus amplification

Monolayers of Vero cells were incubated with viral suspension in RPMI medium containing 2% FBS at 37°C for 2 h. The culture medium was centrifuged at 3,000 rpm for 20 min, and the supernatant was harvested. Virus titers were determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, Poole, UK) assay and plaque assay in Vero cells and are expressed as 50% cell culture inhibitory dose ($CCID_{50}$) and plaque forming units (PFU) per mL. Viruses were stored at 70°C until used.

Cytotoxicity assay

In order to discern whether inhibition of proliferation was due to cytostatic or cytotoxic effect, the viability of Vero cell was measured 48 h post-treatment. The *in vitro* cytotoxic effect was measured by MTT assay colorimetric dye reduction method (Freshney, 1994). Cells (1×10^5 cells/mL) were seeded in 96 well flat-bottomed tissue culture plates (Falcon-Becton Dickinson Labwares, Franklin Lakes, NJ, U.S.A.) in the absence or presence of flavonoid at 37°C for 24 h in a 5% CO_2 humidified incubator. At the end of the incubation, 50 μL of filter sterilized MTT stock solution was added and the plate was incubated for a further 4 h. Dimethylsulfoxide (DMSO, DUCHEFA Biochemie, Netherlands) and Sörensen's buffer (0.1 M glycine, 0.1 M NaCl adjusted to pH 10.5 with 1 M NaOH) was added. The absorbance was detected using an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular devices, Menlo Park, CA, U.S.A.) at 570 nm. The cytotoxicity was measured as the 50% cytotoxic concentration (CC_{50}) which is the concentration of substances to inhibit the growth of cells up to 50% by regression analysis.

Cytopathic effect (CPE) reduction assay

MTT assay was used to determine the titer of the viruses. Vero cells (3×10^4 cells/well) were seeded into a 96-well plate. After one day of Incubation, a confluent monolayer was obtained. After washing the cells with phosphate buffer saline (PBS, Sigma, Poole, UK), 100 μL of tenfold-diluted concentrations of the virus solution, diluted with RPMI 1640 medium supplemented with 2% FBS which was equivalent to 50% cell culture inhibitory dose ($CCID_{50}$), was added to each well and incubated at 37°C for 1 h. Acyclovir (ACV) which is clinically used for the treatment of herpetic disease was used as a positive control. After absorption of the virus, the culture medium was removed and 100 μL of culture medium containing various concentrations of sample was added and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 48 h. After removing the culture medium, MTT assay was carried out as described before and $CCID_{50}$ was measured. The antiviral effective concentration was expressed as the 50% effective concentration (EC_{50}) which is the concentration of the sample required to inhibit virus-induced CPE by 50%. Mock-infected control cells were also prepared simultaneously. Antiviral activity was calculated as follows:

$OD_{(V)} / OD_{(M)} \times 100 = A$

(A: Percentage of the cell treated with the virus (virus control)).

$OD_{(V+S)} / OD_{(M)} \times 100 = B$

(B: Percentage of the cell treated with the virus and sample).

$B \times A = C$

(C: Different value between A and B).

$C / A \times 100 = D$

(D: Percentage of the inhibited cytopathic effect).

$OD_{(V)}$: Optical density of the cells, treated with the virus

(virus control).
$OD_{(M)}$: Optical density of the mock-infected cells only (cell control).
$OD_{(V+S)}$: Optical density of the cells, treated with the virus and samples.

Quantification of HSV for plaque reduction assay

In order to determine the titer of the virus, a plaque reduction assay was carried out as followed: Vero cells (1.5×10^5 cells/mL) were seeded on a 6 well tissue culture plate and after one day of incubation, a confluent monolayer was obtained. After washing the cells with PBS, 200 μL of tenfold-diluted concentrations of the virus solution was infected to each well. The virus was absorbed at 37°C for 1 h, and an agar overlay medium containing 2% FBS was overlaid. After 1-2 days of incubation at 37°C, the monolayers were fixed with 10% formaldehyde, and the nutrient agar was removed. The cells were stained with 1% solution of crystal violet in 70% ethanol. The plate was washed with distilled water, and PFU was determined.

Plaque reduction assay

Antiherpetic activity was evaluated by plaque reduction assay. Briefly, after removal of the cell growth medium, confluent 2-day-old cell monolayer in 6 well plates (Falcon-Becton Dickinson Labwares, Franklin Lakes, NJ, USA) were inoculated with the virus solution equivalent to 150 PFU in the absence or presence of various flavonoid concentrations. After 1 h adsorption, the inoculum was aspirated and 0.4% agar and appropriate concentrations of the flavonoids were added. Three untreated virus controls and one uninfected untreated cell control were included in all assays. Each compound was tested in triplicates. After 2 days of incubation at 37°C, the monolayers were fixed and stained with a solution of 0.4% crystal violet in a mixture of formalin (3% *v/v*) and ethanol (1.67% *v/v*) in distilled water for 24 h. Plaques were counted over a light box after removal of the agar overlay.

Yield reduction assay

Monolayers of Vero cells grown in 6-well plates (Falcon-Becton Dickinson Labwares, Franklin Lakes, NJ, U.S.A.) were infected by adsorption of HSV-1 at a multiplicity of infection (MOI) of 1 PFU per cell at 37°C for 1 h. Cells were washed with MEM containing 2% FBS, and various concentrations of flavonoids which showed strong antiviral activities were selected (ECG and galangin) and added immediately after adsorption. At 16 h after virus inoculation, cells were thoroughly washed with warm medium to remove the flavonoids. Cells in the culture medium were lysed by freezing and thawing (three times), and the supernatant consisting of culture medium and cell lysate was obtained by centrifugation at $400 \times g$ at 4°C for 10 min. Virus titer was determined by plaque forming assay in Vero cells as described above. Vero cells were also pre-incubated with 100 μM of ECG or 100 μM galangin at 37°C for 1 h before HSV-1 adsorption was performed.

RESULTS

Cytotoxic effects of flavonoids

The cytotoxic effect of flavonoids on Vero cells was investigated. The cytotoxicity was expressed as the CC_{50} which is the concentration of substances needed to inhibit the growth of cells up to 50% by regression analysis. When the cells were treated with flavonoids at various concentrations of 0-500 μM for 24 h, most of the flavonoids showed low cytotoxicity except chrysin (CC_{50} = 10 μM, 2.54 μg/mL) (Table II), which indicates that flavonoids did not affect the growth of Vero cells.

Inhibitory effects of flavonoids on the cytopathic effect (CPE)

We carried out a CPE inhibition assay to evaluate anti-herpetic activity and to determine the EC_{50} against HSV-1 and 2 for various flavonoids. The virus titer of HSV-1 and 2 were determined by MTT assay. A diluted HSV concentration which was equivalent to 50% cell culture inhibitory dose was used for seeding virus throughout the experiments. Table II shows the inhibitory effects on the CPE of various flavonoids. Among flavanols, EC and ECG showed a high level of CPE inhibitory activity (2.5 μM (0.725 μg/mL) and 5 μM (2.21 μg/mL), respectively), and among flavanone, naringenin expressed a strong inhibitory effect (5 μM (1.36 μg/mL)) against HSV-1. In addition, among flavonols, quercetin exhibited a high CPE inhibitory activity (5 μM (1.69 μg/mL)), and genistein which is an isoflavone also showed an inhibitory effect (5 μM (1.35 μg/mL)) against HSV-1. Also, EC, ECG, naringenin, quercetin, and genistein showed a potent inhibitory effect on virus-induced CPE (35 μM (10.15 μg/mL), 50 μM (13.51 μg/mL), 50 μM (13.61 μg/mL), 50 μM (22.12 μg/mL), and 50 μM (16.9 μg/mL), respectively) against HSV-2. Although galangin (flavonol) did not show any anti-herpetic activity against HSV-2, it exhibited potent anti-herpetic activity against HSV-1 as shown in Table II (2.5 μM (0.64 μg/mL)). All flavonoids tested exhibited more potent antiherpetic activity on HSV-1 than on HSV-2. Selectivity Index (SI) of the positive control (ACV) against HSV-1 was 10, whereas all of the flavonoids evaluated showed a value of more than 20. Especially, ECG, naringenin, and galangin showed a very high value of SI (150, 100, and 200, respectively) against HSV-1. ECG and naringenin also showed a high level of SI against HSV-2 (15 and 10, respectively).

Table II. CC_{50}, EC_{50}, and SI of flavonoids on Vero cells against HSV-1 and 2 determined by cytopathic effect (CPE) inhibition assay. Acyclovir (ACV) was used as a positive control

Flavonoids	Structure	Toxicity CC_{50} (μM)	Antiviral activity EC_{50} (μM)		Selectivity index (SI)	
			HSV-1	HSV-2	HSV-1	HSV-2
Acyclovir (ACV)	N/A	500	50	50	10	10
(+/-) Catechin hydrate	Flavanol	>1,000	4	N/A	250	N/A
EC	Flavanol	100	2.5	35	40	2.9
ECG	Flavanol	500	4	63	125	7.9
EGC	Flavanol	250	2.5	N/A	100	N/A
EGCG	Flavanol	100	2.5	N/A	40	N/A
Naringenin	Flavanone	750	4	22.5	187.5	33.3
Naringin	Flavanone	1,000	2.5	N/A	400	N/A
Apigenin	Flavone	250	5	N/A	50	N/A
Chrysin	Flavone	10	2.5	N/A	4	N/A
Luteolin	Flavone	100	5	N/A	20	N/A
Rutin	Flavone	10,000	5	N/A	2,000	N/A
Baicalin	Flavonol	1,000	5	N/A	200	N/A
Fisetin	Flavonol	100	2.5	N/A	40	N/A
Galangin	Flavonol	1,000	2.5	N/A	400	N/A
Kaempferol	Flavonol	50	15	N/A	3.3	N/A
Myricetin	Flavonol	100	5	N/A	20	N/A
Quercetin	Flavonol	100	5	35	20	2.9
Genistein	Isoflavone	250	5	50	50	5

CC_{50} is the 50% cytotoxic effect concentration.
EC_{50} is the 50% effective concentration.
Selectivity index (SI) = CC_{50}/EC_{50}.
HSV-1: herpes simplex virus type 1, KOS strain.
HSV-2: herpes simplex virus type 2, G strain.

Antiviral activities of flavonoids against HSV using plaque reduction assay

To investigate the reduction of virus-induced plaques, plaque reduction assay was performed with various flavonoids. A diluted appropriate cell culture inhibitory dose ($CCID_{50}$) was used for seeding viruses throughout the experiment. The inhibitory effects of plaque formation of HSV-1 and 2 in Vero cells by flavonoids are presented in Fig. 2 and Fig. 3. The degree of inhibition was determined as the most effective plaque reduced concentration, which was calculated as the concentration of substances required to reduce virus induced plaque regression analysis. As a result, among flavanols, EC, ECG showed a strong antiviral activity (EC; 80% inhibition at 5 μM, ECG; 85% inhibition at 10 μM), and among flavonols, galangin, and kaempferol showed potent antiviral activity (galangin; 80% inhibition at 10 μM, kaempferol; 90% inhibition at 50 μM) against HSV-1. Also, catechin, EGC, EGCG, naringenin, chrysin, baicalin, fisetin, myricetin, quercetin, and genistein showed moderate inhibitory effects, ranging from 50 to 80%. On the other hand, flavones including apigenin, luteolin and rutin showed 50% or lower inhibition indicating that these flavonoids have little effect on HSV-1. In addition, ECG alone showed a strong inhibitory effect at 100 μM against HSV-2. Overall flavonoids tested exhibited more potent antiherpetic activity on HSV-1 than on HSV-2.

Yield reduction assay

Flavonoids that showed strong antiviral activities were selected and a yield reduction assay was performed with these flavonoids. ECG and galangin showed a potent anti-herpetic activity in both CPE inhibition assay and plaque reduction assay, therefore these flavonoids were chosen. As shown in Fig. 4, yield reduction assays showed a dose-dependent antiviral activity with ECG and galangin against HSV-1. ECG showed a 78% inhibition at a concentration of 100 μM, and 52% inhibition at a concentration of 25 μM. Similarly, when galangin was treated, a 81% inhibition was observed at a concentration of 100 μM, and 45% inhibition was still observed at a concentration of 25 μM. When Vero cells were pre-incubated at 37°C for 1 h in the presence of ECG or galangin at a concentration

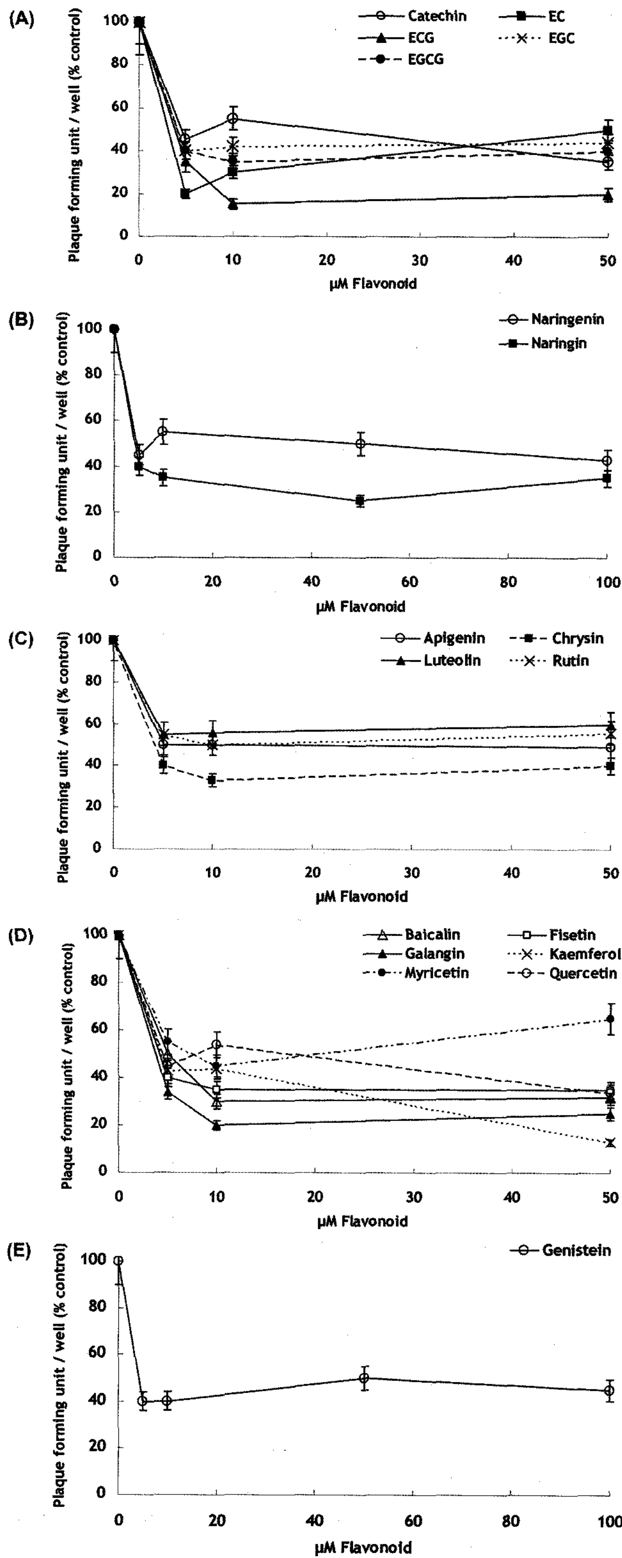

Fig. 2. Inhibitory effects of plaque formation of herpes simplex virus type 1 (HSV-1) in Vero cells by flavonoids (A: Flavanols, B: Flavanones, C: Flavones, D: Flavonols, E: Isoflavone). Results are expressed as percent with respect to virus control (VC) group. Each value is the mean ± S.D. of quadruplicate determination. The data represent the means for four replicate samples of three separate experiments.

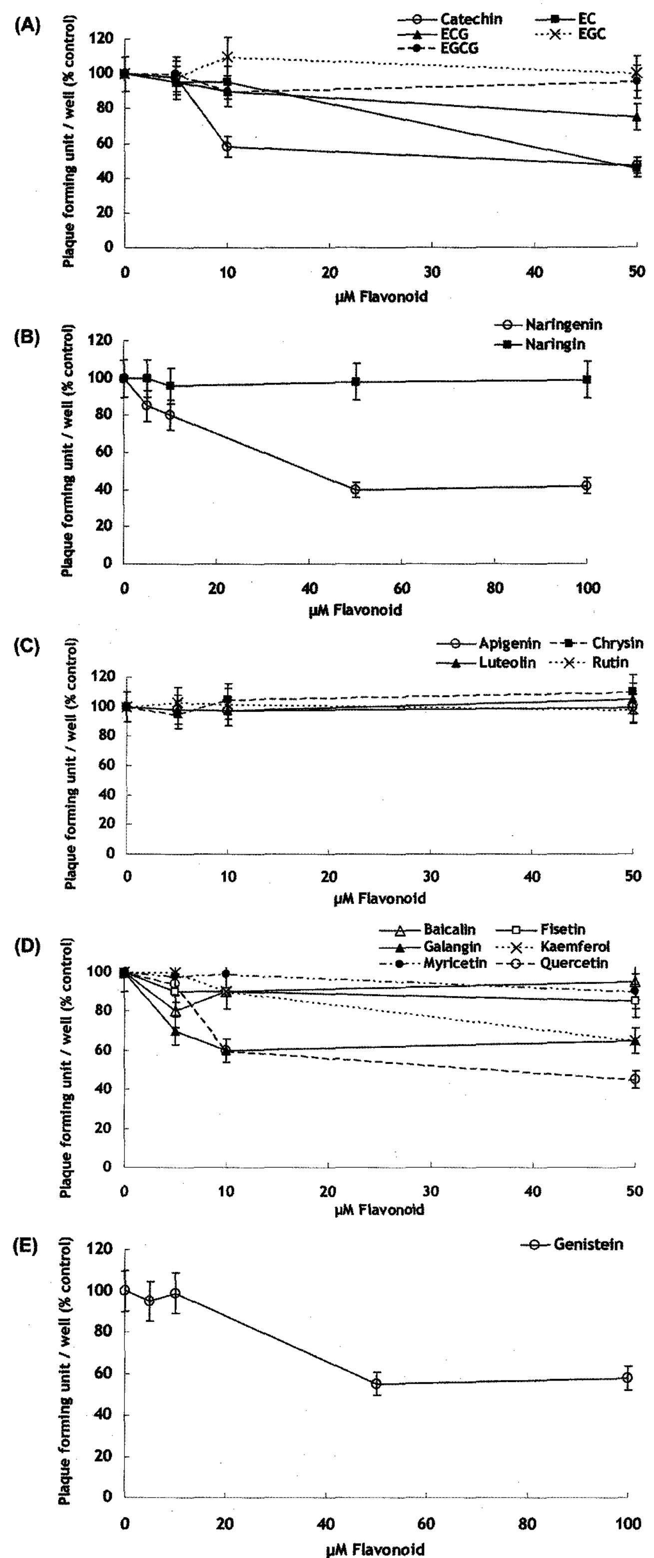

Fig. 3. Inhibitory effects of plaque formation of herpes simplex virus type 2 (HSV-2) in Vero cells by flavonoids (A: Flavanols, B: Flavanones, C: Flavones, D: Flavonols, E: Isoflavone). Results are expressed as percent with respect to virus control (VC) group. Each value is the mean ± S.D. of quadruplicate determination. The data represent the means for four replicate samples of three separate experiments.

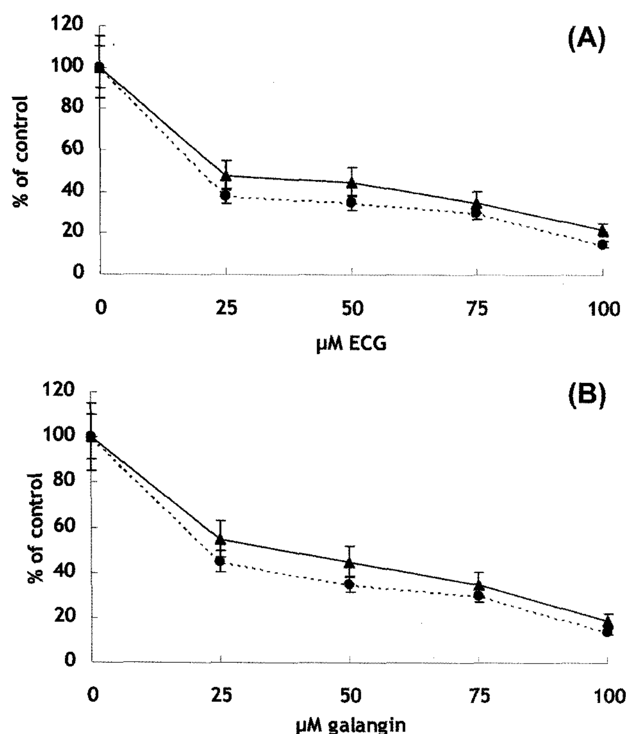

Fig. 4. Antiviral activities of ECG and galangin against HSV-1 as determined by yield reduction assay. Vero cells were infected by adsorption of HSV-1 (MOI 1) for 1 h, and then incubated with serial dilutions of flavonoids in RPMI 1640 for 24 h (-▲-). Alternatively, Vero cells were treated at 37°C for 1 h with (A) ECG or (B) galangin (100 µM) before virus adsorption (-●-), and then infected with HSV-1. After 16 h, the flavonoids were removed, and cells were lysed by three cycles of freezing and thawing, and HSV-1 titer was determined by plaque forming assay. The data represents the mean for four replicates of two separate experiments.

of 100 µM, the percent inhibition was slightly higher. With ECG, a 85% and 62% inhibition was observed at 100 µM and 25 µM, respectively, and with galangin, a 86% and 55% inhibition was observed at 100 µM and 25 µM, respectively.

DISCUSSION

HSV is responsible for a broad range of human infectious diseases. Moreover, HSV infections were reported to be a risk factor for human immunodeficiency virus (HIV) infection. HSV-2 is also known as oncogenic virus which converts cells into tumor cells. The drugs which has clinical effects against HSV includes interferons (IFNs), acyclovir (ACV), vidarabin (ara-A), and ganciclovir (DHPG). However, these drugs have some undesirable complications and also induces drug-resistance viruses (Park *et al.*, 1991).

Up to now, only a few studies have been reported in the antiviral activity of flavonoids. It is known that flavonoids, including quercetin and rutin has antiviral activities against rabies virus, ectromelia virus, and vaccine virus (Betancur-Galvis *et al.*, 2002). In addition, quercetin, morin, luteolin, and fisetin have antiviral activities against pseudorabies virus (Bourne *et al.*, 2000). Naringenin, kaempferol, quercetin, and galangin showed potent antiherpetic activity in Vero cells (Dargan and H., 1986). It is known that antiviral activity of flavonoid is due to the inhibition of reverse transcriptase (RT), inhibition of viral infections via attachment and penetration (Charles *et al.*, 2000), and augmentation of the degree of sulfation (Sarisky *et al.*, 2002).

In this study, we used the most abundant flavonoids in food for screening antiherpes activities including flavanols; (+/-) catechin hydrate, epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG), flavanones; naringenin, naringin, flavones; apigenin, luteolin, chrysin, rutin, isoflavone; genistein, and flavonols; baicalin, fisetin, myricetin, kaempferol, quercetin, galangin. This report clearly demonstrates the antiviral activity of flavonoids against HSV-1 and HSV *in vitro*.

The cytotoxicity of flavonoids on Vero cells were investigated and were expressed as CC_{50}. Most of the flavonoids showed low cytotoxicity (>50 µM) except chrysin, which indicates that flavonoids did not affect the growth of Vero cells. Thus, it seems unlikely that the antiviral effects were due to any cytotoxic effects of flavonoids.

When flavonoids were applied at various concentrations to Vero cells infected by HSV-1 and 2, most of the flavonoids showed inhibitory effects on virus-induced CPE. Among the flavonoids, EC, ECG (flavanols), genistein (isoflavone), naringenin (flavanone), and quercetin (flavonol) showed a high level of CPE inhibitory activity against HSV-1 and 2. On the other hand, galangin which is also a flavonol like quercetin, did not show any anti-herpetic activity against HSV-2, but it showed strong anti-herpetic activity against HSV-1 (Table II). In addition, while EC and ECG (flavanols) showed strong inhibitory activities on HSV-1 and 2, EGC and EGCG, which are also flavanols, did not show any antiherpetic activity. Overall, flavanols and flavonols appeared to be more active than flavones, and the flavonoids tested exhibited more potent antiherpetic activity on HSV-1 than on HSV-2.

Another important criterion for evaluating the antiviral activity is the SI which is the ratio between the 50% cytotoxic concentration and 50% effective concentration (CC_{50}/EC_{50}). If the SI value is more than 4, the compound is considered to have an antiviral effect. SI of the positive control (ACV) against HSV-1 was 10, whereas all of the flavonoids evaluated showed a value of more than 20.

The antiviral activity of flavonoids against HSV-1 and HSV-2 were also examined by a plaque reduction assay. Vero cells in 6-well culture plates were infected with

viruses of $CCID_{50}$ per well in the absence or presence of various concentrations of flavonoids. The degree of inhibition was determined as the most effective plaque reduced concentration, which was calculated as the concentration of substances required to reduce virus induced plaque regression analysis. As seen in Fig. 2 and 3, when HSV-1 and HSV-2 were exposed at 37°C for 1 h to the flavonoids before and during adsorption, Vero cells exhibited a concentration-dependent inhibition of plaque formation compared with the controls. EC, ECG, galangin, and kaempferol showed a strong antiviral activity against HSV-1, and catechin, EGC, EGCG, naringenin, chrysin, baicalin, fisetin, myricetin, quercetin, and genistein showed moderate inhibitory effects. Again, flavanols and flavonols appeared to be more active than flavones. All five flavanol compounds and all six flavonol compounds could reduce the virus plaque whereas among the flavones, only chrysin showed this capability. Since chrysin showed a high cytotoxicity on Vero cells (CC_{50} = 10 μM), the anti-herpetic activity may be due to the cytotoxic effect. The anti-HSV-1 activity of quercetin is controversial since it was found to have an inhibitory effect (Kaul *et al.*, 1985; Mucsi and Pragai, 1985; Wleklik *et al.*, 1988) or to be without any significant activity (Tsuchiya *et al.*, 1985). This discrepancy is probably due to obvious differences between the methodologies used, especially a different HSV- strain and a lower time of incubation (24 h to 48 h instead of 72 h). An oxidative degradation of quercetin in aqueous solution might explain the variations of the activity with the length of the experiment (Vrijsen *et al.*, 1988). Overall, these conclusions are supported by other experimental data (CPE reduction assay) showing that flavonoids have potent antiviral activity against HSV-1. Furthermore, ECG alone showed a strong inhibitory effect against HSV-2 indicating that most of the flavonoids tested exhibits more potent antiherpetic activity on HSV-1 than on HSV-2. In addition, treatment of Vero cells with ECG and galangin (which showed strong antiviral activities) before virus adsorption led to a slight enhancement of inhibition as determined by a yield reduction assay, indicating that an intracellular effect may also be involved.

Taken together, antiviral activities against HSV-1 and 2 by flavonoids were confirmed by CPE inhibitory assay, plaque reduction assay, and yield reduction assay. Among the flavonoids tested, fourteen flavonoids out of eighteen flavonoids possessed a potent anti-herpetic activity. Between flavones, only chrysin showed an antiviral activity, but this may be due to the cytotoxic effect of chrysin against Vero cells. This indicates that flavanols, flavanones, isoflavones, and flavonols are more active than flavones. The results encourages further exploration of the antiviral properties of flavonoids such as the exploration of the *in vivo* activity in animal models infected with HSV or studying the mechanism of various virus inhibition by looking into cell-to-cell spread by plaque development assay.

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