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In vitro antiviral activities of *Caesalpinia pulcherrima* and its related flavonoids

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The aim of this study was to search for new antiviral agents from Chinese herbal medicine. Pure flavonoids and aqueous extracts of *Caesalpinia pulcherrima* Swartz were used in experiments to test their influence on a series of viruses, namely herpesviruses (HSV-1, HSV-2) and adenoviruses (ADV-3, ADV-8, ADV-11). The EC_{50} was defined as the concentration required to achieve 50% protection against virus-induced cytopathic effects, and the selectivity index (SI) was determined as the ratio of CC_{50} (concentration of 50% cellular cytotoxicity) to EC_{50}. Results showed that aqueous extracts of *C. pulcherrima* and its related quercetin possessed a broad-spectrum antiviral activity. Among them, the strongest activities against ADV-8 were fruit and seed (EC_{50} = 41.2 mg/L, SI = 83.2), stem and leaf (EC_{50} = 61.8 mg/L, SI = 52.1) and flower (EC_{50} = 177.9 mg/L, SI = 15.5), whereas quercetin possessed the strongest anti-ADV-3 activity (EC_{50} = 24.3 mg/L, SI = 20.4). In conclusion, some compounds of *C. pulcherrima* which possess antiviral activities may be derived from the flavonoid of quercetin. The mode of action of quercetin against HSV-1 and ADV-3 was found to be at the early stage of multiplication and with SI values greater than 20, suggesting the potential use of this compound for treatment of the infection caused by these two viruses.

Keywords: HSV-1, HSV-2, ADV-3, ADV-8, ADV-11

Introduction

Many drugs have been approved by the US Food and Drug Administration for treatment of viral infections, of which most are synthetic nucleoside analogues. Resistance of virus to synthetic nucleoside analogues has been reported to develop *in vitro* and in *vivo*.[1] It is therefore necessary to find new alternative antiviral compounds. Adenoviral infections can occur throughout the year in all age groups and in many countries. Adenoviral pneumonia has been reported to result in a high mortality rate, especially in children of age below 2 years.[2] Topical 5-iodo-2-deoxyuridine has been used in the chemotherapy of ocular adenoviral infection.[3] Several investigators have reported that some modified nucleoside analogues or cysteine protease inhibitors are effective in inhibiting adenoviral infection *in vitro*.[4,5] However, there is no chemotherapy that has proven effective in preventing or interrupting this virus infection. In order to find more inhibitors for adenoviral infection, we have been looking for inhibitory substances from natural sources.[6] *Caesalpinia pulcherrima* Swartz (Leguminosae) is a common medicinal herb in Taiwan. The different parts of this herb have been used in common remedies for treatment of a number of disorders including pyrexia, menoxenia, wheezing, bronchitis and malarial infection.[7] A recent study of this folk remedy has shown that it possesses antibacterial and antifungal activities.[8] The flower of *C. pulcherrima* contains numerous compounds, such as lupeol, lupeol acetate, myricetin, quercetin and rutin.[9] Lupeol and quercetin have been reported to inhibit proliferation of *Plasmodium falciparum*.[10,11] There are several reports of the efficacy of quercetin against bacteria, fungi and viruses [human immunodeficiency virus (HIV), poliovirus, herpes simplex virus (HSV)], suggesting that it may be an effective antibiotic agent for *C. pulcherrima*.[12–16] Recently, rutin has also been found to inhibit multiplication of parasites, bacteria, fungi and viruses (rotavirus and HSV).[16–20] Some plant-derived flavonoids have been reported to possess activity against HSV[16,21] and in this study, we demonstrate the ability of some naturally occurring flavonoids from a Chinese herb, traditionally used in Chinese medicine, to inhibit the multiplication of HSV and adenoviruses.

Materials and methods

Extraction and purification of compounds

The different parts of *C. pulcherrima* were collected from the southern part of Taiwan. Their authenticity was confirmed by Professor

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Chun-Ching Lin (Graduate Institute of Natural Products, Kaohsiung Medical University) using morphological and anatomical techniques. A voucher specimen of the plant was deposited at the Herbarium of the Graduate Institute of Natural Products of Kaohsiung Medical University. A hot water extract of *C. pulcherrima* was prepared from three parts of the plant according to standard methods with minor modification as previously reported.[22] In brief, dried crude drugs (100 g) were boiled in 1000 mL of distilled water for 1 h, and the decoction obtained was then filtered through gauze. The same procedure was repeated three times. The aqueous extract of three successive extractions was collected, combined and concentrated under vacuum and then lyophilized. The crude dried extract was dissolved in distilled water and pure compounds were suspended in DMSO. Aciclovir, 2′,3′-dideoxycytidine (ddC), DMSO, quercetin (3,3′,4′,5,6-pentahydroxyflavone), rutin (quercetin-3-rutinoside) and cell culture medium RPMI 1640 were purchased from Sigma Chemical Co. XTT (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-5-[(phenylamino)carbonyl-2H-tetrazolium hydroxide]) kits were obtained from Roche Diagnostics GmbH.

Virus and cells

Human skin basal cell carcinoma cell line (BCC-1/KMC), which was established in our laboratory,[23] was used to provide target cells for virus infection in the XTT assay. It was derived from undifferentiated carcinoma cells and grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin G, 100 mg/L streptomycin and 0.25 mg/L amphotericin B. In the antiviral assay, the medium was supplemented with 2% FCS and the above mentioned antibiotics.

The strain of HSV type 1 (HSV-1 strain KOS) used in this study was obtained from the American Type Culture Collection (ATCC), Rockville, USA. HSV-2 strain 196 was kindly provided by Professor W. T. Liu, School of Medical Technology, National Yang-Ming Medical University. The clinical isolates of adenovirus (ADV), ADV-3, ADV-8 and ADV-11, were provided by Dr K. H. Lin, Kaohsiung Medical University Hospital. HSV and ADV were propagated in BCC-1/KMC cells. Virus titres were determined by cytopathic effects in BCC-1/KMC cells and were expressed as 50% tissue culture infective dose ($TCID_{50}$) per mL. All viruses were stored at –70°C until use.

Cytotoxicity

The BCC-1/KMC cells were seeded onto a 96-well plate at a concentration of 1.0×10^5 cells/mL and a volume of 90 µL per well. Different concentrations of crude aqueous extract or pure compounds were applied to culture wells in triplicate. DMSO was used as a negative control. After incubation at 37°C with 5% CO_2 for 3 days, a mixture of 0.1 mL phenazine methosulphate (PMS; electron-coupling reagent) and XTT 1 mg/mL was added to each well with a volume of 50 µL. The trays were further incubated for 2 h to allow XTT formazan production. The absorbances were determined with an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 450 nm and a reference wavelength of 690 nm. Data were calculated as the percentage of inhibition using the following formula: inhibition % = $[100 - (A_t/A_s) \times 100]$%. A_t and A_s refer to the absorbances of the test substances and the solvent control, respectively. The concentration of 50% cellular cytotoxicity (CC_{50}) of test substances was calculated according to Chiang *et al.*[6]

Antiviral assay using XTT method

A sensitive and accurate method for rapid screening of antiviral agents, an automatic XTT tetrazolium-based colorimetric assay, was developed in 1989.[24]

The antiviral activity of *C. pulcherrima* and related flavonoids against HSV-1, HSV-2, ADV-3, ADV-8 and ADV-11 viruses was evaluated by the XTT method.[4,6] BCC-1/KMC cells, treated with trypsin, were seeded onto 96-well plates with a concentration of 1.0×10^5 cells/mL and a volume of 70 µL per well. After incubation at 37°C with 5% CO_2 for 6 h, 20 µL of test virus was added and incubated for another 2 h. Different concentrations of test substances were then added to culture wells in triplicate. The maximum concentration of DMSO (0.1%) was used as a negative control. Aciclovir and ddC were used as a positive control for HSV and ADV assays, respectively. After incubation at 37°C with 5% CO_2 for 3 days, the XTT test was carried out as previously described. Viral inhibition rate was calculated as $(A_{tv} - A_{cv})/(A_{cd} - A_{cv}) \times 100\%$. A_{tv} indicates the absorbance of the test compounds with virus infected cells. A_{cv} and A_{cd} indicate the absorbance of the virus control and the absorbance of the cell control, respectively. The antiviral concentration of 50% effectiveness (EC_{50}) was defined as the concentration which achieved 50% inhibition of virus-induced cytopathic effects. The amount of virus used in each experiment was based on infected target cells of 20–200 $TCID_{50}$ (MOI of 0.002–0.025) of HSV or ADV to produce 50% XTT formazan products as in uninfected control cells.

Dose–response

HSV-1 (25 $TCID_{50}$ per well) or ADV-3 (120 $TCID_{50}$ per well) was absorbed onto confluent monolayers of BCC-1/KMC cells for 2 h. Different concentrations of quercetin were added to culture cells in triplicate at 0, 1 or 2 h after virus infection. After 3 days, XTT test and antiviral activity were carried out as previously described.

Time course

Various concentrations of quercetin were added to culture cells in triplicate at different times pre-infection or post-infection. HSV-1 (25 $TCID_{50}$ per well) or ADV-3 (120 $TCID_{50}$ per well) was inoculated onto confluent monolayers of BCC-1/KMC cells for 2 h. After 3 days, XTT test and antiviral activity were carried out as previously described.

Statistical analysis

The selectivity index (SI) was determined as the ratio of CC_{50} to EC_{50}. The statistically different effects of test compounds on the inhibition of HSV or ADV replication were compared with the control group or compared between different extracts using the Student's *t*-test. The dose-dependent effect of antiviral activity of quercetin was determined by linear regression.

Results

Assessment of anti-HSV activity

Table 1 shows the anti-HSV activity of crude aqueous extracts and flavonoids of *C. pulcherrima.* With the exception of rutin, aqueous extracts of *C. pulcherrima* and quercetin were found to exhibit anti-HSV activity. Among the different parts of this medicinal herb tested, the flower's extract appeared to possess the strongest anti-HSV activity ($P < 0.05$). Quercetin was active against multiplication

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Table 1. Assessment of anti-herpetic activity of *Caesalpinia pulcherrima*

		HSV-1		HSV-2	
Test drug	CC_{50}[a]	EC_{50}[b]	SI[c]	EC_{50}	SI
Aciclovir	126.8	2.8 ± 0.1	45.1	2.2 ± 0.1	58.0
Flower	2751.0	166.8 ± 14.9*,**	16.5	193.1 ± 35.2**	14.2
Stem & leaf	3219.0	202.8 ± 7.7***	16.0	203.1 ± 8.0	15.8
Fruit & seed	3431.0	239.7 ± 15.9	14.3	247.6 ± 13.2	13.9
Quercetin	496.9	22.6 ± 4.2	22.0	86.7 ± 7.4	5.7

[a]The 50% cytotoxic concentration for target cells (BCC-1/KMC) in mg/L.
[b]Concentration of compound in mg/L producing 50% inhibition of virus-induced cytopathic effects of three separate experiments.
[c]Selectivity index (SI) = CC_{50}/EC_{50}.
*Comparison between flower and stem & leaf ($P < 0.05$).
**Comparison between flower and fruit & seed ($P < 0.05$).
***Comparison between stem & leaf and fruit & seed ($P < 0.05$).

Table 2. Assessment of anti-adenoviral activity of *Caesalpinia pulcherrima*

		ADV-3		ADV-8		ADV-11	
Test drug	CC_{50}[a]	EC_{50}[b]	SI[c]	EC_{50}	SI	EC_{50}	SI
ddC	259.2	7.5 ± 0.6	34.6	10.2 ± 1.6	25.3	14.2 ± 1.3	18.3
Flower	2751.0	343.9 ± 52.2*,**	8.0	177.9 ± 52.2*,**	15.5	>1000[d]	
Stem & leaf	3219.0	436.8 ± 19.9***	7.4	61.8 ± 2.3	52.1	>1000	
Fruit & seed	3431.0	483.0 ± 17.0	7.1	41.2 ± 15.5	83.2	>1000	
Quercetin	496.9	24.3 ± 4.9	20.4	39.9 ± 5.4	12.5	44.8 ± 9.4	11.1

[a]The 50% cytotoxic concentration for target cells (BCC-1/KMC) in mg/L.
[b]Concentration of compound (mg/L) producing 50% inhibition of virus-induced cytopathic effects of three separate experiments.
[c]Selectivity index (SI) = CC_{50}/EC_{50}.
[d]Maximum concentration of compound to test did not find EC_{50}.
*Comparison between flower and stem & leaf ($P < 0.05$).
**Comparison between flower and fruit & seed ($P < 0.05$).
***Comparison between stem & leaf and fruit & seed ($P < 0.05$).

of both types of HSV but showed a lower activity in inhibiting HSV-2 replication ($P < 0.05$).

Assessment of anti-adenoviral activity

Table 2 shows the anti-adenoviral activity of crude aqueous extracts and flavonoids of *C. pulcherrima*. With the exception of ADV-11, aqueous extracts of different parts of this folk medicine were active against ADV-3 and ADV-8 replication. Interestingly, among extracts of three parts of the herb tested, all showed the strongest activity against ADV-8, especially fruit and seed, and stem and leaf. Quercetin was found to possess anti-adenoviral activity to inhibit all three viral types with an inhibitory effect (EC_{50}) in the range of 24.3–44.8 mg/L.

Dose–effect of quercetin

In order to confirm the direct activity against virus multiplication, a study was conducted to analyse the dose-dependent effect at three time intervals after viral infection at various concentrations of quercetin. The results showed that quercetin at concentrations between 1 and 60 mg/L exhibited a high correlation between drug concentration and inhibition rate [correlation coefficient (r) > 0.86] (Figure 1).

Time course of quercetin

In order to investigate the mechanism of how quercetin inhibits the infection of herpesviruses and adenoviruses, a study was conducted to investigate the time-course effect at 1 h before and 24 h after the virus infection and of treatment with various doses of quercetin. The results showed that quercetin at concentrations ≥20 mg/L exhibited the greatest inhibition against HSV-1 infection from 0 to 2 h, which was during the early period of virus replication (Figure 2). However, the inhibitory effect of quercetin on ADV-3 infection occurred between 0 and 4 h (Figure 3).

Discussion

The present study has demonstrated that *C. pulcherrima* aqueous extract and its related flavonoid quercetin possess antiviral activity *in vitro*. The antiviral activity of crude drugs from this common Chinese

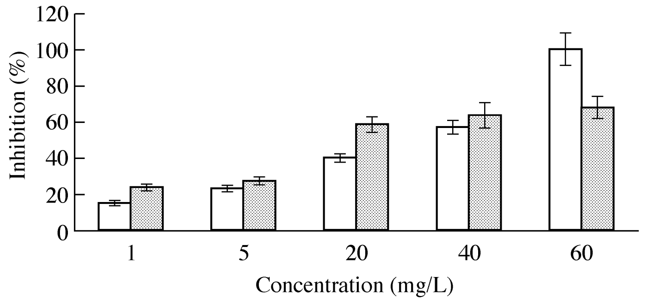

Figure 1. Dose-dependent effect of antiviral activity induced by quercetin. Different concentrations of quercetin were added 1 h after infection of herpesvirus (HSV-1, white bars) or adenovirus (ADV-3, grey bars) to BCC-1/KMC cells at 37°C. After 3 days, inhibition was evaluated by XTT method and expressed as the inhibition rate. The *x*-axis indicates the concentration of quercetin. Each bar represents the mean ± S.E.M. of triplicate samples of three independent experiments. The correlation coefficient (*r*) values from linear regression for HSV-1 and ADV-3 were 0.87 and 0.89, respectively.

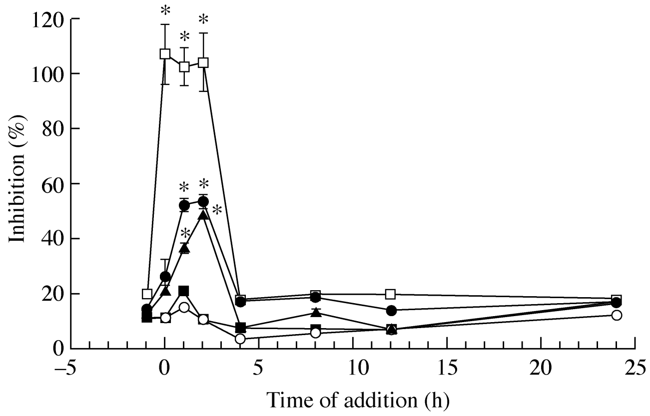

Figure 2. Inhibitory effect of adding quercetin at various times pre-infection or post-infection of herpesvirus (HSV-1) to BCC-1/KMC cells. Different concentrations of quercetin [1 mg/L (open circles), 5 mg/L (filled squares), 20 mg/L (filled triangles), 40 mg/L (filled circles), 60 mg/L (open squares)] were added at various times pre-infection (–1 h), co-infection (0 h) or post-infection (1–24 h) of herpesvirus (HSV-1) to BCC-1/KMC cells at 37°C. After 3 days, inhibition was evaluated by XTT method and expressed as the inhibition rate. The *x*-axis indicates the time course of adding quercetin. Each point represents the mean ± S.E.M. of triplicate samples of three independent experiments. The asterisk indicates a significant difference between test and DMSO control ($P < 0.01$).

medicinal herb was more potent and of a broader spectrum than found in our previous reports.[25,26] According to previous reports, one study showed that rutin was not active against HSV-1,[27] whereas the other demonstrated its positive anti-HSV activity.[16] The results of this study did not confirm the anti-HSV activity of rutin. The difference in results between those studies might be due to the use of different strains of virus.

Quercetin, 3,3′,4′,5,7-pentahydroxy flavone, is one of most widely distributed bioflavonoids in the plant kingdom and is a common constituent of most edible fruits and vegetables. The flower of *C. pulcherrima* also contains quercetin and quercetin-3-rutinoside (rutin).[9] Previous reports of the anti-infective activity of quercetin and rutin showed that they are active against bacteria, fungi, parasites and viruses, suggesting that they may be effective antibiotic agents for *C. pulcherrima.*[12–20] However, our results showed that quercetin possessed a broad spectrum of antiviral activities, whereas rutin did not express the same activity (Tables 1 and 2).

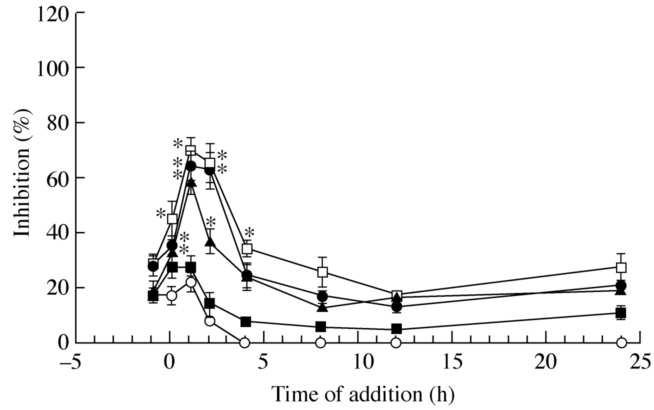

Figure 3. Inhibitory effect of adding quercetin at various times pre-infection or post-infection of adenovirus (ADV-3) to BCC-1/KMC cells. Different concentrations of quercetin [1 mg/L (open circles), 5 mg/L (filled squares), 20 mg/L (filled triangles), 40 mg/L (filled circles), 60 mg/L (open squares)] were added at various times pre-infection(–1 h), co-infection (0 h) or post-infection (1–24 h) of adenovirus(ADV-3) to BCC-1/KMC cells at 37°C. After 3 days, inhibition was evaluated by XTT method and expressed as the inhibition rate. The *x*-axis indicates the time course of adding quercetin. Each point represents the mean ± S.E.M. of triplicate samples of three independent experiments. The asterisk indicates a significant difference between test and DMSO control ($P < 0.01$).

Despite the great advances in the synthetic nucleoside analogues or cysteine protease inhibitors for anti-adenoviral replication, currently there is no proven chemotherapy treatment that interrupts this viral infection.[4,5] New medications such as cidofovir, which is a broad-spectrum nucleoside monophosphate, appear to be effective against the adenoviruses in non-human systems and may have some effect in man.[28,29] However, resistance of adenovirus to cidofovir treatment has also been reported.[30]

Although 5-iodo-deoxyuridine has been clinically applied in treating adenoviral ocular infection,[3] it was found to be quite toxic as its SI value was only 3.4.[4] A popular anti-adenovirus plant drug, ternatin, was reported to have an SI value of 20.[31] Our study shows that three crude drugs from *C. pulcherrima* including flower, stem and leaf, and fruit and seed possess anti-adenoviral activity; the strongest anti-ADV-3 activity was flower with an SI value of 8; strongest anti-ADV-8 activities were stem and leaf, and fruit and seed with SI values of 52.1 and 83.2, respectively. However, quercetin exhibited a broad-spectrum antiviral activity of anti-ADV-3, anti-ADV-8 and anti-ADV-11 with SI values 20.4, 12.5 and 11.1, respectively (Table 2). These findings indicate that the effect of these drugs on adenoviruses is worthy of further investigation to find more potent natural components from this medicinal herb to treat this virus infection.

This study has shown that quercetin possesses broad-spectrum antiviral activities. In order to understand how quercetin inhibits viral replication, dose-dependent and time-course studies of this compound were carried out. Interestingly, quercetin was found to inhibit HSV-1 replication in an obvious dose-dependent manner with EC_{50} 22.6 mg/L and 100% inhibition at concentration 60 mg/L (Figure 2). Quercetin showed that it inhibited ADV-3 multiplication with a similar dose-dependent effect with EC_{50} 24.3 mg/L but a 70% inhibition at concentration 60 mg/L (Figure 3).

According to the results of the time-course study, quercetin was found to possess a similar trend of inhibition of herpesvirus and adenovirus replication. This suggests that the mode of action is not derived from inhibiting the absorption of virus but results from

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inhibition at an early stage of viral replication after infection (Figures 2 and 3).

Among the flavonoids tested, only quercetin possessed significant activity against human herpesviruses and adenoviruses. According to a previous report, rutin (quercetin-3-rutinoside) did not express antiviral activity whereas quercitrin (quercetin-3-rhamnoside) possessed similar activity to quercetin.[25] Therefore, the antiviral activity among the flavonoid glycosides containing the quercetin moiety might be correlated with the species of sugar group at the 3 position.

The present study concludes that *C. pulcherrima*, a herb used in traditional Chinese medicine, and the related quercetin, exhibited potent anti-HSV and -ADV activities. Among them, the crude drugs, namely stem & leaf and fruit & seed, and quercetin, were found to possess the strongest anti-adenoviral activity. As a result of the lack of approved drugs in treating adenoviral infection, these crude drugs and quercetin might be potential therapeutic agents for treating this disease. As indicated by the high SI value ranging between 7.1 and 83.2, these candidate drugs are considered to be less toxic than the current clinically used drug, 5-iodo-deoxyuridine (SI = 3.4). Therefore, the potential of these crude drugs and quercetin for use in treating adenoviral infection merits greater attention.

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