

Antiviral Activity of Eight Commonly Used Medicinal Plants in Taiwan

Lien-Chai Chiang,* Hua-Yew Cheng,† Mei-Chi Liu,*
Wen Chiang‡ and Chun-Ching Lin†

*Department of Microbiology, College of Medicine

†Graduate Institute of Pharmaceutical Sciences, College of Pharmacy, and

‡Department of Clinical Pathology, College of Medicine
Kaohsiung Medical University, Kaohsiung, Taiwan

Abstract: In an effort to find new antiviral agents from natural products, hot water extracts of eight traditionally used medicinal plants in Taiwan were investigated *in vitro* for their activities against adenoviruses (ADV) and herpes simplex viruses (HSV). Results demonstrated that all extracts exhibited antiviral activity with different degrees of potency. Only two extracts were active in suppressing both HSV and ADV infections. Three extracts inhibited only ADV infection whereas one extract blocked only HSV infection. These results suggested that the aforementioned medicinal plants merit further investigation.

Keywords: Medicinal Plant; Anti-HSV Activity; Anti-ADV Activity; Hot Water Extract.

Introduction

Adenoviruses (ADV) are ubiquitous agents and are associated with a wide range of illnesses. They cause ocular, respiratory, gastrointestinal and urinary infections. Usually, ADV infections are mild and resolve without special chemotherapy. However, severe ADV infections have been reported in immunocompromised patients, including patients with leukemia (Zahradnik *et al.*, 1980), AIDS (De Jong *et al.*, 1983), or organ transplantation (Stalder *et al.*, 1977). Also, ADV can cause pneumonia and has been reported to have considerable mortality rate especially in children of age below two years old (Avila *et al.*, 1989; Dudding *et al.*, 1972). Although 5-iodo-2'-deoxyuridine (IDU) and ganciclovir have been used in the chemotherapy of ADV infection, however currently no approved drug is effective in preventing or interrupting ADV infection.

Herpes simplex virus (HSV) is also an ubiquitous agent that causes a variety of diseases in humans ranging in severity from mild to severe, and in certain cases, it may be life threatening. According to an epidemiological survey reported in 1994, the age-adjusted seroprevalence of herpes simplex virus type-2 (HSV-2) in the United States was 20.8% (Fleming *et al.*, 1997). After primary infection, HSV remains latent in the infected hosts for a lifetime. Although some approved nucleoside-based drugs are effective in HSV therapy, they nevertheless fail to modulate the recurrence of the latent virus and become ineffective when resistant mutations occur (Coen, 1994).

Medicinal plants have been used traditionally for treatment of different kinds of ailments, including infectious diseases. Some of them are reported to exhibit antiviral activities (Kaij-a-Kamb *et al.*, 1992; Kurokawa *et al.*, 1993; Vlietinck and Vanden Berghe, 1991). Cragg and his colleagues have pointed out that approximately 60% of the anti-tumor and anti-infective agents that are commercially available or in the late stages of clinical trials today are of natural product origin (Cragg *et al.*, 1997). There is no doubt that traditional medicinal plants can serve as a potential resource in the development of new antiviral agents in future. Since current chemotherapy agents for ADV and HSV infections are few in quantity with limited efficiency, there is a need to search new and more effective antiviral agents for treatment of ADV and HSV infections.

In this study, hot water extracts from eight commonly used medicinal plants in Taiwan were investigated *in vitro* for their anti-HSV and anti-ADV activities. This is the first report on anti-HSV and anti-ADV activities of the hot water extracts of these eight medicinal plants.

Materials and Methods

Plant Materials

The seed and fruit of *Adenanthera pavonia* Linn., the seeds of *Arachis hypogaea* Linn. and *Pisum sativum* Linn., the stem and leaf of *Bauhinia purpurea* Linn. and *Bauhinia variegata* Linn., and the whole plant of *Desmodium caudatum* (Thunb.) DC., *Desmodium triflorum* (Linn.) DC. and *Glycine max* Merr. were collected from the southern regions of Taiwan. Their authenticities were identified and confirmed using morphological and anatomical techniques by Professor C. C. Lin (Graduate Institute of Natural Products, Kaohsiung Medical University, Taiwan). A voucher specimen of the plants was deposited at the Herbarium of the Graduate Institute of Natural Products of Kaohsiung Medical University, Taiwan.

Preparation of the Extracts

Hot water extracts of the medicinal plants were prepared according to the procedures as described earlier by Chang and Yeung with minor modifications (Chang and Yeung, 1988). Briefly, different parts of the medicinal plants were boiled with 1000 ml distilled water for 1 hour. The aqueous was collected and the residual was extracted again with another 1000 ml distilled water. The resulting aqueous extracts were collected, combined, filtered by gauze, concentrated under reduced pressure and then lyophilized to dry.

Acyclovir (ACV) and 2', 3'-dideoxycytidine (ddC) were purchased from Sigma Chemical Co. (USA). The hot water extracts were dissolved in sterile distilled water whereas ACV and ddC were suspended in dimethyl-sulfoxide (DMSO).

Cell and Viruses

The human skin basal cell carcinoma cell line (BCC-1/KMC), established in our laboratory (Chiang *et al.*, 1994), was used as target cells for virus infection. It was derived from undifferentiated carcinoma cells and grown as adherent cells in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin G, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. In the antiviral assay, the medium was supplemented with 2% FCS and the above-mentioned antibiotics. All reagents and mediums used for cell culture were purchased from Gibco BRL (Grand Island, New York).

HSV-1 KOS strain was obtained from the American Type Culture Collection (ATCC), Rockville, USA, and HSV-2 196 strain was provided by Professor W. T. Liu (School of Medical Technology, National Yang-Ming Medical University, Taipei, Taiwan). ADV-3, ADV-8 and ADV-11 were provided by Dr. K. H. Lin (Hospital of Kaohsiung Medical University, Kaohsiung, Taiwan). All viruses were prepared and quantitated on BCC-1/KMC cells and stored in small aliquots at -70°C until use.

Titration of Viruses

BCC-1/KMC cell was seeded in 96-well culture plates at a density of 10^4 cells/well and then incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 24 hours. A serial dilution of virus stock was prepared, and the cells were infected with the dilution of virus. After an additional 72 hours of incubation, the cytopathic effect was recorded. The 50% tissue culture infective dose (TCID₅₀) per ml was calculated as described previously by Reed and Muench (Reed and Muench, 1938).

Antiviral Assay Using XTT Method

The antiviral activity of hot water extracts was evaluated by the XTT method as described previously (Chiang *et al.*, 2002; Weislow *et al.*, 1989). BCC-1/KMC cells, treated by trypsin, were seeded in 96-well culture plates at a volume of 70 µl/well and a concentration of 10^5 cells/ml. After a 24-hour incubation, 20 µl of viruses was added and the infected cells were incubated for another 2 hours. Ten µl tested compound at different concentrations was then added to culture wells in triplicate. The final maximum concentration for DMSO in culture medium was 0.1%. After further incubation at 37°C with 5% CO₂ for 72 hours, the mixture of 0.1 ml PMS (N-methyl dibenzopyrazine methyl sulfate) and 5 mg/5 ml XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) were added to each well to a volume of 50 µl. The trays were re-incubated for an additional 2 hours to allow the production of formazan. Optical densities

were determined with the ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 450 nm and a reference wavelength of 690 nm.

Viral inhibition rate was calculated as $[(OD_{tv} - OD_{cv}) / (OD_{cd} - OD_{cv})] \times 100\%$. OD_{tv} , OD_{cv} and OD_{cd} indicate the absorbance of the test compounds with virus-infected cells, 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 at which 50% cytoprotection level against virus infection was achieved.

Cell Cytotoxic Effect

The cell cytotoxic effect of tested compounds was evaluated with the XTT-based method. It was performed according to the procedures as described above with no virus added. Cell cytotoxic effect of each tested compound was calculated using the following formula: Percent of cell cytotoxic effect = $[1 - (OD_t / OD_s)] \times 100\%$. OD_t and OD_s indicate the absorbance of the test substances and the solvent control, respectively. The concentration of 50% cellular cytotoxicity (CC_{50}) of tested compounds was calculated according to Chiang *et al.* (Chiang *et al.*, 2002).

Statistical Analysis

Data were calculated as mean \pm standard error for three separate experiments. The selectivity index (SI) was calculated 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 by using the Student's t-test.

Results

Antiviral Activity of Eight Medicinal Plants of Taiwan

Results showed that the hot water extracts of eight traditionally used medicinal plants in Taiwan exhibited anti-HSV and anti-ADV activities *in vitro* with different degrees of potency (Table 1). ACV and ddC were served as reference compounds for anti-HSV and anti-ADV assays, respectively.

In the anti-HSV-1 assay, it was observed that *B. variegata* was the most effective agent among the eight extracts in blocking HSV-1 infection. The EC_{50} value of *B. variegata* extract was 182.7 ± 6.3 $\mu\text{g/ml}$. Other extracts possessed EC_{50} values in the range of 290–490 $\mu\text{g/ml}$. The EC_{50} values of *A. pavonia* and *A. hypogaea* extracts were more than 500 $\mu\text{g/ml}$.

In the anti-HSV-2 assay, *A. hypogaea* and *B. variegata* were the two extracts that possessed EC_{50} below 200 $\mu\text{g/ml}$. The anti-HSV-2 activities of *A. pavonia*, *B. purpurea*, *D. caudatum*, *D. triflorum*, *G. max* and *P. sativum* extracts were considered weak with EC_{50} values > 500, 448.7 ± 27.4 , 377.8 ± 17.8 , 496.6 ± 33.8 , 447.3 ± 12.2 and > 500 $\mu\text{g/ml}$, respectively.

The anti-ADV-3 activities of *P. sativum* and *B. variegata* extracts were more potent than the other six extracts with EC_{50} values of 143.1 ± 26.1 and 190.1 ± 8.9 $\mu\text{g/ml}$,

Table 1. Antiviral Activity of Eight Medicinal Plants of Taiwan *In Vitro*

Compounds	Used Part	EC ₅₀ (µg/ml)*				
		Herpes Simplex Viruses		Adenoviruses		
		HSV-1	HSV-2	ADV-3	ADV-8	ADV-11
ACV		2.8 ± 0.1	2.2 ± 0.1	ND	ND	ND
ddC		ND	ND	7.5 ± 0.6	10.2 ± 1.6	13.3 ± 1.2
<i>A. pavonia</i>	Seed and fruit	> 500.0	>500.0	273.9 ± 5.7	116.2 ± 2.5	> 500.0
<i>A. hypogaea</i>	Seed	> 500.0	191.1 ± 20.4	> 500.0	> 500.0	> 500.0
<i>B. purpurea</i>	Stem and leaf	382.5 ± 15.7	448.7 ± 27.4	> 500.0	> 500.0	> 500.0
<i>B. variegata</i>	Stem and leaf	182.7 ± 6.3	117.1 ± 13.6	190.1 ± 8.9	118.1 ± 10.0	> 500.0
<i>D. caudatum</i>	Whole plant	289.6 ± 6.4	377.8 ± 17.8	335.0 ± 27.1	118.8 ± 4.4	> 500.0
<i>D. triflorum</i>	Whole plant	465.2 ± 16.8	496.6 ± 33.8	> 500.0	> 500.0	260.9 ± 13.7
<i>G. max</i>	Whole plant	> 500.0	447.3 ± 12.2	> 500.0	> 500.0	> 500.0
<i>P. sativum</i>	Seed	490.0 ± 62.8	> 500.0	143.1 ± 26.1	87.9 ± 11.1	463.9 ± 30.0

*Concentration that inhibited 50% virus infection.

ACV: Acyclovir, ddC: 2',3'-dideoxycytidine.

Each value represents the mean ± SE of three separate experiments.

ND: Not done.

respectively. The EC₅₀ values for *A. pavonia* and *D. caudatum* extracts were 273.9 ± 5.7 and 335.0 ± 27.1 µg/ml, respectively. Other four extracts showed EC₅₀ values of more than 500 µg/ml.

When tested for anti-ADV-8 activity, four hot water extracts possessed EC₅₀ below 120 µg/ml, whereas the other four extracts had EC₅₀ value higher than 500 µg/ml. The noteworthy tested compound was *P. sativum* extract. *P. sativum* extract was considered to exhibit strong anti-ADV-8 activity with an EC₅₀ value of 87.9 ± 11.1 µg/ml.

Besides ADV-3 and ADV-8, the extracts were also tested for their anti-ADV-11 activity. Only *D. triflorum* was considered to exhibit mild anti-ADV-11 activity with EC₅₀ value of 260.9 ± 13.7 µg/ml. The other seven extracts had EC₅₀ values higher than 450 µg/ml. In general, the tested extracts demonstrated weak in activity to inhibit ADV-11 infection.

Cell Cytotoxic Effect and Selectivity Index of Eight Medicinal Plants of Taiwan

The cell cytotoxic effect of eight tested hot water extracts against BCC-1/KMC cells was shown in Table 2. Overall, all tested hot water extracts showed CC₅₀ values higher than its EC₅₀ values. The CC₅₀ values were ranging from 1328 to 4388 µg/ml.

With the EC₅₀ and CC₅₀ data, the SI of each extract in related assays was calculated by dividing the CC₅₀ with EC₅₀. In the anti-HSV-1 assay, the SI values were in the range of 4.5–9.0 whereas in the anti-HSV-2 assay, they were in the range of 3.0–18.3. For anti-ADV-3, ADV-8 and ADV-11 assays, the SI values were ranging from 4.2 to 30.7, 11.5 to 49.9 and 8.0 to 9.5, respectively.

The most interesting observation in SI value evaluation was the SI value of *P. sativum* in anti-ADV-8 assessment. *P. sativum* had higher SI value (49.9) than that of ddC (25.3). Other

noteworthy SI values were *A. pavonia* in anti-ADV-8 assay, *A. hypogaea* in anti-HSV-2 assay, *B. variegata* in anti-HSV-2 and anti-ADV-8 assays, *D. caudatum* in anti-ADV-8 assay, and *P. sativum* in anti-ADV-3 assay. Here, SI values for all mentioned extracts in related assays were higher than 10.0.

Table 2. Cell Cytotoxic Effect and Selectivity Index of Eight Medicinal Plants of Taiwan

Compounds	CC ₅₀ (µg/ml)*	Selectivity Index (SI) [†]				
		Herpes Simplex Viruses		Adenoviruses		
		HSV-1	HSV-2	ADV-3	ADV-8	ADV-11
ACV	126.8	45.1	58.0	NE	NE	NE
ddC	259.2	NE	NE	34.6	25.3	19.5
<i>A. pavonia</i>	1630.5	NE	NE	6.0	14.0	NE
<i>A. hypogaea</i>	3492.6	NE	18.3	NE	NE	NE
<i>B. purpurea</i>	2389.0	6.2	5.3	NE	NE	NE
<i>B. variegata</i>	1353.5	7.4	11.6	7.1	11.5	NE
<i>D. caudatum</i>	1395.3	4.8	3.7	4.2	11.7	NE
<i>D. triflorum</i>	2080.8	4.5	4.2	NE	NE	8.0
<i>G. max</i>	1328.4	NE	3.0	NE	NE	NE
<i>P. sativum</i>	4388.9	9.0	NE	30.7	49.9	9.5

*Concentration that showed 50% cell cytotoxic effect against BCC-1/KMC cells. Each value represents the mean of three separate experiments.

[†]SI is the ratio of CC₅₀ to EC₅₀.

ACV: Acyclovir, ddC: 2',3'-dideoxycytidine.

NE: Not evaluated.

Discussion

Over the past 50 years, infectious diseases are fast becoming another health crisis for our society. The epidemic of human immunodeficiency virus (HIV) around the world (Stoneburner *et al.*, 1994), the outbreak of Nipah virus in Malaysia and Singapore (CDC, 1999), the death of healthy people caused by an avian strain of influenza virus that had never before infected humans in Hong Kong (CDC, 1997), and the recent outbreak of the severe acute respiratory syndrome (SARS), etc. indicate that the more effective and new antiviral agents are in demand.

In an effort to search for new antiviral agents from natural products, eight commonly used medicinal plants in Taiwan were extracted with hot water and then investigated *in vitro* for their anti-HSV and anti-ADV activities. The antiviral activity was evaluated by XTT assay, which is a simple, fast and efficient method (Kodama *et al.*, 1996; Sudo *et al.*, 1994). This assay not only has lesser steps than the traditional plaque assay, but also avoids the irradiation of radioisotopes. Furthermore, the data of examination were read and printed with an ELISA reader, which is very sensitive and convenient for massive screening.

In this study, *B. variegata* and *D. caudatum* were found to possess a broad spectrum in antiviral activity whereas *G. max* had little effect in inhibiting HSV and ADV infections.

G. max, also named as soybean, is commonly used as food. In China, it is traditionally used to treat chill, fever and headache in cold and influenza, vexation, oppressed feeling in the chest and insomnia (Chinese Pharmacopoeia Commission, 1996). The saponins of *G. max* are found to inhibit the replication of HSV, human cytomegalovirus, influenza virus and HIV type-1 *in vitro* (Nakashima *et al.*, 1989; Hayashi *et al.*, 1997). These previous findings are not consistent with our results. The discrimination in observations between the studies might be due to the lacking of related saponins in the hot water extract used in our study.

A. hypogaea, most commonly known as peanut, is a popular food worldwide. It is traditionally used by the aborigines in Taiwan as a medicinal plant for different kinds of ailments such as moistening the lung, normalizing the function of stomach and spleen, regurgitating the food from stomach, and treatment of beriberi and cough caused by dryness (Chiu and Chang, 1992; Committee on Chinese Medicine Pharmacy, 2000). In this study, *A. hypogaea* was shown to inhibit HSV-2 infection but not HSV-1 and ADV infections. HSV-1 and -2 are classified in the family *Herpesviridae*, subfamily *alphaherpesvirinae*, which also includes the varicella-zoster virus (Roizman, 1996). There is 50% DNA sequence homology between HSV-1 and HSV-2 (Brooks *et al.*, 1995). The inhibitory effect of *A. hypogaea* in HSV-2 multiplication but not HSV-1 replication suggested that the active compound(s) of *A. hypogaea* possessed unique mechanism action(s), which acted only in HSV-2 replication.

During our study, there were three extracts that suppressed ADV infection but not HSV infection; these are *A. pavonia*, *D. triflorum* and *P. sativum*. Among the three extracts, *P. sativum* was the most interesting, as it possessed the lowest EC₅₀ value and the highest SI value. *P. sativum*, also named as garden pea or sugar pea, is another food that is extensively eaten by the Taiwanese. It is traditionally used for cholera morbus, septicemia, inducing diuresis and promoting lactation (Chiu and Chang, 1983). Since ADV pneumonia has been reported to have considerable mortality rate especially in children aged two years and below (Avila *et al.*, 1989; Dudding *et al.*, 1972), the high SI value of *P. sativum* suggested that eating *P. sativum* might be beneficial in preventing ADV infection and, is safe.

Traditional medicinal plants are important in viral treatment not only in their isolated form but also as templates for the formation of analogues with improved activity, and as probes for studying biochemical processes at the molecular level. According to our results, the eight hot water extracts of medicinal plants commonly used in Taiwan were concluded to exhibit anti-HSV and anti-ADV activities *in vitro* at different degrees of potency. These medicinal plants thus merit further studies such as fractionation and clarification of their mechanism of action.

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