In Vitro Anti-herpes Simplex Viruses and Anti-adenoviruses Activity of Twelve Traditionally Used Medicinal Plants in Taiwan

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As an effort to search for new antiviral agents from traditional medicine, the hot water (HW) extract of twelve traditionally used medicinal plants in Taiwan was evaluated for their *in vitro* anti-herpes simplex viruses (HSV; including HSV-1 and HSV-2) and anti-adenoviruses (ADV; including ADV-3, ADV-8 and ADV-11) activities with a XTT-based colorimetric assay. Results showed that the tested HW extracts exhibited anti-HSV and anti-ADV activities at different magnitudes of potency. Among the twelve medicinal plants, *Boussingaultia gracilis* var. *pseudobaselloides* (Basellaceae) and *Serissa japonica* (Rubiaceae) possessed broad spectrum of antiviral activity. *Ardisia squamulosa* (Myrsinaceae) and *Artemisai princeps* var. *orientalis* (Compositae) were more effective in inhibiting ADV-8 replication than the other four viruses. Cell cytotoxic assay demonstrated that all tested HW extracts had CC_{50} values higher than their EC_{50} values. It was concluded that the twelve traditionally used medicinal plants in Taiwan possessed antiviral activity, and some of them merit further investigation.

Key words medicinal plant; Taiwan; antiviral activity; *Boussingaultia gracilis*; *Serissa japonica*

Herpes simplex viruses (HSV) are ubiquitous agents which cause a variety of diseases ranging in severity from mild to severe, and in certain cases, they may became life-threatening, especially in immunocompromised patients. After primary infection, HSV will persist in the host for the latter's entire lifetime, and HSV infection is thus considered as lifelong infection. Nucleoside analogues such as acyclovir (ACV), penciclovir *etc.*, are the only officially approved drugs for the therapy of HSV infection.[1,2] They are usually effective in treatment of primary or recurrent HSV infection. However, the widespread use of nucleoside-based drugs has lead to the emergence of HSV strain resistant to those related drugs, especially among immunocompromised patients. According to the previous surveys, the incidence of ACV-resistant strain among immunocompromised patients is around 5% and reaches 14% among bone marrow transplant recipients.[3—5] The high prevalence of ACV-resistant HSV among these populations suggests that new medication is needed.

Adenoviruses (ADV) are other ubiquitous agents. They are associated with a wide range of illnesses, including ocular, respiratory, gastrointestinal and urinary infections. ADV infection is usually mild and always heals without the need of any special therapy. However, severe ADV infection has been reported in immunocompromised patients, including patients with leukemia,[6] AIDS,[7] or organ transplantation.[8] Furthermore, ADV cause pneumonia have been reported to have considerable mortality rate especially in children of age below 2 years old.[9,10] 5-Iodo-2′-deoxyuridine (IDU), ganciclovir, cidafovir and several cysteine protease inhibitors are reported to inhibit ADV infection, and some of them have been used for ADV infection.[11—14] These agents, however, are either too toxic for use or not approved by governmental agencies for the therapy of ADV infection. In addition, the development of resistance of ADV to related drugs has also been reported in literature.[15] Thus, new and more effective antiviral agents for future therapy in ADV infection are desired.

In our continuous efforts to search for novel antiviral agents from traditional medicinal plants, twelve traditionally used medicinal plants in Taiwan were extracted with hot water (HW) and then investigated for their *in vitro* anti-HSV and anti-ADV activities. This is the first report on the anti-HSV and ADV activities of the HW extract of the related twelve medicinal plants.

MATERIALS AND METHODS

Plant Materials The stem and leaf of *Ardisia squamulosa* PRESL (Myrsinaceae), *Artemisai princeps* PAMP. var. *orientalis* (PAMP.) HARA (Compositae), *Cinnamomum camphora* (LINN.) SIEB. (Lauraceae), *Crossostephium chinense* (LINN.) MAKINO (Compositae) and *Serissa japonica* THUNB. (Rubiaceae), the flower of *Jasminum sambac* (LINN.) AIT. (Oleaceae), and the whole plant of *Basella rubra* LINN. (Basellaceae), *Biden pilosa* LINN. (Compositae), *Boussingaultia gracilis* MIERS var. *pseudobaselloides* BAILEY (Basellaceae), *Drymaria cordata* (LINN.) WILLDENOW (Caryophyllaceae), *Portulaca grandiflora* HOOKER (Portulacaceae) and *Rosa rugosa* THUNB. (Rosaceae) were collected from southern Taiwan. Their authenticity was 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 these plants was deposited at the Herbarium of the Graduate Institute of Natural Products of Kaohsiung Medical University, Taiwan.

Preparation of the Extracts Hot water (HW) extract of the medicinal plants was prepared according to the procedures as described previously by Chang and Yeung with minor modifications.[16] Briefly, different parts of the medicinal plants were boiled with 1000 ml of distilled water for 1 h. The aqueous was collected and the residual was extracted again with another 1000 ml of distilled water. The resulting aqueous extracts were collected, combined, filtered by gauze, concentrated under reduced pressure and then lyophilized to dry.

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Acyclovir (ACV) and 2′,3′-dideoxycytidine (ddC) were purchased from Sigma Chemical Co. (U.S.A.). The HW extracts were dissolved in sterile distilled water whereas ACV and ddC were suspended in DMSO.

Cell and Viruses The human skin basal cell carcinoma cell line (BCC-1/KMC), established in our laboratory,[17] 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 cell culture reagents and media were purchased from Gibco BRL (Grand Island, New York).

HSV-1 KOS strain was obtained from the American Type Culture Collection (ATCC), Rockville, U.S.A., 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 cells were 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% CO_2 for 24 h. A serial dilution of virus stock was prepared, and cells were infected with the dilution of virus. After an additional 72 h of incubation, the cytopathic effect was recorded. The 50% tissue culture infective dose ($TCID_{50}$) per ml was calculated as described previously by Reed and Muench.[18]

Antiviral Assay Using XTT Method The antiviral activity of HW extracts was evaluated by the XTT method as previously described.[19,20] BCC-1/KMC cells, treated by trypsin, were seeded in 96-well culture plates with a volume of 70 μl/well and a concentration of 10^5 cells/ml. After 24 h incubation, 20 μl of 25, 20, 120, 20 and 120 $TCID_{50}$ of HSV-1, HSV-2, ADV-3, ADV-8 and ADV-11 was added, and the infected cells were incubated for another 2 h. Ten microliter of 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%. For every experiment, a parallel virus control was performed to ensure the viral infectivity remained during the experiment. After further incubation at 37 °C with 5% CO_2 for 72 h, 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 with a volume of 50 μl. The trays were reincubated for an additional 2 h to allow the production of formazan. Optical densities were then determined with the EIA reader (Multiskan EX, Labsystems) at a test wavelength of 450 nm and a reference wavelength of 690 nm.

Viral inhibition rate was calculated as $[(ODtv-ODcv)/(ODcd-ODcv)]\times100\%$. ODtv, ODcv and ODcd 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 50% effectiveness concentration (EC_{50}) was defined as the concentration that achieved 50% cyto-protection against virus infection.

Cell Cytotoxic Effect The cell cytotoxic effect of tested compounds toward BCC-1/KMC cells was evaluated with 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 by the following formula: Percent of cell cytotoxic effect$=[1-(ODt/ODs)]\times100\%$. ODt and ODs indicate the absorbance of the test substances and the solvent control, respectively. The 50% cell cytotoxic concentration (CC_{50}) of tested compounds was calculated according to Chiang *et al.*[19]

Statistical Analysis Data were calculated as mean± standard error for three separate experiments. The selectivity index (SI) was calculated as the ratio of CC_{50} to EC_{50}. The Student's unpaired *t*-test was used to calculate *p* values of difference of means between control and the tested samples on the inhibition of HSV or ADV replication. Difference of sample between tested viruses with a *p* value less than 0.05 was considered statistically significant.

RESULTS

Antiviral Activity of Twelve Commonly Used Medicinal Plants in Taiwan Results showed that hot water (HW) extract of twelve commonly used medicinal plants in Taiwan exhibited *in vitro* anti-HSV and anti-ADV activities at different magnitudes of potency (Table 1). ACV and ddC were used as a reference compound for anti-HSV and anti-ADV assays, respectively.

The EC_{50} for twelve tested HW extracts against HSV-1 infection was in the range of 67—484 μg/ml. *B. gracilis* and *S. japonica* showed potent anti-HSV-1 activity with EC_{50} of 80.7±1.5 and 67.5±2.6 μg/ml, respectively. The anti-HSV-1 activity of *A. squamulosa* was at moderate potency with EC_{50} of 168.5±10.4 μg/ml. Other nine HW extracts were either mild or little at activity to suppress HSV-1 infection.

When tested for anti-HSV-2 activity, *S. japonica* showed smallest EC_{50} value. The EC_{50} of *S. japonica* was 92.1±3.8 μg/ml. Beside *S. japonica*, only two HW extracts had an EC_{50} smaller than 200 μg/ml. There were three HW extracts which had EC_{50} values in the range of 200—400 μg/ml, and five HW extracts with EC_{50} values higher than 500 μg/ml.

For anti-ADV-3 activity, there were *B. gracilis* and *S. japonica* that had EC_{50} value smaller than 100 μg/ml. The EC_{50} value of *B. gracilis* and *S. japonica* was 44.1±19.5 and 72.9±12.9 μg/ml, respectively. *A. princeps*, *B. pilosa*, *C. chinense* and *J. sambac* had EC_{50} value between 100—150 μg/ml. *D. cordata* and *P. grandiflora* had an EC_{50} higher than 500 μg/ml.

Our results revealed that four HW extracts possessed EC_{50} smaller than 100 μg/ml when tested for anti-ADV-8 activity. These four HW extracts were *A. squamulosa*, *A. princeps*, *B. gracilis* and *S. japonica* with EC_{50} of 96.7±8.7, 38.6±8.4, 44.7±3.2 and 24.6±8.2 μg/ml, respectively. *B. rubra* and *R. rugosa* had an EC_{50} between 100—200 μg/ml. There were three HW extracts that had EC_{50} higher than 500 μg/ml.

Besides ADV-3 and ADV-8, the extracts were also tested for anti-ADV-11 activity. *B. gracilis* was the most potent extracts with an EC_{50} of 89.8±3.8 μg/ml. Three HW extracts possessed an EC_{50} between 100—200 μg/ml, four HW extracts between 200—300 μg/ml, two HW extracts between

Table 1. *In Vitro* Antiviral Activity of Twelve Commonly Used Medicinal Plants in Taiwan

Compounds	Used part	EC_{50} (μg/ml)[a]				
		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
A. squamulosa	Stem and leaf	168.5±10.4*	214.0±13.1	210.0±4.2	96.7±8.7**,§	254.2±9.8
A. princeps	Stem and leaf	211.0±6.5*	309.9±11.5	139.9±4.4	38.6±8.4**,§	238.0±9.4
B. rubra	Whole plant	>500.0	>500.0	252.4±9.3	168.3±8.5**,§	257.3±12.5
B. pilosa	Whole plant	279.4±17.3	221.8±5.1*	129.5±12.2**	485.5±48.3	182.4±10.6§
B. gracilis	Whole plant	80.7±1.5*	194.7±5.1	44.1±19.5	44.7±3.2§	89.8±3.8
C. camphora	Stem and leaf	483.2±27.5	444.1±25.0	319.2±22.5	284.7±11.9	310.5±25.4
C. chinense	Stem and leaf	237.3±3.5	179.2±16.5*	145.1±9.3**	> 500.0	161.2±12.3§
D. cordata	Whole plant	>500.0	>500.0	>500.0	>500.0	219.1±29.7§
J. sambac	Flower	>500.0	>500.0	119.6±10.6**	290.8±47.8	338.4±11.4
P. grandiflora	Whole plant	>500.0	>500.0	>500.0	>500.0	492.0±111.2
R. rugosa	Whole plant	>500.0	>500.0	213.1±24.2	167.0±12.3**,§	>500.0
S. japonica	Stem and leaf	67.5±2.6*	92.1±3.8	72.9±12.9	24.6±8.2**,§	102.9±5.9

a) Concentration that inhibited 50% virus infection. ACV: Acyclovir; ddC: 2′,3′-dideoxycytidine. ND: Not done. Each value represents the mean±S.E. of three separate experiments. *: $p<0.05$ (compared between HSV-1 and HSV-2); **: $p<0.05$ (compared between ADV-3 and ADV-8); §: $p<0.05$ (compared between ADV-8 and ADV-11).

Table 2. Cell Cytotoxic Effect and Selectivity Index of Twelve Commonly Used Medicinal Plants in Taiwan

Compounds	CC_{50} (μg/ml)[a]	Selectivity Index (SI)[b]				
		Herpes Simplex viruses		Adenoviruses		
		HSV-1	HSV-2	ADV-3	ADV-8	ADV-11
ACV	126.8	45.1	58.0	ND	ND	ND
ddC	259.2	ND	ND	34.6	25.3	19.5
A. squamulosa	1698.7	10.1	7.9	8.1	17.6	6.7
A. princeps	3519.7	16.7	11.4	25.2	91.1	14.8
B. rubra	2358.0	ND	ND	9.3	14.0	9.2
B. pilosa	2705.6	9.7	12.2	20.9	5.6	14.8
B. gracilis	3032.6	37.6	15.6	68.7	67.8	33.8
C. camphora	777.6	1.6	1.8	2.4	2.7	2.5
C. chinense	724.0	3.1	4.0	5.0	ND	4.5
D. cordata	2643.1	ND	ND	ND	ND	12.1
J. sambac	2829.5	ND	ND	23.7	9.7	8.4
P. grandiflora	4853.2	ND	ND	ND	ND	9.9
R. rugosa	2289.9	ND	ND	10.7	13.7	ND
S. japonica	1575.2	23.3	17.1	21.6	63.9	15.3

a) Concentration that showed 50% cell cytotoxic effect against BCC-1/KMC cells. Each value represents the mean of three separate experiments. *b*) SI is the ratio of CC_{50} to EC_{50}. ACV: Acyclovir; ddC: 2′,3′-dideoxycytidine. ND: Not done due to high EC_{50} (>500.0 μg/ml) of HW extract.

300—400 μg/ml, and two HW extracts higher than 450 μg/ml.

Cell Cytotoxic Effect and Selectivity Index of Twelve Commonly Used Medicinal Plants in Taiwan Table 2 shows the cell cytotoxic effect of HW extracts of the twelve medicinal plants. Overall, all HW extracts showed CC_{50} higher than their EC_{50}. These observations indicated that the antiviral activity of HW extracts was not a result of their cytotoxic effect toward cells. The CC_{50} values ranged from 720 to 4860 μg/ml.

With the EC_{50} and CC_{50} data, the selectivity index (SI) was calculated by dividing CC_{50} by EC_{50}. The SI for the anti-HSV-1 assay was in the range of 1.6—37.6, and 1.8—15.6 for the anti-HSV-2 assay. For anti-ADV-3, ADV-8 and ADV-11 assays, the SI ranged from 2.4 to 68.7, 5.6 to 67.8 and 2.5 to 91.1, respectively.

DISCUSSION

Medicinal plants have been traditionally used for different kind of ailments including infectious diseases. Some of them are reported to exhibit antiviral activity in literature.[21—23] According to the Cragg's report, approximately 60% of anti-tumor and anti-infective agents that are commercially available or in the late stages of clinical trials today are of natural product origin.[24] There is therefore no doubt that traditional medicinal plants can serve as a potential resource in the development of new antiviral agents in the future. Since current chemotherapy agents for ADV and HSV infections are either insufficient in quantity or limited in efficiency, there is thus a need to search for new and more effective antiviral agents for future therapy in ADV and HSV infections.

In this study, XTT assay was used for the evaluation of antiviral activity because it is a simple, fast and efficient

method.[25,26] 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 EIA reader. It is therefore very sensitive and convenient for massive screening on antiviral activity of medicinal plants.

Among the twelve tested medicinal plants, two of them were found to exhibit a broad spectrum of antiviral activity. These two medicinal plants were *B. gracilis* and *S. japonica*. *B. gracilis* is also named as *Anredra cordifolia* (Tenore) van Steen. In Taiwan, it is traditionally used as the treatment for gastric pain, cough, diabetes and liver diseases.[27] Previous studies demonstrated that *B. gracilis* showed weak antimutagenic activity.[28] *S. japonica*, also known as Japanese serissa, is commonly used for the treatment of carbuncle on the back and edema in Taiwan.[29] Our studies revealed that *B. gracilis* and *S. japonica* suppressed four (HSV-1, ADV-3, ADV-8, ADV-11) and five (HSV-1, HSV-2, ADV-3, ADV-8, ADV-11) virus infections, respectively.

The HW extracts of these two medicinal plants also showed noteworthy SI value. Although they are less effective than the reference compounds, ACV for HSV assay and ddC for ADV assay, their SI, however, were valuable. For example, the SI of *B. gracilis* against all tested ADV infections was generally higher than that of ddC. Also *S. japonica* had higher SI against ADV-8 infection than that of ddC.

Another interesting observation was the anti-ADV-8 activity of *A. squamulosa* and *A. princeps*. HW extracts of these two medicinal plants had smaller EC_{50} against ADV-8 infection than that of two other tested ADV infections, ADV-3 and ADV-11 ($p<0.05$). Human ADV have at least 51 serotypes and are divided into six groups (A—F) on the basis of their physical, chemical, and biological properties.[30] Their DNA is >90% in homology for the members of a given group but is <20% in homology between the members of different groups. In classification, ADV-3 and ADV-11 are group B ADV, and ADV-8 is group D ADV. The stronger potency of *A. squamulosa* and *A. princeps* against ADV-8 infection than against ADV-3 or ADV-11 infection suggested that these two medicinal plants target some unique properties of ADV-8. Further investigations are necessary to clarify the underlying mechanism action of *A. squamulosa* and *A. princeps* against ADV-8 infection.

Our previous study showed that hot water extract of *Biden pilosa* LINN. var. *minor* (Blume) SHERFF (Compositae) (HWBPLS) possessed *in vitro* anti-HSV-1 and anti-HSV-2 activities.[31] In this study, we showed that hot water extract of *Biden pilosa* LINN. (Compositae) (HWBPL) exhibited anti-HSV and anti-ADV activities. By comparing the EC_{50} values for both medicinal plants, it was revealed that HWBPL was more effective against HSV replication than that of HWBPLS. The EC_{50} values of HWPBL against HSV-1 and HSV-2 infection were 279.4 ($p<0.05$, compared with HWPBLS) and 221.8 μg/ml ($p<0.05$, compared with HWPBLS), whereas those of HWPBLS were 655.4 and 960.0 μg/ml, respectively. Also, the SI values of HWBPL were smaller than those of HWPBLS (The SI values of HWBPL against HSV-1 and HSV-2 were 9.7 and 12.2, and of HWPBLS were >1.53 and >1.04, respectively). Since the same virus strain, host cell, procedures of antiviral activity assessment and extraction method of medicinal plants, *etc.* were used for both studies, we therefore suggest that the continuous investigation on anti-HSV activity of HWBPL is more promising and encouraging than that of HWPBLS. The studies regarding the different anti-HSV activity between HWBPL and HWPBLS will also be conducted in the near future.

In summary, the HW extract of twelve traditionally used medicinal plants in Taiwan was concluded to exhibit anti-HSV and anti-ADV activities at different magnitudes of potency. Among the tested medicinal plants, the anti-HSV and anti-ADV activities of *B. gracilis* and *S. japonica*, and the anti-ADV activity of *A. squamulosa* and *A. princeps* are encouraged for further investigation.

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