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

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Received June 13, 2003; accepted July 22, 2003

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 Artemisia 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 CC50 values higher than their EC50 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 become 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.2,3 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,9 AIDS,7 or organ transplantation.9 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, cidofovir 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 PERSL. (Myrsinaceae), Artemisia 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. (OLACEAE), and the whole plant of Basella rubra LINN. (Basellaceae), Biden pilosa LINN. (Compositae), Boussingaultia gracilis MIERS var. pseudobaselloides BAILEY (Basellaceae), Drymaria cordata (LINN.) WILDENOW (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.
Acyclovir (ACV) and 2',3'-dideoxyctydine (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, 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 concentration for DMSO in culture medium was 0.1%. For every experiment, the viral infectivity remained during the experiment. After an additional 72 h of incubation, the cytopathic effect was exhibited 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<sub>50</sub>) per ml was calculated as described previously by Reed and Muench.¹⁵

**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 at a density of 10<sup>5</sup> cells/well and then incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> 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<sub>50</sub>) per ml was calculated as described previously by Reed and Muench.¹⁵

**Titration of Viruses**  BCC-1/KMC cells were seeded in 96-well culture plates at a density of 10<sup>5</sup> cells/well and then incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> 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<sub>50</sub>) per ml was calculated as described previously by Reed and Muench.¹⁵

**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<sub>50</sub> 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<sub>50</sub> of 80.7±1.5 and 67.5±2.6 μg/ml, respectively. The anti-HSV-1 activity of <i>A. squamulosa</i> was at moderate potency with EC<sub>50</sub> 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, <i>S. japonica</i> showed smallest EC<sub>50</sub> value. The EC<sub>50</sub> of <i>S. japonica</i> was 92.1±3.8 μg/ml. Beside <i>S. japonica</i>, only two HW extracts had an EC<sub>50</sub> smaller than 200 μg/ml. There were three HW extracts which had EC<sub>50</sub> values in the range of 200—400 μg/ml, and five HW extracts with EC<sub>50</sub> values higher than 500 μg/ml.

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

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

Besides ADV-3 and ADV-8, the extracts were also tested for anti-ADV-11 activity. <i>B. gracilis</i> was the most potent extract with an EC<sub>50</sub> of 89.8±3.8 μg/ml. Three HW extracts possessed an EC<sub>50</sub> between 100—200 μg/ml, four HW extracts between 200—300 μg/ml, two HW extracts between 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−ODc)/(ODc−ODc)]×100%. ODt, ODc and ODs indicate the absorbance of the test substances and the solvent control, respectively. The 50% cell cytotoxic concentration (CC<sub>50</sub>) 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<sub>50</sub> to EC<sub>50</sub>. 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.
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>
<thead>
<tr>
<th>Compounds</th>
<th>CC$_{50}$ (μg/ml)$^a$</th>
<th>Selectivity Index (SI)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSV-1</td>
<td>HSV-2</td>
</tr>
<tr>
<td>ACV</td>
<td>126.8</td>
<td>58.0</td>
</tr>
<tr>
<td>ddC</td>
<td>259.2</td>
<td>ND</td>
</tr>
<tr>
<td>A. squamulosa</td>
<td>168.5</td>
<td>10.4*</td>
</tr>
<tr>
<td>A. princeps</td>
<td>211.0</td>
<td>6.5*</td>
</tr>
<tr>
<td>B. rubra</td>
<td>&gt;500.0</td>
<td>ND</td>
</tr>
<tr>
<td>B. pilosa</td>
<td>279.4</td>
<td>17.3</td>
</tr>
<tr>
<td>B. gracilis</td>
<td>80.7</td>
<td>1.5*</td>
</tr>
<tr>
<td>C. camphora</td>
<td>483.2</td>
<td>7.5*</td>
</tr>
<tr>
<td>C. chinense</td>
<td>237.3</td>
<td>3.5</td>
</tr>
<tr>
<td>D. cordata</td>
<td>Whole plant</td>
<td>&gt;500.0</td>
</tr>
<tr>
<td>J. sambac</td>
<td>Flower</td>
<td>&gt;500.0</td>
</tr>
<tr>
<td>E. grandiflora</td>
<td>Whole plant</td>
<td>&gt;500.0</td>
</tr>
<tr>
<td>R. rugosa</td>
<td>Whole plant</td>
<td>&gt;500.0</td>
</tr>
<tr>
<td>S. japonica</td>
<td>Stem and leaf</td>
<td>67.5</td>
</tr>
</tbody>
</table>

$^a$ 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. $^b$ 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 μg/ml) of HW extract.

### 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. According to the Craggs’s report, approximately 60% of antitumor and anti-infective agents that are commercially available or in the late stages of clinical trials today are of natural product origin. 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.\textsuperscript{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 \textit{B. gracilis} and \textit{S. japonica}. \textit{B. gracilis} is also named as \textit{Anredra cordifolia} (Tenore) van Steen. In Taiwan, it is traditionally used as the treatment for gastric pain, cough, diabetes and liver diseases.\textsuperscript{27} Previous studies demonstrated that \textit{B. gracilis} showed weak antimutagenic activity.\textsuperscript{28} \textit{S. japonica}, also known as Japanese serissa, is commonly used for the treatment of carbuncle on the back and edema in Taiwan.\textsuperscript{29} Our studies revealed that \textit{B. gracilis} and \textit{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 \textit{B. gracilis} against all tested ADV infections was generally higher than that of ddC. Also \textit{S. japonica} had higher SI against ADV-8 infection than that of ddC.

Another interesting observation was the anti-ADV-8 activity of \textit{A. squamulosa} and \textit{A. princeps}. HW extracts of these two medicinal plants had smaller EC\textsubscript{50} against ADV-8 infection than that of two other tested ADV infections, ADV-3 and ADV-11 (\textit{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.\textsuperscript{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 \textit{A. squamulosa} and \textit{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 \textit{A. squamulosa} and \textit{A. princeps} against ADV-8 infection.

Our previous study showed that hot water extract of \textit{Biden pilosa} \textsc{Linn.} \textit{var. minor} (Blume) \textsc{Sherff} (Compositae) (HWBPLS) possessed \textit{in vitro} anti-HSV-1 and anti-HSV-2 activities.\textsuperscript{31} In this study, we showed that hot water extract of \textit{Biden pilosa} \textsc{Linn.} (Compositae) (HWBPL) exhibited anti-HSV and anti-ADV activities. By comparing the EC\textsubscript{50} values for both medicinal plants, it was revealed that HWBPL was more effective against HSV replication than that of HWPBLS. The EC\textsubscript{50} values of HWBPL against HSV-1 and HSV-2 infection were 279.4 (\textit{p}<0.05, compared with HWPBLS) and 221.8 \textmu g/ml (\textit{p}<0.05, compared with HWPBLS), whereas those of HWPBLS were 655.4 and 960.0 \textmu g/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 stud-

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  \bibitem{26} Kodama E., Shigeta S., Suzuki T., De Clercq E., \textit{Antiviral Res.}, \textbf{31}, 159—164 (1996).
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