Putranjivain A from *Euphorbia jolkini* inhibits both virus entry and late stage replication of herpes simplex virus type 2 *in vitro*

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**Aims:** To investigate the *in vitro* antiviral properties of putranjivain A, isolated from the whole plant of *Euphorbia jolkini* (Euphorbiaceae).

**Methods and results:** Herpes simplex virus (HSV)-2 strain 196-infected Vero cells were used. It was shown that putranjivain A exhibited antiviral activity with an IC₅₀ of 7.9 ± 1.2 µM using the XTT assay, with the IC₅₀ value increasing with increasing multiplicity of infection. Using the plaque reduction assay, the IC₅₀ and IC₉₀ were 6.3 ± 0.8 and 14.5 ± 3.1 µM, respectively. Putranjivain A showed no cytotoxic effect on cell multiplication at concentrations that achieved antiviral activity. The 50% cell cytotoxic concentration (CC₅₀) was 80.3 ± 14.7 µM, and the selectivity index (SI) (ratio of CC₅₀ to IC₅₀) for the XTT and plaque reduction assays was 10.2 and 12.7, respectively. When tested for virucidal activity, putranjivain A significantly reduced viral infectivity at concentrations of 75 and 100 µM, but not at 50 µM or below. The results of the time-of-addition studies suggested that putranjivain A affected the late stage of HSV-2 replication at 25 µM. Interestingly, putranjivain A also showed inhibition of viral attachment and cell penetration. The combination of putranjivain A and aciclovir produced no interaction.

**Conclusions:** Putranjivain A possesses antiviral activity, inhibiting viral attachment and penetration, and also interfering with the late stage of viral replication.

Keywords: antiviral activity, HSV-2, plants

**Introduction**

Herpes simplex virus (HSV) causes one of the most common viral infections in humans, in whom infection causes a variety of diseases ranging from mild to severe, and, in certain cases, life-threatening illness.

Genital HSV infection is usually caused by HSV-2, and sometimes by HSV-1. Infection with HSV-2 is extremely common worldwide, and epidemiological surveys have indicated a trend of continual increase in infection rate in most countries.¹,² The genital ulcers caused by HSV, which damage the genital mucosal barrier, may facilitate the transmission of HIV.³ Furthermore, the acquisition of herpes by the fetus during pregnancy may lead to neonatal herpes,⁴ which can be severe, and death of the infected neonate or severe neurodevelopmental disability is common.⁶

Aciclovir, famciclovir and foscarnet are effective in the treatment of HSV infections, but reports have indicated that treatment failures result from antiviral-resistant HSV exclusively among immunocompromised patients.⁷–¹² According to the literature, the prevalence of aciclovir-resistant HSV among immunocompromised patients is ~5%, and reaches 14% among bone marrow transplant recipients.⁸,¹² The increasing number of immunocompromised individuals, particularly AIDS and transplant patients, has driven the need for improved antiviral agents to treat diseases caused by herpesviruses.¹³,¹⁴ Although some non-nucleoside inhibitors of herpesviruses have been developed,¹⁵–¹⁷ none is officially approved for HSV therapy.¹⁸,¹⁹ and...
and mode of action of putranjivain A against HSV-2. Previously, Fraction II was repeatedly chromatographed on a MCI-gel CHP 20P acetone:water (4:1, v/v). The extract was concentrated under reduced pressure and then diluted with sterile deionized distilled water before use. The final concentration of DMSO was <0.1%.

Cells and viruses
All reagents and media for cell culture were purchased from Gibco-BRL (Grand Island, NY, USA). African Green Monkey kidney cells (Vero) (ATCC CCR-81) were obtained from the hospital of Kaohsiung Medical University (Kaohsiung, Taiwan). Cells were propagated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum (FCS), penicillin G sodium 200 U/mL, streptomycin sulphate 200 mg/L and amphotericin B 0.5 mg/L. Overlay medium for the plaque assay of HSV-2 consisted of DMEM plus 2% FCS, 1% methylcellulose and antibiotics as described above.

The HSV-2 strain 196 was kindly provided by Dr Lien-Chai Chiang (Department of Microbiology, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan). Its titre was determined by plaque assay and was expressed as plaque-forming units (pfu) per mL. Virus stocks were stored at –80°C until use.

Antiviral assays
(i) XTT assay. The antiviral activity of putranjivain A was assayed using XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulphonic acid) (Sigma) as previously described. Briefly, 10⁴ cells/well were seeded into 96-well culture plates (Falcon; BD Biosciences, Franklin Lakes, NJ, USA). After 4 h of incubation at 37°C with 5% CO₂, cells were infected with HSV-2 at a multiplicity of infection (moi) of 0.5 or 5.0, and then various concentrations of putranjivain A were added. The infected cells were incubated with 2% FCS medium for another 72 h. The medium was then aspirated. Cells were rinsed with PBS and then XTT reagent was added. The plate was re-incubated for an additional 2 h to allow the production of formazan. Absorbances were measured on an EIA reader (Lab Systems; MTX Labs, Vienna, VA, USA) at a test wavelength of 492 nm and a reference wavelength of 690 nm. The antiviral activity of putranjivain A was determined according to the following formula: ²⁸

\[
\text{Antiviral activity} = \frac{(\text{OD}_\text{mock} - \text{OD}_\text{HSV})}{(\text{OD}_\text{mock} - \text{OD}_\text{control})} \times 100\%
\]

where (OD)mock is the absorbance measured for the control untreated HSV-infected cells, and (OD)HSV is the absorbance measured for the control untreated mock-infected cells.

(ii) Plaque reduction assay. Vero cells were seeded into 24-well culture plates (Falcon; BD Biosciences, Franklin Lakes, NJ, USA) at a density of 10⁴ cells/well and incubated at 37°C with 5% CO₂ until they were at least 95% confluent. The cell monolayer was then infected with 100 pfu HSV-2 in the absence or presence of putranjivain A, and incubated for a further 1 h. After 1 h of adsorption, the cell monolayer was overlaid with overlay medium. The overlay medium was removed 2 days later, and the infected cell monolayer was fixed and stained with 10% formalin and 1% Crystal Violet, respectively. The antiviral activity of putranjivain A was determined by the following formula:

\[
\text{Percentage inhibition} = \left(1 - \frac{\text{number of plaque}_{\text{tested}}}{\text{number of plaque}_{\text{control}}}\right) \times 100\%
\]

The minimal concentration of putranjivain A required to reduce the 50% plaque number (IC50) was calculated by regression analysis of the dose–response curves generated from these data. ²⁰

Materials and methods
Test compound
Putranjivain A (Figure 1) was isolated from the whole plant of Euphorbia jolkini as described previously.²⁶ Briefly, fresh whole E. jolkini plants were chopped into small pieces and compounds were extracted with acetone:water (4:1, v/v). The extract was concentrated under reduced pressure and then filtered. The filtrate was subsequently eluted with water/methanol and then with water/acetone to give three fractions. Fraction II was repeatedly chromatographed on a MCI-gel CHP 20P acetone:water (4:1, v/v). The extract was concentrated under reduced pressure and then diluted with sterile deionized distilled water before use. The final concentration of DMSO was <0.1%.

there is a need to find new and more effective antiviral agents to prevent and/or to treat HSV infection.

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Cytotoxicity assay and selectivity index
The cytotoxic effect of putranjivain A on proliferating cells was assessed using an XTT-based method.29 This was performed with procedures similar to the XTT virus assay described above, except that HSV-2 was omitted from the test. The initial seeding cell number was 5.0 × 10^5 cells/well, and after 72 h of incubation, the cell number was increased to ~3.0 × 10^5 cells/well. The 50% cell cytotoxic concentration (CC50) of putranjivain A was calculated according to Cheng et al.29 The selectivity index (SI) was evaluated as the ratio of CC50 to IC50.

Virucidal assay
Virucidal activity of putranjivain A was evaluated as described by Carlucci et al.30 and Hayashi et al.,33 with modification. Briefly, a virus suspension containing 2 × 10^5 pfu HSV-2 was mixed with or without various concentrations of putranjivain A for 6 h at room temperature (–26°C). The sample was then diluted and its residual infectivity was determined by plaque assay.

Time-of-addition studies
The antiviral activity of test samples was evaluated at various time periods up to 24 h according to procedures described by Boulware et al.34 Vero cells were seeded into 12-well culture plates (Nunc; Nalge Nunc International, Rochester, NY, USA) at a density of 2 × 10^5 cells/well, and incubated for 24 h. The cell monolayer was then infected with 1×10^5 pfu/ well HSV-2 and putranjivain A was added into wells either concurrent with HSV-2 infection (0 h) or at intervals of 2, 4, 7 or 12 h post-infection. The highest concentration of putranjivain A was 25 µM. After 24 h of infection, cells were scraped and virus released from cells by freeze-thawing three times. Cell pellets were removed by centrifugation at 1100g for 10 min. The supernatants were divided into small volumes and then stored at –80°C. The virus titre of each supernatant was determined by plaque assay. The percentage inhibition was calculated as the reduction in virus titre observed in infections containing compound compared with that of infections containing deionized water as a solvent control.

Attachment assay
The attachment assay described by De Logu et al.30 was used in this study with a minor modification. Briefly, a Vero cell monolayer was grown in a 24-well culture plate and pre-chilled at 4°C for 1 h. The medium was aspirated and the cell monolayer was then infected with 200 pfu HSV-2 in the absence or presence of serial dilution of putranjivain A. After further incubating the infected cell monolayer at 4°C for another 3 h, the medium was aspirated to remove unabsorbed virus. Cell monolayer was then washed with PBS three times and overlaid with medium containing 1% methylcellulose. The cell monolayer was incubated for a further 48 h before it was fixed and stained. The percentage inhibition was calculated by the following formula:

\[
\text{Percentage inhibition} = \left[1 - \frac{(\text{number of plaque})_{\text{hested}}}{(\text{number of plaque})_{\text{control}}}\right] \times 100\%
\]

Penetration assay
The penetration assay of HSV-2 into Vero cells was performed according to published procedures, with minor modifications.30,33,35 A Vero cell monolayer was grown in a 24-well culture plate and pre-chilled at 4°C for 1 h. The cell monolayer was then infected with 200 pfu HSV-2 and incubated at 4°C for a further 3 h to allow the attachment of HSV-2. After 3 h of incubation, 25 µM putranjivain A was added. The control group contained no putranjivain A. Infected cell monolayer was then incubated at 37°C to maximize the penetration of viruses. At 10 min intervals, the infected cell monolayer was treated with PBS at pH 3 for 1 min to inactivate non-penetrated virus. PBS at pH 11 was then immediately added to neutralize acidic PBS (pH 3). The neutral PBS was removed and the cell monolayer was overlaid with overlay medium. After a further 48 h of incubation, the cell monolayer was fixed and stained. Plaques were counted and the percentage of inhibition of penetration was calculated by the following formula:

\[
\text{Percentage inhibition} = \left[1 - \frac{(\text{number of plaque})_{\text{hested}}}{(\text{number of plaque})_{\text{control}}}\right] \times 100\%
\]

Drug combination assay
The antiviral activity of putranjivain A in combination with aciclovir against HSV-2 was evaluated as described previously, with minor modification.36–38 The XTT assay was conducted as described above, and an moi of 5.0 was used throughout the assays. Effect of the combination of aciclovir and putranjivain A on HSV-2 replication was analysed by using the isobologram method. In this analysis, the IC50 was used to calculate the fractional inhibitory concentration (FIC). The interaction between putranjivain A and aciclovir was interpreted according to the combined FIC index (FIC_{putranjivain A + FIC_{aciclovir} as synergy (≤0.5), no interaction (0.5–4) or antagonism (>4)).39

Statistical analysis
The results were expressed as mean ± S.D. for three independent experiments. Student’s unpaired t-test was used to evaluate the difference between the test sample and solvent control. A P value of <0.05 was considered statistically significant.

Results
Effect of putranjivain A on virus replication and cell growth
Putranjivain A was shown to exhibit antiviral activity with an IC50 in XTT and plaque reduction assays of 7.9 ± 1.2 and 6.3 ± 0.8 µM, respectively (Table 1). The IC90 increased from 7.9 ± 1.2 to 12.4 ± 2.9 µM as the moi increased from 0.5 to 5.0. The IC50 of putranjivain A in the plaque reduction assay was 14.5 ± 3.1 µM.

As determined by XTT assay, putranjivain A exhibited a cytotoxic effect on dividing Vero cells, but only at concentrations higher than the IC50. The CC50 of putranjivain A for Vero cells was 80.3 ± 14.7 µM. Putranjivain A at a concentration of 60 µM exhibited little cytotoxic effect, and ~90% of cells survived (data not shown). The SI in the plaque reduction assay was 12.7. In the XTT assay, the SIs for moi 0.5 and moi 5 were 10.2 and 6.5, respectively.

Effect of putranjivain A on viral infectivity
Figure 2 shows the virucidal activity of putranjivain A. Results demonstrated that putranjivain A had no effect on the viral infectivity at concentrations of 50 µM or below. However, at 75 and 100 µM, putranjivain A significantly killed the virus (P < 0.05). The percentages of inhibition for 0.0, 3.0, 6.0, 12.5, 25, 50, 75 and 100 µM putranjivain A-treated virus were 0.0 ± 4.6, −12.5 ± 7.8, −10.0 ± 12.9, 6.4 ± 8.4, 7.5 ± 6.5, −7.8 ± 3.4, 71.2 ± 2.8 and 99.9 ± 0.0%, respectively. Since putranjivain A concentrations of ≤50 µM showed no virucidal activity, concentrations >50 µM were therefore selected and applied in all the following mode of action studies.

Time-of-addition studies of putranjivain A on viral replication
A time-of-addition experiment was performed to investigate the effect of putranjivain A on virus replication. Compounds were added...
at intervals of 0, 2, 4, 7 and 12 h post-infection, and infected cells were harvested at 24 h post-infection. The effect of putranjivain A on virus yield was determined by plaque assay. The results showed that putranjivain A completely inhibited virus yield when added at 0–12 h. Delaying the time of adding putranjivain A did not affect its antiviral activity (Table 2). A similar result was observed when time-of-addition studies of aciclovir, a known inhibitor of viral DNA replication, were performed. This result indicates that putranjivain A affects the late stage (12 h or later) of HSV-2 infection.

Table 1. Anti-HSV-2 activity, cell cytotoxic effect and selectivity index of putranjivain A on Vero cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Antiviral activity (µM)</th>
<th>Cell cytotoxic effect (µM)</th>
<th>Selectivity index (SI)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>XTT</td>
<td>PRA</td>
<td>XTT</td>
</tr>
<tr>
<td></td>
<td>IC₅₀</td>
<td>IC₉₀</td>
<td>CC₅₀</td>
</tr>
<tr>
<td>Putranjivain A</td>
<td>7.9 ± 1.2</td>
<td>12.4 ± 2.9</td>
<td>6.3 ± 0.8</td>
</tr>
<tr>
<td>Aciclovir</td>
<td>0.8 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

*Antiviral activity was determined by XTT and plaque reduction assays (PRA). Cell cytotoxic effect was determined by XTT assay.

aValues represent the mean ± S.D. for three independent experiments.

bSI is the ratio of CC₅₀ to IC₅₀.

IC₅₀ and IC₉₀ were the concentrations that inhibited 50% and 90% of HSV-2 multiplication, respectively.

CC₅₀ was the concentration that showed 50% cellular cytotoxic effect.

Figure 2. Virucidal activity of putranjivain A (filled circles) and aciclovir (open circles) against HSV-2. The test sample was pre-incubated with HSV-2 at 26°C for 6 h. The residual infectivity was then determined by plaque assay. Each point represents the mean ± S.D. for three independent experiments. *Significant difference between test sample and solvent control (P < 0.05).

Effect of putranjivain A on viral attachment and penetration

According to the results of time-of-addition studies, putranjivain A was demonstrated to affect the late stage of HSV-2 infection. However, putranjivain A was active when it was added to cells concurrently with HSV-2. This observation suggests that putranjivain A might also disturb early events during the first 12 h of HSV-2 replication, including viral attachment, viral penetration and/or viral DNA entering...
Anti-HSV-2 activity of putranjivain A

Table 2. Effect of the putranjivain A treatment time on the anti-HSV-2 activity

<table>
<thead>
<tr>
<th>Time period of incubation (h)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>putranjivain A</td>
</tr>
<tr>
<td>0–24</td>
<td>100.0 ± 0.0*</td>
</tr>
<tr>
<td>2–24</td>
<td>100.0 ± 5.4*</td>
</tr>
<tr>
<td>4–24</td>
<td>100.0 ± 6.1*</td>
</tr>
<tr>
<td>7–24</td>
<td>100.0 ± 2.7*</td>
</tr>
<tr>
<td>12–24</td>
<td>100.0 ± 9.1*</td>
</tr>
</tbody>
</table>

The experimental concentrations of putranjivain A and aciclovir were 25 and 5 µM, respectively. Values represent the mean ± S.D. for three independent experiments. *Significant difference between test sample and solvent control (*P < 0.05).

Figure 3. Effect of putranjivain A (filled circles) and aciclovir (open circles) on HSV-2 attachment to Vero cells. Vero monolayer was pre-chilled at 4°C for 1 h. Two hundred pfu HSV-2 was then used to inoculate the cell monolayer in the absence or presence of test compound and incubated for another 3 h. The percentage of inhibition of test compound was evaluated by plaque assay. Each point represents the mean ± S.D. for three independent experiments. *Significant difference between test sample and solvent control (*P < 0.05).

Discussion

Natural products from plants and microorganisms traditionally have provided the pharmaceutical industry with one of its most important sources of ‘lead’ compounds in the search for new drugs and medicines. Over the past two decades, many compounds isolated from medicinal plants have been found to possess inhibitory activity against the replication of HSV.20,40,41 We are encouraged by the approval of n-docosanol, a naturally occurring antiviral agent, by the US Food and Drug Administration as a topical treatment for herpes labialis.22–24 Thus, research in medicinal plants and ethnopharmacology can be an alternative approach for the discovery of antiviral agents.

The present study demonstrates that putranjivain A has in vitro anti-HSV-2 activity, without significantly affecting cell viability and growth. Its inhibitory effect on HSV-2 multiplication was moi dependent. In addition, pre-treating uninfected cells with putranjivain A prevent the penetration of HSV-2 into cells (Figure 4). Inhibitory effects on viral penetration were observed as early as 10 min after putranjivain A was added.

Combined effect of aciclovir and putranjivain A on antiviral activity

The antiviral activity of various combinations of aciclovir and putranjivain A in various concentrations was evaluated by the isobologram method. Our results demonstrated that the combined effect of putranjivain A and aciclovir showed no interaction with the FIC indices between 0.73 and 0.96 (Table 3). XTT assay showed that none of these drug combinations exhibited a cytotoxic effect against Vero cell growth (data not shown).
and then washing it out did not protect cells from virus infection (data not shown).

Putranjivain A was found to inhibit HSV-2 attachment to cells. This finding is consistent with previous studies. Tannins have been reported to possess a variety of biological activities, including antiviral, antimicrobial, antioxidant, enzyme-inhibiting activities, etc. For antiherpetic activity, it is believed that the inhibitory effect is derived from the binding of tannin molecules to the protein coat of the virus and/or to the host cell membrane. The virus adsorption and the eventual virus penetration is thus arrested. Since putranjivain A is a hydrolysable tannin, it is not surprising that it shows an inhibitory effect on virus attachment.

Besides virus attachment, putranjivain A also appears to inhibit HSV-2 from penetrating into cells: during the penetration experiment, HSV-2 was allowed to attach to cells; however, it did not penetrate. The addition of putranjivain A was observed to block HSV-2 penetration. These observations suggest that putranjivain A affects the virus penetration process possibly through detaching virus that has already bound to the cell, perhaps by the disturbance of viral glycoproteins.

Time-of-addition studies revealed that putranjivain A suppresses HSV-2 multiplication in Vero cells, even when added 12 h post-infection. Since the duration of these experiments was 24 h, only one round of viral replication can occur, suggesting that putranjivain A affected other step(s) of the HSV-2 replication cycle in addition to the effects on virus attachment and penetration. Previous studies showed that samarangenin B, a condensed tannin, can suppress HSV-1 replication in Vero cells. The anti-HSV-1 activity of samarangenin B is mediated through inhibiting viral α gene expression, blocking β gene transcription, and arresting viral DNA synthesis and structural protein expression. Although both putranjivain A and samarangenin B are tannins, further studies are needed to determine whether similar effects apply to putranjivain A, in addition to the underlying mechanistic actions in blocking HSV-2 replication.

Putranjivain A was found to significantly reduce HSV-2 infectivity at concentrations higher than those used in mechanism studies. However, at low concentrations, it showed no virucidal activity. These observations indicate that the mechanisms of action of putranjivain A are not related to its virucidal ability.

As suggested by Cassady & Whitley, future antiviral agents will probably target enzymes or viral factors essential for infection or inhibit other steps in the viral infection cycle, such as viral entry, protein synthesis or capsid assembly. In this study, putranjivain A was found to: (i) reduce viral infectivity at high concentrations (Figure 2); (ii) inhibit virus replication even when added at 12 h after infection, suggesting that it affects the late stage of viral infection (Table 2); (iii) inhibit viral attachment to cells in a dose-dependent manner (Figure 3); and (iv) block viral penetration into cells (Figure 4). Although the combination of putranjivain A and aciclovir resulted in no interaction, the multiple modes of action suggest that further investigation is merited.

Acknowledgements

We thank Professor Itsuo Nishioka, Dr Gen-ichiro Nonaka and Dr Seung-Ho Lee (Faculty of Pharmaceutical Sciences, Kyushu University, Japan) for their help and for supplying us with putranjivain A. We also thank Dr Lien-Chai Chiang (College of Medicine, Kaohsiung Medical University, Taiwan) for providing the HSV-2 strain 196.

### Table 3. Inhibitory effects of putranjivain A in combination with aciclovir on the infection of HSV-2 in Vero cells

<table>
<thead>
<tr>
<th>Compound(s)</th>
<th>Mean IC₅₀ ± s.d.</th>
<th>FICₚₜᵤₐᵣᵣᵢАНₐ + FICₐₛᵢᶜᵢᵢᵥᵐᵣᵢ</th>
<th>Inhibitory effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putranjivain A alone</td>
<td>12.40 ± 2.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aciclovir alone</td>
<td>1.20 ± 0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aciclovir + 3 µM putranjivain A</td>
<td>0.71 ± 0.03</td>
<td>0.82</td>
<td>no interaction</td>
</tr>
<tr>
<td>Aciclovir + 5 µM putranjivain A</td>
<td>0.30 ± 0.04</td>
<td>0.73</td>
<td>no interaction</td>
</tr>
<tr>
<td>Aciclovir + 8 µM putranjivain A</td>
<td>0.19 ± 0.01</td>
<td>0.81</td>
<td>no interaction</td>
</tr>
<tr>
<td>Aciclovir + 10 µM putranjivain A</td>
<td>0.18 ± 0.03</td>
<td>0.96</td>
<td>no interaction</td>
</tr>
</tbody>
</table>

*Results based on three independent experiments.

**FIC**ₚₜᵤₐᵣᵣᵢАНₐ and **FIC**ₐₛᵢᶜᵢᵢᵥᵐᵣᵢ are FICs of putranjivain A and aciclovir, respectively.
References


