In vitro anti-HSV-2 activity and mechanism of action of proanthocyanidin A-1 from Vaccinium vitis-idaea

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Abstract: The aim of this study was to investigate the in vitro anti-HSV-2 activity and mechanism of action of proanthocyanidin A-1, a compound isolated from Vaccinium vitis-idaea Linn (Ericaceae). The results demonstrated that proanthocyanidin A-1 exhibited anti-HSV-2 activity. The IC50 value for the XTT assay was 73.3 ± 14.5 µM and the IC50 and IC90 values for the plaque reduction assay were 41.9 ± 2.0 and 62.8 ± 6.3 µM respectively. Proanthocyanidin A-1 showed no cell cytotoxic effect at concentrations that blocked HSV-2 infection, with a CC50 value of 282.1 ± 27.5 µM. The mechanism studies demonstrated that proanthocyanidin A-1 did not reduce viral infectivity but inhibited viral attachment and penetration and affected the late stage(s) of HSV-2 infection. It was concluded that proanthocyanidin A-1 suppressed HSV-2 infection through many modes of action and thus merits further investigation.

Keywords: Vaccinium vitis-idaea Linn; lingonberry; proanthocyanidin A-1; anti-HSV-2 activity

INTRODUCTION

Vaccinium vitis-idaea Linn (Ericaceae) is a woody, evergreen dwarf perennial shrub distributed worldwide in northern temperate, boreal and subarctic areas. It has been one of the most important food crops in the northern hemisphere since Viking days.1 According to Holloway,2 there are at least 25 common names for V vitis-idaea worldwide.2 Among the more common English names are lingonberry, cowberry, moss cranberry, mountain cranberry, partridgeberry, red whortleberry, alpine cranberry and lingon or lingen.

V vitis-idaea has been reported to have multiple uses. Its fruit has been used in juice, sauce, preserve, candy, jelly, syrup, ice cream, pickle, wine and liqueur manufacture.3–5 The arbutin extracted from its leaves is applied as a medicine for stomach disorders,6 and the flower is dried as a herbal remedy for lung ailments. The plants are used primarily as ornamental ground cover.7 Traditionally, midwives would provide V vitis-idaea juice to delivering mothers under the belief that this might prevent haemorrhage and fever.

Herpes simplex virus type 2 (HSV-2) is a single large double-strand DNA-enveloping virus. It is one of the most common viral infections in humans and is responsible for diseases ranging from gingivostomatitis to keratoconjunctivitis, genital disease, encephalitis and infection of newborn and immunocompromised patients.8 According to epidemiological surveys, the HSV infection rate has been continuously increasing in most countries.9–11 After the primary infection, HSV tends to persist in the neuron of the ganglion.12 Reactivation of latent HSV, which is very common during deficiency of immunity, causes recurrent herpetic infection.

Many plants are currently investigated for their biological activity, and some of them have been used as functional foods. According to previous reports, increasing numbers of people in developed and developing countries are utilising natural products for their primary healthcare needs.13,14 Since V vitis-idaea is an important food in northern hemisphere countries and herpes virus infection is of high prevalence in those areas,9,11 we were thus interested in the anti-HSV-2 activity of V vitis-idaea. In this study a series of experiments was conducted to investigate the in vitro anti-HSV-2 activity and mechanism of action of proanthocyanidin A-1 isolated from V vitis-idaea. This is the
MATERIALS AND METHODS

Test compounds
Proanthocyanidin A-1 (Fig 1) was isolated from dried whole plants of *V. vitis-idaea* as described previously. Briefly, 12.5 kg of *V. vitis-idaea* was extracted with 60% aqueous acetone at room temperature. The acetone was evaporated under reduced pressure and the resulting precipitates were removed by filtration. The filtrate was concentrated and subjected to Sephadex (Pharmacia Fine Chemical Co Ltd, Piscataway, NJ, USA) LH-20 chromatography to yield three fractions (1–3). Fraction 2 was then divided by Sephadex LH-20 chromatography (80% aqueous methanol) to give proanthocyanidin A-1. The resulting precipitates were removed by filtration. The precipitate was evaporated under reduced pressure and the filtrate was concentrated and subjected to Sephadex LH-20 chromatography (80% aqueous acetone) at room temperature. The acetone was evaporated under reduced pressure and the resulting precipitates were removed by filtration. The filtrate was concentrated and subjected to Sephadex LH-20 chromatography (80% aqueous methanol) to give proanthocyanidin A-1. The structure and purity of proanthocyanidin A-1 were confirmed by comparing its spectroscopic and physical data with those of an authentic sample as described by Morimoto et al. Its purity was >95% as determined by HPLC.

The antiviral agent acyclovir (9-[(2-hydroxyethyl)methyl]guanine, ACV) was purchased from Sigma (St Louis, MO, USA). Proanthocyanidin A-1 and ACV were suspended in dimethyl sulfoxide (DMSO) (Sigma) and stored at 4°C for up to 2 weeks. The final concentration of DMSO was less than 1%.

Cell and virus
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). The cells were propagated in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 5% foetal calf serum (FCS), 200 U ml⁻¹ penicillin G sodium, 200 µg ml⁻¹ streptomycin sulphate and 0.5 µg ml⁻¹ amphotericin B. The overlay medium for the plaque assay of HSV-2 consisted of DMEM plus 2% FCS, 1% methylcellulose and antibiotics as described above.

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 the plaque assay and expressed as plaque-forming units (pfu) ml⁻¹. Virus stocks were stored at −80°C until use.

**XTT assay**

The antiviral activity of proanthocyanidin A-1 was assayed using XTT (sodium 3’-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulphonic acid) (Sigma) as described by Weislow et al. Briefly, 10⁴ cells per 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 the cells were infected with HSV-2 at a multiplicity of infection (MOI) of 0.5 or 5.0, then the infected cells were incubated for another 72 h. The medium was then aspirated and XTT reagent was added. The plate was reincubated for an additional 2 h to allow the production of formazan. Optical densities were measured with an EIA reader (Lab Systems, Vienna, VA, USA) at a test wavelength of 492 nm and a reference wavelength of 690 nm. The antiviral activity of proanthocyanidins A-1 and its minimal concentration required to inhibit 50% HSV-2 growth (IC₅₀) were evaluated according to Cheng et al.

**Plaque reduction assay**

Vero cells were seeded into 24-well culture plates (Falcon) at a density of 10⁶ cells per well and incubated at 37°C with 5% CO₂ until reaching at least 95% confluency. The cell monolayer was then infected with 100 pfu of HSV-2 in the absence or presence of proanthocyanidin A-1 and further incubated at 37°C for 1 h with 5% CO₂. 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 with 10% formalin and stained with 1% crystal violet. The minimal concentration of proanthocyanidin A-1 required to reduce the plaque number by 50% (IC₅₀) was calculated according to Cheng et al.

**Cell cytotoxicity assay and selectivity index**

The cytotoxic effect of proanthocyanidin A-1 on proliferating Vero cells was assayed by an XTT-based method. It was performed using procedures similar to the XTT assay, except that HSV-2 was not inoculated. The 50% cell cytotoxic concentration (CC₅₀) of proanthocyanidin A-1 was calculated according to Lin et al. The selectivity index (SI) was evaluated as the ratio of CC₅₀ to IC₅₀.

**Virucidal assay**

The virucidal activity of proanthocyanidin A-1 was evaluated as described by Carlucci et al. A virus suspension containing 2 × 10⁷ pfu of HSV-2 was mixed...
with or without various concentrations of proanthocyanidin A-1 for 6 h at 26 °C. The sample was then diluted and its residual infectivity was determined by the plaque assay.

**Addition time study**

The antiviral activity of proanthocyanidin A-1 was evaluated at various time periods up to 24 h according to procedures described by Boulware et al.21 Briefly, Vero cells were seeded into 12-well culture plates (Nunc) at a density of 2 × 10^5 cells per well and incubated at 37 °C with 5% CO₂ for 24 h. The cell monolayer was then infected with 1 × 10^3 pfu of HSV-2 per well. Aliquots (100 µM) of proanthocyanidin A-1 were added to the wells at 0, 2, 4, 7 and 12 h post-infection. At 24 h post-infection, infected cells were scrapped and viruses were released from the cells by freezing/thawing three times. Cell pellets were removed by centrifugation at 1100 × g for 10 min. The supernatants were divided into small quantities and stored at −80 °C. The virus titre of each supernatant was determined by the plaque assay. The percentage inhibition was calculated as the reduction in virus titre observed in infections containing proanthocyanidin A-1 compared with that of infections containing solvent only.

**Attachment assay**

The attachment assay described by Logu et al22 was used in this study with 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 of HSV-2 in the absence or presence of serial dilutions of proanthocyanidin A-1. After incubating the infected cell monolayer at 4 °C for another 3 h, the medium was aspirated to remove unabsorbed virus. The cell monolayer was then washed with PBS three times and overlaid with 1% methylcellulose medium. The cell monolayer was reincubated at 37 °C for another 48 h before being fixed and stained. The inhibitory effect of proanthocyanidin A-1 on HSV-2 attachment to Vero cell monolayers was calculated.

**Penetration assay**

The virus penetration assay was performed as previously described.22–24 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 of HSV-2 and incubated at 4 °C for another 3 h to allow the attachment of HSV-2 to the cell monolayer. After 3 h of incubation, 100 µM proanthocyanidin A-1 was added. The control group contained no proanthocyanidin A-1. The infected cell monolayer was then incubated at 37 °C to maximise virus penetration. At 10 min intervals the infected cell monolayer was treated with PBS at pH 3 for 1 min to inactivate unpenetrated virus. PBS at pH 11 was then added immediately to neutralise the 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 at 37 °C the cell monolayer was fixed and stained. Plaques were counted and the percentage inhibition of penetration was calculated.

**Statistical analysis**

Results were expressed as mean ± standard deviation for three independent experiments in each of which two measurements were made. Student’s unpaired t-test was used to evaluate the difference between the test sample and the solvent control. A p value of less than 0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

The results showed that proanthocyanidin A-1 exhibited anti-HSV-2 activity (Table 1). The IC₅₀ value for the XTT assay was 73.3 ± 14.5 µM. For the plaque reduction assay the IC₅₀ and IC₉₀ values were 41.9 ± 2.0 and 62.8 ± 6.3 µM respectively.

The cytotoxic effect of proanthocyanidin A-1 on Vero cells was evaluated to ensure that proanthocyanidin A-1 suppressed HSV-2 multiplication without any cell cytotoxic effect. As determined by the XTT assay (Table 1), the CG₅₀ value of proanthocyanidin A-1 was 282.1 ± 27.5 µM. Proanthocyanidin A-1 showed no cell cytotoxic effect at a concentration of 150.0 µM or below (data not shown). The SI values for the XTT and plaque reduction assays were 3.8 and 6.7 respectively. Thus the inhibitory activity of proanthocyanidin A-1 on HSV-2 multiplication was concluded not to be a result of its cytotoxic effect on cells.

In an effort to investigate the mode of action of proanthocyanidin A-1 in inhibiting HSV-2 infection, the virucidal effect of proanthocyanidin A-1 was first

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**Table 1.** Anti-HSV-2 activity, cell cytotoxicity effect and selectivity index of proanthocyanidin A-1 on Vero cells²

<table>
<thead>
<tr>
<th>Compound</th>
<th>Antiviral activity (µM)</th>
<th>Cytotoxicity (µM)</th>
<th>Selectivity index (SI)³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>XTTb IC₅₀ IC₉₀ IC₉₀</td>
<td>XTT CC₅₀</td>
<td>PRA XTT PRA</td>
</tr>
<tr>
<td>Proanthocyanidin A-1</td>
<td>73.3 ± 14.5</td>
<td>62.8 ± 6.3</td>
<td>282.1 ± 27.5 3.8 6.7</td>
</tr>
<tr>
<td>ACV</td>
<td>0.8 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>

²Antiviral activity was determined by XTT assay and plaque reduction assay (PRA). Cytotoxicity was determined by XTT assay.

³Each value represents mean ± standard deviation of three independent experiments.

⁴Ratio of CC₅₀ to IC₅₀.
evaluated. The results showed that proanthocyanidin A-1 exhibited no effect on viral infectivity up to a concentration of 200.0 µM (Table 2).

An addition time experiment was performed to investigate the inhibitory effect of proanthocyanidin A-1 on the viral infectivity cycle. The compounds were incubated at 0, 2, 4, 7 and 12 h post-infection and the infected cells were then harvested at 24 h post-infection. The virus yield was determined by the plaque assay. The results showed that 100.0 µM proanthocyanidin A-1 completely suppressed HSV-2 replication even when added at 12 h post-infection (Table 3). In addition, a similar result was observed with ACV, a known inhibitor of viral DNA synthesis. Since the duration of these experiments was 24 h, only one round of viral replication can occur. Therefore proanthocyanidin A-1 was suggested to affect the late stage (12 h or later) of HSV-2 infection.

The results of the addition time assay demonstrated that proanthocyanidin A-1 was effective in inhibiting viral infection when it was added to the cells concurrently with HSV-2. This observation suggested that proanthocyanidin A-1 may also disturb any first 12 h event(s) of HSV-2 infection, including viral attachment, viral penetration, viral DNA entering the cell nucleus and/or viral α and β gene expression, in addition to affecting the late event(s) of HSV-2 infection. Therefore the effect of proanthocyanidin A-1 on viral attachment and penetration was investigated.

Fig 2 shows the effect of proanthocyanidin A-1 on HSV-2 attachment to Vero cells. Proanthocyanidin A-1 significantly inhibited HSV-2 attachment to the cells in a dose-dependent manner. The percentage inhibition values of 0.0, 1.0, 5.0, 10.0, 15.0 and 20.0 µM proanthocyanidin A-1 were 0.0 ± 0.0, 29.4 ± 6.5, 65.8 ± 14.7, 70.6 ± 3.8, 85.4 ± 17.0 and 91.7 ± 14.4% (p < 0.05) respectively. In contrast, ACV, which is known to be active in affecting only HSV replication, failed to significantly inhibit viral attachment up to 15.0 µM.

Besides viral attachment, the effect of proanthocyanidin A-1 on the penetration of HSV-2 was also investigated. Our results revealed that proanthocyanidin A-1 significantly inhibited HSV-2 from penetrating the cells (Fig 3). The inhibitory effect was observed as early as 10 min after proanthocyanidin A-1 was added.

According to previous studies, HSV attachment is primarily mediated by the viral envelope glycoprotein C (gC), which binds to heparan sulphate residues present on the proteoglycans on the surface of target cells.25 Initial binding is followed by stable attachment, a process which is dependent on the presence of gD.26 Fusion of the membrane between the virion envelope and the plasma membrane of the target cell requires glycoprotein B, D, H or L or a combination thereof.27,28 Our results on viral attachment and penetration suggested that proanthocyanidin A-1 affected viral attachment and penetration possibly through the disturbance of viral glycoproteins and/or the host cell membrane. Nevertheless, further studies are needed to clarify the mechanistic action of proanthocyanidin A-1 in blocking HSV-2 attachment and penetration.

Proanthocyanidins or condensed tannins are high-molecular-weight polymers containing the monomeric

Table 2. Effect of proanthocyanidin A-1 on HSV-2 infectivity

<table>
<thead>
<tr>
<th>Conc (µM)</th>
<th>ACV</th>
<th>Proanthocyanidin A-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>100.0 ± 17.1</td>
<td>100.0 ± 16.5</td>
</tr>
<tr>
<td>0.5</td>
<td>116.5 ± 14.9</td>
<td>ND</td>
</tr>
<tr>
<td>2.5</td>
<td>98.4 ± 8.6</td>
<td>ND</td>
</tr>
<tr>
<td>5.0</td>
<td>94.5 ± 18.3</td>
<td>ND</td>
</tr>
<tr>
<td>10.0</td>
<td>112.5 ± 12.8</td>
<td>ND</td>
</tr>
<tr>
<td>12.5</td>
<td>ND</td>
<td>88.4 ± 19.6</td>
</tr>
<tr>
<td>20.0</td>
<td>100.8 ± 11.3</td>
<td>ND</td>
</tr>
<tr>
<td>25.0</td>
<td>ND</td>
<td>117.5 ± 13.2</td>
</tr>
<tr>
<td>50.0</td>
<td>121.3 ± 14.9</td>
<td>113.4 ± 26.3</td>
</tr>
<tr>
<td>100.0</td>
<td>ND</td>
<td>106.4 ± 20.1</td>
</tr>
<tr>
<td>150.0</td>
<td>ND</td>
<td>90.7 ± 21.8</td>
</tr>
<tr>
<td>200.0</td>
<td>ND</td>
<td>109.3 ± 11.2</td>
</tr>
</tbody>
</table>

Each value represents mean ± standard deviation of three independent experiments. ND, not detected.

Table 3. Effect of addition time of proanthocyanidin A-1 on anti-HSV-2 activity

<table>
<thead>
<tr>
<th>Time period of incubation (h)</th>
<th>ACV</th>
<th>Proanthocyanidins A-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–24</td>
<td>100.0 ± 0.0*</td>
<td>100.0 ± 9.6*</td>
</tr>
<tr>
<td>2–24</td>
<td>100.0 ± 1.2*</td>
<td>100.0 ± 0.0*</td>
</tr>
<tr>
<td>4–24</td>
<td>100.0 ± 0.6*</td>
<td>100.0 ± 6.1*</td>
</tr>
<tr>
<td>7–24</td>
<td>99.9 ± 4.9*</td>
<td>100.0 ± 10.5*</td>
</tr>
<tr>
<td>12–24</td>
<td>99.9 ± 14.9*</td>
<td>100.0 ± 4.7*</td>
</tr>
</tbody>
</table>

The experimental concentrations of ACV and proanthocyanidin A-1 were 5.0 and 100.0 µM respectively. Each value represents mean ± standard deviation of three independent experiments.

* Significant difference between test sample and solvent control (p < 0.05).

Figure 2. Effect of proanthocyanidin A-1 (full circles) and ACV (open circles) on HSV-2 attachment to Vero cells. Each point represents the mean ± SD of three independent experiments. Data points without an error bar indicate that the SD was too small to be shown. An asterisk indicates a significant difference between the test sample and the solvent control (p < 0.05).
unit flavan-3-ol. They are naturally occurring plant metabolites that are widely available in fruits, vegetables, nuts, seeds, flowers and barks. In many cases, these condensed tannins are the active compounds of the medicinal plants from which they can be isolated.29 Recently, oligomeric proanthocyanidin complexes (OPCs), a group of proanthocyanidins, have been identified and are primarily known for their free radical-scavenging and antioxidant activities.30 These compounds also show antibacterial, antiviral, anticarcinogenic, anti-inflammatory, anti-allergic, antilipid peroxidation, anti-platelet aggregation, vasodilative and enzyme-inhibiting activities.29–31 The variety of biological activities of OPCs has made them widely used compounds in the phytopharmaceutical industry.30

Proanthocyanidin A-1 is a member of the OPC family. It has been shown to exhibit superoxide radical-scavenging, complement-modulating, antiviral and antibacterial activities.31–33 In this study, proanthocyanidin A-1 was found to suppress HSV-2 infection through the inhibition of viral attachment and penetration and the disturbance of the late stage of infection. The broad spectrum of biological activities of proanthocyanidin A-1 thus merits further investigation.

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We especially thank Professor Isuo Nishioka, Dr Genichiro Nonaka and Dr Satoshi Morimoto (Faculty of Pharmaceutical Sciences, Kyushu University, Japan) for their help and for providing proanthocyanidin A-1. We also thank Dr Lien-Chai Chiang (College of Medicine, Kaohsiung Medical University, Taiwan) for providing HSV-2 strain 196.

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