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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 IC_{50} value for the XTT assay was 73.3 ± 14.5 μM and the IC_{50} and IC_{90} 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 CC_{50} 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.[2–5] 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

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first report on the mechanism of action of proanthocyanidin A-1 on HSV-2 infection.

MATERIALS AND METHODS

Test compounds

Proanthocyanidin A-1 (Fig 1) was isolated from dried whole plants of *V vitis-idaea* as described previously.[15] 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 (ethanol) into three further fractions (2a–2c). Fraction 2b was further 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*.[15] Its purity was >95% as determined by HPLC.

The antiviral agent acyclovir (9-[(2-hydroxyethoxy)methyl]guanine, ACV) was purchased from Sigma (St Louis, MO, USA). Proanthocyanidin A-1 and ACV were suspended in dimethyl sulphoxide (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 Dulbeco'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.

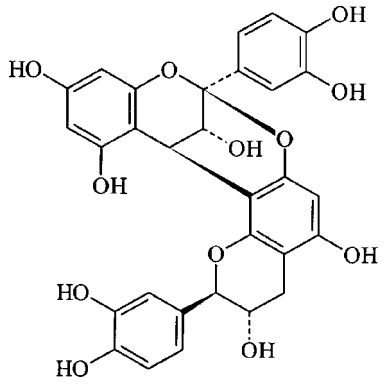

Figure 1. Structure of proanthocyanidin A-1 purified from *Vaccinium vitis-idaea*.

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-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulphonic acid) (Sigma) as described by Weislow *et al*.[16] Briefly, 10^4 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% CO_2 the cells were infected with HSV-2 at a multiplicity of infection (MOI) of 0.5 or 5.0, then various concentrations of proanthocyanidin A-1 were added. 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 (IC_{50}) were evaluated according to Cheng *et al*.[17]

Plaque reduction assay

Vero cells were seeded into 24-well culture plates (Falcon) at a density of 10^5 cells per well and incubated at 37 °C with 5% CO_2 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% CO_2. 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% (IC_{50}) was calculated according to Cheng *et al*.[17]

Cell cytotoxicity assay and selectivity index

The cytotoxic effect of proanthocyanidin A-1 on proliferating Vero cells was assayed by an XTT-based method.[18] It was performed using procedures similar to the XTT assay, except that HSV-2 was not inoculated. The 50% cell cytotoxic concentration (CC_{50}) of proanthocyanidin A-1 was calculated according to Lin *et al*.[19] The selectivity index (SI) was evaluated as the ratio of CC_{50} to IC_{50}.

Virucidal assay

The virucidal activity of proanthocyanidin A-1 was evaluated as described by Carlucci *et al*.[20] A virus suspension containing 2×10^7 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% CO_2 for 24 h. The cell monolayer was then infected with 1×10^5 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 scraped and viruses were released from the cells by freezing/thawing three times. Cell pellets were removed by centrifugation at $1100 \times g$ 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 al*[22] 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 IC_{50} value for the XTT assay was 73.3 ± 14.5 μM. For the plaque reduction assay the IC_{50} and IC_{90} 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 CC_{50} 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

Table 1. Anti-HSV-2 activity, cell cytotoxicity effect and selectivity index of proanthocyanidin A-1 on Vero cells[a]

	Antiviral activity (μM)			Cytotoxicity (μM)		
	XTT[b]	PRA[b]		XTT[b]	Selectivity index (SI)[c]	
Compound	IC_{50}	IC_{50}	IC_{90}	CC_{50}	XTT	PRA
Proanthocyanidin A-1	73.3 ± 14.5	41.9 ± 2.0	62.8 ± 6.3	282.1 ± 27.5	3.8	6.7
ACV	0.8 ± 0.1	0.4 ± 0.1	1.1 ± 0.2	>1000	>1250	>2500

[a] Antiviral activity was determined by XTT assay and plaque reduction assay (PRA). Cytotoxicity was determined by XTT assay.
[b] Each value represents mean ± standard deviation of three independent experiments.
[c] Ratio of CC_{50} to IC_{50}.

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.

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

	Residual infectivity (% of control)	
Conc (μM)	ACV	Proanthocyanidin A-1
0.0	100.0 ± 17.1	100.0 ± 16.5
0.5	116.5 ± 14.9	ND
2.5	98.4 ± 8.6	ND
5.0	94.5 ± 18.3	ND
10.0	112.5 ± 12.8	ND
12.5	ND	88.4 ± 19.6
20.0	100.8 ± 11.3	ND
25.0	ND	117.5 ± 13.2
50.0	121.3 ± 14.9	113.4 ± 26.3
100.0	ND	106.4 ± 20.1
150.0	ND	90.7 ± 21.8
200.0	ND	109.3 ± 11.2

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

Time period of incubation (h)	Inhibition (%)	
	ACV	Proanthocyanidins A-1
0–24	100.0 ± 0.0*	100.0 ± 9.5*
2–24	100.0 ± 1.2*	100.0 ± 0.0*
4–24	100.0 ± 0.6*	100.0 ± 6.1*
7–24	99.9 ± 4.9*	100.0 ± 10.5*
12–24	99.9 ± 14.9*	100.0 ± 4.7*

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 ($p < 0.05$).

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

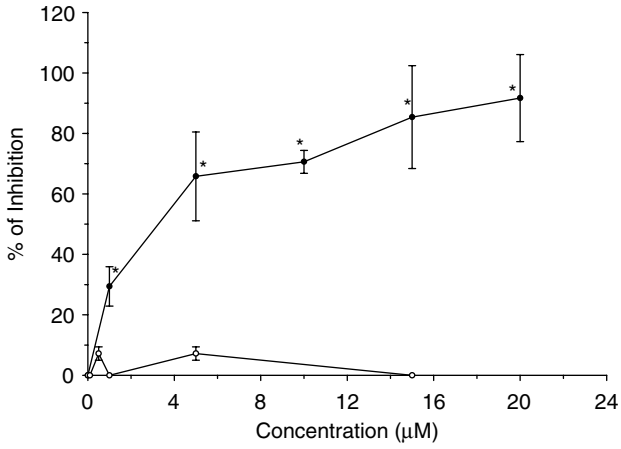

Figure 2. Effect of proanthocyanidin A-1 (full circles) and ACV (open circles) on HSV-2 attachment to Vero cells. Each point represents the mean ± SD of three independent experiments. Data points without an error bar indicate that the SD was too small to be shown. An asterisk indicates a significant difference between the test sample and the solvent control ($p < 0.05$).

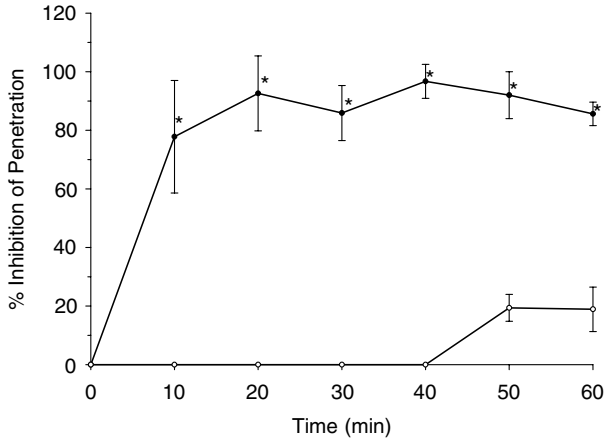

Figure 3. Effect of proanthocyanidin A-1 (full circles) and ACV (open circles) on penetration of HSV-2. Each point represents the mean ± SD of three independent experiments. Data points without an error bar indicate that the SD was too small to be shown. An asterisk indicates a significant difference 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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REFERENCES

1 Stang EJ, Weis GG and Klueh J, Lingonberry: potential new fruit for the northern United States, in *Advances in New Crops*, Ed by Janick J and Simon JE. Timber Press, Portland, OR, pp 321–323 (1990).
2 Holloway PF, Studies on vegetative and reproductive growth of lingonberry (*Vaccinium vitis-idaea* L). *PhD Dissertation*, University of Minnesota, St Paul, MN (1981).
3 Iwagaki H, Ishikawa S, Tamada T and Koike H, The present status of blueberry work and wild *Vaccinium* spp in Japan. *Acta Hort* **61**:331–334 (1977).
4 Liebster G, Experimental and research work on fruit species of the genus *Vaccinium* in Germany. *Acta Hort* **61**:19–24 (1977).
5 Müller HP, The use of *Vaccinium* fruit in the dairy industry. *Acta Hort* **61**:343–347 (1977).
6 Racz G, Fuzi I and Fulop L, A method for determination of the arbutin content of cowberry leaves (*Folium vitis-idaea*). *Rumanian Med Rev* **6**:88–90 (1962).
7 Render A, *Manual of Cultivated Trees and Shrubs*. Macmillan, New York (1940).
8 Whitley RJ, Kimberlin DW and Roizman B, Herpes simplex viruses. *Clin Infect Dis* **26**:97–109 (1998).
9 Armstrong GL, Schillinger J, Markowitz L, Nahmias AJ, Johnson RE, McQuillan GM and St Louis ME, Incidence of herpes simplex virus type 2 infection in the United States. *Am J Epidemiol* **153**:912–920 (2001).
10 Corey L, The current trend in genital herpes. Progress in prevention. *Sex Transm Dis* **21**(2, Suppl):38–44 (1994).
11 Fleming DT, McQuillan GM, Johnson RE, Nahmias AJ, Aral SO, Lee FK and St Louis ME, Herpes simplex virus type 2 in the United States, 1976 to 1994. *New Engl J Med* **337**:1105–1111 (1997).
12 Baringer RJ and Swoveland P, Recovery of herpes-simplex virus from human trigeminal ganglions. *New Engl J Med* **288**:648–650 (1973).
13 Farnsworth NR, Ethnopharmacology and future drug development: the North American experience. *J Ethnopharmacol* **38**:145–152 (1993).
14 Houghton PJ, The role of plants in traditional medicine and current therapy. *J Altern Complement Med* **1**:131–143 (1995).
15 Morimoto S, Nonaka GI and Nishioka I, Tannins and related compounds. LX. Isolation and characterization of proanthocyanidins with a doubly-linked unit from *Vaccinium vitis-idaea* L. *Chem Pharm Bull* **36**:33–38 (1988).
16 Weislow OS, Kiser R, Fine DL, Bader J, Shoemaker RH and Boyd MR, New soluble-formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS-antiviral activity. *J Natl Cancer Inst* **81**:577–586 (1989).
17 Cheng HY, Lin CC and Lin TC, Antiherpes simplex virus type 2 activity of casuarinin from the bark of *Terminalia arjuna* Linn. *Antiviral Res* **55**:447–455 (2002).
18 Scudiero DA, Shoemaker RH, Paull KD, Monks A, Tiemey S, Nofziger TH, Currens MJ, Seniff D and Boyd MR, Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res* **48**:4827–4833 (1988).
19 Lin CC, Cheng HY, Yang CM and Lin TC, Antioxidant and antiviral activities of *Euphorbia thymifolia* L. *J Biomed Sci* **9**:656–665 (2002).
20 Carlucci MJ, Ciancia M, Matulewicz MC, Cerezo AS and Damonte EB, Antiherpetic activity and mode of action of natural carrageenans of diverse structural types. *Antiviral Res* **43**:93–102 (1999).
21 Boulware SL, Bronstein JC, Nordby EC and Weber PC, Identification and characterization of benzothiophene inhibitor of herpes simplex virus type 1 replication which acts at the immediate early stage of infection. *Antiviral Res* **51**:111–125 (2001).
22 Logu AD, Loy G, Pellerano ML, Bonsignore L and Schivo ML, Inactivation of HSV-1 and HSV-2 and prevention of cell-to-cell virus spread by *Santolina insularis* essential oil. *Antiviral Res* **48**:177–185 (2000).
23 Albin R, Chase R, Risano C, Lieberman M, Ferrari E, Skelton A, Buontempo P, Cox S, DeMartino J, Wright-Minogue J,

Jirau-Lucca G, Kelly J, Afonso A, Kwong AD, Rozhon EJ and O'Connell JF, SCH 43478 and analogs: *in vitro* activity and *in vivo* efficacy of novel agents for herpesvirus type 2. *Antiviral Res* **35**:139–146 (1997).

24 Rosenthal KS, Perez R and Hodnichak C, Inhibition of herpes simplex virus type 2 penetration by cytochalasins B and D. *J Gen Virol* **66**:1601–1605 (1985).

25 Tal-Singer R, Peng C, Ponce De Leon M, Abrams WR, Banfield BW, Tufaro F, Cohen GH and Eisenberg RJ, Interaction of herpes simplex virus glycoprotein gC with mammalian cell surface molecules. *J Virol* **69**:4471–4483 (1995).

26 Rajcani J and Vojvodova A, The role of herpes simplex virus glycoproteins in the virus replication cycle. *Acta Virol* **42**:103–118 (1998).

27 Roizman B and Sears AE, Herpes simplex viruses and their replication, in *Virology*, Vol 2, 3rd edn, Ed by Fields BN, Knipe DM, Chanock RM, Hirsch MS, Melnick JL, Monath TP and Roizman B. Lippincott-Raven Publ, Philadelphia, PA, pp 2231–2295 (1996).

28 Spear PG, Entry of alphaherpesviruses into cells. *Semin Virol* **4**:167–180 (1993).

29 Haslam E, Natural polyphenols (vegetable tannins) as drugs: possible modes of action. *J Nat Prod* **59**:205–215 (1996).

30 Fine AM, Oligomeric proanthocyanidin complexes: history, structure, and phytopharmaceutical applications. *Altern Med Rev* **5**:144–151 (2000).

31 De Bruyne T, Pieters L, Witvrouw M, De Clercq E, Berghe DV and Vlietinck AJ, Biological evaluation of proanthocyanidin dimers and related polyphenols. *J Nat Prod* **62**:954–958 (1999).

32 Ho KY, Tsai CC, Huang JS, Lin TC, Hsu YF and Lin CC, Antioxidant activity of tannin compounds from *Vaccinium vitis-idaea* L. *J Pharm Pharmacol* **51**:1075–1078 (1999).

33 Ho KY, Tsai CC, Huang JS, Chen CP, Lin TC and Lin CC, Antimicrobial activity of tannin components from *Vaccinium vitis-idaea* L. *J Pharm Pharmacol* **53**:187–191 (2001).