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Antiherpes simplex virus type 2 activity of casuarinin from the bark of *Terminalia arjuna* Linn.

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Abstract

Casuarinin, a hydrolyzable tannin isolated from the bark of *Terminalia arjuna* Linn. (Combretaceae), was investigated for its antiviral activity on herpes simplex type 2 (HSV-2) in vitro. Results showed that the IC_{50} of casuarinin in XTT and plaque reduction assays were 3.6 ± 0.9 and 1.5 ± 0.2 μM, respectively. The 50% cytotoxic concentration for cell growth (CC_{50}) was 89 ± 1 μM. Thus, the selectivity index (SI) (ratio of CC_{50} to IC_{50}) of casuarinin was 25 and 59 for XTT and plaque reduction assays, respectively. Casuarinin continued to exhibit antiviral activity even added 12 h after infection. During the attachment assay, casuarinin was shown to prevent the attachment of HSV-2 to cells. Furthermore, casuarinin also exhibited an activity in inhibiting the viral penetration. Interestingly, casuarinin was virucidal at a concentration of 25 μM, reducing viral titers up to 100,000-fold. This study concludes that casuarinin possesses anti-herpesvirus activity in inhibiting viral attachment and penetration, and also disturbing the late event(s) of infection.

Keywords: *Terminalia arjuna* Linn.; Casuarinin; Anti-HSV-2 activity; Hydrolyzable tannin; Combretaceae

1. Introduction

Herpes simplex virus type 2 (HSV-2) is a member of the alphaherpesvirus subfamily called the Herpesviridae. It is extremely widespread in the human population. HSV is responsible for a broad range of diseases, ranging from gingivostomatitis to keratoconjunctivitis, genital disease, encephalitis, and infection of newborn and immunocompromised patients (Whitley et al., 1998). After the primary infection, HSV tends to persist in the neuron of the ganglia (Baringer and Swoveland, 1973). Reactivation of latent HSV, which is very common during the deficiency of immunity, causes recurrent herpetic infection.

Acyclovir (ACV), valaciclovir, famciclovir and cidafovir have been used for the treatment of HSV-infection and associated diseases (Cassady

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and Whitley, 1997). However, the efficacy of these drugs is limited by the recent increase in the resistance of virus (Englund et al., 1990) and recurrence of latent virus (Bean, 1992). During the last decade, ACV-resistant herpes simplex virus has been increasingly isolated (Nugier et al., 1992), particularly from immunocompromised patients (Erlich et al., 1989). Thus, there is a need to search for new and more effective antiviral agents as an effort to prevent and/or to treat HSV infection.

Terminalia arjuna Linn. (Combretaceae) is a large woody plant, which has been reported to exhibit antioxidative (Gupta et al., 2001), hypocholesterolaemic (Gupta et al., 2001; Ram et al., 1997), antimutagenic (Kaur et al., 2001), hypolipidemic (Shaila et al., 1998) and antibacterial (Perumal Samy et al., 1998) activities. Previous studies have shown that casuarinin can induce apoptosis in HL-60 cells (Yang et al., 2000). It exhibited inhibitory effect on nitric oxide production by murine macrophage-like cell line, RAW 264.7 (Ishii et al., 1999) and cytotoxic effect on the proliferation of the PRMI-7951 melanoma cells (Kashiwada et al., 1992). Furthermore, it was also shown to inhibit carbonic anhydrase activity (Satomi et al., 1993).

Many hydrolyzable tannins, except casuarinin, have been studied for anti-herpes simplex virus activity (Fukuchi et al., 1989; Takechi et al., 1985). However, only tannic acid had been tested and was shown to inhibit the adsorption of HSV-1 to CV-1 cell (Fukuchi et al., 1989). Thus, in this study, a series of experiments were conducted to investigate the antiviral properties of casuarinin against HSV-2.

2. Materials and methods

2.1. Test compounds

Casuarinin (Fig. 1) was isolated from the bark of *T. arjuna* as described previously (Lin et al., 1996). Briefly, air-dried bark of *T. arjuna* was chipped into small pieces and extracted at room temperature with acetone–water (4:1, v/v). The extract was concentrated under reduced pressure

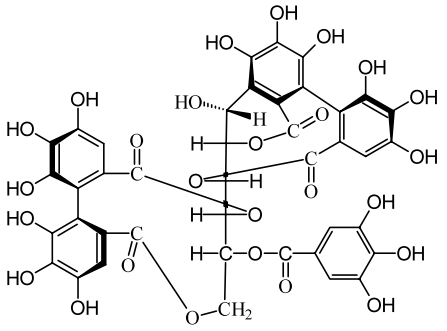

Fig. 1. Chemical structure of casuarinin isolated from the bark of *T. arjuna*.

(about 40 °C) to give an aqueous solution which contained resinous precipitate. After removing the precipitate, the filtrate was concentrated and eluted with a solvent system of methanol–water–acetone to give four fractions. Fraction III was repeated chromatographed on a sephadex LH-20, MCI-gel CHP 20P and Fuji-gel ODS G3 column to obtain casuarinin. The structure of casuarinin was confirmed by comparing its spectroscopic and physical data with the authentic sample as described by Lin et al. (1996).

ACV was purchased from Sigma Company (USA). Casuarinin and ACV were dissolved in sterile de-ionized distilled water.

2.2. Viruses and cells

All cell culture reagents and medium were purchased from Gibco BRL (Grand Island, NY). African green monkey kidney cells (Vero) (ATCC CCR-81) were obtained from the hospital of Kaohsiung Medical University (Kaohsiung, Taiwan). Cells were propagated in Dulbeco's Modified Eagle Medium (DMEM) supplemented with 5% fetal calf serum (FCS), 200 U/ml penicillin G sodium, 200 μg/ml streptomycin sulfate and 0.5 μg/ml amphotericin B. 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 titer was determined by plaque assay and was expressed as plaque forming units (PFU) per ml. Virus stocks were stored at −80 °C until use.

2.3. Antiviral assays

2.3.1. XTT assay

The antiviral activity of casuarinin was assayed using XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid) (Sigma, USA) as described by Weislow et al. (1989). Briefly, 10^4 cells/well were seeded into 96-well culture plates (Falcon). After 4 h of incubation at 37 °C with 5% CO_2, cells were infected with HSV-2 at multiplicity of infection (MOI) = 0.5 and then various concentrations of casuarinin were added. The infected cells were incubated with 2% FBS medium for another 72 h. The medium was then aspirated. Cell was rinsed with PBS and then XTT reagent was added. The plate was reincubated for an additional 2 h to allow the production of formazan. Optical densities were measured with EIA reader (Lab Systems) at a test wavelength of 492 nm and a reference wavelength of 690 nm. The antiviral activity of casuarinin was determined according to the following formula (Pauwels et al., 1988):

$$\text{Antiviral activity (\%)} = \frac{(OD_T)_{HSV} - (OD_C)_{HSV}}{(OD_c)_{mock} - (OD_C)_{HSV}} \times 100\%,$$

whereby $(OD_T)_{HSV}$ is the optical density measured with a given concentration of the casuarinin in HSV-infected cells; $(OD_C)_{HSV}$ is the optical density measured for the control untreated HSV-infected cells; $(OD_C)_{mock}$ is the optical density measured for control untreated mock-infected cells. The minimal concentration of casuarinin required to inhibit 50% HSV-2 growth (IC_{50}) was evaluated according to Weislow et al. (1989).

2.3.2. Plaque reduction assay

Vero cells were seeded into 24-well culture plates (Falcon) at a density of 10^5 cells/well and incubated until reaching at least 95% confluency. Cell monolayer was then infected with 100 PFU HSV-2 in the absence or presence of casuarinin and further incubated for 1 h. After 1 h of adsorption, 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 casuarinin was determined by the following formula:

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

The minimal concentration of casuarinin required to reduce the 50% plaque number (IC_{50}), was calculated by regression analysis of the dose–response curves generated from the data (Logu et al., 2000).

2.4. Cytotoxicity assay

Cellular viability was assayed with XTT (Scudiero et al., 1998). It was performed with the procedures similar as Section 2.3.1, except that HSV-2 was not inoculated. Cytotoxic concentration of casuarinin toward cells was calculated by the following formula:

$$\text{Percent of survival cell} = \frac{OD_T}{OD_C} \times 100\%$$

whereby OD_T and OD_C indicate the absorbencies of tested compounds and solvent control, respectively. The concentration of 50% cellular cytotoxicity (CC_{50}) of tested compounds was calculated according to Weislow et al. (1989).

2.5. Virucidal assay

Virucidal activity of casuarinin was evaluated as described by Carlucci et al. (1999). Briefly, a virus suspension containing 2×10^7 PFU HSV-2 was mixed with or without various concentrations of casuarinin for 6 h at room temperature (about 26 °C). The sample was then diluted and its

residual infectivity was determined by plaque assay.

2.6. 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. (2001). Vero cells were seeded into 12-well culture plates (Nunc) at a density of 2×10^5 cells/well and incubated at 37 °C with 5% CO_2 for 24 h. Cell monolayer was then infected with 1×10^5 PFU HSV-2/well. Ten micromolarity of casuarinin was added into wells concurrent with HSV-2 infection (0 h) or at interval of 2, 4, 7 and 12 h post-infection. After 24 h infection, infected cells were scraped and viruses were released from cells by freeze-thawing for three times. Cell pellets were removed by centrifugation at 3000 rpm for 10 min. The supernatants were divided into small quantity and then stored at −80 °C until use. Virus titer of each supernatant was determined by plaque assay. The percent of inhibition was calculated as the reduction in virus titer observed in infections containing compound compared to that of infections containing de-ionized water as a solvent control.

2.7. Attachment assay

The attachment assay described by Logu et al. (2000) was used in this study with minor modification. Briefly, Vero cell monolayer, which was grown in 24-well culture plate, was pre-chilled at 4 °C for 1 h. The medium was aspirated and cell monolayer was then infected with 200 PFU HSV-2 in the absence or presence of serial dilution of casuarinin. 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 for three times and overlaid with 1% methylcellulose medium. Cell monolayer was incubated at 37 °C or another 48 h before it was fixed and stained. The percentage of inhibition of casuarinin for HSV-2 attachment to Vero monolayers was calculated by the following formula:

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

2.8. Penetration assay

The penetration assay of herpes simplex virus into Vero cells was performed according to procedures in the literature (Albin et al., 1997; Logu et al., 2000; Rosenthal et al., 1985) with minor modifications. Vero monolayer was grown in 24-well culture plate and pre-chilled at 4 °C for 1 h. Cell monolayer was then infected with 200 PFU HSV-2 and incubated at 4 °C for another 3 h to allow the attachment of HSV-2 toward cell monolayer. After 3 h of incubation, 10 μM casuarinin was added. The control group contained no casuarinin. Infected cell monolayer was then incubated at 37 °C to maximize the penetration of viruses. At 10-min interval, infected cell monolayer was treated with PBS at pH 3 for 1 min to inactive unpenetrated virus. PBS at pH 11 was then added immediately to neutralize acidic PBS (pH 3). The neutral PBS was removed and cell monolayer was overlaid with overlay medium. After further 48 h of incubation at 37 °C, cell monolayer was fixed and stained. Plaques were counted and the percentage of inhibition of penetration was calculated by the following formula:

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

3. Results

3.1. Antiviral activity, cellular toxicity and selectivity index of casuarinin

Table 1 shows the effect of casuarinin on HSV-2. The results demonstrated that casuarinin possessed potent antiviral activity in vitro. Its IC_{50} in

Table 1
Anti-HSV-2 activity, cytotoxicity and selective index of casuarinin on Vero cells[a]

Compound	Antiviral activity, IC_{50} (μM)		Cytotoxicity, CC_{50} (μM)	Selective index[c]	
	XTT[b]	PRA[b]	XTT[b]	XTT	PRA
Casuarinin	3.6±0.9	1.5±0.2	89±1	25	59
ACV	0.8±0.1	0.3±0.1	>1000	>1250	>3333

[a] Antiviral activity was determined by XTT and plaque reduction assays. Cytotoxicity was determined by XTT assay.
[b] Values represent the mean±SD of three independent experiments.
[c] Selectivity index was the ratio of CC_{50} to IC_{50}.

XTT and plaque reduction assays was 3.6±0.9 and 1.5±0.2 μM, respectively.

The cytotoxic effect of casuarinin to Vero cell was evaluated to ensure that it showed no cytotoxic effect on cell viability at concentration which blocked HSV-2 infection. As determined by XTT assay (Table 1), the CC_{50} of casuarinin was 89±1 μM. Its selectivity index (SI) (ratio CC_{50}/IC_{50}) for XTT and plaque reduction assays was 25 and 59, respectively.

3.2. Effect of casuarinin on viral infectivity

Although results generated from XTT and plaque reduction assays showed that casuarinin was a HSV-2 inhibitor, they were unable to provide any information on the mode of action of this compound. Therefore, a series of experiments was carried out to determine the stage in which the casuarinin affected the viral life cycle. The effect of casuarinin on viral residual infectivity was investigated with various concentrations of casuarinin; they were mixed directly with HSV-2 for 6 h and then quantitated by plaque assay on Vero cells. As shown in Fig. 2, casuarinin reduced the infectivity of HSV-2 up to 5 $\log_{10}$ PFU at a concentration of 25 μM. However, no significant virucidal effect of casuarinin toward HSV-2 was observed at the concentration of 10 μM. Thus, the concentration of casuarinin at 10 μM or less was selected and used in all the following mode of action studies.

3.3. Time course studies of casuarinin

In order to investigate the stage in which the casuarinin affected the viral life cycle, experiment on the time of addition was performed. The compounds were added at the interval of 0, 2, 4, 7 and 12 h post-infection and infected cell was harvested after 24 h post-infection. Virus yield was determined by plaque forming assay. Casuarinin inhibited at least 90% virus yield when it was added at 0–12 h. Delaying the time of adding casuarinin failed to significantly decrease its antiviral activity. In other words, casuarinin remained active in inhibiting the virus even when added 12 h after infection. A similar result was observed when the time of addition of ACV, a known inhibitor of viral DNA replication was performed. This result indicates that casuarinin affected the late stage (12 h or later) of HSV-2 infection.

3.4. Effect of casuarinin on the viral attachment and penetration

According to the result of time course studies, casuarinin was shown to affect the late stage of HSV-2 infection. However, casuarinin was effective when it was added concurrently with HSV-2 to cells. This observation suggested that casuarinin might disturb any first 12 h event(s) of HSV-2 infection, including viral attachment, viral penetration, and/or the entering of viral DNA into cell nucleus etc. or no. This is in addition to the late event(s) of HSV-2 infection. Results on the effect of casuarinin on the attachment of HSV-2 to cells

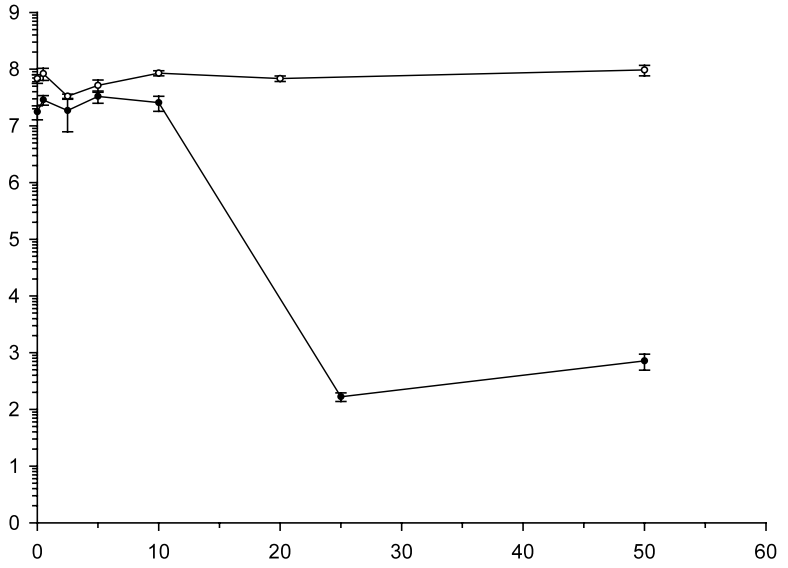

Fig. 2. Virucidal activity of casuarinin (●) and ACV (○) against HSV-2. 2×10^7 PFU HSV-2 was studied on test compounds at various concentrations (0, 0.5, 2.5, 5.0, 10.0 25.0, 50.0 and 0, 0.5, 2.5, 5.0, 10.0 20.0, 50.0 μM for casuarinin and ACV, respectively) for 6 h at room temperature. The residual infectivity of 0, 0.5, 2.5, 5.0, 10.0 25.0 and 50.0 μM casuarinin tested on HSV-2 was $1.8 \times 10^7 \pm 4.9 \times 10^6$, $2.8 \times 10^7 \pm 5.5 \times 10^6$, $1.9 \times 10^7 \pm 1.0 \times 10^7$, $3.3 \times 10^7 \pm 8.0 \times 10^6$, $2.6 \times 10^7 \pm 7.7 \times 10^6$, $1.7 \times 10^2 \pm 2.9 \times 10^1$ and $7.2 \times 10^2 \pm 2.3 \times 10^2$ PFU, respectively. Each point represents the mean ± SD of three independent experiments.

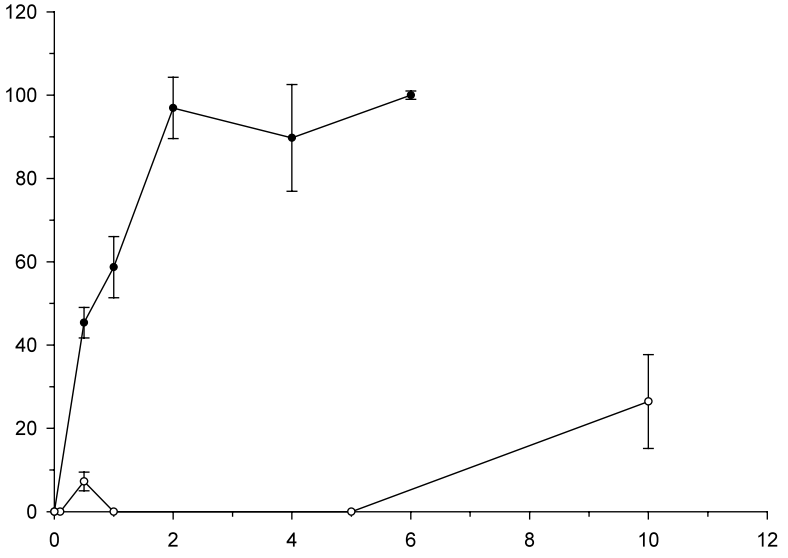

Fig. 3. Effect of casuarinin (●) and ACV (○) on HSV-2 attachment to Vero cells. Vero monolayer was pre-chilled at 4 °C for 1 h. Two hundred PFU HSV-2 was inoculated to Vero monolayer in the absence or presence of test compounds and incubated at 4 °C for another 3 h. Vero monolayer was then washed and overlayed with 1% methylcellulose medium. The percentage of inhibition of test compounds for HSV-2 attached to Vero monolayer was determined by the formula in Section 2.7. Each point represents the mean ± SD of three independent experiments.

are shown in Fig. 3. Attachment was inhibited by casuarinin in a dose-dependent manner. Casuarinin inhibited 96% of the HSV-2 attached to the cells at the concentration of 2 μM. In contrast, ACV, which is commonly known only active in affecting the HSV replication, failed to significantly inhibit virus attachment up to 10 μM. Therefore, it was concluded that casuarinin inhibited the attachment between HSV-2 and Vero cells, and its inhibitory effect was dependent to dose levels.

Besides the attachment, penetration of HSV-2 into cell was also investigated. Our studies revealed that casuarinin possessed activity in preventing the penetration of HSV-2 into cells (Fig. 4). Inhibitory effect of viral penetration was observed as early as 10 min after casuarinin was added, with the inhibition percentage of penetration more than 85%.

4. Discussion

The present study has demonstrated that casuarinin possesses potent activity against HSV-2 in vitro. According to the SI values, it is also confirmed that the antiviral activity of casuarinin was not due to the cytotoxicity of casuarinin toward cells.

Result of virucidal assay showed that casuarinin did not significantly reduce the HSV-2 infectivity at IC_{50} concentrations and at those used in mode of action studies. Therefore, the antiviral activity of casuarinin was concluded not related to its virucidal ability.

Time of addition studies demonstrated that casuarinin continued to exhibit antiviral activity even added 12 h after infection. These observations suggest that casuarinin affected the late event(s) of HSV-2 infection.

Studies on the mode of action have shown that casuarinin inhibited the attachment and penetration of HSV-2 into cells. HSV attachment is mediated by glycoprotein C (gC), which interacts with cell sulfate proteoglycans carrying heparan sulphate carbohydrate (Spear et al., 1992). The stability of attachment between viruses to cells is dependent on the presence of glycoprotein D (gD) (Rajcani and Vojvodova, 1998). Fusion of the membrane between virion envelope and plasma membrane of the target cell requires glycoproteins D, B, H and L, or in combination of all of them

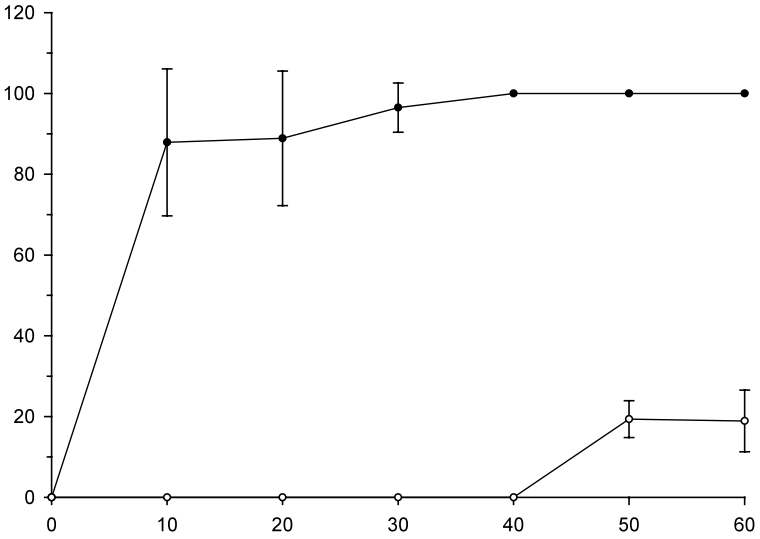

Fig. 4. Effect of casuarinin (●) and ACV (○) on the kinetics of HSV-2 penetration. Vero monolayer was pre-chilled at 4 °C for 1 h and then infected with 200 PFU HSV-2 at 4 °C for another 3 h. After 3 h incubation, 10 μM casuarinin was added. At 10 min interval, extracellular virus was killed by PBS at pH 3 for 1 min. PBS at pH 11 was then added to neutralize acidic PBS. The neutral PBS was removed and overlay medium was added. Each point represents the mean ± SD of three independent experiments.

(Roizman and Sears, 1996; Spear, 1993). According to our results on viral attachment and penetration assays, casuarinin possibly affects the attachment and penetration of viruses into cells through the disturbance of viral glycoproteins.

Many hydrolyzable tannins and condensed tannins have been tested for anti-HSV activity (Fukuchi et al., 1989; Takechi et al., 1985). The mode of antiviral activity of tannic acid was found to inhibit the virus adsorption to the cells (Fukuchi et al., 1989). Nevertheless, no further studies have been reported in elucidating the antiviral mode of tannins. In our studies, casuarinin, a hydrolyzable tannin, has been found to: (a) inhibit directly the HSV to attach to and penetrate into the cells; (b) affect the late event(s) of HSV-2 infection; and (3) exhibit viral inactivation activity at high concentrations.

Over the last decade, the number of immunocompromised patients has increased dramatically. This is the consequence of aggressive chemotherapy regimens, expanding organ transplantation and the rising incidence of human immunodeficiency virus (HIV) infection (Levin, 1993). Some alphaherpesviruses have become resistance to ACV as well as other nucleoside analogues. With the rise of immunocompromised patients and emergence of ACV-resistant herpesviruses, new medications, especially novel antiviral agent, are needed for continuous effective treatment of associated diseases.

As suggested by Cassady and Whitley (1997), future anti-herpesviruses agents will probably target at enzymes or viral factors essential for infection or inhibiting other steps in the viral infection cycle, such as viral entry, protein synthesis or capsid assembly. As casuarinin was shown to inhibit the attachment and penetration of HSV-2, and to disturb the late event(s) of HSV-2 infection, as well as inactivating the HSV-2 infectivity at high concentrations, its many modes of action therefore merit further investigation.

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