ent-Epiafzelechin-(4α→8)-epiafzelechin extracted from Cassia javanica inhibits herpes simplex virus type 2 replication

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INTRODUCTION

Herpes simplex virus (HSV) is a ubiquitous organism that causes infections in human populations throughout the world. It causes a variety of diseases ranging in severity from mild to life-threatening. In this study, ent-epiafzelechin-(4α→8)-epiafzelechin (EEE) extracted from the fresh leaves of Cassia javanica L. agnes de Wit (Leguminosae) was investigated for its in vitro anti-HSV-2 activity using XTT and plaque reduction assays. Results showed that EEE inhibited HSV-2 replication in a dose-dependent manner. The IC50 value was 83±8±10 μM for XTT and 166±8±12 μM for plaque reduction assays, respectively. EEE did not affect the viability and the proliferation of cells at antiviral concentrations. Mechanistic studies demonstrated that EEE prevented HSV-2 from penetrating the cell and also interfered with HSV-2 replication at the late stage of its life cycle. It also disturbed virus attachment but the inhibitory effect was minor. In summary, the conclusion of this study was that EEE exhibits various modes of action in suppressing HSV-2 multiplication.

Abbreviations: ACV, acyclovir; 3,3'-DOGPB, 3,3'-di-O-galloyl prodelphinidin B-2; EEE, ent-epiafzelechin-(4α→8)-epiafzelechin; 3OGPB, 3'-O-galloyl prodelphinidin B-2; SI, selectivity index; XTT, sodium 3-(1-phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid.

Received 7 April 2005
Accepted 11 October 2005

Herpes simplex virus (HSV) is a DNA-enveloped virus and exists as two types: HSV-1 and HSV-2, of which HSV-1 is more frequently associated with oral disease, and HSV-2 is more frequently associated with genital disease. It causes infections that are among the most common human diseases and are worldwide in distribution (Corey, 2002; Smith & Robinson, 2002).

Primary HSV-2 infections can be asymptomatic and unrecognized, or present clinically as typical herpes genitalis. In cases of infection during vaginal delivery, HSV-2 can cause life-threatening neonatal herpes. After primary infection, the virus is always transported in a retrograde fashion along peripheral sensory nerves to the ganglia, which subsequently leads to establishment of persistent (latent) lifetime infection. Proper stimuli can reactivate the latent virus to cause recurrent diseases. HSV infection is, therefore, recognized as a life-long infection.

HSV causes illnesses that are varied and frequently characterized by the formation of vesiculo-ulcerative lesions on the skin and mucus membranes at the infection site. The clinical manifestations range from asymptomatic disease to life-threatening illness, with the most common being genital and orolabial disease. Potential fatal sicknesses include neonatal infection, encephalitis and disseminated infection in patients with defects in cellular immunity (Aurelian, 2000).

Antiviral agents for the treatment of HSV infections have been developed for several decades. The reference compound is acyclovir (ACV), which selectively inhibits HSV DNA replication with low host-cell toxicity. Other used antiviral agents include penciclovir, trifluridine, vidarabine, valacyclovir and famciclovir. For ACV-resistant HSV infection, foscarnet and cidofovir are applied (Chilukuri & Rosen, 2003; Morfin & Thouvenot, 2003). But their toxicities have limited the use of both medicines (Brady & Bernstein, 2004).
The widespread use of ACV has lead to the emergence of HSV strains resistant to ACV. Although the prevalence of ACV-resistant HSV infection is rare among immunocompetent patients (below 1%), it is, however, about 5% among immunocompromised patients and reaches up to 18-4% among bone marrow transplant recipients (Englund et al., 1990; Nugier et al., 1992; Christophers et al., 1998; Danvez-Sztanek et al., 2004).

The increased number of immunocompromised patients, i.e. transplant, cancer or AIDS patients, has driven the need for improved antiviral agents to treat diseases caused by herpesvirus (Griffiths, 1996; Pillay et al., 2000). Although some non-nucleoside inhibitors of herpesvirus have been developed (Liuzzi et al., 1994; Kleymann et al., 2002; Wathen, 2002), none of them are officially approved for HSV therapy (De Clercq, 2001; Naesens & De Clercq, 2001). Consequently, there is still a need to search for novel and more effective antiviral agents to prevent and/or to treat HSV infection in the future.

*Cassia javanica* L. agnes de Wit (Leguminosae) is a traditionally used medicinal plant in China and Southeast Asian countries. It is conventionally believed that the medical herb can reduce fever, decrease the virulence of pathogenic organisms, regulate the flow of ‘chi’ and lubricate the intestines. In China, it is applied to treat gastric pain, cold, malaria, measles, chickenpox and constipation. After he treatment in the treatment of chickenpox, a varicella-zoster virus (VZV)-caused disease (both VZV and HSV-2 are classified in subfamily alphaherpesvirinae), suggest that this plant might also exhibit anti-HSV-2 activity.

In an effort to search for new anti-HSV-2 compounds from natural products, we conducted a series of experiments to investigate the *in vitro* antiviral activity and the mechanism of action of *ent-*epiafzelechin-(4x→8)-epiafzelechin (EEE) extracted from the fresh leaves of *C. javanica*. EEE has not been reported formerly as having anti-HSV activity. This is believed to be the first account of the anti-HSV activity of EEE.

**METHODS**

**Test compound.** Fresh leaves of *C. javanica* (3-5 kg) were extracted with 80% aqueous acetone at room temperature. The acetone was then removed by evaporation, and the resulting aqueous solution yielded precipitates that were removed by filtration. The filtrate was subjected to chromatography over Sephadex LH-20 to give two fractions: fraction 1 and fraction 2. Fraction 2 was subsequently passed over Sephadex LH-20, pre-PACK 500/C18 and Fuji-gel ODS G3 to yield EEE (0-27 g). The structure of EEE (Fig. 1) was determined by comparing its physical and spectral data with published data (Kashiwada et al., 1990). The purity of EEE was >95%.

EEE was purchased from Sigma and putranjivain A was extracted from *Euphorbia jolkini* Bioss (Cheng et al., 2002a). Both samples were used as reference samples. EEE, ACV and putranjivain A were dissolved in DMSO, and then diluted with sterile deionized distilled water before use. The final concentration of DMSO was less than 0-1%.

**Cell and virus.** All reagents and media for cell culture were purchased from Gibco-BRL. African green monkey kidney cells (Vero) (ATCC CCR-81) were obtained from the hospital of Kaohsiung Medical University.

HSV-2 strain 196 was kindly provided by Dr Lien-Chai Chiang (Department of Microbiology, College of Medicine, Kaohsiung Medical University). Its titre was determined by plaque assay and was expressed as p.f.u. ml⁻¹. Virus stocks were stored at −80°C until use.

**Antiviral assays: XTT assay.** The antiviral activity of EEE was assayed using XTT, sodium 3-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid, (Sigma) (Cheng et al., 2002a; Weislow et al., 1989). Briefly, 10⁶ cells per well were seeded into 96-well culture plates (Falcon; BD Biosciences) and incubated for 4 h. Cells were then infected with HSV-2 at m.o.i. 0-5, and various concentrations of EEE were added. After another 72 h of incubation, the minimal concentration of EEE required to inhibit 50% HSV-2 multiplication (IC₅₀) was evaluated according to Cheng et al. (2002a).

**Antiviral assays: plaque reduction assay.** The assay was performed as described previously (Cheng et al., 2002a). Briefly, 10⁵ cells per well were seeded into 24-well culture plates (Falcon; BD Biosciences) and then incubated until reaching at least 95% confluency. The cell monolayer was then infected with 100 p.f.u. HSV-2 in the absence or presence of EEE. After 2 days incubation, the minimal concentration of EEE required to reduce the 50% plaque number (IC₅₀), was calculated by regression analysis of the dose-response curves generated from the data (Cheng et al., 2002a).

**Cytotoxicity assay and selectivity index (SI).** The cytotoxic effect of EEE on growing Vero cells was assayed with an XTT-based method (Lin et al., 2002). The initial seeding cell number was 5×10⁵ p.f.u. HSV-2 in the absence or presence of EEE. After 7 days incubation, the minimal concentration of EEE required to reduce the 50% cytotoxic concentration (CC₅₀) of EEE was calculated as described by Lin et al. (2002). The SI was evaluated as the ratio of CC₅₀ to IC₅₀.

**Virucidal assay.** Virucidal activity of EEE was evaluated as described by Carlucci et al. (1999) with minor modifications. Briefly, a virus suspension containing 2×10⁶ p.f.u. HSV-2 was mixed with or without various concentrations of EEE for 6 h at room temperature.
(about 26°C). The sample was then diluted and its residual infectivity was determined by plaque assay. Putranjivain A was selected as the positive control because of its virucidal activity (Cheng et al., 2004a).

**Time-of-addition studies.** The study was performed as described by Boulware et al. (2001) and Cheng et al. (2004a). Cells, 2 × 10^5 per well, were seeded into 12-well culture plates (Nunc; Nagle Nunc International), and then infected with 1 × 10^3 p.f.u. HSV-2 per well. EEE (250–0 M) was added into wells either concurrent with HSV-2 infection (0 h) or at intervals of 2, 4, 7 and 12 h post-infection. After 24 h of infection, cells were scraped and viruses were harvested. Virus titre was determined by plaque assay, and the percentage of inhibition was calculated.

**Attachment assay.** The attachment assay was performed as described by De Logu et al. (2000) and Cheng et al. (2004a). Briefly, a Vero cell monolayer, which was grown in a 24-well culture plate, was pre-chilled at 4°C for 1 h, and then infected with 200 p.f.u. HSV-2 in the absence or presence of serial dilution of EEE. The plaque assay was performed and the percentage inhibition was calculated.

**Penetration assay.** The penetration assay of HSV-2 into Vero cells was performed according to reported procedures with minor modifications (Albin et al., 1997; Cheng et al., 2004b). A Vero cell monolayer, which was grown in a 24-well culture plate, was pre-chilled at 4°C for 1 h, and then infected with 200 p.f.u. HSV-2. After a further 3 h incubation at 4°C, 250–0 M EEE was added. Infected cells were then incubated at 37°C for 60 min. At 10 min intervals, the infected-cell monolayer was treated with PBS at pH 3 for 1 min, and then immediately neutralized by PBS at pH 11. The percentage inhibition of penetration was calculated based on the reduction of the plaque number.

**Statistical analysis.** Data are presented as the mean ± SD for triplicates of each experiment from three independent determinations. Student’s unpaired t-test was used to test the significance between the test sample and solvent control. A P value of less than 0.05 was considered to be statistically significant.

**RESULTS**

**Antiviral activity, cellular toxicity effect and selectivity index of EEE**

Results generated from XTT and plaque reduction assays (Fig. 2) showed that EEE suppressed HSV-2 multiplication in a dose-dependent fashion. The IC_{50} of EEE on virus replication was 83·8 ± 10·9 and 166·8 ± 12·9 μM for XTT and plaque reduction assays, respectively. ACV, which was used as a positive control, had an IC_{50} of 0·8 ± 0·1 and 0·3 ± 0·1 μM for XTT and plaque reduction assays, respectively. However, 500·0 μM EEE inhibited less than 30% of HSV-1 infection, which suggested that it was less active against HSV-1 than HSV-2 (data not shown).

EEE did not affect the proliferation of Vero cells at antiviral concentrations. With the concentration of 1000·0 μM, about 77·0% cells survived. The CC_{50} of EEE was >1000·0 μM and a SI (ratio of CC_{50} to IC_{50}) of >11·9 and >6·0 was calculated for XTT and plaque reduction assays, respectively.

**Effect of EEE on virus infectivity**

The effect of EEE on HSV-2 infectivity was investigated to determine whether the inhibitory effect of EEE on virus replication was derived from its ability to inactivate the virus or not. Results demonstrated that EEE up to the concentration of 500·0 μM did not affect virus infectivity (data not shown).

**Effect of the EEE treatment time on the antiviral activity**

Table 1 shows that EEE inhibited virus replication when it was added at 0–12 h. Delaying the time of addition of EEE did not affect its antiviral activity. However, adding of EEE at 20 h post-infection did not inhibit HSV-2 infection. A similar result was observed when the time of addition of ACV, a known inhibitor of viral DNA replication, was performed.

**Effect of EEE on virus attachment and penetration**

EEE only slightly hampered HSV-2 attachment to cells. The percentage of inhibition was less than 40·0% despite 250–0 M EEE being applied (data not shown). But it significantly inhibited the penetration of HSV-2 (Fig. 3). EEE prevented less than 70% virus from penetrating into
the cell membrane during the first 30 min period. The inhibition rate was, however, more than 90% at 50–60 min.

**DISCUSSION**

In this study, EEE from *C. javanica* was shown to inhibit HSV-2 replication at concentrations that exhibit little toxic effect on cell viability. However, the pre-treatment of uninfected cells with EEE and then washing it out did not prevent cells from acquiring virus infection (data not shown). Also, similar antiviral activity was observed between EEE pretreated and non-pretreated groups (data not shown). These results suggest that the pre-treatment of EEE did not have a significant effect in suppressing the multiplication of HSV-2.

The initial step of HSV-2 infection is the binding of virus envelope glycoprotein C to the heparan sulfate residues present on the proteoglycans on the surface of the target cells (Tal-Singer et al., 1995). The presence of viral glycoprotein D enhances the binding (Rajcani & Vojvodova, 1998). After binding, membrane fusion between the virus envelope and the plasma membrane of the target cell occurs. This requires the action of a number of viral glycoproteins including glycoprotein B, D, H and L (Spear, 1993; Roizman & Sears, 1996). EEE was found to inhibit virus penetration. It, however, had only slight activity in blocking virus binding. The inhibitory effect on virus penetration gradually amplified as incubation time increased. We, therefore, propose that EEE may affect the penetration of virus into cells through the disturbance of viral glycoproteins.

In addition to virus penetration, EEE was also found to interfere with the late stage of virus infection. This result was anticipated as similar outcomes have been observed in our previous studies of related compounds, 3′-O-galloyl prodolphinin B-2 (3OGPB) (Cheng et al., 2002b) and 3,3′-di-O-galloyl prodolphinin B-2 (33 DOGPB) (Cheng et al., 2003). However, EEE showed a discrepant effect in inhibiting viral attachment and also in reducing viral infectivity as compared to 3OGPB and 33 DOGPB. Both 3OGPB and 33 DOGPB can extensively block HSV-2 attachment to Vero cells and diminish the virus infectivity (Cheng et al., 2002b; Cheng et al., 2003), but EEE had only slight activity.

The diversity of the activity in inhibiting viral attachment is suggested to account for the differentiation of the structure between EEE, and 3OGPB and 33 DOGPB. EEE contains two units of epiafzelechin that are different in configuration [one is (+)-epiafzelechin and the other one is (−)-epiafzelechin], while both 3OGPB and 33 DOGPB consist of two units of (−)-epigallocatechin. Both epigallocatechin and epiafzelechin have the same skeleton structure except that the C-11 and the C-13 of epiafzelechin are substituted by OH groups thus becoming epigallocatechin. The other difference in structure is the presence of the galloyl group at C-3 for 33 DOGPB. EEE lacks the galloyl group at either C-3 or C-3′. From the above, it is supposed that the lower potency of EEE in inhibiting viral attachment may be due to the differences in structural configuration, such as the absence of the OH group at C-11 and C-13, and/or the lack of the galloyl group at C-3 and/or C-3′. Nonetheless, further

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**Table 1. Effect of the EEE treatment time on the anti-HSV-2 activity**

The experimental concentration of EEE and ACV was 250·0 and 5·0 μM, respectively. Each value represents the mean±SD of triplicates of each experiment from three independent determinations.

<table>
<thead>
<tr>
<th>Length of incubation (h)</th>
<th>Inhibition (%)</th>
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<tbody>
<tr>
<td></td>
<td>EEE</td>
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<tr>
<td>0–24</td>
<td>99·9±0·1*</td>
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<tr>
<td>2–24</td>
<td>99·8±0·1*</td>
</tr>
<tr>
<td>4–24</td>
<td>99·6±0·2*</td>
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<tr>
<td>7–24</td>
<td>99·5±0·1*</td>
</tr>
<tr>
<td>12–24</td>
<td>98·9±0·3*</td>
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<tr>
<td>20–24</td>
<td>10·8±0·2</td>
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*Significant difference (P < 0·05) between test sample and solvent control.

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**Fig. 3.** Effect of EEE (○), putranjivain A (□) and ACV (△) on the penetration of HSV-2. Each point represents the mean±SD for triplicates of each experiment from three independent determinations. The asterisk (*) indicates a significant difference between test sample and solvent control (P < 0·05).
investigations are required to elucidate the critical factor among those above that affects viral attachment inhibition.

A different magnitude for the antiviral potency of EEE was detected by XTT and plaque reduction assays, due to the two assays having diverse principles for detecting antiviral activity. The activity of the cellular enzyme mitochondrial dehydrogenase, which converts XTT to formazan, was monitored during the XTT assay. On the other hand, the number of plaques, formed as the result of lysis of virus-infected cells, was calculated by the plaque reduction assay. Similar differences were also observed between the two assays for the antiviral activity of ACV.

In summary, the anti-HSV-2 activity of EEE from C. javanica was mediated through inhibition of viral penetration and disturbance of the late stage (the stage after 12 h but before 20 h) of virus infection. Although EEE is less potent than ACV in suppressing the multiplication of HSV-2, its differences and various mechanisms of action, encourage its future study.

ACKNOWLEDGEMENTS

We thank Dr Lien-Chai Chiang (College of Medicine, Kaohsiung Medical University) for kindly providing the HSV-2 strain 196. We also thank Mr Liang-Tzung Lin for his critical reading and comments on the manuscript.

REFERENCES


