Anti-HSV Activity of Kuwanon X from Mulberry Leaves with Genes Expression Inhibitory and HSV-1 Induced NF-κB Deactivated Properties

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Six stilbene derivatives isolated from Mulberry leaves including Kuwanon X, Mulberrofuran C, Mulberrofuran G, Moracin C, Moracin M 3-O-b-glucopyranoside and Moracin M were found to have antiviral effects against herpes simplex virus type 1 and 2 (HSV-1 and HSV-2) at different potencies except for Mulberrofuran G. Kuwanon X exhibited the greatest activity against HSV-1 I5577 and clinical strains and HSV-2 strain 333 with IC₅₀ values of 2.2, 1.5 and 2.5μg/mL, respectively. Further study revealed that Kuwanon X did not inactivate cell-free HSV-1 particles, but inhibited cellular adsorption and penetration of HSV-1 viral particles. Following viral penetration, Kuwanon X reduced the expression of HSV-1 IE and L genes, and decreased the synthesis of HSV-1 DNA. Furthermore, it was demonstrated that Kuwanon X inhibited the HSV-1-induced nuclear factor (NF)-κB activation through blocking the nuclear translocation and DNA binding of NF-κB. These results suggest that Kuwanon X exerts anti-HSV activity through multiple modes and could be a potential candidate for the therapy of HSV infection.

Key words anti-herpes simplex virus (HSV) activity; Kuwanon X; penetration; gene expression; nuclear factor (NF)-κB; Mulberry

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are prevalent pathogens worldwide. HSV-1 is the main cause of orofacial herpes, cephalitis, corneal blindness, and several disorders of the peripheral nervous system; HSV-2 is usually associated with recurrent genital herpes, meningoencephalitis in neonates and meningitis in adults. Both HSV-1 and HSV-2 can cause latent infections in the neurons system, and then cause lesions at or near the point of entry into the body when they are reactivated.1,2) Recently, it was reported that HSV infections can increase the transmission of human immunodeficiency virus type 1 (HIV-1) and human T-cell leukemia virus type 1 (HTLV-1).3) During the productive infection, a successful HSV replication cycle is dependent on the completion of several steps, including α (immediate-early or IE), β (early or E), γ (late or L) genes and unpaired DNA replication based on sequential expression. The IE genes, such as UL54, encode transacting regulators of gene expression. The E genes, including UL52, are involved in viral DNA synthesis and nucleotide metabolism. The L genes, mainly encode structural proteins, e.g. ICP5 encoding the main capsid protein VP5.4)

The standard therapy for HSV infections are nucleoside analogues, such as acyclovir (ACV) and penciclovir. However, the emergence of HSV strains resistant to ACV remains an important clinical concern in immunocompromised individuals.5) Therefore, it is necessary to develop new antitherpetic agents with mechanisms of action different from that of nucleoside analogues.

Mulberry (Morus alba L.) is widely cultivated in several Asian countries. Its leaves, fruits, twigs and root barks have long been used in Chinese medicine to reduce fever, maintain liver health, improve eyesight and lower blood pressure. Besides, Mulberry leaf is commonly used as silkworm diet. In previous reports, mulberry showed health-promoting effects, including antimicrobial,6) anti-hyperglycemia,7,8) antioxidant and antitumor effects.9) Kuwanon X, a stilbene derivative, was firstly isolated from mulberry tree in 1985.10) This chemical shows good anti-oxidative activity.11) It also is an ADAMTS1 (a disintegrin and metalloprotease with thrombospondin type I motifs-1) inhibitor and possesses anti-arthritis activity.12) To date, there has been no report regarding the antiviral effects of Kuwanon X.

It has been demonstrated that HSV-1 and HSV-2 infections induce strong and persistent nuclear factor (NF)-κB activation in several cell lines.13,14) The activated NF-κB regulates host gene expression leading to reduced virus-induced apoptosis and thus allowing HSV to escape the immune response. Further research indicates that the HSV-dependent NF-κB activation is necessary for initiation of the replication cycle and synthesis of viral protein.15) Suppression of NF-κB transcriptional activity signaling pathway has been demonstrated to inhibit HSV replication.16,17) Under normal circumstances, NF-κB is sequestered in the cytoplasm through binding with its inhibitory partner IκB (including IκBz). IκBz prevents the translocation of NF-κB dimer (most commonly p50/p65) from the cytoplasm into the nucleus where it binds to DNA. In response to a diverse range of stimuli, including viral infection, IκB is phosphorylated, allowing the release and translocation of NF-κB into the nucleus.17)

In a previous study, we demonstrated that ethyl acetate and petroleum ether extracts from 20 species of Mulberry leaves possessed anti-HSV-1 activity.18) In this paper, we identified Kuwanon X and 5 other stilbene derivatives from Mulberry...
leaves with activity against HSV. Among them, Kuwanon X was selected to determine its potential antiviral mechanisms. We analyzed the effect of Kuwanon X on different events of HSV-1 replication as well as its action on NF-κB signaling.

MATERIALS AND METHODS

Chemicals and Reagents Compounds used in this study (Fig. 1) were isolated from Mulberry and their characterization has been reported.²⁹⁻³¹ All reagents were purchased from Sigma Co. (St. Louis, MO, U.S.A.) unless otherwise claimed. Anti-IκB-α, NF-κB-p65, β-actin and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology (Denver, MA, U.S.A.). The HSV VP5, Lamin B antibody and fluorescein isothiocyanate (FITC)-labeled secondary antibody were purchased from Abcam (Cambridge, U.K.).

Viruses and Cells HSV-1 15577 (standard strain) was kindly provided by Dr. Spencer H. S. Lee of Department of Microbiology and Immunology, Dalhousie University, Halifax, Canada. HSV-1 clinical strain was provided by Dr. Paul K. S., Chan of Prince of Wales Hospital, the Chinese University of Hong Kong. HSV-2333 (standard strain) was obtained from Wuhan Institute of Virology, Chinese Academy of Sciences. All virus stocks were stored at −80°C until use.

African green monkey kidney epithelial Vero cells and HeLa cells were obtained from the American Type Culture Collection (ATCC). Both cell lines were cultured in growth medium (GM) which is Dulbecco’s modified Eagle medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), penicillin (100 U/mL; Hyclone), streptomycin (100 U/mL; Hyclone) and 0.22% sodium bicarbonate. The constituents of the maintenance medium (MM) is the same as GM but contains only 2% FBS. Both cells were cultured at 37°C in a humidified 5% CO₂ atmosphere incubator.

Cytotoxicity Assay The cytotoxicity assay was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described.²⁴ Briefly, confluent Vero cells were exposed to various concentrations of samples in MM for 72 h. Then 12 μL of MTT solution (5 mg/mL in phosphate-buffered saline (PBS)) was added to each well. After 4 h’s incubation, the medium and redundant MTT was removed and the formazan crystal in the cells was dissolved by dimethyl sulfoxide (DMSO). The optical densities (OD) were read (570 nm) and the 50% cytotoxic concentration (CC₅₀) was defined as the concentration reducing 50% of cell viability, calculated by regression analysis. The maximal non-cytotoxic concentration (MNCC) was determined as the maximal concentration of the sample that did not exert cytotoxic effect to host cells. It was evaluated from the OD values of non-viable cells.

Plaque Reduction Assay The inhibitory effects of the compounds on HSV replication in Vero cells were evaluated by the plaque reduction assay.²³ Vero cell monolayers were grown in 24-well plates. HSV-1 or HSV-2 (40 plaque forming unit (PFU) per well) was added to the cells and incubated at 37°C for 1.5 h. Cultures were then overlaid with medium containing 1% methylcellulose with or without various concentrations of tested compounds. After 3 d of incubation, cells were fixed with 10% formalin and stained with 1% crystal violet. Plaques were counted and the concentration which reduced plaque numbers by 50% (IC₅₀) was calculated.²⁴ The selectivity index (SI) was calculated as the ratio of CC₅₀ to IC₅₀. ACV was used as a positive control.

Virucidal Assay The virucidal assay was performed as described²⁵,²⁶ with minor modifications. HSV-1 (15577 strain) suspension (4×10⁵ PFU) was pre-incubated with or without various concentrations of samples at 37°C for 2 h. Then the mixtures were diluted (1:1000) and their residual infectivity was determined by plaque reduction assay.

Adsorption and Penetration Assays The effects of Kuwanon X on viral adsorption was performed according to the previously described assay.²⁷ Briefly, Vero cell monolayer was pre-chilled at 4°C and then infected with HSV-1 (40 PFU per well) in the presence or absence of Kuwanon X at indicated concentrations. After incubating at 4°C for 90 min, the cells were washed with cold PBS. Fresh MM was added to each well and cultured at 37°C for 3 d. Viral infection was tested by plaque reduction assay.

Fig. 1. Chemical Structures of Compounds from Mulberry Used for Anti-HSV Studies
In the penetration assay, HSV-1 (40PFU per well) were adsorbed for 2 h at 4°C on pre-chilled Vero cells. After removal of unbound viruses, various concentrations of test compound were added. The temperature was shifted to 37°C to allow penetration for 30 min. Cells were then treated with PBS at pH 3.0 for 1 min to inactivate the non-penetrated viruses, and then PBS at pH 11.0 was added immediately to neutralize the acidic PBS. Following a wash with PBS, the cell monolayer was incubated in fresh MM at 37°C for 3 d and the penetrated viruses detected with the plaque reduction assay.

To further characterize the effect of Kuwanon X on blocking the penetration of HSV-1 into Vero cells, glass cover slips were put into wells of 24-well plate and Vero cells were inoculated on them. When cells were cultured to be monolayer, the plate was pre-chilled at 4°C for 1 h and then cells were infected with HSV-1 at a multiplicity of infection (MOI) of 10 at 4°C for 2 h for viral adsorption. After that, cells were treated with MM or MM with 20 µg/mL of Kuwanon X and immediately followed by induction of viral penetration (37°C temperature shift for 30 min). Unpenetrated viruses were inactivated with acidic PBS for 1 min, neutralized and the cells then fixed with 4% paraformaldehyde for 15 min, permeabilized with PBS−0.1% Triton X-100. The cells were incubated with viral protein VP5 antibody for 1 h followed by 1 h’s incubation with the FITC-labeled secondary antibody at room temperature. Nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) was performed for 20 min. After each step the cover slips were washed repeatedly with PBS, and lastly they were preserved in PBS. Fluorescence was recorded in a confocal laser scan microscope (LSM 510 meta; Zeiss).

**Time of Addition Assay** Time-of-addition assay was performed as the report of Xiang et al. but with some modifications. Briefly, pre-chilled Vero cell monolayer was inoculated with HSV-1 at an MOI of 1, incubated for 60 min at 4°C and then 30 min at 37°C to ensure synchronous viral replication. After removing the virus inoculum, cells were maintained at 37°C and treated with Kuwanon X (20 µg/mL) or ACV (4 µg/mL). At 0, 3, 5, 7, 9, 12, and 16 h post-infection (p.i.), 24 h p.i., the cell cultures were harvested by freeze-thawing three times. Subsequently, virus titration was carried out by plaque reduction assay.

**Real-Time PCR Analysis** For the viral RNA synthesis assay, Kuwanon X, ACV treated or viral control groups were prepared as follows. Vero cells were infected with HSV-1 at an MOI of 5 at 37°C for 1.5 h. Treatments were performed by adding Kuwanon X at a concentration of 20 µg/mL or ACV at a concentration of 4 µg/mL either simultaneously with the virus (simultaneous treatment) or after the viral penetration (any remaining attached virus was inactivated by acidic PBS and then treated with Kuwanon X or ACV, post-penetration treatment). At the indicated time points post-infection, total RNA from the infected cells of each group was extracted using Trizol (Invitrogen). Real-time PCR was conducted to determine the relative expression levels of HSV-1 IE gene UL54, E gene UL52 and L gene UL27 at 3 h, 6 h and 9 h p.i., respectively. RNAs were reverse transcribed to yield single-stranded cDNA by the Prime Script RT reagent Kit (TaKaRa, Japan).

For the viral DNA synthesis assay, Vero cells were infected with HSV-1 (MOI=5) at 37°C for 1.5 h, then Kuwanon X (20 µg/mL) or ACV (4 µg/mL) were added after the viral penetration. Infected cultures were harvested at 24 h p.i. Viral DNA was extracted using a commercial kit (Tiangen, China).

A real-time PCR assay was used for relative quantification of the reverse transcribed cDNA and the obtained viral DNA as described above. The primer pairs for UL54, UL52 and GAPDH were the same as described before. The primer pairs for UL19F is (5'-AAAATCATCGCTGCTGAG-3') and UL19R is (5'-GGGGCGCTTAAAACTGAC-3'). The real-time PCR was performed using the Roche Light Cycler 480 in a total volume of 20 µL containing 2 µL of either cDNA or DNA template, 10 µL of SYBR Premix Ex Taq™ (TaKaRa) and 10 µL of each primer. After initial denaturation at 95°C for 1 min, the amplification was carried out through 40 cycles, each consisting of denaturation at 95°C for 15 s, annealing at 58°C for 15 s, polymerization at 72°C for 40 s, and elongation at 72°C for 2 min. The relative expression was calculated using the formula: \[ \text{Rel Exp} = 2^{-\Delta\Delta C_T} \]

**Electrophoretic Mobility Shift Assay (EMSA)** HeLa cells were infected with HSV-1 (15577) at an MOI of 1 at 37°C for 1.5 h, and then treated with Kuwanon X (20 µg/mL) or ACV (4 µg/mL). Nuclear protein was obtained at 12 h p.i. and 24 h p.i. with Nuclear Protein Extraction kit (Beyotime, China) according to the recommendation. EMSA was performed using the Light Shift Chemiluminescent EMSA Kit (Thermo, U.S.A.), according to the manufacturer’s instructions. Briefly, nuclear protein (10 µg) was pre-incubated in binding buffer which consisting of 2.5% glycerol, 5 mM MgCl₂, 50 mM KCl, 50 µg/mL of poly(deoxyinosinic deoxyctydlyc acid) and 0.05% NP-40 for 10 min at room temperature. After addition of 1 µL of a biotin labeled NF-κB EMSA probe, the incubation was continued for 30 min at room temperature. The mixture was then subjected to 6% non-denaturing polyacrylamide gel electrophoresis (PAGE). DNA was transferred to a nylon membrane, UV-cross-linked and detected by chemiluminescence. The nuclear extract and unlabeled specific dsDNA probe (cold probe) were provided by the manufacturer as competing probe.

**Western Blot Analysis** HeLa cells were infected with HSV-1 (15577) at an MOI of 1 at 37°C for 1.5 h, and then treated with Kuwanon X (20 µg/mL) or ACV (4 µg/mL). At indicated times, total protein extracts were prepared according to the previously described. Nuclear protein was extracted as above and protein concentration was measured using a BCA Protein Assay Kit (Thermo Scientific, U.S.A.). Equal amounts of protein samples were subjected to sodium dodecyl sulfate (SDS)-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, U.S.A.). Membranes were blocked with 5% dry milk in TTBS (TBS with 0.1% Tween 20), incubated with primary antibodies in the same solution, then incubation with HRP-conjugated specific secondary antibodies, proteins were visualized by enhanced chemiluminescence (ECL) plus system (Beyotime Biotechnol., China) and imaged by autoradiography. The β-actin and Lamin B expression were used as the internal control.

**Statistics** Data were analyzed using two-way ANOVA. Results are expressed as the mean±standard deviation (S.D.) with significance at \(p<0.05\), \(p<0.01\) or \(p<0.001\).

**RESULTS**

Cytotoxicity and Anti-HSV Activity of Stilbene De-
rivatives from Mulberry Leaves

As shown in Table 1, Kuwanon X inhibited HSV-1 (15577), HSV-1 clinical strain, and HSV-2 standard strain with IC₅₀ values of 2.2, 1.5, and 2.5 µg/mL, respectively. The CC₅₀ of Kuwanon X was 80.3 µg/mL, so it's selective index (SI) to HSV-1 (15577 and clinical strain) and HSV-2 were all higher than 32. The antiviral activity of 5 other stilbene derivatives from Mulberry against HSV was also analyzed. Mulberrofuran C, Moracin C, Moracin M 3'-O-b-glucopyranoside, and Moracin M showed anti-HSV-1 and HSV-2 activity (IC₅₀ values were between 2.5 and 25 µg/mL) with different cytotoxic effect (CC₅₀ values were higher than 28.0 µg/mL). Mulberrofuran G had low SI value, so it was not a potential antiviral material. Among these 6 compounds, Kuwanon X was chosen for further analysis because of its excellent effect against HSV and relative low cytotoxicity.

Kuwanon X Inhibits Penetration of HSV-1

As shown in Fig. 2(A), Kuwanon X had no significant direct virucidal effect on HSV-1 (15577) even at the concentration of 20 µg/mL. This result indicated that the antiviral activity of the compound was not due to the inactivation of viral particles. In the adsorption and penetration assays, Kuwanon X displayed a moderate inhibitory effect on binding of HSV-1 to host cells (Fig. 2(B)), and it strongly inhibited HSV-1 penetration into host cells in a concentration-dependent manner (Fig. 2(C)). The IC₅₀ values of Kuwanon X in attachment and penetration

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Table 1. Cytotoxicity and Anti-HSV Activity of Stilbene Derivatives from Mulberry⁶

<table>
<thead>
<tr>
<th>Compounds</th>
<th>CC₅₀ (µg/mL)</th>
<th>MNCC (µg/mL)</th>
<th>IC₅₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1 (15577)</td>
<td>HSV-1 (Clinical)</td>
<td>HSV-2 (333)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>80.3±3.2</td>
<td>49.5±3.0</td>
<td>2.2±0.1 (37)</td>
</tr>
<tr>
<td>2</td>
<td>28.0±4.2</td>
<td>18.5±0.4</td>
<td>5.0±0.7 (5.6)</td>
</tr>
<tr>
<td>3</td>
<td>29.1±2.0</td>
<td>11.4±1.9</td>
<td>8.4±0.5 (3.6)</td>
</tr>
<tr>
<td>4</td>
<td>29.3±2.9</td>
<td>15.7±1.3</td>
<td>5.2±0.3 (5.9)</td>
</tr>
<tr>
<td>5</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>12.5±0.9 (&gt;8)</td>
</tr>
<tr>
<td>6</td>
<td>50.0±2.1</td>
<td>24.2±3.5</td>
<td>6.3±0.3 (8)</td>
</tr>
<tr>
<td>ACV</td>
<td>&gt;2500</td>
<td>&gt;200</td>
<td>0.1±0.0 (&gt;25000)</td>
</tr>
</tbody>
</table>

⁶These values represent the mean (µg/mL)±S.D. of three independent experiments. CC₅₀, 50% cytotoxic concentration for Vero cells; IC₅₀, 50% inhibitory concentration; MNCC, maximal non-cytotoxic concentration (µg/mL); Selectivity index values (SI=CC₅₀/IC₅₀); ACV (acyclovir), was used as the positive control.
assays were 9.6 and 3.5 µg/mL, respectively. ACV, as a reference compound, did not affect such stages of HSV-1 infection and was completely nonfunctional in these assays.

An additional penetration assay was conducted by confocal laser scan microscope. Infected cells were treated with or without Kuwanon X, viral penetration induced for 30 min and then stained for HSV-1 VP5. As shown in Fig. 2(D), the fluorescence in the untreated group was stronger than that of cells treated with Kuwanon X (20 µg/mL). Together, these results demonstrated that Kuwanon X could reduce HSV-1 penetration.

Kuwanon X Suppresses HSV-1 Growth Cycle To further investigate which steps of HSV-1 infection are targeted by Kuwanon X, a time-of-addition assay was performed. For that purpose, Kuwanon X (20 µg/mL) and ACV (4 µg/mL) were added to HSV-1 infected Vero cells at different times after infection. At 24 h.p.i., the cultures were harvested and virus titer was calculated. As shown in Fig. 3, the inhibitory effect was significant when Kuwanon X was added from 0 to 7 h.p.i., and then became less significant when Kuwanon X was added at later stages of infection (9-16 h.p.i.). These results indicated that kuwanon X affected the early stage of HSV-1 replication cycle.

Kuwanon X Inhibits HSV-1 RNA and DNA Synthesis HSV-1 infected Vero cells were treated with or without Kuwanon X and then the RNA levels of IE (UL54), E (UL52) and L (UL19) genes were evaluated at 3, 6, and 9 h.p.i., respectively. As shown in Figs. 4A to C, Kuwanon X treatment significantly reduced the expression of UL54 (p<0.001), UL52 (p<0.001) and UL19 (p<0.001) in the simultaneous treatment. However, in the post-infection treatment, Kuwanon X reduced the expression of both UL54 (p<0.01) and UL19 (p<0.01) but not UL52, which suggested Kuwanon X could significantly decrease RNA synthesis of HSV-1 IE and L genes, but did not suppress the RNA synthesis of E gene. These results may be partly due to the inhibitory activities of Kuwanon X during viral adsorption and penetration. Unlike Kuwanon X, ACV could significantly suppress HSV-1 E gene UL52 (p<0.001) and L gene UL19 (p<0.001), but it did not decrease the expression of IE gene UL54 under both the concurrent and post-infection treatments.

To determine whether Kuwanon X is able to affect viral DNA replication, the relative expression levels of viral DNA (amplified by UL52) was detected at 24 h.p.i. by real time PCR. As shown in Fig. 4(D), with the presence of Kuwanon X, HSV-1 DNA replication was significantly inhibited (p<0.001) and the viral DNA copy number was reduced to 14% of the number of viral control, which means Kuwanon X could fundamentally suppress HSV-1 DNA synthesis in Vero cells.

Kuwanon X Blocks HSV-1 Induced NF-κB Activation NF-κB activation is one of the crucial factors in HSV-1 efficient replication. As shown in Fig. 5(A), NF-κB p65 was detected at relatively low levels in normal cells while HSV-1 infection significantly induced p65 nuclear translocation at 12 and 24 h.p.i., in agreement with data from a previous report. However, treatment with Kuwanon X abated HSV-1 induced p65 nuclear translocation. Meanwhile, it inhibited the degradation of IkBα (inhibitor of NF-κB) which is induced by HSV-1 infection (Figs. 5(A, B)). These results suggested Kuwanon X treatment blocked HSV-1 induced activation of NF-κB.

DISCUSSION Mulberry contains a large number of stilbenoids, among which resveratrol has received much attention because of its significant biological effects. Resveratrol and several oligomeric stilbenoids have been found to possess potent activity against HSV. Here the anti-herpetic activities of 6 stilbene derivatives from Mulberry leaves were tested, and five of them showed potent antiviral effect in vitro. Among of them, Kuwanon X was chosen to determine the antiviral mechanisms due to its excellent anti-HSV action.

To explore the influence of the treatment period on the anti-HSV activity of Kuwanon X, the plaque reduction assay was performed firstly under two different conditions (data not shown). Unlike ACV, Kuwanon X was more effective in concurrent treatment (IC_{50}: 2.2 µg/mL) than that in post-infection treatment (IC_{50}: 3.0 µg/mL). These data showed that Kuwanon X inhibits HSV-1 at an early stage. In the present study, we carried out a series of experiments including virucidal assay, inhibition of attachment and penetration assay, and time of addition assay. It was observed that Kuwanon X had no virucidal effect on HSV-1, but could suppress viral attachment and penetration. Previous research have shown that most inhibitors of viral penetration are mainly biopolymers, such as sulfated polysaccharides, poly(4-styrenesulfonic acid-co-maleic acid), and SP-303, while Kuwanon X is a low molecular weight monomeric compound. So the inhibitory effect of Kuwanon X on HSV-1 penetration was further confirmed.
by confocal laser scan microscope assay (Fig. 4D). When the compound was added after the initial binding period at 4°C, it inhibited the stable attachment of HSV-1 on cell surface, suggesting that Kuwanon X could detach viruses that are already cell-bound. The inhibitory effect of Kuwanon X on penetration could be due to the disturbance of viral glycoproteins which are responsible for entry into host cells.

Apart from blocking viral penetration into host cells, Kuwanon X may suppress HSV-1 growth during early period. Firstly, according to the time-of-addition assay, the inhibitory effect was observed when Kuwanon X was added during 0 to 7 h p.i. The period of 3–7 h post-infection is the time for expression of IE and E genes during HSV infection.35) Secondly, based on the real-time RCR analyses, the relative expression level of HSV-1 α (UL54), β (UL52) and γ (UL19) genes were detected when the infected host cells were treated with Kuwanon X for 3, 6 and 9 h p.i., respectively, and the viral DNA synthesis was examined at 24 h p.i. Since Kuwanon X could inhibit HSV-1 enter into host cells, we added the compound following viral entry to investigate the expression level of IE gene and viral DNA synthesis. Our results showed that Kuwanon X reduced the expression of IE gene UL54 (p<0.01) compared to the untreated viral control. The multifunctional ICP27, encoded by the UL54 gene, is essential for HSV replication and is necessary for efficient expression of late viral gene and synthesis of DNA, playing a complex regulatory role.36) The absence of ICP27 results in decreasing accumulation of viral replication proteins, leading to a reduction in viral DNA synthesis.37,38) Accordingly, it is possible that Kuwanon X affects the expression or function of UL54 gene and subsequent blockade of L gene transcription and viral DNA replication. However, the E gene (UL52) was not affected by Kuwanon X.

NF-κB activation is a key regulator of cell proliferation, therefore NF-κB could be an attractive target for therapeutic intervention in viral diseases.13) In fact, inhibiting the activation of NF-κB has been recognized as a common mechanism against viral infection. It is surprising then that Kuwanon X showed no activity against respiratory syncytial virus (RSV) and influenza A virus (data not shown). It is reported that different viruses activate NF-κB through different strategies.13) HSV-1 appears to activate NF-κB in a biphasic way. The first wave of activation is thought to be triggered by the binding of the gD envelope glycoprotein to a cellular receptor, the herpes
 HSV-1 (15577) at an MOI of 1 and then treated with or without Kuwanon X here in reported, further investigation is needed to explore the detailed antiviral mechanism of this compound.

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**Conflict of Interest**
The authors declare no conflict of interest.

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