Orthosiphon stamineus: an Outstanding Anti-HHV1 Herb

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Abstract

Human Herpesvirus type 1 (HHV1) is one of the most important human virus. The emergence of HHV1 drug resistance has created a barrier for successful treatment of infection and thus necessitate exploration for new anti-infective agent. We have previously explored herb known as *Orthosiphon stamineus* (OS) and proved OS has virucidal activity against HHV1. The leaves aqueous extract (OSA) of the plant was observed to have detrimental effects on HHV1 envelope rendering the virus inactive. Here, in this present work we observed and explored OSA potential to treat HHV1 by inhibiting the HHV1 replication cycle. Confirmation of the successful treatment was done through Transmission Electron Microscopy (TEM). Observation on the TEM image showed that there was no production of HHV1 virions inside Vero cells after treatment with OSA as compared to non-treated cells. OSA treated cells retained the structure of the healthy cell. Observation of OSA treated cells at different time point showed that minimum of 2 hours treatment successfully reduced HHV1 plaque formation up to 75%. This post infection mechanism could be through the disruptions of HHV1 early replication stage by OSA. Our finding showed that OSA can be developed further as anti-infective agents for HHV-1.

Keywords: *Orthosiphon stamineus,* Human Herpesvirus type 1, virucidal, TEM

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Introduction

Plants are the main source for bioactive compounds in medicine and catalyst in the exploration of biologically active prototypes towards the production of synthetic drugs [1]. Depending on the method of extraction, plant extracts can contain an enormous variety of active molecules, such as phenolic compounds, essential oils, alkaloids, among others [2]. Working with crude extract can be beneficial in few ways, for example synergism effects within the complex mixtures of different compounds [3]. *Orthosiphon stamineus* (OS) also known as Misai Kucing is a well-known medicinal plant among Malaysians. Pharmacological and phytochemical analysis research on OS has been actively done since decades ago. Some biochemical active compounds isolated from OS extract has shown high potential to be developed as an antiviral agent. Flavanol for example is one of the main chemical group being isolated from various OS extract [5] and research done by Lyu et al. 2005 [4] showed that flavanol group compounds poses high anti-HHV-1 activity. Research by Ripim & Md. Nor 2018 [6] demonstrated that one of OS extract mechanism is by virucidal activity against HHV-1 where the extract destroyed the viral envelope which in turn inhibit the virus from attaching and penetrating into the host cells.

Viral diseases are the primary cause of death among human infectious diseases worldwide [7]. One of the important virus is the Human Herpesvirus type 1 (HHV1) also known as herpes labialis that can cause multiple sites lesion especially mucocutaneous lesions in the oral facial region. In Malaysia, 98.4% of oral lesion cases were reported to be caused by HHV-1 infection [8] with HHV-1 genital infection was observed to increase significantly where Chiam et al. 2010 [9] reported that 42.4% of genital herpes isolated from Obstetric and Genecology Clinic, University Malaya Medical Centre are caused by HHV-1 infection. HHV-1 is a highly contagious infection, which is also common and endemic throughout the world. Based on WHO report in 2012, an estimated 3.7 billion people under the age of 50, or 67% of the population, had HSV-1 infection. Estimated prevalence of the infection was highest in Africa (87%) and lowest in the Americas (40-50%) [10]. To manage the infection in treatment point of view, acyclovir (ACV) is the main drug used to combat the infection. Despite the availability of antiviral chemotherapy, the emergence of ACV-resistance HHV-1 made the treatment efficiency reduced especially in immunocompromised individual. Based on this situation, improving lifestyle activities and exploration of new anti-HHV-1 agent are crucial to stop the cycle of infection. Therefore, the aim of this study is to investigate the potential of OSA as an alternative treatment with different mechanism against HHV-1.

Materials and Methods

Preparation of OS aqueous extract

Preparation of crude extract (OSA) is done by boiling 200g of blended dried OS leaves in 2000 mL deionized water (ddH20) for two hours. Ratio of 1:10 sample to water was used based on previous study [10]. The extract was left to cool at room temperature, filtered and centrifuged at 1200 × g for 15 minutes to remove debris. Next, the extract was filtered using filter paper (Whatman) and was stored at −20 ° C. Extract was then freeze dried to obtain powder form extract.

Post-treatment antiviral activity screening

In the post-treatment assay, treatment with OSA was done after successful infection of cells by HHV-1. To allow successful infection, confluent Vero cells in 12-well plate were infected with 300µL of HHV-1 inoculum containing~100 pfu (plaque forming unit) of the virus and incubated for 2 hours at 37 ° C in the presence of 5% carbon dioxide for adsorption. After 2 hours, 1000µL of methylcellulose (MCS) containing 5% FBS with designated concentration of OSA ranges from 0.012 mg/mL to 0.390 mg/mL was added to each designated well. Each OSA concentration was tested in triplicate. Infected cells that were not treated with OSA served as negative controls while infected cells treated with 10µM acyclovir (ACV) served as positive controls. The plate was further incubated for 48 hours at 37°C in 5% carbon dioxide. After the virus plaques were visible, the plates were stained using crystal violet. The number of plaques was calculated under microscope and the percentage of plaque reduction was calculated using formula 1.

Ultrathin section and TEM viewing

Vero cells containing 5×10^6 cells were cultured in T75, 75cm2 culture tubes (SPL Life Science, Korea). Two samples were prepared: 1) Cells infected with 1000 PFU HHV-1 and treated with 0.39 mg/mL OSA and 2) Cells infected with 1000 PFU HHV-1 without treatment as control. Sample was collected after 16 hours of infection. Cells were harvested and centrifuged at 1500 × g for 10 minutes. Cells pellet were flashed frozen in liquid nitrogen to immediately stop cellular processes. The subsequent procedure was done according to previous study [11] with slight modifications. Briefly, cell pellets were pre-incubated in 2% glutaraldehyde and left for 24 h at 4ºC. Then, glutaraldehyde was removed and pellets were washed with cold PBS for 10 minutes three times. After washing, the pellets were post-stained with osmium tetra oxide for 2 hours. Post-staining washing was done by washing the pellets with cold PBS for 10 minutes three times. After that, the pellets were dehydrated with series of ethanol concentrations: 35%, 50%, 70%, 80%, 90% and 100% ethanol for 10 minutes respectively. The dehydrated pellets were then infiltrated with acetone-resin-mixture at 1:1 ratio for one hour, at 1:3 ratio for two hours and at 0:1 ratio for 24 hours. The pellets were then embedded in an embedding capsule and polymerised in oven at 60ºC for 24 - 48 hours until the sample was fully hardened. Samples were cut using Leica EM UC7. A 30-100 nm thickness sample were placed on copper grid, and stained with uranyl acetate to be viewed under Transmission electron microscope (TEM) (Libra 120, Carl Zeiss, Germany).

Time addition

Time addition assay was performed based on previous study [12] with slight modifications. Monolayer cells were cultured in 12-well plates. Each well containing a total of 2.0×10^5 cells/well were infected with HHV-1 (~100 PFU/well). After 2 hours of adsorption, 1000µL MCS containing 0.39 mg/mL OSA was added at intervals of 2, 4, 6, 8, 10 and 12 hours post-adsorption. The plates were then incubated for 48 hours and stained for plaque counting. The percentage of plaque reduction is calculated as previously described. Infected cells treated with 10µM ACV while was used as a positive control while infected cells without treatment was used as negative control.

Time removal

Cells and infection in time removal assay was done as described in time addition assay. After 2 hours of adsorption, OSA of 0.39 mg/mL (diluted with DMEM in 5% FBS) was added to each plate for treatment at specific time period which were for 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 hours. After designated time of incubation, the extracts were removed and cells were washed with DMEM twice to remove the remaining extracts. MCS was added to each well and incubated 48 hours for plaque to form. The plates were then stained for calculating the percentage of plaque reduction. The positive control was cells treated with 10µM ACV while the negative control was infected cells without treatment.

Statistical analysis of plaque assay

Analysis of variance (one-way ANOVA) was performed based on standard deviation, SD of three different experiments, were calculated against negative controls. Data are considered significant if p-value is less than 0.05 where level of significance was marked as (*).

Results and Discussion

Post-infection treatment activity of OSA

Post-infection treatment test was performed to study the activity of OSA in inhibiting HHV-1 replication process. This current study focus on how OSA act in inhibiting HHV-1 life cycle after successful infection of the cells. Our results showed that OSA at the concentration of 0.39 mg/mL totally inhibited HHV-1 infection in which no plaque formation was observed until the end of incubation period. This inhibition activity was found to be comparable to the positive control used, which is acyclovir at a concentration of 10µM. The plaque reduction activity was observed to be dependant to the OSA concentration used. Antiviral activity was observed to decrease significantly as the extract concentration decreased. Figure 1 showed the percentage of plaque reduction against OSA concentrations in the post-infection treatment test.

We have previously reported OSA activity against HHV1 with different mechanism of antiviral activity. OSA was previously reported to have virucidal activity, an antiviral effect directly towards the virus resulting to inability of the virus to infect due to destruction of the virus outer layer [6]. Thus both finding together proved OSA has more than one antiviral mechanisms.

Acyclovir is a commercial anti-HHV-1 drug discovered in 1978 and its structurally-related compounds, penciclovir, produced in 1980 are both major treatment modalities for treating HHV-1 infection [13]. Although acyclovir, penciclovir and its derivatives are widely used and effective in treating HHV-1 infections in immunocompetent individuals, alternative treatments are important particularly in populations of immunocompromised individuals with increasing episodes of resistance [13, 14]. Nonetheless, both of these drugs have similar antiviral mechanisms towards HHV-1. Both are analogues to natural dioxyguanosine and the activation of this drug requires phosphorylation by viral thymidine kinase which only present in virus-infected cells. Eventually, subsequent phosphorylation is done by cellular kinases that form acyclovir or penciclovir triphosphate. Triphosphate drugs compete with the natural nucleotides that results in the termination of the viral DNA chain [15]. Due to the similar

mechanism of action for both commercial drugs, resistance resulting from mutation of the drugs target site will render both drugs inactive and not effective against resistance strains. Hence, the search for alternative antiviral agent with different mechanism and target that has comparable efficiency as ACV and its derivative is indispensable.

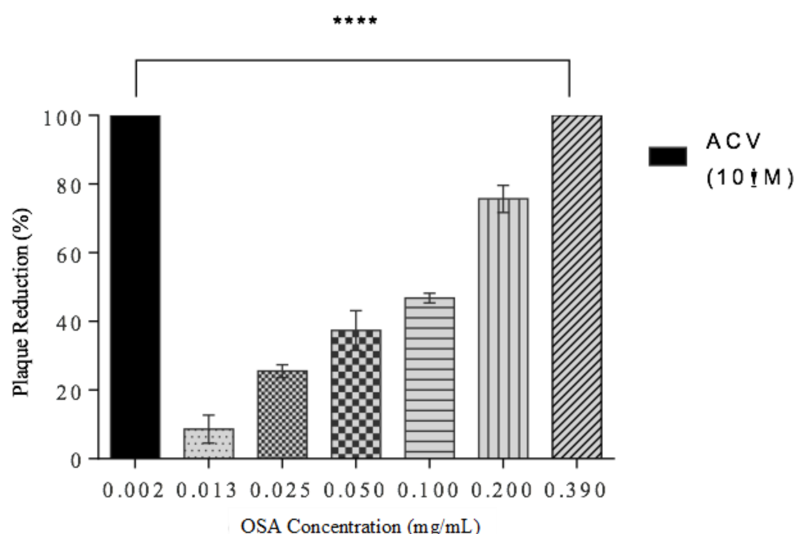

Figure 1. Percentage of plaque reduction by OSA at six different concentrations with positive control acyclovir in post-infection treatment assay. Values represent SD of three independent experiments, calculated based on negative control value, **** $p \leq 0.05$ (one-way ANOVA).

The number of promising viral targets and the class of compounds with substantial anti-herpetic properties has greatly increased over the last decade. However, effective clinical drug against both wild type virus and newly emerged resistance strains has yet to be reported. Since the discovery of acyclovir 4 decades ago, only few drugs have passed the early research. These include Helicase-Primase inhibitors (HPIs) [16], Docosanol, a topical drug that inhibits plasma cell membrane localization and HHV-1 envelope [17] and recent aminopyrimidine BX795 compounds that targeted virus replication [18]. Compounds such as acyclic H2G, carboxylic cyclobutine (lobucavir) and cyclopropane analog (A-5021) do not pass the clinical trial phase and the drugs have been disapproved due to their toxic effects [19]. Thus, ongoing development of anti-HHV-1 agents is required to provide better infection control strategies and to maintain public health.

Morphology of HHV-1 infected cells after treated with OSA under TEM
Effects of early OSA treatment on HHV-1 infected cells

Results observed in post treatment assay was further evaluated using electron transmission microscope (TEM). The morphology of infected treated Vero cells was observed under 100-fold magnification and compared with cells infected with virus without OSA treatment. In order to observe the virus inside the cell, 16-hour period was chosen. From our observation, HHV-1 that were used in this study was observed to produce first plaque between 16-18 hours. Thus, at 16 hours, new virus progenies have been produced in the infected cells, ready to exit and start a new replication cycle in adjacent cells. This hypothesis is supported by out TEM observation.

Based on TEM observations of the infected cell without OSA treatment, at about 16 hour of HHV-1 infection, the host cells were looked transformed and the HHV-1 progenies can be clearly observed (fig. 2 a – c). Observation on OSA treated cell however showed different finding. Based on the morphology of cells treated with OSA at the concentration of 0.39 mg/mL, the host cells morphology was maintained (fig. 3 d-i), the nucleus maintained with compact DNA regions (fig. 3e, labelled 2). In addition, there were no HHV1 like-structure for examples empty capsid proteins, DNA-filled capsids, or capsids that about to exit the nucleus to obtain envelopes were observed. The nucleus which play the important roles in virus production was observed to be intact in treated cells (figure 3d, labelled 4). While the cell morphology without OSA treatment showed changes in the nucleus (fig. 2, c.i) as the result of HHV-1 replication.

Replication of HHV-1 causes chromatin aberration [20], chromatin dispersal that subsequently translocate to the nucleus membrane [21]. This changes can be clearly observed in the sample without OSA treatment in fig. 2 (c.i, arrow). In OSA-treated cells (fig. 3, labelled 2) the nucleolus can be clearly observed with dense DNA region. In addition, OSA treated cells maintained the original shape of nucleus and the nuclear envelope is visible clearly as compared to cells without OSA treatment where the nuclear envelope was observed to have ruptured. Observations using TEM supported the findings where OSA inhibit HHV1 viral replication in post-infection treatment resulting in no plaque formed after the treatment with OSA. This ultrastructure observation also proves that OSA prevented HHV-1 replication at the beginning of the replication since the host cells retained its original structure.

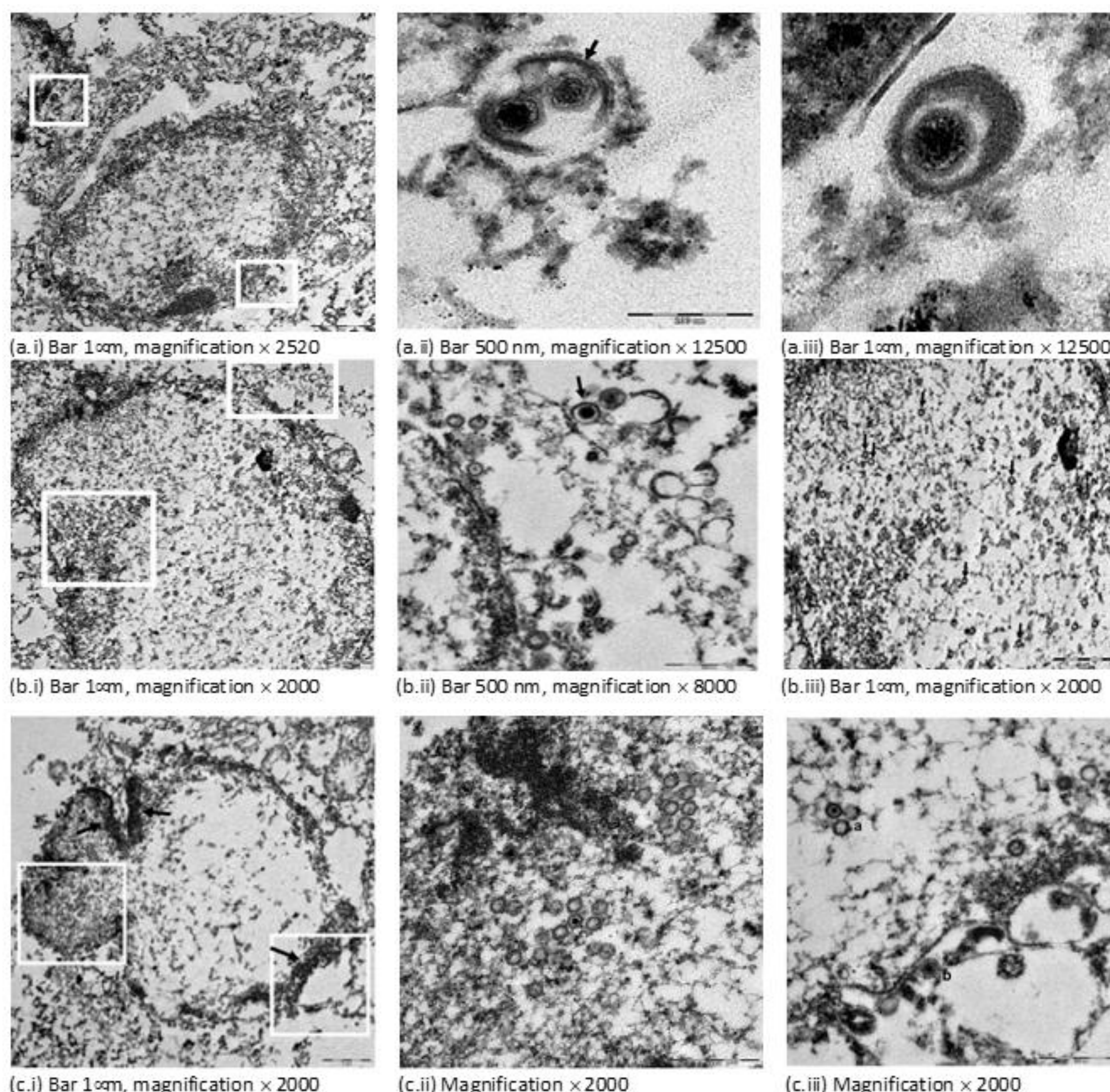

Figure 2. The morphology of Vero cells infected with HHV-1 at 16 h without and with OSA treatment. (a.i), (b.i) and (c.i) show Vero cells changed in morphology as a result of HHV-1 infection. The white box is the image of the virus particles focused on diagrams ii and iii. (a.ii) the capsid leaves the nucleus and obtained primary envelope (arrow) from the nucleus membrane. (a.iii) shows that complete HHV-1 progeny were produced at the 16th hour of infection. (b.i) shows the nucleus of the host cell is filled with HHV-1 capsid. (b.ii) shows an HHV-1 progeny left the nucleus (arrow). (b.iii) shows virus empty capsid proteins (arrows) for the HHV-1 DNA packaging process. (c.i) shows the nucleus of the host cell filled with HHV-1 capsid. In the later stages of infection, the chromatin and nucleoli of the host cell appear degraded and brought to the nuclear membrane region (arrow). (c.ii) shows the capsid produced in the nucleus without the envelope. (c.iii) shows capsid with no envelope within the nucleus (labelled a) and a dark capsid containing DNA while a complete capsid transported outside the nucleus (labelled b).

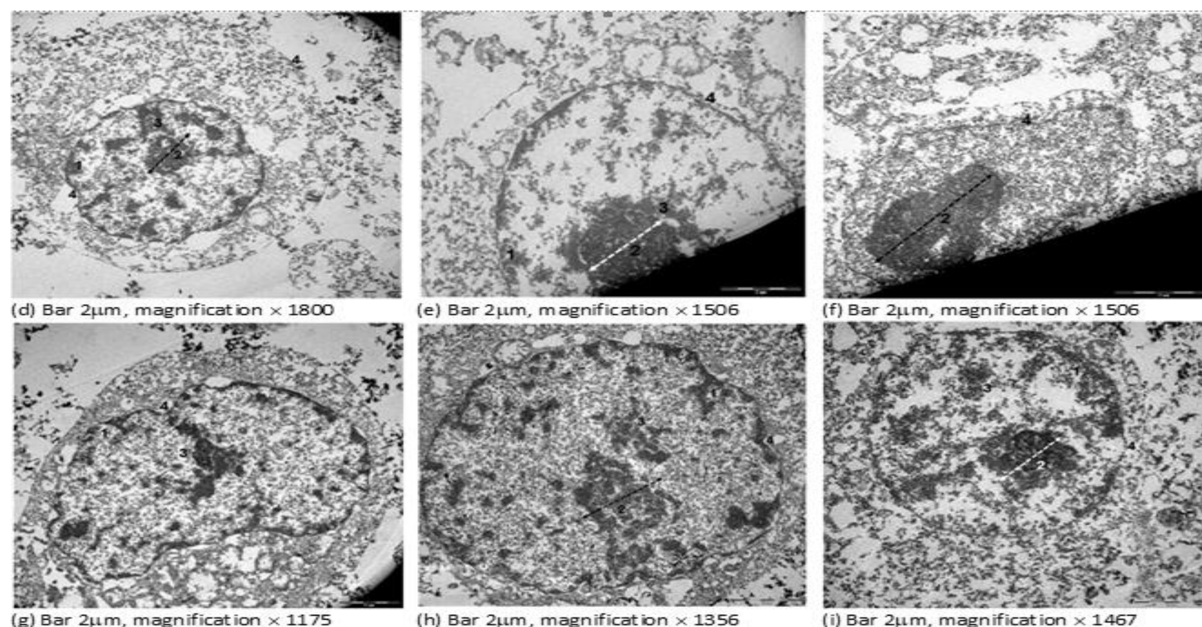

Figure 3. The morphology of Vero cells infected with HHV-1 at 16 h with OSA treatment. Cells still intact in morphology where the cell nucleus can be clearly observed, DNA threads are still compact in the nucleus and virus-like structures are not detectable in any sample images. Label (1) is a tightly packed heterochromatin located on the nuclear envelope. Label (2) shows the nucleolus, a dark area inside nucleus which consist of dense DNA strands. Label (3) shows chromatin related nuclei and label (4) shows the nuclear envelope.

Effects of OSA treatment time and duration on HHV-1 infected cells

Hypothesis that OSA reacted early during viral replication has been further tested in the time addition assay. The treatment with OSA was done at different time intervals to evaluate the most efficient treatment time in preventing HHV1 replication. In this test 0.39mg/mL concentration was used based on post-treatment assay result that showed 100% reduction in plaque formation.

In this assay, two hours after infection is calculated as time point 0 post infection (p.i). Treatment at 0 h showed 100% reduction in plaque in post-infection screening tests. In this time addition assay, the first treatment begins 2 hours after the adsorption process (2 hour p.i). The highest OSA antivirus activity was observed when treatment was given two hours after HHV1 adsorption where the plaque reduction was greater than 70%. Treatment at 4 to 8 p.i showed 50-60% plaque reduction, while the OSA activity was found reduced to less than 50% when treatment was delayed until 10 to 12 hours p.i. Based on the test, OSA activity was found to be dependent on the time of treatment where the earlier the treatment was given, the higher the percentage of plaque reduction observed. Based on these result, early treatment of OSA is hypothesised to disrupt the important stages of viral replication, especially at the early stage of gene expression. OSA treatment in the late stage of infection is found to be less effective in preventing HHV-1 replication. Therefore, OSA activity is most probably targeting early gene expression or early protein. The activity of acyclovir was found to be effective at all time point where the plaque reduction was more than 80% at all treatment. This is expected since ACV mechanism is targeting the DNA replication rather than specific genes or proteins. Figure 4

shows the graph of percentage of plaque reduction against treatment time point in the time addition assay.

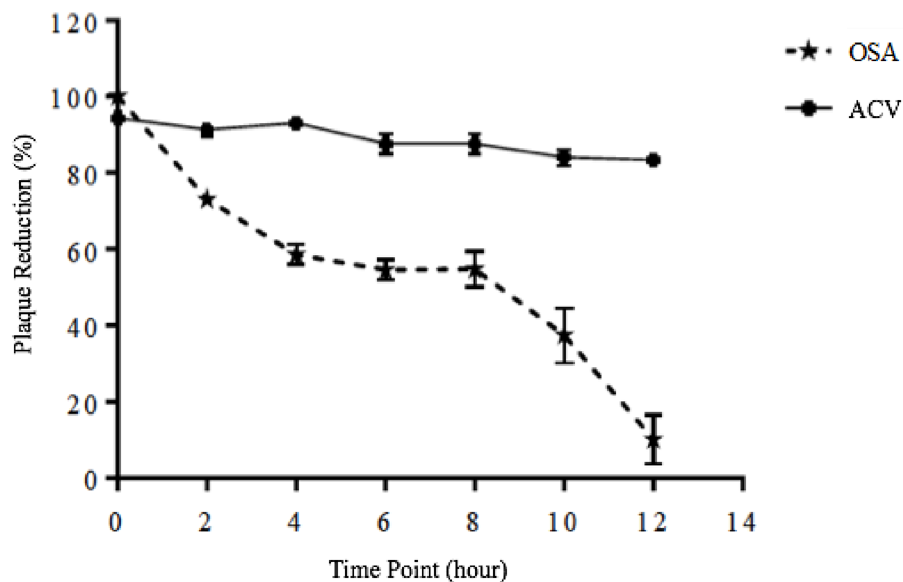

Figure 4. Percentage of plaque reduction in time addition assay.

In the time removal assay, a concentration of 0.39 mg/mL was also used. This test was conducted to determine the minimum duration of treatment required by OSA in inhibiting HHV-1 replication. In this test, OSA treatment was given immediately after two hours of HHV-1 adsorption and removed at certain time points. The results showed that minimal treatment time of OSA for 2 hours post-infection reduced plaque formation by up to 75%. Figure 5 shows the graph of the percentage of plaque reduction against OSA removal time points. In both addition and removal assays, the results found that the first 2 hours were a critical period for OSA treatment of HHV-1-infected cells.

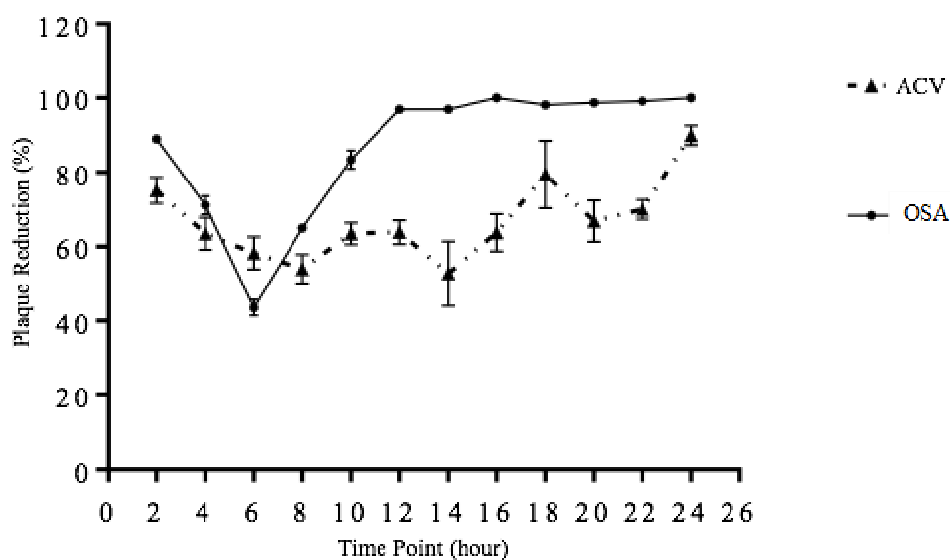

Figure 5. Graph of plaque reduction percentage against time removal time point.

In time addition and removal assays, the purpose of the OSA to be treated after the adsorption process is to see the OSA's ability to inhibit certain stages of HHV-1 replication. After the adsorption process takes place, the HHV-1 capsid is transported to the nucleus with the help of specific proteins such as UL36 and UL37 [22] for the transcription and translation of viral genes. Transcription of the HHV-1 gene occurs sequentially and regulated by viral proteins which begin with the transcription of the immediate early genes (α), the early gene (β) and subsequently the late gene (γ). The transcription and translation cycles of these genes must be maintained to produce an infectious progeny because the proteins produced are related and interdependent. Based on the results of both treatments, OSA is hypothesized to act in the early stages of infection, which most probably interrupt the transcription and translation of early genes, α and β. According to [23], expression of α genes occurs as early as 2-4 hours after infection, whereas expression of β genes occurs between 5-7 hours post-infection and followed by γ genes. Disruption by OSA in the early stages of HHV-1 replication has led to corrupted life cycle of the virus and consequently abbort progeny production.

Conclusion

In this study, OSA was observed to have antiviral effect when given as a post-treatment. However, this current study can only provide preliminary data on OSA mechanism of action. Further study using molecular analysis of gene expression or at protein level could provide further insight of the whole mechanism of action. Nonetheless, OSA showed post-treatment activity that are different from mechanism of action from ACV. Together with virucidal activity reported earlier, OSA has the potential to be developed as antiviral treatment for HHV1.

Equation

Formula 1,

$$Plaquereductionpercentage(\%) = \frac{averageofnegativecontrolplaque - averageoftreatedplaque}{averageofnegativecontrolplaque} \times 100$$

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Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of conflict of interest

The authors have no disclosures to declare.

Compliance with Ethical Standards

The work is compliant with ethical standards.

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