Orthosiphon stamineus water extracts inhibit human herpes virus 1 KOS-1 and acyclovir-resistant strains by virucidal activity and suppressing virus early infection

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ABSTRACT

Aim: To determine the efficacy and mode of action of hot and cold water extracts of Orthosiphon stamineus leaves against two strains of human herpes virus 1 (HHV-1) i.e. KOS-1 and acyclovir (ACV)-resistant UKM-1 (UKM-1) strains.

Methodology and results: Hot and cold water extracts of O. stamineus were not cytotoxic to vero cells as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) assay with 50% cytotoxicity concentration (CC50) values of 3.4 and 3.3 mg/mL respectively. Antiviral activity was determined by plaque reduction assay in post-treatment, pre-treatment and virucidal assays followed by time-addition and time removal assay to relate with the stages during the viral infection cycle. Both extracts displayed antiviral activity against HHV-1 KOS-1 and HHV-1 UKM-1 strains with 50% effective concentration (EC50) values between 0.12-0.15 mg/mL in reducing plaque formation. The calculated selectivity indices (SI) were 23 and 28 for hot and cold water extract respectively, indicating that they have good potential as antiviral agent. The extracts were virucidal towards both HHV-1 KOS-1 and HHV-1 UKM-1 strains which may directly affects the virus structure. This is supported with the fact that exposure of the extracts inhibit viral attachment and penetration to the vero cells. In time of addition assay, both extracts were effective during the early stage of virus infection cycle for HHV-1 KOS-1 strain which is in parallel with the results from the attachment and penetration studies. For HHV-1 UKM-1 strain, contact to the extracts at any time during post-infection inhibits virus replication and also progeny release.

Conclusion, significance and impact of study: Cold and hot water extracts of O. stamineus have good potential as antiviral agent against HHV-1 strain KOS-1 and more importantly against UKM-1 strain which is ACV-resistant. The extracts displayed virucidal effect and inhibition of early virus replication cycle involving viral attachment and penetration to cells.

Keywords: Orthosiphon stamineus leaf extracts, HHV-1 KOS-1 strain, Acyclovir-resistant, HHV-1 UKM-1 strain, antiviral mode of action

INTRODUCTION

Orthosiphon stamineus Benth. (Lamiaceae), locally known as “Misai Kucing” in Malaysia is a traditional medicinal herb utilised in Southeast Asia for the treatment of various inflammatory diseases such as cancer, rheumatism, hepatitis, abdominal pain, hyperlipidemia, psoriasis, diabetes, and kidney stones (Arifullah et al., 2014). The antiviral property of O. stamineus aqueous extract has been demonstrated against a clinical strain of human herpes virus 1 (HHV-1) (Ripim et al., 2018) with virucidal activity as the mode of action (Ripim and Nor, 2018). Acyclovir (ACV) and its sodium salt have been the effective drug of choice against human herpes viruses (HHV-1 and HHV-2, formerly known as herpes simplex virus type 1 or 2, HSV-1 and HSV-2). Unfortunately, these drugs are not capable to eliminate latent HHV infection. ACV-resistant virus strains may develop in prolonged drug application especially in immunocompromised patients resulting in treatment failure (Whitley, 2006). These limitations along with the absence of effective vaccine highlight the fact that new anti-HHV agents are of the highest priority to be developed.

The efficacy and mode of action of either hot or cold-water extracts of O. stamineus previously evaluated in our laboratory (Habbo et al., 2018) have not been demonstrated against HHV-1 reference KOS-1 and ACV-resistant strains. Water extraction is the commonly used method in traditional medicine and metabolites can be highly extracted including those with antiviral activities (Maria John et al., 2014). HHV-1 KOS-1 is less virulent than other HHV-1 strains (Perng et al., 2002) and...
originally isolated from human labial lesion. It was also frequently used to investigate the HHV-1 gene function and pathogenesis (Smith, 1964; Schaffer et al., 1973). The phenotypic and genotypic characterisations of HHV-1 UKM-1 have been described previously by Hussin et al. (2013) originally from a clinical strain that was induced to be ACV-resistant. This study is aimed to evaluate the efficacy of *O. stamineus* cold and hot water extracts towards HHV-1 KOS-1 and UKM-1 strains.

**MATERIALS AND METHODS**

**Plant material and preparation of extracts**

Hot and cold water extracts were prepared according to Habboo et al. (2018). Ground powder of *O. stamineus* leaves (100 g) was added to 1 L of distilled water and boiled to 60 °C for 4 h for hot extraction or left for 72 h at 4 °C for cold extraction. Extracts were centrifuged and filtered through Whatman filter paper No. 2. The filtrates were freeze dried, weighed and stored at 4 °C until used.

**Cells and virus**

African green monkey kidney cells (Vero) (ATCC CCL-81) were purchased from American Type Culture Collection and HHV-1 strains (KOS-1 and UKM-1) were obtained from stock collection at Virology Laboratory, Faculty of Science and Technology, UKM. Vero cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 5% Fetal Bovine Serum (FBS), 100 U/mL Penicillin G, Streptomycin and non-essential amino acids. The cells were incubated at 37 °C in a humidified atmosphere supplemented with 5% CO2.

**Cytotoxicity assay**

A protocol using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as reagent elaborated previously by Mosmann (1983) was followed. The percentage of cell viability was calculated using the following formula:

\[ \text{Cell viability} \% = \frac{OD \text{ of treated cells}}{OD \text{ of untreated cells (control)}} \times 100\% \]

The 50% cytotoxicity concentration (CC50) was defined as the extract concentration that is capable of reducing cell viability by 50%. The CC50 values were computed using regression analysis.

**Post-treatment assay**

Vero cells (2.0 × 10^5 cells) were added to each well of 12-well plates and incubated for 24 h at 37 °C. Subsequently, 100 plaque-forming unit (PFU)/well of virus (HHV-1 KOS-1 or UKM-1 strains) was inoculated onto the monolayers and allowed to be adsorbed for 2 h at 37 °C in a humidified atmosphere supplemented with 5% CO2. The inoculum was removed followed by addition of different concentration of extracts (0.10, 0.15, 0.20, 0.25, 0.30, 0.35 mg/mL) to 1 mL of 1% methylcellulose (MC) as overlay medium which was applied to the infected cells and further incubated under similar conditions as above for 48 h. Plaques were stained using 1% (w/v) crystal violet, dried and enumerated. The percentage of virus reduction was calculated using the following equation:

\[ \text{Plaque reduction percentage, } % = \left( \frac{PC - PT}{PC} \right) \times 100\% \]

where, PC: number of plaques produced in control without treatment; PT: number of plaques produced following treatment with extracts.

**Cells pre-treatment assay**

Cells were pretreated with extracts prior to virus infection according to Harden et al. (2009). Vero cell monolayers were subjected to treatment with various concentrations of non-cytotoxic aqueous extracts ranging from 0.10 to 0.35 mg/mL as determined from the cytotoxicity assay and subjected to incubation for 24 h at 37 °C. Non-treated vero cells served as control. Following removal of media, pretreated cells were inoculated with HHV-1 KOS-1 or UKM-1 strains (100 PFU/well) and subjected to incubation for 2 h at 37 °C. The inoculum was subsequently removed and the overlay 1% MC was added. After 48 h, the number of plaques were enumerated, and plaque reduction percentage calculated using the equation as in the post-treatment assay.

**Virus pre-treatment assay**

The assay was carried out following the procedure described by Cheng et al. (2004). Extracts (0.35 mg/mL) and HHV-1 KOS-1 or UKM-1 strains (1×10^6 PFU) were mixed in serum-free DMEM and subjected to incubation for 1 h at 37 °C in 5% CO2 prior to the dilution of the mixture to achieve the following titers (100, 1000 or 10000 PFU). The monolayers of cells were then inoculated with different virus titers and incubated for another 2 h at 37 °C. The inoculum was aspirated before the overlay 1% MC was added on the infected cells and then subjected to further incubation for 48 h. The residual virus infectivity was detected by the number of plaque formation and computed using the equation as in the post-treatment assay.

**Attachment assay**

The virus was mixed with varying concentrations of aqueous extracts and subjected to incubation for 1 min at...
room temperature. Vero cell monolayer cultured in 12 well culture plate was incubated at 4 °C for 1 h. The medium was decanted, and the cell monolayer was inoculated with 100 PFU/well of HHV-1 KOS-1 or UKM-1 strain prepared as above. The virus-extract mixture was incubated for 3 h at 4 °C and later the mixture was removed. The cell monolayer was washed with PBS, overlaid with 1% MC and subjected to incubation for 48 h. Cells were fixed, stained and plaque number enumerated. Plaque reduction percentage was calculated using the equation above.

Penetration assay

The penetration assay was carried out following procedures previously described by Cheng et al. (2004) with slight modification. Briefly, vero cell monolayers cultivated in 12-well plates were incubated at 4 °C for 1 h. The medium was decanted while the cell monolayer was inoculated with 100 PFU/well of HHV-1 KOS-1 or UKM-1 strains and further subjected to incubation for 3 h at 4 °C. The medium was decanted and replaced with fresh medium, in the absence or presence of 0.35 mg/mL aqueous extracts, and the temperature increased abruptly to 37 °C to maximize penetration of the virus. Virus penetration was allowed to proceed for 30 min intervals (30, 60, 90 and 120 min) before infected cells were treated with PBS pH 3 for 1 min to neutralize the non-penetrating virus. Following washings with serum-free medium for three-times, cells were overlaid with 1% MC and subjected to further incubation for 48 h. Cells were then fixed, stained and the number of plaques was enumerated. Plaque reduction percentage was calculated using the equation above.

Time-of-addition assay

Monolayers of vero cells cultivated in 12 well plates were inoculated with 100 PFU/well of HHV-1 KOS-1 or UKM-1 strains for 2 h at 37 °C in 5% CO2. The inoculum was removed and extracts (0.35 mg/mL) were added after 2, 4, 6, 8, 10 and 12 h post-inoculation with 1% MC was overlaid on the cell monolayer and subjected to incubation for 48 h. The number of plaques was enumerated at each time points and plaque reduction percentage calculated using the equation above.

Time-of-removal assay

Vero cells were subjected to incubation with 100 PFU/well of virus HHV-1 KOS-1 or UKM-1 strains for 2 h at 37 °C in 5% CO2 and the inoculum was removed. Cells were treated with 0.35 mg/mL of aqueous extracts for 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 34, 36, 38, 40, 42, 44 and 48 h before the extract was removed. Cells were overlaid with 1% MC and further subjected to incubation for up to 48 h. The number of plaques was enumerated at each time points and plaque reduction percentage was calculated using the equation above.

Statistical analysis

Experiments were repeated independently at least two times and data are presented as the mean ± standard deviation (SD) of experimental repeats. One-way ANOVA with T-test was used to calculate the P-value and P<0.05 was considered to be statistically significant.

RESULTS

**O. stamineus extracts are not cytotoxic to vero cells**

Figure 1 illustrates the viability of vero cells following exposure to various concentrations of hot and cold water extracts of *O. stamineus* with the calculated CC50 values of 3.4 and 3.3 mg/mL respectively. Cold water extract has a slightly lower CC50 value than hot water extract but with no significant difference in the cytotoxicity at 95% confidence limit (*P*>0.05). The CC50 value for hot water extract is similar to the reported value of 3.4 mg/mL by Alwahid et al. (2015) but different from the value of 0.766 mg/mL reported in Ripim et al. (2018). Crude extract with CC50 value less than 0.01 mg/mL was established as cytotoxic according to King and Jones (2003). The CC50 values of both extracts were greater than 0.01 mg/mL, thus considered as not cytotoxic. Therefore, the subsequent antiviral assays were fixed not to exceed the CC50 values.

**Figure 1:** Viability of vero cells following exposure to various concentrations of *O. stamineus* hot and cold water extracts.

**O. stamineus extracts demonstrated antiviral activity when post-treated to infected cells**

The ability of the extracts to reduce plaque formation when post-treated to infected cells is shown in Figure 2A and 2B. The EC50 values were extrapolated from the plaque reduction percentages in this post-treatment assay and Selective Indices (SI) of both extracts were calculated as shown in Table 1. From the SI values, both hot and cold water extracts of *O. stamineus* showed selectiveness towards the viruses and have potential as antiviral agents. The SI values of both the extracts were higher than 10.
which according to Dargan (1998) possess high potential to be developed as antiviral agent. The post-treatment antiviral activity of both extracts showed dose-related activity especially for HHV-1 KOS-1 strain but not so obvious for HHV-1 UKM-1. More importantly, both extracts are capable of reducing the plaque formation of the ACV-resistant strain, thus showed promising alternative to ACV.

**O. stamineus** extracts demonstrated concentration-dependent antiviral activity when pre-treated to cells prior to infection

Figure 3 shows the percentage of plaque reduction when vero cells were pre-treated with **O. stamineus** extracts prior to virus infection. The pre-treatment reduced the ability of HHV-1 KOS-1 and UKM-1 strains to infect cells but to no greater than 80% inhibition. Extract concentration plays an important role in pre-treatment activity, especially towards KOS-1 infection. ACV has no effect when pre-treated to cells. It is noteworthy that the activity is concentration-dependent; the higher the concentration of extract gives more protection to cells against HHV-1 KOS-1 infection. Pre-treated cells are less infective by HHV-1 UKM-1 regardless of concentration but must be equal or more than 0.15 mg/mL

**O. stamineus** extracts have virucidal activity

Figure 4 shows the percentage of plaque reduction following pre-treatment of the virus with the extracts prior to cell infection to indicate the virucidal effect of **O. stamineus** hot and cold water extracts. The virucidal effect was profound at lower virus titer (100 and 1000

**Table 1**: CC<sub>50</sub>, EC<sub>50</sub> and SI values for hot and cold water **O. stamineus** leaves extracts for HHV-1 KOS-1 and UKM-1 strains.

| Extract | UKM-1 | KOS-1 |
|---------|-------|-------|
|         | CC<sub>50</sub> (mg/mL) | EC<sub>50</sub> (mg/mL) | SI | CC<sub>50</sub> (mg/mL) | EC<sub>50</sub> (mg/mL) | SI |
| Hot     | 3.4   | 0.12  | 28 | 3.4   | 0.15  | 23 |
| Cold    | 3.3   | 0.12  | 27 | 3.3   | 0.13  | 25 |

CC<sub>50</sub>, 50% cytotoxicity concentration; EC<sub>50</sub>, 50% effective concentration; SI, Selective index calculated as CC<sub>50</sub>/EC<sub>50</sub> ratio.
Figure 4: Virucidal activity of hot and cold water *O. stamineus* leaves extracts following exposure to various titers of HHV-1 (A) KOS-1 and (B) UKM-1 strains. ACV, Acyclovir.

PFU) for HHV-1 KOS-1 strain. Virucidal activity of both extracts against HHV-1 UKM-1 was not titer related in having almost 99% plaque reduction regardless of the titer. *O. stamineus* virucidal activity is confirmed in this study regardless of the virus strain and similar mode of action was previously observed by Ripim and Nor (2018).

**O. stamineus** extracts alter virus attachment to cells

The ability of different concentrations of hot and cold water extracts of *O. stamineus* to inhibit viral attachment to the vero cell surface is depicted in Figures 5. Plaque reduction percentage is directly proportional to the concentration of the extracts for HHV-1 KOS-1 strain indicating a dose-related inhibition in viral attachment. However, viral attachment of HHV-1 UKM-1 is not dose-related with the lowest plaque reduction percentage of 88% at 0.2 mg/mL for hot water extract and 91% for cold water extract at similar concentration. ACV has shown no inhibition to the virus attachment.

**O. stamineus** prevents virus penetration to cells

The effect of the extracts on virus penetration was evaluated at different intervals of post-infection as shown in Figure 6. As for HHV-1 KOS-1, exposure to cold water extract reduces the ability of viral penetration that in turn increases the plaque reduction percentage to 87% following exposure of 90 min. Hot water extract inhibits virus penetration 85% when exposed for 120 min. Exposure of hot water extract for 60 min was able to reduce the ability of HHV-1 UKM-1 to penetrate the host cells to 85% plaque reduction. Cold water extract needs 90 min to inhibit viral penetration causing 93% plaque reduction. In comparison, inhibition of the extracts appears to be more effective in attachment than penetration of the virus into the cell. ACV does not inhibit viral penetration when exposed to infected cells.

**O. stamineus** extracts affect antiviral activity at different virus replication stages

Figure 7 shows the effect of extracts addition periodically up to 12 h towards post-infected HHV-1 KOS-1 and UKM-1 strains. About 89% of HHV-1 KOS-1 was inhibited when treated with the cold extract after 2 h post infection and reduces thereafter. As for the hot extract, HHV-1 KOS-1 was also inhibited by 82% for the first 2 h of exposure. It can be deduced that the inhibition of HHV-1 KOS-1 is inversely proportional to the time of exposure. This indicates that the extracts are more effective during the early virus infection cycle of HHV-1 KOS-1 strain which is
parallel with the results from the attachment and penetration studies.

However, for HHV-1 UKM-1 strain, plaque formation was inhibited by the extracts when added at all the different time points. This indicates that the extracts are effective towards HHV-1 UKM-1 strain replication at any time during the 12 h post-infection. As for ACV, no inhibition towards HHV-1 UKM-1 strain was observed at all time points.

**Antiviral activity of *O. stamineus* extracts increase with exposure period**

Results presented in Figure 8 revealed a linear relationship between the exposure periods with extract to infected cells. Plaque reduction percentage was 11% when hot water extract was removed after 2 h incubation from HHV-1 KOS-1 infected cells and increased to 96.6% when extract was removed after 48 hour. Similar trend is shown using cold water extract. From our observation, a complete replication cycle for HHV-1 KOS-1 takes approximately 18 h. Continuous exposure to the extracts causes virus to be inactive due to the virucidal property of the extracts, hence preventing virus replication by not

**Figure 6**: Effect of hot cold water *O. stamineus* leaves extracts on HHV-1 (A) KOS-1 and (B) UKM-1 penetration to vero cells. ACV, Acyclovir.

**Figure 7**: Effect of hot cold water *O. stamineus* leaf extracts added at different time points following infection with HHV-1 (A) KOS-1 and (B) UKM-1. ACV, Acyclovir.

**Figure 8**: Effect of hot and cold water *O. stamineus* leaf extracts removal at different time points towards cells infected with HHV-1 (A) KOS-1 and (B) UKM-1 strains. ACV, Acyclovir.
allowing the attachment and penetration of the virus to cells.

Similar assays were done using hot and cold-water extracts of *O. stamineus* towards cells infected with HHV-1 UKM-1 but the outcome was very different. Plaque formation was not inhibited for up to 16 h. Plaque inhibition was only observed after the 20 h post-exposure which coincided with the release of viral progeny for the first replication cycle (Figure 8B). However, the highest inhibition percentage of hot and cold-water extracts against HHV-1 UKM-1 was only 47% and 49% respectively that were recorded at 48 h post exposure. The outcome of this may relate to the presence of the extracts throughout the experiment towards HHV-1 UKM-1. When the extract was removed, the virus was able to replicate and free to produce plaques. This strain has a slower replication cycle and the progeny release was halted by the extracts from the first replication cycle beginning after 20 hours post infection. On the other hand, ACV did not induce any significant inhibition to HHV-1 UKM-1 throughout the 48 h exposure period. This substantiates the potential of both *O. stamineus* hot and cold water extracts as a potential therapy against HHV-1.

**DISCUSSION**

Natural compounds from plant origin are important source of antiviral compounds against HHV-1 (Khan et al., 2005). Antiviral activity from plant extracts involves complex interactions between phytochemicals and multitarget concept of phytotherapy have been proposed (Efferth and Koch, 2011). Both hot and cold water of *O. stamineus* contains a mixture of flavonoid, terpenoid and steroid with cold water extract has two extra components: saponin and alkaloid (Habboo et al., 2018). Similar phytochemical contents have been reported in *O. stamineus* water extract with rosmanarin and caffeic acids have been determined as the components (olah et al., 2003; Malahubban et al., 2013). Flavonoid content in the extracts was hypothesised to exert the antiviral activity (Ripim et al., 2018). Six flavonoid compounds isolated from the leaves of *O. stamineus* were elucidated as eupatorin, sinensetin, 5-hydroxy-6,7,30,40-tetramethoxyflavone, salvigenin, 6-hydroxy-5,7,40-trimethoxyflavone and 5,6,7,30-tetramethoxy-40-hydroxy-8-C-prenylflavone (Hossain and Mizanur Rahman, 2013).

Previously, aqueous extract of *Melissa officinalis* which contains phenolic compounds such as caffeic acid, p-coumaric acid and rosmanarin acid have been shown to inhibit HHV-1 clinical isolate (Astani et al., 2012), ACV-sensitive and ACV-resistant clinical isolates by inhibition of attachment and penetration (Astani et al., 2014). Rosmanarin acid content in *O. stamineus* leaves water extract may also contribute to the antiviral activity against dengue virus type 2 (Wahab et al., 2018). Flavonoids such as naringenin, caffeic acid, p-coumaric acid are found in *Eleusine indica* and have been previously shown to display virucidal activity (Iberahim et al., 2018). Flavonoids are known to abrogate HHV-1 replication by blocking virus binding to the cell (Bisignano et al., 2017).

Hot and cold water extracts of *O. stamineus* showed profound activities in post-treatment, virucidal, attachment and penetration assays towards both tested strains. The antiviral activity of both extracts also showed almost similar trends in all the tested assays and within different virus strains. Similar antiviral mode of action shown by *O. stamineus* leaf water extract against a HHV-1 clinical strain have been previously reported (Ripim et al., 2018; Ripim and Nor, 2018). Ripim and Nor (2018) have previously shown that the *O. stamineus* leaves extract virucidal activity is associated with the disintegration of HSV-1 virion structure causing interference to the attachment and penetration of the virus into the host cell. Having potent virucidal activity is encouraging since this property has been associated with increased activity in vivo (Carlucci et al., 2004).

Inhibition of HHV-1 plaque in the attachment assay is directly proportional to the concentration of extract for HHV-1 KOS-1 but affects more on the attachment of HHV-1 UKM-1 strain. HHV-1 entry involves complex ligand-receptor interactions and has been shown to be an ideal target for antiviral compounds (Akhtar and Shukla, 2009; Gescher et al., 2011; Iberahim et al., 2016). ACV is an antiviral drug that inhibits viral DNA polymerase specifically during the replication cycle when new viral DNA is synthesized. Viral resistance to ACV represents a particular problem especially among acyclovir-treated immunocompromised individuals (Stranska et al., 2005). HHV-1 UKM-1 has an EC50 value towards ACV of 3.61 (Hussin et al., 2013). This strain has a mutation in the TK gene with specific mutation in the cysteine-active site (C336Y) and in the polymerase gene which prevented the phosphorylation of ACV or DNA chain elongation (Safrin et al., 1994; Swierkoz et al., 2004). ACV does not exert virucidal activity against HHV-1 (Arvin et al., 2007). *O. stamineus* extracts have shown to exhibit virucidal activity through the direct effect to the virus replication without affecting host cells as shown in the cytotoxicity assay. Therefore, *O. stamineus* can be further studied as an alternative treatment against HHV-1 infection using different antiviral mechanism from the current antiviral drug (ACV) in which mutant HHV-1 was developed.

**CONCLUSION**

Hot and cold water extracts of *O. stamineus* have no cytotoxic effect against the vero cells. Both extracts are effective against HHV-1 KOS-1 and UKM-1 strains with virucidal effect, alteration of virus attachment and penetration to the cells. We have provided promising antiv- herpetic candidates which are effective in vitro against HHV-1 especially for a mutant virus resistant towards ACV.

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