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Research

Phaleria macrocarpa Fruit Protein Aqueous Extract Affects Viral Entry, Virucidal Activity, and Progeny Release

Mahmud Yusef Yusef Ismaeel[1,2], Chee Wai Yip[1], Norefrina Shafinaz Md Nor[1], Nazlina Ibrahim[1]*

1. Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
2. Department of Medical Science, Abbs Community College, Hajjah, Yemen
*Corresponding author: nazlina@ukm.edu.my

ABSTRACT

Presence of acyclovir (ACV) resistant virus posed a major problem in treating virus infection. Alternative treatment with the ability to encounter infection of acyclovir-resistant virus is thus needed and possibly with a different mode of action from ACV. Hence, this study evaluates the antiviral effect of *Phaleria macrocarpa* (Scheff.) Boerl fruit protein aqueous extract (PMFPAE) against three different strains of human herpesvirus type-1 (HHV-1) including a clinical strain, a less pathogenic strain (KOS-1), and acyclovir (ACV) resistant mutant (UKM-1). PMFPAE displayed antiviral activity towards all the HHV-1 strains when post-treated with high selective indices (SIs) of 80.6, 50, and 35, respectively. Plaque reduction percentages were reduced in attachment and penetration assays following treatment with PMFPAE indicating the ability to deactivate the early phases of the HHV-1 replication cycle. The virucidal activity was also noted following treatment of the virus with PMFPAE and this is supported by damages to the virus envelope as observed by transmission electron microscopy (TEM). Incubation of virus-treated cells with PMFPAE for 24 hr, reduced progeny release in a dose-dependent manner. The study confirms the antiviral mode of action of *P. macrocarpa* fruit against HHV-1 strains and the ACV-mutant strain includes inhibition during virus entry represented as the early stages of viral replication, virucidal activity, and interfering with progeny release. PMFPAE mode of action is hence different from ACV and worthy for the development of future antiviral drugs.

Key words: Early replication stage, *Phaleria macrocarpa* fruit, progeny release inhibition, virucidal activity

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INTRODUCTION

Human Herpes Virus type I (HHV-1) infects millions worldwide with asymptomatic or minor to life-threatening consequences (Crimi *et al*., 2019). The earliest stage of the virus formerly known as herpes simplex virus (HSV) is febrile vesicles that develop on the skin of the face as cold sores or fever blisters on the lips and also the genitals. The nucleoside analog acyclovir (ACV) is the recommended treatment for HHV infection (Kłysik *et al*., 2018) that targets the viral DNA polymerase in treating HHV infection. The development of herpes virus with ACV resistance was more common in immunocompromised patients and the prolonged management of antiviral medications (Andrei & Snoeck, 2013). The key reason is that the medication only works on active replicating viruses but not against latent HHV-1. These limitations, combined with the lack of an effective vaccination, highlight the urgent need to develop new anti-HHV treatments (Frobert *et al*., 2014). Finding new anti-HHV medicines that work through diverse mechanisms is critical for HHV clinical therapy (Lin *et al*., 2014).

Reports on the antiviral activity of *Phaleria macrocarpa* (Scheff.) Boerl fruit has been mostly limited to HHV-1, including the antiviral effect of *P. macrocarpa* fruit hexane fraction (PMFHF) (Ismaeel *et al*., 2015) and *P. macrocarpa* fruit aqueous extract (PMFAE) (Ismaeel *et al*. 2018b).

Virucidal activity of methanol extract and its fractions against HHV-1 on Vero cells including *P. macrocarpa* fruit chloroform fraction (PMFCF), *P. macrocarpa* fruit ethyl acetate fraction (PMFEAF), and *P. macrocarpa* fruit methanol extract (PMFME) (Ismaeel *et al*., 2018a). Furthermore, the antiviral activity of this plant fruit was produced due to the presence of many phytochemical content in the PMFHF (Ismaeel *et al*., 2015), PMFAE (Ismaeel *et al*., 2018b), and PMFCF, PMFEAF, and PMFME (Ismaeel *et al*., 2018a). *Phaleria macrocarpa* fruit protein aqueous extract (PMFPAE) was reported to affect the early and late replication phases of HHV-1 according to Ismaeel *et al*. (2022). Furthermore, PMFPAE (protein extract) was fractionated into six fractions using gel filtration chromatography (GFC), PMFPAE fractions have different characteristics including non-cytotoxic against Vero cells and these fractions produced potential antiviral activity (Ismaeel *et al*., 2022).

Hence, this study further evaluates the antiviral effect of PMFPAE against the clinical strain (partly reported in Ismaeel *et al*., 2022) and other HHV-1 strains including a non-pathogenic strain KOS-1 (Schaffer *et al*., 1973) and ACV-resistant mutant (UKM-1) (Hussin *et al*., 2013). The hypothesis suggests that the presence of protein and related compounds could potentially play a role in the antiviral efficacy against HHV-1. The effect of PMFPAE on virus attachment, virus penetration, virucidal activity, and virus morphology was determined in light of the need to continue the search for novel antiviral agents.

MATERIALS AND METHODS

Cells and viruses

Vero cells (African green monkey kidney, ATCC CCL-81), HHV-1 strains including a clinical strain, KOS-1 strain, and ACV-resistant mutant (UKM-1) were retrieved from stocks in the Virology Laboratory, Faculty of Science and Technology. Vero cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) mixed with 5% fetal bovine serum (FBS, JR Scientific), non-essential amino acids (Nacalai Tesque), and penicillin/streptomycin 100 U/L (Sigma).

Plant extract preparation

PMFPAE was prepared from *P. macrocarpa* (Scheff.) Boerl fruit according to Ismaeel *et al*. (2022). In brief, ammonium sulfate (Sigma-Aldrich) at 75% (w/v) saturation was used to precipitate protein. The pellet was separated through centrifugation. The obtained pellet was then suspended in deionized water and subsequently subjected to dialyzed for 24 hr at room temperature using acetate buffer with a pH of 5. Following dialysis, the protein solution was freeze-dried to yield PMFPAE, which was stored at -20°C until required for further use.

Plaque inhibition assay

Post-treatment of PMFPAE on all HHV-1 strains was determined in plaque inhibition assay according to Ismaeel *et al*. (2015) and (2022). Vero cells were infected with 100 pfu/well of virus and incubated for 2 hr in 5% CO_2 at 37°C. Different PMFPAE concentrations in the range of 5 to 60 µg/mL, as well as ACV (Sigma-Aldrich) at 4.5 µg/mL, were mixed separately in 1% of methylcellulose (MC) combined with FBS (5%) to cover the infected cells. The cells were incubated at 37°C for 48 hr while ACV was used as a positive control. The virus-infected cells were mock-treated with DMEM as a negative control. The inverted microscope was used to view the plaque, and the percentage of plaque inhibition was determined using the formula below (Souza *et al*. 2008).

$$\text{Plaque inhibition (\%)} = \frac{[(\text{Mean plaques number in untreated control}) - (\text{Mean plaques number in treated sample})]}{(\text{Mean plaques number in untreated control})} \times 100$$

Cell pre-treatment assay

The assay was performed according to Ismaeel *et al*. (2018b) with cells treated with different PMFPAE concentrations in the range of 40 to 60 µg/mL and ACV at 4.5 µg/mL. The incubation period of pre-treated cells was 24 hr at 37°C in a 5% CO_2, and phosphate-buffered saline (PBS) at a pH of 7.4 was used to wash the pre-treated cells after the treatments were removed. After that, at 100 pfu/well, pre-treated cells were directly infected with virus strains and were then incubated at 37°C for 2 hr in 5% CO_2. The HHV-1 strains inocula were removed and covered with MC (1%) mixed with FBS (5%). Furthermore, the cell incubation period was 48 hr at 37°C in a 5% CO_2, and the plaque inhibition percentage was determined using the formula in the plaque inhibition assay.

Attachment assay

PMFPAE doses ranging from 40 to 60 µg/mL were mixed with 100 pfu/mL of three HHV-1 strains. The mix was applied to confluent cells and pre-chilled for 1 hr at 4°C. After being incubated at 4°C for 3 hr with PMFPAE-containing virus-infected cells, the inoculum was discarded. Cells infected with the virus serve as a negative control. Cells were washed with PBS to eliminate the non-absorbed virus. Subsequently, 1% and 5% of MC and FBS were added to the cells and incubated at 37°C in 5% CO_2 for 48 hr. The plaques were counted, and the percentage of plaque inhibition was calculated using the formula stated in the plaque inhibition assay.

Penetration assay

This assay follows the method according to Ismaeel *et al*. (2018b). The cells were first incubated for 1 hr at 4°C. Three HHV-1 strains were separately injected into the cells at a rate of 100 pfu/well. The incubation period of the post-infected cells was 3 hr at 4°C. Added to the cells were given doses ranging from 40 to 60 µg/mL of PMFPAE at intervals of 0.5, 1, 1.5, or 2 hr, followed by a further incubation at 37°C in 5% CO_2. Cells infected with the virus serve as a negative control. The cells were rinsed in PBS for 1 min at pH 3 and pH 7.4. Following that, cells were rinsed three times using DMEM for 1 min. MC (1%) with FBS (5%) were combined and added to the cells. Subsequently, cells were then incubated at 37°C in 5% CO_2 for 48 hr. Plaques were counted, and the percentage of plaque inhibition was determined based on the above formula.

Virucidal assay

Different PMFPAE concentrations in the range of 40 to 60 µg/mL were added to three HHV-1 strains at 10^3 pfu/mL and incubated for 1 hr in 5% CO_2 at 37°C. PMFPAE-virus mix was added to Vero cells and then incubated for 2 hr in 5% CO_2 at 37°C. Untreated control includes cells infected with the virus. The mixtures were removed and then covered with MC (1%) and FBS (5%). The infected virus cells were incubated for 48 hr in 5% CO_2. After the plaques were counted, the percentage of plaque inhibition was determined using the above formula.

Transmission electron microscopy (TEM)

Morphology of the treated virus and infected cells was viewed using TEM. HHV-1 at 10^8 pfu/mL of the three strains was mock-treated or treated with 60 µg/mL PMFPAE for an hr in 5% CO_2 at 37°C. The samples were centrifuged for 5 min at 166×*g* to obtain the virus pellet. The virus-containing pellets were fixed using glutaraldehyde (2%) in 0.1 M phosphate buffer, pH 7.4) for 24 hr at 4°C, and PBS at 0.1 M was used to wash the pellets three times for 10 min. The virus was embedded in resin to prepare for thin sections, according to Fayyad *et al*. *(*2013). Pellets were post-fixed using osmium tetroxide (OsO_4) at 1% at 4°C for 2 hr; PBS at 0.1 M was used to wash the pellets for 10 min. Dehydration was performed using different high concentrations of ethanol at 30%, 50%, 70%, 80%, 90%, and 100%, separately for 10 min each. This is followed by sample submersion in a 1:1 and 1:3 mixture of acetone and resin (100%, Agar 100 resin kit - Agar Scientific, UK) for 1 hr and 2 hr, respectively. The virus was overnight kept in the resin, embedded in the beam capsules in triplicates, and then polymerized at 60°C for 48 hr in an oven. Lastly, sections of resin capsules were cut with an ultramicrotome and gathered on a copper grid. Using a (CM 12 TEM, Philips, Netherlands) operated at 120 kV, the grid preparation of all parts was observed.

Virus yield reduction assay

Virus yield reduction assay follows the method by Saddi *et al*. (2007) with slight modification. Briefly, Vero cells grown in 12-well plates were infected with HHV-1 clinical, KOS-1, and UKM-1 strains at a multiplicity of infection of 1 at 37°C for 2 hr in 5% CO_2. Infected cells were treated with different concentrations of PMFPAE at 40, 50, or 60 µg/mL. ACV at 4.5 µg/mL was used as the positive drug control. Infected cells were incubated for 24 hr and then cell lysed by freezing at -80°C and thawing at least three times. The virus was harvested by centrifugation at 1844 × g (Eppendorf, Model 5804R, Germany) for 10 min at 4°C. The harvested virus was serially diluted and titrated using plaque inhibition assay as above. After 48 hr of incubation, cells were stained and plaques were counted to determine virus yield. Plaque numbers were compared between treated and not treated and the percentage of plaque reduction was calculated using the formula mentioned above.

Statistical analyses

The mean ± standard deviation (SD) was used to express the results. The mean values represent three experiments, each carried out in triplicate. The statistical data analysis was carried out by SPSS one-way ANOVA, with a significant level of (p<0.05).

RESULTS AND DISCUSSION

PMFPAE cytotoxicity and antiviral activity

PMFPAE is not cytotoxic towards Vero cells as an HHV-1 host with a CC_{50} value of 1450 ± 2.0 µg/mL (Ismaeel *et al*., 2022). The values of effective concentration that causes 50% (EC_{50}) of plaque formation are indicated in Table 1. The calculated selective index (SI) was determined from the ratio of CC_{50}/EC_{50} to indicate the potential antiviral effect as shown in Table 1. SI serves as a crucial parameter for evaluating the potential antiviral efficacy of an extract, fraction, or compound, playing a vital role in detecting any potential harmful effects on cellular health (Brezáni *et al*., 2018). The SI values of extract or fraction more than three indicate the possibility of possessing antiviral activity, and SI values greater than 10 show high potential antiviral activity, according to Chattopadhyay *et al*. (2009). Interestingly, the extract showed effectiveness against ACV-resistant strains, although to a lesser extent than clinical and KOS-1 strains.

Table 1. EC_{50} and SI of PMFPAE against HHV-1 clinical, KOS-1, and UKM-1 strains. CC_{50} value of PMFPAE = 1450 ± 2.0 µg/mL

Virus strain	EC_{50} (µg/mL)	SI
Clinical	18 ± 1.2	80.6
KOS-1	29 ± 2.3	50
UKM-1	41 ± 2.5	35

PMFPAE was screened for antiviral activity in a post-treatment assay involving three HHV-1 strains; clinical, KOS-1, and UKM-1 strains (Figure 1). A positive control i.e. known antiviral drug acyclovir (ACV) was also added. Using these three strains allows comprehensive exploration of various aspects of HHV-1 biology, including pathogenesis in the clinical strain of HHV-1, molecular mechanisms which has been extensively studied in the KOS-1 strain, and drug resistance as of UKM-1 strain which is ACV resistant. Thus, contributing to a deeper understanding of this medically significant virus.

PMFPAE has a greater effect on clinical strain compared to KOS-1 and UKM-1 strains. ACV maintained a high plaque inhibition percentage at the tested concentration (4.5 µg/mL). Post-treatment with 60 µg/mL PMFPAE after the virus was infected to cells displayed a significant (p<0.05) reduction in plaque formation towards the strains of HHV-1 KOS-1 or UKM-1 (Figure 1). The SI of PMFPAE is higher compared to previously tested compounds goniothalamin with SI=9.1 (Hussin *et al*., 2022) and *Orthosiphon stamineus* water extract with SI=28 for hot extract and 27 for cold extract (Habboo *et al*., 2020) indicating better potential as antiviral candidate.

Effect on virus attachment and penetration

Further investigation on the PMFPAE was conducted to determine the primary mechanism of action. Virus attachment and penetration are the prerequisite steps in HHV-1 infection. PMFPAE has a dose-dependent attachment to Vero cells activity towards all the tested strains, with the highest plaque inhibition approximately between 70% and 80% at 60 µg/mL of PMFPAE (Figure 2). The HHV-1 plaque inhibition in the attachment assay is directly proportional to the PMFPAE concentrations for HHV-1 three strains. Still, it has a greater effect on the HHV-1 attachment of the UKM-1 strain at 60 µg/mL. The lowest concentration of PMFPAE (40 µg/mL) showed the slightest plaque inhibition against clinical, KOS-1, and UKM-1 of HHV-1 strains, where inhibition percentages were 60%, 57%, and 42%, respectively. In conclusion, PMFPAE has the potential to inhibit the HHV-1 from attaching to its host, which is crucial in the early steps of the replication cycle.

On the other hand, the ability of PMFPAE to inhibit penetration was much higher (84-92%) at 50 and 60 µg/mL when HHV-1 different strains were incubated between 1.5 and 2 hr post-infection (hpi) (Figure 3a, 3b, & 3c). The trend shown in the penetration effect on KOS-1 is lesser than in clinical strain, but the time point in which PMFPAE has the highest plaque inhibition activity was between 1.5 and 2 hpi. Virus inhibition in penetration assay suggests that PMFPAE could prevent the virus penetration into cells irrespective of the tested HHV-1 strains and effectively between 1.5 and 2 hpi. The effect of PMFPAE on the attachment and penetration in this study confirms the earlier observation that PMFPAE affects early phase replication of HHV-1 (Ismaeel *et al*., 2022). A previous study by Ripim *et al*. (2018)

reported that *Orthosiphon stamineus* leaves of aqueous extract (OSLAE) have antiviral activity altered HHV-1 penetration and could affect other proteins involved in the viral cycle phase following entry. In addition, OSLAE treatment is more efficient towards free HHV-1 particles than treatment after the virus has attached to the host cells. The interaction of the different HHV-1 strains with PMFPAE decreases plaque inhibition by not allowing virus penetration.

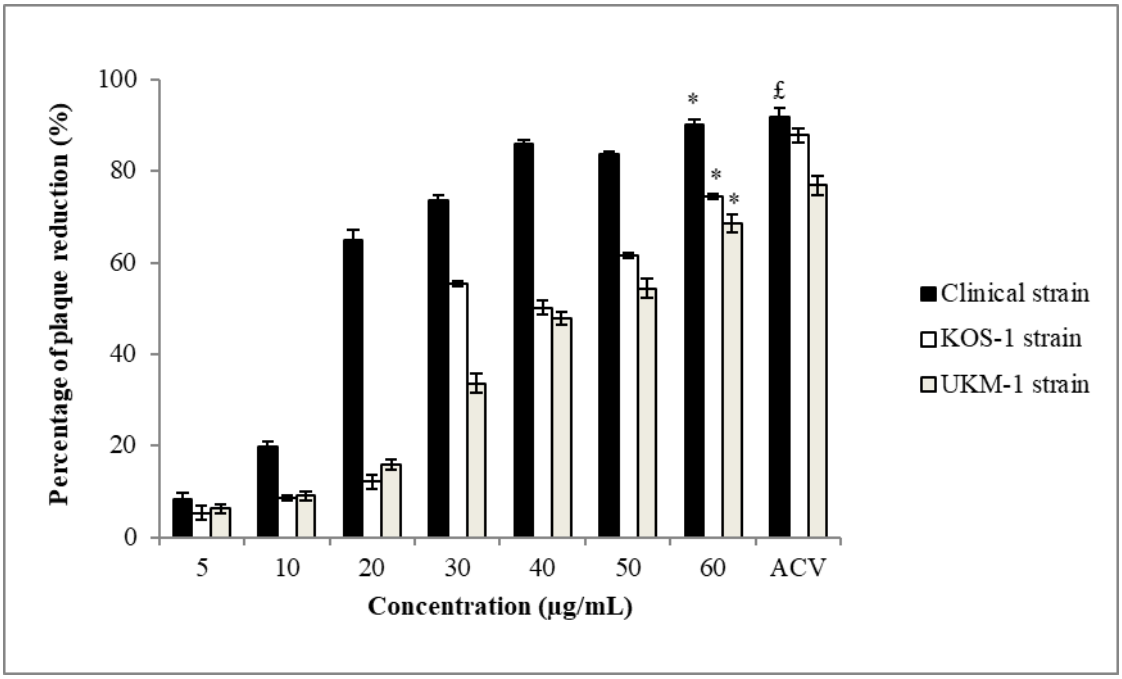

Fig. 1. Efficacy of PMFPAE in post-treatment assay towards three HHV-1 strains.
*Significant plaque inhibition when treated with PMFPAE at 60 µg/mL (p<0.05). The post-treatment activity is almost comparable to ACV at 4.5 µg/mL.£Indicates a significant difference (p<0.05) in plaque inhibition percentage for the KOS-1 strain compared to the UKM-1 strain at ACV 4.5 µg/mL. The values are shown as mean ± SD (n=3).

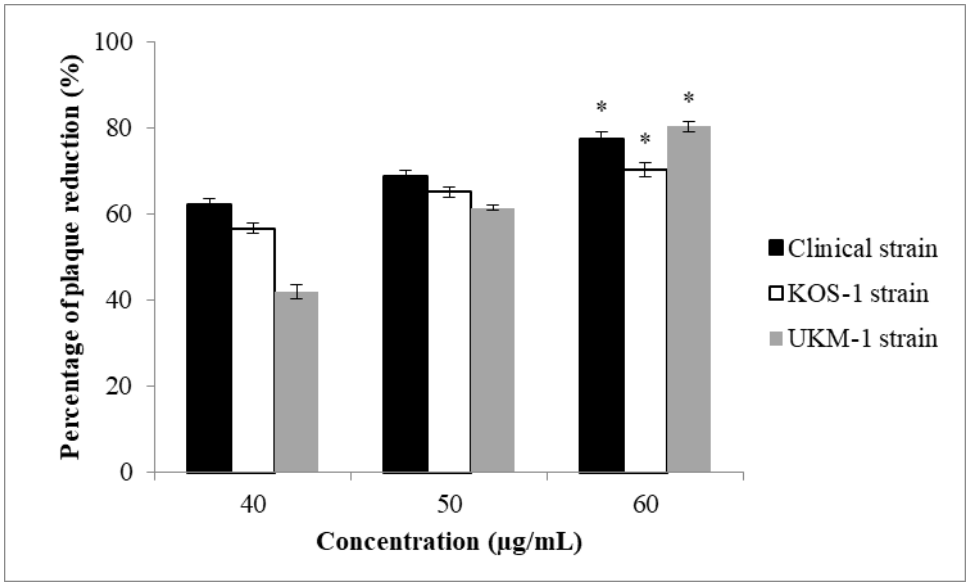

Fig. 2. The effect of PMFPAE on HHV-1 attachment of clinical, KOS-1, and UKM-1 strains to Vero cells. PMFPAE was tested at 40, 50, and 60 µg/mL on Vero cells. *Indicates a significant difference in plaque inhibition when treated with PMFPAE at 60 µg/mL compared to the lower concentrations (p<0.05). The values are presented as mean ± SD (n=3). ACV mechanism of action does not involve preventing virus attachment to host cells. Hence no positive control is added to this assay

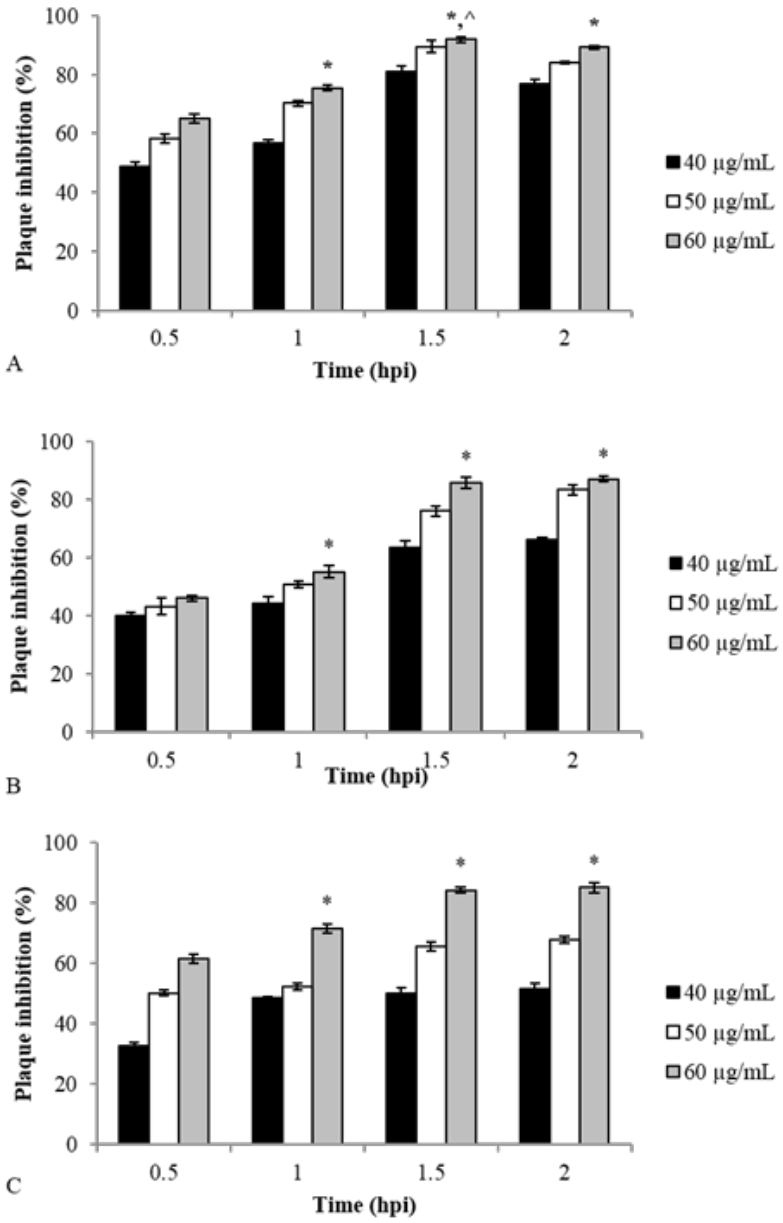

Fig. 3. Effect of PMFPAE on HHV-1 penetration. Panel (A) clinical strain (B) KOS-1 strain, and (C) UKM-1 strain. PMFPAE was evaluated at 40, 50, and 60 µg/mL on Vero cells. ^,*Indicates that PMFPAE at 60 µg/mL significantly inhibits plaque formation (p<0.05). ^Indicates a significant difference in plaque inhibition (p<0.05) at the particular virus penetration time point (hpi). A mean ± SD (n=3) is used to present the values. ACV action does not involve preventing virus penetration from hosting cells; hence no positive control is added in this assay.

Effect on virucidal activity

A virucidal assay was conducted to determine the ability of PMFPAE to interact and physically disrupt HHV-1 particles. The virucidal activity was exhibited by all concentrations tested of PMFPAE towards the clinical strain reaching 90% plaque inhibition at 60 µg/mL of PMFPAE treatment (Figure 4). In addition, virucidal activity was also shown at 60 µg/mL of PMFPAE treatment against the strains of HHV-1 KOS-1 and UKM-1 even though at lower percentage than in the HHV-1 clinical strain. The virucidal activity of PMFCF, PMFEAF, and PMFME has previously been reported by Ismaeel *et al*. (2018a), and this activity is maintained in PMFPAE. The virucidal effect depends on the type of HHV-1 strain, with clinical strains more sensitive compared to KOS-1 or ACV-resistant strains.

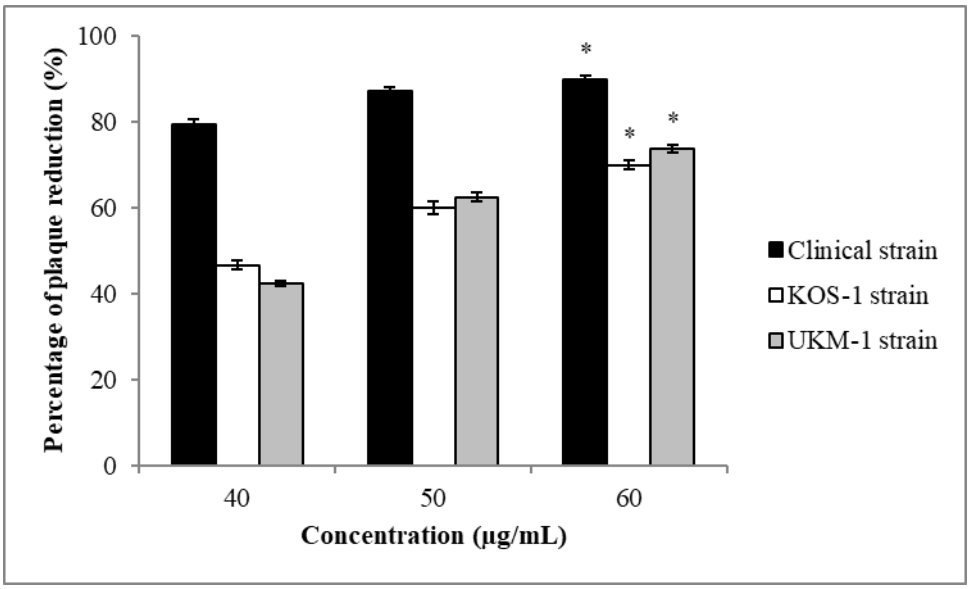

Fig. 4. Virucidal activities of PMFPAE against the HHV-1 clinical, KOS-1, and UKM-1 strains.
*Significant difference when treated with PMFPAE at 50 and 60 µg/mL compared to the lower concentration (p<0.05). The values are represented as the mean ± SD (n=3). ACV mechanism of action does not involve virucidal activity, hence no positive control is added in this assay.

Effect on virus morphology

Further clarification on this activity was done by examining the morphology of PMFPAE-treated virus under transmission electron microscopy (TEM). Intact virus morphology of HHV-1 clinical, KOS-1 or UKM-1 strains was observed in non-treated-infected cells, as shown by blue arrows (Figure 5a, Figure 6a & Figure 7a). Lost or damaged viral envelope in HHV-1 clinical, KOS-1 or UKM-1 strains was observed in PMFPAE-treated at 60 µg/mL, as shown by red arrows (Figure 5b, Figure 6b & Figure 7b). Full loss of envelope HHV-1 particles was observed in clinical strain due to the virucidal activity, which reached to 90% when the PMFPAE-treated virus at 60 µg/mL. The denser capsid is noted in the PMFPAE-treated HHV-1 clinical strain compared to non-PMFPAE-treated viruses. Partially disintegrated or lost in PMFPAE-treated virus observation is parallel to plaque inhibition percentage that accounts for 70% and 74% of virucidal activity in HHV-1 KOS-1 and UKM-1 strains, respectively. The change in morphology is comparable to the HHV-1 morphology and size in TEM analysis from previous studies (Zhou *et al*., 2000; Brown & Newcomb, 2011).

The observation is similar to a previous study where the HHV-1 envelope was destroyed when observed under TEM following treatment with *Marrubium vulgare* hexane fraction (Fayyad *et al*., 2013). PMFPAE appears to be effective for enveloped viruses such as HHV-1 with the ability to cause a virucidal effect and decrease its ability to infect host cells hence blocking the virus's early stages of replication. In conclusion, the virucidal activity has been confirmed by loss or damage to the viral envelope, either entirely or partially lost, when treated with PMFPAE.

Previously it has been shown that flavonoids and terpenoids in *Eleusine indica* play an important role in virucidal activity (Iberahim *et al*., 2015; 2018). *Orthosiphon stamineus* hot and cold water extracts containing alkaloid and flavonoid contents have been tested against HHV-1 strains of KOS-1 and UKM-1 (Habboo *et al*., 2020) that displayed virucidal effect and inhibition of the early virus replication cycle involving viral attachment and penetration to cells similar to *P. macrocarpa* antiviral activities.

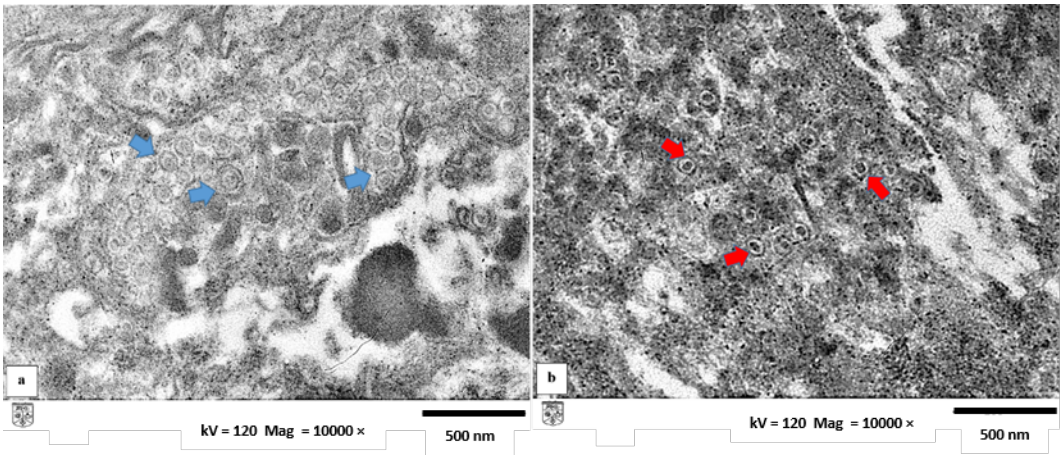

Fig. 5. TEM images of cells infected with HHV-1 clinical strain treated and untreated with PMFPAE embedded in resin. (a) The mock-infected clinical strain of HHV-1 and (b) PMFPAE-treated at 60 µg/mL. The mock-infected virus envelope was intact, but the envelope was disintegrated or lost in PMFPAE-treated viruses. Compared to untreated viruses, treated viruses have denser capsids than non-treated viruses. The blue arrows indicate the virus only, while the red arrows indicate the virus-treated-PMFPAE. Resin is used to embed virus samples. TEM images of samples with a magnification of 10000×.

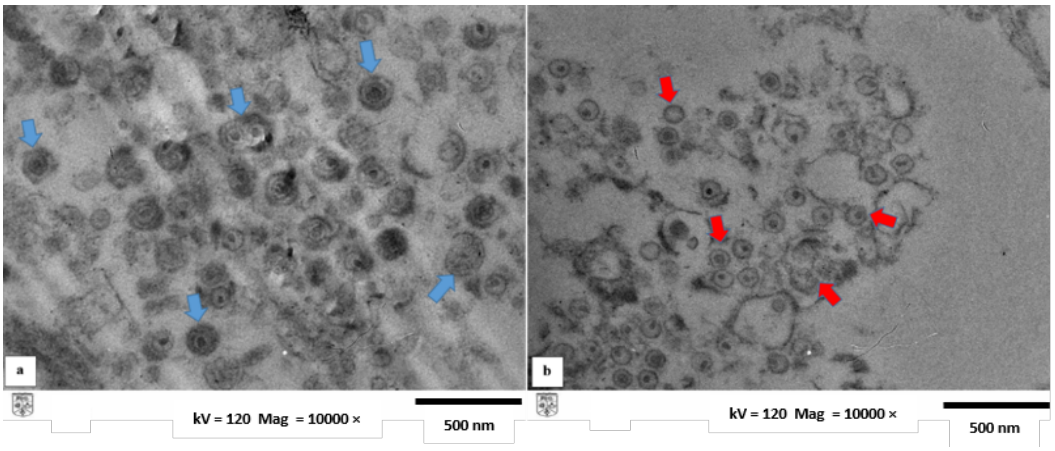

Fig. 6. TEM images of cells infected with HHV-1 KOS-1 strain treated and not treated with PMFPAE embedded in resin. (a) Mock-infected KOS-1 strain of HHV-1 and (b) PMFPAE-treated at 60 µg/mL. The mock-infected virus envelope was intact, but the envelope was disintegrated or lost in PMFPAE-treated viruses. The blue arrows indicate the virus only, while the red arrows indicate the virus-treated-PMFPAE. TEM images of samples with magnification of 10000×.

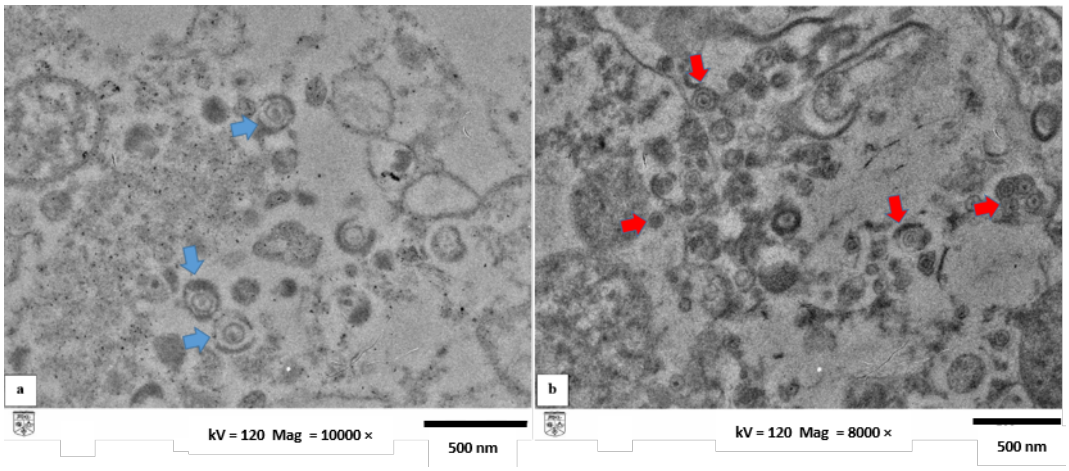

Fig. 7. TEM images of cells infected with HHV- UKM-1 strain treated and not treated with PMFPAE embedded in resin. (a) Mock-infected UKM-1 strain of HHV-1 (b) PMFPAE-treated at 60 µg/mL. The envelope of mock-infected and some PMFPAE-treated viruses seem to be intact. Partially disintegrated or lost in PMFPAE-treated viruses. The blue arrows indicate the virus only, while the red arrows indicate the virus-treated-PMFPAE. TEM images of samples with a magnification of 10000× and 8000×.

The virucidal activity displayed by PMFPAE may provide a broad-spectrum strategy in its antiviral activity. PMFPAE contains proteins (Ismaeel *et al*., 2022) and several other compounds that are capable of reducing the virus strains' ability to attach and penetrate host cells, reducing viral progeny release and directly impacting the viral envelope. PMFPAE has potent antiviral activity against three strains of HHV-1 and can be further explored *in vivo* studies. An important implication of having virucidal activity is that this effect allows antiviral activity towards an array of virus strains that reduces the possibility of the development of drug-resistant viruses (Galdiero *et al*., 2011). Previous fractions from *P. macrocarpa* including CFPMF, EAFPMF, and MEPMF have been reported to show virucidal activity (Ismaeel *et al*., 2018a). A compound associated with the virucidal activity was determined as mangiferin which has been previously described by Du *et al*. (2018). Mangiferin is available in the *P. macrocarpa* extract as determined by Alara *et al*. (2017) and Altaf *et al*. (2013) and so is in our study (unpublished result).

Enveloped viruses such as HHV-1 have phospholipid head groups in the envelopes which is different from the phospholipid found on the host cell membrane (Diamond *et al*., 2021). As observed in the TEM, only the virus envelope solubilises without interfering with the host membrane giving specific activity of PMFPAE towards the HHV particles. The virucidal activity of PMFPAE may reduce virus reinfection, which will not allow virus attachment and penetration. PMFPAE has indeed multiple antiviral strategies against HHV-1 with virucidal activity as the main antiviral activity frequently found in current antivirals and botanical molecules (Álvarez *et al*., 2020). It's important to highlight that various antimicrobial agents can exhibit diverse effects on particular enveloped viruses (Bright & Gilling, 2016).

PMFPAE reduces virus yield

The virus yield reduction assay aims to investigate the effect of PMFPAE on the release of infectious virus progeny. PMFPAE was able to reduce HHV-1 yield in a dose-dependent manner, as shown in Figure 8. PMFPAE was effective in reducing virus progeny of KOS-1 and UKM-1 strains by more than 80% at 60 µg/mL, while the inhibition of virus yield against the clinical strain was 57 ± 0.6%. ACV at 4.5 µg/mL is very effective in reducing HHV-1 clinical and KOS-1 but understandably not effective towards ACV-resistant strain UKM-1. The results showed that PMFPAE could efficiently reduce the virus yield against ACV-resistant strains and moderately for other HHV-1 strains. What we can deduce from the virucidal properties against different strains HHV-1 shown above, exposure of PMFPAE to the viral envelopes of released progeny may be damaged which inhibits the ability to attach and penetrate cells. This contributed to the decreased risk of virus reinfection. The observation substantiates the observation previously that PMFPAE inhibits the late phase of virus replication (Ismaeel *et al*., 2022). The relationship between the virucidal activity of *Eleusine indica* methanol extract and reduced virus reinfection has been indicated previously by Iberahim *et al*. (2018). It was suggested that the modification of the released progeny by exposure to the extract inhibits the attachment and penetration into cells following virus release.

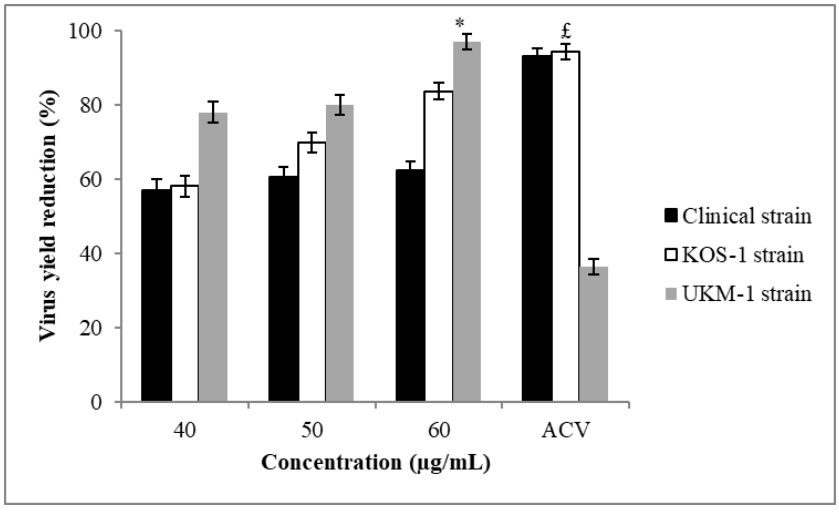

Fig. 8. Virus yield reduction percentage of PMFPAE on HHV-1 clinical, KOS-1 and UKM-1 strains. The virus yield reduction of PMFPAE is higher in the ACV-resistant strain when ACV is not able to reduce virus yield in a similar strain. The PMFPAE was tested at 40, 50, and 60 µg/mL on Vero. Acyclovir (ACV, 4.5 µg/mL) was used as a positive control drug. *Significant yield reduction (p<0.05) by PMFPAE at 60 µg/mL. £Indicates a significant difference in yield percentage between test strains (p<0.05). The values represent the mean ± SD (n=3).

CONCLUSION

PMFPAE has antiviral activity across all strains tested in the study including an ACV mutant strain. PMFPAE inhibits HHV-1 from attaching and penetrating the host which is the crucial step during the early replication cycle. Virucidal activity against HHV-1 strains results from virus envelope solubilization and degradation of the entire HHV particles as part of its mode of action. The reinfection of virus progeny may be halted by exposure to PMFPAE which degrades the virus envelope and reduces the ability to attach and later penetrate the host cells. PMFPAE has a different mode of action from ACV and directly causes damage to HHV-1 but not the host cells. *Phaleria macrocarpa* fruit has the potential for further research in the development of anti-herpes virus drugs, especially for reducing skin infection. Future research should focus on the molecular mechanisms of PMFPAE *in vivo* studies in anti-HSV-1 activity using clinical and ACV-resistant strains. Molecular docking studies can also be included to elucidate the interaction between PMFPAE and viral-inhibited molecules.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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