Hesperetin blocks poxvirus replication by competitively inhibiting binding of the 5' cap of viral mRNA with eIF4E

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Abstract

In this study, hesperetin was shown to inhibit the replication of multiple poxviruses, including buffalopox virus (BPXV), vaccinia virus, and lumpy skin disease virus (LSDV). Hesperetin mainly suppressed viral protein synthesis without affecting other steps of the viral life cycle such as attachment, entry, and budding. In a chromatin immunoprecipitation (CHIP) assay, we further demonstrated that hesperetin-induced reduction in BPXV protein synthesis is due to disruption of the binding of the 5' cap of viral mRNA with the cellular translation initiation factor eIF4E. The molecular docking and MD simulation studies, also confirmed binding of the hesperetin with the cap-binding pocket of eIF4E, in a similar conformation as m7GTP binds. In a BPXV egg infection model, hesperetin was shown to suppress the development of pock lesions on the chorioallantoic membrane, as well as the associated mortality of the chicken embryos. Most importantly, long-term culture of BPXV in the presence of hesperetin did not induce the generation of drug-resistant viral mutants. In conclusion, we for the first time demonstrated the antiviral activity of hesperetin against poxviruses, besides providing novel mechanistic insights into the antiviral action of hesperetin.

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ABSTRACT

In this study, hesperetin was shown to inhibit the replication of multiple poxviruses, including buffalopox virus (BPXV), vaccinia virus, and lumpy skin disease virus (LSDV). Hesperetin mainly suppressed viral protein synthesis without affecting other steps of the viral life cycle such as attachment, entry, and budding. In

a chromatin immunoprecipitation (CHIP) assay, we further demonstrated that hesperetin-induced reduction in BPXV protein synthesis is due to disruption of the binding of the 5' cap of viral mRNA with the cellular translation initiation factor eIF4E. The molecular docking and MD simulation studies, also confirmed binding of the hesperetin with the cap-binding pocket of eIF4E, in a similar conformation as m7GTP binds. In a BPXV egg infection model, hesperetin was shown to suppress the development of pock lesions on the chorioallantoic membrane, as well as the associated mortality of the chicken embryos. Most importantly, long-term culture of BPXV in the presence of hesperetin did not induce the generation of drug-resistant viral mutants. In conclusion, we for the first time demonstrated the antiviral activity of hesperetin against poxviruses, besides providing novel mechanistic insights into the antiviral action of hesperetin.

Keywords: Hesperetin, poxvirus, buffalopox virus, BPXV, eIF4E, m7GTP

INTRODUCTION

Poxviruses are a large family of DNA viruses that are capable of infecting a wide variety of animals including humans. While smallpox has been eradicated, the monkeypox virus has recently become an international concern for human health[1]. Likewise, the lumpy skin disease virus (LSDV) has emerged as the most important pathogen with regards to animal health [2]. The poxviruses are usually host-specific [3]. However, for reasons unknown, they may sometimes break the host tropism to infect unnatural hosts. For example, camelpox virus (CMLV) [4], buffalopox virus (BPXV) [5,6] and monkeypox virus (MPV)[7] have zoonotic implications.

Some poxvirus inhibitors have been described [8-12]. Among these, cidofovir is licenced to treat a variety of DNA viruses[13] whereas Tecovirimat (previously known as ST-246) is the only FDA approved drug that specifically acts against orthopoxviruses [14]. In addition, Brincidofovir, a prodrug of cidofovir, was approved in the United States in 2021 for the treatment of poxviruses. Besides having potential carcinogenic effects[15], these directly virus-acting drugs are prone to induce drug-resistant mutants [16].

We screened a library of small molecule chemical inhibitors targeting host cell's kinases and phosphatases and identified potential candidates with antiviral activity against BPXV. Hesperetin was identified as one of the inhibitors that blocked BPXV replication. Hesperetin ($C_{16}H_{14}O_6$) is the aglycone form of the flavanone glycoside hesperidin ($C_{28}H_{34}O_{15}$). It is a naturally occurring flavonoid found in citrus fruits such as oranges, grapes, and lemons and possesses anti-oxidant and anti-inflammatory properties by regulating various host signalling pathways[17-19]. Recent studies have also demonstrated the antiviral activity of hesperetin against some viral infections such as the chikungunya virus (CHIKV), Zika virus, Sindbis virus and dengue virus (DENV) [20-22]. In this study we extended the antiviral efficacy of hesperetin against poxviruses, besides providing novel mechanistic insights on its antiviral action.

MATERIALS AND METHODS

Cells and viruses

African green monkey kidney (Vero) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with antibiotics and 10% foetal calf serum. Vero cell adapted BPXV (Accession Number VTCC-AVA90) and LSDV (Accession Number VTCC-AVA288) were available at NCVTC Hisar. Vaccinia virus (VV) was procured from the American Type Culture Collection (ATCC). BPXV and VV were quantified by plaque assay in Vero cells and LSDV was quantified by determination of tissue culture infective dose 50/ml ($TCID_{50}$/ml) [23].

Inhibitors

Hesperetin was procured from Cayman Chemical (Ann Arbor, Michigan, USA). FR180204, CGP57380 and 4EGI-1 were procured from Sigma (Steinheim, Germany). The subcytotoxic concentration of ERK inhibitor (FR180204), MNK1 inhibitor (CGP57380) and eIF4E inhibitor (4EGI-1) were 0.2 µg/ml, 0.5 µg/ml and 0.5 µg/ml, respectively, and have been described previously by our group [24].

Antibodies

Anti-BPXV hyperimmune serum produced in rabbits was available at NCVTC, Hisar and has been described before [25]. eIF4E monoclonal antibody (5D11) was procured from Invitrogen (South San Francisco, CA, USA). Anti-β-actin, Anti-Mouse IgG – alkaline phosphatase antibody, and anti-rabbit IgG – peroxidase antibody were received from Sigma-Aldrich (St. Louis, USA).

Cytotoxicity and virucidal activity

The cytotoxicity of hesperetin in Vero cells and virucidal activity against BPXV were determined as described before [26]. Unless otherwise specifically stated, a non-cytotoxic concentration of 12 µg/ml of hesperetin or 0.05% DMSO (vehicle control) was used throughout the manuscript.

Time-of-addition assay

Confluent monolayers of Vero cells, in triplicates, were infected with BPXV at 5 MOI, followed by addition of hesperetin or DMSO (vehicle control) at -0.5 hpi, 1 hpi, 6 hpi, 12 hpi, 18 hpi, 24 hpi, 30 hpi and 36 hpi. Supernatants from the infected cells were collected at 48 hpi and quantified by plaque assay.

Attachment assay

Confluent monolayers of Vero cells, in triplicates, were treated with hesperetin or vehicle control for 1 h, followed by BPXV infection at 5 MOI for 1 h at 4°C. Cells were washed with PBS for five times to remove unattached virus, and cell lysates were prepared by rapid freeze-thaw cycles. The viral titres in cell lysates were quantified by plaque assay.

Entry assay

Confluent monolayers of Vero cells were infected with BPXV at 5 MOI for 1 h at 4°C to permit attachment. After that, cells were washed with PBS, and serum-free DMEM containing hesperetin or DMSO was added. This was followed by incubation at 37°C for 1 h which allowed virus entry. Thereafter, cells were washed again with PBS to remove any extracellular viruses and supplemented with DMEM without any inhibitors. The infectious virus particles released in the supernatant at 48 hpi were titrated by plaque assay.

Budding assay

Vero cells, in triplicates, were infected with BPXV at 5 MOI or mock infected for 1 h, followed by washing with PBS and incubation at 37°C. At 36 hpi, when BPXV presumably starts releasing from the infected cells, hesperetin or DMSO were added, and supernatants were harvested at 30 min and 4 h following the addition of the drug. Virus releases in the supernatants was quantified by plaque assay.

Viral protein synthesis

Vero cells were infected with BPXV at 5 MOI for 1 h. At 3 hpi, hesperetin or DMSO were added, and cells were incubated at 37°C. The cells were scrapped at 24 hpi and subjected to Western blot analysis by using hyperimmune serum raised against BPXV in rabbits.

qRT-PCR

The levels of viral DNA in the infected cells were measured by quantitative real-time PCR (qRT-PCR). Briefly, Vero cells, in triplicates, were infected with BPXV at an MOI of 5 for 1 h, followed by washing with PBS and the addition of fresh DMEM. Hesperetin or DMSO were applied at 3 hpi. Cells were scraped at 30 hpi to quantify BPXV C18L and the house-keeping gene (β-actin) gene as described before[26].

In ovo **antiviral efficacy**

Specific pathogen-free (SPF) embryonated chicken eggs were procured from Indovax Pvt Ltd, Hisar, India. To determine the LD_{50}, 4-fold serial dilutions of hesperetin (concentration ranging from 4000-62.5 µg/egg) or DMSO were inoculated in triplicates via the chorio-allantoic membrane (CAM) route in 10 day old embryonated SPF chicken eggs. The viability of the eggs was examined up to 5 days post inoculation and LD_{50} was determined by the Reed-Muench method [27].

For determination of the EC_{50}, five-fold serial dilutions of hesperetin or DMSO, in triplicates were inoculated in 10 days old SPF embryonated eggs via CAM route, followed by infection of BPXV at 100 EID_{50}. The eggs were examined for 6 days post-infection and CAM were harvested for examination of pock lesions. EC_{50} was determined by Reed-Muench method[27].

Chromatin immunoprecipitation (CHIP) assay

The interaction between BPXV mRNA and eIF4E was performed using the CHIP assay as described previously by our group [28]. Briefly, confluent monolayers of Vero cells in triplicates, were infected with BPXV at 5 MOI for 1 h, followed by washing with PBS. Cells were supplemented with fresh DMEM, and hesperetin or DMSO was added at 3 hpi. At 16 hpi, cells were treated with 1% formaldehyde to covalently cross-link the interacting proteins and nucleic acid for 10 min. The crosslinking was stopped using 125 mM glycine, followed by washing with ice-cold PBS. The cell lysates were prepared in immunoprecipitation buffer and sonicated as described before [28]. The sonicated lysates were centrifuged at 12000 g for 10 min and clarified supernatants were mixed with 10 units of RiboLock RNase Inhibitor (Thermo Scientific, USA), followed by incubation with α-eIF4E (reactive antibody), α-MNK1 (nonreactive antibody), or equivalent volume of IP buffer (beads control) at room temperature. After 45 min, 40 µL (5 ng/µL) of Protein A Sepharose® slurry was incubated with each reaction at 4°C on a rotary platform overnight. The beads were then washed by IP buffer and crosslinking was reversed by addition of 20 mg/ml Proteinase K (followed by incubation at 56 °C for 40 min). The reaction mixture was then centrifuged at 12000 g for 1 min. The supernatant was subjected to RNA isolation, cDNA preparation, and quantitation of the BPXV *M* gene by qRT-PCR.

Preparation of eIF4E for molecular docking and MD simulation

The crystal structures of eIF4E in open (PDB ID – 3TF2) and closed conformation (PDB ID - 1IPC) bound with m7GTP were used for protein visualization, docking studies and molecular dynamics simulation. Prior to analysis, the m7GTP ligand and water molecules were removed, and missing residues in the loop regions of crystal structure were modelled using SWISS-model [29]. The structures were further energy minimised using AMBERff14SB force field [30]followed by the addition of hydrogen atoms and water molecules as described previously [31]. The prepared structures were used for subsequent docking and MD simulation studies.

Molecular docking, protein visualization and Ligplot analysis

The three-dimensional structure of hesperetin was obtained from Pubchem (Pubchem CID - 72281) while the m7GTP structure was taken from the PDB ID - 1IPC. The ligands were energy minimized and subjected to molecular docking using Autodock Vina [32] with eIF4E. First, a grid box was generated around the protein with centre coordinates; X= 46.5, Y= 106 and Z= -16 with the dimensions of the grid box; X= 44.5 Å, Y= 56 Å and Z= 54 Å. Thereafter, global ligand binding searches were performed. The result comprised nine best binding poses for ligands. For further evaluation, the best binding mode for each ligand was selected based on the binding affinity (Kcal/mol), RMSD lower- and upper bond. The protein and generated docked complexes were visualised using Pymol 0.99rc6. To assess the interaction of hesperetin and m7GTP with the eIF4E, Ligplot application was used [33]. The docked complexes that resulted from Autodock Vina were saved in PDB format and uploaded to the Ligplot tool and the intermolecular interactions between the protein and ligands, such as polar and non-polar contacts were obtained in 2D representations.

Molecular dynamics (MD) simulation

To evaluate the stability and protein motion within internal coordinates, MD simulations of complexes with the highest affinities among the ligand and eIF4E were performed through the iMODS webserver (http://imods.chaconlab.org/) [34]. The internal coordinates of the hespetin-eIF4E docked complex, the m7GTP-eIF4E docked complex, and the apo-eIF4E were saved in PDB format and further subjected to MD simulation through the iMODS webserver using normal mode analysis (NMA) for the calculation of the B-factor, structural deformability and eigen values, covariance mapping, and elastic network.

Selection of hesperetin-resistant BPXV mutants

BPXV was sequentially passaged (upto P=40) in Vero cells in the presence of hesperetin or DMSO. For each passage, confluent monolayers of Vero cells were infected with BPXV at an MOI of 0.1 for 1 h, followed by 5 times washing with PBS and the addition of fresh DMEM supplemented with hesperetin (3 µg/ml) or 0.05% DMSO. Supernatants were harvested at ˜96 hpi or after observing a cytopathic effect (CPE) in [?]50% cells. The virus infected cell culture supernatant was used for the next passage (P). A total of 40 such passages were carried out. At the end of P40, the fitness of BPXV-hesperetin-P40 and BPXV-DMSO-P40 viruses was evaluated again in the presence of hesperetin.

RESULTS

Antiviral activity of hesperetin against poxviruses

Before examining the antiviral activity, we first determined a sub-cytotoxic concentration of hesperetin. At a concentration of [?]25 µg/ml, hesperetin had no effect on cell viability **(Fig. 1A).**The higher concentrations of hesperetin were toxic to the Vero cells**(Fig. 1A)** . Therefore, a sub-cytotoxic concentration of 12 µg/ml was used in the subsequent experiments. The cytotoxic concentration 50 (CC_{50}) of hesperetin was determined to be 146.8 µg/ml**(Fig. 1A)** .

To evaluate the antiviral effect, virus yield in hesperetin-treated and untreated cells was determined by plaque assay. As compared to the vehicle-control (DMSO), a sub-cytotoxic concentration of 12 µg/ml resulted in reduced BPXV **(Fig. 1B)** , vaccinia virus (VACV)**(Fig. 1C)** and LSDV **(Fig. 1D)** yield. Further, preincubation of the extracellular virus (BPXV) with hesperetin had no effect on the viral infectivity, which suggested that the antiviral effect of hesperetin is due to inhibition of virus replication in the target cells and not simply due to inactivation of extracellular virions**(Fig. 1E)** .

Hesperetin suppresses BPXV replication at post-entry steps

To narrow down the specific time-points of the BPXV life cycle during which hesperetin may act to inhibit virus replication, we performed a time-of-addition assay where hesperetin was added at various times post infection (-0.5 hpi to 36 hpi) and supernatant was collected at 48 hpi for determination of virus yield. The levels of inhibition in virus yields were comparable, when hesperetin was added to the cells at -0.5 hpi, 1 hpi, 6 hpi or 12 hpi, suggesting that hesperetin may not affect the early stages (attachment, entry) of BPXV replication cycle**(Fig. 2)** . Likewise, virus yields were comparable in cells treated at 30 hpi or 36 hpi with hesperetin, suggesting that hesperetin is unlikely to affect the late stages (budding/release) of the BPXV replication cycle. However, the magnitude of viral inhibition was shown to progressively decrease from 18 hpi to 30 hpi of hesperetin addition, suggesting that hesperetin may act in the middle to pre-budding steps of the virus replication cycle **(Fig. 2)** .

Hesperetin has no effect on BPXV attachment, entry and budding

The effect of hesperetin on BPXV attachment to the host cells was evaluated by infecting the Vero cells at 4, which allowed the attachment of the virus to the cells but restricted viral entry. As shown in **Fig. 3A,** viral titres were comparable in both vehicle control-treated and hesperetin-treated cells, suggesting that hesperetin does not affect BPXV attachment to the host cells. To evaluate the effect of hesperetin on BPXV entry, the virus was first allowed to attach at 4°C in the absence of the drug, followed by incubating the cells at 37°C for 1 h (to allow viral entry) in the presence of hesperetin or vehicle control. As shown in**Fig. 3B,** viral titres were comparable in both DMSO-treated and drug-treated cells, suggesting that hesperetin does not affect BPXV entry into the host cells. To evaluate the effect of hesperetin on virus release, hesperetin was applied when the virus presumably starts budding/release from the infected cells viz; at 36 hpi. As shown in **Fig. 3C,** viral titres were comparable in both DMSO- and drug-treated cells, suggesting that hesperetin does not affect BPXV egress from the infected cells.

Hesperetin treatment reduced the levels of viral DNA, mRNA and protein in the target cells

To evaluate the effect of hesperetin on the synthesis of viral DNA/mRNA/proteins, the drug was applied at 3 hpi, a time-point when the early steps of the BPXV life cycle (attachment and entry) have occurred.

The cells were scraped at 24 hpi to quantify viral DNA/RNA/proteins. As shown in **Fig. 4A,** hesperetin treated cells showed ˜72% reduction in viral mRNA copy numbers as compared to the DMSO-treated cells. Likewise, as compared to the vehicle-control treated cells, hesperetin-treated cells had ˜26 %**DNA (Fig. 4B).** Since reduced levels of viral mRNA may reflect a reduction in the synthesis of viral proteins, levels of viral protein in drug-treated and DMSO-treated cells were also examined. As shown in**Fig. 4C** (upper panel), hesperetin treatment remarkably suppressed the synthesis of viral proteins, whereas levels of the housekeeping control protein β-actin were unaffected **(Fig. 4C, lower panel).**

MAPK/ERK/eIF4E signaling axis is a prerequisite for BPXV replication

Viral mRNA translation in most DNA viruses (including poxviruses) and some RNA viruses occurs in a cap-dependent manner where eIF4E/eIF4G interaction plays a central role in translation initiation[35]. In agreement with the previous findings[35], inhibition of the phosphorylation (activation) of ERK, MNK1 and eIF4E by small molecule chemical inhibitors (FR180204, CGP5738 and 4EGI-1, respectively) resulted in reduced BPXV replication**(Fig. 5)** suggesting the ERK/MNK1/eIF4E signalling axis is prerequisite for translation of BPXV proteins.

Hesperetin disrupts the interaction of viral mRNA with eIF4E

Further, the mechanism underlying the suppression of BPXV protein synthesis by hesperetin was elucidated. We performed a CHIP assay where cell lysates from BPXV-infected and, hesperetin-treated or untreated cells were immunoprecipitated by α-eIF4E and the viral mRNA in the immunoprecipitate was quantified by qRT-PCR. As shown in **Fig. 6,** as compared to DMSO-treated cells, the level of viral mRNA immunoprecipitated by α-eIF4E was significantly low. Further, the nonreactive antibody (α-MNK1) did not immunoprecipitate any viral mRNA. This suggested that e-IF4E specifically reacted with the 5' cap of viral mRNA and that hesperetin blocks the binding of eIF4E and the 5' cap of the viral mRNA.

Molecular docking and molecular dynamic (MD) simulations

In order to further confirm the binding of the hesperetin **(Fig. 7A)** at the 5' cap (m7GTP, **Fig. 7B)**-binding pocket of the eIF4E, we performed protein visualisation, molecular dynamic (MD) simulations, and molecular docking studies **(Fig. 7 and Fig. 8).**We first superimposed the crystal structures of the open **(Fig 7C, grey)** and close (**Fig 7C, green**) conformations of eIF4E using PDB id 3TF2 and 1IPC, respectively. A significant displacement in the loop region ($_{49}$KNDKSKTWQANL$_{60}$) of eIF4E in its mRNA cap-binding pocket was observed **(Fig. 7C).** In agreement with the previous reports [36,37], this flexible loop region adopted a closed conformation with an inward movement of ˜6.4 **(Fig. 7C zoomed view).** This inward movement of the $_{49}$KNDKSKTWQANL$_{60}$loop region (shown in red) provided anchoring points for the mRNA m7GTP cap. Another loop region, $_{203}$ATKSGSTT$_{211}$(shown in orange) critical for the binding of the second nucleotide of the m7GpppA complex also showed an inward shift towards the cap-binding pocket [36]. To confirm these shifts of the loop regions of eIF4E, we performed molecular dynamics simulations using iMODS server in normal mode analysis (NMA). As shown in **Fig. 8A (Upper panel),** we found similar movements in both loop regions of eIF4E.

The molecular docking studies revealed that both m7GTP and hesperetin bind to the same pocket of eIF4E (**Fig. 7D and Fig. 7E**) with binding energies of -8.1 and -7.8 Kcal/mol respectively. In the crystal structure of m7GTP bound eIF4E, the m7GTP is stacked between the Trp56 and Trp102 residues of eIF4E [36]. Similar conformation was also observed in the docking studies of m7GTP with eIF4E (**Fig. 7D** zoomed view). Interestingly, hesperetin also adopted identical stacking conformation between these two residues of eIF4E via its benzopyrone moiety (**Fig. 7E** zoomed view).

Interaction and stability of the hesperetin and eIF4E complex

For a detailed analysis of all the interactions between hesperetin and m7GTP with eIF4E, we constructed Ligplots [33] of the docked structures (**Fig. 7F and 7G**) (Hydrogen bonds are highlighted in a green dashed line with bond lengths indicated in angstrom. The hydrophobic interactions are highlighted in red dashed lines). The methyl group of guanosine moiety which is an important 5'cap modification of mRNA

and critically regulates its fate, interacted with the Trp56 residue of eIF4E via hydrophobic interaction. Remarkably, the methyl group of hesperetin also interacted with eIF4E via its Arg157 and Lys162 residues through non-polar interactions **(Fig. 7G).**

Several polar contacts were also observed in both docked complexes. Most notably, the eIF4E residues Glu103, Arg112 and Asn155 interacted with the nitrogen and oxygen atoms of m7GTP through hydrogen bonds. Owing to the multiple oxygen atoms in its ring structures, hesperetin also interacted with these three residues of eIF4E via hydrogen bonds, besides multiple non-polar contacts.

Overall, m7GTP interacted with eIF4E via 8 polar contacts, and 42 hydrophobic contacts while hesperetin interacted via 5 polar contacts and 49 hydrophobic contacts (**Table 1).**

Table 1. Summary of ligplot results

eIF4E residues	**m7GTP**	**Hesperetin**
Trp56	Hydrophobic	Hydrophobic
Asp90	Polar	
Met101	Hydrophobic	Hydrophobic
Trp102	Hydrophobic	Hydrophobic and polar
Glu103	Polar	Hydrophobic and polar
Arg112	Hydrophobic and polar	Hydrophobic and polar
Asn155	Hydrophobic and polar	Hydrophobic and polar
Arg157		Hydrophobic
Lys162		Hydrophobic and polar
Trp166		Hydrophobic
Gln198	Hydrophobic and polar	
Thr203	Hydrophobic	
Ala204	Hydrophobic and polar	
Thr210	Hydrophobic	
Thr211	Hydrophobic and polar	

To evaluate the stability and physical movement of atoms in docked complexes, we performed molecular dynamics simulations using the iMODS server [34]. The simulations were performed in NMA. The results of the apo-eIF4E, m7GTP-eIF4E and hesperetin-eIF4E complex are depicted in **Fig. 8.** The affine-model **(Fig. 8A, large coloured arrows)** representation and NMA mobility based colour scheme of eIF4E residue were utilized to observe the mobility of eIF4E regions in all three cases. [34]. As shown in **Fig. 8A (upper panel)** , the apo-eIF4E loop regions displayed a bathochromic shift of colors in its backbone (green, yellow and red shades). Besides, wider affine-model (colored arrows) and longer arrow fields (black arrows) also suggested higher mobility of these residues as compared to the eIF4E-m7GTP and eIF4E-hesperetin complexes (**Fig. 8A)** .

To gain further insights into the protein flexibility and mobility, we analysed the deformability and B factor plots, respectively, which were generated by the iMODS server (**Fig 8B and Fig. 8C)** . High peaks in the deformability plot of apo-eIF4E demonstrated flexible regions (**Fig 8B, upper panel)** which diminished after complexion with m7GTP and hesperetin (**Fig 8B, middle and lower panel)** . This was complimented by the B-factor plot (**Fig 8C),** which is proportional to the root mean square (RMS) and demonstrate the stabilities of m7GTP- and hesperetin-docked complexes. Additionally, high eigenvalues of $7.542249e^{-05}$ and $7.541401e^{-05}$ were observed for m7GTP- and hesperetin-docked complexes respectively (**Fig 8D),** which when compare with the eigenvalues of apo-eIF4E ($3.114206e^{-04}$) indicated a higher energy requirement for the deformation of docked complexes. This suggests the stability of docked complexes . The analysis of variance of individual modes (indicating their relative contribution to the equilibrium motions) (**Fig. 8E)** , covariance map (indicating coupling between correlated, uncorrelated and anti-correlated motions residues) (**Fig. 8F)** and elastic network model (correlating to the stiffness of the complex) **(Fig. 8G**) inferred a close

resemblance between the m7GTP-eIF4E and hesperetin-eIF4E binding. In contrast, apo-eIF4E, displayed high mobility and flexibility due to highly uncorrelated and elastic motion between atoms.

Hesperetin provides protection to embryonated chicken eggs against lethal BPXV infection

A wide variety of poxviruses, including BPXV, induce pock lesions on the CAM in embryonated chicken eggs [38,39]. To evaluate the*in ovo* antiviral efficacy of hesperetin against BPXV, we first determined the subcytotoxic concentration of hesperetin in specific pathogen free (SPF) embryonated chicken eggs. As shown in **Fig. 9A,** hesperetin at a dose rate of [?]4000 µg/egg induced mortality in the chicken embryos, whereas no mortality was observed at a dose rate of 1000 µg/egg. The LD_{50} of hesperetin was determined to be 1080.74 µg/egg. For evaluation of the *in ovo* antiviral efficacy, the eggs were infected with BPXV at 100 EID_{50} and treated with the indicated concentrations of hesperetin. As shown in**Fig. 9B,** hesperetin provided protection from BPXV challenge infection in a dose-dependent manner. Moreover, the size of localized pock lesion was also suppressed with increasing concentrations of hesperetin **(Fig. 9C)** . The EC_{50} was calculated to be 22.5 µg/egg and the therapeutic index 48.1.

Evaluation of antiviral drug resistance against hesperetin

We also evaluated the potential of hesperetin to select drug resistant virus mutants under the long-term selection pressure of hesperetin, wherein BPXV was sequentially passaged (P) in the presence of hesperetin or an equivalent volume of DMSO. The infectious virions collected in the supernatant were used for the next round of infection, and the process was repeated until P40. The resulting viruses were named BPXV-P40-hesperetin and BPXV-P40-DMSO. As shown in **Fig. 10,** the levels of suppression of the virus yield by hesperetin in Vero cells were comparable in both BPXV-P40-hesperetin and BPXV-P40-DMSO. This suggested that hesperetin does not select drug-resistant virus mutants.

DISCUSSION

In this study, we demonstrated the *in vitro* antiviral efficacy of hesperetin against multiple poxviruses, which include BPXV, VACV and LSDV. This, together with hesperetin-induced protection in chicken embryos against virulent BPXV, led to the conclusion that hesperetin may potentially be developed as a broad-spectrum antiviral drug against poxviruses. Previous studies have also identified *in vitro*antiviral activity of hesperetin against DENV [20], CHIKV [21], ZIKV [21] and Sindbis virus[22]. The docking and molecular dynamics simulations displayed the effect of hesperetin on the protease 3D models of CHIKV and ZIKV [20]. However, the precise mechanism of the antiviral action of hesperetin remains unknown.

The incubation of cell free virions with hesperetin did not affect the residual infectivity of the virions, which seems to suggest that hesperetin may not be directly targeting viral factors in poxviruses, although this needs further in-depth investigation. We demonstrated that hesperetin treatment mainly suppresses BPXV protein synthesis with a marginal inhibitory effect on the levels of viral DNA and mRNA but without affecting other steps of the viral life cycle such as attachment, entry, and budding. The 5'-cap of mRNA is highly conserved and is an important structural modification critical for eIF4E-mRNA interaction. In order to effectively synthesize viral proteins and to circumvent the action of 5'-3' exonucleases, viral capping mechanisms that generated mRNA capping identical to that of the host were selected during the co-evolution of viruses and hosts [40]. Previous studies have demonstrated that BPXV exploits cap-dependent mechanism of protein translation [24,35,41]. Therefore, we asked whether hesperetin blocks BPXV translation initiation by abrogating the interaction of the 5' cap of viral mRNA and eIF4E. The CHIP assay suggested that hesperetin disrupts binding of the 5' cap (m7GTP binds) of viral mRNA with eIF4E. The molecular docking and MD simulation studies, also confirmed stable binding of the hesperetin with the cap-binding pocket of eIF4E, in a similar conformation as m7GTP binds. This is a novel mechanism wherein hesperetin has been shown to exert its indirect antiviral action by targeting a cellular factor (eIF4E). Further, in agreement with the previous studies[24,41], we demonstrated that BPXV exploits ERK-MNK1-eIF4E signalling to effectively replicate in the target cells. Hesperetin targets this signalling axis by inhibiting the interaction of eIF4E with viral mRNA, which results in the shut off of viral protein translation.

One of the major limitations of antiviral drugs is that they rapidly induce the development of drug-resistant viral mutants[42]. However, as compared to the drugs that directly target viral factors, drugs based on targeting essential cellular factors are considered to have minimal or no tendency to induce antiviral drug resistance [24]. In our study, hesperetin-resistant BPXV mutants were not observed even when the virus was sequentially cultured 40 times in the presence of hesperetin. This seems to be due to the low genetic variability of the host factor (eIF4E), thereby imposing a higher genetic barrier to the generation of resistant viruses [24,25,41].

Since hesperetin can alter cell metabolism, its long-term use could eventually result in cytotoxicity. Therefore, its further validation, long-term *in vivo* efficacy, and clinical trials will be essential before actually introducing it from research into clinical settings.

In conclusion, hesperetin was shown to exert a potent *in vitro*and *in ovo* antiviral efficacy against poxviruses. Mechanistically, hesperetin was shown to competitively inhibit binding of the viral mRNA with eIF4E **(Fig. 11),** thereby blocking viral protein translation. Most importantly, hesperetin was not shown to readily select drug-resistant viral mutants.

DATA AVAILABILITY

Vero cell adapted BPXV was deposited in the National Centre for Veterinary Type Cultures (NCVTC), Hisar with an Accession Number of VTCC-AVA90 which can be accessed at http://ncvtc.org.in/wp-content/uploads/2019/08/Viruses-availble-for-distribution.pdf. Its whole genome sequence is available in GenBank with an Accession Number of MW883892.1.

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REFERENCES

1. Pan D, Nazareth J, Sze S, et al. Transmission of monkeypox/mpox virus: A narrative review of environmental, viral, host, and population factors in relation to the 2022 international outbreak. *Journal of Medical Virology* . 2023;95(2):e28534.

2. Kumar N, Tripathi BN. A serious skin virus epidemic sweeping through the Indian subcontinent is a threat to the livelihood of farmers. *Virulence* . Dec 2022;13(1):1943-1944. doi:10.1080/21505594.2022.2141971

3. Haller SL, Peng C, McFadden G, Rothenburg S. Poxviruses and the evolution of host range and virulence. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* . Jan 2014;21:15-40. doi:10.1016/j.meegid.2013.10.014

4. Bera BC, Shanmugasundaram K, Barua S, et al. Zoonotic cases of camelpox infection in India. *Veterinary microbiology* . Aug 26 2011;152(1-2):29-38. doi:10.1016/j.vetmic.2011.04.010

5. Marinaik CB, Venkatesha MD, Gomes AR, Reddy P, Nandini P, Byregowda SM. Isolation and molecular characterization of zoonotic Buffalopox virus from skin lesions of humans in India. *International journal of dermatology* . May 2018;57(5):590-592. doi:10.1111/ijd.13890

6. Patil DY, George S, Sahay RR, et al. A case of human buffalopox in Malappuram, India: The role of mpox surveillance in 2022. *Journal of Medical Virology* . 2023;95(2):e28580.

7. Alakunle EF, Okeke MI. Monkeypox virus: a neglected zoonotic pathogen spreads globally. *Nature reviews Microbiology* . Sep 2022;20(9):507-508. doi:10.1038/s41579-022-00776-z

8. Caillat C, Topalis D, Agrofoglio LA, et al. Crystal structure of poxvirus thymidylate kinase: an unexpected dimerization has implications for antiviral therapy. *Proceedings of the National Academy of Sciences of the United States of America* . Nov 4 2008;105(44):16900-5. doi:10.1073/pnas.0804525105

9. Yang H, Kim SK, Kim M, et al. Antiviral chemotherapy facilitates control of poxvirus infections through inhibition of cellular signal transduction. *The Journal of clinical investigation* . Feb 2005;115(2):379-87. doi:10.1172/JCI23220

10. Junge RE, Duncan MC, Miller RE, Gregg D, Kombert M. Clinical presentation and antiviral therapy for poxvirus infection in pudu (Pudu puda). *Journal of zoo and wildlife medicine : official publication of the American Association of Zoo Veterinarians* . Sep 2000;31(3):412-8. doi:10.1638/1042-7260(2000)031[0412:CPAATF]2.0.CO;2

11. De Clercq E. Vaccinia virus inhibitors as a paradigm for the chemotherapy of poxvirus infections. *Clinical microbiology reviews* . Apr 2001;14(2):382-97. doi:10.1128/CMR.14.2.382-397.2001

12. Neyts J, De Clercq E. Efficacy of 2-amino-7-(1,3-dihydroxy-2-propoxymethyl)purine for treatment of vaccinia virus (orthopoxvirus) infections in mice. *Antimicrobial agents and chemotherapy* . Jan 2001;45(1):84-7. doi:10.1128/AAC.45.1.84-87.2001

13. Snoeck R, De Clercq E. Role of cidofovir in the treatment of DNA virus infections, other than CMV infections, in immunocompromised patients. *Current opinion in investigational drugs* . Nov 2002;3(11):1561-6.

14. Grosenbach DW, Honeychurch K, Rose EA, et al. Oral Tecovirimat for the Treatment of Smallpox. *The New England journal of medicine* . Jul 5 2018;379(1):44-53. doi:10.1056/NEJMoa1705688

15. Broekema FI, Dikkers FG. Side-effects of cidofovir in the treatment of recurrent respiratory papillomatosis. *European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies* . Aug 2008;265(8):871-9. doi:10.1007/s00405-008-0658-0

16. Romanowski EG, Gordon YJ, Araullo-Cruz T, Yates KA, Kinchington PR. The antiviral resistance and replication of cidofovir-resistant adenovirus variants in the New Zealand White rabbit ocular model.*Investigative ophthalmology & visual science* . Jul 2001;42(8):1812-5.

17. Chen F, Chu C, Wang X, et al. Hesperetin attenuates sepsis-induced intestinal barrier injury by regulating neutrophil extracellular trap formation via the ROS/autophagy signaling pathway. *Food & function* . May 11 2023;14(9):4213-4227. doi:10.1039/d2fo02707k

18. Zaafar D, Khalil HMA, Rasheed RA, Eltelbany RFA, Zaitone SA. Hesperetin mitigates sorafenib-induced cardiotoxicity in mice through inhibition of the TLR4/NLRP3 signaling pathway. *PloS one* . 2022;17(8):e0271631. doi:10.1371/journal.pone.0271631

19. Kong LN, Lin X, Huang C, et al. Hesperetin derivative-12 (HDND-12) regulates macrophage polarization by modulating JAK2/STAT3 signaling pathway. *Chinese journal of natural medicines* . Feb 2019;17(2):122-130. doi:10.1016/S1875-5364(19)30014-7

20. Fatriansyah JF, Rizqillah RK, Yandi MY. Molecular Docking and Molecular Dynamics Simulation of Fisetin, Galangin, Hesperetin, Hesperidin, Myricetin, and Naringenin against Polymerase of Dengue Virus. *Journal of tropical medicine* . 2022;2022:7254990. doi:10.1155/2022/7254990

21. Eberle RJ, Olivier DS, Pacca CC, et al. In vitro study of Hesperetin and Hesperidin as inhibitors of zika and chikungunya virus proteases.*PloS one* . 2021;16(3):e0246319. doi:10.1371/journal.pone.0246319

22. Paredes A, Alzuru M, Mendez J, Rodriguez-Ortega M. Anti-Sindbis activity of flavanones hesperetin and naringenin. *Biological & pharmaceutical bulletin* . Jan 2003;26(1):108-9. doi:10.1248/bpb.26.108

23. Kumar N, Chander Y, Kumar R, et al. Isolation and characterization of lumpy skin disease virus from cattle in India. *PloS one* . 2021;16(1):e0241022. doi:10.1371/journal.pone.0241022

24. Khandelwal N, Chander Y, Kumar R, et al. Antiviral activity of Apigenin against buffalopox: Novel mechanistic insights and drug-resistance considerations. *Antiviral research* . Sep 2020;181:104870. doi:10.1016/j.antiviral.2020.104870

25. Chander Y, Kumar R, Verma A, et al. Resistance Evolution against Host-directed Antiviral Agents: Buffalopox Virus Switches to Use p38-Upsilon under Long-term Selective Pressure of an Inhibitor Targeting p38-alpha. *Molecular biology and evolution* . Sep 1 2022;39(9)doi:10.1093/molbev/msac177

26. Khandelwal N, Chander Y, Rawat KD, et al. Emetine inhibits replication of RNA and DNA viruses without generating drug-resistant virus variants. *Antiviral Research* . 2017;144:196-204.

27. Khandelwal N, Chander Y, Kumar R, et al. Antiviral activity of Apigenin against buffalopox: Novel mechanistic insights and drug-resistance considerations. *Antiviral Research* . 2020;181:104870.

28. Chander Y, Kumar R, Verma A, et al. Resistance evolution against host-directed antiviral agents: Buffalopox virus switches to use p38- under long-term selective pressure of an inhibitor targeting p38-α.*Molecular Biology and Evolution* . 2022;

29. Biasini M, Bienert S, Waterhouse A, et al. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic acids research* . 2014;42(W1):W252-W258.

30. Maier JA, Martinez C, Kasavajhala K, Wickstrom L, Hauser KE, Simmerling C. ff14SB: improving the accuracy of protein side chain and backbone parameters from ff99SB. *Journal of chemical theory and computation* . 2015;11(8):3696-3713.

31. Kumar R, Afsar M, Khandelwal N, et al. Emetine suppresses SARS-CoV-2 replication by inhibiting interaction of viral mRNA with eIF4E.*Antiviral research* . 2021;189:105056.

32. Eberhardt J, Santos-Martins D, Tillack AF, Forli S. AutoDock Vina 1.2. 0: New docking methods, expanded force field, and python bindings.*Journal of chemical information and modeling* . 2021;61(8):3891-3898.

33. Wallace AC, Laskowski RA, Thornton JM. LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions.*Protein engineering, design and selection* . 1995;8(2):127-134.

34. López-Blanco JR, Aliaga JI, Quintana-Ortí ES, Chacón P. iMODS: internal coordinates normal mode analysis server. *Nucleic acids research* . 2014;42(W1):W271-W276.

35. Kumar R, Khandelwal N, Thachamvally R, et al. Role of MAPK/MNK1 signaling in virus replication. *Virus research* . Jul 15 2018;253:48-61. doi:10.1016/j.virusres.2018.05.028

36. Siddiqui N, Tempel W, Nedyalkova L, et al. Structural insights into the allosteric effects of 4EBP1 on the eukaryotic translation initiation factor eIF4E. *Journal of molecular biology* . 2012;415(5):781-792.

37. Tomoo K, Shen X, Okabe K, et al. Crystal structures of 7-methylguanosine 5'-triphosphate (m7GTP)- and P1-7-methylguanosine-P3-adenosine-5', 5'-triphosphate (m7GpppA)-bound human full-length eukaryotic initiation factor 4E: biological importance of the C-terminal flexible region. *Biochemical Journal* . 2002;362(3):539-544.

38. Marennikova S, Shelukhina E, Shenkman L. Role of the temperature of incubation of infected chick embryos in the differentiation of certain poxviruses according to pock morphology. *Acta Virologica* . 1973;17(4)

39. Baxby D. Variability in the characteristics of pocks produced on the chick chorioallantois by white pock mutants of cowpox and other poxviruses. *Epidemiology & Infection* . 1969;67(4):637-647.

40. Ding P, Summers MF. Sequestering the 5'-cap for viral RNA packaging.*BioEssays : news and reviews in molecular, cellular and developmental biology* . Nov 2022;44(11):e2200104. doi:10.1002/bies.202200104

41. Kumar R, Khandelwal N, Chander Y, et al. MNK1 inhibitor as an antiviral agent suppresses buffalopox virus protein synthesis.*Antiviral research* . Dec 2018;160:126-136. doi:10.1016/j.antiviral.2018.10.022

42. Donaldson EF, Deming DJ, O'Rear JJ, Naeger LK. Regulatory evaluation of antiviral drug resistance in the era of next-generation sequencing.*Biomarkers in medicine* . 2015;9(11):1047-51. doi:10.2217/bmm.15.63

Figure legends

Fig. 1 Antiviral efficacy of hesperetin

(A) Cytotoxicity (MTT) assay. The indicated concentration of hesperetin or an equivalent volume of DMSO were incubated with cultured Vero cells for 96 h and cell viability was determined using the MTT assay. (B-D) Antiviral efficacy against poxviruses. Vero cells, in triplicates, were infected with the indicated virus at an MOI of 0.1 in the presence of the indicated concentration of hesperetin or DMSO. The infectious virus particles released in the supernatant at 72 hpi were titrated using plaque assay. Virus yield in hesperetin-treated and DMSO-treated cells in BPXV**(B),** Vaccinia virus **(C)** and LSDV (D) infected cells is shown. (E) Virucidal activity. The indicated concentrations of hesperetin were pre-incubated with BPXV (10^7 pfu/ml) at 37 for 2 h, and residual infectivity of BPXV was determined by plaque assay. The results are representative of three independent experiments. Error bars indicate SD. Pair-wise statistical comparisons were performed using the Student's t-test (*** = P<0.001).

Fig. 2. Time-of-addition assay

Confluent monolayers of Vero cells, in triplicates, were infected with BPXV at an MOI of 5, followed by washing with PBS and hesperetin or DMSO treatment at -0.5 hpi, 1 hpi, 6 hpi, 12 hpi, 18 hpi, 24 hpi, 30 hpi and 36 hpi. Supernatants were harvested at 48 hpi, and infectious virions produced in the supernatant at 48 hpi were quantified by plaque assay.

Fig.3. Effect of hesperetin on BPXV attachment, entry and budding

(A) ***Attachment assay.*** Vero cells, in triplicates, were treated with hesperetin or DMSO for 1 h, followed by BPXV infection at an MOI of 5 for 1 h at 4degC. Thereafter, the cells were washed with PBS to remove the unattached virus, and cell lysates were prepared by the rapid freeze-thaw cycles. The viral titers in cell lysates were quantified by plaque assay. (B) Entry assay. Pre-chilled confluent monolayers of Vero cells were infected with BPXV at 5 MOI for 1 hr at 4degC to permit attachment, followed by washing with PBS and addition of serum-free DMEM containing hesperetin or vehicle control, and incubation at 37degC for 1 h (to allow virus entry). At 1hpi, cells were washed with PBS and supplemented with serum free DMEM without drug. The infectious virus particles released in the supernatant at 48 hpi were titrated by plaque assay. (C) Virus release assay. Vero cells, in triplicates, were infected with BPXV at MOI of 5 for 1 h, followed by washing with PBS and the addition of fresh medium without any inhibitor. The cells were incubated at 37degC. At 36 hpi, when BPXV presumably starts releasing from the infected cells, hesperetin or DMSO were applied. The supernatants were harvested at the indicated time points (0.5 h and 4 h) following the addition of hesperetin and quantified by plaque assay. Values are representative of three independent experiments. Error bars indicate SD. Pair-wise statistical comparisons were performed using the Student's t test. ns = non-significant difference.

Fig. 4. Effect of hesperetin on BPXV mRNA, DNA and protein synthesis

Confluent monolayers of Vero cells, in triplicates. were infected with BPXV at an MOI of 5 for 1 h, followed by washing with PBS and addition of fresh DMEM. Hesperetin or DMSO were added at 3 hpi. Cells were scraped at 24 hpi for quantitation of viral DNA, RNA and proteins (A) mRNA synthesis: Total RNA was isolated from the cells, and cDNA was prepared using oligo (dT) primers. The levels of BPXV *C18L* gene in hesperetin-treated and DMSO-treated cells were quantified using qRT-PCR. (B) Genome synthesis : DNA was isolated from hesperetin-treated or DMSO-treated cells and the BPXV*C18L* gene was quantified by qRT-PCR. (C) Protein synthesis: Cell lysates were prepared in RIPA buffer and subjected to western blot analysis by using anti-BPXV hyper-immune serum (upper panel) or β-actin (housekeeping control) antibody (lower panel). Values are representative of three independent separate experiments. Error bars indicate SD. Pair-wise statistical comparisons were performed using the Student's t-test (** = P<0.01, *** = P<0.001).

Fig.5. ERK-MNK1-eIF4E signaling is a prerequisite for BPXV

Vero cells, in triplicates, were infected with BPXV at an MOI of 0.1. At 1 hpi, cells were washed with

PBS, and fresh medium containing an ERK inhibitor (FR180204: 0.2 μg/ml), MNK1 inhibitor (CGP57380: 0.5 μg/ml) or eIF4E inhibitor (4EGI-1; 0.5 μg/ml) was added. The progeny virus particles released in the infected cell culture supernatant at 48 hpi were quantified by plaque assay. The values are representative of three independent experiments. Error bars indicate SD. Pair-wise statistical comparisons were performed using the Student's t-test (*** = P<0.001).

Fig.6. CHIP assay

Vero cells, in triplicates, were infected with BPXV at an MOI of 5, followed by washing with PBS and addition of serum-free DMEM supplemented with hesperetin or vehicle control. At 16 hpi, cell lysates were prepared as described in method section and incubated with α-eIF4E (reactive antibody), α- MNK1 (nonreactive antibody) or equivalent volume of IP buffer (Beads control). This was followed by incubation with a Protein A Sepharose® slurry. The beads were washed with IP buffer, and cross-linking was reversed using Proteinase K. The reaction mixture was centrifuged and the resulting supernatant was subjected to RNA isolation, cDNA preparation, and quantitation of BPXV *M* gene by qRT-PCR. Values are representative of three independent separate experiments. Error bars indicate SD. Pair-wise statistical comparisons were performed using the Student's t test (*** = P<0.001).

Fig.7. Molecular docking

(A) Structure of hesperetin. (B) Structure of m7GTP. (C) Structure of eIF4E in open (grey, PDB ID – 3TF2) and close (green, PDB ID – 1IPC) conformations. The flexible loop regions of eIF4E in close conformation are highlighted in orange and red. Both eIF4E structures were aligned using Pymol software version 0.99rc6. The bottom loop region of eIF4E (red) in both conformations may be seen moving by 6.4 in a zoomed image.*(D-G).* ***Docking results and Ligplot analysis of m7GTP and hesperetin docked to eIF4E.*** m7GTP (D) and hesperetin (E) binding to the cap-binding pocket of eIF4E. Major eIF4E residues interacting with m7GTP can be seen in the respective zoomed view. Ligplot interaction analysis of the docked complexes of m7GTP-eIF4E **(F)** and hesperetin-eIF4E **(G)**is shown.

Fig. 8. Output of normal mode analysis (NMA) study of apo-eIF4E, m7GTP-eIF4E complex and hesperetin-eIF4E complex by iMODS server

(A) NMA mobility with affine arrows (large, colored) and arrow fields (small). (B) Main chain deformability. (C) B-factor values. (D) Eigenvalue plot. (E) Normal mode variance plot. The blue bars indicate the variance of individual modes while the teal bars indicate cumulative variance. (F) Co-variance map. The correlated, uncorrelated and anti-correlated motions are represented by red, white and blue color respectively. (G) Elastic network model. The dark color represents stiffer springs.

Fig. 9. *In ovo* antiviral efficacy of hesperetin against BPXV

(A) Determination of LD_{50}. Indicated concentrations of hesperetin, in triplicates, were inoculated in 10 day old embryonated SPF chicken eggs. The viability of the embryos was examined for up to 5 days post inoculation and the $LD_{50}$was determined to be 1080.74 μg/egg (Reed-Muench method). (B) Survival curve. Five-fold serial dilutions of hesperetin or DMSO were inoculated, in triplicates, in 10 days old SPF embryonated eggs via the CAM route, followed by infection with BPXV at 100 EID_{50}. The viability of the eggs was examined for six days post-infection. The EC_{50} was calculated to be 22.5 μg/egg **(C)** ***Pock lesions*** . Pock lesions on the CAM of embryonated SPF chicken eggs at day 6 post-BPXV infection are shown.

Fig. 10. Evaluation of hesperetin resistance against BPXV

BPXV was sequentially passaged 40 times in Vero cells in the presence of hesperetin or DMSO. For each passage, confluent monolayers of Vero cells were infected with BPXV at an MOI of 0.1, followed by washing with PBS and the addition of fresh DMEM supplemented with hesperetin (3 μg/ml) or 0.05% DMSO. At the end of P40, the fitness of hesperetin- or DMSO-passaged viruses was evaluated again against hesperetin.

Fig. 11 Schematics of the antiviral mechanism of action of hesperetin against poxviruses

BPXV activates ERK/MNK1/eIF4E signalling. Activated eIF4E binds with the 5' cap of viral mRNA to initiate translation of the viral proteins. Hesperetin competitively blocks binding of eIF4E with the 5' cap of viral mRNA, thereby blocking translation of the viral proteins.

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