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RESEARCH ARTICLE

Antiviral effects of resveratrol against the replication of chikungunya and Japanese encephalitis viruses *in vitro*

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ABSTRACT

Chikungunya virus (CHIKV) and Japanese encephalitis virus (JEV) are mosquito-borne arboviruses that pose significant public health risks, especially in tropical regions like Malaysia. CHIKV is linked to joint and muscle pain, while JEV can cause severe neurological illnesses and encephalitis if untreated. With no specific treatments available, research into potential antivirals is crucial. This study investigates the inhibitory potential of resveratrol (RES) against CHIKV and JEV *in vitro*. Cytotoxicity of RES was assessed on human adenocarcinoma alveolar basal epithelial cells (A549) using the MTS assay, followed by dose-dependent analyses to determine optimal inhibitory concentrations. Antiviral effects were explored through pre-infection, post-infection, virucidal, and anti-adsorption assays, with virus titres measured via plaque and foci-forming assays for CHIKV and JEV, respectively. Results revealed that RES's pre- and post-infection treatments significantly reduced titres of both viruses in a dose-dependent manner. Notably, CHIKV titres were reduced by over 65% ($p < 0.01$) when treated with 100 µM RES, whether administered pre-infection or post-infection. For JEV, a reduction of over 93% ($p < 0.05$) was observed only with post-infection treatment, while pre-infection treatment alone did not yield a significant reduction. In addition, both pre-incubation and anti-adsorption assays for CHIKV and JEV showed no significant results. These findings suggest that RES likely inhibits viral replication by modulating host cellular mechanisms rather than directly targeting the viruses. In summary, this study demonstrates that RES exhibits antiviral properties against CHIKV and JEV replication, underscoring its potential as an effective antiviral agent. However, further *in vivo* studies are needed to fully evaluate its therapeutic potential and efficacy.

Keywords: Infectious diseases; arboviruses; antiviral; natural compound; resveratrol.

INTRODUCTION

Arboviruses, or arthropod-borne viruses, are a diverse group of RNA viruses transmitted to vertebrates by blood-feeding arthropods like mosquitoes, sandflies, and ticks. Over 600 arboviruses have been identified globally, with around 100 capable of infecting humans. Human pathogens in this group include dengue, yellow fever, chikungunya, Zika, and West Nile viruses, which impose substantial global health burdens, especially in tropical and subtropical regions. The risk of arbovirus transmission is increasing due to factors such as climate change, urbanization, and global trade, which disrupt the ecological balance between hosts and vectors (de Souza & Weaver, 2024). The continual emergence and re-emergence of arboviruses highlights the critical need for ongoing research to reduce their impact on both human and animal health.

Chikungunya virus (CHIKV), belonging to the *alphavirus* genus of the *Togaviridae* family, is transmitted by Aedes mosquitoes, particularly *Aedes aegypti* and *Aedes albopictus* (WHO, 2020). This virus is known for causing chikungunya fever, accompanied by symptoms such as excruciating muscle and joint pain, headache, and rashes (CDC, 2022). Since July 2023 to June 2024, approximately 350 000 CHIKV infection cases and over 140 deaths have been reported worldwide (ECDC, 2024). In Malaysia, sporadic CHIKV cases have been reported across the country, with the potential for outbreaks in both rural and urban regions (Wimalasiri-Yapa *et al.*, 2019). A 2019 outbreak in Tanjung Sepat, Malaysia, revealed a high seropositivity rate of 72.5% among residents, indicating significant indoor transmission and a high prevalence of asymptomatic cases (Khor *et al.*, 2023). These occurrences highlight the debilitating nature of the disease, coupled with the virus's rapid transmission, which poses a significant threat to public health.

On the contrary, Japanese encephalitis virus (JEV) belongs to the *flavivirus* genus and is primarily transmitted by the Culex mosquito, specifically *Culex tritaeniorhynchus* (WHO, 2019). This virus is a leading cause of viral encephalitis in many Asian countries, manifesting in symptoms such as fever, headache, weakness, seizures, and coma (Hills *et al*., 2014). In 2023, 2114 new cases of Japanese encephalitis were reported globally, with the majority occurring in the Southeast Asia region, followed by the Western Pacific region (WHO, 2023). In Malaysia, Japanese encephalitis is considered an emerging and re-emerging infectious disease influenced by various factors, including environmental conditions, the population density of susceptible hosts (such as pigs and wading birds), and the presence of competent mosquito vectors (Kumar *et al*., 2018). Although humans can get infected through mosquito bites, they are viewed as dead-end hosts because they do not produce sufficient viremia to infect mosquitoes. While vaccination has reduced the incidence of Japanese encephalitis, the continued presence and genetic diversity of JEV in Malaysia necessitate ongoing surveillance, research into vaccine efficacy across all genotypes, and robust public health strategies to mitigate the risk of future outbreaks.

Resveratrol (RES), also known as trans-3,5,4'-trihydroxystilbene, is a natural compound found in various plants like grapes, peanuts, and berries (Salehi *et al*., 2018). This compound was first identified as a phytoalexin, an antibiotic produced by plants in response to a range of environmental stressors, such as microbial infections, heat, and UV radiation (Chang *et al*., 2011; Koushki *et al*., 2018). RES has garnered significant attention for its wide range of health benefits since its anticancer properties were first discovered in 1997. Research has shown that RES also plays a role in regulating glucose and lipid levels, acting as an antioxidant, reducing inflammation, and modulating the immune system (Meng *et al*., 2020).

Moreover, RES has shown antiviral properties against a variety of animal and human viruses, such as the hepatitis C virus (HCV), dengue virus, cytomegalovirus, respiratory syncytial virus, influenza A virus, Epstein-Barr virus (EBV), herpes simplex virus (HSV), cytomegalovirus, respiratory syncytial virus, human immunodeficiency virus (HIV), duck enteritis virus, and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Abba *et al*., 2015; Chin *et al*., 2023). According to Mohd *et al*. (2019), treating Zika virus-infected cells with RES post-infection led to a significant reduction of over 90% in viral titre and mRNA copy number, suggesting RES's potential as an antiviral compound with replication-inhibiting properties. Previous studies have proposed several antiviral mechanisms of RES. For instance, RES inhibits the replication of dengue and Zika viruses through the retention of nuclear protein high mobility group box 1 (HMGB1) and the upregulation of interferon-stimulated genes (ISGs) (Zainal *et al*., 2017; Chin *et al*., 2022). Additionally, RES has been found to suppress Venezuelan equine encephalitis virus (VEEV), an alphavirus, by interrupting the glycogen synthase kinase-3 (GSK-3) and protein kinase B (AKT) pathways (Lehman *et al*., 2021). RES has also been shown to suppress the synthesis of early intermediate proteins, IE62, which is the primary transcriptional regulatory protein encoded by the varicella-zoster virus (VZV), while also effectively inhibiting the production of viral protein 1 and phosphorylation of pro-inflammatory cytokines in enterovirus 71 (Docherty *et al*., 2006; Yang *et al*., 2015). While RES has demonstrated antiviral activity against other viral infections, its impact on the replication and pathogenesis of CHIKV and JEV remains unexplored. Therefore, the study aims to investigate the antiviral effects of RES against CHIKV and JEV *in vitro*, contributing to a better understanding of its potential as a therapeutic agent for these important arboviral diseases.

MATERIALS AND METHODS

Cells and viruses

Cells used in this study were African green monkey kidney cells (Vero) and human adenocarcinoma alveolar basal epithelial cells (A549). Dulbecco's Modified Eagle Medium (DMEM, Gibco, NY) supplemented with L-glutamine and 10% heat-inactivated foetal bovine serum (FBS, Bovagen, Australia) were used to maintain both cell types. Cells were kept at 37°C in a cell house with 5% CO2. For the RES treatment regimens, A549 cells were used, whereas Vero cells were used for virus propagation and titration. CHIKV and JEV used in the study were provided by Tropical Infectious Diseases Research & Education Centre (TIDREC), University of Malaya, Kuala Lumpur, Malaysia.

Resveratrol

Resveratrol (RES) was purchased from Sigma-Aldrich (St. Louis, MO; R5010; 228.24 g/mol). The stock solution was subsequently diluted to various concentrations (20 µM, 50 µM, 80 µM, 100 µM, and 200 µM) using DMEM supplemented with 2% FBS. These prepared compounds were then stored at -20°C until needed.

Cell cytotoxicity assay

The cytotoxic effects of RES were evaluated using the 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetra-zolium (MTS) assay (Promega, WI). Triplicate experiments were conducted, where both Vero and A549 cells were seeded onto a 96-well plate and incubated overnight. Subsequently, the cells were treated with various RES concentrations (20 µM, 50 µM, 80 µM, 100 µM and 200 µM) for a duration of 72 hours at 37°C and under conditions of 5% CO2. The absorbance was then measured at 490 nm using a TECAN microplate reader. The percentage of cell viability was calculated by comparing the absorbance of treated cells to that of the control (untreated) cells.

Antiviral effects of RES against CHIKV and JEV (Dose-dependent study)

Different concentrations of RES (20 µM, 50 µM, 80 µM, 100 µM, and 200 µM) were administered to a monolayer of A549 cells and incubated for a duration of 4 hours at 37°C in an environment with 5% CO2. Following this treatment, the cells were washed three times with serum-free media (SFM) before being subjected to CHIKV/JEV infection at a multiplicity of infection (MOI) of 1 for 1 hour 30 minutes. Subsequently, the infected cells were subjected to three additional washes with SFM before being provided with fresh DMEM media containing varying concentrations of RES supplemented with 2% heat-inactivated FBS. After 48 hours of incubation, the supernatants were collected. The viral titres were determined using a plaque assay for CHIKV and a foci-forming assay for JEV.

Pre-infection treatment

A549 cells were pre-treated with 100 µM RES for 4 hours prior to CHIKV infection, or with 80 µM RES for 4 hours prior to JEV infection. Following the pre-treatment, the cells were washed three times with SFM and then infected with CHIKV or JEV at a MOI of 1 for 1 hour and 30 minutes. After infection, the cells underwent three additional washes with SFM and were subsequently provided with fresh DMEM media supplemented with 2% heat-inactivated FBS. The supernatants were collected after 48 hours of incubation, and viral titres were determined as previously described.

Post-infection treatment

A549 cells were infected with CHIKV or JEV at a MOI of 1 for 1 hour and 30 minutes. Following infection, the cells were washed three times with SFM and then provided with fresh DMEM media containing 100 µM RES for CHIKV-infected cells or 80 µM RES for JEV-infected cells, supplemented with 2% heat-inactivated FBS. After 48 hours of incubation, the supernatants were collected, and viral titres were assessed as previously described.

Virucidal study

A virucidal assay was performed by pre-incubating CHIKV with 100 µM RES or JEV with 80 µM RES for 1 hour prior to infection of A549 cells. After pre-incubation, the virus-RES mixtures were introduced into A549 cells and incubated at 37°C for 1 hour and 30 minutes. The cells were then washed three times with SFM and subsequently supplemented with DMEM containing 2% heat-inactivated FBS. The cells were then incubated for 48 hours at 37°C in a 5% CO, environment. After the incubation period, the supernatants were collected, and viral titres were determined as previously described.

Anti-adsorption assay

For the anti-adsorption assay, A549 cells were infected with CHIKV or JEV at an MOI of 1 in culture medium containing 100 µM RES for CHIKV or 80 µM RES for JEV. The cells were incubated at 4°C for 1 hour to allow adsorption, followed by washing with SFM. Fresh DMEM supplemented with 2% heat-inactivated FBS was added, and the cells were incubated at 37°C in 5% CO, for 48 hours. Supernatants were then collected, and viral titres were determined as previously described.

Plaque assay

Infected Vero cells were fixed with 4% paraformaldehyde in 1x phosphate-buffered saline (PBS) for 30 minutes at room temperature. Subsequently, the cells were washed with 1x PBS for three times and stained with 1% crystal violet in 20% ethanol for 10 minutes. CHIKV titre was quantified by counting the plaques formed, with results expressed in plaque-forming units per millilitre (PFU/mL). The assay was performed as previously described (Teoh *et al*., 2020).

Focus-forming assay

Infected Vero cells were fixed with 4% paraformaldehyde in 1x PBS for 30 minutes at room temperature, followed by permeabilization with 1% Igepal for 15 minutes. Subsequently, 3% skimmed milk was added to each well to reduce non-specific binding. After overnight incubation at 4°C, the cells were incubated with primary antibody (JEV- positive human serum) at room temperature for 1 hour and 20 minutes. The cells were then incubated with a secondary antibody (anti-human serum) at room temperature for 1 hour. Subsequently, the cells were stained with a metal-enhanced diaminobenzidine (DAB) substrate for 10 minutes in the dark at room temperature. The JEV titre was then quantified by counting the foci formed and expressed in foci forming units per ml (FFU/mL). The assay was performed as previously described (Teoh *et al*., 2013).

Statistical analysis

Graph Pad Prism version 9 (Graph Pad Software Inc., San Diego, CA) was used to assess the absorbance readings and reduction in virus titre. Statistical analyses, including one-way ANOVA, unpaired t-tests, and non-linear regression tests, were conducted using the software. Statistical differences between non-treated and treated groups were represented as follows: ns = P>0.05, *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

RESULTS

Cytotoxicity effects of RES on A549 and Vero cells

MTS assay was utilized to evaluate the cytotoxicity effects of RES on A549 and Vero cells (Figure 1). RES was administered at increasing concentrations of 20 µM, 50 µM, 80 µM, 100 µM and 200 µM to both uninfected and infected cells, with a 72-hour incubation period. The cell viability was then determined by using non-treated cells as a control. The viability of both A549 (Figure 1A) and Vero (Figure 1B) uninfected cells remained above 99% across RES concentrations ranging from 20 µM to 100 µM. However, at 200 µM RES, viability decreased to 76% for A549 cells (Figure 1A) and 80% for Vero cells (Figure 1B). In CHIKV-infected cells, cell viability exceeded 103% for A549 cells (Figure 1C) and 89% for Vero cells (Figure 1D). For JEV-infected cells, cell viability exceeded 90% for A549 cells (Figure 1E) and 70% for Vero cells (Figure 1F). A slight decrease in viability was observed in 20 µM RES-treated JEV-infected Vero cells, though cell viability remained above 70%. Overall, both infected and uninfected cell lines exhibited cell viability greater than 70%, indicating that RES does not cause significant cytotoxicity in either Vero or A549 cells. From the MTS assay, we also determined the CC50 (cytotoxic concentration 50%) values for A549 and Vero cells, which are 448 µM and 774.2 µM, respectively.

Antiviral effects of RES against CHIKV and JEV (Dose-dependent study)

The antiviral effects of RES against CHIKV and JEV were evaluated using simultaneous pre- and post-infection treatments (Figure 2). A549 cells were treated with various concentrations of RES (20 µM, 50 µM, 80 µM, 100 µM, and 200 µM) before and after virus infections, with non-treated and uninfected cells serving as controls. RES treatment resulted in significant CHIKV titre reductions of 21.27% ($p<0.05$), 72.53% ($p<0.0001$), 82.75% ($p<0.0001$), 96.15% ($p<0.0001$), and 91.96% ($p<0.0001$) for 20 µM, 50 µM, 80 µM, 100 µM, and 200 µM RES, respectively, with a slight increase in titres observed at 200 µM (Figures 2A and 2B). For JEV, significant titre reductions of 50.95% ($p<0.0001$), 83.37% ($p<0.0001$), 99.46% ($p<0.0001$), 99.92% ($p<0.0001$), and 100% ($p<0.0001$) were observed at 20 µM, 50 µM, 80 µM, 100 µM, and 200 µM RES, respectively (Figures 2C and 2D). Besides that, the IC50 (half-maximal inhibitory concentration) of RES was determined to be 22.97 µM against CHIKV and 10.40 µM against JEV. These results demonstrate significant reductions in viral titres for both viruses with increasing RES concentrations, suggesting the inhibitory effects of RES against CHIKV and JEV replication. Based on these results, the optimal RES concentrations for subsequent experiments were determined to be 100 µM for CHIKV and 80 µM for JEV.

Inhibitory effects of RES before and after viral entry

The antiviral mechanism of RES was further determined through several other treatment protocols. A549 cells were treated with 100 µM or 80 µM RES either before (pre-infection) or after (post-infection) CHIKV and JEV infections, respectively, at a MOI of 1. For CHIKV-infected cells, pre-infection treatment led to a reduction of over 65% in CHIKV titres compared to non-treated cells (Figures 3A and 3C), indicating a notable decline in virus titres and suggesting potential prophylactic effects. Similarly, post-infection treatment also resulted in a reduction of over 65% in CHIKV titres compared to non-treated cells (Figures 3B and 3D), demonstrating antiviral activity. In contrast, for JEV-infected cells, pre-infection treatment led to a 30% reduction in JEV titres (Figures 4A and 4C), suggesting that RES may not have a strong prophylactic effect against JEV. However, post-infection treatment resulted in a substantial reduction of JEV titres by 93.21% (1 log) (Figures 4B and 4D), indicating strong antiviral activity.

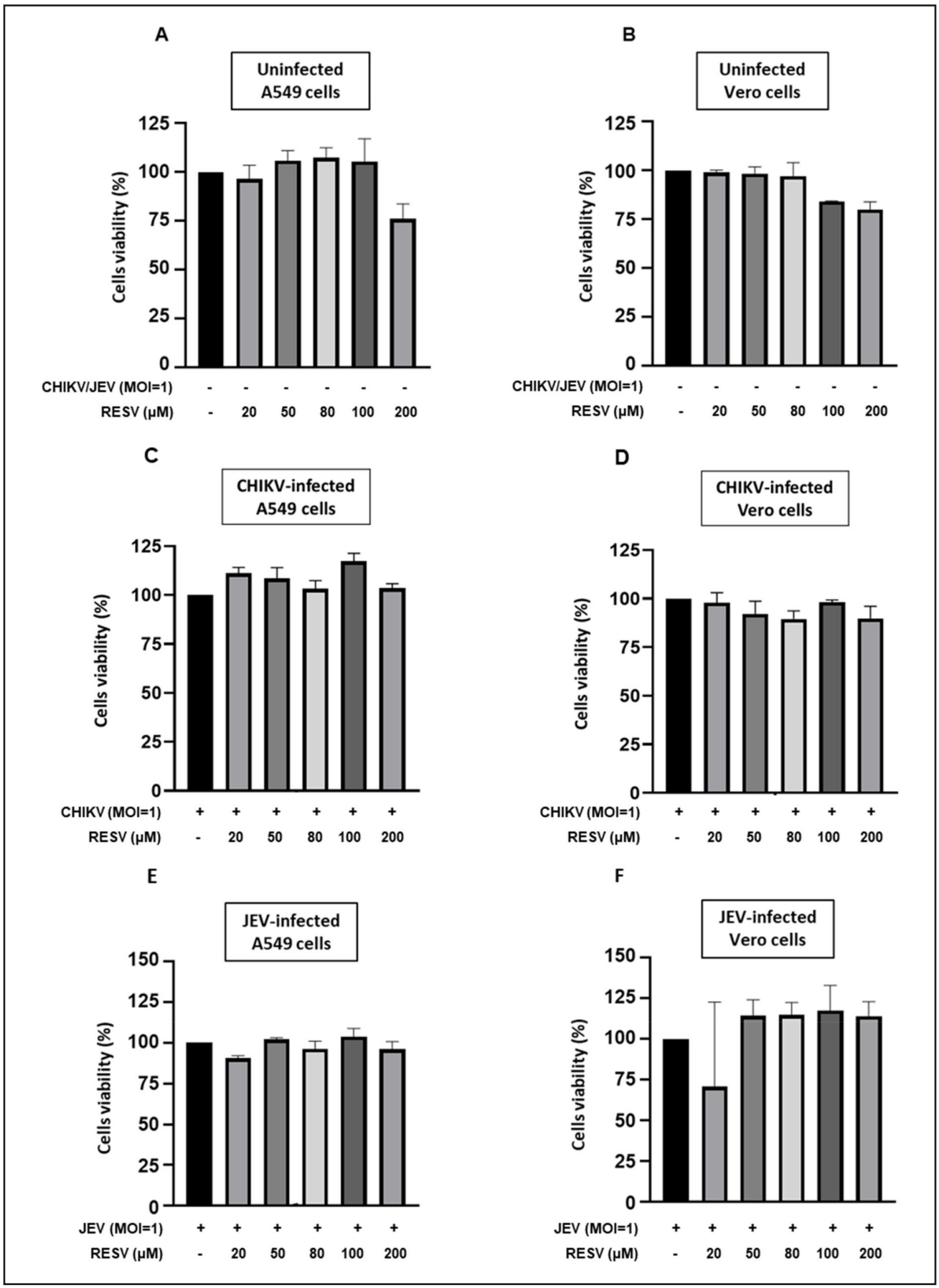

Figure 1. Cytotoxicity effects of RES on both uninfected and infected A549 and Vero cells. The cytotoxicity effects of RES on both uninfected and infected A549 and Vero cells were investigated using the MTS assay. Uninfected A549 (**A**) and Vero cells (**B**) were incubated for 72 hours after treatment with different concentrations of RES (20, 50, 80, 100 and 200 µM). Meanwhile, A549 and Vero cells were infected with CHIKV (**C, D**) or JEV (**E, F**) at an MOI of 1 followed by treatment with different concentrations of RES for 72 hours. The cytotoxicity assay was performed in triplicates and Graph Pad Prism 9 (Graph Pad Software Inc., San Diego, CA, USA, 2020) was used to analyze the data.

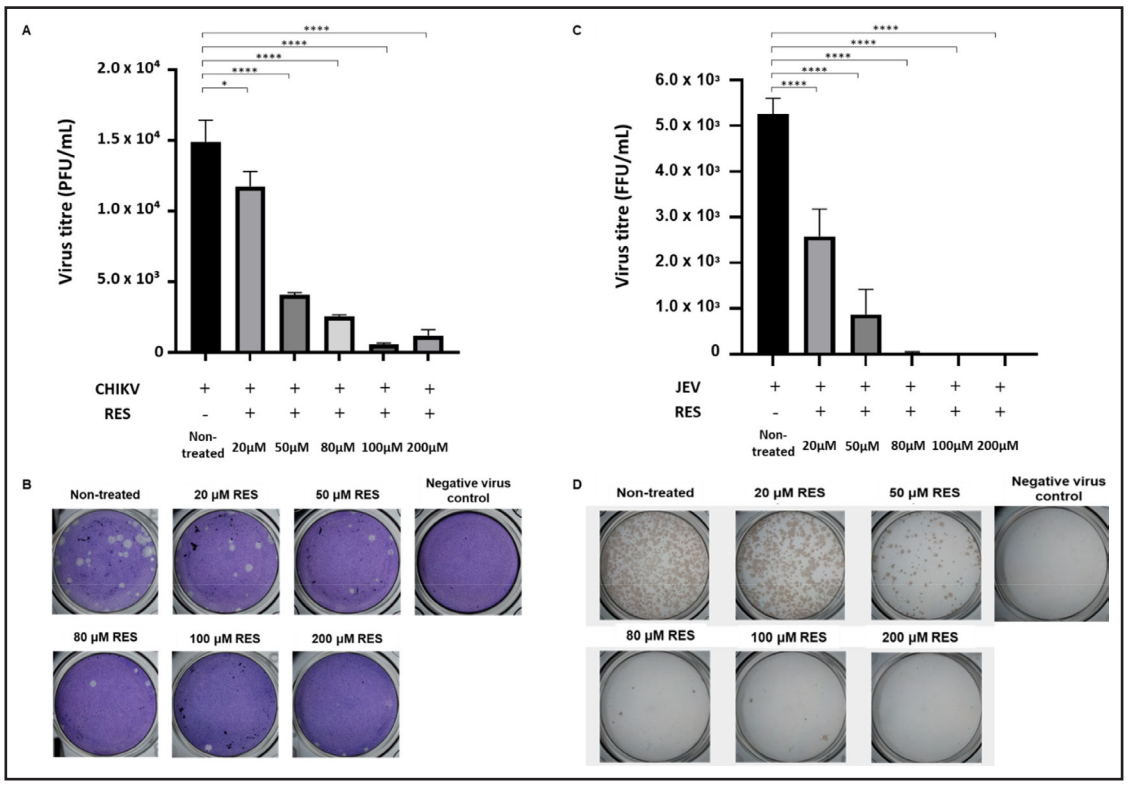

Figure 2. Antiviral effects of RES against CHIKV and JEV (Dose-dependent study). Different RES concentrations (20 µM, 50 µM, 80 µM 100 µM or 200 µM) were treated on A549 cells before and after CHIKV (**A, B)** and JEV (**C, D**) infection at an MOI of 1. The cells were pre-treated with RES for 4 hours, followed by post-infection treatment for an additional 48 hours. The supernatants were collected, and viral titres were determined using a plaque assay for CHIKV and a foci-forming assay for JEV. Graph Pad Prism 9 (Graph Pad Software Inc., San Diego, CA, USA, 2020) was used to analyze and plot the data from duplicate assays. Statistical differences between groups were as follows: *P<0.05, ****P<0.0001.

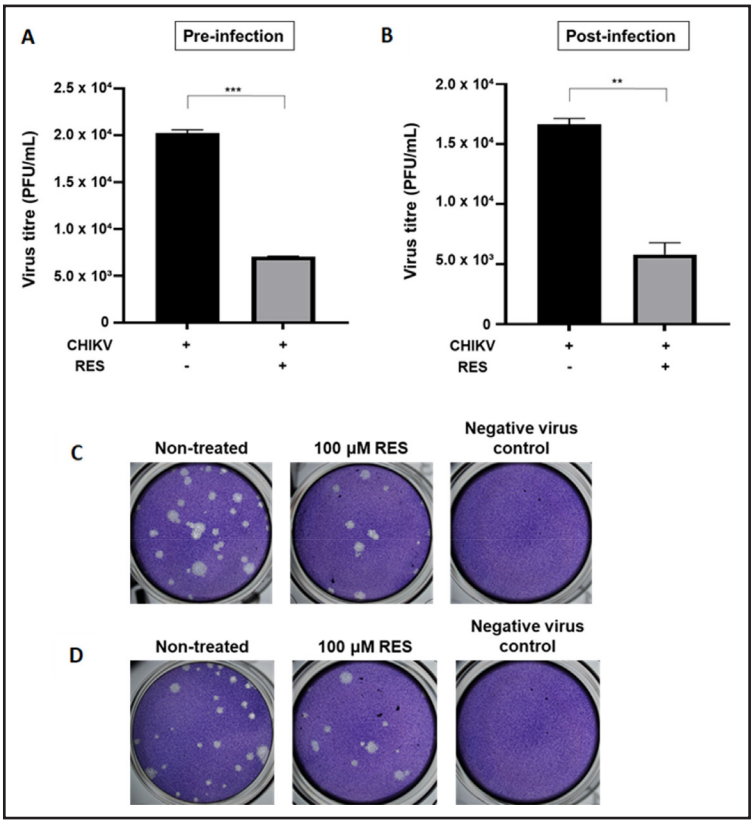

Figure 3. Inhibitory effects of RES before and after CHIKV entry. In this study, A549 cells were treated with 100 µM of RES either before infection (**A,** pre-treatment) or after infection (**B,** post-treatment) with CHIKV at an MOI of 1. Following 48 hours of incubation, the supernatants were collected, and the virus titres were determined using plaque assay. Plaques for both pre-infection (C) and post-infection (D) were observed and compared between non-treated and treated groups. Graph Pad Prism 9 (Graph Pad Software Inc., San Diego, CA, USA, 2020) was used to analyze and plot the data from duplicate assays. Statistical differences between groups were as follows: **P<0.01, ***P<0.001.

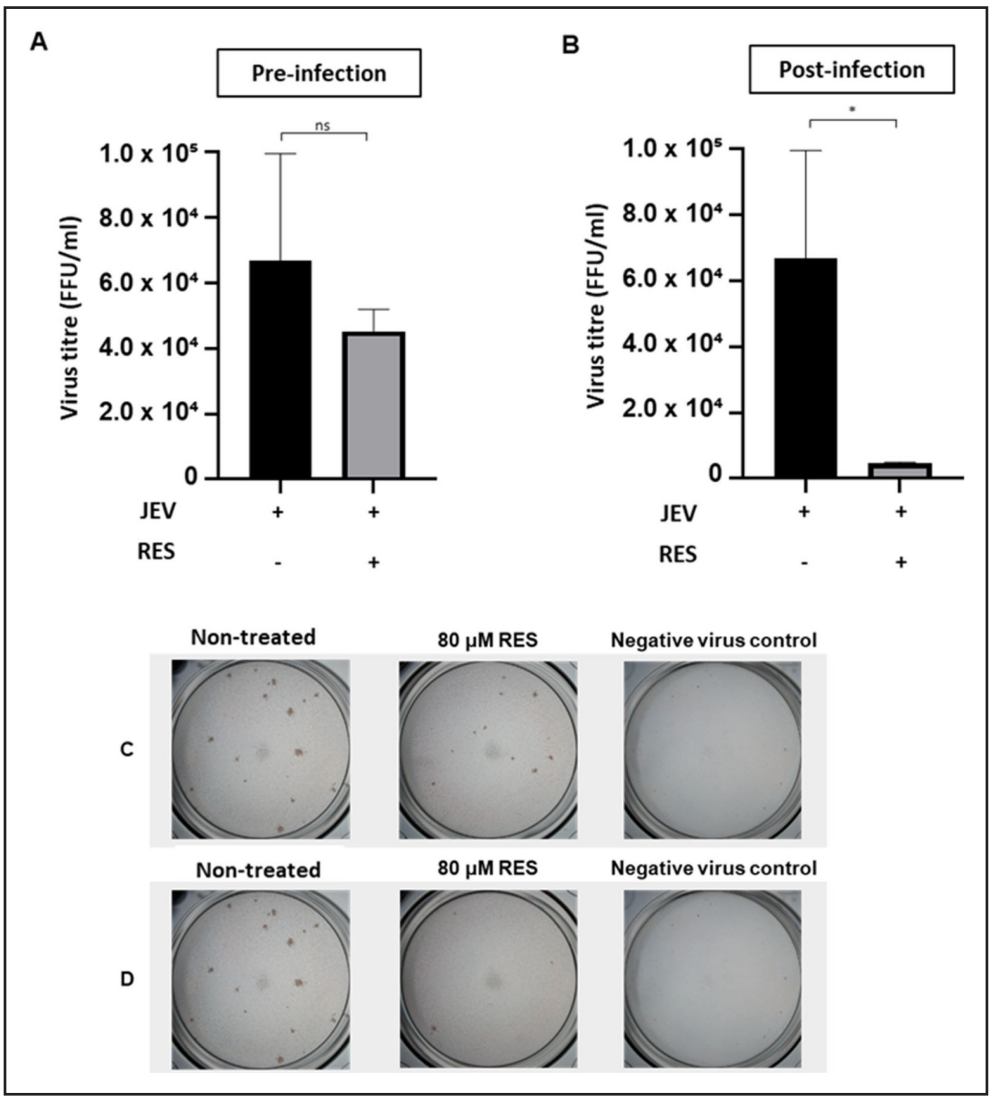

Figure 4. Inhibitory effects of RES before and after JEV entry. In this study, A549 cells were treated with 80 µM of RES either before infection (**A,** pre-treatment) or after infection (**B,** post-treatment) with JEV at an MOI of 1. The supernatants were collected after 48 hours of incubation, and the virus titres were determined by using a foci-forming assay. Foci for both pre-infection (C) and post-infection (D) were observed and compared between non-treated and treated groups. Graph Pad Prism 9 (Graph Pad Software Inc., San Diego, CA, USA, 2020) was used to analyze and plot the data from duplicate assays. Statistical differences between groups were as follows: ns = P>0.05 and **P*<0.05.

Virucidal effects of RES against CHIKV and JEV

In the virucidal assay, both viruses were pre-incubated with RES for 1 hour before infecting A549 cells to evaluate whether RES could directly suppress extracellular CHIKV and JEV particles. The results showed a reduction of less than 10% in CHIKV titres (Figures 5A and 5B) and a 49% reduction in JEV titres (Figures 5C and 5D), indicating only a mild decrease in viral titres for both viruses. Therefore, RES does not appear to directly inhibit extracellular CHIKV/JEV particles, suggesting that its antiviral action is likely due to effects on the cells, preventing viral replication.

Inhibitory activities of RES against CHIKV and JEV binding to host cells

The inhibitory action of RES on the entry and binding of CHIKV and JEV to host cells were investigated through the anti-adsorption assay. A549 cells were infected with CHIKV or JEV at a MOI of 1 in culture medium supplemented with or without the optimal concentration of RES (100 µM for CHIKV-infected cells and 80 µM for JEV-infected cells) for 1 hour at 4°C, a condition that specifically isolates viral binding to host cells by preventing further viral entry. The anti-adsorption assay resulted in a reduction of less than 37% in CHIKV titres (Figures 6A and 6B), while JEV titres showed a 50% increase (Figures 6C and 6D) compared to non-treated cells, indicating only a minor change in viral titres. These findings suggest that RES does not directly inhibit the binding of CHIKV and JEV to host cells.

DISCUSSION

RES has been widely commercialized as dietary supplements due to its potent biological properties. Common sources of RES include red wine extracts, grape seed extracts, and Japanese knotweed extracts, with the latter being particularly noteworthy for its high natural concentration of RES, making it the primary source for many RES supplements available on the market (Nosalova *et al*., 2013; Peng *et al*., 2013). The antiviral properties of RES have been extensively studied against a wide range of viruses. In this study, RES demonstrated significant antiviral effects against both CHIKV and JEV while remaining non-cytotoxic to A549 and Vero cells. Notably, RES at concentrations of 80 µM and 100 µM led to over 90% inhibition of JEV and CHIKV, respectively, indicating the potential of RES as an antiviral agent against both viruses.

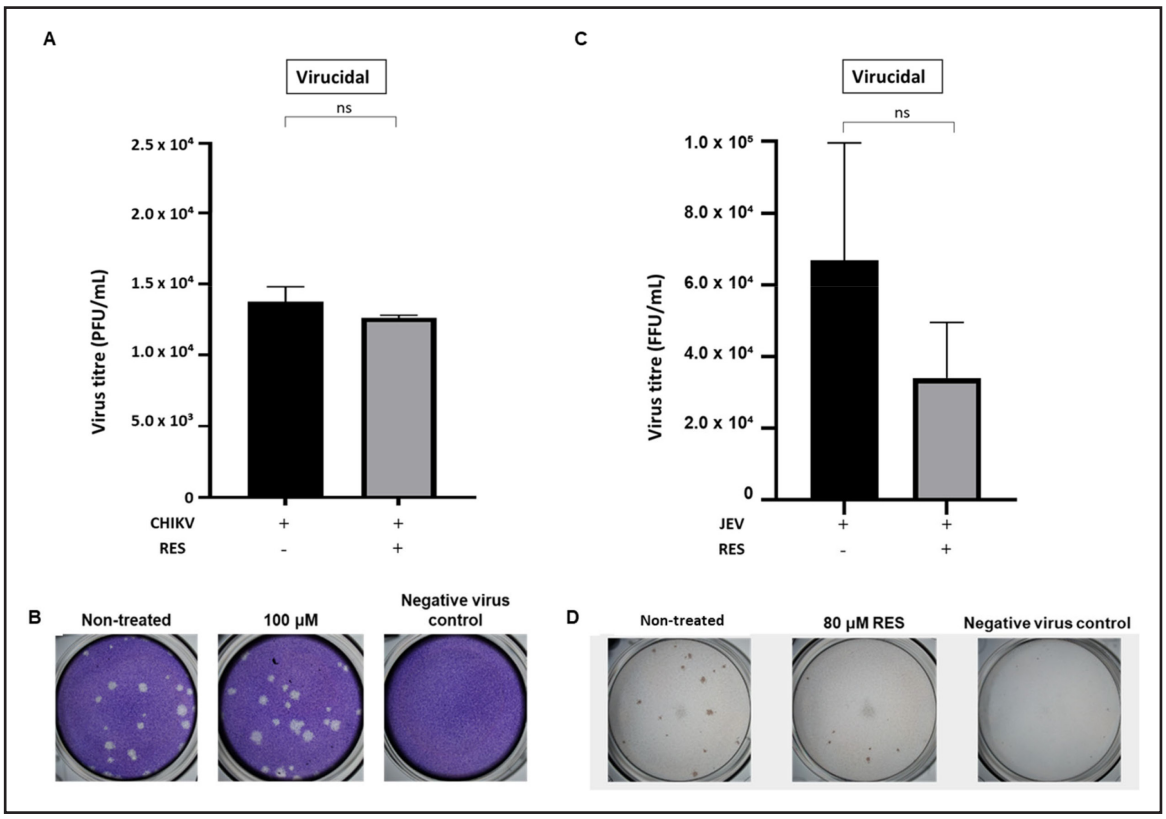

Figure 5. Virucidal effects of RES against CHIKV and JEV. A549 cells were infected with a 1-hour pre-incubated mixture of 100 µM RES-CHIKV or 80 µM RES-JEV at an MOI of 1 for 48 hours. Supernatants from CHIKV-infected and JEV-infected cells were then collected to determine the virus titre using the plaque assay (**A, B**) and foci forming assay (**C, D**), respectively. Graph Pad Prism 9 (Graph Pad Software Inc., San Diego, CA, USA, 2020) was used to analyze and plot the data from duplicate assays. Statistical differences between groups were as follows: ns = P>0.05.

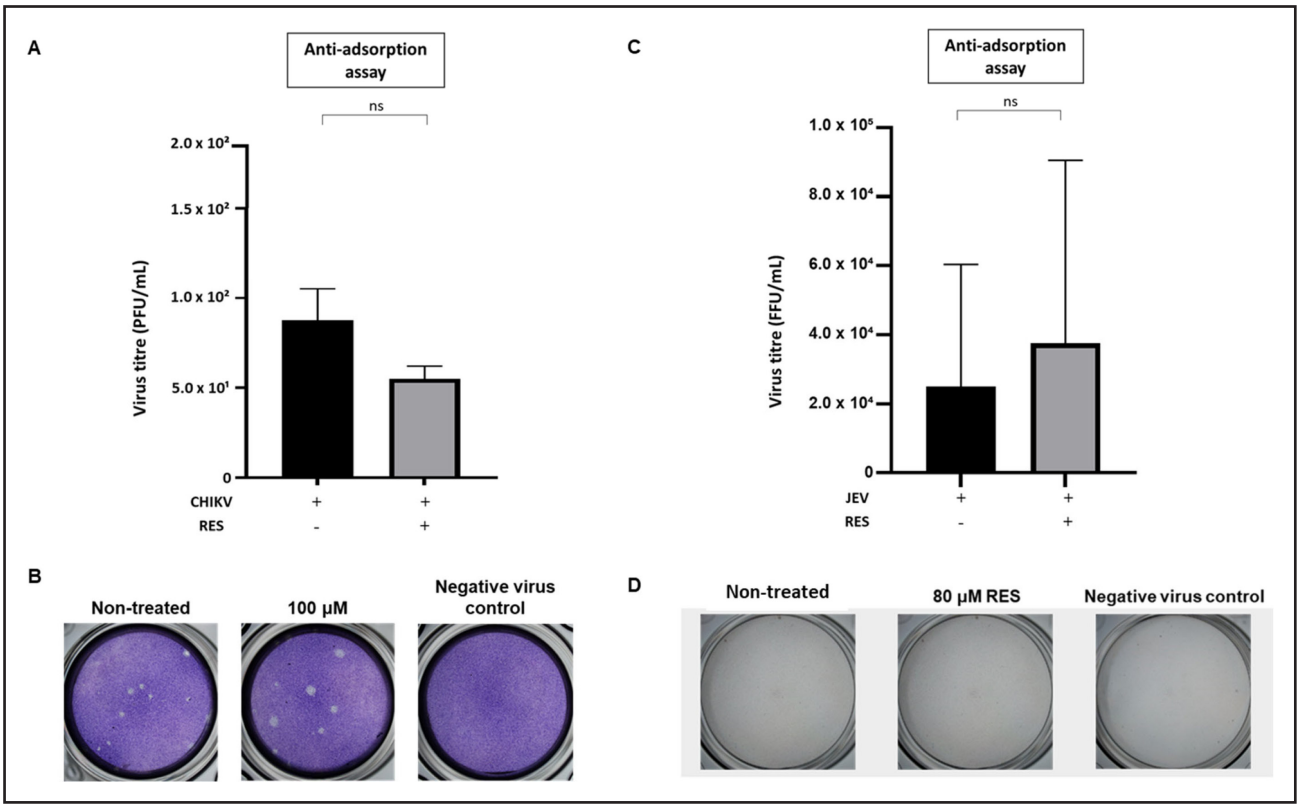

Figure 6. Inhibitory activities of RES against CHIKV and JEV binding to host cells. CHIKV or JEV, pre-incubated with 100 µM or 80 µM RES at 37 C, 5% CO, for 1 hour, were then used to infect A549 cells at an MOI of 1 at 4°C for 1 hour. After 48 hours of incubation, the supernatants were harvested to determine the virus titre through the plaque assay for CHIKV (**A, B**) and foci forming assay for JEV (**C, D**). Graph Pad Prism 9 (Graph Pad Software Inc., San Diego, CA, USA, 2020) was used to analyze and plot the data from 20duplicate assays. Statistical differences between groups were as follows: ns = P>0.05.

RES exhibits both protective and cytotoxic effects, which vary depending on the concentration and the cellular context. For instance, RES at concentrations exceeding 100 µM has been found to induce apoptosis and cause cell cycle arrest in the S phase in breast cancer 4T1 cells, underscoring its role in inhibiting cancer cell proliferation through modulation of cell cycle-related genes (Wu *et al*., 2019). In contrast, RES has protective effects on cell proliferation, as demonstrated by increased viability of human mesenchymal stem cells when treated with RES concentrations ranging from 0.1 to 100 µM (Dai *et al*., 2007). The results from the present study show that both infected and uninfected A549 and Vero cells retained cell viability of over 70% after a 72-hour treatment with RES at concentrations ranging from 20 to 200 µM, consistent with findings from previous *in vitro* studies. For instance, RES demonstrated non-cytotoxicity on Vero cells in 24 and 48-hours treatment regimens at various concentrations (20, 50, 80 and 100 µM), with over 99% cell viability (Mohd *et al*., 2019). Other studies have reported that in cell lines such as Chinese hamster lung fibroblast (V79), human breast adenocarcinoma (MDA-MB 231) and human cervical cancer (HeLa), cell viability remained above 60% even after 24 hours of treatment with 400 µM RES (Göktas *et al*., 2016). Furthermore, *in vivo* studies have shown that RES is safe at doses up to 600 mg per day under controlled conditions, with no notable adverse effects observed (Walle *et al*., 2004; Boocock *et al*., 2007; Nunes *et al*., 2009; Brown *et al*., 2010). Therefore, it is crucial to accurately extrapolate RES doses for future *in vivo* studies and human clinical trials aimed at antiviral purposes.

The antiviral mechanisms of RES are diverse, involving interference with viral replication and modulation of host immune responses. In this study, simultaneous pre- and post-infection treatments with RES led to significant reductions in viral titres for both CHIKV and JEV as RES concentrations increased. Moreover, post-infection treatment with RES alone also resulted in a reduction of viral titres for both CHIKV and JEV. However, pre-infection treatment with RES alone significantly reduced viral titres for CHIKV but did not have the same significant effect on JEV, suggesting that differential antiviral responses may be attributed to variations in the viral entry mechanisms or the specific interactions between RES and the viral components of each pathogen. For example, RES has been found to disrupt the STING/NF-κB signalling pathway to inhibit early HSV-1 infection, reducing neuroinflammation and encephalitis, highlighting its potential as both a preventative and therapeutic agent (Huang *et al*., 2024). On the other hand, the antiviral action of post-infection treatment with RES is primarily attributed to its ability to modulate the host's immune response. Research indicates that post-infection treatment with RES effectively reduces DENV and ZIKV titres by more than 90% in infected cells, primarily by inhibiting the nuclear-to-cytoplasmic translocation of HMGB1, thereby enhancing the expression of type 1 interferon response genes and ISGs such as MXA and IFN-β (Zainal *et al*., 2017; Chin *et al*., 2023).

To elucidate the mechanism by which RES disrupts viral replication, both a virucidal assay and an anti-adsorption assay were conducted. The virucidal assay evaluates RES's direct antiviral activity, specifically its capacity to inactivate the virus outside of host cells. This study revealed that RES was ineffective in inhibiting free circulating CHIKV and JEV particles, as there was no significant reduction of virus titres following virucidal treatment. These findings are consistent with previous studies showing RES did not exhibit virucidal effects against adenovirus (Matias *et al*., 2010) and cytomegalovirus (Evers *et al*., 2004) particles. Conversely, a previous study demonstrated a significant 30% decrease in ZIKV titre and a 68% reduction in mRNA copy number with RES treatment, indicating RES may act directly against extracellular ZIKV particles (Zainal *et al*., 2017). Meanwhile, the anti-adsorption assay was performed to determine whether RES can inhibit the initial stage of the viral infection process, specifically the attachment of the virus to the host cell surface. The result showed no significant changes in CHIKV and JEV titres, indicating that RES was ineffective in preventing virus binding to cells. In contrast, RES has been shown to inhibit human cytomegalovirus binding and entry by obstructing the phosphorylation of the epidermal growth factor receptor (EGFR), which is necessary for productive viral infection (Evers *et al*., 2004). This discrepancy may be attributed to the fact that RES's antiviral mechanisms might differ depending on the specific virus, potentially targeting distinct viral proteins or pathways that are not universally conserved across different viral families.

Based on our findings, RES likely exerts its antiviral properties primarily by modulating immune responses, rather than directly inhibiting viral replication. Specifically, RES was found to inhibit the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, leading to a reduction in the expression of pro-inflammatory cytokines and antiviral genes that are typically upregulated during viral infections (Zhao *et al*., 2017). Furthermore, RES may also suppress the expression of key cytokines such as IL-1β and TNF-α, which helps to mitigate excessive inflammation while promoting a balanced immune response (Ma *et al*., 2015). In addition to these effects, RES's antioxidant properties are additional factors that may enhance cellular resilience against oxidative stress during infections, and recent studies indicate that it may interact with specific viral proteins to inhibit their function (Komaravelli *et al*., 2015). Collectively, these multifaceted mechanisms underscore RES's potential as a supportive agent in managing viral infections by bolstering the host's immune response.

Besides that, RES exhibits distinct advantages and limitations compared to other studied antivirals for CHIKV and JEV, particularly in antiviral efficacy and safety profiles. RES shows promising antiviral activity against CHIKV with an IC_{50} of 22.97 µM, outperforming ribavirin (IC_{50}: 58 µM) and fisetin (IC_{50}: 29.5 µM) but less effective than curcumin (IC_{50}: 3.89 µM) and baicalein (IC_{50}: 6.997 µM) (Powers, 2018). As for JEV, RES also relatively demonstrate lower effective concentrations (IC_{50}: 10.40 µM) but its efficacy against JEV remains less experimentally validated compared to baicalein (IC_{50}: 5.8 µg/mL or 21.45 µM), which exhibit moderate suppression of JEV (Johari *et al*., 2012). The safety profile of RES demonstrates a clear advantage, as the CC50 values reported in our study (774.2 µM for Vero cells and 448 µM for A549 cells) indicate lower cytotoxicity compared to previously published CC50 values for ribavirin (266.3 µM for Vero cells and 205.6 µM for A549 cells) and baicalein (426.5 µM for Vero cells) (Johari *et al*., 2012; Franco *et al*., 2018). Notably, RES's dual antiviral/anti-inflammatory properties and low cytotoxicity provide a safety edge over conventional agents like ribavirin, which often require combination therapies to mitigate toxicity (Hucke & Bugert, 2020). These traits highlight RES's potential as a standalone or adjunct treatment for flaviviruses and alphaviruses.

In conclusion, this study offers an evaluation of the antiviral potential of RES against CHIKV and JEV. RES exhibits significant inhibitory effects on viral replication, particularly in the post-infection context, with over 90% inhibition of JEV and 60% inhibition of CHIKV at effective concentrations of 80 µM and 100 µM, respectively. Despite its lack of direct virucidal activity against free virus particles and ineffectiveness in preventing viral adsorption to host cells, RES's capability to disrupt viral replication post-infection underscores its potential as a therapeutic agent. Moreover, the study highlights the non-cytotoxic nature of RES at antiviral concentrations, further supporting its potential for therapeutic applications. These results contribute to the growing body of evidence supporting RES's role as an antiviral compound

which highlight the need for further *in vivo* studies, as well as a deeper investigation into the specific molecular pathways involved such as HMGB1 translocation, NF-┐B activation, MAPK signalling, and interferon signalling, to validate its efficacy and safety in clinical settings.

Disclosure

The author declares that they have no conflict of interests.

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