



Antiviral Activity of Rhein against *Enterovirus* 71 through Inhibiting Viral Replication and Stability

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Background: *Enterovirus* 71 (EV71) is one of the most prevalent pathogens responsible for hand, foot, and mouth disease in the Asia-Pacific region. Severe EV71 infections can be fatal in children under the age of 5. As of now, there are no proven anti-EV71 drugs available. Rhein is an anthraquinone derivative, mainly derived from rhubarb plants. Several beneficial pharmacological properties of rhein have been linked to anti-inflammatory, antioxidant, anticancer, and antiviral effects. **Aim:** This research aims to evaluate rhein's antiviral activity against *Enterovirus* EV71 *in vitro*. **Methods:** The cytotoxicity of rhein was assessed using a Cell Counting Kit-8 kit. The antiviral activities of rhein were characterized by viral RNA level, protein expression level, and infectious ability using quantitative reverse transcription polymerase chain reaction, Western blot assay, and immunofluorescence assay, respectively. The mechanism by which rhein suppresses virus life cycles was examined utilizing a time-of-addition assay. An inactivation assay was performed to evaluate whether rhein directly impaired the virion stability. **Results:** The findings indicated that rhein exhibited anti-EV71 activity by reducing viral RNA synthesis, protein expression, and infectivity. Rhein demonstrates potent antiviral effects against EV71 at the late-life stage and inhibition of virion stability. **Conclusion:** Our findings strongly support further research into rhein as a potential treatment for EV71.

Key words: *Enterovirus* EV71, antiviral agent, rhein, virus replication, virus life cycle

INTRODUCTION

Enterovirus 71 (EV71) is one of the most important pathogens that cause hand, foot, and mouth disease (HFMD) accompanied by neurological and systemic complications. The epidemic of EV71 has particularly circulated in the Asia-Pacific region and has become a global public health concern over the past decades.¹ Most EV71-infected cases, such as fever, rash, or diarrhea, are asymptomatic and mild. However, severe infections may cause brainstem encephalitis, aseptic meningitis, myocarditis, and cardiopulmonary failure that might be fatal in children under the age of 5.² Owing to unavailable effective therapy, the mortality rate of EV71-caused central nervous system involvement with cardiopulmonary failure is up to 30%–40%.³

EV71 is a positive-sense, single-stranded RNA virus with a genome of approximately 7500 nucleotides. Based on virological classification, EV71 is a species of the *Enterovirus* serotype A in

the genus *Enterovirus* family Picornaviridae.⁴ The nonenveloped EV71 genome encodes four structure protein (VP1, VP2, VP3, and VP4) and seven nonstructure protein (2A, 2B, 2C, 3A, 3B, 3C, and 3D). The viral life cycle of EV71 begins with viral binding to specific receptors, including scavenger receptor B2,⁵ P-selectin glycoprotein ligand-1,⁶ sialic acid-linked glycan,⁷ and CD209.⁸ Subsequently, clathrin-mediated endocytosis occurs, and a viral particle is packaged in an endosome. The lysosome merges with the endosome, and the pH value decreases with the acidosis. With the change of the viral conformation, a pore is formed in the endosome membrane, where viral RNA is released into the cell cytoplasm. Being positive-sensed, viral RNA is directly translated into a polypeptide, which is then cleaved into 11 proteins by the viral proteases. In viral replication complex, RNA-dependent RNA polymerase 3Dpol replicates

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the virus genome. An infectious virus particle is produced after assembling viral RNA into a virus capsid. The infectious viral progeny is released when an infected cell is lysed or using a nonlytic release route.⁹

To prevent the transmission of EV71, it is recommended that individuals develop appropriate hygiene habits, keep social distance, wash their hands frequently, and receive vaccines. It should be noted, however, that in addition to preventive measures, developing new antiviral agents against EV71 is essential to minimize the severity of the illness. As of now, there are no proven anti-EV71 drugs available.

Rhein, a primary bioactive component of rhubarb, is an anthraquinone derivative with several biological effects, including anti-inflammatory, antioxidant, and anti-cancer properties¹⁰ and antidiabetic effects.¹¹ Previous studies also revealed that rhein possessed antiviral activity against the African swine fever virus,¹² Newcastle disease virus,¹³ white spot syndrome virus,¹⁴ influenza A virus,¹⁵ and *Micropterus salmoides* rhabdovirus.¹⁶ Additionally, an anthraquinone derivative, chrysophanol, was reported to be effective in inhibiting *Enterovirus* poliovirus.¹⁷ Further, studies have shown that rhein can alter lipid metabolism.¹⁸ A disruption of the lipid metabolism can lead to a decrease in the replication of the EV71 virus.¹⁹ Based on these findings, the current study aims to investigate *in vitro* whether rhein inhibits EV71 and explore possible mechanisms of antiviral activity.

MATERIALS AND METHODS

Compounds, cells, and virus

Rhein (Figure 1a, chemical structure cited from the website <https://www.chemspider.com/Chemical-Structure.9762.html>) was purchased from ChemFaces and dissolved in dimethyl sulfoxide to prepare a 40 μ M stock solution. Vero cells (ATCC CRL-1586, African green monkey kidney cells) were used in the study. The cells were cultured with Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and maintained at 37°C in a CO₂ incubator. The strain examined in the study was *Enterovirus* (EV71, Tainan/4643/98). Propagation and titration of EV71 were performed using RD (ATCC-CL-136, rhabdomyosarcoma cells) and Vero cells, respectively. Briefly, the virus was added to the monolayer of confluent RD cells in a 10-cm culture plate (multiplicity of infection [MOI] = 0.01). After an 18-h incubation, viral supernatants were collected and stored at -80°C. Viral titer was determined by plaque assay on Vero cells, using a 1.2% methylcellulose overlay; plaques were fixed after 3 days with 4% paraformaldehyde and enumerated by staining with crystal violet.

Cell viability assay

An evaluation of the cytotoxicity of rhein against Vero cells was carried out using the Cell Counting Kit-8 (CCK-8,

DOJINDO Laboratories, Kumamoto, Japan). A monolayer of Vero cells was seeded in the 96-well culture plates overnight. The indicated concentrations of rhein were administrated to the wells in triplicate. A 24-h incubation period was followed by the replacement of the medium with a medium containing 10% CCK-8 reagent. An optical density measurement was conducted at 450 nm on the treated cells using a spectrophotometer (Synergy HT, Biotek, USA). There was a normalization of the cell viability in the compound group compared to the vehicle group.

Quantitative reverse-transcription polymerase chain reaction assay

The total RNA was extracted from infected Vero cells using the Trizol reagent (Ambion). Quantification of viral RNA levels was performed using a 1-step quantitative reverse transcription polymerase chain reaction (qRT-PCR) SYBR kit (Biomax, Cat. No. QRP001) following the manufacturer's instructions. The primer sequences used to detect EV71 and β -actin were as follows: EV71: forward primer 5'-TAGAAGCTGTAAACAACGATCAATAGC-3' and reverse primer 5'-GCGAGCAGCCTATTGATACTCAGTCCGGGG-3' and β -actin: forward primer 5'-AGGCACCAGGGCGTGAT-3 and reverse primer 5'-GCCCACATAGGAATCCTTCTGAC-3'. β -actin was used as an internal control. qRT-PCR was performed in triplicate in 96-well plates using the Roche LightCycler 480 instrument. The relative gene expression was quantified with the 2^{- $\Delta\Delta$ CT} method.

Western blot assay

After the cells had been treated, they were collected and lysed with RIPA buffer (Biomax, Scientific Co., LTD) for analysis. Immunoblots were performed using anti-EV71 VP1 antibody (proprietary) and anti- β -actin as primary antibodies. A rabbit anti-mouse or anti-human horseradish peroxidase-conjugated antibody was used as a secondary antibody. The bands were developed using enhanced chemiluminescence and detected using UVP chemiluminescence detection.

Time-of-drug-addition assay

Rhein (60 μ M) was added to cells relative to a 1-h viral adsorption period in the following groups: co-treatment (co-administration of drug and virus for 1-h viral absorption), posttreatment (administration of drug after 1-h viral absorption), and full-treatment (administration of drug throughout the whole infection period). Following a 23-h incubation period, viral RNA levels were determined using qRT-PCR and were normalized to a vehicle control group.

Immunofluorescence assay

An immunofluorescence assay (IFA) involves fixation, permeabilization, blocking, and staining with primary antibody,

secondary antibody, and counterstain. Briefly, infected cells in the culture plates were fixed in 4% paraformaldehyde for 30 min at room temperature. After that, samples are permeabilized with 0.5% triton X-100. An anti-EV71 VP1 antibody and an Alexa Fluor 488 conjugated rabbit anti-mouse antibody were used as primary and secondary antibodies, respectively, following three washings with PBS and blocking with 5% skim milk. A reference control image was presented using the nuclear dye 4',6-diamidino-2-phenylindole. Fluorescent images were observed under an inverted fluorescence microscope.

Inactivation assay

Rhein was added to the virus solution in a cell-free environment at 37° for 2 h. Afterward, the mixture solution was serially diluted 10-fold, and the virus titer was evaluated to reflect virus activity or virion stability. In the viral titration, similar steps are taken to the IFA. Even so, a medium containing 1.2% methylcellulose was added to the infected cells to restrict the transmission of new virus progeny during the infection period. A virus titer was determined by counting the number of fluorescence foci visible under the inverted fluorescence microscope.

Statistical analysis

GraphPad Prism 6.0 software (GraphPad Software, LLC, 225 Franklin Street. Fl. 26 BOSTON, MA 02110, USA.) was used for statistical analysis. Unless otherwise specified, all data are expressed as the mean $\pm$ standard deviation of at least three independent experiments. Analysis of variance with Dunnett's multiple comparison test was used to assess statistical significance (*present as statistical significance, $*P < 0.05$).

Ethical statement

This study was exempt from the Institutional Review Board review, and the patient consent was also waived by the IRB. This study was performed in accordance with the Declaration of Helsinki.

RESULTS

Rhein exhibited antiviral activity against EV71 infection

First, the antiviral activity was evaluated by administration of rhein in infected Vero cells ($\text{MOI} = 0.01$) at indicated concentrations (0, 20, 40, and 60 μM) in a CO_2 incubator for 24 h. After 24 h of incubation, total RNA was extracted, and qRT-PCR was used to determine viral RNA levels. Compared to the control group, rhein ($\text{EC}_{50} = 5.3 \mu\text{M}$) decreased the levels of EV71 RNA by 75%, 91%, and 96%, respectively, at a concentration of 20, 40, and 60 μM [Figure 1b]. In addition, the viral infectious potential of infected Vero cells treated with rhein was examined using an IFA assay. The results of IFA showed that rhein suppressed EV71 infection dose-dependently [Figure 1c].

As well, rhein decreased viral protein expression as evidenced by a Western blot assay [Figure 1d]. Note that these inhibitory effects of rhein were presented at noncytotoxicity concentrations [Figure 1e]. Overall, it is clear from the results that rhein inhibits EV71 infection effectively.

Rhein suppressed EV71 RNA at the late stage of infection

To further characterize how rhein suppressed the viral life cycle of EV71, a time-of-drug-addition assay was conducted. Briefly, the design of the experiment was divided into four groups: co-treatment group (adding rhein in the cells during the 1-h viral adsorption time), posttreatment group (adding rhein after the 1-h viral adsorption time), full-treatment group (adding rhein throughout the whole infection time), and the group without rhein addition. A timeline of drug administration is shown in Figure 2a. Following the addition of rhein at different stages of the viral infection period, intracellular viral RNA levels were measured over an incubation period. The results indicated that viral RNA levels were significantly reduced in the posttreatment group [Figure 2b], suggesting that rhein had inhibitory effects on the late stage of viral infection. To further examine the antiviral effect of rhein on the postinfection, rhein was added at different postinfection time points and an IFA assay was conducted over an incubation time. The results in Figure 2c confirm that rhein affects viral growth at the late stage, as evidenced by the increase in the percentage of green fluorescence with delayed addition time.

Rhein undermined the EV71 virion stability

It is important to note that the antiviral testing described above was conducted between the virus and cells. A cell-free analysis of the inactivation assay was performed to estimate whether rhein directly inactivates virus particles. Following incubation of virus solution with rhein in an Eppendorf tube at 37°C for 2 h, virion stability was determined using focus formation assays (FFAs). Before FFA, the mixture of virus and rhein was serially diluted 10-fold to avoid residual effects of rhein. As observed by the inactivation assay, the virus titer was significantly reduced at rhein concentrations exceeding 40 μM [Figure 3]. This confirms that rhein has the potential to interfere with viral stability.

DISCUSSION

HFMD is a contagious disease typically caused by EV71 or coxsackievirus A16 and mainly strikes young children under 5 years old.²⁰ In most cases, the symptoms begin with fever, malaise, and rash on the hands, feet, mouth, and throat. Neurological complications, such as viral meningitis or encephalitis, may also occur. Effective antivirals for the treatment of HFMD are not

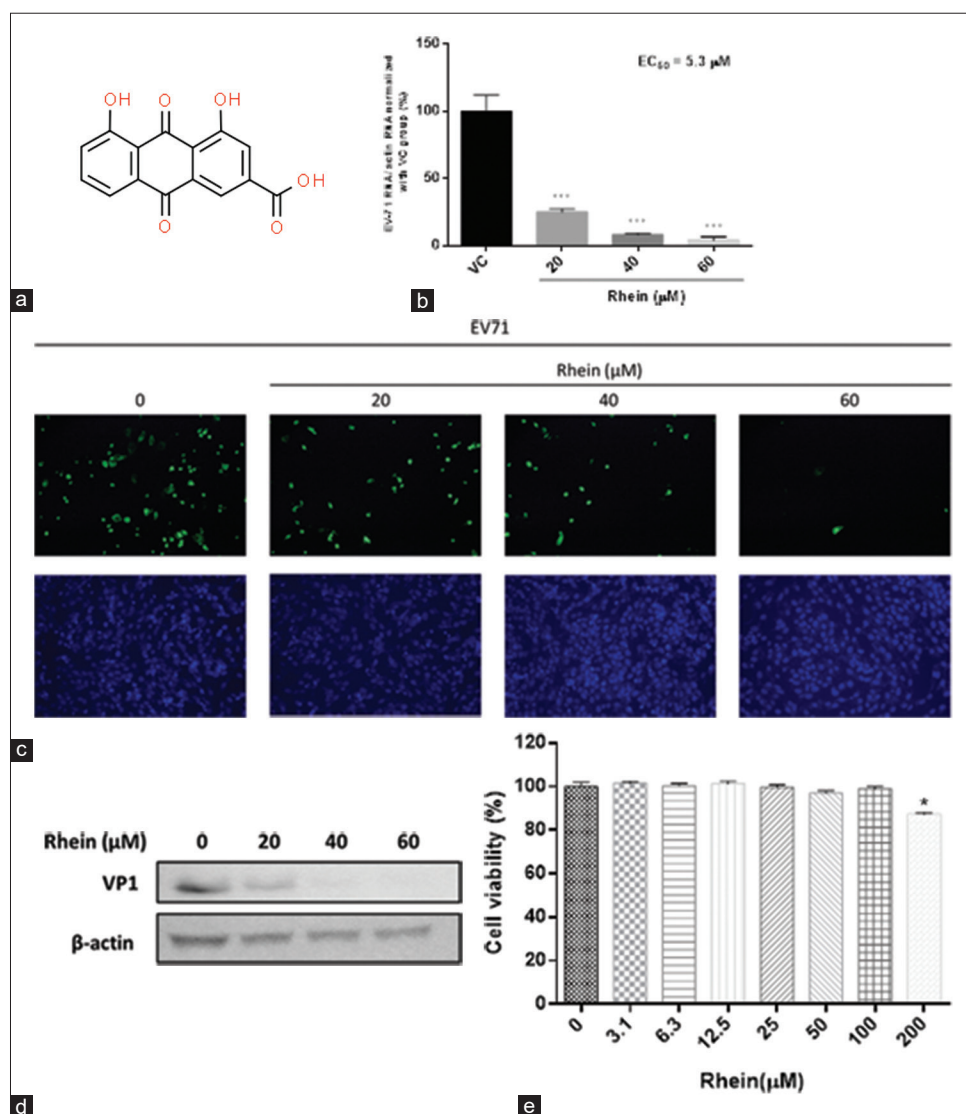


Figure 1: Dose-dependent anti-EV71 activity of rhein. Vero cells were infected with EV71 (multiplicity of infection = 0.01) at 37°C for 24 h at indicated concentrations. Treated cells were harvested and conducted by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), immunofluorescence assay (IFA), and Western blot assays. (a) Chemical structure of rhein (cited from the website <https://www.chemspider.com/Chemical-Structure.9762.html>). (b) The relative viral RNA level of EV71 was determined using qRT-PCR. (c) Inhibitory effects of rhein in Vero cells against EV71 were determined using IFA. (d) Viral protein VP1 was detected by Western blot assay. (e) Cytotoxicity of rhein against Vero cells. The cell viability was determined by a Cell Counting Kit-8 reagent after incubation with rhein for 24 h. The cell viability was normalized in the compound group compared to the vehicle group. Data represent the mean ± standard deviation of at least three independent experiments. * $P < 0.05$ versus 0 group

currently available. Certain natural remedies have been shown to work against the disease by improving symptoms and reducing the duration of the disease.²¹⁻²³ As demonstrated in this study, rhein has antiviral properties against EV71 infection *in vitro*. The inhibitory effects of rhein on EV71 were related to the reduction of viral RNA, protein expression, and viral infectivity. Furthermore, the antiviral mechanisms primarily affect the late stage of infection, according to the time-of-addition assay. The addition of rhein at the postinfection significantly reduced virus growth, suggesting that the inhibitory events involve viral replication, virion assembly, viral maturation, or virion release.

Previous studies have shown that lipid/cholesterol metabolism plays a critical role in EV71 infection, with decreased lipid metabolism resulting in reduced viral replication.^{19,24} One study on the anti-obesity effects of rhein found that it can reduce adipogenic differentiation.²⁵ Hence, rhein's antiviral effects may partly be due to its inhibition of lipid metabolism. In addition, rhein was found to have the ability to inhibit the activation of ERK.^{18,26} ERK is a critical signaling pathway in EV71 infection.^{27,28} It is speculated that rhein may inhibit EV71 replication by inhibiting ERK signaling. Moreover, rhein can suppress heat shock protein 90 (HSP90),²⁹ a crucial host factor

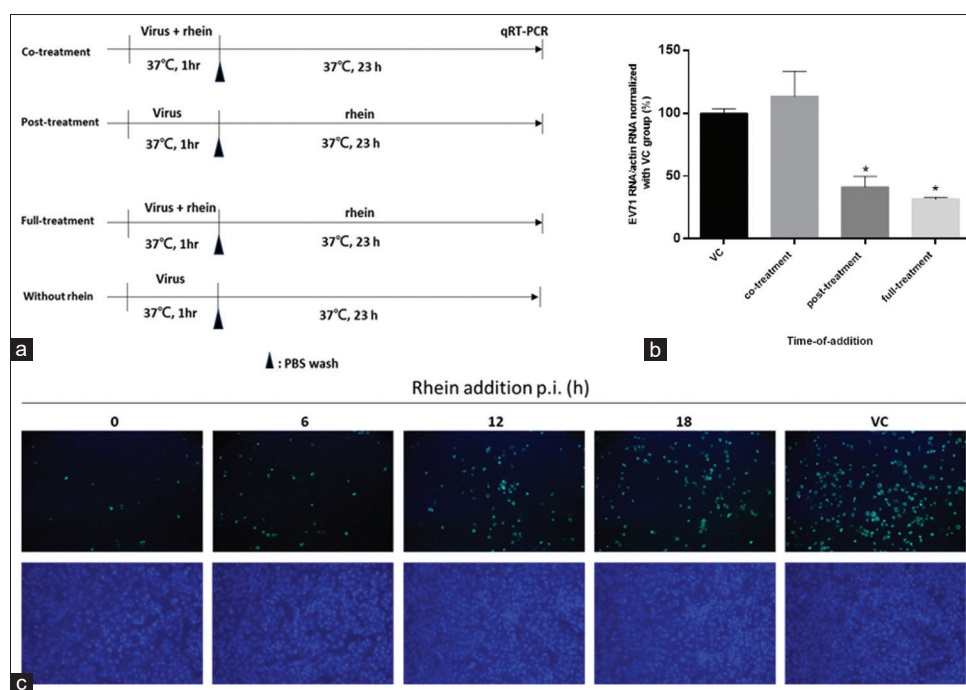


Figure 2: Rhein inhibited EV71 at the late stage of infection. (a) A timeline of time-of-addition. (b) Infected Vero cells (multiplicity of infection = 0.01) were added with rhein (60 μ M) at a distinct time relative to the infection period. Viral RNA levels were determined using quantitative reverse transcription polymerase chain reaction after 24 h. The results showed that rhein suppressed EV71 in the post-treatment group. Data represent the mean $\pm$ standard deviation of at least three independent experiments. * P < 0.05 versus VC group. (c) A delayed addition time of rhein at post-infection and immunofluorescence assay was conducted to evaluate the inhibitory effects of rhein against EV71 growth

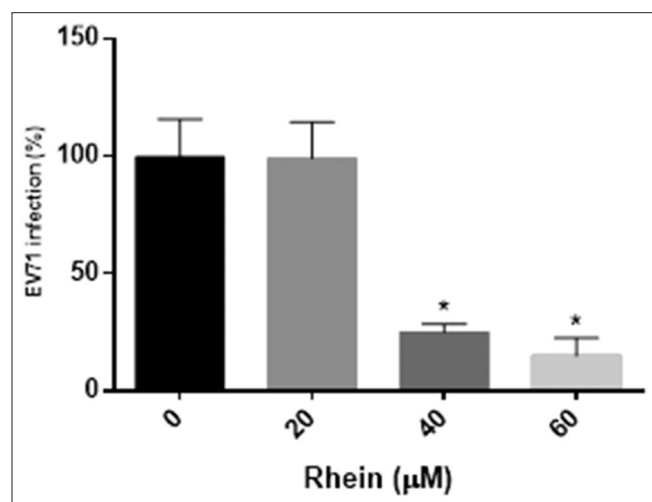


Figure 3: Rhein directly inactivated virion. Virus solution was mixed with rhein at indicated concentrations and incubated at 37°C for 2 h. The viral infectious ability was then serially diluted before titration. Viral titer was determined by plaque assay on Vero cells, using a 1.2% methylcellulose overlay; plaques were fixed after 3 days with 4% paraformaldehyde and enumerated by staining with crystal violet. Each data point represents the mean $\pm$ standard of at least three independent experiments; * P < 0.05

for EV71 replication.³⁰ A putative mechanism is proposed that rhein likely inhibits EV71 replication via the downregulation of HSP90. Further research is needed to investigate these aspects of explaining rhein's anti-EV71 activity.

Based on our results of the inactivation assay, rhein can exert anti-EV71 properties by damaging the viral stability at concentrations above 40 μ M. It is not clear how rhein worked to weaken or to inactivate the viral infectivity. Additional analyses such as negative-stain electron microscopy or surface plasmon resonance may provide more information on molecular interactions.

CONCLUSION

These results demonstrated that rhein exhibited antiviral activity against EV71 *in vitro*. The antiviral mechanisms involved in disrupting viral replication and stability. Consequently, our results extend the antiviral range of rhein and provide evidence for further research into rhein as a possible treatment for EV71.

Data availability statement

The data supporting this study's findings are available from the corresponding author, ZZ Lai, upon reasonable request.

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Conflicts of interest

There are no conflicts of interest.

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