Sophocarpine against enterovirus 71 *in vitro*

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Abstract. Sophocarpine (SCA) is a bioactive alkaloid present in *Sophoraflavescens* Ait. The present study demonstrated that SCA inhibited enterovirus 71 (EV71) infection in Vero cells. The results indicated that the 50% cytotoxicity concentration of SCA for Vero cells was 1,346 μg/ml, and the 50% inhibition concentration of SCA against EV71 was 350 μg/ml. SCA produced a marked inhibitory effect against EV71 when the Vero cells were treated with SCA prior to infection with the virus. Additionally, SCA was effective against EV71 when the Vero cells were infected with EV71 (100x$TCID_{50}$) that had been treated with SCA for 2 h, and was effective when the Vero cells were infected with EV71 (100x$TCID_{50}$) at 37˚C under 5% CO_2 for 2 h prior to treatment with SCA for 2 h. SCA was demonstrated to inhibit the attachment and penetration of EV71 and was more effective at inhibiting attachment. The assay additionally verified that SCA suppressed the replication of viral genomic RNA and indicated that SCA may inhibit EV71 infection *in vitro*.

Introduction

Enterovirus 71 (EV71) is a single-stranded, positive-polarity RNA virus and belongs to the enterovirus genus, *Picornaviridae* family (1). EV71 was initially isolated from the brain of a patient in California in 1969 (2), and it was found to trigger cytopathic effect and cellular apoptosis after infecting microvascular endothelial cells, Jurkat T cells and neuronal cells (3-5). EV71 was one of the major causes of a hand-foot-and-mouth disease (HFMD) epidemic associated with neurological sequelae (6-9). Previously, Du *et al* reported that EV71 induced SH-SY5Y human neuroblastoma cells apoptosis by stimulation of endogenous microRNA let-7b expression (10). As pointed out by Cao and Yue *et al* protective effects of EV71 virus-like particle vaccine against lethal EV71 infection, and genomic and immunologic factors associated with viral pathogenesis in a neonatal mouse model (11,12). In the present study, our data demonstrate that treatment with Sophocarpine (SCA) reduced the viral cytopathic effect on Vero cells. Although vaccines have entered the clinical trial stage (13), drugs are not currently available for the clinical treatment of EV71 infection. Thus, the study of a plant-derived compound against EV71 has important potential.

SCA, an effective compound plant-derived from foxtail-like sophora herb, root and seed, is one of the most abundant alkaloids in *Sophora flavescens*, and its structure is shown in Fig. 1 insert. *Sophora flavescens* has been used as a traditional Chinese medicine for more than 2,000 years for the treatment of fever, throat inflammation, and other diseases. SCA is a white colored crystalline alkaloid monomer with the molecular formula $C_{15}H_{22}N_2O$ and a molecular weight of 246.35. In recent years, studies on SCA have shown an increasing variety of effects, such as anti-arrhythmic, anti-inflammatory, and anticancer effects (14,15). In the present study, we first demonstrate that SCA has the ability to inhibit EV71 induced in Vero cells. Then, we compare the effect of SCA inhibition against EV71 induced in Vero cells when SCA is applied before and after the viral infection is induced. Finally, the experimental result demonstrates that SCA can inhibit viral attachment and RNA replication.

Materials and methods

Chemicals. Vero cells were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. EV71 was obtained from the Centers for Disease Control and

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Table I. PCR primers used for EV71 and 18S.

Symbol	Forward primer	Reverse primer	Tm
EV71	ATTTCAGCAGCTTGGAGTGC	GCAGCCCAAAAGAACTTCAC	45˚C
18S	CGAAACTTTCGTTATTCTTCGC	GTATTCGTCGTTAGGCGTTT	58˚C

Prevention (CDC; Handan, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin, and streptomycin were purchased from Gibco (Carlsbad, CA, USA). 3-[4,5-dimethylthiazol-2-yl]-2.5-diphenyl-tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). SCA was purchased from Baozetang (Suzhou, China). TRIzol Reagent and a reverse transcription kit were purchased from Takara Bio Inc. (Otsu, Japan). Taq DNA Polymerase was purchased from Fermentas (Glen Burnie, MD, USA). An *in situ* cell death detection kit was purchased from Biyuntian Biotech Co., Ltd., (Shanghai, China).

Viruses and cell cultures. EV71 was propagated in Vero cells using DMEM supplemented with 2% FBS. The Vero cells were grown in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 U/ml). The cells were maintained at 37˚C in an incubator under an atmosphere of 5% CO_2.

Virus titers obtained by $TCID_{50}$. The Vero cells (5obt^3 cells/ml) were seeded into a 96-well culture plate (100 μl/well) and incubated for 10 h at 37˚C. Then, the cells were infected with 10-fold serial dilution viral suspensions, which resulted in 8 concentrations. For each concentration, 12 wells were infected with a 50 μl viral suspension and then incubated at 37˚C under 5% CO_2 for 168 h. The virus titer [reported as 50% tissue culture infectious doses ($TCID_{50}$/ml)] was calculated using the Behrens-Karber method (16).

CC_{50} (50% cytotoxicity concentration) assay. Vero cells (1ity^5 cells/ml) were seeded into a 96-well culture plate (100 μl/well) and incubated for 24 h at 37˚C. The cells were then treated with SCA at concentrations of 4,000, 2,000, 1,000, 500, 250, 125, 62.5, 31.25, and 15.625 μg/ml and incubated at 37˚C under 5% CO_2 for 48 h. In the negative control, the cells were not treated with SCA, and for the blank test, DMEM was used. The Vero cells in the control and blank test were incubated with 0.5 mg/ml MTT at 37˚C for 4 h, DMSO was then added (150 μl/well) to dissolve the crystal violet, and the absorbance at 490 nm was measured using an ELISA plate reader. The same procedure was repeated three times, and the concentration of SCA that killed 50% of the cells was the 50% cytotoxicity concentration.

IC_{50} determination by neutralization assay. The Vero cells (1 y ^{5}cells/ml) were seeded into a 96-well culture plate (100 μl/well) and incubated for 24 h at 37˚C. The cells were exposed to a virus suspension containing (100x$TCID_{50}$) and SCA at concentrations of 1,000, 500, 250, 125, 62.5, and 31.25 μg/ml. The cells were then incubated at 37˚C under 5% CO_2 for 48 h, and then an MTT assay was performed. The concentrations of SCA that reduced the virus-induced CPE by 50% were expressed as the IC_{50} (17).

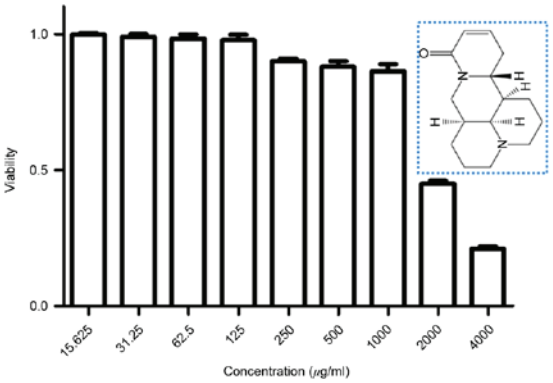

Figure 1. After 2 days of incubation, the cytotoxicity of SCA was determined via an MTT assay. The insert in the blue box is sophocarpine structure. SCA was serially diluted to different concentrations (15.625, 31.25, 62.5, 125, 250, 500, 1,000, 2,000, and 4,000 μg/ml) in triplicate.

Antiviral assays. The Vero cells (1'_E^5 cells/ml) were seeded into a 96-well culture plate (100 μl/well) and incubated for 24 h at 37˚C. To study the antiviral activity of SCA, the Vero cells were divided into 3 groups. In the first experiment, the Vero cells were infected with EV71 (100x$TCID_{50}$) at 37˚C under 5% CO_2 for 2 h and then supplemented with various concentrations of SCA for 2 h. In the second experiment, the Vero cells were infected with EV71 (100x$TCID_{50}$) that had been treated with SCA for 2 h. In the third experiment, the Vero cells were exposed to various concentrations of SCA for 2 h and then infected with EV71 (100x$TCID_{50}$) for 2 h. The same procedure was repeated three times. The cytopathic effect was determined by measuring the cell viability via the MTT assay after 48 h.

Effects of SCA on EV71 attachment. For the attachment assay, the Vero cells (1 nt ^{5}cells/ml) were seeded into a 96-well culture plate (100 μl/well) and incubated for 24 h at 37˚C. The cells were pre-chilled at 4˚C for 1 h, infected with EV71 (100illed$_{50}$) for 2 h at 4˚C to allow for absorption, and then supplemented with SCA at concentrations of 1,000, 500, 250, 125, 62.5, and 31.25 μg/ml. The same procedure was repeated three times. The cytopathic effect was determined by measuring the cell viability via the MTT assay after 48 h (18).

Effects of SCA on EV71 penetration. The Vero cells (1rat^5 cells/ml) were seeded into a 96-well culture plate (100 μl/well) and incubated for 24 h at 37˚C. The Vero cells were pre-chilled at 4˚C for 1 h and then infected with

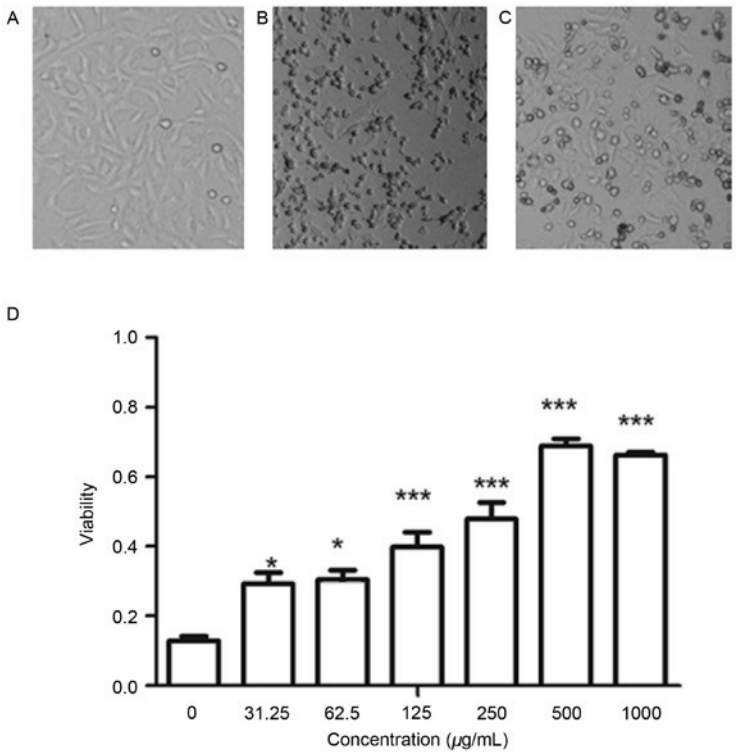

Figure 2. After 2 days of incubation, the cytotoxicity of SCA was determined via an MTT assay. (A-C) Effective concentrations of SCA against EV71 were observed under an inverted microscope. (A) Vero cells. (B) EV71-infected Vero cells. (C) Vero cells exposed to 500 μg/ml SCA (magnification, x40). (D) The Vero cells were exposed to the virus (100xTCID$_{50}$) and SCA. SCA was serially diluted to different concentrations (31.25, 62.5, 125, 500 and 1,000 μg/ml) in triplicate. *P<0.05 and ***P<0.001 vs. control. SCA, sophocarpine; EV71, enterovirus 71.

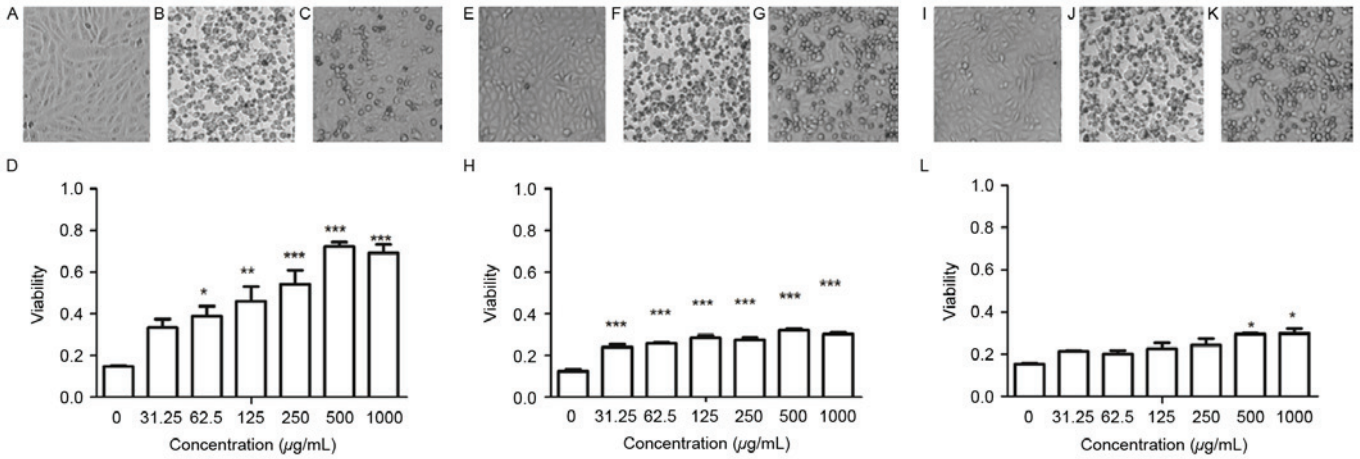

Figure 3. The antiviral activity of SCA using three different treatments. (A-C) Effective concentrations of SCA against EV71 observed under an inverted microscope. (A) Vero cells, (B) EV71-infected Vero cells. (C) Vero cells were exposed to various concentrations of SCA for 2 h and then infected with EV71 (100xTCID$_{50}$) for 2 h (magnification, x40). (D) Vero cells exposed to various concentrations of SCA for 2 h, then were infected with EV71 (100xTCID$_{50}$) for 2 h. SCA was serially diluted to different concentrations (0, 31.25, 62.5, 125, 500 and 1,000 μg/ml) in triplicate.*P<0.05 **P<0.01 and ***P<0.001 compared to the control; (E-G) Effective concentrations of SCA against EV71 observed under an inverted microscope. (E) Vero cells, (F) EV71-infected Vero cells, (G) EV71 was exposed to various concentrations of SCA prior to viral adsorption (magnification, x40). (H) EV71 exposed to various concentrations of SCA before viral adsorption. SCA was serially diluted to different concentrations (0, 31.25, 62.5, 125, 500 and 1,000 μg/ml) in triplicate. ***P<0.001 compared to the control; (I-K) Effective concentrations of SCA against EV71 observed under an inverted microscope. (I) Vero cells, (J) EV71-infected Vero cells and (K) Vero cells were infected with EV71 (100xTCID$_{50}$) at 37˚C under 5% CO_2 for 2 h and then supplemented with SCA (1,000 μg/ml) (magnification, x40). (L) Vero cells were exposed to the virus (100xTCID$_{50}$) for 2 h and then supplemented with SCA. SCA was serially diluted to different concentrations (0, 31.25, 62.5, 125, 500 and 1,000 μg/ml) in triplicate. *P<0.05 compared to the control. SCA, sophocarpine; EV71, enterovirus 71.

EV71 (100withe$_{50}$) for 3 h at 4˚C to allow for EV71 attachment to the cell surface. Subsequently, SCA was applied in concentrations of 1,000, 500, 250, 125, 62.5, and 31.25 μg/ml, and then the cells were incubated at 37˚C to maximize the penetration of EV71. At 10-min intervals, the infected Vero cells were treated with PBS at pH 11 for 1 min to inactivate the viruses that had not penetrated the cells. Then, PBS at pH 3 was immediately added to neutralize the PBS at pH 11.

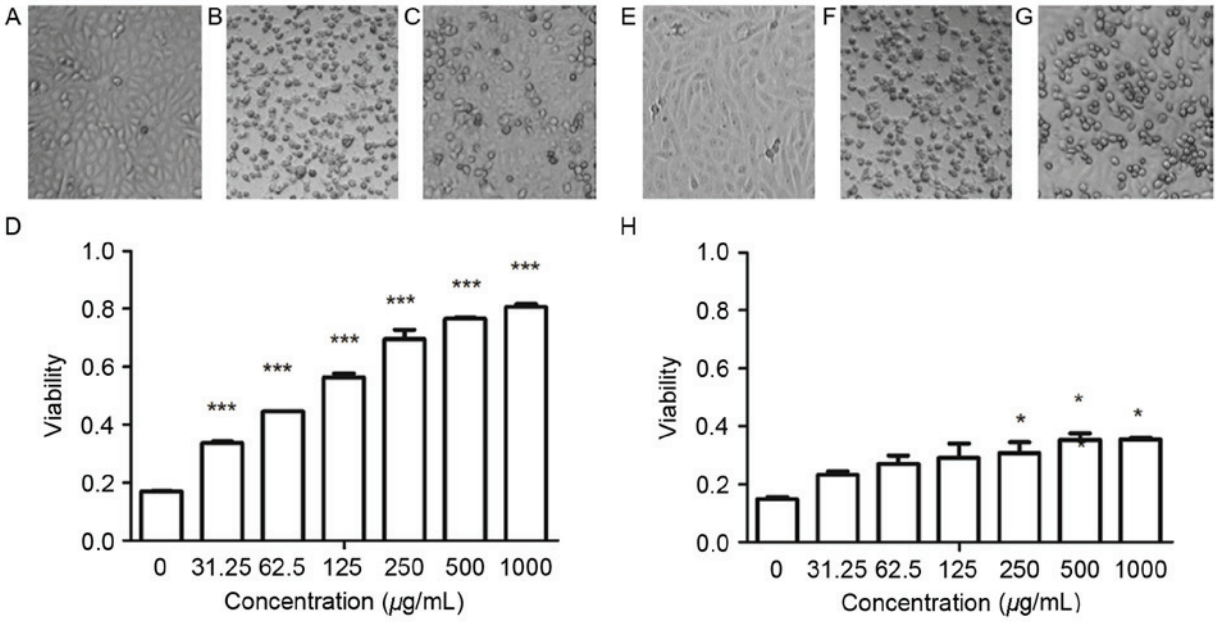

Figure 4. Effects of SCA on EV71 attachment and penetration. Images (A-C) of SCA on EV71 attachment observed under an inverted microscope. (A) Vero cells. (B) EV71-infected Vero cells. (C) Effects of SCA (1,000 μg/ml) on EV71 attachment (magnification, x40). (D) Vero cells were infected with EV71 (100xTCID$_{50}$) under SCA concentrations of 0, 31.25, 62.5, 125, 500 and 1,000 μg/ml, and EV71 (100xTCID$_{50}$) absorption occurred for 2 h at 4˚C. Images (E-G) of SCA on EV71 penetration observed under an inverted microscope. (E) Vero cells, (F) EV71-infected Vero cells. (G) Effects of SCA (1,000 μg/ml) on EV71 penetration (magnification, x40). (H) Vero cells were infected with EV71 (100xTCID$_{50}$) for 3 h at 4˚C to allow for EV71 attachment to the cell surface. Then, varying concentrations of SCA (1,000, 500, 250, 125, 62.5, and 31.25 μg/ml) were added. *P<0.05 and ***P<0.001 compared to the control. SCA, sophocarpine; EV71, enterovirus 71.

The same procedure was repeated three times. The cytopathic effect was determined by measuring the cell viability via the MTT assay after 48 h (19).

Detection of viral RNA by RT-PCR. The Vero cells (4vir^{5} cells/ml) were seeded into a 24-well culture plate (500 μl/well) and incubated for 24 h at 37˚C. The cells were exposed to EV71 (100xTCID$_{50}$) and varying concentrations of SCA and then incubated for 48 h at 37˚C. Subsequently, total RNA was extracted using TRIzol (Takara Bio, Inc.), and cDNA was synthesized from total RNA using reverse transcriptase with random primers and an oligo-dT primer. RT-PCR was performed in a 20 μl reaction mixture containing 2.0 μl of 10xPCR buffer, 1.6 μl of dNTPs (10 mM each), 1.2 μl of Mg^{2+} (25 mM), 0.5 μl of primer F, 0.5 μl of primer R, 0.1 μl of TaqDNA Polymerase, 1.0 μl of cDNA, and 13.1 μl ddH_2O. The 18S gene was used as a reference gene (Table I). A PCR machine (GeneAmp 9700; Applied Biosystems Life Technologies, Foster City, CA, USA) is used to perform the thermal cycle of PCR amplification. The cycling conditions were as follows: 3 min at 95˚C; followed by 30 cycles of 95˚C for 20 sec, 45˚C for 25 sec and 72˚C for 30 sec; and a final extension cycle at 72˚C for 10 min.

Statistical analysis. All data analysis was performed using the Statistical Package for Social Sciences software (version 19.0; SPSS Inc., IL, USA).

Results

Cytotoxicity assay of SCA. SCA induced slight cytotoxicity (0-5%) against the host cells at concentrations of less than 125 μg/ml, and it induced mild cytotoxicity (6-20%) at concentrations between 250 and 1,000 μg/ml and induced over 50% cytotoxicity at concentrations more than 2,000 μg/ml (Fig. 1). The MTT assay showed that the CC_{50} of SCA was 1,346 μg/ml.

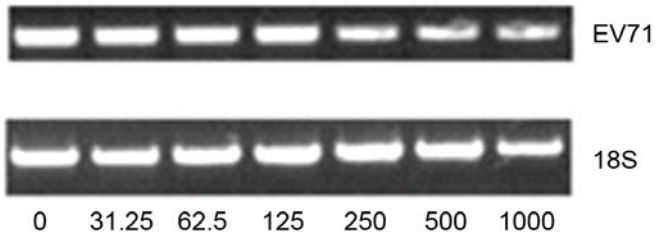

Figure 5. RT-PCR products of EV71 on 1% agarose gel. The Vero cells were exposed to EV71 (100xTCID$_{50}$) and varying concentrations of SCA and then incubated for 48 h at 37˚C. The SCA concentrations were 1,000, 500, 250, 125, 62.5, and 31.25 μg/ml, respectively.

IC_{50} Determination by neutralization assay and selectivity index (SI). Cytotoxicity of SCA was determined using the MTT assay (Fig. 2). SCA was effective (P<0.001; Fig. 2D) against EV71 at a concentration of 250 μg/ml. The morphological features of the Vero cells supplemented with a concentration of 500 μg/ml of SCA were observed under an inverted microscope (Fig. 2A-C). SCA showed high anti-EV71 activity at high concentrations, and the IC_{50} value of SCA was 350 μg/ml. The selective index ($SI=CC_{50}/IC_{50}$) value for the MTT assay was 3.85.

Antiviral assays. The Vero cells were exposed to various concentrations of SCA for 2 h and then infected with EV71

($100xTCID_{50}$) for 2 h (Fig. 3A-D). This experiment indicated that SCA was highly effective against EV71 when it was used to pretreat the Vero cells for 2 h.

When the Vero cells were infected with EV71 ($100xTCID_{50}$) that had been treated with SCA for 2 h (Fig. 3E-H). Fig. 3H shows a slight inhibitory activity at the 31.25 μg/ml and up to 1,000 μg/ml.

The Vero cells were infected with EV71 (100 $cell_{50}$) at 37˚C under 5% CO_2 for 2 h and then supplemented with various concentrations of SCA (Fig. 3I-L). Our experiment demonstrated that SCA was statistically significant against EV71 only at concentrations up to the 500 μg/ml when it was applied after viral adsorption (Fig. 3L).

Effects of SCA on EV71 attachment and penetration. SCA was effective at inhibiting viral infection when it was added prior to viral infection. We hypothesized that SCA may inhibit viral attachment and/or penetration, thereby preventing viral entrance into cells. We found that SCA could significantly inhibit viral attachment at a concentration of 125 μg/ml ($P<0.05$; Fig. 4D). The morphological features of the Vero cells were observed under an inverted microscope (Fig. 4A-C).

Our experiment demonstrated that SCA induced only slight inhibitory effects on EV71 penetration (Fig. 4E-H).

Detection of viral RNA by RT-PCR. The cells were exposed to EV71 ($100ntrat_{50}$) and varying concentrations of SCA and then incubated for 48 h at 37˚C. EV71 RNA was detected by RT-PCR (Fig. 5), and the 18S gene was used as the reference gene. Fig. 5 shows that the EV71 RNA levels continued to decrease when the concentrations of SCA increased.

Discussion

Because of the high incidence and high fatality rates of EV71 in children under six years of age, the development of an effective agent of treatment is urgently required. In China, traditional Chinese medicine was commonly used for treating infectious diseases. Many bioactive compounds exhibited antiviral activity *in vitro* and *in vivo* against human Enterovirus 71, including chebulagic acid (20), GuiQi polysaccharides (21), trans-retinoic acid (22), gallic acid and gramine derivatives (23,24). Increasing evidences have suggested that SCA possible target substance, which can display anti-inflammatory effect in the mammary gland of mice and attenuate liver fibrosis in rats by inhibiting the TLR4 signaling pathway, and contribute to the protection of human immunological liver injury by regulating natural killer (NK) cell activity (25-27). Yang *et al* previously reported clemastanin B from *Isatis indigotica* root that showed useful antiviral activities in the therapy and prevention of human and avian influenza A and B viral infection *in vitro* (28). In this study, we demonstrated that SCA acts as potential drug against EV71 and that SCA is one of the many bioactive compounds of plant origin.

The results of this study indicated that SCA was highly effective against the cytopathicity of EV71 *in vitro*. The research results showed that the 50% cytotoxicity concentration of SCA used singly for Vero cells was 1,346 μg/ml and the 50% inhibition concentration of SCA against EV71 was 350 μg/ml. These findings indicate that SCA did not directly inhibit the cytotoxic effects of EV71 on the cells. The addition of SCA to the Vero cells before viral adsorption significantly suppressed EV71 infection, whereas the inhibitory effect was less significant when SCA was added after viral adsorption and used as a pretreatment on EV71 before viral adsorption. Thus, we supposed that SCA may inhibit viral attachment and/or penetration to prevent the entrance of the virus into the cells. The result suggests that SCA likely targets the EV71 virus by blocking the virus-cell attachment, and the inhibitory effect is likely derived from the binding of SCA molecules to the protein coat of the virus and/or to the host cell membrane.

The present findings suggest that SCA may be an early potential antiviral substance and that as a pretreatment may inhibit viral attachment, but additional investigation may serve to explore the molecular mechanism and provide further clarity.

Acknowledgements

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