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Antiviral effects of two *Ganoderma lucidum* triterpenoids against enterovirus 71 infection

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ABSTRACT

Enterovirus 71 (EV71) is a major causative agent for hand, foot and mouth disease (HFMD), and fatal neurological and systemic complications in children. However, there is currently no clinical approved antiviral drug available for the prevention and treatment of the viral infection. Here, we evaluated the antiviral activities of two *Ganoderma lucidum* triterpenoids (GLTs), Lanosta-7,9(11),24-trien-3-one,15;26-dihydroxy (GLTA) and Ganoderic acid Y (GLTB), against EV71 infection. The results showed that the two natural compounds display significant anti-EV71 activities without cytotoxicity in human rhabdomyosarcoma (RD) cells as evaluated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay. The mechanisms by which the two compounds affect EV71 infection were further elucidated by three action modes using Ribavirin, a common antiviral drug, as a positive control. The results suggested that GLTA and GLTB prevent EV71 infection through interacting with the viral particle to block the adsorption of virus to the cells. In addition, the interactions between EV71 virion and the compounds were predicated by computer molecular docking, which illustrated that GLTA and GLTB may bind to the viral capsid protein at a hydrophobic pocket (F site), and thus may block uncoating of EV71. Moreover, we demonstrated that GLTA and GLTB significantly inhibit the replication of the viral RNA (vRNA) of EV71 replication through blocking EV71 uncoating. Thus, GLTA and GLTB may represent two potential therapeutic agents to control and treat EV71 infection.

1. Introduction

Human enterovirus 71 (EV71) infection is associated with a series of clinical diseases from hand, foot and mouth disease (HFMD) to severe neurological disorders, which are serious threats to children under 6 years of age [1]. Since it was first identified in 1969 [2,3], outbreaks of EV71 infection have been periodically reported worldwide [4–6]. China has recently experienced several HFMD epidemics with a significant number of fatalities occurred among young children [7–10].

However, the pathogenic mechanism of EV71 has not been fully understood and there are currently no approved clinic antiviral drugs available for the prevention or treatment of EV71 infection [11,12]. In clinic, the current common therapies are the treatment of the viral infections with broad-spectrum antiviral drugs, including Ribavirin, Ganciclovir, and Acyclovir, which only partially alleviate the symptoms instead of controlling the infections and usually come with high cytotoxicity. Thus, it is important to develop new specialized drugs and therapies for the control of the viral infection.

The adoptions of natural medicinal compounds and traditional Chinese herbal medicines have been seen across Asian countries for centuries, and also in Western medical treatment and healthcare recently [13,14]. Probing Lingzhi or Reishi medicinal mushroom *Ganoderma lucidum* is widely used as traditional Chinese medicine for a variety of diseases [15]. *G. lucidum* extracts contain a wide spectrum of bioactive substances, such as *G. lucidum* triterpenoids and polysaccharides. These substances have many medical effectiveness and they are the most important active substances for its numerous pharmacological uses, such as chronic bronchitis inflammation, hyperlipidemia, hypertension, neurasthenia, hepatitis, leucopenia, and adjuvant treatment of cancers [16,17].

In this study, we demonstrated that two *G. lucidum* triterpenoids, Lanosta-7,9(11),24-trien-3-one,15;26-dihydroxy (GLTA) and Ganoderic acid Y (GLTB), display significant antiviral effects

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against EV71 infection without cytotoxicity in human rhabdomyosarcoma (RD) cells based on 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assays. In the elucidating the mechanisms by which the natural compounds affect EV71 infection, we revealed that GLTA and GLTB inhibit EV71 infection through interacting with the viral particle and blocking the virus adsorption to the cells. Computer molecular docking suggested that the natural compounds may bind to the viral capsid protein at a hydrophobic pocket. Moreover, we demonstrated that GLTA and GLTB significantly inhibit the replication of the viral RNA (vRNA) of EV71, and further suggested that the two compounds inhibit EV71 replication through blocking EV71 uncoating. Thus, we discovered that GLTA and GLTB may act as potential therapeutic agents to control EV71 infection.

2. Materials and methods

2.1. Natural compounds, viruses, and cells

Two *G. lucidum* triterpenoids, Lanosta-7,9(11), 24-trien-3-one,15;26-dihydroxy (GLTA) and Ganoderic acid Y (GLTB) (Table 1), isolated and purified from crude extracts of Reishi mushroom *G. lucidum*, were obtained from Kunming Institute of Botany, Chinese Academy of Science (Kunming, China). Ribavirin was purchased from Zhonglin Pharmaceutical (Shan'xi, China) and used as a positive control (Table 1).

Human enterovirus 71 (EV71) strain (XiangYang-Hubei-09) (GenBank accession no. JN230523.1) was isolated previously by our group (State Key Laboratory of Virology, Wuhan University, Wuhan, Hubei, China).

Human Rhabdomyosarcoma (RD) cell line was purchased from China Center for Typical Culture Collection (CCTCC) (Wuhan, China). Cells were cultured at 37 °C under 5% CO_2 atmosphere in Minimum Essential Medium (MEM), supplemented with 10% heat-inactivated, virus-free and mycoplasma-free Fetal Bovine Serum (FBS), 100 U/ml of penicillin, 100 μg/ml of streptomycin (GIBCO, Grand Island, NY, USA).

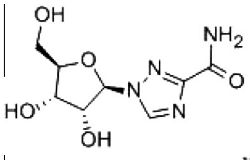
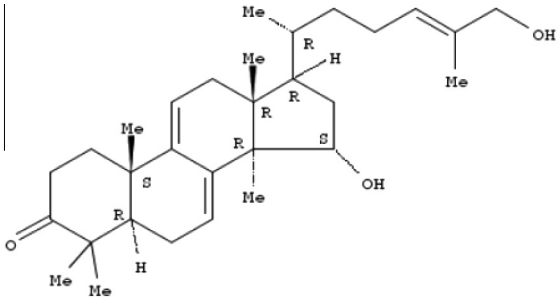
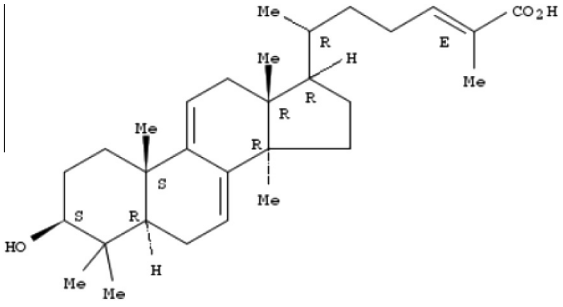
2.2. Cytotoxicity assays and antiviral activity assays

RD cells (1×10^4/well) were plated in 96-well cell culture plate and grown for 24 h, culture media were removed, and cells were washed with PBS and then treated with the antiviral compounds at various concentrations (0, 0.16, 0.8, 4, 20, and 100 μg/ml) in serum-free media for 72 h.

The effects of antiviral compounds on cell viability were evaluated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Culture media were removed, cells were rinsed with PBS twice and added with 5 mg/ml MTT reagent (Promega, Madison, WI) for 4 h, and 150 μl DMSO was added to each well to solubilize the formazan. The Optical Density (OD) at 490 nm was measured using an automatic plate reader (BIOTek, Elx800).

Antiviral activities of the compounds were calculated according to the following formula [18]: antiviral activity (%) = $(ODT)_v$ - $-(ODC)_v/(ODC)_{mock} - (ODC)_v \times 100\%$. $(ODT)_v$ and $(ODC)_v$ represents the optical density of cells infected with EV71 in presence of and in absence of the compounds respectively (index: T = treated, index: C = control). $(ODC)_{mock}$ is the optical density of normal cell without any treatment. The 50% inhibitory concentration (IC50) was calculated by regression analysis.

Table 1
Compounds used in this study.

Drug	Molecular formula	Molecular weight	Purity	2D structure
Ribavirin	$C_8H_{12}N_4O_5$	244	⩾98%	
GLTA	$C_{30}H_{46}O_3$	454	⩾98%	
GLTB	$C_{30}H_{46}O_3$	454	⩾98%	

The information is from Pubchem compound at NCBI website and product descriptions form the company (Zhonglin Pharmaceutical, Shan'xi, PR China) and the original lab in Kunming Institute of Botany, Chinese Academy of Science (Kunming, China).

2.3. Modes of action assays

To explore the mechanisms underline the effects of the compounds, GLTA and GLTB, on EV71 infection, three action modes (prevention action mode, mixture action mode, and treatment action mode) were applied using Ribavirin as a positive control. In the prevention action mode, RD cells were pre-incubated with the compounds, Ribavirin, GLTA, and GLTB, respectively for 1 h at 37 °C, washed with PBS, and then infected with EV71 (0.01 MOI). In the mixture action mode, an equal volume of EV71 suspension and the individual compounds, Ribavirin, GLTA, and GLTB, in MEM were mixed and incubated for 1 h at 37 °C. RD cells were then infected with the mixtures (EV71 at 0.01 MOI). In the treatment action mode, RD cells were first infected with EV71 (0.01 MOI) for 1 h, the supernatants were removed and the remaining viruses were washed away with PBS for three times. The infected cells were then treated with the compounds, Ribavirin, GLTA, and GLTB, respectively. The antiviral activities of the compounds were detected at 48 h posit-infection using MTT assays.

2.4. Molecular docking

The structure of EV71 has been revealed and reported previously [19]. In this study, we used the EV71 structure as a template to design and predicate the capsid structure of EV71 with MODELLER 9v8 and PROCHECK. The dockings of the small molecules, Ribavirin, GLTA, and GLTB, with EV71 capsid were predicated by ChemOffice and running MM2 energy minimization prior to docking. AUTODOCK 4.2 has been used to detect the interaction between these molecules by molecular docking. Polar hydrogens and partial charges were added by MGL tools 1.5. The atomic affinity potential energy was calculated by AUTODOCK supporting software AUTOGRID.

2.5. Quantitative real-time reverse transcription reactions

RD cells were grown in a 24-well culture plate at a density of 6×10^4 cells per well and treated by the different action modes for 12 h. The culture supernatants were removed, the cells were harvested, and total RNA was extracted from treated cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The relative levels of EV71 viral RNA (vRNA) were evaluated by quantitative real-time reverse transcription reaction (qRT-PCR) and normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The sequences of the detection primers are the follows:

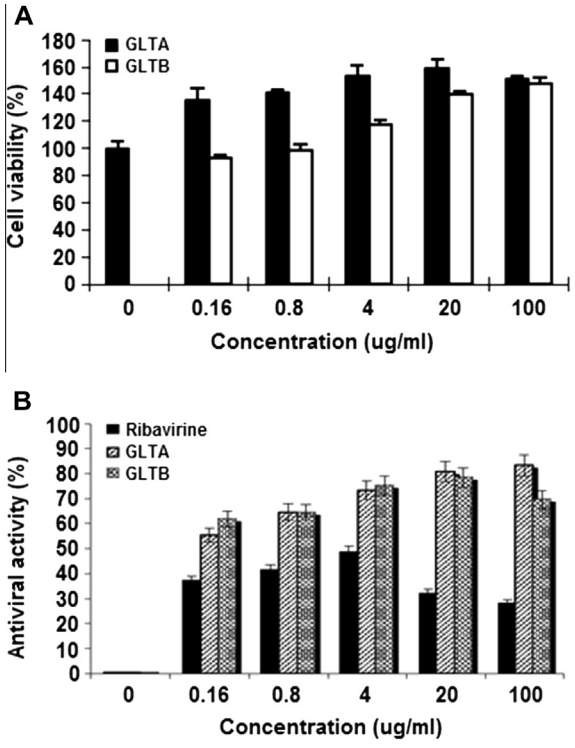


Fig. 1. Evaluation of the Cytotoxicity and the Anti-EV71 Activity of GLTA and GLTB. (A) Evaluation of the cytotoxicity of GLTA and GLTB. RD cells were treated with GLTA and GLTB, respectively, at various concentrations as indicated. The effects of GLTA and GLTB on cell viability were evaluated by MTT assay. The Optical Density (OD) at 490 nm was measured using an automatic plate reader (BIOTek, Elx800). (B) Evaluation of the anti-EV71 activity of GLTA and GLTB. RD cells were infected with EV71 and then treated with Ribavirin, GLTA, and GLTB, respectively, at various concentrations as indicated. The effects of GLTA and GLTB on cell viability were evaluated by MTT assays. The antiviral activities of the compounds were calculated according to the formula: antiviral activity (%) = $(ODT)_v - (ODC)_v/(ODC)_{mock} - (ODC)_v \times 100\%$. The 50% inhibitory concentration (IC50) was calculated by regression analysis. Data are presented as mean ± standard deviation of three independent experiments.

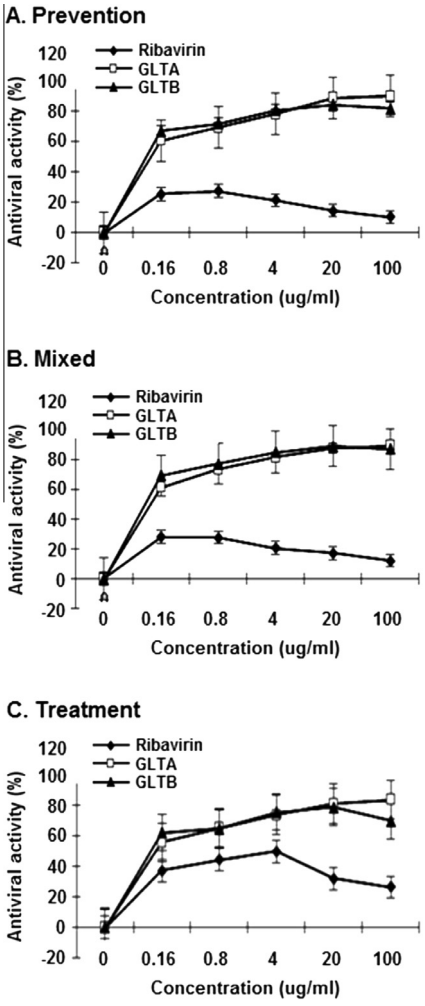


Fig. 2. The modes of action assays for the antiviral compounds. (A) The prevention action mode. RD cells were pre-incubated with the compounds, Ribavirin, GLTA, and GLTB, respectively, and then infected with EV71. (B) The mixture action mode. An equal volume of EV71 suspension and the individual compounds (Ribavirin, GLTA, and GLTB) were mixed, respectively, in MEM and incubated. RD cells were then infected with the mixtures. (C) The treatment action mode. RD cells were infected with EV71 and then treated with the compounds (Ribavirin, GLTA, and GLTB), respectively. The antiviral activities of the compounds were detected at 48 h posit-infection using MTT assays. Data are presented as the mean ± standard deviation ($n = 3$).

EV71-F, 5′-GCAGCCCAAAAGAACTTCAC-3′,EV71-R, 5′-ATTTCAGCAGCTTGGAGTGC-3′;GAPDH-F, 5′-AAGGCTGTGGGCAAGG-3′, GAPDH-R, 5′-TGGAGGAGTGGGTGTCG-3′. PCR products were measured using the Rotor-Gene 2000 Real-Time Cycler and analyzed with Rotor-gene software (Corbett Research). All reactions were performed in triplicate.

3. Results

3.1. GLTA and GLTB display antiviral activities against EV71 infection without cytotoxicity

Before detecting the antiviral activities of the two compounds (GLTA and GLTB) of *G. lucidum* triterpenoids against enterovirus 71 (EV71) infection, the effects of the two compounds on cytotoxicity were evaluated. Human rhabdomyosarcoma (RD) cells were treated with GLTA or GLTB at different concentrations, as indicated. MTT assay showed that both compounds had no cytotoxicity effect on RD cells (Fig. 1A).

To determine the antiviral activities of GLTA and GLTB against EV71 infection, RD cells were infected with EV71 and then treated with GLTA, GLTB, or Ribavirin (an antiviral drug used as a positive control) at different concentrations, as indicated. The antiviral activities of the three compounds were evaluated by MTT assays. Results revealed that the anti-EV71 activities of the three compounds were increased as their concentrations increased from 0.16 to 4 μg/ml (Fig. 1B). The anti-EV71 activity of Ribavirin was decreased at the concentrations of 20 and 100 μg/ml, but the anti-EV71 activities of GLTA and GLTB were still significantly high at the concentrations of 20 and 100 μg/ml (Fig. 1B). These results indicated that GLTA and GLTB display higher anti-EV71 activities than Ribavirin.

3.2. Modes of action of GLTA and GLTB against EV71 infection

The mechanisms by which GLTA and GLTB inhibit EV71 infection were investigated by three different approaches using Ribavirin as a positive control. First approach was the prevention action mode, in which RD cells were pre-incubated with the effective compounds GLTA, GLTB, and Ribavirin, respectively, at different concentrations for 1 h, and then infected with EV71 for 48 h. Antiviral activities of GLTA, GLTB, and Ribavirin were measured by MTT assays. Results showed that anti-EV71 activity of GLTA was increased as the concentrations increased and reached a peak of viral inhibition rate (89.6%) at 20 μg/ml, anti-EV71 activity of GLTB was increased as the concentrations increased and reached a peak of viral inhibition rate (83.8%) at 20 μg/ml, and anti-EV71 activity of Ribavirin was increased as the concentrations increased but reached a peak of viral inhibition rate (27.34%) at 0.16 μg/ml (Fig. 2A). These results suggested that GLTA, GLTB, and Ribavirin prevented EV71 infection, and the prevention effects of GLTA and GLTB are stronger than that of Ribavirin.

Second approach was the mixed action mode, in which EV71 was pre-incubated with GLTA, GLTB, and Ribavirin, respectively, and then infect RD cells. Results showed that anti-EV71 activity of GLTA was increased as the concentrations increased and reached a peak of viral inhibition rate (89.2%) at 20 μg/ml, anti-EV71 activity of GLTB was increased as the concentrations increased and reached a peak of viral inhibition rate (88.8%) at 20 μg/ml, and anti-EV71 activity of Ribavirin was increased as the concentrations increased but reached a peak of viral inhibition rate (28.06%) at 0.16 μg/ml (Fig. 2B). These results indicated that GLTA, GLTB, and Ribavirin may interact with the viral particles and prevent adsorption of virus into host cells, and the effects of GLTA and GLTB are stronger than that of Ribavirin.

Third approach was the treatment action mode, in which RD cells were first infected with EV71, and then treated with the antiviral compounds, respectively. Results showed that GLTA displayed an anti-EV71 effect and the inhibition rate reached to a peak of 83.3% at 20 μg/ml, GLTB exerted an anti-EV71 effect and the inhibition rate reached to a peak of 78.4% at 20 μg/ml, and Ribavirin also exerted an anti-EV71 effect and the inhibition rate reached to a peak of 49.60% at 4 μg/ml (Fig. 2C). These results indicated that GLTA, GLTB, and Ribavirin displayed anti-EV71 effects, and the anti-EV71 effects of GLTA and GLTB are stronger than that of Ribavirin.

3.3. GLTA and GLTB may block the uncoating of EV71, and thus inhibit the viral infection

The crystal structure of EV71 was recently revealed, which showed that the "pocket factor", a small molecule that stabilizes the virus, is partly exposed on the floor of the canyon, and there-

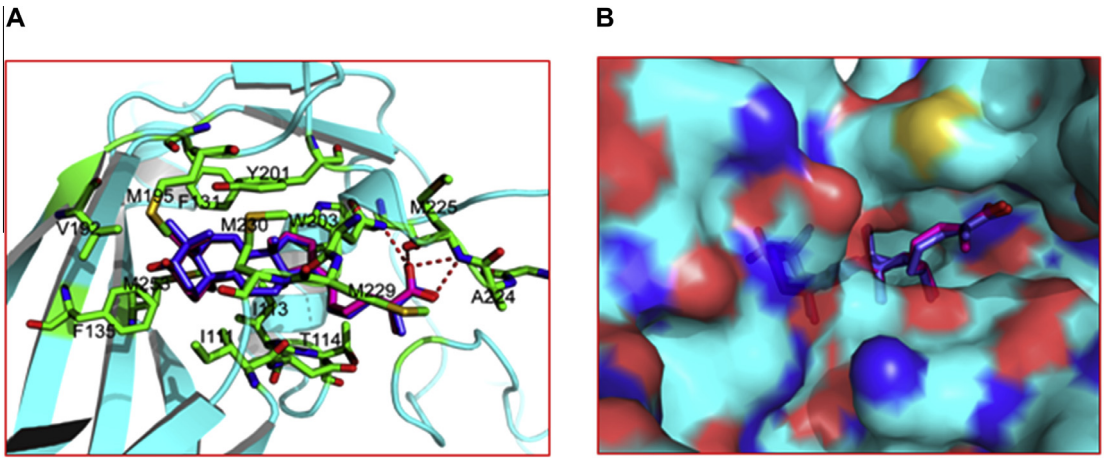


Fig. 3. Molecular docking for the interaction of antiviral compounds with EV71 capsid. (A) Stick conformer diagram. (B) Cartoon conformer diagram. The reported EV71 crystal structure was as a template to design and predicate EV71 capsid structure with MODELLER 9v8 and PROCHECK. The dockings of the small molecules with EV71 capsid were predicated by ChemOffice and running MM2 energy minimization prior to docking. AUTODOCK 4.2 has been used to detect the interaction between these molecules by molecular docking. The atomic affinity potential energy was calculated by AUTODOCK supporting software AUTOGRID. GLTA is magenta, GLTB is slate and EV71 capsid protein secondary structure skeleton is cyan. Amino acid residues interacted with compounds are green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fore, the structure of potential antiviral compound may require a hydrophilic head group to interact with residues at the entrance of the pocket [19]. Thus, we used the EV71 structure as a template to design and predicate the capsid structure of EV71 using MODELLER 9v8 and PROCHECK. To predicate the interaction among EV71 and GLTA, GLTB, and Ribavirin, respectively, we tested the interaction possibility and calculate the binding energy with molecular docking. We initially revealed some possible sites through which the compounds may bind to EV71 capsid using blind docking. The binding energy change was calculated and the highest one was chosen that is most possible site for interacting of the compounds with EV71 capsid. The results illustrated that both GLTA and GLTB could bind stably in the viral capsid mainly through hydrophobic interactions at a hydrophobic pocket (F site) in the capsid of EV71 virion (Fig. 3A).

The high-resolution structures for the mature EV71 virus and natural empty EV71 particles were previously determined, which demonstrated that the empty EV71 particles are dramatically expanded that resembles elusive viral uncoating intermediates, and that hydrophobic capsid pockets within EV71 capsid are collapsed in this expanded particle, providing a mechanism for receptor-binding triggered virus uncoating [20]. In combination with our results, we presumed the two anti-EV71 compounds, GLTA and GLTB, are able to bind to the pocket strongly to inhibit the release of the pocket factor and contribute to viral inactivation, as pocket-factor release seems to be required for the initiation of uncoating (Fig. 3B). It indicated that the two anti-EV71 compounds may block the uncoating during EV71 infection process, thereby, inhibit the viral infection.

3.4. GLTA and GLTB inhibit the replication of the viral RNA of EV71

The mechanisms by which GLTA and GLTB inhibit EV71 replication were further evaluated. RD cells were infected with EV71 and treated with Ribavirin, GLTA, or GLTB using the three different modes of action, respectively. Total RNA was isolated from the treated cells, the viral RNA (vRNA) of EV71 was reverse transcribed into cDNA with specific primers, and the cDNA level was quantified by RT-PCR. Results showed that the levels of EV71 vRNA in the infected and treated cells were reduced in the prevention and mixed action modes, but significant reduced in the treatment action mode, in the presence of Ribavirin (Fig. 4A). However, the levels of EV71 vRNA in the infected cells were reduced in the treatment action mode, significantly reduced in the pre-incubation action mode ($p < 0.05$), and inhibited in the mixed action mode ($p < 0.01$), in the presence of GLTA with the highest inhibition rate of 78.4% at 20 μg/ml (Fig. 4B). Similarly, the levels of EV71 vRNA in the infected cells were reduced in the treatment action mode, significantly reduced in the pre-incubation action mode ($p < 0.05$), but inhibited in the mixed action mode ($p < 0.01$), in the presence of GLTB with the highest inhibition rate of 83.9% at 20 μg/ml (Fig. 4C). These results indicated that GLTA and GLTB display antiviral activities against EV71 vRNA replication mainly by interacting with EV71 viral capsid, and also implicated that GLTA and GLTB inhibit E71 replication may through blocking the uncoating of EV71.

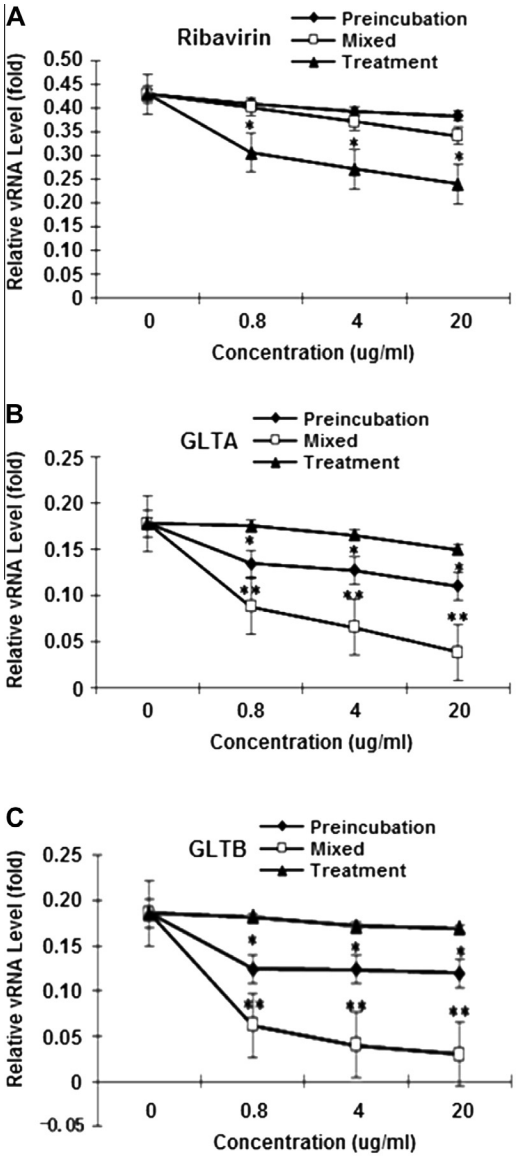


Fig. 4. Inhibition effects of GLTA and GLTB on EV71 vRNA replication. RD cells were infected with EV71 and treated with the compounds, (A) Ribavirin, (B) GLTA, and (C) GLTB, respectively by using the three different action modes, respectively. Total RNA was extracted from treated cells and the levels of EV71 viral RNA (vRNA) were evaluated by qRT-PCR and normalized to the level of GAPDH mRNA. All reactions were performed in triplicate. Data are presented as the mean ± standard deviation ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ relative to non-treated infected cells control.

4. Discussion

EV71, a member of the Picornaviridae family, causes HFMD by spreading through contact with virus-containing body fluids, respiratory droplets, and feces. There are no clinic antiviral drugs available for the treatment of HFMD. The treatments for EV71 infection usually rely on the broad-spectrum antiviral medications that can only relieve part of symptoms. Although immunoglobulin and antiviral agent Ribavirin are commonly used, the efficacy remains uncertain [21,22]. Good personal hygiene, including hand washing and disinfection of surfaces in child care facilities, remains the most effective approaches to reduce the transmission rate of HFMD [23]. Some natural medicinal compounds have demonstrated therapeutic efficacy against the disease by ameliorating the symptoms and shortening the course of the disease [24–26].

In this study, we evaluated the anti-EV71 activities of two *G. lucidum* triterpenoids (GLTs), Lanosta-7,9(11),24-trien-3-one,15;26-dihydroxy (GLTA) and Ganoderic acid Y (GLTB). The results showed that the two natural compounds display significant anti-EV71 activities without cytotoxicity in human RD cells as evaluated by MTT cell proliferation assays. Compared to some medicinal compounds and chemical antiviral drugs, GLTs are pure

Table 2
Binding energy with the F site in EV71 capsid.

Compounds	C1	C8	C17	C56
Binding energy (-kcal/mol)	10.02	9.83	9.6	9.71
Compounds	Pocket factor	Ribavirin	GLTA	GLTB
Binding energy (-kcal/mol)	7.0	5.6	11.95	13.07

All experiments were performed at least twice.

natural ingredients from edible fungi. They are safe, noncytotoxicity, and already been applied in many clinical treatment for a long time [27,28]. We believed that the two natural compounds may be possible to be used in daily life for antiviral therapy.

In addition, the mechanisms by which the two compounds affect EV71 infection were further elucidated by three modes of action using Ribavirin as a positive control. The results revealed that GLTA and GLTB can prevent EV71 infection and inhibit the replication of the vRNA of EV71. Thus, GLTA and GLTB may represent two potential therapeutic agents to control and treat EV71 infection.

Moreover, the interactions between EV71 virion and the two compounds were identified by two approaches. The interactions were first predicated by computer molecular docking, which illustrated that the two GLTs may interact specifically with the hydrophobic pocket factor-binding site (F site), which is a very important site in uncoating process during EV71 infection. The interactions were then elaborated by the results showing that GLTA and GLTB significantly inhibit the replication of the vRNA of EV71 and suggested that the two compounds inhibit EV71 replication through blocking EV71 uncoating. It is interestingly, the F site is an important site in uncoating process during virus infection, which is known as Pocket factor-binding site [20]. In addition, some large-scale disordering small-scale changes occur on the capsid of EV71 lead the viral particle to expended and the release of the pocket factor from a hydrophobic pockets in the EV71 capsid triggered virus uncoating. It have been reported that this hydrophobic pocket factor-binding site (F site) was a therapy target site against EV71 [19,20,29]. Thus, we also randomly selected some candidates for inhibiting EV71 infection from the literature and calculated their binding energy with the F site. The results of molecular docking revealed that the GLTA and GLTB not only have much higher binding energy than the pocket factor, but also may bind more stable to the pocket in viral capsid than other reported EV71 inhibitors (Table 2), indicating these two agents have high effective and therapeutic capabilities to inhibit viral uncoating and prevent EV71 replication. It has been reported that GLTs have immunomodulatory functions [30,31], and combination therapy could be recommended in clinical treatment.

Acknowledgments

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