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RESEARCH ARTICLE

In vitro antiviral activity of medicinal mushroom *Ganoderma neo-japonicum* Imazeki against enteroviruses that caused hand, foot and mouth disease

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ABSTRACT

Hand, foot and mouth disease (HFMD) is a highly contagious viral disease that predominantly affects children younger than 5 years old. HFMD is primarily caused by enterovirus A71 (EV-A71) and coxsackievirus A16 (CV-A16). However, coxsackievirus A10 (CV-A10) and coxsackievirus A6 (CV-A6) are being increasingly reported as the predominant causative of HFMD outbreaks worldwide since the past decade. To date, there are still no licensed multivalent vaccines or antiviral drugs targeting enteroviruses that cause HFMD, despite HFMD outbreaks are still being frequently reported, especially in Asia-Pacific countries. The high rate of transmission, morbidity and potential neurological complications of HFMD is indeed making the development of broad-spectrum antiviral drugs/agents against these enteroviruses a compelling need. In this study, we have investigated the *in vitro* antiviral effect of 4 *Ganoderma neo-japonicum* Imazeki (GNJI) crude extracts (S1-S4) against EV-A71, CV-A16, CV-A10 and CV-A6. GNJI is a medicinal mushroom that can be found growing saprophytically on decaying bamboo clumps in Malaysian forests. The antiviral effects of this medicinal mushroom were determined using cytopathic inhibition and virus titration assays. The S2 (1.25 mg/ml) hot aqueous extract demonstrated the highest broad-spectrum antiviral activity against all tested enteroviruses in human primary oral fibroblast cells. Replication of EV-A71, CV-A16 and CV-A10 were effectively inhibited at 2 hours post-infection (hpi) to 72 hpi, except for CV-A6 which was only at 2 hpi. S2 also has virucidal activity against EV-A71. Polysaccharides isolated and purified from crude hot aqueous extract demonstrated similar antiviral activity as S2, suggesting that polysaccharides could be one of the active compounds responsible for the antiviral activity shown by S2. To our knowledge, this study demonstrates for the first time the ability of GNJI to inhibit enterovirus infection and replication. Thus, GNJI is potential to be further developed as an antiviral agent against enteroviruses that caused HFMD.

Keywords: Antiviral; enteroviruses; *Ganoderma neo-japonicum* Imazeki; hand, foot and mouth disease.

INTRODUCTION

Hand, foot and mouth disease (HFMD) is a common pediatric infectious disease caused by Enterovirus A species (EV-A) within the family of *Picornaviridae*. Enteroviruses are non-enveloped and positive-sense single-stranded RNA viruses (Linden *et al*., 2015). Enterovirus A71 (EV-A71) and coxsackievirus A16 (CV-A16) are the most prevalent etiological pathogens for HFMD (Klein & Chong, 2015). However, CV-A10 and CV-A6 are rapidly emerging and being increasingly reported to cause HFMD outbreaks worldwide such as in Finland (Blomqvist *et al*., 2010), Taiwan (Wei *et al*., 2011), Spain (Bracho *et al*., 2011), France (Mirand *et al*., 2012), Edinburgh in the United Kingdom (Stewart *et al*., 2013), China (Chen *et al*., 2017; Bian *et al*., 2019), Vietnam (Anh *et al*., 2018), Argentina (Cisterna *et al*., 2019), Belem in Northern Brazil (Justino *et al*., 2020) and Uruguay (Lizasoain *et al*., 2020). Children under the age of 5 are the most susceptible and vulnerable groups for HFMD. Although rare, infections with EV-A71 can be associated with severe central nervous system (CNS) complications (Lei *et al*., 2015). In contrast, CV-A16, CV-A10 and CV-A6 associated HFMD are usually self-limited with symptoms such as fever, mouth ulcers and skin rashes on palms and/or soles of the feet (Repass *et al*., 2014). HFMD is highly contagious and easily transmitted from person-to-person via oral-oral and/or fecal-oral routes (Klein & Chong,

2015). Large HFMD outbreaks have been reported worldwide and more recently from countries within the West Pacific region including Malaysia, Singapore, Japan and China (Wu *et al*., 2010; Liu *et al*., 2014), causing severe healthcare burden and disruption.

To date, broad-spectrum antiviral drugs against the enteroviruses that caused HFMD are not available. Despite three EV-A71 inactivated vaccines are currently licensed and used in China (Lin *et al*., 2019), these vaccines only confer protection against EV-A71 but not other enteroviruses (Tan & Chu, 2017; Lin *et al*., 2019). Hence, having broad-spectrum antiviral agents to control the disease transmission and complications is desirable.

In the past decade, *Ganoderma* mushrooms have been reported as one of the best-known medicinal fungus species with a wide range of medicinal properties, including antiviral, anti-cancer, anti-inflammatory and immunomodulatory properties (Sliva, 2003; Li *et al*., 2006; Baby *et al*., 2015; Hapuarachchi *et al*., 2017). *G.lucidum* and *G.tsugae* are the 2 most extensively investigated *Ganoderma* species thus far (Ferreira *et al*., 2015; Huang *et al*., 2019). In contrast, the medicinal properties of *G.neo-japonicum* Imazeki (GNJI) which can be found growing saprophytically on decaying bamboo (*Schizostachyum brachyladium*) clumps in the forests of several countries including Malaysia, Japan, China, Korea and Taiwan, are yet to be fully explored (Tan *et al*., 2015; Subramaniam *et al*., 2017; Subramaniam *et al*., 2020). Its antiviral properties are still unknown as compared to *G. lucidum* (Tan *et al*., 2015).

In this study, we have demonstrated that GNJI crude aqueous extract exerted potent and broad-spectrum *in vitro* antiviral activity against EV-A71, CV-A16, CV-A10 and CV-A6 infection in human primary oral fibroblast cells. Polysaccharides could be one of the active antiviral compounds responsible for the antiviral activities demonstrated in this study. These highlighted the great potential of this medicinal mushroom to be further developed into a broad-spectrum antiviral agent against enteroviruses that caused HFMD and perhaps other viruses as well.

MATERIALS AND METHODS

Cells and viruses

Human primary oral fibroblast (HPOF) cells used in this study were previously isolated from the lip mucosa of a 3-month old infant (Phyu *et al*., 2017). The cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, USA) with Ham's F-12 Nutrient Mixture (F12) (Sigma-Aldrich, USA) in a ratio of 1:1 (DMEM/F12), supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA) and 1 ng/ml fibroblast growth factor (Promega, USA). African green monkey kidney (Vero) cells were grown in DMEM growth medium (DMEM-GM) supplemented with 5% FBS and human rhabdomyosarcoma (RD) cells in DMEM-GM supplemented with 5% FBS and 5% horse serum (HS) (Gibco, USA). EV-A71, CV-A16, CV-A10 and CV-A6 used in this study were previously isolated from children with HFMD. To prepare virus stocks, EV-A71 and CV-A16 were propagated in Vero cells with DMEM maintenance medium (DMEM-MM) supplemented with 2% FBS, and CV-A10 and CV-A6 in RD cells with DMEM-MM supplemented with 2% FBS and 2% HS. The virus titer was determined using a 50% cell culture infective dose ($CCID_{50}$) assay in Vero or RD cells as described previously (Ong *et al*., 2008).

Mushroom extract preparation

GNJI extract powder was prepared as described previously (Subramaniam *et al*., 2017). A total of 5 extracts (S1-S5) were tested in this study. S1 and S2 were crude hot aqueous extracts obtained with 30 mins and 3 hours boiling, respectively, whereas S3 and S4 were 70% and 100% crude ethanolic extracts, respectively. S5 was the GNJI polysaccharides fraction isolated and purified from the crude aqueous extract with 4 hours of boiling. The S1, S2, S3 and S5 powder were dissolved in DMEM to a final concentration of 10 mg/ml, whereas S4 powder was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) to a final concentration of 25 mg/ml. The solutions were filtered through a 0.22 µm pore-size filter (Sartorius, Minisart, Germany), aliquoted and stored at -20°C before use.

Virus titration

50% cell culture infectious dose ($CCID_{50}$) assay was performed as described previously (Ong *et al*., 2008). Briefly, Vero or RD cells were seeded in a 96-well plate overnight at 37°C. Ten-fold serial dilutions of the virus supernatant (10^{-1} to 10^{-8}) in DMEM-MM were prepared and 100 µl of each dilution was inoculated at quadruplicate. The plate was then further incubated for 5 days. $CCID_{50}$ was calculated using the Spearman-Karber method (Karber, 1931).

Determination of maximal non-toxic dose (MNTD)

The maximal non-toxic dose (MNTD) of each crude extract (S1-S4) was determined using Cell Titre 96© Aqueous One Solution Cell Proliferation Assay (MTS) (Promega, USA) and cell morphological observation. MTS assay was performed according to the manufacturer's instructions. Briefly, HPOF cells were seeded in a 96-well plate at cell density of $1.5x10^4$ cells/well before incubating with different concentrations of S1, S2, S3 (0.078 mg/ml to 2.5 mg/ml), S4 (0.003 mg/ml to 0.1 mg/ml), DMSO (diluent for S4, 0.013% to 0.4%) or DMEM (negative control) in quadruplicate. Cells were observed daily for any morphological changes under an inverted microscope. After incubation for 3 days at 37°C, the optical density (OD) in each well was measured using a microplate reader (PowerWave X340, BioTek, USA) at 490 nm wavelength with a reference wavelength of 600 nm. The OD reading is directly proportional to the number of viable cells in the culture. The cell viability graph was presented as percentage of HPOF cells viability against extracts of different concentrations.

Antiviral screening

Non-toxic doses (NTD) of each crude extract (S1-S4) were first screened on EV-A71 using CPE inhibition assay. Extracts that demonstrated complete inhibition of EV-A71 CPE were then selected and tested on CV-A16. Only the extracts that demonstrated 100% CPE inhibition for both viruses were chosen for final testing on CV-A10 and CV-A6. Briefly, HPOF cells were pre-seeded at $1.5x10^4$ cells/well in a 48-well plate overnight at 37°C before incubated with the NTD of each extract for 24 hours at 37°C. The next day, the pre-treated cells were inoculated with 100 $CCID_{50}$ of the virus and incubated for 5 days at 37°C. Untreated, virus inoculated cells were served as virus positive controls while treated and mock-inoculated cells were served as negative cells controls.

In vitro antiviral experiments

Since S2 extract (1.25 mg/ml) demonstrated the most broad-spectrum antiviral activity against all 4 tested enteroviruses, the *in vitro* antiviral activities were further investigated by pre-infection treatment, post-infection treatment and virucidal experiments. All 3 experiments were performed simultaneously in a 96 well-plate with a cell-density of $1.5x10^4$ cells/well. The other extracts were not studied further.

i. Pre-infection treatment experiment

Briefly, HPOF cells were pre-treated with 1.25 mg/ml of S2 overnight (approximately 24 hrs) at 37°C. After overnight incubation, treated and untreated cells were washed twice with PBS before infecting with 100 $CCID_{50}$ of virus. Following pre-absorption for 2 hours at 37°C, unbound viruses were removed, and the cells were washed twice with PBS before replacing with new DMEM/F12 medium. Infected and uninfected cells were observed daily. At 5 days post-infection (dpi), the plate was freeze-thawed 3 times and viral-titrated using the $CCID_{50}$ assay as described.

ii. Post-infection treatment experiment

HPOF cells were infected with 100 $CCID_{50}$ of virus for 2 hours at 37°C. Infected cells were then washed twice with PBS before treated with 1.25 mg/ml of S2. Treated and untreated cells were observed daily. At 5 dpi, the plate was freeze-thawed and viral-titrated as described.

iii. Virucidal experiment

An equal volume of S2 (2.5 mg/ml) was mixed with 200 $CCID_{50}$ of virus and incubated for 1 hour at 37°C. The mixtures were then added onto the HPOF cells and further incubated for 2 hours at 37°C for virus pre-absorption. The virus-S2 mixture was then removed and the cells were washed twice with PBS before replacing with new DMEM/F12 medium. Cells inoculated with virus-S2, virus-DMEM/F12 and DMEM-DMEM/F12 mixtures, respectively, were observed daily. At 5 dpi, the plate was freeze-thawed and viral-titrated as described.

Determination of the post-infection treatment efficacy of S2

To evaluate the efficacy of S2 in inhibiting intracellular virus replication, HPOF cells were seeded and infected as described in the post-infection treatment experiment, except with an MOI of 0.01. Infected cells were treated with 1.25 mg/ml of S2 at 2, 24, 48, or 72 hours post-infection (hpi). At 5 dpi, the plate was freeze-thawed and viral-titrated as described.

Determination of the antiviral activity of GNJI polysaccharides

To determine if polysaccharides isolated from hot aqueous extracts could contribute to the observed S2 antiviral effects, the polysaccharides (S5) (Subramaniam *et al*., 2017) were tested for their antiviral activity against EV-A71 and CV-A16 as described in the post-infection treatment experiment. S5 was also tested for virucidal activity against EV-A71 as described in the virucidal experiment. Briefly, for the post-infection treatment experiment, HPOF cells were seeded and infected with EV-A71 or CV-A16 at MOI of 0.01 as described. Infected cells were treated with 1.25 mg/ml of S5 at 2, 24, 48 and 72 hpi. For the virucidal experiment, EV-A71 ($2x10^3$ $CCID_{50}$) was incubated with an equal volume of S5 (2.5 mg/ml) for 1 hr at 37°C. The virus-S5 mixture was then added onto the HPOF for virus pre-absorption for 2 hrs at 37°C. Uninfected cells treated with 1.25 mg/ml of S5 were set up for cytotoxicity assessment. At 5 dpi, the plate was freeze-thawed and viral-titrated as described.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). All statistical analyses were performed using the MaxStat Lite Statistic Software (MaxStat, Germany). As data were normally distributed (Anderson-Darling Normality Test), a two-tailed unpaired t-test was used to determine the statistical significance between treated and untreated cells. A p-value<0.05 was considered statistically significant.

RESULTS

Determination of maximal non-toxic dose (MNTD)

Based on the MTS (Figure 1) and cell morphology results, S1 (0.078 mg/ml to 0.625 mg/ml), S2 (0.078 mg/ml to 1.25 mg/ml), S3 (0.078 mg/ml to 0.625 mg/ml) and S4 (0.003 mg/ml to 0.025 mg/ml) did not show a significant difference in cell viability (>90% viable) and cell morphology changes between inoculated and uninoculated cells. Therefore,

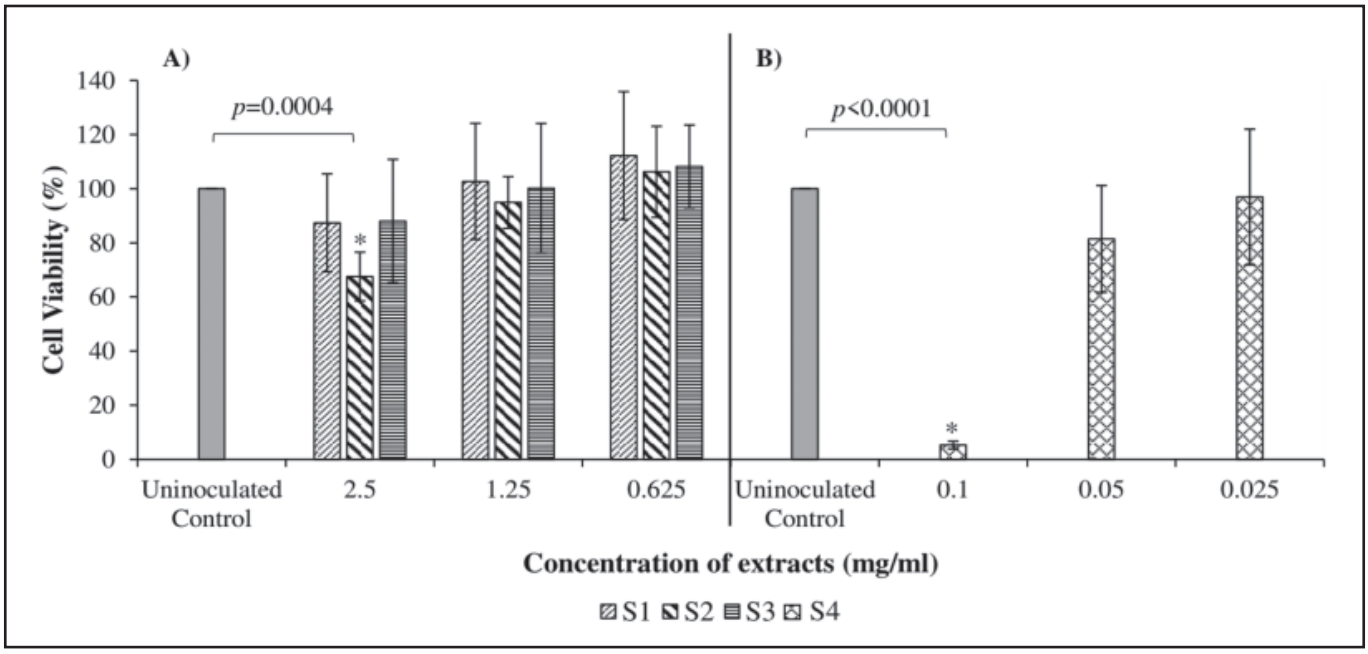

Figure 1. Human oral fibroblast cell viability after exposure to different concentrations of *G.neo-japonicum* Imazeki extracts. Percentage of cell viability determined by MTS assay after 3 days incubation with different concentrations of S1, S2 and S3 (A) and S4 (B). Concentrations lower than 0.625 mg/ml (A) and 0.025 mg/ml (B) are not displayed because the percentage of cell viability is not statistically significant compared to uninoculated control. The percentage of cell viability was expressed as mean percentage ± standard deviation. * = statistically significant (p<0.05).

these concentrations were selected and used for antiviral screening. DMSO present in S4 did not cause cytotoxicity to the cells.

Antiviral screening

MNTD of each crude extract demonstrated antiviral activity against EV-A71 with 100% CPE inhibition (Table 1). Since only the 1.25 mg/ml of S2 demonstrated 100% CPE inhibition for CV-A16 at the second screening, S2 (1.25 mg/ml) was selected for the final testing on CV-A10 and CV-A6. In contrast, 1.25 mg/ml of S2 demonstrated moderate (~60-70%) CPE inhibition in CV-A10 and CV-A6 infected cells (data not shown).

***In vitro* antiviral activity**

In vitro antiviral activity of S2 (1.25 mg/ml) against all 4 viruses was investigated by performing pre-infection treatment, post-infection treatment and virucidal experiments. S2 did not demonstrate pre-infection treatment antiviral activity (Figure 2). In the post-infection treatment experiment, no virus was isolated in EV-A71 (Figure 2A) and CV-A16 (Figure 2B) infected cells. Meanwhile, the virus titers in CV-A10 (2.69 $\log_{10}$ $CCID_{50}$ ± 0.515, p=0.0057) (Figure 2C) and CV-A6 (3.06 $\log_{10}$ $CCID_{50}$ ± 0.125, p=0.01) (Figure 2D) infected cells treated with S2 were significantly lower (p<0.05) compared to the infected untreated controls (4 $\log_{10}$ $CCID_{50}$ ± 0.354 and 3.56 $\log_{10}$ $CCID_{50}$ ± 0.239, respectively).

S2 also demonstrated virucidal activity, but only against EV-A71 (Figure 2A). No virus was isolated in cells inoculated with EV-A71-S2 mixtures (Figure 2A). In contrast, virus titers were not significantly different in cells inoculated with CV-

Table 1. Antiviral screening of S1, S2, S3 and S4 against EV-A71 infection in human oral fibroblast cells

Mushroom extracts	Concentrations (mg/ml)				
S1	0.625 –	0.313 +	0.156 +	0.078 +	
S2	1.25 –	0.625 +	0.313 +	0.156 +	0.078 +
S3	0.625 –	0.313 +	0.156 +	0.078 +	
S4	0.025 –	0.013 +	0.006 +	0.003 +	

1) +: Presence of CPE, 2) –: Absence of CPE.

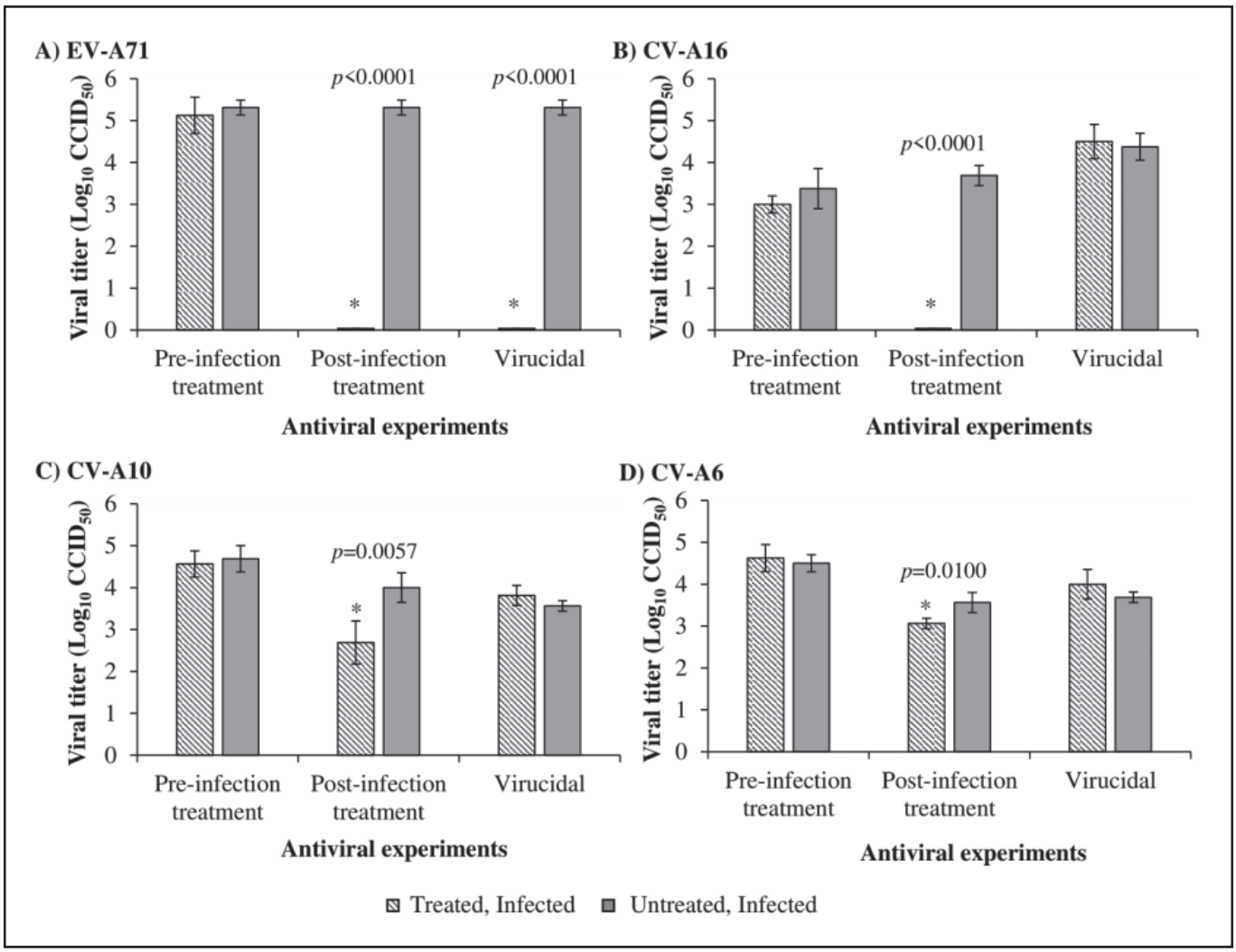

Figure 2. Viral titers of A) EV-A71, B) CV-A16, C) CV-A10 and D) CV-A6 in *in vitro* antiviral experiments. No antiviral activity was demonstrated in the pre-infection treatment experiment. The post-infection treatment experiment showed that S2 was able to inhibit replication of all viruses at 2 hpi. S2 only demonstrated virucidal activity against EV-A71 (A). Virus titers were expressed as the mean $\log_{10}$ $CCID_{50}$ ± standard deviation. * = statistically significant (p<0.05).

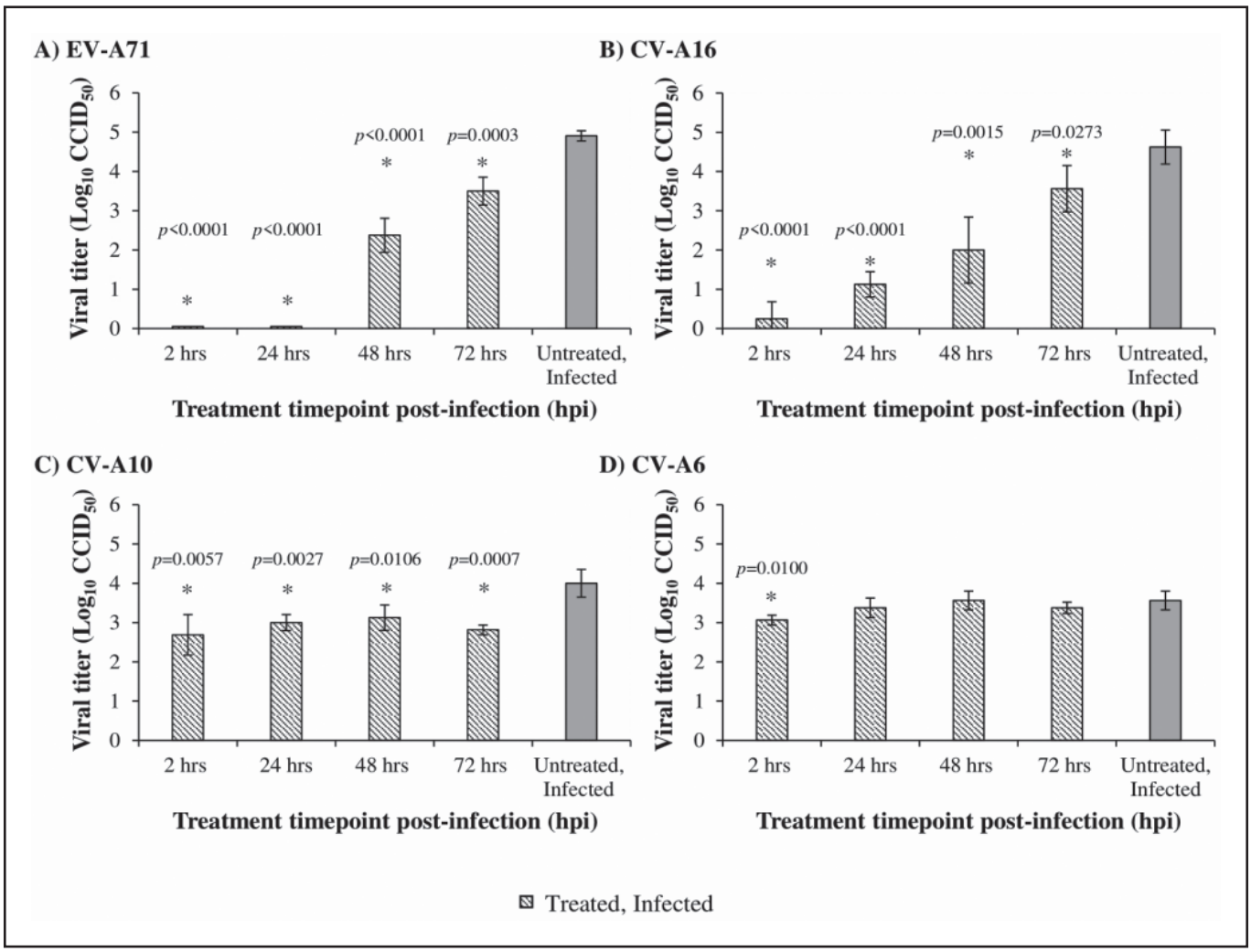

Figure 3. Post-infection treatment efficacy of S2 against A) EV-A71, B) CV-A16, C) CV-A10 and D) CV-A6. From 2 hpi to 72 hpi, significantly lower virus titers were shown in EV-A71, CV-A16 and CV-A10 infected cells treated with S2. CV-A6 virus titer was only significantly lower at 2 hpi. Virus titers were expressed as the mean $\log_{10}$ $CCID_{50}$ ± standard deviation. * = statistically significant (p<0.05).

A16-S2 mixtures (4.5 $\log_{10}$ $CCID_{50}$ ± 0.408, p=0.648) (Figure 2B), CV-A10-S2 mixtures (3.81 $\log_{10}$ $CCID_{50}$ ± 0.239, p=0.1135) (Figure 2C) and CV-A6-S2 mixtures (4 $\log_{10}$ $CCID_{50}$ ± 0.354, p=0.1466) (Figure 2D) compared to virus-DMEM infected controls (4.38 $\log_{10}$ $CCID_{50}$ ± 0.323, 3.56 $\log_{10}$ $CCID_{50}$ ± 0.125 and 3.69 $\log_{10}$ $CCID_{50}$ ± 0.125, respectively).

Post-infection treatment efficacy of S2 against EV-A71, CV-A16, CV-A10 and CV-A6 replication

To further evaluate the post-infection treatment efficacy, EV-A71, CV-A16, CV-A10 and CV-A6 infected HPOF cells were treated with S2 at various hpi. The virus titers of EV-A71, CV-A16 and CV-10 were significantly lowered at all time points (Figure 3A, 3B, 3C). However, virus titer of CV-A6 was only significantly reduced at 2 hpi (Figure 3D).

Antiviral activity of GNJI polysaccharides

HPOF cells infected with EV-A71 and CV-A16 were treated with 1.25 mg/ml of S5 at various hpi similarly as described in the post-infection treatment experiment. The virus titers of EV-A71 were significantly lowered at 2 hpi to 48 hpi (Figure 4A), while virus titers of CV-A16 were significantly reduced at all treatment time points (Figure 4B). Besides, S5 also demonstrated virucidal effect against cell-free EV-A71. Cells inoculated with EV-A71-S5 mixtures have significantly lower virus titers (3.56 $\log_{10}$ $CCID_{50}$ ± 0.375, p=0.0175) compared to virus-DMEM infected control (4.13 $\log_{10}$ $CCID_{50}$ ± 0.144) (Figure 5). No cytotoxic effect was observed in S5 treated, uninfected HPOF cells (data not shown).

DISCUSSION

Developing a safe and effective antiviral agent for HFMD is challenging as the disease could be caused by multiple etiological agents (Klein & Chong, 2015; Wang *et al*., 2017). To date, the research and development of antiviral agents are mainly focused on EV-A71 (Lei *et al*., 2015) even though CV-A10 and CV-A6 associated HFMD are on the rise not only in Asia-Pacific countries but worldwide (Bian *et al*., 2015; Bian *et al*., 2019). Most likely, these emerging HFMD viruses could potentially become the dominant serotypes. A narrow-spectrum antiviral agent will only be beneficial to a small portion of HFMD patients. Hence, this highlighted the importance of developing a broad-spectrum antiviral agent against common and emerging enteroviruses that caused HFMD.

In this study, we have investigated the *in vitro* antiviral activity of GNJI crude extracts obtained from different preparation methods (see Materials and Methods) against 4 HFMD enteroviruses. To our knowledge, this study appears

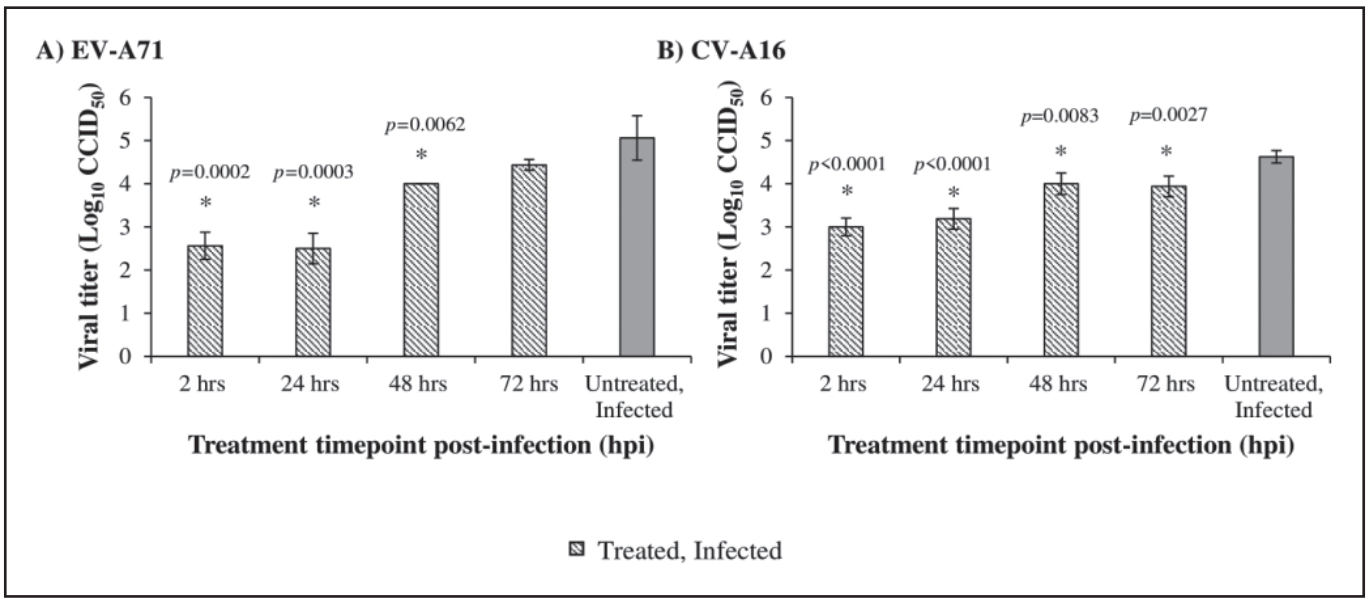

Figure 4. Post-infection treatment efficacy of S5 against A) EV-A71 and B) CV-A16. The virus titers of EV-A71 infected cells treated with S5 at 2 hpi to 48 hpi were significantly lower than untreated control. CV-A16 virus titer was significantly lower at all time points. Virus titers were expressed as the mean $\log_{10}$ $CCID_{50}$ ± standard deviation. * = statistically significant (p<0.05).

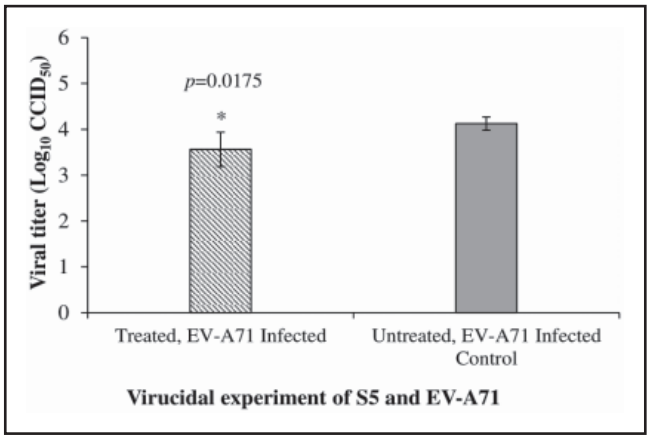

Figure 5. Virucidal activity of S5 against EV-A71. S5 demonstrated virucidal activity against EV-A71. Virus titers were expressed as the mean $\log_{10}$ $CCID_{50}$ ± standard deviation. * = statistically significant.

to be the first study investigating the antiviral activity of GNJI mushroom against enteroviruses.

As demonstrated in the post-infection treatment experiment, S2 was highly effective in inhibiting EV-A71, CV-A16 and CV-A10 replication, even when the infected cells were treated at a late time point (72 hpi). However, S2 was less effective in inhibiting CV-A6 replication. CV-A6 may be able to overcome the antiviral effects of S2 better than the other three enteroviruses, leading to higher viral titers. However, the exact mechanism is unknown and required further investigations to explain the differences observed in our study. As shown in the virucidal experiment, S2 demonstrated virucidal activity against EV-A71 but not to other enteroviruses. We hypothesized that this could be due to the structural differences between EV-A71 virion and other enteroviruses as reported previously (Ren *et al*., 2015; Chen *et al*., 2018; Chen *et al*., 2019). Despite demonstrating good antiviral activities in the post-infection treatment and virucidal experiments, no antiviral activity was detected in the pre-infection treatment experiment, suggesting that S2 could not serve as a prophylaxis agent to prevent EV-A71, CV-A16, CV-A10 and CV-A6 infection. This result also further implied that virucidal effect of S2 against EV-A71 may not be related to the interaction between S2 and the cells, instead, directly with the EV-A71 virus particle itself.

The different antiviral spectrum observed between the crude extracts (S1-S4) could be attributed to the different extraction methods used (extraction solvent, boiling duration, etc.) (Zhang *et al*., 2007; Subramaniam *et al*., 2014). S2 possesses the broadest antiviral spectrum, suggesting that the antiviral bioactive compounds may have a higher solubility in water than in organic solvent. Hot water extraction is regarded as a common and simple method to extract water-soluble polysaccharides from *Ganoderma* mushrooms (Ling-Sing Seow *et al*., 2013; Nie *et al*., 2013; Ferreira *et al*., 2015; Zhu *et al*., 2015; Gong *et al*., 2020). In addition, the antiviral bioactive compounds present in S2 appear to be thermally stable and were not negatively affected during the heating process as demonstrated by the results. However, whether or not this hot water extraction method will negatively affect other non-antiviral compounds requires further investigations.

The prolonged boiling (30 mins vs 3 hrs) may enhance the destruction of fungal cell wall and its subcellular compartment, which will therefore increase the release of water-soluble bioactive compounds such as polysaccharides that are found in abundance in fungal cell wall (Zhang *et al*., 2007; Rop *et al*., 2009). We speculate that the amount and proportion of the extracted antiviral bioactive compound may be relatively lower in S1 as compared to S2. Polysaccharides extracted from *G.lucidum* and *G.pfeifferi* were previously found to exhibit antiviral activities against herpes simplex virus (Hassan *et al*., 2015) and influenza virus type A (Mothana *et al*., 2003), respectively. Similarly, the polysaccharides isolated from GNJI (S5) was found to be able to inhibit the replication of EV-A71 and CV-A16 when treated at later time points post-infection. S5 also demonstrated virucidal activity against EV-A71. These results further suggested that polysaccharides could be one of the active components presents in S2. Although there was a slight difference in the boiling duration

between S5 and S2, it is predicted that S2 would contain polysaccharides as well since both were extracted using the same method. S5 was previously shown to contain β-glucan rich polysaccharides (39.26 mg/100 mg; w/w) (Subramaniam *et al*., 2017). Hence, β-glucan rich polysaccharides may be one of the vital bioactive compounds responsible for the antiviral activity seen in this study.

Besides polysaccharides, triterpenoids are also the common constituent of *Ganoderma* mushrooms (Chen *et al*., 2015; Hapuarachchi *et al*., 2017). Triterpenoids isolated from *G.lucidum* have previously demonstrated virucidal activity against EV-A71 infection in RD cells (Zhang *et al*., 2014). We postulate that the virucidal activity of S2 could be due to the presence of triterpenoids as well. Both polysaccharides and triterpenoids could be part of the constituents of S2 and therefore contributing to its antiviral activity. The speculated bioactive compounds in S2 were not identified or quantified. Thus, further characterization S2 is needed to confirm these speculations.

Although numerous natural compounds have been reported to contain antiviral activity against EV-A71, none of these compounds have demonstrated broad-spectrum antiviral activities against CV-A16, CV-A10 and CV-A6 (Ji *et al*., 2015; Zhao *et al*., 2016). Nonetheless, it has to be addressed that further investigations are necessary to reveal the exact mechanism of antiviral action of S2. The current *in vitro* findings are preliminary and further validation by animal studies are essential to provide more profound insights into the efficacy of this extract in ameliorating the disease and its complications as well.

In conclusion, our findings demonstrated that GNJI S2 aqueous extract is a candidate to be further characterized and developed into an effective, safe and broad-spectrum antiviral agent against enteroviruses that caused HFMD and possibly other medically important human viruses as well.

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Conflict of interests

The authors declared no conflict of interests.

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