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Hederacolchiside C inhibits Enterovirus 71 propagation through activating innate immunity☆

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ABSTRACT

Enterovirus 71 (EV71), a newly emerging life-threatening pathogen induces hand-foot-mouth disease (HFMD), no effective vaccines or specific anti-viral treatments are currently available. In this study, the activity of *hederacolchiside C (HSC)* against EV71 was investigated, and the antiviral mechanism was explored. *HSC* displayed apparent antiviral activity in EV71-infected cells probably through activating the host innate immunity. Comparing with EV71-infected group at 24 hpi, the group pretreated with *HSC* dramatically increased the expression of MAVS, p-IRF3, IRF3 and IFN-β, the innate immune effectors related to innate immunity. In addition, *HSC* displayed stronger antiviral activity in EV71-infected suckling mice in comparison with Ribavirin, a broad-spectrum antiviral drug. The results suggest that *HSC* could have potential as a pharmaceutical drug for HFMD.

Pulsatilla chinensis (Bunge) Regel is a medicinal plant with a long history of clinic usage in China. The roots of *P. chinensis* have been used for the adjunctive treatment of intestinal amebiasis, malaria, vaginal trichomoniasis, bacterial infections, and malignant tumor [1]. *Hederacolchiside C (HSC)* represents one of the active triterpenoid saponins in *P. chinensish*, possessing antitumor, anti-inflammatory, antioxidant and neuroprotective activities [2]. The previous study in our lab demonstrated that *HSC* possessed anti-schistosomal activity against *Schistosoma japonicum in vivo* through reducing the expression of IgG, tumor necrosis factor (TNF)-α, interleukin (IL)-4 and IL-17 [3]. Our further investigation revealed it had strong antiviral activity on Enterovirus 71 (EV71).

EV71, a newly emerging life-threatening pathogen, belongs to the *Enterovirus* genus of the *Picornaviridae* family. It can cause fatal diseases common in infants including hand-foot-mouth disease (HFMD), cardiopulmonary failure, aseptic meningitis, brainstem and/or cerebellar encephalitis, acute flaccid paralysis and encephalomyelitis [4]. The characteristic of tolerance and non-specificity in EV71 mutant makes it difficult to develop effective vaccines or specific anti-viral drugs for the EV71 patients [5]. Recent animal studies and clinical studies suggested that the host immune responses are related to the serious complications of EV71 patients [6]. The importance of suppressing host innate immune responses have been suggested by some viral pathology studies [7]. Because viral proteases EV71 3C can associate with retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), which impair the recruitment of mitochondrial outer membrane adaptor protein MAVS (MAVS; also known as IPS-1, Cardif and VISA) [8] and therefore inhibits the activation of transcription factors interferon regulatory factor (IRF) 3 (IRF3). Furthermore, the expression levels of IFN-β, and TNF-a were decreased in EV71-infected Human rhabdomyosarcoma cells (RD cells) to facilitate the proliferation of EV71 [6].

Many trials have been conducted to screen potential antiviral components from medicinal plants because of low cost, minor side effect and low tendancy to cause resistance [9]. Herein, we report the evaluation of the antiviral activity of *HSC* through the assessment of EV71-infected cells and suckling mice. The results showed

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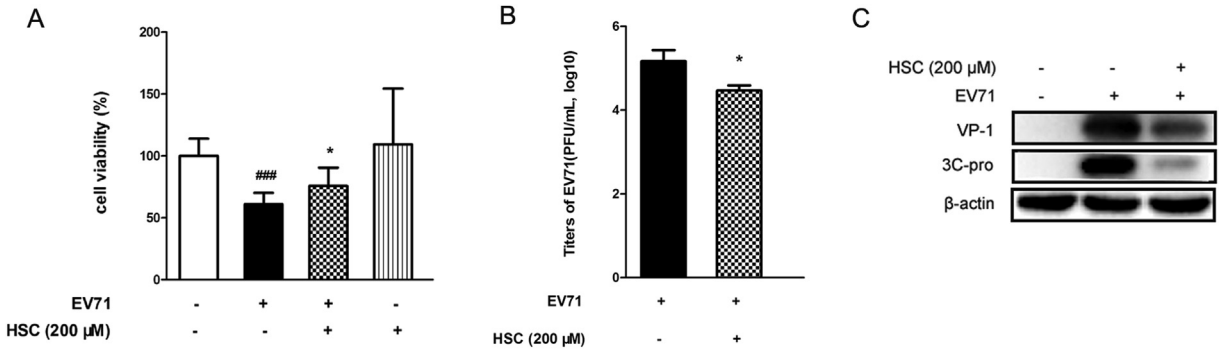

Fig. 1. ***HSC*** **inhibits EV71 propagation without obvious cytotoxicity. A.** Evaluation of the anti-EV71 activity of HSC at 200 μM through MTT assays. The viability of control group (blank cells) was set as 100%. Data are presented as mean ± SD (n = 3). $^{###}p < 0.001$ (compared to (EV71-,HSC-) group) and $^{*}p < 0.05$, (compared to (EV71+,HSC-) group). **B**. RD cells treated with 200 μM HSC before EV71 infection. Samples were harvested at the 24 h post-infection and the viral titers were determined by TCID50 assays. **C.** RD cells treated with 200 μM HSC 24 h before EV71-infected, and then total cell extracts were subjected after 24 h EV71-infenction to Western blot analysis with anti-VP-1 antibody and anti-3Cpro antibody. β-actin was also analyzed as a loading control.

that *HSC* prevented EV71 propagation by activating host innate immunity. Our findings shed new light on the inhibition mechanism of natural products against EV71 viral propagation.

Initially, we conducted experiments in vitro to investigate the effect of HSC on the cell viability of RD cells infected with EV71 by using MTT (10 μL, 5 mg/mL) assays. An administration of the serial concentration of *HSC* (50–2000 μM) on the RD cells showed no cytotoxicity effection in Supplementary (Fig. S1A). Besides, MTT assay evaluated the effective dose of *HSC* on cell viability, showing that the pretreatment of *HSC* (200 μM) prevented apoptosis induced by EV71 (Fig. S1B). The antiviral effect of *HSC* (200 μM) was tested by cytopathic effects (CPE) assays in RD cells which were pretreated with *HSC* 1 h prior to EV71 infection and cultured for 24 h after EV71 infection. As shown in Figure S1C, the RD cells in

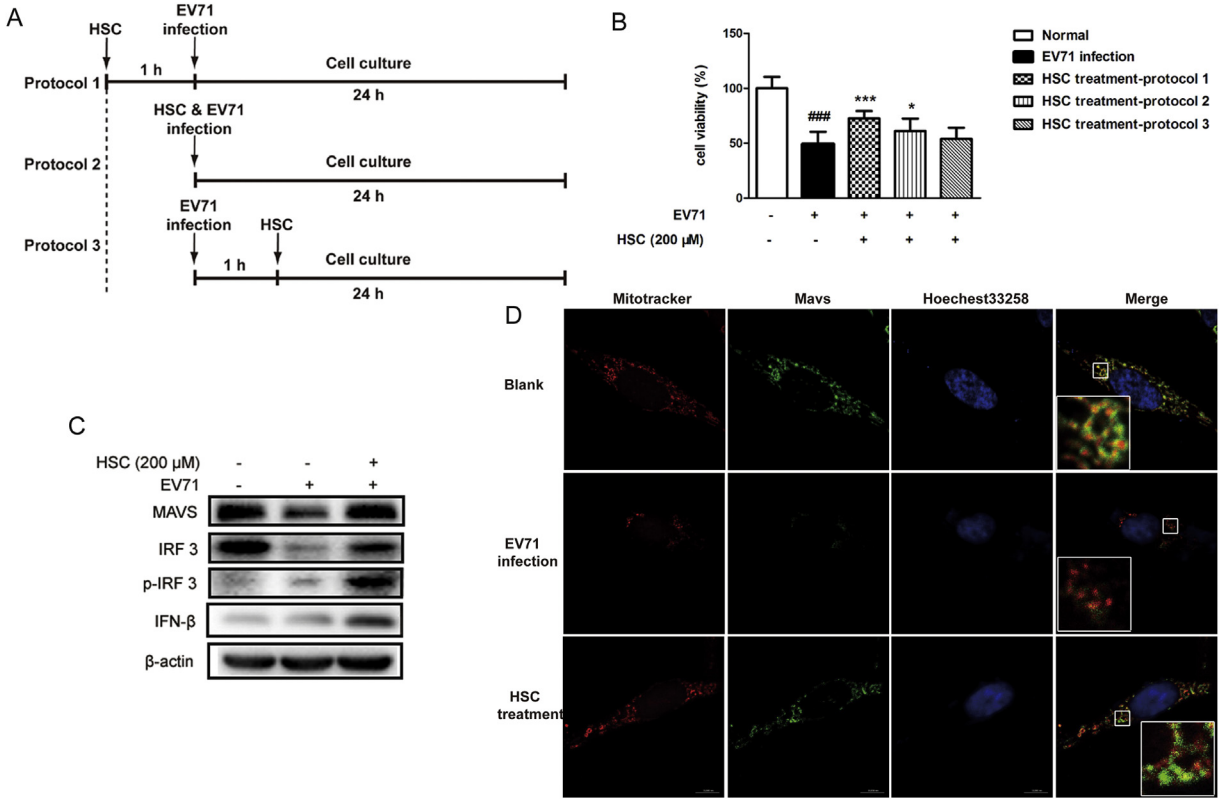

Fig. 2. ***HSC*** **activates innate immune system and suppresses EV71-induced cleavage of MAVS. A.** RD cells were infected with EV71. HSC treatment (200 μM) was performed before or after viral infection as indicated on the figure; **B.** The viability of RD cells according to different protocols **(A)** was detected using MTT assays. The viability of control group was set as 100%. Data are presented as mean ± SD (n = 3). $^{###}p < 0.001$ (compared to (EV71-,HSC-) group) and $^{*}p < 0.05$ and $^{***}p < 0.001$ (compared to (EV71+,HSC-) group); **C.** At the 24 h post-infection time point, the cells were harvested and western blot was used to detect the change of protein related to the innate immune pathway using the anti-MAVS AT107 antibody, IRF 3, p- IRF 3 (ser396) and IFN-β antibodies. β-actin was also analyzed as a loading control. **D.** Representative confocal microscope image showing co-localization of MAVS and mitochondrial in the presence of HSC. At 24 h after treatment with HSC, RD cells were incorporated with mitotracker (red) and immunostained with antibodies against MAVS (green).

normal group attached well on the surface of dishes with spindle-like shape. EV71-infected cells showed obvious CPE, becoming rounded and floating.

Cell viability was reduced to 60.8% in EV71-infected RD cells, however, *HSC* improved the significant cell viability from 60.8% to 75.8% at 24 hpi (hours post-infection) ($p = 0.0023$) (Fig. 1A). Statistically, the *HSC* treatment group (EV71+, *HSC*+) was not significantly low compared to the blank group (EV71-, *HSC*-). These phenomenon indicated that *HSC* show antiviral activity in EV71-infected cells. The supernatants contained EV71 virus stocks of infected cells were measured by TCID50 assay to determine *HSC*'s antiviral potency. As shown in Fig. 1B, the viral titers of EV71 group (EV71+, *HSC*-) and *HSC* treatment group (EV71+, *HSC*+) are $10^{5.2}$ PFU/mL and $10^{4.5}$ PFU/mL ($p = 0.045$) respectively. The viral titers significantly decreased after treating the cells with 200 μM of *HSC* at 24 hpi. To confirm the antiviral effect of *HSC*, we detected a key structural protein of EV71 called VP-1 and another protein 3C protease (3Cpro) which cleaves the EV71 precursor polyprotein into individual proteins [10] by using western blot assays. Consistently, the levels of intracellular VP1 and 3Cpro protein were significantly inhibited by *HSC* (Fig. 1C). Each experiment was performed at least three times. Data statistics was performed with Mantel-Cox test employing Prims software (GraphPad Software, La Jolla, CA, USA) and the density ratio were shown as means ± SD in suplementary (Fig. S2). A two-tailed Student's *t*-test was used to evaluate the data. *p*-Value < 0.05 ($^{*}p < 0.05$) was considered statistically significant.

To investigate the potential mechanisms of *HSC* against EV71 infection, we employed a time-of-addition assay. The administration of 200 μM *HSC* in the infected RD cells followed different protocols respectively (Fig. 2A). The results of cell viability showed that *HSC* pretreatment group (protocol 1) showed the best antiviral effects, while the treatments simultaneously with viral inoculation (protocol 2) or after infection (protocol 3) displayed weaker and weaker antiviral effects (Fig. 2B). Even though there is no significant deference in the cell viability between protocol 1 and protocol 2, the cell viability is higher in *HSC* pretreatment group (protocol 1). These results indicated that *HSC* stimulating cellular defences to inhibit EV71 in RD cells effectively rather than suppressing EV71 production directly or antagonizing viral absorption [11]. Next, we investigated whether the potential antiviral mechanisms of *HSC* against EV71 infection could activate host innate immune system or not. The innate immune effectors inducted by *HSC* were

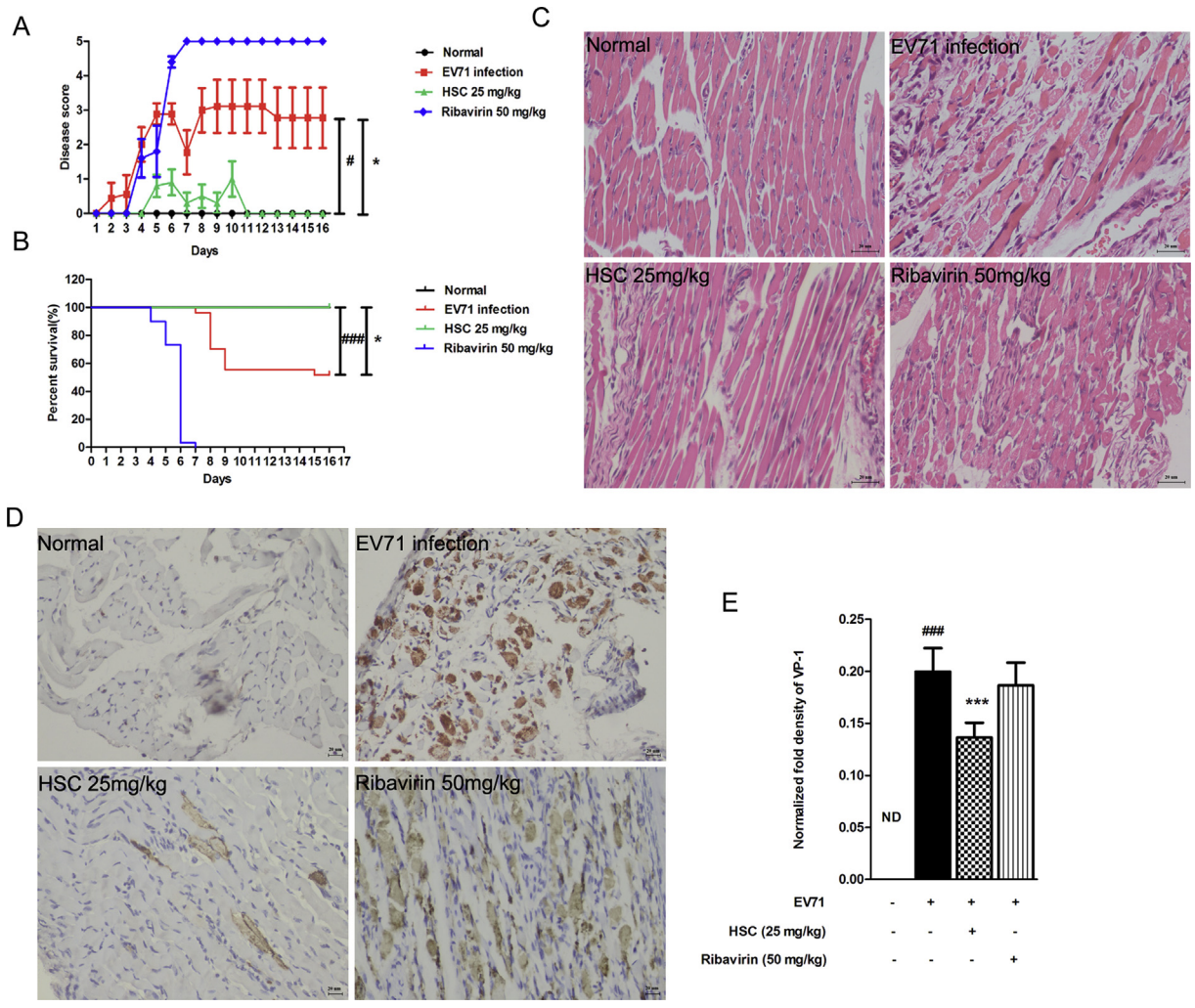

Fig. 3. ***HSC*** **treatment improves survival and inhibits viral propagation in EV71 infected suckling mice model. A and B.** Survival curve (**A**) and clinical scores (**B**) of 2-day-old ICR mice inoculated i.p. with EV71 and treated with saline (n = 10), HSC (25 mg/kg, n = 10) and Ribavirin (50 mg/kg, n = 10). Deaths were calculated into an average score only once at the first observed date. The scoring for ribavirin group was halted after 100% of the mouse died. $^{\#\#}p < 0.01$ (compared to the value of saline control group), $^{*}p < 0.05$ (compared to (EV71+,HSC-) group), $^{***}p < 0.01$ (compared to (EV71+,HSC-) group). **C and D.** Skeletal muscle samples were collected on day 5 post infection and subjected to H&E staining **(C)** and immunohistochemistry **(D)** as described in the material and method section. **E.** The graph results of analysis of immunohistochemistry. Data are presented as mean ± SD (n = 3). $^{\#\#\#}p < 0.001$, $^{***}p < 0.001$.

validated by detecting the protein levels. *HSC* pretreatment dramatically increased the expression of MAVS, p-IRF3, IRF3 and IFN-β (Fig. 2C), when comparing with EV71-infected group at 24 hpi. The density ratio were shown as means ± SD in Suplementary (Fig. S3). These results revealed that MAVS, a vertical adaptor molecule of type I interferon responses, is essential for *HSC* to activate the antiviral innate immunity. MAVS is localized on the outer membrane of mitochondria, and this sub-cellular localization is crucial for its function in antiviral signaling [12]. Therefore, the changes to the cellular distribution of the cleavage products occurred after *HSC* administration were examined by confocal microscopy. The results showed that MAVS co-localized with mitotracker immunostaining green, an RFP-containing mitochondrial target construct, in blank cells. EV71 infection dramatically disrupted this co-localization, however, *HSC* restored this phenomenon (Fig. 2D).

To determine the efficacy of *HSC in vivo*, we employed the EV71 infected suckling mice model which is a common model for studying HFMD [13]. In this model, the observable symptoms appeared on 5th day post infection without drug intervention, characterizing mostly weakness in the hind limbs. Furthermore, the symptoms deteriorated into hind limb or front limb paralysis and reached a peak at 6th–10th days post infection. The muscle tissue is usually taken on the 5th day because the virus multiply in the body at this time and do not cause the death of the suckling mice. *HSC* significantly alleviated above symptoms, as clinical score was markedly lower than that in EV71 group (Fig. 3A, $p < 0.01$ Wilcoxon rank test). As for the mortality of attacked mice, EV71 group yielded a 55.6% survival at the end of the observation while *HSC* group (25 mg/kg) almost showed complete protection (100% survival, Fig. 3B) ($p < 0.01$, Mantel-Cox test). Consistent with previous study, no significant improvement of mortality (0% survival) was observed in positive Ribavirin group (50 mg/kg). Besides, ribavirin treated mice exhibited obvious morbidity at day 4–5 post infection earlier than EV71 group or *HSC* group (Fig. 3B). Histologically, massive myofibril fracture, myocyte disruption and necrotizing myositis with inflammatory infiltrates was observed in the limb muscles from EV71 group. However, the administration of *HSC* (25 mg/kg) significantly improved the integrity of limb muscle structure (Fig. 3C). Immunohistochemistry staining of VP1 was employed to evaluate the extent of viral inhibition caused by *HSC*. Skeletal muscle tissues exhibited the positive staining, as intensive and widespread signal was detected, indicating vigorous virus replication in EV71 group. Shown as Fig. 3D, VP1 expression was largely suppressed by *HSC* in the muscle.

General speaking, the host recognizes viral invasion and activates the innate immune system [14] through the recognition of germline-encoded pattern-recognition receptors (PRRs), resulting in the expression of type I IFNs and proinflammatory cytokines [8]. More recent studies revealed that the interaction of EV71 and MAVS inhibits signal transduction of anti-viral innate immunity to evade the ensuing immune response. As a result, the reduction of full-length MAVS and increase of the cleavage products appeared in the EV71-infected cells. Our results showed that *HSC* treatment could reverse EV71-induced reduction of full-length MAVS. The sub-cellular localization of MAVS on the mitochondria outer membrane is crucial for its function in antiviral signaling. Consistent with previous studies, EV71 infection dramatically disrupted the co-localization between mitochondria and MAVS, however, *HSC* restored this co-localization. Accumulated evidence shows that EV71 infections can restrain the indication of Type I-IFN which is critical in mediating innate immunity defense [15]. In the present study, our results revealed that *HSC* enhanced the induction of IFN-β in EV71-infected cells showing a high-priority target for drug development. To our knowledge, this is the first report on anti-EV71 activities and mechanisms of *HSC* with direct activation of type I IFN response. These results suggested that activation of host immune responses may be the underlying antiviral mechanism of *HSC*. The effect of *HSC* on anti-EV71 activity was assessed using suckling mice infection model. Excitingly, administration of *HSC* strongly suppressed EV71 replication and resulted in improved muscle histology and limb activity.

Taken together, our data suggest that *HSC* is a valuable new candidate for treating HFMD patients and might exert its effect through activation of innate immunity system. Further pharmacological researches are required to understand the relationship between *HSC* 's antiviral abilitise and precise innate immune effector.

Conflicts of interest

All authors of this paper confirm that they have no conflicts of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jiac.2019.07.010.

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