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Fructus Amomi Cardamomi Extract Inhibits Coxsackievirus-B3 Induced Myocarditis in a Murine Myocarditis Model

Yun-Gyeong Lee[1], Jung-Ho Park[2], Eun-Seok Jeon[3], Jin-Hee Kim[4*], and Byung-Kwan Lim[1*]

[1]*Department of Biomedical Science, Jungwon University, Goesan 28024, Republic of Korea*
[2]*Bio-Evaluation Center, Korea Research Institute of Bioscience and Biotechnology, Cheonju 28116, Republic of Korea*
[3]*Cardiac and Vascular Center, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 06351, Republic of Korea*
[4]*College of Herbal Bio-Industry, Daegu Haany University, Gyeongsan 38610, Republic of Korea*

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*Corresponding authors
B.-K.L.
Phone: +82-43-830-8605;
Fax: +82-43-830-8579;
E-mail: bklim@jwu.ac.kr
J.-H.K.
Phone: +82-53-819-1588;
Fax: +82-53-819-1258;
E-mail: jinheekim@dhu.ac.kr

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Coxsackievirus B3 (CVB3) is the main cause of acute myocarditis and dilated cardiomyopathy. Plant extracts are considered as useful materials to develop new antiviral drugs. We had previously selected candidate plant extracts, which showed anti-inflammatory effects. We examined the antiviral effects by using a HeLa cell survival assay. Among these extracts, we chose the *Amomi Cardamomi* (*Amomi*) extract, which showed strong antiviral effect and preserved cell survival in CVB3 infection. We investigated the mechanisms underlying the ability of *Amomi* extract to inhibit CVB3 infection and replication. HeLa cells were infected by CVB3 with or without *Amomi* extract. Erk and Akt activities, and their correlation with virus replication were observed. Live virus titers in cell supernatants and viral positive- and negative-strand RNA amplification were measured. *Amomi* extract significantly increased HeLa cell survival in different concentrations (100–10 μg/ml). CVB3 capsid protein VP1 expression (76%) and viral protease 2A-induced eIF4G1 cleavage (70%) were significantly decreased in *Amomi* extract (100 μg/ml) treated cells. The levels of positive- (20%) and negative-strand (80%) RNA were dramatically decreased compared with the control, as revealed by reverse transcription–PCR. In addition, *Amomi* extract improved mice survival (51% vs 26%) and dramatically reduced heart inflammation in a CVB3-induced myocarditis mouse model. These results suggested that *Amomi* extract significantly inhibited *Enterovirus* replication and myocarditis damage. *Amomi* may be developed as a therapeutic drug for *Enterovirus*.

Keywords: Coxsackievirus B3, myocarditis, plant extract, antiviral effect, *Enterovirus*

Introduction

Coxsackievirus B3 (CVB3) is a positive-stranded RNA virus in the *Enterovirus* genus [28]. CVB3 is the causative pathogen of viral myocarditis and pancreatitis in humans [23]. *Enterovirus* infections are non-symptomatic, but acute myocarditis caused by CVB3 infection can lead to heart failure and progression to dilated cardiomyopathy [5, 6, 9, 10, 20, 26]. However, the details of the mechanism are not fully understood [25]. Both virus-induced damage to the heart and the host immune response play a role in the pathogenesis of CVB3-related myocarditis [7]. *Enterovirus* proteases 2A and 3C are needed for the processing of viral polyprotein, and these enzymes cleave multiple host proteins, which is essential for cell proliferation and survival [7, 25]. Cardiac-specific expression of protease 2A induces dystrophin disruption and development of dilated cardiomyopathy [28]. These results demonstrated that inhibition of protease 2A activity is an effective way to treat CVB3 infection.

Fructus Amomi Cardamomi (Amomi) is the mature fruit of *Amomum villosum* Lour of the family Zingiberaceae. *Amomi* has been reported to suppress mast cell activity and attenuate TNF-α and IL-6 cytokine expression [3, 29].

Amomi strengthens the spleen, and stops vomiting and diarrhea; the latter effect is due to deficiency cold of spleen and stomach. Chrysin (5,7-dihydroxyflavone) is a natural compound, and it showed strong biological activity [11, 21, 22]. Recently, it has been reported that chrysin and its 7-diisopropyl phosphate analog showed a significant anti-enterovirus 71 effect through suppression of the activity of viral 3C protease. Chrysin also shows antiviral activity against CVB3 [22, 24]. In our previous study, we reported the antiviral effect of ORI2 against CVB3 [14]. Plant extracts would be a very powerful agent to inhibit coxsackievirus infection. To develop new antiviral drugs for enteroviruses, we evaluated the ability of plant extracts to regulate cell signaling activity and inhibit virus replication [4, 15].

In this study, we screened a number of plant extracts and selected *Amomi* extract as a candidate for a strong anti-CVB3 agent. This extract inhibited CVB3 replication, having a strong antiviral effect in HeLa cells. We also tested the effects of *Amomi* extract on protease 2A-mediated mouse dystrophin cleavage, Erk and Akt activities, and viral RNA amplification. It significantly improved mice survival from CVB3 infection. These results suggested that *Amomi* extract is a great candidate agent to develop as an anti-enterovirus drug.

Materials and Methods

Viruses

Coxsackievirus B3 was amplified from infectious cDNA of CVB3-H3. The amplified CVB3 titer was determined by the plaque-forming assay in HeLa cells as described previously [13]. CVB3 was maintained and stored at −80°C.

Plant Extracts

We selected candidate plants that had previously been shown to have anti-inflammatory effect. Plant extract was purified from these candidate plants. In brief, dried whole plants were percolated with 95% EtOH for 2 weeks. The extracts were obtained by decompression concentration. Dried plant extract powder was applied to antiviral screening after being resolved by DMSO [14].

In Vitro Screening

HeLa cells were incubated with plant extracts for 30 min and then CVB3 at a multiplicity of infection of 5 was added into well plates. After 18 h incubation, cell survival was measured by a microplate reader at 450 nm wavelength for 2 h after CCK-8 (Dojindo Laboratories, Japan) reagent treatment [30].

Amomi Extract Treatment with CVB3 Infection

HeLa cells were cultured in a 12-well plate (4×10^5 cells/well) at 1 day before the experiment. Then, CVB3 10^4 PFU/ml was added into each well of the plate for 30 min. The plant extract was serially diluted in DMEM (5% FBS, 1% Penicillin) (100 to 1 μg/ml) and then treated, without treated sample used as a virus infection positive control (CON). After 30 h of incubation, we extracted whole proteins and total RNA to perform western blot analysis and RT-PCR.

Western Blot Analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 0.1% SDS, 1% NP40, 150 mM NaCl, 0.5% sodium deoxycholate). Cell extracts were loaded onto 10% SDS-PAGE gels and electrophoresed for 3 h. Protein in the running gel were transferred to PVDF membranes (Amersham Biosciences, USA). After blocking, the membrane was probed with primary antibodies (enterovirus VP1, phosphor-Akt (Ser473), total Akt, phosphor-Erk1/2, total Erk, and GAPDH) for overnight at 4°C and then detected by the Chemi-doc system (Bio-Rad, USA) after ECL solution treatment [17, 18].

Viral RNA Detection

We quantified the viral RNA (positive or negative strand VP1) by RT-PCR. Virus-infected HeLa cell total RNA was extracted by TRIzol reagent (ThermoFisher, USA). Total RNA concentration was measured, and then 1 μg of RNA was used for cDNA synthesis by antisense VP1 primer. The primer sequences were as follows: glyceraldehyde phosphate dehydrogenase (GAPDH): Forward, 5′-GCC AAG GAT ATC CAT GAC AAC T-3′ and Reverse, 5′-CTG CCT GGT CCA GCC ACA GA-3′; VP1: Forward, 5′-CAC TGG GAT TCG TAG ATG TT-3′ and Reverse, 5′-GTC AGC ATG CGT GTA CTT TA-3′. The PCR product was confirmed by DNA electrophoresis using a 2% DNA agarose gel. The gel image was semi-quantified by NIH Imagej software (National Institutes of Health, USA) [30].

Murine CVB3 Myocarditis Mouse Model

The animal experimental protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Samsung Biomedical Research Institute. Five-week-old male Balb/c mice were infected by 2×10^3 plaque-forming units (PFUs) of CVB3 by intraperitoneal injection. Mouse survival was recorded upon 20 days post-infection the termination of experiment [12, 27]. *Amomi* extract (0.5 mg/kg/day) was peritoneal injected for 4 consecutive days from virus infection (CVB3+Amomi group, $n = 20$; CVB3 only, $n = 20$). Tissues (heart and pancreas) were collected at day 14 post CVB3 infection for histologic findings. Heart inflammation level was observed by H&E staining. Animal survival rate was observed upon 20 days, the end of the experiment.

Statistics

The data are presented as the mean ± SEM. All measured data were examined using the Student *t*-test by Prism 4.0 (GraphPad Software, USA). Mice survival was represented by the Kaplan–

Meier survival curve and analysis method. A value of $p < 0.05$ was considered significant [16].

Results

In Vitro Screening of Plant Extracts

We screened various plant extracts by using cell survival screening methods. The survival of CVB3-infected HeLa cells was evaluated by color change after 2 h CCK-8 treatment. We have selected candidate extracts that showed strong antiviral effects. *Amomi* extract showed antiviral effect compared with other plant extracts. In addition, *Amomi* extract showed no cytotoxicity and improved cell survival from CVB3 infection in different doses (100–0.0001 μg/ml; Fig. 1).

Amomi Extract Inhibits Early CVB3 Replication

We investigated the ability of *Amomi* extract to inhibit CVB3 replication. When an enterovirus replicates, it produces its own proteases to cleave the CVB3 polyprotein. However, viral proteases also cleave the host protein eukaryotic translation initiation factor 4 gamma 1 (eIF4G1). This cleavage was observed at an early time after virus infection, during initial replication. *Amomi* extract (100–10 μg/ml) was treated after CVB3 infection. As shown by western blot analysis, eIF4G1 cleavage was significantly lower in *Amomi* extract-treated cells than in the untreated one (upper panel). In addition, the level of virus capsid protein VP1 was dramatically reduced (Fig. 2).

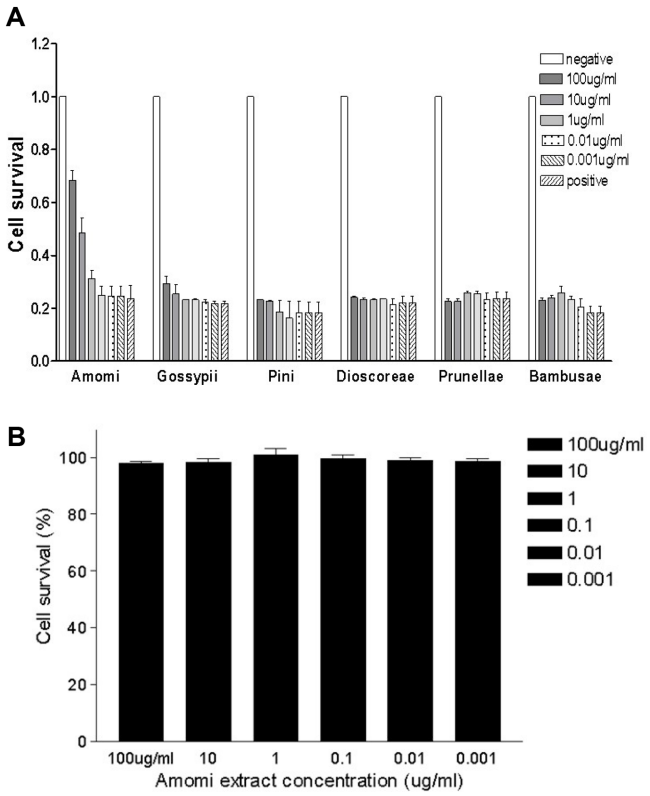

Fig. 1. Anti-enterovirus compounds screened.
(**A**) The antiviral effect of each compound was tested by cell survival assay after CVB3 infection. *Amomi* extract treatment improved HeLa cell survival compared with other plant extracts. Negative: without virus; Positive: virus only. (**B**) Cytotoxicity assay of *Amomi* extract. Cytotoxicity was confirmed by CCK-8 cell survival test.

Amomi Extract Decreases Live Virus Production

We measured live virus production using a plaque-forming assay. The CVB3 titer was measured in supernatants of *Amomi* extract–treated samples. The virus titer was significantly less with *Amomi* extract (100 μg/ml) than in untreated (Fig. 3A). CVB3 replication was directly examined by an immunofluorescence assay with anti-VP1 antibody.

Amomi Extract Inhibits CVB3 Gene Amplification

Virus replication is directly followed by viral gene amplification. Negative-strand RNA is the main regulator of CVB3 replication and an important indicator of virus

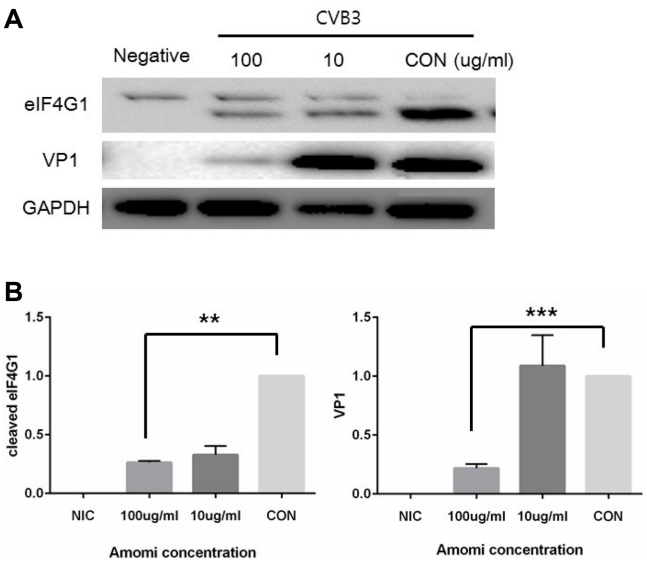

Fig. 2. *Amomi* extract inhibits coxsackievirus B3 (CVB3) replication through protease 2A inhibition.
(**A**) *Amomi* extract was added to HeLa cells following CVB3 infection. Virus protease 2A-induced eIF4G1 cleavage and capsid protein VP1 expression were dramatically reduced by *Amomi* extract in a dose-dependent manner (100–10 μg/ml). (**B**) Western blot results were quantitated by NIH image software and are presented as the mean ± SEM. **, $p < 0.01$; ***, $p < 0.001$.

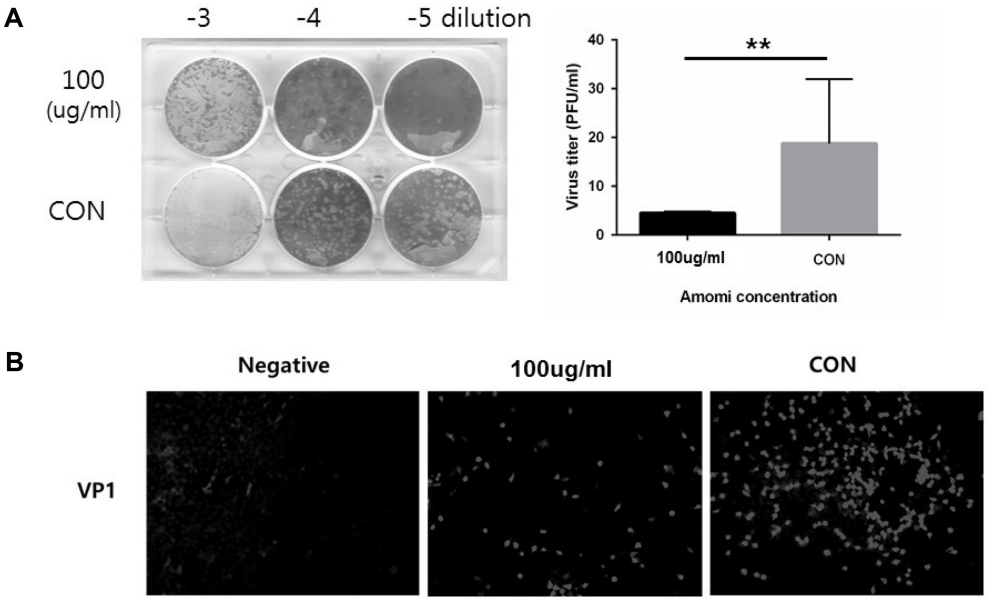

Fig. 3. *Amomi* extract inhibits CVB3 live virus production.
(**A**) CVB3 live virus production was evaluated by cell supernatant plaque forming unit (PFU) assay. *Amomi* extract (100 μg/ml) significantly decreased virus production compared with the untreated sample (CON). (**B**) In immunofluorescent assay, virus-infected cells were dramatically reduced by *Amomi* extract treatment. CVB3 VP1 capsid protein was labeled by red color fluorescence. **, $p < 0.01$.

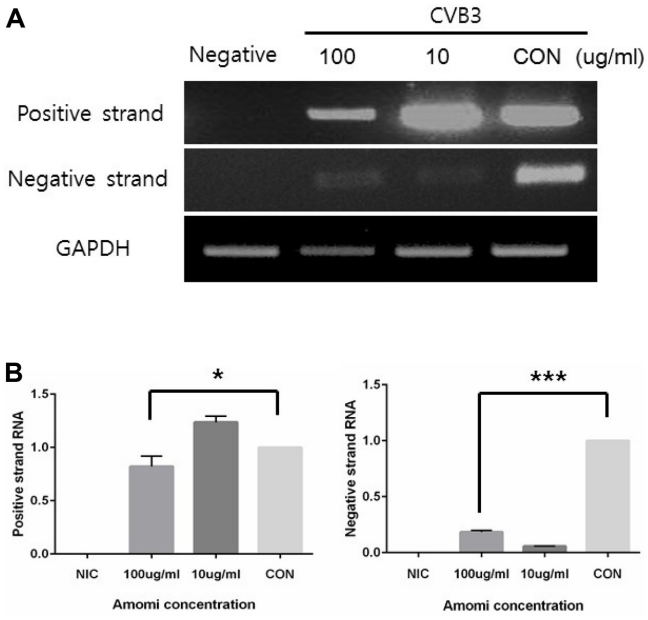

Fig. 4. *Amomi* extract inhibits CVB3 gene amplification.
Virus gene amplification is essential for virus replication. (**A**) CVB3 positive and negative gene numbers were quantified by RT-RCR. Positive-strand RNA was significantly reduced by *Amomi* extract treatment (100 μg/ml) compared with that without treatment (CON). Negative-strand RNA amplification was almost completely blocked. (**B**) All data were quantified by NIH image software and are presented as the mean ± SEM. *, $p < 0.05$; ***, $p < 0.001$.

replication. To observe virus replication directly, viral VP1 genes were quantified by semi-quantitative reverse transcription–PCR (RT-PCR). *Amomi* extract significantly inhibited positive- and negative-strand RNA amplification. These results suggest that *Amomi* extract is affective to inhibit early CVB3 replication (Fig. 4).

Amomi Extract Inhibits Protease 2A and Akt Signaling in HeLa Cells

Enterovirus produces the protein cleavage enzyme protease 2A, which is required for viral protein production. To evaluate the mechanism of the inhibitory effect of *Amomi* extract on CVB3 protease 2A activity. We overexpressed protein Flag-tagged partial mouse dystrophin (Dys-T), which contains the protease 2A cleavage site. Then, CVB3 protease 2A activity was examined upon CVB3 infection by western blot analysis using anti-Flag antibody. *Amomi* extract inhibited Dys-T cleavage by inactivation of CVB3 protease 2A activity (Fig. 5A). CVB3 replication is regulated by host cell signaling molecules such as Erk and Akt activity at early stages of infection [4, 15]. We found that Erk activity was not changed, but Akt activity was significantly decreased by *Amomi* extract treatment (Fig. 5B).

Amomi Extract Improves Mouse Survival in Myocarditis

We tested the in vivo effect of *Amomi* extract in CVB3-

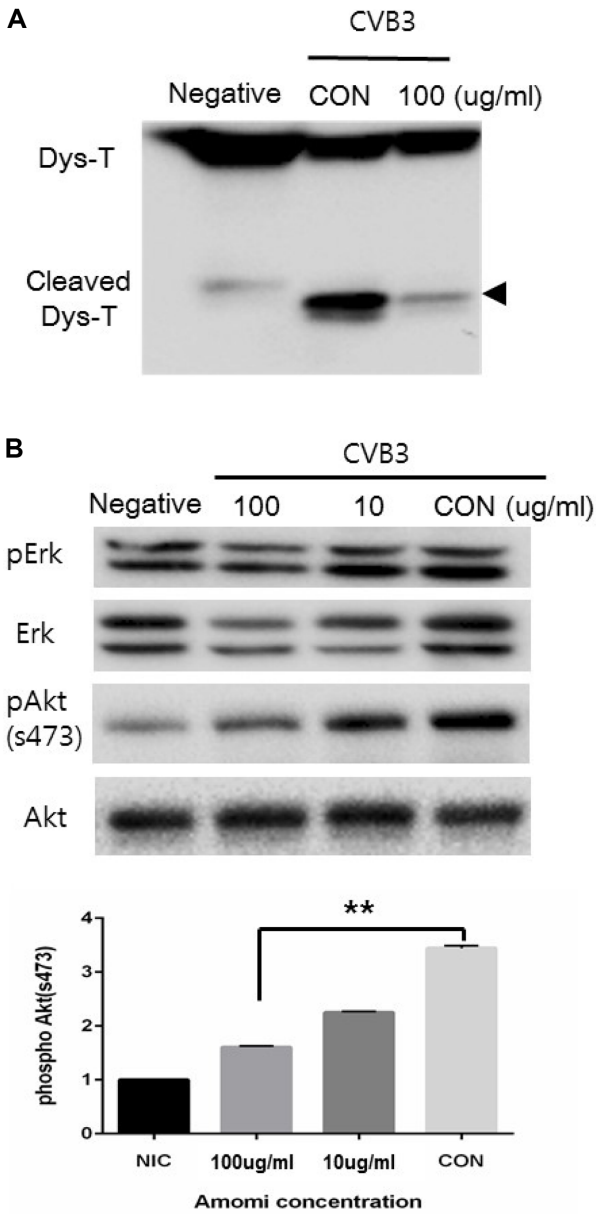

Fig. 5. *Amomi* extract inhibits early replication of CVB3.
Early CVB3 replication was found by protease 2A activity and cell signaling activity regulation. (**A**) CVB3 protease 2A activity was measured by the dystrophin protein (Dys-T) cleavage level. Dystrophin protein cleavage was inhibited by *Amomi* extract. (**B**) Akt signaling activity was significantly decreased, but Erk signaling was not changed. All data were quantified by NIH image software and are presented by the mean ± SEM. **, $p < 0.01$.

infected mice. Intraperitoneal injection of *Amomi* extract for 4 consecutive day, followed by CVB3 infection, decreased mouse mortality and heart inflammation. The survival rate was increased 185% by *Amomi* extract treatment compared with the untreated control group (50% vs. 27%) (Fig. 6A).

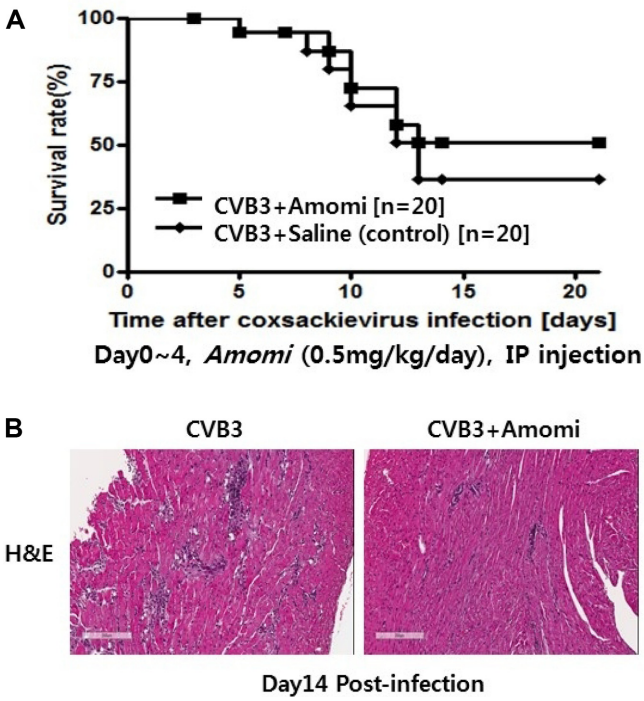

Fig. 6. *Amomi* extract improves mouse survival in a myocarditis model.
(**A**) Animal survival rate was improved 185% in the *Amomi* extract-treated group (CVB3+*Amomi)* compared with the saline-treated control group (50% vs. 27%). (**B**) H&E staining showed inflammation in the mice heart. Inflammatory cell infiltration was decreased in the *Amomi* extract-treated group (CVB3+*Amomi)*.

H&E staining indicated that *Amomi* extract treatment had reduced the myocardium damage and inflammatory cell infiltration at day 14 in CVB3-infected mice heart (Fig. 6B).

Discussion

We found a new antiviral candidate reagent of plant origin that is effective against coxsackievirus B3. Enteroviruses are well known as a common cause of various human infectious diseases [2]. In particular, enterovirus 71 belongs to the same *Enterovirus* genus and is the main causative agent of hand, foot, and mouth disease [24]. Acute CVB3 infection may induce immune responses and lead to direct myocyte damage and cardiomyopathy. [1, 19]. In previous research, to confirm in vivo antiviral reagent against CVB3, a Balb/c viral myocarditis mouse model was used [8, 12, 13]. Moreover, cardiac-specific enterovirus protease 2A expression leads to the development of severe myocarditis [28]. The results suggested that inhibition of early-stage virus replication and protease 2A activity are important to

treat CVB3 infections. Our previous report showed that ORI2 significantly prevented CVB3-induced myocarditis through attenuation of early virus replication [14].

We obtained several candidate plant extracts and evaluated their antiviral activity. We chose *Fructus Amomi Cardamomi (Amomi)* for further testing. *Amomi* extract was tested in CVB3-infected HeLa cells. We found that CVB3 replication and Akt activity were strongly inhibited by *Amomi* extract treatment. *Amomi* extract significantly decreased eIF4G1 cleavage and VP1 expression. *Amomi* also inhibited viral RNA replication and decreased the live virus titer in a high dose. In the presence of *Amomi* extract, there was no cleavage of truncated dystrophin because of inhibition of viral protease 2A. In a CVB3-induced myocarditis mouse model, *Amomi* extract decreased myocarditis and significantly improved the mouse survival rate. These results suggest that *Amomi* extract is able to be developed as a new treatment agent of *Enterovirus* infections.

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