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ORIGINAL ARTICLE

# Yakammaoto inhibits enterovirus 71 infection by reducing viral attachment, internalization, replication, and translation

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**Abstract** Enterovirus 71 (EV71) can cause central nervous system infections with mortality and neurologic sequelae. At present, there is no effective therapeutic modality for EV71 infection. The infection is more common in families with poor socioeconomic status. Therefore, finding a readily available, cost-effective therapeutic modality would be very helpful to these socioeconomically disadvantaged families. Yakammaoto is a cheap and readily available traditional prescription that is proven to have antiviral activity against coxsackievirus B4 (CVB4). CVB4 and EV71 are enteroviruses. In this study, we evaluated the antiviral activity of hot water extract of yakammaoto against EV71. The results of plaque reduction assay and flow cytometry demonstrated that yakammaoto dose dependently inhibited EV71 infection. In addition, reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR results

Conflicts of interest: All authors declare no conflicts of interest.

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showed that yakammaoto reduced viral replication. Western blotting analysis showed that yakammaoto can inhibit viral protein production. Thus, our results suggest that yakammaoto should be considered to manage EV71 infection in the future.


## Introduction

Enterovirus infection is common worldwide. It can cause human diseases with various severity levels. The most severe form of the disease involves infections of the central nervous system (CNS) with mortality and neurologic sequelae, such as flaccid paralysis [1]. Among the various neurotropic enteroviruses, enterovirus 71 (EV71) is perhaps the most catastrophic virus next to poliovirus. After the effective control of poliovirus by vaccination, the importance of controlling EV71 is on the rise. EV71 usually causes symptoms mimicking common airway infections and hand, foot, and mouth disease (HFMD) [2]. However, EV71 can cause brain stem encephalitis, neurogenic shock, and neurogenic pulmonary edema [3,4]. A large outbreak of EV71 occurred in Taiwan in 1998, with reports indicating mortality in 78 cases [3]. Most of the victims were <5 years of age. They all died of CNS infections and cardiopulmonary failure [3]. In addition to this outbreak in Taiwan, a large EV71 outbreak was reported in China [5] and fatal cases were also reported in Asia as recently as 2012 [6,7]. Furthermore, survivors of CNS EV71 infection were found to have neurological sequelae and delayed neurodevelopment [8]. Therefore, effective management of this infection could improve the quality of life of many children. At present, there is no effective therapy available for treating this infection [9]. However, several therapeutic agents are under development [10]. EV71 is transmitted from person-to-person mainly by the fecal—oral route [11]. EV71 is far more easily transmitted in the developing countries because of crowded environments and poor public hygiene. These countries may not have adequate budgets to counteract this disease during an overwhelming outbreak. A cost-effective, readily available, effective therapeutic modality is thus an urgent need.

Yakammaoto (as termed in Japan; Ye-Gan-Ma-Huang-Tang, YGMHT, in Taiwan; and She-Gan-Ma-Huang-Tang or Sheganmahuang Decoction in Mainland China) [12,13] is an inexpensive, readily available, traditional prescription, with reports indicating its use for the management of airway symptoms since the period of the Eastern Han Dynasty of China (25—220 AD). Data of the fingerprint of yakammaoto has been presented in a previous study [12]. Among its nine ingredients, *Ephedra sinica* is helpful against poliovirus I *in vitro* [14] and *Zingiber officinale* has antiviral activity against rhinovirus [15]. EV71, poliovirus, and rhinovirus are all members of the family *Picornaviridae*. Therefore, yakammaoto is expected to show antiviral activities against the members of the family *Picornaviridae*. Yakammaoto has already been proven to be effective against coxsackievirus B4 (CVB4) [12]. Both CVB4 and EV71 are enteroviruses. Therefore, we hypothesized that yakammaoto might be effective against EV71 as well. We used both the human foreskin fibroblast cell line (CCFS-1/KMC) and human rhabdomyosarcoma cell line (RD cells) to test this hypothesis.

## Materials and methods

### Preparation of a hot water extract of yakammaoto

All air-dried ingredients (medicinal plant materials) of yakammaoto were purchased from various herb shops in Southern Taiwan. The authenticity of these materials were confirmed as described previously [12]. A hot water extract of yakammaoto was prepared as follows [12]: 100 g of air-dried ingredients of yakammaoto were decocted for 1 hour with 1000 mL of distilled water repeatedly three times. The decoctions were collected, mixed, filtered through gauze, concentrated under reduced pressure, and lyophilized to dryness. The weight/weight yield of yakammaoto was 17.5%. The extract of yakammaoto was dissolved in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Grand Island, NY, USA) supplemented with 2% or 10% fetal calf serum (FCS) to the final concentrations of 3 μg/mL, 10 μg/mL, 30 μg/mL, 100 μg/mL, and 300 μg/mL for evaluating its bioactivity and up to 3000 μg/mL for evaluating its cytotoxicity prior to the experiments.

### Human cell lines and EV71

Human foreskin fibroblast cell line (CCFS-1/KMC) [16] and rhabdomyosarcoma cell line (RD cell; American Type Culture Collection, ATCC CCL-136, Manassas, VA, USA) were used to culture EV71 (BrCr strain; ATCC VR-784). Human liver hepatocellular carcinoma cell lines, HepG2 (ATCC HB-8065) and Huh7 [17], human larynx epidermoid carcinoma cell line (HEp-2; ATCC CCL-23), lung carcinoma cell line (A549; ATCC CCL-185), and an immortalized proximal tubule epithelial cell line from adult human normal kidney (HK-2; ATCC CRL-2190) were used to test the cytotoxicity of the extract prepared. The reagents and medium used for cell culture were purchased from GIBCO BRL. Cells were propagated at 37°C under 5% $CO_2$ in DMEM supplemented with 10% FCS and antibiotics. The virus was propagated on 90% confluent cell monolayer in DMEM with 2% FCS and antibiotics. The viral titer was determined and expressed as plaque forming units (pfu)/mL. The virus was stored at −80°C until use.

### Cytotoxicity assay

The cytotoxic effects of yakammaoto on proliferating cells were assayed using XTT kit (Roche, Mannheim, Germany) according to the manufacturer's instructions [18]. The 50%

cytotoxic concentration ($CC_{50}$) of yakammaoto was calculated by regression analysis of the dose–response curve generated from the data.

## Evaluation of antiviral effect of yakammaoto in CCFS-1/KMC cells by plaque reduction assay

The antiviral activity of yakammaoto was examined by plaque reduction assay [18] using CCFS-1/KMC cells. In brief, $10^5$ cells/well were plated in 12-well culture plates at 37°C under 5% $CO_2$ for 24 hours and inoculated with a mixture of 200 pfu/well viruses and various concentrations of the yakammaoto extract in triplicate at room temperature for 1 hour. Then, the overlay medium (DMEM + 2% FCS in 1% methylcellulose) was added. The cells were cultured for 3 more days. The monolayer was then fixed with 10% formalin, stained with 1% crystal violet, and the plaques formed were counted. Ribavirin (Sigma-Aldrich, St. Louis, MO, USA) was used as the positive control. The minimal concentration required to inhibit 50% cytopathic effect ($IC_{50}$) of yakammaoto was calculated using regression analysis of the dose–response curve generated from the data.

## Evaluation of the antiviral effect of yakammaoto in RD cells by flow cytometry

RD cells ($1 \times 10^6$/well) were plated overnight, inoculated with a mixture of viruses [multiplicity of infection (MOI) = 0.002] and various concentrations of yakammaoto in triplicate, and cultured for 48 hours. Then, the cells were washed, detached by trypsin to a single-cell suspension, fixed with 4% paraformaldehyde, and permeabilized using −20°C methanol [19]. Viral particles were detected by a 1:200-dilution of commercial monoclonal antibody against viral VP0 and VP2 (MAB979; Chemicon, Temecula, CA, USA) at 4°C overnight, followed by a 1:50-dilution of goat antimouse immunoglobulin G-FITC (Jackson ImmunoResearch, West Grove, PA, USA) at 37°C for 1 hour in the dark. The infection rate was assessed as a function of fluorescein isothiocyanate expression using flow cytometry (BD FACSArray; BD Bioscience, San Diego, CA, USA). $IC_{50}$ was calculated as previously described.

## Time-of-addition assay

The antiviral activity of yakammaoto was examined before and after viral inoculation [18]. In brief, cells were seeded and incubated for 24 hours as previously described. Yakammaoto extract of various concentrations was supplemented at 1 hour or 2 hours before or after viral inoculation. The supernatant was removed before the overlay medium was added. The infected cultures were incubated for 72 hours. After fixation and staining, plaques were counted.

## Evaluation of attachment and internalization of the virus by plaque counting

Yakammaoto was evaluated for its effect on viral attachment [18]. In brief, cells were seeded and incubated for 24 hours. The cells were prechilled at 4°C for 1 hour and the medium was replaced with a mixture of 200 pfu/well virus and various concentrations of yakammaoto extract. After incubation at 4°C for another 3 hours, the free viruses were removed. The cell monolayer was washed with ice-cold phosphate-buffered saline (PBS) three times, covered with overlay medium, incubated for 72 hours, and then examined by plaque counting.

The effect of yakammaoto on viral internalization was examined at 20-minute intervals following viral attachment [18]. In brief, the cell monolayer was grown in 12-well culture plates and prechilled at 4°C for 1 hour. Cells were infected with EV71 (200 pfu/well) and incubated at 4°C for 3 hours to allow for virus binding without internalization. The virus-containing medium was replaced with fresh medium containing various concentrations of yakammaoto extract and the temperature was changed to 37°C. At 20-minute intervals following the temperature shift to 37°C, acidic PBS (pH 3) was supplemented for 1 minute to inactivate the uninternalized virus, followed by addition of alkaline PBS (pH 11) for neutralization. Then, PBS was replaced by fresh overlay medium. After incubation for 72 hours, the cell monolayer was stained and examined by plaque counting.

## Reverse transcription-polymerase chain reaction and quantitative reverse transcription-polymerase chain reaction

The anti-EV71 activity of yakammaoto was further examined using reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR) after viral inoculation. In brief, $1 \times 10^6$/well RD cells or CCFS-1/KMC cells were plated into 6-well culture plates for 24 hours. Various concentrations of yakammaoto were supplemented with EV71 (MOI = 0.002) simultaneously or 1 hour after viral inoculation. After culture for 48 hours (for samples of cells with virus infection and yakammaoto treatment at the same time) or 36 hours (for samples of cells treated with yakammaoto 1 hour after virus infection), viral RNA was extracted from the cell-culture supernatant or cells using QIAamp Viral RNA Mini Kit (Qiagen, Heidelberg, Germany) or RNeasy Plus Mini Kit (Qiagen), respectively. The complementary DNAs (cDNAs) were synthesized from the eluted RNAs using the SuperScript II RNase H-Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). In brief, 0.5 μg RNA was supplemented into a cDNA synthesis master mixture containing 1 μL reverse transcriptase, 2 μL dithiothreitol (DTT), 1 μL 10mM deoxyribonucleotide triphosphate, and 1 μL 10μM EV71 reverse primer (5′-ATTTCAGCAGCTTGGAGTGC-3′) [20]. The reaction was carried out at 42°C for 60 minutes, followed by treatment at 70°C for 15 minutes. Then, 4 μg of cDNA was supplemented into a reaction tube containing 25 μL of 2× *Taq* Master Mix (Vivantis, Saint Petersburg, Florida, USA), 2.5 μL 10μM EV71 forward primer (5′-GCAGCCCAAAAGAACTTCAC-3′), and the same amount of reverse primer. This mixture was then supplemented with nuclease-free water to make up a total volume of 50 μL. The PCR condition was denaturized at 95°C for 10 minutes then followed by 25 cycles of amplification at 95°C (20 seconds), 45°C (25 seconds), 72°C (30 seconds), and the final extension step at 72°C for 10 minutes. The amplification products were analyzed semiquantitatively using 2% agarose gel electrophoresis. Using EZ-VISION DNA Dye (AMRESCO, Solon, OH, USA) as the loading buffer, the

bands in gel electrophoresis were visible at the expected molecular weight (226 bp; visualized using UVDOC HD2 system, Uvitec Cambridge, Cambridge, UK). In quantitative PCR, 2 μg cDNA was supplemented into a reaction tube containing 10 μL Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and 2.5 μL of each primer. These were further supplemented with nuclease-free water to make a total volume of 20 μL. The quantitative PCR condition was as follows: 95°C for 10 minutes, followed by 25 cycles of amplification at 95°C for 15 seconds and 60°C for 1 minute using the Step One Real-Time PCR System (Applied Biosystems). $2^{delta}$ $C_t$ (threshold cycle) calculated as $C_{ttreated} - C_{tuntreated}$, obtained by measuring the differences in $C_t$ between the experimental group and the UV-inactivated EV71 group.

### Western blotting analysis for detecting viral structure proteins

The efficiency of yakammaoto to inhibit viral protein translation was evaluated using Western blotting [21]. In brief, the CCFS-1/KMC or RD cells were supplemented with a mixture of EV71 (200 pfu/well) and various concentrations of yakammaoto or ribavirin (30 μg/mL). To study the effect of treatment of yakammaoto after infection on viral protein translation, cells were treated with the drug 1 hour after infection. After culture, the total cellular protein was extracted using Roche Complete Lysis Kit (Roche Diagnostics GmbH, Indianapolis, IN, USA) and its concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Then, 50 μg of total protein was mixed with 5× sample buffer and boiled for 5 minutes at 95°C [21]. The mixture was separated on a 12% gel using sodium dodecyl sulfate—polyacrylamide gel electrophoresis (Bio-Rad). The separated proteins were transferred to Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and blocked with 5% nonfat milk in Tris-buffered saline with Tween-20. VP0 and VP2 were detected using a 1:1000 dilution of commercial monoclonal antibody (MAB979; Chemicon), followed by a 1:2000 dilution of goat antimouse immunoglobulin G—horseradish peroxidase (HRP) conjugated antibody (Jackson ImmunoResearch, West Grove, PA, USA). The internal control was β-actin, which was assayed using a 1:5000 dilution of monoclonal antibody (Chemicon) and detected using the same secondary antibody mentioned earlier. Target proteins were visualized using Immobilon Western Chemiluminescent HRP substrate (Millipore, Temecula, MA, USA) and their autoradiographs were obtained using UVP ChemiDoc-It 810 Imaging System (UVP, Upland, CA, USA). The density change of target protein was estimated using densitometry (UVP ChemiDoc-It 810 Imaging System VisionWorksLS 7.1). The percentage of density change was calculated from the data of yakammaoto groups normalized by that of β-actin, then divided by the normalized density of viral control.

### Statistical analysis

Results were expressed as mean ± standard deviation. The percentage of control (infection rate; %) was calculated from the data of yakammaoto groups divided by those of the viral control. The relative viral amount (fold change) of qRT-PCR product was calculated from the data of yakammaoto groups divided by those of the UV-inactivated viral control. Data were analyzed with analysis of variance (ANOVA) using JMP 11 software (SAS, Cary, NC, USA). Tukey honestly significant difference test was used to compare all pairs of groups in the ANOVA test. A $p$ value $< 0.05$ was considered statistically significant.

## Results

### Cytotoxicity assay

Yakammaoto contains 12.5% (w/w) *Flos farfarae* (*Tussilago farfara*) [12]. *Flos farfarae* has been found to contain toxic pyrrolizidine alkaloids, mainly senkirkine and traces of senecionine, which are associated with liver injury [22—24]. Therefore, the hepatotoxicity of yakammaoto was examined using HepG2 (Fig. 1A) and Huh7 cells (Fig. 1B) by XTT assay. Yakammaoto at the dose of 3000 μg/mL showed hepatotoxicity after 3 days of treatment. However, after prolonged culture of >7 days, only 300 μg/mL of yakammaoto might be toxic to HepG2 and Huh7 cells ($p < 0.0001$). In HepG2, the estimated $CC_{50}$ values were 2437.5 μg/mL on Day 3, 278.2 μg/mL on Day 7, 259.8 μg/mL on Day 10, and 237.1 μg/mL on Day 14. Yakammaoto was less toxic to Huh7 cells. The $CC_{50}$ values were >3000 μg/mL on Day 3 and Day 7, 1656.4 μg/mL on Day 10, and 235.3 μg/mL on Day 14.

To determine whether yakammaoto was also toxic to other cells, cytotoxicity assays were performed in CCFS-1/KMC (a human foreskin normal fibroblast cell line), RD (a human rhabdomyosarcoma cell line), HK-2 (a human normal renal tubular cell line), A549 (a human lung carcinoma cell line), and HEp-2 (a human larynx epidermoid carcinoma cell line) cells with XTT. Yakammaoto showed minimal cytotoxicity at concentrations of 1000 μg/mL and 30 μg/mL on CCFS-1/KMC cells and RD cells, respectively ($p < 0.05$; Fig. 1C). RD cells were more sensitive to yakammaoto than CCFS-1/KMC cells ($p = 0.0003$). The estimated $CC_{50}$ values were >3000 μg/mL and 191.5 μg/mL in CCFS-1/KMC and RD cells, respectively. Yakammaoto did not show any cytotoxicity against HK-2, A549, and HEp-2 cells (Fig. 1D) up to the concentration of 3000 μg/mL.

### Antiviral effect assay

To determine the anti-EV71 activity of yakammaoto, we performed plaque reduction assay in CCFS-1/KMC cells and flow cytometry in RD cells as demonstrated earlier. Yakammaoto dose dependently reduced plaque formation (Fig. 2A; $p < 0.0001$) with a calculated $IC_{50}$ of 73.3 μg/mL. The results of flow cytometry indicated that yakammaoto also reduced EV71 infection in RD cells (Fig. 2B; $p < 0.0001$) with a calculated $IC_{50}$ of 16.3 μg/mL. Yakammaoto at the dose of 30 μg/mL could decrease >90% EV71 infection in RD cells.

### Time-of-addition assay

To understand whether yakammaoto showed a better anti-EV71 activity when supplemented before or after EV71

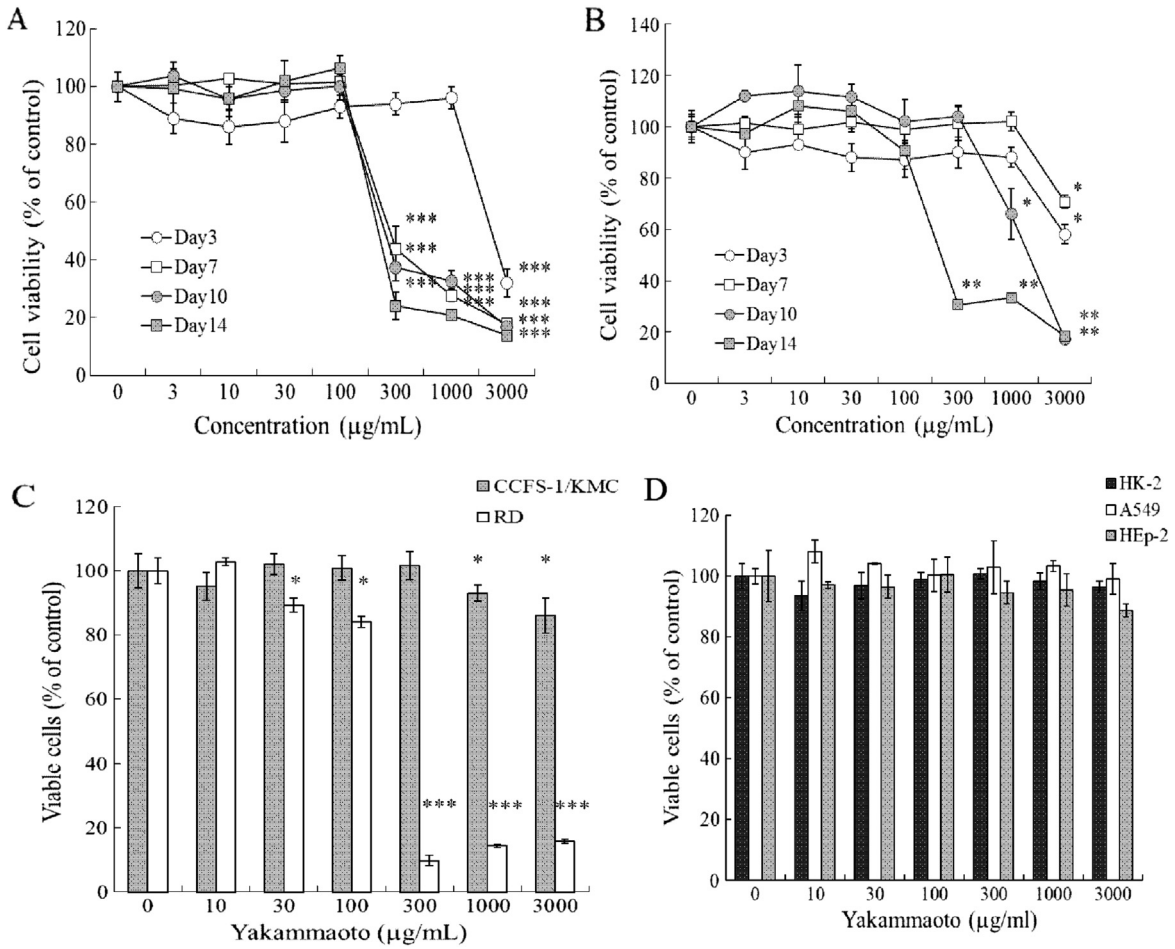


**Figure 1.** Cytotoxicity of yakammaoto: 3000 μg/mL of yakammaoto extract showed a hepatotoxic effect on (A) HepG2 and (B) Huh7 cells; 300 μg/mL yakammaoto might be toxic to HepG2 and Huh7 cells after use for >7 days. However, (C) 300 μg/mL yakammaoto was toxic to RD cells on Day 3. Yakammaoto did not show any cytotoxicity against (D) HK-2, A549 and HEp-2 cells up to the concentration of 3000 μg/mL on Day 3. Data are presented as mean ± standard deviation of six tests. *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$ compared with the negative control.

infection, the time-of-addition assay was performed. Yakammaoto dose dependently (Fig. 3; $p < 0.0001$) and time dependently ($p = 0.027$) inhibited EV71-induced plaque formation in CCFS-1/KMC cells with $IC_{50}$ values of 70.4 μg/mL (2 hours after the infection), 60.6 μg/mL (1 hour after the infection), 88.7 μg/mL (1 hour before the infection), and 79.7 μg/mL (2 hours before the infection). Interestingly, yakammaoto showed its best inhibitory effect when supplemented 1 hour after the EV71 infection.

## Attachment assay and internalization assay

Yakammaoto showed its anti-EV71 activity when given prior to viral inoculation (Fig. 3). Yakammaoto was expected to inhibit viral attachment and/or internalization. To prove these assumptions, an attachment assay and internalization assay were performed. Yakammaoto dose dependently inhibited EV71 attachment on CCFS-1/KMC cells (Fig. 4A; $p < 0.0001$) with a calculated $IC_{50}$ of 95.9 μg/mL. Yakammaoto also dose dependently and time dependently inhibited EV71 internalization (Fig. 4B; $p < 0.0001$). The $IC_{50}$ values of the internalization assay were 112.1 μg/mL (20 minutes), 75.5 μg/mL (40 minutes), and 46.6 μg/mL (60 minutes).

## RT-PCR and qRT-PCR

Yakammaoto showed its anti-EV71 activity when injected after viral inoculation (Fig. 3). Yakammaoto was supposed to work on viral replication and/or protein translation. To prove these assumptions, RT-PCR and qRT-PCR were performed. Yakammaoto dose dependently inhibited the expression of viral RNA in CCFS-1/KMC (Fig. 5A; $p < 0.0001$) and RD cells (Fig. 5B; $p < 0.0001$), both within the cells and in the suspension. EV71 replicated more efficiently in RD cells—100-fold higher in the viral control of the RD cells (Fig. 5B). With less efficient viral replication in CCFS-1/KMC cells, 30 μg/mL yakammaoto could effectively inhibit viral replication (Figs. 5A and 5C). By contrast, it took 100 μg/mL to completely inhibit viral replication within RD cells (Figs. 5B and 5C). Yakammaoto showed similar inhibitory activity on the intracellular EV71 RNA in both CCFS-1/KMC and RD cells as demonstrated by qRT-PCR and RT-PCR assays. However, the results are relatively inconsistent between

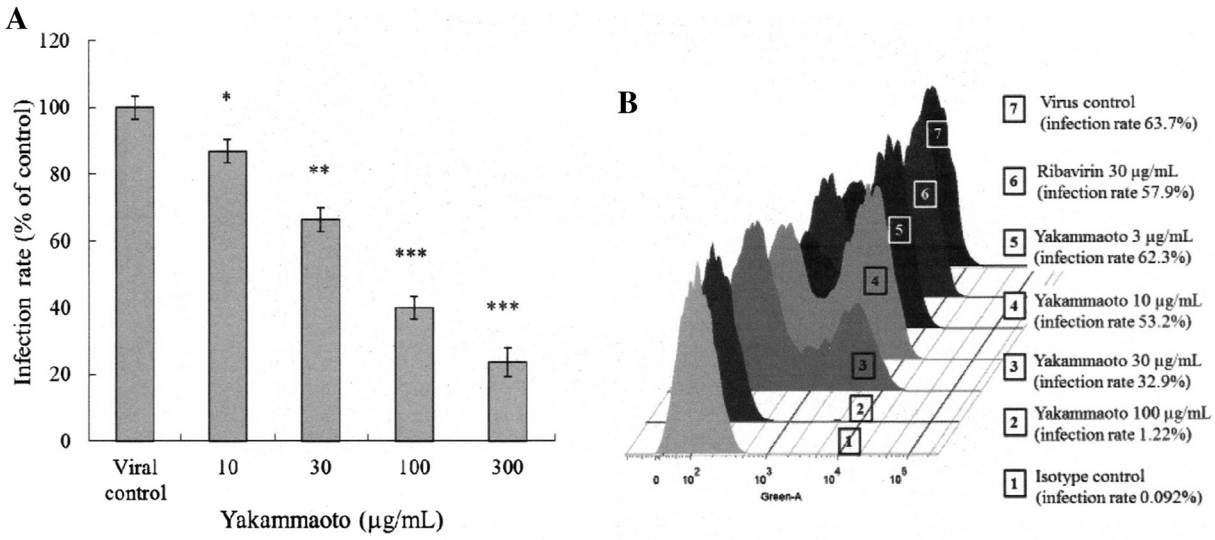


**Figure 2.** Antiviral effect assay. (A) Yakammaoto was dose-dependently effective in reducing EV71-induced plaque formation in CCFS-1/KMC cells. (B) Flow cytometry revealed the anti-EV71 activity of yakammaoto in RD cells. Data are presented as mean ± standard deviation of nine tests in plaque reduction assay. *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$ compared with the viral control. CCFS-1/KMC cells = human foreskin fibroblast cell line; EV71 = enterovirus 71; RD cells = human rhabdomyosarcoma cell line.

qRT-PCR and RT-PCR in the suspension. The results of qRT-PCR assay, but not those of RT-PCR assay, demonstrated that 30 μg/mL, 100 μg/mL, and 300 μg/mL yakammaoto significantly reduced viral amount in the suspension. These discrepancies between qRT-PCR and RT-PCR assays were due to the fact that RT-PCR detects the end product after 25 cycles of amplification, whereas qRT-PCR calculates the threshold cycle. By analyzing the amplification plot of qRT-PCR (data not shown), the threshold cycle can clearly distinguish the viral amounts between groups, whereas the end product was the same at the end of amplification. Therefore, the qRT-PCR results were valid and more convincing, although the results had some discrepancies.

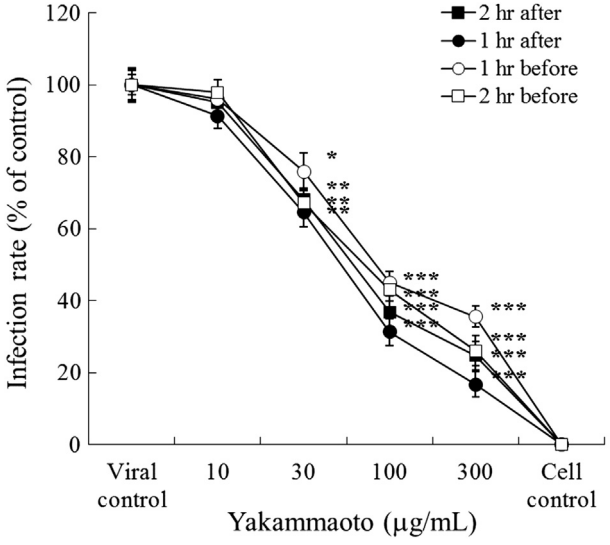


**Figure 3.** Time-of-addition assay. Yakammaoto was dose-dependently and time-dependently effective against EV71 infection in CCFS-1/KMC cells when given prior to or after EV71 inoculation. Data are presented as mean ± standard deviation of nine tests. *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$ compared with the viral control. CCFS-1/KMC cells = human foreskin fibroblast cell line; EV71 = enterovirus 71.

The time-of-addition assay (Fig. 3) results indicated that post-treatment was more effective than pretreatment. This might imply an activity of yakammaoto against viral replication and translation. To confirm that yakammaoto can inhibit viral replication, but not block viral attachment and/or internalization to decrease viral RNA, yakammaoto was supplemented 1 hour after viral inoculation. Yakammaoto effectively inhibited EV71 replication in CCFS-1/KMC cells (Fig. 5D) and RD cells (Fig. 5E) by qRT-PCR. The RT-PCR results (Fig. 5F) were similar to those of qRT-PCR with some discrepancies as noted in the previous experiment (Fig. 5C).

## Western blotting analysis for detecting viral structure proteins

To prove that yakammaoto has an effect on viral protein translation, Western blot assay was performed. The results confirmed that yakammaoto was effective in blocking the expression of VP0 and VP2 at a minimal concentration of 30 μg/mL in CCFS-1/KMC cells (Fig. 6A); 30 μg/mL yakammaoto could also clearly reduce the expression of VP2. However, it took 100 μg/mL to completely block the expression of VP0 and VP2 in RD cells (Fig. 6B). These findings were compatible to those of PCR and suggest that higher concentrations of yakammaoto were needed to block EV71 activities in RD cells than in CCFS-1/KMC cells.

To confirm that the reduction of viral protein expression is due to the inhibition of viral translation, but not due to the blocking of viral attachment and/or internalization,

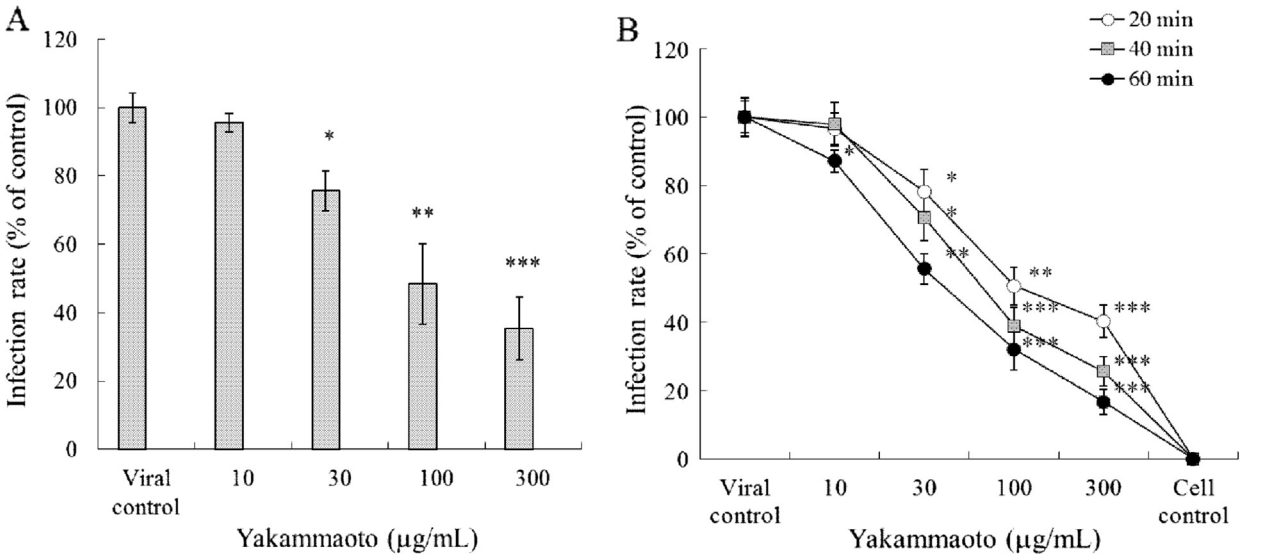


**Figure 4.** Attachment assay and penetration assay. Yakammaoto inhibited (A) viral attachment and (B) internalization in CCFS-1/KMC cells. Data are presented as mean ± standard deviation of nine tests. *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$ compared with the viral control. CCFS-1/KMC cells = human foreskin fibroblast cell line.

yakammaoto was supplemented 1 hour after EV71 inoculation. Yakammaoto dose dependently inhibited EV71 translation in CCFS-1/KMC cells (Fig. 6C) and RD cells (Fig. 6D). It was also found that 30 μg/mL yakammaoto could inhibit >20% viral protein production in both cells. Approximately 100 μg/mL yakammaoto could inhibit 57% VP0 and 62% VP2 production in RD cells, whereas 300 μg/mL yakammaoto could reduce 87% VP0 and 56% VP2 production in CCFS-1/KMC cells.

## Discussion

The current study demonstrates for the first time that yakammaoto could effectively inhibit EV71 infection in both CCFS-1/KMC and RD cells. Yakammaoto is a cost-effective and readily available traditional prescription that can be used to control EV71 infections in socioeconomically disadvantaged families. Being a mixture, yakammaoto could possibly work by inhibiting viral attachment, internalization, replication, and protein translation. In the summer, EV71 infection commonly manifests as HFMD and/or life-threatening cardiopulmonary diseases [3,8,25–28]. Infection of EV71 can spread into the CNS, subsequently causing neuropathy and neural apoptotic cell death. This spread of infection can damage vasomotor and respiratory centers in the medulla, which may lead to autonomic nervous system dysregulation and pulmonary edema [29,30]. However, the neuropathogenesis of EV71 infection is currently unknown. Retrograde axonal spread along the cranial or peripheral nerves is implicated to be the major transmission route for EV71 neuroinvasion. Skeletal muscle was found to serve as an important site for viral replication and entry into the CNS [29,31]. Therefore, inhibition of EV71 infection and its replication in skeletal muscle cells might block the neuroinvasion. Indeed, 10 μg/mL yakammaoto started to decrease the infection rate (Fig. 2B) and viral transcription (Fig. 5B) in RD cells. This inhibition of viral replication was also confirmed by supplementing yakammaoto 1 hour after viral inoculation (Fig. 5E). With a low $IC_{50}$, yakammaoto might help to block the neuroinvasion of EV71. VP0 and VP2 are structural proteins of EV71. VP0 is produced during viral assembly of the replication cycle, which autocleaves into VP2 and VP4 to generate infectious virions [21,32,33]. After cosupplementing with EV71, 30 μg/mL yakammaoto could inhibit the viral protein translation and block the expression of VP0 and VP2 in RD cells (Fig. 6B). Besides, supplementing yakammaoto 1 hour after viral inoculation also confirms this activity. Therefore, yakammaoto could block the viral replication, translation, maturation, and subsequent release and infection. Furthermore, yakammaoto can also prevent EV71 attachment and subsequent penetration into host cells to initiate the second run of infection. Therefore, yakammaoto could be beneficial to manage EV71 infection. Yakammaoto consistently showed its anti-EV71 activity in both human fibroblast and rhabdomyosarcoma cell lines. The results were consistently demonstrated by different methods. Within RD cells, 100 μg/mL yakammaoto can almost completely inhibit EV71 as demonstrated by PCR (Fig. 5C) and flow cytometry (Fig. 2B) results. Therefore, our findings are valid, and support the use of yakammaoto as an alternative choice for treating EV71 infection. Enterovirus infection is more common in families with poor socioeconomic status, living in a crowded space, and with poor hygiene [26]. Yakammaoto is an inexpensive alternative that can reduce the financial impact to manage EV71 infection during endemic periods. If its efficacy can be proven in clinical trials, yakammaoto could become a cost-effective therapeutic modality against EV71 infection for these socioeconomically disadvantaged families.

Yakammaoto contains 12.5% (w/w) flos farfarae [12], which contains toxic pyrrolizidine alkaloids that may result in liver injury [22,24]. Therefore, there are safety issues

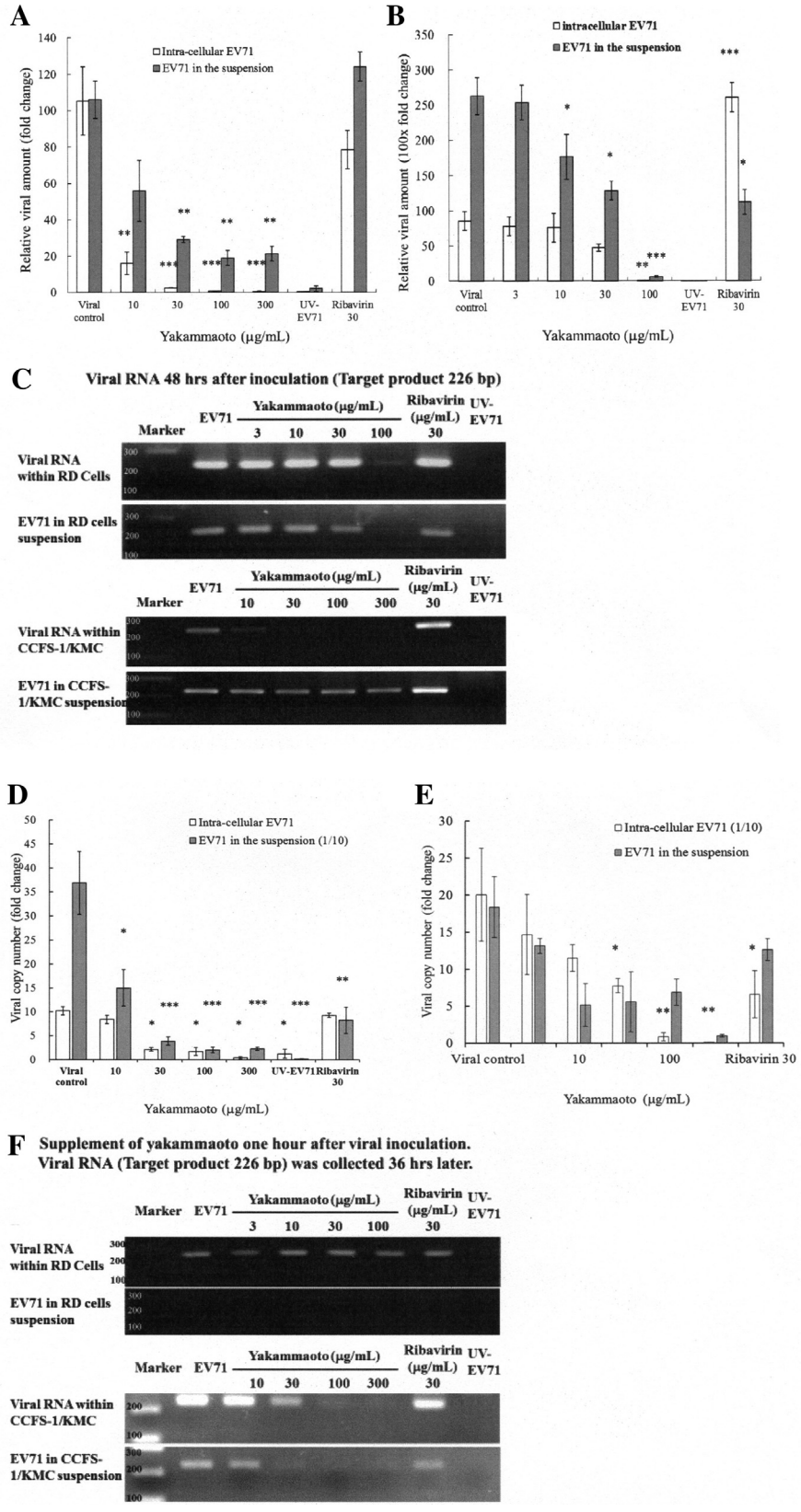


**Figure 5.** Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR) assay. Yakammaoto dose-dependently decreased viral amounts within the cells and in the suspension, compared with those of UV-inactivated EV71 (UV-EV71) groups, by qRT-PCR in CCFS-1/KMC cells [(A) cosupplement and (D) supplemented 1 hour later] and RD cells [(B) cosupplement and (E) supplemented 1 hour later] and by RT-PCR [(C) cosupplement and (F) supplemented 1 hour later]. Please note the different units for the *y* axis between (A) and (B), and the different doses of yakammaoto used in CCFS-1/KMC and RD cells. Data are represented as mean ± standard deviation of three tests. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$ compared with the control group. CCFS-1/KMC cells = human foreskin fibroblast cell line; EV71 = enterovirus 71; RD cells = human rhabdomyosarcoma cell line.

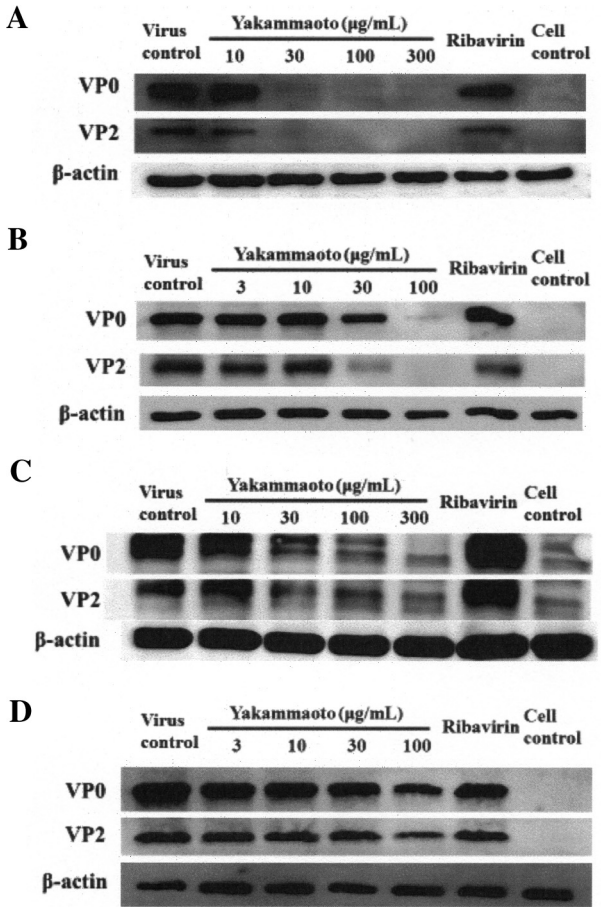


**Figure 6.** Western blot assay. Viral translation is clearly inhibited at the highest tested concentration in both CCFS-1/KMC cells [(A) cosupplement and (C) supplemented 1 hour later] and RD cells [(B) cosupplement and (D) supplemented 1 hour later]. Please note the different doses of yakammaoto used in CCFS-1/KMC and RD cells. CCFS-1/KMC cells = human foreskin fibroblast cell line; RD cells = human rhabdomyosarcoma cell line.

regarding the use of yakammaoto. In studies, yakammaoto did show some toxic effects to hepatocellular carcinoma and rhabdomyosarcoma cell lines at high concentrations, particularly after prolonged treatment (Fig. 1). However, the regular prescription of yakammaoto in traditional use is always at a low dose and for a short duration. Besides, there is an alternative explanation. Yakammaoto may only show its cytotoxic effect on cancer cell lines as several ingredients of yakammaoto and their constituents have antineoplastic activities [34—37]. The actual cytotoxic effects could be lower. Nevertheless, physicians should monitor the liver function and be aware of myalgia. Yakammaoto could still be a useful choice to manage EV71 infection.

In conclusion, yakammaoto is a readily available, inexpensive traditional prescription that could effectively inhibit EV71-induced cell injuries by preventing viral attachment, internalization, replication, and protein translation. The current results support its possible use to manage EV71 infection; however, physicians should carefully monitor the liver function and be aware of myalgia.

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