

# Antiviral Activity of Gallic Acid against Coxsackievirus B3 and Coxsackievirus B4

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Viral infections are capable of inducing reactive oxygen species (ROS) production in the infected cells and antioxidants have been reported to have antiviral activities against many viruses. In this study, an antiviral assay using the cytopathic effect (CPE) reduction method revealed that gallic acid possesses good anti-coxsackievirus B3 (CB3) and coxsackievirus B4 (CB4) activities, reducing the formation of visible CPE. However, ribavirin did exhibit weak anti-CB3 and CB4 activities and was unable to prevent CPE. Therefore, we conclude that the inhibition of CB3 or CB4 production by gallic acid may be due to its general action as an antioxidant.

**Keywords:** Antioxidants, antiviral, coxsackievirus, gallic acid

Many viruses are capable of inducing cell death, leading to lysis of infected cells, morphological changes commonly known as cytopathic effect (CPE), the reactive oxygen species (ROS) production [1]. Antioxidants also have been shown to have antiviral activities against a variety of unrelated viruses [4]. Our previous report showed that gallic acid possesses strong antioxidant activity [2]. Recent studies also have demonstrated that gallic acid possess anti-herpes simplex virus type 1 (HSV-1) [7]. But the antiviral effectiveness of gallic acid against coxsackievirus B3 (CB3) and coxsackievirus B4 (CB4) in vitro has not yet been reported.

Hence, this present study was undertaken to evaluate the antiviral activity against CB3 and CB4, and the effect of gallic acid on CB3 or CB4-induced CPE. Furthermore, Our result suggest that the antiviral activity of gallic acid against CB3 and CB4 is correlated with the antioxidant activity of gallic acid.

In our experiments, sulforhodamine B (SRB) was pur-

chased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were a reagent grade. Gallic acid was isolated from flowers of *Woodfordia fruticosa* in our lab as previously [2], dissolved in dimethyl sulphoxide (DMSO), and stored at  $-20^{\circ}\text{C}$ .

Coxsackievirus B3 (CB3) and coxsackievirus B4 (CB4) were obtained from Chung-cheongnam-Do Health and Environment Research Institute in Korea and were propagated in african green monkey kidney (Vero) cells at  $37^{\circ}\text{C}$ . Vero cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 0.01% antibiotic-antimycotic. Antibiotic-antimycotic, FBS and MEM were supplied by Gibco BRL (Grand Island, NY, USA). The tissue culture plates were purchased from Falcon (BD Biosciences, NJ, USA).

Assays of antiviral activity and cytotoxicity were evaluated by the previous reported SRB method using cytopathic effect (CPE) reduction [3]. Infectivity of virus stock was determined by the SRB method and was determined as infectivity of the virus by SRB ID<sub>50</sub> (50% infective dose). The results were transformed to percentage of controls and were drawn the dose-response curves. Ribavirin was used as positive, and DMSO was used as negative control.

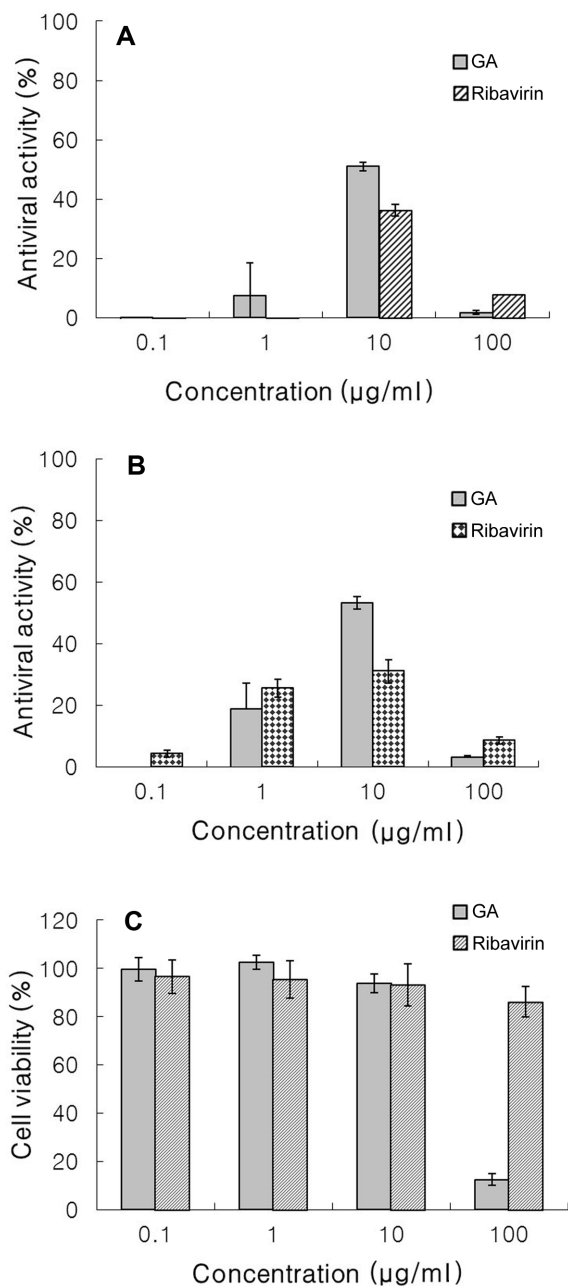
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**Fig. 1. Antiviral activity and cytotoxicity of gallic acid against CB3 and CB4 in Vero cells.** Culture medium 96-well tissue culture plates were removed and the cells were washed with PBS. Then, 0.09 ml of diluted virus suspension and 0.01 ml of medium containing gallic acid or ribavirin of with a 10-fold diluted concentration ranging from 0.1 to 100 µg/ml were added. After incubation at 37°C in 5% CO<sub>2</sub> for 2 days, the antiviral activity was investigated by CPE reduction assay. Results are presented as the mean percentage values obtained from three independent experiments carried out in triplicate ± S.D. (A) Antiviral activity of gallic acid against CB3, (B) antiviral activity of gallic acid against CB4, (C) cytotoxicity of gallic acid in Vero cells. GA, gallic acid.

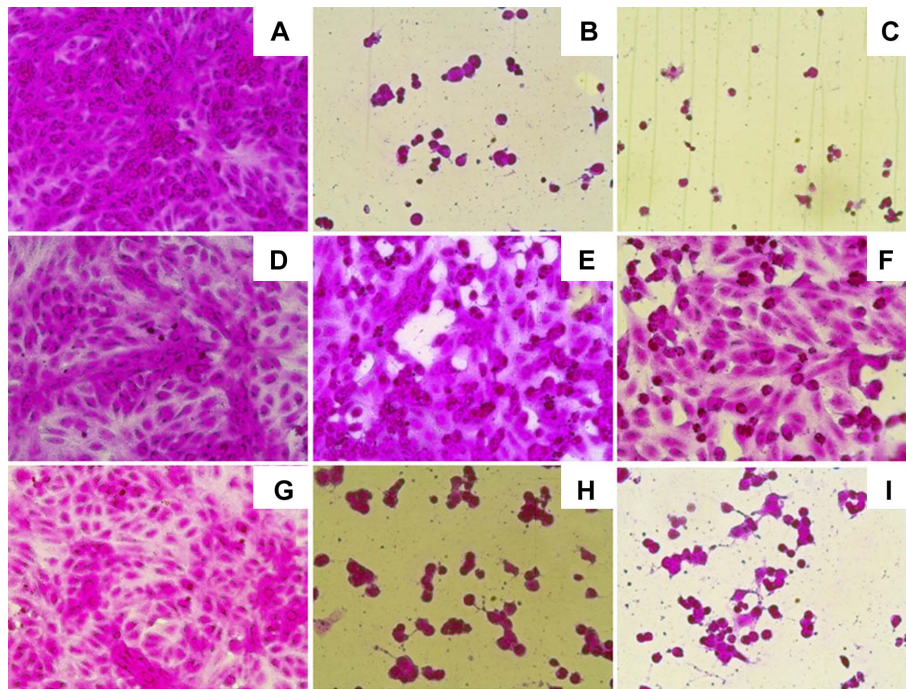
The effect of gallic acid on CB3 or CB4-induced CPE was observed. Briefly, Vero cells were seeded onto a 96-well culture plate at a concentration of  $2 \times 10^4$  cells per well. Next day, medium was removed and washed with PBS. Then, 0.09 ml of diluted virus suspension and 0.01 ml of medium containing gallic acid of 10 µg/ml were added. After incubation at 37°C in 5% CO<sub>2</sub> for 2 days, the morphology of cells was observed under microscope of  $32 \times 10$  magnifications (AXIOVERT10, ZEISS, Germany), and images were recorded.

As shown in Fig. 1A and 1B, gallic acid was investigated for its antiviral activity against CB3 and CB4. The antiviral assays demonstrated that gallic acid possessed good antiviral spectrum against CB3 with antiviral activity of about 51% and CB4 with antiviral activity of about 53% at concentration 10 µg/ml (Fig. 1A and 1B). However, ribavirin did show weak antiviral activity against CB3 and CB4 with 31% at concentration of less than 10 µg/ml (Fig. 1A and 1B). Gallic acid was toxic to Vero cells with cell viability of 12% at concentration of 100 µg/ml and ribavirin showed cell viability of 86% at same concentration (Fig. 1C).

After 2 day infections of Vero cells with CB3 or CB4, Mock cells (Fig. 2A) or cells treated with 10 µg/ml gallic acid (Fig. 2D) or ribavirin (Fig. 2G) showed typical spread-out shapes and normal morphology. At this concentration, no signs of cytotoxicity of gallic acid were observed. Infection with CB3 or CB4 in the absence of gallic acid resulted in a severe CPE (Fig. 2B and 2C). Addition of gallic acid on infected Vero cells inhibited the formation of a visible CPE (Fig. 2E and 2F). However, the addition of ribavirin in CB3 or CB4-infected Vero cell was impossible to prevent CPE (Fig. 2H and 2I). Thus, the CPE of the virus infection is prevented by the presence of gallic acid.

Viral infections frequently result in the generation of oxidative stress in the infected cells [6] and many previous studies showed that interference with the generation of ROS by use of antioxidants can drastically reduce replication of various viruses [8]. Our previous report showed that gallic acid possess strong antioxidant activity higher than that of butylated hydroxy anisol (BHA) [2].

Ribavirin has been used as treat against various DNA and RNA virus infections, although virus-acquired resistance to it was isolated from various virus populations and observed in some patients [5]. In this study, gallic acid is an effective antiviral compound against CB3 and CB4. However, ribavirin didn't exhibit expected antiviral activity.



**Fig. 2. The effects of gallic acid on CB3 or CB4-induced CPE.** Culture medium 6-well tissue culture plates were removed and the cells were washed with PBS. Then, 0.09 ml of diluted virus suspension and 0.01 ml of medium gallic acid or ribavirin of 10  $\mu\text{g}/\text{ml}$  were added. After incubation at 37°C in 5%  $\text{CO}_2$  for 2 days, the morphology of cells was investigated under microscope and a photograph taken. (A) Non-infected cells, (B) CB3-infected cells without gallic acid, (C) CB4-infected cells without gallic acid, (D) non-infected cells with gallic acid, (E) CB3-infected cells with gallic acid, (F) CB4-infected cells with gallic acid, (G) non-infected cells with ribavirin, (H) CB3-infected cells with ribavirin, (I) CB4-infected cells with ribavirin.

In late stages of CB3 and CB4 infections, morphological changes commonly known as CPE, microscopically observed. The morphology of Vero cells after infection with CB3 or CB4 was greatly decreased from that of CB3 or CB4 by addition of gallic acid. However, the addition of ribavirin to CB3 or CB4-infected Vero cell proved to be impossible in preventing CPE.

In conclusion, we present evidence that gallic acid are able to protect cells from coxsackievirus induced death. Gallic acid is potent agents that have been shown to be involved in a number of processes, suggesting that their antiviral effects might be due to its antioxidant functions alone. Nevertheless, further studies are needed to verify the underlying mechanism of gallic acid action in inhibiting CB3 or CB4 infection.

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## 국문초록

### **Gallic acid의 Coxsackievirus B3와 Coxsackievirus B4에 대한 항바이러스 효과**

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바이러스 감염은 감염된 숙주세포에서 활성산소를 유도할 수 있으며, 항산화제들은 많은 바이러스에 대해서 항바이러스 능력을 갖는다고 보고되었다. 본 연구에서 cytopathic effect (CPE) reduction 방법에 의한 항바이러스 활성 평가에서 gallic acid는 현저하게 CPE 형성을 감소시키면서 coxsackievirus B3 (CB3)와 coxsackievirus B4 (CB4)에 대해 항바이러스 활성을 나타내었다. 그러나 rabavirin은 CB3와 CB4에 대해 CPE를 막지 못하면서 약한 항바이러스 활성을 나타내었다. 그럼으로 gallic acid의 CB3와 CB4에 대한 항바이러스 활성은 항산화제 효능 때문인 것으로 사료된다.