

Original Article

The Potential Effect of *Glycyrrhiza Glabra* on Early Step of Influenza Virus Replication

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Abstract

Background and Aims: The emergence of drug-resistant influenza viruses has become a serious threat for human and animal populations. *Glycyrrhiza glabra* (Gg) is a traditional medicine clinically used for the treatment of viral respiratory infection symptoms in most countries. We evaluated the effects of the herb on influenza virus replication in human lung cultured cells (A549) following the determination of cytotoxic concentration 50 of Gg for the cell in culture.

Materials and Methods: Suspensions of influenza virus-infected A549 cells were examined for infectivity up to 48 h after the addition of Gg at various concentrations before and after adsorption of the virus. The possible anti-influenza activity of Gg was also determined using apoptosis detection.

Results: At concentrations ranging from 50 to 400 µg/ml, Gg did not cause a cytotoxic effect against the cells. The increase in viral titers before adsorption and a dose-dependent inhibitory action of Gg after virus adsorption indicated that the herb did not affect influenza virus replication in human epithelial respiratory cells. DNA fragmentation showed that Gg protected cells from influenza virus-induced apoptosis before and after adsorption of the virus.

Conclusion: The findings suggest that Gg cannot directly affect viral HA activity during virus replication. A decrease in virus titer after-treatment of the infected cells with higher concentrations of Gg may interact with cellular signaling factors either involved in viral entry or budding.

Keywords: Influenza A virus, *Glycyrrhiza glabra*, Antiviral activity, Hemagglutinin

Introduction

Hemagglutinin (HA), the receptor-binding glycoprotein of influenza A viruses mediate upper respiratory tract infection in mammals and bird species. After

the viral infection, inflammation progress is due to the secretion of cytokines and chemokines. Thus antiviral therapy and prophylaxis are used to limit the severity and duration of the viral disease.

Two classes of approved anti-influenza drugs are neuraminidase (NA) and M2 ion channel protein inhibitors which can reduce viral spread and inhibit virus replication, respectively (1). Because of high resistance rate, new agents targeted various stages of the viral replication cycle including viral entry and RNA polymerase activities, have been

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developed and are under investigation in late-stage clinical trials (2). In contrast to the medications, herbs have the potential of inhibiting the replication of influenza virus by interfering with multiple targets. According to the WHO report about 80% of population in developing countries use traditional medicines for their primary health care (3). Various herbs have been studied for anti-influenza effects and proposed that they may act in neutralizing the activity of the HA and NA glycoproteins, increasing production of inflammatory cytokines, targeting the cellular factors, and reducing the duration and severity of influenza symptoms (4).

Rapid development of drug-resistance is one of the major problem in the prevention against influenza. Because of some disadvantages of common medications, herbal drugs remedies came more in the center of interest of antiviral treatment. Despite numerous *in vitro* and *in vivo* studies on the antiviral properties of medicinal plants, a few are licensed for clinical use. *Glycyrrhiza glabra* (Gg), commonly known as licorice native to the Middle East, southern Europe, and parts of Asia is one such plant. The roots and rhizomes are the main medicinal parts of the herb and have been widely used to treat cough, colds, bronchitis, inflamed throats, and asthma since ancient times as documented by the Canone of Ibn-Sina.

To date 73 bioactive components including alkaloids, glycosides, phenolic compounds, flavonoids, and saponins as immunomodulator agents have been identified in Gg (5). Among the ingredients, pentacyclic triterpenoid glycoside glycyrrhizic acid (glycyrrhizin, GL) and 18 β -glycyrrhetic acid (GA) have been shown responsible for anti-inflammatory and antiviral properties (6). The mechanism of action is related to the presence of substances and their quantity in the herb. For example, the concentration of GA is varying between 1%-9% depending on the species, geographical location, and methods of extraction (7).

For the past two decades the antiviral activity of the herb for several viruses such as hepatitis B and hepatitis C viruses, herpes virus, human immunodeficiency virus, corona virus, influ-

enza viruses, coxsackie virus, and flavi-viruses has been investigated scientifically (8).

Various mechanisms of action include reducing viral attachment, inhibiting virus gene expression and replication, enhancing host cell activity by blocking the degradation of I κ B, activating T lymphocyte proliferation and/or suppressing host cell apoptosis have been suggested for GL and GA (8-11).

Due to the complexity of replication steps in influenza viruses, the determination of specific antiviral mechanisms for Gg has become difficult. Docking studies have been performed to investigate the anti-influenza mechanism of Gg active components such as GL, glabrone, and other flavonoids on HA, NA, and M viral proteins (12, 13). The computed analysis indicated that the antiviral effect was limited to an early step in the virus replication cycle lead to in conclusion that the potential anti-influenza activity of the herb mediated by an interaction with the virus-cell membrane. In the current study, we screened the inhibitory activity of Gg on replication of influenza virus using the human lung adenocarcinoma (A549) cell as a main target for the viral infection.

Methods

Ethics considerations. The study protocol was approved by the Research Ethics Committee of Azad University of Medical Sciences with the ethics number: IR.IAU.PS.REC.1398.357.

Cell line. A549 (ATCC No CCL-185TM) cells were propagated in Dulbecco's modified Eagle's minimum essential medium (DMEM, Inoclon) supplement with 10% fetal calf serum, penicillin G (100 units/ml) and streptomycin (100 μ g/ml).

Cytotoxicity assay. The amount of 10 g of Gg root powder was dissolved in cold water and boiled at 100°C for 10 min then placed at room temperature for 12 h. The solution was clarified by low-speed centrifugation and sterilized with a 0.22- μ m filter. Seven concentrations of the aqueous solution from 50, 100, 200, 400, 600, 800, and 1000 μ g/ml were made in distilled water. Each of the extracts was separately added to the monolayers of A549 cells (1×10^6 cells/ml) and the

culture plates were incubated for 3 days. The cell viability was determined by MTT colorimetric assay (14). The cytotoxic concentration 50 (CC₅₀), which is the concentration of the extract at which 50% of the cells are viable, was estimated.

Antiviral assay. Two treatment procedures were considered to determine the inhibitory impact of Gg extract on influenza virus replication. In (A) procedure influenza H9N2 virus (acc No. JX456181) at a multiplicity of infection (MOI) of 1.0 PFU/cell was mixed with each herbal extract concentration less than CC₅₀ and incubated 1 h at room temperature. The seeded A549 cells (80-90% confluence) were inoculated with each of the mixture and after 1-h adsorption at 37°C, the virus/extract inoculum was removed and replaced by DMEM. In another procedure (B) A549 cells were inoculated with the virus and following adsorption for 1 h, the inoculum was removed and replaced by DMEM containing the herbal extract of different concentrations. The cultures were incubated at 37°C up to 48 hours post infection (hpi). Appearance of the cytopathic effect (CPE) was checked up to 48 hpi. The virus titer was measured by tissue culture infection dose (TCID₅₀) assay on the basis of the Reed-Muench method at 24 and 48 hpi.

Apoptosis was investigated for determination of the anti-influenza activity of Gg in the early stages of viral infection using DNA fragmentation. The confluent monolayers of A549 cells in both treatment procedures were collected at 8 h-intervals up to 48 hpi.

To extract DNA, the supernatants were centrifuged and the cell pellet suspended in 300 ml of cold cell lysis buffer (Exgene Genomic DNA Micro GeneAll, Korea). The extraction was followed by manufacturer instruction then DNAs were electrophoresed through a 2% agarose gel and stained with SafeRed™ Loading Dye (GeneCopoeia, Inc).

Statistical analysis. Comparisons between groups were made using one-way ANOVA and statistical significance was defined at a *P*-value of <0.05.

Result

The cellular cytotoxicity assay of Gg extract showed this herb was not toxic for A549 cells at concentrations ranging from 50 to 400 µg/ml. Based on the cell viability results (Table 1), the concentration ranges of the extract containing 50% toxicity was between 600- 800 µg/ml. Thus the antiviral activity of Gg extract at concentrations less than the CC₅₀ (50, 100, and 200 µg/ml) was evaluated before and after adsorption.

The H9N2-infected A549 cells showed the marked CPEs at 24 hpi, which extended by detaching the cell up to 48 hpi (Figure 1). Whereas no CPE was observed in A549 cells treated with the mixtures of virus and herb extracts at 24 hpi. The same but delay CPE was seen by a higher dose of Gg extract in another trial.

We found that the viral titers before and after adsorption were lower than those of the H9N2-infected control group in the entire period. Mixing of H9N2 virus with the three concentrations of Gg extract before virus adsorption led to the reduction in viral titers, which was not statistically significant (*P*<0.05) compared to the virus-infected cells (Figure 2). The dose-dependent inhibitory action of Gg on virus replication was shown after virus adsorption. A significant decline in the viral titer observed with 200 µg/ml of the herbal extract at 48 hpi compared to the virus-infected cells (Figure 3). The relative increase in viral titers indicated that Gg did not interact with viral HA activity at early stage of virus replication.

As shown in figure 4, the virus-infected A549 cells were undergoing apoptosis which is a proven feature of influenza infection in the alveolar cell line. Whereas, no detectable DNA fragmentation appeared in the infected cells when treated with Gg. Therefore, treatment of the infected-cells with Gg extract could block the induction of programmed cell death by the influenza virus.

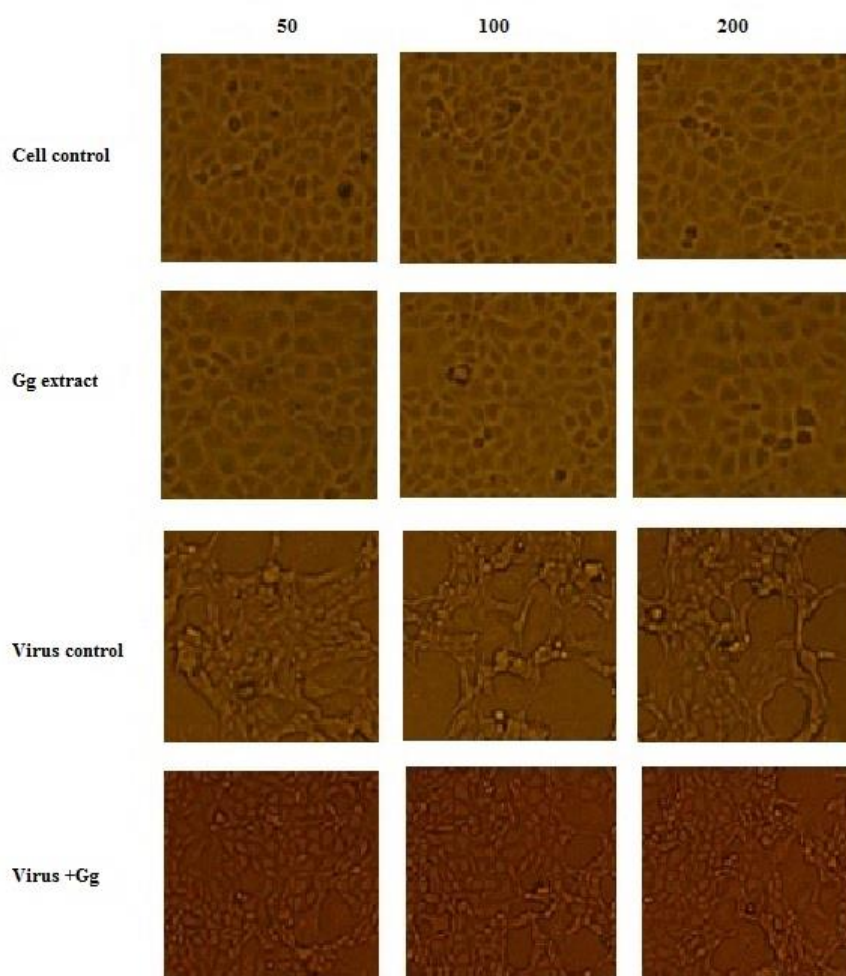


Fig. 1. Cytopathogenicity of A549 cells to influenza virus replication at 48 h after inoculation treated with different concentrations ($\mu\text{g/ml}$) of *Glycyrrhiza glabra* (Gg) extract. Detaching the cell was observed in virus-infected cells compared to the cell and herb extract controls. In virus+Gg treated cells a limited cytopathic effect was observed (20 \times magnification).

Discussion

In the present study, Gg was screened for anti-influenza activity in A549 cells using before and after adsorption approaches. No significant difference in viral infectivity in Gg-pretreated cells was detected. Only the higher concentration of the herb extract affected replication of influenza virus after adsorption of the virus on cultured A549 cells. Replication of viruses at lower concentrations indicated that the inhibitory activity of Gg was not limited to virus replication cycles. Thus the ability of H9N2 virus to replicate in A549 cells indicates Gg could not interfere with viral HA activity. A number of herbal extracts derived from *Alpinia Katsumadai*, *Forsythia suspensa*, and

Ephedra spp have been generally suppressed influenza A virus infection but their mechanisms of action have not been determined (15-17). In some cases, naturals appear to have antiviral activity similar to the common medications like ribavirin. Emerging resistance to the antiviral drugs in the past few years become the major problem in the treatment of influenza.

Efficient treatment highlights the importance of screening the new antiviral activity of herb medicines which targeted other viral factors such as HA (18).

Several critical sites including the receptor-binding site and N-linked glycosylation site of HA, as well as viral entry into respiratory epithelial cells are the potential medicinal

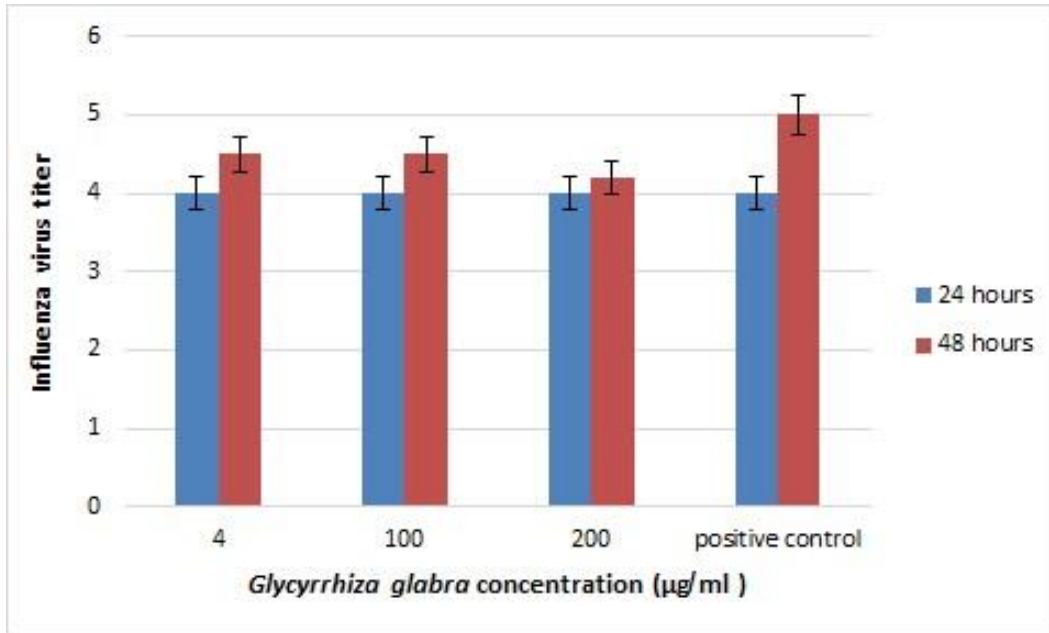


Fig. 2. Evaluation of the inhibitory effect of *Glycyrrhiza glabra* extract on influenza virus replication before virus adsorption. Reduction in viral titers (\log_{10}) was found in the three concentrations of herb extract before virus adsorption ($P < 0.05$) compared to the virus-infected control.

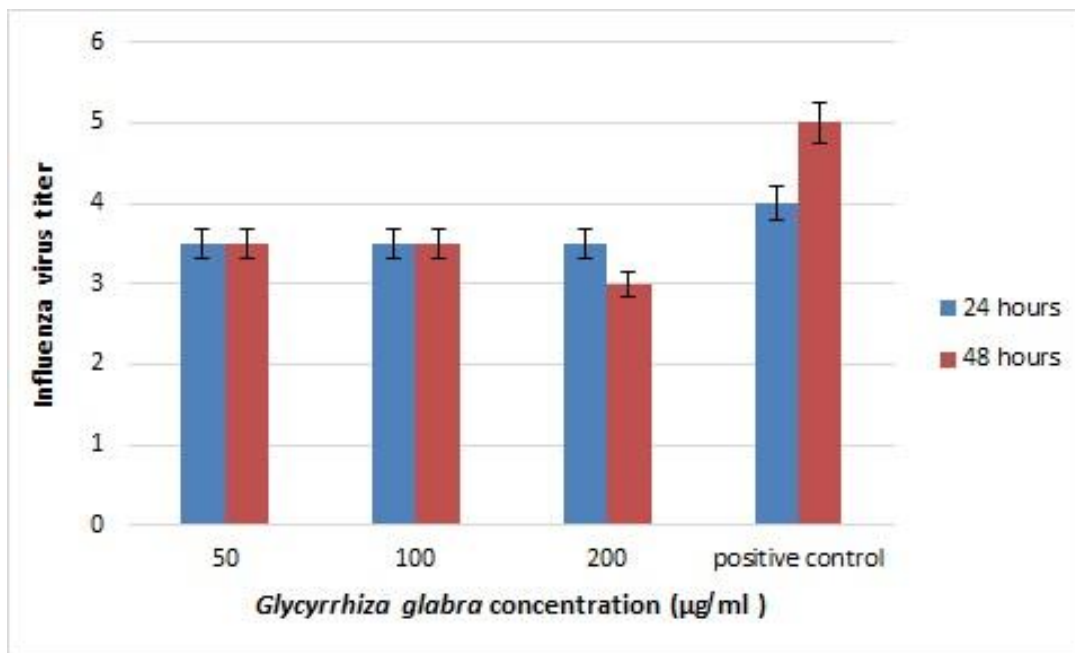


Fig. 3. Evaluation of the inhibitory effect of *Glycyrrhiza glabra* extract on influenza virus replication after virus adsorption. A significant decline in the viral titer (\log_{10}) was observed with the higher concentration of herb extract after virus adsorption ($P < 0.05$) compared to the virus-infected control.

targets for developing antiviral drugs (19, 20). Entry is an essential process to deliver virus ribonucleoprotein into the nucleus following transcription/replication of the viral genome. Interfering with host cell receptor recognition and blocking the receptor binding activity of viral HA protein has been determined for two flavonoids of elderberry extract, catechins

isolated from green tea, curcumin and its derivatives, and rographolide isolated from the stem and leaves of *Andrographis paniculata* (21-24). Similar to our results, GA as the active component of Gg, epigallocatechin-gallate, and dandelion did not directly show anti-influenza activity either by interfering with receptor binding or virus entry (25, 26).

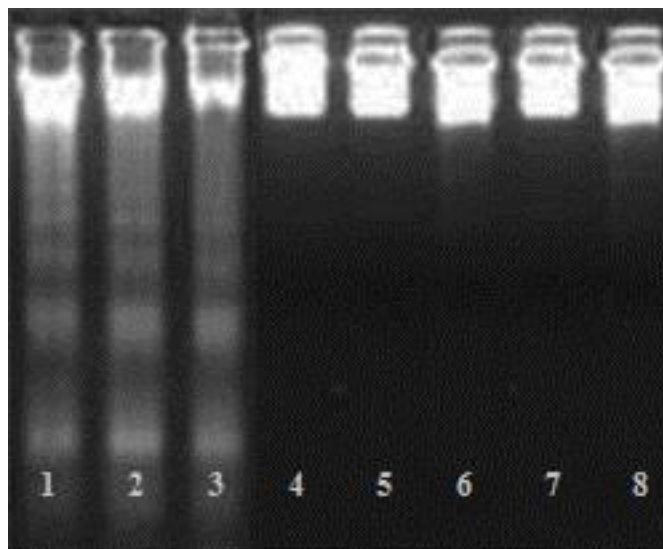


Fig. 4. Induction of apoptosis was inhibited in influenza virus-infected A549 cells treated with 200 $\mu\text{g/ml}$ of *Glycyrrhiza glabra* extract in both pre and post treatments. Lanes 1-3: virus control at 24, 48 and 72 h; lanes 4 and 5: pretreatment at 24 and 48 h; lanes 6 and 7: post treatment at 24 and 48 h; lane 8: the herb extract control.

Table 1: Toxicity evaluation of different concentrations of aqueous extract of *Glycyrrhiza glabra* root on A549 cells.

Concentrations of <i>Glycyrrhiza glabra</i> root ($\mu\text{g/ml}$)	Viable cell percentage		
	24 hours	48 hours	72 hours
0 (control)	98	98	97
50	98	96	96
100	97	95	94
200	96	94	94
400	90	90	87
600	76	76	58
800	72	72	50
1000	36	20	20

This inhibitory activity may be non-specific interactions between the herb components and cell surface receptors or viral envelope proteins.

The roots and leaves of Gg contain a wide spectrum of bioactive constituents that able to interact with various targets ranged from interaction with the virus to down-regulation of host factors such as intracellular signaling cascades (5, 6). Using a computational approach, it has been predicted that flavonoids of Gg have potential as an influenza inhibitor possibly by affecting the virus-cell membrane fusion and blocking viral entry into cells. The pathway is distinct from the currently available

antiviral therapeutics (27). Our cell-based assay for antiviral drug screening revealed Gg root extract could not inhibit the virus-cell receptor binding a step subsequent to virus entry because the herb compounds did not directly interfere with the viral HA binding site on A549 cells. The inhibitory activity of Gg was observed after virus adsorption in the cells which led to a reduction in CPEs and viral titers, and limitation of apoptosis. Induction of apoptosis is a crucial mechanism in the spread of viral progeny. H9N2 virus induced apoptosis signaling via mitochondrial pathway in A549 cells in a dose-dependent manner (14). Gg exhibited the potential to reduce H9N2

spread by limiting the viral-induced apoptosis. These observations lead to the conclusion, that the antiviral activity of Gg is mediated by an interaction with the cell membrane which most likely results in reduced endocytotic activity. Uptake of the virus but the reduction in its titer suggests that Gg can interfere with cellular factors rather than virus entry.

Focusing on molecular and cellular pathways of the virus infectivity cycle leads to prospects for developing novel multi-target antiviral agents. Influenza infections elevate the induction of biologically active cytokines and chemokines include RANTES, MCP-1, MIP-1, pro inflammatory cytokines, and INF- γ as an antiviral cytokine (28). The possible anti-influenza mechanisms of Gg were reported as weak production of CXCL10, IL-6, and CCL5, suppression virus-induced apoptosis, reduction HMGB 1 binding to DNA, and inhibition viral polymerase activity (29, 30). In A549 cells, Gg suppressed influenza virus-induced RANTES secretion. In an animal trial, GA completely protected a group of mice from a lethal dose of influenza virus probably due to the stimulation of INF- γ . The similar results have been found for aloe emodin an active component of Aloe vera, which showed anti-influenza activity by stimulation of cellular mechanisms such as CK2, Nrf2, TLR4, p38/JNK, NF-kb, Galectin-3, STAT1 and INF- γ involved in antiviral pathways. The cytokine suppression property has candidate these herbs for the treatment of viral chronic inflammatory conditions with subsequent stimulation of the antiviral activity. However, Gg is a valuable addition in a formula for respiratory infection, its interactions with anti-hypertensive drugs, potassium-depleting diuretic drugs, and corticosteroids should be considered when higher doses are given (31, 32). In conclusion, Gg can limit the influenza virus replication in human respiratory cells. In agreement with our previous molecular docking results this antiviral potential is not through inhibiting viral HA activity. More assays are needed to confirm the mechanism of anti-inflammatory and immunomodulatory actions of the medicinal herb against influenza infection.

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