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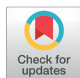

Original Article

Anti-influenza and anti-inflammatory effects of green tea (*Camellia sinensis* L.) extract

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Abstract
Background and aims: Pulmonary complications due to influenza A virus infection, in addition to being caused by the replication of the virus, are partly due to the excess production of pro-inflammatory cytokines. It is therefore helpful to seek out compounds to control the excess production of these cytokines along with administration of antiviral drugs. In this study, the effect of green tea (*Camellia sinensis* L.) extract on the replication of influenza A (H1N1) virus and on gene expression levels of pro-inflammatory cytokines was studied in Madin-Darby canine kidney (MDCK) cells.
Methods: In this experimental study, hydroalcoholic extract of *C. sinensis* leaf was prepared with maceration method. In vitro anti-influenza virus activity of the extract was evaluated by performing hemagglutination (HA) and 50 % tissue culture infectious dose ($TCID_{50}$) assays at 24 and 48 hours of incubation. The gene expression levels of pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin 1 beta (IL-1β) and IL-6 were studied using real-time Polymerase chain reaction (PCR) 24 hours after treatment.
Results: *Camellia sinensis* extract treatment caused a significant decrease in viral titer compared to control virus in a dose-dependent manner (P<0.05). The gene expression levels of IL-6, TNF-α and IL-1β after treatment with the extract decreased significantly compared to the virus control (P<0.05).
Conclusion: Due to its antiviral effect and reducing the gene expression of pro-inflammatory cytokines, *C. sinensis* extract can be used as an adjunctive drug along with current antiviral drugs in severe influenza infection as well as other viral infections, such as coronavirus, to minimize lung damage due to inflammation.
Keywords: Influenza A virus, Pro-inflammatory cytokine, Immunomodulation, *Camellia sinensis* L., Green tea, antiviral

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Introduction

Seasonal influenza causes millions of cases of severe and critical disease and 290 000-650 000 deaths worldwide (1). The most common serious complications of influenza occur in the lungs and are divided into three main categories, primary viral pneumonia, secondary bacterial pneumonia, and exacerbation of chronic diseases (2). Previous research determined that severe complications and ultimately death due to the infection, in addition to being attributable to viral replication, are due to strong immune responses in excess production of pro-inflammatory cytokines against the virus (2-6). It is argued that an excessive pro-inflammatory response in the production of pro-inflammatory cytokines causes acute respiratory pain syndrome, failure in several organs and finally death in cases of severe influenza (7,8). Therefore, regulating the immune response and targeting inflammatory pathways assist in reducing tissue damage and mortality (9). Available anti-influenza drugs only inhibit the mechanisms that are specific to the growth and proliferation of viral proteins and do not control the excess production of pro-inflammatory cytokines. These drugs lead to relative drug resistance and side effects as well (9). Based on this, it seems that medicinal compounds, especially herbal compounds are useful in treating lung infection due to influenza A virus through exhibiting anti-inflammatory and immunomodulatory properties.

Green tea (*Camellia sinensis* L.) is from the *Theaceae* family with green and leathery leaves, white and fragrant flowers. *C. sinensis* powder, which is obtained through drying and grinding the fresh leaves of the plant, does not go through oxidation and fermentation. Extensive research has been conducted to investigate the effects of *C. sinensis* in the prevention or treatment of some diseases, which are mainly due to the presence of polyphenolic compounds, especially catechins, in its leaves (10,11). Epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate are the main compounds of *C. sinensis*. The plant has been reported to produce antioxidant, anti-inflammatory, antiviral, disinfectant, and antibacterial effects as well as to protect against contrast-induced kidney damage (11-15).

Anti-inflammatory and antiviral impacts of *C. sinensis*

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extract have already been demonstrated, but there is not convincing evidence on its effect to control pro-inflammatory factors that exacerbate lung infection with influenza virus. This study was conducted to examine the effect of *C. sinensis* extract on the proliferation of influenza A (H1N1) virus and the control of pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin 1 beta (IL-1β) and IL-6 following infection with the virus in Madin-Darby canine kidney (MDCK) cells.

Materials and Methods

Extraction

The fresh leaves of the plant were procured from a local market and, after authentication, registered in the herbarium of the University (Voucher number: MPSKUMS-206). The leaves were powdered by an electric mill and then the obtained powder was combined with ethanol 70% at 1: 5 for 48 hours; then, the contents were filtered by means of a filter paper. The solvent was concentrated using a rotary evaporator and vacuum pump (at < 40°C), and then the resulting liquid was dried at 37°C. Ultimately, the extract was combined with DMSO and the extract at different concentrations was made by the culture medium. Throughout all steps of the experiment, DMSO was obtained at the final concentration of less than 0.02%.

Cell and virus

Influenza A virus [strain A/Puerto Rico/8/34 (H1N1)] and MDCK cells were obtained from the Influenza Unit, Pasteur Institute of Iran (Tehran, Iran). Stock solution was made from the initial passages of the virus in the vial. After the viral titer was determined by the median tissue culture infectious dose ($TCID_{50}$) assay, the virus was stored as the initial viral seed at -70°C until the subsequent experiments.

Cytotoxicity assay

The MTT assay was done to study the cytotoxic effect of the extract on MDCK cells (cell death percentage) and measure the 50% cytotoxic concentration (CC_{50}). After cell monolayer formed in a 96-well plate, the cells were incubated for 48 hours with different concentrations of *C. sinensis* extract. Next, the cell supernatant was separated and the wells were rinsed with PBS, and then 60 µL of MTT (1 mg/mL) was introduced into each well and incubated for 4 hours at 37°C. After incubation, the contents were gently emptied and 100 µL of dimethyl sulfoxide (DMSO) was introduced into them and the micro plate was incubated at room temperature for 10 minutes. The optical absorbance of the plates was recorded at 570-nm wavelength using an ELISA reader and turned into cell death (%) according to the following formulation (16);

$$\text{Toxicity}(\%) = [1-(At/As)] \times 100$$

where *At* and *As* denote the absorbance of the test substance and the solvent control, respectively.

CC_{50} was calculated using the dose-response curve and regression analysis.

Antiviral assay

After the cell monolayer became confluent in the 12-well micro plate, the cells were rinsed with phosphate-buffered saline (PBS) two times and then the virus at 100 $TCID_{50}$ was introduced. After one hour of incubation at 37°C, the medium containing the virus was removed and the cells were rinsed with PBS. Then, 800 µL of the culture medium containing different non-toxic concentrations of *C. sinensis* extract (concentrations lower than the CC_{50} values) was added to each well along with the virus culture medium (2 µg/mL of TPCK Trypsin and 0.3% BSA). The micro plates were incubated at 37°C for 24 and 48 hours and then the supernatant was collected and the viral titer in each well was calculated by performing hemagglutination (HA) and the $TCID_{50}$ assay (17,18). The results were compared with the control virus.

Hemagglutination assay

The influenza virus is characterized by the ability to cause agglutination in red blood cells. Hemagglutination can be observed by mixing different dilutions of the virus and chicken red blood cells in a micro plate. To perform the assay, a serial dilution of the medium containing influenza virus was prepared and after addition of chicken red blood cells (0.5%) to all the wells, they were incubated for 45 minutes at room temperature. The reverse dilution of the last well that shows complete hemagglutination was considered to represent the viral titer.

TCID50 assay

MDCK cells were cultured in 96-well micro plates, and after the formation of cell monolayer, one virus logarithmic dilution was introduced into the wells so that each concentration was repeated at least 5 times. After leaving the micro plate for 1 hour at 37°C, the cell supernatant medium was removed and the medium containing viral growth medium (2 µg/mL TPCK Trypsin and 0.3% BSA) was added to the wells. The micro plates were incubated for 48 hours at 37°C in 5% CO2. Afterwards, the presence of virus in each well was checked using the HA assay and the results were calculated using Reed and Munch method (19).

Expression levels of cytokine genes

Real-time polymerase chain reaction (PCR) was done to examine the effect of *C. sinensis* on the expression of cytokine genes. For this purpose, a confluent MDCK cells monolayer was made in 6-well micro plates, and the virus at 100 $TCID_{50}$ was introduced into the culture medium. One hour later, the medium including the virus was separated and the culture medium including 3.75, 7.5, 15, and 30 µg/mL of the extract (concentrations lower than the CC_{50} values) and viral growth medium were introduced into the cell. Following a 24-hour

incubation at 37°C, cell supernatant was removed and total intracellular RNA was obtained by Trizol (Sigma-Aldrich, St. Louis, USA) as per the manufacturer's instructions. The RNA was transformed to cDNA by oligo-dT primer and cDNA Synthesis kit (Yekta-Tajhiz-Azma, Iran) as per the manufacturer's guidelines. The expression levels of IL-6, TNFα, and IL-1β genes were measured by SYBR Green Master Mix Kit (Yekta-Tajhiz- Azma, Iran) and Rotor Gene TM 3000 (Corbett, Australia). Primers were designed for real-time PCR and synthesized by Pishgaman Inc. (Tehran, Iran) (Table 1).

The thermal cycling conditions below were used to conduct for PCR: 95°C for 10 minutes and then 45 cycles at 95°C for 15 seconds, 60°C for 20 seconds, and 54 or 72°C for 25 seconds. GAPDH gene was considered to be internal control in real-time PCR. The relative quantitative method ($2^{-\Delta\Delta Ct}$) was applied to identify pronounced changes in pro-inflammatory cytokine mRNA and internal control.

Data analysis

The CC_{50} value was calculated using regression analysis. In the experiments, two independent tests were done in duplicate and the statistical significance of differences was investigated using the non-parametric Kruskal-Wallis test. P value < 0.05 was considered the significance level. Statistical analysis and graphing were done using the GraphPad Prism 6 Demo software.

Results

Extract cytotoxicity

The cytotoxicity of *C. sinensis* extract on MDCK cells was examined 48 hours after treatment using the MTT assay. The CC_{50} was calculated at 78.53 (95% CI: 66.55-92.67) µg/mL (Figure 1).

The effect of C. sinensis extract on the production of viral particles

The cell monolayer including the virus was treated with *C. sinensis* extract at different concentrations for 24 and 48 hours, and then the well supernatants were removed and the viral titer was determined using the HA and TCID50 assays. *C. sinensis* extract caused a significant decrease in viral titer in a dose-dependent manner compared to the control virus ($P < 0.05$, Table 2 and Figure 2).

Table 1. Primers' specification for amplification of the genes

Gene name	Primer sequences (5′ to 3′)	Amplicon length (bp)
TNF	F:5'TTCTTGCCCAAACCGACCCT-3 R:5'CCAGCCCTGAGCCCTTAATTCT-3'	90
IL-6	F:5'GCCCACCAGGAACGAAAGAG-3' R:5'GGAAAGCAGTAGCCATCACCA-3'	113
IL1β	F:5'GCTGCCAAGACCTGAACCAC-3' R: 5'ACAATGACTGACACGAAATGCCTC-3'	158
GAPDH	F:5'GGAGAAAGCTGCCAAATATGACGA-3' R:5'CGAAGGTGGAAGAGTGGGTGT-3'	140

Effect of Camellia sinensis L extract on levels of pro-inflammatory cytokine's mRNA

The gene expression levels of IL-6, IL-1β and TNF-α were investigated in virus-infected cells treated with the extract and untreated, virus-infected cells 24 hours after treatment. The results showed that the expression levels of these genes increased significantly in the virus control compared to the cell control, but the expression level in the treated, infected cells underwent a significant, dose-dependent decrease in comparison to the virus control ($P < 0.05$) (Figure 3).

Discussion

The use of natural compounds in clinical treatments has drawn attention and tried in recent decades. Based on this, extensive studies have also been conducted to confirm the effects of *C. sinensis* for the prevention or treatment of some diseases, which are mainly due to the presence of polyphenolic compounds, especially catechins in the plant's leaves (10,20). In various studies, antioxidant, anti-inflammatory, antiviral, disinfectant, and antibacterial effects of the plant as well as its protective effect against contrast-induced kidney damage have been observed (11-15).

In this study, the effect of *C. sinensis* extract on influenza virus titer was calculated at 24 and 48 hours after treatment using the HA and $TCID_{50}$ assays. With respect to our

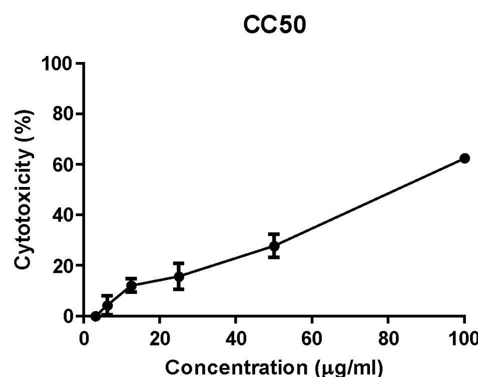

Figure 1. The cytotoxicity of *Camellia sinensis* L. leaf extract on the MDCK cells; cells were incubated with 100, 50, 25, 12.5, 6.25 and 3.125 µg/mL of the extract for 48 hours; the cytotoxicity was measured by MTT assay; data presented as the mean ± SD of three independent experiments. MDCK: Madin-Darby canine kidney

Table 2. Hemagglutination titers of influenza virus in the culture supernatants after treatment with *Camellia sinensis* L. extract at different concentrations

Sample	Concentration (µg/mL)	Log2 HA titer/50 μL supernatant	
		24 h[a]	48 h[a]
Camellia sinensis L. extract	50	0***	0**
	25	0.5 ± 0.55**	1.75 ± 0.83*
	12.5	3 ± 1.26	4.25 ± 0.4
	6.25	4 ± 1.67	5 ± 1
	virus control	7 ± 1.26	8.5 ± 0.5

[a] Hours post-infection.
* $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to virus control, statistical significance was examined by the non-parametric Kruskal-Wallis test.
The data are mean ± SD of three independent experiments.

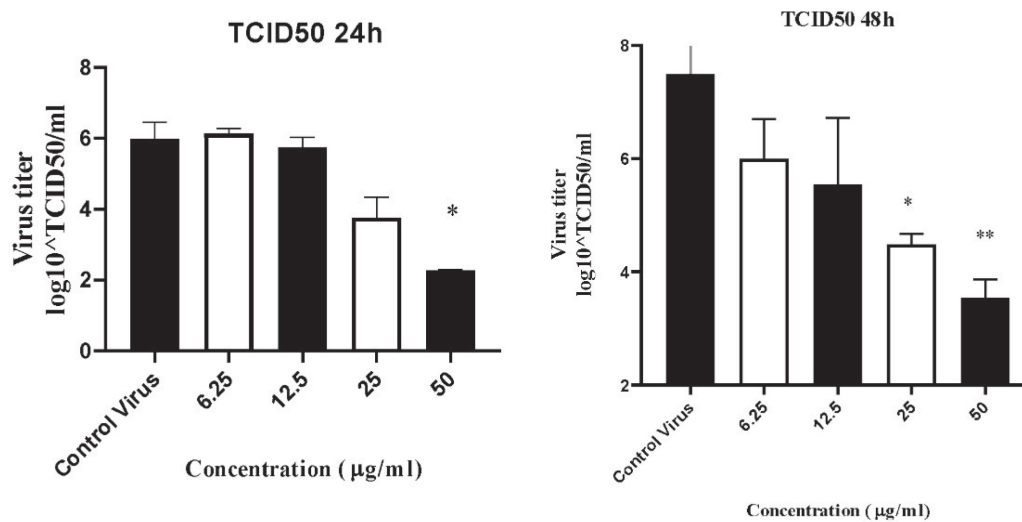

Figure 2. Reduction of influenza virus titers in the culture supernatants by the Camellia *sinensis* L. extract. Influenza-infected MDCK cells were incubated with *Camellia sinensis* L extract at different concentrations for 24 and 48 hours and the supernatants were used for $TCID_{50}$ titration. Statistical significance was examined by the non-parametric Kruskal-Wallis test. The data are mean ± SD of three independent experiments. $TCID_{50}$: 50% tissue culture infectious dose

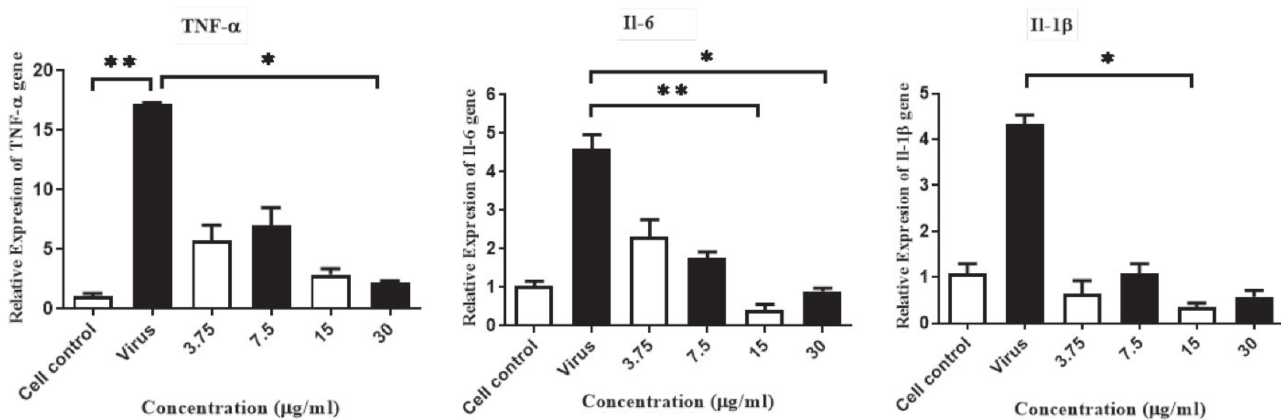

Figure 3. Effects of green tea extract on the mRNA expression of pro-inflammatory cytokines in MDCK cells. MDCK cells were infected with influenza A virus and treated with 30, 15, 7.5 and 3.75 μg/mL of *Camellia sinensis* L. extract and total RNA was extracted at 24 h and 48 h after the virus infection and then the mRNA levels of tumor necrosis factor-alpha (TNF-*α*), interleukin 6 (IL-6) and interleukin 1*β* (IL-1*β*) were determined; mRNA levels were normalized to GAPDH; $P<0.05$ & ** $P<0.01$ in comparison with control viruses, Statistical significance was investigated using the non-parametric Kruskal-Wallis test; data are presented as the median with a range

results, *C. sinensis* extract induced a significant, dose-dependent decrease in viral titer compared to the control virus. *C. sinensis* can act as a source of polyphenols, particularly flavonoids. Catechins are one of the most pronounced flavonoids in the plant (10). Based on the research findings, catechins have so strenuous antiviral effects that they inhibit the proliferation of herpes simplex virus at low concentrations (21). Further examinations revealed that the catechins of *C. sinensis*, especially (-)-epigallocatechin gallate (EGCG), inhibit adenovirus growth at different stages, such as the intracellular development, and virus protease and adenain *in vitro* (22). With regards to the antiviral effects of EGCG, Yamaguchi et al reported that this compound could inhibit HIV-1 virus replication by affecting different stages of its life cycle (23). Polyphenols in *C. sinensis* extract have a wide range of antiviral activities against both RNA and DNA viruses. The antiviral activity of polyphenols due to their antioxidant nature is the inhibition of enzymes involved in virus replication and disruption of their cell membranes. Green tea polyphenols also inhibit virus penetration and attachment to cells, which induces host cell self-defense and affects the activity of a variety of signal transduction pathways (24). It seems that the anti-influenza virus activity observed in our study is related to polyphenols and especially catechins in green tea extract, which needs more studies.

Based on the obtained results, *C. sinensis* extract decreased the expression of TNF-α, IL-6 and IL-1β genes caused by influenza A virus in cell culture in a dose-dependent manner. Various studies have been conducted on the antiviral and anti-inflammatory effects of natural compounds including *C. sinensis*. In the study of Mehrbod et al herbal-marine compound (HESA-A) exhibited an antiviral effect on MDCK cells. The results of that study showed that HESA-A could increase cell viability up to 31% and decrease the HA titer up to 90% in cells exposed to the virus. In addition, the levels of expression and

production of inflammatory cytokines such as TNF-α and IL-6 substantially reduced in the virus-infected cells (25).

In our study, the levels of TNF-α, IL-6 and IL-1β gene expression in MDCK cells increased due to infection with influenza A virus compared to the control cell, which is consistent with the results of the study of Mehrbod et al in which the effect of the influenza virus was investigated on the levels of expression and production of pro-inflammatory cytokines in MDCK cell line (25). The results of both studies showed that the viral infection in the cell increased the expression of TNF-α, IL-6 and IL-1β genes.

In our study, *C. sinensis* extract treatment of virus-infected cells at various concentrations led to a dose-dependent decrease of cytokine expression compared to the control virus. In agreement with the current study, the study of Khalili et al. on the virus-infected MDCK cells revealed that the virus led to a significant increase in pro-inflammatory cytokines such as TNF-α and IL-6, and exposure of virus-infected cells to celastrol at different concentrations led to a significant decrease in the gene expression and proteins of pro-inflammatory cytokines (26). Also in the study of Aghaei et al, the influenza virus induced a significant increase in pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, and exposure of virus-infected cells to punicalagin at different concentrations led to a significant decrease in the gene expression and proteins of proinflammatory cytokines (20). The research of de Lima Mota et al showed that the plant had analgesic and anti-inflammatory properties and could be applied as a natural treatment for chronic inflammatory diseases (27). A previous study showed that *C. sinensis* extract (Oolong tea) due to downregulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression can suppress inflammatory mediators in LPS-stimulated macrophages. In addition, it has been indicated that *C. sinensis* induces anti-inflammatory effects via targeting MAPK and NF-κB pathways in macrophages (28). It seems that nuclear factor kappa B (NF-κB may) induce pro-inflammatory cytokines expression in H1N1-infected MDCK cells and the *C. sinensis* extract may reduce the IL6, TNF-α and Il1-β by interaction by the NF-κB pathway.

Conclusion

Based on the results of the present study, *C. sinensis* extract with a high inhibitory effect against the influenza virus replication substantially reduces the pro-inflammatory cytokines and can be used as an adjunctive drug along with current antiviral drugs in severe influenza infections as well as other viral infections, such as coronavirus, to minimize lung damage due to inflammation.

Author Contributions

Conceptualization: Ali Karimi, Mohammad-Taghi Moradi
Data curation: Majid Asadi-Samani, Dhiya Altememy, Mohammad-Taghi Moradi
Formal Analysis: Majid Asadi-Samani, Mohammad-Taghi Moradi
Funding acquisition: Mohammad-Taghi Moradi.
Investigation: Majid Asadi-Samani, Dhiya Altememy, Mohammad-Taghi Moradi.
Methodology: Majid Asadi-Samani, Dhiya Altememy, Mohammad-Taghi Moradi.
Project administration: Ali Karimi, Mohammad-Taghi Moradi.
Resources: Mohammad-Taghi Moradi.
Supervision: Mohammad-Taghi Moradi.
Validation: Mohammad-Taghi Moradi, Ali Karimi.
Visualization: Majid Asadi-Samani, Mohammad-Taghi Moradi.
Writing – original draft: Majid Asadi-Samani, Dhiya Altememy, Mohammad-Taghi Moradi.
Writing – review & editing: Ali Karimi, Majid Asadi-Samani, Dhiya Altememy, Mohammad-Taghi Moradi.

Conflict of Interest Disclosures

The authors declare that there is no conflict of interests.

Ethical Approval

The ethics approval was obtained from Shahrekord University of Medical Sciences (with the ethical code of IR.SKUMS.REC.1397.229).

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