Phytochemical properties and antiviral effect of green tea (Camellia sinensis) extract on adenovirus in vitro

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Abstract

Background and aims: The lack of effective antiviral drugs for adenoviruses is one of the most important problems in this area. The aim of this study was to investigate the phytochemical properties and antiviral effect of the green tea extract (GTE) on adenovirus in Hep2 cells in vitro.

Methods: In this experimental study, dried leaves of green tea were extracted by maceration. Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant capacity of the extract were measured by Folin-Ciocalteu, aluminum chloride, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) colorimetric methods, respectively. The amounts of some phenolic compounds in the extract were also determined using high-performance liquid chromatography. The toxicity of the extract on Hep2 cells and antiviral activity of the extract on adenovirus were assessed by the MIT colorimetric method. The half-maximum cytotoxicity concentration (CC50) and the 50% inhibitory concentration (IC50) of the extract were calculated as well.

Results: Phytochemical investigations showed that the IC50 of DPPH radical was 42.1 ± 3.2 μg/mL compared with butylated hydroxytoluene (Ic50 of 33.5 ± 3.67 μg/mL). The TPC and TFC of the extract were 74.2 mg GAE/g and 16.3 mg RE/g of the dry extract, respectively. The extract demonstrated the highest amounts of syringic acid, gallic acid, 3,4-dihydroxybenzoic acid, and rutin levels (67.27, 20.12, 7.39, and 2.97 mg/g DW, respectively). Based on the results of cell culture, the CC50 and IC50 of GTE were 103.3 μg/mL and 25.16 μg/mL, respectively.

Conclusion: GTE with phenolic and flavonoid compounds can exert dose-dependent inhibitory effects on adenoviruses.

Keywords: Adenovirus, Camellia sinensis, Antiviral effect, Green tea

Introduction

Viral diseases are among the major health issues in many countries. One of the most important problems in this area is the lack of appropriate antiviral drugs for some viruses such as adenoviruses and the acquisition of resistance to available drugs (1,2). Human adenoviruses are non-enveloped viruses that have double-strand DNA and belong to the genus Mastadenovirus from the Adenoviridae family. Overall, 52 serotypes of these viruses have so far been identified and divided into seven subgroups (A-G). Adenoviruses can multiply and cause disease in the eyes and respiratory, gastrointestinal, and urinary tracts. These viruses cause a wide range of diseases from self-limiting infections and mild diseases, including conjunctivitis and gastrointestinal diseases to some life-threatening infections (3,4). Adenoviruses are spread all over the world and are present all year round and usually do not cause epidemics in the communities. The most common serotypes in clinical specimens include low-numbered respiratory and gastrointestinal types. These viruses are mainly transmitted through the fecal-oral route but are also sometimes transmitted through small respiratory droplets or contaminated objects. Most adenoviral diseases are left undiagnosed because they have no clinical symptoms and many infection cases are asymptomatic (5). Despite many advances in cysteine protease inhibitors and nucleoside synthesis analogues to inhibit adenovirus replication, no chemical treatment has yet been identified to effectively assist in preventing and treating adenovirus infection. Alongside chemical drugs, the advent of antiviral, especially anti-adenoviral, drugs could promise more effective treatments with fewer side effects. Medicinal plants have long been used to treat human diseases, and several herbal medicines have been converted into therapeutic compounds with reportedly remarkable outcomes.

Green tea (Camellia sinensis) belongs to the Theaceae family and has green and leathery leaves, as well as...
white and fragrant flowers. Different types of teas are obtained from this plant depending on the process of auto-oxidation catalysis with polyphenol oxidase and peroxidase. Therefore, teas are classified as non-oxidized (green and white), semi-oxidized (oolong), oxidized (black), and post-fermented (pu-erh tea) types. Green tea powder, which is the result of the process of drying the fresh leaves of the plant, is obtained without going through oxidation and fermentation. Green tea has a variety of compounds, especially from the family of catechins (6,7), which have antioxidant, anti-inflammatory, antiseptic, and hypolipidemic properties, prevent postoperative abdominal adhesions, produce antibacterial effects and protect against contrast-induced nephropathy (6,8-10). The antiviral activity of *C. sinensis* extracts against the herpes simplex virus and influenza virus has been reported in previous research (11). Given the antiviral and antimicrobial effects of green tea, this study aimed to evaluate the phytochemical properties and anti-adenoviral effects of the hydroalcoholic green tea extract (GTE) in vitro.

**Materials and Methods**

**Cells and viruses**

Human adenovirus serotype 5 was procured from a virology laboratory, Shahrekord University of Medical Sciences, Iran. Hep2 cells were purchased from the Pasteur Institute of Iran and cultured in DMEM containing 10% fetal bovine serum.

**Extraction**

To prepare the extract, green tea leaves were purchased from reputable groceries, and after being authenticated by a botanist, were registered in the herbarium of the Medical Plants Research Center, Shahrekord University of Medical Sciences, Iran (Voucher number: MPSKUMS-206). The plant was pulverized by an electric mill, and then the resulting powder was mixed with ethyl alcohol (70%) ratios of 1:5 after 48 hours. The contents of the container were filtered using filter paper and a glass funnel. The solvent was concentrated by a rotary evaporator and vacuum pump (set at <40°C). The concentrated liquid was dried in an oven at 37°C. Finally, the extract was dissolved in DMSO, and different concentrations of the extract were prepared using a culture medium.

**Measuring antioxidant activity by DPPH method**

Antioxidant activity was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) based on the percentage of free radical scavenging. First, different concentrations of the extract were prepared. Two hundred microliters of samples at different concentrations was mixed with 1 mL of a 90 μM DPPH solution to a final volume of 95 mL with the addition of 95% methanol and shaken in the dark for 60 minutes. Then, the absorbance of the samples containing the extract and the control was read at 517-nm wavelength using a UV-Vis spectrophotometer (UNICO 2100: USA). The absorbance was read at 517 nm using the high absorbance of the reaction mixture indicating low free radical scavenging activity. A sample containing methanol and DPPH solution was used as a control. Butylated hydroxytoluene (BHT) was applied as a positive control. The inhibition of free radicals by DPPH was calculated according to the following formula:

\[
\text{Antiradical activity (\%)} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100.
\]

The 50% inhibitory concentration (IC₅₀) value, defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%, was computed based on the linear regression of the plots of the percentage antiradical activity against the concentration of the tested compounds (12).

**Measuring total phenolic content (TPC)**

TPC was measured by the Folin-Ciocalteu colorimetric method, and gallic acid was used as the reference standard. For this purpose, standard solutions of gallic acid were prepared in a solution of 60% methanol, and then 0.5 mL of each solution was transferred to a test tube, to which 0.5 mL of Folin-Ciocalteu 10% solution was added, and 0.4 mL of sodium carbonate 7.5% solution was added after 3-8 minutes. Next, the tubes were left at laboratory temperature for 30 minutes, and the optical absorbance was read by a spectrophotometer at 765-nm wavelength, and finally, a standard curve was plotted accordingly. Then, 0.01-0.02 g of the dried sample was dissolved in 60% methanol to a final volume of 10 mL, and TPC was measured per the above-described method except that the extract solution was added instead of the standard solution. Subsequently, the optical absorbance was added to the standard curve, and the TPC of the extract was obtained in mg gallic acid (GAE) equivalent to g of the dry extract (13).

**Measuring total flavonoid content (TFC)**

TFC was measured by the aluminum chloride colorimetric method. Briefly, 0.5 mL of each extract solution (0.01 g in 10 mL of 60% methanol) was added to 0.5 mL of 2% aluminum chloride and 3 mL of 5% potassium acetate. After 40 minutes, the optical absorbance of samples was read against distilled water at a 415-nm wavelength. Simultaneously, different dilutions of rutin were made, and the standard curve and experiment were prepared per the above-described method. Moreover, the optical absorbance of the samples was compared with the standard curve, and the TFC of the extract was calculated in mg rutin (RE) equivalent to g of the dry extract (14).

**Separation of flavonoids and phenolic acids using high-performance liquid chromatography (HPLC)**

Two flavonoids (quercetin and rutin) and fifteen phenolic acids (gallic acid, 3,4-dihydroxybenzoic acid, syringic...
acid, chlorogenic acid, vanillic acid, caffeic acid, 2,5-dihydroxybenzoic acid, catechin, p-coumaric acid, ferulic acid, chomic acid, rosmarinic acid, salicylic acid, and kaempferol, apigenin) in the extract were analyzed using the HPLC method. The qualitative analysis was performed using reverse-phase HPLC coupled with a UV-visible detector (UV PDA 2800) and column C18 (5 μm particle size, i.d. 250 mm × 4.6 mm). First, 2.5 mg of dry extract was dissolved in 1 mL methanol and water (a ratio of 1:4) to prepare a concentration of 2500 ppm, and then 20 μL of the samples were injected into HPLC and isolated by the fixed phase C18 and the binary mobile phase of deionized water and methanol containing trifluoroacetic acid 0.05%. Detection was performed by scanning from 190 to 800 nm and reading in the range of 280-372 nm. The results were calculated using the calibration curve equation of the analytical standards of each compound to identify and determine the amount. The results were expressed as μg/mg of the extract (15).

**Investigating toxicity by MTT assay**

MTT assay was performed to investigate the extract’s cytotoxic effect on Hep2 cells (percentage of cell death) and to calculate its half-maximum cytotoxicity concentration (CC₅₀) on this cell line. For this purpose, cell suspension containing 10⁴ cells in a volume of 100 μL was added to all wells of a 96-well plate. After 24 hours and cell monolayer formation, cells were incubated with the extract at concentrations of 3.125, 6.25, 12.5, 25, 50, 100, and 200 μg/mL for four days. Then, the cell supernatant was removed and each well was washed with 100 μL of PBS. Subsequently, 60 μL of 1 mg/mL of the MTT solution was added to the wells and incubated for four hours at 37°C. Next, the contents of the wells were gently removed and 100 μL of DMSO was added to them, and the microplate was incubated at room temperature for 10 minutes. The optical absorbance of the plates was read at a 570-nm wavelength. The optical absorbance recorded by the ELISA reader was converted to cell death percentage using the following formula:

Toxicity (%) = (At/As) × 100

where At and As denote the absorbance of the test substance and the solvent control, respectively (16). CC₅₀ or the concentration of the extract that killed 50% of the cultured cells, was obtained by the Probit test.

**Investigating antiviral effects**

Hep2 cells in appropriate numbers were transferred to 96-well plates. After the incubation and formation of a cell monolayer, 100 TCID₅₀ viruses were added to each well and incubated for 2 hours. In the next step, the media on the wells were aspirated, and non-cytotoxic concentrations of the extract (0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, and 100 μg/mL) were added to the wells. Six wells were considered cell control (free of any virus and extract) and six wells were considered virus control (infected with a virus and free of the extract). After 96 hours of incubation under standard cell culture conditions, the MTT assay was performed according to the above-mentioned procedure, and the percentage of viral inhibition at different doses of extracts was calculated concerning virus control using the following formula:

\[
\text{Antiviral activity (\%) = \frac{(Atv−Acv)}{(Acd−Acv)} × 100}
\]

where Atv, Acv, and Acd are the absorbance of test compounds on virus-infected cells, the absorbance of the virus control, and the absorbance of the cell control, respectively. The procedure was performed three times (17). IC₅₀ was calculated as the minimum concentration of the extract with 50% viral inhibition using the Probit test.

**Data analysis**

The CC₅₀ or IC₅₀ and their confidence intervals were obtained by Probit test in SPSS software, version 23. Each experiment was performed in triplicate, and the graphs were plotted using GraphPad Prism 6 software.

**Results**

**Antioxidant capacity, TPC, and TFC**

Phytochemical investigations showed that the IC₅₀ of DPPH radical was 42.1 ± 3.2 μg/mL compared with butylated hydroxytoluene (33.5 ± 3.67 μg/mL). The TPC and TFC of the extract were 74.2 mg GAE/g and 16.3 mg RE/g of the dry extract, respectively (Table 1).

**Flavonoids and phenolic acids in GTE**

Two flavonoids and five phenolic acids were analyzed in the GTE. The results revealed that the major phenolic compounds on virus-infected cells, the absorbance of the test substance and the solvent control, respectively (16). CC₅₀ or the concentration of the extract that killed 50% of the cultured cells, was obtained by the Probit test.

| Sample            | Concentration (μg/mL) | Scavenging of DPPH radical activity inhibition (%) (Mean ± SEM) | DPPH-radical scavenging activity IC₅₀ (μg/mL) |
|-------------------|-----------------------|---------------------------------------------------------------|---------------------------------------------|
| Green tea extract | 70                    | 72.5±2.5                                                      | 42.1±3.2                                    |
|                   | 60                    | 67.1±2.7                                                      |                                             |
|                   | 50                    | 53.9±1.8                                                      |                                             |
|                   | 40                    | 49.1±2.2                                                      | 42.1±3.2                                    |
|                   | 30                    | 43.8±2.6                                                      |                                             |
|                   | 20                    | 35.6±2.3                                                      |                                             |
|                   | 10                    | 26.6±1.9                                                      |                                             |
| BHT               | 20                    | 90.2±1.2                                                      |                                             |
|                   | 10                    | 78.2±3.5                                                      |                                             |
|                   | 50                    | 55.6±0.99                                                    |                                             |
|                   | 25                    | 37±2.35                                                        | 33.5±3.67                                    |
|                   | 12.5                  | 22.9±1.63                                                     |                                             |
|                   | 6.25                  | 15.2±2                                                        |                                             |
|                   | 3.125                 | 12.3±1.7                                                      |                                             |

Note: All results are presented as mean ± SEM of three assays. SEM: Standard mean error; DPPH: 1,1-Diphenyl-2-picrylhydrazyl; BHT: Butylated hydroxytoluene.
and flavonoid compounds in the extract were syringic acid, gallic acid, 3,4-dihydroxybenzoic acid, and rutin levels with 67.27, 20.12, 7.39, and 2.97 mg/g, respectively (Figure 1). On the other hand, chlorogenic acid, vanillic acid, caffeic acid, 2,5-dihydroxybenzoic acid, catechin, P-coumaric acid, ferulic acid, chicoric acid, rosmarinic acid, salicylic acid, quercetin, kaempferol, and apigenin were not detected in GTE.

Cytotoxicity of GTE
The cytotoxicity of GTE at different concentrations (3.125, 6.25, 12.5, 25, 50, 100, and 200 μg/mL) on Hep2 cells was evaluated at 96 hours using an MTT assay. Based on Probit analysis, the concentration of the extract that caused the death of 50% of cultured cells (CC\(_{50}\)) was calculated at 103.3 μg/mL (95% CI: 94.98-112.3). The Probit model demonstrated a significant relationship between the concentration of the extract, and more cell death was found with more increase in the extract concentration (\(P<0.001\), Figure 2).

Anti-viral effect of GTE
The anti-viral effect of GTE at different non-cytotoxic concentrations of the extract (0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, and 100 μg/mL) on adenovirus was evaluated 96 hours post-treatment using an MTT assay. According to the Probit model, the extract reduced the replication of virus-infected cells dose-dependently (\(P<0.001\)). Based on Probit analysis, the IC\(_{50}\) of the extract was calculated at 25.16 μg/mL (95% CI: 23.57-26.86, Figure 3).

Discussion
The present study aimed to evaluate the anti-adenoviral activity of hydroalcoholic GTEs. The results showed that the extract at 103.3 μg/mL had a toxic effect of 50% on Hep2 cells after 96 hours of exposure.

Additionally, it inhibited the proliferation of virus-infected cells in a dose-dependent manner so that the IC50 of the extract was calculated at 25.16 μg/mL.

Several types of compounds have been identified in different types of green tea, the most important of which include secondary metabolism products such as ascorbic acid, terpenes, alkaloids, and especially phenolic compounds, as well as volatile compounds, carotenoids, tocopherols, vitamins A, K, and B, and minerals such as fluorine, potassium, magnesium, iron, manganese, and phosphorus. Primary metabolism products such as polysaccharides, proteins, and lipids have been identified as well (18-20).

In addition, compounds in teas have various biological properties that have been confirmed in several in vivo and in vitro studies (21), the most important of which are antioxidant (8), anti-inflammatory, antiseptic, hypolipidemic, preventive of postoperative intra-
abdominal adhesion, antibacterial, and protective properties against contrast-induced nephropathy (22-24). The anti-herpes effects of *Camellia sinensis* and the adenoviral effects of black tea have also been reported in various studies (11,17,25,26).

*Camellia sinensis* can be considered an important source of polyphenols, especially flavonoids. The most important flavonoids in *C. sinensis* include catechins (7,27). According to the results of some studies, catechins have strong antiviral effects so that they can inhibit herpes virus replication at low concentrations (28, 29). Further studies have shown that catechins in green tea, especially epigallocatechin gallate (EGCG), inhibit virus growth at various stages, including direct inactivation of virus particles, inhibition of intracellular virus growth, and inhibition of the viral protease, adenain, in cell culture (30). Regarding the antiviral effects of EGCG, Yamaguchi et al concluded that this compound could prevent HIV-1 replication by acting on different stages of its life cycle (31).

In the current study, the TPC of GTE and its TFC were calculated at 74.2 mg GAE/g and 16.3 mg RE/g of the dry extract, respectively; therefore, the presence of phenolic and flavonoid compounds in this extract can be the reason for its antiviral effects, which is consistent with the results of other studies. Some studies demonstrated that phenolic compounds have antibacterial and antiviral properties (32,33). Other studies also indicated that flavonoids derived from different plants can exhibit antiviral properties (34-36).

In the present study, the identification of flavonoids and phenolic acids in GTE using HPLC revealed that the extract contains large amounts of flavonoid compounds and phenolic acids, including syringic acid, gallic acid, 3,4-dihydroxybenzoic acid, and rutin. Other compounds such as quercetin, ferulic acid, rosmarinic acid, apigenin, and kaempferol have also been identified in the extract of this plant (6,37,38).

**Conclusion**

According to the results of the current study, the GTE can reduce the proliferation of adenovirus in Hep2 cells in a dose-dependent manner, and the presence of phenolic and flavonoid compounds can be the reason for the antiviral effect of the extract. It is hoped that by conducting further studies and isolating the active ingredients of green tea, this plant will be used as a complementary drug alongside anti-adenoviral drugs in controlling and preventing adenovirus infection.

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**Authors’ contribution**

SM, FJF, and AS: Data acquisition, analysis, and interpretation, and draft writing. AK and ZL: Study designing, and critical revision of the manuscript for important intellectual content. MTM: Study designing, data analysis and interpretation, and supervision. All authors wrote the draft of the manuscript and approved the final manuscript.

**Conflict of interests**

The authors of the present work declare no conflict of interests.

**Ethical approval**

This study was approved by the Ethics Committee of Shahrekord University of Medical Sciences (the ethical code number of IR.SKUMS.REC.1398.005).

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