Iranian Journal of Pharmaceutical Sciences
2022: 18 (1): 77-86
www.ijps.ir

Original Article

Antioxidant and Cytotoxic Effects of Aqueous Extract of Iranian Green Tea (*Camellia sinensis* L.)

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Abstract

Green tea, a common beverage, has several pharmacological effects. In this study, the amounts of phenolic compounds and epigallocatechin (EGC) were determined in an aqueous extract of green tea planted in Lahijan (a city in the north of Iran) by Folin-Ciocalteu method and HPLC, respectively. Antioxidant activity of the extract was measured by DPPH and the FRAP assays. Furthermore, its cytotoxic effect was investigated on HT-29 (colorectal cancer) and 3T3 (normal fibroblast) cell lines, after 24 and 48 h treatment by MTT method. Trypan blue dye exclusion measurement was also done on the HT-29 cells treated with the extract. The results have shown that each gram of the dried extract contains 283.6 ± 10.57 mg total phenolic content and 88.44 ± 1.85 mg EGC. The antioxidant activity of the extract measured by DPPH showed an EC_{50} value of 84.31 ± 4.14 µg/ml. In the FRAP test, the equivalent amount of Iron (Fe) was 4.45 mmol per g of the dried extract. The extract showed toxicity on the HT-29 cell line with an IC_{50} value of 82.45 ± 18.39 µg/ml after 48 h treatment. The data were confirmed by trypan blue dye exclusion test. Based on the results, adding green tea to the diet may have many health benefits.

*Keywords***:** Antioxidant, Antiproliferative, Catechin, DPPH, EGC, FRAP, Green tea.

1. Introduction

Tea (*Camellia sinensis*) is a bushy plant of the *Theaceae* family. Its leaves are used to make a highly consumed beverage all over the world [1]. Tea, especially green tea (the non-fermented form), is full of phenolic compounds. These polyphenols are mostly responsible for its biological activities [2, 3]. Epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) are the most important tea polyphenols [4]. Green tea is mostly consumed in East-Asian countries [5]. Traditionally, it is used to improve health by regulating body temperature and blood sugar, protecting the cardiovascular

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Cite this article as: Mehrabani M., Amirheidari B., Ahmadinia N., Forooghi-Nematollahi M., Sabouri S., Antioxidant and Cytotoxic Effects of Aqueous Extract of Iranian Green Tea (*Camellia sinensis L.*), 2022, 18 (1): 77-86.

system, promoting digestion and treating flatulence. It is also used as a diuretic and an astringent remedy [6].

Recently, antitumorigenic, anti-inflammatory, antioxidative, antibacterial, and antiviral effects have been documented for green tea catechins [7]. Green tea extract and its components have also shown anticancer effects in animal models [8]. Moreover, it reduces DNA damage in yeast by increasing the expression of DNA repair genes [9]. Evidence shows that consumption of green tea may decrease the risk of stomach, breast, and ovarian cancers [10-15]. However, some studies have not been able to associate green tea consumption and incidence of cancers [16-20].

Azadi Gonbad *et al*. evaluated methanolic extracts of 12 different clones of *C. sinensis* from Iran and introduced one of them as a clone with more phenolic compounds and stronger antioxidant activity [21]. It is proved that different extraction methods and solvents as well as the climate of the region where a plant is grown, affects its chemical composition and pharmacological activities [22]. Therefore in this study, the antioxidant and cytotoxic effects of the aqueous extract of green tea cultured in the north of Iran was investigated. Standardization of the extract was done by measuring the amounts of total phenolic content and epigallocatechin (EGC).

2. Materials and Methods

2.1. Chemicals and Cell Lines

Folin-Ciocalteu reagent and EGC were purchased from Merck (Darmstadt, Germany). Gallic acid (GA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), tripyridyltriazine (TPTZ), and ascorbic acid were supplied from Sigma-Aldrich GmbH (Sternheim, Germany). Acetonitrile, methanol, ortho-phosphoric acid, and formic acid of HPLC grade were purchased from Scharlau (Barcelona, Spain). The spray-dried aqueous extract of Lahijan green tea was purchased from Soha Jisa (Mazandaran, Iran).

The human colorectal cancer (HT-29) and mouse normal fibroblast (3T3) cell lines were purchased from the Iranian Biological Resource Center (IBRC, Tehran, Iran). Dulbecco's Modified Eagle's medium (DMEM), Pen/Strep, and trypan blue were bought from Biosera (Boussens, France), FBS from Gibco (NY, USA), and MTT powder from Melford (Ipswich, UK).

2.2. Total Phenolic Content Determination

The total phenolic content of the sample was measured using the Folin-Ciocalteu method with some modifications [23]. A calibration curve of gallic acid solution (10 to 100 mg/L) was prepared. Then, 0.1 ml of the tea extract (1 mg/ml) was mixed with 0.5 ml of 10% Folin-Ciocalteu reagent. After 3-8 minutes, 0.4 ml of a 7.5% sodium carbonate solution was added to the mixture and allowed to stand for 30 min. The absorbance was then measured at 765 nm using a spectrophotometer. The results were calculated using the regression equation of the calibration curve and expressed as mg gallic acid equivalents per g of the sample.

2.3. HPLC Analysis

The amount of EGC was determined by high-performance liquid chromatography (HPLC) method [24]. An ECOM HPLC (Czech

Republic) equipped with an analytical C-18 column of 250 mm × 4.6 mm × 5 µm (Shim-pack, Japan) and a photodiode array detector (PDA) was used for analysis. The sample and solvents were filtered through 0.2 µm and 0.45 µm membrane filters (cellulose nitrate and reverse cellulose membranes, S&S), respectively. For analysis, 20 µl of each sample was injected into the apparatus. A mixture of methanol and 0.02% aqueous solution of ortho-phosphoric acid was used as the gradient solvent, at a flow rate of 1 ml/min. Chromatograms were recorded at 210 nm. All analyses were repeated three times.

2.4. Antioxidant Activity

2.4.1. DPPH Scavenging Activity

The scavenging assay was performed by the method described previously [25], with some modifications. Briefly, 150 µL of a 0.004% DPPH solution was added to the wells of a 96-well microplate, each containing 50 µL of different concentrations (6.25 to 200 µg/mL) of the extract or ascorbic acid (positive control). The microplate was incubated for 20 min in the dark, and the absorbance was measured at 517 nm.

2.4.2. Ferric Reducing/Antioxidant Power (FRAP) Assay

The FRAP method is based on the ability of a compound (antioxidant) to reduce ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}) resulting in the blue colored Fe^{2+}/TPTZ complex. The assay was performed as previously described [26]. In brief, 290 µL of the FRAP reagent (25 mL acetate buffer 300 mM, 2.5 mL TPTZ 10 mM in 40 mM HCl and 2.5 mL $FeCl_3$ 20 mM) was added to 10 µL of the sample (green tea extract) or the positive control (ascorbic acid). The absorbance was measured at 593 nm. An aqueous solution of $FeSO_4$ was used as standard solution.

2.5. Cell Culture

The two cell lines were grown in DMEM supplemented with 10% FBS and 1% pen/strep. Incubation was done at 37 °C, 5% CO_2, and 95% relative humidity. Cells were harvested and used for other procedures at 80% confluency.

2.5.1. MTT Assay

To evaluate the cytotoxicity of the extract, 1×10^4 cells were seeded in each well of a flat-bottom cell culture microplate and incubated for 24 h. The extract was dissolved in dimethyl sulfoxide (DMSO); its concentrations from 0.001 to 1000 µg/ml were prepared by dilution in DMEM (without FBS) and added to each well. Triplicate wells were considered for each concentration. The plate was further incubated for 24 or 48h. MTT powder was dissolved in PBS (pH 7.2) to a concentration of 5 mg/ml. MTT solution (10 µL) was then added to each well and the plates were incubated for 3 to 4 h at 37°C in the dark. Finally, 100 µl DMSO was added to each well (for solubilization of formazan crystals) and cell viability was determined by measuring the average absorbance of each triplicate at 570 nm [27]. The test was repeated three times.

2.5.2. Trypan Blue Dye Exclusion Test

Cells were seeded at 5×10^5 per each well of a flat-bottom 6-well plate and incubated for 24 h. The extract (0.1 to 100 µg/mL) was added to each well and incubated for 24 or 48 h. Triplicate wells were considered for each concentration. The cells were harvested and counted after adding trypan blue [28].

2.6. Statistical Analysis

The median inhibitory concentration at 50% of cells (IC_{50}) values were calculated using GraphPad Prism version 5. Statistical analyses were done using one-way or two-way ANOVA where applicable. *p*-value <0.05 was considered as statistically significant.

3. Results and Discussion

The beneficial effects of green tea including antioxidant, antimutagenic, anticarcinogenic, antihypertensive, antidiabetic, and many other activities have been considered for several years. However, there is a need for further research because of the existing controversy among different reports [3]. Here, an aqueous extract of green tea cultured in the north of Iran (Lahijan) was investigated for the amount of total phenolic compounds and EGC. The antioxidant and antiproliferative effects of the extract were also determined.

3.1. The Amount of Total Phenolic contents and EGC

Each gram of the spray-dried aqueous extract of green tea contained 283.6 ± 10.57 mg gallic acid equivalent (GAE) of total phenolic contents, as determined by the Folin-Ciocalteu method, whereas 88.44 ± 1.85 mg of EGC contents was determined by the HPLC method. The HPLC chromatograms of *C. sinensis* and EGC are presented in Figure 1.

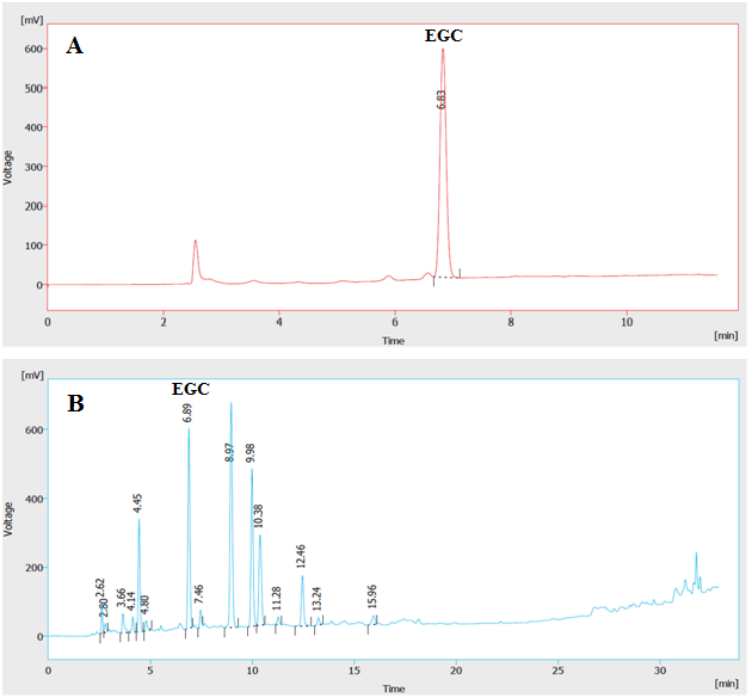

Figure 1. HPLC chromatograms (210 nm) of reference standard (A) and *C. sinensis* extract (B). EGC: (-)-epigallocatechin.

Komes *et al.* reported different amounts of total phenolic compounds in different types of green tea ranging from 880 mg/L to 2560 mg/L. In addition to the type of tea, the amount of total phenolic compounds was influenced by the method of extraction, in a way that water extraction at 100 °C showed the best results [29]. In two other studies, the amount of phenolic acids in the aqueous extracts of green tea was reported to be close to 60 mg/g of the dry extract [30] and 60 GAE/200 µg of the extract [31]. Chong *et al.* reported the amount of gallic acid and EGC as 0.18 and 30 mg per 100 ml of the extract, respectively [9]. Also, an unconcentrated aqueous extract of green tea has yielded 93 mg/g catechins when spray-dried [32]. The amount of EGC reported by Zuo *et al.* for methanolic extracts of four Chinese green teas was between 27.6 to 37.6 mg/g of the dried mass [33]. In general, the amount of gallic acid is thus higher in the black tea, while the amount of catechins is higher in the green tea [3].

3.2. Antioxidant Activity

DPPH was used to evaluate the radical scavenging activity of the extract. The median effective concentration (EC_{50}) value of green tea extract was significantly higher than that of ascorbic acid; however, it was within the acceptable range for antioxidants (Table 1).

The ferric reducing power of the extract and ascorbic acid was measured by the FRAP assay (Table 1). Although the reducing power of ascorbic acid was significantly higher, the power of green tea extract was acceptable. Green tea can hence be considered as a suitable natural antioxidant.

Table 1. Antioxidant capacity (mean ± SD) of the aqueous extract of Lahijan green tea

Compound	DPPH EC_{50} (µg/ml)	FRAP (mmol/g)
Green Tea	84.31 ± 4.14	4.45 ± 0.46
Ascorbic acid	6.59 ± 0.57	11.60 ± 0.57

Several studies have shown that green tea has an antioxidant activity higher than other herbal teas [31, 34, 35]. Based on one study, the EC_{50} of a 2% aqueous infusion was nearly 12 µg/mL calculated by DPPH method [36]. According to Azadi Gonbad *et al.*, the antioxidant activity of 12 tea clones planted in Iran showed the EC_{50} values ranging from 218.24 µg/mL to 234.44 µg/mL as measured by DPPH [21]. In another study, the antioxidant activity of 13 types of green teas displayed EC_{50} values from 0.272 to 1.144 mmol/g of dry plant leaves measured by the FRAP assay. Since the antioxidant activity is directly in correlation with total phenolic contents [37], the variations seen in the reports may reflect disparities between the quality of the green tea samples, which is influenced by the variety, climate, cultivation method, preparation process, and the type of the product, not ignoring data presentation differences (dry plant, extract, infusion, etc). For instance, increases in the daily average temperature will increase the amount of EGC, EC, ECG, and EGCG. Similarly, relative humidity have significant effects on EGC [38]. In addition, it is suggested that the effect of location on catechin contents is higher than the type of cultivar [39].

Furthermore, the extraction method and conditions can strongly affect the antioxidant property of green tea extracts [40].

3.3. Cytotoxicity

The IC_{50} values of the extract on the HT-29 and 3T3 cell lines determined by the MTT assay are presented in Table 2. The results show a moderate cidal effect of the plant extract on the HT-29 cell line after 48 h. This indicates that the extract may have determining effects on the metabolic pathways of the cells. In fact, the antiproliferative effects of aqueous extract of green tea was concentration and time-dependent. The effect of the extract on the 3T3 cells was less than a third of its effect on the cancer cell line. This can suggest the safety of the consumption of the extract on normal cells.

Trypan blue dye exclusion test also revealed that the extract was toxic to the HT-29 cells at 100 µg/ml concentration enough to kill more than 50% of these cells after 48 h treatment (Figure 2). Therefore, it is concluded that the green tea extract might negatively influence cell membrane integrity.

Table 2. The IC_{50} values (mean ± SD) of the aqueous extract of Lahijan green tea on HT-29 and 3T3 cell lines after 24 and 48 h treatment measured by MTT assay and its antioxidant effects measured by DPPH method.

Compound	IC_{50} (µg/ml)			
	HT-29		3T3	
	24 h	48 h	24 h	48 h
Green Tea	492.7 ± 32.81	82.45 ± 18.39	727.2 ± 40.63	315.9 ± 25.11
Doxorubicin	8.79 ± 0.74	3.46 ± 0.66	7.12 ± 0.59	2.63 ± 0.48

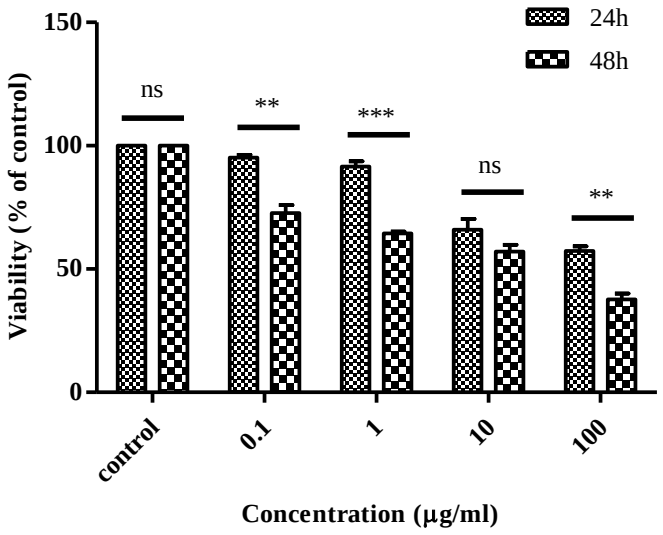

Figure 2. The effect of green tea aqueous extract on HT-29 cell line after 24 and 48 hours treatments measured by trypan blue dye exclusion method. HT-29 Cells were treated with different concentrations of green tea for 24 or 48 hours and the number of live cells was calculated by the Trypan blue dye exclusion method as the percentage of control cells. Mean±SD (N=3). ns: not significant, **: $p<0.01$, ***: $p<0.001$

In previous studies, an aqueous extract of green tea had exerted cytotoxicity on the HepG2 cell line [41]. The aqueous extracts of the green and black tea could also inhibit the growth of 50% of Colo-205 cells at 70 µg/mL, although the green tea extract was more potent [42]. However, Zhao *et al* tested the anticancer effects of the methanolic extracts of some teas on HT-29 cells and found that the green tea was not as effective as the fermented Pu-erh tea (a kind of Chinese tea made from a variant of *C. sinensis*) [43]. Tea components have also shown antiproliferative activity. For instance, EGCG showed anticancer effects on the HT-29 [44] and HepG2 [41] cells. Nonetheless, unlike Valcic *et al.* who reported the antiproliferative effects of EGCG, GC, and EGC on cancer cell lines [45], Ravindranath *et al*. mentioned that EGC does not suppress the growth of prostate and ovarian cancer cell lines in spite of the potent effect of ECG followed by EGCG [46]. Yet, there are several reports of protective effects of green tea consumption against ovarian, prostate, and other types of cancers [3]. Additionally, the green tea extract has been reported to induce DNA repair in yeast, protecting the genome from damage. The total extract has been more effective than EGCG as pure, possibly due to the other components of green tea [9].

4. Conclusion

In this study, the amounts of total phenolic contents and EGC were measured in the aqueous extract of green tea (*C. sinensis*) cultured in Lahijan (Iran). In addition, antioxidant and cytotoxic effects of the extract were evaluated. The results showed the extract contains large amounts of phenolic compounds and EGC. The extract exhibited considerable antioxidant effects. In addition, it showed stronger *in vitro* antiproliferative activity on the cancer cell line as compared to the normal cell line. Therefore, it seems that daily consumption of green tea can have considerable beneficial effects on human health. Further research on this subject is encouraged, especially emphasizing on randomized controlled trials involving human subjects to investigate its clinical outcomes.

Acknowledgments

This study was supported by a grant from Kerman University of Medical Sciences (No. 95000454). The Ethics Approval Code is IR.KMU.REC.1395.685.

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