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Evaluation of Anti-cancer and Pro-apoptotic Activities of Iranian Green Tea Extract Against A549, PC3, and MCF-7 Cancer Cell Lines

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Abstract

Introduction: Green tea contains active polyphenols including catechins. The goal of the current study was to evaluate anti-cancer effects of Iranian green tea extract (IGTE) on 3 human cancer cell lines including A549, PC3, and MCF-7.

Methods: First, *Camellia sinensis* was obtained from Lahijan, a city in the north of Iran and then IGTE was prepared. Next, catechins of IGTE were determined using high-performance liquid chromatography (HPLC). Finally, the cell viability of different cancer cells was evaluated by treatment with IGTE at concentration between 100 and 1000 µg/mL for 72 hours using MTT assay. Cell death of treated cancer cells was assessed by DAPI staining and RT-PCR method.

Results: Our results demonstrated the potential anti-tumor activity of IGTE on MCF-7 cells (IC_{50}= 400 µM), A549 cells (IC_{50}= 500 µM), and PC3 cells (IC_{50}= 600 µM), respectively. Chromatin damages within the nucleus of the treated cancer cells were shown. In addition, we found that IGTE induced apoptosis by up-regulation of Bax (a pro-apoptotic protein) and down-regulation of Bcl2 (an anti-apoptotic protein).

Conclusion: Herein, we showed that IGTE is a potent natural product with anti-tumor activity on breast, lung, and prostate cancer cells. The efficacy of current therapies against cancer is limited by a range of adverse effects, toxicity, and drug resistance; therefore, new therapeutic strategies and more effective agents, particularly with natural origin, are desired and green tea may be a potent candidate in the field of cancer therapy.

Keywords: Iranian green tea extract, HPLC, Cancer cell lines, Apoptosis

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Introduction

Cancer is one of the main causes of human deaths worldwide which is characterized by the abnormal growth of cells and abnormal cells spreading throughout the body. According to the World Health Organization (WHO), lung, breast, and prostate cancers are the most common types of cancers worldwide with the highest death rates.[1] Resistance to anti-cancer drugs and side effects of drugs are major barriers to the success of cancer treatments. Moreover, the increase of various cancers has encouraged the researchers to discover new more effective drugs with the lowest side effects especially from natural products. Among natural products, plants may serve as potent chemotherapeutic agents with less toxicity to normal mammalian tissues and low cost.[2] Tea with high amounts of catechins has been used as a favorite and popular beverage in the world and is considered as a healthy drink with health benefits. All different types of tea are produced from the leaves of *Camellia sinensis* (Theaceae family). Different types of tea result in differences in tea processing.[3,4]

Green tea has been widely studied for its health benefits and potential effects. It has been reported to have anti carcinogenic[5,6] and anti-cardiovascular,[7] inhibition of inflammation.[8,9] Moreover, it has antioxidant activity.[10,11] These benefits result from the catechins in green tea. Green tea catechins (GTCs) are a type of

green tea polyphenols (GTPs) that exist at high levels in green tea. The main GTCs are epigallocatechin-3-gallate (EGCG), epicatechin (EC), epigallocatechin (EGC), and epicatechin-3-gallate (ECG).[12] Several epidemiological studies reported that green tea intake reduced the risk of human cancers.[13,14] Recently, anti-cancer activity of IGTE on Caco-2 colorectal cancer cell line was evaluated.[15]

In this study, the anti-cancer effects of Iranian Green Tea Extract (IGTE) on 3 different human cancer cell lines including human non-small-cell lung cancer A549 cells, human prostate cancer PC3 cells, and human breast cancer MCF-7 cells were investigated. Interestingly, our study is the first report focusing on green tea extract originated from Iran with potential dietary agents for cancer chemoprevention or chemotherapy against 3 common cancers in Iran including breast, lung, and prostate.

Materials and Methods

Sample Preparation and Extraction

The fresh leaves of related plants were collected in March 2018 from Lahijan, Guilan province, Iran. *Camellia sinensis* was recognized in the Department of Pharmacognosy, Tehran University of Medical Sciences and the herbarium was registered as THE-6561. The young leaves were shade dried at room temperature for two weeks. Plant extraction was performed as previously described and stored at -20°C.[16]

HPLC Analysis of Total Catechins in Iranian Green Tea Extract

The total catechin standard was obtained from Sigma (a gift from Dr. D. Bakhshi, University of Guilan, Iran; (+)–Catechin hydrate, C15H14O16, FW 290.3 and 98% purity; Cas No: 88191-48-4). Standard was prepared at a concentration of 100 mg/mL diluted in methanol. The HPLC analysis was performed using a PLATIN blue system (Knauer, Berlin, Germany). HPLC system equipped with a C18 column (5 µm, 150 mm×4 mm) was used for the separation of analytes. The HPLC measurement conditions included flow rate (1 mL/min) and the wavelength (270 nm) at ambient temperature. UV–visible was used as detector in this procedure. The mobile phase for the gradient elution consisted of Solvent A, 10 mM sodium phosphate (pH 2.6), and Solvent B, acetonitrile (93:7, v/v). Both the extract and standard were injected (injection volume: 20 µL) into reverse phase column and identifications were carried out using comparison of retention times and UV spectra of the extract and standard. Each experiment was repeated at least 3 times and run in triplicate.

Cell Lines, Cell Culture, and MTT Assay

Human non-small-cell lung cancer A549 cells, human breast cancer MCF-7 cells, and human prostate cancer PC3 cells were grown in conditions that have been mentioned in our previous experiment.[17]

The cytotoxicity of GTE against cancer cell lines was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide or MTT) assay (MTT assay kit, Bio IDEA, CatNo:BI1017, Iran) according to our previous report.[17]

DAPI Staining Assay

DAPI staining assay was used to determine chromatin changes in A549 cells treated with IGTE for 24 hours. DAPI was purchased from Sigma-Aldrich (USA). All images were taken by an inverted fluorescent microscope (Nikon Eclipse Ti-E).[17]

RNA Extraction, cDNA Synthesis, and Real-time PCR

Total RNA was extracted using 500 µL of Trizol® reagent according to the protocol provided by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA, USA), and cDNA was synthesized using ReveretAid M-Mulv Reverse Transcriptase Kit (Thermo Fisher Scientific, MA, USA). Real-time RT-PCR was then performed to amplify cDNA using SYBR green dye universal master mix (Bioron GmbH, Germany) and the primers (Pishgam Biotech Co. Tehran, Iran) for GAPDH, Bax and Bcl-2 for 40 cycles. Data represent averaged copy number normalized to the GAPDH housekeeping gene. Thermal conditions of the PCR consisted of primary denaturation at 94°C for 2 minutes, 45 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, and amplification at 72°C for 30 seconds. All reactions were triplicated.[18]

Statistical Analysis

Data was analyzed and presented using SPSS version 22.0 (Chicago, IL, USA) and graphs were generated using GraphPad Prism 7 software. The results were expressed as means ± standard deviation (SD). Between-group comparisons were performed using independent sample *t*-test. *P*-value of less than 0.05 was considered statistically significant.[18]

Results

For further confirmation of composition, IGTE was also subjected to HPLC technique. HPLC chromatogram of authentic standards is shown in Figure 1A. The identification of bioactive constituents was done according to the retention time (t_R) obtained from authentic standards under identical HPLC conditions. As shown in Figure 1B, a peak corresponding to catechins in IGTE was identified in the order of catechins (6-7 minutes). In this study, linear regression was used for the measurement of the content, and the assay showed excellent linearity between Y (peak area of the standard polyphenols) and X (concentration of the polyphenols) with the correlation coefficients (R2) in the range of 0.9996 and the quantitative data were analyzed. The quantitative data were analyzed using their related calibration curves.

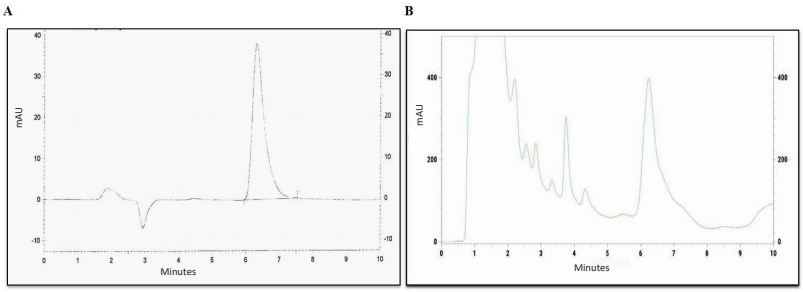

Figure 1. Typical HPLC chromatograms for the separation and analysis of (A) authentic standard of catechins and (B) catechins found in IGTE, as detected by absorbance at 270 nm. Analysis of HPLC was performed as explained in the experimental section.

Effect of IGTE on Viability of Cells

The anti-cancer activities of IGTE against MCF-7, A549, and PC3 cells were evaluated by MTT assay. To do so, we cultured 3 cancer cell lines and then the cells were treated with different concentration of IGTE (100-1000 µg/mL) for 72 hours (Figure 2A, 2B, 2C). We used untreated cells and treated cells with Etoposide as negative and positive controls, respectively. IGTE showed different cytotoxicity against A549, MCF-7, and PC3 cell lines (IC_{50}= 500 µM in A549, 400 µM in MCF-7, and 600 µM in PC3) (Table 1).

The Evaluation of the Changes in Cellular Nuclear Morphology Using DAPI Staining

To further determine the effect of IGTE on apoptotic cell death, the changes in cellular nuclear morphology of MCF-7, A549, and PC3 cell lines exposed to IGTE were examined by DAPI staining. As shown in Figure 3, the characteristic chromatin condensation and nuclear fragmentation were clearly shown in IGTE-treated cells. However, cells without IGTE treatment displayed potential health benefits with a large round nucleus and normal chromatin patterns.

Induction of Apoptosis in A549 Cells by Up-regulation of Bax and Down-regulation of Bcl-2

In order to observe the effect of IGTE on the induction of apoptosis mediated by mitochondrial pathway, a quantitative real-time PCR was conducted on the desired target genes (*Bax, Bcl-2*) in A549 cells lines treated with IGTE for 72 hours at IC50 of 500 µM (Table 2). As shown in Figure 4, IGTE induced apoptosis by increasing mRNA expression of pro-apoptotic Bax and decreasing the expression of anti-apoptotic Bcl-2 in A549 cells lines. GAPDH was used as internal control. Our results indicated that the induction of apoptosis by IGTE in A549 might be attributed to the imbalance of the Bax and Bcl-2 expression.

Discussion

Green tea is a popular and favorite beverage worldwide. The main components of green tea are catechins, a family of polyphenols. EGCG is the major polyphenolic component of dried green tea extracts. In green tea leaves, EGCG accounts for at least 50% of the total catechin content.[19] Many studies have been conducted administrating green tea extracts or pure EGCG and have shown that green tea consumption has beneficial effects on many human diseases. The involvement of several mechanisms in the protective effects of green tea extract and EGCG was demonstrated, including stimulation of anti-oxidant activity and activation of detoxification system,[20,21] alteration of the cell cycle,[22] suppression of mitogen-activated protein kinase (MAPK) and receptor protein kinase (RTKs) pathways,[23,24] induction of apoptosis pathway mediating pro- and anti-apoptotic protein (Bax,

Table 1. IC50 Values of IGTE against Different Cells

Compounds	PC3 Cells	A549 Cells	MCF-7 Cells
IGTE	600±0.015	500±0.017	400±0.010
Etoposide	40±0.046	50±0.029	60±0.009

Note. Data are expressed as mean ± SD of three independent experiments.

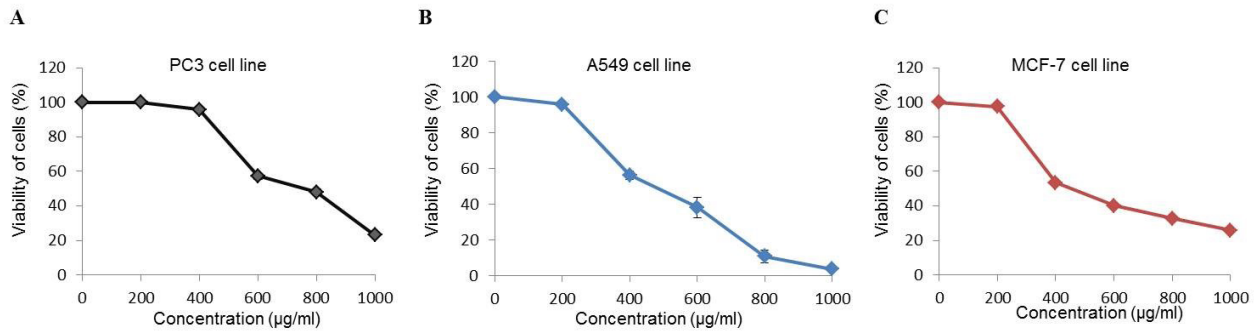

Figure 2. Effects of IGTE on cellular viability in different cancer cells including PC3, A549, and MCF-7 cells were shown (A, B, C). The cells were treated with medium at different concentrations of IGTE for 72 hours.

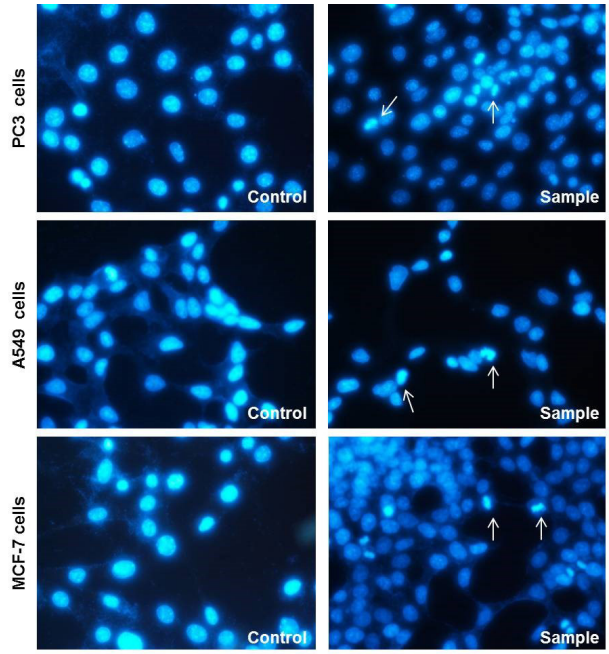

Figure 3. Inverted fluorescent microscopy images of chromatin damages occurred in the nucleus of treated cells with IGTE (as sample) and untreated cells (as control), which have been stained with DAPI in PC3, A549, and MCF-7 cancer cell lines. The experiments were done three times (original microscope magnification, 40X, Scale bar, 10 µm).

Table 2. List of Primers Used in RT-PCR Studies

Gene	Primer Sequence
GAPDH	For 5′-CAA GGT CAT CCA TGA CAA CTT TG-3′ Rev 5′-GTC CAC CAC CCT GTT GCT GTA G-3′
Bax	For 5′-GTC GCC CTT TTC TAC TTT GCC -3′ Rev 5′-CTC CCG CCA CAA AGA TGG TCA-3′
Bcl-2	For 5′-CCC CTC GTC CAA GAA TGC AA-3′ Rev 5′-TCT CCC GGT TAT CGT ACC CTG-3′

Bcl-2, Bcl-XL), and cell cycle regulator proteins (cyclins, CDKs).[25]

In this study, we showed anti-cancer activity of IGTE on different cancer cell lines including MCF-7, A549, and PC3. We demonstrated apoptotic effects of IGTE on human non-small-cell lung cancer A549 cells. IGTE caused a significant increase of Bax and decrease of Bcl-2 mRNA, thereby indicating that intrinsic pathway of apoptosis was induced by IGTE in A549 cells.

Interestingly, several in vivo studies have been done concerning the protective effects of green tea on lung cancer.[26-28] However, some studies did not show the protective effects of green tea.[29-31] It seems that the protective effects of green tea depend on many factors including the dose of green tea consumption and genotype variation in populations. Lin et al studied genotype variations in lung cancer patients and reported a significantly higher tumor incidence in never tea drinkers.[32]

From *in vitro* studies, it was found that EGCG inhibited MMP-2 and -9 expressions in human lung cancer A549 cells.[33] Additionally, an inhibitory effect of EGCG on the migration of bronchial tumor cells in 2D and 3D cell culture models has been shown. EGCG treatment also inhibited the expression of MMP-2 in both mRNA and protein levels and altered the intermediate filaments of vimentin.[34] Moreover, green tea extraction induced expression of actin-binding protein, annexin-I at the

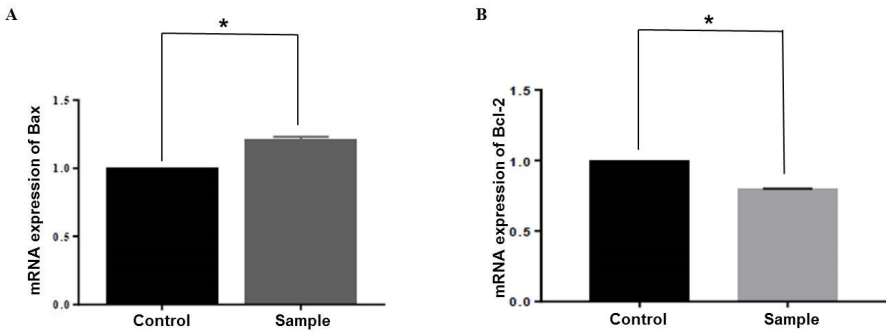

Figure 4. Relative expression of Bax mRNA (A) and relative expression of Bcl-2 mRNA (B) in A549 cells that were treated with IGTE (as sample) and untreated cells (as control) were shown. Data represent mean ± SD of 3 independent experiments. * indicates significance with a P value < 0.05.

transcriptional level in A549 cells. The increased expression of annexin-I correlates with the stimulation of filamentous-actin (F-actin) polymerization, which in turn results in the increase of cell adhesion and decrease of cell motility in A549 cell line.[35]

Conclusion

In summary, the findings of the present study provide evidence for the protective effects of IGTE on lung cancer A549 cell. In vivo experiments will be required to confirm our results. These results may be helpful for interested researchers to design anti-cancer drugs.

Ethical Approval

Not applicable.

Competing Interests

There is no conflict of interests.

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