Alınış / Received: 29.08.2024 Kabul / Accepted: 30.11.2024 Online Yayınlanma / Published Online: 25.04.2025
Araştırma Makalesi / Research Article DOI: 10.22312/sdusbed.1539732

Süleyman Demirel Üniversitesi Sağlık Bilimleri Dergisi
Suleyman Demirel University Journal of Health Sciences

Uncovering the Benefits of Epicatechin for Oxidative Stress in Human Health

İnsan Sağlığında Oksidatif Stres için Epikateşinin Faydalarının Ortaya Çıkarılması

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ABSTRACT

Objective: Epicatechin (EC) is one of the major components of green tea (*Camellia sinensis*) catechins. This study investigated the effect of the amount of epicatechin obtained by brewing green tea under optimal conditions against oxidative stress induced by hydrogen peroxide (H_2O_2). Materials and Methods: In peripheral blood mononuclear cells (PBMCs), the amount of epicatechin determined by brewing green tea under optimum conditions was applied against 250 µM H_2O_2 and cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, total antioxidant status (TAS) levels and total oxidant status (TOS) levels were determined by biochemical analysis, apoptosis-related Bax, Bcl2, p53 expression analysis was determined by quantitative real time polimerase chain reaction (qRT-PCR) method. Results: The cell viability was significantly higher in the H_2O_2+EC group than in the H_2O_2 group ($p< 0.001$). TOS and TAS levels were changed considerably in the H_2O_2+EC group compared to the H_2O_2 group ($p<0.05$ and $p<0.001$, respectively). Bax, p53 expression level decreased in H_2O_2+epicatechin treated cells compared to H_2O_2 treated cells ($p<0.001$ and $p<0.01$ respectively), while Bcl2 expression level increased in H_2O_2+epicatechin treated cells compared to H_2O_2 treated cells $p<0.01$). Conclusion: The results show that the amount of epicatechin obtained from brewing green tea under optimum conditions has a protective effect on peripheral blood mononuclear cells (PBMCs) against H_2O_2 induced oxidative stress. Specifically, it was concluded that epicatechin increased cell viability, decreased oxidative stress markers and modulated the expression of key apoptosis-related proteins, thus promoting cell survival.

Keywords: *Bax, Bcl2, Epicatechin, Oxidative stress, p53*

ÖZ

Amaç: Epikateşin (EC) yeşil çay (*Camellia sinensis*) kateşinlerinin ana bileşenlerinden biridir. Bu çalışmada, yeşil çayın optimum koşullarda demlenmesiyle elde edilen epikateşin miktarının hidrojen peroksit (H_2O_2) tarafından indüklenen oksidatif strese karşı etkisi araştırılmıştır. Materyal ve Metot: Periferik kan mononükleer hücrelerinde (PBMCs), yeşil çayın optimum koşullarda demlenmesi ile belirlenen epikateşin miktarı 250 µM H_2O_2'ye karşı uygulanmış ve hücre canlılığı 3-(4, 5-dimetiltiyazol-2-yl)-2,5-difeniltetrazolyum bromür (MTT) testi ile, total antioksidan seviyeleri (TAS) ve total oksidan seviyeleri (TOS) biyokimyasal analiz ile, apoptoz ile ilişkili Bax, Bcl2, p53 ekspresyon analizi gerçek zamanlı kantitatif polimeraz zincir reaksiyonu (qRT-PCR) yöntemi ile belirlenmiştir. Bulgular: H_2O_2+EC grubunda hücre canlılığı H_2O_2 grubuna göre anlamlı derecede yüksek bulunmuştur ($p<0.001$). TOS ve TAS seviyeleri, H_2O_2 grubuna kıyasla H_2O_2+EC grubunda önemli ölçüde değişmiştir (sırasıyla $p<0.05$ ve $p<0.001$). Bax, p53 ifade düzeyi H_2O_2+epikateşin uygulanan hücrelerde H_2O_2 uygulanan hücrelere kıyasla azalırken (sırasıyla $p<0.001$ ve $p<0.01$), Bcl2 ifade düzeyi H_2O_2+epikateşin uygulanan hücrelerde H_2O_2 uygulanan hücrelere kıyasla artmıştır ($p<0.01$). Sonuç: Sonuçlar, yeşil çayın optimum koşullar altında demlenmesinden elde edilen epikateşin miktarının H_2O_2 kaynaklı oksidatif strese karşı periferak kan mononükleer hücreler (PKMH) üzerinde koruyucu bir etkiye sahip olduğunu göstermektedir. Spesifik olarak, epikateşinin hücre canlılığını artırdığı, oksidatif stres belirteçlerini azalttığı ve apoptozla ilgili anahtar proteinlerin ekspresyonunu modüle ettiği, böylece hücre sağ kalımını desteklediği sonucuna ulaşılmıştır.

Anahtar Kelimeler: *Bax, Bcl2, Epikateşin, Oksidatif stres, p53*

INTRODUCTION

Epicatechin EC is one of the main components of green tea catechins (1). Green tea has characteristics of meta-5,7-dihydroxy groups in chain A and dihydroxy or trihydroxy groups in chain B (2). The B chain seems essential to the antioxidant reactions (3). Antioxidant activity is a molecule or ion's capacity to prevent other molecules' oxidative reactions (4). EC has effective and direct antioxidant activity. By its strong antioxidant capacity, it scavenges free radicals in cells. Compared with vitamin C and vitamin E, the antioxidant capacity of EC is 20 and 50 times greater. The biological activity of ECs is mainly a result of interactions with proteins and lipids. These interactions result in an effect on the levels of oxidants (5).

Oxidative stress, characterised by an imbalance between free radicals and antioxidants, has a significant impact on several health conditions such as infertility, cancer, diabetes, metabolic syndrome, atherosclerosis, neurodegenerative, cardiovascular, gastrointestinal and liver diseases. H_2O_2 is a crucial reactive oxygen species (ROS) and plays a significant role in biological processes. Generally, when it exceeds 50 µM, it causes oxidative damage in tissues and organs and elicits an inflammatory response. Antioxidants are essential for neutralising free radicals and thus preventing oxidative damage. They can be enzymatic or non-enzymatic and work by scavenging free radicals, chelating metal ions or upregulating other antioxidant defences (6).

Green tea is considered one of the healthiest drinks around the globe. Its acceptance in this way is due to its rich structure in polyphenols (7, 8). While many studies have shown that plant-derived flavonoids have excellent antioxidant activity,(9, 10) research on green tea in recent years has focused on the relationship between consumption and disease prevention (7).

The catechins and sensory properties may differ depending on the green tea brewing conditions. Our aim with this study was to understand if the amount of epicatechin determined according to the optimum conditions during green tea brewing has a protective effect on cells against H_2O_2.

MATERIAL and METHOD

Isolation of Human PBMC

Peripheral venous blood was collected from heparin tubes from healthy volunteer who had not been exposed to radiation or any drugs or smoked for six months. The Declaration of Helsinki was followed in the study. This study was approved by the Süleyman Demirel University Medical Faculty Ethics Committee (decision dated 05.12.2023 and numbered 15/270). PBMCS were isolated by Histopaque 1077 (Sigma-Aldrich, Switzerland).
Cell viability was determined to be 98% using trypan blue stain. The medium was changed once every 24 hours (11). The workflow of the study is given in Figure 1.

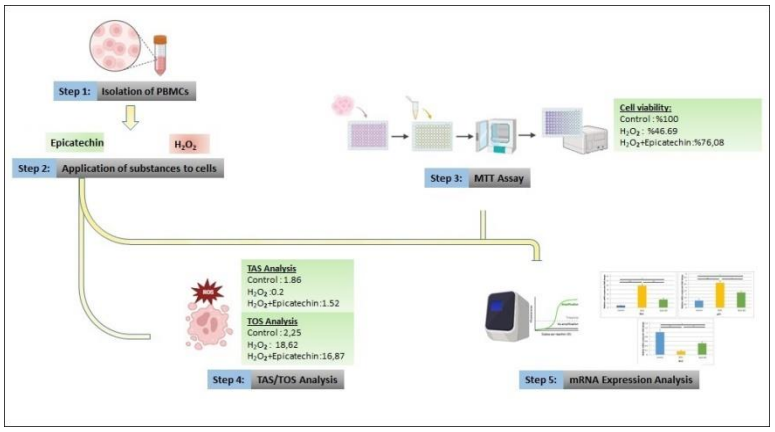

Figure 1: MTT assay, TAS-TOS levels and expression analyses after epicatechin and H_2O_2 treatment of cells

MTT Assay

PBMCs were seeded in 96-well flat-bottomed microplates (Sarstedt AG, Germany) at $1x10^4$ cells/well density. A 5% CO_2 incubator at 37 °C was incubated for 24 h before any treatments.

For the best result of taste and sensory properties, brewing at 85 °C for 3 minutes represents the optimal condition. Under these conditions, an epicatechin maximum of 6.75 mg/100 mL was determined (12). Epicatechin (Sigma Chemical Co., USA) of 67.5 ppm was cultured with cells in an incubator for 24 h (37 °C, 5% CO_2). During the last hour, these cells (except the control group) were incubated with 250 µM H_2O_2 (13).

The medium consisted of the following components: RPMI-1640 (Biological Industries, Israel) medium, 10% FBS (Sigma - Aldrich, USA) and 100 IU/mL penicillin, 100 µg/mL streptomycin (Sigma - Aldrich, USA). MTT (Sigma, USA) final concentration was adjusted to 0.5 mg/mL. The resulting formazan crystals were dissolved in DMSO. A multiscan plate reader (Synergy HTX BioTek, USA) was used to measure cell viability at 570 nm (14). Cell viability was evaluated in percentage relative to the control group, denoted as 100%. Three individual wells were measured per treatment point.

Biochemical Analysis

After the culture step, plates were centrifuged at 1800g for 6 minutes, and the pellet was washed with PBS. An ultrasonic homogenizer was used to homogenize the cells. After the second centrifugation, supernatants were transferred to Eppendorf tubes for analysis (15).
TAS and TOS levels were measured by spectrophotometric method (Beckman Coulter AU 5800, USA) in triplicate with commercial kits (Rel Assay Diagnostics, Türkiye) according to the kit protocol (16). The results were expressed as millimolar Trolox equivalents per liter in TAS and micromolar hydrogen peroxide equivalent per liter (µmol H_2O_2 Eqv/L) in TOS (17).

RT-qPCR Analysis on mRNA

Total RNA extraction, purity and concentration measurement, cDNA extraction were performed similar to our previous study and according to the manufacturer's protocol (18). Primers were designed to detect specific mRNA sequences. The NCBI website was used to test possible primer sequences. Bax (F:5′-CAGGGGCCCTTTTGCTTCA-3′ R:5′-GGAAAAAGACCTCTCGGGGG-3′), Bcl-2 (F:5′-AAAAATACAACATCACAGAGGAAGT-3′ R:5′-TCCCGGTTATCGTACCCTGT-3′), p53 (F:5′-ACCTATGGAAACTACTTCCTGAAA-3′ R:5′-GCTGCCCTGGTAGGTTTTCT -3′) primers were designed to amplify. ACTB (F: 5′-GCCTCGCCTTTGCCGAT-3′ R:5′- AGGTAGTCAGTCAGGTCCCG-3′) expression was used for normalization. The manufacturer's instructions were followed for real-time RT-PCR conditions. The $2^{-\Delta\Delta Ct}$ comparative method was used for relative quantification of gene expression. To determine amplification specificity, qPCR products were evaluated using melting curves. Each sample was run in triplicate.

Statistical Analysis

The results of the expression study were evaluated using SPSS 18.0 statistical analysis software (SPSS Inc., Chicago, IL). One-way ANOVA was used to analyze the results of the expression, MTT, TAS, and TOS levels. LSD and TUKEY tests were used as post-hoc tests. $p<0.05$ was considered to be significant.

RESULTS

MTT Assay Results

Cell viability was significantly lower in the H_2O_2 and H_2O_2+epicatechin groups compared with the control group ($p<0.001$). In the comparison of cell viability in the H_2O_2 group and the H_2O_2+epicatechin group, the H_2O_2+epicatechin group was significantly higher ($p<0.001$) (Figure 2).

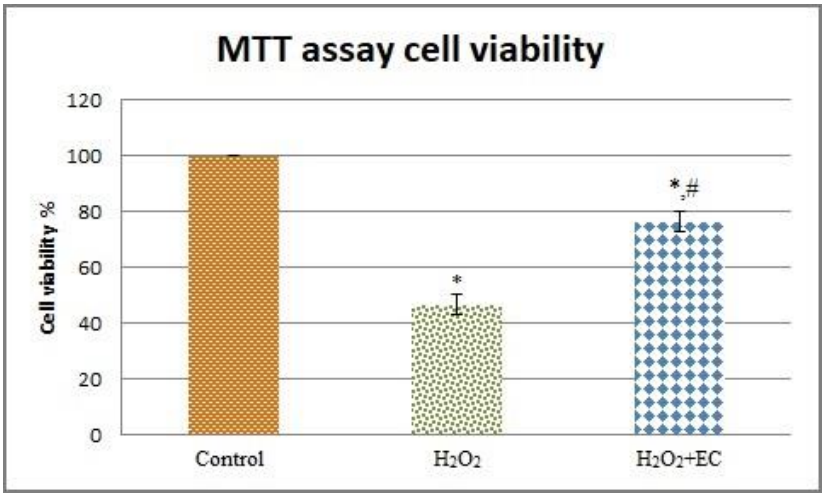

Figure 2: Cell viabilty. Data expressed as mean±SD. Comparision between groups and result were assessed by one-way ANOVA test. *p<0.001 ascompared to the control group, #p<0.001 compared to the H_2O_2-treated group.

Biochemical Results

TOS level was significantly higher in the H_2O_2 and H_2O_2+epicatechin groups than in the control group (p<0.001). TOS level was considerably higher in the H_2O_2 group than in the H_2O_2+epicatechin group (p<0.05). TAS level was significantly lower in the H_2O_2 group than in the control group (p<0.001) and considerably higher in the H_2O_2+epicatechin group (p<0.001). TAS level was significantly higher in the H_2O_2+epicatechin group than in the H_2O_2 group (p<0.001) (Table 1).

Table 1: TOS and TAS levels of PBMCs

Groups	Negative Control	H_2O_2	H_2O_2+EC
TOS (μmol H_2O_2 Eq./L)	2.25±0.74	18.62±0.82*	16.87±0.53*,#
TAS (mmol TroloxEq./L)	1.86±0.08	0.20 ± 0.04*	1.52± 0.04*,##
OSI (μmol $H2O_2$ equiv./lt)/(mmol Trolox equiv./lt x 10)	0.12±0.04	9.36±1.49	1.11±0.02

Data are expressed as mean±SD. One-way ANOVA test was used to assess comparisons between groups and results of oxidative stress markers. LSD tests were used as post-hoc tests. *p<0.001 compared to the control group; #p<0.05, ##p<0.001 compared to the H_2O_2 treated group.

Expression Analysis of Bax, Bcl, p53

The relative mRNA level of Bax in the H_2O_2, H_2O_2+epicatechin groups increased significantly compared to the control group (respectively; p<0.001 and p<0.01) and lower significantly in H_2O_2+epicatechin group compared to the H_2O_2 group (p<0.001). The relative mRNA level of p53 in the H_2O_2, H_2O_2+epicatechin groups increased significantly compared to the control group (respectively; p<0.001 and p<0.01) and lower significantly in H_2O_2+epicatechin group compared to the H_2O_2 group (p<0.01). The relative mRNA level of Bcl-2 in the H_2O_2, H_2O_2+epicatechin groups decreased significantly compared to the control group (respectively; p<0.001 and p<0.01) and lower significantly in H_2O_2+epicatechin group compared to the H_2O_2 group (p<0.01) (Figure 3).

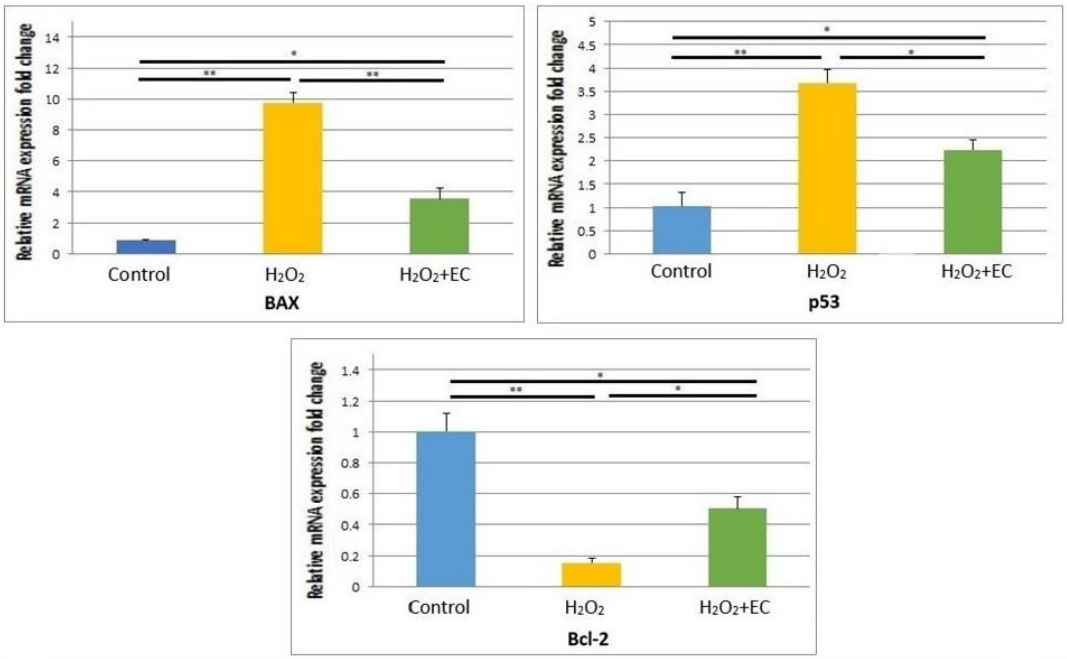

Figure 3: Relative Mrna Expression Results And Statistical Comparison Between Groups.
Bax: Bcl-2-associated X protein; p53: Tumor Protein P53; Bcl2: B-cell lymphoma 2;Values are presented as means±SD. *p<0.01, **p<0.001.

DISCUSSION and CONCLUSION

PBMCs, consisting mainly of lymphocytes and monocytes, are a readily available blood cell fraction with high research potential for testing the effects of dietary intake. At the gene expression level, they reflect the impact of environmental changes. Many studies demonstrated the utility of PBMCs in showing the effects of nutrition, training, and vigorous exercise on mitochondrial oxidative balance, biosynthesis, dynamics, and antioxidant capabilities (19,21). In this context, PBMCs were considered more appropriate for our research.

Oxidative stress is the result of an imbalance between free radicals and antioxidants. It causes changes in the structure of cell membranes, lipids, proteins, lipoproteins, and DNA. Mitochondria are the most important endogenous source of ROS generation, as they play a role in forming ATP through oxidative phosphorylation, which reduces molecular O_2 to H_2O via the electron transport chain (22). A study showed that heavy exercise enhances the oxidative stress-induced apoptosis (23). Another study demonstrated that exopolysaccharide-selenium nanoparticles promoted cell survival by maintaining over 90% cell viability under oxidative stress caused by 0.4 mM H_2O_2 in HepG2 cells (24). H_2O_2 is a source of reactive oxygen species, and functions act to induce oxidative stress (25). H_2O_2 is commonly considered a cytotoxic substance that must be reduced by antioxidant defense enzymes (26).

An ex-vivo study has suggested that green tea consumption prevents LDH oxidation in humans, while Epicatechin also plays a role in the prevention of neurodegenerative diseases such as Alzheimer's and Parkinson's (27). In addition, in an in vivo study, tea catechins were observed to reduce the formation of atherosclerosis in mice with apolipoprotein E deficiency (28), while another study found that the antioxidant capacity of epicatechin in human plasma increased by 40% compared to non-users (29).

Our results show that the treatment with H_2O_2 led to a significant decrease in the viability of the PBMCs. However, epicatechin had a protective effect on cell viability when used against H_2O_2, which causes oxidative stress. In addition, our results showed that the H_2O_2-induced increase in TOS levels in PBMCs decreased with epicatechin treatment, while TAS levels increased.

In a study, it was found that after treatment of mouse granulosa cells and human granulosa cells with H_2O_2, the viability rate of the cells decreased with concentration, while the expression levels of p53 and Bax increased. These findings suggest that H_2O_2-oxidative stress may be a factor in the onset of apoptosis. Because when DNA damage is limited and reversible, cells stop proliferating. Some cells exposed to DNA damage enter the cell cycle. However, when DNA damage is irreparable, cells undergo immediate apoptosis, thereby causing induction of p53 and p21 expression (30). In another study, (-)-epicatechin was found to upregulate death receptors (DR4/DR5) and modulate pro-apoptotic proteins in MDA-MB-231 cells. In contrast, it did not activate the death receptor in MCF-7 cells (31).

Similar to the studies conducted with cell culture in the literature, in our research, Bax, p53 expression increased while Bcl2 expression decreased in cells treated with H_2O_2 only compared to control cells. In H_2O_2+EC treated cells, Bax, p53 expression decreased while Bcl2 expression level decreased compared to the H_2O_2 group. Bax, p53, and Bcl2 genes networks and functions are present in Figure 4 (https://genemania.org/).

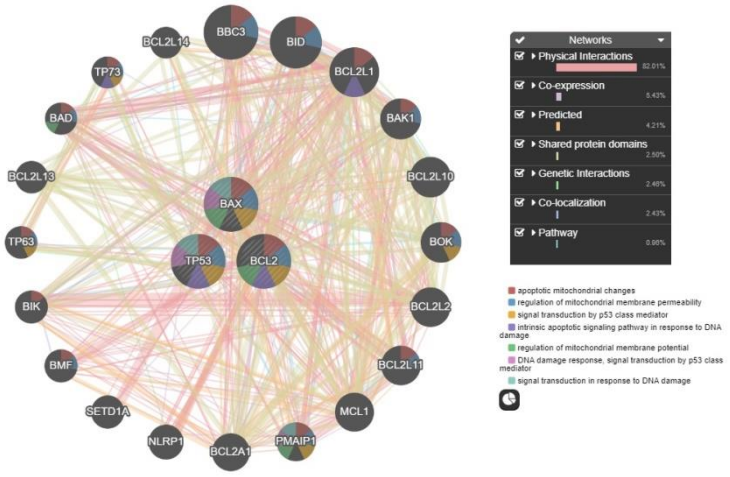

Figure 4: Bax, p53, Bcl2 genes networks and functions

The mechanisms of action of natural antioxidants are unclear. Further research is needed to identify the active target sites. In this direction, in our study, the mechanism by which the amount of epicatechin determined by brewing green tea under optimal conditions plays a protective role against H_2O_2-induced oxidative stress was revealed by genetic and biochemical tests. However, in addition to cellular studies, it is thought that the amount of epicatechin obtained by brewing and consuming green tea under optimal conditions will be insufficient to reduce the effects of oxidative stress in people who exercise excessively. We believe that this study may provide a basis for in vivo studies.

Declaration of Ethical Code: In this study, we undertake that all the rules required to be followed within the scope of the "Higher Education Institutions Scientific Research and Publication Ethics Directive" are complied with, and that none of the actions stated under the heading "Actions Against Scientific Research and Publication Ethics" are not carried out.

This study was approved by the Süleyman Demirel University Medical Faculty Ethics Committee (decision dated 05.12.2023 and numbered 15/270).

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