Research Article

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Investigation of antioxidant, cytotoxic, tyrosinase inhibitory activities, and phenolic profiles of green, white, and black teas

Yeşil, beyaz ve siyah çayın antioksidan, sitotoksik, tirozinaz inhibitör aktiviteleri ve fenolik profillerinin araştırılması

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Abstract

Objectives: To determine of antioxidant, cytotoxic and tyrosinase inhibitory (TI) activities, and phenolic profiles with a new high performance liquid chromatography (HPLC) method of green, white and black teas.
Methods: Antioxidant activities of the teas were examined by means of scavenging of the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and cupric reducing antioxidant capacity (CUPRAC). The phenolic contents were investigated by means of HPLC. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the cytotoxic potential of tea extracts in cancer and normal cell lines. TI activity was investigated against diphenolase (DOPA), using kojic acid as a positive control.
Results: The green tea extract exhibited stronger antioxidant than white and black tea extracts. The green tea contained syringaldehyde, *p*-coumaric acid, benzoic acid, and quercetin. The white tea extract had the highest tyrosinase inhibitor activity. The extracts exhibited higher cytotoxic potential toward cancer cells than normal cells. The methanol extract of green tea had the highest cytotoxic potential, while the water extracts were less toxic.
Conclusion: The green, white, and black teas can be regarded as an important in terms of rich antioxidant, tyrosinase inhibitor, and cytotoxic activities in both the pharmaceutical and food industries.

Keywords: Antioxidants; Black tea; Green tea; White tea; HPLC; Tyrosinase inhibition.

Öz

Amaç: Yeşil, beyaz ve siyah çayın antioksidan, sitotoksik ve tirozinaz inhibitör (TI) aktivitelerini ve yeni yüksek performanslı sıvı kromatografisi (HPLC) metodu ile fenolik içeriklerini belirlemek.
Metod: Çayların antioksidan aktiviteleri; 2,2-difenil-1-pikril hidrazil (DPPH) serbest radikalin süpürülmesi, demir indirgeyici antioksidan güç (FRAP) ve bakır indirgeyici antioksidan kapasite (CUPRAC) ile incelendi. Fenolik içeriği HPLC ile incelendi. MTT yöntemi normal ve kanser hücre hatlarında çay ekstrelerinin sitotoksik potansiyelini değerlendirmek için kullanıldı. TI aktivite, pozitif kontrol olarak kojik asit kullanılarak difenolaza (DOPA) karşı incelendi.

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Bulgular: Yeşil çay ekstresi beyaz ve siyah çay ekstresinden daha güçlü antioksidan aktivite gösterdi. Yeşil çay şiringaldehid, p-kumarik asit, benzoik asit ve kersetin içerdi. Beyaz çay ekstresi en yüksek tirozinaz inhibitör aktivite gösterdi. Ekstreler, kanser hücrelerine karşı normal hücrelerden daha yüksek sitotoksik güce sahipti. Yeşil çayın su ekstresi daha az toksik iken, metanol ekstresi en yüksek sitotoksik güce sahipti.
Sonuç: Yeşil, beyaz ve siyah çay hem farmasötik hem de besin endüstrisinde zengin antioksidan, tirozinaz inhibitör ve sitotoksik aktiviteleri açısından önemli olarak düşünülebilir.

Anahtar Kelimeler: Antioksidanlar; Siyah çay; Yeşil çay; Beyaz çay; HPLC; Tirozinaz inhibisyonu.

Introduction

Reactive oxygen species (ROS) including superoxide anion, hydroxyl radical, hydrogen peroxide, and lipid peroxide are synthesized by various means in vivo [1]. These molecules are extremely reactive and are capable of modifying a range of biological substrates, such as proteins, amines, and deoxyribonucleic acids. These reactions have also been implicated as exacerbating factors in cellular injury and in aging [2]. They also induce lipid peroxidation, a process resulting in loss of food quality [3]. Natural antioxidants obtained from plants are currently the subject of significant interest for the purpose of protecting the human body against attack by free radicals [4]. Polyphenols have emerged as particularly promising natural antioxidants. These chemicals are contained in numerous plant materials, especially vegetables, tomatoes, and teas [4]. The majority of tea on the commercial market comes from the leaf of *Camellia sinensis*. Green tea is particularly popular in Asian countries, such as China and Japan, while black tea is more widely consumed in North America and Europe. White tea is harvested primarily in China, but has also recently been produced in Eastern Nepal, Taiwan, Northern Thailand, and India. Green teas are steamed and roasted, and may be oven-fired in order to prevent enzymatic oxidation. White tea belongs to the group of unfermented teas that do not require panning, rolling or shaking. Black tea undergoes the highest level of enzymatic oxidation. The level of oxidation directly impacts on polyphenolic profile of tea. [5]. Various possible mechanisms have been proposed to explain the scavenging of ROS and free radicals by these polyphenols [6]. These include electron depolarization, the production of intramolecular hydrogen bonds and the rearrangement of the molecular structure [7]. Polyphenols may also obstruct oxidative reactions through the chelation of free copper and iron, capable of catalyzing the development of ROS in the living organisms. Tea consumption has recently increased due to the discovery of numerous health benefits attendant upon regular ingestion. These benefits include a lower risk of cardiovascular disease, activity against some types of cancer, inflammatory bowel disease, neurodegenerative diseases and diabetes, and even weight loss [8].

Tyrosinase is a copper-containing mono-oxygenase responsible for catalyzing melanin synthesis in melanocytes. One of the two major reactions catalyzed by tyrosinase is the hydroxylation of tyrosine oxidation of L-Dopa, a product of O-diphenyl. This oxidation results in a highly reactive intermediate that undergoes further oxidization to produce melanin via the free radical-coupling pathway [9]. Melanin is the major coloring pigment in human skin. It is released by melanocyte cells found in the lowest stratum of the epidermis. Chronic exposure to sunlight, melasma, or other hyper-pigmentation diseases may result in excessive production of melanin. Various depigmenting agents have therefore been developed to counter unsightly skin discoloration. This has in turn encouraged researchers to seek novel and powerful tyrosinase inhibitors for use against discoloring in foodstuffs and as skin whitening agents. Various tyrosinase inhibitors have already been discovered and described [10, 11]. As part of our research into medicinal plants as sources of new enzyme inhibitors with the potential for use as skin whitening agents, we investigated the tyrosinase inhibition potential, phenolic composition, antioxidant activities and cytotoxic potentials of green, white, and black teas from Turkey.

Materials and methods

Chemicals and instrumentation

2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, ethanol, acetic acid, dimethyl sulfoxide and acetonitrile were obtained from Merck (Darmstadt, Germany). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), TPTZ (2,4,6-tripyridyl-s-triazine) and Folin-Ciocalteu's phenol reagent were purchased from Fluka Chemie GmbH (Buchs, Switzerland); and polytetrafluoroethylene membranes (porosity 0.45 μm) for filtration of the extracts were obtained from

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Sartorius (Goettingen, Germany). PC-3 (ATCC® CRL-1435™) and BALB/3T3 clone A31 (ATCC® CCL-163™) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cell culture materials were purchased from MulticellWisent Inc. (Montreal, Canada).

All absorbance measurements were performed with Spectro UV-Vis Double PC-8 auto cell spectrophotometer (Labomed Inc.). All solutions were prepared with deionized water purified in an Elgacan® C114 ultra pure water system deioniser (The Elga Group, Buckinghamshire, England).

The IKA RV 05 Basic (IKA, Werke, USA) rotary evaporator system was used in evaporation. The extraction process was performed with a Heidolp Promax 2020 shaker. All dissolution processes were performed with a Heidolp Reax Top vortex and Elma® Transsonic digital ultra sonic water bath (Germany). The Hanna Instruments microprocessor pH meter was used in pH measurements.

Preparing of the standard solutions

In this study, seven phenolic compounds, *p*-hydroxy benzoic acid, vanillic acid, syringaldehyde, *p*-coumaric acid, sinapic acid, benzoic acid, quercetin were used as standards. Previously, a stock solution of each standard (100 μg/mL) was prepared and filtered through 0.45 μm membranes. A stock solution was prepared by mixing all standard solutions (100 μg/mL). In order to produce a calibration curve, the stock solutions of mixed standards were diluted in a concentration range of 5–100 μg/mL.

Preparing sample solutions

Samples of green, white, and black tea were purchased from herb markets in Trabzon, Turkey, in September 2015. The dried powder of teas (1 g) was weighed and mixed with 20 mL methanol. Each mixture was continuously stirred at room temperature for 24 h. The suspension was removed by centrifugation at 10,000 *g* for 15 min. After centrifugation, the supernatant was concentrated at 40°C in a rotary evaporator (IKA-Werke RV05 Basic, Staufen, Germany). The extracts were redissolved in high performance liquid chromatography (HPLC) grade methanol and filtered through 0.45-μm membranes.

HPLC conditions

Seven standards were used for HPLC analysis; *p*-hydroxy benzoic acid, vanillic acid, syringaldehyde, *p*-coumaric acid, sinapic acid, benzoic acid and quercetin. In order to produce a calibration curve, stock solutions of mixed standards were diluted in a concentration range of 5–100 μg/mL. HPLC analysis of phenolic compounds was performed using a reverse phase column (150 × 4.6 mm i.d, 5 μm) (Waters Spherisorb, Milfort, MA, USA), on a gradient program with a two solvents system [A: 100% methanol; B: 2% acetic acid in water (pH 2.8)] at a constant solvent flow rate of 1.5 mL/min on a HPLC system (Shimadzu Corporation, LC 20 AT, Kyoto, Japan) (Table 1). The injection volume was 20 μL. The signals were detected at 232, 246, 260, 270, 280, 290, 308, and 328 nm by diode array detector (DAD) detection with a column temperature of 25°C. The compounds were identified by comparing retention times and spectral data with those of pure standards. Calibration curves of the standards were used for quantitation.

Table 1: Gradient elution conductions.

Time (min)	Solvent A (%)	Solvent B (%)
0.01	20	80
4.00	30	70
7.00	40	60
8.00	40	60
10.00	45	55
11.00	45	55
12.00	50	50
13.00	50	50
14.00	60	40
15.45	80	20
16.00	Stop	

Validation of the HPLC method

HPLC method validation was evaluated in terms of linearity, detection limits, quantification limits, recovery, precision and selectivity according to ICH guidelines [12]. At least five different concentrations of phenolic compound mixture solutions in the range 5–100 μg/mL were analyzed in five replicates. The peak areas were plotted against each concentration of the mixture solutions to establish a linear regression equation and to determine the correlation coefficient (Table 2). The limit of detection (LOD) and limit of quantification (LOQ) values were assigned using the signal-to-noise method (Table 2). A signal-to-noise ratio (S/N) of three-fold is accepted to evaluate LOD and signal-to-noise ratio of ten-fold is used for to evaluate LOQ (Table 2). Three different concentrations of mix solutions (5, 25, and 50 μg/mL) were applied in triplicate to determine of recovery. The recovery was evaluated as mean and standard deviation of the percentage known quantity (Table 3). Intra-day and inter-day precision were identified

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Table 2: Parameters of linearity, limit of detection (LOD) and limit of quantification (LOQ) and phenolic composition of the methanolic extract of three tea samples.

Phenolic compounds	Retention time (min)	Standard curves	Correlation coefficient (r)	LOD (μg/mL)	LOQ (μg/mL)	Amount (mg/10 g)		
						WT	BT	GT
p-Hydroxy benzoic acid	4.411	y = 56.637x + 7014	0.9998	0.05	0.17	7.01	6.49	–
Vanillic acid	5.102	y = 38.098x + 2538	0.9994	0.08	0.26	–	–	–
Syringaldehyde	6.383	y = 15.237x + 1831	0.9985	0.20	0.66	–	0.56	30.45
p-Coumaric acid	7.437	y = 44.407x – 7600	0.9999	0.07	0.22	–	–	28.09
Sinapic acid	7.947	y = 12.877x – 2206	0.9998	0.25	0.78	–	–	–
Benzoic acid	9.588	y = 14.099x – 934.8	0.9998	0.23	0.71	4.24	–	59.27
Quercetin	14.720	y = 37.337x – 35711	0.9998	0.09	0.27	–	–	20.56

WT, White tea; BT, black tea; GT, green tea.

Table 3: Parameters of precision and recovery values.

Phenolic compounds	RT Precision (% RSD)		% Area Precision (% RSD)		Recovery (%) (Mean ± SD)		
	Intraday	Interday	Intraday	Interday	5 μg/mL	25 μg/mL	50 μg/mL
p-Hydroxy benzoic acid	0.14	0.18	0.62	0.79	101.0 ± 0.76	99.9 ± 0.56	99.5 ± 0.52
Vanillic acid	0.12	0.21	0.51	0.82	100.7 ± 0.27	100.2 ± 1.04	102.1 ± 2.05
Syringaldehyde	0.05	0.16	0.44	0.50	100.2 ± 1.04	102.6 ± 1.41	99.6 ± 1.75
p-Coumaric acid	0.02	0.04	0.26	0.78	99.33 ± 1.97	102.9 ± 1.03	99.5 ± 0.21
Sinapic acid	0.16	0.41	0.65	0.98	102.33 ± 1.95	98.9 ± 0.98	98.3 ± 0.15
Benzoic acid	0.05	0.10	0.36	0.64	102.2 ± 0.21	98.8 ± 0.57	98.2 ± 0.92
Quercetin	0.02	0.07	0.27	1.19	98.9 ± 0.46	99.7 ± 0.69	100.4 ± 0.5

as the relative standard deviation (% RSD) of retention times and % peak areas with three different concentrations, three replicates by the standards mix solutions for two separate days (Table 3). The method selectivity was assessed by evaluating the resolution between the standard peaks. Phenolic compounds were determined using the same HPLC conditions. Sample peaks were detected by comparison with retention times of known phenolic standards. In order to verify the repeatability of the method, the methanolic extract was run in triplicate and the values were expressed as mean and standard deviation of plant concentrations (Table 2).

Determination of antioxidant capacity

The amount of total phenolics in extracts was determined using the Folin-Ciocalteu procedure [13]. Gallic acid was used as a standard, and the total phenolics were expressed as mg of gallic acid equivalents (GAE) per g of 100 g sample. Briefly, concentrations of 0.01, 0.02, 0.03, 0.04 and 0.05 mg/mL of gallic acid were prepared in methanol. Concentrations of 0.1 and 1 mg/mL of tea extract were also prepared in methanol, and 0.5 mL of each sample was placed into test tubes and mixed with 0.5 mL of 0.2 N Folin-Ciocalteu reagent and 1.5 mL of 2% sodium carbonate. The tubes were covered with parafilm and allowed to stand for 2 h at 20°C before the absorbance was at read at 760 nm spectrophotometrically. All measurements were carried out in triplicate. Folin-Ciocalteu reagent was used since it is sensitive to reducing compounds, including polyphenols, and gives a blue color after reaction. This blue color can then be measured using spectrophotometry, giving the total phenolic content (TPC) [14].

Ferric reducing antioxidant power (FRAP) assay is based on measurement of the iron reducing capacities of the extract [15]. In the presence of 2,4,6-tripyridyl-S-triazine (TPTZ), the Fe^{2+}-TPTZ complex displays a blue color which is read at 593 nm. Briefly, 3.0 mL of working FRAP reagent was added to an appropriate volume/concentration of extract. This was then incubated for 4 min at 37°C, and the absorbance was measured at 593 nm against a ferrous sulfate standard. For purposes of comparison, Trolox was also measured under the same conditions as a standard antioxidant compound. The results were expressed as μM Trolox equivalent of g sample.

DPPH radical-scavenging activity is based on the antioxidant's DPPH cation radical scavenging capacity [16].

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Briefly, 0.75 mL of DPPH reagent (0.1 mM in methanol) was added to 0.75 mL of tea extract or standard and vortexed vigorously. This was allowed to stand in the dark for 30 min at room temperature. The discoloration of DPPH was measured spectrophometrically at 517 nm. Percentage inhibition of the discoloration of DPPH by the tea extract was measured by using Trolox as standard, and the values were expressed as SC_{50} (mg sample per mL), the concentration of the samples that causes 50% scavenging of DPPH radical.

The Cupric Reducing Antioxidant Capacity (CUPRAC) levels of extracts were studied using the method described by Apak et al. [17]. Briefly, 1 mL of $CuCl_2$ solution (1.0×10^{-2} M), 1 mL of neocuproine solution (7.5×10^{-3} M) and 1 mL NH_4Ac buffer solution were added to a test tube and mixed. Various concentrations of the extracts were added to the initial mixture for a final volume of 4.1 mL. The test tubes were incubated for 30 min. Absorbance against a reagent blank was measured at 450 nm. The CUPRAC values were expressed as μM Trolox equivalent per gram of sample.

Evaluation of cellular viability

Human prostate adenocarcinoma cells (PC-3) were cultured in Dulbecco's Modified Eagles medium (DMEM) with nutrient mixture F12 (DMEM/F12) containing 10% fetal bovine serum (FBS) and 100 units/mL penicillin and 100 μg/mL streptomycin. Mouse embryonic fibroblast BALB/3T3 cells were incubated in DMEM supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C and 5% CO_2 atmosphere [18].

The MTT test was used to evaluate the relative safety of white, green and black teas. In this test the viable cells reduce the yellow, water-soluble tetrazolium salt to insoluble purple formazan precipitate by succinate dehydrogenase, a mitochondrial respiratory chain enzyme active in viable cells only. For this purpose, cells were plated in a 96-well microplate, incubated with different concentrations of methanol (187–1500 μg/mL) and water (4–7 mg/mL) teas extracts. One percent concentration of DMSO was used as solvent control. Non-exposed cells or phosphate-buffered saline (PBS)-exposed cells were evaluated as growth (negative) controls. After 24 h exposure, 25 μL of 5 mg/mL MTT solution was added for each well and incubated for a further 3 h. The supernatants were then discharged, and 100 μL of DMSO was added to each well and shaken with an orbital shaker for 5 min at 150 rpm. The optical density was read at 590 nm (reference wavelength of 670 nm) using a microplate spectrophotometer. The sample concentration that inhibits 50% of enzyme activity (IC_{50} value) was calculated using this formula [18]:

$$\text{inhibition (\%)} = 100 - [(\text{corrected mean OD sample} \times 100) / \text{corrected mean OD solvent control}].$$

Tyrosinase inhibitory (TI) activity

Tyrosinase inhibitory (TI) activity (EC 1.14.1.8.1, 30 U, mushroom tyrosinase, Sigma) was measured using different concentrations of kojic acid solutions as standard [19]. Briefly, dried and powdered green, white, and black teas were extracted with methanol at room temperature using a shaker. After filtration, the extracts were evaporated to dryness under reduced pressure. The methanol extract was dissolved in 80 μL of phosphate buffer (pH 6.8). The stock sample solution (500 μg/mL) was used to prepare different concentrations of sample solutions (25, 50, and 100 μg/mL). A 96-well microplate and an ELISA microplate reader (VersaMax Molecular Devices, USA) were used for analysis. Wells were composed as A (three wells), 120 μL of phosphate buffer (pH 6.8), and 40 μL of tyrosinase; B (three wells), 160 μL of phosphate buffer (pH 6.8); C (three wells), 80 μL of phosphate buffer (pH 6.8), 40 μL of tyrosinase, and 40 μL of sample solution and D (three wells), 120 μL of phosphate buffer (pH 6.8), and 40 μL of sample solution. The plates were incubated at 23°C for 10 min.

Following incubation, 40 μL of L-DOPA solution was added to each well. The plates were incubated at 23°C for 10 min, and the absorbance of the reaction mixture was determined spectrophotometrically at 490 nm in a microplate reader. The percentage of TI activity was calculated using the following equation:

$$\%\ \text{inhibition} = \left[[(A-B)-(C-D)]/(A-B)\right]\times 100$$

Statistical analysis

Data were expressed means ± standard deviations of three parallel measurements. IC_{50} values were calculated using linear regression analysis (Microsoft Excel programme for Windows, versus 2003). Data were tested using SPSS (Version 22.0, Chicago, IL, USA) software. Statistical differences between two groups were compared using the Mann-Whitney U-test. Correlations were analyzed using Pearson's test. Statistical significance was considered at $p < 0.05$ level.

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Results and discussion

The newly developed and validated HPLC method

Several reports have described the use of HPLC with a DAD for the characterization and quantification of phenolic composition. HPLC is known to exhibit higher robustness, reproducibility, and sensitivity, as well as an interface easily coupled to a great range of detectors [12]. We therefore used the HPLC-DAD system for phytochemical analysis in this study, and seven phenolic compounds were determined in tea samples. The developed and validated HPLC method was assessed by different validation parameters as described above (Tables 1–3).

According to results, the linearity was found to be good with correlation coefficients between 0.9985 and 0.9999 (Table 2). The detection and quantification limits of each phenolic compound are given in Table 2. The LOD and LOQ values indicated that the concentrations ranges applied (5–100 μg/mL) are relevant to the study. The recovery of the method was appropriate in terms of the values ranges of 98.2–102.9%, since acceptable values range between 95% and 105% [20]. The low precision values of relative standard deviation (% RSD) of the retention times and % peak areas indicate the validity of the method within an acceptable range RSD ≤ 2%. All standard peaks were completely separated through the HPLC conditions. This indicated the selectivity of the method. A chromatograms of phenolic standards, and methanolic extracts are shown in Figures 1–4. The amounts of phenolic compounds in the samples are shown in Table 2, and the standard deviation values of repeatability were less than 1.19.

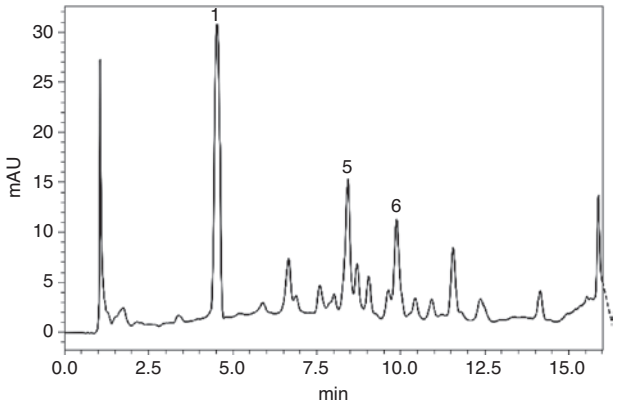


Figure 2: RP-HPLC chromatogram of phenolic standards (50 mg/mL) searched in methanolic extract of white tea detected at 270 nm by DAD. Waters spherisorp ODS2 -C18 column (4.6 × 250 mm, 5 μm), gradient eluent acetic acid/acetonitrile/water, flow rate 1.2 mL/min. Peak identification: (1) *p*-hydroxy benzoic acid, (5) sinapic acid, (6) benzoic acid.

Benzoic acid, sinapic acid, and *p*-hydroxy benzoic acid were determined in white tea, while syringaldehyde, sinapic acid, and *p*-hydroxy benzoic acid were detected in black tea. Green tea contained benzoic acid, syringaldehyde, sinapic acid, and *p*-hydroxy benzoic acid. Syringaldehyde was determined in both black tea and green tea. Stanilkunaite et al. reported that syringaldehyde

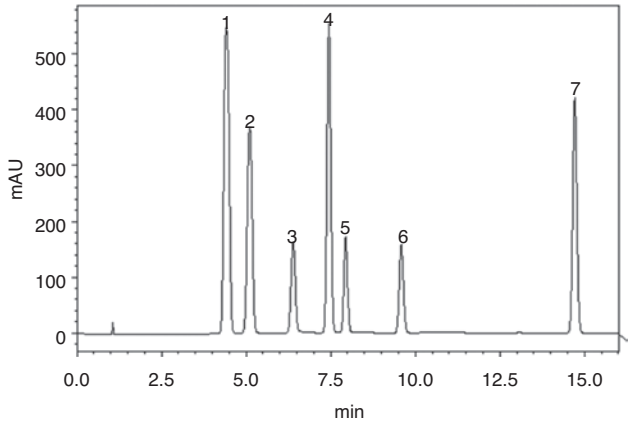


Figure 1: RP-HPLC chromatogram of phenolic standards (25 μM) searched in samples detected at 270 nm by DAD. Waters spherisorp ODS2 -C18 column (4.6 × 250 mm, 5 μm), gradient eluent acetic acid/acetonitrile/water, flow rate 1.2 mL/min. Peak identification: (1) *p*-hydroxy benzoic acid, (2) vanillic acid, (3) syring aldehyde, (4) *p*-coumaric acid, (5) sinapic acid, (6) benzoic acid, (7) quercetin.

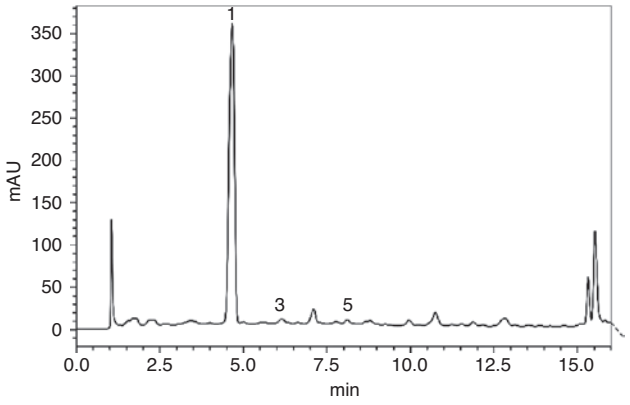


Figure 3: RP-HPLC chromatogram of phenolic standards (50 mg/mL) searched in methanolic extract of black tea detected at 270 nm by DAD. Waters spherisorp ODS2 -C18 column (4.6 × 250 mm, 5 μm), gradient eluent acetic acid/acetonitrile/water, flow rate 1.2 mL/min. Peak identification: (1) *p*-hydroxy benzoic acid, (3) syring aldehyde, (5) sinapic acid.

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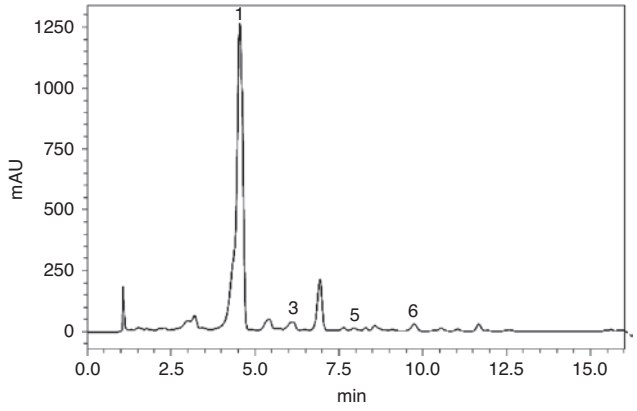


Figure 4: RP-HPLC chromatogram of phenolic standards (50 mg/mL) searched in methanolic extract of green tea detected at 270 nm by DAD.
Waters spherisorp ODS2 -C18 column (4.6 × 250 mm, 5 μm), gradient eluent acetic acid/acetonitrile/water, flow rate 1.2 mL/min. Peak identification: (1) *p*-hydroxy benzoic acid, (3) syring aldehyde, (5) sinapic acid, (6) benzoic acid.

moderately inhibited cyclooxygenase-2 (COX-2) activity with an IC_{50} of 3.5 μg/mL, while syringic acid strongly inhibited COX-2 activity with an IC_{50} of 0.4 μg/mL [21]. COX-2 plays an important role in the inflammatory process. COX-2 is an inducible isoform of cyclooxygenase enzyme responsible for the production of pro-inflammatory prostaglandins [21]. COX-2 inhibitors also play an important role in the treatment of inflammatory disorders, as well as having potential applications for the prevention and treatment of other diseases, such as cancer [22]. On the other hand, the syringaldehyde found in black and green tea is an organic compound that occurs widely in trace amounts in plants. Syringaldehyde has medicinal properties such as antiallergic activities [23]. *p*-hydroxybenzoic acid found in white and black tea is used as a preservative in cosmetics and some ophthalmic solutions. Also, *p*-hydroxy benzoic acid has estrogenic activity [24], and stimulates the growth of human breast cancer cell lines [25].

Total phenolic content of extracts

TPC was determined in comparison with standard gallic acid, and the results were expressed as milligrams of GAE per gram (mg GAE/g) of extract (Table 4). Measurements showed that the methanolic extract of green tea had the highest TPC (Table 4). TPC value increased in the order green tea > black tea > white tea. TPC in methanolic extracts of green tea, black tea, and white tea was 46.9 ± 4.096, 9.3 ± 0.818, and 6.2 ± 0.202 mg of GAE/g, respectively (Table 4).

Polyphenols are widely distributed in plants. With the notable exception of dairy products and mineral water, most beverages are largely made from plant extracts. Beverages are therefore excellent potential sources of polyphenols. Tea is the main source of polyphenol consumed on a daily basis in Turkey. The high level of polyphenols in tea, as well as the characteristics of tea that make it easy to consume in high amounts, may contribute to the supply of significant amounts of antioxidants. Humans are capable of absorbing catechins from tea, which are then metabolized by intestinal flora. High levels of tea consumption and the bioavailability thereof, and the polyphenols contained in tea may play a role in reducing the risk of various human diseases. The highest TPC in this study was determined in methanolic extract of green tea. In another study, the concentrations of total polyphenol in green tea, black tea, Oolong tea, and barley tea were 115, 96, 39, and 9 mg/100 mL, respectively [26]. Differences in methodologies employed in studies, and also factors such as sources and seasonality may cause variations in analytical values [27].

Antioxidant activities of extracts

Polyphenols have been reported to be responsible for the antioxidant activities of extracts. The DPPH, CUPRAC, and FRAP assays have been used to measure antioxidant activity. Tea constitutes a valuable dietary source of

Table 4: The antioxidant activities of methanolic extract of green, white, and black tea.

Test compounds	TPC[a]	FRAP[b]	CUPRAC[c]	DPPH[d]
Green tea	46.9 ± 3.404	1301 ± 5.567	1932 ± 6.244	0.046 ± 0.002
White tea	6.2 ± 0.200[e]	323 ± 1.732[e]	568 ± 4.000[e]	0.307 ± 0.007[e]
Black tea	9.3 ± 0.818[e,f]	247 ± 2.516[e,f]	646 ± 5.131[e,f]	0.254 ± 0.026[e,f]
BHT				0.009 ± 0.001

[a]Total phenolic content expressed in mg of gallic acid equivalent (GAE) per gram of dry plant weight. [b]Expressed as μM trolox equivalents (TE) per gram of dry plant weight. [c]Trolox equivalent antioxidant capacity (TEAC) value expressed in μM trolox equivalents (TE) per gram of dry plant weight. [d]Concentration of test sample (mg/mL) required to produce 50% inhibition of the DPPH radical. [e]Values are significantly different from those of green tea ($p < 0.05$); [f]values are significantly different from those of white tea ($p < 0.05$).

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antioxidants. FRAP values increased in the order green tea > white tea > black tea (Table 4). FRAP values were 1301 ± 4.096 μmol/g for green tea, 323 ± 1.732 μmol/g for white tea, and 247 ± 2.516 μmol/g for black tea. DPPH values increased in the order green tea > black tea > white tea. DPPH scavenging activities were 0.046 ± 0.001 mg/mL for green tea, 0.254 ± 0.026 mg/mL for black tea, and 0.307 ± 0.008 mg/mL for white tea (Table 4). The radical scavenging activity of green tea extract was lower than that of butylated hydroxy toluene (BHT) (0.009 ± 0.001 mg/mL). Green tea, with the highest total polyphenol contents, also exhibited the highest cupric reducing power. The cupric reducing power of green, black, and white tea samples expressed as Trolox equivalent antioxidant capacity (TEAC) were 1932 ± 6.906, 646 ± 5.131, and 568 ± 4.000 μmol/g, respectively (Table 4). Previous studies have reported the in vitro radical trapping antioxidant properties exhibited by black tea and green tea, by tea extracts and by individual polyphenolic compounds contained in tea [28]. Our study reports new data elicited by comparing the in vitro antioxidant/reducing capacities of various types of tea, in the forms in which they would normally be consumed. The FRAP method was used to evaluate antioxidant capacities involving the reduction of ferric tripyridyltriazine (Fe-(III)-TPTZ) complex to a blue-colored Fe(II)-TPTZ with sample antioxidants. For this purpose we used the objective and highly reproducible FRAP assay technique for antioxidant/reducing power. The reduction of ferric iron in FRAP reagent gives a blue product (ferrous-TPTZ complex), the absorbance of which can be read in specimens at 593 nm, with those containing Trolox at a known concentration of 1000 μM. The increased absorbance is an indication of higher reducing power in the FRAP method. In this study, the highest FRAP value was 1301 ± 4.096 μmol/g for green tea. Benzie and Szeto reported FRAP values of 132–654 μmol/g for black teas, 233–532 μmol/g for Oolong teas, and 272–1144 μmol/g for green teas [28]. Natella et al. reported FRAP values of 10.1 mol Fe^{2+}/L for black tea, and 18 mol Fe^{2+}/L for green tea. FRAP values and the phenolic content of various kinds of tea overlapped, exhibiting a 2–3-fold variation among a range of brands of the same kind of tea [29]. We attributed this variation to differences in quality, geographical growing regions, the time of year when the leaves were harvested, and subsequent storage conditions [30]. When antioxidants interact with DPPH they donate an electron or a hydrogen atom to DPPH, thus neutralizing its free-radical character [31]. The radical scavenging activity of DPPH was expressed as IC_{50}, and a lower IC_{50} value indicates higher antioxidant activity. In this study, the highest DPPH scavenging activity was 0.046 ± 0.001 mg/mL for green tea. Anissi et al. reported that coffee (extract) showed DPPH scavenging activity somewhere between those exhibited by green and black teas [32]. Jungmin et al. reported that the DPPH scavenging activity of water extract of black tea was 66.65 ± 1.55 mg ascorbic acid equivalents [33]. These results suggest that green tea may contain the strongest free radical-scavenger compounds.

Table 5: Correlation coefficients, *r*, for relationships between assays.

	CUPRAC	FRAP	DPPH
TPC	0.996[a]	0.988[a]	−0.984[a]
CUPRAC		0.993[a]	−0.983[a]
FRAP			−0.960[a]

[a]Correlation is significant at the 0.01 level. DPPH, 1,1-Diphenyl-2-picrylhydrazyl; FRAP, ferric reducing/antioxidant power; CUPRAC, cupric reducing antioxidant capacity; TPC, total phenol content; TIA, tyrosinase inhibitor activity.

In this study, the green tea, with the highest TPC, also exhibited the highest cupric reducing power. Apak et al. reported the CUPRAC values for Ceylon blended ordinary tea (4.41), green tea with lemon (1.61), English ordinary breakfast tea (1.26) and green tea (0.94), all of which were manufactured from types of *C. sinensis* [17].

These results indicate that polyphenols in tea extracts are largely responsible for their antioxidant activities. Good correlation was also observed between CUPRAC and DPPH radical scavenging activity (r = – 0.983), while correlations between DPPH radical scavenging activity and TPC (r = – 0.984) and DPPH radical scavenging activity and FRAP (r = – 0.960) were slightly weaker than that between DPPH radical scavenging activity and CUPRAC (r = – 0.983). Excellent correlation was also observed among TPC and FRAP (r = 0.998), TPC and CUPRAC (r = 0.996), and FRAP and CUPRAC (r = 0.993) (Table 5). Benzie and Szeto reported similar correlation between polyphenols and antioxidant activity of tea measured using the FRAP method [28].

Evaluation of cellular viability

The cytotoxic potentials of teas, and extracts were evaluated by MTT assay in cancerous (PC-3) and normal (3T3) cell lines. The results show that tea extracts may be cytotoxic at relatively high concentrations. The results also show that methanol extracts may be more toxic than water extracts. The PC-3 cancerous cells were more sensitive (IC_{50} 1.28–3.73 mg/mL) to both water and methanol extracts than 3T3 normal cells (IC_{50} ≥5.43 mg/mL). Green tea extracts have a greater cytotoxic potential than

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Table 6: Evaluation the cellular viability (IC_{50} ± SEM mg/mL) of PC-3 and 3T3 cell lines after exposure to different concentrations (187.5 µg/mL – 7mg/mL) of green, white, black tea, water and methanol extracts.

	Methanol extracts		Water extracts	
	PC-3 cell line	3T3 cell line	PC-3 cell line	3T3 cell line
Green tea	1.283 ± 0.008	24.2%[a]	3.73 ± 0.069	5.43 ± 0.102
White tea	1.408 ± 0.032	27.4%[a]	26.7%[a]	30.4%[a]
Black tea	2.534 ± 0.081	16.95%[a]	19.4%[a]	42.2%[a]

Standard error mean (SEM). [a]IC_{50} value could not calculated, the maximum cell death (mean) was given.

white and black tea extracts. The cytotoxicity potential of teas may be ranked as green tea > white tea > black tea (Table 6).

The toxicity or protective role of tea extracts and chemicals are still controversal. While much research has investigated the protective potential of black, green and white tea and tea extracts against different diseases and toxicity conditions [34–36], other studies have shown that tea and tea extracts may causes harmful effects in normal cells [37, 38]. Our results demonstrate the safety of teas and tea extracts, since only high concentrations may be toxic to cells. In addition, extracts affect cancerous cells more than they do normal cells.

The tyrosinase inhibitory activity of extracts

Tyrosinase is a rate-limiting enzyme that controls the production of melanin. Tyrosinase inhibitors have become increasingly popular because inhibitors are not only used in medical treatment for hyperpigmentation but often used in the cosmetics industries and food processing. The tyrosinase inhibitory (TI) activity may depend on the hydroxyl groups of the phenolic compounds, such as chlorogenic acid, benzoic acid, protocatechuic acid, gallic acid, ellagic acid, kojic acid, resveratrol, and several catechins that may form a hydrogen bond to a site in the enzyme, leading to lower enzymatic activity [39]. According to our literature survey, there are a limited number of studies on green tea, white tea, and black tea [39, 40]. The methanolic extracts of green, white, and black teas were studied in terms of enzyme inhibitory activity against tyrosinase at concentrations of 25, 50, 100, and 500 µg/mL (Table 7). IC_{50} values were 63.53 µg/mL for kojic acid, 72.94 µg/mL for green tea, 181.97 µg/mL for white tea, and 1000 µg/mL for black tea (Figure 5). Jo et al. reported that the methanol extract of green tea pericarp exhibited superior TI activity (IC_{50} = 735.58 ± 24.43 µg/mL) among the extracts. However, water extract of pericarp exhibited weaker TI activity than seed extracts [40]. In this study, methanolic extract of white tea showed a high degree of inhibition against tyrosinase, similarly to the positive control, kojic acid. The extract of white tea exhibited remarkable inhibition against this enzyme (95.33 ± 0.92 µg/mL) and was shown to contain benzoic acid, sinapic acid, and *p*-hydroxy benzoic acid. Its TI potency may perhaps be related to the polyphenols. Sinapic acid, and *p*-hydroxy benzoic acid are phenolic acids contained in green tea, white tea, and black tea and may be described as markers or characteristic compounds. It may therefore be speculated that sinapic acid and *p*-hydroxy benzoic acid found in green tea, white tea, and black tea contribute to skin-whitening effects in

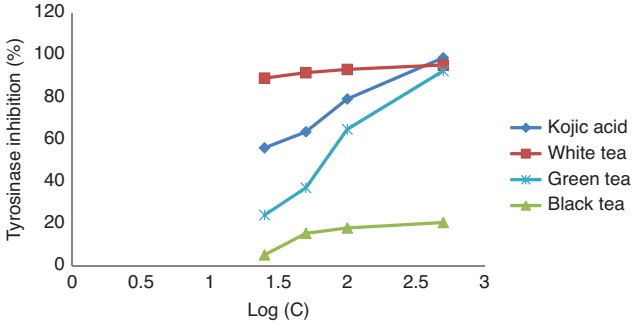


Figure 5: Tyrosinase inhibitor activity of methanolic extract.

Table 7: Tyrosinase inhibitory activity (inhibition ± SEM %) of the extract of the green, white, black tea, and the reference (kojic acid) at 25, 50, 100, 500 µg/mL concentrations.

	Sample I (25 µg/mL)	Sample II (50 µg/mL)	Sample III (100 µg/mL)	Sample IV (500 µg/mL)
Green tea	24.07 ± 2.66	37.04 ± 0.90	64.81 ± 1.61	92.59 ± 2.47
White tea	89.11 ± 1.98[b]	91.77 ± 2.07[b]	93.26 ± 1.05[b]	95.33 ± 0.95
Black tea	5.12 ± 0.24[b,c]	15.38 ± 0.85[b,c]	17.94 ± 0.41[b,c]	20.51 ± 1.00[b,c]
Kojic acid[a]	23.29 ± 0.34	43.37 ± 0.66	71.48 ± 0.65	92.77 ± 0.52

Standard error mean (SEM). [a]Pozitif control for inhibitory activity against tyrosinase. [b]Values are significantly different from those of green tea ($p < 0.05$); [c]values are significantly different from those of white tea ($p < 0.05$).

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cosmetics through their strong antioxidant potential and moderate TI action.

Conclusion

Our findings reveal that extracts prepared from green, white, and black tea growing in Turkey appear to possess significant TI and antioxidant properties, which may be possibly associated with the rich TPC of each of the three teas. Green, white, and black teas may therefore have the potential for use as a raw material by pharmaceutical industries for the preparation of natural drugs, in addition to in food industries. To the best of our knowledge, this is the first study of composition analysis using a validated novel HPLC method of antityrosinase and antioxidant activity in green, white, and black teas.

Conflict of interest: The authors declare that there was no conflict of interest.

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