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POLYPHENOLS, ALKALOIDS AND ANTIOXIDANT ACTIVITY OF DIFFERENT GRADES TURKISH BLACK TEA

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Abstract
The content of polyphenols and alkaloids of seven grades Turkish black tea was investigated as well as their antioxidant activity. Polyphenol and alkaloid contents of teas were determined using HPLC method. Antioxidant activity was evaluated by DPPH radical assay. In tea samples examined, two alkaloids (caffeine and theobromine), two flavan-3-ols (EGCG and ECG), three flavonol glycosides (Q3RG, Q3G and K3RG) and four individual theaflavins (TF-f, TF-3-G, TF-3'-G and TF-3,3'-DG) were identified and quantified. Significant differences were detected in biochemicals content and antioxidant activity between different grades black teas. In general, the contents of alkaloids, Q3RG, Q3G and theaflavins in 1st, 2nd, 3rd and 7th grade teas and the contents of flavan-3-ols in 4th, 5th and 6th grade teas were found to be higher. However, there was no significant difference in K3RG content of the teas. 1st, 2nd, 3rd and 7th grade teas showed also higher antioxidant activity.

Keywords: Black tea, alkaloid, phenolics, tea grade, antioxidant activity

FARKLI SINIF TÜRK SİYAH ÇAYLARININ POLİFENOL VE ALKALOİD İÇERİKLERİ İLE ANTİOKSİDAN AKTİVİTESİ

Özet
Bu çalışmada yedi farklı sınıf Türk siyah çayının polifenol ve alkaloid içerikleri HPLC yöntemiyle, antioksidan aktiviteleri ise DPPH radikal yöntemiyle araştırılmıştır. Çay örneklerinde iki alkaloid (kafein ve teobromin), iki flavan-3-ol (EGCG ve ECG), üç flavonol glikoziti (Q3RG, Q3G and K3RG) ve dört teaflavin bileşiği (TF-f, TF-3-G, TF-3'-G and TF-3,3'-DG) tanımlanmış ve miktarları belirlenmiştir. Farklı sınıf çayların yapısında bulunan bileşiklerin miktarlarının ve bunların antioksidan aktivitelerinin istatistikî olarak anlamlı düzeyde farklı olduğu ortaya konulmuştur. Genel olarak çaylarda bulunan alkaloidlerin, Q3RG, Q3G ve teaflavinler ile antioksidatif aktivitenin 1., 2. 3. ve 7. sınıf çaylarda, buna karşın flavan-3-ol'lerin ise 4., 5. ve 6. sınıf çaylarda daha yüksek olduğu saptanmıştır. K3RG açısından çay sınıfları arasında farklılık görülmemiştir.

Anahtar kelimeler: Siyah çay, alkaloid, fenolik bileşik, çay sınıfı, antioksidan aktivite

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INTRODUCTION

Black tea is the most widely consumed in Turkey which is the sixth largest producer of black tea after China, India, Sri Lanka, Kenya and Indonesia in world tea production (1). In tea trade, the market value depends mainly on the quality of the brewed liquor and the texture or appearance of the made tea (2). In black tea, during the fermentation process most of catechins forming main flavanols of green tea are oxidized and polymerized to theaflavins (TF) and thearubigins (TR) (3, 4) which are responsible for the colour, flavour and brightness of tea (5). The content of individual TF showed a significant correlation with tea taster scores and value (6, 7). Flavonols, present as glycosides, are the other important groups of polyphenols in tea (8) and black tea contains mainly quercetin, kaempferol and myricetin glycosides (9) which contribute to tea antioxidant activity (10). Caffeine, theophylline and theobromine are the main tea alkaloids and also important factors in determining the quality of black tea (3, 11). Caffeine is the most abundant alkaloid, responsible for the briskness of tea (12).

It is well known that green tea catechins have most of health effects. Black tea TF's, oxidation products of catechins, also possess strong antioxidant activity (13) and are considered to be much effective components for the inhibition of carcinogenesis (14). Stewart et al. (15) found that TF retained antioxidant capacity similar to that of (-) epicatechin (EC) monomers. Conversion of catechins to TF during tea fermentation does not significanty alter its free-radical scavenging activity (16).

The levels of individual and total TF of black tea have showed differences depending on the various factors such as plucking season of tea shoots (17) and analytical method (18, 19). On the other hand, in the literature no information is available on the effects of tea grades on the levels of polyphenol and alkaloid of black tea. Therefore, in this study, it is aimed to investigate not only the content of polyphenols and alkaloids but also the antioxidant activity of seven different grades Turkish black tea.

MATERIAL AND METHODS

Plant materials and chemicals

Seven grades (1-7.) black tea samples were collected in triplicate from a tea processing factory in the Eastern Black Sea region. Tea grading was made by using Middleton sieving system which can be fitted with 5 to 10 trays, and the aperture size of the sieves varies from 8 mesh (2.36 mm) to 40 mesh (0.425 mm). Here, five sieve sizes were used with aperture sizes of 8 (largest), 10 (2.0 mm), 12 (1.7 mm), 20 (0.85 mm), and 30 (0.6 mm) mesh. Black teas retained by the 10, 12, and 20 mesh sieves were separated as tea grades 1, 2, and 3, respectively. These teas do not contain any broken parts. Teas passing through the 30 mesh sieve are designated as grade 7 or dust-tea. The largest particles of black tea remaining over the 8 mesh sieve were passed through the breaker crushers and screened again in the same system. Black teas then remaining over the 10, 12, 20, and 30 mesh sieves were classified as grades 4, 5, 6, and 7, respectively. Depending on processing technique and plucking season the approximate percent distrubution of 1^{st} to 7^{th} grades of teas, respectively, were as follows: 2.5; 24; 8.5; 4; 50; 8 and 3. Tea samples were produced by Orthodox + Rotorvane + Orthodox (called as Cay-Kur) method (20).

(+)-Catechin(C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-) epigallocatechingallate (EGCG), (-)-epicatechingallate (ECG), (-)-catechingallate(CG), (+)-gallocatechin (GC) and tea extract containing free theaflavin (TF-f), theaflavin-3-gallate (TF3G), theaflavin-3'-gallate (TF3'G), theaflavin-3,3'-digallate (TF3,3'DG) were purchased from Sigma (St, Louis, Mo. USA). Caffeine, theobromine, quercetin-3-glucoside (Q3G), orthophosphoric acid, HPLC or analytical grade acetonitrile and methanol were purchased from Fluka-Riedel-de Haën (BioChemica Fluka Cheme GmbH Buchs-Switzerland). Kaempferol-3-rhamnosylglucoside (K3RG) was from Chromadex (Santa Ana, ABD). Quercetin-3-rhamnosylglucoside (Q3RG) was from Wako (Pure Chem. Co., Osaka-Japan).

Extraction of tea polyphenols and alkaloids

Ground tea sample (0.2 g) was extracted with 10 ml 80% methanol for 14 h on a horizontal shaker in the dark. The mixture was centrifuged at 14000 g for 10 min. The supernatant was transferred into an screw-capped amber glass vial and stored at -20 °C until analyzed.

Analytical determinations

Content of tea biochemicals was determined by the method described by Turkmen and Velioglu (21). Chromatographic peaks in the samples were identified by comparing their retention times and UV spectra with those of their reference standards and by co-chromatography with added standards. Quantification was performed from the peak area of each component and its corresponding calibration curve.

Preparation of standard solutions

Stock standard solutions were prepared dissolving in 80% methanol except for theobromine, dissolved in distilled hot water, to a concentration of 0.5-1 g/l and kept protected from light at -20 °C for up to 2 months. Each stock solution was then used for the preparation of the diluted solutions (0.25-250 mg/L) for the calibration curves. Working standard solutions were injected into HPLC and peak areas were obtained. Calibration curves were prepared by plotting concentration versus area.

Antioxidant activity

The antioxidant activity of tea samples was measured by using the DPPH assay (22, 23) with some modifications. 50 ml of test sample diluted for fifteenth fold was mixed with an aliquot of 1950 ml of $6x10^{-5}$ M DPPH radical in methanol. The reaction mixture was vortex mixed and let to stand at 25 °C in the dark for 60 min. The decrease in the absorbance at 517 nm was determined using a spectrophotometer using methanol as blank. The antioxidant activity (AA %) was calculated according to the following equation (I):

$$AA(\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100 \qquad (I)$$

where $A_{control}$ is the absorbance of the DPPH solution without sample and A_{sample} is the absorbance absorbance of the test sample.

Standard curve of reference antioxidant ascorbic acid (0-150 mg/ml) was assayed under identical conditions for affinity to scavenge DPPH. Antioxidant activity of samples was transformed to ascorbic acid equivalent (AEAA) defined as g of ascorbic acid equivalents per 100 g tea. Greater values of the AEAA are related to greater antioxidant activity of the sample.

Statistical analysis

All data were expressed as means ± standard deviation of triplicate measurements and analyzed by SPSS for Windows (ver.10.1). One-way analysis of variance (ANOVA) and Duncan's multiple range test were carried out to test any significant differences among different tea grades. Values of $P<0.05$ were considered as significantly different ($\alpha=0.05$).

RESULTS AND DISCUSSION

Analysis of polyphenols and alkaloids in black teas

A typical chromatogram of an extract of black tea is shown in Fig. 1. Among three groups of polyphenols (flavan-3-ols, flavonol glycosides and theaflavins) EGCG and ECG were the major flavan-3-ols identified. Flavonol glycosides determined in teas were Q3RG, Q3G and K3RG. Individual theaflavin compounds were identified as TF-f, TF3G, TF3'G and TF3,3'DG. The main alkaloids identified in tea samples were theobromine and caffeine.

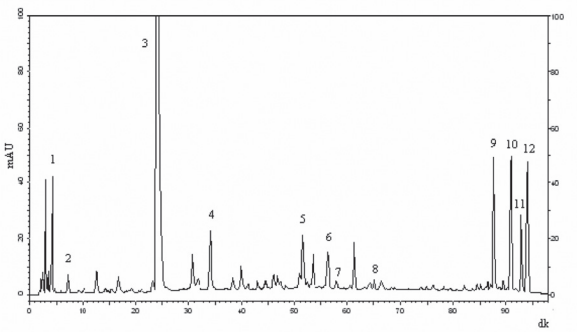

Figure 1 HPLC chromatogram of an extract of black tea

Peak identities:1,GA; 2, teobromin; 3, caffein; 4, EGCG; 5, ECG; 6, Q3RG; 7, Q3G; 8, K3RG; 9, TF-f; 10, TF-3-G; 11, TF-3'-G; 12, TF-3,3'-DG

The content of polyphenols in tea samples significantly varied depending on tea grade. The levels of EGCG ranged from 1.06 mg/g dry weight (DW) in 7th grade tea to 3.16 mg/g DW in 6th grade tea (Table 1) and were in agreement with the results

of previous studies (24-27). However, Wang et al. (27) found lower EGCG contents in Keemun and Sri Lanka black teas, 0.95 (0.48) and 1.16 (0.58) mg/100 ml (mg/g DW), respectively, which could be due to differences in quality of fresh tea leaves, tea processing and analytical methods used.

The levels of ECG ranged from 0.73 mg/g DW in 7th grade tea to 2.54 mg/g DW in 6th grade tea (Table 1), which is in agreement with the study by Sharma et al. (3) who reported the amount of ECG to be 1.0 ve 1.5 mg/g in orthodox and CTC black teas extracted with 70% methanol, respectively. On the other hand, Nishitani and Sagesaka (28) found acetonitrile (50%) extracts from black tea to contain 18 mg/g of ECG, higher than our results. The difference can be due to the same reasons mentioned above for EGCG. The levels of EGCG were higher than those of ECG. Similarly, in literature, the most abundant flavan-3-ol in black teas which were extracted with different solvents such as 40% ethanol, 80% methanol and water was reported to be EGCG (4, 18, 29, 30, 31). With respect to tea grade, the higher amount of flavan-3-ols was found in 4th , 5th and 6th grade teas, followed by 1st, 2nd and 3rd ones which are produced from young tea leaves while 4th , 5th and 6th grade teas are from old ones. This can be attributed to variation in polyphenol oxidase (PPO) enzyme activity of tea leaf components (33). Because, old tea leaves contain lower flavan-3-ols and PPO activity which catalyses the oxidation of the flavan-3-ols and leads to the formation of black tea pigments, namely, TF and TR compared to young ones (2, 17, 20, 34). 7th grade tea which consists of combination of all other teas contained lowest level of flavan-3-ols. Information about polyphenols in different tea grades black tea in the literature is lacking. Therefore, we are unable to compare our results.

Concerning the flavonol glycosides, the content of total flavonol glycosides of teas ranged from 3.04 mg/g DW in 5th grade tea to 3.62 mg/g DW in 7th grade tea (Table 1). In all cases, Q3RG was the most abundant flavonol glycoside, followed by K3RG and Q3G, respectively. Similar results were reported by previous studies (5, 15). However, in the study of Rechner et al. (30), among flavonol glycosides of different brands of black teas Q3RG, Q3G and K3RG were included in the first three order and ranking of their amounts varied with different tea brand. Luximon-Ramma et al. (35) noted that in aqueous methanol extracts of nine different brands Mauritius black teas, as aglycones, more quercetin (1.07-3.29 mg/g DW) and lower kaempferol (0.27-0.70 mg/g DW) were determined, which supports partly our results. While significant difference was detected in Q3RG and Q3G contents of teas, but not in K3RG contents. In general, 1st, 2nd, 3rd and 7th grade teas contained significantly higher contents of Q3RG and Q3G than 4th, 5th and 6th ones, which may be because of the fact that young tea leaves contain higher polyphenol content (16).

In tea samples, the content of total TF compounds varied from 14.18 to 19.95 mg/g DW (Table 2). In literature, total TF contents were reported to be 10.70 mg/g in Ceylon tea (14), average 8.54 mg/g in Chinese teas (24) and 3.57–11.57 mg/g in different black teas (36). These values were lower than

Table1. Flavan-3-ols and flavonol glycosides in different grades black tea (mg/g dry weight)

Tea grade	EGCG	ECG	Q3RG	Q3G	K3RG	Total flavonol glycosides
1	1.18±0.04^{a}	0.94±0.07^{ab}	1.75±0.08^{ab}	0.52±0.02^{cd}	1.10±0.03^{a}	3.38±0.10
2	1.57±0.27^{ab}	1.26±0.13^{bc}	1.74±0.05^{ab}	0.52±0.01^{cd}	1.08±0.03^{a}	3.35±0.10
3	2.05±0.20^{bc}	1.47±0.14^{cd}	1.82±0.07^{bc}	0.55±0.01^{d}	1.08±0.02^{a}	3.45±0.10
4	2.38±0.22^{cd}	1.90±0.09^{de}	1.63±0.08^{ab}	0.45±0.02^{ab}	1.08±0.02^{a}	3.16±0.11
5	3.00±0.30^{e}	2.18±0.24^{ef}	1.53±0.10^{a}	0.44±0.01^{a}	1.06±0.02^{a}	3.04±0.13
6	3.16±0.40^{e}	2.54±0.22^{f}	1.63±0.05^{ab}	0.48±0.04^{abc}	1.05±0.03^{a}	3.16±0.10
7	1.06±0.12^{a}	0.73±0.07^{a}	2.01±0.04^{c}	0.51±0.00^{bcd}	1.09±0.01^{a}	3.62±0.04

Means sharing different letter (a-f) in the same column are significantly different (P<0.05) according to Duncan's test

our results, which may be due to variation in tea variety, tea processing method and extraction and analytical methods used. In all teas, TF-3,3'-DG was the most abundant TF, followed by TF-3-G, TF-f and TF-3'-G in decreasing order, which is in agreement with the results of Caffin et al. (17). On the contrary, Liang et al. (24) reported that in different teas extracted with water, the order of TF contents was as follows: TF-3'-G (2.98 mg/g)> TF-f (2.35 mg/g)> TF-3,3'-DG (2.33 mg/g) and TF-3-G (0.89 mg/g) respectively. Other studies also reported that different black teas extracted with water had lower TF contents and in tea samples, different TF compound was dominant depending on the tea type (37, 38). There was significant difference in total TF contents between different grades teas. 1st, 2nd, 3rd and 7th grade teas contained higher contents of total TF compared to 4th, 5th and 6th ones as observed for flavonol glycoside. This can be attributed to the higher flavan-3-ols content and PPO activity in young tea leaves which form more TF as a result of oxidation.

Caffeine and theobromine in teas were in the range of 23.16- 26.26 mg/g DW and 0.18- 0.20 mg/g DW, respectively (Table 2). Caffeine, the major alkaloid of tea (39), complexes with polyphenols in tea, mainly theaflavins and this complex modifies positively the taste characteristics both caffeine and theaflavins making the tea taste brisker (40). Caffeine contents from this study were in accordance with the previous studies (18, 41). However, 12.32-19.60 mg/g tea leaf (42) and 14.25-16.95 mg/g dry matter (25) of caffeine contents, lower than our results, have been reported. Variation in caffeine content of teas is attributed to differences in plucking season of tea, processing method and variety and structure of leaf (18, 41, 43, 44). Additionally, caffeine content of tea can vary widely depending on the extraction conditions (3, 45). This study showed that tea samples contained much lower theobromine than caffeine. Theobromine levels were also lower than those reported by Khanchi et al. (42) and Sharma et al. (3). While significant difference was detected in caffeine contents of teas, but not in theobromine contents. In general, 1st, 2nd, 3rd and 7th grade teas contained significantly higher content of caffeine than 4th, 5th and 6th ones, which can be because of the fact that there has been more caffeine in young tea leaves than in old ones (17, 43, 44).

Antioxidant activity of black teas

According to the results shown in Table 2, the scavenging activity of black tea extracts on DPPH radical ranged from 8.23 to 8.84 g ascorbic acid/100g tea. DPPH method has been widely used in antioxidant activity studies of plant extracts (46-48). The method is based on the reduction of alcoholic DPPH solutions at 517 nm in the presence of an hydrogen donating antioxidant (49) and polyphenols have been reported to be potent hydrogen donators to the DPPH radical (50, 51) because of their ideal structural chemistry (52). This study showed that 1st, 2nd, 3rd and 7th grade teas had higher activities compared to 4th, 5th and 6th ones. This can be attributed to higher concentration of flavonol glycosides and TF present in these tea extracts due

Table 2. Individual and total TF's, alkaloids (mg/g dry weight) and antioxidant activity (g ascorbic acid/100g tea) of different grades black tea

Tea grade	TF-f	TF-3-G	TF-3'-G	TF-3,3'-DG	Total TF	Caffeine	Theobromine	A.ox. activity
1	3.87±0.24^{bc}	5.49±0.10^{d}	3.38±0.10^{de}	5.84±0.01^{b}	18.58±0.30^{c}	25.97±0.53^{c}	0.20±0.02^{a}	8.84±0.05^{c}
2	3.89±0.31^{bc}	5.27±0.10^{cd}	3.09±0.12^{cd}	5.79±0.03^{b}	18.04±0.49^{c}	24.14±0.41^{ab}	0.19±0.01^{a}	8.56±0.03^{bc}
3	3.68±0.29^{abc}	5.10±0.15^{c}	2.90±0.12^{bc}	5.76±0.11^{b}	17.43±0.46^{c}	24.65±0.33^{b}	0.19±0.01^{a}	8.40±0.17^{ab}
4	3.28±0.23^{ab}	4.72±0.05^{b}	2.56±0.09^{ab}	5.12±0.13^{a}	15.68±0.32^{b}	24.08±0.17^{ab}	0.18±0.01^{a}	8.31±0.10^{ab}
5	2.98±0.17^{a}	4.23±0.09^{a}	2.31±0.05^{a}	4.74±0.16^{a}	14.27±0.09^{a}	23.16±0.33^{a}	0.18±0.01^{a}	8.23±0.08^{a}
6	2.97±0.21^{a}	4.19±0.09^{a}	2.33±0.06^{a}	4.69±0.22^{a}	14.18±0.12^{a}	23.03±0.41^{a}	0.18±0.01^{a}	8.30±0.05^{ab}
7	4.34±0.25^{c}	6.10±0.21^{e}	3.64±0.24^{e}	5.87±0.20^{b}	19.95±0.85^{d}	26.26±0.06^{c}	0.20±0.01^{a}	8.55±0.08^{bc}

Means sharing different letter (a-e) in the same column are significantly different (P<0.05) according to Duncan's test

to the fact that there is a high correlation between antioxidant activity of plant extracts and polyphenols (53-57).

Conclusions

In seven grades Turkish black tea, two alkaloids (caffeine and theobromine), two flavan-3-ols (EGCG and ECG), three flavonol glycosides (Q3RG, Q3G and K3RG) and four individual TF (TF-f, TF-3-G, TF-3'-G and TF-3,3'-DG) were identified and quantified. Significant differences were detected in biochemicals content and antioxidant activity between different grades black teas. In general, the contents of alkaloids, Q3RG, Q3G and TF in 1st, 2nd, 3rd and 7th grade teas and the contents of flavan-3-ols in 4th, 5th and 6th grade teas were found to be higher. However, there was no significant difference in K3RG content of the teas. 1st, 2nd, 3rd and 7th grade teas showed also higher antioxidant activity determined by DPPH radical assay. Therefore, these results showed that 1st, 2nd, 3rd and 7th grade teas had better quality. As explained in materials and methods section, 1st, 2nd, 3rd and a part of 7th grade teas were not passed through crusher were always valuable than others because of containing more buds, so more active compounds.

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