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Letter to Editor

Tea Extracts as Free Radical Scavengers

A. Gramza[1]*, K. Pawlak-Lemańska[2], J.Korczak[1], E. Wąsowicz[3], M. Rudzinska[3]

[1]Department of Food Technology and Human Nutrition, Agricultural University, Wojska Polskiego 31, 60-624 Poznań, Poland
[2]Department of Instrumental Analysis, University of Economics, Al. Niepodległości 10, 60-967 Poznań, Poland
[3] Institute of Food Technology of Plant Origin, Agricultural University, Wojska Polskiego 31, 60-624 Poznań, Poland

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Abstract

Antiradical activity was measured with the use of two different methods of scavenging the stable free radicals $ABTS^{+\bullet}$ and $DPPH^{\bullet}$. Examined tea extracts showed different antiradical activity. Best activity in scavenging $ABTS^{+\bullet}$ expressed as TAA (total antioxidant activity) showed black tea aqueous and ethanol extracts. Green tea extracts were four times less effective. Antiradical activity showed that the lowest concentration needed to scavenge the 50% of initial $DPPH^{\bullet}$ radical (EC_{50}) was green and black tea ethanol extracts. Aqueous extracts showed 50% lower activity than equivalent ethanol extracts. Research proved that antiradical activity of plant extracts is dependent on mechanisms of oxidative activity of free radicals used and the chemical structure of contained antioxidants.

Keywords: free radicals, $ABTS^{+\bullet}$, $DPPH^{\bullet}$, radical scavengers, green and black tea extracts, polyphenols, antioxidant activity.

Introduction

There is growing interest in drinking tea all over the world, which could be connected with polyphenol antioxidative activity, fighting the harmful influence of environmentally generated free radicals [1]. All living animals and plants are permanently on environmental hazard, influenced by such highly reactive molecules as free radicals. Oxidative damage originates from an increase in free radical production either by exogenous radicals such as radiation, pollution and cigarette smoking. Other sources are endogenous sources, such as inflammation, the respiratory burst and xenobiotic killing [2, 3, 4]. Significant free radicals include superoxide, hydroxyl and peroxyl radicals. Radicals are unstable oxygen compounds with an unpaired electron in the atomic electron shell. Since all molecules tend to have complete electron pairs, the radicals react aggressively with other molecules, trapping electrons away from them. These molecules become radicals, starting chain reactions. The endogenous radicals are a result of normal metabolic processes, whereas exogenous radicals, developing outside the body, are recognized as environmental factors, such as UV radiation, stress and air pollution. In a healthy organism the chain reaction is interrupted by antioxidants – radical traps [5]. Living organisms have developed the antioxidative system, enzymatic and non-enzymatic, protecting from toxemia. Research shows much interest in the influence of radicals generated by different environmental factors on human health and food stability, but there still is a need to find substances that would have antiradical properties. Free radicals operating in the cells modify important proteins, damaging the cell membrane and its genotype. Research has shown that all types of organs and tissues are subject to radicals' varied effects [6,7]. Several epidemiological studies have suggested the importance of secondary plant products – polyphenol consumption in reducing the incidence of degenerative diseases [8]. Those substances possess strong antioxidative activity and are counterparts to oxidative stress [9].

*Corresponding author; e-mail: angramza@au.poznan.pl

Much research has focused on naturally occurring plant substances – polyphenols [10, 11, 12]. One polyphenol source is tea leaves (Camelia sinensis L.), consisting of flavonoids, mainly catechins (EGCG, ECG, C, EGC, EC), which undergo oxidation to form theaflavins and thearubigens in the manufacturing of black tea [13, 14, 15, 16]. The number of free hydroxyl groups, the presence of ortho-hydroxylation on B-ring of flavonoid molecules, a C_2-C_3 double bond in C-ring and the presence of 3-hydroxyl groups are listed as main the conditions of antiradical and antioxidant properties [17, 18, 19, 20, 21].

The universally used parameters of free radicals' scavenging ability as well as the evaluation of antioxidant potency are reactions with the radical cation $ABTS^{+\cdot}$ and stable radical $DPPH^{\cdot}$ [22, 23, 24]. The measurement of scavenging ability of $ABTS^{+\cdot}$ is a very effective tool when antioxidant composition of a mixture is not well-known (defined as TAA – total antioxidant activity) as well as the measurement of activity of fine substances or their mixture (defined as TEAC – trolox equivalent antioxidant capacity) [22].

The aim of the research was to qualify the effectiveness of green and black Yunan tea extracts as the $ABTS^{+\cdot}$ and $DPPH^{\cdot}$ radical scavengers. The study involved four Yunan tea extracts: ethanol and aqueous extracts of green and black tea.

Experimental Procedures

Reagents

(+)-catechin (C) (Merck), Folin-Cocalteu reagent was purchased from Fluka, (2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid) diammonium salt (ABTS) (Fluka); microperoxidase-8; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Aldrich); 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (Poland); ethanol POCH (Poland). All of the reagents used were of analytical grade.

Plant Material

Green and black Yunan teas were bought at a specialty tea shop. Extracts were prepared according to the method by Gramza et al. [25]. Aqueous tea extracts were prepared by heating ground tea leaves (100 g) in double-distilled water (1000 ml), followed by stirring for 15 min at 80°C. This procedure was repeated three times. Collected extracts were filtered through filter paper and centrifuged (4500 rpm, 15 min). Then extracts were frozen and lyophilized under a vacuum. Ethanol extracts were prepared by maceration of tea leaves (100 g) with 250 ml of 95% ethanol, at room temperature for 24 hours, then filtered. The procedure was repeated three times. The extracts were collected, filtered and centrifuged (4500 rpm, 15 min). The solvent was evaporated on rotary evaporator (RVO 200A, INGOS). The powdered aqueous and ethanol extracts were kept frozen (-18°C) until further use. Rate of production yield was as follows: green tea ethanol – 12.2% and aqueous extract – 23.1%, black tea ethanol – 6.2% and aqueous extract – 18.8%.

Total Polyphenol Content

The levels of total polyphenols were determined according to the method by Horwitz [26] with the use of Folin-Ciocalteu phenol reagent. The results were expressed as catechin equivalents in mg/g of extract. Standard concentrations of (+)-catechin between 0-600 μg/ml were used to prepare calibration curve.

$ABTS^{+\cdot}$ Free Radical Scavenging Method

The antioxidant activity of tea extracts was measured by TAA assay described by Lemańska et al. [27]. The TAA value is based on stability of the antioxidant to scavenge the blue-green colored $ABTS^{+\cdot}$ radical cation in comparison to scavenging ability of water-soluble vitamin E analogue – Trolox. To generate $ABTS^{+\cdot}$ in phosphate-buffered saline (pH 7.4), microperoxidase-8 (MP8) was used. After mixing, the reaction was initiated by the addition of hydrogen peroxide. The $ABTS^{+\cdot}$ solutions, with stable absorbance at 734 nm for at least 2 h, were used for the determination of TAA assay with tea extracts in ethanol to give the final concentration required. The decrease in absorbance, reflecting the $ABTS^{+\cdot}$ radical scavenging capacity of antioxidant, was plotted against the concentration of the antioxidant. The TAA value represents the ratio between the slope of this plot for scavenging of $ABTS^{+\cdot}$ by the antioxidant, compared to the slope for $ABTS^{+\cdot}$ scavenging by Trolox.

$DPPH^{\cdot}$ Free Radical Scavenging Method

The effect of green and black tea aqueous and ethanol extracts was estimated according to the procedure described by Sanchez-Moreno et al., with slight modifications [24]. An aliquot of ethanol (0.1 ml) solution containing different extracts concentrations (100-1000 ppm), was added to 3.9 ml of $DPPH^{\cdot}$ 0.025 g $litre^{-1}$ in ethanol prepared daily. The decrease in absorbance at 515 nm was measured continuously with data captured at 1min intervals on a Carl Zeiss Spectrophotometer (Jena Optik), until reaction reached plateau. Ethanol was used to zero the spectrophotometer. $DPPH^{\cdot}$ stock solution was stored at 4°C until it was used. The percentage of remaining $DPPH^{\cdot}$ was plotted to obtain the amount of antioxidant needed to decrease the initial concentration by 50%. The time needed to reach the steady state to EC_{50} concentration (T_{EC50}) was calculated graphically. Lower EC_{50} value proves the higher antioxidant ability of studied substra-

tum. The absorbance decrease is connected with the radical scavenging ability by the antioxidants contained in the studied extracts. The faster the absorption decreases, the stronger the antioxidant, possessing higher ability of hydrogen donation [28]. The range of extract concentrations and measurement frequencies were established experimentally. Taking into account that EC_{50} and T_{EC50}, affect the antiradical capacity, antiradical efficiency was defined: AE = 1) $EC_{50} \cdot T_{EC50}$.

Antiradical efficiency parameter allowed dividing the extracts into different antiradical activity groups:

$AE = 1 \cdot 10^{-3}$	low antiradical activity
$1 \cdot 10^{-3} < AE = 5 \cdot 10^{-3}$	medium antiradical activity
$5 \cdot 10^{-3} < AE = 10 \cdot 10^{-3}$	high antiradical activity
$AE > 10 \cdot 10^{-3}$	very high antiradical activity

Statistical Analysis

The results were obtained from a minimum of four independent experiments and averaged. Data were analyzed by the analysis of variance ($p \leq 0.05$) to estimate the differences between values of compounds tested. Results were processed using Statistica 6.0 software.

Results

Total polyphenol content is expressed as catechin equivalent (Figure 1). The level of total phenolic in black and green Yunan tea leaves and extracts varied between 148.6 and 837.7 mg/g ($p < 0.001$).

Results showed that total polyphenol content in tea extracts varied between 837.6 – 245.8 mg/g, and dependent from solvent used for the extraction and tea kind ($p < 0.001$). Highest polyphenol content was observed in extracts with 95% ethanol used; however, this was lower with the use of water. Statistical analysis allowed us to submit significant differences in polyphenol contents in all samples ($p < 0.001$). It was confirmed that ethanol extraction allowed obtaining four times; however, water about twice higher polyphenol content, than it was estimated in green and black tea leaves.

The analysis results of scavenging radical cation $ABTS^{+\cdot}$ ability by tea extracts are presented in Figure 2. The highest antioxidant activity among studied extracts was affirmed for black tea's extracts: aqueous 1256.0 μM/g and ethanol 1206.5 μM/g of dry weight, aqueous and ethanol extracts of green tea, 237.4 μM/g and 342.4 μM/g of dry weight.

The binary analysis of variance showed that green tea extracts possessed the considerably lower ability of scavenging radical cation $ABTS^{+\cdot}$ than the black tea ($p < 0.001$). The influence of applied solvent was not indicated.

The dependence curves of remaining $DPPH^{\cdot}$ radical in the reaction system and Yunan tea extract's concentration in precise time was presented in Figure 3. The analysis of curves permitted affirmation that studied extracts showed the $DPPH^{\cdot}$ radical scavenging ability, dependent on their concentration in the system.

The lowest concentration necessary for scavenging 50% of the $DPPH^{\cdot}$ radical (EC_{50}) was affirmed for green tea ethanol extract (209.61 g/kg) and black (210.70 g/kg). The time for reaching $DPPH^{\cdot}$ radical stable concentration (T_{EC50}) was 19 and 20.25 minutes, respectively. Antiradical activity of tea extracts were presented in Table 1, as AE parameter (antiradical efficiency) [15]. This parameter permits objective comparison of extracts antiradical activity, taking into account its concentration, indispensable for lowering the initial $DPPH^{\cdot}$ radical quantity for 50%, as well as the time of this process.

The antiradical activity analysis permitted classifying tea extracts in low activity group. Green tea ethanol extract showed the highest ability of scavenging studied radicals ($0.25 \cdot 10^{-3}$), the lowest showed green tea aqueous extract ($0.13 \cdot 10^{-3}$). Considerably higher values of AE coefficient were affirmed for ethanol, not aqueous, tea extracts.

The multifactor variance analysis showed that the ability of scavenging $DPPH^{\cdot}$ radicals by Yunan tea extracts depends on extract concentration and was best at 1000 ppm ($p < 0.001$). The ability of $DPPH^{\cdot}$ radical scavenging was also extremely dependent on applied solvent ($p < 0.001$), higher for ethanol extracts.

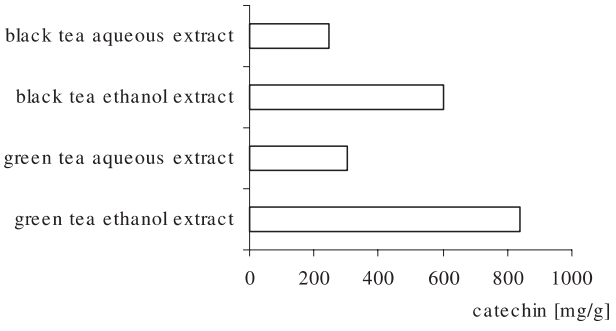

Fig. 1. Total polyphenol contents in green and black Yunan tea extracts [mg catechin/g dry weight extract].

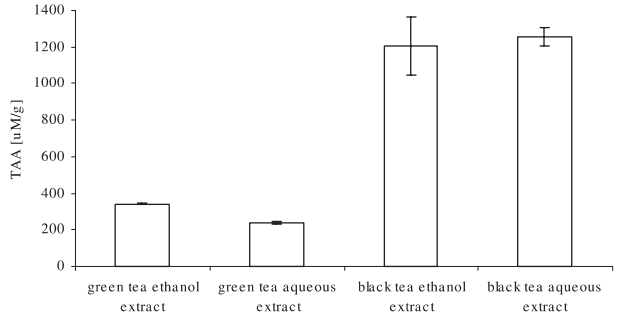

Fig. 2. Scavenging effect of tea extracts on ABTS radical cation.

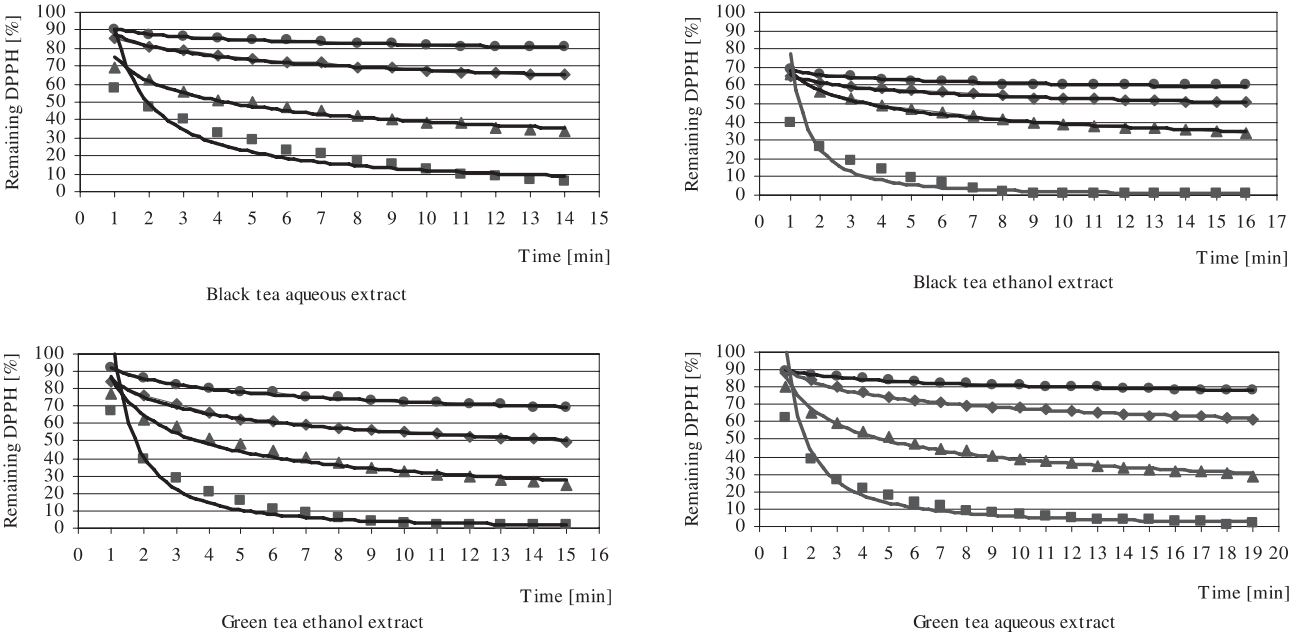

Fig. 3. Hydrogen-donating ability of tea extracts as assessed by the DPPH radical method. ● - 100 ppm, ◆ - 200 ppm,▲ - 500 ppm, ■ - 1000 ppm.

Disscusion of Results

Total Polyphenol Content

Research of polyphenol content in tea leaves and its extracts showed different results. Total polyphenol content in black and green tea leaves measured by Khokhar and Magnusdottir [29] was 80.5-134.9 mg/g and 65.8-106.2 mg/g, respectively. Hoff and Singleton estimated similar levels of polyphenols in green and black tea leaves [30]. However, Manzocco et al. estimated higher polyphenol content in green tea leaves (94.5 mg/g) than in black (80.1 mg/g) [31]. Those results were confirmed in ethanol and aqueous extracts of tea leaves. Total polyphenol content in the present research was significantly higher, confirming the hypothesis that tea leaves and polyphenol content extracts depend on the origin and type of tea.

Present research shows that extraction with ethanol allowed receiving 60% higher content of polyphenols in comparison with water extraction. Nwuha et al. [32] also analyzed the solubility of tea leaf constituents, and found higher solubility of catechins in 99.5% ethanol than in 50% ethanol solution with water. Polyphenol content in ethanol extracts was five to seven times higher than in water extract.

Scavenging Effect of Tea Extracts on $ABTS^{+\cdot}$ Radical (TAA)

Arts et al. [33] studied the ability of $ABTS^{+\cdot}$ radical cation scavenging by commercial tea extracts containing a known type of polyphenolic fraction. Research confirmed considerably higher activity of the green tea extract (7.3 mM TEAC (g of extract) than black tea (5.8 mM TEAC (g of extract). The authors suggest that green tea activity is the result of the presence of considerable quantities of catechins. In black tea, however, catechins occurred in considerably lower quantities. It might be considered; however, that black tea components – tannins, making up 91.5% of the extract – show very strong antiradical proprieties. The above-mentioned observations have not been confirmed in present research. There were affirmed considerably lower radicals scavenging activity of tea extracts. Black tea extracts showed nearly four times higher antiradical activity in comparison to green tea extracts, containing a considerably larger quantity of catechins. Similarly to Arts [33] suggestions, the strong proprieties of $ABTS^{+\cdot}$ radical scavenging by black tea might be alleged by the presence of tannins. Salah et al. [34] measured the antioxidative activity of catechins towards the radicals generated in aqueous phase, ex-

Table 1. Tea extracts antiradical efficiency.

Extract		EC_{50} (g of antioxidant kg^{-1} $DPPH^{\cdot}$)	T_{EC50} (min)	AE ($\cdot 10^{-3}$)	Antiradical efficiency classification
green tea	ethanol	209.61	19.00	0.25	low
	aqueous	349.16	21.25	0.13	low
black tea	ethanol	210.70	20.25	0.23	low
	aqueous	390.12	15.25	0.17	low

pressed as TEAC. The efficiency of $ABTS^{+\cdot}$ radical cation scavenging formed as follows: ECG> EGCG> EGC> EC = C. The analysis of green tea extract and equivalent mixture of component activity was also conducted. It was shown that the TEAC value of green tea extract (3.78 mM/g) was higher than for its components mixture (2.76 mM/g). It was affirmed that ortho-dihydroxy structure in B ring of polyphenols structure is essential in radical stabilization, as well as in metal chelating ability. Moreover, the existing dependence among type of antioxidants and their activity was shown. It was affirmed that nearly 70% of activity might be classified as activity of catechins and their gallic esters. The analysis of tea extracts chemical constitution [5] did not confirm higher antiradical activity of green tea extract, containing considerably more of ECG and EGCG than the black tea extracts. Miller and Rice-Evans [22] studied the theaflavins activity in arrangement with Trolox (TAA) and showed that the scavenging activity of radical cation $ABTS^{+\cdot}$ increases with the esterification degree with gallates.

Pietta et al. [36] affirmed that green tea ethanol extract containing 70% of catechins showed the highest ability of scavenging $ABTS^{+\cdot}$ radical, considerably higher from Ginseng, Ginko Biloba, or grape skin and seed extracts. It was also affirmed that high activity of plant extracts in scavenging the $ABTS^{+\cdot}$ radical is due to the presence of other components, not only catechins. Yet the world literature is poor in information relating to their activities. Gao et al. [37] studied the activity of ethanol and aqueous extracts from wild rose. They affirmed that lipophilic character extracts showed lowest $ABTS^{+\cdot}$ radical cation scavenging ability in comparison to hydrophilic character extracts. The research of Zieliński and Kozłowska [38] confirmed similarly higher activity in scavenging the $ABTS^{+\cdot}$ radical cation by different cereal grains hydrophilic than lipophilic extracts. Above-mentioned ascertainments are consistent with results of present research, were the black tea ethanol extract lipophilic fraction made up 70%, and aqueous 26%, of the extract [35].

Scavenging Effect of Tea Extracts on $DPPH^{\cdot}$ Radical

Yokozawa et al. [12] conducted the analysis of $DPPH^{\cdot}$ free radicals scavenging ability by green and black tea aqueous extracts. They affirmed that green tea aqueous extract showed higher radicals scavenging efficiency than black tea extract. Similarly, Yen and Chen [16] studied the ability of scavenging $DPPH^{\cdot}$ radical by tea extracts and ranked it as follows: aqueous extracts of green tea> red> black. However, Von Gadow et al. [39] studied the free radicals scavenging ability by aqueous extracts of green, oolong and black teas. The results of investigations have permitted us to rank extracts according to their decrescent antiradical proprieties: green> black> oolong tea. Additionally, the best abilities of $DPPH^{\cdot}$ free radicals scavenging was affirmed for (±)-catechin, quercitin and rutin, considerably higher than BHT and α- tocopherol. Present research results do not confirm higher activity of aqueous extract green tea, in which antiradical activity was about 25% lower than activity of black tea extract. Green tea ethanol extract, however, showed about 8% higher $DPPH^{\cdot}$ radicals scavenging activity than black tea extract.

Sanchez-Moreno et al. [24] affirmed that gallic acid showed medium antiradical activity in $DPPH^{\cdot}$ free radicals scavenging test ($2.62 \cdot 10^{-3}$). Quercitin and rutin showed low activity at $0.19 \cdot 10^{-3}$ and $0.21 \cdot 10^{-3}$, respectively. Comparison of the antiradical activity of α-tocopherol ($0.52 \cdot 10^{-3}$) and BHA ($0.10 \cdot 10^{-3}$) was also conducted. High antiradical potential in scavenging of $DPPH^{\cdot}$ radicals was affirmed for EGCG [30]. Hatano et al. [41] and Nanjo et al. [42] affirmed, however, that in spite of gallic acid presence in 3'position of catechins C ring, EGCG activity was not different than EGC and GC. In present research the highest antiradical activity was indicated in green ($0.25 \cdot 10^{-3}$) and black ($0.23 \cdot 10^{-3}$) tea ethanol extracts, despite 10 times higher EGCG content in green tea than black tea ethanol extract [35]. Endo et al. [43] studied the ability of $DPPH^{\cdot}$ free radical scavenging by chlorophyll and pheophitin. It was affirmed that chlorophyll can deliver hydrogen for $DPPH^{\cdot}$ radical reduction as well as scavenge the fatty radicals, formed during lipid oxygenation. Results of presented research confirm the above-mentioned thesis. The analysis of chlorophyll and pheophitin content in tea extracts showed that the antiradical activity in $DPPH^{\cdot}$ system was higher in samples with higher content of these compounds.

Comparison of Yunan tea extracts $DPPH^{\cdot}$ and $ABTS^{+\cdot}$ free radicals scavenging ability is allowed to affirm its diversity. In $ABTS^{+\cdot}$ radical test main activity factor was tea kind, and activity was higher in black tea extracts. In $DPPH^{\cdot}$ test, however, essential factors influencing antiradical activity was a kind of applied solvent and was higher in ethanol extracts. Comparison of both radicals' scavenging abilities is unusually difficult with regard to different action mechanisms of oxidative factors, including free radicals. It was also affirmed that possible antiradical activity of plant extracts was conditioned by antioxidant structures as well as other component interactions.

Abbrevations

ABTS-2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid) diammonium salt; TEAC-Trolox Equivalent Antioxidant Capacity; TAA-Total Antioxidant Activity; EGCG-(-)-epigallocatechin gallate; ECG–(-)-epicatechin gallate; EGC–(-)-epigallocatechin; EC–(-)-epicatechin; C–(+)-catechin; DPPH-2,2-diphenyl-1-picrylhydrazyl; EC_{50}–antioxidant concentration necessary for scavenging 50% of $DPPH^{\cdot}$ radical; T_{EC50}-time of reaching EC_{50} $DPPH^{\cdot}$ radical stable concentration; AE–antiradical efficiency; BHA–butylated hydroxyanisole; BHT–butylated hydroxytoluene.

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