The comparison of effect of catechins and green tea extract on oxidative modification of LDL *in vitro*

Ostrowska J, Skrzydlewska E*

Department of Analytical Chemistry, Medical University of Białystok, Poland

Abstract

Purpose: Green tea due to its content of catechins reveals strong antioxidative activity, which is manifested among others by its ability to inhibit free radical generation, scavenge free radicals as well as chelate transition metal ions that catalyse free radical reactions. The influence of green tea extract, epicatechin (EC), epicatechin galate (ECG) as well as epigallocatechin galate (EGCG) on oxidative modifications of LDL of human blood serum has been examined in the present study.

Materials and methods: This influence has been evaluated by measurement of the concentration of first products of lipid peroxidation – conjugated dienes and lipid hydroperoxides as well as by determining tryptophan and dityrosine content – the markers of protein oxidative modification.

Results: Catechins and green tea abilities to protect lipophilic antioxidant – α-tocopherol against oxidation have been also examined. The results reveal that peroxidation of LDL is markedly prevented by green tea extract and in a slightly weaker way by catechins (EGCG in particular), which is manifested by a decrease in concentration of conjugated dienes, lipid hydroperoxides, MDA, dityrosine and by an increase in tryptophan content. Both green tea as well as catechins (EGCG in particular) have been also revealed to prevent decrease in concentration of α-tocopherol in oxidating conditions.

Conclusions: It can be assumed that green tea and to a lesser degree catechins, protecting the basic antioxidant of LDL-α-tocopherol, prevent oxidative modification of LDL.

Key words: LDL, green tea, catechins, lipid peroxidation, protein oxidative modifications, α-tocopherol.

* CORRESPONDING AUTHOR:
Department of Analytical Chemistry
Medical University of Białystok,
P.O. Box 14, 15-230 Białystok, Poland
Tel: +48 85 7485707; Fax: +48 85 7485707
e-mail: jostrowska@poczta.fm (Justyna Ostrowska)

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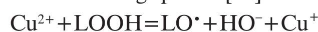
Introduction

A key early event in the development of atherosclerosis is the oxidation of low density lipoprotein via different mechanisms including free radical reactions with both protein and lipid components. The LDL particle (~2.5 MDA) consists of an apolar core of cholesteryl esters (42%) and triglicerides (6%), surrounded by a monolayer of phospholipids (22%), unestrified cholesterol (8%) and one molecule of apolipoprotein B-100 (4536 amino acids, 550 kDa) (22%) [1]. Thus, each LDL particle contains about 1600 molecules of cholesteryl ester, 170 of trigliceryde, 700 of phospholipid and 600 molecules of free cholesterol [2]. Human LDL contains a number of antioxidants that inhibit lipid oxidation, with α-tocopherol the most abudant (~6 α-tocopherol molecules per LDL particle) and other antioxidants (e.g. carotenoids, ubiquinol-10) present in much lower abudance [2].

LDL phospholipids consist of polyunsaturated fatty acids. They have at least one methylene group between double bindings. Elimination of hydrogen atom from methylene groups and displace of double bindings occurs under the influence of oxidizing factors, hydroxyl radical in particular [3]. This transformation results in generation of conjugated dienes which next react with particle oxygen with generation of superoxide radicals. If unpaired electron is localized at the end of a double bindings system it is reduced to lipid hydroperoxides. Lipid hydroperoxides are simple lipid peroxidation products and are relatively constant in the case of transition metal ions absence [4]. Further alterations of peroxidation products, occurring among others on the β-elimination way, lead to decomposition of polyunsaturated fatty acid residues and to generation of few and/or several carbon fragments. Lipid peroxidation final products include cycle ethers, hydroperoxides and carbonyl compounds, such as aldehydes being the most important group among them, including malondialdehyde and 4-hydroxynoneal [5]. Compounds that are lipid peroxidation products may be used as markers of intensification of this process [6]. Among lipid peroxidation products, significant reactivity is revealed particularly by α, β-unsaturated aldehydes, which easily

react with proteins and DNA, and therefore are characterized by toxic and mutagenic properties [7]. Among lipid peroxidation products, 4-hydroxynoneal reveals the strongest toxic activity, whereas malondialdehyde is the most mutagenic [8,9].

The mechanisms by which metal ions stimulate LDL oxidation are poorly understood. Reduced copper ions (Cu^{2+}) catalyze the decomposition of lipid peroxides into alkoxyl radical, a potent oxidizing species [10]:



$Cu^{2+} + LOOH = LO^{\bullet} + HO^{-} + Cu^{+}$

The hydroperoxide-derived radicals next are scavenged by antioxidants. They can also react with polyunsaturated fatty acids (LH) to form carbon-centered radicals ($L^{\cdot}$) that initiate the radical chain reaction of lipid peroxidation [10]. The reaction cycle continues until antioxidants or radical-radical cross-linking reactions terminate lipid peroxidation.

$LO^{\bullet} + LH = LOH + L^{\cdot}$

One possible mechanism of LDL oxidation is the conversion of α-tocopherol to α-tocopherol radical [11,12]. The radical then attack a polyunsaturated fatty acid to initiate lipid peroxidation.

α-tocopherol $+ Cu^{2+} =$ α-tocopherol radical $+ Cu^{+} + H^{+}$

α-tocopherol radical $+ LH =$ α-tocopherol $+ L$.

Reduction of bound Cu^{2+} to Cu^{+} by endogenous α-tocopherol appears to be a key step in initiating LDL lipid peroxidation [11]. This leads to the counter-intuitive proposal that α-tocopherol, normally considered to be an antioxidant, can promote the peroxidation of LDL lipid [12].

Free oxygen radicals may also cause oxidative modification of protein part of LDL – apolipoprotein B-100 [13]. It may undergo oxidative modification as a result of direct reaction of free radicals with amino acid residues or in reaction with carbonyl compounds, generated during degradation of phospholipid peroxidation products – mainly malondialdehyde and 4-hydroxynonenal [14]. These kinds of modifications may even lead to apolipoprotein B-100 particle degradation [2].

To prevent these type of reactions, compounds revealing antioxidative properties, which are demonstrated among others by ability to reduce the amount of free radicals, may be applied. More and more attention has been recently paid to compounds of natural origin. Of particular importance is green tea, which contains abundance of catechins – compounds which reveal strong antioxidative properties [15]. Catechins may reduce of possibilities of occurring oxidative modifications of biologically important compounds, including lipids or proteins.

Therefore the present study has determined the influence of catechins and green tea extract on peroxidation of LDL, through measurement of concentration of succesive lipids peroxidation products – conjugated dienes, lipid hydroperoxides and malondialdehyde as well as oxidative protein modification products – tryptophan and dityrosine. Moreover, the influence of catechins and green tea extract on the protection of the basic lipophoilic antioxidant – α-tocopherol has been examined.

Materials and methods

Determination of catechins in green tea

(-)-epicatechin, (-)-epigallocatechin, (-)-epigallocatechin gallate were obtained from Sigma-Aldrich Chemie Gmbh (Steincheim, Germany). Green tea – Camellia sinensis (Linnaeus) O. Kuntze (standard research blends-lyophilized extract) was provided by TJ Lipton (Englewood Cliffs, NJ). Green tea extract contained epigalloctechin gallate (97 mg/g dried extract), epigallocatechin (82 mg/g dried extract), epicatechin (90 mg/l), epicatechin gallate (15 mg/g dried extract) and caffeic acid (10 mg/g dried extract), determined by HPLC [16].

Isolation of LDL from serum

The LDL fraction was isolated from extraplacental human serum by precipitate method in the presence of heparin and manganese chloride [17]. The protein content in the LDL fraction was measured by the Lowry method [18].

LDL oxidation *in vitro*

The stock LDL solution (5.3 mg protein/ml) was diluted by Tris-HCl buffer (final concentration 0.2 mol/l, pH 7.4) to obtain concentration 150 μg protein/ml). The oxidative modification of LDL was initiated by addition of $CuSO_4$ solution (final concentration 5 μmol/l). To counteract oxidative modification of LDL, epicatechin (EC), epigallocatechine (EGC) and epigallocatechine gallate (EGCG) (final concentration 10 μmol/l) as well as green tea extract (final concentration 3%) were added to samples 5 minutes after Cu^{2+} ions addition. LDL solution (150 μg protein/ml) in Tris-HCl buffer (final concentration 0.2 mol/l, pH 7.4) was the control sample. All samples were incubated for 1, 2, 4, 6, 18, 22 and 30 hours at 37°C. The levels of lipid peroxidation products – conjugated dienes, lipid hydroperoxides, malondialdehyde and of protein oxidative modification products – dityrosine and tryptophan and of α-tocopherol were measured in all samples.

Determination of conjugated dienes

In this method dienes were extracted with chloroform-methanol mixture (2:1; v:v) and were quantitated by their 234 nm absorbance in cyclohexane, relative to cyclohexane blank [19].

Determination of lipid hydroperoxides

Lipid hydroperoxides were determined by a sensitive and specific HPLC method involving chemical conversion 1-naphthyldiphenylphosphine into 1-naphthyldiphenylphosphine oxide, which was injected on RP 18 column eluated by methanol-water mixture (8:2; v:v) at 35°C, the detection was carried at λ=292 nm [20].

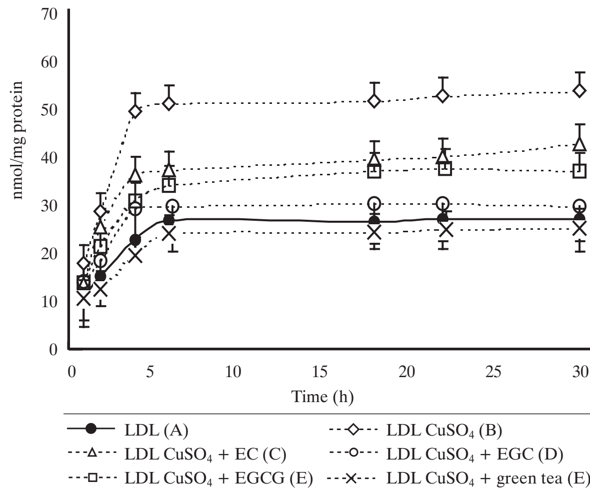
Determination of malondialdehyde

The procedure involves formation MDA with thiobarbituric acid (TBA) adducts and the separation of TBA-MDA adducts on RP 18 column with spectrofluorometric quantification at 532 nm excitation and 553 nm emission. The separation was carried by mixture 40% methanol and 60% phosphorate buffer at pH 7.0 [21].

Determination dityrosine and tryptophan

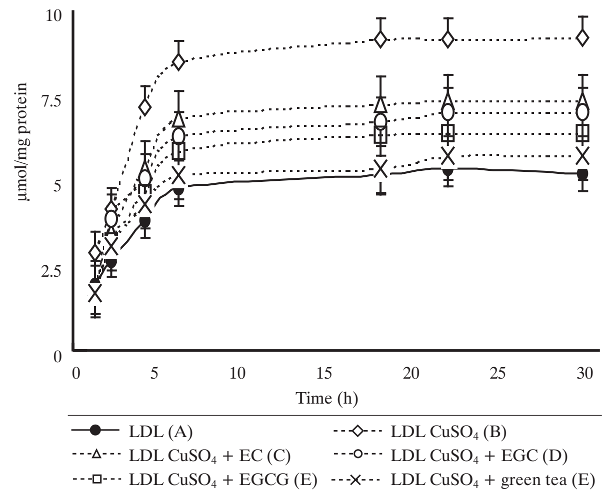
Tryptophan and dityrosine were measured with a spectrofluorometer Hitachi 2500. Signal intensity was calibrated against 0.1 mg/ml quinine sulfate solution in sulfuric acid with fluorescence was assumed as a unit. Fluorescence emission at

Figure 1. **The effect of epicatechin (EC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG) and green tea extract on the level of conjugated dienes in human LDL fraction in presence of 5 μM Cu^{2+} (n=6)**



The values for $p<0.05$ were considered significant:
2 h: A – B, C; B – A, D, F
4 h: A – B, C; B – A, C, D, E, F
6 h: A – B, C, E; B – A, C, D, E, F
18 h: A – B, C, E; B – A, C, D, E, F
22 h: A – B, C, E; B – A, C, D, E, F
30 h: A – B, C, E; B – A, C, D, E, F

Figure 2. **The effect of epicatechin (EC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG) and green tea extract on the level of lipid hydroperoxides in human LDL fraction in presence of 5 μM Cu^{2+} (n=6)**



The values for $p<0.05$ were considered significant:
2 h: A – B; B – A, F
4 h: A – B, C, D; B – A, C, D, E, F
6 h: A – B, C, D, E; B – A, C, D, E, F
18 h: A – B, C, E; B – A, C, D, E, F
22 h: A – B, C, E; B – A, C, D, E, F
30 h: A – B, C, E; B – A, C, D, E, F

338 nm (288 nm excitation) was used as a reflection of tryptophan content, dityrosine content was estimated at 325 nm excitation and 420 nm emission [22].

Determination of α-tocopherol by HPLC

The lipid fraction was extracted from LDL solutions by hexane, and the organic layer was next dryied under nitrogen. The residue was dissolved in ethanol and injected on RP 18 column. The separation was carried by mixture 95% methanol and 5% water with spectrophotometric detection at 294 nm [23,24].

Statistical analysis

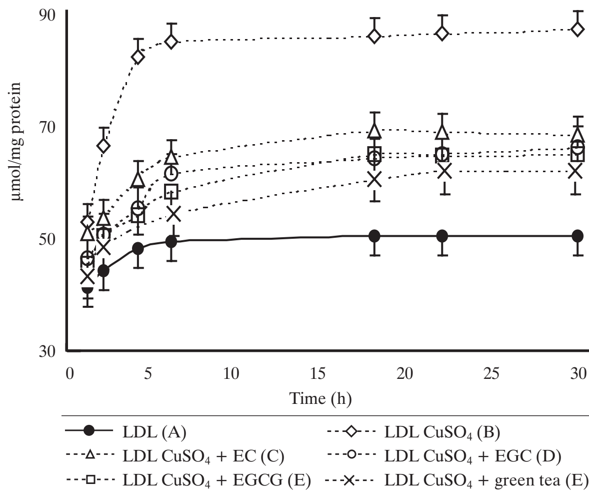
The data obtained in this study are expressed as mean ±SD. The data were analysed by use of standard statistical analyses, one way Student's test for multiple comparisons to determine significance between different groups. The values for $p<0.05$ were considered as significant.

Results

Incubation of LDL with Cu^{2+} ions results in lipid peroxidation. This is manifested by an increase in the amount of primary products of this process – conjugated dienes (up to about 98%) and lipid hydroperoxides (up to about 63%) in comparsion to LDL solution (*Fig. 1* and *Fig. 2*). The level of these compounds increases during 6 hours incubation and after these time its concentration is not changed. However, their level is remarkably lower in the presence of catechins as well as green tea extract. Influenced by green tea, the content of conjugate dienes is decreased by about 54%, and lipid hydroperoxides by about 48% compared to oxidized LDL. The level of final product of lipid peroxidation – malondialdehyde increases up to about 76% during 6 hours in the presence of Cu^{2+} ions, and its concentration is not changed after this time (*Fig. 3*). Catechins and green tea extract remarkably prevent an increase in MDA concentration in LDL solution with Cu^{2+} ions. Lipid peroxidation is the most effectively prevented by epigallocatechin gallate (35% decrease in lipid hydroperoxides concentration and 45% decrease in malondialdehyde concentration in relation to LDL solution with Cu^{2+} ions) and by epigallocatechin (43% decrease in conjugated dienes concentration in relation to LDL solution with Cu^{2+} ions), and the most weakly by epicatechin (26% decrease in conjugated dienes concentration, 10% decrease in lipid hydroperoxides concentration and 25% decrease in malondialdehyde concentration in relation to oxidized LDL).

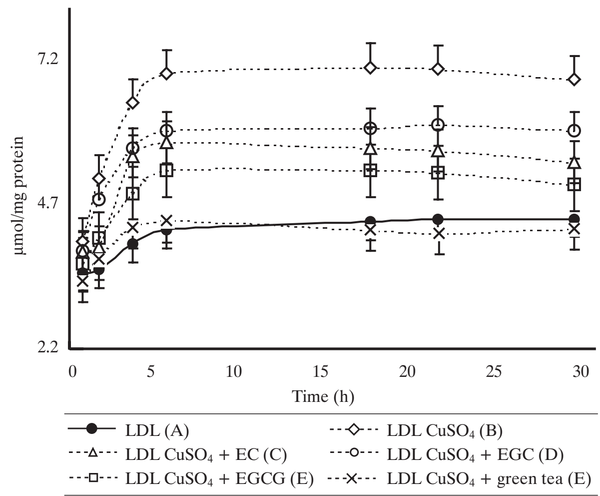
Incubation of LDL solution with Cu^{2+} ions also causes oxidative modifications of protein part of LDL – apolipoprotein B-100. It is manifested by the increase in dityrosine concentration (*Fig. 4*) and by the decrease in tryptophan concentration (*Fig. 5*). Dityrosine content increases by about 93% after 6 hours incubation, and level of tryptophan is decreased by about 41% in relation to the solution containing LDL only. Such significant changes in values of the above parameters do not occur in the presence of catechins and green tea. The supplementation of green tea completely prevents the increase in dityrosine level in LDL fraction due to Cu^{2+} ions oxidation. In the oxidized LDL solution containing green tea dityrosine level is significantly

Figure 3. **The effect of epicatechin (EC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG) and green tea extract on the level of malondialdehyde in human LDL fraction in presence of 5 μM Cu^{2+} (n=6)**



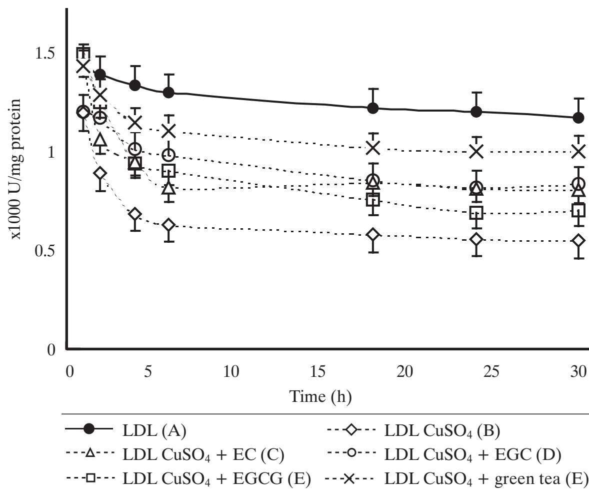
The values for $p<0.05$ were considered significant:
2 h: A – B, C; B – A, D, F
4 h: A – B, C, D; B – A, C, D, E, F
6 h: A – B, C, D, E; B – A, C, D, E, F
18 h: A – B, C, E; B – A, C, D, E, F
22 h: A – B, C, E; B – A, C, D, E, F
30 h: A – B, C, E; B – A, C, D, E, F

Figure 4. **The effect of epicatechin (EC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG) and green tea extract on the level of dityrosine in human LDL fraction in presence of 5 μM Cu^{2+} (n=6)**



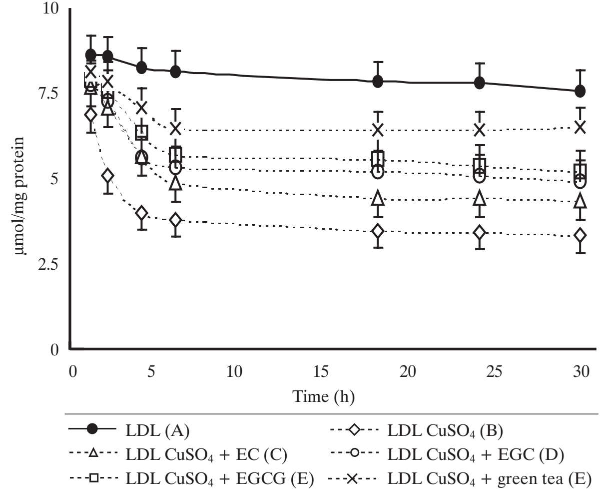
The values for $p<0.05$ were considered significant:
4 h: A – B, C, D; B – A, C, D, E, F
6 h: A – B, C, D, E; B – A, C, D, E, F
18 h: A – B, C, D, E; B – A, C, D, E, F
22 h: A – B, C, D, E; B – A, C, D, E, F
30 h: A – B, C, D, E; B – A, C, D, E, F

Figure 5. **The effect of epicatechin (EC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG) and green tea extract on the level of tryptophane in human LDL fraction in presence of 5 μM Cu^{2+} (n=6)**



The values for $p<0.05$ were considered significant:
2 h: A – B, C; B – A, F
4 h: A – B, C; B – A, D, E, F
6 h: A – B, C, D, E; B – A, C, D, E, F
18 h: A – B, C, E; B – A, C, D, F
22 h: A – B, C, E; B – A, C, D, F
30 h: A – B, C, E; B – A, C, D, F

Figure 6. **The effect of epicatechin (EC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG) i green tea extract on level of α-tocopherol in human LDL fraction in presence of 5 μM Cu^{2+} (n=6)**



The values for $p<0.05$ were considered significant:
2 h: A – B; B – A, F
4 h: A – B, C, D; B – A, D, E, F
6 h: A – B, C, D, E; B – A, D, E, F
18 h: A – B, C, E; B – A, D, E, F
22 h: A – B, C, E; B – A, D, E, F
30 h: A – B, C, E; B – A, D, E, F

decreased (by 57%) while tryptophan content is increased (by about 38%) in relation to concentration of these compounds in oxidized LDL. Among the examined catechins, epigallocatechin gallate reveals the strongest protective action against generation of dityrosine (31% decrease in its concentration) and epigallocatechin against tryptophan decrease (40% increase in tryptophan concentration in relation to LDL solution with Cu^{2+} ions), whereas epicatechin is characterised by the weak-

est protective action against this process (about 8% decrease in dityrosine concentration and 37% increase in tryptophan concentration in relation to oxidized LDL).

Influenced by incubation of LDL with Cu^{2+} ions decrease in α-tocopherol content occurred (*Fig. 6*). The amount of this compound is reduced throughout 4 hours incubation, after which it reaches the constant value. Addition Cu^{2+} ions into the LDL solution causes significant (by about 48%) decrease in α-tocopherol content. Among the applied antioxidants, the green tea extract and epigallocatechin gallate most effectively protect against decrease in α-tocopherol concentration (by 80% and 62% respectively). The catechin that reveals the weakest protective properties against decrease in α-tocopherol content is epicatechin because in its presence concentration of α-tocopherol is increased by about 12% only in relation to LDL solution with Cu^{2+} ions.

Discussion

The present study has revealed that Cu^{2+} ions cause LDL peroxidation. It is manifested by intensified oxidative modification of the lipid and protein LDL part through increase in concentration of conjugated dienes, lipid hydroperoxides, malondialdehyde, dityrosine as well as through decrease in the content of tryptophane and α-tocopherol.

For *in vitro* studies of oxidized LDL, oxidation by copper is frequently used and produces LDL and is similar to LDL oxidized by cells [13]. Copper is more potent than iron in its ability to oxidize LDL *in vitro* [13]. Copper ions are known to induce the formation of radicals in the aqueous phase and they can affect with amino acids of apolipoprotein B-100 [13,25].

A number of antioxidants may act in partly by inhibiting the binding of copper to LDL. Our results are in agreement with previous authors [26] who have demonstrated an antioxidant effect with catechins and particularly green tea extract during copper-mediated oxidation of LDL. Antioxidant capacity of catechins is related to their localization in the LDL particle, as well as to their chemical structures. Catechins contain both lipophylic and hydrophylic moieties, but are mostly hydrophylic and can act as potent inhibitors of LDL oxidation via several mechanisms: scavenging of free radicals by acting as reducing agents, as hydrogen atom donating molecules; chelation of transition metal ions, thereby reducing the metals capacity to generate free radicals; and as well as sparing of vitamin E in the LDL particle, thus protecting LDL from oxidation. Catechins possess penthahydroxypolyphenol structure. Strong antixidative properties of a molecule are due to the presence of at least 5 hydroxyl groups and the double bond of pyrone ring. Such structure has been identified as important for both chelating and radical scavenging activity. The hither to existing examinations have proved, that epigallocatechin gallate and epicatechin gallate reveal the strongest antioxidative properties, and epicatechin – the weaker. The present results confirm these dependences. It has been found that the green tea extract reveals stronger protective activity against lipid peroxidation than single catechins. Therefore the essential property of the catechins is their synergism.

Catechins may inhibit the lipid peroxidation process by scavenging free radicals (mainly owing to orto-dihydroxyphenol structure), as well as by regeneration of α-tocopherol. The present study has revealed, that green tea catechins remarkably prevent decrease in α-tocopherol concentration under the influence of Cu^{2+} ions activity. The literature data suggest that green tea catechins (EGCG in particular) regenerate tocopherol radical to α-tocopherol through the ability to release hydrogen atom to lipid radicals [27]. Moreover, catechins having lower reducing potentials than oxygen free radicals may prevent reduction of α-tocopherol concentration through scavenging oxygen radicals such as hydroxyl radical, superoxide anion, peroxide and lipid radicals [27-31], which occurred in the presence of Cu^{2+} ions. Catechins ability to scavenge radicals is also connected with its di- or trihydroxyl structure of the phenyl ring, which secures stability for radical forms. The present study has demonstrated that epigallocatechin gallate is the most effective catechin, which prevented lipids peroxidation the strongest.

Studying the causes of efficacy of antioxidative activity of EGCG it has been found that it is connected with possessing 3 hydroxyl groups of the phenyl ring [32]. Antioxidative activity of EGCG is also intensified by hydroxyl group estrification in position 3, by gallate acid, since as it has recently been revealed, the gallate acid residue may react with lipids radicals [33].

The present study has demonstrated that also protein part of LDL – apolipoprotein B-100 undergoes oxidative modifications under the influence of Cu^{2+} ions, what was manifested by an increase in dityrosine concentration and decrease in tryptophan concentration. The oxidation products of the lipid components of LDL have been studied extensively, less is known about the oxidation products of apolipoprotein B-100. Metal-catalyzed oxidations have been studied. The functional groups formed by metal-catalyzed oxidations that have been characterized to date have been primarily aldehydes and ketones, but hydroxylated phenylalanine, tyrosine, leucine and valine residues have been reported, along with dityrosine as a product of oxidation [34]. It was shown as well as that Cu^{2+} catalyzes protein oxidation through close binding to tryptophan residues with a site-specific redox reaction that yelds a tryptophan radical and Cu^{+}. Moreover, copper ions are able to bind to the histidine residues on apolipoprotein B-100. The number of binding sites reported varies widely over 2 orders of magnitude [13]. During metal-catalyzed oxidations a redox-active transition metal (e.g. Cu^{2+}/Cu^{+}) binds to a metal-binding site on the protein and Cu^{+} – protein complex reacts with H_2O_2 to produce reactive oxygen species that resemble $HO^{\bullet}$. The reactive oxygen species react with molecular structures in the immediate vicinity of the metal-binding site, resulting in the formation of aldehyde or ketone derivatives. Our results additionally demonstrate that catechins and green tea reveal protective properties in relation to apolipoprotein B-100. They partly prevent dityrosine generation and decrease in tryptophane concentration.

The similar time of studied antioxidants action results from similar antioxidant capacity and similar structure of their particles.

Our results give evidence for the effectiveness of green tea in counteracting changes in antioxidative system and in the lipid peroxidation process that are intensified in the presence of Cu^{2+} ions. It suggests the posibility of its application for neutralizing the consequences of free radicals overproduction in pathological cases.

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