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Comparative Study of Antioxidant Properties and Cytoprotective Activity of Flavonoids

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Abstract—Antioxidant properties and cytoprotective activity of flavonoids (rutin, dihydroquercetin, quercetin, epigallocatechin gallate (EGCG), epicatechin gallate (ECG)) were studied. All these compounds inhibited both NADPH- and CCl_4-dependent microsomal lipid peroxidation, and the catechins were the most effective antioxidants. The I_{50} values calculated for these compounds by regression analysis were close to the I_{50} value of the standard synthetic antioxidant ionol (2,6-di-*tert*-butyl-4-methylphenol). The antiradical activity of flavonoids to $O_2^{\cdot-}$ was studied in a model photochemical system. Rate constants of the second order reaction obtained by competitive kinetics suggested flavonoids to be more effective scavengers of oxygen anion-radicals than ascorbic acid. By competitive replacement all flavonoids studied were shown to be chelating agents capable of producing stable complexes with transition metal ions (Fe^{2+}, Fe^{3+}, Cu^{2+}). The flavonoids protected macrophages from asbestos-induced damage, and the protective effect increased in the following series: rutin < dihydroquercetin < quercetin < ECG < EGCG. The cytoprotective effect of flavonoids was in strong positive correlation with their antiradical activity to $O_2^{\cdot-}$.

Key words: antioxidants, flavonoids, oxidative stress, reactive oxygen species, asbestos, rutin, quercetin, epicatechins

Reactive oxygen species (ROS) which include oxygen-centered radicals ($O_2^{\cdot-}$, $^{\cdot}OH$, $NO^{\cdot}$, $RO^{\cdot}$, $ROO^{\cdot}$) and non-radical molecules (hydrogen peroxide, singlet oxygen, hypochloric acid) are produced by cells as a result of aerobic metabolism. ROS are involved in the organism's normal vital activity, including phagocytosis, regulation of cell proliferation, intracellular signalization, and synthesis of biologically active compounds and ATP [1]. With an insufficiency of the antioxidant protective system or under an intense influence of radical-initiating factors (ionizing radiation, hard ultraviolet radiation, xenobiotics, mineral dust), ROS are hyperproduced and oxidative stress develops. Oxidative stress is a specific feature in pathogenesis of various diseases, including cardiovascular diseases, diabetes, tumors, rheumatoid arthritis, and epilepsy [1]. Although in many cases oxidative stress is not the cause but a symptom of a disease, there is good evidence for preventive and therapeutic effects of natural antioxidants including flavonoids (quercetin, rutin, green tea catechins) [2-4]. Because of their low redox potential ($0.23 < E_7 < 0.75$ V), flavonoids can reduce highly oxidized free radicals with redox potential values of 2.13-1.0 V ($O_2^{\cdot-}$, $^{\cdot}OH$, $NO^{\cdot}$, $RO^{\cdot}$, $ROO^{\cdot}$) [5]. Moreover, flavonoids can suppress the production of ROS due to inhibition of redox enzymes (monooxygenase, cyclooxygenase, lipoxygenase, xanthine oxidase, NADH oxidase) [1, 2] and also bind ions of transition metals which are involved in generation of oxygen radicals by Fenton's reaction [6]. The biological activities of flavonoids (anti-inflammatory, anti-allergic, anti-carcinogenic) is believed to be mainly due to their antioxidative properties, but the contribution of antiradical mechanisms to their biological effects remains unclear. We found earlier a pronounced antioxidative effect of the flavonols quercetin and rutin under conditions of microsomal lipid peroxidation (LPO) *in vitro* [6, 7] and their ability to interact with oxygen-anion radical and thus protect phagocytizing cells against asbestos-induced damage [8, 9]. This work presents a comparative study of antioxidant properties of flavonoids with similar structure: catechins epicatechin gallate and epigallocatechin gallate, flavonols quercetin and rutin, and a flavononol dihydroquercetin. We also attempted to establish the role of antiradical mechanisms in the cytoprotective effect of the compounds studied under conditions of oxidative stress with asbestos-induced damage to phagocytizing cells.

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MATERIALS AND METHODS

Superoxide-dependent reduction of *p*-nitrotetrazolium chloride (85 μM) was performed in 0.175 M phosphate buffer (pH 7.8) containing 0.06 mM EDTA, 0.6 mM N,N,N′,N′-tetramethylethylene diamine, and 6 μM riboflavin [10]. The light source was an LD-20 daylight lamp (20 W) placed at the distance of 20 cm from the specimens. After the illumination the reaction was stopped by introduction into the samples of 0.02 ml of superoxide dismutase (SOD, 5 μg/ml), and the absorption of the specimens was determined at 515 nm.

Lipid peroxidation was induced by addition of 0.3 mM NADPH and was performed at 37°C. During CCl_4-initiated LPO the incubation medium contained 0.05 M phosphate buffer (pH 7.4), 0.02 M KCl, 0.6 mM EDTA, and 3.4 mM CCl_4 dissolved in alcohol at the final concentration of ethanol 2%, and also microsomal protein (1.2 mg/ml). In the course of the NADPH-dependent LPO the incubation medium contained 0.05 M phosphate buffer (pH 7.4), 0.02 M KCl, 10 μM $FeSO_4$, and microsomal protein (1.2 mg/ml). Microsomes were isolated from rat liver by differential centrifugation at 105,000*g* using a VAC 601 centrifuge (Germany). The protein content was determined by the Lowry method. To determine contents of LPO products, the samples (1 ml) were supplemented with 0.5 ml of 30% TCA and 2.5 ml of 0.5% 2′-thiobarbituric acid (TBA), the mixture was kept for 15 min in a boiling water bath, and it was then centrifuged to remove the denatured protein and the absorption was determined at 532 nm.

Peritoneal macrophages were prepared by a modification of a published method [11]. Suspension of macrophages in isotonic phosphate buffer (pH 7.3) contained $5 \cdot 10^6$ cells/ml. Oxidative stress in the macrophages was induced by addition of aqueous suspension of asbestos at the final concentration of 3 mg/ml. The samples were incubated at 37°C for 20 min, and the degree of cell damage was determined by release of lactate dehydrogenase (LDH).

Lactate dehydrogenase activity was determined spectrophotometrically by the rate of NADH utilization in the enzyme-catalyzed back reaction of pyruvate conversion to lactate.

Reduced glutathione content was determined using Ellman's reagent [12].

To assess and compare antiradical, antioxidant, and cytoprotective properties of individual chemical compounds, the parameter I_{50} was used equal to the concentration of antioxidant corresponding to 50% inhibition of processes under study. The I_{50} values were calculated from the dose-effect dependency by regression analysis.

The following reagents were used: epigallocatechin gallate (EGCG), epicatechin gallate (ECG), rutin, quercetin, dihydroquercetin, riboflavin, sodium pyruvate, superoxide dismutase, NADH, thiobarbituric acid from Sigma (USA); *p*-nitrotetrazolium chloride, N,N,N′,N′-tetramethylethylene diamine from Reanal (Hungary); chrysotile asbestos [$Mg_6Si_4O_{10}(OH)_8$] (the length of the fibers was 5-10 μM) was from the Tuva deposit (Russia).

RESULTS AND DISCUSSION

Study of antioxidative activity of flavonoids. Antioxidative properties of the flavonoids (Table 1) were studied on initiation of NADPH- and CCl_4-dependent LPO in microsomal membranes of rat liver. The NADPH-dependent LPO was more likely initiated by ADP-perferryl-ion ($ADP\text{-}Fe^{2+}\text{-}O_2 \leftrightarrow ADP\text{-}Fe^{3+}\text{-}O_2^{\cdot-}$) which was generated with involvement of the NADPH-dependent flavoprotein and could introduce the activated oxygen into molecules of polyunsaturated fatty acids and destroy hydroperoxides produced [13]. In the case of CCl_4-dependent LPO, the initiation stage included the metabolic activation of CCl_4 in the microsomal electron transport chain with involvement of cytochrome P450 and production of free radical intermediates including $CCl_3O_2^{\cdot}$ which could initiate LPO in the absence of iron ions [14].

All flavonoids studied effectively inhibited both NADPH- and CCl_4-dependent LPO, and the degree of inhibition monotonically increased for all flavonoids with increase in their concentration, and this allowed us to calculate the I_{50} values by regression analysis (Table 2) and correctly use these values to comparatively assess the antioxidant effects. It should be noted that the antioxidant effects of ECG and EGCG, which displayed the strongest antioxidative properties, were comparable to the effect of ionol (2,6-di-*tert*-butyl-4-methylphenol), a synthetic antioxidant of hindered phenols.

Antiradical properties of flavonoids to oxygen anion-radical. The interaction of antiradical agents with oxygen anion-radical can be characterized by the rate constant of the second order reaction. In addition to direct methods of determination of this parameter based on measuring the rate of changes in the $O_2^{\cdot-}$ concentration, the method of competitive kinetics is widely used. In this case a constant level of oxygen anion-radical is provided by chemical $O_2^{\cdot-}$-generating systems, and the inhibition of reactions of $O_2^{\cdot-}$-dependent reduction (oxidation) of a test substance is determined relative to a standard. As an $O_2^{\cdot-}$-generating system xanthine oxidase is usually used and cytochrome *c* is used as a test substance. But flavonoids are known to inhibit xanthine oxidase [2], and quercetin and some other flavonoids can directly reduce cytochrome *c*. Therefore, in the present study a riboflavin-containing photosystem [10] was used for generation of oxygen anion-radical and the antiradical effect of flavonoids was assessed by inhibition by these com-

Table 1. Flavonoids under study and their structures

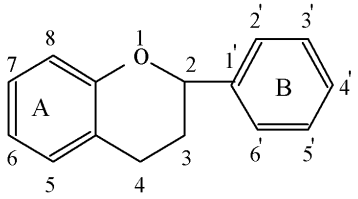
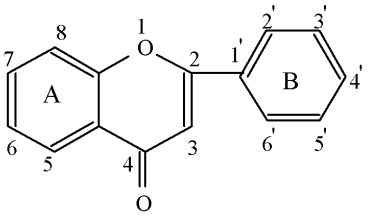
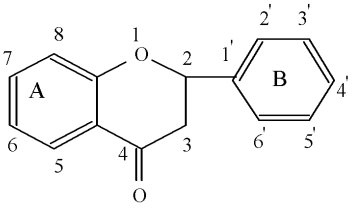
Group	Structure	Compound under study
Flavans (catechins)		EGCG, [3-gallate, 3′,4′,5′,5,7-$(OH)_5$] ECG, [3-gallate, 3′,4′,5,7-$(OH)_4$]
Flavonols		quercetin, [3,3′,4′,5,7-$(OH)_5$] rutin, [3-rutinoside, 3′,4′,5,7-$(OH)_4$]
Flavanonols		dihydroquercetin, [3,3′,4′,5,7-$(OH)_5$]

Table 2. Antioxidant effects of flavonoids (I_{50}) on initiation of LPO in liver microsomes and rate constants of flavonoid reactions with oxygen anion-radical

Flavonoid	NADPH-dependent LPO (I_{50}, μM)	CCl_4-dependent LPO (I_{50}, μM)	$k\times10^5$, $M^{-1}\cdot sec^{-1}$
Rutin	19.0	160.0	0.5
Dihydroquercetin	38.0	110.0	1.5
Quercetin	4.5	6.0	1.7
Epicatechin gallate	2.5	2.5	3.5
Epigallocatechin gallate	2.0	2.5	5.4
Ionol	1.1	1.1	—
Ascorbic acid	—	—	0.7

pounds of the $O_2^{\cdot-}$-dependent reduction of *p*-nitrotetrazolium chloride to diformazan.

Our findings suggested that flavonoids should be effective scavengers of oxygen anion-radical. Based on I_{50} values and with superoxide dismutase as a standard, the rate constants of the second order reaction were calculated for interaction of flavonoids (FL) with oxygen anion-radical (Table 2):

$$k_{FL}/k_{SOD} = I_{50\ (SOD)}/I_{50\ (FL)},$$

$$k_{FL} = k_{SOD} \cdot I_{50\ (SOD)}/I_{50\ (FL)},$$

where $k_{SOD} = 2\cdot10^9\ M^{-1}\cdot sec^{-1}$ [1].

Note that the rate constant of the quercetin reaction with $O_2^{\cdot-}$ determined by us by competitive kinetics (Table 2) is close to the value obtained by other authors by pulse radiolysis and EPR-spectrometry ($0.9\cdot10^5\ M^{-1}\cdot sec^{-1}$) [15]. Our findings showed that all flavonoids studied, except rutin, were more effective scavengers of oxygen anion-radical than the water-soluble antioxidant ascorbic acid. The high antiradical activity of flavonoids to oxygen anion-radical is obviously due to the reactivity of hydroxyl groups in the *m*-position of ring A and *o*-position of

ring B, and the antiradical activity of flavonoids increases with increase in the number of hydroxyl groups in their structure. Thus, the highest activity was found for catechins EGCG and ECG which due to gallation (addition of a gallic acid residue at C_3) acquired additional hydroxyl groups. On the contrary, because glycosylation blocked chemically active groups of quercetin, its glycoside rutin displayed a significant (more than threefold) decrease in antiradical activity, and this is in agreement with data of other authors [5].

Study of chelating properties of flavonoids. The antioxidant effect of flavonoids can be associated with the binding of ions of variable valence metals which play the key role in initiation of free radical reactions [1]. The ability of some flavonoids to produce stable complexes with metal ions can be detected by differential spectroscopy [6]. Figure 1a presents absorption spectra of quercetin and its metallocomplexes which are produced by interaction of the flavonoid with ferric ions. Similar absorption spectra are recorded for the complexing of quercetin and rutin with Cu^{2+} and Fe^{2+}.

The chelating properties of dihydroquercetin and catechins, the interaction of which with metal ions was not accompanied by pronounced spectral changes, were studied by competitive replacement. In these experiments we determined the ability of dihydroquercetin, ECG, and EGCG to displace rutin from its complexes with metals. The ability of EDTA, which is one of most active chelators, to displace rutin and quercetin from their complexes with metals was also determined. By differential spectroscopy and competitive replacement quercetin was found to be the strongest chelator of metal ions of the flavonoids studied. In particular, quercetin was not displaced from the complex with Fe^{3+} by a twofold excess of EDTA (Fig. 1a). In total, the ability of flavonoids for complexing with transition metal ions is described as follows:

Metal	Complexing ability of flavonoids
Fe^{2+}	quercetin > rutin >> ECG = EGCG > dihydroquercetin
Fe^{3+}	quercetin > EGCG = ECG > rutin >> dihydroquercetin
Cu^{2+}	rutin > EGCG = ECG = dihydroquercetin

Thus, all flavonoids studied were chelating agents and could bind ions of variable valence metals producing

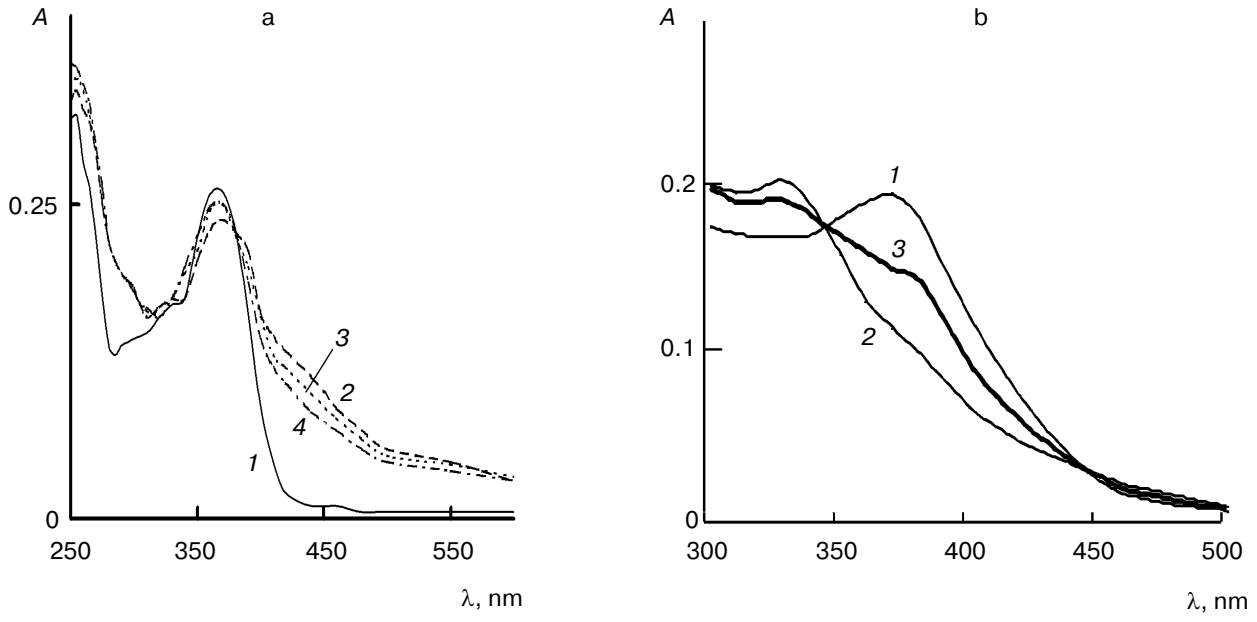


Fig. 1. Typical changes in the absorption spectra of quercetin on complexing with trivalent iron ions (a) and during oxidation in the system containing peritoneal macrophages and asbestos (b). a) The absorption spectrum of quercetin (15 μM) in 0.9% NaCl (*1*), the differential spectrum of Fe^{3+}-quercetin complex against 0.9% NaCl containing Fe^{3+} (*2*), the differential spectrum of Fe^{3+}-quercetin complex just on addition of EDTA (30 μM) against 0.9% NaCl containing Fe^{3+} and EDTA (*3*), the same as spectrum *3* but 15 min after the addition of EDTA (*4*). b) Peritoneal macrophages ($2 \cdot 10^6$ cells/ml) were incubated at 37°C in isotonic phosphate buffer (pH 7.3) containing chrysotile asbestos (2 mg/ml) and 60 μM quercetin. The samples were centrifuged, and the absorption spectrum of the supernatant fluid diluted 2.5-fold was recorded: baseline spectrum of quercetin (*1*), spectrum of quercetin after 20 min of incubation (*2*), spectrum of quercetin after 20 min of incubation in the presence of SOD (100 μg/ml) (*3*).

Table 3. Values of I_{50} and cytoprotective effects of flavonoids on asbestos-induced damage to peritoneal macrophages

Flavonoid	I_{50}, μM	CPE*, arbitrary units
Rutin	275	0.036
Dihydroquercetin	150	0.067
Quercetin	39	0.26
Epicatechin gallate	12	0.80
Epigallocatechin gallate	10	1.00

* The CPE of the most effective cytoprotector EGCG is taken as the unit and CPE of other compounds are calculated by the formula: $CPE_{FL} = 1 \cdot (I_{50\ (EGCG)}/I_{50\ (FL)})$.

stable complexes. However, there was no direct dependence between the inhibition of microsomal LPO by flavonoids (Table 2) and their chelating properties, and this suggested that the chelating of metal ions insignificantly contributed to the antioxidant effect of flavonoids.

Ability of flavonoids to prevent oxidative damage to cells was studied using a model of asbestos-induced oxidative stress in isolated phagocytizing cells (peritoneal macrophages). The incubation of peritoneal macrophages with chrysotile asbestos fibers was earlier shown [11] to sharply increase the production of oxygen anion-radical by the NADPH oxidase complex (respiratory burst) that resulted in damage and lysis of the phagocytizing cells. In our experiments incubation for 20 min of peritoneal macrophages with chrysotile asbestos fibers (3 mg/ml) resulted in a sharp decrease in the level of reduced glutathione (from 7.3 ± 2.0 to 3.1 ± 1.5 μM, $p < 0.01$) and in significant damage and lysis of the cells that was manifested by a decrease in the number of macrophages from $(5.5 \pm 0.6) \cdot 10^6$ to $(1.0 \pm 0.2) \cdot 10^6$ cells/ml ($p < 0.01$) and by the release of a cytoplasmic enzyme LDH into the incubation medium (86 ± 12%, $p < 0.01$).

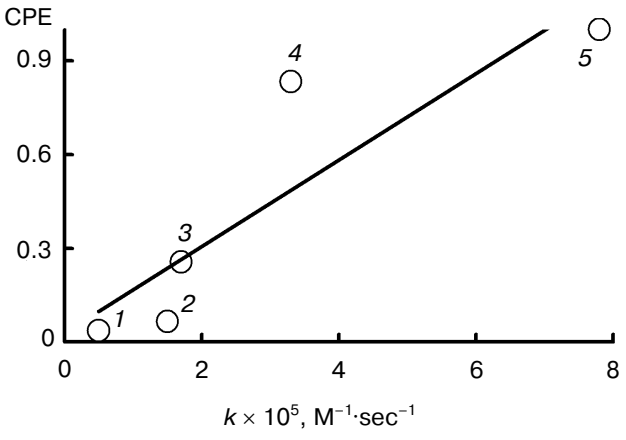


Fig. 2. Dependence between the cytoprotective effect and antiradical properties of flavonoids: rutin (*1*), dihydroquercetin (*2*), quercetin (*3*), ECG (*4*), EGCG (*5*). The correlation coefficient is 0.89 ± 0.24; the regression coefficient is 0.14 ± 0.04, $p = 0.042$.

All flavonoids studied prevented the asbestos-induced damage of peritoneal macrophages which was assessed by entrance of LDH into the incubation medium. The protective effect can be characterized by I_{50} value equal to the flavonoid concentration providing the 50% decrease in the degree of cell damage. Table 3 presents I_{50} values calculated by regression analysis from the corresponding dose–effect dependences. It is more convenient to describe the cytoprotective effect (CPE) in arbitrary units, with CPE of the most effective cytoprotector epigallocatechin gallate taken as the unit and CPE of other flavonoids calculated by the formula:

$$CPE_{FL} = 1 \cdot (I_{50\ (EGCG)}/I_{50\ (FL)}).$$

Data presented in Table 3 show that catechins EGCG and ECG protect the cells against lysis significantly more effectively than quercetin and dihydroquercetin and more than 20-fold more effectively than rutin. Comparison of the CPE values and rate constants of the flavonoid reactions with oxygen anion-radical by correlation analysis shows a direct correlation between the cytoprotective effect of flavonoids studied and their antiradical activity toward $O_2^{\cdot-}$ (Fig. 2), and both these parameters increase in the following series: rutin < dihydroquercetin < quercetin < ECG < EGCG. However, there is no correlation between the cytoprotective effect and the chelating properties of the flavonoids.

It is known that oxidation by oxygen anion-radical of flavonols quercetin and rutin is accompanied by specific changes in their spectra [8]. Similar changes were recorded in the spectra of quercetin (Fig. 1b) and rutin in the presence of asbestos-activated peritoneal macrophages. On addition into the incubation medium of the enzyme SOD (100 μg/ml) catalyzing the dismutation of $O_2^{\cdot-}$ the oxidation of rutin was inhibited virtually completely and the oxidation of quercetin was inhibited by 50% (Fig. 1b). These findings show that flavonoids can scavenge oxygen anion-radical generated during the asbestos-induced respiratory burst in peritoneal macrophages that results in inhibition of Fenton's reaction and other processes with involvement of $O_2^{\cdot-}$ which cause damage and lysis of the cells.

Thus, the studied flavonoids are shown to have high antioxidative activity and prevent the asbestos-induced damage of phagocytizing cells. These findings confirm the key role of $O_2^{\cdot-}$ in the asbestos-induced damage of phagocytizing cells and suggest that the cytoprotective effect of flavonoids is due to their antiradical activity toward oxygen anion-radical.

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