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Experimental evidence that flavonoid metal complexes may act as mimics of superoxide dismutase

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Abstract

Radical scavenging activities of flavonoids rutin, taxifolin, (−)-epicatechin, luteolin, and their complexes with transition metal (Fe^{2+}, Fe^{3+}, and Cu^{2+}) towards superoxide were determined using illumination of riboflavin as source and NBT as detector of $O_2^{\cdot-}$. The scavenger potencies of flavonoid metal complexes were significantly higher than those of the parent flavonoids. To elucidate the mechanism of this phenomenon, the rates of superoxide-dependent oxidation of flavonoids and their metal complexes in photochemical system with riboflavin were examined. It was found for the first time that flavonoids bound to metal ions were much less subjected to oxidation compared with those of free compounds. The findings directly demonstrate superoxide scavenging activity of metal ions in complexes with flavonoids and support earlier suggestions that flavonoid metal complexes may exhibit superoxide dismuting activity.

Keywords: Flavonoids; Rutin; Taxifolin; (−)-Epicatechin; Luteolin; Metal flavonoid complexes; Superoxide; Reactive oxygen species; Superoxide dismutase

Experimental and epidemiological studies have revealed a variety of beneficial effects of dietary flavonoids [1–7]. Health-promoting impact of flavonoids is generally attributed to their antioxidant properties. Indeed, due to their low redox potentials these natural compounds can reduce oxygen and nitrogen free radicals ($O_2^{\cdot-}$, $^{\cdot}OH$, $NO^{\cdot}$, $RO^{\cdot}$, and $ROO^{\cdot}$) [8–11], which are supposedly involved in the causes or manifestation of many diseases [12]. Besides, flavonoids may inhibit the generation of primary oxygen radicals and the following chain oxidation, being effective chelators of transition metal ions [13]. Chelation potency of flavonoids is mainly related to a catechol moiety in the B ring [8,14] while redox behavior of ligands in complexes depends on the presence of the 3-hydroxy group in their structure [14]. The transformation of Fenton's active catalysts into inert forms is probably the main but not the only consequence of metal chelation by flavonoids. Recently, it was found that rutin and taxifolin flavonoid metal complexes possess higher scavenger potencies toward superoxide than parent flavonoids [15,16]. Moreover, transition metals enhance the anti-inflammatory activities of flavonoids [17] and their cytoprotective effects against oxidative injury in isolated cells [16,18]. On these grounds it was suggested that metal complexes of flavonoids acquired additional effective superoxide dismuting metal centers [16,17]. One of the most important consequences of appearance of such dismuting centers has to be the protection of the 3′,4′-dihydroxy substituents of flavonoid molecule from oxidation by ROS. In the present work, we tested this hypothesis using rutin, taxifolin, (−)-epicatechin, luteolin, and their complexes with transition metal (Fe^{2+}, Fe^{3+}, and Cu^{2+}).

Materials and methods

Chemicals

Nitroblue tetrazolium, riboflavin, and tetramethylethylenediamine (TMEDA)[1] were from Reanal (Hun-

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[1] *Abbreviations used:* TMEDA, tetramethylethylenediamine; NBT, nitroblue tetrazolium.

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gary). Rutin, taxifolin, (−)-epicatechin, luteolin, and EDTA were purchased from Sigma (Deisenhofen, Germany). Complexes of flavonoids with metals were prepared in situ by mixing flavonoids and appropriate salts ($FeSO_4 \cdot 7H_2O$; $Fe_2(SO_4)_3 \cdot 9H_2O$; and $CuSO_4$) in water (flavonoid:metal ion ratio was 1:1). In the preliminary study, it was found that flavonoids may bind transition metals in a ratio of 1/1 and such complexes possess stronger antiradical and cytoprotective activity in comparison with that of complexes 2/1. The interaction of metal ions with flavonoids and formation of complexes were followed spectrophotometrically.

Superoxide-mediated reduction of nitroblue tetrazolium by photochemically reduced riboflavin

Reduction of nitroblue tetrazolium (NBT) was carried out at room temperature (22 °C) under fluorescent lighting (20 w, 20 cm). The standard incubation mixture (3.5 ml) contained 6 μM riboflavin, 0.8 mM N,N,N^1,N^1-tetramethylethylenediamine (TMEDA) in 0.016 M phosphate buffer (pH 7.8) and 85 μM NBT. Flavonoids were added in a water:DMSO solution (the final concentration of DMSO not exceeding 0.2%). After 5 min incubation, the reaction was stopped by the light switching off and the addition of 0.05 ml SOD (1 mg/ml) and absorbance was measured at 550 nm. For each flavonoid concentration, control sample containing 0.05 ml SOD solution, which was added before exposure to fluorescent lighting, was analyzed to rule out the possible direct reduction of NBT, by flavonoids and other reducing agents. For estimation of the superoxide-driven reduction of NBT the absorbance of a control sample was subtracted from that of standard reaction mixture.

Superoxide-mediated oxidation of flavonoid metal complexes and uncomplexed ligands by photochemically reduced riboflavin was carried out at room temperature (22 °C) under fluorescent lighting (20 w, 20 cm). The standard incubation mixture was the same as described for the reduction of NBT, except that NBT was omitted. Flavonoids were added as a water:DMSO solution (the final concentration of DMSO not exceeding 0.2%). UV–visible absorption spectra were recorded over the range 250–600 nm with a Cary 50 spectrophotometer (Varian Australia).

Results

Effect of flavonoid metal complexes, free ligands, and metals on superoxide-driven reduction of NBT

Xanthine/xanthine oxidase system is usually used for $O_2^{\cdot-}$ generation and cytochrome *c* is used as a superoxide detector. However, flavonoids are known to inhibit xanthine oxidase [19], besides flavonoids can directly reduce cytochrome *c*. Therefore, in the present study the scavenger efficacy of rutin, taxifolin, luteolin, and (−)-epicatechin as well as their metal complexes and free metal ions towards superoxide was quantitatively evaluated using illumination of riboflavin in the presence of tetramethylethylenediamine. Upon reoxidation on air photochemically reduced flavin generated superoxide anions, which in their turn reduced NBT [20]. The reaction was nearly completely inhibited by SOD at the concentration of 1.5 nM ($I_{50} = 0.15$ nM). Flavonoids and their metal complexes also effectively inhibited NBT reduction and the degree of inhibition is increased in a concentration-dependent manner for all compounds studied (Fig. 1). Among transitional metals only copper

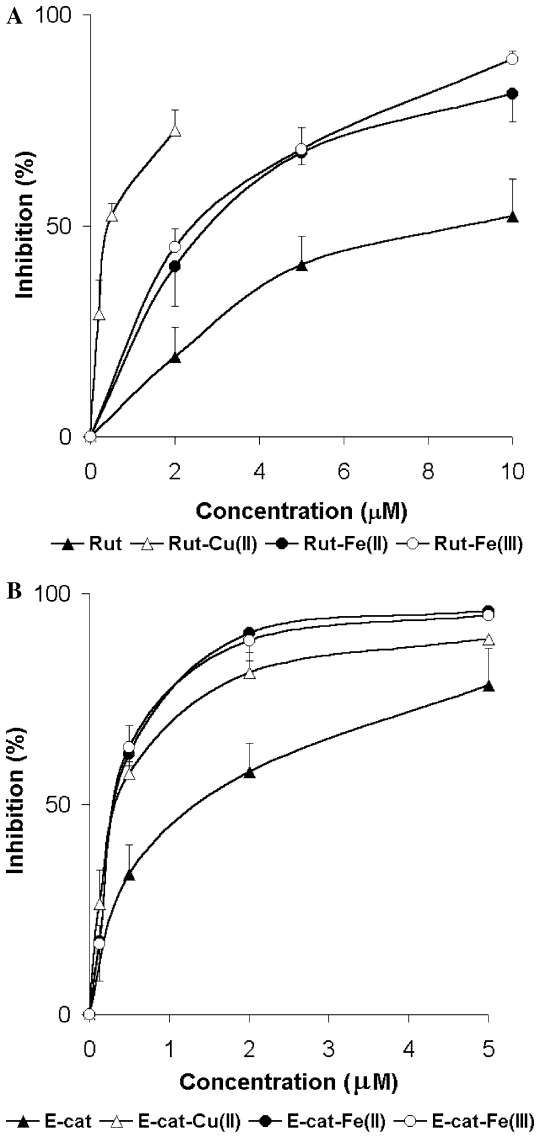

Fig. 1. Effect of flavonoids on reduction of NBT by photochemically generated superoxide. (A) Effect of rutin (Rut) and rutin–copper (Rut–Cu II), rutin–ferrous (Rut–Fe II), and rutin–ferric (Rut–Fe III) complexes; (B) effect of epicatechin (E-cat) and epicatechin–copper (E-cat–Cu II), epicatechin–ferrous (E-cat–Fe II), and epicatechin–ferric (E-cat–Fe III) complexes.

Table 1
Concentrations of flavonoid metal complexes (1:1) and free ligands inhibiting the superoxide-driven reduction of NBT by 50%

Flavonoids	I_{50} values (μM)			
	Free ligands	Complexes with metals		
		Cu^{2+}	Fe^{2+}	Fe^{3+}
Rutin	9.0	0.50	2.7	2.5
Taxifolin	1.9	0.48	0.6	0.55
Luteolin	14.2	0.80	2.5	2.5
(−)-Epicatechin	1.3	0.32	0.3	0.3
EDTA	No inhibition	3.5	6.5	6.7
–Ligand	—	0.22	Slight inhibition at 10 μM	
SOD	0.00015			

ions (Cu^{2+}) were strong inhibitors of the superoxide-driven NBT reduction, while both ferric and ferrous ions were much less effective and inhibited the reduction of NBT at 10 μM concentration only by 12.2 ± 7.1 and $7.3 \pm 6.5\%$, respectively. For all flavonoids, I_{50} values were calculated (Table 1) and compared with those for complexes.

Superoxide-mediated oxidation of flavonoid metal complexes and uncomplexed ligands by photochemically reduced riboflavin

Oxidation of flavonoids was followed spectrophotometrically. UV–visible absorption spectra were recorded over the range of 250–600 nm at 5 min intervals. Oxidation of yellow flavonoids (rutin and luteolin and their metal complexes) was accompanied by bleaching a characteristic visible absorption maximum near 400 nm (band 1), these spectral changes being completely inhibited by 200 ng/ml SOD (Fig. 2A). Oxidation of colorless flavonoids epicatechin, taxifolin, and their metal complexes was monitored via an increase in visible spectra. In the case of epicatechin and epicatechin metal complexes, superoxide-mediated oxidation was accompanied by the appearance of two maxima at 440 nm and at 500 nm completely inhibited by 200 ng/ml SOD (Fig. 2B). It has been shown earlier that taxifolin oxidation by MPO in the presence of a hydrogen peroxide-generating system was accompanied by appearance of broad yellow band at 360–460 nm [21]. However, no essential spectral changes were found over the whole time of taxifolin incubation in photochemical system of $O_2^{\cdot-}$ generation.

The amount of oxidized rutin, luteolin, epicatechin, and their metal complexes during incubation in photochemical system (C_{ox}) was quantitatively evaluated by using the following equation:

$$C_{ox}(\mu mol/L) = \frac{10 \times \Delta At}{\Delta A_{MAX}},$$

where ΔAt is a decrease in absorbance at 400 nm (for rutin, luteolin, and their metal complexes) or an increase

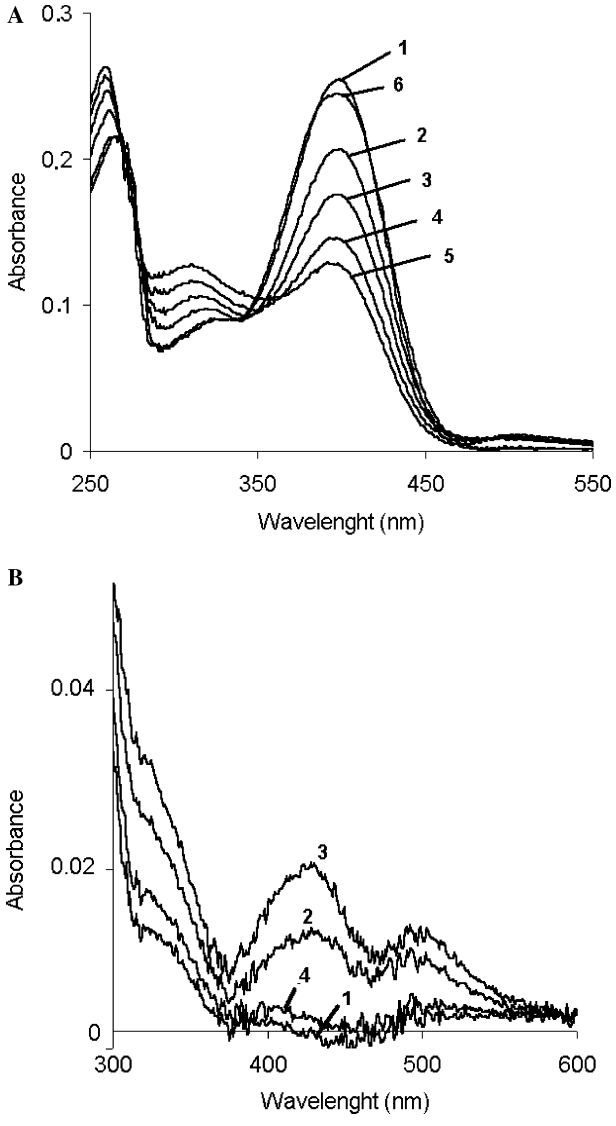

Fig. 2. Typical spectral changes during oxidation of yellow color (A) and colorless (B) flavonoids by photochemically generated superoxide (UV–visible absorption difference spectra versus blank with riboflavin). (A): 1, before reaction; 2, 3, 4, 5 after 10, 20, 30, and 40 min oxidation; 6, after 40 min oxidation with 200 ng/ml of SOD; (B): 1, before reaction; 2, 3 after 10 and 20 min oxidation; and 4, after 20 min oxidation with 200 ng/ml of SOD.

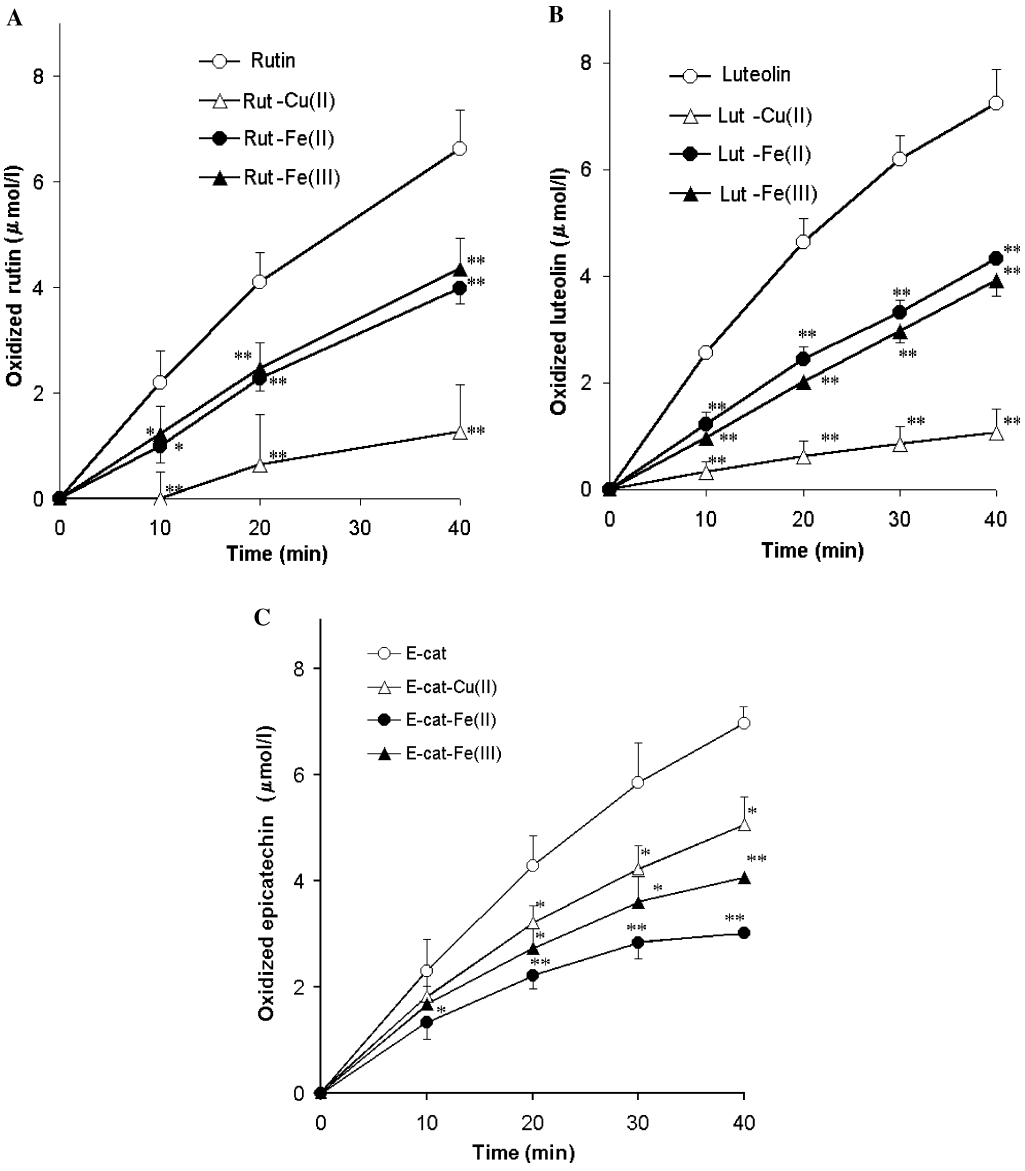

Fig. 3. Time courses of oxidation of rutin and rutin metal complexes (A), luteolin, and luteolin metal complexes (B), and epicatechin and epicatechin metal complexes (C) by photochemically generated superoxide ($C_0 = 10\,\mu M$) $^*P < 0.05$; $^{**}P < 0.01$ (t test versus oxidized free ligand).

in absorbance at 420 nm (for epicatechin and its metal complexes) for time (t) of incubation; ΔA_{MAX} is the same as ΔAt, except that the time of incubation was 120 min (ΔA corresponds to the maximal oxidation of flavonoids).

The time courses of superoxide-mediated oxidation of studied flavonoids and their metal complexes in photochemical system ($C_0 = 10\,\mu M$) are shown in Fig. 3.

Discussion

Superoxide scavenging capacities of rutin, taxifolin, (−)-epicatechin, luteolin, and their complexes with transition metal (Fe^{2+}, Fe^{3+}, and Cu^{2+}) were determined using illumination of riboflavin as a source and NBT as a detector of $O_2^{\cdot-}$. The scavenger potencies of flavonoid metal complexes were manifold more than those of the parent flavonoids. Among metal complexes the most effective were flavonoid copper complexes. However, copper (II) ions were also extremely effective in inhibiting the nitroblue tetrazolium (NBT) reduction by photochemically generated superoxide ($I_{50} = 0.22\,\mu M$). High superoxide dismutase activity of free copper ions and poor activity of copper (II) bound to albumin and other proteins or various low weight compounds were described earlier [12]. In the present study, $O_2^{\cdot-}$scavenger potency of copper bound with albumin was also found to be negligible (data not presented). Copper complex with EDTA was significantly more effective ($I_{50} = 3.5\,\mu M$) however, inhibition of NBT reduction by metal-EDTA complexes might be a

consequence of the interaction of these complexes with a tetrazoinyl radical [22]. Amino acids lysine, histidine, tyrosine [12], salicylates [23,24], macrocyclic polyamine [25], and indomethacin [26] form copper complexes, which are the effective catalysts of $O_2^{\cdot-}$ dismutation. However, the above-mentioned ligands as well as rutin, luteolin, taxifolin, and epicatechin are weak chelators in comparison with EDTA; therefore, copper ions may escape from complexes and react with $O_2^{\cdot-}$ [12]. This should be taken into account estimating the actual SOD catalytic activity of flavonoid copper complexes. Nevertheless, we earlier showed [16] that cytoprotective effects of copper complexes of rutin and taxifolin against superoxide-induced oxidative injury of phagocytic cells were eight and six times higher comparing to parent flavonoids, while free copper (II) ions were totally ineffective. These findings support our suggestion that copper–flavonoid complexes may indeed possess a high superoxide dismutase activity. Unlike copper, ferrous and ferric ions even at high concentration (10 μM) showed only slight inhibition of NBT reduction by photochemically generated superoxide. At the same time iron–flavonoid complexes at 10 μM concentration inhibited NBT reduction practically completely (80–90% and more) and were threefold to nearly sixfold more effective inhibitors than parent flavonoids. Moreover, the catalytic activity of epicatechin complexes with ferrous and ferric ions was the same as that of copper–epicatechin complex $I_{50} = 0.28\,\mu M$, and this value is very close to that for free copper (II) ions.

Important information was obtained from the study of oxidation of flavonoids and their metal complexes by photochemically generated superoxide. For the first time we found that the complexed flavonoids were oxidized significantly lower than free flavonoids. The biggest protection of a flavonoid moiety from oxidation was observed for copper complexes, however in the case of epicatechin all transition metals equally prohibited ligand oxidation. This observation directly demonstrates that metal is the most active antioxidative center in flavonoid–metal complexes, being effective superoxide scavenger with dismuting activity, in accord with the following reactions:

$$Me^{(n+1)+} + O_2^{\cdot-} \rightarrow Me^{n+} + O_2$$

$$Me^{n+} + O_2^{\cdot-} + 2H^+ \rightarrow Me^{(n+1)+} + H_2O_2$$

The findings that the superoxide scavenging properties and oxidative behavior of flavonoid ferric complexes were identical to those of flavonoid ferrous complexes support the above mechanism.

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