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Free radical scavenging abilities of flavonyl-thiazolidine-2,4-dione compounds

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ABSTRACT: Free radical scavenging activity of flavonyl-thiazolidine-2,4-dione compounds has been evaluated using chemiluminescence, electron spin resonance spectroscopy with 5,5-dimethyl-1-pyrroline-1-oxide as spin trap and DPPH (2,2′-diphenyl-1-picrylhydrazyl) method. The examined compounds exhibited 28–50% scavenging superoxide anion radical ($O_2^{\bullet-}$), 16.7–76.7% hydroxyl radical ($HO^{\bullet}$) and 9–40% DPPH radical. Compounds containing carbonyl group in their structure can be considered as antioxidants with high relevance and great biological importance. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: flavone; flavonyl-thiazolidine-2,4-dione compounds; free radicals; scavenging capacity

Introduction

Free radical-mediated lipid peroxidation, oxidative stress and antioxidants are widely discussed in many current research areas. (1–3) Oxygen-centered free radicals such as hydroxyl radical ($HO^{\bullet}$) and superoxide anion radical ($O_2^{\bullet-}$) and other reactive oxygen species (ROS) are the products of normal cellular metabolic processes and pathological stress in human body. (1) They play dual role that is beneficial and harmful in the biology of the cell. (2,3) Under normal conditions ROS and free radicals are effectively eliminated by antioxidant defense systems such as antioxidant enzymes and non-enzymatic factors. (4) However, under pathological conditions, the balance between the generation and elimination of ROS is broken; as a result of these events, biomacromolecules including DNA, membrane lipids and proteins, are damaged by ROS-mediated oxidative stress. The term *oxidative stress* in essence refers to the situation of a serious imbalance between the production of free radicals and the antioxidant defense mechanisms, leading to potential tissue damage. (1,3) Uncontrolled generation of free radicals that attack membrane lipids, protein and DNA is believed to be involved in many health disorders such as diabetes mellitus, cancer and neurodegenerative and inflammatory diseases. (5–9)

Diabetes mellitus, a state of chronic hyperglycemia, is a major cause of serious micro- and macrovascular diseases, affecting, therefore, nearly every system in the body. Growing evidence indicates that oxidative stress is increased in diabetes due to overproduction of ROS and decreased efficiency of antioxidant defenses, a process that starts very early and worsens over the course of the disease. (5,6,8,10,11)

Hyperglycemia is reported to induce oxidative stress through multiple pathways such as redox imbalances secondary to enhanced aldose reductase activity, increased advanced glycation end products, altered protein kinase C activity, especially β-isoforms, prostanoid imbalances and mitochondrial overproduction of superoxide. (12) All these pathways converge in the production of oxidative stress. (8,12,13)

Thiazolidinediones (TZDs) such as pioglitazone and rosiglitazone are a new class of antidiabetic agents that reduce plasma glucose and glucose production and also increase glucose clearance in patients with type 2 diabetes, thus reducing insulin resistance. (10,14) Besides their anti-diabetic potency, these TZDs have been shown to exert antioxidant activity. Pioglitazone inhibits $O_2^{\bullet-}$ radical production in endothelial cells. Rosiglitazone ameliorates the impaired coronary arteriolar dilation in mice with type 2 diabetes by reducing oxidative stress via a mechanism unrelated to its effect on hyperglycaemia. (14)

Flavonoids are a large family of compounds with common chemical structure that have recently attracted considerable interest due to their broad pharmacological and therapeutical effects. They exist extensively in all parts of plants, including fruits, vegetables, seeds, nuts, herbs, flowers and bark. (15) They have been described as health-promoting, disease-preventing dietary supplements which are extremely safe and associated with low toxicity. (16) In addition, traditional herbal medicines having flavones have been recommended for the treatment of diabetes and the antidiabetic activity of several plant extracts has been correlated with their antioxidant properties. As antioxi-

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dants, these compounds may prevent the progressive impairment of pancreatic β-cell function due to oxidative stress, thus reducing the occurrence of type 2 diabetes. (17) As free radical scavengers, flavonoids (15,16,18,19) can act as powerful antioxidants, even more so than the traditional vitamins. (20,21)

In our previous studies, we reported the synthesis and the *in vitro* antidiabetic and aldose reductase inhibitory activity results of the flavonyl-2,4-thiazolidinediones (FTDs) **1–6**. (22) In this paper, the *in vitro* effect of FTDs (Fig. 1) having antidiabetic and aldose reductase inhibitory activity was examined on the reactions generating O_2^-, HO and DPPH radicals.

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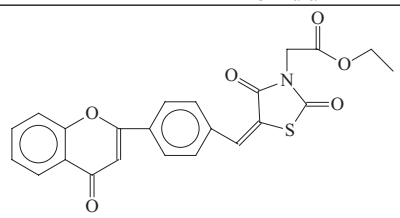
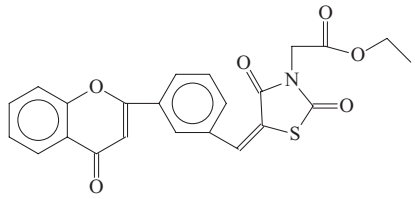
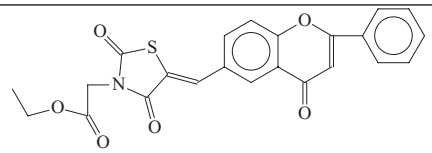
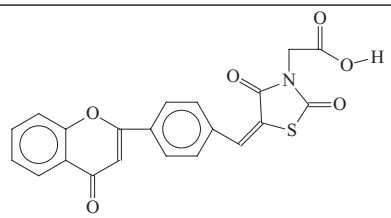
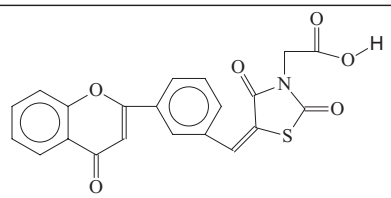
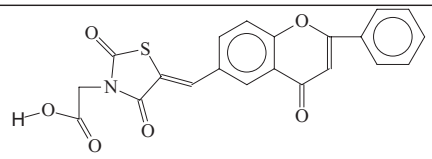
1	
2	
3	
4	
5	
6	

Figure 1. Chemical structures of flavonyl-thiazolidine-2,4-dione compounds (FTDs).

Materials and methods

The compounds **1–3** were synthesized by Knoevenagel reaction of ethyl 2,4-dioxothiazolidine-3-ylacetate with flavone-3′/4′/6-carboxaldehydes using acetic acid–sodium acetate mixture. The compounds **4–6** were obtained with acidic hydrolysis of compounds **1–3**. (22)

Chemicals

Ammonium ferrous sulphate hexahydrate was from Fluka (Buchs, Switzerland); catalase (19,900 U mg^{-1}) from bovine liver and *p*-nitroblue-tetrazolium chloride (NBT) were from Sigma (St Louis, MO, USA). All remaining reagents were purchased from E. Merck (Darmstadt, Germany) and Aldrich (Milwaukee, MI, USA). The reagents were propared fresh daily; dimethylsulfoxide (DMSO) was prepared as a 0.1 mol L^{-1} aqueous solution immediately before use and stored in darkness at –15°C under nitrogen to avoid any oxidation of the spin trap.

Superoxide anion radical scavenging activity

Superoxide anion radical was generated in a KO_2–DMSO system. (23) 18-Crown-6 (1,4,7,10,13,16-hexaoxacyclo-octadecane) (60 mg) in 10 mL anhydrous dimethylsulfoxide (DMSO) was mixed quickly to avoid contact with air humidity with 7 mg of KO_2. The mixture was stirred for 1 h to give a pale yellow solution of $O_2^{\bullet}$, which was stable at room temperature for at least 1 h. The $O_2^{\bullet}$ concentration was determined using a UV spectrum (λ_{max} = 251 nm, $\varepsilon = 2686 \pm 29$ mol cm^{-1}).

Chemiluminescence (CL) measurements were made using an EMI9553Q photomultiplier with an S20 cathode sensitive in the range of 200–800 nm, interfaced with a computer for data acquisition and handling. Reagents were introduced to a thermostated glass cuvette placed in a light-tight chamber. The cuvette was exhausted and washed using a B-169 vacuum system (Büchi, Flawill, Switzerland). The quenching activity was calculated as follows:

Quenching ratio $Q(\%) = [(\Sigma I_0 - \Sigma I_x)/\Sigma I_0] \times 100\%$, where ΣI_0 is the integrated light intensity (CL sum) measured during 3 min $\left(\Sigma I = \int_{t=0}^{t=3} I(t)\mathrm{d}t\right)$ in the absence of a inhibitor (an FTD compound), ΣI_x is the light sum measured in the presence of the inhibitor. Because FTDs were dissolved in DMSO and added to the reaction mixture caused a short-lasting small 'flash' followed by a very small increase in the light emission (data not shown); this reaction was considered as the control reaction.

Hydroxyl radical scavenging activity

Electron spin resonance (ESR) and the spin trap agent 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were used to determine the scavenging hydroxyl radical generated in the Fenton reaction: (24)

$$H_2O_2 + Fe(II) \rightarrow HO^{\bullet} + Fe(III) + HO^- \quad (1)$$

This reaction is accompanied by two other reactions

$$Fe(II) + HO^{\bullet} \rightarrow Fe(III) + HO^- \quad (2)$$

$$H_2O_2 + HO^{\bullet} \rightarrow HOO^{\bullet} + H_2O \quad (3)$$

In the presence of the DMPO trap, $HO^{\bullet}$ is rapidly trapped by this spin trap at high rate constant of 3.4×10^9 mol^{-1} s^{-1}: (25)

$$DMPO + HO^{\bullet} \rightarrow DMPO - \dot{O}H \quad (4)$$

The rate constant for reaction of the $HOO^{\bullet}$ with this nitrone spin trap is very low (~10 mol^{-1} s^{-1}).

The Fenton reaction contained 200 µL of 0.1 mol^{-1} DMPO (aqueous solution), 50 µL of 4 mmol L^{-1} H_2O_2, 50 µL of 80 mmol L^{-1} sodium trifluoroacetate (pH 6.15) and 50 µL of ammonium ferrous sulfate hexahydrate solution at concentration of 0.5 mmol L^{-1}. The reaction mixture was placed in the ESR cavity using a quartz flat cell with an optical pathlength of 0.25 mm. The spin-trapped $DMPO-\dot{O}H$ signal was analyzed 5 min after the addition of ammonium ferrous sulfate hexahydrate on a standard ESR spectrometer operating at 9.3 GHz with 100 kHz modulation of the steady magnetic field.

Tested FTDs were dissolved in DMSO, which is suitable for dissolving water-insoluble samples. (26)

The ability to scavenge the HO radicals was calculated using the following formula:

$$\text{Hydroxyl radical scavenging ratio } R(\%) = [H_0 - H) \,/\, H_0] \times 100\%$$

where H_o and H represent the relative height of the second peak in the ESR spectrum of the spin-adduct from the blank (DMSO) and a sample dissolved in DMSO, respectively.

The conditions of ESR measurement were as follows: microwave power 0.63 mW, modulation amplitude 0.2 mT, time constant 0.1 s, receiver gain 4×10^4, and temperature 293 K.

DPPH radical scavenging activity

Free radical scavenging activity was evaluated according to the method reported by Shimada *et al.* (27) Two milliliters of 0.1 mmol L^{-1} DPPH radical in ethanol was mixed with 2 mL of sample solution (2.5 mmol L^{-1}) in DMSO and incubated at 24°C for 30 min in the dark. The decrease in absorbance at 517 nm was measured against ethanol using a UV–vis Zeiss M-40 spectrophotometer (Zeiss, Germany). Scavenging capacity A (%) was calculated using the following equation:

$$A(\%) = [1 - (A_1 - A_2) : A_o] \times 100\%$$

where A_1 is the absorbance of 2 mL DPPH radical solution and 2 mL sample of a FTD, solution, A_2 is the absorbance of 2 mL sample of a FTD solution and 2 mL ethanol, A_o is the absorbance of 2 mL DPPH radical solution and 2 mL DMSO.

Results and discussion

Within the framework of this paper, the superoxide radical scavenging effect of six novel synthesized compounds was estimated employing the CL from $O_2^{\bullet-}$ radicals generated in DMSO. Superoxide anion radical is a reduced form of molecular oxygen formed by receiving one electron. (28) The radical is known as a precursor of very harmful species to cellular components such as HO radicals as well as singlet oxygen ($^1O_2$) and H_2O_2. In addition, this radical is a one-electron reductant of transition metal ions. (28)

Superoxide anion radical in DMSO elicits strong light emission according to the following reactions: (29–31)

$$O_2^{\bullet-} \rightarrow {}^1O_2 + \text{electron} \quad (5)$$

$$2O_2^{\bullet-} + 2Me_2SO \rightarrow {}^1O_2 + Me_2SO_2 + OH^- + MeSO(CH_2)^- \quad (6)$$

$$2\,{}^1O_2 \rightarrow ({}^1O_2)_2 \rightarrow ({}^3O_2)_2 + \text{light emission} \quad (7)$$

Addition of the $O_2^{\bullet-}$ radical inhibitors leads to a decrease in the light intensity, allowing the evaluation of their antioxidant activity. This reaction has high sensivity and selectivity and has been applied successfully for evaluation of an antioxidative property of the biologically reactive compounds in nonaqueous media. (32)

Typical chemiluminescence intensity–time diagrams in the absence (blank) and in the presence of a antioxidant (sample) were givn in our previous paper. (33)

Four of the six examined FTDs compounds dissolved in DMSO reduced the light sum at least by 28% (range 28–50%) at a concentration of 0.5 mmol L^{-1} (Fig. 2). One of these compounds (denoted as **4**) exhibited the $O_2^{\bullet-}$ radical scavenging activity comparable to that for specific this radical scavenger NBT (1 mmol L^{-1}) and a little lower than (1 mmol L^{-1}) Tiron (1,2-dihydroxy benzene-3,5-disulphonic acid) (56%) at the same concentration. The CL sums for these two inhibitors were accounted for by integrating the area under the kinetic curve $I = f(t)$ after 20 min. The sums were compared with those of the control (without examined compound), considered as 90%. In contrast, under the conditions examined, comounds **1** and **2** increased light emission by about 99 and 207%, respectively.

The enhanced chemiluminescence from the $O_2^{\bullet-}$ radical–DMSO system in the presence of compounds **1** and **2** indicates that both compounds were able to stimulate the $O_2^{\bullet-}$ radical dismutation as follows:

$$2O_2^{\bullet-} + 2H^+ \rightarrow {}^1O_2 + H_2O_2 \quad (8)$$

This means that these compounds might deliver H^+ for reaction (8) that is a source of singlet oxygen. (34) To check if H_2O_2 and $^1O_2$ are generated in reaction (8) the effect of catalase (an enzyme responsible for destruction of H_2O_2 (28,35)) and histidine (a quencher of $^1O_2$ (28)) was examined. An addition of the enzyme at a concentration of 200 µg/mL caused a decrease in the CL sum of about 40%. Also the aqueous solution of histidine (1 mmol L^{-1}) inhibited the light emission by 46%, although water alone at the same volume (5%) as these tested antioxidant H_2O solutions quenched the CL by about 10%. In contrast, an addition of SOD (100 µg mL^{-1}), enzyme which catalyzes the dismutation of $O_2^{\bullet-}$ radical into hydrogen peroxide, (35) to the $O_2^{\bullet-}$–DMSO solution increased the light sum by about 45%. Direct addition of H_2O_2 (0.1 mmol L^{-1}) elicited a time-dependent increment of light emission by about 20% (data not shown).

The antioxidant activities of the individual compound may depend on the structure of the free-radical inhibitors, the substituents present on the rings, e.g. free carboxylic group and the degree of polymerization.

Compounds containing the $-CH_2COOH$ group exhibited the strong direct activity towards the $O_2^{\bullet-}$ radical. This is consistent with a previous finding of Sawyer and Gibian, (36) who reported that weakly acidic $O_2^{\bullet-}$ radical as a weak base can promote the H^+ transfer from organic compounds.

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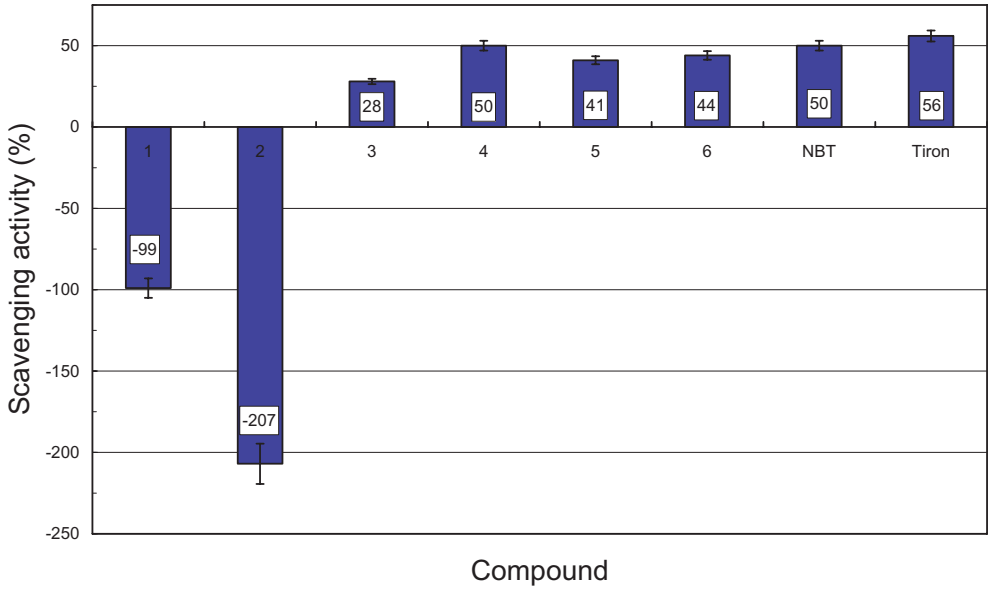


Figure 2. Scavenging effect of the examined flavonyl-thiazolidine-2,4-dione compounds (0.5 mol L^{-1}) on the chemiluminescence from 1 mol L^{-1} O_2^- radicals generated in DMSO. The examined scavengers were dissolved in 0.5 mL DMSO. Temperature 294 K. All data represent means ± SD of three different experiments. Numbers given at the bottom of the figure refer to compounds listed in Fig. 1. The remaining condictions are reported under Materials and Methods.

Among the oxygen-derived free radicals HO radical is the most reactive unstable oxidizing species and can be produced from O_2^- radical and H_2O_2 in the presence of transition metal ions, such as cooper or iron. (28) This species reacts with a variety of organic compounds, including biomolecules such as lipids, proteins, polypeptides and DNA. When a hydroxyl radical reacts with aromatic compounds, its addition across a double bond is reported, and reaction products such as hydroxycyclo-hexadienyl radical and peroxyl radical are thought to be formed. (37) Except for addition reactions, the HO radical may react by hydrogen abstraction and electron transfer reactions. Indeed the reactions of HO radicals with biomolecules result in generation of other radicals, but they are usually of lower toxicity.

The ability of the tested compounds to scavenge the HO radical was monitored using the ESR spin-trapping assay, the technique routinely used for the sensitive and specific detection of short-lived hydroxyl radicals. The Fenton reaction, a well-documented HO radical generator, was utilized to test the ability of tested compounds to scavenge this radical. Hydroxyl radicals trapped by DMPO form spin-adducts, for which a spectrum is shown in Fig. 3A(a). The spectrum exhibits four splitting lines with an intensity ratio of 1:2:2:1 and hyperfine splitting constant of $a_N = a_H = 14.9$ *G*. The parameters are consistent with those values for the DMPO–$\dot{O}$H adduct reported by other authors. (25,38) No ESR signal was measured from the Fenton–DMPO reaction, when one substrate was omitted.

The height of the second peak of the spectrum determined the relative amounts of DMPO–$\dot{O}$H adduct formed in the reaction. When hydroxyl radical scavenger was added, a decrease in the amount of DMPO–$\dot{O}$H adduct was observed.

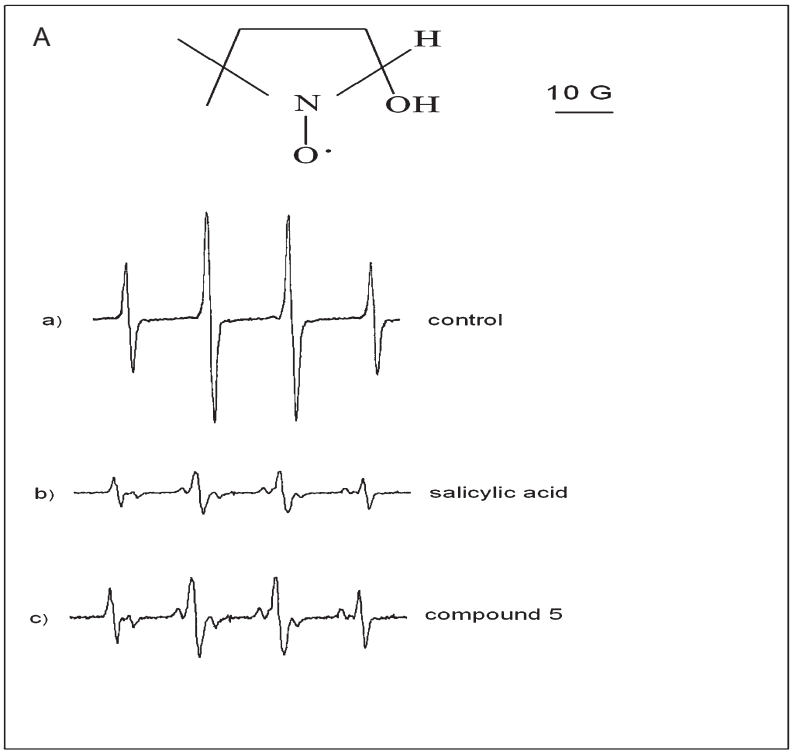
Figure 3A(b and c) shows the ESR spectrum measured in the Fenton–DMPO reaction in the presence of salicylic acid and compound **5**, the OH radical scavenger, respectively. Salicylic acid can remove this radical from the reaction at the reaction rate 1.2×10^{1} L mol^{-1} s^{-1} (39) The use of salicylic acid at concentration 7.2 mmol L^{-1} led to an 80% inhibition. The specificity of the Fenton probe for H_2O_2 and further HO radicals generation was checked by adding catalase (180 µg mL^{-1}). We observed a near-complete prevence of appearance of any ESR signal, whereas in the presence of a heat-denaturateed enzyme the signal was decreased by 5%, indicating that the origin of the DMPO–OH signal was dependent on HO radical generation. This conclusion finds confirmation in a fact that superoxide dismutase (SOD) even at a high concentration did not prevent DMPO–$\dot{O}$H formation.

The scavenging activities of the tested compounds dissolved in DMSO to this Fenton reaction system with DMPO on HO radicals are shown in Fig. 3B. The scavenging abilities were, in descending order, **6** > **5** > **4** > **3** > **1** > **2** > DMSO, and the amplitude decrease ranged from 16.7 ± 3.5 to 76.7 ± 4.6%. The scavenging effect of 0.37 mmol L^{-1} compound **6** (76.7%) was nearly equal to that of the salicylate at concentrartion of 7.2 mmol L^{-1}.

The ability of tested compounds to exhaust their H-donating capacity was examined using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•). The compound is a stable free radical that has a proton free radical with an absorption maximum band around 515–528 nm. The characteristic absorption decreases significantly on exposure due to the proton-donating ability of the antioxidants proportional to the concentration and antioxidant activity of the antioxidant. (39) The DMPPH radical can be used to the primary characterization of the scavenging activity of compounds. (40–43)

According to the conclusion of Sanchez-Moreno, (44) compounds able to scavenge the DPPH radical *in vitro* may also scavenge polyaromatic hydrocarbon cations *in vivo* by donating hydrogen ions or electrons.

The DPPH radical scavenging capacities of tested FTDs are showed in Fig. 4A. For compounds **2**, **4**, **5** and **6** the reaction was biphasic, with a faster decay in the absorbance in the first dozen minutes, followed by a slower step. The DPPH radical scavenging activity of the examined compounds was evident ($p < 0.05$), but



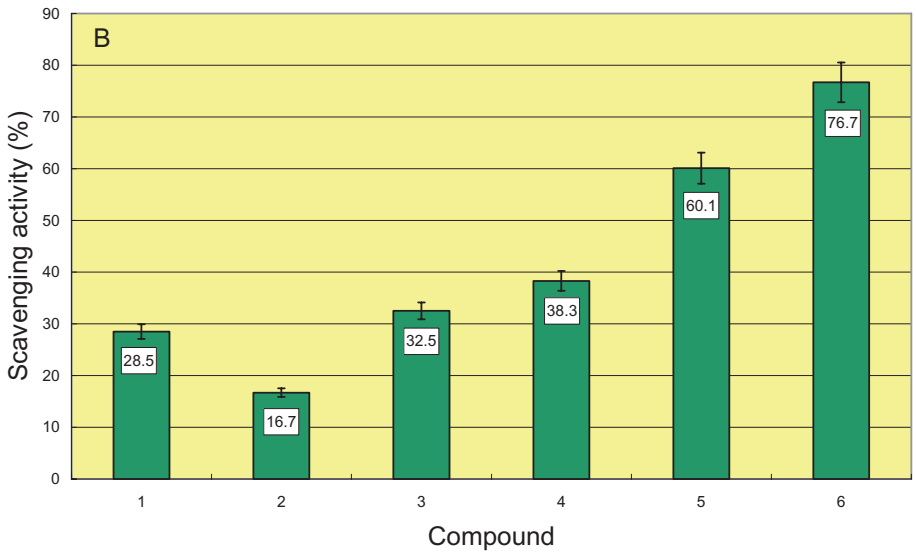


Figure 3. (A) The ESR spectra of the DMPO–OH spin adduct arising in the Fenton reaction: (a) ESR spectrum produced by the reaction mixture containing 50 mol L^{-1} DMPO, 0.5 mol L^{-1} H_2O_2, 1.0 mol L^{-1} sodium trifluoracetate, pH 6.15, 0.5 mL DMSO, and 50 µmol L^{-1} $FeSO_4$ $(NH_4)_2$ SO_4. The reaction was started by an addition of the Fe(II) ion. (b) The signal of DMPO–OH radical quenched by the addition of 7.2 mol L^{-1} salicylic acid dissolved in 0.5 mL DMSO. (c) The DMPO–OH radical signal quenched by the presence of 0.37 mol L^{-1} compound **5** dissolved in 0.5 mL of DMSO. (B) The inhibitory effect of FTD compounds exerted on the DMPO–OH radical formation. The composition of the reaction mixtures was the same as in (A, part c). Data are expressed as means ± SD of three measurements. The numbers in figure and on the *x*-axis refer to the compounds given in Fig. 1. Measurement conditions are reported under the Materials and Methods section.

was lower than ascorbic acid and Trolox at concentrations similar to that used during the compounds examination. The compounds' hierarchy of reducing capacity was **6** > **4** > **5** > **2** > **3** > **1**, ranging from 9 ± 5 to 40 ± 3% (Fig. 4B).

It can be observed that FTDs possessing a free carboxylic group, denoted as **4–6**, exhibited the highest effectiveness in the free radical scavenging power. The –COOH group is capable of readily donating hydrogen electron.

The inhibitory effect examined FTDs containing the $–CH_2–$COOH group on DPPH, O_2^- and HO radicals may represent the direct scavenging activity. Compounds **1–3** could not scavenge free radical as effectively as **4–6**. Other structural properties

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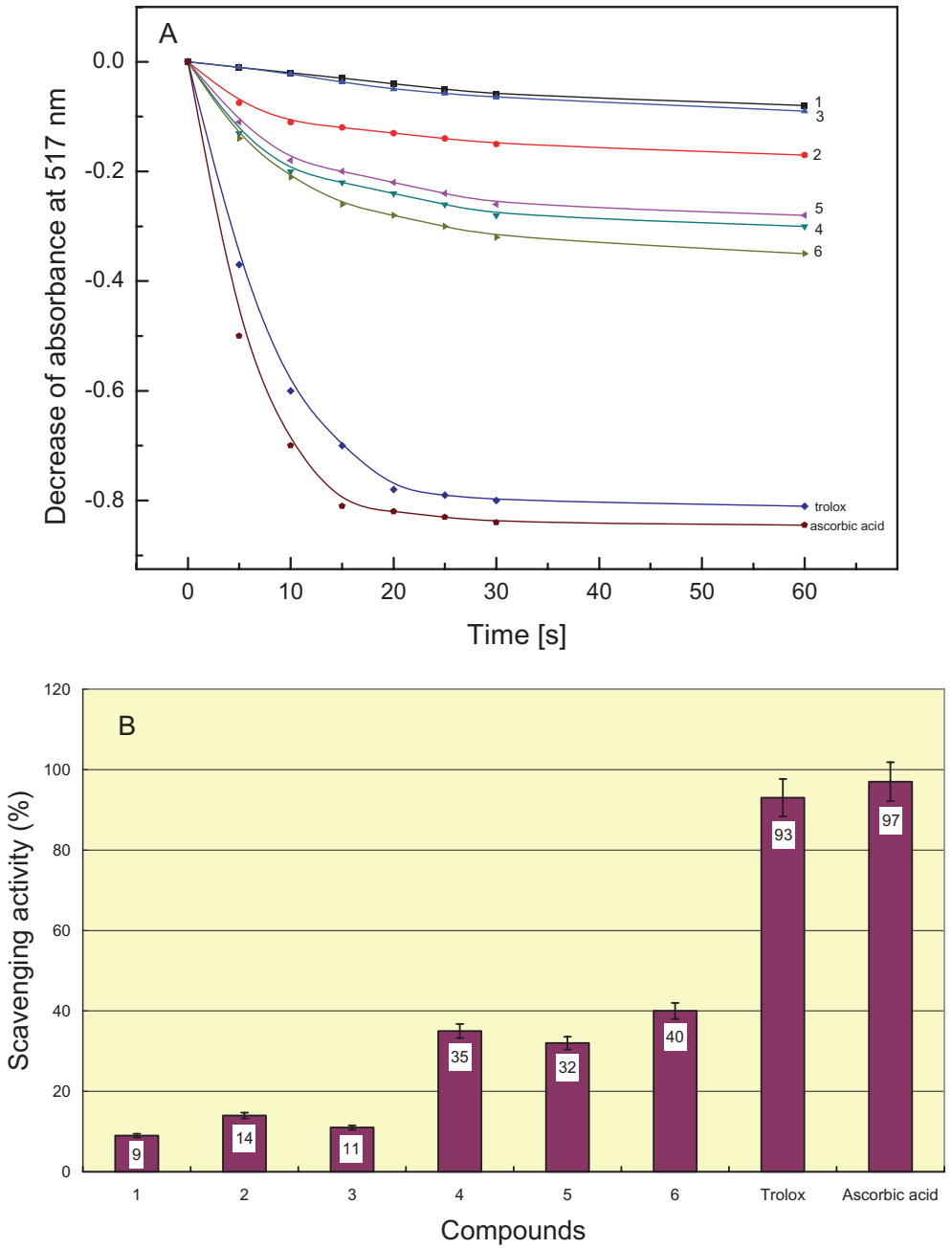

Figure 4. DPPH radical scavenging activity of the FTDs compounds, ascorbic acid 1 mol L^{-1} and Trolox. (A) Kinetic curves of the reaction between DPPH radical and solutions of the examined compounds. (B) The maximal scavenging effect exerted by examined compounds on DPPH$^{\bullet}$. Results are means ± SD of triplicate measurements. Numbers given in the figure refer to compounds listed in Fig. 1. Reaction conditions are described in Materials and Methods.

important for antioxidant nature of the examined compounds include the presence of a C2–C3 double bond in conjugation with 4-oxo function in the C ring. (45) It has been reported that the degree of unsaturation of the C2–C3 bond is an important determinant of high bioactivity of flavonoids. (46) The presence of nitrogen atom in the thiazole ring may also be an active center of FTDs, due to its lone pair electrons. In addition, all examined compounds contain the benzene ring; this also may be valid due to HO and O_2^- radicals abilities for substitution on the ring. (47)

In conclusion, the results obtained in the present study indicated that FTDs have good antioxidant activities. It has been found that among tested compounds those containing the carboxylic group exerted stronger antioxidant effect than FTDs without this group. These findings suggest that there is promise for FTDs to be used successfully in treating diseases that involve free radicals.

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