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Antioxidant properties of ferrous flavanol mixtures

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ABSTRACT

Interaction of metal, especially iron ions with flavanols is considered as an important feature of these compounds and is believed to contribute to their both antioxidant and prooxidant properties. The aim of this study was to examine how Fe^{2+} binding to form a 4:1 (flavanol:Fe^{2+}) mixtures affects the antioxidant properties of flavanols. ABTS* scavenging, protection against fluorescence bleaching induced by AAPH and hypochlorite, protection against lipid peroxidation and protection against hypochlorite-induced hemolysis demonstrated that flavonol-Fe^{2+} mixtures retain antioxidant properties, although, in most cases, they are lower with respect to the flavanols alone. No superoxide dismutase-like or catalase-like activity of the mixtures was revealed.

1. Introduction

Flavanols (flavan-3-ols), the main components of tea extracts, rise increasing interest due to their biological effects. Tea (*Camellia sinensis*) is the second most common beverage in the world next to water; a typical brewed green tea beverage (250 ml) contains 50–100 mg of flavanols (Wei et al., 1999). Tea extracts and pure flavanols has been reported to have antioxidant, antiviral, antibacterial, and anticancer activities, to decrease blood pressure as well as blood glucose level. Lipid metabolism studies have revealed that tea extracts and individual flavanols lower triacylglycerol and total cholesterol concentrations, inhibit hepatic and body fat accumulation, and stimulate thermogenesis (Nagao et al., 2005). Flavanols have been reported to penetrate the blood brain barrier and to protect against neuronal death in a wide array of cellular and animal models of neurological diseases (Mandel, Amit, Reznichenko, Weinreb, & Youdim, 2006). For example, (−)-epigallocatechin gallate (EGC-G) was reported to protect mice from dopamine neuron loss in *substantia nigra* caused by the neurotoxin *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in that model of Parkinsońs disease (Levites, Weinreb, Maor, Youdim, & Mandel, 2001).

Flavanol effects are complex and partly mediated by their properties not directly related to the antioxidant action (Kim, Quon, & Kim, 2014). However, these compounds are, first of all, natural antioxidants and most of their pharmacological actions is considered to be mainly due to their antioxidant activity (Ames, Gold, & Willett, 1995), understood as the ability to scavenge free radicals generated endogenously and formed by various xenobiotics, UV and ionizing radiation. We have recently characterized the antioxidant properties of flavanols demonstrating their excellent antioxidant action against various physiologically relevant oxidants (Grzesik, Naparło, Bartosz, & Sadowska-Bartosz, 2018). Nevertheless, flavanols, like most plant polyphenols possess also prooxidant properties (Hadi, Asad, Singh, & Ahmad, 2000).

Another important property of flavanols is the capacity to bind metal ions, especially iron ions under physiologic conditions. The interaction of flavanols with metal ions may contribute to both prooxidant and antioxidant properties of these compounds. On one hand,

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these compounds may reduce transition metal ions and induce the Fenton reaction (Nakagawa et al., 2002). Hydrogen peroxide, necessary for this reaction, is formed i.a. by autoxidation of catechins (Mochizuki, Yamazaki, Kano, & Ikeda, 2002). The prooxidant activity of flavanols has been associated with their interactions with metal ions. Interaction of EGC-G and (−)-epicatechin (EC) with copper leads to superoxide and hydroxyl radical formation and oxidative DNA degradation. This prooxidant property of flavanols was implied in the apoptogenic and anticancer activity of these compounds (Azam, Hadi, Khan, & Hadi, 2004). On the other hand, sequestering especially iron and copper by flavanols has been recognized as one mode of their antioxidant action (Bao et al., 2013). Under physiological conditions, formation of iron mixtures of flavanols may be of significance, both in the digestive tract where ingested flavanols may interact with iron released from metalloproteins and other sources, and also within the cells. Application of flavanols has been proposed in cases of iron overload (Thephinlap et al., 2007).

Adverse effects of iron binding by flavanols include impairment of intestinal iron absorption and lead to iron deficiency (Kim, Ham, Shigenaga, & Han, 2008; Tamilmani & Pandey, 2016). Nevertheless, high levels of free iron in the gastro-intestinal tract is associated with disease and carcinogenesis (Werner et al., 2011; Radulescu et al., 2012), so iron binding by flavanols in the intestine may be also beneficial.

Iron complex of another flavonoid, quercetin, was reported to be internalized by RKO cells (poorly differentiated colon carcinoma cell line) under conditions of low extracellular iron. The iron complexed to quercetin did not associate with the labile iron pool and cells behaved as though they were iron deficient (Horniblow, Henesy, Iqbal, & Tselepis, 2017). Iron complexes of flavonoids were postulated to be involved in the formation of molecular assemblies due to the facilitation of membrane adhesion and fusion, protein–protein and protein–membrane binding, and other processes responsible for the regulation of cell metabolism and protection against environmental hazards (Tarahovsky, Kim, Yagolnik, & Muzafarov, 2014).

As a significant fraction of flavanols may occur in the form of iron complexes *in vivo*, properties of flavanol-Fe^{2+}complexes are of considerable interest. The aim of this study was to evaluate the antioxidant properties of ferrous mixtures of flavanols in comparison with native compounds. We expected that flavanol ferrous mixtures retain most of the antioxidant activity of parent flavanols. Moreover, we intended to check whether these mixtures show superoxide dismutase or catalase activity. The following flavanols were used in the study: (+)-catechin (C), EC, (−)-epigallocatechin (EGC), (−)-epicatechin gallate (EC-G) and EGC-G. They represent the main flavanols present in the green tea (Zeeb, Nelson, Albert, & Dalluge, 2000). We checked the stoichiometry of flavanol complex formation and, in order to avoid excess iron, 4:1 (flavanol:Fe^{2+}) ferrous mixtures of flavanols were used in this study unless stated otherwise.

2. Materials and methods

2.1. Materials

Nitro Blue Tetrazolium (NBT) produced by BioShop Canada Inc. (Burlington, Ontario, Canada) and dimethyl sulfoxide (DMSO) were purchased from Lab Empire (Rzeszów, Poland). 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH) was from Polysciences (Warrington, PA, USA). Stock solutions of NBT and AAPH were freshly prepared in phosphate buffered saline (PBS) before each experiment. Fluorescein and sodium hypochlorite (NaOCl, 15% active chlorine basis) were obtained from CHEMPUR (Piekary Slaskie, Poland). A stock solution of NaOCl was diluted in 0.1 M NaOH and its concentration was determined spectrophotometrically at 290 nm using the molar absorption coefficient of $\varepsilon_{290nm} = 350\ M^{-1}\,cm^{-1}$ (Morris, 1966). Under such conditions NaOCl exists exclusively as OCl^-. The stock solution of NaOCl was diluted in PBS before use. At pH 7.4 the both forms, HOCl and OCl^- are present in the solution at comparable concentrations. 2-Thiobarbituric acid (TBA) was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany). A stock solution of TBA was prepared in 0.1 M NaOH at a concentration of 0.67%. Selected flavanols and all other reagents, if not mentioned otherwise, were purchased from Sigma (Poznan, Poland) and were of analytical grade. Mohr's salt (purity of 99.997%) was obtained from Sigma-Aldrich. Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Fluorometric and absorptiometric measurements were done in a Tecan Infinite 200 PRO multimode reader or a Spark multimode microplate reader (Tecan Group Ltd., Männedorf, Switzerland). All measurements were performed in triplicate and repeated at least three times on different preparations. Selected flavanols were dissolved in PBS (EGC-G and EGC) or in DMSO/PBS (EC-G, EC, C) (1 ml of 5 mM flavanol solution was obtained by dissolving flavanol in 5 µl DMSO and then 995 µl PBS was added.

2.2. Spectrophotometric titration of flavanols with iron (II)

100 µl samples of 1 mM flavanol solutions were titrated with 5 µl aliquots of 2 mM $Fe_2(NH_4)_2SO_4$ (Mohr's salt) in 1 mM HCl and absorbance was measured at a wavelength corresponding to absorption maximum of the complex, established on the basis of difference in absorbance spectra of 0.5 mM selected flavanol before and after addition of 0.5 mM Mohr's salt solution. From the dependence of absorbance at this wavelength on the amount of Fe^{2+} added, the stoichiometry of complex formation was determined (Fig. 1a–e).

2.3. Preparing flavanol mixtures with iron (II)

Flavanol-Fe^{2+} mixtures were prepared by mixing aqueous Mohr's salt solution with an excess of a flavanol. In order to obtain mixtures for all selected flavanols in the ratio 4:1, 50 µl of 1 mM Mohr's salt in 1 mM HCl was added with 200 µl of 1 mM flavanol solution and incubated for 10 min. For some experiments, flavanol mixtures with Fe^{3+} and Cu^{2+} were prepared in the same manner, using $FeCl_3$ and $CuSO_4$, respectively. An 1:1 mixture of EGC-G-Fe^{2+} was also prepared, using a slight (5%) flavanol excess with respect to Fe^{2+} to avoid appearance of free Fe^{2+}. Then the solutions were diluted with PBS to the final flavanol concentration of 0.5 mM. In experiments concerning superoxide scavenging activity generated by the PMS-NADH, in addition to 4:1 mixtures, 2:1 (flavanol:Fe^{2+}) mixtures were also prepared, as described by Kostyuk, Potapovich, Strigunova, Kostyuk, and Afanas'ev (2004).

2.4. Fourier transform infra-red spectroscopy (FTIR)

Nicolet 6700 FTIR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) were used to evaluate the bonds formed between iron and flavanols. FTIR Spectrometer was controlled by OMNIC software. Samples for FTIR measurement were prepared by mixing a sample (approx. 2 mg) with 200 mg potassium bromide (KBr) powder in an agate mortar then a transparent pellet was prepared with a hand press. FT-IR spectra (KBr pellet) were recorded for the substrates (flavanols and Mohr's salt) and the flavanol-Fe^{2+} mixtures. An average of 32 scans was collected for each measurement at a resolution of 2 cm^{-1}.

2.5. Scanning electron microscopy (SEM)

The morphology of the flavanols and flavanol mixtures with Mohr's salt was visualized using scanning electron microscope (SEM) with energy-dispersive X-ray spectroscopy (EDS) analyzer – Quanta™ 3D 200i (FEI Co. Field Emission Instruments, Hillsboro, OR, USA).

2.6. Measurement of particle hydrodynamic size

The particle size and size distribution in flavanol-Fe^{2+} mixtures in solution were measured by the dynamic light scattering (DLS) technique using a photon correlation spectrometer Malvern Zeta-Sizer Nano-ZS (Malvern Instruments, Worcestershire, United Kingdom) in DTS0012 plastic cells (Malvern Instruments, Worcestershire, United Kingdom). The refraction factor was 1.33, at a detection angle of 90, and a wavelength was set at 633 nm. Selected flavanols and flavanol-Fe^{2+} mixtures were measured at the flavanol concentration of 1 mM. Particle size was measured from the average of 11 × 3 cycles at 25 °C. To analyze the data Malvern software was used.

2.7. Antioxidant activity of flavanols and flavanol-Fe^{2} mixtures in cell-free systems

2.7.1. Antiradical activity: $ABTS^{*}$ scavenging.

The ability of selected flavanols and their Fe^{2+} mixtures to scavenge the 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) radical ($ABTS^{*}$) was measured according to a procedure previously proposed by us (Grzesik et al., 2018).

Appropriate amounts of the studied compounds were added to a solution of $ABTS^{*}$, diluted so that 200 µl of the solution had absorbance of 1.0 in a microplate well, at 734 nm. The decrease in $ABTS^{*}$ absorbance was measured after 1 min ("fast" scavenging) and between 10 and 30 min ("slow" scavenging) of incubation at room temperature

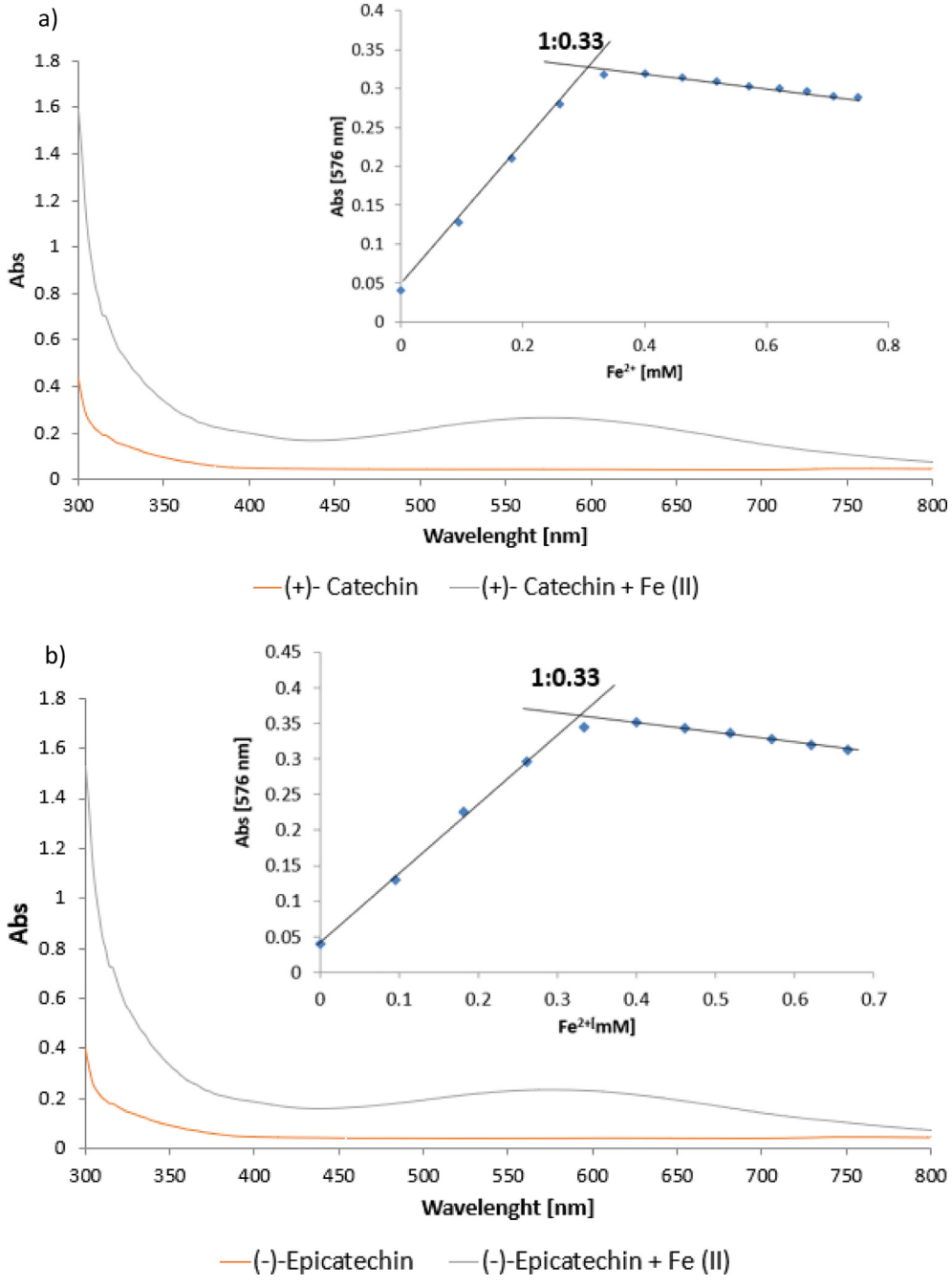

Fig. 1. Absorption spectra of flavanols and their Fe^{2+} mixtures: a) (+)-Catechin, b) (−)-Epicatechin, c) (−)-Epigallocatechin, d) (−)-Epicatechin gallate, e) (−)-Epigallocatechin gallate. Inset: Titration curves obtained from changes in the absorbance of flavanol: Fe^{2+} mixtures at a wavelength corresponding to maximum absorbance of the complex.

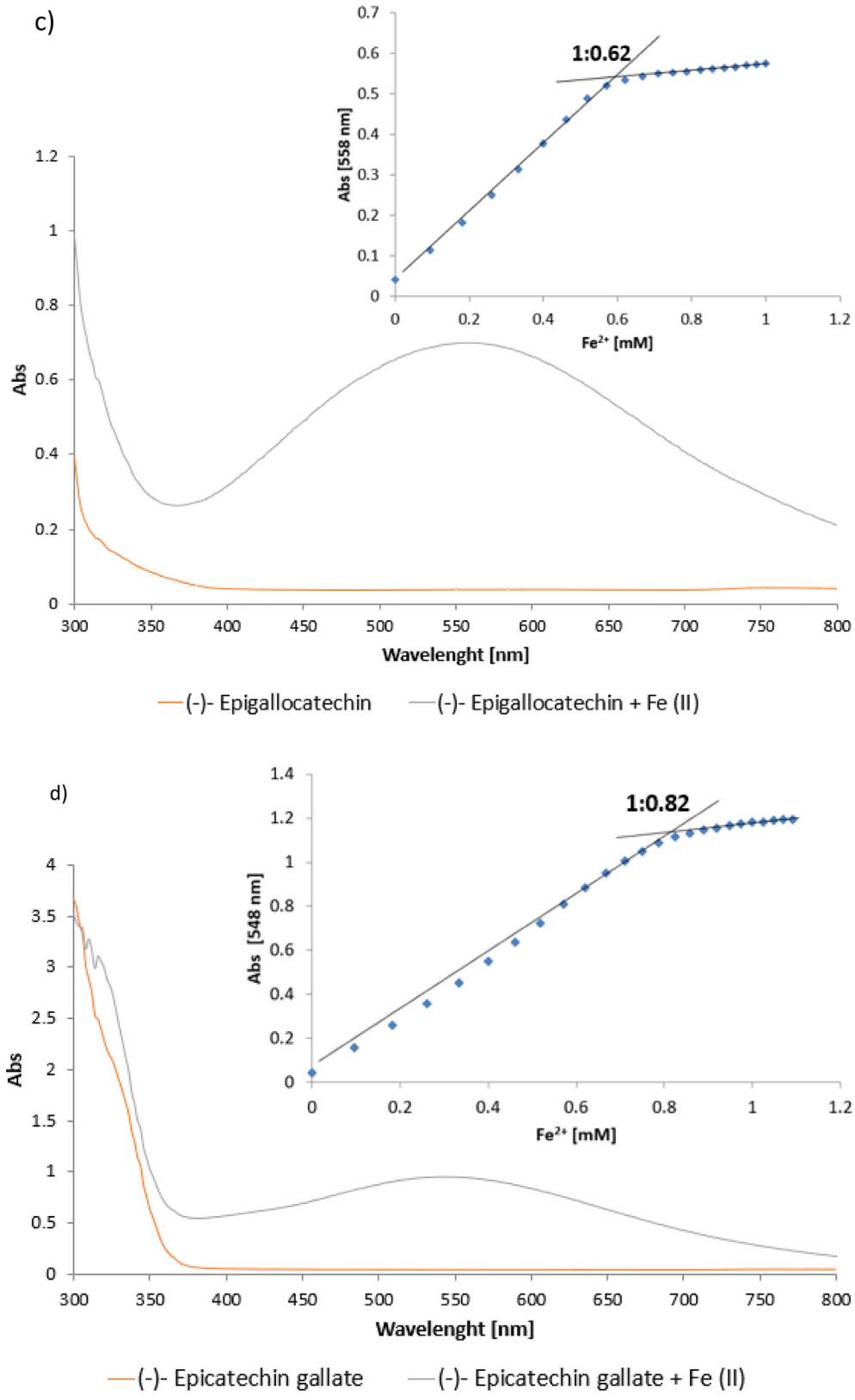

Fig. 1. (*continued*)

(21 ± 1 °C). From the plots of the dependence of absorbance decrease (ΔA) on the compound concentration, the value of ΔA/mM was calculated for the compounds tested.

2.7.2. Protection of fluorescein against bleaching induced by NaOCl or AAPH

Inhibition of fluorescein bleaching was determined with a method proposed by us (Grzesik et al., 2018).

Briefly, aliquot of hypochlorite was added to a microplate well

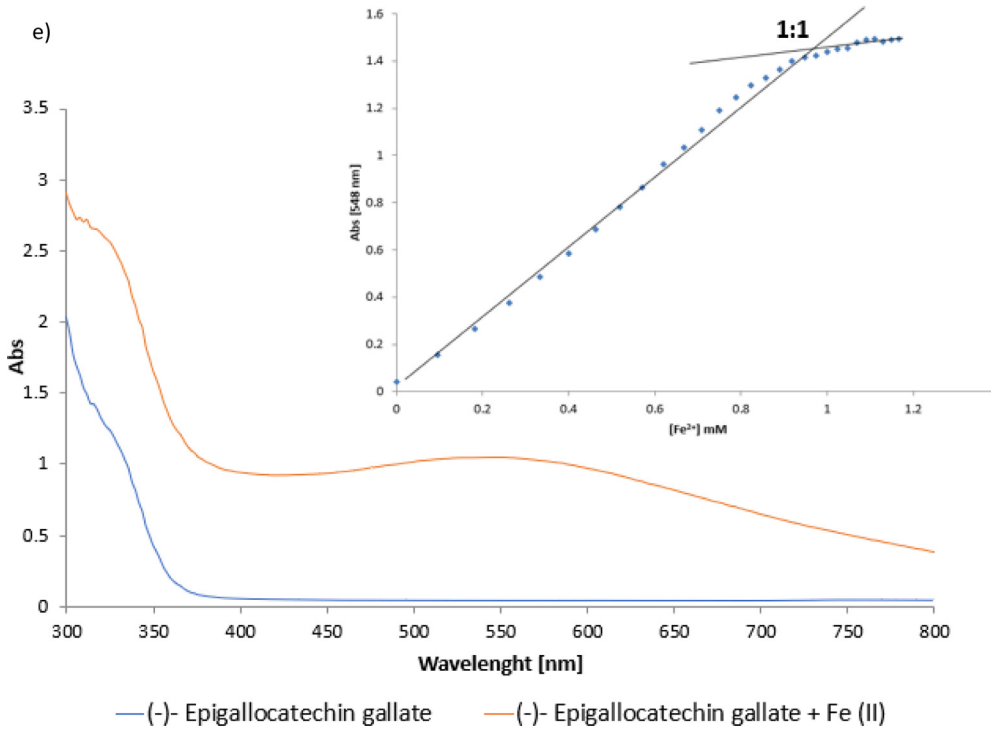

Fig. 1. (*continued*)

containing 100 µl of 0.2 µM fluorescein dissolved in PBS and the solution was mixed immediately. The amount of hypochlorite required to decrease fluorescence down to ca 5–10% of the initial value was determined (1.75 nmol of hypochlorite). Flavanols/flavanol-Fe^{2+} mixtures in a range of concentrations (0.125–5 µM in flavanol), were added to the fluorescein solution before addition of hypochlorite, keeping the volume of the sample constant (100 µl). Fluorescence was measured after 15 min incubation at room temperature at the excitation/emission wavelengths of 485 and 538 nm, respectively.

10 mM AAPH (final concentration) was added to a well containing 0.2 µM fluorescein dissolved in PBS and the solution was mixed immediately. Flavanols/flavanol-Fe^{2+} mixtures in a range of concentrations (0.25–10 µM in flavanol) were added to the fluorescein solution before addition of AAPH, keeping the volume of the sample constant (100 µl). Fluorescence was measured (excitation 485 nm, emission 538 nm) after 1 h incubation at 37 °C. Percent of protection was calculated according to the formula:

$$\%\ Protection = (Fn - Fo)/(Fc - Fo) \times 100\%$$

where F_n – fluorescence of a sample containing fluorescein, hypochlorite/AAPH and a compound studied; F_o – fluorescence of fluorescein treated with hypochlorite/AAPH; F_c – fluorescence of non-treated fluorescein.

From the concentration dependence of protection, the concentrations of compounds providing 50% protection (IC_{50}) against the fluorescein bleaching was calculated.

2.7.3. Superoxide scavenging activity

Superoxide scavenging activity was estimated using several tests:

2.7.3.1. Pyrogallol autoxidation assay. The procedure was modified from Marklund and Marklund (1974). 10 mM pyrogallol solution in 10 mM HCl was added to 50 mM Tris-HCl buffer, pH 8.5, containing flavanols or their Fe^{2+} mixtures in the flavanol concentration range of 50–500 µM. Increase in absorbance at 420 nm was monitored for 10 min.

2.7.3.2. Photochemical reduction of NBT. Flavanols or their Fe^{2+} mixtures were added, at a flavanol concentration range of 1–20 µM, to a solution of 10 mM methionine, 20 µM NBT and 1.5 µM riboflavin. The plates were illuminated for different time periods (up to 2 h) and increase of absorbance at 560 nm was measured (procedure modified from Beauchamp and Fridovich (1971)).

2.7.3.3. Adrenalin autoxidation assay. The procedure was modified from Misra and Fridovich (1972). 10 mM adrenalin solution in 10 mM HCl was added to 50 mM sodium carbonate buffer, pH 10.2, containing flavanols or their 4:1 Fe^{2+}, Fe^{3+} and Cu^{2+} mixtures in the flavanol concentration range of 5–50 µM. Maximal rate of increase in absorbance due to adrenochrome formation was estimated at 480 nm during 5 min.

2.7.3.4. PMS-NADH assay. Superoxide anion was generated nonenzymatically with a phenazine methosulfate (PMS)-NADH system. The reaction mixture consisted of 50 mM sodium carbonate buffer (pH 10.0), 2.5 mM NBT, various amount of sample solution (flavanols/flavanol-Fe^{2+} mixtures) and 8 mM NADH. The reaction was started by the addition of 150 µl of PMS, and the absorbance at 560 nm was recorded for 20 min. As the control, A. dest. was used. The reaction rate was calculated from the proportional increase of absorbance, and scavenging activity of sample was expressed as percent inhibition (modified from Ewing & Janero, 1995).

2.7.4. Hydrogen peroxide scavenging activity

100 µM solution of hydrogen peroxide in 100 mM phosphate buffer, pH 7.4, were added with flavanols/flavanol-Fe^{2+} mixtures to concentrations of 10–50 µM. After various times (15, 30 or 60 min) concentration of hydrogen peroxide was estimated in a reaction with Xylenol Orange (Gay, Collins, & Gebicki, 1999).

Alternatively, flavanols/flavanol-Fe^{2+} mixtures at flavanol concentrations of up to 15 µM were added to 25 mM hydrogen peroxide solution in 100 mM phosphate buffer, pH 7.4. Absorbance of hydrogen peroxide was monitored at 240 nm for up to 30 min.

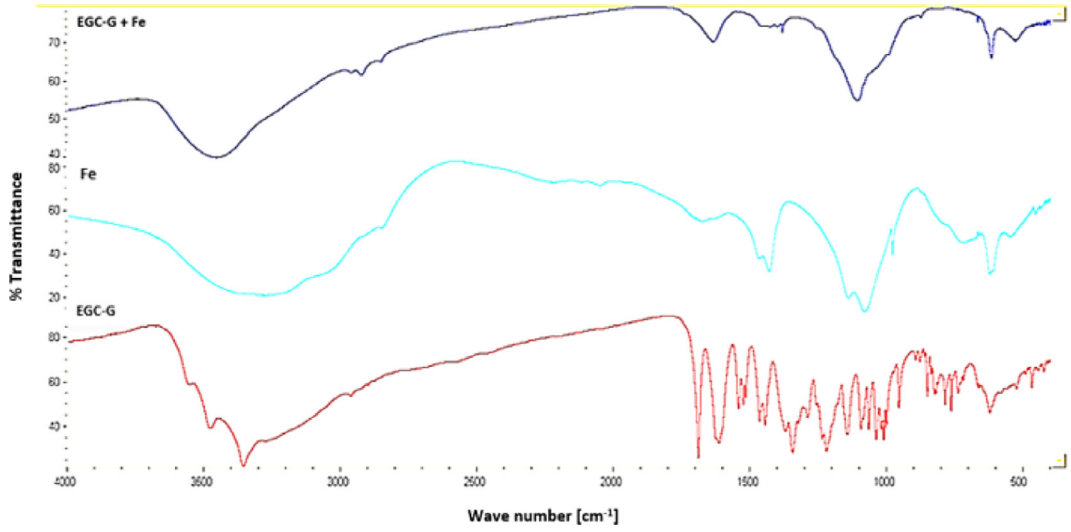

Fig. 2. Comparison of FT-IR spectra of EGC-G-Fe^{2+} mixture, iron, and EGC-G (KBr pellet).

2.7.5. Lipid peroxidation

Protection of egg yolk lipids against peroxidation induced by AAPH and Fe^{2+} was studied. 200 µl of 10% egg yolk suspension in PBS (1 ml of egg yolk suspended vigorous vortexing in PBS) was treated with 10 mM AAPH (final concentration) or 1 mM Fe^{2+} (final concentration) in the presence of flavanols or flavanol/Fe^{2+} mixtures. The concentration range of flavanols/flavanol-Fe^{2+} mixtures was 0.2–1.18 mM or 1–10 µM (with respect to flavanol), for experiments with Mohr's salt and AAPH respectively. The samples were incubated at 37 °C for 3 h under conditions of oxygen access. Then, the samples were added with an equal volume (250 µl) of 10% trichloroacetic acid (TCA) and 250 µl of 0.67% thiobarbituric acid (TBA). The samples were heated at 100 °C for 20 min, cooled, centrifuged, and absorbance of supernatants was measured at 532 nm.

Per cent protection against lipid peroxidation by flavanols/flavanol-Fe^{2+} mixtures was calculated as

$$\%\,\text{Protection} = 100\%[1-(A_n-A_c)/(A_o-A_c)]$$

where A_o – absorbance of sample incubated with AAPH of Fe^{2+} without any flavanol/flavanol-Fe^{2+} mixture, A_n – absorbance of a sample containing a protective agent, A_c – absorbance of a sample not treated with an oxidizing agent.

From the dose-dependent curve relationship of the protection, flavanol/flavanol-Fe^{2+} IC_{50} values were determined.

2.8. Protection against hypochlorite-induced hemolysis

2.8.1. Ethical approval of experiments with blood

The study was approved by the Research Bioethics Committee of the University of Łódź (Poland).

2.8.2. Preparation of erythrocytes

Eight ml of peripheral blood from a healthy donor (lab volunteer, a 40-year-old woman; ISB) was collected in Citrate Tubes containing 3.2% buffered sodium citrate solution and used within the day of its collection. Erythrocytes were isolated by centrifugation for 10 min at 3000 rpm, at 4 °C. The plasma and buffy coat were removed by aspiration. The red blood cells (RBCs) were washed four times with ice-cold PBS. Washed RBCs were suspended to a final hematocrit of 10% in PBS.

2.8.3. Hypochlorite-induced hemolysis

Aliquots of RBC suspensions in PBS were mixed with flavanols/flavanol-Fe^{2+} mixtures at the final flavanol concentration range of 5–25 µM (final volume of 200 µl) and incubated for 15 min with shaking at 37 °C. Then 0.15 mM hypochlorite (final concentration), as optimal concentration to induce hemolysis, was added and turbidity (700 nm) was measured every 2 min for 240 min. The time required to decrease the turbidity to 50% of the initial value (about 1.0) was defined as hemolysis time. Relative hemolysis times were calculated as [100% × (hemolysis time in the presence of a test compound/mean hemolysis time for control sample containing erythrocytes and NaOCl only)].

2.9. Statistical analysis

Statistical significance of differences was evaluated using the Kruskal-Wallis test (when all samples were compared) or paired Student's "t" test (when pairs of results were compared). Statistical analysis of the data was performed using STATISTICA software package (version 13.1, StatSoft Inc. 2016, Tulsa, OK, USA, www.statsoft.com).

3. Results

The flavanols studied differed in the stoichiometry of Fe^{2+} binding, as shown by Fig. 1a–e. (+)-Catechin and EC bound Fe^{2+} at a ratio of 3:1 (flavanol/Fe^{2+}) (Fig. 1a–b). The apparent ratios of Fe^{2+} binding by EGC and EC-G were: 1.61:1 and 1.22:1 respectively, while the apparent ratio of Fe^{2+} by EGC-G was 1:1 (Fig. 1c–e). Thus, mixing Fe^{2+} with fourfold excess of flavanols allowed to form mixtures, in which Fe^{2+} was totally bound by these compounds.

The formation of flavanol-Fe^{2+} complexes was confirmed by FI-IR (KBr pellet) analyses (Fig. 2). For EGC-G, the wave number of spectra of the pure flavanol at 700–900, 1150, 1250 , 1450–1350, 1600–1430, 1700 and 3300–3600 cm^{-1} could be assigned to Ar-H out-of-plane bond, C—O stretch, Ar—O—C, OH bond aromatic, C=C aromatic ring stretch, C=O and O—H stretch respectively. The absorption bands at 1450–1350 cm^{-1} were absent in Fe-EGC-G spectra. A new peak with maximum at wave number at 1382 cm^{-1} was observed in Fe-EGC-G spectra. This band does not appear in the spectrum of Mohr's salt and may be assigned to stretching vibration of Fe—O. The O—H stretching is shifted to higher wave number (Manna, Saha, & Ghoshal, 2014). Analogous results were obtained for other flavanols (not shown).

The vacuum-dried iron-flavanol mixtures were characterized by SEM combined with energy dispersive X-ray (EDX) analysis in order to characterize the morphology of the particles and to examine global their chemical composition. The average particle size of particles was about 5 µm (C with Mohr's salt; Fig. 1Sc–d) or about 10 µm (EC, EC-G,

Table 1
Hydrodynamic diameters d of flavanols and their Fe^{2+} (4:1) mixtures in PBS. Statistical significance of differences between flavanols and their Fe^{2+} mixtures: $^{**}P < 0.01$, $^{***}P < 0.001$.

Compound/Mixture	d [nm]
(+)-C	240.7 ± 24.5
(+)-C + Fe^{2+}	406.7 ± 17.6***
(−)-EC	276.0 ± 9.7
(−)-EC + Fe^{2+}	549.4 ± 25.4***
(−)-EGC	429.6 ± 55.8
(−)-EGC + Fe^{2+}	444.9 ± 28.5
(−)-EC-G	387.8 ± 98.9
(−)-EC-G + Fe^{2+}	760.7 ± 49.5**
(−)-EGC-G	326.6 ± 92.2
(−)-EGC-G + Fe^{2+}	1454.0 ± 51.5***

EG-C and EGC-G with Mohr's salt) (Fig. 1Sf, h, j–k). The presence of iron in the mixture was detected in EDX spectra (not shown).

Taking into account that SEM pictures may be affected by artifacts of drying, we estimated also the hydrodynamic size of particles formed by the flavanols and their mixtures. The results shown in Table 1 demonstrate that the flavanols form aggregates in solution, of average diameters in the nanometer range (< 1 μm) and that addition of Fe^{2+} increases the size of flavanol aggregates. This increase is the most significant in the case of EGC-G, the size of the resulting aggregates exceeding 1 μm (Table 1).

The ABTS* scavenging activity of Fe^{2+} mixture of C was slightly lower in comparison with C, that of EC-Fe^{2+} slightly higher with respect to EC and that of EGC-G-Fe^{2+} mixtures significantly lower with respect to ECG-C (lower for a 1:1 than for a 4:1 mixture). The ABTS* scavenging activities of Fe^{2+} mixtures of EGC and EC-G did not differ significantly from those of respective flavanols (Table 2). Free Fe^{2+} (1–20 μM) did not decrease absorbance of ABTS* (not shown).

Flavanol-Fe^{2+} mixtures of C, EC and EGC retained of the ability of flavanols to protect fluorescein against bleaching induced by AAPH and NaOCl, the IC_{50} values being not changed significantly for the mixtures with respect to those for flavanols alone. The EC-G-Fe^{2+} mixture protected less effectively than EC-G against fluorescein bleaching induced by both agents as evidenced by higher IC_{50} values. Interestingly, the 4:1 EGC-G-Fe^{2+} mixture was more effective than EGC-G in protection against AAPH-induced fluorescein bleaching (for NaOCl-induced fluorescence bleaching, the difference in IC_{50} values was not significant), but the 1:1 mixture was significantly less effective than EGC-G in protection against AAPH-induced and NaOCl-induced fluorescein bleaching. Free Fe^{2+} (0.125–5 μM) did not offer any protection (not shown).

Flavanols as well as their Fe^{2+} mixtures protected against lipid peroxidation induced by AAPH and Fe^{2+}. No significant differences were observed between the mixtures and their parent flavanols except for EC-G (smaller protection against AAPH-induced peroxidation by the Fe^{2+} mixture) and EGC-G (smaller protection against Fe^{2+}-induced peroxidation by the mixture; Table 2).

Flavanols did not inhibit pyrogallol autoxidation. Flavanol-Fe^{2+} mixtures slightly, while flavanol-Fe^{3+} mixtures significantly increased the rate of pyrogallol autoxidation (not shown). Flavanols inhibited dose-dependently photochemical riboflavin-mediated NBT reduction. The extent of reduction decreased in time (probably reflecting flavanol oxidation). Flavanol-Fe^{2+} mixtures showed lower extent of inhibition turning into acceleration of NBT reduction at longer times, as shown for EGC-G in Fig. 2S.

Adrenalin autoxidation at alkaline pH was slightly inhibited by 5–50 μM flavanols, while their Fe^{2+} and Cu^{2+} mixtures did not inhibit this reaction and Fe^{3+} mixtures increased the autoxidation rate, as shown for EGC-G mixtures in Fig. 3S.

Both flavanols and their Fe^{2+} mixtures inhibited PMS-mediated NBT reduction and in general the activities of flavanols and their mixtures were mostly similar. In some cases the inhibitory activity of Fe^{2+} mixtures was higher with respect to flavanols, but in most cases it was lower or not significantly different for 4:1 flavanol:Fe^{2+} mixtures (Table 3).

Table 2
Comparison of antioxidant properties of flavanols and their Fe^{2+} mixtures (4:1) in various assays. For EGC-G, results for a 1:1 mixture are also shown. Statistically significant differences between a flavanol and a corresponding flavanol-Fe^{2+} mixtures: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The statistical significance of differences was evaluated by using the paired Student's "t" test.

Compound/Mixture	ABTS* scavenging activity [mol Trolox equivalents/mol]	Protection against fluorescein bleaching by AAPH (IC_{50}, μM)	Protection against fluorescein bleaching by NaOCl (IC_{50}, μM)	Protection against lipid peroxidation by AAPH (IC_{50}, μM)	Protection against lipid peroxidation by Fe^{2+} (IC_{50}, μM)
(+)-C	2.86 ± 0.05	0.68 ± 0.06	1.43 ± 0.11	9.03 ± 0.10	1121.4 ± 1.9
(+)-C + Fe^{2+}	2.72 ± 0.03*	0.63 ± 0.04	1.30 ± 0.04	7.31 ± 0.64	1123.8 ± 19.4
(−)-EC	2.34 ± 0.08	0.98 ± 0.02	0.55 ± 0.04	3.00 ± 0.24	1086.9 ± 12.9
(−)-EC + Fe^{2+}	2.60 ± 0.03**	0.96 ± 0.02	0.56 ± 0.03	3.90 ± 0.40	1096.3 ± 9.4
(−)-EGC	4.65 ± 0.02	5.35 ± 0.15	1.29 ± 0.01	9.64 ± 0.15	786.3 ± 26.7
(−)-EGC + Fe^{2+}	4.73 ± 0.03	5.31 ± 0.23	1.14 ± 0.05	7.96 ± 0.45*	906.0 ± 29.2
(−)-EC-G	5.19 ± 0.01	1.67 ± 0.05	0.69 ± 0.06	1.93 ± 0.10	821.8 ± 54.8
(−)-EC-G + Fe^{2+}	5.17 ± 0.00	2.07 ± 0.03**	1.05 ± 0.06**	3.14 ± 0.11**	1159.4 ± 22.0
(−)-EGC-G	4.98 ± 0.01	0.98 ± 0.02	0.64 ± 0.02	4.43 ± 0.28	464.2 ± 11.4
(−)-EGC-G + Fe^{2+}	3.96 ± 0.03***	0.78 ± 0.04**	0.58 ± 0.04	4.29 ± 0.21	549.0 ± 37.0*
(−)-EGC-G + Fe^{2+} 1:1	3.48 ± 0.04***	7.56 ± 0.39***	1.49 ± 0.01***	3.40 ± 1.05	591.9 ± 69.5*

Table 3
Protection against PMS-mediated NBT reduction by flavanols and flavanol-Fe^{2+} mixtures. Statistically significant differences between a flavanol and the corresponding flavanol-Fe^{2+} mixture: $^{*}P < 0.05$, $^{**}P < 0.01$.

Compound	Flavanol (3 μM)	[Flavanol:Fe^{2+}] (3 μM:0.75 μM)	Flavanol (7.5 μM)	[Flavanol:Fe^{2+}] (7.5 μM:1.875 μM)	Flavanol (15 μM)	[Flavanol:Fe^{2+}] (15 μM:3.75 μM)
(+)-C	13.46 ± 5.10	23.07 ± 5.11	42.23 ± 4.76	56.17 ± 8.54	63.81 ± 5.59	82.57 ± 5.21*
(−)-EC	37.60 ± 9.21	48.70 ± 4.62	61.43 ± 7.51	73.53 ± 1.96	79.69 ± 0.91	81.31 ± 2.55
(−)-EGC	42.04 ± 6.31	34.45 ± 3.89	78.35 ± 2.75	68.10 ± 6.26*	89.61 ± 0.23	91.11 ± 2.24
(−)-EC-G	8.37 ± 7.21	19.64 ± 2.84*	33.02 ± 3.35	26.69 ± 8.27	71.45 ± 0.26	60.24 ± 8.79
(−)-EGC-G	33.10 ± 2.12	10.11 ± 7.91	61.60 ± 3.41	56.58 ± 3.92	85.90 ± 2.77	73.90 ± 1.03**

We attempted to see whether flavanols and, especially, their Fe^{2+} mixtures have catalase-like activity, incubating them with both low (μmolar) and high (millimolar) concentrations of hydrogen peroxide for up to 1 h. No detectable catalase-like activity of the flavanols or their Fe^{2+} mixtures was found (not shown).

Both flavanols and their Fe^{2+} mixtures protected erythrocytes against lysis induced by HOCl. There were no significant differences between the action of flavanol 4:1 mixtures with respect to flavanols alone, with the exception of C (higher protection by C-Fe^{2+} than C at concentrations of 2.5 μM and 5 μM; Fig. 3a), EC (lower protection by EC-Fe^{2+} than by EC at the concentration of 2.5 μM; Fig. 3b) and EGC-G (higher protection by EGC-G-Fe^{2+} than by EGC-G at the concentration of 1 μM; Fig. 3e). In contrast, 1:1 EGC-G-Fe^{2+} mixture was significantly less effective than EGC-G alone in protection against NaOCl-induced hemolysis, pointing again to different antioxidant behavior of flavanol mixtures of various flavanol:Fe^{2+} ratio (Fig. 3f). Interestingly, Fe^{2+} alone sensitized erythrocytes to the hemolytic action of NaOCl at a low concentration (1 μM) while higher Fe^{2+} concentrations show a weak protective effect (Fig. 3g).

4. Discussion

Flavanol complexes with iron cations are interesting because both components are present in the human body. Flavanols are naturally occurring chemicals found in foods such as fruits, some types of grain, wine and tea (Kim et al., 2008). Iron plays an important role in electron transfer, cellular respiration, cell proliferation and differentiation, and regulation of gene expression. However, iron exposure is directly associated with the pathogenesis of many disorders, such as atherosclerosis, cancer and inflammation, mainly via the production of free radicals (Matsui, Tanaka, & Iwahashi, 2017).

Flavanols are known to chelate metal ions. Chelation activity of flavanols is mainly related to a flavanol groups in the B ring while redox behavior of ligands in complexes depends on the presence of the 3-hydroxy group in their structure. In ring C, the 3-hydroxyl-4-carbonyl and 5-hydroxyl-4-carbonyl groups can also participate in metal chelation (Tarahovsky et al., 2014; Kostyuk et al., 2004). Some flavonoids were found to react with two Fe^{2+} ions at two separate binding sites (Ryan & Hynes, 2007). Additionally, flavonoids can also chelate potentially toxic transition metal ions (Fe^{2+}, Fe^{3+}, Cu^{2+}) preventing metal-catalysed free radical generation reactions. Among these reactions iron chelation is of particular interest since binding of iron to the antioxidant flavonoids can reduce the accessibility of the iron to oxygen molecules and consequently diminish its high toxicity. Iron chelation can also serve as an effective tool in modulating cellular iron homoeostasis, under physiologically relevant conditions (Kostyuk et al., 2004; Tarahovsky, Yagolnik, Muzafarov, Abdrasilov, & Kim, 2012; Tarahovsky et al., 2014; Mladěnka et al., 2011).

There exist diverse data in the literature concerning the stoichiometry of flavonoid:Fe^{2+} interactions (Horniblow et al., 2017; Kostyuk et al., 2004). We titrated the flavanols studied with Fe^{2+} to determine their binding stoichiometry. Although it is often assumed that flavanols form a 1:1 complex with Fe^{2+} (Kostyuk et al., 2004), we found an apparent 1:1 stoichiometry only for EGC-G. An apparent stoichiometry of 3 flavanol molecules/Fe^{2+} was found for C and EC, while for EGC and EC-G apparent fractional stoichiometry between 1:1 and 2:1 was found. This result can be explained as due to heterogeneity of complexes formed; nonetheless it should be taken into account that the flavanols tested were not present as a homogenous solution, but rather as aggregates of a hydrodynamic size of several hundred nm; SEM showed the presence of particles of even higher size (several μm). It should be mentioned that flavanol-iron complexes can change under conditions of flavanol excess and as a function of pH, as it has been demonstrated for other flavonoids (Filipsky, Riha, Hrdona, Vavrowa, & Mladenka, 2013). The presence of such structures must affect the binding properties of flavanols.

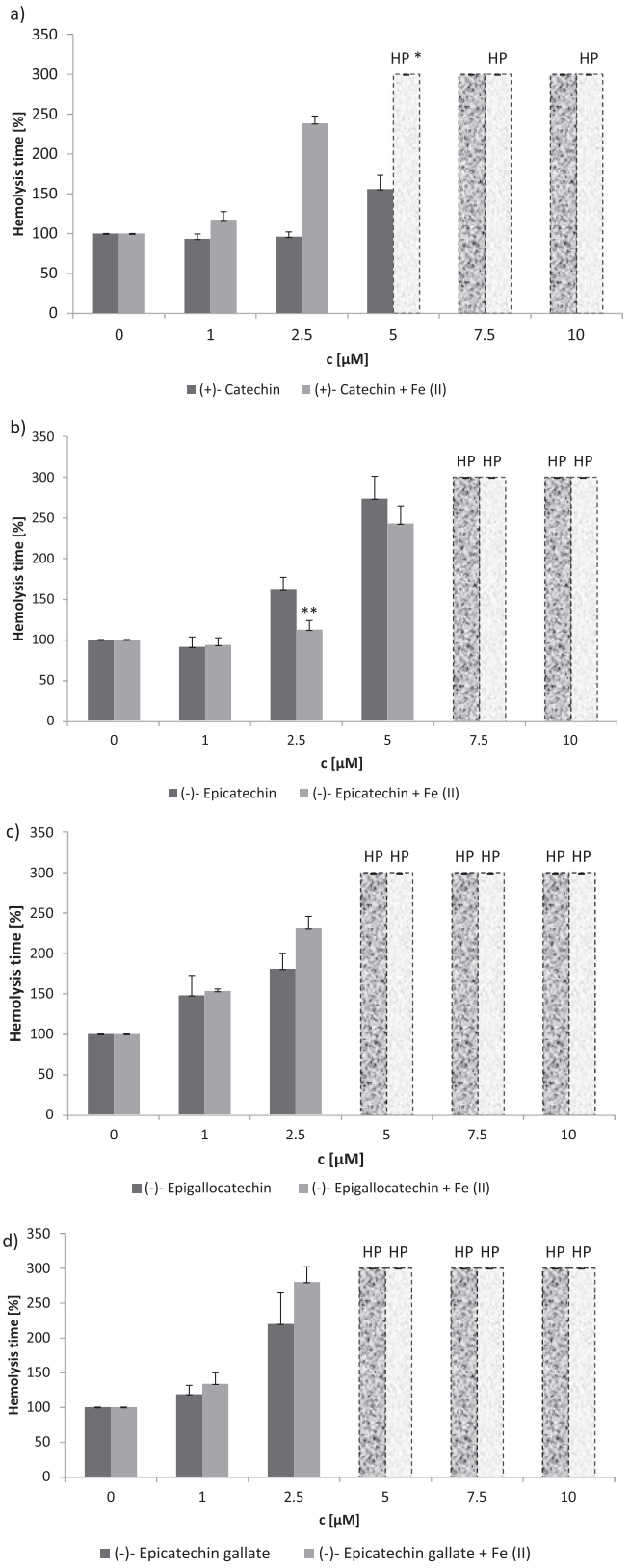

Fig. 3. Protection of erythrocytes against NaOCl-induced hemolysis by flavanols, flavanol-Fe^{2+} mixtures (4:1) and Fe^{2+}.

We prepared 4:1 mixtures of flavanols with Fe^{2+} in order to avoid the occurrence of free Fe^{2+} and mimic the possible *in vivo* situations where flavanols are expected to be in excess over trace amount of Fe^{2+}. Therefore, we studied the effects of flavanol-Fe^{2+} mixtures on a

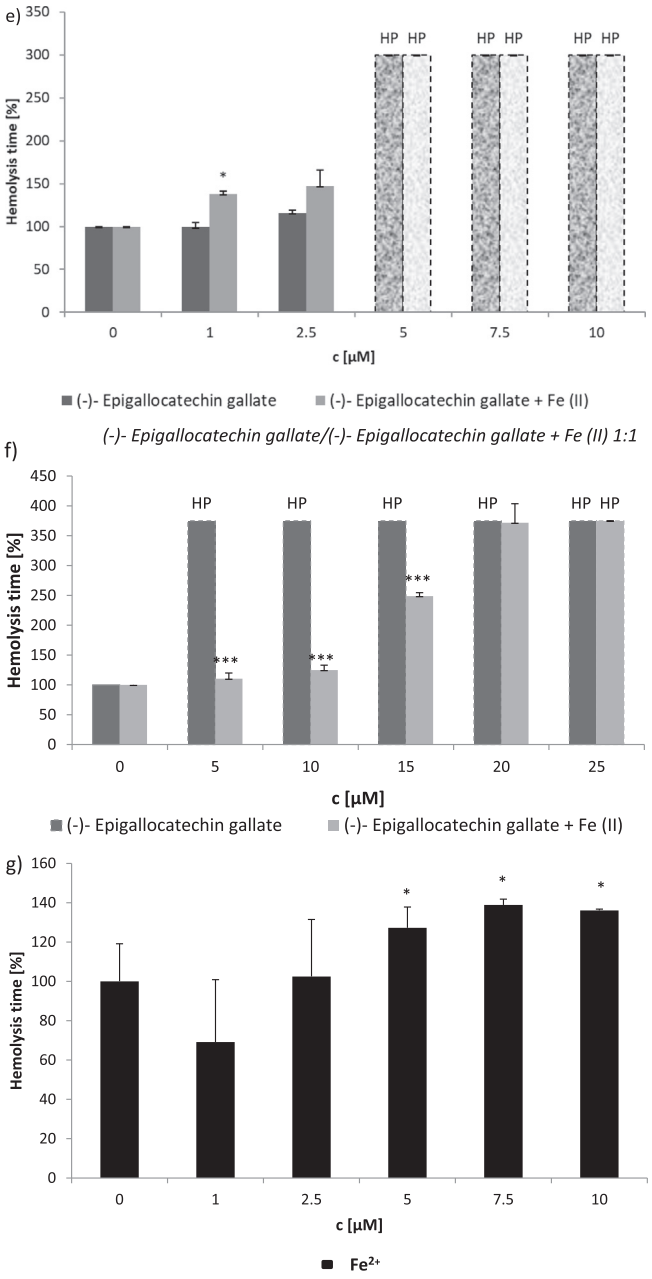

Fig. 3. (*continued*)

background of flavanols alone, so the effects of such mixtures were always compared with those of Fe^{2+}-free flavanols.

There are contradictory data in the literature on antioxidant properties of flavonoids-metal complexes in comparison with uncomplexed flavonoids. Quercetin-Co^{2+} complex (2:1) was found to be more effective than uncomplexed quercetin in scavenging of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH*) (Bukhari, Memona, Tahir, & Bhanger, 2008). However, quercetin-Sn^{2+} complex showed lower ferric reducing antioxidant power (FRAP) values and lower ABTS*-scavenging and DPPH*-scavenging activity than uncomplexed quercetin (Dehghan & Khoshkam, 2012). Similarly, the cadmium complex of quercetin was less effective in scavenging ABTS* and DPPH* than quercetin (Ravichandran, Rajendran, & Devapiriam, 2014). In a non-biological system, the reductive activity of green tea polyphenols for graphene oxide was increased by the presence of iron (Akhavan, Kalaee, Alavi, Ghiasi, & Esfandiar, 2012).

Our results show generally that flavanol-Fe^{2+} show antioxidant activity such as ABTS* scavenging, protection against fluorescein bleaching induced by AAPH and NaOCl and NaOCl-induced hemolysis, and protection against lipid peroxidation comparable to those of flavanols alone. It can be concluded that binding low amounts of Fe^{2+} does not significantly decrease, and in some cases even enhances the antioxidant activity of flavanols. Equimolar EGC-G-Fe^{2+} mixture shows significantly lower antioxidant activity than pure EGC-G and 4:1 EGC-G-Fe^{2+} mixture, as it is seen in decreased ABTS* scavenging, decreased protection against fluorescein bleaching induced by AAPH and NaOCl, and decreased protection of erythrocytes against NaOCl-induced hemolysis. Therefore, flavanols can be expected to act as efficient antioxidants in the gastrointestinal tract in spite of contact with ferrous irons released from the food.

We checked the Fe^{2+} mixtures of flavanols for superoxide dismutase-like activity. Such an activity of flavonoid-metal complexes has been reported. Moridani, Pourahmad, Bui, Siraki and O'Brien (2003) demonstrated superoxide scavenging in a system of hypoxanthine-xanthine oxidase-NBT by 2:1 complexes of flavonoids (including catechin) with Fe^{2+}, Fe^{3+} or Cu^{2+}. This activity was the highest for Cu^{2+}complexes and the lowest, but still discernible for Fe^{2+} complexes. Kostyuk et al. (2004) found that activities of metal complexes of flavonoids, including EC, inhibited NBT reduction by photoactivated riboflavin and IC_{50} values for inhibition of this photoreduction were lower for Cu^{2+}, Fe^{2+} and Fe^{3+} 1:1 complexes than for uncomplexed flavonoids. For EC, the IC_{50} values for inhibition of this reaction were similar for all metal complexes. A postulated mechanism for the pseudo-superoxide dismutase activity of flavanol-iron complexes is bases on a reversible reduction and oxidation of iron bound to hydroxyl groups in the positions 4′ and 5′ of the B ring. This activity was suggested as a reason for the protective effect of the complexes on hepatocytes against hypoxia:reoxygenation injury (Zhao, Khan, & O'Brien, 1998).

We were unable to demonstrate the superoxide dismutase-like activity of iron (and in some systems copper) mixtures of flavanols. Flavanol mixtures did not inhibit (Fe^{2+} mixtures) or accelerated (Fe^{3+} mixtures) pyrogallol and adrenalin autoxidation and were less rather than more effective than flavanols alone in the inhibition of photochemical NBT reduction. In the case of PMS-mediated NBT reduction, Fe^{2+} mixtures of C and EC were in some cases significantly more effective in the inhibition of the reaction than C and EC alone, but the situation was changeable, mainly opposite for Fe^{2+} mixtures of other flavanols. It should be mentioned that all the assays of superoxide dismutase activity employed are indirect and based on complex reactions generally catalyzed by metal ions (Marklund & Marklund, 1974; Misra & Fridovich, 1972; Beauchamp & Fridovich, 1971; Ewing & Janero, 1995) and metal ions chelators are usually included in the assay media. We did not use chelators to avoid metal ion removal from the flavanol mixtures. In such mixture systems, antioxidants may not only scavenge superoxide, but also interfere with reactions leading to superoxide formation, which makes the systems less predictable and complicates interpretation of results. We checked also if Fe^{2+} mixtures acquire catalase activity, but the results obtained do not confirm such a possibility.

It should be remembered that reactions of flavonoids with iron ions are complex and may involve both reduction of Fe^{3+}, as well as oxidation of Fe^{2+} (Yoshino & Murakami, 1998). Therefore, Fe^{3+} ions can appear in flavanol-Fe^{2+} mixtures, especially during prolonged incubation, and may express prooxidant properties, as revealed in this study in the case of pyrogallol autoxidation.

The relevance of this *in vitro* study with respect to the *in vivo* situation may seem limited since, during the absorption in the jejunum, most of flavanols is subject to methylation and *O*-methylglucuronidation (Kuhnle et al., 2000; Del Rio et al., 2013). Nevertheless, interaction of iron with flavanols in the gastrointestinal tract may affect their bioavailability and susceptibility to the action of glucuronyl-transferases, methyl transferases and other metabolizing enzymes. This subject deserves further studies.

In summary, our results demonstrate that flavanol-Fe^{2+} mixtures retain most of antioxidant activity of the parent flavanols so, even after complexing subsaturating amounts of iron, flavanols are still potent antioxidants. However, no gain of antioxidant function of the mixtures was detected.

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Conflicts of interest

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.06.076.

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