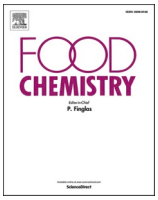
Food Chemistry 462 (2025) 141014

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Different behavior of food-related benzoic acids toward iron and copper

Patrícia Harčárová [a], Zuzana Lomozová [a], Maria Kallivretaki [b,1], Jana Karlíčková [a], Radim Kučera [c], Přemysl Mladěnka [d,*]

[a] The Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmacy in Hradec Králové, Charles University, Akademika Heyrovského 1203, 500 03, Hradec Králové, Czech Republic
[b] The Department of Chemistry, School of Sciences and Engineering, The University of Crete, University Campus-Voutes, 70013 Heraklion, Crete, Greece
[c] The Department of Pharmaceutical Chemistry and Pharmaceutical Analysis, Faculty of Pharmacy in Hradec Králové, Charles University, Akademika Heyrovského 1203, 500 03, Hradec Králové, Czech Republic
[d] The Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Králové, Charles University, Akademika Heyrovského 1203, 500 03, Hradec Králové, Czech Republic

ARTICLE INFO

Keywords:
Chelation
Reduction
Fenton reaction
Hydroxyl radical
Pro-oxidant
Antioxidant
Hemolysis

ABSTRACT

Benzoic acids, which are commonly found in food, are also produced by human microbiota from other dietary phenolics. The aim was to investigate the interactions of 8 food-related benzoic acids with the physiological metals iron and copper under different (patho)physiologically relevant pH conditions in terms of chelation, reduction, impact on the metal-based Fenton chemistry, and copper-based hemolysis. Only 3,4-dihydroxybenzoic acid behaved as a protective substance under all conditions. It chelated iron, reduced both iron and copper, and protected against the iron and copper-based Fenton reaction. Conversely, 2,4,6-trihydroxybenzoic acid did not chelate iron and copper, reduced both metals, potentiated the Fenton reaction, and worsened copper-based hemolysis of rat red blood cells. The other tested compounds showed variable effects on the Fenton reaction. Interestingly, prooxidative benzoic acids mildly protected human erythrocytes against Cu-induced lysis. In conclusion, 3,4-dihydroxybenzoic acid seems to have a protective effect against copper and iron-based toxicity under different conditions.

1. Introduction

Benzoic acid is the simplest aromatic carboxylic acid. Its close derivatives, hydroxybenzoic acids, are the most prevalent and extensively investigated bioactive compounds belonging to plant secondary metabolites (Marchiosi et al., 2020). These simple phenolic acids are present in practically all foods of plant origin and their daily intake is around 200 mg, depending on dietary habits (Silva, Sganzerla, John, & Marchiosi, 2023). There are multiple food and also other relevant plant sources of 4-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid (known also as protocatechuic acid) – for greater detail, see **Table S1**. In addition to occurring as plant secondary metabolites, benzoic acids, together with phenylacetic and phenylpropionic acid derivatives, are the main metabolites of polyphenols produced in the human colon by the microbiota (Murota, Nakamura, & Uehara, 2018; Rupasinghe, 2020; Zhang et al., 2023). For example, the consumption of chocolate, which is rich in polyphenols, increases the urinary excretion of 3-hydroxybenzoic acid in healthy humans (Rios et al., 2003). Similarly, a diet supplemented with red wine polyphenol extract was associated with the urinary excretion of 3-hydroxybenzoic, 4-hydroxybenzoic, and hippuric acids in rats (Gonthier et al., 2003). In addition to the above mentioned, 2,4,6-trihydroxybenzoic acid and 4-methoxysalicylic acid were also identified as colonic metabolites of flavan-3-ols (Sánchez-Patán, Monagas, Moreno-Arribas, & Bartolomé, 2011; Serra et al., 2011). Benzoic acid and its derivatives can also occur in animal tissues and fermented products through microbial activity (del Olmo, Calzada, & Nuñez, 2017).

Moreover, benzoic acid is an important preservative with

Abbreviations: •OH, hydroxyl radical; 2,3-DHBA, 2,3-dihydroxybenzoic acid; 2,5-DHBA, 2,5-dihydroxybenzoic acid; BCS, disodium bathocuproine disulphonate; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; HA, hydroxylamine hydrochloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ROS, reactive oxygen species; TRIS, tris(hydroxymethyl)aminomethane; DTT, DL-dithiothreitol; NAD^+, nicotinamide adenine dinucleotide.

* Corresponding author.
E-mail address: mladenka@faf.cuni.cz (P. Mladěnka).

[1] ERASMUS student at Faculty of Pharmacy, Charles University

https://doi.org/10.1016/j.foodchem.2024.141014
Received 3 July 2024; Received in revised form 20 August 2024; Accepted 25 August 2024
Available online 26 August 2024

antimicrobial activity, which is industrially synthesized and added not only to food, but also to cosmetics, hygiene, and pharmaceutical products (del Olmo et al., 2017; Synowiec, Żyła, Gniewosz, & Kieliszek, 2021; Yun et al., 2019). In fact, it is one of the oldest known and commonly used preservatives (E210 according to EU Regulation on Labelling of Foodstuffs). Its sodium salt (E211) was the first preservative approved by the FDA for use in food products (Lempart-Rapacewicz et al., 2023; Rabiu et al., 2021).

Notwithstanding the fact that many papers have reported the antioxidant effect of hydroxybenzoic acids (Alcalde, Granados, & Saurina, 2019; Biela, Kleinová, & Klein, 2022; Kalinowska et al., 2021; Liu, Du, Beaman, & Monroe, 2020; Moazzen, Öztinen, Ak-Sakalli, & Koşar, 2022; Spiegel et al., 2020), the data on metal interactions are much more limited (Andjelković et al., 2006; Zhou, Yin, & Yu, 2006). Even though copper and iron are essential elements of the human body, these transition metals can participate under some conditions in the Fenton reaction and hence generate the hydroxyl radical, one of the most powerful oxidizing free radicals, which can severely damage biomolecules (Lomozová et al., 2022; Zhao, 2023).

Our hypothesis is that food-related benzoic acids can diminish the iron and copper-based production of the hydroxyl radical and hence subsequently attenuate the consequences of copper-induced hemolysis without chelating these metals. This suggestion is based on a large body of evidence coming from *in vitro* assays showing reactive oxygen species (ROS) scavenging effects of these substances. Even if these compounds can reduce metal ions to a lower oxidation state, which can be associated with promoting the Fenton reaction, we assume that these compounds might block that reaction at the level of hydrogen peroxide or hydroxyl radical by direct scavenging as the prevailing mechanism.

For this reason, the aim of the study was to assess the interaction of 8 structurally derived food-related benzoic acids (**Fig. S1**) with transition metals iron and copper in greater detail at different (patho)physiologically relevant pH levels, from an acidic pH of 4.5 to a neutral pH. As far as we know, no such investigation has ever been carried out. The methods employed to test iron and copper chelation and reduction, and the impact on the iron and copper-based Fenton reaction and copper triggered hemolysis can answer the question if our hypothesis was correct.

2. Materials and methods

2.1. Reagents, solutions, and equipment

The tested compounds (benzoic acid, 3-hydroxybenzoic acid */m*-hydroxybenzoic acid/, 4-hydroxybenzoic acid */p*-hydroxybenzoic acid/, 2,4-dihydroxybenzoic acid, 4-methoxysalicylic acid, 3,4-dihydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid and hippuric acid), ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), cupric sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), cuprous chloride (CuCl), hematoxylin, disodium bathocuproine disulfonate (BCS), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (ferrozine), hydroxylamine hydrochloride (HA), salicylic acid, EDTA disodium salt (Na_2EDTA), hydrogen peroxide (H_2O_2, 30 %), orthophosphoric acid (H_3PO_4, 85 %), catechol, 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA), potassium phosphate monobasic (KH_2PO_4), DL-dithiothreitol (DTT), polyethylene glycol *tert*-octylphenyl ether (Triton X; 10 % solution), sodium lactate, sodium acetate, acetic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), HEPES sodium salt, tris(hydroxymethyl)aminomethane hydrochloride (TRIS hydrochloride), and triethylamine were purchased from Sigma-Aldrich (Munich, Germany). Acetonitrile, methanol, and dimethylsulfoxide (DMSO) were purchased from Thermo Fisher Scientific (Pardubice, Czech Republic), potassium phosphate dibasic (K_2HPO_4), hydrochloric acid (HCl, 35 %), and sodium chloride (NaCl) from Penta (Prague, Czech Republic), nicotinamide adenine dinucleotide (NAD^+) from Toronto Research Chemicals (Toronto, Ontario, Canada), saline from B. Braun (Prague, Czech Republic), and heparin from Zentiva (Prague, Czech Republic). Ultrapure water (Milli-Q RG, Merck Millipore, Burlington, MA, USA) was used throughout this study.

Stock solutions of Cu/Fe salts, ferrozine, BCS (all 5 mM), and HA (100 mM) were prepared in ultrapure water with the exception of CuCl (5 mM), which was dissolved in an aqueous solution of 0.1 M HCl and 1 M NaCl. For red blood cell hemolysis experiments, $CuSO_4 \cdot 5H_2O$ was dissolved in saline at a concentration of 10 mM. Hematoxylin was dissolved in DMSO, and its working solution (0.25 mM) was used within 90 min. The tested compounds were dissolved in DMSO for assessing chelation and red blood cells hemolysis testing, whereas in methanol when employing the high-performance liquid chromatography method.

Chelation experiments and red blood cells lysis *via* measuring lactate dehydrogenase activity were performed in 96-well microplates (BRAND GmbH&Co. KG, Wertheim, Germany). For chelation experiments, the Synergy HT Multi-Detection Microplate Reader spectrophotometer (BioTec Instruments, Inc., Winooski, VT, USA) was used. Lactate dehydrogenase activity was measured by the Hidex Sense Beta Plus Microplate Reader (Hidex, Turku, Finland). For performing the Fenton chemistry analysis, a chromatographic system consisting of the ESA Model 582 Solvent Delivery Module and the ESA coulometric Coulochem III detector was used (both ESA, Chelmsford, MA, USA), equipped with a high-sensitivity analytical cell with two porous graphite working electrodes placed in series in the same cell. The Onyx Monolithic RP C8 column 100 × 4.6 mm (Phenomenex, Torrance, CA, USA) was employed as the stationary phase. The chromatographic system and data integration were performed utilizing the Clarity chromatography software (DataApex, Prague, Czech Republic) (version 9.0.0.82).

2.2. pH conditions

Experiments were performed at four (patho)physiologically relevant pH values (4.5, 5.5, 6.8 and 7.5). Acetate buffers (15 mM of sodium acetate with 27.3 and 2.7 mM of acetic acid, respectively) were used for the two lower pH values, whereas HEPES buffers (15 mM of sodium HEPES with 71.7 and 14.3 mM of HEPES, respectively) were used for pH 6.8 and 7.5. TRIS buffer (5 mM) was used for pH 7.5 instead of the HEPES buffer in the Fenton chemistry experiments. For erythrocyte hemolysis testing, a phosphate buffer was prepared as a mixture of KH_2PO_4 and K_2HPO_4 (2.72 g/100 mL KH_2PO_4 + 3.48 g/100 mL K_2HPO_4) at a pH of 7.8, and a 0.1 M solution of TRIS was used to prepare a buffer at a pH of 8.9.

2.3. Iron and copper chelation and reduction assessment

The underlying principle of these competitive methodologies relies on the fact that indicators (such as ferrozine, BCS, and hematoxylin) function as metal chelators and compete with the tested compounds for binding of the metal. These methods (Carpenter & Ward, 2017; Macáková et al., 2019; Peixoto & Tóth, 2013) hence compare the affinity of the tested compounds for the metal and the stability of their complexes over time due to competition with the indicator. Initially, the tested compounds were mixed with the metal ions to allow for a complex formation, followed by the addition of an indicator to the mixture. To evaluate stability under both immediate and delayed conditions, absorbance was assessed promptly and at predetermined intervals, depending on the method used. An unstable complex loses its metal ion due to the formation of the indicator-metal complex over time, and hence the absorbance is significantly higher when compared to immediate measurement. Additionally, these methods serve for making a reduction assessment, as both BCS and ferrozine exclusively react with ions in their lower valence state (Cu^+, Fe^{2+}).

In all these methods, at least 2 independent stock solutions for each compound were employed. Every measured point (concentration) was the average of at least 2 measurements (in case of a difference exceeding

10 %, an additional 2 measurements with the same concentration diluted from a new stock solution were always carried out). In all compounds, even inactive ones, at least 4 different concentrations were tested. Chelation curves in active compounds were constructed from a minimum of 5 different concentration points.

2.3.1. The ferrozine method

Ferrozine is a specific reagent that forms a magenta-colored complex with Fe^{2+}. This approach is also applicable for evaluating the complete iron ($Fe^{2/3+}$) chelation after the reduction of ferric ions by HA. Different concentrations of benzoic acids dissolved in DMSO were mixed with ferrous or ferric ions in acetate (pH 4.5 and 5.5) or HEPES buffers (pH 6.8 and 7.5) for 2 min. At pH 7.5, HA was introduced to prevent ferrous oxidation. For assessing total iron at pH 4.5, an HA aqueous solution was added after mixing the ferric ions with the tested benzoic acids in order to reduce the remaining Fe^{3+} ions into Fe^{2+} ions. For determining the degree of ferric ion reduction, no HA was added. Subsequently, ferrozine was pipetted, and the absorbance of the resulting purple complex was measured promptly and again after 5 min at λ 562 nm. HA served as the positive control, representing a 100 % reduction (Mladenka et al., 2010).

2.3.2. The hematoxylin method

Hematoxylin forms complexes with cupric ions. DMSO solutions of benzoic acids at different concentrations were mixed with Cu^{2+} ions for 2 min in the presence of a buffer. The mixture was incubated for the next 3 min after the addition of hematoxylin to enable the slow reaction of unchelated copper ions with the indicator. Subsequently, absorbance was measured immediately and then again after an additional 4 min. Different wavelengths were used according to pH: λ 595 nm (pH 5.5), λ 590 nm (pH 6.8), and λ 610 nm (pH 7.5), as reported in our previous paper (Riha et al., 2013). At pH 4.5, hematoxylin has a low affinity to cupric ions, and therefore we did not include testing of the chelation at this pH.

2.3.3. The BCS method

The BCS method is analogous to the ferrozine method, but BCS is specific for cuprous ions. The methodological procedures for both reduction and chelation were nearly identical, with the exception of substituting ferrozine with BCS and incorporating HA (50 μL, 1 mM at pH 6.8/7.5 or 10 mM at pH 4.5/5.5) in all cuprous measurements to maintain copper ions in their reduced state. Measurements were taken at λ 484 nm immediately and again after 5 min. A more detailed methodology can be found in our former study (Riha et al., 2013). In reduction experiments, HA was again used as the positive control.

2.4. The influence on hydroxyl radical generation through the Fenton reaction (HPLC method)

This HPLC method with coulometric detection was used to evaluate the effect of the tested benzoic acids on iron and copper-based Fenton reactions. The method is based on the detection and quantitation of three products of hydroxyl radical ($^{\bullet}OH$) mediated salicylate hydroxylation, namely catechol, 2,3-DHBA, and 2,5-DHBA. The presence of a substance possessing antioxidant and/or chelating properties in the reaction mixture influences the generation of •OH, subsequently leading to alterations in the concentrations of the three measured products. A reduction in catechol, 2,3-DHBA, and 2,5-DHBA production signifies a proportional decrease in •OH generation, indicating the antioxidant potential. Conversely, an elevation in the production of these three analytes suggests increased •OH generation and thus was interpreted in this study as a pro-oxidant effect. The detailed methodology was described in our previous paper (Catapano et al., 2019). In the analytical cell, potential 1 was set to −200 mV and potential 2 to +450 mV, with a range of 200 nA and a + 1.00 V output. The mobile phase was a mixture of an H_3PO_4 buffer (5 mM, pH 2.85) with 1 mM EDTA and acetonitrile (95/5, *v*/v). HPLC analysis was carried out in isocratic mode at a flow rate of 1.0 mL/min and used an injection volume of 20 μL.

Sample preparation - at first, 700 μL of acetate (pH 4.5) or TRIS hydrochloride (pH 7.5) buffer was mixed with 200 μL of methanol (blank) or various concentrations of benzoic acid solutions in methanol. Then, 50 μL of metal ions (Fe^{2+}, Fe^{3+} 1 mM, and Cu^{2+} 40 μM) was added so the molar concentration ratios ranged from 200:1 to 1:20 or 200:1 to 1:100 (metal:benzoic acid). After mixing, 45 μL of salicylic acid (66,67 mM) was added. In the last step, the Fenton reaction was triggered by adding 5 μL of 30 % H_2O_2. The reaction was carried out for 2 min at room temperature. Each investigated ratio was tested using 3 independent stock solutions of the tested compound.

2.5. Red blood cell hemolysis

This assay was performed according to the previous study (Mladenka et al., 2020). Blood samples were obtained either from adult male rats (Wistar Han, Velaz, s.r.o., Prague, Czech Republic) by exsanguination or healthy volunteers (both genders) by venipuncture into plastic tubes containing heparin sodium (170 IU/10 mL; Zentiva, Prague, Czech Republic). The rat exsanguination was performed by a trained researcher in accordance with The Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th edition, revised 2011, ISBN-13: 978–0–309-15,400-0). The blood was used as a by-product from male rats after isolation of the aorta, aimed at testing the vasodilatory effect (approval by the Czech Ministry of the Health No. MSMT-34121_2017–2). The study on healthy volunteers was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Faculty of Pharmacy, Charles University from 31 May 2019. Informed consent was obtained from all healthy volunteers included in this study.

An erythrocyte suspension was incubated with various concentrations of benzoic acids dissolved in DMSO (the final concentration of DMSO was 1 %) and cupric sulfate dissolved in saline at a final concentration of 500 μM for 4 h at 37 °C. The sample was then centrifuged at 1950 ×*g* for 10 min, and 250 μL of the supernatant obtained was used to determine the lactate dehydrogenase activity, a marker of cellular lysis. The remaining supernatant was discarded and replaced with the same amount of lysis buffer (2 mM EDTA, 1 mM DTT, 1 % Triton-X 100, 0.1 M phosphate buffer of pH 7.8). Each sample was thoroughly vortexed and left at room temperature for 20 min to achieve complete lysis of the remaining erythrocytes. Afterward, samples were centrifuged at 6700 ×*g* for 10 min, and 250 μL of supernatant was used to determine lactate dehydrogenase activity.

Lactate dehydrogenase activity was kinetically quantified using the increase in absorbance caused by the conversion of β-NAD^+ to β-NADH, employing a protocol adapted from Chan et al. (Chan, Moriwaki, & De Rosa, 2013). The results were calculated as the percentage of erythrocytes lysed and compared to the positive control, where the solvent DMSO was used instead of the tested compound. The negative control was not incubated with the metal but otherwise treated in the same way as the other samples.

Each tested ratio (benzoic acid:copper) was tested at least 4 times using at least 2 independent stock solutions of the tested compound. Three independent blood donors were always used for each active compound.

2.6. Mathematical calculation and statistical assessment

Data are expressed as means ± SD. All statistical analyses were performed using the GraphPad Prism software version 10.1.2 for Windows (GraphPad Software, USA). For making a comparison of the effects of different concentrations, ANOVA with the *post hoc* Dunnett test was used, while the differences in chelation and reduction activities were analyzed using 95 % confidence intervals of chelation curves and reduction lines.

3. Results

In the first step, all benzoic acids were screened for their metal chelating and reducing ability under different pH conditions using competitive methods. From all 8 substances tested, only 3,4-dihydroxybenzoic acid demonstrated a strong capacity for chelating iron ions (Fig. 1A). Its chelating activity was pH-dependent: as the pH increased, both the number of chelated ions and the stability of the formed complexes increased (**Fig. S2**). 2,4-Dihydroxybenzoic acid and 4-methoxysalicylic acid exhibited a limited ability to chelate iron ions, while the remaining benzoic acids displayed no activity across all tested concentrations and pH values (**Fig. S3**).

Comparable outcomes were observed for the iron-reducing activity. Among all compounds examined, only 3,4-dihydroxybenzoic acid demonstrated the capability to reduce ferric ions (Fig. 1B**; Fig. S4**). This ability was evident at pH 4.5 and 5.5, but not present at the higher pH values of 6.8 and 7.5.

The screening of copper chelation activity with the hematoxylin method suggested that all compounds were able to chelate cupric ions at pH 5.5 and 6.8, while only four of the tested benzoic acids exhibited this activity at a pH 7.5 (**Fig. S5**). 3,4-dihydroxybenzoic acid again demonstrated the highest copper-chelating activity. At pH 7.5, this substance chelated approximately 25 % of the cuprous ions at a concentration ratio of 1:1 (3,4-dihydroxybenzoic acid:Cu^{2+}). However, under more competitive conditions using BCS as an indicator, the chelation activity of all tested benzoic acids toward both cupric and cuprous ions was negligible with the exception of 2,4-dihydroxybenzoic acid and cupric ions (**Fig. S6 and S7**), generally suggesting a low affinity of the tested benzoic acids for copper.

In the next step, copper reduction was investigated. The reduction curves differed among the substances (Fig. 2 **and S8**). Four types of behavior are possible, and all were observed. The reduction scenarios were: a) gradual and rising, b) bell-shaped, *i.e.* after a rise, a decrease was observed, c) no effect, or d) attenuating, *i.e.* spontaneous reduction was contrarily blocked. Two benzoic acid derivatives (3,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid) progressively reduced cupric ions with the former being by far more active reaching complete reduction at ratios below 1:1 under all pH conditions. Three acids (3-hydroxybenzoic acid, 4-hydroxybenzoic acid, and 2,4-dihydroxybenzoic acid) demonstrated a bell-shaped behavior at pH values of 6.8 and 7.5. Three compounds (benzoic acid, 4-methoxysalicylic acid, and hippuric acid) had no effect at pH 4.5 and 5.5, however, they demonstrated the ability to mitigate the spontaneous reduction of cupric ions at elevated pH levels.

For a better overview, chelation and reduction data are summarized in Table 1.

It is almost impossible to predict theoretically the effect of a substance on the production of hydroxyl radicals through the metal-induced Fenton reaction when a compound is both reducing and chelating the metal, and can also directly interact with hydrogen peroxide and/or hydroxyl radicals. In principle, metal chelation should reduce hydroxyl radical formation in the Fenton reaction by eliminating the metal catalyst, whereas metal reduction may induce the opposite effect by restoring the catalyst. Similarly, the direct scavenging effect toward hydrogen peroxide or hydroxyl radical will also attenuate the Fenton reaction. Thus, in the next step, this effect was determined experimentally using an HPLC analysis of hydroxyl radical production. Again, four different behaviors were identified: antioxidant, characterized by a gradual decrease in hydroxyl radical production; pro-oxidant, marked by a gradual increase in hydroxyl radical production; bell-shaped, demonstrating an initial pro-oxidant effect followed by a neutral one; and neutral. The Fenton reactions were triggered by both forms of iron, *i.e.* both ferrous and ferric ions and, in the case of copper, by cupric ions.

In the case of the Fenton reaction triggered by Fe^{2+} ions, most of the tested benzoic acids were unable to stop the production of hydroxyl radicals and, on the contrary, intensified the pro-oxidant action (Fig. 3 **and S9**). This pro-oxidant effect, at least at one pH value and one of the tested concentration ratios, was observed for the 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 4-methoxysalicylic acid and 2,4,6-trihydroxybenzoic acid. Only benzoic acid showed a neutral effect on hydroxyl radical production. A purely antioxidant action was exerted by 3,4-dihydroxybenzoic acid. This effect was observed to be significant even at a concentration ratio of 1:20 at an acidic pH level of 4.5, and from a concentration ratio of 1:2 (3,4-dihydroxybenzoic acid:Fe^{2+}) at neutral pH 7.5. Hippuric acid was antioxidant only at neutral pH at the highest concentration tested (*i.e.* at a concentration ratio of 20:1, hippuric acid:Fe^{2+}, respectively). Otherwise, its influence was neutral.

In the context of the Fenton reaction initiated by Fe^{3+} ions, four substances exhibited a neutral response, namely benzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, and hippuric acid (Fig. 3 **and S9**). The representatives featuring two hydroxy groups on the benzene ring (2,4-dihydroxybenzoic acid and 3,4-dihydroxybenzoic acid) exhibited antioxidant effects under neutral pH conditions and had no effects under acidic pH conditions. For the former, the antioxidant effect was significant from a concentration ratio of 1:1, and for the latter dihydroxy-substituted isomer from 2:1 (dihydroxybenzoic acid:Fe^{3+}). On the other hand, 4-methoxysalicylic acid was pro-oxidant at pH 7.5 whereas it was neutral at pH 4.5. Furthermore, 2,4,6-trihydroxybenzoic acid, possessing three free hydroxy groups on the benzene ring, displayed pro-oxidant properties at both pH levels when the concentration ratio was 1:2 and higher (2,4,6-trihydroxybenzoic acid:Fe^{3+}).

Ultimately, the impact of the examined compounds on the Cu^{2+} ions-

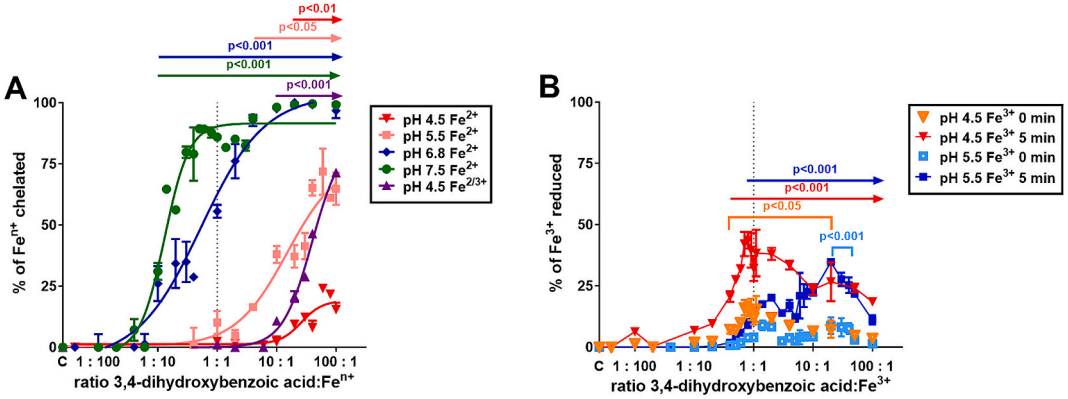


Fig. 1. Iron-chelating and reducing ability of 3,4-dihydroxybenzoic acid tested by the ferrozine method. **A**: Chelation of iron ions. Data represent measurements after 5 min. **B**: Reduction of ferric ions. No reduction was observed at pH 6.8 or 7.5 (data not shown). The length of the arrows or clamps shows the significance *vs.* the control sample (DMSO instead of tested compound, C).

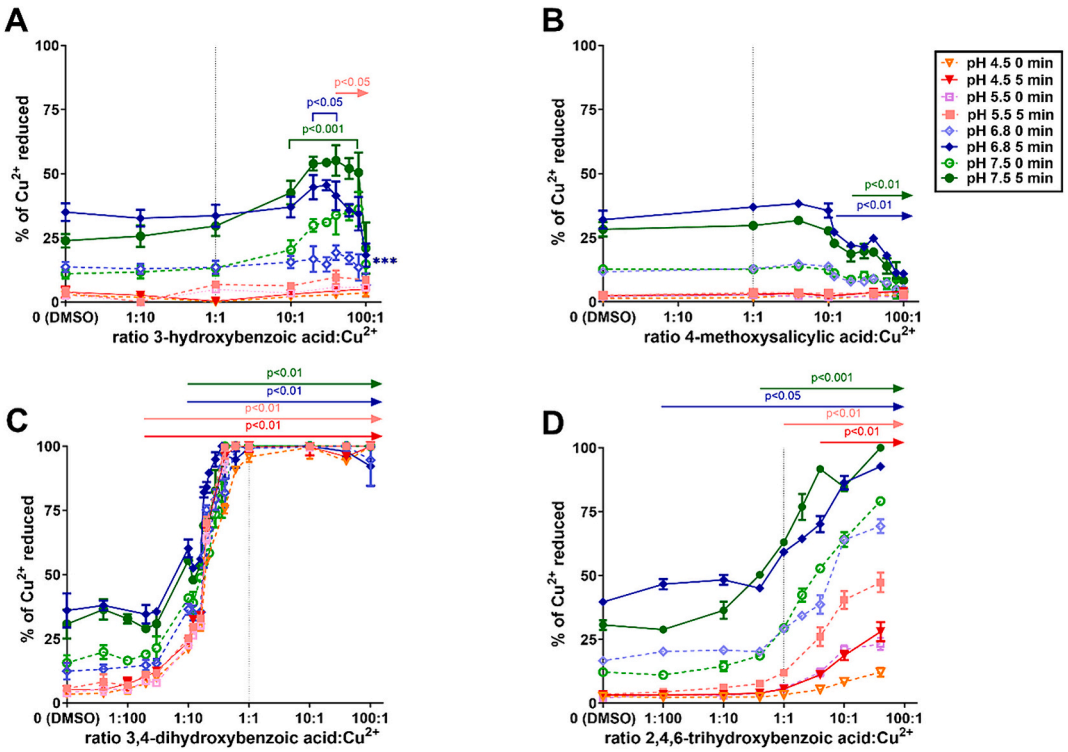


Fig. 2. Representative examples of the curves showing the relationship between the molar concentration ratio (benzoic acid to copper) and the copper reducing potential, measured by the BCS method. **A**: 3-hydroxybenzoic acid, **B**: 4-methoxysalicylic acid, **C**: 3,4-dihydroxybenzoic acid, **D**: 2,4,6-trihydroxybenzoic acid. The data represent means ± SD. Significance *vs.* the control (DMSO) is shown in the figure for the sake of clarity solely for measurements after 5 min. ***$p < 0.001$.

Table 1
Summary of the chelation and reduction effects of the tested derivatives of benzoic acid.

compound	Fe^{2+} chelation				Fe^{3+} ch.	Cu^{2+} chelation (hematoxylin)			Cu^{+}/Cu^{2+} chelation (BCS)	Fe^{3+} reduction				Cu^{2+} reduction			
	pH 4.5	pH 5.5	pH 6.8	pH 7.5	pH 4.5	pH 5.5	pH 6.8	pH 7.5	All pH	pH 4.5	pH 5.5	pH 6.8	pH 7.5	pH 4.5	pH 5.5	pH 6.8	pH 7.5
benzoic acid	×	×	×	×	×	✔✔	✔	×	×	×	×	×	×	×	×	↑↓	↑↓
3-hydroxybenzoic acid	×	×	×	×	×	✔	✔	×	×	×	×	×	×	×	×	↑ˆ	↑↑ˆ
4-hydroxybenzoic acid	×	×	×	×	×	✔✔	✔	×	×	×	×	×	×	×	↑	↑ˆ	↑↑ˆ
2,4-dihydroxybenzoic acid	×	×	✔	×	×	✔✔	✔✔	✔✔	✔[1]×	×	×	×	×	↑	↑	↑ˆ	↑↑ˆ
4-methoxysalicylic acid	×	×	×	✔	✔✔	✔✔	✔✔	✔✔	×	×	×	×	×	×	×	↑↓	↑↓
3,4-dihydroxybenzoic acid	✔	✔✔	✔✔	✔✔	✔✔	✔✔	✔✔	✔✔	×	↑ˆ	↑ˆ	×	×	↑↑	↑↑	↑↑	↑↑
2,4,6-trihydroxybenzoic acid	×	×	×	×	×	✔✔	✔✔	✔✔	×	×	×	×	×	↑	↑	↑↑	↑↑
hippuric acid	×	×	×	×	×	✔✔	✔	×	×	×	×	×	×	×	×	↑↓	↑↓

× – no chelation or reduction; ✔✔ - strong chelation; ✔ weak chelation, *i.e.* at least 20 % chelation at ratios of 10:1 and higher, benzoic acid: metal; ↑↑ strong reduction (at least 50 % at a ratio of 1:1), ↑ weak reduction (significant reduction not fulfilling the criteria for strong reduction); ˆ bell shaped reduction; ↓ - an attenuation of spontaneous reduction.
[1]weak chelation was observed solely at pH 6.8 and 7.5 with cupric ions.
ch.-chelation.

induced Fenton reaction was evaluated under both neutral and acidic pH conditions (Fig. 4 **and S10**). Also in this case, the compounds with two hydroxy groups were able to block the production of hydroxyl radicals and had antioxidant activity. Similarly, 4-hydroxybenzoic acid showed antioxidant activity at both pH values. The last compound with antioxidant activity in this assay was benzoic acid, but this was observed solely at neutral pH as it reduced the production of hydroxyl radicals at concentration ratios from 5:1 to 50:1 (benzoic acid:Cu^{2+}). Two substances, specifically 3-hydroxybenzoic acid and 4-methoxysalicylic acid, exhibited a neutral response at both of the tested pH values. Two substances (2,4,6-trihydroxybenzoic acid and hippuric acid) potentiated the cupric ions-based Fenton reaction under both pH conditions.

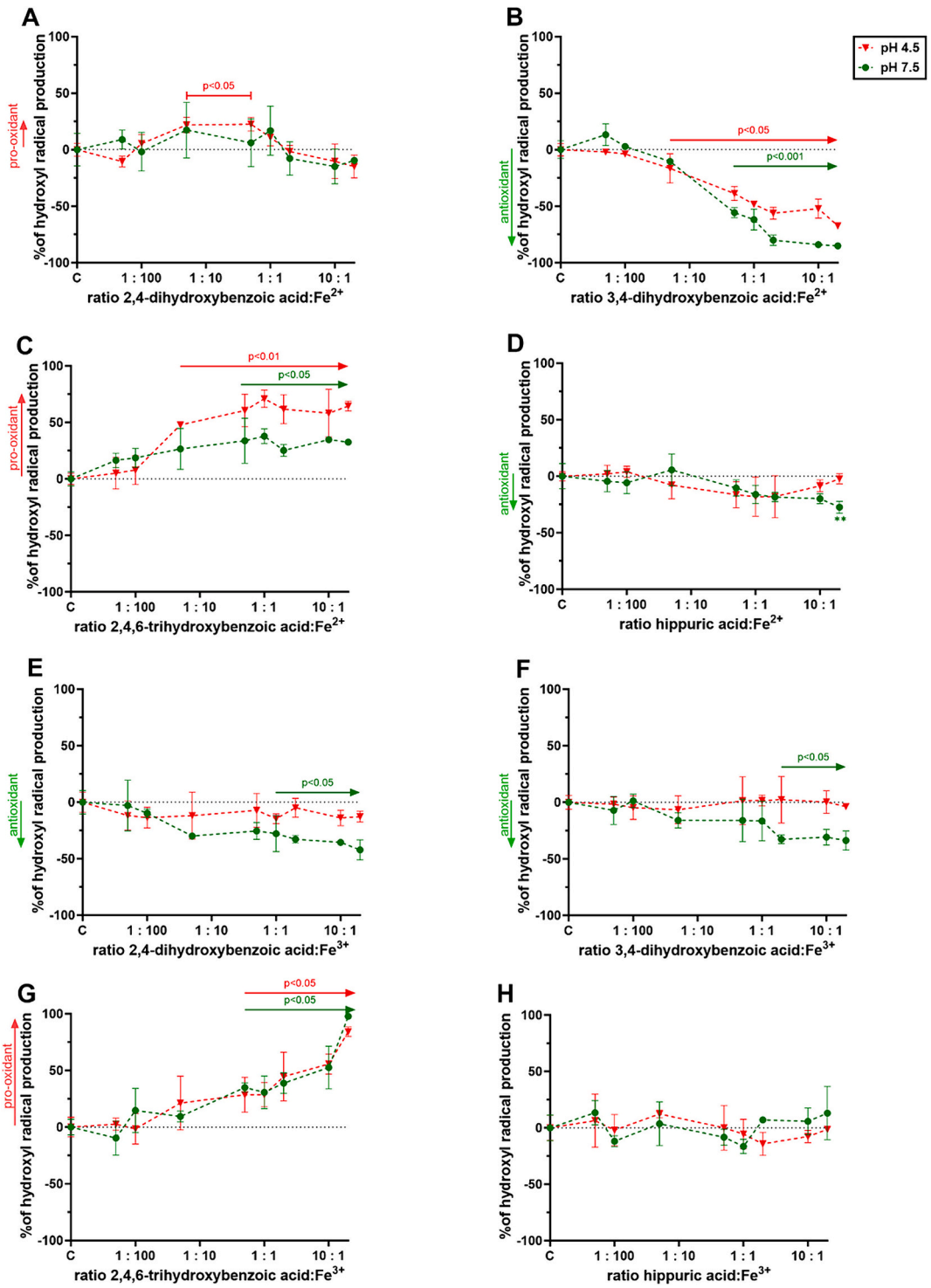


Fig. 3. Examples of the curves showing the level of influence of selected benzoic acids on the ferrous/ferric ions-based Fenton reaction at pH 4.5 and 7.5. **A**: 2,4-dihydroxybenzoic acid and Fe^{2+}, **B**: 3,4-dihydroxybenzoic acid and Fe^{2+}, **C**: 2,4,6-trihydroxybenzoic acid and Fe^{2+}, **D**: hippuric acid and Fe^{2+}, **E**: 2,4-dihydroxybenzoic acid and Fe^{3+}, **F**: 3,4-dihydroxybenzoic acid and Fe^{3+}, **G**: 2,4,6-trihydroxybenzoic acid and Fe^{3+}, **H**: hippuric acid and Fe^{3+}. The data represent means ± SD. Significance *vs.* the control (C: positive blank - the Fenton reaction with the solvent alone) is shown in the figure, for isolated point: $^{**}p < 0.01$.

In the last stage, the impact of selected benzoic acids on copper-induced hemolysis was assessed, as copper, unlike iron, has the ability to cause lysis of erythrocytes at clinically achievable concentrations (Mladenka et al., 2020). Interestingly, almost all the tested substances had a neutral effect on the copper-induced lysis of rat red blood cells (**Fig. S11**). Two compounds, namely 3-hydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid not only failed to protect red blood cells against copper toxicity but also notably worsened the ensuing hemolysis (Fig. 5A, B). The mentioned monohydroxy substituted benzoic acid significantly worsened the impairment only at a concentration ratio of 1:1, whereas the derivative with three hydroxy groups had a concentration-dependent effect. For this reason, these two substances were also tested with human red blood cells. In the case of human erythrocytes, both compounds exhibited a surprisingly mild protective

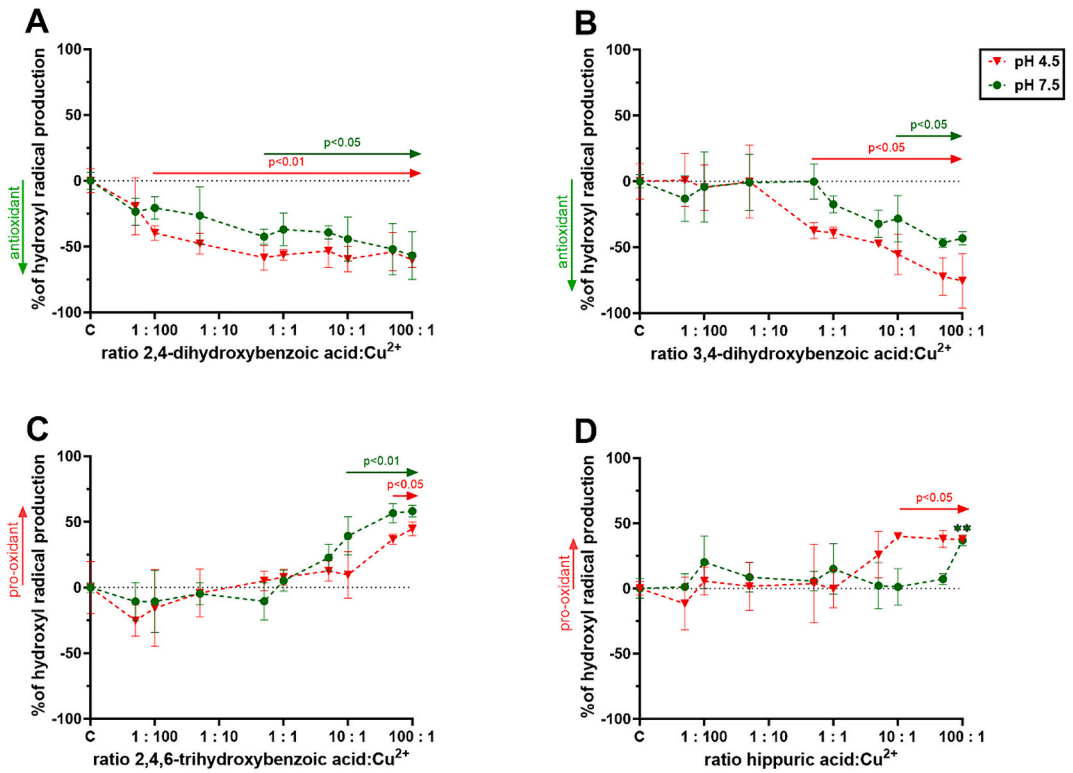


Fig. 4. Examples of the curves showing the level of influence of selected benzoic acids on the cupric ions-based Fenton reaction at pH 4.5 and 7.5. **A**: 2,4-dihydroxybenzoic acid, **B**: 3,4-dihydroxybenzoic acid **C**: 2,4,6-trihydroxybenzoic acid, **D**: hippuric acid. The data represent means ± SD. Significance *vs.* the control (C: positive blank - the Fenton reaction with the solvent alone) is shown in the figure, for the isolated point: $^{**}p < 0.01$.

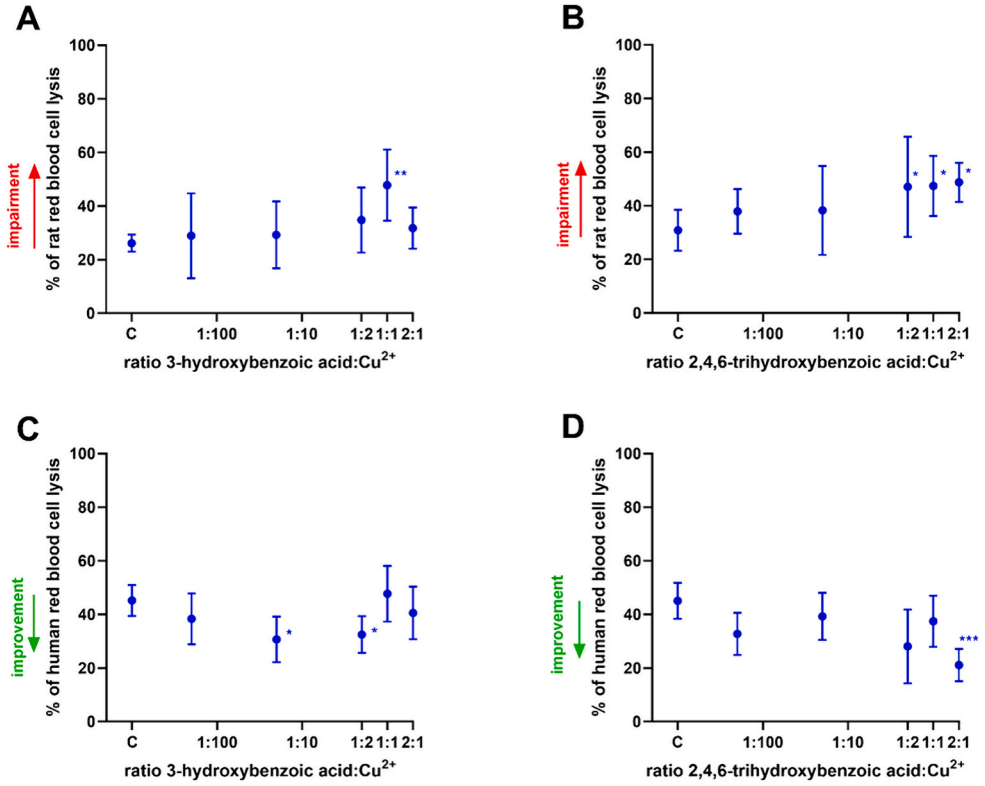


Fig. 5. The effect of 3-hydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid on copper-triggered hemolysis. **A**: 3-hydroxybenzoic acid with rat erythrocytes, **B**: 2,4,6-trihydroxybenzoic acid with rat erythrocytes **C**: 3-hydroxybenzoic acid with human erythrocytes, **D**: 2,4,6-trihydroxybenzoic acid with human erythrocytes. The data represent means ± SD. Significance *vs.* the control (C: copper and solvent) is shown in the figure, for isolated points: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

effect, shielding red blood cells against damage caused by copper (Fig. 5C, D).

The summary of all results is shown in Table 2.

For making an easier comparison with other similarly oriented studies, the effects of benzoic acids were compared with that of the flavonol quercetin. Briefly, quercetin was a more potent iron chelator at pH 4.5 and 5.5 than 3,4-dihydroxybenzoic acid, but their effects were comparable from the statistical point of view under slightly acidic and neutral conditions (**Fig. S12**). Quercetin was a more potent ferric reducing agent than 3,4-dihydroxybenzoic acid at pH 4.5, while the situation was precisely the opposite at pH 5.5 (**Fig. S13**). Quercetin was definitely a more potent copper chelator, but its cupric reducing effect was comparable to that of 3,4-dihydroxybenzoic acid (**Fig. S14–15**). However, in relation to the copper-driven Fenton chemistry, quercetin pro-oxidative effects reproduced that of 2,4,6-trihydroxybenzoic acid (**Fig. S16**), but quercetin was clearly protective toward Cu-driven rat erythrocyte lysis in contrast to all of the tested benzoic acids here (**Fig. S17**).

4. Discussion

4.1. The interaction of benzoic acids with copper and iron

Physiologically, iron and copper are tightly regulated by complexation with different storage, transport, and other proteins. However, under several pathological conditions, they can be released from these bonds and participate in ROS generation, including the Fenton reaction. As both the chelation and reduction of metals can influence this process, both interactions need to be studied. Based on previous studies with other structurally similar compounds and limited chelation data on benzoic acids (Friggeri et al., 2015; Pan et al., 2022; Shubina, Kozina, & Shatalin, 2021), we hypothesized that their chelation activity is low with the possible exception of benzoic acids with catechol moiety at neutral or slightly acidic pH. Indeed, experimental data confirmed that compounds without a hydroxyl group or with one isolated hydroxyl group (benzoic acid, 3- and 4-hydroxybenzoic acids, hippuric acid) did not chelate either iron or copper under highly competitive conditions. The only potent iron chelator was 3,4-dihydroxybenzoic acid, which possesses the mentioned catechol moiety. Its effects were clearly more expressed under neutral or slightly acidic conditions. This is likely the result of the deprotonation of hydroxyl group(s) under neutral conditions in contrast to acidic pH. The copper chelating effect of this dihydroxyderivative was observed solely under mildly competitive conditions (hematoxylin) but not in a more competitive assay (BCS). This agrees with a previous non-competitive study, where 3,4-dihydroxybenzoic acid derivatives formed metal complexes with Fe^{3+}, Fe^{2+}, and Cu^{2+} under non-competitive conditions (Friggeri et al., 2015). In our set of compounds, three compounds have an *o*-hydroxy group in the position to a carboxylic group (2,4-dihydroxybenzoic acid, 4-methoxysalicylic acid, and 2,4,6-trihydroxybenzoic acid). Some limited chelation effects of the former two compounds were observed, suggesting that *ortho* carboxyl-hydroxy moiety is also able to form some metal complexes but has a lower affinity to metals than *o*-dihydroxy moiety. Interestingly, the presence of an additional hydroxy group in position 6 apparently even decreased the chelation potential, as 2,4,6-trihydroxybenzoic acid was not able to chelate either iron or copper under more competitive conditions.

Contrarily, it was hypothesized that all hydroxybenzoic acids would be able to reduce metals, as reducing properties are often, albeit incorrectly, considered synonymous with antioxidant effects. There are previous studies that showed that some benzoic acid derivatives reduce ferric ions at highly acidic pH levels (the FRAP method) and the data mostly correlated with voltametric measurements (Alcalde et al., 2019; Spiegel et al., 2020). These results reported 3,4-dihydroxybenzoic acid as clearly the most potent. 2,4,6-Trihydroxybenzoic acid demonstrated a low level of activity, whereas in the case of 3- and 4-hydroxybenzoic acid, very low or no activity was reported. 4-hydroxybenzoic acid was slightly more potent than 3-hydroxybenzoic acid (Spiegel et al., 2020), and this was also observed in our study with cupric reduction (Fig. 2 and Fig. S8). The fact that 3,4-dihydroxybenzoic acid proved to be the most effective among the compounds examined highlights the significance of the catechol moiety not just in chelation but also in metal reduction. The second most effective substance showing a progressive ability to reduce Cu^{2+} ions as a result of increasing concentration was 2,4,6-trihydroxybenzoic acid. These findings suggest that the reducing activity of benzoic acids could be related to the number of free hydroxyl groups in their structure. However, apparently also the localization of these hydroxyl groups is important as can be documented by the CUPRAC assay, which is based on cupric to cuprous ion reduction and hence principally similar to the mentioned FRAP assay (Kalinowska et al., 2021). The impact of hydroxyl groups was already observed for flavonoids, where some studies have shown that the reducing activity of copper seems to depend largely on the number of hydroxyl groups in the molecule (Mira et al., 2002). At the same time, this conclusion is supported by the fact that benzoic acids bearing at least one free hydroxyl group were able to exhibit at least a small reducing effect. The exception is 4-methoxysalicylic acid, where the presence of a free hydroxyl group on the benzene ring might be mitigated by the presence of a methoxy group. Contrarily, benzoic and hippuric acids, having no free hydroxyl group on the aromatic ring, decreased the reduction. This finding is of particular

Table 2
Summary of the effects of tested derivatives of benzoic acid.

compound	chelation of $Fe^{2+/3+}$	reduction of Fe^{3+}	chelation of $Cu^{+/2+}$ (BCS)	reduction of Cu^{2+}	Fe^{2+} triggered Fenton reaction	Fe^{3+} triggered Fenton reaction	Cu^{2+} triggered Fenton reaction	Cu^{2+} induced rat red blood cells lysis
benzoic acid	×	**neutral**	×	**decrease**	**neutral**	**neutral**	**antioxidant**	**neutral**
3-hydroxybenzoic acid	×	**neutral**	×	**(˄)**	**pro-oxidant**	**neutral**	**neutral**	**impairment**
4-hydroxybenzoic acid	×	**neutral**	×	**(˄)**	**pro-oxidant**	**neutral**	**antioxidant**	**neutral**
2,4-dihydroxybenzoic acid	✔	**neutral**	✔	**(˄)**	**pro-oxidant**	**antioxidant**	**antioxidant**	**neutral**
4-methoxysalicylic acid	✔	**neutral**	×	**decrease**	**pro-oxidant**	**pro-oxidant**	**neutral**	**neutral**
3,4-dihydroxybenzoic acid	✔	**(˄)**	×	**progressive**	**antioxidant**	**antioxidant**	**antioxidant**	**neutral**
2,4,6-trihydroxybenzoic acid	×	**neutral**	×	**progressive**	**pro-oxidant**	**pro-oxidant**	**pro-oxidant**	**impairment**
hippuric acid	×	**neutral**	×	**decrease**	**antioxidant**	**neutral**	**pro-oxidant**	**neutral**

× – not present, ✔ - present, **(˄)** – bell-shaped.

importance as attenuation of spontaneous cupric reduction was associated in our previous studies with copper chelation (Lomozová et al., 2022). As both compounds are weak copper chelators, the mechanism is likely different. It is possible that these compounds can act as oxidants blocking cupric reduction by the BCS.

A comparison of the ferric and cupric reducing potential among the tested benzoic acids showed a greater number of compounds exhibiting a reducing capacity for copper ions in comparison to iron ions. Only 3,4-dihydroxybenzoic acid showed a moderate reducing activity toward Fe^{3+} ions, whereas the others had no effect at all. This observation is not exactly surprising as it can be elucidated by considering the standard reduction potentials of the metals. Specifically, the standard reduction potential of the Cu^{2+}/Cu^{+} couple (+0.15 V) is significantly lower than that of the Fe^{3+}/Fe^{2+} couple (+0.77 V). These findings align with prior studies conducted on flavonoids (Lomozová et al., 2022; Pan et al., 2022).

4.2. The anti- or pro-oxidant impact of benzoic acids in vitro

There are many papers confirming benzoic acids as antioxidants due to their scavenging activity toward stable radicals. Contrarily, there is a lack of studies analyzing the impact of benzoic acids on ROS generation in the presence of an excess of physiological metals. Moreover, it is currently impossible to predict the behavior of compounds showing both chelation and reduction as well as direct ROS scavenging properties. Even benzoic acids with a single hydroxyl group are at least partly active scavengers of synthetic radicals according to some papers (Alcalde et al., 2019; Moazzen et al., 2022). In fact, a recent paper showed that benzoic acid, in particular 3,4-dihydroxybenzoic acid, can directly neutralize hydrogen peroxide (Liu et al., 2020). Our experiments seemed to correspond with this finding as only 3,4-dihydroxybenzoic acid exhibited antioxidant (or at worst neutral) activity in all experiments assessing its effect on hydroxyl radical production from the transition metals-induced Fenton reaction. On the other hand, 2,4,6-trihydroxybenzoic acid exhibited consistent pro-oxidant behavior across all tests, as evidenced by its promotion of hydroxyl radical production. As far as we know, there are generally no studies which reported pro-oxidation by benzoic acids with the exception of one paper (Kalinowska et al., 2021). It should also be emphasized that pro-oxidant benzoic acids in that paper were potent scavengers of the stable radicals $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ as well as iron and copper reductants.

In our study, different pH levels and different metals had an apparently important impact on the outcomes of the Fenton reaction assay. The sole exception was the mentioned 3,4-dihydroxybenzoic acid, which decreased the extent of this reaction under all conditions employed. The change of the localization of the hydroxyl groups modified the outcomes as 2,4-dihydroxybenzoic acid with hydroxyl groups in a *meta*-position was pro-oxidant in the case of Fe^{2+} ion-induced Fenton reactions. When comparing 2,4-dihydroxybenzoic acid with 4-methoxysalicylic acid, the importance of a free hydroxyl group was evident. These two representatives differ only by one methylation of the hydroxy group at position 4. While 2,4-dihydroxybenzoic acid was able to block the production of hydroxyl radicals from Fe^{3+} and Cu^{2+} ions-triggered Fenton reactions and thus had an antioxidant effect, 4-methoxysalicylic acid did not have this ability and in the case of ferric ions, it was even pro-oxidant. These results are supported by another study comparing the free radical scavenging capacity of 3,4-dihydroxybenzoic acid and its two derivatives with a methylated hydroxyl group in either positions 3 or 4 (vanillic and isovanillic acids) by a DPPH assay (Chen et al., 2020). 3,4-dihydroxybenzoic acid showed a significantly higher radical scavenging activity than its derivatives with the methoxy groups, whereas the effects of these derivatives were comparable.

Based on the published literature and our results, we can trace the possible mechanism responsible for the effect of benzoic acids on the metal-driven Fenton reaction. It seems that the direct scavenging potential of 3,4-dihydroxybenzoic acid toward hydrogen peroxide and/or hydroxyl radicals can overcome the negative impact of the metal reduction potential on the Fenton reaction. The chelating effect toward iron and copper is likely of lower importance in this case as this compound strongly chelated iron solely under neutral conditions but it blocked the Fenton reaction mediated by iron at low pH levels as well as by copper, which was not substantially chelated by this compound. As the other tested benzoic compounds have apparently even lower metal chelating activity, their final effect on the Fenton reaction seems to be the result of opposing properties – direct ROS scavenging and metal reduction. At least in the case of 2,4,6-trihydroxybenzoic acid, the strong Cu^{2+} reduction apparently prevailed over the direct ROS scavenging potential and was hence directly transformed with intensification of the Fenton reaction. As most compounds potentiated the ferrous ion-based Fenton reaction, their direct scavenging activity toward hydrogen peroxide or hydroxyl radicals must be relatively weak. Contrarily, their low impact on ferric ion-triggered Fenton reactions apparently agrees with their low ferric reduction properties, as the Fenton reaction runs solely with reduced iron ions, so ferric ions must first be reduced to ferrous in order to be able to catalyze this reaction. Interestingly, the same compounds were able to reduce copper-based Fenton reactions, and this together with their inability to chelate copper ions indicated that there might be some mechanistic differences between iron and copper-based Fenton reactions. To summarize, our initial hypothesis was only partly correct, as some compounds under certain conditions behaved as pro-oxidants in the Fenton reactions.

4.3. The impact of benzoic acids on copper-driven hemolysis

To assess whether the results from the Fenton reaction experiments can be extended to a more complex model, we conducted copper-mediated oxidation experiments using isolated rat erythrocytes. Copper-induced hemolysis mimics a biologically significant condition observed in patients with Wilson's disease. Free copper levels in these individuals can range between 1.6 and 3.1 mM and can be higher than 3.1 mM in symptomatic patients (Lucena-Valera, Ruz-Zafra, & Ampuero, 2023). These concentrations are much higher than those used in our experiment. Unfortunately, it turned out that none of the tested substances could protect rat red blood cells against copper toxicity. On the contrary, two substances (3-hydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid) exacerbated the impairment. These results do not agree with the cytoprotective effect of 3,4-dihydroxybenzoic acid on copper-induced toxicity observed in primary rat hepatocytes (Ahamed, Javed Akhtar, Majeed Khan, & Alhadlaq, 2023). A likely reason for the discrepancy between the *in vitro* Fenton reaction and the *ex vivo* copper-triggered hemolysis is the different mechanisms as the Cu-based hemolysis might be mediated by superoxide instead of hydroxyl radicals (Aaseth, Korkina, & Afanas'ev, 1998). Regardless, the action of these two above-mentioned pro-oxidant acids was then verified in human red blood cells to confirm or refute their toxic effects. In these experiments, however, both substances were no longer harmful; instead, they were mildly protective. We currently have no explanation for this phenomenon, but there are differences between human and rat erythrocytes (da SilveiraCavalcante, Acker, & Holovati, 2015), and we cannot exclude the negative effect of the anesthetic drug urethane, which was used before blood collection in rats.

4.4. Study limitation

This study is purely an *in vitro* and *ex vivo* study, hence *in vivo* confirmation is missing. Bioavailability is not likely an issue, as at least some small phenolics including benzoic acids reach relatively high concentrations in the blood in contrast to parent flavonoids. Contrarily, we did not assess any blends of benzoic acids as food is a mixture of different compounds which can interact together. Future mechanistic studies also need to investigate the differences in the mechanism of action in relation to metal toxicity as various cells might react in

different way and hence our results cannot be generalized to all tissues.

5. Conclusions

This study is the first study reporting a complex relationship between a series of structurally related food-relevant benzoic acids and the transition metals iron and copper. It was found that relatively subtle differences in the substitution of the benzene ring can markedly impact the behavior of these simple phenolics toward transition metals and its biological consequences. 3,4-Dihydroxybenzoic acid behaved in all assays as protective or at worse neutral in terms of its influence on metal-based toxicity. On the other hand, 2,4,6-trihydroxybenzoic acid was shown to potentiate the toxicity of both iron and copper under certain conditions. These results can bring an important clinical message as 3,4-dihydroxybenzoic acid and hippuric acid are very important metabolites formed by human microbiota and reach relatively high concentrations in humans. This study opened the question of the potential utility of benzoic acids in pathophysiological conditions associated with metal overload. At the very least, 3,4-dihydroxybenzoic acid seems to be safe in relation to metal excess, but *in vivo* confirmation is still needed and should be performed in the future. The situation is, however, slightly different with hippuric acid. This compound was mostly protective, but its impact in copper overload, like in Wilson's disease, might be negative and will require further investigations. This paper also brings another important outcome in relation to food: benzoic acid, frequently used as a food preservative, did not have a negative effect in any assay.

Future studies in real food matrices should be performed, and as the chelation of metals can also take place in the GIT. Its impact on metal absorption under metal overload conditions should be studied as well. We also reiterate the need for the of use of different assays to confirm the safety and beneficial effects of different food-related compounds as the tested compounds can apparently behave very promising in one assay but negatively in other assays or under specific but clinically relevant conditions.

Funding

This open-access paper was supported by The project New Technologies for Translational Research in Pharmaceutical Sciences /NETPHARM, project ID CZ.02.01.01/00/22_008/0004607, co-funded by the European Union/. P.H. thanks to Charles University (SVV 260 664).

CRediT authorship contribution statement

Patrícia Harčárová: Writing – original draft, Investigation, Data curation. **Zuzana Lomozová:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Data curation. **Maria Kallivretaki:** Writing – original draft, Investigation, Data curation. **Jana Karlíčková:** Writing – review & editing, Investigation, Data curation. **Radim Kučera:** Writing – review & editing, Supervision. **Přemysl Mladěnka:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Research data for this paper are available at https://zenodo.org/records/13467045.

Acknowledgement

We thank theBESTtranslation for its English revision.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.141014.

References

Aaseth, J., Korkina, L. G., & Afanas'ev, I. B. (1998). Hemolytic activity of copper sulfate as influenced by epinephrine and chelating thiols. *Acta Pharmacologica Sinica, 19*(3), 203–206. Retrieved from http://www.chinaphar.com/article/view/8867.

Ahamed, M., Javed Akhtar, M., Majeed Khan, M. A., & Alhadlaq, H. A. (2023). Protocatechuic acid mitigates CuO nanoparticles-induced toxicity by strengthening the antioxidant defense system and suppressing apoptosis in liver cells. *Journal of King Saud University - Science, 35(3), Article 102585*. https://doi.org/10.1016/j.jksus.2023.102585

Alcalde, B., Granados, M., & Saurina, J. (2019). Exploring the antioxidant features of polyphenols by spectroscopic and electrochemical methods. *Antioxidants, 8*(11), 523. https://doi.org/10.3390/antiox8110523

Andjelković, M., Van Camp, J., De Meulenaer, B., Depaemelaere, G., Socaciu, C., Verloo, M., & Verhe, R. (2006). Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. *Food Chemistry, 98*(1), 23–31. https://doi.org/10.1016/j.foodchem.2005.05.044

Biela, M., Kleinová, A., & Klein, E. (2022). Phenolic acids and their carboxylate anions: Thermodynamics of primary antioxidant action. *Phytochemistry, 200, Article 113254*. https://doi.org/10.1016/j.phytochem.2022.113254

Carpenter, C. E., & Ward, R. E. (2017). Iron determination by Ferrozine method. In S. S. Nielsen (Ed.), *Food analysis laboratory manual* (3st ed., pp. 157–159). Cham: Springer.

Catapano, M. C., Protti, M., Fontana, T., Mandrioli, R., Mladenka, P., & Mercolini, L. (2019). An original HPLC method with coulometric detection to monitor hydroxyl radical generation via Fenton chemistry. *Molecules, 24*(17), 3066. https://doi.org/10.3390/molecules24173066

Chan, F. K., Moriwaki, K., & De Rosa, M. J. (2013). Detection of necrosis by release of lactate dehydrogenase activity. *Methods in Molecular Biology, 979*, 65–70. https://doi.org/10.1007/978-1-62703-290-2_7

Chen, J., Yang, J., Ma, L., Li, J., Shahzad, N., & Kim, C. K. (2020). Structure-antioxidant activity relationship of methoxy, phenolic hydroxyl, and carboxylic acid groups of phenolic acids. *Scientific Reports, 10*(1), 2611. https://doi.org/10.1038/s41598-020-59451-z

Friggeri, L., De Vita, D., Pandolfi, F., Tortorella, S., Costi, R., Di Santo, R., & Scipione, L. (2015). Design, synthesis and evaluation of 3,4-dihydroxybenzoic acid derivatives as antioxidants, bio-metal chelating agents and acetylcholinesterase inhibitors. *Journal of Enzyme Inhibition and Medicinal Chemistry, 30*(1), 166–172. https://doi.org/10.3109/14756366.2013.866657

Gonthier, M. P., Cheynier, V., Donovan, J. L., Manach, C., Morand, C., Mila, I., & Scalbert, A. (2003). Microbial aromatic acid metabolites formed in the gut account for a major fraction of the polyphenols excreted in urine of rats fed red wine polyphenols. *The Journal of Nutrition, 133*(2), 461–467. https://doi.org/10.1093/jn/133.2.461

Kalinowska, M., Gołębiewska, E., Świderski, G., Męczyńska-Wielgosz, S., Lewandowska, H., Pietryczuk, A., & Lewandowski, W. (2021). Plant-derived and dietary hydroxybenzoic acids—A comprehensive study of structural, anti−/pro-oxidant, lipophilic, antimicrobial, and cytotoxic activity in MDA-MB-231 and MCF-7 cell lines. *Nutrients, 13*(9), 3107. https://doi.org/10.3390/nu13093107

Lempart-Rapacewicz, A., Kudlek, E., Brukało, K., Rapacewicz, R., Lempart, Ł., & Dudziak, M. (2023). The threat of food additive occurrence in the environment—A case study on the example of swimming pools. *Foods, 12*(6), 1188. https://doi.org/10.3390/foods12061188

Liu, J., Du, C., Beaman, H. T., & Monroe, M. B. B. (2020). Characterization of phenolic acid antimicrobial and antioxidant structure–property relationships. *Pharmaceutics, 12*(5), 419. https://doi.org/10.3390/pharmaceutics12050419

Lomozová, Z., Hrubša, M., Conte, P. F., Papastefanaki, E., Moravcová, M., Catapano, M. C., & Mladěnka, P. (2022). The effect of flavonoids on the reduction of cupric ions, the copper-driven Fenton reaction and copper-triggered haemolysis. *Food Chemistry, 394, Article 133461*. https://doi.org/10.1016/j.foodchem.2022.133461

Lucena-Valera, A., Ruz-Zafra, P., & Ampuero, J. (2023). Wilson's disease: Overview. *Medicina Clínica, 160*(6), 261–267. https://doi.org/10.1016/j.medcle.2022.12.004

Macáková, K., Catapano, M. C., Tvrdý, V., Klimková, K., Karlíčková, J., & Mladěnka, P. (2019). Hematoxylin assay of cupric chelation can give false positive results. *Journal of Trace Elements in Medicine and Biology, 52*, 29–36. https://doi.org/10.1016/j.jtemb.2018.10.022

Marchiosi, R., dos Santos, W. D., Constantin, R. P., de Lima, R. B., Soares, A. R., Finger-Teixeira, A., & Ferrarese-Filho, O. (2020). Biosynthesis and metabolic actions of simple phenolic acids in plants. *Phytochemistry Reviews, 19*(4), 865–906. https://doi.org/10.1007/s11101-020-09689-2

Mira, L., Fernandez, M. T., Santos, M., Rocha, R., Florencio, M. H., & Jennings, K. R. (2002). Interactions of flavonoids with iron and copper ions: A mechanism for their

antioxidant activity. *Free Radical Research, 36*(11), 1199–1208. https://doi.org/10.1080/10715760210000016463

Mladenka, P., Karlickova, J., Hrubsa, M., Veljovic, E., Muratovic, S., Carazo, A., & Duric, K. (2020). Interaction of 2,6,7-trihydroxy-xanthene-3-ones with iron and copper, and biological effect of the most active derivative on breast cancer cells and erythrocytes. *Applied Sciences, 10*(14), 4846. https://doi.org/10.3390/app10144846

Mladenka, P., Macakova, K., Zatloukalova, L., Rehakova, Z., Singh, B. K., Prasad, A. K., & Saso, L. (2010). *In vitro* interactions of coumarins with iron. *Biochimie, 92*(9), 1108–1114. https://doi.org/10.1016/j.biochi.2010.03.025

Moazzen, A., Öztinen, N., Ak-Sakalli, E., & Koşar, M. (2022). Structure-antiradical activity relationships of 25 natural antioxidant phenolic compounds from different classes. *Heliyon, 8*(9), Article e10467. https://doi.org/10.1016/j.heliyon.2022.e10467

Murota, K., Nakamura, Y., & Uehara, M. (2018). Flavonoid metabolism: The interaction of metabolites and gut microbiota. *Bioscience, Biotechnology, and Biochemistry, 82*(4), 600–610. https://doi.org/10.1080/09168451.2018.1444467

del Olmo, A., Calzada, J., & Nuñez, M. (2017). Benzoic acid and its derivatives as naturally occurring compounds in foods and as additives: Uses, exposure, and controversy. *Critical Reviews in Food Science and Nutrition, 57*(14), 3084–3103. https://doi.org/10.1080/10408398.2015.1087964

Pan, Y., Qin, R., Hou, M., Xue, J., Zhou, M., Xu, L., & Zhang, Y. (2022). The interactions of polyphenols with Fe and their application in Fenton/Fenton-like reactions. *Separation and Purification Technology, 300, Article 121831*. https://doi.org/10.1016/j.seppur.2022.121831

Peixoto, P. S., & Tóth, I. V. (2013). Increased sensitivity spectrophotometric flow analysis method for copper determination in water samples. *Analytical Letters, 46*(18), 2899–2911. https://doi.org/10.1080/00032719.2013.821655

Rabiu, S., Abubakar, M. G., Sahabi, D. M., Makusidi, M. A., Dandare, A., & Bello, J. H. (2021). Benzoic acid based beverages: Health implications. *Asian Food Science Journal, 20*(4), 93–105. https://doi.org/10.9734/afsj/2021/v20i430290

Riha, M., Karlickova, J., Filipsky, T., Macakova, K., Hrdina, R., & Mladenka, P. (2013). Novel method for rapid copper chelation assessment confirmed low affinity of D-penicillamine for copper in comparison with trientine and 8-hydroxyquinolines. *Journal of Inorganic Biochemistry, 123*, 80–87. https://doi.org/10.1016/j.jinorgbio.2013.02.011

Rios, L. Y., Gonthier, M. P., Rémésy, C., Mila, I., Lapierre, C., Lazarus, S. A., & Scalbert, A. (2003). Chocolate intake increases urinary excretion of polyphenol-derived phenolic acids in healthy human subjects. *The American Journal of Clinical Nutrition, 77*(4), 912–918. https://doi.org/10.1093/ajcn/77.4.912

Rupasinghe, H. P. V. (2020). Special issue "flavonoids and their disease prevention and treatment potential": Recent advances and future perspectives. *Molecules, 25*(20), 4746. https://doi.org/10.3390/molecules25204746

Sánchez-Patán, F., Monagas, M., Moreno-Arribas, M. V., & Bartolomé, B. (2011). Determination of microbial phenolic acids in human faeces by UPLC-ESI-TQ MS. *Journal of Agricultural and Food Chemistry, 59*(6), 2241–2247. https://doi.org/10.1021/jf104574z

Serra, A., Macia, A., Romero, M.-P., Anglès, N., Morelló, J., & Motilva, M.-J. (2011). Metabolic pathways of the colonic metabolism of procyanidins (monomers and dimers) and alkaloids. *Food Chemistry, 126*, 1127–1137. https://doi.org/10.1016/j.foodchem.2010.11.145

Shubina, V. S., Kozina, V. I., & Shatalin, Y. V. (2021). Comparison of antioxidant properties of a conjugate of taxifolin with glyoxylic acid and selected flavonoids. *Antioxidants, 10*(8), 1262. https://doi.org/10.3390/antiox10081262

Silva, A., Sganzerla, W., John, O., & Marchiosi, R. (2023). A comprehensive review of the classification, sources, biosynthesis, and biological properties of hydroxybenzoic and hydroxycinnamic acids. *Phytochemistry Reviews, 1-30*. https://doi.org/10.1007/s11101-023-09891-y

da SilveiraCavalcante, L., Acker, J. P., & Holovati, J. L. (2015). Differences in rat and human erythrocytes following blood component manufacturing: The effect of additive solutions. *Transfusion Medicine and Hemotherapy, 42*(3), 150–157. https://doi.org/10.1159/000371474

Spiegel, M., Kapusta, K., Kołodziejczyk, W., Saloni, J., Żbikowska, B., Hill, G. A., & Sroka, Z. (2020). Antioxidant activity of selected phenolic acids–ferric reducing antioxidant power assay and QSAR analysis of the structural features. *Molecules, 25*(13), 3088. https://doi.org/10.3390/molecules25133088

Synowiec, A., Żyła, K., Gniewosz, M., & Kieliszek, M. (2021). An effect of positional isomerism of benzoic acid derivatives on antibacterial activity against *Escherichia coli*. *Open Life Sciences, 16*(1), 594–601. https://doi.org/10.1515/biol-2021-0060

Yun, S. S., Kim, J., Lee, S. J., So, J. S., Lee, M. Y., Lee, G., & Kim, M. (2019). Naturally occurring benzoic, sorbic, and propionic acid in vegetables. *Food Additives & Contaminants: Part B, 12*(3), 167–174. https://doi.org/10.1080/19393210.2019.1579760

Zhang, L., Wu, T., Zhang, Y., Chen, Y., Ge, X., Sui, W., & Zhang, M. (2023). Release of bound polyphenols from wheat bran soluble dietary fiber during simulated gastrointestinal digestion and colonic fermentation in vitro. *Food Chemistry, 402, Article 134111*. https://doi.org/10.1016/j.foodchem.2022.134111

Zhao, Z. (2023). Hydroxyl radical generations form the physiologically relevant Fenton-like reactions. *Free Radical Biology and Medicine, 208*, 510–515. https://doi.org/10.1016/j.freeradbiomed.2023.09.013

Zhou, K., Yin, J.-J., & Yu, L. (2006). ESR determination of the reactions between selected phenolic acids and free radicals or transition metals. *Food Chemistry, 95*(3), 446–457. https://doi.org/10.1016/j.foodchem.2005.01.026