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*Review*

# Metal Ions, Metal Chelators and Metal Chelating Assay as Antioxidant Method

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**Abstract:** Heavy metals are essential for a wide range of biological processes, including the growth and reproduction of cells, synthesis of biomolecules, many enzymatic reactions, and the body's immunity, but their excessive intake is harmful. Specifically, they cause oxidative stress (OS) and generate free radicals and reactive oxygen species (ROS) in metabolism. In addition, the accumulation of heavy metals in humans can cause serious damage to different organs, especially respiratory, nervous and reproductive and digestive systems. Biologically, metal chelation therapy is often used to treat metal toxicity. This process occurs through the interaction between the ligand and a central metal atom, forming a complex ring-like structure. After metals are chelated with appropriate chelating agents, their damage in metabolism can be prevented and efficiently removed from the body. On the other hand, heavy metals, including Zn, Fe and Cu, are necessary for the suitable functioning of different proteins including enzymes in metabolism. However, when the same metals accumulate at levels higher than the optimum level, they can easily become toxic and have harmful effects toward biomolecules. In this case, it induces the formation of ROS and nitrogen species (RNS) resulting in peroxidation of biological molecules such as lipids in the plasma membrane. Antioxidants have an increasing interest in many fields due to their protective effects, especially in food and pharmaceutical products. Screening of antioxidant properties of compounds needs appropriate methods including metal chelating assay. In this study, a general approach to the bonding and chelating properties of metals is described. For this purpose, the basic principles and chemical principles of metal chelation methods, both in vivo and in vitro, are outlined and discussed. Hence, in the main sections of this review, the descriptions related to metal ions, metal chelating, antioxidants, importance of metal chelating in biological system and definitions of metal chelating assays as widely used methods to determine antioxidant ability of compounds are provided. In addition, some chemical properties, technical and critical details of the used chelation methods are given.

**Keywords:** metals; antioxidants; metal chelating; antioxidant activity; antioxidant methods



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## 1. Introduction

### *1.1. Heavy Metals*

Metal ions are necessary for the continuation of the vital functions of living organisms. For thousands of years, people have widely used metals for daily needs without considering their drawbacks and consequences. As a result, in addition to destroying the entire ecosystem, metal ions pollute the water resources and have seriously affected plant and animal life. Today, metal pollution is mostly caused by mining, industrial sewage, urban wastes, acid rain, fossil fuel residues, fertilizers and pesticides [1].

Heavy metals (HMs) are elements with a density above 5 g/mL [2,3]. They have been used in many different areas for thousands of years. The most common ones are Cr, Pb, Cd, Hg, Cu and Zn. In addition, as can be included in this group due to its similar physical and chemical properties to heavy metals [4–6]. Fe, Co and Mn are less common

heavy metals. HMs can be examined under two groups as essential and non-essential HMs according to their toxicity. Essential HMs, including Zn, Cu, Fe and Co, are less effective or relatively harmless at low quantity. However, non-essential HMs, including Cd, Hg, As, and Cr are highly toxic even at low quantities [7,8]. On the other hand, HMs, including Zn, Cu and Fe, are obligatory for the biological activities of different proteins and enzymes as cofactors in distinct biological and physiological processes. For instance, Cu, Fe, Zn and Co demonstrate a vital role in the use of oxygen in the electron ($e^-$) transport chain, cell growth and differentiation, many enzymatic reactions, synthesis of biomolecules and the continuity of the immune system. The excess of HMs in the cytoplasm can disrupt the intracellular redox balance and also cause changes in the cytoplasm's pH, alter protein conformation and inhibit enzyme's function. This situation can easily lead to cell dysfunction, necrosis or apoptosis. In addition, HMs can interact with proteins thiol, carboxyl and imidazole groups [9,10].

Iron (Fe) is the best example of the metals that are taken or exposed to excessively in our daily life. An average person includes 4–5 g of elemental iron. Two-third of this amount exists in the hemoglobin as oxygen transporter protein and another one-third is stored in the iron-keeping proteins including hemosiderin or ferritin [11]. Fe exists in cytochromes, hemoglobin, myoglobin, and is essential for many enzymes, including peroxidases, catalase, succinate dehydrogenase, aconitase, aldehyde oxidase and oxygenase [3,12]. However, although the human body can tolerate relatively high iron levels, excess iron is quite toxic. Metal poisoning has become quite common in young children as a result of excessive iron intake due to iron-enriched food supplements. In addition, acute poisoning is less widespread in adults, but chronic Fe overload is usually encountered in β-thalassemia patients due to the mandatory and regular intake of whole blood transfusions [11,13]. The fatally accumulated iron level primarily affects the heart and liver. The regular evacuation of metal ions can be increased by the application of a convenient sequestering agent. Desferrioxamine B is one of the current drugs of choice for $Fe^{3+}$ removal. However, Desferrioxamine B is able to reduce the available iron level to about ten times of the normal level [11,13,14]. In humans, iron overloads can be decreased by the management of agents that can compete with the transferrin protein, which binds and transfers metal ions. As described in this study, drugs that have a higher $Fe^{3+}$ affinity than transferrin for effective chelation may damage natural iron stores under physiological conditions. Enterobactin as natural siderophore had high affinity toward $Fe^{3+}$ ions [15]. Recent studies have proven that siderophores, which are small molecules produced by some microorganisms, make iron soluble and thus usable by plants. Siderophores from microbial origin are good iron chelating agents primarily due to powerful iron chelating components, such as hydroxamate, catecholate and α-hydroxocarboxylates. On the other hand, phytosiderophores such as mugineic acid and its derivatives are polydentate ligands with carboxylate and amine groups as metal chelators. Gramibactin has been reported to effectively form $Fe^{3+}$ as an impressive example for a new group of diazeniumdiolate siderophores established on its ability to isolate iron. In the aforementioned study, it was reported that gramibactin forms quite stable complexes with $Fe^{3+}$ ions in a broad pH range (Table 1) [16]. Although there are many reasons for Fe pollution, it occurs especially with the corrosion of water pipes. The groundwater and soil are contaminated through industrial and agricultural human wastes including Fe. In addition, today, intense air pollution from the steel industry includes particulate iron and iron oxide together. Vomiting, nausea, diarrhea, abdominal pain, lethargy, and dehydration are the most common symptoms of iron toxicity [17,18].

Aluminum (Al) is the second plentiful metal in the earth's crust and constitutes about 8% of the total mineral quantity. It has an important place today because it is widely used in different industries. The acceptable daily intake limit of Al in humans is about 3 to 10 mg. Therefore, excessive and irregular intake of Al causes dangerous effects for living creatures [19]. $Al^{3+}$ toxicity is a major factor in living organisms. Due to its chemical properties, $Al^{3+}$ leads to an imbalance of free radical metabolism, resulting in the oxidative injury of polysaccharides, proteins, nucleic acids and membrane lipids, and disrupts the

normal cell activities. Al toxicity is still not completely unraveled at the molecular level, but some potential mechanisms have been detailed. For example, it is known that Al exhibits an important pro-oxidant effect in living systems [20]. Al toxicity induces an excessive increase in ROS levels. Especially, it promotes different neurodegenerative diseases including dementia and encephalopathy in humans. This toxicity also causes serious damage to biomolecules. The presence of Al in living systems creates different toxic effects. Another effect is the change in the natural structure and roles of proteins and enzymes in the glycolysis and TCA pathways, cells, tissues, central nervous system (CNS), and other organs [21]. Al as a strong Lewis acid prefers oxygen donor ligands, including phosphates, nucleotides, carboxylates and nucleic acids. It promotes hyperphosphorylation of normal proteins. In a recently proposed paradigm, it has been suggested that Al can interact directly with the backbone of proteins. In this study, it was suggested that Al coordinates directly to the carbonyl oxygen and protonated peptide nitrogen, occurring in stable structures with a 5-membered ring that forms strong covalent bonds, and can interact directly with the backbone of proteins [22]. It was reported that the patients affected by Al intoxication were treated successfully with the ethylenediaminetetraacetic acid (EDTA) as chelating agent over a short period (Table 1) [23].

Copper (Cu) is compulsory for some metabolic enzymes, including cytochrome c oxidase, superoxide dismutase (SOD), tyrosinase, ceruloplasmin and dopamine-β-hydroxylase [19]. ROS can occur when liver cells are exposed to copper overload, and this is generally considered a critical event leading to cell death. Wilson's disease (WD) is a defect which blocks the body from getting rid of excessive Cu. In people with WD, copper accumulates in the brain, liver and the other organs, especially the eyes. Whereas a small quantity of dietary Cu is sufficient to stay healthy, too much Cu quantity is toxic. In this case, excessive $Cu^{2+}$ is effectively bounded either by ligands containing both hard and soft donors. The leading drug used for this purpose in the treatment of WD is penicillamine. This drug is a molecule including both types donor atoms and selectively binds $Cu^{2+}$ ions [15]. The ternary H-point standard addition method is simultaneously used to determine $Cu^{2+}$ ions using murexide as chromogenic reagent. Murexide, a reddish-purple compound, has attracted much attention due to its application in chemical analysis and spectrophotometric fields (Table 1) [24]. In addition, murexide as a metal ion indicator is used as a chromogenic reagent for the traditional spectrophotometric determination of some metals, especially copper. Furthermore, different complexation reactions were performed between murexide and $Co^{2+}$, $Cu^{2+}$, $Ni^{2+}$, $Cd^{2+}$, $Zn^{2+}$, and $Pb^{2+}$ ions and recorded by a spectrophotometric technique (Table 1) [25]. In the murexide method, a simple and sensitive spectrophotometric method, spectrophotometric detection of $Cu^{2+}$ with murexide, which is the ammonium salt of purpuric acid, had been developed. This method depends on the formation of a stable yellow greenish colored complex (at pH 5.0), which had a maximum absorption at 476 nm. Murexide is used in analytical chemistry for complexometric titrations, most often as a complexometric indicator for $Ca^{2+}$ ions, but also for $Co^{2+}$, $Cu^{2+}$, $Ni^{2+}$, $Cd^{2+}$, $Zn^{2+}$ and $Pb^{2+}$ and rare earth metals [26].

**Table 1.** Some metals and their chelating agents.

| Metals | Binding Agent | References |
|---|---|---|
| $Pb^{2+}$ | Murexide | [25] |
| $Cd^{2+}$ | Murexide | [25] |
| $Hg^{2+}$ | Dimercaprol | [27] |
| $Cu^{2+}$ | Murexide | [28] |
| | Penicillamine | [29] |
| | EDTA | [15] |
| $Ni^{2+}$ | Murexide | [25] |
| $Zn^{2+}$ | N,N,N′,N′-tetrakis(2-pyridylmethyl)-ethylenediamine | [30] |

**Table 1.** *Cont.*

| Metals | Binding Agent | References |
|---|---|---|
| $Fe^{3+}$ | Desferrioxamine | [31] |
| | Enterobactin | [15] |
| | Deferoxamine | [32] |
| | Gramibactin | [16] |
| $Al^{3+}$ | EDTA | [26] |
| $Co^{2+}$ | Murexide | [26] |
| $Mn^{2+}$ | Desferrioxamine | [26] |
| $Ca^{2+}$ | Calbindins | [23] |

Zinc (Zn) is the fourth most widespread metal in use after Fe, Al and Cu. It is stored and transferred in metallothionein and essential for the function of over three hundred enzymes and thousand transcription factors. Approximately 2–4 g Zn is distributed throughout the human body. An increase in the amount of Zn in the living environment can cause serious negative effects on living organisms. Zn homeostasis in the human body is controlled by the small intestines. Zn is stored in specific synaptic vesicles by glutamatergic neurons in the brain and modulates neuronal excitement [33].

Since Zn has a flexible coordination geometry, they allow the conformation of the proteins they are in to change rapidly. Thus, biological reactions take place faster. The best example of Zn-containing enzymes is the carbonic anhydrase enzyme family, which can reversibly convert carbon dioxide ($CO_2$) and water to bicarbonate ($HCO_3^-$) [34–36]. In addition, Zn is a cofactor of many metalloenzymes, including anhydrases, oxidases, dehydrogenases, and peroxidases. It plays a crucial role in the arrangement of nitrogen metabolism, cell proliferation, auxin synthesis and photosynthesis in plants [33]. This reagent readily permeates cell membranes and forms a stable 6-coordinate complex with Zn [30]. Furthermore, Zn is essential for folding of protein, configurational and conformational changes of proteins as well as DNA replication, growth hormones and fertility [37]. The most common intracellular Zn chelator is N,N,N′,N′-tetrakis(2-pyridylmethyl)-ethylenediamine (Table 1).

Cobalt (Co), which is necessary for all animal metabolisms, is also an important element for the synthesis of cobalamin and vitamin $B_{12}$. Especially, bacteria in the ruminants' stomach convert Co salts into vitamin $B_{12}$, [38]. Heavy metal pollution in water and soil has increased rapidly in recent years due to different reasons [3]. Similar to some earlier metals, a fully elucidated mechanism of Co toxicity has not been defined in general. In some studies, Co's high affinity for sulfhydryl (-SH) groups in biomolecules has been linked to oxidation and degradation of Krebs cycle intermediates, as well as damage to the transporting system resulting in enhanced intracellular $Ca^{2+}$ ions [39].

Although As, Hg and Cd are not very active elements, they stimulate OS by inhibiting SOD, affecting antioxidants and binding to sulfhydryl group (-SH) of proteins. As they exist in trivalent form and, thus, induce OS by oxidation-reduction reactions. Because of their multivalent states, they also affect acid-base and methylation reactions. Hg toxicity has been shown to cause OS, enzyme inactivation, inflammation and autoimmunity. However, the specific molecular mechanisms of Hg toxicity have not yet been fully elucidated. On the other hand, Pb indirectly causes the OS by generating free radicals and ROS and decreases the antioxidant capacity of the cells. Prenatal exposure to As, Hg and Cd can result in brain dysfunction and neuronal diseases [40].

Although food is the main source of mercury poisoning, fish and dental amalgams are also considered as the most important sources of Hg exposure. People who consume a lot of fish meat from polluted waters may be at increased risk of Hg exposure. Fetuses have a looser blood-brain barrier than adults. Therefore, mercury in the mother's bloodstream can reach the brain of the fetus. Therefore, pregnant women should avoid consuming fish caught from polluted waters [41–43]. Hg toxicity can cause many ailments, including hypertension, heart and kidney dysfunction [44,45].

Although Calcium (Ca) is not a heavy metal, it is tightly bound by calbindins, which are a putative class of $Ca^{2+}$-binding proteins. Calbindins belong to the $Ca^{2+}$ messenger system, which reply to the transitory in intracellular $Ca^{2+}$ concentration (Table 1). A structural property of calbindins is their functional domain, which consists of two interacting binding sites. So, they have cooperative binding [46]. A similar situation to calbindins-$Ca^{2+}$ co-binding is also observed between laurate and human serum albumin (HSA) that is commonly used as standard protein in biochemical assays [47–50]. It is known that HSA binds to a wide range of ligands, especially fatty acids. In addition, in another study, multiple binding equilibria were searched for HSA and laurate binding using by a dialysis-exchange method [46].

### *1.2. The Importance of Metal Chelating in Biological Systems*

Metal chelating therapy is the most important and primary clinical treatment in case of heavy metal poisoning. Chelation is the process of linking existing ions or molecules of a ligand to a central metal atom or ion through an acyclic or ring-like coordination bond. A ligand is a molecule or ion with two or more atoms, which can easily donate two electrons to form a covalent bond. Ligands can be classified in three different ways based on the properties of the bond between the ligand and the covalent atom. The complexes' stability varies with the metal ions and ligand interactions. Although Hg and Pb ions have higher affinity for sulfur and nitrogen than for oxygen ligands, the opposite is the case for Ca atoms. In addition, these differences that occur in affinity procedures, are the basic principle in the selection of chelating agents [3,51]. For this purpose, some drugs, such as dimercaprol, EDTA, deferoxamine, penicillamine, dimercaptosuccinic acid and their analogues, are used as chelating agents, which are widely used in the treatment of metal toxicities [3].

### *1.3. Reactive Oxygen Species (ROS) and Oxidative Stress (OS)*

Oxygen is a highly reactive atom and a powerful oxidizing agent that can easily form oxides with many other elements and compounds. In the atmosphere, it exists in the ground state and undergoes a gradual reduction process [52–55]. Molecular oxygen contains a pair of electrons with parallel spins located in two separate anti-bonding orbitals. Therefore, it can easily accept two electrons from any ordinary electron donor [56–58]. In addition, redox reactions are a very important metabolic process in living organisms, where electrons can be easily transferred from one species to another. This process is the basic reaction in most biological systems. In this case, the series of chemical reactions in living organisms uses molecular oxygen in the air for oxidation and, as a result, provides an immediate usable form of energy such as ATP [59–61]. Oxygen is commonly used in reduction-oxidation reactions and the enzymatic biocatalysis process in cells and tissues. Furthermore, it has interatomic electron transfer ability. It is an important structural element for aerobic creatures and living metabolism. In addition, it is the final electron acceptor in the electron transport system [62–65]. So far, everything is very normal, but the main problem arises when the electron flow becomes disconnected. This situation results in the formation of free radicals having an odd electron. Free radicals are highly unstable and active reagents against molecules and intermediates [66–68]. These unstable and short-lived species derive from the three basic elements of oxygen, sulphur and nitrogen. For example, ROS include hydroxyl (HO·), superoxide anion ($O_2 \cdot^-$), alkoxyl (RO·), nitric oxide (NO·), peroxyl (ROO·) and lipid hydroperoxides (LOO·) radicals. Of these $O_2 \cdot^-$, NO· and LOO· had less reactivity [69–71]. In addition, in living systems, hydrogen peroxide ($H_2O_2$), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl) are nonradical ROS forms [70,71]. In addition, elemental ions such as $Fe^{2+}$ can initiate ROS production in living systems [72,73]. OS occurs as a result of an imbalance between ROS production and antioxidant system. OS disrupts a number of cellular functions and leads to different pathological events in organisms [74–76]. This situation leads to oxidative modification of proteins, DNA, RNA, and lipids [77,78]. OS has long been known to pose an increased risk for many diseases, including cancer,

arthrosclerosis, diabetes, aging, arthritis and some neurodegenerative diseases [79,80]. Nevertheless, antioxidants have a very important role in health by inhibiting oxidative processes and reducing the harmful effects of ROS [81].

When HMs accumulate at toxic levels in the human body, they cause serious hazardous effects in different organs, including the nervous, respiratory, reproductive and digestive systems [38]. As a result of this situation, in the plasma membrane, lipid peroxidation occurs and stimulates the formation of RNS and ROS. Transition metals such as Fe and Cu also trigger Fenton and Haber–Weiss reactions and the formation of ROS such as OH· [82–84]. In the presence of metal ions and $O_2$, $H_2O_2$ can form OH· by the renowned Fenton reaction [85]. On the other hand, the Haber–Weiss reaction produces OH· from $O_2^{\bullet -}$ and $H_2O_2$, which is catalyzed by ferrous ions. The reaction was first suggested by Fritz Haber and his student [86]. In later studies, it was determined that both reactions are the main sources of radicals and responsible for the cellular damage.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet} \text{ (Fenton reaction)}$$

$$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + H_2O + OH^{\bullet} \text{ (Haber–Weiss reaction)}$$

The metal ions chelation can be important in order to avoid ROS formation and radical production which can induce damage to biomolecules. In addition, natural metal chelating compounds including phenolics and flavonoids are desired over synthetic chelating agents, which are associated with the problem of toxicity [87].

### *1.4. Antioxidants*

Antioxidants block the harmful effects of ROS and are divided into two main groups, natural and synthetic, prevent free radicals from harming the body by catching and neutralizing them [88–90]. Antioxidants are described as substances that effectively scavenge ROS, positively regulate antioxidant defense systems or inhibit ROS production [91,92]. In addition, antioxidants can prolong the shelf life of products by delaying the lipid peroxidation process during processing and storage, preventing deterioration of pharmaceutical and food products [93–95]. Synthetic antioxidants have long been preferred over natural antioxidants due to their higher performance and high availability, stability and low cost [96]. As seen in Figure 1, the most preferred and used synthetic antioxidants are butylated hydroxyanisole (BHA) and hydroxytoluene (BHT), propyl gallate (PG) and tert-butylhydroquinone (TBHQ) [97–99].

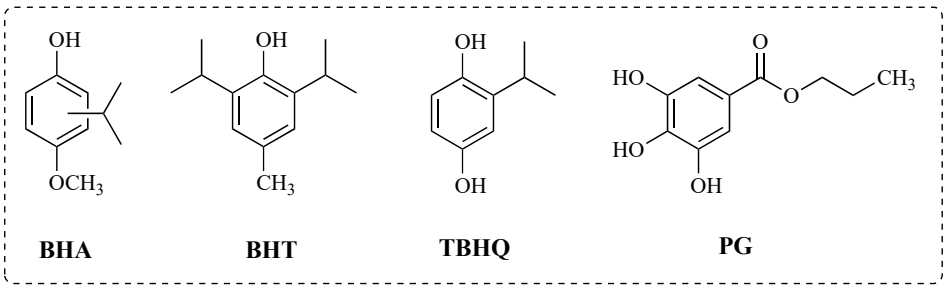


**Figure 1.** The chemical structures of the most commonly used synthetic antioxidants.

Although these synthetic antioxidants are widely used, it has been reported that they cause some health problems, such as fatty liver, carcinogenesis, skin allergies and gastrointestinal system problems in long-term use [79,100,101]. For this reason, consumers prefer natural antioxidants more in their daily diets and are worried about being exposed to the undesirable effects of synthetic antioxidants. Unlike synthetic antioxidants, natural antioxidants, which are known to be safer, are obtained from different plants, including fruits, herbs, spices and vegetables [102–104]. Therefore, the increasing popularity of natural antioxidants may cause more food consumers and manufacturers to replace synthetic

ones [105,106]. For example, aqueous tea, anise and fennel extracts were used as natural antioxidant sources due to their rich content of various components, including tannins, catechins, theines and flavonoids [107–109]. However, the quality and antioxidant capacity of natural antioxidant and extracts depend not only on the quality of the natural source, but also on the applied processes and technologies for the extraction. In addition, the safety of proven natural antioxidants has been determined taking into account information on chemical compounds and potential cumulative effects assessed by the results of toxicity studies [79,110,111].

### *1.5. Metal Chelating Ability*

All living things need transition metals to maintain their vital functions. Metal ions have different functional roles in biological systems. In most cases, metals are tightly bound by forming coordination bonds with metalloproteins, and proteins acquire a three-dimensional structure in this way [112,113]. For instance, the chemical affinity of metal ions can be used by proteins to produce enzymes with strong catalytic activity. Metal chelating is usually considered as a the most putative and common antioxidant method. Antioxidants have been reported to have an effective Fe-binding ability due to their functional groups that perform metal binding. The interaction of Fe ions with antioxidant compounds may also alter their biological effects including antioxidant properties [114,115]. As can be seen in Figure 2A, the proposed chelating sites for Fe to taxifolin, as a known antioxidant, is the 4-oxo, 5-OH groups between the heterocyclic and the A rings, the catechol moiety of B ring and the 3-OH groups, 4-oxo group in the heterocyclic ring (Figure 2A). These groups in the taxifolin molecule prevent the formation of the iron ions-ferrozine complex. So, taxifolin can chelate $Fe^{2+}$ before ferrozine or by 2,2′-bipyridine regents, which had a high affinity for $Fe^{2+}$. In this way, taxifolin converts ferrous ions into metal complexes or sterically inhibits interactions between lipid intermediates and metals. Taxifolin can possibly chelate more than one $Fe^{2+}$ ion over functional hydroxyl (-OH) and carbonyl (-C=O) groups [116]. Metallic centers stabilize the semiquinone-metal complex [117]. Binding selectivity can be generated by a reagent on two or more substrates or by two or more positions on the same substrate. This is successed by electronic and steric factors between receptors and substrates. The binding of chemical species depends on completely different affinities. Functional biomolecules can generally act as multiple linker site systems. [15]. In addition, selective binding is very important in chelation therapy. There are different approaches about metal binding affinities. Another approach was taken by Harris and coworkers [13]. In this approach, the metal ion concentration in solution was used for comparison the relative efficacy of different tris catecholate ligands against $Fe^{3+}$ ions [11]. In another study, it was reported that the addition of a diamino unit to the pyrolic tripodal architecture significantly increased their binding ability when compared to parent aminopyrolic receptors [118]. The most common step, when investigating interactions between binding types, is the evaluation of binding affinities. A quantitative assessment of binding affinities is based on the evaluation of binding constants. However, a binding constant fully describes a reagent's affinity for a ligand only when a complex is formed. Sometimes, a cooperative effect can be observed in the binding of any atom or group to a binding or chelating agent. The best example of this is demonstrated by the binding affinity of oxygen to hemoglobin, which is responsible for transporting oxygen in the bloodstream to peripheral tissues by forming a reversible complex. For this aim, the formation of four different complexation stages including the uptake of four $O_2$ has been identified [46]. The first $O_2$ can bind to the $Fe^{2+}$ ions contained in a heme prosthetic group in each monomer of a tetrameric hemoglobin. The stability constant for the coordination of first $O_2$ to deoxyhemoglobin is relatively low; however, when a second $O_2$ binds to the heme, the oxygen affinity enhances, letting the other oxygens such as the third and fourth $O_2$ to bind more easily. This is because the coordination of oxygen changes the shape of the binding site of hemoglobin. This steric and conformational modification is transmitted to the remaining monomer subunit in the tetrameric hemoglobin, where it stimulates conformational modification in the other heme

regions, thus making it easier for oxygen to bind to these regions. In addition, this effect is called the cooperative effect as well as the positive homotropic allosteric effect [15].

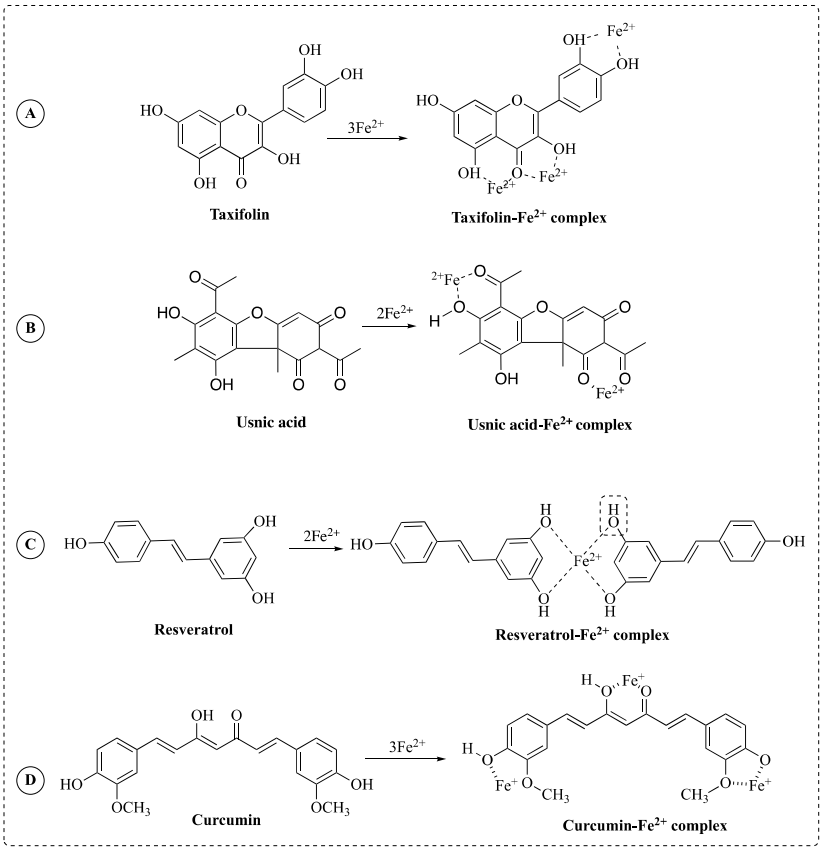


**Figure 2.** The suggested ferrous ions ($Fe^{2+}$) binding mechanism of taxifolin (**A**), usnic acid (**B**), resveratrol (**C**) and curcumin (**D**).

In addition, curcumin, which is used as a food ingredient and is abundant in ginger and turmeric, chelates $Fe^{2+}$ ions and prevents the formation of the $Fe^{2+}$-ferrozine complex. In this way, curcumin can capture iron ions with a high binding affinity such as ferrozine. It has been suggested that a curcumin molecule binds three $Fe^{2+}$ ions, as seen in Figure 2D. It has been reported that curcumin chelates iron ions with biological active -OH and -$OCH_3$ groups [119]. In addition, the compounds containing functional groups such as C=O and C–OH can easily bind metal ions. In another study, Kazazica et al. showed that kaempferol binds to $Fe^{2+}$ and $Cu^{2+}$ ions. They also stated that this binding was mediated by functional -OH and -$OCH_3$ groups [120]. Compounds containing two or more -OH, -COOH, -SH, -$OCH_3$, -C=O, -$PO_3H_2$, -$NR_2$, -O- and -S- functional groups in a suitable function-structure configuration can easily chelate $Fe^{2+}$ ions [121–124]. In another study, Fiorucci and coworkers showed that quercetin, as an abundant phenolic compound in plants, had similar metal chelating ability [125]. Recently, the possible $Fe^{2+}$ binding mechanism of usnic acid was proven by our research group [126]. It was reported that usnic acid prevented the formation of the complex of $Fe^{2+}$-ferrozine (Figure 2B). As shown, usnic acid can chelate $Fe^{2+}$ ions with -OH and -COOH groups attached to the phenolic ring. In another effective study, it was observed that resveratrol binds $Fe^{2+}$ ions on their -OH groups at *meta* positions [127]. In this way, it has been reported that the main antioxidant ability of resveratrol, a strong and natural antioxidant, may be related to its iron binding capacity. In this study, it was clearly demonstrated that resveratrol binds $Fe^{2+}$ and interferes to form the $Fe^{2+}$-ferrozine complex.

One of the strategies for estimating the chelation capacity is to measure free iron ions ($Fe^{2+}$) using a chelating agent such as ferrozine or 2,2′-bipyridine (Figure 3), forming

an easily detectable complex by spectroscopic analysis. Metal chelators form complexes and reduce the reactivity of metals such as iron, making them inactive [128]. The main contribution to metal binding is because of the catechol moiety, as sampled by the more pronounced bathochromic shift produced by Cu binding to quercetin when compared to the chelating ability of kaempferol [129]. Flavonoids show bioavailability by chelating excess metal ions in the human body. Such a metal chelating effect of flavonoids plays an important role in the detoxification of other HMs, such as Cr, Sn, Cd and Pb as well as binding excess Al. The chelating agents effectively chelate the toxic metal ions forming the complexes [114,130].

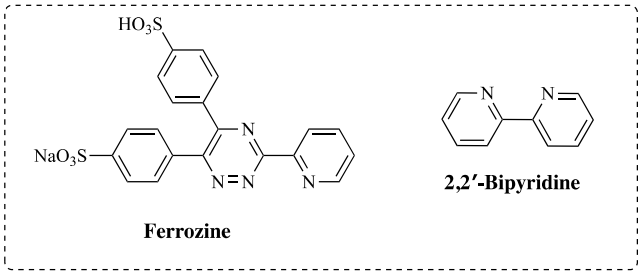


**Figure 3.** The chemical structures of ferrozine and 2,2′-bipyridine as metal chelator.

$FeCl_2$ and $FeSO_4$ are generally used as sources of ferrous ion ($Fe^{2+}$). The decrease in absorbance at 485 nm for 2,2′-bipyridine or 562 nm for ferrozine after transferring the metal ion to an antioxidant indicates the formation of the metal-antioxidant complex [131]. The metal chelating capacities of the metal chelated-antioxidant are measured in this way [132]. Both reagents form complex structures with unbonded $Fe^{2+}$, thus, a decrease in the amount of $Fe^{2+}$-ferrozine or $Fe^{2+}$-2,2′-bipyridine complexes formed after adding antioxidant chelating reagent. Complexes of $Fe^{2+}$ with ferrozine or 2,2′-bipyridine form a red colored chromophore with maximum absorbance at 562 and 485 nm, respectively. Although EDTA is not an antioxidant molecule, it is used as a standard metal chelator in antioxidant practices because it has a high chelating capacity. In fact, in many studies, the metal chelation abilities of antioxidants or extracts are expressed as EDTA equivalents [133]. These measurements are affected by both the formation constants of the antioxidant-$Fe^{2+}$ and $Fe^{2+}$-ferrozine or $Fe^{2+}$-2,2′-bipyridine complexes [134]. From this point of view, it seems that a weak metal chelator could not prevent the Fenton reaction in vivo. Despite everything, this reaction may be a suitable evaluation for the iron chelating ability of an antioxidant.

$$3\text{Ferrozine} + \text{Fe}(H_2O)_6^{2+} \rightarrow \text{Fe} - (\text{Ferrozine})_3^{4-} + 6H_2O$$

Metal chelating ability is very important as it reduces the metal concentration, which has a catalytic effect in lipid peroxidation. In addition, metal chelating agents are considered as secondary antioxidants because they decrease the redox potential and thus stabilize the oxidized metal ions [135].

## 2. Antioxidant Methods

Several antioxidant assays have been developed for measurement and investigation of antioxidant capacity of food, pharmaceutical, medicinal and biological materials. Until now, the most common and effective methods are inhibition of autoxidation of linoleic acid emulsion (Thiocyanate method) [136], ORAC and TRAP assays [59], ferric ions ($Fe^{3+}$) reducing assay (FRAP) [108,137], $Fe^{3+}$-$Fe^{2+}$ transformation assay [138], cupric ions ($Cu^{2+}$) reducing assay (Cuprak method) [139], Folin–Ciocalteu reducing assay [140], DPPH$^{\bullet}$ [141], ABTS$^{\bullet+}$ [142], DMPD$^{\bullet+}$ [139] and superoxide anion radical scavenging assays [143] and putative $Fe^{2+}$ binding assay, which are described in detail in the present study. As is known, most of the methods use the same principle: a redox active compound or synthetic-colored

radical is produced; then, the ability of a biological sample to scavenge or reduce the redox-active compounds is measured by a spectrophotometer, applying a suitable standard to measure the antioxidant ability [144].

## 3. Metal Chelating Assays

### 3.1. Metal Chelating Assay by Ferrozine Reagent

The described spectroscopic methods were developed taking into account the binding affinity between a reagent and a metal ion such as $Fe^{2+}$. The $Fe^{2+}$ chelation by Ferrozine was evaluated by the Dinis method [145]. Briefly, a different quantity of sample and standard compounds was transferred to a 0.05 mL of $FeCl_2$ solution (2 mM). In this way, the interaction between the sample and $Fe^{2+}$ is supplied, that is, $Fe^{2+}$ ions are chelated by the sample. The reaction was started by adding 0.2 mL of ferrozine reagent (5 mm) and the mixture was stirred and left standing at 25 °C for 10 min. Then, absorbance values of solution were recorded at 562 nm [146].

### 3.2. Metal Chelating Assay by 2,2′-Bipyridine Reagent

The $Fe^{2+}$ chelating by 2,2′-bipyridine are generally performed according to the method of Re et al. [147]. Briefly, different quantities of the sample and standard compounds were transferred to a solution of 0.25 mL $FeSO_4$ (2 mM). Thus, the interaction of the sample and $Fe^{2+}$ ions is ensured. So, $Fe^{2+}$ ions are chelated by the sample. Then, 1.5 mL of 0.2% bipyridyl solution dissolved in 1 mL of Tris-HCl solution (pH 7.4) and HCl (0.2 M) were transferred to the mixture, sequentially. After the solution was incubated for 30 min, 2.5 mL of ethyl alcohol and 0.63 mL of deionized water were transferred. Their absorbances were recorded at 522 nm against the blank consisting of the Tris-HCl buffer [148].

### 3.3. The Percentage Metal Chelating

The percentage chelating ability of samples and standards is determined using the following equation:

$$\text{Percentage chelating effect (\%)} = [(A_0 - A_1)/A_0)] \times 100$$

where $A_0$ and $A_1$ are the absorbances of the control and sample, respectively. The control did not include $FeCl_2$, ferrozine, and 2,2′-bipyridine [149].

### 3.4. The Importance of $IC_{50}$ Value in Binding Affinity

The $IC_{50}$ value is commonly used in biochemistry to compare metal chelating [11]. $IC_{50}$ values describe binding affinity quantitatively. This parameter is widely used in biochemical applications and is an alternative approach based on a suitable principle. The idea of evaluating half of the binding property does not require extra knowledge of binding constants. The lower $IC_{50}$ value indicates the higher binding affinity and the easier it is to use. For all these reasons, the $IC_{50}$ value is the most practical way to evaluate binding affinities. In most cases, it reports on biological effects [14,46]. In functional antagonists' studies, the $IC_{50}$ value of a drug is the concentration required to inhibit half of the maximum biological activity of the agonist by formation a dose-response curve. It can be determined by examining the effect of different concentrations of antagonists. The $IC_{50}$ value is not a direct indicator of binding affinity, but at least for competitive agonists and antagonists, $IC_{50}$ and affinity are correlated with the Cheng–Prusoff equation [150], which is given below.

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_i}} \text{ (Cheng–Prusoff equation)}$$

In this formula, the Ki value can be easily calculated from the $IC_{50}$ value for a competitive inhibitor in single-substrate enzymatic reactions. In this formula, $IC_{50}$ is the half maximal concentration of the competitive inhibitor and shows a 50% inhibition. S is the substrate concentration. $K_m$ is the Michaelis–Menten constant of the substrate for enzymatic

reaction. The $IC_{50}$ value of a compound may vary depending on experimental conditions and parameters such as temperature and pressure, but Ki is a relatively stable value [151].

## 4. Conclusions

Heavy metals have different functional roles in biological systems and are necessary for many different biological events, such as cell growth, development and proliferation, synthesis of biomolecules, catalysis of many enzymatic reactions and immunity of the body; however, excessive uptake of metals by different pathways is extremely harmful and can create applications that cannot be repaired. In addition, heavy metals such as $Fe^{2+}$ can simplify the production of ROS in living systems. The metal chelating ability of the agents used for this purpose can be extremely valuable for antioxidant properties. Antioxidants play a crucial to reduce oxidative damage and hazardous effects of ROS. Metal chelating activity is one of the most applied methods in food, biological and pharmaceutical applications. In this review article, metals, heavy metals, the effects of excessive metal exposure, the importance of metal chelating in biological systems, reactive oxygen species, OS, Antioxidants, metal chelating ability, antioxidant methods, and two distinct in vitro metal chelating assays were explained in details.

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