# The bioactivity of Poplar root extracts as a novel treatment for iron overload in a THP-1 cell culture model

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A Manuscript Submitted in Partial Fulfillment of the Requirements for the Degree of
**BACHELOR OF BIOLOGY HONOURS**
In the Faculty of Biology

Julianne Anderson, 2022, University of Victoria

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## Abstract

Iron is an essential element for all living beings. However, either too little or too much iron can be detrimental. Non-protein bound iron is highly reactive due to its' ability to easily accept and donate electrons. This makes iron a powerful and essential component in many metabolic and physiological processes, but it also poses a potential oxidative danger. Consequently, iron uptake, storage, and recycling are tightly regulated under normal circumstances. In cases of systemic iron overload, excess iron in the body causes extensive damage to DNA, proteins, and other macromolecules, eventually leading to tissue and organ damage. The current leading treatment for diseases of iron overload in humans is chelation therapy, in which iron chelators, molecules that bind and sequester iron, are administered. However, chelator treatments can have cytotoxic effects, driving the search for novel chelator treatment options. In my research, the potential bioactivity of iron chelators present in poplar root extracts were investigated in a simple iron-overload model using a human leukemia monocytic THP-1 cell line. Root extracts were harvested from poplars grown under iron reduced conditions, which we hypothesise to stimulate biosynthesis of chelators, and iron normal conditions as controls. The impact of these growth conditions on root chelator production was examined. To assess the potential of poplar root extracts as a novel bioactive chelators for iron-overload treatment in humans, we measured the intracellular iron content of THP-1 cells under iron overload conditions and after treatment with the clinically used iron chelator deferoxamine in comparison to root extracts. Intracellular iron normalized to protein concentrations as a proxy for cell number. Intracellular iron concentration, measured using ferrozine, was increased in response to chronic iron treatment relative to control cells grown under iron normal conditions. Relative to cells treated with chronic iron overload, it was found that DFO reduced intracellular iron content of chronically iron overloaded cells by 33% ($p = 0.020$). Iron normal and iron reduced poplar root extracts also reduced iron content by 17% and 15% on average, but these trends were not significant. There was no significant difference between the iron content of cells treated with either poplar root extract and DFO. Unexpectedly, there was no difference in the iron content of cells treated with extracts from roots of plants grown under iron normal and iron reduced poplar conditions ($p = 0.878$). Trypan

blue staining illustrated that chelator treatments had little effect on cell viability, indicating low cytotoxicity of root extracts. Future investigations of the potential bioactivity of iron chelators present in poplar root extracts should examine the chelating abilities of poplar root extracts with increasing concentration. Furthermore, metabolic characterization of the root extracts would help identify individual compounds of interest, and to improve the effectiveness of the screening process.

## Introduction

### *Iron homeostasis in humans*

Iron is an essential element for all living cells (Skibsted, 2016). Iron is an important cofactor in proteins that catalyze essential metabolic reactions, such as in electron transport chains of the mitochondria and chloroplasts (Romheld and Marschner, 1981; Geissler and Singh, 2011). In animals, iron is most often incorporated into prosthetic groups of proteins that are crucial to red blood cell formation, oxygen transport, and pathogen defense (Ganz, 2013). Mammals, including humans, get most of their iron through their diet as heme or non-heme iron. Heme iron is found in meat and fish, derived from hemoglobin. Non-heme iron is mostly associated with nuts, seeds, and vegetables, though it is also present in animal products. Dietary iron is absorbed by enterocytes of the gastrointestinal tract as food is digested (Knutson, 2017). Once absorbed and processed within the enterocyte, the majority of iron is either stored intracellularly in enterocytes bound to ferritin, a protein that sequesters iron, or exported to the blood via ferroportin, a transmembrane transport protein, as ferric iron ($Fe^{3+}$) (Richardson and Ponka, 1997; Knutson, 2017). In the blood plasma, the exported $Fe^{3+}$ is immediately bound by transferrin, a plasma protein that is able to bind two $Fe^{3+}$ ions. In the sink cell, the $Fe^{3+}$-transferrin complex binds to the cell surface transferrin receptor and is taken into the endosome by receptor-mediated endocytosis (Sendamarai *et al*., 2008). Within the endosome, the iron is released from ferritin and is reduced by ferrireductases to ferrous iron ($Fe^{2+}$). The $Fe^{2+}$ is then transported back across the endosomal membrane by divalent metal transporter 1, and is stored in cytosolic ferritin (Knutson, 2017; Katsarou and Pantopoulos, 2020).

The majority of systemic iron is present in hemoglobin (Ganz, 2013). Hematoblasts, pluripotent stem cells of the blood, synthesize hemoglobin and differentiate into erythrocytes (Knutson, 2017). In the differentiation process, called erythropoiesis, $Fe^{3+}$ previously bound to transferrin is incorporated into hemoglobin of newly formed erythrocytes, which remain in circulation until they are degraded and recycled by macrophages (Knutson, 2017; Katsarou and Pantopoulos, 2020). Hemoglobin is required for the transport of oxygen throughout the body. Oxygen is bound by the heme group of hemoglobin and is delivered to tissues as blood is circulated (Geissler and Singh, 2011). The remainder of iron is incorporated into myoglobin in the muscle or in other tissues as metalloproteins (Katsarou and Pantopoulos, 2020).

In humans, iron uptake and storage are primarily regulated by the peptide hormone hepcidin (Steinbicker and Muckenthaler, 2013). Hepcidin regulates iron storage by causing the internalization and degradation of ferroportin, effectively preventing the efflux of intracellular iron stores (Steinbicker and Muckenthaler, 2013; Katsarou and Pantopoulos, 2020). Hepcidin synthesis is upregulated by high concentrations of transferrin in the plasma, high concentrations of iron in the liver, and inflammation (Knutson, 2017). Hepcidin synthesis is downregulated, and thus iron release stimulated, when there is an increased rate of erythropoiesis (Steinbicker and Muckenthaler, 2013; Knutson, 2017). The presence of iron transport proteins, such as transferrin, also prevents excess iron from accumulating in the body. Non-protein bound iron (iron that is not stored in ferritin, transported by transferrin or being used in enzymes or hemoglobin) is highly reactive and cytotoxic due to its' ability to easily accept and donate electrons (Crichton et al., 2002; Ganz 2013). Under normal physiological conditions, only one third of transferrin is bound to iron (Katsarou and Pantopoulos, 2020). The remaining two thirds of transferrin form a buffer to bind iron as it enters circulation via ferroportin on enterocytes, hepatocytes or macrophages, thereby preventing any cytotoxic effects and tissue damage that excess iron may cause (Knutson, 2017; Katsarou and Pantopoulos, 2020).

Compared to iron-deficiency, iron-overload is a fairly rare condition. This may be the reason that regulated iron excretion pathways don't exist in humans and iron loss is typically non-specific, while iron uptake and homeostasis is strictly regulated (Hallberg, 2001). Most iron

is lost in small amounts by the loss of blood and the shedding of dead skin cells. While a lack of iron excretion pathways is generally a beneficial design due to the limited bioavailability of iron, it can also cause major problems if normal iron homeostasis is disrupted (Ganz, 2013; Skibsted, 2016). When iron accumulates to elevated levels, existing mechanisms to sequester excess iron are overwhelmed. This results in excess iron in circulation and subsequent cytotoxic effects.

### *Diseases of iron overload*

Excess iron accumulation causes tissue damage as iron cations participate in the self-renewing Fenton reaction and produce reactive oxygen and nitrogen species (ROS, RNS) (Crichton *et al*., 2002; Xie *et al*., 2016; Yu *et al*., 2020). ROS and RNS readily react with many macromolecules, causing cellular damage (Di Meo *et al*., 2016; Xie *et al*., 2016). One ROS produced by the Fenton reaction is the highly reactive hydroxyl radical, which reacts with DNA and proteins, and causes self-renewing lipid peroxidation reactions (Crichton *et al*., 2002; Li *et al*., 2018). Furthermore, iron catalyzed formation of ROS can accumulate in tissues and can eventually result in dysfunction of major organs such as the heart, liver, spleen, pancreas or brain (Steinbicker and Muckenthaler, 2013).

Iron overload caused by low hepcidin levels and subsequent ferroportin overexpression is called hemochromatosis in humans (Steinbicker and Muckenthaler, 2013). Hemochromatosis may be caused by inherited or acquired mutations of genes directly involved in iron homeostasis (primary iron overload), or by diseases that indirectly lead to an accumulation of iron (secondary iron overload) (Papanikolaou *et al*., 2005; Ganz *et al*., 2013; Steinbicker and Muckenthaler, 2013). Primary hemochromatosis is most often caused by mutated HFE proteins (human homeostatic iron regulator protein), which help to regulate hepcidin, or ferroportin proteins that are unresponsive to hepcidin (Papanikolaou *et al*., 2005; Ganz *et al*., 2013; Steinbicker and Muckenthaler, 2013). With low or non-functional hepcidin, or unresponsive ferroportin, the body is not able to decrease ferroportin concentrations in response to systemic signals of high iron content. This unresponsiveness results in the continued efflux of iron from intracellular stores despite normal systemic iron concentrations (Steinbicker and Muckenthaler, 2013; Knutson, 2017). Furthermore, unresponsive hepcidin or ferroportin cause continued iron uptake at high levels in the duodenum (Steinbicker and Muckenthaler, 2013). Due to the

disruption of mechanisms that regulate iron uptake and circulation, systemic iron concentrations surpass storage and binding capabilities, and iron accumulates in tissues such as the liver, heart, and pancreas.

Acquired or secondary hemochromatosis may also be caused by symptoms of other diseases, for example genetic anemias, in which erythropoiesis is inefficient and iron absorption in the gut is overstimulated (Papanikolaou *et al*., 2005; Ganz, 2013; Steinbicker and Muckenthaler, 2013). Genetic anemias are most commonly caused by mutations in the genes for hemoglobin production that limit erythrocyte production (Ganz, 2013). Insufficient or non-functional erythrocytes signal the body to increase iron absorption in the duodenum, but because the synthesis of erythrocytes is compromised, this iron cannot be incorporated into erythrocytes at a sufficient rate, therefore resulting in excess iron accumulation. Genetic anemias are therefore distinct from other anemias, which are caused by nutritional iron deficiency and subsequent reduced erythropoiesis rates. Still, the systemic response to either condition is increased iron uptake and mobilization.

In many genetic anemias, iron absorption is upregulated despite high iron concentrations due to significant deficiency in erythroid biogenesis and hemoglobin biosynthesis (Papanikolaou *et al*., 2005). Again, due to the lack of major iron excretion pathways in mammals, continued absorption of iron causes systemic iron concentrations to overwhelm mechanisms to sequester and recycle excess iron. Inefficient erythropoiesis and increased iron absorption are both characteristic of beta-thalassemia, a comparably common form of a genetic anemia (Papanikolaou *et al*., 2003; Mobarra *et al*., 2016). In more severe forms of the disease, such as transfusion-dependent beta-thalassemia, mutations greatly reduce the ability of the patient to synthesize functional hemoglobin or erythrocytes (Steinbicker and Muckenthaler, 2013). In these cases, frequent blood transfusions are required to compensate for the loss of oxygen transport, further exasperating iron overload. In all diseases of iron overload, excess iron can cause damage to major organs and to the endocrine system (Poggiali *et al*., 2012).

### *Treatments for diseases of iron overload*

Iron chelation therapy is currently a leading treatment for diseases of iron overload (Christensen *et al*., 2001; Mobarra *et al*., 2016). The goal of iron chelator therapy is to introduce an exogenous supply of small molecular weight chelators, such that non-protein bound iron may be sequestered and excreted as an organo-metal complex into the urine or bile, therefore shielding surrounding tissues from the cytotoxic effects of non-protein bound iron. Several iron chelators have been developed for treatment of iron overload. Some commonly used clinical iron chelators include deferiprone (DFP) and deferoxamine (DFO) (Mobarra *et al*., 2016). DFP is administered orally and is often given to patients that have not had sufficient responses to other chelator treatments. DFO is a hexadentate chelator that is administered as a subcutaneous or intravenous injection (Poggiali *et al*., 2012). Because of its widespread use as a clinical chelator treatment for diseases of iron overload, DFO was used as a positive control chelator treatment in this thesis.

DFO has been clinically approved for long-term use in combatting iron overload and is used to treat transfusion-dependent beta-thalassemia (Poggiali *et al*., 2012; Mobarra *et al*., 2016). DFO binds to iron at 1:1 molar ratio, and binds to excess iron in the muscle, in the liver, and to previous erythrocyte iron being recycled by macrophages (Mobarra *et al*., 2016). After binding to excess iron, DFO molecules are expelled from the body via urine or bile (Christensen *et al*., 2001; Mobarra *et al*., 2016). DFO has been shown to significantly decrease intrahepatic and serum iron concentrations, consequently decreasing iron deposition in tissues and improving endocrine function (Christensen *et al*., 2001; Poggiali *et al*., 2012).

Despite DFO's ability to decrease systemic iron concentrations, DFO's effectiveness as a treatment is burdened by uncomfortable administration and side-effects (Christensen *et al*., 2001; Poggiali *et al*., 2012; Mobarra *et al*., 2016). Due to a short plasma half-life, DFO must be administered by injection multiple times each day for 5-7 days per week (Poggiali *et al*., 2012). Cytotoxic effects of DFO at high concentrations further impede its' success as a treatment for iron overload (Christensen *et al*., 2001; Poggiali *et al*., 2012; Mobarra *et al*., 2016). Alongside common side-effects of many medications such as nausea and dizziness, high dosages of DFO have been associated with visual and auditory neurotoxicity, bone toxicity, and organ failure

(Christensen *et al*., 2001; Poggiali *et al*., 2012; Mobarra *et al*., 2016). These cytotoxic effects place constraints on the possible dosages that may be used to safely treat diseases of iron overload without inflicting significant damage to the body from the chelator's effects. Because DFO binds to iron at a 1:1 molar ratio, ideal dosages are therefore not attainable (Christensen *et al*., 2001). The toxic effects of iron chelator treatments such as DFO drive the search for novel iron chelator treatments that may offer a safer and easier alternative treatment for people with diseases of iron overload.

Throughout history, plants have been used extensively as medicines and treatments for a variety of ailments (Petrovska, 2012). Plants still offer a valuable source for medicinal compounds, as exemplified in the widely used heart medication digitalis, derived from the foxtail plants of the genus *Digitalis* (Ehle *et al*., 2011). Consequently, plant-derived compounds are a logical new frontier for the search for novel chelators

### *Iron uptake in plants*

Despite being the most abundant heavy metal in the earths' crust, iron is poorly soluble in soils, and is therefore a limiting nutrient for plants (Marschner and Rengel, 2007). Low soil mobility drives plants to alter their root development and biochemistry to increase iron capture efficiency (Romheld and Marschner, 1981; Marschner *et al*., 1986; Subramani *et al*., 2021). There are two distinct strategies to mitigate iron deficiency in plants, both of which involve exudation of chelating compounds from the roots into the surrounding soil. Strategy II, commonly seen in graminaceous monocots (grasses), involves the secretion of phytosiderophores into the surrounding soil (Marschner *et al*., 1986). Phytosiderophores are iron chelators that are secreted into the rhizosphere and are taken back up into the plant tissues once bound to iron (Marschner *et al*., 1986). The phytosiderophore-iron complexes re-enter plant roots via a transport system that is specific to plants that utilize strategy II (Marschner *et al*., 1986; Marschner and Rengel, 2007). Strategy I, seen in dicots and non-graminaceous monocots, is characterized by increased plasma membrane-bound iron reductase activity, acidification of the rhizosphere by increased proton pumping, and secretion of iron-chelating phenolic compounds into the soil (Marschner *et al*., 1986; Marschner and Rengel, 2007). Acidification of the rhizosphere increases iron solubility in the soil water, and the

rate of iron uptake of the roots is improved by an increase in their iron reducing capacity at the root surface (Romera *et al*., 1992; Marschner and Rengel, 2007). Iron reducing capacity is essential in the ability of plants to take up iron from the soil, as ferric iron ($Fe^{3+}$) is the predominant form of iron in soils, but strategy I plants take up iron in the ferrous ($Fe^{2+}$) form (Romheld and Marschner, 1981).

It has been demonstrated that plants that utilize iron uptake strategy I increase iron chelator production in their roots in response to decreased iron solubility or availability (Romheld and Marschner, 1981; Marschner *et al*., 1986). This suggests that strategy I plants, when grown in iron deficient soils, may provide an even greater source of iron chelators than those grown in iron normal soils. Furthermore, while strategy II plants mainly use phytosiderophores that are mugineic acid derivatives, strategy I plants use a greater variety of chelators, therefore increasing the chemical diversity of the candidate pool (Suzuki *et al*., 2021).

Current research into plant-derived chelator treatments have been focused on strategy II plants and are therefore somewhat specific to siderophore effectiveness in iron overload treatment. For example, *Triticum aestivum Linn*., a monocot that employs iron uptake strategy II, has been illustrated as effective in decreasing systemic iron concentrations in mice experiencing iron overload (Das *et al*., 2012). This demonstrates that plant-derived iron chelators can offer a potential treatment of iron overload in mammals. Further research is needed to investigate the effectiveness of iron chelators produced by plants that employ iron uptake strategy I and the potential for increased chelator yield from strategy I plants grown in iron reduced soils. Poplar is one such strategy I plant and is the focal species of this thesis.

Poplar is rich in abundance and variety of phenolic compounds (Pannucci *et al*., 2022). Phenols and polyphenols have high ROS scavenging and antioxidant abilities, and have proven effective in binding metal ions, such as iron (Li *et al*., 2014). A high concentration and diversity of phenolics make poplar a good plant to use as a chelator treatment. Furthermore, the entire genome of poplar has been sequenced, and their secondary metabolic pathways are comparably well-described (Tuskan *et al*. 2004). The easy growth of poplar cuttings in hydroponic systems enables the manipulation of iron availability and root harvest, making poplar ideal for practical experimental purposes as well. Overall, the high content of iron-

binding compounds, well-established secondary metabolic pathways, and low-maintenance growth conditions of poplar make this model plant ideal for our research purposes.

### *Goals and approaches*

*Populus* species are rich in salicinoids, flavonoids, phenolic esters and amides, and numerous and other phenolic compounds produced through the phenylpropanoid pathway, many of which have iron chelating abilities (Veach *et al*., 2019). For example, tannins are compounds produced in the phenylpropanoid pathway that has been shown to have iron chelating abilities (Phiwchai *et al*., 2018). The high content of phenols and polyphenols in poplar, as well as well-described pathways from which they are synthesized, make this genus a promising candidate for the isolation of novel phytochelators (Tuskan *et al*., 2004; Pannucci *et al*., 2022).

Before being tested extensively on animal models and going through expensive and time-consuming clinical trials, novel drugs are first tested in much more simple cell culture systems. In my project, poplar root extracts are tested on cells of the THP-1 cell line. THP-1 cells are a human leukemia monocytic cell line that are commonly used as an innate immune system model of monocytes and macrophages (Bocchietto *et al*., 2007; Chanput *et al*., 2014). Monocytes aid in inflammatory responses and are the first line of defence in sequestering iron away from pathogens (Byrd and Horwitz, 1989). Furthermore, monocytes can differentiate into macrophages, which are a crucial component of iron homeostasis due to their role in red blood cell recycling (Byrd and Horwitz, 1989; Shan *et al*., 2008). THP-1 cells are frequently used in biomedical studies due to their immortality and representation of human peripheral monocytes *in vitro*. Their role in iron metabolism and representation of human monocytes make THP-1 cells ideal for our research purposes.

My research aims to quantify iron chelation abilities of phytochemicals derived from hybrid poplar (*Populus trichocarpa* x *deltoides* H11/11) roots on human monocytes experiencing iron overload. We hypothesize that phenolic compounds present in poplar root extracts will reduce intracellular iron content of THP-1 cells grown exposed to chronic iron treatment. Furthermore, we hypothesize that root exudates of poplars grown in conditions

with low iron availability will have a greater chelating capabilities than root exudates of poplar grown under iron normal conditions. We assess the potential of poplar root exudates to successfully mitigate iron overload in human cells to inform further experiments on the development of novel bioactive iron-chelators.

My research also aims to investigate the effect of soil iron availability of the iron chelating properties of *P. trichocarpa* x *deltoides* root extracts. We are attempting to stimulate iron-chelating metabolite production in poplar roots by depriving the growing plants of iron. We hypothesize that root phenolic production, or at least iron-chelator production, will increase when exposed to iron deficiency in comparison to poplars grown with normal iron availability.

## Methods

*Disclaimer on collaborative work*: This project was performed in close collaboration with Sarah Lane, PhD candidate in the Ehlting lab. Poplar growth, root extract preparation, and the phenolic Folin-Ciocalteu assay were performed by Sarah Lane, but the methods are described here for completeness. Cell culturing, iron and chelator treatments, ferrozine assays, and data analysis were performed by Julianne Anderson.

### *Chemicals and reagents*

Chemicals and reagents were purchased from Gibco, Fischer Scientific or Sigma-Aldrich, unless otherwise specified. Details of reagents and chemicals used are found in Appendix A.

### *Poplar growth and root extract preparation*

Poplar cuttings (collected from dormant trees located on the Centre for Forest Biology Compound at the University of Victoria) were grown in controlled environmental chambers at 21°C with 16 hours of 300 µE light per day in a spray aeroponics system to develop roots. Modified ½ Hoagland's [6000 µM $KNO_3$, 4000 µM $Ca(NO_3)_2$, 1000 µM $NH_4H_2PO_4$, 2000 µM $MgSO_4$, 46 µM $H_3BO_3$, 9 µM $MnCl_2$, 0.8 µM $ZnSO_4$, 0.1 µM $Na_2MoO_4$ 0.3 µM $CuSO_4$, and 254.8 µM [Fe(III)citrate]$NH_4$ containing 200 µM available Fe(III)] nutrients diluted in distilled water

was used as a growth solution. A volume of 1000 µM MES (2-(N-morpholino)ethanesulfonic acid) was added to growth solutions to maintain a constant pH of 6.4. To stimulate root formation, 0.5 µM 1-naphthaleneacetic acid was added to growth medium until roots began to form in at least 80% of the poplar cuttings.

To reduce media volumes and to enable biological replicates, plants were transferred to 1 L amber glass jars in modified ½ Hoagland's nutrients as above for three days after the plants had sufficient root mass. Plants were placed into 8 groups with approximately equal root masses, then randomly assigned to either control (n = 4) or iron reduced (n = 4) treatments. Control poplars were grown for one week in modified ½ Hoagland's nutrients as above. Iron reduced poplars were grown for one week in above media with 25.48 µM [Fe(III)citrate]$NH_4$ containing 20 µM available Fe(III). Media was changed daily and aerated constantly.

The roots of each plant were harvested, flash frozen in liquid nitrogen and stored at -80°C until needed for extract preparation. Frozen root tissue was ground to a fine powder in liquid nitrogen. Root extracts were prepared by adding 1.5 mL of UPLC grade MeOH to each sample of 200 mg ground frozen root tissue. Samples were homogenized with glass beads using a Precellys 24 Tissue Homogenizer at 5000 x g for 2 x 45 seconds. Samples were sonicated in a 75T VWR water bath sonicator for 10 minutes. Samples were centrifuged in a Thermo scientific CL 2 centrifuge for 15 minutes at 15000 x g, and the supernatant was removed and set aside. A volume of 1.0 mL of MeOH added to each sample and samples were homogenized, sonicated, centrifuged, and the supernatant was removed and set aside for a second time. This was repeated once more, such that the total volume of supernatant collected from each sample was approximately 3.5 mL. The methanol extract of each tissue sample was dried in a SpeedVac overnight and weighed. The difference between the initial and post-extract mass of the tissue samples served as an extract mass measurement.

A small amount of sample was analysed for total phenolic content using the Folin-Ciocalteu method, as described by Schwarz (2017). Briefly, a standard curve was prepared from serially diluted 2 mg/mL gallic acid. A volume of 20 µL of root extract samples were incubated with 100 µL of Folin reagent for 5 minutes. Samples were centrifuged at 16100 x g for 5

minutes. Their supernatant was transferred to a 96-well plate and absorbances were measured at 730 nm in a VICTOR$^{TM}$ X5 2030 multilabel plate reader (Perkin Elmer, model 2030-0050).

Root tissue extracts were resuspended in a small volume of 30% ethanol in phosphate-buffered saline (PBS) and centrifuged at 15000 x g for 5 minutes. The supernatant was set aside and sterile filtered into sterile tubes. Root extracts were stored at -20°C under darkened conditions until needed.

### *Cell passaging and iron treatment*

Protocols for cells passaging and iron treatment were developed by Sarah Lane.

THP-1 cells were maintained from the American Type Culture Collection (ATCC). THP-1 (TIB-202) cells in prepared RPMI media containing 2 mM GlutaMAX, 1% (v/v) penicillin-streptomycin, and 10% (v/v) fetal bovine serum were passaged every 2-3 days to maintain a concentration of 25 x $10^5$ cells/mL. Cell morphology and health were microscopically investigated, and cells were counted at every passage to quantify cell viability and concentration. Cells were cultured in T75 tissue culture flasks at 37$^o$C and 5% $CO_2$ in a FormaScientific incubator (model 3158).

Iron citrate and citrate blank solutions were prepared by adding 2.79 mL AAS iron standard (1001 mg/L ± 4 mg/L) and 1.0 mL of 0.2 M MOPS (3-(N-morpholino)propanesulfonic acid) buffer to 1.0 mL 1 M citrate dropwise in succession while stirring constantly. The pH of the solution was adjusted by adding NaOH dropwise until a pH of 7.4 was reached. In succession, 1.29 mL 0.5 M NaOH and 0.210 µL dd$H_2O$ were added while stirring constantly. The final solution had a final pH of 7.4. A citrate blank was prepared in the same manner using 2% $HNO_3$ solution instead of AAS.

Iron treated and no-iron control cells had iron citrate or citrate added to their media such that the culture media had a final concentration of 20 µM iron citrate or citrate, respectively (Figure 1). Iron treatment was continued for 12 days, over five passages.

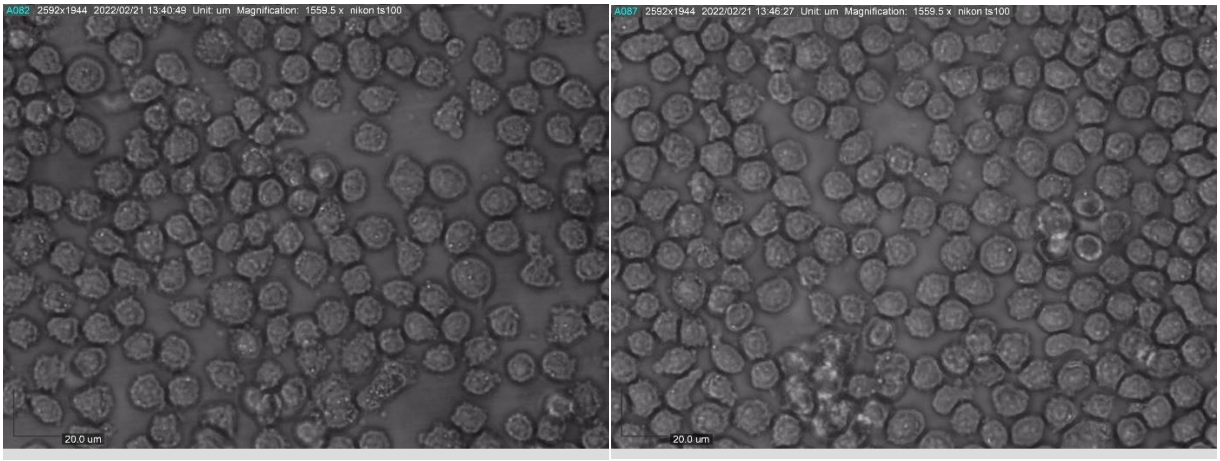


**Figure 1.** THP-1 monocytic cell line cells grown under iron overloaded (20 µM iron citrate; left) and iron normal conditions (20 µM citrate right), as pictured with a Nikon ts100 camera.

## *Chelator preparation and chelator treatment*

A deferoxamine (DFO) solution was prepared such that it had a final concentration of 10 mM DFO in 30% EtOH in PBS solution. Cell cultures were transferred to a 6 well plate at a concentration of $6x10^6$ cells per well. Each well plate was treated with a chelator or blank such that each well had a final concentration of 30 µM DFO, 30 µM gallic acid equivalents (GAE) of root extracts, or an equal volume of blank solvent. Cells were incubated on an orbital shaker at 60 x g in an incubator at 37°C and 5% $CO_2$ for 18 hours.

Cell cultures were removed from well plates. Wells were rinsed with 2 mL PBS and the wash was added to the original cell media. One random sample was taken from each growth condition to investigate cell viability. Cells were centrifuged at 1200 x g and 21°C in a Thermo Scientific CL 2 centrifuge for 5 minutes, and the supernatant was removed. Wells were rinsed with 3 mL 500 µM EDTA in PBS and the wash was added to the cell pellet. Cells were centrifuged at 1200 x g for 5 minutes. The supernatant was removed, and 6 mL PBS was added to the cell culture. Cells were centrifuged at 1200 x g for 5 minutes, and supernatant was removed. This step was repeated one more time. Cells were resuspended in 80 µL PBS, and 5 µL (6.25%) of each sample was set aside for protein determination. The remainder of cells in PBS were centrifuged at 1200 x g for 5 minutes, and supernatant was removed. Dry pellets were stored at -20°C.

Cell viability counts were preformed from the random sample of each chelator treatment collected after 18 hours of chelator treatment using trypan blue staining and hemocytometer counting.

### *Ferrozine and protein assays*

Cell samples were thawed and 100 µL Cell-lytic M was added to each sample. Cell samples were incubated on an orbital shaker for 2 hours at 200 x g at 25°C. Serial dilutions of 300 µM $FeCl_3$ were prepared, and 100 µL Cell-Lytic M was added to each standard. A volume of 100 µL 10 mM HCl was added to each sample. A volume of 50 µL 1.4 M HCl and 50 µL 284.8 mM $KMnO_4$ in $ddH_2O$ were added to each cell lysate and standard. Samples and standards were incubated at 60°C for 2 hours in the dark.

Iron detecting reagent was prepared such that the final 10 mL solution had a concentration of 6.5 x $10^{-3}$ M neocuproine, 6.8 x $10^{-3}$ M ferrozine, 2.5 M ammonium acetate, and 1.0 M ascorbic acid.

Cell lysates and iron standards were cooled to room temperature, and 30 µL of iron detecting reagent was added to each tube. Tubes were incubated at 21°C for 30 minutes under darkened conditions. Tubes were centrifuged at 15000 G for 5 minutes, and 280 µL of supernatant from each tube was transferred into a 96-well plate in duplicate. The well plate was kept in the dark at 25°C for half an hour. Absorption of samples were measured at 550 nm in a VICTOR™ X5 2030 multilabel plate reader. Protein concentrations of cell samples were determined by performing a Pierce™ BCA protein assay according to manufacturer instructions.

### *Data analysis*

The absorbances of iron and protein standards were graphed in standard curves, and linear regression analyses results were used to interpolate iron and protein concentrations from cell lysates in MS Excel. The original iron and protein concentrations of cell lysates were calculated by correcting for dilution factors. The ratio of iron to protein concentrations were calculated for each sample. Relative iron content (%) to cells treated with iron overload was calculated for each growth condition. Averages and standard errors of the relative iron content

(%) in intracellular iron concentration to iron overload conditions were calculated from four replicates per growth condition (n = 4) in MS Excel.

Summary statistics of the raw intracellular iron concentrations (ng/mL) and relative iron content (%) compared to iron overload conditions were generated for each chelator treatment in R Studio (R Core Team, 2019). Grubb's outlier tests were performed with $\alpha = 0.05$. Grubbs tests on the control and CrFe cells used 10 degrees of freedom, DFO-treated cells had their outliers tested with 9 degrees of freedom, and iron normal and iron reduced poplar root extract -treated cells had their outliers tested with 6 degrees of freedom. A Kruskal-Wallis test was performed on all collected data to investigate if the mean relative iron content differed across all chelator treatments. Paired Wilcoxon rank sum exact tests were performed to investigate the differences in relative iron content (%) between chelator treatments. All Wilcoxon rank sum tests used $\alpha = 0.05$.

Average cell mortality (%) was calculated for each chelator treatment from total cell counts and dead cell counts for each experimental replicate. Averages were calculated from four replicate counts per chelator treatment per experiment. Calculated average cell mortalities were graphed in a stacked bar plot using R studio. Kruskal-Wallis rank sum tests were performed in R studio to investigate the differences in average cell mortality between chelator treatments and between experiment replicates of each chelator treatment. Kruskal-Wallis tests investigating the differences between chelator treatments used $\alpha = 0.05$ and 3 degrees of freedom. Kruskal-Wallis tests investigating the differences between experimental replicates of chelator treatments used $\alpha = 0.05$ and 1 degree of freedom.

## Results

*Populus trichocarpa* x *deltoides* (H11/11) cuttings were rooted aeroponically and grown in hydroponics under either iron normal or iron deficient conditions. Root tissues were harvested from four biological replicates each of iron reduced and iron normal poplars (n = 4) and frozen prior to use. For practical reasons, the root tissues of iron reduced poplars and iron normal poplars were each pooled to generate a single root sample for each growth condition. Phenolics assays quantified the total phenolic content of methanol-soluble root extracts. For

each experimental replicate using THP-1 cells (b - d), a fresh methanol extract from each pooled root sample was prepared. Root extracts of poplars grown under iron normal conditions contained 371.4 µg/mL total phenolics in experiment b, and 355.6 µg/mL total phenolics in experiment c, and 321.5 µg/mL total phenolics in experiment d (figure 2). Root extracts of poplars grown under iron reduced conditions contained 338.9 µg/mL total phenolics in experiment b, 405.8 µg/mL total phenolics in experiment c, and 384.76 µg/mL total phenolics in experiment d. Total phenolics data were converted to gallic acid equivalents (GAE), a unit used as a proxy for the total phenolic content of extracts by comparing the total phenolic content of root extracts to that of the phenolic chelator gallic acid. All poplar extracts were diluted to allow using a final concentration of 5.103 µg phenolics/mL, or an equivalent of 30 µM gallic acid, in subsequent human suspension THP-1 cell assays.

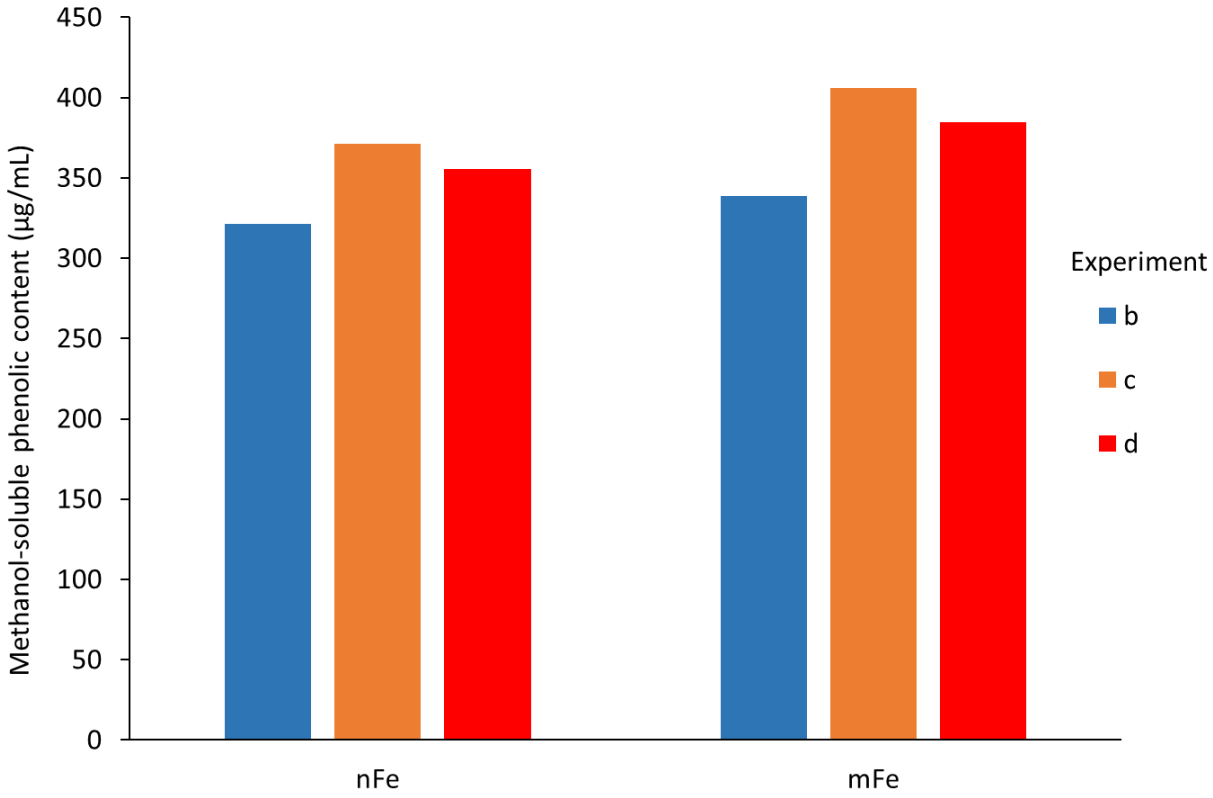


**Figure 2.** The total methanol-soluble phenolic content (µg/mL) of pooled root extracts of poplars grown under iron normal (nFe) and iron reduced (mFe) poplar extracts, as measured across three experimental batches (colour coded as indicated).

THP-1 suspension cells were grown under excessive iron or iron normal treatment for 12 days. Iron normal cells were not treated with a chelator and served as a no-treatment control.

Iron-treated cells were subsequently treated with either no chelator (CrFe), deferoxamine (DFO), iron normal poplar extract (nFe), or iron reduced poplar extract (mFe) for 18 hours. Following chelator or blank treatment, assays were performed to quantify protein and intracellular iron concentrations.

A total of four independent biological replicate experiments were performed (experiments a – d). Four replicates per treatment were performed per experiment, for a total of 72 samples tested. Experiment a was performed without iron normal (nFe) or iron reduced (mFe) poplar extract treatments to reduce sample numbers in this first experimental set. Experiments b, c, and d included the complete set of treatments, that was i) iron normal and iron reduced poplar root extract treatments on iron-exposed cells, ii) cells treated without iron or a chelator (Ctrl), iii) chronically iron-treated cells (CrFe), and iv) iron-exposed cells treated with the clinical chelator deferoxamine (DFO). Experiments b and c were full biological replicates. Experiment d was excluded from data analysis due to the protein assays from these samples being oversaturated and outside of the linear range determined by a standard curve. The saturation of the standard curve resulted in underestimation of protein concentration of these samples. See appendix B for supplementary information regarding this decision. Any data points that were extrapolating iron concentrations past the range of the iron standard curve were also excluded. Following the removal of outliers and samples that were removed due to error, data from a total of 48 samples were analyzed.

A Kruskal-Wallis rank sum test concluded that there was differences in the means of intracellular iron content between chelator treatments ($p = 9.1 \times 10^{-5}$, $df = 4$). These differences were further investigated in a pairwise fashion using Wilcoxon rank sum exact tests.

Intracellular iron concentration was normalized to the average iron content measured in chronically iron-treated cells, which served as a model for a disease-state. Each experimental replicate (a to c) was normalized to their respective chronically-iron treated cells. Comparison of the relative iron content (%) between treatments illustrated that excessive iron treatment elevated intracellular iron content, as expected. All chelator treatments decreased the intracellular iron content of iron-treated cells. However, no chelator treatment decreased

intracellular iron contents back to levels seen in control cells grown under iron normal conditions. Control cells had the least variation of all treatments. In contrast, there was high variation in the intracellular iron concentration of the chronically iron-treated cells. Chelator-treated cells had similarly high levels of variation. There was also variation across replicate experiments. For example, all chelator treatments had highly reduced intracellular iron concentrations in experiment b in comparison to other experimental replicates.

Exposing cells to chronic iron treatment and no chelator resulted in an almost fivefold increase in intracellular iron content compared to control cells, which was a significant difference (figure 3). Chronically iron-treated cells were used to normalize the iron content of all treatments, and therefore were set to a mean relative iron content of 100% in each experiment. Control cells had a mean relative iron content of 19.2 ± 0.2% (mean ± SE). DFO-treated cells had a mean relative iron content of 67.0 ± 7.5%, or a mean 33.0% decrease in iron content compared to chronically iron-treated cells, which was a significant decrease ($p = 0.020$) (table 1). However, the relative iron content of DFO-treated cells was still significantly higher than that of control cells. Cells treated with iron normal poplar extracts had a mean relative iron content of 83.1 ± 8.8% and cells treated with iron reduced poplar extracts had a mean relative iron content of 85.0 ± 16.6% (figure 3). Cells treated with either poplar root extract had the greatest variation of intracellular iron content of all treatments and cells treated with iron reduced poplar had the greatest variation in iron content. Despite a mean reduction of about 15%, the iron content of cells treated with either poplar extract did not significantly differ from chronically iron-treated cells, or from the DFO-treated cells. In all experimental replicates, the cells treated with either poplar root extract had significantly higher relative iron content compared to the control cells grown under iron normal conditions. There was also no significant difference in the intracellular iron concentration when comparing cells treated with root extracts of poplar grown under iron normal or iron reduced conditions.

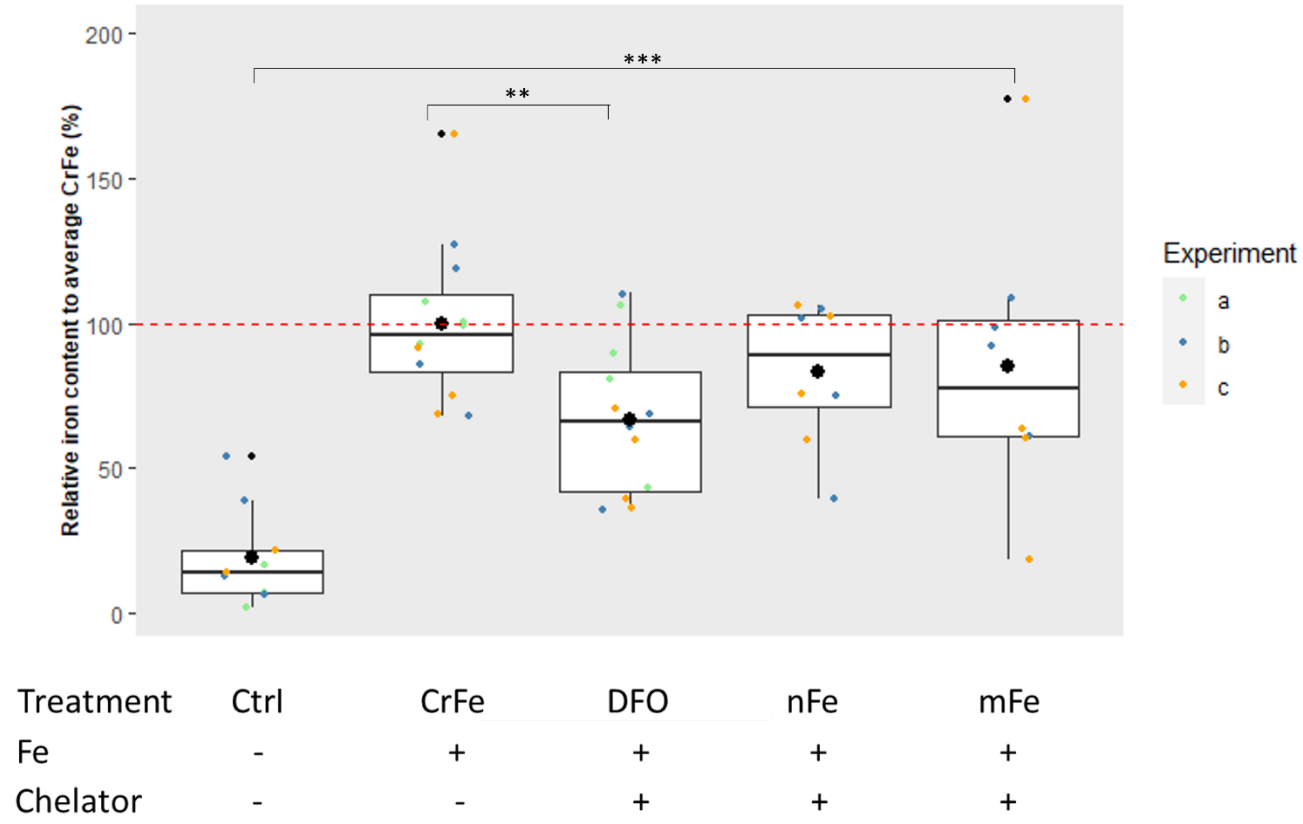


**Figure 3.** Boxplot and strip chart of the intracellular iron content (%) in human THP-1 cells relative to the mean iron concentration in cells exposed to iron overload (CrFe, set to 100% for each experiment separately). Relative iron content is shown for control cells grown under iron normal conditions (Ctrl), cells treated with the clinical iron chelator deferoxamine (DFO, 30 µM), root extract from poplar plants grown under iron normal conditions (nFe, 30 µM GAE), or under iron reduced conditions (mFe, 30 µM GAE). Mean values for each treatment were calculated from values of all experiments. Measurements were taken across three experimental batches (colour coded as indicated) with four replicate measurements of cells under each treatment per experiment (for a total of n = 12). Differences between chelator treatments were investigated using paired Wilcoxon rank sum exact tests. All Wilcoxon rank sum exact tests used data from all experimental replicates and $\alpha = .05$ (*** = $p < 0.005$, ** = $p < 0.05$). A horizontal line illustrates the mean iron content in CrFe cells set to 100%.

**Table 1.** P-values of pairwise Wilcoxon rank sum exact test comparing the intracellular iron content (%) relative to CrFe cells between cells under chronic iron treatment (CrFe), control cells (Ctrl), deferoxamine-treated cells (DFO), iron normal poplar extract-treated cells (nFe), and iron reduced poplar extract-treated cells (mFe). All Wilcoxon rank sum tests were run in R studio using pooled data from all experimental replicates (n = 3) and $\alpha = .05$.

| | CrFe | Ctrl | DFO | mFe |
|---|---|---|---|---|
| Ctrl | $6.80 \times 10^{-5}$ | - | - | - |
| DFO | 0.020 | $6.80 \times 10^{-4}$ | - | - |
| mFe | 0.340 | 0.001 | 0.525 | - |
| nFe | 0.525 | $6.80 \times 10^{-4}$ | 0.340 | 0.878 |

One cell sample was taken per treatment per experimental replicate for trypan blue cell viability counts. Cell viability staining illustrated very low levels of cell mortality in experiment b, but overall a greater degree of cell mortality in experiment c (figure 4). There was high variability in average cell mortality (in %) between experiments. Average cell mortality was not significantly different between treatments ($p = 0.881$, $\chi^2 = 0.667$, $df = 3$), but was significantly different between experimental replicates ($p = 0.021$, $\chi^2 = 5.334$, $df = 1$). The largest increase in mean cell mortality from experiment b to c was seen in the cells treated with root extracts from poplars grown under iron reduced conditions, which had 0.47% mean cell mortality in experiment b and 37.46% in experiment c.

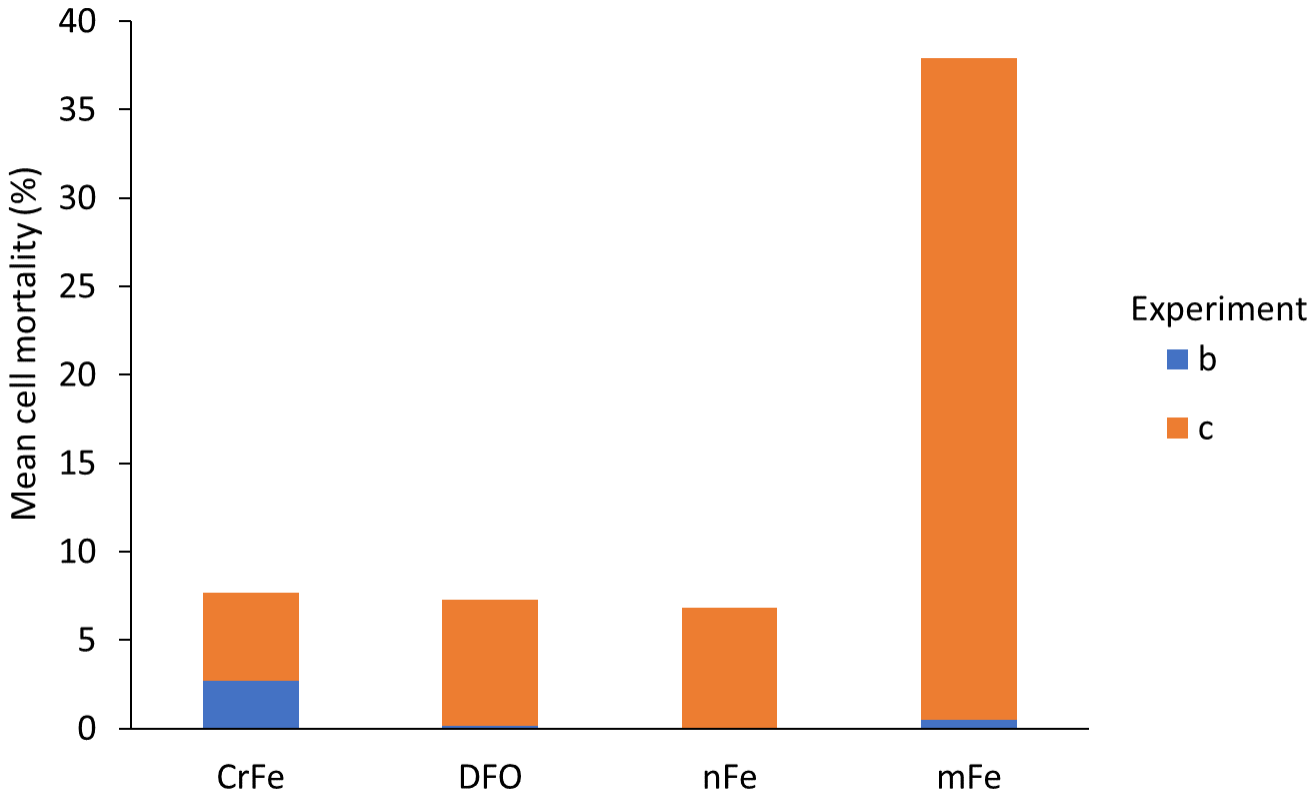


**Figure 4.** Bar graph of the mean cell mortality (%) after plating cells under iron overload (CrFe), cells treated with deferoxamine (DFO), with root extract from poplar plants grown under iron normal conditions (nFe), or with root extract from poplar plants grown under iron reduced conditions (mFe), as counted with a trypan blue stain on a hemocytometer. Measurements were taken across two experimental replicates. Averages were calculated from four cell counts per chelator treatment per experiment (n = 4).

## Discussion

Currently available clinical chelator treatments for diseases of iron overload are accompanied by side effects (Christensen *et al*., 2001; Poggiali *et al*., 2012; Mobarra *et al*., 2016). For this reason, there is great need for the development of novel chelator treatments that elicit less cytotoxic effects while remaining effective in their iron chelating ability, or as an adjunct chelator that would allow dose reductions of current clinical chelators. Plants offer one such frontier for the search of novel chelator treatments, as they have been used medicinally since ancient times and many are still used in modern medicine (Ehle *et al*., 2011; Petrovska,

2012). The main objective of this research was to investigate the potential for phenolic compounds derived from *Populus trichocarpa* x *deltoides* (H11/11) roots to successfully mitigate iron overload in mammals using a human cell culture model. I found that poplar root extracts reduced intracellular iron in THP-1 cells, but this reduction was small, and due to large variation, was not significant. I also compared the iron chelating abilities of poplar root extracts from iron normal and iron reduced plants to investigate the impact of iron availability on the phenolic production and chelating ability of poplar roots. In contrast to the hypothesis that iron deficiency would increase iron chelator biosynthesis in poplar roots, no difference in chelator bioactivity was found when comparing the iron content of cells treated with poplar root extracts of plants grown in iron normal and iron reduced conditions.

It was clear that intracellular iron content of THP-1 cells was increased in response to chronic iron treatment. Cells that were chronically treated with iron and not exposed to a chelator had an almost fivefold mean increase in intracellular iron content in comparison to control cells. Iron normal and iron reduced poplar root extracts reduced iron content by 16.9% and 15.0% on average, but these decreases were not significant. Unexpectedly, there was no difference in the iron content of cells treated with iron normal and iron reduced poplar root extracts. Trypan blue staining illustrated that chelator treatments had little effect on cell viability, indicating low cytotoxicity of root extracts, though there was high variability in cell viability counts between experimental replicates.

Cells treated with iron normal and iron reduced poplar extracts decreased intracellular iron contents of cell iron overload by 17% and 15% on average, respectively. The mean intracellular iron contents of cells treated with either extract were not significantly different from the DFO-treated cells. This was expected, as we know that poplar extracts contain an abundance of phenolics, many of which have chelating abilities, and would therefore expect these extracts to behave as chelators of a similar strength to DFO (Marschner *et al*., 1986; Marschner and Rengel, 2007; Pannucci *et al*., 2022). However, the reductions in intracellular iron content by the root extracts were not found to be significant from cells under iron overload. The lack of significance of these results was likely due to the high level of variation in cells treated with the poplar extracts. Notably, the variation was greater in cells treated with

root extracts from iron reduced poplars than in cells treated with root extracts from iron normal poplars. The modest iron chelating abilities of poplar extracts and the high variation seen in each treatment were unexpected, and both results may be due to the variety of phenolics present in the root extracts, and how these phenolics varied between experimental replicates.

The phenolic concentration of root extracts that cells were treated with (5.103 µg phenolics/mL, or 30 µM GAE) was determined from previous experiments. In the preliminary results of these experiments, 5.103 µg phenolics/mL had bioactive chelating potential comparable to 30 µM DFO. However, these preliminary results were performed on N2A cells and lacked sufficient replication. For this reason, I opted to perform a large number of replicates, in independent experiments and in biological replicates within each experiment. In future experiments, a new comparison of root extracts and DFO chelating bioactivity should be performed to reassess the root extract concentrations given to THP-1 cells.

Further impacting the response of cells to poplar extracts is the variability of compounds within the extracts. When calculating the phenolic contents of root extracts, we quantify the phenolic content of the extracts based on gallic acid equivalents (GAE) but it remains unknown what fraction of these compounds actually do have chelating ability, due to the diversity and abundance of phenolic compounds in poplar extracts (Blainski *et al*., 2013; Pannucci *et al*., 2022). The detection of specific phenolic compounds was outside of the scope of this project, and so we cannot be sure how the phenols were distributed in the extracts. For example, a large amount of our phenolic content may have been on a single compound, or they may be distributed relatively evenly across many compounds.

Furthermore, since new plant extracts were prepared for each experimental replicate, there was likely variation in the make up and concentrations of the phenolic compounds in each new extract. This variation may account for differences in the iron chelating abilities of poplar extracts between experimental replicates. The greater variation present in the iron reduced poplar extracts may also be explained by these plants producing a greater variety of phenolics in their roots in response to reduced iron availability (Romheld and Marschner, 1981;

Marschner *et al*., 1986). To account for this variation, the identities of phenolic compounds in the root extracts should be investigated through mass spectrometry and chromatography. By identifying the compounds present in root extracts prior to cell treatment, specific plants may be selected for chelator extraction based on their chemical profile, and dosage calculations may be more standardized. Furthermore, these results should be validated through dose response analyses.

There was no significant difference in the iron content of cells treated with iron normal and iron reduced poplar extracts. This was unexpected, as it was hypothesized that poplars grown under iron reduced conditions would increase iron chelator production in their roots, and therefore have a greater iron chelating capacity than poplar grown in iron normal conditions (Romheld and Marschner, 1981; Marschner *et al*., 1986). The similarity of intracellular iron content between the poplar extract treatments was impacted by the overall unexpectedly low impact of both root extracts and high variance of our samples. The high variance may be attributed to variance in phenolic content of extracts, as described previously. Furthermore, if this were not a time-constrained experiment, increased sample sizes of our poplar root extract-treated cells may impact these results by decreasing standard errors and therefore increasing precision of our results. Future experiments with even higher numbers of replicates should be performed to confirm my findings.

Deferoxamine (DFO) reduced intracellular iron content of critically iron-treated cells by 33.0% on average. The reduction of intracellular iron content in chronic iron-treated cells by DFO was expected, as DFO is a widely used clinical chelator and has been shown extensively to chelate iron effectively (Crichton et al., 2002; Mobarra et al., 2016). Further supporting this result, DFO has been demonstrated to decrease intracellular non-heme iron content in iron-overloaded human erythrocytes by approximately 40% at a 50 µM/mL dose by Das et al. (2012), though this was not a significant decrease. The smaller decrease of intracellular iron by DFO in my results may be attributed to the dosing used in my experiments. My cells were treated with a lower dose of 30 µM/mL, and DFO chelates iron in a dose dependent manner (Das et al., 2012). A concentration of 30 µM/mL DFO was chosen for my experiment based on a preliminary DFO dose response curve in which 30 µM/mL had similar iron chelating activity to

compared plant root extracts. DFO-treated cells also had significantly greater mean iron content than control cells. This result was expected, as DFO did not significantly decrease blood iron concentrations in similar experiments at a 50 µM/mL dose (Das et al., 2012). However, DFO was illustrated to decrease blood iron levels to control levels in the same experiment at 100 µM/mL and 200 µM/mL doses. Future experimental replicates including higher doses of DFO may therefore supplement the results of this experiment.

My experimental design aimed to rescue chronically iron overloaded cells, which may be more difficult than preventing iron overload by chelators. Though a chronic iron treatment model simulates disease conditions well, it may be useful to expose cells to iron and chelators at the same time. Simultaneous iron and chelator treatments would test the ability for root extracts to prevent iron uptake instead of testing their ability to reduce iron content in already iron overloaded cells.

Trypan blue viability staining illustrated very low levels of cell mortality in experiment b, but a higher degree of cell mortality in experiment c, with high variability between experimental replicates. Overall, these results suggest that chelator treatments had little effect on cell viability, indicating low cytotoxicity of root extracts. However, cells treated with iron reduced poplar extracts had a sharp increase from 0.47% mean cell mortality in experiment b to 37.46% mean cell mortality in experiment c. Notably, these results were obtained from only one cell sample per treatment over two experimental replicates. Therefore, more replicates of cell viability counts should be performed prior to any conclusions being drawn. Further investigations of cell viability post-chelator treatment using more sensitive and specific cellular stains, such as Calcein AM assays (Ramirez *et al*., 2010), are currently being established in the lab.

In the future, this experiment may be replicated with multiple doses of poplar root extracts to investigate if they chelate iron in a dose-dependent manner. It appears that owing to the variability in root extracts, despite highly controlled growth conditions, dose response curves may have to be established for each root sample used. Investigation of poplar root extracts using chromatography and mass spectrophotometry such that specific phenolic compounds can be identified should also be done, so that chelator content of root extracts can be quantified

and concentrated. Overall, the experimental results would also be greatly supplemented by further biological replicates to increase the sample sizes and decrease variance of our samples.

## Conclusion

With the limitations imposed by the experimental conditions, poplar root extracts do not appear to be powerful sources of bioactive iron chelators. However, further experimental replicates are needed to validate this result. The observed bioactivity of poplar root extracts as iron chelators was likely impacted by the great variation in phenolic compounds present in poplar roots. Trypan blue staining illustrated that chelator treatments had little effect on cell viability, indicating low cytotoxicity of root extracts. However, this assay had high variability and a limited sample size, so further investigations of cell viability following chelator treatment are being performed. An investigation of phenolic compounds in root extracts through mass spectrophotometry and chromatography would help identify the phenolic chelators in root extracts, and to improve the effectiveness of poplar extracts as chelator treatments.

## Acknowledgments

I would like to thank Sarah Lane for growing the poplar plants, preparing root extracts, performing phenolics assays on the root extracts, and developing experimental protocols, as well as for being an amazing mentor. Thank you to my advisors, Dr. Juergen Ehlting and Dr. Patrick Walter for your guidance and support throughout my honours research and thank you to Dr. Raad Nashmi for letting me use the tissue culture room in his lab to perform cell culturing.

## Appendix A

**Table I**. Critical reagents table

| Reagent | Company | Catalog number |
|---|---|---|
| 1-naphthaleneacetic acid | Phytotechnology Laboratories | N600 |
| AAS iron standard | Fischer Scientific | 16596 |
| Ammonium acetate | Caledon | 1220-1-80 |
| Ascorbic acid | Sigma-Aldrich | 255564 |
| BCA protein assay | Pierce | 23227 |
| $Ca(NO_3)_2$ | Bio Basic Canada inc. | CB0258 |
| Cell-lytic M | Sigma-Aldrich | C2978 |
| $CuSO_4$ | Fisher Scientific | AC197715000 |
| EDTA | Bio Basic Canada inc. | 17892 |
| [Fe(III)citrate]$NH_4$ | Sigma-Aldrich | F5879 |
| Ferrozine | Sigma-Aldrich | 160601 |
| Fetal bovine serum | Gibco | F1051 |
| Folin reagent | Sigma-Aldrich | F9252 |
| Gallic acid | Arcos Organics | 410860050 |
| GlutaMAX | Gibco | 35050061 |
| $H_3BO_3$ | Bio Basic Canada inc. | BB0044 |
| HCl | Fisher Scientific | 351280-500 |
| $HNO_3$ | VWR international | BDH3044 |
| $KMnO_4$ | Sigma-Aldrich | 223468 |
| $KNO_3$ | Fisher Scientific | P263-500 |
| MeOH | Fisher Scientific | A408-1 |
| MES | Bio Basic Canada inc. | MB0341 |
| $MgSO_4$ | Fisher Scientific | M63-500 |
| $MnCl_2$ | Caledon | 4930-1 |
| MOPS buffer | Bio Basic Canada inc. | MB0360 |
| $Na_2MoO_4$ | Arcos Organics | 206371000 |
| $NH_4H_2PO_4$ | EMD Millipore | 231-448-7 |
| Penicillin-streptomycin | Gibco | 15140122 |
| T75 tissue culture flasks | Falcon® | 08-772-1F |
| THP-1 cells | ATCC | TIB-202 |
| $ZnSO_4$ | Arcos Organics | 205982500 |

## Appendix B

Our standard sample of 2000 ug/mL protein was saturated in the plate reader, as seen in figure I, resulting in an underestimation of its' concentration. Because of this, 1.054 nm was the maximum absorbance represented in our standard curve. A large portion of my experimental data from this experimental replicate had protein absorbances that were greater than 1.054 nm, as seen in table I, and their protein concentrations were therefore underestimated using the equation of this standard curve. We would expect that an underestimation of protein concentration would subsequently overestimate iron concentrations once data has been converted to an iron to protein ratio. This result was especially observed in the iron to protein ratios of cells treated with root extracts of plants grown under iron normal conditions.

Furthermore, iron to protein ratios in our CrFe-treated cells were highly variable and the average was much greater than those seen in all other experimental replicates, as illustrated in table II. One data point was removed from the CrFe data, as it was greater than three times the interquartile range of this dataset and was thus considered an extreme outlier (Daniel, 2005). Due to the extreme values in the CrFe cells, the relative iron content of cells under the other treatments were skewed, as seen in figure II.

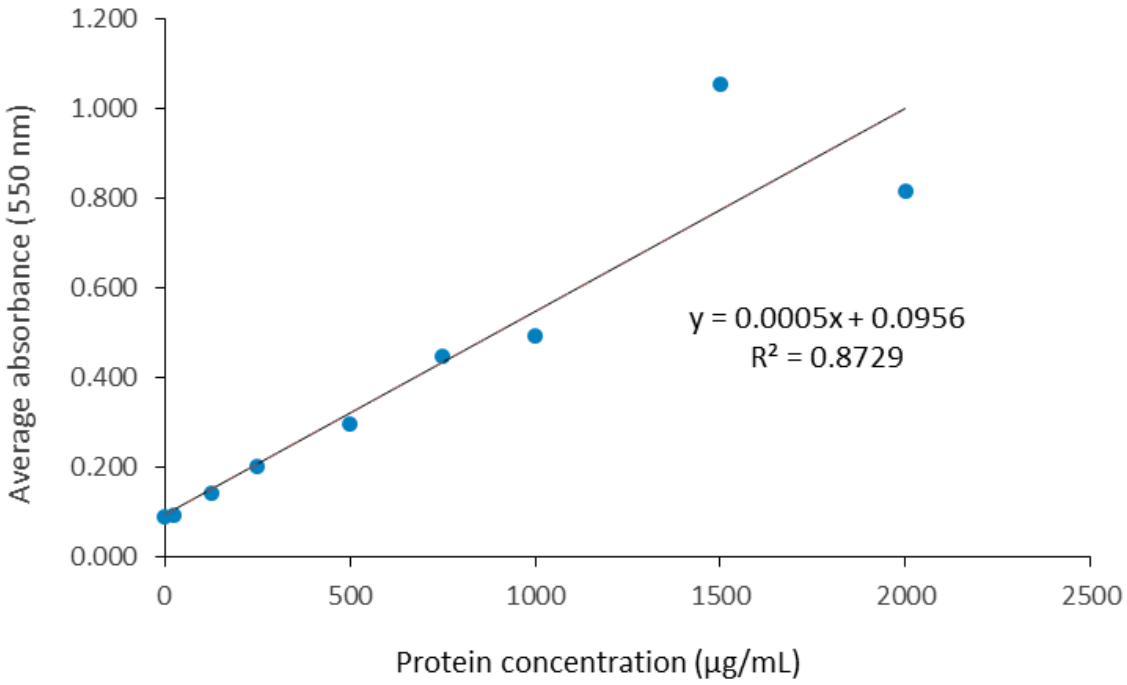


**Figure I.** A standard curve of bovine serum protein concentrations, as measured in a VICTOR™ X5 2030 multilabel plate reader at 550 nm, used to calculate protein concentrations of cell samples in an experimental replicate (d). Average protein absorbances for each standard were calculated from the readings of two replicates per sample (n = 2).

**Table II.** Raw values of protein absorbances of control cells (Ctrl), cells under iron overload (CrFe), cells treated with deferoxamine (DFO), normal poplar extract (nFe), or iron reduced poplar extract (mFe), as measured in a VICTOR™ X5 2030 multilabel plate reader at 550 nm. Illustrated in red are all values that are extrapolating above the range of our standard curve.

| Treatment | Absorbance 1 | Absorbance 2 | Average absorbance |
|---|---|---|---|
| Ctrl | 0.642 | 0.527 | 0.585 |
| | 1.1676 | 0.721 | 1.198 |
| | 1.079 | 0.811 | 0.945 |
| | 0.962 | 0.934 | 0.948 |
| CrFe | 0.840 | 0.759 | 0.800 |
| | 0.576 | 0.574 | 0.575 |
| | 0.453 | 0.457 | 0.455 |
| | 0.948 | 0.948 | 0.948 |
| DFO | 0.616 | 0.617 | 0.616 |
| | 0.578 | 0.577 | 0.578 |
| | 1.529 | 1.195 | 1.362 |
| nFe | 0.853 | 0.825 | 0.839 |
| | 1.031 | 1.086 | 1.058 |
| | 0.967 | 1.649 | 1.308 |
| | 1.104 | 1.137 | 1.120 |
| mFe | 0.582 | 0.589 | 0.585 |
| | 1.095 | 1.090 | 1.093 |
| | 0.689 | 0.707 | 0.698 |
| | 0.662 | 0.662 | 0.662 |

**Table III.** The values of iron concentrations and iron to protein ratios of four replicate samples of CrFe-treated cells.

| Iron concentration (nmol/mL) | Iron to protein ratio (nmol/mg) | Average | Standard error |
|---|---|---|---|
| 36.14902 | 1.486122 | 7.093622 | 3.378494 |
| 24.27595 | 1.10408 | | |
| 206.947 | 11.79526 | | |
| 405.2251 | 13.98902 | | |

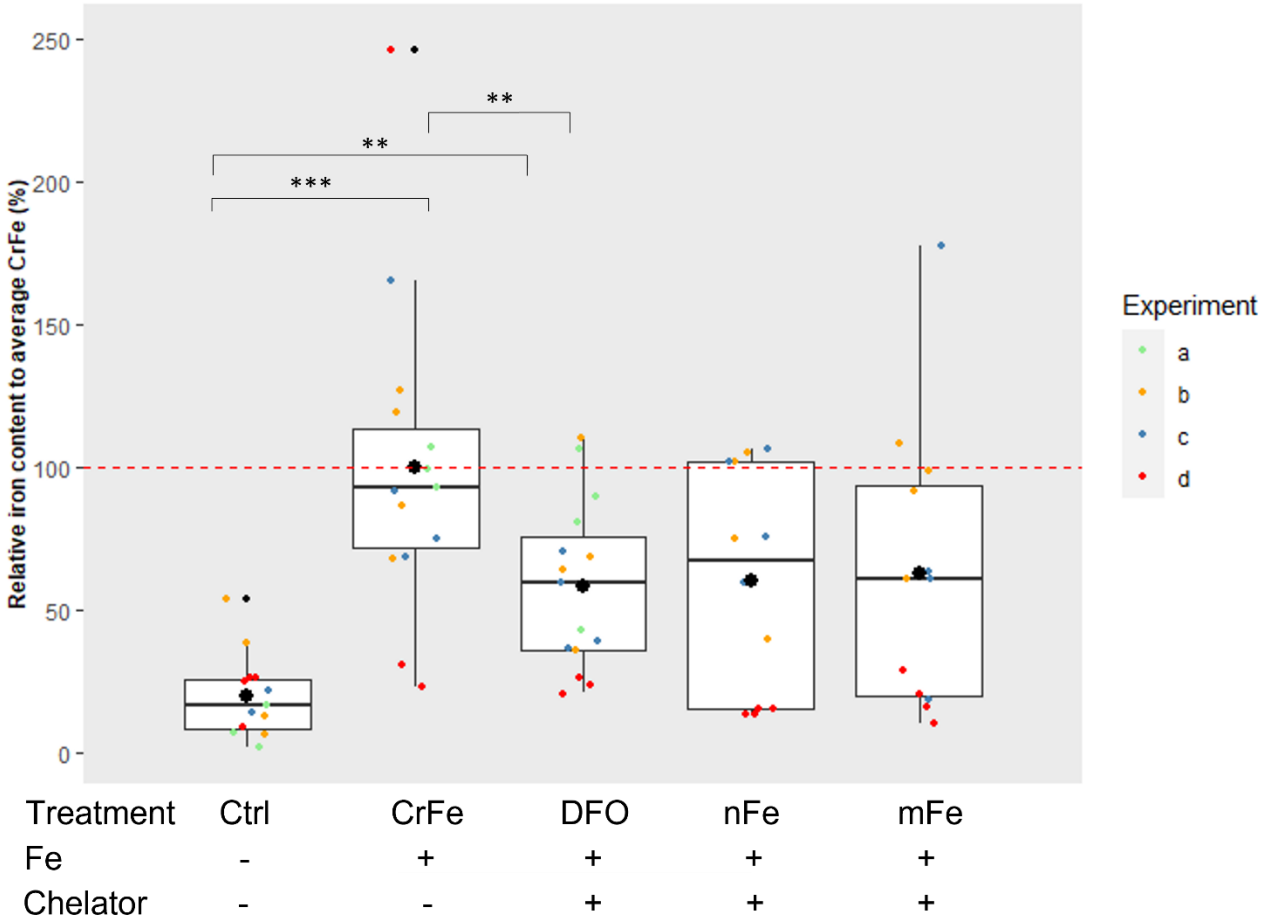


**Figure II.** Boxplot and strip chart of the relative iron content (%) to the average CrFe intracellular iron concentration of control cells (Ctrl), cells under iron overload (CrFe), cells treated with deferoxamine (DFO), normal poplar extract (nFe), or iron reduced poplar extract (mFe). Mean values for each treatment were calculated from pooled values of all experiments. Measurements were taken across three experimental replicates with four replicate measurements of each chelator treatment per experiment (n = 4). Differences between chelator treatments were investigated using paired Wilcoxon rank sum exact tests. All Wilcoxon rank sum exact tests used pooled data from all experimental replicates and $\alpha = .05$ (*** = $p < 0.005$, ** = $p < 0.05$). A horizontal line illustrates a relative iron content to average CrFe of 100%.

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