The Effect of synthetic iron-chelators and flavonoids on siderophore-producing *P. fluorescens*

Thousand Oaks High School

Abstract

Although every living organism needs a certain amount of iron to survive, about a quarter of people suffer from iron abnormalities. This experiment focuses on free radical pathogenicity that is enhanced by siderophore-producing bacteria and is a key factor in many chronic and infectious diseases. The synthetic iron-chelating drug deferiprone (L1) and two natural iron-chelating flavonoids, quercetin and catechin, were tested against the growth of *P. fluorescens* using optical density (OD). M9 minimal media and two concentrations of iron (III) citrate were used to control iron media concentration and siderophore production. Along with bacterial growth, siderophore presence in the iron deficient media was measured by OD. The chelators inhibited bacterial growth at both

concentrations of iron media, although there appears to be little correlation between iron concentration in media and chelator-induced growth inhibition.

Purpose

The purpose of this experiment was to test whether deferiprone (L1), quercetin, and catechin will be effective against the growth and siderophore production of the bacteria *P. fluorescens*, as assessed through optical density (OD) and a standard curve, in both high and low iron media.

Hypothesis

Quercetin, catechin, and L1 are predicted to have dose-dependent inhibitory effects on the growth of *P. fluorescens* in both high and low concentrations of iron media. Pyoverdine is expected to increase with higher concentrations of chelators.

Control

The two growth curves of each iron media concentration were used as control groups.

Variables

Variables included the concentration of iron in media, the type of chelator added, and the concentration of chelator added.

Introduction

All living things require certain levels of iron in the body for physiological function, growth and development. Specifically, the average daily iron intake from foods and supplements is 13.7–15.1 mg/day in children aged 2–11 years, 16.3 mg/day in children and teens aged 12–19 years, and 19.3–20.5 mg/day in men and 17.0–18.9 mg/day in women older than 19 (Dietary Supplement Fact Sheet, 2016). However, a quarter of the human population worldwide is affected by iron abnormalities. There are four main iron abnormalities: iron overload, iron deficiency, chronic-disease induced anemia, and free radical pathogenicity. Iron deficiency and iron overload have available comprehensive treatments, but there is no current

comprehensive treatment for free radical pathogenicity (Kontoghiorghe, Kolnagou, & Kontoghiorghes, 2015), due to the nature of radical oxidative damage and competing bacteria-produced mechanisms such as siderophores (see: *Siderophores*) (Cornelis & Andrews, 2010).

Free Radical Pathology

Free radicals, in regards to physiology, are molecules with at least one unpaired electron and high instability; they are essential for the normal activity of a wide spectrum of biological processes (Bergendi, Beneš, Ďuračková, & Ferenčik, 1999). The human body produces free radicals in the cellular redox process, when cells use oxygen to generate energy. These self-produced by-products of cellular respiration are highly reactive and make up a class of molecules called Reactive Oxygen Species (ROS)

and Reactive Nitrogen Species (RNS), and their production can be influenced by several exogenous factors, including essential transition metals (Fe, Cu, Zn, *etc*), that act as catalysts for their production in cells. Free radicals are beneficial in low concentrations when serving as intracellular messengers for processes such as regulation of blood flow and neural activity, and even can combat disease via their production through phagocytes to destroy invading pathogens (Pham-Huy, He, & Pham-Huy, 2008). In high concentrations, however, free radicals can cause cell damage when they bind to important cell structures, such as lipids, proteins, and nucleic acids, to take up their electrons. This process of cell damage, also called oxidative damage, interferes with cellular respiration and cell maintenance.

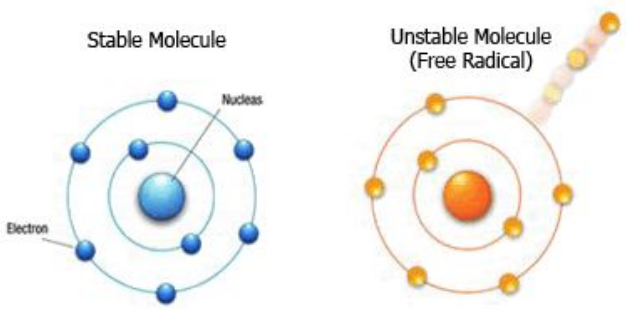


Fig. 1: A stable molecule has all paired electrons while a free radical has one unpaired electron, which makes it highly reactive

In order to keep low levels of free radicals, the body must neutralize or destroy excess free radicals through the use of antioxidants. The primary role of antioxidants is to give electrons to free radicals in order to neutralize them. After antioxidants neutralize radicals however, they are oxidized themselves. Some antioxidants are produced intrinsically, but most of the antioxidants in the body are derived from extrinsic sources, primarily from food. Many vitamins and minerals,

including Vitamin C (ascorbic acid), as well as phytochemicals found in plants and teas, including catechin and quercetin, are antioxidants (Nimse & Pal, 2015). When there is an imbalance in antioxidant and free radical levels, cells are damaged through oxidative stress.

An imbalance of antioxidant vs. free radicals levels can be induced by a number of factors, and can be seen in particular in immunodeficient hosts. This is why free radical damage is seen predominantly in chronic and infectious diseases. In fact, much of the cell degeneration and cell aging in certain chronic diseases, such as kidney disease and Alzheimer's, is attributed to free radical oxidative damage (Held, 2014).

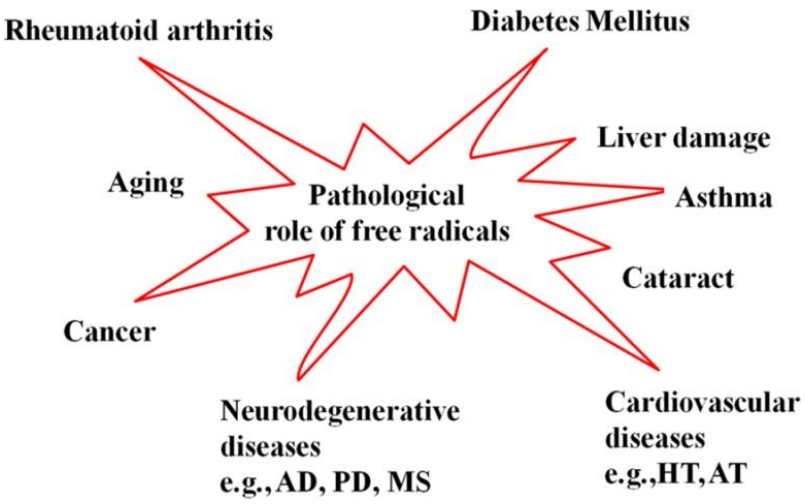


Fig. 2: Diagram depicting the pathological role of free radicals in various diseases and pathological conditions

All aerobic living things, including bacteria, also produce and are influenced by free radicals and antioxidants. The same imbalance discussed previously can induce oxidative stress on aerobic bacteria, and the human body uses iron to induce oxidative damage and cell degeneration in infectious diseases (Kashmiri & Mankar, 2014).

Hypoferremia and Iron Homeostasis in Infectious Diseases

Free radicals and iron also play a role in numerous infectious diseases. The body utilizes a concept called iron-targeted nutritional immunity induce anaemia in infectious bacteria. The body destroys the bacteria through iron depletion and excessive free radical production, which creates oxidative damage in the bacteria and limits the bacteria's ability to harm the host. The body uses two different pathways to induce hypoferremia (anaemia) in the infectious disease: the hepcidin-dependent pathway and a hepcidin-independent pathway. In the hepcidin-dependent pathway, the body will increase its production of hepcidin, a metabolic iron-regulating mechanism, to reduce iron plasma and induce hypoferremia of the infectious disease. There is another hepcidin independent pathway for hypoferremia in infections as well, where iron

chelation may be used in order to correct an iron abnormality caused by an infectious disease (Ganz & Nemeth, 2009).

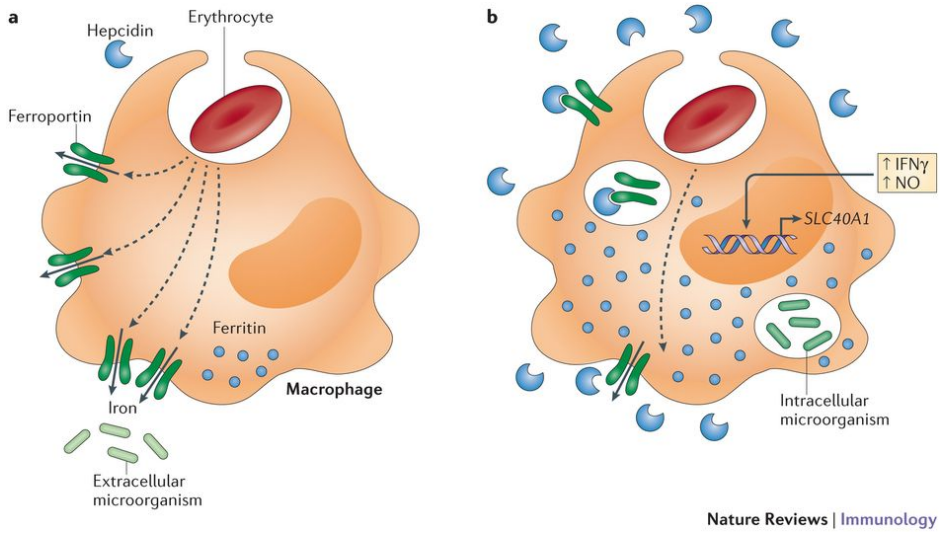


Fig. 3: Hepcidin regulation of iron uptake in macrophages to restrict iron availability for microorganisms a) with low hepcidin concentration and b) high hepcidin concentration

Siderophores

Certain bacteria have adapted to attempt to survive hypoferremia by producing siderophores. Siderophores are specific peptides and proteins that are produced by bacteria and

are used to facilitate bacterial iron uptake in iron deficient conditions by binding and transporting iron in microorganisms. Siderophores themselves have attracted interest from researchers for their potential uses as possible biopesticides and detoxifying chemical wastes (Djibaoui & Bensoltane, 2005). Siderophores are produced by bacteria in low levels of essential metals such as iron, and their production decreases as more metal is present (Hohnadel & Meyer, 1986). This experiment will focus on siderophores as indicators of bacterial growth and iron availability, which will also give some indication of chelator efficacy. In theory, if more iron is chelated, the production of siderophore should increase.

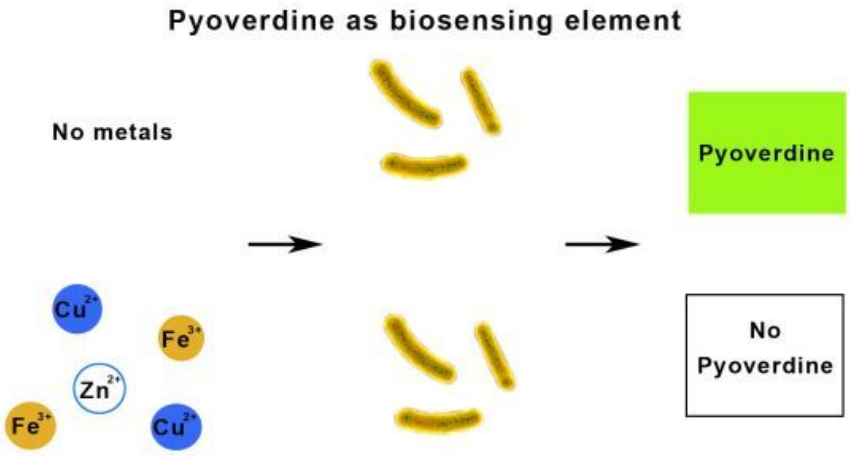


Fig. 4: Diagram showing the activation of pyoverdine in low/no metal conditions

Pseudomonas fluorescens

Pseudomonads, the type of bacteria used in this experiment, include a wide range of both pathogenic and beneficial pseudomonads that produce pyoverdine, a high-affinity siderophore that enhances growth under iron deficiency (Ringel, Draeger, & Brueser, 2016). *P. fluorescens* are popular subjects for testing in free radical pathology because although they themselves are not pathogens, they have similar structures and

mechanisms of pathogenic strains. *P. aeruginosa* is an opportunistic pathogen that causes infection and uses several types of iron-uptake mechanisms for its survival in different iron-limited environments, including the siderophore pyoverdine (*Fig. 1)* that is present in *P. fluorescens* (Kirienko, Revtovich., & Kirienko, 2016).

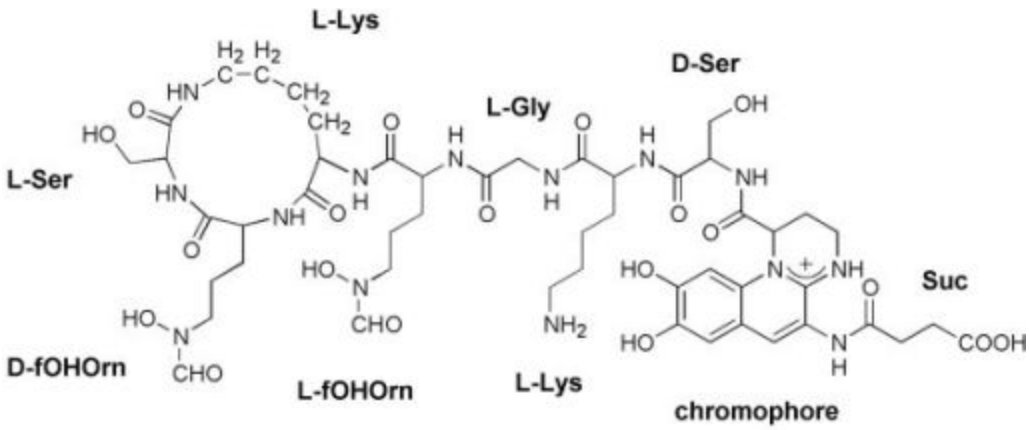


Fig. 5: Structure of pyoverdine from P. fluorescens sold at Sigma Aldrich

Iron Chelators

Iron chelators are molecules that bond to iron and may transport it, and may be a key component in treating iron abnormalities. There are natural and weaker iron chelators such as ascorbic acid, which may help with minor iron deficiency and be helpful as an adjuvant to oral supplements of iron. However, there are also siderophore chelators (pyoverdine), phytochemicals with chelating properties (quercetin and catechin), and synthetic iron-chelator drugs. L1, a drug tested in this experiment, is used as a synthetic iron chelator in the treatment of thalassemia, a serious iron-overload condition where life expectancy is a few years if left untreated. In principle, however, iron chelators can remove, add, or exchange iron. This means that L1 cannot only treat iron overload, but it also can treat other iron abnormalities.

L1 is one of five main synthetic chelating drugs. The three others are deferoxamine (DFO), deferasirox (DFRA), ethylenediaminetetraacetic acid (EDTA), and diethylenetriaminepentaacetic acid (DTPA) (Figure 2) (Kontoghiorghe, Kolnagou, & Kontoghiorghes, 2015).

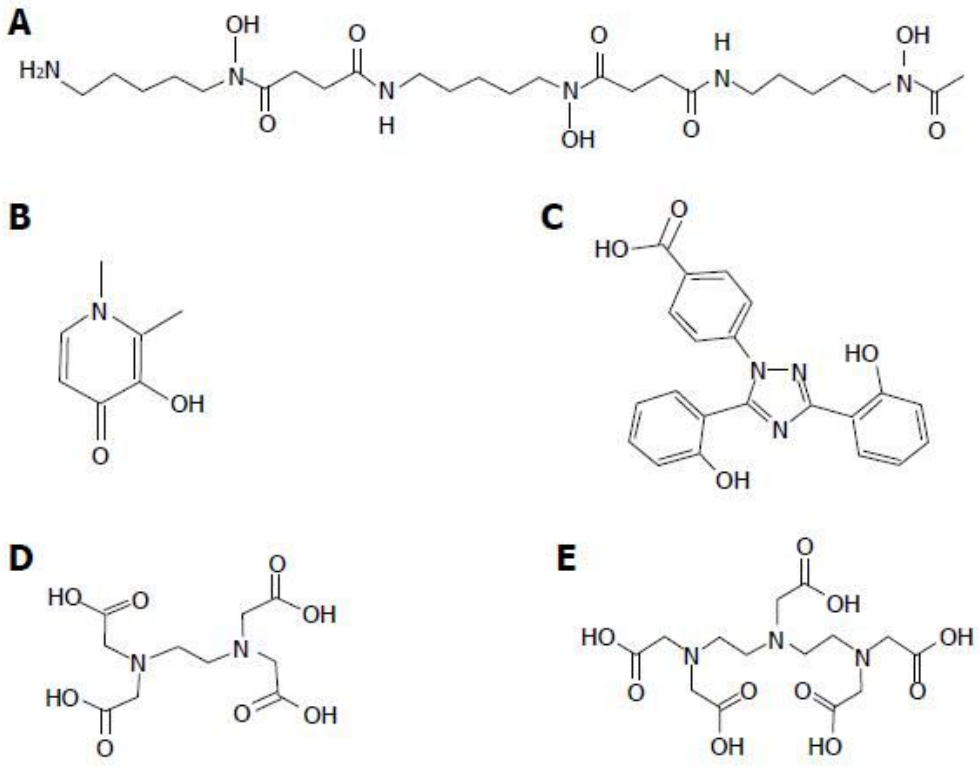
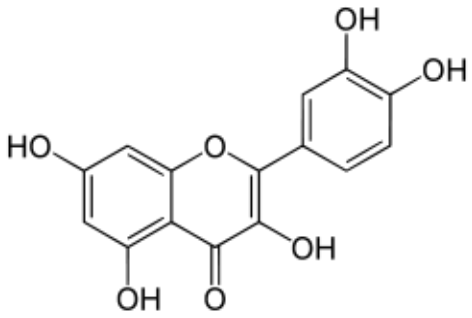


*Fig. 6: Structures of the main iron chelating drugs: deferoxamine (DF) (**A**), deferiprone (L1) (**B**), deferasirox (DFRA) (**C**), EDTA (**D**), and DTPA (**E**).*

Deferoxamine was omitted in this experiment because it is a bacterial product and thus has been seen to be taken up by bacteria to facilitate iron uptake and growth. Deferasirox was not used in this experiment because of controversy surrounding its safety (Thompson *et. al*, 2012). EDTA is not an effective iron chelator because it forms an open complex with iron and allows for oxidative stress (Flora, & Pachauri, 2010). DTPA is also not used, but has shown promising signs of iron chelation. In fact, it is used in a diluted form of 10% to prevent a so-called "yellow leaf" disease, or chlorosis, in aquaponics (growing plants and fish in the same environment) (Walworth, 2006).

Flavonoids

Recent research has revealed that flavonoids may be useful metal chelators as well. Flavonoids are one of the most common phytochemicals found in plants. In fact, catechin is found in green tea and quercetin in buckwheat as well as green tea and berries, among other foods. Most flavonoids have metabolic properties, and some have insecticide and antifungal properties as well. Catechin, quercetin, and rutin exhibit strong antioxidant properties towards iron (Cherrak et al, 2016). This experiment will test their bacterial inhibitory ability in response to elevated levels of iron, which will test their chelating abilities as well.



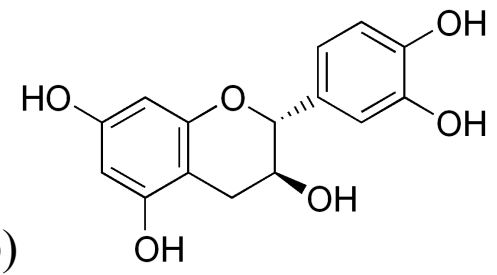
a) b) 

Fig. 7: Chemical structures of quercetin (a) and catechin (b).

Materials

- 100 mg Deferiprone (L1) (UCLA)
- 1 g Catechin Hydrate (96 %, Sigma Aldrich, #22110)
- 1 g Quercetin (98%, Samsara Herbs)
- Slant culture *P. fluorescens* (Flinn Scientific, Inc, Catalog #LM 1009)
- 10 g iron (iii) citrate (Flinn Scientific, Inc, Catalog #F0057)
- 50 mL DMSO (universal dissolving agent) (Flinn Scientific, Inc, Catalog #D0051)

- M9 minimal media (Na_2HPO_4, NH_4Cl, KH_2PO_4, NaCl, $MgSO_4$, $CaCl_2$, 10% glucose)
- 1.5, 2, and 3 mL microcentrifuge tubes
- 2 96-well plates
- Cuvettes
- Micropipette and tips
- V5000 Visible Spectrophotometer
- accuSkan FC Electronic plate reader
- Eppendorf Centrifuge 5418
- Analytical balance
- Weighing boat
- Spatula
- Micropipettes and tips
- Sterile loop

- Serological pipet
- 250-ml Erlenmeyer flasks
- Beakers

Methods

(Methods were conducted in lab room E8 at Thousand Oaks High School under the supervision of Dr. Nikki Malhotra.)

Preparing P. fluorescens Culture

A small amount of a culture solution (Flinn Scientific) was transferred to a flask filled with 50 mL LB media. The culture was incubated at 25º C for 2-3 days, and 30 mL of media was changed out regularly to ensure a consistent standing culture.

Preparation of M9 Minimal Media

500 mL of the M9 minimal media was prepared according to the recipe by Barrick Labs. This method included combining Na_2HPO_4, NH_4Cl, KH_2PO_4, NaCl, $MgSO_4$, $CaCl_2$, and 10% glucose for a total of 500 mL of media.

400 μM and 4 μM Iron Media Preparation

10 mL of a 0.1 M iron (iii) citrate stock solution was made by dissolving 0.246 g of iron (iii) citrate powder (Flinn Scientific) in warm water. 2.8 mL of the 0.1 M stock was added to 70 mL of M9 minimal media to reach a final concentration of 400 μM iron media. In a serial dilution, 0.07 mL of the 400 μM iron media was added to fresh minimal media to make 70 mL of 4 μM iron media. These treatments were done to ensure testing

was done on cultures that produced the maximum and the minimum amount of pyoverdine, respectively.

Growth curves

50 mL of M9 minimal media was used to create two growth curves with 400 μM and 4 μM iron concentration, respectively. First, 2 mL of the *P. fluorescens* running culture was centrifuged and washed with M9 minimal media twice according to previous methods (Kasai, Hori, & Goodman, 1990), and then transferred to prepared 400 or 4 μM media. The OD of two samples for 4 μM iron media and one sample for 400 μM iron media was taken at 0, 1, 2, 24, 48, and 96 hours after inoculation. The values were plotted on a graph, and error bars

were created through calculations of standard error. This data was used as a control.

Dilutions of L1

L1 was weighed out and dissolved in warm water using 1.5 mL microcentrifuge tubes in stock concentrations of 0.02 M, 0.05 M, 1 M, and 1.5 M (100X of final concentration). The concentrations were added to both the high iron media and low iron media trials.

Preparation of Flavonoids

For maximum solubility, the flavonoids were dissolved in 100% DMSO. As previously described, 100X stocks of 0.02 M,

0.05 M, 1 M, and 1.5 M were made in 1.5 mL microcentrifuge tubes and stored at 25º C.

Adding P. fluorescens

When L1 and the flavonoids were added to bacterial culture for testing, they were diluted to 1X 0.2 mM, 0.5 mM, 1 mM, and 1.5 mM. In order to dilute the 100X stock to 1X, 10 µL of 100X stock chelator was added to 990 µL of bacterial culture to reach 1 mL of chelator and bacteria. The chosen concentrations were based on a previous experiment (Cherrak et al, 2016). All samples were analyzed in triplicate.

Optical Density (OD) Analysis

The OD of each treatment sample was measured at ≈ 600 nm after 24 hours and compared to the control to test for growth inhibition. For the 400 μM iron media treatment samples, a V5000 Visible Spectrophotometer was used to take the ODs. For the 4 μM iron media treatments, 300 μL of each treatment sample was transferred to a 96 well-plate, which was run through an electronic plate reader (accuSkan FC) at 590 nm. The OD of each sample was recorded.

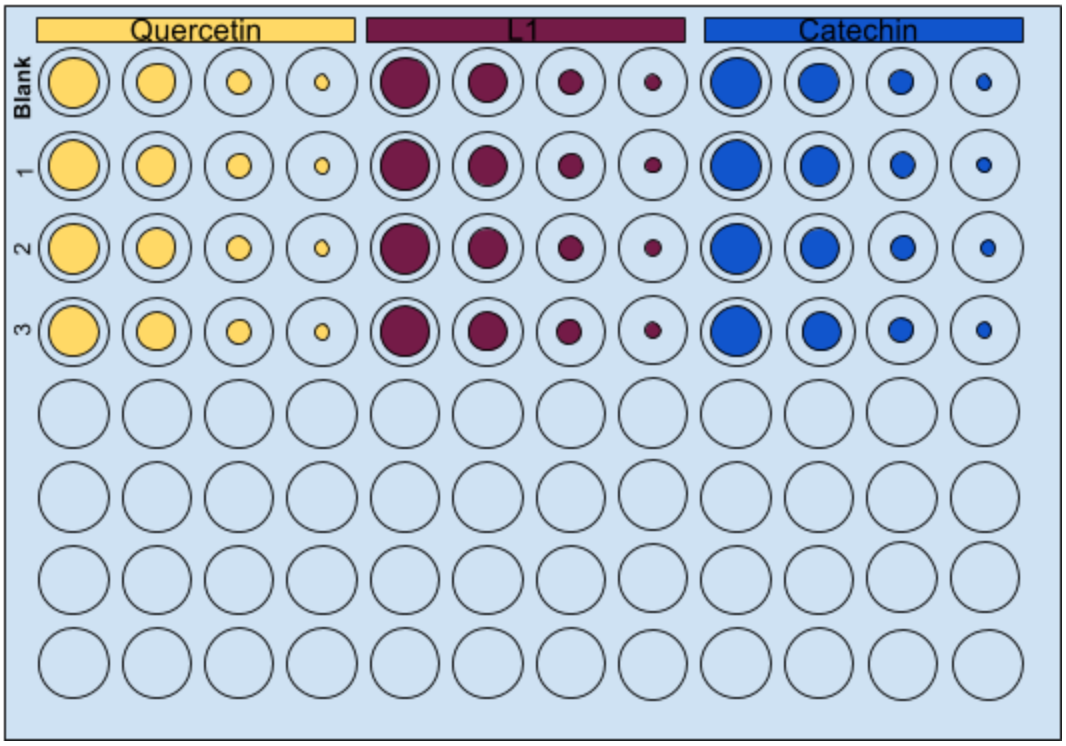


Fig 8: 96 well plate layout with treatments for low iron media. From left to right: (quercetin) 1.5 mM, 1 mM. 0.5 mM, 0.2 mM, (L1) 1.5 mM. 1 mM. 0.5 mM, 0.2 mM, (catechin) 1.5 mM, 1 mM, 0.5 mM, 0.2 mM. From top to bottom: blank with chelator, sample 1, sample 2, sample 3.

Siderophore/Pyoverdine

The low iron media treatment group was expected to maximize pyoverdine production (Djibaoui & Bensoltane, 2005). Therefore, the samples of the 4 μM treatment, which included 48 samples of triplicates of the three chelators at the four concentrations, were centrifuged at 16000 rpm for 2 minutes. The supernatant of each sample was pipetted into a 96 well plate and the plate was run through the plate reader at 405 nm to measure pyoverdine presence at 24 hrs.

Trials

Each treatment with each concentration of chelator was conducted in triplicate for a total of 96 samples. All data was analyzed as the averages of all 3 trials with standard deviation and standard error.

Results

Growth Curves

The growth curves served as control groups, and were plotted using time vs. average optical density (OD). The growth of *P. fluorescens* appeared to be more vigorous but not more rapid in the 400 µM iron media than in the 4 µM iron media.

Table 1: A control group, or growth curve, was made for each iron media concentration, 4 µM (a) and 400 µM (b). Absorbance of media was taken at 0, 1, 2, 3, 24, 48, and 96 hours (hour 3 omitted in 400 µM iron media growth curve).

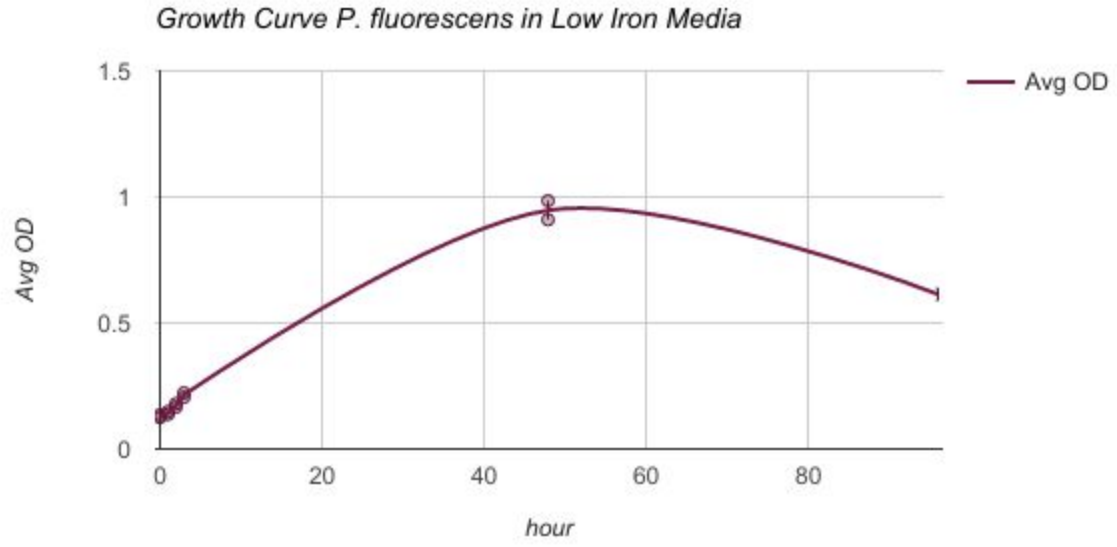
a)

Absorbance of Growth Curve in Low Iron Media						
Hour	**0**	**1**	**2**	**3**	**48**	**96**
	0.132	0.14	0.174	0.218	0.927	0.714
	0.128	0.146	0.172	0.21	0.968	0.514
avg	0.13	0.143	0.173	0.214	0.9475	0.614

b)

Absorbance of Growth Curve in High Iron Media					
Hour	**0**	**2**	**24**	**48**	**96**

	0.04	0.12	1.1	1.47	0.932



a)

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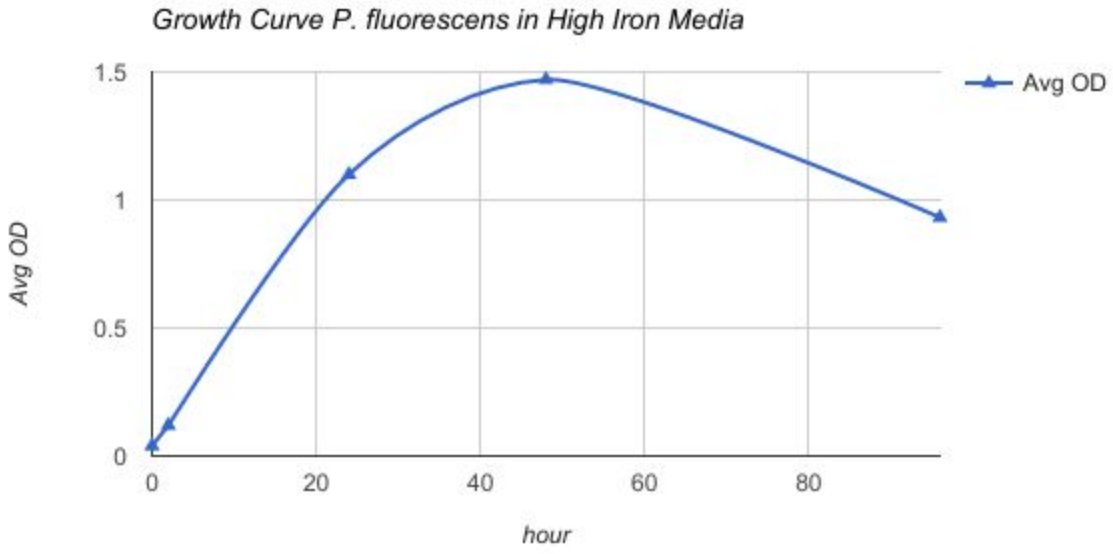


Fig. 9: The growth curve of P. fluorescens in 4 µM iron media (a) The growth curve of P. fluorescens in 400 µM iron media (b)

Dose-Response of Chelators Vs. Bacterial Growth

The growth inhibition of each chelator at 0.2, 0.5, 1, and 1.5 mM was quantified using the absorbance of each sample after 24 hours of incubation in both low (table 1) and high (table 3) iron media concentrations. The average absorbance values for each concentration of chelator were compared to the control group absorbance value after 24 hours. Quercetin, catechin, and L1 appeared to inhibit bacterial growth at both low and high iron media concentrations. L1 and catechin exhibited lower absorbance as the concentration of chelator increased, while quercetin had little correlation between absorbance and concentration of chelator. Standard deviation and error were conducted for each concentration of chelator in both low (Table

2) and high (Table 4) iron media, and average standard error was used to create error bars for each chelator in the graphs.

Table 2: The absorbance of samples was recorded and the averages were calculated for each concentration in 4 μM iron media.

Low Iron Media Bacterial Absorbance (590 nm)					
Chelator	**Concentration**	**Absorbance Trials 1-3**			**Avg. Absorbance**
Quercetin	0.2 mM	0.2539	0.2793	0.2592	0.2628
	0.5 mM	0.07990	0.08860	0.02250	0.06367
	1 mM	0.3042	0.2427	0.3477	0.2982
	1.5 mM	0.6429	0.3797		0.5113
L1	0.2 mM	0.1795	0.1928	0.1776	0.1833
	0.5 mM	0.1662	0.1437	0.1311	0.147
	1 mM	0.1163	0.1146	0.1217	0.1175
	1.5 mM	0.1191	0.09210	0.1436	0.1183
Catechin	0.2 mM	0.2131	0.2473	0.2618	0.2407
	0.5 mM	0.2384	0.2288	0.2389	0.2354
	1 mM	0.2978	0.3040	0.2611	0.2876
	1.5 mM	0.2137	0.1750	0.1866	0.1918

Table 2: Standard deviation, average standard deviation, standard error, and average standard error were calculated for each chelator in 4μM iron media treatments.

Low Iron Media Absorbance Data					
Chelator	Concentration	Standard Deviation (SD)	Avg SD	Standard Error (SE)	Avg SE
Quer	0.2 mM	0.0133993781	0.0720454505	0.00773613455	0.04159551483
	0.5 mM	0.03591578112		0.0207359859	
	1 mM	0.05275651619		0.03045898882	
	1.5 mM	0.18611050048		0.10745090501	
L1	0.2 mM	0.00828190799 3	0.0138825973 2	0.00478156180 9	0.0080151213 02
	0.5 mM	0.01778116982		0.01026596318	
	1 mM	0.00370720020 1		0.00214035303 4	
	1.5 mM	0.02576011128		0.01487260718	
Cate	0.2 mM	0.02500526611	0.0720454505	0.01443679712	0.0106441684 4
	0.5 mM	0.00569239258 4		0.00328650439 1	

	1 mM	0.02318670596		0.01338685093	
	1.5 mM	0.01986059751		0.01146652132	

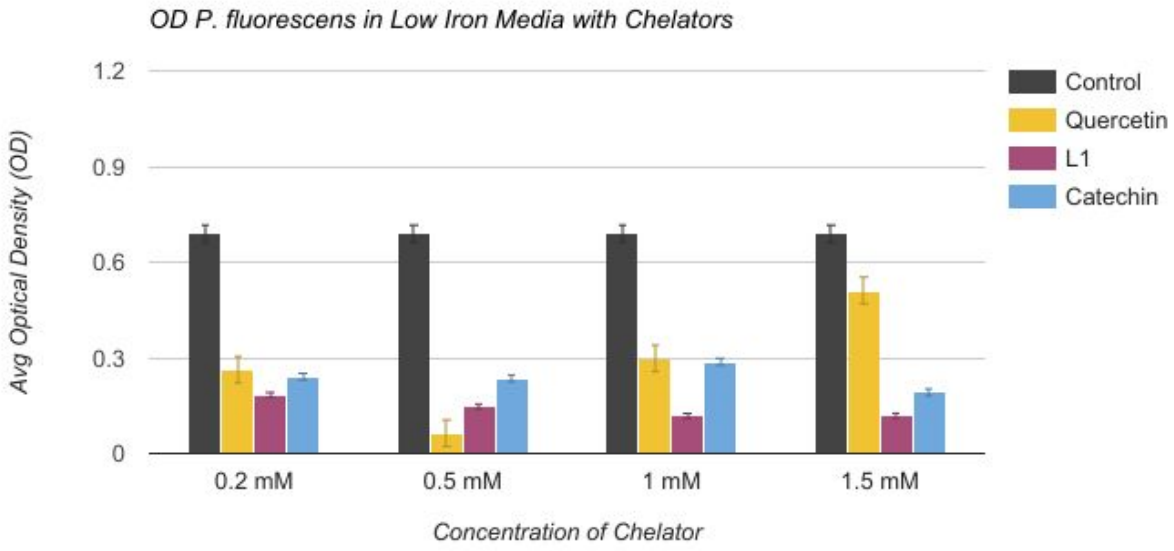
Table 3: The absorbance of samples was recorded and the averages were calculated for each concentration in 400 µM iron media.

High Iron Media Bacterial Absorbance (600 nm)					
Chelator	Concentration	Absorbance Trials 1-3			Avg. Absorbance
Quercetin	0.2 mM	0.494	0.488	0.514	0.4987
	0.5 mM	0.06	0.136	0.024	00.0733
	1 mM	0.602	0.554	0.602	0.586
	1.5 mM	0.342	0.078	0.494	0.3047
L1	0.2 mM	0.166	0.248	0.176	0.1967
	0.5 mM	0.142	0.118	0.14	0.1333
	1 mM	0.044	0.046	0.032	0.0407
	1.5 mM	0.166	0.092	0.12	0.126
Catechin	0.2 mM	0.138	0.128	0.15	0.1387
	0.5 mM	0.102	0.138	0.172	0.1373
	1 mM		0.12	0.038	0.079
	1.5 mM	0.072	0.024	0.029	0.0417

Table 4: Standard deviation, average standard deviation, standard error, and average standard error were calculated for each chelator in 400 µM iron media treatments.

High Iron Media Absorbance Data Statistical Analysis					
Chelator	Concentration	Standard Deviation (SD)	Avg SD	Standard Error (SE)	Avg SE
Quer	0.2 mM	0.01361371857	0.07725060963	0.007859884	0.04460066014
	0.5 mM	0.05717808438		0.033011782	
	1 mM	0.02771281292		0.016	
	1.5 mM	0.2104978226		0.121530746	
L1	0.2 mM	0.04473626419	0.0257469704	0.025828494 17	0.04460066014
	0.5 mM	0.01331665624		0.007688375063	
	1 mM	0.00757187779 4		0.004371625683	
	1.5 mM	0.03736308338		0.021571588625	

Cate	0.2 mM	0.0110151 4109	0.032597 69684	0.0063595 94676	0.020701 19909
	0.5 mM	0.0350047 6158		0.0202100 0852	
	1 mM	0.0579827 5606		0.041	
	1.5 mM	0.0263881 2864		0.0152351 9318	



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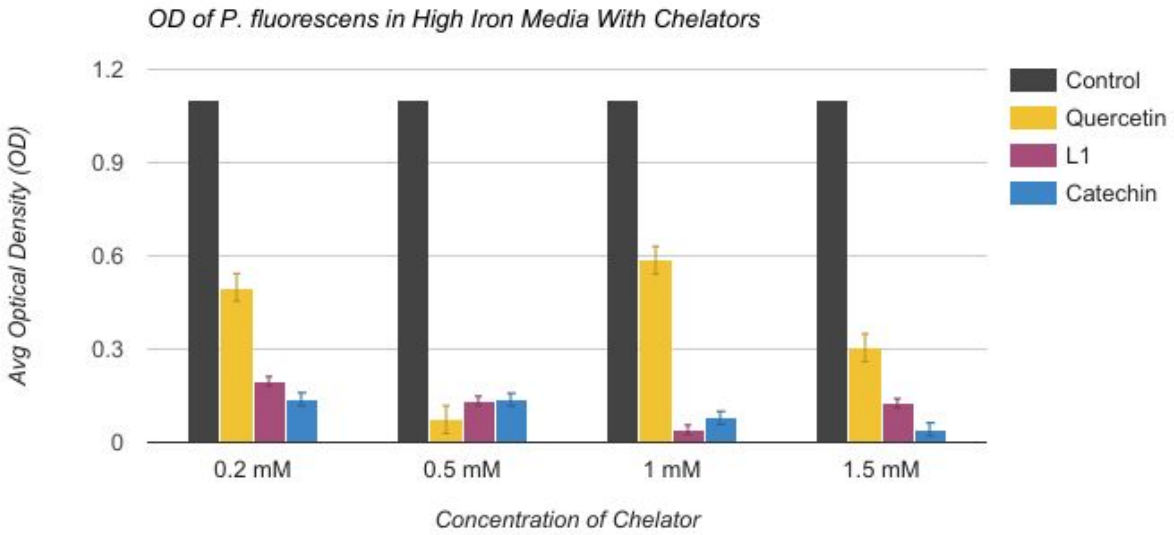


b)

Fig. 10: a) The OD of the standard curve was compared to the OD of samples inoculated with each concentration of chelator in 4 µM iron media. b) The OD of the standard curve was compared to the OD of samples inoculated with each concentration of chelator in 400 µM iron media.

Pyoverdine Measurements in Iron Deficient Media

The presence of the siderophore that *P. fluorescens* produces, pyoverdine, was measured and plotted on a concentration vs. absorbance line graph (fig. 3). For catechin, the amount of pyoverdine could have either slightly declined

with concentration of chelator, or it may have remained constant due to the standard error. For L1, there was a slight trend as the concentration of L1 increased, the amount of pyoverdine decreased. However, the absorbance slightly increased from 1 mM to 1.5 mM. There may have been error in recording the absorbance for 0.2 mM L1, and the amount could have been close to constant or slightly increasing. For quercetin, the graph shows a steady increase in absorbance until 1 mM of the chelator, and then a decline in absorbance from 1 mM to 1.5 mM quercetin. Due to the nature of bacterial production of siderophores (see discussion), the decreasing point at 1.5 mM

quercetin may be due to an error.

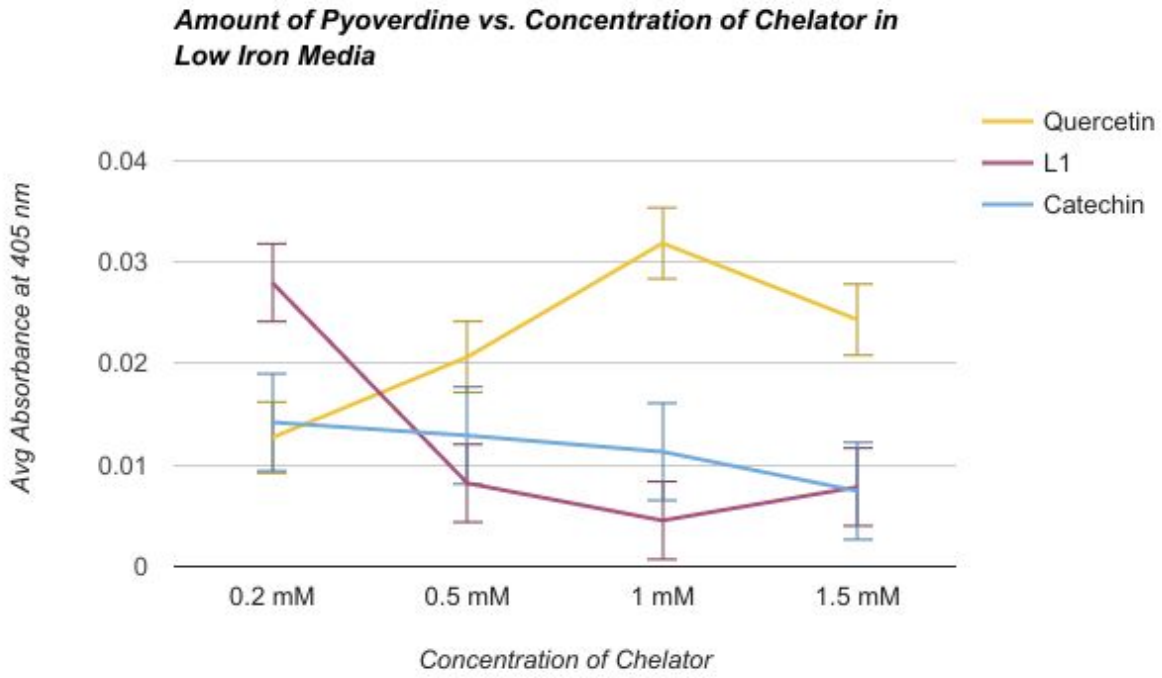
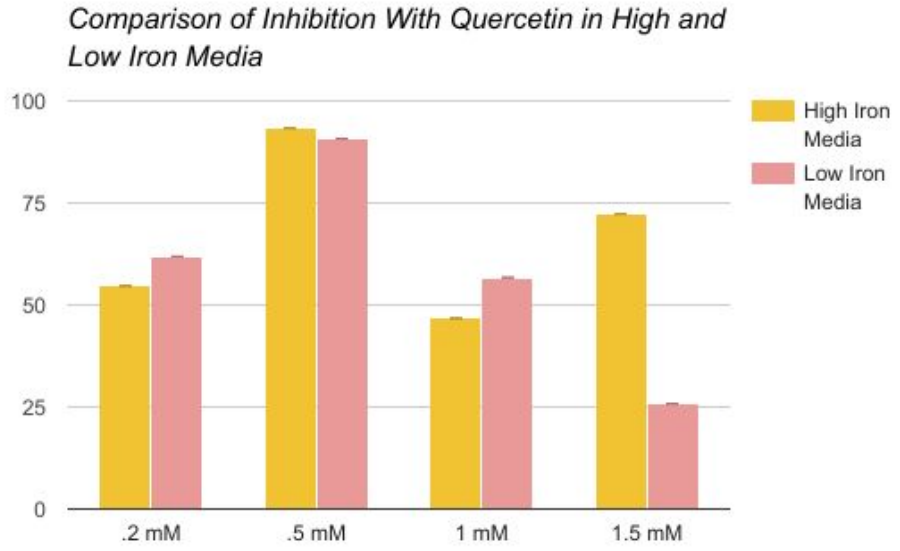


Fig. 11: The optical density was taken of each sample at 405 nm after 24 of incubation to determine pyoverdine presence and the absorbance at each concentration of chelator was compared.

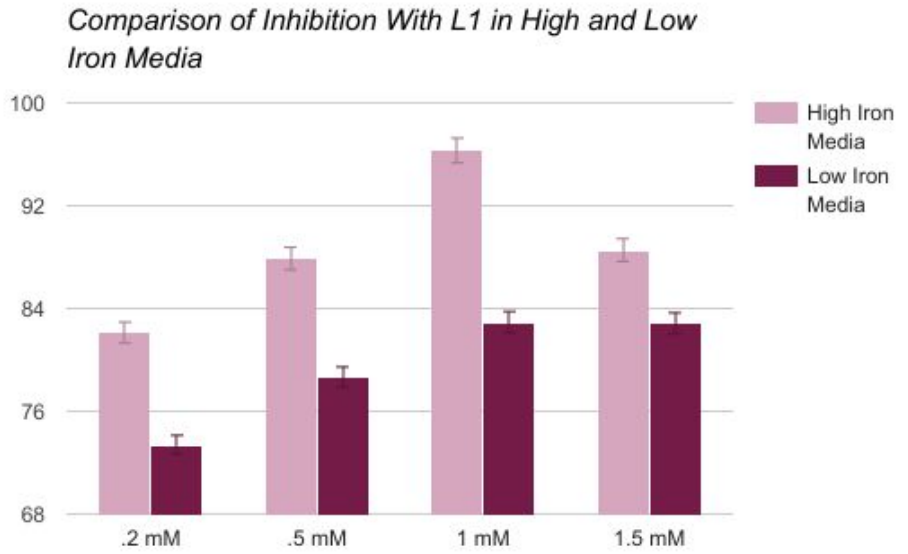
% Growth Inhibition

The growth inhibition for each chelator was compared in the low and high concentration iron medias. For quercetin (a), there seems to be no discernable correlation between inhibition

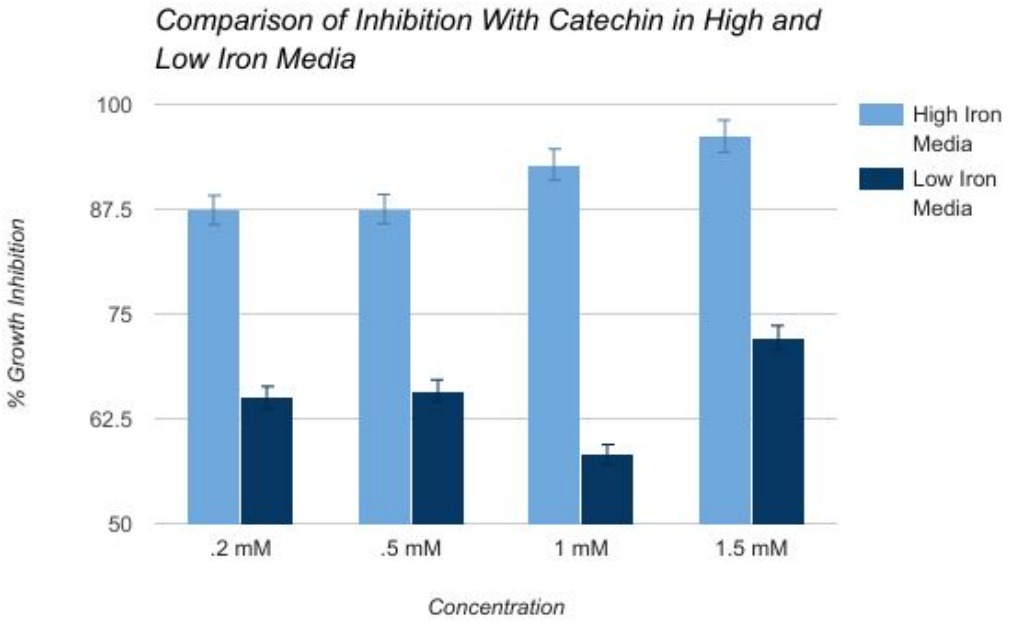
in the 400 µM versus the 4 µM iron medias. For L1 (b) and catechin (c), the % growth inhibition appears to be higher in 400 µM iron media than in 4 µM iron media. L1 exhibited a decrease in % growth inhibition after a concentration of 1 mM, suggesting a maximum effectiveness at 1 mM. L1 (b) had a maximum percent growth inhibition of 96.3% at 1 mM in high iron media, and catechin (c) had a maximum of 88% inhibition.



a)



b)



c)

Fig. 12: The percent growth inhibition for each chelator in high and low iron concentration media was determined by comparing the treatment absorbance to the control absorbance.

Discussion

Growth Curve Analysis

The more vigorous but similar rate of bacterial growth in 400 μM iron (high iron) media as compared to growth in the 4 μM iron (low iron) media indicates that while iron may facilitate more bacterial growth within a certain time frame, additional iron may not influence the rate of bacterial growth.

Dose Response Analysis

The data suggests that there is a dose-dependent effect on growth inhibition in both high and low iron media concentrations, and that bacterial growth was inhibited by the addition of an iron chelator in media with a known iron

concentration. Catechin appeared to reached its maximum efficacy at 1.5 mM, the highest concentration of chelator tested. L1, however, appeared to plateau in efficacy. In both high and low iron concentrations, L1 exhibited maximum inhibitory effects at 1 mM and did not increase bacterial inhibition at 1.5 mM. This is similar to many other pharmaceutical drugs, which also plateau in efficacy in certain concentrations. Quercetin did not appear to have a discernable trend in efficacy for maximum growth inhibition. It was expected that inhibition would increase with each increasing concentration of chelator, but quercetin did not follow this model. Other variables that could influence quercetin's deviation from the data set include interaction sites on the bacteria and quercetin, and experimental sources of error.

Pyoverdine Assay Analysis

Although pyoverdine measurements were recorded for low iron concentration media, further analysis is required to relate chelator concentration to pyoverdine production (see further work). In general, however, it was expected that pyoverdine amounts would increase at higher concentrations because siderophore-producing bacteria use siderophores when under iron deficiency induced stress. Therefore, the bacteria was expected to produce pyoverdine in the iron deficient media, and it was expected to increase pyoverdine production when there was more chelator added because of less iron available. The error bars on catechin and L1 indicate that the amount of pyoverdine may have stayed constant rather than decreased with chelator concentration, and the trend for quercetin indicates that pyoverdine production increased with concentration up until 1.5

mM. The decrease in pyoverdine at 1.5 mM may have been due to a technical error with the plate reader, or a methodical error with taking the supernatant.

Percent Growth Inhibition Analysis

There are two possible explanations for the higher percent growth inhibition in the iron supplemented media. Because there is less iron available for the bacteria at low iron media concentrations, the bacteria may have grown less overall. Thus, the magnitude of the percent difference in growth inhibition would be smaller. Additionally, siderophores are not produced at high iron concentrations, and therefore there would not have been competition between the chelators and the siderophores. This would mean that the chelators had 1) more available iron to

chelate because of the high iron concentration in the media, and 2) the chelators could bind of iron without the iron uptake competition of siderophores. L1, again, plateaued in percent growth inhibition at 1 mM. This is further evidence that maximum effectiveness for L1 is reached at 1 mM. Furthermore, the percent of growth inhibition reached up to 96% with catechin and up to 88% with L1, as compared to the control. This suggests that both flavonoids and synthetic chelators may be effective growth inhibitors against siderophore-producing bacteria.

Conclusion

Ultimately, the data provides evidence that iron chelators may be effective in inhibiting growth of siderophore-producing

bacteria. It is not certain whether iron chelators influence the production of siderophores, but some evidence suggests that there may be a correlation between chelator concentration and siderophore presence, and between siderophore presence and bacterial growth inhibition. Iron media concentration does appear to have somewhat of a correlation with percent growth inhibition with each chelator. Catechin is the best model for this observation. Models and statistical analysis may be used to explain qualifications in the data, but the experiment would need to be repeated for further analysis.

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Sources of Error

One critical source of error was the precipitation of quercetin into the media. Although the samples were warmed and dissolved in 100% DMSO, precipitation of quercetin in water based M9 media could not be prevented. One possible solution would be the addition of a cyclodextrin, a carrier that increases solubility of compounds and chemicals in water (Sigma Aldrich). However, the cyclodextrin would decrease the concentration of available quercetin.

One optical density sample reading was taken for the 400 μM iron media control growth curve. This prevented any statistical analysis of significance that would have included an ANOVA between the values for each iron media concentration. A new growth curve would be made with triplicate sample readings, and then an ANOVA test could be conducted.

This experiment would need to be repeated to ensure accurate sample readings using the electronic plate reader for each iron media concentration and a statistical analysis to compare significance of growth inhibition.

Further Work

Siderophore/Chelator Correlation

Both external chelators and pyoverdine need to be added to treatments with known iron media concentrations to test the effect of iron concentration on siderophore production. Extensive time-course optical density analysis would be conducted.

L1/Flavonoid Combinations

Flavonoids have the potential to be adjuvants to L1 (Kontoghiorghes & Kontoghiorghe, 2016), and so they would be tested in combination in previous methods.

Free Radical Scavenging Ability

The antioxidant/antiradical properties of the flavonoids and synthetic drugs would be estimated by the radical scavenging activity method using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) and compared to that of a standard, such as ascorbic

acid. The absorbance at 515 nm would be measured over time (every 30 seconds) on a spectrophotometer until the reaction time reached 16 minutes. The percentage of remaining DPPH• as assessed by the absorbance at 515 nm would be plotted to evaluate the EC50 (concentration of the antioxidant required to scavenge the initial DPPH• concentration by 50%) [51–53]. Low EC50 values reflect high antioxidant capacities of the considered systems.

HPLC Detection of Oxidative Damage

Levels of oxidized GSH vs. GSSG are used to indicate the amount of oxidative damage. First, the samples of the cell culture would be homogenized using a high shear homogenizer. Then, bacterial cultures would be then centrifuged, and the bacterial culture supernatants would be processed with the

sample preparation. The filtrates would be analyzed by HPLC (Szkop & Bielawski, 2013).

GSH, GSNO, and GSSG are detected using HPLC. A 4-channel electrochemical array would be employed for the simultaneous detection of GSH, GSNO, and GSSG. The mobile phase would be composed of 25 m*M* monobasic sodium phosphate, 0.5 m*M* 1-octane sulfonic acid (ion-pairing agents), and 2.5% acetonitrile, pH 2.7. The pH for the mobile phase should be adjusted with 85% phosphoric acid. A flow rate of 1 mL/min would be used with a C18 column. With 2.5% of acetonitrile in the mobile phase, the retention times for GSH generally appears at ~5 min, GSNO ~16 min, and GSSG ~20 min. This method was adapted from (Yap, Sancheti, Ybanez, Garcia, Cadenas, & Han, 2010).

(Word Count: 4448)

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