# Supplementation of Antioxidants with Curcumin, Quercetin, and L-glutathione to Reduce Dopaminergic Neurodegeneration, Alpha-synuclein Accumulation, and Decreased Motility Associated with Parkinson's in *Caenorhabditis elegans*

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**Abstract**

Parkinson's disease (PD) is a progressive neurodegenerative disorder that diminishes motor functioning and affects over 10 million people worldwide. PD is exacerbated by an imbalance between reactive oxygen species (ROS) and cellular antioxidant activity which increases oxidative stress. This oxidative stress leads to dopaminergic neurodegeneration, Alpha-synuclein accumulation and decreased motility due to unstable free radicals damaging and denaturing cellular structure. The purpose of this study was to examine the effectiveness of curcumin (25µM, 50µM, 100µM), quercetin (50µM, 100µM, 200µM), and L-glutathione (0.1mM, 1mM, 10mM) in reducing dopaminergic neuron degeneration and alpha-synuclein accumulation in fluorescently tagged Parkinson's induced *C. elegans.* BZ555 strain *C. elegans* express green fluorescent protein (GFP) where the level of fluorescence signals the amount of tagged dopaminergic neurons. OW13 strain *C. elegans* express yellow fluorescent protein (YFP) where the level of fluorescence indicates the amount of tagged Alpha-synuclein. Both strains were exposed to 10mg/L copper sulphate ($CuSO_4$) for 24 hours and then treated for 72 hours with either curcumin, quercetin, or L-glutathione. Fluorescence images were captured using a Zeiss Axiovert fluorescence microscope under 250x magnification, and analyzed through ImageJ, and the corrected total cellular fluorescence equation was calculated using an equation. Motility and physical reflexes were measured by the locomotion and liquid thrashing assays. The means of each assay were analyzed through One Way ANOVA followed by a post-hoc Scheffe test ($p<0.05$). Curcumin treated BZ555 GFP showed the most significant increase in dopaminergic neuron levels and L-glutathione treated OW13 YFP showed the most significant inhibition of Alpha-synuclein levels. The L-glutathione treated group was also the most efficient in significantly increasing motility and reflexes of neurotoxin exposed *C. elegans*. It's postulated that *C. elegans* treated with curcumin, quercetin, and L-glutathione experienced a balance between reactive oxygen species and cellular antioxidant activity which reduces oxidative stress, a main factor in the exacerbation of Parkinson's.

## I. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that diminishes motor functioning and affects over 10 million people worldwide. (Centers for Disease Control, 2019) Patients diagnosed with PD can experience a wide range of symptoms including tremors, slowness of movement, impaired balance and coordination, stiffness, and several other symptoms that can severely affect one's life. (National Institute of Neurological Disorders and Stroke, 2019) Rates of PD have increased from 18.2 between 1976 and 1985 to 30.4 between 1996 and 2005 with the greatest increase in men over 70. (Savica et al. 2016) Parkinson's risk and prevalence increases with age and with life expectancy on the rise, PD diagnosis is projected to increase.

Parkinson's disease symptoms worsen as the disease progresses, which is due to the neurodegenerative effects of the disorder. Motor degeneration caused by PD leads to symptoms typically beginning on one side of the body, but as the disease progresses it can affect both sides and eventually lead to the loss of motor function. The two main factors in the pathology of PD is the loss of dopaminergic neurons in the substantia nigra and the development of Lewy bodies in the brain tissue. (National Institute of Neurological Disorders and Stroke, 2019)

Movement control is established by interactions between nerve cells in the central nervous system. (Triarhou, 2013) The neurons that are specifically designed for movement communication are located in the substantia nigra. The substantia nigra is a major region of the brain that controls balance and movement. The neurotransmitter dopamine, also known as 3,4-Dihydroxytyramine, is a chemical released by neurons to send signals to other neurons. Dopamine controls movement and is used by the substantia nigra to send signals to the corpus striatum which results in smooth, purposeful movement. Dopamine is produced by dopaminergic neurons in the brain from tyrosine through the addition of a hydroxyl group which transforms it to L-dopa and then removes a carboxylic acid group resulting in dopamine. Once produced, dopamine is stored in a synaptic vesicle until action potentials, which is the change in electrical potential associated with the passage of an impulse along the membrane of a neuron, induce the release of dopamine. (Triarhou, 2013)

The cause of motor related symptoms in Parkinson's patients is largely due to the progressive loss of dopamine-producing neurons in the substantia nigra. (National Institute of Neurological Disorders and Stroke, 2019) Dopaminergic neurons (DA) begin to die when the cell's energy producer, mitochondria, fail. The loss of dopaminergic neurons decreases dopamine signaling to the striatum which contributes to the motor symptoms of PD. (Cooper et al, 2018) The average Parkinson's patient loses over 60 to 80 percent or more of the dopamine-producing cells in the substantia nigra before symptoms appear. People with Parkinson's do not develop symptoms until later in life which indicates that age plays a role in the

development of the disease. As patients age, mitochondrial function and cellular metabolism decline resulting in dopamine neurons dying. (Parkinson, 2019)

In affected brain cells of people with PD, there is a strong presence of Lewy bodies, which are abnormal aggregates of protein that develop inside nerve cells and contribute to the progression of the disease. (U.S. National Library of Medicine) The protein that is deposited in these Lewy bodies is alpha-synuclein, which is a protein that is abundant in the brain and found mainly at the endings of neurons. Alpha-synulcien is encoded by the SNCA gene which is a gene that can make PD heritable. (Fu et al. 2014) The function of alpha-synuclein is not completely understood, however, it may play a role in the release of dopamine. In Parkinson's, the alpha-synuclein protein misfolds and begins to accumulate forming Lewy bodies. (The Cure Parkinson's Trust, 2019)

This accumulation is very damaging to cellular components and causes cell death, including dopaminergic neurons. Alpha-synuclein accumulation spreads from diseased cells to healthy cells through synapses, a connection between two cells, resulting in the exacerbation of Parkinson's. (Longhena et al. 2017) It has been found that abnormal alpha-synuclein deposition occurs early in PD and may play a key cause in the pathogenesis of PD. A promising strategy in treating PD could be reducing and altering alpha-synuclein to nonpathogenic. Alpha-synuclein accumulation is harmful to cellular structure because it attacks synaptic vesicles leadings to leaking of neurotransmitters like dopamine which results in oxidative stress. (Stefanis, 2012)

Oxidative stress occurs when an imbalance is formed between production of reactive oxygen species (ROS) and cellular antioxidant activity. (Hwang, 2013) Oxidative damage is the harm sustained by cells and tissues that are unable to keep up with free radical production. The presence of ROS generating enzymes including tyrosine hydroxylase and monoamine oxidase makes dopaminergic neurons prone to oxidative stress. The major sources of oxidative stress generated for dopaminergic neurons is believed to be the ROS produced during dopamine metabolism, mitochondrial dysfunction, and neuroinflammation. (Hwang, 2013) Due to the cellular activity of oxidative enzymes, free radicals are produced which are unstable atoms that can damage cells. The accumulation of oxidative stress caused by aging and free radicals can cause accumulation of alpha-synuclein which exacerbates PD. (Kumar et al. 2012)

As of right now there is no effective cure for PD. (Fu et al. 2014) Currently,the most popular treatment for PD is levodopa, which is converted to dopamine in the brain. Levodopa, however, become less effective over time and is limited by significant side effects. The most effective therapeutic route for PD involves finding compounds that can both improve the disease and prevent its symptoms. Natural products could potentially protect dopaminergic degeneration while acting as therapeutic agents in the pathogenesis of alpha-synuclein. (Stefanis, 2012)

Curcumin, a compound ($C_{21}H_{20}O_6$) in the plant turmeric, has been linked to reducing inflammation (Woodman et al, 2016). Turmeric is made up of 4 curcuminoids including curcumin, demethoxycurcumin, bisdemethoxycurcumin, and cyclocurcumin. Curcumin which is a curcuminoid of turmeric, is suggested to reduce inflammation by being intervened with the upregulation of the peroxisome proliferator-activated receptor-y (PPAR-y) activation. (Jacob et al, 2008) Curcumin also exhibits antioxidant and neuroprotective properties in neurological disorders. Since the imbalance between reactive oxygen species (ROS) generation and cellular antioxidant activity is a leading cause of oxidative stress which leads to PD, curcumin's antioxidant properties may decelerate the progression of PD. (Nguyen et al. 2018)

Quercetin, a compound ($C_{15}H_{10}O_7$) is a flavonoid that is found in fruits and vegetables. (Warnsmann et al. 2018) Quercetin actively balances cellular reactive oxygen species (ROS) levels and has a cyto-protective function. Costa et al. (2016) found that quercetin has neuroprotective effects and is able to scavenge free radicals, reducing ROS. Antioxidant properties have been shown to reduce oxidative stress in *C. elegans* strains BZ555 and OW13. (Cooper et al, 2018)

L-glutathione reduced is an antioxidant present in almost every cell in the body, playing a role in the detoxification of drugs and xenobiotics. Reduced glutathione (GSH) acts as a hydrogen donor in the detoxification of hydrogen peroxide. As a dietary supplement, GSH possesses various systemic effects such as improvement of liver abnormalities, improvement of diabetic complication, protection from viral infection, and antitumor activity. (Weschawalit, 2017) Morgan et al. 2010 found that L-glutathione prevented oxidative stress and suppressed ROS formation in Caenorhabditis elegans.

Testing the therapeutic value of possible treatment compounds is made efficient through the use of inexpensive in vivo assays involving *Caenorhabditis elegans*. (Fu et al, 2014) *Caenorhabditis elegans* are nematodes or roundworms that live in the soil and feed on bacteria (Alton, 2006). A versatile cuticle surrounds *C. elegans* on the outside with neurons and the hypodermis separated from the musculature by a thin extracellular matrix known as the basal lamina. The muscles receive contribution from the neurons by sending muscle arms to motor neuron processes in the nerve ring. The somatic muscular system consists of striated tissue. The non-striated muscle consists of tissue that don't have sarcomeres. The adult body is anatomically simple with about 1000 somatic cells. *C. elegans* are well studied, have 8 dopaminergic neurons and have a PD-related homologous gene. (Alton, 2006)

*C. elegans* are also transparent and can be transgenically produced to be fluorescent to express human alpha-synuclein and dopaminergic neurons. *C. elegans* can be exposed to neurotoxins to induce Parkinson's which results in a useful pharmacological model of PD. Neurotoxins cause toxicity by increasing ROS leading to cellular toxicity, damage, and neuronal loss. (Cooper et al. 2018) Copper sulphate ($CuSO_4$) for example, is a toxin used by Mashock et al. (2016) to induce neurodegenerative

effects in *C. elegans*. Food-borne exposure of neurotoxins has been shown to be the primary, and most toxic, route of exposure in *C. elegans* (Mashock et al. 2016)

The purpose of this study was to examine the effectiveness of curcumin (25µM, 50µM, 100µM), quercetin (50µM, 100µM, 200µM ), and L-glutathione (0.1mM, 1mM, 10mM) in reducing dopaminergic neuron degeneration and alpha-synuclein accumulation in fluorescently tagged Parkinson's induced *C. elegans.* Both BZ555 strain *C. elegans* expressing green fluorescent protein tagged dopaminergic neurons and OW13 strain *C. elegans* expressing yellow fluorescent protein tagged Alpha-synuclein were exposed to 10mg/L copper sulphate ($CuSO_4$) for 24 hours and then treated for 72 hours with either curcumin, quercetin, or L-glutathione. Based on the research of Satapathy et al. (2016), Javed et al. (2019), and Morgan et al. (2010), it was hypothesized that curcumin, quercetin, and L-glutathione would each significantly restore dopamine levels, reduce alpha-synuclein accumulation, and improve motility in Parkinson's induced *C. elegans* due to their potent antioxidant properties.

## II. Methodology

**All of the below protocols were completed by the competition entrant.**

The first part of this experiment measured the dopaminergic neuron fluorescence of BZ555 *Caenorhabditis elegans* control, 24 hour copper sulphate exposure only, 25µM, 50µM, and 100µM 72 hour curcumin treated, 50µM, 100µM, and 200µM 72 hour quercetin treated, and 0.1mM, 1mM, and 10mM 72 hour L-glutathione treated. After 72 hour treatment, *C.elegans* were transferred onto a slide for 15 minutes and paralyzed with ethanol, the slide was then viewed under 250x total magnification at an excitation wavelength of 500 nm and an emission wavelength of 535 nm. Images were captured of three worms in each group with a canon DSLR camera attached to a ZEISS Axiovert 40 CFL fluorescent microscope with a green fluorescent protein filter (GFP).

The next part of this experiment measured the Alpha-synuclein accumulation of OW13 *Caenorhabditis elegans* control, 24 hour copper sulphate exposure only, 25µM, 50µM, and 100µM 72 hour curcumin treated, 50µM, 100µM, and 200µM 72 hour quercetin treated, and 0.1mM, 1mM, and 10mM 72 hour L-glutathione treated. After 72 hour treatment, *C.elegans* were transferred onto a slide for 15 minutes and paralyzed with ethanol, the slide was then viewed under 250x total magnification at an excitation wavelength of 500 nm and an emission wavelength of 535 nm. Images were captured of three worms in each group with a canon DSLR camera attached to a ZEISS Axiovert 40 CFL fluorescent microscope with a yellow fluorescent protein filter (YFP).

The fluorescence intensities of both GFP and YFP groups was quantified using ImageJ and the corrected total cellular fluorescence equation (CTCF) and then analyzed in IBM SPSS V.26. After determining which concentrations had the greatest effects, a locomotion and liquid thrashing assay was

conducted using BZ555 *C. elegans* exposed to the control which was just agar, 24 hr 10mg/L copper sulphate only, 72 hour quercetin treated, 72 hour curcumin treated, and 72 hour L- glutathione treated.

**Caenorhabditis elegans Strains:** Strains BZ555 and OW13 were generously donated by the Caenorhabditis Genetics Center (CGC). *C. elegans* strain BZ555 is tagged with green fluorescent proteins expressed specifically in dopaminergic neurons. *C. elegans* strain OW13 is tagged with yellow fluorescent proteins expressed specifically in alpha-synuclein.

**Culturing and Maintenance of *Escherichia coli* OP50:** Luria broth powder (Carolina Biological) was dissolved in water in a ratio of 250g/L. The solution was stirred with a glass rod in an Erlenmeyer flask over a hot plate. Luria broth was sterilized in the autoclave for 25 minutes at 121°C at STE. Once fully sterilized, the broth was transferred into previously sterilized vials. Next, an inoculating loop was sterilized by heating it in a Bunsen burner flame, and utilized to transfer a small amount of OP50 E. coli from the culture vial to a Luria broth vial. These vials were incubated at room temperature (37°C) overnight. (Carolina, 2018).

***Caenorhabdistis elegans* Food Source Preparation:**

The new *E. coli* was removed from the incubator and the mouth of the vial was sterilized using a Bunsen burner. A sterile cotton tip applicator was opened and used to swab 1.0mL fluid inoculum onto a nematode growth media (NGM) plate. The NGM plate was then placed in the incubator (Lab-Line Imperial II Incubator) overnight to ensure growth. This process was done on several petri dishes (Carolina, 2018).

**Culturing and Maintenance of *Caenorhabditis elegans*:** To culture the *C. elegans*, *E. coli* OP50 inoculated plates were removed from the 37 °C incubator. A sterile disposable scalpel was removed from its package. The *C. elegans* culture was cut into blocks of $1cm^3$. The blocks were then transferred to one of the inoculated plates and placed upside down so that the worms could directly contact the agar. This process was repeated for the other inoculated plates. Then, the scalpel was disposed into the autoclave disposal bag. The *C. elegans* cultures were placed in an area with no air drafts and at room temperature and the original *C. elegan*s stock was kept as an extra stock. (Carolina, 2018).

**Age Synchronization:** In order for results to be comparable, each assay had to be done exactly the same. Age synchronization was performed on *C. elegans* before all assays. First, 6.0 mL of M9 solution was pipetted onto a plate of *C. elegans* to dislodge the worms from the plate. Then, 5.0 mL of worms were transferred into a tube where it was centrifuged (1,000 x g for 1 minute) to create a worm pellet at the bottom of the tube. There was a blank micro test tube in the centrifuge to balance out the circular motion. Then the supernatant was taken out carefully. Next the worms were washed with 20% alkaline hypochlorite (5.0mL) and after centrifuged, the supernatant was removed again. Next the worms were washed with 10 mL M9 buffer in the centrifuge (1,000 x g for 1 minute). The supernatant was

removed and new M9 was inserted and then the tube was put onto an orbital shaker (Cole Parmer) for 24 hours. 24 hours later, *C. elegans* (L4 stage) were placed onto a petri dish and were ready to use in assays (Satapathy et al. 2016).

**Copper Sulphate Exposure:** Age synchronized *C. elegans* were placed onto unseeded agar plates containing 10mg/L copper sulphate for 24 hour exposure. *C. elegans* that were not receiving treatment were placed on an unseeded agar plate for 72 hours. Groups that were receiving treatment were then transferred onto agar plates with specific variables and concentrations for 72 hours.

**Chemical Administration:** Age synchronized *C. elegans* were placed onto the specific agar plates. Curcumin, quercetin, and L-glutathione treated groups were placed onto agar plates that were made with the associated chemical. Curcumin, quercetin, and L-glutathione were all purchased from Sigma-Aldrich. Curcumin and quercetin were diluted with Dimethyl Sulfoxide and l-glutathione was diluted with distilled water. To treat groups of copper sulphate exposed *C. elegans*, chemicals were integrated into the agar using a micropipette. In this experiment *C. elegans* groups were treated for 72 hours by being exposed to 25µM, 50µM, and 100µM of curcumin, 50µM, 100µM, and 200µM of quercetin, and 0.1mM, 1mM, and 10mM of L-glutathione reduced.

**Fluorescence Microscopy Dopaminergic Neurodegeneration Assay:** Assay of dopaminergic degeneration was performed in *C. elegans* exposed to either copper sulphate alone or treated with a concentration of curcumin, Quercetin, and L-glutathione. A single L4 *C. elegans* was transferred using a sterilized platinum wire into 1 mL of ethanol (40%) on a microscope well slide to paralyze the specimen, and a cover slip was placed on top of the ethanol. After 15 minutes, the slide was viewed under the fluorescent microscope. In this assay a ZEISS Axiovert 40 CFL fluorescent microscope with a green fluorescent protein filter having an excitation wavelength of 500 nm and an emission wavelength of 535 nm was utilized to observe fluorescently tagged dopaminergic neurons in *C. elegans* at 250x magnification. A Canon DSLR camera with an 8” shutter speed and 3200 ISO was also used to capture photographs.

**Fluorescence Microscopy Alpha-synuclein Accumulation Assay:** Assay of alpha-synuclein accumulation was performed in animals exposed to either copper sulphate alone or treated with a concentration of curcumin, Quercetin, and L-glutathione. A single L4 *C. elegans* was transferred using a sterilized platinum wire into 1 mL of ethanol (40%) on a microscope well slide to paralyze the specimen, and a cover slip was placed over the ethanol. After 15 minutes, the slide was viewed under the fluorescent microscope. In this assay a ZEISS Axiovert 40 CFL fluorescent microscope with a yellow fluorescent protein filter having an excitation wavelength of 500 nm and an emission wavelength of 535 nm was utilized to observe fluorescently tagged Alpha-synuclein in OW13 strain *C. elegans* at 250x

magnification. A Canon DSLR camera with an 8” shutter speed and 3200 ISO was also used to capture photographs.

**Quantification of Fluorescence:** After images were captured using the canon DSLR, photos were uploaded to an Apple Macbook Pro with ImageJ software. Images were analyzed through ImageJ, where the fluorescence represents the level of dopaminergic neurons in GFP tagged BZ555 *C. elegans* and Alpha-synuclein accumulation in YFP tagged OW13 *C. elegan*s. With the numbers generated from ImageJ analysis, fluorescence was quantified through the corrected total cellular fluorescence (CTCF), equation 1.

[CTCF = Integrated Density - (Area of Selected Cell × Mean Fluorescence of Background Readings)] (Equation 1)

This allowed both a qualitative measurement of mutant SOD1 aggregation in the images and a quantitative measurement of the CTCF equation outputs.

**Locomotion Assay:** First, age synchronized groups of BZ555 control, 24 hr copper sulphate exposed, 72 hour quercetin treated, 72 hour curcumin treated, and 72 hour L- glutathione treated were placed on an unseeded agar plate. Then, worms in resting position, were stimulated using a platinum worm pick. The *C. elegans* were tapped with a platinum worm pick for one second. The worms performed body bends which were then recorded in a 60 second trial. A 60 second video of *C. elegans* were recorded using a video phone with the SnapZoom attachment.

**Liquid Thrashing Assay:** BZ555 groups were tested in the liquid thrashing assay. Groups of age synchronized BZ555 control, 24 hr copper sulphate exposed, 72 hour quercetin treated, 72 hour curcumin treated, and 72 hour L- glutathione treated were placed on an unseeded petri dish. Next, 3 mL of M9 buffer was placed in the center of the dish and spread to the entirety of the dish. *C. elegans* then thrashed showing stimulation as a result of the application of the M9 buffer. The number of thrashes performed was recorded during a 60 second video of *C. elegans* using a video camera with the SnapZoom attachment to the compound microscope.

**Data Analysis:** For the Locomotion assay, each scored *C. elegans* was considered as a trial, and the mean number of body bends across all trials of a group was calculated. Similarly, for the Liquid Thrashing assay, each scored *C. elegans* was considered as a trial, and the mean number of body thrashes across all trials of a group was calculated. Each *C. elegans* in both the dopaminergic neurons and alpha-synuclein fluorescence microscopy assays was also considered a trial, and the mean of all corrected total cell fluorescence (CTCF) output values of a group was calculated. (Fitzpatrick, 2014) Mean ± SD are presented on bar graphs using Microsoft Excel for each assay. All data was analyzed using a One Way ANOVA followed by a post-hoc Scheffe test ($p < 0.05$) using the IBM SPSS version 25 software.

## III. Results

Dopaminergic Neuron Degeneration Fluorescence

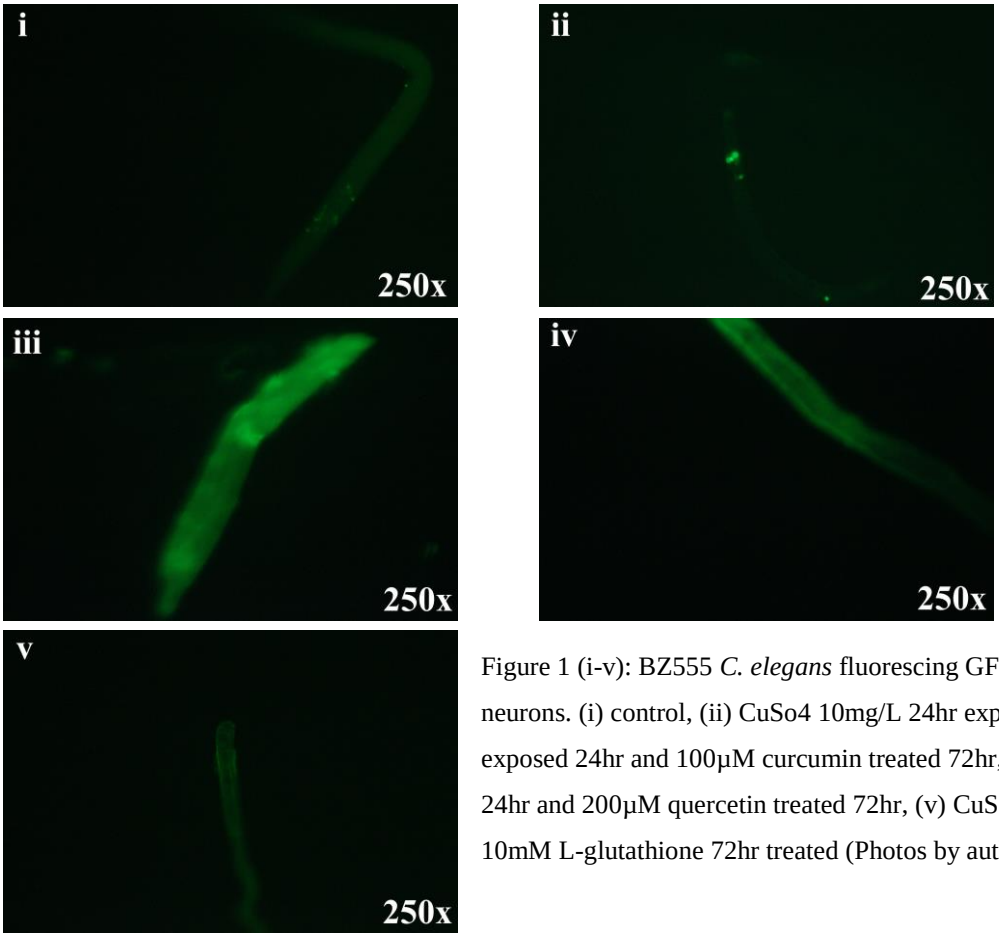


Figure 1 (i-v): BZ555 *C. elegans* fluorescing GFP tagged dopaminergic neurons. (i) control, (ii) CuSo4 10mg/L 24hr exposed, (iii) CuSO4 exposed 24hr and 100µM curcumin treated 72hr, (iv) CuSO4 exposed 24hr and 200µM quercetin treated 72hr, (v) CuSO4 exposed 24hr and 10mM L-glutathione 72hr treated (Photos by author)

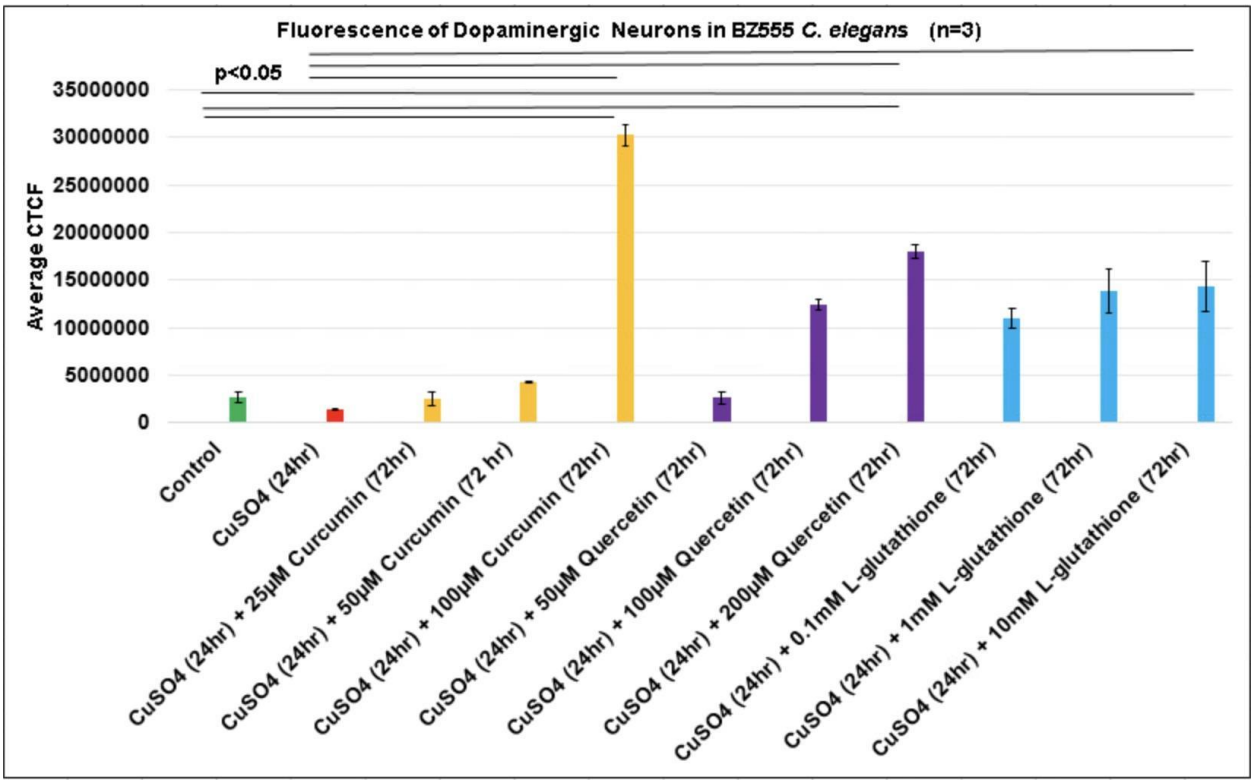


Figure 2: Mean ± SD GFP tagged dopaminergic neuron fluorescence intensities for BZ555 *C. elegans* in CTCF units. Statistical significance determined by a One-Way ANOVA with a post-hoc Scheffe test ($p<0.05$). (Graph by author)

The fluorescence microscopy green fluorescent protein (GFP) assay measured the amount of dopaminergic neurons present in GFP tagged BZ555 *C. elegans*. In this assay, a higher fluorescence indicates a higher amount of dopaminergic neurons where as a lower fluorescence correlates to neurodegeneration. By interpreting the qualitative data seen in figure 1, the 100µM curcumin treated group had the brightest fluorescence suggesting it to be the most effective in increasing/protecting dopaminergic neuron production. The values in Figure 2 are derived from picture analysis using ImageJ and the CTCF equation. *C. elegans* CTCF was quantified after 24 hour copper sulphate exposure, 25µM, 50µM, and 100µM 72 hour curcumin treated, 50µM, 100µM, and 200µM 72 hour quercetin treated, and 0.1mM, 1mM, and 10mM 72 hour L-glutathione treated. Over three trials, the mean fluorescence intensity for the control BZ555 *C. elegans* was 2,748,734 CTCF. The mean fluorescence for the 24 hour copper sulphate exposure was 1,463,632 which indicates neurodegeneration compared to the control and is seen in figure 1 (ii). $CuSO_4$ exposed *C. elegans* treated with 25µM curcumin averaged 2,565,472 CTCF. *C. elegans* treated with 50µM curcumin averaged 4,316,244 CTCF. *C. elegans* treated with 100µM curcumin had a significantly higher CTCF, 30,248,935, than *C. elegans* only exposed to $CuSO_4$

and a significantly higher CTCF than the control group. *C. elegans* treated with 50µM quercetin averaged 2,637,756 CTCF and *C. elegans* treated with 100µM quercetin averaged 12,400,978 CTCF. *C. elegans* that were treated with 200µM quercetin had a significantly higher CTCF, 18046401, than both the control and $CuSO_4$ only group. The group treated with 0.1mM L-glutathione averaged a CTCF of 11,028,378 and the group treated with 1mM L-glutathione averaged a CTCF of 13,825,252. Finally, the group treated with 10mM glutathione had a significantly higher CTCF, 14,300,010, than both the control and $CuSO_4$ only group. The 100µM curcumin, 200µM quercetin, and 10mM L-glutathione groups were the most effective concentrations from the three treatment variables which is why they are later used in the locomotion and liquid thrashing assay.

Alpha-synuclein Accumulation Fluorescence

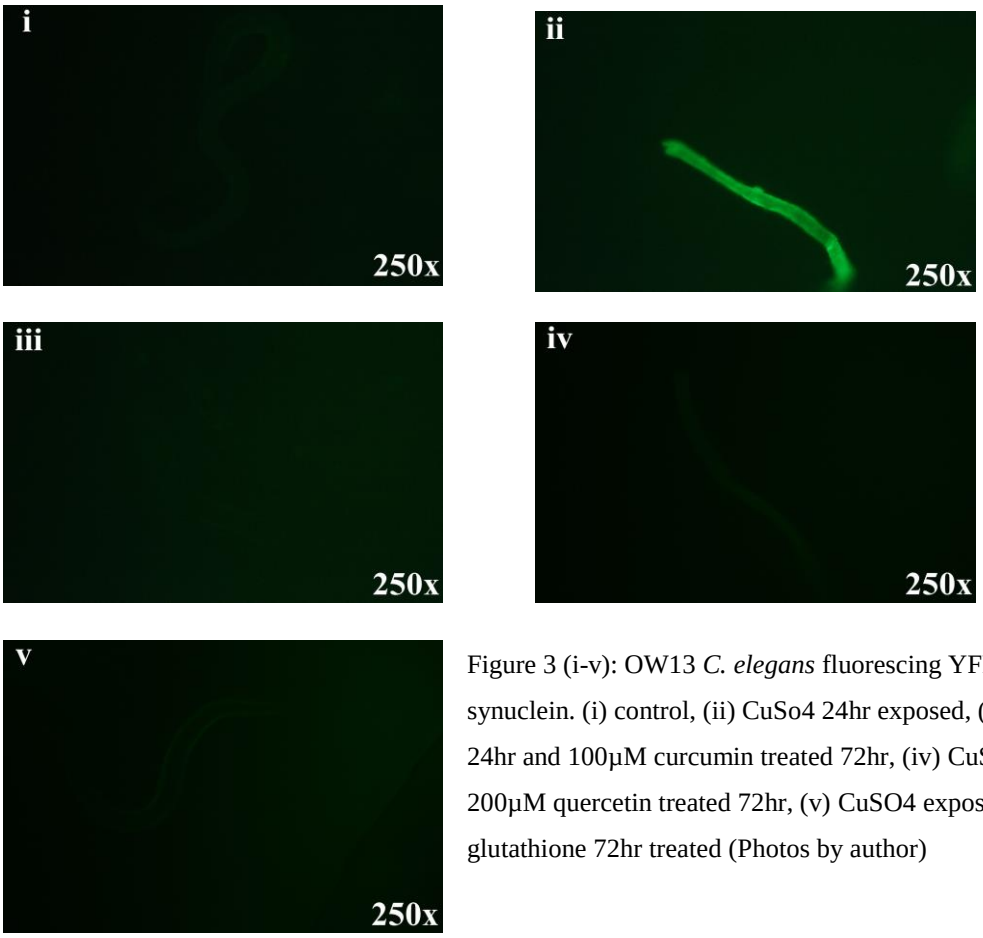


Figure 3 (i-v): OW13 *C. elegans* fluorescing YFP tagged Alpha-synuclein. (i) control, (ii) CuSo4 24hr exposed, (iii) CuSO4 exposed 24hr and 100µM curcumin treated 72hr, (iv) CuSO4 exposed 24hr and 200µM quercetin treated 72hr, (v) CuSO4 exposed 24hr and 10mM L-glutathione 72hr treated (Photos by author)

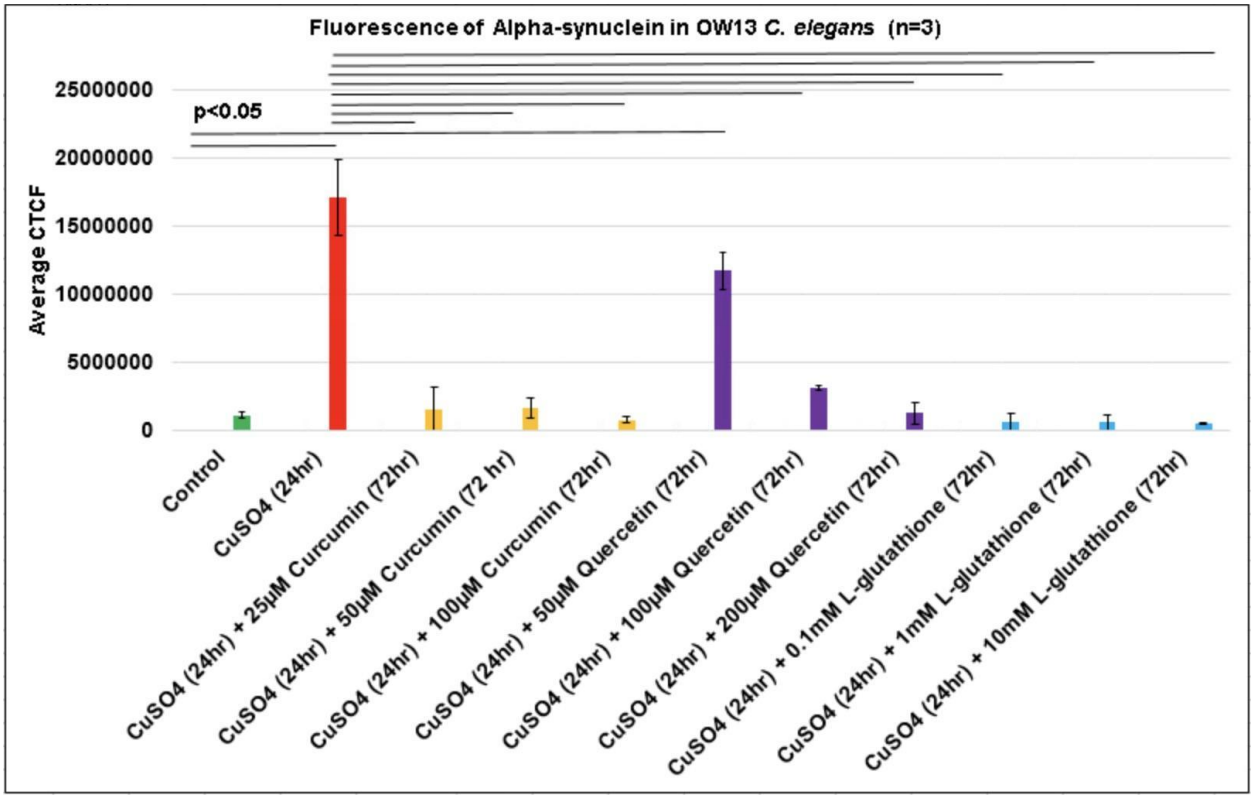


Figure 4: Mean ± SD YFP tagged Alpha-synuclein accumulation fluorescence intensities for OW13 *C. elegans* in CTCF units. Statistical significance determined by a One-Way ANOVA with a post-hoc Scheffe test ($p<0.05$). (Graph by author)

The fluorescence microscopy yellow fluorescent protein (YFP) assay measured the amount of Alpha-synuclein accumulation present in YFP tagged OW13 *C. elegans.* In this assay, a higher fluorescence indicates a higher buildup of Alpha-synuclein where as a lower fluorescence correlates to low amounts of Alpha-synuclein. By interpreting the qualitative data seen in figure 1, the 10mM L-glutathione treated group had the lowest fluorescence suggesting it to be the most effective in decreasing/preventing Alpha-synuclein accumulation.The values in Figure 4 are derived from picture analysis using ImageJ and the CTCF equation. *C. elegans* CTCF was quantified after 24 hour copper sulphate exposure, 25µM, 50µM, and 100µM 72 hour curcumin treated, 50µM, 100µM, and 200µM 72 hour quercetin treated, and 0.1mM, 1mM, and 10mM 72 hour L-glutathione treated. Qualitatively speaking, Figure 3 (ii) is much brighter than figure 3 (i) suggesting that $CuSO_4$ exposure increases Alpha-synuclein. Over three trials, the mean fluorescence intensity for the control OW13 *C. elegans* was 1,165,510 CTCF which was significantly lower than the CTCF for the $CuSO_4$ only group. The control group which was not exposed to $CuSO_4$ has a lower CTCF which indicates less Alpha-synuclein accumulation compared to the CuSO4 24 hour exposed group. The mean fluorescence for the 24 hour copper sulphate exposure was 17,049,301. $CuSO_4$ exposed *C. elegans* treated with 25µM curcumin

averaged 1,631,652 CTCF which was significantly lower than the $CuSO_4$ only group. *C. elegans* treated with 50µM curcumin averaged 1,659,907 CTCF, significantly lower than the $CuSO_4$ only group. *C. elegans* treated with 100µM curcumin had a significantly lower CTCF, 824,581, than *C. elegans* only exposed to $CuSO_4$. *C. elegans* treated with 50µM quercetin averaged 11,715,968 CTCF which was however, significantly higher than the control CTCF. *C. elegans* treated with 100µM quercetin had a significantly lower average CTCF than the untreated $CuSO_4$ exposed group by averaging 3,140,003 CTCF. *C. elegans* treated with 200µM quercetin also had a significantly lower CTCF than the untreated $CuSO_4$ exposed group with an average CTCF of 1,340,270 units. Finally, the groups treated with 0.1mM (659,586 CTCF), 1mM (641,156 CTCF), and 10mM (537,781 CTCF) L-glutathione all averaged significantly lower CTCF values than the $CuSO_4$ only group.

Locomotion

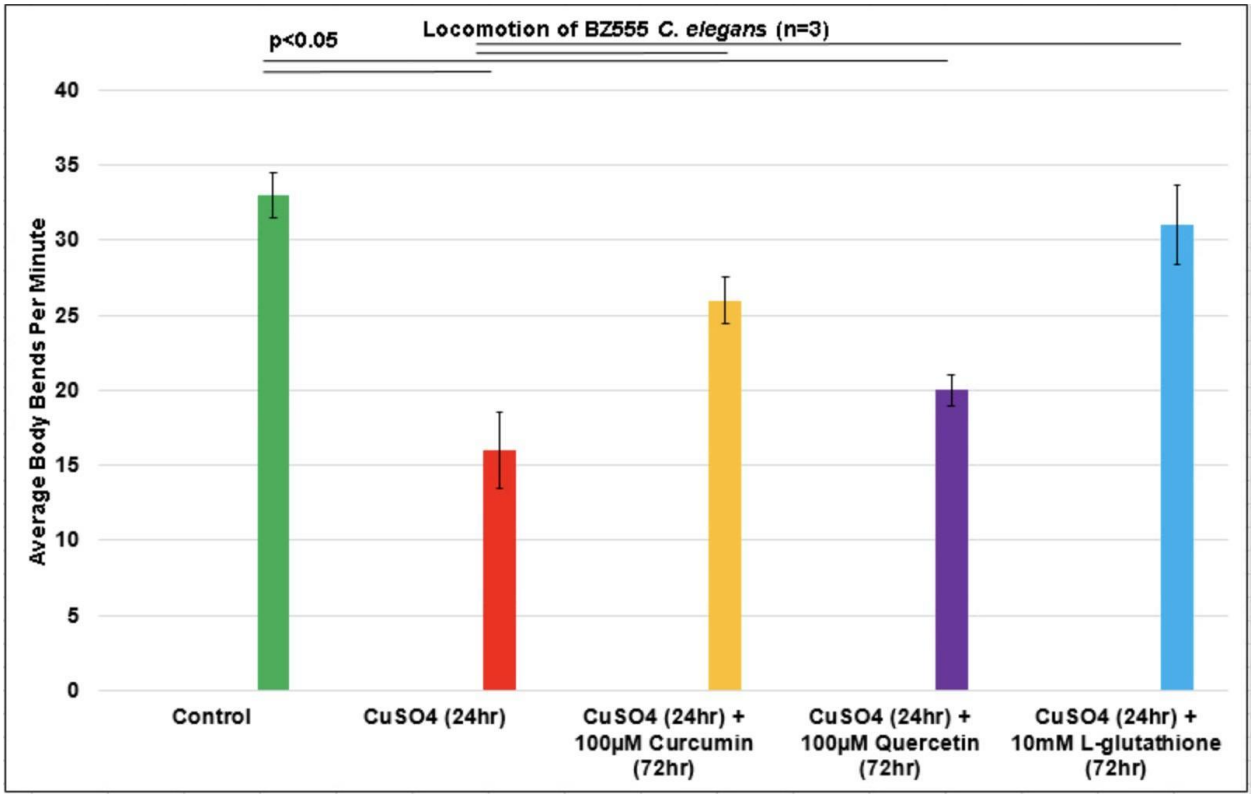


Figure 5: Mean ± SD number of body bends per minute for BZ555 *C. elegans.* Statistical significance determined by a One-Way ANOVA with a post-hoc Scheffe test ($p<0.05$). (Graph by author)

The locomotion assay measured the body bends of BZ555 *C. elegans* control, 24 hour copper sulphate exposed, 72 hour 100µM curcumin treated,72 hour 200µM quercetin treated, and 72 hour 10mM L-glutathione treated. The number of body bends were measured during a 60 second stimulation period where a platinum wire was utilized to probe the *C. elegans*. The BZ555 control group averaged 33 bends per minute which was significantly higher than the $CuSO_4$ only group (16 body bends per minute). *C.*

*elegans* treated with 100µM curcumin for 72 hours averaged 26 body bends per minute, significantly higher than the $CuSO_4$ only group. *C. elegans* treated with 200µM quercetin for 72 hours averaged 20 body bends per minute which was actually significantly lower than the control. Finally, *C. elegans* exposed to 10mM L-glutathione for 72 hours and averaged 31 body bends per minute which was significantly higher than the $CuSO_4$ only group.

Liquid Thrashing

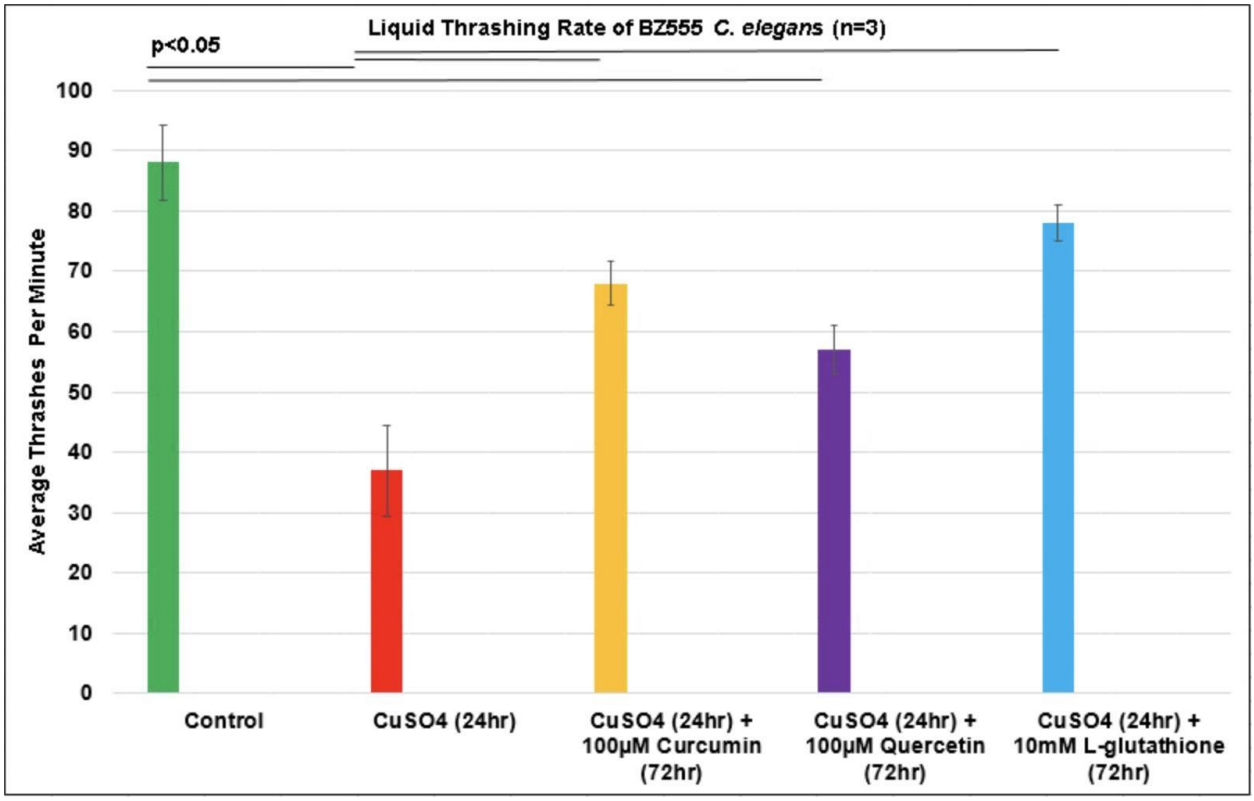


Figure 6: Mean ± SD number of liquid thrashes per minute for BZ555 *C. elegans.* Statistical significance determined by a One-Way ANOVA with a post-hoc Scheffe test ($p<0.05$). (Graph by author)

The liquid thrashing assay measured the average thrash counts of BZ555 *C. elegans* control, 24 hour copper sulphate exposed, 72 hour 100µM curcumin treated, 72 hour 200µM quercetin treated, and 72 hour 10mM L-glutathione treated. The number of thrashes were measured during a 60 second period where 3mL of M9 buffer was pipetted into the center of the agar plate which influenced a swimming behavior. The BZ555 control group averaged 88 thrashes per minute while the copper sulphate exposed group only averaged 37 per minute which was significantly lower. *C. elegans* treated with 100µM curcumin for 72 hours averaged a significantly higher 68 thrashes per minute than the $CuSO_4$ only group. *C. elegans* treated with 200µM quercetin for 72 hours averaged 57 thrashes per minute but this is significantly lower than the $CuSO_4$ only group. Finally, Then, C. *elegans* exposed to 10mM L-glutathione for 72 hours averaged 78 thrashes which was significantly higher than the $CuSO_4$ only group.

## IV. Discussion

The purpose of this study was to examine the effectiveness of curcumin (25µM, 50µM, 100µM), quercetin (50µM, 100µM, 200µM), and L-glutathione (0.1mM, 1mM, 10mM) in reducing dopaminergic neuron degeneration and alpha-synuclein accumulation in fluorescently tagged *C. elegans* induced with Parkinson's from copper sulphate exposure. Both BZ555 strain *C. elegans* expressing green fluorescent protein tagged dopaminergic neurons and OW13 strain *C. elegans* expressing yellow fluorescent protein tagged Alpha-synuclein were exposed to 10mg/L copper sulphate ($CuSO_4$) for 24 hours and then treated for 72 hours with either curcumin, quercetin, or L-glutathione.

It was hypothesized that treatments of curcumin, quercetin, and L-glutathione would each significantly restore dopaminergic neuron levels, reduce alpha-synuclein accumulation, and improve motility in Parkinson's induced *C. elegans* due to their potent antioxidant properties. All treatment groups were exposed to variables on top of agar plates for 72 hours which allowed for the molecules to be consumed by the *C. elegans* and have enough time for the antioxidant properties to function. The results of the dopaminergic degeneration fluorescence assay support the hypothesis because groups that were exposed to 100µM curcumin, 200µM quercetin, and 10mM L-glutathione all experienced a significant increase in dopaminergic neurons compared to the untreated copper sulphate group. The results of the Alpha-synuclein accumulation fluorescence assay support the hypothesis because copper sulphate exposed groups treated with 25µM, 50µM, and 100µM curcumin, 100µM and 200µM quercetin, and 0.1mM, 1mM, and 10mM l-glutathione all produced a significantly lower CTCF value indicating a reduction in Alpha-synuclein when compared to the untreated copper sulphate group. The results of the locomotion assay support the hypothesis as well since copper sulphate exposed groups treated with 100µM curcumin and 10mM L-glutathione had significantly higher body bends per minute compared to the untreated copper sulphate exposed group. The results of the liquid thrashing assay also support the hypothesis because copper sulphate exposed groups treated with 100µM curcumin and 10mM L-glutathione had significantly higher thrashes per minute than the untreated copper sulphate exposed group.

Despite very little research studying copper sulphate's effect on neurodegeneration, this study shows that copper sulphate decreased dopaminergic neurons which is why it was used for the entirety of the experiment to induce Parkinson's associated symptoms. Copper sulphate exposed *C. elegans* experienced a significant decrease in dopaminergic neurons in the fluorescent GFP assay and a significant increase in Alpha-synuclein accumulation in the fluorescence YFP assay which indicates neurodegeneration. Copper sulphate most likely acted as a neurotoxin by producing hydroxyl radicals at the exterior of cells causing damage to membranes. (Mashock et al. 2016) The copper sulphate molecules could have depleted cellular energy through decreasing mitochondria function which would result in an

increase in oxidative stress and overall depletion of dopaminergic neurons. Excess levels of heavy metals can also inhibit cellular metabolic activity and lead to the disruption of protein structure. Since copper sulphate is believed to act as a neurotoxin by disrupting protein structure with damaging cell membranes with hydroxyl radicals, Alpha-synuclein may accumulate from copper sulphate's damage to protein structure which could possibly create Lewy bodies. (Mashock et al. 2016) These neurodegenerative effects of copper-sulphate also decreased motility in exposed *C. elegans*, further supporting that copper sulphate is a neurotoxin. (Mashock et al. 2016)

The trends seen in the GFP and YFP fluorescence assays further support the antioxidant and anti-inflammatory properties of curcumin, quercetin, and L-glutathione. Increases in oxidative stress can lead to ROS and cellular damage including neurodegeneration of dopamine, but when antioxidants are consumed, free radicals are neutralized which limits cell damage. Antioxidants improve mitochondrial oxidant stress and lowers oxidized dopamine which allows dopaminergic neurons to function normally, alleviating the symptoms of Parkinson's. (Northwestern, 2017)

By interpreting the qualitative data seen in figure 1, the 100µM curcumin treated group (iii) had the brightest fluorescence suggesting it to be the most effective in increasing and protecting dopaminergic neuron production. As curcumin concentrations increased in the dopaminergic neuron fluorescence assay, CTCF values increased which shows that the higher the curcumin concentration, the stronger of the effects on dopaminergic neurons. The 100µM curcumin group's CTCF was over 21 times as high as the GFP untreated group which shows how effective curcumin is at increasing dopaminergic neurons. Satapathy et al. (2016) also found that a curcumin concentration of 50µM significantly increased dopaminergic levels in neurotoxin exposed *C. elegans*. Curcumin most likely increased dopaminergic neurons with its powerful antioxidant properties which could have mitigated cellular damage induced by oxidative stress. (Satapathy et al. 2016) Curcumin was also significantly effective in lowering Alpha-synuclein in copper sulphate exposed *C. elegans*. In the fluorescence microscopy YFP assay *C. elegans* treated with 25µM curcumin averaged 1,631,652 CTCF which was significantly lower than the $CuSO_4$ only group. *C. elegans* treated with 50µM curcumin averaged 1,659,907 CTCF, significantly lower than the $CuSO_4$ only group, and *C. elegans* treated with 100µM curcumin had a significantly lower CTCF, 824,581, than *C. elegans* only exposed to $CuSO_4$. This significant reduction in Alpha-synuclein suggests that curcumin is effective in treating Alpha-synuclein accumulation which is a key risk of Parkinson's. In the locomotion and liquid thrashing assay, *C. elegans* treated with 100µM curcumin for 72 hours averaged 26 body bends per minute, significantly higher than the $CuSO_4$ only group and *C. elegans* treated with 100µM curcumin for 72 hours averaged a significantly higher 68 thrashes per minute than the $CuSO_4$ only group. Since curcumin increases dopaminergic neurons which correlate to movement, the

curcumin treated groups likely increased experienced an increase in movement due to an increase in dopamine.

The data from this study also suggests that the antioxidant quercetin is effective in increasing dopaminergic neurons and reducing Alpha-synuclein accumulation. *C. elegans* that were treated with 200µM quercetin had a significantly higher CTCF, 18,046,401, than both the control and untreated $CuSO_4$ group in the GFP fluorescence assay. This shows that quercetin is effective in increasing dopaminergic neurons in a neurotoxin exposed model organism, *C. elegans*. In the YFP fluorescence assay, *C. elegans* treated with 100µM quercetin had a significantly lower average CTCF than the untreated $CuSO_4$ exposed group by averaging 3,140,003 CTCF. *C. elegans* treated with 200µM quercetin also had a significantly lower CTCF than the untreated $CuSO_4$ exposed group with an average CTCF of 1,340,270 units. Javed et al. (2019) found that flavonoids similar to querectin reduced oxidative stress in a *Caenorhabdtis elegans* which further supports querectin's antioxidant capabilities.

Finally, the results of this study affirm that L-glutathione is an effective antioxidant to increase dopaminergic neurons, reduce Alpha-synuclein accumulation, and improve motility in Parkinson's induced *C. elegans*. In the GFP fluorescence assay, groups treated with 10mM glutathione had a significantly higher CTCF, 14,300,010, than both the control and untreated $CuSO_4$ group. In the YFP fluorescence assay, groups treated with 0.1mM (659,586 CTCF), 1mM (641,156 CTCF), and 10mM (537,781 CTCF) L-glutathione all averaged significantly lower CTCF values than the untreated $CuSO_4$ group. This clearly shows that L-glutathione reduces Alpha-synuclein accumulation as it was significant in every group. The locomotion and liquid thrashing assay also found that *C. elegans* exposed to 10mM L-glutathione for 72 hours and averaged 31 body bends per minute which was significantly higher than the untreated $CuSO_4$ group and *C. elegans* exposed to 10mM L-glutathione for 72 hours averaged 78 thrashes which was significantly higher than the untreated $CuSO_4$ group. Morgan et al. (2010) also found that L-glutathione had neuroprotective effects on *Caenorhabditis elegans* exposed to neurotoxins.

Although this study was very successful in exploring the capabilities of natural antioxidants to be used as supplements in Parkinson's induced *C. elegans*, there were some limitations during the study. First, copper sulphate was efficient in inducing neurodegenerative effects in *C. elegans*, but not as effective as oxidopamine which is a widely used neurotoxin to induce PD in model organisms. Oxidopamine couldn't be used in this study due to its potential risk to humans. Another limitation was contamination. Due to air sporulation, the agar would become contaminated and begin to mold. This was a problem because when the agar would mold, *C. elegans* could no longer live on the agar. This was resolved by creating a more rigours parafilm regiment which included parafilming in the fume hood to decrease the amount of time the agar was exposed to the air. Lastly, the *E.coli* culture would sometimes become too dense and unable to spread on the agar. This was problematic because the *E.coli* was the main

food source for the *C. elegans.* This was resolved by creating more frequent cultures of *E.coli* and parafilming the mouths of the vials.

Overall this study found that curcumin, quercetin, and L-glutathione significantly increased dopaminergic neurons, attenuated Alpha-synuclein accumulation, and restored motility and reflexes in Parkinson's induced *Caenorhabtids elegans*. These findings ultimately suggest supplementing antioxidants as a potential treatment for Parkinson's disease.

## V. Conclusion

Curcumin, quercetin, and L-glutathione all significantly increased dopaminergic neurons, attenuated Alpha-synuclein accumulation, and restored motility and reflexes in Parkinson's induced *Caenorhabtids elegans*, supporting the hypothesis. The curcumin concentration of 100µM was the most effective trial in increasing and protecting dopaminergic neurons as it significantly increased the CTCF over 21 times as high as the untreated group. The L-glutathione concentration of 10mM was the most effective trial in reducing Alpha-synuclein accumulation as it significantly reduced the CTCF by over 31 times. The L-glutathione concentration of 10mM was also most effective in increasing motility and reflexes of copper sulphate exposed *C. elegans* as it significantly increased body bends by 15 per minute and thrashes by 41 per minute. Curcumin and L-glutathione were most likely effective in restoring dopaminergic neurons and inhibiting Alpha-synuclein accumulation because of their potent antioxidant properties. It's postulated that *C. elegans* treated with curcumin, quercetin, and L-glutathione experienced a balance between reactive oxygen species and cellular antioxidant activity which reduces oxidative stress, a main factor in the exacerbation of Parkinson's.

## VI. Future Studies

In future experiments curcumin, quercetin, and L-glutathione will be compared to levodopa which is the currently used medication to treat Parkinson's by replacing dopamine. Levodopa, however, has been found to have restrictive side effects and wear off after elongated treatment. Natural antioxidants like the chemicals studied in this experiment may be more efficient is increasing dopaminergic neuron levels than levodopa. The addition of levodopa in this experiment would help suggest whether or not antioxidants should be considered in future treatments of Parkinson's. Future studies will also involve *C. elegans* pre-treated with antioxidants and a longer exposure period to examine the protective functions of antioxidants. Finally, a chi squared statistical analysis test will be used to analyze similarity to see if dopaminergic neurons or movement is restored.

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