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Characterization of Manganese-Induced Neurodegenration in C. elegans Treated with Winterberry Leaf Extract

Brendan Moline

CHARACTERIZATION OF MANGANESE-INDUCED NEURODEGENERATION IN C. ELEGANS TREATED WITH WINTERBERRY LEAF EXTRACT

by

Brendan Campbell Moline

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Advisory Committee:

Jennifer Newell-Caito, Lecturer, Molecular and Biomedical Sciences, Advisor
Edward Bernard, Senior Lecturer, Undergraduate Program Coordinator, and Graduate Coordinator, Molecular and Biomedical Sciences
Margaret Killinger, Professor of Honors, Rezendes Preceptor for the Arts, Honors College

ABSTRACT

Neurodegeneration is a condition present in Alzheimer's disease (AD) and Parkinson's disease (PD) in which the cells of the nervous system experience loss of function and death. Around the world, each year PD and AD affect 6.2 million and 29.8 million people, respectively, with the exact causes remaining unknown. Manganese (Mn) is a transition metal which is essential for human survival in trace concentrations. However, overexposure to Mn can induce neurodegeneration through the accumulation of reactive oxygen species and the eventual onset of oxidative stress. An extract produced from winterberry leaves (*Ilex verticillata*) exhibits antioxidant properties as it has been shown to protect against Mn-induced oxidative stress in the nematode worm, *Caenorhabditis elegans*. Due to this observed response in *C. elegans*, it was hypothesized that the winterberry leaf extract (WLE) could offer protection against neurodegeneration of dopaminergic neurons. To evaluate dopaminergic integrity and its effect on nematode behavior, a wild-type *C. elegans* strain was treated with several concentrations of WLE and manganese chloride ($MnCl_2$). A motility assay with the wild-type N2 worm strain was anticipated to produce a dose-dependent increase in movement upon treatment with WLE. Mn-exposed worms pre-treated with WLE were expected to also have an increase in movement. In a 1-nonanol dopamine-dependent repulsion assay, worms pre-treated with WLE were predicted to repulse faster from 1-nonanol exposure compared to Mn-treated worms. The expected results of a progeny assay would show that pre-treatment of worms with WLE would increase the percentage of hatched progeny compared to the Mn control. Overall, our hypothesis is that pre-treatment with WLE may offer *C. elegans*

protection against Mn-induced dopaminergic neurodegeneration and merits further exploration as a potential alternative medicine which could be used to treat people affected by neurodegenerative disorders.

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LITERATURE REVIEW

Winterberry (*Ilex verticillata*) is a deciduous holly that is primarily distributed within the eastern regions of the United States and southeastern portions of Canada. It can be found in wetland environments such as swamps, marshes, bogs and along ponds, brooks, and streams. The berries of the plant can be used for decorative purposes in gardens, especially during the winter months. This plant is affected by few diseases and pests, making it easy to maintain. The berries themselves are poisonous to humans, though their leaves were historically ground and mixed into a tea by Indigenous people for medicinal purposes. This use of the leaves in a tea led to the coining of the term "fever bush" for its use in treating fever. Additional uses of *I. verticillata* as means of treatment include the use of its bark as a tea for "craziness," diarrhea, fever, parasites, liver and skin disorders, and its roots for treatment of hay fever (Strauch, 1999).

The *Ilex* genus is one of the most diverse genera of plants, ranging from evergreen trees to holly and encompassing hundreds of species. Members of the genus have demonstrated a richness in the concentration of bioactive compounds, specifically polyphenols and alkaloids (Gan, et al., 2018). Structurally, polyphenols such as ellagitannins and flavonoids are composed of several benzene rings bonded to at least one hydroxyl group (Figure 1), while alkaloids express high diversity in structure while containing nitrogen atoms. The species *Ilex paraguariensis*, which is used to make Yerba Mate tea, contains the polyphenol chlorogenic acid at a concentration greater than 90 mg equivalents per gram of dried leaves once extracted from tea (Heck & Mejia, 2007). In addition to chlorogenic acid, there are at least 46 unique polyphenols identified in *I. paraguariensis* products, primarily derivatives of hydroxycinnamic acid and flavonols

(Gan, et al., 2018). When the tea is consumed, the polyphenols exhibit antioxidant properties by reducing the concentration of reactive oxygen species (ROS), natural byproducts of oxygen metabolism, in a peroxidase-like action. This prevents the occurrence of oxidative stress in various bodily systems caused by the build-up of ROS. Additionally, catechin polyphenols in dietary green tea supplements have indicated a degree of protection against the development of chronic diseases like chronic obstructive pulmonary disease (COPD), diabetes, and various forms of cancer through their role as antioxidants (Khan & Mukhtar, 2019).

Research in the Newell laboratory has characterized much about the winterberry leaf extract (WLE) and its effect on the nematode *Caenorhabditis elegans*. The total phenolic content (TPC) of the extract was quantified through the use of a colorimetric Lowry assay with a gallic acid standard curve (Ainsworth & Gillespie, 2007). The TPC of the extract was calculated as 524.45 ± 1.82 mg, though the specific polyphenols present are unknown (McKinnon, et al., 2022). Due to the presence of polyphenolic compounds within WLE, it is possible that the extract could be used as an antioxidant to protect against neurodegeneration that occurs from oxidative stress caused by the metal manganese.

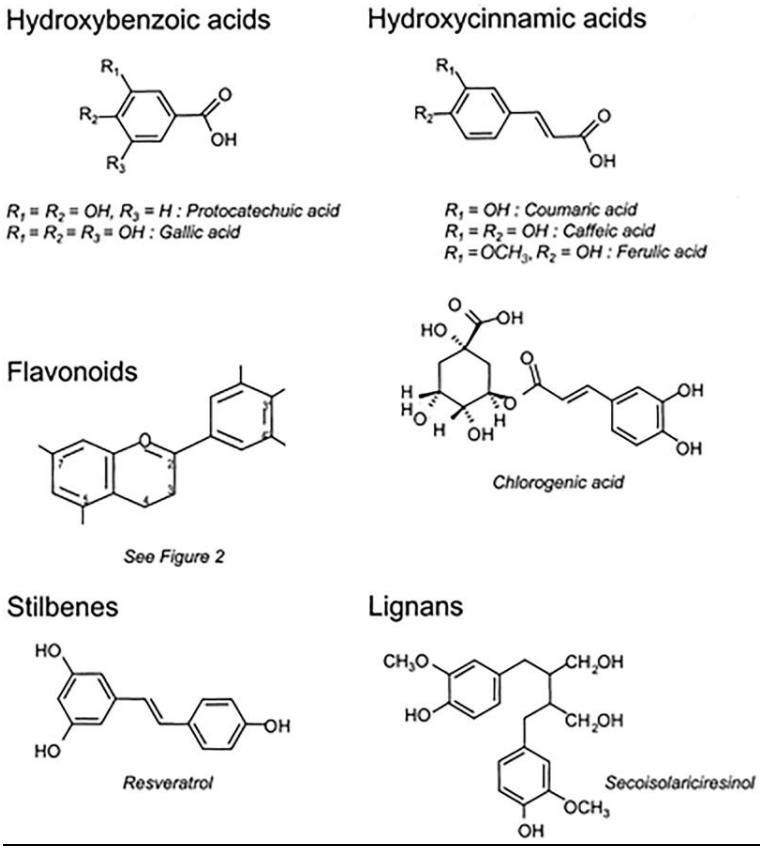

Figure 1. Structure of some of the primary classes of polyphenols, exhibiting the wide variation in structure. Image credits Manach, et al., 2004.

Neurodegenerative Diseases

Neurodegenerative diseases are conditions that occur in the nervous system as a direct result of progressive breakdown of neuronal structure and function. Due to the eventual death of the neurons and their inability to regenerate, these conditions are irreversible and are an increasing cause of mortality annually. Alzheimer's disease is an example of one of these common neurodegenerative diseases, estimated to affect 24 million people globally in 2018 and making up 60 – 80% of all dementia-related cases (Erkkinen, et al., 2018). In comparison, Parkinson's disease is estimated to occur within 8-18 out of every 100,000 person-years and is the second most prevalent neurodegenerative disease,

Huntington's disease occurs in approximately 2.7 out of 100,000 people, and amyotrophic lateral sclerosis (ALS) occurs in about 4.5 out of 100,000 people (Erkkinen, et al., 2018). Likewise, the number of people with dementia was estimated to be 50 million worldwide as of 2019, which is anticipated to increase to approximately 139 million by the year 2050 according to current trends (World Health Organization, 2021). There are several risk factors for these diseases ranging from aging (often described as the most significant risk factor) to genetic, environmental (exposure to metals and chemical substances), and lifestyle-associated causes (obesity, smoking, diet). In order to combat these diseases, billions of dollars have been used to fund research to identify potential causes, treatments, and cures. There have been no cures identified to date, but treatments have been identified to manage symptoms of each condition. These include surgical techniques like deep brain stimulation (DBS) in which an electrode is implanted into a target region of the brain and electrical signals are sent via a neurostimulator to act upon the region in a controlled manner. Additionally, pharmaceuticals like L-DOPA, a dopamine precursor molecule, have been used to supply healthy dopaminergic neurons when dopamine neurotransmitters (signaling molecules) are in short supply (NICE, 2017).

The Importance of Dopaminergic Neurons

Neurons are cells which represent the simplest building block of the nervous system of animals, consisting of dendrites which receive signals from surrounding cells, a cell body (soma) which processes the signal, and an axon which transmits information to the terminal boutons, which then transmits a signal to a neighboring cell (Figure 3). Dopaminergic neurons are associated with the cranial reward system in addition to

executive functions, motivation, movement, and cognition. Neurons of this type synthesize the neurotransmitter dopamine which acts as a biochemical messenger to surrounding cells (Alcaro, et al., 2007). The dopamine system is divided into the mesolimbic, mesocortical, nigrostriatal, and tuberoinfundibular pathways, with each pathway fulfilling its own unique processes. The substantia nigra is located within the nigrostriatal pathway and is important in proper brain function, being divided into the pars reticulata which contains GABAergic neurons, and the pars compacta which contains dopaminergic neurons. Although only about 3 – 5% of the substantia nigra is composed of dopaminergic neurons, they contain high levels of dopamine that plays an important role in movement and the reward system (Chinta & Andersen, 2005). It is these neurons that Parkinson's disease targets and destroys, producing symptoms of bradykinesia, tremors, and changes in posture as the striatum, which the substantia nigra pars compacta projects to, becomes deprived of dopamine. Other neurodegenerative disorders, like Alzheimer's disease, Huntington's disease, and ALS, target other neurotransmitter pathways within the nervous system, producing different cognitive and motor symptoms than those associated with the dopaminergic pathways.

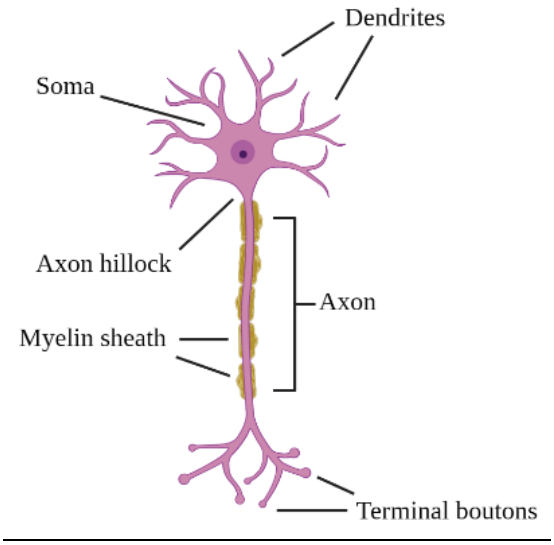

Figure 2. Basic anatomy of a neuron. Information enters the cell through dendrites before being processed in the cell body (soma). The processed information leaves the soma through the axon hillock in the form of electrical impulses. Axons wrapped in an insulating myelin sheath allows for rapid signal transmission to the terminal boutons, where neurotransmitters or an electrical signal are released from the synapse onto the dendrites of surrounding neurons. Image generated through BioRender, 2023.

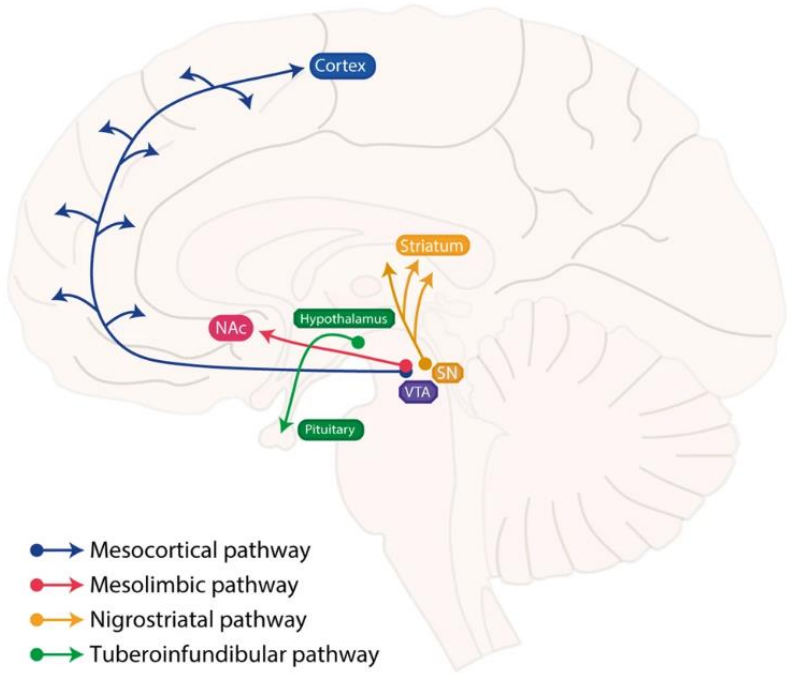

Figure 3. Graphic representation of dopamine pathways within the human brain. Image credits Klein, et al., 2019.

The substantia nigra is not the only region where Parkinson's disease affects dopaminergic neurons. The ventral tegmental area, which is located in the mesocorticolimbic system (a combination of the mesolimbic and mesocortical pathways), experiences dopaminergic neuron degeneration to a lesser degree than the substantia nigra (Chinta & Andersen, 2005). Further lesions in the noradrenergic locus ceruleus and ascending cholinergic pathways may also occur in Parkinson's, which may result in cognitive disorders like dementia, which occurs in approximately 30% of Parkinson's cases (Aarsland, et al., 1996).

Unfortunately for Parkinson's disease, there are no cures that exist, though treatments are available to manage symptoms. The L-DOPA family is one of the leading treatments for people affected with the disease as the substance is a precursor molecule for dopamine (Figure 4). Due to its lack of a charge, it is able to penetrate the blood-brain barrier, where it can enter the substantia nigra pars compacta and is converted to dopamine (Chinta & Andersen, 2005). However, it is unable to replace lost cells, therefore only making it and other drugs effective for a limited time. Dopamine agonists - molecules that act on dopamine receptors and mimic the neurotransmitter to activate dopaminergic neurons - are another category of drugs used to treat Parkinson's (Borovac, 2016). Surgical procedures are an alternative method to treat Parkinson's, and stimulation of the thalamus through deep brain stimulation represent some of the non-drug treatments of the disease (Chinta & Andersen, 2005). In order to treat these conditions properly, however, it is important to understand likely sources for the condition, such as Mn metal toxicity as a result of environmental exposure.

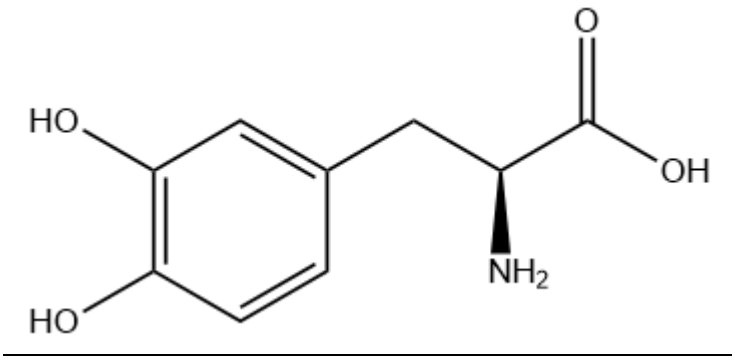

Figure 4. Structure of the dopamine precursor L-DOPA. Image generated through ChemDraw, 2023.

Manganese and Reactive Oxygen Species

The element manganese (Mn) is an essential element in organisms that is involved with enzyme synthesis and activation in addition to metabolism regulation, its role as a coenzyme, reproduction, immunity, and digestion. The main source of the element Mn is obtained in the diet from shellfish, nuts, legumes, rice, and even water, though these often occur in trace amounts (Harvard, 2023). Manganese superoxide dismutase (MnSOD) is an enzyme that reduces oxidative stress within mitochondria and is the primary antioxidant for superoxide (Li & Yang, 2018). Although the concentration of Mn is regulated through homeostasis, significant abnormalities in its concentration – as with other trace elements such as copper, zinc, and selenium – may result in the occurrence of metabolic and neurological diseases. Decreased levels of Mn result in a lack of Mn available to produce MnSOD, enhancing the accumulation of the superoxide ion radical species (Figure 5). Conversely, increased exposure to Mn may result in manganism, a neurotoxic condition characterized by accumulation of Mn in the brain. This often occurs in welders and individuals who work in Mn mines or associated processing factories and may manifest

through non-Parkinson's bradykinesia, rigidity, and tremors (Settivari, et al., 2009). As Mn is also a known oxidant by forming manganese oxides and free radicals, increased accumulation of the metal would correspond with an increase in reactive oxygen species, damaging cellular structures which would culminate in the appearance of disorders (Zeiner, et al., 2021).

Reactive oxygen species (ROS) are oxygen radicals that are produced upon the reduction of oxygen with electrons, resulting in molecules that are highly reactive. Examples of such radicals include superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl ($^{\cdot}OH$) as seen in Figure 5 (Dryden, 2018). These ROS are normal products of biochemical respiration reactions with oxygen that occur through several mechanisms including within the mitochondria via oxidative phosphorylation, in peroxisomes through biomolecule catabolism, and various enzymatic reactions (Brieger, et al., 2012). ROS are used in signaling and homeostasis mechanisms, ranging from ion channel regulation to the termination of the inflammatory immune response (Brieger, et al., 2012). ROS have also exhibited a degree of protection against infection from bacteria and fungi through its role in the immune response (Dryden, 2018; Brieger, et al., 2012). However, excessive production of ROS from dysfunction or impairment of these structures results in the occurrence of oxidative stress in which antioxidant molecules are unable to eliminate these substances.

During conditions of oxidative stress, in which there is an imbalance between oxidative species and antioxidant molecules and systems, there are high concentrations of free radicals and other oxidizing agents, which can react with metals like manganese to create damaging species. As seen in Figure 6, ROS can lead to oxidative damage to

biological macromolecules such as protein carbonylation, lipid peroxidation, and introduction of 8-oxo-guanine in DNA and RNA (Pisoschi & Pop, 2015; Singh, et al., 2019). This eventually culminates in mitochondrial dysfunction, which is characterized by the inability to metabolize energy sources like glycogen. As a result, the production and concentration of the energy molecule adenosine triphosphate (ATP) decreases. When mitochondrial dysfunction is present, innate immune responses like the p38MAPK pathway in *C. elegans* activate to provide protection against the onset of neurodegeneration (Chikka, et al., 2016). This works in conjunction with another pathway, SKN-1, which is similarly activated under oxidative stress and mitochondrial dysfunction (Figure 6)(Palikaras, et al., 2015). Diseases such as cancer, Alzheimer's, and hypertension may manifest, and cell death ultimately occurs through apoptosis (Brieger, et al., 2012). Additionally, oxidative stress is also associated with infertility in males and aging in mammals, including humans.

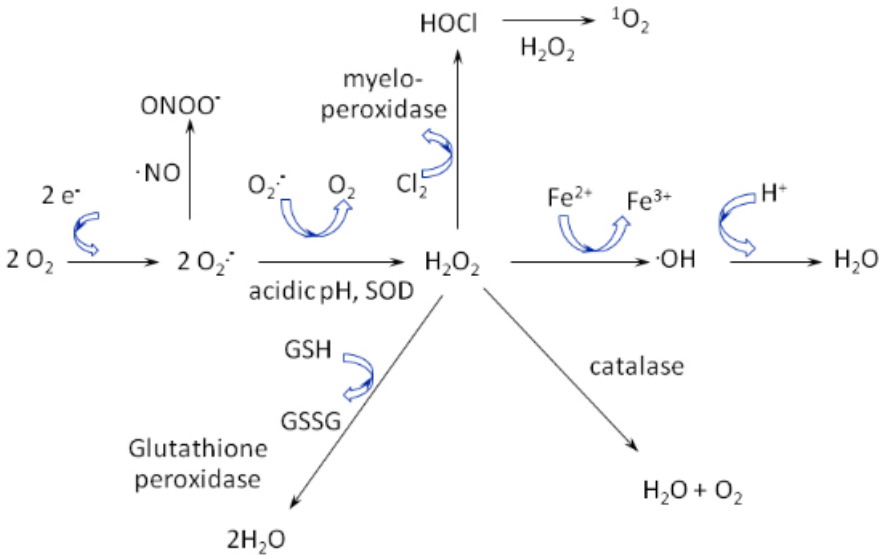

Figure 5. Generation and transition of ROS demonstrating their reactivity. Image credits Brieger et. al, 2012.

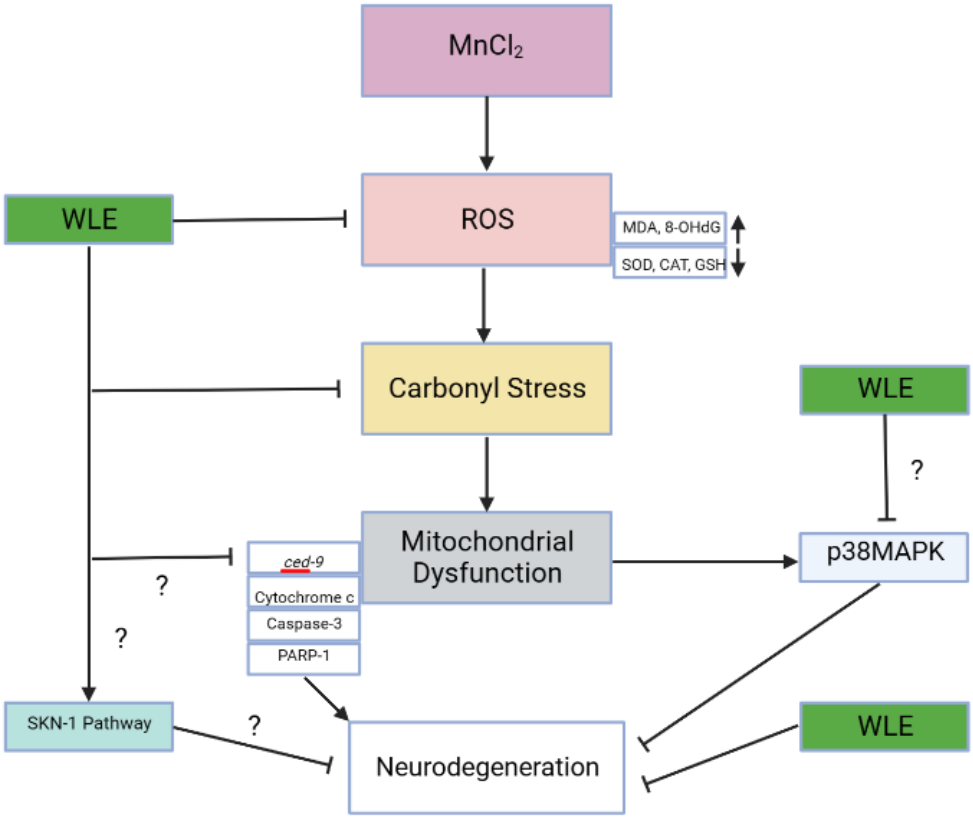

Figure 6. Manganese-induced oxidative stress in *C. elegans*. Question marks indicate experiments which have not been conducted but could represent future areas of research. Image generated through BioRender, 2023.

C. elegans as a Model Organism

In order to devise new, less-costly methods of treatment for neurodegenerative disorders, the use of suitable model organisms is invaluable to evaluate the effects of the proposed methods. Such methods include drugs and natural treatments that are tested on the models before they can be applied for human use. *Caenorhabditis elegans* is a small nematode that is naturally found in humid and temperate climates, primarily in the United States, southern Canada, Western Europe, and in smaller islands throughout the rest of the world. During the last several decades, the nematode has become an attractive model

organism due to several factors which include but are not limited to: a small size of up to 1 millimeter, short lifespan (only a few days between hatch and adult stages), transparency, high reproductive rate, and highly manipulable genome, allowing researchers to perform several experiments with easily manipulated strains within a short amount of time (Ruszkiewicz, et al., 2018). Additionally, approximately 80% of its genome is homologous to that of humans. The nematode was initially isolated in 1900 by Emile Maupas and initial research was performed in the 1940s by Ellsworth Dougherty and Victor Nigon (Maupas, 1900; Nigon, 1949; Dougherty, et al., 1959). It was not until 1963 that Dr. Sydney Brenner proposed using *C. elegans* as a model for genetics research, providing the impetus for greater study and widespread usage of the nematode (Brenner, 1963; Brenner, 1974).

The anatomy of *C. elegans* is rather similar to other species in the phylum *Nematoda*. Its body exhibits bilateral symmetry with no segmentation, and contains a cuticle, mouth, pharynx, gonad, and intestine, all of which make up the most basic anatomy of the species (Figure 7)(Wallace, et al., 1996). The pseudocoelom (body cavity) is filled with fluid and certain organ systems that are also found in humans, but lacks circulatory and respiratory systems like all nematodes. The presence of a simple nervous system that shares many basic characteristics with those of higher animals has made the organism a suitable and ideal candidate for neural research. *C. elegans* are either male or hermaphroditic, the latter being able to produce both sperm cells and oocytes despite having a female body (Starostina, et al., 2007). However, males are much less common than females, only occurring in one out of every thousand nematodes (Brenner, 1963).

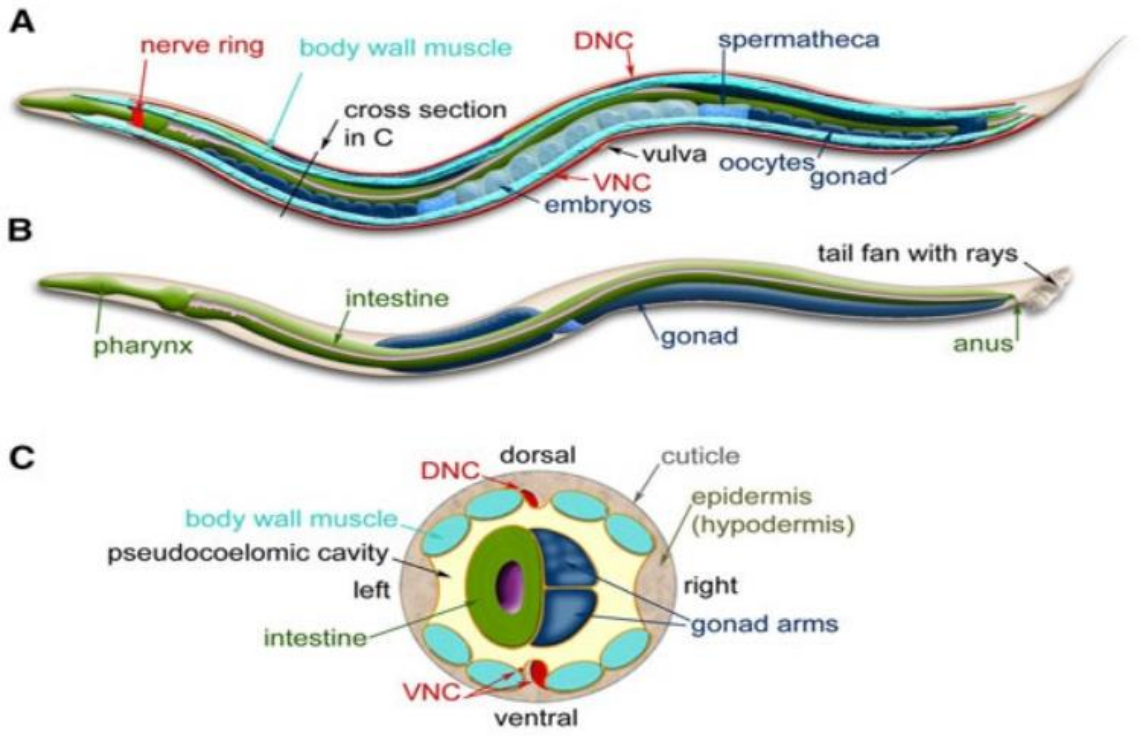

Figure 7. Lateral representation of the anatomy of a *C. elegans* hermaphrodite (A) and male (B) accompanied by a cross-sectional view of the hermaphrodite (C). Image credits Corsi, et al., 2015.

C. elegans has a lifespan that is marked by three different life stages: embryonic, larval, and adult. The larval stage is further divided into four unique stages, L1 through L4, respectively. At 22℃, eggs laid by hermaphrodites hatch only hours after they are laid, where the nematodes enter the L1 larval stage. This stage is significant as acute treatment at this point allows for the visualization and measurement of treatment effects throughout the entire nematode life cycle. The nematodes then proceed through the L2, L3, and L4 larval stages until they enter the adult stage approximately 55 hours after the eggs were initially laid (Li, et al., 2014). It is important to note that the growth period is extended in acute conditions where food is absent, with the potential for developmental arrest at each larval stage in addition to Dauer diapause and adult reproductive diapause

(Baugh & Hu, 2020). Overall, the wild-type *C. elegans* strain N2 has an average lifespan of approximately 16-17 days, though different strains may have longer or shorter lifespans (Muschiol, et al., 2009).

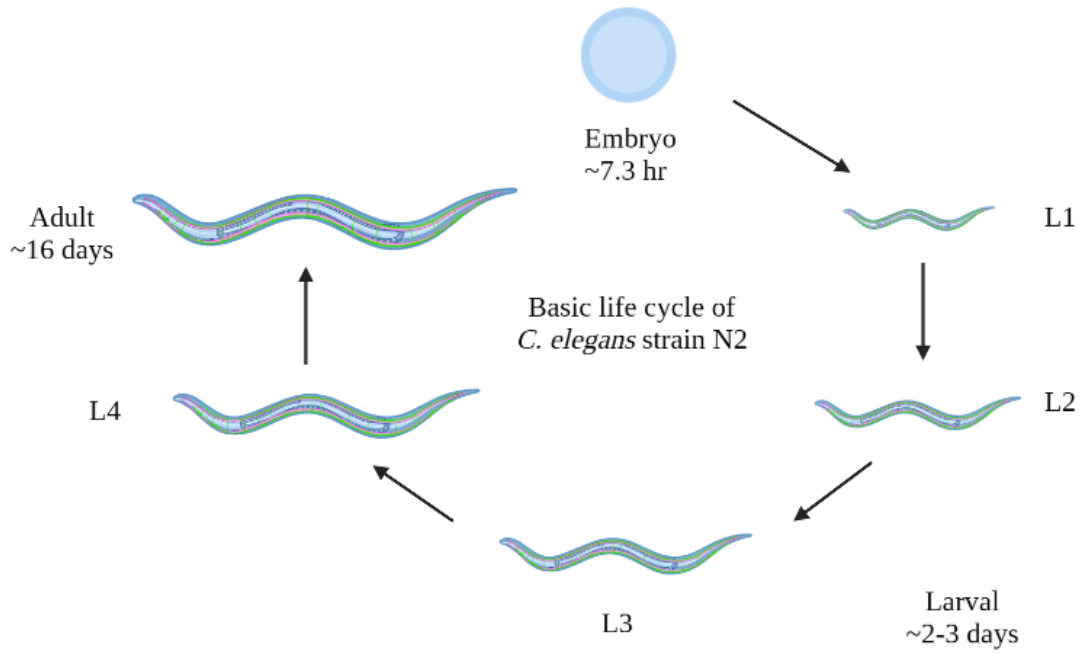

Figure 8. Life cycle of the nematode *C. elegans* N2 strain. Figure generated through BioRender, 2023.

Environmental factors can significantly affect the growth of *C. elegans*. The worms grow best between temperatures of 16℃ and 25℃, optimally at 20℃ (Stiernagle, 2006). Increased rates of growth as high as 2.1 times have been observed at 25℃ in comparison to 16℃, at which rate of growth is much lower. When exposed to temperatures outside the optimal range for growth, the nematodes become susceptible to temperature-induced mutations, such as the *hsp-6* mutation for example, that can affect systems responsible for growth and reproduction. The bacterial diet of the nematodes can also influence the development of the worms and most laboratory diets of *C. elegans* are of the

OP50 or NA22 strains of *Escherichia coli* (Zhang, et al., 2017; Ke, et al., 2020). Each strain has specific uses: the OP50 strain is most beneficial when viewing nematodes as it does not produce a thick growth layer on plates containing nematode growth media (NGM), while the NA22 strain is best for producing large numbers of nematodes to be used for experiments.

C. elegans and Manganese-Induced Neurodegeneration

As *C. elegans* contains a simple nervous system, homologues of Parkinson's-associated genes, and all of the genes and neurons required for dopaminergic neurotransmission, it is a useful model for studying Mn-induced neurodegeneration (Settivari, et al., 2009). There are eight dopaminergic neurons in the nematodes, primarily concentrated within the head. Research into the effect of acute and chronic Mn treatment on *C. elegans* at the L1 stage reveals the induction of neurodegeneration in dopaminergic neurons which is observable through a fluorescence assay (Chen, et al., 2014). This is specifically done by tagging dopaminergic neurons or their receptors with a fluorescent protein such as GFP or mCherry before performing microscopy. The fluorescent signal generated is used to quantify and evaluate the level of activity at the neuron. Neurodegeneration is achieved because Mn reacts with oxygen to produce ROS throughout the body (Figure 6). When these ROS have accumulated to a greater than normal concentration, they start to oxidize lipids, sugars, and proteins through cleavage, producing reactive carbonyl species (RCS) (Pisoschi & Pop, 2015; Iacobini, et al., 2022). As RCS continue to accumulate, they start to damage cellular structures (proteins, nucleic acids, etc.) directly in conjunction with ROS. In the mitochondria, an organelle responsible for cellular metabolism, this damage leads to the presentation of mitochondrial

dysfunction in which the organelle is unable to properly metabolize energy sources. This leads to the generation of additional ROS and mutations within the mitochondrial DNA (mtDNA), amplifying the effect within the mitochondria and inducing damage in other cellular structures (Lin & Beal, 2006). If this occurs within neurons, it may lead to the onset of neurodegenerative symptoms.

As neurodegeneration occurs within the dopaminergic neurons of *C. elegans*, alterations of dopamine-dependent behavior within the nematodes have been observed (Ruszkiewicz, et al., 2018). One study revealed that acute treatment with manganese chloride ($MnCl_2$) at the L1 stage produced a deficit in memory and adaptive learning within the olfactory system of *C. elegans* while acknowledging that exposure to the element is responsible for impairment in neurological pathways (Raj, et al., 2021). This is reflective of studies in which children exposed to large concentrations of Mn either pre- or postnatally exhibited lower IQ scores and increased potential for neurobehavioral problems (Ruszkiewicz, 2018). As dopaminergic neurons are associated with movement and feeding in *C. elegans*, acute treatment with $MnCl_2$ at the L1 phase has resulted in impairment of these systems. The basal slowing response is a dopamine mediated activity in which nematode locomotion is decreased in the presence of a bacterial food source (Sawin, et al., 2000). Acute treatment with $MnCl_2$ has interfered with this activity, signified by a smaller change in the number of body bends while on and off food. Similarly, the dopamine-controlled behavior of nematode swim-to-crawl transition has exhibited impairment upon acute treatment with $MnCl_2$, with a delayed transition time stemming from damaged neurons (Ijomone, et al., 2016; Chen, et al., 2013). Other associated movement-based behaviors including area searching and tap withdrawal response are also affected by the

Mn-induced degeneration of dopaminergic neurons. Additionally, acute treatment with $MnCl_2$ has been shown to reduce nematode egg-laying behavior, which is controlled by dopamine (Schetinger, et al., 2019). With fewer eggs laid, brood size decreases as fewer progeny hatch from the eggs. While *C. elegans* and other animals contain corrective pathways that are activated by ROS accumulation and mitochondrial dysfunction, these pathways could eventually become compromised, necessitating the use of supplemental treatments (Figure 6).

Previous data has shown an acute exposure (30 min) to 50 mM $MnCl_2$ at the L1 stage has the potential to double the concentration of ROS in cells of *C. elegans* compared to animals not treated with $MnCl_2$ (Settivari, et al., 2009). Acute pre-treatment of *C. elegans* with WLE and 5 mM $MnCl_2$ in the Newell laboratory has demonstrated a dose-dependent decrease in the concentration of ROS within the nematodes while also increasing the concentration of the antioxidant molecule glutathione (GSH), supporting that antioxidant polyphenolic compounds can protect against ROS accumulation (McKinnon, et al., 2022). An increase in mean nematode lifespan from 3 to 7 days was also observed. As WLE extends lifespan and protects against the accumulation of ROS in *C. elegans* when acutely treated with $MnCl_2$, it was hypothesized that WLE could provide protection against neurodegeneration, specifically within dopaminergic neurons. Cases with large acute doses (often severe and one-time) or smaller, chronic doses (often less-severe and continuing) have led to neurodegenerative effects that resemble Parkinson's disease in chronic cases, both of which occur due to damage in the basal ganglia (Li & Yang, 2018; Chen, et al., 2014). Due to the role that Mn toxicity has in the

destruction of dopaminergic neurons, which contributes to Parkinson's disease, it is hypothesized that neurodegeneration stemming from Mn-induced oxidative stress could be mitigated by WLE.

INTRODUCTION

Neurodegenerative disorders are one of the most concerning public health issues in the world today. In 2019, it was estimated that approximately 55 million people worldwide were living with dementia, a number that is expected to increase to 139 million by the year 2050 (World Health Organization, 2021). Due to variability between the numerous disorders and the characteristics of each individual person, there is no single cause for each condition, and there are currently no cures that exist. This fuels the search for potential treatments, and there are a variety of treatments available today to manage symptoms of these disorders. Such treatments range from pharmaceutical drugs in the L-DOPA family to surgical procedures like deep brain stimulation (DBS), which are both used to treat symptoms of Parkinson's disease (NICE, 2017). However, some of these treatments could become rather expensive for companies and the patient. For example, direct medical costs of a two year regimen of DBS was approximately €88,950 (about $100,500 USD) in 2017 (Ooms, et al., 2017). As a result, natural and historical medical treatments may be valuable alternatives for treating these neurodegenerative disorders.

Winterberry leaf extract (WLE) provides an opportunity to study a potential natural treatment for neurodegenerative conditions that is cost-effective and is rooted in Indigenous medicine in which it was used to treat fever, skin conditions, and "craziness." Since winterberries are a holly often found in wetlands and grasslands in the wild, and in many ornamental gardens, people living in climates suitable for the plant are able to cultivate their own leaves for personal use. However, literature on the biochemical processes of winterberries is very limited, providing the opportunity to perform novel research to characterize the holly. Unpublished data from the Newell laboratory at the

University of Maine reveals that winterberry leaves are rich in polyphenolic compounds that successfully decrease the concentration of Mn-induced accumulation of reactive oxygen species (ROS) – natural oxygen metabolism byproducts – within *C. elegans* while also increasing the concentration of the antioxidant glutathione (GSH) (McKinnon, et al., 2022). Manganese was selected as the oxidant of study due to its potential to induce neurodegeneration through the generation of ROS. Similarly, *C. elegans* was selected as the model organism for neurodegeneration due to its short lifespan and ability to produce multiple generations within a short period of time, in addition to other characteristics like an easily manipulable genome, transparency (to visualize internal structures), and small size. As many fundamental functions of the *C. elegans* nervous system are homologous to those of the human nervous system, we are able to easily study comparable systems in humans that are of greater complexity.

The goal of this thesis project attempted to expand upon the current knowledge of WLE while establishing its potential for treatment of neurodegenerative disorders. It was hypothesized that WLE would decrease the concentration of ROS within *C. elegans* upon treatment with $MnCl_2$, thus producing a degree of protection against neurodegeneration within the nematodes. The effects of WLE and $MnCl_2$ on the activity of dopaminergic neurons were characterized through the swim-crawl transition and basal body movement behavioral assays. These two assays are rooted in the understanding that dopamine is an important mediator of nematode mobility. A repulsion assay with 1-nonanol was performed to indirectly measure the concentration of dopamine within the nematodes.

A progeny assay was performed to establish a Mn-induced toxic effect through quantifying the percentage of larva that hatch from eggs and evaluate a dopaminergic effect on egg-laying behavior.

METHODS

Preparation of Winterberry Leaf Extract

To prepare the winterberry leaf extract, *Ilex verticillata* leaves were collected from Bangor City Forest and dried at 50°C for 16 hours. Unused leaves were stored at -80°C. Ten grams of the dried leaves were macerated with a mortar and pestle to rupture the cells before being transferred to a flask containing 100 mL of a 50% acetone solution. The mixture was placed in a shaker at 200 rpm at 37°C for 1 hour. The shaking step was repeated three times in total and the resultant extract was filtered with a Buchner funnel and Whatman filter paper. The filtered extract went through fractional distillation at 60°C to remove the acetone and the final volume of extract was divided into 1.5 mL aliquots and stored at -80°C. The concentration of polyphenolic compounds was measured using the colorimetric Lowry assay on a microtiter plate reader courtesy of Dr. Julie Gosse (Ainsworth & Gillespie, 2007). A gallic acid standard curve was used to calculate the concentration of polyphenolic compounds within the extract.

Treatment of *C. elegans* with Winterberry Leaf Extract

Three experiments were designed in order to characterize the extent of neurodegeneration within *C. elegans* after acute treatment with the winterberry leaf extract and $MnCl_2$. Due to the nature of the nematodes, several replicates of each experiment were conducted. Wild-type *C. elegans* strain N2 was used for the three experiments detailed in this section. The nematode strain was grown and maintained on 150 mm 8P plates containing a lawn of NA22 strain *Escherichia coli* bacteria as a food source. The plates were incubated at 18°C once seeded with *C. elegans* to encourage non-mutational growth.

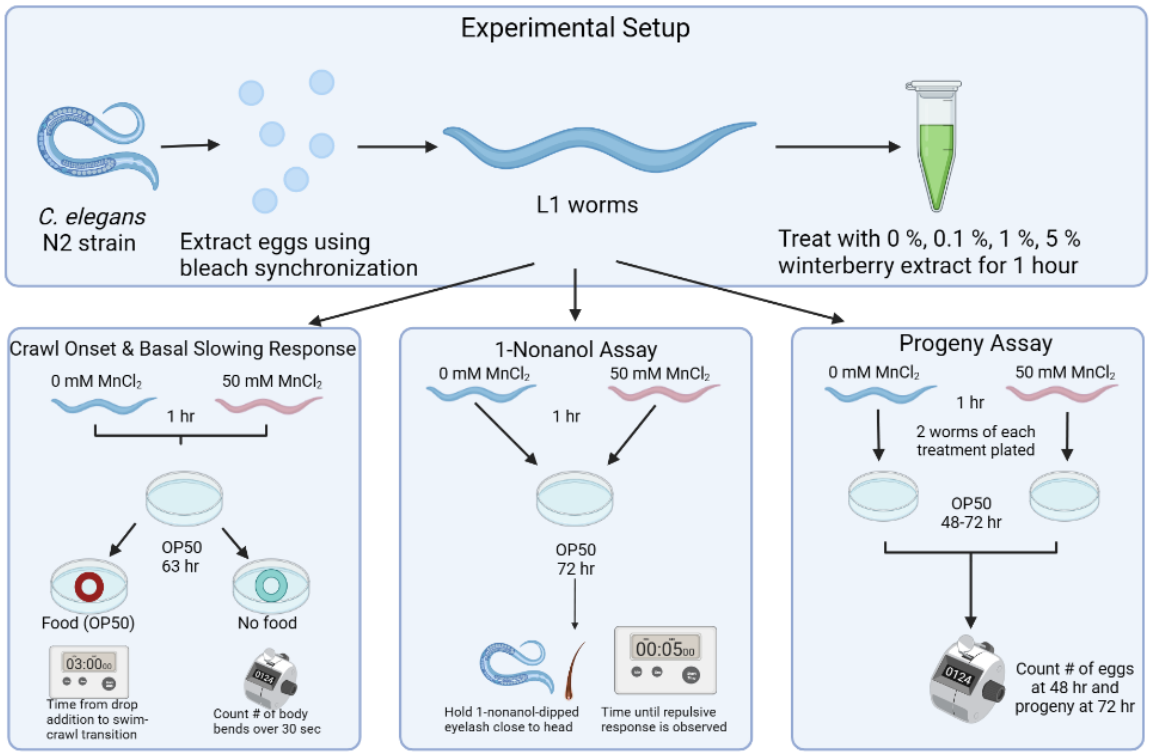

Figure 9. Graphic representation of the experimental setup. Image generated through BioRender, 2023.

Bleach Synchronization

Bleach synchronization was carried out to eliminate age-induced variation within the experimental results. In each experiment, nematodes were washed off 8P plates with ddH_2O and pipetted into a 15 mL conical tube. The nematodes were centrifuged at 3000 rpm for 1 minute. After centrifugation, the solution was aspirated to 1-2 mL (dependent on pellet size) and a second wash of the plate with ddH_2O was added to the tube before centrifugation. The nematodes were washed twice with 15 mL ddH_2O. Lysogeny of the nematodes was performed by shaking with a bleaching solution for 7 minutes to extract eggs. 2 µL solution was pipetted onto a microscope slide to confirm that eggs had been extracted from the adult nematodes. M9 reagent was added to 15 mL and centrifuged

at 3000 rpm for 1 minute before the solution was aspirated to the pellet and new M9 was added to wash the eggs. After a second M9 wash and aspiration, 10 mL of a 30% sucrose solution was added to the tube and centrifuged at 1600 rpm for 10 minutes to separate the eggs from the rest of the solution. Eggs were pipetted from the uppermost 3 mL of the solution and added to a fresh 15 mL conical tube with 12 mL ddH_2O. The eggs were centrifuged at 3000 rpm for 3 minutes and the water aspirated to 1 mL. The eggs were poured onto a NGM plate without bacteria and incubated at 18°C for 16 hours.

Nematode Treatment

L1 larval *C. elegans* were washed off the synchronization plate with 5 mL 85 mM NaCl and pipetted into a 15 mL conical tube. After centrifugation at 2500 rpm for 1 minute, the salt solution was aspirated to 2 mL and vortexed. Two samples of 2 µL were pipetted onto a microscope slide and the number of nematodes present was used to calculate the volume containing 1000 nematodes. NaCl was added to or aspirated from the conical tube as needed. Volumes of 85 mM NaCl and *C. elegans* were added to two sets of four polyethylene glycol (PEG) coated Eppendorf tubes, and winterberry leaf extract was added to the tubes in concentrations of 0%, 0.1%, 1%, and 5%, with a total volume of 1 mL in all tubes. The tubes were rotated at 20 rpm for 1 hour before centrifugation at 7500 rpm for 30 seconds. The treatment mixture was aspirated to 200 µL and 1 mL of fresh 85 mM NaCl was added to each tube, vortexed, and centrifuged. This washing was performed twice. After the final aspiration, the nematodes were treated with 50 mM $MnCl_2$. The tubes were rotated at 20 rpm for 1 hour before centrifugation at 7500 rpm for

30 seconds. The nematodes were washed twice in the same manner as the extract, aspirated to 200 µL, vortexed, and plated onto prepared NGM plates seeded with OP50-1 strain *E. coli* as a food source. The plates were incubated at 18°C for 48-72 hours.

Transition to Crawling Assay & Basal Body Bends

Two mobility-based behavioral assays were performed to evaluate dopaminergic integrity (Vidal-Gadea, et al., 2011; Ijomone, et al., 2016). Approximately 72 hours post-treatment, day 1 N2 strain adult *C. elegans* nematodes treated with 0 M $MnCl_2$ and 50 mM $MnCl_2$ were washed off OP50-1 plates with 1 mL S-Basal buffer and pipetted into PEG-coated Eppendorf tubes. The tubes were centrifuged at 7500 rpm for 30 seconds and tubes were aspirated to 200 µL. Fresh S-Basal buffer was added to each tube and the nematodes were washed twice with the buffer in the same manner as the preceding treatment procedure. After washing, the tubes were aspirated and vortexed. A 5 µL drop of each treatment was pipetted onto plates containing either a ring of OP50-1 (food) or no bacteria (no food) and viewed under a light microscope. Ten nematodes were timed from the placement of the drop until their transition of movement from swimming to crawling on the plate. After waiting for 5 minutes to allow for acclimation to the plate environment, the ten nematodes were timed for 30 seconds and the number of S-shaped bends performed by each nematode was recorded. The change in body bends (ΔBB) was calculated as the difference between the number of body bends over 30 seconds between treatments plated without food and treatments plated with food.

1-Nonanol Mobility Assay

1-nonanol assay was performed to indirectly measure dopamine concentration in *C. elegans* treated with WLE and $MnCl_2$. Prior research suggests that repulsion times to 1-nonanol are faster in nematodes with normal concentrations of dopamine while nematodes with inadequate levels of dopamine experience increased repulsion times (Sammi, et al., 2019). Twenty wild-type N2 nematodes washed for the crawl onset and basal body bends assays were plated on NGM plates in absence of a bacterial food source. After waiting 10 minutes for acclimatization, an eyelash was dipped in 1-nonanol and held close to the heads of the nematodes. Care was taken to prevent pick contact with the nematode and the agar in order to prevent desensitization; nematodes that experienced premature contact with the pick were disregarded. Nematodes were timed from the initial placement of the pick near the head until a repulsive action was observed.

Progeny Assay

A progeny assay was carried out to evaluate the impact of WLE and $MnCl_2$ on neurons regulating *C. elegans* reproduction. Treatment with $MnCl_2$ has previously shown a decrease in brood size of the nematodes through the disruption of neurons containing the neurotransmitters dopamine and serotonin (Schetinger, et al., 2019). These neurotransmitters express inhibitory and excitatory actions on egg-laying, respectively, and are also responsible for learning, locomotion, and pharyngeal pumping mechanisms (Dempsey, et al., 2005). This assay not only measures the effect on egg-laying and hatching, but it also acts as a further measure of nematode behavior. Immediately after treatment, two wild-type N2 nematodes from each treatment were transferred with a

platinum worm pick to NGM plates containing OP50-1 and incubated at 18°C. Approximately 48 hours post-treatment, the plates were placed onto a transparent grid and the number of eggs in five squares was counted and averaged. This average was multiplied by the total number of squares encompassed by the plate to provide an estimate of the total number of eggs on the plate. After an additional 24 hours of incubation at 18°C, the number of progeny on each plate was counted using the same grid counting method as the previous day.

RESULTS

Basal Slowing Response Results

Initially, the *C. elegans* nematodes exhibited no Mn-dependent change in transition time from swimming to crawling after plating onto NGM plates with and without food. Exposure to a food-deprived environment did not produce a dose-dependent decrease in the transition time for nematodes treated with extract and $MnCl_2$, with a decrease in the transition time from 221 seconds for the control treatment to approximately 175 seconds for the 5% extract and 50 mM $MnCl_2$ treatment (Table 1A). However, the transition time in nematodes treated with only 50 mM $MnCl_2$ was faster (around 175 seconds) than the untreated control, contrary to the expected increase in time. The treatments that were exposed to a food-rich environment of OP50-1 bacteria revealed no statistically significant change in nematode transition time among the treatments. However, unlike the food-deprived treatments, there is some recovery in the transition times for the extract and $MnCl_2$ treatments compared to the controls (Table 1B). The 220-second transition time for the $MnCl_2$ control, while not statistically significant, was moderately faster than the 240-second time recorded for the untreated control.

An assay measuring the body bends of the *C. elegans* nematodes after the crawl transition assay did not produce statistically significant results. In a food-deprived environment, treatment with WLE alone slightly decreases the number of S-shaped body bends within the nematodes, averaging approximately 2.25 bends within 30 seconds for non-$MnCl_2$ treatments (Table 2A). Some recovery is also observed in treatments with WLE and 50 mM $MnCl_2$ when compared to the $MnCl_2$-only treatment. However, the standard

error indicates that the results are not significant. Similar results were also observed when an OP50-1 food source was present, with all treatments experiencing a slight increase in body bends (approximately 0.5 – 1 bend) versus the food-deprived treatments (Table 2B). The results are ultimately not statistically significant. The change in body bends (ΔBB) between treatments in food-rich and food-deprived environments was not significant ($p > 0.05$), with the difference of only a single S-shaped body bend at most with large error (Table 3).

Crawl Onset (sec) - Food	Control	0.1WLE	1WLE	5WLE	Mn	0.1WLE+50Mn	1WLE+50Mn	5WLE+50Mn
Mean	221.5	190.25	179.75	184	175.75	176	186.75	176.5
Std dev	101.56279	58.380219	33.509949	46.115796	13.93736	43.535426	21.975365	8.1034972
Std err	50.781394	29.19011	16.754974	23.057898	6.9686799	21.767713	10.987682	4.0517486

Crawl Onset (sec) +Food	Control	0.1WLE	1WLE	5WLE	Mn	0.1WLE+50Mn	1WLE+50Mn	5WLE+50Mn
Mean	240.75	173.25	159.75	191.75	220	182.5	180.75	191
Std dev	84.255069	36.436017	28.523382	29.033027	44.773504	40.435133	33.049206	47.377913
Std err	42.127535	18.218008	14.261691	14.516514	22.386752	20.217567	16.524603	23.688957

Table 1. Mean transition time of *C. elegans* movement from swimming to crawling on NGM plates seeded with and without an OP50-1 food source. (A) Transition time for day 1 adult nematodes in the absence of OP50-1 are separated by treatment. (B) Transition time for day 1 adult nematodes in the presence of OP50-1 are separated by treatment. Standard deviation and standard error was calculated where N=4.

Body Bends/ 30 sec - Food	Control	0.1WLE	1WLE	5WLE	Mn	0.1WLE+50 Mn	1WLE+50 Mn	5WLE+50Mn
Mean	2.375	1.875	2.45	1.85	3.125	2.85	2.375	2.5
Std dev	1.0045729	0.6849574	1.3625956	0.6806859	0.9215024	1.3674794	1.6194135	1.6472199
Std err	0.5022864	0.3424787	0.6812978	0.340343	0.4607512	0.6837397	0.8097067	0.8236099

Body Bends/30 sec +Food	Control	0.1WLE	1WLE	5WLE	Mn	0.1WLE+50Mn	1WLE+50Mn	5WLE+50Mn
Mean	2.95	2.825	3	2.575	3.6	3.275	2.85	2.825
Std dev	1.7406895	1.1176612	1.4651507	1.332604	1.339154	1.5348724	1.3127579	0.9215024
Std err	0.8703448	0.5588306	0.7325754	0.666302	0.669577	0.7674362	0.656379	0.4607512

Table 2. Mean number of S-shaped body bends in *C. elegans* on NGM plates seeded with and without an OP50-1 food source over 30 seconds. (A) Number of body bends for day 1 adult nematodes in the absence of OP50-1 are separated by treatment. (B) Number of body bends for day 1 adult nematodes in the presence of OP50-1 are separated by treatment. Standard deviation and standard error was calculated where N=4.

ΔBody Bends/30 sec	Control	0.1WLE	1WLE	5WLE	Mn	0.1WLE+50Mn	1WLE+50Mn	5WLE+50Mn
Mean	0.575	0.95	0.55	0.725	0.475	0.425	0.475	0.325
Std dev	1.602862	0.5802298	0.8812869	0.670199	1.4244882	1.1441882	0.5737305	1.1441882
Std err	0.801431	0.2901149	0.4406435	0.3350995	0.7122441	0.5720941	0.2868652	0.5720941

Table 3. Treatment of N2 nematodes with WLE and Mn produced little change in the basal slowing response. Data expressed as change (Δ) in body bends during a 30 second interval.

Mobility Response to 1-Nonanol

Exposure of *C. elegans* to 1-nonanol resulted in nematodes displaying a repulsive response to the fatty alcohol. Nematodes treated with the control exhibited an average repulsion time of approximately 2.9 seconds, while nematodes treated with 50 mM $MnCl_2$ exhibited a noted increase in response time, averaging 4 seconds (Table 4). There appears to be a slight dose-dependent decrease in repulsion time when treated with 0.1% WLE, as the treatment exhibited a repulsion of approximately 3.8 seconds, which is marginally faster than the repulsion time of the $MnCl_2$-only treatment. However, the results from all treatments are not significant ($p > 0.05$), though they do suggest that WLE does not demonstrate an adverse effect on *C. elegans*.

Reaction Time (sec)	Control	0.1WLE	1WLE	5WLE	Mn	0.1WLE+50 Mn	1WLE+50 Mn	5WLE+50 Mn
Mean	2.925	3.1875	2.625	3.1875	4.1625	4.1625	3.775	4.1
Std dev	1.3407088	1.9228343	0.8568353	1.9245671	2.4094865	2.525	2.7204473	3.0391885
Std err	0.6703544	0.9614171	0.4284176	0.9622835	1.2047432	1.2625	1.3602236	1.5195942

Table 4. Mean reaction time of *C. elegans* when exposed to the fatty alcohol 1-nonanol on NGM plates without an OP50-1 food source. Standard deviation and standard error was calculated where N=4.

Progeny Assay Results

The two L1 nematodes from each treatment that were plated produced upwards of 120 eggs by the time eggs were counted approximately 2 days post-treatment. While the total number of eggs and hatched progeny varied by treatment (Tables 5-6), it was important to determine the percentage of eggs that hatched. Nematodes treated with only 50 mM $MnCl_2$ expressed a dramatic decrease in the number of eggs laid and experienced recovery when treated in conjunction with WLE in a decreasing dose-dependent manner

(Table 5). Despite the variation in egg totals, the percentage of eggs that hatched expressed a moderate degree of uniformity. While lower concentrations of WLE (0.1% and 1%) have no significant effect on the percentage of eggs that hatch, it is important to note that treatment with 5% WLE decreased this percentage (Table 6). The untreated control only had a hatching percentage slightly over 50% with error, while the $MnCl_2$-only treatment unexpectedly had a hatching percentage of 60% with some error. In comparison, the treatments with 5% WLE had hatching percentages around 35 – 37% with error.

Number of Eggs	Control	0.1WLE	1WLE	5WLE	Mn	0.1WLE+50Mn	1WLE+50Mn	5WLE+50Mn
Mean	71.5	74	78.5	38	24.5	91	47	37
Std dev	16.263456	14.142136	7.7781746	53.740115	30.405592	32.526912	35.355339	5.6568542
Std err	11.5	10	5.5	38	21.5	23	25	4

Table 5. Mean number of eggs laid on NGM plates seeded with OP50-1 food source during the progeny assay. Standard deviation and standard error was calculated where N=2.

Percentage Hatched	Control	0.1WLE	1WLE	5WLE	Mn	0.1WLE+50Mn	1WLE+50Mn	5WLE+50Mn
Mean	0.5314685	0.6148649	0.5923567	0.5263158	0.5918367	0.7197802	0.3510638	0.3648649
Std dev	0.4692747	0.2267582	0.4207816	0.3721615	0.3074377	0.2665333	0.0955308	0.3883077
Std err	0.3318273	0.1603423	0.2975375	0.2631579	0.2173913	0.1884675	0.0675505	0.274575

Table 6. Percentage of *C. elegans* eggs that hatch into progeny on NGM plates seeded with an OP50-1 food source. Standard deviation and standard error was calculated where N=2.

DISCUSSION & FUTURE DIRECTIONS

The extract produced from winterberry leaves is promising in treating people who are experiencing neurodegenerative disorders upon acute exposure to Mn. While not intended as a cure, WLE could provide a natural treatment that is easy to cultivate in one's yard and is rooted in its usage by Indigenous people. Prior research within the Newell laboratory has revealed the presence of polyphenols within winterberry leaves which exhibit an antioxidant effect within *C. elegans,* though the specific polyphenols present have not been identified (McKinnon, et al., 2022). Given the active polyphenol chlorogenic acid is present in the related species *Ilex paraguariensis* and other species of *Ilex*, it is hypothesized that it may be present in *Ilex verticillata*, though further research will be required to isolate and characterize the polyphenols in *I. verticillata* (Heck & Mejia, 2007).

Since acute abnormal Mn exposure has exhibited the capability to damage the dopaminergic neurons, it was hypothesized that it would increase the swim-crawl transition time. Prior research has demonstrated that dopamine is important for the initiation of crawling after swimming as genetic damage to each dopaminergic neuron has a significant impact on the ability of the nematodes to transition from swimming to crawling (Vidal-Gadea, et al., 2011). While the transition time results were not what was expected, the absence of statistical analysis and significance serves as an indicator that a conclusion cannot be made at this time. Additional trials and an analysis to establish the significance of the results should be performed, while it would also be valuable to consider ways to improve the assay. It would be important to reconsider plating 10 nematodes at once and timing the nematodes together as the timer would run continuously until all 10 nematodes

transitioned to crawling (Tables 1A-B). This method may lead to error in the experimental results; therefore, plating each nematode alone and timing individually may produce less error. Another likely cause of error may stem from the environmental conditions in which the assay is performed. Any temperature variations in the air and in the NGM plates, as well as the quality of the NGM media, may affect the absorption rate of the droplet containing the nematodes. To reduce the possibility of environmental effects on the absorption of the nematode droplet during the swim-crawl transition assay, all replicates should be carried out at the same room temperature and with minimal variation between the thickness, freshness, and temperature of the NGM plates. Nematodes should also be plated individually instead of a group to prevent interaction between the nematodes which may impact the results obtained. Ultimately, while there was an observed trend of slight recovery in treatments containing food, the results were not significant for the assay, necessitating further trials to reduce the error.

The results obtained in the nematode basal body bends assay contrasts with established literature as it was expected that nematodes with dopaminergic damage would experience a significant increase in the number of body bends, both in the presence and absence of the OP50-1 food source (Table 2A-B; Ijomone, et al., 2016). Conversely, nematodes with intact dopaminergic neurons would experience a decrease in the number of body bends. While there was a minor change in body bends observed between nematodes in food-rich and food-deprived environments, the lack of a proper statistical analysis of the results prevents the formation of a conclusive statement. In order to fully characterize the effect of WLE and Mn on *C. elegans* movement, it would be important to

consider performing further trials as necessary to reduce standard error and increase the significance of the results before any conclusions or claims can be made.

C. elegans that were treated with $MnCl_2$ appeared to exhibit a delayed repulsion response when exposed to 1-nonanol (Table 4), a fatty alcohol that is used in perfumes and flavoring, yet is toxic when concentrated. This trend is expected as nematodes in a similar experiment with perfluorooctane sulfonate (PFOS), which induces oxidative stress in *C. elegans*, exhibited a dose-dependent delay in response time upon exposure to 1-nonanol (Sammi, et al., 2019). This was reflective of negatively-affected dopamine-dependent behavior. While the results from WLE and Mn treatment did not reveal a significant dose-dependent effect on the reaction time of *C. elegans* due to the absence of proper statistical analysis, they do suggest that WLE does not have an adverse effect on the nematodes, providing a promising avenue for further trials to establish and increase the significance of the delayed response.

Based on the results of the progeny assay, it would be important to perform further treatments in order to fully characterize the effect of WLE and Mn treatment on nematode egg-laying and hatching behavior due to the low number of replicates performed. While it was anticipated that the number of eggs laid would decrease with 50 mM Mn treatment and recover when treated in conjunction with increasing WLE concentration, the results did not offer any conclusive effect on nematode egg-laying behavior (Table 5). It is not known specifically which neurons may have been affected upon treatment, though the effects may have been greater in serotonergic neurons rather than dopaminergic neurons, due to the former's role in promoting egg-laying and the latter's inhibitory role (Weinshenker, et al., 1995; Dempsey, et al., 2005). It may be possible that the nematodes

experienced different growth rates between the treatments, impacting the number of eggs and progeny counted at the set times (Gubert, et al., 2016). Treatment with 0.1% and 1% WLE exhibited some recovery in the percentage of eggs that hatched but the error and absence of proper statistical analysis was not enough to make a conclusion about the results, including whether or not egg-laying and hatching behaviors are independent of each other. When treated with 5% WLE with and without $MnCl_2$, the percentage of eggs hatched noticeably decreased (Table 6). This is likely attributed to the concentration of the treatment being near the WLE LD_{50} value of 5.87% as determined by the Newell laboratory at the University of Maine (McKinnon, et al., 2022).

WLE has been shown to induce a dose-dependent decrease in the concentration of ROS within *C. elegans* while also increasing the concentration of the antioxidant compound glutathione (GSH) (McKinnon, et al., 2022). While there is error and absence of significance among the results, this does not necessarily suggest that neurodegeneration is not happening or that WLE is ineffective. It would be important to consider other effects of $MnCl_2$ treatment that may appear in *C. elegans* and produce similar results if dopaminergic neurons were not experiencing neurodegeneration. Two effects that are observed within oxidative stress are carbonyl stress and mitochondrial dysfunction, both of which are likely to occur before the onset of neurodegeneration (Figure 6). While WLE decreased the concentration of ROS and the magnitude of carbonyl stress in the nematodes, the degree of which it affects mitochondrial dysfunction is currently unknown.

To evaluate the possibility of mitochondrial dysfunction having an effect on the results, an ATP testing kit should be used to measure the concentration of mitochondrial ATP in the nematodes in order to better characterize the molecular effects of WLE and Mn

on mitochondria. Although it may be nonspecific for cell types within *C. elegans*, it could characterize the observed results as stemming either from mitochondrial dysfunction or neurodegeneration. Further research should also examine the effects of WLE and $MnCl_2$ on the SKN-1 pathway, which is activated under conditions of oxidative stress and mitochondrial dysfunction (Palikaras, et al., 2015). By promoting synaptic function and stress resistance, it should express a natural inhibitory effect on neurodegeneration (Blackwell, et al., 2015). Additionally, certain parts of the *C. elegans* innate immune system, such as the p38MAPK pathway, provide protection against dopaminergic neurodegeneration when mitochondrial dysfunction is present (Chikka, et al., 2016). As a result, it would be of interest to study the effect of WLE and Mn on the functioning of these pathways.

The assays that were performed should be repeated to reduce the error seen in the treatments, allowing for more conclusive arguments to be made about the effects of WLE and Mn on *C. elegans*. Ideally, 6-7 replicates should be performed for each experiment; only 2-4 replicates were performed for each experiment in the time that was available. Additionally, imaging of neurons and receptors tagged with fluorescent proteins would allow for visualization and quantification of neuronal activity, and would supplement the above assays. As WLE and leaves from related *Ilex* species have been shown to reduce ROS and increase GSH in *C. elegans*, the former may still be a useful candidate for protecting against dopaminergic neurodegeneration induced by Mn. To summarize, these assays did not reveal many significant effects that WLE may have on dopaminergic neurons and no conclusive statement of WLE protection against neurodegeneration can be made at this time. Further research is needed to narrow down

what is responsible for the effects observed. However, the experiments that were performed, combined with the Newell laboratory results, support that WLE has beneficial effects within *C. elegans* globally, and a better understanding of the processes that occur with treatment would be able to justify the potential of winterberry leaves as a natural treatment for humans.

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AUTHOR'S BIOGRAPHY

Brendan Campbell Moline was born on January 2, 2001, in Danbury, Connecticut. Raised in Newtown, Connecticut and the towns of Orland and Northport in Maine, he graduated from Belfast Area High School in 2019. He enrolled at the University of Maine that fall, initially pursuing a Bachelor of Science in biochemistry before switching his major to a Bachelor of Science in microbiology in 2021, additionally picking up a minor in neuroscience. Brendan is a member of the Phi Beta Kappa honors society and a recipient of the Maine Top Scholars Award.

Upon graduating from the University of Maine in May of 2023, Brendan will be continuing his education at graduate school, where he is planning to obtain his doctorate in the pharmaceutical sciences. Outside of academics, he enjoys discovering new music and expanding his knowledge in the field of numismatics.