IN VIVO NEUROPROTECTIVE EFFECTS OF CINNAMON BIOACTIVE COMPOUNDS IN *C. ELEGANS* AND *D. MELANOGASTER*

by

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figures **Page**

CHAPTER I

REVIEW OF THE LITERATURE

Introduction

Neurodegenerative diseases (NDs), such as Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis, Huntington's disease, and Friedreich's ataxia, are devastating age-related conditions that have become one of the primary public health concerns of recent decades. Over the last century, the world experienced dramatic increases in life expectancy, and the aging population is expected to continue to expand; the population of individuals over age 65 in the world is projected to increase from 524 million in 2010 to 1.5 billion by the year 2050.[1,2] While the remarkable rise in life expectancy represents a great societal achievement, it is tempered by one of the most devastating and costly ramifications of an expanding older population, an increase in prevalence of neurodegenerative diseases.

NDs are characterized by the progressive and irreversible loss of neurons in selective regions of the brain. In afflicted individuals, symptoms typically manifest as memory loss, anxiety, and depression, which evolve to severe motor dysfunction, profound cognitive deterioration, and loss of independent function. The risk of developing a ND rises sharply with age; the number of people with a neurodegenerative disease is low at younger ages, but the prevalence of people suffering from neurodegeneration doubles every 5 years after age 65.[3] AD, the most common form of NDs, currently affects about 10 percent of the population over age 65 and 47 percent of

adults aged over 85 years.[3] NDs typically involve a slow decline in human function that results in an eventual need for constant care and assistance with the most basic activities of daily life, consequently generating a substantial social and financial burden. In the 2010 World Alzheimer's Report, it was estimated that the worldwide cost for medical expenses, social care by community professionals, and informal care by friends and family for individuals with a ND exceeded $600 billion.[1] As the world is experiencing ever-longer life expectancies, the prevalence of these diseases are also expected to rise. Alzheimer's Disease International projected that over 115 million people in the world will be living with a ND by 2050.[4] Currently, the etiology behind most NDs remains largely unknown and the available treatment options are unable to slow, reverse, or cure the underlying neurodegeneration and natural course of disease. As such, more effective interventions to prevent or treat neurodegeneration are critical to promoting an active and healthy aging population and preventing the potential dramatic rise in the prevalence of NDs

Alzheimer's Disease

AD is the most common ND in the world.[5] This disease involves pathological events that affect the anatomy, biology, and function of selective regions of the brain.[6] Specifically, widespread neurodegeneration is observed in the cortex and hippocampus.[7] AD is clinically characterized by a progressive loss in cognitive function that typically begins with memory loss, anxiety, and depression. As the disease progresses, the symptoms evolve to severe motor dysfunction, profound cognitive deterioration, and loss of independent function.[6] Pathologically, AD involves a cascade of events that lead to modifications in the metabolism of the amyloid precursor protein (APP) and the tau

protein.[6] These changes result in the characteristic development of extracellular amyloid beta-protein (Aβ) deposits and intraneuronal neurofibrillary tangles (NFTs), respectively.[5,6] Consequently, common cell signaling pathways are affected and result in neuronal network dysfunction, neuronal loss, neurotransmitter failure, and cell death.[6]

Amyloid Beta-protein Deposits

Aβ exists in the cerebrospinal fluid and sera as a normal byproduct of APP metabolism.[8] APP is a transmembrane protein expressed ubiquitously in cells, indicating that it has a normal biological role; yet, its function is unknown.[8] However, in cases of AD, APP is found to be aberrantly processed to yield soluble Aβ oligomers and insoluble Aβ fibrils that aggregate extracellularly in the brain.[8] The metabolism of APP normally occurs through a cleavage by α-secretase, a membrane-bound protease that acts on APP within the Aβ domain, therefore it does not produce the Aβ peptide.[6,9] For unknown reasons, in AD, APP is processed in an alternate pathway that involves two subsequent cleavages.[9] In this pathway, referred to as the amyloidgenic pathway, the enzyme β-secretase first cleaves APP, releasing a membrane bound C-terminal fragment consisting of 99 amino acid residues, referred to as C99.[6] Gamma-secretase subsequently cleaves C99 and releases Aβ, typically containing 40, 42, or 43 amino acids, into the transmembrane domain.[6] The Aβ peptide can oligomerize to form soluble oligomers, but may also converge to form insoluble fibrils in a beta-sheet conformation that are deposited extracellularly.[9] It has recently been found that the soluble Aβ oligomers accumulate in synapses and behave as a pathogenic ligand to membrane proteins, which disrupts synaptic transmission and downstream events that are required for memory formation.[4] The insoluble Aβ fibrils that form are deposited as plaques extracellularly and

contribute to neurodegeneration in several ways. Aβ plaques are thought to directly contribute to synaptic dysfunction by altering synaptic plasticity.[6] The plaques are thought to promote oxidative stress by decreasing antioxidant enzymes, increasing free radical production, and/or causing mitochondrial dysfunction.[5] Additionally, inflammation surrounding the plaque is believed to promote the degeneration of nearby neurons.[6] Aβ plaques are also thought to disrupt cellular functions by interacting with cell membranes and promoting oxidation.[8] Furthermore, Aβ plaques form poorly selective channels in the lipid bilayer that disrupts the membrane potential responsible for generating action potentials, which results in an influx of calcium into the cell that promotes apotosis.[8]

Neurofibrillary Tangles

NFTs are intraneuronal protein aggregates primarily composed of the hyperphosphorylated cytoskeletal protein, tau. The main function of tau relates to microtubule stability. Tau is intimately involved in maintaining the balance between assembly and disassembly of the microtubules, a function that is essential to the stability of the cytoskeleton and to the integrity of neurons.[8] The phosphorylation state of tau modulates the stability of the microtubules and is regulated by various protein kinases and phosphatases. However, in AD, tau is found to be aberrantly hyperphosphorylated, rendering it incapable of microtubule interaction, consequently impacting normal neuronal functions, morphology, and viability.[8] Recent evidence suggests that, in addition to being a microtubule stabilizer, tau may regulate neuronal excitability and serve as a master regulator of the trafficking of molecules within the cell that contribute to synaptic function.[10] These additional functions are also disrupted by the hyperphosphorylation of

tau and contribute to the dysfunctional regulation of neuronal signaling and synaptic function common to AD. This is evidenced by a strong correlation between the hyperphosphorylated tau and a decrease in presynaptic protein expression.[11] Interestingly, studies have shown that reduction in the levels of tau prevents the neurotoxic effects of Aβ oligomers, suggesting that the damage induced by Aβ oligomers on the synapses may be mediated by the aberrant phosphorylation of tau. In addition, both oxidative stress and soluble Aβ oligomers are believed to promote the actions of the kinases ERK, p38, and JN, which are capable of phosphorylating tau.[10] The hyperphosphorylation of tau promotes its aggregation and represents the main component of the insoluble NFTs characteristic of AD.[6]

Pathological Mechanisms

Convincing evidence reveals that oxidative stress plays a key pathogenic role in the dysfunction and death of neurons that drives NDs. Free radicals are highly reactive and unstable molecules formed constantly in cells and must be neutralized by an antioxidant defense system to maintain redox homeostasis.[12] Oxidative stress occurs when there is an imbalance between the production of free radicals and the ability of the cells to neutralize these toxic compounds.[12] When redox homeostasis is lost, the overabundant free radicals react with and damage cellular components with destructive consequences, such as lipid peroxidation, enzyme inactivation, nucleic acid breakage, and altered membrane fluidity.[8] Oxidative stress can cause damage to all biomolecules and eventually leads to cell death, if not resolved.[8] The brain is particularly susceptible to conditions of oxidative stress since it consumes approximately 20-30% of the oxygen inspired.[7] Ninety percent of free radicals formed in the cell are produced by the

mitochondria, an organelle that neurons are highly reliant on for oxidative phosphorylation and the maintenance of membrane polarity.[12] Additionally, the brain contains a high concentration of polyunsaturated fatty acids (PUFAs) that are particularly vulnerable to oxidation.[7] Although oxidative stress is known to be a key factor in the pathology of AD, it remains unclear if it is a cause or result of the disease.[8] Evidence suggests that oxidative stress promotes the formation of Aβ plaques and NFTs; however, it has also been observed that the presence of these protein aggregates promotes oxidative stress.[8] This relationship creates a detrimental cycle of free radical production that overwhelms the antioxidant defense system.[8] Many proteins have been identified that are damaged by oxidative stress in AD, including proteins involved in cell signaling, neuronal communication, antioxidant defense, regulation of neurotransmitters, pH regulation, energy metabolism, phosphorylation of tau, and processing of APP.[7] The oxidative damage to these important proteins disrupts their activity and contributes to the molecular dysfunction seen in AD.[7] An increase in lipid peroxidation has also been observed in the brains of individuals with AD.[8] The peroxidation of the particularly susceptible PUFAs in the cell membrane result in alterations in the membrane composition and is thought to contribute to the decreased expression of the nicotinic acetylcholine receptors commonly seen in AD.[8] In addition, byproducts of lipid peroxidation such as HNE and acrolein are capable of covalently modifying proteins and inhibiting enzyme activity.[8] HNE has also been shown to upregulate the expression of β-secretase, thus promoting the processing of APP to form Aβ peptide.[8] Oxidative stress can also cause modifications to DNA, including strand breakage, cross-linking, base modification, and oxidation of deoxyribose.[12] Oxidative damage to mitochondrial DNA

further promotes oxidative stress by causing modifications in the electron transport chain that lead to the leakage of electrons.[8]

Originally, inflammation was thought to be a consequence of neurodegeneration, but recent evidence suggests that it may also be a primary initiating factor.[13] The persistent formation of plaques, along with progressive neuronal damage lead to the overactivation of microglia, the immune cells of the CNS. The overactive microglia release large amounts of pro-inflammatory and/or cytotoxic factors, which can cause damage to the neurons and further activate microglia, resulting in a vicious cycle that causes uncontrolled and prolonged inflammation which drives neurodegeneration.[14]

Glutamate, the primary excitatory neurotransmitter in the CNS, activates several receptors located in pre- and post-synaptic terminals and on astrocytes surrounding synapses, including the N-methyl-D-aspartate receptor (NDMA).[15] The extracellular levels of glutamate and other excitatory neurotransmitters are strictly regulated to maintain relatively low concentrations (1-10 μM) to allow for sufficient activation of glutamate receptors and to protect against accumulation of glutamate and consequent over-activation of the receptors.[15] Higher extracellular concentrations (10-100 μM) can lead to death of the cells expressing glutamate receptors.[15] The latter is a pathology referred to as 'excitotoxicity' that is implicated in many neurodegenerative diseases, including AD. In the brains and platelets of AD patients, a decrease in glutamate uptake has been observed.[16,17] While the expression of glutamate transporters in the brain tends to decrease with age, a more rapid decrease is observed in patients with AD.[17,18] Reduced glutamate uptake has also been reported in transgenic mice expressing age-dependent tau protein accumulations, which was associated with motor dysfunctions; interestingly,

theses changes preceded the tau accumulations, suggesting that dysfunctions in the glutamate neurotransmitter system may be involved in tauopathy pathogenesis.[19]

Risk Factors

The greatest risk factor for the development of AD is age.[5] It is thought that the increase in oxidative stress that occurs with aging constitutes a large part of the risk. Vascular diseases, such as hypertension, hypercholesterolemia, and diabetes also increase the risk of AD.[5] On top of vascular complications, diabetes poses an additional risk for AD due to the effects of hyperinsulinemia and insulin resistance on amyloid metabolism.[5] This correlation is evidenced by a significantly high incidence of AD development among individuals with diabetes. It has been suggested that hyperinsulinemia and insulin resistance leads to decreased clearance and increased deposition of the Aβ peptide.[20] Additionally, impaired insulin-signaling results in abnormal glucose and energy metabolism, oxidative stress, mitochondrial dysfunction, and hyperphosphorylation of tau via increased activity of glycogen synthase kinase.[5,9] Although the majority of cases of AD are sporadic and without certain cause, approximately 10% of cases can be ascribed to genetic susceptibility.[8] Mutations in APP, presenilin 1, and presenilin 2 located on chromosome 21, 14, and 1, respectively, have been identified to promote the abnormal processing of APP that leads to aggregation.[8] Additionally, inheritance of the ε4 allele of the apolipoprotein E gene on chromosome 19 increases the risk for the development of AD.[8]

Parkinson's Disease

PD is the second most common neurodegenerative disease that affects .3% of the population of industrialized countries.[13] The prevalence increases to 1% of the population over 60 years of age, and to 4% of the population over 80 years of age.[13] PD involves a progressive loss of neurons in the brain manifesting into characteristic motor and non-motor symptoms. Motor symptoms include akinesia, bradykinesia, rigidity, resting tremor, postural instability, and gait impairment.[21] Non-motor symptoms include anosmia, depression, anxiety, sleep disorders, gastrointestinal symptoms, autonomic dysfunction, and cognitive impairment.[21]

Dopaminergic Pathway and Lewy Bodies

PD is a neurodegenerative disease of the central nervous system hallmarked by the degeneration of dopaminergic neurons, dopamine deficit, and the presence of Lewy bodies in remaining neurons.[22] Although there are signs of pathology in multiple neuronal systems in PD, the cardinal symptoms developed are ascribed to the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and subsequent loss of the neurotransmitter dopamine.[14] The SNpc is critical for the control of motor function, since it promotes voluntary movement and inhibits involuntary movement by affecting the basal ganglia loops, a process mediated by dopamine receptor D1 activation and D2 inactivation.[14] Neurodegeneration in this dopaminergic pathway leads to dopamine depletion, which has shown to alter the proportions of the D1- and D2-receptors.[23] Specifically, the loss of dopamine results in reduced activity of the D1-receptor and increased activity of the D2-receptor.[23] These pathological changes in the

dopamine receptors alter the balance in the basal ganglia loops and result in the characteristic motor symptoms of the disease.[14] Lewy bodies are found in the cytoplasm of the remaining neurons of the SNpc; however, they are also found in other non-dopaminergic neurons, including neurons of the amygdala, basal nucleus of Meynert, hippocampus, and brain stem.[14] The presence of Lewy bodies in these non-dopaminergic neurons indicates that they play a role in the development of the non-motor symptoms associated with PD.[13] Alpha-synuclein (α-syn) has been identified as the primary protein aggregate found in Lewy bodies.[21] The exact cause of aggregation is unclear; however, overexpression of the protein, genetic mutations, and oxidative stress are thought to contribute.[21] Alpha-syn has been identified as a key factor implicated in PD, because it is plays an important role in controlling the function of dopaminergic neurons; thus, the dysfunction of this protein at dopaminergic synapses is thought to contribute to the initiation of neurodegeneration.[24] Additionally, it is suggested that the aggregation of α-syn has a pathogenic role in PD through exerting negative effects on cell membrane and proteasomal functionality, disrupting gene expression regulation, influencing cell signaling and cell death pathways, promoting inflammation, and modifying the storage and release of dopamine.[21]

Pathological Mechanisms

No single pathogenic event has been found to be the primary factor in the development of PD.[21] Rather, the process appears to be multifactorial involving several mechanisms that act synergistically in a complex manner to foster neurodegeneration.[21] Similar to AD, mechanisms involved in the pathogenesis of PD include oxidative stress, inflammation, and excitotoxicity.[21] Oxidative damage to lipids, proteins, and nucleic

acids has consistently been observed in the SNpc of individuals with PD.[21] The cause of increased oxidative stress in PD is unclear, but it has been suggested that mitochondrial dysfunction and accelerated dopamine metabolism play a role.[21] The exact cause of mitochondrial dysfunction in PD is also unknown, but results in further production of free radicals, presumably by defects in the electron transport chain that lead to the leakage of electrons.[14] Dopamine metabolism is accelerated in PD, and contributes to oxidative stress through the concurrent production of free radicals such as quinones and peroxides.[14] The vulnerability of dopaminergic neurons to neurodegeneration in PD compared to other neuronal systems may be linked to unique morphological and physiological properties.[14] The dopaminergic neurons consist of long, narrow, branched projections that have higher basal energy requirements than other neurons, rendering them more susceptible to damage caused by mitochondrial dysfunction.[14] Additionally, dopaminergic neurons are particularly susceptible to oxidative stress due to a high rate of oxygen and calcium metabolism, elevated iron concentrations, and reduced levels of antioxidants, specifically glutathione.[14] Inflammation is another mechanism involved in PD, as evidenced by microglia activation in the striatum and elevated levels of pro-inflammatory cytokines in the cerebrospinal fluid and basal ganglia in individuals with PD.[21] Microglia can become activated in PD in response to toxins, protein aggregates, or damaged neurons and become chronically active as a result of positive feedback from dying neurons, as seen in AD. Excitotoxicity is also implicated in PD. Dopaminergic neurons have particularly high levels of receptors for glutamate, the principle excitatory neurotransmitter of the central nervous system, rendering them vulnerable to glutamate-

mediated toxicity.[21] As with AD, over activation of the NDMA receptors by glutamate leads to increased intracellular calcium levels, which promotes apoptosis.[21]

Risk Factors

The etiology behind PD is largely unknown, but there are some genetic and environmental factors identified that contribute to the development of the disease.[25] Similar to AD, age represents the biggest risk for developing PD.[25] Environmental factors, such as the neurotoxin MPTP and certain pesticides are known to cause PD through the inhibition of complex 1 in the mitochondria.[12] The active metabolite of MPTP, MPP^+, exerts toxic effects on the dopaminergic neurons causing mitochondrial membrane potential impairment, altered calcium handling, excessive free radical production, and ultimately cell death.[21] Rotenone is an example of a pesticide that also exhibits detrimental toxic effects on complex 1 in the mitochondria.[13] Other environmental risk factors include carbon monoxide poisoning, exposure to solvents, and hydrogen sulfide intoxification.[13] Although rare, various genetic mutations have been identified to cause PD in about 5-10% of cases.[25] Specifically, mutations in α-syn, parkin, DJ-1, and LRRK2 are associated with the development of PD.[12] These mutations are believed to cause mitochondrial dysfunction and increased oxidative stress, and thus contribute to the development of PD.[12]

Limitations of Pharmacological Management of Neurodegenerative Diseases

Alzheimer's Disease

At present, there is no cure for AD. The current treatments offer palliative therapy and provide temporary improvement in cognitive function, but do not impact the

underlying neurodegeneration. The pathological cascade of events triggered in AD is asynchronous; as such, there are viable neurons that can be therapeutic targets at each stage of the disease progression.[6] Thus, the current treatment options focus on maximizing the activity of the neurotransmitter systems in remaining functional neurons.[6] Much effort is being directed toward the development of novel treatments for AD that can modify the disease process by targeting principal upstream pathological mechanisms.[9,26] Currently, the treatment targets being investigated involve decreasing production of Aβ; enhancing the clearance of Aβ; interrupting aggregates; protecting tau from hyperphosphorylation; and neuroprotective agents that focus on regeneration and development of neurons, mitochondrial activity, and the viability of neuronal membranes.[9] Many of these new treatment candidates have progressed to large clinical trials, but have unfortunately proved unsuccessful in treating AD.[26] The current FDA approved pharmacological agents for the treatment of AD are cholinesterase inhibitors and memantine.[26]

The degeneration of cholinergic neurons and diminished acetylcholine levels are consistently observed in AD patients, which consequently disrupts the cholinergic pathway and leads to a rapid loss of memory and learning capacity that is characteristic of AD.[27] Cholinesterase inhibitors target the cholinergic neurotransmitter system in order to maximize the availability of acetylcholine at the synaptic cleft.[6] Specifically, they work in the forebrain regions to inhibit the action of acetylcholinesterase, which is a serine hydrolase that functions at neuromuscular junctions and synapses within the cholinergic pathway to rapidly hydrolyze acetylcholine and effectively terminate the nerve impulse.[28] Inhibition of acetylcholinesterase prevents the hydrolysis and

inactivation of acetylcholine, thus increasing the amount of acetylcholine for neurotransmission in the remaining viable cholinergic neurons.[5] The basis for the use of memantine in the treatment of AD is related to the glutamate-mediated excitotoxicity that occurs in the disease process.[29] Memantine is an amantadine derivative that functions as an uncompetitive voltage-dependent antagonist of the N-methyl-D-aspartate receptor that is able to selectively block the activation of the receptors under uM concentrations of glutamate, but permits the normal physiologic stimulation of the receptors in response to mM glutamate concentrations.[30] This action of memantine maintains normal stimulation of the receptors required for memory and learning processes, but prevents overstimulation that can result in cell death.[29]

While cholinesterase inhibitors and memantine are currently the only approved treatment options available for individuals with AD, these drugs only address the secondary effects resulting from neurodegeneration, but do not affect the underlying pathology. Consequently, treatment can delay symptomatic progression by several months, but do not impact the natural course of disease.[11] Moreover, clinical studies have shown that cholinesterase inhibitors and memantine have inconsistent effects on behavioral dysfunctions and limited impact on activities of daily living.[31,32] In fact, the effect of cholinesterase inhibitors on cognitive and behavioral have shown to be so modest that its clinical significance has been debated.[33,34] Beyond these limitations, the current treatment options also confer the risk of side effects. Cholinesterase inhibitors are commonly associated with diarrhea, nausea, vomiting, muscle cramps, poor appetite, sleep disturbances, dizziness, and syncope.[35,36] For memantine, potential side effects

include, dizziness, cough, fatigue, confusion, headache, pain, vomiting, dysnea, and hypertension.[29]

Parkinson's Disease

Similar to AD, the current treatment options for PD are unable to slow the progression of disease or reverse lost functions.[25] Therefore, the current approaches to treatment of PD aim to ameliorate the resulting symptoms and improve the quality of life of patients afflicted with this neurodegenerative disease.[13] Treatment provides only symptomatic relief, since nothing can currently be done to slow or halt the disease progression.[13,21] Since diminished levels of dopamine are a primary pathological factor in PD, current pharmacologic treatments are used to increase dopamine levels in the brain by altering dopamine metabolism.[37] The current pharmacologic agents target various steps in the pathway of dopamine metabolism and include levodopa, dopamine receptor agonists, catechol-O-methyltransferase inhibitors (COMT-I), and monoamine oxidase-B inhibitors (MAO-B).

Levodopa is the naturally occurring dopamine precursor and is considered the gold standard in the treatment of PD.[38] Exogenous levodopa can cross the blood-brain barrier, where it is rapidly taken up by the striatal dopaminergic neurons and converted to dopamine. Thus, levodopa counteracts the endogenous dopamine deficit in PD and helps ameliorate the resulting motor dysfunctions that occur. However, due to peripheral metabolism of levodopa, large doses are required to obtain sufficient effects, which causes severe side effects such as hypotension, nausea, diaphoresis, and vomiting.[39] As such, co-administration with a dopa-decarboxylase inhibitor is required to decrease the

peripheral metabolism of levodopa and reduce the dosage required. Dopamine receptor agonists are a class of pharmaceutical agents that contain a moiety similar to dopamine in their structure that allows direct stimulation of the dopamine receptors, which can also mitigate the dopamine deficit implicated in PD. When levodopa is taken with a dopa-decarboxylase inhibitor, catechol-O-methyltransferase (COMT) represents the main enzyme that metabolizes levodopa via the addition of a methyl group.[40] For this reason, COMT inhibitors are often used along with levodopa in order to maximize the amount of levodopa available for uptake into the brain. Monoamine oxidase (MAO)-B inhibitors target the action of MAO-B, an enzyme involved in the catabolism of both free intraneuronal dopamine as well as in releasable stores.[39] Therefore, inhibiting the actions of MAO-B increases the pool of dopamine in the brain available for dopaminergic neurotransmission, and is thus the therapeutic action of MAO-B inhibitors.

Although Levodopa is considered the highest standard for treating PD, there are several limitations. The potential side effects of levodopa include sedation, hypotension, hallucinations, nausea, motor fluctuations, and dyskinesias.[41] Dyskinesias and motor fluctuations are unfortunate potential side effects that relate to the "on-off" phenomenon that can occur with levodopa usage, which refers to a rapid and unpredictable switch from severe PD symptoms to a relatively non-symptomatic state, followed by a dyskinetic state of involuntary movement.[41] These complications are thought to be a consequence of the short half-life of levodopa that necessitates frequent administrations, resulting in inconsistent and pulsatile stimulation of the dopamine receptors.[23] Due to the progressive degeneration of the dopaminergic neurons, the ability to synthesize, store, and release dopamine diminishes through the course of the disease.[23] Therefore, the

dopaminergic neurons become increasingly reliant on the dopamine supplemented by levodopa, thus becoming more sensitive to factors such as the short half-life.[39] As treatment continues, the symptoms become more frequent and severe, and only remain effective for approximately 10 years.[41] Dopamine receptor agonists have a longer half-life than levodopa which reduces the risk of dyskinesia; however, they have shown to become ineffective as the disesase progresses. Side effects of the dopamine receptor agonists include confusion, vomiting, nausea, dyspepsia, hypotension, abdominal pain, insomnia, dyskinesia, and somnolence.[42] The class of dopamine receptor agonist that are ergot derivatives pose a risk for additional side effects including erythromelalgia, vasoconstrictive events, and retoperitoneal or cardiac fibrosis.[42] Most metabolism of the dopamine receptor agonists occur in the liver; however, pramipexole is an exception, which is excreted in the urine via renal tubular secretion in the urine, which may cause interactions with drugs that are excreted in the same manner, such as trimethoprim, procainamide, and cimetidine.[43] In addition to levodopa, COMT is involved in the metabolism of many compounds that contain a catechol moiety; as such, COMT inhibitors may alter the actions of drugs that are metabolized by COMT, such as norepinephrine, epinephrine, isoproterenol, apomorphine, and isoetherine.[44] COMT inhibitors administered with a non-selective monoamine oxidase (MAO) inhibitor such as phenelzine or tranylcyproime may limit catecholamine metabolism, as these drugs inhibit the two main enzymes responsible involved in catecholamine metabolism.[44] As COMT inhibitors potentiate the effects of levodopa, it has the potential to increase the orthostatic hypotension side effect that may occur with levodopa treatment.[44] MAO inhibitors also confer the risk of side effects; as a monotherapy, side effects that are commonly observed

include arthralgia, depression, flu syndrome, and dyspepsia.[45] As adjunctive therapy with levodopa, hypotension, dyskinesia, weight loss, vomiting, abdominal pain, dry mouth, and constipation commonly occur.[45]

Despite intensive research efforts over many decades, the etiology underlying these complex and multifactorial diseases is poorly understood, which is reflected by the limited efficacy of current pharmacologic treatment options. The drugs available treat the biochemical sequelae of neurodegeneration; deficits of dopamine and acetylcholine are a consequence of dopaminergic and cholinergic neuronal death, respectively, whereas dysfunctional glutamate signaling is linked to degeneration in the cortical region.[9] However, there are no treatment options that modulate upstream pathologies underlying neurodegeneration. Consequently, the drugs currently in use offer modest symptomatic improvement, but the effects are temporary and do not impact the ultimate outcome of the disease.

Natural Alternatives, Especially From Dietary Sources

Neurodegenerative diseases involve a progressive loss in human function, which imparts a severe individual and familial burden. As discussed, present medications only address single pathologies that manifest as a result of neurodegeneration and are associated with many side effects. As such, there is a critical need for safe and effective therapies that modulate principle upstream causative factors and prevent or slow the progression of disease.

Natural products have been recognized for their health promoting effects for thousands of years. Plants produce a vast collection of secondary products, such as

alkaloids, phenolic compounds, and terpenes that have shown health benefits in a variety of conditions including cancer, cardiovascular disease, and neurodegenerative diseases.[46-49] Central to the health benefits of plant-based products is a complex mixture of structurally diverse phytochemicals that act synergistically to promote cellular health and survival.[50] Based on the wide range of conditions that phytochemicals have been used to treat in traditional medicine, it is suggested that a main function of these compounds is to maintain or regain homeostasis among critical systems within the cell. For example, phytochemicals, such as catechins and epicatechins, have shown to modulate the antioxidant defense system and inflammatory network, which are mechanisms commonly seen to be dysregulated and implicated in the pathogenesis of many chronic diseases.[51] Medicinal herbs, such as Ginseng, *Ginko bilboa*, and St John's Wort, are a rich source of phytochemicals and have been used to treat specific health conditions; however, these plants are not commonly consumed and may pose a risk for toxicity due to unique phytochemicals that are not typical to human diets. *Ginko bilboa,* for instance, has been used to enhance memory and treat neuronal disorders; however, the leaves and seeds contain the neurotoxin, ginkotoxin, which can induce seizures and cause unconsciousness.[52,53] In contrast, dietary sources of phytochemicals have been commonly consumed throughout history and offer minimal risk of toxic side effects. Numerous epidemiological and intervention studies have shown that the consumption of common fruits, vegetables, whole grains, herbs, and spices correlates with a reduced risk of chronic diseases, which has further corroborated the safety and efficacy of dietary phytochemicals.[51,54-56]

In the search for more effective and safe treatment options for neurodegenerative diseases, several dietary sources of phytochemicals have been investigated. Historical usage in traditional medicine, along with modern research has identified several key products with potential benefits in neurodegenerative diseases. For example, curcumin, a phenolic compound found in turmeric, has shown to reduce cerebral lipid peroxidation and enhance the activity of glutathione in rats.[57] *In vitro*, curcumin has inhibited NF-kB mediated inflammation and protected against Aβ- induced cell death in a human neuroblastoma cell line.[58] Curcumin has also been shown to protect against Aβ induced memory deficits and reduce Aβ deposits in rats.[59,60] Cinnamon is one of the oldest spices used in traditional medicine, and has shown to modulate multiple mechanisms involved in neurodegeneration. *In vivo,* cinnamon has shown to reduce oxidative stress and neuroinflammation.[61,62] Additionally, cinnamon has reduced Aβ aggregation and improved cognitive function in an AD mouse model.[63] Blueberries have also recently emerged as a potential neuroprotective agent; preliminary studies have suggested that supplementation with blueberries in aged animals improves memory and motor function.[64,65] The neuroprotective effects of blueberry may be due to the high concentration of anthocyandins, which have been identified in the brains of rats in regions that are important in cognitive function, including the neocortex and hippocampus, after supplementation with blueberry.[65] Phenolic compounds in green tea have also shown to protect neurons from oxidative and metabolic stress. Green tea has protected against 6-hydroxydopamine induced damage to dopaminergic neurons in a Parkinson's disease rat model, and reduced neurotoxicity in a Huntington's disease model.[66-68]

Among these dietary sources that have shown promising neuroprotective activity, cinnamon shows particular merit for further research into its therapeutic potential for neurodegenerative diseases. Significant evidence suggests that cinnamon bioactives have potent health promoting effects through modulating critical pathways that promote cellular survival, including the insulin signaling pathway, antioxidant defense system, and apoptotic pathways.[69-71] In addition to neurodegenerative diseases, studies have shown the therapeutic benefits of cinnamon in cardiovascular disease, cancer, oxidative stress, inflammatory diseases, and diabetes.[48,69,72,73] The pathogenesis of neurodegenerative diseases involves complex interactions between multiple mechanisms, including oxidative stress, inflammation, and proteotoxicity, which have yet to be ameliorated by pharmaceutical drugs with single targets. Therefore, identifying the neuroprotective bioactive compounds in cinnamon and their actions may allow them to be exploited to offer a therapeutic option that targets the underlying pathological mechanisms of neurodegenerative diseases.

Cinnamon

Introduction

Cinnamon has been used as a culinary spice and an agent in traditional systems of medicine since ancient age.[46] Mentioned in Chinese texts dating back to 2800 B.C.E, cinnamon may be one of the oldest spices used in traditional herbal medicine.[74] The spice was used as an ingredient in embalming fluid used for the mummification process in ancient Egypt, and frequently referenced in the bible, including instructions for its use in preparing holy ointments and perfumes. Once worth fifteen times as much as silver,

cinnamon was held in high esteem and was considered one of the most valuable herbal medicines among Greeks and Romans.[75] Cinnamon has been used throughout history to treat a broad range of conditions, including anorexia, dyspepsia, bloating, arthritis, asthma, cough, toothaches, wounds, diarrhea, fever and psoriasis.[76] In Indian Ayurvedic medicine, impaired digestion and metabolism, peptic ulcer, intestinal tract inflammation, vomiting, impotence, dry mouth, sinusitis/rhinitis, scorpion bites, and worm infestations were treated by cinnamon bark oil as a single drug.[76] The dried inner stem bark of cinnamon is employed for dysentery, indigestion, flatulence, and wheezing in Siddha medicine.[76] Cinnamon has been used in traditional Chinese medicine to treat many conditions, including the "thirsty disease," an old term used in China referring to diabetes.[69] In a text written around 50-70 C.E., the Greek physician Dioscorides mentioned that cinnamon cleared the eyes, provoked urine, brought down the menses, comforted the stomach, soothed the bites and stings of venomous creatures, and reduced inflammation in the kidneys and intestines.[75] The extensive medicinal applications of cinnamon throughout history give a clear indication the importance and appreciation of the spice in traditional herbal medicine.

The term "cinnamon" refers to the genus *Cinnamomum,* which belongs to the family of Lauraceae and comprises over 250 species of evergreen trees and shrubs.[46] Of these, *Cinnamomum verum* and *Cinnamomum cassia* are the most common in the food and pharmaceutical industries. Referred to as the "true cinnamon," *C. verum* is native to the central hills of Sri Lanka and South India, but is also cultivated in parts of Africa (the Seychelles and Madagascar), South America, Indonesia, and the West Indies.[46] Among the species of *Cinnamomum*, cinnamon produced from the bark of *C. verum* cultivated in

Sri Lanka is considered the highest quality.[75] *C. cassia*, or "Chinese cinnamon" originated in Southeast Asia and was subsequently introduced into South America, Hawaii, and Indonesia.[77] *Cinnamomum tamala*, also known as Indian cassia, is native to northeastern India and is now cultivated widely throughout Australia, South America, the Pacific region, and tropical and sub-tropical Asia. Another important species, *Cinnamomum burmannii* or Indonesian cassia, is cultivated in West Sumatra and the Java region.[78] Currently, about 60% of the worlds supply of cinnamon is cultivated along the coastal belt of Sri Lanka. The cinnamon plant is maintained as a bush about 2-3 m tall during cultivation. The plant is ready for harvest at around two years of age, at which it is about 2 m tall, around 8-12 cm wide at the base, and has about 3-4 shoots. The leaves of the cinnamon plant are about 7-18 cm long and have an ovate-oblong shape.[46] Along the stems of the plant are branched clusters of white flowers, and the fruit is a small purple berry about 1 cm in diameter.[46] The most valuable products are derived from the inner bark of cinnamon shoots. The peeling of cinnamon bark is accomplished by traditional methods that require skilled workers and specialized tools.[75] The bark is most commonly processed to form cinnamon quills; the outer bark is stripped off and typically disposed of, and the inner bark is peeled off and allowed to curl into tubes, which are systematically dried in the sun and shade for a specified period of time. Quillings are also produced from the bark and consist of splits and broken pieces of the quills. The small pieces and shavings of the bark that are not used in quill production are sold as featherings. Scraping off the tough bark on thicker stems that cannot be peeled produces cinnamon chips.[75] Leaf and bark oil of the cinnamon plant are also important products, which are produced by distillation of the leaves and bark, respectively.[46] Cinnamon

oleoresin is a dark brown, concentrated and viscous liquid that is prepared by solvent extraction of the bark.

Cinnamon is most commonly used as a spice to flavor foods. It is found in confections, liquors, canned fruit, beverages, baked goods, marinades, teas, soups, sauces, and chewing gum.[76] It is the classic flavor of apple pie and an important spice in many doughnuts, pastries, cakes, and cookies.[74] Cinnamon imparts a synergistic effect with sugar to enhance the sweetness sensation through its complementary aroma. It is a popular ingredient in chocolate in Spain and is among the many spices used to make mulled wine. Cinnamon quills are often added to infuse beverages such as tea or hot chocolate. Cinnamon is also used in the preparation of savory dishes; in Mexico, it is an ingredient in the famed *mole* sauce served over chicken and is one of the spices in the Chinese 5-spice blend.[76] It is commonly added to rice dishes, meat curries, fish, and vegetables in Sri Lanka. The bark and leaf oils of cinnamon are commonly used in the preparation of sodas and liquors. The leaf oil is generally cheaper than bark oil and is commonly used in flavoring confections and in the preparation of synthetic vanillin. The bark oil is important in the food industry in flavoring meat, confections, and various processed foods. Cinnamon oleoresin is also a commodity in the flavoring industry. Cinnamon is often added to foods for its antimicrobial activity to delay spoilage and can also help mask unpleasant odors and flavors in food and drugs.[76]

Bioactive Compounds

The wide range of health benefits that cinnamon has been associated with for thousands of years can be ascribed to its vast collection of phytochemicals. Synthesized

in response to pathogen invasion or environmental stresses such as UV radiation, phytochemicals are products of secondary metabolism in plants that serve to increase overall cell viability. Driven by fundamental properties of their underlying chemical structure, these compounds exert many biological activities beyond nutrition, such as free radical scavenging and modulating cell signaling pathways. The phytochemistry of cinnamon is largely represented by volatile components found in all parts of the plant, which generally posses the same collection of compounds in varying proportions. The exact composition of cinnamon varies depending on the species, geographical origin, and processing methods; however, the major phytochemicals in cinnamon can be broadly classified as phenolic compounds and terpenes.

Phenolic Compounds

Phenolic compounds represent one of the largest and most ubiquitously distributed class of secondary metabolites in plants. Chemically, phenolic compounds contain one or more aromatic rings which contain varying numbers of hydroxyl groups.[79] Further classified by their structure, phenolic compounds can range from simple (phenolic acids, flavonoids) to complex polymerized molecules (tannins, melanins, lignins).[80] Flavonoids constitute one of the largest classes of phenolic compounds; as such, phenolics can be classified as flavonoids and non-flavonoids for simplicity. In a study by Lan et al., 80% methanol and 50% acetone extracts of cinnamon showed the highest concentration of phenolic compounds (148 and 186mg GE/g, respectively) among black peppercorn, nutmeg, rosehip, and oregano leaf.[50] The main component of cinnamon bark oil is *trans*-cinnamaldehyde (97.7%), whereas eugenol is the primary component in leaf oil (87.3%).[81,82] Cinnamaldehyde and eugenol belong to a class of non-

flavonoid phenolic compounds referred to as phenylpropanoids that are characterized by a three-carbon propene moiety and six-carbon aromatic phenyl ring derived from the simple phenolic acid, cinnamic acid. E-cinnamyl acetate is another phenylpropanoid found in the volatile oil of cinnamon fruit stalks, fruits, and flowers at 37%, 44-55%, and 48%, respectively. Other predominate phenylpropanoids in cinnamon include cinnamyl alcohol, ferulic acid, and 3-phenylpropyl acetate.[83,84] Coumarin is another non-flavonoid found in cinnamon, which is characterized by a phenol and pyrone ring composing a two-ring structure. Coumarin is found in low amounts in *C. verum* (.05% of leaf oil), whereas it is present in higher concentrations (6.36% of leaf oil) in *C. cassia* and is used as a marker compound to distinguish between species.[83] Cinnamon is also a rich source of flavonoids, which represent a large and diverse class of phenolic compounds that share a common structure of an oxygen-containing pyrene ring (C ring) connecting two benzene rings (A and B rings), and differ in the oxidation state of ring C.[85] Catechin and epicatechin have been found in cinnamon at concentrations of 16.1 and 7.25 μg/g of dry weight (DW), respectively.[86] These compounds belong to a class of flavonoids referred to as flavanols, which have a common flavan ring structure and differ from each other only in the spatial orientation of a hydroxyl group. Quercetin, Quercitrin, and kaempferol have also been detected, with the highest concentration found in leaf oil.[83] Characterized by a ketone group on carbon 4 of ring C, these compounds are collectively referred to as flavonols. Proanthocyanidins, or condensed tannins, are polymers of catechin and/or epicatechin, and have also been identified at a concentration of 23mg/g in cinnamon powder.[87] They are present in two forms, B-type, which has a single linkage between carbon 4 and 6 or carbon 4 and 8, or A-type, which has an additional ester bond between

carbon 2 and 7.[87] Specifically, Cinnamtannin B-1 is an A-type proanthocyanidin identified in cinnamon at 5.47 mg/g extract, whereas procyanidin B2 is a B-type proanthocyanidin found at 1.52 mg/g of cinnamon extract.[87,88] The acetylated tannin, epigallocatechin gallate (EGCG) is composed of epicatechin derivitized with gallic acid and has been found in cinnamon extract at 358 μg/g.[84] Key phenolic compounds and their chemical structures found in cinnamon are represented in Table 1.

Table 1. Major Phenolic Compounds in Cinnamon

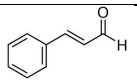
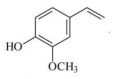
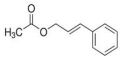
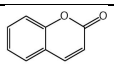
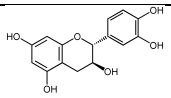
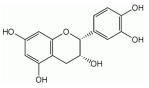
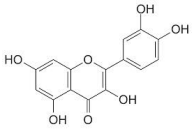
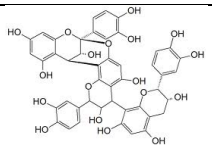
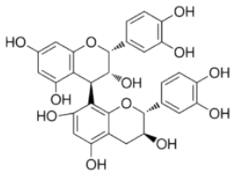
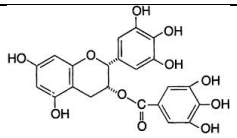
Class	**Compound**	**Structure**
Non-flavonoids		
Phenylpropanoid	Cinnamaldehyde	
	Eugenol	
	Cinnamyl acetate	
Coumarin	Coumarin	
Flavonoids		
Flavanol	Catechin	
	Epicatechin	
Flavonol	Quercetin	

Table 1 Continued		
Class	**Compound**	**Structure**
Proanthocyanidins	Cinnamtannin B-1	
	Procyanidin B2	
Acetylated tannins	Epigallocatechin gallate	

Terpenes

The cinnamon plant produces large amounts of terpenes, which are the largest class of secondary metabolites produced in plants. Terpenes account for about 90% of the total weight of cinnamon root bark oil, 10% of the weight of stem bark oil, and 8% of the weight of leaf oil.[75] The synthesis of terpenes begins with a branched condensation reaction in which the keto group of acetyl CoA joins with a second acetyl CoA molecule to yield β-hydroxy-β-methyl glutaryl CoA, which is subsequently converted to a 5 carbon isoprene unit.[83] Terpenes are ultimately formed from the union of these isoprene units and vary in their chemical complexity depending on the number of isoprene units contained.

Among the monoterpenes present in cinnamon leaf oil, linalool, piperitone, α-phellandrene, and 1,8-cineole represent 8.5%, 3.3%, 2%, and .7%, respectively, of the

compounds in cinnamon leaf oil.[81] Camphor is the major compound (56.2 %) in root bark oil, followed by 1,8-cineole (11.7%).[46,83] α-terineol is a monoterpene alcohol comprising 4.4% of the compounds in cinnamon bark oil. Numerous sesquiterpenes have also been identified in the volatile oils of cinnamon. B-caryophyllene is a bicyclic sesquiterpene found at 2% in leaf oil and 6% in bark oil.[81,89] Cinnamon bark oil also contains the sesquiterpenes δ-cadinene (.9%), α-copaene (.8%), α-amorphene (.5%), α-guaiene (1.5-7%), α-humulene (1.3%), and α-muurolene (.1-1.8%).[81,82] Aromadendrene and bicyclogermacrene are sesquiterpenes present in cinnamon leaf oil at 1.1% and 4%, respectively.[81] Several non-volatile diterpenes have also been detected among the genus. These include cinnzeylanine[1] and cinnzeylanine[2] and their derivatives, anhydrocinnzeylanine, anhydrodioxocinnzeylanol, andhydrocinnzeylanol, dioxocinnzeylanine, dioxocinnzeylanol, and trioxocinnzeylanol.[75] Cinncassiols are also a group of non-volatile diterpenes in cinnamon and include Cinncasiol A, B, C1, C2, C3, D1, D2, D3, D4, E, and their corresponding glucosides.[75]

Neuroprotective Effects of Cinnamon

NDs are characterized by progressive damage and destruction of neuronal networks in selective regions of the brain, which leads to severe behavioral and cognitive dysfunction. Currently, these diseases affect over 10 million individuals in the world each year, and the prevalence is projected to rise by 20% in the next decade.[90] While the pathology of NDs is complex and multifactorial, protein aggregation and a loss of homeostasis in oxidative and inflammatory processes in the brain have been identified as key causative factors. Interestingly, the prevalence of NDs among the Asian population is much lower than the Western population, and it has been indicated that this phenomenon

may be related to differential consumption of spices, including cinnamon.[61] As such, many investigations have been inspired to determine the neuroprotective role of cinnamon in these diseases.

Neuroinflammation

Neuroinflammation is a mechanism common to the progression of several neurodegenerative diseases including AD, PD, and Multiple Sclerosis.[91] Progressive neuronal damage and various disease-specific stimuli, such as protein aggregates or toxins, can lead to chronically active microglia, which are the immune cells of the central nervous system.[21] While microglial activation plays an important role in the CNS through scavenging foreign compounds, a neurodegenerative environment creates overactive microglia and causes a detrimental cycle of prolonged and uncontrolled inflammation that promotes neurodegeneration.[37] The potential inhibition of neuroinflammation by cinnamon and its bioactive compounds was investigated using a lipopolysaccharide (LPS)-activated microglia culture assay.[61] Results of this study indicated that cinnamon suppresses the production of the pro-inflammatory compounds nitric oxide, TNF-α, IL-1β, and IL-6 in the activated microglia and the activation of the transcription factor, NF-κB, required for their expression.[61] In order to determine the bioactive compounds in cinnamon contributing the anti-inflammatory effect, an assay was performed with pure compounds and revealed cinnamaldehyde as the most potent inhibitor inflammation, followed by 2-methoxycinnamaldehyde, α-methyl cinnamaldehyde, eugenol, and cinnamyl alcohol. Similar effects on attenuating the inflammatory response were observed with sodium benzoate, a major metabolite of cinnamon. In mouse microglia with LPS-induced inflammation, sodium benzoate inhibited the expression of pro-

inflammatory compounds (IL-1B and TNF-a), inducible nitric oxide synthase, and several surface markers (CD68, CD11c, and CD11b). Additionally, they found that sodium benzoate inhibited the DNA-binding capability of NF-kB, which may explain the anti-inflammatory effects observed.[91]

Tau Aggregation

Cinnamon has also shown to attenuate disease-specific pathologies. AD is characterized by intraneuronal neurofibrillarly tangles (NFTs) and the deposition of amyloid-beta (Aβ) plaques extracellularly in the brain. *In vitro,* cinnamon extract has shown to significantly inhibit tau aggregation.[92] Proanthocyanidins possess structural and chemical properties that facilitate their biologically activity; in particular, they have a high affinity towards binding unstructured proteins, particularly those rich in proline. Physical studies revealed they can bind the primary chain of peptides via hydrophobic interactions and hydrogen bonding with either the prolyl ring or the carbonyl moiety of a prolyl linkage.[92] Since the tau protein has regions with high concentrations of proline and exhibits little structure, it was hypothesized that proanthocyandins may be the key compounds in cinnamon contributing to the inhibition of tau aggregation. As such, an A-linked proanthocyandin trimer was identified by reverse phase high-performance liquid chromatography (HPLC), purified, and tested for tau aggregation inhibition. Results indicated a significant inhibition compared to control; however, the inhibition was four times less than the whole cinnamon extract, indicating that the A-linked proanthocyanidin trimer was not the only compound in cinnamon with inhibitory activity. Since repeating units of epicatichin constitute the structure of the A-linked proanthocyanidin trimer, pure epicatechin was then tested for inhibitory activity; however, there was no inhibitory

activity observed. To determine if the collective total of proanthocyanidins in cinnamon were responsible for the inhibitory effect, the researchers examined tau aggregation treated with cinnamon extract void of proanthocyanidins. The cinnamon extract was incubated with polyvinylpyrrolidone (PVP), a polyproline structural analog that binds tightly to proanthocyanidins with high specificity, and was used to test the aggregation of tau. Results revealed that the cinnamon extract treated with PVP retained 20% of the inhibitory activity of the whole cinnamon extract. HPLC analysis confirmed the extract was void of proanthocyanidins and displayed a major peak that was identified as *trans*-cinnamaldehyde; a subsequent experiment was performed and indicated that *trans*-cinnamaldehyde also showed significant inhibitory activity. While cinnamon, proanthocyanidins, and cinnamaldehyde inhibited the aggregation of tau, it is unclear if tau aggregation is a protective measure against cellular toxicity; soluble oligomers of tau have previously been associated with toxicity. As such, the products resulting from the inhibition of tau aggregation were tested for toxicity to rat hippocampal neurons in culture. The products resulting after treatment of tau with cinnamon extract, A-linked proanthocyanidin trimer, and cinnamaldehyde in aggregatory conditions were administered to hippocampal neurons at 2 and 5 μM, since the concentration of tau within a neuron is estimated to be 2 μM. In each case, no toxicity was observed.

Amyloid Beta-protein Formation

The Aβ plaques that are characteristic of AD form as a result of the abnormal processing of the amyloid precursor protein (APP) to yield an Aβ peptide, typically containing 40, 42, or 43 amino acids. The Aβ peptides are released into the extracellular environment result have a high tendency to coalesce into insoluble fibrils and aggregate

into plaques. The presence of these plaques is toxic; they promote oxidative stress, disrupt synaptic transmission, and cause inflammation.

Cinnamon extract has demonstrated inhibition of Aβ oligomer formation *in vitro* and *in vivo*.[63] Initially, the morphology and kinetics of Aβ fibril formation were investigated to determine the mechanism by which cinnamon may acting in the amyloidgenic pathway.[63] $A\beta_{42}$ was incubated with the cinnamon extract at increasing concentrations.[63] Separation of the reaction mixtures by SDS-PAGE followed by a western blot analysis indicated a dose-dependent inhibition of the Aβ oligomer formation. The tendency of the Aβ oligomers to form fibrils was also examined; $A\beta_{40}$ and $A\beta_{42}$ oligomers were incubated with increasing concentrations of the aqueous cinnamon extract and monitored for nine days. Results showed that the oligomers incubated with cinnamon showed a delay in fibril formation compared to control. The Aβ fibrils that formed were also found at lower levels and smaller in diameter and length for the oligomers that were treated with cinnamon. The study then investigated the effects of the cinnamon extract on Aβ-mediated cytotoxicity in a cultured cell line. Rat neuronal PC12 cells were treated with $A\beta_{42}$ fibrils, $A\beta_{42}$ oligomers, or $A\beta_{40}$ oligomers and various concentrations of the cinnamon extract. Cell viability was monitored, and results revealed that cinnamon dose-dependently inhibited the Aβ-mediated cytotoxicity.

The *in vitro* effects observed were then examined *in vivo*. First, a transgenic model of *Drosophila melanogaster* expressing the human Aβ42 in their nervous system were fed either regular Drosophila medium or medium containing .75 mg/ml of the cinnamon extract. The AD flies that were not supplemented with cinnamon showed a lifespan of 16 days, which was significantly lower than the control group (flies that did

not express Aβ42), which lived for 25 days; however, AD flies that were treated with cinnamon showed a lifespan that was not significantly different from the control group. The locomotive defects in the AD flies were also ameliorated with cinnamon supplementation. At days 4-10, the AD flies consuming cinnamon showed a climbing ability nearly identical to the control flies, whereas the AD flies that were not consuming cinnamon showed a climbing ability of only 30-60%. Subsequent analysis of the brains of the flies revealed the presence of Aβ tetramers in AD flies that were not consuming cinnamon, which were not present in the cinnamon-treated AD flies. Additionally, a 20% increase in Aβ monomers was observed in the cinnamon treated AD flies when compared to AD flies that were not consuming cinnamon. These results indicate that cinnamon supplementation resulted in a significant phenotypic recovery of the AD flies, possibly by inhibiting the formation of Aβ oligomers and fibrils.

These effects were then examined in another *in vivo* assay using an aggressive AD transgenic mouse model that express five familial AD mutations. The AD model mice were administered 100μg/ml of the cinnamon extract in their water for 120 days, and monitored for changes in cognitive function and the presence of Aβ oligomers and plaques. Results showed a significant improvement in memory for the cinnamon-treated AD mice compared to the mice that were not treated with cinnamon. In fact, the cinnamon-treated AD mice showed a cognitive performance almost identical to the non-transgenic control mice. After the behavioral assays were completed, the mice were sacrificed in order to extract the soluble and insoluble Aβ fractions from their brains. Analysis revealed remarkable differences between the cinnamon treated AD mice and the non-treated AD mice. There was a 60% reduction in the levels of the Aβ oligomers in the

cinnamon treated AD mice, the number and average size of the Aβ plaques in the cinnamon-treated mice was reduced, total plaque load was 42% less in the cinnamon-treated mice, and the total insoluble Aβ fractions were reduced by 35% in the cinnamon-treated mice compared to the AD mice that were not consuming cinnamon. In effect, this data indicates that cinnamon supplementation improved cognitive performance and reduced Aβ deposition in the transgenic AD mice.[63]

A-synuclein

PD is the second most common neurodegenerative disease in the world, affecting over 1% of individuals over 65 years old.[93] Pathologically, PD is characterized by the progressive degeneration of dopaminergic neurons and the presence of Lewy body inclusions in the brain. The primary protein aggregate in Lewy bodies is α-synuclein (α-syn). For unknown reasons, in PD, α-syn monomers coalesce into oligomers and subsequently aggregate into insoluble amyloid fibrils, a phenomenon that causes inflammation, altered storage and release of dopamine, gene expression dysregulation, and negative effects on cell membrane and proteasomal function.[21,93] Cinnamon has shown to inhibit the oligomeric and fibrillar assembly of α-syn *in vitro* and *in vivo*.[93] The study first investigated the influence of cinnamon extract on α-syn formation. The protein was incubated with increasing concentrations of cinnamon extract and the fibrillation process was observed for 5 days; results suggested a significant dose-dependent inhibition of fibrillation. Interestingly, when cinnamon extract was added to pre-formed α-syn fibrils, a reduction in the amount of fibrils was observed, indicating that cinnamon extract was promoting the disassembly of the α-syn fibrils. The investigation continued with an *in vivo* experiment using a *Drosophila melanogaster* fly model that had been

genetically manipulated to over-express the mutated A53T α-syn gene in their nervous system. After 9 days, 87% of the wild type flies showed climbing ability, whereas only 60% of the PD model flies showed climbing ability; however, PD model flies that were supplemented with cinnamon (.75 mg/mL) showed 78% climbing ability. The most significant effect of cinnamon supplementation in climbing ability was observed on day 19: PD model without cinnamon (30%), PD model consuming cinnamon (56%), and wild type (66%). Quantification of α-syn accumulation in the PD model fly brain was subsequently carried out by immunostaining the brains with an anti α-syn antibody; analysis revealed a significant decrease of the α-syn aggregates in the flies that had been treated with cinnamon.

Diabetes

Epidemiological studies have revealed a significant correlation between T2DM and the development of dementia, mild cognitive impairment, and AD.[94] Around the year 2000, this concept gained much interest in the scientific community, particularly with the dramatic increase in the rates of T2DM, obesity, and AD.[94] By 2005, a wealth of data was available and elucidated several interrelationships among these diseases: brain insulin resistance and deficiency occurs progressively in AD; experimental T2DM and obesity animal models show cognitive impairment; insulin signaling is required for neuronal survival; experimentally induced brain insulin resistance and deficiency causes cognitive impairment and neurodegeneration; and clinical trials testing insulin sensitizer agents showed improved cognitive function in AD patients.[94] Since numerous studies indicate that cinnamon can improve peripheral insulin sensitivity in T2DM and delay neurodegenerative processes as previously discussed, it can be hypothesized that

cinnamon may ameliorate the pathological relationship between T2DM and dementia. Anderson et al investigated this hypothesis by studying rats fed a Hfa/Hfr diet to cause insulin resistance, with (cinnamon group) or without (control group) cinnamon (20g cinnamon/kg diet), and measuring behavioral changes and mRNA expression of genes associated with insulin signaling and AD.[47] Results showed that cinnamon prevented the decreased peripheral insulin sensitivity observed in the control group, in congruence with studies previously discussed. The cinnamon group also showed more activity in a Y maze and less anxiety in an elevated plus maze assay. A down regulation of GLUT1 and GLUT3 in the hippocampus and cortex was observed in the control group, but was not seen in the cinnamon group. Levels of mRNA encoding glycogen synthase kinase-3 (GSK-3), a downstream target in the insulin-signaling pathway associated with Aβ deposition and NFT formation, were increased in the control group but not the cinnamon group.[47] mRNA levels of protein kinase B, a key enzyme in the insulin-signaling pathway, were significantly increased in the cinnamon group compared to the control group. Additionally, the control group showed increases in AD associated mRNA expression, including PTEN, APP, and Tau, which were not observed in the cinnamon group. This data suggests that cinnamon alleviated the negative impacts of insulin resistance on cerebral insulin signaling, behavior, and risk for AD.[47]

Previous Research

Effect of Cinnamon Extract on *In Vivo* Stress Response Signaling

In our previous research, the effects of (1 mg/ml, 5 mg/ml, and 10 mg/ml) of an aqueous extract of cinnamon on the insulin/ insulin like (INS/ILS), nuclear factor-E2-

related factor 2/ Kelch-like ECH-associated protein (Keap1)- antioxidant response element (Nrf2/ARE), Hypoxia inducible factor (HIF)/Apoptosis, heat shock protein/ unfolded protein response (HSP/UPR) and mitogen activated protein kinase (MAPK), were evaluated *in vivo* in the nematode *C. elegans*.

INS/ILS Signaling Pathway

The INS/ILS signaling cascade is a critical pathway required for homeostasis among the basic functions of cells, including energy metabolism, cell growth, mitogenesis, and apoptosis.[95] Accumulating evidence suggest a prominent role of the targets in the INS/ILS signaling pathway in human pathology, including diabetes, aging, cancer, and neurodegenerative diseases.[95] The effects of insulin and IGF promote glucose metabolism, cell growth, and proliferation; however, INS/ILS signaling also results in a decrease in the cellular stress response capability via inhibition of the transcription factor Forkhead Box O (FOXO). In previous experiments, it was found that cinnamon treatment in *C. elegans* resulted in an overall upregulation in the INS/ILS signaling pathway. Interestingly, relatively consistent levels of the redox-sensitive antioxidant enzyme catalase (*ctl* in *C. elegans*) (1mg/mL: RF=1.008, p=.380; 5mg/mL: RF=1.022, p=.187; 10mg/mL: RF=.909, p=.032) suggested that cinnamon treatment resulted in physiologic redox homeostasis, despite an upregulation in INS/ILS signaling.[96] As such, it was hypothesized that cinnamon may modulate other pathways involved with stress response signaling, which was investigated in subsequent experiments

Nrf2/ARE Signaling Pathway

Oxidative stress is a pathological state implicated in the initiation and progression of numerous diseases, including neurodegenerative diseases. A major protective mechanism against oxidative stress is the activation of the transcription factor, Nrf2, which enables the expression of antioxidant and detoxifying enzymes that can assist in maintaining or restoring redox homeostasis.[97] In *C. elegans,* it was found that cinnamon treatment resulted in an overall upregulation in the Nrf2 pathway in a dose-dependent manner; the levels of Nrf2 (1mg/mL: RF=1.13, p=.001; 5mg/mL: RF=1.435, p=.002; 10mg/mL: RF=1.680, p=.000) and several downstream genes, including glutathione disulfide reductase, (1mg/mL: RF=1.13, p=.000; 5mg/mL: RF=1.426, p=.000; 10mg/mL: RF=1.168, p=.119), thioredoxin (1mg/mL: RF=1.138, p=.000; 5mg/mL: RF=1.729, p=.000; 10mg/mL: RF=2.141, p=.169), and thioredoxin reductase (1mg/mL: RF=1.959, p=.000; 5mg/mL: RF=2.948, p=.000; 10mg/mL: RF=3.405, p=.000) were significantly higher when compared to control.[96] As the Nrf2 pathway is critical for protecting cells against oxidative stress, the activation of this pathway by cinnamon may offer significant benefits in pathological conditions such as neurodegenerative diseases.

HIF-1 Signaling Pathway

The effects of cinnamon on several genes important in HIF-1 signaling, apoptosis, and autophagy regulation were also evaluated. Hypoxia is a condition that occurs in numerous pathological conditions, such as diabetes, inflammatory disorders, cancer, and Alzheimer's disease.[98] Hypoxic stress must be rapidly resolved in cells to avoid detrimental imbalances in their energy metabolism.[98] As such, hypoxia induces a

signaling pathway that is dependent upon the activation HIF-1.[98] It was found that cinnamon resulted in a marginal increase in HIF-1 signaling (1mg/mL: RF=.936, *p*=.040; 5mg/mL: RF=1.429, *p*=.010; 10mg/mL: RF=1.972, *p*=.000).[96] For apoptosis, however, the effects of cinnamon were inconsistent. It was indicated that fractionation of cinnamon may be beneficial in neurodegenerative diseases, since different fractions may have varying concentrations of autophagy and apoptotic proteins.

HSP/UPR Pathways

Accumulation and/or aggregation of unfolded proteins lead to intracellular toxicity and apoptosis. Consequently, it is a pathology associated with several diseases, including cardiovascular and neurodegenerative diseases.[99] As such, cells are equipped with important mechanisms to protect against protein toxicity, including heat shock proteins and the unfolded protein response (UPR). In response to cinnamon treatment in *C. elegans*, an increase in the expression of genes related to heat shock proteins was observed, including a 16 kD heat shock protein (*hsp16.2* in *C. elegans*) (1mg/mL: RF=.426, *p*=.034; 5mg/mL: RF=1.230, *p*=.042; 10mg/mL: RF=1.216, *p*=.000) and a gene share promoter region, *phsp* (1mg/mL: RF=2.339, *p*=.000; 5mg/mL: RF=3.417, *p*=.000; 10mg/mL: RF=4.048, *p*=.000).[96] This data indicated that cinnamon enhanced the capability of cells to handle misfolded proteins. Interestingly, genes involved in the UPR were found to be downregulated when compared to control. To see if the UPR pathway might be blocked, a gene that is expressed when the UPR is blocked, *abu-1*, was evaluated, which was found to be downregulated when compared to control; as such, it is suggested that UPR was not blocked and there was less ER stress occurring in cinnamon-treated worms.

MAPK Signaling Pathway

The mitogen-activated protein kinases (MAPKs) mediate the communication of extracellular signals to responses within the cell. The effect of cinnamon on several genes important in MAPK signaling pathways was previously evaluated. It was found that cinnamon treatment in *C. elegans* did not result in an overall increase in MAPK signaling; rather, just an increase in p38 MAPK (*pmk-1*) gene expression was observed (1mg/mL: RF=1.146, *p*=.144; 5mg/mL: RF=1.579, *p*=.001; 10mg/mL: RF=1.646, *p*=.007).[96] As reported, our previous studies indicated an upregulation in the INS/ILS pathway in response to cinnamon, but disturbances in redox balance were not observed. It has been shown that p38 MAPK can phosphorylate and increase the activity of Nrf2, which would promote the expression of important antioxidant enzymes and thus contribute to redox homeostasis.[52]

TGF-β Signaling Pathway

The transforming growth factor-β (TGF-β) signaling pathway consists of a diverse group of developmental elements and growth factors involved in the regulation of cell proliferation and differentiation. The role of the TGF-β signaling pathway in neurodegenerative diseases has been controversial. Downregulation of key components of the pathway have been reported in the brains of Alzheimer's disease patients; however, overactivation of the pathway is associated with amyloid β plaque deposition and impaired clearance. In response to cinnamon, a marginal upregulation of TGF-β levels was observed (1mg/mL: RF=.837, *p*=.000; 5mg/mL: RF=1.017, *p*=.089; 10mg/mL: RF=.131, *p*=.005), which may have benefits in neurodegenerative diseases; however,

careful regulation of this pathway and proper concentration of cinnamon are important factors.[96]

Effect of Cinnamon on Neurodegenerative Disease Like Symptoms

The effects of varying concentrations of an aqueous cinnamon extract on models of Alzheimer's and Parkinson's disease symptoms were evaluated *in vivo* and *in vitro*.

Alzheimer's Disease

To investigate the effects of cinnamon on Alzheimer's disease like symptoms, a transgenic strain of *C. elegans* containing a mutation that expresses the human amyloid β protein in the body and muscle of the worm in response to heat shock was used. An aqueous extract of cinnamon was added to the treatment plates at a concentration of 0.1%, and the time post-induction in which 50% of worms were paralyzed (PT_{50}) was calculated. However, it was reported that cinnamon treatment did not significantly influence the time to PT_{50} when compared to control.

Parkinson's Disease

Dysregulation in the maintenance of extracellular concentrations of the primary excitatory neurotransmitter, glutamate, can lead to excessive activation of the NDMA receptor, which can result dopaminergic cell death.[15] The effects of an aqueous extract of cinnamon on L-glutamate (LG) and NMDA induced excitation neurotoxicity in chick primary neuronal retina culture were previously evaluated. It was reported that cinnamon, treatment protected against glutamate-mediated excitation neurotoxicity; after 48 hours, cell viability in the neural retina culture treated with 0.1% (v/v) and 0.25% (v/v) were

121% (p <0 .05) and 128% ($p < 0.05$), respectively, compared to control. Studies have shown that activating the insulin signaling pathway has neuroprotective effects in the nigrostriatal DA system; as such, the upregulation in the insulin signaling pathway we previously observed may have contributed to the neural retinal cell protection by cinnamon.[100] Additionally, TGF-β signaling has anti-apoptotic properties and has shown to reduce overexcitation of the NDMA receptor.[101] Therefore, the increase in TGF-β signaling in response to cinnamon may have also contributed to the neuroprotective effect.

The effects of cinnamon on MPP^+ induced dopaminergic neurodegeneration were also evaluated previously. MPP^+ is the active metabolite of the neurotoxin, MPTP, that is known to selectively damage dopaminergic neurons and induce severe Parkinson's disease symptoms in humans. In *C. elegans*, MPP^+ also damages dopaminergic neurons and causes similar symptoms, including paralysis and increased mortality. To investigate, worms were administered MPP^+ and cinnamon at a concentration of 0.1% (for treatment worms), and the mobility of the worms was scored until all worms were paralyzed. However, there was no significant difference between the time to PT_{50} among treatment and control worms.

Gap in Knowledge

Based on the literature and results from investigations previously conducted in our lab, it is evident that cinnamon has potential as a neuroprotective agent. However, there are several limitations that prevent a comprehensive understanding of cinnamon's mechanism of action and therapeutic efficacy in neurodegenerative diseases. The primary

limitations of the current evidence relate to the complex and multifactorial nature of neurodegenerative diseases and the multifarious phytochemical composition of cinnamon, which have yet to be fully considered in investigations regarding cinnamon's neuroprotective potential.

First, the majority of studies have been conducted using a whole cinnamon extract. While this has provided evidence that cinnamon exerts neuroprotective effects, knowledge regarding the actions of specific compounds underlying cinnamon's bioactivity is limited. Cinnamon is a source of a vast and diverse collection of phytochemicals, which may have overlapping, complementary, or antagonistic neuroprotective functions; therefore, the identification of key compounds contributing to the observed beneficial effects is essential to elucidating and maximizing cinnamon's therapeutic potential. Secondly, most *in vivo* studies have investigated the effects of a whole cinnamon extract on alleviating phenotypic aberrations in neurodegenerative disease models. However, phenotypic symptoms of neurodegenerative diseases manifest as a result of complex interactions among several pathogenic factors occurring upstream; therefore, this approach is too simplistic to assess how cinnamon modulates the disease process. In fact, very few studies have investigated the biochemical and molecular mechanisms by which cinnamon exerts neuroprotective effects; those that have were conducted *in vitro*, which provides preliminary evidence regarding cinnamon's actions, but leaves a gap in knowledge regarding its mechanistic actions *in vivo*. Moreover, there are even fewer studies that have characterized the biological functionality of specific compounds contributing to cinnamon's bioactivity. As such, a more comprehensive evaluation of the molecular mechanisms behind cinnamon bioactives *in vivo* in

neuroprotection is imperative to understanding the therapeutic potential of cinnamon for neurodegenerative diseases.

Objective

The objective of this study was to elucidate the neuroprotective effects of bioactive compounds in cinnamon using *in vivo* model systems.

Specific Aim 1: Evaluate the effects of the polar acidic and polar neutral fractions of cinnamon on the expression of important genes relevant to stress response signaling pathways and on experimentally induced neurodegeneration in *C. elegans*

Specific Aim 2: Investigate the effects of cinnamon bioactives on human amyloid-beta$_{1\text{-}42}$ induced paralysis and chemically-induced Parkinson's disease-like neurotoxicity in *C. elegans.*

Specific Aim 3: Evaluate the molecular effects of cinnamon bioactive compounds on locomotion and lifespan in transgenic *Drosophila* models for Alzheimer's and Parkinson's disease.

CHAPTER II

PRELIMINARY INVESTIGATION OF CINNAMON FRACTIONS ON STRESS RESPONSE SIGNALING AND NEURODEGENERATION IN *C. ELEGANS*

Introduction

To provide a more comprehensive understanding of the actions and therapeutic efficacy of cinnamon in neurodegenerative diseases, we expanded our research to include the *in vivo* effects of bioactive compounds in cinnamon on stress response signaling and on neurodegenerative disease like symptoms. To this effect, we have evaluated the actions of two polar fractions of cinnamon on important signaling pathways relevant to neurodegenerative diseases and on a *C. elegans* model of Parkinson's disease.

Methods

Polar Acidic and Polar Neutral Fractions

Aqueous extracts of cinnamon (Penzey's Ceylon Cinnamon, ground, Sri Lanka) were prepared by heating 1.5g of the spice in 30 ml of distilled water for 30 minutes at 60°C. The mixture was vacuum-filtered through Whatman filter paper and filter sterilized. Solvent fractionation (represented in Figure 1) was then used to fractionate the aqueous extract based on polarity to yield polar acidic (PA), polar basic (PB), and polar neutral (PN) fractions.

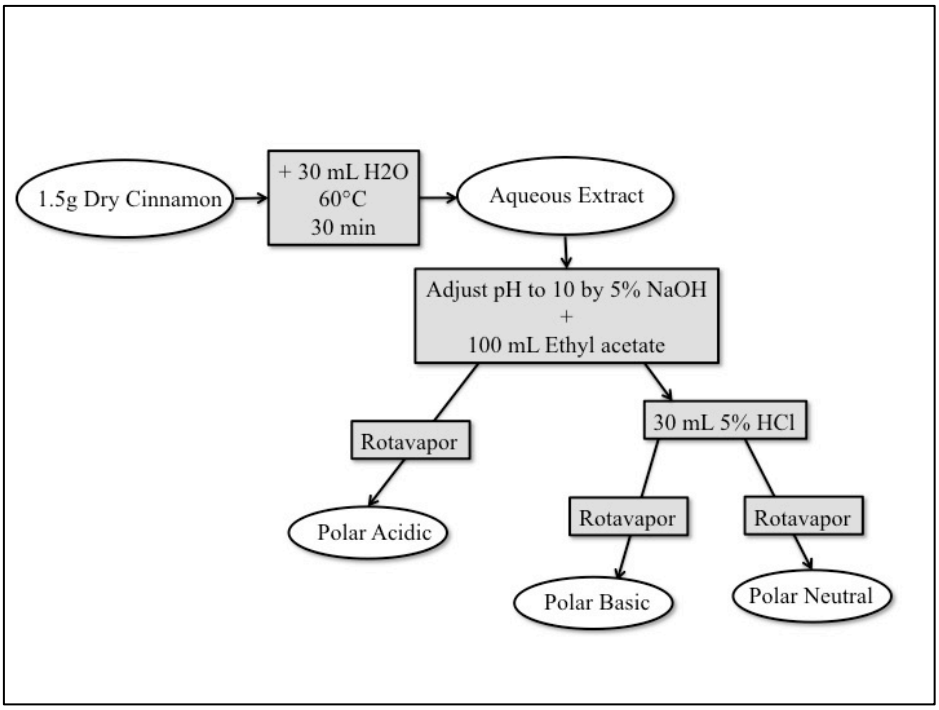


Figure 1. Method of Solvent Fractionation of Cinnamon to Separate Samples of Varying Polarity and Charge.

C. elegans Strains and Maintenance

Wild type and transgenic strains of *C. elegans* were obtained from the Caenorhabditis Genetics Center (CGC) (University of Minnesota, Minneapolis, MN). Worms were kept at 20 °C and maintained on 35 mm culture plates with Nematode Growth Medium (NGM) (1.7% agar, 0.3% NaCl, 0.25% Peptone, 1 mM CaCl, 1 mM MgSO4, 5 mg/l cholesterol, 2.5 mM KPO4 at 16–20 °C, Brenner, 1974). A peristaltic pump was used to pour the NGM into the culture plates (4.5 ml for 35 mm plates). After solidifying for 36 h, the NGM culture plates were then inoculated with 50 µl of Escherichia coli OP50 (CGC, University of Minnesota, Minneapolis, MN) overnight cultures and placed in a 37 °C incubator for 8 h. To maintain the strains of C. elegans, 2-3 young adult worms were picked onto fresh NGM plates every 4-7 days.

Age Synchronization

Prior to each assay, the worms were age synchronized. Onto each NGM culture plate, ten worms at the L4 strains (F0) were transferred and allowed to progress to adulthood and lay eggs. The adult worms were then removed from the plates and the remaining eggs hatched (F1) and were allowed to grow to the L4 stage. The L4 worms of the F1 generation were then transferred to fresh NGM plates and allowed to reach adulthood and lay eggs. Adult worms were again removed from the plates, and the resulting progeny (F2) were used for experimentation.

In Vivo Stress Response Signaling Studies in *C. elegans*

The effects of the polar acidic (PA) and polar neutral (PN) fractions of cinnamon on the modulation of stress response signaling pathways important in neurodegenerative diseases were evaluated using transgenic *C. elegans* models. Specifically, changes in the expression of FOXO, Nrf2, Sirtuin 2, and several of their target downstream genes (Table 2) in response to the PA and PN fractions of cinnamon were investigated.

Worms were age synchronized on 35mm culture plates with Nematode Growth Medium (NGM) (1.7% agar, 0.3% NaCl, 0.25% Peptone, 1M CaCl, 1M MgSO4, 5mg/mL Cholesterol in ethanol, 1M KPO4) inoculated with 25 µl of *E. coli* OP50 overnight cultures. L1 worms (F_2) generation were collected from the plate by washing with S-complete liquid media and transferred into a sterile falcon tube by pipette. Five mg/ml of concentrated OP50 and 0.1% (v/v) cinnamon fractions (PA and PN) were added to the falcon tube; S-complete was added in place of cinnamon fractions for control. A total volume of 50 µl of the mixture was transferred to each well of a sterile 96 well plate

and incubated at 20°C. Upon maturation to the L4 stage, the worms were transferred onto a 2 ml layer of solidified 1% phytagel in a 35mm culture plate for imaging.

Table 2. List of *C. elegans* Strains Used to Evaluate the Effects of Polar Acidic and Polar Neutral Fractions of Cinnamon Genes Relevant to the FOXO, Nrf2, and Sirtuin 2 Pathways.

Strain	Gene	Human Homolog	Wormbase ID
GR1352	*Daf-16a*	Forkhead Box O (FOXO)	WBGene00000912
LG326	*Skn-1*	Nrf2	WBGene00004804
CF1553	*Sod-3*	Iron/Manganese superoxide dismutase	WBGene00004932
CF2266	*Ins-7*	Insulin/Insulin growth factor (IGF)-1-like peptide	WBGene00002090
BC13632	*Sod-4*	Copper/Zinc superoxide dismutase	WBGene00004933
CL2070	*Hsp16.2*	16-kD heat shock protein; αB crystalline driven (D)	WBGene00002016
CL2166	*Gst-4*	Glutathione S-Transferase (GST)	WBGene00001752
UL3294	*Sir-2.1*	Sirtuin 2	WBGene00004800
UL3351	*Sir-2.1*	Sirtuin 2	WBGene00004800
OE3010	*Trx-1*	Thioredoxin (TRX)	WBGene00015062
GA800	*ctl*	Catalase (ctl-1, ctl-2 and ctl-3)	WBGene00000831

Five to ten microliters of 25 mM sodium azide solution in Magnesium buffer was added to immobilize the worms and direct *in vivo* fluorescence imaging of GFP was performed using the Nikon SMZ1500 fluorescence microscope with a Ri1CCD camera

(Nikon, Japan). Relative fluorescence, with respect to control, was quantified from the corrected total fluorescence of randomly selected L4 worms using the National Institute of Health's Image J software (Sigma-Aldrich, St. Louis, MO). Fold change in relative fluorescence was calculated between controls and each treatment.

MPP+ Induced Neurotoxicity in *C. elegans*

The effects of the PA and PN fractions of cinnamon in ameliorating Parkinson's disease like symptoms were investigated in *C. elegans*. The neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces dopaminergic neurodegeneration and Parkinson's disease in humans. MPTP is lipophilic and is therefore able to cross the blood-brain barrier. Within cells, MPTP is metabolized to the toxic compound, 1-methyl-4-phenylpyridinium (MPP^+), which is taken up by dopaminergic neurons. Once in the neurons, MPP^+ causes the inactivation of complex 1 of the electron transport chain in the mitochondria. As a result, there is a depletion of ATP levels and a significant increase in free radical production, which causes damage and death of the neurons. In *C. elegans*, MPP^+ also selectively damages the dopaminergic neurons and causes paralysis and increased mortality.

Wild type *C. elegans* was obtained from the CGC. The neurotoxin, MPP^+, was used to induce dopaminergic neurodegeneration and Parkinson's disease like symptoms. All strains were maintained as previously mentioned.[102] The worms were age synchronized; adult *C*. *elegans* were picked onto NGM plates, allowed to lay eggs, and removed. Upon hatching and maturation of the eggs to L1 generation, the worms were washed by pipette with S-basal liquid media (0.59% NaCl, 5% 1M KPO4, 5 mg/ml Cholesterol in ethanol) into a sterile 50 ml Falcon tube. S-complete [97.7% S-basal, 1%

potassium citrate, 1% trace metals, 0.3% $CaCl_2$, 0.3% $MgSO_4$] liquid media was then added to the tube with concentrated *E. coli* OP50 yielding a 5 mg/ml food supply (Lewis & Fleming, 1995). To each well on a 96 well plate, a total volume of 50 µl was added [20µl of IC_{90} MPP^+, 30µl S-complete (5 mg/ml *E. Coli* OP50, and 17-23 worms)]. For treatment worms, the PA and PN fractions of cinnamon were added to the S-complete at a concentration of 0.1% to the 30 µl portion of well volume along with the *E. coli* OP50; cinnamon PA and PN fractions were prepared as mentioned previously. To ensure statistical validity, a minimum of eight wells with at least 100 worms were used.

After 24 hours incubation the wild type (N2) worms were scored for mobility, worms were placed in three categories, wildtype movement, uncontrolled movement, or paralyzed. The worms were scored every subsequent 12 hours until all worms were paralyzed.

Statistical Analysis

A one-way ANOVA was used to evaluate the statistical signifigance ($p<0.05$) for gene expression studies and for the MPP+ induced neurotoxcity assay in C. elegans. The Breslow-Gehan-Wilcoxon test was used to measures differences in survival for the Alzheimer's disease assay. Lifespan survival curves were determined and the statistical differences were evaluated using the Log rank test (Mantel Cox). Each test used Graphpad prism Software 6.0 (GraphPad Software, Inc. San Diego, CA).

Results

In Vivo Stress Response Signaling Studies in *C. elegans*

Overall, it was found that the bioactivity of the cinnamon fractions was less pronounced than what we observed with the whole cinnamon extract. The expression of

daf-16a, the *C.elegans* ortholog of Forkhead Box O (FOXO), did not significantly change in response to PA (RF=.95, *p*=.554) or PN (RF=1.08, *p*=.418) relative to control. In response to PA, the levels of *skn-1*, the C. elegans otholog of Nrf2, were not significantly different compared to control (RF=1.08, *p*=.418), whereas an upregulation was observed in response to treatment with PN (RF=1.45, *p*=.002). The levels of *sod-3* were downregulated after treatment with PA (RF=.72, *p*=.053), whereas relatively consistent levels were observed with PN treatment (RF=.825, *p*=.146) in comparison to control. *Sod-4* levels did not significantly change in response to PA (RF=1.02, *p*=.171); however, we observed a marginal increase in *sod-4* levels with PN treatment (RF=1.06, *p*=.007). PA treatment resulted in a downregulation in *ins-7* levels (RF=.768, *p*=.035), but the levels did not significantly change in response to PN treatment (RF=.913, *p*=.501). We observed relatively consistent levels of *hsp16.2* in response to PA treatment (RF=1.03, *p*=.185), but found an upregulation *hsp16.2* with PN treatment (RF=1.15, *p*=.001). We did not find changes in the levels of *gst-4* with either PA (RF=1.02, *p*=.750) or PN (RF=.98, *p*=.763) treatments compared to control. We evaluated two strains expressing *sir2.1*, and in each case, we saw consistent levels in response to PA (RF=.963, *p*=.227; RF=.979, *p*=.269) and marginal increases with PN treatment (RF=1.07, *p*=.045; RF=1.07, *p*=0.00). Treatment with PA did not significantly change the levels of *trx-1* (RF=1.03, *p*=.160), whereas we observed a slight upregulation in response to PN (RF=1.05, *p*=.026). For *ctl*, we observed an upregulation with both PA (RF=1.40, *p*=.031) and PN (RF=1.32, *p*=.015) treatments. (Table 3).

Table 3. Effect of Polar Acidic and Polar Neutral Fractions of Cinnamon on Fold-Change in Gene Expression Relative to Control.

Gene	PA			PN		
	RF	**SE**	***p* value**	**RF**	**SE**	***p* value**
Daf-16a	1.08	.009	.421	.95	.006	.554
Skn-1	1.08	.013	.418	1.45	.014	.002
Sod-3	.72	.010	.053	.825	.002	.146
Ins-7	.768	.004	.035	.913	.002	.501
Sod-4	1.02	.013	.171	1.06	.006	.007
Hsp16.2	1.03	.010	.185	1.15	.005	.001
Gst-4	1.02	.005	.750	.98	.007	.763
Sir-2.1	.963	.004	.227	1.07	.006	.045
Sir-2.1	.979	.007	.269	1.07	.013	0.00
Trx-1	1.03	.008	.160	1.05	.008	.026
Ctl	1.40	.015	.031	1.32	.009	.015

MPP+ Induced Neurotoxicity in *C. elegans*

After 48 hours, 92% of worms exposed to MPP^+ without cinnamon treatment were paralyzed. In worms that were exposed to MPP^+, but co-treated with 0.1% PA cinnamon, 92% paralysis was also observed after 48 hours. For worms that were treated with 0.1% PN cinnamon and MPP^+, it was found that only 65% of worms were paralyzed after 48 hours; thus, a 27% inhibition of paralysis in PN-treated worms when compared to control was observed.

Alzheimer's Disease Assay in *C. elegans*

After treatment with PA, it was found that worms had an accelerated rate of paralysis (PT50=30; p= <0.0001) when compared to control (PT50=30). However, treatment with PN conferred protection against paralysis (PT50=32; p= <0.0001), when compared to control (PT50=30).

CHAPTER III

IN VIVO NEUROPROTECTIVE EFFECTS OF CINNAMON BIOACTIVE COMPOUNDS IN *C. ELEGANS* AND *D. MELANOGASTER*

Introduction

Bioactive Compounds

Numerous investigations elucidating the effects of cinnamon on a wide range of pathologies, including cardiovascular disease, diabetes, cancer, neurodegeneration, and oxidative stress have provided insight on key bioactive compounds underlying cinnamon's health promoting effects. Evidence suggests that the bioactivity of cinnamon is largely ascribed to phenolic compounds, including cinnamic acid, cinnamate, cinnamaldehyde, and proanthocyanidins. As such, these compounds were evaluated to determine their mechanism of action and therapeutic potential in neurodegenerative diseases. A summary of these bioactive compounds and their reported health effects is represented in Table 4.

Table 4. Investigations on the Health Benefits of Bioactive Compounds in Cinnamon.

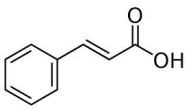
Compound	**Pathology**	**Dose/Model**	**Result**	**Reference**
Cinnamic acid 	Oxidative stress	15, 30, or 60 mg/kg in mice	↓Cyclophosphamide-induced oxidative stress (MDA) ↑SOD, CAT, GST	[70]
	CVD	37.5, 75, or 150 mg/kg in rats	↓Severity of myocardial ischemia ↓TNF-α and IL-6	[103]

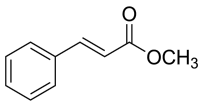
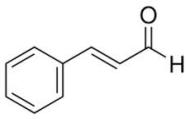
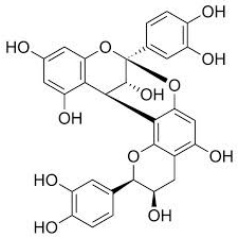
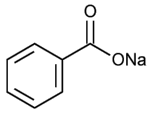
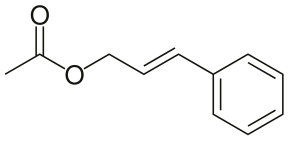
Table 4 Continued				
		Rat thoracic aorta	Relaxed aortic strips pre-contracted with phenylephrine	[104]
		HUVECs	↑ Phosphorylation of eNOS ↑NO production ↑cGMP and PKG	[104]
Ethyl cinnamate 	Oxidative stress	0.1g/100g diet in rats	↓ oxidative stress induced by high cholesterol diet (MDA) ↑CAT, Gpx	[105]
	CVD	.1g/100g diet in rats	↑HDL and ↓ hepatic TG and cholesterol levels, and HMG CoA Reductase activity in high cholesterol fed rats	[105]
Cinnamaldehyde 	Cancer	Human colon cancer cells	↑Nrf2 ↑HO-1 and ϒ-GCS ↓cellular damage from H_2O_2- induced genotoxicity	[49]
		Human hepatoma cancer cell lines Hep3B and HepG2	↓ Growth and proliferation of cancer cells ↑ Apoptosis via activation of capsase 3	104
		22.5, 45, or 90 mg/kg in rats	↓Severity of myocardial ischemia ↓TNF-α and IL-6	[103]

Table 4 Continued

		J774 A.1 macrophages	↓secretion of TNF-α and IL-1β	[106]
		Endothelial cells	↓Adhesion of of TNF-α induced monocytes to endothelial cells ↓Expression of VCAM-1 and ICAM-1 via inhibition of NFkB	[107]
	NDs	LPS-stimulated microglia	↓ NO, TNF-α, IL-1β, and IL6	[61]
		Tau	↓ Tau aggregation	[92]
Proanthocyanidins 	T2DM	200 or 300 mg/kg in diabetic mice	↓ Blood glucose levels	[2]
		Insulin-resistant HepG2 cells Normal HepG2 cells	↑Extracellular glucose uptake	[2]
	NDs	Tau	↓ Tau aggregation	[92]
Sodium benzoate 	NDs	2.5 to 10 mg/ml in mice Mouse microglia	↓ Inflammation and clinical symptoms of MS ↓ proinflammatory molecules	
Cinnamyl acetate 		LPS-stimulated RAW 264.7 cells	↓ NO production and PGE2 production	[108]

Cinnamic Acid

Cinnamic acid is a simple phenolic acid, which contains a basic chemical structure that serves as a precursor for numerous other phenolic compounds.[83] Along with proanthocyanidins, cinnamaldehyde, and coumarin, cinnamic acid has been identified as one of the primary compounds in cinnamon water extract.[109] In addition to cinnamon, cinnamic acid and its derivatives have been found in oats, whole wheat, apples, and coffee.[110] The antioxidant activity of cinnamic acid has been demonstrated in mice treated with cyclophosphamide, a common chemotherapeutic agent that causes many side effects, including oxidative stress.[70] As an adjuvant with cyclophosphamide, cinnamic acid enhanced endogenous antioxidant enzyme activity and decreased the levels of lipid peroxidation. Cinnamic acid has also shown therapeutic effects in cardiovascular disease; supplementation (37.5, 75, or 150 mg cinnamic acid/kg/d) decreased the levels of the inflammatory compounds TNF-α and IL-6 after isoproterenol-induced myocardial ischemia in rats.[103] Modulation of tumor cell growth via inhibition of NF-κB by cinnamic acid and its derivatives has also been observed.[110]

Ethyl Cinnamate

Another phenolic compound, cinnamate, has been found in cinnamon at a concentration of .48% of cinnamon bark oil.[82] Cinnamate has shown therapeutic effects in oxidative stress and cardiovascular disease; in rats fed a high cholesterol diet, cinnamate supplementation (.1g/100g of diet) showed protection against oxidative stress, as indicated by enhanced endogenous antioxidant activity and reduced lipid and protein oxidation.[105] Furthermore, cinnamate increased HDL, decrease hepatic triglyceride and

cholesterol levels, and reduced HMG-CoA reductase activity in high-cholesterol fed rats.[105] Interestingly, these effects were significantly more pronounced compared to rats treated with lovastatin. In human endothelial cells, cinnamate and its derivatives showed anti-inflammatory activity by inhibiting the TNF-α mediated expression of cell adhesion molecules.[111]

Cinnamaldehyde

Found to be the major component of cinnamon bark oil (97.7%) and key flavor compound in cinnamon, cinnamaldehyde is an α,β-unsaturated aldehyde that belongs to a class of phenolic compounds referred to as phenylpropanoids.[81] Significant evidence suggests that cinnamaldehyde is highly bioactive and contributes to many health-promoting effects of cinnamon. Previous investigations have linked the cellular effects of cinnamaldehyde to the antioxidant, anti-inflammatory, anti-diabetic, and anti-cancer activities of cinnamon.[81,112-115] In macrophages, cinnamaldehyde has shown to reduce LPS-mediated inflammation through inhibiting NF-κB transcriptional activity.[115] Doses of cinnamaldehyde (22.5, 45, or 90 mg/kg/d) reduced the levels of inflammatory compounds (TNF-α and IL-6) after isoproterenol-induced myocardial ischemia in rats.[103] This effect was accompanied by a less severe myocardial ischemia event, as indicated by a less dramatic ST-segment elevation, when compared to untreated rats.[103] Cinnamaldehyde also showed cardioprotective effects in endothelial cells; a reduction of TNF-α induced monocytes to endothelial cells and decreased expression levels of VCAM-1 and ICAM-1 were observed.[107] Cinnamaldehyde was found to be a potent activator of Nrf2 in HUVECs, which was an activity ascribed to its unique chemical properties that allow it to behave as a Michael acceptor.[49] Anti-cancer activity by

cinnamaldehyde has been demonstrated in human hepatoma cancer cell lines, in which it inhibited the growth and proliferation of cancer cells and promoted apoptosis through the activation of capsase 3.[49] Protective effects for neurodegenerative diseases have also been shown; cinnamaldehyde reduced the levels of inflammatory compounds (nitric oxide, TNF-α, IL-1β, and IL6) in LPS-stimulated microglia and reduced tau aggregation *in vitro*.[61,92] Immunomodulation by cinnamaldehyde has also been reported. In *V. vulnificu* and *S. agalactiae*-infected zebrafish, cinnamaldehyde supplementation decreased the expression levels of pro-inflammatory compounds (IL-1β, IL-6, IL15, NF-κB, COX-2, and TNF-α), and increased the levels of IL-10, an immunosuppressive cytokine with important functions in maintaining homeostasis in the immune system by reducing the production of pro-inflammatory cytokines.[116]

Proanthocyanidins

Also known as condensed tannins, proanthocyanidins are polymers of catechin and/or epicatechin, which are further classified based on linkages of the monomeric units. B-type proanthocyanidins are derived from single covalent linkages between monomeric units, whereas A-type proanthocyanidins have double linkages and an additional ester linkage between carbon 2 and carbon 4.[109] Proanthocyanidins are commonly found in apples, cocoa beans, cinnamon, blueberries, and grapes.[117] In cinnamon powder, proanthocyanidins have been identified at a concentration of 23 mg/g.[118] Proanthocyanidins are considered the primary compounds contributing to the anti-diabetic effects of cinnamon. In an *in vitro* investigation, proanthocyanidins were found to be the most effective compounds in cinnamon for reducing the misfolding of human islet amyloid polypeptide, which is a pathological factor associated with pancreatic β-cell

dysfunction.[109] *In vivo*, cinnamon has shown to lower blood glucose levels. In diabetic mice, proanthocyanidins were identified as the major compounds in cinnamon contributing to the observed glucose-lowering effect.[87] Additionally, in an investigation on tauopathies, proanthocyanidins were identified as a major bioactive compound in cinnamon contributing to the inhibition of tau aggregation.[92] Subsequent isolation and testing for inhibitory activity confirmed that the purified proanthocyanidins were effective in inhibiting tau aggregation.[92]

Sodium Benzoate

While most commonly found in foods as an added preservative, sodium benzoate is also found naturally in foods including apricots, cranberries, cinnamon, and apples. In cinnamon, benzoic acid is formed from the oxidation of the simple phenolic compound, cinnamic acid, and is primarily found as a sodium salt, referred to as sodium benzoate. In addition to well-documented safety and low risk of toxicity, sodium benzoate has shown to confer several health benefits. In stimulated human peripheral blood mononuclear cells, sodium benzoate suppressed the Th1-type immune response by reducing the activity of the pro-inflammatory cytokine, IFN-gamma.[119] In a mouse model, treatment with sodium benzoate significantly reduced the clinical symptoms of experimentally induced multiple sclerosis after just four days.[120] Additionally, the expression of two proteins (E- and P-selectins) important for the sticking of microglial adhesion molecules to the vasculature was reduced in the central nervous system of these mice after treatment with sodium benzoate. Supplementation with sodium benzoate has also reduced the expression of proinflammatory cytokines in mouse microglia after stimulation with LPS,

Abeta peptides, IL-1B, and MPP+, important proinflammatory stimuli associated with neurodegenerative diseases.[91]

Cinnamyl Acetate

Cinnamyl acetate has been found to be a major component of cinnamon fruit and leaf oil that has shown anti-inflammatory activity.[108,121] *In vitro*, cinnamyl acetate reduced the production of nitric oxide in macrophages after stimulation with LPS.[108]

To further elucidate the mechanism of action underlying the neuroprotective effects of cinnamon, this study investigated five pure bioactive compounds on experimentally induced neurodegeneration in *C. elegans* and *Drosophila*. More specifically, *C. elegans* was used to evaluate the efficacy of bioactive compounds in abrogating chemically-induced neurotoxicity and human amyloid-beta$_{1-42}$ induced paralysis. In *Drosophila*, the molecular effects of cinnamon bioactives on lifespan and behavior in transgenic and chemically-induced models of Alzheimer's and Parkinson's disease were investigated.

Methods

C. elegans Strains and Treatment

Wild type and transgenic *C. elegans* (CL4176) carrying heat shock protein (hsp) promoter fusions of the human amyloid-β-42 peptide were obtained from the *Caenorhabditis* Genetics Center (CGC) (University of Minnesota, Minneapolis, MN). Worms were grown on 35 mm culture plates with Nematode Growth Medium (NGM) (1.7% agar, 0.3% NaCl, 0.25% Peptone, 1M CaCl, 1M MgSO4, 5mg/mL Cholesterol in

ethanol, 1M KPO4) and maintained at 20 °C (with the exception of CL4176, which was maintained at 16 °C). Using aseptic techniques, NGM was poured into the culture plates (4.5 ml for 35 mm) and allowed to solidify for 48 h. *Escherichia coli* OP50 overnight cultures (25 µl) (CGC, University of Minnesota, Minneapolis, MN) was used to inoculate the NGM culture plates, which were then incubated for 8 h at 37 °C.

An initial experiment was conducted in wild type worms to determine the concentration of bioactive compounds to be used in the subsequent assays. Cinnamon bioactives were added to each well of a 96 well plate at concentrations ranging from 0-100 µg/ml and survival was monitored every two days. Concentrations that did not exhibit a risk for toxicity and were used for the present investigation are as follows: ethyl cinnamate (25 µg/ml), cinnamic acid (15 µg/ml), cinnamyl acetate (10 µg/ml), cinnamaldehyde (20 µg/ml), and sodium benzoate (25 µg/ml).

Age Synchronization

Prior to each assay, the worms were age synchronized. Onto each NGM culture plate, ten worms at the L4 strains (F0) were transferred and allowed to progress to adulthood and lay eggs. The adult worms were then removed from the plates and the remaining eggs hatched (F1) and were allowed to grow to the L4 stage. The L4 worms of the F1 generation were then transferred to fresh NGM plates and allowed to reach adulthood and lay eggs. Adult worms were again removed from the plates, and the resulting progeny (F2) were used for experimentation.

Alzheimer's Disease Assay in Transgenic *C. elegans*

Transgenic *C. elegans* (CL4176) containing a mutation that expresses human amyloid-$\beta_{1\text{-}42}$ in the muscle tissue upon heat shock were used to model AD. The worms were kept at 16°C on 35mm NGM culture plates inoculated with 25 µl of *E. coli* OP50. Treatment plates were prepared by adding 10 ug/ml cinnamyl acetate, 15 ug/ml cinnamic acid, 20 ug/ml cinnamaldeyhde, 25 ug/ml ethyl cinnamate, or 25 ug/ml sodium benzoate to the NGM media prior to pouring; control plates were prepared without extract in the NGM media. The plates were inoculated with 25 µl of *E. coli* OP50; compounds were added to the *E. coli* OP50 at respective concentrations for treatment plates. To ensure the food supply was standardized, the plates were placed in a Strategene UV Stratalinker and subjected to a UV dose sufficient to arrest the growth of the *E. coli* OP50.[4] Age synchronized L4 worms of F_2 generation were transferred to the plates, allowed to lay eggs, and removed. When the eggs matured to L3 stage, they were transferred to a 25°C incubator to initiate the expression of amyloid-$\beta_{1\text{-}42}$. Beginning at 20 hours post-transfer, the mobility of the worms were scored every two hours until all worms were paralyzed.

MPP+ Induced Parkinson's Disease Assay in *C. elegans*

Wild type C. elegans (N2) were maintained on 35mm NGM culture plates at 20°C. Age synchronized L3 worms were transferred into a sterile falcon tube by washing with S-complete. Five mg/ml of E. coli OP50 was added to ensure a sufficient food supply; cinnamon bioactive compounds were added at a concentration of 10 µg/ml (cinnamyl acetate), 15 µg/ml (cinnamic acid), 20 µg/ml (cinnamaldehyde) and 25 µg/ml (sodium benzoate or ethyl cinnamate); S-complete was added in place of the compounds

for the control group. Forty microliters of the resulting suspensions were mixed with 10 µl of the neurotoxic molecule 1-methyle-4-phenylpyridinium (MPP+) to initiate dopaminergic neurodegeneration and facilitate symptoms reflective of PD.[2] The mobility of the wild type worms (N2) was scored every 12 hours and the total number of paralyzed worms, as measured by failure to respond to touch (via worm pick) and absence of pharyngeal pumping, was quantified after 48 hours.

Table 5. Genotype of *C. elegans* Strains Used for Experimentation.

C. elegans		
Model	**Strain**	**Genotype**
Neurotoxicity (MPP+)	N2	*Wild type*
Amyloid-β-induced paralysis	CL4176	*smg-1(cc546) I; dvIs27 X*

Drosophila Strains and Treatment

The stocks of flies used for experimentation were obtained from the Bloomington Stock Center (Department of Biology, Indiana University, Bloomington, IN, USA). Strains were reared on Formula 4-24® Instant *Drosophila* Medium (Carolina Biological Supply, Burlington, NC) and kept at 25°C and 75% humidity with a 12-hour light/dark cycle. The stock of flies was maintained in flasks with 40 ml of media, to which 60 ml of water was added. After the media solidified, flies were anesthetized with CO_2 and transferred into the fresh media. For experimental diets, 5 ml of media was provided in vials with 7.5 ml of water. A list of strains used for experimentation is listed in Table 6.

Similar to *C. elegans,* an initial evaluation was conducted to determine the concentrations of the bioactive compounds to be used for experimentation. Age-synchronized wild type flies were transferred into vials with media containing concentrations of the compounds that ranged from .5 to 100 mg/ml. Fresh media was provided every three days and survival was monitored every two days. The concentrations that did not pose a risk for toxicity and were used for subsequent experimentation include: ethyl cinnamate (0.5 mg/ml), cinnamic acid (0.5 mg/ml), cinnamyl acetate (0.5 mg/ml), cinnamaldehyde (0.5 mg/ml), and sodium benzoate (5.0 mg/ml).

Table 6. List of *D. melanogaster* Strains and Their Genotype.

Category	Strain	Genotype
Wild type	1	Canton-S
Drivers	458	P^{120}elav[C155]
	2077	w[*]; P^{120}2
Parkinson's disease	8147	w[*]; P{w[+mC]=UAS-Hsap\SNCA.A30P}40.1
	8148	w[*]; P{w[+mC]=UAS-Hsap\SNCA.A53T}15.3
Alzheimer's disease	32038	P{w[+mC]=UAS-Abeta1-42.G}1, w[*]

Amyloid-β_{1-42} Induced Neurodegeneration Assay in *Drosophila*

Strains of *D. melanogaster* developed for the UAS/GAL4 tissue-specific expression system (STR-32038 and STR-458) were used to model Alzheimer's disease. Adult flies carrying the transgene that expresses the 42 amino acid version of human amyloid-β (STR-32038) under the control of UAS were transferred into flasks (200 flies

per flask) and allowed to lay eggs; after nine days, adult flies were removed and newly emerged female flies were collected within eight hours of eclosion to ensure virginity. The female flies were then paired with at least 200 male driver flies (STR-458) that express the GAL4 transcription factor. The male offspring of the F1 generation were collected (n=120; 1-3 days old) and used for experimentation. Male flies were randomly divided into six groups and placed into vials containing Instant *Drosophila* Medium as described above. The bioactive compounds were provided in the water prior to pouring into the media. Final concentrations of the compounds provided in the experimental diets were as follows: 0.5 mg/ml of cinnamaldehyde, cinnamic acid, cinnamyl acetate, or ethyl cinnamate; sodium benzoate was added at a concentration of 5.0 mg/ml. Fresh medium was provided every three days. Survival was monitored and deaths were recorded every two days. For the molecular analysis, the same experiment was preformed with a separate set of flies, which were frozen on days 0 and 30 and stored in -80 °C until later analysis.

A-synuclein induced Neurodegeneration Assay in *Drosophila*

Parkinson's disease was modeled using strains *of D. melanogaster* developed for the UAS/GAL4 tissue-specific expression system. Flies (STR-8147) carrying the UAS-regulated transgene that expresses human mutant A30P for α-synuclein were transferred into flasks (200 flies per flask) and allowed to lay eggs; after nine days, adult flies were removed (STR-8147) and newly emerged female flies were collected within eight hours of eclosion to ensure virginity. The female flies were crossed with male driver flies (STR-458) that express the GAL4 transcription factor. The male offspring of the F1 generation were collected (n=120; 1-3 days old) and used for experimentation. Male flies

were randomly divided into six groups and placed into vials containing Instant *Drosophila* Medium as described above. The bioactive compounds were provided in the water prior to pouring into the media. Cinnamon bioactive compounds were provided in the medium at concentrations mentioned previously. Control diets were provided using standard protocol as described above. Diets were refreshed every three days. Survival was monitored and deaths were recorded every two days. A separate group of flies were subjected to the same experiment and frozen on days 0, 15, and 30 and stored in -80 C for later analysis.

Rotenone Induced Neurotoxicity Assay in *Drosophila*

Administration of rotenone in *D. melanogaster* was used to model neurotoxicity. Fly culture medium was prepared using Formula 4-24® Instant *Drosophila* Medium (Carolina Biological Supply, Burlington, NC) through methods previously described (Carolina *Drosophila* manual). Cinnamon bioactives were provided in the diets as previously mentioned. Newly eclosed wild type (STR-1) male flies (n=175; 1-3 days old) were randomly separated into 7 groups of 25 flies each. Two groups of flies served as blank (10% sucrose) and control (500mM rotenone in 10% sucrose) and were placed in vials containing 5 ml of Instant *Drosophila* Medium and 7.5 ml of sterilized water. Cinnamon bioactive compounds were added at concentrations described above for the experimental groups. Neurotoxicity was studied in the flies through exposure to the neurotoxin rotenone. Every three days, flies were starved in empty vials and subsequently transferred into vials containing a filter paper saturated with 1 ml of 10% sucrose (blank) or 1 ml of 500 mM rotenone in a 10% sucrose solution (control and experimental groups). After 24 hours, the flies were transferred into vials containing filter paper

saturated with 1 ml of sterilized water for a wash out period and subsequently transferred into vials with a fresh supply of their respective diets. The flies were monitored for survival every two days. The survival curves were plotted versus time and the groups were compared by the Kaplan-Meier method. For assessment of the expression of superoxide dismutase, catalase, and α-synuclein, a separate experiment was performed as described above, and flies were sacrificed at days 0 and 15 and stored at -80 °C for subsequent analysis.

Climbing Assay

The effects of cinnamon bioactives on climbing ability were evaluated in *Drosophila* models of rotenone-induced neurotoxicity and α-synuclein-induced neurodegeneration. Age-synchronized male and female flies (n=25; 1-3 days old) were transferred into vials with respective treatments and subjected to the same experimental conditions as previously described. Flies were transferred into a graduated cylinder without anesthetization and superimposed against a ruler for reference. The cylinder was tapped to ensure all flies were at the bottom and a picture was taken (Cannon EOS 50D 15.1 MP Digital SLR) after three seconds. This process was repeated three times on each specified time point. For the rotenone-induced neurotoxicity experimental flies, the climbing assay was performed at days 1, 5, 10, 15, and 20; for the α-synuclein-induced neurodegeneration fly model, this assay was performed every 7 days until all flies had deceased.

Sample Extraction for Biochemical Analysis

Flies that were collected in each experiment were homogenized in 1.5 ml of cold

homogenizing buffer (20mM Tris-HCl, .2 M sucrose, 2 mM $MgCl_2$, 1 mM PMSF, 8 M urea) using the Mini-Beadbeater-1 (Biospec Products) and subsequently centrifuged (10 °C, 14,000 RPM, 5 minutes).[122] The supernatant was collected centrifuged again. Aliquots of 250 µl were transferred into new tubes and stored in -20 °C. The sample extracts were subsequently used for determination of catalase activity, superoxide dismutase activity, hydroperoxide concentration, and western blot analysis.

Protein Concentration Determination

Protein concentration in each sample was quantified using the Bio-Rad DC protein assay. One to two mg/ml of bovine serum albumin was used as a reference.

Catalase Assay

Catalase is an important antioxidant that catalyzes the decomposition of hydrogen peroxide into water and molecular oxygen. For each *Drosophila* experiment, an assay kit (Caymen Chemical, Ann Arbor, MI, USA) was used to measure catalase activity in each sample extract. The assay uses the peroxidatic function of catalase to measure enzymatic activity. Aliphatic alcohols such as methanol donate electrons to specifically to catalase as opposed to other peroxidatic enzymes. Therefore, the formaladehyde produced as a result of the reaction between methanol and catalase was used to measure catalytic activity. Purpald (4-amino-3hydrazino-5-mercapto-1,2,4-triazole) reacts with aldehydes and changes from colorless to purple; therefore, purpald was added in order to measure formaldehyde concentration.

Superoxide Dismutase Assay

SOD is another important antioxidant implicated in neurodegenerative diseases as it is involved in the detoxification of the superoxide radical. Low levels of SOD are specifically observed in the pathophysiology of PD. Mutations in SOD are also associated with the development of ALS, further indicating the important role of SOD in the pathogenesis of neurodegeneration.[123] SOD activity was measured using an assay kit (Caymen Chemical, Ann Arbor, MI, USA). The kit utilizes xanthine oxidase to generate superoxide radicals in the sample. The amount of SOD required to disumtate 50% of the radicals (one unit of SOD) was measured by the addition of a tetrazoluim salt that reacts with superoxide to form a formazan dye. The flies collected in each experiment were homogenized as described above; the supernatant was then diluted 10 million times in lysis buffer and used to measure to SOD activity in each sample. Into each well of a 96 well plate, 10 µl of sample was added to 200 µl of the diluted Radical Detector. Samples were measured in duplicate. Xanthine oxidase (20 µl) was then added to initiate the reaction. The microplate was then incubated on a shaker for 30 minutes at room temperature. After incubation, the absorbance was read at 450 nm using a plate reader (BioTek Instruments, Winooski, VT). The units of SOD in each sample were normalized based on total protein content.

Lipid Hydroperoxide Assay

The concentrations of lipid hydroperoxides in each sample were quantified using an assay kit (Caymen Chemical, Ann Arbor, MI, USA). Lipid hydroperoxides are unstable and react with ferrous ions to yield ferric ions. Thus, the principle of this assay is

to detect ferric ions via addition of the chromagen, thiocyanate, which indicates the concentration of lipid hydroperoxides in the sample. To avoid potential sources of error, including the reactivity of hydrogen peroxide present in the samples with ferrous ions and the presence of ferric ions in the samples, the samples were first extracted in chloroform according to the assay protocol. The chloroform extract of each sample was then used in the lipid hydroperoxide assay. The hydroperoxide concentration in each sample (μM) was normalized to total protein concentration.

Western Blot Analysis

The sample extracts from the *Drosophila* (Alzheimer's and Parkinson's) and *C. elegans* (Alzheimer's) assays were used in western blot analysis to determine the concentration of amyloid-β and α-synuclein in the Alzheimer's and Parkinson's transgenic experimental samples, respectively. SDS-PAGE was used to separate the proteins in each sample on 15% (Amyloid-β) or 10% (A-synuclein) polyacrylamide gels for two hours and subsequently transferred to a PVDF membrane. The membranes were then blocked for two hours at room temperature with 5% blot (Santa Cruz Biotechnology, Santa Cruz, CA) in Tris-glycine, 1X buffer (PBST), 0.05% Tween, at pH 8.4. The membranes were then incubated with primary anti-β-amyloid, 1-16 (1:1,000) antibody (BioLegend) or primary anti-α-synuclein (1:500) antibody (BD Biosciences) in 5% blotto/1X PBSTwith 0.05% Tween at 4 °C for 12-15 hours. The membranes were then incubated in the secondary anti-mouse IgG-peroxidase (1:5,000) antibody (Sigma) in 5% blotto/1X PBST with 0.05% Tween at room temperature for one hour. Washing with 1X PBST (4 x 10 minutes) followed incubations in both primary and secondary antibodies.

Western Lightning® Plus-ECL, enhanced chemiluminescent plus substrate (PerkinElmer, Waltham, MA) was utilized to detect immunoreactivity.

Real-time PCR

Flies (n=3-10) were weighed and subsequently homogenized using a rotor-stator homogenizer. RNA was purified using the RNeasy Mini kit (Qiagen, Louisville, KY) according to the manufacturer's protocol. cDNA was then synthesized from these samples using SuperScript® III Reverse Transcriptase with Oligo$(dT)_{20}$ primers (Life Technologies, Carlsbad, CA). Analysis of gene-expression was performed using SYBR green QPCR with the StepOnePlus™ Real Time PCR System (Life Technologies, Grand Island, NY). The $2^{-\Delta\Delta CT}$ method was employed to determine the relative levels of gene expression. The primers used for each sample set are represented in Table 7. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured for each sample and was used to normalize the expression of each target gene.

Table 7. List of Target Genes and Their Sequence to Measure Gene Expression.

Target gene	**Primer sequence**
Amyloid-β-forward	CTA CGC TAT GAC AAC ACC GC
Amyloid-β-reverse	AGA CTT TGC ATC TGG CTG CT
A-synuclein-forward	ATG GAT GTA TTC ATG AAA GG
A-synuclein-reverse	TTA GGC TTC AGG TTC GTA G
SOD-forward	GCG GCG TTA TTG GCA TTG

Table 7 Continued	
SOD-reverse	ACT AAC AGA CCA CAG GCT ATG
GAPDH-forward	CCA CTG CCG AGG AGG TCA ACT AC
GAPDH-reverse	ATG CTC AGG GTG ATT GCG TAT GC

Statistical Analysis

The Breslow-Gehan-Wilcoxon test was used to measures differences in survival for the Alzheimer's disease assay. Lifespan survival curves were determined and the statistical differences were evaluated using the Log rank test (Mantel Cox). Each test used Graphpad prism Software 6.0 (GraphPad Software, Inc. San Diego, CA).

Results

Alzheimer's Disease Assay in Transgenic *C. elegans*

The Alzheimer's disease assay was conducted in two subsequent experiments. In the first assay, 50% of the control worms were scored as paralyzed or dead (PT_{50}) 28.4 h after the heat shock. It was found that treatment with sodium benzoate significantly extended the time to reach PT_{50} (30.4 h; $p < .0001$). Additionally, treatment with cinnamyl acetate also delayed paralysis (PT_{50} = 28.2 h; $p = 0.001$); however, supplementation with cinnamic acid did not have a significant effect on the time to reach PT_{50} (28.2 h; $p = 1.07$). In a subsequent assay, 28.9 h after the heat shock, 50% of the control worms were scored as dead or paralyzed. It was found that treatment with either cinnamaldehyde (PT_{50}=30 h; $p < 0.0001$) or ethyl cinnamate (PT_{50}= 30.8; $p < 0.001$)

significantly delayed the amyloid-beta$_{1-42}$ induced paralysis. A summary of results is represented in Table 8.

Table 8. Time to Paralysis in the Alzheimer's Disease Assay in *C. elegans.*

Treatment (CL4176)	Median Survival (h)	PT50 (h)	P-value	Effect
Assay 1				
Control	30	28.4		
Cinnamic Acid	26	28.2	0.107	↔
Cinnamyl Acetate	30	29	0.001	↑
Sodium Benzoate	30	30.4	<0.0001	↑
Assay 2				
Control	28	28.9		
Cinnamaldehyde	30	30	<0.0001	↑
Ethyl Cinnamate	30	30.8	<0.001	↑

MPP+ Induced Parkinson's Disease Assay in *C. elegans*

After inducing dopaminergic neurodegeneration in *C. elegans* by administration of the neurotoxin MPP+, 50% of control worms were found to be paralyzed or dead at 52.3 hours. In worms that were co-treated with sodium benzoate, the time to reach PT_{50} was significantly delayed (69 h; $p = 0.0028$). However, no significant differences were found between control and worms treated with ethyl cinnamate (52.6 h; $p = 0.7525$), cinnamic acid (48.2 h; $p = 0.1444$), or cinnamaldehyde (59.3 h; $p = 0.2375$), whereas

treatment with cinnamyl acetate increased the rate of paralysis (39.9 h; $p < 0.0001$). Results are represented in table 9.

Table 9. MPP+ Induced Neurotoxicity Assay in *C. elegans.*

Treatment (MPP+)	Median Survival (h)	PT50 (h)	P-value	Effect
Control	48	52.3		
Ethyl Cinnamate	48	52.6	0.7525	↔
Cinnamic Acid	36	48.2	0.1444	↔
Cinnamyl Acetate	36	39.9	<0.0001	↓
Cinnamaldehyde	48	59.3	0.2375	↔
Sodium Benzoate	60	69	0.0028	↑

Effect of Cinnamon Bioactive Compounds on the Survival of Experimental Models of Neurodegeneration in *Drosophila*

Amyloid-β_{1-42} Induced Neurodegeneration Assay in Drosophila

A transgenic *Drosophila* model developed to produce human amyloid-β1-42 was used to assess the effect of cinnamon bioactives on lifespan. Treatment with sodium benzoate (PT_{50}= 60; p = <0.0001), cinnamic acid (PT_{50}= 54.6; p = 0.0301), or cinnamyl acetate (PT_{50}= 58; p = 0.0071) significantly increased survival when compared to control (PT_{50}= 50). There were no significant differences in survival after treatment with ethyl cinnamate (PT_{50}= 48; p = 0.2584) or cinnamaldehyde (PT_{50}= 52.93; p = 0.0747), when compared to control. The results are depicted in Table 10.

Table 10. Effect of Cinnamon Bioactives on the Survival of a *Drosophila* Model of Amyloid-beta Induced Neurodegeneration.

Amyloid-β_{1-42} induced neurodegeneration				
Treatment	**Median Survival (d)**	**PT50 (d)**	**P-value**	**Effect**
Control	50	49.1		
Ethyl Cinnamate	48	46.8	0.2584	↔
Cinnamic Acid	58	54.6	0.0301	↑
Cinnamyl Acetate	57	58	0.0071	↑
Cinnamaldehyde	54	52.93	0.0747	↔
Sodium Benzoate	60	63.18	<0.0001	↑

A-synuclein Induced Neurodegeneration

The effect of cinnamon bioactive compounds on the survival of a transgenic Drosophila model developed to produce the A30P mutant of α-synuclein was evaluated. Forty-four days after eclosion, 50% of the control flies were dead. Significant increases in survival were found in flies treated with ethyl cinnamate (PT_{50} = 49.6 d; p = 0.0298), cinnamyl acetate (PT_{50} = 54.9 d; p = 0.0033), cinnamaldehyde (PT_{50}= 54.3 d; p = 0.0007), and sodium benzoate (PT_{50} = 55.6 d; p = 0.0001). Treatment with cinnamic acid did not confer any significant difference in survival when compared to control (PT_{50} = 47.5 d; p = 0.1757). Results are summarized in Table 11.

Table 11. Effect of Cinnamon Bioactives on the Survival of A-synuclein Induced Paralysis in *Drosophila*

A-synuclein-induced neurodegeneration				
Treatment	**Median Survival (d)**	**PT50 (d)**	**P-value**	**Effect**
Control	44	44		
Ethyl Cinnamate	48	49.6	0.0298	↑
Cinnamic Acid	46	47.5	0.1757	↔
Cinnamyl Acetate	58	54.9	0.0033	↑
Cinnamaldehyde	52	54.3	0.0007	↑
Sodium Benzoate	55	55.6	0.0001	↑

Rotenone Induced Neurotoxicity

Exposure to the neurotoxin rotenone was used to induce Parkinson's disease-like neurotoxicity. After 26 days, 50% of control flies were scored as dead. However, there were delays in the time to reach PT50 in flies treated with cinnamyl acetate (PT_{50}= 28.8 d; $p < 0.0001$), cinnamaldehyde (PT_{50}=40 d; $p = 0.0005$), or sodium benzoate (PT_{50}=44.3; $p < 0.0001$). There were no significant changes in survival when compared to control in flies treated with ethyl cinnamate (PT_{50}=31.4 d; $p = 0.0884$) or cinnamic acid (PT_{50}=43.2 d; $p = 0.222$). Results are depicted in Table 12.

Table 12. Survival of Wild Type Flies After Rotenone Induced Neurotoxicity in *Drosophila.*

Rotenone-Induced Neurotoxicity			
Treatment	**Median Survival (d)**	**P-value**	**Effect**
Control	26		
Ethyl Cinnamate	35	0.0884	↔
Cinnamic Acid	34	0.222	↔
Cinnamyl Acetate	48	<0.0001	↑
Cinnamaldehyde	49	0.0005	↑
Sodium Benzoate	50	<0.0001	↑

Climbing Assay

A climbing assay was used to measure the effects of cinnamon bioactive compounds on behavior in flies after rotenone-induced neurotoxocity and a-synuclein induced neurodegeneration. Due to observations in flies serving as blank (wild type flies that were not exposed to rotenone), the data was not quantified. Future research will include the optimization of the assay in order to assay behavior.

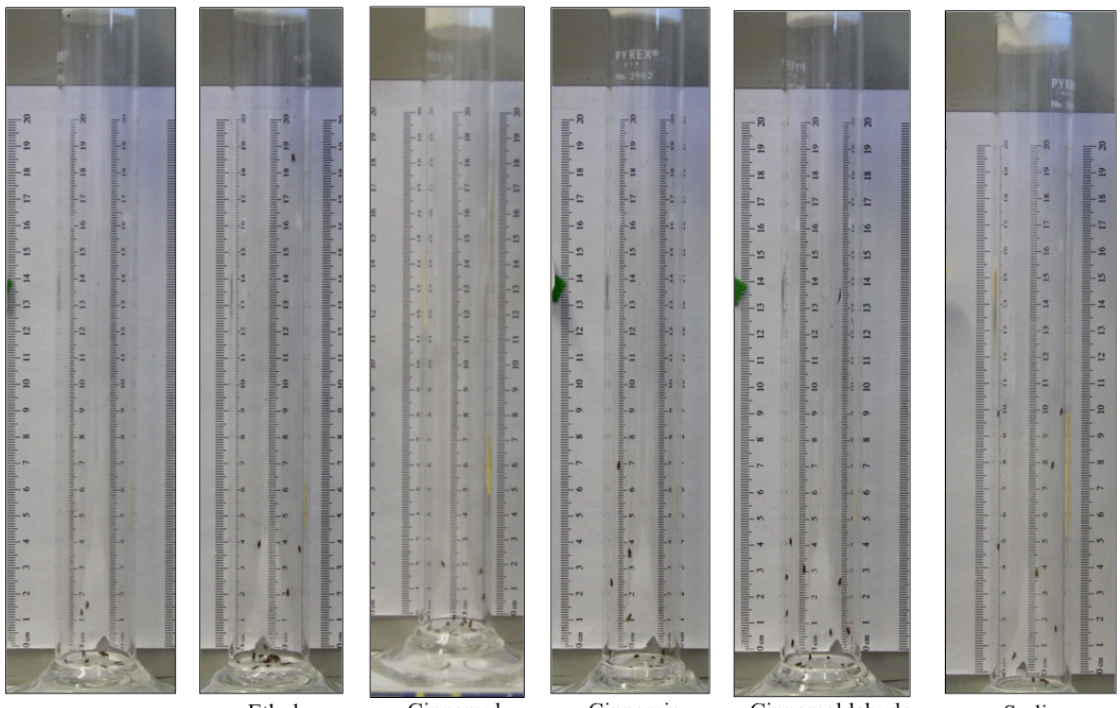


Figure 2. Climbing Ability of Flies After Rotenone Induced Neurotoxicity on Day 10.

Biochemical Evaluation of Cinnamon Bioactives on Experimental Models of Neurodegeneration in Drosophila

Amyloid-beta Induced Neurodegeneration

After amyloid-beta induced neurodegeneration, the catalytic activity of ethyl cinnamate, and sodium benzoate were lower than control; the activity in samples of flies treated with cinnamyl acetate, cinnamic acid, and cinnamaldehyde were increased. For catalase activity, levels were increased in ethyl cinnamate, cinnamyl acetate, and cinnamic acid, while catalase activity was reduced in cinnamaldehyde and sodium benzoate samples. The levels of lipid hydroperoxides were elevated in each treatment

group except for sodium benzoate, which was less than control (Table 13). Significant differences (p = <0 .05) are represented in bold.

Table 13. Biochemical Analysis After Amyloid-beta Induced Neurodegeneration in *Drosophila.*

Amyloid-beta induced neurodegeneration			
Treatment	**SOD (FC)**	**Catalase (FC)**	**LPO (FC)**
Control	1	1	1
Ethyl Cinnamate	0.89	**1.57**	**1.29**
Cinnamyl Acetate	**1.37**	**1.95**	**1.84**
Cinnamic Acid	**1.66**	**2.44**	**2.56**
Cinnamaldehyde	1.12	0.95	**1.25**
Sodium Benzoate	**0.81**	0.89	**0.84**

A-synuclein Induced Neurodegeneration

For the a-synuclein induced neurodegeneration samples that were frozen on day 15, levels of SOD activity were higher than control in flies treated with ethyl cinnamate, cinnamyl acetate, cinnamic acid, and sodium benzoate, whereas the levels were similar between control and cinnamaldehyde. Catalase activity was reduced in flies treated with ethyl cinnamate, while treatment with cinnamyl acetate, cinnamic acid, and cinnamaldehyde were reduced. Levels between sodium benzoate and control were similar (Table 14). Significant differences (p = <0 .05) are represented in bold.

Table 14. Biochemical Analysis After A-synuclein Induced Neurodegeneration in *Drosophila* on Day 15.

A-synuclein induced neurodegeneration **Day 15**		
Treatment	**SOD (FC)**	**Catalase (FC)**
Control	1	1
Ethyl Cinnamate	**1.27**	**0.72**
Cinnamyl Acetate	**1.61**	**1.47**
Cinnamic Acid	**2.31**	**1.75**
Cinnamaldehyde	1.00	1.10
Sodium Benzoate	**1.66**	1.01

On day 30, SOD activity level was elevated in all treatment groups when compared to control. Catalase was increased in ethyl cinnamate, cinnamyl acetate, cinnamic acid, and slightly higher in sodium benzoate. Reduced levels of cinnamaldehyde were observed in cinnamaldehyde (Table 15). Significant differences (p = <0 .05) are represented in bold.

Table 15. Biochemical Analysis After A-synuclein Induced Neurodegeneration in *Drosophila* on Day 30

A-synuclein induced neurodegeneration Day 30		
Treatment	**SOD (FC)**	**Catalase (FC)**
Control	1	1
Ethyl Cinnamate	1.11	**1.50**
Cinnamyl Acetate	**1.53**	**2.48**
Cinnamic Acid	**1.83**	**1.15**
Cinnamaldehyde	**1.98**	**0.81**
Sodium Benzoate	**1.57**	1.06

Rotenone Induced Neurotoxicity

Samples of flies treated with rotenone were frozen on day 15. SOD and catalase levels were reduced in each treatement group, with the exception of flies treated with cinnamyl acetate, in which the levels of catalase were similar to control (Table 16). Significant differences (p = <0 .05) are represented in bold.

Table 16. Biochemical Analysis After Rotenone Induced Neurotoxicity in *Drosophila.*

Rotenone-induced neurotoxicity			
Treatment	**SOD (FC)**	**Catalase (FC)**	**LPO (FC)**
Control	1	1	1
Ethyl Cinnamate	**0.67**	**0.50**	**1.19**
Cinnamyl Acetate	**0.43**	1.01	**0.80**
Cinnamic Acid	**0.32**	**0.43**	**0.81**
Cinnamaldehyde	**0.64**	0.87	**1.24**
Sodium Benzoate	**0.48**	**0.67**	1.14

Western Blot Analysis

The levels of amyloid-beta and a-synuclein were evaluated through western blotting. For samples of flies with amyloid-beta induced neurodegeneration, higher-molecular weight bands were observed; however, after additional controls were analyzed, the bands appeared to be due to non-specific binding. Similar problems were observed with antibodies for a-synuclein, therefore, future studies will evaluate these proteins through western blotting with different antibodies.

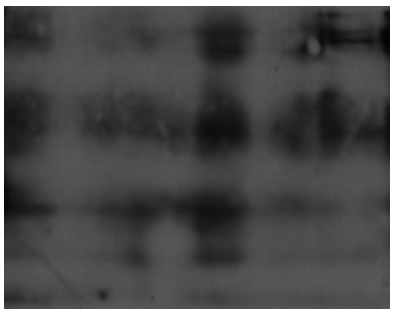


Figure 3. Western Blot Analysis for Amyloid-beta

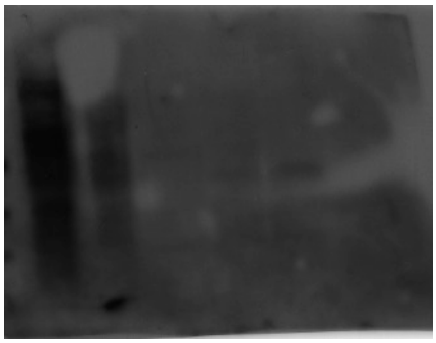


Figure 4. Western Blot Analysis for A-synuclein

Real-time PCR

Changes in gene expression were measured for amyloid-beta and a-synuclein for the Alzheimer's and Parkinson's disease assay in *Drosophila*, respectively. SOD gene expression levels were measured in each of the *Drosophila* assays. Changes in gene expression are expressed as fold changes with respect to control, which was designated a value of one and represented in Tables 17-19.

Table 17. Expression of SOD and Amyloid-beta Relative to Control (Day 30).

Amyloid-beta Induced Neurodegeneration				
	SOD		**A-beta**	
Treatment	**Fold Change**	**Standard Error**	**Fold Change**	**Standard Error**
Control	1		1	
Ethyl Cinnamate	**4.33**	0.26	0.937	0.053
Cinnamyl Acetate	**1.63**	0.083	**1.151**	0.054
Cinnamic Acid	**0.45**	0.019	**11.045**	0.434
Cinnamaldehyde	**4.60**	0.287	**0.674**	0.0391
Sodium Benzoate	**1.23**	0.063	**0.662**	0.0315

Table 18. Expression of SOD and A-synuclein After A-synuclein Induced Neurodegeneration (Day 15)

A-synuclein Induced Neurodegeneration				
	SOD		**A-synuclein**	
Treatment	**Fold Change**	**Standard Error**	**Fold Change**	**Standard Error**
Control	1	1	1	1
Ethyl Cinnamate	**1.70**	0.008	**3.56**	0.007
Cinnamyl Acetate	**1.70**	0.013	**6.02**	0.011
Cinnamic Acid	**4.00**	0.022	**25.60**	0.048
Cinnamaldehyde	1.05	0.005	**2.63**	0.006
Sodium Benzoate	1.04	0.002	**2.15**	0.006

Table 19. Expression of SOD After Rotenone Induced Neurotoxicity

Rotenone Induced Neurotoxicity		
	SOD	
Treatment	**Fold Change**	**Standard Error**
Control	1	1
Ethyl Cinnamate	0.460	0.002
Cinnamyl Acetate	1.059	0.004
Cinnamic Acid	0.669	0.002
Cinnamaldehyde	0.002	9.28E-06
Sodium Benzoate	2.458	0.010

CHAPTER IV

EFFECT OF CINNAMON BIOACTIVE COMPOUNDS ON NEURODEGENERATION IN *C. ELEGANS* AND *D. MELANOGASTER*

Discussion

Effect of Polar Acidic and Polar Neutral Fractions of Cinnamon on Models of Neurodegeneration and Stress Response Signaling in *C. elegans*

Previous experiments in our lab and the current literature suggest that cinnamon is neuroprotective. Various mechanisms of neuroprotection could be possible, including compounds that have antioxidant and anti-inflammatory actions. We have used the polar acidic and polar basic fractions of cinnamon to evaluate the possible effects of cinnamon on experimental models of neurodegeneration and stress response signaling in *C. elegans*.

In an experimental model of Alzheimer's disease, we looked at the effects of PA and PN on a strain of C. elegans developed to produce human amyloid-beta in the skeletal muscle, which consequently induces paralysis. We found that the PN fraction significantly extended the time to PT50, where as no significant differences were observed. We also looked at MPP+ induced paralysis in C. elegans, and again found that PN increased time to PT50 (30% when compared to control), while no significant protection after treatment with the PA fraction.

Neurodegeneration is a progressive loss of neurons that is characterized by protein aggregation and uncontrolled cycles of oxidative stress and inflammation. Therefore, the mechanisms by which the cell responds to stress are extremely critical and

have been of much interest in neurodegenerative research. We have looked at the effects of the polar basic and polar acidic fractions of cinnamon on the expression of several genes involved in stress response signaling including Nrf2, Superoxide dismutase (SOD), Sirtuin 2, heat shock protein, and catalase. Nrf2 is a transcription factor that is essential for the expression of genes such as phase-II detoxifying enzymes and antioxidants. Heat shock proteins were evaluated because of their role in protein folding and reducing protein aggregation. SOD is a powerful antioxidant that is critical for the clearance of the toxic species, superoxide. Similarly, catalase is an important antioxidant involved in the detoxification of hydrogen peroxide. Sirtuin-2 is a protein that has shown anti-aging properties and has been widely studied for lifespan extension. Overall, our results were less conclusive than stress response signaling studies using a whole cinnamon extract. However, we did find that the PN fraction of cinnamon conferred more significant increases in the expression of Nrf2, SOD (Fe/Mn), Sirtuin 2, 16 kD heat shock protein, and catalase when compared to changes observed with the PA fraction. PA significantly downregulated SOD (Fe/Mn) and upregulated catalase. Results are represented in Table 3.

These data lead us to conclude that there may be neuroprotective bioactive compounds in the PN fraction of cinnamon. Therefore, we continued our investigation with smaller phenolic compounds that might be present in the PN fraction, including ethyl cinnamate, cinnamyl acetate, cinnamic acid, cinnamaldehyde, and sodium benzoate.

Effect of Cinnamon Bioactive Compounds on Experimental Models of Neurodegeneration

As previously discussed, AD is a multifactorial disease which pathological characteristics including a loss of synapses, amyloid-beta plaque deposits, NFTs, oxidative stress, and inflammation. Formed from the aberrant processing of APP, amyloid beta is a peptide that typically contains 38-42 amino acids and has a tendency to form neurotoxic oligomers, fibrils, proto-fibrils, and aggregates. Among these peptides that are released, Amyloid-beta$_{1-42}$ is considered to be the most neurotoxic. Originally, the extracellular amyloid-beta aggregates were thought to be the most toxic species of amyloid-beta. However, emerging evidence suggests that the smaller amyloid beta oligomers may be the most neurotoxic form of the peptide. It has been found that the amyloid-beta oligomers can be inserted into the lipid bilayer and begin a chain reaction of lipid peroxidation. Investigations on amyloid-beta neurotoxicity revealed two additional hydrophobic amino acids contained in amyloid-beta$_{1-42}$ that are not present in the less-toxic species, amyloid-beta$_{1-40}$. The enhanced hydrophobicity of amyloid-beta$_{1-42}$ may enhance the peptide's association with the unsaturated fatty acids of the cell membrane, possibly explaining the increased neurotoxicity associated with amyloid-beta$_{1-42}$. New evidence regarding the metabolism of amyloid-beta has also recently emerged. While it was thought that amyloid-beta was formed exclusively by the altered processing of the extracellular domain of APP, it is now suggested that intracellular production and trafficking of amyloid-beta occurs, and may have more significant implications in the pathogenesis of AD than does the extracellular production and deposition of amyloid-beta.

PD is a neurodegenerative disease that is characterized by degeneration of dopaminergic neurons and the presence of protein aggregates. Similar to AD, PD a primary protein aggregate has been identified, referred to as a-synuclein. While the etiology behind the aggregation of a-synuclein has yet to be fully elucidated, oxidative stress has is believed to play an important role.[24] Dopaminergic neurons are inherently susceptible to oxidative stress due to the oxidative by-products produced during dopamine metabolism. Exposure to environmental toxins, such as MPP+ and rotenone, have been known to cause Parkinson's disease; MPP+ is selectively taken up by dopaminergic neurons, whereas rotenone can cause more nonspecific damage. However, due to the susceptibility of dopaminergic neurons to oxidative stress, rotenone exposure causes Parkinson's disease like symptoms.

We have used two transgenic models of Drosophila that were developed to produce human amyloid-beta and the A30P variant of a-synuclein to study the effects of cinnamon bioactive compounds on Alzheimer's and Parkinson's disease, respectively. We have also used Drosophila to study the effects of cinnamon bioactives on rotenone-induced neurotoxicity. In flies with amyloid-beta induced neurodegeneration, we observed significant increases in lifespan after treatment with cinnamyl acetate, cinnamic acid, and sodium benzoate. The most significant increase was observed with sodium benzoate, which lead to a 20% increase in lifespan compared to control (Table 10). We also found significant extensions in lifespan in flies with a-synuclein-induced neurodegeneration after treatment with ethyl cinnamate, cinnamyl acetate, cinnamaldehyde, and sodium benzoate. Similar to results observed with amyloid-beta-

induced neurodegeneration; sodium benzoate conferred the most significant increase in lifespan (26% compared to control) (Table 11). After rotenone-induced neurotoxicity in wild type flies, we found highly significant increases in lifespan after treatment with cinnamyl acetate, cinnamic acid, cinnamaldehyde, and sodium benzoate. Again, treatment with sodium benzoate resulted in the greatest increase in lifespan, extending survival nearly two-fold compared to control (Table 12). This is in congruence with our previous study on MPP+ induced neurotoxicity in *C. elegans*; sodium benzoate delayed paralysis by 30% compared to control.

Because oxidative stress is intimately involved in the pathogenesis of neurodegeneration, we evaluated the catalytic activity of two important antioxidants, SOD and catalase in order to determine possible mechanisms by which the cinnamon bioactives were modulating lifespan in the flies. We also investigated the mRNA levels of SOD to determine possible transcriptional modulation. Additionally, we assessed the extent of lipid peroxidation in the flies by quantifying lipid hydroperoxides. For amyloid-beta induced neurodegeneration, a significant increase in catalytic activity of SOD was observed in flies treated with cinnamyl acetate and cinnamic acid. The mRNA levels were elevated after treatment with ethyl cinnamate, cinnamyl acetate, cinnamaldehyde, and sodium benzoate, whereas mRNA was reduced after treatment with cinnamic acid. The activity of catalase was increased in ethyl cinnamate-, cinnamyl acetate-, and cinnamic acid-treated flies. With the exception of sodium benzoate, all treatment samples had elevated levels of lipid hydroperoxides relative to control; however, treatment with sodium benzoate resulted in significantly reduced lipid hydroperoxide levels (Tables 13 and 17). For flies with a-synuclein-induced neurodegeneration, SOD activity was higher

in all treatment groups compared to control. SOD mRNA levels were increased in ethyl cinnamate, cinnamyl acetate, and cinnamic acid. Catalase activity was higher in ethyl cinnamate, cinnamyl acetate, and cinnamic acid samples. In each case, cinnamic acid treatment sample values were the highest. Lipid hydroperoxide levels were similar to control in each treatment group (Tables 14 and 18). In flies that were treated with rotenone, the catalytic activity of SOD and catalase was reduced for all treatment samples. The mRNA levels of SOD were reduced after treatment with cinnamyl acetate and cinnamaldehyde, whereas a 2.5 fold increase was observed with sodium benzoate samples, when compared to control. Lipid hydroperoxides were reduced after treatment with cinnamyl acetate and cinnamic acid, while elevated levels were observed in cinnamaldehyde and ethyl cinnamate treatment groups (Tables 16 and 19).

There are several mechanisms by which cinnamon bioactive compounds may extend lifespan and delay paralysis in *Drosophila* and *C. elegans*, respectively. Bioactive compounds have been known for thousands of years to promote health and reduce the risk of disease. Known bioactivity of plant-based products that may confer neuroprotection include, but are not limited to, antioxidant, anti-inflammatory, and anti-diabetic activity. Our results and the current literature suggest that cinnamon bioactive compounds may confer neuroprotection by modulating oxidative stress, inflammation, and macroautophagic processes.

Oxidative Stress

While reactive oxygen species (ROS) are a product of normal aerobic metabolism and serve important functions as signaling molecules, excessive production or reduced

capacity to neutralize these compounds leads to oxidative stress.[124] High levels of ROS result in damage to DNA, lipids, and proteins. The superoxide radical is formed from one-electron reductions of molecular oxygen and is commonly produced during mitochondrial dysfunction.[124] Perhaps the most toxic ROS is the hydroxyl radical, which rapidly reacts with unsaturated fatty acids of the lipid bilayer and begins a chain reaction of lipid peroxidation. Hydrogen peroxide is another ROS formed from the dismutation of the superoxide radical catalyzed by superoxide dismutase; while it does not have an unpaired electron, hydrogen peroxide is easily converted to the hydroxyl radical through Fenton reactions.[124]

We observed a reduction in the catalytic activity of superoxide dismutase and catalase in flies treated with rotenone, whereas moderate increases in catalytic activity were observed in flies with amyloid-beta induced neurodegeneration after treatment with cinnamic acid and cinnamyl acetate. Supplementation with each cinnamon bioactive in flies with a-synuclein induced neurodegeneration resulted in a net increase in antioxidant activity. These observations may be due the biphasic nature of the antioxidant defense system in response to differential levels of oxidative stress. Under subtoxic oxidative conditions, the antioxidant defense system is upregulated in cells in order to restore redox homeostasis. When oxidative stress becomes toxic and overwhelms the antioxidant defense system, however, the cell is unable to compensate for the extensive oxidative damage and apoptosis is induced. Cinnamon bioactives may offer neuroprotection by the upregulation of key antioxidants during subtoxic oxidative environments, as seen with a-synuclein induced neurodegeneration. During higher oxidative conditions, cinnamon

bioactives showed lower antioxidant activity, indicating a more homeostatic redox environment relative to control.

Inflammation

Upon activation in response to protein aggregation or neuronal damage, microglia produce proinflammatory molecules in order to scavenge the toxic cargo. While inflammation is a protective and essential process, a detrimental overactivation of microglia has been observed in neurodegenerative diseases. Elevated levels of pro-inflammatory cytokines are commonly found in the SN and striatum in the brains of postmortem PD patients.[21] NFkB is a transcription factor that is required for the expression of many pro-inflammatory molecules. Inhibition of NFkB has been identified as a potential therapeutic target for reducing neuroinflammation in neurodegenerative disesases. *In vitro*, a whole cinnamon extract has blocked NFkB activation and reduced the levels nitric oxide, IL-1B, and TNF-a.[61] In particular, sodium benzoate has shown to prevent the expression of inducible nitric oxide synthase and pro-inflammatory cytokines (TNF-a and IL-1B) in microglia after experimentally induced inflammation; inhibition of NF-kB binding capability was also observed and may be contributing to the enhanced neuroprotection observed after treatment with sodium benzoate.[91]

Autophagy

Abnormal protein handling has become increasingly recognized as a primary event in the pathogenesis of neurodegenerative diseases. Proteins that are altered or misfolded are generally identified and delivered lysosome for degradation via three different autophagic pathways: chaperone-mediated autophagy (CMA), microautophagy,

and macroautophagy.[125] Each pathway ultimately delivers cellular cargo to the lysosome for degradation, but differs in the mechanism of delivery. Macroautophagy is responsible for the bulk degradation of proteins and organelles. This process involves the sequestration of cytoplasmic cargo within a double-membrane vesicle, referred to as an autophagic vesicle (AV), and subsequent fusion with the lysosomal membrane. Cellular cargo contained within the AV is then released into the lysosome for degradation by acidic lysosomal hydrolases.[126] An abnormal number of AVs within degenerating neurons gave the first indication that autophagic dysfunction plays a role in the pathogenesis of neurodegenerative diseases. It remains unclear if the elevated number of AVs is a result of an upregulation of the autophagic system as an attempt to clear aggregated proteins or if there is a dysfunction in the AV-lysosomal fusion leading to an accumulation of AVs. However, recent investigations have identified several implications of autophagic dysfunction in neurodegenerative diseases.[127]

While amyloid-beta plaques were originally thought to be formed exclusively from the abnormal processing of APP extracellularly, current research has revealed that amyloid-beta is also produced intracellulary.[126] Furthermore, it is suggested that the intracellular production and subsequent trafficking of amyloid-beta may have more implications in the neurodegenerative process than the extracellular amyloid-beta aggregates. Considering the role of macroautophagy in protein trafficking and degradation, autophagic dysfunction has been investigated as a potential explanation of amyloid-beta toxicity. Manipulations to the macroautophagic process have been applied in order to elucidate the nature of these processes; induction of autophagy has reduced intracellular amyloid-beta load and improves cognitive function in mice. On the other

hand, inhibition of autophagy has increased intracellular amyloid-beta levels and reduced extracellular amyloid-beta load, suggesting an important role of autophagy in amyloid-beta secretion. Significantly, AVs are also a source of intracellular amyloid-beta production. AVs contain the enzymes and precursor (APP) required for amyloid-beta production; consequently, the accumulation of AVs observed in degenerating neurons may contribute to intracellular amyloid-beta accumulation and toxicity.[127]

Depending on the conformation, a-synuclein can be degraded via the ubiquitin-proteasome system (UPS) or autophagy. While fibrillar forms can be delivered to the proteasome, only soluble forms of the protein can be degraded; consequently, the fibrillar forms of a-synuclein that are delivered can become stuck within the proteasome and prevent further proteosomal activity.[125] When targeted for degradation via the CMA pathway, a-synuclein mutants bind tightly to the lysosomal membrane; however, it has been shown that the a-synuclein protein, albeit bound to the membrane with high affinity, is not translocated into the lysosome for degradation. Rather, it remains bound and consequently disrupts the uptake of other cellular proteins targeted for degradation, including other pathogenic a-synuclein proteins. It has been found that acute disruption of these pathways leads to an upregulation of the macroautophagic pathway. Chronic and persistent disruption of these pathways, however, results in constitutive upregulation of macroautophagy, but not without consequence; under these circumstances, cells are unable to futher activate the macroautophagic in response to stress.[125]

Previous experiments in our lab have indicated that treatment with a whole cinnamon extract in *C. elegans* leads to an upregulation in several genes important in autophagic processes. In the present study, significant increases in lifespan in flies

afflicted with amyloid-beta- and a-synuclein-induced neurodegeneration were observed. While the precise implications of intracellular production, trafficking, and secretion of amyloid-beta and a-synuclein have not been fully elucidated, evidence suggests that impaired autophagic processes play a significant role in the pathogenesis of neurodegenerative diseases. In five models of neurodegeneration, we observed the most significant extensions in lifespan (*Drosophila*) and delayed paralysis (*C. elegans*) after treatment with sodium benzoate. A recent study by Hazan et al. showed that sodium benzoate directly effects intracellular membrane trafficking in *Saccharomyces cerevisiae*. The upregulation of autophagy has been identified as a potential therapeutic strategy for neurodegenerative diseases; as such, the modulation of autophagic processes by cinnamon bioactive compounds, sodium benzoate in particular, may be contributing to observed neuroprotective effects.

Conclusion

The results of the present study indicate that cinnamon bioactive compounds have neuroprotective effects, as evidenced by lifespan extension in five models of neurodegeneration. It is suggested that key bioactive compounds in cinnamon, including ethyl cinnamate, cinnamyl acetate, cinnamic acid, cinnamaldehyde, and sodium benzoate may exert these effects by modulating antioxidant activity and expression, depending on the type of stress occurring.

Among the compounds investigated, sodium benzoate conferred the most significant increases in lifespan and protection against oxidative stress. These findings are in congruence with recent *in vitro* studies suggesting sodium benzoate has significant

neuroprotective effects.[91,120,128] Sodium benzoate has improved cognition in individuals with early-phase AD. [129] Antipsychotic effects have also been observed in a schizophric mouse model.[130] There are several mechanisms by which sodium benzoate could be exerting neuroprotective effects. It is a d-amino acid oxidase inhibitor, which could protect against dopamine degradation in Parkinson's disease.[130] It is also a known hydroxyl radical scavenger, which may protect against oxidative stress.[131] In yeast, sodium benzoate modulates autophagy, which may have important implications considering the emerging evidence of the role of autophagy in AD.[132 133]

Our results and the current literature indicate that more research into the neuroprotective effects of cinnamon phytochemicals and metabolites, sodium benzoate in particular, is highly merited. Evaluation of post-translational modification of proteins would enable a better understanding of the mechanism of action. Additionally, an investigation of synergistic effects of varying compounds would further elucidate cinnamon's mechanism of neuroprotection and therapeutic potential for neurodegenerative diseases.

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