Doctoral Dissertation (Shinshu University)

Study on the screening of neuroprotective compounds from agricultural products using a *Caenorhabditis elegans* model

（線虫モデルを用いた農業生産物からの神経保護物質の探索に関する研究）

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ABBREVIATIONS

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

AD: Alzheimer's disease

APP: Amyloid precursor protein

Aβ: Amyloid β

BACE: β-site APP cleaving enzyme

C. elegans: *Caenorhabditis elegans*

CGC: Caenorhabditis genetic center

CRISPR: Clustered regularly interspaced short palindromic repeats

DPPH: 1,1-diphenyl-2-picrylhydrazyl

FoxO: Forkhead box O transcription factor

GDNF: Glial cell line-derived neurotrophic factor

GFP: Green fluorescent protein

GLE: *Glochidion littorale* leaves extract

GSH: Glutathione

GSSG: Oxydized glutathione

H_2DCF-DA: 2',7'-dichlorodihydrofluorescein-diacetate

HGT: Heat-dried green tomato

HSP: Heat shock protein

IGF: Insulin growth factor

IIS: Insulin/insulin-like growth factor signaling

LC-MS: Liquid chromatography-mass spectrometry

MPP^+: 1-Methyl-4-phenylpyridinium ion

MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MRP: Maillard reaction product

NFTs: Neurofibrillary tangles

NGM: Nematode growth media

Nrf2: Nuclear factor erythroid 2-related factor 2

PD: Parkinson's disease

PINK1: PTEN-induced putative kinase 1

PTEN: Phosphatase and tensin homolog

RNAi: RNA interference

ROS: Reactive oxygen species

SN: Substantia nigra

SNpc: Substantia nigra pars compacta

SOD: Superoxide dismutase

TNF: Tumor necrosis factor

α-syn: α- synuclein

6-OHDA: 6-hydroxydopamine

ABSTRACT

The longevity and neuroprotective effect of dry green tomato and *Glochidion littorale* extract on *Caenorhabditis elegans* were investigated in this doctoral dissertation.

In Chapter 1, a literature review is included, and the objectives of this thesis are highlighted. Aging is considered to have effects on various molecular pathways and is a risk factor for neurodegenerative pathologies such as Alzheimer disease (AD) and Parkinson disease (PD). Oxidative stress is one of the main paths leading to aging and age-related diseases. Compounds that exhibit strong antioxidant power seem to be efficient countermeasures to oxidative stress. Hence, plants rich in antioxidant molecules are considered important.

The Maillard reaction which is a reaction between amino acids or protein and reducing sugar in the presence of heat, occurs every day with or without human intervention. The reaction leads to the formation of browning compounds, flavor, and Maillard reaction products (MRPs). Some MRPs have been reported to have strong antioxidant properties, but their neuroprotective effects have yet to be explored. As tomato is produced and consumed worldwide, dried tomatoes might be a good source of MRPs. In addition, numerous plants used in traditional medicine in Southeast Asian countries are considered beneficial for health. Several species from the genus Glochidion have been investigated for their flavonoid rich content. *Glochidion littorale* leaves are used as folk medicine against general injuries and diseases in countries such as Thailand. However, there are few reports concerning their neuroprotective effects.

Chapter 2 investigates the anti-aging and the neuroprotective effects of MRP fractions derived from dried tomatoes (*Solanum lycopersicum L.*). *Caenorhabditis elegans* was used as an *in vivo* model. The results showed that MRP fraction has a strong radical scavenging activity, as evaluated through DPPH and ABTS assays. Aqueous extracts of green tomatoes and their MRP fractions

showed a significant lifespan extension effect in *C. elegans*. Furthermore, the MRP fraction protected worms against H_2O_2 damage and 1-methyl-4-phenylpyridinium ion (MPP^+) induced neurotoxicity in *C. elegans*.

In Chapter 3, the neuroprotective effect of *Glochidion littorale* leaf extract (GLE) on *C. elegans* and the probable mechanisms involved are investigated. Specifically, the antioxidant activity of GLE was evaluated *in vivo* by inducing an oxidative stress in worms and by measuring the intracellular reactive oxygen species (ROS) using a fluorescent probe (2',7'-dichlorodihydrofluorescein-diacetate). Compared with the control, the GLE treated worms showed significantly reduced ROS intensity and increased oxidative stress resistance. Thereafter, the neuroprotective effects were evaluated in wild type *C. elegans* and transgenic mutant (*daf-2* and *daf-16* mutant) worms. These results suggest a neuroprotective effect of GLE and the possible involvement of DAF-16 transcription factor in both stress resistance and the neuroprotection pathway.

Finally, Chapter 4 provides a summary and conclusion of the study. Our findings suggest that MRP fraction from dry green tomatoes exerts possible lifespan extension and neuroprotective effects on *C. elegans*. Second, our findings suggest an oxidative stress resistance and possible neuroprotective effects of GLE in *C. elegans* via activation and translocation of the transcription factor DAF-16 in the nucleus. These agricultural products may be a promising preventive strategy for age-related decline in the elderly.

CHAPTER 1: INTRODUCTION

1. Background

For many years, aging has been considered as a physiological condition, which favors the onset of many chronic diseases. However, the exact mechanism involved and the relationship between aging and related pathologies are still object of scientific research [1]. Neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) are also increased with age [2]. As neurons age, they show signs of increased oxidative stress, disturbances in mitochondrial function, and accumulation of misfolded proteins, which are exacerbated in AD and in PD. Yet, a direct link between mechanisms of aging and the onset of such neurodegenerative disorders is still missing [2-3]. A plethora of scientific evidence has clearly indicated that an increased generation of reactive oxygen species (ROS) that is not counterbalanced by an internal defense mechanism results in oxidative stress and the manifestation of neurodegenerative diseases [4-5].

Due to their health beneficial effects in animal models and low toxicity, in recent years, natural antioxidant compounds have become attractive sources for aging and neurodegenerative diseases drug development [6]. Antioxidant compounds can be largely found in fruits and vegetables. Some biochemical reactions such as the Maillard reaction can also induce the production of some antioxidant compounds [7-8]. When vegetables are treated at low temperature, prooxidants are generated, whereas treating at high temperature decreases the prooxidants and increases antioxidant properties due to the production of Maillard reaction products (MRPs). Such antioxidant activity of the MRPs comes from the high molecular weight brown compounds that are formed in the advanced stages of the reaction [9].

Among the models available for investigating aging and age-related diseases, the nematode *Caenorhabditis elegans* is getting more and more attention from researchers. The hermaphrodite

nematode has its neuronal network fully mapped [10]. The capacity for immediate assessment for the presence or absence of specific neurons in a living organism is a key attribute in using this system to study neurodegenerative disorders. Accumulating evidences showed that *C. elegans* is a powerful tool to study Insulin/insulin-like growth factor signaling (IIS) which could lead to new discoveries that would inform investigations into mammalian IIS – a prediction borne out shortly thereafter by the revelation that the *C. elegans* FoxO transcription factor DAF-16 is a major target of IIS [11]. Subsequent studies have revealed new regulatory components and mechanisms involved in IIS regulation of a variety of biological processes including oxidative stress, aging and neuroprotection. Moreover, many of the downstream transcriptional targets that control these processes have been identified. Considering that oxidative stress is believed to be involved in many pathologies related to aging, we hypothesized antioxidant rich plant extracts could slowdown aging and prevent neurodegeneration, and *C. elegans* model would be useful for screening neuroprotective compounds.

2. Literature review

2.1. Neurodegenerative diseases

Neurodegenerative diseases which could be hereditary or sporadic are conditions that result in the progressive loss of the structure and function of neurons as well as neuronal death. Neurodegenerative disorders are associated with high morbidity and mortality, and few effective options are available for their treatment [12]. Thus, many studies have been conducted focusing on natural compounds present in food as important molecules against neurodegenerative diseases such as PD. Neurodegenerative diseases are becoming increasingly prevalent in the aging populations of industrialized nations, going hand in hand with the increase in life expectancy [13].

Although many pathologies are classified under this term, AD and PD are the most common neurodegenerative diseases that affect a large population around the globe [4]. Aging and oxidative stress are currently recognized as key factors that cause the pathophysiological features of these diseases. The inevitable aging process may augment ROS formation and accumulation, which activate several neurodegenerative pathways and lead to neuronal loss [4]. As neurons age, they show signs of increased oxidative stress, disturbances in mitochondrial function and accumulation of misfolded proteins, which are exacerbated in AD, and in PD. However, a direct link between mechanisms of aging and the onset of such neurodegenerative disorders is still under study [3].

2.2. Alzheimer's disease

AD is the neurodegenerative disorder most usually associated with age-related dementia and is etiologically multifactorial. Histologically, AD is characterized by extensive neurodegeneration, extracellular deposition of amyloid-β peptide (Aβ) forming senile plaques, and intraneuronal accumulations of hyperphosphorylated microtubule-associated protein tau, the neurofibrillary tangles (NFTs) [3].

Neurons in the human brain have tremendous oxygen consumption and metabolic rates. For this reason, neurons rely on mitochondria that are found in abundance in brain tissues for the production of energy by oxidative phosphorylation. Mitochondria are the central sites of ROS, as natural byproducts of the oxidative phosphorylation cascade, and excessive production of ROS is usually offset by the normal homeostasis function of mitochondria [4].

Aβ appears as a solitary molecule but tends to form small clusters that are soluble and able to travel freely in the brain and eventually forms plaques that are hallmarks of AD. Recent studies have highlighted the effect of Aβ on mitochondrial respiratory function, including mitochondrial complexes I–IV activities and mitochondrial oxygen consumption. Chen and Yan (2010) have

shown that Aβ can penetrate the mitochondrial membrane and interact with internal mitochondrial protein abnormalities and decrease the activity of the mitochondrial electron transfer chain, the citric acid cycle, and the generation of ROS [14]. Moreover, Cha et al., (2012) have demonstrated that exposure of the hippocampal cell line of mice (HT22 cells) to exogenous $A\beta_{1-42}$ induced identical morphological alterations as those observed in AβPP/PS1 double transgenic mice [15]. $A\beta_{1-42}$ accumulation in mitochondria induces cellular toxicity and leads to cellular death. Mitochondrial associated amyloid precursor protein (APP) forms a complex with the translocase of the outer mitochondrial membrane 40 (TOM40) import channel and the translocase of the inner mitochondrial membrane 23 (TIM23) import channel and causes the inhibition of nuclear-encoded cytochrome c oxidase subunits IV and Vb proteins [16]. Following this event, cytochrome c oxidase activity is decreased, and the generation of hydrogen peroxide is increased in mitochondria. This finding correlated with a higher level of regional dissemination of mitochondrial APP in AD-vulnerable areas, including the frontal cortex, hippocampus, and amygdala [16].

The deposition of Aβ peptide, the main component of senile plaques in neuronal cells, is known as "amyloidopathy." It is becoming clear that the extracellular aggregation of Aβ plaques is closely associated with widespread neuronal atrophy and the concomitant damage of synapses in different brain regions that results in gradual neuronal death and memory loss. It has been reported that several environmental factors, including oxidative stress, drug and brain injury, and genetic variants, can contribute to the pathogenesis of AD [16-17]. The amyloid cascade hypothesis is the most accepted and well understood mechanism of deposition of the amyloid beta protein in Alzheimer's pathology [15-17]. APP is a large transmembrane protein that produces Aβ that consists of either 40 or 42 amino acids. $A\beta_{40}$ is a commonly found protein molecule whereby $A\beta_{42}$ is highly toxic to neuronal cells. In physiological states, the membrane glycoprotein APP plays a

crucial role in synapse formation and neuronal activity and transmission via two proteolytic pathways: “nonamyloidogenic” and “amyloidogenic” pathways [17]. In the “nonamyloidogenic” pathway, soluble APPα is released to the extracellular compartment through APP cleavage that involves α- and γ-secretases. On the other hand, the “amyloidogenic” pathway is triggered by β-secretase 1 mediated cleavage that produces soluble extracellular APPβ, which is subsequently cleaved by a γ-secretase complex that releases Aβ into the extracellular space. Perturbation of the physiological pathway may occur in the event of mutations and changes in the expression of APP, β-site APP cleaving enzyme 1 (BACE-1), IDE, Apo-E, and neprilysin lead to the progressive accumulation of Aβ and the manifestation of AD [18].

Aerobic cells are inherently equipped with natural enzymatic and nonenzymatic antioxidant systems that prevent the accumulation of ROS [18-19]. The enzymatic antioxidant system is a vital component that comprises enzymes such as superoxide dismutase (SOD), glutathione peroxidase, and catalase, which catalyzes the ROS to less toxic molecules and thereby plays a key role in preventing lipid peroxidation. The superoxide anions, which are produced during normal cell signaling processes, are converted to hydrogen peroxide (H_2O_2) and oxygen molecules (O_2) by SOD [19]. On the other hand, glutathione peroxidase functions in detoxifying hydroperoxides, including DNA and lipid hydroperoxides, into water and alcohol molecules. This enzyme catalyzes the reduction of hydroperoxides at the expense of reduced glutathione (GSH) to produce oxidized glutathione (GSSG) and reduced hydroperoxide. Subsequently, the GSSG is recycled to GSH by GSH reductase and NADPH. In addition, catalase detoxifies H_2O_2 molecules, which are converted to oxygen and water molecules. Oxidative stress in cells activates the transcription of antioxidant enzymes through the antioxidant response element (ARE) and the binding of members of the Cap-N-Collar family of transcription factors, including Nuclear factor (Nrf)1 and Nrf2 [19].

Activated Nrf2 by oxidative stress enters the nucleus and escalates the transcription of ARE-containing genes. These genes cause the expression of antioxidant enzymes, such as, SOD, GPx (GSH peroxidase), and CAT (catalase). Subsequently, these enzymes will catalyze the detoxification of ROS and protect neuronal cells from damage. Glycogen synthase kinase-3β participates in phosphorylation of tau protein and induces accumulation of neurofibrillary tangles (NFT) that inhibits Nrf2. Besides inhibiting Nrf2 activity, alpha-synuclein and NFT stimulate oxidative stress via ROS production [19].

Neuroinflammation is a complex sequence of inflammatory events in nervous tissue that are triggered in response to infection, trauma, and toxic substances. In the past 20 years, a wealth of information has emerged associating aging and AD with neuroinflammation. In addition to neuronal cells, microglia and astrocytes are major cells that are involved in inflammatory events in the central nervous system. The initial discovery was made by McGeer et al., (1988) by demonstrating the presence of reactive microglia in substantia nigral region of postmortem brain tissue of PD patients [20]. As a consequence of persistent microglial activation that surrounds both Aβ and tau tangles, the glial cells lose their homeostatic function and attain a proinflammatory characteristic and intensify neuronal damage. In the event of neuroinflammation, inflammatory mediators, such as TNF-α, IL-6, IL-1β, and cyclooxygenase-2, are found in serum and brain samples of AD patients [20].

2.3. Parkinson's Disease

PD is a progressive neurodegenerative disorder commonly affecting elderly people worldwide. It affects around 0.3% of the general population and 1–3% of the population over the age of 65 and its number is going to rise from 8.7 to 9.3 million by 2030 [21]. The PD symptoms were described two hundred years ago by James Parkinson in 1817. Manifestations of PD primarily

include dysfunctions of the somatomotor system (i.e., rigidity, bradykinesia, postural instability, gait dysfunction and tremors). At its core, PD involves progressive degeneration of the nigrostriatal dopaminergic pathway with substantial loss of substantia nigra pars compacta (SNpc) neurons and depletion of dopamine [22]. Impairments in non-motor functions are often accompanied (such as dementia, hyposmia and gastrointestinal alterations) during the disease course [22-24].

Protein aggregation is a biological phenomenon which involves intracellular or extracellular accumulation of misfolded proteins [25]. Such protein aggregates are the pathological hallmark of PD pathology involving accumulation of α-synuclein (α-syn) protein in the form of Lewy bodies [26]. Another hallmark of PD pathology is related to ROS. The mitochondrial electron transport chain is the main source of generating the intracellular ROS and hence, an important target for the detrimental effects of ROS. Cellular metabolism depends on the continuous supply of ATP from the mitochondria which indicates that any damage which impairs the function of the mitochondrial respiratory chain would have an effect on cell viability. To protect the cells from oxidative damage, mitochondria contain a defense system to neutralize the ROS and repair the ROS-induced damage. ROS generation together with the release of pro-apoptotic proteins from the mitochondrial inter membrane space could trigger the activation of different cell death pathways [27]. In PD pathology the nigrostriatal dopaminergic pathway gets affected, since, the nigrostriatal dopaminergic neurons are in significant oxidative stress, due to redox cycling of catechols, which leads to increased generation of ROS [28]. Reduction in the level of GSH in SNpc of pre-symptomatic PD suggests that oxidative damage occurs much earlier than the actual neuronal loss [29]. Interplay between mitochondrial functional impairment and oxidative stress is further suggested by the dysfunction of mitochondrial complex I due to chronic depletion of antioxidant glutathione [30]. Various studies have confirmed the increased levels of oxidative damage in the SNpc of PD patients

including DNA damage, lipid peroxidation, protein oxidation, decrease in reduced glutathione and increased iron deposition [30-31]. Evidences have suggested that oxidative stress has significant impact on PD pathology [27]. Although oxidative stress has emerged as an imperative mechanism in disease, still no antioxidant treatment has proven as curative or preventive therapy for PD clinically. Studies have suggested that instead of an external supply of antioxidant, an up regulated endogenous antioxidant would be more beneficial. In addition the use of antioxidant as combined therapy might also be more effective as discussed elsewhere [27].

In vitro studies in human neuroblastoma SHSY5Y cells and primary mesencephalic culture also showed the involvement of apoptotic death of dopaminergic neurons [28]. The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA) treatment to primary culture of MN9D (i.e. dopaminergic neuronal cell line) and mesencephalic dopaminergic neurons also resulted in the release of cytochrome-c from mitochondria and caspases activation [29]. 6-OHDA treated SHSY5Y cells have shown that cytochrome-c release and caspase-3 activation can be prevented by the over-expression of bcl-xl [30]. Interestingly another study showed the increased activity of caspase-9 and 3 after 6-OHDA treatment but not with MPP^+ treatment which could be prevented by co-treatment with inhibitor z-VAD or with N-acetyl cysteine [29]. Withdrawal of trophic factors is another important event in neurodegenerative disorders leading to apoptotic neuronal death. Different trophic factors such as brain derived neurotrophic factor, nerve growth factor, neurotrophin-3, 4 and 5 deprivations cause initiation of apoptosis in different *in vitro* systems [32-38].

PD mechanism is summarized in Figure 1-1.

2.4. *C. elegans* as a model organism for neurodegenerative diseases

The nematode *Caenorhabditis elegans* was first introduced by Sydney Brenner in the mid-1960s as a model organism to study development and neurobiology [39]. Nowadays, *C. elegans* is used to study a vast array of biological processes, including apoptosis, gene regulation, cell metabolism, cell fate regulation, embryogenesis, stress signaling, and aging. As a model organism, the worm provides numerous advantages. It is remarkably simple to culture in the laboratory on a diet of *E. coli*, and rapidly develops within 3 days post-hatching into fertile adult animals at 20°C. A single adult self-fertilizing hermaphrodite can produce approximately 300 offspring, rendering it amendable to the large-scale growth of thousands of animals for high-throughput screening [40]. Furthermore, *C. elegans* is transparent, and expression of fluorescent transgenes readily allows for the live study of cellular processes. In the context of disease research, the worm is highly amenable to genetic manipulation and features extensive sequence homology with mammalian genes, making it a valuable system for studying human disease [41]. Both RNA interference (RNAi) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology are well established in *C. elegans*, enabling the use of knock-down (RNAi), knock-out, and knock-in approaches (both CRISPR/Cas9) to study the role of individual genes and gene clusters [42-43]. More recently, *C. elegans* became the first organism to have every neuron and its' connections – termed "connectome" – mapped [44]. The sum of these features renders *C. elegans* an ideal model organism for probing the molecular basis of protein aggregation in NDs.

C. elegans provides a powerful *in vivo* genetic system to study the effects of neurotoxic Aβ through transgene analysis [45]. Many transgenic strains have been generated in which a signal sequence followed by the human Aβ sequence is expressed in all cells, in all neurons, in specific subsets of neurons, or in muscle cells. *C. elegans* expressing human $A\beta_{3\text{-}42}$ in muscle tissue showed an age-dependent paralysis at 20°C; paralysis occurred more rapidly and more severely

when $A\beta_{1-42}$ was produced at 25°C [46-48]. The level of muscle paralysis was significantly decreased when insulin signaling was decreased. Furthermore, inhibiting daf-16/FOXO and hsf-1, which encodes a heat shock protein transcription factor, reversed the effects of decreased insulin signaling [46], [49]. Hence, the paralysis effects of Aβ correlates with age and is dependent on insulin signaling.

Among the various cellular and transgenic models available for use in studying the pathology of PD, *C. elegans* offers several distinct advantages enabling researchers to dissect avenues leading to this disease. While mammals have billions of neurons in their brain, and even fruit flies have ~10,000 neurons, the adult *C. elegans* hermaphrodite has exactly 302 neurons throughout its body, with fully mapped neuronal circuitry [44]. Of the 302 neurons, 8 neurons are dopaminergic in the hermaphrodite, which include 6 anterior dopaminergic neurons (4 CEP neurons and 2 ADE neurons) and 2 posterior DA neurons [50]. With *C. elegans* being a completely transparent animal, neurons are easily visualized by simply expressing a fluorescent protein (e.g., GFP), as previously described by Chalfie and colleagues (1994), who later shared the 2008 Nobel Prize in Chemistry for his groundbreaking research using GFP [39]. This is particularly useful when studying neurodegenerative diseases, such as PD, in that neuronal cell death can be readily observed and quantified within living organisms. The capacity for immediate assessment for the presence or absence of specific neurons in a living organism is a key attribute in using this system to study neurodegenerative disorders.

Some well-known pharmacological PD models in mammalian systems include the classical and highly selective neurotoxin 6-OHDA, as well as MPTP and its metabolite, MPP^+. These toxins result in decreased ATP production, increased ROS production, and increased apoptosis of dopaminergic cells [50-51]. Similarly, herbicides and pesticides such as rotenone, paraquat, and

maneb are also commonly used as PD models that result in increased ROS production and altered mitochondrial energetic [52]. A newer pharmacological model involves utilization of lipopolysaccharide (LPS), an inflammagen that causes production of reactive nitrogen species in dopaminergic cells via microglial activation, as another molecular feature of PD is nigrostriatal inflammation [53]. Similarly, in *C. elegans*, exposure to pesticides, 6-OHDA and MPTP/MPP^+ all result in increased oxidative stress, ATP depletion, and disrupted mitochondria that appear with the hallmark dopaminergic neurodegeneration [54–56]. Although the use of LPS as a PD model has not been well studied in worms, the role of other environmental toxins, such as various heavy metals, has recently emerged in *C. elegans* literature. As an example, manganese (Mn) exposure results in dopaminergic neurodegeneration that is associated with increased oxidative stress [46]; with exposure to antioxidant compounds reversing the Mn-induced ROS induction [57-59]. Therefore, *C. elegans* model system has now been validated for use in the study of the role of oxidative stress in dopaminergic neurodegeneration [60].

2.5. Glochidion littorale

Glochidion genus belongs to the Euphorbiaceae family and comprises more than 300 species around the world [61]. The genus is distributed in tropical Asia, the Pacific islands, and Malaysia, with a few in tropical America and Africa. More than twenty species can be found in Vietnam, and most are used as food and traditional medicine in the treatment of influenza, dysentery, impaludism, rheumatoid arthritis, dyspepsia etc [62]. *Glochidion littorale* leaves (Figure 1-2) are commonly used as an infusion for the treatment of stomach ache [63].

2.6. Maillard reaction

The Maillard reaction has been named after the French physicist and chemist Louis Camille Maillard (1878–1936) who initially described it. It is often defined as nonenzymatic browning

reaction. While foods are processed or cooked at high temperatures, a chemical reaction occurs between amino acids and reducing sugars which generate different flavors and brown color. So, it is often used in the food industry for giving food a different taste, color, and aroma [64].

Consumption of diets rich in fruits and vegetables renders many health benefits to us. However, processing method plays an important role in dictating the magnitude of the beneficial health effects obtained from fruits and vegetables. Depending on treatment temperature, furoylmethyl derivatives have been found in processed vegetables and fruits like orange juices [65] and processed tomato products [66], and also in dehydrated carrots [67]. It has been shown that dehydrated carrot contains significantly high amount of furoylmethyl derivatives compared to carrot juices, baby carrot, or tinned carrot. It is suggested that the processing time during the heat treatment plays an important role for furoylmethyl derivatives formation [67]. Dueik and Bouchon (2011) have reported that vacuum frying carrot chips, and potato and apple slices can help significantly to retain their total carotenoids and ascorbic acid levels [68]. However, it should be mentioned here that MRPs can also exhibit prooxidant properties [68-69].

MRPs can prevent the enzymatic browning reaction caused by polyphenol oxidase [70]. Plant derived products, such as fruits and vegetables, produce many endogenous phenolic compounds during postharvest handling and processing. These compounds are oxidized by oxidoreductase enzymes like polyphenol oxidases and tyrosinases. This reaction, in turn, generates highly reactive quinonic compounds that are condensed and polymerized to produce brown pigments and thereby decrease the quality of the food product. MRPs can prevent this enzymatic process at the initial step of this reaction and thereby help to maintain the product quality. Besides anti browning, MRPs have also been shown to render antiallergenic properties for cherry derived allergens [71].

3. Objectives

The main objective of this research was to investigate the neuroprotective effects of plant extracts (green tomato and *Glochidion littorale*) using *C. elegans* as an *in vivo* model. Specifically, the antioxidant properties were evaluated *in vitro* and *in vivo*, and the neuroprotective effects were examined by using neurotoxins in worms.

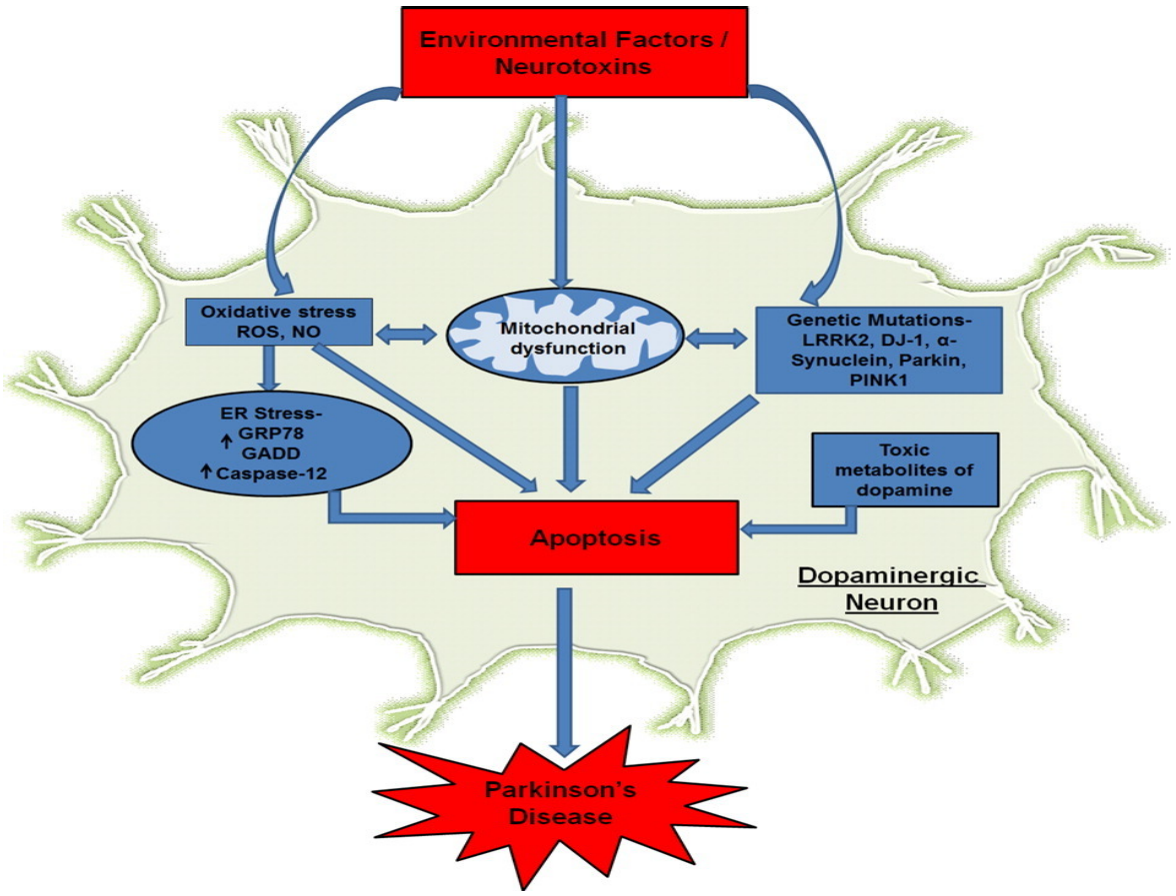

Figure 1-1. Mechanism involved in Parkinson's Disease (PD) pathology. Adapted from [26]. Pictorial presentation showing the involvement of different neurodegenerative mechanisms like oxidative stress, genetic mutations, endoplasmic reticulum (ER) stress, mitochondrial dysfunction and apoptosis in dopaminergic neurons after exposure to environmental factors/neurotoxins.

Figure 1-2. Leaves and fruits of *Glochidion littorale.*

CHAPTER 2

Neuroprotective effects of heat-dried green tomato extract and its Maillard reaction products in *C. elegans*

1. Abstract

Maillard reaction products (MRPs) produced during thermal processing and storage of foods have received considerable attention because of their potential antioxidant activity. We investigated whether heat-dried tomato (*Solanum lycopersicum L.)* extracts and their MRPs confer longevity and neuroprotective effects in *C. elegans*. Heat-drying of mature green tomato resulted in increased absorbance at 294 nm and 420 nm, which indicated the presence of MRPs. Heat-dried green tomato extracts and its MRP fraction exhibited DPPH and ABTS radical scavenging activities and significantly extended the lifespan of *C. elegans*. The MRP fraction protected against β-amyloid-, 1-methyl-4-phenylpyridinium (MPP^+)-, and H_2O_2-induced neurotoxicity in *C. elegans*. These results suggest that the dry processing of unripe green tomato induces MRP formation that might protect against neurodegeneration.

2. Introduction

Aging is characterized by a general physiological decline over time and may involve disruptions in oxidative stress response, aberrant cell signaling, and mitochondrial dysfunction. It is now suspected that this progressive deterioration of cellular functions during aging may significantly increase the risk for several cardio-metabolic and neurodegenerative disorders, including AD and PD [72]. Therefore, elucidating molecular mechanisms relevant to aging and age-associated pathologies to facilitate discovery of therapeutic targets and clinical or dietary intervention that can promote healthy aging has emerged as a vibrant area of rigorous scientific study.

The roundworm *C. elegans*, a small (~1 mm total length), free-living, and soil-dwelling nematode, is among the most widely used models for aging-related research. Their rapid life cycle coupled with high reproductive capacity makes *C. elegans* a suitable tool for mutagenesis and compound screening. In AD research, specific strains, such as transgenic *C. elegans* strains that express high levels of human β-amyloid (Aβ) under the control of a muscle-specific promoter (CL2006), are often used [73]. This transgenic AD model relates Aβ expression and cellular toxicity to an easily visible progressive paralytic phenotype, and thus greatly facilitates the evaluation of pharmacological intervention strategies. CL2006 is an ideal model to investigate the therapeutic potential of AD treatment drugs because this strain constitutively expresses Aβ in the body wall muscles [74]. *C. elegans* also has a short adult lifespan and is an established model for biological aging [74–76]. Recently, a 1-methyl-4-phenylpyridinium (MPP^+) toxicity-induced PD model in *C. elegans* that can recapitulate many key features of PD, including dopaminergic neuron degeneration was established. This *C. elegans* model is increasingly used to screen neuroprotective agents *in vivo* [77-78].

During thermal processing and food storage, some biochemical reactions, such as the Maillard reaction, occur and form components that could be beneficial for health. The Maillard reaction occurs between amino acids or proteins and reducing sugars in heat-treated foods such as bakery products and coffee [8], [79-80]. This results in the formation of Maillard reaction products (MRPs) that have a positive effect on flavor, color, and texture [81]. Some MRPs are reported to have beneficial antioxidative and antibacterial effects [81-82].

Fruits and vegetables contain various healthy compounds. Tomato is one of the most important crops globally valued at 124.6 billion US dollars annually [6] representing the largest sector of the fleshy fruit market. It is widely used as plant model due to its short generation time, and well-studied genetic, biochemical, and physiological properties. Rich in beneficial phytochemicals, tomato fruit are delicate, develop and ripen quickly and are used at various stages of their development either whole, or for various processing purposes including canned goods, pastes, sauces, juices, etc [83]. The growth of tomato fruits goes through different phases. The early phase of fruit development is characterized by high metabolic activity and a rapid cell division of the tissue, whereas at a later developmental phase the cells expand. Fruit ripening begins when seeds are completely formed and the fruit reaches its final size [84]. This ripening process involves a series of coordinated events including changes at the physiological and biochemical levels [85]. During the initial phases of tomato ripening, chloroplasts differentiate into chromoplasts. This plastid transition is accompanied by the expression of specific genes involved in chromoplast formation and the subsequent synthesis of enzymes correlated with ripening [86].

As sink organs, fruits are dependent on the translocation of sucrose, amino acids, and organic acids to the developing fruit cells. The rate of import of these photo assimilates from the leaves is governed by the metabolic activity of the fruit [87]. In the case of the tomato, green fruit cells

contain most of the photosynthetically active chloroplasts that give the developing fruit its green appearance, and play a significant role in carbon dioxide scavenging [88]. In developing fruits, about 70% of the total amino acid content found in the pericarp belongs to the glutamate family [89]. γ-Aminobutyric acid (GABA) was the predominant N-form (almost 60% of the total amino acid molar content) at the earlier growing stages of the tomato fruit and glutamine (ca. 30%) in mature green fruits [90]. Many compounds such as sucrose, malate, GABA, glutamine and others are higher in mature green tomato compared to ripened red tomato [91].

Many studies suggest that there is high content of tomatidine and specific amino acids, including Asn, Ser, Pro, Tyr, and Val, in unripe green tomatoes, although their amounts decrease as the fruit ripens [89-91]. Thus, we hypothesized that dry heat treatment of green tomatoes can produce MRPs with high antioxidant activity that might confer longevity and neuroprotective effects. In this study, we investigated the effect of dried tomato extract and its MRPs on wild-type *C. elegans* as a model organism. Furthermore, the neuroprotective effects of MRPs against β-amyloid, MPP^+, and H_2O_2 neurotoxins on *C. elegans* were evaluated.

3. Materials and Methods

3.1. Materials

Tomato (*Solanum lycopersicum* L. 'Reika') was obtained from the Education and Research Center of Alpine Field Science, Shinshu University (Nagano, Japan). In this study, green tomato (mature unripe tomato) and red tomato (mature ripe tomato) were harvested on 46 and 59 days after plant flowering, respectively. All reagents were of analytical grade. 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and MPP^+ were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

3.2. Preparation of dried tomato and its extract

The green and red tomato fruits were washed, sliced to 3 mm thickness, and dried in a constant temperature oven (DKN812, Yamato, Tokyo, Japan) at 40°C for 7 days or freeze-dried using a freeze-dryer (FDU-1100, EYELA, Tokyo, Japan). All samples were ground into powder using an A10 basic analytical grinder mill (IKA, Staufen, Germany), passed through a 0.5 mm particle size sieve, and stored at −25°C until use. Tomato extract was prepared by adding 30 mg ground tomato to 30 mL distilled water. The solution was then mixed at 12 000 rpm for 2 min using a Polytron PT 3100 (Kinematica AG, Lucerne, Switzerland) and sonicated for 10 min. The extract was finally filtered through 0.22 μm membrane filters and stored at –25 °C until use.

3.3. Browning intensity

The absorbances of tomato samples at 294 nm and 420 nm were measured using a UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan) according to the method described by Ganesan et al., (2014) with slight modifications [81]. Briefly, 10 mg/mL freeze-dried or heat-dried tomato samples were prepared in distilled water. The solution was then homogenized at 12 000 rpm for 2 min using a Polytron PT 3100 (Kinematica AG) and sonicated for 10 min. The extract was finally filtered through a 0.45 μm membrane filter, and the absorbance measured at 294 nm and 420 nm.

3.4. Reducing sugar content

Reducing sugar in the dried tomato extract was measured by the dinitrosalicylic acid method (Deshavath et al., 2020). Standard glucose solutions were used to develop a calibration curve. The reducing sugar content was calculated as per g dry weight of the dried tomato sample.

3.5. Free radical scavenging activity of tomato samples

The capacity of dried tomato extract and collected fraction (F1, F2, and F3) to scavenge free radicals was assessed using ABTS and DPPH assays (Blois, 1958) [92]. The results were expressed as µg 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent/mg sample.

3.6. *C. elegans* maintenance

All *C. elegans* strains and their food, *E. coli* OP50, were obtained from the Caenorhabditis Genetics Center (CGC) (Minneapolis, MN, USA). Wild-type N2 and AD model CL2006 worms were used for lifespan assays. The worms were maintained according to the standard protocols described by Stiernagle T. (2006) at 20°C on nematode growth medium (NGM) agar plates containing *E. coli* OP50 [93]. S-complete solution was prepared according to the method described by Solis and Petrascheck (2011) [94].

3.7. Lifespan assay using wild type *C. elegans*

Wild type *C. elegans* lifespan was determined according to the method described by Amrit et al., (2014) [95]. Briefly, 12 larval stage 4 (L4) of the *C. elegans* N2 strain from a synchronized population were transferred to eight NGM plates and allowed to become adults in the presence or absence of tomato extract (1 mg/mL) or collected fraction (F1, F2, and F3, 500 µg/mL) at 20°C. Worms were scored as dead or alive every other day. Missing worms were censored based on their lifespan count. Significance was determined by the log-rank test, and the mean was calculated by averaging the days of *C. elegans* death for each condition. The results were obtained from three independent experiments (50–120 worms/treatment in each experiment).

3.8. Lifespan assay using CL2006 strains

CL2006 strains were used to assess paralysis according to the method described by Diomede et al. (2013) [96] with some modifications. Briefly, 12 larval stage 4 (L4) nematodes from a

synchronized population were transferred to eight NGM plates and allowed to become adults in the presence or absence of F1 (0.5 mg/mL) at 16°C. Worms were transferred every other day until all nematodes were dead. The number of worms paralyzed was scored starting from L4 larval stage (day 0), and for each consecutive day until all worms were dead. Significance was determined by the log-rank test, and the mean was calculated by averaging the days of *C. elegans* death for each condition. The results were obtained from three independent experiments (50–120 worms/treatment in each experiment).

3.9. MRP preparation using gel filtration chromatography

Dried tomato samples (40 mg/mL) were dissolved in 50 mM ammonium bicarbonate buffer (pH 7.5). The sample solution was vortexed, sonicated for 10 min, and centrifuged at 7 000 g for 15 min. The supernatant was filtered through a 0.45 μm membrane filter and loaded onto a HiPrep 26/60 Sephacryl S-200 HR gel filtration column (GE Healthcare, Chicago, IL, USA) on an ÄKTA Pure 25 chromatography system (GE Healthcare). Elution was carried out with 50 mM ammonium bicarbonate buffer (pH 7.5) at a flow rate of 0.5 mL/min, and the absorbance was measured at 280 nm. The column bed void fraction was determined from the peak retention volume of blue dextran, and each fraction (F1, F2 and F3) of the applied samples was collected as MRPs and freeze-dried.

3.10. Neurotoxicity assay

MPP^+ induced neurotoxicity in wild type *C. elegans* was performed using the method described by Lu et al., (2010) [78] with some modifications. L1 larvae were added to 96 well plates at an average of 12 worms per well in a 40 μL solution containing OP50. A 952 μM MPP^+ solution and the tested tomato sample dissolved in S-complete solution were added to achieve a final volume of 50 μL per well. L1 larvae were incubated with MPP^+ alone or in the presence of various concentrations of MRP fraction. Forty-eight hours after exposure to MPP^+, worm viability was

visually inspected under a stereomicroscope. The results of the MPP^+-treated groups were normalized and expressed as a percentage of the normal controls. The results were obtained from three independent experiments (80–130 worms/treatment in each experiment).

3.11. Oxidative stress assays

Oxidative stress was induced by H_2O_2 in wild type *C. elegans*. L1 larvae were added to 96 well plates at an average of 12 nematodes per well in a 40 µL solution containing *E. coli* OP50. A 2 mM H_2O_2 solution and the tested tomato sample dissolved in S-complete solution were added to achieve a final volume of 50 µL per well. L1 larvae were incubated with H_2O_2 alone or in the presence of various concentrations of F1. Forty-eight hours after exposure to H_2O_2, worm viability was visually inspected under a stereomicroscope. The results from the H_2O_2-treated groups were normalized and expressed as a percentage of normal controls. The results were obtained from three independent experiments (80–130 worms/treatment in each experiment).

3.12. Statistical analysis

Data are expressed as mean ± standard deviation (SD) for each group. Significant difference between groups was assessed using one-way ANOVA, followed by Tukey's post hoc comparison test. Statistical significance was set at $p < 0.05$, $p < 0.01$, or $p < 0.001$. For lifespan assays, *C. elegans* survival was plotted using Kaplan–Meier survival curves and analyzed by log-rank tests using Graph Pad Prism software (version 9.01, San Diego, USA).

4. Results

4.1. Browning intensity and radical-scavenging activity of dried tomato extract

To confirm that the Maillard reaction occurs during the tomato drying process, the browning intensity of the dry samples was determined by measuring the UV absorbance at 294 nm and 420 nm (Table 2-1). The results showed that heat-dried samples had higher absorbance readings than

freeze-dried samples. No marked differences were observed between the green and red tomato samples. On the other hand, the heat-dried samples had lower amount of reducing sugar than freeze-dried samples. These results suggest that during the heat-treatment, the MRP was produced via Maillard reaction between reducing sugars and proteins or amino acids. The free radical-scavenging activity in the tomato samples was determined using the DPPH and ABTS methods. The results showed that green tomato samples possessed higher free radical-scavenging activity than the red tomato samples using both assays. Furthermore, the heat-dried green tomato (HGT) extract exhibited the highest antioxidant properties among the tested samples.

4.2. Effect of crude dried tomato extract on *C. elegans* lifespan

Extracts of dried tomato fruit (1 mg/mL) were added to agar plates containing the standard *C. elegans* laboratory diet. The median *C. elegans* lifespan increased significantly when treated with the HGT extract (Figure 2-1). Treatment with freeze-dried green tomato extract resulted in a slight decrease in the median lifespan for unknown reasons. There was no significant difference between treatment with control and extract samples from freeze-dried and heat-dried red tomatoes. These results suggest that the extract obtained from the HGT possesses lifespan-extending effects in *C. elegans*.

4.3. MRP isolation from HGT extract

To isolate MRPs from HGT extract, the extract sample was subjected to gel filtration chromatography using Sephacryl S-200. Three fractions (F1, F2, and F3) were obtained (Figure 2-2). F1 was eluted in the column void volume (MW > 250 kDa) and showed the highest UV absorbance at 294 nm and 420 nm among the fractions (Table 2-2). The free radical-scavenging activity of F1 was also higher than that of F2 and F3.

4.4. Effect of chromatographic fractions isolated from HGT-derived on *C. elegans* lifespan

Next, the effect of chromatographic fractions of HGT extract on the *C. elegans* lifespan was investigated. Compared with the control, F1 extended the median and maximum lifespan (Figure 2-3A), but there was no significant difference among F2, F3, and control (Figure 2-3B). These results suggest that MRPs contained in F1 may extend the lifespan of wild-type *C. elegans*.

4.5. Neuroprotective effects of HGT-derived MRP in *C. elegans*

To investigate the effect against Aβ-induced toxicity, we used transgenic CL2006 *C. elegans* expressing human Aβ specifically in the body wall muscle. Compared with the control, F1 significantly extended the median and maximum CL2006 lifespan compared to the control (Figure 2-4A). The median lifespan was increased by 32% after treatment with F1 (Figure 2-4B).

Next, investigation on the protective effect against MPP^+-induced neuronal injury was done. The survival rates indicated a protective effect of F1 (Figure 2-5). MPP^+ treatment resulted in a remarkable decrease in survival rate. However, F1 treatment with MPP^+ increased the survival rate in a dose-dependent manner. Additionally, the survival rate was significantly decreased by H_2O_2 treatment. Like previous experiments, F1 treatment increased survival in a dose-dependent manner (Figure 2-6).

5. Discussion

Many studies have focused on the antioxidant activity of MRPs in processed foods such as roasted coffee beans and bakery products [79], [97]. In this study, we isolated MRPs with high antioxidant activity from HGT fruit. In the Maillard reaction, reducing sugars react with amino

acids or proteins to form complex MRPs in three major stages: early, intermediate, and final stage [98-99]. UV absorbance at 294 nm is often used to indicate the intermediate MRPs, while the final stage is monitored by measuring the absorbance at 420 nm (browning intensity). Most significant increases in the absorbances at 294 nm and 420 nm were observed in HGT. An increment in the antioxidant activity of MRP is closely related to browning [100], suggesting that the Maillard reaction proceeded to the final stage. On the other hand, freeze-dried samples also showed high absorbance at 294 and 420 nm, indicating MRP production. Maillard reaction begins in the initial stages of ripening and progresses during fruit storage [101]. Since the antioxidant activities of freeze-dried tomato samples was lower than those of dry-heated tomato samples, the antioxidant activities might be attributed by brown polymers which were formed at final stages of Maillard reaction. During tomato fruit ripening, Asn, Ser, Pro, Tyr, and Val content declines, whereas Asp and Glu content increases [102]. Echavarría et al., (2013) reported that MRPs derived from Trp and Lys show the highest absorbance at 420 nm, and MRPs from Tyr showed the highest free radical-scavenging activity among all tested MRPs [98]. The major sugars of tomato are glucose and fructose [103], and the amount of reducing sugars increased during fruit ripening [104]. Since the dried green tomato samples exhibited higher antioxidant activity than the dried red tomato samples, amino acid metabolism during tomato fruit ripening might mainly affect the antioxidant activity of MRPs produced from each tomato sample.

As far as the lifespan experiments are concerned, worms treated with HGT extract had a longer median lifespan than control *C. elegans*. To further understand the reason for this lifespan extension, HGT extract was analyzed by gel filtration chromatography. Among the three collected fractions, only F1 showed a high absorbance at 420 nm and strong free radical scavenging activity. These results suggest that F1 contains high molecular weight MRPs derived from the HGT extract.

During the past decade, the *C. elegans* AD model has been extensively used to study the protective effects of natural products against AD. Interestingly, supplementation with F1 extended the lifespan of CL2006, a transgenic *C. elegans* strain that constitutively expresses Aβ in muscle cells. Coffee extracts also protect against Aβ toxicity in a transgenic *C. elegans* AD model, possibly by the activation of skn-1 (Nrf2 transcription factor in mammals) [105]. Nrf2 activation can induce an increased antioxidant response, thereby increasing stress resistance and lifespan. Oxidative stress is associated with aging and age-related diseases [106]. Accumulating evidence indicates that aging is mainly caused by free radical damage [107]. This study demonstrates that MRPs obtained from HGT increased the lifespan and attenuated H_2O_2-induced neurotoxicity, which results from mitochondrial dysfunction resulting in cell death. In contrast, HGT-derived MRPs showed a potential neuroprotective effect in MPP^+-treated *C. elegans*. MPP^+ is a dopaminergic neuronal toxin that induces oxidative stress and apoptosis. Therefore, further research is necessary to better understand the underlying mechanisms responsible for neuroprotection against Aβ accumulation and oxidative stress.

In conclusion, we demonstrated that unripe green tomato derived MRPs have antioxidant activities that increase longevity and extend neuroprotective effects against various neurotoxins, such as Aβ, MPP^+, and H_2O_2 in *C. elegans*. These findings provide useful information for developing beneficial compounds that promote longevity and healthy aging. The potential extension of lifespan and neuroprotective effects of HGT extract might be the result of antioxidant capacities of the MRPs formed during the drying process. Further investigation is necessary to clarify the detailed mechanism of action.

Table 2-1. Browning intensity and antioxidant properties of dried tomato extracts

	Absorbance		Reducing sugar (g of glucose/g dry weight)	DPPH (μg Trolox eq/mg)	ABTS (μg Trolox eq/mg)
	294 nm	420 nm			
Freeze-dried green tomato	0.863 ± 0.000 [a]	0.666 ± 0.001 [b]	0.137 ± 0.001 [c]	23.501 ± 0.130 [c]	95.723 ± 1.765 [c]
Heat-dried green tomato	1.290 ± 0.002 [b]	0.744 ± 0.001 [d]	0.071 ± 0.000 [a]	37.133 ± 0.252 [d]	99.821 ± 1.013 [d]
Freeze-dried red tomato	0.822 ± 0.010 [a]	0.413 ± 0.009 [a]	0.153 ± 0.001 [d]	16.750 ± 0.231 [a]	89.860 ± 1.708 [a]
Heat-dried red tomato	1.105 ± 0.010 [b]	0.708 ± 0.000 [c]	0.121 ± 0.002 [b]	17.373 ± 0.522 [b]	92.244 ± 0.282 [b]

Different letters show significant differences at $p < 0.05$.

Table 2-2. Browning intensity and antioxidant properties of the fractions isolated from heat-dried immature tomato.

Fraction	Absorbance		DPPH (mg Trolox eq/mg)	ABTS (mg Trolox eq/mg)
	294 nm	420 nm		
F1	0.721 ± 0.027 [c]	0.884 ± 0.000 [b]	746.355 ± 29.461	195.426 ± 6.923 [b]
F2	0.175 ± 0.000 [b]	0.050 ± 0.000 [a]	ND	9.691 ± 1.761 [a]
F3	0.038 ± 0.000 [a]	ND	ND	ND

Different letters show significant differences at $p < 0.05$. ND: Not detected

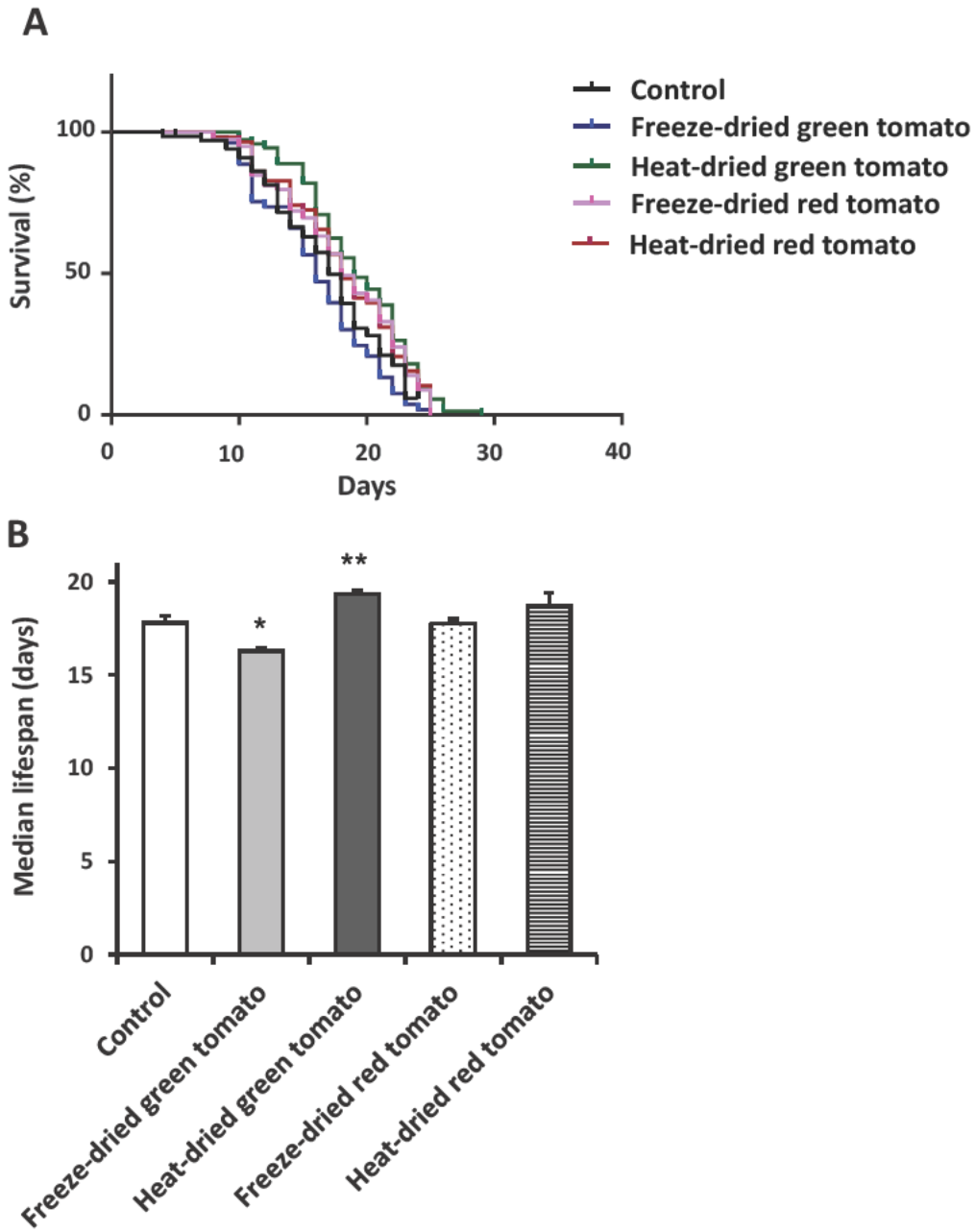

Figure 2-1. Effect of crude dried tomato extracts on the *C. elegans* lifespan. (A) Lifespan curve from a single experiment, representative of n = 3 independent experiments. (B) Median lifespan. Data shown as mean ± standard deviation, significance was reported by ANOVA and subsequent Dunnett multiple as compared with control; $^{*}p < 0.05$, and $^{**}p < 0.01$; as compared with the untreated blank group.

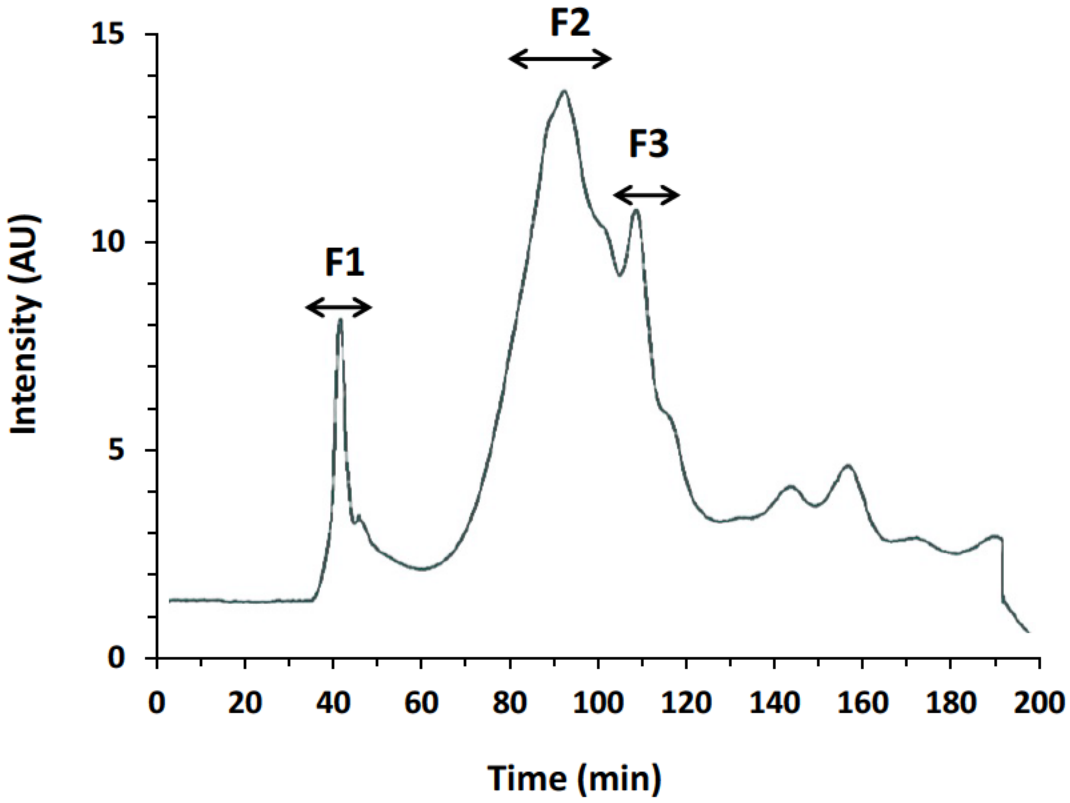

Figure 2-2. Gel filtration chromatography of heat-dried green tomato (HGT) extract. The sample was dissolved to 40 mg/mL in ammonium bicarbonate buffer (pH 7.5) and applied to a HiPrep 26/60 Sephacryl S-200 HR gel filtration column. Elution was carried out at a flow rate of 0.5 mL/min and the absorbance at 280 nm was measured.

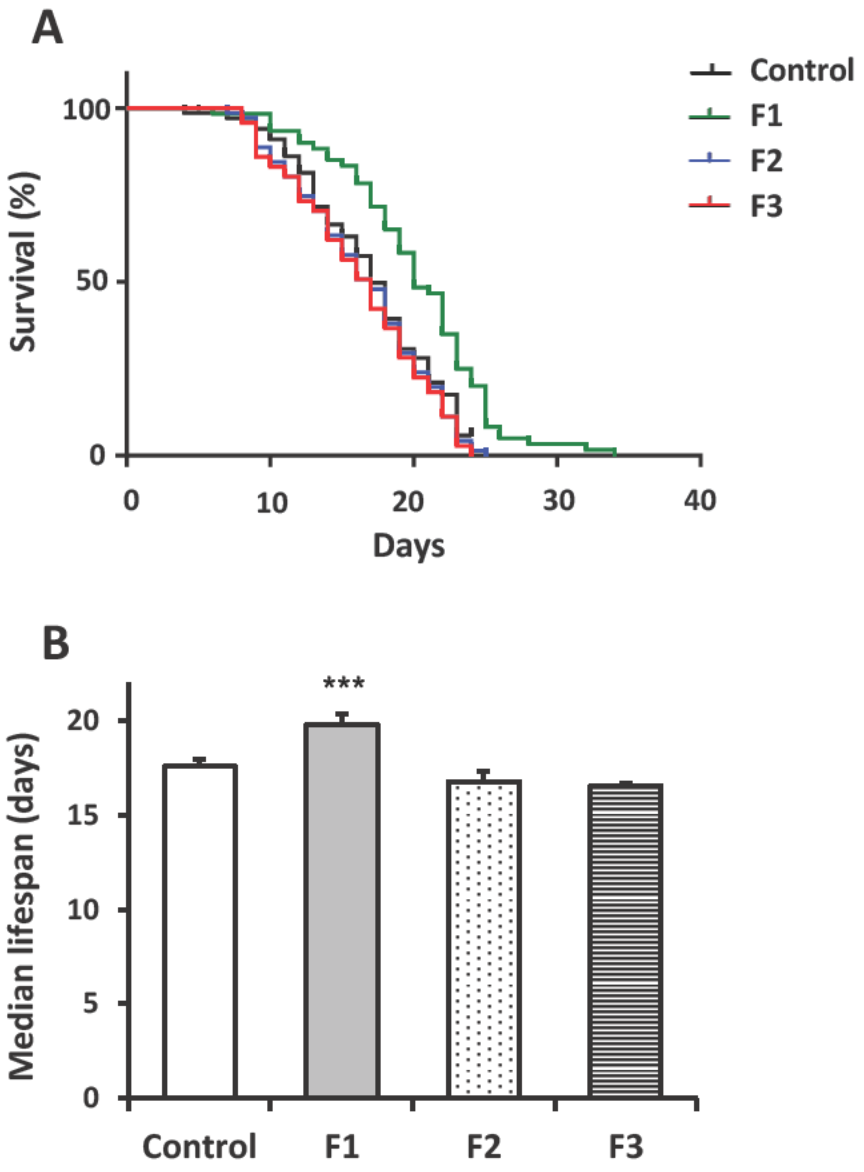

Figure 2-3. Effect of heat-dried green tomato (HGT) extract fractions on the *C. elegans* lifespan. (A) Lifespan curve from a single experiment, representative of n = 3 independent experiments. (B) Median lifespan. Data shown as mean ± standard deviation, significance was reported by ANOVA and subsequent Dunnett multiple as compared with control; $^{***}p < 0.001$.

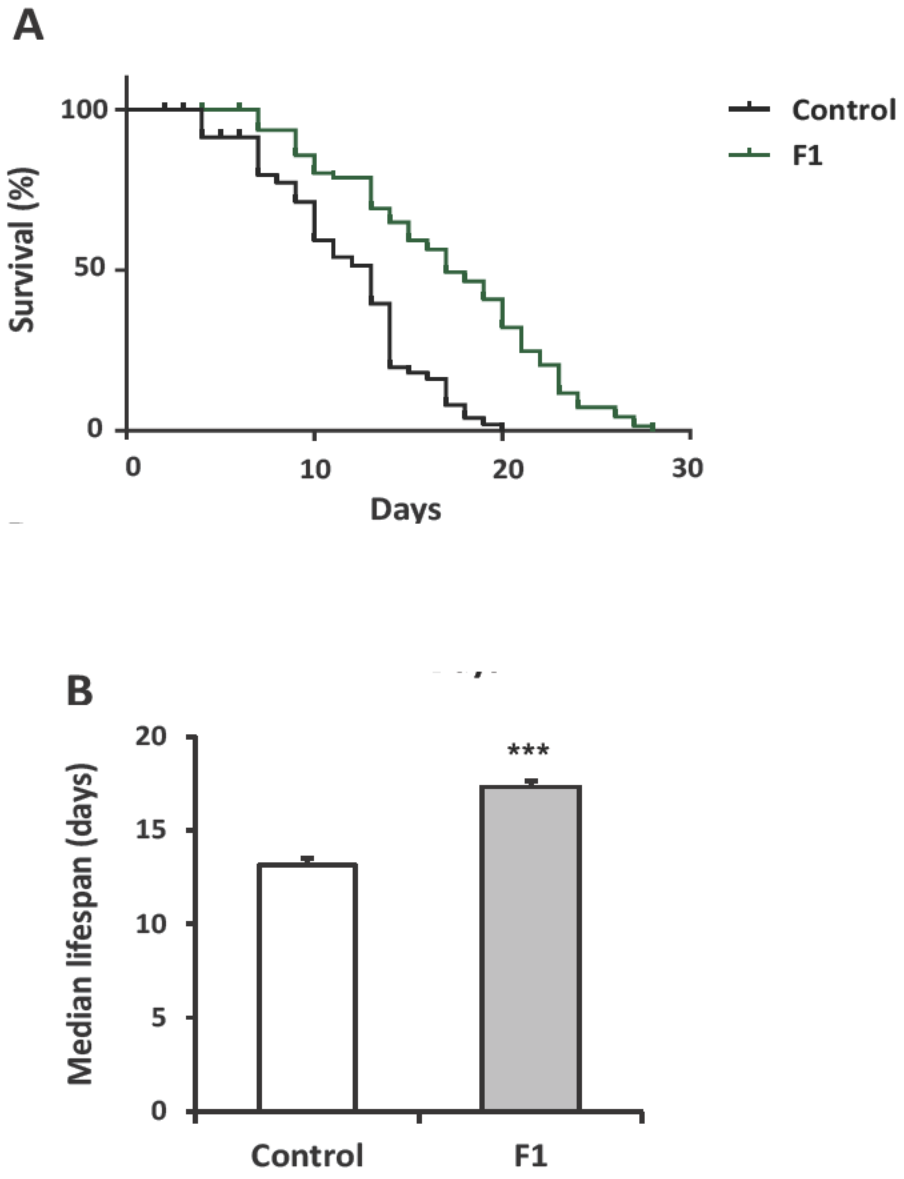

Figure 2-4. Effects of Maillard reaction products isolated from HGT extract on the lifespan of CL2006 transgenic *C. elegans*. (A) Lifespan curve from a single experiment, representative of n = 3 independent experiments. (B) Median lifespan. Data shown as mean ± standard deviation, significance was reported by t-test; $^{***}p < 0.001$.

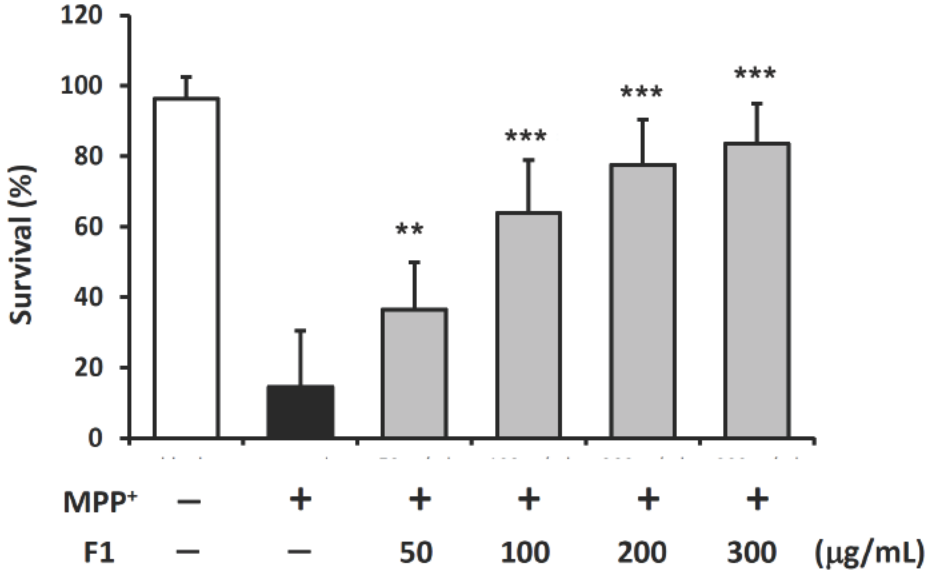

Figure 2-5. Effects of Maillard reaction products isolated from Heat-dried green tomato (HGT) extract on MPP^+-induced neurotoxicity in *C. elegans*. Each experiment was repeated independently at least three times. The results are presented as mean ± SD. ** $p < 0.01$, *** $p < 0.001$ compared to control.

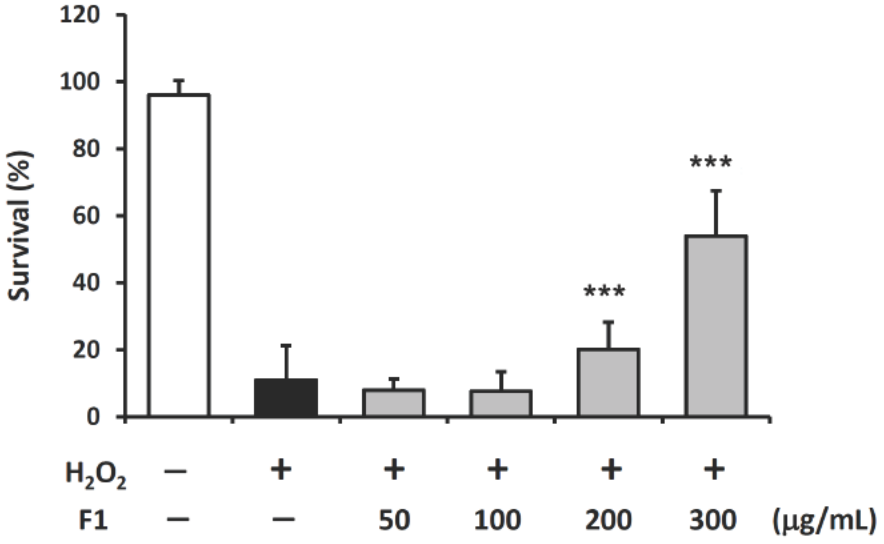

Figure 2-6. Effects of Maillard reaction products isolated from Heat-dried green tomato (HGT) extract on H_2O_2-induced neurotoxicity in *C. elegans*. Each experiment was repeated independently at least three times. The results are presented as mean ± SD. *** $p < 0.001$ compared to control.

CHAPTER 3

Neuroprotective effects of *Glochidion littorale* leaf extract in *Caenorhabditis elegans*

1. Abstract

A number of plants used in folk medicine in Thailand and Eastern Asia are attracting interest due to the high bioactivities of their extracts. The aim of this study was to screen the edible leaf extracts of 20 plants found in Thailand and investigate the potential neuroprotective effects of the most bioactive sample. The total phenol and flavonoid content, and 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity were determined for all 20 leaf extracts. Based on these assays, *Glochidion littorale* leaf extract (GLE), which showed a high value in all tested parameters, was used in further experiments to evaluate its effects on neurodegeneration in *Caenorhabditis elegans*. GLE treatment ameliorated H_2O_2-induced oxidative stress by attenuating accumulation of reactive oxygen species and protected the worms against 1-methyl-4-phenylpyridinium-induced neurodegeneration. The neuroprotective effects observed may be associated with the activation of the transcription factor, DAF-16. The LC-MS analysis showed that m/z of compounds in GLE corresponded to several phenolic compounds, including myricetin, coumestrin, chlorogenic acid, and hesperidin, which may play a key role in neuroprotection. This study reports the novel neuroprotective activity of GLE which may be used to develop treatments for neurodegenerative diseases such as Parkinson's syndrome.

2. Introduction

Neurodegenerative disorders including AD and Parkinson's disease PD pose major health and financial concerns to global health care organizations [108]. Although human lifespan has increased in the last few decades in industrialized countries, the prevalence of age-related diseases has also increased. The incidence of late-onset disorders such as neurological disruptions is expected to increase rapidly over the next few decades. Therefore, it is crucial to encourage studies and perform clinical trials on compounds that may have the potential to cure, prevent, or at least delay the onset of neurodegenerative diseases [109-110]. In PD pathogenesis, an increased production of ROS plays a key role in the loss of dopaminergic neurons [111]. Therefore, the reduction of oxidative stress is considered a promising therapeutic approach in PD treatment [112]. The MPP^+, which inhibits mitochondrial complex I activity, can induce PD-like symptoms in humans and animal models [113].

The use of *Caenorhabditis elegans* as an *in vivo* model provides certain advantages in the study of PD [39], [114]. The nematode is simple, inexpensive, and has a short life cycle. It supports studies involving large-scale analysis. Moreover, the neuronal network of *C. elegans* has been mapped completely [44], [115]. It contains 8 DA neurons and PD-related homologous genes [116]. Neurodegeneration, which mimics parkinsonian symptoms, can be induced in *C. elegans* via treatment with neurotoxins such as MPP^+ [116].

Polyphenols are known to be among the most abundant antioxidants in the human diet [117]. It has also been established that oxidative processes are involved in many pathologies including neurodegeneration, cancer, diabetes, cardiovascular and anti-inflammatory diseases. Hence, finding polyphenols exhibiting antioxidant properties from natural sources could contribute towards preventing or treating those pathologies. This study focused on extracts from the edible

leaves of plants found in Thailand. Most varieties cultivated widely in northern and southern Thailand have been used as folk medicine against general injuries and diseases; however, there are few reports concerning their neuroprotective effects.

In this study, we first screened the extracts of edible leaves from 20 plants cultivated in Thailand and assessed their phenolic and flavonoid contents, and their DPPH radical-scavenging activity. The effects of *Glochidion littorale* leaf extract (GLE), which showed a high value in all tested parameters, were evaluated on *C. elegans* with neurodegeneration. Furthermore, the potential pathways involved in the neuroprotective effect of GLE were examined, along with the identification of the main components in GLE.

3. Materials and methods

3.1. Materials

The leaves of 20 different plants (Table 3-1) were obtained from a local market in Chiang Rai, Thailand. All reagents were of analytical grade. DPPH and 2',7'-dichlorodihydrofluorescein-diacetate (H_2DCF-DA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

3.2. Preparation of leaf extracts

Leaf samples were frozen in liquid nitrogen and then powdered samples (5 g) were mixed in 100 mL of distilled water at 45°C for 30 min, following sonication using a Branson SLPe Sonifier (Branson, North Billerica, MA, USA) at 35 kHz. The extract was filtered and freeze-dried to obtain a powdered sample.

3.3. Total phenolic content

The Folin-Ciocalteu method was used to determine the total phenolic content. Briefly, 11.4 µL of the extract (1 mg/mL) was mixed with 227.3 µL of 2% (w/v) Na_2CO_3 solution and then the mixture was allowed to stand at room temperature for 2 min. After addition of 11.4 µL of 10% (w/v) Folin-Ciocalteu reagent. The incubation in the dark was conducted for 30 min. Subsequently, the absorbance was measured at 750 nm using a using a microplate reader (Nivo 3F Multimode Plate Reader, PerkinElmer, Waltham, MA, USA). Gallic acid was used as a standard for the calibration curve. The total phenolic content was expressed as gallic acid equivalents (mg gallic acid equivalent/g of plant extract).

3.4. Total flavonoid content

The aluminum chloride colorimetric method was used to measure the total flavonoid content. Briefly, 25 µL of the extract (2 mg/mL) was mixed with 7.5 µL of 5% (w/v) $NaNO_2$ solution and

152.5 µL of distilled water. After 6 min, 15 µL of 10% (w/v) $AlCl_3$ solution was added and allowed to stand for 5 min. Then, 50 µL of 1 M NaOH solution was added to the mixture. Subsequently, the mixture was incubated in the dark for 15 min and the absorbance was measured at 510 nm using a microplate reader. The total flavonoid content was calculated by generating a calibration curve using quercetin as a standard, and the results were expressed as quercetin equivalent (mg quercetin equivalent/g of plant extract).

3.5. Free radical-scavenging activity

The capacity to scavenge free radicals was assessed using DPPH assays [92]. Briefly, 100 µL of the extract (1 mg/mL) were mixed with 100 µL of DPPH solution. After 30 min, the absorbance was measured at 517 nm using a microplate reader. The results were expressed as percentage of inhibition of the DPPH radicals.

3.6. *C. elegans* maintenance

Wild-type N2, CF1038 (*daf-16(mu86) I*), CB1370 (*daf-2(e1370) III*), and TJ356 (*zIs356 [daf-16p::daf-16a/b::GFP + rol-6(su1006)]*) strains and their diet, *Escherichia coli* OP50, were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN, USA). According to the standard protocols, N2, CF1038, and TJ356 strains and CF 1370 strain were maintained at 20°C and 15°C, respectively, on NGM agar plates containing heat inactivated *E. coli* OP50 [93]. S-complete solution was prepared according to previously described literature [95].

3.7. Oxidative stress assay

Oxidative stress was induced by treating wild-type (N2) and daf-16 mutant (CF1038) worms with H_2O_2. L1 larvae were added to 96-well plates at an average of 15 nematodes per well in a 40 µL solution containing *E. coli* OP50. Five mM of H_2O_2 solution and the tested GLE dissolved in S-complete solution were added to achieve a final volume of 50 µL per well. L1 larvae were

incubated for 48 h with H_2O_2 alone or in the presence of various concentrations of GLE, and worm viability was visually inspected under a stereomicroscope. The results from the H_2O_2-treated groups were normalized and expressed as a percentage of normal controls. The results were obtained from three independent experiments (100–160 worms/treatment in each experiment).

3.8. Intracellular ROS levels

Intracellular ROS levels were determined using the H_2DCF-DA probe. L1 larvae of wild-type N2 worms were treated with 5 mM H_2O_2 and GLE at different concentrations in S-solution for 48 h in black 96-well plates; each well comprised a minimum of 100 worms. Worms were subsequently incubated with 25 μM H_2DCF-DA in the dark at 20°C for 1 h. After incubation, the fluorescence intensity was measured at wavelengths of 485/530 nm using a Powerscan HT microplate reader (DS Pharma Biomedical, Osaka, Japan).

3.9. Neurotoxicity assay

Neurotoxicity was induced by treating wild type (N2) and transgenic (CF1038 and CB1370) worms with MPP^+. L1 larvae were added to 96-well plates (15 worms/well) in a 40 μL solution containing *E. coli* OP50. Worms were then incubated with 50 μL of 0.75 mM MPP^+ alone or in the presence of different concentrations of the tested sample for 48 h. After incubation, worm viability was visually inspected under a stereomicroscope. Live worms were counted every 12 h post treatment until no live worms remained. The results of the MPP^+-treated groups were normalized and expressed as a percentage of the normal controls. The results were obtained from three independent experiments (80–130 worms/treatment in each experiment).

3.10. Nuclear localization of DAF-16

Transgenic *C. elegans* TJ356, which expresses a DAF-16-GFP fusion protein, was used to examine the intracellular distribution of DAF-16. L1 stage nematodes were treated with GLE for

48 h at 20°C. The worms were then transferred to a 2% agarose pad on a glass slide and anesthetized by adding one drop (approximately 20 µL) of 25 µM sodium azide to the agarose pad. The expression of GFP was examined via fluorescence microscopy (EVOS fl; Advanced Microscopy Group, Bothell, WA, USA). The mean fluorescence intensity of DAF-16 in the nuclei was analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

3.11. Phytochemical profiling using LC-MS

The leaf extract was analyzed using the LCMS-8040 (Shimadzu). Mass spectra were acquired over a range of m/z 50 – 1000 using the Q3 scan mode. The solution was injected onto an Inertsil ODS-3 (250 mm 2.1 mm, 5 µm, GL Sciences, Tokyo Japan) at a column temperature at 40°C using a gradient of (A) 0.1% formic acid and (B) acetonitrile/water (80/20) containing 0.1% formic acid. The following gradient with a flow rate of 0.2 mL/min was used: 0–100% B (0–45 min), 100% B (45–50 min), and 0% B (50–60 min). Compounds were putatively identified by matching the experimental m/z values to the library of theoretical calculated m/z values in databases, including the Human Metabolome Database and the METLIN database.

3.12. Statistical analysis

Data were expressed as the mean ± standard deviation for each group. Significant difference between two groups was assessed using the *t*-test, whereas difference between three and more groups was assessed using one-way ANOVA, followed by Tukey's post-hoc comparison test. Statistical significance was set at $p < 0.001$ and $p < 0.0001$. For lifespan assays, *C. elegans* survival was plotted using Kaplan–Meier survival curves and analyzed via log-rank tests using GraphPad Prism software (version 9.01; GraphPad software, San Diego, CA, USA).

4. Results

4.1. Screening of Thai plant leaves

Crude extracts of edible leaves from plants cultivated in Thailand were prepared by ultrasonication. The leaf extracts of 20 plants were screened for their phenolic and flavonoid contents, and antioxidant activity by DPPH radical-scavenging assay. Few of the tested samples, such as *Glochidion sphaerogynum* and *Mentha piperita,* were found to possess high radical-scavenging activity with low phenolic and flavonoid content, whereas certain samples, such as *Clinacanthus nutans* and *Ocimum citriodorum*, exhibited the opposite trend (Table 3-1). The leaf extract of *G. littorale* showed high DPPH radical-scavenging activity as well as high phenolic and flavonoid content. Therefore, the bioactivities associated with *G. littorale* were further investigated.

4.2. GLE enhanced resistance against oxidative stress via DAF-16 in *C. elegans*

The effect of GLE on the survival of N2 worms under oxidative stress was investigated. Treatment with H_2O_2 (5 mM) induced 75% death in the control group, whereas co-treatment with 50 μg/mL and higher concentrations of GLE was associated with a high survival rate (Figure 3-1A). Among the tested concentrations of GLE, 100 μg/mL and 200 μg/mL were associated with the highest survival rates (82.0% and 88.2%, respectively). Therefore, these two concentrations were used in subsequent experiments. To evaluate the antioxidant effect of GLE *in vivo*, the intracellular ROS levels were measured in wild-type nematodes using H_2DCF-DA, a well-known fluorescence probe for detecting intracellular ROS production. Significant decreases in the fluorescence intensities in the GLE-treated groups were observed compared to that in the untreated group (Figure 3-1B), confirming the antioxidant property of GLE.

As the transcription factor DAF-16 is known to play a key role in regulating oxidative stress [11], it was hypothesized that GLE may target DAF-16. The *C. elegans* strain CF1038, which is a DAF-16 loss-of-function mutant strain, was used to determine the survival rate of worms treated with and without GLE. In H_2O_2-induced oxidative stress, GLE treatment did not increase the survival rate of transgenic worms (Figure 3-1C).

4.3. GLE treatment reduced the lethality of MPP^+-induced DA neurotoxicity via DAF-16 in *C. elegans*

C. elegans possesses 8 DA neurons [78]. Selective degeneration of these DA neurons was evaluated after exposure to MPP^+. The treatment of wild-type N2 worms with 0.75 mM MPP^+ resulted in a remarkable decrease in survival (Figure 3-2). However, co-treatment with GLE significantly increased the survival of the worms. The effect of GLE treatment on *daf-16* mutant worms was investigated. As shown in Figure 3-3 and Table 3-2, GLE treatment did not increase the survival of these worms after exposure to MPP^+ compared to that in the control group. These results suggest that DAF-16 may be required for mediating the neuroprotective effect of GLE in *C. elegans*. Next, a DAF-2 loss-of-function mutant strain, CB1370, was used to determine whether DAF-2 was involved in the observed neuroprotective effects. As shown in Figure 3-4 and Table 3-3, the median and maximum survival significantly increased in *daf-2* mutant worms treated with GLE.

4.4. Effect of GLE on DAF-16 localization

It has been demonstrated that DAF-16 activation is regulated by its nuclear accumulation [118]. Subsequently, we investigated whether GLE could induce the nuclear accumulation of DAF-16 in a transgenic strain TJ356 that expresses a DAF-16::GFP fusion protein. Results showed that after

48 h of incubation with 100 µg/mL GLE, the green fluorescence intensity of DAF-16 in the nucleus increased significantly compared to that in the untreated group (Figure 3-5).

4.5. Phytochemical characterization in GLE

LC-MS was conducted for profiling the phytochemicals in GLE and its results are presented in Figure 3-6. The chromatographic peaks were identified by comparing the MS data with databases based on the search of m/z values of molecular ion peaks in the positive mode $[M + H]^+$. Consequently, myricetin, coumestrin, chlorogenic acid, and hesperidin were detected as the major compounds (Table 3-4).

5. Discussion

Plant extracts are a rich source of natural bioactive compounds. Many studies have evaluated plant extracts used in Southeast Asian countries including Thailand where these extracts are components of folk medicine [119-121]. In this study, the extracts of 20 edible plant leaves from Thailand were screened, and *G. littorale* was selected for further investigation because it showed high phenol content, flavonoid content, and radical-scavenging activity. Several studies have investigated various species of the genus *Glochidion* [119–122]; however, there are few studies concerning the functional properties and constituents of *G. littorale.* Our data showed that GLE protected *C. elegans* against H_2O_2-induced oxidative stress by reducing intracellular ROS accumulation. This might have been due to the high content of phenolic compounds such as flavonoids which are known to possess strong antioxidant activity [123]. These findings are similar to those obtained by Duangjan et al., (2019) who showed that *G. zeylanicum* leaf extracts can protect *C. elegans* against oxidative stress [62]. The IIS pathway regulates growth, stress responsiveness, and longevity in *C. elegans* [124-125]. We found that *daf-16* null mutant *C. elegans* treated with GLE were susceptible to oxidative stress. This result suggests that the

antioxidant effect of GLE in reducing oxidative stress in nematodes is possibly involved in not only radical scavenging activity but also the regulation of DAF-16 transcription factor.

The protective effects of GLE against MPP^+-induced toxicity in *C. elegans* were examined. DA neurons in nematodes take up MPP^+ mainly via high-affinity DA transporters, which is similarly observed in mammals. The accumulation of MPP^+ inside the neurons inactivates the mitochondrial complex I of the respiratory chain and induces cell death [57], [126-127]. GLE treatment was found to significantly reduce the lethality associated with MPP^+ treatment in wild-type worms. The IIS pathway is modulated by insulin-like peptides through the DAF-2 receptor in *C. elegans* [128]. Under normal conditions, the IIS pathway inhibits the phosphorylation of DAF-16 and prevents its nuclear translocation. In *daf-2* null mutants, the GLE-treated group survived longer than the control group. In contrast, no difference in survival was observed between the control group and GLE-treated group containing *daf-16* null mutant worms. It is known that downregulated DAF-2 signaling facilitates the entry of DAF-16 into the nucleus where it can upregulate the expression of target genes, and control stress resistance and longevity [129]. This may explain why *daf-2* mutant worms treated with GLE showed a relatively higher survival. Furthermore, an increased nuclear accumulation of DAF-16 in worms treated with GLE was observed using transgenic TJ356 DAF-16::GFP *C. elegans*. Cumulatively, these results indicated that GLE might have exhibited its neuroprotective effects via activation of DAF-16.

LC-MS profiling led to the identification of 11 phytochemical compounds in GLE. Myricetin identified in the main peak of GLE is a flavonoid widely found in many plants and is well known to exhibit protective effects against oxidative stress. A previous study has demonstrated that myricetin extended the lifespan of *C. elegans* by diminishing stress-induced ROS accumulation and the pro-longevity effects of myricetin were dependent on DAF-16 [130]. Chlorogenic acid has

been also reported to exhibit pro-longevity effects via the attenuation of oxidative stress in *C. elegans* [131]. Considering these findings, the neuroprotective effects of GLE were mainly induced by flavonoid such as myricetin, and GLE might be a good candidate for management of neurodegenerative diseases. However, it is to be noted that a confirmation of the observed phytochemical compounds should be done through LC-MS/MS and check whether the ion patterns of identified peaks coincide with those of the authentic phenolic compounds.

In conclusion, our study demonstrated that GLE possessed strong antioxidant activity which reduced oxidative stress in *C. elegans*. The extract also showed neuroprotective activity against MPP^+-induced neurotoxicity in *C. elegans*. Various experiments performed using different transgenic worms suggested the possible involvement of the DAF-16 transcription factor in the observed neuroprotection. The high content of phenolic compounds including flavonoids present in GLE may be responsible for the observed stress resistance and neuroprotective properties. Further studies should identify the target genes involved in the neuroprotection mechanism.

Table 3-1. Properties of the plants investigated in this study.

No.	Scientific name	Phenolic content (mg GAE[1]/g)	Flavonoid content (mg quercetin/g)	DPPH[2] radical scavenging activity (%)
1	*Clinacanthus nutans*	3.306	1.799	41.058
2	*Gymnema inodorum*	1.183	0.959	33.890
3	*Glochidion sphaerogynum*	0.709	0.781	51.705
4	*Anethum graveolens*	0.825	0.387	29.367
5	*Spilanthes acmella*	0.552	0.243	18.580
6	*Acacia pennata*	5.031	1.563	51.914
7	*Mentha piperita*	1.107	0.761	59.151
8	*Glochidion littorale*	20.104	4.527	78.984
9	*Ocimum sanctum* Linn.	0.131	0.076	45.442
10	*Ocimum basilicum* Linn.	1.342	0.771	51.635
11	*Ocimum* × *citriodorum*	2.446	1.612	39.666
12	*Azadirachta indica*	13.744	2.725	79.819
13	*Morus Alba*	6.190	4.019	54.488
14	*Moringa oleifera*	1.696	5.696	18.928
15	*Psidium guajava* Linn.	3.414	2.937	61.865
16	*Melientha suavis* Pierre	2.263	2.433	40.362
17	*Pandanus amaryllifolius*	1.409	1.050	36.395
18	*Zanthoxylum limonella*	3.128	1.469	46.555
19	*Piper sarmentosum*	0.584	0.487	24.217
20	*Citrus maxima*	11.690	2.461	79.193

[1] GAE, gallic acid equivalent; [2] DPPH, 2,2-diphenyl-1-picrylhydrazyl.

Table 3-2. Survival of *daf-16* mutant *C. elegans* treated with MPP^+.

Survival time	MPP^+ [1]	MPP^+ + GLE (100 µg/mL)	MPP^+ + GLE (200 µg/mL)
Median (h)	48.0 ± 1.2	48.0 ± 1.7	48.0 ± 1.6
Maximum (h)	72.0 ± 1.5	72.0 ± 2.1	72.0 ± 1.8

[1] MPP^+, 1-methyl-4-phenylpyridinium.

Table 3-3. Survival of *daf-2* mutant *C. elegans* treated with MPP^+.

Survival time	MPP^+ [1]	MPP^+ + GLE (100 µg/mL)	MPP^+ + GLE (200 µg/mL)
Median (h)	60.0 ± 2.6	84.0 ± 3.9 ***	84.0 ± 4.0 ***
Maximum (h)	108.0 ± 5.3	180.0 ± 5.5 ****	192.0 ± 9.6 ****

***$p < 0.001$, ****$p < 0.0001$ vs MPP^+-treated worms.

Table 3-4. Compounds identified from the chromatogram of GLE.

Peak	Retention time (min)	$[M + H]^+$ (m/z)	Identified compounds	Theoretical mass	Mass error (ppm)
1	8.7	431.0973	Coumestrin	430.0900	6
2	8.8	299.2005	All-trans-3,4-didehydro-retinoic acid	298.1933	1
3	9.0	248.2009	Lycopodine	247.1936	3
4	9.2	289.0707	2-Hydroxynaringenin	288.0634	10
5	10.0	166.1226	Hordenine	165.1154	1
6	15.3	355.1024	Chlorogenic acid	354.0952	7
7	18.3	611.1970	Hesperidin	610.1898	5
8	20.7	449.1078	Quercitrin	448.1006	17
9	21.2	459.0922	Epigallocatechin gallate	458.0849	3
10	25.3	319.0448	Myricetin	318.2370	5
11	26.2	465.1028	Isoquercitrin	464.0955	3
12	26.6	465.3575	Unknown	–	–
13	27.3	567.3038	Unknown	–	–

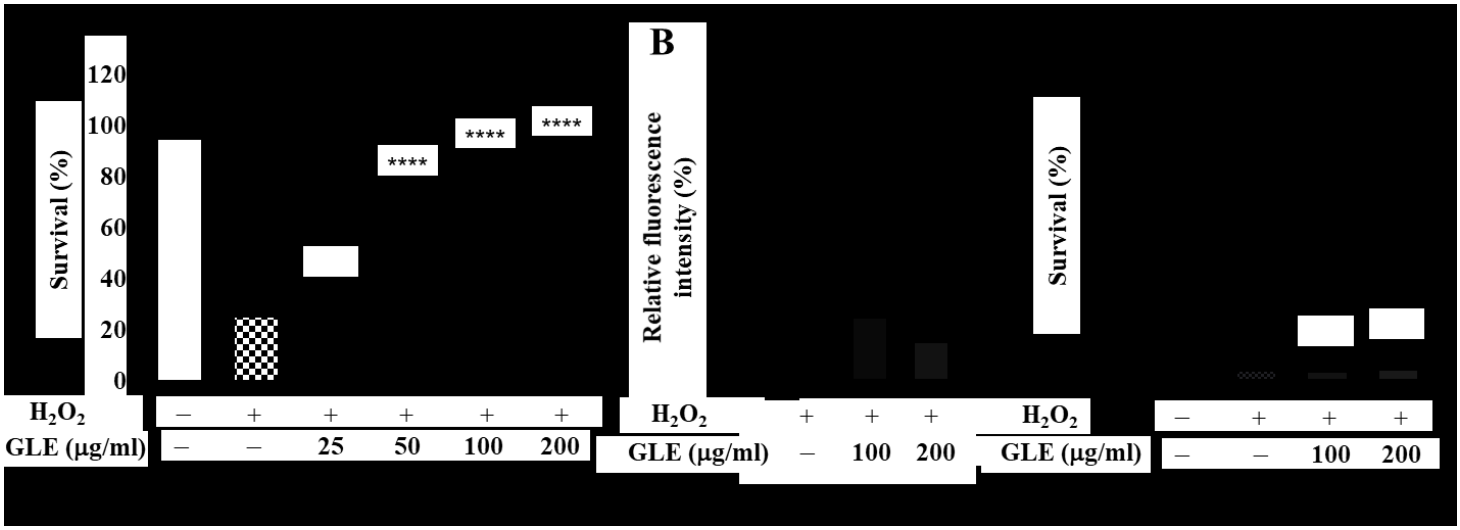

Figure 3-1. Effect of *Glochidion littorale* leaf extract (GLE) on stress resistance in wild-type and *daf-16* mutant *Caenorhabditis elegans*. A) Effect of GLE against H_2O_2-induced toxicity in wild-type worms. B) Intracellular reactive oxygen species (ROS) contents in wild-type worms. C) Effect of GLE against H_2O_2-induced toxicity in *daf-16* mutant worms. Experiments were performed in triplicate. Data are presented as mean ± standard deviation (SD). $^{****}p < 0.0001$ compared to H_2O_2-treated worms.

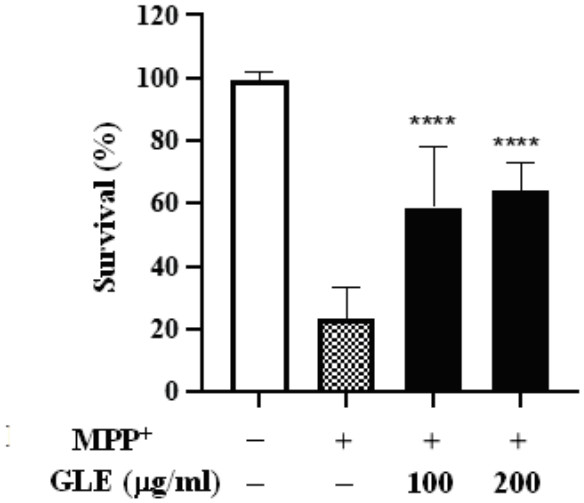

Figure 3-2. Effect of GLE on 1-methyl-4-phenylpyridinium ion (MPP^+)-induced neurotoxicity in N2 *C. elegans.* The effects of GLE (100 and 200 µg/mL) on MPP^+-induced toxicity were evaluated. Experiments were performed in triplicate. Data are presented as mean ± SD. $^{****}p < 0.0001$ compared to MPP^+-treated worms.

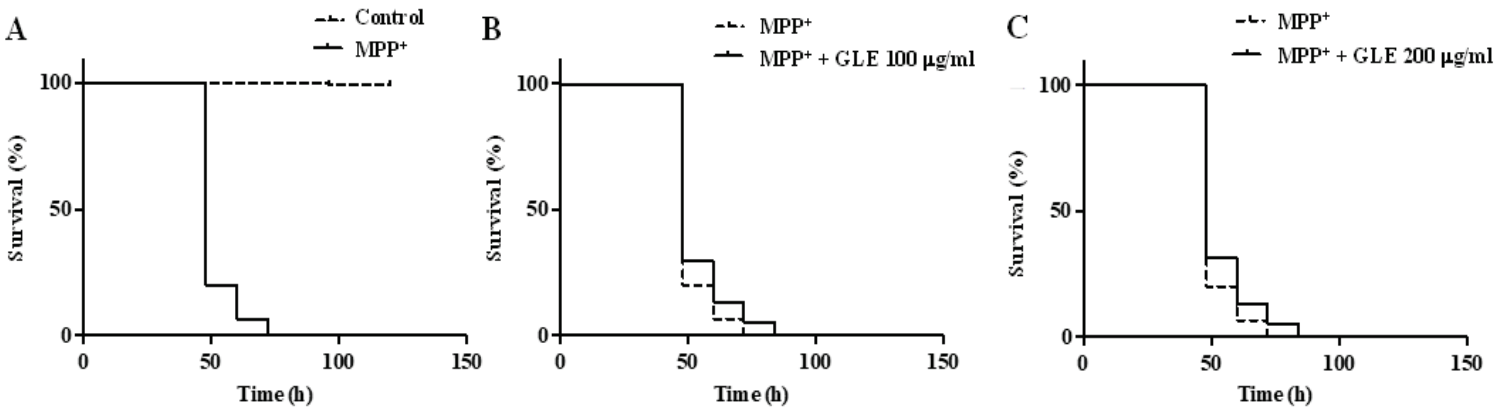

Figure 3-3. Effect of GLE on MPP^+-induced neurotoxicity in *daf-16* mutant *C. elegans*. A) Lifespan curve of worms in the presence or absence of MPP^+. B) Lifespan curve of worms with MPP^+-induced toxicity treated with 100 µg/mL GLE. C) Lifespan curve of worms with MPP^+-induced toxicity treated with 200 µg/mL GLE. Each experiment was repeated independently at least thrice and one of the representative data is shown.

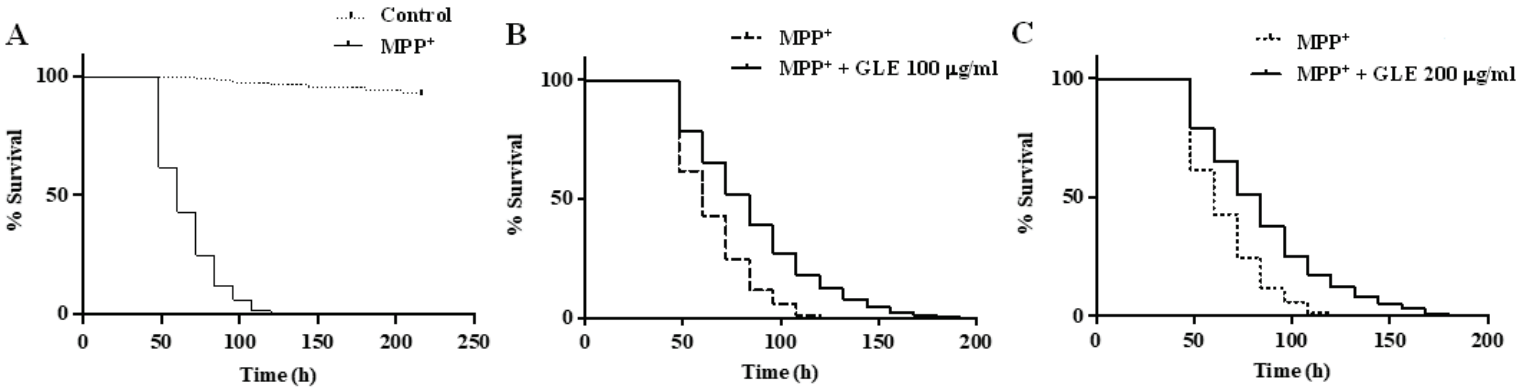

Figure 3-4. Effect of GLE on MPP^+-induced neurotoxicity in *daf-2* mutant *C. elegans*. A) Lifespan curve of worms in the presence or absence of MPP^+. B) Lifespan curve of worms with MPP^+-induced toxicity treated with 100 µg/mL GLE. C) Lifespan curve of worms with MPP^+-induced toxicity treated with 200 µg/mL GLE. Each experiment was repeated independently at least thrice and one of the representative data is shown.

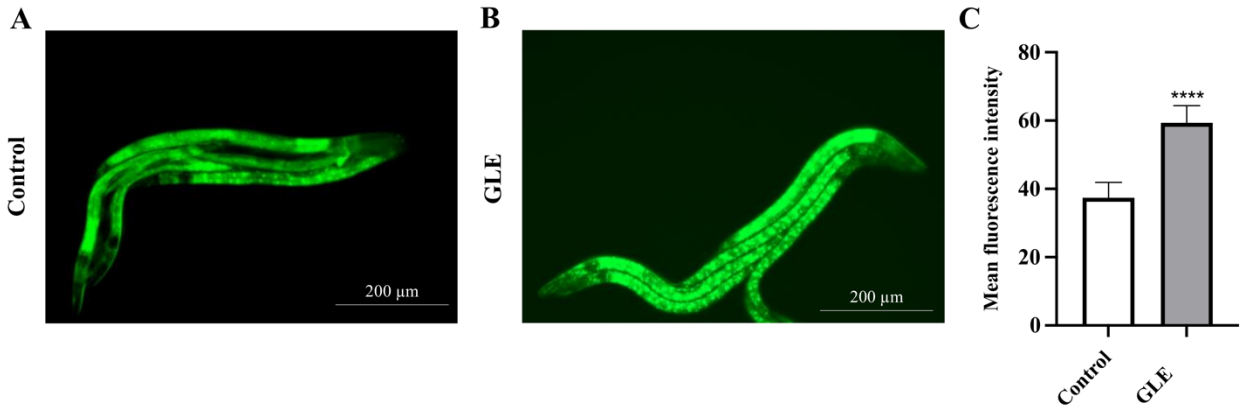

Figure 3-5. Effect of GLE on DAF-16 localization. A) Untreated worms. B) Worms treated with 100 µg/mL GLE. C) Quantification of DAF-16::GFP nuclear accumulation in GLE and GLE-free conditions. The scale bar shows 200 µm. Each experiment was repeated independently at least thrice. Significant differences were analyzed using the *t*-test method; $^{****}p < 0.0001$ as compared with control.

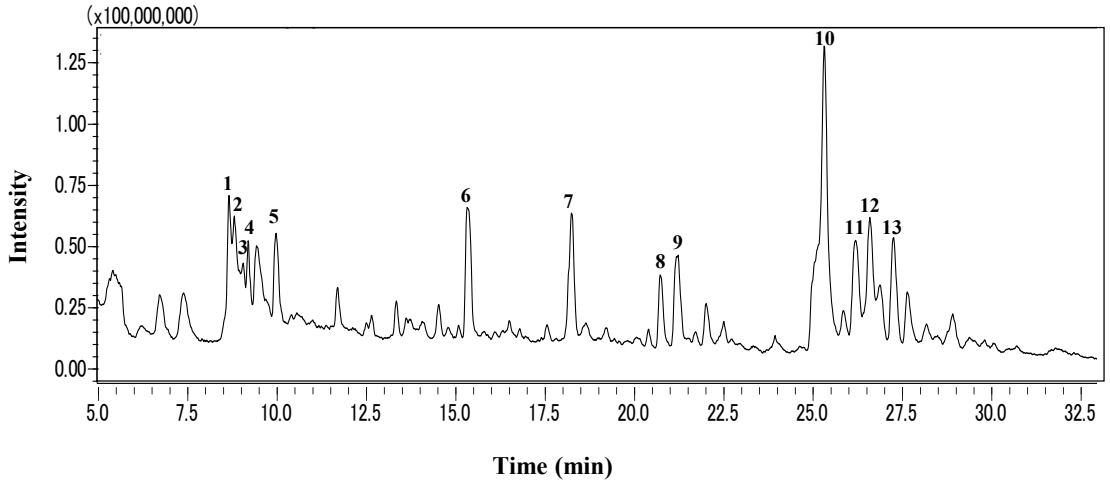

Figure 3-6. LC-MS profile of GLE. The total ion chromatogram was obtained by a triple quadrupole mass spectrometer operated in the positive electrospray ionization mode.

CHAPTER 4

Summary and Conclusion

This study was conducted to assess the neuroprotective effects of some plant extracts. In the first study, lifespan extension and neuroprotective effect of dry tomato extract were evaluated using *C. elegans*. Maillard reaction was induced through drying process at 40 °C for 7 days and formation of MRPs was confirmed by measuring the UV absorbance at 420 nm. Green tomato samples and its MRPs fraction showed a stronger radical-scavenging activity compared to red tomato samples. Lifespan assay showed that extracts of dried green tomato and its MRPs fraction increased significantly the median and maximum lifespan of *C. elegans*. To investigate the effect against Aβ-induced toxicity, we used transgenic CL2006 *C. elegans* expressing human Aβ specifically in the body wall muscle. Compared with the control, MRP fraction significantly extended the median and maximum lifespan of CL2006 worms. Interestingly, MRP fraction treatment with MPP^+ (a dopaminergic neuronal toxin that induces oxidative stress and apoptosis) protected the worms from an obvious neurodegeneration by increasing their survival rate in a dose-dependent manner. As aging is mainly caused by free radical damage, it was hypothesized that the MRP fraction which seems to contain high molecular weight compounds, might extend lifespan of worms by reducing those free radicals.

For the second study, the neuroprotective effect of GLE on *C. elegans* and the probable mechanism involved have been investigated. The effect of GLE on N2 *C. elegans* survival under oxidative stress was evaluated. The result showed that worms treated with GLE increased significantly ($p < 0.0001$) oxidative stress resistance compared to the untreated control group. A significant decrease in ROS fluorescence intensity in GLE treated group compared to the untreated group was observed. Repeating the oxidative stress experiment using *daf-16* mutant worms

showed no stress resistance in GLE treated group compared to the untreated group. As DAF-16 transcription factor is known to play a key role in regulating oxidative stress, we concluded that the transcriptional activity of DAF-16 might be a prerequisite before GLE to induce stress resistance in *C. elegans*. Similarly, GLE protected worms against MPP^+ induced neurotoxicity however, it failed to show any protection effect in daf-16 mutant. Additionally, GLE increased the nuclear accumulation of DAF-16 in *C. elegans*. DAF-16/FoxO is one of the main components of the IIS. The activation of the DAF-2/insulin receptors induces a cascade of reactions leading to the phosphorylation of DAF-16 which remains inactivate in the cytoplasm. GLE might reduce DAF-2 activity which would induce dephosphorylation of DAF-16 and facilitate its nuclear translocation. In the nucleus DAF-16 will participate in regulation of oxidative stress resistance, longevity, and neuroprotection.

In conclusion, the antioxidant properties observed in both MRP fraction in tomato and GLE might be responsible for the longevity and neuroprotection effects observed in this study. The extracts might act via the IIS pathway by downregulating DAF-2 receptor and inducing the nuclear accumulation of DAF-16 transcription factor. Both extracts used in this study provided interesting data for further investigations in aging and age-related diseases research.

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