Neuroprotective Screening of Natural Phenolics in an MPTP-induced Model of Parkinson's Disease using Zebrafish Larvae

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Abstract

This study investigates the neuroprotective effects of Ascorbic Acid (AA), Vanillic Acid (VA), and Ferulic Acid (FA) in a zebrafish model of Parkinson's Disease (PD), induced by MPTP. The research focuses on dopaminergic neurons, assessing responses to these compounds in terms of gene expression, mitochondrial dynamics, cellular stress responses, and cardiac health. Using quantitative polymerase chain reaction (qPCR) and Immunohistochemistry (IHC), this study examines the influence of AA, VA, and FA on dopaminergic function and neuronal health. AA was found to enhance dopaminergic function, indicated by upregulation of the dopamine transporter (DAT) gene and an increase in eGFP+ dopaminergic cells, suggesting improved dopamine reuptake and potential neuroprotection. AA also positively affected mitochondrial dynamics and stress response pathways, countering MPTP-induced dysregulation. VA influenced dopaminergic pathways and showed a nuanced effect on mitochondrial dynamics, though its impact on cardiac abnormalities was limited. FA displayed significant neuroprotective effects through modulation of gene expression related to dopaminergic neurons and mitochondrial dynamics, with extended benefits to cardiac health. These findings suggest that AA, VA, and FA may serve as potential protective interventions for Parkinson's Disease, reflecting various beneficial effects at the molecular and cellular levels. However, the translation of these results to practical clinical applications requires further investigation. This study contributes to the understanding of neuroprotective strategies in neurodegenerative diseases, highlighting the need to consider interactions between physiological systems. The direct applicability of these results in clinical PD settings warrants extensive future research.

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List of Abbreviations

AADC- Aromatic Amino Acid Decarboxylase

AA- Ascorbic Acid

ACVS- Animal Care and Veterinary Service

BBB- Blood Brain Barrier

cDNA- complementary DNA

CNS- Central Nervous System

DA- Dopamine

DAnergic- Dopaminergic

DAT- Dopamine Transporter

Drp1- dynamin-related protein 1

Dyn2- dynamin-2

Dpf- day post fertilization

ETC- electron transport chain

ER- endoplasmic reticulum

ef1a- elongation factor 1 alpha

FA- Ferulic Acid

Fis1- mitochondrial fission protein 1

GFP- green fluorescent protein

L-DOPA- L-Dihydroxyphenylalanine

MAO- Monoamine Oxidase

Mff- mitochondrial fission factor

Mfn- Mitofusin

Mid49 and Mid51- dynamics protein 49 and 51

MPP+- 1-methyl-4-phenylpyridinium

MPTP- 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MSNs- Medium Spiny Neurons

mtDNA- mitochondrial DNA

NAc- Nucleus Accumbens

NPT- Neuroprotective treatment

Opa1- optic atrophy-1

PD- Parkinson's Disease

PINK1- PTEN-induced kinase 1

PNS- Peripheral Nervous System

ROS- reactive oxygen species

rpl13a- ribosomal protein L 13a

SNpc- Substantia Nigra pars compacta

TH- Tyrosine Hydroxylase

VA- Vanillic Acid

VMAT2- Vesicular Monoamine Transporter 2

VTA- Ventral Tegmental Area

vDC- ventral diencephalic

6-OHDA- 6-hydroxydopamine

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1. Introduction

1.1.Dopamine Biosynthesis

Dopamine (DA), a small catecholaminergic neurotransmitter, exerts a significant influence on both the central nervous system (CNS) and the peripheral nervous system (PNS). Its involvement in a diverse range of behaviors, from basic movements to complex experiences like pleasure and memory, has placed it in the forefront of vertebrate neurological research for decades (Wise, 2004). The biosynthesis of DA begins in the liver, where the enzyme phenylalanine hydroxylase catalyzes the conversion of dietary phenylalanine to tyrosine. This tyrosine is carried through the bloodstream to the brain, crossing the blood-brain barrier (BBB), and entering dopaminergic (DAnergic) neurons. Within these neurons' cytosol, tyrosine hydroxylase (TH) introduces a hydroxyl group to the meta position of tyrosine, forming dihydroxyphenylalanine (L-DOPA) (Figure 1). This step is crucial and often rate-limiting due to its oxygen requirements and the need for tetrahydrobiopterin, a vital cofactor for TH (Daubner et al., 2011; Ayano G, 2016). Notably, DA negatively regulates TH through a feedback mechanism, competing with tetrahydrobiopterin.

Aromatic amino acid decarboxylase (AADC or DOPA decarboxylase) further converts L-DOPA to cytosolic DA. This DA is actively transported into pre-synaptic vesicles by the vesicular monoamine transporter 2 (VMAT2), accumulating there for release following action potentials of DAnergic neurons. The released DA interacts with G-protein coupled DA receptors located extensively throughout the CNS and PNS (Elsworth and Roth, 1997). After release, any remaining

DA in the synaptic cleft can be reabsorbed by the dopamine transporter (DAT), facilitating its re-packaging into vesicles or its degradation by enzymes like monoamine oxidase (MAO) or glial cells outside the neuron (Meiser et al., 2013) (Figure 1). It's worth noting that neurons containing dopamine beta-hydroxylase and phenylethanolamine N-methyl transferase can convert DA to norepinephrine and epinephrine, respectively, but their location and function significantly differ from that of DAnergic neurons (Wong, 2006).

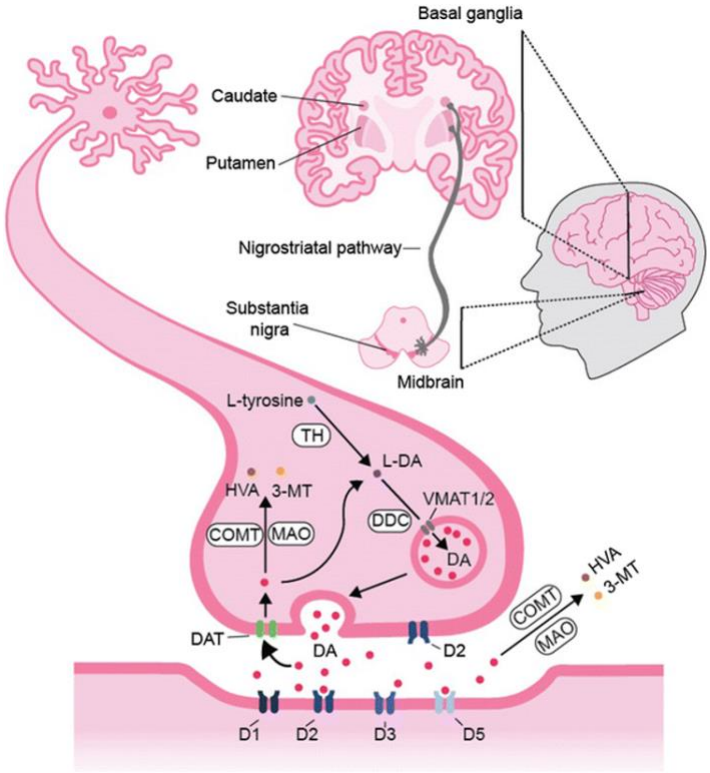


Figure 1- Schematic representation of the dopaminergic nigrostriatal pathway and dopamine metabolism in the human brain (Fernanda Lopes et al., 2017). The diagram illustrates the synthesis, release, and recycling of dopamine (DA) within the substantia nigra and its projection to the basal ganglia, specifically the caudate and putamen. Key enzymes involved in dopamine metabolism

such as tyrosine hydroxylase (TH), DOPA decarboxylase (DDC), monoamine oxidase (MAO), and catechol-O-methyltransferase (COMT) are highlighted, along with dopamine transporters (DAT) and receptors (D1, D2, D3, D5). This pathway is critical in understanding the neurobiological basis of Parkinson's disease and other movement disorders.

1.2.Dopaminergic Systems, Functions and Pathways

The functioning of the dopamine systems plays a crucial role in a variety of basic biological processes, ranging from bodily movement to inner drive. Consequently, any disruption in the transmission of dopamine signals can give rise to a wide array of severe mental and neurodegenerative disorders (Reeves et al., 2002; Dunlop and Nemeroff, 2007). Despite constituting less than 1% of the total brain cell population, midbrain dopamine-producing neurons are the primary originators of dopamine release into the central nervous system, profoundly influencing the overall balance of neurological functions (Arias-Carrión et al., 2010). These dopamine-producing neurons are widely scattered but are grouped into clusters, with varied populations found prominently in regions such as the olfactory bulbs, substantia nigra, striatum, thalamus, ventral tegmentum area, nucleus accumbens, and other specific parts of the midbrain, forebrain, and diencephalon (Chinta and Andersen, 2005). The study of neural circuits has identified four distinct pathways that regulate different aspects of behavior and emotion.

The mesolimbic pathway originates in the ventral tegmental area (VTA) of the midbrain and consists of extended fiber-like dopamine-producing projections. These projections terminate at various locations, including the nucleus accumbens (NAc) in the ventral striatum, olfactory

tubercle, pyriform cortex, and the amygdala. Additionally, there are other endpoints characterized by delicate axonal branching found in nuclei of the thalamus, habenula, and hypothalamus (Adinoff, 2004). Researchers propose that the primary function of this pathway is to modulate feelings of reward, motivation, stress, and desire. This means that activities like eating, mating, and pleasurable experiences all trigger the release of dopamine through the mesolimbic pathway (Gardner and Ashby, 2000; Adinoff, 2004). Notably, this circuit is particularly vulnerable to substances that induce stimulation, which can contribute to the development of addiction. Numerous studies have demonstrated that both the nucleus accumbens and the ventral tegmental area are widely dispersed and possess receptors for substances like nicotine, cannabinoids (Tsou et al., 1998), opioids (Margolis et al., 2014), and dopamine transporters (which are targeted by psychostimulants like cocaine and amphetamines; Brodie and Dunwiddie, 1990). Given the cognitive implications linked to the mesolimbic pathway, a significant portion of antipsychotic medications used to mitigate episodes of schizophrenia target dopamine receptors that are distributed throughout structures within this pathway (Adinoff, 2004; Volkow and Morales, 2015) (Figure 2).

Named appropriately, **the mesocortical pathway** comprises dopamine fibers connecting the medial region of the VTA to the medial prefrontal, cingulate, and perirhinal cortices, as well as to the septo-hippocampal regions in the brain. Intrinsically linked to the mesolimbic system, this pathway primarily governs functions related to cognitive control, motivation, and learning aimed at achieving goals (Hauser et al., 2017). However, it's important to note that these circuits don't always share the same roles (Quessy et al., 2021). A study highlighted this distinction by optogenetically inhibiting the mesocortical and mesolimbic pathways in mice, revealing that

inhibition of the mesocortical pathway made the mice more susceptible to social defeat and depression, while inhibition of the mesolimbic pathway made them more resilient to these stressors (Chaudhury et al., 2013). In human research, Hauser and colleagues further illuminated the distinct functions of the dorsomedial prefrontal cortex and the NAc in the context of predicting rewards and effort. They discovered that the dorsomedial prefrontal cortex primarily encodes effort prediction errors and estimations of apathy, but not reward prediction errors, while the opposite was observed for the ventral striatum (Hauser et al., 2017). Nevertheless, disruptions and irregularities in dopamine transmission within this pathway have been demonstrated to lead to psychiatric symptoms resembling those of schizophrenia and bipolar disorder. Additionally, cognitive impairments characteristic of other dopamine-related neurodegenerative conditions such as memory deficits and depression, commonly seen in Parkinson's patients, can arise from dysfunction in this pathway (Figure 2).

The tuberoinfundibular pathway is situated along the hypothalamo-pituitary tract and plays a vital role in regulating the neuroendocrine system. This pathway originates from dopamine synthesized in the arcuate nucleus of the hypothalamus. Neurons from this nucleus send upward projections to the median eminence of the pituitary gland, and dopamine is also actively transported through vessels connecting the hypothalamus and pituitary. Within this circuit, dopamine functions more like a hormone than a neurotransmitter. Its continuous activity suppresses the release and secretion of prolactin, a hormone essential for reproductive functions, by binding to D2 receptors on the anterior pituitary lobe. Prolactin has a significant role in regulating maternal lactation and care (Grattan, 2015). In mice, research has revealed that during the nursing phase, dopamine-producing neurons experience an increase in membrane potential

oscillation speed and depolarization events. This, in turn, leads to a decrease in dopamine levels during periods of high prolactin associated with lactation (Thörn Pérez et al., 2020). Following the weaning stage, the hyperpolarized state quickly returns to the baseline observed in mice that have not given birth, showcasing the adaptability and plasticity of dopamine-related signaling within one of the many neural pathways (Hodson et al., 2012; Thörn Pérez et al., 2020) (Figure 2).

The nigrostriatal pathway originates in the substantia nigra pars compacta (SNpc) within the midbrain and projects neurons that extend to form the basal ganglionic loop. The basal ganglia comprises a collection of subcortical nuclei that influence cortical motor signals received through the thalamus, forming the thalamocortical system (Lanciego et al., 2012). The efferent dopamine-producing neurons from the SNpc target the dorsal striatum, caudate nucleus, and putamen. In these regions, there exists a complex network of unmyelinated dopamine-producing branches and presynaptic terminals. Due to its close proximity to the brainstem and the specific structures involved, dopamine release within this pathway regulates aspects of movement and sleep. The balance between involuntary and voluntary movement is intricately managed by the interplay of indirect and direct signaling pathways within this system. The direct pathway is characterized by its excitatory dominance. Dopamine released from the SNpc binds to D1 receptors on striatal medium spiny neurons (MSNs) in the dorsal striatum. This binding suppresses GABAergic activity in the internal segment of the globus pallidus, exciting the motor cortex through glutamatergic signaling, ultimately leading to movement. On the contrary, the indirect pathway functions to inhibit motor activity. Dopamine binding to D2 receptors on striatal MSNs triggers an inhibitory signal that travels from the external segment of the globus pallidus to the subthalamic nucleus, which in turn stimulates the internal segment of the globus pallidus. Activation of the internal

globus pallidus, rich in GABA, leads to reduced cortico-thalamic signaling by increasing thalamic inhibition. It's important to note that the MSNs described above belong to distinct subpopulations within the striatum. Some exclusively express D1 receptors, others exclusively express D2 receptors, and a smaller group expresses both D1 and D2 receptors. Although these subpopulations exhibit unique characteristics in terms of dendritic spine density and neurodegeneration in mice, the exact role of MSNs expressing both D1 and D2 receptors in the direct and indirect control of movement remains unclear (Gagnon et al., 2017). Considering their impact on locomotion, dopamine-producing neurons in the SNpc are significantly affected in Parkinson's Disease (PD) and other movement disorders. In PD, the reduction in dopamine output from the SNpc leads to an amplified inhibitory response in the cortico-thalamic pathway and reduces glutamate input to the cerebral cortex, resulting in the characteristic rigid and tremor-related symptoms (Sulzer et al., 2016) (Figure 2).

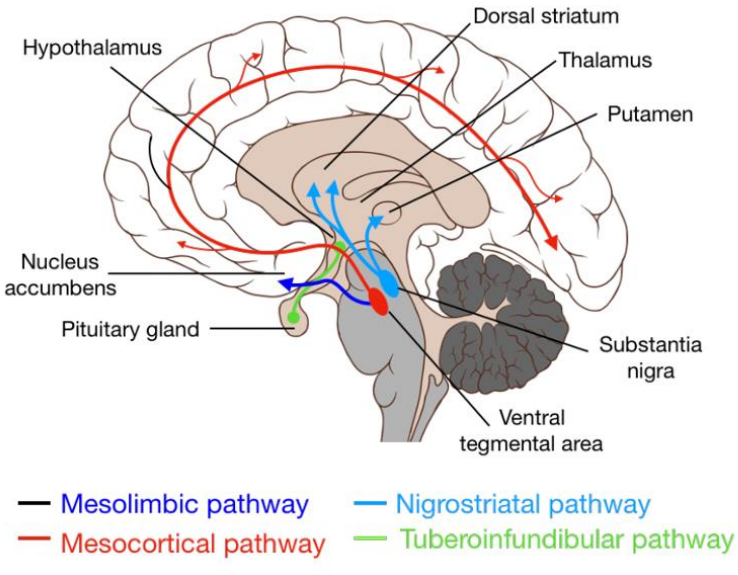


Figure 2- Illustration of major dopaminergic pathways in the human brain (Lauri Nummenmaa, 2020). This diagram depicts the mesolimbic, mesocortical, nigrostriatal, and tuberoinfundibular pathways, highlighting their origins and projections. The mesolimbic pathway (red) originates from the ventral tegmental area (VTA) and projects to the nucleus accumbens, influencing reward and pleasure. The mesocortical pathway (red) extends from the VTA to the prefrontal cortex, affecting cognition and executive function. The nigrostriatal pathway (blue) connects the substantia nigra to the dorsal striatum, playing a key role in movement control. Lastly, the tuberoinfundibular pathway (green) runs from the hypothalamus to the pituitary gland, regulating hormone release. These pathways are crucial for understanding the functional and behavioral aspects of dopaminergic neurotransmission.

1.3.Parkinson's Disease

Parkinson's disease (PD) stands as the second most prevalent neurodegenerative disorder worldwide. This condition is characterized by the progressive degeneration of dopaminergic neurons situated in the SNpc. The loss of this neuronal population leads to an imbalance in the nigrostriatal pathway, resulting in reduced dopamine (DA) levels in the striatum. Consequently, individuals experience various symptoms, including bradykinesia, resting tremors, and rigidity, which are typical manifestations of Parkinson's disease (Fernanda Martins Lopes et al., 2017).

To explore the molecular and cellular mechanisms underlying neurodegeneration in Parkinson's disease (PD), a wide array of experimental methods has been employed. These include genetic analyses involving human subjects, investigations using animal models, studies utilizing

cell cultures, and biochemical experiments focusing on specific proteins (W. Philip Bartel et al., 2020). Research indicates that PD arises from a combination of factors, encompassing age, sex, genetics, environment, and mitochondrial influences. Symptoms of Parkinson's commonly manifest after the age of 65, with a higher prevalence observed in males compared to females in a 3:2 ratio (Charlotte A Haaxma et al., 2007). Given the uncertain cause of PD and the fact that 85% of cases are sporadic, it is likely that a combination of genetic and environmental factors, some of which are described below, contribute to its development.

Genetic factors play a significant role in Parkinson's disease (PD), with both monogenic and polygenic contributions to its pathophysiology. Several mutated genes have been identified in association with PD, including PARKIN, PINK1, DJ-1, PARL, ATP13A2, SNCA, and LRRK2. Notably, mutations in PARKIN, PINK1, DJ-1, PARL, and ATP13A2 are considered recessive, while mutations in SNCA and LRRK2 are dominant (Christine Klein and Ana Westenberger, 2012). Many of these genes are directly related to mitochondrial function, maintenance, and regulation. Notably, PINK1, PARKIN, and ATP13A2 play crucial roles within healthy mitochondria, working together to balance mitophagy (the selective removal of damaged mitochondria) and apoptosis (programmed cell death) by controlling processes of fission and fusion (Alicia M. Pickrell and Richard J. Youle, 2015). The intricate interplay of these genetic factors highlights the importance of mitochondrial homeostasis in the context of PD development and progression.

Environmental factors play a crucial role in Parkinson's disease, and significant insights have emerged from studies on neurotoxin-induced Parkinsonism, particularly the discovery of 1-

methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is a lipophilic compound that easily crosses the blood-brain barrier (BBB) and accumulates near glia cells. Within the brain, monoamine oxidase B (MAO-B) metabolizes MPTP into its active neurotoxic form, 1-methyl-4-phenylpyridinium (MPP+) (Figure 3). This MPP+ is then transported into dopaminergic cells by dopamine transporters (DAT), where it disrupts ATP synthesis and mitochondrial respiration. Furthermore, MPP+ increases superoxide production in complex I of the electron transport chain (ETC) (Serge Przedborski et al., 2000). MPTP has been extensively utilized to simulate PD-related symptoms in various animal models, including mice, rats, and primates (Kim Tieu, 2011). Subsequent studies have revealed that other commonly used substances like rotenone, 6-OHDA (6-hydroxydopamine), and paraquat can induce PD-like symptoms through similar mechanisms to MPTP (Amanpreet S. Dhillon et al., 2008; Karuppagounder S. Saravanan et al., 2005; D. Hernandez-Baltazar et al., 2017). These neurotoxins have provided valuable insights into the environmental factors that can contribute to the development and manifestation of Parkinson's disease.

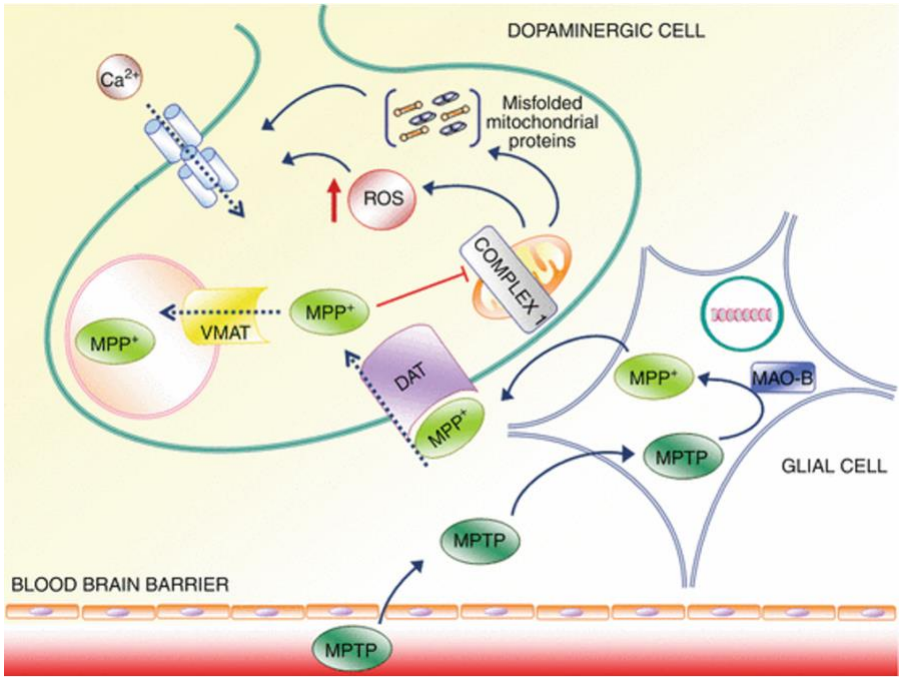


Figure 3- Mechanistic overview of MPTP neurotoxicity in dopaminergic neurons (Pasquali et al., 2014). This diagram illustrates the metabolic conversion of MPTP to MPP+ within glial cells, mediated by the enzyme MAO-B, and the subsequent transport of MPP+ into dopaminergic neurons. Once inside the neurons, MPP+ inhibits mitochondrial complex I, leading to increased production of reactive oxygen species (ROS) and disruption of cellular energy metabolism. The diagram also depicts the role of the dopamine transporter (DAT) and vesicular monoamine transporter (VMAT) in the internalization and sequestration of MPP+, respectively. Additionally, it shows the protective role of the multidrug resistance protein (MRP) in exporting MPP+ out of the cells, and the impact of calcium ions on neuronal vulnerability. This cascade underscores the pathophysiological basis for dopaminergic cell death in Parkinson's disease models.

1.4.Mitochondrial Dynamics

Mitochondria are dynamic cellular structures that intricately manage the energy requirements of the cell through two primary mechanisms: glycolysis and oxidative phosphorylation. Particularly in highly active cells, such as neurons, the emphasis is often on the latter process. Neurons have a high metabolic demand to uphold membrane potentials, facilitate neurotransmission, and maintain rhythmic patterns. Consequently, any disruption in the functioning of the mitochondrial network can result in severe neurological consequences (Dauer and Przedborski, 2003). Among neurons, a specific subgroup that is notably vulnerable to damage and oxidative stress is the substantia nigra pars compacta DAnergic neurons. As previously mentioned, these neurons extend into a complex axonal network within the striatum, forming

around 1 to 2 million synapses in humans (Misgeld and Schwarz, 2017) and 200 to 400 thousand in rats (Moss and Bolam, 2008; Matsuda et al., 2009). This considerable synaptic density could elucidate why mitochondrial dysfunction prominently features in various types of Parkinson's disease, including monogenic, polygenic, and idiopathic cases.

Apart from their primary role in generating ATP through respiration, mitochondria serve as versatile and essential organelles involved in several crucial processes. These functions encompass the regulation of reactive oxygen species (ROS), handling of calcium ions (Ca2+), and the determination of cellular destiny (Contreras et al., 2010). In the context of cellular fate determination, mitochondria actively participate in orchestrating pathways related to apoptosis and mitophagy as responses to diverse environmental signals (Youle and Bliek, 2012).

In the process of apoptosis, mitochondria regulate the equilibrium between anti- and proapoptotic signaling through the Bcl-2 and Bax mechanisms, which govern the release of cytochrome-c. This cytochrome-c is situated in the intermembrane space, situated between the inner and outer mitochondrial membranes. Its release into the cytosol occurs when proapoptotic Bax is recruited and localized to the outer mitochondrial membrane. This binding of Bax triggers a signal for the assembly of a complex that includes Apoptotic peptidase activating factor 1 (Apaf-1), an adaptor molecule within the apoptosome, and the initiator Caspase-9. This complex formation facilitates the activation of downstream effector caspases such as Caspase-3, which play a pivotal role in cellular demise (Baliga and Kumar, 2003). This sequence of events has been demonstrated both in vitro, where recombinant Bax prompts cytochrome-c release in cultured mitochondria, and in vivo, where mice lacking Caspase-9 failed to generate the apoptosomal

complex and activate Caspase-3 (Kuida et al., 1998). Collectively, these investigations enhance our understanding of the underlying mechanisms and emphasize the essential role of all components in the orchestration of apoptosomal formation and mitochondrial-mediated apoptosis.

Alternatively, mitophagy serves as a cellular safeguard mechanism to selectively eliminate damaged mitochondria through a process referred to as autophagy (Ding and Yin, 2012). Autophagy, a conserved evolutionary process, employs the lysosomal pathway to break down intracellular components, spanning from organelles to detrimental proteins or aggregates (Vijayakumar and Cho, 2019). In the context of mitochondria, their sequestration involves initiating the ubiquitination of the outer mitochondrial membrane, which subsequently attracts the autophagosome complex responsible for engulfing the impaired organelle. This process, termed mitophagy, is set in motion by the intrinsic signaling of PINK1/Parkin during stress conditions. When subjected to stress, mitochondria undergo a conformational shift or fission event that fragments larger mitochondria into smaller bodies. These fragmented bodies exhibit a diminished membrane potential, leading to the accumulation of PINK1 on the outer mitochondrial membrane. Subsequently, PINK1 recruits Parkin, enabling the translocation and ubiquitination of various mitochondrial substrates targeted for autophagosomal degradation. One of these targets subject to Parkin-mediated ubiquitination is the mitochondrial fusion proteins, Mitofusin (Mfn) 1 and 2. This exacerbates the isolation of mitochondria and hampers the surrounding network's ability to integrate these reduced bodies, further impeding potential recovery (Tanaka et al., 2010). The precise mechanism of PINK1 signaling to Parkin remains somewhat elusive. While certain researchers posit that indirect phosphorylation of the kinesin anchoring substrate Miro in drosophila is responsible (Wang et al., 2011), others suggest that it may arise from the direct

phosphorylation or anchoring of Parkin by PINK1 (Sha et al., 2010). Regardless, the effectiveness of this pathway hinges on both factors, as Parkin fails to associate with the outer mitochondrial membrane without PINK1, and the absence of Parkin leads to a lack of phagosomal activation. Nevertheless, in healthy conditions, these processes are infrequent due to the swift proteolytic cleavage of PINK1 by enzymes such as Parl prior to Parkin recruitment (Merhi et al., 2021).

As mentioned earlier, mitochondria possess the ability to undergo structural adjustments in reaction to environmental signals or the cellular metabolic requirements. These alterations involve transitions between states of fission and fusion. Fission tends to occur in glycolytic settings characterized by rapid cell proliferation or as a protective response. Conversely, oxidative phosphorylation is prevalent in cells that are healthy, actively demanding, and differentiated (Seo et al., 2018).

During the process of fragmentation, several accessory or adaptor proteins play a role, including mitochondrial dynamics protein 49 and 51 (Mid49 and Mid51), mitochondrial fission factor (Mff), and mitochondrial fission protein 1 (Fis1). These proteins collectively facilitate the recruitment of dynamin-related protein 1 (Drp1, known as Dnm1 in yeast) to the outer mitochondrial membrane. Drp1 is a pivotal player in this mechanism, and its deficiencies result in elongated and overly fused mitochondrial structures (Qian et al., 2012). Subsequent to Drp1 recruitment, the process involves membrane constriction accompanied by the assembly of dynamin-2 (Dyn2), a crucial factor for the final scission event. The specific sites of cleavage are determined through microdomains of contact with the endoplasmic reticulum (ER) (Aoyama-

Ishiwatari and Hirabayashi, 2021). Additionally, the ER releases proteins that promote fission, such as INF-2, which has been observed to "prime" the mitochondria prior to Drp1 binding.

In contrast, the process of mitochondrial fusion is regulated by proteins known as fusogens, which include Mfn1, Mfn2, and optic atrophy-1 (Opa1; referred to as Mgm1 in yeast). These fusogens collaborate to bring adjacent mitochondrial structures together through a series of stages: tethering, docking, and eventual fusion of the two outer mitochondrial membranes via GTP hydrolysis. Mfn1 and Mfn2 play essential roles in this process by forming complexes that can be either homo- or heterotypic, leading to the merging of outer mitochondrial membranes. Simultaneously, Opa1 and cardiolipin (CL) work synergistically to facilitate the integration of inner mitochondrial membranes and bring about the remodeling of cristae between the fused mitochondria (Ban et al., 2017). Notably, mutations in the genes encoding Mfn or Opa1 machineries have been associated with various effects, including the fragmentation of mitochondria and the formation of spherical structures, along with compromised cristae development (Meeusen et al., 2006).

Fusion serves a crucial role in facilitating the recovery of damaged mitochondrial DNA (mtDNA) or mitochondrial compartments, whereas fission is essential for segregating faulty segments, marking them for subsequent processes like mitophagy or apoptosis (Youle and Bliek, 2012). mtDNA is notably more susceptible to mutations, around 10 to 20 times greater than nuclear DNA (gDNA), making mitochondria more prone to oxidative damage. This situation establishes a negative feedback loop: stress leads to more mutations, and an increased mutation load heightens vulnerability to stress. The aging process intensifies this cycle, possibly accounting for the prevalence of mitochondrial dysfunction as a foundational factor in conditions like Parkinson's

disease (PD) and other neurodegenerative disorders. While numerous animal models have been employed to replicate these effects in vivo, the zebrafish has rapidly emerged as a promising model in this domain. This is due to its preserved genetics, neural circuitry, and behavioral characteristics, which parallel those of higher-order mammals.

1.5.Zebrafish (Danio Rerio), as a Good Model to Study Dopamine Neurons

In recent years, the zebrafish (Danio rerio) has emerged as a valuable model organism for studying motor disorders and neurodegenerative diseases. The neuronal network responsible for controlling zebrafish movement is well understood, and the molecular mechanisms involved are remarkably similar to those governing human movement. Despite notable morphological distinctions between mammals and fish, intriguingly, researchers have discovered that over 70% of human disease genes have functional counterparts, or homologs, in zebrafish (Kerstin Howe et al., 2013). This striking similarity offers exciting opportunities to leverage the zebrafish model in uncovering insights into human neurological conditions and advancing therapeutic strategies.

Indeed, despite the morphological differences between zebrafish and humans, numerous developmental pathways are functionally conserved between them. Zebrafish possess the remarkable ability to establish a fully formed cerebral dopaminergic system that holds relevance for understanding Parkinson's disease pathogenesis. Specifically, the dopaminergic neurons in humans, projecting from the substantia nigra pars compacta to the striatum, are believed to be homologous to zebrafish neurons extending from the posterior tuberculum through the ventral diencephalic (vDC) and basal telencephalon. Within the vDC, the dopaminergic clusters have been

classified as clusters 5, 6, 8, 11, 12, and 13 (V. Sallinen et al., 2009). This remarkable similarity in the dopaminergic system provides an excellent opportunity to utilize zebrafish as a valuable model for studying Parkinson's disease and gaining insights into its underlying mechanisms.

While PD symptoms have predominantly been investigated in mammalian models in vivo, zebrafish present a unique opportunity to explore these mechanisms in real-time. Zebrafish possess remarkable regenerative capabilities and neurogenesis, allowing them to regenerate cells after injury or death. Unlike mammalian brains, which have limited adult neurogenesis, zebrafish have numerous proliferating ventricular progenitors that support their regenerative potential. Studying the zebrafish brain can thus provide valuable insights into the mechanisms responsible for broad adult neurogenesis and aid in developing effective treatments for humans (Matthew Gemberling et al., 2013). Moreover, zebrafish offer several advantages for research due to their short generation times (about 3 months) and highly transparent ex utero development. These features enable rapid and straightforward gene manipulation using gene-silencing, gene-editing, and knockdown methods. Direct imaging during ex utero development allows the study of various phenomena at different developmental stages. Additionally, zebrafish have a high fecundity, producing hundreds of embryos at a time, facilitating the evaluation of pharmacological and therapeutic potentials at a rapid rate (Caghan Kizil & Michael Brand, 2011). This combination of unique traits makes zebrafish an invaluable model organism for investigating Parkinson's disease and exploring potential therapeutic interventions.

Our laboratory has successfully generated a transgenic zebrafish line, namely Tg(dat:eGFP), which hold great relevance for studying the dopaminergic system in Parkinson's

disease (PD) research (Sandra Noble et al., 2015). In Tg(dat:eGFP), we incorporated a green fluorescent protein (GFP) cassette into the dat gene's cis-regulatory elements. As a result, the expression of GFP becomes localized specifically to dopaminergic (DA) neurons, facilitating their visualization and study.

1.6.Neuroprotective Compounds

As of now, a cure for DA (dopaminergic) cell death remains elusive due to the complex pathological mechanisms associated with it. While promising neuroprotective compounds have been identified in preclinical research, effectively transitioning them into clinical trials has proven challenging (Salamon et al., 2019). Nonetheless, there are several pharmacological treatments available to help slow down the progression of the disease. Some of these treatments include MAO-B (monoamine oxidase B) inhibitors, dopamine receptor agonists, NMDA (N-methyl-D-aspartate) receptor antagonists, and iron chelators. These substances work by influencing various aspects of cellular mechanisms, ultimately reducing stress in the cells (Salamon et al., 2019). Although they may not offer a cure, these pharmacological interventions can provide significant benefits in managing the symptoms and progression of Parkinson's disease. Research in this area continues to seek new and effective treatments to improve the quality of life for individuals living with PD.

Ascorbic acid (AA), the reduced form of vitamin C, has demonstrated its potential in mitigating oxidative stress damage in both in vitro and in vivo systems. While most mammals synthesize endogenous AA in the liver, humans and some other species rely on exogenous

supplementation due to the lack of this ability. Despite being widely distributed throughout the body, AA accumulates to high concentrations (2-10mM) in the brain. Its entry into the brain is facilitated by sodium vitamin C and glucose transporters that enable it to cross the blood-brain barrier (Nualart et al., 2014). The dietary dependence of AA became well-known in the 1930s when it was used to alleviate the clinical manifestations of scurvy. Subsequent studies have revealed that AA plays a significant role in various cellular processes, including ROS (reactive oxygen species) detoxification (Arrigoni and De Tullio, 2002), cholesterol metabolism (Ginter, 1975), hippocampal synaptic plasticity (Fraga et al., 2020), and functioning as a cofactor for enzymatic redox cycling (Krishnan et al., 2009). In the context of neurological health, recent research using zebrafish has highlighted AA's potential to alleviate neurotoxicity induced by substances such as butachlor, deltamethrin, lead, and neomycin. These studies observed reduced morphological abnormalities, hatching defects, and mortality, along with normalized expression levels of genetic markers indicative of oxidative stress, such as superoxide dismutase (sod) and catalase (cat) (Wu et al., 2015; Xiang et al., 2018; Paduraru et al., 2021). These findings underscore the potential neuroprotective effects of ascorbic acid and its significance in addressing oxidative stress-related damage in the brain.

Ferulic acid (FA) is a natural phenol renowned for its antioxidizing properties. Found as one of the primary active components in traditional Chinese medicines, FA has been extensively studied for its impact on various cellular processes. Among these processes are free-radical scavenging, where it efficiently neutralizes harmful free radicals (Ogiwara et al., 2002), as well as its role in regulating lipid metabolism (Son et al., 2010), exhibiting anti-inflammatory effects (Cheng et al., 2008), and modulating anti-cancer signaling pathways (Kawabata et al., 2000). In

recent years, the potential of FA treatment has expanded to investigate its effects in neuroprotection. Research has shown that FA can effectively protect rodent neurons and PC-12 cell cultures against cytotoxicity induced by hypoxia and ischemia (Koh, 2013; Ren et al., 2017). These findings highlight the promising neuroprotective properties of ferulic acid, suggesting its potential therapeutic value in safeguarding neural cells and countering conditions related to hypoxia and ischemia-induced damage. Continued research in this field may uncover additional neuroprotective mechanisms and enhance our understanding of FA's role in addressing neurodegenerative diseases and related disorders.

Vanillic acid (VA), a plant-derived compound, has emerged as a promising therapeutic agent with antioxidative properties, effectively mitigating the impacts of oxidative stress in various models and processes. VA is produced as a byproduct during the conversion of ferulic acid into vanillin. Notably, VA has demonstrated its potential in modulating healthy and disease states in various physiological functions. In the cardiac context, VA has shown therapeutic effects in cardiac conditions (Kumar et al., 2011). Additionally, in the neurological field, VA has been found to have beneficial effects in addressing neurological disorders (Khoshnam et al., 2018). Furthermore, VA has shown promise in influencing metabolic processes (Salau et al., 2020) and regulating inflammatory responses (Stanely Mainzen Prince et al., 2011). The versatility of vanillic acid's therapeutic effects across multiple systems holds great potential for its application in addressing a wide range of health conditions. As research continues to unveil the mechanisms underlying VA's beneficial effects, it may offer new possibilities for therapeutic interventions and treatment strategies in various medical fields.

1.7.General Objective and Hypothesis

It is our goal to screen neuroprotective effects of natural phenolics in an MPTP-induced Model of PD using Zebrafish. Based on the evidence for antioxidant properties of AA, VA and FA, we hypothesize that pre-treatment of zebrafish with these natural phenolics will have a positive impact on DA neuron health within an MPTP-induced PD model. We will address this hypothesis through the following objectives:

1. Quantify and compare differential expression of genes associated with biosynthesis of DA, apoptosis of DA neurons, mitochondrial fission and fusion following administration of candidate neuroprotectants in an MPTP-induced model of PD using zebrafish.

2. Validate gene expression data using immunohistochemical quantification of DAnergic neurons in an MPTP-induced model of PD using zebrafish treated with the candidate neuroprotectants.

These objectives are essential steps in assessing the potential neuroprotective effects of the natural phenolics on DA neurons and gaining insights into their mechanisms of action in the MPTP-induced PD model. The utilization of zebrafish as a model organism offers distinct advantages in terms of translational research and allows for a rapid screening of potential therapeutic candidates. our study has the potential to contribute significantly to our understanding of neuroprotective agents and may pave the way for future treatments targeting Parkinson's disease.

2. Methods and Material

2.1.Zebrafish Care and Husbandry

All experimental procedures conducted in this study were approved by the University of Ottawa and adhered to the guidelines set forth by the Canadian Council Animal Care and the Animal Care and Veterinary Service (ACVS). To obtain the necessary larvae for the research, adult zebrafish from the Tg(*dat*:eGFP) transgenic line were bred. In Tg(*dat*:eGFP), a green fluorescent protein (GFP) cassette is incorporated into the *dat* gene's cis-regulatory elements. As a result, the expression of GFP becomes localized specifically to dopaminergic neurons, facilitating their visualization and study. The zebrafish were housed under controlled conditions, including a temperature of 28.5°C, with a 14-hour dark and 10-hour light cycle. The tank water was recirculated, and appropriate E3 media was used for the embryos, containing the following concentrations: 13mM NaCl, 0.5mM KCl, 0.02mM Na2HPO4, 0.04mM KH2PO4, 1.3mM CaCl2, 1.0mM MgSO4, and 4.2mM NaHCO3. These conditions provided a suitable and controlled environment for the zebrafish and their developing embryos during the study.

2.2.Neuroprotective Treatment and MPTP Exposure

During the experiment, zebrafish larvae were exposed to different neuroprotectants, including Ascorbic acid (AA), Vanillic acid (VA), and Ferulic Acid (FA), along with MPTP in 6-well plates. These plates were maintained at room temperature throughout the entire treatment period. The pre-treatment exposures to the neuroprotectants were as follows:

- Ascorbic acid (AA) was administered at the optimal concentration of 50µM
- Vanillic acid (VA) was administered at the optimal concentration of 250µM
- Ferulic Acid (FA) was administered at the optimal concentration of 100µM

These optimal concentrations were identified in our laboratory as part of an unpublished honors project conducted in 2021.

These pre-treatment exposures took place from 1-day post-fertilization (dpf) to 3dpf. Subsequently, the zebrafish larvae were exposed to 0.25mM (LC50 concentration) MPTP from 4dpf until the time of analysis. The exposure mediums containing the neuroprotectants and MPTP were refreshed daily to maintain the appropriate concentrations and ensure consistent exposure throughout the experiment (Figure 4). This timeline allows for a comprehensive evaluation of the neuroprotective effects of the natural phenolics (AA, VA, and FA) in the MPTP-induced model of Parkinson's disease in zebrafish.

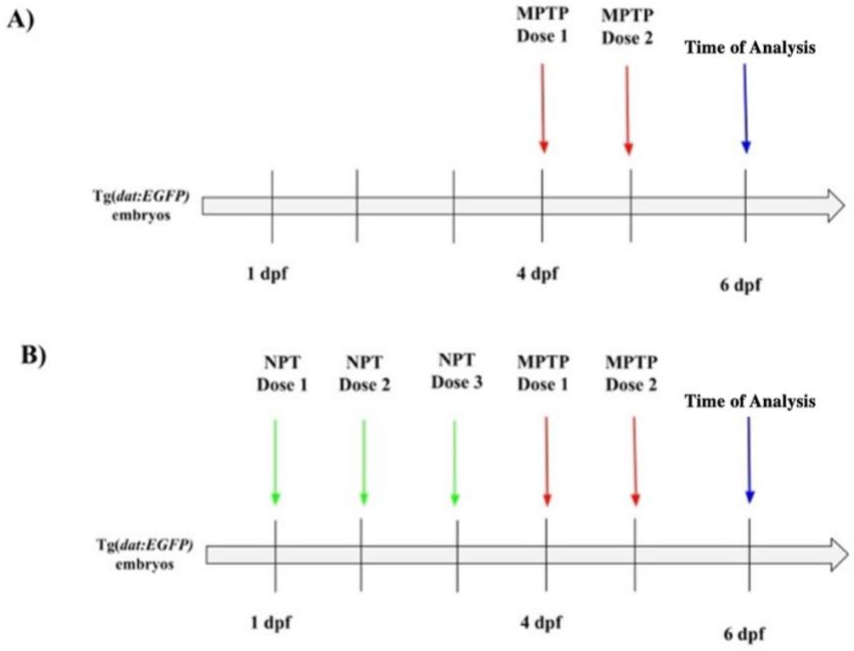


Figure 4- Timeline of the neuroprotective treatment and MPTP neurotoxin exposure in zebrafish larvae. A) Positive control (MPTP-treated larvae): The larvae in this group were treated with MPTP only. From the time of hatching until 4 days post-fertilization (dpf), the larvae were maintained in E3 Embryo medium. Subsequently, two doses of MPTP were administered: one dose at 4 dpf and another dose at 5 dpf. B) Neuroprotective treatments (NPT): In this group, neuroprotective doses (NPT) were administered daily at 1 dpf, 2 dpf, and 3 dpf. At 4 dpf, the larvae received the first dose of MPTP neurotoxin, followed by the second dose at 5 dpf. At 6 dpf, the larvae were collected for further analysis.

Additionally, in the experiment where zebrafish were treated with FA alone, the larvae were exposed to 100µM FA from 1 dpf until 3 dpf and housed in a 6-well plate. After this period, from 4dpf onwards, the larvae were transferred to E3 medium, which they remained in until the analysis was conducted at 6dpf. To ensure consistent conditions, the exposure mediums for the

larvae were refreshed every day, maintaining the targeted concentrations throughout the experiment.

2.3.RNA Extraction, cDNA Synthesis, and qRT-PCR

RNA extraction was performed from three biological replicates, each containing 20 whole zebrafish larvae collected at 6 days post-fertilization (dpf) following the manufacturer's TRIzol protocol (InVitrogen). To assess the quality of the RNA, samples were run on a bleached agarose gel, visualizing the presence of distinct 18S and 28S bands. For further experimentation, only samples with 260/280nm absorption ratios ranging from 1.8 to 2.1 were considered to ensure RNA purity and concentration, as determined using the NanoDrop 1000 spectrophotometer (ThermoFisher). Subsequently, the RNA samples were reverse transcribed into complementary DNA (cDNA) using the iScript™ cDNA Synthesis Kit (Bio-Rad). In the reaction mix, 5µL of SsoFastTM EvaGreen® Supermix (Bio-Rad), 0.4µL of forward primer, 0.4µL of reverse primer, 0.2µL of nuclease-free water, and 4µL of cDNA were combined. The reactions were run on the Bio-Rad CFX96 instrument, and the Maestro software was used for data analysis.

Normalized quantification of the number of *th1* (gene identifier number: ZDB-GENE-990621-5), *dat* (ZDB-GENE-010316-1), *pink1* (ZDB-GENE-041212-53), *parkin* (ZDB-GENE-050417-109), *fis1* (ZDB-GENE-050506-92), *mfn1* (ZDB-GENE-121101-4), *opa1* (ZDB-GENE-041114-7), and *p53* (ZDB-GENE-990415-270)transcripts was achieved using the comparative Cq (quantification cycle) method with three technical replicates and two reference genes: ribosomal protein L 13a *(rpl13a)* and elongation factor 1 alpha *(ef1a).* The oligonucleotide primer sequences

used in the experiments are listed in Table 1. This rigorous experimental protocol ensures the reliable and accurate assessment of gene expression levels, providing valuable data to study the impact of neuroprotective treatments on the expression of *th1, dat, pink1, parkin, fis1, mfn1, opa1,* and *p53* genes in the zebrafish model of Parkinson's disease.

Table 1- List of primers designed for qRT-PCR.

Primer	Forward Sequence (5′-3′)	Reverse Sequence (5′-3′)	Reference
th1	GACGGAAGATGATCGGAGACA	CCGCCATGTTCCGATTTCT	(Chen et al. 2016)
dat	AGACATCTGGGAAGGTGGTG	ACCTGAGCATCATACAGGCG	(Barreto-Valer et al. 2012)
pink1	GGCAATGAAGATGATGTGGAAC	GGTCGGCAGGACATCAGGA	(Chen et al. 2016)
parkin	GCGAGTGTGTCTGAGCTGAA	CACACTGGAACACCAGCACT	(Sarath Babu et al. 2016)
fis1	CCCTGAACCTTCCAGTGTTT	GTCTCTGGAAACGGGTCCTT	(Chen et al. 2016)
mfn1	CTGGGTCCCGTCAACGCCAA	ACTGAACCACCGCTGGGGCT	(Chen et al. 2016)

opa1	GCTTGAGCGCTTGGAAAAGGAA	TGGCAGGTGATCTTGAGTGTTGT	(Chen et al. 2016)
p53	ATATCCTGGCGAACATTTGG	ACGTCCACCACCACCATTTGAAC	(Chen et al. 2016)
rpl13a	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	(Tang et al. 2007)
ef1a	CTGGAGGCCAGCTCAAACAT	ATCAAGAAGAGTAGTACCGCTAGCATTAC	(Tang et al. 2007)

2.4.Immunohistochemistry on Whole Mount Zebrafish Larvae

Zebrafish were raised to the appropriate developmental stage, manually dechorionated and euthanized with an overdose of MS-222. They were then fixed in a 4% solution of fresh electron microscopy grade paraformaldehyde (ThermoFisher Scientific) dissolved in phosphate buffered saline (137mM NaCl, 2.7mM KCl, 4.3mM Na2HPO4, 1.47mM KH2PO4, PBS) overnight at 4°C, then rinsed thrice in PBS and permeabilized for 15 minutes in PBS with 0.1% Triton X-100 (PBSTx). Samples were then blocked overnight at 4°C in a 10% fetal bovine serum (Ambion) in PBS, followed by an incubation overnight at 4°C in primary antibody solution [anti-GFP (AS-55887, Anaspec)] diluted to a 1:500 concentration in 1% fetal bovine serum in PBSTx. Samples were then washed thrice for 15 minutes at room temperature in PBSTx, then incubated overnight

at 4°C in secondary antibody solution [goat anti-mouse Alexa 488 conjugate (A-11001, ThermoFisher Scientific) diluted to a 1:1000 concentration in 1% fetal bovine serum in PBSTx. Samples were again washed thrice for 15 minutes at room temperature in PBSTx following secondary antibody incubation and imaged with Nikon A1RsiMP Confocal microscope as described in the following.

2.5.Microscopy and Image Analysis

At 6 dpf, treated zebrafish larvae were mounted dorsal side up on slides using a 1% low-melting point agarose solution. Larvae were continuously re-hydrated while mounted using system water. Imaging was conducted using the Nikon A1siMP confocal microscope with a 10× water immersion objective. Larvae were scanned using the laser at a wavelength of 488 nm to excite eGFP. Images were obtained in a 2μm interval Z-stack that was processed and compiled to produce a three-dimensional image. The total cell numbers for clusters 8, 12, and 13 were determined in 3-D to avoid repeated counts of the same cell and by two independent researchers in a blinded fashion to remove bias. Maximum intensity projection images used for the study were produced using the NIS-Elements software (Nikon). Cell counts were performed using Fiji (ImageJ) software and were performed by two independent researchers in a blinded approach to remove personal bias.

2.6.Statistical Analysis

Statistical analysis was done using the software Microsoft Excel. eGFP+ cell counts were quantified from 10–15 zebrafish, and gene expression data were collected from three pools of 20 embryos. Data bars are shown as the mean ± the standard error of the mean. Cell quantification

between two independent groups was conducted using a one-way ANOVA. Conversely, for comparisons involving two related sets of observations, a two-way ANOVA employed. Additionally, gene expression data were calculated using a one-way ANOVA. Statistical significance was determined when p-value < 0.05 and was indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns= not significant ($p > 0.05$).

3. Results

3.1. Quantitative RT-PCR Results

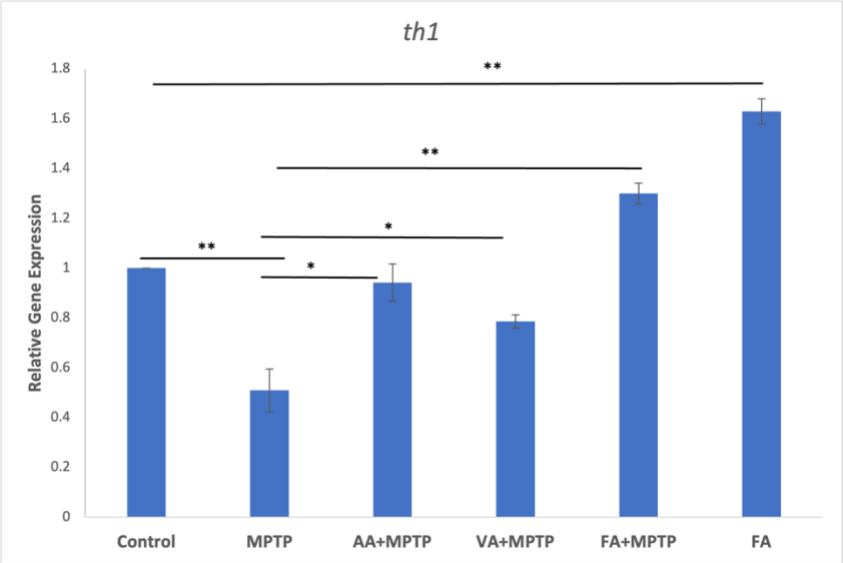
Dopaminergic neurons, being highly metabolically active cells that depend on oxidative phosphorylation to produce energy, are especially vulnerable to any changes in oxidative metabolism. Consequently, disruptions in mitochondrial function can have significant implications for the health and behavior of dopaminergic neurons. In order to investigate the potential neuroprotective effects on mitochondria and dopamine (DA) homeostasis, we analyzed the variations in gene expression related to mitochondrial fission and fusion, as well as the biosynthesis of DA, and the survival and apoptosis of dopaminergic neurons. The results of the study can be found in the following sections.

3.1.1. Natural Phenolic Compounds Exhibit a Protective Effect on the Expression of Dopamine Biosynthesis Genes in Zebrafish Larvae Exposed to MPTP.

The impact of various neuroprotective agents on DA neurons in a zebrafish model of Parkinson's disease was analyzed, focusing on gene expression changes in *th1* and *dat* (figure 5). MPTP treatment led to a significant decrease in both *th1* and *dat* gene expression: about 49.1% and 86.2% respectively compared to the control. However, pre-treatment with different agents showed varied effects. AA Pre-treatment resulted in a substantial increase in gene expression compared to MPTP-treated zebrafish. *Th1* gene expression rose by approximately 85%, and *dat* expression increased by around 8.8-fold. However, when compared to the control group, *th1* showed a slight decrease (5.7%), while *dat* exhibited a 21.2% increase. VA Pre-treatment led to about a 54% increase in *th1* expression and a 3-fold rise in *dat* expression compared to the MPTP group. However, *th1* expression decreased by 21.4% and *dat* by 57% compared to the control group.

FA pre-treatment showed the most significant positive effects, with *th1* expression increasing approximately 2.5-fold and *dat* expression about 9.9-fold compared to MPTP-treated group. Relative to the control group, there was a 29.9% increase in *th1* expression and a 36.9% increase in *dat* expression. Notably, FA pre-treatment led to higher *th1* and *dat* gene expression than the control group. As a result, our study focused on exploring the effect of FA on the expression levels of *th1* and *dat* genes in scenarios where MPTP was not present. During the experiment, zebrafish larvae underwent exposure to 100μM FA starting from 1dpf until 3dpf, housed in a 6-well plate. After this period, from 4dpf onwards, the larvae were transferred to E3 medium, which they remained in until the analysis was conducted at 6dpf. To ensure consistent conditions, the exposure mediums for the larvae were refreshed every day, maintaining the targeted concentrations throughout the experiment. Further investigation into FA's standalone effects

(without MPTP) revealed a marked increase in gene expression: *th1* by 62.9% and *dat* by 71.7%. However, MPTP co-treatment with FA attenuated these increases, reducing the *th1* and *dat* expression elevations by 52% and 48.5%, respectively.



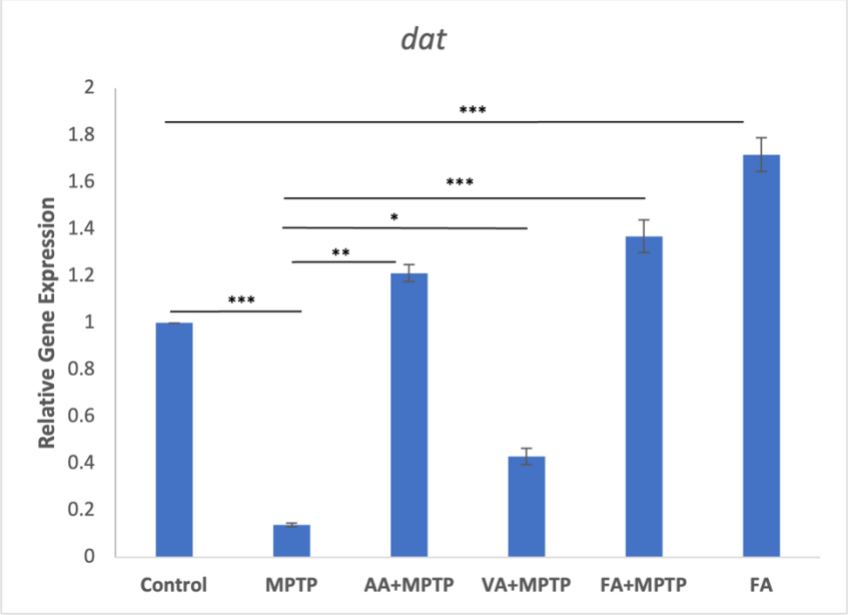


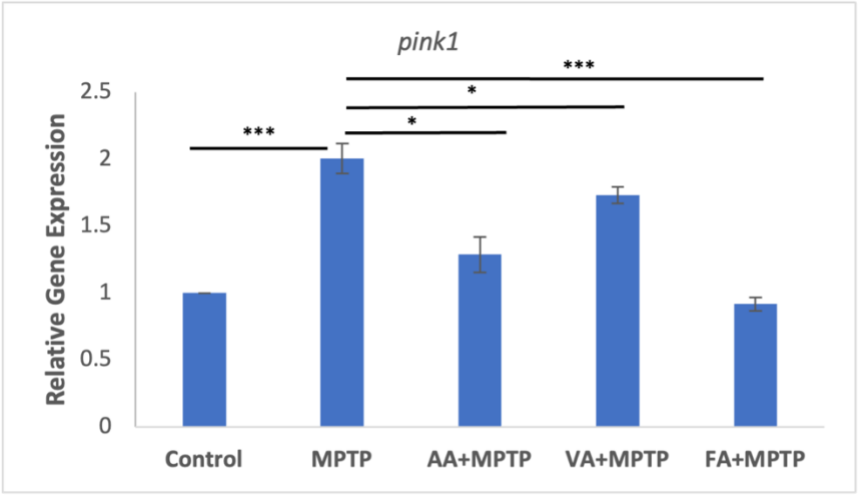
Figure 5- Effects of neuroprotective compounds on *th1* and *dat* gene expression. Relative gene expression of *th1* and *dat* analyzed between Control, MPTP, ascorbic acid, vanillic acid and ferulic acid. (*n*= 3 pools of 20 larvae). Bars represent the Mean ± the SEM. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), ns= not significant ($p>0.05$).

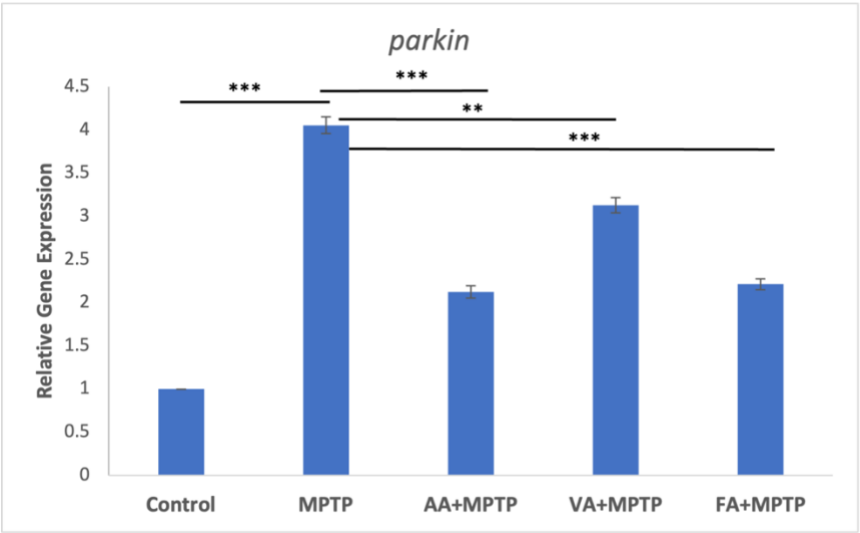
3.1.2. Natural phenolic Compounds Show a Protective Effect on the Expression of Mitochondrial Fission Genes in Zebrafish Larvae Exposed to MPTP.

In this study, we assessed the effects of various neuroprotective agents on mitochondrial fission in a zebrafish model of Parkinson's disease by measuring the gene expression levels of *pink1*, *parkin,* and *fis1* (Figure 6). Relative to the control group, MPTP treatment resulted in significantly increased expression of *pink1* (100.5%), *parkin* (305.5%), and *fis1* (49.28%). Pre-treatment with AA reduced the expression levels of *pink1, parkin,* and *fis1* by 35.65%, 47.5%, and 41% respectively, compared to MPTP treatment group. Similarly, VA pre-treatment decreased these genes' expression by 13.7%, 22.8%, and 2.03%, respectively, while FA pre-treatment showed a reduction of 54.2%, 45.4%, and 41%, respectively.

These results highlight the potential of AA and VA in counteracting MPTP-induced upregulation of *pink1* gene expression, with FA showing a more substantial inhibitory effect. Additionally, all three neuroprotective agents—AA, VA, and FA—were effective in reducing *parkin* gene expression induced by MPTP, with AA and FA having a more pronounced effect than VA. These findings suggest that these natural phenolics may modulate *parkin* gene expression and merit further research as potential neuroprotective agents for Parkinson's disease. Furthermore, the

study showed that MPTP significantly increases *fis1* gene expression, which is considerably reduced by AA and FA pre-treatments, indicating their neuroprotective properties. In contrast, VA had a minimal effect on *fis1* expression. Overall, these natural phenolics appear to influence mitochondrial dynamics and could be valuable in developing neuroprotective strategies for Parkinson's disease.


pink1
Relative Gene Expression
2.5
2
1.5
1
0.5
0
***
*
*
***
Control
MPTP
AA+MPTP
VA+MPTP
FA+MPTP


parkin
Relative Gene Expression
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
***
***
**
***
Control
MPTP
AA+MPTP
VA+MPTP
FA+MPTP

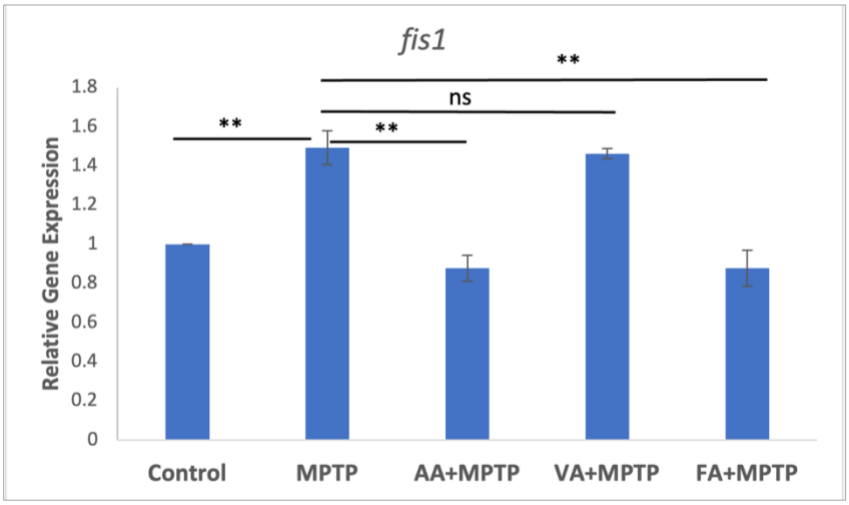

fis1
Relative Gene Expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
ns
**
Control
MPTP
AA+MPTP
VA+MPTP
FA+MPTP

Figure 6- Effects of natural phenolics compounds on mRNA level of genes related to mitochondrial fission. Relative gene expression of *pink1, parkin,* and *fis1* analyzed between Control, MPTP, ascorbic acid, vanillic acid and ferulic acid. (*n*= 3 pools of 20 larvae). Bars represent the Mean ± the SEM. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), ns= not significant ($p>0.05$).

The study examined the effects of potential neuroprotective agents on the gene expression of *mfn1* and *opa1*, which are associated with mitochondrial fusion, as shown in Figure 7. In the group treated with MPTP, a decrease of 15% in *mfn1* and 10% in *opa1* gene expression was observed compared to the control. This reduction in *mfn1* expression was not statistically significant. Pre-treatment with AA led to a 24.7% increase in *mfn1* and an 8.8% increase in *opa1* expression relative to the MPTP group, though these increases were not statistically significant. Similarly, pre-treatment with VA resulted in a 29.4% rise in *mfn1* and a 2% rise in *opa1* expression compared to MPTP, but these changes were also not statistically significant. Contrastingly, FA pre-treatment showed an 11.7% increase in *mfn1* and a 16% increase in *opa1* expression relative to MPTP treatment. However, the increase in *mfn1* expression was not statistically significant.

In summary, the tested neuroprotective agents (AA, VA, and FA) did not demonstrate a significant impact on the gene expression of *mfn1* and *opa1*, indicating that they might not notably influence mitochondrial fusion in the context of this study.

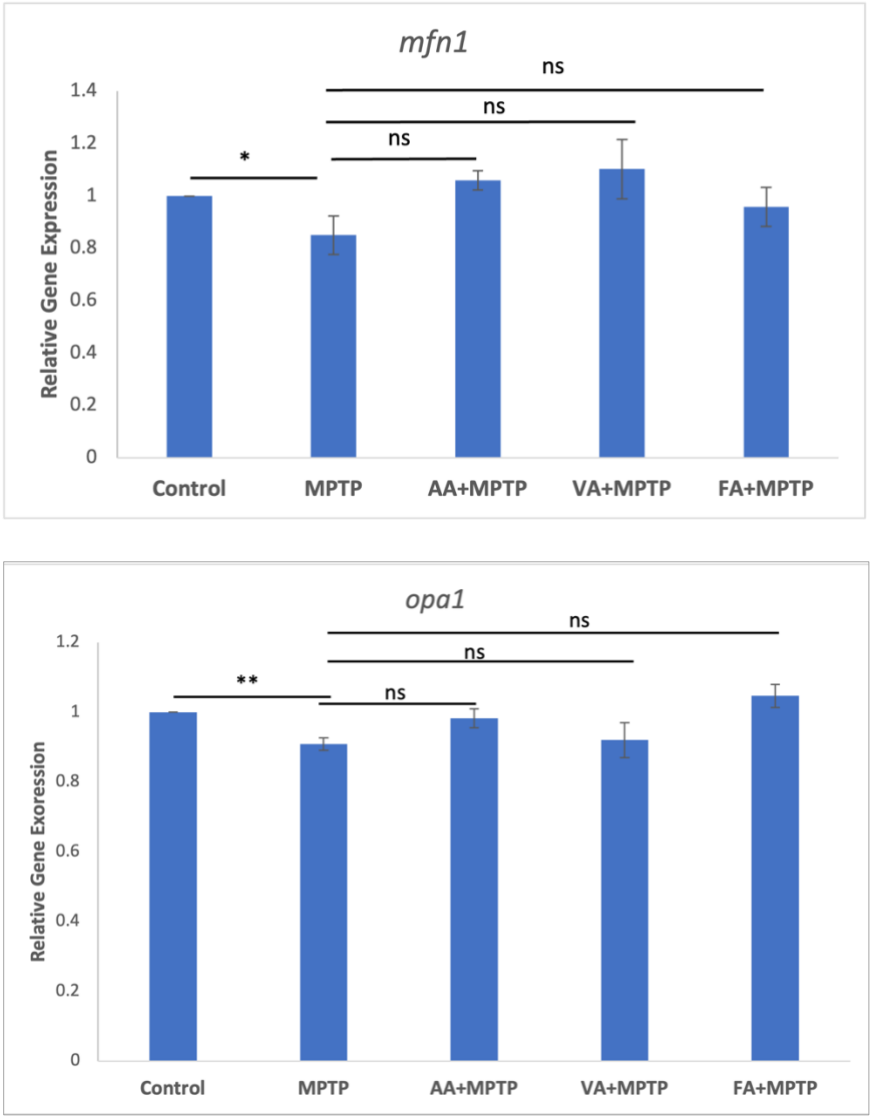


Figure 7- Effects of natural phenolics compounds on mRNA level of genes related to mitochondrial fusion. Relative gene expression *mfn1* and *opa1* analyzed between Control, MPTP, ascorbic acid, vanillic acid and ferulic acid. (*n*= 3 pools of 20 larvae). Bars represent the Mean ± the SEM. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), ns= not significant ($p>0.05$).

3.1.3. Natural Phenolic Compounds Exert a Protective Effect on the Expression of *p53* Gene Involved in DA Neuron Apoptosis in Zebrafish Larvae Exposed to MPTP.

Figure 8 illustrates a significant increase in *p53* gene expression, by about 79.5%, following MPTP treatment compared to the control group's baseline. This substantial rise implies that MPTP treatment may have induced cellular stress or damage, leading to the activation of *p53*, a key regulator of cellular stress responses and DNA damage repair mechanisms. In contrast, pre-treatment with AA showed a 34.7% decrease in *p53* expression compared to the MPTP group, suggesting a mitigating effect of AA on *p53* activation. Similarly, VA pre-treatment resulted in a 15.7% reduction in *p53* expression relative to MPTP, indicating a comparable negative impact on *p53* levels. Furthermore, FA pre-treatment exhibited a more pronounced effect, with a 51.17% decrease in *p53* expression compared to MPTP treatment, reinforcing the trend observed with AA and VA.

Overall, the gene expression analysis underscores varied responses of the *p53* gene to different treatments. The pronounced increase in *p53* expression following MPTP treatment highlights its potential role in inducing cellular stress or damage. In contrast, pre-treatments with AA, VA, and FA consistently led to decreased *p53* expression, with reductions of approximately 34.7%, 15.7%, and 51.17% respectively, suggesting that each agent might modulate *p53* activity in distinct ways. The results particularly emphasize the potential of FA pre-treatment in significantly lowering *p53* expression, aligning with the effects observed for AA and VA.

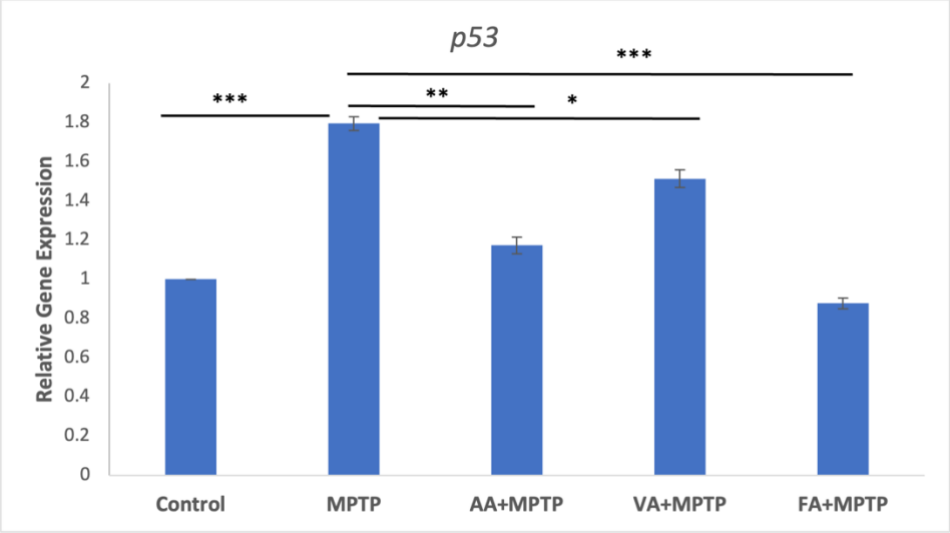


Figure 8- Effects of neuroprotective compounds on mRNA level P53 gene (related to apoptosis). Relative gene expression of *p53* analyzed between Control, MPTP, ascorbic acid, vanillic acid and ferulic acid. (*n*= 3 pools of 20 larvae). Bars represent the Mean ± the SEM. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), ns= not significant ($p>0.05$).

3.2.Zebrafish Morphology Following Neurotoxic and Neuroprotectant Exposures

In our study, we conducted an evaluation of cardiac edema in response to MPTP exposure and the administration of neuroprotective compounds. For each experimental group, we carefully examined 20 larvae at 6dpf and replicated the entire process twice, engaging two to three distinct investigators in each trial to ensure the reliability and accuracy of our findings. By employing this rigorous experimental design, we aimed to comprehensively assess the potential effects of MPTP and the neuroprotective compounds on cardiac development in larvae. The utilization of multiple repetitions enhances the robustness of our results and strengthens the validity of any conclusions drawn from the data.

3.2.1. Exposure to MPTP and Pre-treatment with Neuroprotectants Resulted in Cardiac Abnormalities in Larval Zebrafish.

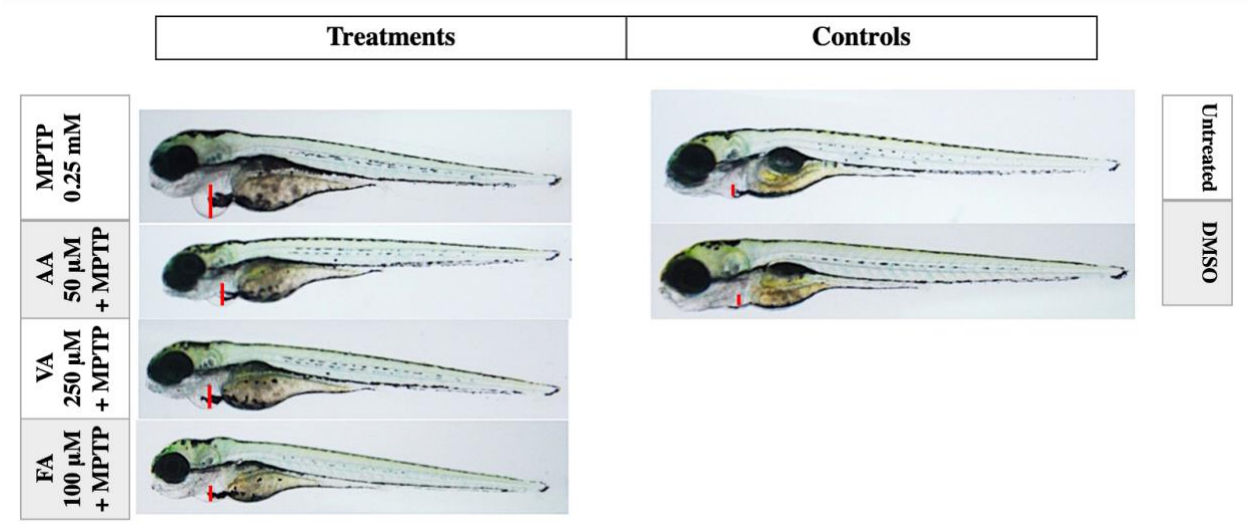


Figure 9- Zebrafish morphology following MPTP and neuroprotectants exposures. Larvae were exposed to 50µM AA, 250µM VA, 100µM FA starting at 1dpf, and 0.25 mM MPTP starting at 4dpf and imaged at 6dpf. Control larvae were exposed to 0.1% DMSO or left untreated. Cardiac area is depicted by a red line. Based on the length of the red line, we classified the type of cardiac edema. If the length was <100 µm, it was categorized as a normal heart. If it fell within the range of 100-150 µm, it was designated as mild cardiac edema. If the length exceeded 150 µm, it was classified as severe cardiac edema. Refer to Table 2 for the frequency of each morphological malformation.

Table2- Summary of impacts of MPTP and natural phenolics exposures in zebrafish cardiac defects.

	Severe cardiac edema	**Mild cardiac edema**	**Normal heart**
Control	0/20	0/20	20/20
0.1% DMSO	0/20	0/20	20/20
MPTP, 0.25 mM	12/20	2/20	6/20
AA 50 μM +MPTP	4/20	6/20	10/20
VA 250 μM +MPTP	10/20	3/20	7/20
FA 100 μM +MPTP	2/20	5/20	13/20

In the control groups, detailed in figure 9 and table 2 of our study, we observed optimal health and normal cardiac development in all larvae. Notably, there were no instances of cardiac edema or other abnormalities in heart morphology in these groups. This crucial finding establishes a solid baseline for our study, confirming that the experimental conditions and procedures themselves did not inherently induce cardiac edema in the larvae. The absence of any cardiac abnormalities in the control groups thus serves as a reliable reference point for comparing and evaluating the impact of various treatments – MPTP, AA, VA, and FA – on cardiac development in our experimental larvae.

Upon comparing the cardiac outcomes between the control and the MPTP-exposed larvae, significant differences emerged. While the control group maintained normal, healthy cardiac

development without any signs of cardiac edema or malformation, a marked difference was noted in the MPTP-exposed group. Here, a considerable majority, about 70% (14 out of 20 larvae), displayed cardiac edema. In-depth analysis, we found that out of these 20 larvae, 12 exhibited severe cardiac edema, and 2 demonstrated mild edema. This clear distinction underlines the detrimental impact of MPTP on cardiac development, manifesting as a high incidence of cardiac edema in the experimental group.

In the AA pre-treatment group (50μM), we observed distinct cardiac outcomes when compared with the MPTP-exposed larvae. Among the AA-pre-treated larvae, cardiac edema was present in 50% of cases (10 out of 20 larvae), with 4 showing severe edema and 6 mild. Interestingly, the other half of the AA group exhibited normal cardiac development, similar to the control groups. This contrasts with the MPTP group, where 70% showed cardiac edema. The AA-pre-treatment group, therefore, displayed a significantly lower proportion of cardiac edema compared to the MPTP-exposed group. These findings suggest that AA pre-treatment may exert a milder impact on cardiac development than MPTP exposure, potentially offering some degree of protection or mitigation against cardiac edema induced by MPTP. The observation that half of the AA group had normal cardiac development, which is a higher percentage than in the MPTP group, further supports this inference.

Turning to the results with 250μM VA pre-treatment, we noted differences in cardiac outcomes when compared to the MPTP-exposed larvae (figure 9, table 2). Out of 20 VA pre-treated larvae, 13 (65%) displayed cardiac edema – with 10 showing severe and 3 mild cases – while the remaining 7 larvae (35%) exhibited normal cardiac development similar to the control

group. This comparison reveals that the VA pre-treatment group had a slightly lower proportion of cardiac abnormalities compared to the MPTP group. However, the reduction was not pronounced enough to suggest a significant mitigative effect of VA on MPTP-induced cardiac abnormalities. These results indicate that VA pre-treatment may not have strong protective properties against the detrimental cardiac effects induced by MPTP exposure.

Lastly, in the case of 100μM FA pre-treatment, the outcomes were more promising. Here, 35% of the larvae (7 out of 20) exhibited cardiac edema – with 2 showing severe and 5 mild cases – while a majority, 65% (13 out of 20), displayed normal cardiac development, paralleling the control group. The lower incidence of cardiac edema in the FA group suggests that FA may have a mitigating effect on this specific outcome compared to MPTP exposure. The results indicate that pre-treatment with FA can effectively reduce the impact of MPTP exposure on cardiac abnormalities, suggesting that FA possesses protective properties against the detrimental effects of MPTP on cardiac development.

In conclusion, while all pre-treatments (AA, VA, and FA) exhibited some degree of impact on mitigating MPTP-induced cardiac edema, the extent and efficacy of this mitigation varied. AA and FA, in particular, showed more promising results in reducing the incidence of cardiac abnormalities compared to MPTP exposure, indicating their potential as protective agents against the harmful cardiac effects induced by MPTP.

3.3.Immunohistochemistry Results

To determine the effect of MPTP and neuroprotective compounds (AA, VA, and FA) on the number of DAnergic neurons in the ventral diencephalon (vDC) region of the brain, we treated larvae according to the timeline of neuroprotective treatment and MPTP neurotoxin as shown in figure 4. The results of the study can be found in the following.

3.3.1. MPTP Reduces Number of Dopamine Neurons

In response to exposure to 0.25 mM MPTP, larvae exhibited a noteworthy reduction in the total count of eGFP-positive neurons, particularly within specific clusters of the ventral diencephalon (vDC) (Figure10- C, Figure11- A). Specifically, clusters 8, 12, and 13 displayed substantial decreases of 63%, 59.8%, and 70.7%, respectively, in the number of eGFP-positive neurons. Cumulatively, the vDC area demonstrated a significant overall decline, revealing a 65% decrease in the total count of eGFP-positive neurons. These findings underscore the impact of 0.25 mM MPTP exposure on the vulnerability of eGFP-positive neurons in the specified vDC clusters, suggesting a potential susceptibility of these neuronal populations to the neurotoxic effects induced by MPTP.

3.3.2. Ascorbic Acid, Vanillic Acid and Ferulic Acid Pre-treatments Can Improve Impact of MPTP on the Number of Dopamine Neurons.

After pre-treatment with 50μM of AA, larvae showed an increase in the overall count of eGFP-positive neurons in specific clusters of vDC (Figure10- D, Figure11- B). Clusters 8, 12, and 13 exhibited increases of 31%, 12.6%, and 6%, respectively, in the number of eGFP-expressing dopaminergic (DAergic) cells compared to the control groups. Overall, 14.85% more eGFP-positive neurons were found in the vDC area compared to the control group. There was also a significant increase in eGFP-positive dopaminergic (DAergic) cells in AA-treated larvae in comparison to MPTP-treated larvae, with 254%, 180%, and 262% enhancements in clusters 8, 12, and 13, respectively. Overall, the number of eGFP-positive DAergic cells in the vDC region increased impressively by 3.3-fold (229.4%) compared to the MPTP group. Accordingly, pre-treatment with 50μM of AA demonstrated a notable neuroprotective effect in larvae, leading to increased counts of eGFP-positive neurons.

Following pre-treatment with 250μM of VA, larvae exhibited a reduction in the overall count of eGFP-positive neurons within specific clusters of vDC (Figure10- E, Figure11- C). Compared to the control group, clusters 8, 12, and 13 showed a decrease in eGFP-expressing dopaminergic (DAergic) cells by 34%, 41.5%, and 30.7%, respectively. As a result, there was a 35% decrease in eGFP-positive neurons in the vDC area overall. However, VA pre-treated larvae showed a notable increase in eGFP-expressing dopaminergic (DAergic) cells compared to MPTP-treated larvae, with enhancements of 78%, 45%, and 137% in clusters 8, 12, and 13, respectively. Globally, the number of eGFP-positive DAergic cells in the vDC region exhibited a substantial 86% increase compared to the MPTP group.

After being pre-treated with 100 mM of FA, larvae displayed an increase in the number of eGFP-positive neurons in certain clusters of vDC (Figure10- F, Figure11- D). It was found that

clusters 8, 12, and 13 had increased levels of eGFP-expressing dopaminergic (DAergic) cells, as compared to the control group, by 27%, 20%, and 17%, respectively. This led to an increase of 20.8% in the number of eGFP-positive neurons in the area of the vDC, as a result of the increased eGFP expression. Furthermore, FA pre-treated larvae demonstrated a significant increase in the number of dopaminergic (DAergic) cells expressing eGFP when compared with larvae treated with MPTP. Specifically, clusters 8, 12, and 13 exhibited an increase of 243.75%, 198.8%, and 301.3%, respectively. According to our data, the number of eGFP-positive DAergic cells in the vDC region experienced a significant 245.8% (or 3.4-fold) increase in comparison to the MPTP group in general.

In the pre-treatment group with FA, the quantity of eGFP-positive DAergic cells exceeded that of the control group. Consequently, we conducted an investigation into the impact of FA on the quantity of these cells using immunohistochemistry (IHC) in the absence of MPTP. Throughout the experiment, zebrafish larvae were exposed to 100μM of FA from 1dpf to 3dpf in a 6-well plate. Following this initial exposure, the zebrafish larvae were subsequently exposed to E3 medium from 4dpf until the time of analysis at 6dpf. As part of the experiment, the exposure mediums were renewed daily to maintain the specified concentrations. Following treatment with 100μM of FA, larvae displayed an increase in the total number of eGFP-positive neurons within particular clusters of vDC (Figure10- G, Figure11- D). Dopaminergic (DAergic) cells expressing eGFP were significantly increased in clusters 8, 12, and 13 compared to the control group by 53.7%, 33%, and 14%, respectively. Therefore, there was an overall increase of 31% in eGFP-positive neurons in the vDC area. In conclusion, after treatment with 100μM of FA, larvae demonstrated a substantial 31% increase in eGFP-positive neurons in the vDC region, indicating a pronounced impact of FA on enhancing dopaminergic cell populations.

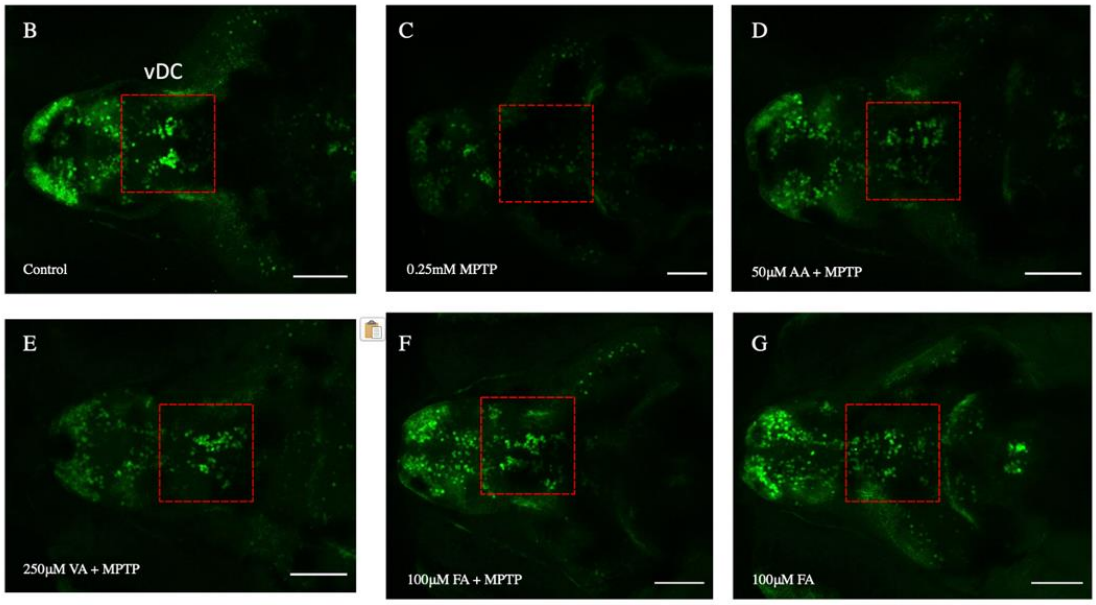


Figure 10- Effects of MPTP neurotoxin and neuroprotective agents (AA, VA, and FA) on ventral diencephalic (vDC) DAnergic clusters of 6dpf Tg (*dat*:eGFP) zebrafish larvae. A) Schematic representation of eGFP neuronal clusters 8, 12, and 13 within the vDC: Cluster 8 in yellow, cluster 12 in green, and cluster 13 in blue. Dorsal view with anterior to the left. B-G) eGFP+ cells within the vDC through confocal fluorescent imaging following exposure to 0.25 mM MPTP, 50µM AA, 250µM VA, and 100µM FA (n = 10-15). All images were acquired using a maximum projection of 2µm z-stacks, scale bar = 100µm.

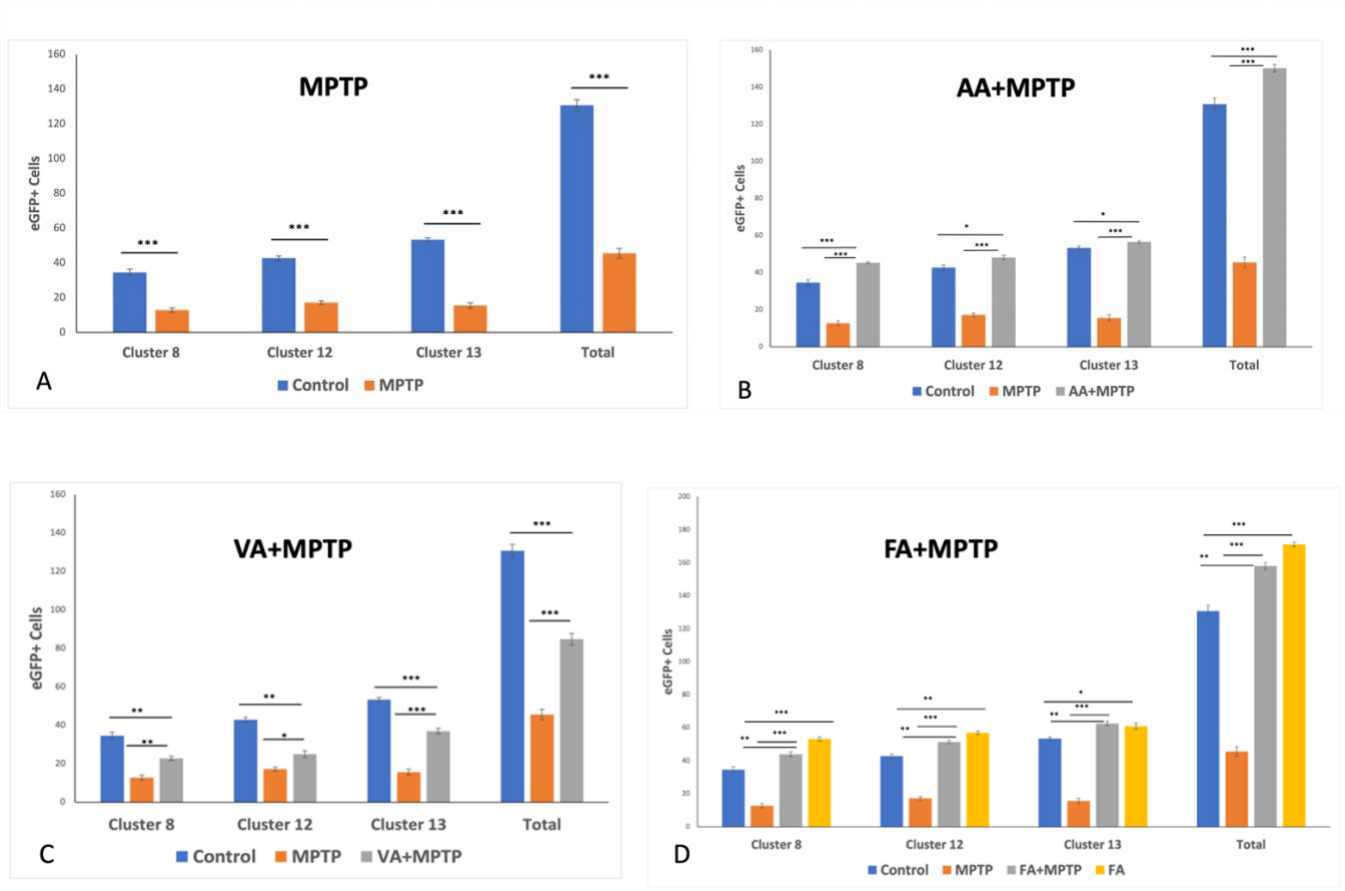


Figure 11- Quantification eGFP+ cells within the vDC through confocal fluorescent imaging following exposure to A) 0.25 mM MPTP, B) 50µM AA, C) 250µM VA, and D) 100µM FA (n = 10-15). Bars represent the Mean ± the SEM. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

4. Discussion

Very few research studies have been conducted on zebrafish to view the effects of neuroprotection in MPTP-induced PD models. Studies on other disease animal models have suggested that Vanillic Acid (VA), Ascorbic Acid (AA), and Ferulic Acid (FA) possess antioxidative and anti-inflammatory properties, potentially protecting neurons. Our study aimed to investigate whether these substances could prevent the death of dopamine (DA) neurons, a hallmark of PD progression, in a zebrafish model exposed to MPTP. We analyzed the impact of AA, VA and FA on the expression of genes related to DA biosynthesis, DA neuron apoptosis, and mitochondrial dynamics, and conducted immunohistochemical assessments of DA neurons. Each compound demonstrated a notable capacity to counteract the neurodegenerative effects of MPTP, underscoring their potential as neuroprotective agents in PD management. This provided us with preliminary insights into the neuroprotective capabilities of these compounds against PD.

According to the findings of the study on the efficacy of Ascorbic Acid (AA), Vanillic Acid (VA), and Ferulic Acid (FA) in the MPTP-induced zebrafish model of Parkinson's disease, several key insights emerge regarding their potential as neuroprotective agents. The study's observations on gene expression modulation across different pathways provide a comprehensive view of how each compound might contribute to mitigating neurodegeneration and cellular stress, central aspects of Parkinson's disease pathology. The study observed that AA, VA, and FA varied in their efficacy to modulate *dat* and *th1* gene expression in the MPTP-induced zebrafish model of Parkinson's disease. AA demonstrated a substantial upregulation, with an 8.8-fold increase in *dat* and an 85% increase in *th1*, suggesting its potential in mitigating neurodegeneration. VA, with a

3-fold increase in *dat* and 54% in *th1*, showed moderate effectiveness. FA stood out with a remarkable 9.9-fold and 2.5-fold increase in *dat* and *th1*, respectively, surpassing even the control levels. These numeric differences highlight the distinct molecular mechanisms of these compounds, underscoring FA's potential as the most effective neuroprotective agent in this model.

Additionally, in examining the neuroprotective properties of AA, VA, and FA, our study has provided significant insights into the modulation of genes associated with mitochondrial fission, a key factor in the pathogenesis of Parkinson's disease. MPTP treatment alone led to a marked increase in the expression levels of *pink1*, *parkin*, and *fis1* genes—by 100.5%, 305.5%, and 49.28%, respectively, indicating a profound disturbance in mitochondrial dynamics. Pre-treatment with AA showed a reduction in the expression of these genes by 35.65%, 47.5%, and 41%, respectively, when compared to the MPTP treatment group. VA also led to a decrease in gene expression, but to a lesser extent, with reductions of 13.7%, 22.8%, and 2.03% for *pink1*, *parkin*, and *fis1*, respectively. Notably, FA pre-treatment exhibited a more substantial inhibitory effect with a reduction of 54.2% for *pink1*, 45.4% for *parkin*, and 41% for *fis1* gene expression levels, relative to the MPTP group. These findings suggest that while all three compounds possess neuroprotective capabilities, FA may exert a stronger regulatory influence over mitochondrial fission processes in dopaminergic neurons under toxic stress induced by MPTP. Interestingly, the varying degrees of efficacy observed among these agents in regulating *pink1* and *parkin* gene expression underscore potential differences in their mechanisms of action or bioavailability, which could be pivotal in their neuroprotective roles. Furthermore, the ability of AA and FA to considerably reduce *fis1* expression, coupled with the minimal effect of VA, highlights the potential for selective neuroprotective interventions targeting specific aspects of mitochondrial

dysfunction in Parkinson's disease. As such, these natural phenolics, particularly FA, could be valuable in the development of neuroprotective strategies for mitigating the progression of Parkinson's disease.

In the following, the impact of neuroprotective agents Ascorbic Acid (AA), Vanillic Acid (VA), and Ferulic Acid (FA) on the expression of *mfn1* and *opa1* genes in the context of mitochondrial fusion was assessed in an MPTP-induced model of Parkinson's disease in zebrafish. Following the induction of Parkinsonian pathology by MPTP, a decrease in the expression of *mfn1* by 15% and *opa1* by 10% was indicative of mitochondrial fusion impairment. AA slightly upregulated *mfn1* by 24.7% and *opa1* by 8.8%, suggesting some protective capability. VA's treatment increased *mfn1* by 29.4% and *opa1* by 2%, yet without definitive impact. FA's treatment led to 11.7% and 16% increases in *mfn1* and *opa1*, respectively, indicating a potential effect on fusion processes, though the significance of *mfn1* changes remained unclear. The data suggest that while the tested compounds may confer some degree of neuroprotective effect, their influence on mitochondrial fusion is not as pronounced as one might expect. This could imply a limited role for AA, VA, and FA in the specific modulation of *mfn1* and *opa1* expression, or it may reflect the complexity of the mitochondrial dynamics that are affected in Parkinson's disease. Given these findings, it may be concluded that the capacity of these neuroprotective agents to modulate genes associated with mitochondrial fusion does not significantly contribute to their overall protective mechanisms in the MPTP model of Parkinson's disease in zebrafish. Further research might explore the possibility of synergistic effects with other compounds or investigate alternative pathways through which these agents may exert their neuroprotective effects.

Furthermore, our analysis revealed a significant upregulation of *p53* gene expression by about 79.5% following MPTP treatment when compared to the control, indicating MPTP's potential role in inducing cellular stress that leads to apoptosis. In the face of this stress, pre-treatments with AA, VA, and FA have shown to modulate this response. AA pre-treatment resulted in a 34.7% decrease in *p53* expression, suggesting its capacity to mitigate cellular stress response. VA also contributed to a reduction, but to a lesser extent, with a 15.7% decrease in *p53* levels. FA, however, had the most substantial effect, demonstrating a 51.17% reduction in *p53* expression compared to the MPTP group. These findings suggest that while all three agents have some impact on the *p53* pathway, FA might be particularly effective in modulating cellular stress responses in this model of Parkinson's disease.

Integrating the latest findings on the neuroprotective efficacy of AA, VA, and FA concerning dopaminergic neuron preservation in the vDC region with previous insights offers a holistic understanding of their potential against Parkinson's disease. The study meticulously bridges the gap between molecular genetic alterations and tangible neuroprotective outcomes, casting light on the comprehensive role these agents play in combating neurodegeneration. The neuroprotective efficacy of AA, VA, and FA in the context of dopaminergic neuron preservation in the vDC region was a key focus of our study. After MPTP administration, a substantial decrease in the population of eGFP-positive neurons was observed, indicating significant neurodegeneration. Pre-treatment with AA showed a notable reversal of this effect. Specifically, there was an impressive 3.3-fold increase in the number of eGFP-positive DAergic cells compared to the MPTP group. This suggests that AA has a potent neuroprotective effect, significantly countering the neurotoxicity of MPTP. VA, while less effective than AA, still demonstrated a

significant protective impact. Compared to MPTP-treated larvae, VA pre-treatment led to increases in dopaminergic neurons, resulting in an overall 86% increase. This indicates that VA, albeit to a lesser extent than AA, can mitigate MPTP-induced dopaminergic neuron loss. FA exhibited the most substantial neuroprotective effect among the tested compounds. Not only did it markedly increase the number of dopaminergic neurons in response to MPTP exposure, with increases ranging from 243.75% to 301.3% in specific clusters, but it also enhanced neuron counts beyond the control group's levels. This finding is particularly noteworthy as it suggests that FA might not only protect against neurodegeneration but also promote the growth or survival of dopaminergic neurons. These results collectively underscore the potential of these neuroprotective agents, particularly FA, in preserving and enhancing dopaminergic neuron populations in Parkinson's disease models. Such findings are instrumental in guiding future therapeutic strategies for Parkinson's disease, where dopaminergic neuron preservation is crucial. However, it is essential to consider these results in the context of a zebrafish model and the need for further studies to validate the efficacy of these compounds in mammalian models or clinical settings.

The study presents two critical aspects of neuroprotection in the context of Parkinson's disease: the expression levels of the *dat* gene and the actual counts of dopaminergic (DAergic) neurons in the vDC region of zebrafish. Notably, these two parameters offer complementary insights into the effects of neuroprotective agents Ascorbic Acid (AA), Vanillic Acid (VA), and Ferulic Acid (FA). On one hand, the expression levels of the *dat* gene, which plays a crucial role in dopamine neurotransmission, exhibited significant modulation upon treatment with these neuroprotective agents. The variations in *dat* gene expression suggested a differential capacity of these agents to influence the molecular pathways critical for dopaminergic neuron function. On

the other hand, the actual neuron counts provided a more direct measure of the neuroprotective efficacy of these agents. The substantial increases in the number of DAergic neurons in the vDC region following treatments, particularly with FA, indicated a potent capacity to not only protect but possibly also to promote the survival and growth of these neurons. This was especially evident with FA, which showed not only a protective effect against MPTP-induced neurodegeneration but also an enhancement beyond the baseline levels observed in the control group. The comparison of these two sets of results offers a nuanced understanding of neuroprotection. While changes in gene expression can indicate molecular alterations and potential neuroprotective mechanisms, the neuron counts provide a more tangible measure of the efficacy of these treatments in preserving neuron populations. The correspondence between the upregulation of *dat* gene expression and the increase in neuron counts, particularly in the case of FA, suggests a link between molecular changes at the gene level and actual neuroprotective outcomes in terms of neuron survival.

In the next step, our investigation into the cardioprotective effects of AA, VA, and FA in the context of MPTP-induced neurotoxicity in zebrafish reveals insightful findings. The control groups displayed optimal cardiac health, providing a baseline against which the impacts of MPTP and the neuroprotective agents could be measured. The MPTP group exhibited a significant occurrence of cardiac edema, with 70% of the larvae affected, indicating a pronounced adverse effect of MPTP on cardiac development. In comparison, AA pre-treatment showed a reduced incidence of cardiac edema, present in 50% of the larvae. This suggests a mitigative impact of AA on cardiac edema, although it did not completely prevent cardiac abnormalities. The fact that half of the AA group maintained normal cardiac development, in contrast to the predominantly affected MPTP group, points to the potential cardioprotective properties of AA. The VA pre-treatment

group displayed cardiac edema in 65% of the larvae, a slight improvement compared to the MPTP group. However, this reduction was not marked enough to signify a strong protective effect against MPTP-induced cardiac damage. Thus, VA's role in cardiac protection appears to be limited within the parameters of this study. FA pre-treatment presented the most effective cardioprotective outcome. A lower incidence of cardiac edema was observed in the FA group, with only 35% of the larvae affected, and a majority exhibiting normal cardiac development. This indicates that FA might provide significant protection against MPTP-induced cardiac abnormalities, demonstrating its potential as a potent neuroprotective agent with cardioprotective capabilities. Conclusively, while all three agents showed some degree of effect in mitigating MPTP-induced cardiac edema, the varying degrees of efficacy highlight their distinct protective mechanisms. Notably, AA and FA emerged as more effective in reducing cardiac abnormalities, with FA displaying a particularly strong protective effect. These results enhance our understanding of the multifaceted impact of neuroprotective agents, not only in neuroprotection but also in preserving cardiac health in Parkinson's disease models, underscoring the intricate link between neurological and cardiac health in this disease context.

Our integrative study demonstrates that AA, VA, and FA exhibit potent neuroprotective and cardioprotective effects in a zebrafish model of Parkinson's disease. For AA, we observed a 3.3-fold surge in dopaminergic neurons, echoing the substance's role in combating oxidative stress-related neurotoxicity, as also supported by literature documenting its enzymatic and metabolic functions (Wu et al., 2015; Xiang et al., 2018; Paduraru et al., 2021; Nualart et al., 2014). This finding is significant, considering AA's facilitated transport across the blood-brain barrier, which may underpin its neuroprotective efficacy. Similarly, VA's capacity to increase dopaminergic

neuron survival and reduce cardiac abnormalities aligns with its antioxidative properties. Such dual benefits in neurological and cardiac health, as demonstrated by reduced cardiac edema in our study, are supported by its documented roles in neurological disorders and cardiac conditions (Khoshnam et al., 2018; Kumar et al., 2011; Salau et al., 2020; Stanely Mainzen Prince et al., 2011). Our research further extends to FA's impact, showcasing its significant enhancement of dopaminergic neuron counts and cardioprotective effects. FA's potent neuroprotective capability dovetails with its known antioxidizing properties and effectiveness against free radical damage (Ogiwara et al., 2002; Koh, 2013; Ren et al., 2017; Son et al., 2010; Cheng et al., 2008). The collective findings from our study suggest that while AA, VA, and FA share common neuroprotective and antioxidative traits, they may operate through different mechanisms in various systems and against different neurotoxic stressors. This underscores their potential as multifaceted protective agents, especially for conditions like Parkinson's disease, and highlights the need for more nuanced investigations into their specific actions and efficacy across neurodegenerative and cardiovascular diseases.

This research could suggest a cautious approach towards clinical applications in Parkinson's disease. The potential of ascorbic acid, vanillic acid, and ferulic acid as neuroprotective agents, due to their antioxidative properties, shows that they may provide a means to manage oxidative stress in neurons. However, the translation from zebrafish models to human treatment is complex and requires extensive further study. If effective, these compounds could offer a dual benefit for neurological and cardiac health, potentially slowing disease progression and improving patient quality of life. The research underscores the importance of continued exploration into these compounds' mechanisms and therapeutic viability. While the zebrafish

model offers valuable insights, its translatability to human Parkinson's disease is a significant limitation of this study. Future research could focus on comparative studies across different models, including mammalian systems, to better understand the compounds' effects. Long-term studies could observe the progression and potential long-term benefits or side effects of these neuroprotective compounds. Ultimately, progressing to human clinical trials will be critical to establish the efficacy and safety in humans, taking into account the complexities of human neurodegenerative diseases.

5. Conclusion

This study represents a significant contribution to the field of neurodegenerative disease research, specifically focusing on Parkinson's disease. The investigation into the neuroprotective properties of AA, VA, and FA in a zebrafish model exposed to MPTP has yielded promising results that enhance our understanding of PD's molecular dynamics and potential health-promoting interventions. Our findings suggest that all three compounds – AA, VA, and FA – exhibit neuroprotective and, to a lesser extent, cardioprotective properties. These effects are particularly evident in their capacity to prevent the death of dopamine neurons, a hallmark of PD. This neuroprotection is likely due to their antioxidative and anti-inflammatory properties, with each compound showing varying degrees of efficacy and potential mechanisms of action. FA stood out as the most effective neuroprotective agent, demonstrating substantial protective effects against neurodegeneration and cardiac abnormalities. Its strong antioxidative properties suggest a significant role in combating oxidative stress, a key factor in PD pathology. AA also showed notable neuroprotective capabilities, particularly in enhancing dopaminergic neuron survival,

possibly due to its facilitation across the blood-brain barrier. VA, while less effective than FA and AA, still exhibited protective qualities, underscoring its potential as a neuroprotective agent. These findings, while promising, highlight the necessity for further research. The complexity of translating results from zebrafish models to human applications cannot be overstated. Future studies should focus on exploring the specific pathways through which these compounds exert their neuroprotective effects. Comparative studies across different animal models, including mammalian systems, are essential to gain a comprehensive understanding of these compounds' effects in various biological contexts. Moreover, long-term studies observing the progression of neurodegenerative diseases and potential side effects of these compounds will be crucial. Ultimately, clinical trials in humans will be necessary to establish the efficacy and safety of these potential treatments for Parkinson's disease.

In conclusion, this research opens new avenues for the development of neuroprotective approaches for Parkinson's disease. The antioxidative properties of AA, VA, and FA, their effects on mitochondrial dynamics, and their potential to modulate key genes and neuron survival present a promising basis for future protective strategies. However, the journey from laboratory research to clinical application is long and requires a meticulous approach to ensure safety and efficacy in human patients. As we continue to unravel the complexities of neurodegenerative diseases, studies like this lay the groundwork for innovative treatments that could significantly improve patient outcomes in Parkinson's disease and potentially other neurodegenerative disorders.

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