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Evaluation of the Neuroprotective Role of Astaxanthin against Cisplatin-Induced Neurotoxicity in Mice Models

by

Samin Khan

A thesis submitted in partial fulfillment for the
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in the

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Abstract

Disruption of nervous systems in humans or animals due to direct or indirect effects of chemicals is accompanied by neurotoxicity. Cisplatin is a chemotherapeutic drug that is widely used in the treatment of cancer; however, due to its undesirable neurotoxic effects, including neuroinflammation, neuronal degeneration, and cognitive dysfunction in the nervous system, its use is limited. Astaxanthin (ASTA), a natural xanthophyll carotenoid, has been identified as a powerful antioxidant and anti-inflammatory agent that has emerged as a promising neuroprotective agent. This study was designed to explore the anti-neurotoxic properties of Astaxanthin on cisplatin-induced neurotoxicity in vivo, using histopathological, network pharmacology, and molecular docking approaches. Initially, neurotoxicity was induced by administering cisplatin (2.3 mg/kg, i.p) to all the experimental groups except the normal. Astaxanthin (5, 10, or 25 mg/kg, i.p) and mecobalamin (1 mg/kg, i.p) were injected 30 minutes before cisplatin induction. Neurobehavioral parameters were assessed using the Open Field Test (OFT) and Inverted Screen Test (IST). The histopathological changes in the cisplatin-induced hippocampus and cerebellum were evaluated by hematoxylin and eosin (H & E) staining. Similarly, the level of TNF- α and IL-1 β in the hippocampus and cerebellum was measured using an Enzyme-Linked Immunosorbent Assay (ELISA). Furthermore, the levels of nitric oxide (NO) and malonaldehyde (MDA) were quantified in the cisplatin-induced hippocampus and cerebellum. Network pharmacology and computational analysis were carried out to investigate the underlying molecular signaling in cisplatin-induced neurotoxicity. The results indicated that astaxanthin markedly improved cisplatin-induced neurobehavioral parameters dose dependently. It was noted that Astaxanthin significantly suppressed histopathological changes following cisplatin induction. Moreover, Astaxanthin significantly reduced cisplatin-induced enhanced levels of proinflammatory cytokines (TNF- α and IL-1 β) and oxidative stress parameters (NO and MDA). The therapeutic targets were determined by network pharmacology, the signaling pathways were enriched, and the molecular docking confirmed the interactions between Astaxanthin

and 6 hub proteins: Tumor Necrosis Factor (TNF), Interleukin-6 (IL-6), Peroxisome Proliferator-Activated Receptor Gamma (PPAR γ), Mitogen-Activated Protein Kinase 14 (MAPK14), Mitogen-Activated Protein Kinase 3 (MAPK3), and Mitogen-Activated Protein Kinase 1 (MAPK1).

Keywords: Astaxanthin, Cisplatin, Neurotoxicity, Neuroinflammation, Network Pharmacology, Molecular Docking.

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Abbreviations

ANOVA	Analysis of Variance
BCA	Bicinchoninic Acid
CGA	Chlorogenic Acid
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme-Linked Immunosorbent Assay
H&E	Hematoxylin and Eosin
HRP	Horseradish Peroxidase
IAEC	Institutional Animal Ethics Committee
IL-1β	Interleukin-1 Beta
IL-6	Interleukin-6
i.p.	Intraperitoneal
NF-κB	Nuclear Factor Kappa B
SD	Standard Deviation
SEM	Standard Error of the Mean
TNF-α	Tumor Necrosis Factor Alpha

Symbols

n	Number of animals per group
%	Percentage
mg	Milligram
μg	Microgram
pg	picogram
mg/kg	Milligram per kilogram
μL	Microliter
mL	Milliliter
cm	Centimeter
s	Second
$^{\circ}C$	Degree Celsius
*	$p < 0.05$ (statistically significant)
**	$p < 0.01$ (highly significant)
***	$p < 0.001$ (very highly significant)
#	$p < 0.05$ vs control group
##	$p < 0.01$ vs control group
###	$p < 0.001$ vs control group
$\pm$	Plus or minus (used with mean $\pm$ SD or mean $\pm$ SEM)

Chapter 1

Introduction

1.1 The Nervous System: Components and Functions

In the human body, the most complex and exceptionally ordered system is the NS. It is responsible for regulating and coordinating nearly all physiological and behavioral activities. It acts as a complex communication system that interprets sensations, analyzes signals, and produces an appropriate response to internal or external stimuli. The NS is essential in maintaining homeostasis and regulating higher cognitive functions like learning, memory, perception, emotional control, motor coordination, and behavior. It requires the proper functioning of specific cells and intricate neural networks to transmit and receive electrical and chemical signals [1–3].

1.1.1 Structural Organization of the NS

The NS is organized into two principal parts: the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS comprises the brain and spinal cord and is the site of integration, interpretation, and coordination of neural information. The brain is responsible for cognition, emotions, memory, voluntary

movement, and sensory perception, and the spinal cord links the brain to the periphery and is responsible for many reflex actions. The PNS consists of peripheral ganglia, spinal nerves, and cranial nerves, which link the CNS with muscles, organs, and sensory receptors throughout the body. It is then divided into the Somatic NS, involved in voluntary movement and conscious sensation, and the autonomic NS, which controls automatic roles such as heart rate, respiration, digestion, and glandular activity. The autonomic NS consists of three divisions: sympathetic and parasympathetic, as shown in Figure 1.1 [1, 2].

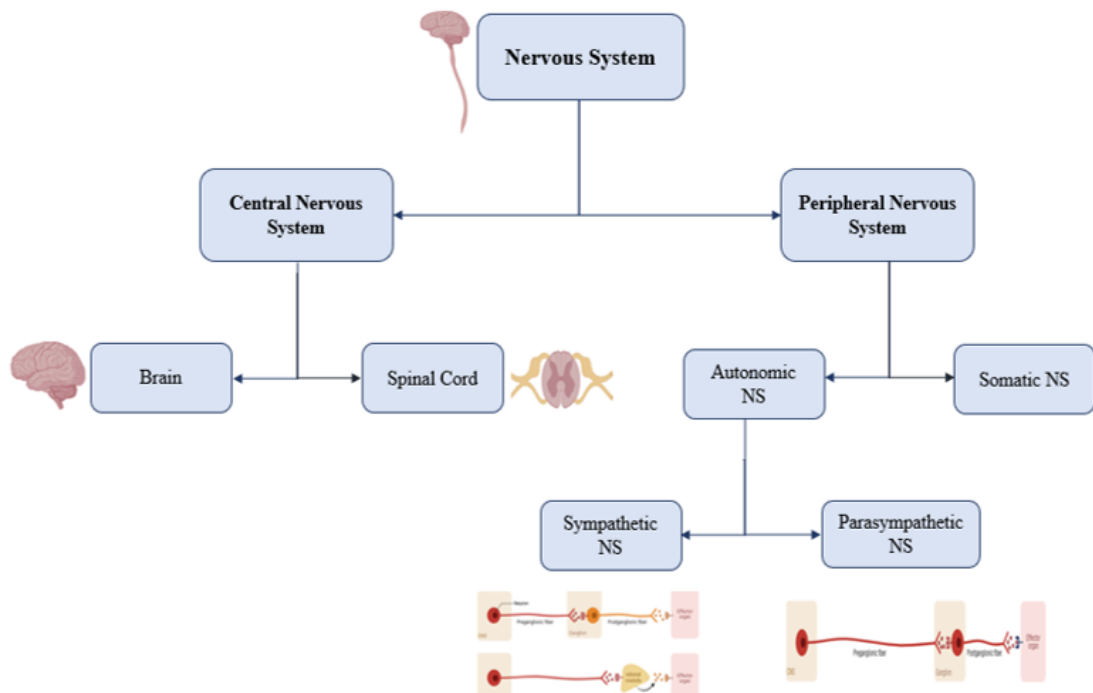


FIGURE 1.1: Division of the Nervous System into the Central Nervous System and Peripheral Nervous System

1.1.2 Cellular Division of the NS

The NS consists mainly of nerve cells (neurons) and supporting cells (glial cells) at the cellular level [4]. Neurons are highly specialized excitable cells and the integral units of the NS. They send, receive, and produce electrical signals called action potentials. A single neuron is composed of a cell body (soma), which processes incoming information, a dendrite, which sends the information to the cell body, and an axon, which carries a signal to the target cell through a synapse. Neurons release chemicals called neurotransmitters inside the synaptic cleft that bind to

receptors on another neuron or on an effector cell. This allows information to be delivered quickly and accurately throughout the NS [4, 5].

Neuroglia, or glial cells, support neurons in structure, metabolism, and immunity, and are essential for maintaining neuronal integrity and homeostasis. The most important glial cells in the CNS are astrocytes, oligodendrocytes, microglia, and ependymal cells [6]. In the PNS, Schwann cells are the major glial cells. Astrocytes maintain the extracellular balance of ions, supply metabolic support to the tissue, help maintain the blood–brain barrier, and recycle neurotransmitters [5]. Myelin sheaths are formed by oligodendrocytes and Schwann cells and wrap around axons, enhancing the conduction of nerve impulses. In the CNS, microglia are cells with innate immunity in normal tissue that respond to injury and infection. The ventricles of the brain are lined by ependymal cells, which are responsible for the formation and movement of cerebrospinal fluid. [1, 2, 6].

1.1.3 Neurotransmission and Chemical Signaling

The functioning of the NS depends on complex excitatory and inhibitory neurotransmission. Glutamate is the main excitatory neurotransmitter in the CNS, and is supposed to play a part in learning, memory, and synaptic plasticity. The main inhibitory neurotransmitter that regulates neuronal excitability is gamma-aminobutyric acid (GABA). Dopamine, serotonin, acetylcholine, glycine, and norepinephrine are other important neurotransmitters that regulate motor activity, cognition, autonomic functions, mood, and sensory processing. The NS's normal functioning depends on the balance of neurotransmitter activity, and disturbances in that balance lead to a variety of neurodegenerative and neuropsychiatric diseases [1, 2, 4].

1.1.4 Vulnerability of NS to Damage and Neurotoxicity

The NS, although highly organized, is very vulnerable to damage and the effects of toxic agents. Neurons have very high metabolic requirements and require a

constant supply of oxygen and glucose. They are also relatively weak regenerators in the adult CNS and have weaker antioxidant defenses, making them more vulnerable to toxic damage and oxidative stress.

Neurotoxicity is produced by the presence of environmental toxins, heavy metals, pesticides, infections, and some therapeutic drugs, which can disrupt neuronal signaling, induce neuroinflammation, cause structural damage, and ultimately lead to neurological dysfunction [1, 7].

1.2 Neurotoxicity

Neurotoxicity is the harmful effect of a chemical, biological, or physical substance on the structure or function of the NS. It may be caused by direct damage to neurons or by indirect damage resulting from changes in glia, neurotransmitter systems, mitochondrial function, axonal transport, or intracellular signaling pathways. Neurotoxicity can be acute or chronic and can affect either the CNS or the PNS [7, 8].

Neurotoxic damage can affect multiple pathways simultaneously, resulting in neuropathy (neuronal death), axonopathy (axonal degeneration), myelinopathy (myelin damage), or synaptic dysfunction. These changes can lead to cognitive impairment, memory loss, behavioral disturbances, motor dysfunction, sensory dysfunction, or autonomic dysfunction. [8].

1.3 Background of Chemotherapy - Induced Neurotoxicity

Chemotherapy is a widely used treatment against various types of cancers. Despite its clinical effectiveness, it is associated with severe side effects, among which one of the most common is neurotoxicity. Neurotoxicity results in structural and functional disturbances in both the CNS and PNS, leading to various neurological

and behavioral complications. [9]. In recent years, chemotherapy-induced CNS neurotoxicity, also called chemobrain, has been widely recognized, manifesting as impairments in memory, attention, learning ability, and executive functioning [10]. From clinical and experimental studies, it is evident that patients receiving chemotherapy develop persistent long-term cognitive and emotional disturbances even after treatment ends, thereby significantly affecting their quality of life [11]. Chemotherapeutic drugs like platinum-based drugs, taxanes, and vinca alkaloids have been reported to contribute to neurotoxicity through direct neuronal injury and through indirect pathways like neuroinflammatory, oxidative, and apoptotic pathways, and glial cell activation. The mechanisms involved are complicated and include oxidative stress, mitochondrial impairment, neuroinflammation, decreased neurogenesis, and apoptotic pathway activation [9]. Brain regions such as the hippocampus and cerebellum are most susceptible to chemotherapeutic damage, resulting in both cognitive impairments and motor disturbances [10, 12, 13]. Despite increasing awareness of these complications, effective strategies to prevent or manage chemotherapy-induced neurotoxicity remain limited, emphasizing the need for further research into novel neuroprotective agents targeting oxidative and inflammatory pathways [9, 14].

1.3.1 Types of Neurotoxicity

1.3.1.1 Chemical-Induced Neurotoxicity

It is one of the most general categories and includes damage to the neurons caused by exposure to an external chemical. These are often caused by exposure to heavy metals (including lead, mercury, arsenic) [15], pesticides (organophosphates, organochlorines) [16, 17] industrial solvents, exposure to environmental toxins, and therapeutic medications like chemotherapeutic drugs [18].

Pathophysiologically, these chemicals are linked to inducing oxidative stress, mitochondrial defect, impaired axonal transport, DNA damage, and altered neurotransmission. Clinically, it presents with cognitive impairment, memory loss,

motor dysfunction, sensory neuropathy, and behavioral abnormalities, depending on the agent, dosage, and duration of exposure [7, 8, 16–18].

The clinical presentation of chemical-induced neurotoxicity varies significantly depending on the specific chemical involved, the dose, treatment duration, and the level of development of the NS. Symptoms may include cognitive impairment, memory deficits, behavioral abnormalities, motor dysfunction (including tremors, bradykinesia, and ataxia), sensory neuropathy characterized by paresthesia, numbness, and neuropathic pain, and in severe cases, seizures or encephalopathy. [7, 16]. To investigate these effects, several well-established experimental animal models of chemical-induced neurotoxicity have been developed. These models are widely used in preclinical research to replicate human conditions and evaluate neuroprotective agents.

a. Cisplatin-Induced Neurotoxicity Mode

The cisplatin-induced neurotoxicity model is one of the most extensively used experimental models for studying chemotherapy-induced peripheral and central neurotoxicity [9, 19, 20]. In rodents, cisplatin reliably produces several disease processes, such as oxidative stress, mitochondrial impairment, neuroinflammation, DNA damage, and apoptotic neuronal injury [9, 21–23]. The effects of experimental treatment include decreased locomotor activity and motor coordination, cognitive impairments, anxiety, and peripheral neuropathy [22, 24]. Histopathological changes such as neuronal degeneration, gliosis, vacuolation, and structural damage in areas of the brain, such as the hippocampus and cerebellum, have also been consistently reported [22, 25]. The model has been widely employed to assess the neuroprotective activity of anti-oxidant and anti-inflammatory agents, natural products such as astaxanthin [25].

b. Rotenone-Induced Neurotoxicity Model

The rotenone-induced neurotoxicity model is the most popular for inducing Parkinson's disease-like degeneration in experimental rodents [26].

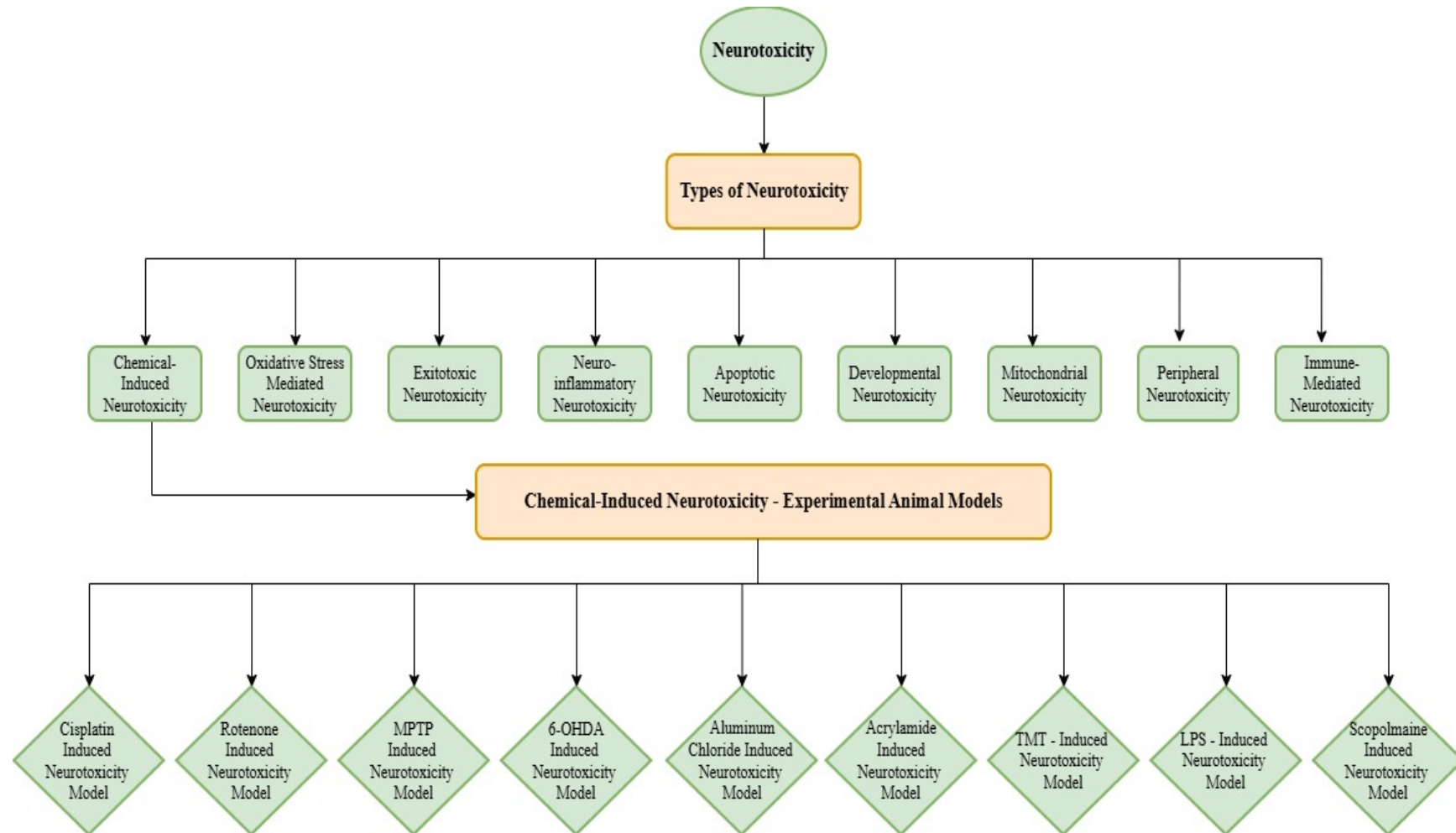


FIGURE 1.2: Types of Neurotoxicity and Chemical-Induced Neurotoxicity Models

Rotenone is a highly lipophilic pesticide and a potent inhibitor of the mitochondrial complex I that readily crosses the blood–brain barrier [27]. The inhibition of complex I causes ATP depletion, an rise in ROS, oxidative stress, and mitochondrial impairment; in the substantia nigra pars compacta, dopaminergic neurons are selectively degenerated [28, 29]. Chronic exposure to rotenone also induces aggregation of α -synuclein and neuroinflammatory responses, which are similar to the pathological variations seen in Parkinson’s disease [28, 30]. Commonly observed clinical symptoms in experimentally treated animals are Parkinsonian symptoms, such as a decrease in spontaneous locomotor activity, postural disturbances, and motor coordination impairment [29, 31]. This model can reproduce both behavioral and neuropathological symptoms of Parkinson’s disease and has thus been widely used to study mitochondrial impairment, oxidative stress-mediated neuronal damage, and therapeutic efficacy of antioxidant and neuroprotective drugs [32, 33].

c. MPTP-Induced Neurotoxicity Model

The MPTP-induced neurotoxicity model is a broadly used and well-established investigational model of Parkinson’s disease because of its simplicity, reproducibility, and high clinical relevance when compared with other toxin-induced models [34]. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a potent neurotoxin that can cross the BBB and is metabolized into its lethal metabolite MPP⁺ (1-methyl-4-phenylpyridinium) by monoamine oxidase-B (MAO-B) [35].

MPP⁺ selectively binds to the dopamine transporters, is transported into dopaminergic neurons, and binds to mitochondrial complex I, causing ATP depletion, oxidative stress, mitochondrial impairment, neuroinflammation, and, in the substantia nigra pars compacta, dopaminergic neurons are degenerated [35, 36].

Experimental animals treated with MPTP have characteristic Parkinsonian symptoms such as decreased locomotor activity, muscular rigidity, and motor coordination problems [37]. The MPTP model has been widely used to understand the mechanisms of dopaminergic neurodegeneration and to assess the effectiveness of

neuroprotective treatments due to its high pathophysiological relevance and validation [36].

d. 6-Hydroxydopamine Induced Neurotoxicity Model

The 6-hydroxydopamine (6-OHDA) neurotoxin is broadly used to make experimental models of Parkinson's disease, effectively reproducing the key cellular processes involved in PD pathology, including oxidative stress, neurodegeneration, neuroinflammation, and neuronal expiry by apoptosis [38].

6-OHDA has been used in unilateral injections, causing motor deficits such as bradykinesia, akinesia, and rotational behavior, which are characteristic of human Parkinson's disease [39, 40]. The model also shows dose dependency: with different doses of 6-OHDA (from 0.5 μ g to 20 μ g), the dopaminergic cell loss and behavioral impairments vary, allowing researchers to mimic different stages of the disease [41, 42].

The 6-OHDA model does not fully mimic all symptoms of Parkinson's disease, but is highly reproducible and has been shown to have selective effects on dopaminergic neurodegeneration and represents an invaluable model for evaluating the mechanisms of the disease and for assessing therapeutic interventions [43, 44].

e. Aluminum Chloride-Induced Neurotoxicity Model

The aluminum chloride-induced neurotoxicity model is a widely used experimental approach for studying Alzheimer's disease-like pathology in rodents [45]. After a long period of exposure, chronic administration of Aluminum chloride causes oxidative stress, neuroinflammation, and cholinergic dysfunction, which results in the buildup of amyloid beta and phosphorylated tau proteins in the brain tissue [45–47]. This leads to deterioration of neurons, particularly in the hippocampus and cerebellum, and the treated animals show marked cognitive dysfunction, memory loss, and anxiogenic behavior [45, 48]. This model is a valued tool for evaluating the neuroprotective potential of different therapeutic compounds and can be used to determine the effectiveness of treatments for neurodegenerative diseases [49, 50].

f. Acrylamide-Induced Neurotoxicity Model

Acrylamide is a potent neurotoxic agent that exerts multiple effects on the NS in several experimental models and human populations. Occupational and dietary exposure to acrylamide results in peripheral neuropathy characterized by gait abnormalities, muscle weakness, ataxia, and sensory dysfunction in both humans and laboratory animals [51, 52]. The neurotoxic effects include disruption of axonal transport, protein adducts on synaptic proteins, and induction of oxidative stress, which cause neuronal degeneration [53–55].

The effects of acrylamide exposure in experimental models include motor dysfunction, changes in neurotransmitter levels such as acetylcholine and dopamine, and neuroinflammatory responses mediated by the stimulation of the NLRP3 inflammasome [54, 56]. The consistency of results from zebrafish, rodent, and human studies signifies the robustness of the acrylamide model of neurotoxicity and its value for studying neuroprotective therapeutic targets [57, 58].

g. Trimethyltin - Induced Neurotoxicity Model

This neurotoxicity model is a widely accepted experimental model for studying selective neurodegeneration of the hippocampus and limbic system dysfunction. TMT specifically targets cells of the hippocampus, including the CA1 and CA3 regions, and causes severe structural and functional damage to the region [59, 60]. The neurotoxicity caused by TMT can be mediated by several interrelated mechanisms such as mitochondrial impairment, neuroinflammation, excitotoxicity, and caspase-dependent apoptotic pathways [61].

Investigational studies have revealed that the administration of TMT not only leads to microglial activation but also to stimulation of pro-inflammatory signaling pathways, such as activation of the NLRP3 inflammasome, which increases the release of pro-inflammatory cytokines and progressive neuronal damage [62].

Animals exposed to TMT exhibit characteristic behavioral abnormalities such as hyperactivity, aggression, cognitive impairment, learning and memory deficits, anxiety-like behavior, and seizure susceptibility [59]. Further, neuroimaging and

histopathological data show significant atrophy of the hippocampus, loss of neurons, gliosis, and degeneration in the limbic brain regions [63].

The TMT model is widely used for studying mechanisms of limbic neurodegeneration and the neuroprotective effects of pharmacological agents and regenerative therapies, such as metformin and stem cell therapies, with the latter showing reproducible behavioral and pathological changes [64, 65].

h. Lipopolysaccharide Induced Neurotoxicity Model

The LPS model is a well-known experimental model of neuroinflammation-induced neurotoxicity in which microglia and astrocytes are stimulated through the toll-like receptor 4 (TLR4) pathway [66–68]. This activation also induces pro-inflammatory effects such as cytokine secretion, an increase in ROS production, and disruption of neuronal homeostasis [69–71].

The induced neuroinflammation renders the LPS model useful for neurodegenerative disease research, and some applications have been reported in Alzheimer's disease and amyotrophic lateral sclerosis research [72, 73] and is specific in Parkinson's disease modeling [74].

i. The Scopolamine-induced Neurotoxicity Model

This is a well-known and broadly used model to study cognitive deficits and mechanisms associated with Alzheimer's disease. Scopolamine is a non-selective muscarinic acetylcholine receptor antagonist that impairs central cholinergic function and causes severe impairments in spatial learning, recognition memory, and associative memory. The deficits are very similar to those seen in Alzheimer's disease [75, 76].

Scopolamine induces cognitive impairment, oxidative stress, neuro-inflammatory responses, and mitochondrial defects, especially in the hippocampus and cortex, which results in synaptic dysfunction and neuronal injury [77–79]. The model is used broadly for the screening of potential precognitive and neuroprotective drugs and for the elucidation of the role of the cholinergic systems in memory [78].

1.3.1.2 Oxidative Stress-Mediated Neurotoxicity

Oxidative Stress-Mediated Neurotoxicity corresponds to the excess production of ROS and Reactive Nitrogen Species (RNS) in relation to the antioxidant action of the endogenous antioxidant systems.[80]. The leading causes are environmental toxins and toxic chemicals used for cancer treatment (such as cisplatin) [18], and mitochondrial toxins.

On the pathophysiological level, excess ROS results in lipid peroxidation of neuronal membranes, protein oxidation, damage of mitochondria, and fragmentation of DNA, which in turn induces neuronal apoptosis or necrosis. Neurons have a high metabolic demand and low antioxidant reserves, making them particularly susceptible.

In clinical terms, this type manifests as a gradual cognitive decline, motor dysfunction, and neurodegenerative symptoms characteristic of Parkinson's disease, Alzheimer's disease, and chemotherapy-induced psychological damage [18, 80, 81].

1.3.1.3 Excitotoxic Neurotoxicity

Excitotoxic Neurotoxicity states neural injury produced by over-activation of excitatory glutamate receptors [82]. The main causes are stroke, traumatic brain injury, status epilepticus, and exposure to excitotoxins like kainic acid.

Pathophysiologically, overactivation of NMDA and AMPA receptors results in an immense influx of calcium, which stimulates damaging enzymes (proteases, phospholipases, endonucleases), mitochondrial overload, ROS production, and finally necrotic or apoptotic cell death [83, 84].

Its clinical manifestations include seizures, neuronal loss in susceptible areas of the brain (e.g., hippocampus and cortex), and cognitive defects & epilepsy [82–86].

1.3.1.4 Neuroinflammatory Toxicity

Neuroinflammatory Toxicity results from chronic activation of glial cells, particularly microglia and astrocytes, in response to toxic insults or neuronal damage. These can be due to neurotoxic chemicals, infections, and systemic inflammation. Pathophysiologically, stimulated microglia release pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) and nitric oxide (NO) and other ROS and establish a self-perpetuating circle of inflammation and oxidative stress in surrounding neurons. Acute neuroinflammation might be a protective process; chronic activation promotes neurodegeneration. In clinical practice, it is associated with cognitive impairment, depressed mood, motor dysfunction, and neurodegenerative disorders [87–89].

1.3.1.5 Apoptotic Neurotoxicity

Apoptotic neurotoxicity is a final common pathway of many kinds of neurotoxicity due to programmed neuronal cell death. It can be caused by many factors, including oxidative stress [90], mitochondrial toxins [91], and excitotoxicity [86, 92]. Pathophysiologically, it is regulated through intrinsic (mitochondrial) pathways, including cytochrome c release, stimulation of caspase-3, an rise in the Bax/Bcl-2 ratio, and DNA fragmentation [90]. Apoptosis is energy-dependent and causes little inflammation, compared to necrosis. Pathologically, it causes a progressive loss of neurons, leading to functional loss in the affected brain areas and, consequently, cognitive impairments and motor dysfunctions [90, 92–95].

1.3.1.6 Developmental Neurotoxicity

Developmental Neurotoxicity is defined as a negative impact on the developing after exposure in the prenatal or early postnatal period. The sources are mostly lead, methylmercury, arsenic, alcohol (ethanol), and some pesticides [96]. Pathophysiologically, these agents disrupt neuronal proliferation, migration, differentiation, synaptogenesis, and myelination [96, 97].

Exposures, even at low levels, can cause permanent changes in brain architecture and connections. The clinical features include rational disability, attention deficit/hyperactivity disorder (ADHD), learning disabilities, autism spectrum illnesses, and persistent motor impairments into adulthood [97–100].

1.3.1.7 Mitochondrial Neurotoxicity

Mitochondrial Neurotoxicity happens when toxicants directly damage the structure and function of the mitochondria. These include chemotherapeutics, rotenone, and MPTP [101, 102].

Pathophysiologically, the inhibition of electron transport chain complexes leads to decreased ATP production, increased ROS leakage, disturbed calcium homeostasis, and the mitochondrial permeability transition pore is open, leading to cell death. Neurons have very high energy requirements, and as a consequence, mitochondrial impairment quickly disrupts neuronal survival and synaptic transmission [102, 103].

In clinical studies, it has been closely associated with Parkinson’s disease, Huntington’s disease, and chemotherapy-induced fatigue and memory loss [101, 104, 105].

1.3.1.8 Peripheral Neurotoxicity

Peripheral Neurotoxicity is a side effect that affects the peripheral NS, particularly the sensory and motor nerves. It is most commonly caused by chemotherapeutic agents (cisplatin, paclitaxel, vincristine) [106, 107], diabetes, and certain industrial chemicals.

Pathophysiologically, it includes axonal degeneration, demyelination, damage to dorsal root ganglia, and mitochondrial toxicity in long axons [106, 108]. The clinical features observed in patients are chemotherapy-induced peripheral neuropathy (CIPN), which involves distal sensory loss, paresthesia, neuropathic pain, decreased reflexes, and muscle weakness [106, 109, 110].

1.3.1.9 Immune-Mediated Neurotoxicity

Immune-Mediated Neurotoxicity results from an abnormal immune reaction against the NS. The causes are certain drugs, such as immune checkpoint inhibitors, infections, and autoimmunity [111]. The brain damage is pathologically mediated by excessive cytokine and autoantibody production, as well as infiltration of immune cells [112, 113].

Chronic microglial activation further exacerbates injury. It can be clinically manifested as encephalitis [114], demyelinating syndromes [115], cognitive dysfunction, or psychiatric symptoms [115–117].

1.4 Cisplatin: Mechanism and Clinical Use

The chemical structure of cisplatin, a platinum-based anticancer drug commonly used as a first-line treatment for various cancers, including head, neck, bladder, and lung cancer as shown in Figure 1.3 [118, 119].

Once cisplatin enters the cell, cellular aquation occurs because of the low concentration of intracellular chloride. It becomes highly reactive and binds to DNA covalently as it reacts to the N7 site of purine bases, especially guanine, resulting in the formation of intra-strand and inter-strand DNA crosslinks. These DNA adducts lead to structural distortion of the DNA helix, thus inhibiting replication and transcription of DNA, and leading to cell cycle capture mainly at the G2/M stage. When DNA damage becomes irreparable, cisplatin activates apoptosis through the intrinsic mitochondrial pathway [119–121].

In addition to the classical pathway, cisplatin disrupts mitochondrial function, leading to an overproduction of ROS and depletion of antioxidant defenses, such as glutathione and superoxide dismutase [122]. The resulting oxidative stress triggers the inflammatory cascade, including NF- κ B and MAPK, which facilitates the production of inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6 [123]. All

these interrelated pathways eventually result in apoptosis through mitochondrial signaling pathways [122–124].

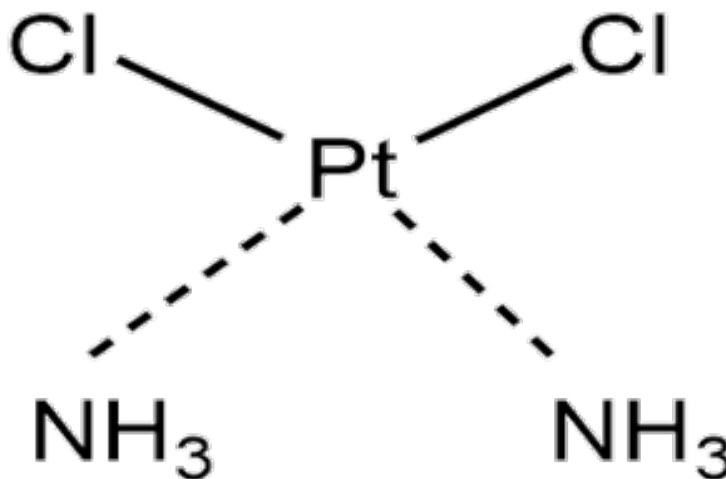


FIGURE 1.3: Chemical structure of Cisplatin

1.5 Cisplatin Toxicities Overview

Although cisplatin has an extraordinary antineoplastic effect, it is associated with a broad range of dose-limiting toxicities that pervade different organ sites. They involve nephrotoxicity, which is the most well-known adverse effect [125], ototoxicity, hepatotoxicity, cardiotoxicity, hematological toxicity, and gastrointestinal disturbances, as shown in Figure 1.4.

Oxidative stress, inflammation, and programmed cell death caused by cisplatin accumulation in non-target tissues are largely attributed to the underlying mechanisms of these toxicities [125, 126].

In addition to all these side effects, neurotoxicity has drawn growing interest because it has major effects on the PNS and CNS, resulting in prolonged neurological and cognitive impairments [9].

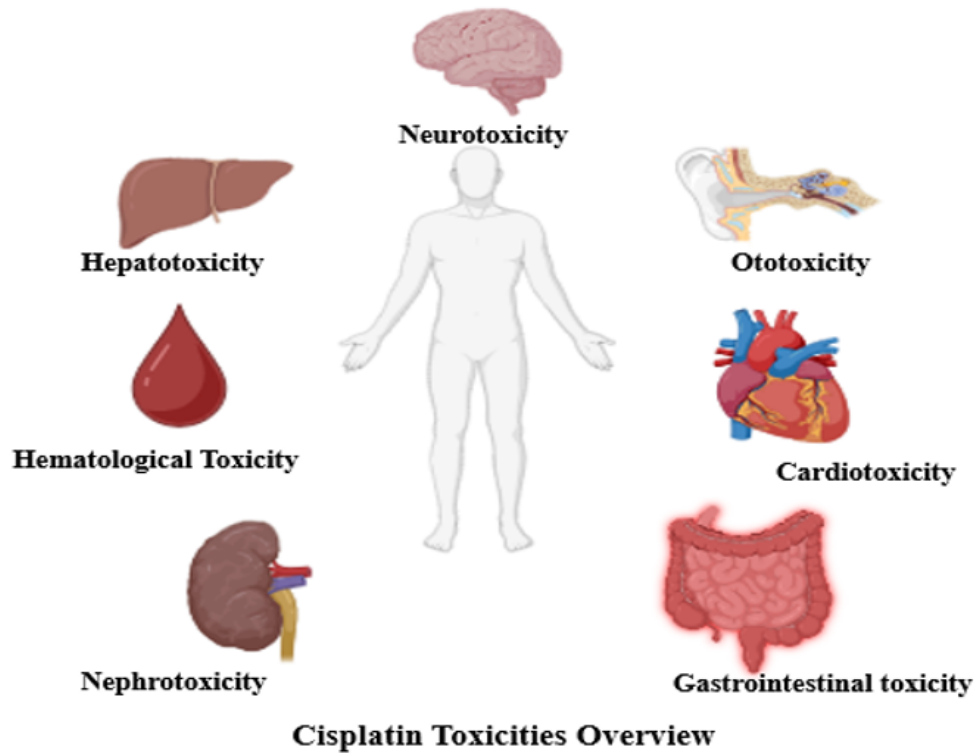


FIGURE 1.4: Cisplatin Toxicities Overview

1.5.1 Cisplatin-Induced Neurotoxicity

Neurotoxicity is one of the serious dose-limiting toxicities induced by Cisplatin, which impacts the NS and leads to a serious decline in the quality of life of patients with cancer [9]. Although traditionally associated with peripheral neuropathy, there is a recent increasing body of evidence that cisplatin has significant changes on the central NS, contributing to cognitive dysfunction, behavioral alterations, and structural brain changes [12]. The neurotoxicity of cisplatin is progressive and, in most cases, irreversible, and therefore, the clinical presentations and the mechanisms involving the neurotoxicity of cisplatin are paramount to understanding [9, 127].

1.5.2 Clinical Manifestations

The neurotoxicity associated with cisplatin has clinical manifestations that include the peripheral and central NS. Numbness, tingling, and neuropathic pain,

which are mainly experienced in the extremities, are some of the characteristics of peripheral neuropathy [127]. Moreover, central neurotoxicity manifests through cognitive impairment, such as memory, attention, learning, and executive dysfunction, often known as chemotherapy-related cognitive impairment or chemobrain. Such changes in behavior, which may include anxiety, depression, and a decrease in motor coordination, have been reported. Remarkably, trauma to certain brain parts, including the hippocampus, causes memory impairment, but cerebellar dysfunction can result in motor coordination and balance issues, and new cognitive disturbances linked with cerebellar cortical interconnections [9, 10, 12].

1.5.3 Pathophysiological Mechanisms

The pathogenesis of cisplatin-induced neurotoxicity is complex and involves multiple interconnected molecular mechanisms. Cisplatin exerts its neurotoxic effects primarily through the formation of deoxyribonucleic acid (DNA) adducts, which not only interfere with nuclear DNA but also impair mitochondrial DNA integrity, leading to mitochondrial defects and reduced adenosine triphosphate (ATP) production. This energy deficit ultimately triggers neuronal apoptosis. In addition, cisplatin promotes an immense rise in ROS, resulting in oxidative stress characterized by increased lipid peroxidation, as proved by a rise in malondialdehyde (MDA) levels, and depletion of endogenous antioxidant defenses such as catalase and glutathione peroxidase (GPx). These effects are further exacerbated by the downregulation of the nuclear factor erythroid 2-related factor 2 (Nrf2)–antioxidant response element (ARE) signaling pathway, which plays a crucial role in cellular antioxidant defense mechanisms [9, 128].

Concurrently, cisplatin induces a pronounced neuroinflammatory response through activation of nuclear factor kappa B (NF- κ B) signaling pathways, causes an increased production of pro-inflammatory cytokines, including tumor necrosis factor - α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6), as well as an increase in production of antiapoptotic biomarkers like caspase, Bax, and Bcl-2 [129]. These combined processes contribute to neuronal damage, synaptic dysfunction,

and structural alterations in critical brain regions such as the hippocampus and cerebellum, as shown in Figure 1.5 [9, 130]

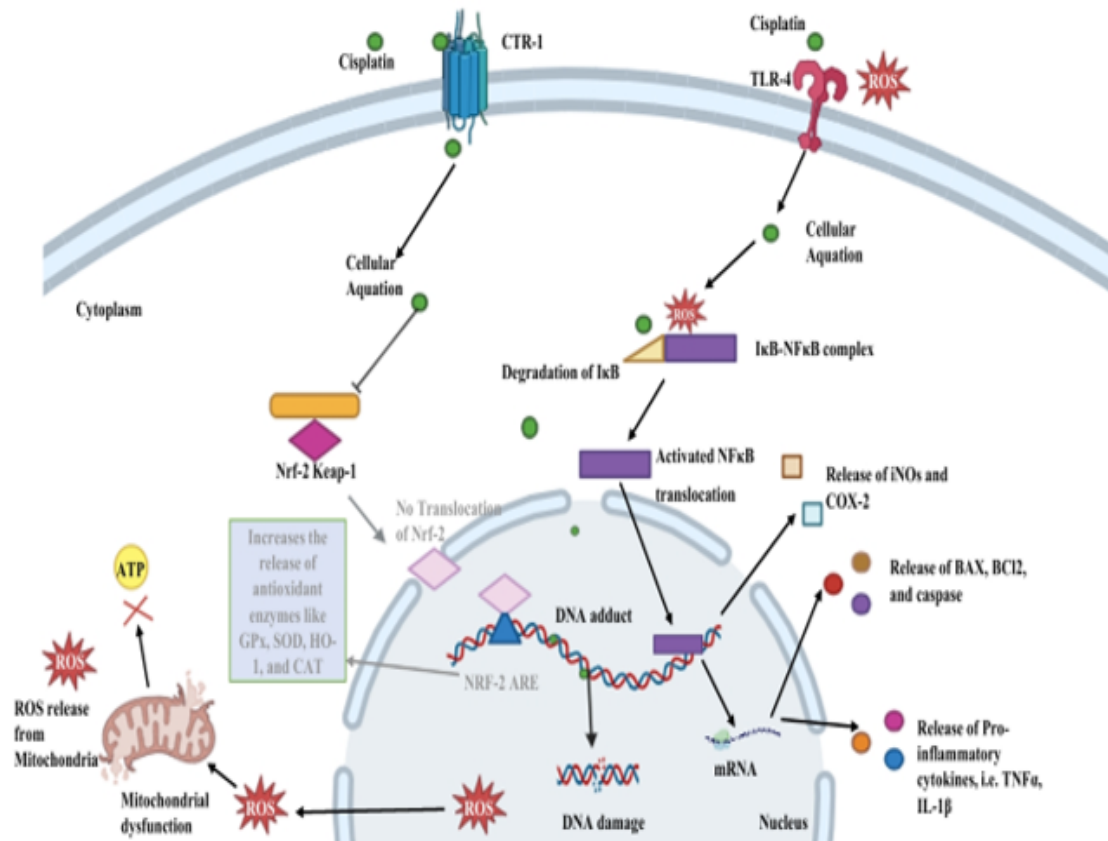


FIGURE 1.5: Cisplatin Toxicities Overview

The figure shows that Cisplatin enters cells either passively or actively and, through cellular aquation enter nucleus and forms DNA adduct so cell cycle arrest. Also, an increase in ROS production causes degradation of the IκB-NFκB complex, and activated IκB translocate bind to DNA, and resulted in the production of pro-inflammatory cytokines and apoptotic biomarkers and the release of COX-2 and iNOs.

ROS leads to lipid peroxidation and causes mitochondrial impairment, no ATP production, and also leads to apoptosis of cells. Cisplatin hinders the Nrf-2 and Keap-1 pathway, leading to no translocation of Nrf-2 and no production of antioxidant enzymes.

1.6 Role of Oxidative Stress and Neuroinflammation in Neurodegeneration

Neuroinflammation and oxidative stress are interconnected processes that can be considered central to the pathophysiology of neurodegenerative diseases and neurotoxicity caused by chemotherapy. Oxidative stress results from the inability of the endogenous antioxidant defense mechanisms to respond to excessive generation of ROS/RNS [131]. Increased ROS levels in the brain cause lipid peroxidation, protein oxidation, and DNA damage, as the brain is highly exposed to oxidative stress because of high oxygen consumption and high lipid content. Such consequences are usually associated with increased MDA levels and reduced action of antioxidant enzymes, including superoxide dismutases (SOD), catalase, and glutathione peroxidase (GPx). Nrf2-ARE pathway is a key protective pathway that keeps the expression of antioxidant genes in check, but is downmodulated during pathological conditions, which only increases oxidative damage [9, 128, 132].

Simultaneously, under the effect of oxidative stress, neuroinflammatory mechanisms are activated, which involve the stimulation of microglia and astrocytes, causing an increase in pro-inflammatory cytokines, including TNF, IL-1, and IL-6. This inflammatory cascade is significantly triggered by the activation of NF- κ B signaling pathways, which augments further oxidative stress development in a vicious cycle of neuronal injury [133].

Those processes lead to synaptic dysfunction, impaired neurogenesis, and neuronal apoptosis [9], especially true in the susceptible parts of the brain, like the hippocampus and cerebellum [9, 133]. Oxidative stress and neuroinflammation have combined effects, which eventually result in stage-related neuronal loss and impairments. Thus, targeting these interacting pathways is an attractive therapeutic approach to chemotherapy-induced central NS toxicity and the prevention and management of neurodegenerative disease.

1.7 Limitations of Current Treatment Strategies

Although important developments have been made in the treatment of cancer, the prevention and treatment of cisplatin-induced central neurotoxicity remain a significant clinical need. There is no FDA-approved pharmacological agent or standard therapy for protecting the central NS against cisplatin toxicity. Currently, some methods are available to control Cisplatin-induced neurotoxicity, but they interfere with the treatment plan by dose reduction or temporary discontinuation of chemotherapy strategies that can compromise anticancer efficacy and patient survival [134].

Symptomatic treatment (e.g., analgesics or antidepressants to treat pain, mood changes, or cognitive complaints) only offers partial and short-term relief without addressing the underlying neuronal damage. A range of neuroprotective candidates, such as antioxidants and anti-inflammatory agents, have been subjected to preclinical models and early clinical trials. Their clinical translation has, however, been poor due to their variation in efficacy, lack of ability to penetrate the blood-brain barrier (BBB), and the possibility of disrupting the antitumor effect of cisplatin [135].

The complexity is further increased by the fact that cisplatin-induced neurotoxicity is multifactorial, including oxidative stress, mitochondrial impairment, neuroinflammation, and apoptotic pathways that simultaneously put brain areas at risk. This complication makes single-target interventions inappropriate. There is also significant inter-individual variability, driven by age, genetics, cumulative dose, and comorbidities, which further complicates reliable prevention. The presence of cognitive deficits and other central impairments often continues to affect quality of life for months or years after treatment, without any solid, large-scale randomized controlled trials proving a definite neuroprotective effect [9, 127, 135].

All these limitations emphasize the need for new agents that can target various pathological pathways, pass the BBB safely and maintain the therapeutic outcomes of cisplatin.

1.8 Astaxanthin: Chemical and Pharmacological Overview

Astaxanthin is a naturally occurring xanthophyll carotenoid renowned for its vibrant red-orange pigment and exceptional biological activities. Chemically, it is designated as 3, 3'-dihydroxy- β , β' -carotene-4, 4'-dione (molecular formula $C_{40}H_{52}O_4$, molecular weight 596.84 g/mol). It has a structure consisting of two terminal 2-ionone rings bonded together by a long-conjugated polyene chain of 13 double bonds, at the 3,3' and 4,4' positions, with hydroxyl and keto groups, respectively, as shown in Figure 1.6. These structural modifications provide a characteristic polar-nonpolar-polar orientation, allowing astaxanthin to cross cell membranes and respond well to both hydrophilic and lipophilic environments, characteristics uncommon to most other carotenoids [136, 137].

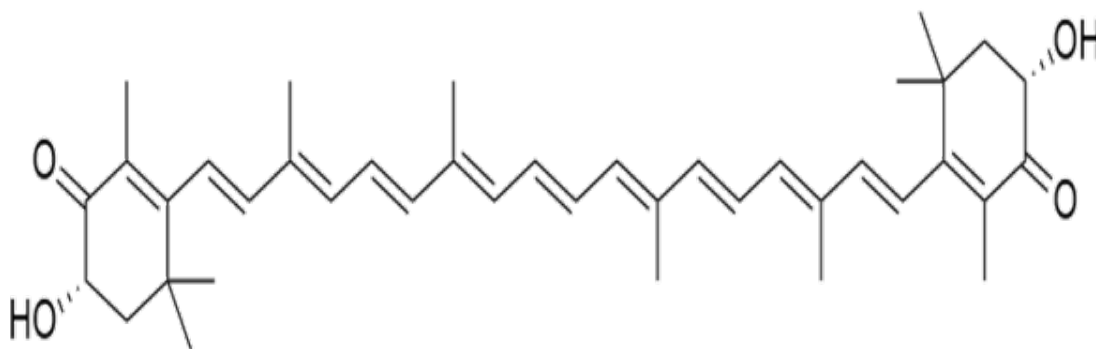


FIGURE 1.6: Chemical Structure of all trans Astaxanthin

Astaxanthin is found in the microalga *Haematococcus pluvialis* (which may reach as much as 3-5 percent of dry weight in stress conditions), and some yeasts (*Phaffia rhodozyma*), marine bacteria, and crustaceans, including shrimp, krill, and salmon, where it imparts its characteristic coloration. Astaxanthin frequently occurs in the esterified form (as a mono- or di-ester with fatty acids) or in particular stereoisomers in nature, with the (3S,3'S) isomer predominating in algal sources [136, 138, 139].

Astaxanthin is pharmacologically one of the most effective antioxidants with a higher scavenging potential of free radicals than β -carotene, vitamin E, or lutein [140]. Its antioxidant effect is due to the capacity to suppress singlet oxygen and

neutralize ROS, and it can prevent lipid peroxidation and oxidative damage to cellular components. In addition to direct antioxidative action, astaxanthin regulates important signaling pathways, including the Nrf-2 pathway [140], which increases the expression of endogenous antioxidant enzymes, including heme oxygenase-1 (HO-1), SOD, and catalase. It also has effective anti-inflammatory activities by inhibition of NF- κ B signaling pathways and downregulating the synthesis of pro-inflammatory cytokines [138–140].

Significantly, in the context of central NS therapies, astaxanthin is capable of bypassing the BBB, enabling it to directly provide neuroprotective effects in brain tissue. Its complexity of action, including antioxidant, anti-inflammatory, anti-apoptotic, and mitochondrial-protective effects, has made it a promising candidate in reducing oxidative stress and neuroinflammation in many neurological diseases without the safety risks that synthetic compounds typically present [139, 141]. This pharmacological profile and chemical versatility render astaxanthin highly applicable to the multifactorial nature of cisplatin-induced central neurotoxicity, as is the case with the current research.

1.9 Neuroprotective Potential of Astaxanthin

Astaxanthin has become a very promising neuroprotective compound owing to its complex pharmacology, particularly its capacity to regulate oxidative stress, neuroinflammation, and apoptosis [142]. Since these mechanisms are key in cisplatin-induced neurotoxicity, astaxanthin has received significant interest as a promising therapeutic candidate [143].

Its high antioxidant properties allow it to directly scavenge ROS and decrease lipid peroxidation, thus maintaining cell integrity. Moreover, astaxanthin promotes the endogenous antioxidant defense systems by increasing the activities of enzymes, including SOD, catalase, and GPx, mainly via the Nrf2 signaling pathway [144, 145].

In addition, astaxanthin exhibits pronounced anti-inflammatory action by suppressing NF- κ B activation, thereby reducing pro-inflammatory cytokines such as TNF- α , IL-1 beta, and IL-6 [145, 146]. It is also important for sustaining mitochondrial activity by reducing oxidative stress and maintaining mitochondrial membrane potential, thereby preventing energy depletion and neuronal apoptosis [145].

Moreover, astaxanthin has anti-apoptotic effects, such as regulating apoptotic protein expression, inhibiting Bax, and activating anti-apoptotic signals [147].

Notably, astaxanthin can penetrate the BBB and thus has protective activity in the central NS, specifically in areas like the hippocampus and cerebellum, which are highly vulnerable to oxidative and inflammatory damage. It has also been shown through experimental research that astaxanthin enhances cognitive function, suppresses neuronal death, and alleviates behavioral deficiencies in different models of neurotoxicity [144, 148]. All these results indicate the therapeutic promise of astaxanthin in central NS toxicity caused by chemotherapy as a multi-targeted neuroprotective factor.

1.10 Study Rationale and Knowledge Gap

Cisplatin is one of the most important chemotherapeutic agents used in the management of multiple malignancies, but its application in clinical practice is largely constrained by its dose-related toxicities, especially neurotoxicity, which impairs both the peripheral and central NSs. New evidence also suggests that cisplatin-induced neurotoxicity is mediated by complex interactions of oxidative stress, mitochondrial defects, neuroinflammation, and apoptosis that result in cognitive impairment and neuronal damage of essential brain areas, including the hippocampus and cerebellum. Although there is a growing awareness of these complications, present treatment measures are largely ineffective since they are predominantly symptomatic and do not specifically address the underlying molecular pathways.

Astaxanthin is a natural and powerful antioxidant and anti-inflammatory carotenoid substance that has shown considerable neuroprotective effects by regulating important signaling pathways, such as Nrf2 activation and NF- κ B inhibition. The above properties render it a good candidate for countering neuronal damage caused by oxidative stress and inflammation. Furthermore, the fact that it can cross the BBB and exhibit protective properties in the central NS additionally enhances its therapeutic applicability.

Thus, the current study aims to discover the neuroprotective properties of astaxanthin against neurotoxicity induced by cisplatin with special emphasis on its modulatory effects on oxidative stress and neuroinflammatory pathways in the brain region like hippocampus and cerebellum. The purpose of this study is to offer experimental data on the use of astaxanthin as a multi-target therapeutic agent in the prevention or mitigation of cisplatin-induced central NS toxicity.

1.11 Research Hypothesis

The hypothesis is that astaxanthin has a neuroprotective effect against cisplatin-induced neurotoxicity, which mitigates behavioral changes, histopathological alterations, oxidative stress, neuroinflammation, and enhances functional outcomes in the central NS, especially in the hippocampus and cerebellum.

1.12 Novelty and Significance of Study

The current research is of great importance since it examines a new treatment method of alleviating chemotherapy-related neurotoxicity, namely, the central NS. Although earlier studies have focused mainly on peripheral neuropathy, this study is unique in that it examines the effects of cisplatin on key brain areas like the hippocampus and cerebellum, thus filling a gap in the literature on chemotherapy toxicity. Moreover, the research assesses the neuro-protective potential of astaxanthin as a multi-target agent that can act to regulate both oxidative stress and

neuroinflammatory pathways at the same time. This study will combine biochemical, histopathological, and mechanistic studies to offer a complete understanding of the protective properties of astaxanthin. The results of the study can help improve safer adjunct therapy in cancer treatment, which in the end can enhance the quality of life of patients and increase the therapeutic use of natural antioxidants in neuroprotection.

Chapter 2

Literature Review

2.1 Overview of Chemotherapy - Induced Neurotoxicity

Neurotoxicity during chemotherapy is a critical clinical challenge in cancer care, especially when considering the increased cancer survival rates and extended patient lifespan [110]. A significant percentage of patients who use chemotherapeutic agents develop neurotoxicity, with a prevalence of up to 75% for cognitive deficits and 30-40% for peripheral neuropathy, depending on the form of drug, dosage, and period of treatment [149]. Previous research mainly focused on peripheral NS involvement, but recent findings have pointed to the crucial role of chemotherapy on the CNS, also known as chemotherapy-induced cognitive impairment or chemobrain [9, 150]. The condition is defined by the lack of memory, attention, executive functions, and processing speed, which can last long beyond the therapy course [9].

Clinical and experimental evidence is gradually mounting to indicate that neurotoxicity caused by chemotherapy is not confined to temporary functional changes but can be accompanied by permanent structural and molecular brain changes [151]. The most noticeable changes include those of the hippocampus, a very vital part of the brain in learning and memory, and the cerebellum [150], which

is getting more and more known to contribute to motor coordination as well as cognitive processing [9]. The pathophysiology is multifactorial and characterized by oxidative stress, neuroinflammation, mitochondrial defects, and activation of apoptotic processes, which all play a role in neuronal damage and dysfunctional neural connectivity [9, 152].

Considering the high impact of these neurotoxic effects on the life quality and functional independence of patients [110], there has been an increase in the desire to learn more about the scope and the biology behind chemotherapy-induced CNS toxicity. This knowledge is critical in the formulation of specific therapeutic interventions that prevent or reduce neurotoxic injury without affecting the effectiveness of chemotherapeutic agents against cancer [152, 153].

2.2 Clinical and Experimental Evidence of Cisplatin Neurotoxicity

Traditionally, neurotoxicity induced by cisplatin has been defined under the term of peripheral neuropathy, but accumulating clinical and experimental evidence has now shown that its neurotoxic effects have a far wider reach into the CNS. Over the past years, more and more attention has been paid to chemotherapy-induced cognitive impairment (chemobrain) that comprises memory, attention, and executive impairments. According to a review [10] published in the International Journal of Molecular Sciences, cisplatin can cross the BBB and directly impact brain structures, especially the hippocampus, which causes neurogenesis impairment and neuronal loss [10].

Equally, CNS-based research reveals that chemotherapy, such as cisplatin, causes sustained cognitive impairment that can persist for several months or years following the administration. A neuropathological review indicates that cisplatin interferes with hippocampal synaptic activity, decreases neuronal plasticity, and leads to neuroinflammation, which causes long-term cognitive deficits [12]. Together, these data indicate that neurotoxicity induced by cisplatin not only affects

peripheral nerves but also has complex central mechanisms that result in structural and functional changes to the brain.

2.2.1 Clinical Evidence

There has been a growing clinical literature that patients undergoing cisplatin-based chemotherapy develop central neurological complications in addition to peripheral neuropathy. They are typically mentioned as chemotherapy-induced cognitive impairment and encompass impairments in memory, attention, processing speed, and executive functioning. There is evidence to suggest that these impairments can be long-lasting following treatment withdrawal, which implies long-term CNS involvement [12].

Furthermore, it has been observed clinically that a high percentage of patients receiving cisplatin chemotherapy report cognitive disturbances during and following therapy, with some studies reporting that as many as 60-75 percent of patients experience short-term or long-term cognitive deficits. Such deficits are frequently followed by emotional and behavioral alterations, such as anxiety and depression, which further undermine the patient's quality of life [22, 154].

Alongside the cognitive symptoms, there is clinical and translational evidence that cisplatin can cause structural and functional modifications in the brain, especially in regions of the brain that are involved in cognitive functions, like the hippocampus. The findings underscore the increased awareness of CNS toxicity as a clinically significant outcome of cisplatin therapy [154].

2.2.2 Experimental Animal Models

The CNS toxicity of cisplatin has been strongly supported by experimental animal models. The administration of cisplatin in rodents has been reported to affect learning and memory by causing poor performance in their behavioral tests, such as maze tests and object recognition assays. These impairments in cognitive

functions are closely linked to structural and molecular changes in the hippocampus [10]. At the cellular level, experimental research illustrates that cisplatin causes neuroinflammation, oxidative stress, and mitochondrial impairment as well as apoptosis of brain tissues. A recent experimental study published in *Frontiers in Pharmacology* indicated that cisplatin resulted in a significant rise in hippocampal concentrations of pro-inflammatory cytokines (TNF- α , IL-6), reactive oxygen species, and lipid peroxidation, and deterioration of mitochondrial activities, which ultimately led to neuronal damage and cognitive deficits [22]. Additionally, animal research shows that cisplatin impairs synaptic plasticity and decreases neurogenesis in the hippocampus important for learning and memory [9]. There is also growing evidence that other brain regions, like the hippocampus and cerebellum, are also involved, which means cisplatin-induced neurotoxicity impacts various neural pathways [155].

2.3 Brain Region-Specific Effects of Cisplatin

2.3.1 Hippocampus

The hippocampus is among the most susceptible brain regions to cisplatin-induced neurotoxicity, owing to its critical roles in learning, memory, and neurogenesis. Experimental research has repeatedly shown that exposure to cisplatin induces significant structural and functional changes in the hippocampus. In an experiment [156], the administration of cisplatin meaningfully reduced the cell proliferation of the hippocampal cells and improved the expression of pro-apoptotic genes, which are indicators of impaired neurogenesis and augmented neuronal cell death. These results indicate that cisplatin interferes with the regeneration ability of the hippocampus, which plays an important role in the formation of memory. Besides its impact on neurogenesis, cisplatin has also been revealed to affect the integrity of synapses in neurons of the hippocampus. According to a study carried out by [157], even low-dose cisplatin caused a huge loss of dendritic spines and decreased synaptic density, which caused impaired neuronal communication and cognitive

impairments. The observed structural changes go hand in hand with learning and memory deficits in behavioral studies. Moreover, oxidative stress and neuroinflammation are closely associated with cisplatin-induced hippocampal damage.

According to a review published by *Frontiers in Pharmacology* [9], elevated ROS production and inflammatory pathways lead to neuronal apoptosis and loss of synaptic plasticity in the hippocampus. The overall result is indicative of the hippocampus as one of the primary targets of central neurotoxicity caused by cisplatin [9, 156, 157].

2.3.2 Cerebellum

The cerebellum is traditionally linked with motor coordination, but is currently also known to be involved in cognitive and emotional processes, which is why it is a suitable target in chemotherapy-induced neurotoxicity. New experimental data show that cisplatin causes structural and functional changes in the cerebellum, which also leads to motor and cognitive defects. The exposure of animals to cisplatin has revealed that neuronal degeneration, cerebellar architecture disruption, oxidative stress escalation, and motor coordination impairment are associated with exposure. Recent experimental research suggests a cisplatin dose-related rise in oxidative stress, neuronal apoptosis, histopathological injury (Purkinje cell degeneration and cortical layer thinning), and inflammatory responses in cerebellar tissue [128, 158].

These alterations reflect the pathogenic processes in the hippocampus and possibly explain the impairments of balance, coordination, and mental processing. Moreover, expanding the neurotoxicity literature, including that of the *International Journal of Molecular Sciences* [10].

It suggests that brain dysfunction as a result of chemotherapy is a multi-neural circuitry, including cerebellar pathways. The presence of the cerebellum also upholds the idea that cisplatin-induced neurotoxicity is a global CNS event and not a localized action [10, 128, 158].

2.4 Molecular Mechanisms of Cisplatin-Induced Neurotoxicity

Neurotoxicity caused by cisplatin is a multifaceted condition comprising a combination of molecular and cellular processes that all play a role in causing dysfunction in the neurons within the central NS. Evidence from experimental research and peer-reviewed studies suggests that the main elements of this neurotoxic cascade are oxidative stress, neuroinflammatory signaling, mitochondrial impairment, and apoptotic processes [9, 127]. The production of ROS and impairment of endogenous antioxidant defenses in the brain tissue have been consistently observed with exposure to cisplatin, resulting in the oxidative damage of lipids, proteins, and nucleic acids [9, 10].

Simultaneously, glial cells are activated and release pro-inflammatory cytokines, which facilitate neuronal injury and synaptic impairment, which further leads to damage of the central NS [10, 127]. Mitochondrial defects has also been proposed as an important contributing factor, with studies suggesting impaired cellular energy metabolism and increased oxidative burden in neuronal tissues [9]. These changes are often linked to the activation of apoptotic pathways, which causes neuronal loss and inhibited neurogenesis, especially in such areas as the hippocampus [156]. Notably, these processes are very well intertwined, and oxidative stress and inflammation serve as upstream factors that modulate pathways of mitochondrial functioning and cell survival [127]. Although the exact role of each pathway may differ between experimental models, existing data suggest a multifactorial model of cisplatin-induced neurotoxicity with overlapping and reinforcing mechanisms that ultimately result in dysfunction of the central NS [159].

2.4.1 Oxidative Stress

Oxidative stress is a common mechanism of cisplatin-induced central CNS neurotoxicity. Animal experiments show that cisplatin administration increases ROS, causing peroxidation of lipids, oxidation of proteins, and imbalances in neuronal

cellular functions. An extensive review published in *Frontiers in Pharmacology* [9] reports that oxidative stress is a key mechanism underlying chemotherapy-induced neurotoxicity, with elevated ROS associated with neurotoxicity and cognitive impairment.

Experimental data in targeted areas of the brain also support this. In mice, the administration of cisplatin led to a substantial rise in lipid peroxidation and a considerable reduction in antioxidant enzymes, including SOD and GPx, in *The Journal of Basic and Applied Zoology* [128], thereby confirming the involvement of an oxidative stress-mediated mechanism of neurotoxicity. This oxidative stress is accompanied by severe histopathological changes in the cerebellar cortex, such as Purkinje cell degeneration and disorganization of the cell structure [128].

Similarly, laboratory studies in rodents have shown that cisplatin-induced oxidative stress is involved in structural damage and dysfunction of both the hippocampus and cerebellum, which are key areas involved in learning and memory and motor coordination, respectively [160]. These studies consistently demonstrate increased oxidative markers alongside decreased antioxidant defense systems, supporting the role of ROS-mediated damage in neuronal degeneration [127, 158, 160].

2.4.2 Neuroinflammation

Neuroinflammation is emerging as an important aspect in cisplatin neurotoxicity in the central NS. Review articles and experimental studies indicate that cisplatin treatment can stimulate glial cells like microglia and astrocytes to release numerous pro-inflammatory cytokines, TNF- α , IL-1 β , and IL-6. A review article in *Frontiers in Pharmacology* [9] highlights neuroinflammatory signaling as one of the key mechanisms that contribute to chemotherapy-induced neurotoxicity, particularly in conjunction with oxidative stress. These findings are supported by experimental evidence that shows increased expression of cytokines in brain tissues treated with cisplatin. Experimental models show elevated levels of TNF- α , IL-1 β and IL-6 in areas such as the hippocampus, suggesting localized inflammatory processes that are linked to cognitive dysfunction [10, 127].

Notably, a recent experimental study published in *Psychopharmacology* [129] found that cognitive impairments following cisplatin administration were linked to increased levels of pro-inflammatory cytokines, further reinforcing the impact of neuroinflammation in central neurotoxicity. Beyond the hippocampus, recent findings indicate that neuroinflammation may also impact other parts of the brain, such as the cerebellum, in which inflammatory and oxidative changes have been linked to neuronal injury and histopathological damage in animal models [128].

2.4.3 Mitochondrial Dysfunction

Mitochondrial dysfunction has been proposed as an important contributing mechanism in cisplatin-induced neurotoxicity, particularly in the central NS, where neurons have high energy demands. Preclinical studies suggest that cisplatin exposure interrupt's mitochondrial function, which causes diminished energy metabolism and amplified oxidative burden in neuronal tissues. Reviews such as those published in *Frontiers in Pharmacology* [9] and *International Journal of Molecular Sciences* [127] mitochondrial impairment as a key component of the broader neurotoxic cascade associated with chemotherapy.

Experimental evidence indicates that mitochondrial impairment in cisplatin - treated models is characterized by reduced ATP production, disruption of mitochondrial membrane potential, and amplified production of ROS, which together contribute to neuronal stress and injury [10, 127].

These alterations are particularly relevant in brain regions such as the hippocampus, where high metabolic activity makes neurons more vulnerable to energy deficits and oxidative damage.

In addition, mitochondrial impairment is closely linked with other pathological processes, including oxidative stress and apoptotic signaling. While specific molecular events such as mitochondrial DNA damage or release of pro-apoptotic factors have been proposed in the literature, their consistent demonstration in CNS-focused cisplatin models remains limited, and thus, they should be interpreted as

potential rather than universally established mechanisms [9].

2.4.4 Apoptosis

Apoptosis plays a significant role in cisplatin-induced neurotoxicity and central NS cell death. Animal studies using rodent models have shown that cisplatin treatment results in increased neuronal death, especially in the hippocampus, which is vital for learning and memory [156].

Specifically, cisplatin therapy has been shown to inhibit neuronal proliferation and to alter the expression of genes for apoptosis, such as a reduction of anti-apoptotic and an increase of pro-apoptotic gene expression, which activate cell death programs [156].

Apoptosis is regulated by upstream factors. Experimental and review evidence suggest that oxidative stress and neuroinflammation portray the loss of neuronal homeostasis and cell death pathways induced in models of cisplatin-induced neurotoxicity [9, 127].

Apoptotic changes have also been observed in combination with morphological changes (e.g., loss of dendritic spines, synaptic dysfunction) and cognitive dysfunction [10, 157].

2.5 Biomarkers of Neurotoxicity

Biochemical markers are frequently used in lab studies to assess cisplatin neurotoxicity and to understand the mechanisms of cisplatin-induced injury. The literature suggests that markers of oxidative stress, neuroinflammation, and apoptosis are particularly valuable for evaluating CNS damage [161]. These biomarkers not only help to define the neurotoxic mechanisms but also play an important role in the assessment of the neuroprotective effects of potential treatments. Overall, experimental evidence indicates that changes in oxidative stress, inflammation,

and apoptosis can be robustly measured using biochemical methods, providing a comprehensive approach to study cisplatin-induced neurotoxicity [22, 161].

2.5.1 Oxidative Stress Biomarkers

Biomarkers of oxidative stress are well-documented markers of cisplatin-induced neurotoxicity in experimental studies. Many studies have shown that cisplatin treatment alters redox balance, resulting in elevated generation of ROS and oxidative stress [22, 162]. Increased markers of lipid peroxidation have been reported in the brain after cisplatin exposure, suggesting increased oxidative damage [22].

Simultaneously, changes have been observed in oxidative defense mechanisms, as reflected in alterations in antioxidant enzymes, such as SOD, as well as increased expression of the transcription factor Nrf-2, both of which indicate a response to oxidative stress. These findings collectively suggest a shift toward oxidative imbalance, rendering neuronal cells more susceptible to damage. Moreover, cisplatin disrupts mitochondrial complex I function, exacerbating cellular dysfunction and oxidative stress [22].

2.5.2 Neuroinflammatory Biomarkers

Biomarkers of neuroinflammation offer valuable insights into the inflammatory responses in cisplatin-induced damage. In experimental models, cisplatin treatment activates glial cells, promoting the release of pro-inflammatory cytokines, which play a role in the dysfunction of neurons [161]. In particular, TNF- α , IL-6, and NF- κ B have been identified as the primary markers of neuroinflammation [22].

Elevations of these inflammatory molecules have been consistently reported in the hippocampus of cisplatin-treated rodents, especially in areas involved in cognitive processing [22]. These neuroinflammatory responses are accompanied by cognitive impairments and are key mechanisms of cisplatin-induced neurotoxicity. While there is variability in the magnitude and distribution of these responses in different

studies, the overall findings suggest that neuroinflammatory biomarkers are good predictors of cisplatin-induced toxicity in the CNS and can be used to assess the effectiveness of drugs targeting inflammatory pathways [161].

2.5.3 Apoptotic Markers

Apoptotic biomarkers are extensively used to assess neuronal cell death associated with cisplatin-induced neurotoxicity. Recent evidence from experimental models indicates that cisplatin treatments trigger activation of apoptotic pathways, leading to loss of nerve cell in critical brain areas, such as the hippocampus [156, 157].

Changes from apoptotic to anti-apoptotic proteins are frequently observed, suggesting activation of programmed cell death pathways with increased expression of pro-apoptotic genes (Bik, Bid, Bok, Trp53p2, Card6) and decreased expression of anti-apoptotic proteins (Bcl2a1) [156].

These apoptosis-related changes are frequently accompanied by morphological changes, such as loss of dendritic spines and synaptic dysfunction, which have been implicated in memory deficits in cisplatin-treated animals [157].

The molecular mechanism of apoptosis, however, seems to vary, with several signaling pathways being activated, such as both caspase-dependent and caspase-independent, depending on the experimental model used [163]. Nonetheless, apoptosis is consistently recognized as a downstream consequence of cisplatin neurotoxicity [161].

2.6 Histopathological and Behavioral Findings

Histopathological and behavioral analyses are critical for assessing the structural and functional effects of cisplatin-induced neurotoxicity in animal models. Histopathological assessment, including hematoxylin and eosin (H&E) staining, has consistently shown that cisplatin treatment causes extensive neuronal damage

in areas of the brain, including the hippocampus and cerebellum [128, 156]. Described changes include degeneration of neurons, disorganization of cells, and damage to Purkinje cells in the cerebellar cortex, indicating structural damage corresponding to cognitive and motor impairments [128]. A recent study in BMC Pharmacology and Toxicology reported dose-dependent increases in neuronal necrosis, apoptosis, and structural disorganization in hippocampal regions following cisplatin exposure, confirming its central neurotoxic effects [154].

Behavioral tests also provide functional confirmation of these changes. For example, the OFT is commonly used to assess locomotor and exploratory behaviors. Research has shown that cisplatin exposure results in decreased locomotion, exploration, and altered behavior, suggesting CNS impairment. It is believed that these changes are indicative of dysfunction in brain areas responsible for cognitive function and the regulation of emotions, such as the hippocampus [164]. Tests of motor coordination and neuromuscular performance often include the rotarod test. Experimental research suggests that cisplatin treatment may result in motor impairment; however, results are not consistent [118, 164].

2.7 Current Treatment and Challenges Against Cisplatin-Induced Neurotoxicity

Cisplatin-induced neurotoxicity is a dose-limiting unexpected result in cancer therapy, but the existing treatment strategies are mostly preventative and symptomatic. Clinical practice mainly relies on minimizing cumulative cisplatin dose, avoiding the combined use of other neurotoxic drugs, and establishing routine neurological surveillance to monitor for the development of neurotoxic symptoms [134]. These strategies are considered the cornerstone of clinical practice; however, they do not directly reverse established neuronal damage.

Drug-based therapies have been widely explored, but yield limited results. Currently, duloxetine is the only agent that has demonstrated consistent efficacy and

been recommended by guidelines for the treatment of painful peripheral neuropathy induced by chemotherapy [165]. On the other hand, widely used drugs like gabapentin, pregabalin, and amifostine have shown mixed or insufficient effectiveness in large clinical trials, restricting their clinical use [166].

Numerous neuroprotective drugs have also been tested in an effort to prevent cisplatin-induced nerve damage. Antioxidants such as vitamin E, N-acetylcysteine and other free radical scavengers have been explored due to their ability to reduce oxidative stress and restore the redox balance. While such agents commonly show neuroprotective effects in preclinical studies, their clinical effects have been variable and inconsistent, and so they are not incorporated into routine clinical practice [167].

Likewise, various pharmacologic agents have been investigated for their effects on different mechanisms. Sodium thiosulfate has shown some success in mitigating cisplatin-induced ototoxicity, and compounds including corticosteroids, statins, and D-methionine have reported variable efficacy in certain patient cohorts. But systematic reviews suggest that the quality of evidence remains poor, and none of these compounds offer consistent neuroprotection [168].

There has been a recent focus on mechanism-based approaches, such as modulation of the oxidative stress response, mitochondrial protection, anti-inflammatory pathways, and apoptosis. While these approaches show promise in preclinical studies, their clinical use is hindered by poor pharmacokinetics, off-target effects, and potential antagonism of cisplatin's anti-cancer effects [161].

Crucially, despite extensive research, no universally accepted or FDA-approved treatments are available for the inhibition or treatment of cisplatin-induced central and peripheral neurotoxicity. As such, treatment options are largely supportive and consist of dose adjustments, symptom management, and regular neurological assessments. Additionally, recent findings have shown that many promising neuroprotective therapies do not yield consistent or reproducible results in experimental and clinical studies, suggesting that cisplatin-induced neurotoxicity is likely to be multifactorial [169].

Astaxanthin is a naturally derived xanthophyll carotenoid with an extensive variety of pharmacological effects and potential therapeutic benefits in neurodegenerative and neurotoxic diseases. The unique molecular structure with a conjugated polyene chain and terminal functional groups with polar nature enables it to interact with both hydrophilic and lipophilic environments within biological membranes. This property has been suggested to account for its greater antioxidant activity than other carotenoids [137].

2.8 Astaxanthin: Advanced Pharmacological Insights

There is considerable literature supporting the potent antioxidant activity of astaxanthin, which involves scavenging ROS, suppressing peroxidation of lipids, and preventing damage to cellular components [170]. *In vitro* experiments have shown that astaxanthin upregulates antioxidant enzymes, including SOD, catalase, and GSH. Moreover, activation of the Nrf2 pathway [171] has been shown to play a crucial role in achieving redox balance [170, 172]. But inconsistent results indicate these effects may be dose, time, and model dependent.

Beyond its antioxidant action, astaxanthin has also been shown to have potent anti-inflammatory effects. Research suggests it regulates inflammatory pathways, including by suppressing NF- κ B activity, leading to decreased expression of pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6 [173].

These pharmacological actions are vastly linked to the context of cisplatin-induced neurotoxicity, where chronic neuroinflammation contributes to neuronal dysfunction and degeneration [171, 173].

Astaxanthin also exhibits anti-apoptotic effects and promotes neuronal survival. Preclinical data indicate that it regulates the expression of pro- and anti-apoptotic proteins, and potentially inhibits the stimulation of caspases. Moreover, its mitochondrial protective effects have been suggested to be crucial in maintaining

cellular energy balance and preventing apoptosis-related neuronal damage. These actions suggest that it could be a broad-spectrum neuroprotective agent [170, 174].

Crucially, pharmacokinetic and experimental data indicate that astaxanthin can enter the BBB, allowing it to directly influence the CNS. Experimental studies have shown beneficial effects on neuronal integrity, synaptic plasticity, and cognitive function, particularly in brain areas like the hippocampus. But some reports have also shown improvement in motor function, suggesting that protective effects may extend to the cerebellum as well [172, 175].

2.9 Mechanistic Evidence of Astaxanthin

Astaxanthin has been widely studied for its neuroprotective effects through its modulation of various processes involved in the injury. The literature suggests that its key mechanisms of action include regulation of oxidative stress, inhibition of inflammatory pathways, and modulation of apoptosis. These processes are interrelated and play important role in the progress of cisplatin-induced neurotoxicity. Several studies demonstrate the role of specific signaling pathways, such as Nrf2 and NF- κ B, which support the multimodal pharmacology of astaxanthin. Nrf2 is the master regulator of the antioxidant defense system, activating protective genes (such as HO-1 and NQO1) that neutralize oxidative damage in neurons. NF- κ B, in contrast, is the key transcription factor driving neuroinflammation by upregulating pro-inflammatory cytokines (TNF- α , IL-1 β), so the balance between the two pathways determines whether neurons are protected or injured following a toxic insult such as cisplatin [172, 176, 177].

2.9.1 Antioxidant Mechanism

The antioxidant activity is one of the major mechanisms of action of astaxanthin. *In vitro* studies have revealed that astaxanthin can scavenge ROS, suppress lipid peroxidation, and boost endogenous antioxidants, including SOD, catalase, and glutathione [170, 172].

Apart from its direct antioxidant properties, astaxanthin has also been shown to modify redox-sensitive signaling pathways, such as the Nrf2 pathway. Nrf2 activation upregulates antioxidant and protective genes such as heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1), which helps to preserve redox balance. Research has shown that astaxanthin increases Nrf2 translocation and expression of antioxidant genes [176, 178]. Additionally, there is some evidence that astaxanthin can perform antioxidant functions via both Nrf2-dependent and independent pathways, suggesting a broader cytoprotective function [171].

2.9.2 Anti-inflammatory Mechanisms

Astaxanthin has also been widely described to have anti-inflammatory effects via NF- κ B pathways. NF- κ B is a key transcription factor for inflammatory responses, and its stimulation results in the release of pro-inflammatory cytokines. Current studies have shown that astaxanthin prevents NF- κ B activation and nuclear translocation, and reduces the expression of cytokines, including IL-6 and IL-1 β [176, 177]. The interaction between Nrf2 and NF- κ B pathways has also been highlighted in recent literature, suggesting that astaxanthin may regulate inflammatory responses through pathway crosstalk [176].

2.9.3 Anti-apoptotic Mechanisms

Astaxanthin has also been reported to modulate apoptosis, a key factor in the neurotoxicity-induced loss of neurons. *In vitro* studies have confirmed that astaxanthin influences the expression of apoptotic proteins, such as Bax, Bcl-2, and caspase-3, which are involved in the regulation of cell death and survival [170, 172].

It has been confirmed that astaxanthin downregulates pro-apoptotic markers (Bax, caspase-3) and upregulates anti-apoptotic markers (Bcl-2). It has also been reported to maintain mitochondrial membrane potential, indicating a protective effect against mitochondrial apoptosis [178]. It should be noted that some of the literature on its impact on apoptosis comes from studies examining its potential

anticancer properties, in which it has been shown to induce apoptosis in cancer cells [179].

2.10 Research Gap and Rationale

Cisplatin-induced neurotoxicity has been well studied, yet there is a lack of effective neuroprotective interventions. The disease is linked with oxidative stress, neuroinflammation, and apoptosis; however, treatment is largely limited to dose adjustment and supportive care, with no effective treatment agent. Astaxanthin, with its antioxidant, anti-inflammatory, and anti-apoptotic effects, has drawn interest as a potential neuroprotective agent and is effective in preclinical studies using various animal models. However, additional research is needed. The majority of studies examine general neuroprotection or peripheral neuropathy, with little research on cisplatin-induced CNS toxicity. Moreover, many studies focus on single mechanisms rather than multiple mechanisms.

Moreover, there are few studies that combine biochemical, behavioral, and histopathologic analyses to fully comprehend the mechanisms of neuroprotection, especially in the hippocampus and cerebellum. Therefore, the present study will evaluate the neuroprotective properties of astaxanthin against cisplatin-induced neurotoxicity by evaluating oxidative stress, inflammatory markers, and apoptosis as well as behavioral and histopathological changes. This integrated approach is expected to provide more comprehensive and translational evidence for its therapeutic potential.

2.11 Aim and Objectives of the Study

2.11.1 Aim

Our study aims to investigate the neuroprotective effect of astaxanthin against cisplatin-induced neurotoxicity in mice.

2.11.2 Objectives

- i. To assess the effect of astaxanthin on histopathological changes in the brain.
- ii. To evaluate the anti-inflammatory potential of astaxanthin against cisplatin-induced neuroinflammation.
- iii. To evaluate the effect of astaxanthin on cisplatin-induced oxidative stress markers (MDA and NO).

To investigate the binding interaction of astaxanthin on Nrf2 and NF- κ B markers

Chapter 3

Methods and Materials

3.1 Experimental Animals

Adult male BALB/c mice weighing 25-35 g were used in the study. They were sourced from the Animal House of Capital University of Science and Technology, Islamabad. Upon arrival, the general health of the mice was examined, and only those that appeared healthy were used in the study.

The study protocol was approved by the Institutional Animal Ethics Committee of Capital University of Science and Technology, Islamabad (Approval No. IAEC/CUST/2025/19). The experimental protocol was conducted in accordance with institutional ethical guidelines and international standards, including those of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

The mice were randomly allocated into six groups ($n = 10$) by computer. The study groups comprised: a control normal saline, a group given cisplatin (2.3 mg/kg), a +ve control group (cisplatin 2.3 mg/kg and mecobalamin 1 mg/kg), and three experimental groups (cisplatin 2.3 mg/kg and astaxanthin 5 mg/kg, 10 mg/kg, and 25 mg/kg, respectively) as shown in figure 3.1.

BALB/c mice were selected because they are a well-validated, genetically consistent model for studying cisplatin-induced neurotoxicity, with established behavioral, biochemical, and histopathological protocols that closely mirror mammalian CNS responses. This model also allows ethically approved, reproducible in vivo testing at a scale and cost suited to this kind of neuroprotection study.



FIGURE 3.1: Experimental male BALB/c mice

3.2 Housing Conditions

The mice were kept in groups in laboratory cages, which were stacked on racks in a well-ventilated room. The cage room was controlled to maintain a temperature of $24 \pm 1^{\circ}\text{C}$, relative humidity of 50-60%, and a 12-hour light/dark cycle (08:00 to 20:00) using an automatic timer. Adequate ventilation was provided to enable airflow and promote a hygienic, pathogen-free environment.

The cages were provided with autoclaved wood shavings as bedding, which were changed every 48 hours or sooner if needed, to keep the cages clean and reduce ammonia levels. The cages were regularly cleaned and sanitized with 70% ethanol.

Measures were taken to avoid crowding by keeping the number of animals per cage appropriate, so that they could freely move and exercise. In general, the animals were housed under controlled conditions for their well-being and to ensure consistency in the experimental conditions.

3.3 Food Composition

The animals were nourished with a standard laboratory diet in the form of nutritionally adequate pellets. The diet was formulated using locally available ingredients, which allowed for quality control and ensured consistency and reproducibility of the experimental results, as shown in Table 3.1.

TABLE 3.1: Composition of animals' food

Food Item	Percentage (%)	5.0 Kg	10.0 Kg
Wheat Flour	50	2.5 Kg	5 Kg
Chokar (Bran)	15	750 g	1.5 Kg
Fish Meal	15	750 g	1.5 Kg
Soybean Meal	10	500 g	1 Kg
Dry Skimmed Milk	5	250 g	500 g
Vitamins/Minerals*	5	250 g	500 g

3.4 Acclimatization

All mice were given a seven-day acclimatization period in the animal facility before the start of experimental procedures. This allowed the mice to adapt to the new housing conditions, such as temperature, humidity, light/dark cycle, and handling. Acclimatized mice were kept in groups of ten in conventional polycarbonate cages with corn cob bedding. The housing conditions were controlled (temperature $24 \pm 1^\circ\text{C}$, 55-60% relative humidity, 12-hour light/dark cycle with lights on from 08:00 to 20:00). The mice had ad libitum access to a standard pelleted feed and filtered water. Health checks were conducted daily to monitor body weight, grooming, activity, and signs of sickness or suffering. To reduce stress and enhance adaptation, mice were handled for 5-10 minutes per cage per day. This approach not only alleviated anxiety symptoms but also facilitated easier handling during subsequent phases, such as drug delivery and behavioral testing. Following the acclimatization period, the mice were arbitrarily allocated to different experimental groups created on their body weight to ensure equal representation of all groups.

3.5 Mice Handling

Animal handling was conducted in compliance with the ethical guidelines of the Ethics Committee of Capital University of Science and Technology (CUST), Islamabad. Mice were given one week for acclimatization to the laboratory environment and to minimize the stress factor before the start of the experimental work. Mice were closely monitored for any signs of disease, discomfort, pain, or unusual behavior, and were habituated to gentle handling by the researcher.

To facilitate identification, mice were marked on the tail with non-toxic permanent ink; cages were also labeled. Mice were allocated into experimental groups using a simple allocation procedure to prevent bias. During handling, care was taken to minimize stress. Mice were held by grasping the tail base while allowing them to hold on to the cage lid, then gripping the scruff of the neck with the thumb and forefinger. The tail was held in place with the little finger to allow for control without discomfort or constriction of breathing, as shown in Figure 3.2.

During the study period, the mice were weighed daily, and their food, water, grooming, and activity patterns were noted. If an animal showed clear signs of distress or illness, it was sacrificed via cervical dislocation in line with ethical guidelines.



FIGURE 3.2: Mice Handling

3.6 Mice Bedding

Mice were kept in commercial polycarbonate cages ($30 \times 20 \times 15$ cm) with corn cob bedding as the substrate. This bedding material was chosen for its absorbency, odor absorbency, low dust, and non-toxicity, which allows for animal comfort and stability in experimental parameters. The bedding was maintained at a depth of 2-3 cm to allow for burrowing and nesting.

Cages and bedding were cleaned and changed every day to prevent the build-up of ammonia and other contaminants. When cages were cleaned, mice were housed in clean cages using animal-friendly methods to reduce distress. Bedding was sterilized by autoclaving before use to prevent contamination and to ensure that experimental outcomes were not affected. Using the same type of bedding material for all groups ensured consistent environmental conditions among the groups and prevented variability that may affect biochemical or physiological outcomes.

3.7 Randomization

To avoid bias and ensure valid experimental findings, animals were randomly allocated to treatment groups before the experiment began. Random allocation was made using a computer-generated random number, with grouping by body weight to ensure equal numbers in each group.

To identify individual mice, non-toxic ink was applied to their tails. We adopted a simple marking system that comprised circular bands of different numbers and sizes. For instance, Animal #1 was marked with one small circular band around the tail, and Animal #2 with two small circular bands, separated by equal intervals. Animal #3 had three small circular bands, while Animal #4 had four small circular bands. Animal #5 was identified by one thick circular band, and Animal #6 by one thick circular band and one small band. Animal #7 had one thick circular band followed by two small circular bands. Animal #8 was marked with one thick circular band and three small circular bands. Animal #9 was marked with

one thick circular band and four small circular bands, and Animal #10 with two thick circular bands, with each band separated by a small gap. Variations in band size and numbers were used to distinguish all animals within a cage. This enabled the identification of each animal within a cage and the recording and comparison of specific behavioral and biochemical information.

3.8 Euthanasia Methods

After the experimental period was complete, animals were euthanized to obtain samples of the brain for biochemical and histopathological studies. All methods were conducted in accordance with institutional ethical guidelines to reduce pain and discomfort.

3.8.1 Cervical Dislocation

Cervical dislocation was used as the method of euthanasia. This method was chosen for its speed, effectiveness, and suitability for further biochemical and histological investigations because it does not involve chemical interference by anesthetics. The method was only carried out by trained staff. Cervical dislocation was performed by first holding the mouse gently by the base of its tail. It was then placed on a table, and a stainless steel or wooden spatula was firmly positioned across the skull base. The tail was then pulled sharply, resulting in instantaneous cervical dislocation of the vertebrae from the skull. This caused immediate separation of the brainstem/spinal cord, leading to unconsciousness in a matter of seconds, followed by cessation of breathing and cessation of the heartbeat. The absence of respiration and heartbeat was taken to indicate death.

3.8.2 Concussion

When cervical dislocation was not possible, concussion followed by cervical dislocation was used. This approach ensured humane euthanasia while maintaining

tissue quality for post-mortem analyses. A concussion was induced by striking the head with a hard object to induce immediate brain damage and render the animal unconscious. This method was carried out by a trained individual to guarantee accuracy. After a concussion, cervical dislocation was immediately applied to render irreversible cessation of all vital functions, as concussion meets the standards of stunning but does not qualify for complete euthanasia. Euthanasia was established by absence of breathing and heartbeat, as well as loss of corneal reflexes. After euthanasia, brain tissues (hippocampus, cerebellum) were promptly collected on ice for biochemical and histopathological studies.

3.9 Chemicals and Drugs

All chemicals and drugs used in this study were of standard analytical grade and were obtained from commercial suppliers. The test compounds, all trans Astaxanthin, Cisplatin, and Mecobalamin, were prepared fresh daily before administration to ensure stability and efficacy, as shown in Table 3.2.

TABLE 3.2: List of chemicals and drugs used in the study, along with their sources and suppliers

Sr. No.	Category	Chemical/Substance	Source
1	Test Compound	all trans Astaxanthin	Sigma-Aldrich, USA
2	Drug	Cisplatin	Pharmedic Laboratories (Pvt.) Limited
3	Drug	Mecobalamin	Sigma-Aldrich, US
4	Solvent	Dimethyl Sulfoxide (DMSO)	
5	Solvent	Normal Saline (0.9% NaCl)	Amson Pharmaceuticals, Pakistan
6	Buffer	Phosphate Buffered Saline (PBS)	Sigma-Aldrich, USA
7	Buffer	RIPA Buffer	
8	ELISA Kit	Mouse IL-1 β ELISA Kit	Biosharp, China
9	ELISA Kit	Mouse TNF- α ELISA Kit	
10	Protein Assay Kit	BCA Protein Assay Kit	
11	Staining	Hematoxylin	Sigma-Aldrich, USA

Table 3.2 continued from previous page

Sr. No.	Category	Chemical/Substance	Source
12	Staining	Eosin	
13	Solvent	Xylene	
14	Solvent	Ethanol	
15	Fixative	Formalin (10% neutral buffered)	

3.10 Cisplatin-Induced Neurotoxicity Model

A mouse model of cisplatin-induced neurotoxicity was developed in accordance with a well-established and widely accepted model that mimics the major biochemical, histological, and behavioral characteristics of chemotherapy-induced neurotoxicity. Cisplatin, a platinum-based antineoplastic agent, is reported to cause neurotoxicity via a combination of oxidative stress, neuroinflammation, and apoptotic mechanisms, which result in both functional and morphological changes in the CNS.

Cisplatin was dissolved in normal saline containing 2% DMSO and injected intraperitoneally (i.p.) at a dosage of 2.3 mg/kg body weight. The volume of injection was kept at 10 mL/kg. The schedule was chosen based on a previously reported protocol to induce stable neurotoxic effects with minimal death rate. Six groups (n = 10) of animals were assigned randomly. The normal control group was given normal saline with 2% DMSO. The -ve control group received cisplatin (2.3 mg/kg). The +ve control group received mecobalamin (1 mg/kg), with intraperitoneal administration 30 minutes before administering cisplatin (2.3 mg/kg).

The groups receiving astaxanthin were given 5 mg/kg, 10 mg/kg, and 25 mg/kg. In all cases, astaxanthin was administered 30 minutes before cisplatin. This approach was chosen to investigate whether astaxanthin could prevent cisplatin-induced neurotoxicity. The intervention was administered over the duration of the experimental protocol, with animals being checked daily for toxicity, behavioral changes, or discomfort. Neurotoxicity was evaluated by changes in locomotor activity, motor

coordination, and behavior, as well as by subsequent biochemical and histopathological evaluation. Neurotoxicity and treatment effects were assessed at specific time points through behavioral testing. This approach permitted the comparison of control, cisplatin-induced, standard drug-treated, and astaxanthin-treated animals, which allowed for the assessment of neuroprotective effects at different concentrations.

The astaxanthin doses (5, 10 and 25 mg/kg) were selected based on previously reported neuroprotective and safety data in the literature, using a low-to-high range to establish a clear dose-response relationship. The cisplatin dose (2.3 mg/kg) was based on established protocols known to reliably induce neurotoxicity with minimal mortality.

3.11 Behavioral Assessments

All groups of animals were subjected to behavioral testing to assess functional deficits caused by cisplatin-induced neurotoxicity. The tests aimed to evaluate alterations in motor activity, coordination, and overall performance. Testing was performed in a dimly lit and quiet environment between 09:00 and 17:00 to avoid circadian effects. Animals were acclimatized to the testing room for at least 30 minutes prior to each test to reduce stress. To prevent interference, behavioral tests were conducted in a sequence that ranged from least to most stressful tasks.

3.11.1 Open Field Test

The Test was conducted to evaluate spontaneous motor activity and exploration, which are often disrupted in models of neurotoxicity. This test used a square arena (40 cm × 40 cm × 35 cm) made of opaque acrylic, divided into central and peripheral zones. The mice were then placed one by one into the middle area and given 5 minutes to move about the arena freely. The test was video recorded from above with a digital video camera. The main outcome measure was

the distance traveled (cm) in the test period, which represents locomotor activity. Decreased activity and exploration were taken as signs of cisplatin-induced neurotoxicity. 70% ethanol was used between each test to clean the apparatus and remove potential odors and biases, as shown in Figure 3.3.



FIGURE 3.3: Open Field Test

3.11.2 Inverted Mesh or Grid Test or Kondziela's Inverted Screen Test

The inverted mesh/grid test (also called Kondziela's inverted screen test) was performed to assess muscle strength, gripping, and motor coordination, which are often affected by cisplatin-induced neurotoxicity. This test involved a square mesh (approximately 43 cm $\times$ 43 cm) with 12 mm grids, surrounded by a wooden frame to prevent animals from escaping the mesh. Animals were transferred to the testing room 10-20 minutes before the test to acclimatize them and ensure they were alert. The mouse was carefully placed on the wire mesh screen at its center, and the stopwatch was started. The mesh screen was then inverted over a period of

about 2 seconds with the head down, and held firmly above a soft surface of 40-50 cm to avoid injuries in the event of a fall. The duration until the mouse released its grasp was measured with an extreme period limit of 60 seconds, at which point the test was terminated if the animal was still clinging. A shorter duration of hanging was thought to reflect motor dysfunction and muscular weakness due to cisplatin neurotoxicity. Sufficient recovery periods were allowed between tests by placing the mice back into their cages to prevent exhaustion and ensure that the results were valid as shown in Figure 3.4.

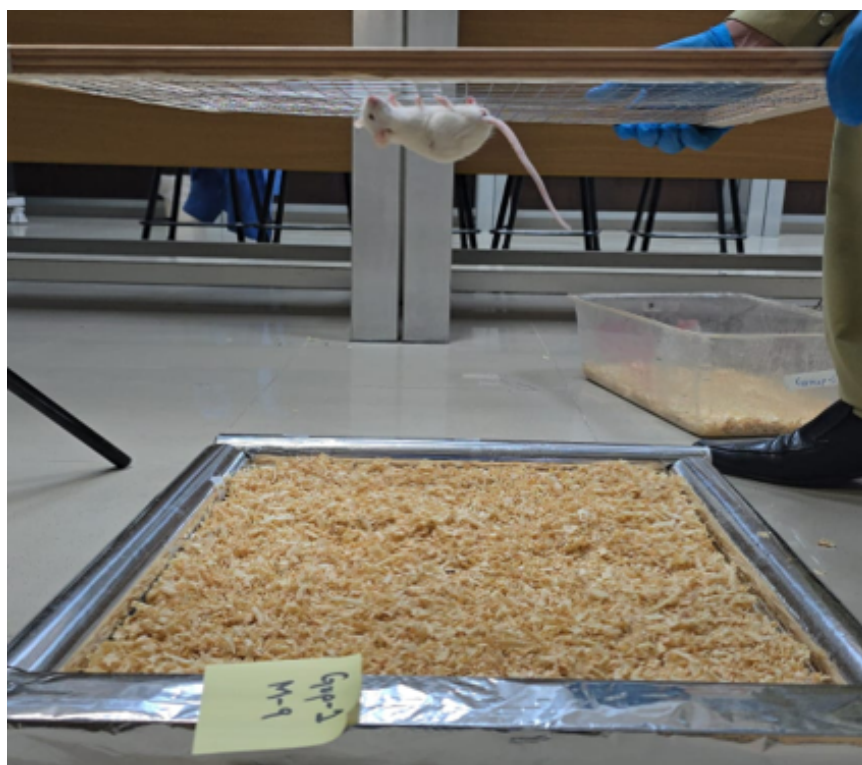


FIGURE 3.4: Kondziela's Inverted Screen Test

3.12 Experimental Design and Grouping of Animals

After the acclimatization period, mice were equally distributed into six groups ($n = 10$) for experimentation. The study was designed to assess the neuroprotective outcome of astaxanthin at varying doses (5, 10, and 25 mg/kg) against cisplatin-induced neurotoxicity, with mecobalamin (1 mg/kg) as the positive control. Drugs

were injected intraperitoneally (i.p.) Neurotoxicity was induced by treatment with cisplatin (2.3 mg/kg), and astaxanthin or mecobalamin were given 30 minutes before cisplatin to determine their neuroprotective effects. The treatment schedule involved two phases of treatment with intervening rest periods. During the first phase (day 1 to day 5), on day 0, baseline behavioral tests were performed, and mice were treated accordingly. On day 5, two hours after the dose, the animals were tested for early signs of neurotoxicity and the effects of the treatments. A recovery period (day 6 to day 10) was then provided, during which the animals received no treatment.

Cisplatin-induced behavioral deficits develop and progress over days as the toxin accumulates, not within hours, so testing 2 hours post-dose on days 5, 15, and 20 captures peak treatment effects across the two dosing phases. Hourly testing would not reflect this cumulative, longer-term neurotoxic process and would add confounding stress from repeated handling.

The second phase (days 11 to 15) involved a re-administration of the treatments. Neurotoxicity was evaluated again on day 15, two hours after treatment. From day 16 to day 20, the animals were permitted a second resting period. The last behavioral testing was executed on day 20. Animals were sacrificed on day 21, and brain tissues were taken for biochemical and histopathology studies. This method allowed us to test the neuroprotective efficacy of astaxanthin in the short and long term, as presented in Table 3.3.

TABLE 3.3: Experimental design: Dosing of animals and behavioral test

Study Phase	Treatment	Assessments
Day 0		Behavioral Tests (OFT + IST)
Day 1 – Day 5	Dosing Phase I Normal Saline (Control) Cisplatin (Negative Control) Mecobalamin + Cisplatin (Positive Control) Astaxanthin 5 mg/Kg + Cisplatin Astaxanthin 10 mg/Kg + Cisplatin Astaxanthin 25 mg/Kg + Cisplatin	
Day 5		Behavioral Tests (OFT + IST) (2 hrs. post-dose)
Day 6 – Day 10	Resting Period	

Table 3.3 continued from previous page

Study Phase	Treatment		Assessments
Day 11 – Day 15	Dosing	Phase Normal Saline (Control)	
	II		
		Cisplatin (Negative Control)	
		Mecobalamin + Cisplatin (Positive Control)	
		Astaxanthin 5 mg/Kg + Cisplatin	
		Astaxanthin 10 mg/Kg + Cisplatin	
		Astaxanthin 25 mg/Kg + Cisplatin	
Day 15			Behavioral Tests (OFT + IST) (2 hrs. post-dose)
Day 16 – Day 20	Resting Period		
Day 20			Behavioral Tests (OFT + IST) (2 hrs. post-dose)
Day 21	Euthanasia		Tissue Collection (Brain, Cerebellum, Hippocampus)

3.13 Study Design

This study combines both *in vivo* and *in silico* methods to explore the neuroprotective properties of astaxanthin (ASTA) against cisplatin neurotoxicity. Male BALB/c mice were used for the *in vivo* study, in which they were injected with cisplatin (2.3 mg/kg, i.p.) after 30 minutes of treatment with astaxanthin (5, 10, and 25 mg/kg, i.p.) or mecobalamin (1 mg/kg, i.p.). Evaluation of neuroprotective efficacy was performed by behavioral assessment using the OFT AND IST, and Histopathological changes were analyzed in sections of the hippocampus (CA1, CA3, and dentate gyrus) stained with hematoxylin and eosin (H&E); biochemical estimation of TNF- α , IL-1 β , MDA, and NO in the hippocampus and cerebellum. The *in silico* workflow involved identification of protein targets that are associated with astaxanthin and neurotoxicity, screening of common targets, construction of protein-protein interaction (PPI) network, follow-up Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses, identification of hub genes and molecular docking of astaxanthin with six core hub proteins (TNF, IL6, PPARG, MAPK14, MAPK3, and MAPK1) for the purpose of validating their interaction and elucidating the molecular mechanism underlying the neuroprotective effect of astaxanthin.

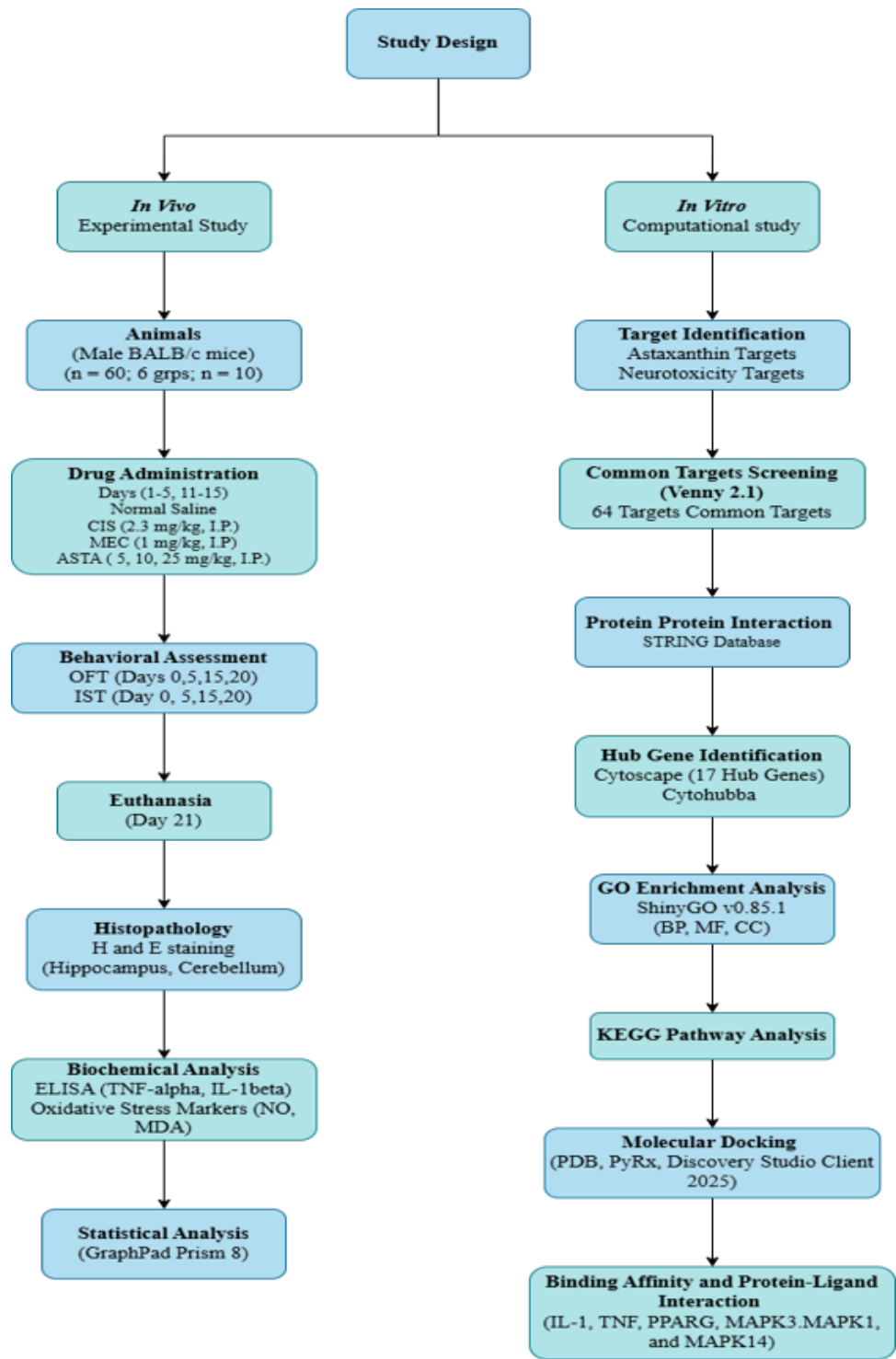


FIGURE 3.5: Study Design

The left side displays the *in vivo* timeline, highlighting the stages from grouping to euthanasia, including treatment groups and behavioral tests, histopathological and biochemical analysis. The right shows the *in silico*, workflow, starting with target prediction and gene databases, followed by PPI analysis, identification of

hub genes, pathway enrichment, and docking, with the use of clean databases and molecular icons.

3.14 Drug Administration

To calculate the dose for cisplatin, astaxanthin, and mecobalamin, mice were weighed before drug administration. Drugs were administered as daily fresh solutions prepared in 2% dimethyl sulfoxide (DMSO) in normal saline to ensure stability and consistency among the groups. Drugs were delivered via intraperitoneal (i.p.) injection with a sterile 1 mL syringe and 26-gauge needle. The injection volume was standardized at 10 mL/kg body weight for all groups to maintain consistency. The time of drug administration was standardized to the same time of day (09:00 am to 12:00 pm) to avoid the impact of circadian rhythm.

For this experiment, 2.3 mg/kg cisplatin (i.p.) was used to induce neurotoxicity. The positive control group was administered mecobalamin (1 mg/kg, i.p.), while the treatment group was administered astaxanthin at doses of 5 mg/kg, 10 mg/kg, and 25 mg/kg (i.p.), respectively. Mecobalamin and astaxanthin were injected 30 minutes before the administration of cisplatin to assess their neuroprotective effects. The control group was injected with the same amount of vehicle (2% DMSO/normal saline) at the same time. The dosing regimen was carried out in two phases: from day 1 to day 5 and from day 11 to day 15, with rest intervals in between. This enabled evaluation of the treatment effects on acute and long-term cisplatin-induced neurotoxicity as shown in Figure 3.5.



FIGURE 3.6: Intraperitoneal administration of drug

3.15 Dose Preparation

Mice were weighed before drug administration. Drugs were made up in 2% DMSO in normal saline every day in a sterile manner to maintain stability. Administration of the drugs was done via the intraperitoneal (i.p.) method with the use of a sterile 1 mL syringe and 26-gauge needle. All the injections involved a volume of 10 mL/kg body weight in all cases as shown in Table 3.4. Drug doses for individual mice were calculated according to the following formula:

$$\text{Dose of Mouse (mg)} = \text{Dose level} \left(\frac{\text{mg}}{\text{kg}} \right) \times \text{Body weight of mouse (kg)} \quad (3.1)$$

The body weight of each mouse was recorded before dosing and used individually in the calculation. The corresponding injection volume (in mL) was then determined as:

$$\text{Injection volume per mouse (mL)} = \text{Body weight (kg)} \times \frac{10 \text{ mL}}{\text{kg}} \quad (3.2)$$

Drug solutions were prepared at appropriate concentrations (mg/mL) such that:

$$\text{Concentration} \left(\frac{\text{mg}}{\text{mL}} \right) = \frac{\text{Dose per mouse (mg)}}{\text{Injection volume per mouse (mL)}} \quad (3.3)$$

TABLE 3.4: Dose preparation of Cisplatin, Mecobalamin, and Astaxanthin for I.P. administration

Group	Compound	Dose (mg/kg)	Avg. Weight (kg)	Mouse Dose per Mouse (mg)	Total Mice	Total Dose for Group (mg)
I	Saline	10 mL/kg	0.03	0.3 mL	10	30.0 mL
II	Cisplatin	2.3 mg/kg	0.03	0.069	10	6.9
III	Mecobalamin	1 mg/kg	0.03	0.03	10	3
	Cisplatin	2.3 mg/kg	0.03	0.069	10	6.9
IV	Astaxanthin (Low)	5 mg/kg	0.03	0.15	10	15
	Cisplatin	2.3 mg/kg	0.03	0.069	10	6.9

Table 3.4 continued from previous page

Group	Compound	Dose (mg/kg)	Avg. Weight (kg)	Mouse Dose per Mouse (mg)	Total Mice	Total Dose for Group (mg)
V	Astaxanthin (Medium)	10 mg/kg	0.03	0.3	10	30
	Cisplatin	2.3 mg/kg	0.03	0.069	10	6.9
VI	Astaxanthin (High)	25 mg/kg	0.03	0.75	10	75
	Cisplatin	2.3 mg/kg	0.03	0.069	10	6.9

Animals were injected daily between 9:00 am and 12:00 pm. Astaxanthin and mecobalamin were administered 30 minutes before cisplatin. The saline (vehicle) group was given 10 mL/kg of a mixture of 40 μ L DMSO and 960 μ L normal saline. Astaxanthin and mecobalamin were initially dissolved in 40 μ L DMSO and then diluted to the desired concentration with 960 μ L normal saline. The same vehicle was used to prepare cisplatin. All solutions were vortex-mixed before use. Prepared drug solutions were stored at 4°C in sterile Eppendorf tubes and administered within 24 hours. The drugs were administered intraperitoneally using a sterile 1 mL syringe with a 26-gauge needle and a volume of 10 mL/kg of body weight. Astaxanthin and mecobalamin were given 30 minutes before cisplatin, as shown in Table 3.5.

TABLE 3.5: Preparations of Solutions

Compound	Dose (mg/kg)	Concen- tration (mg/mL)	Preparation
Vehicle (Control)	10 mL/kg	-	40 μ L DMSO + 960 μ L normal saline (2% DMSO)
Cisplatin	2.3 mg/kg	0.23 mg/mL	0.23 mg cisplatin dissolved in 1 mL of 2% DMSO in normal saline
Mecobalamin (Positive Control)	1 mg/kg	0.1 mg/mL	0.1 mg mecobalamin dissolved in 1 mL of 2% DMSO in normal saline
Astaxanthin (Low)	5 mg/kg	0.5 mg/mL	0.5 mg astaxanthin in 40 μ L DMSO + 960 μ L normal saline

Table 3.5 continued from previous page

Compound	Dose (mg/kg)	Concen- tration (mg/mL)	Preparation
Astaxanthin (Medium)	10 mg/kg	1.0 mg/mL	1.0 mg astaxanthin in 40 μ L DMSO + 960 μ L normal saline
Astaxanthin (High)	25 mg/kg	2.5 mg/mL	2.5 mg astaxanthin in 40 μ L DMSO + 960 μ L normal saline

3.16 Tissue Preparation

On day 21, after the last behavioral test (day 20), mice were sacrificed for brain tissue collection. Animals were sacrificed by cervical dislocation under controlled stress-free conditions. Once the animals were euthanized, the skull was opened quickly, and the whole brain was removed. Regions of the brain of interest, such as the hippocampus and cerebellum, were dissected on a glass plate on ice to avoid protein degradation. Dissected tissues were rinsed with cold phosphate buffer solution (PBS, pH 7.4) to remove traces of blood or debris. Those tissues that were to be used in biochemical analysis were immediately frozen in liquid nitrogen at -80°C . Tissue samples intended for histopathology were fixed in 10% neutral buffered formalin for at least 48 hours at room temperature. To extract proteins, brain tissues frozen in liquid nitrogen were homogenized in ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing a cocktail of protease inhibitors (1:100). Tissues were homogenized using a mechanical homogenizer at 4°C with brief pulses to avoid warming of the sample. The mixtures were then centrifuged at 14,000 rpm for 30 minutes at 4°C . The supernatant, which contained soluble proteins, was then carefully collected and stored at -80°C for further biochemical and ELISA experiments.

3.17 Histopathological Examination

Histopathological analysis was conducted on brain sections (hippocampus and cerebellum) to assess structural and morphological changes in response to cisplatin-induced neurotoxicity and potential protective response to astaxanthin intervention. Tissues from the brain that had been fixed in 10% neutral buffered formalin for a minimum of 48 hours were embedded in paraffin wax using conventional histological techniques. The tissues were dehydrated in a series of ethanol baths (70%, 80%, 95%, and absolute ethanol), cleared in xylene, and then infiltrated with melted paraffin wax. The tissues were subsequently embedded in paraffin, and coronal sections of around 5 μm were cut using a rotary microtome. The sections were attached to clean glass slides and dried overnight at 37°C.

For H&E staining, the slides were deparaffinized in xylene, rehydrated in a series of decreasing ethanol concentrations, and then washed with distilled water. The slides were then stained with Harris hematoxylin for 5-7 minutes, followed by a tap water wash. They were differentiated in 1% acid alcohol for several seconds to clear the nuclei and then blued in 0.2% ammonia water. Then, counterstaining of the cytoplasm was done using 1% eosin Y for 30-60 seconds. The sections were then dehydrated in increasing concentrations of ethanol, cleared with xylene, and mounted with a permanent mounting medium.

The sections were then examined through a light microscope with 10 $\times$ and 40 $\times$ magnification. Particular focus was placed on the hippocampus (especially CA1, CA3), dentate gyrus, and cerebellar structure (such as the Purkinje cell layer). The sections were examined for various parameters such as neuronal integrity, karyolysis, cytoplasmic vacuolation, neuronal degeneration, and evidence of gliosis. Photomicrographs were taken for documentation and to compare various experimental groups.

3.18 Biochemical Assays

3.18.1 Determination of Proteins by Bicinchoninic Acid Assay

The total protein content in brain tissue homogenates from the hippocampus and cerebellum was measured with a bicinchoninic acid (BCA) protein assay kit (Beyotime, China) according to the kit instructions. The BCA method is a popular colorimetric protein assay that relies on the ability of proteins, in an alkaline environment, to reduce cupric ions (Cu^{2+}) to cuprous ions (Cu^+). These ions then bind to bicinchoninic acid to produce a purple-colored complex, the absorbance of which is related to the concentration of protein and can be measured by visible light spectrometry at 562 nm. For the preparation of the standard curve, a series of bovine serum albumin (BSA) standards (0, 25, 50, 100, 200, 400, 600, 800, and 1000 $\mu\text{g}/\text{mL}$) was run. The BCA working reagent was prepared fresh by mixing Reagent A and Reagent B in a 50:1 ratio just before the assay. To conduct the assay, 25 μL of each standard and diluted tissue homogenate sample was added to a 96-well microplate in duplicate for better precision and accuracy. Then, 200 μL of the BCA working reagent was added to each well. The microplate was shaken to mix the reagents and samples, and then incubated at 37°C for 30 minutes to allow the reaction to proceed to completion.

The plate was then cooled to room temperature, and the absorbance at 562 nm was read on a microplate reader (Thermo Fisher Scientific, USA). The average absorbance values of the BSA standards were used to develop a standard curve by plotting them against their concentrations. The concentration of proteins in the tissue extracts was then determined by plotting their absorbance values on the standard curve using linear regression. The absolute protein concentrations were expressed in $\mu\text{g}/\text{mL}$ and used to normalize the following biochemical parameters. After measuring the amount of cytokines by ELISA, the protein concentration was subsequently adjusted for the total protein content in each sample by multiplying

by the following formula:

$$\text{pg/mg protein} = \left(\frac{\text{Cytokine (pg/mL)}}{\text{Total protein } (\mu\text{g/mL})} \right) \times 1000 \quad (3.4)$$

3.18.2 Estimation of Cytokines by Enzyme - Linked Immunosorbent Assay

The levels of pro-inflammatory cytokines, TNF- α and IL-1 β in the hippocampus and cerebellum homogenates were quantified by sandwich ELISA (Biosharp, China). ELISA is a highly sensitive and specific immunologic assay, which uses the antigen-antibody recognition principle to quantify the presence of a protein in a sample.

In sandwich ELISA, specific capture antibodies against the target cytokines are coated onto the microplate and selectively bind the antigen of interest in the sample. This is followed by the addition of an enzyme-linked secondary antibody, typically conjugated with horseradish peroxidase (HRP), which binds to the captured antigen and enables signal generation.

The enzyme-substrate reaction results in a change in color upon addition of a chromogenic substrate, and the absorbance is measured and is proportional to the level of the cytokine in the sample.

This approach is ideal for evaluating neuroinflammatory responses underlying cisplatin-induced neurotoxicity, as elevated TNF- α and IL-1 β levels are commonly used as indicators of neuroinflammatory activation in the CNS.

3.18.2.1 Reagent Preparation

ELISA reagents were brought to room temperature before use to allow for optimal reaction conditions. The 20 $\times$ wash buffer provided was diluted with distilled water to make a 1 $\times$ solution.

Lyophilized cytokine standards were reconstituted as per the manufacturer's instructions; in short, the vials were initially centrifuged to bring the contents to the bottom of the vial and then reconstituted with the standard diluent provided to generate the highest standard concentration.

Then, a series of dilutions was made to prepare standard curves (usually 0-1000 pg/mL for TNF- α and 0-500 pg/mL for IL-1 β). The sample diluent was used without dilution. Brain homogenates from the hippocampus and cerebellum were diluted 1:2 with sample diluent to ensure that the number of cytokines in the samples was within the linear range of the ELISA assay.

3.18.2.2 ELISA Assay Procedure

TNF- α and IL-1 β concentrations were determined using Biosharp Mouse ELISA kits (TNF- α : Cat. No. BSEM-004-96T; IL-1 β : Cat. No. BSEM-015-96T), according to the instructions. The assay was performed using 96-well microplates that were pre-coated with monoclonal capture antibodies against the cytokines. Standards, samples, and controls were run in duplicate to ensure reproducibility.

In the assay, 50 μ L of the standards, samples, and controls were applied to the appropriate wells. The plate was tapped to ensure even distribution and then incubated at 37°C for 30 minutes to allow the antigens to bind with the antibodies. Wells were emptied and washed five times with 300 μ L of washing buffer to eliminate all the unbound substances after the incubation process.

Then, 50 μ L of horseradish peroxidase (HRP)-labeled detection antibody was added to the wells, and the plate was incubated for a further 30 minutes at 37°C. This allowed the formation of an antigen-antibody "sandwich". The wells were washed again to remove any unbound detection antibody. To develop the color, 50 μ L of chromogen solution A (containing hydrogen peroxide) and 50 μ L of chromogen solution B (containing tetramethylbenzidine, TMB) were added to each well. The plate was gently mixed and incubated for 15 minutes at 37°C in the dark to allow the HRP enzyme to convert TMB into a blue product. A stop solution (50 μ L of 2 M sulfuric acid) was then added to each well to stop the

reaction and turn the color from blue to yellow. The optical density (OD) was read at 450 nm in a Thermo Scientific Multiskan FC microplate reader within 15 minutes of the stop solution being added to the wells. This protocol was followed for the TNF- α and IL-1 β assays to measure the concentration of cytokines in the hippocampus and cerebellum.

3.18.2.3 Measurement of Absorbance and Concentration

The absorbance (optical density or OD) at 450 nm of the wells was measured using a microplate reader within 15 minutes of adding the stop solution to maintain the reliability and consistency of the colorimetric signal.

A standard curve was generated by plotting the mean absorbance of the standards versus their concentrations. Linear regression was used to determine the equation of the standard curve and the coefficient of determination (R^2), which confirmed the linear relationship and validity of the assay.

The absorbance values of TNF- α and IL-1 β in the hippocampal and cerebellar tissue samples were used to determine their concentrations from the standard curve. To standardize the measurement and enable comparison between the different treatment groups, the levels of cytokines were expressed relative to the total protein concentration, measured earlier by the BCA method.

3.18.2.4 Quality Control

To improve accuracy and reliability, all ELISA readings were performed in duplicate, and duplicate variability was assessed. The coefficient of variation (CV) for duplicate measurements was kept below 10% for all samples. Appropriate blanks (containing sample diluent only) were also included in each assay plate to account for background absorbance.

The standard curve was only accepted if its linearity was good, with a correlation coefficient (R^2) greater than or equal to 0.99. Plates with non-acceptable standard curve criteria were repeated to ensure the validity of the experimental results.

3.19 Oxidative Stress Parameters

3.19.1 Measurement of MDA Level

To measure lipid peroxidation in hippocampal and cerebellar tissue, the thiobarbituric acid reactive substances method was used as previously described method [180]. In a reaction mixture consisting of 0.1 M phosphate buffer, trichloroacetic acid (TCA), and thiobarbituric acid, samples were incubated. Following that step, the reaction mixture was heated and cooled, then centrifuged; its absorbance was recorded at 532 nm, and the results were expressed as a percentage.

3.19.2 Measurement of NO level

To evaluate the NO level in hippocampal and cerebellar tissue, the established Griess reagent method were used [180]. Sample supernatant was mixed with 1:1 of Griess reagent 1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride). Pink azo dye was developed, that is analyzed spectrophotometrically at 548 nm. For the standard calibration curve, sodium nitrite was used and the NO concentration was stated as a percentage.

Oxidative stress was targeted because it is the primary upstream trigger that initiates the neuroinflammatory and apoptotic cascade in cisplatin-induced neurotoxicity, so measuring MDA and NO directly reflects both disease severity and treatment efficacy. Antioxidant enzymes were not measured separately, as the oxidative stress markers already capture the net redox outcome relevant to the scope of this study.

3.20 *In Silico* Studies: Network Pharmacology and Molecular Docking

To identify the possible molecular mechanisms that mediate the neuroprotective effects of astaxanthin in cisplatin-induced neurotoxicity, *In-Silico* analysis was conducted using an integrated network pharmacology and molecular docking strategy. Potential protein targets of astaxanthin were discovered using publicly available databases, such as SwissTargetPrediction and PharmMapper, with the search limited to Homo sapiens to guarantee biological relevance. Extra relevant targets were obtained using GeneCards, intersecting with neurotoxicity genes from OMIM and GeneCards. A list of the identified targets was filtered, eliminating duplicates, and a curated list of unique human proteins was obtained to proceed with analysis.

The STRING database was used to construct protein-protein interaction (PPI) networks, and the networks were visualized and analyzed using Cytoscape (version 3.10.4). Network centrality parameters like degree, betweenness, and closeness were used to identify key hub genes. ShinyGO (version 0.85.1) was used with a false discovery rate ($FDR < 0.05$) to perform functional enrichment analysis, such as Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.

Based on the results of network pharmacology analysis, molecular docking studies were performed to verify the interactions between astaxanthin and six hub targets, such as IL-6, TNF- α , PPAR γ , MAPK14, MAPK3, and MAPK1. Protein structures were retrieved from the Protein Data Bank (PDB), prepared using PyRx, and docked with astaxanthin using AutoDock Vina algorithm. Protein–ligand interactions were analyzed using Discovery Studio Client 2025 software, identifying key amino acid residues and interaction patterns involved in the neuroprotective effects of astaxanthin against cisplatin-induced neurotoxicity, with the best docking conformations being selected based on the lowest binding energy values.

3.20.1 Network Pharmacology

To explore the possible molecular mechanisms of astaxanthin neuroprotective activity against cisplatin-induced neurotoxicity, this network pharmacology study was performed. The potential target proteins of astaxanthin were screened by the SwissTargetPrediction (<https://www.swisstargetprediction.ch>), PharmMapper (<https://www.lilab-ecust.cn/pharmmapper/>) and GeneCards (<https://www.genecards.org>) databases. 100 human targets were retrieved from SwissTargetPrediction, 25 human targets were retrieved from PharmMapper and 2 more were retrieved from GeneCards. Following the merging of the datasets and the exclusion of any duplicated targets, 127 unique targets associated with astaxanthin were identified.

The keyword “neurotoxicity” were used to search OMIM (<https://www.omim.org/>) and GeneCards (<https://www.genecards.org>) databases for genes associated with cisplatin-induced neurotoxicity. After data integration and de-duplication, 3,418 genes associated with disease were found. Venny 2.1 (<https://bioinfogp.cnb.csic.es/tools/venny>) was used to identify the common targets as possible targets involved in the neuroprotective action of astaxanthin between the 127 targets associated with astaxanthin and 3,418 targets associated with neurotoxicity.

Finally, these 64 overlapping targets were imported to the STRING database (<https://string-db.org/>) using medium level of confidence (score >0.4) to create a protein–protein interaction network (PPI) with the organism set to only Homo sapiens. The resulting interaction data were exported and imported into Cytoscape 3.10.4 (https://cytoscape.org/release_notes_3_10_4.html) for visualization and network topology analysis. The CytoHubba plugin was used to identify hub genes by three topological algorithms: Degree Centrality (DC), Betweenness Centrality (BC) and Closeness Centrality (CC).

Top 20 genes were selected for each algorithm and ranked separately based on their centrality scores. Using Venny 2.1, a Venn intersection analysis was conducted for the three most popular gene lists, with a top-20 list for each. Based on the above analysis, 13 genes were identified as common hub genes, which were found to be

associated with all three topological methods, suggesting their possible role in the therapeutic mechanism of astaxanthin against cisplatin-induced neurotoxicity.

3.20.1.1 Gene Ontology

Common genes were used for Gene Ontology (GO) enrichment analysis through ShinyGO v0.85.1 (<https://bioinformatics.sdstate.edu/go/>), an archived version of Ensembl Release 104. The analysis was carried out to determine the significantly enriched functional categories in the Biological Processes (BP), Molecular Functions (MF), and Cellular Components (CC) categories in *Homo sapiens*. The BP, MF, and CC enrichment analysis was conducted independently with default parameters and a statistical significance of a false discovery rate (FDR) < 0.05 . Enriched GO terms were ranked according to P-value, and the most significant terms were visualized based on gene count, fold enrichment, and $-\log_{10}(\text{FDR})$.

3.20.1.2 Kyoto Encyclopedia of Genes and Genomes Pathway Analysis

To evaluate significantly enriched signaling pathways of the common target genes of astaxanthin, pathway enrichment analysis was carried out with ShinyGO v0.85.1 based on KEGG. *Homo sapiens* was used as the reference species for the analysis, and statistical significance was defined as $\text{FDR} < 0.05$.

For detailed interpretation and visualization of the pathways, the top 20 pathways with the lowest p-value were selected. Bar plots and bubble plots generated through ShinyGO were used to represent pathway enrichment scores, including $-\log_{10}(\text{FDR})$, gene ratio, and fold enrichment.

3.20.2 Molecular docking

The molecular docking experiments were carried out to confirm the predicted interactions resulting from network pharmacology analysis by docking astaxanthin

with the key hub proteins identified from the protein–protein interaction (PPI) network.

The seven core targets identified are IL-6, TNF- α , PPAR γ , MAPK14, MAPK3, MAPK1, and NFkB1, which were determined by the CytoHubba analysis using Degree, Betweenness, and Closeness centrality scores. These targets were selected due to their proven role in neuroinflammation, oxidative stress, apoptosis, and the signaling pathways responsible for cisplatin-induced neurotoxicity.

3.20.2.1 Recovering Protein Structure from Protein Data Bank

The three-dimensional crystal structures of target proteins were obtained from the Protein Data Bank (PDB) library (www.rcsb.org) in PDB format. The selected protein structures included IL-6 (PDB ID: 1N26), TNF- α (PDB ID: 2AZ5), MAPK1 (PDB ID: 1TVO), MAPK3 (PDB ID: 2ZOQ), MAPK14 (PDB ID: 5ML5), PPAR γ (PDB ID: 6MS7), and NFkB1 (PDB ID: 1NKB). The proteins chosen were those that are biologically relevant and play a key role in the underlying molecular mechanisms that cause cisplatin-induced neurotoxicity.

3.20.2.2 Preparation of Protein Macromolecule

The retrieved protein structures were prepared for the molecular docking procedure with the AutoDock Vina algorithm integrated into the software PyRx, and then analyzed with the software Discovery Studio Client 2025. All water molecules, co-crystallized ligands, ions and other heteroatoms were removed before docking to prevent possible interference with ligand binding. The Kollman and Gasteiger charges were calculated with AutoDock tools in PyRx and the prepared proteins were saved as pdbqt format. The proteins were structurally validated using Ramplot web server (www.ramplot.in) with the help of Ramachandran plot and the structural integrity of the proteins and the active site region were studied further by Discovery Studio Client 2025.

3.20.2.3 Preparation of Ligand

Astaxanthin 3D structure was retrieved from PubChem and imported into PyRx for 3D molecular docking studies. Energy minimization was carried out using the Universal Force Field (UFF) to obtain a stable conformation with minimum steric hindrance. Gasteiger charges were calculated and rotatable bonds were defined to allow flexibility of the ligands during docking simulations. The optimized structure of the ligand was then converted to a pdbqt file to be used in the docking analysis.

3.20.2.4 Ligand–Protein Molecular Docking

For the molecular docking analysis, the AutoDock Vina engine integrated to PyRx was used to determine the binding affinity of astaxanthin to the chosen target proteins. The grid box parameters were determined according to the active-site regions of each protein, for complete coverage of the ligand-binding pocket. Docking studies with default AutoDock Vina parameters were carried out, and many binding conformations were generated for protein–ligand complexes. The docking poses were ranked by the binding energy (kcal/mol) reported by the docking software, and the pose having the lowest binding energy was selected for further study. The resulting docked complexes were visualized and represented graphically using Discovery Studio Client 2025.

3.20.2.5 Post-Docking Analysis and Visualization

Discovery Studio Client 2025 (version 25.1.0) was used to analyze the selected docked complexes to study the molecular interactions between astaxanthin and the target proteins. To allow for detailed binding pattern analysis, 2D and 3D interaction maps were created. Interactions such as hydrogen-bond, hydrophobic interactions, π – π interactions, alkyl interactions and van der Waals force were recognized and described.

In addition, the amino acids that are in the active site areas of the selected protein targets and are associated with the binding of the ligand were investigated

in terms of binding stability and comparison of the binding profiles of the astaxanthin molecule. These analyses enabled the understanding of the possible neuroprotective mechanisms of action that can be attributed to astaxanthin against cisplatin-induced neurotoxicity.

3.21 Statistical Analysis

All results were analyzed by GraphPad Prism version 8.0 (GraphPad Software, Inc., La Jolla, CA, USA). All data were presented as mean $\pm$ standard error of the mean (SEM) for each experimental group. For behavioral assessments and biochemical parameters one-way ANOVA was used to compare differences among experimental groups. Post-hoc test of Tukey was used for pairwise comparison between groups. Molecular docking studies were performed to determine the best interaction possibilities among the different protein targets with the binding affinity (GlideScore). A p-value < 0.05 was considered statistically significant. The following significance levels were used: *p < 0.05 , **p < 0.01 , and ***p < 0.001 . Behavioral and biochemical experiments were done on $n = 10$ animals per group. Analysis was conducted in a blinded fashion with the investigator analysis blinded to group assignment to reduce bias.

Chapter 4

Results

4.1 Behavioral Assessment

To assess the neuroprotective effect of astaxanthin against cisplatin-induced neurotoxicity in mice, behavioral tests were performed on Day-0, Day-5, Day-15 and Day-20 of the experimental protocol. The OFT was employed to observe locomotor activity and anxiety-like behavior, and the IST was used to measure muscle strength and coordination. Data are presented as mean $\pm$ standard deviation (n = 10 in each group). Neurotoxicity was induced using two doses of cisplatin (2.3 mg/kg, i.p.) on Days 1-5 and Days 11-15. The 1 mg/kg, i.p. dose of MEC and the 5, 10, and 25 mg/kg, i.p. doses of ASTA were injected 30 minutes before injection of the cisplatin.

4.1.1 Effect of Astaxanthin on Locomotor Activity in the OFT

All groups showed comparable baseline locomotor activity on Day-0 with no significant differences observed that of Normal Saline: 1566 ± 66 cm; CIS: 1552 ± 66 cm; MEC (1 mg/kg) + CIS: 1558 ± 66 cm; ASTA (5 mg/kg) + CIS: 1565 ± 65 cm; ASTA (10 mg/kg) + CIS: 1546 ± 70 cm; ASTA (25 mg/kg) + CIS: $1565 \pm$

68 cm). Hypolocomotion was observed in the cisplatin (CIS, 2.3 mg/kg) injected group as compared to the normal saline injected group, as evidenced by a significant decrease ($p < 0.05$) in distance travelled. Distance travelled was significantly reduced by CIS to 1223 ± 47 cm compared to 1559 ± 47 cm in the normal saline control group ($###p < 0.001$) on Day-5. This deficit became more pronounced on Day-15 by CIS: 1017 ± 43 cm vs Normal Saline: 1563 ± 43 cm; ($###p < 0.001$) and on Day-20 by CIS: 834 ± 43 cm vs Normal Saline: 1559 ± 43 cm; ($###p < 0.001$).

MEC (1 mg/kg) + CIS gave moderate protection against cisplatin-induced hypolocomotion. It significantly increased distance travelled compared to the CIS group on Day-5 by 1403 ± 74 cm ($***p < 0.001$), Day-15 by 1364 ± 71 cm ($***p < 0.001$), and Day-20 by 1364 ± 57 cm ($***p < 0.001$). ASTA (5 mg/kg) + CIS provided moderate protection, significantly attenuating the CIS-induced reduction on Day-15 by 1232 ± 79 cm ($***p < 0.0001$) and Day-20 by 1179 ± 57 cm ($***p < 0.0001$), while the effect on Day-5 was 1242 ± 75 cm and was not statistically significant. ASTA (10 mg/kg) + CIS showed stronger protection, significantly reversing the CIS-induced hypolocomotion on Day-5 by 1500 ± 76 cm ($***p < 0.001$), Day-15 by 1373 ± 88 cm ($***p < 0.001$), and Day-20 by 1338 ± 98 cm ($***p < 0.001$). ASTA (25 mg/kg) + CIS showed the greatest improvement, nearly normalizing locomotor activity. It significantly increased distance travelled to 1512 ± 78 cm on Day-5 ($***p < 0.001$), 1496 ± 77 cm on Day-15 ($***p < 0.001$), and 1493 ± 67 cm on Day-20 ($***p < 0.001$) compared with the CIS group, thereby restoring values close to normal saline control levels. The results indicated that the 25 mg/kg dose of ASTA was the most effective in ameliorating cisplatin-induced hypolocomotion in a dose-dependent manner in the OFT as shown in figure 4.1.

The locomotor activity parameters in the OFT are shown as bar graphs in the above figure. The distance travelled was significantly reduced in the group that received cisplatin (2.3 mg/kg) compared to the normal saline group. These changes were dose-dependently reversed by pre-treatment with MEC (1 mg/kg) and ASTA (5, 10 and 25 mg/kg), with the highest dose (25 mg/kg) of ASTA providing the

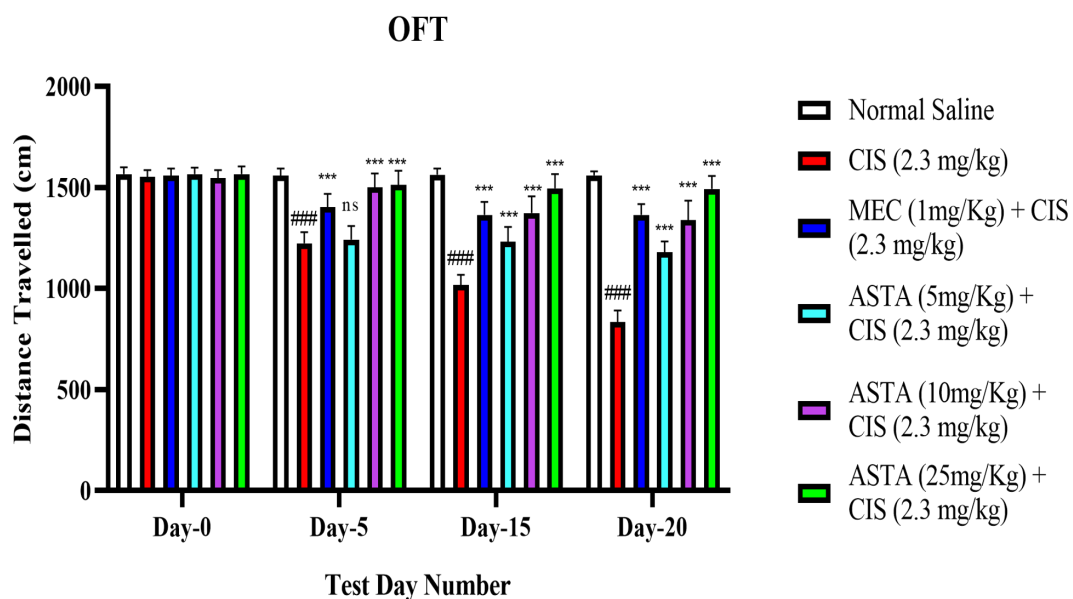


FIGURE 4.1: Distance travelled in the OFT displayed the effects of Astaxanthin

greatest recovery. The results are presented as means $\pm$ SD ($n = 10$). Mean differences were statistically significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared to the CIS group, and $\#p < 0.05$, $\##p < 0.01$, and $\###p < 0.001$ compared to the control group. ns = not significant.

4.1.2 Effect of ASTA on Muscle Strength and Co-ordination in the Kondziela IST

All groups showed comparable baseline muscle strength and coordination on Day-0 with no significant differences observed (Normal Saline: 60.00 ± 1.3 s; CIS: 60.00 ± 1.3 s; MEC (1 mg/kg) + CIS: 60.00 ± 1.3 s; ASTA (5 mg/kg) + CIS: 60.00 ± 1.3 s; ASTA (10 mg/kg) + CIS: 60.00 ± 1.3 s; ASTA (25 mg/kg) + CIS: 60.00 ± 1.3 s). There was no significant difference in hanging time between the cisplatin (CIS, 2.3 mg/kg) group and the normal saline group on any of the test days. On Day-5, hanging time decreased marginally in CIS to 57.70 ± 1.3 s as compared to 59.50 ± 1.3 s in the normal saline group, which was within the normal physiological range. Similarly, on Day-15, CIS: 58.10 ± 1.3 s vs Normal Saline: 59.30 ± 1.3 s and Day-20, CIS: 58.80 ± 1.3 s vs Normal Saline: 59.60 ± 1.3 s,

no statistically meaningful differences were observed. These results indicate that cisplatin in the dose level of 2.3 mg/kg causes no, or at least negligible, peripheral neuromuscular toxicity with regard to muscle strength and coordination.

There was also no significant difference between the MEC (1 mg/kg) + CIS and the control treatment group throughout the study. The MEC (1 mg/kg) + CIS treatment group was not significantly different from the CIS group during the study period. ASTA (5 mg/kg) + CIS maintained hanging time at 59.20 ± 1.3 s on Day-5, 59.00 ± 1.3 s on Day-15, and 59.40 ± 1.3 s, on Day-20. ASTA (10 mg/kg) + CIS showed values of 59.80 ± 1.3 s on Day-5, 59.60 ± 1.3 s on Day-15, and 59.90 ± 1.3 s on Day-20. ASTA (25 mg/kg) + CIS recorded 59.50 ± 1.3 s, on Day-5, 59.70 ± 1.3 s on Day-15, and 59.60 ± 1.3 s on Day-20. The overall results from the IST performed by Kondziela show that the dose of cisplatin used does not appear to be causing significant peripheral toxicity, as shown in Figure 4.2.

The Inverted Screen Test was included to assess whether cisplatin-induced neurotoxicity extends to peripheral neuromuscular function, rather than to screen for drug side effects. The absence of a significant deficit in hanging time in the cisplatin group indicates that, at 2.3 mg/kg, cisplatin's toxicity is predominantly central (hippocampal/behavioral) rather than peripheral, which is consistent with the Open Field Test, histopathological, and biochemical findings, and confirms that astaxanthin and mecobalamin did not cause any adverse motor impairment at the doses tested.

Bar graphs of muscle strength and coordination as part of the IST were shown above. No significant difference was seen in hanging time between cisplatin (2.3 mg/kg) and normal saline. Results are expressed as mean $\pm$ SD ($n = 10$).

4.1.3 Neuroprotective Effects of Astaxanthin on Cisplatin-Induced Hippocampal Damage

The protective role of astaxanthin (ASTA) against cisplatin-induced neurotoxicity was evaluated using hematoxylin and eosin (H&E) staining to assess the

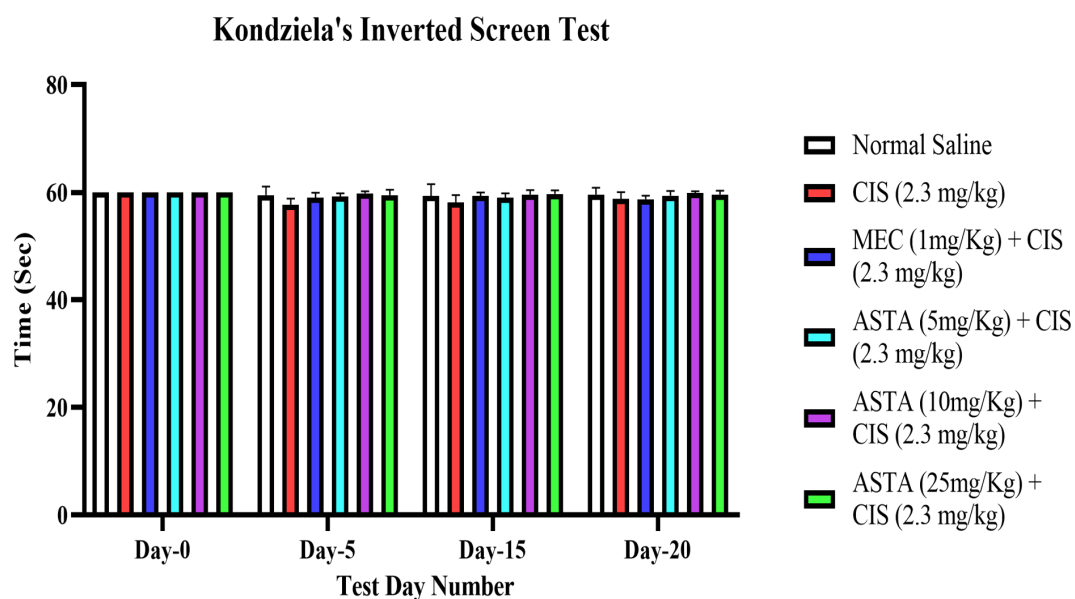


FIGURE 4.2: The effects of ASTA on hanging time in Kondziela's Inverted Screen Test

histopathological alterations in the hippocampus. The hippocampus is a region of the brain that is very sensitive to oxidative stress, neuroinflammation, and neuronal degeneration following cisplatin treatment, and which has a significant role in learning, memory, and cognitive processing. CA1, CA3, and the dentate gyrus (DG) are regions of particular interest due to their critical roles in memory formation and synaptic plasticity. The positive control was mecobalamin (MEC; 1 mg/kg), and the ASTA was given as a pretreatment 30 minutes before the injection of cisplatin. Hippocampal sections were examined using light microscopy at a $4\times$ magnification, as shown in Figure 4.3.

The normal saline group showed intact hippocampal cytoarchitecture and distinct CA1, CA3, and dentate gyrus areas. The pyramidal cell layers of the CA1 and CA3 region were compact and well organized, and the dentate gyrus region showed continuous and densely packed granular cell layers. The arrangement of the neurons was consistent, and there were no signs of structural abnormalities or degeneration in the overall architecture of the hippocampus.

The cisplatin-treated group, on the other hand, showed pronounced histopathological changes throughout the hippocampus. In the CA1 and CA3 regions, there

was a marked disruption of normal cellular organization, a decrease in the density of neurons, widespread vacuolation, and a significant degree of karyolysis, which suggests severe neuronal injury and degeneration. The dentate gyrus showed loss of normal structural integrity, disorganization of the granular layer, and reduced cell density. Overall, cisplatin administration resulted in substantial deterioration of hippocampal architecture compared with the normal control group, consistent with neurodegenerative changes induced by oxidative stress and inflammatory damage.

Histopathological damage caused by cisplatin was significantly reduced after pretreatment with mecobalamin (1 mg/kg). CA1 and CA3 regions demonstrated better maintenance of cellular organization, decreased degenerative changes, and decreased vacuolation, with better neuronal density. Likewise, structural organization and cellular integrity were restored in the dentate gyrus. Even though there were still some mild changes that were observed, the overall architecture of the hippocampus was significantly enhanced when compared with the cisplatin-treated group.

ASTA (25 mg/kg) before cisplatin treatment led to significant neuroprotective effects against the damage to the hippocampus induced by cisplatin. The group treated with astaxanthin showed significant preservation of the cytoarchitecture of the hippocampus, with better organization of the CA1 and CA3 pyramidal layers, and restoration of dentate gyrus organization.

Compared with the cisplatin-treated group, the cellular density was increased, and vacuolation was significantly reduced, with signs of karyolysis significantly decreased. The overall histological appearance of the hippocampus of the ASTA-treated group was close to the normal control group and was comparable with that of the group treated with mecobalamin, suggesting significant protection from cisplatin-induced neuronal damage.

These histopathological changes are consistent with the behavioral changes observed and offer morphological proof of the neuroprotective effects of astaxanthin against cisplatin-induced neurotoxicity. Preservation of CA1, CA3, and dentate

gyrus regions indicates that astaxanthin is effective in preserving normal structural organization and preventing neuronal degeneration in the hippocampus.

The neuroprotective effect is likely due to the strong antioxidant, anti-inflammatory, and anti-apoptotic activities of astaxanthin that, together, help to reduce the oxidative injury and/or neuronal damage associated with cisplatin administration. In general, the results of H&E reveal that ASTA can provide significant protection against the damage to the hippocampus caused by cisplatin and promote survival of the neurons.

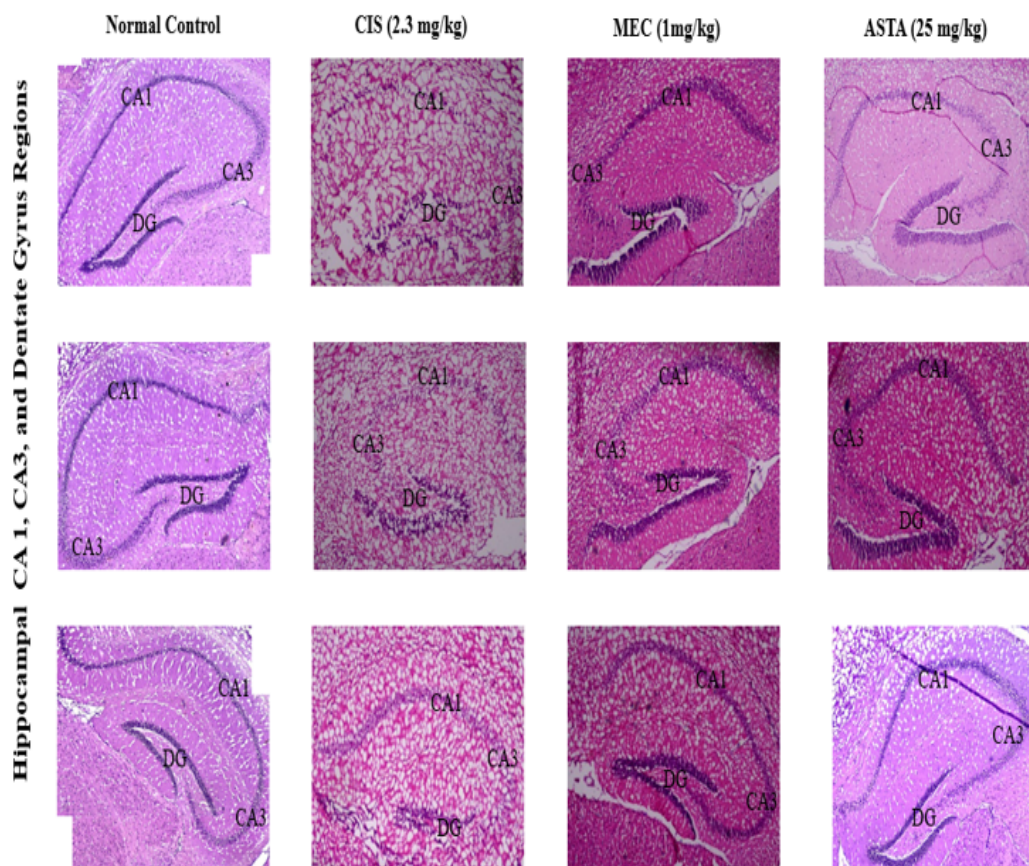


FIGURE 4.3: Representative photomicrographs of H&E-stained hippocampal sections showing the CA1, CA3, and dentate gyrus regions.

Representative sections (4 $\times$) from the Normal Control, Cisplatin (CIS 2.3 mg/kg), Mecobalamin (MEC 1 mg/kg) + Cisplatin (2.3 mg/kg), and ASTA (25 mg/kg) + Cisplatin (2.3 mg/kg) groups. In the normal control group, the hippocampus had normal architecture, including distinct CA1 and CA3 pyramidal layers and a small

dentate gyrus. The administration of cisplatin caused significant neuronal damage, including neuronal loss, vacuolation, karyolysis, and disorganization of hippocampal organization. These pathological changes were significantly reduced, and the architecture of the neurons was maintained by pre-treatment with mecobalamin (MEC) 30 minutes before the cisplatin administration.

The ASTA-treated group showed significant recovery of the hippocampal morphology with the restoration of the neuronal organization and cell density almost identical to that of the normal control group. Labels indicate the CA1, CA3, and dentate gyrus (DG) regions. CA1: Cornu Ammonis 1; CA3: Cornu Ammonis 3; DG: Dentate Gyrus.

4.1.4 Enzyme-Linked Immunosorbent Assay

4.1.5 Effects of Cisplatin and Astaxanthin on Total Protein Content in Brain Tissues Assessed by BCA Assay

Total protein concentration of hippocampal and cerebellar tissue lysates was measured before ELISA by the BCA assay to standardize the amount of protein loaded in each experimental group. The results showed that administration of cisplatin resulted in a significant reduction in total protein concentration in the hippocampus and cerebellum when compared to the normal saline control group, which suggests substantial neuronal damage, loss of synapses, and neurodegenerative changes due to cisplatin toxicity.

This reduction was dose-dependently prevented by co-administration with astaxanthin, which indicated a restoration of protein integrity and partial reversal of cisplatin-induced neurotoxicity.

In treatment groups, there was a dose-dependent increase in the preservation of total protein content, which was better than in the controls in some areas. These protein values were then used to normalize the ELISA data and were expressed

as pg/mg protein to reduce variations resulting from different tissue damage as shown in table 4.1.

TABLE 4.1: Total Protein Concentration in Hippocampus and Cerebellum Determined by BCA Assay

Group			Hippocampus ($\mu\text{g/ml}$)		Cerebellum ($\mu\text{g/ml}$)		Interpretation
Normal Saline (Control)			492.67 585.75	–	454.53 555.23	–	Normal protein integrity and cellular homeostasis in both brain regions.
Cisplatin (2.3 mg/kg)			169.21 333.99	–	237.87 259.23	–	Marked reduction in total protein content, indicating substantial neuronal damage and neurotoxicity.
Cisplatin + Mecobalamin (1 mg/kg)			497.39 540.08	–	494.20 541.50	–	Significant restoration of protein levels, demonstrating effective neuroprotection against cisplatin-induced damage.
Cisplatin + Astaxanthin (5 mg/kg)			330.48 376.84	–	224.02 249.52	–	Partial recovery of protein content, suggesting mild neuroprotective effects.
Cisplatin + Astaxanthin (10 mg/kg)			406.41 432.79	–	390.45 434.69	–	Moderate restoration of protein levels, indicating dose-dependent neuroprotection.
Cisplatin + Astaxanthin (25 mg/kg)			533.87 578.41	–	537.74 572.02	–	Near-complete restoration of protein content to control levels, indicating strong neuroprotective efficacy.

4.1.6 Effect of Astaxanthin on Tumor Necrosis Factor - Alpha Levels

The administration of Cisplatin led to a significant increase in the concentration of TNF- α both in the hippocampus and in the cerebellum, which suggests the establishment of a marked neuroinflammatory response (### $p < 0.001$) when compared with the normal saline group. In the hippocampus, the cisplatin-induced increase

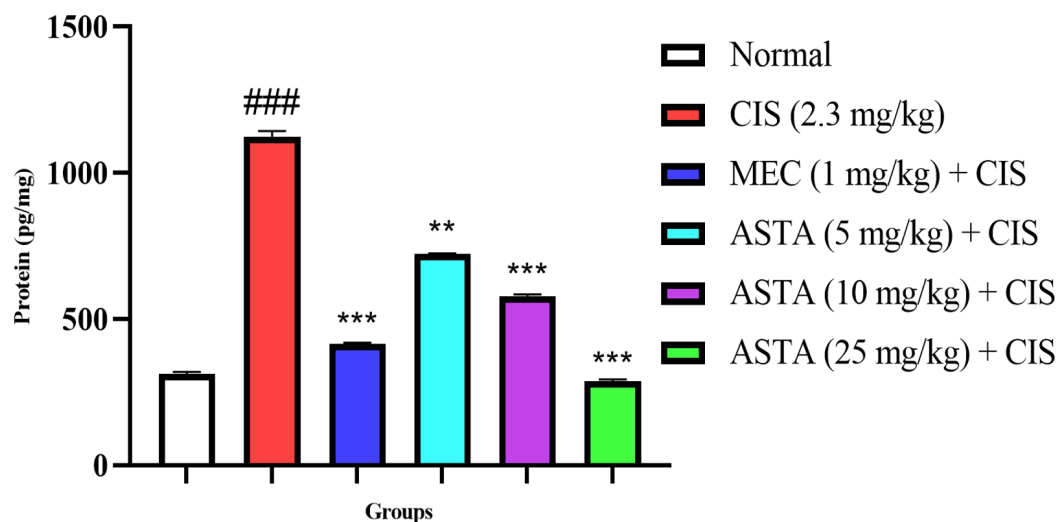
in the amount of TNF- α was around fourfold, and in the cerebellum, it was also increased, supporting the inflammatory effect of cisplatin in several brain areas.

Cisplatin pretreatment with mecobalamin (1 mg/kg) significantly reduced the elevation of the pro-inflammatory cytokine, TNF- α , in the hippocampus and cerebellum (**** $p < 0.001$ vs cisplatin group), showing considerable anti-inflammatory activity. Treatment with astaxanthin also decreased the level of TNF- α in a dose-dependent manner. The lowest dose of ASTA (5 mg/kg) produced a moderate reduction in TNF- α but remained significantly higher than normal control values.

ASTA at 10 mg/kg also decreased the levels of TNF- α in both brain regions, and the concentration of 25 mg/kg was more effective in decreasing brain TNF- α levels, reducing the levels almost to the same level as those of the normal control and the mecobalamin-treated groups.

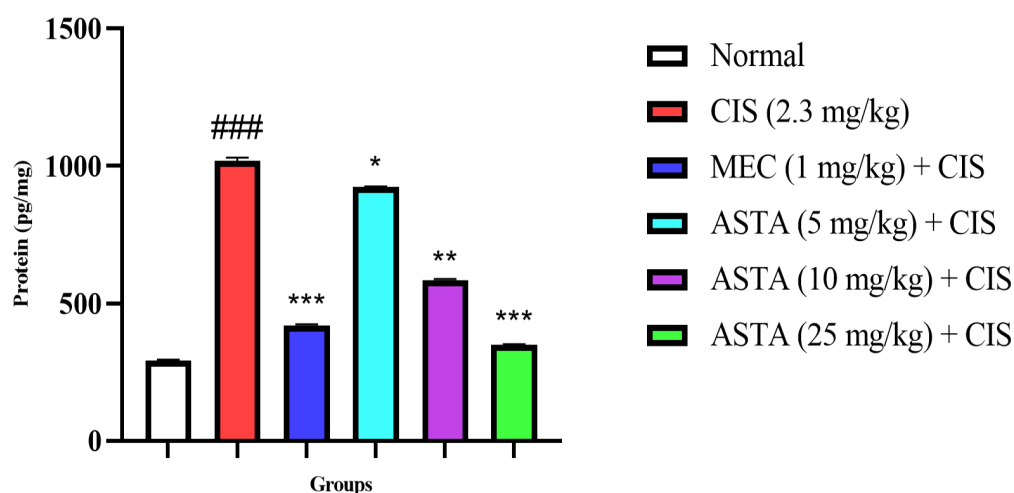
The results indicate that astaxanthin can effectively reduce neuroinflammation induced by cisplatin by inhibiting the production of TNF- α , with the best protection in the hippocampus and cerebellum achieved at 25 mg/kg in both the hippocampus and cerebellum as shown in figure 4.4.

TNF-Alpha Normalized (Hippocampus)



(A)

TNF-Alpha Normalized (Cerebellum)



(B)

FIGURE 4.4: Effect of ASTA on the levels of TNF- α determined by ELISA assay in the hippocampal and cerebellar tissues

As shown in the above figure, the concentration of (pg/mg protein) of TNF- α protein in hippocampal and cerebellar tissues. The level of TNF- α was significantly higher in the cisplatin group (2.3 mg/kg) than in the normal saline group. Pretreatment with mecobalamin (1 mg/kg) and ASTA (5 mg/kg, 10 mg/kg, 25 mg/kg) was found to markedly reduce the concentration of TNF- α , with the highest ASTA dose (25 mg/kg) having the most significant decrease and moving towards normal values of the cytokine. All data reported are mean $\pm$ SD ($n = 10$). Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs cisplatin group; ### $p < 0.001$ vs normal saline control group.

4.1.7 Effect of Astaxanthin on Interleukin-1 Beta Levels

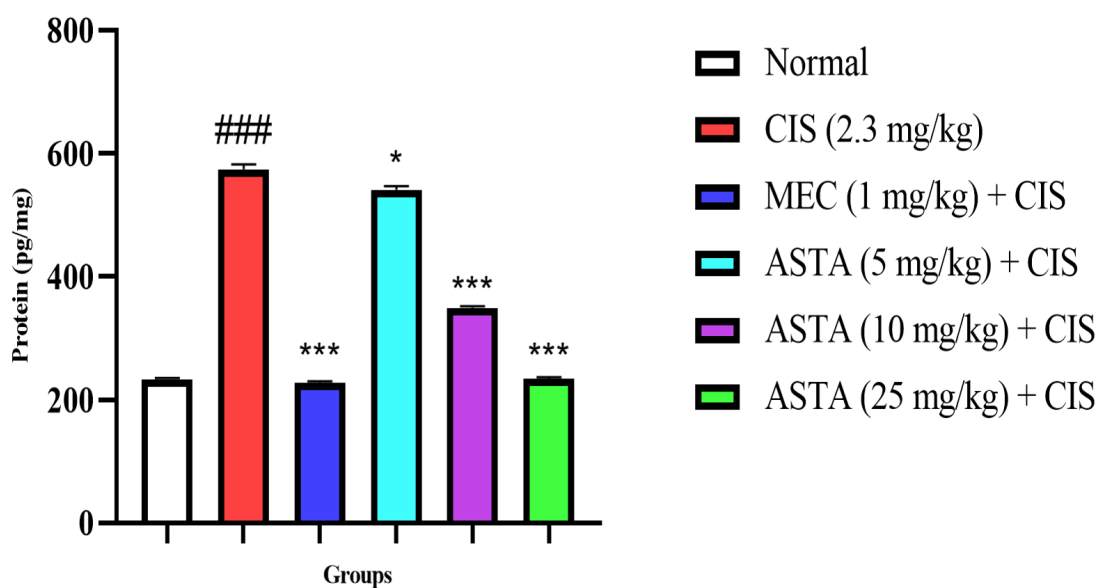
Treatment with cisplatin also resulted in significantly higher levels of IL-1 β in the hippocampus and cerebellum than those seen in the normal saline group (### $p < 0.001$), further supporting the development of an active inflammatory response

that accompanies cisplatin-induced neuronal damage. High levels of IL-1 β are a sign of increased microglial activation and activation of neuroinflammatory pathways, leading to neuronal dysfunction and degeneration.

Mecobalamin (1 mg/kg) pretreatment markedly decreased the level of IL-1 β in both brain regions ($***p < 0.001$ vs the cisplatin group). Similarly, ASTA treatment produced a dose-dependent reduction in IL-1 β levels. The 10 mg/kg dose offered more protection and reduced cytokine levels significantly, whilst the 5 mg/kg dose was only partially suppressive of IL-1 β . The highest dose (25 mg/kg) of ASTA resulted in the greatest decrease in IL-1 β levels, bringing the levels back to those of the normal saline and mecobalamin administration groups.

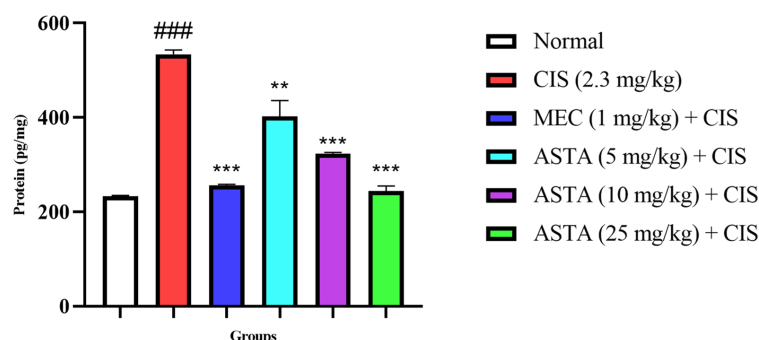
The reduction of IL-1 β level in both hippocampus and cerebellum tissue was uniform; thus, the production of pro-inflammatory cytokines was suppressed and protected against cisplatin-induced neuroinflammation by the action of ASTA as shown in figure 4.5.

IL-1beta Normalized (Cerebellum)



(A)

IL-1 β Normalized (Hippocampus)



(B)

FIGURE 4.5: Effect of ASTA on IL-1 β concentrations in the hippocampal tissue and cerebellar tissue by ELISA

The above figure shows the concentration of IL-1 β (pg/mg protein) in the tissues of the hippocampus and cerebellum. Compared with the normal saline control group, cisplatin (2.3 mg/kg) resulted in a significant increase in the expression of IL-1 β . This elevation was significantly reduced by pretreatment with mecobalamin (1 mg/kg) and ASTA (5, 10, and 25 mg/kg), with ASTA 25 mg/kg showing the greatest inhibitory effect and bringing the cytokine levels close to normal values. The data are presented as mean $\pm$ SD ($n = 10$). Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs cisplatin group; ### $p < 0.001$ vs normal saline control group.

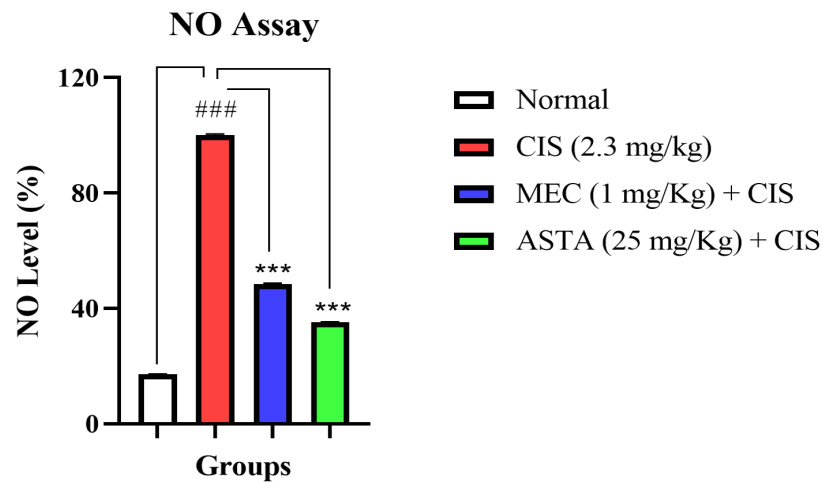
4.2 Effect of Cisplatin and Astaxanthin on Oxidative Stress Parameters in the Hippocampus and Cerebellum

To assess the antioxidant activity of astaxanthin against oxidative stress caused by cisplatin, levels of NO and MDA in hippocampal tissue were quantified. NO is a well-established marker for nitrosative stress, and MDA is a good marker of lipid peroxidation and oxidative damage of the cell membranes.

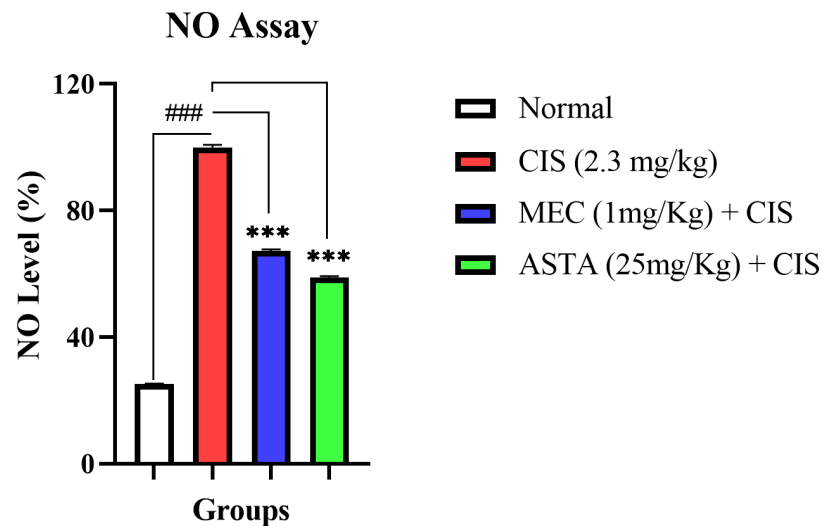
4.2.1 Effect of Astaxanthin on NO level

Compared to the normal control group, severe nitrosative stress in the hippocampus was observed after administration of cisplatin (2.3 mg/kg). The NO level in the normal control group was $17.14 \pm 0.12\%$, while the cisplatin treatment group had $100.00 \pm 0.41\%$ (### $p < 0.001$), which is about 5.8-fold higher. This increase was significantly reduced with pretreatment with mecobalamin (1 mg/kg), and the NO level was lowered to $48.50 \pm 0.30\%$ (** $p < 0.001$ vs. cisplatin group), which was about 51.5% less than the cisplatin group. Pretreatment with Astaxanthin (25 mg/kg) showed maximum protection, reducing NO levels to $35.23 \pm 0.16\%$ (** $p < 0.001$ vs. cisplatin), which is roughly 64.8% less than for cisplatin. The results demonstrated that astaxanthin is more effective at reducing the generation of NO and nitrosative stress, caused by cisplatin treatment, than mecobalamin, as shown in Figure 4.7A.

Cisplatin (2.3 mg/kg) treatment led to a significant increase in NO concentration in the cerebellum as compared to normal saline. This suggests that there is a significant nitrosative stress in this brain region. The NO level in the normal control group was $25.13 \pm 0.20\%$ and in the cisplatin treatments group the NO levels were significantly elevated to $99.99 \pm 0.77\%$ (### $p < 0.001$) corresponding to about a fourfold increase. This is a huge increase in the nitrosative damage of the cerebellar tissue due to cisplatin. Pretreatment with mecobalamin (1 mg/kg) significantly reduced the cisplatin-induced increase in NO levels (down to $67.26 \pm 0.49\%$ (** $p < 0.001$ vs. cisplatin group), which is about 32.7% less than the cisplatin treated group). Interestingly, Astaxanthin (25 mg/kg) pretreatment showed superior protecting effects, lowering the level of NO to $58.81 \pm 0.48\%$ (** $p < 0.001$ vs. cisplatin group) which corresponds to about a 41.2% reduction when compared with the cisplatin group. Astaxanthin treated group exhibited significantly more reduction in NO level than mecobalamin treated group which demonstrates that the anti-nitrosative activity of astaxanthin is significant. These results show the mitigation of the cisplatin-induced nitrosative stress in the cerebellum with highest dose of astaxanthin that shows the highest protection in the cerebellum in the figure 4.6B.



(A)



(B)

FIGURE 4.6: Effect of astaxanthin on the level of NO in the hippocampal tissue and cerebellar tissue.

After cisplatin administration (Figure 4.6). Cisplatin (2.3 mg/kg) significantly increased the levels of NO as compared to normal controls. These elevations were significantly reduced by pretreatment with mecobalamin (1 mg/kg), and more so by astaxanthin (25 mg/kg). Data are presented as Mean $\pm$ SD. ### $p < 0.001$ vs. Normal saline; *** $p < 0.001$ vs. Cisplatin.

Together, the figures show that cisplatin causes substantial nitrosative stress in both the hippocampus and the cerebellum, with prominent increases in NO levels.

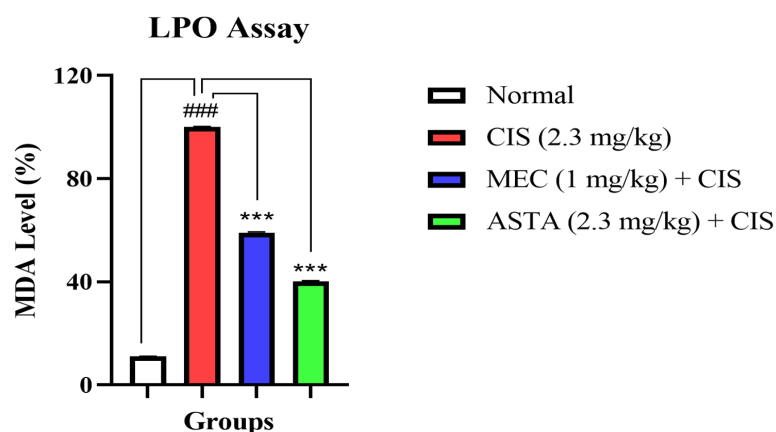
Astaxanthin (25 mg/kg) significantly inhibited the cisplatin-induced NO elevation in both brain regions, and provided better protection than mecobalamin. The dose-dependent and consistent decrease in NO in the hippocampus as well as in the cerebellum suggests that astaxanthin is a potent anti-nitrosative agent with promising neuroprotective effects in the context of chemotherapy-induced nitrosative damage.

4.2.2 Effect of Astaxanthin on Lipid Peroxidation Levels

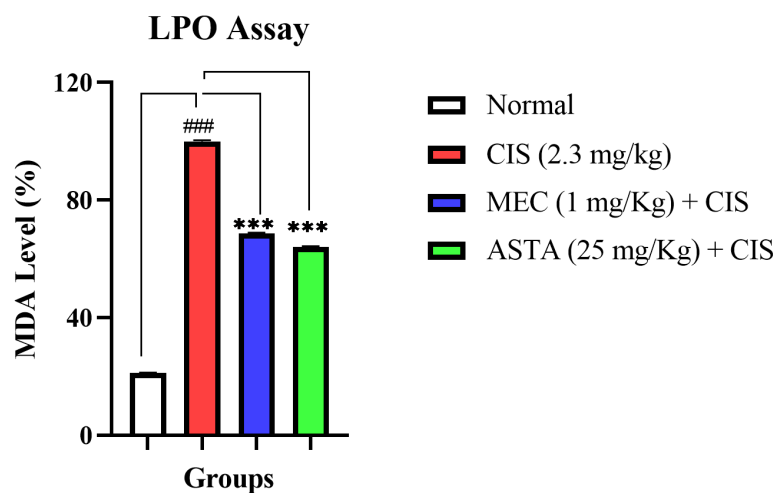
Lipid peroxidation was significantly enhanced (using MDA) in cisplatin-treated animals. The normal control group displayed an MDA level of $11.08 \pm 0.07\%$, while the cisplatin group reached $100.00 \pm 0.23\%$ (### $p < 0.001$). When treated with Mecobalamin (1 mg/kg), the MDA level was significantly reduced to $59.05 \pm 0.27\%$ (** $p < 0.001$ vs. cisplatin), which is about a 41% reduction. A greater reduction was obtained with Astaxanthin (25 mg/kg), which reduced the MDA levels by 60% of that found in the cisplatin group ($40.24 \pm 0.22\%$ (** $p < 0.001$ vs. cisplatin)). The results of this study indicate the strong protective effect of astaxanthin against the oxidative damage of membranes induced by cisplatin, as shown in Figure 4.7A.

The cisplatin-treated group showed a significant increase in MDA concentration in the cerebellum relative to the normal saline group, suggesting a widespread lipid peroxidation and oxidative damage to membrane structures. The normal control group had a MDA level of $21.12 \pm 0.18\%$, whereas the cisplatin-treated group had a significantly higher level of MDA at $99.99 \pm 0.38\%$ (### $p < 0.001$) which is nearly 5-fold higher. This massive rise is likely due to oxidative damage to neuronal membranes in the cerebellum caused by cisplatin. The cisplatin-induced rise in MDA level was significantly reduced by pretreatment with mecobalamin (1 mg/kg) and the MDA level was reduced to $68.71 \pm 0.25\%$ (** $p < 0.001$ vs. cisplatin group), which is about a 31.3% decrease from the cisplatin treated group. It shows that the cerebellum had moderate protection against cisplatin induced lipid peroxidation by mecobalamin and that Astaxanthin (25 mg/kg) pretreatment had

a more potent protective effect as the MDA level was significantly reduced to $64.03 \pm 0.30\%$ (** $p < 0.001$ vs. cisplatin group) which is about 36.0% reduction compared to the cisplatin group. In the present study, the group treated with astaxanthin exhibited a higher level of attenuating the lipid peroxidation level compared to the group treated with mecobalamin, suggesting that astaxanthin has strong antioxidant activity in the membranes as illustrated in Figure 4.7B.



(A)



(B)

FIGURE 4.7: Effect of astaxanthin on the level of MDA in the hippocampal tissue and cerebellar tissue.

After cisplatin administration (Figure 4.7). Cisplatin (2.3 mg/kg) significantly increased the levels of both NO and MDA as compared to normal controls. These elevations were significantly reduced by pretreatment with mecobalamin (1 mg/kg),

and more so by astaxanthin (25 mg/kg). Data are presented as Mean $\pm$ SD. ### $p < 0.001$ vs. Normal saline; *** $p < 0.001$ vs. Cisplatin.

The 25 mg/kg dose consistently showed the strongest and most significant neuroprotective effect across the behavioral and histopathological results, so it was selected as the representative optimal dose for the more resource-intensive biochemical assays. This is standard practice once a clear dose-dependent trend has already been established from the other endpoints.

These findings together indicate that cisplatin causes substantial lipid peroxidation and oxidative membrane injury in both the hippocampus and the cerebellum, as reflected by the substantial increase in the levels of MDA. Astaxanthin (25 mg/kg) strongly inhibited the increase in MDA in both the brain regions, demonstrating more protection than mecobalamin in the inhibition of cisplatin induced MDA elevation.

The dose- and region-dependent decrease in MDA levels suggests strong antioxidant and membrane-protective effects of astaxanthin and suggests its therapeutic potential for reducing the oxidative damage caused by chemotherapy in the CNS.

4.3 In Silico Studies

4.3.1 Network Pharmacology

4.3.1.1 Potential Targets of Astaxanthin and Neurotoxicity-Associated Genes

The SwissTargetPrediction, GeneCards, and PharmMapper databases were used to identify potential therapeutic targets for astaxanthin. A total of 127 unique human protein targets were obtained after combining and removing duplicate entries from the three databases. The antioxidant defense, inflammatory regulation, lipid metabolism, signal transduction, apoptosis, and cellular stress response pathways

were the major areas associated with these targets, reflecting the multi-target pharmacological properties of astaxanthin.

The GeneCards and OMIM gene databases were used to gather the genes associated with neurotoxicity by searching for the term “Neurotoxicity”. A total of 3,418 neurotoxicity-related genes were identified after data integration and removal of duplicate entries. The genes included in these pathways exhibited oxidative stress, neuroinflammation, neuronal apoptosis, mitochondrial impairment, synaptic signaling, and neurodegeneration. A total of 64 overlapping genes were found between the astaxanthin targets and the genes associated with neurotoxicity; the present findings indicate that this number of genes may be potential therapeutic targets for the neuroprotective effects of astaxanthin against cisplatin-induced neurotoxicity.

4.3.1.2 Venn Diagram Analysis

Venn diagram analysis was carried out in Venny-2.1 software to find the common targets between astaxanthin-related proteins and neurotoxicity-associated genes as shown in the figure 4.8.

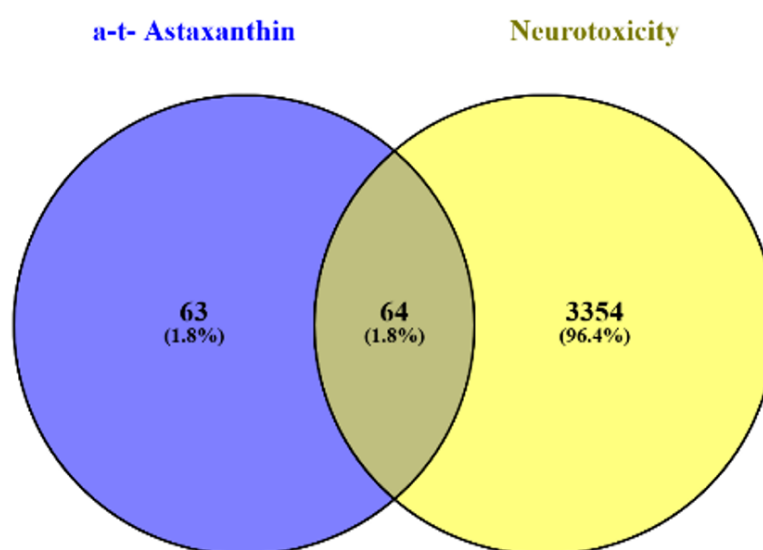


FIGURE 4.8: Venn diagram the intersection between the predicted all trans astaxanthin and neurotoxicity

The analysis showed that 63 targets were specific to astaxanthin, while 3,354 genes were specific to neurotoxicity. There was a total of 64 "overlapping" targets identified between the two datasets.

These common targets indicate that astaxanthin could affect multiple molecular pathways in the pathogenesis of neurotoxicity. Many of these targets are linked to inflammatory signaling, the regulation of oxidative stress, apoptosis, cellular survival and neuronal function.

Thus, the commonly recognized overlapping genes offer a mechanistic framework for the possible neuroprotective effects of astaxanthin against cisplatin-induced neuronal damage.

a- t-astaxanthin (all trans astaxanthin) targets (127 genes from SwissTargetPrediction, GeneCards, and PharmMapper) and the neurotoxicity-associated genes (3,418 genes from GeneCards and OMIM) is shown in the Venn diagram below. There are 64 molecular targets common to both sets, which could be involved in the neuroprotective effects of astaxanthin against neurotoxicity.

There are 64 molecular targets common to both sets, which could be involved in the neuroprotective effects of astaxanthin against neurotoxicity.

4.3.1.3 Protein-Protein Interaction Network

The 64 common targets were uploaded to STRING database (Homo sapiens, confidence score > 0.4) to build the protein-protein interaction (PPI) network. The interaction data was then displayed and analyzed using Cytoscape version 3.10.4.

The network showed high interconnectivity among the identified targets, suggesting that these proteins are involved in highly coordinated biological processes that are related to neurotoxicity.

Several proteins were found to play key roles in the network and exhibited high connectivity with other nodes, suggesting a potential role in regulating neuroinflammatory and oxidative stress pathways as shown in the figure [4.9](#).

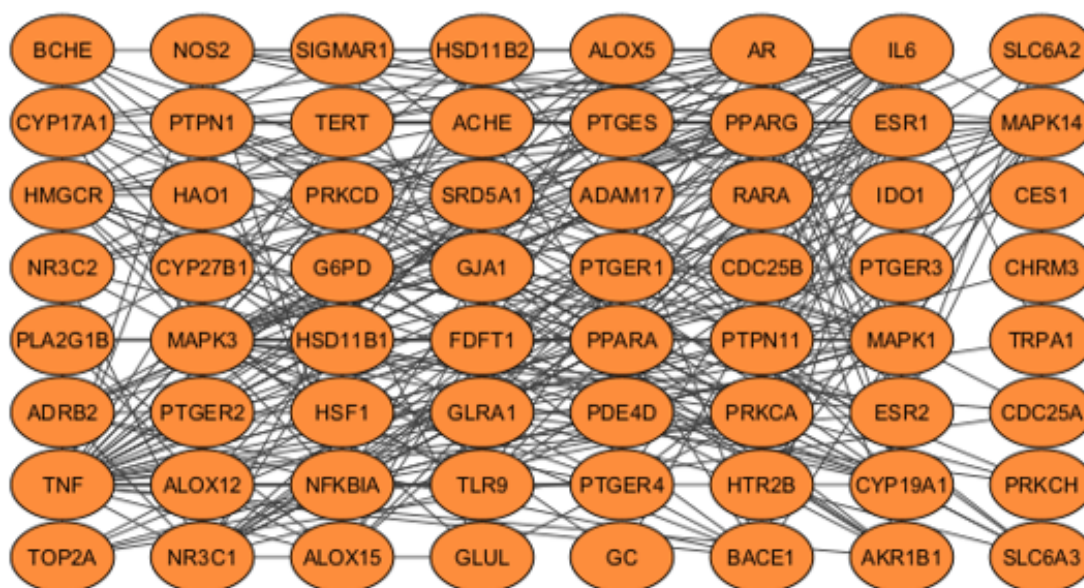


FIGURE 4.9: Protein-protein interaction network obtained by using the STRING database (confidence ≥ 0.4 , Homo sapiens) from the 64 common targets identified between astaxanthin-related targets and neurotoxicity-associated genes with Cytoscape version 3.10.4.

The topological analysis of the protein-protein interaction (PPI) network (Table 4.1) revealed the highly connected network architecture of the 64 common targets between astaxanthin and neurotoxicity. A total of 64 nodes and 315 edges were identified in the network, representing functional interactions among the target proteins. On average, a protein interacted with 9.844 other proteins per protein, which indicates a high level of connectivity in the network and efficient communication between the different signaling pathways involved in neurotoxicity. However, the network diameter of 6 and radius of 3 suggested that the network was relatively compact and that information could be efficiently passed between distant nodes. Moreover, the characteristic path length was found to be 2.243, which indicated that the majority of proteins can be reached by interacting with a small number of proteins, facilitating fast spreading of biological signals across the network.

The clustering coefficient was 0.516, which showed a high level of local clustering, suggesting that proteins were strongly connected into functional modules involved in similar biological processes. The network density of 0.156 showed that the network was moderately dense but had a biological relevance, and the network

heterogeneity value of 0.827 showed that there were proteins with different connectivity levels, including a number of highly influential hub proteins. Moreover, the network centralization value of 0.461 indicated moderate centralization of the network around key regulatory targets. A single connected component indicated that there was no isolated sub-network of proteins among the proteins that were integrated in a single network. Together, these topological features indicate that the targets identified are involved in highly coordinated biological processes of oxidative stress, neuroinflammation, apoptosis, mitochondrial impairment, and neuronal survival, as shown in Table 4.2.

TABLE 4.2: Statistics of the PPI network

Statistic	Value
Number of nodes	64
Number of edges	315
Avg. number of neighbors	9.844
Network diameter	6
Network radius	3
Characteristic path length	2.243
Clustering coefficient	0.516
Network density	0.156
Network heterogeneity	0.827
Network centralization	0.461
Connected components	1
Analysis time (sec)	0.201

4.3.1.4 Hub Gene Identification

Topological analysis was performed using the CytoHubba plugin on CytoScape (version 3.10.4) to identify the most influential targets in the protein-protein interaction (PPI) network. To define the importance of each node in the network, three centrality algorithms were used: Degree Centrality (DC), Betweenness Centrality (BC), and Closeness Centrality (CC). Degree centrality reveals highly connected

proteins that participate in local network interactions; betweenness centrality reveals proteins that act as critical bridges controlling information flow between different regions of the network; closeness centrality reveals proteins that can communicate to other proteins quickly across the network.

The top 20 genes were independently ranked using each of the centrality measures. Venn Diagram analysis was then conducted to find common genes in the three centrality ranks: Degree, Betweenness, and Closeness. These common genes were identified as 13 genes in the intersection analysis, namely TNF, IL6, PPARG, MAPK3, MAPK1, ESR1, MAPK14, NR3C1, HMGCR, PPARA, PRKCA, ESR2 and ADRB2 (Figure 4.10). These genes showed high topological importance across the three algorithms, and were thus regarded as the core genes that regulate the neuroprotective mechanisms of astaxanthin against neurotoxicity.

The degree value for each identified hub gene was calculated and the top two genes with the highest degree values were TNF and IL6, which had 38 interactions with the other identified genes, and suggested their central role in neuroinflammatory signaling. IL6 also had the highest betweenness centrality value (625.40), indicating that it is a key bottleneck protein that controls the interactions between various signaling pathways. In addition to a high degree value (31), PPARG had a high betweenness centrality (360.79), indicating its important function in anti-inflammatory and antioxidant responses. Likewise, MAPK3, MAPK1 and MAPK14 had very high centrality scores suggesting functions in cellular stress response, apoptosis, and inflammatory pathways, respectively. Additionally, other important hub genes such as PPARA, NR3C1, ESR1, ESR2, HMGCR, PRKCA, and ADRB2 are known to play roles in metabolic homeostasis, steroid hormone signaling, cellular survival, and neuroprotective responses. Together, these hub genes are significantly linked to biological mechanisms involved in cisplatin-induced neurotoxicity such as neuroinflammation, oxidative stress, mitochondrial impairment, apoptosis, and neuronal survival. The fact that these targets are centrally located in the PPI network indicates that modulation of these targets may play a significant role in the neuroprotective effects of astaxanthin, as shown in Table 4.3.

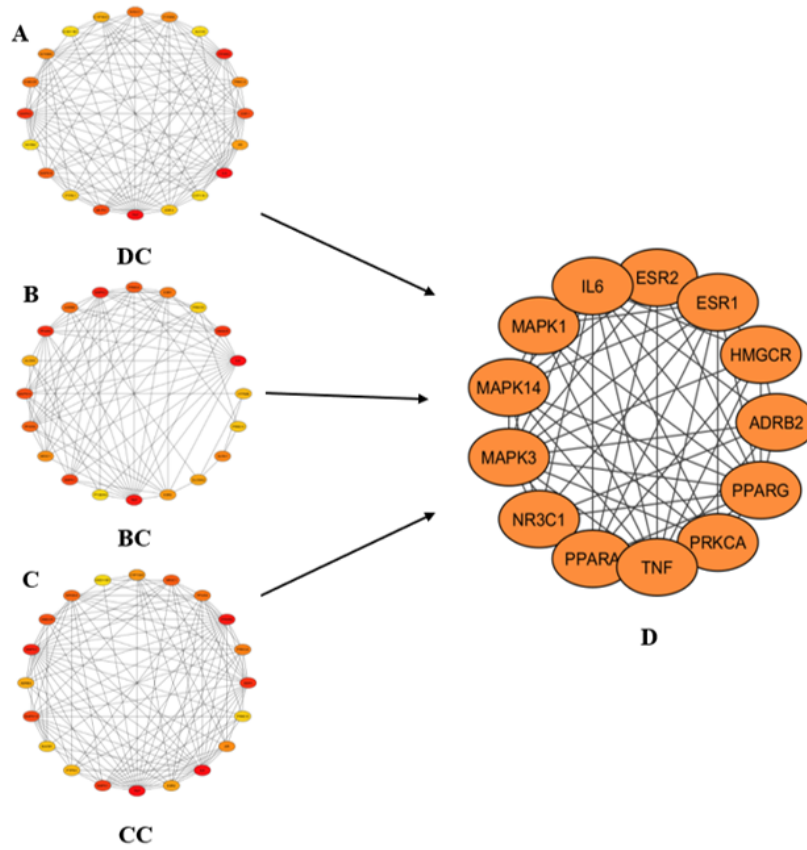


FIGURE 4.10: Multi-centrality topological analysis and identification of hub genes in the Astaxanthin-Neurotoxicity PPI network.

TABLE 4.3: Centrality Matrices for the 13 Common Hub Genes

Rank (by Degree)	Gene Symbol	Degree	Betweenness	Closeness
1	TNF	38	566.9	50.08
1	IL6	38	625.4	50.08
3	PPARG	31	360.79	46.58
4	MAPK3	30	384.38	46.08
5	MAPK1	22	232.64	41.25
5	ESR1	22	136.19	41.58
7	MAPK14	19	229.33	39.67
8	NR3C1	18	115.25	39.25
8	HMGCR	18	282.18	39.42
10	PPARA	17	213.88	38.92
10	PRKCA	17	227.13	38.67
15	ESR2	12	109.01	36.17
17	ADRB2	11	183.08	35.83

(A) Top 20 genes ranked by Degree Centrality (DC), which identifies proteins

with high degree centrality, meaning that they are interconnected and have a large number of local interactions in the network. (B) Top 20 genes by Betweenness Centrality (BC), proteins that act as critical bridges and that regulate information flows among different modules of the network. (C) Top 20 genes by Closeness Centrality (CC), proteins with high global accessibility and high communication potential between them in the network. (D) Core hub gene network constructed from the top 20 genes in the intersection of genes identified by the DC analysis, BC analysis, and CC analysis.

A total of 13 common hub genes were identified: TNF, IL6, PPARG, MAPK3, MAPK1, ESR1, MAPK14, NR3C1, HMGCR, PPARG, PRKCA, ESR2 and AD-RB2. These genes had a high topological importance in all three-centrality metrics, and were deemed as significant targets for regulation that might be associated with the neuroprotective effects of astaxanthin against neurotoxicity.

4.3.1.5 Enrichment Analysis for Gene Ontology

Gene Ontology (GO) enrichment analysis was then performed using ShinyGO v0.85.1 under three ontological classes: Biological Processes (BP), Molecular Functions (MF), and Cellular Components (CC). The analyses were carried out separately for each category, using default parameters (species: Homo sapiens; significance threshold: $FDR < 0.05$). Enriched terms were sorted by p-value, and lollipop plots were generated to represent fold enrichment, gene count, and statistical significance ($-\log_{10}(FDR)$).

The enriched GO terms showed a high degree of coherence with known pathomechanisms of cisplatin-induced neurotoxicity, and the neuroprotective mechanisms of astaxanthin such as lipid mediator biosynthesis, regulation of neuroinflammation, membrane/organelle homeostasis, receptor-mediated signaling, and oxidative stress response cascades in all three dimensions of GO (Figure 4.11).

a. Biological Processes

The top-fold enriched terms within the BP domain were lipoxin biosynthetic process and lipoxin metabolic process with the greatest fold enrichment, followed by regulation of chronic inflammatory response, negative regulation of cholesterol storage, negative regulation of glial cell apoptotic process, Bergmann glial cell differentiation, cellular response to estrogen stimulus, negative regulation of lipid storage, heat generation, negative regulation of lipid localization, negative regulation of miRNA transcription, regulation of miRNA metabolic process, regulation of ossification, and regulation of lipid localization (Figure 4.11A).

In the context of this present study, these processes are mechanistically interesting, biologically enriched ones. Astaxanthin may facilitate resolution-phase inflammation by modulating signaling pathways derived from arachidonic acid, as lipoxins are endogenous pro-resolving lipid mediators, which are biosynthetically and metabolically enriched. The high contribution of terms associated with the suppression of glial apoptosis and astaxanthin's ability to induce Bergmann glial differentiation further indicate that astaxanthin maintains the viability and structural integrity of glial cells under cisplatin-induced cytotoxic stress, which is a vital factor in maintaining the viability of neurons. Furthermore, the enrichment of miRNA transcriptional regulation pathways suggests that post-transcriptional gene regulatory networks are likely downstream effectors of astaxanthin's neuroprotective effects, and lipid homeostasis-related terms highlight the well-established antioxidant activity of astaxanthin, which tends to maintain the integrity of membrane lipid composition under oxidative stress.

b. Cellular Components

Significant enrichment of nuclear envelope lumen, pseudopodium, neuronal cell body membrane, endolysosome, cell body membrane, caveola, lipid droplet, membrane raft, endoplasmic reticulum (ER) lumen, membrane microdomain, receptor complex, perinuclear region of cytoplasm, neuronal cell body, somatodendritic compartment, endoplasmic reticulum membrane, ER subcompartment, nuclear outer membrane-endoplasmic reticulum membrane network, and organelle subcompartment (Figure 4.11B).

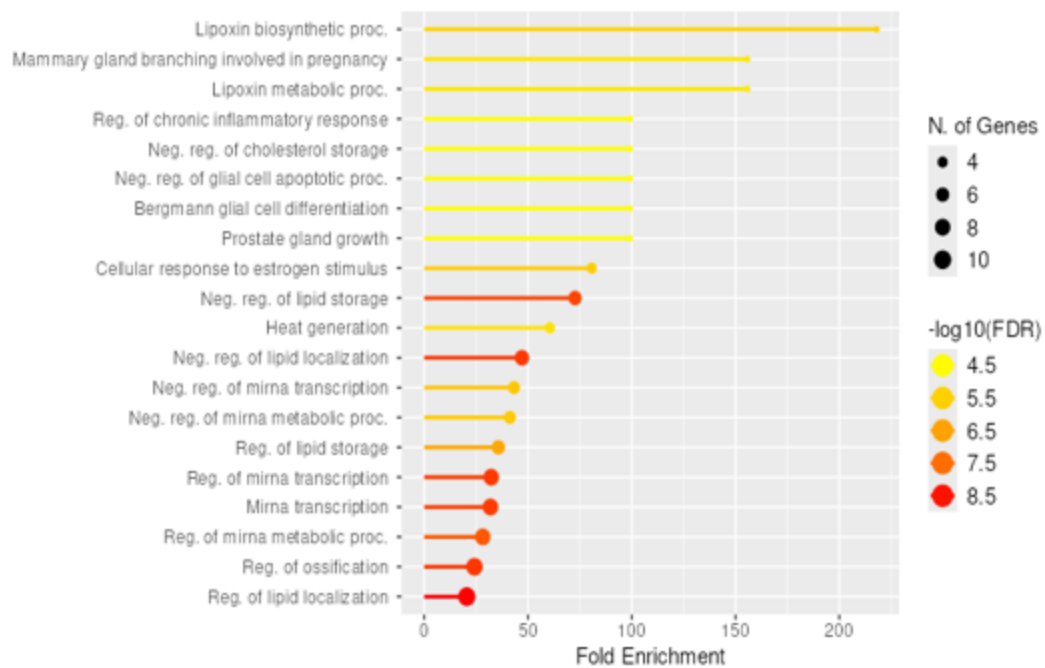
These cellular localizations have mechanistic relevance, in particular to cisplatin neurotoxicity. This enrichment of ER-associated compartments, such as the ER lumen, ER membrane, and nuclear outer membrane ER network indicates that the overlapping targets are likely enriched where ER stress and the unfolded protein response are activated, which is well established in neurons after platinum-based drug exposure. Enrichment of lipid rafts, caveolae, and lipid droplets implicates membrane microdomain-dependent signaling and lipid peroxidation defense as central to astaxanthin's mechanism of action. Moreover, the term neuronal cell body and somatodendritic compartment suggests that the neuroprotective network identified is spatially concentrated in areas most sensitive to cisplatin-induced damage to the axon and the somatodendritic compartment, respectively.

c. Molecular Functions

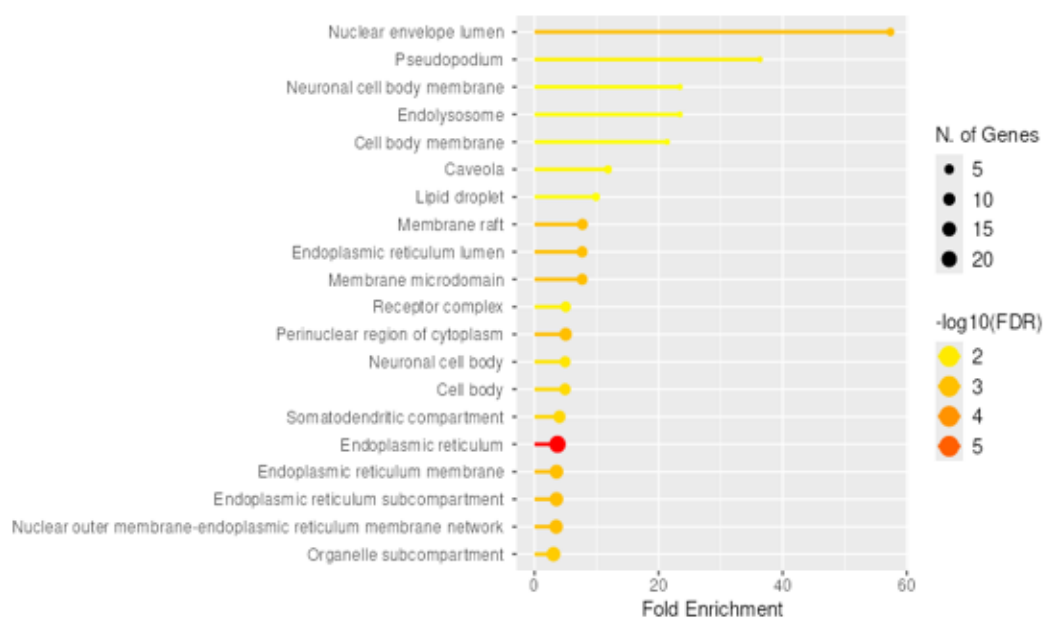
A group of functionally distinct but mechanistically related terms was identified via MF enrichment analysis: Prostaglandin E receptor activity, Cholinesterase activity, Diacylglycerol-dependent calcium-independent serine/threonine kinase activity, Linoleate 13S-lipoxygenase activity, Arachidonate 15-lipoxygenase activity, D3 vitamin binding, Arachidonate 12(S)-lipoxygenase activity, Prostaglandin receptor activity, Dopamine: sodium symporter activity, Norepinephrine: sodium symporter activity, Nuclear estrogen receptor activity, Prostanoid receptor activity, Estrogen response element binding, NFAT protein binding, Icosanoid receptor activity, Interleukin-6 receptor binding, Vitamin D binding, Nuclear steroid receptor activity, Nuclear receptor activity, Ligand-activated transcription factor activity (Figure 4.11C). The significant involvement of eicosanoid-related activities, such as prostaglandin / prostanoid receptor activity and several lipoxygenase isoforms, also supports the central role of arachidonic acid metabolism and inflammatory lipid signaling in the pathogenesis of cisplatin-induced neurotoxicity and therapeutic interference by astaxanthin.

The upregulation of neurotransmitter transporter activities (dopaminergic and noradrenergic sodium symporters) identifies targets within monoaminergic neurotransmission pathways that are commonly altered in chemotherapy-induced cognitive and sensory deficits. Specifically, the enrichment of nuclear receptor

binding, along with nuclear receptor activities such as estrogen, steroid, and ligand-activated transcription factor activities, indicates that astaxanthin may have transcriptional reprogramming effects via hormone-responsive regulatory elements, which regulate genes involved in cellular redox homeostasis, apoptotic resistance, and neuroinflammation.



(A)



(B)



(C)

FIGURE 4.11: Gene Ontology enrichment analysis.

ShinyGO v0.85.1 was used to conduct Gene Ontology (GO) enrichment analysis for the 64 common overlapping targets, presented in three panels: Biological Processes (A), Cellular Components (B), and Molecular Functions (C) with $FDR < 0.05$. Each panel displays a bubble plot of the most enriched words by P-value (high $-\log_{10}(FDR)$ for words within significant categories) and a lollipop plot of fold enrichment (x-axis), number of genes (bubble size), and significance (color gradient).

4.3.1.6 KEGG Pathway Enrichment Analysis

The 64 common targets were obtained by the intersection of the astaxanthin-related targets and the neurotoxicity-associated genes and were then analyzed for pathway enrichment using ShinyGO v0.85.1. Pathways with $FDR < 0.05$ were defined as significantly enriched pathways, and the most highly enriched pathways were sorted by statistical significance and fold enrichment. The analysis indicated that astaxanthin could also have neuroprotective effects via several biological pathways relating to inflammation, oxidative stress, apoptosis, immune regulation, neurotransmission, and cellular survival [4.12](#).

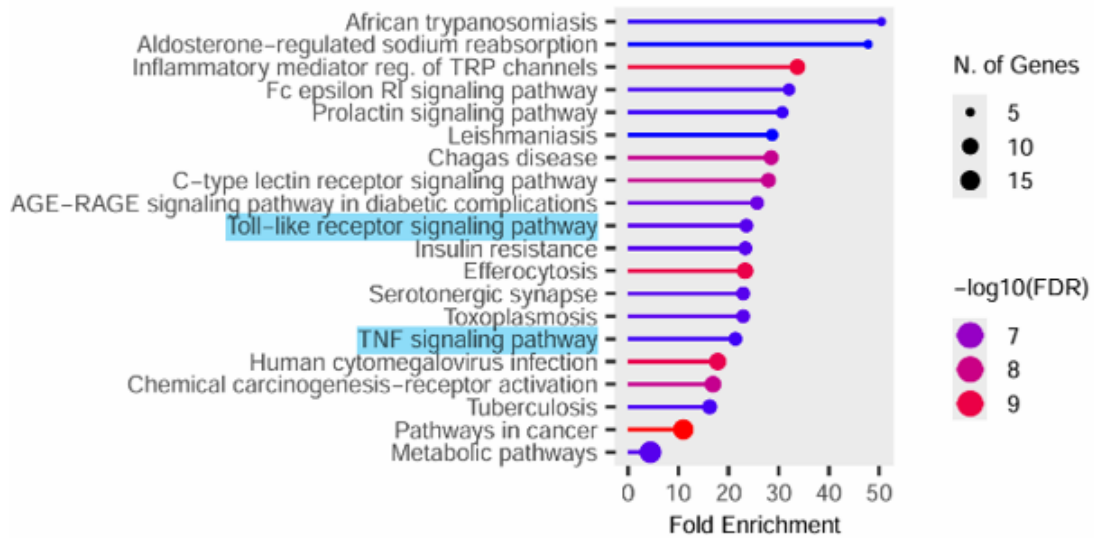


FIGURE 4.12: Lollipop plot of KEGG pathway enrichment.

The lollipop plot of KEGG pathway enrichment analysis of 64 common targets for astaxanthin and neurotoxicity was generated as shown in the above figure. Lollipop size indicates the number of genes enriched, the color indicates the statistical significance (FDR), and the x-axis indicates the fold enrichment. Enrichment of pathways associated with inflammation, immune regulation-associated pathways, TLR signaling, and TNF signaling is highlighted.

The significantly enriched pathway was Pathways in Cancer ($\text{FDR} = 1.65 \times 10^{-10}$; Fold Enrichment = 10.99) with key genes IL6, PPARG, MAPK1, MAPK3, ESR1, ESR2, PRKCA, and NFKBIA.

While designated as a cancer pathway, it also includes multiple signaling pathways that regulate cell proliferation, apoptosis, oxidative stress responses, and inflammatory regulation, which are also linked to neurotoxicity, as shown in Table 4.4.

TABLE 4.4: Top 20 enriched KEGG pathways

Pathway	nGenes	Fold Enrichment	Enrichment FDR
Pathways in cancer	16	10.99	1.65E-10
Inflammatory mediator regulation of TRP channels	9	33.71	6.73E-10
Efferocytosis	10	23.29	1.15E-09

Table 4.4 continued from previous page

Pathway	nGenes	Fold ment	Enrich- ment	Enrichment FDR
Human cytomegalovirus infection	11	17.84		1.39E-09
Chagas disease	8	28.50		1.41E-08
Chemical carcinogenesis-receptor activation	10	16.90		1.41E-08
C-type lectin receptor signaling pathway	8	27.95		1.41E-08
AGE-RAGE signaling pathway in diabetic complications	7	25.69		2.94E-07
Metabolic pathways	19	4.43		4.02E-07
Toll-like receptor signaling pathway	7	23.55		4.02E-07
Serotonergic synapse	7	22.91		4.02E-07
Insulin resistance	7	23.33		4.02E-07
Toxoplasmosis	7	22.91		4.02E-07
Fc epsilon RI signaling pathway	6	32.06		4.93E-07
Tuberculosis	8	16.24		4.93E-07
TNF signaling pathway	7	21.37		5.30E-07
Prolactin signaling pathway	6	30.71		5.65E-07
African trypanosomiasis	5	50.47		5.96E-07
Aldosterone-regulated sodium reabsorption	5	47.81		7.26E-07
Leishmaniasis	6	28.68		7.26E-07

The pathway Inflammatory Mediator Regulation of TRP Channels showed significant enrichment (Fold Enrichment = 33.72) and comprised the genes: MAPK14, PRKCA, PTGER2, PTGER4 and TRPA1, which are associated with modulation of nociception, neuroinflammation and neuronal excitability. Likewise, two of the most significantly enriched pathways were TNF signaling pathway (Fold Enrichment = 21.38) and TLR signaling pathway (Fold Enrichment = 23.55), both of which included the most prominent hub genes, such as those involved in inflammation signaling, including TNF, IL6, MAPK14, MAPK1, MAPK3, and NFKBIA, further emphasizing that the inflammatory signaling pathway is a key mechanism in cisplatin-induced neurotoxicity. The AGE-RAGE signaling pathway in diabetic

complications (Fold Enrichment = 25.70) was also significantly enriched, suggesting an involvement of oxidative stress-mediated cellular damage. This pathway was enriched with genes that have previously been associated with the generation of reactive oxygen species and neuronal injury, including TNF, IL6, MAPK1, MAPK3, MAPK14, PRKCA, and PRKCD.

In addition, enrichment of the Serotonergic Synapse pathway (Fold Enrichment = 22.92) suggests a potential modulation of neurotransmitter signaling mediated by astaxanthin, and pathways like Fc epsilon RI signaling, C-type lectin receptor signaling, and Efferocytosis indicate the involvement of immune and inflammatory mechanisms in the observed neuroprotective effects. Together, these results indicate that the anti-cisplatin neuroprotective mechanism of astaxanthin may be multi-target and multi-pathway, involving inhibition of inflammation mediated by TNF, modulation of MAPK signaling, attenuation of oxidative stress, modulation of immune response, and preservation of neuronal signaling pathways, as shown in Table 4.5.

TABLE 4.5: KEGG pathways enrichment analysis of top 20 pathways

ID	Pathway Name	Count	Genes	Enrichment FDR
hsa05200	Pathways in cancer	16	ESR1, ESR2, IL6, AR, NFKBIA, NOS2, PPARG, PRKCA, MAPK1, MAPK3, PTGER1, PTGER2, PTGER3, PTGER4, RARA, TERT	1.65E-10
hsa04750	Inflammatory mediator regulation of TRP channels	9	MAPK14, ALOX12, HTR2B, PRKCA, PRKCD, PRKCH, PTGER2, PTGER4, TRPA1	6.73E-10
hsa04148	Efferocytosis	10	MAPK14, ALOX5, ALOX15, PPARG, MAPK1, MAPK3, PTGER2, PTGER4, PTPN11, ADAM17	1.15E-09
hsa05163	Human cytomegalovirus infection	11	MAPK14, IL6, NFKBIA, PRKCA, MAPK1, MAPK3, PTGER1, PTGER2, PTGER3, PTGER4, TNF	1.39E-09
hsa05142	Chagas disease	8	MAPK14, IL6, NFKBIA, NOS2, TLR9, MAPK1, MAPK3, TNF	1.41E-08

Table 4.5 continued from previous page

ID	Pathway Name	Count	Genes	Enrichment FDR
hsa05207	Chemical carcinogenesis-receptor activation	10	ADRB2, ESR1, ESR2, AR, PPARA, PRKCA, MAPK1, MAPK3, VDR, CDC25A	1.41E-08
hsa04625	C-type lectin receptor signaling pathway	8	MAPK14, IL6, NFKBIA, PRKCD, MAPK1, MAPK3, PTPN11, TNF	1.41E-08
hsa04933	AGE-RAGE signaling pathway in diabetic complications	7	MAPK14, IL6, PRKCA, PRKCD, MAPK1, MAPK3, TNF	2.94E-07
hsa01100	Metabolic pathways	19	CYP17A1, CYP19A1, FDFT1, AKR1B1, ALOX12, ALOX5, ALOX15, G6PD, GLUL, HMGCR, HSD11B1, HSD11B2, IDO1, NOS2, PDE4D, PLA2G1B, HAO1, SRD5A1, PTGES	4.02E-07
hsa04620	Toll-like receptor signaling pathway	7	MAPK14, IL6, NFKBIA, TLR9, MAPK1, MAPK3, TNF	4.02E-07
hsa04726	Serotonergic synapse	7	ALOX12, ALOX5, ALOX15, HTR2B, PRKCA, MAPK1, MAPK3	4.02E-07
hsa04931	Insulin resistance	7	IL6, NFKBIA, PPARA, PRKCD, PTPN1, PTPN11, TNF	4.02E-07
hsa05145	Toxoplasmosis	7	MAPK14, ALOX5, NFKBIA, NOS2, MAPK1, MAPK3, TNF	4.02E-07
hsa04664	Fc epsilon RI signaling pathway	6	MAPK14, ALOX5, PRKCA, MAPK1, MAPK3, TNF	4.93E-07
hsa05152	Tuberculosis	8	MAPK14, IL6, NOS2, TLR9, MAPK1, MAPK3, TNF, VDR	4.93E-07
hsa04668	TNF signaling pathway	7	MAPK14, IL6, NFKBIA, MAPK1, MAPK3, ADAM17, TNF	5.30E-07
hsa04917	Prolactin signaling pathway	6	MAPK14, CYP17A1, ESR1, ESR2, MAPK1, MAPK3	5.65E-07
hsa05143	African trypanosomiasis	5	IL6, IDO1, TLR9, PRKCA, TNF	5.96E-07
hsa04960	Aldosterone-regulated sodium reabsorption	5	HSD11B2, NR3C2, PRKCA, MAPK1, MAPK3	7.26E-07
hsa05140	Leishmaniasis	6	MAPK14, NFKBIA, NOS2, MAPK1, MAPK3, TNF	7.26E-07

a. Toll-Like Receptor Signaling Pathway

TLR signaling pathway was identified as a significantly enriched KEGG pathway among the common overlapping molecular targets of astaxanthin and cisplatin-induced neurotoxicity. Six key nodes are drawn in red, indicating their central role in this neuroinflammatory pathway, as shown in the pathway diagram (Figure 4.13): TLR9, TNF- α , IL-6, I κ B α , ERK, and p38. The red rectangles represent the key genes/proteins with high enrichment scores in the network pharmacology analysis of the targets of astaxanthin. These include critical nodes, like TLR9, TNF- α , IL-1 β , IL-6, and downstream effectors in NF- κ B, MAPK, and IRF pathways.

As for cisplatin-induced neurotoxicity, cellular stress induces DAMP release, leading to activation of TLR9, which activates two important downstream arms via TRAF6 and TAK1 in the MyD88-dependent cascade. In the NF- κ B arm, the phosphorylation and degradation of I κ B α allows NF- κ B to stimulate the upregulation of the transcription of TNF- α and IL-6, which contributes to neuroinflammation, glial activation, and neuronal apoptosis. At the same time, the MAPK arm activates phosphorylation of ERK and p38, which increases the expression of inflammatory genes and carries out pro-apoptotic programs in injured neurons exposed to cisplatin.

Astaxanthin acts at multiple levels of the pathways, including stabilizing I κ B α to inhibit nuclear translocation of NF- κ B, decreasing inflammatory amplification through the MAPK pathway, and dampening damage to the neurons through a reduction in the production of cytokines. All these nodal modulations of TLR9-initiated signaling mechanisms provide compelling mechanism-based evidence of the neuroprotective efficacy of astaxanthin against cisplatin-induced neuroinflammation and oxidative injury.

b. TNF Signaling Pathway

The TNF signaling pathway was identified as a significantly enriched KEGG pathway among the common overlapping molecular targets of astaxanthin and cisplatin-induced neurotoxicity.

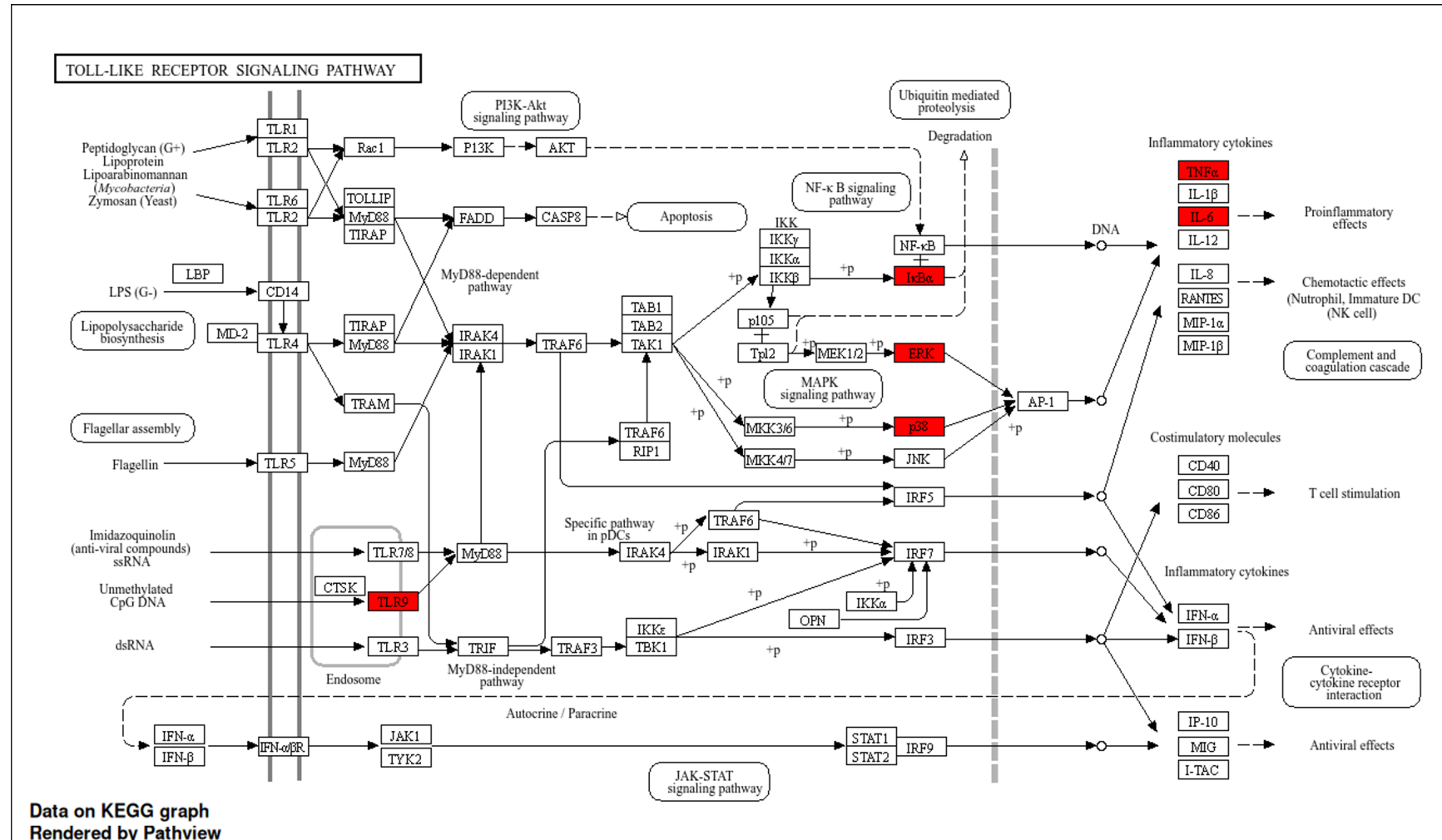


FIGURE 4.13: Toll-Like Receptor Signaling Pathway (visualized in the KEGG database via Pathview).

The nodes highlighted in the pathway diagram (Appendix I) are put in the spotlight, including the following: TNFR1, TNFR2, TNF, TACE, IL-1 β , IL-6, I κ B α , ERK1/2, and p38, which are located in both arms of the pathway, emphasizing their roles in the development of cisplatin-induced neuroinflammation and neuronal injury.

In cisplatin-induced neurotoxic conditions, TNF is proteolytically released to trigger the activity of TNFR1 through two important arms: the TRADD-TRAF2 / 5-TAK1 and the second involving TRAF3. In the NF- κ B arm, IKK phosphorylation and degradation of I κ B α frees NF- κ B to upregulate IL-1 β and IL-6, leading to production of pro-inflammatory cytokines, activation of glia, and neuronal apoptosis.

At the same time, activation of the MAPK arm also promotes the phosphorylation of ERK1/2 and p38, leading to an increase in the neuroinflammatory signaling pathway and the execution of apoptotic pathways in damaged neurons. In addition, TNFR1 activates a complex of RIP1, FADD, and CASP8/10 (the ripoptosome) and RIP1, RIP3, and MLKL (the necrosome), linking inflammatory signaling to apoptotic and necroptotic neuronal death.

In the TNFR2 arm, convergent highlighting of the target proteins I κ B α and p38 indicates that the targets of astaxanthin are shared on both receptor arms, and that the TRAF1/2/3-NIK-IKK ϵ and MKK3, p38 signaling pathways also influence neuronal survival decisions.

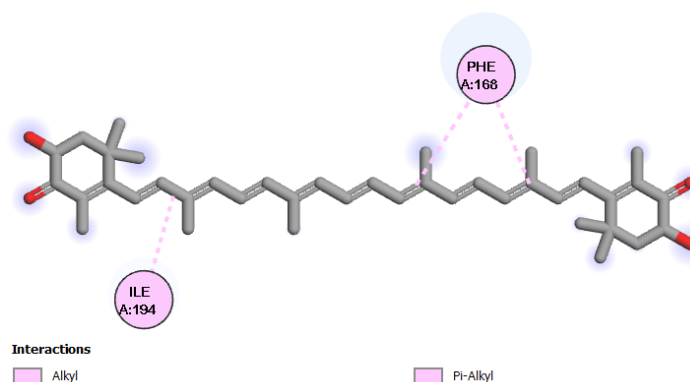
Astaxanthin acts at multiple points in both the TNFR1 and TNFR2 pathways, upstream, midstream, and effector levels, thus delivering mechanistically coherent, multi-layered neuroprotection from cisplatin-induced neuroinflammation, apoptosis, and necroptosis.

The red rectangles represent the key genes/proteins with high enrichment scores in the network pharmacology analysis of the targets of astaxanthin. These include critical nodes, like TNFR1, TNFR2, TNF, TACE, IL-1 β , IL-6, I κ B α , ERK1/2, and p38, which are located in both arms of the pathways.

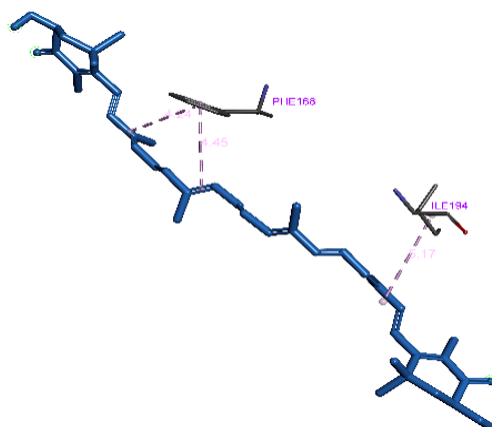
4.3.2 Molecular Docking Analysis of Astaxanthin with Core 6 Hub Proteins

To validate the network pharmacology results and verify the multi-target binding potential of astaxanthin in relation to cisplatin-induced neurotoxicity, molecular docking studies were done using AutoDock Vina integrated in PyRx on the six most important core hub proteins: IL6, TNF, PPARG, MAPK14, MAPK3, and MAPK1. The proteins were prioritized based on their key functions in neuroinflammation (IL6, TNF), MAPK signaling and oxidative stress response (MAPK14, MAPK3, MAPK1), and metabolic regulation (PPARG) as shown in the PPI network, GO/KEGG enrichment, and drug-target-pathway network. The binding affinities from all docking simulations were negative, indicating stable, thermodynamically favorable interactions. These binding energies ranged from -7.1 to -9.8 kcal/mol, indicating a strong affinity for the protein due to the lipophilic nature of carotenoid pigments and the potential for multiple hydrophobic interactions and hydrogen bonding within the protein's active pockets, as shown in the figure 4.14. The results are consistent with the strong antioxidant and anti-inflammatory bioactivity of astaxanthin and suggest that it may be able to interact with the inflammatory, oxidative stress, and apoptotic pathways that are activated by cisplatin neurotoxicity.

1. Protein: IL6 PDB ID: 1N26

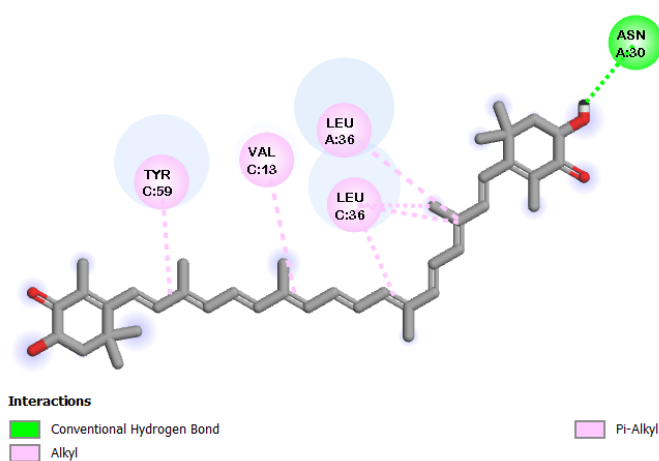


(2D)

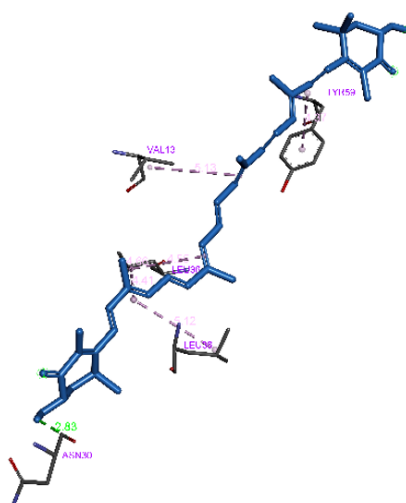


(3D)

2. Protein: TNF PDB ID: 2AZ5

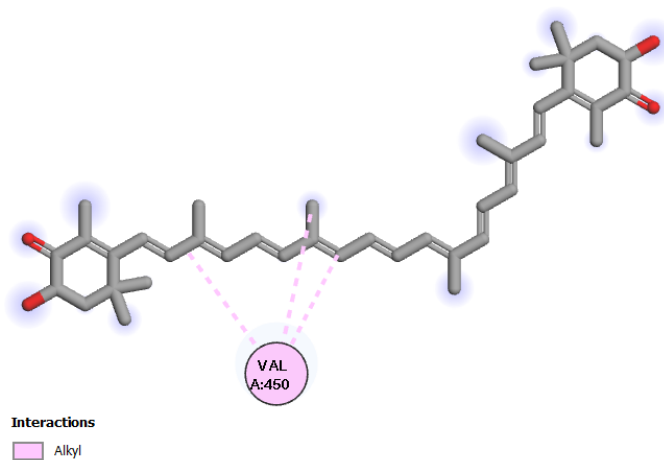


(2D)

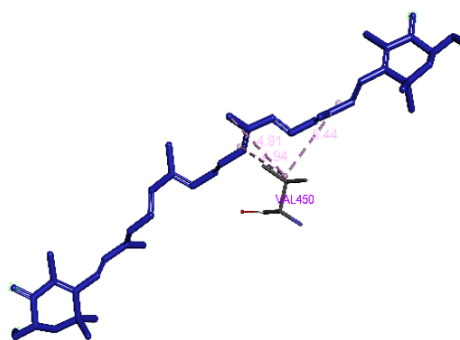


(3D)

3. Protein: PPARG PDB ID: 6MS7

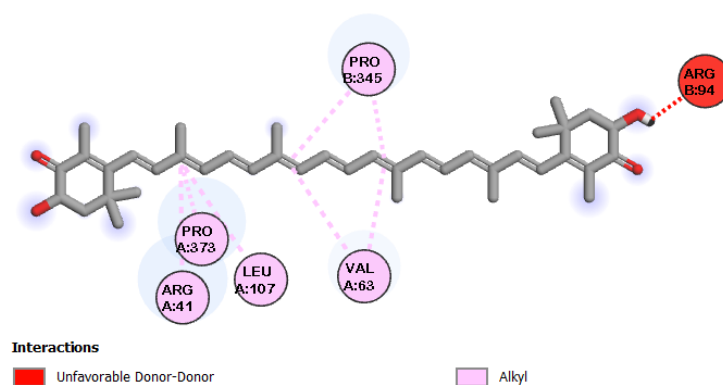


(2D)

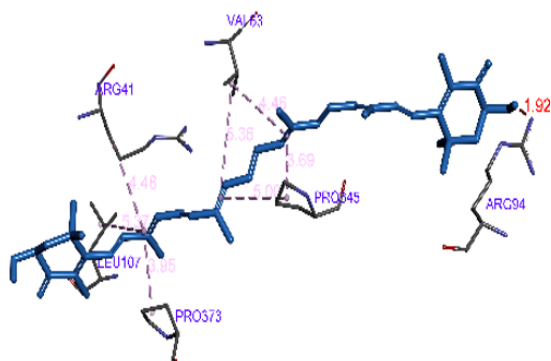


(3D)

4. Protein: MAPK3 PDB ID: 2ZOQ

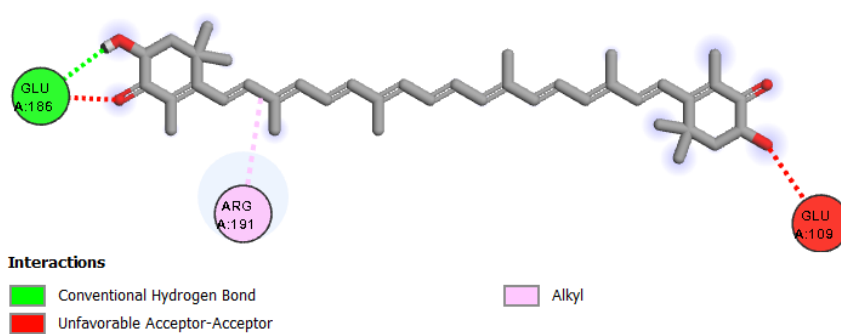


(2D)

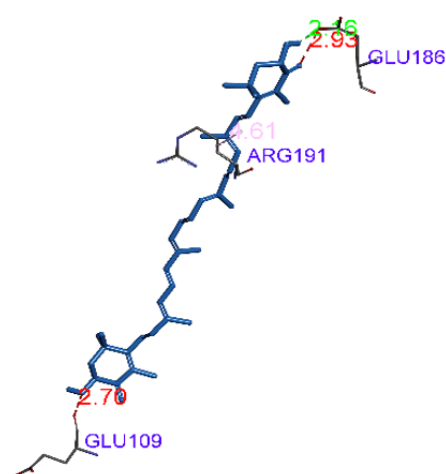


(3D)

5. Protein: MAPK1 PDB ID: 1TVO



(2D)



(3D)

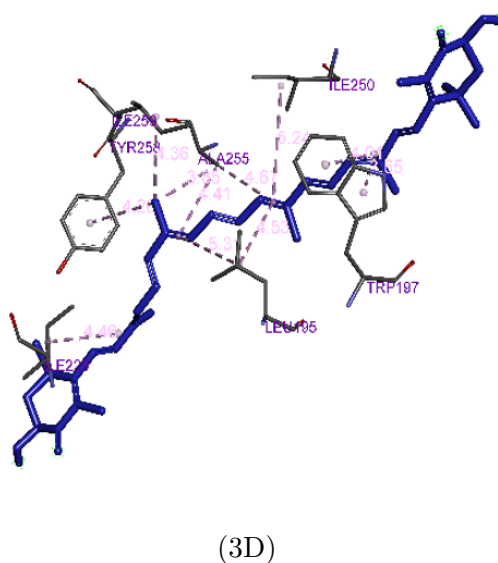
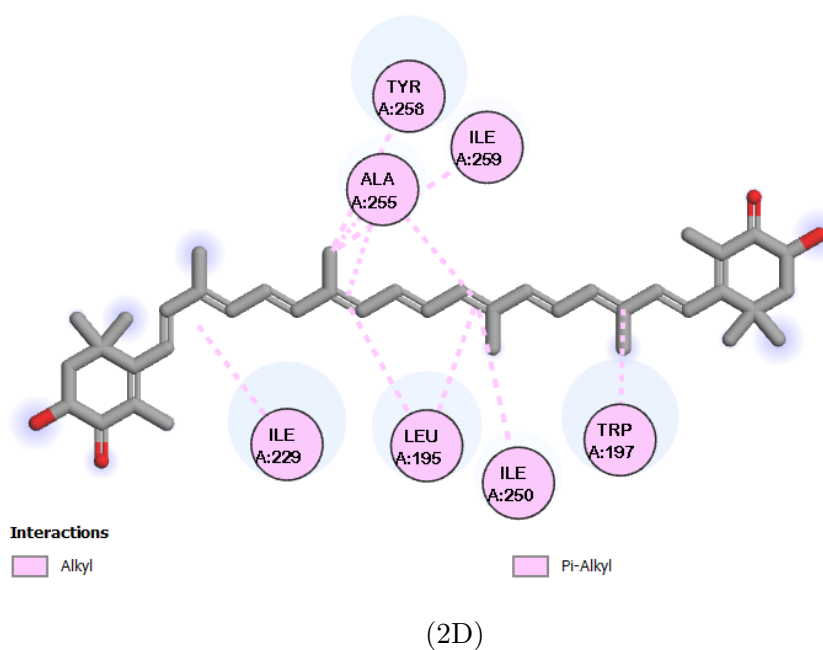
6. Protein: MAPK14 PDB ID: 5ML5

FIGURE 4.14: Molecular Docking visualization of Astaxanthin binding with six key hub proteins

Molecular docking visualization of Astaxanthin binding with the six key hub proteins. IL6 (1N26, -7.1 kcal/mol), TNF (2AZ5, -9.8 kcal/mol), (C) PPARG (6MS7, -7.6 kcal/mol), (D) MAPK14 (5ML5, -7.8 kcal/mol), (E) MAPK3 (2ZOQ, -7.8 kcal/mol), (F) MAPK1 (1TVO, -9.1 kcal/mol). Left panels show 2D binding poses; right panels show 3D interaction diagrams.

The complexes demonstrate stable binding primarily mediated by hydrophobic (alkyl and pi-alkyl) interactions along with conventional hydrogen bonds with key residues. The images validate the multi-target neuroprotective properties of astaxanthin against cisplatin-induced neurotoxicity by targeting the binding pockets of various key proteins involved in inflammation, stress response, and signaling processes. The strongest binding affinity was observed for Astaxanthin for TNF (-9.8 kcal/mol) and MAPK1 (-9.1 kcal/mol). Such high affinities underscore its remarkable ability to target central players of neuroinflammation and MAPK-mediated stress signal transduction. The docking results confirmed the network pharmacology predictions since the binding of astaxanthin is energetically favorable and direct to the identified hubs. The interactions are mainly hydrophobic and hydrogen-bonding, and the results indicate that astaxanthin can be used to inhibit pro-inflammatory cytokine signaling pathways (TNF, IL6) and MAPK-mediated pathways of oxidative stress and apoptosis involved in the neurotoxicity effect of cisplatin. The results are in line with the known neuroprotective effects of astaxanthin and offer robust computational support for its therapeutic potential. The detailed docking parameters such as key interacting residue, bond length, and binding affinity are summarized in Table 4.6.

TABLE 4.6: Molecular Docking: Binding Properties of Astaxanthin with 6 Core hub proteins

Protein	PDB ID	Residues involved in bonding	Bond length (Å)	Binding Interactions	Binding Affinity (kcal/mol)
IL6	1N26	PHE168, ILE194	4.24, 4.45, 5.17	Pi-Alkyl, Alkyl	-7.1
TNF	2AZ5	ASN30, TYR59, VAL13, LEU36	2.83 (H-bond), 4.37, 4.41, 4.55, 5.12, 5.13	Conventional Hydrogen Bond, Pi-Alkyl, Alkyl	-9.8
PPARG	6MS7	VAL450	4.91, 4.94, 5.44	Alkyl, Pi-Alkyl	-7.6
MAPK14	5ML5	TYR258, ILE259, ALA255, ILE229, LEU195, ILE250, TRP197	3.65, 4.01, 4.36, 4.41, 4.46, 4.65, 5.24, 5.31	Pi-Alkyl, Alkyl, Hydrophobic	-7.8
MAPK3	2ZOQ	ARG94, ARG41, LEU107, VAL63, PRO373, PRO345	1.92, 3.69, 3.95, 4.46, 4.48, 5.00, 5.36, 5.37	Pi-Alkyl, Alkyl, Unfavorable Donor-Donor	-7.8
MAPK1	1TVO	GLU186, GLU109, ARG191	2.46, 2.70, 2.93 (H-bonds), 4.61	Conventional Hydrogen Bond, Alkyl	-9.1

Chapter 5

Discussion

Our study focuses on the neuroprotective properties of astaxanthin against cisplatin - induced neurotoxicity, as evident from behavioral tests, biochemical studies, histopathological analysis, and computational methods. The results showed that astaxanthin possesses several action mechanisms for the effect of reducing cisplatin-induced neurotoxicity, the highest dose level (25 mg/kg) being the most effective. The behavioral tests in this work showed that cisplatin administration caused significant motor deficits in distance travelled in the OFT, but not in the Kondziela IST for muscle strength and coordination. This type of neurotoxicity is similar to previous studies, where currently used doses of cisplatin did not cause a prominent effect on peripheral neuromuscular function, but did affect dopaminergic/motor pathways. The hypolocomotion observed is continuous, as stated by Grimmig *et al.* (2017), who confirmed that neurotoxic insults in mice lead to decreased locomotor activity and that astaxanthin supplementation can reverse these deficits [181]. The dose-dependent protection of astaxanthin in our study is consistent with the study done by Grimmig *et al.* (2015), in which they showed that astaxanthin supplementation improves cognitive function in the hippocampus and spatial memory in mice [182]. Significantly, muscle strength and coordination were preserved during the IST, which suggests that the neurotoxicity from the administered dose of cisplatin is not peripheral, but rather CNS neurotoxicity,

according to the results of Wu *et al.* (2015), who showed that the neuroprotective effect of astaxanthin in CNS architectures is higher than in peripheral sites [183].

The H&E staining results show that the administration of cisplatin caused significant histopathological changes in the hippocampus, such as loss of neurons, vacuolation, karyolysis, and disturbance of the hippocampal architecture in various regions. The results agree with the known neurotoxicity of cisplatin on the hippocampus and with previous studies reporting damage to the hippocampus after chemotherapy in patients that has been associated with cognitive dysfunction. The protective action of astaxanthin (25 mg/kg) against the architecture of the hippocampus is significant and similar to mecobalamin (+ve ctrl grp), and indicates that astaxanthin could have neuroprotective effects equivalent to those of other drugs. The preservation of cellular organization and neuronal density in the astaxanthin-treated group agrees with Lu *et al.* (2015), who report that, suggesting that astaxanthin's neuroprotective effects have been extended to other types of neuronal injuries [184]. Our histopathological observations also corroborated the results of Lobos *et al.* (2016), who revealed that astaxanthin is able to save main hippocampal nerve cells from the noxious effects of A β -oligomers, indicating that the neuroprotective effects of astaxanthin could be direct protection of hippocampal neuronal structures from various toxicities [185]. The organization of dentate gyrus and the preservation of pyramidal cell layers in CA1 and CA3 areas are of particular importance due to the role these areas play in learning, memory and cognitive processing, and are morphologically relevant to the behavioral improvements seen in our study, which is uniform with the outcomes of Grimmig *et al.*, (2019) that astaxanthin was found to modify perceptive function and synaptic elasticity in young and aged mice [182].

Cisplatin therapy was found to significantly raise pro-inflammatory cytokines (TNF- α and IL-1 β) in both brain areas (hippocampus and cerebellum), as shown by ELISA results obtained in our study. This finding is in line with the known neuroinflammation that occurs in CIPN, and with previous studies that reported an inflammatory response after chemotherapy using platinum-based drugs. The marked decrease in total protein content seen in the hippocampus and cerebellum

with cisplatin treatment indicates that there is significant neuronal damage and loss of synapses, which again is steady with earlier neurotoxicity studies that have revealed the neurotoxic effects of cisplatin on CNS structures. The decreasing effects of TNF- α and IL-1 β upon astaxanthin dose in our study are definitely in favor of the anti-inflammatory properties of this compound and, as such, give biochemical proof for the therapeutic potential. The present outcomes were steady with the findings of Kim *et al.* (2010), which showed that astaxanthin suppresses the production of IL-6 in activated microglia by inhibiting ERK-, MSK-, and NF- κ B-mediated signal transduction and immunoinflammatory effects [186]. In addition, our results are reliable with those of Ye *et al.* (2012) in showing that astaxanthin can suppress MPP+-induced oxidative stress in PC12 cells through the HO-1/NOX2 axis, suggesting that astaxanthin can regulate inflammatory response in neuronal cells by multiple interconnected pathways [187].

The improvement of total protein in the hippocampus and cerebellum following astaxanthin treatment indicated maintenance of the integrity of the neurons and protein homeostasis, which is necessary for normal neuronal functions and synaptic transmission. This outcome is in accordance with the account of Grimmig *et al.* (2019) on the ability of astaxanthin to reduce neurotoxicity in aged Parkinson's disease mouse models while maintaining protein expression of neurons, indicating that the neuroprotective activity of astaxanthin is similar across models of neuronal injury [182]. The biochemical evidence (BCA assay) obtained in our study is in addition to the aforementioned behavioral and histopathological data, which further support the multi-modal protective effect of astaxanthin. These findings on the effect of astaxanthin on behavior further validate the clinical relevance of neuroprotective activity.

Oxidative stress is a major mechanism underlying cisplatin-induced neurotoxicity. In our study, hippocampal NO concentrations and MDA levels were significantly affected by the administration of cisplatin (2.3 mg/kg) with a significant increase of NO and MDA levels as compared with normal controls, suggesting that neuronal membranes were severely damaged by nitrosative and oxidative stress. This is steady with the outcomes of Chtourou *et al.* (2015) who found that treatment

with cisplatin greatly increased the levels of MDA, NO, ROS, protein carbonyls, and TNF- α in the striatum of rats, and that this led to inflammation and programmed cell death through NF- κ B and p53 pathways. As in their model, behavioral deficits, as well as neuronal degeneration in the hippocampus, accompanied our biochemical changes [188].

Pretreatment with mecobalamin significantly reduced both markers; astaxanthin (25 mg/kg) even produced even greater reductions ($\sim 65\%$ for NO and $\sim 60\%$ for MDA), bringing levels closer to normal. The hepatoprotective effect of astaxanthin on CCl₄-induced rats by Islam *et al.*, 2017 is similar to the protection results on MDA and NO, the restoration of SOD and CAT activities, the inhibition of inflammation, and improvement of liver tissue histopathology [189].

Likewise, in another study by Xuan *et al.* (2016), astaxanthin markedly reduced MDA and ROS production, reduced the expression of NF- κ B and caspase-3, and increased the expression of the HO-1 in the L-NAME induced preeclampsia model, protecting against placental inflammation and oxidative damage [190]. The consistent results of the various studies indicate that astaxanthin can break lipid peroxidation chains, scavenge ROS/RNS, and stabilize membranes, all mechanisms which are directly related to our hippocampal results. These observations are corroborated by our network pharmacology analysis (implicating pathways such as the TNF, MAPK and AGE-RAGE pathways) and molecular docking data (significant binding potential to TNF, MAPKs, etc.). In conclusion, the neuroprotective activity of astaxanthin in our cisplatin model is consistent with, and supports, the anti-oxidant and anti-inflammatory properties described in these studies, further supporting the ability of astaxanthin to maintain the integrity of neurons in chemotherapy-induced oxidative stress.

Based on the results of the in-silico analysis in our study, 64 common genes between the astaxanthin targets and the genes associated with neurotoxicity were found, offering a framework for the neuroprotective effects of astaxanthin and supporting the idea that it acts through several molecular pathways. The results indicated that the identified targets were highly interconnected, having a network diameter of 6 and a distinctive path length of 2.243, which suggested efficient

signal transmission throughout the network and that the identified targets may be an integrated system rather than discrete molecular entities. The multi-target pharmacological properties of astaxanthin, as described by Wu *et al.* (2015), who reviewed the neuroprotective capacity of astaxanthin in several neurological diseases and pointed out its ability to act on several pathological pathways at the same time, are steady with the outcomes of this study [183].

Through centrality analysis, 13 hub genes (TNF, IL6, PPARG, MAPK3, MAPK1, ESR1, MAPK14, NR3C1, HMGCR, PPARA, PRKCA, ESR2, and ADRB2) were identified as the key genes that are associated with the neuroprotective effects of astaxanthin and offer rational explanations of the underlying mechanisms of its therapeutic action. The number of high degree values of TNF and IL6 (38 interactions for each) further highlights the importance of inflammatory signalling and the possibility of regulating these pathways by astaxanthin, which was reflected in the biochemical data obtained from the ELISAs. The anti-inflammatory effect of astaxanthin is consistent with that of Zhou *et al.* (2015), which showed that astaxanthin can inhibit inflammation in diabetic mice and relieve cognitive impairment [191].

The GO enrichment analysis showed strong enrichment of biological processes associated with lipoxin biosynthesis, regulation of chronic inflammatory reaction, and negative regulation of the apoptotic method of glial cells, indicating the particular cellular functions that are modulated by astaxanthin. Intriguingly, the enrichment of genes related to lipid mediators of anti-inflammatory resolution, such as lipoxins, is especially noteworthy because these endogenous mediators are pivotal for inflammatory resolution and tissue repair, thereby highlighting astaxanthin's ability not only to dampen inflammatory responses but also to promote inflammatory resolution and tissue repair. The highest contribution of terms involved in the inhibition of glial apoptosis and the induction of Bergmann cell differentiation by astaxanthin further suggests that astaxanthin preserves the viability and structural integrity of glial cells during cytotoxic stress induced by cisplatin, playing a crucial role in maintaining neuronal viability. These results are consistent with the well-known anti-inflammatory and neuroprotective properties of astaxanthin

and are in line with the review by Galasso *et al.* (2018), which suggested that astaxanthin can act as a neuroprotective agent in different neurological disorders [144].

The KEGG pathway enrichment analysis described various important enriched pathways, including the TLR signaling pathway and the TNF signaling pathway, which play an important part in neuroinflammation and neuronal damage, respectively, and are of special interest in the reference of cisplatin-induced neurotoxicity. Visualization of these pathways with key nodes highlighted (TLR9, TNF- α , IL-6, I κ B α , ERK, and p38) gives insight into the mechanism of anti-inflammatory modulation of these pathways by astaxanthin, while also revealing that this compound acts at multiple levels within these pathways to promote neuroprotection. The discovery of the AGE-RAGE signaling pathway in diabetic complications indicates that there is a role for oxidative stress-induced cellular damage in the neurotoxic process, a mechanism similar to the one known to be involved in the neurotoxicity of cisplatin. The results are aligned with Kim *et al.* (2010) that astaxanthin downregulates the release of pro-inflammatory cytokines via NF- κ B-mediated signals in activated microglia, and provide a comprehensive molecular framework to understand the neuroprotective mechanisms of astaxanthin [186].

The molecular docking outcomes revealed that astaxanthin exhibited high binding activities with the six core hub proteins, whose binding energy ranges from -7.1 to -9.8 kcal/mol, indicating that the molecular docking data were in good agreement with the results predicted by the network pharmacology. Among the proteins tested, the highest binding affinity to astaxanthin was found for TNF (-9.8 kcal/mol) and MAPK1 (-9.1 kcal/mol), indicating a specific affinity for proteins associated with neuroinflammation and MAPK-mediated stress signal transduction, both of which show a main role in the pathobiology of cisplatin-induced neurotoxicity. These findings confirm the results predicted by network pharmacology and indicate that astaxanthin has a multi-target neuroprotective effect, which means that astaxanthin can interact directly with important molecular targets related to the neurotoxic cascade. The binding interactions are dominated by hydrophobic (alkyl and pi-alkyl) interactions and hydrogen bonds, which are typical features of

carotenoid pigments, and offer some clues about the molecular foundation of the anti-neurodegenerative properties of astaxanthin. Astaxanthin binds strongly to TNF and MAPK1, which might explain how astaxanthin has been demonstrated to decrease levels of pro-inflammatory cytokines and to preserve the structure of neurons, and could be a mechanism for these effects. The results are consistent with Wang *et al.* (2010), who showed that astaxanthin regulates the expression of heme oxygenase-1 via the ERK1/2 pathway, and that this regulation results in protection against β -amyloid-induced cytotoxicity [192]. These findings are in line with the concept that astaxanthin directly interacts with some key molecules in the neuroprotective pathway.

A comparison of our results to those in which aged animals were used to investigate the effect of astaxanthin indicates significant implications for age-related susceptibility to neurotoxicity and the effectiveness of agents that are neuroprotective. It is worthwhile to note that there are age-related physiological changes that influence the response to therapeutic interventions, as Grimmig *et al.* (2017) showed that astaxanthin has a better neuroprotective effect in young mice compared to aged mice. This elevated vulnerability of the aged animal to neurotoxic insult and the different microglial response to treatment highlights the importance of dosing and therapeutic strategies appropriate to the animal's age, which would imply that higher doses or longer treatment duration might be required in older and/or compromised animals [181]. The results of our study on cisplatin-induced neurotoxicity also indicate that astaxanthin's protective effects could be affected by the organism's age and physiological state, as well as the dose; the highest dose (25 mg/kg) showed the most beneficial effects, suggesting that in more challenging physiological conditions, higher doses may be required.

Comparing astaxanthin to the positive control (mecobalamin) in our study, both agents appear to have neuroprotective effects, albeit in different ways. Astaxanthin at the dosage of 25 mg/kg yielded a similar or higher degree of protection against cisplatin-induced neurotoxicity in several parameters, such as restoration of hippocampal architecture and reduction of pro-inflammatory cytokine levels, whereas moderate protection was observed in the case of mecobalamin. This is

in accordance with the study of Al-Amin *et al.* (2016) that showed that astaxanthin could improve the spatial memory loss and oxidative stress in the neurons induced by aluminum chloride, and may therefore serve as a promising alternative or complementary agent for neuroprotection [193].

The multi-target pharmacological properties found during our network pharmacology analysis recommend that the neuroprotective activities of using astaxanthin may be broader than those of single-target agents, while the neuroprotective effects of astaxanthin could be more effective in certain parameters due to its ability to affect multiple aspects of inflammatory signaling, as well as responses to oxidative stress and apoptotic pathways.

The comprehensive evidence from our study allows the possible clinical use of astaxanthin as a neuroprotective agent for chemotherapy-induced neurotoxicity. As reviewed by Wu *et al.* (2015), astaxanthin has a good safety profile, and it passes the BBB, which makes it a potential option for neuroprotection in patients with cancer who are undergoing cisplatin-based chemotherapy [183].

The translation from the preclinical to the clinical setting, however, takes into account the dosing regimens, the timing of the administration, and possible interactions with other chemotherapeutic drugs, as these interactions can be complex in patients being treated with several drugs. Future work should look into how much and when astaxanthin should be given during cisplatin-based chemotherapy, and consider the impact that astaxanthin might have on the anti-tumor activity of cisplatin. Furthermore, the neuroprotective effects of astaxanthin should be evaluated in patients with neurological diseases, since various metabolic and drug disposition changes with age could impact the pharmacokinetics and pharmacodynamics of astaxanthin. Astaxanthin is a potent antioxidant that may be used as a nutritional supplement for cancer prevention or reduction of chemotherapy-induced neurotoxicity, especially for the use of platinum-based chemotherapy drugs for cancer treatment. More long-term safety studies and well-made medical trials are desired to establish its clinical usefulness. Overall, our study offers broad support for the neuroprotective role of astaxanthin against cisplatin-induced neurotoxicity, with dose-dependent effects on behavior, neuroinflammation, neuronal

architecture, and multi-target modulation of molecular pathways. The study outcomes support the potential use of astaxanthin as a neuroprotective agent in the context of chemotherapy-induced neurotoxicity, but there is a need for further clinical studies to prove its efficiency and safety in humans.

5.1 Limitations

Although the neuroprotective activity of astaxanthin against cisplatin-induced neurotoxicity has been shown to be promising, there are several limitations.

First, the study was done only in a murine model, and so the results may not fully capture the complex pathophysiological processes in human patients who undergo cisplatin chemotherapy. Second, the sample size was relatively small, possibly limiting the ability to generalize the results.

Third, the investigation was mainly focused on the behavioral performance, histopathological alterations, pro-inflammatory cytokines, oxidative stress parameters, network pharmacology and molecular docking, which did not include other important biochemical markers involved in apoptosis, mitochondrial impairment and neurotransmitter imbalance.

Fourthly, the targets and pathways that were predicted using network pharmacology and molecular docking were not validated by gene expression or protein expression studies, including RT-PCR, Western blotting, or immunohistochemistry. Besides, the study was conducted over a short period of experimentation, and the effect of astaxanthin on chronic cisplatin exposure on the neurological system has not been examined.

Last but not least, the pharmacokinetic parameters, brain bioavailability, and the possible interactions between astaxanthin and cisplatin were not evaluated. Hence, more mechanistic and translational research is needed to completely evaluate the therapeutic efficacy of astaxanthin in the treatment of chemotherapy-induced neurotoxicity.

Chapter 6

Conclusion and Future Perspective

6.1 Conclusion

In the present study, the neuroprotective activity of astaxanthin (ASTA) against cisplatin-induced neurotoxicity was evaluated using behavioral, histopathological, biochemical, network pharmacology, and molecular docking approaches. Behavioral tests showed that treatment with cisplatin was effective in inducing behavioral changes such as locomotor activity and motor coordination deficits. These behavioral deficits were found to be remarkably and significantly improved in a dose-dependent fashion with pretreatment using 25 mg/kg of astaxanthin, which showed effects similar to or greater than those of mecobalamin. Histopathological analysis of the hippocampus showed increased neuronal degeneration, disorganization of cells, vacuolation, and karyolysis after cisplatin injection. Astaxanthin, in contrast, maintained the architecture of the neurons, prevented neurodegenerative changes, and enhanced neuronal survival, suggesting significant neuroprotection from cisplatin-induced structural damage. Neuroinflammation was also found to play a role in the neuronal damage caused by cisplatin, as biochemical analysis showed a greater raise in pro-inflammatory cytokines in both hippocampal and cerebellar tissues. The levels of all these inflammatory mediators were

significantly decreased after the Astaxanthin treatment, reflecting strong anti-inflammatory activity. In addition to reducing neuroinflammation, astaxanthin effectively attenuated oxidative stress induced by cisplatin, as demonstrated by the significant reduction in MDA and NO levels in the hippocampus. Cisplatin administration markedly increased lipid peroxidation and nitrosative stress, reflecting excessive ROS generation and neuronal oxidative damage. Pretreatment with astaxanthin (25 mg/kg) greatly reduced these oxidative stress biomarkers to normal ranges, indicating potent antioxidant activity. Network pharmacology analysis indicated several common targets and several hub genes, such as TNF, IL6, PPARG, MAPK14, MAPK3, and MAPK1, suggesting astaxanthin may act by numerous targets and pathways.

GO and KEGG enrichment analysis also directed that the protective effects of astaxanthin were involved in inflammatory signaling pathways, TNF signaling, TLR signaling, AGE-RAGE signaling, and MAPK-mediated pathways. The molecular docking analysis revealed high binding affinities of astaxanthin to the hub proteins, such as TNF, MAPK1, PPARG, MAPK14, MAPK3, and IL6, which were steady with the network pharmacology outcomes and provided mechanistic evidence for direct modulation of inflammation-related targets. This study indicates that astaxanthin has the ability to alleviate cisplatin-induced neurotoxicity by reducing neuroinflammation, maintaining neuronal integrity, and regulating several molecular signaling pathways. On the basis of these findings, astaxanthin possibly will be a promising neuroprotective compound against chemotherapy-induced neurological dysfunction.

6.2 Future Perspectives

These study results have established a solid foundation for further exploration on the use of astaxanthin in the treatment of chemotherapy-induced neurotoxicity. The molecular mechanisms of the neuroprotective effects of astaxanthin require further investigation and should be fully understood by measuring apoptotic substances, neurotrophic factors, and inflammatory pathways. The hub genes

and pathways identified by network pharmacology should be validated experimentally with advanced molecular methods, including quantitative RT-PCR, Western blotting, immunohistochemistry, and immunofluorescence, using these genes and pathways. Future studies on astaxanthin should also focus on the influence of astaxanthin on BBB integrity, neurotransmitter systems, synaptic plasticity, and mitochondrial function, leading to a more complete understanding of astaxanthin's neuroprotective effects.

In addition, there is a need for long-term testing to evaluate the ability of astaxanthin to offer long-term protection from chronic neurological side effects of cisplatin treatment. In addition, future studies could involve the co-administration of astaxanthin with currently known neuroprotective drugs (e.g., antioxidants) for potential synergistic effects. Further studies on pharmacokinetics and bioavailability are needed to optimize the dosage regimen and achieve better therapeutic effectiveness. In addition, transcriptomic, proteomic, and metabolomic analyses could reveal novel targets and signaling pathways that show roles in the neuroprotective activity of astaxanthin. Ultimately, well-designed clinical trials in cancer patients receiving cisplatin chemotherapy will be necessary to convert these preclinical results into clinical practice and establish astaxanthin as a safe and effective adjunct therapy for the inhibition of chemotherapy-induced neurotoxicity.

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Appendix - 1

Behavioral Test Parameters (Mean $\pm$ SD, n = 10 per group)

Group	OFT Travelled (cm)	Distance Kondziela's (sec)	IST
Normal Saline (Control)	1561 $\pm$ 3.276	59.60 $\pm$ 0.29	
Cisplatin (2.3 mg/kg)	1157 $\pm$ 307.8	58.65 $\pm$ 1.08	
Cisplatin + Mecobalamin (1 mg/kg)	1422 $\pm$ 92.35	59.25 $\pm$ 0.56	
Cisplatin + Astaxanthin (5 mg/kg)	1305 $\pm$ 175.9	59.40 $\pm$ 0.43	
Cisplatin + Astaxanthin (10 mg/kg)	1439 $\pm$ 99.54	59.83 $\pm$ 0.17	
Cisplatin + Astaxanthin (25 mg/kg)	1517 $\pm$ 33.39	59.70 $\pm$ 0.21	

Appendix - 2

Normalized TNF- α and IL-1 β levels in the hippocampus and Cerebellum (pg/mg protein)

Group	TNF- α (pg/mg protein)		IL-1 β (pg/mg protein)	
	Hippocampus	Cerebellum	Hippocampus	Cerebellum
Normal Saline (Control)	292.3 $\pm$ 1.82	313.6 $\pm$ 3.62	232.6 $\pm$ 2.24	233.2 $\pm$ 2.19
Cisplatin (2.3 mg/kg)	1018 $\pm$ 3.85	1123 $\pm$ 4.12	534 $\pm$ 8.76	573.9 $\pm$ 4.86
Cisplatin + Mecobalamin (1mg/kg)	419.6 $\pm$ 2.6	415.6 $\pm$ 1.84	256.4 $\pm$ 2.01	227.6 $\pm$ 1.53
Cisplatin + Astaxanthin (5mg/kg)	923.2 $\pm$ 1.45	722.5 $\pm$ 1.23	402.54 $\pm$ 3.33	540.9 $\pm$ 3.52
Cisplatin + Astaxanthin (10mg/kg)	584.8 $\pm$ 1.86	576.8 $\pm$ 2.81	323.22 $\pm$ 2.55	349.0 $\pm$ 1.62
Cisplatin + Astaxanthin (25mg/kg)	348.8 $\pm$ 1.72	287.4 $\pm$ 3.36	243.8 $\pm$ 10.8	234.2 $\pm$ 1.37

Appendix - 3

Standard Curve Data for TNF- α ELISA

Concentration (pg/ml)	Absorbance (450 nm)
1000	0.338
500	0.214
250	0.145
125	0.109
62.5	0.097
31.25	0.089
0	0.071

Appendix - 4

Standard Curve Data for IL-1 β ELISA

Concentration (pg/ml)	Absorbance (450 nm)
1000	1.463
500	0.779
250	0.474
125	0.293
62.5	0.154
31.25	0.11
0	0.06

Appendix - 5

Total Protein Concentration by BCA Assay (used for ELISA normalization)

Group	Hippocampus ($\mu\text{g/ml}$)	Cerebellum ($\mu\text{g/ml}$)
Normal Saline (Control)	492.67 – 585.75	454.53 – 555.23
Cisplatin (2.3 mg/kg)	169.21 – 333.99	237.87 – 259.23
Cisplatin + Mecobalamin (1mg/kg)	497.39 – 540.08	494.20 – 541.50
Cisplatin + Astaxanthin (5mg/kg)	330.48 – 376.84	224.02 – 249.52
Cisplatin + Astaxanthin (10mg/kg)	406.41 – 432.79	390.45 – 434.69
Cisplatin + Astaxanthin (25mg/kg)	533.87 – 578.41	537.74 – 572.02

Appendix - 6

Percentage of Oxidative stress biomarkers (NO and MDA) in hippocampal and cerebellum tissue

Group	NO Level (%) (Mean $\pm$ SD)		MDA Level (%) (Mean $\pm$ SD)	
	Hippocampus	Cerebellum	Hippocampus	Cerebellum
Normal Saline (Control)	17.14 $\pm$ 0.12	25.13 $\pm$ 0.20	11.08 $\pm$ 0.07	21.12 $\pm$ 0.18
Cisplatin (2.3 mg/kg)	100.00 $\pm$ 0.41	99.99 $\pm$ 0.77	100.00 $\pm$ 0.23	99.99 $\pm$ 0.38
Cisplatin + Mecobalamin (1mg/kg)	48.50 $\pm$ 0.30	67.26 $\pm$ 0.49	59.05 $\pm$ 0.27	68.71 $\pm$ 0.25
Cisplatin + Astaxanthin (25mg/kg)	35.23 $\pm$ 0.16	58.81 $\pm$ 0.48	40.24 $\pm$ 0.22	64.03 $\pm$ 0.30

Appendix - 7

Standard Curve Data for NO Assay

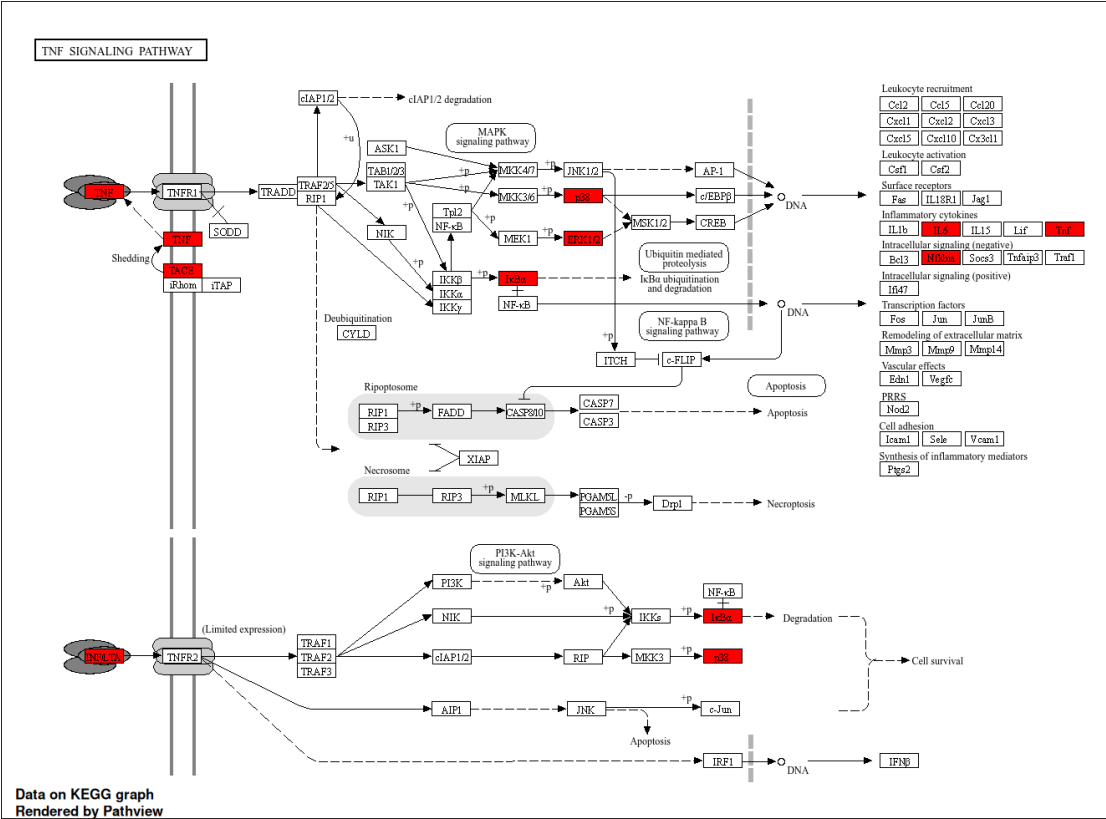
Concentration ($\mu\text{g/ml}$)	Absorbance (532 nm)
100	1.9
50	1.07
25	0.61
12.5	0.45
6.25	0.29
3.125	0.18
1.562	0.16
0	0.04

Appendix - 8

Standard Curve Data for MDA Assay

Concentration ($\mu\text{g/ml}$)	Absorbance (532 nm)
100	1.48
50	0.78
25	0.59
12.5	0.37
6.25	0.25
3.125	0.17
1.562	0.15
0	0.04

Appendix - 9



TNF Signaling Pathway (visualized in the KEGG database via Pathview).