NEUROPROTECTIVE EFFECTS OF WILD BLUEBERRIES AND SEA CUCUMBER EXTRACTS

By

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Abstract

Neurodegenerative diseases involve gradual loss of functional neurons leading to cell death. In Parkinson's disease (PD) for instance, there is a loss of dopaminergic neurons, with corresponding bradykinesia, tremor at rest, and rigidity. Idiopathic factors such as increased oxidative stress, glial activation, and mitochondrial changes also contribute to PD. Polyphenols present in wild blueberries and saponins in sea cucumbers have been reported to exert neuroprotective effects. To explore the neuroprotective effects of blueberries in C57BL/6 mice, the lipophilic neurotoxin MPTP was administered subcutaneously, and behavioural tests and relevant protein measurements were carried out. Compared to MPTP-treated mice given a normal diet, MPTP-treated mice given a 5% blueberry enriched diet showed higher tyrosine hydroxylase levels in the midbrain and cortex. Furthermore, there were no differences in behaviour across treatment as measured in the rotarod, open field, or light/dark box. Likewise, to study neuroprotective effects of sea cucumber and aqueous blueberry studies were conducted in cell cultures. Substantia nigra (SN), rat neocortical (RNC), and C57BL/6 mixed cultures were treated with/without 100 μM glutamate or 100 ng/mL alpha-synuclein for 24 hours with/without the extracts. The sea cucumber extracts with glutamate exposure showed higher cell counts in SN and RNC cells; the sea cucumber + aqueous blueberry showed higher cell counts in RNC and mixed culture. Overall, wild blueberries, as well as sea cucumber extracts, possess neuroprotective properties.

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List of abbreviations

℃	Degree Celsius
6-OHDA	6-Hydroxydopamine
AMP	Adenosine monophosphate
AMPA	α-Amino-3-hydroxy-5-methylisoxazole-4-propionate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BB	Blueberry
BBB	Blood brain barrier
Ca^{2+}	Calcium
cAMP	Adenosine 3',5'-cyclic monophosphate
CDK5	Cyclin dependent kinase 5
CNS	Central nervous system
CO_2	Carbon dioxide
COMT	Catechol-o-methyltransferase
DA	Dopamine
DAPI	4', 6-diamidino-2- phenylindole
DAT	Dopamine transporter
dH_2O	Distilled water
DJ-1	Protein deglycase
DNA	Deoxyribonucleic acid
DOPAC	Dihydroxyphenylacetic acid

DOPAL	Dihydroxyphenylacetaldehyde
ETC	Electron transport chain
$FADH_2$	Flavin adenine dinucleotide
G	Gauge
g	Gram
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
H_2O	Water
H_2O_2	Hydrogen peroxide
HBSS	Hank's Buffered Salt Solution
$HO_2^{\bullet}$	Hydroperoxyl radical
HRP	Horseradish peroxidase
HRS	Hours
HSD	Honestly significant difference
HVA	Homovanillic acid
i.p.	Intraperitoneal
IgG	Immunoglobulin G
IL	Interleukin
kg	Kilogram
L	Litre
L-DOPA	Levodopa
LB	Lewy body
LN	Lewy neurites

LRRK2	Leucine rich receptor kinase 2
MAO	Monoamine oxidase
MAO-B	Monoamine oxidase B
mg	Milligram
mL	Millilitre
mM	Millimolar
mm	Millimetre
MPDP+	1-Methyl-4-phenyl-2,3-dihydropyrinidinium
MPP+	1-Methyl-4-phenylpyridinium ion
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mt DNA	Mitochondrial deoxyribonucleic acid
N_2	Nitrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
ng	Nanogram
NGS	Neuronal growth supplement
NMDAR	N-methyl-D-aspartate receptor
NO	Nitric oxide
NOS	Nitric oxide synthase
$O_2^{\bullet -}$	Superoxide radical
$OH^{\bullet}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffer saline
PD	Parkinson's disease
PenStrep	Penicillin/streptomycin
PFA	Paraformaldehyde
PINK1	PTEN-induced kinase 1
PLL	Poly-L-lysine
RIPK1	Receptor-interacting serine/threonine-protein kinase 1
RNC	Rat neocortical cells
RNS	Reactive nitrogen species
$RO^{\bullet}$	Alkoxyl radicals
$ROO^{\bullet}$	peroxyl radicals
ROS	Reactive oxygen species
rpm	Rotations per minute
s.c.	Subcutaneous
SC	Sea cucumber
SDS	Sodium dodecyl sulfate
ser-129	Serine-129
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
TBST	Tris Buffered Saline with Tween
TNF	Tumor necrosis factor
TRAP1	tumor necrosis factor receptor associated protein 1

UI	Uncertainty interval
v/v	volume/volume
VMAT 2	Vesicular monoamine transporter 2
Zn^{2+}	Zinc
µg	Microgram
µL	Microlitre
µm	Micrometer
µM	Micromolar

Chapter 1 - Introduction

1.1. Neurodegenerative diseases

Neurodegenerative disease is a term used to group several chronic progressive central nervous system (CNS) disorders. Common neurodegenerative disorders include Parkinson's disease (PD), with loss of dopaminergic nigrostriatal neurons (Poirier et al., 1965), Huntington disease, with loss of spiny medium-sized striatal neurons (Graveland et al., 1985), and Alzheimer's disease, with diffuse cerebral atrophy (Ichimiya et al., 1986).

The pathogenesis of neurodegenerative diseases shares common features, including abnormal accumulation and aggregation of disease-specific proteins, which may spread from one cell or region in the brain to another, inducing protein misfolding and aggregation in those previously unaffected areas (Braak et al., 2003; Kidd, 1964; Terry et al., 1964; Woodard, 1962). In neurodegenerative disease, a significant increase in neuronal loss is seen compared to age-matched controls correlating to longitudinal examination as diseases progress (Dijkstra et al., 2014; West et al., 1994). There are various pathways involved in the death of neurons, including apoptosis, necrosis, autophagic cell death, and excitotoxicity (Alberdi et al., 2002; Anglade et al., 1997; Matakas et al., 1973; Mochizuki et al., 1996; Nixon et al., 2005; Smale et al., 1995; Stefanis et al., 2001).

Apoptosis is programmed cell death, including cell shrinkage, chromosome condensation, and deoxyribonucleic acid (DNA) fragmentation (Ankarcrona et al., 1995; Kerr, 1971; Wyllie, 1980). Apoptosis is incited extrinsically via death receptor activation

or intrinsically via DNA damage, p53 activation, and upregulation of pro-apoptotic factors on the Bcl-2 family (Eizenberg et al., 1996; Martin, 2000; Oltval et al., 1993; Shaw et al., 1992; Wu et al., 1997). Both of these pathways alter mitochondrial membrane permeability, executing apoptosis in a caspase-dependent or independent manner (Marzo et al., 1998; Susin et al., 1999).

Necrosis is characterized by cell swelling leading to loss of cell membrane integrity and leakage of cellular content (Ruffolo, 1964). In necroptosis, death receptors such as tumor necrosis factor (TNF) alpha receptor 1 upon activation lead to the recruitment of proteins, including cellular inhibitors of apoptosis 1 and 2, receptor-interacting serine/threonine protein kinase 1 (RIPK1) (Hitomi et al., 2008; Ting et al., 1996). Uncontrolled necrosis is proposed to exist in the case of an ischemic brain injury. The neurons are deprived of oxygen and glucose supply in severe ischemia, resulting in adenosine triphosphate (ATP) depletion and Na^+/K^+ activated ATPase inhibition (Nagafuji et al., 1992). There is an increase in non-synaptic glutamate release leading to excitotoxicity (Benveniste et al., 1984). There is also an increase in the influx of calcium ions (Ca^{2+}) into neurons, changing permeability of the mitochondrial membrane and causing calpain activation (Ankarcrona et al., 1995; Hong et al., 1994; Yamashima et al., 2003).

Autophagic cell death is triggered by many factors such as nutrient starvation (Moriyasu et al., 1996; Takeshige et al., 1992), reactive oxygen species (ROS) (Scherz-Shouval et al., 2007), calcium (Høyer-Hansen et al., 2007), and adenosine monophosphate (AMP)-activated protein kinase (Høyer-Hansen et al., 2007). In autophagic cell death, organelle swelling, cytoplasm vacuolation, and membrane

disruption occur, leading to local inflammation (Hornung et al., 1989; Schweichel et al., 1973). Massive autophagy causes irreversible cellular atrophy, which leads to cell death, for further explanation see review by (Clarke, 1990).

1.2. Parkinson's disease

James Parkinson first described PD in his essay, An Essay on the Shaking Palsy, published in 1817, reprinted in (Parkinson, 1969). PD is a debilitating condition that predominately affects dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Bernheimer et al., 1973; Poirier et al., 1965). In addition, microglial activation has also been associated with dopaminergic neuron loss induced by 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al., 1984), annonacin, rotenone (Sherer et al., 2003), and lipopolysaccharide (Hunter et al., 2009) in various animal models of PD.

A global burden study carried out in 2017 stated the incidence of PD globally was 1.02 million (95% uncertainty interval (UI) 0.85-1.22 million) and prevalence was 8.52 million (95% UI 7.03-10.18 million) (James et al., 2018). A combined cohort study in North America among the age group ≥ 45 years showed the prevalence of PD was 572 per 100,000 (95% confidence interval 537 – 614) with an estimated projection to 1,238,000 by 2030 (Marras et al., 2018). In 2016, the male to female ratio of age-standardized prevalence rates of PD was 1.40 (95% UI 1.36 – 1.43) (Dorsey et al., 2018).

The main clinical features of PD include bradykinesia, rigidity, and tremor at rest (reprinted in (Parkinson, 1969)). These clinical features are only after dopamine (DA) deficiency in the striatal nucleus has reached a higher degree, such as DA decrease in

caudate nucleus is 70-80% (Bernheimer et al., 1973). Besides motor symptoms, non-motor symptoms such as depression, anxiety, sleep disturbances, fatigue, constipation, olfactory dysfunction and orthostatic hypotension are also seen (Jost et al., 1994; Mathers et al., 1989; Shulman et al., 2002; Struck et al., 1990; Vanderhaeghen, 1970; Ward et al., 1983).

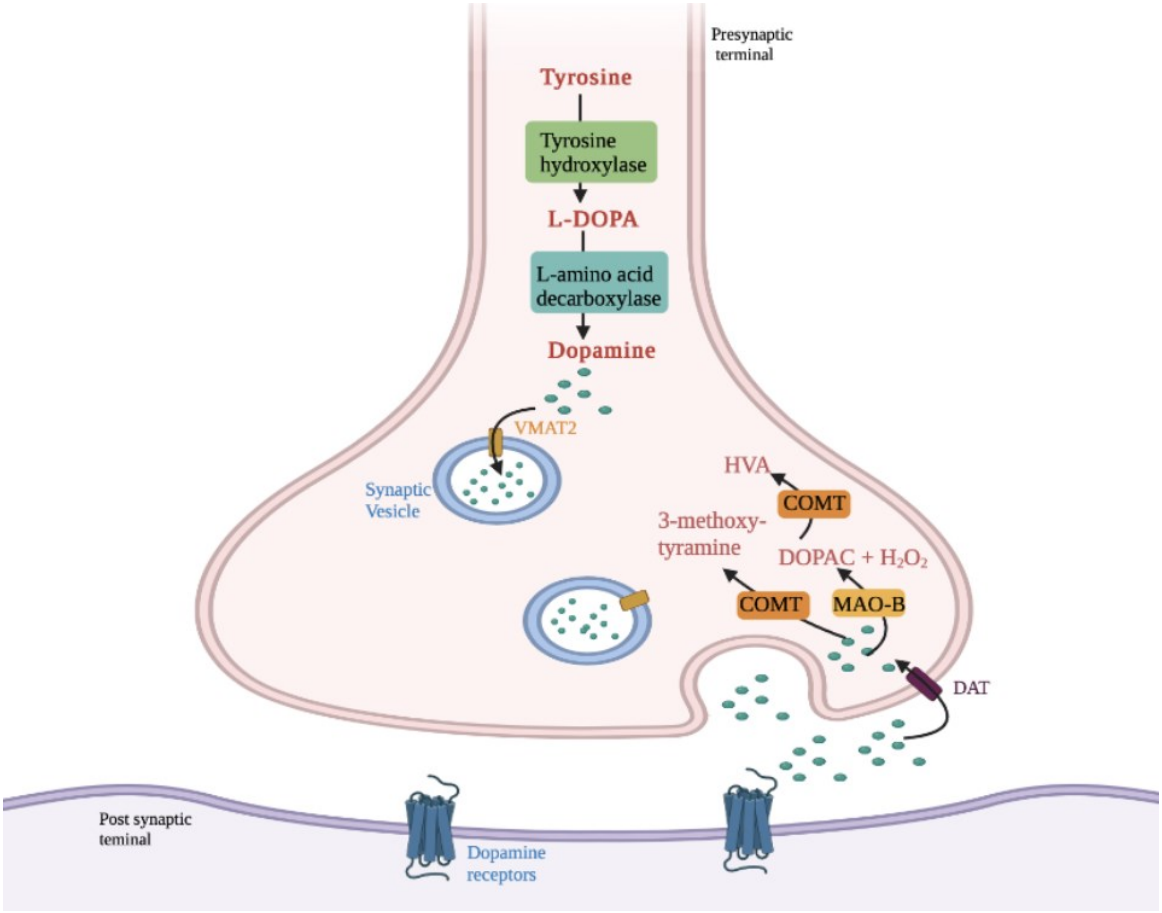

Figure 1: Bio-synthesis pathway of dopamine. In the presence of tyrosine hydroxylase, a rate limiting enzyme, tyrosine is converted into L-DOPA which leads to the formation of Dopamine by L-amino acid decarboxylase. VMAT2 uptakes the dopamine and stores it in synaptic vesicle. Dopamine gets metabolised by MAO-B and COMT into HVA and 3-methoxytyramine respectively. See text for further details. Figure created using biorender.com.

DA is a catecholamine neurotransmitter synthesized from the amino acid tyrosine (Nagatsu et al., 1964) (see Figure 1), responsible for regulating motor neurons, reward and pleasure, motivation, and spatial memory function (Franklin, 1978; Routtenberg et al., 1969; Simon et al., 1986). In the cytosol, tyrosine hydroxylase, the rate-limiting enzyme (Levitt et al., 1965), converts tyrosine to levodopa (L-DOPA) (Nagatsu et al., 1964) in the presence of co-factors O_2 and Fe^{2+}. In the presence of the co-factor pyridoxal phosphate, L-DOPA is converted into DA by L-amino acid decarboxylase (Gunsalus et al., 1944). DA may also be formed in the SN via the activity of cytochrome P450 2D6 from p-tyramine (Hiroi et al., 1998). Synthesized DA is transported to and stored in synaptic vesicles via vesicular monoamine transporter 2 (VMAT 2) (Nirenberg et al., 1996). The acidic environment of vesicles prevent DA oxidation (Mani, 2009). In a non-acidic environment or the presence of monoamine oxidase B (MAO-B), DA is converted into 3,4 – dihydroxyphenylacetaldehyde (DOPAL) (Mattammal et al., 1993), which further converts into 3,4- dihydroxyphenylacetic acid (DOPAC) and H_2O_2 in the presence of aldehyde dehydrogenase (Rosengren, 1960). Also, catechol-O-methyltransferase (COMT) can convert DOPAC into homovanillic acid (HVA) and DA into 3-methoxytyramine. In PD, the amount of HVA and 3-methoxytyramine correlates with disease progression (Bernheimer et al., 1973). The catechol ring in DA can undergo autooxidation and produce super oxide radical and hydrogen peroxide (H_2O_2), which may react with iron increasing the oxidative stress in the cell (Herlinger et al., 1995).

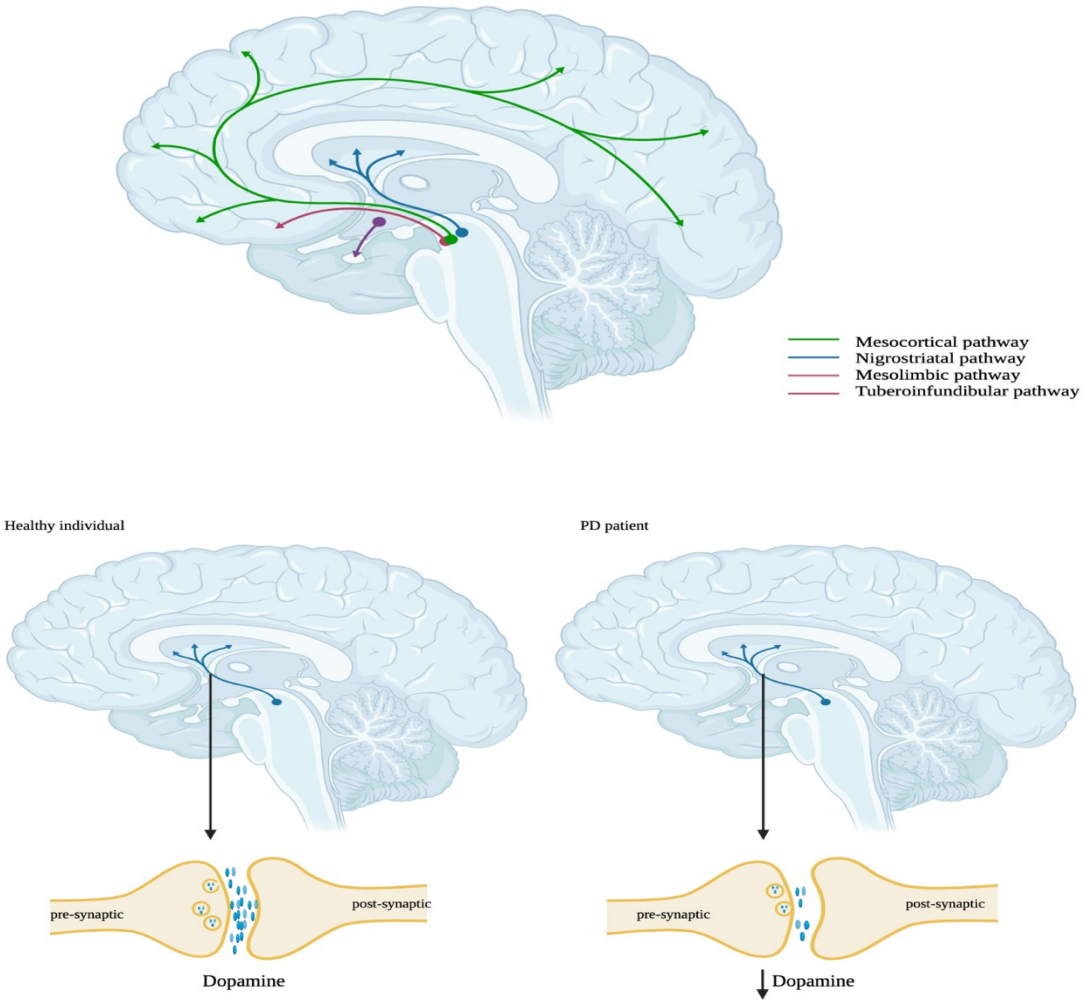

Figure 2: Relationship between dopaminergic pathways and Parkinson's disease. Dopaminergic pathways run from the substantia nigra pars compacta to the basal ganglia and dorsal striatum (caudate nucleus and putamen). Degeneration of the nigrostriatal pathway results in decrease dopamine causing various motor symptoms associated with PD. Figure created using biorender.com.

The distinct morphological change in PD brains is seen in the transverse section of the brainstem with a decrease in darkly pigmented areas in the SNpc and locus coeruleus (Greenfield et al., 1953). The major pathological hallmark of PD includes loss of

melanin-containing neurons of the SN and formation of intracytoplasmic Lewy body (LB) inclusions with alpha-synuclein and ubiquitin and Lewy neurites (LN) with the neuronal projections of similar inclusions (Mann et al., 1982; Woodard, 1962).

In stages 1 and 2 of Braak PD pathology, LB lesions are visible in the dorsal motor nucleus, anterior olfactory nucleus, and reticular formation. In stage 3, the disease progresses to the SNpc, and melanized neuronal loss is observed. In stage 4, the disease extends to the locus coeruleus, amygdala, and temporal limbic cortex. In stages 3 and 4, the clinical motor symptoms start to appear. Lastly, at stages 5 and 6, the prefrontal cortex, primary sensory and the motor area are involved (Braak et al., 2003).

1.2.1. Pathogenesis of Parkinson's disease

The most common neurodegenerative causes of parkinsonism are believed to be alpha-synucleinopathies (Spillantini et al., 1997), and tauopathies (Lynch et al., 1994; Spillantini et al., 1997). The various factors contributing to PD include mitochondrial function changes (Bindoff et al., 1989; Schapira et al., 1989), protein aggregation (Woodard, 1962), increased oxidative stress (Spina et al., 1989), glial activation (McGeer et al., 1988), and apoptosis (Mochizuki et al., 1996).

Oxidative stress

Oxidative stress is an imbalance between oxidants and antioxidants, which favors oxidants, disrupting redox signaling or causing molecular damage (Sies, 2015). The neurons in the brain are constantly exposed to ROS such as H_2O_2 (Boveris et al., 1973),

superoxide anion ($O_2^{\bullet-}$) (Du et al., 1998; Fabian et al., 1995), hydroxyl radical ($OH^{\bullet}$) (Haber et al., 1934), as well as reactive nitrogen species (RNS) such as nitric oxide (NO), nitroxyl radical, and peroxynitrite ($ONOO^-$) (Padmaja et al., 1993) due to endogenous or exogenous exposure to oxidative stress such as disease or stimuli (Abrahams et al., 2019; Dallé et al., 2018).

The significant contributors to ROS at the mitochondrial level include the electron transport chain while ionizing radiation, pollutants and energy transfer reactions also contribute (Boveris et al., 1973; Hiura et al., 2000; Thomas et al., 1989). $O_2^{\bullet-}$ reacts with NO to form $ONOO^-$ (Beckman et al., 1990) which readily nitrates and performs deactivation of various enzymes and DNA (Whiteman et al., 2002). Metals such as iron in ferrous form react readily with H_2O_2 to form highly reactive $OH^{\bullet}$ radicals through the Fenton reaction, known as the Haber-Weiss cycle as it was first described by these scientists (Haber et al., 1934), and in ferric form catalyzes the production of $OH^{\bullet}$ from H_2O_2 and $O_2^{\bullet-}$, which pathologically leads to PD (Dexter et al., 1989). Similarly, NO, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Hoffman et al., 1980) and monoamine oxidase (MAO) (Simonson et al., 1993) also contribute to oxidative stress.

Similarly, RNS cause lipid peroxidation, protein oxidation, and damage to DNA (Görsdorf et al., 1990; Paxinou et al., 2001; Radi et al., 1991). Nitric oxide synthase (NOS) has three different isoforms, neuronal NOS (Bredt et al., 1990), endothelial NOS (Busse et al., 1990), and inducible NOS, which is widely expressed in the SNpc in PD patients (Hunot et al., 1996). Nitrogen dioxide generally forms by reaction between peroxyradical and nitric oxide (Padmaja et al., 1993). The nitration of aromatic amino

acids such as tyrosine to form 3-nitrotyrosine also results in the generation of RNS (Bian et al., 2003). NO and $ONOO^-$ also inhibit complex I via S-nitrosylation, nitration, and protein thiol formation (Clementi et al., 1998).

Neuroinflammation

Neuronal degeneration occurs due to pathological interaction with glial cells, microglia, astrocytes, or peripheral immune cells' infiltration, such as macrophages and lymphocytes (McGeer et al., 1988; McGeer et al., 1988; Schneider et al., 1988). Microglia, CNS resident macrophages, constitute the frontline defense of the innate immune system, and secrete neurotrophic factors, repair neurons, and remove toxic substances (Sierra et al., 2016). Microglia shift towards classical pro-inflammatory M1 phenotype (He et al., 2016) or immunosuppressive and cytoprotective M2 phenotypes in response to inflammatory conditions (Wu et al., 2010). Damage associated molecular proteins, such as neuromelanin (Wilms et al., 2003), released by dying neurons, or pro-inflammatory mediators secreted by astrocytes (He et al., 2016), alpha-synuclein (Zhang et al., 2005), and signals from toll-like receptors (Bsibsi et al., 2002), favors acquisition of M1 pro-inflammatory phenotype. M1 microglia have amoeboid morphology, upregulation of major histocompatibility complex II, and increment in pro-inflammatory mediators, interleukin (IL), IL-1ß, IL-6 and TNF alpha (Giulian et al., 1986; Hayes et al., 1988; McGeer et al., 2002; Woodroofe et al., 1991). Thereby, microglia alters the permeability of the blood brain barrier (BBB) and induce brain infiltration reinforcing local inflammatory responses (Kangwantas et al., 2016; Labus et al., 2018; Maruo et al.,

1992; Mayhan, 2002; Nishioku et al., 2010). Also, induction of expression of gene encoding components of NADPH oxidase and generation of ROS and NO takes place, potentiating chronic inflammation (Zhang et al., 2005).

Astrocytes (A1 type) are abundant in PD (Miklossy et al., 2006; Mirza et al., 1999) and lose their function of nurturing neurons, synapse, and phagocytosis of altered synapse and myelin debris (Christopherson et al., 2005; Chung et al., 2013; Liddelow et al., 2017). A1 astrocytes also secrete unknown neurotoxic factors which promote the death of neurons and oligodendrocytes (Liddelow et al., 2017). On the other hand, A2 astrocytes generally result after ischemia, upregulates neurotrophic factors and are neuroprotective (Liddelow et al., 2017; Miyazaki et al., 2011).

Mitochondrial dysfunction

The first link between mitochondrial dysfunction and PD was discovered in the 1980s when users exposed to MPTP exhibited parkinsonian phenotype and loss of nigral neurons (Langston et al., 1983, 1984).

Complex I acts at the entry point for electrons into electron transport chain (ETC) from the mitochondrial matrix in oxidative phosphorylation. Superoxide radicals are produced due to single electron transfer to oxygen in the respiratory chain (Loschen et al., 1971; Turrens et al., 1980), which gets converted into H_2O_2 by superoxide dismutase 2 (Boveris et al., 1975; McCord et al., 1969) and is ultimately detoxified by catalase. In the presence of Fe^{2+}, H_2O_2 is converted into $OH^{\bullet}$ (Haber et al., 1934), thus damaging cellular components. Superoxide generation occurs due to decreased ATP production, high proton

force (Korshunov et al., 1997), reduced coenzyme Q pool (Lambert et al., 2004), and a high NADH/NAD^+ ratio (Kussmaul et al., 2006). Also, during reverse electron transport, the amount of ROS is increased (Turrens et al., 1980).

Reduction in complex I increases ROS, affecting mitochondrial DNA (mtDNA) (Richter et al., 1988) that codes for subunits of the complex I enzyme. Deletions or changes in mtDNA increase their susceptibility toward oxidative stress in nigral neurons (Bender et al., 2006). The entry of calcium through L-type channels increases DA metabolism in the SNpc and produces basal mitochondrial oxidant stress increased during cell death and aging (Mosharov et al., 2009; Nedergaard et al., 1993; Striessnig et al., 2006).

Leucine Rich Repeat Kinase 2 (LRRK2) plays a vital role in cytoskeletal maintenance (Meixner et al., 2011), autophagy (Alegre-Abarrategui et al., 2009), and immune responses (Moehle et al., 2012). LRRK2 mutations are responsible for increased kinase activity (Sheng et al., 2012), and are associated with pathogenesis of PD (Zimprich et al., 2004). LRRK2 has been suggested to interact with peroxiredoxin 3, a mitochondrial antioxidant, and mutations could affect its radical scavenging ability (Angeles et al., 2011). Similarly, mitochondrial oxidative stress (Takahashi-Niki et al., 2004), DA oxidation (Burbulla et al., 2017), and alpha-synuclein accumulation (Xu et al., 2017) is potentiated due to homozygous mutation of protein deglycase (DJ-1) (Burbulla et al., 2017; Strobbe et al., 2018). Also during high oxidative stress, PTEN-induced kinase 1 (PINK1) degradation in mitochondria is impeded, causing its accumulation which later recruits Parkin (Narendra et al., 2010) and induces E3 ubiquitin ligase activity (Imai et al., 2000). Parkin adds ubiquitin chains in mitochondrial membrane resulting in engulfment

and degradation, also termed mitophagy (Chan et al., 2011; Heo et al., 2015; Poole et al., 2008).

Alpha-synuclein

Alpha-synuclein is a presynaptic protein (Jakes et al., 1994; Maroteaux et al., 1988) comprising a hydrophobic non-amyloid component responsible for protein aggregation (Bisaglia et al., 2006; Jakes et al., 1994; Uéda et al., 1993), and a polar c-terminal, mediating interactions with other proteins, ligands, and metal ions, and is responsible for post-translational modification (Jakes et al., 1994). Alpha-synuclein undergoes extensive post-translational modifications such as nitration, DA modification and phosphorylation (Anderson et al., 2006). In a PD brain, around 90% of total alpha-synuclein undergoes phosphorylation at serine-129 (ser-129), while in a healthy brain, only around 4% of total alpha-synuclein undergoes such phosphorylation (Fujiwara et al., 2002).

Structurally, alpha-synuclein exists in two forms: an unstructured soluble form and a membrane-bounded helical form, which are in equilibrium with one another (Eliezer et al., 2001). Therefore, alpha-synuclein can react with anionic lipids bringing conformational changes forming aggregates that can interfere with mitochondrial and lysosomal functions, vesicular homeostasis, autophagy, and microtubule transport (Pacheco et al., 2015).

Oligomers convert into insoluble fibrillar aggregates with cross beta-sheet conformation. LB and lewy neurites contain filamentous aggregates of phosphorylated

and ubiquitinated alpha-synuclein (Spillantini et al., 1998*).* The alpha-synuclein aggregation pathway comprises the lag phase, forming competent aggregation nucleus (Uversky et al., 2001), the elongation phase, converting nucleus into protofibrils, and the stationary phase, forming amyloid fibrils from soluble proteins, and follows first order kinetics (Wood et al., 1999).

1.3. Neurotoxins

1.3.1. MPTP neurotoxin

MPTP has represented a significant contribution to understanding and treating PD after identifying selective toxicity in the nigrostriatal tract by Langston et al. in the mid-1980s when young drug users from Northern California showed parkinsonism due to injecting a street drug-containing MPTP, a contaminant (Ballard et al., 1985; Langston et al., 1983). MPTP, highly lipophilic, crosses the blood-brain barrier and is transformed into 1-methyl-4-phenylpyridinium ion (MPP^+) in the presence of monoamine oxidase B (MAO-B) in a two-step reaction (Heikkila et al., 1984; Javitch et al., 1985; Langston et al., 1984; Markey et al., 1984). At first, MAO-B catalyzes two-electron oxidation of MPTP, producing an intermediate, 1-methyl-4-phenyl-2,3-dihydropyrinidinium ($MPDP^+$), which being unstable, undergoes an unstable phase spontaneous dismutation forming MPP^+ and MPTP (Castagnoli et al., 1985). MPP^+ is released into the extracellular space and possesses high affinity towards the dopamine transporter (DAT), which carries MPP^+ into dopaminergic neurons of SNpc (see Figure 3) (Javitch et al., 1985; Shen et al., 1985). Inside the neurons, MPP^+ enters synaptic vesicles and via active

transport, accumulates in the mitochondrial matrix (Ramsay et al., 1986; Ramsay et al., 1986). Subsequently, MPP^+ inhibits complex I (NADH-ubiquinone oxidoreductase) (Nicklas et al., 1985; Ramsay et al., 1987), reducing midbrain ATP by around 20% in vivo and increasing ROS, leading to cell death (Chan et al., 1991; Salach et al., 1984).

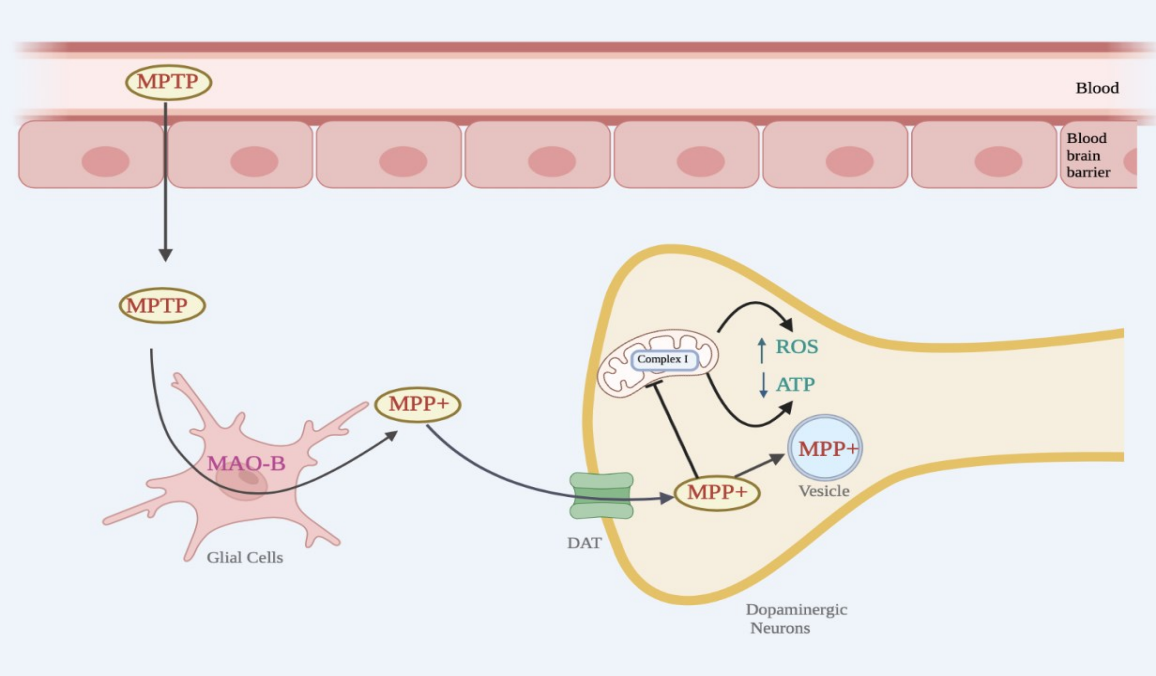

Figure 3: The mechanism of action of MPTP causing degeneration of dopaminergic neurons. MPP^+ formed from MPTP in the presence of MAO-B enters dopaminergic neuron via DAT, which inhibits mitochondrial complex I, increases ROS production and decreasing ATP, leading to the death of dopaminergic neurons. Figure created using biorender.com.

MPTP mouse model

MPTP is the only known dopaminergic neurotoxin capable of causing a clinical picture in animals such as mice (Heikkila et al., 1984), monkey (Burns et al., 1983), and humans (Langston et al., 1983), which is relatively indistinguishable from PD after its

systemic administration, unlike other neurotoxins such as 6-hydroxydopamine (6-OHDA) or rotenone, which require stereotaxic frames or surgery on live animals (Ferrante et al., 1997; Mendez et al., 1975; Shibuya et al., 1981). It has been shown that strain, sex, age, body weight, and even the source/supplier affect the sensitivity and reproducibility of the lesion of MPTP in mice (Giovanni et al., 1991; Gupta et al., 1986; Heikkila, 1985; Miller et al., 1998; Ricaurte et al., 1987; Sershen et al., 1985; Sonsalla et al., 1986; Sundström et al., 1990). A study has shown that female mice present differences in sensitivity to MPTP with regards to variation in brain MAO-B activity during the oestrus cycle (Unzeta et al., 1994). Also, a study by (Antzoulatos et al., 2010) showed that female mice exhibited higher mortality rates than males. A protocol by (Jackson-Lewis et al., 2007) suggests using male mice weighing at least 22g and at least 8 weeks of age for most reproducible results.

The most common routes of MPTP administration are intraperitoneal (i.p.) and subcutaneous (s.c.) injection. At the same dose, the s.c injection results in a more severe dopaminergic lesion than an i.p injection as it avoids first-pass hepatic metabolism (Chiba et al., 1988). Although many injection regimens are found in the literature, the best-characterized regimen is an acute regimen, four i.p. injections at a dose of 20mg/kg with 2 hours intervals, (Jackson-Lewis et al., 2007) and sub-acute, one i.p. injection of 30 mg/kg MPTP daily for five consecutive days (Tatton et al., 1997).

Besides MPTP, other neurotoxins such as 6-OHDA (Betarbet et al., 2000), and lipopolysaccharide (Hunter et al., 2009), and mouse genetic models using alpha-synuclein, LRRK2 (Li et al., 2010), mitopark mouse (Good et al., 2011), and a rat genetic model (Dusonchet et al., 2011; Zhou et al., 2011) are other models of PD. In addition,

alternative models using drosophila (Feany et al., 2000), C. elegans (Braungart et al., 2004), Zebrafish (McKinley et al., 2005) are used to study PD.

1.3.2. Alpha-synuclein neurotoxin

Previous studies have shown that oligomeric and fibrillary alpha-synuclein aggregates are toxic and potentially cause cell death *in vitro* and *in vivo* (El-Agnaf et al., 1998; Periquet et al., 2007; Volpicelli-Daley et al., 2011). The prion-like hypothesis states that pathological forms of alpha-synuclein are transferred from diseased cells into neighboring intact cells, seeding the aggregation of native alpha-synuclein in healthy cells, thus spreading the pathology throughout the brain (Desplats et al., 2009; Olanow et al., 2009). The *in vitro* models of synucleinopathies mainly consist of modeling alpha-synuclein aggregation, using alpha-synuclein expressing cells, and adding alpha-synuclein species exogenously, as reviewed in (Alam et al., 2019; Delenclos et al., 2019). In extracellular models, alpha synucleins are directly added to cell culture, where they interact with cell membranes (Masaracchia et al., 2018). Furthermore, it may lead to alterations of membrane structure and integrity, bringing changes in transmembrane receptor functions. Alpha-synuclein monomers pass easily through the membrane via diffusion, while the oligomers and fibrils are taken up by dynamin-dependent endocytosis or micropinocytosis (Lee et al., 2008; Shearer et al., 2021). These internalized aggregates induce the formation of insoluble aggregates in the host cells leading to synaptic dysfunction and death (Volpicelli-Daley et al., 2011). Additionally, the exogenous alpha-synuclein affects cytoskeletal integrity via various mechanisms. It leads towards actin

filament stabilization, and microtubule destabilization (Bellani et al., 2014) (Gąssowska et al., 2014). Also, exogenous alpha-synuclein potentiates defects in axon elongation, and neuron growth (Tilve et al., 2015). Extracellular alpha-synuclein causes over activation of cyclin dependent kinase 5 (CDK5), increase in ROS, and dysregulation in Ca^{2+} (Czapski et al., 2013; Wang et al., 2010).

Alpha-synuclein oligomers exert many pathogenic effects, including increased intracellular Ca^{2+}, and seed intracellular alpha-synuclein aggregation (Danzer et al., 2007, 2009). The oligomers impair the autophagy-lysosomal degradation system and the ubiquitin-proteasomal system (Vekrellis et al., 2011). It has also been reported that the alpha-synuclein oligomers generated *in vitro* in primary neuronal cultures cause transmembrane seeding of alpha-synuclein aggregation in a time- and dose-dependent manner (Danzer et al., 2009).

1.3.3. Glutamate neurotoxin

The term excitotoxicity was coined by John Olney in 1969 while discussing the brain lesions that resulted from feeding monosodium glutamate to mice (Olney, 1969). Excitotoxicity refers to cell death due to the toxic activities of excitatory amino acids. Generally, neuronal excitotoxicity refers to the damage and death of neurons resulting from extensive exposure to glutamate and the associated influx of ions into the cells, as glutamate is the major excitatory neurotransmitter in the CNS (Curtis et al., 1959).

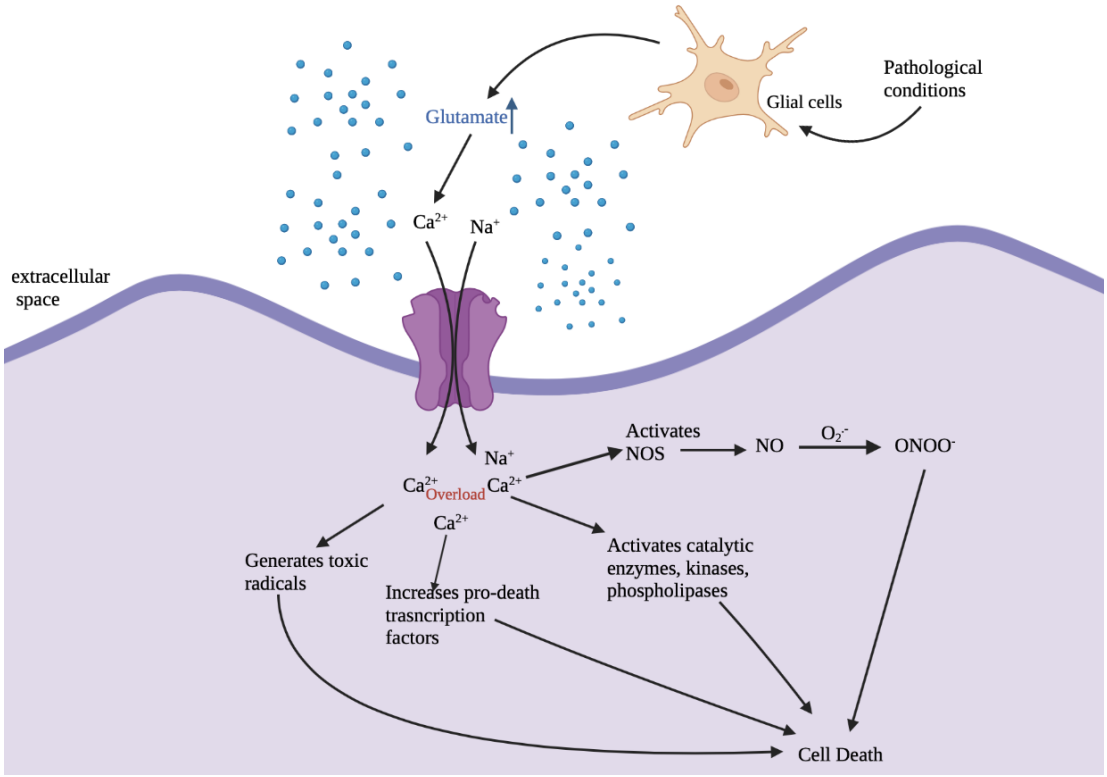

Figure 4: The mechanism of glutamate mediated cell death. Excess glutamate causes increased influx of Ca^{2+}, which results in cell death via generation of toxic radicals, increment in pro-death transcription factors, and activation of catalytic enzymes, kinases, phospholipases and NOS. Figure created using biorender.com.

Glutamate acts on specific membrane receptors which can be classified into two groups:

A) ligand-gated ion channels, including N-methyl-D-aspartate receptors (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), and kainic acid receptors, as reviewed in (Hollmann et al., 1994; Honoré, 1989).

B) Metabotropic receptors including quisqualate, ibotenate and 1-amino-4,5-cyclopentane-trans-1,3-dicarboxylate, as reviewed in (Méndez et al., 2004; Pin et al., 1995).

Glutamate stimulates the N-methyl-D-aspartate receptors (NMDARs), causing neuronal death via necrosis and apoptosis (Choi et al., 1988). The increased extracellular glutamate levels result in excess activation of Ca^{2+} permeable NMDARs, leading to Ca^{2+} overload and excitotoxicity (Choi, 1987) (Figure 4). Under various pathological conditions, the activated microglia and reactive astrocytes enhance glutamate release. A higher level of intracellular Ca^{2+} activates catalytic enzymes, produces toxic radicals, and impairs cellular energy production, inducing cell death (Novelli et al., 1988).

Also, Ca^{2+} permeable AMPA receptors have been known to cause selective neuronal death by increasing Ca^{2+}/Zn^{2+} entry, Ca^{2+} buffering mechanism, receptor desensitization, and receptor distribution (Vieira et al., 2010). Magnesium ions are found to block glutamate binding to NMADRs (Mayer et al., 1984). The metabotropic glutamate receptor present post synaptically act via potentiation of NMDAR-medicated Ca^{2+} influx, and those in pre-synapses act via inhibition of adenosine 3',5'-cyclic monophosphate (cAMP) formation and reduction of influx of Ca^{2+} through NMDARs (Lau et al., 2010).

1.4. Natural products

Natural products are compounds derived from various natural sources that may possess biological activities (Xie et al., 2015). Some herbal extracts such as *Hibiscus asper Hook. f.* (Foyet et al., 2011; Hritcu et al., 2011), *Carthamus tinctorius L* (Ablat et al., 2016), and *Ginkgo biloba L* (Yang et al., 2016) have shown positive effects in animal models of PD.

1.4.1. Wild blueberries

The term blueberry (BB) refer to small fruit that belongs to plants in the genus of *Vaccinium*, in the family Ericaceae (Vander Kolet, 1988). BB contains a wide variety of polyphenols. They are rich in a wide array of flavonoids such as anthocyanins, glucosides, galactosides, and arabinosides of five anthocyanidins: delphinidin, malvidin, petunidin, cyanidin and peonidin (Bombardelli et al., 1977; Spinardi et al., 2019); and flavanols, quercetin, myricetin, iraricitrin, kaempferol, isorhamnetin, syringetin, catechin, epicatechin (Cho et al., 2005; Sellappan et al., 2002; Vrhovsek et al., 2012). They also contain phenolic acids such as gallic acid, caffeic acid, p-coumaric acid, ferulic acid, ellagic acid, vanillic acid, ferulic acid, and chlorogenic acid (Sellappan et al., 2002; Taruscio et al., 2004; Zadernowski et al., 2005); and stilbenes such as resveratrol (Lyons et al., 2003). BB also possesses a high content of vitamin C, selenium, calcium, sodium, zinc, iron, and manganese (Dróżdż et al., 2018; Sinelli et al., 2008; Walker et al., 2006; Zhang et al., 2014). The polyphenol content of BB is affected by the type of BB species, fruit ripeness, size, growing climate, environment, and storage after harvest (Kalt et al., 1999; Kalt et al., 1996; Mallik et al., 2017; Prior et al., 1998; Wang et al., 2008). Various species of berries grown in Newfoundland and Labrador contain a wide array of anthocyanin and flavonols with a high antioxidant capacity (Hossain et al., 2016). In ethanolic and methanolic extracts of *V. angustifolium* the anthocyanins, delphinidin-3-galactoside, delphinidin-3-arabinoside, delphinidin-3-glucoside, cyanidin-3-galactoside, petunidin-3-galactoside, malvidin-3-galactoside, malvidin-3-glucoside, peonidin-3-

glucoside; and falvonols such as myricetin-3-rahmnoside, quercetin-3-galactoside were detected (Hossain et al., 2016).

Figure 5: Picture of blueberry plant taken by Dr. John Weber.

BB is found to be protective against cardiovascular diseases (Ahmet et al., 2009) and acts as an anti-inflammatory agent (Huang et al., 2014), antioxidant (Vyas et al., 2013), antitumoral (Pan et al., 2007), anti-obesity (Song et al., 2013), and anti-angiogenic agent (Atalay et al., 2003).

1.4.2. Sea cucumber

Sea cucumber (SC), an echinoderm belonging to class Holothuroidea, is considered a valuable marine food and an essential source of medicine (Zhao et al., 2018). SCs are elongated tubular or flattened soft-bodied invertebrates habitually found in benthic areas and deep seas having leather skin that ranges from a few millimeters to a meter in length (Mondol et al., 2017). A study on *Holothuria leucospilota* by (Malaiwong et al., 2019) comprised of chemical and nuclear magnetic resonance analysis, indicated the presence of alkaloids, flavonoids, terpenoids, phenols, steroids, saponins (triterpenoids), and glycosides as major constituents. This study also showed SC extracts

suppressed alpha-synuclein aggregation in a transgenic PD model, and promoted neuroprotection in 6-OHDA treated worms (Malaiwong et al., 2019).

SCs are rich in nutrients such as Vitamin A, B1, B2, D, and E, for detail see review by (Chen, 2003). Also, SCs contain calcium, magnesium, iron and zinc (Barzkar et al., 2017; Sroyraya et al., 2017; Wen et al., 2010). Saponins, triterpene glycoside, are the major secondary metabolites produced by SC, which act as a basis of their chemical defense (Delia et al., 1977; Kamyab et al., 2020). The triterpene portions of saponin is derived from lanost-9(11)en-3β-ol (Kerr et al., 1995). The glycosides present in SC have a lactone cycle in the aglycone units, which includes: holostane type glycoside comprising of 3β-hydroxyholost-9(11)-ene aglycone skeleton (Silchenko et al., 2005), and holostane type glycoside comprising of 3β-hydroxyholost-7-ene (Girard et al., 1990). The glycosides without any lactone cycle in the aglycone units are also present and known as nonholostane type glycoside (Silchenko et al., 2005). The glycone part is covalently connected to C-3 of the aglycone, and comprises of xylose, 3-O-methyl-D-xylose, quinovose, D-glucose, and 3-O-methyl-D-glucose (Girard et al., 1990; Silchenko et al., 2005).

Cucumaria frondosa, commonly known as Pheonix SC or orange footed SC, are widely present in the Indo-West Pacific, and the North East coast of Scotland, Shetland, and Orkney, and the West Atlantic (Bordbar et al., 2011). It is also most abundant and widely distributed in the cold waters of the North Atlantic Ocean (Hossain et al., 2020). The common glycosides produced by *C. frondosa* include: Frondoside A2-1, A2-2, A2-3 A2-6 (Silchenko et al., 2005); Frondoside A2-7, Frondoside A2-8 (Silchenko et al.,

2005); Frondoside A7-1, A7-2, A7-3, A7-4, Isofrondoside C (Silchenko et al., 2007), Frondoside A (Girard et al., 1990), Frondoside A1 (Avilov et al., 1993), and Frondoside B (Findlay et al., 1992). Besides saponins, *C. frondosa* also contains polyphenols such as flavonoids (Mamelona et al., 2007).

C. frondosa has shown potential chemopreventive properties for colon carcinogenesis (Janakiram et al., 2010). *C. frondosa* is also found to have anti-tumor properties (Sajwani, 2019), antioxidant properties (Mamelona et al., 2007), is effective in acute leukemias (Sajwani et al., 2017), inhibits breast cancer metastasis (Ma et al., 2012), exhibits neuroprotective effects and shows cognitive improvements (Wu et al., 2014).

1.5. Secondary metabolites

Secondary metabolites are those substances produced by organisms to make them competitive in their own environment (Williams et al., 1989). Secondary metabolites can be classified into various categories based on different features such as chemical structure, solubility, and biosynthetic pathways. The most common classification of secondary metabolites is based on the presence or absence of nitrogen-containing groups in their chemical structure, as shown in Figure 6, reviewed in (Katerova et al., 2017).

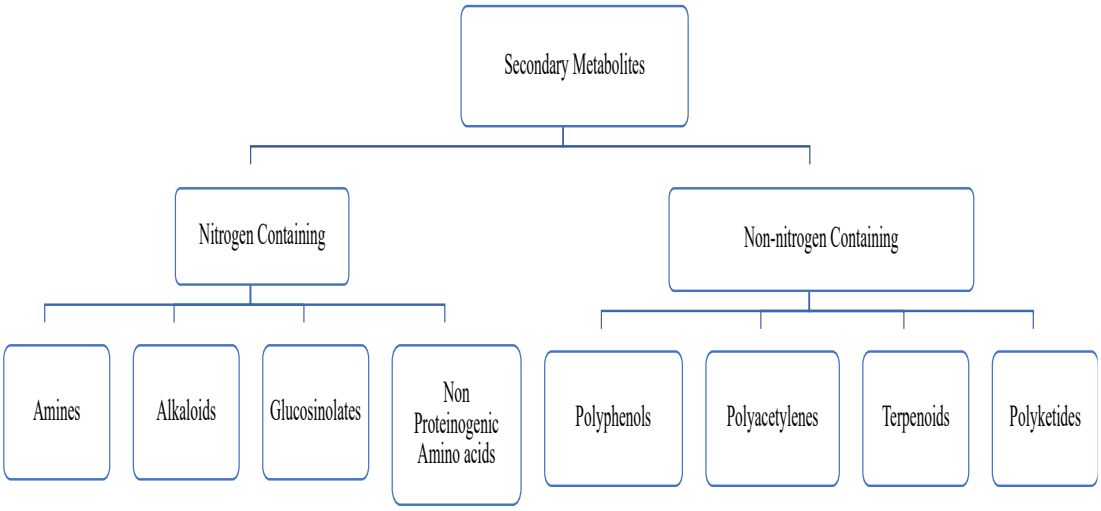

Figure 6: Classification of the secondary metabolites in plants.

1.5.1. Polyphenols

Polyphenols, previously known as vegetable tannins, can be defined as plant secondary metabolite which are derived exclusively from the shikimate-derived phenylpropanoid and/or the polyketide pathway(s) lacking any nitrogen based functional group in their basic structure and features more than one phenolic ring, for detail see review by (Quideau et al., 2011). Polyphenols are the most abundant dietary bioactive compounds found with primary sources being fruits and vegetables (Dick et al., 1987; Joseph et al., 2003), coffee (García et al., 1985), red wine (Mattivi, 1993), black and green teas (Roberts et al., 1951), chocolate (Adam et al., 1931) and extra virgin olive oil (Visioli et al., 1998). Based on their carbon skeleton, polyphenols are divided into phenolic acids, flavonoids, stilbenes, and lignans, and are shown in Figure 7, as reviewed

in (Han et al., 2007; Pandey et al., 2009). Phenolic acids are further classified into hydroxycinnamic acid and hydroxybenzoic acid. Flavonoids are the largest group of dietary polyphenols, comprising up to 60% of these compounds with around 4000 varieties. Flavonoids are classified into flavones, flavonols, flavanones, flavan-3-ols, anthocyanins, dihydroflavonols, and isoflavones based on the degree of unsaturation and substitution pattern, see reviews by (Dias et al., 2021; Pandey et al., 2009). On the other hand, Stilbenes are only present in low quantities in a diet and are structurally made up of the 1,2-diphenylethylene ring, see review by (Han et al., 2007). Lignans are products of oxidative dimerization of two or more phenylpropanoid units, see review by (Durazzo et al., 2018).

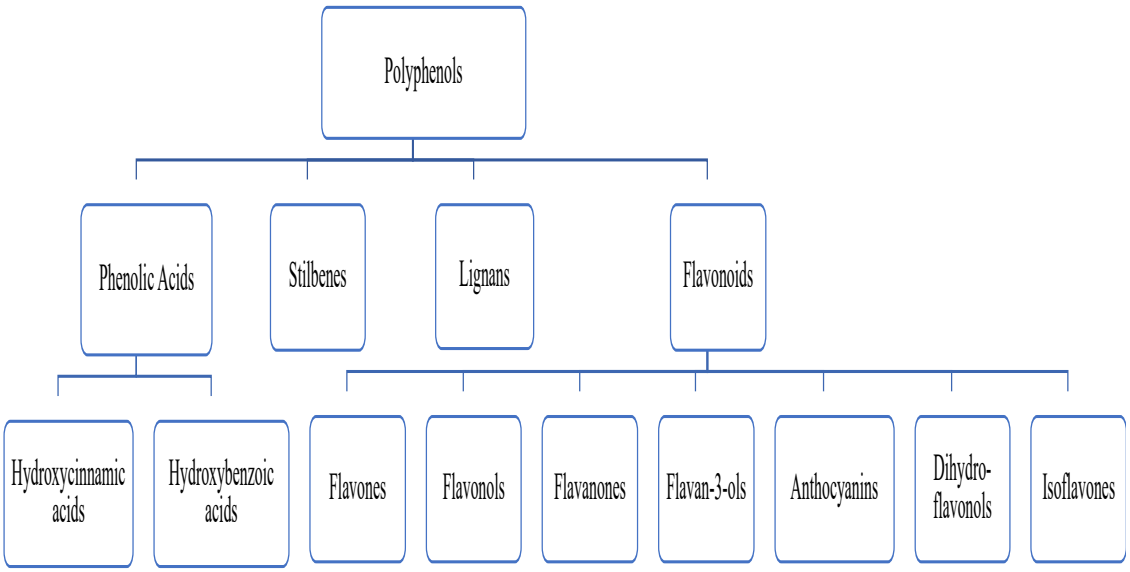

Figure 7: Classification of the polyphenols based on the nature of their carbon skeleton.

Polyphenols show potential effects on human health and are widely known for their antioxidant (Ho et al., 1992; Visioli et al., 1998), antidiabetic (Al-Awwadi et al., 2004; M.C et al., 2002), anti-inflammatory (Liu et al., 2013), anti-cancer (Wang et al., 1989), anti-obesity (Han et al., 2003), antiplatelet (Bertelli et al., 1995), anti-mutagenic (Mukhtar et al., 1992; Wang et al., 1989), as well as cardiovascular (Pace-Asciak et al., 1995) and neuroprotective properties (Joseph et al., 2003; Vyas et al., 2013).

1.5.2. Saponins

Saponins are characterized based on the presence of steroidal/ triterpenoid aglycones and one or more sugar chains, see review (Faizal et al., 2013). Saponins are complex amphipathic glycosides present in more than 100 families of plants and in marine sources such as starfish and SCs (Bedour et al., 1964; Cheng et al., 2017; Ha et al., 2014; Maier et al., 1993; Nigrelli et al., 2006). Saponins are mainly found in chickpeas (Cheng et al., 2017), black beans (Guajardo-Flores et al., 2012), elephant garlic (Morita et al., 1988), oats (Yang et al., 2016), fenugreek (Bedour et al., 1964), horse chestnut (Zhao et al., 2003), starfish (Maier et al., 1993), SC (Nigrelli et al., 2006) and ginseng (Shibata et al., 1963). Saponins are glycosides with one or more sugar chains on a triterpene or steroid aglycone backbone called sapogenin, see review (Faizal et al., 2013). The most common monosaccharides include D-glucose, D-galactose, D-glucuronic acid, L-rhamnose, L-arabinose, S-xylose, and D-fucose (Girard et al., 1990). Due to lipid-soluble aglycones and water-soluble sugar chains, they are amphiphilic, for more information see the review by (Lorent et al., 2014). Saponins also show a wide variety of

biological activities such as antioxidant (Guillaume et al., 1994), antiviral (Amoros et al., 1987; Subba Rao et al., 1974), anti-cancer (Yang et al., 2016), hypoglycemic (Pascale et al., 2018), hypocolesterolemic (Sauvaire et al., 1991) neuroprotective (Hou et al., 2018; Zhang et al., 2012), and anti-inflammatory effects (Guillaume et al., 1994; Wei et al., 2004).

1.6. Hypothesis

For this study, I hypothesized the following:

- The MPTP treated group will suffer behavioural deficits and neuronal damage to a greater extent than the control group, while the MPTP BB treated group will exhibit neuronal protection, making it comparable to the controls.
- In the cell cultures, the extracts of sea-cucumber and BB will help combat the oxidative stress and neuroinflammation induced by the neurotoxins, glutamate and alpha-synuclein, and decrease cell loss.

1.7. Objectives

The objectives of my study are as follows:

- To study the impact of a BB-enriched diet on motor coordination in the MPTP mouse model of PD.
- To determine the extent of changes in protein concentration of alpha-synuclein and tyrosine hydroxylase in the brain tissues of the MPTP mouse model of PD.

- To test if the dietary intake of wild blueberries decreases the extent of dopaminergic neuron loss in a mouse model of PD.
- To investigate the neuroprotective effects of sea-cucumber and BB extracts in substantia nigra cells, cortical cells, and mouse mixed cultures when exposed to glutamate and alpha-synuclein.

Chapter 2 - Materials and Methods

2.1. MPTP mouse model of PD

A total of 36 male mice of the C57BL/6 strain (Charles River) were used in this study. The mice were two months old and weighed ~22 g at the start of the study. The experiments were conducted at the Health Sciences Centre Animal Care Facility, and the Institutional Animal Care Committee of Memorial University approved all of the procedures used in the study involving mice.

2.1.1. Housing and feeding behavior

The mice were housed individually with free access to regular chow (9.00 to 15.00 hours (hrs)). Every day at 15.00 hrs, the chow was replaced by a freshly prepared mash (7 g) of either rodent chow and water in the ratio of 1:1 or a mash of ground BB, rodent chow, and water. The diet was supplied from the start of the study, July 17, 2019, till the day before the sacrifice of mice, August 20, 2019.

2.1.2. Injection regimen

After five days on the diet, the mice, aged 66 days, underwent a series of subcutaneous injections for five days, modified from (Wang et al., 2015). The first group on a regular diet received an injection of sterile water and served as the control group for the study. The second and third groups were injected with 15 mg/kg MPTP dosed by weight. The group receiving MPTP injections and on a regular diet were named the

MPTP group, and the final group that received MPTP injection and was on an enriched BB diet were named the MPTP BB group.

2.1.3. Behavioural analysis

The mice in the three different groups, control (n=12), MPTP (n=12), and MPTP + BB (n=12), were tested starting on the 7th day post-injection on three different behavioural tests (see Figure 8). The rotarod test on constant rotations per minute (rpm) was carried out on test day 1, August 2, 2019, and the rotarod on accelerating rpm was carried out on Aug 5, 2019, test day 2. The open field and light/dark box tests were carried out on August 7, 2019, and August 8, 2019, respectively.

Rotarod

Each mouse was placed on a rotating rod at a constant rpm of 15 for a maximum of 2 minutes. The latency to fall was recorded. Each mouse was given a maximum of 3 trials with 30 minutes between each trial. On the next day, the mice were again exposed on a rotating rod that accelerated from 4 rpm to 40 rpm (constant rate = 0.6rpm/s) for a maximum of 5 minutes, and the latency to fall was recorded. Each mouse was given a minimum of 3 trials with 30 minutes between each trial.

Open field

The open field test was carried out in a 48cm x 48cm x 48cm wooden box. In order to determine the amount of time the mice spent in the centre of the box relative to

the perimeter, a square was marked off by tape 10 cm from the walls of the box. Mice were placed in the centre of the box and allowed to freely explore for 5 minutes. Duration in the centre, velocity, and distance traveled was calculated (Adamec et al., 2006) with the EthoVision XT10 tracking system (Noldus, Wageningen, Netherlands).

Light/dark box

The LD boxes were made from two grey Plexiglas (0.5 cm thick) boxes (length: 32 cm, width: 10.5 cm, height: 14.5 cm). The dark box had an opaque, acrylic Plexiglas removable top, metal mesh floor and black painted walls. The light box had a clear Plexiglas removable lid with ventilation holes and a 9W, 550 lm light was positioned 5.1 cm above it. Mice were placed into the light side of a LD box and were allowed to explore for 5 min. Video recordings were taken from above the light box for analysis. The total time and the number of entries in the light side of the box was determined manually from videos by a researcher blind to treatment (Adamec et al., 2006; Fifield et al., 2013, 2015).

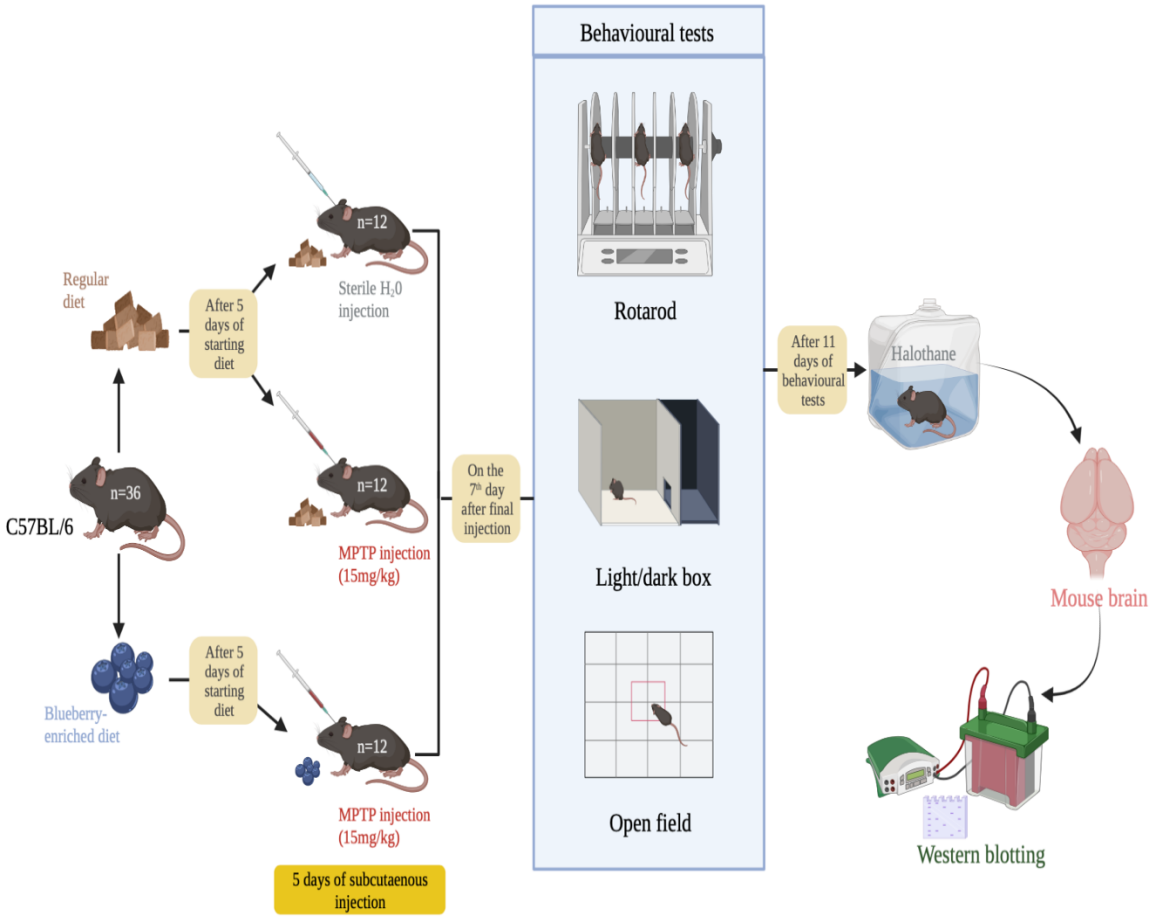

Figure 8: Outline of the experimental design for the MPTP mouse model of PD used in this study. Figure created using biorender.com.

2.1.4. Brain tissue analysis

After 11 days of behavioural tests, the mice were euthanized using halothane, and the brain was removed. The midbrain and cortical regions of the brain were separated and flash-frozen using liquid nitrogen. All of the samples were stored at -80 °C until used for further investigation.

Brain tissue preparation

The brain samples were removed from the -80 °C freezer and placed on ice. In a mortar and pestle, liquid nitrogen (N_2) was added to the brain samples which were gently broken down into smaller pieces. Then, 100g of the tissues were placed into a Percellys tube on ice, and 900 µL of lysis buffer (for 10mL lysis buffer, one protease inhibitor tablet was added). This mixture was then bead beat homogenized at 6500 rpm, one run cycle for 25 seconds. The tubes were then placed on a shaker rack for constant agitation at 4 °C for 2 hours, then the tubes were centrifuged for 20 minutes at 4 °C, 12000 rpm, and placed directly on ice. Lastly, the supernatant was aliquoted and placed at -80°C.

Western blotting

The cortical and midbrain tissue lysates (40 µg/well) were separated by SDS-PAGE (8.5% or 12.5%) under reducing conditions and transferred to a nitrocellulose membrane (Bio-Rad, USA). The blots were blocked using a blocking solution (5% non-fat dried milk in TBST) for 1 hour at room temperature. After blocking, the blots were incubated overnight at 4 °C with anti-tyrosine hydroxylase antibody (1:1000, v/v; catalog #AB152, lot #3638897) (EMD Millipore Crop., USA) and anti- glyceraldehyde 3-phosphate dehydrogenase (GADPH antibody) (1:1000, v/v; catalog #ab181603, lot #GR200347-34) (Abcam, USA), as the loading control.

The blots were blocked with 4% paraformaldehyde (PFA) prior to blocking with the blocking solution for alpha-synuclein detection. After blocking, these blots were incubated overnight at 4 °C with anti-alpha synuclein antibody (1:1000, v/v; catalog

#ab51253, lot#GR3317474-4) (Abcam, USA), and anti-alpha/beta-tubulin polyclonal antibody (1: 1000, v/v; catalog #2148S, lot #8) (Cell Signaling, USA), as the loading control.

After primary antibody incubation, all blots were washed three times with TBST and incubated with horseradish peroxidase (HRP) conjugated-secondary antibody Anti-rabbit IgG (1:3000, v/v; catalog #7074S, lot #29; Cell Signaling, USA) for two hours at room temperature. The protein bands were visualized with the commercial Western Lightning chemiluminescence kit (HRP-catalyzed system, Perkin Elmer, USA) and imaged using Image quant LAS 4000. The quantification of the blots was carried out using ImageJ software.

2.1.5. Statistical Analysis

The analyses for all the behavioral tests and western blotting were conducted using one-way analysis of variance (ANOVA), with a limit for significance at alpha < 0.05. All of the data were analyzed using SPSS (IBM SPSS Statistics 27, Armonk, USA)

2.2. Cell Culture

BB fruit from Fogo island and sea-cucumber extract supplied by Oceans Pride Fisheries Ltd. was used for the cell culture experiments. All cell culture experiments were carried out under a biosafety cabinet, except for the dissection of pups and heating media in a water bath at 37 °C. The mixed cultures of neurons were prepared from dissected C57BL/6 strain mouse-pups from our inhouse breeding colony in the Health Sciences

Centre Animal Care Facility, while pure cultures of substantia nigra and rat cortical neurons were purchased from ScienCell Research Laboratories (Carlsbad, CA). The Institutional Animal Care Committee of the Memorial University approved all the protocols involving the breeding colony and dissection of mouse pups.

2.2.1. Mixed cell cultures

Dissection Plate

At least 24 hours prior to dissection, 24-well Costar® culture plates were coated with 10 µg/mL poly-L-lysine (PLL) solution and incubated at 37 °C and 5 % Carbon dioxide (CO_2) to ensure that the cells adhered to the flat bottom wells. On the day of dissection, each well was washed twice with sterile H_2O before coating.

Dissection

Preparation: All of the tools were rinsed using 70% ethanol. Two tubes containing 10 mL of dissection media, comprising of 100:1 ratio of Hank's Buffered Salt Solution (HBSS) and penicillin/streptomycin (PenStrep), were placed on ice. Two small petri-plates were also taken and filled with ice-cold dissection media.

Procedure: The C57BL/6 mouse pups used were one to three days old (P1-3). The mouse-pup was decapitated quickly using large scissors. First, the skin on the skull was cut posterior to anterior, and then laterally, by going into the foramen magnum on both sides, and then cutting towards the front of the head. The skin was peeled back, and the visible skull was cut medically. The brain was carefully scooped out and put in the

petri-plate containing ice-cold dissection media. The meninges from the brain were carefully removed using a dissection scope and fine forceps, followed by removal of the cerebellum using a scalpel. The brain's remaining parts were placed in the previously chilled tubes containing dissection media and centrifuged at 1000rpm at 4 °C for 3 minutes. The supernatant was removed, and 10 mL of ice-cold dissection media was added and centrifuged under the same conditions. Then, the supernatant was removed and a solution containing 0.8 mL 0.25% Trypsin-EDTA and 9.2 mL HBSS was added. The mixture was incubated at 37 °C for 15 minutes. The supernatant was removed and again centrifuged with dissection media at 4 °C, 1200 rpm for 5 minutes. Fresh 4 mL of growth media, comprised of 40 mL Neuro cell media (ScienCell) and 4 mL Horse serum, was added, and cells were triturated using 18 G and 221/5 G needles 15 times to break the larger aggregates. The solution was filtered through a 70 μm cell strainer, and a final volume of 25 mL was made using the growth media.

Culture

For each well of the 24-well Costar® culture plates, 1 mL of the prepared cell solution was added and incubated at 37 °C and 5% CO_2 for 10-12 days. Twenty-four hours from the initial plating, the media was removed entirely and replaced with 1 mL of new media per well. Then, after every two days, 450 μL of the media from each well was removed and replaced with 500 μL of new media.

2.2.2. Rat neocortical cells

The rat neocortical cells (RNC) were kept frozen at -80 °C prior to use. The plates were coated with PLL solution at 37 °C and 5 % CO_2 overnight. The neuronal media (ScienCell) was prepared by reconstituting 500 mL sterile neuronal media, 5 mL Neuronal Growth Supplement (NGS), and penicillin/streptomycin solution. Also, 5 % amphotericin B was added to the neuronal media. The optimal growth period for RNC cells was 7-10 days. Each vial of RNC contained >1x 10^6 cells per mL with an optimal density of between 20,000-25,000 cells/cm^2.

The total µL of cells/number of wells = X µL per well

X µL per well/ Surface area of well = cells/cm^2

For 24-well Costar® culture plates, surface area of one well = 1.9 cm^2, resulted in ~41,700 cells per well per plate and ~21,929 cells/cm^2 with 1 mL media. Thus, for every 1 mL per well, 41.70 µL of RNC cells were added and incubated at 37 °C and 5% CO_2. After 24 hours, the media was completely removed and replaced with 1mL of new media per well. Then after every two days, 450 µL of the media from each well was removed and replaced with 500 µL of new media.

2.2.3. Substantia nigra cells

The SN cells were frozen at -80 °C prior to use. The plates were coated with PLL solution at 37°C and 5 % CO_2 overnight. The neuronal media (ScienCell) was prepared by reconstitution of 500 mL sterile neuronal media, 5 mL NGS, and pen/strep solution. Also, 5 % amphotericin B was added to the neuronal media. The optimal growth period

for SN cells was 7-10 days. Each vial of SN contained >1x 10^6 cells per mL with an optimal density of between 20,000-25,000 cells/cm^2.

The total µL of cells/number of wells = X µL per well

X µL per well/ Surface area of well = cells/cm^2

For 24-well Costar® culture plates, surface area of one well = 1.9 cm^2, resulted in ~41,700 cells per well per plate and ~21,929 cells/cm^2 with 1 mL media. Thus, for every 1 mL per well, 41.70 µL of SN cells were added and incubated at 37 °C and 5% CO_2. After 24 hours, the media was completely removed and replaced with 1mL of new media per well. Then after every two days, 450 µL of the media from each well was taken out and replaced with 500 µL of new media.

2.2.4. Cell Treatments

After the cells were differentiated, they were given various treatments and incubated at 37 °C and 5% CO_2 for additional 24 hours. The following day, the cells were fixed, stained, and analyzed. Various stock solutions of glutamate, alpha-synuclein, aqueous BB, and SC extracts were prepared and stored at 4 °C.

Control: For the control treatment, 10 µL of sterile distilled water (dH_2O) was added per mL of media

SC extract:

SC extract (Oceans Pride Fisheries Ltd., Lower Wedgeport, Nova Scotia) was prepared by dissolving the powder in a solvent of 80% acetone and 20% dH_2O (v/v). The final concentration of the solution prepared was 66.8 mg/mL. The lab research group previously determined that a concentration of 1 µL/mL could elicit neuroprotective effects and had high antioxidant capacity (unpublished data).

- 1 µL of SC extract per mL of media

Aqueous BB:

Between 10-20 frozen BBs were thawed at room temperature and mashed using a mortar and pestle. The mixture was filtered through a sterile filter into a 50 mL tube. This solution was used in combination with sea-cucumber extracts alone or with glutamate and alpha-synuclein. The lab research group previously determined that the concentration of 1 µL/mL could elicit neuroprotective effects (Vyas et al., 2013).

- 1 µL of BB extract per mL of media

Glutamate:

To make a stock solution of 10 mM, 14.7 mg L-glutamic acid (Research Biochemicals Inc.) was added to 10 mL of sterile dH_2O. The lab researchers previously determined that the concentration of 100 µM glutamate was an optimal concentration to cause significant cell loss after 24-48 hours (Debnath-Canning et al., 2020; Vyas et al., 2013).

- 10 µL of 10 mM glutamate stock per mL media for a final concentration of 100 µM glutamate

Glutamate + aqueous BB + SC:

- 10uL of 10 mM glutamate stock per mL media for a final concentration of 100 µM glutamate
- 1 µL of BB extract per mL of media
- 1 µL of SC extract per mL of media

Alpha-synuclein:

The stock solution of alpha-synuclein (Abcam) was prepared was diluting 2 mg/mL alpha-synuclein. The lab researchers previously determined the concentration of 100 ng/mL as an optimal concentration to cause neuro-inflammation and decrease in cell viability (Debnath-Canning et al., 2020).

- 1 µL of alpha-synuclein stock per mL of media for a final concentration of 100 ng/mL alpha-synuclein.

Alpha-synuclein + aqueous BB + SC:

- 1 µL of alpha-synuclein stock per mL of media for a final concentration of 100 ng/mL alpha-synuclein.
- 1 µL of BB extract per mL of media
- 1 µL of SC extract per mL of media

2.2.5. Fixing, Staining, and Imaging

The media was removed, and the wells were washed twice with phosphate buffer solution (PBS) and fixed for 20 minutes using 4% paraformaldehyde (PFA). Then permeabilization of cultures was carried out using 0.2% Triton X for 10 minutes and then wells were washed twice with PBS. The cells were then dehydrated with 70% ethanol, followed by dehydration with 99% ethanol. After the cells were dried entirely, 5 µL of VectaShield mounting medium with the fluorescent stain 4', 6-diamidino-2- phenylindole (DAPI) was added to the wells and sealed with 15 mm glass coverslips.

All of the Costar® cell culture images were captured with a Zeiss A1 Observer inverted microscope with a PCO.Pixelfly qe CCD camera at a magnification of 10X using PCO Camware software. A total of five images were taken from separate locations in each well, then cells were counted manually by two individuals, and the counts were averaged.

2.2.6. Statistical analysis

The analyses for all of the cultured cells were conducted using one-way ANOVA with post-hoc tukeys HSD, with a limit for significance at alpha < 0.05. All of the data were analyzed using SPSS (IBM SPPS Statistics 27, Armonk, USA).

Chapter 3: Results

3.1. MPTP mouse model of PD

3.1.1. Rota rod

The behavioural test involving the rotarod apparatus was carried out to characterize changes in the motor function at the fixed 15 rpm (Figure 9) and the accelerating rpm (Figure 10). A one-way ANOVA comparing three different groups at a constant 15 rpm revealed no significant differences in the latency to fall between the groups ($F(2,33) = 0.378$, $p = 0.688$). Similarly, the one-way ANOVA comparing three different groups at an accelerating rpm also revealed no significant differences in the latency to fall between the groups ($F(2,33) = 0.554$, $p = 0.580$).

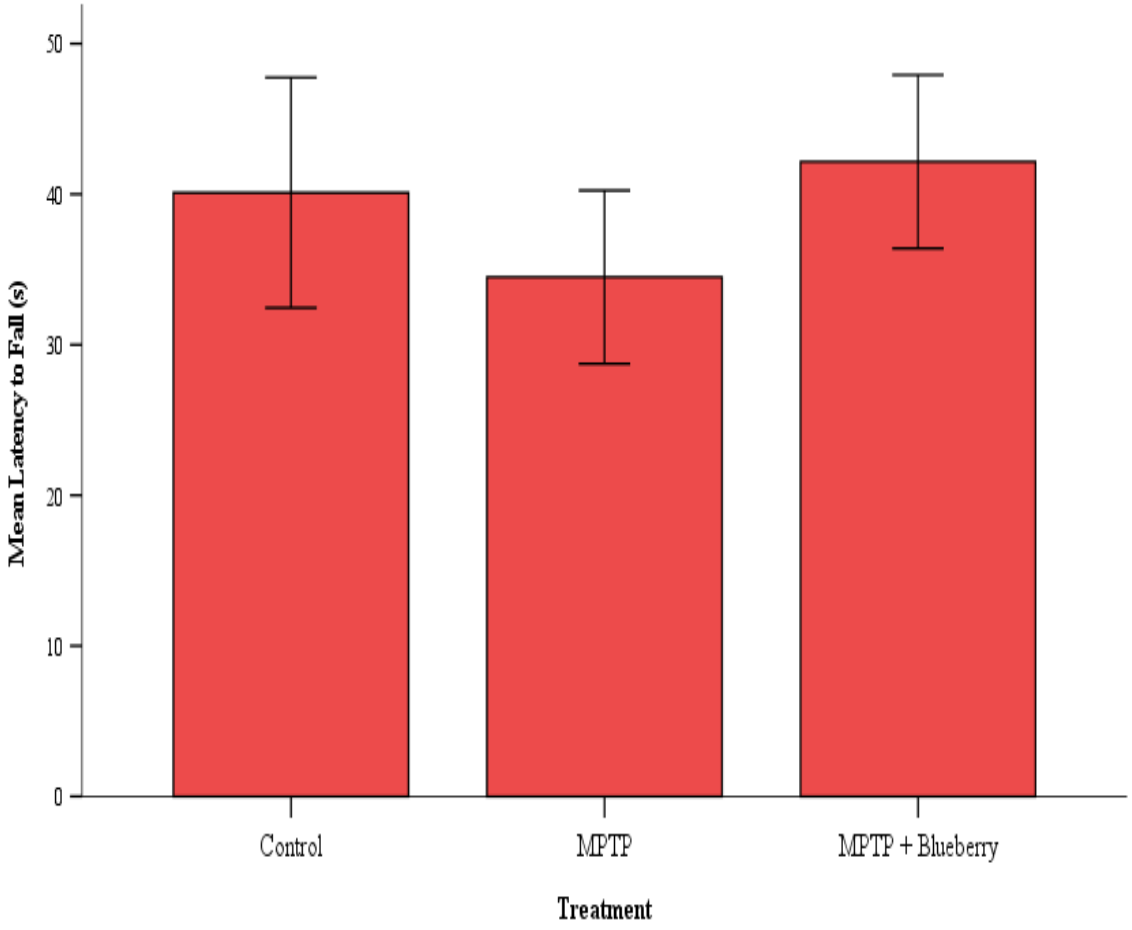

Figure 9: The average time spent by the mice on a rotarod apparatus at a constant rpm of 15. Increased latency corresponds with greater motor abilities. Data is expressed as average time in seconds ± standard error, n=12 per group. Not statistically significant at p=0.05, One-way ANOVA.

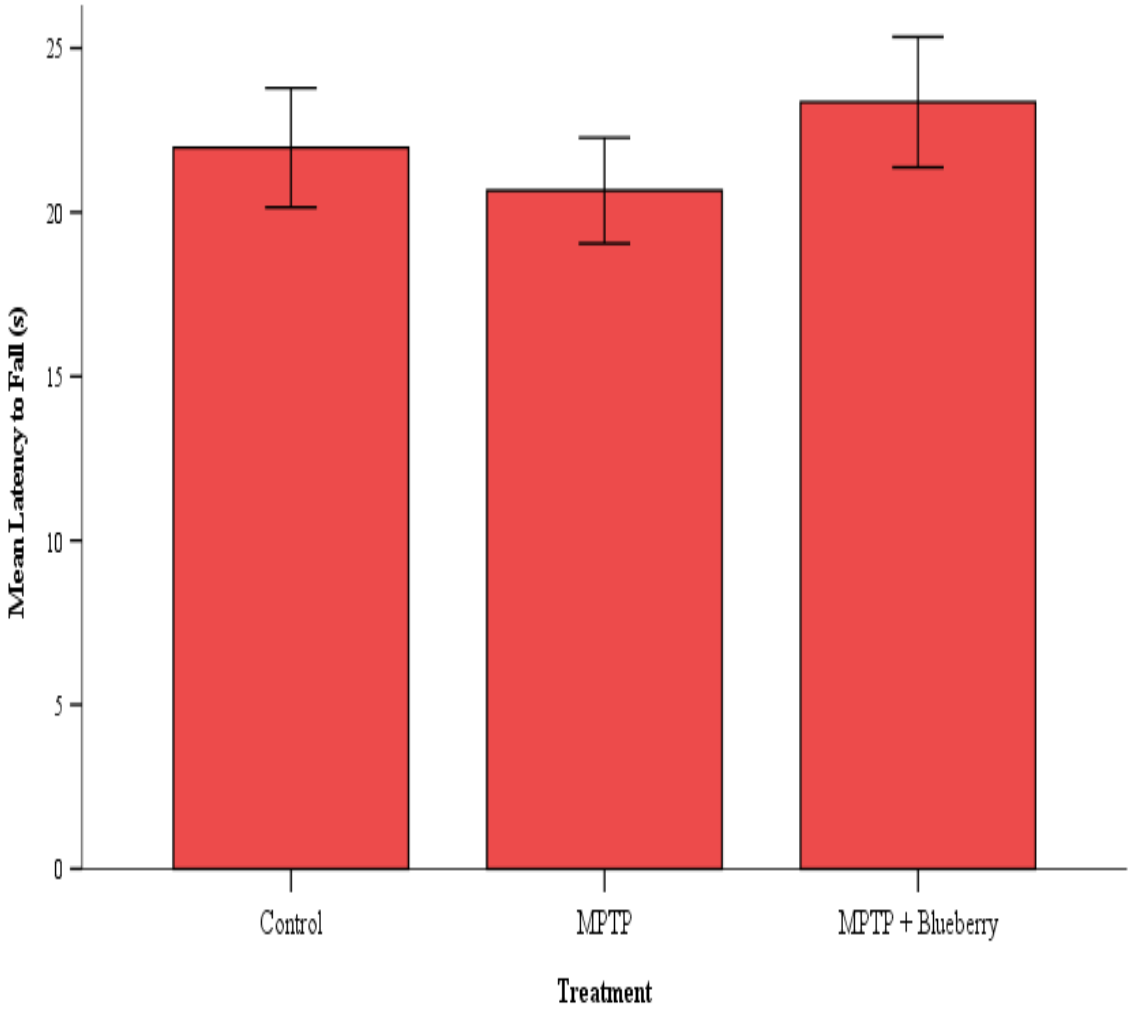

Figure 10: The average time spent by the mice on a rotarod apparatus at an accelerating 4-40 rpm. Increased latency corresponds with greater motor abilities. Data is expressed as average time in seconds ± standard error, n=12 per group. Not statistically significant at p=0.05, One-way ANOVA.

3.1.2. Open field

When mice are allowed to spend 5 minutes freely in an open box, this can also be used as a measure of motor function. A one-way ANOVA comparing the mean velocity (Figure 11) of the three different groups revealed no significant differences ($F(2,29) = 2.036$, $p = 0.149$). Similarly, the one-way ANOVA comparing the total distance travelled (Figure 12) between the three different groups revealed no significant differences ($F(2,29) = 2.051$, $p = 0.147$). The one-way ANOVA showed no significant differences ($F(2,32) = 2.243$, $p = 0.123$) comparing total time spent in the centre (Figure 13).

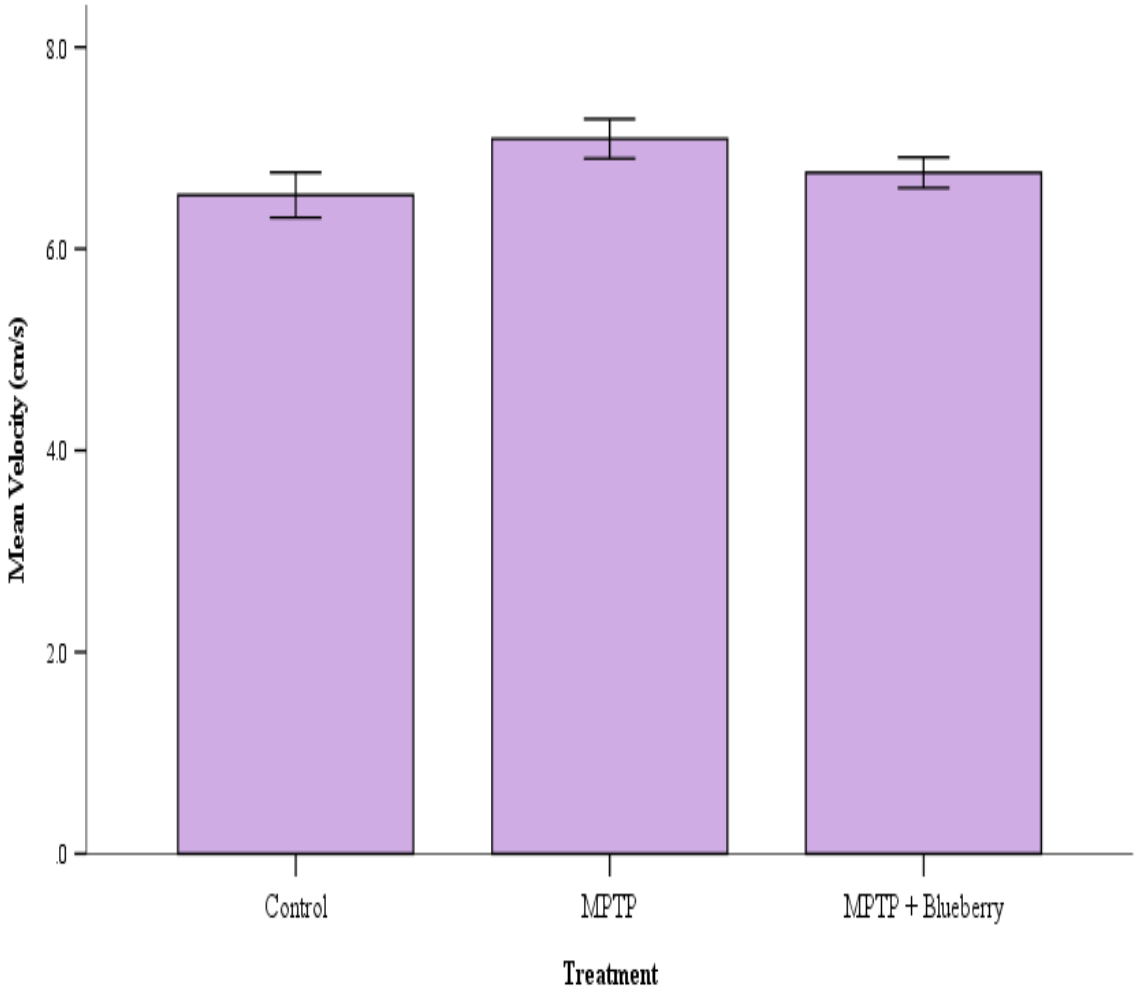

Figure 11: The mean velocity of each treatment group in the open field. The data is determined by using a behaviour tracking software, EthoVision XT10, and is expressed as mean velocity in cm/s ± standard error, n=12 per group. Data expressed as mean velocity in cm/s ± standard error. Not statistically significant at p=0.05, One-way ANOVA.

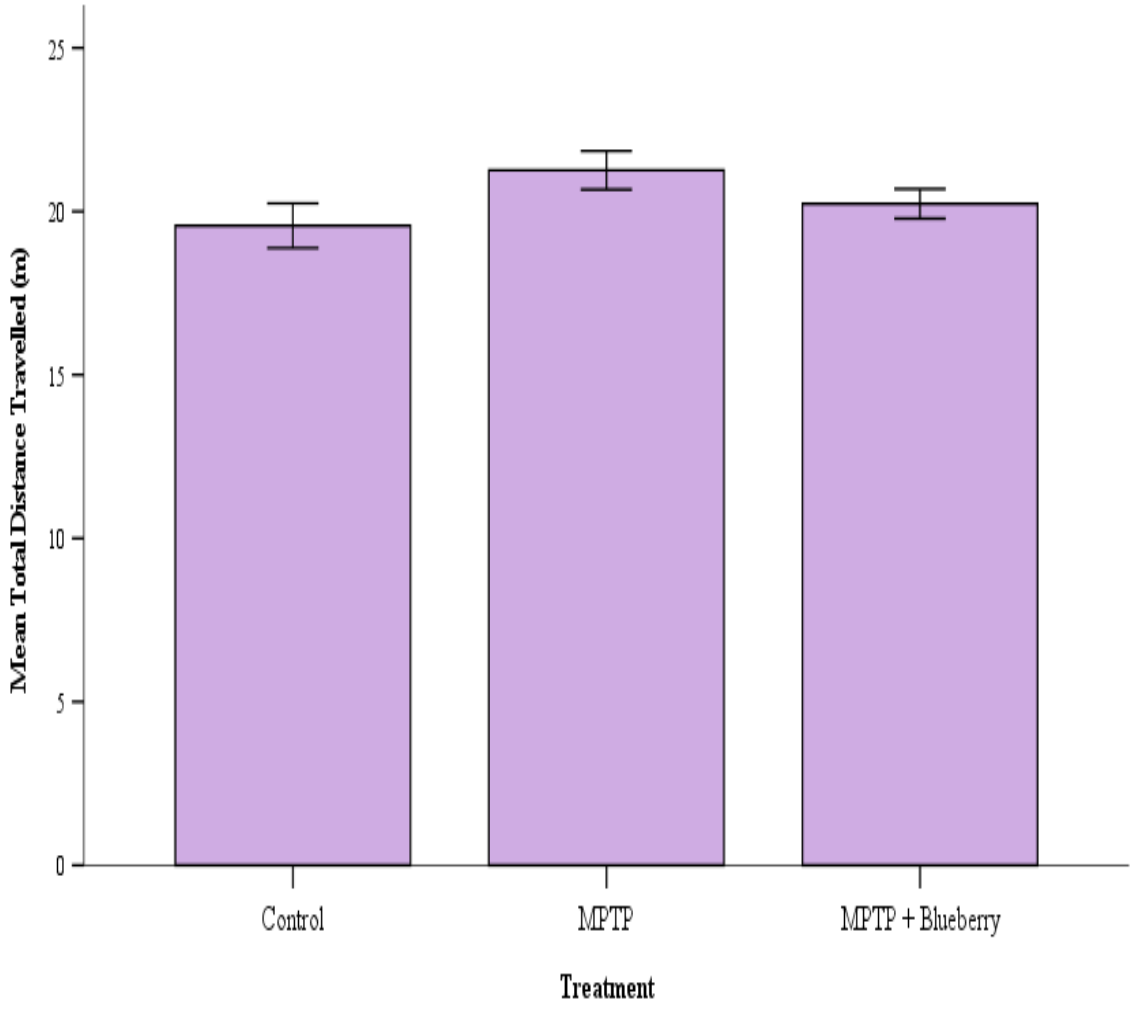

Figure 12: The average distance moved by the mice in an open field. The data is determined by using a behaviour tracking software, EthoVision XT10, and is expressed as distance travelled in m ± standard error, n=12 per group. Not statistically significant at p=0.05, One-way ANOVA.

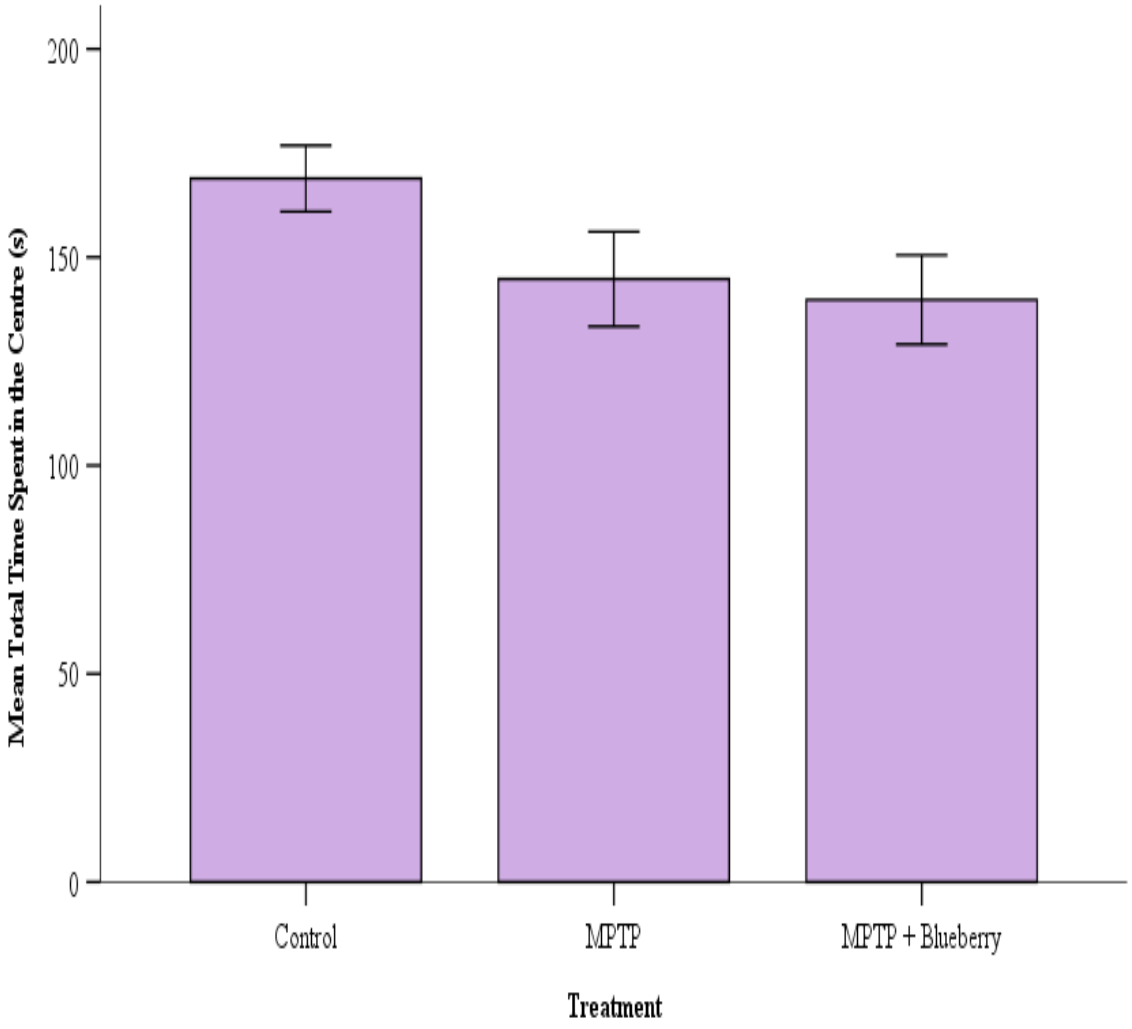

Figure 13: The average time spent by the mice in the centre of an open field. The data is determined by using a behaviour tracking software, EthoVision XT10, and is expressed as time spent in s ± standard error, n=12 per group. Not statistically significant at p=0.05, One-way ANOVA.

3.1.3. Light/dark box

The light/dark box can be used to measure anxiety-like behavior. A one-way ANOVA comparing the three different groups on time in the light side revealed no significant differences between the groups ($F(2,32) = 2.052$, $p = 0.145$), see Figure 14. There were no changes in the frequency of mice entering the light side among the treated groups.

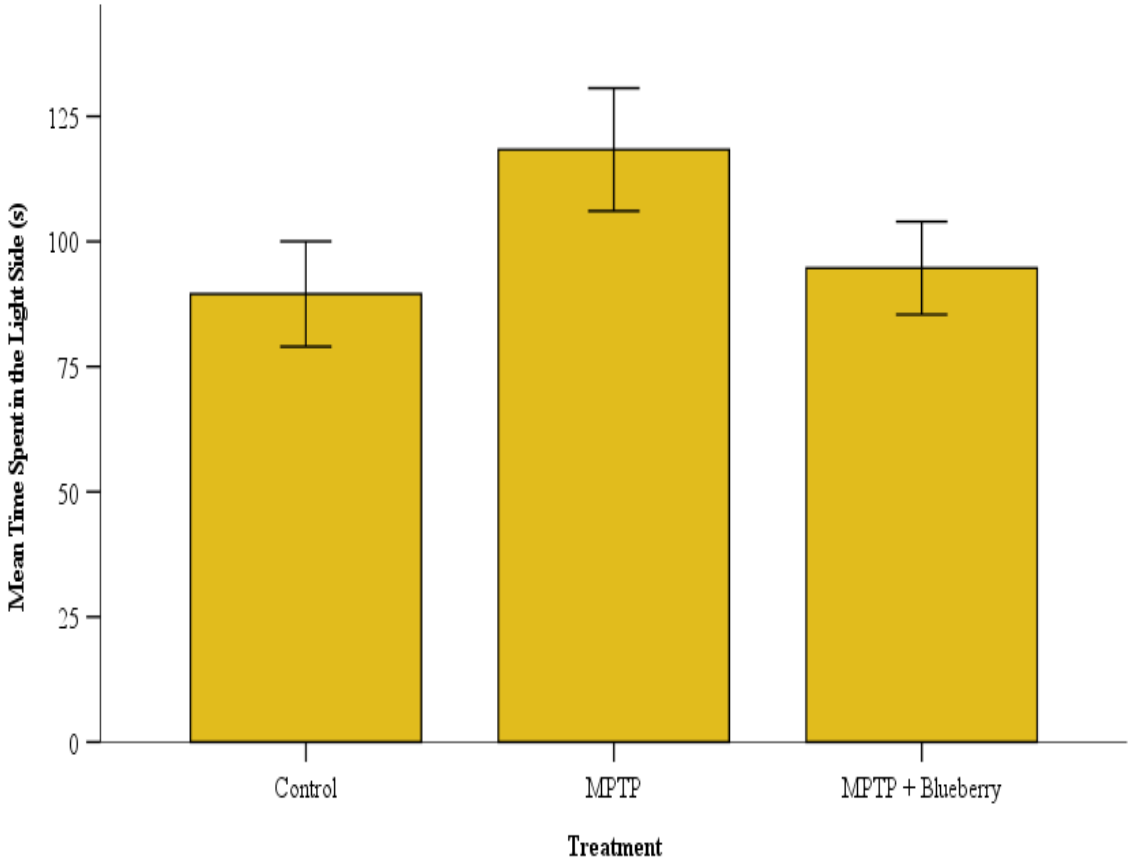

Figure 14: The average time spent by the mice in the light side of a dark and light box. Increased duration in the dark arm signifies anxiety like behavior. Data is expressed as time spent in seconds ± standard error, n=12 per group. Not statistically significant at p=0.05, One-way ANOVA.

3.1.4. Western Blot Protein Quantification

Western blotting was used to quantify the amount of tyrosine hydroxylase and alpha-synuclein in the control, MPTP, and MPTP BB treated groups. Tyrosine hydroxylase is the rate-limiting enzyme involved in the process of synthesis of DA. Alpha-synuclein is the major protein accumulated in the SNpc seen in postmortem analysis of the brain of patients.

ANOVA testing for comparing the expression of tyrosine hydroxylase in the cortical portion of the mouse brain (Figure 15) showed no significant differences in the protein content between the three groups ($F(2,14) = 1.585$, $p = 0.240$). In contrast, the midbrain one-way ANOVA comparison showed a significant difference in the tyrosine hydroxylase protein concentration between the three groups ($F(2,15) = 4.032$, $p = 0.040$). The post-hoc analysis with Tukey-HSD was carried out to determine which groups were significantly different. The control and MPTP groups in the mid brain were found significantly different with the value $p = 0.045$. However, the p-values between control and MPTP + BB was 0.890, and between MPTP and MPTP + BB was 0.104, see Figure 16. Summarizing in the whole brain (Figure 17), the tyrosine hydroxylase concentration was significantly different between the three groups ($F(2,28) = 5.498$, $p = 0.010$). The post-hoc Tukey HSD showed a significant difference between control and MPTP ($p=0.011$), and between MPTP and MPTP + BB ($p=0.042$), while no difference in the control and MPTP + BB group ($p=0.722$).

The comparison of the alpha-synuclein protein concentration between the three different groups showed no significant differences in the cortical region ($F(2,14) = 0.707$,

p= 0.510), Figure 18, in the mid brain region (F (2,14) = 1.164, p= 0.341),Figure 19, as well as in the entire brain (F (2,32) = 1.736, p= 0.192), Figure 20.

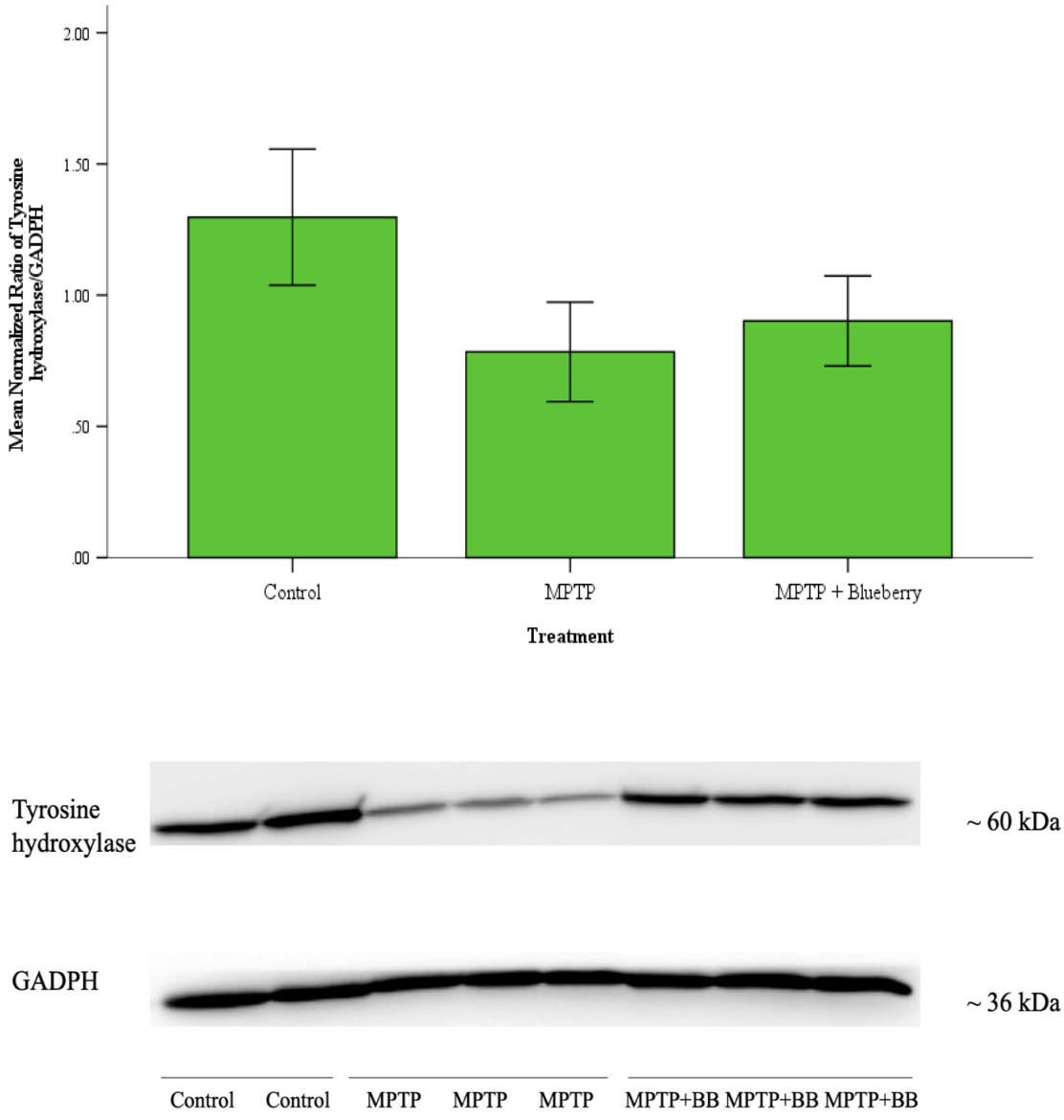

Figure 15: Protein expression of tyrosine hydroxylase in the cortex of mouse brain tissue. The protein expression is normalized using GADPH, the loading control. Top: Quantitative analysis of tyrosine hydroxylase as determined by western blotting. Bottom: Tyrosine hydroxylase and GADPH representative blots of protein expression. Data are expressed as mean normalized ratio ± standard error, n=5-6 per group. Not significant at $p < 0.05$, One-way ANOVA.

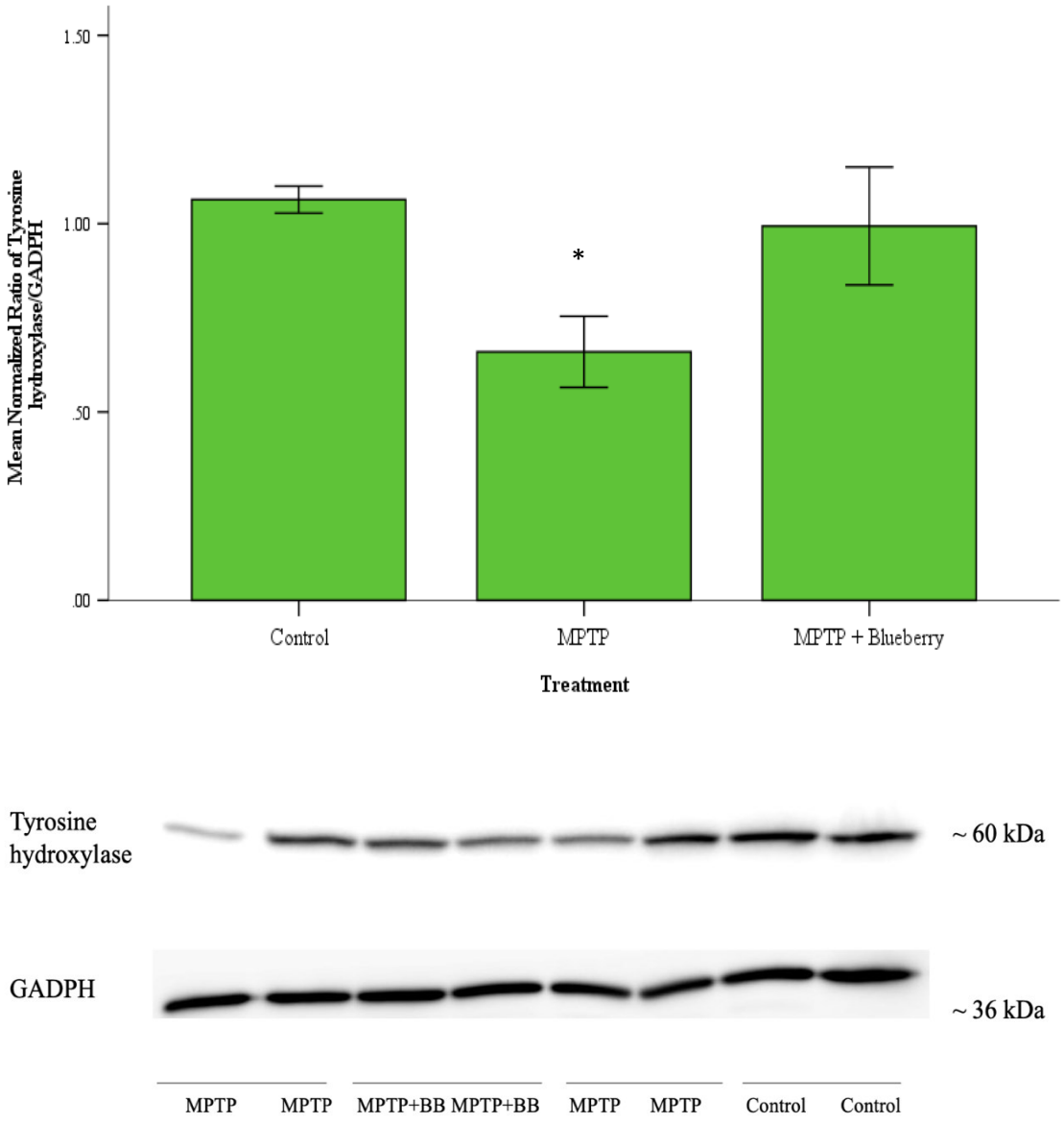

*Figure 16: Protein expression of tyrosine hydroxylase in the mid brain cells of mouse brain tissue. The protein expression is normalized using GADPH, the loading control. Top: Quantitative analysis of tyrosine hydroxylase as determined by western blotting. Bottom: Tyrosine hydroxylase and GADPH representative blots of protein expression. Data are expressed as mean normalized ratio± standard error, n=6 per group. *, p< 0.05 VS control (one-way ANOVA with Tukey's post hoc analysis).*

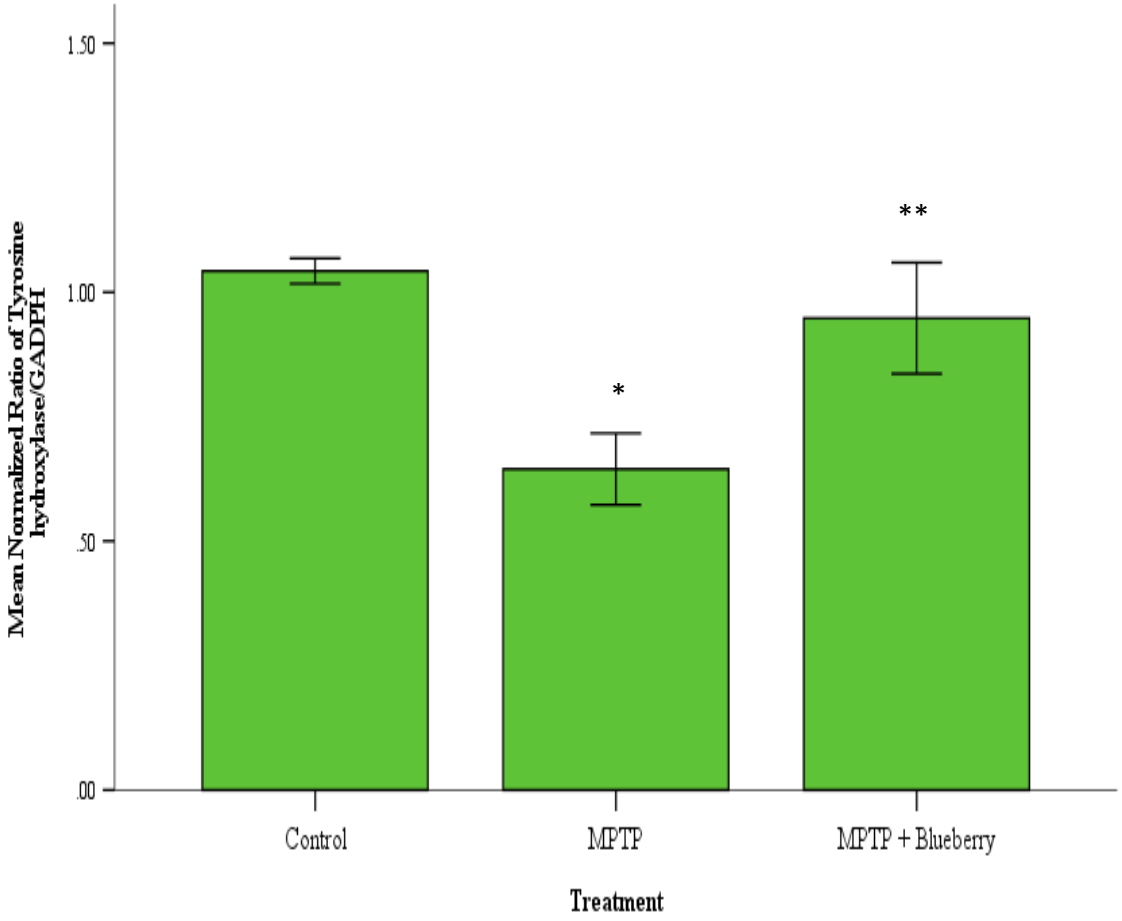

*Figure 17: Protein expression of tyrosine hydroxylase in the entire brain of the mice. The protein expression is normalized using GADPH, the loading control. Quantitative analysis of tyrosine hydroxylase as determined by western blotting. Data are expressed as mean normalized ratio± standard error, n=9-12 per group. *, p< 0.05 VS control; ** < 0.05 VS MPTP only (one-way ANOVA with Tukey's post hoc analysis).*

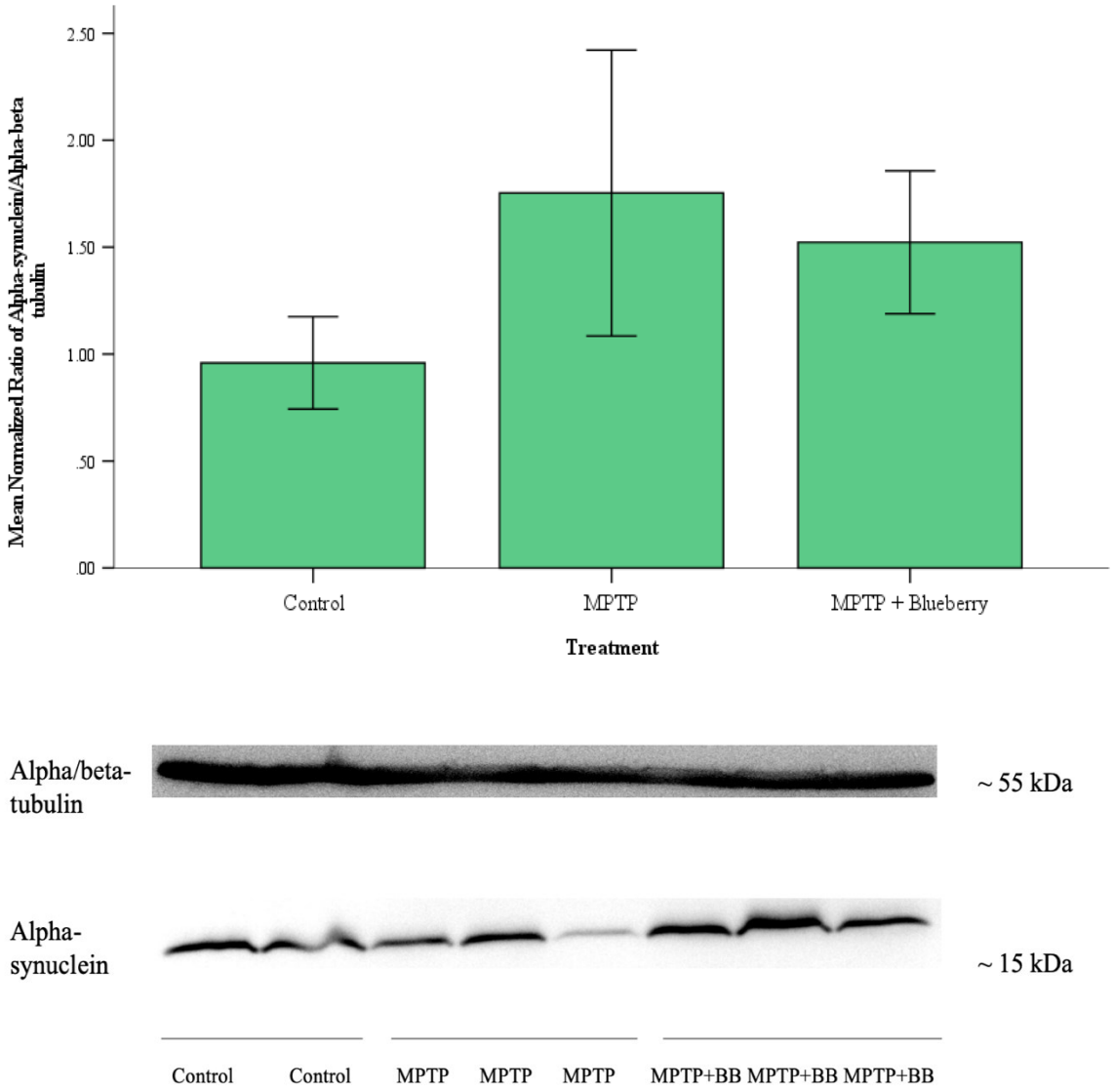

Figure 18: Protein expression of alpha-synuclein in the cortex of mouse brains. The protein expression is normalized using alpha-beta tubulin, the loading control. Top: Quantitative analysis of alpha-synuclein as determined by western blotting. Bottom: Alpha-synuclein and alpha/beta-tubulin representative blots of protein expression. Data are expressed as mean normalized ratio± standard error, n=5-6 per group, not significant at $p<0.05$, One-way ANOVA).

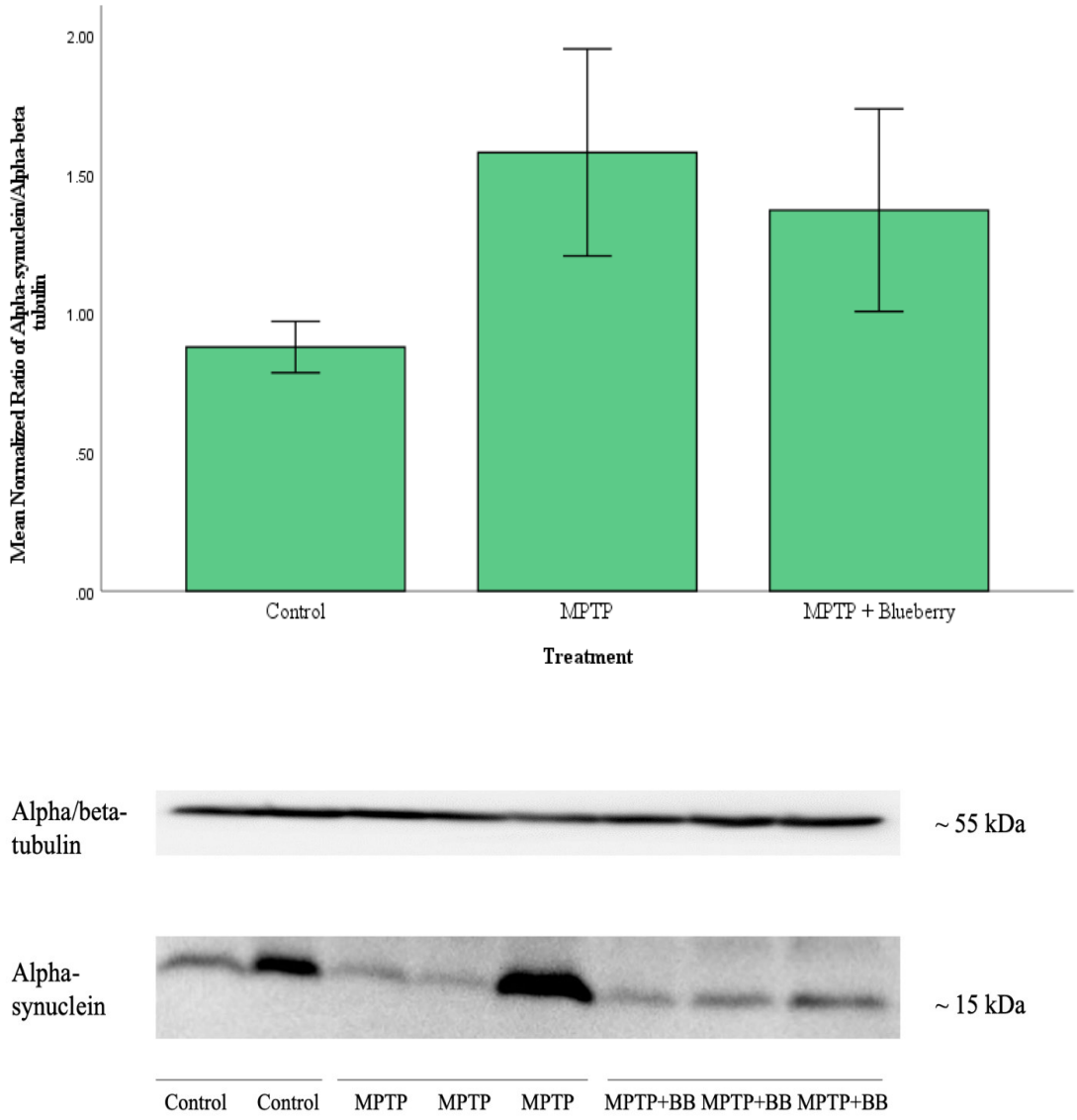

Figure 19: Protein expression of alpha-synuclein in the mid brains of mice. The protein expression is normalized using alpha-beta tubulin, the loading control. Top: Quantitative analysis of alpha-synuclein as determined by western blotting. Bottom: Alpha-synuclein and alpha/beta-tubulin representative blots of protein expression. Data are expressed as mean normalized ratio± standard error, n=5-6 per group, not significant at $p<0.05$, One-way ANOVA).

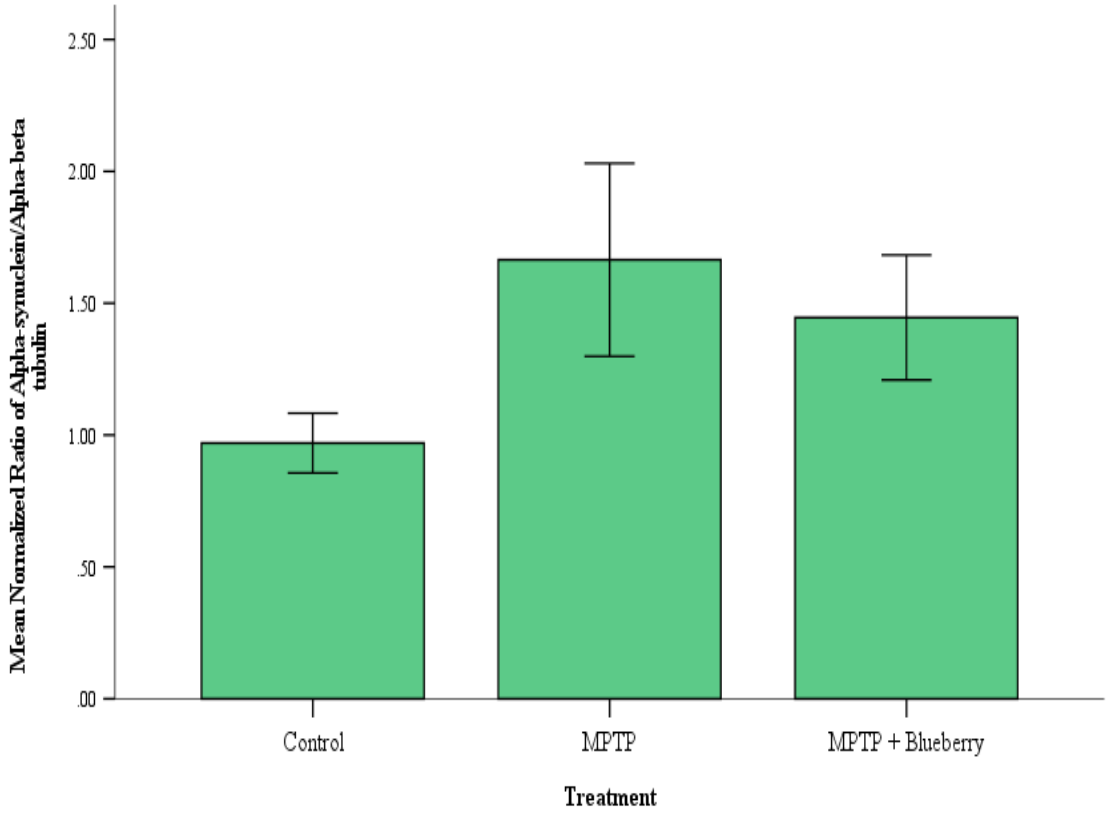

Figure 20: Protein expression of alpha-synuclein in the whole brain of the mice. The protein expression is normalized using alpha-beta tubulin, the loading control. Quantitative analysis of alpha-synuclein as determined by western blotting. Data are expressed as mean normalized ratio± standard error, n=11-12 per group. Not significant at $p<0.05$, One-way ANOVA.

3.2. Cell culture experiments

For experiments in cell cultures, DAPI-stained nuclei were counted, and the results were expressed as percentage of control. The SN cells (Figure 21) treated with glutamate resulted in cell loss with only 38.7% of control remaining. The glutamate + SC treated group seemed to inhibit cell death averaging 89.7% of control, and glutamate + SC + aqueous BB averaged 70.4% of control (see Figure 22). Similarly, the alpha-synuclein-treated cells also suffered cell death, resulting in an average cell count of 71.5% of control. Upon treatment, alpha-synuclein + SC seemed protective and averaged 87.9% control, while alpha-synuclein + SC+ aqueous BB also averaged 76.6% of control (see Figure 23). These SN cells under certain treatment conditions showed significant differences between the groups ($F(6,41) = 4.802$, $p = 0.01$). The post-hoc analysis with Tukey-HSD was carried out to determine which groups were significantly different. The post hoc analysis showed that control and glutamate ($p = 0.000$), and glutamate and glutamate + SC ($p = 0.010$) had significant differences. Other treatments group were not significant to each other. SC and BB extracts present with alpha-synuclein in SN cells also showed no significant differences, alpha-synuclein vs alpha-synuclein + SC ($p = 0.895$), alpha-synuclein vs alpha-synuclein + SC + aqueous BB ($p = 1.000$) (for detail p values see Appendix-4).

Similarly, the RNC treated with glutamate resulted in cell death, causing the average cell count to decrease to 40.9% of control, shown by reduced DAPI-stained nuclei count (Figure 24). The treatment including glutamate + SC treated group averaged 146.6 % of control, and glutamate + SC + aqueous BB averaged 152.7 % of control (see Figure 25). Similarly, the alpha-synuclein-treated cells averaged 70.8% of control, alpha-

synuclein + SC averaged 98.3% control, and alpha-synuclein + SC + aqueous BB averaged 131.7 % of control (see Figure 26). The one-way ANOVA for the RNC showed a significant difference between the treatment groups ($F(6,33) = 4.082$, $p = 0.004$). The post-hoc Tukey's HSD showed that glutamate and glutamate + SC ($p = 0.015$) and glutamate + SC + aqueous BB ($p = 0.005$) had significant differences. However, control and glutamate ($p = 0.278$), glutamate + SC ($p=0.658$), glutamate + SC + aqueous BB ($p = 0.458$) showed no significant differences. Similarly, glutamate + SC and glutamate + SC + aqueous BB showed no significant differences ($p = 1.000$). Also, alpha-synuclein and alpha-synuclein + SC ($p = 0.981$) and alpha-synuclein + SC + aqueous BB ($p = 0.474$) showed no significant differences (for detail p values see Appendix-4).

The mixed cultures (Figure 27) treated with glutamate decreased cell count to 60.6% of the control group. When treated with glutamate enriched with SC, the cell count increased to 147.0% of control. Similarly, glutamate + SC + aqueous BB had a cell count of 122.2% of control, and glutamate + aqueous BB increased to 168.8% of control. The one-way ANOVA for the mixed cultures showed a significant difference between the treatment groups ($F(4,15) = 4.642$, $p = 0.012$). The post-hoc Tukey's HSD showed a significant difference between glutamate and glutamate + SC ($p = 0.046$) and glutamate + aqueous BB ($p = 0.010$) (see Figure 28). While, the other groups were not significant to one another, for detail p values see Appendix-4.

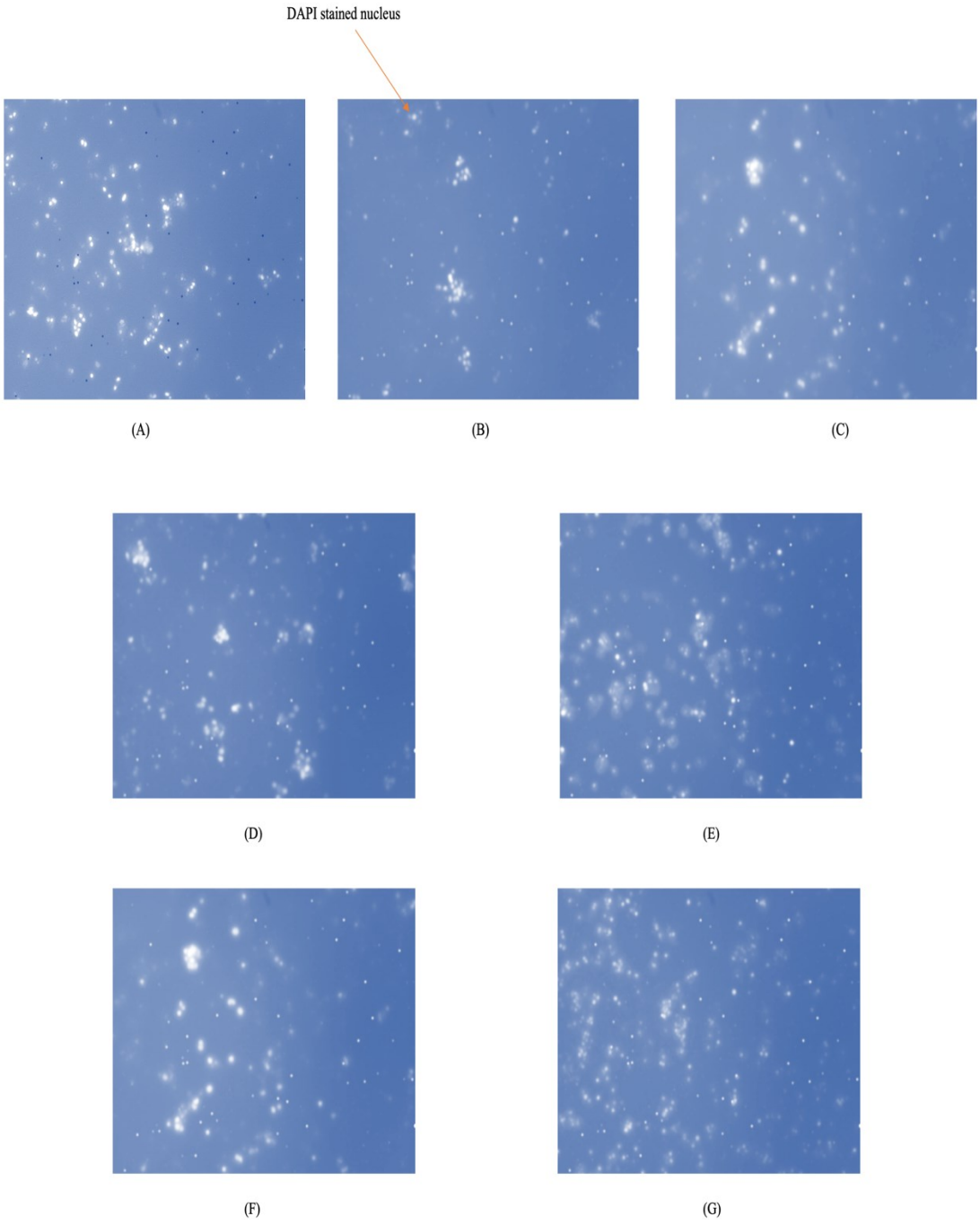

Figure 21: Representative figures of DAPI-stained nuclei in substantia nigra cells with different treatment groups. (A) Control (B) Glutamate (C) Alpha-synuclein (D) Glutamate + Sea cucumber (E) Glutamate + Sea cucumber + Aqueous blueberry (F) Alpha-synuclein + Sea cucumber (G) Alpha-synuclein + Sea cucumber + Aqueous blueberry.

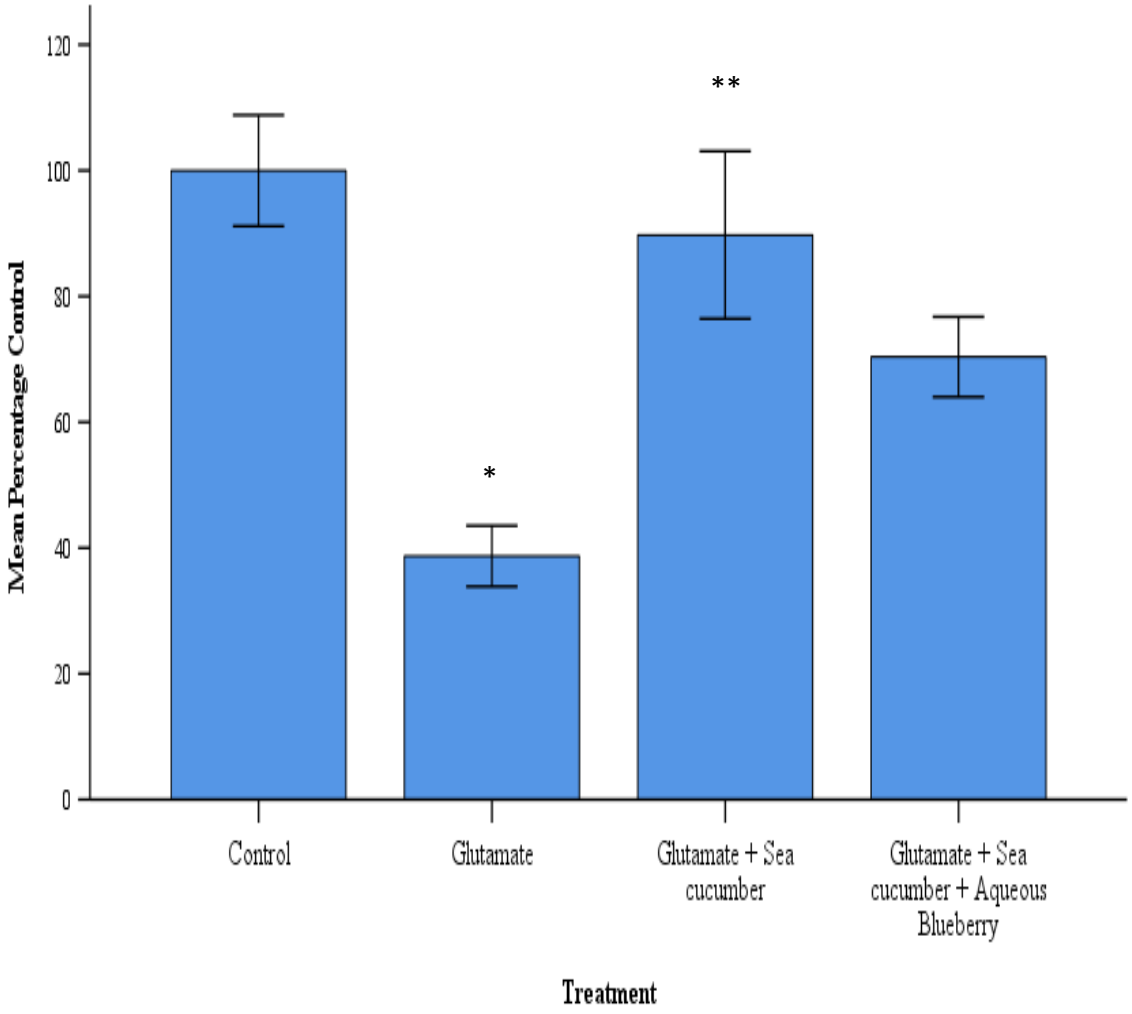

*Figure 22: Substantia nigra cells treated with 100 µM glutamate for 24 hrs in the presence or absence of extracts from sea-cucumber and aqueous blueberry. The graph represents the effect of sea-cucumber and aqueous blueberry on glutamate mediated cell death. The amount of DAPI-nuclei was quantified, and data are expressed as percent of control values± standard error, n=6-10. * p< 0.05 VS control; ** p< 0.05 VS glutamate only (one-way ANOVA with Tukey's post hoc analysis).*

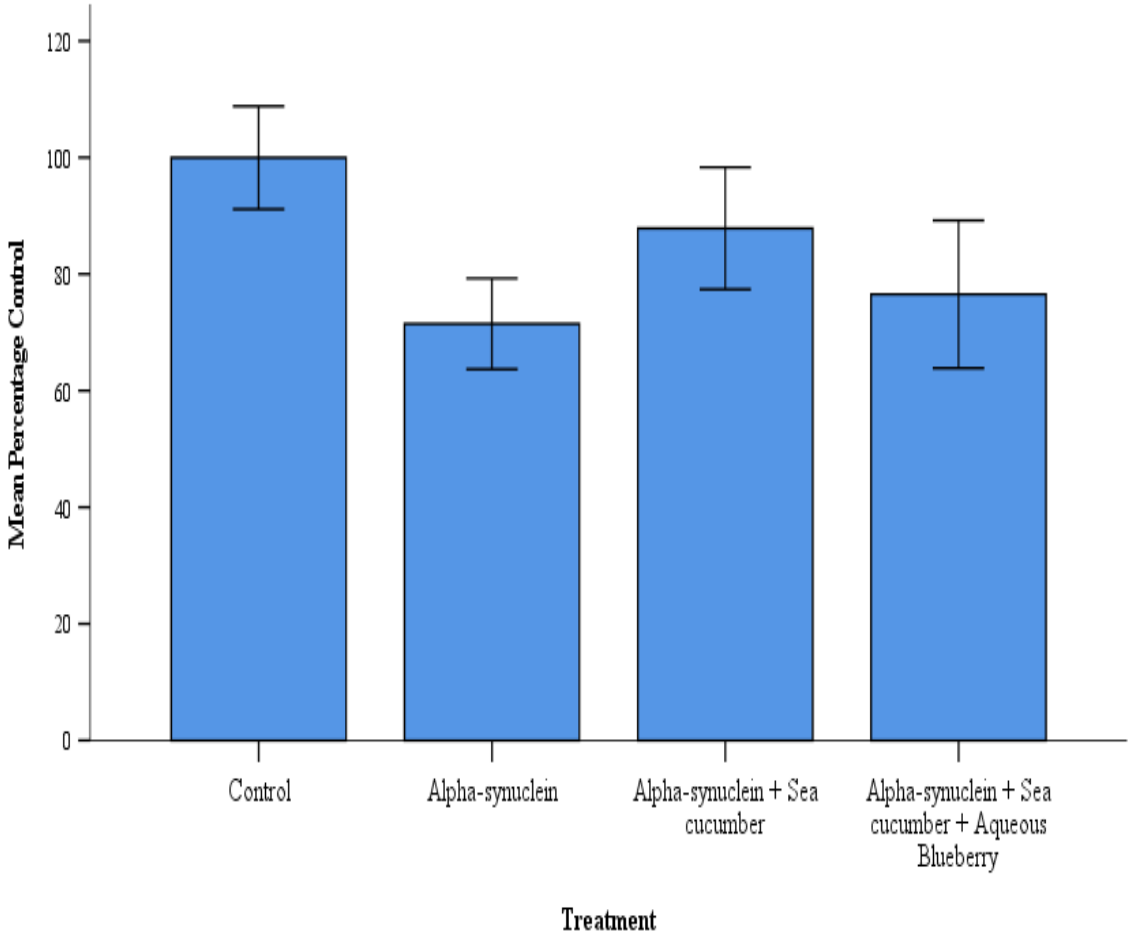

Figure 23: Substantia nigra cells treated with 100 ng/mL of alpha-synuclein for 24 hrs in the presence or absence of extracts from sea-cucumber and aqueous blueberry. The graph represents the effect of sea-cucumber and aqueous blueberry on alpha-synuclein mediated cell death. The amount of DAPI-nuclei was quantified, and data are expressed as percent of control values± standard error, n=6-10. Not significant, One-way ANOVA.

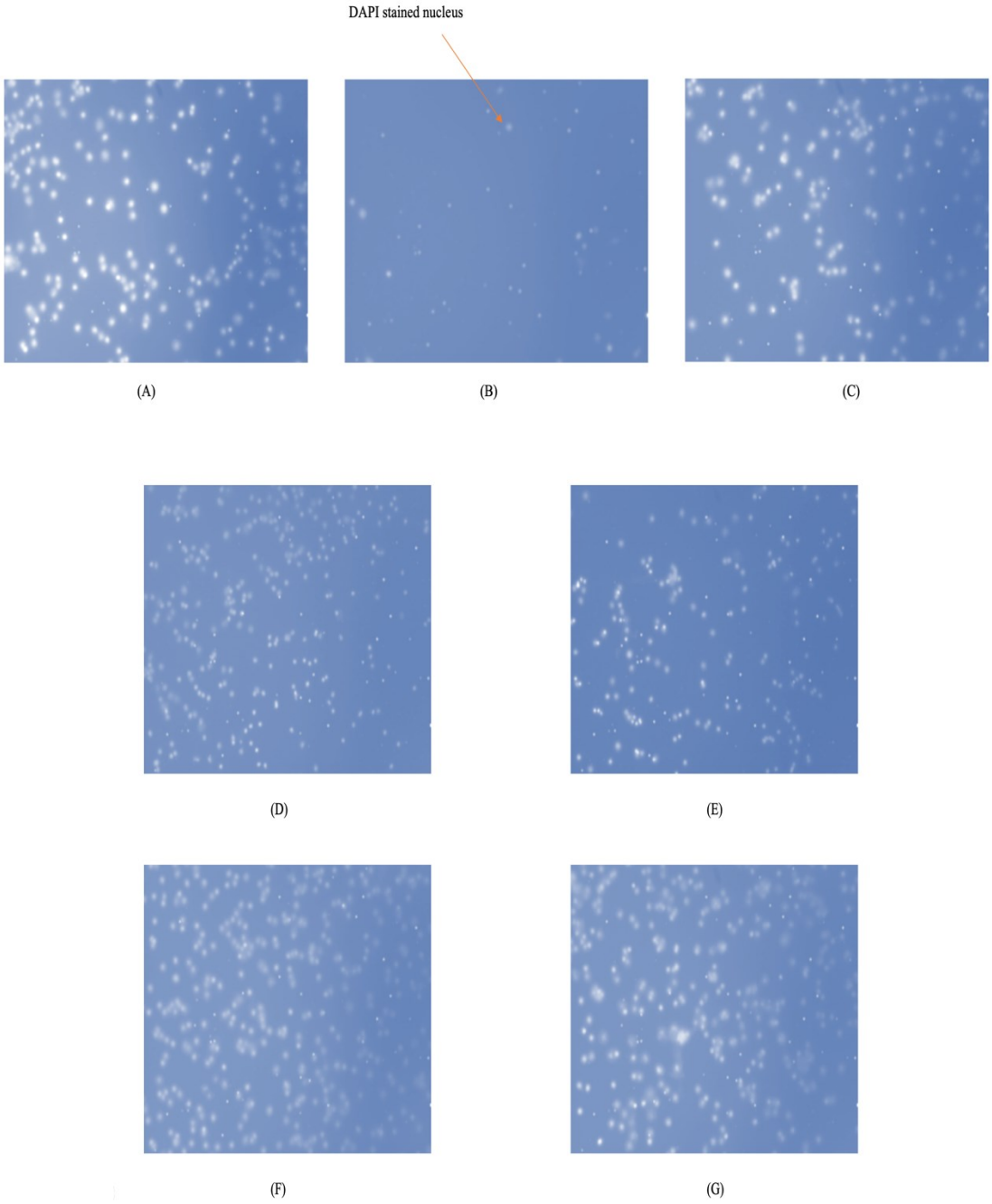

Figure 24: Representative figures of DAPI-stained nuclei in rat neocortical cells with different treatment groups. (A) Control (B) Glutamate (C) Alpha-synuclein (D) Glutamate + Sea cucumber (E) Glutamate + Sea cucumber + Aqueous blueberry (F) Alpha-synuclein + Sea cucumber (G) Alpha-synuclein + Sea cucumber + Aqueous blueberry.

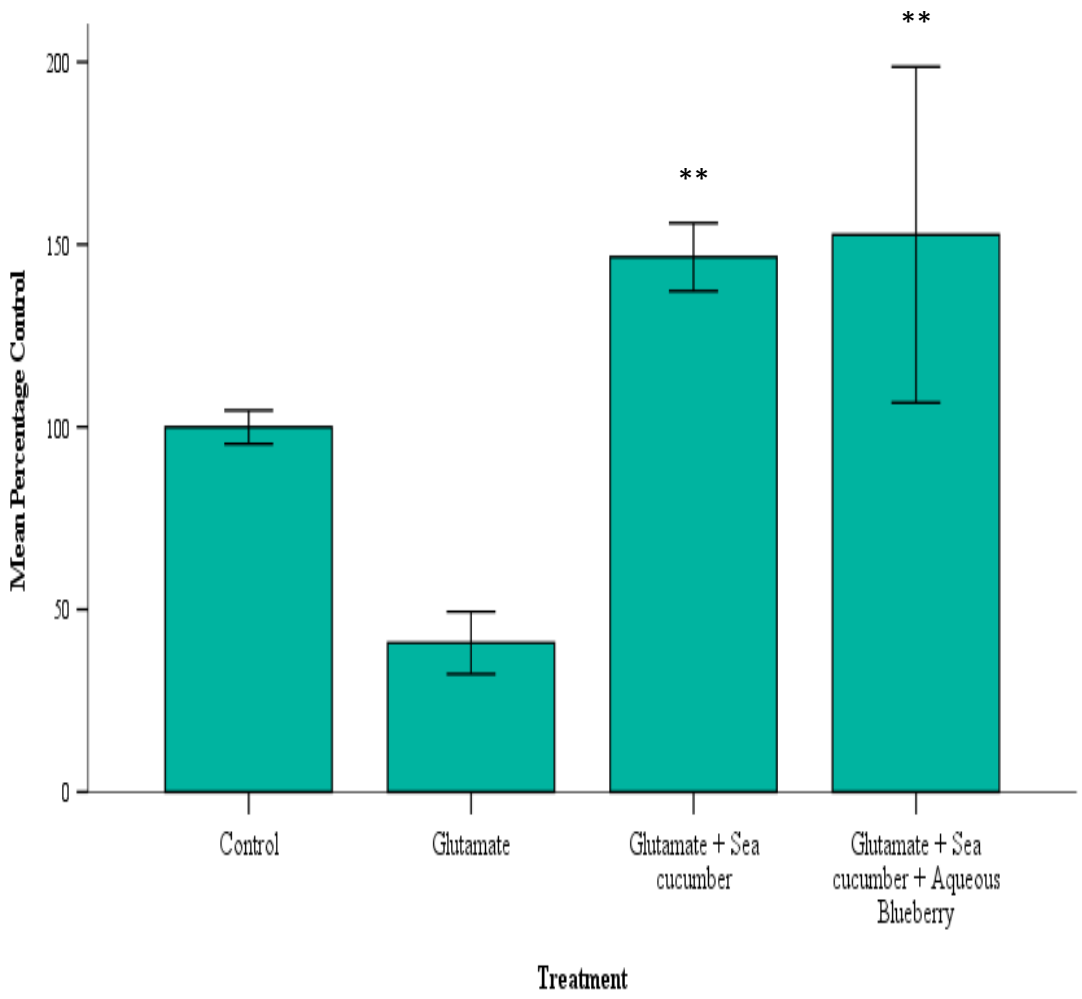

*Figure 25: Rat neocortical cells were treated with 100 µM glutamate for 24 hrs in the presence or absence of extracts from sea-cucumber and aqueous blueberry. The graph represents the effect of sea-cucumber and aqueous blueberry on glutamate mediated cell death. The amount of DAPI-nuclei was quantified, and data are expressed as percent of control values± standard error, n=5-8. ** p < 0.05 VS glutamate only (one-way ANOVA with Tukey's post hoc analysis).*

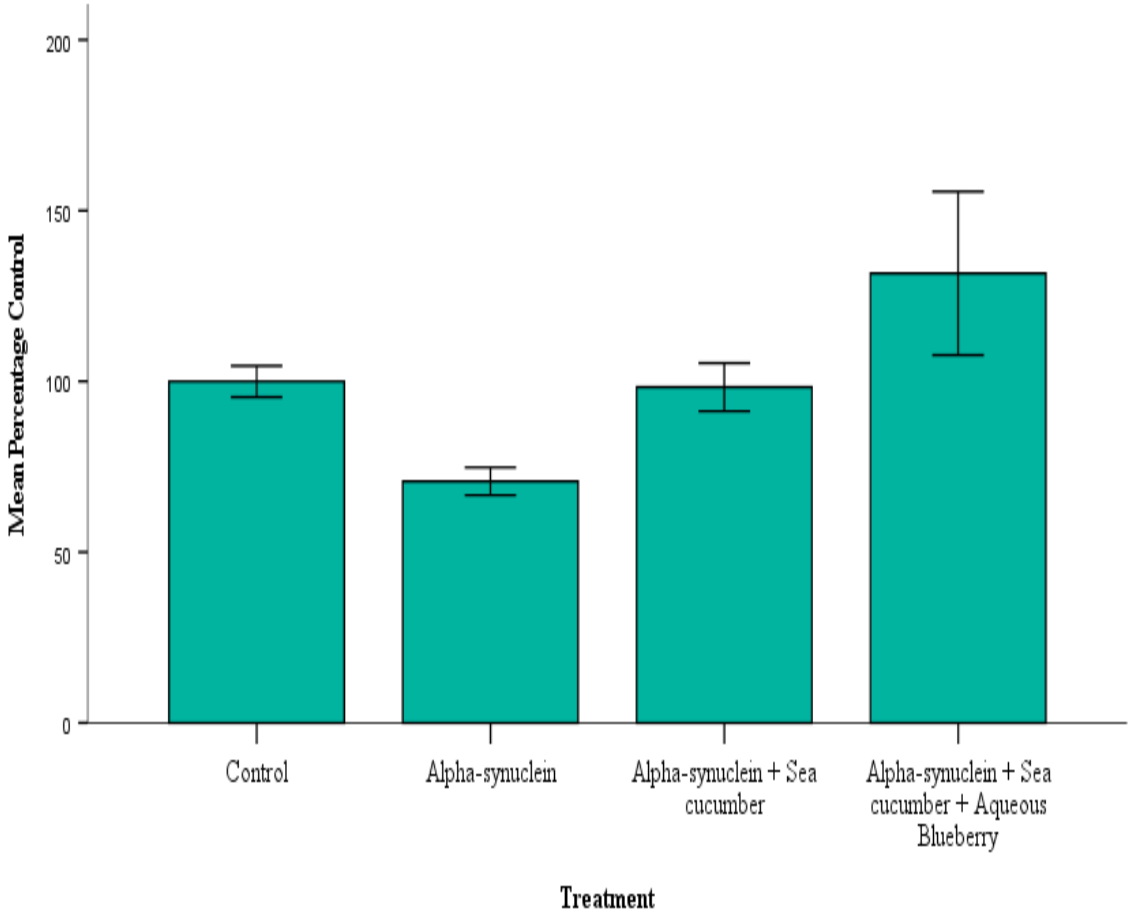

Figure 26: Rat neocortical cells treated with 100 ng/mL alpha-synuclein for 24 hrs in the presence or absence of extracts from sea-cucumber and aqueous blueberry. The graph represents the effect of sea-cucumber and aqueous blueberry on alpha-synuclein mediated cell death. The amount of DAPI-nuclei was quantified, and data are expressed as percent of control values± standard error, n=4-8. Not significant, one-way ANOVA.

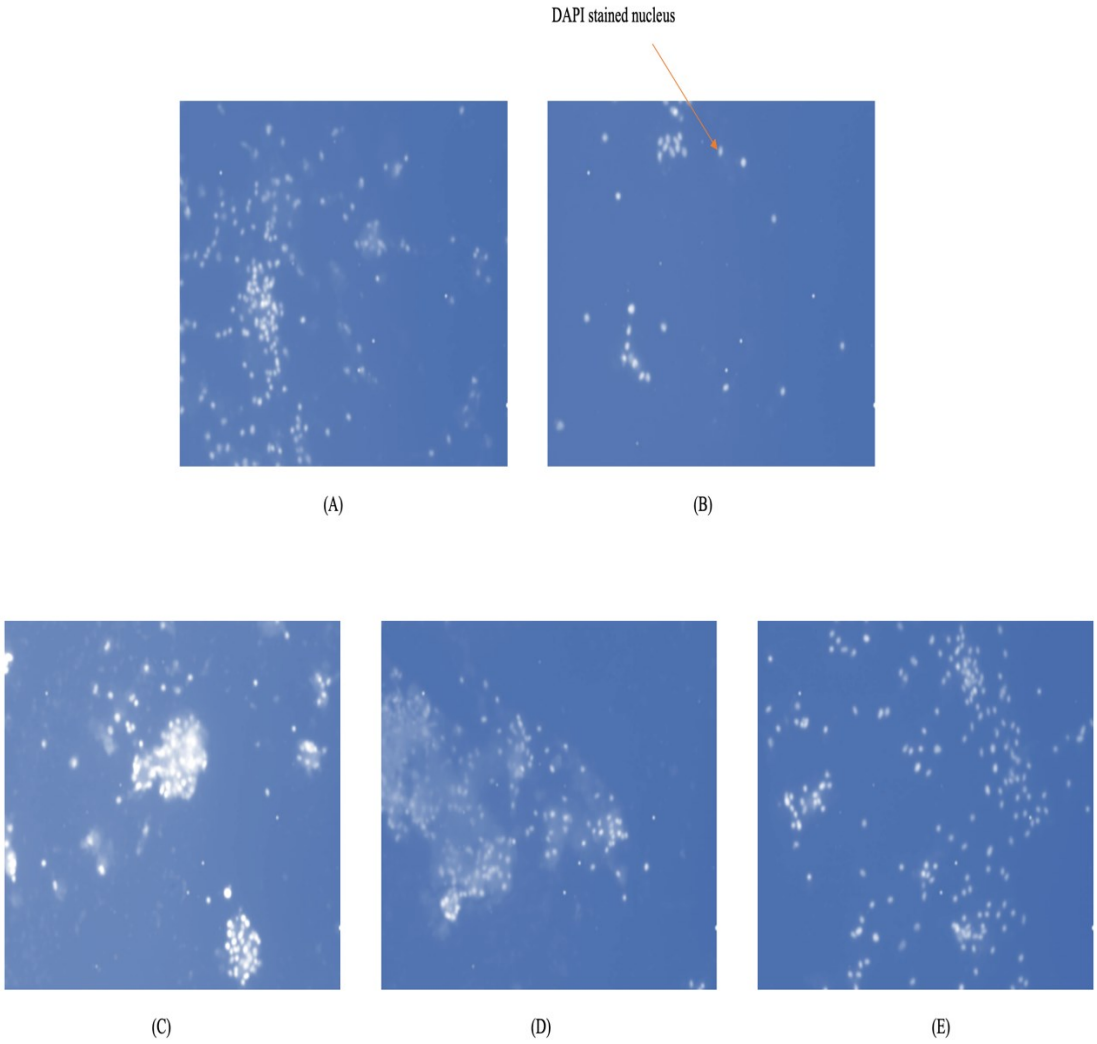

Figure 27: Representative figures of DAPI-stained nuclei in mouse brain mixed cultures with different treatment groups. (A) Control (B) Glutamate (C) Glutamate + Sea cucumber (D) Glutamate + Sea cucumber + Aqueous blueberry (E) Glutamate + Aqueous blueberry.

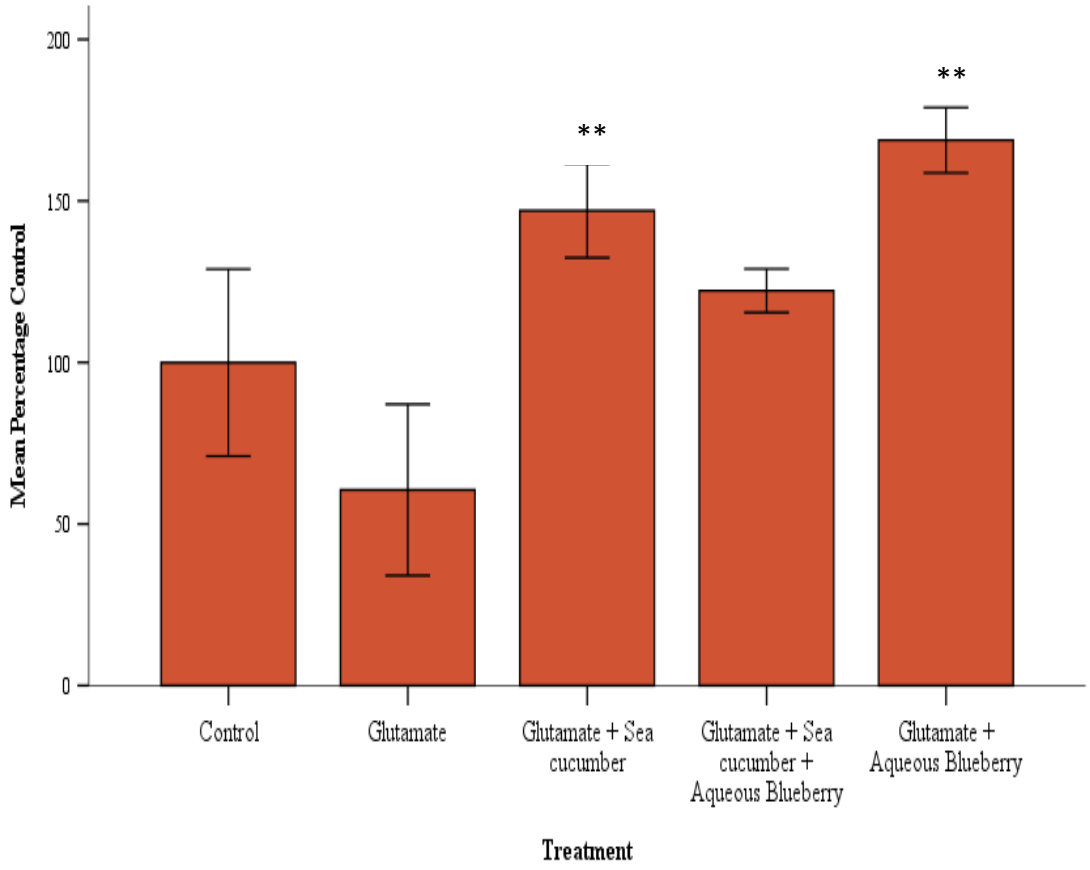

*Figure 28: Mouse brain mixed culture cells treated with 100 µM glutamate for 24 hrs in the presence or absence of extracts from sea-cucumber and aqueous blueberry. The graph represents the effect of sea-cucumber and aqueous blueberry on glutamate-mediated cell death. The amount of DAPI-nuclei was quantified, and data are expressed as percent of control values± standard error, n=4. ** p< 0.05 VS glutamate only (one-way ANOVA with Tukey's post hoc analysis).*

Chapter 4: Discussion

To study the neuroprotective effects of BB the MPTP mouse model of PD was used where C57BL/6 strain male mice were injected with the MPTP neurotoxin, and behavioural and cellular protein changes were analyzed. Overall, there were no significant behavioural changes observed among the treated groups. At the same time, the tyrosine hydroxylase levels were significantly improved in the mid-brain region of the group receiving the BB enriched diet.

The cellular studies involved SN, RNC, and mixed cultures treated with or without 100 μM glutamate or 100 ng/mL alpha-synuclein in the presence or absence of SC and aqueous BB extracts. The purpose of this study was to explore the neuroprotective effects of these extracts. The SC extracts with glutamate exposure increased cell counts in all three types of cell cultures compared to glutamate. Meanwhile, SC + aqueous BB significantly improved cell counts in RNC and mixed cultures compared to glutamate.

4.1. MPTP mouse model of PD

The behavioural tests with MPTP lesioned mice show variation among different inbred strains, apparently lacking the reproducibility of some behavioural results (Deacon et al., 2007; Wahlsten et al., 2003). Also, MPTP injected mice may show considerable recovery from injections; mice look normal, thus requiring challenging behavioral tests to unravel deficits (Willis et al., 1987). The rotarod and open field tests provide a reliable measure of motor function for MPTP injected mice (Hutter-Saunders et al., 2012).

Traditionally, assessment via rotarod has been used to understand motor coordination in mice, where mice are placed on a horizontal rod rotating at fixed or accelerating conditions (Deacon, 2013). The sensitivity and reproducibility of an accelerated rotarod are reportedly more remarkable than those obtained with constant speed rotarod (Jones et al., 2011). Also, it is reported that fewer animals can obtain a statistically significant result and give a more accurate measure of motor coordination as it avoids any effect on memory (Jones et al., 2011). Our results do not indicate any significant differences among the treatment group, suggesting MPTP-treated mice may have recovered functionally or the lesion induced may have been moderate. The lack of measurement of pre-treatment performance may have caused variation in the results. The study suggests that pre-treatment performance and using a rat size rod instead of mouse size may reduce the degree of variations similar to pervious findings (Hutter-Saunders et al., 2012). An open field test is used to measure anxiety and movements in a box (Kraeuter et al., 2019; Seibenhener et al., 2015). The major limitation in an open field can be due to variabilities such as time, novel object and light conditions (DeFries et al., 1966; Russell et al., 1973; Seibenhener et al., 2015; Valle, 1970). Our study does not indicate any difference in distance traveled or the time spent in the centre by the mice in the three different treatment group, suggesting similar results as above.

Recent data suggested that MPTP-treated mice have increased anxiety-like behaviours as measured in the elevated plus-maze tests (Cunha et al., 2017), and light/dark box (Mitsumoto et al., 2019). Hence, we measured anxiety-like in the light/dark box in MPTP-treated mice. Our results show no significant difference among the three

treatment groups, suggesting that the MPTP group did not display obvious signs of anxiety.

The major limitation in the behavioural studies are its inherent complexity requiring consideration of aspects such as motivation in the test, sources of variability, the interaction between the animals and experimenter, and sensory modality required by animals to solve the task (HÃ¥nell et al., 2014; Sorge et al., 2014; McCall et al., 1969; Schmitt et al., 1998). Also, factors such as housing conditions, testing conditions, lab environment and the sex and strain of animals affect the reproducibility the test (Chesler et al., 2002; Crabbe et al., 1999; Mandillo et al., 2008; Saré et al., 2021).

Studies have shown that mice aged 8-12 months have extensive neurodegeneration while younger animals aged 6-8 weeks have relatively less degeneration in SN after MPTP administration (Ricaurte et al., 1987). Also, MPTP injection showed 63% depletion in the younger group while 87% depletion in older mice (Ricaurte et al., 1987). It has been suggested that aging influences the vulnerability of dopaminergic neurons in the SN of mice due to the neurodegenerative effects of MPTP (Ricaurte et al., 1987). As in our study, mice were eight weeks old when we started the experiment and approx.12 weeks when we sacrificed them, which might explain a lesser extent of neurodegeneration. Some studies have shown that changes in behaviour and locomotor functions produced by MPTP in the mouse are of much shorter duration. MPTP-treated mice have been reported to appear relatively normal within a week after MPTP administration, as reviewed in (Heikkila et al., 1989). This suggests that the damage created by MPTP may have been reversed partially, so changes in behaviour were not significant.

Some studies have shown that MPTP results in massive loss of dopaminergic neurons in the SN and striatum of mice. For example, tyrosine hydroxylase activity has been reduced in the SN and striatum of MPTP-treated mice using western blotting quantification and immunohistochemistry (Guo et al., 2016; Wang et al., 2019). Another study showed that after nigral lesions TH activity was reduced 40-75 percent on lesion side of the caudate nucleus and putamen than the corresponding intact side (Goldstein et al., 1969). Treatment with polyphenols such as theaflavin (Anandhan et al., 2012) and resveratrol (Abolaji et al., 2018) have improved tyrosine hydroxylase activity in MPTP treated mice due to their antioxidant capacity. BB supplementation has also shown reduced TH loss in the striatum of 6-OHDA-induced PD model rats (Parra-Paz et al., 2021). Also, supplementation of fetal locus coeruleus with BB has shown enhanced survival of TH-positive neurons in ventral mesencephalic tissues (Berglöf et al., 2009). A similar effect is seen in the levels of TH in this study. As shown in Figure 16, the TH level is significantly reduced in the mid-brain, reflecting damage in the DA synthesis pathway. As shown in Figure 17, the MPTP-induced reduction in TH levels in the whole brain were significantly improved in the BB enriched group.

Alpha-synuclein aggregates are highly phosphorylated at *ser-129* in PD patients. Small amounts of phosphorylated alpha-synuclein are present in the soluble fractions of both normal and diseased brains, suggesting Lewy body-associated forms are produced during the normal metabolism of alpha-synuclein (Anderson et al., 2006). However, detection of alpha-synuclein during immunoblotting is challenging as the protein can get detached from the membrane during incubation. Therefore, studies have shown that the addition of 4% PFA before blocking the blots has increased immunodetection of alpha-

synuclein (Lee et al., 2011; Newman et al., 2013; Sasaki et al., 2015; Wang et al., 2019). In addition, some studies have shown that the levels of alpha-synuclein increased after injection of MPTP. For example, the use of catalpol prevents such MPTP-induced increments in the level of alpha-synuclein (Wang et al., 2019). In this study, although the level of alpha-synuclein seems to be increased after MPTP induction, the difference is not statistically significant. However, the trends in this result, as shown in Figure 18, Figure 19, and Figure 20 are similar to the results in the literature (Wang et al., 2019).

Even though no significant behavioural deficits were seen in our study, there were differences in the level of TH in the brain tissues. Even though any obvious behavioural phenotype cannot be picked up, the changes in brain tissues occurred. This phenomenon is similar to actual PD. When the significant PD symptoms are exhibited, there is a significant amount of cell loss already, such as only after DA decreases to 70-80% significant motor changes occur as mentioned earlier in the introduction (Bernheimer et al., 1973).

Various natural products such as echinacoside (extracted from *Cistanche deserticola)* (Liang et al., 2019), and catalpol (extracted from the roots *of Rehmania radix*) (Wang et al., 2019) have shown neuroprotective effects in the MPTP mouse model of PD. The polyphenol resveratrol, present in red wine and grapes, has improved motor function and pathological changes induced in MPTP-treated mice (Guo et al., 2016) due to its antioxidant and anti-inflammatory properties (Abolaji et al., 2018). Similarly, the polyphenol theaflavin, found in black tea, also protects MPTP induced mice from neurodegeneration due to its anti-oxidative and anti-apoptotic activity (Anandhan et al., 2012). Studies have shown that supplementation with dietary BB extract improved

dopamine neuron survival and rotational behaviors such as in rats with embryonic transplanted DA neurons (McGuire et al., 2006).

4.2. Cell culture experiments

Plant polyphenols, as well as saponins, are found to be effective free radical scavengers as well as potent anti-inflammatory agents. Polyphenols in various species of berries have been found to be neuroprotective in various cellular studies (Debnath-Canning et al., 2020; Vyas et al., 2013). A study has shown that externally added glutamate could induce oxidative stress in primary cortical neurons when measured by cellular ROS detection assay kits and superoxide dismutase assay kits (Cong et al., 2016). Upon measurement after 24 hours of treatment with 100 μM of glutamate, rat brain cell cultures showed ~23% cell loss (Vyas et al., 2013). In our study, exogenously administered glutamate elicited excitotoxicity, resulting in higher cell death represented by DAPI stained nuclei. The addition of protectants such as SC extract alone or in combination with aqueous BB prevented cells from undergoing toxicity in the presence of excess glutamate. It showed that potentially the scavenging factors present in the extracts were able to prevent cell death. In cellular cultures of microglial cells, BB fruits and leaves have shown decreased inflammation and inhibition of cell death (Debnath-Canning et al., 2020). Other polyphenol-rich sources, such as green tea, can inhibit glutamate-induced ROS release, showing its antioxidant properties in cultured primary cortical neurons (Cong et al., 2016). Similarly, the effect of eicosapentaenoic acid-enriched phospholipids from *C. frondosa* on oxidative damage in PC12 cells showed decreased

leakage of lactate dehydrogenase and increased intracellular total antioxidant and superoxide dismutase capacity revealing its neuroprotective properties via inhibition of the mitochondria-dependent apoptotic pathway (Wu et al., 2014).

Alpha-synuclein accumulation is one of the pathological hallmarks of PD. These aggregates are insoluble damaging DA neurons and eliciting motor dysfunction. Exogenously added alpha-synuclein at a concentration of 100 ng/mL was able to induce toxicity at the cellular level (Debnath-Canning et al., 2020). Alpha-synuclein may have induced cell death, as shown by the number of DAPI stained nuclei; however, not as significantly as glutamate. The addition of protectants such as SC extract alone or combined with aqueous BB prevented cell death caused by alpha-synuclein administration likely due to their free radical scavenging capacity.

In our cellular experiments, the extracts of SC and SC + Aqueous BB are seen to protect against glutamate-induced toxicity to a greater extent than control in RNC and mixed cultures. However, SN cells treated with these extracts were not as protective in glutamate-induced toxicity, indicating SN cells may be more sensitive to glutamate than other cell types. Also, the mouse mixed cultures contain glial cells along with the neurons unlike RNC and SN cells which only contain pure neurons, resulting in our mixed cultures providing better protection to the glutamate induced toxicity. Upon changes in homeostasis, glial cells, and microglia move to an activated stage leading to proliferation, migration in the direction of chemoattractants (Wang et al., 2015; Yao et al., 1990), and phagocytosis to clear any damage tissues, as reviewed in (Jäkel et al., 2017; Penfield, 1925).

In some previous studies in the Weber Lab, when the extracts of BB and SC were added to normal cells without injury, the cell counts were higher than control. This suggests that extracts themselves are good for brain health in general, not just when there is an insult, such as toxins (unpublished data). The major limitation in some of the cell culture studies could be the sample sizes. As we can see with some of the trends in our results, increasing sample sizes may result in more significant differences among the treatment groups.

4.3. Future directions

While conducting MPTP studies in the future, carrying out the MPTP injections following a sub-acute regimen at a higher dose could help degenerate more reproducible results (Tatton et al., 1997). Familiarizing the mice with the test devices prior to the study, noting the value for normalizing the test results later, and taking it as a pretest performance, could help define critical behavioural data (Hutter-Saunders et al., 2012). Also, using slightly older mice while carrying out experiments in mouse models could be an advantage, as studies have shown that the extent of neurodegeneration is more remarkable in older mice (Ricaurte et al., 1987). Performing IHC to locate cellular changes and flowcytometric/HPLC techniques for quantification instead of western blotting could be helpful, particularly for small molecular weight protein like alpha-synuclein.

While carrying out cell culture work in the future involving SC extracts, a detailed HPLC analysis could be carried out in order to identify the compounds present and

characterize their potential role in neuroprotection, similar to previous lab studies involving BB (Hossain et al., 2016). Also, the result of combining SC and BB was different from our expectation as the combination was not as effective as using them alone. Therefore, a detailed HPLC analysis of both extracts could give a clear picture of the components present and potentially clarify their chemical interactions.

Chapter 5: Conclusion

The MPTP mouse model is based on mitochondrial dysfunction in PD, reducing complex I activity in brain cells. The MPTP mouse study showed changes in the levels of tyrosine hydroxylase, and alpha-synuclein levels in the MPTP injected mice, which was recovered when treated with a blueberry-enriched diet. Our study did not show significant changes in the behavioural analysis; however, our results were similar to a previous report in the literature (Wang et al., 2019). Previous studies using motor function tests to measure nigrostriatal damage have also shown conflicting results suggesting the motor function assessment may not be successfully reproduced on mice intoxicated with MPTP (Hirst et al., 2008; Hutter-Saunders et al., 2012; Sundström et al., 1990). Therefore, it indicates that my results are similar to previous studies. Although no significant behavioural deficits were present, significant changes in TH levels in brain tissues were seen, indicating that cellular changes in the brain occurred even though a behavioural phenotype was not evident. This is a very similar phenomenon to actual PD, whereby the time significant PD symptoms are observed, a significant amount of cell loss has already occurred (Bernheimer et al., 1973).

The cell culture experiments with pure SN neurons, rat cortical neurons, and mixed cultures of neurons and glia showed a higher number of DAPI stained nuclei upon treatment with sea-cucumber extracts, aqueous blueberry, or in combination upon addition with neurotoxins, glutamate (100 μM), or alpha-synuclein (100 ng/mL). The secondary metabolites such as phenols and saponins present in these natural products act in various ways to prevent neurodegeneration. Overall, these results suggests that dietary supplementation with these natural products may be effective in neuroprotection,

however, additional studies seem necessary to determine a promising or a more precise conclusion.

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Appendices

Appendix-1: Diet Calculation of MPTP mouse model of PD

Blueberry Enriched Diet:

Total = 12 mice

Blueberry = 4.55 g

dH_2O = 43.23

Powdered Chow = 43.23 g

Normal Diet:

Total = 24 mice

dH_2O = 87.5 g

Powdered Chow = 87.5 g

Each mouse received 7g of the freshly prepared mash. In the calculation for mash preparation, 1 or 2 extra mouse was taken into consideration.

Appendix-2: Recipes for western blotting

Separating Gel:

	8.5%	10%	12.5%
dH_2O	8.54 mL	7.54mL	5.88 mL
Separating gel buffer	5.0 mL	5.0 mL	5.0 mL
20%SDS	100 µL	100µL	100 µL
Acrylamide	5.67 mL	6.67mL	8.33 mL
TEMED	10 µL	10 µL	10 µL
2.8%APS	660 µL	660 µL	660 µL

Stacking Gel:

dH_2O	9.5 mL
Stacking gel buffer	1.5 mL
20%SDS	60 µL
Acrylamide	1.8 mL
TEMED	35 µL
2.8%APS	400 µL

Sample buffer (5X):

dH_2O	1.0 L
Tris HCL	50mM
SDS	5%
Glycerol	10%
B-mercaptoethanol	5%
Bromophenol blue	0.125%

1XTGS:

Glycine	57.7 g
Tris base	12.1 g
SDS	4.0 g
dH_2O (Final volume)	4L

Transfer buffer:

Tris base	12.1 g
Glycine	57.7 g
MeOH	800 mL
dH_2O (Final volume)	4L

Blocking solution:

1X TBS	40 mL
Tween 20	20 µL
Milk powder (non-fat)	2 g

1X TBST

1.0 M Tris HCl pH 7.6	20 mL
5.0 M NaCl	56 mL
10% Tween 20	20 mL
dH2O (make the volume)	2L

Primary Antibody:

Anti-tyrosine hydroxylase	1:1000
Anti-alpha synuclein	1:1000
Anti-alpha/beta tubulin *(Loading Control)*	1:1000
Anti-GADPH *(Loading Control)*	1:1000

Secondary Antibody

Goat anti rabbit	1:3000

Appendix-3: SPSS output of MPTP mice model

Latency to fall in an accelerated rotarod

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	43.574	2	21.787	0.554	0.580
Within Groups	1298.87	33	39.360		
Total	1342.444	35			

Latency to fall in a rotarod at 15rpm

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	377.951	2	188.975	0.378	0.688
Within Groups	16480.519	33	499.410		
Total	16858.469	35			

Mean velocity of the mouse in an open field

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.703	2	0.852	2.036	0.149
Within Groups	12.133	29	0.418		
Total	13.837	31			

Total distance travelled in an open field

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	15.761	2	7.880	2.051	0.147
Within Groups	111.399	29	3.841		
Total	127.16	31			

Total time spent in the centre of an open field

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5495.53	2	2747.765	2.243	0.123
Within Groups	39193.764	32	1224.805		
Total	44689.293	34			

Total time spent in the light arm of light/dark box

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5640.372	2	2820.186	2.052	0.145
Within Groups	43975.654	32	1374.239		
Total	49616.026	34			

Normalized tyrosine hydroxylase/GADPH in the mid brain of MPTP mice model

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.560	2	0.280	4.032	0.040
Within Groups	1.041	15	0.069		
Total	1.600	17			

Post Hoc Tukey HSD

		p value
Control	MPTP	0.045
Control	MPTP + BB	0.890
MPTP	MPTP + BB	0.104

Normalized tyrosine hydroxylase/GADPH in the cortex of MPTP mice model

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.820	2	0.410	1.585	0.240
Within Groups	3.619	14	0.259		
Total	4.439	16			

Normalized tyrosine hydroxylase/GADPH in the whole brain of MPTP mice model

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.847	2	0.423	5.498	0.010
Within Groups	2.156	28	0.077		
Total	3.003	30			

Post Hoc Tukey HSD

		p value
Control	MPTP	0.011
Control	MPTP + BB	0.722
MPTP	MPTP + BB	0.042

Normalized alpha-synuclein/alpha-beta tubulin in the mid brain of MPTP mice model

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.381	2	0.691	1.164	0.341
Within Groups	8.307	14	0.593		
Total	9.688	16			

Normalized alpha-synuclein/alpha-beta tubulin in the cortex of MPTP mice model

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.786	2	0.893	0.707	0.510
Within Groups	17.69	14	1.264		
Total	19.476	16			

Normalized alpha-synuclein/alpha-beta tubulin in the whole brain of MPTP mice model

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.873	2	1.437	1.736	0.192
Within Groups	26.475	32	0.827		
Total	29.348	34			

Appendix-4: SPSS output of cell culture experiments

Different treatment conditions in SN cells

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	17697.844	6	2949.641	4.802	0.001
Within Groups	25183.701	41	614.237		
Total	42881.544	47			

Post Hoc Tukey HSD

		p value
Control	Glutamate	0.000
Control	Glutamate + SC	0.984
Control	Glutamate + SC + Aqueous BB	0.262
Glutamate	Glutamate + SC	0.010
Glutamate	Glutamate + SC + Aqueous BB	0.271
Glutamate + SC	Glutamate + SC + Aqueous BB	0.822
Control	Alpha-synuclein	0.253
Control	Alpha-synuclein + SC	0.962
Control	Alpha-synuclein + SC + Aqueous BB	0.535
Alpha-synuclein	Alpha-synuclein + SC	0.895

Alpha-synuclein	Alpha-synuclein + SC + Aqueous BB	1.000
Alpha-synuclein + SC	Alpha-synuclein + SC + Aqueous BB	0.985
Glutamate	Alpha-synuclein	0.196
Glutamate	Alpha-synuclein + SC	0.015
Glutamate	Alpha-synuclein + SC + Aqueous BB	0.113
Glutamate + SC	Alpha-synuclein	0.836
Glutamate + SC	Alpha-synuclein + SC	1.000
Glutamate + SC	Alpha-synuclein + SC + Aqueous BB	0.967
Glutamate + SC + Aqueous BB	Alpha-synuclein	1.000
Glutamate + SC + Aqueous BB	Alpha-synuclein + SC	0.881
Glutamate + SC + Aqueous BB	Alpha-synuclein + SC + Aqueous BB	0.999

Different treatment conditions in RNC

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	60796.281	6	10132.714	4.082	0.004
Within Groups	81918.603	33	2482.382		
Total	142714.885	39			

Post Hoc Tukey HSD

		p value
Control	Glutamate	0.278
Control	Glutamate + SC	0.658
Control	Glutamate + SC + Aqueous BB	0.458
Glutamate	Glutamate + SC	0.015
Glutamate	Glutamate + SC + Aqueous BB	0.005
Glutamate + SC	Glutamate + SC + Aqueous BB	1.000
Control	Alpha-synuclein	0.943
Control	Alpha-synuclein + SC	1.000
Control	Alpha-synuclein + SC + Aqueous BB	0.919
Alpha-synuclein	Alpha-synuclein + SC	0.981
Alpha-synuclein	Alpha-synuclein + SC + Aqueous BB	0.474

Alpha-synuclein + SC	Alpha-synuclein + SC + Aqueous BB	0.951
Glutamate	Alpha-synuclein	0.945
Glutamate	Alpha-synuclein + SC	0.532
Glutamate	Alpha-synuclein + SC + Aqueous BB	0.053
Glutamate + SC	Alpha-synuclein	0.228
Glutamate + SC	Alpha-synuclein + SC	0.774
Glutamate + SC	Alpha-synuclein + SC + Aqueous BB	0.999
Glutamate + SC + Aqueous BB	Alpha-synuclein	0.126
Glutamate + SC + Aqueous BB	Alpha-synuclein + SC	0.626
Glutamate + SC + Aqueous BB	Alpha-synuclein + SC + Aqueous BB	0.992

Different treatment conditions in mouse brain mixed cultures

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	28203.901	4	7050.975	4.642	0.012
Within Groups	22784.767	15	1518.984		
Total	50988.669	19			

Post Hoc Tukey HSD

		p value
Control	Glutamate	0.620
Control	Glutamate + SC	0.459
Control	Glutamate + SC + Aqueous BB	0.924
Control	Glutamate + Aqueous BB	0.143
Glutamate	Glutamate + SC	0.046
Glutamate	Glutamate + SC + Aqueous BB	0.219
Glutamate	Glutamate + Aqueous BB	0.010
Glutamate + SC	Glutamate + SC + Aqueous BB	0.893
Glutamate + SC	Glutamate + Aqueous BB	0.929
Glutamate + SC + Aqueous BB	Glutamate + Aqueous BB	0.468

Appendix-5: A representation of different sections of a mouse brain

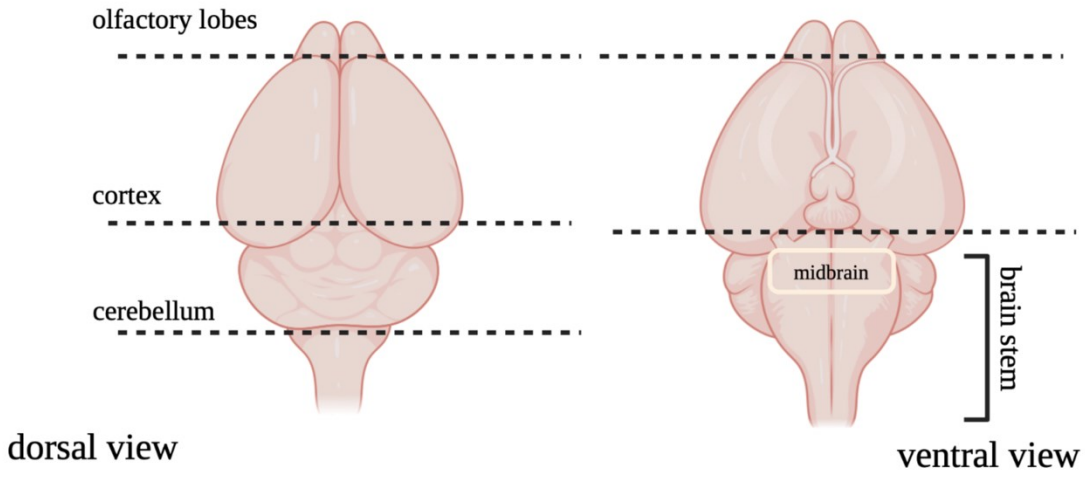

Figure A1: A representation of different sections of a mouse brain. The dotted line represents the dissection and separation of various parts. Left: The dorsal view represents cortex, cerebellum, and olfactory lobes of a mouse brain. Right: The ventral view represented brain stem, highlighting the mid brain. Figure created using biorender.com.