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Neuroprotective Activities of Acai Berries (*Euterpe* sp.) Against Monosodium Glutamate Induced Toxicity in Neural Cells

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Author declaration

I hereby declare that this dissertation and the material contained therein as my own, unless otherwise stated. This thesis has not previously been submitted to this university or any other for a degree award:

Signature

Date 06/ 03/2023

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- **Scientific contributions outside of this thesis**

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Abstract

Although L-glutamate (L-Glu) is not an essential amino acid, it is a vital excitatory neurotransmitter that plays a critical role in brain function. An excess of L-Glu levels over 1 mM within excitatory synaptic cleft has been linked to neurotoxicity in neurodegenerative diseases (NDDs) and stroke. Monosodium glutamate (MSG) is a highly used food enhancer and food additive worldwide, and excessive consumption of it can cause extreme levels of L-Glu to build up in the cerebrospinal fluid (CSF), leading to neuronal death.

The native South American palm berry known as the acai berry (*Euterpe* sp.) is a potential nutraceutical that contains several bio-active phytochemicals with multi-pharmacological effects. Several studies in recent years have shown that acai berries and their bioactive contents can relieve inflammation, act as antioxidants, prevent carcinogenesis, and protect the nervous system.

The first thesis aim was to conduct a systematic review of existing literature to examine how L-Glu affects neuronal viability and its implications for NDDs. A systematic review of 71 studies that met eligibility criteria found that exposure to L-Glu *in vitro* or *in vivo* is associated with multiple pathogenic mechanisms that affect neuronal viability, including oxidative stress, antioxidant defence decline, neuroinflammation, neurotransmitter levels dyshomeostasis, aberrant protein aggregation, excitotoxicity, mitochondrial malfunction, calcium level dyshomeostasis, and abnormalities in neuronal histology, cognitive function, and animal behaviour. Thus, identifying and understanding these diverse mechanisms might enable the design of more effective and efficient agents targeting multiple L-Glu-based pathways for NDDs.

Moreover, the following research project aimed to evaluate L-Glu (0.137 - 100 mM), acai aqueous and ethanolic extracts (0.001 µg/mL - 1000 µg/mL) toxicity and investigate the neuroprotection effects of acai berry extracts. The evaluation was carried out using various methods, including dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays to assess cell viability, and to assess mitochondrial function the adenosine 5′-triphosphate (ATP) bioluminescent, and

mitochondrial membrane potential (MMP) measurements as well as oxidative stress measurement using the 2,7-dichlorodihydrofluorescein diacetate (DCFHDA) assay. A whole-cell patch-clamp assay was conducted to investigate the effects of L-Glu and acai berry extracts on NMDARs. This study investigated human neuroblastoma cells SH-SY5Y and differentiated human rhabdomyosarcoma cells TE671. The impact of acai berry extracts and L-Glu toxicity was also investigated in differentiated SH-SY5Y cells and human cortical neural progenitor cells (ReNcell CX) via MTT assay. L-Glu caused a significant reduction in cell viability ($p < 0.01$ - $p < 0.0001$), ATP levels ($p < 0.05$ - $p < 0.0001$), and MMP level ($p < 0.05$ - $p < 0.0001$) and increased ROS production ($p < 0.05$ - $p < 0.0001$) in human undifferentiated SH-SY5Y and differentiated TE671 cells. Whole-cell patch-clamp recordings showed that L-Glu and glycine (Gly) administration did not activate currents in SH-SY5Y cells, while activated currents were observed in differentiated TE671 cells. Although acai berry extracts alone had some adverse effects high concentration at 1-1000 µg/mL reduced cell viability, ATP, and MMP level and increased ROS production in undifferentiated SH-SY5Y and differentiated TE671 cells. However, the co-application of acai berry extracts to L-Glu provided neuroprotection against L-Glu with sustained cell viability, decreased LDH production, restored ATP levels, preserved MMP levels and reduced ROS levels. Moreover, the results of this thesis showed novel results that the acai berry aqueous extract significant inhibits (0.001, 100, 1000 µg/mL, $p < 0.001$, $p < 0.0001$, and $p < 0.0001$, respectively) L-Glu + Gly-activated currents in differentiated TE671 cells in a concentration-dependent manner. Furthermore, differentiated SH-SY5Y cells showed no significant reduction in viability after exposure to L-Glu (0.137 - 100 mM), but ReNcell CX cells experienced significant ($p < 0.0001$) toxicity impacts after exposure to L-Glu at concentration range 0.03 - 100 mM. In ReNcell CX cells, aqueous acai berry extract (0.0001 - 100 µg/mL) co-applied to neurons exhibited significant ($p < 0.05$ - $p < 0.0001$) neuroprotection against L-Glu. Thus, this study showed that acai berries contain nutraceuticals with antioxidant and anti-excitotoxicity properties, making them a potentially beneficial dietary component to prevent pathological deficits caused by excessive levels of L-Glu.

The third project in this dissertation is designed to evaluate the potential nutraceutical benefits of acai berry extracts *in vitro* by examining their ability to inhibit cholinesterase enzymes (ChE) and scavenging free radicals by using 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assays. Furthermore, several antioxidant effects were assessed, including hydrogen peroxide (H_2O_2) or hydroxyl radical ($OH^{\bullet}$) scavenging, nitric oxide radical ($NO^{\bullet}$) scavenging, lipid peroxidation (LPO) inhibition, and ferric ion reduction (Fe^{+3}). Total polyphenols (TPC) and flavonoids (TFC) were determined in both acai extracts. In addition, acai berry extracts were fractionated and analyzed by liquid chromatography mass spectrometry (LC-MS) to identify phytochemicals that may possess anti-cholinesterase and antioxidant properties. Acai berry extracts showed novel action in their ability to inhibit acetyl- and butyryl-cholinesterase with estimated IC_{50} of 0.001 μg/mL, and 6.378 mg/mL, respectively. Moreover, both acai extracts exhibited effective concentration-dependent antioxidant and free radical scavenging properties. The acai ethanolic extract showed the most potent antioxidant capability and exhibited the highest phenolic and flavonoid contents 101.39 ± 4.61 milligram gallic acid equivalents/gram of acai berry extracts (mg GAE/g) and 11.78 ±1.42 milligram quercetin equivalents/gram of extract (mg QUER E/g), respectively. Fractionation and analysis of acai berry extracts with LC-MS identified several phytochemicals that may have provided antioxidants and anti-cholinesterase effects. Therefore, acai berry extracts could be a potentially dietary supplements that reduce the pathogenic impairments seen in AD.

Taken together, the data in this thesis suggest that excessive L-Glu intake has adverse effects, including neuronal tissue degradation, similar pathomechanisms found in NDDs and stroke. Moreover, this thesis reveals that acai berry extracts may include neuroprotective compounds and could be exploited to develop drugs to treat NDDs and strokes, which are linked to excessive buildup and toxicity of L-Glu. In addition, acai extracts may present a novel approach for prospective AD therapy if further fractionated due to their novel anti-cholinesterase and antioxidant properties. However, it is still necessary to validate these findings using human primary cell lines, 3D neuronal models, and *in vivo* models to explore the influences of L-Glu and acai berry extracts exposure in humans.

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List of abbreviations

ABTS	2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADI	Acceptable daily intake
α-KGDH	α-ketoglutarate dehydrogenase
$AlCl_3$	Aluminium chloride
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AMPK	5′AMP-activated protein kinase
ANOVA	Analysis of variance
$apo^{E-/-}$	Apolipoprotein E deficient
APP	Amyloid precursor protein
Arc	activity-regulated cytoskeleton-associated protein
aSyn	α-synuclein
ATCI	Acetylthiocholine iodide
ATD	Amino-terminal domain
$ATF2^{pp}$	Activating transcription factor 2 phosphorylated
ATP	Adenosine triphosphate
Aβ	Amyloid beta protein
$A\beta_{1-42}$	Amyloid $beta_{1-42}$
Bax	Bcl-2-associated X protein
BBB	Blood-brain barrier
BChE	Butyrylcholinesterase
Bcl-2	B-cell lymphoma-2
BDNF	Brain derived growth factor
bFGF	Basic fibroblast growth factor
BHA	Butylated hydroxyanisole
BP	Blood Pressure
Bsn	Bassoon protein
BuChE	Butyrylcholinesterase
bw	Body weight
Ca^{2+}	Calcium ion
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CCL2	chemokines (chemokine (C-C motif) ligand 2
CCl_4	Carbon tetrachloride
CDC	Centers for disease control and prevention
ChAT	Choline acetyltransferase
ChE	Cholinesterase
ChEIs	Cholinesterase inhibitors
CHOP	CCAAT/enhancer-binding protein homologous protein
CK	Creatine kinase

Cl^-	Chloride ion
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
CO_2	Carbon dioxide
COMT	Catechol-O-methyl transferase
COX-2	Cyclooxygenase-2
CPK	Creatine phosphokinase
CPK-BB	Creatine phosphokinase isoenzymes BB
CsCl	Caesium chloride
CSF	Cerebrospinal fluid
CTD	C-terminal tail
CVOs	Circumventricular organs
CXCL1	Chemokine (C-X-C motif) ligand 1
Cyt C	Cytochrome C
DAergic	Dopaminergic
DAMPs	Damage-associated molecular patterns
DCFHDA	2′,7′-Dichlorofluorescin diacetate
DIV	Day *in vitro*
Dlg4	Discs large MAGUK scaffold protein 4
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNQX	6,7-dinitroquinoxaline-2,3-dione
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
DTC	d-tubocurarine
DTNB	5,5'-Dithiobis-(2-Nitrobenzoic Acid)
dUTP	Deoxynucleotidyl transferase
EAAT	Excitatory amino acid transporters
EC_{50}	Half maximal effective concentration
ECF	Extracellular fluid
ECG	Electrocardiogram
EFSA	European Food Safety Authority
EGF	Epidermal growth factor
EGR1	Early growth response 1
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ENS	Enteric nervous system
EPSPs	Excitatory postsynaptic potentials
ER	Endoplasmic reticulum
ERKs	Extracellular signal-regulated kinases
FAO	Food and agriculture organization
Fas-L	Fas ligand
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
FCR	Folin–Ciocalteu reagent
FDA	Food and drug administration
Fe^{2+}	Ferrous ions

Fe^{3+}	Ferric ions
FGF	Fibroblast growth factor
FJB	Fluoro jade B
FRAP	Ferric-reducing antioxidant power
FTY	Fingolimod
GA	Glatiramer acetate
GABA	γ-aminobutyric acid
GAD	Glutamic acid decarboxylase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAT	GABA transporters
GCLC	Glutamate cysteine ligase catalytic subunit
GFAP	Glial fibrillary acidic protein
GluR	Glutamate receptors
Gly	Glycine
GPCRs	G-protein-coupled receptor
GPx	Glutathione peroxidase
GR	Glutathione reductase
GRP78	Glucose regulatory protein 78
GSH	Glutathione
GSSG	Oxidised glutathione
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
HBSS	Hanks' balanced salt solution
hAChE	Human acetylcholinesterase
hBuChE	Human butyrylcholinesterase
HCl	hydrogen chloride
HCN-1A	Human cortical neuronal cells
HCO^{3-}	Bicarbonate
HD	Huntington's disease
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
[3H]-Glu	[3H]-glutamate
3H-GABA	[3H]gamma-aminobutyric acid
HMGB1	High-mobility group box 1
HO-1	Heme oxygenase-1
H_2O_2	Hydrogen peroxide
HSP70	70 kDa heat shock protein
[3H] TBOB	[3H]-t-butylbicycloorthobenzoate
Htt	Huntingtin protein
i.g.	Maternal intragastric
i.p.	Intraperitoneal injection
Iba-1	Ionised calcium-binding adaptor molecule 1
IC_{50}	50% inhibition concentration
ICD	International classification of disease
ICH	Intraparenchymal or intracranial or intracerebral haemorrhages
IFN-β	Interferon-β
IFN-γ	Interferon gamma

iGluR	Ionotropic glutamate receptors
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-18	Interleukin18
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin 1 beta
IL-4	Interleukin 4
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
IP_3	Inositol triphosphate synthesis
IPSP	Inhibitory postsynaptic potential
JNK	c-Jun N-terminal kinase
K^+	Potassium ions
KA	Kainic acid
KAR	Kainate receptors
KCNQ	Potassium channels
kDa	Kilodalton
LACI	Lacunar infarction
LBD	Ligand-binding domain
LC-MS	Liquid chromatography mass spectrometer
LDH	Lactate dehydrogenase
L-Glu	L-glutamate
LPO	Lipid peroxidation
LPS	Lipopolysaccharide
mAbs	Monoclonal antibodies
mAChR	Muscle acetylcholine receptors
MAPK	Mitogen-activated protein kinase
MAs	monoamines
MC I	mitochondrial complex I
MC V	mitochondrial complex V
MDA	Malondialdehyde
ME	Median eminence
MeHg	Methylmercury
MEL	Mean escape latency
MFN2	Mitofusin 2
Mg^{2+}	Magnesium ion
mGluR	Metabotropic glutamate receptors
mGluR5	Metabotropic glutamate receptor 5
MHC	Histocompatibility complex
MK-801	Dizocilpine
MMP	Mitochondrial membrane potential
MMPs	Matrix metalloproteinases
Mn-SOD	Manganese superoxide dismutase
MPO	Myeloperoxidase
mRNA	Messenger RNA

MS	Multiple sclerosis
MSG	Monosodium L-glutamate
MSNs	Medium spiny neurons
MT	Mechanical thrombectomy
mtDNA	Mitochondrial DNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWM	Morris water maze
Na^+	Sodium ion
NaCl	Sodium chloride
Na^+-K^+- ATPase	Sodium potassium adenosine triphosphatase
nAChR	Nicotinic acetylcholine receptors
ND	Not determined
NDDs	Neurodegenerative diseases
NDUFS7	NADH: Ubiquinone Oxidoreductase Core Subunit S7
NDUFS8	NADH: Ubiquinone Oxidoreductase Core Subunit S8
NF200	Neurofilament protein 200
NFTs	Neurofibrillary tangles
NF-κB	Nuclear factor kappa B
NLRP3	Nod-like receptor protein 3
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO^-	Nitrite
$NO^\bullet$	Nitric oxide radical
NOX2	NADPH-oxidoreductase-2
GluN2B	N-methyl-D-aspartate receptor subunit 2B
Nrf2	Nuclear factor erythroid 2-related factor 2
NRSF	Neuron-restrictive silencer factor
NS	Not significant
NT	Neurotransmitter
NTD	N-terminal domain
O_2	Oxygen
$O^{2-\bullet}$	Superoxide
OCSP	Oxfordshire community stroke project
O-GlcNAcylation	O-linked-N-acetylglucosaminylation
$OH^\bullet$	Hydroxyl radical
8-OHdG	8-hydroxy-2′-deoxyguanosine
$ONOO^-$	Peroxynitrite
OVLT	Vascular organ of the lamina terminalis
OxLDL	Oxidized low-density lipoprotein
OXPHOS	Oxidative phosphorylation
p.o.	*per os*, (orally)
p38 MAPK	p38 mitogen-activated protein kinase
PACI	Partial anterior circulatory infarction
p-AMPK	Phosphorylated AMP-activated protein kinase

PAMPs	Pathogen-associated molecular patterns
PARP	Poly (adenosine diphosphate (ADP)-ribose) polymerase
PARP-1	(ADP ribose) polymerase-1
PBS	Phosphate buffered saline
PCC	Protein carbonyl content
PCO2	Partial pressure of carbon dioxide
PD	Parkinson's disease
pERK	Phospho-extracellular signal-regulated kinase
p-ERK 1/2	Phospho-extracellular signal-regulated kinase 1/2
PGE2	Prostaglandin E2
Pi	Pineal gland
PLA2s	Phospholipases A2
p-MAPKs	Phosphorylated mitogen-activated protein kinases
p-NF-$_k$B	Phosphorylated nuclear factor kappa-light-chain-enhancer of activated B cells
PNS	Peripheral nervous system
POCI	Posterior circulation infarction
polyQ	Polyglutamine
pp38	Phosphorylated-p38 mitogen-activated protein kinase
PPMS	Primary progressive multiple sclerosis
PRMS	Progressive relapsing multiple sclerosis
PRRs	Pattern recognition receptors
PSA-NCAM	Polysialylated neural cell adhesion molecule
PSEN1	Presenilin 1
PSEN2	Presenilin 2
PST	Polysialyltransferase
p-tau	Phosphorylated-tau protein
PTMs	Post-translation modifications
RA	Retinoic acid
RCS	Reactive chlorine species
ReNcell CX cells	Human cortical progenitor neuronal stem cell line
REST	RE1-silencing transcription factor
rFVIIa	Recombinant factor VII
GSH	Glutathione (reduced)
RIP	Necrosis receptor-interacting protein
RIP kinase 1	Receptor-interacting protein kinase 1
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RRMS	Relapsing-remitting multiple sclerosis
s.c.	Subcutaneous
S1PR	Sphingosine-1-phosphate receptors
SAH	Subarachnoid haemorrhage
SAT2	System A transporter 2
SCO	Subcommissural organ
SDH	Succinate dehydrogenase

SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SFO	Subfornical organ
SH-SY5Y cells	Human neuroblastoma SH-SY5Y cell line
SK-N-BE(2)	Human neuroblastoma cell lines
SN1	Transporter system N1
SNP	Sodium nitroprusside
SNpc	Substantia nigra pars compacta
SOD1	Superoxide dismutase-1
SPMS	Secondary progressive multiple sclerosis
STICH	Surgical trial in intracerebral haemorrhage
TBA	2-thiobarbituric acid
TBOB	t-Butylbicycloorthobenzoate
TCA	Trichloroacetic acid
TDP-43	Trans-activation response DNA binding protein 43
TE671	The rhabdomyosarcoma cell line
TERT	Telomerase reverse transcriptase
TFC	Total flavonoid content
TGF-β	Transforming growth factor beta
TH	Tyrosine hydroxylase
TIA	Transient ischemic attack
TMD	Transmembrane domin
TNF-α	Tumour necrosis factor-α
t-PA	Tissue plasminogen activator
TPC	Total phenolic content
TUNEL	Terminal deoxynucleotidyl transferase (dUTP) nick
UK	United Kingdom
US	United States
v/v	Volume/volume
VAChT	Vesicular ACh transporter
VDCC	Voltage-dependent Ca^{2+} channels
VGLUTs	Vesicular glutamate transporters
VIAAT	Vesicular inhibitory amino acid transporter
VMAT2	Vesicular monoamine transporter type 2
w/v	Weight/volume
WHO	World health organization
X_c^-	Cystine/glutamate antiporter system
XO	Xanthine oxidase
Zn^{2+}	Zinc ion

Chapter 1
Introduction and Background

Chapter 1. Introduction and background

1.1 Glutamate and glutamatergic neurons

In the central nervous system (CNS), L-glutamate (L-Glu) is the most important excitatory neurotransmitter which facilitates communication and signaling between neurons (Chayat and Yedidya 2012; Leibowitz *et al.* 2012; Purves *et al.* 2018). L-Glu is a non-essential amino acid and the most abundant free amino acid in the CNS, accounting for about 60% of total brain neurotransmitter activity (Chayat and Yedidya 2012; Leibowitz *et al.* 2012). Glutamatergic neurons are prominent in the brainstem, cerebral cortex, cerebellum, hippocampus, and retina (Theibert 2020). They are also found in the thalamus, hypothalamus, basal ganglia, limbic system, and spinal cord (Theibert 2020). Like other signalling substances, L-Glu signalling effect does not depend on the chemical nature of L-Glu but on how cells are programmed to respond when exposed to it (Mehta *et al.* 2013). Several critical roles for L-Glu in the development and function of the normal brain have been recognized, including a vital regulator in the process of communication between neurons, plasticity development in the CNS and serving as a fuel reserve in the absence of glucose (regular energy supplier) (Mehta *et al.* 2013; Theibert 2020). Additionally, glutamate contributes to learning and memory processes, transmitting sensory information, encoding information, brain motor control and coordination, cognition, emotion, spatial recognition, awareness, and long-term potentiation (Theibert 2020; Willard and Koochekpour 2013). Moreover, it can act as a neurotrophic factor during CNS development (Cameron *et al.* 1998; McDonald and Johnston 1990). Its action occurs in three distinct compartments inside the brain: presynaptic neurons, postsynaptic neurons and glial cells (Mehta *et al.* 2013). Hence, L-Glu plays a critical role in brain development, and its health effects are highly dependent on strict homeostasis, keeping its quantities under their toxic level in the brain extracellular fluid (ECF) (Leibowitz *et al.* 2012).

L-Glu can be obtained from the diet or synthesized in neurons from local precursors such as glutamine (Gln) (Purves *et al.* 2018; Theibert 2020). System A transporter 2 (SAT2) transports Gln into presynaptic terminals, where it is converted to L-Glu by the

mitochondrial enzyme glutaminase (Figure 1.1) (Purves *et al.* 2018). Transamination of 2-oxoglutarate, an intermediate in the Krebs cycle, can also be used by neurons to synthesize L-Glu from glucose (Purves *et al.* 2018; Theibert 2020). The vesicular glutamate transporters (VGLUTs) transport synthesized L-Glu in the presynaptic cytoplasm into synaptic vesicles (Purves *et al.* 2018; Theibert 2020). Once the neuron is depolarized, the stored L-Glu within the vesicles is released into the synaptic cleft (Chayat and Yedidya 2012). At that time, the excitatory amino acid transporters (EAATs) remove the remaining L-Glu from the synaptic cleft after it is released (Purves *et al.* 2018). Some EAATs are present in glial cells but others reside in presynaptic terminals, and they are a group of five distinct L-Glu co-transporters that rely on sodium ions (Na^+) for their function (Purves *et al.* 2018). An astrocyte cytoplasmic enzyme called glutamine synthetase converts L-Glu delivered into glial cells via EAATs into Gln (Purves *et al.* 2018). The transporter system N1 (SN1) then transports Gln out of glial cells, and SAT2 transfers it back into the neuron terminals to be converted back to L-Glu (Purves *et al.* 2018). Generally, these events are identified as the glutamate-glutamine cycle (refer to Figure 1.1) (Purves *et al.* 2018; Theibert 2020). This cycle permits glial cells and presynaptic terminals to work together to ensure an adequate supply of L-Glu for synaptic transmission while also allowing postsynaptic L-Glu activity to be quickly terminated (Purves *et al.* 2018; Theibert 2020).

Moreover, EAATs are found on brain-blood endothelium cell abluminal membrane (Hawkins 2009; Hawkins *et al.* 2010). These EAATs provide unidirectional movement of L-Glu from the brain to blood (Zaragozá 2020). Furthermore, recent research shows that the cystine/glutamate antiporter, or system x_c^-, is also involved in regulating extracellular and intracellular L-Glu concentrations (De Bundel *et al.* 2011; Farhat *et al.* 2021; Martis *et al.* 2020; Massie *et al.* 2011). The cystine/glutamate antiporter is a membrane-bound Na^+-independent amino acid transporter made up of a heavy chain component (4F2hc) that is shared by all amino acid transporters and a light chain unique subunit, xCT (De Bundel *et al.* 2011; Farhat *et al.* 2021; Martis *et al.* 2020). In the system x_c^-, intracellular L-Glu is exchanged for extracellular cystine at a molar ratio of 1:1, allowing antioxidant

glutathione (GSH) to be synthesized as well as non-vesicular L-Glu to be released (De Bundel *et al.* 2011; Farhat *et al.* 2021; Martis *et al.* 2020; Massie *et al.* 2011).

Thus, the glutamate-glutamine cycle, EAATs, and system X_c^- are all mechanisms for maintaining L-Glu homeostasis in the brain (Hawkins 2009; Hawkins and Viña 2016). The L-Glu levels in different areas in normal physiological conditions are summarised in Table 1.1 (Featherstone 2010; Hawkins and Viña 2016; Meldrum 2000; Schultz *et al.* 2020). However, numerous studies demonstrated that L-Glu accumulation in various disease conditions, such as neurodegenerative disease (NDDs), and its excess release after stroke onset causes a toxicity cascade leading to neuronal death (Belov Kirdajova *et al.* 2020; Mehta *et al.* 2013).

Table 1.1: The L-glutamate (L-Glu) levels in the brain and blood.

Location	Estimated concentration
CSF	<1 μM
Brain ECF	0.5 - 5 μM
Plasma	30 - 100 μM
Synaptic cleft	2 - 1,000 μM
Glutamatergic neuron cytoplasm	5 - 10 mM
Brain (homogenate)	5 - 15 mmol/Kg
Synaptic vesicle	100 mM

Abbreviations: CSF, cerebrospinal fluid; ECF, extracellular fluid

1.2 Glutamate receptors (L-GluR)

L-Glu can bind and activate only the cells with its receptor proteins ("glutamate receivers") on their surfaces (Mehta *et al.* 2013). Two receptor classes for L-Glu are recognized, ligand-gated ion channels (ionotropic glutamate receptors (iGluR)) and G-protein coupled (metabotropic glutamate receptors (mGluR)) (Purves *et al.* 2018; Sharma 2015; Theibert 2020). The difference between the glutamatergic receptors in response speed after activation shows that iGluR mediates rapid synaptic transmission while mGluR mediates slower and longer-lasting responses (Chayat and Yedidya 2012; Purves *et al.* 2004; Purves *et al.* 2018).

There are three ionotropic receptor subtypes: *N*-methyl-D-aspartate receptors (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR), and kainate receptors (KAR) (Purves *et al.* 2004; Purves *et al.* 2018; Theibert 2020). These sub-receptors are based on selective activation agonists, which are like L-Glu but are not naturally found in the brain: NMDA, AMPA, and KA (Mark *et al.* 2001; Theibert 2020). All iGluR are tetrameric protein compounds that create selective cation channels that allow Na^+ and potassium ions (K^+) to pass and a significant amount of calcium (Ca^{+2}) primarily through NMDAR (Bettler and Mulle 1995; Christensen *et al.* 2016; Theibert 2020; Zarei and Dani 1994). The general architecture of all iGluR subunits is similar, with a long (>500 amino acid) N-terminal extracellular domain, four membrane segments (M1,3,4 are transmembrane α-helices, whereas, M2 is a re-entrant loop called the pore-loop), and a variable-length intracellular, mainly disordered C-terminal tail (CTD) (Dawe *et al.* 2020; Traynelis *et al.* 2010). The amino-terminal domain (ATD; also referred to as N-terminal domain "NTD") and the agonist or ligand-binding domain (LBD) are two globular, self-interacting regions in the extracellular area (Figure 1.2) (Dawe *et al.* 2020).

The mGluR is a G-protein-coupled receptor (GPCR) that triggers neuromodulatory responses by activating G proteins that control ion channels and second messenger systems (Theibert 2020). Presynaptic and postsynaptic neurons express iGluR and mGluR; however, mGluR is also expressed on the extrasynaptic and perisynaptic membranes (Theibert 2020). Released L-Glu works as a neurotransmitter stimulating

glutamatergic receptors (ionotropic NMDAR, AMPAR and KAR or mGluR) (Chayat and Yedidya 2012).

1.2.1 α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR)

Like all iGluR, AMPAR are created by combining four protein subunits, which can produce many isoform receptors in many ways (Purves *et al.* 2018; Theibert 2020). GluA1-GluA4 are the four subunits of AMPAR, and each subunit confers its functional properties (Purves *et al.* 2018; Theibert 2020; Traynelis *et al.* 2010). Most AMPAR have two GluA2 subunits and a GluA1, GluA3, or GluA4 dimer (Theibert 2020). AMPA binds with highest affinity to AMPAR (Purves *et al.* 2018; Theibert 2020). There are several domains in each component of the AMPAR subunit, including an extracellular glutamate-binding domain and a transmembrane domain that functions as an ion channel (Purves *et al.* 2018; Theibert 2020). Figure 1.3 shows how these subunits are arranged into a tetrameric form (Purves *et al.* 2018). AMPAR have an asymmetrical extracellular structure that appears different when seen from the front and side surfaces (Figure 1.3) (Purves *et al.* 2018). As the subunits of the AMPAR pass through the plasma membrane, their large extracellular domains gradually narrow to form a Y-shaped receptor (Figure 1.3) (Purves *et al.* 2018). L-Glu and other ligands interact within the opening of the extracellular ligand-binding domains, which have a distinctive "clamshell" structure (Purves *et al.* 2018). The helices and pore-loop in the transmembrane domain create the channel pore and convergence of M3 helices at extracellular end closes the pore when L-Glu is not coupled to the receptor (Purves *et al.* 2018). The clamshell structure "shuts" when L-Glu is bound; this action allows the gate helices inside the transmembrane domain to shift, allowing the channel pore to open, as a result of this Na^+ and K^+ flow down their electrochemical gradients (Purves *et al.* 2018; Theibert 2020). One of the rare AMPAR tetramers without GluA2 dimers has been identified as Ca^{2+}-permeable (Theibert 2020). GluA2 regulates Ca^{2+}-permeability of AMPAR through RNA editing that involves converting glutamine (Gln; Q) in GluA2 mRNA to arginine (Arg; R) as a result of post-transcriptional modifications (Wright and Vissel 2012). A Ca^{2+}-impermeable AMPAR is one containing the edited GluA2(R) subunit, whereas a Ca^{2+}-permeable receptor is one lacking or containing the unedited GluA2(Q) (Wright and

Vissel 2012). A negative electrostatic potential forms with the N-terminus of the M2 loop as result of Gln existence that causes cations attraction (Wright and Vissel 2012). However, diffusion prevention of divalent cation through the channel occurs when Gln is replaced by a large positively charged Arg (Wright and Vissel 2012). A flip or a flop variant of AMPAR is generated by alternative splicing of a 115-base pair region encoding 38 amino acid sits in the extracellular ligand binding domain and before the transmembrane domain M4 (Sommer *et al.* 1990). In flip and flop polypeptides segment, only 9 to 11 amino acids are changed, and synaptic transmission mediated by AMPAR may be functionally heterogeneous as a result of this alternative splicing (Sommer *et al.* 1990). There is no direct involvement of this splice variance region in ligand binding, but it seems to modulate receptor desensitization and channel conductance (Gomes *et al.* 2008). Moreover, during development, each splice variant expresses at a different level and theses splice variance can also alter the sensitivity of AMPAR to allosteric modulation (Pei *et al.* 2009; Quirk and Nisenbaum 2003).

1.2.2 Kainate receptors (KAR)

Kainate receptors (KAR) are selectively activated by kainate, which was originally isolated from a red alga *Digenea simplex* (Dawe *et al.* 2020; Theibert 2020). As a convulsant, kainate provokes seizures by activating KAR and possibly activating AMPAR even though it has lower affinity for the agonist binding site in the AMPAR (Theibert 2020). KAR and AMPAR share many similarities; however, KAR are considerably less prevalent and poorly defined than AMPAR (Theibert 2020). KAR are made of tetramers, including GluK1 to GluK5 subunits and are activated more slowly than AMPAR, allowing Na^+ and K^+ to pass through the channel pore (Mayer 2021; Theibert 2020). However, when studying iGluR, researchers usually classify AMPAR and KAR together as non-NMDAR due to their similar properties and functions (Theibert 2020). AMPAR and KAR are functionally distinct from NMDAR in terms of antagonism due to their common sensitivity to quinoxalinedione antagonists such as 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) (Dawe *et al.* 2020).

1.2.3 *N*-methyl-D-aspartate receptors (NMDAR)

The L-Glu agonist NMDA mainly activates NMDAR, which are tetramers of four GluN1 to GluN3 subunits (Purves *et al.* 2018; Theibert 2020). The glutamate-binding subunit is GluN2, while the glycine-binding subunits are GluN1 and GluN3 (Purves *et al.* 2018). Typically, two glutamate-binding subunits (GluN2) and two glycine-binding subunits (GluN1) are usually found in NMDAR tetramers (Purves *et al.* 2018). NMDAR subunits have the clamshell-shaped ligand-binding domains that bind L-Glu or glycine (Gly) and transmembrane domains that create the channel pore and gate, similar to AMPAR and KAR subunits (Figure 1.4) (Purves *et al.* 2018; Theibert 2020). A region in the extracellular border, close to the transmembrane domain, is thought to bind Ca^{2+} and facilitate its permeability through NMDAR (Figure 1.4) (Purves *et al.* 2018). When L-Glu and Gly are bound to their ligand-binding domains of NMDAR, this causes a conformational shift that opens the channel pore; however, some antagonist binding displaces the ligand-binding domains, preventing channel opening (Purves *et al.* 2018).

The physiological features of NMDAR distinguish them from AMPAR and KAR (Purves *et al.* 2018; Theibert 2020). The first remarkable characteristic of NMDAR is that they require a co-agonist in addition to L-Glu, such as the amino acid Gly or D-serine (Purves *et al.* 2018; Theibert 2020). A depolarized postsynaptic membrane is required for magnesium ions (Mg^{2+}) or zinc ions (Zn^{2+}) expelling and cations influx through the NMDAR pore in addition to L-Glu and co-agonist binding (Purves *et al.* 2018; Theibert 2020). In response to L-Glu binding, NMDAR are permeable to Na^+ and K^+ but mostly to Ca^{2+} (Purves et al. 2018; Theibert 2020b). As a result, NMDAR-mediated excitatory postsynaptic potentials (EPSPs) raise Ca^{2+} levels in the postsynaptic neuron, with Ca^{2+} functioning as a second messenger to activate intracellular signalling cascades (Purves *et al.* 2018).

At the resting membrane potential (polarized), NMDAR contain an internal binding site for Mg^{2+} or Zn^{2+}, resulting in a voltage-dependent block of the pore (Purves *et al.* 2018; Theibert 2020). Membrane depolarization dislodges and repels Mg^{2+} or Zn^{2+}, permitting Na^+, K^+, and Ca^{2+} to flow through the channel (Purves *et al.* 2018; Theibert 2020).

NMDAR require L-Glu, a co-agonist, and membrane depolarization caused by AMPAR or other excitatory neurotransmitter (NT) responses, to be activated (Purves *et al.* 2018; Theibert 2020).

The NMDAR is inhibited by a number of types of antagonists, including competitive antagonists (whose action involves both the Gly or L-Glu sites), channel blockers (acting at the ion channel pore), or noncompetitive - allosteric modulators (acting at specific modulation sites like GluN2B-containing receptor amino terminal domains) (Monaghan and Jane 2009). D-2-amino-5-phosphonovalerate (D-APV) became one of the first competitive antagonists at the L-Glu binding site and dizocilpine (MK-801), memantine, and ketamine act as channel blockers (Monaghan and Jane 2009). Ifenprodil, the first subunit-specific NMDAR antagonist identified, belongs to a class of noncompetitive inhibitors (Monaghan and Jane 2009).

1.2.4 Metabotropic glutamate receptors (mGluR)

Eight distinct subtypes of metabotropic L-Glu receptors (mGluR) are arranged into three groups (I-III) based on their downstream signalling (Purves *et al.* 2018; Theibert 2020). The $G_{\alpha q/11}$ family of G proteins is activated by the group I mGluR (mGluRI and mGluR5), which activates phospholipase C (PLC), and is mainly found in postsynaptic areas (Theibert 2020). Groups II (mGluR2 and mGluR3) and III (mGluR4 and mGluR6 to mGluR8) mGluR inhibit adenylyl cyclase by coupling through the $G_{\alpha i/o}$ family of G proteins and are primarily engaged in presynaptic inhibition (Theibert 2020). mGluR are GPCR that function in the hippocampus, cerebellum, cerebral cortex, and other parts of the brain, as well as in the peripheral nervous system (PNS), and are involved in slow synaptic transmission and neuromodulation via either excitation or inhibition of postsynaptic neurons (Purves *et al.* 2018; Theibert 2020). Although all GPCR have a transmembrane domain that traverses the membrane seven times, mGluRs are physically unusual because they are dimers of two identical subunits (Figure 1.5) (Purves *et al.* 2018). Each subunit has a ‘venus flytrap’ domain, a glutamate-binding domain that resembles the clamshell-shaped ligand-binding domains seen on iGluR (Figure 1.5) (Purves *et al.* 2018). A linker domain rich in the amino acid cysteine connects this venus

flytrap domain to the transmembrane domain (Purves *et al.* 2018). The venus flytrap domains seal when L-Glu binds to them, and the associated rotation causes the transmembrane domains to twist and activate the receptor (Purves *et al.* 2018). Intracellular signalling is initiated when G-proteins dissociate from the activated receptor (Purves *et al.* 2018). mGluRs can modulate NMDAR, and mGluR dysregulation has been linked to the aetiology of neurodegenerative diseases like Parkinson's disease (Theibert 2020).

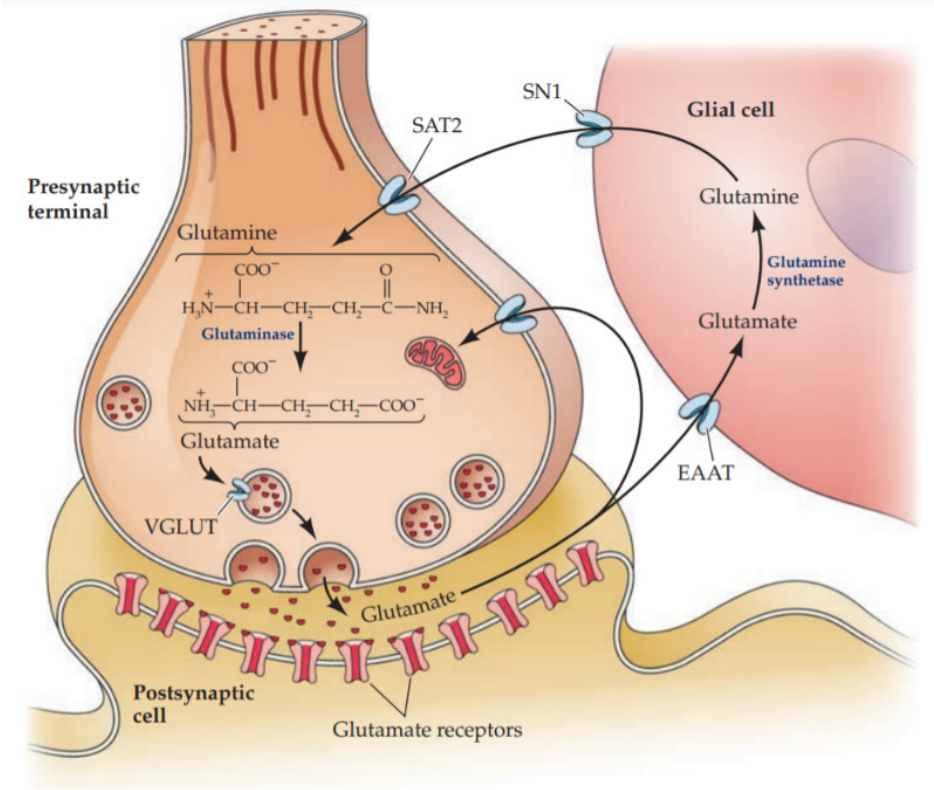

Figure 1.1: Synthesis and cycling of L-glutamate between neurons and glia (Purves *et al.* 2018).

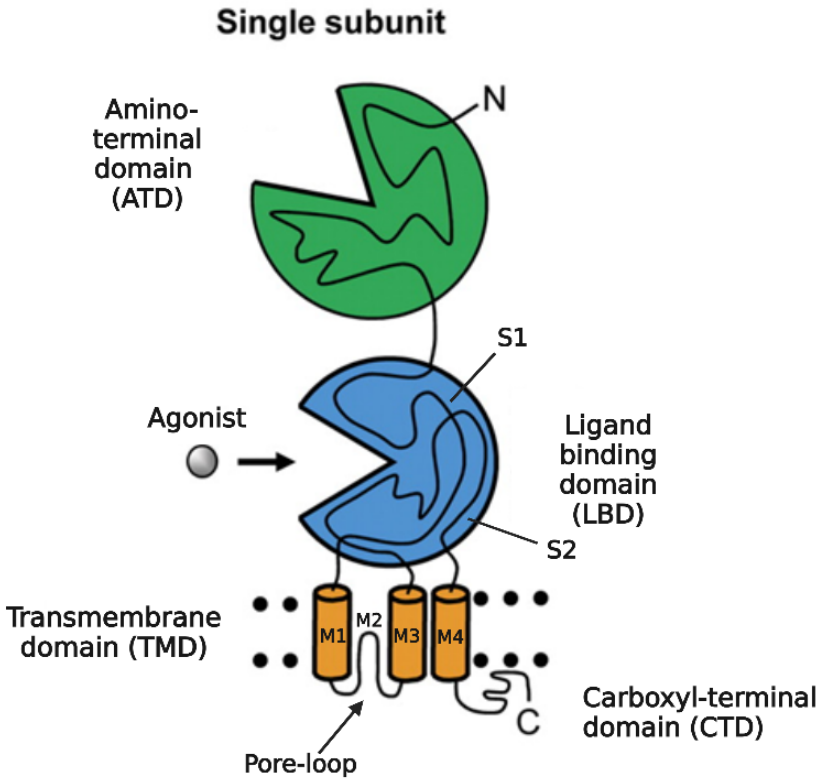

Figure 1.2: Ionotropic glutamate receptor (iGluR) tetramer structure. There are four distinct semiautonomous domains on each iGluR subunit, regardless of the subtype. These include the extracellular amino-terminal domain (ATD), the extracellular ligand binding domain (LBD), the transmembrane domain (TMD), and an intracellular carboxyl-terminal domain (CTD). LBD consists of two amino acid segments called S1 and S2. Three membrane-spanning helices (M1, M3, and M4) and a membrane re-entrant loop (M2) constitute the TMD (Hansen *et al.* 2010; Traynelis *et al.* 2010). The figure was created using BioRender (https://www.biorender.com/).

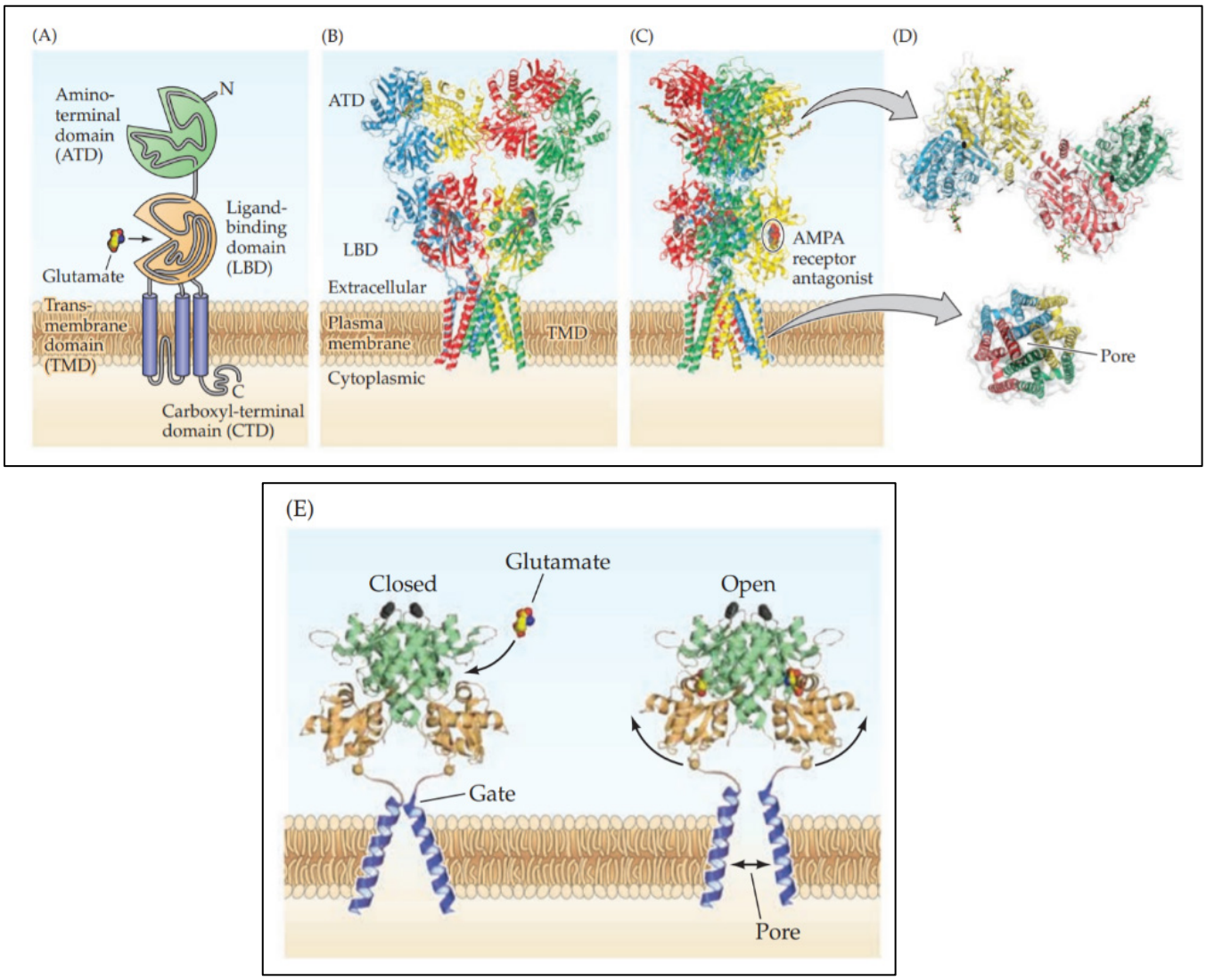

Figure 1.3: The AMPAR structure. (A) structural domains of an AMPAR subunit. The amino-terminal domain (ATD) and the ligand-binding domain (LBD) make up the majority in each subunit of the extracellular portion. An intracellular carboxyl-terminal domain (CTD) binds the receptor to intracellular proteins, while a transmembrane domain (TMD) is part of the ion channel pore (B-D). The AMPAR crystallographic structure. The four subunits are each represented by a distinct colour. (B) The Y shape of the AMPAR is seen from this angle. (C) The asymmetrical proportions of the receptor are seen after turning it 90 degrees. (D) Two different images of the AMPAR in cross-section. (E) L-Glu regulation of the AMPAR model (Purves *et al.* 2018).

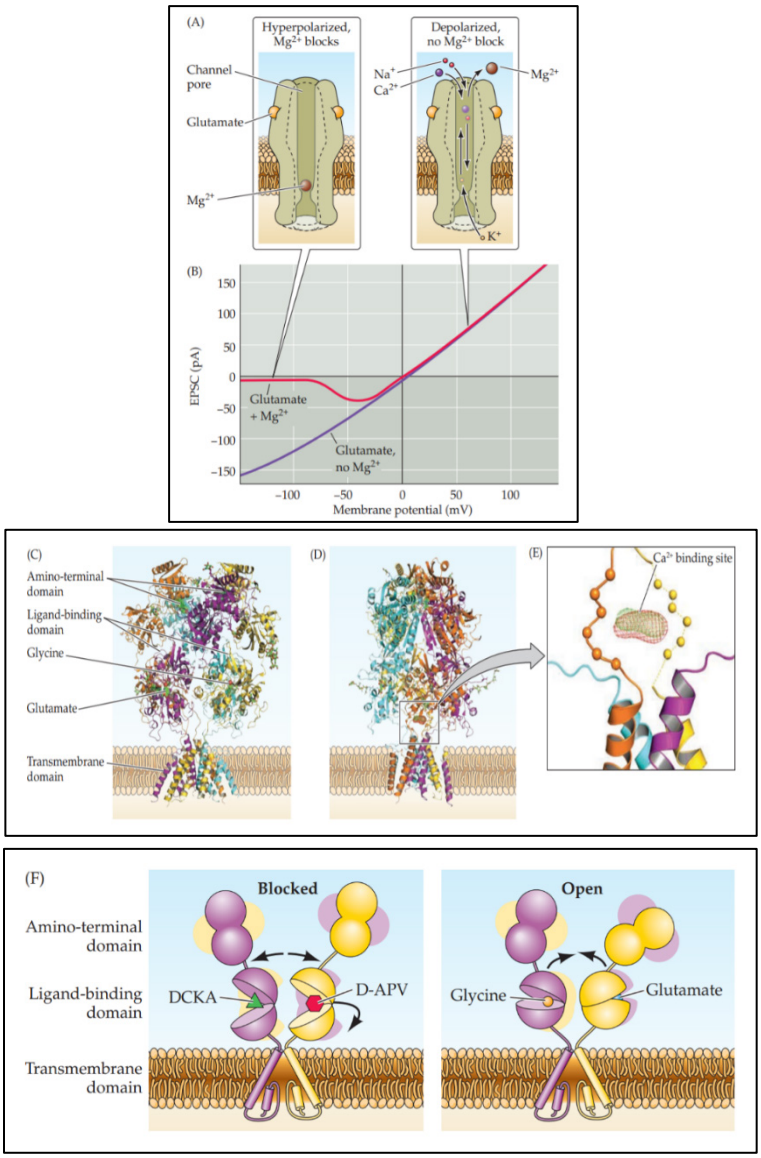

Figure 1.4: The NMDAR function and structure. (A) Mg^{2+} blocks the NMDAR pore in a voltage-dependent manner. (B) Current flowing via glutamate-activated NMDAR has a voltage dependency due to Mg^{2+} block. (C–E) In the NMDAR crystallographic structure, GluN1 subunits are orange and yellow, while GluN2 subunits are cyan and purple. (C) The NMDAR has an ATD, LBD, TMD, and CTD, similar to the AMPAR. (D) The position of the probable Ca^{2+} binding site is revealed by rotating the receptor 90 degrees. (E) In the extracellular domain of the receptor, a close-up image of the potential Ca^{2+} binding site (red and green mesh). (F) The NMDAR gating model. When bound to competitive antagonists, such as 5,7-dichlorokynurenic acid (DCKA) and D-AP5 (D-APV) (left), or agonists (Gly and L-Glu, right), the receptors amino-terminal domain and ligand-binding domain sections are proposed to shift (arrows) (Purves *et al.* 2018; Mulabagal and Calderón 2012; Pacheco-Palencia *et al.* 2008; Poulose and Shukitt-Hale 2013; Poulose *et al.* 2012; Schauss *et al.* 2006b).

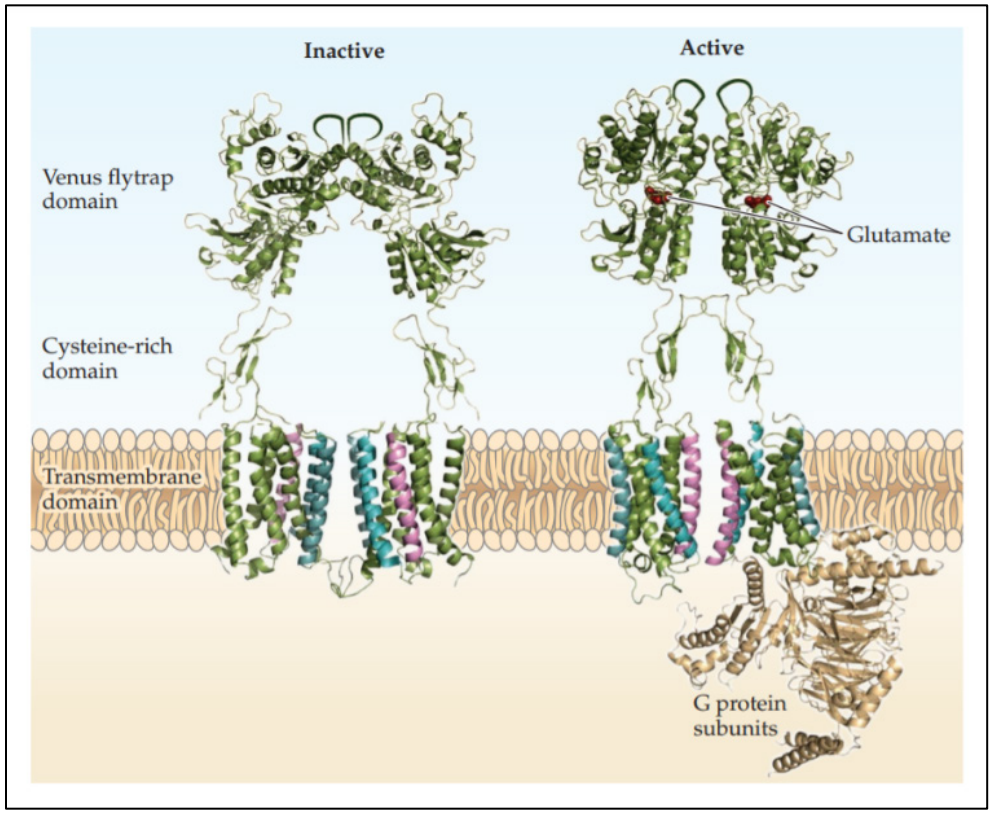

Figure 1.5: Metabotropic glutamate receptors (mGluR) structure (Purves *et al.* 2018).

1.3 Neurodegenerative diseases (NDDs)

Worldwide, NDDs are the most prevalent and serious health issue affecting the elderly and are responsible for a considerable portion of mortality and morbidity (Erkkinen *et al.* 2018). In the United Kingdom (UK), approximately one million people suffer from NDDs, and more than 50 million people suffer globally (Alzheimer's-Association 2019; Harvard-NeuroDiscovery-Center ; Neurological-Alliance 2019; World-Alzheimer-Report 2018). The annual cost of treating people with neurodegenerative illnesses is estimated to be around €130 billion (Zahra *et al.* 2020). These diseases are of global concern as their prevalence is continuously increasing and is associated with massive economic and social burdens (Féger and Hirsch 2015).

NDDs are heterogeneous disorders commonly recognized by the gradual, irreversible loss of specific groups of neurons (Obuobi *et al.* 2016). Although neurogenesis occurs at a limited level in adult human brains (Bergmann *et al.* 2015), these progressive neural degradations cause movement disabilities and a chronic decline in cognitive functions in NDDs patients (Batista and Pereira 2016). Patients with NDDs exhibit symptoms due to neural dysfunction, such as gradual memory loss and changes in their behaviour, language, learning and emotions (Batista and Pereira 2016).

Although some neurodegenerative disorders affect different areas in the brain and exhibit distinctive characteristics at the genetic and phenotypic level (Montero-Odasso *et al.* 2017; Woolley *et al.* 2011), the cellular and molecular aetiology of these diseases are considerably similar (Gitler *et al.* 2017; Hervás *et al.* 2012; Vadakkan 2016). To date, the pathological mechanisms of development of NDDs, including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and multiple sclerosis (MS), are not entirely understood (Kim *et al.* 2015). Many studies, however, have identified the cellular aetiology of these disorders, which are oxidative stress, chronic excitotoxicity, impaired mitochondria, chronic neuroinflammatory and irregular accumulation of protein in brain tissues (Chen *et al.* 2012; Denzer *et al.* 2016; Kovacs 2016; Lewerenz and Maher 2015). There are many different types of NDDs, below are some examples of the most prevalent diseases.

1.3.1 Alzheimer's disease (AD)

Dementia syndrome manifests as memory loss, difficulty speaking, psychological and psychiatric alterations that interfere with daily functioning (Burns and Iliffe 2009). Dementia is caused mostly by Alzheimer's disease (AD), a common NDD that accounts for 70 - 80% of all dementia cases (Alzheimer's-Association 2018; Boughey and Graff-Radford 2007). AD is estimated to affect 35 million people worldwide, and the prevalence is expected to reach 66 million by 2030 and over 115 million by 2050 (Banovic *et al.* 2018).

This progressive degenerative brain disease can be identified by clinical and histopathological hallmarks (Boughey and Graff-Radford 2007). The early symptoms of the disease include memory loss for new events or conversations, lacking concern, depression, and sleep disorders, and more advanced symptoms such as difficulty in communicating, speaking, swallowing and walking, as well as being unable to sense direction (Kumar *et al.* 2022). Later stages include incontinence, primitive reflexes, and caregiver dependency (Kumar *et al.* 2022). The histopathological components are plaques which are composed of the amyloid beta protein filaments (Aβ) that deposit in the extracellular space around neurons in the brain and neurofibrillary tangles (NFTs), which are intraneuronal warped threads of hyperphosphorylated tau protein (p-tau) (Alzheimer's-Association 2018; Boughey and Graff-Radford 2007; Goedert and Spillantini 2006; Reitz and Mayeux 2014). Transmembrane amyloid precursor protein (APP) is proteolytically cleaved by protease enzymes; α-sectretase, β-secretase and γ-secretase, to produce the $A\beta_{40}$ and $A\beta_{42}$ isoforms of the Aβ peptide, which consists mainly of 40–42 amino acids (Zheng and Koo 2011). In normal conditions, Aβ has several physiological functions, such as synaptic activity control; however, in AD, it aggregates and oligomerises, ultimately forming fibrous, insoluble plaques that alter neuronal connections and synaptic function resulting in neurodegeneration (Schaffert and Carter 2020). Human p-tau exists in six isoforms ranging from 352 to 441 amino acids and is mainly found in neuronal axons, where it is involved in microtubule stabilization and neuronal transport (Schaffert and Carter 2020). Moreover, tau, the primary component of NFTs, is abnormally phosphorylated in AD, resulting in structural changes

that mitigate its ability to bind microtubules and promote its accumulation into combined spiral fibers (Schaffert and Carter 2020). As a consequence of these extreme changes from the normal neural structure, the neural cells in the brain are damaged and die (Alzheimer's-Association 2018).

Moreover, the human Presenilin 1 (*PSEN1*) and 2 (*PSEN2*) genes codes for presenilins, which are transmembrane proteins that are found in intracellular membrane organelles such as the endoplasmic reticulum (ER), nuclear envelope, and Golgi apparatus and are responsible for developmental morphogenesis, unfolded protein responses, and the processing of selected proteins like the β-amyloid precursor protein (Fraser *et al.* 2000). However, studies reported that mutations of this protein cause the overproduction of long-tailed amyloid β-peptides or/and presenilin complexes due to its interaction with β-catenin, consequently resulting in familial Alzheimer's disease development (Fraser *et al.* 2000).

Biochemical examination of tissue biopsies taken from AD patients illustrated a selective neurotransmitter pathology, especially in the presynaptic cholinergic system (Francis *et al.* 1999). This was confirmed by decreased acetylcholine (ACh) synthesis and choline acetyltransferase (ChAT) activity in those patients (Francis *et al.* 1999). In addition to cholinergic transmission dysfunction and ACh depletion, AD patients' cerebral cortex and hippocampus display altered acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activity (Marucci *et al.* 2021; Nordberg *et al.* 2013). It was observed that AChE activity was reduced in some brain regions while BuChE activity was increased or unchanged (Ciro *et al.* 2012; Perry *et al.* 1978). As a consequence of these factors, cholinergic circuits degenerate, contributing to the progressive decline in memory and cognitive function that is regularly seen in AD patients (Nordberg *et al.* 2013).

1.3.2 Parkinson's disease (PD)

PD is the world second most common NDD; there were 6.1 million PD patients worldwide in 2016; however, this disease is predicted to affect 9 million people by 2030 (G.B.D-Neurology-Collaborators 2019; Xicoy *et al.* 2017). PD has motor symptoms such as bradykinesia, resting tremor, rigidity, and postural instability and non-motor symptoms

including sleeping problems, mood disorders, anosmia, cognitive difficulties, pain, and sensory disorders that deteriorate with increasing the age, resulting in a need for assistance with all day-to-day activities (Erkkinen *et al.* 2018; Xicoy *et al.* 2017).

PD's pathological hallmark is degradation or loss of dopaminergic neurons, which have a cell body in the substantia nigra pars compacta (SNpc) region and their nerve terminals projecting to the striatum (Teismann and Schulz 2004). Clinical investigation identifies that approximately 60-70% of SNpc neurons are lost before symptoms develop (Postuma *et al.* 2010). Moreover, Lewy body accumulation is a significant pathophysiology feature of the disease, which is where abnormal intracellular proteins, including aggregated α-synuclein, accumulate (Davie 2008; Goedert *et al.* 2013; Teismann and Schulz 2004; Xicoy *et al.* 2017). The cytosolic α-synuclein found within neurons is a 140 amino acid protein that plays an essential function in synaptic signalling due to its location at neuronal presynaptic terminals (Schaffert and Carter 2020). α-synuclein oligomer and/or fibril accumulation found in familial and sporadic cases of PD (Schaffert and Carter 2020).

1.3.3 Amyotrophic lateral sclerosis (ALS)

ALS also known as motor neuron disease or Lou Gehrig's disease, is a progressive disease characterized by the loss of upper and lower motor neurons in the brain and spinal cord (Lamptey *et al.* 2022; Masrori and Van Damme 2020). The worldwide incidence of ALS in recent studies ranged from 0.6 to 3.8 per 100,000 person-years, while it was higher in Europe and ranged from 2.1 to 3.8 per 100,000 person-years (Longinetti and Fang 2019). ALS individuals have a wide range of differences in terms of their onset age, place of onset, and rate of disease progression, however, most patients die within three years after symptom onset due to respiratory failure (Masrori and Van Damme 2020). Clinically, affected individuals develop progressive weakness and wasting of muscles with dysarthria, dysphagia, dysphonia or, not often, masseter weakness (Masrori and Van Damme 2020). Further to the motor impairments, some patients develop a few non-motor symptoms like frontotemporal dementia and can be identified as alteration in behaviour, executive function impairment, and/or language

impairment (Beswick *et al.* 2022; Masrori and Van Damme 2020). In addition to symptoms such as pain, sleep disturbances, and fatigue, approximately 35%–40% of patients experience mild behavioural and cognitive changes (Beswick *et al.* 2022; Masrori and Van Damme 2020). The histopathological hallmark of ALS is trans-activation response DNA binding protein 43 (TDP-43), which accumulates in the cytoplasm of degenerating neurons in both familial and sporadic forms (Suk and Rousseaux 2020). Normally, TDP-43 resides in the nucleus and regulates RNA metabolism; however, in ALS, it accumulates in the cytoplasm as a truncated phosphorylated protein aggregate (Mackenzie and Rademakers 2008; Suk and Rousseaux 2020).

Moreover, other proteins, such as the antioxidant enzyme superoxide dismutase-1 (SOD1), have also been reported to form aggregations in familial and sporadic ALS (Rotunno and Bosco 2013). SOD1 is a copper- and zinc-dependent metalloenzyme consisting of 153 amino acids and two noncovalently linked subunits and is 32 kDa in size (Pansarasa *et al.* 2018). In addition to the cytoplasm, SOD1 is found in nuclei, mitochondria, and lysosomes (Pansarasa *et al.* 2018). Its vital role is to eliminate the free radicals that cause oxidative stress via the dismantling of highly reactive superoxide radicals ($O_2^{\bullet-}$) into oxygen (O_2) and less harm hydrogen peroxide (H_2O_2) (Pansarasa *et al.* 2018). In familial ALS caused by SOD1 mutations, abnormal post-translational processing is thought to lead to protein misfolding, aggregation, and neuronal inclusion bodies (Schaffert and Carter 2020). In addition, patients with sporadic ALS are found to have misfolded SOD1 (Schaffert and Carter 2020).

In addition, the human gene C9orf72 encodes the protein C9orf72 that is found in many areas of the brain and involved in the regulation of vesicular membrane transport and autophagy (Pang and Hu 2021; Yang *et al.* 2020). The hexanucleotide repeat (GGGGCC (G_4C_2)) mutations in the C9orf72 gene are the most common cause of 40% of familial ALS as well as 6% of sporadic ALS (Pang and Hu 2021; Lai and Ichida 2019; Yang *et al.* 2020). Researchers found that ALS patients may have up to over hundreds of repeats of G_4C_2 in comparison with healthy people who have only 2 to 23 repeats (Pang and Hu 2021; Yang *et al.* 2020). As a result of the vast G_4C_2 repeat expansion in C9orf72, both of

the predominant neurotoxic and neurodegenerative mechanisms causing ALS are induced: impaired autophagy in proteostasis (loss-of-function) and protein misfolding aggregates (gain-of-function) (Lai and Ichida 2019; Yang *et al.* 2020).

1.3.4 Huntington's disease (HD)

HD is a fatal neuropsychiatric degenerative disorder resulting from a dominant inheritance, and it typically appears between 35 and 50 years of age (Roth 2019). The global estimated prevalence of HD ranges between 0.4 and 13.7 cases per 100,000 people (Baig *et al.* 2016). In normal conditions the huntingtin protein (Htt) is a 3144-amino-acid monomeric protein that regulates synaptic activity and is necessary for embryonic neurogenesis (Young 2003). In HD patients, the Htt gene that is found in the short arm of the fourth chromosome is mutated due to 40 or more trinucleotide repetitions of cytosine-adenine-guanine (C-A-G) which encodes the amino acid Gln (Roth 2019). This mutation results in a polyglutamine (polyQ) expansion in the Htt protein's extreme N terminus (Harding *et al.* 2019). A histological characteristic of HD is the production of intraneuronal aggregated and inclusion bodies of PolyQ-expanded Htt (Arrasate and Finkbeiner 2012). Proteostasis induction, transcription impairment, mitochondrial dysfunction, cellular energy imbalance, synaptic dysfunction, and axonal transport damage ultimately result in neuronal death all hypothesised to be caused by polyQ-expanded Htt (Arrasate and Finkbeiner 2012; Han *et al.* 2010). HD is distinguished by two types of neurons that are primarily affected, which are the GABAergic medium spiny neurons (MSNs) in the striatum, and dopaminergic projections from the SNpc to the striatum (Kim *et al.* 2021). The main clinical aspects of HD include movement impairments, notably choreatic dyskinesias and voluntary movement impairment, as well as neuropsychiatric disorders, which include changes in behaviour and personality and cognitive decline (Roth 2019).

1.3.5 Multiple sclerosis (MS)

MS is the most prevalent autoimmune NDDs causing significant physical or cognitive deficits and neurological consequences in young adults (Filippi *et al.* 2018; McGinley *et*

al. 2021). MS usually occurs in people between the ages of 20 and 40, but it also affects children, while about 2-10 % develop MS beyond 50 years (Filippi *et al.* 2018; Ghasemi *et al.* 2017; Inaloo and Haghbin 2013). Since 2013, MS prevalence has increased globally, with an estimated 2.8 million individuals living with MS worldwide in 2020 (35.9 per 100,000 population) (Walton *et al.* 2020). MS is not considered a genetic disease, with familial MS accounting for about 13% of all MS phenotypes; however, some genetic changes through environmental triggers are thought to raise the likelihood of acquiring the illness (Filippi *et al.* 2018).

The hallmark pathological feature of MS is the presence of demyelinating lesions in the brain and spinal cord grey and white matter (Dobson and Giovannoni 2019; Filippi *et al.* 2018). The principal pathogenesis causes of myelin sheath destruction in MS involves the widespread inflammation in the white and grey matter tissues of the CNS caused by T lymphocyte, B lymphocyte and macrophage infiltrations, and oligodendrocyte apoptosis (Dobson and Giovannoni 2019; Filippi *et al.* 2018; Ghasemi *et al.* 2017).

MS has four distinct clinical courses, each with its particular treatment strategy and disease prognosis; relapsing-remitting multiple sclerosis (RRMS), secondary progressive multiple sclerosis (SPMS), primary progressive multiple sclerosis (PPMS) and progressive relapsing multiple sclerosis (PRMS) (Filippi *et al.* 2018; Ghasemi *et al.* 2017). At the outset of symptoms, 87 % of patients are diagnosed with RRMS, defined by unstable acute attacks followed by periods of remission; after that, most develop progressive, permanent disability SPMS (Filippi *et al.* 2018; Ghasemi *et al.* 2017). Furthermore, 10% of MS patients have a disease course that includes a persistent deterioration in neurological function without recovery, categorised as PPMS (Filippi *et al.* 2018; Ghasemi *et al.* 2017). Lastly, PRMS is the least frequent variety of MS, affecting about 5% of individuals and causing symptoms such as eye pain and double vision, sexual, digestive, and urinary system dysfunction, disorientation, and depression (Filippi *et al.* 2018; Ghasemi *et al.* 2017). MS presents a variety of clinical symptoms that depend on where the demyelinating lesions are located in the CNS, including vision problems, paresthesia, movement disability, fatigue, rigidity, mental illnesses, and

psychological issues, as well as digestive and urinary system problems (Filippi *et al.* 2018; Ghasemi *et al.* 2017; McGinley *et al.* 2021).

Furthermore, several pieces of evidence demonstrated the link between neuroinflammation and neurodegeneration in MS and protein aggregations such as Aβ, tau, APP, and bassoon protein (Bsn) (Schaffert and Carter 2020). The scaffold protein Bsn consists of 3926 amino acids and is found in the cytoskeletal matrix of presynaptic neurons, where it is involved in regulating the synaptic function; however, in MS, it is mislocalized and accumulates in neurons, causing neurotoxicity (Schaffert and Carter 2020).

In addition to providing examples of the most common NDDs and giving critical information about each disease, Table 1.2 below summarizes approximate global prevalence, predicted change in prevalence, frequency of genetic and sporadic forms, damaging area and protein, the average age at onset, average life expectancy after onset, as well as the clinical symptoms of major NDDs.

Table 1.2: Overview of the most common neurodegenerative diseases (Bäckström *et al.* 2018; Baig *et al.* 2016; G.B.D-Neurology-Collaborators 2019; de Lau *et al.* 2005; Dommershuijsen *et al.* 2020; Gan *et al.* 2018; Ghosh and Tabrizi 2018; Kirkwood *et al.* 2001; Kumar *et al.* 2022; Lassmann 2018; Liang *et al.* 2021a; Myers and Chakraborty 2011; Reitz and Mayeux 2014; Roth 2019; Schaffert and Carter 2020; Stephenson *et al.* 2018; Tysnes and Storstein 2017; Walton *et al.* 2020).

Disease /area of comparison	Amyotrophic lateral sclerosis (ALS)	Parkinson's disease (PD)	Multiple sclerosis (MS)	Alzheimer's disease (AD)	Huntington's disease (HD)
Global prevalence	0.6 to 3.8:100,000	100-200:100,000	35.9:100,000	593:100,000	0.4-13.7:100,000
Predicted change in prevalence	↑69% in 25 years	Double in 25 years	↑2·4% per year	↑3·3% per year (triple by 2050)	↑15 - 20% per decade
Sporadic cases	90 - 95%	>90%	80 - 90%	>98%	3%
Familial cases	5 - 10%	<10%	10 - 20%	<2%	97%
Damaged areas	Motor neurons in the cortex, brain stem, and spinal cord	Substantia nigra in basal ganglia and thalamus	Cortex, basal ganglia, brain stem, spinal cord	Cortex, hippocampus	Basal ganglia, cortex
Mutated proteins	TDP-43, SOD1, c9orf72	α-synuclein	-	APP, presenilins	Htt
Proteins aggregates	SOD1, TDP-43	α-synuclein (Lewy bodies)	Aβ, tau, APP, Bsn	Aβ (plaques), tau (tangles)	Htt
Mean age at diagnosis	47 - 52 (familial) 58 - 63 (sporadic)	60 - 70	20 - 40	1-5% early onset AD < 65, 95-99% late-onset AD > 65	35 - 50
Mean survival from time of diagnosis	3 - 5 years	6.7 - 9.1 years	6 - 7 years	5.8 years	15 - 20 years
Signs/symptoms at presentation	Weakness Spasticity Cramps Fasciculations	Fasciculations Rigidity Rest tremor	Optic neuritis Paresthesias Limb weakness	Memory loss for new events or conversations, lacking concern,	Involuntary motor abnormalities, primarily chorea. abnormal extraocular movements,

Disease /area of comparison	Amyotrophic lateral sclerosis (ALS)	Parkinson's disease (PD)	Multiple sclerosis (MS)	Alzheimer's disease (AD)	Huntington's disease (HD)
		Postural instability	Impaired coordination	depression, and sleep disorders	brisk muscle stretch reflexes, diminished rapid alternating movements, short-term memory and visuospatial functioning
Signs/symptoms of advanced disease	Dysphagia Respiratory failure Sialorrhea Pain	Dysphagia Levodopa-induced movement disorders Sialorrhea	Spasticity Pain Bowel/bladder dysfunction Fatigue Depression	Difficulty in communicating, speaking, swallowing and walking, unable to sense direction, incontinence, primitive reflexes, dependence in the activities of daily living	Global subcortical dementia, bradykinesia, spasticity, dysarthria, dysphagia, incontinence, and dependency in the activities of daily living
Clinical course	Progressive	Progressive	Relapsing-remitting Primary progressive Secondary progressive	Progressive	Progressive

1.3.6 The cellular and molecular aetiology of NDDs

Oxidative damage is a common cytopathology of several NDDs. This damage is caused by an excessive accumulation of highly reactive free radicals associated with an impaired oxidant defence system, which is unable to adequately prevent this buildup of radicals (Markesbery and Carney 1999; Sayre *et al.* 2001). Free radicals, such as 'reactive oxygen (ROS), nitrogen (RNS) and chlorine species (RCS)', are defined as 'any atom or molecule that has one or more unpaired electrons' (Florence 1995). Typically, a low concentration of free radicals is essential for normal cellular functions; on the other hand, they are harmful if found out of their normal place or in high abnormal concentrations (Florence 1995; Schreck and Baeuerle 1991). Data indicates that neural cells undergo necrotic or apoptotic death when they fail to adequately respond to oxidative stress (Halliwell and Whiteman 2004; Perry *et al.* 1998a; Perry *et al.* 1998b). Cellular death by ROS occurs through the alteration of the critical biomolecules (lipid, protein and nucleic acids) that can seriously affect cell health and viability or induce a variety of cellular responses through the generation of secondary reactive species (Halliwell and Whiteman 2004). Evidence demonstrates that the CNS is more vulnerable to free radical damage than other organs due to the high level of O_2 intake by the brain, abundant lipid content, specifically polyunsaturated fatty acids, and low levels of antioxidant enzymes (Chance *et al.* 1979; Floyd and Carney 1992; Shivasharan *et al.* 2013; Uttara *et al.* 2009). Therefore, oxidative stress is a key pathomechanism contributing to neuronal degradation.

Another cellular aetiology contributing to NDD development is 'excitotoxicity', which is neuron degeneration caused by excessive or prolonged activation of excitatory receptors by the excitatory amino acid L-Glu (Lau and Tymianski 2010). It has been found that L-Glu accumulates in various NDD patients, causing a toxicity cascade leading to neuronal death (Mehta *et al.* 2013; Belov Kirdajova *et al.* 2020). This pathological mechanism commonly occurs via iGluR; it involves AMPAR and KAR which mainly influx Na^+, and NMDAR which influx significant amounts of Ca^{2+} (Lodge 2009). Two different excitotoxic pathways are defined; acute and delayed forms of cytotoxic events due to the

overactivation of L-Glu receptors (Choi *et al.* 1987; Kato *et al.* 1991; Rothman and Olney 1986). The acute form occurs during minutes of excitatory amino acid exposure and is characterised by excessive neuron depolarization via the opening of membrane cation channels leading to chloride ion (Cl^-) and passive Na^+ influx, followed by an influx of water causing neuronal swelling, osmotic lysis and neuronal necrosis (Rothman *et al.* 1987).

In contrast, delayed neuronal degeneration is Ca^{2+}ion-dependent and occurs after several hours of L-Glu exposure (Choi 1992). In this excitotoxic pathway, a massive influx of extracellular Ca^{2+}, together with any Ca^{2+} release triggered from intracellular stores, increases cytosolic free Ca^{2+} and initiates a cascade-like effect leading to cell death (Choi 1987). The excessive influx of Ca^{2+} through NMDAR and AMPAR (Bonfoco *et al.* 1995; Choi and Rothman 1990) or following mGluR activation (Farooqui and Farooqui 2009) leads to necrotic cell death through damage by the generated free radicals and activation of Ca^{2+} dependent catabolism enzymes (Norenberg and Rao 2007). Moreover, this also triggers transcriptional activation of apoptotic programs (Londono *et al.* 2012; Pellegrini-Giampietro *et al.* 1997).

Mitochondrial dysfunction has been implicated in neuron death in all NDDs. Mitochondrial damage leads to impaired energy generation, imbalanced Ca^{2+}concentration, mitochondrial DNA (mtDNA) alteration, increased ROS and release of pro-apoptotic factors, ultimately resulting in cell death via apoptosis or necrosis (Beal 1998; Krieger and Duchen 2002; Wu *et al.* 2019).

Chronic neuroinflammation is a prominent pathological aspect of NDDs. The chronic activation of immune cells in the CNS through peripheral immune cell infiltration through the blood brain barrier (BBB) is common to all NDDs, regardless of their etiologies (Stephenson *et al.* 2018). CNS inflammation (what is referred to as "neuroinflammation") can have several effects, including neuroprotective, neuro-regenerative, and neurodegenerative outcomes, depending on the timing, location, and duration (Wareham *et al.* 2022). It is a physiological response that occurs in the CNS, and it can be triggered by a variety of exogenous and endogenous insults, including normal ageing, dementia, autoimmune reactions, trauma, strokes, hypertension, depression,

diabetes, tumours, infections, traumatic brain injury, toxins, and medicines (Ahmad *et al.* 2022b; Chen *et al.* 2017; Kempuraj *et al.* 2016; Lucas *et al.* 2006).

A crucial distinction must be made between neuroinflammation associated with disease and injury and the type of inflammation related to non-pathological conditions (Wohleb and Godbout 2013). Non-pathological neuroinflammation occurs in situations without significant disruption of the BBB, peripheral immune cell (such as leukocytes, including T cells and macrophages) infiltration, or clear damage to the CNS (Hanna *et al.* 2022; Wohleb and Godbout 2013). This is also known as an acute inflammatory response or low-grade inflammatory response, which results from the activation of innate immunity in which glial cells (microglia and astrocytes) are activated immediately upon receiving harmful stimuli (Hanna *et al.* 2022; Kempuraj *et al.* 2016; Wohleb and Godbout 2013). This is followed by the release of brief cytokines and chemokines that facilitate better defence against foreign invaders and develop adaptive and beneficial physiological changes (Hanna *et al.* 2022; Kempuraj *et al.* 2016; Wohleb and Godbout 2013). However, pathological neuroinflammation is caused by diseases, traumatic CNS injuries, CNS infections, and autoimmunity, which trigger immune responses that disrupt neuronal function, cause significant tissue damage, and higher infiltrate peripheral immune cells (Wohleb and Godbout 2013). As well-known as chronic neuroinflammation, its manifestations include prolonged and increased inflammatory reactions, high levels of inflammatory mediators, and accumulation of activated inflammatory cells (Kempuraj *et al.* 2016). Additionally, this pathological type of inflammation causes oedema, in which neuron fluid retention results in neuron destruction and eventually dies (Ahmad *et al.* 2022b; Disabato *et al.* 2016). The most severe consequences of neuroinflammation in the CNS are oedema and tissue swelling, as the brain can only swell to a limited extent due to restraints by the bony skull and may result in pathological increases of intracranial pressure, which disrupts microcirculation and exacerbates tissue damage caused by ischemia (Lassmann 2015). When inflammatory stimuli persist, they can cause a failure in the normal resolution mechanism or continues activation of the inflammatory cells, contributing to the development of NDDs (Hanna *et al.* 2022; Kempuraj *et al.* 2016).

There is ample evidence that proteotoxicity is one of the consequences of neurodegeneration in NDDs. Two crucial mechanisms enable cells to maintain the quality of their proteins, including the ubiquitin-proteasome system and chaperones-mediated autophagy (Li *et al.* 2022). However, in NDDs, disrupted normal protein function and subsequent proteinopathies correlate with disturbed cellular processes, such as protein translation (genetic mutation), post-translational modification, and/or protein degradation or clearance (Lontay *et al.* 2020). Whether the form of NDD progression is sporadic or familial, protein toxicity is a unifying characteristic (Flagmeier *et al.* 2016; Hunter and Brayne 2018; Mori *et al.* 2013). Typically, NDDs are characterized histopathologically by abnormal intra- or extracellular aggregates of degradation-resistant proteins in specific brain areas (Ruz *et al.* 2020).

Neurons affected by toxic proteins undergo cell death due to impairment of the transcription process, mitochondrial function, nucleocytoplasmic transport, and protein/RNA quality control system (Figure 1.6) (Gasset-Rosa *et al.* 2017; Lustbader *et al.* 2004; Mateju *et al.* 2017; Tai *et al.* 2012). Many data suggest that the neurons or brain area affected by aggregated or misfolded protein can be spread to other neurons or regions in the brain (Desplats *et al.* 2009; Kaufman *et al.* 2016; Ren *et al.* 2009; Westergard *et al.* 2016). Therefore, toxic protein triggers the progress of neurodegeneration in most NDDs.

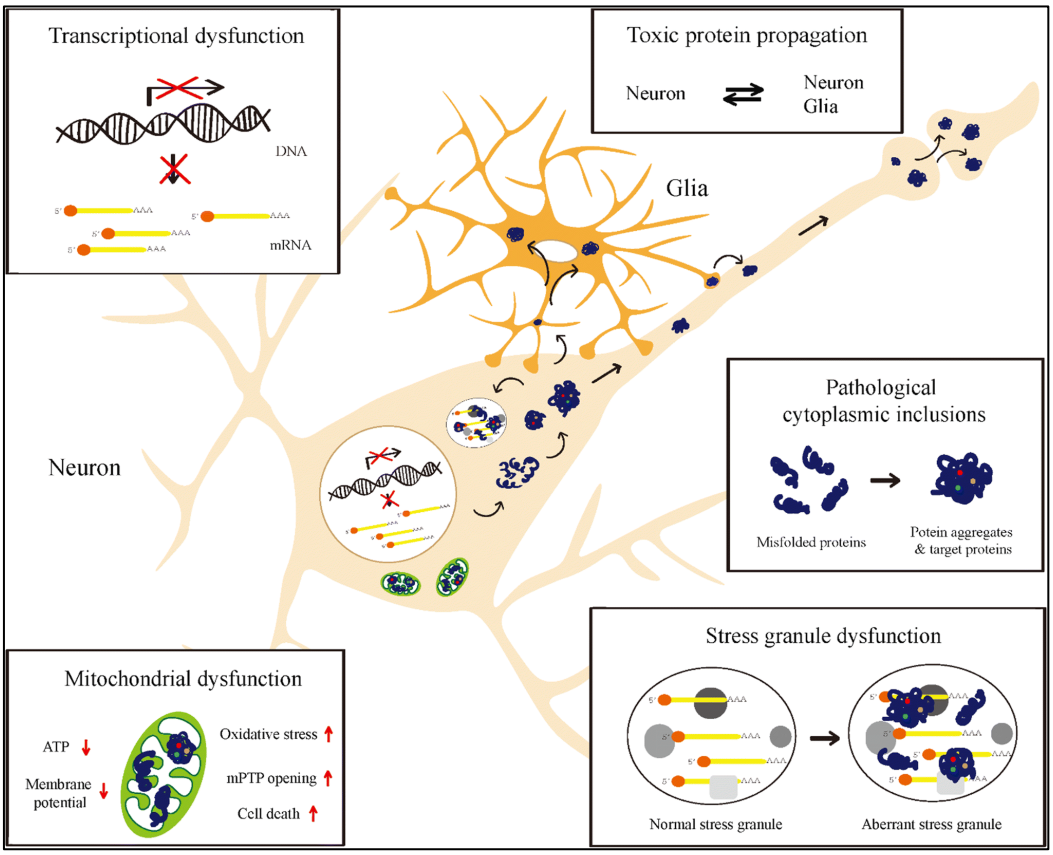

Figure 1.6: Protein toxicity in neurodegenerative diseases. This diagram illustrates the mechanisms of protein toxicity on neuronal cells. Specific compartments in neurons such as the nucleus, mitochondria, cytoplasm, and stress granules are affected by toxic protein aggregation. Toxic disease protein can be propagated and cause the spreading of the disease pathology to other nearby neurons (Chung *et al.* 2018). mPTP, Mitochondrial permeability transition pore.

1.3.7 The risk factors of neurodegenerative diseases

Research has categorised the risk factors that influence the development of NDDs into non-modifiable and modifiable categories (Farooqui and Farooqui 2016; Podcasy and Epperson 2016). Non-modifiable factors, such as age, family history, genetic susceptibility, gender, and race/ethnicity, cannot be altered by medical involvement or personal behaviour (Baumgart *et al.* 2015; Beydoun *et al.* 2022; Edwards III *et al.* 2019; Howell *et al.* 2017; Podcasy and Epperson 2016). In contrast, modifiable factors, such as unhealthy lifestyles like smoking, excessive alcohol consumption, and inadequate diet, can be changed or modified to lower the chance of progressive cognitive decline (Edwards III *et al.* 2019; Farooqui and Farooqui 2016; Podcasy and Epperson 2016). Age is the prominent risk factor for NDDs; studies demonstrate that most people with AD dementia are 65 years or older, and the prevalence of NDDs increases with age (Alzheimer's-Association 2019; Hebert *et al.* 2013; Hou *et al.* 2019).

Family history is another critical risk factor for developing NDDs; for instance, researchers have determined that AD incidence increases in individuals with first-degree relatives with AD (Green *et al.* 2002; Loy *et al.* 2014). Collective evidence suggests that mutations in some genes are closely associated with NDD incidence (Chung *et al.* 2018; García and Bustos 2018); for instance, the correlation between familial AD and mutations in three genes: *APP*, *PSEN1* and *PSEN2* (Cacace *et al.* 2016; Dorszewska *et al.* 2016; Guerreiro *et al.* 2012). Moreover, the prevalence of NDDs, such as AD, PD, ALS, HD, and MS, is gender-dependent, and the severity of the disease varies significantly between the sexes (Trojsi *et al.* 2020; Ullah *et al.* 2019). According to the studies, whether NDDs are more prevalent in women or men may be influenced by life expectancy (women live longer than men), biological factors (hormonal factors, epigenetic factors, and frailty), cognitive factors (women and men generally perform differently on cognitive tests), and gender-specific social roles (educational and occupational opportunities, post-retirement roles) (Andrew and Tierney 2018). It was reported that gender and sexes play an independent role in the development of NDDs, with men having a higher risk of PD, ALS, and AD, while females have a higher risk of HD and MS (Trojsi *et al.* 2020; Ullah *et al.* 2019; Zielonka and Stawinska-Witoszynska 2020). Moreover, race/ethnicity

appears to influence the prevalence of NDDs. However, several estimates indicate that the highest diagnostic frequency was observed in individuals who identified as White (Bruzelius *et al.* 2019; Dahodwala *et al.* 2009; Kister *et al.* 2021; Lennon *et al.* 2022; Rechtman *et al.* 2015).

Recently, sufficiently strong evidence from several studies suggests that many environmental factors, such as metals, pesticides, solvents, electromagnetic fields, brain injuries, lifestyles, dietary factors/food additives, and other neurotoxic agents are considered risk factors for NDDs (Farooqui 2015; Jakaria *et al.* 2018; Modgil *et al.* 2014; Pang *et al.* 2019). In addition, groups of acidic amino acids, such as L-Glu and D-aspartic acid, occurring naturally or as additives in the diet, can act as excitotoxins and have been identified as NDD developing agents (Mattson 2003; Meldrum 1993; Olney 1990; Olney *et al.* 1979). Furthermore, cognitive impairments can be influenced by many other modifiable factors such as hypercholesterolemia, diabetes mellitus, obesity, and hypertension (Boughey and Graff-Radford 2007; Silva *et al.* 2019b).

1.3.8 Neurodegenerative diseases current and potential treatment

The World Health Organization (WHO) has identified cardiovascular disease, cancer, and NDDs as the three major causes of death in developed countries (Rowinska-Zyrek *et al.* 2015). To date, no cure has been found for NDDs, and existing treatments target a specific area of the brain and can ameliorate symptoms without stopping disease progression, thereby resulting in permanent disability or death of those afflicted (Hussain *et al.* 2018). Table 1.3 represents the various drugs used for the treatments of NDDs, a comparison of their pharmacologic effects, and reported side effects.

In addition to previously FDA approved medications for treating mild-to-moderate AD symptoms including cholinesterase inhibitors (ChEIs) and the NMDAR antagonist, memantine (Grand *et al.* 2011; Massoud and Léger 2011; Winslow *et al.* 2011), FDA recently approved two new drugs which are monoclonal antibodies targeting Aβ, lecanumab and aducanumab (US-FDA 2021, 2023). Even though the medications slow the progression of the disease, none of them can prevent or stop it, nor can they provide long-term relief (Budd Haeberlein *et al.* 2022; Féger and Hirsch 2015; Hardy and

Mummery 2023; Li *et al.* 2019; McDade *et al.* 2022; Yiannopoulou and Papageorgiou 2013). These treatments can cause adverse effects (Table 1.3) (Atri 2019; Qiao *et al.* 2023; Rahman *et al.* 2023; Winslow *et al.* 2011; Yiannopoulou and Papageorgiou 2020). The actions of ChEIs and NMDAR antagonists involve modulation of neurotransmitter systems, such as those of ACh and L-Glu, respectively (Massoud and Léger 2011; Winslow *et al.* 2011). The mechanism of action of human immunoglobulin 1 (IgG1) drugs involves selectively targeting Aβ aggregates and promotes its clearance. Lecanemab targets soluble Aβ aggregates (oligomers and protofibrils) while aducanumab binds to neuritic Aβ plaques and Aβ oligomers by specifically binding to amino acids 3 - 7 of the Aβ peptide (Arndt *et al.* 2018; Linse *et al.* 2020; Söderberg *et al.* 2023; Söllvander *et al.* 2015).

Currently, PD is a non-reversible condition, and there is no cure, and available medications offer only modest relief of symptoms, helping to improve the quality of the patients' life (Zahoor *et al.* 2018). However, managing PD is primarily about restoring the brain dopamine levels (Lamptey *et al.* 2022; Zahoor *et al.* 2018). Most people with PD are treated with combined levodopa and carbidopa (Lamptey *et al.* 2022; Zahoor *et al.* 2018). In addition, entacapone and tolcapone inhibit catechol-O-methyl transferase (COMT), causing the methylation loss of levodopa (Table 1.3) (Lamptey *et al.* 2022; Zahoor *et al.* 2018). Moreover, two drug classes of PD are also used; dopaminergic agonists are drugs that mimic the effect of dopamine, while monoamine oxidase inhibitors preserve the dopamine in the brain by preventing oxidative deamination (Lamptey *et al.* 2022; Zahoor *et al.* 2018).

More than 40 randomized controlled trials conducted in patients with ALS in the past decade have failed to demonstrate any drugs with a benefit on disease progression or survival, illustrating the complexity of the disease (Mitsumoto *et al.* 2014). Two medications are approved for ALS patients: riluzole, a L-Glu-receptor antagonist (KAR, NMDAR, and AMPAR) (Albo *et al.* 2004; Debono *et al.* 1993), and edaravone, which works as a free-radical scavenger (Lamptey *et al.* 2022). According to clinical studies, ALS patients treated with riluzole live 3 to 4 months longer than those treated with a placebo (Masrori and Van Damme 2020).

Unlike PD, the HD results from an overactivity of dopaminergic nigrostriatal pathways and current treatments focus on symptomatic management (Kim *et al.* 2021). Two United States (US) food and drug administration (FDA) approved treatments for HD are tetrabenazine and deutetrabenazine (Table 1.3) (Kim *et al.* 2021). One major adverse effect of HD treatment is that tetrabenazine may raise the risk of suicide in HD patients (Kim *et al.* 2021).

MS patients are treated mainly with immunosuppressants and immunomodulators, and are primarily effective against the inflammatory component of the disease (Wei *et al.* 2021). The FDA currently approves the following treatments for MS: interferon-β (IFN-β), glatiramer acetate (GA), teriflunomide, fingolimod (FTY), mitoxantrone, natalizumab, dimethyl fumarate (Tecfidera), and alemtuzumab (Table 1.3) (Wei *et al.* 2021).

To conclude, treatments for NDDs have limited effectiveness because the diseases have complex aetiology and pathogenesis (Slanzi *et al.* 2020). NDDs are currently treated with therapeutic drugs that do not provide a complete cure and only produce a limited, sometimes even temporary, symptomatic relief without addressing the progressive loss of neurons combined with these disorders. Considering the multifactorial pathoetiology of NDDs, conventional drug discovery approaches targeting one pathomechanism may not be ideal for disease-modifying therapeutic development for these diseases (Van der Schyf 2011). Moreover, these treatments are connected with several unpleasant severe events (Table 1.3). Therefore, discovering a novel therapy with multi-activities targeting several pathological issues with no or little side effect, and can stop or reverse the disease, has been considered by many neuroscientists.

Moreover, broad evidence shows that persistent oxidative stress is the key factor in developing and maintaining the progressive neurodegeneration process in NDDs (Chen *et al.* 2012; Singh *et al.* 2019; Solleiro-Villavicencio and Rivas-Arancibia 2018). Several studies demonstrated that post-mortem brains of NDD patients, including AD, PD, ALS, HD, and MS, exhibit oxidative injury markers (Beal 2003; Dexter *et al.* 1989; Dias *et al.* 2013; Kreilaus *et al.* 2016; Le Gall *et al.* 2020; Lovell and Markesbery 2007; Polidori *et al.* 1999; Sorolla *et al.* 2008; Tanaka and Vécsei 2020; Youssef *et al.* 2018). Evidence supports the hypothesis that chronic oxidative stress can lead to mitochondrial

dysfunction, protein aggregation, and neuroinflammation that can trigger a cascade of events leading to cell death in multiple NDDs (Chen *et al.* 2012; Singh *et al.* 2019; Solleiro-Villavicencio and Rivas-Arancibia 2018). Therefore, finding an agent targeting oxidative stress with no adverse effects can help in the treatment of NDDs.

Table 1.3: Summarizes the currently approved drugs for NDDs, their mechanisms of action, and reported side effects (Budd Haeberlein *et al.* 2022; Cada *et al.* 2013; Cruz 2018; Huntington-Study 2016; Jankovic and Clarence-Smith 2011; Kim *et al.* 2021; Lamptey *et al.* 2022; Leyden and Tadi 2021; Loma and Heyman 2011; Masrori and Van Damme 2020; Qiao *et al.* 2023; Rahman *et al.* 2023; Rollins and Blumenthal 2016; Silvestrelli *et al.* 2006; Solopova *et al.* 2023; Wei *et al.* 2021; Yiannopoulou and Papageorgiou 2020; Zahoor *et al.* 2018).

Diseases	Approved drugs	Mechanism of action	Adverse effects
Alzheimer's disease (AD)	Donepezil	Rapid, reversible inhibitors of AChE	Gastrointestinal-like nausea, vomiting, diarrhoea, sleep disturbances, bradycardia, cardiac conduction defects, and syncope.
	Galantamine	Rapid, reversible inhibitors of AChE	
	Rivastigmine	Slowly reversible inhibitor of AChE and BuChE	
	Memantine	Noncompetitive antagonist of the NMDA-type L-Glu receptor	Dizziness, headache, confusion.
	Lecanumab	Reduce Aβ plaques and prevent Aβ deposition	Nausea, vomiting, diarrhea, anorexia, dizziness, depression, and headache. Brain oedema and microhaemorrhage or hemosiderin deposition into brain parenchyma. Seizures
	Aducanumab		
Parkinson's disease (PD)	Levodopa	Decarboxylated in the brain to dopamine	Dyskinesias, severe on-off motor fluctuations, gastrointestinal disturbances, nausea, vomiting, orthostatic hypotension, and neuropsychiatric features such as anxiety and hallucinations.

Diseases	Approved drugs	Mechanism of action	Adverse effects
	Apomorphine hydrochloride Pergolide Pramipexole dihydrochloride Ropinirole hydrochloride Rotigotine	Dopamine agonists	Nausea, vomiting, dry mouth, insomnia, peripheral oedema, constipation, fainting, hallucinations, sleepiness, compulsive, impulsive behavioural problems (impulse control disorder). Withdrawal syndrome (anxiety, panic attacks, insomnia, irritability, dysphoria, agitation, fatigue, orthostatic hypotension, diaphoresis, and drug cravings). Pergolide; withdrawn as a treatment in 2007, risk of pericardial, retroperitoneal, and pleural fibrosis.
	Carbidopa	Decarboxylase inhibitors: Peripheral DOPA-decarboxylase inhibitor (Prevent peripheral breakdown of levodopa).	Carbidopa-induced dyskinesias, including "facial twitching and head bobbing.
	Selegiline, rasagiline	Monoamine oxidase inhibitors (inhibit the oxidative deamination of dopamine in the brain).	Gastrointestinal side effects, aching joints, depression, fatigue, dry mouth, insomnia, dizziness, confusion, nightmares, hallucinations, flu-like symptoms, indigestion, and headache.
	Entacapone, tolcapone	Catechol-O-methyltransferase (COMT) inhibitor that blocks the peripheral conversion of levodopa to 3-O-methyD	Dyskinesias, sleepiness, nausea, loss of appetite, diarrhoea, dizziness, orange urine discolouration, hallucinations, abdominal pain, headaches, confusion, dry mouth, and chest pain. Tolcapone cause hepatotoxicity.
Amyotrophic lateral sclerosis (ALS)	Riluzole	L-Glu receptor antagonist	Nausea, diarrhoea, fatigue, dizziness and liver problems.
	Edaravone	Free radical scavanger	Contusions, gait disturbance, headache, dermatitis, eczema, and respiratory disorder.

Diseases	Approved drugs	Mechanism of action	Adverse effects
Huntington's disease (HD)	Tetrabenazine	Inhibits the VMAT2 and causes presynaptic depletion of monoamine	Depression, sedation, fatigue, akathisia, anxiety, nausea, parkinsonism, increase the risk of suicide.
	Deutetrabenazine	VMAT2 inhibitor structurally related to tetrabenazine	Cholecystitis, agitated depression, somnolence, increase the risk of suicide.
Multiple sclerosis (MS)	Interferon IFN-β : IFN-β-1a IFN-β-1b	Inhibition of T cell activation and proliferation reduction in matrix metalloproteinase activity. Reduces the production of proinflammatory cytokines and induces anti-inflammatory cytokines.	Skin reactions; erythema, itching necrosis, influenza symptoms, muscle pain, joint pain, chills, headache, and body weakness.
	glatiramer acetate (GA)	Immunomodulator: Inhibits antigen-specific murine T cell hybridomas. Increases cytokine levels such as interleukin 10 (IL-10), tumour necrosis factor-α (TNF-α), and interleukin 4 (IL-4). Increasing anti-inflammatory action via increases expression of Foxp3 in $CD4^+$ $CD25^+$T regulatory cells. Shifts reactive proinflammatory T helper 1 lymphocyte (Th1) population to an anti-inflammatory T helper 2 (Th2).	Skin reaction at the injection site, fat atrophy, blushing, chest pain, palpitations, urticaria, and dyspnea.
	Teriflunomide	Dihydroorotate dehydrogenase inhibitor; reduce DNA synthesis, inhibits T and B cell proliferation and production of cytokines, and inhibits	Diarrhoea, nausea, thinning of hair (alopecia), increased alanine aminotransferase (liver damage).

Diseases	Approved drugs	Mechanism of action	Adverse effects
		intracellular adhesion molecule production.	
	fingolimod - (FTY)	Sphingosine 1-phosphate receptor (S1PR) receptor modulator: inhibit the lymphocytes from entering CNS. Regulate the expression of S1PR on the surface of oligodendrocytes and neurons neuroprotection and repair.	Cardiac autonomic nervous dysfunction, high infection rates (herpes infection), melanoma, eye problems; macular oedema Animal studies; teratogenicity, embryonic lethality; organ defects (permanent truncus arteriosus and ventricular septal defects).
	Mitoxantrone-	Immunosuppressant and a topoisomerase II inhibitor; inhibit the DNA synthesis via embeds into DNA bases molecular inhibit the antigen presentation via T and B cells, reduce pro-inflammatory cytokine, and enhance the anti-inflammatory responses.	Cardiotoxicity, hair loss, constipation, abnormal liver function, bone marrow suppression, mild alopecia, nausea, possible transient bluish discolouration of the sclera and urine.
	Natalizumab	Anti -Integrin α4, or CD49 monoclonal antibody; binds and blocks the interaction between integrin α4 (CD49) in lymphocytes and their ligand in endothelial cells, thus preventing the lymphocyte entrance to CNS through BBB.	Headache, urinary tract infection, abdominal pain, fatigue, joint pain, gastroenteritis.
	Alemtuzumab	Anti-CD52 monoclonal antibody: induces complete depletion of CD52 bearing cells such as T and B cells, increases the secretion of brain derived growth factor (BDNF).	Rash, headache, fever, antibody-mediated autoimmune diseases such as idiopathic thrombocytopenic purpura, Graves' disease and Goodpasture syndrome.
	Dimethyl fumarate (Tecfidera)	Immunomodulators; Activation of the Nrf2 pathway regulates the antioxidant glutathione in T cells, antioxidant	Flushing, abdominal pain, diarrhoea, skin itching, rash, nausea, erythema, vomiting Pruritus.

Diseases	Approved drugs	Mechanism of action	Adverse effects
		genes activation, promotes Th1 transformation to Th2.	

1.4 Stroke

The term stroke describes the disruption of the blood supply to the brain either by occlusion or rupture of the CNS blood vessels that results in brain damage (Caplan and Simon 2015). Stroke had been misplaced as a blood vessel disease in the international classification of disease (ICD) since 1955, but in 2018, it was reclassed as a neurological disease in the new ICD-11 (Kuriakose and Xiao 2020; Shakir 2018). Globally, approximately 13.7 million people are affected by stroke, and 5.5 million die every year, making it the second leading cause of death worldwide (Kuriakose and Xiao 2020). Annually, over 113,000 British citizens suffer a stroke, and there are around 1 million stroke survivors in the UK today (King *et al.* 2020). The number of strokes in the UK per year is expected to increase by 60% within 20 years, and the number of stroke survivors will nearly double in that period (King *et al.* 2020). Stroke has a vast socio-economic cost; the annual cost of long-term care, rehabilitation and lost employment is estimated at £25.6 billion (Hurford *et al.* 2020).

Strokes are classified into haemorrhage and ischemia, and the ischemia is significantly more common than haemorrhage, with over 80% of stroke cases being ischemic infarctions (Caplan and Simon 2015; Kuriakose and Xiao 2020). A haemorrhage occurs when blood leaks into the skull, inside the brain, or surrounding tissues, accounting for 10% to 20% of strokes each year (Caplan and Simon 2015; Chen *et al.* 2014). Ischemic strokes occur because of insufficient blood supply to the brain leading to deficiency in sugar and O_2 which are essential for maintaining brain function (Caplan and Simon 2015).

1.4.1 Haemorrhagic stroke

Two types of haemorrhagic stroke have been identified, which are intracerebral and subarachnoid haemorrhage (Caplan and Simon 2015; Shinohara 2008). The most common type of haemorrhagic stroke is intracerebral (ICH) or parenchymatous, intraparenchymal haemorrhages caused by bleeding as a result of small blood vessels or arterioles rupturing into the brain parenchyma resulting in disruption and splitting of

crucial connections in grey and white matter (Caplan and Simon 2015). Hematomas are local blood leakage and their presence cause changes in brain normal structure (Caplan and Simon 2015). The parenchymatous or intraparenchymatous haemorrhages appear in a specific area of the brain, and their damage level is determined by bleeding location, speed, amount, and pressure (Figure 1.7) (Caplan and Simon 2015). Regularly, oedema forms around the hematomas, increasing brain tissue damage (Caplan and Simon 2015). In large haemorrhages, the pressure inside the skull squeezes vital brainstem regions, which often results in death (Caplan and Simon 2015).

In the subarachnoid haemorrhage (SAH), the blood leaks into the fluid around the brain and collected under the arachnoid membrane that lies over the pia mater (Figure 1.7) (Caplan and Simon 2015). Blood leakage into the subarachnoid space might occur due to a vascular abnormality such as a ruptured artery or vein or as a consequence of an ICH or an extreme bleeding tendency (Caplan and Simon 2015; Shinohara 2008). For instance, an aneurysm is caused by an abnormality in the blood vessel wall, which causes blood to leak rapidly into the spinal fluid, circulating around the brain and spinal cord (Caplan and Simon 2015). The quick release of blood at high-pressure results in brain dysfunction (Caplan and Simon 2015). The patient suffers from severe sudden-onset headache, frequently accompanied by vomiting, and may stare, drop to the knees, or become disoriented and unable to remember (Caplan and Simon 2015).
In contrast to subarachnoid haemorrhages, the hematoma in ICH is limited and causes loss of function in the area affected by the local blood collection (Caplan and Simon 2015). For instance, when bleeding occurs in the left cerebral hemisphere, the patient will have weakness and loss of feeling in their right limbs and normal speech, whereas bleeding into the cerebellum will induce dizziness and loss of balance (Caplan and Simon 2015). Traumatic head injuries to blood arteries are the most common cause of subdural and epidural haemorrhages (Figure 1.7) (Caplan and Simon 2015). Subdural haemorrhages are caused by bleeding from veins between the arachnoid membrane and the dura mater, whereas epidural haemorrhages are caused by bleeding from the meningeal arteries (Caplan and Simon 2015). The symptoms in individuals with epidural haemorrhages frequently appear immediately after a head injury because the blood

accumulates considerably faster when it releases from arteries than from veins (Caplan and Simon 2015). Patients with subdural haemorrhages may experience headaches and brain dysfunction after weeks from the head injury because veins bleed slowly (Caplan and Simon 2015).

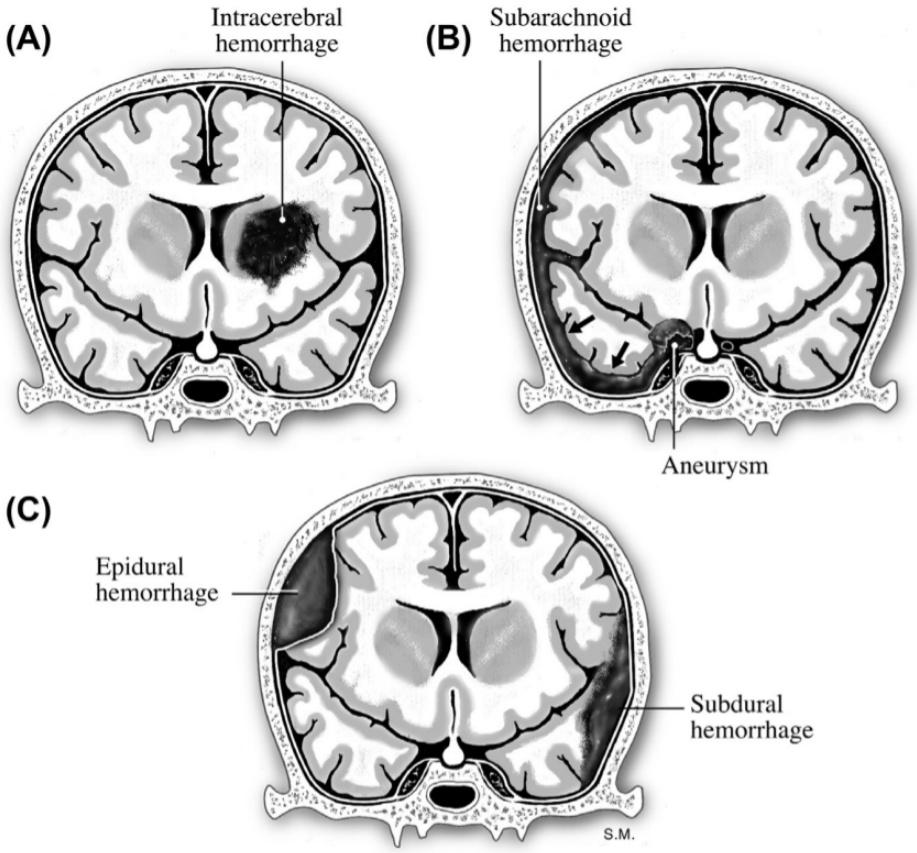

Figure 1.7: A representation of different types of haemorrhages in the brain. (A) The white dot shows the intracerebral (intraparenchymatous) bleeding in the right basal ganglia. (B) Aneurysm (white dot)-caused blood leakage surrounding the brain (black arrows). (C) The lines indicate blood collections in the subdural space (between the arachnoid and dural membranes) and an epidural haemorrhage outside the dura (Caplan and Simon 2015).

1.4.2 Ischemic stroke

The term "ischemia" refers to a decrease in blood flow to the brain, and "infarction" is defined as the death of brain tissue due to prolonged ischemia (Caplan 2005). Ischemia can be classified into three types based on the cause and mechanism of decreased blood flow to the brain: thrombosis, embolism, and systemic hypoperfusion (Caplan and Simon 2015; Velez *et al.* 2020). Thrombosis occurs due to an irregularity in a blood vessel that supplies a brain region, limiting blood flow through that vessel (Figure 1.8, A) (Caplan and Simon 2015; Velez *et al.* 2020). An embolism occurs as a result of local brain vessel blockage by a small portion of thrombi (blood clot) or bacteria or cholesterol that travels from buildup found in another place in the body (Figure 1.8, B) (Caplan and Simon 2015; Velez *et al.* 2020). During embolism, similarly to a primary vascular lesion ("thrombosis"), the blocked vessel causes localized ischemia and infarction to a specific brain region (Caplan and Simon 2015). Contrary to focal ischemia, which is localized blood flow reductions caused by thrombosis or embolism, systemic hypoperfusion occurs when the general blood flow to the head decreases and is known as global ischemia (Figure 1.8, C) (Caplan and Simon 2015; Kanyal 2015; Sacco *et al.* 2013; Velez *et al.* 2020). Many factors contribute to systemic hypoperfusion, such as abnormal rhythms, cardiac arrests, cardiac failures, hypotension, bleeding, and dehydration, leading to inadequate blood perfusion into the brain (Caplan and Simon 2015).

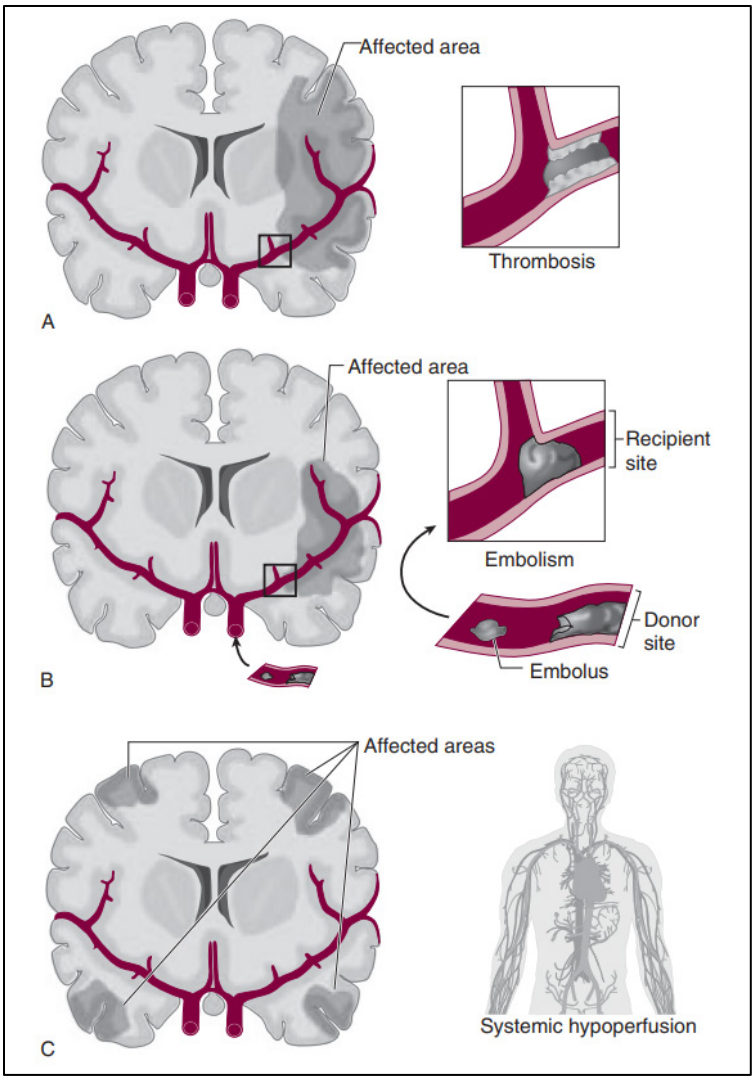

Figure 1.8: Represents the different types of brain ischemia/infarction. (A) Thrombosis: A thrombus in an atherosclerotic artery leads to a brain infarction. (B) Embolism, and (C) systemic hypoperfusion (Caplan and Liebeskind 2016).

When an ischemic stroke occurs, neurologic deficits vary according to the vascular area involved (Goldman and Schafer 2020). The patient with a brain embolism or thrombosis usually has a blocked artery, which results in focal brain deficits, such as weakness in one-half of the body, similar to those resulting from local brain hematomas (Caplan and Simon 2015).

Contrary to focal ischemia, the generalized decrease in blood flow caused by systemic hypoperfusion results in global ischemic stroke with more widespread abnormalities (Caplan and Simon 2015; Velez *et al.* 2020). Global ischemic patients commonly present with diffuse neurologic symptoms, significantly diminished consciousness (Sacco *et al.* 2013), proximal arm and leg weakness and numbness (Saint and Chopra 2018). In addition, the patients exhibited light-headedness, dizziness, confusion, impaired arousal and awareness, reduced vision, and hearing loss; also, patients appeared pale and weak (Caplan and Simon 2015; Hoesch *et al.* 2008). Moreover, severe or persistent global cerebral ischemia can be associated with neurological complications, such as comas, vegetative states, seizures, motor deficits, and brain death (Hoesch *et al.* 2008).

1.4.3 Cellular and molecular pathophysiology

Although either ischemia or haemorrhage can cause stroke, it always indicates an infarct of neuronal tissue regardless of the initial infarct volume (Naranjo *et al.* 2013; Sacco *et al.* 2013). Blood supply disruption, either by occlusion or rupture of CNS blood vessels, resulting in insufficient O_2 and glucose delivery to maintain cellular homeostasis, causes a variety of interconnected pathways, which are multifaceted and complex, initiating ischemic infraction (Doyle *et al.* 2008; Fisher and Savitz 2022; Saenger and Christenson 2010). This hypoperfusion of the brain, either gradual or sudden, initiates an ischemic cascade, causing diminished cellular energy, excitotoxicity, oxidative/nitrosative stress, ion homeostasis disruption, BBB breakdown, intracellular Ca^{2+}overload, cytotoxic and vasogenic oedema, peri-infarct depolarization, inflammation that eventually induces apoptosis, necrosis, or death of neurovascular unit-constituent cells (neurons, astrocytes, pericytes, and vascular endothelial cells) (Martínez-Coria *et al.* 2021; Saenger and Christenson 2010).

As cerebral blood flow is impaired, glucose and O_2 levels decrease, which results in a decrease in energy adenosine triphosphate (ATP) and an increase in lactate, which produces a reduction in cell pH (acidosis) (Schurr 2002). Low ATP levels lead to sodium/potassium pump (Na^+/K^+) dysfunction and Ca^{2+} homeostasis impairment (Hu and Song 2017). An excessive inflow of positively charged Na^+ occurs due to the Na^+/K^+ pumps dysfunction, leading to neuron depolarization (Hu and Song 2017). In response to neuron depolarization, the excitatory neurotransmitter L-Glu is released (Doyle *et al.* 2008). Excessive L-Glu binds to its receptors and initiates an excitotoxic event, which increases Ca^{2+} and Na^+ influx into neurons and leads to neuron death via apoptosis or necrosis (Belov Kirdajova *et al.* 2020; Doyle *et al.* 2008). Moreover, L-Glu depolarizes neurons, which indirectly activates the voltage-dependent Ca^{2+} channels (VDCCs) and increases the Ca^{2+} influx (Barron and Kim 2019; Norenberg and Rao 2007). Moreover, high-level extracellular L-Glu blocks the X_c^- system, resulting in ROS accumulation, and the cell becomes incapable of removing this excessive ROS (Lewerenz *et al.* 2013). Nitric oxide synthase (NOS) activates during the stroke and generates the potent oxidant peroxynitrite ($ONOO^-$) from $O^{2-\bullet}$ and nitric oxide ($NO^\bullet$) (Lo *et al.* 2003; Martínez-Coria *et al.* 2021; Modak and McCullough 2017). That causes nitrosative stress which leads to DNA damage, alters protein structure 'nitrosylation', and poly-ADP-ribose polymerase (PARP-1) activation resulting in caspase-independent apoptosis (Lo *et al.* 2003; Martínez-Coria *et al.* 2021; Modak and McCullough 2017). In addition, the ischemic brain region accumulation of lactate during anaerobic metabolism that induces acidosis, triggers Ca^{2+}-permeable acid-sensing ion channels and further contributes to Ca^{2+} dysregulation (Xiong *et al.* 2006). The catabolic enzymes, such as proteases, endonucleases, and lipases, are stimulated by a high-level Ca^{2+} influx, which causes the release of cytokines and other inflammatory mediators, eventually resulting in neuron death (Jaffer *et al.* 2011). L-Glu promotes Na^+, Ca^{2+} influx resulting in water influx, swelling and oedema of neuronal cell bodies (cytotoxic oedema) and shrinking of extracellular space that triggers neuron necrosis (Martínez-Coria *et al.* 2021; Song and Yu 2014; Xing *et al.* 2012). ROS are key inducers of inflammatory cytokines such as interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), interferon gamma (IFN-γ) and neurovascular protease (Glial cell-derived matrix metalloproteinases (MMPs)) production

by microglia, leukocytes, and neurons (Biswas 2016; Jaffer *et al.* 2011; Takata *et al.* 2021). As inflammatory mediators like ROS, cytokines, and nitric acid are rapidly produced in brain tissues and they play role in neuron injury (Doyle *et al.* 2008; Kanyal 2015). The BBB leakage and disruption occurs when these neuroinflammatory mechanisms are activated, altering cytokine levels, adhesion molecule expression, and tight-junction components (Yang *et al.* 2019a). As a result of BBB breakdown, cerebral oedema (vasogenic oedema) develops from plasma protein extravasation and increased brain pressure (Belov Kirdajova *et al.* 2020; Martínez-Coria *et al.* 2021; Takata *et al.* 2021).

1.4.4 The causes and risk factors of stroke

In both ischemic and hemorrhagic stroke, there are nonmodifiable risk factors such as age, sex, genetics, and race/ethnicity, as well as modifiable risk factors such as hypertension, hyperlipidemia, diabetes, atrial fibrillation, smoking, alcohol and drug abuse, poor diet, and physical inactivity (Boehme *et al.* 2017; Kuriakose and Xiao 2020). Stroke incidence increases with age, with the risk doubling after 55 years old; however, the global incidence of strokes among people aged 20–54 years jumped from 12.9% to 18.6% between 1990 and 2016 (Boehme *et al.* 2017; Kuriakose and Xiao 2020). It found that the woman's incidence is higher at a younger age, whereas a man's incidence increases a little with advancing age (Boehme *et al.* 2017; Kuriakose and Xiao 2020). There is a link between the higher stroke risk among younger women due to pregnancy, postpartum state, and hormonal contraceptive use (Boehme *et al.* 2017; Kuriakose and Xiao 2020).

The most prominent risk factor for stroke is parental or family history; research found stroke risk increases by 30% when there is a family history (Boehme *et al.* 2017). In addition, there are many other genetic alterations which result in specific disorders that increase the risk of stroke development, such as familial syndrome, cerebral autosomal dominant arteriopathy, and sickle cell anaemia (Boehme *et al.* 2017; Kuriakose and Xiao 2020). Racial disparities have long been documented in stroke studies; for instance, studies show that Black people face a double risk of stroke and have a higher mortality rate than their counterpart Whites (Boehme *et al.* 2017). The modifiable risk factors such

as hypertension, hyperlipidemia, diabetes, atrial fibrillation, smoking, alcohol and drug abuse, poor diet, and physical inactivity all contribute to increasing the risk of stroke development (Boehme *et al.* 2017; Kuriakose and Xiao 2020). Moreover, evidence demonstrates that dietary intakes of non-essential amino acid L-Glu, such as found in the food additive monosodium L-glutamate (MSG), is associated with stroke incidence (Bayram *et al.* 2022; Ma *et al.* 2018; Nagata *et al.* 2015; Samuels 2020; Zheng *et al.* 2016).

1.4.5 Stroke prevention and therapy approaches

Modifying risk factors is a significant way to prevent strokes, whereas managing stroke results from addressing its pathophysiology (Davies and Delcourt 2021; Diener and Hankey 2020). Generally, it is recommended that people modify their lifestyle by consuming a healthy diet, losing weight, stopping smoking, and participating in regular physical activity to reduce stroke risk (Diener and Hankey 2020). Moreover, treating health issues such as hypertension, hyperglycemia, hyperlipidaemia, and atrial fibrillation are more effective ways as the primary prevention of stroke (Diener and Hankey 2020). ICH treatment is designed to avoid further hematoma expansion, reduce high blood pressure (BP) and intracranial hypertension, and restore coagulation (Boccardi *et al.* 2017). Providing hemostatic therapy within 3 to 4 hours of onset may reduce hematoma growth after ICH and possibly prevent further bleeding (Mayer 2003). Several surgical techniques have been considered for supratentorial ICH; however, the international surgical trial in intracerebral haemorrhage (STICH) did not find that early surgery was beneficial over initial conservative treatment for the patients (Sahni and Weinberger 2007).

The SAH treatments are similar to ICH and are intended to prevent further hematoma growth, reduce high BP and intracranial hypertension, and restore coagulation. Vasospasm is one of the most recognized difficulties of a SAH, which increases the risk of ischemic injury and doubles mortality risk (Patel *et al.* 2021). Hence, Ca^{2+} channel blockers are commonly prescribed, but long-term management is necessary due to the long duration of vasospasm risk, which can last up to 21 days (Patel *et al.* 2021). There are two surgical approaches to a SAH treatment to remove the blood and/or occlusion of

the bleeding site, including endovascular coiling and open surgical clipping (Patel *et al.* 2021).

The goal in the treatment of ischemic stroke aimed to limit the insult by achieving rapid reperfusion and interrupting the pathophysiological cascade leading to the damage caused by ischemic neuronal damage (Jaffer *et al.* 2011). However, the currently available treatment strategies are limited and only achieve the first goal, stopping ischemia and helping reperfusion to brain tissue (Hurd *et al.* 2021).

In the hour following ischemia, two layers of cerebrovascular tissues develop the inside core, which contains neuronal necrosis, and the outer penumbra, which displays a reduced amount of ischemia and the available therapeutic intervention can restore the cells within the ischemic penumbra (Jaffer *et al.* 2011). To date, the only FDA-approved treatment for symptomatic cerebral ischemia is intravenous administration of tissue plasminogen activator (t-PA) such as alteplase within 3-4 hours after symptom onset (Hurd *et al.* 2021; Jaffer *et al.* 2011). t-PA has thrombolytic action and helps reestablish cerebral blood flow (Hurd *et al.* 2021; Jaffer *et al.* 2011). According to several studies, t-PA exhibits neuroprotective action involving the prevention of zinc-induced cell death and oxygen-glucose deprivation as well as the activation of mammalian target of rapamycin and the Janus kinase signal transducer and activator of transcription signalling pathways (Hurd *et al.* 2021). However, due to the prolonged (over 4 hours) administration of t-PA following ischemia, some adverse effects have been observed, such as the breakdown of the BBB accompanied by hemorrhagic complications and neurotoxicity due to diffusion of t-PA into the brain parenchyma (Hurd *et al.* 2021; Jaffer *et al.* 2011). Another widely available treatment is mechanical thrombectomy (MT), which involves physically removing the clot using stent-retriever devices within 6 - 8 hours of the onset of an ischemic stroke (Hurd *et al.* 2021). Even though MT provides a more extended time window than thrombolysis after the onset of stroke, it has many limitations, including being unable to target more distal occlusions and developing significant side effects (Hurd *et al.* 2021).

All stroke treatments target the symptoms only, such as bleeding, clot formation or ischemia, high BP, and intracranial hypertension. Moreover, none of these treatments interferes with pathological events such as excitotoxicity, oxidative/nitrosative stress, ion

imbalance, and mitochondrial dysfunction in ischemic brain tissue, resulting in massive brain damage. Therefore, to solve these problems, introducing a new treatment approach such as antioxidant treatment or L-Glu and the NMDAR antagonist could provide neuronal protective or/and regeneration and could contribute to the treatment strategies for stroke (Jaffer *et al.* 2011; Kuriakose and Xiao 2020).

1.5 Monosodium L-glutamate (MSG)

MSG is a sodium salt of L-Glu and it is a white crystalline powder soluble in water and ether but not in alcohol, acetone, benzene, methanol, or acetic acid (Farhat *et al.* 2021; Henry-Unaeze 2017). MSG can also be called Ajinomoto or Chinese salt or sodium 2-aminopentanedioate (Ahanger *et al.* 2021). Naturally, MSG is found in a variety of protein foods, which is relatively little in animal proteins, while high in vegetable proteins (Campbell 2014; Henry-Unaeze 2017).

L-Glu was first isolated chemically in 1866 from wheat proteins by Ritthausen (Henry-Unaeze 2017). In 1908 a Japanese scientist, Professor Kikunae Ikeda, discovered salts of glutamic acid and identified its unique umami taste (Jinap and Hajeb 2010). Since then, the sodium salt of L-Glu has been added to food to enhance its flavour (Onaolapo *et al.* 2016). Sugar beet molasses and carbohydrate sources (such as corn and sugar beet) have been used to produce MSG since the 1950s (Farhat *et al.* 2021; Henry-Unaeze 2017). A genetically modified bacteria secreting glutamic acid through their cell walls were used later in 1957 to produce MSG in the US (Kazmi *et al.* 2017). MSG can be supplemented as a “pure’’ or “hidden ingredient” of yeast extracts or hydrolyzed proteins, both of which contain high L-Glu content (Populin *et al.* 2007). Figure 1.9 represents the chemical structures of glutamate, glutamic acid, and MSG.

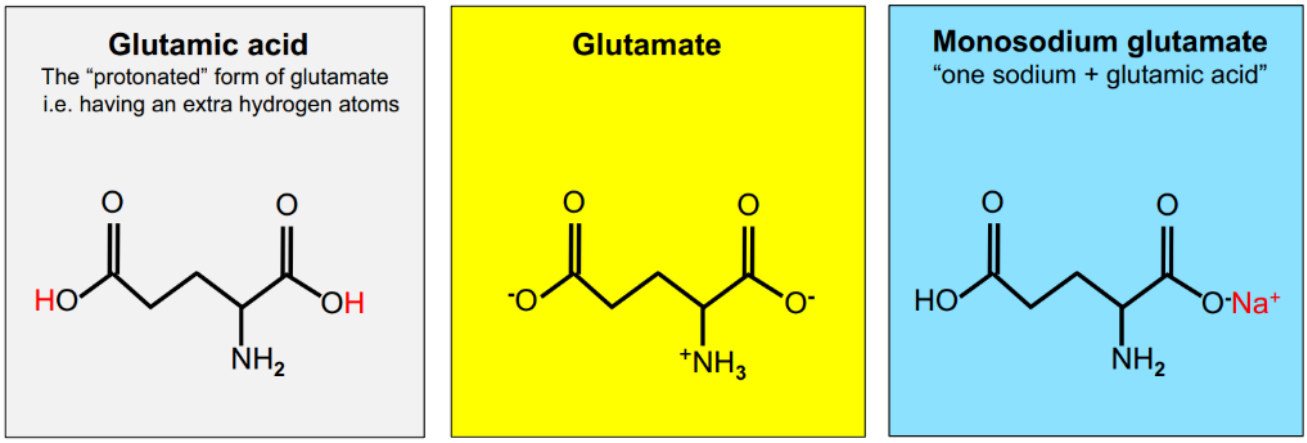

Figure 1.9: The chemical structures of different forms of glutamate. Glutamic acid, glutamate, and monosodium glutamate (MSG) (Levite 2017).

Generally, the food industry adds L-Glu to food products as MSG (Cynober 2018), encoded as E 621. Dissolving MSG salts in aqueous solution results in dissociation of the compound and produces free L-Glu in the anionic form of glutamic acid (Cynober 2018; Sano 2009). There are several ways that humans can become exposed to MSG. The main route is orally, but there are other routes, such as subcutaneous and inhalant (Chakraborty 2019). Free L-Glu can be introduced into the human body via natural food, food additives, or seasoning. Currently, MSG can be found in frozen entrees, crackers, canned tuna, soups, processed meats, cosmetics, and dietary supplements (Kazmi *et al.* 2017). Toddlers are exposed to MSG in milk formula, infant food, cooked/mashed and canned fruits and vegetables (Kazmi *et al.* 2017; Mortensen *et al.* 2017; Nepalia *et al.* 2017).

Furthermore, L-Glu can enter the body through vaccines (Kazmi *et al.* 2017). The Centers for Disease Control and Prevention (CDC) indicates on their website that MSG is added to the ingredients of some vaccines to act as stabilizers and preservers under unsuitable conditions such as exposure to heat, light, acidity, and humidity (CDC 2018). Moreover, MSG is sprayed on crops and can easily be inhaled (Chakraborty 2019). Some countries have approved the spraying of Autigro (30% MSG) on fresh fruit and vegetable crops as a fertilizer (Chakraborty 2019; Kazmi *et al.* 2017).

An important concept in neuroscience is that exogenous L-Glu, consumed in the diet, can accumulate in the brain, posing a risk to neuronal survival. Considerable evidence demonstrates that consumption of MSG-rich meals by human and animals elevates L-Glu levels in blood and muscles (Bertrand *et al.* 1995; Loï and Cynober 2022; Moro *et al.* 2000). Similar research exploring the effect of MSG consumption on human adults found that MSG 150 mg/kg consumption resulted in a quick, substantial rise in plasma L-Glu (Graham *et al.* 2000). This is also supported by a recent study that plasma L-Glu concentration significantly increases after oral ingestion of high doses of MSG by healthy participants (Shimada *et al.* 2015). Additionally, plasma levels can rise significantly if very large MSG concentrations of more than 5 g are ingested (Campbell 2014).

Moreover, current research also demonstrates that highly daily intake of MSG raises blood L-Glu (Hussein *et al.* 2017). An *in vivo* study detected that oral administration of

low-dose MSG significantly increases plasma L-Glu levels (Onaolapo *et al.* 2016). To date, the available literature suggests that plasma/blood and CSF glutamate concentrations are strongly correlated in animals and humans, in healthy individuals and patients with NDDs (Chayat and Yedidya 2012; Dimogerontas *et al.* 2016; Hashimoto 2018; Leibowitz *et al.* 2012). This means that elevation of plasma L-Glu levels could raise the L-Glu concentrations in the brain ECF. This is reinforced by one interesting finding by Viña *et al.* (1997), that L-Glu enters the brain slowly through the BBB, and the BBB's permeability to L-Glu was greater in infant rats than adults.

Moreover, Viña et al. (1997) illustrate that the L-Glu freely enters the brain circumventricular organs (CVOs) without inhibition. The CVOs act as windows due to their containing fenestrated capillaries through which molecules can move freely from blood circulation into the CNS or vice versa (Fry and Ferguson 2009). Moreover, CVOs have extensive and efficient connections with all important autonomic control centers in the brain, the medulla and hypothalamus (Fry and Ferguson 2009). A recent study supports this hypothesis of L-Glu elevation in the brain following MSG treatment, reporting that either orally or intraperitoneally, MSG administration raised the amount of L-Glu neurotransmitters in both treated neonate and adult rats (Akataobi *et al.* 2021). Therefore, it seems to be reasonable that dietary MSG could pass into the brain tissue, raise the L-Glu level and potentially induce neurotoxicity, a risk that is increased in infants, which have incompletely developed BBB.

In the human CNS, L-Glu works naturally as an excitatory neurotransmitter, signalling and communicating between neurons (refer to section 1.1) (Chayat and Yedidya 2012; Leibowitz *et al.* 2012). The concentration of L-Glu within the excitatory synaptic cleft normally rises to the relatively high level of ≈1 mM after the arrival of an action potential at the presynaptic nerve terminal, but it only remains at this level for a few milliseconds and returns to normal levels, due to association with high-affinity transporters expressed on neurons and glia (Iovino *et al.* 2020; Moussawi *et al.* 2011). Thus, the primary concern of L-Glu is that it can act as a toxin when its concentration increases and remains high for an abnormally long duration, as occurs in certain pathological conditions (Singh 2018). In the case of abnormal L-Glu accumulation, the L-Glu can initiate excessive or

prolonged activation of neuronal L-Glu receptors, which trigger intracellular signalling cascades initiating apoptosis or necrosis death pathways of excited neurons (Ankarcrona *et al.* 1995; Farhat *et al.* 2021; Olney *et al.* 1997; Purves *et al.* 2004). This phenomenon is named glutamatergic excitotoxicity, and it was first described by Lucas and Newhouse (1957), who serendipitously noticed that subcutaneous administration of MSG to infant mice damaged the neurons in the retina. Considering this evidence, Olney and his coworkers argued that L-Glu damages neurons by a process analogous to the transmission, which occurs in exciting glutamatergic synapses, and they invented the term excitotoxic to refer to this pathological effect (Olney and Sharpe 1969; Olney 1969, 1971; Olney *et al.* 1972; Olney 1994). Olney later extended this discovery by illustrating that oral or intraperitoneal administration of L-Glu can cause acute neuronal degeneration throughout the brain (Olney and Sharpe 1969; Olney 1969, 1971; Olney *et al.* 1972; Olney 1994). Numerous studies have strongly supported this hypothesis of extensive neuronal devastation mediated by L-Glu neurotoxicity in more recent decades (Choi 1988; Greenamyre 1986; Monno *et al.* 1995; Populin *et al.* 2007; Roy *et al.* 1995; Sattler and Tymianski 2001).

The early theory that pathologic elevation of L-Glu levels can trigger excitotoxicity and death of neurons opened the door to a whole research field involving L-Glu in NDDs and stroke aetiology. L-Glu neurotoxicity is correlated with several acute and chronic NDDs such as stroke or trauma (Rothman and Olney 1986; Suzuki *et al.* 2022; Tiwari *et al.* 2016), AD (Hynd *et al.* 2004; Zumkehr *et al.* 2015), PD (Iovino *et al.* 2020; Wang *et al.* 2020; Zhang *et al.* 2016), MS (Bolton and Paul 2006; Rajda *et al.* 2017; Stojanovic *et al.* 2014), ALS (Fomin *et al.* 2018; Plaitakis and Constantakakis 1993; Rothstein *et al.* 1992), and HD (Estrada Sánchez *et al.* 2008; Zeron *et al.* 2001). Moreover, many animal models and human clinical studies demonstrate that several acute and chronic NDDs are associated with pathologically raised L-Glu levels (Chayat and Yedidya 2012; Madeira *et al.* 2018; Suzuki *et al.* 2022). Additionally, MSG has been reported by various authors as a co-factor or an aggravating factor for NDDs, e.g. AD, ALS and HD or stroke (Banerjee *et al.* 2021; Loï and Cynober 2022; Madhavadas *et al.* 2014; Meldrum 2000). Hence, in

many acute and chronic CNS diseases, excitotoxicity contributes to neuronal degeneration (Farhat *et al.* 2021; Mehta *et al.* 2013).

Furthermore, investigating L-Glu toxicity is a continuing concern for many researchers due to its implication for NDDs (refer to Chapter 3). Moreover, many researchers are still interested in researching L-Glu toxicity because of its role in stroke incidence. It is hypothesized that MSG could increase Na^+ levels in the blood, causing hypertension that might lead to stroke. Even though the Na^+ content in MSG is relatively low, using MSG to season foods increases the daily Na^+ intake (Bartoshuk *et al.* 1974; Fernstrom 2007; Hermann 2017). Several studies found that MSG administration via diet or injection in animals significantly elevated Na^+ levels in the blood (Ilegbedion *et al.* 2013; Olarotimi 2020, 2021; Sadek *et al.* 2016). In addition, recent research supports the concept that MSG intake is linked to arterial hypertension (Cunha *et al.* 2010; Karlen-Amarante *et al.* 2012; Konrad *et al.* 2012; Shi *et al.* 2011). In the UK and internationally, there is a well-established link between Na^+ consumption and the risk of high BP (hypertension), which is a risk factor in the development of cardiovascular diseases such as heart disease and stroke (Dong 2018; Grillo *et al.* 2019; Robert *et al.* 2020). Moreover, a case-cohort study involving a randomized, controlled dietary intervention study found that dietary L-Glu intake was associated with stroke risk in human (Zheng *et al.* 2016). Similarly, research of combined cohorts found that greater L-Glu consumption was related to an increased risk of total and cardiovascular death, including stroke in humans (Ma *et al.* 2018). Thus, there is a clear line between MSG consumption and stroke progress risk in human.

The complex cascade of excitotoxicity steps leading to neuronal death via L-Glu accumulation in various NDDs, and the result of excess L-Glu release after stroke onset, are described in Chapter 3 (Belov Kirdajova *et al.* 2020; Mehta *et al.* 2013).

In summary, MSG use is on the rise worldwide, and its safety is debatable. It should be used with caution and in accordance with the authorised doses given by various organisations to avoid its harmful effects. As a result of understanding the pathomechanism of excess L-Glu accumulation, there is a need to find an agent that has the potential to prevent and/or reverse mitochondrial dysfunction, oxidative stress, Ca^{2+} imbalance, and synaptic impairment caused by MSG exposure, as well as protect against

the consequences of cell imbalance. This might open the way for novel drug development exploration methodologies and therapeutic targets for NDDs and stroke.

1.6 Acai berry and neuroprotection

Acai berry is a fruit of tropical palm trees belonging to the *Euterpe* genus, natively found in the Amazon region of South America and a few Caribbean islands (Figure 1.10) (Schauss 2010). *Euterpe edulis*, *Euterpe precatoria*, and *Euterpe oleracea* are three species generating edible fruit which were discovered in the Amazon region (Schauss 2010). The *Euterpe oleracea* has a high free radical scavenging capacity *in vitro* and discovered by Alexander Schauss in 1995 (Schauss *et al.* 2006a; Schauss 2015). Since then, this novel berry has received much attention among food scientists, being called a 'superfood' (Schauss 2010). *Euterpe oleracea* berry is a small round palm fruit, 1 to 2 cm in diameter, containing a single, dark coloured seed (Schauss 2015). A thin layer of edible purple pulp covers the seed (Schauss 2015). In the Para State of Brazil, acai palms are extensively distributed and cultivated (Gallori *et al.* 2004), covering over 12 million hectares of flooded forest land near the Amazon River, and over 120,000 tons of the fruit is processed annually for its pulp (Schauss 2015).

As a result of the highly rich bioactivate nutritional and phytochemical composition of acai berry, its pulp has been extensively examined (Kang *et al.* 2011; Schauss *et al.* 2006a). Acai berry pulp composition analysis found that it contains various biologically active phytochemicals and ample amounts of mono- and polyunsaturated fatty acids, which are not found in most fruits and other berries (Poulose *et al.* 2012). Additionally, acai berry is a protein-rich fruit with high energy and nutritional value (Jesus *et al.* 2018). The phytochemicals found in acai pulp are anthocyanins, proanthocyanidins, flavonoids and carotenoids (Figure 1.11) (Kang *et al.* 2012; Schauss *et al.* 2006a).

There are a variety of extraction methods and solvents that can be used to isolate active agents such as polyphenols, with the polarity of the solute of interest determining the degree of solvation (Altemimi *et al.* 2017). In particular, an independent investigation found that water as a solvent provided the best yields of polyphenols and flavonoids when compared to methanol and ethanol alone for acai berry phytochemical extractions

(Chung 2012). A further study found that a hydroalcoholic extraction (50% ethanol) appeared to yield even higher levels of phenolic content than using of 100% ethanol or water only (López de Dicastillo *et al.* 2019). In another study, methanol and ethanol fractions of acai pulp were found to be particularly rich in anthocyanins, whereas acetone fractions were considered rich in other phenolic compounds (Poulose *et al.* 2012). Therefore, this thesis investigated different extraction methods of acai berry to compare phytochemical yields and potential neuroprotective effects.

A considerable body of evidence has been gathered demonstrating that acai berry extract and its bioactive content exhibit many pharmacological activities such as anti-inflammatory, antioxidant, anticarcinogenic, and neuroprotective properties (Ajit *et al.* 2016; Cadoná *et al.* 2021; Del Pozo-Insfran *et al.* 2006; Kang *et al.* 2010; Pacheco-Palencia *et al.* 2008; Schauss *et al.* 2010; Spada *et al.* 2009; Torma *et al.* 2017; Wong *et al.* 2013; Xie *et al.* 2012). Experiments demonstrated that acai berry extracts confer its neuroprotection by showing antioxidant and anti-inflammatory activities, inhibiting toxic protein aggregation, and restoring Ca^{2+} homeostasis and mitochondrial function (De Almeida Magalhães *et al.* 2020). Therefore, investigations have demonstrated that acai berries can target multiple mechanisms and exert synergistic effects on signalling pathways, thereby reducing multiple components of NDDs and stroke pathology. Additionally, acai berry exhibited antidepressive and anticonvulsant activities, which may benefit people with neurological disorders (De Almeida Magalhães *et al.* 2020). The neuromodulatory effects of acai berry diet supplementation on the vital brain areas that influence memory, cognition, and overall brain function is discussed below.

There is growing interest in acai fruits due to their broad usage in the food and cosmetics industries and their pharmaceutical potential. Acai berry parts such as pulp, leaves, roots, and seed oil were actually studied for pharmacological utilization, indicating specific biological activities based on their chemical composition. The potential health benefits of acai fruits are illustrated by many cell-based, animal, and clinical studies (De Almeida Magalhães *et al.* 2020), although there are limited experiments investigating acai's impact on brain health or cognitive function and these are summarized below (Table 1.4 and 1.5).

Figure 1.10: Acai (*Euterpe oleracea*) palms with numerous stems in each clump and a single cluster of purple-colored fruits (De Oliveira and Schwartz 2018).

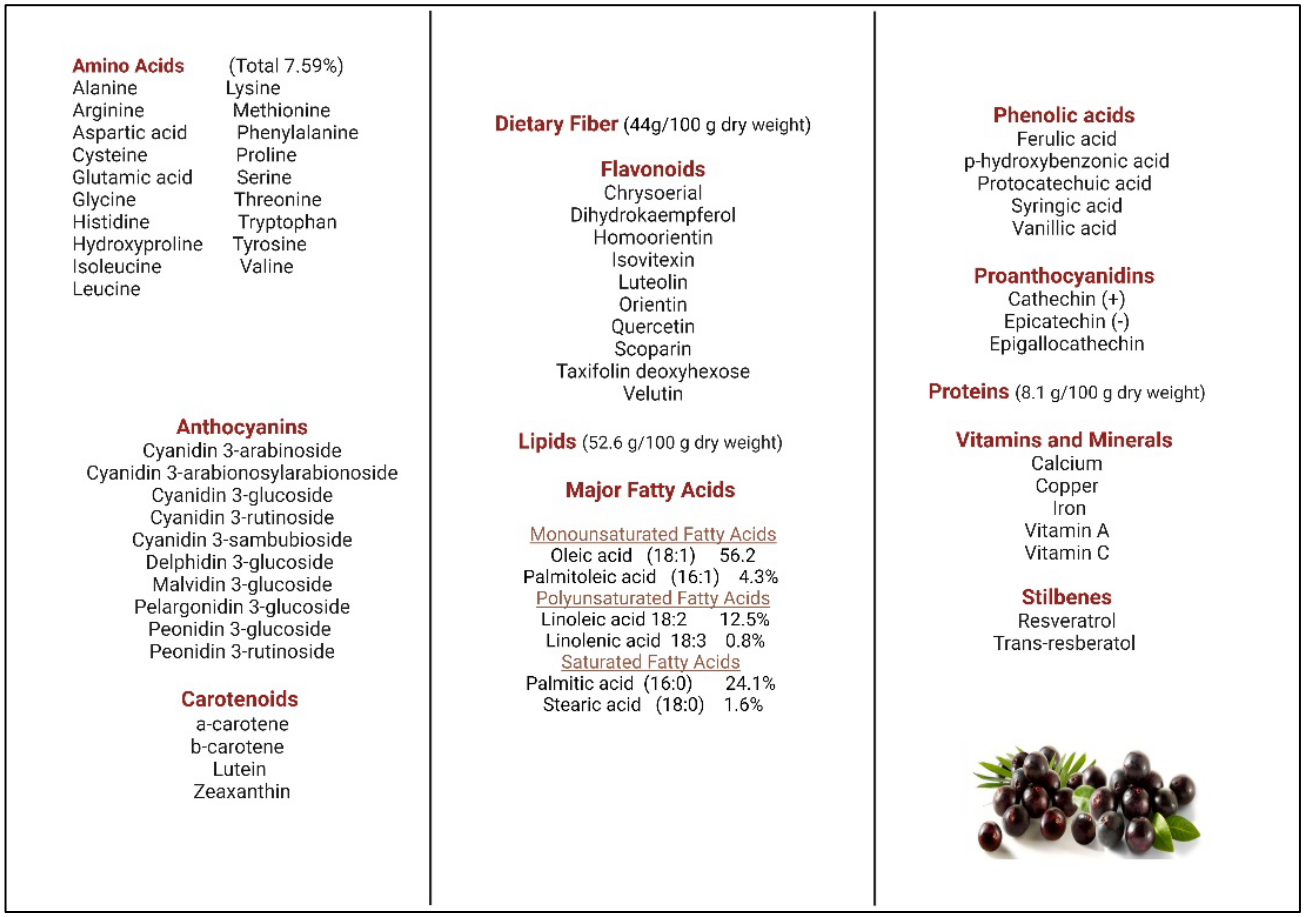

Figure 1.11: Phytochemicals and nutrient composition identified to date of the Amazonian acai berry whole fruit pulp were fractionated with solvents of various polarities such as ethanol, methanol, chloroform, ethyl acetate, petroleum ether, and acetone (Kang *et al.* 2010; Mulabagal and Calderón 2012; Pacheco-Palencia *et al.* 2008; Poulose and Shukitt-Hale 2013; Poulose *et al.* 2012; Schauss *et al.* 2006b). The figure was created using BioRender (https://www.biorender.com/).

Table 1.4: Recent findings of the neuroprotective roles of acai berry in *in vitro* models.

Major observed effects after acai exposure	Level of Significance	Dose	Model	Reference
↓ NF-κB after velutin exposure in RAW-Blue cells induced by LPS and OxLDL.	$p < 0.05$	Velutin acai extract; 5 µM, 2.5 µM, 1.25 µM, 0.625 µM used in LPS induced cells. Velutin acai extract; 10 µM, 5 µM and 2.5 µM on OxLDL induced cells.	RAW-Blue mouse macrophage cell lines induced by LPS or OxLDL.	Kang *et al.* (2011).

↓ NO when compared with LPS treated microglia. ↓ iNOS when compared with LPS treated microglia. ↓ TNFα expression when compared with LPS alone.	$p < 0.05$ in acai ethanol, ethyl acetate, and acetone fraction. $p < 0.001$ in methanol extract $p < 0.001$ in all acai fractions $p = 0.009$ in ethyl acetate fraction, $p = 0.016$ for acetone fraction, $p < 0.001$ for methanol and ethanol fractions	The acai extract concentrations ranged from 50 to 1000 µg/mL for the methanol, ethyl acetate, and acetone fractions and from 10 to 250 µg/mL for the ethanol fraction.	BV-2 murine microglial cells toxicity induced by LPS	Poulose *et al.* (2012).
↓ p38-MAPK phosphorylation in LPS induced microglia.	$p < 0.001$			
↓ NF-κB phosphorylation except for the ethyl acetate fraction versus LPS alone.	$p < 0.001$			
↓ COX-2 expression in LPS induced microglia versus LPS alone.	$p < 0.001$			
↓ ROS by all acai genotypes in H_2O_2-treated cells.	$p < 0.05$	Hydroethanolic extracts from six acai (*Euterpe oleracea*) genotypes (L09P09, L22P13, BRS-PAMISTA, L11P09, L06P13 and L04P16)	Human neuroblastoma cell line SH-SY5Y.	Torma *et al.* (2017).

		and an available commercial pulp at concentrations 0.5, 5.0 and 50 μg/mL.		
↓ TNF-α and IL-6 production by velutin in LPS-treated RAW 264.7 and C57BL/6 macrophages. ↓ NF-κB activation by velutin in LPS-treated RAW 264.7 macrophages. Inhibiting the degradation of NF-κB by velutin in LPS-treated RAW 264.7 macrophages. Inhibiting p38-MAPK and JNK phosphorylation by velutin addition in LPS-treated RAW 264.7 macrophages.	$p < 0.05$ $p < 0.05$ ND ND	Velutin isolated from the pulp of acai at 2.5 to 20 μM (flavones as controls: luteolin, apigenin and chrysoeriol).	RAW 264.7 peripheral macrophages and mouse C57BL/6 peritoneal macrophages with inflammation induced by LPS.	Xie *et al.* (2012).
↑ NDUFS7 and NDUFS8 expression. ↓ ROS levels and LPO in both experimental designs.	Before $p < 0.001$ or after $p < 0.01$ rotenone exposure $p < 0.001$	Acai freeze-dried hydroalcoholic extract 5 μg/mL was added before and after rotenone.	Human neuroblastoma cell line (SH-SY5Y) toxicity induced via rotenone exposure.	Machado *et al.* (2016).
↓ Cellular proliferation vs LPS control. ↓ ROS generation vs LPS control. ↓ Pro-inflammatory cytokines vs LPS control.	$p < 0.05$ $p < 0.05$ $p < 0.05$	1 μg/mL of Freeze-dried hydroalcoholic acai extract.	BV-2 microglia cell line activated by LPS	De Souza *et al.* (2020).

↓ Caspase when compared to LPS control.	$p < 0.05$			
↓Cellular proliferation in LPS-activated microglia to the negative control level (cells with normal media).	ND	0.001 - 1000 µg/mL of freeze-dried hydroalcoholic acai extract	Microglia EOC 13.31 cell line inflammatory induced by LPS or/and nigericin	Cadoná *et al.* (2021).
↓NO and ROS levels in LPS-activated microglia to the negative control level constituted of cells and normal media.	ND			
↓ NO levels after acai treatment of nigericin treated microglia like negative control.	ND			
↓NLRP3-infammasome induced in microglia via LPS as well as microglia induced by LPS and nigericin to negative control level.	ND			
↓ Caspase-1 and IL-1β expression levels in LPS and nigericin activated microglia to negative control.	ND			
↑ ATP levels similar to negative control, which were dropped by LPS and nigericin activation.	ND			
↓ Macrophage activation when compared with PHA-treated cells.	$p < 0.001$	Freeze-dried hydroalcoholic acai extract 0.001 - 1000 µg/mL.	Macrophage cell line RAW 264.7 inflammation induced by phytohemagglutinin (PHA)	Machado *et al.* (2019)
↓ ROS induced by PHA.	$p < 0.01$ at 100 µg/mL $p < 0.001$ at 0.001 - 10 µg/mL and 1000 µg/mL			

↓NO generated by PHA.	$p < 0.05$ at 1000 µg/mL, $p < 0.01$ at 0.001, 0.01, 0.1 and 500 µg/mL, and $p < 0.001$ at 0.005, 0.05, 1, 10, 100 µg/mL			
↓ Interferon-gamma (IFN-γ) when compared with PHA-treated cells.	$p < 0.01$			
↓ IL-1 β, IL-6, and ↓TNF-α when compared with PHA-treated cells.	$p < 0.001$			
↑ IL-10 which was reduced via PHA.	$p < 0.001$			
↓ NLRP3 inflammasome protein levels that were induced by PHA.				
↓ Caspase 1,3,8 which were increased via PHA.	$p < 0.001$			
↓ Caspase 8 which were increased via PHA.	$p < 0.05$			
↓ NO production and iNOS expression that caused by LPS-exposure.	$p < 0.01$	2% of lyophilized acai pulps.	BV-2 murine microglial cells were pretreated with 10% blood serum from rats fed acai then inflammation induced	Carey *et al.* (2017).
↓ TNF-α in microglia activated by LPS exposure.	$p < 0.05$			

			by LPS.	
↓ NF-κB activity in LPS-induced DI TNC1 astrocytes. ↑ ARE activity to 2-3 fold by acai alone. ↑ ARE activity in the presence of LPS to 10-fold by acai exposure. ↑ Antioxidant pathway Nrf2 expression, reaching 3-4 fold in untreated DI TNC1 astrocytes. ↑ Antioxidant pathway HO-1 expression, reaching 3-4 fold in untreated DI TNC1 astrocytes.	$p < 0.001$ $p < 0.001$ $p < 0.001$ $p < 0.05$ at acai of 12.5 μg/mL, and $p < 0.001$ at 25 and 50 μg/mL $p < 0.05$ at 12.5 μg/mL acai, and $p < 0.01$ 25 and 50 μg/mL acai.	Hydroalcoholic acai extract 6.25, 12.5, 25, and 50 μg/mL.	Rat astrocyte (DI TNC1) cell line stably transfected with the NF-κB or Nrf2-Antioxidant Response Element (ARE) constructs.	Ajit *et al.* (2016).
↓ Toxicity of Ca^{2+} influx caused by dopamine application. ↓ The bafilomycin A1-induced build-up of autophagic vacuoles. Reversed the reduction in the length of primary	$p < 0.05$ $p = 0.001$	1 - 5 μg/mL of aqueous extracts of freeze-dried acai pulp.	Rodent Primary hippocampal neurons (HT22).	Poulose *et al.* (2014).

basal dendrites caused by wortmannin.	$p < 0.05$			
↑ Neuronal viability following $A\beta_{1–42}$ exposure.	$p < 0.01$ at 5 µg/mL, and $p < 0.05$ at 50 µg/mL	0.5, 5 and 50 µg/mL aqueous extract of freeze-dried acai pulp and skin powder.	Rat PC12.	Wong *et al.* (2013).
Fibril inhibition and alteration on $A\beta_{1–42}$ morphology.	$p < 0.05$			
↑ [^{3}H] TBOB binding to $GABA_A$ receptors in cortical neurons.	$p < 0.05$ at 5% acai and $p <$ 0.001 at 25% acai	0 - 25% commercial clarified *Euterpe oleracea* Martius juice from Amazon Dreams (Belém, Pará, Brazil) in Hank's buffer (250 µL final volume).	Primary cultures of neocortical neurons and cortical astrocytes	Arrifano *et al.* (2018).
↑ [^{3}H] flunitrazepam binding to $GABA_A$ receptors by acai at concentration 25% in cortical neurons.	$p < 0.01$			
↓ [^{3}H] GABA uptake in cortical neurons by acai at concentration 25%.	$p < 0.05$			
↓ [^{3}H] GABA uptake in astrocytes.	$p < 0.01$ at 10% and $p < 0.001$ at 25% acai.			

Abbreviations: $A\beta_{1-42}$, amyloid beta$_{1-42}$; ATP, adonise diphosphate; COX-2, cyclooxygenase-2; GABA, gamma-aminobutyric acid; H_2O_2, Hydrogen peroxide; HO-1, heme oxygenase-1; [3H] TBOB, [3H]-t-butylbicycloorthobenzoate; IL-1β, interleukin 1 beta; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; LPO, lipid peroxidation; LPS,

lipopolysaccharide; ND, not determined; NDUFS7, NADH: Ubiquinone Oxidoreductase Core Subunit S7; NDUFS8, NADH: Ubiquinone Oxidoreductase Core Subunit S8; NF-κB, nuclear factor kappa B; NLRP3, NLR family pyrin domain containing 3; NO, nitric oxide; Nrf2, nuclear factor erythroid 2-related factor 2; OxLDL, oxidized low-density lipoprotein; p38-MAPK, p38 mitogen-activated protein kinase; ROS, reactive oxygen species; TNF-α, tumour necrosis factor-α.

Table 1.5: acai berries neuroprotective effects in *in vivo* models.

Major observed effects	Level of Significance	Dose and duration	Model	Reference
↑ Chemotaxis response in CL2355 strain.	$p < 0.001$ at 100 μg/mL acai.	Fresh acai pulp was extracted using 80% methanol 50, 100, 200 μg/mL for 48 hours.	*Caenorhabditis elegans* strain CL2355, which express $A\beta_{1-42}$ and impairs their chemosensory system.	Peixoto *et al.* (2016b).
↓ PolyQ aggregation when compared with AM141 control worms.	$p < 0.01$ at 50 μg/mL and $p < 0.001$ at 100 and 200 μg/mL		Transgenic worms (AM141) as Huntington's disease model which express polyQ	
↓ Intracellular ROS accumulation in N2 worms.	$p < 0.001$			
↓ Protein oxidation levels in N2 worms.	$p < 0.05$ at 300 μg/mL			

			Caenorhabditis elegans strain N2 (wild type)	
Improved performance in the cognitive testing in aged rats.	$p < 0.05$	2% of lyophilized acai pulps for 8 weeks.	19-month-old Fischer 344 rats.	Carey *et al.* (2017).
Protected against behavioural alterations caused by PTZ.	$p < 0.001$ and $p < 0.01$	10 µL/g /d of acai juice for 4 days.	Male Swiss mice. Seizure model induced by pentylenetetrazol (PTZ)	Souza-Monteiro *et al.* (2015).
Prevented electrocortical changes induced by PTZ.	$p < 0.001$			
↓ LPO in the cerebral cortex that induced by PTZ.	$p < 0.05$			
The total energy intake, carbohydrate, protein, total lipids, and metabolic equivalent of task were unchanged after acai consumption.	ND	200 g/d of acai pulp for 4 weeks.	Thirty-five healthy women.	Barbosa *et al.* (2016).
↓ ROS level when compared with its level before acai intake.	$p = 0.004$			
↑ Total antioxidant capacity of polymorphonuclear cells (PMN) cells by 104% compared to before acai intake.	$p < 0.001$			
↑ CAT activity after acai intake when compared with the baseline results.	$p < 0.001$			

↓ Protein carbonyl after acai intake when compared with the baseline results.	$p = 0.027$			
↑ Sulfhydryl groups after acai when compared with the same groups at baseline.	$p < 0.001$			
↓ NADPH-oxidoreductase-2 (NOX2) in aged animals fed with acai-enriched diets.	$p < 0.05$, EO and EP	Freeze-dried acai powder = 2 % of the diet for 8 weeks. *Euterpe precatoria* (EP) and *Euterpe oleracea* (EO).	19-month-old male Fischer rats (aged rats).	Poulose *et al.* (2017).
↓ NF-κB in acai-consumed aged rats.	$p \leq 0.01$ EO, and EP			
↑ Glutathione S-transferase (GST) and SOD were observed in acai fed aged rats.	$p < 0.05$, EO and EP			
↑ Nrf2 transcription factor expression in acai fed rats.	$p < 0.05$, EO and EP			
Prevented CCl_4 inhibition of creatine kinase activity in rats.	$p < 0.01$ in cerebral cortex, and hippocampus, while $p < 0.001$ in cerebellum	Acai frozen pulp via oral gavage at a dose of 7 µL/g /d for 14 days.	Male Wistar rat experimental model of hepatic encephalopathy provoked by CCl_4. The animals also presented neurological symptoms.	De Souza Machado *et al.* (2016).
↓ LPO induced by CCl_4 in rat's cerebellum and cerebral cortex.	$p < 0.05$			
↓ Heightened carbonyl levels induced by CCl_4 in rats.	$p < 0.01$ in cerebellum, and $p < 0.001$ in			

	cerebral cortex and hippocampus			
↑ CAT activity which was reduced in rat brain by CCl_4.	$p < 0.05$ in hippocampus and $p < 0.01$ in cerebellum			
↑ SOD activity which was reduced in CCl_4 - treated rats.	$p < 0.05$ in cerebral cortex, and $p < 0.01$ in cerebellum and hippocampus			
Improved neurobehavioral disturbance caused by MeHg when compared with MeHg only treated group.	$p < 0.05$	Clarified acai juice, 10 µL/g /d for 8 days.	Male Swiss mice. Toxicity induced by MeHg.	Crespo-López *et al.* (2019).
↓ LPO which was elevated by MeHg.	$p < 0.05$			
↓ The elevated level of NO^- in MeHg treated animals to similar to those of the control with when compared with MeHg only treated group.	$p < 0.05$			
Prevented the reduction of TERT mRNA expression in the brain by MeHg.	$p < 0.001$			

Acai juice reduced the effects of mercury exposure while having no effect on mercury levels in the CNS.	ND			
Prevented the increase of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) caused by CCl_4 in the serum of rats.	$p < 0.05$	7 µL/g /d of acai juice for 14 days.	Wistar rat model for hepatic encephalopathy induced by CCl_4. The animals used in these experiments presented neurological symptoms associated with hepatic encephalopathy.	De Souza Machado *et al.* (2015).
↓ TNF-α, IL-1β and IL-18 levels in the cerebral cortex, hippocampus, and cerebellum which was induced by CCl_4.	$p < 0.05$			
Prevented the anhedonia-like state induced by LPS.	$p < 0.01$	10 µL/g /d body weight of acai juice for 4 days.	Mouse model of depressive-like behavior induced by LPS.	Souza-Monteiro *et al.* (2019).
↑ Muscle activity which was completely inhibited by LPS.	$p < 0.05$			
Prevented immobility or the absence of response to stimulus caused by LPS.	$p < 0.001$			
↓ The LPO which was generated by LPS exposure.				

↓ NO^- levels that was induced in the hippocampus by LPS exposure.	$p < 0.05$ in striatum and prefrontal cortex, and $p < 0.001$ in hippocampus
↑ TERT mRNA expression, illustrating its anti-aging effect.	$p < 0.01$
↑ TERT mRNA expression in all tested brain areas which was reduced by LPS treatment.	$p < 0.01$ in the hippocampus, and $P < 0.001$ in striatum and prefrontal cortex $p < 0.001$

Abbreviations: $A\beta_{1\text{-}42}$, amyloid $beta_{1\text{-}42}$; CAT, catalase; CCl_4, carbon tetrachloride; COX-2, cyclooxygenase-2; IL-18, interleukin18; IL-1β, interleukin 1 beta; LPO, lipid peroxidation; LPS, lipopolysaccharide; MeHg, methylmercury; ND, not determined; NF-κB, nuclear factor kappa B; NO^-, nitrite; Nrf2, nuclear factor erythroid 2-related factor 2; polyQ, polyglutamine; ROS, reactive oxygen species; SOD, superoxide dismutase; TERT mRNA, telomerase reverse transcriptase; TNF-α, tumour necrosis factor-α.

1.6.1 Antioxidant effects of acai berry

Acai berry polyphenolic-rich extract has effective and direct scavenging activities against most ROS (Kang *et al.* 2012; Peixoto *et al.* 2016b). Recently, many findings have shown that acai berry extract is capable of regulating the antioxidant/pro-oxidant status (Barbosa *et al.* 2016; de Liz *et al.* 2020; Peixoto *et al.* 2016a; Yamaguchi *et al.* 2015). The chemical contents of acai berry, such as carotenoids, ascorbic acid, and phenolic compounds, are responsible for their effective antioxidant actions (Machado *et al.* 2016; Mathew *et al.* 2015).

An acai berry rich diet could modulate oxidative stress and enhance endogenous antioxidant enzyme defence through decreased pro-oxidant NADPH-oxidoreductase-2 (NOX2) and increased expression of nuclear factor erythroid 2-related factor 2 (Nrf2) in the hippocampus and frontal cortex of elder rat brains (Poulose *et al.* 2017). A study on a hepatic encephalopathy animal model with neurological symptoms illustrated that 14 days of acai berry treatment prevented creatine kinase (CK) activity inhibition, antioxidant enzyme catalase (CAT) activity reduction, oxidative damage involving increasing levels of LPO and protein carbonyl groups in the cerebral cortex, hippocampus, and cerebellum (De Souza Machado *et al.* 2016). Moreover, the frozen pulp was able to restore the decrease in SOD activity in the hippocampus (De Souza Machado *et al.* 2016). A study investigating the antioxidant activity of different acai berry genotypes and commercial pulp found that all hydroethanolic extracts had a potent scavenging property in reducing ROS produced by H_2O_2 in the human neuroblastoma SH-SY5Y cell line, and no difference in the antioxidant activity was seen between different genotypes by 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and deoxyribose assays (Torma *et al.* 2017). Moreover, acai berry extract addition resulted in a significant reduction in ROS to the negative control levels in lipopolysaccharide (LPS)-activated microglia (Cadoná *et al.* 2021; De Souza *et al.* 2020). The behavioural analysis revealed that the consumption of commercial acai berry juice could improve neurobehavioral disturbance as a consequence of methylmercury (MeHg) as well as reduced LPO and NO^- level induced by MeHg (Crespo-López *et al.* 2019). In this study, reduction of telomerase reverse transcriptase (TERT) mRNA expression in the brain as a

consequence of mercury exposure was prevented by acai berry consumption. Thus, these studies give valuable evidence about the protection potential of acai berries against oxidative stress on brain cells, which could have a role in the treatment and/or prevention of NDDs.

1.6.2 Anti-inflammatory effects of acai

One of the most evident pharmacological activities of acai berry, which has been recorded in many works of literature, is its anti-inflammatory effect. *In vitro* evaluation of acai berry on an inflammatory macrophage model induced via phytohemagglutinin demonstrated its anti-inflammatory potential through antioxidant pathway and modulation of nod-like receptor pyrin containing 3 (NLRP3) inflammasome proteins as well as a decrease of all pro-inflammatory cytokines and increase of anti-inflammatory cytokine IL-10 levels (Machado *et al.* 2019). Two percent acai berry administration to the diets of ageing rats displayed a reduction of proinflammatory transcription factor nuclear factor κB (NF-κB) in the hippocampus (Poulose *et al.* 2017). Carey *et al.* (2017) reported that aged rats fed with acai berry showed improved performance in cognitive testing compared with control rats. Blood serum from the same rats also had attenuated LPS-induced inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), NO, and TNF-α production in BV-2 murine microglial cells. Acai berry exposed an inflammatory modulation by reducing TNF-α, interleukin 1 beta (IL-1β), and interleukin 18 (IL-18) levels in the brains of rat models of hepatic encephalopathy that presented many neurological symptoms (De Souza Machado *et al.* 2015).

Moreover, Poulose *et al.* (2012) confirmed that acai berry extracts attenuated oxidative- and inflammatory-stress-induced signals on BV-2 microglial cells subjected to LPS through reduced NO release and decreased levels of inducible iNOS, COX-2, p38-mitogen-activated protein kinase (MAPK), TNF-α, and NF-κB. Similarly, acai berry extract was capable of reducing increased proinflammatory cytokines, IL-1β, IL-6, and TNF-α in LPS-activated BV-2 microglial (De Souza *et al.* 2020). Data from Kang *et al.* (2011) indicated that five flavonoids were isolated from acai berry pulp, and only one of them, velutin, was able to inhibit the activation of inflammatory mediator factor NF-κB in RAW-Blue cells induced by LPS. Investigating the modulatory effects of velutin

isolated from acai berry on LPS-induced proinflammatory cytokines showed that it has the most potent inhibitory effects compared to other structurally similar flavones against NF-κB, p38 mitogen-activated protein kinase (p38 MAPK), and c-Jun N-terminal kinase (JNK) phosphorylation, consequently reducing the TNF-α and IL-6 production (Xie *et al.* 2012). A study exploring acai berry regulation of the oxidative/proinflammatory (NF-κB) and anti-oxidative (Nrf2) pathways in DI TNC1 astrocytes showed a reduction of LPS-induced NF-κB activity and induction of the anti-oxidation pathway through Nrf2 and heme oxygenase-1 (HO-1) expression (Ajit *et al.* 2016). Moreover, acai berry extract decreased cellular proliferation, IL-1β and restored NO in inflammatory activated microglia (Cadoná *et al.* 2021). Thus, it suggests the vital role of antioxidant-rich acai berry in the regulating and inhibition of the inflammatory response.

1.6.3 Calcium homeostasis

An *in vitro* study, acai berry pre-treatment protected rat primary hippocampal neurons from dopamine-induced Ca^{2+} dysregulation (Poulose *et al.* 2014). Despite the need for further investigations to address the acai berry role in intraneuronal Ca^{2+} regulation, the results from previously conducted experiments suggest that quercetin and myricetin (other flavonoids) regulated intraneuronal Ca^{2+} concentration (Oyama *et al.* 1994) and these bioflavonoids are richly found in acai berry pulp (Moura *et al.* 2018; Poulose *et al.* 2014). Since the majority of NDDs are associated with intracellular Ca^{2+} elevation, finding substances that can maintain and/or restore Ca^{2+} homeostasis gives promise to the prevention of these diseases.

1.6.4 Recovering of the mitochondrial function

Acai berry extract restored the impaired ATP levels in LPS and nigericin inflammatory inducted microglia (Cadoná *et al.* 2021). In the same study, acai berry extract significantly lowered the pro-apoptotic caspase 1 level to the control level (Cadoná *et al.* 2021). Increased levels of pro-apoptotic proteins, such as caspase 1, 3, and 8, were reduced in LPS exposed BV-2 microglia after acai berry extract treatment (De Souza *et al.* 2020). An *in vitro* investigation pointed out that acai berry hydroalcoholic extract was able to reverse mitochondrial dysfunction induced by rotenone exposure in neuronal-like

SH-SY5Y cells (Machado *et al.* 2016). In this study, functional recovery of the mitochondrial electron transport chain in neurons was mainly by overexpression of nuclear mitochondrial complex I subunit genes (NDUFS7 and NDUFS8) (Machado *et al.* 2016).

1.6.5 Protection from toxic protein accumulation

Acai berry aqueous extract pre-treatment improved cell viability following exposure to human amyloid-β protein 1–42 ($A\beta_{1–42}$) (Wong *et al.* 2013). Moreover, in this study, acai berry extracts exhibited the most fibril inhibition and alteration of $A\beta_{1–42}$ morphology when compared with pure phenolics (Wong *et al.* 2013). Acai berry aqueous extract pre-treatment of neurons significantly reversed the basal dendrite length reduction and autophagy dysfunction induced by autophagy inhibitors such as bafilomycinA1 or wortmannin (Poulose *et al.* 2014). Similarly, a diet supplemented with 2% of acai berry exhibited upregulation of autophagy markers in the hippocampus and frontal cortex of ageing rat brains (Poulose *et al.* 2017). In mutant strain *Caenorhabditis elegans* CL2355, which expressed $A\beta_{1–42}$ and have an impaired chemosensory system, pre-treatment with extract of acai berry enhanced the chemotaxis response and decreased both polyglutamine (polyQ) aggregation and protein oxidation levels (Peixoto *et al.* 2016b). These studies collectively reinforced that acai berry extracts protect against the excessive accumulation of misfolded cytotoxic proteins, which are pathological hallmarks of many NDDs. Thus, acai berry improves the protein homeostasis through molecular mechanisms and consequently attenuates neurotoxicity.

1.6.6 Anticonvulsant properties

It is reported that there is an association between the common neurodegenerative dementia syndromes and epileptic seizure phenomena, particularly in AD, PD, prion diseases, and HD (Larner 2010). Acai berry juice was able to protect against behavioural changes and reduce oxidative stress caused by seizures induced by pentylenetetrazol (PTZ) administration in mice (Souza-Monteiro *et al.* 2015). Hence, this study suggested that acai berry juice displayed anticonvulsant effects and additional neuroprotective effects against LPO connected with seizures. Similarly, acai berry treatment at 5%-25%

concentration on primary cultures of cortical neurons and astrocytes showed its potential in the treatment of seizures and epilepsies by improving GABAergic neurotransmission (Arrifano *et al.* 2018). In this study, acai berry was able to interact with the $GABA_A$ receptor through increased agonist flunitrazepam binding and decreased antagonist t-Butylbicycloorthobenzoate (TBOB) binding as well as inhibiting γ-aminobutyric acid (GABA) reuptake, consequently leading to the accumulation of endogenous GABA in the synaptic cleft and enhanced inhibitory neurotransmission in the brain.

1.6.7 Antidepressant and anti-ageing effects of acai

In many NDDs, patients develop depression symptoms in some stage of disease progression (Baquero and Martín 2015). This substantially leads to increased cognitive and motor symptom impairment, morbidity, stress on families, and the cost of illness (Baquero and Martín 2015). Since many synthetic anti-depression medications have various side effects, such as nausea, anxiety, drowsiness, insomnia, and sexual dysfunction, it is considered critical in finding a new antidepressant herb with fewer side effects (Alamgeer *et al.* 2013). However, *in vivo* investigation by Souza-Monteiro *et al.* (2019) on mouse models of depressive-like behaviour showed improvements in electromyographic measurements and prevention of the despair-like and anhedonia behaviours after acai berry treatment. In the same study, acai berry decreased the oxidative stress that developed in the depressive mouse model, thus protecting against hippocampal neuron loss. Moreover, this research highlighted that acai treatment caused an increase in TERT mRNA expression, illustrating its anti-ageing and neuroprotective action.

To conclude, acai pulp fractions have shown promising beneficial effects and multi-target properties in regulating all five pathological processes (1) oxidative stress, (2) chronic inflammation, (3) mitochondrial dysfunction, (4) Ca^{2+} level elevation due to excitotoxicity and (5) accumulation of misfolded or aggregated proteins, which have interdependent mechanisms (Figure 1.12). Moreover, acai berry extracts give hope in controlling other neurological disorders through other neuromodulatory properties such as anticonvulsant, antidepressant and antiaging. This offers valuable insights into the acai

berry pulp and its considerable pharmacological potential in treatment of NDDs and stroke.

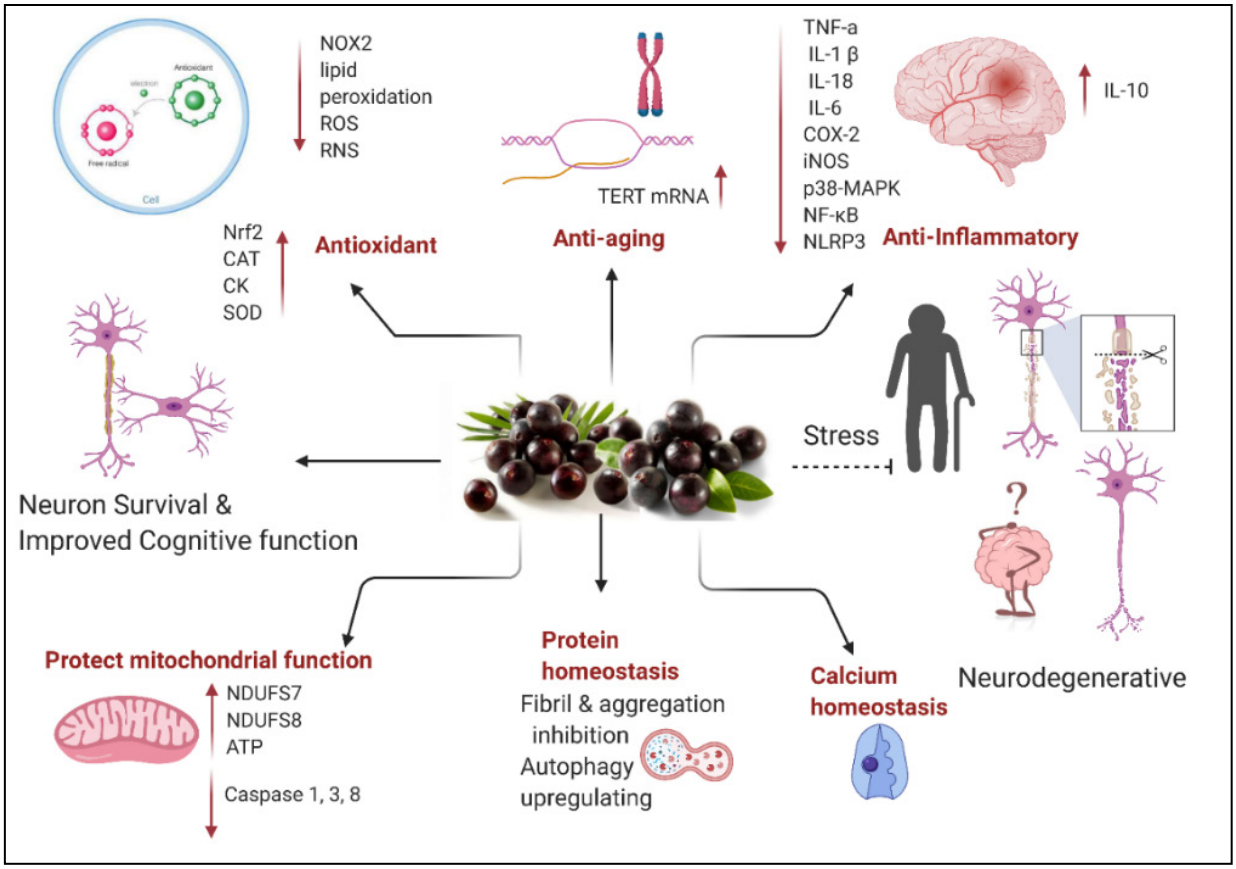

Figure 1.12: Known neuroprotective actions of *Euterpe sp*. fruits. Acai berry extracts can prevent neurodegeneration and maintain neurons' survival and cognitive function via the number of mechanisms summarized in this figure. First, the potential antioxidant action of acai berry extract involves decreasing oxidative stress markers such as NOX2, LPO, ROS, and RNS, and increasing antioxidant defence systems like Nrf2, CAT, CK, and SOD. Second, the anti-ageing action resulted in an increase in the TERT mRNA. Third, the anti-inflammatory activity of acai caused a reduction in inflammatory markers such as TNF-α, IL-1β, IL-18, IL-6, COX-2, iNOS, p38-MAPK, NF-κB, and NLRP3, while a rise in anti-inflammatory agent IL-10. Fourth, acai berry extracts can maintain the mitochondrial function via repairing the ATP production impairment and induce the expression of NDUFS7 and NDUFS8 while reducing the levels of the pro-apoptotic proteins, including caspases 1, 3, and 8. Fifth, acai berry extracts help maintain the neurons' protein and calcium homeostasis. Abbreviations: ATP, adonise diphosphate; CAT, catalase; CK, creatine kinase; COX-2, cyclooxygenase-2; IL-*10*, interleukin *10*; IL-18, interleukin 18; IL-1β, interleukin 1 beta; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; NDUFS7, NADH: Ubiquinone Oxidoreductase Core Subunit S7; NDUFS8, NADH: Ubiquinone Oxidoreductase Core Subunit S8; NF-κB, nuclear factor kappa B; NLRP3, NLR family pyrin domain containing 3; NOX2, NADPH-oxidoreductase-2; Nrf2, nuclear factor erythroid 2-related factor 2; p38-MAPK, p38 mitogen-activated protein kinase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TERT mRNA, telomerase reverse transcriptase; TNF-α, tumour necrosis factor-α. Figure created using BioRender (https://www.biorender.com/).

1.7 *In vitro* models to study human neurotoxicity and neuroprotection

Neurobiology and neuroscience fields have been greatly enhanced by the ability to use *in vitro* model systems (Breier *et al.* 2010). Cells in culture provide an effective platform for studying basic physiological processes of nervous system cells, such as protein characterisation, molecular mechanisms, and understanding disease and infection pathology (Giordano and Costa 2011; Shipley *et al.* 2016). They have also been used to conduct pilot drug testing and examine the neurotoxic effects of chemicals (Giordano and Costa 2011; Shipley *et al.* 2016).

1.7.1 Human neuroblastoma SH-SY 5Y cell line

The SH-SY5Y cell line is a thrice-subcloned cell line derived from the parental SK-N-SH neuroblastoma cell line (Biedler *et al.* 1978). In the early 1970s, the SK-N-SH cells were initially established from a bone marrow biopsy of a four-year-old female with neuroblastoma cancer, which had sympathetic adrenergic ganglial origin (Biedler *et al.* 1978). Since the early 1980s, the SH-SY5Y cell line has been extensively used as a model for neurons because these cells have several neuronal biochemical and functional characteristics (Barbosa *et al.* 2015). The SH-SY5Y cell line is a relatively homogeneous neuroblast-like cell line (Xie *et al.* 2010).

The continuously dividing SH-SY5Y cell line is used as a model to study NDDs such as AD (Abemayor and Sidell 1989; Agholme *et al.* 2010) and PD (Xie *et al.* 2010). These cell lines are used because a stable karyotype consisting of 47 chromosomes was identified for these cells, and they can be differentiated from a neuroblast-like state into mature human neurons via culturing in different agents such as retinoic acid (RA) or phorbol esters and specific neurotrophies such as brain derived growth factor (BDNF) (Shipley *et al.* 2016).

Significant differences were noticed between undifferentiated and differentiated SH-SY5Y cells. In the undifferentiated state, non-polarised cells multiply rapidly and develop very few short processes (Gimenez-Cassina *et al.* 2006; Kovalevich and Langford 2013). These cells become polarized and proliferate slowly, developing a long-branched process when differentiated (Gimenez-Cassina *et al.* 2006; Kovalevich and

Langford 2013). Moreover, researchers studied excitotoxicity in this cell line and detected the expression of NMDAR and other L-Glu receptors in undifferentiated and differentiated cells (Goldie *et al.* 2014; Korecka *et al.* 2013; Kulikov *et al.* 2007; Lee *et al.* 2015; Nair *et al.* 1996; Pizzi *et al.* 2002; Sun and Murali 1998).

1.7.2 Human rhabdomyosarcoma cell line TE671

The TE671 cell line was first thought to have been established by McAllister *et al.* (1977) from a 6-year-old Caucasian girl with a tumour of the left cerebellum; a biopsy was made and cultured before X-rays or chemotherapy was performed. These undifferentiated cells were derived from a cerebellar medulloblastoma showing 47 chromosomes and displaying polygonal and fusiform phenotypes with short, unbranched processes (McAllister *et al.* 1977). This cell line was first described as human medulloblastoma cell line TE671 and was found to have neuron-like characteristics, including functional nicotinic acetylcholine receptors (nAChR) (Syapin *et al.* 1982). Moreover, a study reported that TE671 cell line differentiation using dibutyryl adenosine 3':5' -cyclic monophosphate (db-cAMP) induced morphological evidence of differentiation, including process formation and markedly increased neuronal maturation marker (Sasaki *et al.* 1986). Similar changes after db-cAMP exposure were detected in addition to reduction in nAChR expression (Bencherif and Lukas 1991; Siegel and Lukas 1988).

However, a later study revealed that this cell line expressed subunits identical to those of muscle acetylcholine receptors (mAChRs) rather than nAChRs (Schoepfer *et al.* 1988). Researchers found that the TE671 human medulloblastoma and rhabdomyosarcoma cell line RD shared several phenotypic characteristics with striated muscle cells (Stratton *et al.* 1989). Moreover, two studies confirmed that TE671 and the rhabdomyosarcoma cell line RD are similar (Chen *et al.* 1989; Stratton *et al.* 1989). Consequently, the TE671 cell line ends up on the list of possible cross-contamination cell lines (Capes-Davis *et al.* 2010). This contamination may be because TE671 cells came from the same laboratory that had established a rhabdomyosarcoma cell line from a 7-year-old girl's pelvic sarcoma (McAllister *et al.* 1969). However, researchers have employed the TE671 cell line as a source of nAChRs with properties similar to those of human muscle (Brier *et al.* 2003;

Franciotta *et al.* 1999; Patel *et al.* 2020). In addition, emerging evidence demonstrated the expression of active and functional L-Glu receptors in the undifferentiated TE671 cell line (Brocke *et al.* 2010; Luksch *et al.* 2011; Stepulak *et al.* 2009; Wojciech *et al.* 2001). Therefore, this implied the value of this cell line as a model to examine L-Glu toxicity.

1.7.3 Human cortical neuronal progenitor cell culture (ReNcell CX)

Several studies of neurotoxicants have used human neuroprogenitor cells as models (Donato *et al.* 2007). ReNcell CX cells isolated by ReNeuron Group (Guildford, Surrey, UK) are a human neuroprogenitor cell model used extensively to assess chemical effects on developmental neurotoxicity (Donato *et al.* 2007). ReNcell CX lines are available commercially via Merck Millipore and are self-renewing, multipotent, and phenotypically and genotypically stable due to immortalization by retroviral transduction with the c-myc oncogene (Breier *et al.* 2010; Donato *et al.* 2007). Initially, this immortalized human neural progenitor cell line was derived from the cortical region of brain tissue of a 14-week gestation human foetus, and they proliferate linearly with a doubling time of 20-30 hours as a monolayer on laminin (Breier *et al.* 2010; Donato *et al.* 2007). The karyotype of these cells is normal diploid (46, XY), which is normal for male cells (Breier *et al.* 2010; Donato *et al.* 2007). The undifferentiated cells are small polygonal-shaped cells, have few processes, and express the neural stem cell marker nestin (Donato *et al.* 2007). These cells can be differentiated by removing the growth factors from the growth media to different types of neurons, astrocytes and oligodendrocytes (Donato *et al.* 2007).

1.8 Hypothesis and aims

As an excitatory neurotransmitter, L-Glu works in the brain as a signal to aid neurons in communicating (Chayat and Yedidya 2012; Leibowitz *et al.* 2012; Purves *et al.* 2018). However, considerable evidence demonstrates that neuronal death in NDDs and stroke is associated with L-Glu accumulation and induction of toxicity (Belov Kirdajova *et al.* 2020; Fomin *et al.* 2018; Hynd *et al.* 2004; Mehta *et al.* 2013; Plaitakis and Constantakakis 1993; Rothman and Olney 1986; Rothstein *et al.* 1992; Tiwari *et al.* 2016; Zeron *et al.* 2001; Zumkehr *et al.* 2015). MSG is used extensively worldwide as a

food enhancer or food additive. High daily intake of MSG results in accumulation and raises the glutamic acid level in the blood and CSF, which poses a risk to neural cell survival. It is reported that MSG induces neuron damage through excitotoxicity, free radical generation, antioxidant content reduction, mitochondrial dysfunction, Ca^{2+} imbalance, and deterioration of synaptic transmissions (Bernardo *et al.* 2017; Cong *et al.* 2016; Dar *et al.* 2018; Lv *et al.* 2017). Since the acai berry is rich in several bio-active phytochemicals with multi-pharmacological characteristics, it may represent a potential treatment strategy for NDDs and stroke. Therefore, this study hypothesizes that using plant extracts, such as acai berry, could prevent L-Glu-neurotoxicity through its multitarget activities. This research assumes that the acai berry could preserve cell viability, moderate excitotoxicity, limit mitochondrial dysfunction, preserve antioxidant content, and normalize synaptic transmission. Moreover, this research presumes that the acai berry could contain compounds that are potential candidates for drug development and a possible novel therapeutic to treat NDDs and stroke.

This thesis will concentrate on the following aspects outlined below:

1. In view of this concern regarding excessive exogenous L-Glu, in addition to possible accumulation at the synaptic cleft and the potential impact on neuronal survival (Niaz *et al.* 2018; Singh 2005), systematic research of the literature was conducted to provide a comprehensive, unbiased analysis of the effects of L-Glu on neuronal viability and its implications for the pathogenesis of NDDs such as AD, PD, MS, ALS, and HD.
2. Excessive L-Glu release from neurons plays a significant role in neuron death in both NDDs and strokes as well as exogenous L-Glu contributes to neuron toxicity. However, the native South American palm acai berry (*Euterpe sp*) is a potential source of dietary phytochemicals beneficial to health. Therefore, the following study aimed to evaluate L-Glu toxicity, acai aqueous and ethanolic extracts safety, and investigate neuroprotection effects through combined incubation of L-Glu and acai berry extracts. The evaluation was done using different assessments: to evaluate cell viability (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays

were completed; oxidative stress measured using the 2,7-dichlorodihydrofluorescein diacetate (DCFHDA) assay; mitochondrial function evaluation via the adenosine 5′-triphosphate (ATP) bioluminescent assay; mitochondrial membrane potential (MMP) measurement; as well as to examine the effects on *N*-methyl-D-aspartate receptors (NMDAR) using whole-cell patch-clamp assays. This was investigated in the SH-SY5Y human neuroblastoma cell line and differentiated TE671 human rhabdomyosarcoma cell line. In addition, a cell viability MTT assay was done for differentiated SH-SY5Y cells and human cortical neuronal progenitor cell culture (ReNcell CX) to investigate L-Glu toxicity and acai berry extract effects.

3. One of the main pathomechanisms in several NDDs is oxidative stress and current treatments are symptomatic relief and do not stop or reverse the progressive neural death. Moreover, combined with a dysfunction of ACh-releasing neurons in the brain, AD contributes substantially to cognitive decline. Furthermore, it has been shown that targeting cholinesterase enzymes (ChE) via inhibition allows for the maintenance of cholinergic activity and symptomatic improvement over the course of AD. Therefore, the fifth chapter of this thesis aimed to consider an *in vitro* evaluation of the nutraceutical potential of acai berry aqueous and ethanolic extracts on their ability to inhibit ChE scavenge free radicals via 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) or 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assays. The antioxidant potential was assessed via hydrogen peroxide (H_2O_2) or hydroxyl radical ($OH^{\bullet}$) scavenging, nitric oxide radical ($NO^{\bullet}$) scavenging, lipid peroxidation (LPO) inhibition and the ability to reduce ferric ions (Fe^{+3}). Total polyphenol (TPC) and flavonoid (TFC) contents were determined in both aqueous and ethanolic acai berry extracts. In addition, acai berry extracts were fractionated and analyzed by liquid chromatography mass spectrometer (LC-MS) to identify phytochemicals that may be responsible for anti-cholinesterase and antioxidants properties.
4. The last chapter discusses the thesis significant novel results, prospective consequences, future research options, and the study shortcomings.

Chapter 2
Materials and methods

Chapter 2. Materials and methods

2.1 Systematic review

The systematic review was conducted based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Page *et al.* 2021a; Page *et al.* 2021b).

2.1.1 Search scheme

A systematic electronic database search was performed from 6 to 16 October 2020 on Medline (OvidSP), Embase (OvidSP), Web of Science Core Collection, and PubMed. The aim was to identify research studies that reported the influence of L-Glu upon neuronal viability and resulting NDDs. Controlled search vocabularies (MeSH) were used and involved a combination of the following: (a) excitotoxic* amino acid OR L-glutamate OR glutamate neurotoxic* OR L-glutamic acid OR monosodium glutamate; (b) neurodegenerative diseases OR nerve degeneration OR neurodegenerat* OR neurotoxicity; (c) mitochondrial dysfunction OR protein toxicity, excitotoxicity OR oxidative damage OR inflamma* OR neuroinflammat*. Hand searching of related papers generated by bibliography screening was also conducted.

2.1.2 Data extraction and collection

Data were extracted from eligible articles, and information collected for the following variables: authors, year of publication, *in vitro* or *in vivo* study, dose or concentration of L-Glu, route of application, toxicity assay and method, overall study outcome, and conclusions.

2.1.3 Eligibility criteria

All search results (n = 4060) were imported into EndNote (Clarivate Analytics), and automatic deduplication was performed. A manual check of the title and abstract screening was then undertaken to identify studies considered relevant to the prespecified inclusion criteria.

All *in vitro* findings that were original studies directly investigating the effect of L-Glu on molecular mechanisms chiefly associated with the pathology of AD, PD, MS, ALS, or HD, and in which L-Glu influenced neuronal viability, were included. Similarly, all *in vivo* (animal) evidence that focused upon the direct impact of L-Glu on neuronal molecular processes that resulted in diseases, such as that observed in NDDs, specifically AD, PD, MS, ALS, or HD, was included. There was no time restriction for the studies included in this systematic review.

Studies were excluded if they focused on L-Glu toxicity in organs other than the brain or were focused on NDDs other than those specified above, were published in a language other than English, were performed with nonhuman neurons or tissue, or were review articles, editorials, or conference abstracts.

2.2 Chemicals and reagents

Table 2.1, 2.2, 2.3, and 2.4 list the reagents, chemicals, kits and plasticwares used for all the experiments discussed in this thesis.

Table 2.1: Reagents and chemicals used for the cell culture.

Reagent	Product Number	Supplier	Storage Conditions
Accutase	SCR005	Sigma-Aldrich, Poole, UK	−20°C After thawing can be stored for up to 2 months at 4°C
All-*trans*-retinoic acid (RA)	R2625	Sigma-Aldrich, Poole, UK	−20°C

Reagent	Product Number	Supplier	Storage Conditions
Basic fibroblast growth factor (bFGF)	GF003	Sigma-Aldrich, Poole, UK	−20°C
Calcium chloride	21114	Honeywell, Fisher Scientific, UK	Room temperature
Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone	C2920	Sigma-Aldrich, Gillingham, UK	2 - 8°C
Cesium chloride	C3032	Sigma-Aldrich, Gillingham, UK	Room temperature
Cesium hydroxide solution	232041	Sigma-Aldrich, Poole, UK	Room temperature
D-Glucose anhydrous	50-99-7	Fisher-scientific, UK	< 30°C
2′,7′-Dichlorodihydrofluorescein diacetate	D6883	Sigma-Aldrich, Poole, UK	−20°C
Dimethyl sulphoxide (DMSO) for cell culture	BP231100	Fisher-Scientific, UK	Room temperature
(3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) (MTT)	M5655	Sigma-Aldrich, Poole, UK	2 - 8°C
6,7-Dinitroquinoxaline-2,3-dione	0189	TOCRIS, UK	Room temperature

Reagent	Product Number	Supplier	Storage Conditions
Dizocilpine (MK-801)	391A	Research Biochemicals Incorporated	Room temperature
Dulbecco's Modified Eagle Medium/F12	DF-042-B	Sigma-Aldrich, Merck Gillingham, UK	2 - 8°C, away from light
d-tubocurarine chloride	T2379	Sigma-Aldrich, Poole, UK	0 - 5°C
Dulbecco′s Modified Eagle′s Medium - high glucose	D6546	Sigma-Aldrich, Gillingham, UK	2 - 8°C
Epidermal growth factor (EGF)	GF001	Sigma-Aldrich, Poole, UK	−20°C
Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid	E4378	Sigma-Aldrich, Gillingham, UK	Room temperature
F12 Nut mix (Ham) (1X)	217650-29	Life Technologies, Paisley, UK	2 - 8°C
Freeze-dried acai pulp and skin powder	-	NaturaleBio (Organic product under EU Directive 834/2007, purchased via Amazon.co.uk)	2 - 8°C
Glycine	G8898	Sigma-Aldrich, Poole, UK	Room temperature

Reagent	Product Number	Supplier	Storage Conditions
Hanks' Balanced Salt Solution (HBSS) (1X)	14170088	Life Technologies, Paisley, UK	15 - 30°C
Hanks' Balanced Salt Solution	H8264	Sigma-Aldrich, Poole, UK	2 - 8°C
Heat inactivated Foetal Bovine Serum (FBS)	F9665	Sigma-Aldrich, Poole, UK	−5 - −20°C
Hydrochloric acid solution	84435	Honeywell, Fisher-Scientific, UK	Room temperature
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid	H4034	Sigma-Aldrich, Gillingham, UK	Room temperature
Kainic acid	K0250	Sigma-Aldrich, Gillingham, UK	2 - 8°C
Laminin	CC095	Sigma-Aldrich, Gillingham, UK	−20°C
L-Glutamic acid monosodium salt monohydrate	49621	Sigma-Aldrich, Gillingham, UK	Room temperature
L-Glutamine (100x)	G7513	Sigma-Aldrich, Gillingham, UK	−20°C

Reagent	Product Number	Supplier	Storage Conditions
Magnesium chloride	26123	BDH chemicals Ltd., Poole, England	Room temperature
Minimum Essential Medium Eagle (EMEM)	M4655	Sigma-Aldrich, Poole, UK	2 - 8°C
MitoTracker™ Green FM,	M7514	Thermo-fisher, UK	−20°C
N6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt	D0627	Sigma-Aldrich, Poole, UK	−20 °C
N-Methyl-D-aspartic acid	0114	TOCRIS, UK	Room temperature
Non-Essential Amino Acid Solution (NEAA) (100X), without L-Glutamine	M7145	Sigma-Aldrich, Poole, UK	2 - 8°C
Penicillin/ streptomycin (Penicillin 10,000 units/ml streptomycin 10 mg/ml)	P4333	Sigma-Aldrich, Poole, UK	−5 - −20°C
Phosphate buffered saline pH 7.4 (1X)	10010-015	Life Technologies, Paisley, UK	15 - 30°C
Poly-D-lysine	A-003-E	Sigma-Aldrich, Poole, UK	−20°C

Reagent	Product Number	Supplier	Storage Conditions
Potassium chloride	31248	Honeywell, Fisher-Scientific, UK	Room temperature
ReNcell CX maintenance medium	SCM005	Sigma-Aldrich, Poole, UK	−20°C until ready to use. Upon thawing, the maintenance medium should be stored at 2-8°C
ReNcell CX NSC freezing medium	SCM007	Sigma-Aldrich, Poole, UK	−20°C
Sodium chloride	S9625	Sigma-Aldrich, Poole, UK	Room temperature
Trizma base	T1503	Sigma-Aldrich, Poole, UK	Room temperature
Trypan blue	T10282	Fisher-Scientific, UK	18 - 25°C
Trypsin-EDTA with phenol red (0.05%)	25300062	Life technologies, Paisley, UK	−5 - −20°C

Table 2.2: List of the kits used for the study.

Kit	Product Number	Supplier	Storage Conditions
ATP bioluminescence	11699695001	Roche, Sigma-Aldrich, Gillingham, UK	−20°C
CyQUANT™ LDH cytotoxicity	C20301	Thermo-fisher, UK	−5 - −30°C

Table 2.3: Reagents and chemicals used for the acai berry extract experiments.

Reagent/ chemical	Product number	Supplier	Storage conditions
Acetylcholinesterase from Electrophorus electricus (electric eel)	C3389	Sigma, Irvine, UK	−20°C
Acetylcholinesterase from human	C1682	Sigma-Aldrich, UK	−20°C
Acetythiocholine iodide	A5751	Sigma-Aldrich, UK	2 - 8°C
Aluminium chloride	237078	Sigma-Aldrich, UK	Room temperature
α-tocopherol	T3251	Sigma-Aldrich, UK	2 - 8°C
Azamethiphos-oxon	35575-96-3	QMX Laboratories Ltd., Thaxted, UK	Room temperature
2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid Cation	194430	Sigma-Aldrich, UK	2 - 8°C

Reagent/ chemical	Product number	Supplier	Storage conditions
Bovine brain extract type I, Folch fraction I	B1502	Sigma, UK	−20°C
Butylated hydroxyanisole	B1253	Sigma-Aldrich, UK	Room temperature
Butyrylcholinesterase from equine serum	C7512	Sigma, Irvine, UK	−20°C
Butyrylcholinesterase from human	B4186	Sigma-Aldrich, UK	2 - 8°C
Butyrylthiocholine iodide	203989	Sigma-Aldrich, UK	2 - 8°C
2-Deoxy2-ribose sugar	533-67-5	Thermo-Fisher, Stafford, UK	2 - 8°C
Dimethyl sulphoxide	D8418	Sigma-Aldrich Poole, UK	Room temperature
2,2-Diphenyl-1-picrylhydrazyl or di(phenyl)-(2,4,6 trinitrophenyl)iminoazanium	D9132	Sigma-Aldrich, UK	2 - 8°C
5,5′-Dithiobis(2-nitrobenzoic acid)	D8130	Sigma-Aldrich, UK	Room temperature
Ethanol absolute, ≥99.8% (vol.)	40347H	Honeywell International Inc., UK	Room temperature
Ethopropazine hydrochloride	1094-08-2	(QMX Laboratories Ltd., Thaxted, UK)	−20°C
Ethylenediaminetetraacetic acid	E5134	Sigma-Aldrich Poole, UK	Room temperature
Ferric chloride	157740	Sigma-Aldrich, UK	Room temperature

Reagent/ chemical	Product number	Supplier	Storage conditions
Freeze-dried acai pulp and skin powder	-	NaturaleBio (Organic product under EU Directive 834/2007, purchased via Amazon.co.uk*)*	Cool and dry place
Gallic acid	G7384	Sigma-Aldrich, UK	Room temperature
Glacial acetic acid	64-19-7	Scientific Laboratory Supplies (Nottingham, UK)	Room temperature
Hydrochloric acid	231-5957	Thermo-Fisher Scientific, Loughborough, UK	Room temperature
Hydrogen peroxide	H1009	Sigma-Aldrich Poole, UK	2 - 8°C
L-ascorbic acid	A92902	Sigma-Aldrich, UK	Room temperature
Methanol	M/4056/17	ThermoFisher Scientific, Loughborough, UK	Room temperature
Naphthalene diamine dichloride	1465-25-4	Thermo-Fisher, Stafford, UK	Room temperature
phosphate buffer (Gibco™ PBS, pH 7.4)	10010023	Thermo-Fisher, Stafford, UK	15 - 30°C
Phosphate buffered saline pH 7.4 (1X)	10010-015	Thermo-Fisher Scientific Rochester, UK	15 - 30° C

Reagent/ chemical	Product number	Supplier	Storage conditions
Phosphomolybdic/phosphotungstic acid complexes (Folin–Ciocalteu reagent) or Folin & Ciocalteu′s phenol reagent	F9252	Sigma-Aldrich, UK	Room temperature
Potassium acetate	P1190	Sigma-Aldrich, UK	Room temperature
Potassium ferricyanide	P3667	Sigma-Aldrich, UK	Room temperature
Potassium persulfate	216224	Sigma-Aldrich, UK	Room temperature
Quercetin	Q4951	Sigma-Aldrich, UK	Room temperature
Sodium carbonate	451614	Sigma-Aldrich, UK	Room temperature
Sodium dodecyl sulfate	L3771	Sigma-Aldrich Poole, UK	Room temperature
Sodium hydroxide	72068	Sigma-Aldrich, UK	Room temperature
Sodium nitroprusside	567538	Sigma-Aldrich, UK	10 - 30°C
Sulphanilamide	S9251	Sigma-Aldrich, UK	2 - 8°C
2-Thiobarbituric acid	T5500	Sigma-Aldrich, UK	Room temperature
Trichloroacetic acid	76-03-9	Thermo-Fisher Scientific, UK	Room temperature
Tris base	T1503	Sigma-Aldrich Poole, UK	Room temperature

Table 2.4: Plasticware used for the study.

Plasticware	Catalog Number	Supplier
Cell scraper	CC7600-0250	Starlab, UK
Corning® Costar® Stripette® serological pipettes, individually paper/plastic wrapped	CLS4488-200EA	Sigma-Aldrich, UK
Corning™ Centrifuge Tube Top Vacuum Filters, CA Membrane	09-761-34	Thermo-Fisher Scientific, Rochester, UK
Greiner 96 well plates, polystyrene	M2936	Sigma-Aldrich, UK
Millex-GP Syringe Filter Unit, 0.22 µm, polyethersulfone, 33 mm, gamma sterilized	SLGP033RS	Merck life science, UK
Sterile, clear bottom, tissue culture treated 96 well plates	167425	Thermo-Fisher Scientific, Rochester, UK
Sterile, clear bottom, tissue culture treated 6 well plates	CC7682-7506	STARLAB International GmbH, Hamburg, Germany
Sterile, clear bottom, tissue culture treated black 96 well plates	165305	Thermo-Fisher Scientific Rochester, UK
Vented cap, Angled neck T25 flasks	130189	Thermo-Fisher Scientific, Rochester, UK

Plasticware	Catalog Number	Supplier
Vented cap, Angled neck T75 flasks	130190	Thermo-Fisher Scientific, Rochester, UK

2.3 Cell culture and treatments

The study was conducted using different cell lines defined in Table 2.5.

Table 2.5: SH-SY5Y, TE671, and ReNcell CX cell loading density.

	SH-SY5Y		TE671		ReNcell CX	
Type of cell culture ware	**Loading volume**	**Total number of cells**	**Loading volume**	**Total number of cells**	**Loading volume**	**Total number of cells**
T25 flask	5 mL	250,000	5 mL	250,000	5 mL	350,000
T75 flask	15 mL	1,000,000	15 mL	1,000,000	15 mL	1,500,000
96 well plate	200 µL/well	10000/ well	100 µL/well	10000/ well	200 µL/well	30000/ well
6 well plate	2 mL/ well	100,000/ well	1 mL/ well	200,000/ well	-	-

2.3.1 Human neuroblastoma SH-SY5Y cell line culture

The undifferentiated human neuroblastoma SH-SY5Y cell line (ECACC 94030304) was purchased from the European Collection of Authenticated Cell Cultures (ECACC). SH-SY5Y cells were grown in 1:1 Minimum Essential Medium Eagle (EMEM) and Ham's F12 Nut mix (EMEM/F12) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids (NEAA), 1% penicillin–streptomycin solution in a 95% humid atmosphere of 5% CO_2 and 37°C. These conditions were maintained for all the cellular assays. SH-SY5Y cells were cultured in T75 flasks until they reached 70-80% confluence at which time they were passaged by rinsing the cells with Hanks' balanced salt solution (HBSS) and then they were detached by incubating with 3 mL of 0.25% trypsin-EDTA for 3 minutes in an incubator at 37°C. Then, an equal volume of FBS was added to terminate the activity of trypsin-EDTA. Cells were harvested into a 15 mL falcon tube, centrifuged at 1000 rpm for 5 minutes and the supernatant removed. Cells were then washed twice in 10 mL HBSS and centrifuged at 1000 rpm for 5 minutes to pellet the cells. After that, the supernatant was discarded, and the cells resuspended in EMEM/F12 medium. The cells were counted using a Countess™ II FL automated cell counter and seeded in EMEM/F12 medium. The experiments were performed using cell passage number 13 - 15 to reduce morphological and genetic changes that can arise from cells that have been extensively passaged.

The SH-SY5Y cells were differentiated according to Amat-Ur-Rasool *et al.* (2021), and de Medeiros *et al.* (2019). In 96-well plates pre-coated with 50 µg/mL poly-D-Lysine (PDL), SH-SY5Y cells were grown in the same media indicated above which contained 10% FBS and the same culture conditions. Once cells reached 60% confluence, growth media was replaced with differentiation media consisting of 1:1 EMEM and EMEM/F12 medium containing 1% FBS, 1% NEAA, 2 mM glutamine, 1% penicillin–streptomycin solution, and 10 µM all-*trans*-retinoic acid (RA). A light microscope was used to monitor differentiation of neurons through regular replacement of differentiation media every other day for six days. A 5 mM stock of RA was prepared in absolute ethanol, and 10 µM RA was added to differentiation media at the time of changing the media under light-

protected conditions to avoid the photodegradation of RA. It has been shown in many studies that differentiation of SH-SY5Y cells by RA produces more neuron-like cells with neurite outgrowths (de Medeiros *et al.* 2019; Kovalevich and Langford 2013).

2.3.2 Human rhabdomyosarcoma cell line TE671 culture

The human undifferentiated rhabdomyosarcoma cell line TE671 was obtained from the European Collection of Authenticated Cell Cultures (ECACC; catalogue no. 89071904). TE671 cells were grown as monolayer cultures in 75 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, 2 mM glutamine, 1% penicillin–streptomycin solution at 37°C in a humidified 5% CO_2 and 95% air environment. As soon as the cells reached 80-90% confluence, they were passaged by rinsing them with PBS, then they were detached by incubating them in 0.25% trypsin-EDTA for 3 minutes at 37°C. After that, trypsin-EDTA activity was terminated by adding an equal volume of DMEM media. In a 15 mL falcon tube, cells were harvested and centrifuged at 1000 rpm for 5 minutes to remove the supernatants and the cells resuspended in DMEM media. Cells were counted using countess^{TM} II FL automated cell counter and seeded as described in the table 3.3. When TE671 cells reached 60% confluency, they were differentiated in serum-free DMEM containing 2 mM L-glutamine, 1% Penicillin/Streptomycin, and 400 µM N6,2′-O-dibutyrylladenosine 3′,5′-cyclic monophosphate sodium salt (dbcAMP) as a differentiation agent. Cells were monitored under the microscope to observe the differentiated cells, which exhibit characteristic features of neuronal subtypes, such as long and branched processes.

2.3.3 Human cortical neuronal progenitor cell culture (ReNcell CX)

Undifferentiated ReNcell CX cells (SCC007) are an immortalized human neural progenitor cell line and were purchased from Merck Millipore. The ReNcell CX neural stem cells were grown in maintenance medium, supplemented with 20 ng/mL fibroblast growth factor, 20 ng/mL epidermal growth factor, 100 µg/mL penicillin, and 100 µg/mL streptomycin (referred to as complete media) in a humid atmosphere of 5% CO_2 and 95% air at 37°C. The monolayer cultures were maintained by plating the cells at a density of

1.5 million cells in T75, 75 cm^2 or 350,000 cells in T25, 25 cm^2 plastic tissue culture flasks precoated with 20 µg/mL laminin in Dulbecco's Modified Eagle Medium/F12. ReNcell CX cells were cultured until they reached confluence (70 - 80%) then detached by incubating with accutase for 3 minutes in the incubator. Cells were then resuspended in the maintenance medium and centrifuged at 300 x g for 5 minutes to pellet the cells. Then, the supernatant was discarded, and the cells resuspended in complete medium. The cells were counted using a Countess™ II FL automated cell counter and seeded in complete medium. The cells were incubated for 48 hours before carrying out the assays to ensure complete attachment to the plate and to obtain 70 - 80% confluence.

2.3.4 L-glutamic acid (L-Glu), acai berry aqueous and ethanolic extracts preparation and cell treatments

L-Glu stock solution was prepared by dissolving its monosodium salt monohydrate crystals in culture medium with final concentrations obtained by dilution of stock solutions in normal growth media and pH was checked (7 to 7.5). The acai berry aqueous extract was prepared using methods as described in Wong *et al.* (2013). The commercially available freeze-dried acai berry pulp and skin powder purchased from NaturaleBio (Organic product under EU Directive 834/2007) were weighed and extracted by dissolving directly in culture media with vigorous vortexing. Then, the extract was centrifuged at 400 rpm and filtered using a 0.20 µm syringe filter to obtain a clear solution. The final extract concentrations were obtained by diluting the stock solution (2000 µg/mL) in media. An ethanolic extract was prepared by maceration of 300 mg/mL of freeze-dried acai berry pulp and skin powder in 70% ethanol for 48 hours. The macerated sample was shaken three times daily to assist solvation. Then, the solution was filtered using a bottle top filter. Filtrates were dried at 45 - 50°C for 24 hours in a water bath to obtain the ethanolic dry extracts (Machado *et al.* 2016; Nwidu *et al.* 2018). A stock solution was prepared by dissolving the dry ethanolic extract in culture media at a concentration of 2000 µg/mL and filtered using a 0.20 µm syringe filter to obtain a clear solution.

Cells were seeded and then grown to approximately 70 - 80% confluence after 48 hours in a culture plate, and then the toxicity of L-Glu evaluated by incubating with a broad concentration range (0.137 - 100 mM) in SH-SY5Y cells and TE671 cells while in ReNcell CX cells using concentrations range (0.03 - 100 mM) for 24 and 48 hours. To evaluate the most effective concentration of acai berry extract, the SH-SY5Y and TE671 cells were exposed to a broad concentration range (0.001 µg/mL - 1000 µg/mL or 0.001 µg/mL - 10 µg/mL) in the LDH assay under two periods of incubation, 24 and 48 hours. The ReNcell CX cells were exposed to different concentrations (0.0001 - 1000 µg/mL) under the same periods of incubation. Acai berry extract was added in combination with L-Glu concentrations of 11 mM or 100 mM to test its neuroprotective capability. Cells without the addition of L-Glu and acai berry extracts were used as negative controls (indicated as 0).

2.4 (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assays

This colorimetric assay was first developed by Mosmann (1983) and used to determine the cell viability by measuring the purple coloured product (Buranaamnuay 2021). This product is yielded as a result of the conversion of the yellow coloured water-soluble tetrazolium solution (MTT) into water-insoluble purple formazan crystals by the action of active mitochondrial dehydrogenase within viable cells (Figure 2.1) (Buranaamnuay 2021; Kamiloglu *et al.* 2020). MTT reduction has been associated with co-enzyme nicotinamide adenine dinucleotide (NADH), which has a central role in cytoplasmic glycolysis and oxidative phosphorylation in mitochondria (Buranaamnuay 2021). These intracellularly formed crystals are released through the cell membrane by adding solvent, such as dimethyl sulfoxide (DMSO), and can subsequently be easily quantified colourimetrically (Buranaamnuay 2021; Srivastava *et al.* 2018). Therefore, formazan colour intensity correlates with the number of metabolically active cells (Buranaamnuay 2021).

The MTT assay was used to determine the cell viability of undifferentiated and differentiated SH-SY5Y cells, differentiated TE671 cells, and undifferentiated ReNcell CX cells following exposure to L-Glu, acai extracts or the combination of both agents.

Briefly, cells were plated in 96-well plates and after 48 hours treated with specific compounds for predetermined times as detailed above (Section 2.3.4). MTT was added to each well at a final concentration of 0.5 mg/mL, and the plates were incubated for 2 hours at 37°C and 5% CO_2. The MTT solution with culture medium was then removed and DMSO was added, then plates were shaken for 5 - 10 minutes to dissolve the formazan crystals. The absorbance was measured at 570 nm with DMSO as the blank using a Varioskan™ LUX multimode microplate reader (ThermoFisher, Waltham, US). The absorbance of the control group (absence of test compound) was considered 100% of cell viability.

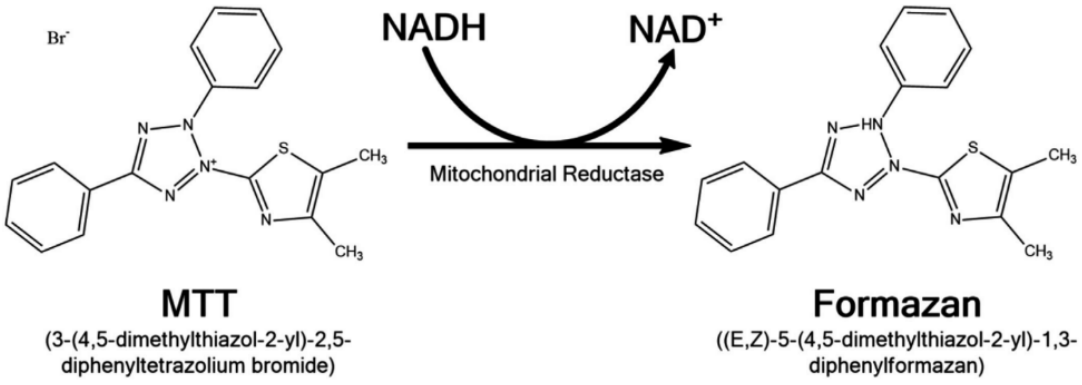

Figure 2.1: The principle of the MTT assay involving the formation of purple formazan crystals (Kamiloglu *et al.* 2020).

2.5 Lactate dehydrogenase (LDH) assay

The stable cytoplasmic enzyme LDH is found in all cell types (Wang *et al.* 2012). In the situation of cell plasma membrane damage, the LDH releases rapidly into the cell culture media (Wang *et al.* 2012). Therefore, the quantitative determination of LDH reflects the toxicity of various molecules and drugs (Kamiloglu *et al.* 2020; Wang *et al.* 2012). The LDH in the media can be quantified by a two-step enzymatic reaction, as presented in Figure 2.2 (Kamiloglu *et al.* 2020; Wang *et al.* 2012). Firstly, oxidation of lactate to pyruvate as a result of LDH catalysis by reduction of NAD^+ (nicotinamide adenine dinucleotide) to NADH (Kamiloglu *et al.* 2020; Wang *et al.* 2012) occurs. Then, with NADH, H^+ and diaphorase enzyme, a tetrazolium salt (iodonitrotetrazolium or 2-(4-iodophenyl)-3-(4-nitrophenyl)-5- phenyl-2H-tetrazolium (INT)) is reduced to a red coloured water soluble formazan salt product that can be easily measured colourimetrically Figure 2.2 (Kamiloglu *et al.* 2020; Wang *et al.* 2012).

The toxicity of L-Glu, acai berry extracts, or the combination of both was determined by the quantitative determination of LDH that was released into the cell culture media as a result of cell plasma membrane damage (Wang *et al.* 2012). The level of LDH released from damaged cells into the medium was measured using an LDH cytotoxicity assay kit (C20300, Invitrogen, Thermo-Fisher Scientific, UK) according to the manufacturer's instructions. Briefly, SH-SY5Y cells and differentiated TE671 cells were treated with L-Glu or acai berry extracts as described above (Section 2.3.4), with each assay point conducted in three replicates and three independent assays performed. A volume of 50 µL of the cell-free culture media from each treatment was transferred into a new 96-well plate. Then, 50 µL of reaction mixture provided by the LDH assay kit was added to each sample well and gently mixed, and the mixture was incubated at room temperature for 30 minutes in the dark, after which 50 µL of stop solution was added to each sample well and mixed gently. In the same plate, an LDH positive control from the kit was used. The plates were read at 490 nm and 680 nm using a spectrophotometer (Varioskan™ LUX multimode microplate reader, ThermoFisher, Waltham, US). The 680-nm absorbance value (instrument background signal) was subtracted from the 490-nm absorbance value

to calculate LDH activity. The OD values for each of the treatments were normalized to the mean of the negative control and the % LDH production was determined.

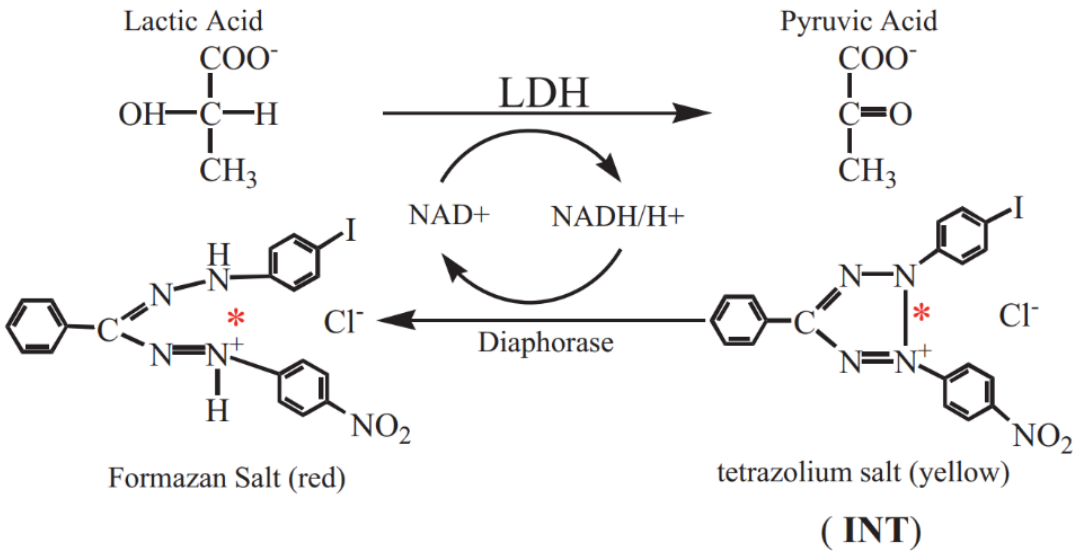

Figure 2.2: Two enzymatic reactions to quantitative LDH activity (Wang *et al.* 2012). INT, Iodonitrotetrazolium or 2-(4-iodophenyl)-3-(4-nitrophenyl)-5- phenyl-2H-tetrazolium.

2.6 Adenosine 5′-triphosphate (ATP) bioluminescent assay

One of the most common methods to estimate the number of living, metabolically active cells is by measuring cellular ATP content using firefly luciferase enzyme because the ATP is required for cellular viability and functionality (Crouch *et al.* 1993; Riss *et al.* 2016). In the case of loss of cell membrane integrity by injury, ATP synthesis in the cells is decreased, and endogenous ATPase is rapidly released to deplete any remaining ATP from the cytoplasm (Lee *et al.* 2012; Riss *et al.* 2016). Therefore, the status of cellular toxicity can be determined using rapid and accurate methods to measure the intracellular ATP content (Lee *et al.* 2012; Kamiloglu *et al.* 2020). This assay is based on the principle of the light production process in some living organisms called bioluminescence, where ATP is the key element of the chemical conversion and production of light in these organisms (Chollet and Ribault 2012; Kamiloglu *et al.* 2020). The bioluminescence mechanism in light-producing organisms, such as fireflies, is a multistep process and includes ATP, luciferin substrate, O_2 and Mg^{2+} (Chollet and Ribault 2012; Kamiloglu *et al.* 2020). The chemical reaction in the ATP bioluminescence assay involves the luciferase enzyme that catalyses luciferin oxidation and induces light, the intensity of which is directly proportional to the level of ATP in the sample (Kamiloglu *et al.* 2020; Riss *et al.* 2016). The two-step reaction (refer to Figure 2.3) begins with the conversion of firefly D-luciferin into the enzyme-bound luciferil adenylate complex by luciferase enzyme in the presence of ATP and Mg^{2+} (Chollet and Ribault 2012). The next step is the oxidative reaction which includes the conversion of luciferil complex to oxyluciferin (Chollet and Ribault 2012; Kamiloglu *et al.* 2020). Subsequently, the excited oxyluciferin is stabilised by rapid energy loss to produce the stable form that results in light emission (Chollet and Ribault 2012; Kamiloglu *et al.* 2020).

SH-SY5Y cells and differentiated TE671 cells were seeded in 6-well plates, grown to 80 - 90% confluency, and then treated with L-Glu or acai berry extracts or both as detailed in Section 2.3.4. After treating the cells for the desired time, the cells were rinsed three times with ice-cold PBS before being scraped into 500 µL of Tris/EDTA assay buffer (100 mM Trizma base and 4 mM ethylenediaminetetraacetic acid (EDTA), pH 7.75) through agitation on ice for 5 minutes then scraping the cells into the buffer. The isolated

cell suspension was then added to 9 mL of boiling Tris/EDTA buffer and incubated at 100 °C for 10 minutes. Samples were then centrifuged at 1000 x g, and the supernatant was transferred to a new Eppendorf and kept on ice until needed. ATP levels were determined in control and treated SH-SY5Y cells using an ATP Bioluminescence assay Kit, CLS II (11699695001, Sigma-Aldrich, Poole, UK). As described in the manufacturer's instructions, ATP standards were prepared over a concentration range of 1 x 10^{-5} to 1 x 10^{-10} M. Assay samples (100 µL volume) were transferred into a white opaque 96-well microplate for quantitation, 100 µL of luciferase reagent was added to each well and then well luminescence measured using a Varioskan™ LUX multimode microplate reader (ThermoFisher, Waltham, US) with an integration time of 1 second. The ATP content in control and treated samples was interpolated from the ATP standard curve. Lysis assay buffer alone was used as a blank, with values subtracted from the test samples. The corrected luminometric measurements from test samples were normalized to the mean of the control and expressed as a percentage relative to the negative control. Three separate experiments were carried out for each assay data point and each assay was performed in triplicate.

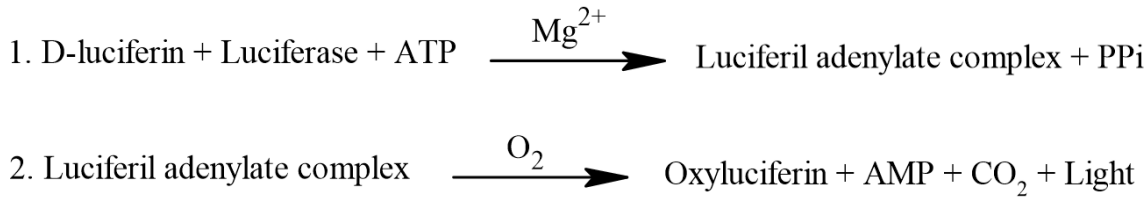

Figure 2.3: Luciferin/luciferase system of the ATP-bioluminescence assay. The light emission is photons of yellow-green light (550 to 570 nm) (AMP; adenosine monophosphate, PPi: inorganic pyrophosphate, CO_2; Carbon Dioxide) (Chollet and Ribault 2012).

2.7 Measurements of mitochondrial membrane potential (MMP)

The MMP is generated when various ions, including protons, are distributed across the inner and outer membranes of mitochondria as part of the energy production in oxidative phosphorylation process (Chu *et al.* 2021). It is essential to maintain MMP for optimal cellular function, and its abnormality is associated with NDDs, such as AD and PD (Chu *et al.* 2021; Liang *et al.* 2021b). In this regard, fluorescent probes have been designed to enable mitochondrial biology researchers to detect MMP, thus offering a valuable research tool (Chu *et al.* 2021; Liang *et al.* 2021b). In this assay, the MitoTracker Green probe was used, a lipophilic compound with a positive charge that is capable of passing through cell membranes and selectively accumulating in the negatively charged mitochondrial membranes (Keij *et al.* 2000; Presley *et al.* 2003). This assay relies on the chemical reaction between two chloromethyl groups in MitoTracker Green with thiol groups in cysteine residues in mitochondrial proteins to form a stable thioether conjugate (Presley *et al.* 2003). Therefore, the fluorescent intensity can be used to measure the MMP and detected via fluorescence microplate reader (Buckman *et al.* 2001; Keij *et al.* 2000; Liang *et al.* 2021b; Xiao *et al.* 2016). An effective mitochondrial uncoupler, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), is used as a positive control in this assay (Park *et al.* 2002). It depolarizes MMP and preventing mitochondrial ATP synthesis by inhibiting oxidative phosphorylation and triggering proton transport through mitochondrial inner membranes (Park *et al.* 2002).

The measurement of functionally active mitochondrial membranes was performed with minor modifications (Al-Ghafari *et al.* 2019). SH-SY5Y cells and differentiated TE671 cells were seeded in 96-well plates and grown to 80 - 90% confluency before treatment with L-Glu, acai berry extracts, or both, as described in Section 2.3.4. After the cells were treated for 24 or 48 hours, media were removed, and cells incubated with a staining solution of 50 nM MitoTracker® Green ^{FM} at 37°C for 30 minutes. The staining solution was removed, and fresh PBS added. The fluorescence was measured using a Varioskan™ LUX multimode microplate reader (ThermoFisher, Waltham, US), using 490 nm excitation and 516 nm emission filters. Untreated cells stained with 50 nM MitoTracker®

Green ^{FM} were used for negative controls, and wells with non-stained cells used as blanks. A positive control, FCCP (300 µM for SH-SY5Y cells or 100 µM for differentiated TE671 cells) was incubated with cells for 24 hours to induce membrane uncoupling. Fluorescent measurements were calculated after a subtraction of the means of blank values for each treatment and normalized to the mean of the negative control and expressed as a percentage.

2.8 2,7-dichlorodihydrofluorescein diacetate (DCFHDA) assay

Human bodies are susceptible to pathological conditions such as inflammation, cancer, and NDDs resulting from oxidative stress, which occurs when ROS or RNS overwhelms endogenous detoxifying ability (Kim and Xue 2020). DCFHDA staining method is used to determine cellular ROS levels in adherent cells (Kim and Xue 2020; Nova *et al.* 2020). In this assay the non-fluorescent DCFHDA reagent is easily diffused across the plasma membrane of the cell where the esterase cleaves off acetyl groups, generating more hydrophilic 2′,7′-dichlorodihydrofluorescein (DCFH) (Kim and Xue 2020; Nova *et al.* 2020). The oxidation action of ROS converts DCFH to highly fluorescent 2′,7′-dichlorofluorescein (DCF), which emits green fluorescence that can be measured spectrofluorometrically at 485 nm and 530 nm excitation, and emission wavelength respectively (Figure 2.4) (Kim and Xue 2020; Nova *et al.* 2020).

The generation of cellular ROS was used as an indicator of neurotoxicant-induced oxidative stress. Neurons exposed to L-Glu, acai berry extracts, or a combination were assayed for ROS production. SH-SY5Y cells and differentiated TE671 cells were seeded in 96-well black edge plates and grown to 80 - 90% confluency. Cells were incubated with L-Glu and acai berry extracts at concentrations detailed above (Section 2.3.4) and then 50 µM DCFHDA fluorescent dye added in normal media for 3 or 6 hours at 37°C. H_2O_2 at 500 µM for 30 minutes was used as a positive control for ROS generation, untreated cells stained with 50 µM DCFHDA used as a negative control, and non-stained cells used as blanks. Cells were then washed with PBS and the florescence signal emitted at 535 nm with a 485 nm excitation were measured in PBS using a Varioskan™ LUX multimode microplate reader (ThermoFisher, Waltham, US). The treatment and control

measurements were corrected via subtraction of the blank and normalized to the mean value from the negative control wells. ROS production was expressed as a percentage relative to the negative control. Data was generated from three separate experiments with each assay point performed in triplicate.

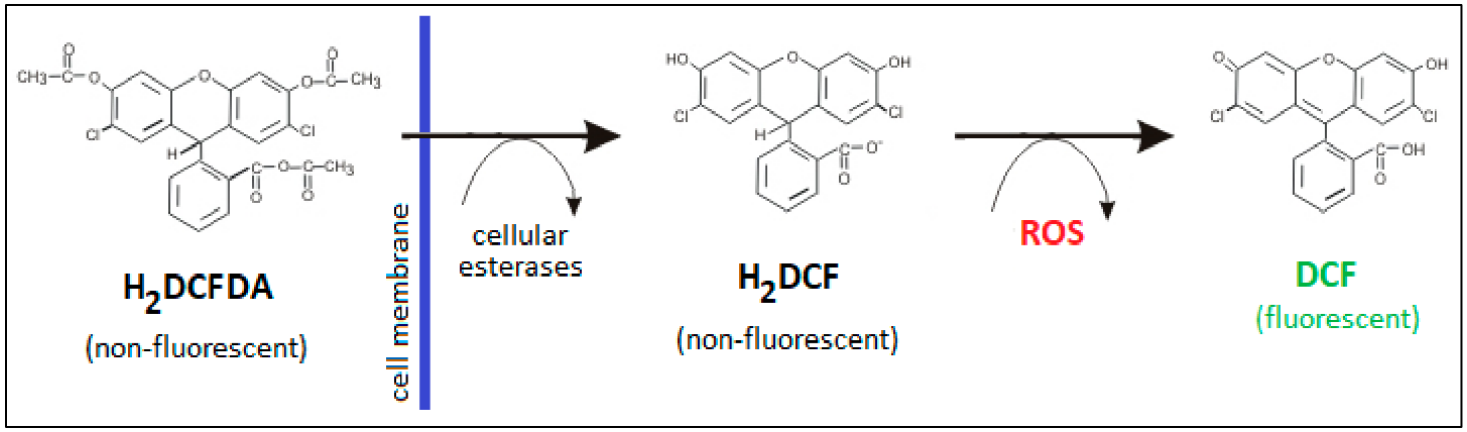

Figure 2.4: Measurement of ROS level with DCFHDA staining method (Nova *et al.* 2020).

2.9 Whole-cell patch-clamp assay

Ion channel activities are typically studied using an electrophysiology technique known as patch clamping (Rubaiy 2017). The purpose of using this technique is to gain a deeper understanding of the normal neuronal functions as well as their pathophysiology (Rubaiy 2017). The technique is based on using a glass micropipette filled with electrolyte solution and containing a recording electrode to make a tight seal on the surface of the cell and record whole-cell electric currents (ion movement across a cell membrane) while holding the membrane voltage constant via a feedback amplifier (clamp) (Hill and Stephens 2021; Rubaiy 2017). A negative pressure is used to make a very fine aperture on a cell membrane that creates a high-resistance micropipette-cell membrane seal (gigaseal) (Polder *et al.* 2005; Rubaiy 2017). A highly sensitive converter of current to voltage can be used to record any ions that are flowing into or out of the cell membrane (Polder *et al.* 2005). The value of whole-cell recording is not limited to clamping membrane voltage and measuring ionic currents, but it also allows to manipulate solution on the extracellular side by modifying bath solutions or applying a rapid pharmacological treatment (Van Hook and Thoreson 2014).

Whole-cell patch-clamp electrophysiology was performed on undifferentiated and differentiated SH-SY5Y cells and differentiated TE671 cells at a holding potential of −50 mV using an Axopatch 200A patch-clamp amplifier (Axon Instruments, US) and output recorded to a PC disk using a data acquisition device (National Instruments, NI PCI-6221/BNC-2110) and WinEDR V3.9.1 software (Dr John Dempster, Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, UK). SH-SY5Y cells and differentiated TE671 cells grown on glass coverslips were placed in a perfusion chamber and continuously perfused with mammalian Ringer (135 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 5 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 10 mM D-glucose, pH 7.4 with NaOH) at a flow rate of ~5 mL/min. A programmable micropipette puller (P-97, Sutter Instrument Co., US) was used to create patch-pipettes using borosilicate glass capillaries (1B150F-4, World Precision Instruments, UK). A solution of 140 mM caesium chloride (CsCl), 11 mM ethylene glycol-bis(β-aminoethyl

ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM $CaCl_2$, 5 mM NaCl, 5 mM HEPES, pH 7.2 with CsOH, was used to fill Patch-pipettes, creating resistances of ~5 MΩ. A DAD-12 Superfusion system (Adams & List Associates, New York, US) was used to perfuse 3 mM L-Glu + 10 µM glycine (Gly) solution as 1 - 2 s pulses for undifferentiated and differentiated SH-SY5Y cells.

In differentiated TE671 cells, the fast perfusion system was used to test different compounds; (1) 3 mM L-Glu + 10 µM Gly, (2) 100 µM kainic acid (KA) (3) 100 µM N-Methyl-D-aspartic acid (NMDA) + 10 µM Gly, (4) 300 µM L-Glu + 10 µM Gly, (5) 1000 µg/mL acai aqueous extract, (6) 300 µM L-Glu + 10 µM Gly + 1 µM dizocilpine (MK-801), (7) 300 µM L-Glu + 10 µM Gly + Mg^{+2} , (8) 300 µM L-Glu + 10 µM Gly + different acai aqueous extract concentration (0.001, 1, or 1000 µg/mL), (9) 1000 µg/mL acai aqueous extract + 1 µM d-tubocurarine (DTC), (10) 1000 µg/ml Acai + 10 µM 6,7-dinitroquinoxaline-2,3-dione (DNQX).

Compressed nitrogen was used to pressurize the perfusion system and solutions were applied at a pressure of 200 mm/Hg. 20 cells were examined for each cell line.

2.10 Preparation of aqueous and ethanolic extracts of acai berry (*Euterpe oleracea*)

The aqueous extract (10 mg/mL) was prepared using methods as described by Wong *et al.* (2013). The commercially available freeze-dried acai pulp and skin powder purchased from NaturaleBio (Organic product under EU Directive 834/2007, purchased via Amazon.co.uk) was weighed and extracted by dissolving in PBS and by vigorous vortexing. The extract was centrifuged at 400 rpm and filtered using a 0.20 µm syringe filter to obtain a clear solution and stored at 4 °C. An ethanolic extract of acai berry was prepared by the maceration of 300 mg/mL of the freeze-dried acai pulp and skin powder in 70% ethanol for 48 h. The macerated sample was shaken three times per day for two days to assist solvation and then filtered using a bottle-top filter. Filtrates were dried at 45 - 50 °C for 24 h in a water bath to obtain the ethanolic dry extract. The dried extract was reconstituted in PBS at a concentration of 10 mg/mL, filtered using a 0.20 m syringe filter, and kept at 4 °C.

2.11 Cholinesterase (ChE) activity assessments

This colourimetric assay was used to determine the ChE activity on tissue extracts, homogenates, cell suspensions, etc., and it is based on a method developed by Ellman *et al.* (1961). The method principle involves producing thiocholine as a result of AChE hydrolysis of acetylthiocholine, thereby, with 5,5'-dithiobis-(2- nitrobenzoic acid) (DTNB) forms a yellow-coloured product, the anion of 5-thio-2-nitro-benzoic acid (Figure 2.5) (Ellman *et al.* 1961). The absorbance of this coloured product can be measured at 412 nm in a spectrophotometer (Ellman *et al.* 1961). Low enzyme concentrations or small amounts of tissue can be quantified using this method, which is highly sensitive (Ellman *et al.* 1961).

The ability of the acai berry extracts to inhibit the activity of AChE and BuChE was assessed in a 96-well microtiter plate. From a total volume of 200 µL, a 10 µL of acai berry aqueous or ethanolic extracts (concentration range from 1×10^{-6} µg/mL to 1000 µg/mL) or positive control was mixed with 150 µL of 0.38 mM 5 DTNB, 3 µL of ChE enzyme, and 33 µL of PBS. The ChE enzymes used were 0.5 U/mL of AChE enzyme from *Electrophorus electricus* (electric eel) or 0.5 U/mL of BuChE enzyme from equine serum or 1 U/mL of human AChE (hAChE) enzyme or 1 U/mL of human BuChE (hBuChE) enzyme. Samples were incubated for 20 min at room temperature; then, the reaction was initiated by the addition of 4 µL of 35 mM of acetylthiocholine iodide (ATCI) substrate for AChE or butyrylthiocholine iodide (BTCI) substrate for BuChE, and the absorbance was measured at 412 nm every 30 s for 5 min using a Varioskan™ LUX multimode microplate reader (ThermoFisher, UK). The controls were the enzymes without addition of extracts and reagent blanks were performed in the absence of AChE or BuChE. Ten µL of positive (inhibitor) control for AChE assays was an organophosphate pesticide, azamethiphos at 5 mM, capable of the irreversible inhibition of AChE (Carter *et al.* 2007). For BuChE assays a 10 µL of ethopropazine hydrochloride at 5 mM was used as a recognised inhibitor of BuChE (Dorling *et al.* 2019). The percentage of AChE or BuChE activity remaining after incubation with acai berry extracts was calculated relative to the enzyme only (the negative control), which was designated as 100% enzymatic activity. The acai berry extract concentrations producing

50% inhibition (IC_{50}) of AChE or BuChE activity were determined. The assays were performed in duplicate for at least three independent experiments, after which a mean was calculated.

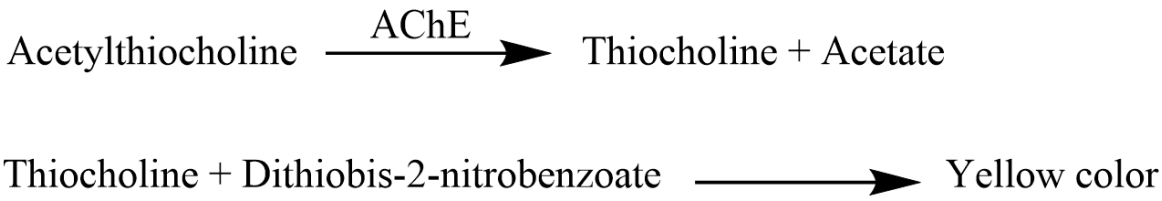

Figure 2.5: The chemical principle for assessing cholinesterase activity based on the Ellman method (Ellman *et al.* 1961).

2.12 Reference compounds used in antioxidant activity assessments.

A reliable reference should satisfy certain requirements, such as being stable, inexpensive, soluble in test solvent and structurally related to the tested compounds (Nenadis *et al.* 2007). A variety of recognized antioxidant compounds were used as standards in this thesis for antioxidant activity assessments, including L-ascorbic acid, α-tocopherol, gallic acid, butylated hydroxyanisole (BHA) and quercetion (Fukumoto and Mazza 2000; Nenadis *et al.* 2007). Ascorbic acid, also known as vitamin C, is six-carbon lactone and a water-soluble antioxidant that functions as a cofactor for multiple enzymes and as powerful radical scavenger (Charlton *et al.* 2023). The phenolic antioxidant vitamin E, also commoly called α-tocopherol, has bicyclic chromanol ring structure with a C_{13} tail that makes it a powerful antioxidant (Charlton *et al.* 2023). Gallic acid (3,4,5-trihydroxybenzoic acid) is a partially hydrophobic secondary polyphenolic metabolite found in plants, making it a natural antioxidant (Charlton *et al.* 2023). The synthetic compound BHA, which consists of two isomers 3-*ter*-butyl-4-metoxyphenol and 2-*tert*-butyl-4-metoxyphenol, is an authorized food preservative, and is used in pharmaceuticals and cosmetics (Sánchez-Gallego *et al.* 2011). The flavonoid quercetin (3,3′,4′,5,7-penthahydroxyflavone) is a prominent and ubiquitous phenolic antioxidant in food (Sánchez-Gallego *et al.* 2011).

2.13 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

This method, established by Blois (1958), was used to assess the antioxidant activity of compounds and their ability to act as free radical scavengers or hydrogen donors (Blois 1958; Kedare and Singh 2011; Moon and Shibamoto 2009). It is a rapid, inexpensive, sensitive, and simple procedure (Blois 1958; Kedare and Singh 2011; Moon and Shibamoto 2009). DPPH is a commercially available stable organic nitrogen radical compound characterized by a deep purple colour and intense absorption at 517 nm (Moon and Shibamoto 2009). This assay is based on the theory that the odd electron of a nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to form hydrazine (Kedare and Singh 2011). The DPPH radical changes from purple to colourless solution after it has absorbed hydrogen from an antioxidant (Figure 2.6)

(Moon and Shibamoto 2009). Therefore, reducing UV absorption at 517 nm can indicate antioxidant activity (Moon and Shibamoto 2009).

The antioxidant capacities of acai berry aqueous and ethanolic extracts over the concentration range of 0.01 - 4000 µg/mL were evaluated by monitoring the ability to reduce the stable free radical di(phenyl)-(2,4,6 trinitrophenyl)iminoazanium (DPPH). DPPH was dissolved in ethanol at a final concentration of 0.1 mM. For a total final volume of 200 µL, 160 µL of DPPH was added to 20 µL of either acai berry extract or L-ascorbic acid, α-tocopherol, or gallic acid as positive control antioxidants, and the material was mixed with 20 µL of distilled water. Antioxidant standards were evaluated using the same concentration range as the acai berry extracts. The mixture was incubated for 40 min in the dark at 37 °C, and then the absorbance was read using a Varioskan™ LUX multimode microplate reader (ThermoFisher, Stafford, UK) at 517 nm as an endpoint measurement. Antioxidant activity was calculated as a percentage of the DPPH radical scavenging activity according to the following equation:

$$\text{DPPH scavenging activity (\%)} = (A0 - A1)/A0 \times 100$$

Where A0 is the absorbance of the control without extract or positive control, and A1 is the absorbance of the sample.

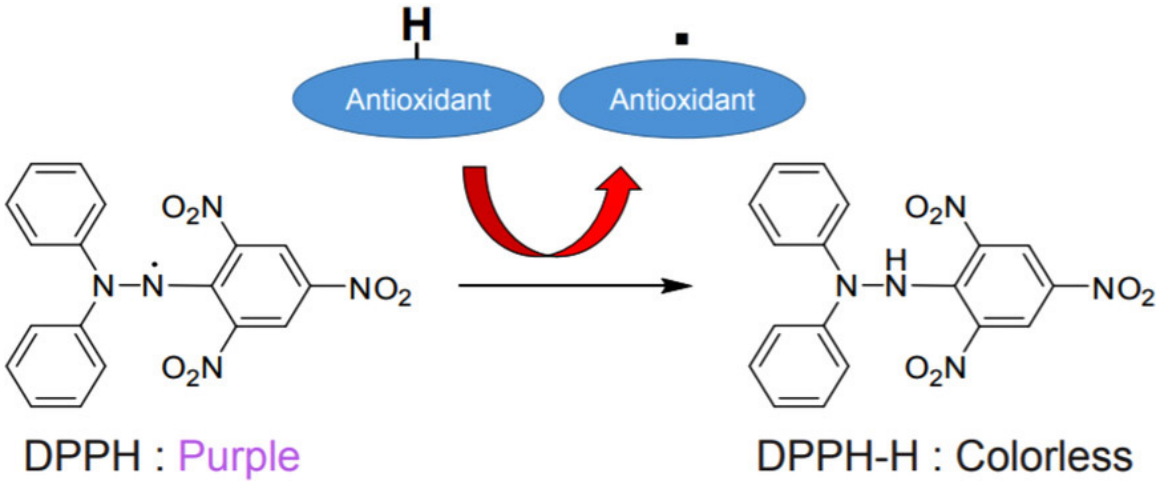

Figure 2.6: The chemical principle for DPPH assay. Formation of DPPH-H by reaction between DPPH˙ radical and antioxidant hydrogen (Xiao *et al.* 2020).

2.14 Radical 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid cation ($ABTS^{\bullet+}$) scavenging activity

This method is used to screen for antioxidative activity of compounds in lipophilic and hydrophilic foods and beverages (Moon and Shibamoto 2009). The principle of this assay is based on the formation of the monocation ABTS radical ($ABTS^{\bullet+}$) via oxidation of ABTS and by losing an electron from its nitrogen atom by potassium persulfate giving the ABTS radical with its bluish-green colour that absorbs at 743 nm (Figure 2.7) (Xiao *et al.* 2020). In the case of adding hydrogen donating antioxidants, nitrogen absorbed hydrogen atoms in the solution, result in solution decolourization and a decrease in absorbance (Xiao *et al.* 2020). Therefore, ABTS decolourization is used to determine the antioxidant activity of natural products by measuring cation radical reduction, which presents as a percentage of $ABTS^{\bullet+}$ scavenging activity (%) (Moon and Shibamoto 2009).

The ability of the acai berry extracts to scavenge $ABTS^{\bullet+}$ was determined according to the procedure of Acharya (2017), with some modifications. Briefly, ABTS (7 mM) and potassium persulfate (2.45 mM) solutions were prepared in distilled water. A working solution was then prepared by combining 3 mL of each stock solution and letting them react for 12 - 16 h in the dark at room temperature (25 °C). The interaction of ABTS with potassium persulfate led to the formation of $ABTS^{\bullet+}$ (Acharya 2017). The solution was then diluted by mixing 1 mL $ABTS^{\bullet+}$ solution with 25 mL of PBS to obtain an absorbance of 0.70 at 750 nm, as monitored using a Varioskan™ LUX multimode microplate reader (ThermoFisher, Stafford, UK). A total of 200 µL reaction mixture per well was assessed, comprised of 190 µL of $ABTS^{\bullet+}$ solution followed by 10 µL of standard (L-ascorbic acid, α-tocopherol, or gallic acid) or acai berry extracts at a concentration range of 1–1000 µg/mL. The plate was shaken for 10 s at medium speed and incubated for 5 min in the dark. Then, the absorbance was measured at 750 nm. The activity of the acai berry extracts was compared with other antioxidant standards, i.e., L-ascorbic acid, α-tocopherol, and gallic acid. The ability of the extracts to scavenge $ABTS^{\bullet+}$ was calculated using the following equation:

$$ABTS^{\bullet+} \text{ scavenging activity (\%)} = (A0 - A1)/A0 \times 100$$

Where A0 is the absorbance of the control, and A1 is the absorbance of the sample.

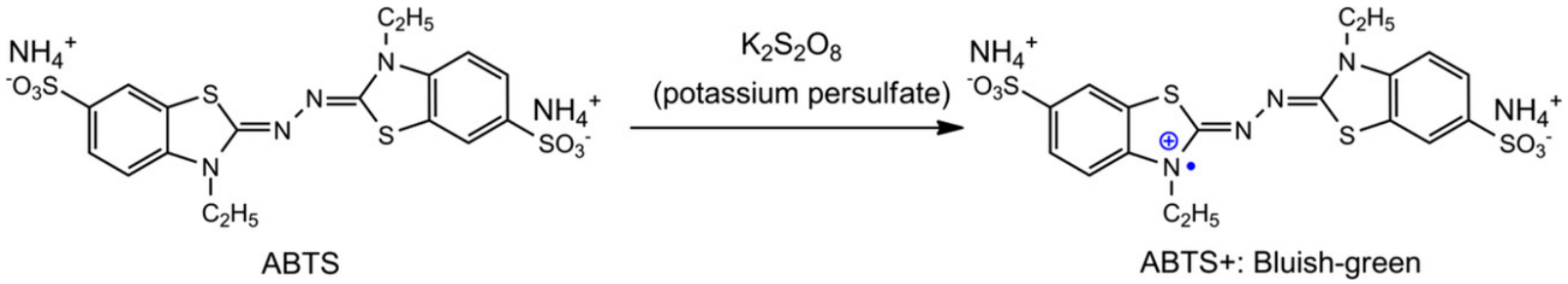

Figure 2.7: The formation of a stable ABTS radical from ABTS and potassium persulfate reaction (Xiao *et al.* 2020).

2.15 Hydrogen peroxide (H_2O_2) scavenging activity

Humans are exposed to H_2O_2 either directly through mitochondrial metabolism or indirectly from the environment (Phaniendra *et al.* 2015). H_2O_2 is widely considered a cytotoxic agent, and the rapid breakdown of H_2O_2 can produce a $^{\bullet}OH$ that can initiate lipid peroxidation and cause protein and DNA damage (Phaniendra *et al.* 2015). This assay is based on the theory that H_2O_2 can naturally absorb UV light at 230 nm, and this feature can be used to determine this compound spectrophotometrically (Apak *et al.* 2022). In the presence of antioxidant radical scavengers, H_2O_2 is neutralized to H_2O via protons (H^+) or electron donation, causing the measured absorbance to drop (Adjimani and Asare 2015; Apak *et al.* 2022). Therefore, the reduction in absorbance measurement reflects the scavenging ability of natural compounds on H_2O_2. Although there is a considerable chance that numerous UV-absorbing organic compounds can interfere with the UV procedure, taking a blank measurement can overcome this problem and produce accurate and precise results (Apak *et al.* 2022).

The H_2O_2 scavenging ability of the acai berry extracts was measured using the method of Alam *et al.* (2013), with modifications. In 200 µL total solution per well of a 96-well plate, 180 µL of 40 mM H_2O_2 solution prepared in PBS was added, followed by 20 µL of standard (α-tocopherol, or gallic acid) or acai berry extracts at a concentration range of 1 - 4000 µg/mL. The mixture was incubated for 10 min, and the absorbance was read at 230 nm using a Varioskan™ LUX multimode microplate reader (ThermoFisher, Stafford, UK). The following equation was used to calculate the percentage of H_2O_2 scavenging:

$$H_2O_2 \text{ scavenging activity (\%)} = (A0 - A1)/A0 \times 100$$

where A0 is the absorbance of the control, and A1 is the absorbance of the sample.

2.16 Hydroxyl radical (˙OH) scavenging activity

Free radicals such as hydroxyls belong to ROS and can react with polyunsaturated fatty acids in cell membrane phospholipids, causing damage to cells (Haida and Hakiman 2019). The principle of this experiment is based on a Fenton's reaction, which involves the Fe^{3+}– ascorbic acid–EDTA–H_2O_2 system to produce hydroxyl radicals (Brizzolari *et al.* 2017). In this assay, Fe^{3+} is reduced to Fe^{2+} by ascorbic acid and produces ˙OH through the "Fenton reaction" with H_2O_2 (Brizzolari *et al.* 2017). In the presence of ˙OH, 2-deoxyribose molecules are degraded, resulting in a mixture that, when heated at acidic pH, generates malondialdehyde (MDA) and other carbonyl reactive species, known as MDA-like products, which can be identified by reacting with 2-thiobarbituric acid (TBA) (Figure 2.8) (Adjimani and Asare 2015; Brizzolari *et al.* 2017; Treml and Šmejkal 2016). A pink chromogen is formed in this reaction, which is spectrophotometrically measured at 532 nm (Adjimani and Asare 2015; Brizzolari *et al.* 2017; Treml and Šmejkal 2016). Antioxidants and other ˙OH scavengers can interfere with the production of MDA and MDA-like compounds because the scavenger competes with sugar for ˙OH (Brizzolari *et al.* 2017). Therefore, the pink colour and the absorbance is reduced reflecting the antioxidant action of compounds.

The ˙OH scavenging activity of acai berry extracts was evaluated using the method described by Bajpai *et al.* (2015), with modifications. In a total volume of 200 µL, the reaction mixture contained 50 µL of 12 mM 2-deoxy2-ribose sugar, 20 µL of 1 mM ferric chloride ($FeCl_3$), 20 µL of 1 mM EDTA, 20 µL of 1 mM L-ascorbic acid, 50 µL of 8 mM H_2O_2, 30 µL of PBS, and 10 µL of standard (gallic acid) or acai berry extracts at a concentration range of 1 - 4000 µg/mL. A volume of 40 µL of 2.8% trichloroacetic acid (TCA) and TBA (0.5% in 0.025 M sodium hydroxide solution) was added to the reaction mixture after 45 min at 37 °C, and the mixture was incubated at 85 °C for 15 min to generate a pink chromogen that resulted from the reaction of TBA with degraded sugar, i.e., a 'malondialdehyde-like' compound (Halliwell and Gutteridge 1981). After cooling, 200 µL of the sample was transferred to a 96-well microtiter plate, and the absorbance was measured at 532 nm using a Varioskan™ LUX multimode microplate reader (ThermoFisher, Stafford, UK). Gallic acid was used as a reference standard. The

percentage of inhibition activity was determined using the same formula as for the DPPH radical scavenging activity (Section 2.13).

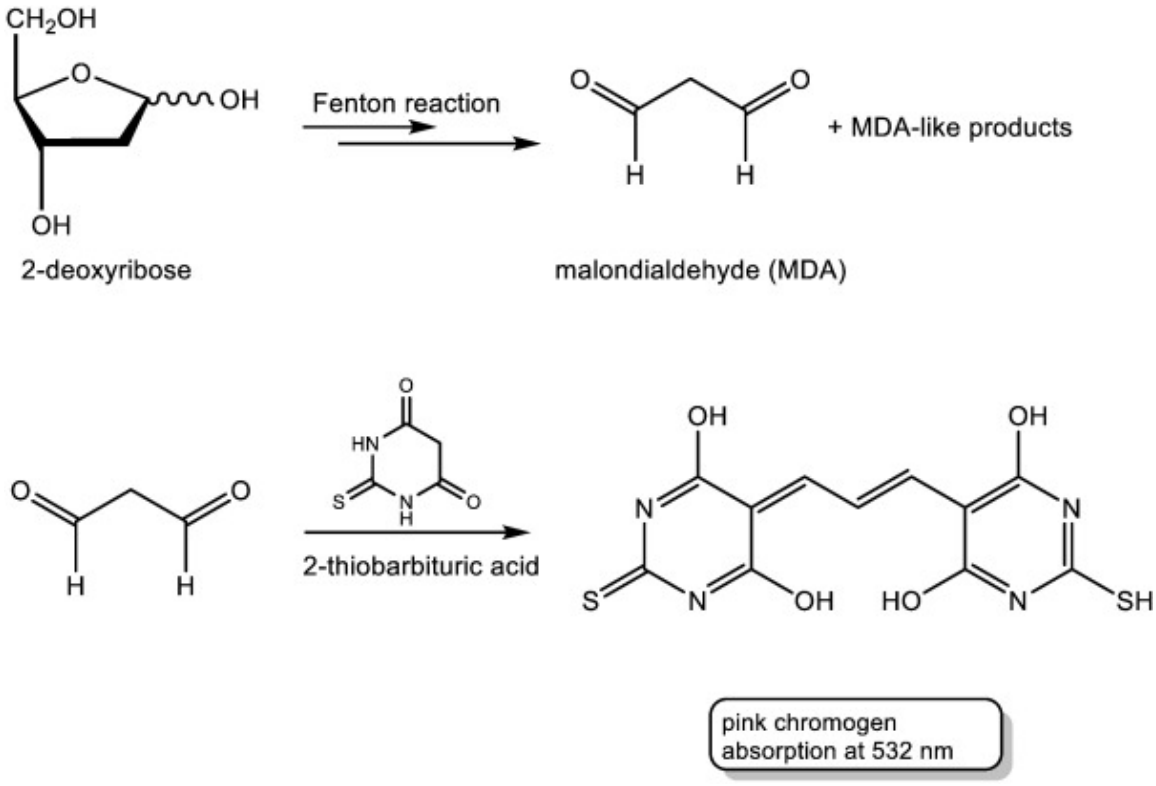

Figure 2.8: The reaction scheme for deoxyribose assays. 2-deoxyribose reacts with the ˙OH radical to yield pink chromogen $(TBA)_2$-MDA (Brizzolari *et al.* 2017).

2.17 Nitric oxide radical (˙NO) scavenging activity

The ˙NO combination with other radicals can produce highly toxic $ONOO^-$ responsible for protein oxidation, nitration, LPO, mitochondrial dysfunction, and cellular death (Radi 2018). The test is based on ˙NO radical production by sodium nitroprusside (SNP) decomposing in an aqueous solution at pH (7.2) (Alam *et al.* 2013; Siddeeg *et al.* 2021). The Griess reagent containing 0.33% sulphanilamide and 0.1% naphthalene diamine dichloride can be used to determine ˙NO quantities under aerobic conditions, as ˙NO reacts with oxygen to produce nitrates (Figure 2.9) (Alam *et al.* 2013; Siddeeg *et al.* 2021). Nitrite ions are reduced when ˙NO scavenging molecules are present, which lowers their spectrophotometric absorbance (Siddeeg *et al.* 2021).

The procedures described by Jimoh *et al.* (2019) and Unuofin *et al.* (2018) were adapted for the determination of the capability of the acai berry extracts to scavenge ˙NO radicals. Briefly, 2 mL of 10 mM SNP in PBS was combined with 0.5 mL of acai berry extracts or BHA at concentrations of 0.1 - 500 µg/mL. After 150 min of incubation at 25 °C, 0.5 mL of the solution was combined with 0.5 mL of Griess reagent, which was prepared by mixing 1 mL of 0.33% sulphanilamide reagent (in 20% glacial acetic acid) and 1 mL of 0.1% naphthalene diamine dichloride at room temperature ~20 °C for 5 min. Following a 30 min incubation period at room temperature, 150 µL of the mixture was transferred to a 96-well plate, and the absorbance was measured at 540 nm using a Varioskan™ LUX multimode microplate reader (ThermoFisher, Stafford, UK). A negative control was prepared using a water-based solution instead of the extract or standard BHA. Using the same formula as the DPPH radical scavenging activity (Section 2.13), the percentage of the nitric oxide scavenging activity was calculated.

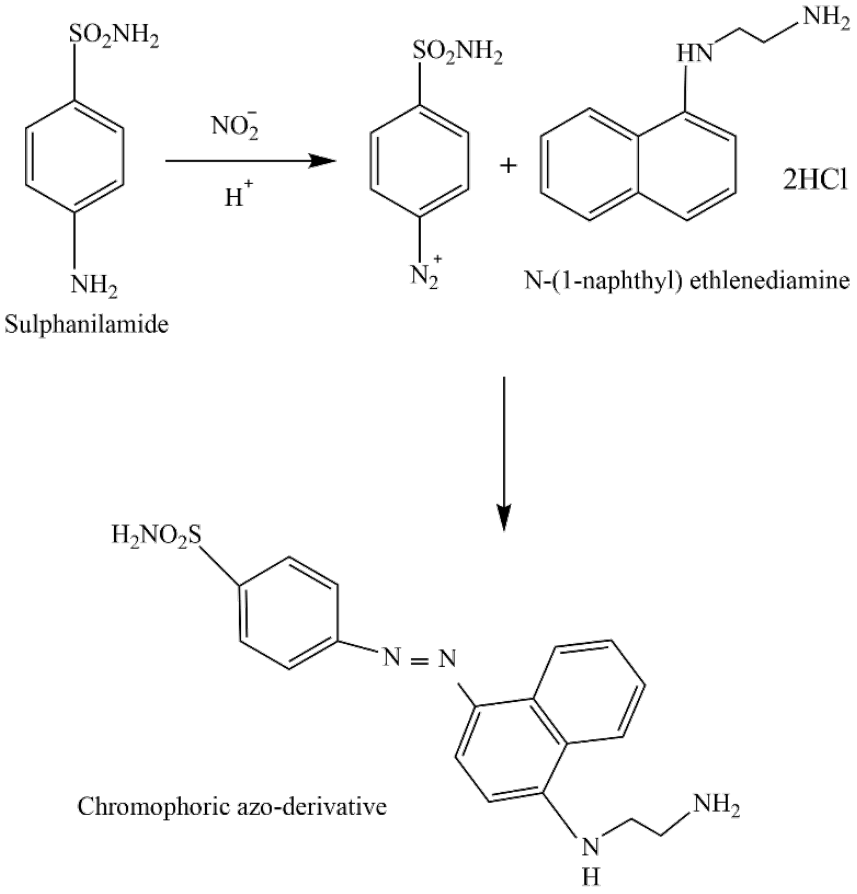

Figure 2.9: The principle of nitric oxide radical scavenging activity assay (Siddeeg *et al.* 2021).

2.18 Lipid peroxidation (LPO) inhibitory activity

A high oxygen consumption rate, significant levels of oxidizable unsaturated lipids, oxidative metals such as iron, and lowered antioxidative defence mechanisms contribute to the brain's vulnerability to oxidative damage (Escames *et al.* 1997; Khan *et al.* 2014). ROS such as $O^{2\bullet-}$ anions, $^{\bullet}OH$, and the H_2O_2 radical trigger LPO, which damages cell membranes and produces numerous secondary products that are neurotoxic, resulting in neuronal death via necrosis or apoptosis (Angelova *et al.* 2021). Moreover, it has been shown that LPO contributes to developing many NDDs, including AD (Angelova *et al.* 2021).

This test relies on SNP decomposing to release $^{\bullet}NO$ at physiological pH~7, which can react with lipid in bovine brain extract and induce LPO (Escames *et al.* 1997; Khan *et al.* 2014). This reaction results in MDA production, a degradative product of LPO and oxidative stress marker (Escames *et al.* 1997; Khan *et al.* 2014). When heated in an acidic solution, MAD combines with TBA to create a unique compound that produces a pink colour (Abeyrathne *et al.* 2021). The colour may be detected using a visible light spectrophotometer at 532 - 535 nm (Abeyrathne *et al.* 2021). The MDA are reduced in the presence of $^{\bullet}NO$ scavenging, thereby reducing spectrophotometric absorbance (Escames *et al.* 1997).

The ability of acai berry extracts to inhibit LPO was assessed using a method modified from that described by Akomolafe *et al.* (2013). Briefly, 100 µL of 5 mg/mL bovine brain extract type I Folch fraction I (Sigma, B1502) was mixed with 30 µL of PBS, 40 µL of distilled water, and 100 µL of acai berry extracts or standard (BHA) at a concentration range of 0.1 - 1000 µg/mL, with 100 µL of 5 mM SNP as the prooxidant. After a 2 h incubation at 37 °C, 300 µL of 8.1% sodium dodecyl sulphate (SDS), 500 µL of acetic acid, and 500 µL of 0.8% TBA were added. This mixture was incubated at 85 °C for 45 min to induce the formation of the MDA coloured product. A volume of 200 µL of the samples was transferred to a 96-well microtiter plate after cooling, and the absorbance was measured at 532 nm using a Varioskan™ LUX multimode microplate reader (ThermoFisher, Stafford, UK). The percentage inhibition of the formation of MDA

was calculated according to the equation for the DPPH radical scavenging activity (Section 2.13).

2.19 Ferric-reducing antioxidant power (FRAP) assay

The bioactive compound reducing power represents its electron-donating capability and can be used to determine its antioxidant activity (Gülçin 2015). The assay theory is based on measuring the capacity of antioxidant compounds to directly reduce Fe^{3+}-ferricyanide complexes $Fe[(CN)_6]_3$ to the ferrous (Fe^{2+}) $Fe[(CN)_6]_2$ form (Figure 2.10) (Gülçin 2015). Depending on the reducing power of the antioxidant, the test solution's yellow colour changes to green or blue in this assay (Gülçin 2015). Thus, the production of Perl's Prussian blue can be used to calculate the amount of reduction, and greater absorbance at 700 nm reflects a more extensive ferric reducing capability (Gülçin 2015).

The ability to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) was used to estimate the reducing capacity of acai berry extracts. The acai berry berry extracts concentrations were assessed over a concentration range of 0.001 - 8000 µg/mL. Each assay data point contained 4 µL of acai berry extracts, 400 µL of phosphate buffer, and 250 µL of 1% potassium ferricyanide. After the incubation of the mixture at 50 °C for 20 min, 250 µL of 10% TCA was added. The samples were centrifuged at 3000 rpm for 10 min. Then, 100 µL of the supernatant was transferred to a 96-well microtiter plate and mixed with 100 µL of double-distilled water and 20 µL of freshly prepared (0.1%) $FeCl_3$ solution. Then, the formation of Perl's Prussian blue was measured at 700 nm, according to Nwidu *et al.* (2018) using a Varioskan™ LUX multimode microplate reader (ThermoFisher, Stafford, UK). The positive control was L-ascorbic acid.

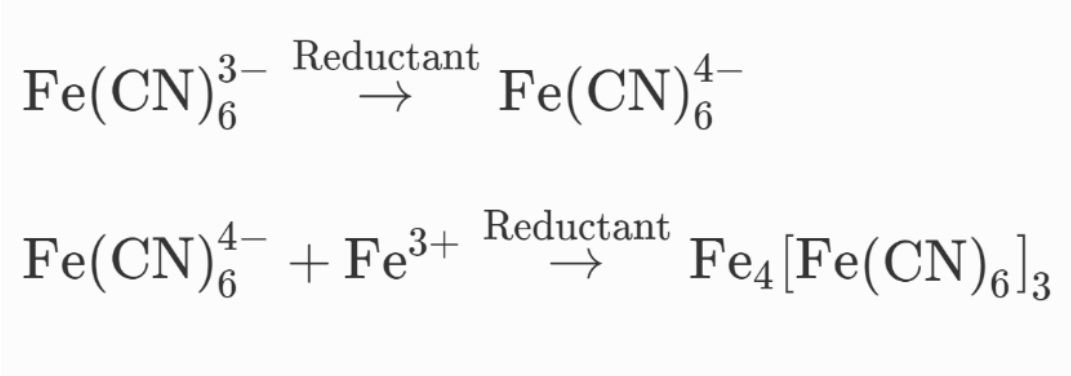

Figure 2.10:The principle of ferric-reducing antioxidant power (FRAP) assay (Gülçin 2015).

2.20 Total phenolic content (TPC) determination

An established method for measuring the total phenolic content in plant-derived foods and biological samples is the Folin–Ciocalteu test (Munteanu and Apetrei 2021). In this assay, electrons are transferred from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes (Folin–Ciocalteu reagent (FCR)) under alkaline conditions, resulting in a detectable blue chromophore with the maximum absorption at 765 nm via spectrophotometer (Figure 2.11) (Ford *et al.* 2019; Munteanu and Apetrei 2021). A phenolic antioxidant donates an electron to the molybdenum ion in the complex, transforming it from Mo^{6+} to Mo^{5+} (Munteanu and Apetrei 2021).

Based on the FCR method, the total phenolic content in acai berry extracts was determined spectrophotometrically according to Nwidu *et al.* (2018). A concentration range of 15.63 - 3000 µg/mL was used to evaluate the acai berry extracts. Each assay data point of total volume 200 µL within a 96-well plate contained 20 µL of acai berry extract, 90 µL of water, and 30 µL of FCR. Then, the mixture was shaken vigorously in a plate reader for 8 min. Then, 60 µL 7.5% sodium carbonate solution was added, and the plate was incubated at 40 °C on a shaking incubator for 30 min. The plate was read in a spectrophotometer at 760 nm using a Varioskan™ LUX multimode microplate reader (ThermoFisher, Stafford, UK). The positive control was gallic acid, using a concentration range of 15.63 - 1000 µg/mL to generate a standard curve for the quantification of the total phenolic content of the acai berry extracts. The total phenolic content was determined as milligram gallic acid equivalents/gram of acai berry extracts (mg GAE/g).

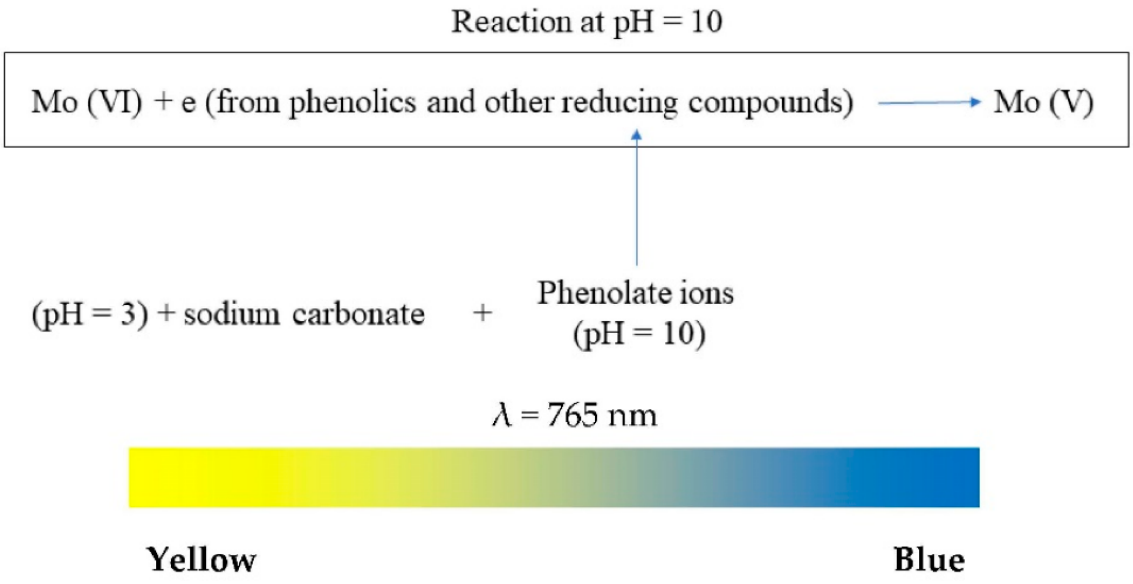

Figure 2.11: The chemical reaction in Folin-Ciocalteu method. A blue colour produces by reacting phenolic compounds with phosphotungstic and phosphomolybdic acids in an alkaline solution (Bibi Sadeer *et al.* 2020).

2.21 Total flavonoid content (TFC) determination

This colourimetric method is based on the principle that aluminium chloride ($AlCl_3$) forms acid-stable complexes with flavone and flavonol C-4 keto groups and their C-3 or C-5 hydroxyl groups (Chang *et al.* 2002). Furthermore, $AlCl_3$ produces acidic compounds with orthodihydroxyl groups in flavonoid A- or B-rings (Figure 2.12) (Chang *et al.* 2002). As a result of this reaction, a complex compound forms with a change in solution colour to yellow, which can be detected spectrophotometrically (Mabry *et al.* 1970). As a standard for flavonoid determination, quercetin is a flavonoid from the flavonol group with the C-4 keto group and C-3 or C-5 hydroxy group (Chang *et al.* 2002).

The total flavonoid contents of the acai berry extracts were assessed via the colourimetric procedure described in Nwidu *et al.* (2018). A total volume 220 µL within a 96-well plate, 20 µL of acai berry extracts or quercetin as standard or positive control, over a concentration range of 15.63 - 3000 µg/mL, was mixed with 100 µL of 10% aluminium chloride solution and 100 µL 1 M potassium acetate. After a 30 min incubation at room temperature, the plate was read using a Varioskan™ LUX multimode microplate reader (ThermoFisher, Stafford, UK) at 415 nm. Total flavonoid content was expressed as milligram quercetin equivalents/gram of extract (mg QUER E/g).

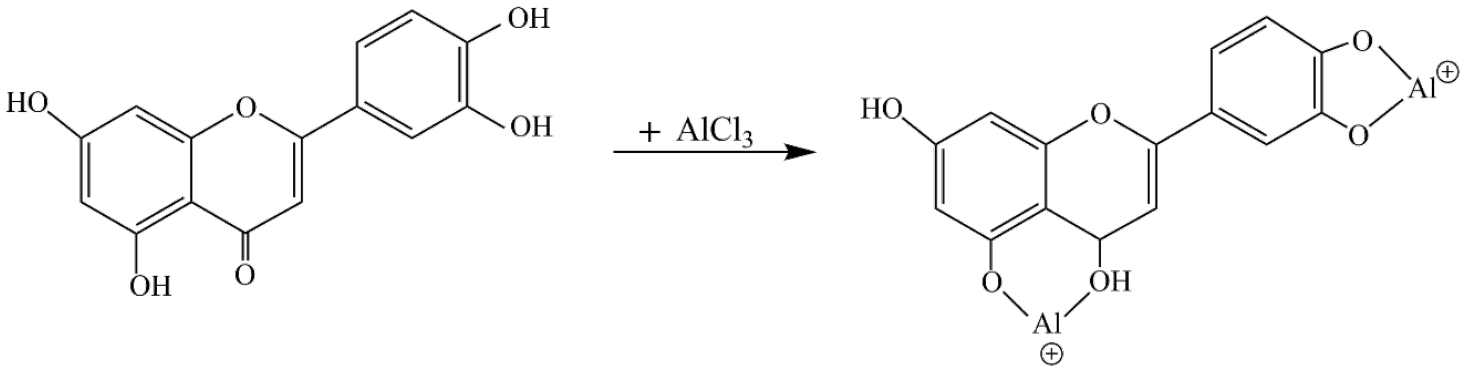

Figure 2.12: The principle of flavonoid content determination assay. Colorimetric determination is based on the formation of complexes between flavonoids and aluminium ($AlCl_3$) (Sepahpour *et al.* 2018).

2.22 Liquid chromatography- mass spectrometry (LC-MS)

LC-MS is a powerful analytical technique combining liquid chromatography (LC) and mass spectrometry (MS) that provides the ability to separate, identify, and quantify unknown and known compounds as well as to study molecule structures and properties (Korfmacher 2005).

Acai berry aqueous and ethanolic extractions were prepared as described in a previous section 2.10, but the initial concentration used was 500 mg/mL. Final products were prepared for analysis in methanol at a concentration range of 10 - 300 mg/mL. Acai berry extracts were analysed using a Dionex UltiMate 3000 high-performance liquid chromatography (HPLC) system coupled to a Q-Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with a heated electrospray ionization (HESI) source (Thermo Fisher Scientific, Hemel Hempstead, UK) as described in (Abdelrazig *et al.* 2020). Injection volumes of 10 µL were used for sample analysis and were maintained at 4°C during the analysis. The samples were injected for chromatographic separation into a ZIC-pHILIC column (150 × 4.6 mm; 5 µm particle size; Merck SeQuant, Darmstadt, Germany) while the temperature was maintained at 45°C. Mobile phase A was composed of 20 mM ammonium carbonate in water (pH 9.1), and mobile phase B was 100% acetonitrile. The initial mobile phase was 20% A with a flow rate of 300 µL/min, which increased to 95% A after 8 min. After 11 min, the flow rate was raised to 400 µL/min, and the mobile phase A proportion was reduced to 20%. The proportion of solvent A was then maintained for re-equilibration, and after 13 - 14 min, the flow rate was decreased again to 300 µL/min.

Full MS profiling with simultaneous ESI+ and ESI– switching was used over the m/z range of 70 - 1050 and at resolution of 70,000. The probe and capillary temperatures were maintained at 150 and 275°C, respectively. The following setups were employed: sheath gas 40, auxiliary gas 5, sweep gas 1, 1 x 10^6 automatic gain control (AGC) target, and maximum injection time 100 ms. The positive and negative modes of ionization used were with a spray voltage of +4 kV or -4 kV, respectively. Parallel reaction monitoring (PRM) data-independent tandem MS/MS spectra were produced on the ions in the

inclusion list, which consisted of all of the compounds of interest in both positive and negative ionization mode at a resolution of 17,500, with AGC target 2×10^5, maximum injection time 100 ms, normalized collision energy 35, isolation window 4.0 m/z, default charge state 1 (Xcalibur v 4.2.28.14, Thermo Fisher Scientific, Hemel Hempstead, UK). The compounds in the acai berry extracts were identified using Compound Discoverer 3.3 software (Thermo Scientific, UK) by matching accurate masses in PlantCyc database, the retention times and accurate masses in the authentic standards (within 0.5 min shift), and MS^2 fragmentation patterns in the mzCloud database. The LC-MS analysis of acai berry extracts was performed by Ayman AlSaadi and Dr Alison Whitby under the supervision of Dr Dong-Hyun Kim in the center for Analytical Bioscience, School of Pharmacy, University of Nottingham.

2.23 Statistical analysis

Results were expressed as means ± standard error of the mean (SEM) in each treatment and control group. Non-linear regression analysis was used to calculate the concentration of L-Glu producing 50% of maximum inhibitory (IC_{50}) effects on cells viability and the concentration of acai berry extracts producing 50% inhibition (IC_{50}) of ChE activity and free radicals. The statistical analysis comparing different groups was performed using one-way ANOVA tests with Tukey's or Dunnett's multiple comparisons post-test via PRISM v7 (GraphPad Software Inc., San Diego, CA, USA. www. graphpad.com). The two-way ANOVA with Tukey's post-hoc analysis was used to evaluate the effect of time (24 hours versus 48 hours) of L-Glu and acai bery extracts exposure in cells and to compare different effects of acai aqueous and ethanolic extracts in ChE activity. A *p*-value of below 0.05 was defined as the level of statistical significance for all analyses.

Chapter 3

L-glutamate (L-Glu) toxicity effects on neuron cells

Chapter 3. Is L-Glutamate toxic to neurons and thereby contributes to neuronal loss and neurodegeneration? A systematic review

3.1 Introduction

Excessive L-Glu levels can contribute to neuronal damage and degradation through different aetiology processes, including oxidative stress, excitotoxicity, mitochondrial dysfunction, inflammation, and protein aggregation, and thereby contribute to the progression of NDDs (Chen *et al.* 2012; Denzer *et al.* 2016; Kovacs 2016; Lewerenz and Maher 2015). It therefore remains critical to brain health that the levels of L-Glu remain optimal. However, exogenous free L-Glu can be introduced to the human body through the diet, for example, as natural foods or food additives (Cynober 2018; Onaolapo *et al.* 2016; Populin *et al.* 2007). Vaccines could also be a source for L-Glu entry into the body (CDC 2018).

In view of this concern regarding the incorporation of excessive exogenous L-Glu, and the potential for accumulation at the synaptic cleft and associated risk to neuronal survival (Niaz *et al.* 2018; Zanfirescu *et al.* 2019), this systematic research was undertaken to provide a comprehensive, unbiased analysis of the effects of L-Glu on neuronal viability and implications for the pathology of NDDs, such as AD, PD, MS, ALS, and HD. Understanding acute L-Glu neurotoxicity may be useful for understanding the molecular mechanisms that drive neuronal loss in NDDs, as well as other diseases associated with L-Glu levels, such as stroke.

3.2 Results

The primary database search resulted in a total of 4043 articles, and then hand searching for relevant papers added a further 17 related papers. After removing duplicates, 2467 papers were then excluded based upon the title and abstract screening. This yielded 864 articles, and these were subjected to full-text assessment.

A total of 793 of these studies were then excluded based on unfulfilled predefined eligibility criteria and for the following reasons: not relevant (n = 94), review (n = 45), animal *in vitro* studies (n = 583), non-English language (n = 4), animal or human neurons in another organ: retina (n = 32), cochlea, heart, liver (n = 5), focus on different neurological diseases (n = 22), predictive data from a virtual experimental system (n = 1), L-Glu mixed with another compound (n = 3), modified or transgenic human neurons, human APP mutation, and senescence by X-irradiation (n = 4). This resulted in a total of 71 articles that met the inclusion criteria. These results are shown as a flowchart detailing the stages of study retrieval and selection based on PRISMA (Figure 3.1). Of the 71 included studies, most were *in vivo* studies (n = 47), while 23 studies were *in vitro*, and only 1 study used mixed methods.

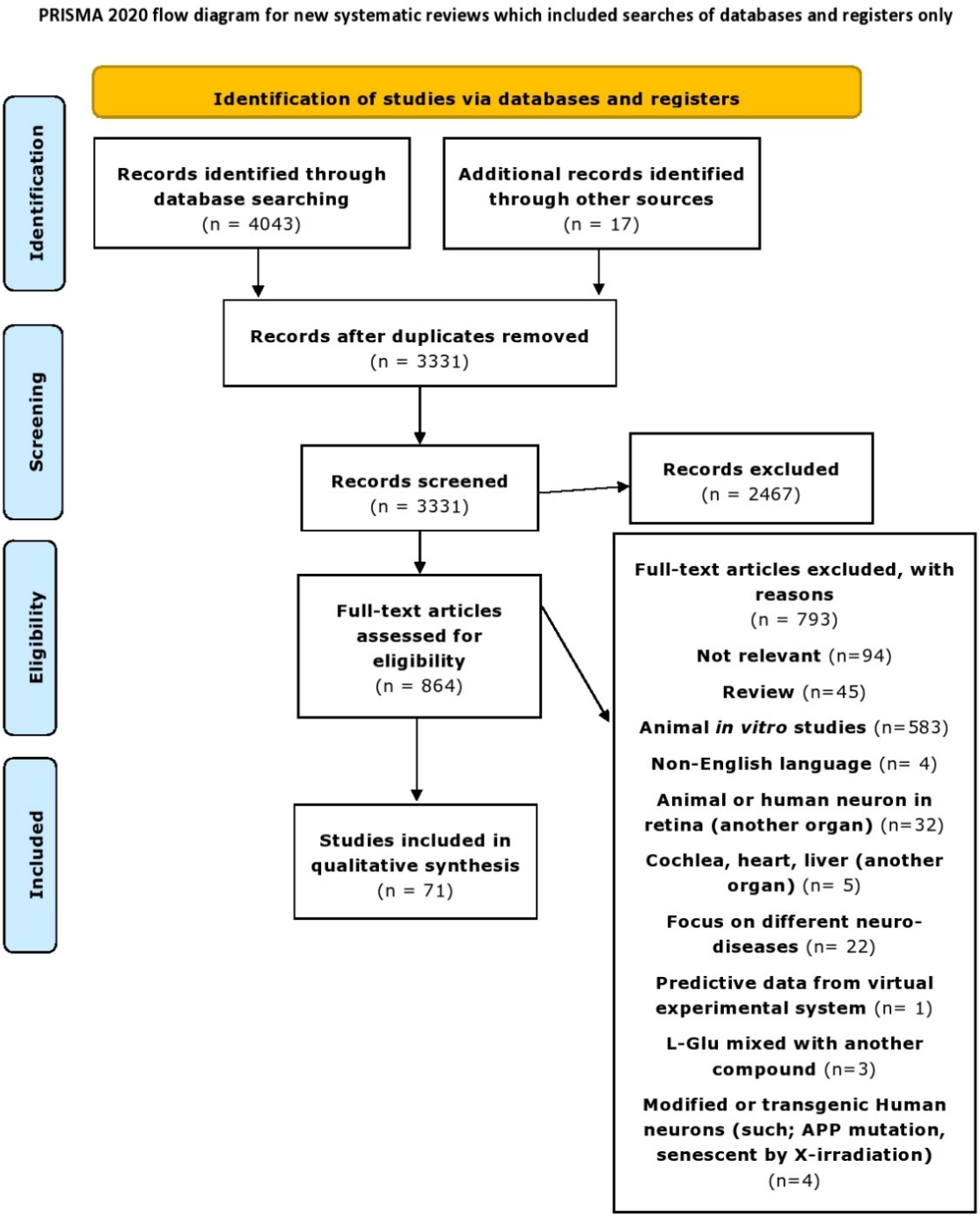

Figure 3.1: Preferred reporting items for systematic reviews and meta-analyses (PRISMA) flowchart illustrating the processes of data collecting and selection (Moher *et al.* 2009)**.**

3.2.1 *In vitro* studies evaluating L-Glu toxicity in human neurons

In vitro studies were only included if they considered a direct effect of L-Glu on human neurons. A number of different neuronal models were considered: human neuroblastoma cells (SH-SY5Y) (n = 15) (Bebitoglu *et al.* 2020b; Bharate *et al.* 2018; Brizi *et al.* 2016; De Oliveira *et al.* 2019; Hu *et al.* 2012; Jeong *et al.* 2014; Lee *et al.* 2019; Li *et al.* 2017a; Nampoothiri *et al.* 2014; Petroni *et al.* 2013; Shah *et al.* 2016; Sun *et al.* 2016; Xin *et al.* 2019; Yang *et al.* 2019b; Yuksel *et al.* 2019; Zhu *et al.* 2016), SK-N-BE (n = 1) (Fallarini *et al.* 2009), IMR-32 (n = 1) (Kataria *et al.* 2012), HCN-1A human cortical neurons (n = 2) (Occhiuto *et al.* 2008; Palumbo *et al.* 2012), human embryonic stem cell (HESC) line H9 (n = 1) (Gupta *et al.* 2013), HB1.F3 human neural stem cells (NSCs) (n = 1) (Yon *et al.* 2018), and primary human fetal brain tissue (14- to 18-week aborted foetuses) (n = 2) (Chao and Hu 1994; de Vera *et al.* 2008), with studies listed in chronological order (Table 3.1). Studies utilised L-Glu directly or as MSG with concentrations ranging from 1.6 μM to 100 mM. L-Glu was prepared by dissolving in culture media directly or as a stock solution in phosphate-buffered saline (PBS) or 1 M hydrogen chloride (HCl) or dimethyl sulfoxide (DMSO). Most of the treatments were for 24 h, although study treatments of 0.5 h to 6 d were also undertaken.

Table 3.1: L-glutamate *in vitro* studies' outcome summary.

Study reference	*In vitro* model	L-Glu treatment and duration	Study outcomes	Level of significance
Hu *et al.* (2012)	SH-SY5Y undifferentiated	8 mM; 0.5 h or 12 h	↓ cell viability	$p < 0.01$
			↑ LDH levels	$p < 0.01$
			Morphological alterations	ND
			↑ apoptosis	ND
			↑ Bax expression	$p < 0.01$
			↓ Bcl-2 expression	$p < 0.01$
Petroni *et al.* (2013)	SH-SY5Y undifferentiated	1 mM; 6 h or 24 h	↓ cell viability	$p < 0.05$
			↑ tau protein phosphorylation	NS
Jeong *et al.* (2014)	SH-SY5Y undifferentiated	0.01 - 6 mM (MSG); 24 h	↓ cell viability (5 and 6 mM)	$p < 0.01$ - 0.001
			Morphological changes: pyknosis, nuclear condensation, and cytoplasmic shrinkage	ND
Nampoothiri *et al.* (2014)	SH-SY5Y undifferentiated	5 - 80 mM; 48 h	↓ cell viability	Undifferentiated $p < 0.001$

	and differentiated			Differentiated
	SH-SY5Y differentiated	20 mM; 48 h	↓ cell viability	$p < 0.05$ - 0.001
			↑ apoptosis	$p < 0.05$
			↓ neurite length	$p < 0.01$
			↑ ROS	$p < 0.001$
Brizi *et al.* (2016)	SH-SY5Y undifferentiated	1 - 100 mM; 24 h	↓ cell viability	$p < 0.001$ ND
			↑ apoptosis (50 mM)	$p < 0.01$ $p < 0.01$, $p < 0.001$
			↓ growth (50 and 80 mM)	ND ND
			↑ ROS (50 mM)	
			Morphological alteration in neurons and nuclear material	
Shah *et al.* (2016)	SH-SY5Y undifferentiated	10 - 30 mM; 3 h 30 mM; 3 h	↓ cell viability	$p < 0.05$ - 0.001
			↑ apoptosis	$p < 0.001$
			↑ p-AMPK protein	$p < 0.001$
			↓ Nrf2 protein	$p < 0.01$
			↓ HO-1 protein	$p < 0.01$
			↑ ROS	

			↑ p-NF-$_k$B protein	$p < 0.001$
			↑ COX-2 protein	$p < 0.001$
			↑ caspase-3 protein	$p < 0.001$
				$p < 0.001$
Sun *et al.* (2016)	SH-SY5Y undifferentiated	10 - 50 mM; 1, 2, 4, 6 or 8 h	↓ cell viability	$p < 0.05$
			↑ apoptosis and necrosis	$p < 0.05$
			↑ Ca^{2+}	$p < 0.05$
			↓ MMP (10 or 15 mM)	$p < 0.05$
			↑ MMP (25 or 50 mM)	$p < 0.05$
			↑ RIP kinase 1 protein	$p < 0.05$
Zhu *et al.* (2016)	SH-SY5Y undifferentiated	5 - 40 mM (MSG); 24 h 20 mM (MSG); 24 h	↓ cell viability	$p < 0.05$ - 0.01
			↑ apoptosis	$p < 0.01$
			↑ ROS	$p < 0.01$
			↑ Bax protein	ND
			↓ Bcl-2 protein	ND
			↓ MMP	$p < 0.01$

			↓ cytosolic cytochrome c protein	ND
			↑ mitochondrial cytochrome c protein	ND
			↑ cleaved caspase-9 protein	ND
			↑ cleaved caspase-3 protein	ND
Li *et al.* (2017a)	SH-SY5Y undifferentiated	10 mM; 24 h	↓ cell viability	ND
			↑ LDH	ND
			↑ ROS	$p < 0.05$
			↑ PCC	$p < 0.05$
			↑ LPO	$p < 0.05$
			↓ SOD	$p < 0.05$
			↓ CAT	$p < 0.05$
			↓ MMP	ND
			↓ ATP	ND
			↑ mitochondrial PCC	ND
			↑ apoptosis	ND
			↑ Bax protein	ND
			↑ cleaved caspase-3 protein	ND

			↓ Bcl-2 protein	ND
			↑ p-MAPKs protein	ND
Bharate *et al.* (2018)	SH-SY5Y differentiated	250 μM; 24 h	↓ cell viability	ND
De Oliveira *et al.* (2019)	SH-SY5Y undifferentiated	10 - 80 mM; 24 h	↓ cell viability (40 - 80 mM)	$p < 0.05$
		80 mM; 24 or 6 h	↑ cleaved PARP level	$p < 0.05$
			↑ DNA fragmentation	$p < 0.05$
			↑ LPO	$p < 0.05$
			↑ protein nitration	$p < 0.05$
			↑ PCC	$p < 0.05$
			↓ protein thiol	$p < 0.05$
			↑ 8-OHdG level	$p < 0.05$
			↓ MMP	$p < 0.05$
			↓ ATP	$p < 0.05$
			↑ MC I and V activities	$p < 0.05$
			↓ mitochondrial enzyme activities	$p < 0.05$
			↑ Bax protein	$p < 0.05$

			↑ cytosolic cytochrome c content	$p < 0.05$
			↓ mitochondrial cytochrome c content	$p < 0.05$
			↑ caspase-9 activity	$p < 0.05$
			↑ caspase-3 activity	$p < 0.05$
			↑ $O_2^{-\bullet}$	$p < 0.05$
			↑ $NO^{\bullet}$	$p < 0.05$
			↑ ROS	$p < 0.05$
Lee *et al.* (2019)	SH-SY5Y undifferentiated	12.5 - 100 mM; 3 h	↓ cell viability	$p < 0.01$
			Nuclear condensation	ND
			↑ DNA fragmentation	$p < 0.01$
	SH-SY5Y differentiated	100 mM; 3 h	↑ AChE activity	$p < 0.01$
	SH-SY5Y undifferentiated		↓ GSH level	$p < 0.01$
			↓ SOD protein	$p < 0.01$
			↓ GPx protein	$p < 0.01$
			↑ pp38 protein	$p < 0.01$
			↑ Bax protein	$p < 0.01$

			↑ cleaved caspase-3 protein	$p < 0.01$
			↑ cleaved PARP protein	$p < 0.01$
			↓ Bcl-2 protein	$p < 0.01$
Xin *et al.* (2019)	SH-SY5Y undifferentiated	0.1 - 100 mM; 12 h	↓ cell viability	ND
			↑ ROS	ND
Yang *et al.* (2019b)	SH-SY5Y undifferentiated	10 mM; 24 h	↓ cell viability	ND
			↑ LDH	ND
			↑ ROS	ND
			↑ LPO	ND
			↓ SOD, GPx activities, and GSH level	ND
			↓ MMP and ATP	ND
			↑ Ca^{2+}	ND
			↑ CHOP, GRP78 proteins, and caspase-4 activity	ND
			↑ NLRP3 protein	ND
			↑ IL-1β and IL-6	ND
			↑ Bax/Bcl-2 ratio	ND

			↑ cleaved caspase-1 and caspase-3 proteins	ND
			↑ p-MAPKs protein	ND
			↑ apoptosis	ND
Yuksel *et al.* (2019)	SH-SY5Y undifferentiated	80 mM (MSG); 24, 48 or 72 h	↑ cell toxicity	ND
			↑ LPO	$p < 0.001$
			↓ SOD activity	$p < 0.05$
			↓ GSH level	$p < 0.001$
			↑ TNF-α	$p < 0.001$
			↑ caspase 3 and caspase 9 mRNA	$p < 0.001$
Bebitoglu *et al.* (2020b)	SH-SY5Y undifferentiated	1 - 50 mM; 3 h or 24 h	↓ cell viability (15-50 mM)	$p < 0.05$ - 0.0001
			↓ CAT	$p < 0.05$
			↓ SOD	NS
			↑ H_2O_2	$p < 0.0001$
			↑ LPO	$p < 0.0001$
			Morphological alteration	ND
Fallarini *et al.* (2009)	Differentiated SK-N-BE(2)	1 mM; 24 h	↑ LDH	$p < 0.01$

			↑ Ca^{2+}	$p < 0.01$
			↑ c-fos and c-jun mRNA	$p < 0.01$
Kataria *et al.* (2012)	Differentiated IMR-32 human neuroblastoma	0.06 - 10 mM; 24 h	Morphological changes, cell shrinkage, and rounding	ND
			↓ cell viability	$p < 0.05$
			↑ LDH	$p < 0.05$
			↓ NF200 mRNA and protein (0.25 and 0.5 mM)	$p < 0.05$
			↑ HSP70 mRNA and protein (0.25 and 0.5 mM)	$p < 0.05$
			↑ PSA-NCAM expression (0.5 mM)	$p < 0.05$
			↑ PST mRNA (0.25 and 0.5 mM)	$p < 0.05$
Occhiuto *et al.* (2008)	Differentiated HCN-1A cell line	0.01 - 5 mM; 24 h	↓ cell viability	ND
		0.1 mM; 24 h	↓ cell viability	$p < 0.01$
			↑ LDH	$p < 0.05$
			Neuron morphological alteration	ND

Palumbo *et al.* (2012)	Differentiated HCN-1A cell line	0.1 mM; 24 h	↑ LDH	$p < 0.05$
			↓ cell viability	$p < 0.05$
			Neuron morphological alteration	ND
			↑ Ca^{2+}	$p < 0.01$
Gupta *et al.* (2013)	Human embryonic stem cell (HESC) line H9	20 - 200 μM; 24 h	↑ neuronal death (20–80 μM)	ND
			↑ Ca^{2+} influx (200 μM)	$p < 0.001$
Yon *et al.* (2018)	Human neural stem cell (NSC) culture HB1.F3	0.8 - 50 mM (MSG); 2 h	↑ LDH	ND
		2.5 mM (MSG); 2 h	↑ LDH	$p < 0.05$
			↑ NF-κB mRNA	$p < 0.05$
			↑ TNF-α mRNA	$p < 0.05$
			↑ IL-6 mRNA	$p < 0.05$
			↑ iNOS mRNA and protein	$p < 0.05$
			↑ COX-2 mRNA and protein	$p < 0.05$
			↑ TGF-β protein	$p < 0.05$
			↑ HMGB1 protein	$p < 0.05$

Chao and Hu (1994)	Human fetal brain tissue	1.6 - 5000 µM; 6 d	↑ LDH	ND
			↓ GABA uptake (as marker of GABAergic neuron integrity)	ND
de Vera *et al.* (2008)	Human fetal cortical brain tissue 14–42 DIV	1 - 10 mM; 24 h	↑ neuronal death (5 mM at 26, 32, and 42 DIV)	$p < 0.05$
			↑ swelling of astrocyte nuclei (5 mM at 32 DIV)	$p < 0.05$

Abbreviations: AChE, acetylcholinesterase; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ATP, adenosine triphosphate; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2; Ca^{2+}, calcium ions; CAT, catalase; CHOP, CCAAT/enhancer-binding protein homologous protein; COX-2, cyclooxygenase-2; DIV, day *in vitro*; DNA, deoxyribonucleic acid; FJB, fluoro jade B; GPx, glutathione peroxidase; GRP78, glucose regulatory protein 78; GSH, glutathione; *GSSG,* oxidised glutathione; GST, glutathione-S-transferase; GABA, gamma-aminobutyric acid; HCN-1A line, human cortical neuronal cells; HMGB1, high-mobility group box 1; HO-1, heme oxygenase-1; H_2O_2, hydrogen peroxide; HSP70, 70 kDa heat shock protein; IL-1β, interleukin 1β; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; L-Glu, L-glutamate; LPO, lipid peroxidation; MC I, mitochondrial complex I; MC V, mitochondrial complex V; MMP, mitochondrial membrane potential; mRNA, messenger RNA; MSG, monosodium glutamate; ND, not determined; NF200, neurofilament protein 200; NLRP3*,* Nod-like receptor protein 3; NMDAR, N-methyl-D-aspartate receptor; NO^-, nitrite; $NO^\bullet$, nitric oxide radical; Nrf2, nuclear factor erythroid 2-related factor; NS, not significant; $O_2^{-\bullet}$, superoxide radical; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; p38 MAPK, p38 mitogen-activated protein kinase; p-AMPK, phosphorylated AMP-activated protein kinase; PARP, poly (adenosine diphosphate (ADP)-ribose) polymerase; PCC, protein carbonyl content; p-MAPKs, phosphorylated mitogen-activated protein kinases; p-NF-$_k$B, phosphorylated nuclear factor kappa-light-chain-enhancer of activated B cells; pp38, phosphorylated-p38 mitogen-activated protein kinase; PSA-NCAM, polysialylated neural cell adhesion molecule; PST, polysialyltransferase; RIP kinase 1, receptor-interacting protein kinase 1; ROS, reactive oxygen species; SH-SY5Y, neuroblastoma cell line; SK-N-BE(2), human neuroblastoma cell lines; SOD, superoxide dismutase; TGF-β, transforming growth factor beta; TNF-α, tumour necrosis factor-α. Note: Malondialdehyde (MDA) level used as an index of lipid peroxidation (LPO).

3.2.1.1 L-Glu exposure reduces neuronal viability

L-Glu effects on neuronal cell proliferation, viability, and cytotoxicity utilised several assay types that measured cellular metabolic activity, cytolysis, DNA fragmentation, the release of structural proteins, and stress markers, along with cell death via apoptosis or necrosis. L-Glu administration to differentiated and undifferentiated SH-SY5Y cells resulted in decreased cell viability in a concentration-dependent manner (over the concentration range of 5, 10, 20, 40, and 80 mM), with undifferentiated cells more vulnerable to L-Glu exposure (Nampoothiri *et al.* 2014). The lowest concentrations shown to induce neurotoxicity were 250 µM L-Glu for differentiated SH-SY5Y cells (Bharate *et al.* 2018), whereas for undifferentiated SH-SY5Y cells, 12.5 - 100 mM L-Glu for 3 h still resulted in a significant concentration-dependent reduction of cell viability (Bebitoglu *et al.* 2020b; Lee *et al.* 2019; Shah *et al.* 2016). Similarly, an 8 h incubation of 15–25 mM L-Glu to undifferentiated SH-SY5Y cells caused a significant reduction in cell viability (Sun *et al.* 2016). Reduced cell viability was similarly observed for undifferentiated SH-SY5Y cells after exposure to L-Glu at concentrations from 1 to 100 mM for 12 or 24 h (Bebitoglu *et al.* 2020b; Brizi *et al.* 2016; Hu *et al.* 2012; Jeong *et al.* 2014; Li *et al.* 2017a; Petroni *et al.* 2013; Xin *et al.* 2019; Yang *et al.* 2019b; Zhu *et al.* 2016). However, by contrast, undifferentiated SH-SY5Y cells were not affected at concentrations lower than 40 mM L-Glu for 24 h, according to a study by De Oliveira *et al.* (2019).

L-Glu induced a loss of cell viability that reflected the length of exposure time from 2 to 24 h (Jeong *et al.* 2014; Sun *et al.* 2016), but toxicity was greater after 24 h rather than 48 or 72 h (Yuksel *et al.* 2019). Human fetal neurons displayed progressive loss throughout 6 days after L-Glu, as evidenced by microscopic examination (Chao and Hu 1994). For other primary fetal cortical neurons and stem cells, a significant reduction of cell viability was only observed in older cultures (de Vera *et al.* 2008; Gupta *et al.* 2013), as well as in HCN-1A and IMR-32 cell lines for which a

significant loss of viability was observed after a 24 h incubation (Kataria *et al.* 2012; Palumbo *et al.* 2012).

L-Glu caused a loss of neuronal membrane integrity and release of cytosolic lactate dehydrogenase (LDH) into the cell culture medium as an alternative means to quantify cell viability. L-Glu at a concentration range of 0.06 to 10 mM significantly increased LDH release in primary or neuronal cell lines (Chao and Hu 1994; Fallarini *et al.* 2009; Hu *et al.* 2012; Kataria *et al.* 2012; Li *et al.* 2017a; Palumbo *et al.* 2012; Yang *et al.* 2019b). An L-Glu concentration range from 0.8 to 50 mM applied to human neural stem cells caused significant LDH leakage in a concentration-dependent way and was maximal at 12.5 mM, indicative of saturation of cytotoxicity (Yon *et al.* 2018).

Another marker of L-Glu toxicity was DNA fragmentation, and this increased after 80 mM L-Glu administration for 24 h to SH-SY5Y cells (De Oliveira *et al.* 2019).

L-Glu exposure resulted in structural damage, such as reduced expression of neurofilament 200 (NF200) protein and a marker of plasticity, polysialylated neural cell adhesion molecule (PSA-NCAM) (Kataria *et al.* 2012). L-Glu also triggered the expression of endoplasmic reticulum (ER) stress markers and other stress response proteins. The expression level of ER stress-related proteins, such as CCAAT/enhancer-binding protein homologous protein (CHOP), glucose regulatory protein 78 (GRP78), and caspase-4, was significantly increased after 10 mM L-Glu addition to SH-SY5Y cells for 24 h (Yang *et al.* 2019b). Additionally, the stress signal 70 kDa heat shock protein (HSP70) was elevated following 0.25 and 0.5 mM L-Glu addition to IMR-32 cells (Kataria *et al.* 2012).

L-Glu induced cell apoptosis at a concentration of 8 (Hu *et al.* 2012), 10 (Li *et al.* 2017a; Yang *et al.* 2019b), 20 (Nampoothiri *et al.* 2014; Zhu *et al.* 2016), 25 (Sun *et al.* 2016), 30 (Shah *et al.* 2016), 50, and 80 mM (Brizi *et al.* 2016). However, one study reported that no cellular apoptotic response was observed in response to 5- and

10-mM L-Glu treatment (de Vera *et al.* 2008). L-Glu-induced apoptosis was associated with an elevation of the expression of c-fos and c-jun genes in SH-SY5Y cells (Fallarini *et al.* 2009).

Exposure to L-Glu at 20 and 50 mM caused an increase in the percentage of necrotic neurons and upregulated the expression of the key signalling molecule, necrosis receptor-interacting protein (RIP) kinase 1, but not RIP kinase 3 (Sun *et al.* 2016).

3.2.1.2 L-Glu exposure impairs cellular oxidant defence and stimulates oxidative stress

Five cell-based studies reported that L-Glu exposure impaired the endogenous antioxidant defence system. Relatively high concentrations of L-Glu (above 10 mM) caused significantly decreased activities of superoxide dismutase (SOD) and CAT, and depleted cellular GSH (Lee *et al.* 2019; Li *et al.* 2017a; Yang *et al.* 2019b; Yuksel *et al.* 2019). However, one study reported that although 15 mM L-Glu significantly reduced CAT activity, there was only a slight and nonsignificant decrease in SOD activity (Bebitoglu *et al.* 2020b). Research using a human neuron model reported that L-Glu exposure at 10, 20, and 30 mM for 3 h also significantly impaired the expression of the antioxidant defence Nrf2/HO-1 (nuclear factor erythroid 2-related factor-(Nrf2-)/heme oxygenase-1 (HO-1)) axis (Shah *et al.* 2016).

At pathological concentrations of L-Glu (≥10 mM), there was also an induction of cellular oxidative stress. Eight studies reported a significant rise in the production of ROS (Brizi *et al.* 2016; De Oliveira *et al.* 2019; Li *et al.* 2017a; Nampoothiri *et al.* 2014; Shah *et al.* 2016; Xin *et al.* 2019; Yang *et al.* 2019b; Zhu *et al.* 2016). Hydrogen peroxide (H_2O_2) levels also rose after L-Glu application (Bebitoglu *et al.* 2020b), and plasma levels of malondialdehyde (MDA), an indicator of LPO, were raised after 10, 15, and 80 mM L-Glu treatments (Bebitoglu *et al.* 2020b; De Oliveira *et al.* 2019; Li *et al.* 2017a; Yang *et al.* 2019b; Yuksel *et al.* 2019). Incubation with 10

and 80 mM L-Glu induced protein oxidation, evidenced as increased levels of protein carbonyl content (PCC) (De Oliveira *et al.* 2019; Li *et al.* 2017a). Furthermore, L-Glu-induced protein oxidation was detected via depletion of protein thiols and increased protein nitration or 3-nitrotyrosine content (De Oliveira *et al.* 2019). DNA oxidative damage was also evident as increased 8-hydroxy-2′-deoxyguanosine (8-OHdG) content (De Oliveira *et al.* 2019).

3.2.1.3 L-Glu enhances acetylcholinesterase (AChE) activity

A single study investigated the *in vitro* effects of L-Glu on AChE activity in differentiated SH-SY5Y cells, and this increased significantly after a 100 mM exposure for 3 h (Lee *et al.* 2019).

3.2.1.4 L-Glu exposure triggers mitochondria dysfunction and neuronal apoptosis

L-Glu administration at concentrations of 8, 10, 20, 30, 80, and 100 mM resulted in mitochondria impairment and increased mitochondria-related apoptotic factors, such as cleaved poly (adenosine diphosphate (ADP)-ribose) polymerase (PARP) (De Oliveira *et al.* 2019; Lee *et al.* 2019), caspase-3 (De Oliveira *et al.* 2019; Lee *et al.* 2019; Li *et al.* 2017a; Shah *et al.* 2016; Yang *et al.* 2019b; Yuksel *et al.* 2019), caspase-9 (De Oliveira *et al.* 2019; Yuksel *et al.* 2019; Zhu *et al.* 2016), and Bcl-2-associated X protein (Bax) (De Oliveira *et al.* 2019; Hu *et al.* 2012; Lee *et al.* 2019; Li *et al.* 2017a; Zhu *et al.* 2016). L-Glu also triggered the downregulation of antiapoptotic B-cell lymphoma-2 (Bcl-2) expression (Hu *et al.* 2012; Lee *et al.* 2019; Li *et al.* 2017a; Zhu *et al.* 2016), upregulation of the Bax/Bcl-2 ratio (Yang *et al.* 2019b), and release of other proapoptotic proteins, such as cytochrome c (De Oliveira *et al.* 2019; Zhu *et al.* 2016).

Levels of phosphorylated mitogen-activated protein kinase (MAPK) forms: p38 (Lee *et al.* 2019; Li *et al.* 2017a; Yang *et al.* 2019b), p54 (c-jun N-terminal kinase) (JNK)

(Li *et al.* 2017a; Yang *et al.* 2019b), and p42/44 extracellular signal-regulated kinases (ERKs) (Li *et al.* 2017a) were upregulated after L-Glu exposures, contributing to the activation of neuronal apoptosis.

The activity of the mitochondrial enzymes aconitase, α-ketoglutarate dehydrogenase (α-KGDH), succinate dehydrogenase (SDH), complex I (NADH dehydrogenase), and complex V was significantly reduced after exposure to L-Glu at 80 mM (De Oliveira *et al.* 2019). ATP levels declined after L-Glu exposure (De Oliveira *et al.* 2019; Li *et al.* 2017a; Yang *et al.* 2019b), as did the mitochondrial membrane potential (MMP) (De Oliveira *et al.* 2019; Li *et al.* 2017a; Sun *et al.* 2016; Yang *et al.* 2019b; Zhu *et al.* 2016). However, one study reported a heightened MMP at L-Glu concentrations of 25 or 50 mM, and this may contribute to neuronal cell death (Sun *et al.* 2016). Mitochondrial protein carbonyl content and 5′ AMP-activated protein kinase (AMPK) activity were enhanced due to L-Glu exposure, indicative of oxidative stress and apoptosis, respectively (Li *et al.* 2017a; Shah *et al.* 2016).

3.2.1.5 L-Glu exposure stimulates excitotoxicity and alters neuronal calcium levels

Six *in vitro* studies considered the role of L-Glu in excitotoxicity via its action as an excitatory neurotransmitter to damage neurons through overactivation of its receptors. After 24 h exposure to 1 and 0.1 mM L-Glu, there was an excessively high intracellular accumulation of Ca^{2+} (Fallarini *et al.* 2009; Palumbo *et al.* 2012). Similarly, there was a concentration-dependent elevation of intracellular Ca^{2+} concentration after exposure to L-Glu (15-25 mM) for 1 h, but this was not statistically significant at concentrations of 10 and 50 mM (Sun *et al.* 2016). Ca^{2+} ion influx into neurons was significantly increased at 10 mM L-Glu (Yang *et al.* 2019b). Likewise, 6- and 8-week-old cultures of human embryonic stem cell (HESC)-derived neurons developed increasing Ca^{2+} influx in response to extracellular L-Glu application, in contrast to early week neurons, which were unresponsive to L-Glu

(Gupta *et al.* 2013). These 8-week cultures had increased expression of NMDA and AMPA receptor subunits (Gupta *et al.* 2013). A functional assessment of GABAergic neurons displayed concentration-dependent decreases in ^{3}H-GABA uptake in response to exposure to L-Glu (1.6 to 5000 μM) for 6 days (Chao and Hu 1994).

3.2.1.6 L-Glu exposure triggers neuroinflammation

Five cell-based studies suggested an association of L-Glu with neuroinflammation. There was increased expression and activity of early matrix metalloproteinases (MMPs) following L-Glu (Kataria *et al.* 2012). Activation of proinflammatory markers, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and COX-2, was upregulated after 30 and 2.5 mM L-Glu exposures, respectively (Shah *et al.* 2016; Yon *et al.* 2018). In addition, iNOS was increased following exposure to 2.5 mM L-Glu (Yon *et al.* 2018). There was a marked increase in Nod-like receptor protein 3 (NLRP3) protein inflammasomes in response to L-Glu stimulation (Yang *et al.* 2019b). At 2.5 and 10 mM L-Glu, interleukin 6 (IL-6) was induced (Yang *et al.* 2019b; Yon *et al.* 2018). TNF-α was increased at L-Glu concentrations of 2.5 and 80 mM (Yon *et al.* 2018; Yuksel *et al.* 2019) and interleukin 1β (IL-1β) at 10 mM L-Glu (Yang *et al.* 2019b). Other cytokine mediators of inflammation, such as transforming growth factor-β (TGF-β) and high-mobility group box 1 (HMGB1), were significantly increased in response to L-Glu (Yon *et al.* 2018).

3.2.1.7 L-Glu exposure affects the morphological characteristics of neurons

Nine studies reported morphological alterations to neurons following L-Glu administration, with alterations to nuclear size, chromatin condensation, and nuclear fragmentation (Bebitoglu *et al.* 2020b; Brizi *et al.* 2016; de Vera *et al.* 2008; Hu *et al.* 2012; Jeong *et al.* 2014; Lee *et al.* 2019), as well as signs of toxicity, particularly as cell body shrinkage and dendritic retraction (Brizi *et al.* 2016; Hu *et al.* 2012; Jeong *et al.* 2014; Kataria *et al.* 2012; Nampoothiri *et al.* 2014; Palumbo *et al.* 2012).

3.2.1.8 Protein aggregation

One article that investigated the relation between L-Glu treatment and toxic protein aggregation reported that SH-SY5Y exposed to 1 mM for 6 h resulted in a build-up of tau protein phosphorylation, but this was not statistically significant (Petroni *et al.* 2013).

3.2.2 *In vivo* studies evaluating L-Glu toxicity

In vivo (animal) data focused on the direct impact of L-Glu on neuronal molecular processes that resulted in disease, such NDDs. Different animal models were used to evaluate L-Glu neurotoxicity, including albino Wistar rats (n = 22) (Abdel Moneim *et al.* 2018; Babu *et al.* 1994; Burde *et al.* 1971; Chaparro-Huerta *et al.* 2005; Chaparro-Huerta *et al.* 2008; Del Río and Massieu 2008; Dief *et al.* 2014; Ferger *et al.* 1998; Fouad *et al.* 2018; Hashem *et al.* 2012; Hazzaa *et al.* 2020; Hussein *et al.* 2017; Khalil and Khedr 2016; Mejía-Toiber *et al.* 2006; Rivera-Cervantes *et al.* 2004; Rivera-Cervantes *et al.* 2015; Sadek *et al.* 2016; Segura Torres *et al.* 2006; Shivasharan *et al.* 2013; Singh *et al.* 2003; Swamy *et al.* 2013; Thonda *et al.* 2014), Sprague–Dawley rats (n = 13) (Babu *et al.* 2011; Bodnár *et al.* 2001; Calis *et al.* 2016; Firgany and Sarhan 2020; Greene and Greenamyre 1995; Kim *et al.* 2013; Kumar and Babu 2010; Kumar *et al.* 2010; Morales and Rodriguez 2012; Shah *et al.* 2015; Shah *et al.* 2016; Yang *et al.* 1998; Yang *et al.* 2017), other rats (n = 1) (Hamza *et al.* 2019), Swiss albino mice (n = 3) (Burde *et al.* 1971; Mohan *et al.* 2017; Onaolapo *et al.* 2016), wild-type mice (n = 5) (Estrada-Sánchez *et al.* 2009; Estrada-Sánchez *et al.* 2010; Guemez-Gamboa *et al.* 2011; Wang *et al.* 2015; Zou *et al.* 2016), Kunming mice (n = 2) (Ma *et al.* 2007; Yu *et al.* 2006), CD-1 mice (n = 1) (Penugonda and Ercal 2011), *Caenorhabditis elegans* nematodes (n = 1) (Chu *et al.* 2020), and ephyrae of *Aurelia aurita* (n = 1) (Spangenberg *et al.* 2004). Moreover, there were different routes of L-Glu administration in these *in vivo* studies: orally (*p.o.*) (n = 10) (Abdel Moneim *et al.*

2018; Burde *et al.* 1971; Dief *et al.* 2014; Hamza *et al.* 2019; Hashem *et al.* 2012; Hussein *et al.* 2017; Khalil and Khedr 2016; Mohan *et al.* 2017; Onaolapo *et al.* 2016; Penugonda and Ercal 2011), subcutaneous (*s.c.*) injection (n = 12) (Babu *et al.* 1994; Bodnár *et al.* 2001; Burde *et al.* 1971; Chaparro-Huerta *et al.* 2005; Chaparro-Huerta *et al.* 2008; Dief *et al.* 2014; Firgany and Sarhan 2020; Rivera-Cervantes *et al.* 2004; Rivera-Cervantes *et al.* 2015; Sadek *et al.* 2016; Segura Torres *et al.* 2006; Shah *et al.* 2015), intraperitoneal (*i.p.*) injection (n = 8) (Calis *et al.* 2016; Fouad *et al.* 2018; Hazzaa *et al.* 2020; Shah *et al.* 2016; Shivasharan *et al.* 2013; Singh *et al.* 2003; Swamy *et al.* 2013; Thonda *et al.* 2014), maternal intragastric (*i.g.*) (n = 2) (Ma *et al.* 2007; Yu *et al.* 2006), stereotactic injection via intrastriatal or cerebral cortex injection (n = 12) (Babu *et al.* 2011; Del Río and Massieu 2008; Estrada-Sánchez *et al.* 2009; Estrada-Sánchez *et al.* 2010; Greene and Greenamyre 1995; Guemez-Gamboa *et al.* 2011; Kim *et al.* 2013; Kumar and Babu 2010; Kumar *et al.* 2010; Mejía-Toiber *et al.* 2006; Yang *et al.* 2017; Zou *et al.* 2016), microdialysis (n = 3) (Ferger *et al.* 1998; Morales and Rodriguez 2012; Yang *et al.* 1998), brain infusion via a cannula (n = 1) (Wang *et al.* 2015), and also L-Glu to animals via living media (n = 2) (Chu *et al.* 2020; Spangenberg *et al.* 2004). Furthermore, the data from current research found that different parts of the nervous system were examined to evaluate L-Glu neurotoxicity. The number of studies used the whole of brain homogenates (Bodnár *et al.* 2001; Calis *et al.* 2016; Fouad *et al.* 2018; Hazzaa *et al.* 2020; Penugonda and Ercal 2011; Sadek *et al.* 2016; Shivasharan *et al.* 2013; Swamy *et al.* 2013), cerebral cortex (Babu *et al.* 2011; Chaparro-Huerta *et al.* 2008; Dief *et al.* 2014; Estrada-Sánchez *et al.* 2009; Hamza *et al.* 2019; Hashem *et al.* 2012; Hussein *et al.* 2017; Kim *et al.* 2013; Kumar and Babu 2010; Kumar *et al.* 2010; Mohan *et al.* 2017; Rivera-Cervantes *et al.* 2004; Segura Torres *et al.* 2006; Thonda *et al.* 2014; Yang *et al.* 1998; Yang *et al.* 2017; Zou *et al.* 2016), hippocampus (Chaparro-Huerta *et al.* 2005; Dief *et al.* 2014; Hazzaa *et al.* 2020; Khalil and Khedr 2016; Ma *et al.* 2007; Onaolapo *et al.* 2016; Rivera-Cervantes *et al.* 2015; Shah *et al.* 2015; Shah *et*

al. 2016; Shivasharan *et al.* 2013; Swamy *et al.* 2013; Thonda *et al.* 2014; Yu *et al.* 2006), striatum (Chaparro-Huerta *et al.* 2008; Del Río and Massieu 2008; Estrada-Sánchez *et al.* 2009; Estrada-Sánchez *et al.* 2010; Ferger *et al.* 1998; Greene and Greenamyre 1995; Guemez-Gamboa *et al.* 2011; Mejía-Toiber *et al.* 2006; Morales and Rodriguez 2012), cerebellum (Singh *et al.* 2003; Thonda *et al.* 2014), forebrains (Abdel Moneim *et al.* 2018), hypothalamus (Babu *et al.* 1994; Burde *et al.* 1971), circumventricular organs (Babu *et al.* 1994), spinal cord (Firgany and Sarhan 2020; Wang *et al.* 2015), cerebral hemisphere, brain stem, diencephalon (Singh *et al.* 2003), pituitary gland (Bodnár *et al.* 2001), neural circuits (Chu *et al.* 2020), and rhopalia (Spangenberg *et al.* 2004). Table 3.2 shows the results of *in vivo* research classified based on the animal's species and strain.

Table 3.2: L-glutamate *in vivo* studies' outcome summary.

Reference	Species and Strain, Size of L-Glu Treatment Group	L-Glu Treatment and Duration (Dose, Route of Drug Application)	Study Outcomes	Level of Significance
Babu *et al.* (1994)	Wistar rats (n = 6)	MSG; 4 mg/g, *s.c.*; 10 days postnatally (PD 1 - 10)	↑ LPO ↑ CAT activity ↓ sulfhydryl levels	$p < 0.01$ $p < 0.025$ $p < 0.05$
Ferger *et al.* (1998)	Albino Wistar rats (n = 5) adults male (350 g)	L-Glu 50 mM, striatum microdialysis	↑ $OH^{\bullet}$	ND
Singh *et al.* (2003)	Wistar rats (n = 3) male (160–180 g) 3 - 4 months old	MSG 4 mg/g/day, *i.p.*; 6 consecutive days	↓ Mn-SOD activity ↓ CAT activity (mitochondrial) ↓ GSH content ↑ GPx content ↑ LPO ↑ uric acid	$p < 0.001$ $p < 0.01$ - 0.001 $p < 0.05$ - 0.001 $p < 0.05$ - 0.001 $p < 0.02$ - 0.001 $p < 0.05$ - 0.01
Rivera-Cervantes *et al.* (2004)	Wistar rats (n = 6)	MSG; 4 mg/g, *s.c.*; PD 1, 3, 5, 7	↑ neurons' histologic changes and degeneration ↑ Gliosis ↑ GluN1 mRNA subunit of NMDAR ↑ GluR2 mRNA subunit of AMPAR ↑ $ATF2^{pp}$ protein (for p38 MAPK activation)	$p < 0.001$ ND $p < 0.001$ $p < 0.001$ $p < 0.001$
Chaparro-Huerta *et al.* (2005)	Wistar rats (n = 8)	MSG: 4 mg/g, *s.c.*; PD 1, 3, 5, 7	↓ glial size and processes ↑ apoptosis ↑ TNF-α mRNA and protein ↑ IL-1ß mRNA and protein ↑ IL-6 mRNA and protein	ND $p < 0.001$ $p < 0.05$, and $p < 0.001$ $p < 0.001$ $p < 0.001$

Reference	Species and Strain, Size of L-Glu Treatment Group	L-Glu Treatment and Duration (Dose, Route of Drug Application)	Study Outcomes	Level of Significance
Mejía-Toiber *et al.* (2006)	Wistar rats (n = 3 - 9) male (250 - 320 g)	1 µmol/µL, intrastriatal injection; 0.5 µL/min for 2 min	↑ striatal lesions ↓ ATP level (after 6 h but not at 3 h) ↑ LPO level (after 5 or 24 h)	$p < 0.0005$ ND $p < 0.05$
Segura Torres *et al.* (2006)	Wistar rats (n = 5)	MSG; 4 mg/g, *s.c.*; PD 1, 3, 5, 7	↑ GluR2 protein subunit of AMPAR at PD 8 ↓ GluR2 protein subunit of AMPAR at PD 14 ↑ REST mRNA at PD 8 and 14 ↑ Fas-L and Bcl-2 mRNA at PD 8	ND ND $p \leq 0.01$ - 0.001 $p \leq 0.01$
(Chaparro-Huerta *et al.* 2008)	Wistar rats (n = 8)	MSG; 4 mg/g, *s.c.*; PD 1, 3, 5, 7	↑ apoptosis ↑ nuclear material condensed ↑ TNF-α mRNA ↑ IL-1ß mRNA ↑ IL-6 mRNA	$p < 0.05$, $p < 0.001$ ND $p < 0.05$ - $p < 0.001$ $p < 0.001$ $p < 0.001$
Del Río and Massieu (2008)	Wistar rats (n = 4 - 7) male (250 - 300 g)	1 µL (500 nmoles), intrastriatal injections; rate of 0.5 µL/min	↑ brain lesions ↑ calpain activation protein	ND $p \leq 0.05$
Hashem *et al.* (2012)	Albino rats (n = 10) adult male (150 - 200 g) 3 - 6 months old	MSG; 3 g/kg/day, *p.o.*; 14 days	Neurons' morphological alterations Darkly stained cytoplasm of Purkinje cells Shrunken darkly stained nuclei ↑ neurons degeneration ↑ inflammatory cells	ND

Reference	Species and Strain, Size of L-Glu Treatment Group	L-Glu Treatment and Duration (Dose, Route of Drug Application)	Study Outcomes	Level of Significance
			↑ GFAP immunoreactivity in the astrocytes of granular layer	
Shivasharan *et al.* (2013)	Wistar rats (n = 6) adult female (190 - 220 g)	MSG; 2 g/kg/day, *i.p.*; 7 days	↓ locomotor activity	$p < 0.001$
			Decreased hippocampus layer, darkly stained shrunken cells, and mildly separated interconnected neuropil fibres	ND
			↑ NO^-	$p < 0.001$
			↑ LPO level	$p < 0.001$
			↓ GSH level	$p < 0.001$
			↓ GST activity	$p < 0.001$
			↓ CAT activity	$p < 0.05$
			↓ total thiols level	$p < 0.01$
Swamy *et al.* (2013)	Wistar albino rats (n = 6) (50 - 200 g) of either sex	MSG 2 g/kg/day, *i.p.*; 7 days	↑ behavioural alterations and reduced locomotor activity	$p < 0.05$
			Marked cerebral oedema, neuronal eosinophilia, nuclear pyknosis, and neuronal karyorrhexis	ND
			↑ Ca^{2+}	$p < 0.05$
			↑ Na^+	$p < 0.05$
			↓ K^+	$p < 0.05$
			↓ GABA	$p < 0.05$
			↓ GSH level	$p < 0.05$
			↓ SOD activity	$p < 0.05$

Reference	Species and Strain, Size of L-Glu Treatment Group	L-Glu Treatment and Duration (Dose, Route of Drug Application)	Study Outcomes	Level of Significance
			↓ CAT activity	$p < 0.05$
			↑ LPO levels	
Dief *et al.* (2014)	Wistar rats (n = 6) male (40 - 60 g) 5 weeks old	MSG; 2 g/kg/day, *p.o.*; 10 consecutive days	↑ anxiety behaviour	$p < 0.05$
			↓ working memory	$p < 0.05$
			↓ AMPK protein	$p < 0.05$
		MSG; 4 g/kg/day, *s.c.*; 10 alternate days	↑ Fas-L protein	$p < 0.05$
			↑ Aβ (1-42)	$p < 0.05$
Thonda *et al.* (2014)	Wistar rats (n = 6) adult female (180 - 220 g)	MSG 2g/kg/day, *i.p.*; 7 days	↓ locomotor activity	$p < 0.001$
			↓ grip strength	$p < 0.01$
			↓ memory retention	$p < 0.01$
			↓ motor coordination and body balance	$p < 0.001$
			Hippocampal pyramidal cells' degeneration with intact neuropil fibres	ND
				$p < 0.01$
				$p < 0.001$
			↓ body weight	$p < 0.01$
			↑ LPO levels	$p < 0.001$
			↓ CAT activity	$p < 0.01$
			↓ SOD activity	
			↓ GSH level	
Rivera-Cervantes *et al.* (2015)	Wistar rats (n = 4 - 5)	MSG; 4 mg/g, *s.c.*; PD 1, 3, 5, 7	↑ alterations and loss in the hippocampal neurons at PD 8, 10, 12, and 14	$p < 0.01$
				$p < 0.001$
			↑ TUNEL-positive cells at PD 8, 10, and 14	$p < 0.001$
			↑ NMDAR subunit GluN1 mRNA at PD 10, 12, and 14	$p < 0.001$

Reference	Species and Strain, Size of L-Glu Treatment Group	L-Glu Treatment and Duration (Dose, Route of Drug Application)	Study Outcomes	Level of Significance
			↑ AMPAR subunit GluR1 mRNA at PD 12 and 14	$p < 0.001$
			↓ AMPAR subunit GluR2 mRNA and protein at PD 10 and 14	$p < 0.01$
			↑ NRSF mRNA at PD 8 and 14	$p < 0.001$
			↑ $ATF2^{pp}$ protein	
Khalil and Khedr (2016)	Wistar rats (n = 8) male (120 - 150 g) 12 weeks old	MSG; 4 mg/kg/day, *p.o.*; 4 weeks	↑ L-Glu level	$p < 0.01$
			↑ AChE activity	$p < 0.001$
			↑ TNF-α level	$p < 0.001$
			↑ GluN2B mRNA	$p < 0.001$
			↑ mGluR5 mRNA	NS
Sadek *et al.* (2016)	Albino Wistar rats (n = 8) male (130 - 160 g) 2 months old	MSG; 5 mg/kg/day, *s.c.*; 4 weeks	↑ LDH	$p < 0.05$
			↑ Na^+	$p < 0.05$
			↓ K^+	$p < 0.05$
			↑ LPO level	$p < 0.05$
			↑ GST activity and mRNA	$p < 0.05$
			↑ CAT activity and mRNA	$p < 0.05$
			↑ SOD activity	$p < 0.05$
			↓ GSH level	$p < 0.05$
			↑ AChE activity	$p < 0.05$
			↑ Bax mRNA	$p < 0.05$
			↓ Bcl-2 mRNA	$p < 0.05$
			↑ Serum ChE level	$p < 0.05$
			↑ CPK activity	$p < 0.05$
			↑ CPK-BB activity	$p < 0.05$
Hussein *et al.* (2017)	Albino rats (n = 6) male (100 - 130 g)	MSG; 100 mg/kg/day, *p.o.*; 2 months	Pathological damage to brain tissue	ND

Reference	Species and Strain, Size of L-Glu Treatment Group	L-Glu Treatment and Duration (Dose, Route of Drug Application)	Study Outcomes	Level of Significance
			↑ LPO level	$p < 0.01$
			↑ $NO^{\bullet}$	$p < 0.001$
			↓ SOD activity	$p < 0.001$
			↓ CAT activity	$p < 0.001$
			↓ GSH level	$p < 0.01$
			↑ Aβ (1-42)	$p < 0.001$
			↓ AChE activity	$p < 0.01$
			↑ serotonin level	$p < 0.001$
			↑ dopamine level	$p < 0.001$
			↑ L-Glu level	$p < 0.001$
			↑ Ca^{2+}	$p < 0.05$
			↑ Na^{+}	$p < 0.05$
			↓ K^{+}	$p < 0.05$
			↑ 8-OHdG in the brain DNA	$p < 0.01$
Abdel Moneim *et al.* (2018)	Albino rats (n = 20) male (45–70 g) 5 - 6 weeks old	High; MSG 1.66 g/kg/day, *p.o.*; 30 days Low; MSG 0.83 g/kg/day, *p.o.*; 30 days	↓ cognitive functions	$p < 0.001$
			↓ serotonin level (high-dose MSG)	$p < 0.001$
Fouad *et al.* (2018)	Albino rats (n = 12) adult male (250 - 300 g) 2 months old	2 g/kg/day, *i.p.*; 7 days	↓ spontaneous alternation behaviour (spatial working memory)	$p < 0.05$
			↑ MEL in the MWM	$p < 0.05$
			↓ time spent in target quadrant (MWM)	$p < 0.05$
			↑ cytochrome c mRNA	$p < 0.05$

Reference	Species and Strain, Size of L-Glu Treatment Group	L-Glu Treatment and Duration (Dose, Route of Drug Application)	Study Outcomes	Level of Significance
			↑ caspase-3 level	$p < 0.05$
			↑ LDH	$p < 0.05$
			↑ Aβ (1-42)	$p < 0.05$
Hazzaa *et al.* (2020)	Wistar albino rats (n = 10) male (40 g) 1 month old	MSG 4 g/kg/day, *i.p.*; 7 days	↓ locomotor activity	$p < 0.001$
			↓ spatial memory	$p < 0.05$–0.001
			↑ morphological alteration in hippocampus neurons	$p < 0.05$
			↑ LPO level	$p < 0.05$
			↑ caspase-3 protein	ND
			↓ SOD activity	$p < 0.05$
			↑ GFAP protein	$p < 0.05$
			↓ Ki-67 protein	$p < 0.05$
			↑ calretinin protein	$p < 0.05$
Greene and Greenamyre (1995)	Sprague–Dawley rats (n = 5) male (200 - 250 g)	L-Glu 0.3 M intrastriatal injection; 2 µL of solution at 0.5 µL/min (0.6 µmoles)	↑ lesion	$p < 0.01$
Yang *et al.* (1998)	Sprague–Dawley rats (n = 10) male (280 - 350 g)	1.5 or 15 mM L-Glu, cortex microdialysis; 2 µL/min 20–180 min	↑ LPO at 1.5 and 15 mM	ND
Bodnár *et al.* (2001)	Sprague–Dawley rats (n = 7 - 9) of both sexes	MSG; 4 mg/g, *s.c.;* PD 2, 4, 6, 8, 10	↑ degradation of TH-positive (dopaminergic) neurons of hypothalamic arcuate nucleus	$p < 0.05$
Kumar and Babu (2010)	Sprague–Dawley rats (n = 6)	1 µmole/1 µL, cerebral cortex	↓ pyramidal neurons' size	ND
			↑ condensed nuclei	$p < 0.001$

Reference	Species and Strain, Size of L-Glu Treatment Group	L-Glu Treatment and Duration (Dose, Route of Drug Application)	Study Outcomes	Level of Significance
	male (300 - 350 g) 3 months old	injection	↑ Ca^{2+}	$p < 0.001$
			↑ LPO level	$p < 0.001$
			↑ ROS	$p < 0.001$
			↓ SOD activity	$p < 0.001$
			↓ CAT activity	$p < 0.001$
			↓ GSH level	$p < 0.05$
			↓ GR activity	$p < 0.001$
			↑ TNF-α level	$p < 0.001$
			↑ IFN-γ level	$p < 0.05$
			↑ $NO^{\bullet}$	$p < 0.001$
			↓ MMP	$p < 0.001$
Kumar *et al.* (2010)	Sprague–Dawley rats (n = 6) male (300 - 350 g) 3 months old	1 µmole/1 µL, cerebral cortex injection	↓ pyramidal neurons' size	ND
			↑ condensed nuclei	$p < 0.001$
			↑ ROS	$p < 0.001$
			↑ $ONOO^{-}$	$p < 0.001$
			↓ MMP	$p < 0.001$
			↓ GSH	$p < 0.001$
			↑ Ca^{2+}	$p < 0.001$
			↑ nNOS mRNA	$p < 0.001$
			↑ iNOS mRNA	$p < 0.001$
			↑ caspase-3 mRNA	$p < 0.001$
			↑ caspase-9 mRNA	$p < 0.001$
			↓ Bcl-2 mRNA	ND
			↑ Bax mRNA	ND
			↓ Bcl-2/Bax ratio mRNA	$p < 0.001$
Babu *et al.* (2011)	Sprague–Dawley rats (n = 6) male (300 - 350 g)	1 µmole/1 µL, cerebral cortex injection	↑ LPO level	$p < 0.001$
			↑ ROS	$p < 0.001$
			↓ SOD activity	$p < 0.05$

Reference	Species and Strain, Size of L-Glu Treatment Group	L-Glu Treatment and Duration (Dose, Route of Drug Application)	Study Outcomes	Level of Significance
	3 months old		↓ CAT activity	$p < 0.05$
			↓ GSH level	$p < 0.05$
			↓ GR activity	$p < 0.05$
			↑ TNF-α level	$p < 0.001$
			↑ IFN-γ level	$p < 0.001$
			↑ $NO^{\bullet}$	$p < 0.001$
			↓ MMP	$p < 0.001$
			↑ Ca^{2+}	$p < 0.001$
			↑ caspase-3 mRNA	ND
			↑ caspase-9 mRNA	ND
			↑ iNOS mRNA	ND
			↑ nNOS mRNA	ND
Morales and Rodriguez (2012)	Sprague–Dawley rats (n = 5, 7, 8) male (300 - 350 g)	L-Glu in Ringer solution of 2 µL/min, striatum microdialysis; 60 min	↑ astrogliosis	$p < 0.001$
			↑ L-Glu level	$p < 0.001$
			↑ alanine level	$p < 0.001$
			↓ glutamine level	$p < 0.001$
Kim *et al.* (2013)	Sprague–Dawley rats (n = 6) male (300 - 350 g) 3 months old	1 µmol/1 µL, cerebral cortex injection	↑ LPO level	ND
			↑ Ca^{2+}	ND
			↑ ROS	ND
			↓ GSH level	ND
			↓ SOD activity	ND
			↓ CAT activity	ND
			↓ GPx level	ND
			↓ GR level	ND
			↑ TNF-α level	ND
			↑ IFN-γ level	ND
			↑ NO^{-}	ND
			↑ NADPH oxidase activity	ND

Reference	Species and Strain, Size of L-Glu Treatment Group	L-Glu Treatment and Duration (Dose, Route of Drug Application)	Study Outcomes	Level of Significance
			↑ nNOS mRNA	ND
			↓ MMP	ND
			↑ p-ERK1/2 mRNA	ND
			↑ caspase-3 mRNA	ND
Shah *et al.* (2015)	Sprague–Dawley male rats (n = 10) male (18 g) pups at PD 7	5 mg/kg, *s.c.*; 4 or 12 h, PD 7	↑ L-Glu level	$p < 0.01$ - 0.0001
		10 mg/kg, *s.c.*; 4 or 12 h, PD 7	↑ Bax protein	ND
			↓ Bcl-2 protein	ND
			↑ Bax/Bcl-2 ratio	$p < 0.01$ - 0.0001
			↑ cytochrome c protein	$p < 0.01$ - 0.0001
			↑ cleaved caspase-3 protein and level	$p < 0.01$ - 0.0001
			↑ p-AMPK protein (high dose)	$p < 0.0001$
			↑ FJB positive neurons (high dose)	$p < 0.0001$
			↑ cleaved PARP-1 protein	$p < 0.01$ - 0.0001
Calis *et al.* (2016)	Sprague–Dawley rats (n = 7) Female (250–300 g) 3 - 4 months old	MSG; 2 g/kg/day, *i.p.*; 7 days	No neuron degeneration in pyramidal and granular neurons in the brain cortex	ND
			No effect on SOD level	NS
			↓ LPO level	$p < 0.001$
Shah *et al.* (2016)	Sprague–Dawley rats (n = 5) (18 g) pups at PD 7	10 mg/kg, *i.p.*; 2, 3, 4 h	↑ DNA fragmentation	$p < 0.001$
			↑ L-Glu	$p < 0.05$ - 0.001
			↑ AMPAR protein	$p < 0.05$ - 0.001
			↑ p-AMPK protein	$p < 0.05$ - 0.001
			↑ p-NF-$_k$B protein	$p < 0.05$ - 0.001
			↓ Nrf2 protein	$p < 0.05$ - 0.01
			↑ CaMKII protein	$p < 0.001$
			↓ GSH level	$p < 0.001$

Reference	Species and Strain, Size of L-Glu Treatment Group	L-Glu Treatment and Duration (Dose, Route of Drug Application)	Study Outcomes	Level of Significance
			↓ GSH/GSSG ratio	$p < 0.001$
			↑ GFAP	$p < 0.001$
			↑ microglia Iba-1	$p < 0.001$
			↑ ROS	$p < 0.001$
			↑ COX-2 protein	$p < 0.001$
			↑ TNF-α protein	$p < 0.001$
			↑ caspase-3 protein	$p < 0.001$
			↓ HO-1 protein	$p < 0.001$
Yang *et al.* (2017)	Sprague–Dawley rats (n = 6) adult male (270 - 320 g)	1 M/1 µL, cerebral cortex injection	↑ TUNEL-positive cells	ND
			↑ caspase-3 protein and activity	ND
			↑ calpain protein and activity	ND
			↑ Bax protein	ND
			↓ Bcl-2 protein	ND
			↓ Bcl-2/Bax ratio	ND
			↑ ROS	ND
			↑ NO	ND
			↑ TNF-α level	ND
			↑ IFN-γ level	ND
			↑ IL-1β level	ND
			↑ NOX activity	ND
			↑ LPO level	ND
			↓ SOD activity	ND
			↓ GR activity	ND
			↓ GSH level	ND
			↓ CAT activity	ND
			↑ iNOS protein	ND
			↑ nNOS protein	ND

Reference	Species and Strain, Size of L-Glu Treatment Group	L-Glu Treatment and Duration (Dose, Route of Drug Application)	Study Outcomes	Level of Significance
			↓ Nrf2 protein	ND
			↓ HO-1 protein	ND
			↓ GCLC protein	ND
			↓ ATP level	ND
			↓ Na^+-K^+-ATPase level	ND
			↓ cytochrome c oxidase activity	ND
Firgany and Sarhan (2020)	Sprague–Dawley rats (n = 10) male (365 g) 18 months old	MSG; 4.0 g/kg/day, *s.c.*; 10 days	Remarkable morphological alteration in motoneurons and neuroglia	ND
			↑ caspase-3 activity	$p < 0.001$
			↑ LPO level	$p < 0.001$
			↑ IL-1β level	$p < 0.001$
			↑ IL-6 level	$p < 0.001$
			↑ TNF-α level	$p < 0.001$
			↑ IFN-γ level	$p < 0.001$
			↓ IL-10 level	$p < 0.001$
			↓ SOD activity	NS
			↓ CAT activity	$p < 0.001$
			↓ GFAP level	$p < 0.001$
			↑ $ATF2^{pp}$ protein	
Hamza *et al.* (2019)	Rats (n = 8) adult male (200 - 250 g)	MSG high; 17.5 mg/kg/day, *p.o.*; 30 days MSG low; 6 mg/kg/day, *p.o.*; 30 days	MSG high dose: Large area of haemorrhage and necrotic areas of the brain with the congested area with degeneration in some glial	ND
			↓ catecholamine level	$p \leq 0.05$
			↓ dopamine level	$p \leq 0.05$

Reference	Species and Strain, Size of L-Glu Treatment Group	L-Glu Treatment and Duration (Dose, Route of Drug Application)	Study Outcomes	Level of Significance
			↓ serotonin level	$p \leq 0.05$
			↓ AChE activity	$p \leq 0.05$
			↓ thiol level	$p \leq 0.05$
			↓ SOD activity	$p \leq 0.05$
			↓ CAT activity	$p \leq 0.05$
			↓ GPx activity	$p \leq 0.05$
			↓ GSH level	$p \leq 0.05$
			↓ BDNF level	$p \leq 0.05$
			↑ COX-2 activity	$p \leq 0.05$
			↑ PGE2 level	
			MSG low dose: Moderate area of haemorrhage and necrosis in the brains	ND
Burde *et al.* (1971)	Wistar rat 4 day old and Swiss albino mice 10 day old (n = 2 - 9)	MSG; 1 mg/g, 4 mg/g, *p.o., or s.c.*; 5 h MSG; 4 mg/g, 2 mg/g, *p.o., or s.c.*; 5 h	↑ lesions in arcuate of the hypothalamus Lesions more extensive in rat than mice ↑ necrosis of neuron of arcuate Perikaryon swelling loss of cytoplasmic density and nuclear pyknosis	ND
Onaolapo *et al.* (2016)	Swiss mice (n = 10) adult male (20 - 22 g)	MSG; 10, 20, 40, and 80 mg/kg/day, *p.o.*; 28 days L-Glu; 10 mg/kg/day, *p.o.*; 28 days	MSG: ↑ brain weight (40 and 80 mg/kg) ↑ neurons' morphological alteration (MSG and L-Glu) ↑ glial cell number	$p < 0.01$ $p < 0.05$ $p < 0.05$

Reference	Species and Strain, Size of L-Glu Treatment Group	L-Glu Treatment and Duration (Dose, Route of Drug Application)	Study Outcomes	Level of Significance
			↑ L-Glu plasma level (40 and 80 mg/kg)	$p < 0.001$
			↑ glutamine plasma level (40 and 80 mg/kg)	$p < 0.01$
			↓ SOD level (20, 40, and 80 mg/kg)	$p < 0.01$ $p < 0.001$
			↓ CAT level (40 and 80 mg/kg)	$p < 0.002$
			↑ NO• (all doses)	
Mohan *et al.* (2017)	Swiss albino mice (n = 5 for some experiments) of either sex (18 - 22 g)	MSG; 1000 mg/kg/day, *p.o.*; 14 days	↓ onset of immobility delayed	$p < 0.05$
			↓ total immobility period	$p < 0.05$
			↓ brain weight	$p < 0.05$
			Neurodegeneration characterised by deformed brain layers, pyknosis, and neuronal cell vacuolisation	ND
			↓ SOD activity	$p < 0.05$
			↓ CAT activity	$p < 0.05$
			↓ RGSH	$p < 0.05$
			↑ LPO level	$p < 0.05$
Estrada-Sánchez *et al.* (2009)	Wild-type mice (n = 3 - 4) female 10 - 14 weeks old	500 nM/ 0.5 µL, intrastriatal injection; 3 and 24 h	↑ striatal lesions at 10 and 14 weeks	$p \leq 0.05$
			↑ FJB-positive degenerating neurons	$p \leq 0.05$
Estrada-Sánchez *et al.* (2010)	Wild-type mice (n = 3 - 6) female 10 weeks old	500 nM/0.5 µL, intrastriatal injection; 24 h	↑ lesions	$p \leq 0.05$

Reference	Species and Strain, Size of L-Glu Treatment Group	L-Glu Treatment and Duration (Dose, Route of Drug Application)	Study Outcomes	Level of Significance
Guemez-Gamboa *et al.* (2011)	Wild-type mice (n = 3 - 7)	MSG; 500 nM/0.5 µL, intrastriatal injection; rate of 0.175 µL/min	↑ microglia activation	ND
			↑ NT-positive cells (NT; nitrosylated proteins the index of oxidative damage)	$p < 0.05$
			↑ striatal lesions	$p < 0.001$
			↑ FJB-positive degenerating neurons	$p < 0.001$
			↑ ROS	$p < 0.001$
			↑ NADPH oxidase activity	$p < 0.05$
			↑ calpain protein	$p < 0.05$
Wang *et al.* (2015)	Wild-type mice (n = 3) male 4 - 6 months old	10 mM at a flow rate of 1 µL/h, left lateral ventricle, brain infusion cannula; 7 days	↑ motor neurons clumping or fragmented nuclei	ND
			↑ mitochondrial fragmentation	$p < 0.001$
			↓ MFN2 protein	$p < 0.05$
			↑ cleaved caspase-3-positive neurons	ND
Zou *et al.* (2016)	Wild-type mice (n = 6) (25 - 30 g) 4 - 5 months old	MSG; 0.25 M, 0.2 µL injected on each side of the parietal cortex	↑ lesion	$p < 0.01$
Yu *et al.* (2006)	Kunming mice (n = 8 - 10) female, pregnant 7 weeks old	MSG; 1, 2, 4 g/kg/day, *i.g.*; at days 17 - 19 days of pregnancy	↑ hyperactivity from open field test	$p < 0.0001$ - 0.0004
			↓ the memory retention and Y-maze discrimination learning capacities	$p < 0.0001$
			↑ hippocampal lesions	ND
			↑ [^{3}H]-Glu uptake	ND
			↓ Bcl-2 protein	ND
			↑ caspase-3 protein	ND

Reference	Species and Strain, Size of L-Glu Treatment Group	L-Glu Treatment and Duration (Dose, Route of Drug Application)	Study Outcomes	Level of Significance
Ma *et al.* (2007)	Kunming mice (n = 11-13) adults 8 weeks old	MSG; 1, 2, 4 g/kg/day, *i.g.*; 10 days	↓ discrimination learning and memory using Y-maze test	$p = 0.0006$
			Hippocampal lesions' intracellular oedema, degeneration and necrosis of neurons, and hyperplasia	ND
Penugonda and Ercal (2011)	CD-1 mice (n = 4) adult male (38 - 40 g)	2000 mg/kg/day, *p.o.*; 1 week	↓ GSH level	NS
			↑ LPO level	$p < 0.05$ - 0.005
			↑ PLA2s activity	$p < 0.05$ - 0.001
Chu *et al.* (2020)	*Caenorhabditis elegans (*wild-type) nematodes (n = 30)	20 mM, animals living media; 24 h	↑ damaged locomotory ability	$p < 0.05$
			↑ ROS	ND
			↑ $O_2^{-\bullet}$	ND
			↓ GSH level	ND
Spangenberg *et al.* (2004)	Ephyrae of *Aurelia aurita* (n = 2 - 4)	MSG; 5 mM, animals living media (artificial sea water); 1 - 24 h	↑ impaired pulsing and swimming motility	$p < 0.05$
			↓ pulsing rates	$p < 0.05$
			↑ Ca^{2+}	$p < 0.05$
			↑ ROS	$p < 0.05$
			↑ $NO^{\bullet}$	ND

Abbreviations: AChE, acetylcholinesterase; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; AMPK, 5′AMP-activated protein kinase; $ATF2^{pp}$, activating transcription factor 2 phosphorylated; ATP, adenosine triphosphate; Aβ, amyloid beta; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2; BDNF, brain-derived neurotrophic factor; Ca^{2+}, calcium ions; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CAT, catalase; ChE, cholinesterase; COX-2, cyclooxygenase-2; CPK, creatine phosphokinase; CPK-BB, creatine phosphokinase isoenzymes BB; DNA, deoxyribonucleic acid; Fas-L, Fas ligand; FJB, fluoro jade B; GABA, gamma-aminobutyric acid; GCLC, glutamate cysteine ligase catalytic subunit; GFAP, glial fibrillary acidic protein; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; *GSSG*, oxidised glutathione; GST, glutathione-S-transferase; ^{3}H-

GABA, [^{3}H]gamma-aminobutyric acid (GABA); [^{3}H]-Glu, [^{3}H]-glutamate; HO-1, heme oxygenase-1; *i.g.*, maternal intragastric; *i.p.*, intraperitoneal injection; Iba-1, ionised calcium-binding adaptor molecule 1; IFN-γ, interferon gamma; IL-1β, interleukin 1 beta; IL-10, interleukin 10; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; K^+, potassium ions; LDH, lactate dehydrogenase; L-Glu, L-glutamate; LPO, lipid peroxidation; MEL, mean escape latency; MFN2, mitofusin 2; mGluR5, metabotropic glutamate receptor 5; MMP, mitochondrial membrane potential; Mn-SOD, manganese superoxide dismutase; mRNA, messenger RNA; MSG, monosodium glutamate; MWM, Morris water maze; Na^+, sodium ions; Na^+-K^+-ATPase, sodium potassium adenosine triphosphatase; ND, not determined; GluN2B, N-methyl-D-aspartate receptor subunit 2B; NMDAR, N-methyl-D-aspartate receptor; nNOS, neuronal nitric oxide synthase; NO^-, nitrite; $NO^\bullet$, nitric oxide; Nrf2, nuclear factor E2-related factor 2; NRSF, neuron-restrictive silencer factor; NS, not significant; $O_2^{-\bullet}$, superoxide; $OH^\bullet$, hydroxyl radicals; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; *ONOO*$^-$, peroxynitrites; p-AMPK, phosphorylate AMP-activated protein kinase; PARP-1, (ADP ribose) polymerase-1; PD, postnatal day; p-ERK 1/2, phospho-extracellular signal-regulated kinase; PGE2, prostaglandin E2; PLA2s*, phospholipases A2*; p38 MAPK, p38 mitogen-activated protein kinases; p-NF-$_k$B, phosphorylate nuclear factor kappa-light-chain-enhancer of activated B cells; *p.o.*, orally; REST, RE1-silencing transcription factor; RGSH, reduced glutathione; ROS, reactive oxygen species; *s.c.*, subcutaneous injection; SOD, superoxide dismutase; TH, tyrosine hydroxylase; TNF-α, tumour necrosis factor alpha; TUNEL, terminal deoxynucleotidyl transferase (dUTP) nick end labeling. Note: malondialdehyde (MDA) level is an index of lipid peroxidation (LPO).

3.2.2.1 Administration of L-Glu directly to animals

3.2.2.1.1 L-Glu administration reduces neuronal viability

Neuron injury enzyme markers, such as serum creatine phosphokinase (CPK) and creatine phosphokinase isoenzymes BB (CPK-BB), were significantly higher in the brains of rats injected *s.c.* with L-Glu (Sadek *et al.* 2016). LDH released from damaged tissue was significantly elevated in the brains of rats injected with L-Glu *i.p.* or *s.c.* (Fouad *et al.* 2018; Sadek *et al.* 2016). Additionally, the Ki-67 protein, a marker of actively proliferating neurons, was significantly declined in brain tissue after L-Glu administration to rats *i.p.* (Hazzaa *et al.* 2020). High fluoro jade B (FJB)-stained neurons and overactivation of poly (adenosine diphosphate (ADP)-ribose) polymerase (PARP-1) were observed, two labels of degenerating neurons, as a result of a high dose of exogenous L-Glu to the brain (Shah *et al.* 2015). There was also a significant reduction in BDNF following L-Glu treatment at 17.5 mg/kg dose *p.o.*, although this was not significant at a 6 mg/kg dose (Hamza *et al.* 2019).

3.2.2.1.2 L-Glu administration impairs cellular oxidant defence and stimulates oxidative stress

Delivery of a high dose (≥17.5 mg/kg) of L-Glu by either *p.o.* or *i.p.* routes significantly reduced brain SOD and CAT levels (Hamza *et al.* 2019; Hussein *et al.* 2017; Mohan *et al.* 2017; Onaolapo *et al.* 2016; Singh *et al.* 2003; Swamy *et al.* 2013; Thonda *et al.* 2014), although a relatively low L-Glu dose (6 mg/kg) was without effect on these enzymes (Hamza *et al.* 2019). However, low-dose L-Glu (*s.c.* injection of 5 mg/kg) resulted in a significantly increased expression of SOD and CAT genes (Sadek *et al.* 2016). L-Glu at 4 g/kg by *s.c.* and *i.p.* routes significantly reduced the activity of SOD in the CNS (Firgany and Sarhan 2020; Hazzaa *et al.* 2020). Conversely, a relatively high dose of L-Glu (2 g/kg *i.p.*) was without effect on SOD activity (Calis *et al.* 2016). However, a high dose of L-Glu (4 g/kg injected *s.c.*) induced CAT (Babu *et al.* 1994), whereas 4 g/kg via an *i.p.* injection reduced CAT activity (Shivasharan *et al.* 2013).

Brain GSH level was significantly declined after L-Glu treatment by *p.o.* (Hamza *et al.* 2019; Hussein *et al.* 2017; Mohan *et al.* 2017), *s.c.* (Sadek *et al.* 2016), or *i.p.* routes (Shah *et al.* 2016; Shivasharan *et al.* 2013; Singh *et al.* 2003; Swamy *et al.* 2013; Thonda *et al.* 2014). In addition, a relatively high oral dose of L-Glu (2 g/kg) was also able to reduce cellular GSH levels but not significantly (Penugonda and Ercal 2011).

A similar contrary result was observed after L-Glu was taken orally (17.5 mg/kg), as this induced inhibition of glutathione peroxidase (GPx) activity (Hamza *et al.* 2019), while a high dose (4 g/kg) administered *i.p.* resulted in a significant boost of GPx activity (Singh *et al.* 2003). Similarly, glutathione-S-transferase (GST) activity was inhibited after L-Glu treatment *i.p.* (Shivasharan *et al.* 2013); however, its activity and gene expression were significantly increased in response to *s.c.* L-Glu injection (Sadek *et al.* 2016).

The modest antioxidant uric acid was significantly reduced but only after a significant drop in the concentration of other antioxidants (Singh *et al.* 2003). This study also showed a significant elevation in brain uric acid content in response to *i.p.* L-Glu injection (Singh *et al.* 2003).

L-Glu *i.p.* resulted in the suppression of the transcription factor nuclear factor E2-related factor 2 (Nrf2), which regulates the expression of antioxidant proteins (Shah *et al.* 2016). There was also a significant decline in thiol levels in *p.o.* and *i.p.* L-Glu-treated groups (Hamza *et al.* 2019; Shivasharan *et al.* 2013), but not after a low *p.o.* dose (6 mg/kg) (Hamza *et al.* 2019). However, the activities of the brain antioxidant enzymes myeloperoxidase (MPO) and xanthine oxidase (XO) were unaffected following L-Glu administration (Hamza *et al.* 2019).

Administration of L-Glu *in vivo* by *p.o.*, *s.c.*, or *i.p.* routes triggered the accumulation of LPO products (Babu *et al.* 1994; Firgany and Sarhan 2020; Hazzaa *et al.* 2020; Hussein *et al.* 2017; Mohan *et al.* 2017; Penugonda and Ercal 2011; Sadek *et al.* 2016; Shivasharan *et al.* 2013; Singh *et al.* 2003; Swamy *et al.* 2013; Thonda *et al.* 2014), but L-Glu *s.c.* at 0.004 mg/kg dose showed no significant differences in LPO levels within the frontal cortex (Babu *et al.* 1994). Furthermore, a single study reported significantly reduced LPO levels following L-Glu injection *i.p.* (Calis *et al.* 2016).

There was a significant elevation of RNS after L-Glu via *p.o.* or oral cannula or *i.p.* injection (Hussein *et al.* 2017; Onaolapo *et al.* 2016; Shivasharan *et al.* 2013). Relatively high levels of ROS were apparent after L-Glu was given *i.p.* (Shah *et al.* 2016). L-Glu administrated *p.o.* also significantly elevated 8-OHdG levels in rat brains, a biomarker of oxidative damage to DNA (Hussein *et al.* 2017).

3.2.2.1.3 L-Glu administration influences acetylcholinesterase (AChE) activity

AChE activity was significantly reduced at relatively higher doses of L-Glu (17.5 mg/kg, *p.o.* (Hamza *et al.* 2019) or 100 mg/kg, *p.o.*) (Hussein *et al.* 2017), whereas a low dose of L-Glu (6 mg/kg, *p.o.*) did not affect AChE activity (Hamza *et al.* 2019). However, by contrast, AChE activity was significantly enhanced by L-Glu at 4 mg/kg (*p.o.*) or 5 mg/kg (*s.c.*) (Khalil and Khedr 2016; Sadek *et al.* 2016).

3.2.2.1.4 L-Glu administration influences neurotransmitter levels

L-Glu administration (4 and 100 mg/kg, *p.o.*; 10 mg/kg, *i.p.*; 5 and 10 mg/ kg, *s.c.*) significantly elevated brain L-Glu neurotransmitter levels (Hussein *et al.* 2017; Khalil and Khedr 2016; Shah *et al.* 2015; Shah *et al.* 2016), but not at doses of 10, 20, 40, and 80 mg/kg, *p.o.* (Onaolapo *et al.* 2016), or 2 g/kg, *p.o.*, or 4 g/kg, *s.c.* (Dief *et al.* 2014). Dopamine levels were significantly reduced after a 17.5 mg/kg dose of L-Glu (*p.o.*) and 100 mg/kg dose via oral gavage (Hamza *et al.* 2019; Hussein *et al.* 2017). Similarly, brain serotonin was significantly elevated after L-Glu at 100 mg/kg, *p.o.* (Hussein *et al.* 2017), but also significantly declined following L-Glu at 17.5 mg/kg, 0.83 g/kg, and 1.66 g/kg, *p.o.* (Abdel Moneim *et al.* 2018; Hamza *et al.* 2019). The neurotransmitters noradrenaline and adrenaline also declined after administration of L-Glu at 17.5 mg/kg (*p.o.*) (Hamza *et al.* 2019). Catecholamine (noradrenaline and adrenaline), dopamine, and serotonin levels were not altered at an L-Glu dose of 6 mg/kg *p.o.* (Hamza *et al.* 2019).

3.2.2.1.5 L-Glu administration triggers neuronal apoptosis

The administration of L-Glu by *s.c.* and *i.p.* routes induced activation and phosphorylation of AMP-activated protein kinase (AMPK) in some (Shah *et al.* 2016; Shah *et al.* 2015) but not all studies (Dief *et al.* 2014). L-Glu (*s.c.*) induced a significant

upregulation of phosphorylated activating transcription factor 2 (ATF2) expression and associated p38 MAPK activity (Firgany and Sarhan 2020; Rivera-Cervantes *et al.* 2004; Rivera-Cervantes *et al.* 2015). The number of terminal deoxynucleotidyl transferase (dUTP) nick end labeling (TUNEL)-positive neurons (indicative of apoptosis) was significantly increased after L-Glu treatment (*s.c.* or *i.p.*) (Chaparro-Huerta *et al.* 2005; Chaparro-Huerta *et al.* 2008; Rivera-Cervantes *et al.* 2015; Shah *et al.* 2016). There was also a significant upregulation of the proapoptotic Bax and downregulation of antiapoptotic Bcl-2 genes in rats treated with L-Glu by *s.c.* (Sadek *et al.* 2016; Shah *et al.* 2015) and *i.g.* (Yu *et al.* 2006) routes. However, one study reported that Bcl-2 mRNA expression in the brain was increased as a consequence of *s.c.* L-Glu injection (Segura Torres *et al.* 2006).

Apoptosis signalling caspase-3 protein was significantly increased in its expression in the brain in response to L-Glu injected *s.c.* (Firgany and Sarhan 2020; Shah *et al.* 2015), *i.p.* (Fouad *et al.* 2018; Hazzaa *et al.* 2020; Shah *et al.* 2016) in rat, or *i.g* in mice. (Yu *et al.* 2006). L-Glu injected *i.p.* or *s.c.* initiated a significant rise in the cytochrome c gene expression (Fouad *et al.* 2018) and release (Shah *et al.* 2015) in rat brain tissue. Administration of L-Glu *p.o.* and *s.c.* induced a rise of the Fas ligand as an apoptosis mediator (Dief *et al.* 2014; Segura Torres *et al.* 2006).

3.2.2.1.6 Excitotoxicity, calcium level, and other ions in the brain

Rats administered L-Glu via *p.o.* and *s.c.* routes exhibited significantly increased expression of specific NMDAR subunits, including GluN2B and GluN1, in several brain areas (Khalil and Khedr 2016; Rivera-Cervantes *et al.* 2004; Rivera-Cervantes *et al.* 2015). In addition, *s.c.* and *i.p.* L-Glu induced significantly higher expression of AMPAR subunits, GluR1 (Rivera-Cervantes *et al.* 2015) and GluR2 (Rivera-Cervantes *et al.* 2004; Segura Torres *et al.* 2006; Shah *et al.* 2016). Furthermore, *s.c.* L-Glu treatment significantly increased the level of expression of the GluR2 transcription regulator known as neuron-restrictive silencer factor (NRSF) mRNA levels or RE1-silencing transcription factor (REST) (Rivera-Cervantes *et al.* 2015; Segura Torres *et al.* 2006). There was also a nonsignificant increase in the expression of the metabotropic glutamate receptor 5

(mGluR5) gene in the brain in response to *p.o.* L-Glu (Khalil and Khedr 2016). As a consequence of L-Glu excitotoxicity, its administration *i.p.* into animals resulted in a significant reduction in the level of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (Swamy *et al.* 2013).

Significant elevation of Ca^{2+} levels was observed in the brains of rodents treated with L-Glu *p.o.* (Hussein *et al.* 2017) or *i.p.* (Hazzaa *et al.* 2020; Shah *et al.* 2016; Swamy *et al.* 2013). In the L-Glu *i.p.* treated group, the Ca^{2+} level was detected by strong calretinin immune reactivity in neurons and upregulation of the expression level of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Hazzaa *et al.* 2020; Shah *et al.* 2016). This was associated with heightened Na^{+} levels and reduced K^{+} levels (Hussein *et al.* 2017; Sadek *et al.* 2016; Swamy *et al.* 2013).

3.2.2.1.7 Neuroinflammation

The brain or spinal cord tissue of rats exhibited a significant elevation in the levels of neuroinflammatory cytokines, including TNFα, after *p.o.*, *s.c.*, or *i.p.* applied L-Glu (Chaparro-Huerta *et al.* 2005; Chaparro-Huerta *et al.* 2008; Firgany and Sarhan 2020; Khalil and Khedr 2016; Shah *et al.* 2016). Additionally, relatively elevated levels of the cytokines IL-1ß and IL-6 were detected in the cervical spinal cord and brain of rats *s.c.* injected with L-Glu (Chaparro-Huerta *et al.* 2005; Chaparro-Huerta *et al.* 2008; Firgany and Sarhan 2020). Likewise, *s.c.* L-Glu-treated rats displayed a substantial rise in the amount of spinal cord IFN-γ with a significant decline in the anti-inflammatory cytokine IL-10 as compared with controls (Firgany and Sarhan 2020).

Administration of L-Glu *i.p.* to rats revealed a significant induction of glial cell activation, which was identified by an upsurge in the glial fibrillary acidic protein (GFAP) immunodetection (Hazzaa *et al.* 2020; Shah *et al.* 2016) and a marker for microglial activation, Iba-1 (Shah *et al.* 2016). A study that analysed the morphometrics of GFAP-stained astrocytes showed that *s.c.* application of L-Glu resulted in considerable shrinkage of the astrocyte surface area in the spinal cords of rats (Firgany and Sarhan 2020). Moreover, gliosis was detected in response to *i.p.* L-Glu administration to rats, the glial cells' reactive change in response to brain damage (Hazzaa *et al.* 2020). L-Glu via

i.p. and *p.o.* routes at doses of 10 and 17.5 mg/kg induced the activities of the proinflammatory mediators COX-2 (Hamza *et al.* 2019; Shah *et al.* 2016) and prostaglandin E2 (PGE2), respectively (Hamza *et al.* 2019). However, no difference was found in these proinflammatory mediators after a low *p.o.* L-Glu dose of 6 mg/kg (Hamza *et al.* 2019).

In addition, the activity and phosphorylation of proinflammatory nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) were upregulated in response to *i.p.* L-Glu rat injection (Shah *et al.* 2016). Furthermore, L-Glu given by oral gavage to animals was associated with inflammation in mouse brains via the enhanced activity of phospholipase A2 (PLA2) (Penugonda and Ercal 2011).

3.2.2.1.8 Histology alteration

Histological and histomorphometric changes in several brain areas were the result of *p.o.*, *s.c.*, or *i.p.* L-Glu administration, consistent with neuronal degeneration (Bodnár *et al.* 2001) via necrosis or apoptosis and nuclear pyknosis (Burde *et al.* 1971; Firgany and Sarhan 2020; Hamza *et al.* 2019; Hashem *et al.* 2012; Hazzaa *et al.* 2020; Hussein *et al.* 2017; Mohan *et al.* 2017; Onaolapo *et al.* 2016; Rivera-Cervantes *et al.* 2004; Rivera-Cervantes *et al.* 2015; Swamy *et al.* 2013). In addition, the brain tissue following the application of L-Glu *p.o.*, *s.c.*, or *i.p* exhibited areas of haemorrhage (Hamza *et al.* 2019), congestion (Hamza *et al.* 2019; Hussein *et al.* 2017), accumulation of inflammatory cells (Bodnár *et al.* 2001; Hashem *et al.* 2012; Hazzaa *et al.* 2020; Rivera-Cervantes *et al.* 2004), oedema (Hussein *et al.* 2017; Swamy *et al.* 2013), neuronal cytoplasmic vacuolisation (Firgany and Sarhan 2020; Hazzaa *et al.* 2020; Mohan *et al.* 2017; Onaolapo *et al.* 2016; Rivera-Cervantes *et al.* 2004), and neuronal eosinophilia (Swamy *et al.* 2013). Additionally, *i.p.* L-Glu treatment caused morphological changes to neuropil fibres (Shivasharan *et al.* 2013; Thonda *et al.* 2014) and neuronal karyorrhexis (Swamy *et al.* 2013). Treatment with L-Glu by *i.g.* route caused brain tissue alterations, including oedema, neuron death via necrosis, and hyperplasia (Ma *et al.* 2007; Yu *et al.* 2006). However, two studies indicated that L-Glu *p.o.* (10 and 20 mg/kg) and *i.p.* (2g/kg) had no effects on the neuronal features of brain tissue (Calis *et al.* 2016; Onaolapo *et al.* 2016).

3.2.2.1.9 Behaviour and cognitive function

The negative impact of *p.o.*, *s.c.*, or *i.g.* L-Glu administration on cognitive functions and the induction of the impairment of learning ability has been extensively demonstrated (Abdel Moneim *et al.* 2018; Dief *et al.* 2014; Fouad *et al.* 2018; Ma *et al.* 2007; Yu *et al.* 2006). Similarly, poor memory retention was observed in animals following L-Glu, *i.p* (Hazzaa *et al.* 2020; Thonda *et al.* 2014), causing locomotor impairment (L-Glu, *i.p.* or *i.g.*) (Hazzaa *et al.* 2020; Shivasharan *et al.* 2013; Swamy *et al.* 2013; Yu *et al.* 2006) or coordination abnormalities (L-Glu, *i.p.*) as well (Thonda *et al.* 2014). In contrast, one study reported that treatment by L-Glu *p.o.* or *s.c.* caused neurobehavioral deficits but without any sign of motor or coordination abnormalities (Dief *et al.* 2014). In addition, L-Glu, *p.o.* or *s.c.*, triggered behavioural phenotypic changes, including increased anxiety (Dief *et al.* 2014; Mohan *et al.* 2017), while aggressiveness and loss of muscle strength were detected after L-Glu, *i.p.*, treatment (Swamy *et al.* 2013; Thonda *et al.* 2014).

3.2.2.1.10 Protein aggregation

A significant increase in Aβ protein accumulation by more than twofold in rat brain tissue was reported after L-Glu administration through *i.p.* (7 days) or *p.o.* (2 months) or *p.o. and s.c.* (10 days) routes (Dief *et al.* 2014; Fouad *et al.* 2018; Hussein *et al.* 2017).

3.2.2.1.11 Brain weight

One study indicated that relative brain weight was increased significantly after *p.o.* L-Glu was introduced at 40 and 80 mg/kg in mice (Onaolapo *et al.* 2016). However, a higher *p.o.* dose of 1000 mg/kg L-Glu caused a significant decrease in relative brain weight in mice (Mohan *et al.* 2017). This was supported by another study showing that the total protein of brain tissue was reduced significantly in high-dose 2 g/kg, *i.p.*, L-Glu-treated rats (Thonda *et al.* 2014).

3.2.2.2 L-Glu administration directly to animal brains

A total of 16 *in vivo* studies administrated L-Glu directly into brain tissue through microdialysis, stereotactic injection, or brain infusion canal (Table 3.2).

3.2.2.2.1 Antioxidant and oxidative stress markers

Four studies reported that L-Glu stereotactic administration into the cerebral cortex induced significant reductions in the endogenous antioxidant defences: SOD, CAT, GSH, and glutathione reductase (GR) (Babu *et al.* 2011; Kim *et al.* 2013; Kumar and Babu 2010; Yang *et al.* 2017). Similarly, cerebral cortex GSH levels considerably declined after L-Glu stereotactic injection (Kumar *et al.* 2010). In addition, the cerebral cortex GPx was reduced in L-Glu-treated animals (Kim *et al.* 2013). Moreover, the expression of the antioxidant regulatory proteins nuclear factor erythroid 2-related factor 2 (Nrf2), glutamate cysteine ligase catalytic subunit (GCLC), and heme oxygenase-1 (HO-1) was significantly decreased in the cerebral cortex of the L-Glu-treated group (Yang *et al.* 2017).

L-Glu administration through microdialysis (15 mM) or stereotactic injection (1 µL of 1 µM or 1 M) significantly enhanced the LPO level in the cortex and striatum of rats (Babu *et al.* 2011; Kim *et al.* 2013; Kumar and Babu 2010; Mejía-Toiber *et al.* 2006; Yang *et al.* 1998; Yang *et al.* 2017). Similarly, 1.5 mM L-Glu solution increased the LPO level by approximately 100 %, but this was not statistically significant (Yang *et al.* 1998). There is also experimental evidence of elevated LPO levels detected 3 h after intrastriatal injection with L-Glu (Mejía-Toiber *et al.* 2006). Furthermore, stereotactically injected L-Glu induced significantly high levels of ROS formation in the brain striatum and cerebral cortex (Babu *et al.* 2011; Guemez-Gamboa *et al.* 2011; Kim *et al.* 2013; Kumar and Babu 2010; Kumar *et al.* 2010; Yang *et al.* 2017). There was also elevated nitric oxide ($NO^{\bullet}$) production in the cerebral cortex after stereotactic L-Glu administration (Babu *et al.* 2011; Kim *et al.* 2013; Kumar and Babu 2010; Yang *et al.* 2017) and increased mRNA expression of iNOS (Babu *et al.* 2011; Kumar *et al.* 2010; Yang *et al.* 2017) and nNOS (Babu *et al.* 2011; Kim *et al.* 2013; Kumar *et al.* 2010; Yang *et al.* 2017). L-Glu exposure significantly enhanced NADPH oxidase (NOX) activity in the cerebral cortex and striatum (Guemez-Gamboa *et al.* 2011; Kim *et al.* 2013; Yang *et al.* 2017). An elevated number of neurons positive for the marker of oxidative damage, nitrosylated proteins, were detected in the striatum of animals subjected to L-Glu (Guemez-Gamboa *et al.* 2011). Cortical neuron peroxynitrite ($ONOO^{-}$) production level was significantly higher

in animals injected with L-Glu (Kumar *et al.* 2010). The perfusion of L-Glu into the striatum induced the formation of 2,3-dihydroxybenzoic acid (2,3-DHBA), reflecting a significant increase in the levels of hydroxyl radicals ($HO^{\bullet}$) (Ferger *et al.* 1998).

3.2.2.2.2 Neurotransmitter levels

Introduction of L-Glu into the striatum via microdialysis induced intracellular L-Glu build-up in astroglia and alanine but a reduced glutamine level in the neurons (Morales and Rodriguez 2012).

3.2.2.2.3 Mitochondrial dysfunction and apoptosis

L-Glu, stereotactically injected into the cerebral cortex, significantly reduced the MMP of cortical neurons (Babu *et al.* 2011; Kim *et al.* 2013; Kumar and Babu 2010; Kumar *et al.* 2010). In addition, a significantly diminished mitochondrial ATP level was recorded in animals receiving L-Glu in one study (Yang *et al.* 2017), but this was not statistically significant in another study (Mejía-Toiber *et al.* 2006). L-Glu perfusion into the left lateral ventricle significantly caused mitofusin 2 (MFN2) decline and mitochondrial fragmentation in spinal cord neurons (Wang *et al.* 2015). Furthermore, L-Glu stereotactically injected into the cerebral cortex induced mitochondrial dysfunction by causing sodium potassium adenosine triphosphatase (Na^{+}-K^{+}-ATPase) level reduction and a decline in the activity of mitochondrial cytochrome c oxidase (Yang *et al.* 2017).

L-Glu administration via stereotactic injection or infusion cannula induced the proapoptotic protein caspase-3 levels in the brain and spinal cord neurons (Babu *et al.* 2011; Kim *et al.* 2013; Kumar *et al.* 2010; Wang *et al.* 2015; Yang *et al.* 2017). In contrast, one study reported that caspase activation was not affected following intrastriatal L-Glu injection (Del Río and Massieu 2008). Animals stereotactically injected with L-Glu exhibited higher production of the proapoptotic protein caspase-9 (Babu *et al.* 2011; Kumar *et al.* 2010; Yang *et al.* 2017). Stereotactically injected L-Glu reduced antiapoptotic Bcl-2 gene expression and elevated pro-apoptotic Bax gene expression, such that the Bcl-2/Bax ratio was considerably reduced (Kumar *et al.* 2010; Yang *et al.* 2017). Additionally, the levels of phospho-extracellular signal-regulated

kinase (pERK) rose, leading to apoptosis in the cerebral cortex after stereotactic administration of L-Glu (Kim *et al.* 2013).

3.2.2.2.4 Calcium level

Stereotactic administration of L-Glu into the cerebral cortex resulted in significant elevation of intracellular levels of Ca^{2+} (Babu *et al.* 2011; Kim *et al.* 2013; Kumar and Babu 2010; Kumar *et al.* 2010), and the Ca^{2+}-dependent protease calpain was also triggered in response to L-Glu stereotaxic injection (Del Río and Massieu 2008; Guemez-Gamboa *et al.* 2011; Yang *et al.* 2017).

3.2.2.2.5 Neuroinflammation

Studies into the influence of L-Glu on neuroinflammation observed that its introduction into the brain triggered reactive astrogliosis (Morales and Rodriguez 2012) and microglial activation (Guemez-Gamboa *et al.* 2011). In contrast, a report evaluating continuous L-Glu administration concluded that it did not activate astrocytes or microglia in spinal cords (Wang *et al.* 2015). However, stereotactic injection of L-Glu induced the production of the proinflammatory cytokines TNF-α, IL-1β, and IFN-γ in the cerebral cortex (Babu *et al.* 2011; Kim *et al.* 2013; Kumar and Babu 2010; Yang *et al.* 2017).

3.2.2.2.6 Histological abnormalities

Studies that examined brain sections showed lesions in the striatum after L-Glu was introduced at concentrations of 500 nM and 0.3 M (Del Río and Massieu 2008; Greene and Greenamyre 1995). Lesions were observed after histological examination of sections from the brain cortex and striatum following L-Glu exposure at concentrations of 500 nM, 1 μM, and 0.25 M (Estrada-Sánchez *et al.* 2009; Estrada-Sánchez *et al.* 2010; Guemez-Gamboa *et al.* 2011; Mejía-Toiber *et al.* 2006; Zou *et al.* 2016). Data showed a higher number of degenerating fluoro jade-positive neurons in the striatum of L-Glu-exposed rodents (Estrada-Sánchez *et al.* 2009; Guemez-Gamboa *et al.* 2011). A histological study indicated that the cerebral cortex pyramidal neurons were significantly smaller in size in L-Glu-treated rats (Kumar and Babu 2010; Kumar *et al.* 2010). Another study reported an elevated number of neurons with positive terminal deoxynucleotidyl

transferase (dUTP) nick end labeling (TUNEL) in the cerebral cortex, indicating DNA fragmentation (Yang *et al.* 2017). However, brain infusion with L-Glu at 10 mM did not trigger any pathohistological features or alteration in brain tissue (Wang *et al.* 2015).

3.2.2.2.7 Behaviour and cognitive function

Stereotactic injection of 0.25 M L-Glu into the parietal cortex had no effects on learning ability when assessed via a Morris water maze (MWM) test (Zou *et al.* 2016). Similar results were found in a study reporting that a perfusion of 50 mM L-Glu via microdialysis into the striatum did not alter the locomotor activity or circling behaviour (Ferger *et al.* 1998).

3.2.2.2.8 Electroencephalogram (EEG)

Research assessing the electrical activity of the brain reported that after 500 nM/0.5 μL L-Glu stereotaxic injection, mice brains exhibited regular electrical activity (Estrada-Sánchez *et al.* 2009).

3.2.2.3 L-Glu administration directly to an animal's living media

L-Glu addition to the media of living animals induced oxidative stress through increased production of ROS (Chu *et al.* 2020; Spangenberg *et al.* 2004), superoxide ($O_2^{-\bullet}$) (Chu *et al.* 2020), and $NO^{\bullet}$ (Spangenberg *et al.* 2004). Depletion of GSH was detected following L-Glu administration to the growth medium of the *Caenorhabditis elegans* nematode (Chu *et al.* 2020). Furthermore, 5 mM L-Glu in the artificial seawater of the ephyrae of *Aurelia aurita* raised the Ca^{2+} level in the animal's sensory organ, rhopalium (Spangenberg *et al.* 2004). Administration of L-Glu into the media of *C. elegans* and the ephyrae of *Aurelia aurita* also affected the locomotory ability of these animals (Chu *et al.* 2020; Spangenberg *et al.* 2004).

3.3 Discussion

Several organisations, such as the USA Food and Drug Administration (FDA), the European Food Safety Authority (EFSA), and the Joint FAO/WHO Expert Committee on Food Additives (JECFA), have continually reasserted the safety of L-Glu (Zanfirescu *et al.* 2019). However, the EFSA panel re-evaluated L-Glu safety in 2017 and suggested that exposure to L-Glu that exceeded the acceptable daily intake (ADI) of 30 mg/kg bw per day in all age groups is linked to adverse health effects (Mortensen *et al.* 2017; Roberts *et al.* 2018). Nutritional analytical studies suggest that the daily intake of free L-Glu in humans is greater than 1 g and can reach 10 g/day, equivalent to 170 mg/kg bw, for a 60 kg person (Beyreuther *et al.* 2007; Maluly *et al.* 2017). As a result of the widespread global consumption of L-Glu, it remains a contentious issue, as excessive exposure has been associated with defects in neurophysiological function in both human and animal research (Ahanger *et al.* 2021).

This review considered whether the levels of L-Glu have pathogenic consequences for neurons and could therefore contribute to the development of certain NDDs, such as AD, PD, MS, ALS, and HD. This chapter highlighted the experimental evidence that supports the involvement of L-Glu in a number of toxic cellular mechanisms. L-Glu has an impact upon neuronal viability, oxidative stress, endogenous antioxidant defence, neuroinflammation, neurotransmitter levels, aberrant protein accumulations, excitotoxicity, mitochondrial dysfunction, intracellular Ca^{2+} levels, neuronal morphology, animal behaviour, and cognitive function.

However, two major mechanisms underpin L-Glu toxicity: receptor-mediated excitotoxicity and non-receptor-mediated oxidative stress, and they are integrated in parallel in neurons, as shown in Figure 3.2 (Mattson 2003; Lewerenz *et al.* 2013). In the receptor-mediated excitotoxicity, there is an excess of Ca^{2+} influx into neurons as a result of L-Glu overactivation of its receptors (AMPAR, NMDAR, and KAR) (Mattson 2003; Vincent and Mulle 2009) and due to the activation of VDCCs (Mattson 2007). Furthermore, stimulation of mGluR increases the synthesis of inositol triphosphate (IP_3) and the release of Ca^{2+} from ER stores (Mattson 2003). Pathologically high levels of Ca^{2+} ions result in the activation of Ca^{2+}-dependent protease enzymes, which can degrade

proteins in neurons, such as cytoskeletal proteins, and generate oxidative stress (Mattson 2003). Oxidative stress promotes neuron death by damaging crucial cell components, such as the cell membrane, proteins, and DNA (Mattson 2003). Ca^{2+} also mediates mitochondrial dysfunction, resulting in the release of proapoptotic proteins (Mattson 2003), and causes ER stress-induced cell death (Bahar *et al.* 2016). The X_c^- system can be involved in the non-receptor-mediated oxidative stress (Lewerenz *et al.* 2013). An excess of extracellular L-Glu blocks the X_c^- system, and this results in reduced cysteine, which is a required component for the production of the major cellular antioxidant, GSH (Lewerenz *et al.* 2013). This causes oxidative glutamate toxicity, also known as oxytosis, which causes cell death by oxidative stress via ROS accumulation (Lewerenz *et al.* 2013). Oxidative stress promotes the activation of numerous pathways, resulting in proinflammatory cytokine production and neuroinflammation (Figure 3.2) (Biswas 2016).

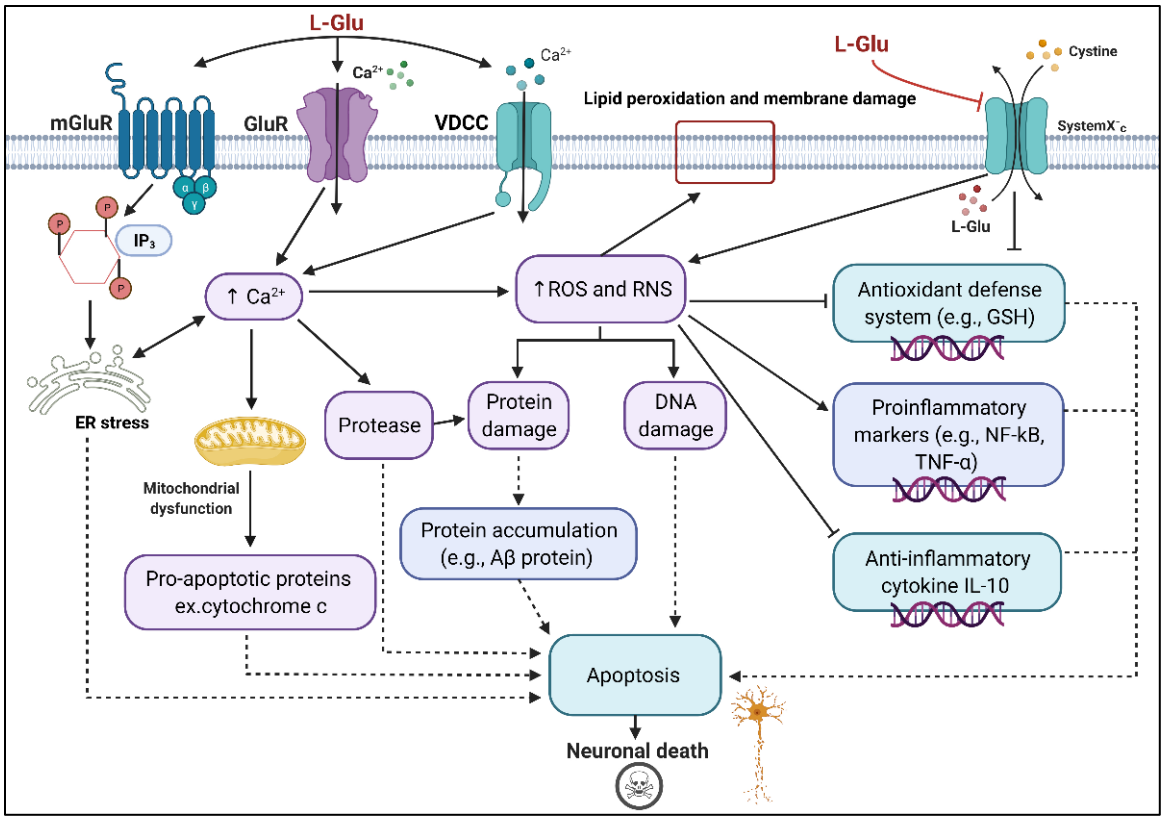

Figure 3.2: Schematic summary of the molecular mechanisms of L-Glu neurotoxicity. The binding of L-glutamate (L-Glu) to ionotropic L-Glu receptors (GluR) opens receptor channels, resulting in calcium ion (Ca^{2+}) influx. L-Glu also causes indirect opening of voltage-dependent Ca^{2+} channels (VDCCs) and further Ca^{2+} influx. L-Glu activation of metabotropic glutamate receptors (mGluR) results in increased inositol triphosphate (IP_3) synthesis, triggering Ca^{2+} release from endoplasmic reticulum (ER) stores. Collectively, a pathological level of Ca^{2+} ions triggers ER impairment and Ca^{2+}-dependent protease activation, which contributes to cellular protein damage and mitochondrial dysfunction, and neuronal apoptosis. High Ca^{2+} accumulation in neurons also leads to the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). L-Glu inhibits the X_c^- system, resulting in cystine depletion, an essential element for the production of the cellular antioxidant, glutathione (GSH). This impairs the endogenous antioxidant defence system and further induces redox stress. ROS and RNS cause lipid peroxidation, protein, and deoxyribonucleic acid (DNA) damage and induce the production of markers of inflammation such as nuclear factor kappa-light-chain-enhancer of activated B cell (NF-kB) activation, production of tumour necrosis factor-α (TNF-α), and inhibition of the production of the anti-inflammatory cytokine, interleukin 10 (IL-10), which collectively contributes to neuroinflammation and neuronal death. Protein modification and damage can also result in the accumulation of toxic proteins, such as amyloid beta (Aβ).

3.3.1 Common L-Glu neurotoxic pathways *in vitro* and *in vivo*

3.3.1.1 Cellular and molecular changes

The experimental evaluation of both cell-based studies and animal (*in vivo*) findings supports a toxic effect of L-Glu with diminished neuronal viability (Bebitoglu *et al.* 2020b; Bharate *et al.* 2018; Brizi *et al.* 2016; Chao and Hu 1994; de Vera *et al.* 2008; Gupta *et al.* 2013; Hamza *et al.* 2019; Hazzaa *et al.* 2020; Hu *et al.* 2012; Jeong *et al.* 2014; Kataria *et al.* 2012; Lee *et al.* 2019; Li *et al.* 2017a; Nampoothiri *et al.* 2014; Palumbo *et al.* 2012; Petroni *et al.* 2013; Sadek *et al.* 2016; Shah *et al.* 2015; Shah *et al.* 2016; Sun *et al.* 2016; Xin *et al.* 2019; Yang *et al.* 2019b; Yuksel *et al.* 2019; Zhu *et al.* 2016) and release of LDH (Chao and Hu 1994; Fallarini *et al.* 2009; Fouad *et al.* 2018; Hu *et al.* 2012; Kataria *et al.* 2012; Li *et al.* 2017a; Palumbo *et al.* 2012; Sadek *et al.* 2016; Yang *et al.* 2019b; Yon *et al.* 2018). L-Glu induces apoptosis via influencing the activity of several apoptotic factors, such as caspases-3 and 9, cytochrome c, and activation of intracellular signalling pathways, including MAPKs, AMPK, PARP, Fas ligand, Bax, and Bcl-2 (Dief *et al.* 2014; Hu *et al.* 2012; Kumar *et al.* 2010; Lee *et al.* 2019; Li *et al.* 2017a; Segura Torres *et al.* 2006; Yang *et al.* 2017; Yu *et al.* 2006; Zhu *et al.* 2016) (Table 3.2). Furthermore, cell growth and proliferation were perturbed, and markers of cellular structural changes and injury were observed (Hamza *et al.* 2019; Hazzaa *et al.* 2020; Kataria *et al.* 2012; Sadek *et al.* 2016; Yang *et al.* 2019b). Cellular damage from L-Glu was in part derived from the generation of redox stress and depletion of the antioxidant defence system. This included reduced SOD (Babu *et al.* 2011; Hamza *et al.* 2019; Hussein *et al.* 2017; Kim *et al.* 2013; Kumar and Babu 2010; Lee *et al.* 2019; Li *et al.* 2017a; Mohan *et al.* 2017; Onaolapo *et al.* 2016; Singh *et al.* 2003; Swamy *et al.* 2013; Thonda *et al.* 2014; Yang *et al.* 2017; Yang *et al.* 2019b; Yuksel *et al.* 2019), CAT (Babu *et al.* 2011; Hamza *et al.* 2019; Hussein *et al.* 2017; Kim *et al.* 2013; Kumar and Babu 2010; Lee *et al.* 2019; Li *et al.* 2017a; Mohan *et al.* 2017; Onaolapo *et al.* 2016; Singh *et al.* 2003; Swamy *et al.* 2013; Thonda *et al.* 2014; Yang *et al.* 2017; Yang *et al.* 2019b; Yuksel *et al.* 2019), and GSH activities (Babu *et al.* 2011; Chu *et al.* 2020; Hamza *et al.* 2019; Hussein *et al.* 2017; Kim *et al.* 2013; Kumar and Babu 2010; Kumar *et al.* 2010; Lee *et al.* 2019; Li *et al.* 2017a; Mohan *et al.* 2017; Sadek *et al.* 2016; Shah *et al.*

2016; Shivasharan *et al.* 2013; Singh *et al.* 2003; Swamy *et al.* 2013; Thonda *et al.* 2014; Yang *et al.* 2017; Yang *et al.* 2019b; Yuksel *et al.* 2019); inhibited GR (Babu *et al.* 2011; Kim *et al.* 2013; Kumar and Babu 2010; Yang *et al.* 2017), GPx (Hamza *et al.* 2019; Kim *et al.* 2013), and GST (Shivasharan *et al.* 2013); impact upon the expression of antioxidant regulatory proteins, Nrf2 (Shah *et al.* 2016; Yang *et al.* 2017), HO-1 (Shah *et al.* 2016; Yang *et al.* 2017); and GCLC (Yang *et al.* 2017), and thiol levels (Hamza *et al.* 2019; Shivasharan *et al.* 2013).

There were significant accumulations of ROS (Babu *et al.* 2011; Brizi *et al.* 2016; Chu *et al.* 2020; De Oliveira *et al.* 2019; Guemez-Gamboa *et al.* 2011; Kim *et al.* 2013; Kumar and Babu 2010; Kumar *et al.* 2010; Li *et al.* 2017a; Nampoothiri *et al.* 2014; Shah *et al.* 2016; Spangenberg *et al.* 2004; Xin *et al.* 2019; Yang *et al.* 2017; Yang *et al.* 2019b; Zhu *et al.* 2016), RNS (Babu *et al.* 2011; Guemez-Gamboa *et al.* 2011; Hussein *et al.* 2017; Kim *et al.* 2013; Kumar and Babu 2010; Kumar *et al.* 2010; Onaolapo *et al.* 2016; Shivasharan *et al.* 2013; Spangenberg *et al.* 2004; Yang *et al.* 2017; Yon *et al.* 2018), $O^{2-\bullet}$ (Chu *et al.* 2020), $HO^{\bullet}$ (Ferger *et al.* 1998), and H_2O_2 content (Bebitoglu *et al.* 2020b), as well as LPO build-up, following L-Glu exposure (Babu *et al.* 1994; Babu *et al.* 2011; Bebitoglu *et al.* 2020b; De Oliveira *et al.* 2019; Firgany and Sarhan 2020; Hazzaa *et al.* 2020; Hussein *et al.* 2017; Kim *et al.* 2013; Kumar and Babu 2010; Li *et al.* 2017a; Mejía-Toiber *et al.* 2006; Mohan *et al.* 2017; Penugonda and Ercal 2011; Sadek *et al.* 2016; Shivasharan *et al.* 2013; Singh *et al.* 2003; Swamy *et al.* 2013; Thonda *et al.* 2014; Yang *et al.* 1998; Yang *et al.* 2017; Yang *et al.* 2019b; Yuksel *et al.* 2019). Moreover, oxidative damage to proteins (De Oliveira *et al.* 2019; Guemez-Gamboa *et al.* 2011; Li *et al.* 2017a) and DNA (De Oliveira *et al.* 2019; Hussein *et al.* 2017) was induced *in vitro* and *in vivo* after L-Glu.

L-Glu induced a significant elevation in AChE activity *in vitro* and with relatively high L-Glu dosing *in vivo* (Khalil and Khedr 2016; Lee *et al.* 2019; Sadek *et al.* 2016). However, in contrast, AChE activity was reduced in other *in vivo* studies conducted at lower L-Glu dosing (Hamza *et al.* 2019; Hussein *et al.* 2017). A number of brain neurotransmitter levels were impaired after L-Glu application *in vivo*, such as L-Glu (Hussein *et al.* 2017; Khalil and Khedr 2016; Morales and Rodriguez 2012; Shah *et al.*

2015; Shah *et al.* 2016), dopamine (Hamza *et al.* 2019; Hussein *et al.* 2017), serotonin (Abdel Moneim *et al.* 2018; Hamza *et al.* 2019; Hussein *et al.* 2017), noradrenaline, and adrenaline (Hamza *et al.* 2019).

Both *in vivo* and *in vitro* studies demonstrated L-Glu impairment of mitochondrial function, which was observed through ATP (De Oliveira *et al.* 2019; Li *et al.* 2017a; Yang *et al.* 2017; Yang *et al.* 2019b) and MMP (Babu *et al.* 2011; De Oliveira *et al.* 2019; Kim *et al.* 2013; Kumar and Babu 2010; Kumar *et al.* 2010; Li *et al.* 2017a; Sun *et al.* 2016; Yang *et al.* 2019b; Zhu *et al.* 2016) diminution (Table 3.1 and Table 3.2). Furthermore, there was a reduction in the activity of a number of critical mitochondrial enzymes after L-Glu treatment (De Oliveira *et al.* 2019; Yang *et al.* 2017). L-Glu impaired mitochondrial fusion and triggered mitochondrial fragmentation in spinal cord neurons (Wang *et al.* 2015). As a consequence of L-Glu excitotoxicity, its administration *in vivo* and *in vitro* triggered increased intracellular Ca^{2+} levels (Babu *et al.* 2011; Fallarini *et al.* 2009; Gupta *et al.* 2013; Hazzaa *et al.* 2020; Hussein *et al.* 2017; Kim *et al.* 2013; Kumar and Babu 2010; Kumar *et al.* 2010; Palumbo *et al.* 2012; Shah *et al.* 2016; Sun *et al.* 2016; Swamy *et al.* 2013; Yang *et al.* 2019b). This resulted in Ca^{2+}-dependent protease calpain production (Del Río and Massieu 2008; Guemez-Gamboa *et al.* 2011; Yang *et al.* 2017), along with increased expression of L-Glu receptor subunits, including NMDAR (Gupta *et al.* 2013; Khalil and Khedr 2016; Rivera-Cervantes *et al.* 2004; Rivera-Cervantes *et al.* 2015) and AMPAR (Gupta *et al.* 2013; Rivera-Cervantes *et al.* 2004; Rivera-Cervantes *et al.* 2015; Segura Torres *et al.* 2006; Shah *et al.* 2016) subunits. Although the change in mGluR5 expression was nonsignificant, upregulation of the L-Glu receptors' expression resulted in Ca^{2+} release from intracellular stores, which triggered enzymatic overactivation, such as protein kinases, and led to cellular protein and membrane breakdown (Khalil and Khedr 2016). Furthermore, inhibitory GABA homeostasis was impaired after L-Glu exposure *in vivo* and *in vitro* (Chao and Hu 1994; Swamy *et al.* 2013).

Advanced activation of several proinflammatory markers, such as nuclear factor NF-kB (Shah *et al.* 2016), COX-2 (Hamza *et al.* 2019; Shah *et al.* 2016; Yon *et al.* 2018), IL-6 (Chaparro-Huerta *et al.* 2005; Chaparro-Huerta *et al.* 2008; Firgany and Sarhan 2020;

Yang *et al.* 2019b; Yon *et al.* 2018), TNF-α (Babu *et al.* 2011; Chaparro-Huerta *et al.* 2005; Chaparro-Huerta *et al.* 2008; Firgany and Sarhan 2020; Khalil and Khedr 2016; Kim *et al.* 2013; Kumar and Babu 2010; Shah *et al.* 2016; Yang *et al.* 2017; Yon *et al.* 2018; Yuksel *et al.* 2019), and IL-1β (Chaparro-Huerta *et al.* 2005; Chaparro-Huerta *et al.* 2008; Firgany and Sarhan 2020; Yang *et al.* 2017; Yang *et al.* 2019b), were reported after L-Glu administration *in vivo* and *in vitro*. Additional inflammatory mediators, such as activation of MMPs (Kataria *et al.* 2012), NLRP3 inflammasomes (Yang *et al.* 2019b), TGF-β, and HMGB1, were a consequence of neuronal culture exposure to L-Glu (Yon *et al.* 2018). Administration of L-Glu triggered the activity of the inflammatory biomarkers PGE2 (Hamza *et al.* 2019), PLA2s (Penugonda and Ercal 2011), and IFN-γ (Babu *et al.* 2011; Firgany and Sarhan 2020; Kim *et al.* 2013; Kumar and Babu 2010; Yang *et al.* 2017), and a decline in the anti-inflammatory cytokine IL-10 was observed in L-Glu-treated rats (Firgany and Sarhan 2020).

Collectively, NDDs are characterised histopathologically by the accumulation of extracellular, cytosolic, or nuclear protein oligomers and fibrils, and the formation of these is influenced by an array of protein PTMs (Schaffert and Carter 2020). For AD, the accumulation of extracellular Aβ peptide and intracellular hyperphosphorylated tau is thought to be toxic and contribute to neurodegeneration. L-Glu application triggered a significant increase in Aβ (1-42) accumulation in the brain tissue of rats (Dief *et al.* 2014; Fouad *et al.* 2018; Hussein *et al.* 2017), and increased levels of Aβ (1-40 and 1-42) were also observed in 3-month-old rats after neonate L-Glu administration (Jin *et al.* 2018a). L-Glu induced a nonsignificant increase in tau phosphorylation *in vitro* (Petroni *et al.* 2013), but neonatal exposure to MSG increased tau phosphorylation in approximately 3-month-old rats (Jin *et al.* 2018b) and 3- and 6-month-old mice (Hassaan *et al.* 2019; Špolcová *et al.* 2015). L-Glu also stimulated increased tau translation (Kobayashi *et al.* 2019).

Aberrant and neurotoxic protein aggregation could also be increased in response to other molecular mechanisms induced by L-Glu, including protein damage by redox stress such as oxidation of thiol groups and amino acids, and increased PCC (Levy *et al.* 2019), increased protein nitration, and altered levels of phosphorylation (Figure 3.3).

The common L-Glu neurotoxicity pathways reported *in vitro* and *in vivo* are summarised in Figure 3.4.

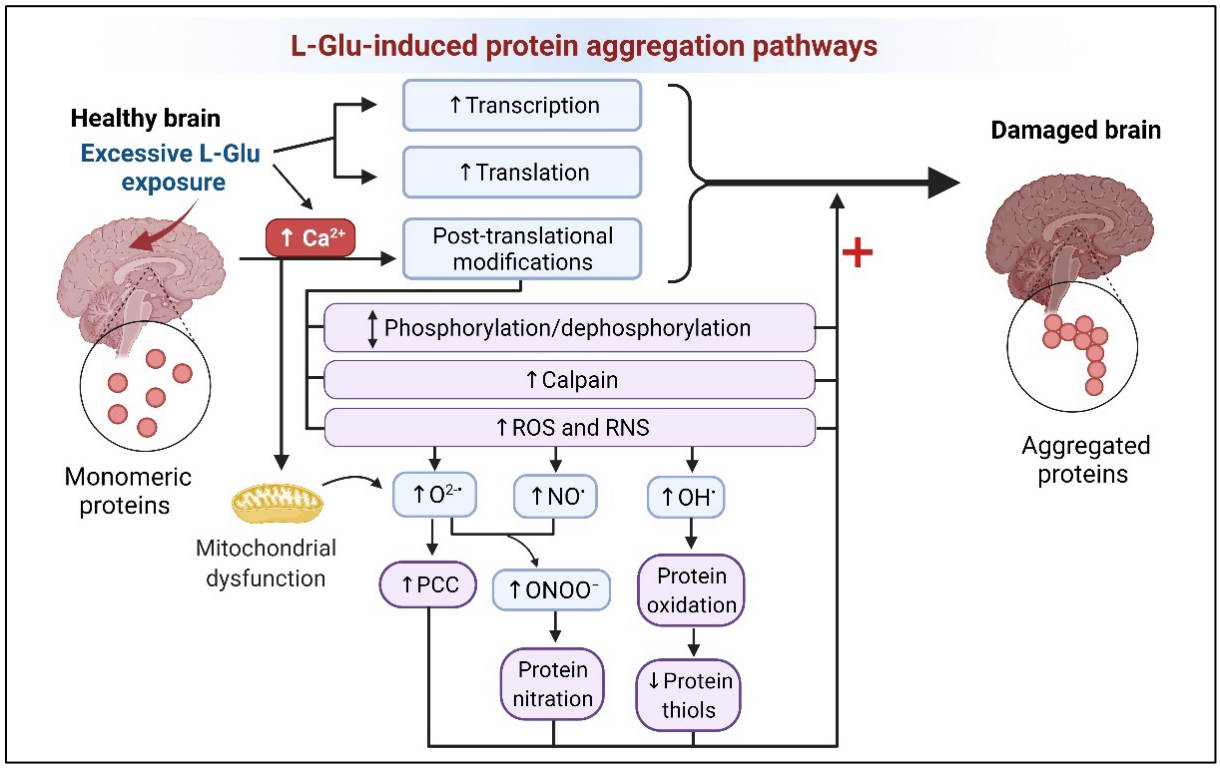

Figure 3.3: L-Glu induction of neurotoxic protein aggregation mechanisms reported from *in vitro* and *in vivo* model studies. Excessive L-Glu exposure can potentially induce protein aggregation in neurons via an influence on transcription, translation, or protein post-translation modifications (PTMs). High L-Glu in the synaptic cleft induces excitotoxicity, resulting in high Ca^{2+} influx into neurons, and this triggers protein PTMs. PTMs arise via the activation of kinases and/or phosphatases affecting the levels of protein phosphorylation/dephosphorylation, calpain activation, or increased oxidative stress (ROS or RNS production). Excessive Ca^{2+} also causes mitochondrial dysfunction, resulting in ROS leakage, including $O^{2-\bullet}$, which causes protein oxidation and protein carbonylation. $O^{2-\bullet}$ ions also interact with NO produced by nitric oxide synthase to produce reactive nitrogen species, such as $ONOO^-$, which covalently modify proteins via protein nitration. L-Glu also increases the production of $OH^\bullet$, which can also cause protein oxidation and oxidation of protein thiols. Collectively, these PTMs could alter protein conformation and promote misfolding and protein aggregation. Abbreviations: Ca^{2+}, calcium ions; $NO^\bullet$, nitric oxide; $O^{2-\bullet}$, superoxide; $OH^\bullet$, hydroxyl radical; $ONOO^-$, peroxynitrite; PCC, protein carbonyl content; RNS, reactive nitrogen species; ROS, reactive oxygen species. This figure was generated using BioRender (https://www.biorender.com/).

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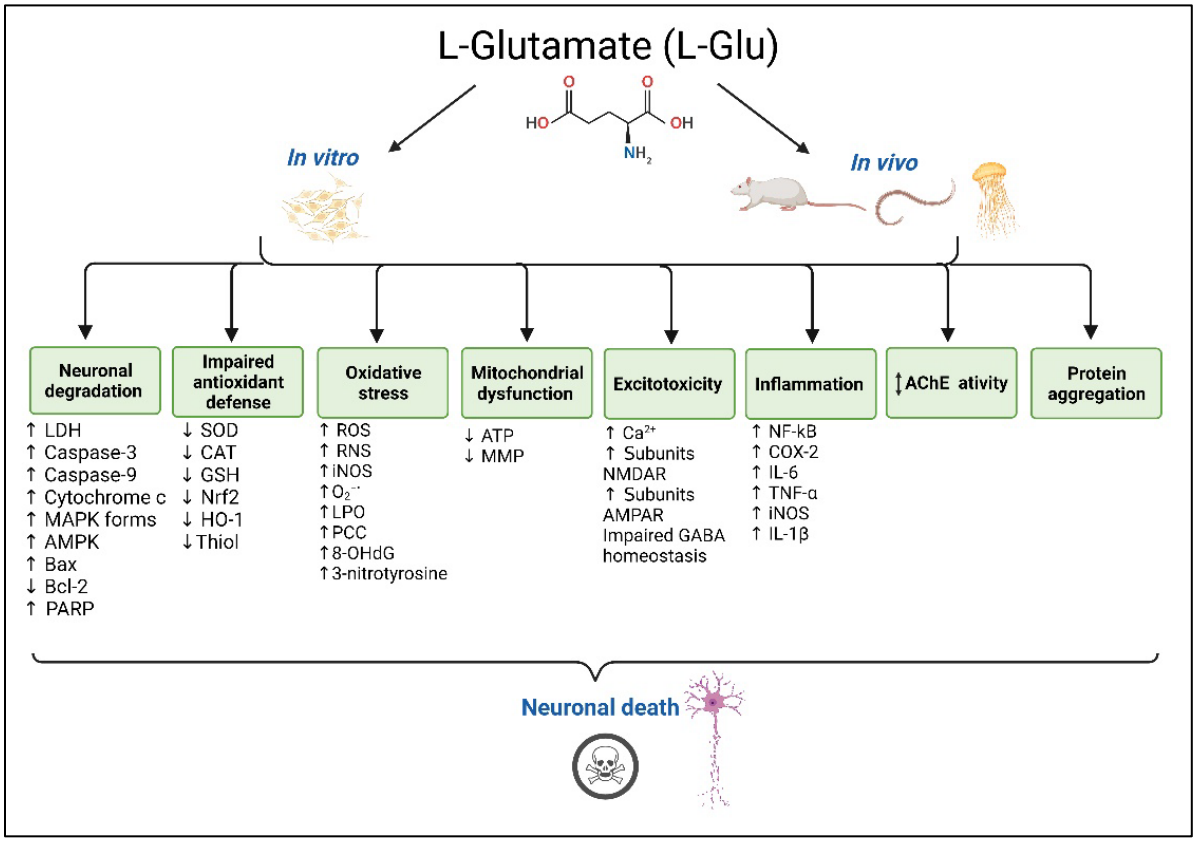

Figure 3.4: Schematic summary of the shared neurotoxicity mechanisms of L-Glu reported from *in vitro* and *in vivo* model studies. Abbreviations: AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; AMPK, 5′ AMP-activated protein kinase; ATP, adenosine diphosphate; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2; Ca^{2+}, calcium ion; CAT, catalase; COX-2, cyclooxygenase-2; GABA, gamma-aminobutyric acid; GSH, glutathione; HO-1, heme oxygenase-1; IL-1β, interleukin 1 beta; IL-6, interleukin 6; iNOS, nitric oxide synthase; LDH, lactate dehydrogenase; LPO, lipid peroxidation; MAPK, mitogen-activated protein kinase; MMP, mitochondrial membrane potential; NF-κB , nuclear factor kappa-light-chain-enhancer of activated B cells; NMDAR, N-methyl-D-aspartate receptor; Nrf2, nuclear factor erythroid 2-related factor 2; $O_2^{\bullet-}$, superoxide; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; PARP, poly (adenosine diphosphate (ADP)-ribose) polymerase; PCC, protein carbonyl content; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumour necrosis factor-α. This figure was created using BioRender (https://www.biorender.com/).

3.3.1.2 Neural structure changes

Significant alteration in morphological characteristics was observed after L-Glu introduction *in vivo* and *in vitro*, including changes to the neuronal cell body, nucleus, and processes (Bebitoglu *et al.* 2020b; Brizi *et al.* 2016; Burde *et al.* 1971; de Vera *et al.* 2008; Firgany and Sarhan 2020; Hamza *et al.* 2019; Hashem *et al.* 2012; Hazzaa *et al.* 2020; Hu *et al.* 2012; Hussein *et al.* 2017; Jeong *et al.* 2014; Kataria *et al.* 2012; Kumar and Babu 2010; Kumar *et al.* 2010; Lee *et al.* 2019; Mohan *et al.* 2017; Nampoothiri *et al.* 2014; Onaolapo *et al.* 2016; Palumbo *et al.* 2012; Rivera-Cervantes *et al.* 2004; Rivera-Cervantes *et al.* 2015; Swamy *et al.* 2013; Yang *et al.* 2017).

3.3.2 Other neuropathology observed after L-Glu administration *in vivo*

In addition to the common neuropathological outcomes evidenced with *in vitro* models, there were additional results obtained from *in vivo* models.

3.3.2.1 Brain structural changes

Brain haemorrhaging and neuroinflammatory cell aggregation were observed in several *in vivo* studies following L-Glu induction (Bodnár *et al.* 2001; Hashem *et al.* 2012; Hazzaa *et al.* 2020; Rivera-Cervantes *et al.* 2004), and L-Glu administration triggered gliosis *in vivo* (Guemez-Gamboa *et al.* 2011; Hazzaa *et al.* 2020; Morales and Rodriguez 2012; Shah *et al.* 2016).

3.3.2.2 Changes in behaviour and cognition

Reports addressing the influence of excessive levels of L-Glu on animal behaviour and cognitive ability demonstrated its influence on memory (Hazzaa *et al.* 2020; Thonda *et al.* 2014), locomotor coordination (Chu *et al.* 2020; Hazzaa *et al.* 2020; Shivasharan *et al.* 2013; Spangenberg *et al.* 2004; Swamy *et al.* 2013; Yu *et al.* 2006), and learning ability impairment (Abdel Moneim *et al.* 2018; Dief *et al.* 2014; Fouad *et al.* 2018; Ma *et al.* 2007; Yu *et al.* 2006), as well as behavioural phenotype changes, including anxiety (Dief *et al.* 2014; Mohan *et al.* 2017) and aggressiveness (Thonda *et al.* 2014; Swamy *et al.* 2013) (Table 3.2).

3.3.2.3 Brain weight and homeostasis changes

L-Glu systematic application caused a significant increase (Onaolapo *et al.* 2016) or decrease (Mohan *et al.* 2017) in the relative weight of mice brains. Alterations in brain ionic homeostasis, such as Na^+ and K^+ ion levels, were also observed (Hussein *et al.* 2017; Sadek *et al.* 2016; Swamy *et al.* 2013).

3.3.3 Study limitations

A common limitation in most of the models used to study L-Glu neurotoxicity is that the time used to monitor L-Glu neurodegeneration is typically short and included studies lasting hours and days to a few months, whereas in humans, excessive exogenous L-Glu exposure may be prolonged for years, in keeping with the gradual neuronal degradation that typifies NDDs. However, an understanding of acute L-Glu neurotoxicity may still be relevant to provide an insight into the molecular mechanisms that drive neuronal loss in NDDs and have an application to other diseases affected by L-Glu levels, such as stroke.

As summarised in Table 3.1, L-Glu neurotoxicity has been confirmed by human neuron studies *in vitro*. However, evidence from these studies showed that different neuronal cell lines have different reactions to L-Glu exposure. The SH-SY5Y cell line was the most resistant to L-Glu toxicity, whereas the most sensitive were primary neurons from human foetuses. This may be explained by the fact that in some cell lines, the L-Glu toxicity may occur through overactivation of specific L-Glu receptors, whereas in other cell lines lacking such receptors, the neurotoxicity may be manifested via alternative pathomechanisms, such as induction of redox stress (Kritis *et al.* 2015). Furthermore, the length of time associated with stimulation and the L-Glu concentration are important factors that will influence cell survival and death (Kritis *et al.* 2015), and these vary between studies and, for some, may represent the application of supraphysiological L-Glu concentrations (Sheldon and Robinson 2007). Additional variability between studies arose from differences in the preparation of L-Glu stock solutions either in cell culture media, PBS, or DMSO. Furthermore, *in vitro* studies invariably used isolated cells and therefore lack a blood–brain barrier (BBB) and the neuronal heterogeneity associated with the whole brain (Barbosa *et al.* 2015).

The findings of laboratory trials on animals reported many adverse impacts of L-Glu on neurological systems (Table 3.2). Most of the *in vivo* studies only considered acute neuronal damage, a few hours or days after the last treatment, whereas long-term effects and damage were much less often studied, with only five investigations performed that considered long-lasting neuronal damage after weeks to a month from the last L-Glu dose (Babu *et al.* 1994; Bodnár *et al.* 2001; Dief *et al.* 2014; Hazzaa *et al.* 2020; Singh *et al.* 2003; Yu *et al.* 2006; Zou *et al.* 2016). Surprisingly, neuronal damage was still evident after these potential periods of recovery, and the mechanisms of neurotoxicity were similar and sustained.

There were variability and limitations associated with the experimental design of some of the *in vivo* studies, including small sample sizes, broad and sometimes nonphysiological dosing, and a range of administration routes (Zanfirescu *et al.* 2019). Furthermore, the current review has highlighted that the majority of *in vivo* studies have utilised male animals, and a range of parts of the nervous system have been investigated (cerebral cortex, hippocampus, cerebellum, cerebral hemisphere, brain stem, diencephalon, forebrains, hypothalamus, circumventricular organs, spinal cord, pituitary gland, neural circuits, and rhopalia). However, a commonality of mechanisms exists, such that L-Glu neurotoxicity was similar in both sexes and analogous in the different regions analysed. Nevertheless, it appears that the route of exogenous L-Glu administration influences its neurotoxic potential *in vivo*. In the absence of gastrointestinal tract metabolism through L-Glu administration via *s.c.* and *i.p.* routes, or when administered directly into the brain through microdialysis or stereotactic or intrastriatal injection or via brain infusion canal, extensive neurotoxicity was evident at relatively low doses. Certainly, in humans, the L-Glu neurotoxic effects on the brain could in part be mitigated by reduced circulatory levels after gastrointestinal metabolism following oral ingestion (Ganesan *et al.* 2013).

Some of the studies in this review evaluated L-Glu toxicity at prenatal (Ma *et al.* 2007; Yu *et al.* 2006) and recent postnatal time points (Babu *et al.* 1994; Bodnár *et al.* 2001; Burde *et al.* 1971; Chaparro-Huerta *et al.* 2005; Chaparro-Huerta *et al.* 2008; Rivera-Cervantes *et al.* 2004; Rivera-Cervantes *et al.* 2015; Segura Torres *et al.* 2006; Shah *et al.* 2015; Shah *et al.* 2016). The brain of the foetus or newborn animals may be more

vulnerable to L-Glu damage than adult models due to an immature BBB (Samuels 2020). Furthermore, the studies that used *C. elegans* and the ephyrae of *Aurelia aurita* also lack a functional BBB, allowing L-Glu to readily diffuse into the neurological system and cause neurotoxic effects (Chu *et al.* 2020; Spangenberg *et al.* 2004).

3.4 Summary and conclusions

In summary, excessive L-Glu intake can have pathological consequences that result in the degeneration and death of neuronal tissue. The neurotoxicity of L-Glu is mediated by multiple cellular mechanisms, including induction of redox stress and depletion of antioxidant defence, mitochondrial dyshomeostasis, excitotoxicity, neuroinflammation, altered neurotransmitter levels, and influencing of the expression and aggregation potential of key proteins involved in neurodegenerative diseases. An improved understanding and appreciation of these diverse mechanisms should enable the design of more suitable agents, such as antioxidants, that can mitigate multiple elements of acute, subacute, or even chronic neurotoxicity and neuronal damage. Furthermore, clinical, and epidemiological studies may be needed to assess the potential harm to the public from excessive intake of exogenous L-Glu.

Chapter 4

Acai berry (*Euterpe sp*) extracts are neuroprotective against L-glutamate-induced toxicity by limiting mitochondrial dysfunction, cellular redox stress and excitotoxicity.

Chapter 4. Acai berry (*Euterpe* sp) extracts are neuroprotective against L-glutamate-induced toxicity by limiting mitochondrial dysfunction, cellular redox stress and excitotoxicity.

4.1 Introduction

The findings of this thesis demonstrated that excessive L-Glu intake have pathological consequences resulting in the degeneration and death of neuronal tissue that has been linked to NDDs and strokes (Chapter 3, Figure 3.4). Furthermore, animal models and human clinical studies have demonstrated that neurodegeneration detected in NDDs, and stroke correlates with pathologically raised L-Glu levels (Andersen *et al.* 2021; Madeira *et al.* 2018; Suzuki *et al.* 2022). Thus, targeting the pathways downstream of excessive L-Glu accumulation such as mitochondrial dysfunction, oxidative stress, and excitotoxicity might reduce the adverse consequences of excessive L-Glu and its impact on neurodegeneration in NDDs and stroke (Kuriakose and Xiao 2020; Prentice *et al.* 2015).

Acai berries (*Euterpe oleracea*) have neuroprotective properties include antioxidant and anti-inflammatory, as well as the ability to preserve proteins, calcium homeostasis, and mitochondrial function (Machado *et al.* 2016; Poulose *et al.* 2012; Poulose *et al.* 2014; Wong *et al.* 2013). Therefore, this chapter investigated the potential of the acai berry extracts to provide neuroprotection against excessive L-Glu-induced cell death using the human neuroblastoma SH-SY5Y cell line and differentiated human rhabdomyosarcoma cell line TE671. Moreover, the ability of acai berry extracts to prevent or limit L-Glu-induced mitochondrial damage and loss of function, and accumulation of ROS was also examined, and whether L-Glu neurotoxic effects were mediated by activation of ionotropic Glu-receptors. Additionally, L-Glu toxicity and the effects of acai berry on differentiated SH-SY5Y cells and human cortical neuronal progenitor cells (ReNcell CX cells) were investigated.

4.2 Results

4.2.1 Exposure of neuronal cells to L-Glu reduces cell viability

Exposure of undifferentiated SH-SY5Y cells to L-Glu at a concentration range of 0.137 - 100 mM for 24 or 48 hours reduced cell viability from 11.1 mM but this did not reach significance until 100 mM. Cell viability was significantly reduced after a 100 mM L-Glu exposure for 24 hours (29.7% reduction, $p < 0.001$), and 48 hours (47% reduction, $p < 0.0001$) (Figure 4.1A). There was no significant difference in cell viability between 24 and 48 hours of L-Glu exposure. The IC_{50} for L-Glu treatment was estimated as 148.6 mM after 24 hours and 93.16 mM after 48 hours. Interestingly, low L-Glu concentrations of 0.137 - 11.1 mM for 24 hours and 0.137 - 3.7 mM for 48 hours promoted a moderate but insignificant increase (approximately 10–14%) in absorbance values for the MTT assay compared to vehicle treated cells, consistent with increased cell proliferation (Figure 4.1A).

In general, 24- and 48-hour exposures to acai berry aqueous or ethanolic extracts were not toxic to neuronal cells at concentrations ranging from 0.001 - 1000 µg/mL (Figure 4.1B). Relatively low concentrations of aqueous extracts (0.01 - 100 µg/mL) triggered increased absorbance values in MTT assays, indicative of increased cell proliferation, although this did not reach significance (Figure 4.1B).

The neurotoxicity of L-Glu was confirmed via the induction of extracellular LDH (Figure 4.1C). A significant increase in LDH production was observed on L-Glu exposure for 48 hours compared with 24 hours exposure hours ($p < 0.0001$). After a 24 hour exposure, L-Glu increased LDH release at a concentration of 100 mM, and after a 48 hour exposure, L-Glu at 11.1, 33.3, and 100 mM induced significantly increased LDH production of 19% ($p < 0.001$), 32% ($p < 0.0001$), and 128% ($p < 0.001$), respectively (Figure 4.1C).

Mostly, acai berry extracts were not toxic to undifferentiated SH-SY5Y cells, however, a slight increase of LDH production (approximately 11%) from control levels was observed after a 24 hour exposure to 1 µg/mL of the aqueous extract (Figure 4.1D). Likewise, the acai berry ethanolic extract significant increased LDH release at concentrations of 0.01 to 1 µg/mL after 24 hour exposures (13-20% increase) and at 0.1 and 1 µg/mL after 48 hours (30 and 20% increase, respectively) (Figure 4.1D).

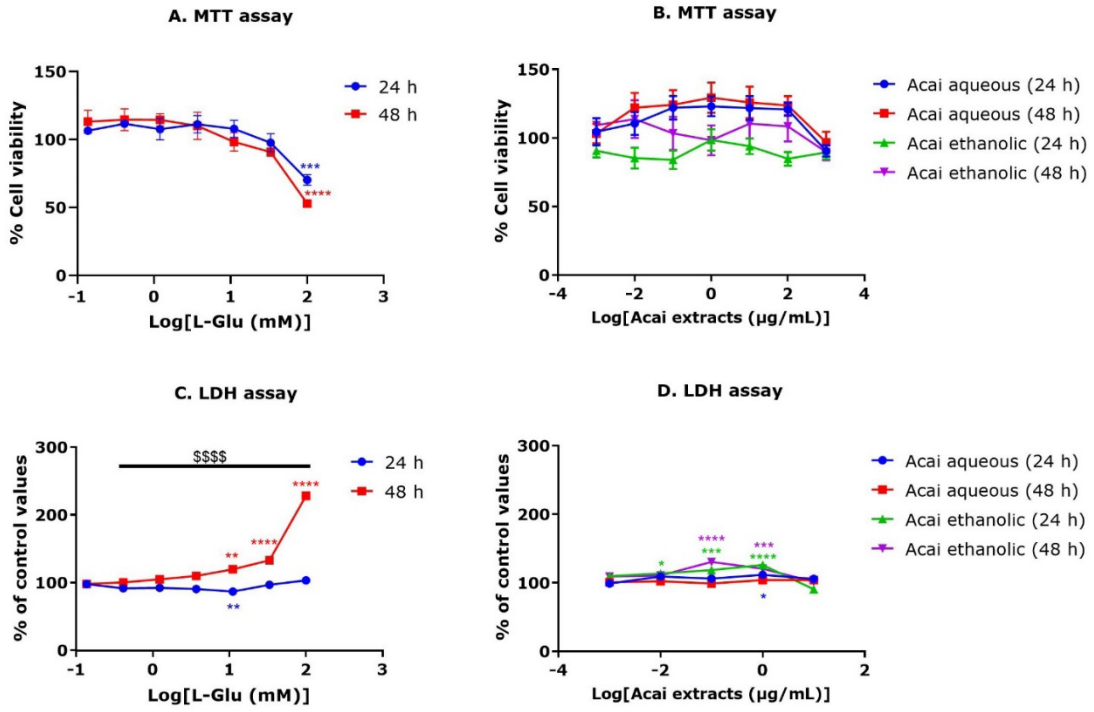

Figure 4.1: Cytotoxicity of L-Glu and acai berry extracts. Undifferentiated SH-SY5Y cells were treated with L-Glu or acai berry extracts for 24 or 48 hours and cytotoxicity determined using MTT (A,B) or LDH assays (C,D). Data points are means ± SEM of three independent experiments in triplicates (n = 3 per experiment). Statistical evaluation was done using One-way ANOVA followed by Dunnett's multiple comparisons post-test. For statistically significant changes: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ vs control untreated cells. The two-way ANOVA followed by Tukey's multiple comparisons post-test was used to evaluate the effect of time on L-Glu or acai berry extract exposure. The marked significance $^{\$\$\$\$}p < 0.0001$ represent the effect of L-Glu for 24 vs 48 hours.

As seen in Figure 4.2A, a L-Glu exposure to differentiated SH-SY5Y cells at a concentration range of 0.137 - 1.23 and at 100 mM for 24 hours reduced cell viability by (20-30%). In contrast, between 18-30 % increase in cell viability was observed after 3.7 - 33.33 mM L-Glu treatment for 24 hours. However, these results indicated earlier were not statistically significant. Remarkably, 48 hours of L-Glu treatment at concentration range (0.412 - 33.33) significantly induced an approximately 30 - 50% increase in cell viability compared to untreated cells (Figure 4.2A). Mostly, a range of concentrations between 0.001 and 1000 µg/mL of acai berry extracts did not have a significant toxic effect on differentiated SH-SY5Y cells when exposed for 24 and 48 hours (Figure 4.2B). However, 3 - 7% of cell viability reduction was observed after acai berry aqueous extract treatment at concentration range of 0.001 and 1000 µg/mL for 24 hours where more reduction about 7 - 24% after 48 hours treatment, but these results were not statistically significant (Figure 4.2B). Likewise, acai berry ethanolic extract caused an approximate 10 - 20% reduction in cell viability after 24 hours exposure. Whereas the 48 hours treatment of acai berry ethanolic extract induced a slight increase in viability around 3-7% at concentration range of 0.001 and 100 µg/mL but, at concentration of 1000 µg/mL caused 20% reduction in cell viability. However, all results indicated here were not statistically significant (Figure 4.2B).

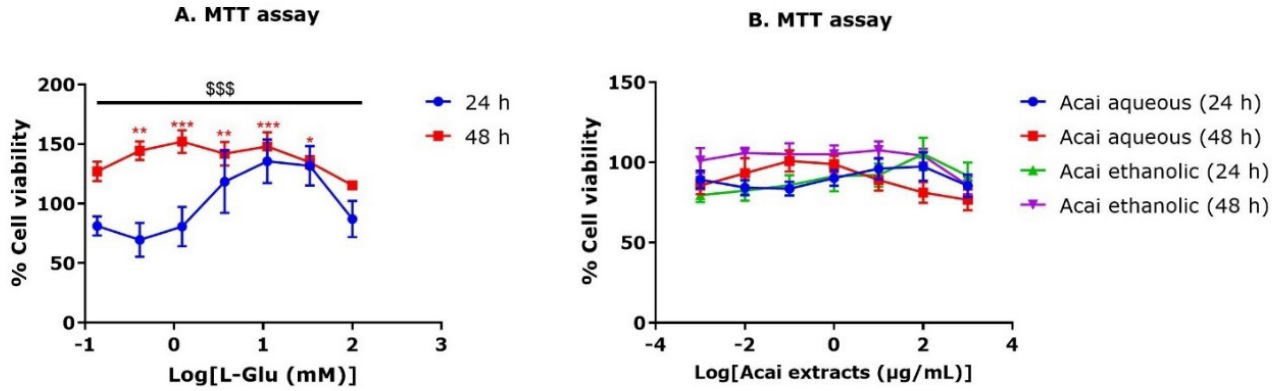

Figure 4.2: Cytotoxicity of L-Glu and acai berry extracts. Differentiated SH-SY5Y cells were treated with L-Glu or acai berry extracts for 24 or 48 hours and cytotoxicity was determined using MTT. Data points are means ± SEM of three independent experiments in triplicates (n = 3 per experiment). Statistical evaluation was done using One-way ANOVA followed by Dunnett's multiple comparisons post-test. For statistically significant changes: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ vs control untreated cells. The two-way ANOVA followed by Tukey's multiple comparisons post-test was used to evaluate the effect of time of L-Glu or acai berry extract exposure. The marked significance $^{\$\$\$}p < 0.001$ represented the effect of L-Glu for 24 vs 48 hours.

The impact of L-Glu on the viability of differentiated TE671 cells was determined using the MTT assay. A significant reduction in cell viability was observed after 24 hours when compared with 48 hours exposure ($p < 0.0001$). As shown in the Figure 4.3A, L-Glu influences cell metabolic activity in a concentration-dependent manner by means of a curve that exhibits various degrees of deviation from 100% viability. L-Glu at concentration range of 0.137 - 100 mM significantly decreased cell viability approximately 10 - 25 % after 24 hours exposure. In contrast, a 48 hour treatment of differentiated TE671 cells by L-Glu at concentration 0.137–1.23 mM did not exhibit any toxicity. Interestingly, 3.7 mM did significantly induce the cell viability (14% increase, $p = 0.0047$). Moreover, a significant loss of cell viability was observed after 48 hours L-Glu treatment at concentration of 33.33 and 100 mM (17% reduction, $p = 0.0008$, and 72% reduction, $p < 0.0001$, respectively). As a result of L-Glu treatment, the IC_{50} was estimated after 24 hours as 88.01 mM and after 48 hours as 67.03 mM.

MTT assay was used to investigate the impact of acai berry extracts treatment on differentiated TE671 cells growth and metabolic activity. Notably, a significant reduction was observed in cell viability with increased time exposure to acai berry aqueous and ethanolic extracts ($p < 0.0001$). However, acai berry aqueous extracts for 24 hours exposure at concentration 0.001 - 1000 µg/mL showed an increase in cell viability in differentiated TE671 cells of about 2 - 14% compared to the control; however, it was not statistically significant (Figure 4.3B). A slight decrease in cell viability was detected after 48 hours acai berry aqueous extract treatment of about 4-6% at concentrations 0.001 - 1 µg/mL but this was not statistically significant. A significant reduction of about 13 - 33% was seen after acai berry aqueous extract exposure for 48 hours at concentration 10, 100, and 1000 µg/mL ($p < 0.001$, $p < 0.0001$, $p < 0.0001$, respectively) (Figure 4.3B). Similarly, a significant cell viability reduction was observed at high concentrations (1 – 1000 µg/mL) of acai berry ethanolic extract after 24 (about 14 - 28% decrease), and 48 hours (14 - 77% decrease). A lower concentration of 0.001 µg/mL for 48 hours caused a significant reduction of about 16%, $p = 0.005$.

The production of LDH after L-Glu exposure confirmed its neurotoxicity on differentiated TE671 cells (Figure 4.3C). When L-Glu was administered to neurons at a concentration of 0.137 - 100 mM for 24 hours, LDH release from neurons increased at 100 mM only, but this finding was not statistically significant. Surprisingly, LDH production was significantly reduced (13 - 15%, $p = 0.01$, $p = 0.0062$, $p = 0.0021$, respectively) after 24 hours L-Glu exposure at concentrations of 3.7 - 33.33 mM compared with control cells. However, L-Glu exposure for 48 hours promoted LDH production in a concentration dependent manner with statistically significant increases at L-Glu concentrations of 33.33 and 100 mM (36%, and 136%, increase respectively) compared with control untreated cells. Compared with 24 hours of exposure to L-Glu, 48 hours exposure produced a significant increase in LDH production ($p < 0.0001$).

As shown in Figure 4.3D, mostly acai berry extracts had no toxic effects on differentiated TE671 cells. Remarkably, a reduction in LDH production was noticed after 24 hours acai berry aqueous extract introduction at concentrations of 1 and 10 µg/mL. Likewise, compared with untreated control cells, the acai berry ethanolic extract significantly decreased the loss of cell viability (reduced LDH levels) at a concentration of 10 µg/mL for 24 hours. Statistically significant increases in LDH production were observed after 48 hours of exposure to acai aqueous extract compared with 24 hours exposure ($p < 0.0001$).

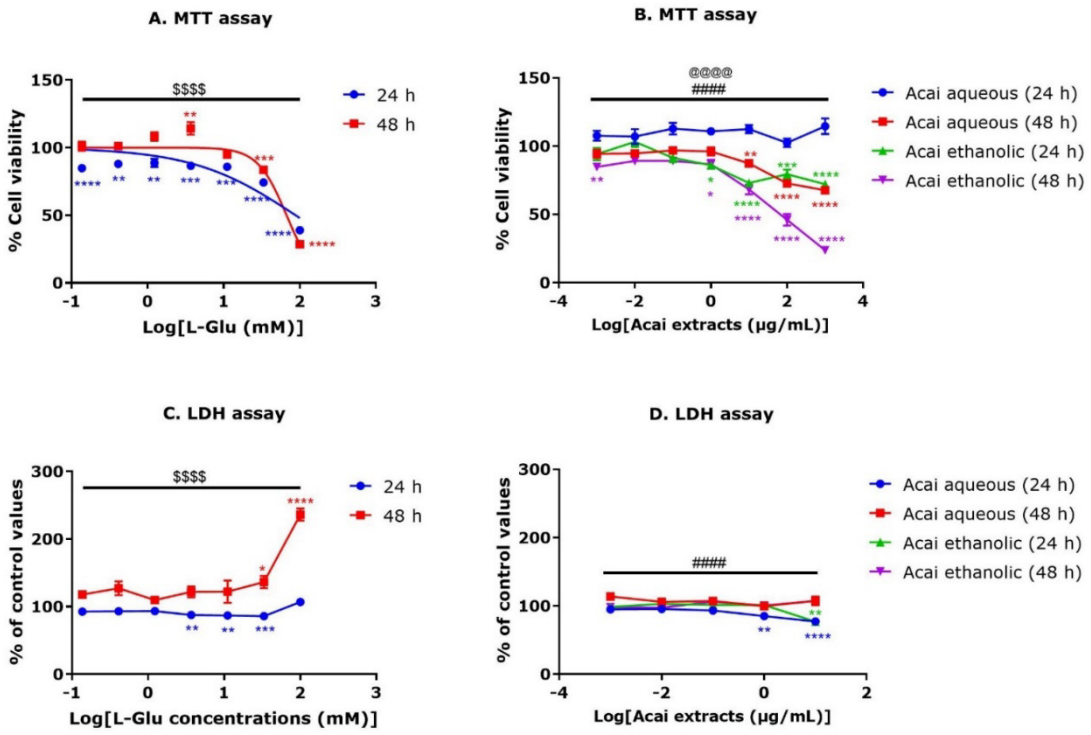

Figure 4.3: Cytotoxicity of L-Glu and acai berry extracts. Differentiated TE671 cells were treated with L-Glu or acai berry extracts for 24 or 48 hours and cytotoxicity was determined using MTT (A,B) or LDH assays (C,D). Data points are means ± SEM of three independent experiments in triplicates (n = 3 per experiment). Statistical evaluation was done using One-way ANOVA followed by Dunnett's multiple comparisons post-test. For statistically significant changes: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ vs vehicle control untreated cells. The two-way ANOVA followed by Tukey's multiple comparisons post-test was used to evaluate the effect of time in L-Glu or acai berry extracts exposure. The marked significance $^{\$\$\$\$}p < 0.0001$ represented the effect of L-Glu for 24 vs 48 hours while $^{\#\#\#\#}p < 0.0001$ acai aqueous extract 24 vs 48 hours and $^{@@@@}p < 0.0001$ acai ethanolic extract 24 vs 48 hours.

The MTT test detected cytotoxic effects of L-Glu on ReNcell CX cell viability. A significant reduction was observed in cell viability after 24 hours exposure to L-Glu when compared with 48 hours exposure ($p < 0.0001$). The 0.03 - 100 mM L-Glu treatments for 24 hours all caused a significant decrease in cell viability with an approximate 27 - 40% decline when compared to the control (Figure 4.4A). By comparison, there was no significant effect of 0.03 - 10 mM L-Glu concentrations on survival after 48 hours when compared with non-treated cells. An impact on cell viability was observed after 48 hours exposure to 30 and 100 mM L-Glu, which induced an approximately 23% and 51%, reduction in neuron growth, respectively. L-Glu treatment resulted in an IC_{50} of 86.88 mM after 24 hours and 88.67 mM after 48 hours.

The cell viability response of ReNcell CX cells to different concentrations (0.01 - 1000 µg/mL) of acai berry aqueous extract was analysed by MTT assay. Generally, 24 and 48 hours exposure to acai berry aqueous extract exhibited no-toxic impacts on neuron cells at concentrations ranging from 0.01 µg/mL - 100 µg/mL (Figure 4.4B). Addition of acai berry aqueous extract on ReNcell CX cells at 1000 µg/mL for 24 hours caused a slight non-significant decrease of approximately 12% in neuron viability, and this was significant (16% reduction) after 48 hours exposure (Figure 4.4B).

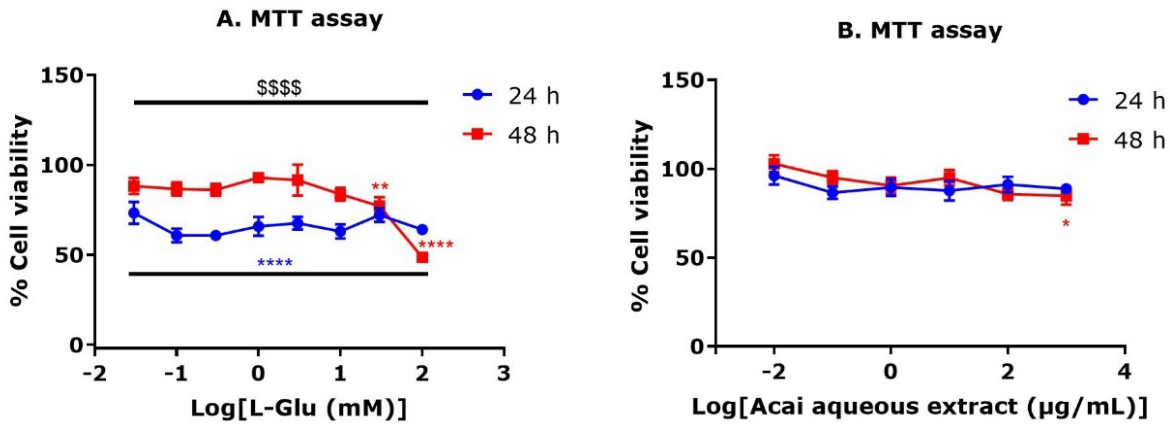

Figure 4.4: Effects of L-Glu and acai berry aqueous extract on ReNcell CX cells viability using MTT assay. ReNcell CX cells were treated with L-Glu (A) or acai berry aqueous extract (B) for 24 or 48 hours. Data are means ± SEM of three independent experiments in triplicates (n = 3 per experiment). Statistical evaluation was done using One-way ANOVA followed by Dunnett's multiple comparisons post-test. *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$ vs control (0) in the absence of L-Glu or acai berry extracts. The two-way ANOVA followed by Tukey's multiple comparisons post-test was used to evaluate the effect of time in L-Glu exposure. The marked significance $^{\$\$\$\$}p < 0.0001$ represented the effect of L-Glu for 24 vs 48 hours.

4.2.2 Certain concentrations of acai berry extracts provide neuroprotection against L-Glu-induced decreased cell viability

The neuroprotective capability of acai berry aqueous and ethanolic extracts against neurotoxicity induced by 100 mM L-Glu exposure for 24 and 48 hours were evaluated using an MTT assay in undifferentiated SH-SY5Y cells. Acai berry aqueous extract at concentrations of 0.01, 1, and 10 µg/mL increased cell viability by 11-27% after a 24 hour exposure (Figure 4.5A). The acai aqueous extract at concentrations ranging from 0.001-100 µg/mL was not neuroprotective against L-Glu after a 48 hour exposure, and the highest concentration of acai berry aqueous extract (1000 µg/mL) caused a further reduction in cell viability by approximately 20% when compared with L-Glu only (Figure 4.5B). Acai ethanolic extracts were not protective against 100 mM L-Glu after 24 hours, however, after 48 hours of exposure, 100 µg/mL significantly protected against L-Glu neurotoxicity and increased cell viability by 23% ($p < 0.0001$) (Figure 4.5C,D).
LDH levels were not significantly affected by L-Glu exposure after 24 hours (results not included), therefore, neuroprotection experiments at this time point were not conducted. There was a concentration dependent inhibition of 11 mM L-Glu-induced LDH production after a 48 hour co-application with both acai aqueous or ethanolic extracts (Figure 4.5E,F). The acai berry ethanolic extract co-incubated with L-Glu caused a significant reduction of LDH production induced by L-Glu of approximately 20% at concentrations of 1 and 10 µg/mL ($p < 0.01$ and $p < 0.05$, respectively) (Figure 4.5F). However, neither of the acai berry extracts was neuroprotective against the higher concentration of 100 mM L-Glu (Figure 4.5G,H).

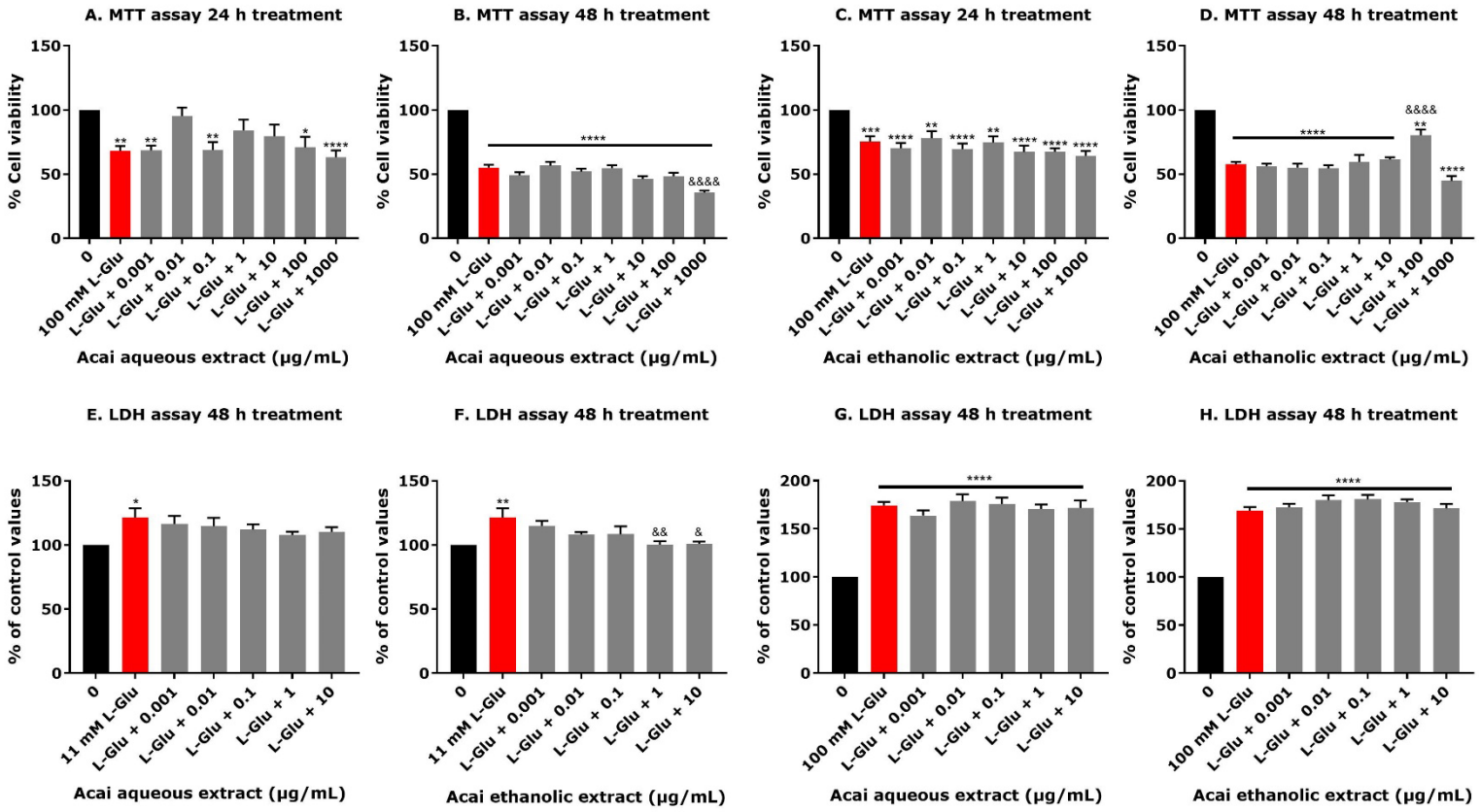

Figure 4.5: Neuroprotective effects of acai berry extracts in undifferentiated SH-SY5Y cells. The cytotoxicity of 11 or 100 mM L-Glu in combination with acai berry extracts was determined after 24 or 48 hours using MTT (A-D) or LDH assays (E-H). Data are means ± SEM of three independent experiments (n = 3 per experiment). Statistical evaluation was performed using one-way ANOVA followed by Tukey's multiple comparisons post-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs control in the absence of L-Glu and acai extract. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, and $^{\&\&\&\&}p < 0.0001$ vs L-Glu alone.

MTT assays were used to evaluate the neuroprotective ability of different concentrations of acai berry aqueous extract against neurotoxicity induced by 0.137 or 100 mM L-Glu exposure for 24 or 48 hours in differentiated TE671 cells. After 24 hours, all concentrations of acai berry aqueous extract promoted survival of cells by about 5 –34% that was reduced via 0.137 mM L-Glu alone, with a significant increase observed at lower concentrations of acai berry extract (0.001 - 1 µg/mL), even more than control cell levels ($p = 0.0197$ - $p < 0.0001$) (Figure 4.6A). A 100 mM L-Glu incubation for 24 hours induced about a 60% loss of cell viability that was prevented after applying acai berry aqueous extract which showed a concentration dependent protection against L-Glu with a significant protection of approximately 15-36 % vs L-Glu alone, at higher concentrations (1 - 1000 µg/mL) (Figure 4.6B). Acai berry aqueous extract at high concentrations of 100 and 1000 µg/mL demonstrated some protection against 100 mM L-Glu neuron death after 48 hours of exposure with about 11% increase in viability over L-Glu alone (Figure 4.6C).

All acai berry aqueous extract concentrations significantly reduced LDH production (about 40 % reduction, $p < 0.0001$) induced via 100 mM L-Glu application for 48 hours to control level (Figure 4.6D). Furthermore, the toxicity triggered via 100 mM L-Glu incubation for 48 hours was reduced after acai ethanolic extract treatment (above 17%, reduction) with significant results at a concentration range of 0.001 - 10 µg/mL (Figure 4.6E).

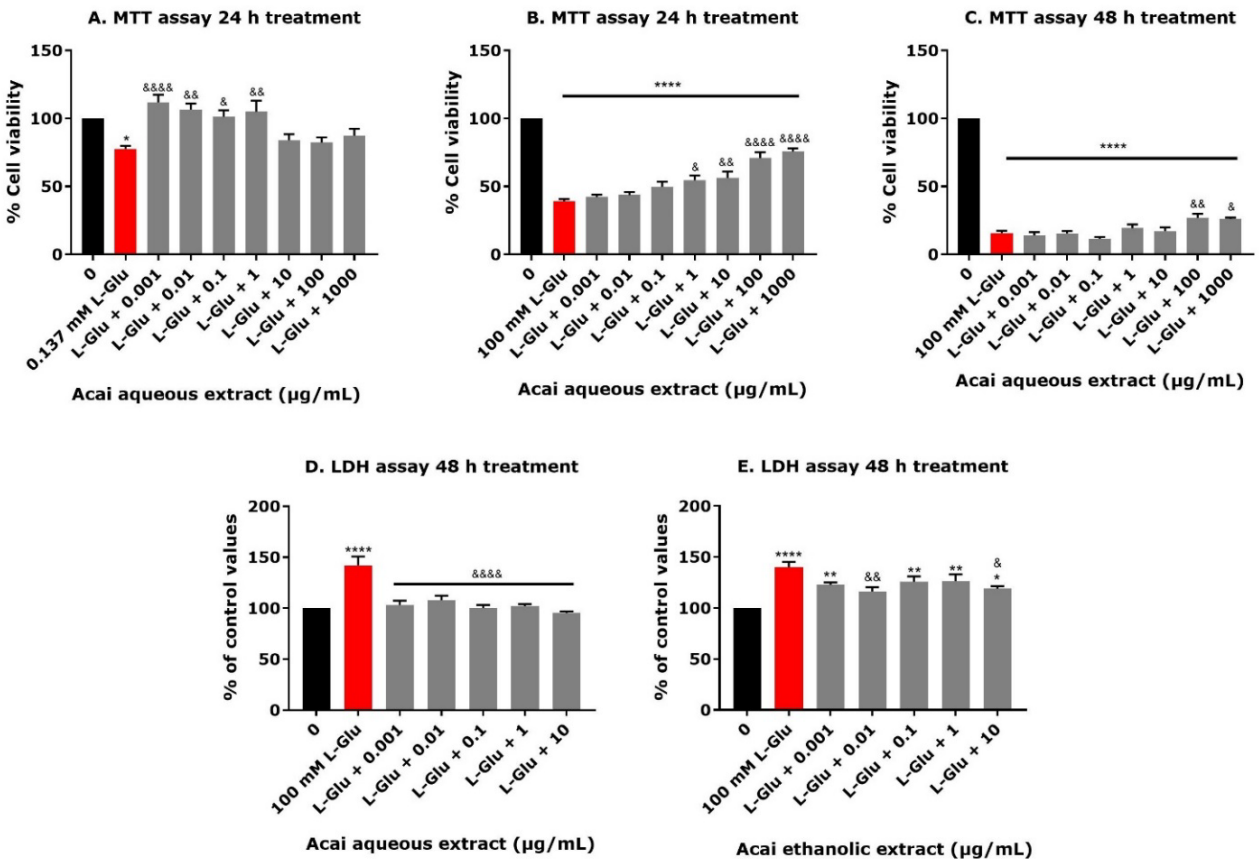

Figure 4.6: Neuroprotective effects of acai berry extracts. The cytotoxicity on differentiated TE671 cells exposed to 0.137 or 100 mM L-Glu and different concentrations of acai berry extracts were determined after 24 or 48 hours using an MTT (A-C) or LDH assays (D,E). Data are means ± SEM of three independent experiments (n = 3 per experiment). Statistical evaluation was done using One-way ANOVA followed by Tukey's multiple comparisons post-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ vs control in the absence of L-Glu and acai extract. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, and $^{\&\&\&\&}p < 0.0001$ vs 0.137- or 100-mM L-Glu.

MTT assay results showed that all concentrations of acai berry extracts exhibited protection from L-Glu toxicity when its aqueous extracts (0.0001 µg/mL - 100 µg/mL) were combined with L-Glu at 0.1 or 0.3 mM for 24 hours in ReNcell CX cells. All concentrations of acai berry extract co-applications significantly increased cell viability by approximately 15 - 40% after 24 hours exposure when compared with 0.1 mM L-Glu alone (Figure 4.7A). Likewise, significant increases in neuron viability were observed after acai berry aqueous extract co-addition at 0.0001-100 µg/mL concentration with 0.3 mM L-Glu for 24 hours (Figure 4.7B). Co-application of acai berry aqueous extract at a concentration range of 0.0001 - 0.01 µg/mL with 0.3 mM L-Glu for 24 hours caused a concentration dependent increase in cell viability of around 8 - 24% compared to control levels.

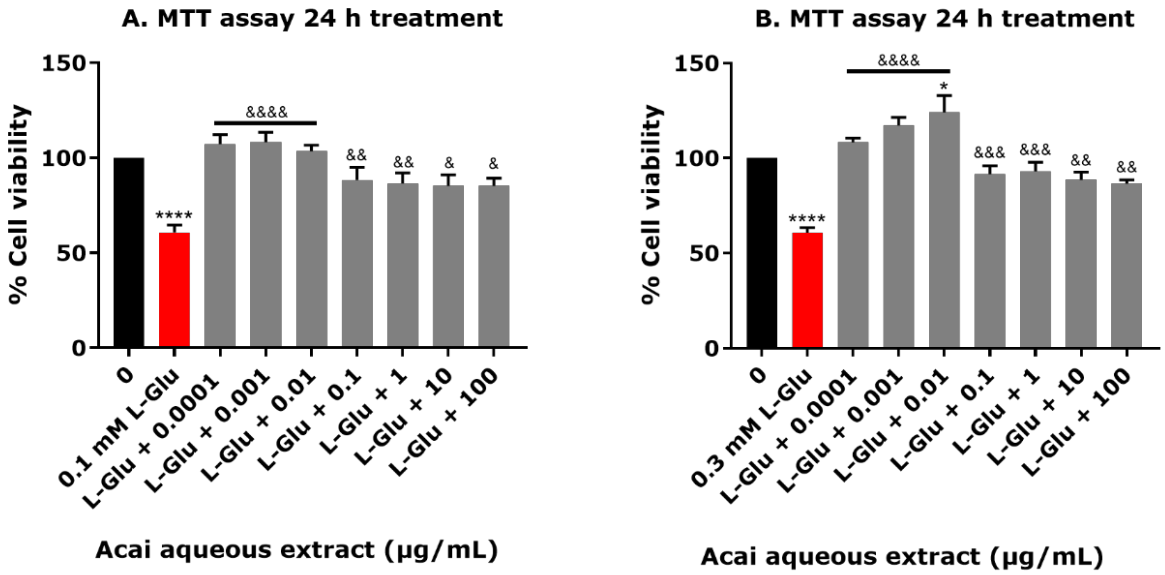

Figure 4.7: Neuroprotection of co-application of different concentrations of acai berry aqueous extract for 24 hours against 0.1 mM (A) or 0.3 mM (B) L-Glu in ReNcell CX cells. Data are means ± SEM of 3 independent experiments (n = 3 per experiment). Statistical evaluation was done using One-way ANOVA followed by Tukey's multiple comparisons post-test. *$p < 0.05$ and ****$p < 0.0001$ vs control (0) in the absence of L-Glu or acai berry extracts. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, and $^{\&\&\&\&}p < 0.0001$ vs 0.1 or 0.3 mM L-Glu.

4.2.3 Acai berry extracts preserve ATP levels that were diminished after L-Glu treatment

The application of L-Glu at 11.1 - 100 mM for 24 hours caused significant reductions in cellular ATP levels in undifferentiated SH-SY5Y cells, with a 33 - 55% decline and an estimated IC_{50} of 85.72 ± 26.82 mM (Figure 4.8A). A 48 hour incubation with L-Glu induced a higher depletion of cellular ATP and one initiated at a lower concentration of L-Glu (3.7 mM) to 87.3% ± 3.3 ($p < 0.05$), and reduced to 13.9% ± 0.64 ($p < 0.0001$) at the higher concentration of 100 mM L-Glu; with an estimated IC_{50} of 52.17 ± 4.26 mM (Figure 4.8A). A slight reduction in ATP levels was observed after acai berry extract application (Figure 4.8B). A 24 hour exposure to acai berry aqueous extract at 0.01 and 0.1 µg/mL had no effect on ATP levels but after 48 hours these caused a significant reduction in ATP levels by approximately 30 % (Figure 4.8B). Acai berry ethanolic extract similarly reduced ATP levels at concentrations above 0.01 µg/mL for a 24 hour exposure or above 0.1 µg/mL after 48 hours (Figure 4.8B). There was no statically significant difference in ATP level reduction with time exposure to L-Glu or acai berry extracts.

Generally, acai berry extracts provided protection from the decline in ATP levels caused by 11 or 100 mM L-Glu. Greater protection from 11 mM L-Glu was observed with the acai berry aqueous extract at the relatively low concentration range of 0.01 - 10 µg/mL, which significantly increased ATP levels by approximately 20%, and similarly, the acai berry ethanolic extract preserved ATP levels over the concentration range of 0.01-1 µg/mL (Figure 4.8 C,D). Protection against 100 mM L-Glu was evident with the acai berry aqueous extract at concentrations ranging from 0.01 - 1 µg/mL and with the acai berry ethanolic extract at concentrations of 0.01 and 0.1 µg/mL (Figure 4.8 E,F). However, the highest concentration of the acai berry aqueous extract (1000 µg/mL) further reduced ATP levels (Figure 4.8E).

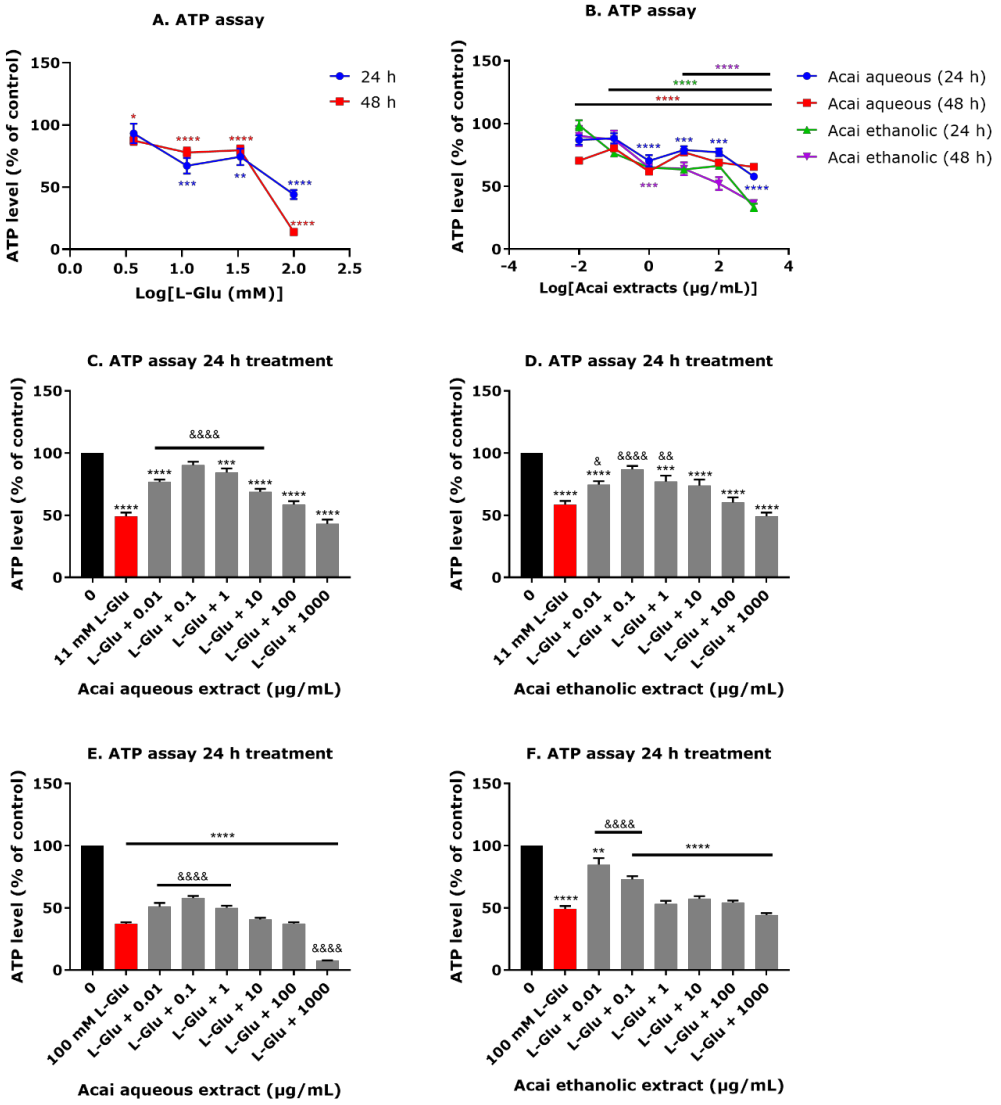

Figure 4.8: The effects of L-Glu and acai berry extracts on ATP levels. Undifferentiated SH-SY5Y cells were exposed to L-Glu (A) or acai berry extracts (B) for 24 or 48 hours or exposed to 11 or 100 mM L-Glu and different concentrations of acai berry extracts for 24 hours (C-F), and ATP levels quantified. Values were normalized to the negative control providing an ATP level percentage relative to the vehicle control. Results were expressed as the mean ± SEM for three separate experiments at each concentration (n = 3 per experiment) and analysed using one-way ANOVA with Dunnett's multiple comparisons. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ vs control (0) in the absence of L-Glu and acai berry extracts. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, and $^{\&\&\&\&}p <$ 0.0001 vs L-Glu alone. The two-way ANOVA followed by Tukey's multiple comparisons post-test was used to evaluate the effect of time of L-Glu or acai berry extracts exposure.

Figure 4.9A shows L-Glu induced a concentration dependent reduction in ATP level in differentiated TE671 cells. Compared to 24 hours of exposure to L-Glu, 48 hours of exposure caused a significant reduction in ATP level ($p < 0.0001$). L-Glu treatment for 24 hours at concentrations of 1.2 - 100 mM triggered a decrease in ATP level with significant decreases seen at 11.11, 33.3, and 100 mM with 22, 30, and 68% decline, respectively, and an estimated IC_{50} of 58.17 ± 10.18 mM (Figure 4.9A). A significant reduction in ATP level in a concentration dependent way was demonstrated after L-Glu 48 hours treatment at 0.137 - 100 mM with 30-60% drop in ATP level and estimated IC_{50} of 18.79 ± 4.578 mM (Figure 4.9A).

Concentration dependent reduction in ATP level was detected after acai berry extract application (Figure 4.9B). Exposure to acai berry aqueous extract for 24 hours reduced the level of ATP significantly compared to 48 hours exposure ($p < 0.0001$). Acai berry aqueous extract at concentrations 0.01 - 1000 µg/mL with 24 hours incubation caused a significant decrease in cellular ATP of approximately 23 - 69 % and a similar significant reduction after 48 hours observed at a concentration range of 0.1 to 1000 µg/mL (Figure 4.9B). The acai ethanolic extract treatment for 24 hours induced a significant drop in ATP levels at only high concentrations of 1 - 1000 µg/mL with 21 - 70% decline, and after 48 hours exposure a reduction was observed in the same concentration range with 32 - 43% decline (Figure 4.9B).

Figure 4.9 C-F show that a 24 hour exposure of acai berry extracts protected against ATP level declines caused by 11 or 100 mM L-Glu induction. Low concentrations (0.01 - 1 µg/mL) of acai berry aqueous and ethanolic extracts significantly increased ATP levels by approximately 20 - 30% that were decreased by 11 mM L-Glu application alone (Figure 4.9 C,D). Acai berry aqueous extract at concentrations of 0.01 and 0.1 µg/mL significantly improved 100 mM L-Glu-reduced ATP levels in TE671 cells by 27% and 15%, respectively (Figure 4.9E). While acai berry ethanolic extract at a concentration range of 0.01 - 10 µg/mL significantly attenuated ATP reduction induced by 100 mM L-Glu (Figure 4.9F).

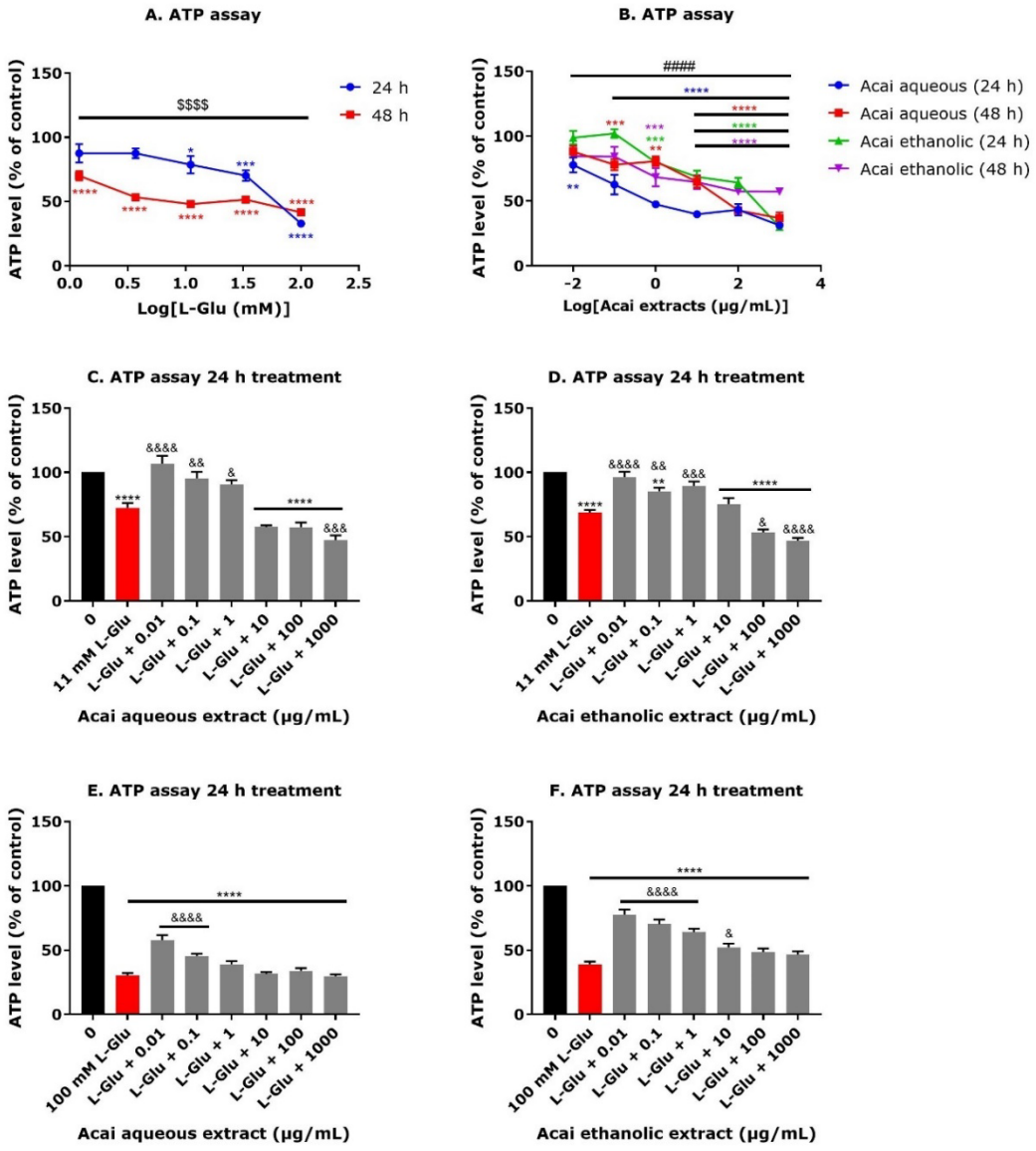

Figure 4.9: L-Glu and acai berry extract effects on ATP levels in differentiated TE671 cells. ATP levels in differentiated TE671 cells exposed to L-Glu (A) or acai berry extracts (B) for 24 or 48 hours and ATP levels after exposure to 11 or 100 mM L-Glu and different concentrations of acai berry extracts (C-F) for 24 hours. Values were normalized to the negative control providing an ATP level percentage relative to the vehicle control. Results were analysed using one way ANOVA with Dunnett's multiple comparison and expressed as the mean ± SEM for three separate experiments at each concentration (n = 3 per experiment). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ vs control (0) in the absence of L-Glu or acai berry extracts. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, and $^{\&\&\&\&}p < 0.0001$ vs L-Glu group. The two-way ANOVA followed by Tukey's multiple comparisons post-test was used to evaluate the effect of time of L-Glu or acai berry extract exposure. The marked significance $^{\$\$\$\$}p < 0.0001$ represented the effect of L-Glu for 24 vs 48 hours while $^{\#\#\#\#}p < 0.0001$ acai aqueous extract 24 vs 48 hours.

4.2.4 Acai berry extracts restored the MMP level that was reduced after L-Glu treatment

L-Glu at concentrations of 3.7 - 100 mM for 24 hours caused a significant reduction of MMP values in undifferentiated SH-SY5Y cells whereas an extension of the incubation period to 48 hours resulted in a return to control values (Figure 4.10A). For the acai berry extracts, only the aqueous extracts (0.001 - 1000 µg/mL) reduced the MMP (by approximately 30% after 24 hours), but values returned to control levels after a 48 hour exposure (Figure 4.10B). Acai berry extracts were protective against the reduced MMP in response to 11 and 100 mM L-Glu. A 24 hour exposure of the acai berry aqueous extract at concentrations of 0.001-100 µg/mL significantly restored MMP to control levels when compared to 11 mM L-Glu alone (Figure 4.10C). Likewise, the acai berry ethanolic extract restored the MMP to approaching control levels after a 24 hour 11 mM L-Glu treatment, with significant changes at concentrations of 0.001, 1, and 10 µg/mL (Figure 4.10D). For reductions of the MMP after 100 mM L-Glu, only the lowest concentration (0.001 µg/mL) of the acai berry aqueous extract resulted in a significant restoration of the MMP level (Figure 4.10E), whereas all of the acai berry ethanolic extract concentrations showed protection against 100 mM L-Glu effects on the MMP (Figure 4.10F).

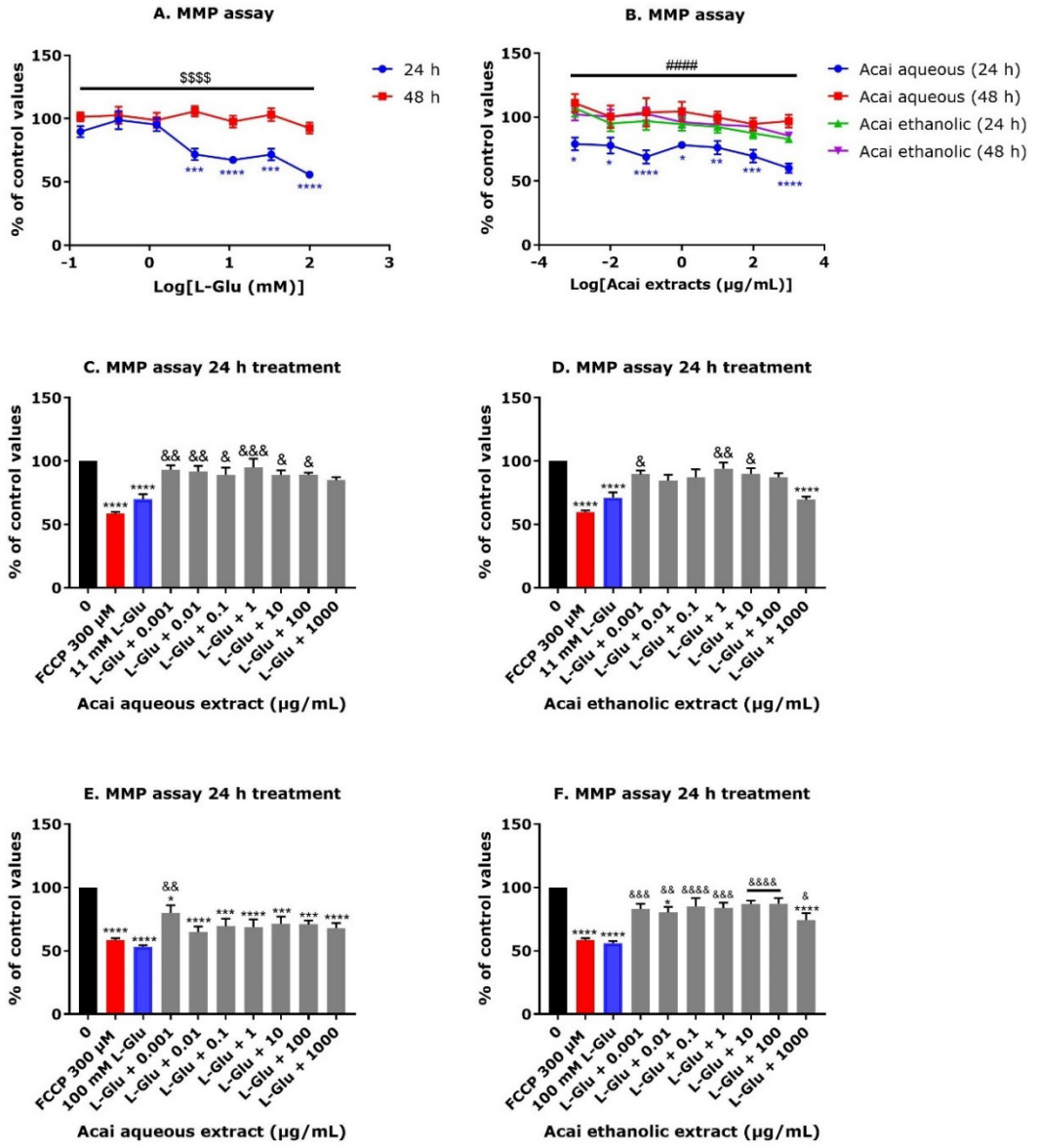

Figure 4.10: The effects of L-Glu and acai berry extracts on MMP levels. Undifferentiated SH-SY5Y cells were exposed to L-Glu (A) or acai berry extracts (B) for 24 or 48 hours or exposed to 11 or 100 mM L-Glu with different concentrations of acai berry extracts for 24 hours (C-F), and the MMP level quantified. As a positive control, cells were treated for 24 hours with 300 µM FCCP. Data values presented are means ± SEM for three separate experiments at each concentration (n = 3 per experiment). Statistical evaluation was done using One-way ANOVA followed by Dunnett's multiple comparisons post-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ vs control (0) in the absence of L-Glu, acai berry extracts and FCCP. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, and $^{\&\&\&\&}p < 0.0001$ vs L-Glu alone. The two-way ANOVA followed by Tukey's multiple comparisons post-test was used to evaluate the effect of time of L-Glu or acai berry extract exposure. The marked significance $^{\$\$\$\$}p < 0.0001$ represented the effect of L-Glu for 24 vs 48 hours while $^{\#\#\#\#}p < 0.0001$ for acai berry aqueous extract 24 vs 48 hours.

As Figure 4.11A demonstrates, L-Glu decreased MMP level in differentiated TE671 cells. MMP levels were significantly lower with increased time of exposure to L-Glu ($p < 0.0001$). Exposure to L-Glu at concentrations in the range 0.412 - 100 mM for 24 hours significantly decreased the MMP level by approximately 12-20% (Figure 4.11A). A greater decrease was observed after introduction of L-Glu at concentrations of 0.137 - 100 mM for 48 hours with 10 - 30% MMP level reduction.

Acai berry extracts had some influences to MMP levels as shown in Figure 4.11B. Acai berry aqueous extract at concentrations of 0.001 - 1000 µg/mL significantly induced 10 - 45% reduction in MMP level after a 24 hour incubation. A 48 hours incubation of acai berry aqueous extract at the same concentrations showed a similar effect on the MMP level; a significant concentration dependent reduction (13 - 45%). Moreover, a high concentration (100 µg/mL) of acai berry ethanolic extract application for 24 hours promoted a decline of the MMP level by 22% and a significant further reduction was noted at 1000 µg/mL with 51% decline; in the same way, 48 hours exposure to 100 and 1000 µg/mL caused significant reductions of 40% and 69%, respectively.

A 24 hour incubation of acai berry extracts prevented MMP reduction induced by 11 mM L-Glu on differentiated TE671 cells as shown in Figure 4.11 C,D. Acai aqueous extract at concentrations of 0.001 - 10 µg/mL significantly increased L-Glu-reduced MMP level by 20 - 26% (Figure 4.11C). Furthermore, 100 µg/mL acai berry aqueous extract showed an increase in the reduced MPP level, but the result was not statistically significant. In contrast, acai berry aqueous extract at 1000 µg/mL was incapable of protecting against L-Glu-induced MMP reduction. Acai berry ethanolic extract at 0.01 - 10 µg/mL showed a significant concentration dependent increase in L-Glu diminished MMP level by approximately 20 - 35% as illustrated in Figure 4.11D. However, a high concentration (1000 µg/mL) of acai berry ethanolic extract failed to protect from L-Glu-induced MMP reduction and more of an MMP reduction was observed.

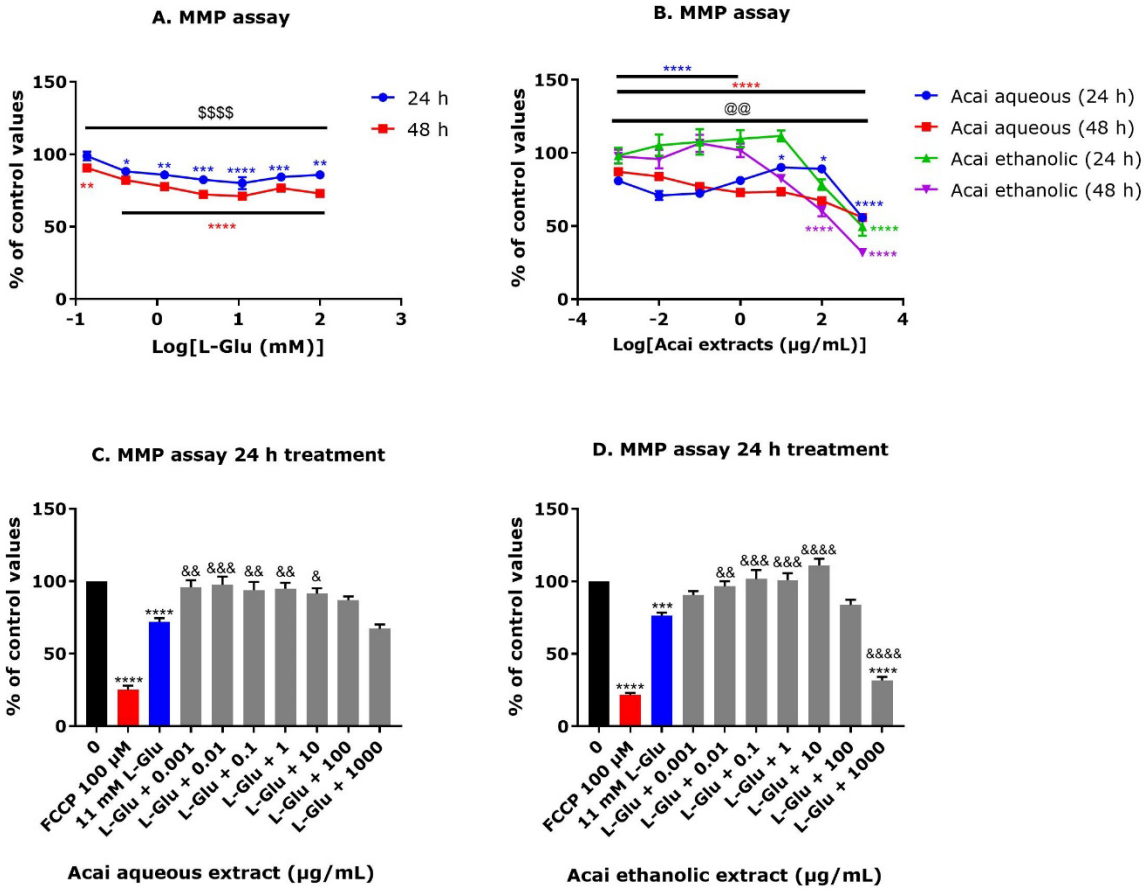

Figure 4.11: The effect of L-Glu and acai berry extracts on MMP levels in differentiated TE671 cells. MMP levels after exposure to L-Glu (A) or acai berry extracts (B) for 24 and 48 hours. MMP level in differentiated TE671 cells exposed to 11 mM L-Glu and different concentrations of acai berry extracts after 24 hours; acai berry aqueous (C) and ethanolic (D) extracts. As a positive control, cells were treated for 24 hours with 100 µM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP). The data presented are means ± SEM for three separate experiments at each concentration (n = 3 per experiment). Statistical evaluation was done using One-way ANOVA followed by Dunnett's multiple comparisons post-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ vs control (0) in the absence of L-Glu or acai berry extracts or FCCP. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, and $^{\&\&\&\&}p < 0.0001$ vs L-Glu group. The two-way ANOVA followed by Tukey's multiple comparisons post-test was used to evaluate the effect of time in L-Glu or acai berry extracts exposure. The marked significance $^{\$\$\$\$}p < 0.0001$ represented the effect of L-Glu for 24 vs 48 hours while $^{@@}p < 0.01$ for acai berry ethanolic extract 24 vs 48 hours.

4.2.5 Acai berry extracts significantly reduced ROS production induced by L-Glu

The levels of ROS induced after undifferentiated SHSY-5Y cell exposure to L-Glu were quantified using a DCFHDA assay. Treatment of cells with L-Glu for 3 hours induced ROS in a concentration dependent way, which reached significance at concentrations of 11.1, 33.3, and 100 mM (56, 50, and 91% increase from controls, respectively) (Figure 4.12A). Greater ROS levels were observed after 6 hours L-Glu exposure at lower concentrations of 0.412 - 11.1 mM by approximately 54 - 115 % (Figure 4.12A). In general, an incubation with acai berry extracts for 3 and 6 hours showed no increase in ROS production except for the 0.1 µg/mL of the aqueous extract for 6 hours as well as 0.01 and 1000 µg/mL concentrations of the ethanolic extract for 3 hours and 0.01 µg/mL for 6 hours (Figure 4.12B). The acai berry extracts exhibited antioxidant effects and reduced the ROS induced by L-Glu to levels similar to controls and for the ethanolic extracts; co-exposure displayed a concentration dependent reduction of ROS levels (Figure 4.12 C,D).

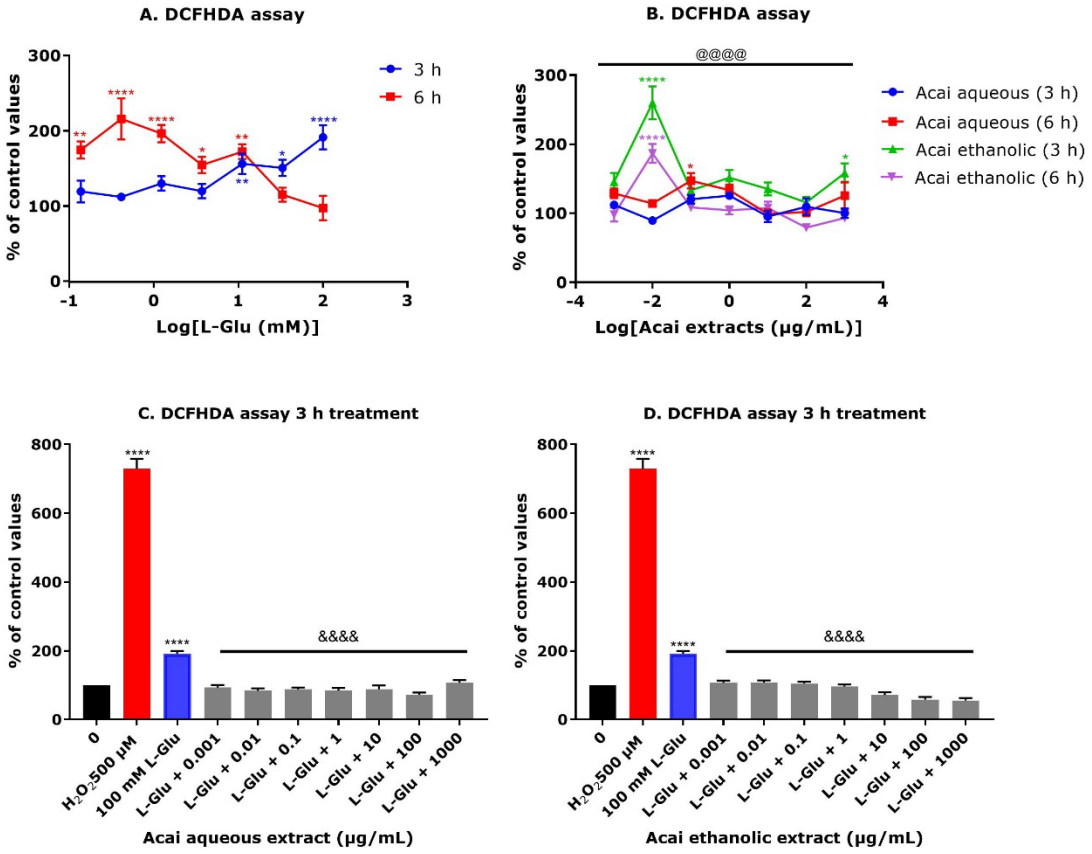

Figure 4.12: L-Glu and acai berry extract effects on cellular ROS levels. Undifferentiated SH-SY5Y cells were treated with L-Glu (A), acai berry extracts (B), or 100 mM L-Glu with different concentrations of acai berry extracts (C,D), and the levels of ROS quantified using a DCFHDA assay. Hydrogen peroxide (H_2O_2) at 500 µM for 30 minutes was used as a positive control for ROS generation. Data values presented are means ± SEM for three separate experiments at each concentration (n = 3 per experiment). Statistical evaluation was done using One-way ANOVA followed by Dunnett's multiple comparisons post-test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$ vs control in the absence of L-Glu, acai berry extracts, and H_2O_2. $^{\&\&\&\&}p < 0.0001$ vs L-Glu alone. The two-way ANOVA followed by Tukey's multiple comparisons post-test was used to evaluate the effect of time of L-Glu or acai berry extract exposure. The marked significance $^{@@@@}p < 0.0001$ is for acai berry ethanolic extract 3 vs 6 hours.

ROS levels were significantly increased with increased time of exposure to L-Glu ($p < 0.0001$). Concentration-dependent ROS generation was observed in differentiated TE671 cells exposed to L-Glu at a concentration range of 0.137 - 100 mM for 3 h and 6 h (Figure 4.13A). After 3 hours L-Glu incubation triggered significant increases in ROS production around 40 - 58% at concentrations of 11.1 - 100 mM. After 6 hours incubation, all L-Glu concentrations (0.137 - 100 mM) caused significant rises in ROS production in a concentration dependent manner starting from 70 - 183% increase compared to the control level.

Figure 4.13B illustrates that acai berry extracts induced some ROS production. Acai berry aqueous extract after a 3-hour exposure at concentrations of 0.001 - 10 µg/mL, significantly increased ROS levels in differentiated TE671 cells by 60 - 96% above control level. However, after 6 hours exposure of acai berry aqueous extract at concentrations in the range 0.001 - 1000 µg/mL, there was no induced ROS production in differentiated TE671 cells. Acai berry ethanolic extract at concentration of 1 - 1000 µg/mL significantly induced ROS levels by 65 - 216% after 3 hours exposure (Figure 4.13B). In contrast, only 1000 µg/mL of acai berry ethanolic extract for 6 hours exposure significantly increased ROS levels in cells by approximately 271% of control.

Acai berry extract protected neurons from ROS-induced via L-Glu as shown in Figure 4.13 C-F. Co-application of acai berry aqueous extract at a concentration range of 0.001 - 10 µg/mL caused a significant concentration dependent decrease in ROS level induced via 6 hours exposure to 0.137 mM L-Glu by 40 - 90% compared with L-Glu only treated cells (Figure 4.13C). An incubation with 0.137 mM L-Glu induced ROS and this was reduced by co-addition of acai berry ethanolic extract at concentrations of 0.1 - 100 µg/mL, with significant reduction observed at 100 µg/mL with a 48% decrease (Figure 4.13D). ROS produced as a result of 6 hours exposure to 100 mM L-Glu was significantly decreased by 89 - 228% in a concentration dependent manner by co-supplement of acai berry aqueous extract (Figure 4.13E). Likewise, acai berry ethanolic extracts at concentrations of 0.001 - 1000 µg/mL significantly reduced ROS levels induced by 100 mM L-Glu by 166 - 223% (Figure 4.13F).

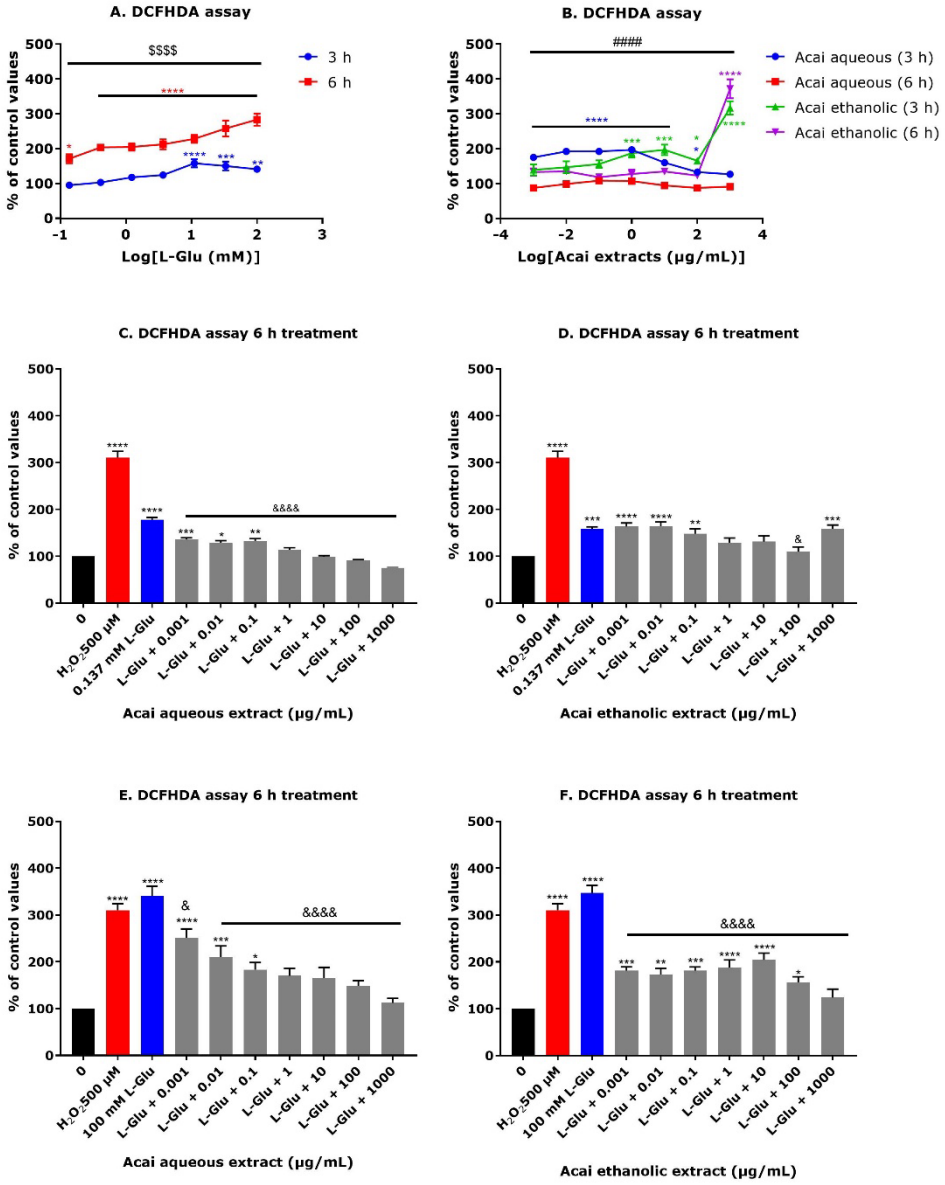

Figure 4.13: L-Glu and acai berry extract effects on ROS levels in differentiated TE671 cells measured by DCFHDA assay. ROS levels after L-Glu (A), or acai berry extract (B), exposure for 3 or 6 hours. ROS level production in differentiated TE671 cells exposed to 0.137 or 100 mM L-Glu and different concentrations of acai berry extracts after 6 hours; acai berry aqueous extract (C,E), and ethanolic extract (D, F). Hydrogen peroxide (H_2O_2) at 500 µM for 30 minutes was used as a positive control. The data presented are means ± SEM for three separate experiments at each concentration (n = 3 per experiment)**.** Statistical evaluation was done using One-way ANOVA followed by Dunnett's multiple comparisons post-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ vs control in the absence of L-Glu or acai berry extracts or H_2O_2. $^{\&}p < 0.05$ and $^{\&\&\&\&}p < 0.0001$ vs L-Glu group. The two-way ANOVA followed by Tukey's multiple comparisons post-test was used to evaluate the effect of time of L-Glu or acai berry extract exposure. The marked significance $^{\$\$\$\$}p < 0.0001$ represented the effect of L-Glu for 3 vs 6 hours while $^{\#\#\#\#}p < 0.0001$ is for acai berry aqueous extract 3 vs 6 hours.

4.2.6 Whole-cell patch-clamp assay

In order to examine the presence of functionally active iGluR in undifferentiated and differentiated SH-SY5Y cells, 20 cells of each type were examined via the whole cell patch clamp technique with exposure to 3 mM L-Glu + 10 μM Gly. Whole-cell patch-clamp recordings were obtained at a holding potential of −50 mV in Mg^{2+}-free perfusion solution. However, typical inward currents did not appear following the administration of the agonists in both SH-SY5Y cells (Figure 4.14).

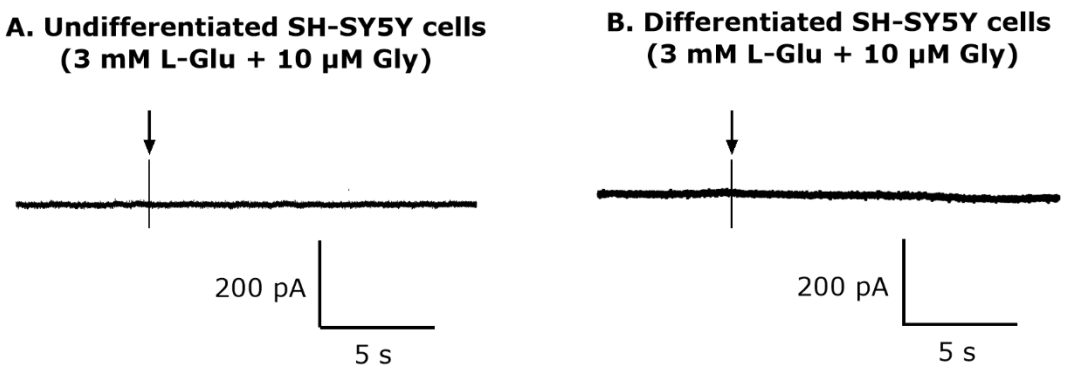

Figure 4.14; Patch clamp recordings of whole-cell current responses. (A) No inward current was recorded after the application of 3 mM L-Glu + 10 µM glycine (Gly) to undifferentiated and differentiated SH-SY5Y cells using whole-cell patch-clamp recordings at a holding potential of −50 mV (n = 20).

The expression and functional properties of iGluRs in differentiated TE671 cells were also examined using whole-cell patch clamp technique. Patch clamp recordings from differentiated TE671 cells were made in the whole cell configuration at a holding potential of −50 mV and using Mg^{2+}-free perfusion solution. Significant inward currents appeared following administration of 3 mM L-Glu + 10 μM Gly, 100 μM KA, and 100 μM NMDA+ 10 μM Gly (Figure 4.15A). The results showed that two populations of cells developed after TE671 differentiation using dbcAMP; in the first one, cells generated a large inward current upon exposure to 300 μM L-Glu + 10 μM Gly and much lower inward current after application of acai aqueous extracts at 1000 μg/mL (Figure 4.15B). The second population of cells showed the contrary activities (Figure 4.15C). As Figure 4.16 A - C shows, the 300 μM L-Glu + 10 μM Gly-activated currents were inhibited via application of NMDAR antagonists; MK-801 and Mg^{2+} resulting in a reduced response by approximately 65% and 45%, respectively. Acai berry aqueous extract caused concentration dependent inhibition of the 300 μM L-Glu + 10 μM Gly-activated currents with an estimated IC_{50} of 119.6 ± 1.544 μg/mL (Figure 4.16 B-D). The 1000 μg/mL acai berry aqueous extract activated currents were inhibited via a nAChR antagonist (DTC) and the AMPAR and KAR competitive antagonist, (DNQX) by 51%, and 56%, respectively (Figure 4.17 A,B).

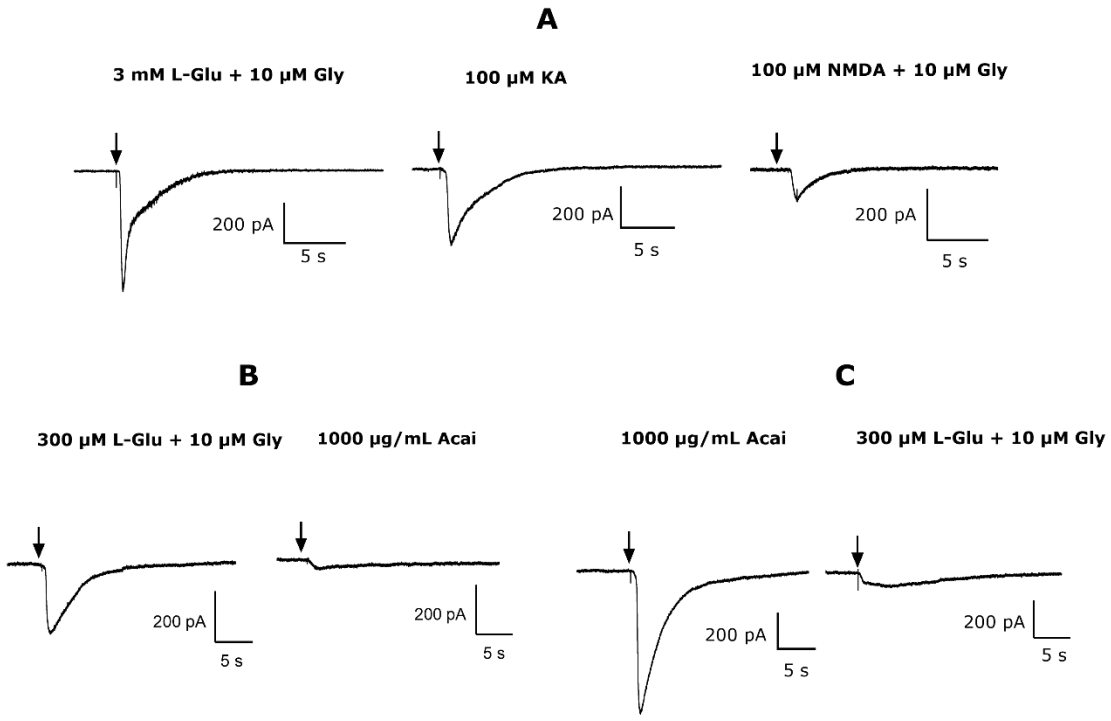

Figure 4.15: The inward current was recorded after the application of 3 mM L-Glu + 10 µM Gly, 100 µM kainic acid (KA), and 100 µM NMDA + 10 µM Gly (A). 300 µM L-Glu + 10 µM Gly or 1000 µg/mL acai aqueous extracts (B,C) to differentiated TE671 cells using whole-cell patch-clamp at a holding potential of −50 mV (n = 20). Arrows indicate application of agonists.

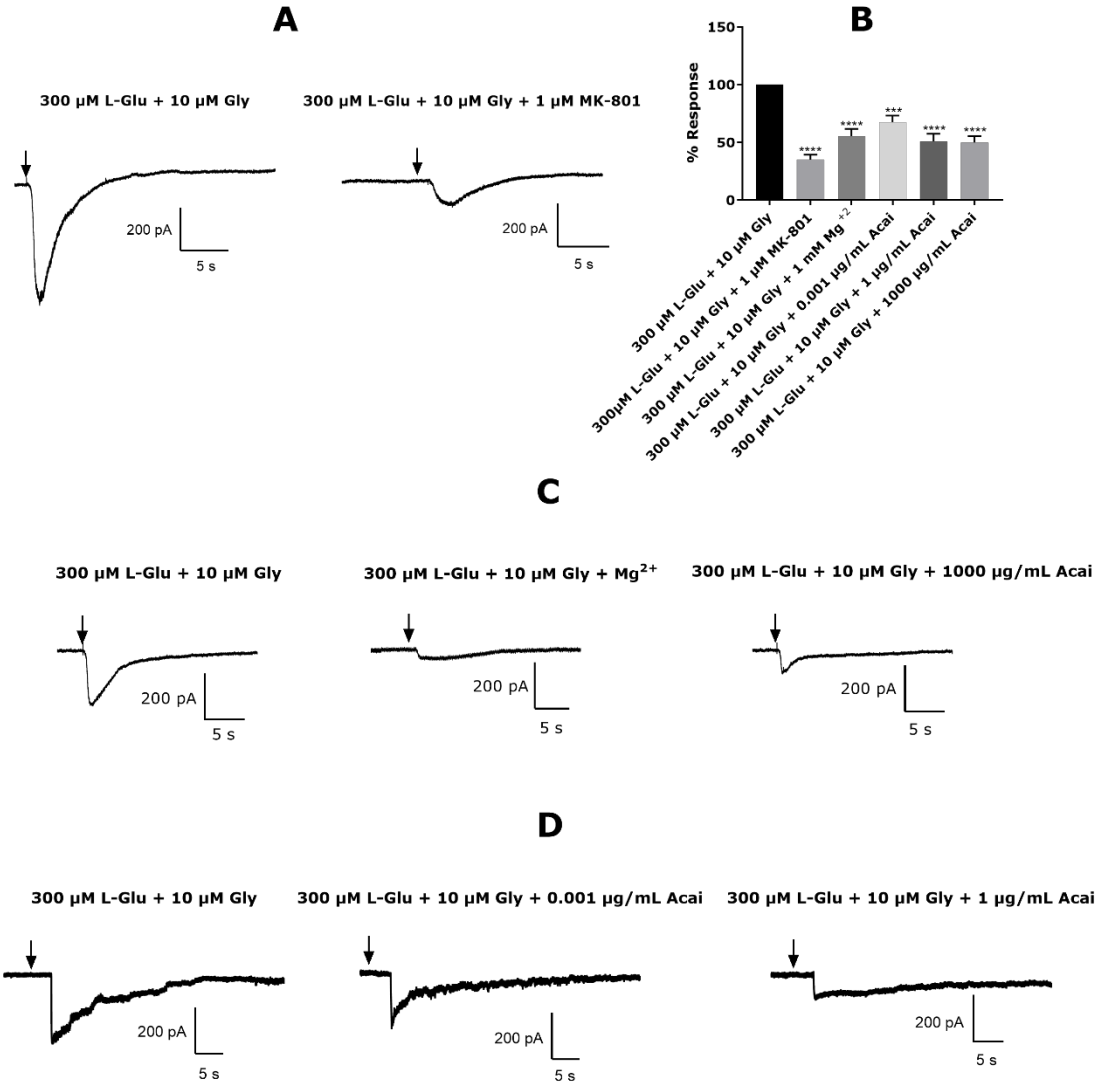

Figure 4.16: The inward current was recorded after the application of different compounds to differentiated TE671 cells using whole-cell patch-clamp at a holding potential of −50 mV. (A) 300 µM L-Glu + 10 µM Gly, and 300 µM L-Glu + 10 µM Gly + MK-801. (B) The histograms presented are means of % normalized current ± SEM for 20 cells recorded (n = 20). ***$p < 0.001$ and ****$p < 0.0001$ vs control cells exposure to 300 µM L-Glu + 10 µM Gly (C) 300 µM L-Glu + 10 µM Gly + Mg^{2+}, 300 µM L-Glu + 10 µM Gly + 1000 µg/mL acai aqueous extract (D) 300 µM L-Glu + 10 µM Gly, 300 µM L-Glu + 10 µM Gly + 0.001 µg/mL acai aqueous extract, 300 µM L-Glu + 10 µM Gly + 1 µg/mL acai aqueous extract. Arrows indicate application of agonists.

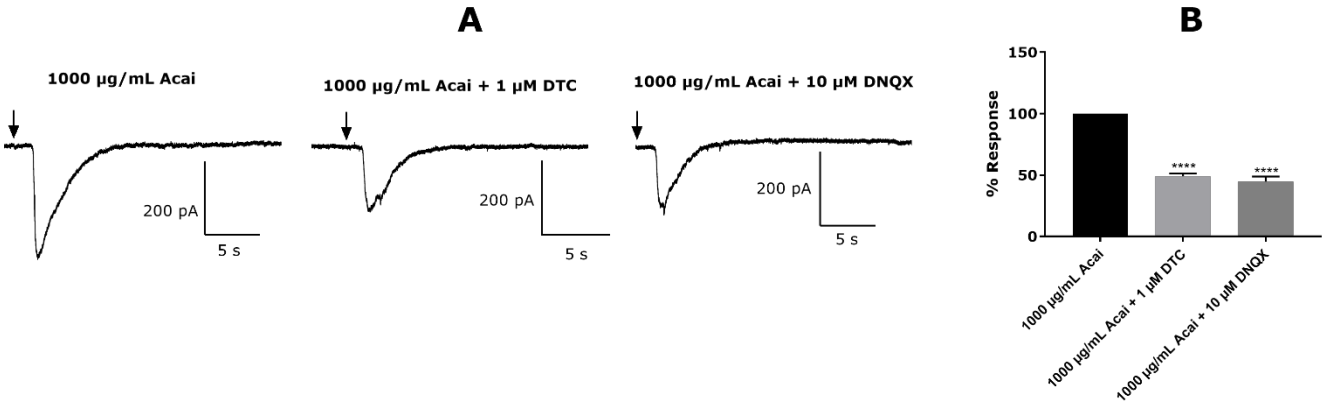

Figure 4.17: The inward current was recorded after the application of (A) 1000 µg/mL acai aqueous extract, 1000 µg/mL acai aqueous extract + 1 µM DTC, 1000 µg/mL acai aqueous extract + 10 µM DNQX to differentiated TE671 cells using whole-cell patch-clamp at a holding potential of −50 mV. (B) The histograms presented are means of % normalized current ± SEM for 20 cells recorded (n = 20). ****$p < 0.0001$ vs control cells exposure to 1000 µg/mL acai aqueous extract. Arrows indicate application of agonists.

4.3 Discussion

The pathological process of several NDDs and stroke has been linked to abnormal over-stimulation of glutamatergic neurotransmitter systems (Estrada Sánchez *et al.* 2008; Madeira *et al.* 2018; Rajda *et al.* 2017; Suzuki *et al.* 2022; Van Damme *et al.* 2005; Verma *et al.* 2022; Wang and Reddy 2017; Wang *et al.* 2020; Zeron *et al.* 2001). Previous studies have reported that high concentrations of L-Glu may trigger neural death via excitotoxicity and/or oxidative injury (Lewerenz *et al.* 2013; Mattson 2003). The result of this chapter showed that L-Glu induced a significant decline of the survival of undifferentiated SH-SY5Y cells at the elevated concentration of 100 mM for 24- and 48-hours, as observed using an MTT assay. However, using an LDH assay, a more definitive method able to distinguish cytostatic from cytocidal effects, significant toxicity was observed at lower concentrations (from 11.1 mM) of L-Glu. Moreover, MTT assay showed that L-Glu exposure reduced cell viability in differentiated SH-SY5Y cells at a concentration range of 0.137 - 1.23 and 100 mM for 24 hours. In contrast, L-Glu increased cell viability in differentiated SH-SY5Y cells after 48 hours of L-Glu treatment in the concentration range 0.412 - 33.33 mM. Furthermore, 24 hours exposure of L-Glu induced a concentration dependent decrease in cell viability in differentiated TE671 cells while only high concentrations (33.33 and 100 mM) caused significant loss of cell viability after 48 hours exposure. However, LDH level was increased confirming the L-Glu toxicity after 24 hours exposure only at high concentration of 100 mM in differentiated TE671 cells while after a 48 hours exposure the LDH production was increased at 33.33 and 100 mM. In the ReNcell CX cells, L-Glu (0.03 - 100 mM) exposure for 24 hours induced significant decreases in cell viability, however, only high concentrations (30 and 100 mM) significantly reduced cell viability after 48 hours exposure. L-Glu also impacted cellular bioenergetics with depletion of ATP levels and the MMP, and induction of ROS in undifferentiated SH-SY5Y cells and differentiated TE671 cells.

Acai berry extracts displayed little or no significant neurotoxicity and were able to counter the L-Glu neurotoxicity, with increased survival of L-Glu treated cells after 24 and 48 hours. The neuroprotective character of the acai berry extracts was further

validated via their ability to limit the L-Glu depletion of cellular ATP levels and MMP, as well as counter L-Glu induced redox stress, monitored as elevated ROS. Lastly, the toxicity of L-Glu was not mediated via excitotoxicity and activation of iGluRs in undifferentiated SH-SY5Y cells since patch-clamp recording did not detect their functioning. However, the differentiated TE671 cells exhibited iGluRs expression and functionality and so they may play a role in L-Glu induced toxicity in these cells. Other independent studies have confirmed that L-Glu is toxic to neuronal cells and induces cell death at a concentration range from 0.1 - 400 mM (Bernardo *et al.* 2017; Brizi *et al.* 2016; De Oliveira *et al.* 2019; Lee *et al.* 2019; Li *et al.* 2017a; Sun *et al.* 2010; Xin *et al.* 2019; Yang *et al.* 2019b; Yuksel *et al.* 2019). By comparison, incubation with the acai berry extracts alone at a concentration range of 0.01 - 100 µg/mL increased the cell viability in undifferentiated and differentiated SH-SY5Y cells. Similarly, acai berry extracts at concentration 0.001 - 1000 µg/mL showed an increase in cell viability in differentiated TE671 cells. This is consistent with the presence of agents capable of stimulating cell proliferation, and this observation is supported by other studies (Cadoná *et al.* 2021; Machado *et al.* 2016). Selective concentrations of the acai berry extracts possibly contain additional chemical(s) that were neurotoxic under these incubation conditions, most notably the 1 µg/mL aqueous extract and concentrations of 0.01 to 1 µg/mL for ethanolic extracts, as these evoked slight increases in extracellular LDH production in undifferentiated SH-SY5Y cells.

Furthermore, a relatively high concentration of acai berry aqueous extract (10 to 1000 µg/mL) induced cell decline as did concentrations of 0.001 and 10 - 1000 µg/mL of acai berry ethanolic extract using the MTT assay in differentiated TE671 cells. The rationale for why only selective concentrations of either aqueous or ethanolic extracts are able to induce mild toxicity is yet to be established and will need to be assessed after further fractionation of the extracts. Other studies have validated acai berry extract safety (Arrifano *et al.* 2018; De Souza *et al.* 2020; Poulose *et al.* 2012; Torma *et al.* 2017), however, similar to this chapter findings, certain concentrations of extracts as well as relatively high concentrations reduced cell viability (Arrifano *et al.* 2018; De Souza *et al.* 2020; Poulose *et al.* 2012; Torma *et al.* 2017). A limited number of the acai berry extracts were neuroprotective against the L-Glu-induced decline in cell viability measured via

MTT or LDH assays, but this was also not concentration dependent in undifferentiated SH-SY5Y cells (Figure 4.5 A-D). In contrast, MTT or LDH assays showed that most acai berry extracts prevent L-Glu-induced cell viability decline in differentiated TE671 cells (Figure 4.6 A-E) and ReNcell CX cells (Figure 4.7 A,B).

The neurotoxicity of L-Glu was in part mediated by an impact on cellular bioenergetics with reduced ATP production, consistent with other studies (De Oliveira *et al.* 2019; Li *et al.* 2017a; Yang *et al.* 2019b). The decline in ATP levels correlated with L-Glu concentration and similarly, the low-level toxicity of acai berry extracts and reduced ATP levels was concentration dependent in undifferentiated SH-SY5Y cells (Figure 4.8 A,B) and in differentiated TE671 cells (Figure 4.9 A,B). Hence, neuroprotection provided by the acai berry extracts was predominantly mediated at relatively low extract concentrations, with further toxicity evident from the coincubation of L-Glu with the highest acai berry extract concentrations in undifferentiated SH-SY5Y cells (Figure 4.8 C-F) and in differentiated TE671 cells (Figure 4.9 C-F).

L-Glu-induced damage to mitochondria was evident from lowered MMP values with 24 hour effects that correlated with L-Glu concentration in undifferentiated SH-SY5Y cells and 24 and 48 hours effects in differentiated TE671 cells. Other studies have similarly demonstrated L-Glu effects on MMP levels and functionality of mitochondria in SH-SY5Y cells (De Oliveira *et al.* 2019; Li *et al.* 2017a; Yang *et al.* 2019b; Lee *et al.* 2019; Yuksel *et al.* 2019; Sun *et al.* 2016; Zhu *et al.* 2016). Mild toxic effects of the aqueous acai berry extract were apparent from reduced MMP levels, and these were predominantly concentration dependent in undifferentiated SH-SY5Y cells. In differentiated TE671 cells, there was a concentration dependent decline in MMP level after acai aqueous extract exposure, however, the ethanolic extract reduced MMP only at high concentrations. The majority of the aqueous extracts and several of the ethanolic extracts were neuroprotective and able to restore the reduced MMP in response to 11 mM L-Glu, and surprisingly, the ethanolic extracts ably restored the MMP with the 100 mM L-Glu treatments in undifferentiated SH-SY5Y cells (Figure 4.10 C-F). In differentiated TE671 cells, the acai berry extract low concentrations (0.001 to 10 µg/mL) were capable

of reinstatement of the diminished MMP level in response to 11 mM L-Glu exposure (Figure 4.11 C,D).

Damage to mitochondria promotes the release of ROS that can damage cellular proteins, lipids, and DNA, and contribute to signaling for cell death (Kowalczyk *et al.* 2021; Wu *et al.* 2019). In keeping with L-Glu-induced oxytosis after a 3 hour exposure, ROS levels were directly correlated with L-Glu concentration in undifferentiated SH-SY5Y cells however, after 6 hours exposure, ROS levels were gradually reduced specifically at higher concentrations of 33.3 and 100 mM and this may be due to involvement of free radicals in the process of cellular component injuries. A 3 or 6 hour exposure of L-Glu induced a concentration dependent increase of ROS levels in differentiated TE671 cells. The acai berry ethanolic extracts induced more ROS than their aqueous extract counterparts at each of the assayed concentrations, but aside from a single anomalous data point at 0.01 µg/mL and at the highest concentration of 1000 µg/mL, these did not reach significance in undifferentiated SH-SY5Y cells. However, a low concentration of acai aqueous extract and high concentration of ethanolic extract induced ROS in differentiated TE671 cells. Both aqueous and hydroethanolic extracts of the acai berry have potent antioxidant activity (Cadoná *et al.* 2021; De Souza *et al.* 2020; Torma *et al.* 2017), in keeping with their abilities to ameliorate the L-Glu induced production of ROS and oxidative stress in undifferentiated SH-SY5Y cells (Figure 4.12 C,D) and (Figure 4.13 C,D) in differentiated TE671 cells (Machado *et al.* 2016; Spada *et al.* 2009).

Collectively, the acai berry aqueous and ethanolic extracts displayed differential neuroprotective effects, some of which were concentration dependent. This likely reflects differences in phytochemicals recoveries between the solvents and this was confirmed after liquid chromatography-mass spectrometry (LC-MS) analysis of extract fractions (refer to Table 5.3). Polar solvents such as methanol and ethanol are effective at extracting phenolic compounds from plants, some of which are useful antioxidants (Altemimi *et al.* 2017). However, the yield of polyphenols and flavonoids from acai berries was higher with water than methanol or ethanol alone (Chung 2012). Furthermore, many studies have previously reported that certain phytochemicals have neuroprotective properties such as protocatechuic acid (Szwajgier *et al.* 2017; Zhang *et*

al. 2015), syringic acid (Ogut *et al.* 2022; Szwajgier *et al.* 2017), vanillic acid (Ullah *et al.* 2020), gallic acid (Szwajgier *et al.* 2017), 4-hydroxybenzoic acid (Winter *et al.* 2017), chlorogenic acid (Szwajgier *et al.* 2017), luteolin (Khan *et al.* 2020) , quercetin (Khan *et al.* 2020), taxifolin (Inoue *et al.* 2019), quercetin-3-O-rutinoside (rutin) (Khan *et al.* 2020), and isoorientin (Li *et al.* 2020) and these have been identified in the acai berry extracts analysis (refer to Table 5.3). This implies that these acai berry extracts may retain a range of phytochemicals that have antioxidant and neuroprotective effects that could provide neuroprotection against excessive L-Glu associated with NDDs and stroke (refer to Table 5.3).

Earlier studies detected the expression of NMDA and other L-Glu receptors in undifferentiated and differentiated SH-SY5Y cells (Goldie *et al.* 2014; Korecka *et al.* 2013; Kulikov *et al.* 2007; Lee *et al.* 2015; Nair *et al.* 1996; Pizzi *et al.* 2002; Sun and Murali 1998). However, in the present study, the whole cell patch clamp recordings showed no response to combined L-Glu and Gly administration in both SH-SY5Y cells. A possible explanation for this discrepancy is that neuroblastoma cell lines are tumoral in origin, which means their phenotype might change over time, since long-term culturing can change a cell line's genetic and molecular profiles (Prasad *et al.* 2023; Shipley *et al.* 2016). Moreover, the cultivation technique used may have a significant impact on the expression, processing, and localization of iGluRs, which could affect the results of the experiment. Studies demonstrated that difference in culture methods, such as generating of mature neurons using various doses of differentiating agents or various periods of treatment time, have a significant impact on SHSY-5Y phenotypes, including the presence of active ion channels and neurotransmitter receptors (Lopez-Suarez *et al.* 2022; Xicoy *et al.* 2017). Another possible explanation for the lack of functional NMDAR in differentiated SHSY5Y in this thesis is that the approach used did not incorporate BDNF with the RA differentiation agent. It was found that RA differentiation followed by BDNF treatment resulted in fully matured neurons and intense changes in gene expression (Cheung *et al.* 2009; Encinas *et al.* 2000; Goldie *et al.* 2014; Hromadkova *et al.* 2020). Thus, this thesis suggested that the L-Glu toxicity in undifferentiated cells may not be caused by excitotoxicity mediated by activation of iGluRs (Figure 4.14 A,B).

The results from this chapter demonstrate that there was a responsive current to L-Glu and Gly stimulation, KA, and NMDA and Gly in dbcAMP-differentiated TE671 cells (Figure 4.15A). Furthermore, the L-Glu + Gly-activated currents were inhibited by NMDAR antagonists such as MK-801 and Mg^{2+} (Figure 4.16 A,C). These results confirmed the expression of iGluR in differentiated TE671 cells. These results were supported by the finding that the administration of an AMPAR agonist evoked a current response in undifferentiated TE671 cells, and the expression of NMDAR, AMPAR, KAR and mGluR subunits was demonstrated in another study (Stepulak *et al.* 2009). Likewise, another investigation determined the expression of iGluR subunits; GluA4, GluN1, GluK5 and GluN2D in the same cell line (Luksch *et al.* 2011). Furthermore, another study has supported the expression of GluN1 and the GluA2/3 subunits of the AMPAR (Wojciech *et al.* 2001). Therefore, this evidence confirmed that the iGluRs are expressed in TE671 cells and that the mechanism of L-Glu toxicity to neurons in this study is potentially via excitotoxicity in which the L-Glu receptors are over-stimulated.

Patch clamp results found that two cell types developed after TE671 cell differentiation; in the first type of cells, when exposed to L-Glu and Gly they produced a large inward current whereas when cells were exposed to acai berry aqueous extract this produced a smaller inward current (Figure 4.15B). The other cell type showed opposite behavior, with higher inward current after acai berry extract exposure and lower with L-Glu + Gly (Figure 4.15C). This may be due to some cells not fully differentiated and the results suggest that L-Glu receptor is more expressed in cells after they are fully differentiated. db-cAMP induced differentiation in the TE671 cell line, resulting in process formation and increased neuronal maturation markers (Sasaki *et al.* 1986). However, research has found that in addition to neurite extension and morphological changes, the expression of nAChR was reduced after db-cAMP differentiation agent addition (Bencherif and Lukas 1991; Siegel and Lukas 1988).

In addition, results from this chapter confirmed that acai berry aqueous extract produced concentration dependent inhibition of the L-Glu + Gly-activated currents in differentiated TE671 cells (Figure 4.16 B-C). Acai berry extract may contain pharmacologically active substance(s) that may be responsible for this inhibition. Several plants and herbal

products have been reported to block glutamatergic excitation mediated through NMDAR (Afshari *et al.* 2020; Liang *et al.* 2013; Marchetti *et al.* 2011; Silva *et al.* 2019a). Previous investigations have found that these plant products have enormous diversity of naturally occurring compounds, such as flavonoids, alkaloids, terpenoids and fatty acids that bind to receptors and act as antagonists (Afshari *et al.* 2020; Liang *et al.* 2013; Marchetti *et al.* 2011; Silva *et al.* 2019a). Furthermore, in previous studies, NMDA antagonist activity and prevention of L-Glu excitotoxicity was reported for several plant phytochemicals such as chlorogenic acid (Ahmad *et al.* 2022a), quercetin, garlic acid, protocatechuic acid, and vanillic acid (David *et al.* 2019) and the LC-MS results from this thesis revealed that acai berry extracts contain these phytochemicals (refer to Table 5.3). Despite the fact that the mechanism of action of the compound(s) in acai berry extracts is still unclear, it is possible that they block NMDAR function through interactions at specific site(s) in the receptor and act as uncompetitive antagonists since they achieve the active site(s) only after NMDAR activation (Liang *et al.* 2013). However, more experiments are needed to isolate, purify, and characterise acai berry extracts to identify the active agent(s) responsible on attenuating NMDAR activated currents.

Additionally, this chapter demonstrated that acai aqueous extract-activated currents were inhibited after brief exposure to a nAChR antagonist (DTC) and AMPAR and KAR competitive antagonist (DNQX) in differentiated TE671 cells (Figure 4.17 A,B). Therefore, this suggested that the acai berry extract can activate the nAChR, AMPAR and KAR. nAChR activation could arise from the supplementation of caffeine in acai berry powder which, in low concentrations, can act as a nAChR agonist and it has been added in many acai commercial products (Fabiani *et al.* 2018; Ulbricht *et al.* 2012). The stimulation of AMPAR and KAR could be due to the presence of L-Glu in acai berry chemical matrix as previously reported (Bichara and Rogez 2011) and detected in this thesis by LC-MS (Table 5.3). Even though, LC-MS results identified numbers of phytochemicals in acai berry extracts (Table 5.3), and more experiments directed toward the analysis and purification of the chemical composition of extracts are needed to confirm these suggestions.

A further factor to consider in the MSG model is the exogenous Na^+ loading results since extensive Na^+ uptake causes neuronal swelling and lysis due to necrotic excitotoxic death (Liao *et al.* 2020). In addition, cells shrink when hyperosmolarity conditions are present, since existing high Na^+ in the extracellular space results in water efflux and cell shrinkage (Liao *et al.* 2020). However, a study distinguished the MSG excitotoxicity from Na^+ hyperosmolarity using real time polymerase chain reaction (RT-PCR) and found that comparing with control and NaCl (hyperosmolar stress positive control)-treated brain slices, 100 mM MSG reduced expression of excitation-related transcripts including discs large MAGUK scaffold protein 4 (Dlg4), and early growth response 1 (EGR1) mRNA expression, and decreasing nNOS expression (Liao *et al.* 2020). The immediate early gene of EGR1 is a marker of excitation activity, while Dlg4 is a synaptic scaffolding protein linked with NMDAR that promotes nNOS to create NO (Liao *et al.* 2020). The reduction in these excitation-related mRNAs may be due to oxidative stress induced by MSG, which affects the stability and transcription of neuronal mRNA, or it may be caused by neuronal death, ultimately decreasing the number of neurons contributing to excitation-related proteins (Liao *et al.* 2020). Therefore, it appears that MSG's toxicity is not caused by hyperosmolar sodium stress, but rather by L-Glu toxicity, even at high concentrations.

To conclude, although acai berry extracts alone exhibited mild adverse effects on undifferentiated SH-SY5Y cells and differentiated TE671 cells, co-incubation of certain acai berry extract concentrations with L-Glu was neuroprotective and limited the L-Glu induced loss of cell viability and restored mitochondrial function and ameliorated oxidative stress in both cell lines in addition to excitotoxicity in differentiated TE671 cells. Given the important role of excessive L-Glu accumulation and toxicity in NDDs and stroke, extraction of the active neuroprotective agent(s) from acai berry extracts may prove useful chemicals able to limit disease development and/or provide a therapeutic treatment. However, the chapter results are preliminary and only provides *in vitro* data with whole extracts, therefore, future testing of the active ingredients within acai berries will need to be undertaken *in vitro* and confirmed using *in vivo* experimental models before consideration of their application to humans.

Chapter 5

Anti-cholinesterase and antioxidant activities of commercially available acai berry (*Euterpe* sp.) powder

Chapter 5. Anti-cholinesterase and antioxidant activities of commercially available acai berry (*Euterpe* sp.) powder

5.1 Introduction

Even though AD medications can potentially slow disease progression, none can prevent or stop the course of the disease in addtion these treatments can cause adverse side effects (refere to Table 1.3). Moreover, the actions of AD drugs such as ChEIs, NMDAR antagonist and human immunoglobulin were designed to target specific mechanisms such as neurotransmitter activity modulation or Aβ aggregation (Table 1.3). However, these drugs were not originally developed to resolve other pathological mechanisms implicated in the development and/or progression of AD, such as tissue damage from oxidative stress, which has been detected in *post-mortem* brain tissue from AD patients. Hence, a search continues for other novel treatment strategies with additional activities that target cholinergic deficits as well as other elements of AD pathology, with the expectation of reduced side effects.

BuChE is capable of hydrolyzing ACh and compensates for a depleted level of AChE in AD patients, resulting in continued depletion of ACh and breakdown of cholinergic circuits. Finiding drugs that target and inhibit both AChE and BuChE may be more clinically effective in combating the elevated levels of BuChE in AD patients.

Moreover, the findings of this thesis demonstrated that excessive L-Glu intake have pathological consequences resulting in the degeneration and death of neuronal tissue that has been linked to NDDs and strokes)Chapter 3, Figure 3.4). That cellular damage from L-Glu was caused in part by a significant rise in AChE activity, as well as the generation of redox stress and depletion of the antioxidant defence system. Thus, finding agent(s) able to mitigate L-Glu neurotoxicity as well as inhibit ChE in addition to its potent antioxidant activity could help treat or prevent AD.

The south American acai berries (*Euterpe oleracea*) have been extensively studied for their pulp due to its potential health benefits. Therefore, this chapter aimed to analyse the nutraceutical potential of *Euterpe* sp. (in the form of aqueous and ethanolic extracts) for

possible development as an AD treatment via the ability to inhibit AChE and BuChE; the study also aimed to further delineate its antioxidant capabilities. Lastly, acai berry extracts were fractionated and analyzed by liquid chromatography mass spectrometer (LC-MS) to identify phytochemicals that may be responsible for anti-ChE and antioxidants activities.

5.2 Results

5.2.1 Acai berry extracts inhibit cholinesterases

The electric eel AChE activity was inhibited significantly more by acai berry aqueous extract when compared with ethanolic extract ($p < 0.0001$). The acai berry aqueous extract significantly inhibited electric eel AChE activity in a concentration-dependent manner over concentrations ranging from 1×10^{-5} µg/mL to 0.01 µg/mL, but no further inhibition of AChE activity was observed at concentrations higher than 0.01 µg/mL (Figure 5.1A). The concentration of acai berry aqueous extract that produced 50% of the maximum inhibition (IC_{50}) of AChE activity was estimated as 0.001 µg/mL using non-linear regression. The maximum inhibition effect (E_{max}) of acai berry aqueous extracts on AChE activity was 50%. In contrast, the acai berry ethanolic extract showed a limited inhibition (1 - 19%) of AChE, and only at the relatively high extract concentrations of 100 and 1000 µg/mL, however, this was not statistically significant (Figure 5.1A). Acai berry ethanolic extract had a 19.3% E_{max} versus AChE activity.

Similarly, inhibition of equine BuChE activity was significantly greater by acai berry aqueous extract than ethanolic extract ($p < 0.001$).The acai berry aqueous extract was also primarily a concentration-dependent inhibitor of BuChE from equine serum over concentrations ranging from 1×10^{-5} µg/mL to 0.01 µg/mL (Figure 5.1B). However, similar to the inhibition of AChE, a point of saturation was detected, with the further inhibition of BuChE observed only at the highest concentration examined, i.e., 1000 µg/mL (43.6%). The concentration of acai berry aqueous extract that produced half maximal inhibition of BuChE activity (IC_{50}) was estimated as 6378 µg/mL using non-linear regression whereas the E_{max} was 34.7%. Similar to the inhibition of AChE, the acai berry ethanolic extract also showed a limited inhibition of BuChE, with only marginal

inhibition levels (12 - 15%) at concentrations of 0.01 µg/mL or higher, and these effects were not statistically significant (Figure 5.1B). The maximum inhibition effect (E_{max}) of the ethanolic acai berry extracts on BuChE activity was 14.8%.

As shown in Figure 5.2 A, acai berry aqueous extract inhibited electric eel AChE in a kinetic assay while the ethanolic extract had a limited inhibition especially at high concentrations (Figure 5.2B). The activity of BuChE from equine serum was kinetically inhibited by the acai berry aqueous extract, as shown in Figure 5.2C. The acai berry ethanolic extract influence on enzyme kinetics is presented in Figure 5.2D.

The acai berry aqueous and ethanolic extracts at a concentration range of 0.001 - 1000 µg/mL did not exhibit inhibitory activity against hAChE (Figure 5.3A). In contrast, the acai berry aqueous and ethanolic extracts showed significant inhibitory activity against hBuChE and there were significant inhibition effects by ethanolic extracts ($p < 0.05$) (Figure 5.3B). Acai berry aqueous extract caused about 20-35% inhibition of hBuChE. Acai berry ethanolic extract caused significant higher inhibition on hBuChE activity approximately 38 - 55%, $p < 0.0001$, with $IC_{50} = 134.1$ µg/mL (Figure 5.3B). The maximal induction effect (E_{max}) of the acai berry aqueous and ethanolic extracts on hBuChE activity was 36.4% and 52%, respectively.

Acai berry aqueous and ethanolic extracts did not exhibit inhibitory activity against hAChE in a kinetic assay (Figure 5.4A,B). hBuChE activity was kinetically inhibited by the acai berry aqueous and ethanolic extracts, as shown in Figure 5.4C,D.

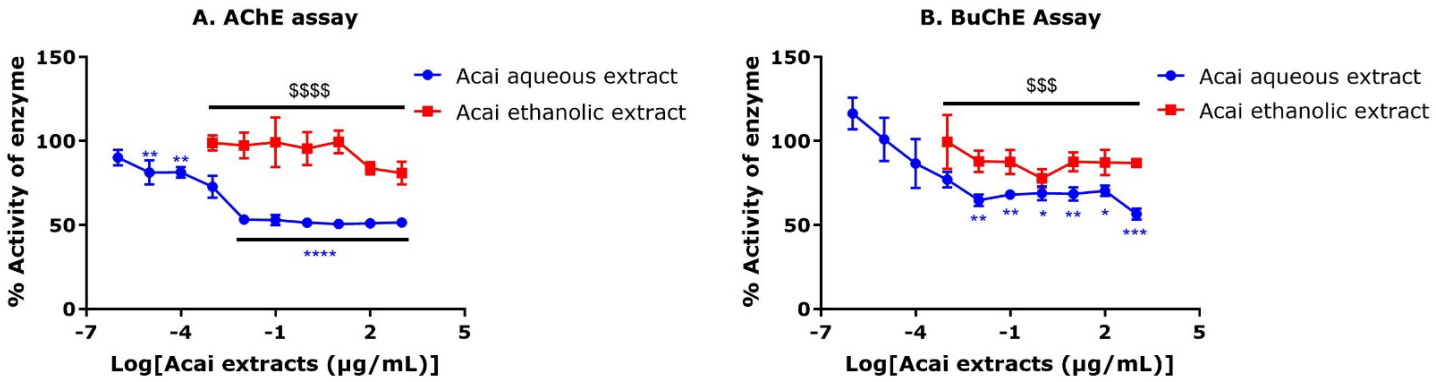

Figure 5.1: Cholinesterase inhibition by acai berry aqueous and ethanolic extracts. Electric eel AChE inhibitory activity of acai berry aqueous and ethanolic extract (A). Equine BuChE inhibitory activity of acai berry aqueous and ethanolic extract (B). The data presented as means ± SEM for three independent experiments in duplicate assays at each extract concentration (n = 2 per experiment). For marked significance * $p <$ 0.05, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ vs control untreated enzyme. The marked significance represented $^{\$\$\$}$ $p < 0.001$, and $^{\$\$\$\$}$ $p <$ 0.0001 for the effect of acai aqueous vs acai ethanolic extract.

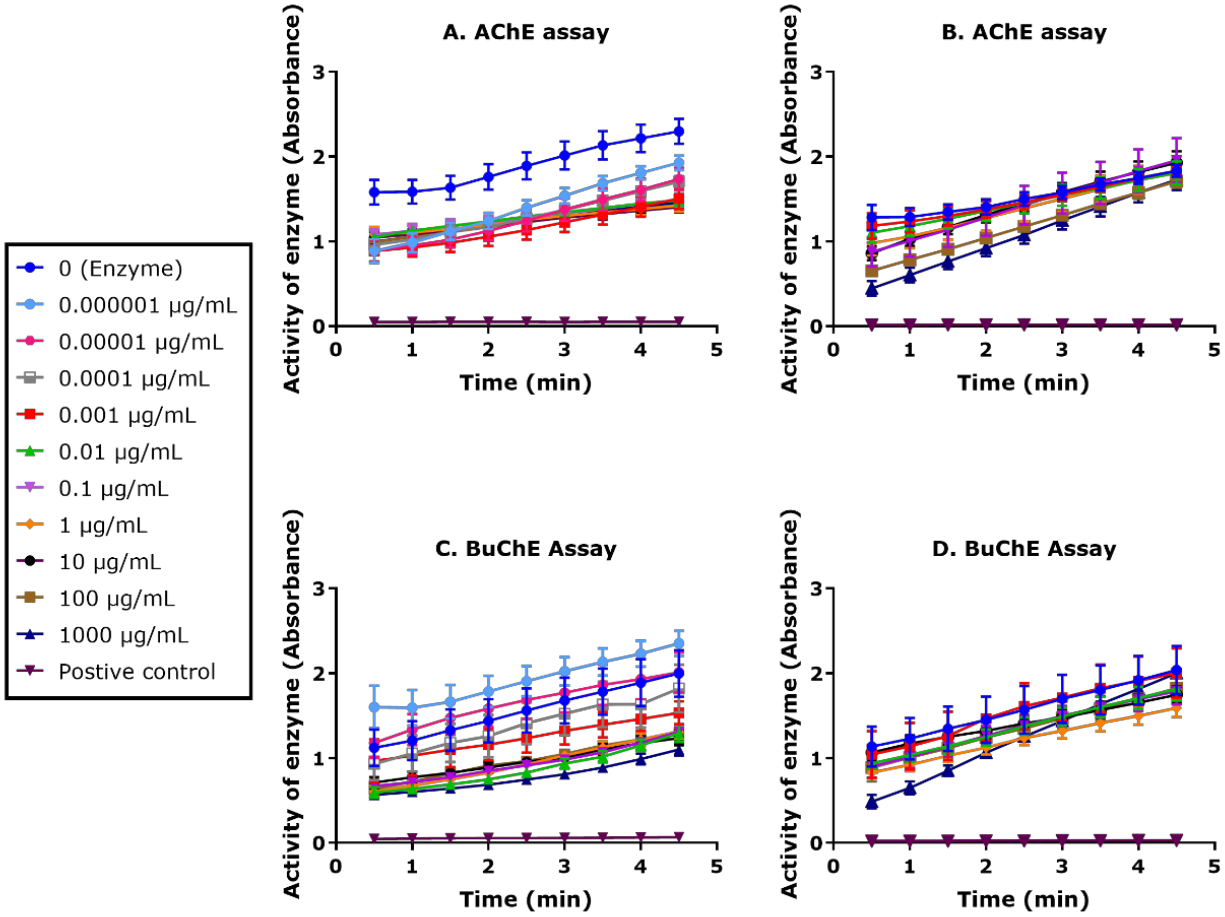

Figure 5.2: The Ellman assay was used to determine the influence of acai berry extracts on cholinesterase inhibitory activity kinetics. AChE inhibitory activity kinetics of acai berry aqueous extract (A) and acai berry ethanolic extract (B). BuChE inhibitory activity kinetics of acai berry aqueous extract (C) and acai berry ethanolic extract (D). For the positive control inhibitors, 5 mM azamethiphos and 5 mM ethopropazine hydrochloride were used for AChE and BuChE, respectively. The data presented as means ± SEM for three independent experiments in duplicate of each extract concentration (n = 2 per experiment).

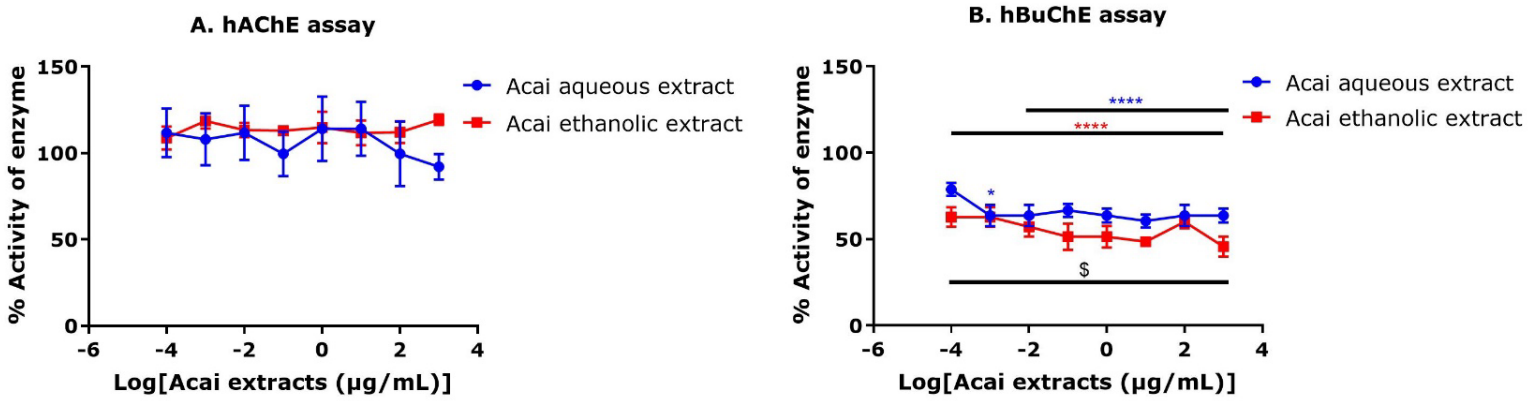

Figure 5.3: Human cholinesterase inhibition by acai berry aqueous and ethanolic extracts. hAChE inhibitory activity of acai berry aqueous and ethanolic extract (A). hBuChE inhibitory activity of acai berry aqueous and ethanolic extract (B). The data presented as means ± SEM for three independent assays in duplicate at each extract concentration (n = 2 per experiment). Marked significance *$p < 0.05$, and ****$p < 0.0001$ for treated vs control untreated enzyme. The marked significance represented $^{\$}$ $p < 0.05$ for the effect of acai aqueous vs acai ethanolic extract.

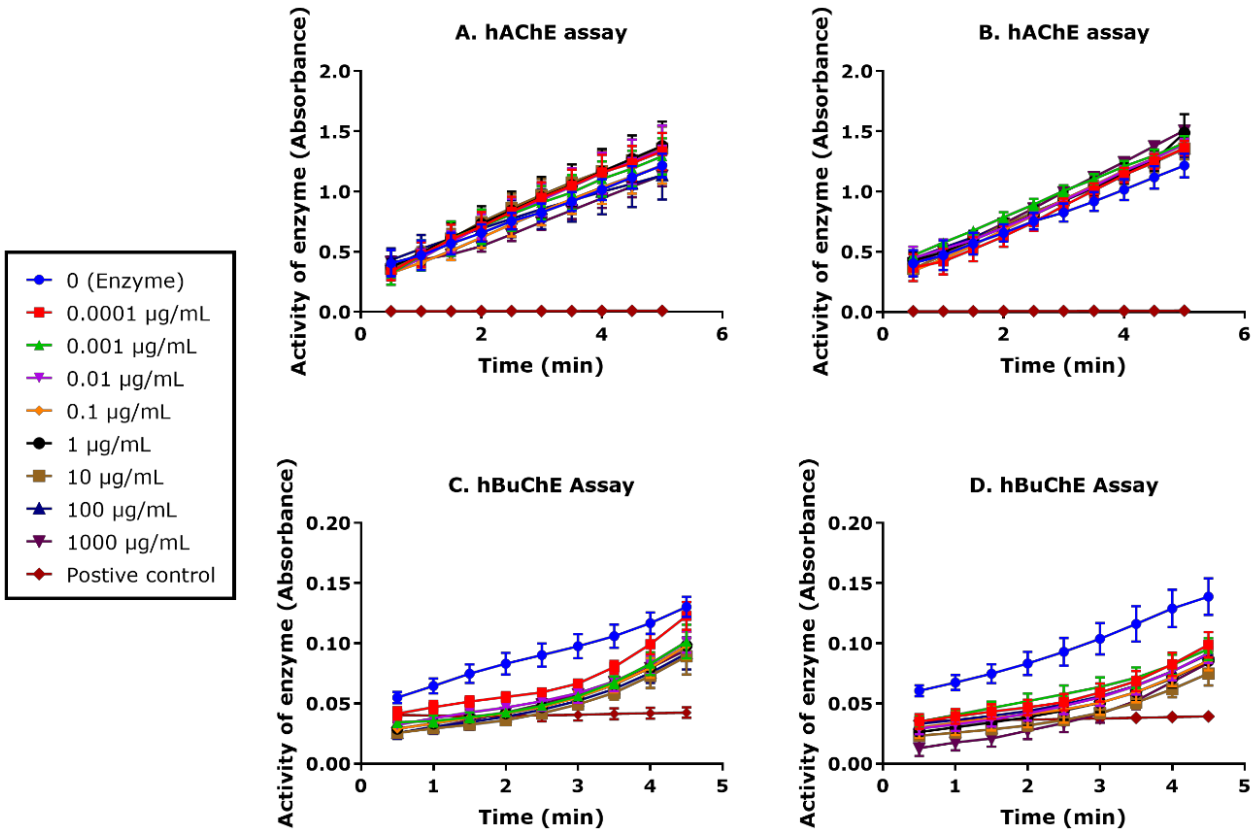

Figure 5.4: The Ellman assay was used to determine the influence of acai berry extracts on human cholinesterase inhibitory activity kinetics. hAChE inhibitory activity kinetics of acai berry aqueous extract (A) and acai berry ethanolic extract (B). hBuChE inhibitory activity kinetics of acai berry aqueous extract (C) and acai berry ethanolic extract (D). For the positive control inhibitors, 5 mM azamethiphos and 5 mM ethopropazine hydrochloride were used for hAChE and hBuChE, respectively. The data presented as means ± SEM for three independent experiments in duplicate at each extract concentration (n = 2 per experiment).

5.2.2 Acai berry aqueous and ethanolic extracts exhibit 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

The ability of aqueous and ethanolic extracts of acai berry to act as free radical scavengers was assessed using a DPPH assay. Both extracts displayed DPPH radical scavenging abilities and had similar concentration-dependent curves. At the lower end of the concentrations examined (from 0.01 µg/mL to 10 µg/mL), the aqueous extract displayed a similar antioxidant capability to that of α-tocopherol, and the ethanolic extract surpassed that of α-tocopherol and gallic acid (Figure 5.5). The aqueous extract had a lower percentage of activity than the ethanolic extract over the concentration range of 100 - 4000 µg/mL (Figure 5.5). The antioxidants α-tocopherol (vitamin E), L-ascorbic acid (vitamin A), and gallic acid all displayed higher free radical scavenging over the concentration range of 100 - 4000 µg/mL. The concentration of each of the agents that produced a 50% inhibition of free radical levels (IC_{50}) was calculated by non-linear regression as 11.550 mg/mL for the acai aqueous extract and 791 µg/mL for the ethanolic extract. By comparison, for α-tocopherol, L-ascorbic acid, and gallic acid, the IC_{50} values were 50 µg/mL, 115 µg/mL, and 8 µg/mL, respectively.

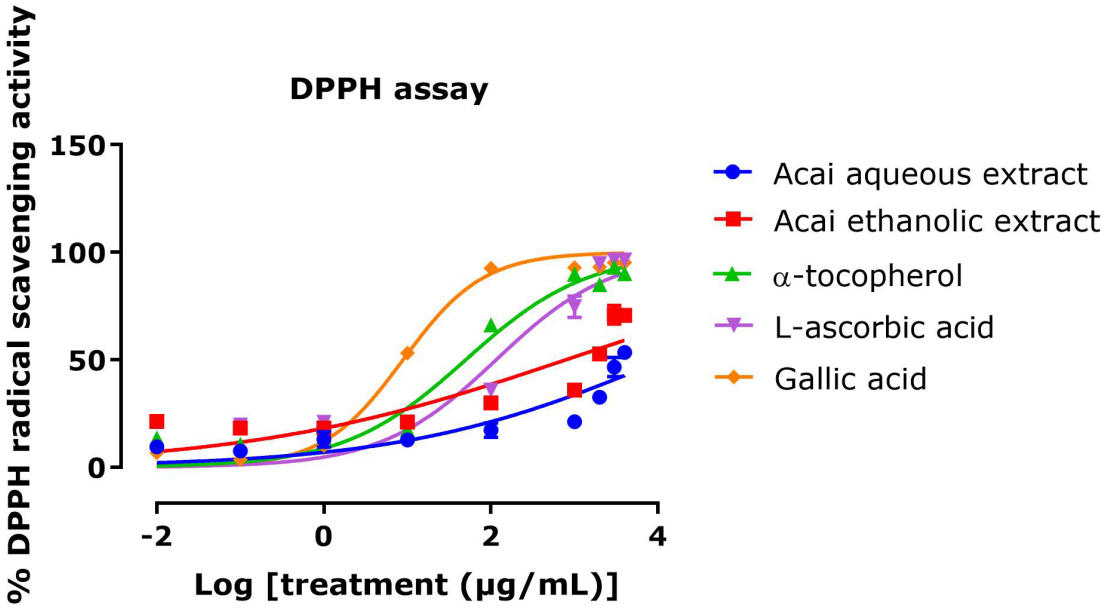

Figure 5.5: DPPH radical scavenging activity of acai berry aqueous and ethanolic extracts. Acai berry antioxidant activity was assessed via the percentage inhibition (radical scavenging) of DPPH over a concentration range of 0.01 - 4000 µg/mL. The data presented as means ± SEM for three independent experiments in duplicate at each extract concentration (n = 2 per experiment).

5.2.3 Acai berry aqueous and ethanolic extracts exhibit 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid radical cation ($ABTS^{\bullet+}$) scavenging activity

Acai berry aqueous and ethanolic extracts exhibited $ABTS^{\bullet+}$ scavenging activity in a concentration-dependent manner that was approximately linear over the concentration range of 1 - 1000 µg/mL (Figure 5.6). The acai berry ethanolic extract displayed a greater antioxidant capacity than either L-ascorbic acid or α-tocopherol, with an IC_{50} of 461.6 µg/mL compared to 690 µg/mL and an estimated 1270 µg/mL for L-ascorbic acid and α-tocopherol, respectively. Gallic acid exhibited the greatest scavenging activity, with an IC_{50} of 8 µg/mL. The acai berry aqueous extract showed the lowest antioxidant capacity, with an estimated IC_{50} of 30.541 mg/mL.

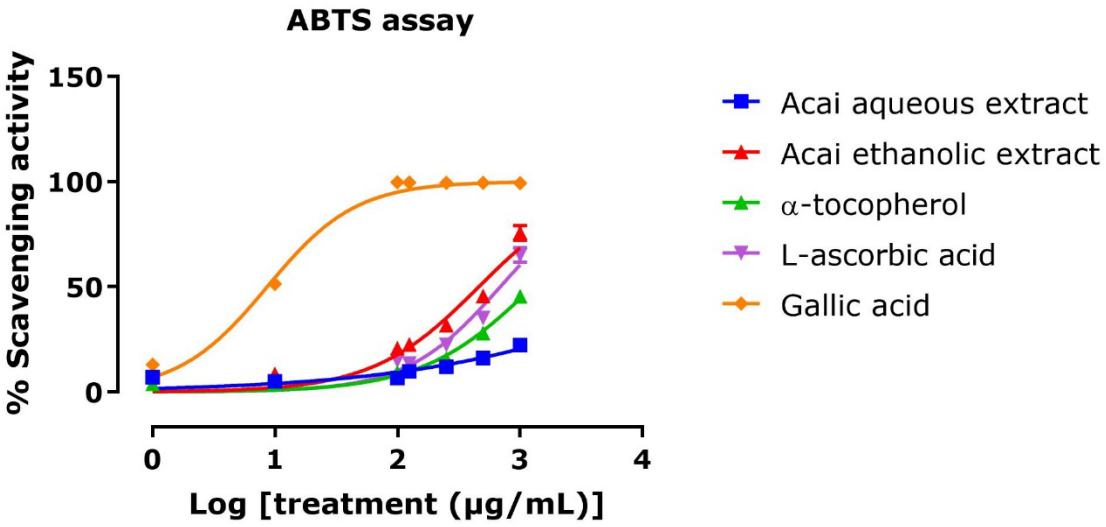

Figure 5.6: $ABTS^{\bullet+}$ scavenging activity of acai berry aqueous and ethanolic extracts. Acai berry antioxidant activity was assessed as a percentage inhibition (radical scavenging) of ABTS over a concentration range of 1 - 1000 µg/mL. The data presented as means ± SEM for three independent experiments in duplicate at each extract concentration (n = 2 per experiment).

5.2.4 Acai berry aqueous and ethanolic extracts exhibit hydrogen peroxide (H_2O_2) scavenging activity

Both acai berry extracts displayed moderate but concentration-dependent H_2O_2 scavenging activity (Figure 5.7), with an inhibition percentage of 20 - 30% and estimated IC_{50} values of 7.803 mg/mL for the aqueous extract and 1.479 mg/mL for the ethanolic extract. The maximum inhibition effect (E_{max}) of acai berry aqueous and ethanolic extracts on H_2O_2 were 35% and 21.8%, respectively. α-Tocopherol and gallic acid were more potent H_2O_2 radical scavengers, with IC_{50} values of 1.847 mg/mL and 0.405 mg/mL, respectively.

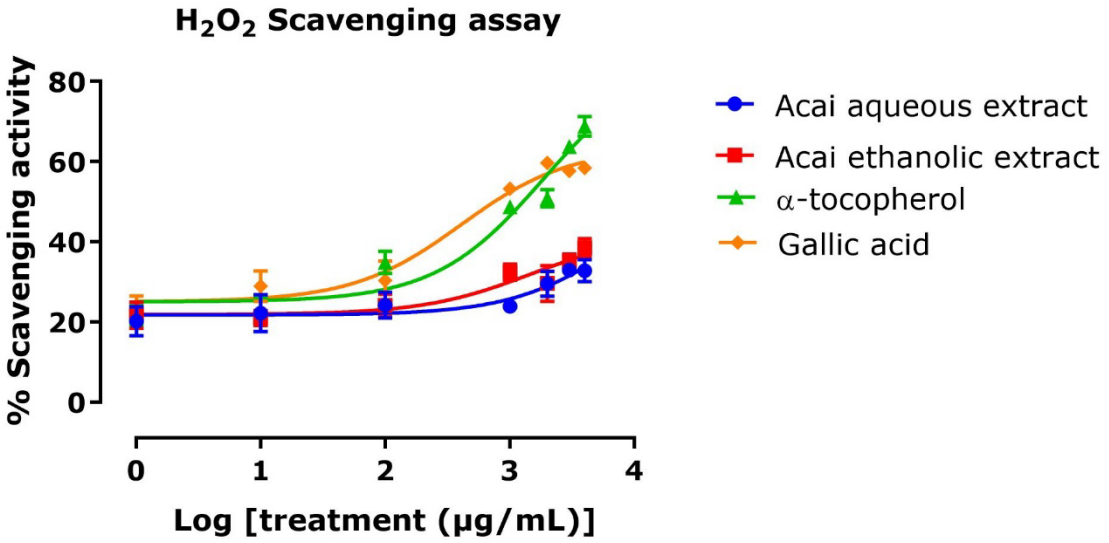

Figure 5.7: H_2O_2 scavenging activity of acai berry aqueous and ethanolic extracts. Acai berry antioxidant activity was assessed as a percentage of the scavenging activity of H_2O_2 over a concentration range of 1 - 4000 µg/mL. The data presented as means ± SEM for three independent experiments in duplicate at each extract concentration (n = 2 per experiment).

5.2.5 Acai berry aqueous and ethanolic extracts exhibit hydroxyl radical (˙OH) scavenging activity

Both acai berry extracts exhibited ˙OH scavenging activity in a concentration-dependent manner, as shown in Figure 5.8. In comparison to the acai berry aqueous extract, the acai berry ethanolic extract showed higher antioxidant action, with an IC_{50} of 946 µg/mL, while the IC_{50} of acai berry aqueous extract was estimated as 11.604 mg/mL. Gallic acid was a potent ˙OH radical scavenger, with an IC_{50} of 0.7 µg/mL.

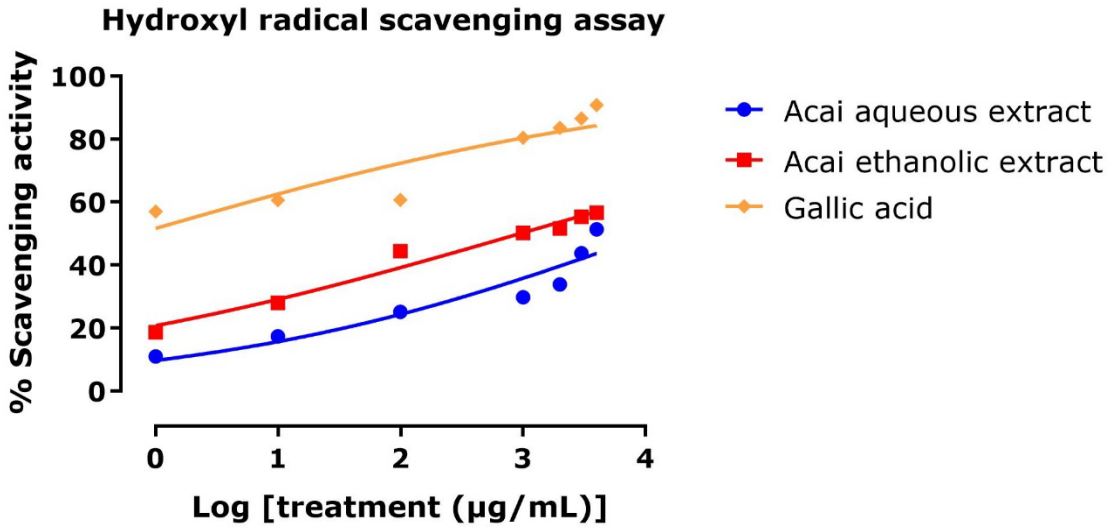

Figure 5.8: Hydroxyl radical scavenging activity of acai berry aqueous and ethanolic extracts. Acai berry antioxidant activity was assessed via the percentage of the scavenging of ˙OH. Results were expressed as the mean ± SEM for three independent experiments in two replicates at each concentration (n = 2 per experiment).

5.2.6 Acai berry aqueous and ethanolic extracts exhibit nitric oxide (•NO) scavenging activity

The percentage of •NO scavenging increased in proportion to the concentration of the acai berry extracts, as displayed in Figure 5.9. The acai berry ethanolic extract exhibited greater inhibition activity than the aqueous extract; it had an estimated IC_{50} of 0.17 mg/mL, whereas the estimated IC_{50} of the aqueous extract was 0.16 mg/mL. The maximum inhibition effect (E_{max}) of acai berry aqueous and ethanolic extracts on •NO were 35.6% and 46%, respectively.

The standard, BHA, displayed a higher scavenging ability at lower concentrations, but these reached saturation such that the estimated IC_{50} was 0.082 mg/mL, which was lower than either of the two acai berry extracts.

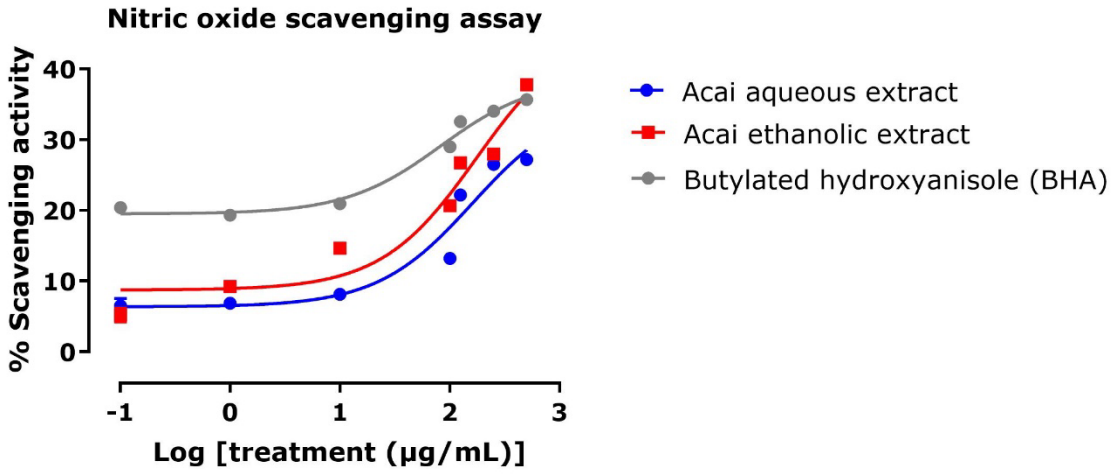

Figure 5.9: Nitric oxide (•NO) scavenging activity of acai berry aqueous and ethanolic extracts. Acai berry antioxidant activity was assessed via the percentage of the scavenging of •NO. Results were expressed as the mean ± SEM for three independent experiments in two replicates at each concentration (n = 2 per experiment).

5.2.7 Acai berry aqueous and ethanolic extracts exhibit lipid peroxidation (LPO) inhibitory activity

The concentration-dependent inhibition of LPO was observed after incubation with either the acai berry aqueous or ethanolic extract, as shown in Figure 5.10. Both extracts displayed relatively moderate anti-lipid peroxidation in comparison with BHA. The acai berry aqueous extract displayed greater antioxidant activity than the ethanolic extract, with estimated IC_{50} values of 4.862 mg/mL and an estimated IC_{50} of 438.8 mg/mL, respectively; BHA had an IC_{50} of 4 µg/mL. At the highest concentration examined, i.e., 1000 µg/mL, the inhibition of lipid peroxidation was 36.5% ± 0.51 and 26.8% ± 1.60 for the acai berry aqueous and ethanolic extracts, respectively, and 82.8% ± 0.16 for BHA. Aqueous and ethanolic extracts of acai berries had maximum inhibition effects (E_{max}) of 39.5% and 27.3%, respectively.

The IC_{50} values and the maximum inhibition effect (E_{max}) for each of the cholinesterase and antioxidant assays are included in Table 5.1.

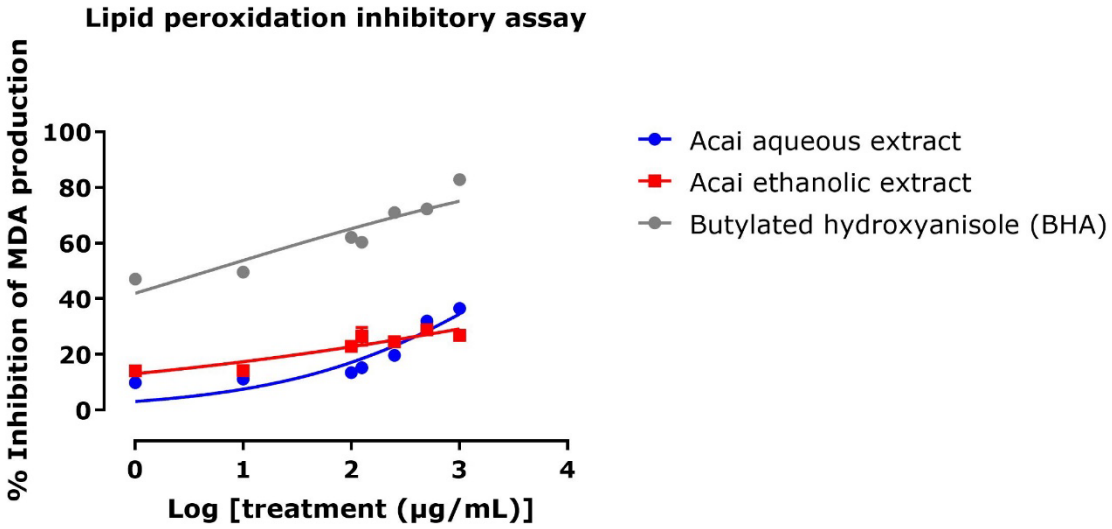

Figure 5.10: Lipid peroxidation inhibitory activity of acai berry aqueous and ethanolic extracts. Acai berry antioxidant activity was assessed via the percentage of the inhibition of malondialdehyde (MDA) production. Results were expressed as the mean ± SEM for three independent experiments in two replicates at each concentration (n = 2 per experiment).

Table 5.1: The approximate IC_{50} values (mg/mL) and E_{max} (%) of acai berry aqueous extract, acai berry ethanolic extract, α-tocopherol (vitamin E), L-ascorbic acid (vitamin A), gallic acid, and butylated hydroxyanisole (BHA) for the AChE, BuChE, DPPH, ABTS, H_2O_2, $^{\bullet}OH$, $^{\bullet}NO$, and LPO assays.

Sample		AChE	BuChE	hAChE	hBuChE	DPPH	ABTS	H_2O_2	$^{\bullet}OH$	$^{\bullet}NO$	LPO
Acai aqueous extract	IC_{50}	1×10^{-6}	6.378	NA	NA	11.551	30.541	7.803	11.604	0.160	4.862
	E_{max}	50	34.7	NA	36.4	NA	31	35	NA	35.6	39.5
Acai ethanolic extract	IC_{50}	NA	NA	NA	NA	0.791	0.462	1.479	0.946	0.175	438.8
	E_{max}	19.3	14.8	NA	44.3	NA	NA	21.8	57.5	46	27.3
α-tocopherol	IC_{50}	-	-	-	-	0.05	1.270	1.847	-	-	-
	E_{max}					89.8	NA	-			
L-ascorbic acid	IC_{50}	-	-	-	-	0.115	0.690	-	-	-	-
	E_{max}					110.8	NA				
Gallic acid	IC_{50}	-	-	-	-	0.008	0.008	0.405	0.001	-	-
	E_{max}					94.37	99.4	60	93.9		
Butylated hydroxyanisole	IC_{50}	-	-	-	-	-	-	-	-	0.082	0.004
	E_{max}									38.7	119

(-), Not evaluated; E_{Max}, maximum effect; NA, not available.

5.2.8 Acai berry aqueous and ethanolic extracts exhibit reducing power activity

The direct reduction of $Fe[(CN)_6]_3$ to $Fe[(CN)_6]_2$ provides a determination of the reducing capacity of a plant compound (Gülçin 2015). The reducing capacity of acai berry aqueous and ethanolic extracts was concentration-dependent but relatively low compared to L-ascorbic acid over the 1000 - 8000 µg/mL concentration range, as shown in Figure 5.11. The acai berry ethanolic extract exhibited more antioxidant capacity than the aqueous extract. However, at the lower concentrations of 0.001 - 10 µg/mL the reducing capacities were limited for the two acai berry extracts and similar and matched that of L-ascorbic acid (Figure 5.11).

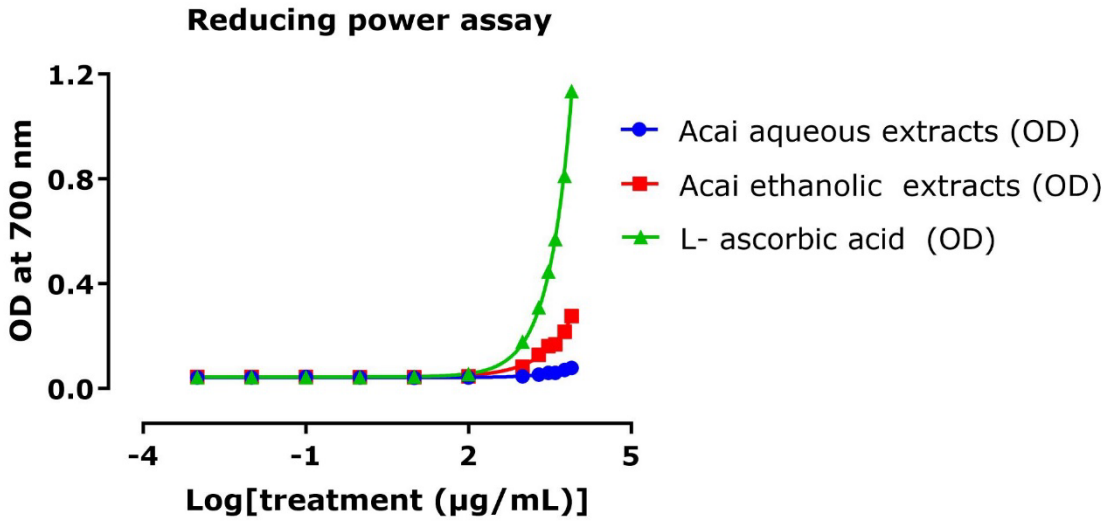

Figure 5.11; Reductive capacity of different concentrations of plant extracts from acai berry. Plant reducing power was measured by the ability to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron (OD density change at 700 nm). The positive control was L-ascorbic acid (vitamin C). Results were presented as the mean ± SEM for three independent experiments in two replicates at each concentration (n = 2 per experiment).

5.2.9 Total phenolic and total flavonoid content of acai berry extracts

The total phenolic content (TPC) and total flavonoid content (TFC) of the acai berry aqueous and ethanolic extracts were quantified and are included in Table 5.2.

The total phenolic content of the extracts was calculated using the gallic acid curve and regression equation presented in Figure 5.12A. Concentration-dependent increase of phenolic content was observed in acai berry aqueous and ethanolic extracts (Figure 5.12B), with most phenolic content in ethanolic extract (Figure 5.12C).

Figure 5.13A illustrates the standard quercetin curve and regression equation used for the calculation of the total flavonoid content of the acai berry extracts. The flavonoid content of acai berry aqueous and ethanolic extracts increased in a concentration dependent way (Figure 5.13B). Acai berry ethanolic extract exhibited the greatest amounts of flavonoid content (Figure 5.13C).

Table 5.2:Total phenolic (TPC) and flavonoid (TFC) contents of acai berry aqueous and ethanolic extracts.

Acai Berry Extracts	Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mg QUER E/g)
Acai aqueous extracts	19.42 ± 0.40	1.26 ± 0.11
Acai ethanolic extracts	101.39 ± 4.61	11.78 ±1.42

Values represent means ± SEM for three independent experiments in duplicate at each concentration (n = 2 per experiment). GAE: gallic acid equivalent; mg QUER E/g: milligram quercetin equivalents/gram of extract.

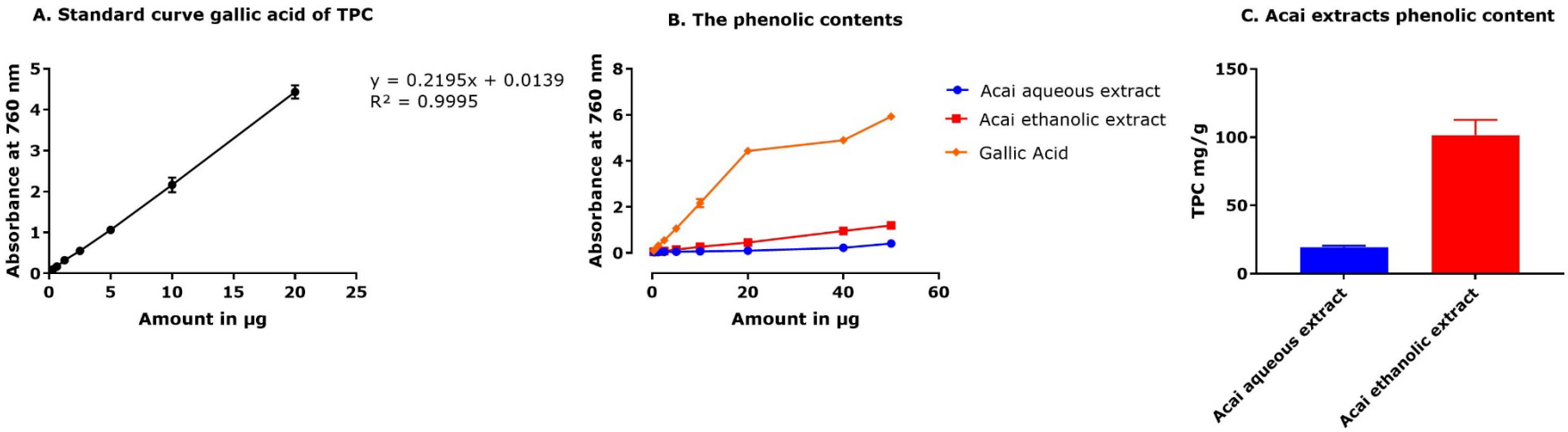

Figure 5.12: (A) Standard curve of gallic acid (TPC). (B) Total phenolic content of different compound amount (0.3 - 50 in µg) of acai berry aqueous and ethanolic extracts and reference gallic acid. (C) The total phenolic content of acai berry extracts. Results were presented as the mean ± SEM for three independent experiments in two replicates at each concentration (n = 2 per experiment).

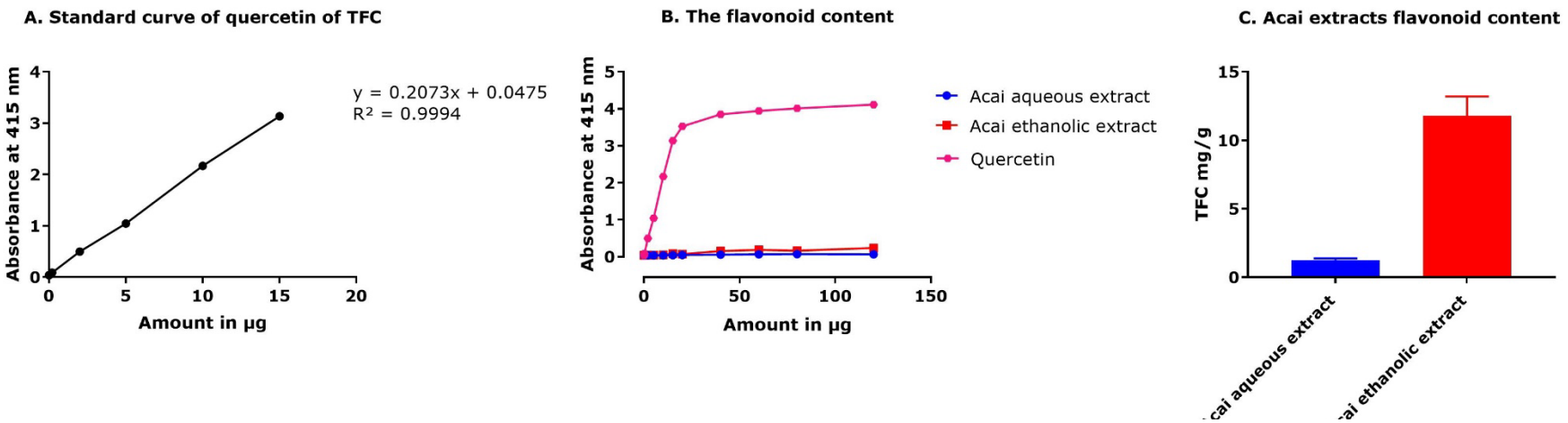

Figure 5.13: (A) Standard Curve of Quercetin (TFC). (B) Total flavonoid content of different compound amounts (0.002 - 120 in µg) of acai berry aqueous and ethanolic extracts and quercetin. (C) The total flavonoid content of acai berry extracts. The data were presented as the mean ± SEM for three independent experiments in two replicates at each concentration (n = 2 per experiment).

5.2.10 Liquid chromatography-mass spectrometry (LC-MS)

LC-MS analysis of acai berry extracts revealed the presence of an extensive range of phytochemicals, including phenolic compounds, flavonoids, lignans, proanthocyanidins, monoterpenoids, norisoprenoids, as well as fatty acids and amino acids (Table 5.3). Differences in the compound profiles between aqueous and ethanolic extractions were evident, with improved recovery of phenolic compounds, phenolic acids and amino acids in PBS, in contrast to flavonoid and fatty acid enrichment in the ethanolic extracts (Table 5.3, Appendices A1).

Table 5.3: LC-MS results of acai berry extracts from this thesis and anti-cholinesterase or antioxidant activities of each chemical compounds that were detected in previous studies (Amat-Ur-Rasool *et al.* 2020; Babiaka *et al.* 2020; Bolouri-Moghaddam *et al.* 2010; Ding *et al.* 2013; Ercan and Dogru 2022; Olennikov *et al.* 2017; Xu *et al.* 2012).

Name of compound	Chemical structure	Molecular weight	Acai berry extracts		Cholinesterase inhibitor activity		Antioxidant activity
			PBS	Ethanol	AChE	BuChE	
Phenolic compounds and phenolic acids:							
Protocatechuic acid	$C_7H_6O_4$	154.12	Detected	Detected	✓	✓	✓
Syringic acid	$C_9H_{10}O_5$	198.17	Detected	Detected	✓	✓	✓
Vanillic acid	$C_8H_8O_4$	168.15	Detected	Detected	✓	✓	✓
Gallic acid	$C_7H_6O_5$	170.12	Detected	Not Detected	✓	✓	✓
4-Hydroxybenzoic acid	$C_7H_6O_3$	138.12	Detected	Detected	✓	✓	✓
Benzoic acid	$C_7H_6O_2$	122.12	Detected	Not Detected	✓	✓	✓
2,5-Dihydroxybenzoic acid	$C_7H_6O_4$	154.12	Detected	Detected	-	-	✓
Chlorogenic acid	$C_{16}H_{18}O_9$	354.31	Detected	Detected	✓	✓	✓
Flavonoids:							
Dihydrokaempferol	$C_{15}H_{12}O_6$	288.25	Detected	Detected	-	-	✓
Luteolin	$C_{15}H_{10}O_6$	286.24	Not Detected	Detected	✓	✓	✓
Quercetin	$C_{15}H_{10}O_7$	302.23	Not Detected	Detected	✓	✓	✓

Name of compound	Chemical structure	Molecular weight	Acai berry extracts		Cholinesterase inhibitor activity		Antioxidant activity
			PBS	Ethanol	AChE	BuChE	
Taxifolin deoxyhexose or Taxifolin	$C_{15}H_{12}O_7$	304.25	Detected	Detected	✓	-	✓
Quercetin-3-O-rutinoside (rutin)	$C_{27}H_{30}O_{16}$	610.5	Not Detected	Detected	✓	✓	✓
Quercetin 3-O-glucoside (Isoquercitrin)	$C_{21}H_{20}O_{12}$	463	Not Detected	Detected	✓	✓	✓
Kaempferol rhamnoside	$C_{21}H_{20}O_{10}$	432.4	Detected	Not Detected	-	-	✓
Isoorientin	$C_{21}H_{20}O_{11}$	448.4	Not Detected	Detected	✓	✓	✓
Lignans:							
(+)-isolariciresinol	$C_{20}H_{24}O_6$	360.4	Detected	Detected	-	✓	✓
(+)-lariciresinol	$C_{20}H_{24}O_6$	360.4	Detected	Detected	✓	✓	✓
Dihydroconiferyl alcohol	$C_{10}H_{14}O_3$	182.22	Detected	Detected	-	-	-
Proanthocyanidins:							
Catechin (+)	$C_{15}H_{14}O_6$	290.27	Not Detected	Detected	✓	✓	✓
Monoterpenoids:							
(+)-menthiafolic acid	$C_{10}H_{16}O_3$	184.23	Detected	Not Detected	-	-	-

Name of compound	Chemical structure	Molecular weight	Acai berry extracts		Cholinesterase inhibitor activity		Antioxidant activity
			PBS	Ethanol	AChE	BuChE	
(E,Z)-2,6-dimethyl-2,6-octadiene-1,8-diol	$C_{10}H_{18}O_2$	170.25	Not Detected	Detected	-	-	-
Norisoprenoids:							
(-)-loliolide	$C_{11}H_{16}O_3$	196.24	Not Detected	Detected	✓	✓	✓
Major fatty acids:							
Monounsaturated fatty acids							
Oleic acid	$C_{18}H_{34}O_2$	282.5	Detected	Detected	-	-	✓
Palmitoleic acid	$C_{16}H_{30}O_2$	254.41	Not Detected	Detected	-	-	-
Polyunsaturated fatty acids							
Linoleic acid	$C_{18}H_{32}O_2$	280.4	Not Detected	Detected	✓	-	✓
Linolenic acid	$C_{18}H_{30}O_2$	278.4	Not Detected	Detected	✓	✓	✓
Saturated fatty acids							
Palmitic acid	$C_{16}H_{32}O_2$	256.42	Not Detected	Detected	✓	✓	-
Stearic acid	$C_{18}H_{36}O_2$	284.5	Not Detected	Detected	-	-	-
Amino acids:							
Alanine	$C_3H_7NO_2$	89.09	Detected	Detected	✓	-	-
Lysine	$C_6H_{14}N_2O_2$	146.19	Detected	Not Detected	✓	-	✓

Name of compound	Chemical structure	Molecular weight	Acai berry extracts		Cholinesterase inhibitor activity		Antioxidant activity
			PBS	Ethanol	AChE	BuChE	
Arginine	$C_6H_{14}N_4O_2$	174.2	Not Detected	Detected	✓	-	✓
Methionine	$C_5H_{11}NO_2S$	149.21	Detected	Not Detected	×	-	✓
Phenylalanine	$C_9H_{11}NO_2$	165.19	Detected	Detected	×	-	-
Proline	$C_5H_9NO_2$	115.13	Detected	Detected	✓	-	-
Glutamic acid	$C_5H_9NO_4$	147.13	Detected	Detected	×	-	-
Serine	$C_3H_7NO_3$	105.09	Detected	Detected	✓	-	-
Glycine	$C_2H_5NO_2$	75.07	Detected	Detected	-	-	-
Threonine	$C_4H_9NO_3$	119.12	Detected	Detected	-	-	-
Histidine	$C_6H_9N_3O_2$	155.15	Detected	Detected	×	-	✓
Tryptophan	$C_{11}H_{12}N_2O_2$	204.22	Detected	Detected	×	-	✓
Tyrosine	$C_9H_{11}NO_3$	181.19	Detected	Detected	✓	-	✓
Isoleucine	$C_6H_{13}NO_2$	131.17	Detected	Detected	-	-	-
Valine	$C_5H_{11}NO_2$	117.15	Detected	Detected	✓	-	-
Leucine	$C_6H_{13}NO_2$	131.17	Not Detected	Detected	×	-	-
Other compounds:							
Cellotetraose	$C_{24}H_{42}O_{21}$	665	Not Detected	Detected	-	-	-
Sucrose	$C_{12}H_{22}O_{11}$	341	Detected	Detected	-	-	✓

Name of compound	Chemical structure	Molecular weight	Acai berry extracts		Cholinesterase inhibitor activity		Antioxidant activity
			PBS	Ethanol	AChE	BuChE	
Quinic acid isomer 1	$C_7H_{12}O_6$	191	Detected	Detected	-	-	✓
Ascorbic acid (Vitamin C)	$C_6 H_8 O_6$	176.03	Detected	Detected	-	-	✓

Abbreviation: PBS, phosphate buffered saline; (×), not observed, (-), no data; (✓) Recognized or published.

5.3 Discussion

This chapter evaluated the nutraceutical and, hence, the therapeutic potential of acai berry extracts based on their ability to act as ChEIs, as well as their additional benefits of free radical scavenging and antioxidant activities. The current first-line treatment for mild-to-moderate AD is to treat the cholinergic deficit experienced by AD patients via a transient inhibition of AChE to increase the signal longevity of the neurotransmitter ACh (Ferreira-Vieira *et al.* 2016; Massoud and Léger 2011; Winslow *et al.* 2011). However, ChEI treatment can induce adverse reactions and only addresses one component of the AD disease pathology (insufficient ACh levels), whereas multiple elements of cellular dysfunction may contribute to the disease, including oxidative stress (Barbagallo *et al.* 2015; Cai *et al.* 2011; Cummings *et al.* 2019; Lovell and Markesbery 2007; Su *et al.* 2008; Sutherland *et al.* 2013; Winslow *et al.* 2011; Yiannopoulou and Papageorgiou 2020; Youssef *et al.* 2018). Hence, there is an unmet need to tackle disease aetiology, for example, through a multipronged treatment strategy (Amat-Ur-Rasool *et al.* 2021; Cummings *et al.* 2019). Indeed, animal models of AD have demonstrated improvements in cognitive function and behavioural defects after antioxidant therapy (Collins *et al.* 2022; Wang *et al.* 2021).

In experiments using ChE of non-human origin, aqueous extract from the acai pulp inhibited both electric eel AChE (estimated IC_{50} of 0.001 μg/mL) and, to a lesser extent, equine BuChE (estimated IC_{50} of 6.378 mg/mL), suggesting that acai berry extract may contain an inhibitory agent(s). Interestingly, the agent(s) binding the cholinesterases presumably reached a point of saturation at an approximate concentration of 0.01 μg/mL, such that further enzymatic inhibition was limited. This may result from finite binding at the esteratic and/or peripheral binding sites of AChE or BuChE (Bajda *et al.* 2013; Dvir *et al.* 2010; Rosenberry 2010; Silva *et al.* 2020). Alternatively, this could be as result of acai berry extracts binding to an allosteric site found peripherally on AChE or BuChE, reducing the binding affinity but not completely preventing it (Bourne *et al.* 2003; Macdonald *et al.* 2012). This question can be probed further once the active agent(s) in acai berry extracts is/are purified. By comparison, the ethanolic extract displayed minimal anti-AChE and anti-BuChE activities; there were only minor reductions in

activity evident at the highest concentrations of extract, and these did not reach significance. This may reflect the solvation of the agent(s) within water alone (rather than ethanol) since water is a more polar solvent than ethanol. Ultimately, a range of extraction methods and solvents may be needed to isolate active agents such as polyphenols, with the solvation of specific phytochemical(s) governed by the polarity of the solute of interest (Altemimi *et al.* 2017). Specifically, for acai berry phytochemical extractions, an independent study reported that water as a solvent produced the highest yields of polyphenols and flavonoids as compared with methanol and ethanol alone (Chung 2012), although this may be improved further if a hydroalcoholic extraction is undertaken (50% ethanol) (López de Dicastillo *et al.* 2019). Herein, the benefit of an aqueous extraction as a method for the possible isolation of soluble ChEIs and their future purification and identification.

On the other hand, according to this chapter findings, neither aqueous nor ethanolic acai berry extracts had any inhibitory effects on hAChE, whereas both extracts showed significant inhibitory effects on hBuChE. However, acai berry extracts exhibited different inhibitory effects on AChE and BuChE enzymes of animal and human origin; overall, it is demonstrated here that acai berry extracts contain ChEI(s). Moreover, this is more explained by several studies that demonstrated the interspecies differences in AChE and BuChE inhibition via using different ChEI (Bosak *et al.* 2008; Kasteel *et al.* 2020; Khan *et al.* 2008). In order to explain this, the structure of AChE and BuChE between animals and humans may be similar but not identical, thus inhibiting differences between species. Eventually, the results here need to be confirmed by *in vitro* human cell models.

Chemicals able to target and simultaneously inhibit both AChE and BuChE, rather than AChE alone, may offer improved clinical efficacy to combat the increased levels of BuChE in AD patients, with a reduction in side effects (Amat-ur-Rasool *et al.* 2022; Li *et al.* 2017b; Mushtaq *et al.* 2014; Zhou and Huang 2022). However, neither of the drugs currently approved by the US FDA, namely donepezil and galantamine, are potent BuChE inhibitors (BuChE IC_{50} values of 5 μM and 12.6 μM, respectively), whereas rivastigmine, the FDA approved drug, was originally extracted from a medicinal plant, is a relatively potent AChE and BuChE inhibitor (IC_{50} values of 4 nM and 13 nM,

respectively) (Ogura *et al.* 2000; Tang and Han 2006). It will be of interest in future studies to determine whether the same agent(s) within the acai berry aqueous extract is/are responsible for inhibiting both AChE and BuChE, and to determine the relative potency of the inhibitor(s). A comparison of the anti-AChE activity of the extracts/fractions of 54 plant species used guidelines that considered an IC_{50} < 20 µg/mL as high potency, moderate potency as an IC_{50} > 20 µg/mL but < 200 µg/mL, and low potency as an IC_{50} > 200 µg/mL but < 1000 µg/mL (Dos Santos *et al.* 2018). The cut-offs for potency related to the average IC_{50} value for galantamine, reported in the literature as approximately 2 µM (or 0.575 µg/mL), multiplied by a factor of 10 (Dos Santos *et al.* 2018). Accordingly, the aqueous extract of acai fruits, with an IC_{50} of 0.001 µg/mL, was an extract of high potency and therefore has potential use as an effective ChEI.

In addition to ChEI activities, the acai berry extracts displayed useful radical scavenging and antioxidant activities; they were able to scavenge DPPH, ABTS, $^{\bullet}OH$, H_2O_2, and $^{\bullet}NO$ free radicals, and they exhibited ferric ion reduction. Similarly, other independent studies have reported the high antioxidant capacity of the acai berry against $O^{2\bullet-}$ and peroxyl radicals (RO_2) (Schauss *et al.* 2006a). Acai berry also displays useful neuroprotective activity, and it can prevent rotenone-induced oxidative damage (Machado *et al.* 2016). Acai pulp also reduced nitrite radicals (NO^{2-}) in mouse brain BV-2 microglial cells *in vitro* (Poulose *et al.* 2012). Acai flower and spike fractions (as well as the fruit) also contain agents able to inhibit $^{\bullet}NO$ production (Matheus *et al.* 2006). In addition, in a pilot study with human volunteers, the antioxidant capacity of plasma was elevated by 2.3- and 3-fold after the consumption of acai juice and pulp, respectively (Mertens-Talcott *et al.* 2008). It is also promising to note that an *in vivo* study demonstrated that an acai-rich diet reduced markers of oxidative stress in brain regions of aged rats (Poulose *et al.* 2017).

The current chapter also demonstrated that acai berry extracts have anti-LPO effects, in support of studies *in vitro* (Schauss *et al.* 2006a) and *in vivo* (Spada *et al.* 2009). The significant inhibition of LPO was also detected post-consumption of a juice blend (of which acai berry was the predominant ingredient) in a pilot study with human volunteers (Jensen *et al.* 2008).

Research has considered the chemical composition of the acai berry and its antioxidant potential and has detected the presence of numerous polyphenols and flavonoids, such as anthocyanins (Jensen *et al.* 2008; Kang *et al.* 2011; Machado *et al.* 2016; Matheus *et al.* 2006; Mertens-Talcott *et al.* 2008; Pacheco-Palencia *et al.* 2008) (refer to Appendices A2). Phytochemicals such as these may provide the basis for the acai berry extract to neutralise free radicals and potentially limit oxidative stress, such as that associated with AD aetiology, but further *in vitro* and *in vivo* studies are required to confirm the potential use of acai berry extracts as a treatment option for AD. Ultimately, a diet that incorporates acai berries may provide the ongoing benefit of a diet rich in antioxidants along with the possibility of sustained cholinergic signalling that may limit the likelihood of developing or indeed the progression of NDDs such as AD.

Further investigation of the phytochemical composition of the acai berry extracts was conducted using LC-MS analysis. This revealed several compounds found in acai berry aqueous and/or ethanolic extracts with recognized antioxidant and anti-ChE activities such as protocatechuic acid, syringic acid, vanillic acid, 4-hydroxybenzoic acid, chlorogenic acid, quercetin, taxifolin, quercetin-3-O-rutinoside, isoorientin and (+)-lariciresinol (Table 5.3) (Amat-Ur-Rasool *et al.* 2020; Babiaka *et al.* 2020; Bolouri-Moghaddam *et al.* 2010; Ding *et al.* 2013; Ercan and Dogru 2022; Olennikov *et al.* 2017; Xu *et al.* 2012). This suggests that acai berry extracts may contain a variety of phytochemicals with dual anticholinesterases and antioxidant properties that may protect against neurodegeneration associated with AD. However, these acai berry extracts and phytochemicals will require further *in vivo* testing in order to assess their therapeutic potential.

Chapter 6

General discussion

Chapter 6. General discussion

6.1 Summary of findings and discussion

Exogenous free L-Glu can be introduced to the human body through many routes, mainly orally, in addition to subcutaneous or inhalant ways (Chakraborty 2019). MSG is a sodium salt of glutamic acid that gives free L-Glu in an aqueous solution (Cynober 2018; Farhat *et al.* 2021; Henry-Unaeze 2017; Sano 2009). There is an ongoing debate regarding the hazards and risks of MSG consumption through food and whether or not it results in brain disease conditions. The EFSA panel re-evaluated L-Glu safety in 2017, and the panel concluded that the daily intake of L-Glu exceeding 30 mg/kg bw per day is associated with adverse health outcomes (Mortensen *et al.* 2017; Roberts *et al.* 2018). As a result of some misconceptions about the amount of MSG that should be used, MSG use still remains controversial (Zanfirescu *et al.* 2019). Neuroscientists consider that exogenous L-Glu in the diet can accumulate in the brain and threaten the survival of neurons. This hypothesis of L-Glu elevation in the brain is supported by a recent study reporting that neonates and adults treated with MSG raised their levels of L-Glu, whether administered orally or intraperitoneally (Akataobi *et al.* 2021). Therefore, the consumption of dietary MSG could possibly pass into the brain tissue, raise the L-Glu level and cause neurotoxicity, a risk that is higher in infants with inadequate BBB development.

Furthermore, researchers continue to investigate L-Glu toxicity because of its implications for NDDs. The excess levels of L-Glu contribute to a variety of etiological processes, including oxidative stress, excitotoxicity, mitochondrial dysfunction, inflammation, and protein aggregation, leading to neuronal damage and degradation, which contributes to the progression of NDDs (Chen *et al.* 2012; Denzer *et al.* 2016; Kovacs 2016; Lewerenz and Maher 2015). As a result of the identified pathomechanisms associated with excess L-Glu accumulation, it is essential to find agents that can prevent and/or reverse these adverse effects of cellular imbalance. This thesis may lead to the development of novel therapeutic methods that target NDDs and stroke.

There has been an increase in interest in herbal medicines in recent years because phytochemicals contents can have long-term health benefits or therapeutic effects

(Elufioye *et al.* 2017; Iriti *et al.* 2010; Khazdair *et al.* 2019; Kumar and Khanum 2012). A novel fruit called 'acai' is found on tropical palm trees of the *Euterpe* genus that grows in the Amazon region of South America and a few Caribbean islands (Schauss 2010). Acai berries have been called a 'superfood' since they were discovered due to their high nutritional value and phytochemical content, as well as its pulp that has been widely studied for its health benefits (Kang *et al.* 2011; Schauss *et al.* 2006a; Schauss 2010). Furthermore, despite some toxicity effects caused by high acai berry extract concentrations, several *in vitro* and *in vivo* toxicity evaluations of acai berry extract have documented its safety and absence of toxic effects (Benatrehina *et al.* 2018; Caiado *et al.* 2017; Choi *et al.* 2017; Marques *et al.* 2017; Monge-Fuentes *et al.* 2017; Schauss *et al.* 2010; Spada *et al.* 2008; Udani *et al.* 2011). Additionally, several studies have demonstrated the neuroprotective properties of acai berries (Machado *et al.* 2016; Poulose *et al.* 2012; Poulose *et al.* 2014; Wong *et al.* 2013). Thus, the acai berry appears to be a promising dietary agent capable of neuroprotection or perhaps even preventing neurodegeneration.

Therefore, this thesis focused on the neurotoxicity evaluation of L-Glu and the neuroprotective effects of acai berry against L-Glu toxicity. The L-Glu neurotoxicity and its associations with the NDDs pathogenesis such as AD, PD, MS, ALS, and HD in this thesis was evaluated by conducting a systematic review. Different molecular assessments were conducted including MTT, LDH, ATP, MMP and DCFHDA assays, that were used to assess L-Glu toxicity, acai berry extract safety and investigate neuroprotection effects through co-application of acai berry extracts with L-Glu. Furthermore, the effects on iGluRs were conducted using the whole-cell patch-clamp assay. Pharmacological assessments of acai berry aqueous and ethanolic extracts were performed to determine their ability to inhibit ChE enzymes and scavenge free radicals via DPPH or ABTS assays. In addition, efficacy in scavenging H_2O_2 or $^{\bullet}OH$, inhibiting LPO, and reducing Fe^{+3} was assessed as well as both acai extracts were tested for polyphenol and flavonoid content. LC-MS was used to identify phytochemicals that may be responsible for the anticholinesterase and antioxidant properties of acai berry extracts.

In light of these concerns that the excessive exogenous L-Glu may accumulate at the synaptic cleft and cause neuronal death (Niaz *et al.* 2018; Zanfirescu *et al.* 2019), Chapter 3 explored the effects of exogenous L-Glu on neuronal viability and implications for NDDs pathology, such as AD, PD, MS, ALS, and HD, by conducting a systematic, unbiased research investigation. This study found from a total of 71 included studies that both cell-based and animal findings supported L-Glu involvement in a number of toxic cellular mechanisms. First, L-Glu diminished neuronal viability and induced the release of LDH. Furthermore, several apoptotic factors and intracellular signalling pathways are activated by L-Glu that result in apoptosis. In addition, markers of cellular structural changes and injury were observed, along with perturbations in cell growth and proliferation. The generation of redox stress and depletion of the antioxidant defence system are some of the mechanisms contributing to cellular damage caused by L-Glu. Following L-Glu exposure, oxidative damage to proteins, lipid and DNA was induced both *in vitro* and *in vivo* due to significant accumulation of ROS and RNS.

The effects of L-Glu on AChE activity were observed both *in vitro* and *in vivo*, and a number of brain neurotransmitters were adversely affected, including L-Glu, dopamine, serotonin, noradrenaline, and adrenaline levels. *In vivo* and *in vitro* studies evidenced that L-Glu impaired mitochondrial function. It has been shown that L-Glu excitotoxicity led to an increase in intracellular Ca^{2+} levels and the release of intracellular Ca^{2+} from intracellular stores. This resulted in increased expression of L-Glu receptor subunits, Ca^{2+}-dependent proteases such as calpain, as well as enzymatic overactivation, such as protein kinases, resulting in cellular protein and membrane breakdown. Additionally, L-Glu exposure also impaired the inhibitory GABA homeostasis *in vitro* and *in vivo*. Furthermore, L-Glu administration led to advanced activation of several proinflammatory markers and mediators as well as a decline in anti-inflammatory cytokines *in vivo* and *in vitro*. L-Glu application triggered toxic protein aggregations such as extracellular Aβ peptide accumulation *in vivo* and intracellular hyperphosphorylated tau *in vivo* and *in vitro* and these can contribute to neurodegeneration. Both *in vivo* and *in vitro* L-Glu exposure altered neuronal morphological characteristics, including the cell body, nucleus, and processes. There was evidence of haemorrhaging and neuroinflammatory cell aggregation in animal brains following L-Glu induction, and

excessive levels of L-Glu were linked to animal behavioural changes and cognitive impairments. In addition, systematic L-Glu administration changed the relative weight and ionic balance of animal brains. Thus, a high intake of L-Glu can consequently lead to pathological outcomes that results in neuronal degeneration and death. In light of the improved understanding of these pathological mechanisms, there is the possibility to design less toxic and potentially more effective agents, including antioxidants, which can target multiple components of neurotoxicity and neuronal damage.

The focus of the fourth chapter was to investigate the toxicity of L-Glu and the acai berry extracts and their neuroprotection potential against excessive L-Glu-induced neuronal toxicity, using the SH-SY5Y human neuroblastoma cell line, the differentiated TE671 human rhabdomyosarcoma cell line, and the ReNcell CX human neural progenitor cell line. In undifferentiated SH-SY5Y cells, only high L-Glu concentration reduced neuron viability significantly after 24 hours or 48 hours. In contrast, differentiated SH-SY5Y cells showed a nonsignificant reduction in cell viability at lower concentrations for 24 hours. A concentration-dependent reduction in cell viability after L-Glu exposure for 24 hours in differentiated TE671 cells was observed and only a high concentration induced cell viability reduction after 48 hours. Furthermore, all L-Glu concentrations induced cell viability decline after 24 hours exposure and after 48 hours only high concentrations exhibited toxicity impacts in ReNcell CX cells. There have been a number of studies that have shown this variation in L-Glu effect on neuron viability (Bebitoglu *et al.* 2020b; Lee *et al.* 2019; Shah *et al.* 2016; Sun *et al.* 2016). A variation in L-Glu toxicity is explained in this thesis systematic review by the fact that some cell lines exhibit L-Glu toxicity as a result of overactivation of specific L-Glu receptors, whereas other cell lines do not have such receptors, and the neurotoxicity is manifested through alternative pathomechanisms, such as induction of redox stress refer to Chapter 3. No further experiments were done using differentiated SH-SY5Y cells due to their resistance to L-Glu toxicity. Similar observations found in an earlier study that reported differentiated SH-SY5Y cells were less susceptible to L-Glu toxicity than undifferentiated cells (Nampoothiri *et al.* 2014). Additionally, experiments planned to be performed with ReNcell CX cell lines in 2020 had to be stopped due to Covid-19 restrictions.

However, L-Glu exposure resulted in an increase in LDH production and a decline in ATP levels in undifferentiated SH-SY5Y cells and differentiated TE671 cells. Similar results were observed in earlier studies in which L-Glu induced cell viability reduction (Bebitoglu *et al.* 2020b; Bharate *et al.* 2018; Brizi *et al.* 2016; de Vera *et al.* 2008; De Oliveira *et al.* 2019; Gupta *et al.* 2013; Hu *et al.* 2012; Jeong *et al.* 2014; Kataria *et al.* 2012; Lee *et al.* 2019; Li *et al.* 2017a; Nampoothiri *et al.* 2014; Occhiuto *et al.* 2008; Petroni *et al.* 2013; Shah *et al.* 2016; Sun *et al.* 2016; Xin *et al.* 2019; Yang *et al.* 2019b; Yuksel *et al.* 2019; Zhu *et al.* 2016), LDH level increase (Chao and Hu 1994; Fallarini *et al.* 2009; Hu *et al.* 2012; Kataria *et al.* 2012; Li *et al.* 2017a; Occhiuto *et al.* 2008; Yang *et al.* 2019b; Yon *et al.* 2018), as well as ATP level decline (De Oliveira *et al.* 2019; Li *et al.* 2017a; Yang *et al.* 2019b). The results from this thesis also showed that L-Glu decreased MMP levels and increased ROS production in undifferentiated SH-SY5Y cells and differentiated TE671 cells, similar to previous studies (Brizi *et al.* 2016; De Oliveira *et al.* 2019; Li *et al.* 2017a; Nampoothiri *et al.* 2014; Shah *et al.* 2016; Sun *et al.* 2016; Xin *et al.* 2019; Yang *et al.* 2019b; Zhu *et al.* 2016).

There are two possible mechanisms through which this toxicity is caused; one is excitotoxicity and the other is oxidative stress. Therefore, whole-cell patch clamping was used to test for the presence of iGluRs in order to understand the mechanisms underlying L-Glu toxicity. According to the results from Chapter 4, stimulation with L-Glu and Gly did not result in a current response in undifferentiated SH-SY5Y cells, while a significant current in response to L-Glu and Gly, KA, and NMDA and Gly stimulation in dbcAMP-differentiated TE671 cells was observed. Therefore, it appears that L-Glu toxicity might be mediated by oxidative damage via inhibition of the X_c^- system functions or mitochondrial dysfunction caused by excessive Ca^{2+} build-up via activation of mGluR or ROS build-up in undifferentiated SH-SY5Y cells, but not through NMDAR overactivation (Atlante *et al.* 2001; Bebitoglu *et al.* 2020a; Duchen 2000; Jantas *et al.* 2008; Lewerenz *et al.* 2013; Mattson 2003; Norenberg and Rao 2007; Weber *et al.* 2010). However, in differentiated TE671 cells, it appears that not only oxidative damage and mitochondrial dysfunction are responsible for L-Glu toxicity, but also excitotoxicity via NMDAR, where the L-Glu receptors are over-stimulated causing numerous cytotoxic

events culminating in the death of neurons (Luksch *et al.* 2011; Stepulak *et al.* 2009; Wojciech *et al.* 2001).

The results of this study demonstrated that acai berry extracts increased the survival rate of undifferentiated SH-SY5Y and differentiated TE671 cells, similar to earlier studies showing that acai application improved cell viability and proliferation (Cadoná *et al.* 2021; Machado *et al.* 2016). Even though acai berry extracts increased LDH and ROS levels in both cells and reduced ATP and MMP levels, our study confirmed that this extract offers neuroprotection against L-Glu toxicity. Co-application of acai berry extracts increased cell viability, ATP and MMP levels, which had been decreased after L-Glu application alone; in addition, it decreased LDH and ROS production that were induced by L-Glu application alone. Several studies have reported the acai berry extracts ability to inhibit cell viability loss, and modulate mitochondrial dysfunction and oxidative stress (Cadoná *et al.* 2021; De Souza *et al.* 2020; Machado *et al.* 2016; Torma *et al.* 2017; Wong *et al.* 2013). Thus, much established evidence demonstrates the neuroprotective properties of acai berries.

According to this thesis findings, acai berry aqueous extract inhibited L-Glu + Gly-activated currents in a concentration-dependent way in differentiated TE671 cells. It has been reported that NMDAR-mediated glutamatergic excitation can be blocked by various plants and herbal products (Afshari *et al.* 2020; Liang *et al.* 2013; Marchetti *et al.* 2011; Silva *et al.* 2019a). In light of the fact that NMDAR excitotoxicity is associated with NDDs and stroke pathology, finding agents that inhibit the NMDAR activity may be helpful in treating or preventing these diseases (Belov Kirdajova *et al.* 2020; Doyle *et al.* 2008). These novel findings open the avenue for identifying acai berry's active component(s) and using them to treat NDDs and stroke.

The fifth chapter of the thesis focused on examining the nutraceutical potential of *Euterpe* sp. (in the form of aqueous and ethanolic extracts) regarding its ability to inhibit AChE and BuChE as its antioxidant potential as a potential AD treatment. The results suggested that acai berry aqueous extract may contain an inhibitory agent(s) that is able to inhibit both electric eel AChE and equine BuChE. An ethanolic extract displayed

minimal anti-AChE and anti-BuChE activity such that at the highest concentration examined, only minor reductions in activity were evident. There is no doubt that a range of extraction methods and solvents may be required in order to isolate active agents like polyphenols, with the solvation of specific phytochemicals being determined by the polarity of the solute (Altemimi *et al.* 2017). However, neither the aqueous nor ethanolic extracts of acai berries displayed any inhibitory effects on hAChE, while both extracts inhibited hBuChE significantly. Hence, collectively, the acai berry extracts showed varied inhibitory effects on AChE and BuChE enzymes that derived from both humans and animals so the acai berry extracts may contain ChEI agent(s). This is further explained by several studies that showed interspecies variations in AChE and BuChE inhibition (Bosak *et al.* 2008; Kasteel *et al.* 2020; Khan *et al.* 2008). Furthermore, instead of just targeting AChE, drugs that concurrently target and inhibit both AChE and BuChE may be more clinically effective in combating the elevated levels of BuChE in AD patients (Amat-ur-Rasool *et al.* 2022; Li *et al.* 2017b; Mushtaq *et al.* 2014; Zhou and Huang 2022). However, in future research it will be interesting to find out if the same agent(s) in the acai berry aqueous extract is/are responsible for inhibiting both AChE and BuChE, as well as to find out the inhibitor agent(s) potency.

The acai berry extracts also showed beneficial radical scavenging and antioxidant properties in addition to ChEI activities; these included reducing ferric ions, LPO and scavenging DPPH, ABTS, $^{\bullet}OH$, H_2O_2, and $^{\bullet}NO$ free radicals. Similar to these research results, numerous studies have noted that the acai berry retains considerable antioxidant capacity against ROS and oxidative stress (Machado *et al.* 2016; Poulose *et al.* 2012; Schauss *et al.* 2006a; Spada *et al.* 2009). The finding of this research showed that in addition to potent antioxidant capacity, the acai berry extracts display high phenolic and flavonoid contents. According to multiple studies, the acai berry antioxidant capacity is attributed to the presence of various polyphenols and flavonoids, including anthocyanins (Jensen *et al.* 2008; Kang *et al.* 2011; Machado *et al.* 2016; Matheus *et al.* 2006; Mertens-Talcott *et al.* 2008; Pacheco-Palencia *et al.* 2008). An extensive range of phytochemicals was identified in acai berry extracts using the LC-MS analysis and these compounds may be responsible for anticholinesterases and antioxidant properties of the acai berry extracts (Table 5.3). Furthermore, plants such as *Eucalyptus globulus*,

Origanum vulgare, and *Moringa oleifera* shared some phytochemical compositions, including chlorogenic acid, and rutin, with acai berry extracts, which demonstrated their potent antioxidant and anticholinesterase properties (Amat-Ur-Rasool *et al.* 2020; de Torre *et al.* 2022; Nwidu *et al.* 2018; Xu *et al.* 2019). According to this thesis, acai berry extracts can be fractionated, and their components identified so that they may be used as drug substitutes for AD treatments in the future.

6.2 Implications of the study

In general, the results presented in this thesis have made a significant contribution to our knowledge by building on existing evidence of the L-Glu toxicity and its implication to neuronal death that is observed in NDDs and stroke. It validated and confirmed an existing theory that L-Glu toxicity can be mediated via excitotoxicity and/or oxidative injury. Moreover, this thesis provided novel findings demonstrating that acai berry extracts have anti-excitotoxicity and anti-cholinesterase activities.

Multiple cellular pathomechanisms are involved in L-Glu neurotoxicity, including inducing redox stress and depleting antioxidants, mitochondrial dysfunction, excitotoxicity, neuroinflammation, neurotransmitter levels alteration, and aggregation of aberrant proteins and these can often be observed in NDDs. Likewise, L-Glu excessive release after stroke is associated with pathological mechanisms including excitotoxicity, oxidative stress, ion imbalance, and mitochondrial dysfunction. Therefore, this thesis provides a better understanding of these diverse pathomechanisms and that may facilitate the development of more effective therapies that have the potential to mitigate multiple aspects of neurotoxicity and neurodegeneration.

Conventional drug approaches that target one pathomechanism may not be ideal for disease-modifying therapeutic development for NDDs and stroke. Stroke treatments for example do not interfere with pathological events indicated above and were designed to deal with symptoms only, such as bleeding, clot formation or ischemia, high BP, and intracranial hypertension. Thus, this thesis demonstrated that acai berry extracts have multi-activities including maintaining cell viability and mitochondrial function, in addition to antioxidant, anti-excitotoxicity, and anti-cholinesterase activities. In light of those findings acai berry extracts appear to be capable of modifying or attenuating L-Glu

toxicity; this study suggests they may contain neuroprotective agent(s). Therefore, bioactivity-guided fractionation of acai berry extracts might explore a potent compound for NDDs and stroke treatment that could be designed in a dosage form with the collaboration of pharmaceutical companies.

This study provides a substantial basis for future research, it may open new avenues for investigating acai berry extract safety, efficacy, and ability to cross the BBB to reach neurons in the brain through *in vivo* animal studies and clinical trials. Further studies could be conducted on the potential effects of acai berry extracts on brain tissue and cognitive function in patients with NDDs and stroke.

6.3 Limitations of the study and future work

Even though this thesis has achieved its main aims and objectives, it is essential to emphasise its limitations. This research employed cost- and time-efficient reasonably high throughput assays to assess the neurotoxicity of L-Glu and acai berry extracts in two cell lines; human neuroblastoma SH-SY5Y cells and a differentiated human TE671 cells. After MTT assessment, differentiated SH-SY5Y cells in this study demonstrated resistance to L-Glu toxicity; hence, no additional experiments were conducted. There was an opportunity to evaluate the neurotoxicity of L-Glu and acai berry extracts using a human neural progenitor cell line (ReNcell CX), which more closely resembles the complexity of the CNS and representation of three cell lineages when differentiated (Donato *et al.* 2007). However, due to the impact of COVID-19, further experiments which were planned to be done in 2020 could not be completed. Nonetheless, the trials conducted using undifferentiated SH-SY5Y cells and differentiated TE671 cells have achieved the objectives of this dissertation.

The human undifferentiated SH-SY5Y and differentiated TE671 cell lines employed in this investigation could only represent one homogenous neuronal-like cell population and so might not accurately represent the response from a mixed cellular population or their intercellular interactions that are found in human brains (Bencherif and Lukas 1991; Sasaki *et al.* 1986; Siegel and Lukas 1988; Xie *et al.* 2010). Thus, primary cell cultures with mixed neuronal populations may be used in future studies.

L-Glu receptor expression is crucial because this study focused on L-Glu excitotoxicity; however, the results of this thesis showed no response to combined L-Glu and Gly administration in SH-SY5Y cells. This might be due to differences in cultivation methods, or because neuroblastoma cells are tumoral in origin and their phenotype might change over time. Therefore, it may result in NMDAR and other iGluRs being nonfunctional when examined via the whole cell patch clamp technique. Nevertheless, the expression of functional iGluRs in TE671 cell lines was confirmed earlier, and it was examined in this study (Brocke *et al.* 2010; Luksch *et al.* 2011; Stepulak *et al.* 2009; Wojciech *et al.* 2001). As a result, this thesis suggested the relevance of differentiated TE671 cells as a model for examining the toxicity of L-Glu.

In addition, this thesis had some shortcomings due to the use of *in vitro* experiments and models. L-Glu neurotoxicity experiments in this thesis typically used *in vitro* models that are exposed to the chemical for short periods of time. While these acute L-Glu neurotoxic effects may not be observed in humans, they may still be relevant to understanding the molecular mechanisms behind neuronal loss in NDDs and may be applicable to other diseases that are affected by L-Glu levels, like stroke. Two significant drawbacks of this study, which are frequently cited as issues with all *in vitro* investigations, are the lack of a BBB and gastrointestinal tract metabolism. Even though the BBB acts as a physical barrier that prohibits molecules from entering the brain, there are regions of the brain known as circumventricular organs (CVOs) that allow for the unrestricted movement of larger molecules (Hawkins and Viña 2016; Montagne *et al.* 2015; Standring 2015). Undoubtedly, in humans, the L-Glu neurotoxicity and the effects of acai berries on the brain could be partially offset by lower circulating levels once gastrointestinal metabolism occurs after oral consumption (Ganesan *et al.* 2013; Gavhane and Yadav 2012). However, the focus of recent research was on determining the direct effects of L-Glu and acai berry extracts on neuronal cells.

The complexity of the CNS makes it difficult to completely correlate L-Glu and acai berry extract studies in both cell lines with human conditions, as well as not being able to cover all the mechanisms involved in NDDs, such as neuroinflammation and protein toxicity, with these two cell models and a limited number of measurement endpoints.

Consequently, there is still a need to validate the current data with human primary cell cultures and neuronal models or to develop *in vivo* models to observe behaviour and responses to L-Glu and acai berry extract exposure in humans.

In addition, there may be research limitations associated with using only one commercially available acai powder; therefore, further studies with non-commercial whole fruits may be undertaken in the future. The main limitation of the acai berry study that aimed to analyse the nutraceutical potential of *Euterpe* sp for possible development as an AD treatment is that the work was performed *in vitro,* and *in vivo* analyses, particularly those that mimic NDDs such as AD, are needed in the future. Even though this study demonstrated that the acai berry contained phytochemicals that likely contribute to its anti-ChE and antioxidant effects, it did not pinpoint the chemical(s) responsible for these effects. Therefore, acai extracts could be further separated in the future and their components identified for their potential use as a medication alternative for AD therapy because of their potent ChEI activity and strong antioxidant characteristics.

6.4 Conclusions

In conclusion, this body of work has begun to fill several key gaps in the existing studies of how L-Glu impacts neurons and that the neuroprotection by acai berry provides relevant findings to understand the molecular basis of L-Glu toxicity. It has also implicated acai berry as a treatment to address pathomechanisms found in NDDs and stroke, which have been associated with excessive L-Glu buildup and toxicity.

A unique insight provided by this thesis into L-Glu-based pathways for NDDs and stroke may lead to more effective preventive and management strategies. Furthermore, this thesis reported novel mechanisms of acai berry extract neuroprotection that can target multiple pathomechanisms in NDDs and stroke. Acai berry neuroprotection action includes inhibition of ChE, preventing L-Glu excitotoxicity via NMDAR, besides attenuating its consequences such as oxidative stress, impaired bioenergetics, and induction of apoptosis.

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Appendices

A1: Identification of the most significant phytochemicals in acai berry extracts

Name	Formula	Molecular Weight	Retention Time (RT) [min]	Method of identification	Peak area (Acai berry 100 mg/mL PBS)	Peak area (Acai berry 100 mg/mL Ethanol)
Protocatechuic acid	$C_7H_6O_4$	154.0268	6.93	m/z	142062302.5	246395905.3
Syringic acid	$C_9H_{10}O_5$	198.0530	4.56	m/z, MS/MS	749817613.8	208146241.8
Vanillic acid	$C_8H_8O_4$	168.0425	6.63	m/z, MS/MS	158073252.1	1342599037
Gallic acid	$C_7H_6O_5$	170.0218	6.92	m/z, MS/MS	1187899740	556385573.7
4-Hydroxybenzoic acid	$C_7H_6O_3$	138.0319	7.58	m/z, MS/MS	8562874.571	9213014.457
Benzoic acid	$C_7H_6O_2$	122.0372	5.01	m/z, MS/MS	806239570.3	76215847.7
2,5-Dihydroxybenzoic acid	$C_7H_6O_4$	154.0268	8.98	m/z, MS/MS	2378542397	6607458764
Chlorogenic acid (3-Caffeoylquinic acid)	$C_{16}H_{18}O_9$	354.0951	7.05	m/z, MS/MS	41254658.1	19719117.49
Dihydrokaempferol	$C_{15}H_{12}O_6$	288.0635	6.89	m/z	3563931656	2805932354
Luteolin	$C_{15}H_{10}O_6$	286.0479	6.84	m/z, MS/MS	74301063.41	637974914.8
Quercetin	$C_{15}H_{10}O_7$	302.0427	6.97	m/z, MS/MS	8801456.333	1931877453
Taxifolin deoxyhexose or Taxifolin	$C_{15}H_{12}O_7$	304.0584	8.24	m/z	51900906.3	208060416.2

Name	Formula	Molecular Weight	Retention Time (RT) [min]	Method of identification	Peak area (Acai berry 100 mg/mL PBS)	Peak area (Acai berry 100 mg/mL Ethanol)
Quercetin-3-O-rutinoside (Rutin)	$C_{27}H_{30}O_{16}$	610.1519	7.40	m/z, MS/MS	6216840.247	108429854
Quercetin 3-O-glucoside (Isoquercitrin; Quercetin-3β-D-glucoside)	$C_{21}H_{20}O_{12}$	464.0950	7.43	m/z, MS/MS	14580914.02	68978660.78
Kaempferol rhamnoside	$C_{21}H_{20}O_{10}$	432.1048	6.89	m/z	94244594.75	87161024.38
Isoorientin	$C_{21}H_{20}O_{11}$	448.0996	7.91	m/z	180885039	586612888.6
(+)-Lariciresinol	$C_{20}H_{24}O_6$	360.1576	4.39	m/z	3040047885	1776128019
Dihydroconiferyl alcohol	$C_{10}H_{14}O_3$	182.0945	4.47	m/z	474006188.5	267566942.9
(+)-Catechin	$C_{15}H_{14}O_6$	290.0786	9.39	m/z	442004.644	6244069.64
(+)-menthiafolic acid	$C_{10}H_{16}O_3$	184.1100	6.82	m/z	50566410.83	26581930.46
(E,Z)-2,6-dimethyl-2,6-octadiene-1,8-diol	$C_{10}H_{18}O_2$	170.1310	4.23	m/z	62969783.44	29811330.21
(-)-Loliolide	$C_{11}H_{16}O_3$	196.1100	4.22	m/z	251552539.7	689110550.8
Oleic acid	$C_{18}H_{34}O_2$	282.2558	3.43	m/z	9614398.93	1.74306E+11
Palmitoleic acid	$C_{16}H_{30}O_2$	254.2248	3.52	m/z, MS/MS	2047260.509	1535403535
Linoleic acid	$C_{18}H_{32}O_2$	280.2401	3.43	m/z, MS/MS	41818790.91	37018332011
Linolenic acid	$C_{18}H_{30}O_2$	278.2246	3.46	m/z	12785017.32	9157058614
Palmitic acid	$C_{16}H_{32}O_2$	256.2404	3.49	m/z, MS/MS	341768031.3	69914808961
Stearic acid	$C_{18}H_{36}O_2$	284.2715	3.40	m/z	238365663	5230510217

Name	Formula	Molecular Weight	Retention Time (RT) [min]	Method of identification	Peak area (Acai berry 100 mg/mL PBS)	Peak area (Acai berry 100 mg/mL Ethanol)
Alanine	$C_3H_7NO_2$	89.0477	8.72	m/z, MS/MS, RT	1204252982	942337311.8
Lysine	$C_6H_{14}N_2O_2$	146.1055	12.12	m/z, MS/MS	862218767.6	8177305.396
Arginine	$C_6H_{14}N_4O_2$	174.1116	7.94	m/z, MS/MS	11587352.55	1192295220
Methionine	$C_5H_{11}NO_2S$	149.0511	7.73	m/z, MS/MS, RT	34288157.81	3052384.554
Phenylalanine	$C_9H_{11}NO_2$	165.0791	7.12	m/z, MS/MS	4348060351	903228280.5
Proline	$C_5H_9NO_2$	115.0633	8.13	m/z, MS/MS, RT	14951886340	8002696625
Glutamic acid	$C_5H_9NO_4$	147.0532	8.09	m/z, MS/MS, RT	2656643104	462904369.4
Serine	$C_3H_7NO_3$	105.0426	9.00	m/z, MS/MS, RT	317494512.7	115778449.5
Glycine	$C_2H_5NO_2$	75.0320	9.00	m/z, MS/MS, RT	36455496.26	2414696.613
Threonine	$C_4H_9NO_3$	119.0582	8.58	m/z, MS/MS, RT	1520425794	435342369.3
Histidine	$C_6H_9N_3O_2$	155.0695	8.61	m/z, MS/MS, RT	431822467	2537021.976
Tryptophan	$C_{11}H_{12}N_2O_2$	204.0901	8.12	m/z, MS/MS, RT	194890773.7	146363293
Tyrosine	$C_9H_{11}NO_3$	181.0741	8.40	m/z, MS/MS, RT	671881817.7	253308033.8
Isoleucine	$C_6H_{13}NO_2$	131.0946	7.63	m/z, MS/MS, RT	3029317492	2131796773
Valine	$C_5H_{11}NO_2$	117.0789	7.57	m/z, MS/MS, RT	13980494046	16844933194
Leucine	$C_6H_{13}NO_2$	131.0947	7.46	m/z, MS/MS, RT	6961405160	3547352224

Name	Formula	Molecular Weight	Retention Time (RT) [min]	Method of identification	Peak area (Acai berry 100 mg/mL PBS)	Peak area (Acai berry 100 mg/mL Ethanol)
Cellotetraose (beta-D-Glucopyranosyl-(1->4)-beta-D-glucopyranosyl-(1->4)-beta-D-glucopyranosyl-(1->4)-beta-D-glucopyranose)	$C_{24}H_{42}O_{21}$	666.2226	8.82	m/z, MS/MS	1469922.742	3737516.065
Sucrose	$C_{12}H_{22}O_{11}$	342.1156	8.65	m/z, MS/MS, RT	206116691.1	423947867.1
Quinic acid	$C_7H_{12}O_6$	192.0636	7.66	m/z, MS/MS	39234440929	23463016974
Ascorbic acid (Vitamin C)	$C_6H_8O_6$	176.0323	6.77	m/z, MS/MS	1122656150	670491945.4

Abbreviation: MS/MS, Tandem mass spectrometry; m/z, Mass-to-charge ratio; PBS, phosphate buffer saline; RT, Retention time.

A2: Chemical compounds detected in acai berry extracts from previous studies with possible cholinesterase inhibitor activity and recognized antioxidant activity

(Ahmed *et al.* 2021; Alcalde *et al.* 2019; Alqurashi *et al.* 2016; Ambigaipalan *et al.* 2020; Ayaz *et al.* 2017; Bajpai *et al.* 2017; Bernal-Mercado *et al.* 2018; Birsan *et al.* 2021; Budryn *et al.* 2022; Másedas *et al.* 2019; Chambers *et al.* 2019; Choi *et al.* 2014; Chunhakant and Chaicharoenpong 2019; Conforti *et al.* 2009; da Silveira *et al.* 2017; Dos Santos *et al.* 2018; El-Hassan *et al.* 2003; Fang *et al.* 2010; Geiss *et al.* 2019; Gulaboski *et al.* 2013; Hayes *et al.* 2011; He *et al.* 2018; Jabir *et al.* 2018; Jung *et al.* 2014; Jung *et al.* 2022; Kang *et al.* 2011; Karaoglan and Koca 2020; Kawabata *et al.* 2002; Khan *et al.* 2018; Khaw *et al.* 2018; Kikuzaki *et al.* 2002; Kikuzaki *et al.* 2019; Kim *et al.* 2016; Kissling *et al.* 2005; Kobus-Cisowska *et al.* 2019; Kucukboyacı *et al.* 2010; Li and Seeram 2011; Li *et al.* 2021; Luo *et al.* 2006; McCann *et al.* 2015; Mishra *et al.* 2003; Müller *et al.* 2011; Murillo *et al.* 2019; Oh *et al.* 2021; Okello and Mather 2020; Orhan *et al.* 2007; Platzer *et al.* 2021; Pohanka 2014; Rohmah *et al.* 2022; Saito *et al.* 2004; Salau *et al.* 2020; Schauss *et al.* 2006b; Sordon *et al.* 2019; Spagnol *et al.* 2019; Sultana and Khalid 2010; Szwajgier and Borowiec 2012; Tang *et al.* 2016; Tena *et al.* 2020; Tung *et al.* 2007; Uriarte-Pueyo and Calvo 2011; Veselova *et al.* 2007; Wang *et al.* 2018; Wei *et al.* 2016; Woo *et al.* 2011; Wu *et al.* 2022; Xu *et al.* 2012; Xu *et al.* 2017; XueJiao *et al.* 2021; Yamaguchi *et al.* 2015; Yoshida and Niki 2003; Yoshikawa *et al.* 2013; Yu *et al.* 2019; Zeng *et al.* 2020; Zhang *et al.* 2014; Zhang *et al.* 2020; Zhao *et al.* 2017).

Compounds in acai berry extract	Cholinesterase inhibitor activity		Antioxidant activity
	AChE	BuChE	
Phenolic compounds and phenolic acids:			
Ferulic acid	✓	✓	+ (DPPH, $ABTS^{\bullet+}$)
Protocatechuic acid	✓	✓	+ (DPPH, $ABTS^{\bullet+}$, FRAP)
Syringic acid	✓	✓	+ (DPPH, $ABTS^{\bullet+}$)
Vanillic acid	✓	✓	+ (DPPH, $ABTS^{\bullet+}$, FRAP)
Gallic acid	✓	✓	+ (DPPH, $ABTS^{\bullet+}$, FRAP)
4-Hydroxybenzoic acid	✓	✓	+ (DPPPH, $ABTS^{\bullet+}$)
Benzoic acid	✓	✓	+ (DPPH)
Coumaric acid	✓	✓	+ (DPPH, $ABTS^{\bullet+}$)
3,4'-dihydroxy-3'-methoxypropiophenone	-	-	-
Protocatechuic acidmethyl ester	-	-	+ (DPPH)
3,4-Dihydroxybenzoic acid	-	-	+ (DPPH, $ABTS^{\bullet+}$, FRAP)
2,5-Dihydroxybenzoic acid	-	-	+ (DPPH, $ABTS^{\bullet+}$, FRAP)
Chlorogenic acid	✓	✓	+ (DPPH, $ABTS^{\bullet+}$, FRAP)
Caffeic acid	✓	✓	+ (DPPH, $ABTS^{\bullet+}$, $O^{2\bullet-}$)
Trans-cinnamic acid	✓	-	+ (DPPH, $ABTS^{\bullet+}$)
Ellagic acid	✓	✓	+ (DPPH, $ABTS^{\bullet+}$, FRAP)
3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-1-Propanone	-	-	-
Lignans:			
(+)-isolariciresinol	-	✓	+ (DPPH)
(+)-5-methoxy-isolariciresinol	-	-	+ (DPPH)
Erythro-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-Hydroxypropyl)-2-methoxyphenoxy]-1,3-propanediol	-	-	+ (SOD)
Threo-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxypropyl)-2-methoxyphenoxy]-1,3-propanediol	-	-	+ (SOD)
(-)-(7R,8S)-dihydrodehydroconiferyl alcohol	-	-	
(+)-(7R,8S)-5-methoxy-dihydrodehydroconiferyl alcohol	-	-	-
(+)-lariciresinol	✓	✓	-
(+)-pinoresinol	✓	-	+ (DPPH, $ABTS^{\bullet+}$, $^{\bullet}OH$, FRAP, SOD)
(+)-syringaresinol	✓	-	+ (DPPH)
3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone	-	-	+ (DPPH)
3,4'-dihydroxy-3'-methoxypropiophenone	-	-	+ (DPPH)
Dihydroconiferyl alcohol	-	-	-
Protocatechuicacid methyl ester			

Compounds in acai berry extract	Cholinesterase inhibitor activity		Antioxidant activity
	AChE	BuChE	
	-	-	-
		-	+ (DPPH, $ABTS^{\bullet+}$)
		-	
Amino Acids:			
Alanine	✓	-	-
Lysine	✓	-	+
Arginine	✓	-	+
Methionine	ND	-	+
Aspartic acid	✓	-	-
Phenylalanine	ND	-	-
Cysteine	✓	-	+
Proline	✓	-	-
Glutamic acid	ND	-	-
Serine	✓	-	-
Glycine	-	-	-
Threonine	-	-	-
Histidine	ND	-	+
Tryptophan	ND	-	+
Hydroxyproline	-	-	+
Tyrosine	✓	-	+
Isoleucine	-	-	-
Valine	✓	-	-
Leucine	ND	-	-
Anthocyanins:			
Cyanidin 3-arabinoside	-	-	+ (ROS)
Cyanidin 3-arabionosylarabionoside	-	-	-
Cyanidin-3-O-glucoside	ND	-	+ (DPPH, ORAC)
Cyanidin-3-O-rutinoside	-	-	+ (H_2O_2, FRAP, $^{\bullet}NO$)
Pelargonidin-3-O-glucoside	-	-	+ (LPO, $O^{2\bullet-}$)
Peonidin-3-O-rutinoside	-	-	-
Cyanidin 3-sambubioside	-	-	-
Delphinidin 3-glucoside	-	-	+ (LPO)
Malvidin 3-glucoside (Oenin chloride)	-	-	+ (DPPH, ORAC)
Peonidin 3-glucoside	-	-	-
Proanthocyanidins:			
Catechin (+)	✓	✓	+ (DPPH, $ABTS^{\bullet+}$, FRAP)
Epicatechin (-)	✓	✓	+ (DPPH, $ABTS^{\bullet+}$)
Epigallocatechin	✓	✓	+ (DPPH, $ABTS^{\bullet+}$)
Carotenoids:			
α-carotene	-	-	+ (αTEAC, LPSC)

Compounds in acai berry extract	Cholinesterase inhibitor activity		Antioxidant activity
	AChE	BuChE	
β-carotene	-	-	+ (DPPH, $ABTS^{\bullet+}$)
Lutein	✓	-	+ (DPPH, $ABTS^{\bullet+}$, FRAP)
Zeaxanthin	-	-	+
Flavonoids:			
Apigenin	✓	✓	+ (DPPH, $ABTS^{\bullet+}$, ORAC)
Dihydrokaempferol	-	-	+ (DPPH, $ABTS^{\bullet+}$, FRAP)
Isovitexin	✓	✓	+ (DPPH, ORAC)
Luteolin	✓	✓	+ (DPPH, $ABTS^{\bullet+}$, FRAP, ORAC)
luteolin-C-8 -glucoside (orientin)	-	-	
luteolin-6-C-glucoside (homo-orientin)	-	-	+ (DPPH, $ABTS^{\bullet+}$, FRAP)
Quercetin	✓	✓	+ (DPPH, $ABTS^{\bullet+}$, FRAP)
Scoparin	-	-	+ (DPPH, $ABTS^{\bullet+}$)
Taxifolin deoxyhexose or Taxifolin	-	-	-
Velutin	-	-	+ (DPPH, $ABTS^{\bullet+}$)
Quercetin-3-O-rutinoside (rutin)	✓	✓	+ (ORAC)
Vitexin	✓	✓	+ (DPPH, $ABTS^{\bullet+}$)
Kaempferol rutinoside	✓	✓	+ (DPPH, ORAC)
Kaempferol rhamnoside	-	-	+ (DPPH, $ABTS^{\bullet+}$, ROS)
Isoorientin	✓	✓	+ (DPPH, $ABTS^{\bullet+}$, ROS)
Crisoeirol	-	-	+ (DPPH, ORAC)
5,4'-dihydroxy-7, 3',5'-trimethoxyflavone	-	-	-
Luteolin diglycoside	-	-	+ (ORAC)
Procyanidin dimers			-
Chrysoeriol	-	-	+ (DPPH, $ABTS^{\bullet+}$)
	-	-	+ (LPO, $O^{2\bullet-}$)
Benzoquinone:			
2,6-dimethoxy-1, 4-benzoquinone	✓	✓	+ ($O^{2\bullet-}$)
Monoterpenoids:			
(+)-menthiafolic acid	-	-	-
(E,Z)-2,6-dimethyl-2,6-octadiene-1,8-diol	-	-	-
(E,E)-2,6-dimethyl-2,6-octadiene-1,8-diol	-	-	-
Norisoprenoids:			
(-)-loliolide	✓	✓	+ (DPPH, $ONOO^-$)
(4R)-4-[(1E)-3-hydroxy-1-butenyl]-3,5,5-trimethyl-2-cyclohexen-1-one	-	-	-
Lipids sterols:			
ß-sitosterol	✓	✓	+ (DPPH, $ABTS^{\bullet+}$, H_2O_2, LPO)
Campesterol	-	-	

Compounds in acai berry extract	Cholinesterase inhibitor activity		Antioxidant activity
	AChE	**BuChE**	
Sitgmasterol	✓	-	+ (LPO) + (LPO)
Major Fatty Acids: Monounsaturated Fatty Acids			
Oleic acid	-	-	+ (ROS)
Palmitoleic acid	-	-	-
Polyunsaturated Fatty Acids			
Linoleic acid	✓	-	+ (DPPH, $ONOO^-$)
Linolenic acid	✓	✓	+ (DPPH, $ONOO^-$)
Saturated Fatty Acids			
Palmitic acid	✓	✓	-
Stearic acid	-	-	-
Stilbenes:			
Trans-resveratrol	✓	✓	+ (DPPH, $ABTS^{\bullet+}$, ORAC)
Vitamins and minerals:			
Calcium	✓	-	-
Copper	✓	-	-
Iron	✓	-	-
Vitamin A	-	-	+ (DPPH, FRAP)
Vitamin C	-	-	+ (DPPH, $ABTS^{\bullet+}$, $O^{2\bullet-}$)

Abbreviation: α-TEAC, α-tocopherol equivalent antioxidant capacity; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; H_2O_2, hydrogen peroxide; $^{\bullet}OH$, hydroxyl radical scavenging assay; LPO, lipid peroxidation; LPSC, luminol-chemiluminescence peroxyl radical scavenging capacity; ND, not detected; $^{\bullet}NO$, nitric oxide; $O^{2\bullet-}$, superoxide anion radical; $ONOO^-$, peroxynitrite; ORAC, oxygen radical absorption capacity; ROS, reactive oxygen species; SOD, superoxide radical scavenging assay; (-), no data; (✓), detected or recognized; (+), observed.