Potential Neuroprotective effects of Blueberry and Lingonberry fruits and Leaves

A dissertation submitted in partial fulfillment to the School of Graduate Studies of the requirement for the

Degree of Master of Science in Pharmacy

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May 2014

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Abstract

Production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are natural processes occurring in the brain. However, overproduction of ROS and RNS may occur during aging and contribute to neurodegenerative diseases and disorders such as stroke. Phenolic compounds constitute a large class of phytochemicals that are widespread in the plant kingdom and known to have antioxidant capacities. This study aimed to determine the radical scavenging capacity and reducing power, as well as the polyphenolic content in fruits and leaves of blueberries and lingonberries growing in Newfoundland. We determined the potential neuroprotective effect of extracts against glutamate-mediated excitotoxicity, which is believed to contribute to disorders such as stroke and neurodegenerative diseases. We found that extracts of fruits and leaves of blueberry and lingonberry plants have high levels of total soluble phenolics, anthocyanins, tannins, and flavonoids. Overall, the levels of these compounds were significantly higher in the leaves of these plants versus the fruits. Total antioxidant capacity, in terms of radical scavenging activity and reducing power, were much higher in the leaves of both plants as compared to their fruits. We then tested the effects of the extracts against glutamate-mediated excitotoxicity, a pathological process partially involving overproduction of ROS and RNS. Brain-derived cortical cell cultures from neonatal rat pups were prepared and grown for 9-16 days *in vitro*. Cells were exposed to glutamate (100 µM) for 24 hours. Glutamate-exposed cells displayed morphological alterations such as disrupted cell bodies, and increased dark punctae, which is often indicative of condensed nuclei and delayed cell death. Glutamate caused a ~23% cell loss after 24 hours as determined by the amount of DAPI-positive nuclei. A specific NMDA receptor blocker (AP5) was used to determine the contribution of this receptor type to cell damage. While lingonberry fruit extract did not provide protection from glutamate toxicity, blueberry fruit extracts were

protective. Cultures treated with leaf extracts of lingonberry and blueberry showed no cell loss in the presence of glutamate, indicating a strong protective effect of both the leaf extracts. The overall greater protective effect of leaf extracts was in correlation with the levels of phenolics and antioxidant capacity. Treatment with AP5 (NMDA receptor blocker) gave us positive results and the cell death caused by 100 µM glutamate was suppressed, but the trend was not clear enough to make any conclusion. Recent studies have analyzed antibody staining against alpha-synuclein, a protein involved with Parkinson's disease, after glutamate exposure. These studies helped us raise a set of questions on the various factors leading to increased expression of alpha-synuclein and allowed us to get some preliminary data. I have measured levels of lactate in the culture media from different conditions which may be indicative of altered cell health. Overall, these findings suggest that berries or their components may provide protection to the brain from various pathologies such as stroke, trauma and neurodegenerative diseases. This protective effect of berry extracts may be due to a decrease in oxidative stress, nitrosative stress, or other damaging mechanisms caused by exposure to glutamate.

Dedicated to my Parents

Acknowledgement

I am very grateful and would like to express my gratitude and appreciation to my supervisor Dr. John T. Weber for his continuous support and guidance. This thesis would not have been possible without his help and patience. I am very thankful to him for his good advice and support which he provided to me during my academic and research work. I would also like to thank my committee members, Dr. Mohsen Daneshtalab and Dr. Andrei Igamberdiev for their assistance and guidance during my research work. I thank the School of Pharmacy, Memorial University of Newfoundland for having the faith in me and providing me with all the necessary equipment's in the lab with the financial support from the School of Graduate Studies (SGS) to complete my project. I am extremely grateful to all the staff members of the School of Pharmacy and the Neuroscience group in the Division of BioMedical Sciences of Memorial University for the support and help throughout my graduate program. It would be very unfair if I do not thank Poorva Vyas for all the support she provided to me during my entire program and research. I also would like to express my sincere appreciation to my fellow lab mates Lyudamila Chibrikova and Mattew Lamont for helping me whenever I needed them and also creating a good academic and social environment in the lab. My special thanks to all graduate students in the School of Pharmacy and the Neuroscience group in the Division of BioMedical Sciences of Memorial University who created a very good atmosphere for learning and communicating.

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Symbols and Abbreviations

AD – Alzheimer's disease

$AlCl_3$ – Aluminium chloride

AMPA - 2-amino, 3-(3-hydroxy- 5-methylisoxazol-4-yl) propionate

AP5 or APV - ((2R)-amino-5-phosphonovaleric acid; (2R)-amino-5-phosphonopentanoate)

AsA – Ascorbic acid equivalents

ATP- Adenosine tri phosphate

BBB – Blood brain barrier

BDNF - Brain derived neurotropic factor

BME - Basal Medium Eagles

$[Ca^{2+}]_i$ - Intracellular free calcium levels

CE - Catechin equivalent

CNS – Central nervous system

CO_2 – Carbon doixide

CSF – Cerebrospinal fluid

DAPI - 4', 6-diamidino-2-phenylindole

dH_2O - Distilled water

DIV - Days in vitro

DLB – Dementia with Lew bodies

DNA – De-oxy ribonucleic acid

DPPH - 2, 2-diphenyl-1-picrylhydrazyl

EAAT - Excitatory amino acid transporter

EDTA - Ethylene diamine tetra-acetic acid

ER – Endoplasmic reticulum

$FeCl_3$ – Ferric chloride

GAE - Gallic acid equivalents

Gln – Glutamine

GPx – Glutathione peroxidase

GR - Glutathione reductase

GSH - Reduced glutathione

GSSG - Oxidized glutathione

HBSS - Hank's buffered salt solution

HCl – Hydrochloric acid

H_2O_2 – Hydrogen peroxide

IP_3 - Inositol-trisphosphate

MAP-2 - Micro-tubule-associated protein-2

mGluRs - Metabotropic glutamate receptors

$NaNO_2$ – Sodium nitrite

NaOH – Sodium hydroxide

NMDA - N-methyl D-aspartate

NO – Nitric oxide

$NO2^{\bullet}$ – Nitrogen dioxide

$O_2^{\bullet-}$ – Superoxide radical

$^{\bullet}OH$ – Hydroxyl radical

$ONOO^-$ – Peroxynitrite

PBS – Phosphate buffer solution

PD – Parkinson’s disease

PFA - Paraformaldehyde

PLO - Poly-L-Ornithine

PNS – Peripheral nervous system

PI – Propidium Iodide

RNA – Ribonucleic acid

RNS – Reactive oxygen species

ROS – Reactive nitrogen species

SE – Standard error

SGS – School of Graduate Studies

SOD – Superoxide dismutase

TBI – Traumatic brain injury

TCA - Trichloroacetic acid

Trypsin-EDTA – Trypsin- Ethylene diamine tetra-acetate

UPR - Unfolded protein response activation

vGluT – Vesicular glutamate transporter

CHAPTER 1

INTRODUCTION

1.1 Nervous System and Brain

Vertebrates have a single nervous system which is divided into the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS consists of the brain and spinal cord (Seeley, Stephens and Tate, 2006) which controls movement, sensation, higher brain functions and other homeostatic functions (Amerman, 2010). The brain is one of the most complex organs in the body which is protected within a skull. The brain and spinal cord are both covered by a set of connective tissue layers called the meninges. The three meninges protecting the brain are the dura matter (outermost), arachinoid matter (middle) and the pia matter (innermost). There is plasma like fluid in the space between the pia matter and the cerebral hemispheres surrounding the brain called the cerebrospinal fluid (CSF), which is formed by blood filtration. The spaces in the brain are called the ventricles and are filled with the CSF. The CSF has various functions such as making the brain buoyant, cushioning the brain, providing nutrients and hormones to the brain and is also involved in removal of waste (Campbell *et al*., 2005). The brain mainly consists of the cerebral hemispheres (cerebrum), the diencephalon (thalamus, hypothalamus and epithalamus) and the cerebellum. The cerebrum is the largest portion of the brain and the outermost region of this portion is called the cerebral cortex, which is further divided into right and left hemispheres (Kandel, Schwartz and Jessell, 2000). The two major kinds of cells found in the brain are neurons and glial cells. Glial cells are primarily a supportive tissue for the neurons. Brain cells and especially neurons require effective antioxidant protection for two main reasons:

1. They exhibit higher oxygen consumption compared to other tissues (Braughler and Hall,

1989) and

2. Formation of reactive oxygen and nitrogen species can significantly alter their function.

1.2 Oxidative stress, Reactive Oxygen and Nitrogen Species

In healthy cells, molecular oxygen is taken up and then gets converted to superoxide radical (O_2•−) in the mitochondria. This O_2•− has two fates in the cells (See **Fig 1.1**). The first fate is to be converted to peroxynitrite radical (ONOO−) in presence of nitric oxide (NO) and the next is in presence of a natural antioxidant enzyme, superoxide dismutase (SOD) where hydrogen peroxide (H_2O_2) is formed. Hydrogen peroxide can further be neutralized in the presence of different antioxidant enzymes like catalase and glutathione peroxidase (GPx) and produce no harm to the cell. But, one possible mechanism converts H_2O_2 in a series of reactions called Fenton's reaction to produce hydroxyl radical (•OH), which is harmful to cell. Reactive oxygen species (ROS) include O_2•−, hydroxyl radicals, and non-radical derivatives of oxygen, such as H_2O_2, whereas examples of reactive nitrogen species (RNS) include NO, ONOO− and nitrogen dioxide (NO_2•) (Halliwell, 2006). These ROS and RNS are formed in the cells as a part of normal metabolic processes contributing to regulatory roles in normal functioning of metabolic pathways. However, when there are insufficient levels of natural antioxidant enzymes or antioxidants in the cells, overproduction of ROS and RNS takes place. Oxidative stress can be described as an imbalance between oxidants (ROS and RNS) and antioxidants in the body (Simonion and Coyle, 1996). Another way to define oxidative stress is the metabolic state of imbalance between the generation of reactive oxygen species (ROS) and their detoxification (Heck, 1968). The stress caused by the over production of RNS is called nitrosative stress.

Oxidative and nitrosative stresses are natural processes occurring in the body due to injury, aging or some abnormal conditions. The brain is very complex and has a very high rate of

oxygen consumption, making it susceptible to oxidative stress (Braughler and Hall, 1989). The brain is particularly prone to oxidative damage mainly because of high energy demand of neuronal cells as compared to other cells ultimately causing mitochondria to produce more ROS (Slemmer et al., 2008). Constant exposure of the brain to relatively high levels of ROS and RNS is mainly due to a high metabolic rate of oxygen, which is associated with a high rate of O_2•− leakage from the mitochondrial electron transport chain (Halliwell, 2006). Since mitochondria utilize the vast majority of O_2 uptake in order to generate adenosine-tri-phosphate, (ATP), it is unavoidable to control the production of ROS during respiration (Rhoads et al., 2006). Mitochondria is both a potential source and victim of free radicals (Sastre et al., 2003) and mitochondrial decay contributes to aging (Atamna et al., 2002; Ames, 2004). Identified mutations in mitochondrial DNA have been found to cause neurological dysfunctions (Gao et al., 2008). Production of these free radicals in the brain is more common because the brain cells do not possess strong antioxidant defense systems needed to prevent the damage caused by oxidative stress. Very low levels of catalase activity are present in the brain and other antioxidant enzymes, superoxide dismutase and glutathione peroxidase, are also present in moderate quantity (Lau et al., 2005). All of these factors together contribute to oxidative damage and later results in apoptosis. Apoptosis or programmed cell death is a natural mechanism in which damaged cells are eliminated, so as to prevent its spreading to the adjacent cells (Kroemer and Dallaporta, 1998; Hoeberichts and Woltering, 2003). Excess apoptosis can cause atrophy. Excessive ROS and RNS mediated damage to cellular proteins, DNA, lipids and cell membranes in human beings is responsible for aging (Sastre et al., 2000), neurodegenerative diseases (Simonion and Coyle, 1996) and diseases such as mutagenesis, cardiovascular diseases (Khan and Baseer, 2000), cancer (Kawanishi et.al., 2001), and several other disorders.

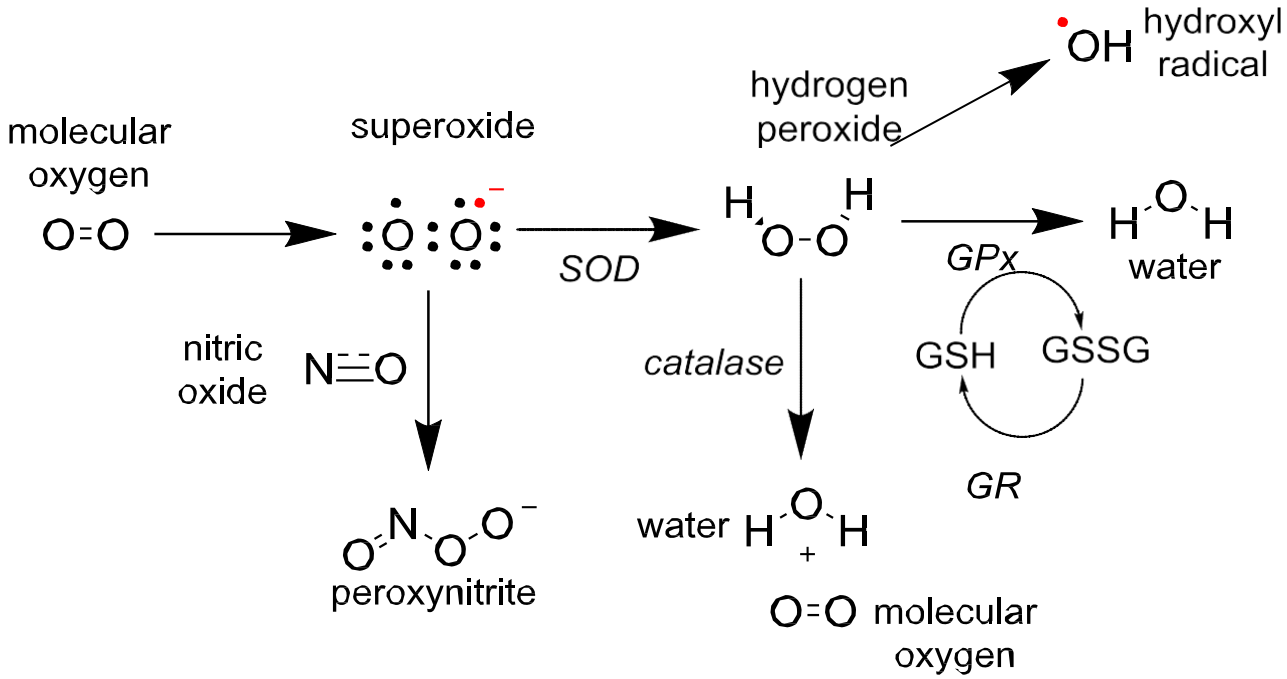

Fig 1.1 Critical ROS and RNS pathways in mammalian cells Molecular oxygen can be transformed into superoxide ($O_2^{\bullet -}$) radicals by various enzymes, particularly in mitochondria. Superoxide can combine with nitric oxide (NO•) to form peroxynitrite (ONOO-), or can be dismutated by superoxide dismutase (SOD) to form hydrogen peroxide (H_2O_2). Hydrogen peroxide can be neutralized by catalase, by glutathione peroxidase (GPx), or can be transformed to a hydroxyl radical (•OH) by the Fenton reaction (not shown). GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione. To combat or overcome the presence of excess reactive species, the level of antioxidants in the brain has to be increased (Modified from Slemmer *et al*., 2008).

1.3 Brain Aging and Neurodegenerative Diseases

Prolonged oxidative stress causes impairment in normal brain function which is known as brain aging (Harman, 1955; Harman, 1994; Lau *et al.*, 2005; Harman, 2006). Dr. Denham Harman's free radical theory of aging states a small amount of injury everyday leads to aging of the brain, and also suggests that the lifespan of an organism could be increased by reducing radical production and increasing radical scavenging (Kehrer and Smith, 1994). Brain aging is a major risk factor and eventually leads to neurodegenerative diseases.

Neurodegenerative diseases can be a result of various factors such as genetic defects, defective proteins, fibre accumulation or prolonged oxidative stress (brain aging) in a specific region of the brain. One common feature of most neurodegenerative diseases is the aggregation of mis-folded proteins (Lausted *et al*., 2013). Protein folding or mis-folding takes places in the endoplasmic reticulum (ER) and is termed ER stress. Some neurodegenerative diseases like Alzhiemer's disease (AD) and Parkinson's disease (PD) involve ER stress (accumulation of mis-folded proteins) and leads to unfolded protein response activation (UPR) (Haynes *et al*., 2004). This UPR tries to normalize the function of the cell and activate signaling pathways which cause protein folding. If protein folding is not achieved then production of ROS takes place, which ends up in oxidative stress and cell death. A decrease in misfolded proteins in the ER causes a decrease in oxidative stress. Different proteins are involved with each disease and these mis-folded proteins are believed to cause the progression of the disease (Frost and Diamond, 2009; De Calignon *et al*., 2012; Hardy J*et al*., 2012). Some of the common neurodegenerative diseases are AD and PD (Gandhi *et al*., 2012). A plethora of literature is available on the different mechanisms and factors of these diseases but a treatment is still not known.

1.3.1 Parkinson's disease and Lewy Body Dementia

Neurodegenerative disorders are known to be very debilitating. PD is the second most common neurodegenerative disease after AD (Lees *et al*., 2009), which is characterized by rigidity, tremors, postural instability, slow movements and many other symptoms (Ross *et al*., 2004). Degeneration of dopaminergic neurons in the substantia nigra of the midbrain results in PD (Forno, 1996). The second most common form of dementia after AD is Dementia with Lewy bodies (DLB) (Zaccai *et al*., 2005). The symptoms of DLB overlap with those of AD, PD and other disorders involving Lewy body formation (Nervi *et al*., 2011) but the three most prominent symptoms are recurrent visual hallucinations, Parkinsonism, and fluctuating cognition. The common pathological characteristic of both PD and DLB are the formation of Lewy Bodies (Lewy, 1912; Tretiakoff, 1919; Okazaki *et al*., 1961; Kosaka *et al*., 1976). Lewy Bodies are composed of alpha-synuclein and ubiquitin (Spillantini *et al*., 1997). They are usually found in the substantia nigra and locus coeruleus of the brain stem in the case of DLB, but they are also found in the limbic system and cortical areas of the brain (Collerton *et al*., 2003; Zupancic *et al*., 2011; Nakatsuka *et al*., 2013). Pathologically, aggregates of alpha synuclein protein are seen in the Lewy bodies in various regions of the brain in PD (Spillantini *et al*., 1997). Alpha synuclein is a 140 amino acid containing soluble protein (Olanow and Brundin, 2013) which is located in pre-synaptic terminals (Lee and Trojanowski, 2006). Folding and mis-folding of this protein causes changes in the cell (Stefani and Dobson, 2003) such as accumulation of ROS, and oxidative stress. Both neurodegenerative and neurological diseases involve oxidative stress. Two other common neurological disorders which also involve oxidative stress are traumatic brain injury (TBI) and stroke.

1.4 Traumatic Brain Injury

TBI refers to any kind of physical or mechanical damage to the brain. It is one of the major causes of mortality and disability worldwide (Jennet, 1996). The patho-physiology of this kind of injury is complicated and is said to consist of two phases. The first phase is called the primary (mechanical) damage phase and the second is called the secondary (delayed) phase. The primary phase occurs at the moment of injury. It includes contusion, laceration, diffuse axonal injury and intracranial hemorrhage which take place immediately after the injury (Weber, 2012). Post traumatic production of free radicals takes place within a few minutes after the mechanical injury. This free radical production plays a major role in the secondary phase of TBI (Ohta *et al.*, 2012). The secondary (delayed) damage phase includes processes which occur at the time of injury but do not appear clinically for days, weeks or months later after the injury. It includes the activation of degrading enzymes, swelling, and ischemia (Weber, 2004). One possible reason for the secondary injury is an imbalance in calcium homeostasis (Weber, 2004). This leads to excessive intracellular calcium levels in the CNS which is again responsible for the release of free radical species and causes damage to proteins, lipids, nucleic acids, and cell membranes (McIntosh and Sapolsky, 1996). Overall this process ends up causing cell death and pathological changes in cells which may progress into other neurodegenerative diseases. Lactate is a by-product of glycolysis and is a biochemical indicator of anaerobic metabolism. Lactate measurements after different time frames of TBI can give an estimate about the health of cells. Apart from neurodegenerative and neurological disorders, there are other conditions which eventually result in production of reactive species. One such condition is the presence of excess glutamate at receptors, which is termed glutamate excitotoxicity.

1.5 Glutamate Excitotoxicity

There are various amino acids required by the body. Some are essential and others are non- essential. Essential amino acids are the amino acids, which are not synthesized in the body and have to be supplemented through diet. Some of the essential amino acids are phenylalanine, threonine, tryptophan, methionine, valine, and lysine (Young, 1994). Non-essential amino acids are the amino acids which can be synthesized by the body itself and they need not be supplemented through diet. Some of the non-essential amino acids are alanine, aspargine, glutamine, glutamic acid, serine and tyrosine. Glutamic acid (glutamate) is one such non-essential amino acid present in the body (Chen *et al*., 2009). Glutamate is primarily an excitatory neurotransmitter (Yu *et al*., 2008) and also the most abundant neurotransmitter in the CNS, which is necessary for long-term potentiation, learning, memory, and other cognitive functions (Chen *et al*., 2009). Released glutamate interacts with three classes of ionotropic glutaminergic receptors namely N-methyl D-aspartate (NMDA), 2-amino, 3-(3-hydroxy- 5-methylisoxazol-4-yl) propionate (AMPA) and Kainate receptors (Reidel *et al*., 2003). Glutamate also reacts with metabotropic glutamate receptors (mGluRs). Activation of NMDA receptors leads to depolarization which, releases the Mg^{2+} block in the receptor and allows the transfer of Ca^{2+} ions (Spandou et al., 2007). AMPA receptors are co-expressed with NMDA receptors after the release of Ca^{2+} (Chen and Lipton, 2006). The primary function of NMDA receptors is the permeability of Na^{+} but not Ca^{2+}. Prolonged activation of the excitatory glutamate receptors, excessive glutamate in the synapse or decreased re-uptake of glutamate from synapses leads to the onset of a condition which is referred to as excitotoxicity (Lipton *et al*., 2008; Vincent and Mulle, 2009; Dong *et al*., 2009) (See **Fig 1.2**). In normal conditions, the neurons try to inhibit the activity of pumps responsible for toxicity and bring back glutamate levels to a normal

physiological concentration (Gottlieb et al., 2003). However, intense exposure to glutamate hyper-activates the receptors and increases intracellular Ca^{2+} levels which then undergo a cascade of reactions to finally lyse the cell (Mehta *et al.*, 2012). Cell lysis is a major contributing factor in various neuro-degenerative diseases and other disorders such as TBI and stroke (Mehta *et al.*, 2012). Therefore, the presence of excess glutamate at the receptors or decreased reuptake of glutamate results in increased levels of intracellular calcium. This excess intracellular calcium could significantly alter the health of cells.

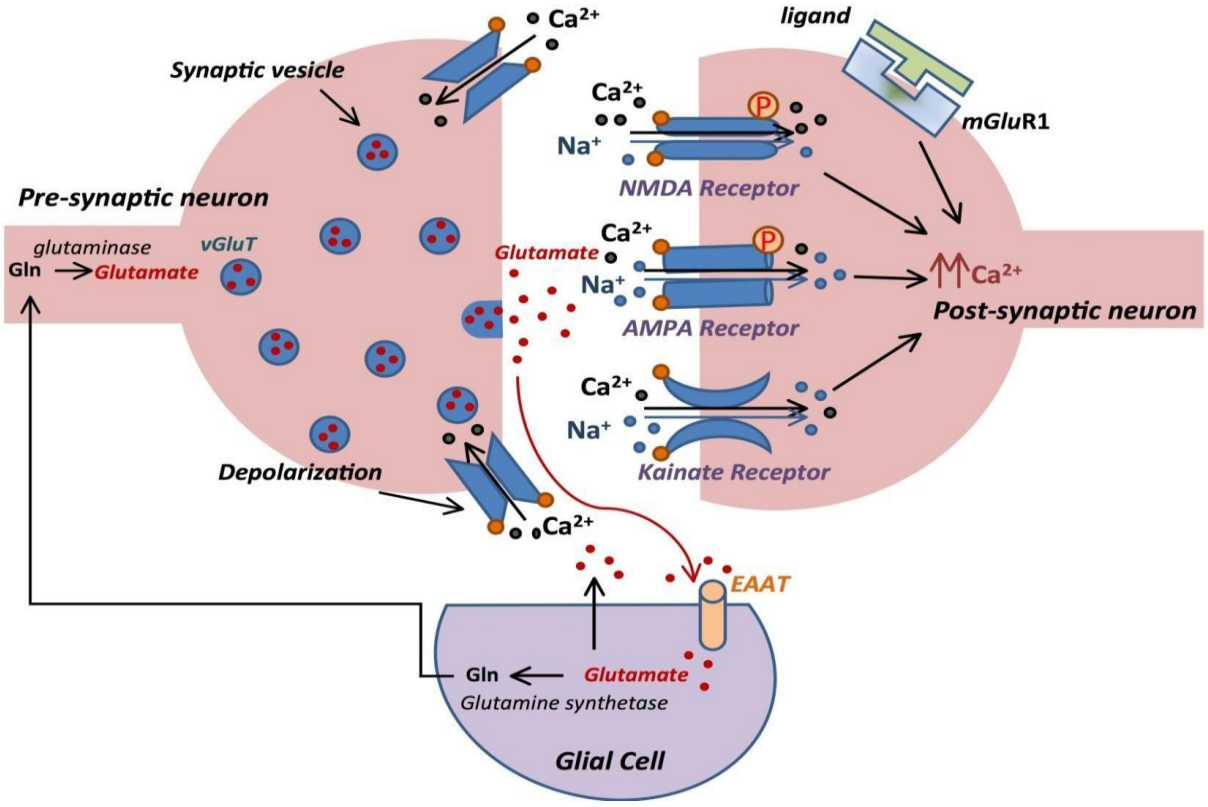

Fig 1.2 Glutamate-mediated neurotransmission and excitotoxicity Glutamate is packaged into synaptic vesicles by the vesicular glutamate transporter (vGluT), which are then released upon depolarization and influx of extracellular Ca^{2+}. Glutamate activates ionotropic receptors (i.e. AMPA, kainite, and NMDA receptors) on post-synaptic neurons that causes an influx of Na^{+} and Ca^{2+}. Glutamate activates metabotropic receptors (mGluRs), which leads to the production of inositol-trisphosphate (IP_3) and can cause the release of Ca^{2+} from internal stores. Glial cells typically clear the synapse of glutamate by uptake through an excitatory amino acid transporter (EAAT). Glutamate is then converted to Glutamine (Gln) and then shuttled back to neurons, which convert Gln back to glutamate. The increases in Na^{+} and Ca^{2+} are important for normal physiological functions in the brain. However excessive Na^{+} influx may lead to ionic disturbances and cell swelling, while excess Ca^{2+} influx can trigger several detrimental processes in cells including an increase in oxidative and nitrosative stress.

1.6 Calcium mediated cell death

Alterations in the regulation of Ca^{2+} are a leading factor for various diseases such as myocardial infarction, stroke, AD and other nervous system disorders (Trump and Berezesky, 1995). Overproduction of Ca^{2+} or excessive intracellular Ca^{2+}, adjuvant generation of free radicals, and reduction of cell energy metabolism play a vital role in the pathogenesis of ischemic stroke and TBI (Choi, 1988; Meldrum and Garthwaite, 1990; Coyle and Puttfarcken, 1993; Lipton and Rosenberg, 1994). Excessive Ca^{2+} leads to activation of different pathways (See **Fig 1.3**) which can cause cell death. Some enzymes like endonucleases, proteases, phospholipase A_2, and NO synthase are involved in these pathways. Production and activation of calpains affects the cytoskeletal structures further damaging the integrity of the cell (Slemmer *et al*., 2008). Ultimately, this can lead to neuronal death. Activation of Ca^{2+}-dependent endonucleases can induce DNA cleavage that further causes cell death (Trump and Berezesky, 1995; McConkey and Orrenius, 1996; Toescu, 1998). Calcium related mitochondrial damage is another pathway of cell destruction (Kristián and Siesjö, 1998). Calmodulin and Phospholipase A_2 activation involves the production of excess free radicals post-injury which causes cell damage (Slemmer *et al*., 2008). Other enzymes like caspases are also activated due to increased levels of Ca^{2+}. Induction of an apoptotic cascade takes place due to caspase activation (Slemmer *et al*., 2008). Overall, excessive intracellular free calcium levels ($[Ca^{2+}]_i$) activate various degradative pathways, which mostly lead to cell death by reversible or irreversible phases. Reduction of these excessive intracellular calcium levels and finally controlling the oxidative stress or cell death might be possible with the help of specific glutamate receptor blockers. Additionally, it is well known that oxidative stress can be controlled by antioxidants. These antioxidant compounds may reduce the intracellular levels of Ca^{2+} and thus protect the cells against damage and dying.

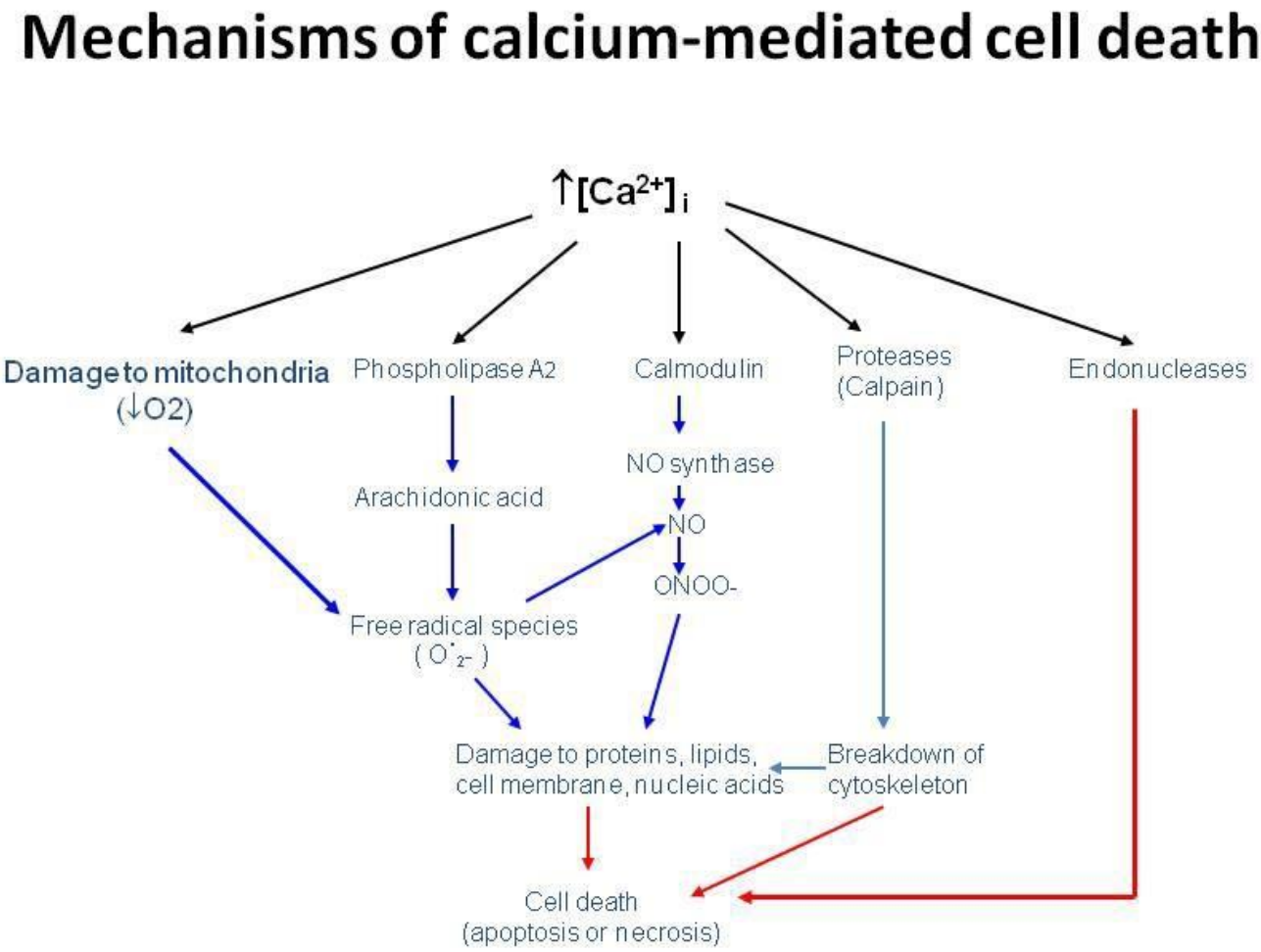

Fig 1.3: Summary of the mechanisms of neuronal damage and death caused by elevated $[Ca^{2+}]_i$ after traumatic injury. NO, nitric oxide; ONOO- , peroxynitrite; $O_{2\,-\cdot}$, superoxide (modified from Weber, 2004).

1.7 Antioxidants

To combat or overcome oxidative stress and increased levels of intracellular Ca^{2+}, the level of antioxidants in the brain needs to be increased. Some antioxidants naturally found in our body are glutathione, catalase, and glutathione peroxidase. Glutathione, a tri-peptide consisting of glutamate, cysteine, and glycine is highly important for normal functioning of cells, scavenging radicals (Bains *et al*., 1997), maintaining thiol redox state (Schafer *et al*., 2001) and reducing sulphur. Reduction of glutathione is said to cause neurodegeneration (Schulz *et al*., 2000). There are many factors involved in the synthesis of GSH. While performing *in-vitro* experiments, mixed cultures are helpful as astroglial cells reduce cystine to cysteine and release it to adjacent neurons (Dringen *et al*., 1999). This provides neurons with cysteine, which is a precursor for the synthesis of GSH. Transport of cysteine can take place in the presence of an excitatory amino acid transporter (EAAC1). Neurons lacking in EAAC1 transporters show a great decrease in cysteine transportation which in turn cuts down the production of glutathione (Dringen *et al*., 1999), a natural antioxidant essential to eliminate oxidative stress. There are five types of sodium dependent excitatory amino acid transporters (EAATs); (EAAT1, EAAT2, EAAC1 or EAAT3, EAAT4 and EAAT5) each with specific functions and locations (Maragakis *et al*., 2004). A common function of most of these transporters is to regulate the extracellular glutamate levels in the CNS. Some of these transporters are mostly distributed to astrocytes and others to Purkinje cells in the cerebellum, but EAAC1 is widely expressed throughout the nervous system in neurons (Maragakis *et al*., 2004).

The reduced glutathione levels leads to oxidative stress which further causes neural deficits, age-dependent abnormalities, neurodegeneration, death of cells, and other

abnormalities (Andersen *et al*., 2004). Therefore, reduction of natural antioxidants in the brain leads to oxidative stress conditions and further cell death. To overcome and avoid such a situation, consumption of antioxidants available in nature might act as an addition for the natural antioxidants and prevent cell death.

1.7.1 Health and effects of Nutraceuticals

The US Department of Health and human services, along with the US Department of Agriculture, published in a report named "Healthy People 2010" that every person above the age of two years should eat a minimum of two servings of fruit daily (US Department of Health and Human Services, 2005). Diet and exercise are two very important factors for a better mind and health. Consumption of junk food can reduce the healing effect of the brain as well as cause mental decline over the years whereas consumption of healthy food and regular exercise increases the ability of the brain to fight against damage, improve cognitive abilities, and facilitate synaptic transmission (Gomez *et al*., 2008; 2011). The term nutraceutical was originally defined by Dr. Stephen DeFelice as being daily nutritional supplements like food or a part of food, which have beneficial effects in treating or preventing disease (Kalra, 2003). There has been an increasing demand for nutraceuticals as plant derived supplements are considered to be much safer than synthetic drugs (Raskin *et al*., 2002). Various dietary components affect health in different ways. A healthy diet, which consists of omega-3-fatty acids, curcumin or other polyphenolic compounds tends to elevate the levels of brain derived neuro-tropic factor (BDNF), which is useful for the brain in maintaining neuronal circuitry and managing learning and memory (Gomez *et al*., 2011). A decrease in levels of BDNF leads to various disorders in the brain such as AD, PD and Schizophrenia (Gomez *et al*., 2011). It is a well-established fact that higher intake of a diet

containing β-carotene shows positive results in individuals with risk of cancer (Hennekens, 1986) and coronary heart disease (Poppel *et al*., 1994). Docosahexaenoic acid, a component Omega-3-fatty acid, is very essential for the recovery of damaged neuronal membrane (Jones *et al*., 1997). Polyphenols are another broad class of phytochemicals that are widespread in the natural kingdom and are available in berry plants in high concentrations. As the name suggests, these polyphenols have multiple phenol groups, and they are powerful antioxidants. These phenolic compounds are the secondary metabolites of the plants which are derived from phenylalanine and tyrosine (Shahidi and Naczk, 2004; Naczk and Shahidi, 2006). They are useful for the plant defence system against stress and pathogens (Shahidi and Naczk, 2004; Naczk and Shahidi, 2006). Polyphenols have potential beneficial effects such as anti-inflammatory, anticancer, antifungal, antimicrobial, and antiulcer properties (Rahman *et al*., 2007). Chronic diseases, such as cardiovascular disease and AD can be improved with an intake of phenolic compounds and anthocyanins containing fruits and green leafy vegetables (Joshipura *et al*., 2001; Huxley and Neil, 2003; Castrejón *et al*., 2008; Krikorian *et al*., 2010). They are widely available in fruits and vegetables (plants) and show beneficial effects on the brain. Berry crops are very rich sources of polyphenolic antioxidants, particularly flavanoid compounds (Zeng *et al*., 2003) and can be used in our diet to provide the beneficial effects of antioxidants. These berries are considered to be nutraceuticals as they are a part of food and have positive health effects. There are various kinds of berry plants and each of it has its own beneficial effect based on its chemical profile.

1.8 Berries and Leaves

Berries are small fleshy fruits which are low in calories but high in moisture. They contain both soluble and insoluble fibres (US Department of Agriculture National Nutrition

Database). The fruits contain low levels of sodium and large amounts of potassium and natural antioxidants, such as vitamins C and E. They are naturally rich in micronutrients: folic acid, calcium, selenium, α- and β-carotene, and lutein. Berries have been reported to have diverse health promoting phytochemicals and are very rich sources of polyphenolics especially flavonoids, phenolic acids, anthocyanins, and proanthocyanidins (Zheng and Wang, 2003). An enormous body of research has been published suggesting that the dietary consumption of berries has positive effects on human health and diseases (Sreeram, 2008; Battino *et al*., 2009). The health promoting properties of berries are gaining continued interest not only as antioxidants, but also because of their bioactive properties *in vivo* (Sreeram *et al*., 2006; Sreeram, 2008). Berries of *Vaccinium* species are especially known for their antioxidant activities and have been extensively studied. The protective effects of berries have been attributed to their phenolic content which further determines its antioxidant capacity. Antioxidant potential of a compound can be defined as the potential to suppress the effect of free radicals or ROS that are responsible for causing oxidative damage to cells. Several mechanisms by which antioxidant compounds suppress oxidative stress are: 1) By scavenging free radicals, 2) by metal chelation 3) by breaking the chain reaction of auto-oxidation and 4) by a reduction mechanism (Brewer, 2011). The three commercially important berry crops of *Vaccinium* species are blueberry (*Vaccinium angustifolium),* cranberry (*Vaccinium oxycoccos* and *Vaccinium macrocarpon)* and lingonberry (*Vaccinium vitis-idaea*). Other berries of the *Vaccinium* species include bilberry (*Vaccinium myritillus*), red huckleberry (*Vaccinium parvifolium*), sparkleberry (*Vaccinium arboreum)* and creeping blueberry (*Vaccinium crassifolium).* The characteristic feature of these berries is fleshy fruit with high ascorbate and anthocyanins levels (Yao and Vieira, 2007). It was reported that these fruits possess several beneficial properties such as antioxidant, anti-tumor, anti-ulcer,

and anti-inflammatory activity (Zafra-Stone *et al.,* 2007). Owing to their high phenolic levels, berries have been shown to exhibit a wide range of biological effects such as anticarcinogenic effects (Knekt *et al.,* 1997) and prevention of cardiovascular diseases (Hertog, 1993; Keli *et al*., 1996). Apart from the nutritional value, berries serve as protection against several other diseases and disorders by such mechanisms as cancer suppressive actions (Knekt *et al.,* 1996; Mertens-Talcott *et al*., 2003; Olsson *et al.,* 2004; Juranic *et al*., 2005), alleviating coronary heart disease (Hertog *et al.,* 1993; Knekt *et al.,* 1996), stroke (Keli *et al*., 1996), and some other degenerative diseases resulting from oxidative stress (Ames *et al.* 1993). Cranberry proanthocyanidins have been shown to maintain urinary tract health (Foo *et al.,* 2000a; Foo *et al.,* 2000b; Howell *et al.,* 2005). A variety of species from this family are utilised as commercially important fruit crops, and medicinal plants (Jaakola *et al*., 2001). A plethora of literature is available about the protective effects of wild blueberries in animal model systems (Norton *et al.,* 2005; Del Bo' *et al*., 2010; Kristo, *et al*., 2012). According to some recent reports, consumption of wild blueberries for six weeks significantly decreases oxidative stress which causes oxidized DNA, and increased resistance to damage (Riso *et al*., 2012). In particular, the potential of berries and their constituents to protect the brain from aging and neurodegenerative diseases has gained increased attention in recent years. For example, dietary supplementation with polyphenol containing fruits can decrease age-related behavioral deficits in rats (Shukitt *et al*., 2005). In a recent study conducted with a mouse model of AD, treatment with berries rich in polyphenols decreased the extent of behavioral abnormalities associated with the disease (Vepsäläinen *et al*., 2012). Other experimental studies have shown that rats fed a diet enriched with blueberries can protect the brain against oxidative stress and associated learning deficits (Duffy *et al*., 2008). Surprisingly, a diet enriched with blueberries has been

demonstrated to later protect animals from the damage induced by ischemic stroke (Sweeney *et al*., 2002; Wang *et al.*, 2005).

Leaves are an important part of the plants which are mostly responsible for photosynthesis. Several research groups have reported on the health benefits of leaves. One of the most common uses of leaves is in the preparation of beverage, Tea. Various studies suggest some of the health benefits of both green and black teas in cardiovascular, anti-diabetic, cancer and anti-aging effects (Khan and Mukhtar, 2013). Leaves of Aloe vera have been traditionally used for its cosmetic value (Gupta and Malhotra, 2012). Ethanolic extract of *Olea Europea* L. leaves have shown antithrombotic effect in rabbits (Dub and Dugani, 2013). Leaves of *Brassica Juncea* are known to be of therapeutic importance for the cognitive disorders associated with diabetes or oxidative stress to the brain (Thakur *et al*., 2013). Another study showed the beneficial effects of antioxidants present in leaves and flowers of *Erica australis* L. (Nunes and Carvalho, 2012). Interestingly, results in this study showed that leaves of this plant had double the antioxidant activity than the flowers and the radical scavenging activity of leaves was almost three times that of flowers.

Newfoundland and Labrador is rich in blueberry and lingonberry plants. These berries are well known to be rich in antioxidants and that made us select these plants for my study. We drew our interest towards leaves as well because less research has been carried out on them and some studies have shown their beneficial effects. We believed that leaves and berries of these plants would be high in polyphenolic content.

1.8.1 Blueberry

(Image taken from the website free-extras.com)

Blueberries are flowering plants belonging to the genus *Vaccinium.* These plants have dark-purple berries which contain high amounts of phenolic compounds, including anthocyanins, chlorogenic acids, flavonols, and procyanidins, which result in high antioxidant activity and provide health benefits (Cho *et al*., 2004; Huang *et al*., 2012). The anthocyanins from blueberries are considered to be nature's most potent antioxidants and have demonstrated properties that extend well beyond suppressing free radicals (Srivastava *et al*., 2007). Consumption of blueberries helps in maintaining healthy blood flow via several mechanisms including normal platelet aggregation, healthy low-density lipoprotein (LDL) oxidation, and maintenance of endothelial function (Kalt *et al*., 2008; Shaughnessy *et al*., 2009). Blueberries are very famous in North America for their nutritional and medicinal value (Kalt and Dufour, 1997). Wild blueberries showed protection against cardiovascular disorders in various animal models (Norton *et al*., 2005; Kalea *et al*., 2009; Del Bo' *et al*., 2010; Kristo *et al*., 2010). Age-related motor and cognitive decrements in rats could be reversed by using lowbush blueberries (Joseph *et al*., 1999). A diet containing

blueberry extracts also showed reduction in plaque formation in the case of an AD animal model (Joseph *et al*., 2003). The above studies have shown the positive health effects of blueberry fruits in humans and in in *vitro* models. There is much less information about the beneficial effects of blueberry leaves. Crude phenolic extracts of blueberry leaves were found to have shown antioxidant activity in a β-carotene linoleate model system and the radical scavenging effect was found to be very high as well (Naczk *et al*., 2003). Some studies have also shown beneficial effects against diabetes (Cignarella *et al*., 1996) and fatty liver disorders (Yuji *et al*., 2013). Lingonberry plants were the second variety of berry I was interested in.

1.8.2 Lingonberry

(Image taken from the website www.naturephoto-cz.com)

Lingonberry (also known as partridgeberry) that grows natively in Newfoundland and Labrador, Canada is a low shrub, bearing bright red fruits. Various studies show that they have higher antioxidant activity when compared to other berry fruits such as blackberry, blueberry,

raspberry and strawberry (Wang *et al*., 2000, Zheng *et al*., 2003). Lingonberry has been used as a medicinal plant and there are several reports on its health benefits. Lingonberry plants are a very rich source of antioxidants especially phenolic compounds. Lingonberries have shown potential antimicrobial effects and have also shown beneficial effects against urinary tract infection pertaining to its high arbutin levels (Larsson *et al.,* 1993; Ho *et al*., 2001). Reports have shown that lingonberry exhibits anticancer activity and that its extract can potentially induce apoptosis of human leukemia HL-60 cells (Bomser *et al*., 1996; Wang *et al*., 2005). Gonorrhoea, a sexually transmitted disease, has been treated by lingonberry (Duke and Ayensu, 1985). Lingonberry helps in curing gastric diseases and lowering cholesterol levels (Dierking and Beerenobst, 1993). A study done on women showed that lingonberry juice has a protective effect against recurrent urinary tract infections (Kontiokari *et al*., 2001). A recent study reveals the protective effects of anthocyanins from lingonberry against damage caused by radiation (Fan *et al*., 2012). Leaves of lingonberry have diuretic effects and have also shown astringent properties (Chiej, 1984). Similar to blueberry leaves, much less information about the beneficial effects of lingonberry leaves using animal models is available in literature.

1.9 Hypotheses and Objectives of the Study

In summary, diet is one of the most important determining factors for the health of an individual. A diet rich in antioxidants is considered to be good for the brain. Berries, especially blueberries and lingonberries, are rich in antioxidants. Berries are highly rich in phenolic compounds, flavanoids and vitamin C. According to the literature, it is believed that antioxidants help prevent and repair the stress condition that has developed due to oxidation, a natural process that occurs during normal cell function in the brain. Few cells become damaged during oxidation and the oxygen and nitrogen molecules are converted into free radicals, which can start a series of chain reactions to harm more cells. Antioxidants have the potential to reduce this overproduction of free radicals. Thus, loss of the reactive species or free radicals has a tendency to reduce brain aging, counteract neurodegenerative diseases, and improve the health of the brain. Uncontrolled activity of free radicals can result in oxidative stress, which can further aggravate disease conditions like AD, and PD, and disorders such as TBI and stroke.

I believe that our berry and leaf extract samples are rich in polyphenols, anthocyanins, flavonoids, and tannins. I also suspect high levels of antioxidant capacities in our extract samples. I wanted to test the effects of these extracts in systems of neurological disorders like TBI and glutamate excitotoxicity in the brain, which involve oxidative stress. My hypotheses are:

(1) Berry extracts exert neuroprotective effects against glutamate excitotoxicity and

(2) Berry extracts decrease the levels of glutamate induced up-regulation of alpha-synuclein in rat brain cultures.

The major objectives of the study are:

- To carry out extractions of the berry and leaf samples, perform biochemical analysis and compare the total antioxidant capacity of blueberry and lingonberry fruits and leaves.
- To determine the biological (neuroprotective) effects of blueberry and lingonberry fruits and leaves in rat brain cultures, using a model of glutamate excitotoxicity.
- To obtain preliminary data and examine the effects of different berry extracts on neurons which have up-regulated alpha-synuclein as a result of glutamate treatment.

The minor objectives of the study are:

- To determine the effect of the NMDA receptor blocker, AP5, against glutamate excitotoxicity because I suspect that one mechanism through which berry extracts inhibit cell death is by blocking the NMDA receptor induced excessive intracellular Ca^{2+}.
- To test the effect of blueberry fruit extract in a TBI model (94A cell injury controller) in rat brain cultures.
- To perform lactate measurements for different samples in order to get an additional estimation about the altered health of the cells.

CHAPTER 2

METHODS

2.1 Extract Preparation

Plant materials used in the present study are fruits and leaves of wild low bush blueberry (*Vaccinium angustifolium* Aiton) and lingonberry (*Vaccinium vitis idaea*). Blueberry samples were collected near Fort Amherst, St. John's, NL, Canada in September 2011. Lingonberry samples were collected at Bauline, St. John's, NL, Canada in October 2011. Samples were harvested and stored at -20 ºC within an hour of collection. Fruit and leaf samples of each kind were divided into three sections before extraction. Sample extraction of each section was carried out from the collected leaf and fruit samples on different days in 80% (v/v) acetone with 0.2% formic acid in the ratio 1:2 and was subjected to 30 min shaking on ice. This extraction procedure was used because acetone and formic acid were previously found to be the best extraction solvents among ethanol, methanol and acetonitrile at various aqueous mixtures with different shaking periods (Vyas *et al*, 2013). The sample mixture was then centrifuged at 20,000 *g* for 20 min at 4ºC. Supernatants were collected and the procedure was repeated with the residue. Both the supernatants were mixed together and the final concentrations for leaf and fruit samples were 25 mg/ml and 250 mg/ml of fresh weight respectively. So, based on the extraction day, fruit and leaf samples were divided and termed as "n". The "n" value for our fruit and leaf extraction and assays was three (n=3). The cell culture experiments were carried out after the samples were filtered through a sterile filter of 40 μm pore size.

2.2 Biochemical assays

Blueberry and Lingonberry leaf and fruit extracts were further diluted ten times for biochemical assays.

2.2.1 Determination of total soluble phenolics

Total soluble phenolic content in both leaves and fruits was determined using Folin-Ciocalteu reagent as described by Chandrasekara and Shahidi (2011) with some modifications. 0.5 ml of Folin-Ciocalteu reagent was added to centrifuge tubes containing 0.5 ml of extracts and vortexed. One ml of saturated sodium carbonate solution was added to each tube to neutralize the reaction. The final volume was adjusted to 10 ml by water and vortexed for 30 sec. The reaction mixtures were kept in the dark for 35 min at room temperature and then centrifuged at 4,000 *g* for 10 min. The absorbance was measured at 725 nm. The total soluble phenolic content of each sample was determined using gallic acid standard curve and expressed as milligrams of gallic acid equivalents (GAE) per g of berry or leaf fresh weight.

2.2.2 Determination of total anthocyanin content

Total anthocyanin content of samples was measured by the pH differential method described by Foley and Debnath (2007). KCl and sodium acetate buffers were prepared at pH 1.0 and 4.5 respectively. One ml of extract was added to four ml of KCl. In the same way, one ml of extract was added to four ml of sodium acetate. These mixtures were kept in dark for 15 min at room temperature. Absorption at 510 nm and 700 nm was measured in buffers at pH 1.0 and pH 4.5 and the difference between the two values was used to determine total anthocyanin content. Results are expressed as GAE.

2.2.3 Determination of total flavonoid content

Total flavonoid content was measured by the aluminum chloride colorimetric assay (Zhishen *et al*., 1999). One ml of extract or standard solution of catechin (0.5 mg/ml) was mixed with 4 ml of water, followed by addition of 0.3 ml 5% $NaNO_2$, of 0.3 ml of 10% $AlCl_3$ (after 5 min) and 2 ml of 1 M NaOH (one minute later), the volume was adjusted (with water) to 10 ml. The absorbance was measured at 510 nm. Total flavonoid content was expressed as µmol of catechin equivalent (CE) per g of leaf or fruit.

2.2.4 Determination of total proanthocyanidin (tannin) content

Proanthocyanidin contents of extract samples were determined by the method developed by Chandrasekara and Shahidi (2011). Five ml of 0.5% vanillin-HCl reagent were added to 1 ml of extract, mixed thoroughly and incubated at room temperature for 20 min. A separate bank for each sample was read with 4% HCl in methanol. The absorbance was read at 500nm, and the content of proanthocyanidins was expressed as µmol of catechin equivalent (CE) per g of leaf or fruit.

2.2.5 Determination of total Antioxidant activity

Total antioxidant capacity of samples was determined by the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay which was conducted according to the method of Brand-Williams *et al* (1995) with some modifications. The stock solution of 1 mM DPPH in methanol was diluted to 60 µmol, 1.9 ml of the latter was mixed with 0.1 ml leaf extract, shaken vigorously and left in the dark for 20 mins. The absorbance was read at 515 nm. The scavenging capacity was expressed as % of inhibition of DPPH consumption. The Gallic acid standard curve was used to express the results as GAE.

2.2.6 Determination of reducing power

The reducing power of extracts was determined by the method described by Chandrasekara and Shahidi (2011). The medium of the assay was 200 mM phosphate buffer (pH 6.6) with 1% potassium ferricyanide. 2.5 ml of diluted extract was added to the assay buffer and incubated for 20 min at 50ºC and then 2.5 ml of 10% trichloroacetic acid (TCA) was added to the assay, mixed and then centrifuged for 10 min at 1750 g. 2.5 ml of supernatant was transferred to empty tubes and combined with 2.5 ml of de-ionized water and 0.5 ml of 0.1% $FeCl_3$. The absorbance was measured at 700 nm, and the results were expressed as ascorbic acid equivalents using appropriate standard curves.

2.3 Cell culture Experiments

2.3.1 Poly-L-ornithine plate coating

Poly-L-Ornithine (PLO-500 µg/mL; Sigma) promotes the adhesion of cells to culture wells. To prepare the PLO solution, 100 mg of PLO was mixed with 10 ml of distilled water to achieve a 10 mg/ml solution. 300 µl of this solution was then aliquotted to centrifuge tubes (2 ml) and then these were stored at -80ºC. The PLO coating solution was made by mixing 6 ml of distilled water with 1 aliquot (300 µl) of PLO for each plate. In the case of Costar 24 well polystyrene plates (Corning, NY, USA), 250 µl of PLO coating solution was then added to each well, and for injury plates, 1 ml of PLO coating solution was added to each well. Both culture plates were coated with PLO solution 1 day prior to the cell culture preparation and plates were incubated in a humidified incubator at 37 ºC with 5% CO_2.

2.3.2 Solution preparation for Dissection

2.3.2.1 Hank's buffered salt solution

Hank's buffered salt solution (HBSS) is without calcium chloride, magnesium chloride and magnesium sulphate. HBSS is a solution which helps in maintaining pH and osmotic balance. It also provides the cells in the culture with organic ions and required water content. Five ml of Penstrep (Pencillin-streptomycin an antibiotic−antimycotic solution at a final concentration of 100 units/mL penicillin G, 100 µg/mL streptomycin sulfate, and 250 ng/mL amphotericin B) (Sigma, St. Louis, MO, USA), was added to 500 ml of HBSS to decrease the chances of bacterial or fungal contamination.

2.3.2.2 Trypsin-EDTA 0.1%

Trypsin (an enzyme used for protein hydrolysis)-Ethylene diamine tetra-acetate-complexing/chelating agent (EDTA) is a reagent or solution, which is used to digest the divalent cations from the culture and help in detachment of the cells. EDTA alone does this but combination with Trypsin gives much better results. Trypsin-EDTA was dissolved in HBSS containing Penstrep to obtain 10 µg/ml. 100 ml of Trypsin-EDTA was added to 100 ml of HBSS containing 1 ml of Penstrep to make a solution of 10µg/ml and stored in -18ºC.

2.3.2.3 Growth Media Preparation

First, growth media free of horse serum was prepared by adding 5 ml of sterile PenStrep solution, 8 ml glucose solution (Sigma), 5 ml sodium pyruvate solution (GIBCO), and 5 ml of N_2 supplement (GIBCO) into a 500 ml bottle of sterile Basal Medium Eagles (BME) (GIBCO, Grand Island, NY, USA) and was stored at 4 ºC. Then, to make growth media containing 10% HS (GIBCO), 4.5 ml HS was added to 45 ml growth media in a 50 ml labeled Falcon tube,

which was stored at 4ºC. The solution was sterile-filtered prior to use.

2.3.2.3.1 Solutions for Growth media preparation

Glucose solution - 32.5 gm of D (+/-) Glucose was dissolved in 67.5 ml of distilled water and stored at 4ºC.

Sodium pyruvate solution - 550 mg of sodium pyruvate was mixed with 50 ml of Distilled water and stored at 4ºC.

2.3.2.4 Culture media Preparation

Serum free culture media was prepared using BME (500 ml- GIBCO) containing 5 ml of PenStrep solution, 8 ml glucose solution (Sigma), 5 ml sodium pyruvate solution (GIBCO), 5 ml of N2 supplement (GIBCO) and 10 ml of B27 (GIBCO) supplements. N2 and B27 supplements are preferably added at the end. Medium was then sterilized through a bottle filter and stored at 4ºC.

2.3.3 Dissection

The brains of 1-3 day old rat pups, supplied by Animal Care Services, were dissected and cultures were prepared as described below. The dissection protocol was approved by the Institutional Animal Care Committee of Memorial University (protocol 12-20-JW). The centrifuge was switched on an hour prior to the start of the dissection in order for it to cool to 4ºC. The rat pups were euthanized and brains were removed from the skull and placed in 15 ml cold HBSS solution supplemented with 10 mg/ml PenStrep in a small petri dish, which was put on ice. The cerebellum was cut and discarded. The right and left cortical hemispheres were separated by a scalpel. Then the corpus callosum, the blood vessels, and membranes surrounding the brain were removed under microscopic light. The hemispheres free of blood vessels were

then placed in a centrifuge tube containing 10 ml of cold HBSS. Before centrifuging the cell aggregates were dissociated to smaller pieces by gently using a serological pipette (10 triturations). The tissue was then washed (centrifuged) at 1000 rpm at 4ºC for 3 min and the supernatant removed and 10 ml of fresh HBSS was added. The tissue was again washed at 1000 rpm at 4 ºC for 3 min, and the supernatant was discarded. At this point, 0.8 ml of 0.25% Trypsin-EDTA in HBSS supplemented with 9.2 ml of fresh HBSS was added. This tube was incubated for 15 min at 37ºC. After incubation, the tube was centrifuged at 1000 rpm at 4ºC for 3 min. Again the supernatant was discarded and 10 ml fresh growth media was added to it. This tube was now centrifuged at 1200 rpm at 4ºC for 5 min. The tube was taken out of the centrifuge and supernatant was discarded. Four ml of fresh growth media was added and cells were triturated using a 5 ml serological pipette until all large aggregates were dissociated. It was further triturated with the same pipette equipped with a 100 µL plastic pipette tip. Now the suspension was filtered through a 70 µm nylon strainer into a 50 ml plastic tube. The suspension was diluted with growth media up to 12 ml if using two 24 well plates. The PLO coated plates were then removed from the incubator. The PLO was removed and the wells were washed twice with distilled water (1 ml for injury plate and 300 µL for a 24 well plate). One ml of the suspension was transferred to each well (injury plate) or 250 µL was transferred to each well in a 24 well plate. The cultures were then stored in a humidified incubator (37ºC, 5% CO_2). Cells were grown for 9-16 days in vitro (DIV) for the experiments. Media was changed regularly twice a week with culture media.

2.3.4 Cell Treatments

Cells which were between 9 to 16 DIV were used for treatments. The cells were treated with different conditions as described below.

2.3.4.1 Solution Preparation and treatment

2.3.4.1.1 Glutamate solution (100 µM final concentration)

14.7 mg of glutamic acid was dissolved in 10 ml of distilled water. 30 µl of this solution was added to 300 µl of culture media in the cell culture wells to give a final concentration of 100 µM. Glutamate was dissolved in sterile distilled water (dH_2O). Cell cultures were exposed to glutamate (100 µM) in a volume of 3 µl per 0.3 ml of cell culture media, and control cultures received an equivalent volume of sterile dH_2O.

2.3.4.1.2 **Solvent** (acetone+ 0.2% of formic acid)

We first tested whether the solvent used in the extraction of blueberry and lingonberry fruits and leaves had any effect on cell cultures before treating cells with the extract itself, and determined the appropriate amount of solvent that did not affect the cultures. Cell cultures were exposed to 1 µl of the solvent per 0.3 ml of culture media.

2.3.4.1.3 Berry Extracts

The cell culture experiments were carried out after the samples of extracts were filtered through a sterile filter of 40 µm pore size. Cell cultures were treated with 1 µl lingonberry and blueberry fruit extracts (250 mg/ml) and leaf extract (25 mg/ml) at the time of glutamate exposure, and were treated with the leaf and fruit extracts for 24 hr. We found that cultures treated with 1 µl of solvent alone had no significant change in cell number after 24 hr. For each treatment plate, at least two control treatments were performed using dH_2O as well as two glutamate treatments. Experiments were performed in at least three separate culture preparations and each condition was represented by at least six samples.

2.3.5 Dose Response

Different glutamate solutions were prepared and the cultures were treated with three different concentrations, which were 10 µM, 100 µM and 1 mM. Glutamate was dissolved in dH_2O. Cell cultures received glutamate solution and the control wells received an equivalent amount of dH_2O. For achieving a concentration of 10 µM, a stock solution of 1 mM (1.97 mg of glutamate in 10 ml of dH_2O) was made. 3 µl of this solution was added to 0.3 ml of the media. For achieving a concentration of 100 µM and 1 mM, a stock solution of 10 mM (19.71 mg of glutamate in 10 ml of dH_2O) was made. 3 µl and 30 µl of this stock solution was added to 0.3 ml of media to get 100 µM and 1 mM concentration respectively. The treatment was for 24 hours which was followed by staining.

2.3.6 APV Experiments

AP5 or APV ((2R)-amino-5-phosphonovaleric acid; (2R)-amino-5-phosphonopentanoate) (TOCRIS) is a selective NMDA receptor antagonist that competitively binds to the ligand binding site on the receptor and inhibits the action of ligand (glutamate). Two different concentrations of APV – 30 µM and 100 µM were selected based on the literature and a common stock solution was made. A stock solution of 1 mM was made by adding 1.97 mg of D-APV to 10 ml of distilled water. To achieve a concentration of 30 µM in the media, 9 µl of APV was added to 291 µl of culture media. Another concentration of 100 µM was achieved by mixing 30 µl of APV with 270 µl of culture media. Addition of APV solutions alone was performed to see if APV had any effect on the cells. In normal conditions, these two different concentrations of APV were mixed with 100 µM glutamate and added to the media. In the case of 30 µM APV, 3 µl of 1 mM glutamate and 9 µl of APV was added to 288 µl of culture media. In the case of 100 µM APV, 3 µl of 1 mM glutamate and 30 µl

of APV was added to 267 µl of culture media. The treatment was for 24 hours and then it was followed by the staining protocol.

2.3.7 In vitro traumatic injury

In a set of experiments we used a model 94A Cell Injury Controller developed by Ellis *et al*. (1995) (Bioengineering Facility, Virginia Commonwealth University, Richmond, VA, USA) for injuring the cells. For this set of experiments, the cell cultures were grown on deformable Silastic membranes in 6-well FlexPlates. For treatment, after addition of fresh 300 µl of fresh culture media, a 50 ms pulse of compressed air is delivered to the wells, which deforms the Silastic membrane and adherent cells to varying degrees controlled by pulse pressure as shown in Fig 2.1. The extent of cell injury is dependent on the degree of deformation, or stretch (Ellis *et al*, 1995). This is followed by the staining technique 24 hours later.

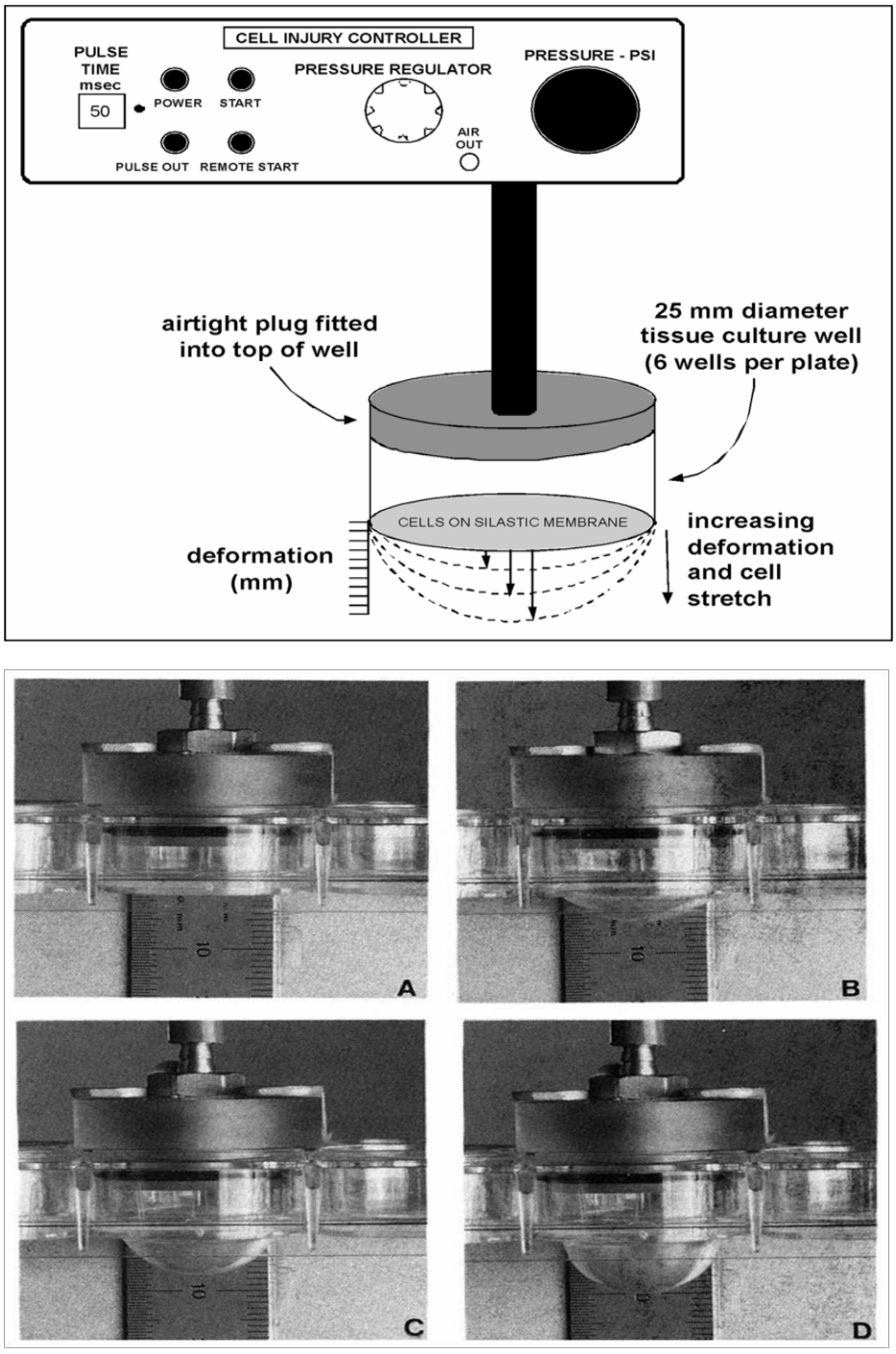

Fig 2.1 Cell injury controller

Cell cultures are grown on deformable Silastic membranes in 6-well FlexPlates. A 50 ms pulse of compressed air is delivered to the wells, which deforms the Silastic membrane and adherent cells to varying degrees controlled by pulse pressure. The extent of cell injury is dependent on the degree of deformation, or stretch (Ellis *et al.*, 1995).

2.3.8 Staining, Fixing and Imaging of cell cultures

After 24 hrs of cell treatment (control, 100 µM glutamate, 100 µM glutamate with berry extracts, injury, injury with berry extracts), 250 µl of culture media was removed from each treated well in case of 24-well plate. Then 250 µl of propidium iodide (PI) solution was added to each well in the dark. After 5 mins, PI was removed and wells were washed with PBS pH 7.4. 4% Paraformaldehyde fixative solution (250 µl) was added to each well and was allowed to stand for 20 minutes. Wells were washed twice with PBS solution and once with 100% ethanol to remove any excess moisture. 10 µl of 4', 6-diamidino-2-phenylindole (DAPI) was added and cells were then covered with cover slips. DAPI is a fluorescent stain that binds strongly to A-T rich regions in DNA. It is used extensively in fluorescence microscopy. DAPI can pass through an intact cell membrane, therefore it can be used to stain both live and fixed cells. PI can only pass through damaged cell membrane. Therefore, DAPI stain gives us the total count of cells in the images taken and PI on the other hand indicates dying or damaged cells only. Images were captured using a Zeiss Observer A1 microscope and a Pixelfly qe CCD camera (pco, Kelheim, Germany). Five random images per well were taken using different filters and a magnification of 200x. These images were then counted manually. Images of DAPI positive cells were captured with a DAPI optical filter set. At least two investigators who were blind to the treatment conditions counted the amount of cells in each image. Each well on a given day is considered to be "n", which consist of the average counts of five captured images. The mean value of both the readings for each well was generated and calculated accordingly. Data on the number of DAPI- positive cells is expressed as % of control values for each given experimental day. Condensed nuclei data is expressed as the % of the total amount of DAPI-positive cells that contained condensed nuclei within each condition. The % of condensed nuclei was calculated using the program Image J (Rasband *et al*., 1997).

Representative images of cellular morphology in control and glutamate-treated cultures were also captured using differential interference contrast microscopy.

2.3.8.1 Preparation of Propidium iodide solution

Propidium iodide solution was made by diluting 3 µl of propidium iodide in 10 ml of PBS solution in the dark.

2.3.8.2 Preparation of Paraformaldehyde Fixative

400 ml of distilled water was taken in a 1000 ml beaker under a fume hood and was heated to 70ºC and then 40 g paraformaldehyde (PFA) was added and the solution was stirred well, as the solution appeared cloudy. Cold 10 N NaOH was added slowly drop wise to the solution until it cleared. 500 ml of 0.2 M cold Phosphate buffer was added to it. Then distilled water was added to the solution to bring the final volume up to 1000 ml making it 4% PFA concentration. This solution was filtered using a bottle filter and stored at 4ºC.

2.3.9 Alpha-Synuclein Experiments: Staining and Imaging

After 24 hrs of cell treatment, 250 µl of culture media was removed from each treated well in the case of 24-well plates. All wells were washed twice with Phosphate Buffer Solution (PBS). 4% PFA fixative solution (250 µl) was added to each well and was allowed to stand for 20 min. Wells were then permeabilized with 0.2% Triton X for 10 min. This was followed by washing once with PBS solution. Blocking solution was added to incubate the cultures for 30 min. After removal of this solution, cultures were incubated with primary antibodies to microtubule-associated protein-2 (MAP-2) and alpha-synuclein in 10% blocking solution diluted in PBS for 1 hour. The cultures were again washed twice

with PBS. Cultures were then incubated with secondary antibodies such as Alexa 488 and Alexa 595 in 10% blocking solution diluted in PBS for 1 hour. The wells were again washed with PBS twice. Cultures were at last washed with 70% and 100% ethanol to remove any excess moisture. After the cultures dried, 10 µl of DAPI was added and cells were then covered with cover slips. Images were captured using a Zeiss Observer A1 microscope and a Pixelfly qe CCD camera (pco, Kelheim, Germany). Five random images per well were taken using different filters and a magnification of 200x. These images were then counted manually. Images of DAPI positive cells were captured with a DAPI optical filter set.

2.3.10 Lactate Measurements

Lactate measurements were also performed in media from cortical cultures. Cultures received a complete media change just before glutamate treatment and treatment in the presence of extracts, and samples of media were removed from cultures 24 hours after glutamate exposure and stored at −20°C until measurements. Lactate concentrations in media were determined electrochemically with a biochemical analyzer (Analox Instruments, Ltd, UK). Data are presented as mmol/l. All experiments were completed using three separate culture preparations between 9 and 16 DIV.

2.4 Statistical analysis

All of the biochemical experiments were repeated at least three times. Data in the text and the figures are expressed as means ±SE of three replicates. Statistically significant differences were determined by the non-parametric unpaired t-test using the statistical program SPSS (IBM Inc.). In all cases the confidence coefficient was set at 0.05. The data for the bioactivity (cell culture) experiments were analyzed with one-way ANOVA ($p < 0.05$) followed by

Tukey's multiple comparisons test using the statistical program GraphPad Prism (La Jolla, CA, USA). Data represented in figures are expressed as means ± SE of at least 6 wells per condition. Significance was also set at $p < 0.05$ for these experiments.

CHAPTER 3

RESULTS

A portion of the results described here have been published in the article named "Chemical analysis and effect of blueberry and lingonberry fruits and leaves against glutamate-mediated excitotoxicity" from the *Journal of Agriculture and Food Chemistry*. The list of authors are Vyas, P., Kalidindi, S., Chibrikova, L., Igamberdiev, A. U., & Weber, J. T. (2013) where Vyas P. and Kalidindi S. have contributed equally.

3.1 Biochemical assays

The content of total soluble phenolics, flavonoids, anthocyanins and tannins was found to be significantly higher in lingonberry fruits as compared to blueberry fruits (**Fig. 3.1**). This same trend was observed for leaves except for flavonoid content, in which the leaves of blueberry had a higher content as compared to those of lingonberry. The content of total soluble phenolics, flavonoids, anthocyanins and tannins was significantly higher in the leaves of both species versus the fruits ($p<0.05$). This latter finding correlated well with total radical scavenging capacity and reducing power, in which the leaves had much higher activity compared to fruits (**Fig. 3.2**). The reducing power of the leaves of both plants was similar, but the radical scavenging capacity was significantly higher in lingonberry leaves versus blueberry leaves ($p<0.05$). There was no significant difference between the blueberry and lingonberry fruits in scavenging activity and reducing power.

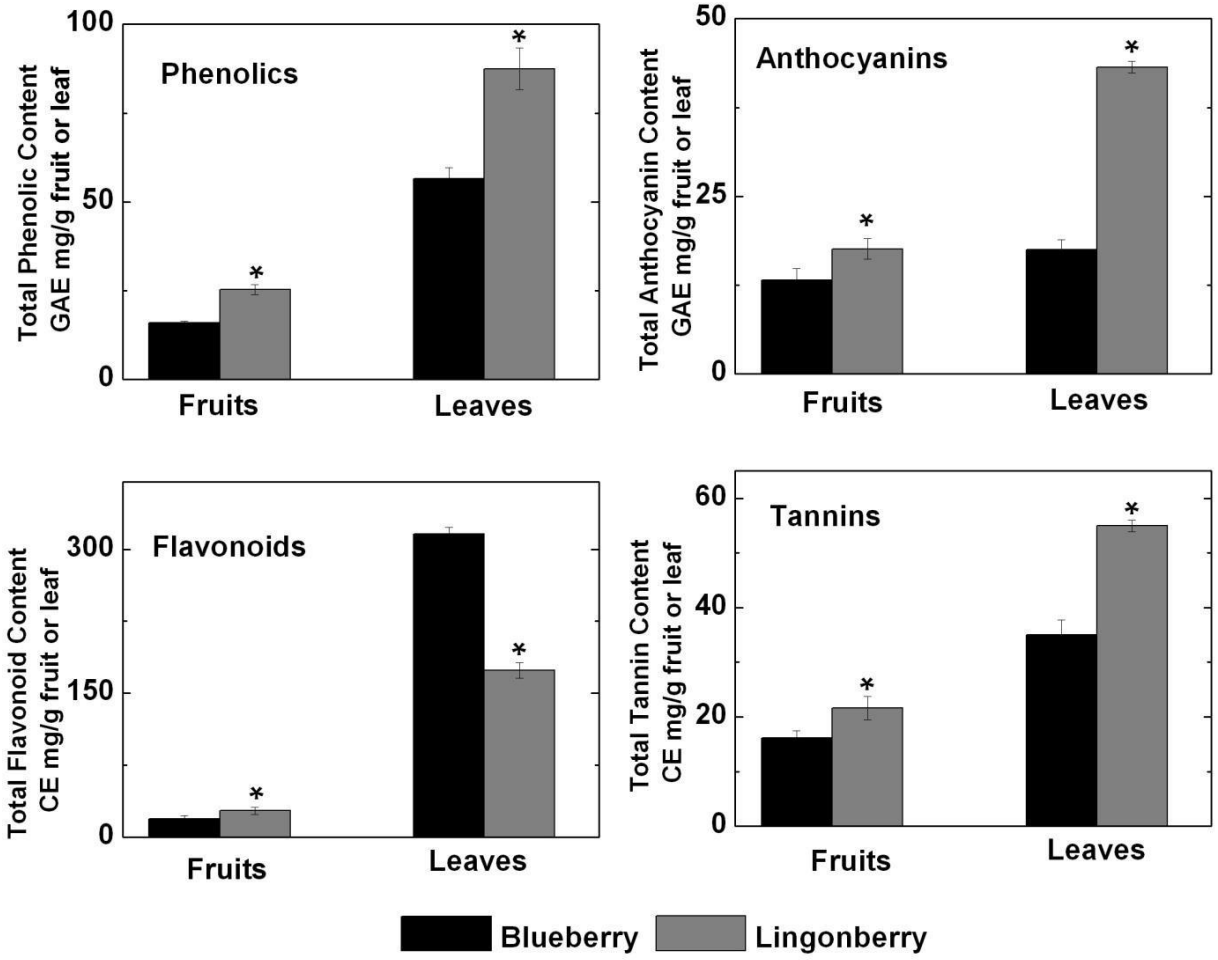

Fig 3.1 Total phenolic, anthocyanin, flavonoid and tannin content in extracts. Total phenolic, anthocyanin, flavonoid and tannin content in wild blueberry and lingonberry extracts from fruits and leaves found in Newfoundland. Data is expressed as the mean ± SE. n=3 for each experiment. "n" value represents the sample extracted on a particular day. *-indicates values differ significantly from blueberry data at $p<0.05$ (non-parametric unpaired t-test). The content of total soluble phenolics, flavonoids, anthocyanins and tannins was significantly higher in the leaves of both species versus the respective fruits. GAE: Gallic acid equivalents; CE: catechin equivalents. (In Vyas, Kalidindi, Chibrikova, Igamberdiev and Weber, 2013)

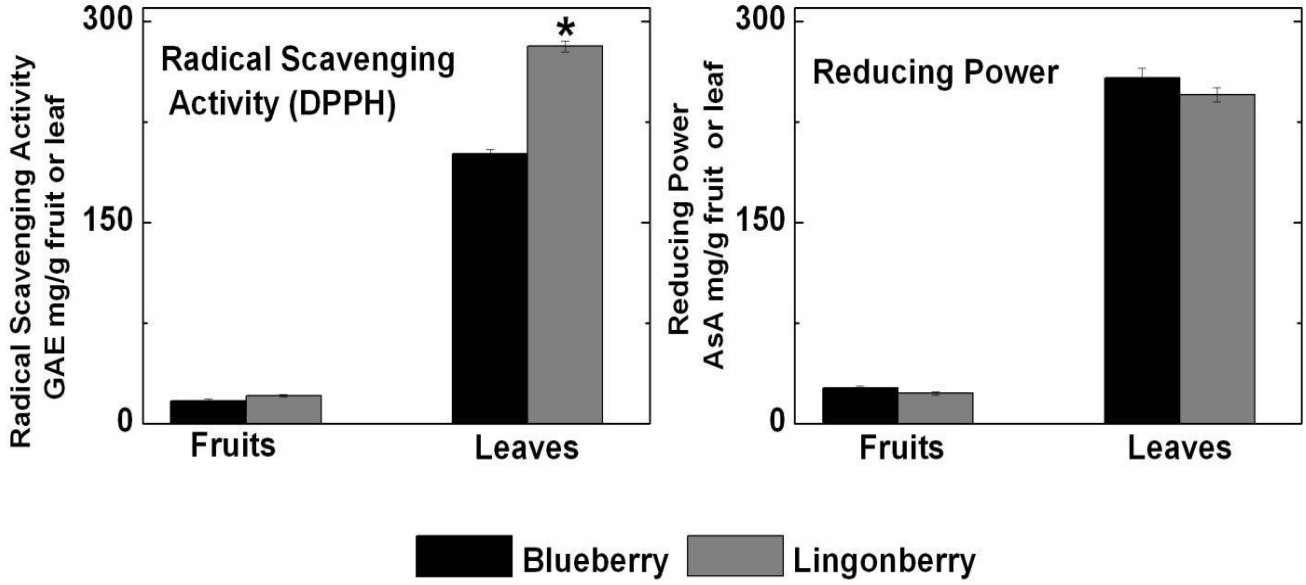

Fig 3.2 Total radical scavenging activity and reducing power. Total radical scavenging activity and reducing power in blueberry and lingonberry extracts from fruits and leaves. Data is expressed as the mean ± SE. n=3 for each experiment. "n" value represents the sample extracted on a particular day. *-indicates values differ significantly from blueberry data at $p<0.05$ (non-parametric unpaired t-test). GAE: Gallic acid equivalents; AsA: ascorbic acid equivalents. (In Vyas, Kalidindi, Chibrikova, Igamberdiev and Weber, 2013)

3.2 Cell culture experiments

In preliminary studies I found that treatment of cultures with 1 µl of solvent alone had no significant change in cell number after 24 hours and 10 µM glutamate produced a cell loss of 17.0 ± 5.7 %, which was not statistically significant. 1 mM glutamate treatment did not cause much cell death (**Fig 3.3**). However, 100 µM glutamate treatment for 24 hours caused a significant loss of cells of 23.1 ± 5.1 %, therefore I used a concentration of 100 µM glutamate for the remaining experiments. Analysis of cultures using light microscopy indicated that the cells in control (untreated) cultures had intact cell bodies and that cells consistent with neuronal morphology also displayed intact, smooth neurites (**Fig. 3.4**) indicating healthy cells. After treatment with glutamate for 24 hours, many cells had disrupted cell bodies, and there was an increase in dark punctae, which may be indicative of condensed nuclei in dead or dying cells (Chen *et al*., 2000).

I stained a set of cultures with PI and DAPI. PI is a fluorescent dye which stains both RNA and DNA. Representative images of PI and DAPI were taken under various conditions (**Fig 3.5**). PI staining indicates cell death or damaged cells. I did not quantify the PI data because of a condition called "co-localization" which means dual staining. Nucleolar organizer regions, telomeres and centromeric regions have high affinity for PI whereas other chromosomal regions have high affinity for both PI and DAPI. When both PI and DAPI stains are added to the cultures we observe a unique differential staining pattern along the chromosomes producing a purplish color (**Fig 3.6**). I believed that this purple color on the cells would alter our counts and the data might not be accurate.

Therefore, I quantified the potential neuroprotective effects of the berry extracts by counting the amount of DAPI-stained cells that were present in cultures under various conditions (e.g. **Figs. 3.3, 3.7 and 3.8**). Treatment with glutamate appeared to increase the

amount of brighter, condensed nuclei in cultures, which is often indicative of delayed cell death (Weber *et al*., 2012) (**Fig 3.7**). Glutamate exposed cell cultures treated with blueberry and lingonberry fruit extracts showed 112.9 ± 15.1% and 79.1 ± 12.4 % of control values respectively, indicating high protection from blueberry fruit, but no protection from lingonberry fruit (**Fig 3.8**). Cultures treated with leaf extracts of blueberry and lingonberry showed 101.8 ± 5.1% and 106.9 ± 8.6 % of control values respectively, which was significantly different versus glutamate treatment alone, indicating a highly protective effect of the leaf extracts from both species (**Fig 3.8**). I quantified the percentage of cells displaying condensed nuclei in the same cultures in which we determined the extent of cell loss. Glutamate caused a trend towards an increase in the amount of condensed nuclei after 24 hr of exposure, but this trend was not statistically significant. Interestingly, there also appeared to be a trend towards increase in condensed nuclei in cultures treated with blueberry or lingonberry leaf extract, but again these findings were not statistically significant.

I next used a selective NMDA receptor blocker (APV) instead of the berry extracts to determine if one possible mechanism of action of the berry extracts is by inhibiting Na^+ and Ca^{2+} entry through this receptor type. Two different doses of APV were selected based on the literature search. We quantified the % of cortical cells in different conditions (control, 100 µM glutamate, 30 µM APV, 100 µM APV, 30 µM APV + glutamate and 100 µM APV + glutamate) and tried to compare them. 100 µM glutamate as expected caused significant cell death. Other treatments did cause some cell death but none of this was significant **(Fig 3.9).** I could not give a firm conclusion about the mechanism of action at this point but NMDA receptor blockade might be one of many possible mechanisms by which berry extracts provide protection.

Another set of experiments were performed using the 94A controller injury model which caused stretch to the cells. I quantified the % of cortical cells which were injured and compared them with the control cells as well as with injured cells in presence of blueberry fruit extract. Injured cells showed 31.7 ± 7.7 % of the control cells, whereas the injured cells in the presence of the blueberry fruit extract showed 109.4 ± 15.2 % of control cells which was significant (**Fig 3.10**). This suggested a high protective effect of blueberry fruit against traumatic injury in rat brain cultures.

The measurement of lactate from the cultures would give an estimate of the metabolic changes and health of the cells. Lactate was measured in culture media 24 hours after treatment with 100 µM glutamate and glutamate in the presence of various extracts, which was then compared with the lactate in the control cells. The lactate levels for glutamate treated wells showed a trend towards a decrease when compared to the control cells, and glutamate treatment in the presence of various fruit and leaf extracts showed a lean towards an increase in lactate levels, which were similar to the control levels (**Fig 3.11**). However, none of these findings were found to be statistically significant.

Alpha-synuclein is a protein found in neurons. This protein agglomerates to form insoluble fibrils which are known as Lewy bodies (Wakabayashi *et al*., 2007) in certain conditions. Cell cultures which were treated with 100 µM glutamate, glutamate in the presence of berry extracts, injured and injured in presence of berry extracts were stained with alpha-synuclein antibody, MAP-2 and DAPI to examine the expression of the protein alpha-synuclein. I observed that with injury and glutamate treated cultures, there was an increased expression of alpha-synuclein in the neurons (**Fig 3.12**). Representative images of alpha-synuclein stained glutamate with berry treated cultures are shown (**Fig 3.13**).

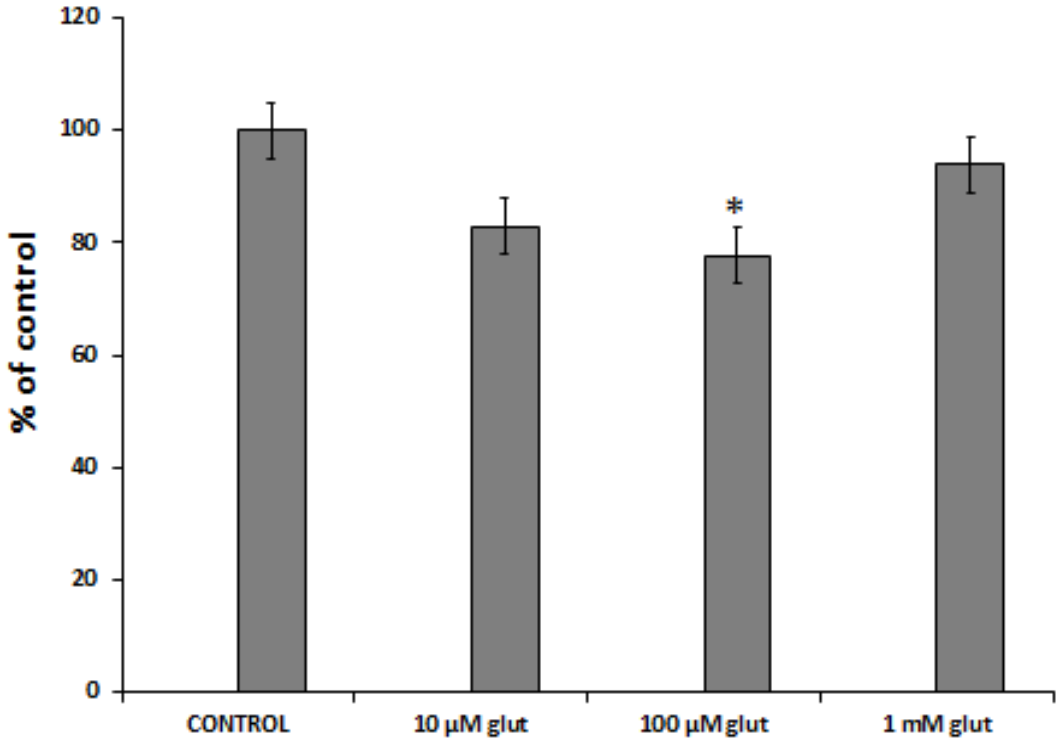

Fig 3.3 Glutamate Dose Response. Dose response data for glutamate induced cell death by different concentrations of glutamate. Cells in culture were treated with 10 µM glutamate, 100 µM glutamate and 1 mM glutamate alone. Staining and imaging with DAPI was carried out after 24 hour treatment with different concentrations of glutamate. The amount of DAPI-positive nuclei was quantified and the data is expressed as % of control values ± SEM. n= 9-11 (n refers to the wells on a given day). Cell loss or the amount of DAPI-positive nuclei for glutamate treated cells was quantified and expressed. 100 µM glutamate treatment showed a significant loss of cells whereas 10 µM and 1mM glutamate induced cell death was not significant. *$P<0.05$ when compared with the control.

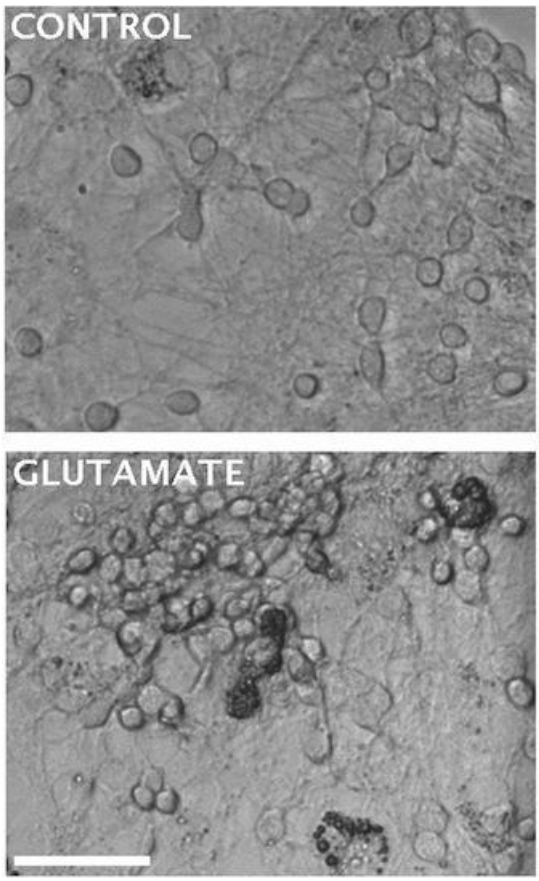

Fig 3.4 Morphology of cell cultures. Light microscopic images of untreated cells (control; top) and cells treated with 100 μM glutamate for 24 hours (bottom). Note that cells in control cultures had intact cell bodies and that cells consistent with neuronal morphology displayed intact, smooth neurites. After treatment with glutamate for 24 hours, many cells had disrupted cell bodies, and there was an increase in dark punctae. Scale bar = 50 μm and applies to both images. Cell cultures are 12 DIV. (In Vyas, Kalidindi, Chibrikova, Igamberdiev and Weber, 2013)

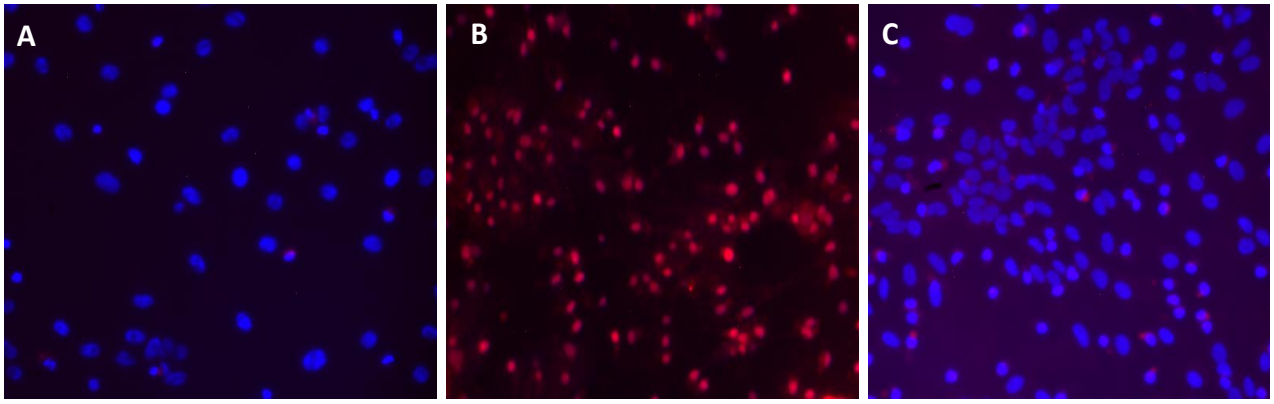

Fig 3.5 DAPI and PI stained cortical cells. Representing images of cortical cells stained with both DAPI (blue) and PI (red) in (A) control conditions, (B) after 24 hour treatment with 100 μM glutamate and after (C) 24 hour treatment with 100 μM glutamate in the presence of berry extracts. The presence of PI in the cells indicates damaged cells or dying cells.

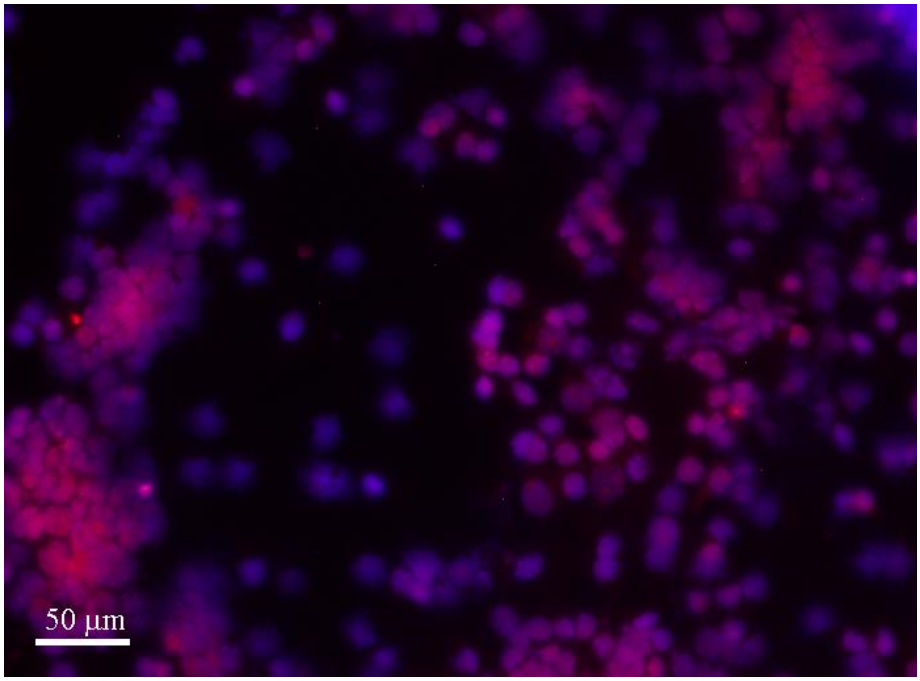

Fig 3.6 PI and DAPI Co-localization. An image showing cortical cells stained with both DAPI (blue) and PI (red). The image shows a phenomenon called "co-localization" (PI and DAPI both stains the same cells). The cells appear purplish in color. For this reason, we were unable to quantify PI data with confidence.

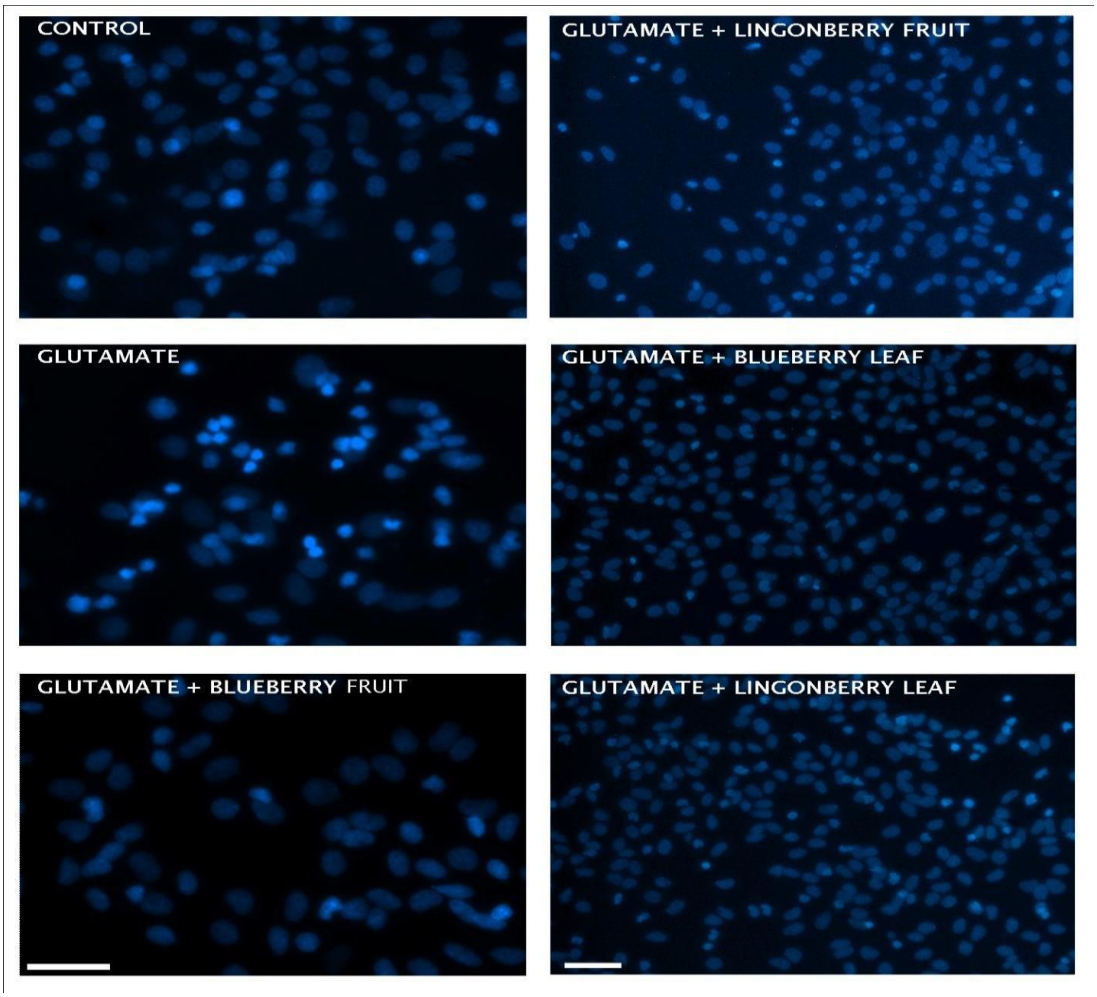

Fig 3.7 DAPI staining in cell cultures. Representative images of cortical cells stained with DAPI (blue) in control conditions, after 24 hour treatment with 100 μM glutamate, and after 24 hour treatment with 100 μM glutamate in the presence of blueberry and lingonberry fruit and leaf extracts. Note the presence of several condensed nuclei after treatment with glutamate, which may be indicative of delayed cell death. Images on the right are at a magnification of 200 X, while images on the left have been enlarged in order to better represent nuclear morphology. Both scale bars = 50 μm. The scale bar in the glutamate + blueberry fruit images applies to all three images on the left, while the scale bar in the glutamate + lingonberry leaf image applies to the three images on the right. Cell cultures in images on the left are 16 DIV, while those on the right are 15 DIV. (In Vyas, Kalidindi, Chibrikova, Igamberdiev and Weber, 2013)

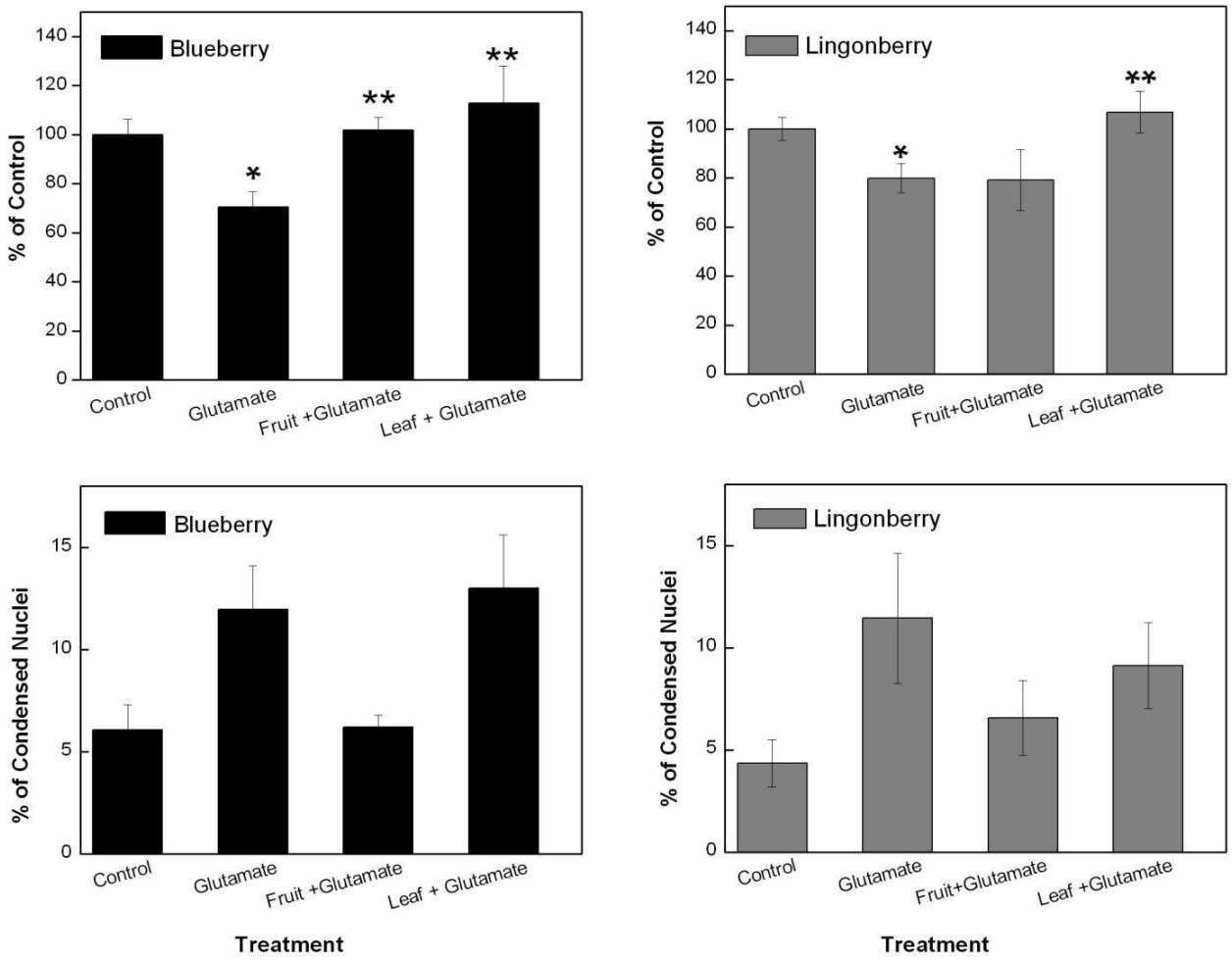

Fig 3.8 Effects of blueberry and lingonberry fruit and leaf extracts on glutamate-mediated cell death. Summary of the effects of blueberry (left) and lingonberry (right) fruit and leaf extracts on glutamate-mediated cell death. Cells were treated with 100 μM glutamate alone or in the presence of extracts. Top graphs: the amount of DAPI-positive nuclei was quantified and data is expressed as % of control values. Data is expressed as % of control values ± SEM. n=6-16 11 (n refers to the wells on a given day). *p<0.05 vs. control; **p<.05 vs. glutamate only (one-way ANOVA with Tukey's post-hoc analysis). Bottom graphs: the percentage of nuclei that exhibited a condensed morphology in the same culture wells used to generate the data in the top graphs. (In Vyas, Kalidindi, Chibrikova, Igamberdiev and Weber, 2013)

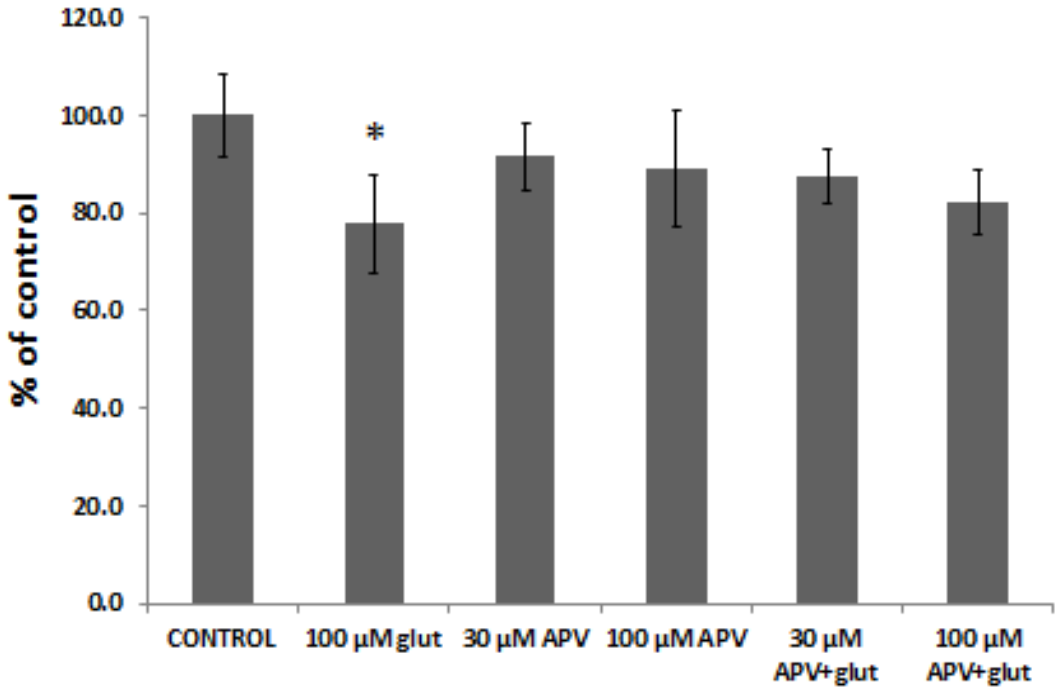

Fig 3.9 Effect of NMDA receptor blockade against glutamate excitotoxicity. APV is a selective NMDA receptor blocker. Here, a series of experiments were carried out by treating cells with two different concentration of APV (30 µM and 100 µM) alone and in the presence of glutamate. 100 µM glutamate was significant when compared to the control and caused a cell death of approximately 23 % of cells. Data is expressed as % of control values ± SEM. n=9-11 (n refers to the wells on a given day). *P<.05 when compared with control.

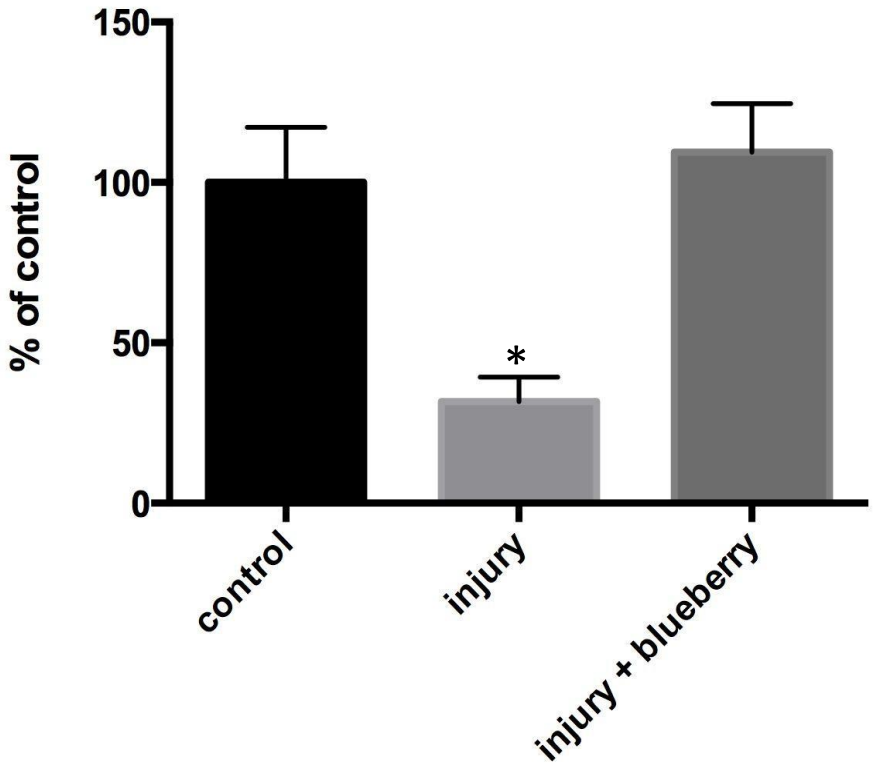

Fig 3.10 Effect of Blueberry fruit against traumatic injury. Summary of the effects of blueberry fruit extracts on traumatic injury-mediated cell death. Cells were injured with a 94A controller (injury model) alone or in the presence of extracts. The stretch (31 %) caused by the injury model was equivalent to 5.5 mm deformation. Data is expressed as % of control values ± SEM. n=4 (n refers to the wells on a given day). *P<.01 compared with both control and blueberry treated cells.

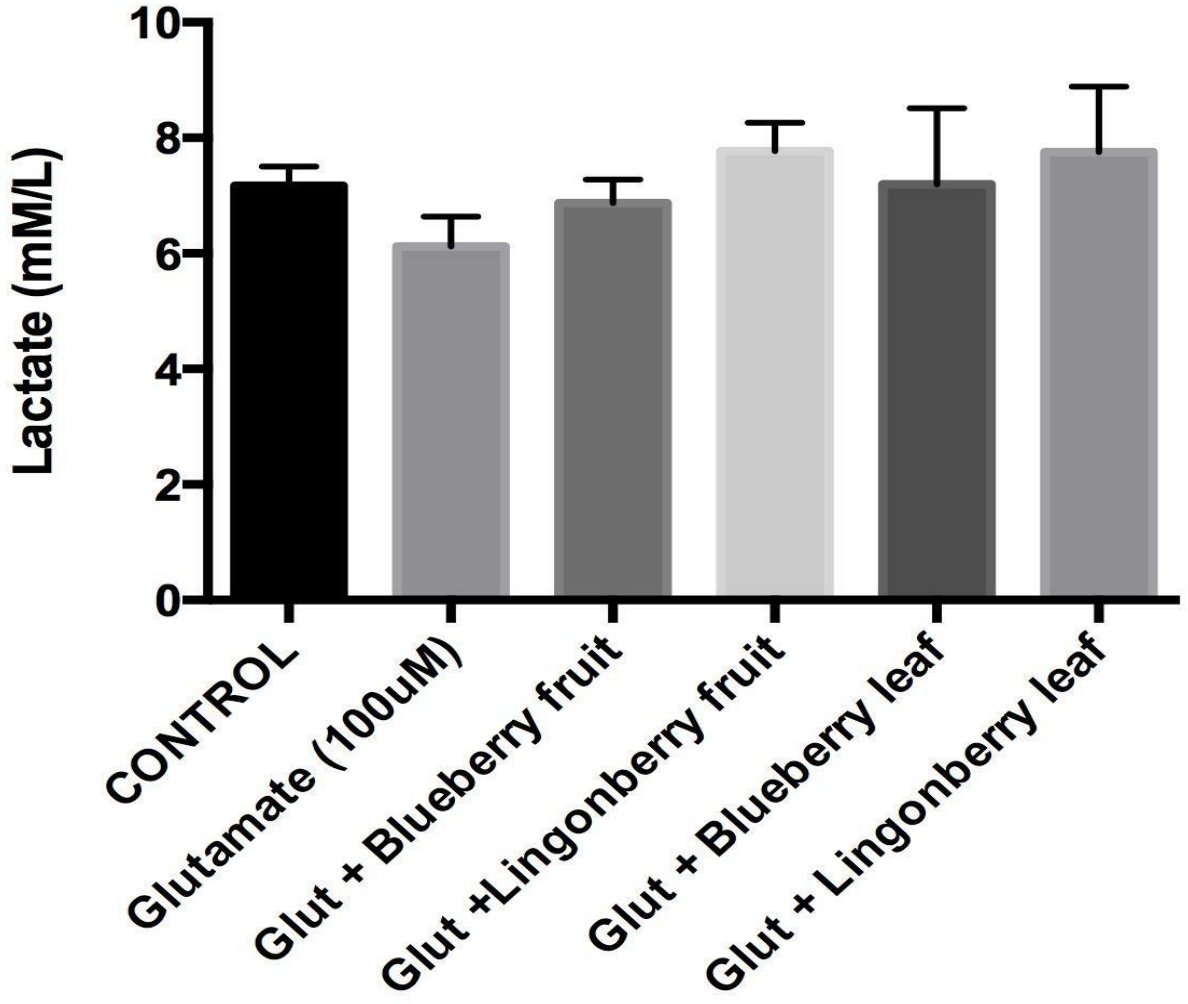

Fig 3.11 Lactate Analysis. Release of lactate in the culture media 24 hours after treatment with dH_2O (control), 100 µM glutamate and glutamate in the presence of different extracts was measured. Data is expressed as % of control values ± SEM. All conditions had n=22 for control and n=6-14 for experimental conditions (n refers to the wells on a given day). No significance was observed with one-way ANOVA and Tukey's multiple comparison test.

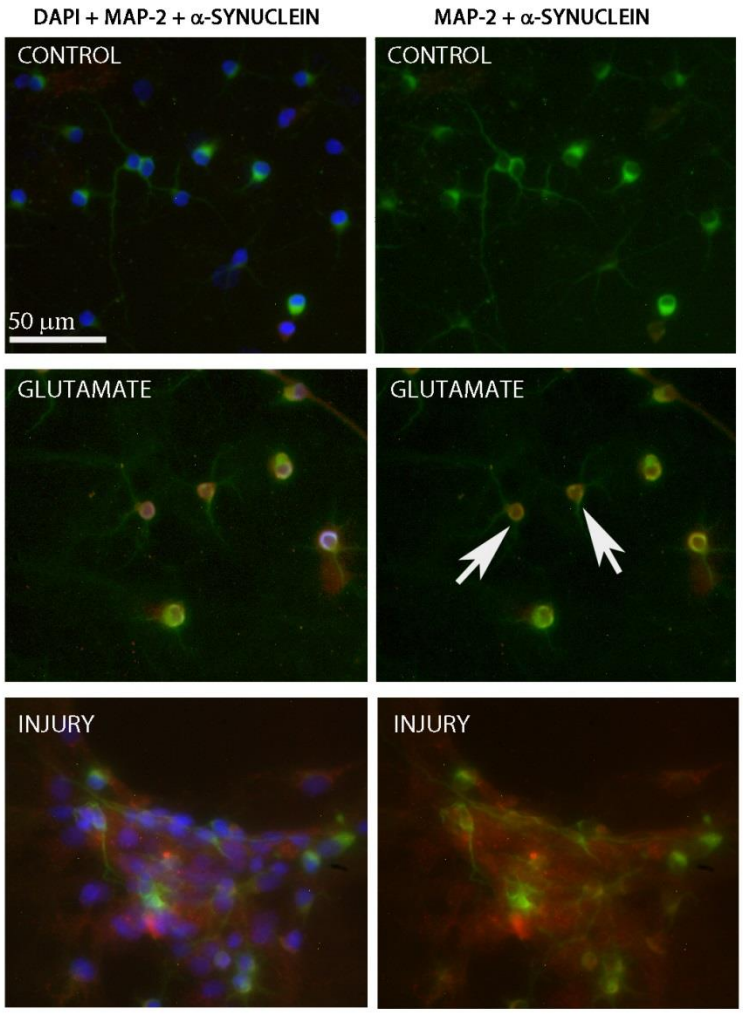

Fig 3.12 Alpha Synuclein expression in cortical cells. Representative images of rat brain cortical cells stained with DAPI (blue), MAP-2 (green) and alpha-synuclein (red) in control conditions, after 24 hour treatment with 100 μM glutamate, and after 24 hour of injury. We can observe the presence of alpha-synuclein in the neurons after treatment with glutamate and after injury compared to control conditions, which may indicate increased expression of the protein alpha-synuclein. The arrows point to neurons which show the presence of alpha-synuclein in the cell bodies. Images are at a magnification of 200x and the scale bars = 50 μm.

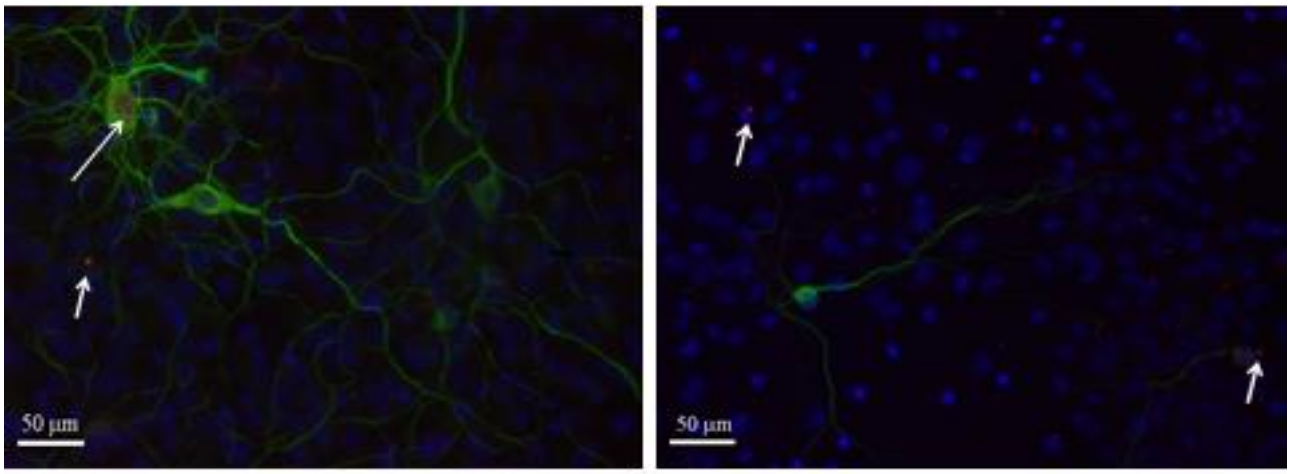

Fig 3.13 Alpha Synuclein in berry treated cells in the presence of glutamate. Representative images of rat brain cortical cells stained with DAPI (blue), MAP-2 (green) and alpha-synuclein (red) 24 hours after treatment with berry extracts + glutamate. We can see the presence of alpha-synuclein in a few neurons and glial cells (pointed to by arrows) after treatment with glutamate + berry extracts, which may indicate decreased expression of the protein alpha-synucelin.

CHAPTER 4

DISCUSSION

In this study, we collected and assayed the contents of blueberry and lingonberry fruits and leaves. We detected high levels of polyphenolic compounds, such as anthocyanins, flavonoids, and tannins, in blueberries and lingonberries (partridgeberries) growing natively in Newfoundland. When we compared the two fruit extracts the lingonberry extract had much higher anthocyanins, flavonoids, and tannins, making it much richer in polyphenolic content. We observed that the leaves of both species have a higher level of phenolic compounds compared to the respective fruits. The leaves were found to have much higher total antioxidant capacity than the fruits, as indicated by radical scavenging activity and reducing power, which confirms previous findings for lingonberry (Vyas, Debnath and Igamberdiev, 2013). These biochemical data are generally in line with the biological activities of the extracts on glutamate-exposed rat brain cultures with some alterations. For example, although the fruit extract of blueberries was highly protective against glutamate toxicity in rat brain cultures, lingonberry fruits were not protective. However, the extracts from leaves of both plant species were highly neuroprotective in our cell culture model. This could be because of the direct correlation of the involvement of ROS and RNS during glutamate excitotoxicity (Wang and Michaelis, 2010) and the role of polyphenols in scavenging ROS (Fang *et al*., 2002). The fruits and leaves of blueberry and lingonberry plants are rich in polyphenolic content and all extracts except the lingonberry fruit extract have shown protection against glutamate excitotoxicity.

Glutamate, which is a major endogenous excitatory neurotransmitter in the mammalian

CNS, plays an important role in long term potentiation, learning, and memory under normal physiological conditions (Chen *et al.*, 2009). Glutamate also plays an important role in normal neurophysiology, such as those involved in a variety of cognitive functions (Rahn *et al*., 2012), and is involved with synaptic plasticity, believed to be the cellular mechanism of learning and memory (Kim and Linden, 2007; Lamont and Weber, 2012). The appropriate levels of glutamate are important as it should be present in the correct concentration in order to produce its normal physiological effects. However, increased glutamate levels result in glutamate-mediated excitotoxicity, which can lead to cell damage and death (Goto *et al*., 2009) and contribute to impaired signaling and cell death (Greenwood *et al.*, 2007). This phenomenon could be due to excessive glutamate release in the synapse, over-activation of glutamate receptors, or inadequate uptake of glutamate by glial cells through transporters. This pathological process can contribute to brain aging and neurodegeneration over the course of many years, but there is a possibility that it can occur very rapidly during severe insults such as those seen in stroke and TBI (Nakamura *et al*., 2010; Weber, 2004). Excitotoxicity generally causes an excessive elevation of intracellular Ca^{2+} levels (Chen *et al*., 2000; Mehta *et al*., 2012), which in turn causes changes in the functioning of neurons and can over activate several Ca^{2+} dependent enzymes leading to changes in regular cellular processes (Nicholls *et al*., 2004; Weber, 2004; Wojda *et al*., 2008). This leads to a cascade of reactions which may all lead to cell death. As a consequence of the activation of calcium-dependent enzymes such as xanthine oxidase (Atlante *et al*., 2000), phospholipase A_2 (Wood *et al*., 2003; Weber, 2004) and nitric oxide synthase, free radicals including ROS and RNS are produced in cells (Mehta *et al*., 2012). Excitotoxicity is also responsible for oxidative dysfunction in mitochondria (Rego and Oliveira, 2003), which could lead to further ROS generation. Therefore, in our experiments when cell cultures were exposed to a high concentration of glutamate,

receptors were over activated, which likely led to oxidative and nitrosative stress contributing to cell death. However, elevated levels of Ca^{2+} can also activate other enzymes, such as endonucleases and proteases, which can degrade DNA and proteins, respectively (Weber, 2004). Therefore, there is a possibility that cell death may have occurred through mechanisms other than oxidative and nitrosative stress, and the protective effect of our berry extracts may not have been due to antioxidant and/or anti-nitrosative properties. Some studies in cultures support antioxidant or anti-nitrosative mechanisms against glutamate toxicity. For example, Ahn *et al*. (2011) found that 100 μM glutamate causes hippocampal cell death by altered calcium signaling and nitrosative stress. Another recent study by Lee *et al*. (2012) showed that 100 μM glutamate caused oxidative stress by excessive release of calcium. In our culture system, we found that neurons generally constitute only ~12% of the total cells, with the remaining cells mostly made up by glial cells, specifically astrocytes. After an exposure of 100 μM glutamate we observed a total cell loss of ~23%. This suggests that most of the cell loss was due to the death of glial cells rather than the neurons. It is possible that glial cells died due to excessive glutamate uptake, and cell swelling, however, for example, Chen *et al*. (2000) found that oxidative stress was the primary reason for the death of astrocytes in the cultures. Therefore, this finding suggests that berry extracts could likely protect glia through antioxidant properties. Our qualitative finding of increased dark punctae and altered morphology in glutamate-treated cells and the presence of disrupted cell bodies is also consistent with the response of astrocytes exposed to glutamate toxicity (Chen *et al*., 2000). In a set of experiments we tested the effect of 10 μM and 1 mM glutamate on cell viability. 1 mM did not cause much cell loss while 100 μM glutamate caused maximum cell death. This decrease in cell death with a higher glutamate dose might be due to super-saturation of glutamate. Excess glutamate in culture media may have been coming out of solution and not taken up by the cells.

Another possible reason may be down regulation of glutamate receptors in the presence of excessive glutamate which causes lesser cells to be available to actively take up glutamate leading to a decrease in cell death.

Many polyphenolic compounds have antioxidant capacity and are potent free radical scavengers, and a plethora of literature is available on the antioxidant capacities of phenolic compounds (Kähkönen and Heinonen, 2003; Chandra *et al*., 2004; Naczk and Shahidi, 2006) and their protective effects (Kong *et al*., 2003; Rossi *et al*., 2003). Other groups have found similar findings compared to ours using nervous system cell culture models to assess potential neuroprotective effects of various polyphenolic compounds. For example, Ahn *et al*. (2011) found that a proanthocyanidin extract from grapes could inhibit hippocampal cell death by decreasing nitrosative stress. Lee *et al*. (2012) found that antioxidants found in acorn extract could inhibit calcium spikes and thus control glutamate induced excitotoxicity. Similar results have been found with other antioxidant compounds such as carotenoids from *Pittosporum tobira*. These carotenoids have been shown to protect rat cortical cells against exposure to 100 μM glutamate for 24 h (Moon *et al*., 2010), the same treatment protocol used in the current study. Another study showed that Apigenin, a flavonoid, could inhibit the excessive $[Ca^{2+}]_i$ induced by glutamate treatment (Han *et al*., 2008). An extract from the fruit of *Alpinia oxyphylla* was shown to protect cortical neurons against exposure to 30 μM glutamate and also had an effect on condensed nuclei, similar to our findings (Yu *et al*., 2003). However, in a model of glutamate toxicity in hippocampal cultures, a seed extract of *Cassia obtusifolia* provided no protection to either neurons or glia (Drever *et al*., 2008). Interestingly, nicotine also showed protective effects against glutamate induced neurototoxicity by inhibiting the formation of excess NO which was triggered due to excessive calcium influx (Kaneko *et al*., 1997). These studies indicate that many plant species containing antioxidants

offer protection against glutamate excitotoxicity in neurons.

It is very interesting that out of all of the four extracts only the extract from lingonberry fruit did not show any protection against toxicity in our model. The reason for this might be due to the actual chemical composition of the lingonberry fruit versus the other fruit and leaf extracts. Although our biochemical assay shows that lingonberry fruit had a higher overall polyphenolic content when compared with that of blueberry fruit, the specific compounds in each extract is unknown to us. It is also possible that there are some specific compounds present in the blueberry fruit and other leaf extracts which are absent in the lingonberry fruit extract and they are responsible for the protection against glutamate toxicity. For example, Bhuiyan *et al*. (2011) found that when cell cultures were exposure to 50 µM glutamate it killed 40% of cortical neurons. But when a specific polyphenol (cyanidin-3-glucoside) was added, there was no protection. The same cyanindin-3-glucoside provided protection against oxygen glucose deprivation in primary cortical neurons (Bhuiyan *et al*., 2011). Another study done by Bhuiyan *et al.* (2012) found that specific anthocyanins extracted from black soya bean seed coat did not protect against glutamate excitotoxicity in neuronal cells but interestingly they found that treatment of cells with 1 µM of MK801 (non-competitive NMDA receptor antagonist) controlled the glutamate induced cell death. It is known that not all compounds or polyphenols can cross the blood brain barrier (BBB). Mostly, nutrients and highly lipophilic compounds have a tendency to cross the endothelial layer and reach the brain. Previous studies have shown that some dietary polyphenols can cross the BBB, (Vouzour *et al*., 2012) and some flavonoids are specifically known to cross the BBB (Faria *et al*., 2011, Faria *et al*., 2014). Anthocyanins specifically have been detected in brain tissue after oral administration to rodents (Andres-Lacueva *et al*., 2005; Talavera *et al*., 2005; El-Mohsen *et al*., 2006). Estimates of specific anthocyanins in brain tissue are generally in the subnanomolar

range (~0.2−0.25 nmol/g tissue) (Talavera *et al*., 2005; El-Mohsen *et al*., 2006). Berry anthocyanins have also been detected to cross the BBB (Milbury and Kalt, 2010). Direct comparisons with the above studies may not be appropriate as we used the whole extract and not specific anthocyanins in the current work. There is a possibility that the specific anthocyanins in the whole extract are responsible for such an effect. The final concentration of the extracts we added was 0.833 μg/mL of fruit extract and 0.083 μg/mL of leaf extract. We have previously conducted chemical analysis of commercially available lingonberry extracts (unpublished data) and have found that these lingonberry extracts contain an estimated 63.7 mg of cyanidin-3-galactoside/100 mg of fresh extract weight. Assuming that our fresh lingonberry extracts contain a similar amount of this compound, this would translate to the cultured cells being exposed to approximately a 10 nM concentration of fruit extract and 1 nM in leaf extract. Talavera *et al*. (2005) detected a level of another cyanidin compound (cyanidin-3-glucoside) of 0.25 nmol equivalent/g of tissue. Therefore, the amount of extract that we added to cultures is likely slightly higher than what might be achieved in the brain after oral administration. A study was performed on the bioavailability of various polyphenolic compounds and the compounds which were maximally absorbed were isoflavones and gallic acid, followed by flavanones. In addition, the polyphenolic compounds contained in our extracts may not be the predominant forms that would actually enter the brain, as a recent study found that although anthocyanins have a fairly high bioavailability, they undergo significant metabolism, producing diverse metabolites (Czank *et al*., 2013). A recent study has shown that anthocyanin and particularly its metabolites can cross the BBB and reach the CNS (Faria *et al*., 2014). Although we did not administer our extracts orally, we feel that our system is useful in screening specific polyphenols at various concentrations to test for neuroprotective potential and study the mechanisms of action of protection. The mechanism

of action through which the extracts caused the protection is unknown. Similarly, the actual mechanism for glutamate induced cell death is also not known.

I believe that some glutamate receptor blockers can be used to prevent the excessive influx of calcium into neurons via the NMDA receptors and further inhibit the cascade of reactions which cause cell death based on the general mechanism of action. In this case, selective NMDA blockers would be of much importance in accordance with the calcium entry. A previous study showed that NMDA antagonists such as Morphinan DX blocked glutamate induced toxicity (Choi *et al*., 1987). Another study done by Choi *et al*. (1988) has shown that APV in different concentrations provides protection against glutamate induced toxicity in cortical cultures. Choi *et al*. (1988) showed that influx of calcium through over activation of NMDA receptors was the major reason for cell death in cortical cultures. Therefore, we used APV, instead of the berry extracts, to determine the contribution of the NMDA receptor type to cell damage. Based on a literature review, we selected two doses of APV which were 30 µM and 100 µM. As previously seen, treatment of cultures with 100 µM glutamate caused a significant amount of cell death. Treatment of cultures with APV in the presence of glutamate gave us positive results and the cell death caused by 100 µM glutamate was suppressed to an extent. Treatment with only APV showed a trend towards cell death when compared with control levels. Unfortunately none of our data on NMDA receptor blockade was significant so we cannot justify that berry extracts provide protection against glutamate excitotoxicity solely by blocking NMDA receptors. A study reported that a water soluble extract from *Celastrus paniculatus* inhibited oxidative stress in rat cortical cultures produced due to glutamate by altering the function of glutamate receptors (Godkar *et al*., 2004). A recent study showed that a compound from a plant called *Psoralea corylifolia* blocked the cell death associated with retinal damage by controlling oxidative stress which was due to NMDA activation, calcium influx and a cascade of reactions leading to cell

death (Kim *et al*., 2013). However, NMDA receptor blockade may be one of many mechanisms of protection provided by the berries because as per our results APV + glutamate did suppress cell death to a certain level. Therefore, future studies are aimed at analyzing the threshold of dietary consumption of berry products and potential neuroprotection. Also, they should be aimed at isolating the individual compounds in the extracts and their quantification. These isolated compounds may be tested specifically for their protective effects against glutamate excitoxicity.

TBI is a form of damage to the brain initiated by an external force. The model used in our laboratory for injuring cells and mimicking the trauma created during TBI uses the 94A Cell Injury Controller, which creates a stretch induced trauma in mixed cultures. The stretch (31 %) caused by the injury model is equivalent to 5.5 mm deformation. Cell death induced by trauma was measured 24 hours later and cause a significant amount of cell death (more than 60% when compared to % of control). TBI is followed by oxidative stress which worsens the condition. Therefore, addition of antioxidants may reduce the stress levels observed in TBI as seen with the neuroprotective effects of oxyresveratrol from *Smilacis chinae* rhizome in rat brain cultures (Ban *et al*., 2006). A study performed by Weber *et al*., 2012 also showed the potential neuroprotective effects of oxyresveratrol isolated from *Morus alba* L. against TBI. Therefore, cells were injured with the stretch injury model alone or in the presence of blueberry fruit extracts in order to see whether the antioxidant rich blueberry fruits are beneficial. Studies have previously shown the neuroprotective effects of blueberry extracts (Yang and Jiang, 2010), and a plethora of literature is available about blueberry fruits and their neuroprotective effects (Giacalone *et al*., 2011). Treatment of cultures with blueberry fruit extracts immediately after stretch injury displayed neuroprotection of cells. To our surprise, there was no cell loss and the number of cells was almost equal to the % control of cells when cells were stretched in the

presence of extracts. Although we expected our extract to cause some neuroprotection, a complete protection was unexpected. Future studies involving specific polyphenols isolated from blueberry fruits, for example cyanidin-3-glucoside and their effects against TBI might give us the exact mechanism of action or pathways responsible for this protective effect.

Lactate is a by-product of glycolysis and is a biochemical indicator of anaerobic metabolism. Measurement of lactate from the mixed cultures after various treatment conditions would give an estimate of the metabolic changes and health status of the cells (Choi *et al*., 1988). When lactate levels decreased after 24 hour glutamate treatment, we expected that to be an indication of alteration in the metabolic state of cells. After treatment with glutamate in the presence of berry extracts, we observed a slight variation in the lactate levels. Interestingly however, the lactate levels for the extracts + glutamate were much closer to the control levels when compared with that of the glutamate treatment. These results suggest that measurement of lactate levels in different treatment conditions could give us a better idea regarding the condition of the cells, but unfortunately none of this data was statistically significant. However, it is possible that if different time frames were used for measurement such as 15 minutes or 48 hours, then lactate levels may be significantly altered. Measurement of the lactate/pyruvate ratio and pyruvate levels specifically could give us a better picture regarding the metabolic changes (Weber *et al*., 2012). An increase in concentration of glutamate and the extracts might also lead to a significant change in lactate levels. Measurement of lactate and pyruvate levels using microdialysis and high performance liquid chromatography (HPLC) has been used to determine metabolic changes in rat brain (Hallstrom *et al*., 1989). Other studies have shown that lactate levels were used for measuring metabolic changes in ischemic conditions (Hilliered *et al*, 1989). Therefore, future studies may involve measuring lactate and pyruvate levels and their

ratio at different treatment time frames in order to give a better indication about cell health.

Alpha-synuclein is a 140 amino acid containing protein which is located mostly in neurons. Over expression of this protein is considered to be a hallmark for various neurodegenerative diseases such as PD, AD and DLB. The actual mechanism of action of this protein to cause neurodegeneration is not very clear (Nemani *et al*., 2010). We believe that glutamate induced excitotoxicity or injury also leads to over expression of alpha-synuclein which may further be a secondary factor or a link to PD. We used an alpha-synuclein antibody immunohistochemistry staining technique for our experiments in order to detect the presence of alpha-synuclein in the cultures. Cultures injured by the 94A controller or treated with glutamate would cause a condition like TBI or glutamate excitotoxicity, respectively, based on our previous experiments in the lab. As expected after antibody staining with alpha-synuclein, MAP-2 and DAPI, we could capture some great images which represented injured or dying cells with over expression of alpha-synuclein (bead formation), neurons and all cortical cells respectively. These images helped us believe our prediction that glutamate or injury can lead to an increased expression of alpha-synuclein protein (Fig 3.11), although the exact reason for this over expression is unknown. It is not known what happens due to this increased expression of alpha-synuclein in glutamate treated or injured cortical cultures. Interestingly, a study showed that alpha-synuclein released due to induction by valproic acid could cause neuroprotection against glutamate induced excitotoxicity in rat cerebellar cultures (Leng and Chuang, 2006). So the actual relationship between glutamate excitotoxicity and alpha-synuclein is not clearly understood based on our experiments and the literature. The immunostaining experimental technique is a qualitative way of determining alpha-synuclein. The berry extracts were used in the presence of glutamate to determine its effect on alpha-synuclein expression. After the treatment some very good images were able to be captured which showed very few neurons

with alpha-synuclein and most other neurons looked healthy. This might be an indication that berry extracts have some effect on the levels of alpha-synuclein but a definitive comment cannot be made at this point regarding exactly what occurs. However, an increased level of expression of alpha-synuclein in cortical cultures after injury or glutamate treatment is certain. Future studies involving other more quantitative techniques such as Western blot analysis would be a better way to more specifically measure increased expression of alpha-synuclein after injury or glutamate treatment.

CONCLUSIONS

Overall, the findings suggest that consumption of blueberries and lingonberries could have a positive effect on the human brain. The high polyphenolic content and antioxidant capacity of these two species could be potentially beneficial for the prevention of disorders such as stroke and TBI, and I have provided further evidence that these species can be beneficial for these brain disorders. Studies have shown that a diet rich in fruits and vegetables, as well as blueberry specifically would be very healthy for the brain. An intake of a small quantity of blueberries daily (approximately one cup) by humans could be very beneficial for neuronal age deficits (Galli *et al*., 2002). It is possible that the consumption of fresh or frozen berries or a tea made from the leaves of the plants, as well as dietary supplements produced from the extracts of the fruits and/or leaves could slow brain aging or inhibit the development of neurodegenerative disorders. Therefore, ingestion of berries or supplements made from leaves could possibly increase the antioxidant and antinitrosative capacity of the brain. Other studies have shown that rats fed a diet enriched with blueberries can protect the brain against oxidative stress (Duffy *et al*., 2008) as well as stroke (Wang *et al*., 2000; Sweeney *et al*., 2002), which suggests that the outcome from such typically debilitating disorders can be improved if the antioxidant levels in the brain are increased through diet. Therefore, future studies should involve performing detailed chemical analysis of these extracts and quantification of the isolated compounds to get a better idea about the individual compounds which are present in a high concentration. Toxicological studies specifically carried out on berry leaves could be very helpful as leaves were found to be very potent and are considered to be toxic in general when compared with the berry fruits. Lactate measurements could act as a marker for the health of the cells depending on the time-point of measurement. Lastly, antibody staining to alpha

synuclein demonstrated expression of this protein in neurons, which is associated with both glutamate treated and injured cells, but further experiments are needed in order to determine the effects of berry extracts on alpha-synuclein expression.

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