

Chemical Analysis and Neurobiological Effects of Newfoundland Wild Blueberries

by

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Abstract

Antioxidants may be beneficial for the treatment of certain neurological diseases since oxidative stress and inflammation are believed to contribute to neurological degenerative disorders. We performed an analysis of the biochemical attributes and neurobiological activity of extracts found in *Vaccinium* species. Wild blueberries, native to Newfoundland and Labrador (NL), were collected from different locations. Biochemical assays were performed to determine the phenolic content of extracts and antioxidant capacity. To quantify and identify major anthocyanins in extracts, High-Performance Liquid Chromatography Mass-Spectroscopy analysis was performed. Microglial cells isolated from mouse brains were treated with glutamate or α -synuclein to induce inflammatory responses, and treatment with extracts was conducted to assess neuroprotective effects. Results showed that wild blueberry fruits and leaves native to NL are high sources of antioxidants. Leaves have an overall significantly higher level of antioxidants compared to the fruits. Utilizing HPLC-MS, more anthocyanins were identified in blueberry fruit than leaves, but the anthocyanins in leaf extracts were present in higher quantity. Cell culture experiments demonstrated the preventative role blueberry fruits and leaves had on activated microglial cells mimicking conditions in the neurodegenerative conditions. Overall, findings showed that both fruit and leaf extracts significantly reduced the inflammatory response of brain cells seen in certain neurodegenerative disease.

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List of Symbols and Abbreviations

Ab: Antibody

A β : Amyloid beta

AD: Alzheimer's disease

AIS: Alcohol insoluble solids

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

APP: Amyloid precursor proteins

BS: Bovine serum

BSA: Bovine serum albumin

CHO: Carbohydrate

CE: Catechin equivalent

CNS: Central nervous system

CO₂: Carbon dioxide

CD-4: Cluster of differentiation-4

DAD: Diode array detector

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

dH₂O: Distilled water

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DPPH: 2, 2-diphenyl-1-picrylhydrazyl

DTT: Dithiothreiol

ER: Estrogen receptor

ESI: Electrospray ionisation

FCS: Fetal calf serum

FeCl₃: Ferric chloride

G: Gravitational force

GAE: Gallic acid equivalents

GFAP: Glial fibrillary acidic protein

H/DIC: High magnification differential interference contrast

HPLC-MS: High performance liquid chromatography mass-spectroscopy

HSC: Health Sciences Centre

IL-1 β : Interleukin-1 beta

IL-6: Interleukin 6

MHC: Major histocompatibility complex

MUN: Memorial University of Newfoundland

m/z: mass/charge

NADPH: Nicotinamide adenine dinucleotide phosphate

NEM: N-ethylmaleimide

NL: Newfoundland and Labrador

NMDA: N-Methyl-D-aspartate

NO: Nitric oxide

PBS: Phosphate buffer solution

PD: Parkinson's disease

PFA: Paraformaldehyde

PLL: Poly-L-Lysine

PLO: Poly-L-Ornithine

RBP4: Retinol binding protein 4

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

SE: Standard error

SNpc: Substantia nigra pars compacta

TAA: trichloroacetic acid

TBI: Traumatic brain injury

TBS: Tris-base saline

TFC: Total flavonoid content

TNF- α : Tumour necrosis factor alpha

TPC: Total soluble phenolic content

TTC: Total tannin content

Chapter 1: Introduction

1.1 General

In order to treat, prevent, or slow down the progression of neurodegenerative diseases associated with aging of the brain, rich amounts of antioxidants such as polyphenols found in *Vaccinium* berry species have been reported to be an effective alternative or addition to medicinal products (Chu *et al.*, 2011). Over the years, polyphenols have been credited a lot of attention due to their ability to reduce stress from oxidation and inflammation (Li *et al.*, 2014), and the possible benefits they could exert on human health in the form of nutraceuticals. An extensive analysis of Newfoundland and Labrador (NL) blueberry leaves and fruits has yet to be conducted, therefore, it is important to analyze the neurobiological activity and biochemical constituents of compounds detected in *Vaccinium* species found here in the province. A significant portion of the research in our lab focuses on locally grown berries native to NL, their constituents, and how both the fruits and the leaves affect the neurological activity of specific brain cells. According to Hirsch & Hunot (2009), many of the neurodegenerative conditions show neuro-inflammation, which is an inflammation that occurs in the brain and is a contributor to the insults in the central nervous system (CNS). Microglia, also known as the brains immune cells (Saura *et al.*, 2003), are believed to play an important role in neuro-inflammation. In specific neurodegenerative diseases, clusters of the protein α -synuclein, and excessive levels of the neurotransmitter glutamate are often found in the brain (Stefanis; 2012, Choi; 1988). Recent studies from our research group have shown that native lowbush blueberry fruits and leaves collected in NL protect rodent brain cells exposed to high levels of glutamate (Vyas *et al.*, 2013b). Therefore, it is to our

interest to further research the *in vitro* models of neuro-inflammation in microglial and neuronal cells treated with glutamate and α -synuclein.

1.2 Nutraceuticals

Botanical nutraceuticals or drugs can be defined as materials that are plant derived with medicinal benefits to prevent or treat disease and exceed nutritional requirements (Raskin *et al.*, 2002). Plants are considered a low-budget and safe alternative to synthetic sources to make nutraceutical products. Studies have shown different types of neuroprotective properties these supplements have contributed based on their variety at a metabolic and biochemical level (Mecocci *et al.*, 2014). These include dietary supplements as well as synthetically made products from natural food sources. Studies on nutraceuticals are still emerging, being a relatively new area of research, even though natural derivatives of dietary products have been used for centuries as therapeutic agents.

The use of nutraceuticals may decrease and prevent incidences of certain neurological conditions, along with increasing the quality and longevity of life without depending on expensive medication (Rahman, 2003). It is important to identify the components of plants that can benefit and improve neurological functions of human brain, as these natural sources of therapeutic agents have potential in preventing and slowing down the progress of neurodegeneration. For example, resveratrol, which is a non-flavonoid polyphenol, has shown positive effects on *in vivo* models of neurodegeneration (Pirola & Frojdo, 2008). The sources of resveratrol include the seeds and skin of fruits such as grapes. Xu *et al.* (2010) showed in animal models that resveratrol increased the level of the neurotransmitter serotonin in the hippocampus, which is an important

component in mood enhancement. It was found that resveratrol inhibited noradrenaline and specific serotonin re-uptake in rats. This study indicates the antidepressant role resveratrol can play in the CNS. Another type of polyphenolics known as flavonoids, have also counteracted cognitive decline associated with age (Beking & Vieira, 2011). Due to this type of evidence, flavonoid-rich dietary interventions have emerged, including the tea *Camellia sinensis* (Chan *et al.*, 2006) and the cocoa *Theobroma cacao* (Dinges, 2006), as well as blueberry supplements (Joseph *et al.*, 1999). These flavonoid enriched foods and beverages have been shown to have a beneficial effect on learning and memory. The catechins found in green tea (Li *et al.*, 2009) and blueberries (Andres-Lacueva *et al.*, 2005) have been suggested to preserve memory and learning via kinases and transcription factors that play a key role in neurogenesis (Aberg *et al.*, 2000), which can be defined as the procedure of generating neurons that are functional from other precursors (Ming & Song, 2005).



Figure 1.1: Lowbush wild blueberry species (*Vaccinium angustifolium*).

1.3 Blueberry

The lowbush wild blueberry species used in this study, known as *Vaccinium angustifolium* (Figure 1.1), is known to be one of the richest sources of phenolic compounds (Francis, 1989), with one of the highest contents of antioxidants of a variety of fruits and vegetables tested (Camire, 2000). The awareness of blueberry health benefits has expanded the blueberry industry and its products worldwide. Blueberries are now found as fresh, frozen, processed, dried, canned and in preserved forms (Routray & Orsat, 2011). Fresh blueberries, in general, consist of 84% water, 9.7% carbohydrates, 0.6% protein and 0.4% fat (Michalska & Lysiak, 2015). Blueberries are good sources of polyphenols (Seeram, 2006). Huang *et al.* (2012) showed that the dry weight of blueberries contained 9.44 mg gallic acid/g of total phenolic content and a total flavonoid content of 36.08 mg rutin/g, which was the highest when compared to other berry fruits. The lowbush blueberries are also noted for their higher level of *in vitro* antioxidant capacity in comparison to highbush blueberries that have been cultivated (Kalt *et al.*, 1999a). External factors such as timing of harvest play an important role in the content of antioxidants found in blueberries. For example, depending on the cultivar and how mature the berries are, the outer layer thickness of a blueberry can differ greatly. Polyphenols are mostly located directly underneath the epidermis, in varied amounts (Lee & Wrolstad, 2004). It is important to analyze the different biochemical components of blueberries, as outlined in Figure 1.2.

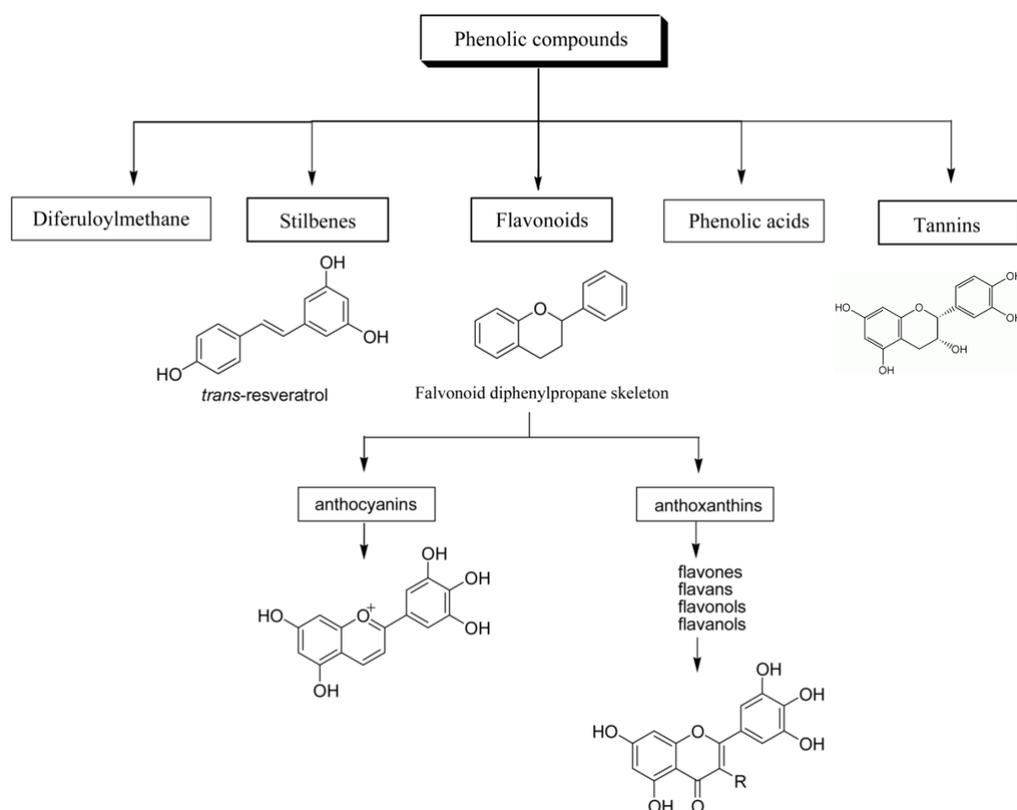


Figure 1.2: Classification and structures of polyphenols.

Adapted from “Dietary Polyphenols and Their Biological Significance” by Han *et al.*, 2007.

1.3.1 Phenolics

The structure of a phenolic compound consists of aromatic rings with one or more hydroxyl group attached (see Figure 1.2). There are more than 8,000 known phenolic structures, ranging from tannins, which are polymerized, to simple molecules known as phenolic acids (Dai & Mumper, 2010). Different classes of polyphenols exist based on their number of phenol rings and what structural element is present for binding them together (D’Archivio *et al.*, 2007). Secondary metabolites, which are organic compounds

such as terpenes, phenolics and nitrogen containing compounds, play a key role in defending plants against ultraviolet radiation and parasites (Schafer & Wink, 2009). Since phenolics are present in most of the plant organs, they constitute a major part of plant foods such as fruits and vegetables, and beverages such as tea and wine (Dai & Mumper, 2010).

1.3.2 Flavonoids

Flavonoids are polyphenols that make up a large portion of secondary metabolites, and the major flavonoids found in berries are flavonols and anthocyanins (Cho *et al.*, 2004). They consist of 15 carbon atoms and have six subgroups due to their structural variations, according to how the central C-ring is oxidized. They are the largest group of phenolic compounds that occur naturally, which act as antioxidants and protect cells from various forms of cell degeneration (Sulaiman & Balachandran, 2012). The common carbon skeleton found in flavonols include a diphenyl propane and two benzene rings joined together by a three carbon chain that is linear (D'Archivio *et al.*, 2007). Some common flavonoid includes catechin, measured as catechin equivalent (CE), which is found in tea, and cyanidin-glycoside, which is an anthocyanin of rich quantity found in berry fruits (D'Archivio *et al.*, 2007). Catechin and cyanidin-glycoside are often used as a known standard when quantifying phenolics. Evidence suggests that consuming flavonoids from plant derived sources could provide protection from many adverse health conditions, including heart disease (Knekt *et al.*, 1997) and stroke (Keli *et al.*, 1996).

1.3.3 Tannins

Another group of polyphenols found in the diet are tannins, which are highly polymerized, and consist of two types. The first type is hydrolysable tannins, which contain glucose and another polyol in the center, esterified with either Gallic acid or hexahydroxyphenic acid (Kashiwada *et al.*, 1992). The second type is condensed tannins, which are also known as proanthocyanidins due to the fact that they could be broken down to anthocyanin through an acid-catalyzed oxidation reaction (Dávalos & Lasunción, n.d.). The variety in tannin structurally allows for many oxidative linkages to be formed (Koleckar *et al.*, 2008). They play a role in the astringent characteristics found in fruits such as berries and beverages such as tea. Since proanthocyanidins have high molecular weight, there is potential that their absorption through the human gut may be limited (D'Archivio *et al.*, 2007).

1.3.4 Anthocyanins

Anthocyanins are one of the six subgroups of plant polyphenols of flavonoids, also responsible for the colours found on blueberries (Veberic *et al.*, 2009), specifically the red, blue and purple colour of the berries (Dai & Mumper, 2010). The colour is dependent on the pH; red at a pH < 2 as flavylium anhydride forms, changing to a blue colour as the pH increases and quinone-type anhydro bases form, and becoming colourless at a high pH due to extreme alkaline conditions causing the pigment to break down (Chu *et al.*, 2011). They are mainly found on the skin of fruits, where the anthocyanin content increases as the fruit becomes riper. Anthocyanins can scavenge free-radicals, chelate trace metals, and reduce deoxyribonucleic acid (DNA) oxidation

and degradation of lipids (Hosseinian & Beta, 2007). The anthocyanins found in the leaves function as a defense mechanism against photoinhibition (Hoch *et al.*, 2001). Photoinhibition occurs when photosynthetic capacity decreases due to excess energy of carbon reduction that is utilized due to photosynthetic tissues being illuminated (Powles, 1984). The anthocyanin levels accumulating in berries are influenced by a variety of factors. For example, in two consecutive harvest times, it was observed that anthocyanin levels fluctuated up to 30% (Kalt *et al.*, 1999b). In some parts of the world, blueberry anthocyanins are produced as a food colorant (Espin *et al.*, 2000). Anthocyanins are water soluble, and occur as glycosides of their aglycone form, known as anthocyanidin (D'Archivio *et al.*, 2007). The content of anthocyanin can depend on environmental factors such as temperature, the level of nitrogen and phosphorous in the soil, and the amount of solar radiation it experiences (Chu *et al.*, 2011). Blueberries contain levels of around 4000 parts per million of a combination of anthocyanidins, including delphinidin, malvidin, petunidin, cyanidin and peonidin (Aiyer *et al.*, 2011a), as identified through mass spectrometry. Cyanidin is responsible for the red and purple colors, and delphinidin is responsible for the blue colours (Hosseinian & Beta, 2007). Lowbush blueberries contain anthocyanins in both acetylated and non-acetylated forms (Routray & Orsat, 2011).

Methods to quantify phenolic compounds in extracts from plants can depend on the chemical characteristic of the analyte, the standards selected and the effect interfering substances have on the phenolics (Naczki *et al.*, 2006). According to Aiyer *et al.* (2011b), berries have phytochemicals that interfere with some of the pathways that can lead to cancer development. The estrogen receptor (ER) signalling pathway, according to Clarke

et al. (2001), plays a key role in breast cancer. In breast cancer diagnostics, over 70% of breast tumours detected are ER positive cells (Clarke *et al.*, 2001). Antioxidants, specifically the anthocyanidins in berries, can lower the level of estrogen. This is done by binding to the ER in breast cancer cells, which reduces 17 β estradiol (E2)-induced estrogen response elements-luciferase expression (Schmitt & Stopper, 2001), although the dosage, type of cell and specific anthocyanidin involved still needs to be determined. According to Sasaki *et al.* (2007), the anthocyanin; cyanidin-3-glucoside, improved cases of hyperglycemia by reducing the expression of retinol binding protein 4 (RBP4) in mouse white adipose tissue, of type 2 diabetes. This was also accompanied by the reduction of tumour necrosis factor alpha (TNF- α) in the white adipose tissue. Talavera *et al.* (2005), found that the anthocyanin, cyanidin 3-glucoside was capable of crossing the blood brain barrier in a rat diet study.

1.4 Antioxidants and oxidative stress

Oxidative stress is a condition that can occur when there are excessive free radicals, such as reactive oxygen or nitrogen species that overcomes antioxidant capacity (Dai & Mumper, 2010). When an imbalance occurs between free radical production and the defences of antioxidants, oxidative stress results and can be associated with damaging key components in the body including lipids, nucleic acids and proteins (McCord, 2000). Free radicals are molecules that have an unpaired electron and can exist independently. Many of these free radicals can donate or accept electrons from other molecules (see Figure 1.3), allowing them to function as either reductants or oxidants (Cheeseman & Slater, 1993). Many free radicals are noted to be highly reactive and capable of damaging

molecules such as DNA, lipids, carbohydrates (CHO) and proteins in the cellular nucleus and membrane (Young & Woodside, 2001). Oxidative stress and mitochondrial impairment are now considered to be one of the main underlying mechanisms contributing to neurodegeneration (Sanders & Greenamyre, 2013).

A stable molecule that can donate an electron to a free radical and neutralize it is called an antioxidant. They can do this by acting as a metal chelator, scavenge free radicals, inhibit enzymes and donate hydrogen or electrons (Frie *et al.*, 1988). Antioxidants function to inhibit or delay damage caused to the cells by scavenging free radicals (Halliwell, 1995). Natural sources of antioxidants such as polyphenols are known to scavenge reactive oxygen species (ROS) and improve the antioxidant system in cells, therefore they are considered potential candidates to be neuroprotective agents (Kelsey *et al.*, 2011). Studies have shown that blueberry extracts containing rich polyphenol contents prevented oxidative stress due to hypobaric hypoxia (Zepeda *et al.*, 2013), which shows potential for people undergoing conditions that lead to oxidative stress. Antioxidants from both natural and synthetic sources are added to medicine and food containing oils and fats to protect them against oxidation, but consumer preference favored antioxidants of natural sources that have lower volatility and higher stability in comparison to synthetic antioxidant products (Papas, 1999). Some of the proposed mechanisms of how the antioxidant properties of phenolics can be mediated include scavenging radicals, such as ROS and reactive nitrogen species (RNS), as well as inhibiting enzymes or chelating metals that are involved in production of free radicals and upregulating the defense mechanism of antioxidants (Cotelle, 2001).

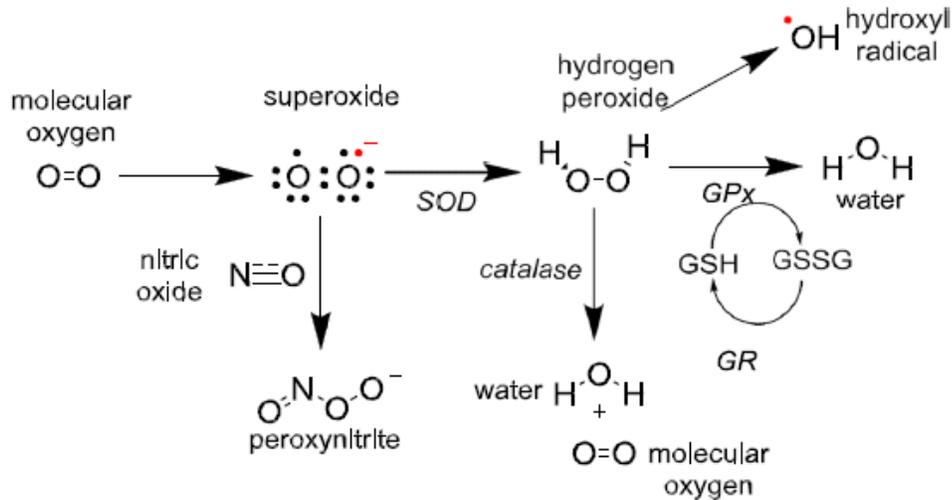


Figure 1.3: ROS and RNS pathway in mammalian cells. Reprinted from “Antioxidants and free radical scavengers for the treatment of stroke, traumatic brain injury and aging” by Slemmer, J. E., *et al.*, 2008.

1.5 Microglia activation in the central nervous system

The CNS has two main components, the brain and the spinal cord, which are responsible for interpreting sensory input and sending out commands for motor response based on current conditions, reflex and past experience (Marieb & Hoehn, 2007). The brain is composed of different cells, including neurons, astrocytes, oligodendrocytes, glia and microglia (Temburni & Jacob, 2001). Microglia are ovoid shaped cells that contain long processes that function as the immune cells of the CNS (Ghosh, 2007). When the brain is in a healthy state, microglial cells are described to be resting, while activated microglia show structural changes such as motile branching (Stence *et al.*, 2001). Approximately 12% of brain cells are comprised of myeloid cells, which further develop

into microglia (del Rio Hertege, 1932). During a healthy state, these cells are highly ramified with a low profile phenotype in the adult brain (Sierra *et al.*, 2010). When alterations in the environment occur causing damage to the neurons, microglial cells show signs of morphological change. They proliferate and become phagocytic (Saura *et al.*, 2003). This process is known as microglial activation, which is a response to protect the neural tissues (Kreutzberg, 1996). Activation can cause programmed cell death during brain development or when there is an injury in the CNS (Perry *et al.*, 2010). It has been suggested that microglial over activation could result in increased neuronal damage during neurodegenerative diseases (Lull & Block, 2010). There is also evidence suggesting that microglia are involved with other glial cells and neurons during activation (Stoll, 1999). Since this can result in producing neurotoxic species, if microglial cells are chronically activated, they could contribute to the pathogenesis of disorders such as Alzheimer's disease (AD). Once activated, microglia can also display abnormal functional plasticity and transform phenotypically to macrophage-like similarities (Ghosh, 2010). According to Shukitt-Hale *et al.* (2008), when rats consumed a 2% blueberry diet over the span of 8 weeks after using kainic acid to induce microglial activation, cognitive performance had improved significantly while microglial activation decreased.

1.5.1 The role of reactive oxidative speceis and cytokines in neuro-inflammation

Neurotoxic ROS are produced by microglia through stimulating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity (Block *et al.*, 2007b). The polyphenols in blueberries influence activation of microglial cells both *in vivo* and *in vitro*. A study looked at the effect of blueberries on lesions on the brain, specifically, the

subcortical nuclei called the basal nuclei/ganglia. These basal ganglia are present within the cerebral white matter of the brain (Marieb & Hoehn, 2007). The corpus striatum of the basal ganglia was induced to cause lesions in a rat study, which caused the activation of microglial cells. Once these rats were given a diet enriched in blueberries, results showed a reduction in activated microglia within the brain region (Stromberg *et al.*, 2005). Morphological changes in microglia include producing neurotrophic and neurotoxic factors such as TNF- α and ROS (McGeer *et al.*, 2002).

During neuro-inflammation, microglial cells are activated and pro-inflammatory factors (Pocock and Liddle, 2001), also known as cytokines, are released. Cytokines are small proteins that have various functions, including cellular communication; released for signalling (autocrine and paracrine) and regulating cell growth and survival (Hanisch, 2002). Pro-inflammatory factors released by activated microglia include cytokines such as interleukin-1beta (IL-1 β), and free radicals such as nitric oxide (NO) and superoxide, and studies have shown that when many of these factors are produced in excess, neurons can become damaged (Boje & Arora, 1992; McGuire *et al.*, 2001). In the CNS, two key cytokines important for this study are TNF- α and interleukin-6 (IL-6). Cytokines influence the hormonal release from the adrenal or pituitary glands, which can cause activation of the endocrine axis by means of the hypothalamic nuclei (Turnbull & Rivier, 1999). Functions necessary for homeostasis, such as temperature control and food consumption, are targets for actions produced by cytokines (Vitkovic *et al.*, 2000). By releasing cytokines and other mediators, microglia could also influence communication between neurons of the hypothalamus and the neurosecretory cells in the signalling pathways between microglia and neurons (Stern & Filosa, 2013).

TNF- α is a ligand belonging to the TNF family, which is also known as cachectin (Idriss & Naismith, 2000). TNF- α is a product of dendritic cells, monocytes/macrophages or lymphocytes. TNF- α is an important part of inflammation and pathological conditions such as neurological pain, especially after injury (Hanisch, 2002). Neurological or neuropathic pain is recognized when nociceptive damage prolongs past the level of damage to the actual nerve and tissue surrounding it (Leung and Cahill, 2010). TNF- α mediated glutamate release has been observed from astrocytes (Bezzi *et al.*, 2001). A nonpolar blueberry fraction blunts NADPH oxidase activation in neuronal cells exposed to TNF- α . NADPH-oxidase can cause oxidation in human neuroblastoma cells that has been exposed to TNF- α (Chakraborty *et al.*, 2010). When treated with 5 μ g/ml of blueberry extract, research showed that oxidation levels reduced to almost control levels by inhibiting the assembling of NADPH oxidase enzyme (Gustafson *et al.*, 2012).

Expression of cytokines, along with microglia activation in the hippocampus, has resulted in the inhibition of proliferating neural precursors during neurogenesis (Ekdahl *et al.*, 2003). This could be due to soluble factors such as IL-6 being released (Monje *et al.*, 2003), since the elevation of IL-6 from activated microglia has been associated with the disruption of neural maturation and division (Ekdahl *et al.*, 2003). IL-6 is a pro-inflammatory cytokine that works to coordinate an inflammatory response in order to limit infectious disease from spreading. In the CNS, IL-6 is involved in sleep, increasing pain perception and inducing fevers. During early insults within the CNS, microglia tend to primarily release IL-6 (Raivich *et al.*, 1999). There is evidence to suggest that the immunologic response after traumatic brain injury (TBI) or stroke could be due to the release of cytokines as well, such as TNF and interleukins (Morganti-Kossmann *et al.*,

1992; Stelmasiak *et al.*, 2000). Since microglia are very sensitive to changing environments, their response to any form of infection or injury is to rapidly become activated. Once activated, they can upregulate surface receptors such as major histocompatibility complex (MHC) (Kreutzberg, 1996). The role microglial cells play in immune surveillance is important, as they present antigens of MHC class II that are expressed to cluster of differentiation-4 (CD-4) positive T cells, and have similar antigens with hematopoietic macrophages (Fedoroff *et al.*, 1997).

1.5.2 Pathological conditions associated with microglial activation

Aspects of cognition that are influenced by nutrition include many neurological disorders, such as AD and Parkinson's disease (PD), (Associate Parliamentary Food and Health Forum, 2008). Specific nutritional components such as antioxidants, have been considered one approach for slowing down the progression of AD (Swaminathan & Jicha, 2014). Initially based on a post-mortem analysis of the brain of patients with AD and PD, it was suggested that microglial activation was involved in these neurodegenerative diseases (Liu & Hong, 2003).

According to Arendt (2001), people with AD show unusual production levels of amyloid precursor proteins (APP), which progresses into amyloid beta (A β) aggregation forming plaques which is a predominant hall mark of AD. Evidence suggests that activated microglia were present in the cortical regions of AD brains, co-localizing with neuritic plaques (Rogers *et al.*, 1988). According to Paulson *et al.* (2008), this disease is characterized by neurofibrillary tangles, synapse loss, and the hallmark trait of A β peptide deposits. The A β peptides stimulate microglia, which produces factors such as TNF- α

which causes a neurotoxic cascade which tends to recruit more microglia (Hanisch, 2002). The brains of AD patients have shown a large amount of inflammation, where cytokines such as IL-6 and TNF- α have been detectable in the brain tissue, postulated to cause neuronal structure loss (Akiyama *et al.*, 2000). Also, patients under nonsteroidal anti-inflammatory drug treatment were observed to have a lower prevalence of AD (McGeer & McGeer, 1999; Akiyama *et al.*, 2000). Cytokines such as IL-1 and TNF- α causing inflammation can further increase the progression of AD (Perry *et al.*, 2001), which shows the relationship between the pro-inflammatory factors released by microglial cells and the development of AD.

PD is characterized by symptoms such as tremor and rigidity (de Lau & Breteler, 2006). The pathological characteristics of PD include the dopaminergic neurons decreasing in the substantia nigra *pars compacta* (SNpc) and activated macrophages (Lowe & Leigh, 2002), along with Lewy bodies, which are inclusion bodies present intracellularly, surrounded by α -synuclein aggregates (Matsumoto *et al.*, 2010). Early onset and familial PD is seen in a small fraction (<5%), and most clinical symptoms and sporadic cases of PD usually progress over the later decades (Olanow & Tatton, 1999). In PD, activated microglia and other inflammatory mediators are shown to surround nigral aggregates, which are immunoreactive to α -synuclein (Yamada *et al.*, 1992). Pro-inflammatory cytokines could also play a role, as Wahner *et al.* (2007) found that the risk of developing PD doubled for patients if they carried the homozygous variant genes of TNF- α position -308, and the susceptibility of PD could be influenced by the polymorphism of the TNF- α gene.

1.5.3 Inducers of neuroinflammation: α -synuclein and glutamate

α -synuclein is part of the family of synuclein neuronal proteins that localize predominantly to the presynaptic terminals (George, 2002), which is found in the pathogenesis of PD and other disorders related to neurodegeneration. PD patients have shown accumulation of nigral aggregates and Lewy bodies, immunoreactive to the protein α -synuclein, which were often present and surrounded by activated microglia (Yamada *et al.*, 1992). Lewy bodies are cytoplasmic inclusions that are characterized in PD patients. It has been suggested that microglia activation in PD patients could be due to α -synuclein aggregates released from dopaminergic neurons that are dying, which could potentially initiate inflammation in order to eliminate α -synuclein that is released (Zhang *et al.*, 2005).

Glutamate is an important excitatory neurotransmitter in the mammalian nervous system, and increases during conditions such as TBI *in vivo* (Palmer *et al.*, 1994). According to Purves *et al.* (2001), glutamate is a neurotoxic substance, which acts on metabotropic and ionotropic receptors. These include Kainate and 2-amino, 3-(3-hydroxy-5-methylisoxazol-4-yl) propionate (AMPA) receptors, all non-selective cation channels that allow K^+ , Na^+ and sometimes Ca^{2+} to pass through (Purves *et al.*, 2001). Glutamate also acts on neurons via N-Methyl-D-aspartate receptor (NMDA) receptors which results in an increased calcium influx and cell death (Blanke & VanDongen, 2009). Neurotoxicity mediated by glutamate has been observed in neurodegenerative conditions such as brain injury (Hanisch & Kettenmann, 2007). Since glutamate is known to activate receptors such as calcium permeable ion channels (Weber, 2012), cell cultures derived from rodent brains have undergone glutamate induced toxicity, where these neurons and

glial cells were damaged by an increased stimulation from neurotransmitters such as glutamate (Vyas *et al.*, 2013). This process mimics the pathology of neurodegenerative disorders by inducing nitrosative and oxidative stress (Slemmer *et al.*, 2008). Excess glutamate, produced due to synaptic activity is usually taken up by astrocytes under normal conditions. When certain pathological conditions occur, the uptake of glutamate by astrocytes is impaired (Persson *et al.*, 2005). The term “excitotoxicity”, which was labelled by Olney (1969), is the process of glutamate being released in large amounts and overstimulating amino acid receptors that are excitatory. This excessive glutamate release is observed in cell death and injury of the CNS (Lau & Tymianski, 2010). After injury, there can be an elevation in calcium due to leakage via damaged cell membranes or voltage gated calcium channels being activated (Young, 1992). When free calcium levels are increased, it can cause the activation of enzymatic reactions which can eventually cause cell death (Wojda *et al.*, 2008), and this may be an important mechanism that influences secondary damage to neurons after insult to the brain (Weber, 2012).

1.6 Hypotheses:

Based on biochemical assays and high performance liquid chromatography mass-spectroscopy (HPLC-MS) analysis for blueberry fruits and leaves, I hypothesize that blueberry leaves will have higher amounts of polyphenolics (flavonoids, tannins) and anthocyanins than fruits. I also hypothesize that due to past literature supporting the ability of anthocyanins to cross the blood brain barrier, blueberry fruits and leaves will have a neuroprotective effect on microglia cells by reducing the amount of activation induced by glutamate and α -synuclein, and by sustaining healthy microglia cells.

1.6 Objectives of the study

The purpose and aims of this research project are:

- To conduct extractions and assays of blueberry fruits and leaves from seven different locations within St. John's and the surrounding area in order to investigate overall levels of polyphenols and their biochemical activity.
- To perform a pilot study to determine if polyphenols, specifically the anthocyanins in blueberries can cross the blood brain barrier of mice at a detectable level.
- To quantify and identify major anthocyanins detected in blueberries and mouse tissue using HPLC-MS.
- To determine the potential neuroprotective effect that blueberries have on the inflammatory response mediated by microglia, after induction with glutamate or α -synuclein.

Chapter 2: Materials and Methods

2.1 Plant extraction procedure

2.1.1 Collection and storage

For this project, the material used for fruit and leaf studies included wild lowbush blueberries (*Vaccinium angustifolium*). The blueberry fruit and leaf samples were collected from seven different sites, including Pippy Park, Signal Hill, Johnson Geo Centre, Fort Amherst, Blackhead, Marine Drive and Middle Cove in NL during the month of September, 2014 (See Figure 2.1 and Table 2.1). Blueberry fruits and leaves were harvested and stored at -20°C within the first hour of collecting, then after 40 minutes, transferred to a -80°C freezer for storage to prevent degradation of phenolic compounds, since past literature found that phenolic content can remain the same or even increase in cold storage, depending on the cultivar (Connor *et al.*, 2002).

Table 2.1: Geographical information on collection sites of Newfoundland blueberry fruits and leaves

Location	Legend from Figure 2.1	Latitude	Longitude
Signal Hill		47.5701° N	52.6819° W
Johnson Geo Center		47.5730° N	52.6900° W
Blackhead		47.5299° N	52.7094° W
Fort Amherst		47.5635° N	52.6825° W
Pippy Park		47.5824° N	52.7333° W
Marine Drive		47.6622° N	52.7097° W
Middle Cove		47.6404° N	52.6865° W

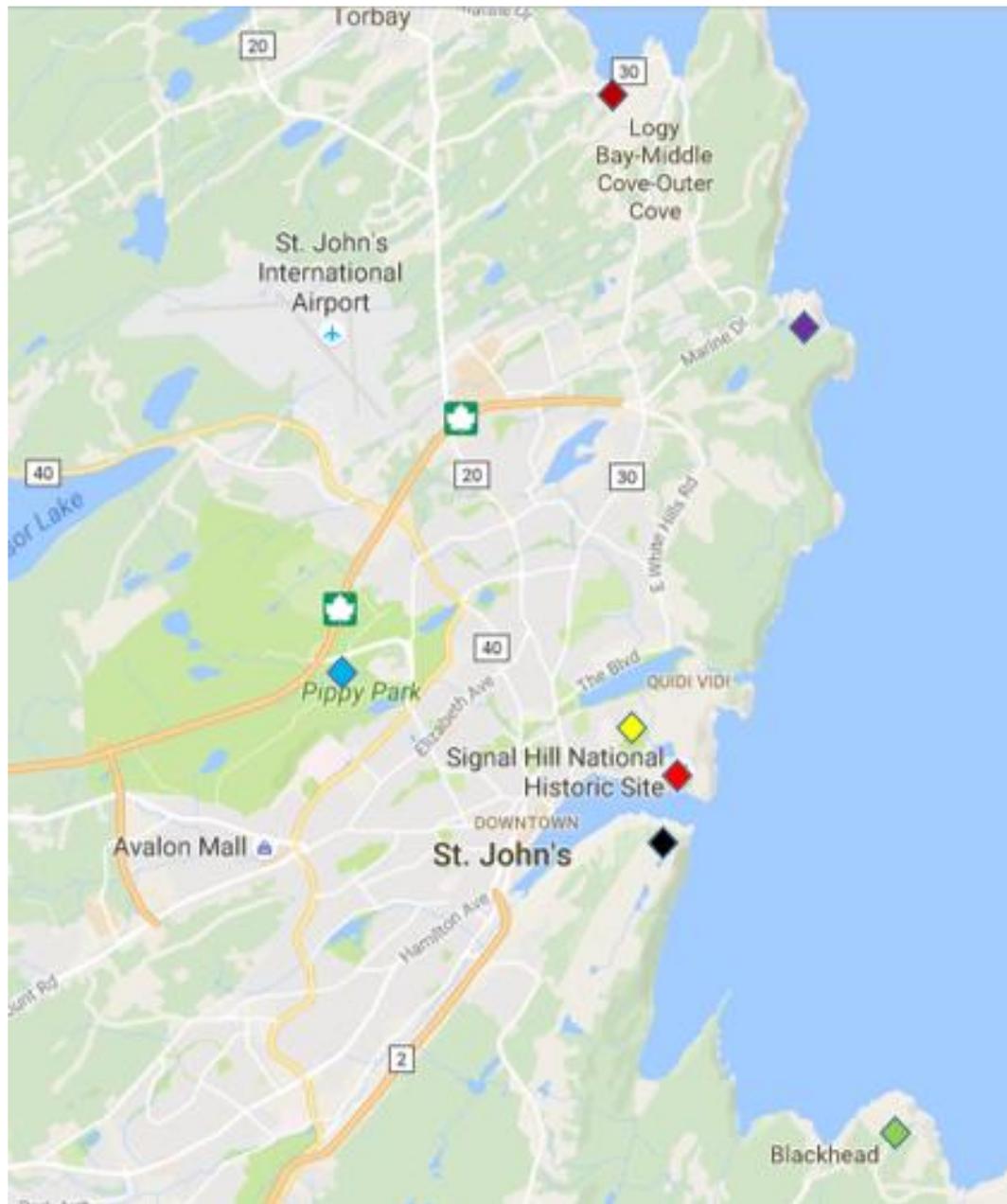


Figure 2.1: Collection sites of wild Newfoundland blueberry fruits and leaves

Image modified from Google Maps (2016). Blueberry collection site represented by 

Legend is available on Table 2.1.

2.1.2 Fruit and leaf extraction to determine phenolic compounds

Fruit extraction

A stock solution was prepared consisting of 80% (v/v) acetone with 0.2% formic acid and 19.8% distilled water (dH₂O). Based on past literature, these solvents were determined to be the best extract solvent when compared to various mixtures of solutions (Vyas *et al.*, 2013a). Blueberry fruits were grinded using a mortar and pestle. Since weight of specific sample varied slightly, in order to have a consistent concentration, a stock solution was added to blueberry fruits at a ratio of 1:2. This mixture was then vortexed to homogenize, then shaken on ice for 30 minutes. The solution was then centrifuged at 20 000 G (gravitational force) for 20 minutes at 4°C and the supernatant was collected. The entire procedure was repeated using the residue pellets, then supernatants combined. The final concentration was further adjusted to give 250 mg/ml solvent of fresh weight, which is the weight of the specimen including its water content.

Leaf extraction

Liquid nitrogen was added to the leaf sample, followed by grinding to fine powder. Liquid nitrogen was used due to the efficiency of breaking down the tough cellulose structures in the leaves, compared to other methods of grinding the sample. The above stock solution was added to leaf samples at a ratio of 1:2, vortexed to homogenize, then shaken on ice for 30 minutes. The mixture was then centrifuged at 20 000 G for 20 minutes at 4°C and the supernatant was collected. The entire procedure was repeated using the residue, and then supernatants combined. The sample was then diluted, giving a final concentration of 25 mg/ml of fresh weight.

2.2 Blueberry biochemical assays

Leaf and fruit extracts were diluted an additional 10X for this portion of the experiment, to a final concentration of 25 mg/ml of fruit and 2.5 mg/ml of leaf fresh weight. All biochemical experiments were replicated three times.

2.2.1 Total soluble phenolic content

Colorimetric reactions using the Folin-Ciocalteu method was used to quantify the polyphenols in both fruits and leaves as outlined by Vyas *et al.* (2013b). The polyphenols react with Folin-Ciocalteu reagent, a redox reagent, to form a blue color complex that can be quantified by a spectrophotometer. This was done by using phenolate ion to reduce the phosphotungstate-phosphomolibdenum complex to change the colour to blue. When the sample extract consists of more phenolics, this reduction process increases, which causes the blue colour to become darker and increase the absorbance value (Arbianti *et al.*, 2007). 500 μ l of Folin-Ciocalteu reagent was added to 500 μ l of 2.5 mg/ml of leaf or 25 mg/ml of fruit sample and vortexed. In order to neutralize the reaction, 1 ml of saturated sodium carbonate solution was added to a final volume and was adjusted to 10 ml with dH₂O, then vortexed for 30 seconds. The mixtures were then incubated in the dark for 35 minutes at room temperature, centrifuged at 4000 G for 10 minutes, and the absorbance was read on a spectrophotometer at 725 nm. The total phenolic content (TPC) was then determined using Gallic acid as a standard and expressed as GAE or ‘Gallic Acid Equivalents’ (Blainski *et al.*, 2013). The unit of measurement was expressed as milligrams of GAE per gram of sample fresh weight.

2.2.2 Total flavonoid content

Flavonoid content is measured using an aluminum chloride colorimetric assay as outlined by Vyas *et al.* (2013b). One ml of sample or standard solution of catechin (0.5 mg/ml) was added to 0.3 ml of 5% NaNO₂. After 5 minutes, 0.3 ml of 10% aluminum chloride (AlCl₃) was added and after 1 minute, 2 ml of 1 M NaOH was added and adjusted with 24 ml of dH₂O. This was vortexed and incubated in the dark for 15 minutes at room temperature and centrifuged for 5 minutes at 4000 G. The absorbance was measured at 510 nm and the total flavonoid content (TFC) was expressed as μmol of CE per gram of sample.

2.2.3 Total tannin/ proanthocyanidin content

This assay uses vanillin as a reagent which reacts with the A-ring of flavonols, forming a chromophore. The absorbance in the solution is directly proportional to the number of flavonols present (Waterman & Mole, 1994). The structure of vanillin consists of an aromatic aldehyde that reacts with the ring structure of flavonols from the extract to yield a red colour product. According to the method developed by Chandrasekara and Shahidi (2011), one ml of sample was added to 5 ml of 0.5% vanillin-hydrogen chloride (HCl) reagent and incubated in the dark for 20 minutes. The standard used was catechin (Price *et al.*, 1978). The absorbance was read at 500 nm, and total tannin content was expressed as μmol of CE per gram of sample.

2.2.4 Total antioxidant capacity

Total antioxidant capacity was determined using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay, which is a stable free radical that is violet in colour, adopted from the protocol outlined by Brand-Williams *et al.* (1995) with some modifications (Vyas *et al.*, 2013b). When DPPH solution is dissolved with a substance that can donate a hydrogen atom, it yields a reduced product, indicated by the loss of violet colour originally present in DPPH solution. This method can be used to assess the properties of parts of plants that can scavenge free radicals. This procedure is dependent on the spectrophotometric measurement of DPPH concentration changes that occurs due to a reaction with an antioxidant (Pyrzynska & Peka, 2013). A sample unit of 100 μ l was added to 1900 μ l of 0.06 mM of DPPH stock and vortexed. This was incubated in the dark for 20 minutes and read on the spectrophotometer at 515 nm. The unit of measurement for scavenging capacity was expressed as ‘% of inhibition of DPPH consumption’ and the results were expressed as GAE against the Gallic acid standard curve.

2.3 Bioavailability of blueberry polyphenols in mice (pilot study)

The mice used in this study were of the C57bl/6 strain purchased from Charles River Laboratories and housed under standard conditions (12 hour light and 12 hour dark cycle). They were 7 week old males, weighing between 20-22 grams initially, with a total of 18 mice. The experiment was conducted at the Health Sciences Centre (HSC) Animal Care facility, and all procedures using mice were approved by the Institutional Animal Care Committee of Memorial University of Newfoundland (MUN). Mice were

acclimatized for 5 days, and were given standard mice chow and water for the entire study. They were housed in cages consisting of three mice per cage, and divided into three groups consisting of 6 mice. The three diet groups were control, leaf and fruit, all administered via oral gavage.

2.3.1 Sample extraction for leaves and fruits for mice feed

Frozen berry fruit or leaf samples of 20-30 grams were used to feed mice directly via oral gavage. Leaf and fruit samples were thawed at room temperature, then homogenized into a fine paste. The extraction procedure used different solvents in diet study compared to other experiments. In order to extract fruit and leaf extract for diet study, only dH₂O was used to dilute the concentration, since we did not want other chemicals to cause unnecessary side effects in our mice during the diet study. According to previous studies (Andres-Lacueva *et al.*, 2005; Kalt *et al.*; 2008, Milbury & Kalt, 2010), we adjusted the solution to 2% fruit extract using dH₂O as the solvent. Since our earlier biochemical analysis results showed that 10 times the polyphenolic content was found in leaves compared to plants, we determined 0.2% leaf extract should be used. Homogenate was placed in a syringe and supernatant filtered into a clean vial using a 0.45 µm filter, since that allowed for extracts to be small enough to pass through the syringe during oral gavage.

2.3.2 Monitoring

Via oral gavage, 0.1 ml of the 2% berry or 0.2% leaf extract was administered to the mice every day for 14 days around 10 am. The control mice were given 0.1 ml of

dH₂O in place of extract. The mice were monitored for those 14 days consecutively, where mice were weighed on a daily basis, and consumption of food and water was monitored.

2.3.3 Dissection and organ extraction

The food was removed from each mouse 4 hours prior to surgery. The anesthetization procedure involved placing a mouse into a sealed container with a paper towel lightly soaked with halothane/isoflurane. In order to ensure full anesthetization, tapping the mouse on the eye to ensure the blink reflex was suppressed, was performed after one minute of anesthetization. Mice were decapitated around the same time of day using an animal guillotine and organs of interest extracted included the brains (cortex and cerebellum), right and left kidneys, heart, and liver (in HSC 3415, with protocols 14-24-JW and 15-31-JW). All organs were placed in a liquid nitrogen bath for 1 minute to be flash frozen, put into labeled tubes and placed into a -80 °C freezer until use.

2.4 HPLC and MS Analysis of berries and mouse organs

In order to determine which anthocyanins were present in the leaf and fruit extracts, and if they were absorbed and detectable in the mouse organs after consumption, a HPLC-MS analysis was conducted. We chose to focus on anthocyanins because we found that past experiments in our lab were able to detect them in different local berries and we wanted to narrow down the compounds available in blueberries native to NL. In order to quantify the amount of certain anthocyanins detectable in the leaves, fruits, and

mouse organs, two standards of known anthocyanins were used: delphinidin 3-*O*-glucoside and cyanidin-3-galactoside.

2.4.1 Extract preparation for HPLC

Preparation of extracts was performed using methods outlined by Cho *et al.* (2004) with modifications. Berry samples were homogenized into paste using a mortar and pestle. Leaf samples were ground into a fine powder using liquid nitrogen. Homogenate was placed into a lyophilizer and freeze-dried overnight until dehydrated. Twenty mL of extraction solution (methanol/water/formic acid 60:37:3 v/v/v) was mixed with 5 grams of fruit/leaf powder. This mixture was then sonicated for 30 minutes in a water-bath sonicator, and then centrifuged at 2600 G for 30 minutes at 4°C. A 4 mL aliquot of the supernatant was collected and placed in a rotary evaporator at 45 °C until at least 95% of extract solvent was removed. The sample was then re-suspended in 1 mL of 3% formic acid in water solution and filtered through a 0.45 µm pore filter.

2.4.2 Standard and calibration curves

Two grams of anthocyanin reference standards (delphinidin 3-*O*-glucoside and cyanidin-3-galactoside) was dissolved in 10 mL of 3% formic acid to make a final concentration of 200 µg/ml. Serial dilutions were conducted to obtain concentrations of 150 µg/ml, 100 µg/mL, 50 µg/mL, and 25 µg/mL and 12.5 µg/mL of the reference standard stock solutions. Each standard solution was injected separately under the same HPLC conditions in order to create calibration curves for the reference compounds. The aim was to generate a linear calibration curve with $R^2 \geq 0.98$.

2.4.3 HPLC parameters

An Agilent 1100 Series HPLC system equipped with a G1311A quaternary pump (Serial Number DE40926119) was used. Fruit and leaf extracts from two locations, which showed the highest levels of polyphenols in the biochemical analysis, were injected (about 15 μ l) into the HPLC system. Separation was conducted using a 3.9 mm x 150 mm C18 (5 μ m) column. Prior to each injection, the system was equilibrated for 20 minutes. A Diode Array Detector (DAD) was used to detect flavonols at 360 nm and anthocyanins at 520 nm. The liquid chromatography flow rate was at 0.8 ml/min and the temperature was at 25 °C. The mobile phase consisted of a linear gradient of 5% formic acid (A) and methanol (B) from 2% methanol to 60% methanol for 60 min at a rate of one ml/min.

2.4.4 MS parameters

The parameters for MS were similar to the design developed by Cho *et al.* (2004), where both positive and negative electrospray ionization (ESI) were utilized. The capillary voltage was 4.0 kV, nebulizing pressure of 30.0 psi, temperature of 300 °C, drying gas flow rate of 9.0 ml/min. The performance was at 1.0 second/cycle with a scanner mass range of 10-1000 m/z (mass/charge). For identification, HPLC chromatographs and their MS values were compared to values accessible in the literature.

2.4.5 Tissue sample preparation for HPLC analysis

A stock solution was prepared of acidified methanol, using methanol (80% v/v), acetic acid (2.5% v/v) and ascorbic acid (15% v/v), adjusted with dH₂O. The volume of this stock solution differed based on the organ and the weight of the organ. The extraction

procedure used different solvents compared to extraction procedures for other portions of this project due to the fact we were using animal tissues instead of plant tissues, therefore different solvents used in past literature were observed to be effective. The animal tissues were weighed, and minced ground using a glass mortar and pestle. Depending on the type and weight of tissue, the amount of stock solution added differed. For one gram of brain tissue, 4 ml of acidified methanol solution was added to grind the organ and collect the residues on the glass vase and homogenizer. For one gram of liver, lungs, kidneys, 2 ml of acidified methanol was added to homogenize the mixture and an additional 2 ml to clean the homogenizer, making up a final volume of 4 ml. The sample was transferred into a 10 ml conical glass tube and vortexed for 5 minutes, then it was centrifuged at 3000 G for 30 min at 4°C. The supernatant was transferred into a clean tube, then the procedure was repeated two more times to extract the leftover residues with 4 ml acidified methanol and vortexed for 5 minutes. The mixture was again centrifuged at 3000 G for 30 min at 4°C. In order to evaporate the organic solvents, a rotary evaporator was used to yield a final volume of 400 µl, and placed in a seal amber vial for HPLC analysis.

2.5 Cell culture experiments

2.5.1 Microglial cell culture process

Microglia were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) which were isolated from postnatal day two C57BL/6 mouse brains, and cryopreserved and frozen prior to use at the HSC.

Microglia medium

Microglia medium (ScienCell) was prepared at the HSC cell culture lab, which consisted of 500 ml of sterile basal medium (MACS Neuro Medium), 5 ml microglia growth supplement, 25 ml of bovine serum and 5 ml of penicillin/streptomycin solution, and stored at 4°C.

Poly-L-ornithine

In order to promote cells to adhere to the culture wells for the first set of experiments, Poly-L-Ornithine (PLO-500ug/ml; Sigma), was used to coat in 12 of the wells in 24 well plates. Two types of plates were used; Falcon® and Costar®.

PLO coating solution

A PLO solution was prepared using 10 ml of distilled water added to 100 mg of PLO in order to get 10 mg/ml of solution. From this solution, 300 µl was placed in 1.5 ml centrifuge tubes and stored at -80°C. The coating solution was made by adding 5.7 ml of distilled water to 300 µl of PLO, to make a PLO concentration of 500 µg/ml solution, then vortexed. For the 24 well polystyrene plates, 12 wells received 250 µl of coating solution. The culture plates were coated in half of the wells a day prior to cell culture experiments, and incubated at 37°C in a humidified incubator with 5% carbon dioxide (CO₂).

Culturing cells

In the first experiment, the PLO was rinsed twice with 0.5 ml of sterile water per well the following day, and 1 ml of microglial medium was added to each well. Primary microglial cells were plated (20 µl per well) at a seeding density of $\geq 10,000$ cells/cm² per well in a 24 well plate, in both PLO coated and uncoated plates. After the first set of

treatments, wells were no longer treated with PLO explained further in the ‘Discussion’ section. In the second set of experiments, the plates were coated at a seeding density of $\geq 30,000$ cells/ cm^2 per well, with 27.8 μl per well. The most recent set of experiments had a seeding density of $\geq 43,000$ cells/ cm^2 per well, with 40 μl of cells per well.

The culture was left undisturbed in the incubator for 24 hours after initiation, and medium was changed the next day (by removing 1 ml of old media and replaced with 1ml of fresh media) to remove the residual preservative; dimethyl sulfoxide (DMSO) and unattached cells from the culture. Media was then refreshed every other day (0.5 ml of media replaced) until treatment experiments started.

2.5.2 Cell treatments

Once microglial cells were plated in wells and progressed to more elongated cells, they were treated with various conditions. From each final stock solution, 500 μl was administered to the treatment well. Blueberry fruit and leaf extracts collected from Pippy Park, which were tested in all biochemical assays, were used in all treatments in cell culture. Stock solutions of the following conditions were first made: solvent, control, 10 μM glutamate, 100 μM glutamate, α -synuclein, glutamate and fruit, glutamate and leaf, α -synuclein and fruit, and α -synuclein and leaf.

Solvent treatment

In order to ensure the solvent from the blueberry extracts had no effect on the cells before treatment with blueberries, the solvent alone was used to treat the cells in the first few experiments. The solvent consisted of 80% acetone and 0.2 % formic acid and 19.8% dH_2O and filtered.

Control treatment

A stock solution of 2.0 ml media was added to 25 μ l of dH₂O and 2.5 μ l of solvent treatment.

10 μ M glutamate treatment

For some of the earlier set of experiments, this concentration of glutamate was also used to find an optimal glutamate concentration causing neuro-inflammation. 2.5 μ l of the 10 mM L-glutamic acid stock solution was added to 2.5 ml of the culture media per well to get a final concentration of 10 μ M.

100 μ M glutamate treatment

A stock solution of 10 mM glutamate was made, where 10 ml of sterile dH₂O was added to 14.7 mg of L-glutamic acid (Research Biochemicals Inc.) and filtered. 25 μ l of 10 mM stock solution was placed in 2.5 ml of media in the culture well, which gave a final concentration of 100 μ M.

α -synuclein treatment

A stock solution of α -synuclein was made by dissolving 100 μ g/ml of α -synuclein into 5 ml of dH₂O. 2.5 μ l of this stock solution was added to 2.5 ml of microglia media to yield a final concentration of 100 ng/ml.

Glutamate and blueberry fruit treatment

Twenty μ l of the 10 mM L-glutamic acid stock solution was added to 2 ml of the culture media and 1 μ l of blueberry sterile filtered fruit extract per well, giving a final concentration of 12.5 μ g/ml for fruit extracts.

Glutamate and blueberry leaf treatment

Twenty μl of the 10 mM L-glutamic acid stock solution was added to 2 ml of the culture media and 1 μl of blueberry sterile filtered leaf extract per well. This gave a final concentration of 1.25 $\mu\text{g/ml}$ for leaf extracts.

α -synuclein and blueberry fruit treatment

Two μl of the α -synuclein stock solution was added to 2 ml of the culture media and 1 μl of blueberry sterile filtered fruit extract per well.

α -synuclein blueberry leaf treatment

Two μl of the α -synuclein stock solution was added to 2 ml of the culture media and 1 μl of blueberry sterile filtered leaf extract per well.

All stock solution of different treatments was then vortexed and incubated in the water bath (37°C) for 10 minutes. Old media was removed and each well was given 0.5 ml of specified treatment.

2.5.3 Cell culture staining and immunolabeling

The cells were all treated for 24 hours in appropriate treatment conditions, then culture media was removed from all treated wells. The media from each well was saved in separate Eppendorf tubes and stored at -80°C. All chemical solutions used in this part of the experiment is further explained in Table 2.2.

Table 2.2: Chemical preparation and purpose for fixing and staining cultured cells

Chemical	Preparation and use/purpose
<i>0.2 M Phosphate Buffer</i>	In order to make 4 litres of this solution, 92 grams of sodium phosphate dibasic was added to 20.96 grams of sodium phosphate monobasic. These chemicals were dissolved in 3.8 L of dH ₂ O, pH was adjusted to 7.4 using 10 N NaOH (in order to increase pH) or concentrated HCl (in order to decrease pH). In order to top up to a final volume of 4000 ml, dH ₂ O was added.
<i>Phosphate Buffer Solution (PBS)</i>	In order to make 1 litre of this solution, 500 ml of 0.2 phosphate buffer was added to 500 ml of dH ₂ O and 9 grams of NaCl.
<i>Paraformaldehyde Fixative (PFA)</i>	In order to prepare this fixative, 400 ml of dH ₂ O was placed in a 1000 ml beaker and heated to 70°C under the fume hood. Then we added 40 grams of PFA and solution was stirred until no longer cloudy in appearance. The solution of 10 N NaOH, which was cold, was added by drops until the solution became clear, then 500 ml of 0.2 M cold Phosphate buffer was added. In order to bring the final volume to 1000 ml, dH ₂ O was added to the solution, making a final concentration of 4% PFA. Using a bottle filter, the solution was filtered and stored at 4°C.
<i>Blocking Solution</i>	This solution contained 2 grams of 2% Bovine Serum Albumin (BSA) combined with 2 ml of 2% Fetal Calf Serum (FCS), 200

	mg of 0.2% fish skin gelatin and 98 ml of PBS. This was stirred for one hour, and stored at 4°C.
<i>Antibodies</i>	The primary antibodies (Ab) used in our experiment include F4/80, Iba-1 and glial fibrillary acidic protein (GFAP) antibodies. F4/80 and Iba-1 antibody binds to microglial cells, which expresses antigens on the microglial cell surface. GFAP is expressed by astrocytes, and used in this project to determine if any astrocytes are present in the cell culture. Secondary antibodies include Goat anti-rabbit Alexa Fluor® 488 which fluoresces green and conjugated goat anti-mouse Alexa Fluor®594 which fluoresces red.
<i>Antibody Solution 1</i>	This solution contained 1.125 ml of PBS, 0.125 ml of Bovine Serum (BS), 25 µl of F4/80 and 5 µl of GFAP or 2.7 ml PBS, 0.3 ml BS, 6 µl Iba-1 Ab.
<i>Antibody Solution 2</i>	This solution contained 1.125 ml of PBS, 0.125 ml of BS, 4.2 µl of F4/80 and 2.5 µl of GFAP.
<i>Antibody Solution 3</i>	This solution contained 3.825 ml of PBS, 0.425 ml of BS, 14.2 µl Alexa 488 (fluorescing green) and 14.2 µl of Alexa 594 (fluorescing red).

The wells were washed twice with 0.5 ml phosphate buffer solution (PBS) (pH 7.4). Then 300 µl of 4% paraformaldehyde (PFA) fixative solution was added to each

well, and removed after 20 minutes. Cells were then permeabilized using 0.2 % Triton X-100 (Sigma), and the solution was removed after 10 minutes. Cell wells were washed once with PBS, then incubated with blocking solution (250 μ l per well), which coats cells in order to block non-specific binding. The blocking solution was removed after 30 minutes, and specific wells were incubated with 250 μ l of primary antibody for one hour, then the solutions were removed. The cultures were washed twice with PBS, then incubated with secondary antibody solution (250 μ l per well) in the dark for one hour. The remaining protocols for the experiment were conducted under the dark fume hood. After solution was removed, cells were washed with 0.5 ml PBS two times, then dehydrated with 250 μ l of 70% ethanol, followed by dehydration with 250 μ l of 100% ethanol to remove all excess moisture. Approximately 15 μ l of VectaShield mounting medium with the fluorescent stain 4', 6-diamidino-2-phenylindole (DAPI) was added to wells, which binds to adenine-threonine regions in DNA. This stain can be used to label live and fixed cells due to its ability to pass through the cell membrane. The cells were covered with glass cover slips and stored at -20°C.

All images of cells were captured using a Zeiss Observer A1 microscope, Pixelfly qe CCD camera at a magnification of 40X (pco, Kelheim, Germany) and Axiovision software. The images were taken from 5 different sections of the well, then the cells counted manually. The differential interference contrast microscope was used to observe the morphology of cells in various treatments by capturing light microscopy images. Images were also captured using a confocal microscope by Dr. Noriko Daneshtalab using 16 well slides. The same procedures were followed for cells on slides, with the exception of the amount. 100 μ l of media and 8.7 μ l of cells were plated per well, and each well was

given 100 μ l of target treatment. During the staining and fixing procedure, 100 μ l all solutions were given per well.

Immunocytochemistry

In order to quantify the cell death of microglial cells, immunolabeling using F4/80 and GFAP was conducted (Weber *et al.*, 2012), Along with this, overall cell counting was performed which was determined by DAPI staining, using the program Axiovision.

Experimental changes:

Poly-L-Lysine (PLL) has a positively charged amino acid chain that improves adhesion of cells by changing the surface charges on the substrates of the culture (Cornbrooks, 1986), and it was recommended to use this instead of PLO as a coating agent to promote the adhesion of cells in culture. It is also encouraged to use this surface treatment to increase the survival rate of primary cells in culture. F4/80 was substituted by Iba1 Ab in later experiments, due to this new Ab labelling the cells better and giving a stronger fluorescence. Primary Ab solution now contained 0.3 ml of BS, a 2.7 ml of PBS and 6 μ l of Iba1 Ab. Secondary Ab solution contained 0.35 ml of BS, 3.15 ml PBS and 11.7 μ l of Alexa 488 (goat anti-rabbit).

In experiment 5, Vectashield was not available, so cells were mounted using 50/50 glycerol and dH₂O. The primary antibody consisted of 3.6 ml PBS, 0.4 ml BS and 20 μ l F4/80 Ab and the secondary Ab consisted of 4.05 ml tris-base saline (TBS), 0.45 ml BS and 15 μ l of Alexa 594 anti-rat Ab and 3 μ l of DAPI.

2.6 Statistical analysis

The experiments for biochemical analysis were all repeated three times and the results are expressed as mean \pm standard error (SE) of all three replicates. Using two-sample assuming unequal variance t-test (Microsoft Excel), statistically significant differences were determined between fruits and leaves. One-way ANOVA and Duncan's Multiple Range Variance Test (SPSS 39, IBM Inc.) were used to determine differences between different locations. The cell culture experiments were analyzed with one-way ANOVA ($P < 0.05$) followed by the post hoc Tukey's Multiple Comparison Test (SPSS 39, IBM Inc.). Results are expressed as means \pm SE.

Chapter 3: Results

3.1 Biochemical assay of blueberries collected from various sites

In order to determine and identify the levels of phenolic content of different samples collected from various locations, and to directly compare the fruits with the leaves from each location, different phenolic tests were conducted. Fruit samples from Middle Cove, Signal Hill and Johnson Geocentre were not tested because during the time of sample collection, there was a limited amount of healthy berry fruits available from these specific locations. Although locations were chosen within St. John's and surrounding area, it gave us a perspective on the variety of phenolic compounds that can exist within a similar region due to genotype and environmental factors. Each biochemical assay experiment was graphed to show the overall comparison between leaves and fruits, then individually graphed based on location to show the various significant difference amongst fruits or leaves collected from locations that were geographically close together, yet provided significant differences in phenolic content. The leaves, overall, had a higher content than fruits of lowbush blueberries when assessing the total phenolic content (Figure 3.1), the total flavonoid content (Figure 3.2), total tannin (Figure 3.3) and total antioxidant content (Figure 3.4). The only exception to this trend was found in berries from Blackhead, in which the fruits had higher tannin content than leaves (Figure 3.3). When measuring the total soluble phenolics, leaf extracts from Pippy Park (Figure 3.1.1) and fruit extracts from Marine Drive showed the highest soluble phenolic content (Figure 3.1.2). Fruits from Pippy Park (Figure 3.2.1) and leaves collected from Pippy Park (Figure 3.2.2) had the highest content of flavonoids. Leaf samples collected from Pippy Park (Figure 3.3.1) and fruits collected from Marine Drive

(Figure 3.3.2) had the highest tannin levels. The total antioxidant capacity was the highest in leaves collected from Signal Hill (Figure 3.4.1) and fruits collected from Fort Amherst (Figure 3.4.2). All overall significant differences determined between leaves and fruits were analyzed by using two-sample T-test assuming unequal variances ($P < 0.05$). The significant differences between samples of different locations was calculated by One-way ANOVA, followed by Duncan's Multiple Range Test (Table 3.1 and 3.2). Data are expressed as mean \pm SE; where experiments were performed with three replicates. Within the same column, different letters (a,b,c,d,e) indicate statistical significance at $P < 0.05$. Values represented by the same letter indicate there are no significant differences within samples from those locations. Table 3.1 and table 3.2 further summarizes the mean value of different biochemical characteristics of blueberry fruits or leaves of different locations, showing the significant difference amongst extracts that were collected from nearby sites within St. John's and surrounding areas. Table 3.1 show leaves from Fort Amherst had the highest TPC, Pippy Park leaves had the highest TFC and TTC, and leaves collected from Signal Hill had the highest total antioxidant content. Table 3.2 show fruits from Marine Drive had the highest TPC and TTC, Pippy Park fruits had the highest TFC, and fruits collected from Fort Amherst had the highest total antioxidant content.

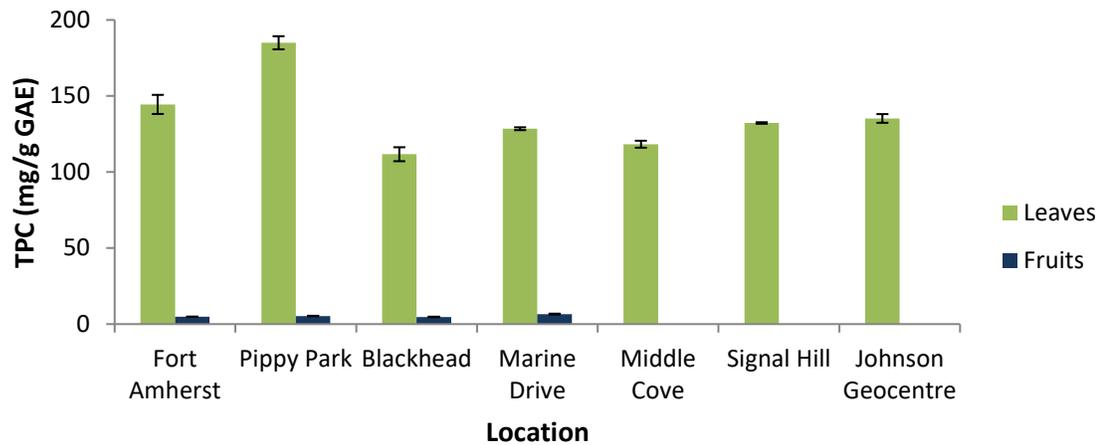


Figure 3.1 - Total phenolic content (TPC) of blueberry leaf and fruit extracts. The data are expressed as the mean \pm SE. GAE: Gallic acid equivalent. All experiments were repeated three times.

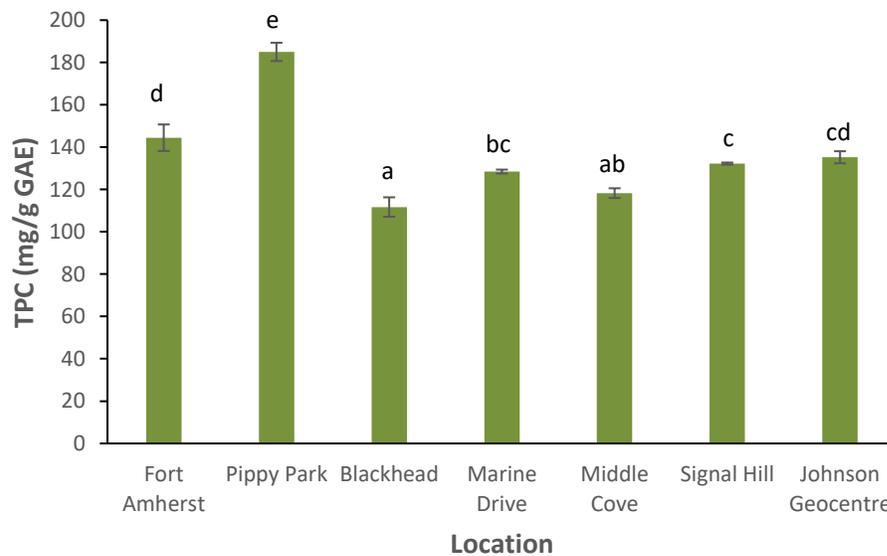


Figure 3.1.1 - Total phenolic content (TPC) of blueberry leaf extracts. The data are expressed as the mean \pm SE. All experiments were repeated three times. Data are the same as that presented in Figure 3.1.

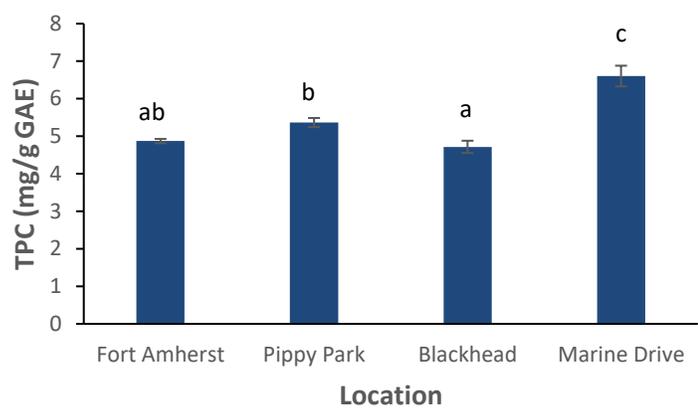


Figure 3.1.2 - Total phenolic content (TPC) of blueberry fruit extracts. The data are expressed as the mean \pm SE. All experiments were repeated three times. Data are the same as that presented in Figure 3.1.

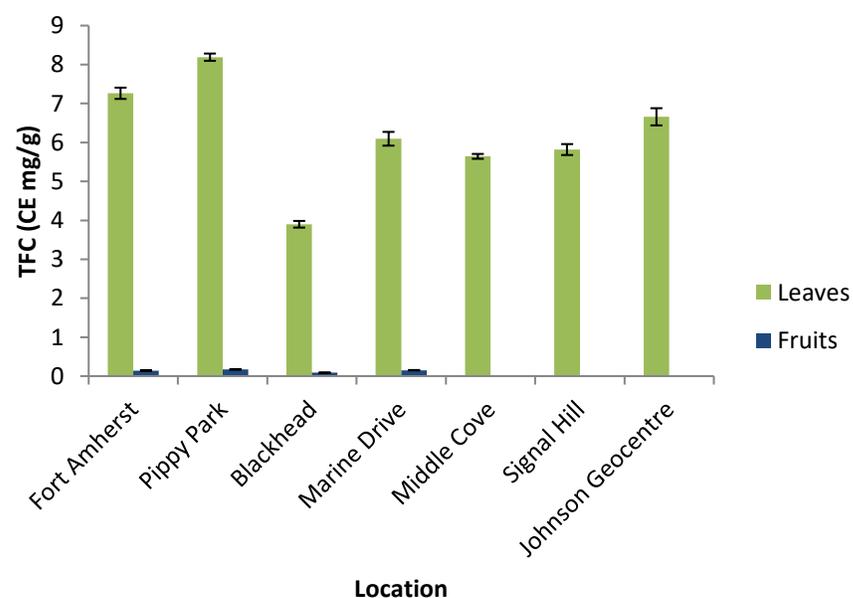


Figure 3.2 - Total flavonoid content (TFC) of blueberry leaf and fruit extracts. The data are expressed as the mean \pm SE. All experiments have been repeated three times. CE: catechin equivalent.

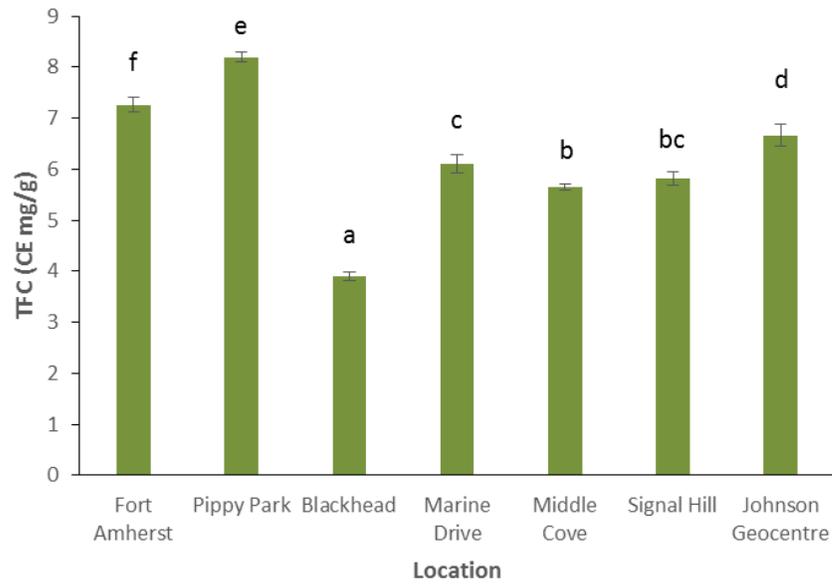


Figure 3.2.1 Total flavonoid content (TFC) of blueberry leaf extracts. The data are expressed as the mean \pm SE. The experiment has been repeated three times. Data are the same as that presented in Figure 3.2.

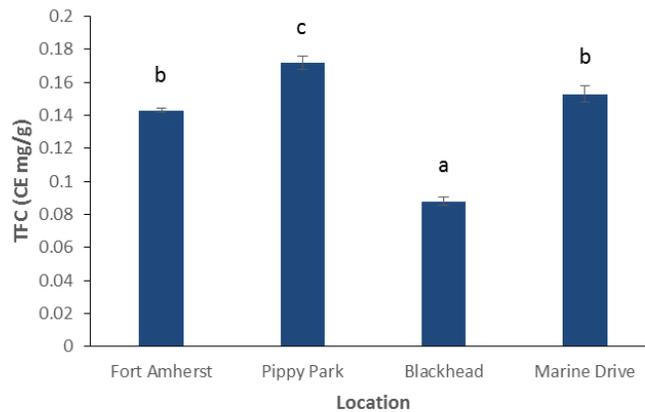


Figure 3.2.2 - Total flavonoid content (TFC) of blueberry fruit extracts. The data are expressed as the mean \pm SE. The experiment was repeated three times. Data are the same as that presented in Figure 3.2.

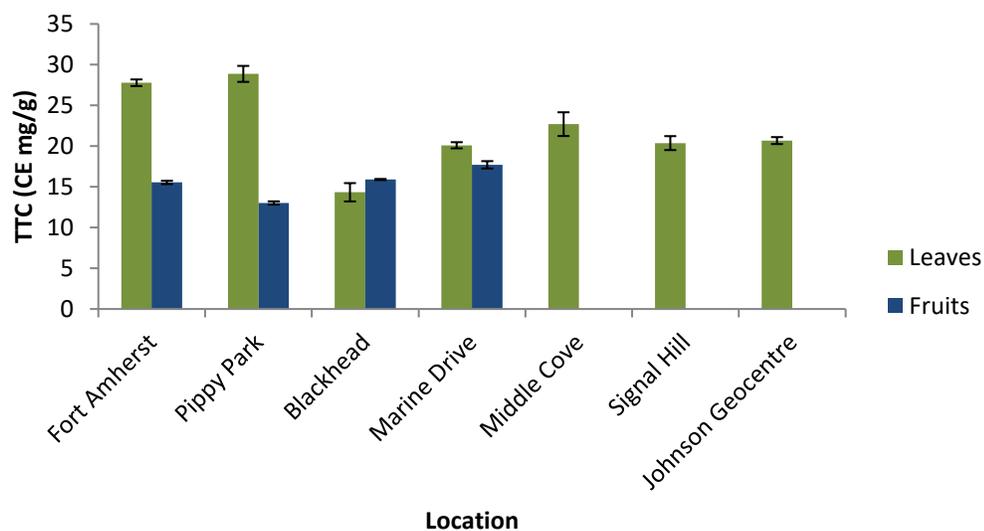


Figure 3.3 - Total tannin content (TTC) of blueberry leaf and fruit extracts. The data are expressed as the mean \pm SE. The experiment was repeated three times.

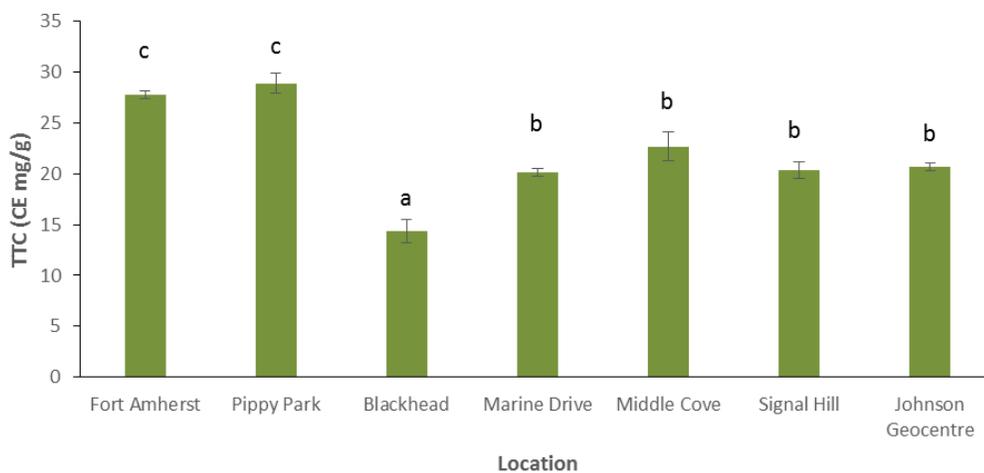


Figure 3.3.1 - Total tannin content (TTC) of blueberry leaf extracts. The data are expressed as the mean \pm SE. The experiment was repeated three times. Data is the same as that presented in Figure 3.3.

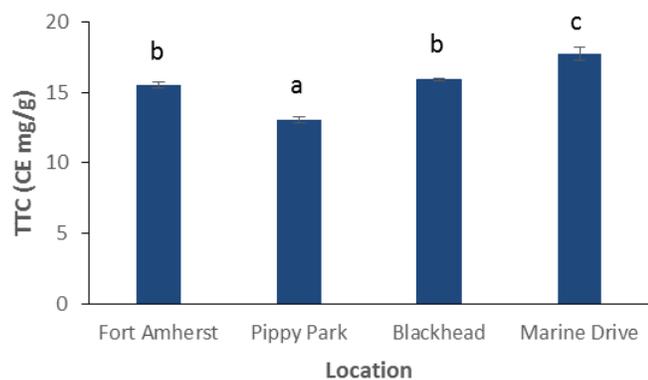


Figure 3.3.2 - Total tannin content (TTC) of blueberry fruit extracts. The data are expressed as the mean \pm SE. The experiment was repeated three times. Data is the same as that presented in Figure 3.3.

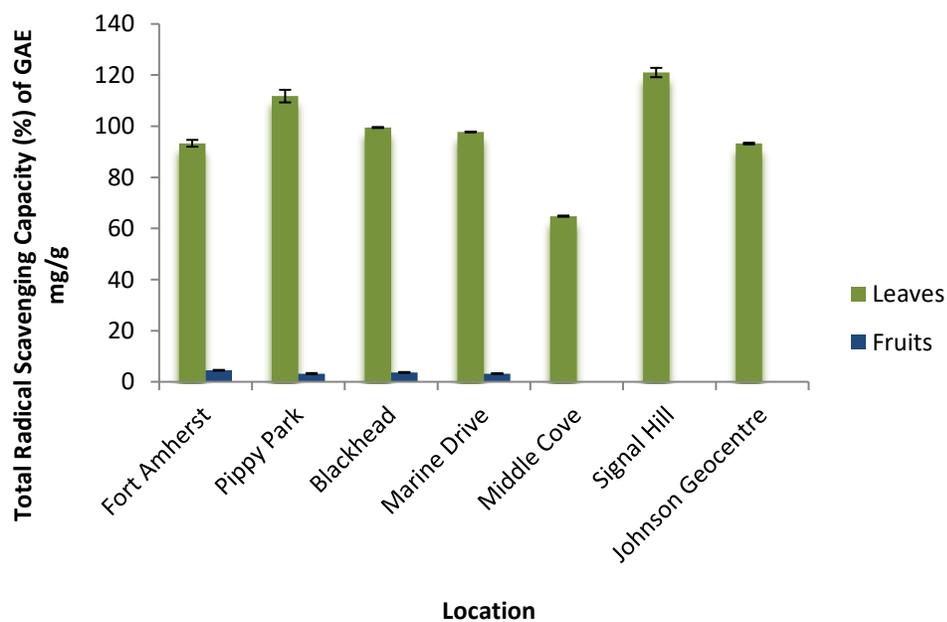


Figure 3.4 - Total antioxidant content of blueberry fruit and leaf extracts. The data are expressed as the mean \pm SE. The experiment was repeated three times.

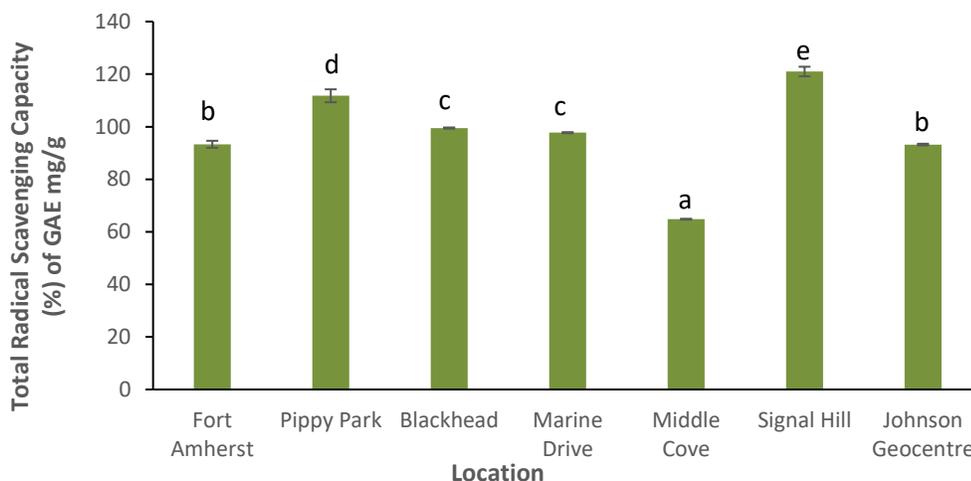


Figure 3.4.1 - Total antioxidant content of blueberry leaf extracts. The data are expressed as the mean \pm SE. The experiment was repeated three times. Data are the same as that presented in Figure 3.4.

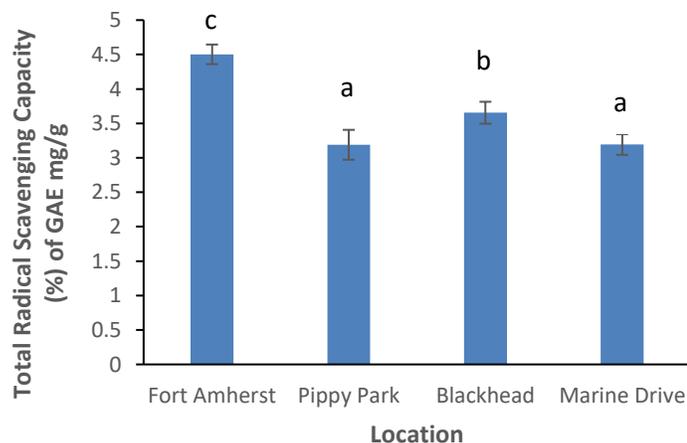


Figure 3.4.2 - Total antioxidant content of blueberry fruit extracts. The data are expressed as the mean \pm SE. The experiment was repeated three times. Data are the same as that presented in Figure 3.4.

Table 3.1: The mean value of different biochemical characteristics of blueberry leaves of different locations

Sample Location	Total Phenolic Content	Total Flavonoid Content	Total Tannin Content	Total Antioxidant
Marine Drive	128.47 ^{bc} ± 0.93	6.09 ^c ± 0.18	20.1 ^b ± 0.41	97.73 ^c ± 1.32
Fort Amherst	144.45 ^d ± 6.27	7.26 ^f ± 0.15	27.78 ^c ± 0.98	93.32 ^b ± 0.41
Blackhead	111.71 ^a ± 4.59	3.90 ^a ± 0.09	14.3 ^a ± 1.12	99.49 ^c ± 0.98
Pippy Park	184.99 ^e ± 4.31	8.19 ^e ± 0.09	28.9 ^c ± 0.38	111.77 ^d ± 1.12
Signal Hill	132.22 ^c ± 0.50	5.82 ^{bc} ± 0.14	20.4 ^b ± 1.46	120.99 ^e ± 0.38
Johnson Geocentre	135.23 ^{cd} ± 2.86	6.66 ^d ± 0.22	20.7 ^b ± 0.85	93.22 ^b ± 1.46
Middle Cove	118.28 ^{ab} ± 2.29	5.64 ^b ± 0.06	22.69 ^b ± 0.43	64.82 ^a ± 0.85

Data are expressed as means ± SE; n=3.

Within the same column, different letters (a,b,c,d,e) indicate statistical significance at $P < 0.05$. Values represented by the same letter indicate there are no significant differences within samples from those locations. According to this data, leaves from Fort Amherst had the highest TPC, Pippy Park leaves had the highest TFC and TTC, and leaves collected from Signal Hill had the highest total antioxidant content.

Table 3.2: The mean value of different biochemical characteristics of blueberry fruits of different locations

Sample Location	Total Phenolic Content	Total Flavonoid Content	Total Tannin Content	Total Antioxidant
Marine Drive	6.60 ^c ± 0.28	0.15 ^b ± 0.01	17.70 ^c ± 0.21	3.19 ^a ± 0.14
Fort Amherst	4.87 ^{ab} ± 0.06	0.14 ^b ± 0.001	15.54 ^b ± 0.19	4.50 ^c ± 0.21
Blackhead	4.72 ^a ± 0.16	0.09 ^a ± 0.003	15.89 ^b ± 0.07	3.65 ^b ± 0.16
Pippy Park	5.37 ^b ± 0.12	0.17 ^c ± 0.004	13.02 ^a ± 0.45	3.18 ^a ± 0.11

Data are expressed as means ± SE; n=3.

Within the same column, different letters indicate statistical significance at P<0.05. Fruits from Marine Drive had the highest TPC and TTC, Pippy Park fruits had the highest TFC, and fruits collected from Fort Amherst had the highest total antioxidant content.

3.2 Identifying and quantifying levels of anthocyanin in extracts using HPLC and MS analysis

In order to identify which anthocyanins were present in the extracts, we conducted mass spectrometry on the leaves and fruit samples from locations that tested for the highest phenolic content as determined by the biochemical analysis. Since biochemical assays determined that blueberry samples collected from Pippy Park and Fort Amherst had the highest overall phenolic content, we chose to identify major anthocyanin content of samples from those two locations. In order to identify the major anthocyanins, we matched the m/z with the literature values obtained from Cho *et al.* (2004) and Esposito *et al.* (2014), which are summarized throughout table 4.1 to table 4.4. Also, figure 4 shows a representative image of an HPLC-MS chromatogram which allowed us to detect the peaks and retention time, significant to identify anthocyanin components. It was found that fruits (Table 4.1 and 4.2) had more detectable anthocyanin compounds than leaves (Table 4.3 and 4.4).

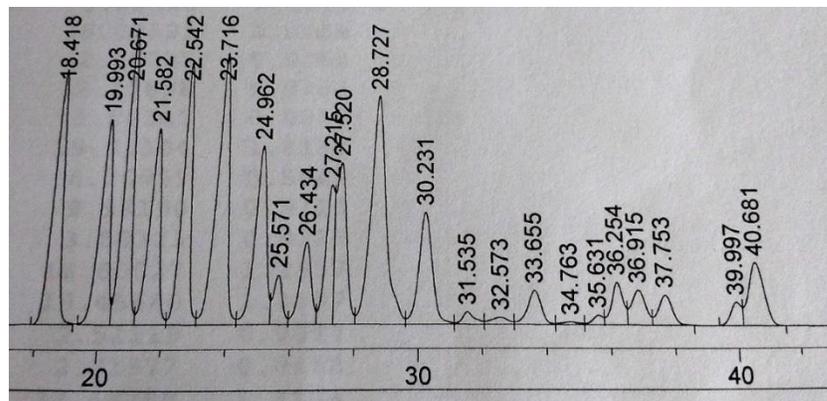


Figure 4. Representative image of HPLC chromatogram. Each retention time correlates with the peak used to identify the anthocyanin compound.

Table 4.1 Major compounds identified in blueberry fruit extracts from Pippy Park using mass spectrometry at positive electrospray ionisation (ESI)

Peak number	HPLC retention time (minutes)	Compound Identified	m/z value in literature	m/z value
1	20.2	Delphinidin-3-galactoside	465	464.9
2	21.7	Delphindin 3-glucoside	465	464.8
3	22.4	Cyanidin-3-galactoside	449	448.8
4	23.2	Delphinidin-3-arabinoside	435	434.8
5	24.2	Cyanidin-3-glucoside	449	448.8
6	26.6	Petunidin-3-galactoside	479	479.1
7	28.8	Peonidin 3-galactoside	463	462.9
8	29.2	Malvidin-3-galactoside	493	492.8
9	31.9	Peonidin-3-glucoside	463	462.9

*literature value obtained from Cho *et al.* (2004) and Esposito *et al.* (2014)

Table 4.2 Major compounds identified in blueberry fruit extracts from Fort Amherst using mass spectrometry at positive ESI

Peak number	HPLC retention time (minutes)	Compound Identified	m/z value in literature	m/z value
1	18.4	Delphinidin 3-galactoside	465	465.0
2	19.9	Delphinidin 3-glucoside	465	465.0
3	20.7	Cyanidin 3-galactoside	449	449.1
4	21.6	Delphinidin-3-arabinoside	435	435.0
5	23.7	Cyanidin 3-glucoside	449	449.1
6	24.9	Petunidin-3-galactoside	479	479.1
7	25.6	Peonidin-3-galactoside	463	463.1
8	26.4	Petunidin-3-arabinoside	449	449.0
9	28.7	Malvidin-3-galactoside	493	493.1
10	30.2	Peonidin-3-glucoside	463	463.1

*literature value obtained from Cho *et al.* (2004) and Esposito *et al.* (2014)

Table 4.3 Major compounds identified in blueberry leaf extracts from Pippy Park using mass spectrometry at positive ESI

Peak number	HPLC retention time (minutes)	Compound Identified	m/z value in literature	m/z value
1	22.2	Cyanidin-3-galactoside	449	447.9
2	26.3	Cyanidin-3-glucoside	449	446.8
3	34.7	Petunidin 3-galactoside	479	477.4
4	35.6	Peonidin 3-galactoside	463	463
5	38.5	Peonidin 3-arabinoside	433	433.4
6	39.9	Malvidin 3-arabinoside	463	461.6

*literature value obtained from Cho *et al.* (2004) and Esposito *et al.* (2014)

Table 4.4 Major compounds identified in blueberry leaf extracts from Fort Amherst using mass spectrometry at positive ESI

Peak number	HPLC retention time (minutes)	Compound Identified	m/z value in literature*	m/z value
1	19.6	Cyanidin-3-galactoside	449	449.0
2	20.9	Cyanidin-3-glucoside	449	447.0
3	28.8	Petunidin 3-galactoside	479	479.0
4	34.5	Peonidin 3-galactoside	463	463.0
5	36.1	Peonidin 3-arabinoside	433	433.0
6	38.1	Malvidin 3-arabinoside	463	463.0

*literature value obtained from Cho *et al.* (2004) and Esposito *et al.* (2014)

Quantitative analysis using HPLC

We chose two specific standards based on past experiments conducted in our lab, delphinidin 3-glucoside and cyanidin 3-galactoside. The two HPLC readings allowed us to quantify these specific anthocyanin compounds based on the standards. Using MS, we were able to determine that in extracts collected from both locations, the leaves had a higher amount of the anthocyanin; cyanidin-3-galactoside (Table 4.6) in comparison to the fruits (Table 4.5). Although fruit extracts contained more compounds of anthocyanins that were detected at these specific wavelengths (See Tables 4.1 through 4.4), when quantifying them, the leaves contained higher levels of the compounds (Table 4.6) compared to the fruits (Table 4.5). The retention times differed slightly in order to identify the same compound, based on the instrument and its parameters. As long as the chronological order of the compounds detected matches the literature values, these compounds can be identified. Also, we were able to determine that there was no detectable amount of delphinidin-3- glucoside present in the leaves (Table 4.6), although in the fruits, this anthocyanin was present at a greater amount from fruits from Pippy Park in comparison to fruits from Fort Amherst (Table 4.5).

Table 4.5 Anthocyanin levels detected in blueberry fruit extracts using mass spectrometry at positive ESI

Location	HPLC retention time (minutes)	Compound Identified	Concentration in Injected Sample $\mu\text{g/ml}$ (determined by standard at 520 nm)	Amount of anthocyanin per gram berry fruit weight (mg/100g)
Pippy Park	21.7	Delphinidin 3-glucoside	248.3	99.3
Pippy Park	22.4	Cyanidin 3-galactoside	69.0	27.6
Fort Amherst	19.9	Delphinidin 3-glucoside	133.1	53.2
Fort Amherst	20.7	Cyanidin 3-galactoside	65.4	26.1

Table 4.6 Anthocyanin levels detected in blueberry leaf extracts using MS at positive ESI

Location	HPLC retention time (minutes)	Compound Identified	Concentration in Injected Sample $\mu\text{g/ml}$ (determined by standard at 520 nm)	Amount of anthocyanin per gram berry leaf weight (mg/100g)
Pippy Park	22.2	Cyanidin-3-galactoside	288.0	115.2
Fort Amherst	19.6	Cyanidin-3-galactoside	442.6	177.0

3.3 Pilot bioavailability diet study

Although the amount of polyphenols in blueberry extracts could be determined using biochemical experiments, it was important to start exploring the bioavailability of these extracts in the body. Therefore, we conducted a dietary pilot study to detect how much extract was absorbed in different organs, with specific focus on the brain, using a mouse model. A two-week trial diet study was conducted to observe how much leaf and fruit of lowbush blueberries collected from Pippy Park would be absorbed in different organs of C57bl/6 mice. This strain of mice was used for our experiment as our lab aims to use a Parkinson's disease model of the same strain for research in the future. Samples were chosen from Pippy Park because according to the results from biochemical assays, these extracts had one of the overall higher levels of phenolic content. Mice were housed three in each cage, separated according to control, 0.2% leaf consumption and 2% fruit consumption via oral gavage. All mice were given the standard amount of food and water. Four mice died during this study due to perforation in the esophagus and aspiration due to extracts being administered accidentally via the trachea and entering the lungs. This was due to two main factors. The first was improper techniques of conducting oral gavage on my part, the second factor was due to the fact that the syringe given by the animal care staff was an inaccurate size for the animal weight, which caused perforation in the mouse lungs when administered by an animal care technician. Two mice from the control group and two mice from the leaf extract group died during this portion of the study. Excluding the four mice that died during this trial study, results showed that there was no significant weight gain or loss in the control (Figure 5.1), fruit (Figure 5.3) or leaf (Figure 5.2) group. A comparison of all three groups showed no significant weight change from the

initial to the final date of the trial study (Figure 5). Food and water intake varied each day, and there was no obvious pattern unless a mouse in that cage expired, which resulted in lower levels of overall food (Figure 5.4) and water intake (Figure 5.5). In order to analyze if the blueberry fruit or leaf extract was absorbed by different organs in the body, the mouse tissues were further tested using HPLC-MS to detect, identify and quantify specific anthocyanin compounds from blueberries in the biological tissues. Unfortunately, results indicated that there were no detectable anthocyanin compounds found in the mouse organs using HPLC-MS, therefore there is no data to be shown for that portion of the experiment.

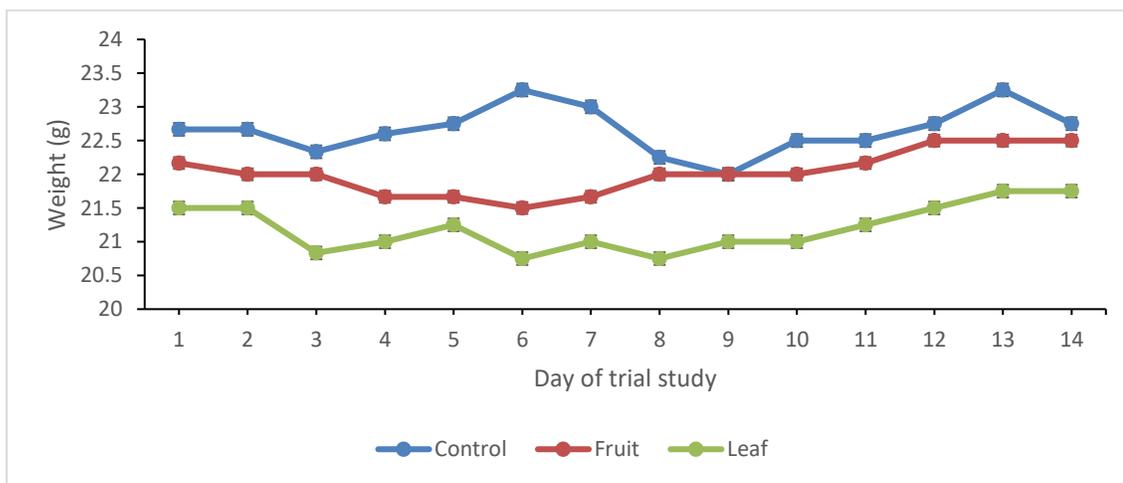


Figure 5 - Average weight change of C57bl/6 mice. Mice of control group and leaf group (C3, L3 and L6) expired July 23rd, mouse of control group (C1) expired July 24th, 2015. Although there is a variation in the weight of mice from different groups, it is not a significant difference. Overall, the weight did not fluctuate more than one gram for any group from initial to final day of study. Data is expressed as mean \pm SE.

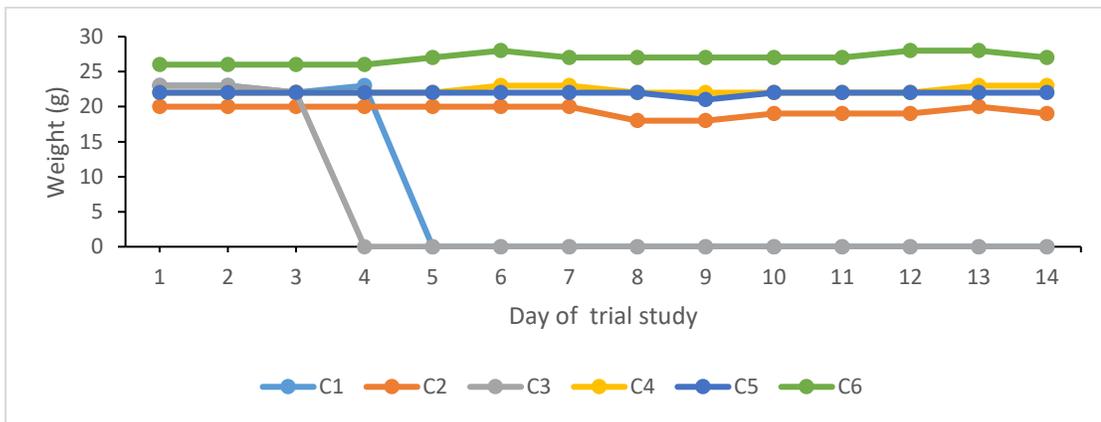


Figure 5.1 - Weight change of C57bl/6 mice of the control group. C3 expired July 23rd, C1 expired July 24th, 2015. C represents the control group that were not administered blueberry fruit or leaf extract. Weight did not vary drastically for mice over the trial study of two weeks.

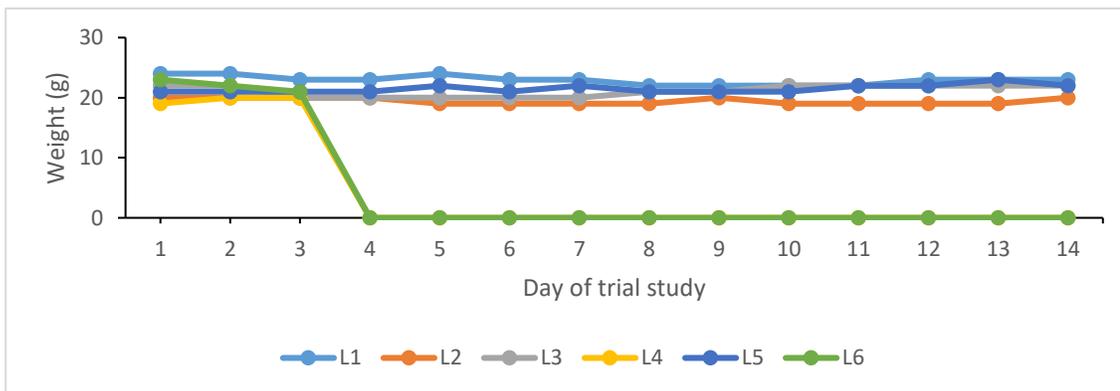


Figure 5.2 - Weight change of C57bl/6 mice consuming 0.2% blueberry leaf extract. L4 and L6 expired July 24th, 2015. L represents the mice administered 0.2% leaf extract via oral gavage. Weight did not vary drastically for mice over the trial study of two weeks.

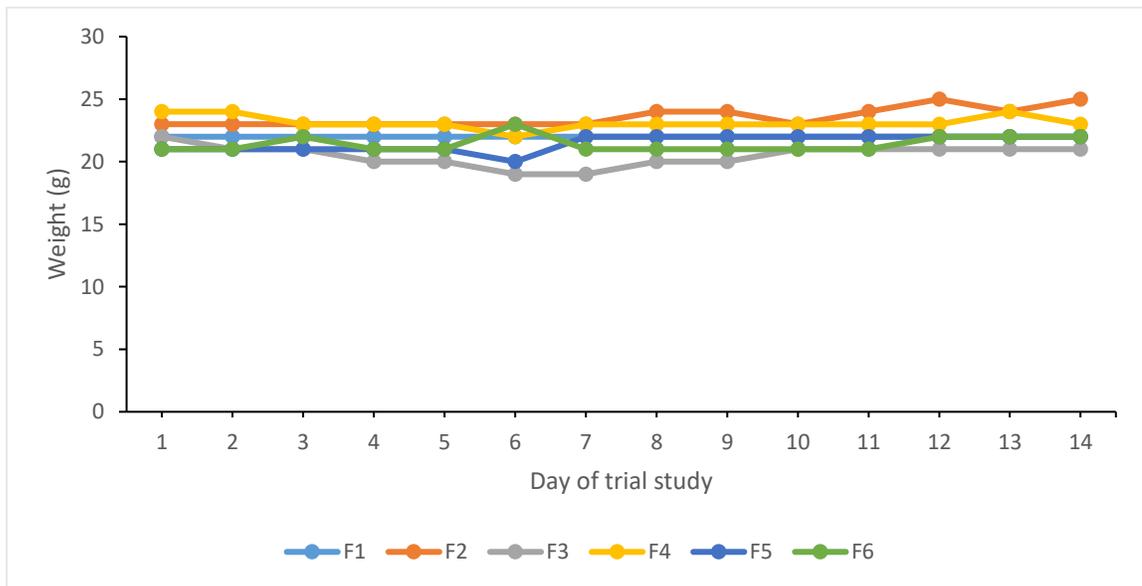


Figure 5.3 - Weight change of C57bl/6 mice administered 2% blueberry fruit extract. F represents mice administered 2% fruit extract via oral gavage. Weight did not vary drastically for mice over the trial study of two weeks.

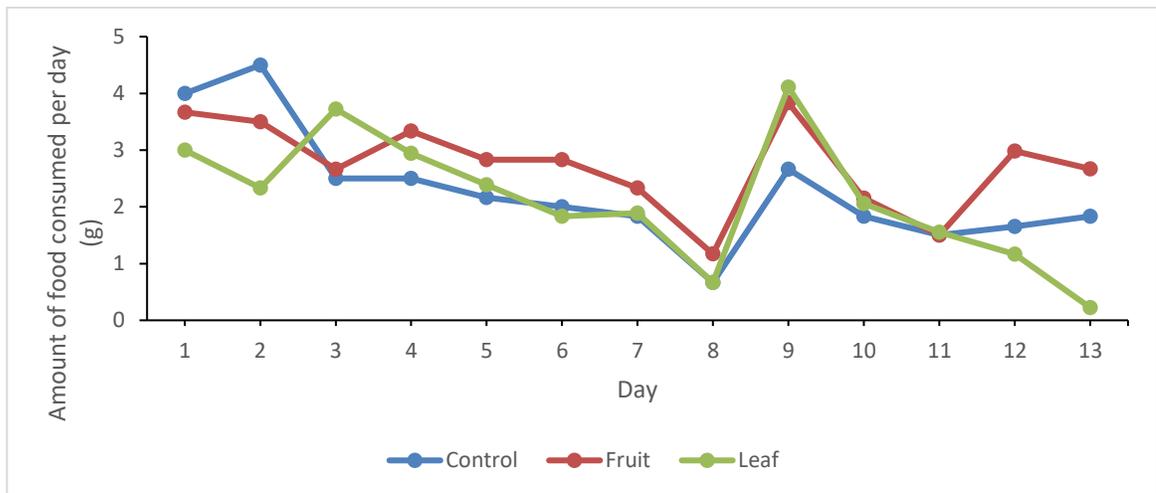


Figure 5.4 - Average food consumption of C57bl/6 mice administered 2% blueberry fruit extract (fruit), 0.2% leaf extract (leaf), or no extract (control). Average food consumption decreased for all three groups of mice by the end of the study, with mice consuming leaf extract having the greatest decline in food intake. These values take into consideration that two mice in the control group and two mice in the leaf group expired early in the trial study.

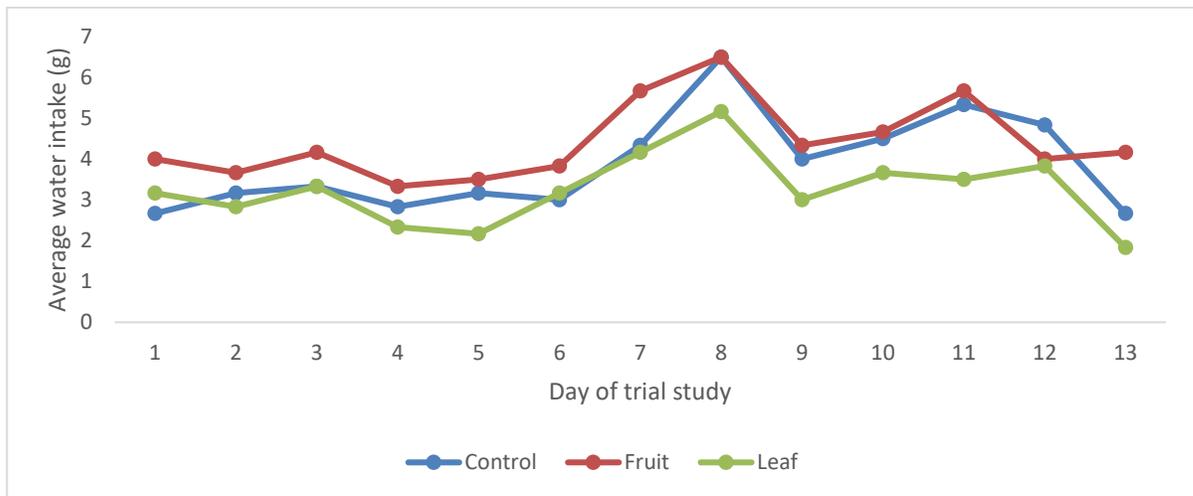


Figure 5.5 - Average water consumption of C57bl/6 mice administered 2% blueberry fruit extract (fruit), 0.2% leaf extract (leaf), or no extract (control).

Average water consumption for all control and leaf groups of mice decreased by the end of the study, with mice consuming fruit extract having no significant change in water intake. These values take into consideration that two mice in the control group and two mice in the leaf group expired early in the trial study.

3.4 Cell culture

After determining the rich phenolic content in blueberries using biochemical assays and HPLC-MS, we wanted to test the neuro-protective capabilities these extracts had on the brain, specifically microglial cells. We chose to use fruit and leaf extracts from Pippy Park, which showed to have one of the highest overall phenolic levels. We quantified the cells that could offer potential neuroprotection by counting the cells stained with DAPI in all treatments. All cell counts were performed with two individuals to ensure accuracy. Light microscopy images (Figure 6) and confocal images (Figure 6.1) captured microglia morphology in control conditions with elongated shape, and activated condition, treated with α -synuclein to have a 'fried egg' shape. Dark punctae was also visible in cells treated with glutamate for 24 hours. Mixed morphology is also apparent in confocal images of cells treated with blueberry extract (Figure 6.1). Figure 6.2 showed 10 μ M and 100 μ M glutamate treatment decreased the average % of control cells. We also used the solvent in which extracts were prepared as a treatment condition to ensure that the solvent used for the berry extracts was not harming the cells (Figure 6.2). Results showed no statistically significant difference between control and solvent treatments. Blueberry fruit and leaf extracts from Pippy Park were then tested further and results from Figure 6.3 showed that glutamate and α -synuclein caused a significant percentage of cell damage evident by the decrease in DAPI-stained nuclei. Since there was also a significant decrease in % of control cells when blueberry fruit extract added to cells treated with glutamate, no significant neuroprotective effects was determined. There was an increase in average % of control cells with blueberry leaf extract added to cells treated with glutamate indicating a neuroprotective effect. The average % of control cells present also

increased once the blueberry fruit and leaf extracts were added to conditions treated with α -synuclein, indicating a neuroprotective role of blueberries (Figure 7.3).

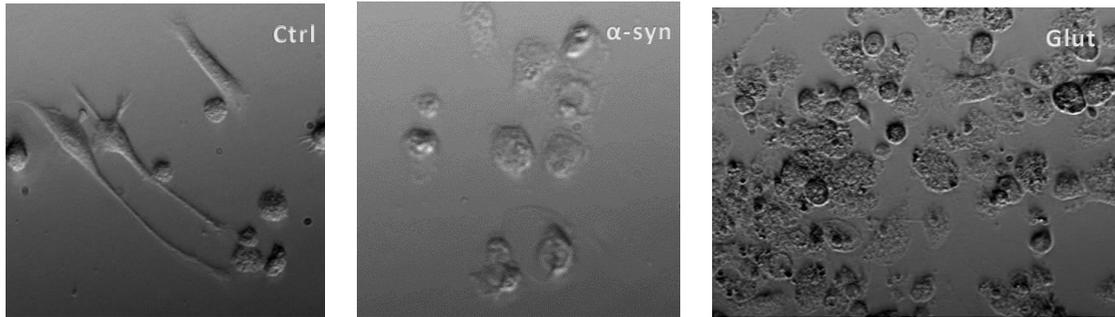


Figure 6. Light microscope cell culture representative images. Microglial cell images captured at 80X magnification using light microscopy after 24 hours of treatments of various conditions. Cell morphology captured using high magnification differential interference contrast (H/DIC). Healthy cells in control conditions are observed to be more elongated and activated cells treated with α -synuclein are observed to be a flatter, irregular shape. Cells treated with glutamate are observed to have dark punctae present indicating cell disruption and irregular circular shape morphology. (Ctrl=Control, Glut=100 μ M glutamate, α -syn=100 ng/ml α -synuclein).

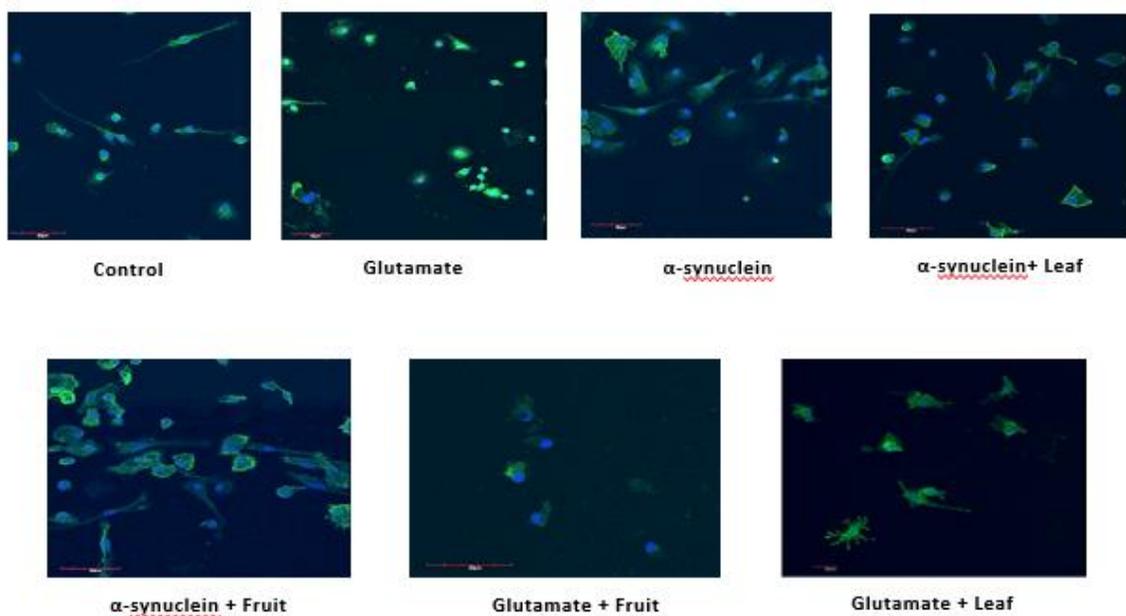


Figure 6.1 Confocal cell culture representative images. Microglial cell images captured by Dr. Noriko Daneshtalab using confocal microscopy. Cells have been treated for 24 hours with 100 ng/ml α -synuclein or 100 μ M glutamate, or either condition plus 12.5 μ g/ml blueberry fruit extract or 1.25 μ g/ml leaf extract. Microglia are labelled with antibodies Iba1 and Alexa 488. Cells treated with α -synuclein or glutamate are in an activated state ('fried egg' shape) and both activated and healthy states (more elongated) are observed when treated with blueberry extracts based on morphology. (Glut=100 μ M glutamate, α -syn=100 ng/ml α -synuclein).

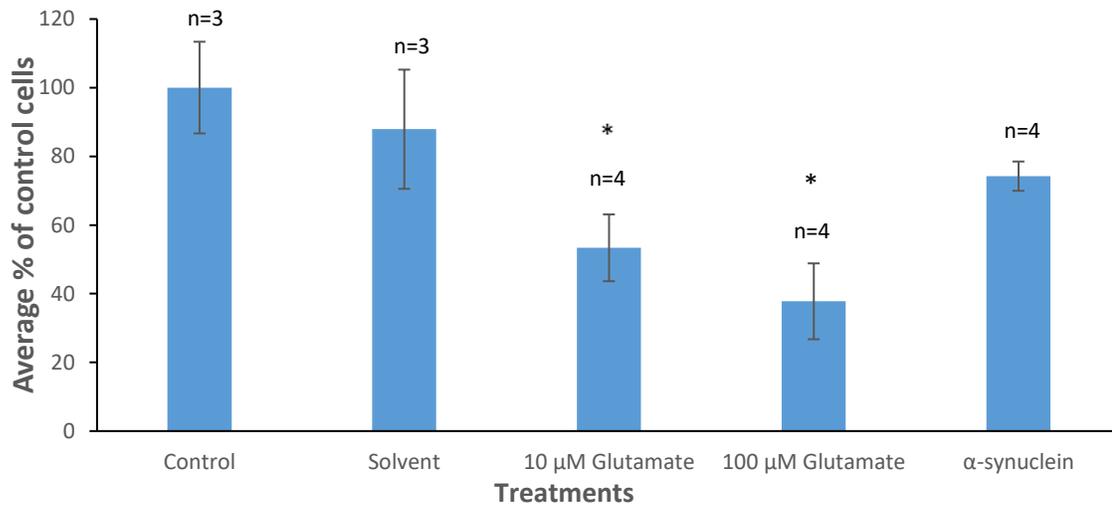


Figure 6.2 - Average percentage of living microglial cells undergoing different treatments. Cell count was calculated as a percentage of the control cells. The % of control cells was determined by quantifying the amount of DAPI-stained nuclei. (α -synuclein concentration = 100 ng/ml). Statistical significant difference (*) was determined between control and treatment wells by One-way ANOVA, followed by Tukey's test ($P < 0.05$). Data are expressed as means \pm SE). n=3-4 (n indicates the number of wells on day of experiment). Primary microglial cells were plated at a seeding density of $\geq 30,000$ cells/cm² per well.

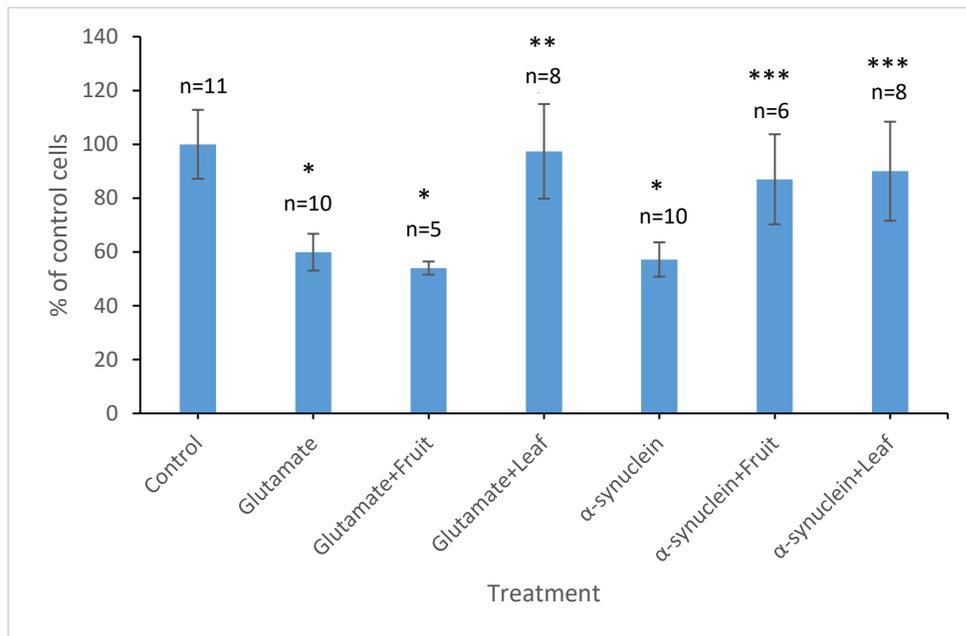


Figure 6.3 – The neuroprotective role of blueberry extracts on microglial cells

undergoing activation. Primary microglial cells were plated at a seeding density of $\geq 40,000$ cells/cm² per well. The % of control cells was determined by quantifying the amount of DAPI-stained nuclei. Statistical significant difference ($p < 0.05$) was determined between control and treatment wells (*), between 100 μ M glutamate and 100 μ M glutamate plus 12.5 μ g/ml fruit extract or 1.25 μ g/ml leaf extract (**), and between 100 ng/ml α -synuclein and 100 ng/ml α -synuclein plus 12.5 μ g/ml fruit extract or 1.25 μ g/ml leaf extract wells (***). All data was calculated by One-way ANOVA, followed by Tukey's test ($p < 0.05$). Data are expressed as average % of the control \pm standard error (SE). n=5-11 (n indicates the number of wells on day of experiment). Experiments were repeated three times.

Chapter 4: Discussion

Blueberry fruits and leaves from seven different locations grown in the St. John's area of NL were collected. Extracts from these samples were analyzed using the biochemical assays for this research project. It was found that blueberry fruit and leaf extracts were high in phenolic compounds, such as tannins, anthocyanins, flavonoids and total antioxidant capacity. In all of the biochemical assay experiments with the exception of tannins, leaves had a significantly higher content of total phenolic and flavonoid content. The blueberry leaves also had a higher level of total antioxidant capacity, measured as the total radical scavenging capacity, which indicates that their ability to scavenge free radicals is higher in comparison to the berry fruits, which is consistent with previous findings (Vyas *et al.*, 2013). In order to get more specific results, the mean value of different biochemical analyses of blueberry fruits and leaves from different locations was calculated, and determined which locations were statistically similar and different from one another, where within the same column, different letters indicate statistical significance at $P < 0.05$ (Table 3.1 and 3.2). According to the statistical analysis of the data obtained for blueberry fruits, the TPC was the highest in samples from Marine Drive, the TFC was highest for Pippy Park, the TTC was the greatest in samples from Marine Drive and the total antioxidant content was the highest in samples from Fort Amherst. For the blueberry leaves, samples from Pippy Park had the highest TPC, TFC and TTC. Leaves collected from Signal Hill had the highest antioxidant capacity. When analyzing the biochemical assays from different locations, there are significant differences between samples from some locations, while there is no significant difference found between other locations. This could be due to factors such as agronomic conditions, light, temperature,

and even genotype could influence some antioxidant components (Hosseinian *et al.*, 2007) and these factors could differ based on locations these samples were collected from. This is also supported by previous literature where blueberry of the same cultivars grown in different locations and different blueberry cultivars grown in the same location had significantly different antioxidant activity, suggesting the influence of genotype and environmental factors (Connors *et al.*, 2002). It is also evident to see that in most cases, the blueberry leaves have overall higher TPC, TFC, TTC and total antioxidant capacity compared to their respective fruits, which is also supported in previous findings (Vyas *et al.*, 2013).

Since we determined that there are high levels of overall antioxidants within the leaves and fruits of blueberries, next we wanted to determine the bioavailability of these compounds in the body by conducting a rodent diet study. In a two-week pilot study, C57bl/6 mice were monitored and administered water for the control group, 0.02% leaf extract, or 2% fruit extract via oral gavage. The extracts were chosen from the Pippy Park location, which showed one of the highest overall levels of polyphenols in the biochemical assays. During the study, two mice in the control group and two mice in the leaf group died early due to either perforation of syringe in the esophagus or when aspiration occurred (the syringe was placed in the trachea and filled lungs with fluid). Results showed that there was no significant weight gain or loss among any of the groups, which matches with previous findings in mice that were fed a blueberry supplemented diet (Adams *et al.*, 2011) and rats administered a 2% blueberry fruit diet (Randeiro *et al.*, 2012), although there was a decrease in food and water consumption in mice of the leaf group. This could be due to the loss of appetite after being administered leaf extract,

which may not appeal to their taste buds while the fruit extract may be more appealing since other studies have added blueberry fruit extract to feed that has been successfully consumed by the mice (Andres-Lacueva *et al.*, 2005; Aiyer *et al.*, 2011a). The lack of significant change in weight could also be due to the fact that although administered different extracts, the amount of food and water administered was consistent throughout the entire study and there were no noticeable changes in intake overall. A longer trial study will have to be conducted in the future in order to observe if the extract influenced water or food intake, or cause any significant changes in the weight of these mice. We used the same concentration of 2% blueberry fruit extract as stated in other research investigating fresh blueberry fruits (Willis *et al.*, 2010), although their experiments included rats of different age groups and longer trials which focused on quantifying other factors such as microglial activation in the hippocampus. Previous literature showed that the anthocyanin content in blueberries was 7.2 ± 0.5 mg/g dry matter (Lohachompol *et al.*, 2004), so we can estimate a 2% diet blueberry fruit diet for mice would contain ~0.72 mg of anthocyanin since we used 0.1 g of blueberry fruit per dose. Since there is limited data on leaf extracts in diet studies, future research is necessary to determine the concentration of anthocyanin present in blueberry leaves.

According to Aiyer *et al.* (2011a), blueberry fruits contain a moderate amount of anthocyanin (~ 4000 parts per million), and we analyzed them further in our berry samples. In order to quantify the amount of anthocyanins and identify the types of anthocyanins absorbed by different organs in mice and the amount detected in our extracts, we ran all samples using HPLC-MS. We chose fruits and leaves from Fort Amherst and Pippy Park, which had the highest levels of antioxidants as determined by

the biochemical assays. We ran HPLC-MS on extracts from these two locations, and identified the anthocyanins according to previous methods similar to Cho *et al.* (2004) and Esposito *et al.* (2014). We identified the following 10 different anthocyanin compounds in blueberry fruits (Table 4.1 and Table 4.2) from the two locations using different solvents; Delphinidin 3-galactoside, Delphinidin 3-glucoside, Cyanidin 3-galactoside, Delphinidin-3-arabinoside, Cyanidin 3-glucoside, Petunidin-3-galactoside, Peonidin-3-galactoside, Petunidin-3-arabinoside, Malvidin-3-galactoside and Peonidin-3-glucoside. In previous studies analyzing anthocyanins in lowbush blueberry fruits using HPLC-MS, many of these anthocyanins were also detected (Barnes *et al.*, 2009). In comparison, we detected and identified six different anthocyanin compounds in the blueberry leaves (Table 4.3 and Table 4.4); Cyanidin-3-galactoside, Cyanidin-3-glucoside, Petunidin 3-galactoside, Peonidin 3-galactoside, Peonidin 3-arabinoside, Malvidin 3-arabinoside. This analysis on leaves is still relatively new research, as other literature suggests that more studies on anthocyanins are necessary in the vegetative parts and leaves of plants (Naczka *et al.*, 2006). The detection of anthocyanins confirmed the presence of these bioactive compounds in both the blueberry leaf and fruit extracts. In order to quantify these compounds, we used two standards that were used in our lab in previous experiments; cyanidin-3-galactoside and delphinidin-3-*O*-glucoside. With the contribution from our summer student Scott Unruh, we were able to identify that out of the two anthocyanins, only cyanidin-3-galactoside was detectable in the blueberry leaves. When comparing extracts from both locations, we found that the leaves and the fruits from Pippy Park had a higher quantity of the anthocyanins cyanidin-3-galactoside and delphinidin-3-glucoside (Table 4.5 and Table 4.6). Confirming that anthocyanins were

present in the berry and leaf extracts, we then proceeded to test if anthocyanins from blueberries were present in organs of mice from the bioavailability diet study. There were no detectable traces of anthocyanins present in the organs we analyzed, including the brains (cortex and cerebellum), right and left kidneys, heart, and liver. Although there is literature to support that anthocyanin bioavailability in organs are detectable in diet studies involving rodents (Andres-Lacueva *et al.*, 2005), our research focuses on wild blueberry fruit and leaves native to NL, where the entire extract is used for consumption, not specific anthocyanins or powdered forms with controlled phenolic components. Past studies looked at blackberry diets in rats where they showed that native anthocyanins from berries were detected in stomach with no metabolites, indicating that the anthocyanins absorbed were intact. Methylated forms of anthocyanins from the diet were detected in the brain of rats, indicating that they could permeate the blood brain barrier (Talavera *et al.*, 2005). Therefore, we tested brain material to observe detectable anthocyanins in blueberry fed mice.

Limitations for this portion of the experiment includes several factors. For example, the instrumentation used to detect HPLC-MS has never been used on animal tissue before, therefore detection of anthocyanins could have interference due to other proteins and organic material present in the sample. Using whole blueberries in the feed instead of a controlled diet with measured levels of anthocyanins could also play a role in undetectable amounts of anthocyanins in tissues. The presence of metabolites in the extracts could also play a role in the lack of anthocyanin detection since metabolites increase the complexity of studying phytochemicals of berries and affects their bioavailability. For example, how polyphenols are metabolized in the body and how the

metabolites already present in the gut and liver influence the effectiveness of the polyphenols *in vivo*. Since anthocyanidins conjugate with different sugar moieties to produce anthocyanins, this influences how the gut metabolizes and absorbs the compound (Wu *et al.*, 2005). The bioavailability of different polyphenols differs as well, and those mostly in our diet do not always have the best bioavailability profiles (D'Archivio *et al.*, 2007). Also, there could be a loss of organic material during the extraction process since mouse organs are of such a small quantity, which could have resulted in loss of detectable anthocyanin content.

In a previous study conducted in our lab by Vyas *et al.*, 2013a, rat brain cell cultures consisting of glial cells and neurons were exposed to glutamate toxicity. Their results found that leaf extract from both lingonberry and blueberries showed neuroprotection, while only blueberry fruits showed neuroprotection against glutamate toxicity. Dosages for glutamate exposure and berry extract for cell culture were established previously in our lab (Vyas *et al.*, 2013a). Therefore, we focused solely on blueberry extracts to determine their protective role in microglial cells of mice. For the first set of experiments with cell culture, plates were coated with PLO as recommended by the supplier for greater cell adherence, but results showed that there was not a great difference in cell attachment in wells coated or uncoated with PLO, therefore for the remaining sets of experiments, the wells were left uncoated. In order to activate the microglial cells, glutamate and α -synuclein were used. We chose α -synuclein as past experiments have observed that in response to this cytosolic protein, there is an inflammatory response indicated by release of pro-inflammatory cytokines in astrocytes and neurons (Lee *et al.*, 2010). The dosage for α -synuclein was previously established by

the research team Dr. Weber was a part of in Germany, conducted at the University of Marburg (data not published). Light microscopy images (Figure 6) with representative images of microglial cells distinctly demonstrated different morphology of healthy and activated cells. Microglia in control conditions had an elongated shape while activated cells treated with α -synuclein were observed to have a 'fried egg' shape. Cells treated with glutamate for 24 hours had an increase in dark punctae, indicating disruption of cell bodies. The presence of dark punctae and irregular circular shape morphology was also observed previously in our lab where glial and neuronal cells were treated with glutamate (Vyas *et al.*, 2013). Results from Figure 6.2 showed that both 10 μ M and 100 μ M glutamate treatment significantly decreased ($P<0.05$) the average % of control (untreated) cells, although 100 μ M decreased slightly more cells (although not significantly), therefore we narrowed it down to 100 μ M glutamate to treat microglia cultures in subsequent experiments to stay consistent with previous work conducted in our lab. We also used the solvent in which extracts were prepared as an independent treatment condition in the beginning to ensure that the solvent used for the berry extracts was not harming the cell growth. As there was no statistically significant difference ($P<0.05$) between control and solvent treatments, further experiments no longer tested with 'solvent' treatment independently, since the solvent used to dissolve the berry and leaf extract had no negative effect on the cells' integrity. Blueberry fruit and leaf extracts from Pippy Park were then tested further, which, according to results from biochemical assays, showed to contain one of the highest constituents of phenolics on activated microglial cell cultures. Results from Figure 6.3 showed that glutamate and α -synuclein used for cell activation caused a significant percentage of cell damage evident by the decrease in

DAPI-stained nuclei, indicating the effect of these pro-inflammatory factors on cell loss. There was also a significant decrease in % of control cells when blueberry fruit extract was added to cells treated with glutamate, which indicates that blueberry fruit shows no significant neuroprotective effects on these cells. This could suggest certain concentrations of blueberry fruit extract could be ineffective to microglial cells, as previous literature has found certain concentrations of the antioxidant oxyresveratrol reduces viable glial cells following mild traumatic injured (Weber *et al.*, 2012). Results showed that there was an increase in the number of viable cells once blueberry leaf extract was added to cells treated with glutamate indicating a neuroprotective effect. There was also a greater average % of control cells present once the blueberry fruit and leaf extracts were added to conditions treated with α -synuclein, indicating a neuroprotective role blueberries can have on activated microglial cells (Figure 6.3). One possibility is that blueberry anthocyanins are able to reduce the amount of inflammatory mediators that are released, which in turn reduced damage caused by oxidation as seen by Youdim *et al.* (2002) in an atherosclerosis study. Many neurological disorders such as PD and AD are associated with oxidative stress, which could lead to severe cellular damage, necrosis and apoptosis (Routray & Orsat, 2011), and more emerging evidence supports the idea that microglia could potentially be a source of ROS which causes neuronal damage leading to neurodegenerative diseases (Block *et al.*, 2007b). Therefore, the important antioxidant properties in blueberries may be highly beneficial for the brain. As results show, by being able to inhibit cell death with blueberry extracts that have been damaged by α -synuclein, there is potential to slow down the progress of neurological diseases associated with α -synuclein localization. Previous literature found that

blueberries decreased activation of microglial cells in intraocular hippocampal grafts (Willis *et al.*, 2011) but also increased newly generated cells in the hippocampus of rats after supplementation with blueberries (Casadesus *et al.*, 2004), which agrees with our data after fruit and leaf extracts were added to cells treated with α -synuclein. This regeneration of cells could also potentially slow down neurogenesis progression.

Chapter 5: Conclusions and future directions

This study analyzed the nutraceutical properties of blueberries native to Newfoundland and Labrador. Biochemical assays were able to compare the different phenolic contents between blueberry fruits and leaves. Results showed higher phenolic, flavonoid, tannin and total antioxidant content in leaves when compared to fruits. We were also able to determine that although samples were collected from nearby locations within St. John's and the surrounding area, the phenolic content varied significantly between the fruits and the leaves collected from different locations. The HPLC-MS results showed anthocyanins in the fruits and leaves, indicating that leaves had a higher quantity of specific anthocyanins while fruits had a greater number of detectable anthocyanin compounds. The cell culture study indicated that fruits and leaves have an overall positive effect on reducing inflammation of activated microglial cells in the brain, which is significant for future neurological studies.

One of the limitations of this research is that the extraction procedure for the diet study differed greatly from the other experiments, which also used different solvents. This is an inconsistency in our experiment, but difficult to control until further experiments find common solvents for extractions that are safe and effective in multiple experimental approaches. Additionally, although these initial results are significant to research in the neuroprotective properties of blueberries, future studies will be needed to establish toxicity levels, particularly for blueberry leaves. Due to limited previous investigations on blueberry leaves, more research is needed before establishing dosage and efficacy of these extracts.

The dietary recommendations of the Canadian Food Guide were set to focus on health management and disease prevention. The reference values coded for these recommendations were considered sufficient to meet the needs of a majority of the Canadian population. Now, with emerging evidence on genetic predispositions and mutations, environmental factors, and diverse backgrounds in terms of race and culture, these guidelines are too general for the growing population. With allometric conversions from different rodent studies to humans, research has shown that even one to two cups of blueberries a day could have health benefits (Adams *et al.*, 2011). With the increase in detection of neurodegenerative diseases, it is necessary to turn focus towards nutraceutical products that could be synthesized as supplements or added to traditional meals in order to effectively target prevention and reduced incidences of neurological problems. Treatments that are phytochemical-based could be taken into clinical trials in the future for neurological conditions, due to the fact that phytochemicals have low toxicity levels and their bioavailability is high (Mecocci *et al.*, 2014). Future directions involving human trials would present stronger evidence relating the role of nutrients to cognitive function. This could also help to determine if short term or long term use of nutraceuticals would be effective in reducing the progression and incidence of neurodegenerative diseases.

It is important not to group the functions of antioxidants, but to analyze the role individual components of antioxidants play in protecting and regenerating brain cells. By identifying these key roles of different antioxidant components, it will be easier to isolate the function and mechanism of action required to protect the brain. Future studies are needed to analyze the role of different isolated antioxidants present in blueberry fruits and

leaves, as well as how they are metabolized and absorbed in the body, to better understand their roles in neuroprotection. It is important to recognize and understand how these natural sources of antioxidants could further slow the progress or prevent certain neurodegenerative diseases.

In essence, blueberry fruits and leaves, in a variety of forms from powders to tea leaves, have great potential and could have a huge impact on future treatments regarding neurodegenerative health issues.

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