CHEMICAL ANALYSIS AND POTENTIAL NEUROPROTECTIVE EFFECTS OF NEWFOUNDLAND BERRIES

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Chemical analysis and potential neuroprotective effects of Newfoundland berries

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

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Abstract

The research in our laboratory involves the study of nutraceuticals, which are foods claimed to have a medicinal effect on human health. Powdered samples of bilberry, blueberry, black currant and lingonberry were provided from Natural Newfoundland Nutraceuticals (NNN Inc., Markland Newfoundland). Whole berries were also collected. For the analysis, various solvent extractions of the berry samples were conducted. The extract solutions were analyzed using a liquid chromatography-mass spectrometry (LC-MS) method for compound detection. Two distinct elution regions for anthocyanins and flavonols were obtained with near base line separation of different compounds. For proper identification of these compounds, two standards (delphinidin-3-glucoside and cyanidin-3-galactoside) were run to obtain a standard retention peak with MS value. LC-MS analysis revealed that various polyphenolic compounds, such as flavonoids (e.g. anthocyanins and flavonols), are present in these species of berries. In addition, our analysis revealed that bilberry and blueberry extracts appear to contain a greater array of antioxidant compounds when compared to black currant and lingonberry extracts. We have also conducted some quantitative analysis in order to compare compounds from native Newfoundland berries to those detected in these species from other regions. The bilberry extract was examined for neuroprotection by applying them to cultured rat brain cortical cells. Cells were injured using an *in vitro* model of trauma. High levels of lactate were measured after injury, showing that cells were unhealthy. When extracts were added to the cells 15 minutes before injury lactate levels dropped significantly. Aging experiments showed that up to day 17 cell cultures demonstrated no significant death of neurons, but by day 20 there was a dramatic loss of neurons. For glial cells, proliferation was noticed by day 17, but by day 20 there was no significance further change in glial cells. Extracts were added on days 17, 18 and 19 *in vitro*. By counting neurons and glia on day 20 it was found that no noticeable effect was shown on the glia and neurons with the addition of the extracts. Therefore, bilberry extracts appear to have a more prominent effect on trauma than on aging in cell cultures.

Dedicated to

My parents and my wife

Acknowledgement

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Table of Contents

List of Tables

List of Figures

Symbols and Abbreviations

AD	Alzheimer's disease
ATP	Adenosine tri-phosphate
CAT	Catalase
DAD	diode array detector
DAPI	4', 6-diamidino-2-phenylindole
DIV	Days *in vitro*
EtOH	Ethanol
ESI	electrospray ionization
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulphide
HPLC	High performance liquid chromatography
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low density lipoprotein
MeOH	Methanol
MAP-2	Anti-microtubule-associated protein 2
MS	Mass spectrometry
NNN	Natural Newfoundland Neutraceuticals
NOS	Nitric oxide synthase
OXY	Oxyresveratrol

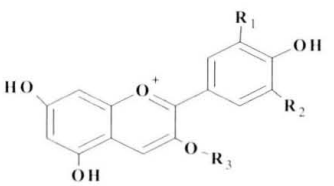
PD	Parkinson's disease
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSD	Relative standard deviation
SOD	Superoxide dismutase
TBI	Traumatic brain injury

Chapter 1

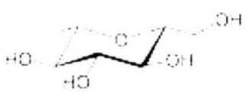
INTRODUCTION

1.1 General introduction

Flavonoids refer to polyphenolic compounds (Figure 1.1) defining a wide range of secondary plant metabolites (Cho et al., 2004), namely "anthocyanins, flavonols, flavones, catechins and flavonones" (Robards et al., 1997; Mikkonen et al., 2001). In berries, anthocyanins and flavonols are found in the glycosylated patterns (Cho et al., 2004). One of the important aspects of flavonoids is that they act as free radical scavengers (Dragovic-Uzelac et al., 2010). Notably, certain flavonoids, particularly if they have a catechol structure, have copper complexation properties (Brown et al., 1998). It is assumed that the type of sugar moiety (Wang et al., 1997), degree of glycosylation (Wang et al., 1997) and acylation of anthocyanin glucosides influence the antioxidant capacity of flavonoids (Tamura et al., 1994; Matusufuji et al., 2003).



$R_1 = R_2 = R_3 = H$: Pelargonidin
$R_1 = OH, R_2 = R_3 = H$: Cyanidin
$R_1 = R_2 = OH, R_3 = H$: Delphinidin



$R_1 = R_2 = OH, R_3 =$: Delphinidin-3-glucoside
$R_1 = OCH_3, R_2 = OH, R_3 = H$: Petunidin
$R_1 = OCH_3; R_2 = OCH_3, R_3 = H$: Malvidin

Figure 1.1: Common anthocyanidin structures

Generally, anthocyanins account for those "red, blue and purple pigments" found in "many fruits, fruit juices, wines, leaves and flowers" (Cooney et al., 2004). As anthocyanins are chiefly responsible for the colour of flowers and fruits, they certainly play a vital role in attracting animals for pollination and seed dispersal (Strack and Wray, 1993). Fruits usually contain six anthocyanidins, namely, delphinidin, cyanidin, pelargonidin, peonidin, petunidin and malvidin (Goiffon et al., 1991), while cyanidin is most commonly found. Blueberries contain all the anthocyanidins except pelargonidin (Robards and Antolovich, 1997).

Anthocyanins not only serve as a source of natural food colourant but they can be used as protection in cases where free radical species cause damage to the human body (Cho et al., 2004). It is estimated that the daily consumption of anthocyanins is as much as 180 - 215 mg in the U.S. (Kühnau, 1976), as they are widely distributed in fruits and vegetables (Table 1.1) (Wang et al., 1997).

Table 1.1 Total anthocyanin content in some common fruits and vegetables [a, b]

Fruits and vegetables	Anthocyanins mg/ 100 g of fresh weight
Blackberry	83-326
Blueberry	25-495
Raspberry	
Black	214-428
Red	20-60
Sweet cherry	350-450
Cranberry, whole	78
Juice	18-87
Strawberry [c], whole	7-30
Juice [d]	21-333
Red grapes	30-750
Red wine [e]	100-1000
Currant	
Black	250
Red	12-19
Apple (Scugog)	10
Red cabbage [f]	25
Red onion	9-21

[a] All values are expressed on fresh weight basis (mg/ 100 g) except values for juice and wine, which are expressed in mg/ L. [b] Data from Mazza and Miniati (1993) unless noted differently. [c] Kikoku et al. (1995). [d] Bakker et al. (1994). [e] Glories (1988). [f] Timberlake and Henry (1988).

1.1.1 Biological activity of anthocyanins

Flavonoids have been shown to exhibit a vast range of biological activities based on *in vitro* studies, including antioxidant properties (Bravo, 1998). A major role of antioxidants is to inhibit cellular damage caused by oxidative stress which can cause cancer as well as degenerative diseases of aging such as cardiovascular disease and even brain dysfunction (Ames et al., 1993). Various studies suggest that anthocyanin potentially plays a role to protect humans from other degenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD) (Kay, 2004). *In vitro* studies demonstrate that anthocyanins have considerable antioxidant properties and also act as oxygen radical scavengers (Bohm et al., 1998). It has been assumed that oxidative stress plays an important role in cellular cholesterol accumulation whereas antioxidants prevent low density lipoprotein (LDL) oxidation, which ultimately decreases the formation of plaques in the arteries (Kaplan and Aviram, 1999).

Additionally, as Kay (2004) found in an extensive literature review, consumption of anthocyanin enhances the hydrophilic and lipophilic blood/plasma antioxidant capacity. It has been found that, depending on different glycosylation arrangements, anthocyanidins can either enhance or decrease antioxidant activity (Kähkönen and Heinonen, 2003). Since berries containing flavonoids have antioxidant properties, this biological activity of flavonoids explains their role in prevention of diseases.

1.2 Bilberry, blueberry, black currant and lingonberry

The following description of all four berries i.e. bilberry, blueberry, black currant and lingonberry enables us to know about their anthocyanin content, antioxidant properties and also their role in various diseases. However, through analysis of these berries it is possible to determine what specific types of compounds are present and how they act as antioxidants. Section 1.3 provides information about the analytical method and solvent system for this analysis.

Bilberry: Bilberries belong to the genus *Vaccinium* and usually the plant is 20-60 *cm* high. The fruit is black and has potential health benefits because of its astringent property and its ability to act as a diuretic. Besides this, it has been suggested that its leaves help to reduce blood sugar levels. (Petri et al., 1997).

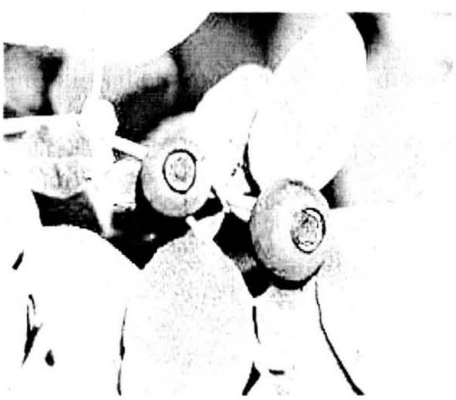


Figure 1.2: Bilberry

Blueberry: Blueberry plants also belong to the genus *Vaccinium* and produce dark-blue, purple or black coloured fruits. These plants are mainly native to North America (Neto,

2007). Kalt et al. (1999) revealed that there is a strong correlation between the antioxidant capacity and the total anthocyanin content of high and low bush blueberries. They also found that these two blueberries have three times the antioxidant benefit of strawberries and raspberries (Nicoue et al., 2007). Further, these researchers indicate that anthocyanins in blueberries offer a much greater antioxidant contribution (0.4-9.4%) than does ascorbic acid. (Kalt et al., 1999).

Figure 1.3: Blueberry

Black currant: Black currants (*Ribes nigrum* L) possess a variety of phenolics as well as "high antioxidant activity" (Anttonen et al., 2006). Black currant is also considered to be rich in vitamin C and in polyphenolics (Määttä-Riihinen et al., 2003). *In vitro* research has indicated that the phenolics present in black currants prevent oxidative damage to neuroblastoma and promyelocyte cells (Anttonen et al., 2006).

Figure 1.4: Black currant

Lingonberry: Lingonberries, which like blueberries and black currants contain phenolic compounds, are favoured by many Northern Europeans, and are made edible in a variety of ways such as jam, jelly, pie etc. (Ek et al., 2006). Commercial harvesting is done in Newfoundland, where the berries are known as "Partridgeberries" (Hendrickson, n.d.). With the average harvest of 96,500 kg a year, Newfoundland provides more of these berries than any other North American source (Hendrickson, n.d.). Lingonberry is becoming popular as a natural remedy in treating infections of the urinary tract (Ek et al., 2006).

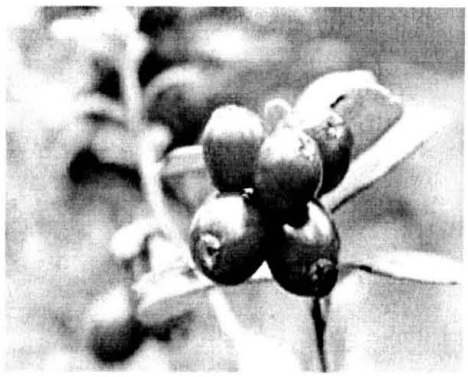


Figure 1.5: Lingonberry

1.3 HPLC analysis of anthocyanins

Currently, the phenolic compounds present in the four kinds of berries under examination, are most generally extracted with "aqueous mixture of methanol, ethanol and acetone" (Kapasakalidis et al., 2006). Additionally, as Merken and Beecher (2000) indicate that for reverse-phase HPLC the mobile phase is "acetonitrile and/or methanol" in combination with water containing an acid. The authors add that sometimes tetrahydrofuran is used as an organic solvent in the mobile phase. The most common variation found in the mobile phases was the addition of acid, which was used to reduce peak tailing. Formic or acetic acid is used most of the time (Merken et al., 2000).

It has been found that when anthocyanin is dissolved in acidic solvents, flavylium ions are produced. When examined using ESI mass spectrometer, it is evident that this process creates molecular cations, M^+ (Cooney et al., 2004). It is difficult to quantify anthocyanins by spectroscopic means as the ratio of coloured to colourless forms of anthocyanins, the solution pH and presence of co-pigments influence their molar absorptivity (Clifford, 2000).

Currently, the LC-MS method is considered to be the most acceptable method for anthocyanin analysis. Organic solvents, namely acidified water, methanol and acetone, are usually used for anthocyanin extraction, but acidified methanol shows the "highest extraction efficiency (70-100%)" (Hosseinian et al., 2007).

In addition to being able to detect these compounds, it is wise to know about the various biological functions that have an impact in cell destruction and cause of diseases. For this, it is important to know about redox homeostasis-a specific biological function described in section 1.4.

1.4 Mechanisms of maintenance of redox homeostasis

Redox homeostasis refers to a balance between reactive oxygen species (ROS) and various kinds of scavengers present in the biological system (Dröge, 2002). The mechanism of redox homeostasis depends upon "ROS mediated induction of signal cascades" (Dröge, 2002), which plays a role in increasing the antioxidative enzyme concentration as well as in the amount of glutathione (an important endogenous antioxidant) available in the cell (Dröge, 2002). In aerobic organisms energy is produced in the mitochondria via the electron transport chain (Valko et al., 2007). Additional ROS, produced in the mitochondria, "can damage cellular lipids, proteins or DNA" (Valko et al., 2007). In living organisms "redox regulation" creates a balance between the beneficial and deleterious impacts of free radicals and in these ways protects cells from certain oxidative stresses while maintaining "redox homeostasis" (Dröge, 2002). Schafer and Buettner (2001) report that the presence of disease can also influence the redox

potential. However, an imbalance between cellular ROS production and antioxidant activity of cells may lead to various disease states (Thannickal and Fanburg, 2000; Djordjević et al., 2006). Only knowing redox homeostasis is not sufficient enough to understand it properly. For this, it is better to have a good knowledge of ROS and RNS as described in section 1.4.1 and 1.4.2.

1.4.1 Reactive oxygen species

In biological systems, ROS have both beneficial and detrimental effects. It has been found that formation of a superoxide radical occurs upon addition of one electron to molecular oxygen (Miller et al., 1990). Superoxide being produced through metabolic processes is known as the primary ROS, which can produce secondary ROS by interacting with other molecules using "enzyme or metal catalyzed processes" and can further interact in-directly through enzyme or metal catalyzed processes (Valko et al., 2007). Superoxide is mainly produced in the cellular mitochondria. "The electron transport chain", Valko et al. (2007) pointed out, is where most of the ATP in the cells of mammals is generated – ATP being a chemical necessary for survival of mammalian life. While energy transduction proceeds, the superoxide is formed with premature leakage of some electrons to oxygen. (Valko et al., 2007). De Gray is cited as reporting that superoxide in its protonated form is known as simple peroxyl radical and is generally expressed as " hydroperoxyl radical or perhydroxyl radical" (Valko et al., 2007). However, fatty acid peroxidation is initiated by hydroxyl peroxy radicals, through "two

parallel pathways [i.e.] fatty acid hydroperoxide (LOOH)-independent and LOOH-dependent" (Aikens and Dix, 1991).

1.4.2 Reactive nitrogen species

$NO^{\cdot}$ is considered to be a reactive nitrogen species (RNS) and its antibonding $2\pi^{*}_{v}$ orbital contains one unpaired electron. Certain enzymes known as nitric oxide synthases (NOSs) are responsible for production of nitric oxide radicals in the cell (Ghafourifar and Cadenas, 2005). Additionally, $NO^{\cdot}$ plays an important role in a large number of physiological functions such as "neurotransmission, blood pressure regulation, defense mechanisms, smooth muscle relaxation and immune regulation" (Bergendi et al., 1999). $NO^{\cdot}$ also has an impact on the central nervous system as it influences neurotransmission and "synaptic plasticity" (Valko et al., 2007). It has been observed that $NO^{\cdot}$ reacts with oxygen and water present outside the cell and forms two anions, namely nitrate and nitrite (Valko et al, 2007).

Nitrosative stress occurs when a system produces excess RNS, but the system is neither capable of neutralizing nor discarding those extra RNS molecules that are produced (Klatt and Lamas, 2000; Ridnour et al., 2004). Nitrosylation reactions can occur due to nitrosative stress (Valko et al, 2007). This can bring change in the structure of proteins and thus can disrupt normal protein function.

Superoxide, as well as $NO^{\cdot}$, is also generated by the body's immune system when inflammation occurs. At this phase, a reaction occurs between $NO^{\cdot}$ and superoxide which produces peroxynitrite anion ($ONOO^{-}$). Peroxynitrite has a deleterious effect on DNA

and also can lead to oxidation of lipid. (Carr et al., 2000). So, it can be assumed that $NO^{\cdot}$ toxicity occurs while it combines with "superoxide anions" (Valko et al., 2007). In addition it is also important to understand oxidative stress in order to prevent cellular damage as described in section 1.4.3

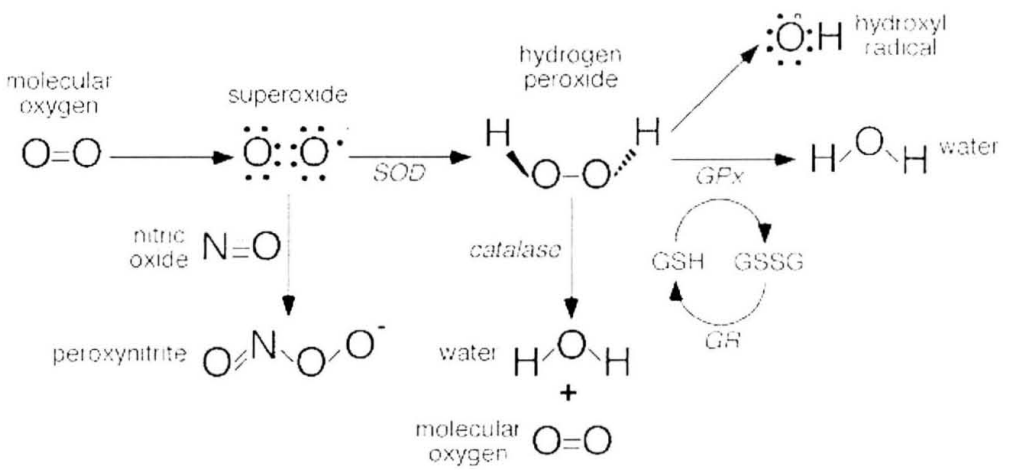
1.4.3 Oxidative stress

ROS is generally produced by normal metabolic processes of cells. ROS, when produced excessively, can have detrimental effects on lipid, proteins or DNA present in the cell (Valko et al., 2007). Freeman et al. (1982) and Halliwell et al. (1985) report that the cellular defense mechanism has a role in neutralizing ROS production in the cell. However, about 1% ROS do not get neutralized by the cellular systems and cause damage to cellular components. This phenomenon, in which ROS are not totally neutralized and cause harmful effects to the cellular body is known as oxidative stress (Lau et al., 2005). By targeting this aspect of metabolism cellular damage can be decreased in a number of diseases.

As oxidative stress causes cellular damage due to excessive ROS production, and the berries researched are considered to have antioxidant properties, the berries under examination could protect the cells and also prevent various diseases. Thus, it is also very important to know about antioxidants present in the living system and how they prevent oxidative stress.

1.5 Antioxidants

There are several different mechanisms that cells use to combat oxidative stress. These include preventive and repair mechanisms, as well as physical and antioxidant defenses. There are several different types of enzymatic antioxidant systems in particular which include "superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT)" (Valko et al., 2007) (See Figure 1.6). There are also non-enzymatic antioxidants namely α-tocopherol (Vitamin E), ascorbic acid (Vitamin C), Glutathione (GSH) as well as carotinoids and flavonoids (Valko et al., 2007). Upon oxidation glutathione is converted to glutathione disulphide (GSSG). Glutathione is available as a soluble antioxidant in various cellular components such as cytosol, nuclei, and mitochondria (Valko et al., 2007). GSH is generated due to the activity of "glutamate–cysteine ligase and glutathione synthetase" (Valko et al., 2007).



From Slemmer et al., 2008

Figure 1.6: Production of superoxide, peroxynitrite and hydrogen peroxide

(SOD-Superoxide dismutase, GSH- Glutathione, GSSG- Glutathione disulphide, GPx- Glutathione peroxidase, GR- Glutathione reductase)

However, in mitochondria GSH is present through an "inner membrane transport" system (Valko et al., 2007). Maintenance of redox regulation of protein sulphydryls is carried out by GSH present in the nucleus. This is essential for repairing of DNA inside the cells. The GSH and GSSG ratio also helps to define oxidative stress produced in the organism. It has been found that GSSG in high concentration has negative effects on many enzymes (Nogueira et al., (2004); Jones et al., (2000)). Masella et al. (2005) mentioned that the active form of vitamin C and E (a potent antioxidant) can be revived by glutathione. However, Valko et al. (2007) mention that both enzymatic and non enzymatic antioxidants play various roles in protecting against oxidative stress.

1.6 Oxidative stress and brain aging

Brain aging refers to decay in normal brain function due to various factors such as oxidative stress over a long period of time (Lau et al., 2005). Different evidence shows that oxidative stress has a detrimental effect on the brain. The brain uses about 20% of the oxygen consumed by biological systems. The brain cannot defend properly against various forms of oxidative stress due to lack of antioxidants present within it. The brain shows less catalase function and two antioxidant enzymes - namely superoxide dismutase and glutathione peroxidase - are also present in limited quantity (Lau et al., 2005). Moreover, iron and ascorbate are present in a higher amount in the brain, and play a vital role in lipid peroxidation (Lau et al., 2005). Accumulation of iron also occurs with the aging of the brain. This can make it more likely to produce ROS (Slemmer et al., 2008).

However, when mouse brains show age-related decline in GSH, this means there are increases in oxidative stress due to age (Slemmer et al., 2008). It has been found that neuroinflammation can decrease normal brain activity, which can cause aging of the brain. The neuroinflammation caused by oxidative stress can cause neurological disorders like AD and PD (Lee et al., 2010; Tansey and Goldberg, 2010). During normal aging, some behavioural deficiencies occur, and for this oxidative stress as well as inflammation are potentially responsible (Lau et al., 2005). Through oxidative phosphorylation and the electron transport chain, ATP is produced, which causes ROS and RNS production. If mitochondrial ROS and RNS production are high in aged neurons then this can be caused by cellular dysfunction. This can be harmful for mitochondria or other cellular components like nuclear DNA and proteins (Slemmer et al., 2008).

1.7 Effects of polyphenols and berries on brain aging

It has been well established that oxidative stress has a vital role in aging and brain aging in particular. The excessive ROS produced in oxidative stress is responsible for cellular damage. However, AD and PD are linked to excessive oxidative stress (Lau et al., 2005). As previously mentioned, cellular defense mechanisms have an important role in neutralizing ROS but they cannot neutralize all excessive cellular ROS. For that reason, it is wise to have nutritional antioxidants to fight against oxidative stress. It has been observed that natural products such as fruits and vegetables enriched with polyphenolic compounds help to enhance brain activity (Lau et al., 2005). It has also been found that rodents treated with blueberries and other berries show neuroprotection and more brain

function than normal (Lau et al., 2005; Giacalone et al., 2011). From our research and the research of others, it can be said that the berries enriched with polyphenolic compounds can give neuroprotection and thus can slow the progression of brain aging.

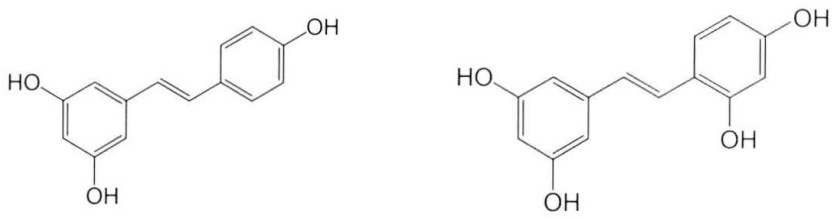
1.8 Traumatic brain injury and its perspective

Traumatic brain injury (TBI) refers to the brain damage that occurs due to several insults either physical or mechanical. At present, TBI has become one of the major causes of death of people in the west (Jennet 1996). In the UK around 200-300 per 100,000 people need to be hospitalized per year as a result of TBI (McGregor and Pentland, 1997). The situation is more numbers in South Africa and southern Australia (Hillier et al., 1997). A recent study in Ontario suggests that mild TBI may have an incidence rate as high as 535/100,000 people per year (Ryu et al., 2009). TBI mainly consists of two phase i.e. primary and secondary damage phases. Primary damage happens at the beginning of the trauma which includes injury and hemorrhage, whereas secondary damage does not appear immediately. Secondary damage also causes alterations in neuronal functions and includes activation of different enzymes, swelling, as well as ischemia. Thus it causes brain damage. (McIntosh et al., 1996; Bouma et al., 1992; Van santBrink et al., 2002). However, for secondary damage ischemia is considered as one of the important functional matters due to injury.

Due to trauma, excessive $NO^{\cdot}$ is produced and thus glutamatergic membrane receptors get over-activated (Weber 2004). The extra amount of $NO^{\cdot}$ created in the cell reacts with $O_2^{\cdot -}$ and forms $ONOO^{-}$, which is very harmful to cellular components

(Dawson, 1999: Liu et al., 2002). NOS gets over-activated after injury and also $O_2^{\cdot -}$ is produced by arachidonic acid metabolism and mitochondrial damage. SOD becomes neutral due to saturation. Therefore, a higher amount of $ONOO^-$ forms, causing harm to neurons (Slemmer et al., 2008).

Different experimental evaluations of TBI show that free radical scavangers or antioxidants, have positive effects on the brain following a stroke (Slemmer et al., 2008). Berries contain flavonoids having antioxidant properties, which suggests that they can have an impact on brain trauma or injury, and stroke as well. The hydroxyl stilbene oxyresveratrol (OXY) is a potent antioxidant obtained from mulberry wood (*Morus Alba* L.) (Weber et al., 2012). It has a similar structure to that of resveratrol but has one additional hydroxyl group at position 2 on the stilbene molecule (Slemmer et al., 2008). Due to this difference, the antioxidant capacity of OXY is higher than that of resveratrol, as suggested by Lorenz et al. (2003). OXY crosses the blood brain barrier (BBB) in a low amount. However, Breuer et al. (2006) found that OXY penetrates the BBB to more of an extent in animal brains suffering from stroke. It has also been observed that when neurons suffer damage through stroke, OXY gives some protection (Andrabi et al., 2004). Recent studies from our lab have found that OXY has a role in protecting cortical neurons from trauma using an *in vitro* model of mechanical strain injury, which is further described in section 1.9 (Ellis et al., 1995; Weber et al., 2012).



I II

Figure 1.7: Chemical structure of Resveratrol (I) and Oxyresveratrol (II)

Studies are now showing that diet can affect the outcome of neurological syndromes as damaging as TBI and stroke. For example, Sweeney et al. (2002) showed that feeding rats a diet enriched with blueberries for just six weeks decreased damage produced by ischemic stroke. In another study researchers documented how three groups of rats whose diets were supplemented with blueberries, spinach, and spirulina, respectively, all suffered less brain cell loss and were better able to recover lost function following artificially induced ischemia, than rats in a non-supplemented control group (Wang et al., 2005). Recently, Cole et al. (2010) proved that branched chain amino acids (leucine, isoleucine and valine) could improve the recovery of rats after TBI *in vivo*. This finding shows that diet or dietary constituents can affect the nature of outcome after TBI. Taken together, these findings suggest that antioxidant compounds found in various species of berries may protect against insults as severe as TBI.

1.9 Cell culture techniques and *in vitro* trauma model

Growing dissociated cells from nervous system tissue is a common technique used in neuroscience research. Cells can be isolated from various areas of the brain in rodents (Figure 1.8), including the hippocampus, cerebellum and cortex and then grown in a

culture system (Slemmer et al., 2002; Slemmer et al., 2004; Engel et al., 2005). Neurons and glial cells can be grown for several days in culture wells and viability can be measured. Berry extracts can be applied in order to investigate the pharmacological effects on cell death and aging.

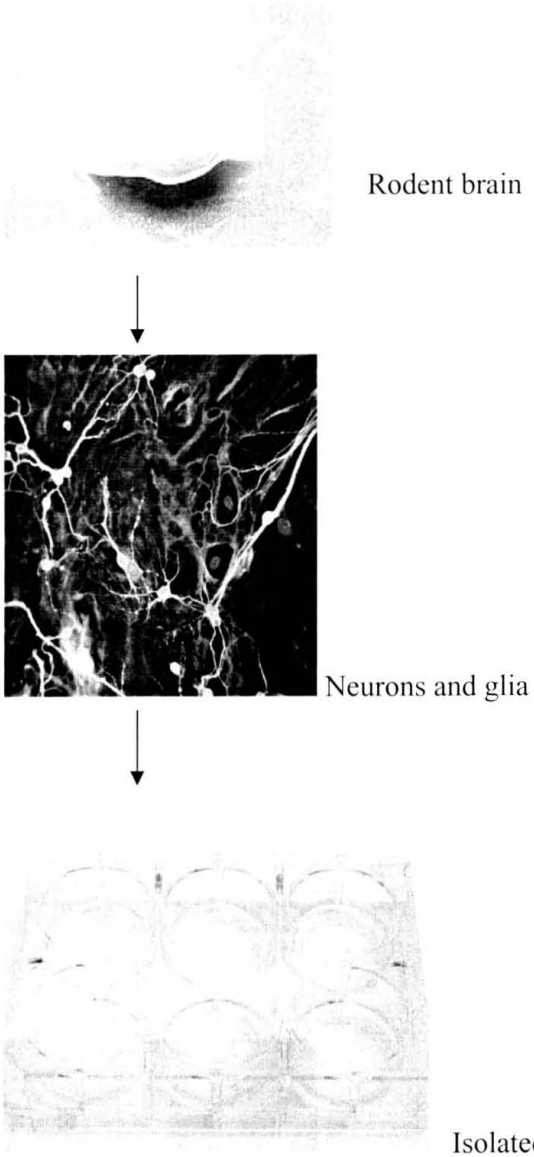


Figure 1.8: Preparation of cortical cultures. Cells are dissected from rat brains and grown in culture wells.

For injury, cell cultures are grown on deformable silastic membranes in 6-well FlexPlates (as shown in Figure 1.8). A 50 ms pulse of compressed gas is delivered to the wells, which deforms the silastic membrane and adherent cells to varying degrees controlled by pulse pressure (Figure 1.9). The extent of cell injury depends on the degree of deformation, or stretch. This model was developed by Ellis et al. (1995). An example of control (uninjured) and injured cell cultures is shown in Figure 1.10.

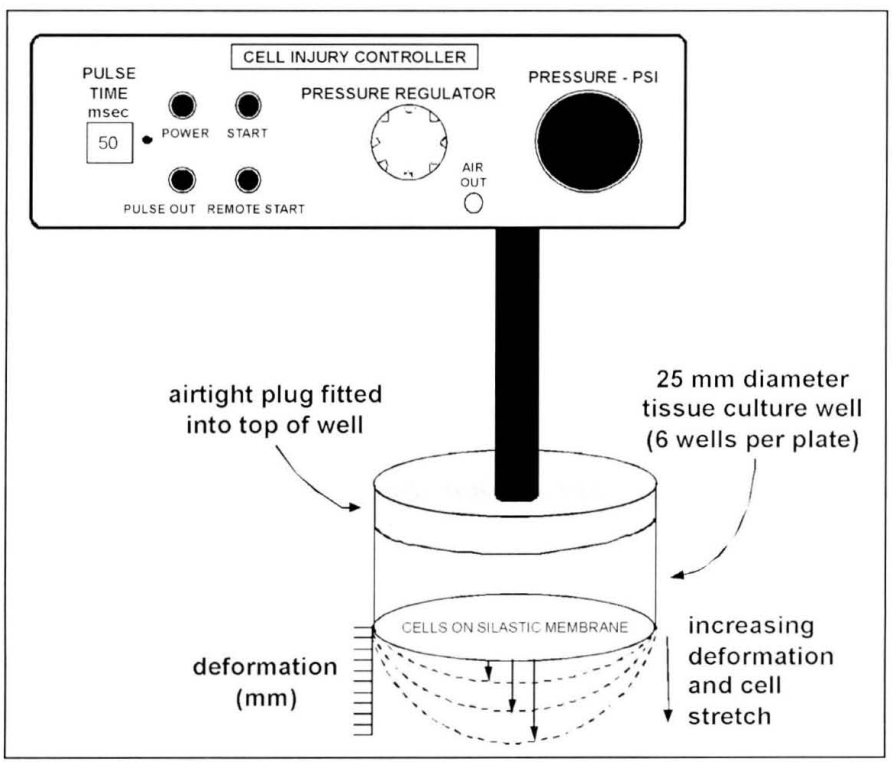


Figure 1.9: *In vitro* trauma model (From Ellis et al., 1995)

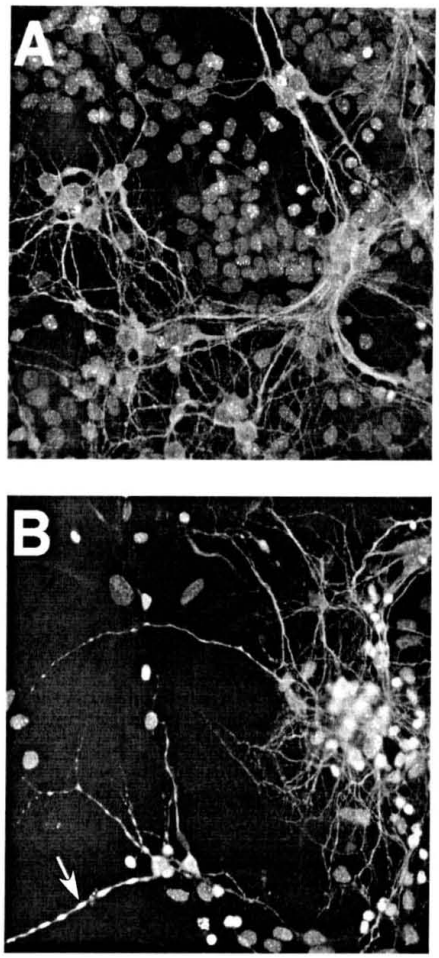


Figure 1.10: Neurons and glia in mixed cultures A: Representative image of microtubule associated protein (MAP-2) stained neurons (red) and DAPI-labeled nuclei (blue, representing neurons and glia) from an uninjured culture well, demonstrating intact soma and smooth neurites. **B:** Image from a well 24 hr post injury. Arrow points to beaded neurites. (Images are from Engel et al., 2005)

1.10 Study objectives

The aims of this study were to:

- analyze the various chemical components present in extracts of Newfoundland and Labrador berries;
- quantify the amounts of specific compounds i.e. delphinidin-3-glucoside and cyanidin-3-galactoside in berry extracts; and
- determine the biological activity of bilberry extracts against traumatic injury and aging in cell cultures derived from rat brain.

Note that the amount of specific compounds in berry extracts has been determined by running standards and quantifying the amount from standard curves. In this research work, a suitable solvent was chosen for extraction of the berries and then various components were identified. A major problem is that while running samples on the LC-MS trap machine the samples showed different peak retention times. So, for those circumstances, several samples have been run to confirm the correct retention time. The use of organic solvents in RP-HPLC has permitted the separation of several active compounds from different berries.

After chemical analysis, some pharmacological studies have been carried out using cell cultures of rat brain cortical cells. This work reveals information about the neuroprotective potential of berry extracts. In cell culture, rat brain cells have been grown and trauma has been induced using a mechanical *in vitro* model. In some experiments, bilberry extracts were added to cell culture media before injury. Measurement of lactate has been performed,

as well as analysis of markers of cell death, in order to evaluate protective properties of berries. In addition, neurons and glia have been counted in order to determine the effects of berries on cell aging *in vitro*.

Chapter 2

Materials and Methods

2.1. Materials:

1. Berry samples: Powdered samples of bilberry, blueberry, black currant and lingonberry were donated by Natural Newfoundland Nutraceuticals (NNN), Markland, NL. Fresh samples of blueberries were collected from Pippy Park in St. John's, Newfoundland and central Newfoundland.

2. Standard samples delphinidin-3-glucoside and cyanidin-3-galactoside, were purchased from Chromadex Inc. Santa Ana, California, USA.

3. Solvents: a) methanol (HPLC grade), ethanol (95%), acetone (HPLC grade) and formic acid were from Caledon Laboratories, Georgetown, Ontario, Canada. b) Conc. hydrochloric acid was from Sigma Aldrich, Catalogue no.435570, Oakville, Ontario, Canada.

4. Culture materials: a) basal medium Eagles (BME), GIBCO, Grand Island, NY containing 10% horse serum (GIBCO); b) 10 µg/mL gentamycin (Sigma, St. Louis, MO, USA); c) 0.5% glucose (Sigma); d) 1mM sodium pyruvate (GIBCO) and 1% N_2 supplements (GIBCO); e) Flexplates (FlexCell, Hilsbourough, NC); f) Poly-L-ornithine (Sigma).

5. Immunohistochemistry materials: a) Paraformaldehyde (Acros, New Jersey, USA); b) TritonX (Sigma); c) 4', 6-diamidino-2-phenylindole (DAPI) (Invitrogen, Eugene, Oregon, USA); d) anti-microtubule-associated protein 2 (MAP2) antibody (Sigma); e) Alexa-

594-conjugated-goat-anti-mouse antibody (Invitrogen, Eugene, Oregon, USA); f) VectaShield (Vector Laboratories Inc., Burlingame, CA 94010).

2.2 Methods:

2.2.1 Preparation of bilberry, blueberry, black currant and lingonberry extract from powdered berry samples

For preparation of each extract, 5.0 g of sample was weighed exactly and transferred into a 125 mL conical flask. Then, 20.0 mL of extract ion solution ($MeOH/H_2O/HCOOH$ = 60:37:3) was added using a 100 mL measuring cylinder. Up to this point we used the methodology similar to that of Cho et al., (2004). Sonication was performed for 30 minutes using a Fisher Inc. (USA) sonicator (model FS110H). After sonication the volume of the extract was 18±1 mL. The sample extract was transferred into a 50 mL Falcon tube and centrifuged for 30 minutes at 3000 rpm (rotor size 26 cm), after which the solid was precipitated at the bottom of the tube. The entire extraction process was repeated twice with solid samples. Supernatant was collected and subjected to rotary evaporation under vacuum using a Büchi rotary evaporator at 45°C until 95% of its original volume was achieved. This was done due to the fact so that no solvent will remain in the sample solution. Four mL of the sample solution was then combined with one mL of 3% formic acid using 5 mL volumetric flask as was conducted in Cho et al. (2004). Finally, the solution was filtered using a 0.45 micrometer nylon filter.

2.2.2 Preparation of extracts obtained from wild blueberry from central Newfoundland and Pippy Park, St. John's, Newfoundland

Samples of wild blueberries were collected and blended to make puree, and 10 g of the puree was placed in a 125 mL conical flask. From this point the same methodology was followed as that of section 2.2.1.

2.2.3 Preparation of delphinidin and cyanidin standards

Both delphinidin-3-glucoside (1.4 mg) and cyanidin-3-galactoside (1.5 mg) were dissolved in 10.0 mL of 3% formic acid. A serial dilution was prepared for both of the standards for the concentrations of 25 µg/mL, 50 µg/mL, 100 µg/mL, 150 µg/mL and 200 µg/mL using 3% formic acid solution. For each different concentration the retention time peak was determined three times. The peak was identified using a liquid chromatography mass spectrophotometer (LC-MS) instrument. Finally, the relative standard deviation (RSD) was determined for each concentration.

2.2.4 HPLC analysis of the berry extract samples

Berry extract samples (15 µL) were analyzed using an Agilent 1100 series HPLC system equipped with a model G1311A Quaternary pump (SL No. DE 40926119). Separation was carried out using a 3.9 mm x 150 mm Symmetry C18 (5 µm) column (SL No. W31761L 008) (Agilent technologies). The system was equilibrated for 20 min at the

primary gradient before each injection. LC flow rate was one mL/min. Detection wavelengths for flavonols used were 360 nm and for anthocyanins 520 nm. For our analysis, we have used similar MS parameters to those developed in Cho et al., (2004). For MS we used the ESI mode. Both positive and negative ionization modes were applied. The parameters were capillary voltage 4.0 kV, nebulizing pressure 30.0 psi, drying gas flow 9.0 mL/min and the temperature was 300°C. Data collection was performed at 1.0s per cycle with a mass range of m/z 100-1000.

2.2.5 Cell culture

Primary cortical cell cultures (10% neurons, 90% glia) were prepared from rat pups (P1-P3). Dissociated cortices were diluted in serum-containing growth media [basal medium Eagles (BME; GIBCO, Grand Island, NY) containing 10% horse serum (GIBCO), 10 µg/mL gentamycin (Sigma, St. Louis, MO), 0.5% glucose (Sigma), 1mM sodium pyruvate (GIBCO) and 1% N_2 supplements (GIBCO)] to a concentration of 500, 000 cells per mL. Cells were plated in 1 mL aliquots onto collagen-coated six-well FlexPlates (FlexCell, Hilsbourough, NC), coated overnight with poly-L-ornithine (500 µg/mL; Sigma). All cultures were maintained in a humidified incubator (5% CO_2, 37°C) until use in experiments. Neuron-enhanced cultures were obtained by replacing half of the media two days after plating and then twice a week thereafter, with serum-free media containing 2% B27 (GIBCO). Glia formed a confluent monolayer and adhered to the membrane substrate while neurons adhered to the underlying glia. Cells were used for experiments after 10-20 days *in vitro* (DIV).

2.2.6 Cell injury

Primary cortical cultures were stretch-injured using a model 94A Cell Injury Controller developed by Ellis et al., (1995). In brief, this stretch-injury entails a rapid and transient deformation in the silastic membrane of the FlexPlate caused by a 50-ms pulse of compressed gas. The degree of injury depends on the extent of deformation or stretch of the membrane. Based on previous work, we used levels of cell stretch equivalent to 5.5 mm deformation (31% stretch) and 6.5 mm deformation (38%), which are defined as mild and moderate levels of injury, respectively.

2.2.7 Immunohistochemistry and cell counts

Immunohistochemistry uses specific antigen-antibody binding to identify antigenic sites in a tissue or subcellular region. This project makes use of the indirect method of antibody labeling. In this indirect method, two sets of antibodies are used together. First, the primary antibody (which is not conjugated to any label) is applied. The secondary antibody is applied next. The secondary antibody reacts with the primary antibody. The primary antibody used in these experiments is a monoclonal antibody. It is highly specific, but requires the use of a secondary antibody conjugated to a fluorescent label, which is designed to react with the primary antibody.

Cell cultures were fixed with 4% paraformaldehyde for 20 min at 15 min after injury, 24 h after injury, or at various DIV (for aging experiments similar to the techniques of Engel et al., 2005). Cells were permeabilized with 0.2% TritonX for 10 minutes. Triton essentially works by putting holes in the cell membranes so the antibodies are able to

enter. Blocking solution was then added and cells held for 30 minutes. Blocking solution prevents non-specific binding to the antibody. Cultures were incubated in primary antibody, anti-microtubule-associated protein 2 (MAP2, 1:500), for one hour. Cultures were next incubated with secondary antibody (Alexa-594-conjugated goat-anti-mouse, 1:300) for one hour. Cultures were dehydrated with ethanol and mounted with VectaShield mounting medium with 4', 6-diamidino-2-phenylindole (DAPI). The anti-MAP2 is a mouse monoclonal antibody that labels the dendrites and somas of the neurons and DAPI labels the nuclei of all cells. Fluorescent images were captured and analyzed using a Zeiss Examiner D1 microscope. MAP2-positive neurons and total cell numbers were counted at a magnification of 100x or 160x in five continuous fields. An average of these five images was determined as the cell number or amount of MAP-2 positive neurons in that specific well.

2.2.8 Biochemical measurements

One hundred μL of media was removed from culture wells at either 15 min or 24 h after injury and stored at -20˚C until the day of measurement. Levels of lactate in media were determined in two samples (5 μL each) from each 100 μL media sample using a a GM7 biochemical analyser (Analox Instruments®, Lunenburg, MA). The GM7 analyser functions by measuring the oxygen change when oxidoreductase enzymes (oxidases) react with their substrates under controlled, semi-anaerobic conditions. Oxygen change is measured with an oxygen sensor, with the total change and rate-of-change both being directly proportional to the substrate that was initially present. L-lactate:oxidoreductase

catalyses the oxidation of L-lactate to pyruvate, and under the controlled conditions of the GM7 analyser, the maximum rate of oxygen consumption is directly related to lactate concentration. The lactate levels determined in both 5 µL samples were then averaged to provide an estimate of lactate levels for each culture well. Data is expressed as mmol/L.

2.2.9 Data analysis

The data associated with delphinidin-3-glucoside and cyanidin-3-galactoside standards were statistically analyzed and the percentage RSD was determined. For concentrations of 25 µg/mL, 50 µg/mL, 100 µg/mL, 150 µg/mL and 200 µg/mL for the delphinidin-3-glucoside standard, percentage RSD was calculated as 0.15%, 0.32%, 0.24%, 0.02% and 0.55%, while for the cyanidin-3-galactoside standard with the same concentration levels the percentage RSD was calculated as 1.13%, 1.04%, 1.39%, 1.66% and 0.58%.

The data associated with cell cultures were analyzed using the statistical program Graphpad prism (Graphpad Software Inc., San Diego, CA). Data were computed as mean ± standard error of the mean (SEM) values. Statistical significance was established by one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test. Data are considered significant at $p < 0.05$.

Chapter 3

Results

Chemical analysis

3.1 Analysis of bilberry extracts

In this chapter we describe chemical analysis and our findings for various compounds present in the berry samples analyzed. Various HPLC chromatograms and their corresponding MS values were compared with values available in the literature for identification purposes. We were able to confirm and quantify two compounds detected by running two standards. For the chemical analysis, various solvent extraction methods were used and we tried to determine how the solvent systems act in extracting different compounds, as well as their role in varying HPLC retention time. However, we tried to keep the MS parameters the same for all analyses.

In chapter two, we have described the method used by Cho et al. (2004) for extraction of different berry samples. However, this method does not give satisfactory extraction for all berry samples. Due to this, for the analysis of the bilberry extracts, the sample was first treated with acidified methanol (15% HCl in methanol). During the analysis the sample solvent ratio was 1:8. The pH was adjusted to 1.0 (25°C) using 1M HCl. We used the methodology similar to that developed by Hosseinian and Beta (2007). The sample solvent mixture was sonicated for 30 minutes and the mixture was transferred to a 50 mL Falcon tube, which was centrifuged at 3000 rpm (rotor size 26 cm) for 45

minutes. Supernatant was collected and subjected to rotary evaporation until 95% of its original volume was achieved, which was then spun at 3000 rpm for another 20 minutes. This was done so that most of the solvent was evaporated. The supernatant obtained was filtered using a 0.45 µm nylon filter prior to analysis using the LC-MS instrument. As described before we used the LC-MS parameters for our analysis. Mobile phases used were: A) 5% formic acid and B) 100% methanol. Eluent gradient was a) 0 minute - 2%B; then gradually b) 60 minute - 60%B. Different chemical compounds were identified from the HPLC peaks as shown in Figure 3.1 and summarized in Table 3.1. The HPLC peaks were identified based on comparison of detected and calculated molecular ion as mentioned widely in the literature and also due to lack of standards (Cho et al., 2004; Zhang et al., 2004).

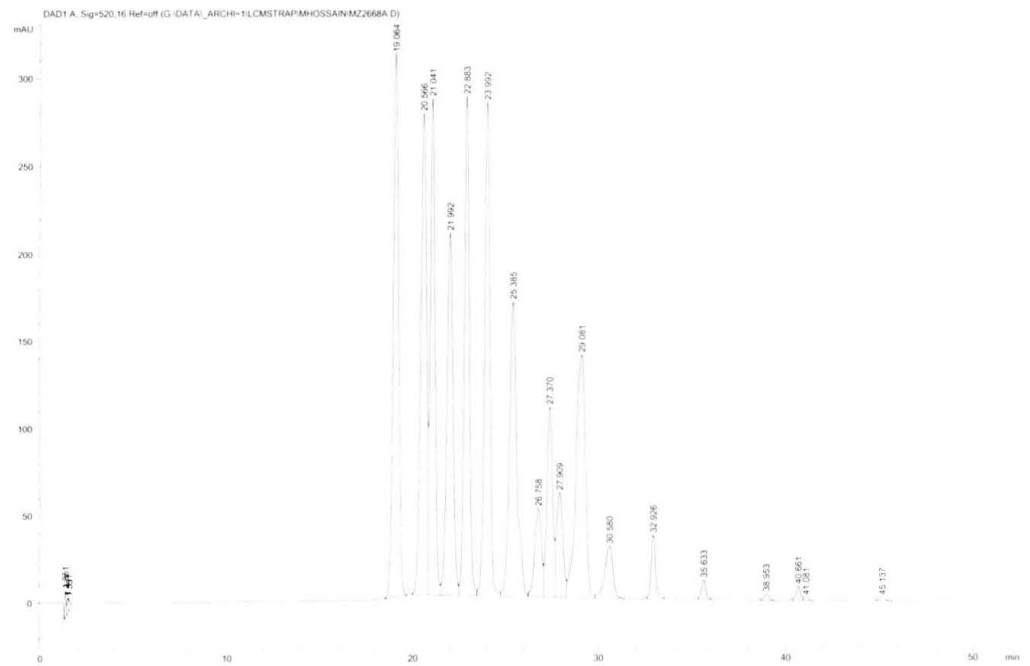


Figure 3.1: HPLC peaks detected in bilberry MeOH/HCl extracts

Table 3.1: Compounds identified in bilberry methanol extracts

No.	HPLC RT(min)	Identification	m/z values	m/z values in literature[1,2]
		Anthocyanin	$[M]^+$	$[M]^+$
1	19.06	Delphinidin-3-galactoside	465.1	465
2	21.04	Cyanidin-3-galactoside	449.1	449
3	21.99	Delphinidin-3-arabinoside	435.1	435
4	22.88	Cyanidin-3-glucoside	449.1	449
5	23.99	Cyanidin-3-arabinoside	419.1	419
6	25.39	Petunidin-3-galactoside	479.1	479
7	27.37	Peonidin-3-galactoside	463.1	463
8	29.08	Malvidin-3-galactoside	493.1	493
9	30.58	Peonidin-3-glucoside	463.1	463

There were some other unknown peaks in the HPLC chromatogram which could not be identified. [1]Cho et al. (2004), [2]Zhang et al. (2004)

In a separate experiment, bilberry samples were treated with extraction solvent [MeOH/H_2O/HCOOH (60:37:3 v/v/v)] as described by Cho et al., (2004). Sonication was then performed for 30 minutes and the mixture was transferred to a 50 mL Falcon tube, which was centrifuged at 3000 rpm (rotor size 26 cm) for 30 minutes. Supernatant was collected and subjected to rotary evaporation until 95% of its original volume was achieved, which was rotated (rpm 45) for 2 hours at 45°C. The extract was then filtered using a 0.45 µm nylon filter. Finally, bilberry extracts were analyzed using HPLC-MS. The volume of the sample injected into the column was 15 µL. Mobile phase and eluent

gradient were the same as for the previous analysis of bilberry. Conditions for MS were the same as other analyses. An example of HPLC peaks obtained from bilberry extracts is shown in Figure 3.2. Chemical compounds identified are summarized in Table 3.2. The HPLC peaks were identified based on comparison of detected and calculated molecular ion as mentioned in the literature (Cho et al., 2004; Zhang et al., 2004).

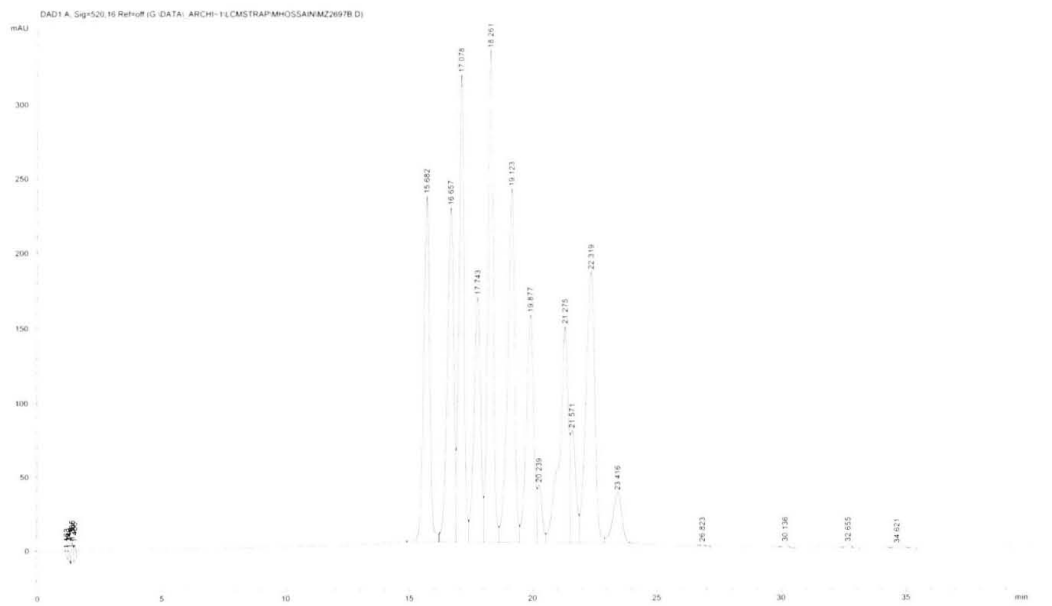


Figure 3.2: HPLC peaks detected in bilberry MeOH/H_2O/HCOOH extracts

Table 3.2: Compounds identified in bilberry MeOH/H_2O/HCOOH extracts

No.	HPLC RT(min)	Identification	m/z value	m/z value in the literature[1,2]
		Anthocyanin	$[M]^+$	$[M]^+$
1	15.68	Delphinidin-3-galactoside	465.1	465
2	16.66	Delphinidin-3-glucoside	465.1	465
3	17.08	Cyanidin-3-galactoside	449.1	449
4	17.74	Delphinidin-3-arabinoside	435.1	435
5	18.26	Cyanidin-3-glucoside	449	449
6	19.12	Cyanidin-3-arabinoside	419	419
7	19.88	Petunidin-3-glucoside	479.1	479
8	21.28	Peonidin-3-galactoside	463.2	463
9	22.32	Malvidin-3-galactoside	493.2	493
10	23.42	Malvidin-3-arabinoside	463	463

There were some other unknown peaks in the HPLC chromatogram which could not be identified. [1]Cho et al. (2004), [2]Zhang et al. (2004)

The two different solvent systems were used to determine different compounds present in the bilberry extracts. It was assumed that the compounds which were not extracted in the first solvent system (MeOH/HCl) used may be isolated in the second solvent system (MeOH/H_2O/HCOOH) and more easily detected.

3.1.1 Data analysis and calibration curve for delphinidin-3-glucoside standard:

To construct a calibration curve for the delphinidin-3-glucoside standard, data were collected at the concentrations of 25 µg/mL, 50 µg/mL, 100 µg/mL, 150 µg/mL and 200 µg/mL. Standard deviation and percentage RSD was also calculated. This data is summarized in Table 3.3.

Table 3.3 Data analysis for delphinidin-3-glucoside standard

Conc (µg/mL)	A1	A2	A3	Avg	STDEV	%RSD
25	518.39	516.88	517.36	517.54	0.77	0.15
50	1205.07	1198.53	1198.40	1200.66	3.82	0.32
100	3008.31	2993.82	3000.83	3000.99	7.24	0.24
150	4240.77	4233.19	4241.82	4238.60	0.77	0.02
200	6934.86	6984.83	7010.61	6976.76	38.51	0.55

The %RSD value for all five concentrations of 25 µg/mL, 50 µg/mL, 100 µg/mL, 150 µg/mL and 200 µg/mL is well below 5%, which seems to be very good for a creation a standard curve. The average values of all five peak areas have been plotted against all five concentrations as shown in Figure 3.3 and a linear equation was obtained. The R^2 value was also determined.

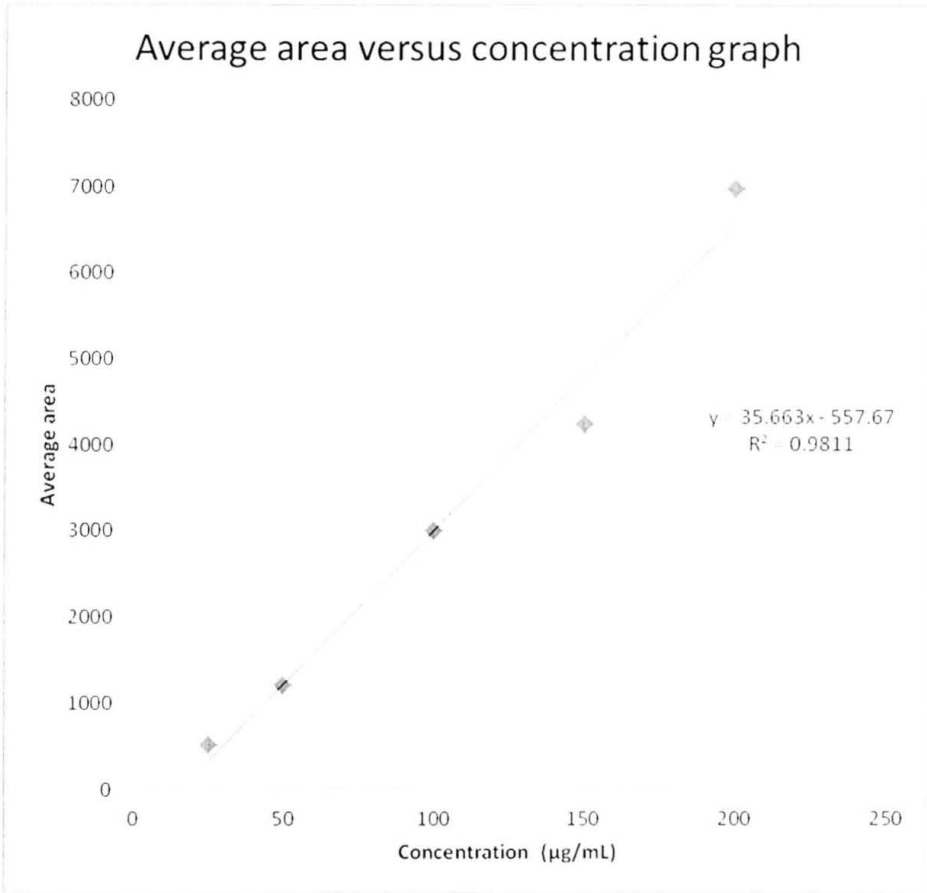


Figure 3.3: Average area versus concentration graph from HPLC analysis of delphinidin-3-glucoside standard.

3.1.2 Data analysis and calibration curve for cyanidin-3-galactoside standard:

A calibration curve for the cyanidin-3-galactoside was created using the data from standard solutions at concentrations of 25 µg/mL, 50 µg/mL, 100 µg/mL, 150 µg/mL and 200 µg/mL. Standard deviation and percent RSD was also calculated. This data is summarized in Table 3.4.

Table 3.4 Data analysis for cyanidin-3-galactoside standard

Conc (µg/mL)	A1	A2	A3	Avg	STDEV	%RSD
25	1304.93	1330.79	1330.7	1322.14	14.91	1.13
50	2166.99	2127.25	2129.98	2141.41	22.2	1.04
100	3508.19	3586.07	3600.33	3564.86	49.6	1.39
150	4179.5	4292.41	4309.57	4260.49	70.67	1.66
200	5395.9	5339.62	5390.53	5375.35	31.06	0.58

As the relative standard deviation value for all five concentrations of 25 µg/mL, 50 µg/mL, 100 µg/mL, 150 µg/mL and 200 µg/mL is below 5%, it seems to be acceptable for a standard curve. The average value of all five peak areas have been plotted against all five concentrations as shown in Figure 3.4 and a linear equation was obtained. The R^2 value was also determined.

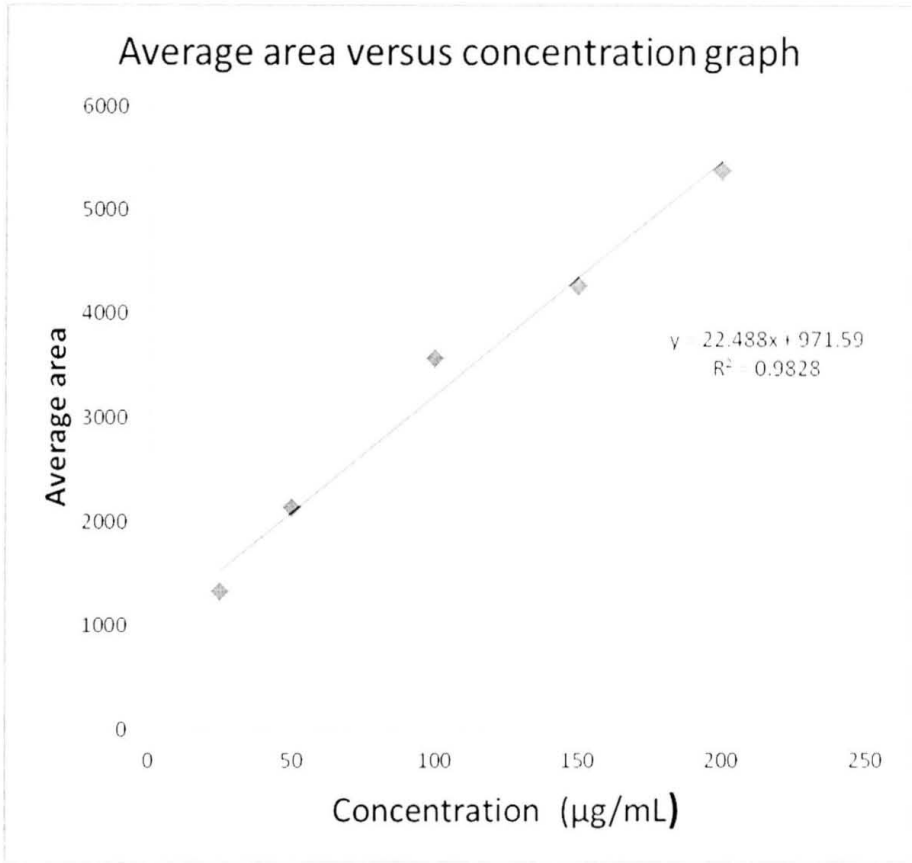


Figure 3.4: Average area versus concentration graph from HPLC analysis of cyanidin-3-galactoside standard

3.1.3 Confirmation and quantification of delphinidin-3-glucoside and cyanidin-3-galactoside in bilberry extracts:

In chemical analysis, identification alone is not sufficient enough to explain the presence of certain compounds in a sample. It needs confirmation and quantification as well. So for that reason standards were run, and compounds identified in the sample were compared with that of standard with respect to retention time and concentrations and thus confirmed and quantified. In our berry analysis, for confirmation and quantification purposes we have run two standards i.e. delphinidin-3-glucoside and cyanidin-3-galactoside. It was of interest to carry out analysis with additional standards but lack of availability and cost was a barrier. The two compounds chosen were identified in our different analyzed berry extracts and deemed significant. Delphinidin-3-glucoside and cyanidin-3-galactoside standards were subjected to LC-MS analysis to obtain their retention times. The LC conditions were the same for all analyses. The values of the retention times for delphinidin-3-glucoside and cyanidin-3-galactoside standards were 16.38 and 17.02 respectively (Figure 3.5 and 3.6).

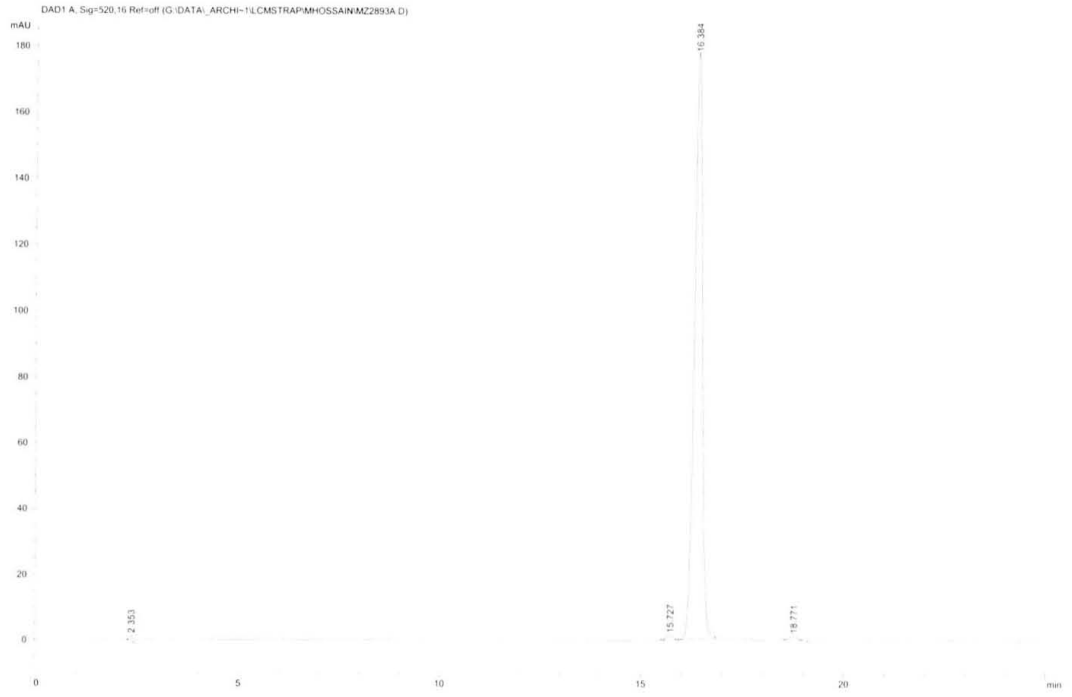


Figure 3.5: HPLC chromatogram of delphinidin-3-glucoside standard

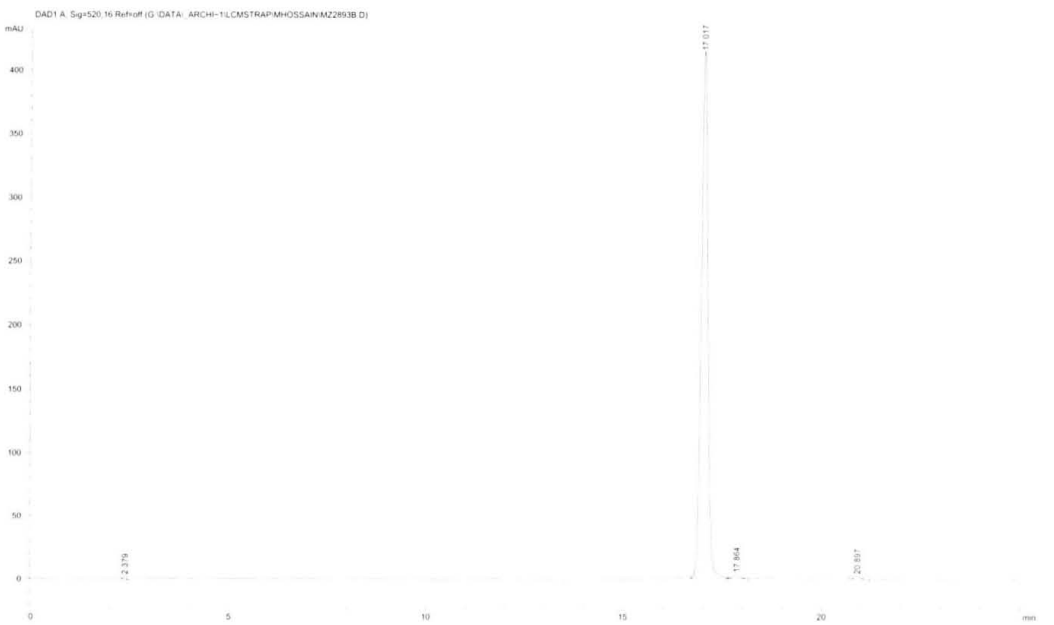


Figure 3.6: HPLC chromatogram of cyanidin-3-galactoside standard

It was found that delphinidin-3-glucoside and cyanidin-3-galactoside identified in bilberry extracts have similar retention times to that of the standards. Therefore, the retention times and m/z i.e. $[M+H]^+$ values also provide further confirmation of these two compounds. Further quantification was performed for these two compounds from the calibration curve of the standard. The retention time of the sample peak for delphinidin-3-glucoside = 16.66 minutes as shown in Figure 3.2. From HPLC analysis the peak area obtained = 4073.88. From the graph (Figure 3.3) after calculation the concentration = 129.87 µg/mL. The sample (5.07 g) was dissolved in 20.0 mL extraction solution. Therefore, in 20 mL solution the concentration is:

= (129.87x20 µg)

= 2597.4 µg

=2.6 mg

Thus, the concentration of delphinidin-3-glucoside in 100 g of extract is 51.8 mg or 0.051%.

The retention time of the sample peak for cyanidin-3-galactoside = 17.08 minutes as shown in Figure 3.2. From HPLC analysis the peak area obtained = 4451.68. From the graph (Figure 3.4) after calculation the concentration = 154.75 µg/mL. Sample amount = 5.07 g. Extraction solvent amount = 20 mL. Therefore, in 20 mL solution the amount of cyanidin-3-galactoside present = 3095.06 µg or 3.10 mg

Therefore, the concentration of cyanidin-3-galactoside in the sample is 0.061%

3.2 Analysis of blueberry extracts

The blueberry extracts were first treated with 40 mL of 2M HCl in ethanol. Hydrolysis was performed at 90°C in an oil bath for 90 minutes to break the glycoside linkage. We used a method similar to Nyman et al., (2001). Hydrolysis was conducted to produce the flavylium ion (aglycon part) which is identified in the MS system. After hydrolysis, the extract solution was diluted and filtered using a 0.45 micrometer nylon filter. The sample was then analyzed using a LC-MS instrument. Conditions for MS were the same as those of other analyses. An example of HPLC peaks obtained from blueberry extracts is shown in Figure 3.7. Different compounds identified are summarized in Table 3.5. The HPLC peaks were identified based on comparison of detected and calculated molecular ions as mentioned in the literature (Cho et al., 2004, Hosseinian and Beta, 2007). It is to be noted that there is a lack of reproducibility in exact retention times as these may be influenced by room temperature, column, solvent system etc.

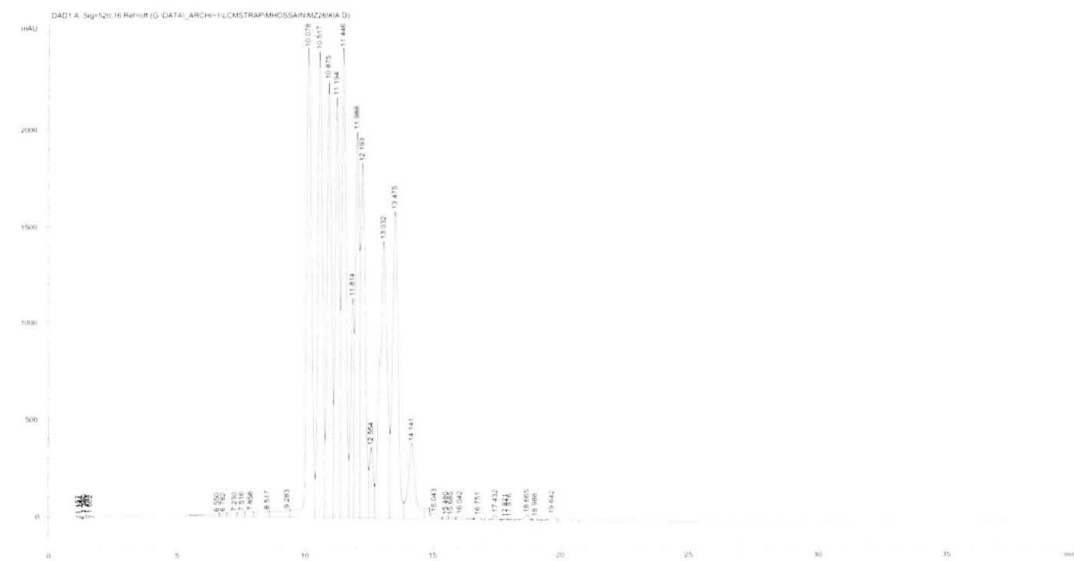


Figure 3.7: HPLC peaks for different components of blueberry ethanol extracts

Table 3.5: Various chemical compounds identified in blueberry ethanol extracts

No.	HPLC RT(min)	Identification	m/z value	m/z value in literature[1,2]
		Anthocyanins	$[M]^+$	$[M]^+$
1	10.08	Delphinidin-3-galactoside	465.1	465
2	10.52	Delphinidin-3-glucoside	465.1	465
3	10.88	Cyanidin-3-galactoside	449.1	449
4	11.19	Delphinidin-3-arabinoside	435.1	435
5	12.19	Petunidin-3-glucoside	479.1	479
6	13.48	Malvidin-3-glucoside	493.1	493
7	14.14	Peonidin-3-glucoside	463.1	463

There were some other unknown peaks in the HPLC chromatogram which could not be identified.
[1]Cho et al. (2004), [2]Hosseinian and Beta. (2007)

In a separate experiment, blueberry extracts were first treated with acidified methanol (20 mL) [MeOH in 1M HCl (85:15 v/v)]. In this experiment the sample solvent ratio was 1:8. We used the method similar to that of Hosseinian et al. (2007). This experiment was conducted in order to evaluate whether any additional compounds would be detected using acidified methanol rather than acidified ethanol as the solvent due to polarity. Acidified methanol also acts as a very good solvent system for anthocyanin compounds. The pH was adjusted to 1.0 and for this 1M HCl was used. Sonication was performed at room temperature for 45 minutes. Supernatant was collected and subjected to rotary evaporation until 95% of its original volume was achieved, which was spun at 3000 rpm for 20 minutes. The extract was filtered using a 0.45 μm nylon filter. Finally, blueberry

extracts were analyzed using a LC–MS instrument. The method is similar to Cho et al. (2004). Various chemical compounds were identified from the LC peaks as shown in Figure 3.8 and summarized in Table 3.6. The HPLC peaks were identified based on comparison of detected and calculated molecular ion as mentioned in the literature. (Cho et al, 2004; Hosseinian and Beta, 2007).

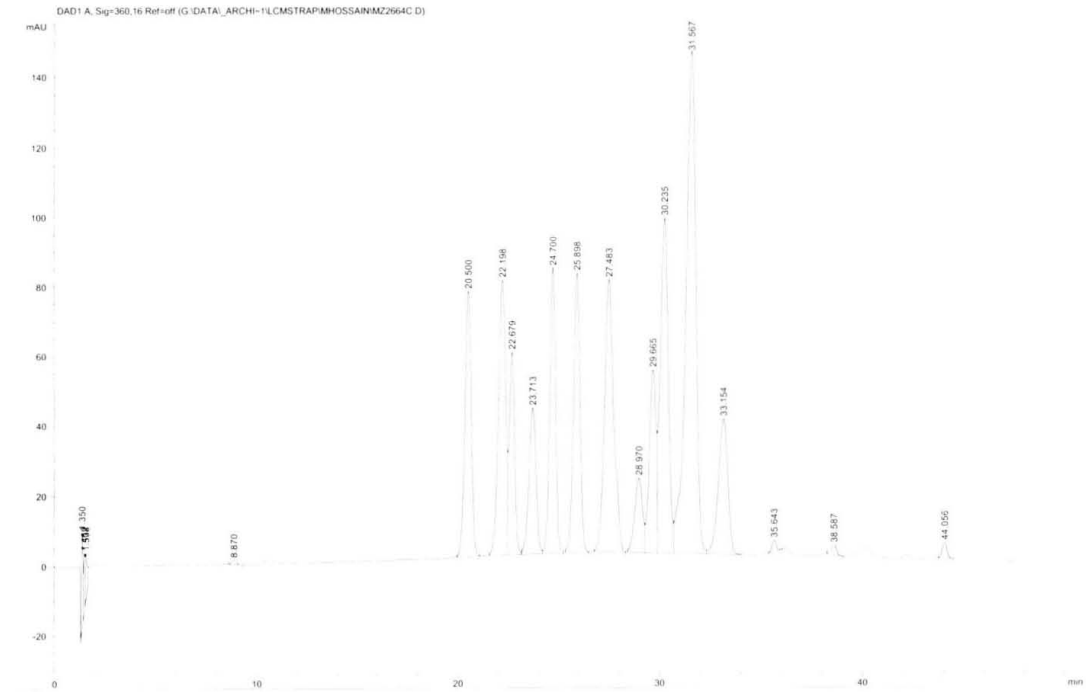


Figure 3.8: HPLC peaks for different components of blueberry methanol extracts

Table 3.6: Compounds identified in blueberry methanol extracts

No.	HPLC RT(min)	Identification	m/z value	m/z value in literature[1,2]
		Anthocyanins	$[M]^+$	$[M]^+$
1	22.68	Delphinidin-3-galactoside	465.1	465
2	23.71	Delphinidin-3-arabinoside	435.1	435
3	24.70	Cyanidin-3-galactoside	449.1	449
4	25.90	Petunidin-3-galactoside	479.1	479
5	30.24	Malvidin-3-galactoside	493.1	493
6	33.15	Peonidin-3-glucoside	463.1	463
		Flavonols	$[M]^-$	$[M]^-$
7	20.50	Myricetin-3-rhamnoside	463.0	463
8	22.20	Quercetin-3-galactoside	463.0	463

There were some other unknown peaks in the HPLC chromatogram which could not be identified. [1]Cho et al. (2004), [2]Hosseinian and Beta (2007)

3.3 Analysis of black currant extracts

For the analysis of the black currant extracts, the samples were first treated with acidified methanol. The pH was adjusted to 1.0 (25°C) and 1M HCl was used for this purpose. The method is the same as previously described (p. 30). Sonication was performed for 30 minutes, then sample solutions were transferred to a 50 mL Falcon tube, which was

centrifuged at 3000 rpm for 45 minutes. Supernatant was collected and subjected to rotary evaporation until 95% of its original volume was achieved. Centrifugation was performed at 3000 rpm for another 20 minutes. The extract was then filtered using a 0.45 µm nylon filter before it was analyzed using LC-MS. We have used the same mobile phase gradients as with other sample. The chromatogram is as shown in Figure 3.9. Various chemical compounds identified in black currant are summarized in Table 3.7. The HPLC peaks were identified based on comparison of detected and calculated molecular ions as mentioned in the literature (Anttonen et al., 2006; Cho et al., 2004).

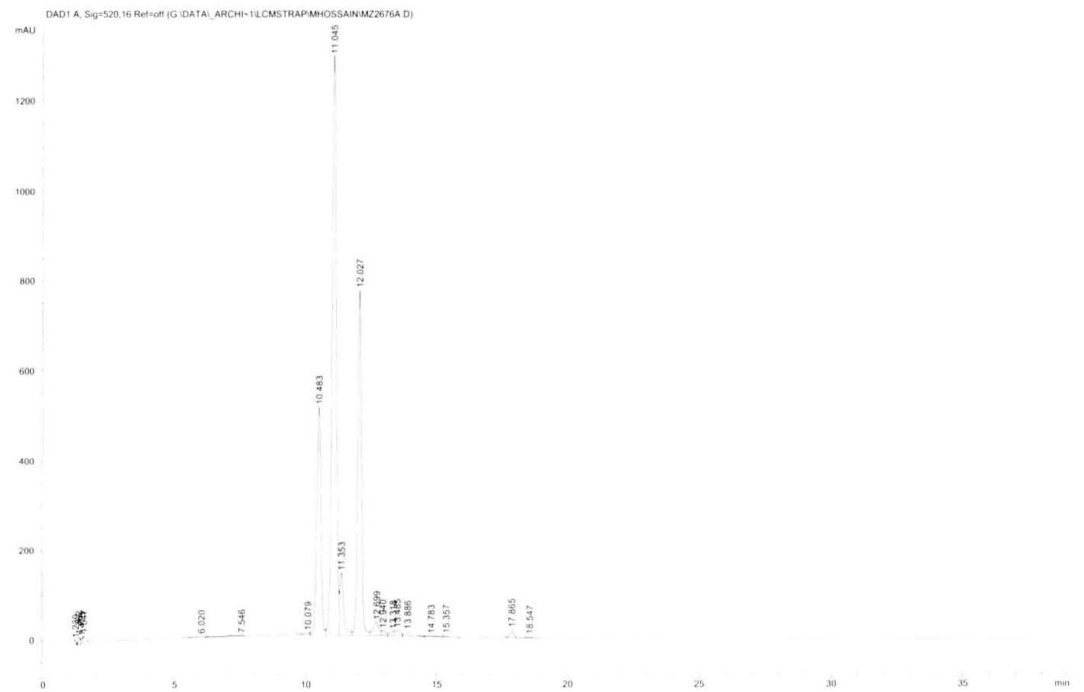


Figure 3.9: HPLC peaks for different components of black currant methanol extracts

Table 3.7: Compounds identified in black currant methanol extracts

No.	HPLC RT(min)	Identification	m/z values $[M]^+$ or $[M+H]^+$	m/z values in literature[1,2] $[M]^-$ or $[M-H]^-$
1	1.55	chlorogenic acid	353.0	353
2	10.48	myricetin-3-rhamnoside	463.1	463
3	11.05	quercetin-3-rutinoside	609.2	609
4	12.03	kaempferol rutinoside	595.2	595

There were some other unknown peaks in the HPLC chromatogram which could not be identified. [1]Anttonen et al. (2006), [2]Cho et al. (2004)

In a separate experiment the black currant sample was treated with 70% aqueous acetone having 0.01M HCl (20.0 mL) which was sonicated for 20 minutes. We used a method similar to that of Anttonen et al., (2006). Extraction was further performed two times with 20.0 mL of solvent for 10 minutes. Extraction mixtures were subjected to centrifugation at 3000 rpm for 10 minutes. Acetone from the extracts was removed using a rotary evaporator. Final volume was adjusted to 25.0 mL. The extract was filtered using a 0.45 μm nylon filter prior to analysis using LC-MS. Analysis parameters were the same as those of our other analyses. Different LC peaks were detected as shown in Figure 3.10. The compounds identified in black currant extracts by this method are listed in Table 3.8.

The HPLC peaks were identified based on comparison of detected and calculated molecular ions as mentioned in the literature (Anttonen et al., 2006; Cho et al., 2004).

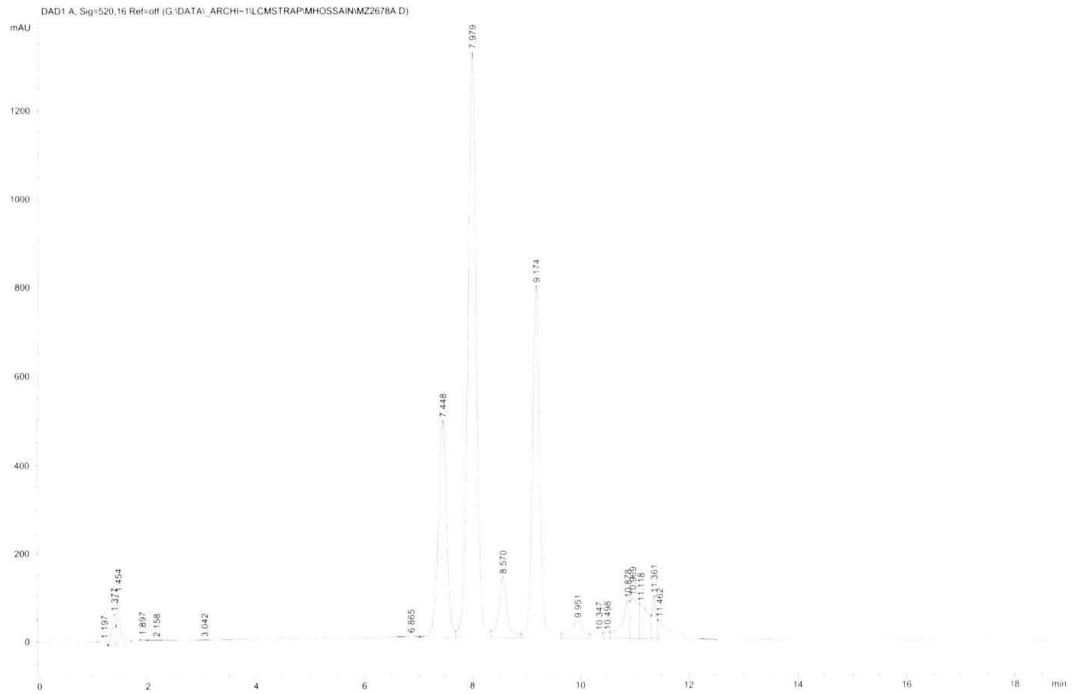


Fig 3.10: HPLC peaks for different components of black currant acetone extracts

Table 3.8: Different chemical compounds detected in black currant acetone extracts

No.	HPLC RT(min)	Identification	m/z values $[M]^-$ or $[M+H]^+$	m/z values in literature[1,2] $[M]^-$ or $[M-H]^-$
1	1.45	chlorogenic acid	353.0	353
2	7.45	quercetin glucoside	465.1	465
3	8.57	kaempferol glucoside	449.1	449

There were some other unknown peaks in the HPLC chromatogram which could not be identified.
[1]Anttonen et al, (2006), [2]Cho et al, (2004)

3.4 Analysis of lingonberry extracts

For the analysis of the lingonberry extracts, the sample was first treated with extraction solvent ($MeOH/H_2O/HCOOH$). The method is similar to that of Cho et al. (2004). Next, sonication was performed for 30 minutes. The sample solution was then transferred to a 50 mL Falcon tube, which was centrifuged at 3000 rpm for 30 minutes. About 5% of the sample extract was evaporated using a rotary evaporator. Four mL of the extract was resuspended in 1 mL of 3% formic acid. Then, the extract was filtered using a 0.45 μm nylon filter. Finally, lingonberry extracts were analyzed using a LC-MS instrument. The LC-MS parameters were the same as for other extracts. After the analysis the LC peaks of different chemical compounds were obtained as shown in Figure 3.11. Various chemical compounds identified in lingonberry extracts are summarized in Table 3.9. The HPLC peaks were identified based on comparison of detected and calculated molecular ions as mentioned in the literature (Ek et al., 2006).

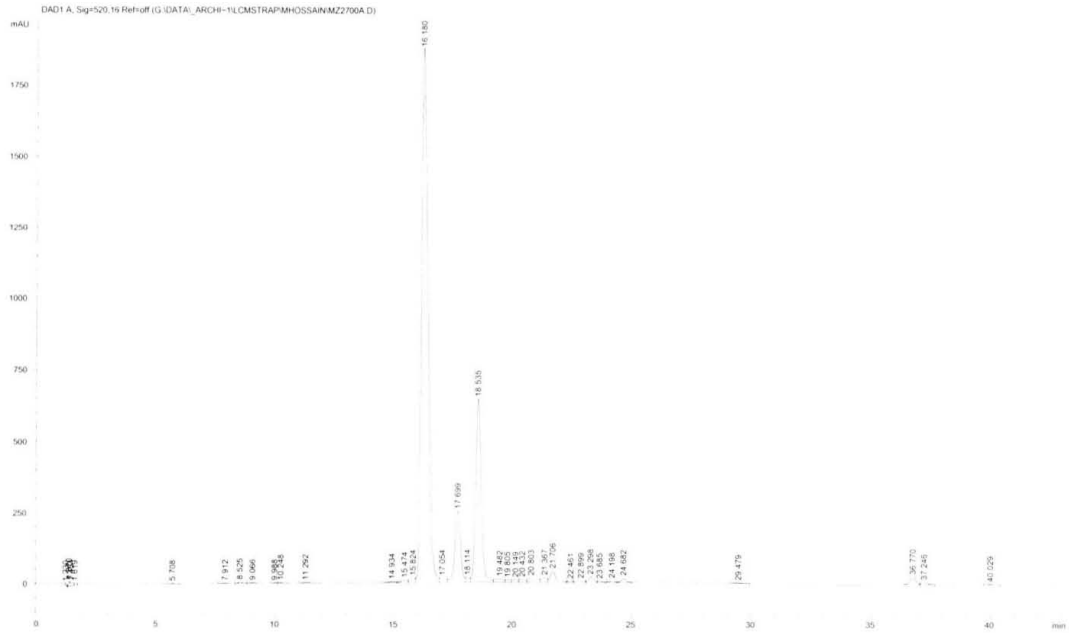


Figure 3.11: HPLC peaks for different components of lingonberry MeOH/H_2O/HCOOH extracts

Table 3.9: Compounds identified in lingonberry MeOH/H_2O/HCOOH extracts

No.	HPLC RT(min)	Identification	m/z values		m/z values in literature[1]
			$[M-H]^-$	$[M+H]^+$	$[M+H]^+$
1	16.18	Cyanidin-3-glucoside	447.1	449.1	449
2	17.70	Cyanidin-3-galactoside		449.1	449
3	18.54	Cyanidin-3-arabinoside		419.1	419

There were some other unknown peaks in the HPLC chromatogram which could not be identified.
[1]Ek et al. (2006)

3.4.1 Confirmation and quantification of cyanidin-3-galactoside in lingonberry extracts.

It was found that the retention time for LC peaks of cyanidin-3-galactoside present in lingonberry extracts was similar to the retention time for LC peaks of the cyanidin-3-galactoside standard. This further confirms that this compound is present in lingonberry extracts. For quantification of this compound:

- Sample analysis data for cyanidin-3-galactoside:

Retention time of sample peak: 17.70

From HPLC analysis peak area obtained: 3978.62

From the graph (Figure 3.4) after calculation the concentration is = 133.71 µg/mL

Therefore, in 100 g of extract the amount of cyanidin-3-galactoside is 66.33 mg or 0.06633 g.

So, the percentile of cyanidin-3-galactoside in the sample is 0.066.

In another experiment using a similar method to that of Ek et al. (2006), sample (5.01g) was treated with methanol (20 mL), which was sonicated for 1 h. Sample solution was transferred to a 50 mL Falcon tube and was centrifuged at 3000 rpm for 10 minutes. Then the solution was diluted in a 1:2 ratio with ultra pure water prior to analysis. The extract was filtered using a 0.45 µm nylon filter. Finally, the extract was analyzed using a LC-MS instrument. The mobile phase was: A) 0.1% formic acid, and B) methanol. Eluent gradient was 1. %B-0 min- 6% 2. %B- 20 min- 12% 3. %B- 30 min-55%. MS conditions were the same as previously used. A range of LC peaks have been detected, as shown in Figure 3.12. The various chemical compounds identified in lingonberry extracts

by this method are summarized in Table 3.10. The HPLC peaks were identified based on comparison of detected and calculated molecular ions as mentioned in the literature (Ek et al., 2006). We have used this methodology to establish a primary analysis guideline for further chemical analysis of lingonberry.

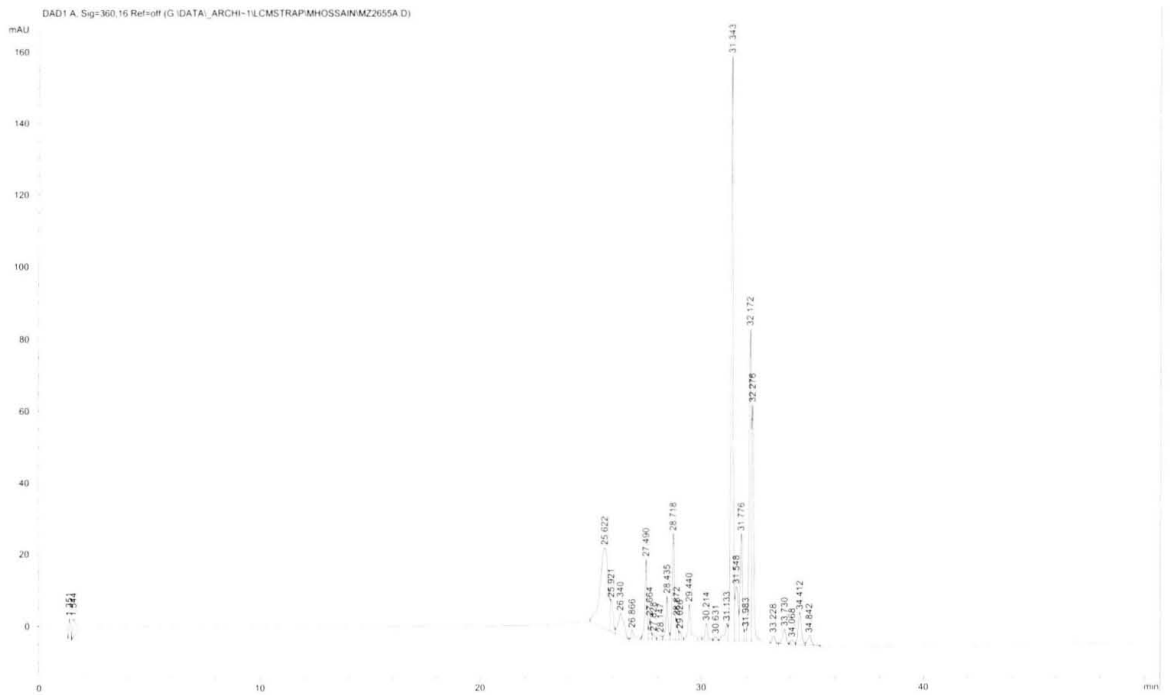


Figure 3.12: HPLC peaks of lingonberry methanol extracts

Table 3.10: Different chemical compounds identified in lingonberry methanol extract

No.	HPLC RT(min)	Identification	m/z value	m/z value in literature[1]
		Anthocyanins	$[M+H]^+$	$[M+H]^+$
1	25.62	Cyanidin-3-glucoside	449.2	449
2	25.92	Cyanidin-3-galactoside	449.1	449
3	27.49	Proanthocyanidin A	577.1	577
4	31.34	Quercetin-3-glucoside	465.1	465
5	32.28	Quercetin-3-O-α arabinoside	435.1	435

There were some other unknown peaks in the HPLC chromatogram which could not be identified. [1]Ek et al, (2006)

3.5 Analysis of blueberries found in central Newfoundland

For the analysis of the blueberries from central Newfoundland, the sample was first treated with extraction solvent ($MeOH/H_2O/HCOOH$) using a method similar to that developed by Cho et al., (2004). We have followed the same extraction methodology as previously described. MS conditions were the same as those of other analyses which, are similar to those of Cho et al. (2004). After the separation of blueberry extracts, peaks for a number of compounds were obtained (Figure 3.13). The various compounds identified in the blueberry extracts are summarized in Table 3.11. The HPLC peaks were identified based on comparison of detected and calculated molecular ions as mentioned in the literature (Cho et al., 2004; Hosseinian and Beta, 2007).

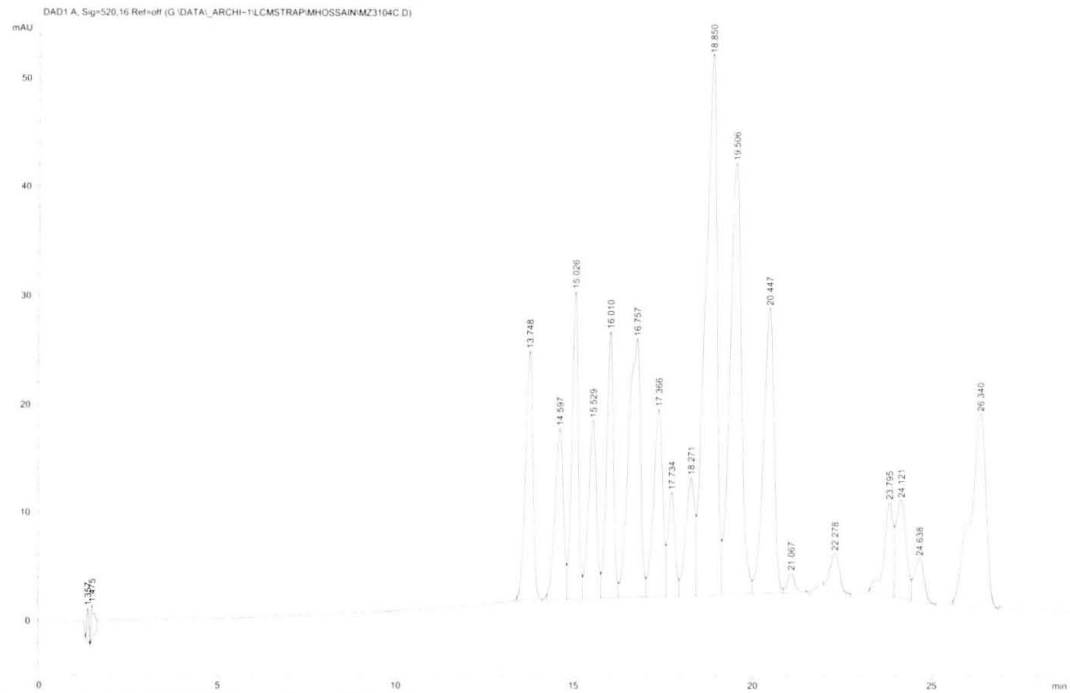


Figure 3.13: HPLC peaks of blueberry extracts from central Newfoundland

Table 3.11: Compounds identified in blueberry extracts from central Newfoundland

No.	HPLC RT(min)	Identification	m/z values	m/z values in literature[1,2]
		Anthocyanins	$[M]^+$	$[M]^+$
1	15.03	Cyanidin-3-galactoside	449.4	449
2	15.53	Delphinidin-3-arabinoside	435.4	435
3	16.01	Cyanidin-3-glucoside	449.4	449
4	16.76	Cyanidin-3-arabinoside	419.3	419
5	17.73	Peonidin-3-galactoside	463.5	463
6	18.85	Malvidin-3-galactoside	493.6	493
7	19.50	Malvidin-3-glucoside	493.6	493
8	20.44	Malvidin-3-arabinoside	463.5	463

There were some other unknown peaks in the HPLC chromatogram which could not be identified. [1]Cho et al. (2004), [2]Hosseinian and Beta (2007)

3.6 Analysis of blueberries found in Pippy Park, St. John's, Newfoundland

For the analysis of the blueberries found in Pippy Park, St. John's, Newfoundland, the sample was first treated with extraction solvent ($MeOH/H_2O/HCOOH$). We used the same method as described previously. MS conditions were the same as other analyses. After the analysis, the HPLC peaks of different chemical compounds were obtained as shown in figure 3.14. Various chemical compounds of blueberries identified are summarized in Table 3.12. The HPLC peaks were identified based on comparison of detected and calculated molecular ions as mentioned in the literature. (Cho et al., 2004; Hosseinian and Beta, 2007).

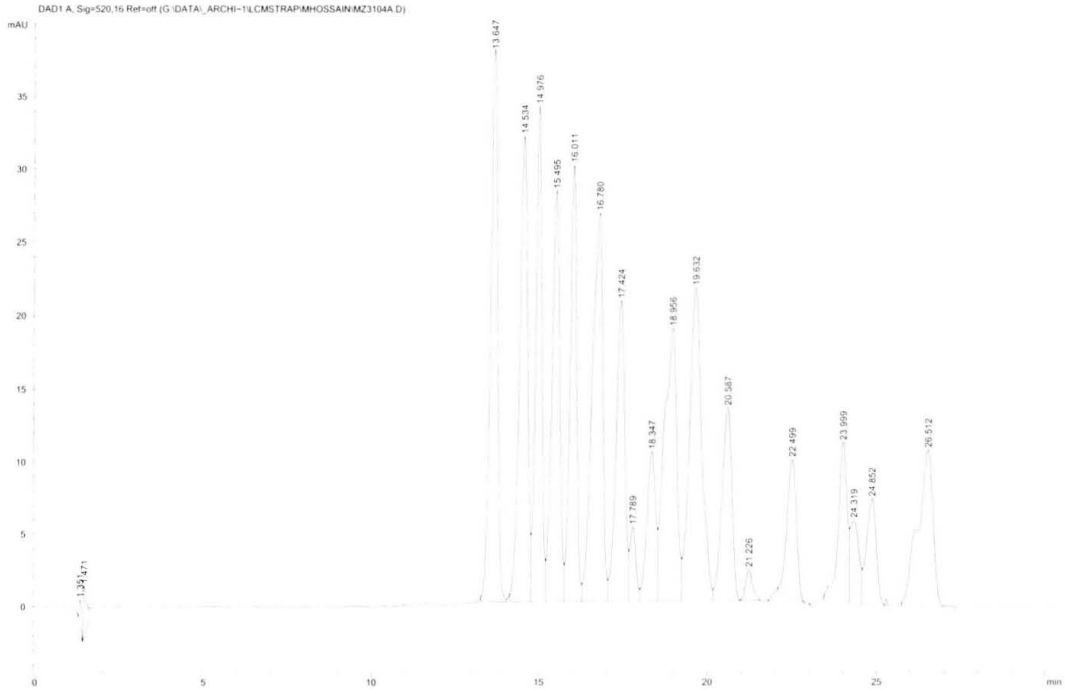


Figure 3.14: HPLC peaks of blueberries found in Pippy Park, St. John's, Newfoundland

Table 3.12: Compounds identified in blueberries from Pippy Park, St. John's, Newfoundland

No.	HPLC RT(min)	Identification	m/z value	m/z values in literature[1,2]
		Anthocyanins	$[M]^+$	$[M]^+$
1	14.98	Cyanidin-3-galactoside	449.4	449
2	15.50	Delphinidin-3-arabinoside	435.4	435
3	16.01	Cyanidin-3-glucoside	449.4	449
4	16.78	Cyanidin-3-arabinoside	419.3	419
5	17.42	Petunidin-3-glucoside	479.5	479
6	18.34	Petunidin-3-arabinoside	449.4	449
7	18.96	Malvidin-3-galatoside	493.6	493
8	20.59	Malvidin-3-arabinoside	463.5	463

There were some other unknown peaks in the HPLC chromatogram which could not be identified. [1]Cho et al. (2004), [2]Hosseinian and Beta (2007)

Table 3.13: Summary of compounds present in different berry extracts analyzed from Natural Newfoundland Nutraceuticals.

Compounds	Bilberry	Blueberry	Black currant	Lingonberry
Delphinidin-3-galactoside	X	X		
Delphinidin-3-glucoside	X	X		
Delphinidin-3-arabinoside	X	X		
Cyanidin-3-galactoside	X	X		X
Cyanidin-3-glucoside	X			X
Cyanidin-3-arabinoside	X			X
Petunidin-3-arabinoside		X		
Petunidin-3-glucoside	X	X		
Petunidin-3-galactoside	X	X		
Peonidin-3-galactoside	X			
Peonidin-3-glucoside	X	X		
Malvidin-3-galactoside	X	X		
Malvidin-3-glucoside		X		
Malvidin-3-arabinoside	X			
Chlorogenic acid			X	
Myrecitin-3-rhamnoside		X	X	
Quercetin-3-galactoside		X		
Quercetin-3-rutinoside			X	
Kaempferol rutinoside			X	
Quercetin glucoside			X	
Kaempferol glucoside			X	
Proanthocyanidin A				X
Quercetin-3-glucoside				X
Quercetin-3-O-α arabinoside				X

Table 3.14: Comparison of compounds present in blueberry extract of Natural Newfoundland Nutraceuticals, Central Newfoundland and Pippy Park, St. John's, NL.

Compounds	Blueberry (NNN)	Blueberry (Central Newfoundland)	Blueberry (Pippy Park, St. John's, NL)
Delphinidin-3-galactoside	X		
Delphinidin-3-glucoside	X		
Delphinidin-3-arabinoside	X	X	X
Cyanidin-3-galactoside	X	X	X
Cyanidin-3-glucoside		X	X
Cyanidin-3-arabinoside		X	X
Petunidin-3-arabinoside	X		X
Petunidin-3-glucoside	X		X
Petunidin-3-galactoside	X		
Peonidin-3-galactoside		X	
Peonidin-3-glucoside	X		
Malvidin-3-galactoside	X	X	X
Malvidin-3-arabinoside		X	X
Malvidin-3-glucoside	X	X	
Myrecitin-3-rhamnoside	X		
Quercetin-3-galactoside	X		

Chapter 4

Results

Biological activity of bilberry extract

We conducted some initial experiments in order to screen bilberry extracts for neuroprotective effects against traumatic injury and aging. After chemical analysis, we chose bilberry extract first to investigate its neuroprotective effects. This was in order to maintain the experimental sequence of bilberry, blueberry, black currant and lingonberry analysis. This is an initial screening using dissociated cell cultures from brain tissue. Bilberry extracts were applied to cultures before mechanical injury in order to observe the effects on cellular lactate levels. Effect of cell aging in culture was also determined.

4.1 Effect of bilberry extracts on cellular lactate levels after injury

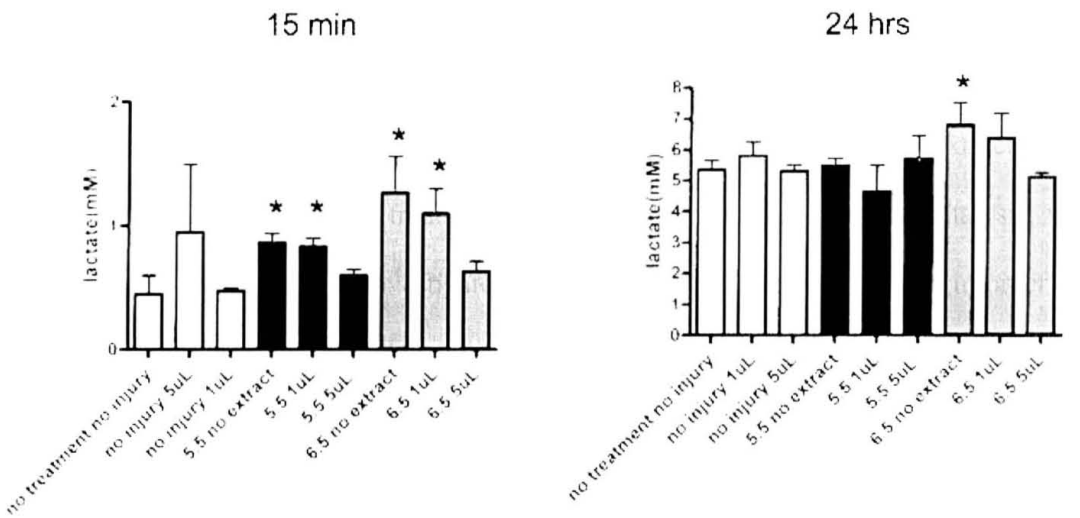


Figure 4.1: Effects of bilberry extracts on cellular lactate levels 15 minutes and 24 hours after injury

For cortical injury, trauma was induced in neurons and glial cells. Two different injury levels of 5.5 mm and 6.5 mm deformation were used. Bilberry extracts [extracted using acidified methanol ($MeOH/H_2O/HCOOH$)] were added to cell cultures 15 min before inducing the different levels of injury. For lactate measurements, a high level of lactate release into the media was observed by 15 min after injury at both injury levels (Figure 4.1), suggesting that cells were unhealthy. While 1 μL of extract had no effect, when 5 μL extract was added to the cell cultures 15 minutes before injury, lactate levels were not significantly elevated. At 24 hours after injury, no significant differences were found in 5.5 mm injury versus control wells. After 6.5 mm injury there was still elevated lactate levels at 24 hr, however there was no significant elevation of lactate levels when extracts were added to the cultures. (*$p<0.05$ vs control; n=6-8)

4.2 Effect of aging in culture

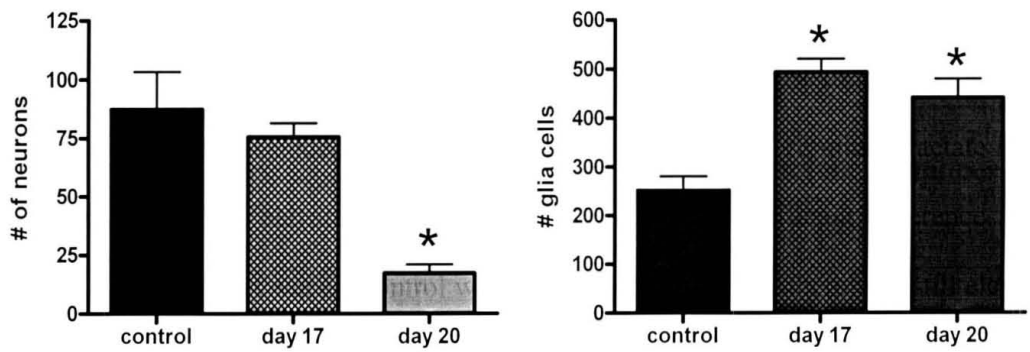


Figure 4.2: Effect of aging in culture

Aging experiments showed that up to day 17 *in vitro* cell cultures demonstrated no significant death of neurons (Figure 4.2). However, by day 20 there is a dramatic loss of neurons. For glial cells proliferation was noticed by day 17, but by day 20 there is no significance change in glial cells compared to day 17. Control represents neurons and glial cells counted at 10-11 DIV (*$p<0.05$ vs. control; n=6-10).

4.3 Effects of bilberry extracts on aging in culture

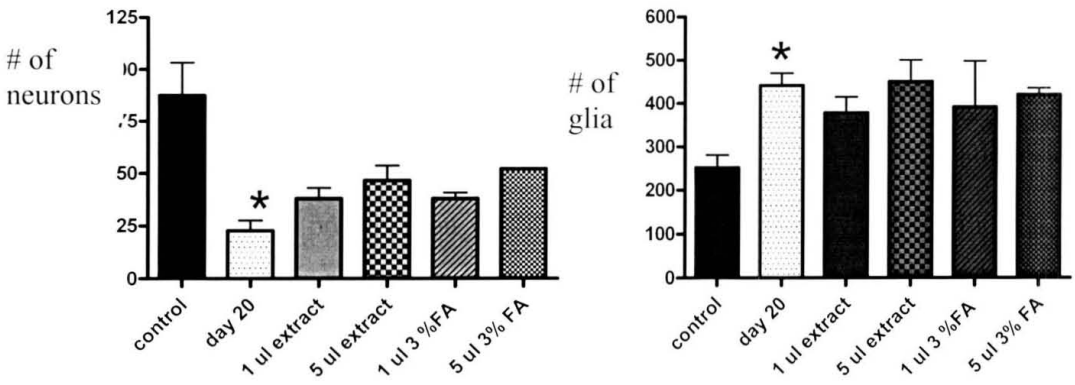


Figure 4.3: Effects of bilberry extracts on aging in culture

In Figure 4.3 control represents neurons and glia counted at 10-11 DIV. Extracts (One µL or 5 µL) were added on days 17, 18 and 19. One µL or 5 µL of acidified methanol ($MeOH/H_2O/HCOOH$ = 60: 3: 37 v/v/v) solution was added to some cultures as a control. Neurons and glia were counted on day 20 *in vitro* (*$p<0.05$ vs. control; n= 2-12). No statistically significant effect was found on the amount of glia and neurons with the addition of the extracts in the aging model.

Chapter 5

Discussion

The objective of this research was to analyze the chemical composition of different berries found in Newfoundland and Labrador and to begin to study their biological impact on neuroprotection and brain aging. Extracts of four berries including bilberry, blueberry, black currant and lingonberry were analyzed. The gradient method developed in combination with the Symmetry C18 column provided excellent separation of anthocyanins in bilberry, blueberry, black currant and lingonberry. During extraction different solvent systems were used. This was performed in order to obtain maximum compound extraction. HPLC-MS conditions remained the same for all analyses.

In bilberry, a total of 10 compounds were identified. All of the compounds detected were anthocyanins. The compounds identified are listed in Table 3.2 (p. 34) with corresponding DAD peaks shown in Figure 3.2 (p. 33). These findings are consistent with a previous report in which 11 anthocyanidins were detected in bilberry extract powder (Zhang et al., 2004). Nyman and Kumpulainen (2001) conducted analysis on bilberries found in Finland and reported that six anthocyanidins are present: pelargonidin, cyanidin, petunidin, peonidin, delphinidin and malvidin. In our analysis of bilberry, we found delphinidin, cyanidin, petunidin, peonidin and malvidin present in the glycosidic form, only pelargonidin was not detected. In another report by Lätti et al. (2008), they showed that 15 anthocyanins are present in bilberries grown in Finland. However, they used a different extraction technique although the analytical conditions were the same. The

extraction solvents used were MeCN-MeOH (85:15 v/v) (solvent A) and 8.5% aqueous HCOOH (solvent B). Therefore, it is possible that this different extraction system led to the identification of more compounds in their bilberry samples. Kähkönen et al. (2003) reported that 15 anthocyanins are present in bilberry grown in Finland. This shows the same number of anthocyanins as that of the analysis conducted by Lätti et al. (2008). The extraction solution used was MeCN/TFA/H_2O in a ratio of 49.5:0.5:50 v/v/v. For analysis the mobile phases used were 10% Formic acid (solvent A) and 100% MeCN (solvent B). So, this shows that different extraction systems and the same analytic conditions did not have much impact on the number of compounds as identified in Finland bilberry.

In blueberry powder, a total of 8 compounds were identified. Among them, six compounds are anthocyanins and two are flavonols. The compounds identified are listed in Table 3.6 (p. 45) with corresponding HPLC peaks shown in Figure 3.8 (p. 44). Cho et al. (2004) reported that blueberry genotypes including two commercial cultivars found in the USA, Bluecrop known as Northern Highbush and Ozarkblue, known as Southern Highbush, contained 17 anthocyanins, and in Bluecrop blueberry six flavonols were present. However, chlorogenic acid was also identified in the berry extract as reported by Cho et al. (2004). This suggests that more compounds may be present in blueberries found in the USA as compared to that of Newfoundland. This may be due to species, climate, soil and collection time, as the extraction method and analysis conditions were the same. In blueberries found in central Newfoundland (collected during August and stored in the refrigerator at 4° C for 5 days before analysis), eight compounds have been identified. The compounds identified are listed in Table 3.11 (p. 54) with corresponding

HPLC peaks shown in Figure 3.13 (p. 53). Whereas, in blueberries collected from Pippy Park, St. John's, Newfoundland, eight anthocyanins have been identified. These berries were collected during August and stored in the refrigerator at 4° C for 6 days before analysis.

The compounds identified are listed in Table 3.12 (p. 56) with corresponding HPLC peaks shown in Figure 3.14 (p. 55). There was a slight difference in the compounds found in blueberries from central Newfoundland and Pippy Park, St. John's, Newfoundland. This may be due to location, time of year of harvesting, soil, temperature and subspecies. The amount of different anthocyanins detected in our extract powder sample is less than previously reported for blueberries found in Quebec (Nicoue et al., 2007) and Manitoba (Hosseinian and Beta, 2007). This may due to the fact that those studies were conducted in berries versus extract powder, or to differences in the natural composition of berries. Nicoue et al. (2007) reported that five anthocyanidins were present in Quebec wild blueberry, namely delphinidin, cyanidin, peonidin, petunidin and malvidin, while pelargonidin was absent. For extraction purposes ethanol was used as the solvent. The mobile phase used for chromatographic separation was 5% formic acid and methanol. Comparing our analysis with this report shows that pelargonidin was not identified in either Quebec wild blueberries or Newfoundland blueberries.

In black currant, only four compounds were identified, which were chlorogenic acid (3-caffeoylquinic acid), myrecitin-3-rhamnoside, quercetin-3-rutinoside, kaempferol rutinoside listed in Table 3.7 (p. 47) with HPLC data in figure 3.9 (p. 46). This represents a different profile of compounds compared to previous work conducted on black currant

species growing in Finland (Nyman et al., 2001; Mikkonen et al., 2001; Anttonen and Karjalainen, 2006). Slimestad and Solheim (2002) worked on black currant berries available in Norway and reported that 15 anthocyanins are present in those berries. Extraction was performed by aqueous methanol for 24 hours. For the HPLC A mobile phase the solvent was 5% formic acid and for the B phase they used 75% methanol/ 2.5% formic acid. The different extraction process and HPLC conditions may be the reason that the number of compounds identified in berries found in Norway was more than in our findings. Määttä et al. (2003) identified eight anthocyanin compounds in acidified methanolic extracts of black currant available in Sweden. Mikkonen et al. (2001) identified four compounds, myricetin, morin, quercetin and kaempferol, in black currant grown in Finland. For identification of compounds they used the same HPLC parameters utilized by Määttä et al. (2003). The results of Mikkonen et al. (2001) show similarity with our findings. The mobile phases used in their study were 1% formic acid (A) and acetonitrile (B). However, we are most likely not accurately reporting all of the phenolic compounds present in our black currant extracts due to the fact that we were unable to obtain adequate peak separation with higher retention times in our samples.

In lingonberry, three compounds were identified, which were cyanidin-3-glucoside, cyanidin-3-galactoside, and cyanidin-3-arabinoside as listed in Table 3.9 (p. 50) with HPLC data in Figure 3.11 (p. 49). A previous report conducted on lingonberries growing in Finland identified as many as 28 compounds present in methanolic extracts in that species (Ek et al., 2006). Comparing HPLC conditions we found that Ek and co-workers (2006) used the similar analytical conditions as used by our lab. We are unsure of the

explanation for this difference in the number of compounds, considering we obtained excellent peak separation during analysis of the extract and the drying method used to produce the original extract maintains at least 94% (label claimed) of the bioactive compounds intact. It is well known that the chemical composition differs significantly depending on soil, subspecies, climates etc. As these berries were grown in Newfoundland compared to Finland, it is possible that due to different conditions the number of compounds may vary.

Anthocyanins are unstable compounds, therefore the availability of standards is poor and the price is high. For the determination of anthocyanins in the glycoside form, it is very difficult to obtain standards for every anthocyanin form present in the sample. We ran standards for two anthocyanin compounds, delphinidin-3-glucoside and cyanidin-3-galactoside as shown in Figure 3.5 (p. 39) and Figure 3.6 (p. 40), and tested the levels of these compounds in samples of extracts from bilberries. Both of these compounds are reported to have high antioxidant activity (Kähkönen et al., 2003). It was revealed that in Newfoundland bilberries delphinidin-3-glucoside and cyanidin-3-galactoside were present at 51.8 mg/100 g and 61.04 mg/100 g respectively. We also successfully tested levels of cyanidin-3-galactoside in lingonberries and detected levels of 63.66 mg/100 g in this species. Levels of delphinidin-3-glucoside and cyanidin-3-galactoside have been analyzed in Saskatoon berries (263.76 mg/100 g and not detected respectively) and in wild blueberries (84.39 mg/100 g and not detected, respectively) available in Manitoba (Hosseinian and Beta, 2007). Therefore, in comparison to other berries growing in Canada, Newfoundland bilberries appear to have a lower content of delphinidin-3-

glucoside compared to certain species of Manitoba berries, but they have a high content of cyanidin-3-galactoside. Newfoundland lingonberries also have high levels of cyanidin-3-galactoside, which is noteworthy since this compound is considered to have fairly high antioxidant activity (Veridiana et al., 2008). Overall, the results suggest that various species of berries growing natively in Newfoundland and Labrador contain a wide array of anthocyanin and flavonol compounds with high levels of antioxidant capacity. Further investigation is required to confirm other polyphenols potentially present in these species of berries and to quantify levels of the various antioxidant compounds.

For extraction we utilized various solvent systems in order to investigate which solvent system would show the highest efficiency of extraction. This is due to the fact that in one solvent system a maximum amount of compounds may not be extracted, which could be extracted in other solvent systems. For bilberry we used two extraction methods. In one method acidified methanol (15% HCl in methanol) was used. We found that in this extraction method nine compounds were detected. Whereas in another method where we used formic acid in methanol a total of 10 compounds have been detected. So, this variation between the numbers of compounds identified in bilberry extract is due to different solvent systems.

Bilberry extracts were examined for neuroprotection against trauma and aging by applying them to cultured rat brain cortical cells. We tested bilberry extracts rather than those from other species due to the fact that we completed the chemical analysis of bilberries before other berries. Two different levels of 5.5 mm and 6.5 mm deformation were used, which produce mild and moderate injury, respectively. Counting the number

of neurons and glia 24 h after injury we found that no noticeable effect was shown with the addition of the extracts in the trauma model. For lactate measurement, a high level of lactate was observed after injury, showing that cells were not healthy. It has been revealed that lactate is released during anaerobic metabolism and is elevated in cell injury, thus it acts as a marker for injury (Cureton et al., 2010). Extracts were added to the cells 15 minutes before injury; lactate levels dropped significantly in both 5.5 mm and 6.5 mm injury in the presence of extracts. Aging experiments showed that up to day 17 cell cultures demonstrated no significant death in neurons, but by day 20 there is a dramatic loss of neurons. For glial cells, proliferation was noticed by day 17, but by day 20 there was no significance change in glial cells compared to day 17. For determining the effect of bilberry extracts on aging in culture, the number of neurons and glia were counted at 10-11 DIV. Extracts were added on days 17, 18 and 19. One μL or 5 μL of acidified methanol ($MeOH/H_2O/HCOOH$ = 60: 3: 37 v/v/v) solution was added to some cultures as a control. There were no significant effects of the extracts on the number of neurons and glia when evaluated on day 20 *in vitro*.

It is possible that we may need to add extracts earlier than 15 min before trauma to see more positive results. There have been more positive results reported *in vivo*. For example, Sweeney et al. (2002) showed that rats fed blueberries for 6 weeks were protected from stroke. Perhaps extracts from other berries would be more protective against aging or trauma since the blueberry extract we analyzed have several compounds with antioxidant properties. Black currant and lingonberry extracts may also show significant results in neuroprotection against trauma and aging as they also have potential

antioxidant compounds. Further evaluation is needed to prove this.

Although extracts were added directly to cell cultures, normally they would have to cross the blood brain barrier (BBB) to produce their effects. Andres-Lacueva et al. (2005) showed in their research that certain compounds present in blueberries were found in rat brain cells after feeding them a diet with blueberry extract. This demonstrates that compounds present in blueberries can cross the BBB to produce their effects. Through chemical analysis we tried to identify different chemical compounds present in Newfoundland berries, and to analyze biological activity of berry extracts in regard to neuroprotection. As only bilberry extract has been evaluated for biological activity, other extracts are to be analyzed in the future in order to determine more positive results.

Conclusions

From the berry extract analysis various chemical components were identified. Among the four extracts, bilberry, blueberry, black currant and lingonberry, it was observed that bilberry and blueberry contain the highest amount of compounds with reported antioxidant activity compared to that of black currant and lingonberry. Two compounds, i.e. delphinidin-3-glucoside and cyanidin-3-galactoside were further confirmed and quantified in bilberry and lingonberry. It was found that the bilberry extract had only a minor effect on cell death following traumatic injury. However, it was also revealed that bilberry extract decreases lactate release after injury, suggesting that the compounds in bilberries have a positive effect on cell health after injury. Analysis of aging in neurons *in vitro* shows that bilberry extracts do not have any significant effect. There was also no significant effect of bilberry extracts on glial cells with aging. Further investigation is required to decipher the effects of various species of berries on brain aging and protection from trauma. In our research we have performed a biological investigation for bilberry extract only. So, in the future further biological studies can be performed on blueberry, black current, and, lingonberry extracts. In our analysis we have confirmed only two compounds. However, more compounds which were detected can be confirmed in the future by using additional standards and their neuroprotective effects can also be determined.

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