Neuroprotective Properties of Partridgeberry (*Vaccinium vitis-idaea* L. var. minus Lodd) Polyphenols

by

Khushwant Singh Bhullar

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Dedications

Father Kirpal (the Merciful) has ordered thus: Whatever a child wants, he shall be given.

(**Guru Arjan Dev Ji**).

Dedicated to Biji Surinder Kaur Ji, at whose lotus feet

the writer imbibed sweet elixir of

Holy Naam-The Word

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ABSTRACT

This study aimed at the extraction and biochemical characterization of polyphenols of partridgeberry (*Vaccinium vitis-idaea* L. var. minus Lodd) found in Southern Labrador, Canada and the investigation of their cytoprotective and neuroprotective characteristics. Three partridgeberry polyphenol fractions (PPFs) were prepared which were rich in either, anthocyanins, flavan-3-ols and flavonols. The fractions rich in flavan-3-ols and flavonols, were potent antioxidants, and displayed effective attenuation of amyloid-beta sensitized neural injury in primary neurons *in vitro*, possibly through superoxide dismutase and catalase hyperactivity. Furthermore, pre-exposure of primary neurons to these PPFs significantly increased their viability following Oxygen Glucose Deprivation/Reperfusion (OGD/R) injury. ELISA analysis revealed that the levels of TNF-α, IL-6, HIF-1α and PPAR-γ were also modulated towards neuroprotection. These properties make the flavan-3-ols- and flavonols-rich PPFs suitable aspirants for preventive nutritional intervention against brain disorders such as Alzheimer's disease and ischemic stroke, for which there is currently no medicinal remedy.

LIST OF ABBREVIATIONS USED

AA	Ascorbate
AAPH	2,2'-Azobis (2-amidinopropane) dihydrochloride
AChE	Acetylcholinesterase
AD	Alzheimer's Disease
Akt	Protein Kinase B
$AlCl_3$	Aluminum Chloride
ANOVA	Analysis of Variance
APAF1	Apoptotic Protease Activating Factor 1
APOE	Apolipoprotein E
APP	Amyloid Precursor Protein
ARE	Antioxidant Response Element
ATCC	American Type Culture Collection
Aβ	Amyloid-beta
Bad	The Bcl-2-Associated Death Promoter
Bax	Bcl-2-Associated X Protein
BBB	Blood Brain Barrier
BCL2	B-cell CLL/lymphoma 2
BSA	Bovine serum albumin
BuChE	Butyrylcholinesterase
CAT	Catalase
CI	Cerebral Ischemia
CRD	Completely Randomized Design
CUR	Curcumin
DCFH-DA	2', 7'-dihydrodichlorofluoresceindiacetate
DMAC	4-dimethylaminocinnamaldehyde
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenylpicrylhydrazyl
DTNB	5,5′-dithio-bis(2-nitrobenzoic acid)

DW	Dry Weight
EGCG	Epigallocatechin Gallate
ELISA	Enzyme-Linked Immunosorbent Assay
FRAP	Ferric Reducing Ability of Plasma
FW	Fresh Weight
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric Acid
HIF-1α	Hypoxia-Inducible Factor 1-Alpha
HO-1	Heme oxygenase
IL	Interleukin
Keap1	Kelch-like ECH-associated protein 1
LCMS	Liquid Chromatography–Mass Spectrometry
LDH	Lactate Dehydrogenase
MAPK	Mitogen-Activated Protein Kinase
MMPs	Matrix metalloproteinases
MRM	Multiple Reactions Monitoring Mode
MTS	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MΩ	Megaohm
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NFTs	Neurofibrillary Tangles
Nrf2	Nuclear factor erythroid 2-related factor 2
OGD/R	Oxygen Glucose Deprivation-Reperfusion
ORAC	Oxygen Radical Absorbance Capacity
PI3K	Phosphoinositide 3-kinase
p38	Protein 38 or tumor protein 38
p53	Protein 53 or tumor protein 53
PPARγ	Peroxisome proliferator-activated receptor gamma
PPFs	Partridgeberry Polyphenol fractions
Q	Quercetin
ROS	Reactive Oxygen Species

SAS	Statistical Analysis System
SI	Selectivity Index
SOD	Superoxide Dismutase
STAT	Signal Transducers and Activators of Transcription
TFC	Total Flavonoid Content
TGF-β	Transforming Growth Factor Beta
ThT	Thioflavin T
TNF-α	Tumor Necrosis Factor Alpha
tPA	Tissue Plasminogen Activator
UPLC-ESI-MS/MS	Ultra-Performance Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry

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CHAPTER 1: INTRODUCTION

1.1. Research Rationale

In the mid 1950's, Denham Harman presented the free radical theory of aging that implicated free radicals in aging and the degenerative diseases. His theory was suggestive of the synergistic role of molecular oxygen and the oxidative enzymes in the origin of free radicals and their subsequent cellular damage (Harman, 1956). The free radical theory gained credibility over the 1980-90s, as the research united two strands of significant scientific inquiry: the interplay between cellular oxidants and aging, and the potential role of antioxidants in promoting human health (Beckman & Ames, 1998). Many decades after Harman proposed his theory, the role of antioxidants in extending mammalian lifespan was explained by Schriner et al. (2005), as they proved the role of endogenous antioxidants in mitochondrial re-genesis and attenuation of reactive oxygen species (ROS) *in vivo*. Since the 1990s, scientists have explored numerous research facets in the area of free radical biology, including but not limited to:

• Understanding the role of ROS in human health and disease;

• Antioxidants and their pharmacological relevance;

• Identification of antioxidants from dietary sources;

• Evaluation of plant polyphenols as exogenous antioxidants;

• Determining the therapeutic role of polyphenols in aging and degenerative diseases; and

• Prospects of polyphenols for neuroprotection.

Although a significant amount of research has been conducted in the disciplines of antioxidants and polyphenols, a research gap remains in the development of clinically applicable, polyphenol-based antioxidant therapy for prevention and treatment of neurodegenerative diseases.

Neurodegenerative diseases are a critical burden on human health, and share the common features of progressive loss of brain structure, nervous system dysfunction and synaptic loss. These diseases not only impact human health but also represent an extensive economic burden; for example, the current cost to Canadians for treating Alzheimer's disease is more than $15 billion, which is expected to rise by ten-fold, in the coming generation (Alzheimer's Association, 2012). As yet, there is no cure for diseases like Alzheimer's disease and stroke, due to the complex pathology of the diseases and the convergence of multiple factors such as diet, genetics, environment and age in the pathogenesis of these ailments (Winner, Kohl, & Gage, 2011). However, this same complexity provides multiple therapeutic targets, and various strategies, including antioxidant therapy, aimed at attenuating the oxidative stress associated with the neurodegenerative diseases.

There is a significant amount of experimental and clinical evidence that implicates mitochondrial dysfunction and oxidative stress as central players in neurodegenerative diseases. For example, the net production of mitochondrial ROS has a major role in the cognitive decline (Lin & Beal, 2006).

In light of these facts, a natural polyphenol-based antioxidant therapy, for preventing or delaying the onset of neural disorders like Alzheimer's disease and stroke may exert a variety of beneficial biochemical and pharmacological effects. Polyphenols are a group of plant secondary metabolites which are recognized as antioxidants, and are ubiquitous to fruits and vegetables. Habitual consumption of dietary polyphenols from Ginkgo biloba, green tea, and red wine has demonstrated neuroprotective properties against Alzheimer's disease and stroke (Lin, 2011). Dietary polyphenols are also known to attenuate the oxidative stress contributing the pathology of neurodegenerative disorders (Panickar & Anderson, 2011). The pharmacological properties of

polyphenols have led to their accelerated use in therapeutic and dietary strategies to combat the oxidative stress and mitochondrial dysfunction associated with disorders of the central nervous system. Although a wide range of studies have implicated *Vaccinium* species of berries as potential neuroprotective agents, the use of Canadian partridgeberry (*Vaccinium vitis-idaea* L. var. minus Lodd) remains unexplored. This thesis addresses an initial aspect of developing a neuroprotective nutraceutical aimed at neurodegenerative disorders. Specifically, this thesis involves the extraction of polyphenols from partridgeberry and the assessment of antioxidant properties of partridgeberry polyphenol preparations (*in vitro*, using both chemical substrates and human cell models), and providing an evaluation of their neuroprotective potential using rat primary cortical and hippocampal neurons.

Partridgeberry was chosen for this thesis research for several following reasons including:

• Partridgeberry has not yet been explored for its potential neuro-pharmacological relevance;

• Partridgeberry contains an abundance of bioactives, including phenolic acids, flavonoids, anthocyanidins, catechins and proanthocyanidins (Ek et al, 2006);

• Partridgeberry contains higher concentrations of polyphenols than bakeapple (or cloudberry) and other berries, however, the results are preliminary (Li, 2013); and

• There is recent evidence of high anthocyanin content in partridgeberry grown in Oregon, USA (Lee & Finn, 2012).

Solvent-based extraction and fractionation was chosen to isolate class specific polyphenols (Dai & Mumper, 2010). As Alzheimer's disease and stroke triggers cortical thinning and hippocampal volume loss, the use of cell models of primary cortical and hippocampal neurons can offer *in vitro* screening for both synthetic and natural neuroprotective agents. The current study aims to fill a research gap in our understanding of the polyphenols present in the

Canadian partridgeberry and develop methodology for polyphenol extraction, and access the cytoprotective activity, and neuroprotective potential.

1.2. Research Hypotheses

Hypothesis 1: Liquid-liquid separation and reverse phase column chromatography fractionation will result in specific polyphenols subclass-rich fractions.

Hypothesis 2: Exposure of human fibroblasts (WI-38 cells) to partridgeberry polyphenols before peroxyl radical insult will prevent or reduce cell death and membrane damage.

Hypothesis 3: Pretreatment with partridgeberry polyphenols will protect primary rat cortical and hippocampal neurons against Aβ toxicity *in vitro*.

Hypothesis 4: Partridgeberry polyphenol pretreatment will attenuate cellular damage *in vitro* in rat primary cortical neurons subjected to Oxygen Glucose Deprivation/Reperfusion (OGD/R).

1.3. Research Objectives

The overall objective of this research is to establish a bio-analytical procedure to isolate the polyphenols from partridgeberry, measure their antioxidant and cytoprotective potential *in vitro*, and to evaluate their potential as neuroprotective agents.

Objective 1 Identify the appropriate solvent system(s) to extract polyphenols from partridgeberry and establish a fractionation process to generate polyphenol sub-classes rich fractions.

Objective 2 Measure the *in vitro* antioxidant and cytoprotective properties of polyphenol-rich partridgeberry fractions.

Objective 3 Investigate neuroprotective properties of polyphenol preparations using rat primary cortical and hippocampal cells subjected to the Aβ toxicity *in vitro*.

Objective 4 Investigate the neuroprotective properties of partridgeberry fractions using rat primary cortical neurons subjected to Oxygen Glucose Deprivation/Reperfusion (OGD/R) injury.

1.4. Organization of Thesis

Chapter 1-Introduction: Describes the research rationale, research hypothesis, thesis objectives and organization of the thesis.

Chapter 2-Background: Provides a brief summary of antioxidants and their pharmacological relevance. Discussed in this chapter are the neurodegenerative diseases, their prevalence and epidemiology, cellular models of Alzheimer's disease and ischemic stroke, and neuroprotective potency of polyphenols, along with current literature on partridgeberry.

Chapter 3-Antioxidant and cytoprotective properties of polyphenol-rich partridgeberry (*Vaccinium vitis-idaea* L. var. minus Lodd) extracts and fractions: Presents the findings of objective1 and 2, which involved identifying the appropriate extraction solvent, isolation of polyphenol sub-classes from partridgeberry, along with the assessment of their antioxidant and cytoprotective abilities *in vitro*.

Chapter 4-Partridgeberry polyphenols protect primary cortical and hippocampus neurons against Aβ toxicity: relevance to Alzheimer's disease: Presents the findings of objective 3, which involved investigating the neuroprotective ability of partridgeberry polyphenol fractions against Aβ toxicity in rat primary cortical and hippocampal neurons *in vitro*.

Chapter 5-Partridgeberry polyphenols protect primary cortical neurons from oxygen–glucose deprivation–reperfusion induced injury via suppression of inflammatory adipokines and regulation of HIF-1α and PPARγ: Presents the findings of objective 4, which involved studying the ability of novel partridgeberry polyphenol fractions to protect rat primary cortical neurons against to Oxygen Glucose Deprivation-Reperfusion (OGD/R) stress.

Chapter 6-Conclusions, Synopsis and Future recommendations: Presents overall results of the thesis and key findings from the current results. Recommendations for future work with respect to the signal transduction/genetic studies and animal studies are also provided.

CHAPTER 2: BACKGROUND

2.1. Oxidative Stress and Antioxidants

Oxidative stress, a resultant pathology due to overproduction of free radicals and a simultaneous decline in the cellular antioxidant defense system, contributes to DNA damage, enzyme inactivation, lipid peroxidation and protein oxidation (Jomova et al, 2010). Oxidative stress is implicated as an underlying cause or an ancillary factor in the pathology of several disorders including cancer, neurodegeneration, diabetes and rheumatoid arthritis (Tang et al, 2011). As a primary source of ROS, mitochondria play a key role in the onset of oxidative stress, and are also the principle target for antioxidant therapy. Apart from cellular antioxidant defense, exogenous or dietary antioxidants are also critical in maintaining the cellular redox balance and homeostasis (Halliwell, 2011). Dietary antioxidants are nutritional substances that may protect cells from oxidative stress and attenuate the overproduction of ROS. Chemically, antioxidants are nucleophilic reductants that interact with oxidants (electrophiles) and prevent the further oxidation of other molecules by donating their electrons (Forman, Davies, & Ursini, 2014). However, research has shown that the mode of antioxidant action is not solely limited to the scavenging of free radicals, as they alleviate oxidative stress through multiple mechanisms, including direct scavenging of ROS, signal transduction, and modulation of both oxido-reductases and local oxygen tension (Day, 2014).

A large body of literature supports the pharmacological relevance of antioxidants, particularly polyphenols, against oxidative stress. Resveratrol, a stilbene member of plant polyphenols, is a potent *in vitro* and *in vivo* antioxidant, as it attenuates oxidative stress in both animal and cell model studies by reducing the production of free radicals (Chang et al, 2012; Mikuła-Pietrasik et al, 2012). Similarly, quercetin and EGCG have also shown protection against

oxidative stress and related disorders by engaging various signaling pathways to attenuate oxidative stress (Liu et al, 2012; Xie et al, 2012). Apart from *in vitro* and *in vivo* evidence, clinical evidence also suggests the antioxidant potential and therapeutic attributes of the polyphenols. A 13-year long clinical study showed that a higher intake of antioxidant polyphenols, including flavonoids and phenolic acids, helps in improving memory and slowing the course of brain aging (Kesse-Guyot et al, 2012). It can be concluded that polyphenols are potent *in vitro* and *in vivo* antioxidants, and that their clinical use for antioxidant therapy is a promising approach to attenuate oxidative damage occurring in neurological and other oxidative stress related disorders.

2.2. Alzheimer's Disease and Stroke

Neurological disorders such as Alzheimer's disease (AD) and stroke represent a key clinical problem in developed countries, and are major economic burdens for the health care system in Canada and United States (Herrmann, 2010; Hung et al, 2010). Amyloid-β (Aβ) peptides derived from amyloid precursor protein (APP) via γ-secretase and β-secretase cleavage are classical histopathological hallmarks of AD. Along with Aβ, tau protein alteration in neuronal microtubules also contributes to the pathology of AD, as its abnormal phosphorylation and aggregation triggers pathological events which cause neuronal dysfunction in AD brain (Ittner & Götz, 2011). The accumulation of Aβ monomers, oligomers, and fibrils in the brain triggers the formation of plaques and neurofibrillary tangles, leading to the loss of memory and cognitive functions (Hung et al, 2010). The Aβ deposits first originate in the neocortex and allocortical brain regions, then spread to the diencephalic nuclei, the striatum and finally to the cerebellum, causing severe AD (Thal et al, 2002). Apart from the classical hallmarks, AD is also characterized by elevated peripheral blood cytokine concentrations for interleukin (IL)-6, tumor

necrosis factor alpha (TNF-α), IL-1β, transforming growth factor beta (TGF-β), IL-12 and IL-18 suggestive of a pro-inflammatory immune response in AD pathology (Swardfager et al, 2010). As Aβ and related oxidative stress plays a crucial role in the onset and progression of AD, thus its attenuation via pharmaceutical/nutraceutical interference is a valid therapeutic approach.

Stroke, another neurological disorder is the fourth leading cause of mortality, death and adult disability, and a heavy socio-economic burden in Canada and United States (Canadian Stroke Network, 2011; Towfighi & Saver, 2011). Stroke occurs either due to disruption of the blood supply to the brain (ischemic stroke) or bleeding into or around the brain due to a ruptured artery (intracerebral or subarachnoid hemorrhage). Of the total stroke incidences, about 80% are caused by the ischemic injury in brain (Canadian Stroke Network, 2011). Stroke is also accompanied by inflammation and immune system dysfunction, triggering the loss of 2 million neurons and 14 million synapses within a minute of its incidence (Saver, 2006). Like AD, inflammatory cytokines including TNF-α, IL-1β and IL-6 play immunomodulatory role in the stroke pathology, as their activation leads to the accelerated neural death during stroke (Tuttolomondo et al, 2008). The current treatment of stroke employs thrombolysis for breakdown of blood clots, i.e. use of tissue plasminogen activator (tPA) for reversing stroke damage by removing blood clots, if used within 4.5 hours of symptom onset (Chapman et al, 2014). The epidemiology of stroke presents a major health care challenge as only 8% of the total stroke patients in Canada receive tPA treatment while one-third of hospitalized stroke patients die within year of stroke (Canadian Stroke Network, 2011).

2.3. Cellular models of neurodegenerative diseases

Neurodegeneration is a generic term used to define the loss of brain function, usually culminating in the loss of cognition and neuronal death (Lin & Beal, 2006). Currently,

developing an effective treatment therapy of neurodegenerative diseases presents a major scientific challenge. The process of identifying drug candidates for therapeutic targets generally proceeds through cell model studies assays, which help to translate candidate molecules to the clinically effective neuroprotective agents (Chen & Herupp, 2008). The use of an *ex vivo* primary cortical and hippocampus cell culture system offers a reliable screening tool for both synthetic molecules and natural phytochemicals (Ansari et al, 2009; Kang & Kim, 2007). Since the first report in the 1970s, primary neurons have been used in multiple studies in neuropharmacology and nutraceutical development (Mains & Patterson, 1973). Primary cortical and hippocampal neurons are well established for screening drug candidates for Alzheimer's disease (Aβ induced cell toxicity) and ischemic stroke (oxygen glucose deprivation) studies (Wang, Zhang & Du, 2012; Chang et al, 2013). Multiple phytochemicals like curcumin, resveratrol and quercetin have been assessed for their neuroprotective potential using primary rat cortical and hippocampus cells (Wang, Zhang and Du, 2012, Panickar & Anderson, 2011). Apart from primary neurons, adult hippocampal slice cultures, pluripotent stem cells and bioengineered stem cells are also used as cellular models for studying the neurodegeneration (Ahlgren et al, 2011; Yuan & Shaner, 2013).

The use of *in vitro* cell culture has multiple advantages over *in vivo* studies, such as simplicity and thorough control of the model system. The *in vitro* neuron culture system also minimizes the complex inter-neural, endocrine and immune system interactions. Compared to *in vivo* studies, cell model system serves as a simple, relatively economic and rapid choice for screening large number of drug candidates. In addition to these advantages, *in vitro* cell model studies provide information to rapidly assess the drug toxicity available to cultured neurons. Furthermore,

primary neuron cultures provide cells directly from the desired animal species, as well allowing the screening and toxicological analysis of drug candidates on specific regions of the brain.

2.4. Polyphenols as neuroprotective agents

Polyphenolic compounds are potent antioxidants and exhibit neuroprotective properties, including therapeutic action in both AD and stroke (Bhullar & Rupasinghe, 2013). A variety of foods, including polyphenol-rich green and white tea extracts have shown the inhibition of acetylcholinesterase along with protection of primary cortical neurons against Aβ-induced cytotoxicity (Okello, Leylabi, & McDougall, 2012; Qin, Cheng, & Yu, 2012). In transgenic mouse model studies, polyphenol extracts from grape seeds have shown tinhibition of the oligomerization of Aβ peptides and abnormal tau protein folding, thus reducing the cognitive impairments in transgenic mice (Liu et al, 2011). Resveratrol, a polyphenol abundant in grapes and red wine, also inhibits the Aβ fibril formation and neurotoxicity, by attenuating inducible nitric oxide synthase production (Huang et al, 2011). However, quercetin and rutin, unlike resveratrol not only inhibit the Aβ formation, but also disaggregate the Aβ fibrils in AD rodent models (Jiménez-Aliaga et al, 2011). Interestingly, a phenolic acid compound, ferulic acid exhibited stronger neuroprotection against Aβ toxicity, than quercetin, a powerful antioxidant flavonoid, possibly through its potent antioxidant ability (Jagota & Rajadas, 2012). Similarly, in transgenic mouse model studies, tannic acid and 7,8-dihydroxyflavone have been shown to attenuate the deposition of Aβ and improve cognitive abilities, by decreasing cleavage of β-carboxyl terminal amyloid precursor protein (APP) fragment and inhibiting Aβ synthesis (Mori et al, 2012; Devi & Ohno, 2012). Another polyphenol, liquiritigenin (flavanone) also improves brain function in mice model of AD, as it inhibits the astrocytosis and the Notch-2 expression, as the latter can contribute to neuronal decay (Liu et al, 2011). The ability of polyphenols to

improve neural redox homeostasis by elevating antioxidant enzymes, targeting multiple signaling pathways and reducing Aβ toxicity supports their therapeutic utility for age related disorders like AD and dementia.

Apart from AD, various epidemiological studies suggest that a diet rich in polyphenols can extend neuroprotection and lower the risk and severity of stroke (Ashafaq et al, 2012). Experimental evidence using rodent and cellular models indicates the neuroprotective potential of dietary polyphenols, particularly in cerebral ischemia. Green tea polyphenols have been found to protect neurons against hypoxia-induced ischemic injury by controlling inflammation cascade and oxidative stress (Panickar, Polansky, & Anderson, 2009). Quercetin has also been found to attenuate ischemic injury by controlling calcium and sodium ion dysregulation and lipid peroxidation in neurons possibly through its inhibitory action against MMPs and free radical damage in neurons (Yao et al, 2010; Pandey et al, 2012). Rutin, another flavonoid, has been found to control neural damage in CI through down-regulation of p53, a protein which induces neural necrosis in stroke (Khan et al, 2009). Baicalin, also a flavonoid, has been shown to reduce the ischemic stroke damage by targeting multiple therapeutic targets like caspase-3, oxidative stress, p38 mitogen activated protein kinase (MAPK) (Cao et al, 2011). Similarly, resveratrol has been shown to extend protection against ischemic injury by improving brain energy metabolism and attenuating oxidative stress (Li et al, 2011). The experimental data reveal that polyphenols may prevent, attenuate or slow via multiple mechanisms, the course of stroke and age related neural disorders. Since the risk for stroke increases with age, consumption of a polyphenol rich diet presents itself as an important preventive strategy.

2.5. Polyphenol extraction from plant materials

The extraction of polyphenols is a primary step in utilizing plant phytochemicals for the development of nutraceuticals or pharmaceutical active compounds. Polyphenols can be extracted from plant materials by employing multiple biochemical strategies of separatory chemistry i.e. pre-treatment, extraction, and purification. The pre-treatment step mainly comprises of the homogenization, milling or grinding of plant samples to obtain the crude extracts, rich in polyphenols (Dai & Mumper, 2010). In order to prepare crude plant extracts, organic solvents with varying polarity are used due to their high efficiency, easy applicability and documented success. Many organic solvents, such as methanol, ethanol, acetone, ethyl acetate, and their aqueous combinations have been extensively used for extracting polyphenols from plant materials (Rupasinghe, Kathirvel, & Huber, 2011). These organic solvents are efficient in extracting or partitioning class-specific polyphenols, for example: methanol and aqueous acetone for lower and high molecular weight polyphenols respectively (Fromm, Bayha, Carle, & Kammerer, 2012). Apart from the solvent type, the recovery of polyphenols is also influenced by the extraction time, temperature and acidification of organic solvents (Biesaga & Pyrzyńska, 2013; Thoo et al, 2010). Since there is no universal solvent for polyphenol recovery, in order to obtain the best yield of polyphenols, a preliminary experiment with multiple solvent systems is advisable.

The next step of extraction comprises of selecting an efficient extraction method while maintaining the stability of polyphenols. Over past years, microwave and ultrasound-assisted extractions along with use of compressed fluids as extracting agents have replaced conventional methods such as maceration and soxhlet extraction (Mason, Chemat, & Vinatoru, 2011). The ultrasound-assisted extraction (UAE) method used in our experiments is the most common extraction method due to the lack of complex procedure, short extraction time and low

degradation of polyphenols. The crude extracts obtained following the extraction step may contain large amounts of sugars/carbohydrates, lipoidal material and low concentration of the polyphenols. In order to remove the undesired compounds, especially sugars, a purification process is followed using solid phase extraction with preconditioned C18 columns/cartridges (Dai & Mumper, 2010). The unwanted constituents are removed by washing the aqueous sample (in C18 columns/cartridges) with acidified water and then the desired polyphenols are obtained by elution with organic solvents like methanol, ethanol or aqueous acetone (Ena, Pintucci, & Carlozzi, 2012). Column chromatography and liquid-liquid separation are widely used to isolate class specific polyphenols by employing various organic solvents and their aqueous combinations as eluents (Vidal, Riekkola, & Canals, 2012). Column chromatography and liquid-liquid separation can be used for isolation of class specific polyphenols such as anthocyanins, procyanidins, flavan-3-ols, phenolic acids and flavonol glycosides (Cao et al, 2010; Olszewska, 2012).

2.6. Partridgeberry (*Vaccinium vitis-idaea* L. var. minus Lodd)

Partridgeberry, also known as lingonberry or cowberry, is an evergreen dwarf shrub found in various Scandinavian countries, Canada, and US (Lee & Finn, 2013). Rich in anthocyanins and numerous phenolics, it is an important fruit crop; medicinal plant and ornamental landscape cover (Debnath & Sion, 2009). There have been extensive studies on European cultivars of partridgeberry (Ek et al, 2006), and individual polyphenolic compounds in cultivars have been studied using multiple analytical techniques (Szakiel et al, 2012). These studies have shown that European partridgeberry is rich in phenolics including procyanidin A2, catechin, epicatechin, quercetin-3-*O*-α-arabinofuranoside, kaempferol-pentoside, and kaempferol-deoxyhexoside (Ek et al, 2006; Jungfer et al, 2012). Apart from the *in vitro* antioxidant activity, European

partridgeberry also improved the redox homeostasis and favorably affected the antioxidant defense enzymes in rats *in vivo* (Mane et al, 2010). However, the Canadian partridgeberry, grown in Newfoundland and Labrador, remains relatively unexplored. The Atlantic Cool Climate Crop Research Centre at St. John's, Newfoundland and Labrador was established to study local fruit crops. To date, Canadian partridgeberry research at the institute has concentrated on propagation techniques, genetic diversity, anthocyanin content, and antioxidant activity (Vyas, Debnath, & Igamberdiev, 2013; Sion, Debnath, & Bishop, 2010; Debnath & Sion, 2009). However, a comprehensive study on the Canadian partridgeberry, demonstrating a standardized polyphenol extraction methodology, LCMS analysis, antioxidant and cytoprotective activity, and neuroprotective potential, has so far not been conducted. This thesis aims at filling this gap and and exploring the pharmacological prospects of the partridgeberry.

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CHAPTER 3: ANTIOXIDANT AND CYTOPROTECTIVE PROPERTIES OF POLYPHENOL-RICH PARTRIDGEBERRY (*VACCINIUM VITIS-IDAEA* L. VAR. MINUS LODD) EXTRACTS AND FRACTIONS

3.0 Abstract

Partridgeberry (Vaccinium vitis-idaea) is a polyphenol-rich berry of the Ericaceous family, grown in Newfoundland and Labrador. The objectives of this study were to identify extraction solvents for the maximum recovery of polyphenols, to establish a fractionation procedure for the isolation of major sub-classes of polyphenols, and to evaluate the antioxidant and cytoprotective properties of the partridgeberry polyphenol preparations. The results showed that acidified 70% acetone was the ideal solvent for the maximum recovery of polyphenols from the partridgeberry. Further, aqueous two-phase extraction, column chromatography and UPLC-MS/MS were employed to produce three partridgeberry polyphenol fractions (PPF 1-3), rich in either, anthocyanins, flavan-3-ols or flavonols. All three PPF were potent antioxidants and showed cytoprotective activity through the activation of Nrf2 pathway, scavenging of reactive oxygen species, and inhibition of cellular death. The current study suggests that the partridgeberry has numerous potential health implications in both prevention and amelioration of various diseases involving oxidative stress.

3.1. Introduction

Continuous production of reactive oxygen species (ROS) by the mitochondrial electron transport chain causes damage to mitochondria and important biomolecules, and initiates pathological processes in human body (Circu & Aw, 2010). The ROS, including superoxide anion radicals (O_2-•), hydroxyl radical ($OH^{\bullet}$), hydroperoxyl radicals ($HO_2^{\bullet}$), and lipid peroxyl radicals cause irreversible damage to cellular macromolecules including membrane lipids, proteins and nucleic acids (Ray, Huang, & Tsuji, 2012). The excessive production of ROS is indicative of oxidative stress leading to the cellular damage and accelerated Aging (Circu & Aw, 2010; Ray, Huang, & Tsuji, 2012). Oxidative stress and continuous ROS production also trigger the activation of cell signaling pathways contributing to the pathology of cancer, diabetes, inflammation, neurological disorders and obesity (Reuter et al, 2010). Dietary antioxidants, especially fruit polyphenols, have been used extensively with a view to attenuate oxidative damage and its pathological manifestations. Polyphenols are naturally occurring plant secondary metabolites found in fruits, vegetables, wines and other plant-based dietary sources (Wang, Camp, & Ehlenfeldt, 2012). There are over 25,000 different types of polyphenols, divided into many sub-classes including phenolic acids, flavonoids, stilbenes and lignans (Wang, Camp, & Ehlenfeldt, 2012). Epidemiological studies associated with disease risk have also suggested that habitual consumption of dietary polyphenols offer protection against occurrence of cancer, cardiovascular disorders, diabetes and osteoporosis (Graf, Milbury, & Blumberg, 2005).

Many investigations have demonstrated that berries of *Vaccinium* species are a good source of polyphenols and exhibit a wide range of biological activities, including antioxidant, anticancer and antimicrobial activities (Su, 2012; Wang, Camp, & Ehlenfeldt, 2012). *Vaccinium* berries have exhibited their therapeutic potential *in vitro* and *in vivo*, both in rodent models and

humans (Su, 2012). Accumulating scientific evidence on the health benefits of berries has led to an escalated interest in investigation of wild type *Vaccinium* species for their therapeutic potential. Partridgeberry (*Vaccinium vitis-idaea* L. var. minus Lodd) also known as cowberry and lingonberry, is commonly found in Scandinavian countries and North America. Similar to other *Vaccinium* species of berries, partridgeberry has also shown a wide spectrum of beneficial biological properties that may be related to its polyphenol constituents (Heinonen, 2007). Partridgeberry is a low (2-12 cm) evergreen *ericaceous* shrub found throughout Newfoundland and Labrador of Canada in diverse topographical regions. Though the production and propagation of Canadian partridgeberry has been reported (Debnath & McRae, 2001), to our best knowledge there is no scientific literature documenting the phenolic profile, antioxidant potential or cytoprotective ability of Canadian partridgeberry. In the current study, we investigated the optimum extraction methods for polyphenols, their concentrations along with the *in vitro* antioxidant and cytoprotective properties of partridgeberry found in southern Labrador region of Canada.

3.2. Materials and methods

3.2.1 Plant Materials and chemicals

Fruit of the partridgeberry (*V. vitis-idaea* L. var. minus Lodd) harvested during 2012 from the southern Labrador area (51°43', W 56°26') was provided by the Department of Natural Resources of the Government of Newfoundland and Labrador, Canada. Fluorescein, Folin-Ciocalteu reagent, gallic acid, 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ), Trolox, quercetin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric chloride, phosphate buffer and all standards, unless stated otherwise, were obtained from Sigma-Aldrich (St. Louis, MO). 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Walco Chemical Products Co

Inc., (Buffalo, NY). Formic acid, DMSO, 6-well and 96-well microplates were purchased from Fisher Scientific (Ottawa, ON). Sterile 96- and 6-well assay plates, pipettes and cell culture flasks were obtained from BD Biosciences (Mississauga, ON). CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay kit and CytoTox-ONE™ homogeneous membrane integrity assay kit was obtained from Promega (Madison, WI).

3.2.2 Extraction and fractionation

Partridgeberry fruits were stored in a -20°C freezer until extraction and further analysis. Frozen berries were thawed at room temperature. The extraction process was divided into two stages, the first stage comprised of small scale initial extraction of berries (10 g per extraction) and their phytochemical analysis for selecting the appropriate extraction solvent system. The second stage comprised of the large scale bulk extractions (500 g per extraction) and their subsequent fractionation to obtain specific polyphenols-rich fractions. The extraction of phenolic compounds from different plant/biological samples is affected by the solubility of samples along with the polarity of extraction solvents. Therefore, it is important to assess different solvents with varying dielectric constants to select an ideal solvent for extracting different classes of phenolic compounds from partridgeberry. Eleven solvent types (**1-11**), differing in their polarity were used for the initial extraction and solvent selection. Various analytical parameters were assessed with the aim of selecting the best solvent resulting in maximum recovery of a wide range of polyphenols from partridgeberry.

3.2.2.1 Initial extraction and solvent system selection

The solvents selected, based on literature for optimizations of polyphenol extraction, were: 100% methanol, 70% methanol, 70% methanol (2% formic acid), 100% ethanol, 70% ethanol, 70% ethanol (2% formic acid), 100% acetone, 70% acetone, 70% acetone (2% formic acid), 70%

ethyl acetate (2% formic acid), 100% water. Ten grams of berries were mixed with 100 mL of each solvent for a brief extraction solvent test. The samples were extracted using a blender (Black and Decker Type I blender, Applica Consumer products, Inc Miramar, FL) and then sonicated in an ultrasonication bath (VWR 750D, VWR International, Mississauga, ON) in three cycles of 15 minutes at 28°C with 15 min intervals b/w each cycle. Following sonication, berry extracts were centrifuged for 10 min at 3000 rpm using a centrifuge (Sorvall ST16, Thermo Scientific, Hamburg, Germany). The supernatant was collected and *in vitro* analysis for phenolic composition and antioxidant activity was performed as stated in experimental procedures below.

3.2.2.2 Large scale extraction and fractionation

The large scale extraction and fractionation was performed after selecting the optimum extraction solvent. Frozen berries (500 g) were extracted with 2 L of solvent (acetone:water:formic acid, 70:28:2, v:v:v) using a blender (Black and Decker Type I, Applica Consumer products, Inc Miramar, FL) and then sonicated in an ultrasonication bath (VWR 750D, VWR International, Mississauga, ON) in three cycles of 15 minutes at 28°C. Following the sonication, berry extracts were centrifuged for 10 min at 3000 rpm using a centrifuge (Sorvall ST16, Thermo Scientific, Hamburg, Germany). The extracts were collected in a round bottom flask and dried using a rotary evaporator (Buchi R-200, Buchi Corporation, New Castle, DE) and then reconstituted to 500 mL with an ethyl acetate and water solution (50:50, v:v). The ethyl acetate and water solution was then subjected to aqueous two-phase liquid-liquid separation using a 1 L separatory funnel. The berry extract in the separatory funnel was allowed to stand for 24 h as polyphenols separated into two layers based on their polarity. The upper ethyl acetate layer and lower aqueous layer were carefully removed into different flasks by opening the PTFE stopcock. At the interface between two layers of polyphenol solutions, the stopcock of separatory

funnel was carefully closed to prevent mixing of the layers. The separated layers were stored at 4°C. After liquid-liquid separation, both separated layers were slowly dried using a rotary evaporator (Buchi R-200, Buchi Corporation, New Castle, DE). The upper organic layer was reconstituted in 50 mL of ethyl alcohol:water mixture (50:50) and aqueous layer in 50 mL of ultrapure water. The purified organic layer in 50% ethanol was used for further fractionation in 400 g C_{18} adsorbent resin (Sorbent SP-207-05 sepabead resin brominated, Sorbent Tech., Norcross, GA) packed into a chromatography column (46 × 2.3 cm, length and internal diameter) and equilibrated with ultrapure water (specific resistance 18 MΩ cm^{-1}) and 50% ethanol. Berry extracts were washed with water to remove sugars, until the sugar percentage reached less than 1% as measured using a digital refractometer (Model no. 300016, Sper Scientific, Scottsdale, AZ). The phenolic compounds were fractionated using a step-wise gradient of varying concentration of aqueous ethanol. The polyphenols-rich fractions (F1-F15) were obtained using 500 mL of 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 90% and 100% ethanol. All the fractions were collected in amber bottles and stored at 4°C till further analysis. The extraction and fractionation procedures were repeated three times to collect sufficient amounts of phenolic fractions for biochemical assays and the fractions were characterized using ultra-performance liquid chromatography electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS).

3.2.3 UPLC-ESI-MS/MS analysis

UPLC-ESI-MS/MS analysis was conducted using the method described by Sekhon-Loodu et al, 2013. Phenolic compounds present in partridgeberry samples were separated using the UPLC system (Model Waters Aquity CHA, Waters Corp, Milford, MA) equipped with an Aquity BEH C_{18} (100 mm × 2.1 mm, 1.7 µm) column and C_{18} guard column (Waters Corp, Milford, MA).

The flow rate of the UPLC system was maintained at 300 μL min^{-1} with a total 12 minute run time for each sample. The injection volume of sample was maintained at 2 μL. All the standards and samples were prepared in 100% methanol and their concentrations were used as follows: 0.20 - 20 mg/L of catechin, epicatechin, epigallocatechin, chlorogenic acid, caffeic acid, ferulic acid, phloridzin, quercetin (Q), Q-3-*O*-galatoside, Q-3-*O*-rutinoside and Q-3-*O*-glucoside. The anthocyanin standard samples were also prepared in methanol and their concentrations were used as follows: 0.25 - 25 mg/L of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-galactoside. MS-MS analysis was carried out using a Micro-mass Quattro Micro API MS/MS system. Electrospray ionization (ESI), in negative ion mode (ESI-), was used for the analysis of the flavonols, flavan-3-ol, and phenolic acid compounds. ESI in positive ion mode was used for anthocyanins. Mass spectrometry conditions used for the analysis were as follows: capillary voltage 3000 V, nebulizing gas (N_2) at a temperature of 375°C at a flow rate of 0.35 mL min^{-1}. The cone voltage (25-50 V) was optimized for each compound. Individual compounds were identified using the multiple reactions monitoring mode (MRM), using specific precursor-production transition: m/z 301→105 for Q, m/z 463→301 for Q-3-*O*-glucoside and Q-3-*O*-galactoside, m/z 609→301 for Q-3-*O*-rutinoside, m m/z 289→109 for catechin, m/z 290→109 for epicatechin, m/z 353→191 for chlorogenic acid, m/z 179→135 for caffeic acid, m/z 305→125 for epigallocatechin, m/z 457→169 for epigalloatechingallate, m/z 317→245 for phloridzin, m/z 329 → 234 for phloritin, m/z 193→134 for ferulic acid, m/z 331→242 for cyanidin-3-*O*-glucoside and m/z 303→229 for cyanidin-3-*O*-galactoside.

3.2.4 Preparation of partridgeberry polyphenol fractions

After selection of the solvent for the highest recovery of phenolics from partridgeberry, the extracts were subjected to various separation and chromatography techniques for the extraction

of different classes of polyphenols. The detailed methodology as shown in Fig 1 and described in section 3.2 and 3.2.1 was used to prepare polyphenol-rich partridgeberry fraction (PPF) rich in different classes of partridgeberry polyphenols.

3.2.5 Total phenolic content

Total phenolic content (TPC) was measured using the Folin Ciocalteu assay as outlined by Rupasinghe, Erkan, & Yasmin (2009). The phenolic content of berry extracts was expressed as gallic acid equivalents per 100 g fresh weight (FW) while TPC of PPFs was expressed as gallic acid equivalent per g dry weight (DW).

3.2.6 Total flavonoid content

Total flavonoid content (TFC) was measured using the aluminum chloride colorimetric method as outlined by Rupasinghe, Erkan, & Yasmin (2009). The total content of berries was expressed as quercetin equivalents per 100 g FW while TFC of PFs was expressed as Quercetin equivalent per g dry weight (DW).

3.2.7 Total proanthocyanidin content

An improved 4-dimethylaminocinnamaldehyde (DMAC) colorimetric method as described by Prior et al (2010) was used with slight modifications to measure total proanthocyanidins (PAC) in berry extracts. The extraction solvents used to extract PAC are described in section 2.3.1. Ethanol was acidified by adding 6N H_2SO_4 to 100 mL of ethanol (80%) in a glass bottle. DMAC reagent (50 mg) was added to H_2SO_4 acidified ethanol and added in 3:1 ratio to analyzed samples. Catechin at 20, 100, 250, 500 and 750 µM concentrations was used as standard reference compound. The results (concentration of PAC) were expressed as µmole catechin equivalent per 100 g FW while that of PPF was expressed as catechin equivalent per g dry weight (DW).

3.2.8 The ferric reducing ability of plasma (FRAP) assay

FRAP assay is based on the ferric to ferrous ion reduction at low pH by antioxidants. The assay was performed as described by Rupasinghe, Erkan, & Yasmin (2009). The antioxidant capacity of berries extracts was expressed as Trolox equivalents per 100 g fresh weight while that of PPF as Trolox equivalents per g dry weight (DW).

3.2.9 The oxygen radical absorption capacity (*ORAC) assay*

ORAC assay is an antioxidant capacity determination assay based ability of antioxidants to protect flourescein against peroxy radical generator 2,2'-azobis (2-amidinopropane) dihydrochloride. The assay was performed according to previously described method by Rupasinghe, Erkan, & Yasmin (2009). The antioxidant capacity of berries using ORAC assay was also expressed as μmole Trolox equivalents per g dry weight (DW).

3.2.10 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

DPPH radical scavenging assay uses DPPH, a stable free radical for measuring antiradical ability of candidate antioxidants. DPPH after accepting hydrogen from a donor antioxidant loses its characteristic deep purple color and becomes colorless. The assay was performed as described by Sharma & Bhat (2009) and results were expressed as IC_{50} values (inhibitory concentration of berry fractions decreasing the absorbance of DPPH solution by 50%).

3.2.11 Cell Culture

Human fibroblasts or WI-38 cells (ATCC® CCL-75™) were obtained from Cedarlane Labs (Burlington, ON) and maintained in 75 cm^2 culture flasks. Fibroblasts were grown at 37°C in a humidified incubator (VWR International, Mississauga, ON) supplied with 95% air and 5% CO_2. WI-38 cells were grown in DMEM medium, supplemented with 10% FBS and 100 mg/L of penicillin and streptomycin. Medium of growing cultured cells was changed every 48 hours. In

order to conduct specific experiments, cells were collected from the culture flask using EDTA trypsin. The detailed information involving cell experiments is described in experimental sections.

3.2.12 Peroxyl radical-induced cellular stress model

2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH; peroxyl radical generator) based cellular model for oxidative stress as outlined by He et al (2013) was used with modifications. Briefly, 2×10^4 WI-38 cells were seeded in 96-well plates containing 100 µL of growth media. Oxidative stress was induced by addition of 100 µL of 30 mM AAPH to WI-38 cells. To assess the cytoprotective effects of partridgeberries, the fibroblasts (WI-38 cells) were incubated with berry extracts before inducing oxidative insult using peroxyl radical generated by AAPH. The effects of oxidative stress were analyzed by measurement of cytotoxicity and cell viability in polyphenol-treated fibroblasts with respect to individual assay controls. The model was verified by an assay control (negative control) having no AAPH-induced oxidative stress and a vehicle control.

3.2.13 Cell Viability Assay

Cell viability was assessed using the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). The assay was performed according to the manufacturer's instructions after optimization of cell number for the assay. Briefly, 1×10^4 WI-38 cells suspended in 100 µL of growth media were seeded in 96-well sterile plates (BD International, Mississauga, ON). Cells were pretreated with crude extract and three partridgeberry polyphenol fractions separately (Fig 1) and then subjected to AAPH-induced oxidative stress. After induction of oxidative stress, the viability of cells was measured by pipetting 20 µl of the MTS solution into each well of the plate containing fibroblasts in culture medium. The plate was

incubated for 2 hours at 37°C in a humidified, 5% CO_2 atmosphere and absorbance was read at 490 nm using FLUOstar OPTIMA plate reader (BMG Labtech Inc., Offenburg, Germany). The results were expressed as the percent viability relative to control cells.

3.2.14 LDH Release Assay

Cytotoxicity analysis was conducted using the CytoTox-ONE™ homogeneous membrane integrity assay (Promega Corporation, Madison, WI). Briefly, 2×10^4 cells were seeded using 100 µL media in a 96-well tissue culture plate. Cells were pretreated with crude extract and four partridgeberry polyphenol preparations for 24 h and oxidative stress was induced using 30 mM AAPH for 24 hours. Plates were removed from 37°C incubator and placed at room temperature for 30 minutes. All remaining steps for membrane damage assessment assay were performed according to the manufacturer's instructions. The absorbance value was read using FLUOstar OPTIMA plate reader (BMG Labtech Inc. Offenburg, Germany) at wavelength of 590 nm. The percentage membrane damage was expressed as the release of the enzyme lactate dehydrogenase from cells with respect to control cells.

3.2.15 Reactive oxygen species (*ROS) analysis*

ROS were analyzed using methodology outlined by Wang & Joseph (1999). Briefly, WI-38 cells were seeded in a 24-well sterile plate at density of 2×10^5 cells per well. Cells were allowed to adhere for 24 hours and then incubated with four partridgeberry polyphenol preparations for 12 h prior to oxidative insult. After incubation, cell growth media containing the berry fractions was removed and cells were washed twice with PBS. Cellular growth media was changed to FBS-free medium and 5 μM of fluorescein was added to cells, and plate was again placed in incubator for 5 minutes. After 5 minutes, 30 mM AAPH was added to the wells of 96-well plate at temperature of 37°C. The 96-well plates were immediately placed in fluorescence plate reader at

an excitation wavelength of 490 nm and an emission wavelength of 510 nm. Fluorescence was measured at interval of 60 minutes (initial reading at 0 minute and final reading at 60 minutes) and ROS were estimated as percentage inhibition of flourescein with respect to an assay control.

3.2.16 Protein content

Total protein content was determined in the cells using the Bradford assay (Bio-Rad Protein Assay kit). The assay was performed according to the manufacturer's instructions (Bio-Rad, Mississauga, ON). Standards at five different concentrations were prepared using the bovine serum albumin and absorbance was read at 595 nm using FLUOstar OPTIMA plate reader (BMG Labtech Inc., Offenburg, Germany). The results were expressed as the percentage inhibition of protein oxidation by PPF with respect to assay control (untreated cells).

3.2.17 Peroxide detection assay

Peroxides were measured by using the Thermo Scientific Pierce quantitative peroxide assay kit (Fisher Scientific Canada, Nepan, ON) according to the manufacturer's directions. Briefly, 2×10^5 WI-38 cells were seeded in a 6-well sterile plate. Seeded cells were then treated with 100 and 1000 µg/mL of partridgeberry polyphenol preparations separately for 24 h followed by oxidative insult using 30 mM of AAPH. The peroxides were measured following the manufacturer's instructions and absorbance was read at 595 nm using FLUOstar OPTIMA plate reader (BMG Labtech Inc. Offenburg, Germany). The results were expressed as percentage inhibition of peroxide radicals with respect to a positive control (untreated cells).

3.2.18 Nuclear factor erythroid 2-related factor 2 (Nrf2) ELISA

Nrf2 ELISA was performed using a commercially available kit (MyBioSource, Inc., San Diego, CA). Analysis was performed according to the manufacturer's instructions. Briefly, 2×10^5 WI-38 cells were grown in 6-well tissue culture plates using complete growth media. The cells were

then pretreated with crude extract and three partridgeberry fractions (Fig 1) for 24 h and subjected to AAPH-induced oxidative stress for 12 h (Section 2.12). The adherent cells were detached using EDTA-trypsin and collected by centrifugation. Collected cells were washed thrice with cold PBS and subjected to ultrasonication for 3 cycles of 10 seconds. Cellular debris was removed by centrifuging cells at 4000 rpm and cell lysate was immediately used to perform the experiment. The results for Nrf2 ELISA assay were expressed as ng/mL of Nrf2 in cell lysate.

3.2.19 Statistical analysis

Completely randomized design (CRD) was used and all experiments were done in triplicates (n=3) unless stated otherwise. All the results were expressed as mean ± SD (standard deviation). Statistical comparison of the means was performed using one-way ANOVA, followed by Tukey's test at $p < 0.05$ confidence interval using the statistical analysis system (SAS Institute, Cary, NC).

3.3. Results

3.3.1 Initial extraction and solvent selection

3.3.1.1 Total phenolic content

The Folin-Ciocalteu assay indicated that the highest total phenolics were present in solvent **10** of the ethyl acetate: water: formic acid (80:18:2) extraction method [25 µM GAE/g fresh weight (FW)] followed by other extraction solvents ($p \leq 0.05$) (Table 1). The lowest total phenolics were obtained through anhydrous methanol (**1**) and water (**11**) assisted extraction procedures ($p \leq 0.05$). However, all other extraction solvents (**2-9**) were statistically similar in their total phenolic

content ($p \leq 0.05$). Overall, the extraction using solvents with lower dielectric constant led to higher yields of total phenolics.

3.3.1.2 Total flavonoid content

Total flavonoid content obtained by the aluminum chloride ($AlCl_3$) colorimetric method indicated the highest flavonoid content (19.8 μM QE/100 g FW) in polyphenols extracted by solvent 6 (EtOH:H_2O:FA, 70:28:2, v:v:v) followed by solvent 9 and solvent 5 ($p \leq 0.05$) (Table 1). Unlike the total phenolic content, solvents 10, 1 and 11 exhibited the lowest potential ($p \leq 0.05$) as favorable solvents for flavonoid extraction. Interestingly, the addition of formic acid to solvents significantly improved the flavonoid extraction from partridgeberries ($p \leq 0.05$).

3.3.1.3 Total anthocyanin content

Table 1 summarizes all total anthocyanin values obtained from the different extraction solvents used, and indicates the influence of extracting solvent on total anthocyanin contents. The highest amount of the total anthocyanin was obtained by using solvent **10** [ethyl acetate: water: formic acid (80:18:2)] while solvent **11** was the weakest in its ability to extract anthocyanins from partridgeberries. Similar to the total phenolic and flavonoid results, the acidification of solvents, except methanol, resulted in improved extraction of target polyphenols (anthocyanins). Also, the solvents with lower dielectric constants were more suitable for extraction of anthocyanins from partridgeberries.

3.3.1.4 Total proanthocyanidin content

The results shown in Table 1 indicate significant differences ($p \leq 0.05$) in total proanthocyanidins contents between different extracts. Solvent 9 gave the highest proanthocyanidin content when compared with other solvents ($p \leq 0.05$), followed by solvent 1, solvent 4 and solvent 3 while

solvents 10 and 11 exhibited the lowest amount of extracted total proanthocyanidins in partridgeberry extracts. Similar to the earlier results, solvent 11 with the highest dielectric constant resulted in poor extraction of total proanthocyanidins from partridgeberry.

3.3.1.5 Ferric reducing antioxidant power (FRAP)

All the solvent extracts reduced the Fe^{3+}-tripyridyltriazine to Fe^{2+}-tripyridyltriazine and exhibited potent antioxidant activity (Table 1). The strongest antioxidant capacity ($p \leq 0.05$) was shown by solvent **9** followed by solvent **5** and solvent **1**. Continuing the previous trend solvent **11** exhibited the lowest antioxidant activity among all extracts which was in agreement to its phenolic and flavonoid content.

3.3.1.6 Selection of solvent system

In the current study, 11 different solvent types were used to identify the best solvent for extracting a wide range of polyphenols from partridgeberries. All berry extracts were rich in polyphenols but the concentration of polyphenols was significantly dependent on the solvent polarity and its acidification. An increase in the percentage of ethanol or methanol had no significant effect on extraction efficacy (Table 1). A similar trend was observed with acetone as there was only a marginal difference between the extraction efficacies of its hydrous and anhydrous counterparts. However, acidification of extraction solvents, particularly acetone, indicated the strong influence on extraction of polyphenols. Among compared solvent systems, acidified hydrous acetone (**9**) provided the greatest recovery of total phenolics, flavonoid, proanthocyanidins and exhibited the strongest antioxidant activity. The only limiting factor in the solvent **9** assisted polyphenol extraction was the lower content of anthocyanins compared to solvent **10**. However, this limitation can be overlooked due to poor efficacy of solvent **10** in

extraction of total flavonoids, proanthocyanidins along with weak antioxidant activity. These results may indicate that 70% acetone acidified with 2% formic acid is more appropriate to be used for extraction of phenolic compounds from partridgeberryies. On the other hand, water (**11**) is the least effective solvent for extracting the phenolic compounds despite its safety as solvent for human consumption.

3.3.2 Preparation of partridgeberry polyphenol fractions

The crude extract was obtained by extracting 500 g of berry in 2 L of acetone:formic acid:water mixture (70:2:28). The extract was nearly dried using Buchi R-200 rotary evaporator at 40°C and then completely freeze dried using stoppering tray dryer (Dura stop, Kinetic thermal systems, Stone Ridge, NY) for 48 hours. The powdered extract was weighed and stored in a -20°C freezer until further analysis. The first partridgeberry polyphenol fraction (PPF1) was obtained from aqueous partition (B) as shown in Supplementary data. The PPF1, rich in anthocyanins, was collected from the separatory funnel and freeze dried in a similar fashion as the crude extract. The powdered PPF1 was also weighed and stored in a -20°C freezer. The next two fractions, PPF2 (rich in flavan-3-ols by pooling F1-F5) and PPF3 (rich in flavonols by pooling F6-F10), were obtained after column chromatography was applied to the ethyl acetate partition (A) using a five percent gradient to obtain fractions 1-15 (Supplementary Data). The fractions were collected thrice and were evaporated using a rotary evaporator at 40°C, and then completely freeze dried using a stoppering tray dryer for 48 hours.

3.3.2.1 Analysis of polyphenol fractions using UPLC-ESI-MS/MS

Fifteen phenolic compounds were quantified by UPLC-ESI-MS/MS based on their retention time and external calibration curves (Table 2). Flavan-3-ol such as catechin, (-)-epigallocatechin (EGC) and (-)-epicatechin (EC) were concentrated in F1-F5. The catechin was concentrated in

fractions **1-5** while the highest amount of catechin was in fraction **4** ($p \leq 0.05$). The concentration of EGC was very low and was distributed over fractions **1-4,** while EC was also concentrated in the same fractions ($p \leq 0.05$). The highest content of both EC and catechin was found in fraction **4**. All other fractions, i.e. fractions **6-15** had very low content of flavan-3-ol compounds ($p \leq 0.05$). These fractions (F1-F5) were pooled, the solvent was evaporated and freeze dried to prepare flavan-3-ol-rich fraction (PPF2).

Flavonols, including quercetin (Q), Q-3-*O*-glucoside, Q-3-*O*-galactoside, and Q-3-*O*-rutinoside, were abundant in fractions number 6-10, which were eluted with 40% to 65% ethanol (Table 2). Fraction **6** (45% EtOH) and fraction **7** (50% ethanol) exhibited the highest amount of Q-3-*O*-glucoside and Q-3-*O*-galactoside ($p \leq 0.05$). The remaining fractions displayed the lowest range of the quantified flavonol ($p \leq 0.05$) when compared statistically. The highest amount of phloridzin, a dihydrochalcone, was detected in fractions **7** and **8** ($p \leq 0.05$) while the remaining fractions exhibited trace amounts of the dihydrochalcones. The next group of phytochemicals analyzed using UPLC was phenolic acids (Table 2). The results showed that phenolic acids, including caffeic acid, ferulic acid and chlorogenic acid, were abundant in different fractions as shown in Table 2. Fractions 6-10 were combined, evaporated and freeze dried to obtain flavonol-rich fraction (PPF3), also abundant in dihydrochalcones. All the polyphenol preparations were weighed into new autoclaved vials and a stock solution of 1000 µg/mL was prepared for *in vitro* biochemical and cell studies.

3.3.4 Spectrophotometric analysis of four polyphenol preparations

3.3.4.1 Total phenolic content

The results showed the highest total phenolics ($p \leq 0.05$) in flavan-3-ol- (PPF2) followed by flavonol-rich (PPF3) partridgeberry fractions (Table 3). Based on the dry weight of crude extracts and fractions, the lowest total phenolic content was shown by the crude extract, marginally followed by the anthocyanin fraction (PPF1) of partridgeberry ($p \leq 0.05$). The concentration of total phenolics in PPF2 was ~3.5 times higher than crude extract while PPF3 also exhibited around 3 times higher phenolics, compared to its crude counterpart. However, the crude extract and the anthocyanin fraction were not statistically different in their total phenolic content.

3.3.4.2 Total flavonoid content

The flavonoid content in four polyphenol preparations was assayed using the aluminum chloride ($AlCl_3$) colorimetric method (Table 3). PPF3 exhibited the highest flavonoid content among all polyphenol preparations while the crude extract displayed the lowest content of flavonoids ($p \leq 0.05$). The PPF3 exhibited ~10 times higher total flavonoid content compared to the crude partridgeberry extract. The PPF3 was followed by PPF2, with 7 times higher flavonoid content compared to the crude fraction. Similar to the total phenolic content results, crude extract and PPF1 differed marginally in their total flavonoid content at the assayed concentration ($p \leq 0.05$). The higher total flavonoid content measured in PPF2 by the $AlCl_3$ colorimetric method may be related to formation of aluminum chloride assisted acid stable complexes either at C-3 or C-5 hydroxyl group of flavonols, thus giving higher flavonoid content than expected (Chang, Yang, Wen, & Chern, 2002).

3.3.4.3 Total anthocyanin content

The results confirmed that the highest anthocyanin content ($p \leq 0.05$) occurred in PPF1, which exhibited ~7 times higher concentrations of total anthocyanins than that of the crude extract (Table 3). The PPF2 was followed by PPF3 with the lowest anthocyanin content among all partridgeberry fractions ($p \leq 0.05$). PPF2 had only 2.4% of the anthocyanins content of PPF1, thus confirming the effective separation of anthocyanins using liquid-liquid separation.

3.3.4.4 Total proanthocyanidins

Among the four polyphenol preparations, total proanthocyanidin content was greatest in PPF2, followed by PPF3 ($p \leq 0.05$) (Table 3). The crude extract and the anthocyanin-rich fraction displayed the lowest total proanthocyanidin content compared to the other two polyphenol-rich fractions ($p \leq 0.05$). In comparison to the crude extract and PPF1, the PPF2 and PPF3 consisted of about 3.5 times higher total proanthocyanidin content.

3.3.4 Antioxidant activity of polyphenol preparations

3.3.4.1 FRAP values

The antioxidant capacity measured by FRAP assay was the greatest in PPF3 followed by PPF2 ($p \leq 0.05$) (Table 4). However, the lowest antioxidant capacity was exhibited by the anthocyanin-rich fraction (PPF1) followed by the crude extract ($p \leq 0.05$). Both PPF2 and PPF3 exhibited twice the antioxidant capacity compared to PPF1. These results were confirmed at two concentrations and followed the similar trend observed for total phenolic and total flavonoid content of polyphenol preparations indicating the contribution of the polyphenols to the total antioxidant capacity of tested samples.

3.3.4.2 ORAC values

The strong antioxidant capacity measured by ORAC was exhibited by PPF2 and PPF3 when compared to the other two preparations (Table 4). Similar to FRAP assay results, the lowest ORAC value ($p \leq 0.05$) was observed in the PPF1 followed by the crude extract.

3.3.4.3 DPPH radical scavenging activity

The strongest anti-radical activity was exhibited by PPF2 ($p \leq 0.05$) (Table 4), followed by PPF3 ($p \leq 0.05$). The lowest anti-radical activity was exhibited by the crude extract, with ~700 times lower activity compared to PPF3. Similar to PPF3, PPF2 also exhibited ~187 times stronger activity than the crude extract. PPF1 was also a weak scavenger of free radicals compared to the other two fractions. The anti-radical activity of PPF1 was around 645 and 173 times weaker, compared to the PPF3 and PPF2, respectively. The results of anti-radical activity obtained by DPPH radical scavenging assay were in agreement with the results of FRAP and ORAC analyses as they confirmed the antioxidant properties of PPFs. However, DPPH results distinctly showed greater antiradical activity of PPF2 and PPF3 in comparison to the crude extract and PPF1 ($p \leq 0.05$).

3.3.5 Concentration of phenolic compounds measured by UPLC-ESI-MS/MS

The phenolic compounds present in the polyphenol preparations were determined using the UPLC-MS/MS technique (Table 5). Compared to the crude extract, PPF1 exhibited higher concentrations of anthocyanins including cyanidin-3-*O*-glucoside and cyanidin-3-*O*-galactoside ($p \leq 0.05$). However, the concentration of other phenolic classes in both crude extract and PPF1 was statistically similar ($p \leq 0.05$). PPF2 exhibited the highest concentration of flavan-3-ols among all the fractions as concentration of catechin and epicatechin was 62 and 43 times higher compared to its crude counterpart ($p \leq 0.05$). The PPF3 indicated the highest concentrations of

quercetin and related compounds as the concentrations of quercetin-3-*O*-galactoside and quercetin-3-*O*-glucoside in PPF3 were 58 and 52 times higher than the crude extract. Furthermore, the concentration of phloridzin was highest in PPF3 ($p \leq 0.05$) and exhibited higher concentration of total flavonols compared to both PPF1 and PPF2.

3.3.6 Cell studies

3.3.6.1 Cell viability

In order to characterize the potential cytoprotective activity of partridgeberry fractions, the cellular viability of peroxyl radical exposed WI-38 cells was evaluated using the MTS assay (Table 6). The exposure to AAPH generated peroxyl radical injury depleted the cell viability of WI-38 cells by 94%. However, the pre-exposure to partridgeberry polyphenols maintained the cell viability of WI-38 cells following oxidative insult. Among the polyphenol preparations, the strongest cytoprotective potential was exhibited by PPF2 ($p \leq 0.05$). When exposed to PPF2 at concentration of 1000 mg/L, WI-38 cells exhibited 95 percent cellular viability which was followed by 94 percent cell viability after PPF3 treatment, at the same concentration. However, PPF3 treatment at 100 mg/L concentration exhibited higher cellular viability compared to PPF2, at the same concentration ($p \leq 0.05$). The crude extract and anthocyanin fraction (PPF1) exhibited lower cell viability at both assayed concentrations compared to the other fractions ($p \leq 0.05$). PPF2 and PPF3 possessed the greatest cytoprotective ability against peroxyl radical-induced oxidative damage.

3.3.6.2 Membrane damage

Increased extracellular lactate dehydrogenase (LDH) is a biomarker of oxidative stress and disrupted cell integrity during the lipid peroxidation and oxidative stress. The LDH release assay

results showed that membrane damage in WI-38 cells ranged from 6.3% to 22.1% compared to a positive control (Table 6). The results showed that among the polyphenol preparations, PPF2 exhibited the greatest inhibition of LDH release caused by peroxyl radicals at 30 mM AAPH ($p \leq 0.05$). Interestingly, the crude extract and PPF1 displayed strong inhibition of membrane damage in WI-38 cells *in vitro*. However, contrary to the previous results, PPF3 emerged as a weak inhibitor of membrane damage in comparison to the crude extract and other partridgeberry fractions ($p \leq 0.05$).

3.3.6.3 ROS inhibition

The increased levels of intracellular ROS in WI-38 cells by AAPH induced oxidative injury were attenuated by exposure to partridgeberry polyphenols. The results showed that PPF3 and PPF2 exhibited the highest inhibition of ROS in WI-38 cells ($p \leq 0.05$) (Table 6). These fractions, at both assayed concentrations, were the most potent ($p \leq 0.05$) in their ability to limit the production of ROS, followed by PPF1 ($p \leq 0.05$) and the crude extract. These results followed the trend of cellular viability and antioxidant capacity assays, which established PPF2 and PPF3 as potent antioxidants.

3.3.6.4 Total protein content

Total protein content in WI-38 cells was assayed for the dual purpose of ELISA protein quantification and as an index of oxidative damage. There was a significance difference ($p \leq 0.05$) in the protein levels of cells treated with the four polyphenol preparations (Table 6). The analysis revealed that the oxidative insult decreased the level of protein content in control cells from 150 to 95.1 $\mu g\ mL^{-1}$ (data not shown). Subsequently, the polyphenol pretreatment atenuated the cellular protein oxidation by oxidative insult in WI-38 cells compared to the untreated cells. The

analysis showed that PPF3 and PPF2 were the most effective fractions in preventing the protein oxidation induced by peroxyl radical exposure, followed by PPF1 and the crude extract ($p \leq 0.05$). Continuing the earlier trend, crude partridgeberry extract and PPF1 exhibited the weakest potentials as antioxidants *in vitro* ($p \leq 0.05$).

3.3.6.5 Peroxide radical inhibition

The ability of the polyphenol preparations to mitigate oxidative stress caused by peroxyl radical in WI-38 cells was further examined by quantifying the inhibition of oxidative damage. The results indicated the strongest inhibition of peroxy radical damage in WI-38 cells by PPF2 and PPF3 ($p \leq 0.05$) at a concentration of 1000 µg/mL (Table 6). On the other hand, PPF1 also exhibited a strong ability to limit the production of peroxyl radicals in WI-38 cells at the same concentration. Similar to the ROS inhibition results, the crude extract at the concentration of 100 µg/mL displayed the lowest peroxide radical scavenging capacity ($p \leq 0.05$).

3.3.7 Nrf2 ELISA

The Nrf2 ELISA analysis (Fig. 2) revealed the cellular mechanism of the enhanced cytoprotective ability of polyphenol-exposed WI-38 cells. The first phase of the Nrf2 studies focused on the activation of antioxidant pathways in the absence of oxidative stress. The incubation of WI-38 cells with polyphenols significantly increased the intracellular levels of Nrf2, compared to the vehicle control ($p \leq 0.05$). The Nrf2 level in control cells i.e. without polyphenols treatment was 1.14 ng/mL while the levels of PPF-treated cells ranged between 9.04-13.76 ng/mL. The highest amount of Nrf2 activation was observed in the flavonol-rich fraction (12.91 mg/mL) while the anthocyanin-rich fraction exhibited the lowest amount (9.04 ng/mL) of the intracellular Nrf2. Curcumin, a known Nrf2 activator was chosen for comparison

and exhibited elevated levels of Nrf2 (13.35 ng/mL) in WI-38 cells compared to control, prior to oxidative stress ($p \leq 0.05$). Curcumin was a stronger Nrf2 activator compared to the partridgeberry crude extract and anthocyanin-rich fraction, thus exhibiting strong antioxidant activity *in vitro* ($p \leq 0.05$). In the second phase, Nrf2 levels were measured after the polyphenol treatment followed by the peroxyl radical induced oxidative stress. The phase two Nrf2 levels in control cells i.e. without polyphenol treatment after oxidative stress were 2.30 ng/mL while there was a sharp increase in the levels of Nrf2 in polyphenol treated cells ($p \leq 0.05$). The flavonol-rich fraction exhibited the highest amount (24.14 ng/mL) of intracellular Nrf2 ($p \leq 0.05$), followed by the flavan-3-ol-rich fraction ($p \leq 0.05$) which exhibited 19.63 mg/mL of Nrf2. In contrast, the anthocyanin-rich fraction maintained the lowest amount of Nrf2 among all studied fractions ($p \leq 0.05$). Following the trend observed in phase I, curcumin exhibited the ability to activate Nrf2 and a stronger antioxidant potential compared to the crude extract and anthocyanin-rich fractions ($p \leq 0.05$). Overall, the flavan-3-ol-rich and flavonol-rich fractions of partridgeberry exhibited a greater potential to activate Nrf2 in WI-38 cells, in comparison to curcumin ($p \leq 0.05$). These results showed that the polyphenol preparations may activate the antioxidant response pathway through possible translocation of Nrf2 to nucleus.

3.4. Discussion

Increased use of dietary antioxidant-rich foods has gained much attention due to their ability to activate cytoprotective enzymes and extend health benefits via scavenging of free radicals (Reuter et al, 2010). The aim of this study was to evaluate the antioxidant and cytoprotective properties of polyphenol preparations of partridgeberries. Selecting the right solvent affects the total amount and class of polyphenols extracted as the acidified organic solvents with weak organic acids (0.5-3%) are recommended for the maximum extraction of polyphenols. The

higher amount of acids in extraction medium may result in the conflicting hydrolysis and degradation of polyphenols in plant materials (Dai & Mumper, 2010). In addition, the ultrasound-assisted extraction used in the current study, further facilitates the recovery of polyphenols by breaking the cell wall of plant materials. In the first phase of the study, the extraction solvents with high polarity led to higher recovery of polyphenols and subsequently exhibited greater antioxidant activity based on dry weight basis of extracts. Acidified 70% acetone was identified as the ideal solvent that facilitated the highest recovery of polyphenols from fresh partridgeberries. Various earlier studies have reported that 70% acetone resulted in higher recovery of polyphenols from plant based polyphenol sources including green tea leaves, peas and peanuts, as compared to other solvents like methanol, ethanol and water (Chavan, Shahidi, & Naczk, 2001; Drużyńska, Stępniewska, & Wołosiak, 2007). Apart from 70% acetone, 50% acetone has also displayed the capability of recovering higher amounts of polyphenols from plant sources as compared to methanol, ethanol and water (Suresh et al., 2013). Overall, our observations were in accordance with previous reports suggesting that the solvents with high polarity were optimum for extraction of polyphenols from fruits (Zhou & Yu, 2004; Yu, Ahmedna, & Goktepe, 2005). The extraction of bioactive compounds such as polyphenols from plant sources is the primary step in the preparation of dietary supplements or nutraceuticals.

In the next phase of the study, separation of partridgeberry polyphenols into three distinct fractions was achieved by employing liquid-liquid separation and solid-phase fractionation techniques. The liquid-liquid partitioning and/or solid phase extraction helps to remove polar non-phenolic compounds such as sugars, organic acids resulting in purification of fractionated products (Dai & Mumper, 2010). Multiple reports have used aqueous two-phase extraction to separate polyphenol classes, especially anthocyanins, based on their affinity for water (Liu et al.,

2013; Wu et al., 2011). In the current study, the liquid-liquid partitioning resulted in migration of >96% anthocyanins into the aqueous phase, indicating the successful separation of two classes of polyphenols (anthocyanin and non-anthocyanins). The elution of polymeric anthocyanins using liquid-liquid separation results in reduced contamination of anthocyanins in the non-aqueous phase, thus exhibiting the higher sensitivity of the extraction method. The ethyl acetate fraction (non-anthocyanin) was further used to obtain the flavan-3-ol and flavonol-rich fractions. Several authors have reported the use of a similar strategy to fractionate polyphenol mixtures into class specific polyphenols extracts. A similar distribution of apple polyphenols into fractions was earlier reported by Sekhon-Loodu et al (2013), where the gradient range of alcohol was used to separate various phenolic classes. Another report by Wilson et al (2006) also reported the separation and fractionation of wild blueberry extracts (*Vaccinium angustifolium*) using gradients of organic solvents to obtain class specific polyphenols.

In the subsequent phase of the current study, the flavan-3-ol- and flavonol-rich fractions were found to be more powerful radical scavenger and antioxidant agents *in vitro*, than the corresponding crude extract. The presence and higher concentration of catechins and quercetin glycosides are strong contributory factors to the antioxidant activity of these fractions. Our findings are similar to an earlier report where the class specific fractions emerged as potent antioxidants *in vitro* (Sekhon-Loodu et al., 2013). Similarly, a study by Chen et al (2013) also reported the high antioxidant activity of flavonol glycosides from sea buckthorn compared to other extracts.

Further, the crude extract and three partridgeberry fractions also indicated cytoprotective action against the peroxyl radical-induced oxidative damage of WI-38 cells. Similar to the antioxidant activities measured for these fractions, the flavan-3-ol- and flavonol-rich fractions

also exhibited higher cytoprotective ability, which can be related to individual cytoprotective polyphenol constituents such as quercetin glycosides (Ramyaa & Padma, 2013) and catechins (Banach, Dong, & O'Brien, 2009). As phenolic acids and flavonoids are relatively easily absorbed into human circulation, compared to anthocyanins (Graf, Milbury, & Blumberg, 2005), the flavan-3-ol and flavonol-rich fractions may play a significant role in attenuating oxidative stress *in vivo* (Banach, Dong, & O'Brien, 2009; Ramyaa & Padma, 2013). Similar to the current observations, other berries of *Vaccinium* species have also exhibited cytoprotective ability (Del Bo et al., 2010) leading to attenuation of lipid peroxidation and cellular damage. Finally, the activation of the Nrf2 pathway by the partridgeberry polyphenol preparations confirmed that these natural antioxidants not only scavenge free radicals but also trigger signal transduction pathways leading to possible activation of multiple genes involved in antioxidant response. It is important to note that Nrf2 under oxidative stress dissociates from Kelch-like ECH-associated protein 1 (Keap1) and binds with antioxidant response element (ARE) in the nucleus to trigger expression of antioxidant enzymes (Motohashi & Yamamoto, 2004). Similar to the antioxidant and cytoprotective abilities, flavan-3-ol- and flavonol-rich fractions were potent activators of Nrf2 in WI-38 cells. This may be related to fact that these fractions contain known activators of Nrf2 such as quercetin and catechins (Ramyaa & Padma, 2013; Singh, Shankar, & Srivastava, 2011). Overall, partridgeberries contained distinct polyphenol sub-classes and exhibited antioxidant, free radical scavenging ability and cytoprotection properties *in vitro*.

3.5. Conclusion

Vaccinium species of berries are a rich source of dietary polyphenols with strong antioxidant and cytoprotective properties. In the current study, acidified hydrated acetone (70:28:2) provided the greatest recovery of polyphenols from fresh partridgeberries. Anthocyanin-, flavan-3-ol- and

flavonol-rich fractions obtained from partridgeberries exhibited strong antioxidant and cytoprotective abilities against peroxyl radical-induced cell death in WI-38 cells. All the polyphenol fractions were potent antioxidants *in vitro* and significantly reduced the cell death and membrane damage caused by oxidative stress in WI-38 cell model system. Among the three fractions, flavan-3-ol- and flavonol-rich fractions exerted relatively higher antioxidant and cytoprotective properties than the crude extract and the anthocyanin-rich fraction. Partridgeberry polyphenols were also found to enhance antioxidant enzyme system through the activation of Nrf2 pathway *in vitro*. The current study warrants further mechanistic exploration of the Nrf2-ARE pathway and suggests that the partridgeberry derived polyphenol-rich extracts may represent a potential antioxidant therapy in humans, particularly in view of their potent antioxidant and cytoprotective effects.

3.6. References

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Table 1 Total phenolics, total flavonoids, total anthocyanins, total proanthocyanidins and antioxidant capacity of various partridgeberry extracts

Solvent	Total Phenolics (µmole GAE/100 g FW)	Total Flavonoids (µmole QE/100 g FW)	Total Anthocyanins (µmole C3GE/100 g FW)	Total Proanthocyanidins (µmole CE/100 g FW)	FRAP (µmole TE/100 g FW)
Methanol (**1**)	21.42±1.02^{b}	11.18±0.12^{e}	111.46±0.31^{b}	4.68±0.13^{b}	8.12±0.22^{abc}
Methanol-Water (70:30) (**2**)	22.67±0.08^{ab}	13.97±0.75^{d}	106.36±0.25^{bcd}	3.81±0.03^{cd}	7.31±0.43^{cde}
Methanol-Water-FA (70:28:2) (**3**)	23.97±1.41^{ab}	12.91±0.24^{d}	61.95±0.10^{cde}	4.50±0.11^{bc}	7.93±0.12^{bcd}
Ethanol (**4**)	22.81±0.69^{ab}	13.26±1.13^{d}	107.56±0.26^{bc}	4.81±0.14^{b}	6.35±0.45^{f}
Ethanol-Water (70:30) (**5**)	22.75±0.53^{ab}	18.39±0.34^{abc}	77.98±0.60^{fg}	3.35±0.13^{d}	8.28±0.26^{ab}
Ethanol-Water-FA (70:28:2) (**6**)	22.47±0.64^{ab}	19.84±0.25^{a}	94.36±0.43^{de}	3.31±0.24^{d}	7.07±0.37^{def}
Acetone (**7**)	23.65±0.65^{ab}	17.11±0.63^{c}	85.22±0.24^{ef}	3.18±0.61^{d}	7.35±0.41^{cde}
Acetone-Water (70:30) (**8**)	21.82±2.52^{ab}	17.94±0.52^{bc}	85.22±0.22^{ef}	3.56±0.47^{d}	6.85±0.35^{ef}
Acetone-Water-FA (70:28:2) (**9**)	24.02±1.64^{ab}	19.32±0.24^{ab}	87.33±0.38^{ef}	5.90±0.32^{a}	8.85±0.38^{a}
EA-Water-FA (80:18:2) (**10**)	25.02±0.71^{a}	7.93±0.58^{f}	165.39±0.81^{a}	2.19±0.11^{e}	6.51±0.32^{f}
Water (**11**)	22.27±0.78^{ab}	10.67±0.26^{e}	70.82±0.64^{g}	2.17±0.19^{e}	6.20±0.38^{f}

$^{a\text{-}g}$Different superscripts within the column indicates significant differences ($p \leq 0.05$). FA, formic acid; FW, fresh weight ; µmole, micromolar; GAE: gallic acid equivalents; QE: quercetin equivalents; Total anthocyanins, expressed as µmole of cyanidin 3-glucoside equivalents (C3GE)/100 g FW; total proanthocyanins, expressed as µmole of catechin equivalents (CE)/100 g FW; FRAP: ferric reducing ability of plasma; TE: trolox equivalents

Table 2 Concentration (mg/L) of major sub-classes of polyphenols in fractions of partridgeberry measured using UPLC-MS/MS.

Fraction (% Ethanol)	**Total Flavonol**	**Total Dihydrochalcones**	**Total Flavan-3-ols**	**Total Hydroxycinnamic acid**
F1 (20%)	2.51±0.22^{e}	0.24±0.01^{c}	36.48±1.04^{b}	3.32±0.08^{e}
F2 (25%)	0.36±0.02^{f}	0.02±0.00^{d}	23.68±0.14^{c}	0.54±0.00^{f}
F3 (30%)	0.66±0.19^{f}	ND[1]	38.94±1.32^{a}	1.71±0.01^{f}
F4 (35%)	2.18±0.18^{e}	ND	43.14±2.3^{a}	2.08±0.00^{f}
F5 (40%)	6.24±0.42^{d}	0.06±0.00^{d}	26.30±0.91^{c}	16.76±1.01^{c}
F6 (45%)	25.26±0.68^{a}	0.39±0.00^{b}	11.34±0.52^{d}	28.78±2.15^{b}
F7 (50%)	27.76±0.46^{a}	0.62±0.00^{a}	2.50±1.82^{e}	39.46±3.22^{a}
F8 (55%)	23.32±0.2^{b}	0.78±0.10^{a}	0.72±0.05^{e}	39.54±1.81^{a}
F9 (60%)	13.02±0.41^{c}	0.42±0.00^{b}	0.42±0.01^{e}	19.68±2.00^{c}
F10 (65%)	4.1±0.02^{d}	0.08±0.00 ^{d}	0.26±0.01^{e}	4.72±0.60^{d}
F11 (70%)	1.91±0.1^{e}	0.04±0.00^{d}	0.42±0.02^{e}	1.16±0.01^{ef}
F12 (75%)	1.36±0.2^{f}	0.02±0.00^{d}	0.56±0.02^{e}	2.91±0.21^{e}
F13 (80%)	1.66±0.2^{e}	0.02±0.00^{d}	0.36±0.01^{f}	4.12±0.19^{e}
F14 (90%)	0.57±0.0^{e}	0.02±0.00^{d}	0.04±0.0^{f}	2. 17±0.22^{e}
F15 (100%)	0.34±0.0^{g}	0.02±0.00^{d}	0.08±0.0^{f}	0.32±0.00^{f}

Aceone:water:formic acid (70:28:2, v:v:v) was used as solvent for extraction of polyphenols from partridgeberry. All results are expressed as average (n=3) mg of polyphenolic compounds per L of fractionation product. Different letters in each column are significant different ($p \leq 0.05$) as obtained by Tukey's test. ND[1]- Not Detected; Total Flavonol: quercetin galactoside; quercetin-glucoside; quercetin rutinoside; quercetin. total dihydrochalchones: phloridzin and phloritin; Total Flavan-3-ols: (-)-epigallocatechin; catechin; (-)-epicatechin, Total Hydroxycinnamic acid: caffeic acid; ferulic acid; chlorogenic acid.

Table 3
Concentration of polyphenolic sub-classes (mg/g DW) measured spectrophotometrically for four polyphenol preparations of partridgeberry.

Polyphenol Preparation	TPC*	TFC§	TAC†	TPr.C‡
Crude	149.18±2.89^{d}	62.86±2.62^{cd}	3.96±0.04^{b}	184.17±7.94^{c}
PPF1	217.86±8.17^{cd}	86.70±2.17^{c}	29.01±0.09^{a}	201.83±10.79^{c}
PPF2	520.29±10.04^{a}	481.59±5.61^{b}	0.07±0.01^{c}	689.92±12.85^{b}
PPF3	440.16±8.65^{b}	624.61±9.58^{a}	2.72±0.02^{c}	767.88±9.05^{a}

$^{a-g}$ Different letters in each column indicate significant differences at $p \leq 0.05$ as obtained by Tukey's test (n=6). TPC: Total phenolic content; TFC: Total flavonoid content; TAC: Total anthocyanin content; TPr.C: Total proanthocyanidins content; PPF1: Anthocyanin-rich fraction; PPF2: Flavan-3-ol-rich fraction; PPF3:Flavonol-rich fraction.
Analysis was conducted at the concentration of 1000 µg/mL of assayed samples.
*Results are expressed as mg of gallic acid equivalents/g dry weight of partridgeberry fractions
§ Results are expressed as mg of quercetin equivalents/g dry weight of partridgeberry fractions
† Results are expressed as mg of cyaniding-3-glucoside equivalents/g dry weight of partridgeberry fractions
‡ Results are expressed as mg of catechin equivalents/g dry weight of partridgeberry fractions

Table 4
The antioxidant potential as measured by FRAP, ORAC and DPPH assays.

Polyphenol Preparation	Conc (µg/mL)	FRAP (µmole TE/ L)	ORAC (µmole TE/ L)	DPPH IC_{50} (µg/mL)
Crude	100	425.97±4.12^{g}	770.95±26.64^{f}	146.31^{d}
	1000	1086.88±26.88^{c}	1055.25±53.72^{d}	
PPF1	100	576.05±12.73^{f}	560.59±44.12^{g}	135.57^{c}
	1000	696.80±8.84^{e}	867.87±25.43^{e}	
PPF2	100	966.65±46.52^{d}	1545.34±42.32^{b}	0.21^{a}
	1000	1296.95±62.35^{b}	1773.23±33.84^{a}	
PPF3	100	1327.41±37.81^{b}	1078.13±16.93^{d}	0.78^{b}
	1000	1484.01±76.77^{a}	1290.54±15.57^{c}	

Different letters in each column indicate significant differences at $p \leq 0.05$ as obtained by Tukey's test (n=6).Values followed by different letters in a column are significantly different ($P < 0.05$) by Tukey's test. Among all the observed factors (2×2 factorial design), there was significant effect of interaction ($p<0.001$), which was used for statistical mean separation and letter grouping. FRAP: Ferric reducing ability of plasma; ORAC: Oxygen radical absorbance capacity; DPPH: The 2,2-diphenylpicrylhydrazyl

Table 5
Concentration of polyphenolic sub-classes (mg/g DW) measured by UPLC-MS/MS in four polyphenol preparations of partridgeberry.

Polyphenol Preparation	Total Flavonols	Total Dihydrochalchones	Total Catechins	Total Anthocyanins
Crude	9.71±0.18^{c}	8.13±1.04^{b}	12.34±1.36^{b}	56.51±7.14^{b}
PPF1	6.61±0.56^{c}	6.12±0.49^{b}	7.71±0.67^{b}	155.19±3.39^{a}
PPF2	24.58±1.09^{b}	56.86±6.84^{b}	384.24±2.86^{a}	7.06±0.62^{c}
PPF3	271.35±12.62^{a}	279.18±7.62^{a}	52.72±1.73^{b}	9.64±0.96^{c}

The extraction solvent, Acetone-Water-FA (70:28:2) was used as the solvent for extraction of polyphenols from partridgeberry. All results are expressed as mg of polyphenolic compounds per L of fractionation product. Different letters in each column are significant different ($p \leq 0.05$). Total Flavonol: quercetin-3-*o*-glucoside; quercetin; quercetin-3-*o*-rutinoside. Total Dihydrochalchones: phloridzin and phloritin; Total catechins: (-)-epigallocatechin; catechin; (-)-epicatechin, epigallocatechin-3-gallate; (-)-epicatechin-3-gallate.Total Anthocyanins: cyanidin-3-*O*-glucoside and cyanidin-3-*O*-galactoside.

Table 6
Cytoprotective and antioxidant activity of partridgeberry polyphenol preparations in human fibroblasts (WI-38 cells) against peroxyl radical-induced oxidative stress.

Polyphenol Preparation	Concentration (μg/mL)	Cell Viability (%)	Membrane Damage (%)	ROS Inhibition (%)	Protein Oxidation Inhibition (%)	Peroxide radical Inhibition (%)
Crude	100	55.93±3.62^{d}	10.94±2.73^{B}	30.11±3.56^{d}	31.14±1.37^{c}	49.78±0.77^{e}
	1000	83.58±7.01^{a}	18.29±2.72^{A}	70.56±4.79^{b}	24.03±0.39^{bc}	70.26±3.55^{bc}
PPF1	100	44.03±5.43^{e}	6.32±1.94^{B}	50.51±4.69^{c}	32.55±1.32^{c}	58.41±0.41^{d}
	1000	80.19±2.03^{b}	13.54±4.36^{A}	72.93±3.27^{b}	25.56±2.97^{c}	77.13±4.46^{b}
PPF2	100	66.41±4.98^{c}	5.62±2.15^{B}	73.54±5.23^{b}	74.39±2.58^{b}	70.02±0.85^{bc}
	1000	94.52±3.68^{a}	18.92±2.9^{A}	95.14±16.33^{a}	84.52±2.56^{a}	85.85±4.37^{a}
PPF3	100	81.32±4.07^{b}	12.31±2.78^{B}	78.66±3.35^{b}	76.35±1.83^{b}	65.29±1.11^{c}
	1000	93.99±4.54^{a}	22.07±6.45A	94.44±3.57^{a}	86.19±3.95^{a}	80.03±5.60^{a}

$^{a\text{-}f}$Different letters in each column (except for % membrane damage) are significant different ($p \leq 0.05$) as obtained by Tukey's test. Data are means ± standard deviation of six independent replicates (n=6).
$^{A\text{-}B}$Among all the observed factors (2×2 factorial design), there was significant effect of interaction ($p < 0.001$) except membrane damage analysis, which was used for statistical mean separation and letter grouping.
All the percentage measurements are relative to the control of no pre-incubation with polyphenol preparations. PPF1: Anthocyanin-rich fraction; PPF2: Flavan-3-ol-rich fraction; PPF3: Flavonol-rich fraction.

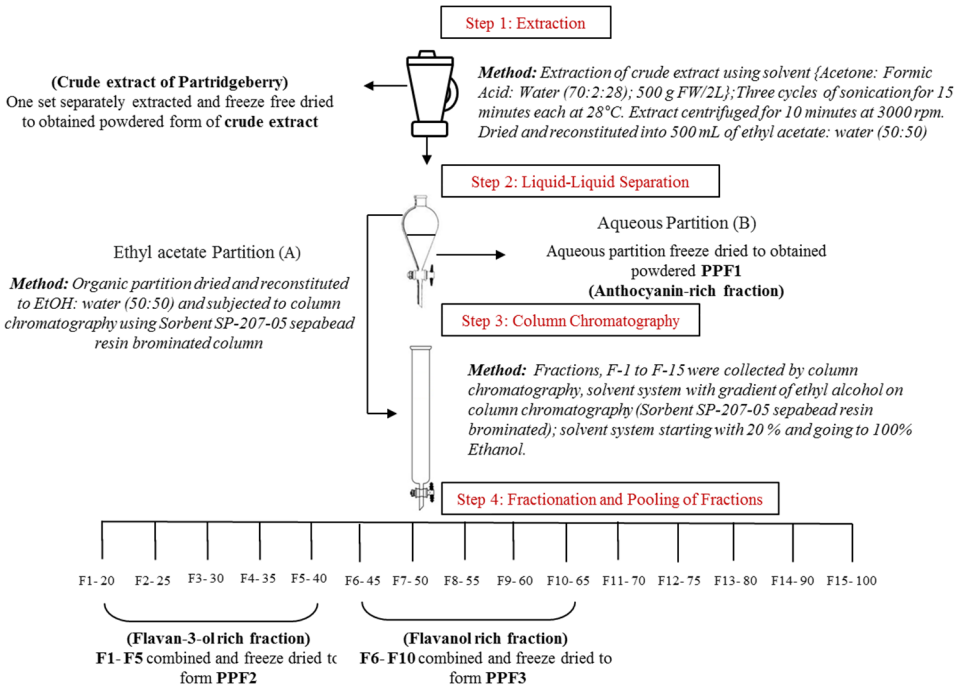

Figure 1. Method of obtaining crude extracts, and 3 fractions of partridgeberry derived polyphenols. Crude extract was obtained using extraction solvent (Acetone:water:formic acid, 70:28:2, v:v:v) PPF 1 (Anthocyanin fraction from hydrous layer of water:ethyl acetate 1:1), PPF2 (Flavan-3-ol-rich fraction obtained by merging partitions 1-5), PPF3 (Flavonol-rich fraction obtained by merging fractions 6-10). Extraction solvent corresponds 70% Acetone + 2% Formic acid. PPF1: Anthocyanin-rich fraction; PPF2: Flavon-3-ol-rich fraction; PPF3: Flavonol-rich fraction.

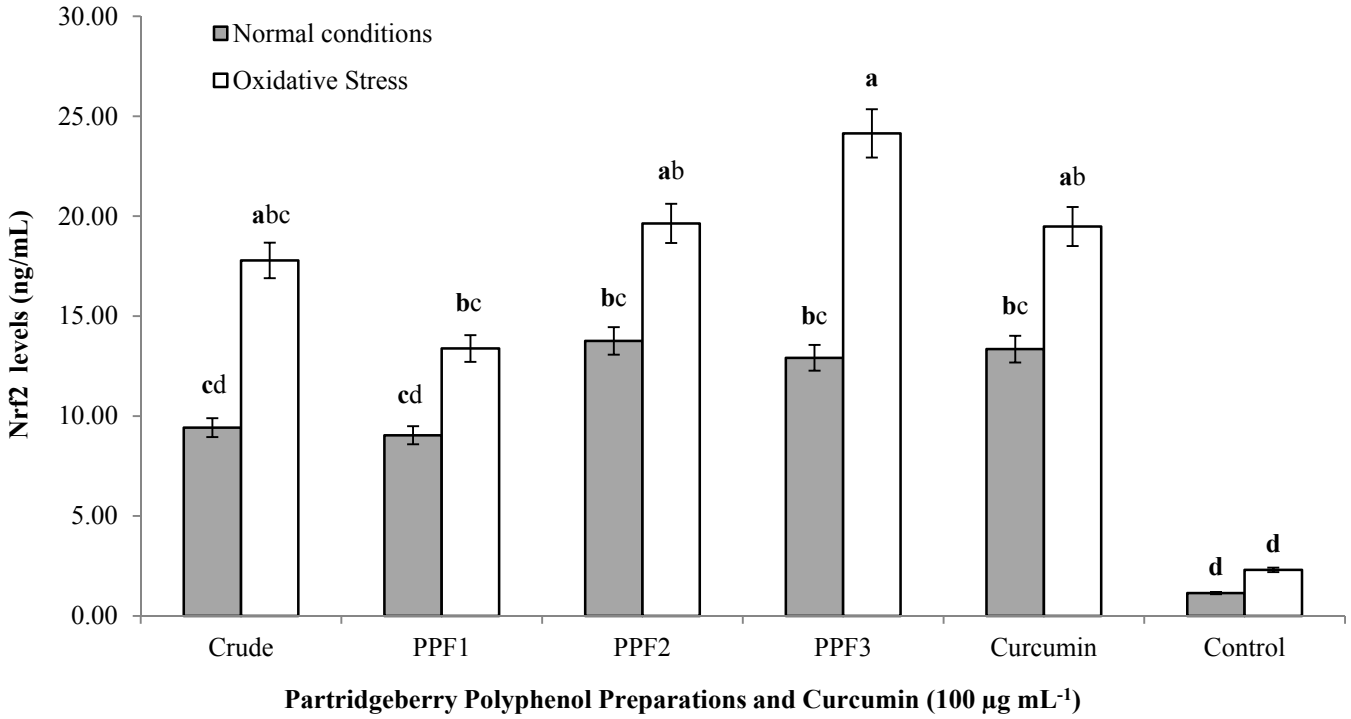

Figure 2. Transcriptional induction of Nrf2 in fibroblasts incubated with partridgeberry polyphenol preparations (100 µg mL^{-1}) or curcumin (100 µg mL^{-1}) for 24 h. Nrf2 levels are expressed as ng/mL of cell lysate. Data is representative of the six replicates (n=6) for each experiment. PPF1: Anthocyanin-rich fraction; PPF2: Flavan-3-ol-rich fraction; PPF3: Flavonol-rich fraction.

CHAPTER 4: PARTRIDGEBERRY POLYPHENOLS PROTECT PRIMARY CORTICAL AND HIPPOCAMPAL NEURONS AGAINST Aβ TOXICITY: RELEVANCE TO ALZHEIMER'S DISEASE

4.0 Abstract

As the population ages, Alzheimer's disease (AD) will become a major health burden worldwide. Aβ deposition elicits a toxic effect on neurons and plays a crucial role in the aetiology and/or progression of AD. Recently, a growing body of evidences suggests that polyphenols derived from plant sources may modulate the pathophysiology of AD, possibly through antioxidant mechanism(s). The present study was aimed to investigate the neuroprotective potential of polyphenols of partridgeberry (*Vaccinium vitis-idaea* L. var. minus lodd) and elucidate the mechanism by which they confer protection against Aβ toxicity in rat primary neurons *in vitro*. The pre-treatment of rat primary cortical and hippocampal neurons with partridgeberry polyphenols (10-200 μg mL^{-1}) significantly attenuated Aβ-induced cell death and membrane damage. The flavan-3-ol- and flavonol-rich fractions of the partridgeberry exhibited the strongest ability to maintain cell viability (EC_{50} 5.9 μg mL^{-1}) and prevent lactose dehydrogenase release (IC_{50} 0.01 μg mL^{-1}) ($p \leq 0.05$). Similar to the maintenance of cellular viability, the flavan-3-ol- and flavonol-rich fractions also amplified the greatest activity of SOD and catalase among all polyphenol preparations exposed to neurons ($p \leq 0.05$). All four partridgeberry polyphenol preparations reduced the intracellular Aβ levels by 7-15 folds, and initiated Aβ clearance from neurons as compared to untreated cells ($p \leq 0.05$). Partridgeberry derived polyphenol preparations; especially the flavonol-rich fraction (IC_{50} 97.1 μg mL^{-1}) significantly inhibited the *in vitro* acetycholinesterase activity ($p \leq 0.05$), indicating potential pharmacotherapy application in AD. These findings suggest that partridgeberry polyphenols,

especially flavan-3-ols- and flavonols-rich fractions, could be of importance in prevention and/or treatment of AD and other oxidative stress-related diseases.

4.1. Introduction

Alzheimer's disease (AD) is a progressive and fatal neurodegenerative disorder that affects millions of people worldwide through pathological decline in brain health and cognitive abilities. As of the year 2010, about 35.6 million people suffer from AD and it is predicted that the incidence of AD may increase to 81.1 million by year 2040 (Prince et al., 2013). The pathology of this chronic disease includes a reduction in the size of temporal and frontal lobes of the brain, along with loss of memory and cognitive abilities due to degeneration of neurons. There are multiple hallmarks of AD, but principally the presence of amyloid-β (Aβ) plaques and neurofibrillary tangles (NFTs) are the major characteristics of the disorder (Liang-Hao et al., 2013). The Aβ plaques are characterized as the deposits of fibrils and amorphous aggregates of Aβ peptides while the NFTs are intracellular fibrillar aggregates of the hyperphosphorylated tau protein (Takahashi et al., 2010). The presence of the Aβ plaques and NFTs in the cortical and hippocampal regions of brain directly affect, and consequently impair, the ability of learning and trigger memory loss (Guillozet, Weintraub, Mash, & Mesulam, 2003). During the progression of AD, the concentrations of neurotransmitters including acetylcholine are reduced through the increased enzymatic concentrations of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). These enzymes degrade acetylcholine and create a cholinergic deficit, thus adding to pathological alterations in the brain of an AD patient (Ballard, 2002). Apart from these classical hallmarks, the brain in AD experiences increased oxidative stress and invasion of reactive oxygen species (ROS) leading to mitochondrial dysfunction. These mitochondrial abnormalities further initiate disruptions in energy metabolism, calcium homeostasis and trigger metal ions accumulation in Aβ plaques (Jomova, Vondrakova, Lawson, & Valko, 2010).

Currently, there is no complete cure for AD and drugs such as cholinesterase inhibitors (e.g. donepezil), used for treating symptoms such as memory loss, provide only modest and temporary symptomatic relief (Prince et al., 2013). Other prospective treatment strategies are also aimed at a possible reduction of Aβ production using medications such as β- or γ-secretase inhibitors, anti-inflammatory drugs, tau therapy, apolipoprotein E (APOE) therapy and Aβ vaccination (Citron, 2010). However, the clinical use of these medications is associated with side effects including headache, nausea, vomiting, loss of appetite and dizziness, along with treatment failure in the advanced stages of the disease (Clodomiro et al., 2013). Interestingly, for the last two decades after the credence of free radical hypothesis of AD, one vital approach for AD therapeutics has focused on modulating oxidative stress damage in brain. Therefore, in order to combat the disease onset and symptom progression in AD along with medication side effects, bioactive natural phytochemicals have gained attention for their nutritional intervention. Emerging evidence has indicated that polyphenols and other phytochemicals occurring ubiquitously in fruits, vegetables and some medicinal plants may ameliorate neuronal signal transduction and communication, and attenuate Aβ engendered oxidative stress and neural toxicity, both *in vitro* and *in vivo* (Bhullar & Rupasinghe, 2013; Choi, Lee, Hong & Lee, 2012; Park et al., 2008; Joseph et al., 2003).

Fruits of the partridgeberry (*Vaccinium vitis-idaea* L. var. minus lodd) grown in Newfoundland and Labrador, Canada are a polyphenol-rich wild berry species with strong antioxidant and cytoprotective characteristics (See chapter 3). Oxidative stress, cholinergic deficit and Aβ toxicity are all closely associated with the pathology of AD. As a result, the polyphenol-rich partridgeberry dietary intervention, owing to its sound antioxidant potential, is thought to be beneficial in the attenuation of the AD symptoms. However, to date, the effects of

partridgeberry on Aβ toxicity and related oxidative damage have not been elucidated either *in vitro* or *in vivo*. Therefore, the present study was performed to investigate the potential of partridgeberry polyphenols against the Aβ-induced cytotoxic effects in rat primary cortical and hippocampal neurons along with its assessment as a prospective cholinesterase(s) inhibitor *in vitro*.

4.2. Materials and methods

4.2.1 Materials

Acetylcholinesterase (AChE) from the electric eel (*Electrophorus electricus)*, butyrylcholinesterase (BChE) from equine serum, galanthamine hydrobromide, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), acetylthiocholineiodide, butyrylthiocholine iodide (BTCI), Dimethyl sulfoxide (DMSO), Hydrochloric acid (HCl), powdered $A\beta_{1-42}$, Thioflavin T, dichlorofluorescein, Iron(II) chloride, FerroZine, poly-D-lysine and sodium citrate buffer solution (pH 8.0) were obtained from Sigma-Aldrich (St. Louis, MO). Sterile 96, 24 and 6 well poly-D-lysine coated assay plates, pipettes and cell culture flasks were obtained from BD Biosciences (Mississauga, ON). CellTiter 96® AQ_{ueous} non-radioactive cell proliferation assay kit and CytoTox-ONE™ homogeneous membrane integrity assay kits were obtained from Promega (Madison, WI).

4.2.2 Preparation of partridgeberry fractions

Partridgeberry polyphenol fractions (PPFs) were prepared as described earlier in Chapter 3. Briefly, the crude partridgeberry extract was obtained by extracting 500 g of berry fruits in 2 L of acetone: formic acid: water mixture (70:2:28). The crude extract was dried using a rotary evaporator (Buchi R-200, Buchi Corporation, Asheville, NC) and stored in a -20°C freezer until further analysis. Another batch of the crude extract was obtained again, nearly dried using a

rotary evaporator (Buchi R-200, Buchi Corporation, Asheville, NC) and then reconstituted into 1000 mL of 50% aqueous ethyl acetate. The 50% aqueous ethyl acetate solution was then subjected to aqueous two-phase liquid-liquid separation using a 1 L separatory funnel. This extract was allowed to stand for 24 h in a separatory funnel as the polyphenolic constituents separated into two layers based on their polarity. The upper ethyl acetate layer (non-anthocyanin) and lower aqueous layer (anthocyanin-rich) were then carefully removed into separate flasks by opening the PTFE stopcock. The separated fractions were then immediately stored at 4°C and later dried using a rotary evaporator (Buchi R-200, Buchi Corporation, New Castle, DE). The powdered form of the extracts were weighed and stored in a -20°C freezer until further analysis. The first partridgeberry polyphenol fraction (PPF1), rich in anthocyanins was obtained from aqueous partition (B) as shown in Chapter 3 (Fig 1, Chapter 3) and freeze dried to obtain powdered form of the polyphenol-rich fraction. The powdered PPF1 was then weighed and stored in a -20°C freezer. The next two PPFs (rich in flavan-3-ols and flavonols) were obtained after column chromatography technique was applied on the ethyl acetate partition (Fig 1, Chapter 3) using C18 column chromatography and eluted with 5% gradient of ethanol to obtain fractions 1-15. After LCMS quantification, fractions 1-5 and 6-10 were combined separately and freeze dried to obtain PPF2 (flavan-3-ol-rich) and PPF3 (flavonol-rich), respectively. All PPFs were weighed to obtain stock solutions of 1000 μg mL^{-1} using DMSO for *in vitro* biochemical and cell studies.

4.2.3 Selection of assay concentrations

In the current study, we systematically tested partridgeberry polyphenols at a wide range of concentrations, ranging from 10-1000 μg mL^{-1}, for their potential anti-Aβ activity *in vitro*.

4.2.4 Cell culture

Primary rat cortex and hippocampal neurons were obtained from Life Technologies Inc. (Burlington, ON) and cultured according to the manufacturer's instructions. Briefly, the cells were cultured in neurobasal™ medium supplemented with 200 mM glutamax™-I and 50X B27 supplement (Life technologies, Burlington, ON). Cells were plated on to a poly-D-lysine (4.5 μg/cm^2) coated cell cultures flasks, and 24- and 6-well plates. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in an incubator (VWR International, Mississauga, ON). After 24 hours of incubation, half of the growth medium was aspirated from each well and replaced with fresh medium. Cells were cultured every second day by replacing half of the medium from each well with fresh medium. The primary rat hippocampal neurons were supplemented with 25 μM L-glutamate up to the 4th day in culture for optimal growth and maintenance of neurons. Overall, the neurons were cultured using standard aseptic conditions on poly-D-lysine (4.5 μg/cm^2) coated plates and exposure of culture to light was avoided.

4.2.5 AChE and BuChE inhibition assay

The *in vitro* AChE and BuChE inhibition assays were performed according to the methodology outlined by Ellman, Courtney & Featherstone (1961). The absorbance was quantified at 410 nm wavelength using BioTek, Power wave XS2 spectrophotometer (BioTek, Winooski, VT). The results were expressed as *in vitro* percentage inhibition of cholinesterase(s) with respect to an assay control.

4.2.6 Aβ (1-42) toxicity and treatment model

The Aβ toxicity was induced in growing cortex and hippocampal neurons on the day of the experiment after changing the growth media. The cells were cultured according to standard conditions and were first given fresh neurobasal media, and incubated for 2 h at 37°C in an incubator (VWR International, Mississauga, ON). Afterwards the cells were exposed to freshly

solubilised $A\beta_{1-42}$ peptides (50 μM) for 24 h and then given fresh medium containing or not PPFs at different concentrations. The experiment was conducted using treatment of PPFs against $A\beta_{1-42}$ peptide toxicity.

4.2.7 Aβ firbril formation assay

The Thioflavin T (ThT) dye fluorescence assay was used to quantify the formation and inhibition of amyloid fibrils in the presence of PPFs. The assay was performed as per the initial report (Hudson, Ecroyd, Kee, & Carver, 2009) and fluorescence measurements were performed using the FLUOstar OPTIMA plate reader (BMG Labtech Inc. Offenburg, Germany). The analysis was performed using both Aβ cell model and real time *in situ* ThT fluorescence assay. The results were expressed as mean ± SD of the percentage inhibition of the Aβ fibril formation activity with respect to an assay control.

4.2.8 Cell viability and injury assay

Cell viability was assessed using the CellTiter 96® AQ_{ueous} non-radioactive cell proliferation assay (Promega, Madison, WI). Briefly, 2×10^4 cortex and hippocampal neurons were seeded separately using 500 μL neurobasal media in a 24-well tissue culture plate. After the induction of Aβ stress, the cellular viability was measured by pipetting 50 μL MTS solution into each well of the plate containing neurons in culture medium. The plate was incubated for 3 hours at 37°C in a humidified, 5% CO_2 atmosphere and absorbance was read at 490 nm using FLUOstar OPTIMA plate reader (BMG Labtech Inc., Offenburg, Germany). The proliferation indices were normalized to assay controls and results were expressed as EC_{50} values using regression analysis. Cell injury analysis was conducted using the CytoTox-ONE™ homogeneous membrane integrity assay (Promega Corporation, Madison, WI). Cells were pre-treated with the crude extract and three partridgeberry fractions for 24 h. All remaining steps for membrane damage assessment

assay were performed according to the manufacturer's instructions. The absorbance value was read using FLUOstar OPTIMA plate reader (BMG Labtech Inc. Offenburg, Germany) at wavelength of 490 nm. The LDH release assay results were expressed as the percentage of LDH release with respect to positive control and IC_{50} values obtained using regression analysis.

4.2.9 $A\beta_{1-42}$ peptide quantification

The amount of rat beta-Amyloid (1-42) in cortex and hippocampal neurons after Aβ stress was quantified using SensoLyte® anti-rat $A\beta_{1-42}$ quantitative ELISA kit (Anaspec Inc, CA). The ELISA was performed following the manufacturer's instructions and absorbance was read at wavelength of 490 nm using a BioTek, Power wave XS2 spectrophotometer (BioTek, Winooski, VT). The results were expressed as concentration of Aβ (pg mL^{-1}) in comparison to the vehicle control.

4.2.10 Intracellular ROS assay

Intracellular ROS were monitored in cortex and hippocampal neurons after Aβ stress using the 2', 7'-dihydrodichlorofluoresceindiacetate (DCFH-DA) assay as outlined by Wang & Joseph (1999). After PPF pre-treatment and Aβ stress, DCFH-DA was added to the cell culture plates at a final concentration of 5 μM. Fluorescence was quantified after 40 minutes of incubation in dark and ROS were estimated with respect to fluorescein degradation. The fluorescence was measured at an excitation wavelength of 490 nm and an emission wavelength of 510 nm using FLUOstar OPTIMA plate reader (BMG Labtech Inc. Offenburg, Germany). The results were expressed as mean percentage inhibition of ROS for six replicate determinations with respect to the assay control.

4.2.11 Superoxide dismutase (SOD) and catalase assay

The levels of endogenous antioxidant enzymes SOD and catalase were measured using commercially available kits (Cayman chemicals, Ann Arbor, MI). SOD activity in cortex and hippocampal neurons after Aβ stress and PPF treatments was assessed by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine. The measurement of catalase activity in cell lysates is based on the reaction of catalase with methanol in the presence of an optimal concentration of H_2O_2 resulting in formaldehyde formation. The formaldehyde produced was further measured using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen which changed from colorless to a purple color by forming bicyclic heterocyclic compound with aldehydes. The results of enzyme activity were expressed as nmol/min/mL SOD and catalase activity in the neuronal lysates.

4.2.12 Cellular Morphology

Cell morphology was examined under a contrast-phase microscope (Nikon Eclipse TS100, Nikon, Japan) and images were taken at different stages of cell culture.

4.2.13 Statistical analysis

All experiments were performed in hexaplicates (n=6) unless stated otherwise and the statistical significance of means was assessed by one way analysis of variances (ANOVA) using SAS software (SAS Institute, NC).

4.3. Results

4.3.1 AChE and BuChE inhibition values

Our first inquiry was whether the two cholinesterases were affected by the partridgeberry polyphenol preparations. To answer this question, we used an *in vitro* substrate assay to monitor changes in hydrolysis of acetylcholine in the absence or presence of four PPFs. The berry fractions were assayed at seven different concentrations and IC_{50} values for the enzyme

inhibition were obtained using regression analysis (Table 7). The IC_{50} values for AChE inhibition ranged between 97.1-204.3 μg mL^{-1}. The crude extract (IC_{50} 204.3 μg mL^{-1}) of partridgeberry displayed the weakest AChE inhibition, while flavonol-rich fraction (PPF3) (IC_{50} 97.1 μg mL^{-1}) exhibited the greatest AChE inhibition ($p \leq 0.05$). The crude extract of partridgeberry was closely followed by flavan-3-ol-rich fraction (PPF2) of partridgeberry (IC_{50} 192.9 μg mL^{-1}) while the anthocyanin-rich fraction (PPF1) exhibited significantly higher (IC_{50} 109.6 μg mL^{-1}) AChE inhibition compared to its crude and PPF2 counterparts ($p \leq 0.05$). Similar to AChE inhibition, fractionation significantly improved BuChE inhibitory ability of PPFs *in vitro* ($p \leq 0.05$). The IC_{50} values for BuChE inhibition ranged between 241.0-1014.2 μg mL^{-1}, indicating the stronger selectivity of PPFs towards AChE than the non-specific cholinesterase enzyme, BuChE. The strongest BuChE inhibition was exhibited by the PPF3 (IC_{50} 241.0 μg mL^{-1}) while the crude extract exhibited the strongest BuChE inhibition (IC_{50} 1014.2 μg mL^{-1}) ($p \leq 0.05$). Compared to the crude extract, PPF3 was about 18-time stronger BuChE inhibitor *in vitro*. The PPF3 was closely followed by the PPF2 fraction of partridgeberry (IC_{50} 255.2 μg mL^{-1}) while PPF1 exhibited significantly weaker (IC_{50} 772.9 μg mL^{-1}) BuChE inhibition than PPF2 and PPF3 ($p \leq 0.05$). Interestingly, both PPF2 and PPF3 exhibited strong AChE inhibition ($p \leq 0.05$), but displayed low selectivity index for the BuChE. The selectivity index (SI) calculation showed that crude partridgeberry extract, exhibited the highest selectivity index for AChE compared to BuChE, resulting in stronger inhibition of acetylcholine hydrolysis in comparison to butyrylcholine.

4.3.2 $A\beta_{1-42}$ firbril formation values

The Thioflavine T (ThT) staining assay was conducted to assess anti $A\beta_{1-42}$ activity of partridgeberry fractions (100 μg mL^{-1}) using real time *in situ* analysis and primary rat cortical

and hippocampal neurons *in vitro*. The real time *in situ* analysis using substrate showed that all berry fractions were potent anti $A\beta_{1-42}$ agents (Table 8) while classical amyloid fibrils were observed fluorometrically in samples of untreated $A\beta_{1-42}$. The strongest anti-Aβ activity was displayed by PPF2 and PPF3 ($p \leq 0.05$), followed by PPF1 and the crude extract of partridgeberry ($p \leq 0.05$). The assay was repeated for anti-Aβ activity in the primary rat cortical and hippocampal neurons. In contrast to the results from real time *in situ* analysis, the anti-Aβ activity of the crude extract and the PPF1 was significantly reduced in the cell model of Aβ stress ($p \leq 0.05$). The crude extract of partridgeberry exhibited ~6 times weaker anti-Aβ activity in the rat primary cortical neurons while the PPF1 also lost 8-fold of its real time *in situ* activity. The strongest anti-Aβ activity was displayed by PPF2 and PPF3 ($p \leq 0.05$), which also maintained their strong anti-Aβ activity in neurons *in vitro*. The anti-Aβ effects of the crude extract and the PPFs were also similar in the rat primary hippocampal neurons. Following the earlier trend, both the crude extract and the PPF1 exhibited very weak anti-Aβ activity in rat primary hippocampal neurons, as they lost around 4-5 times of their real time *in situ* anti-Aβ activity. As expected, the strongest anti-Aβ activity in rat primary hippocampal neurons was displayed by PPF2 and PPF3 of partridgeberry ($p \leq 0.05$). Overall, the PPF2 and PPF3 showed the strongest anti-Aβ activity as they blocked Aβ-fibril formation in both real time *in situ* analysis and in the cell model experiments *in vitro*, but no statistically significant differences were observed between both fractions ($p \leq 0.05$).

4.3.3 Cell viability values

The results depicting the ability of PPFs to inhibit $A\beta_{1-42}$ firbril formation suggested that PPFs might be useful in attenuation of Aβ-mediated neural toxicity (Fig 3). The cell viability assay following Aβ stress in primary rat cortical and hippocampal neurons was performed using MTS

assay (Promega, Madison, WI). The exposure of primary cortical and hippocampal neurons to freshly solubilised $A\beta_{1-42}$ peptides (50 μM) for 24 h resulted in the depletion of 71% and 94% cellular viability in the respective assay controls. This procedure was selected as it sought to determine whether pre-treatment of PPFs, which from our previous observations should maintain cell viability following Aβ-toxicity. The partridgeberry fractions were analysed at five different concentrations (10, 20, 50, 100, 200 μg mL^{-1}) and EC_{50} values were obtained from the emerging trends using regression analysis. Treatment with PPFs significantly improved the cellular viability of primary rat cortical and hippocampal neurons compared to untreated control cells *in vitro* ($p \leq 0.05$). The EC_{50} values for the primary cortical neurons ranged between 5.9-62.2 μg mL^{-1}. The strongest neuroprotective ability was exhibited by PPF2 and PPF3 (EC_{50} 5.9-7.7 μg mL^{-1}) while PPF1 was the weakest neuroprotective fraction (EC_{50} 62.2 μg mL^{-1}) of partridgeberry *in vitro* ($p \leq 0.05$). The active polyphenol preparations, PPF2 and PPF3 were followed by the crude extract of partridgeberry (EC_{50} 13.2 μg mL^{-1}) in its ability to maintain the cell viability following Aβ stress ($p \leq 0.05$). The cell viability studies using primary rat hippocampal neurons displayed EC_{50} values in the range of 7.3-34.2 μg mL^{-1}. Similar to primary cortical neurons, the strongest inhibition of membrane damage was exhibited by PPF2 and PPF3 (EC_{50} 7.3-8.9 μg mL^{-1}) while PPF1 was the weakest neuroprotective fraction (IC_{50} 34.2 μg mL^{-1}) of partridgeberry ($p \leq 0.05$). Following the previous results, the crude extract of partridgeberry (IC_{50} 18.7 μg mL^{-1}) closely followed the PPF2 and PPF3 in its ability to maintain cell viability following Aβ stress *in vitro* ($p \leq 0.05$). Overall, the results showed that partridgeberry polyphenol preparations were potent neuroprotective agent *in vitro* and sustained the cellular viability following Aβ stress in both primary rat cortical and hippocampal neurons.

4.3.4 Membrane damage values

The effect of Aβ stress on neural membrane damage *in vitro* and its attenuation by partridgeberry fractions was next examined using LDH release assay (Promega, Madison, WI). Similar to the cell viability studies, the crude extract and three partridgeberry fractions were analysed at five different concentrations (10, 20, 50, 100, 200 μg mL^{-1}) and IC_{50} values were calculated from the results (Fig 4). When $A\beta_{1-42}$ solution (50 μM) was prepared and incubated with neurons for 24 h, it was cytotoxic, and displayed around 69-71% elevation in LDH release in primary cortical and hippocampal neurons respectively. The results showed that the pre-treatment of primary rat cortical and hippocampal neurons with partridgeberry fractions prior to Aβ stress resulted in significantly lower LDH release compared to the untreated cells ($p \leq 0.05$). The IC_{50} values for LDH release inhibition in rat primary cortical cells ranged between 0.01-22.5 μg mL^{-1}. The strongest inhibition of membrane damage was exhibited by PPF2 and PPF3 (EC_{50} 0.01-0.03 μg mL^{-1}) while PPF1 was the weakest neuroprotective fraction (EC_{50} 22.5 μg mL^{-1}) of partridgeberry ($p \leq 0.05$). Similar observations were made in experiments with primary hippocampal neurons, as PPF2 was the strongest inhibitor of neural membrane damage (EC_{50} 0.6 μg mL^{-1}) while PPF1 exhibited the weakest ability (EC_{50} 17.5 μg mL^{-1}) to inhibit Aβ-induced membrane damage ($p \leq 0.05$). Interestingly, the PPF3 (EC_{50} 9.2 μg mL^{-1}) of partridgeberry exhibited weaker neuroprotective potency in hippocampal neurons ability than the crude extract ($p \leq 0.05$) *in vitro*. In summary, all berry fractions significantly attenuated Aβ-induced membrane damage in both primary rat cortical and hippocampal neurons *in vitro*, while PPF2 was a highly effective inhibitor of LDH release attributable to Aβ-induced membrane damage.

4.3.5 Intracellular ROS values

ROS are implicated in multifactorial pathogenesis of AD as they trigger the alteration of mitochondrial function and increase lipid peroxidation. All partridgeberry polyphenol fractions

and the crude extract were tested at concentration of 100 μg mL^{-1} for their ability to inhibit ROS following Aβ stress in the primary rat cortical and hippocampal neurons *in vitro* (Fig 5). The explosive ROS production following Aβ injury was suggestive that the binding of Aβ to the neural membrane may have triggered the intracellular ROS generation. The results showed that all partridgeberry fractions significantly attenuated the oxidative stress compared to the untreated control ($p \leq 0.05$). ROS inhibition in primary rat cortical neurons by partridgeberry fractions ranged between 59.6-92.1 % *in vitro*. The strongest inhibition of ROS was exhibited by PPF2 and PPF3 (92.1-88.1 %) while PPF1 exhibited the weakest inhibition of ROS *in vitro* ($p \leq 0.05$). Similarly, the PPF3 also exhibited the highest inhibition of ROS (91.6%) in primary rat hippocampal neurons *in vitro* ($p \leq 0.05$). The PPF3 was closely followed by PPF2 and the crude extract in their ability to attenuate ROS in hippocampal neurons ($p \leq 0.05$). Similar to the membrane and viability studies, the PPF1 showed the lowest ($p \leq 0.05$) ability to inhibit ROS following Aβ stress in neurons *in vitro*. The strong ROS inhibition (>90%) at 100 μg mL^{-1} suggests that either PPF3 or PPF2 preconditioning alone can dissuade the majority of ROS produced via Aβ insult, bypassing the requirement of a post injury antioxidant stimulation.

4.3.6 Superoxide dismutase (SOD) and catalase activity

Since ROS, oxidatively activated during Aβ-injury, were significantly attenuated by PPFs, we hypothesized whether SOD and catalase were activated in both cell types, owing to the increased concentration of exogenous activators of antioxidant pathway. In the current study, we observed that the partridgeberry fractions (100 μg mL^{-1}) protected the neurons *in vitro* against β-amyloid induced cell death and triggered the release of antioxidant enzyme, SOD. The release of SOD in partridgeberry extract treated primary cortical neurons ranged from 8.8-17.3 nmol/min/mL while the control and untreated cells exhibited 4.8 and 6.8 nmol/min/mL enzymatic activity

respectively (Fig 6. A). The highest SOD activity was observed in PPF2 followed by PPF3 ($p \leq 0.05$), and the *in vitro* enzymatic activity of both the fractions was twice as compared to the untreated cells. Following the earlier trend of weak cytoprotective ability, PPF1 emerged as the weakest activator of SOD in both primary cortical and hippocampal neurons ($p \leq 0.05$), while PPF3 and the crude extract exhibited the strongest release of SOD in primary rat hippocampal cells followed by PPF2 ($p \leq 0.05$). Catalase (CAT) or glutathione peroxidase is a crucial antioxidant enzyme as it detoxifies H_2O_2 produced during metabolic processes and extends cytoprotective therapy in Alzheimer's disease and ischemia-reperfusion injury (Yan, Wang, & Zhu, 2013). In the current study, the antioxidant effect of partridgeberry fractions against Aβ through induction of catalase enzyme was studied in the primary rat cortical and hippocampal neurons. The results showed that the antioxidant candidate fractions induced the catalase enzyme in primary rat neurons against Aβ stress ($p \leq 0.05$). The total catalase activity in partridgeberry extract treated primary cortical cells ranged between 2.3-5.4 nmol/min/mL while the control and untreated neurons exhibited 1.0 and 0.5 nmol/min/mL enzymatic activity respectively (Fig 6. B). The strongest induction of catalase was exhibited by the PPF2 (5.4 nmol/min/mL) of partridgeberry ($p \leq 0.05$) while PPF3 (4.5 nmol/min/mL) followed the leading fraction in its ability to express catalase *in vitro* ($p \leq 0.05$). The potent antioxidants, PPF2 and PPF3 demonstrated 11 and 9 times higher catalase activity than the untreated control respectively. However, the crude extract and PPF1 demonstrated the weakest activity of catalase in primary rat cortical neurons *in vitro* ($p \leq 0.05$). Similarly, the primary rat hippocampal neurons were also protected against Aβ through activation of catalase. The PPF2 treated neurons, exhibited the strongest (~12 times higher) catalase activity compared to the Aβ injury control, followed by PPF3 ($p \leq 0.05$). Similar to SOD results and catalase observations in cortical neurons, the PPF1

exhibited the weakest ability to trigger catalase release *in vitro* ($p \leq 0.05$). Collectively, these results suggest that in PPF2 and 3-treated neurons, the possible dismutation and detoxification of superoxide anion and H_2O_2 produced during Aβ stress, are most likely caused by hyperactivity of SOD and catalase.

4.3.7 β-Amyloid (1-42) peptide concentration

Next, we studied whether PPFs (100 μg mL^{-1}) affect Aβ peptide levels in rat primary neuronal cells using ELISA method. As the Aβ peptides have a crucial role in the pathogenesis of AD, therefore lowering their concentrations/levels serve as a crucial drug target in AD therapy. Partridgeberry polyphenol preparations were assayed for their ability to attenuate Aβ peptides in rat primary cortical and hippocampal neurons (Fig. 7). The results showed that the treatment of cells with partridgeberry fractions (100 μg mL^{-1}) significantly lowered the concentration of Aβ peptides in both rat cortical and hippocampal neurons ($p \leq 0.05$). The strongest anti-Aβ activity in primary cortical cells was exhibited by PPF3 and PPF2 fractions of partridgeberry ($p \leq 0.05$). These class specific polyphenol-rich fractions displayed ~7-fold downregulation of Aβ concentration *in vitro* (Fig. 7. A). Compared to PPF2 and PPF3, both the crude extract and PPF1 displayed the weak attenuation of Aβ peptides in rat primary cortical neurons ($p \leq 0.05$). The cell suspensions of primary cortical neurons were also analyzed for the Aβ concentration and results showed very high anti-Aβ activity of partridgeberry fractions *in vitro* ($p \leq 0.05$). The Aβ concentration in the cell suspensions treated with partridgeberry fractions ranged between 19.28-28.31 pg/mL. The strongest anti-Aβ activity was exhibited by PPF3 ($p \leq 0.05$), resulting in 15-fold downregulation of Aβ concentration than the untreated control. Following the primary cortical neurons results, the partridgeberry fractions significantly lowered the Aβ concentration ($p \leq 0.05$) in the primary hippocampal cells. The strongest anti-Aβ activity in hippocampal cells

was exhibited by PPF2 followed by PPF3 fraction of partridgeberry ($p \leq 0.05$). These fractions displayed 14- and 10-fold downregulation of Aβ concentration *in vitro* respectively (Fig. 7.B). Compared to PPF2 and PPF3, the PPF1 displayed the weakest anti-Aβ activity in rat primary hippocampal neurons ($p \leq 0.05$). The cell suspensions analysis also showed very high anti-Aβ activity of partridgeberry fractions *in vitro*. The Aβ concentration in the cell suspensions treated with partridgeberry fractions ranged between 66.1-300.2 pg/mL. Interestingly, the PPF1 which displayed the weakest cellular anti-Aβ activity, exhibited the strongest anti-Aβ activity in hippocampal cell suspension followed by the PPF2 and PPF3 ($p \leq 0.05$), possibly due to higher concentration in suspension due to poor cellular uptake.

4.3.8 Cellular morphology

The inspection of the short-term effects of Aβ injury and PPFs treatment on cell morphology and viability was conducted using the phase contrast microscopy (Fig. 8). Neurons treated with Aβ displayed changes in cellular morphology that commenced within 1 h of Aβ injury, resulting in gradual and irreversible loss of cellular morphology. Aβ treatment induced the cellular morphological changes such as cell shrinkage, plasma membrane rupture, along with loss of dendritic branches, indicating the degeneration of cellular machinery (Fig. 8.B). However, all neurons treated with PPFs remained stable and no noteworthy cytoskeletal or morphological damage was observed during 24 h of imaging. Amongst all PPFs treatment, neurons treated with the crude extract and PPF1 showed minor changes in cellular morphology and low neuron density (Fig. 8–C,D), as compared to vehicle control (Fig. 8–A). However, the PPF2 and PPF3 treated neurons, in agreement to previous results, demonstrated strong protection against Aβ toxicity (Fig. 8–E,F). The neurons treated with these two fractions, inhibited the Aβ -induced

changes in cellular morphological and viability. These results were in accordance with the MTS assay; however, it is unclear if Aβ-induced neural death is an apoptotic or necrotic process.

4.4. Discussion

Currently, the exact pathogenesis of AD is unclear, but a large body of scientific literature supports the free radical hypothesis of AD, i.e. that Aβ and its related oxidative stress plays a crucial role in the development of AD (Sultana et al., 2011). The alterations in the redox homeostasis during AD demote the activities of antioxidant enzymes like SOD and CAT, leading to oxidative stress, and impaired cognition and memory deficits. This is the first report showing that partridgeberry derived polyphenol preparations induce cellular preconditioning against Aβ in an *in vitro* model of rat primary hippocampus and cortical neurons. A proposed mechanism by which PPFs can exhibit neuroprotective ability in primary rat cortical and hippocampal cells is depicted in Fig. 9. The experimental evidence obtained in the current study to support the proposed PPF-induced neuroprotection is discussed below.

First, in the present report, an *in vitro* AD model was established using the primary cortical and hippocampal neurons by employing $A\beta_{(1-42)}$ stress, as the cortex and hippocampus are the key brain components involved in the memory formation and encoding of the spatial information. This model confirmed that Aβ-triggers neuronal death, increases ROS production and initiates membrane damage in neurons. The results indicate that partridgeberry polyphenols are able to protect both the cortical and hippocampal neurons exposed to the Aβ-induced toxicity *in vitro*. The results demonstrate that partridgeberry polyphenols can inhibit Aβ-mediated neural damage and cell death by attenuating the production of free radicals, and rejuvenating the neural antioxidant defense system. A significant feature of the current study was the strong anti-Aβ activity of partridgeberry polyphenols at low concentrations, depicting their efficacy for the

reduction of Aβ pathology in primary neurons, confirming previous reports, which concluded that the polyphenols at low concentration may be useful for the attenuation of AD symptoms and/or progression (Bhullar & Rupasinghe, 2013).

Secondly, the present findings regarding cellular viability and membrane cytotoxicity were similar to the previous studies that show the ability of polyphenols to attenuate Aβ toxicity and exert strong neuroprotective effects. Brewer et al. (2010) found that 125 and 500 μg mL^{-1} of blueberry extract reduced the Aβ neurotoxicity and extended protection against ROS in a cellular model of AD. Similarly, another study found that anthocyanin-rich bilberry (*Vaccinium myrtillus* L.) and blackcurrant (*Ribes nigrum*) at 0.25 to 31 μg mL^{-1} concentrations displayed significant inhibitory effects on soluble $A\beta_{40}$ and $A\beta_{42}$ levels in human SH-SY5Y neuroblastoma cells, and also improved the spatial learning in transgenic mice (Vepsäläinen et al., 2013). A similar report has also shown that cinnamon extract administration at 20- 40 μg mL^{-1} was able to rectify the neurotoxicity and neuronal damage in both neuronal PC12 cells and AD fly model (Frydman-Marom et al., 2011). Our research outcomes were also consistent with other reports, where polyphenol-rich extracts, from diverse plant sources such as walnuts (Muthaiyah, Essa, Chauhan, & Chauhan, 2011), turmeric (Shytle et al., 2012) and green tea (Okello, 2011) attenuated the Aβ-induced cognitive dysfunction in both cell and mouse models. Apart from the crude polyphenol extracts, their polyphenol constituents such as quercetin (Ansari et al., 2009), catechins (Haque et al., 2008), and phenolic acids (Harvey et al., 2011) have also demonstrated potent inhibition against β-amyloid induced neurotoxicity. In line to our mechanistic findings, the increased levels of neural SOD and CAT to combat Aβ related oxidative damage has been also shown previously, which indicate the upregulation of Nrf2/ERk1/2 pathway (Hritcu et al., 2014; Zhao et al., 2012). AD is exacerbated by ROS which hamper the subsequent upregulation of neural SOD as a

defence/compensatory mechanism against oxidative stress (De Leo et al., 1998). Therefore, one of the interesting findings of this study was the rise in the neural SOD, CAT activity and ROS scavenging following treatment with partridgeberry polyphenols. The antioxidant mechanism of partridgeberry polyphenols can be explained theoretically as a reaction of upregulated SOD with elevated superoxides (due to Aβ) to form diatomic oxygen and hydrogen peroxide, which is further converted to water by catalase (Yan, Wang & Zhu, 2013). As mitochondrial dysfunction is implicated in AD, our findings showing the simultaneous upregulation of SOD and CAT by partridgeberry polyphenols presents itself as a unique therapeutic option to attenuate the oxidative pathology of AD. Thirdly, our ELISA and Aβ-firbril formation assay show that PPFs can inhibit aggregation or promote its disaggregation at low concentrations (100 μg mL^{-1}). The $A\beta_{1-42,}$ a key toxic amyloidogenic form of Aβ formed fewer fibrils (as observed fluorometrically) in the presence of PPFs, whereas increased incubation of PPFs promoted clearance of Aβ-peptides in both cell types and suspensions. Microscopic evidence, illustrating fewer non-viable neurons in PPF-treated samples, supports cytoprotective, anti-Aβ aggregation ability of PPFs, while ELISA data with a completely different method confirmed the findings (Fig 6).

Finally, the restoration of cholinergic activity through AChE inhibition is also a crucial strategy of pharmacotherapy in AD as the principal function of AChE is to terminate the nerve impulse flow in the synapse by hydrolyzing acetylcholine. The overexpression of AChE is observed in the AD brain, which causes impaired memory and learning. Drugs from plant sources such as galantamine and taucrine, have been widely used for improving brain function through AChE inhibition, but no study has explored the anticholinesterase activity of partridgeberry polyphenols. Our results show that the flavonol-rich fraction (PPF2) from partridgeberry inhibited the AChE *in vitro*, in a dose-dependent manner. On the other hand, the

crude berry extract and other fractions indicated weak AChE inhibitory activity *in vitro*. Conversely, all the partridgeberry polyphenol preparations, including the crude extract exhibited very weak BChE inhibition activity *in vitro*. As AChE is abundant in the blood and in neural synapses as compared to BuChE, therefore the strong inhibition of the former cholinesterase by PPFs advocates the prospective use of partridgeberry polyphenols in attenuation or prevention of cholinergic deficit in AD. These findings are in accordance with the previous reports which indicated a strong anticholinesterase potential of polyphenol-rich *Vaccinium* and other fruits. For example, Papandreou et al. (2009) showed that wild blueberry (*V. angustifolium*) extract, abundant in polyphenols, improved the brain antioxidant status and displayed inhibition of AChE activity in adult mice. Similarly, polyphenols of rabbiteye blueberry (*V. virgatum*) also inhibited ROS mediated neuronal death and exhibited strong AChE inhibition in *in vivo* experiments (Jeong et al., 2013).

Overall, this study suggests that polyphenol-rich partridgeberry fractions, especially PPF2 and PPF3 at 0.01–8.9 μg mL^{-1} concentrations, exerted a protective effect on $A\beta_{(1–42)}$ induced toxicity in cortical and hippocampal neurons *in vitro*. These fractions, abundant in flavan-3-ols and flavonols respectively, exhibited the highest neuroprotection, possibly through higher cellular uptake than the crude and anthocyanin-rich fractions which are abundant in non-phenolics, especially sugars. However, the metabolic breakdown of flavan-3-ols and flavonols, during *in vivo* digestion raises questions regarding their efficacy (at low concentrations in plasma) for clinical use. As polyphenols, especially flavan-3-ols and flavonols, undergo extensive metabolism and biotransformation, the pharmacokinetic evidence suggests that these extracts/fractions may only attain very low-micromolar plasma levels in animal or human subjects following oral administration (Landete, 2012). The substantial amounts of polyphenols

that enter the large intestine and are subjected to extensive metabolism by the intestine microflora and the metabolites are then re-absorbed into the bloodstream before excretion (Possemiers, Bolca, Verstraete, & Heyerick, 2011). Various bioavailability studies in mice show that the bulk of the polyphenolic constituents were excreted, but small amounts of metabolites reach the brain. Interestingly, these low traces of polyphenols make their way through the blood brain barrier and exhibit neuroprotective effects through multiple mechanisms (Faria et al., 2010). The re-absorption of metabolized polyphenols explains the neuroprotective potential of polyphenols, despite their low bioavailability following extensive metabolism (Landete, 2012). Authors also suggest that the synergistic effects of the absorbed intact polyphenols and their colon metabolites might present an enhanced neuroprotective effect at low concentrations. In light of these facts, an *in vivo* study using mice model to further explore the bioavailability and the efficacy of polyphenol-rich partridgeberry extracts for neuroprotection in AD is under scheduling. Taken together, the results from the current report provide a plausible antioxidant mechanism by which partridgeberry polyphenols prevent cell death of cortical and hippocampal neurons following Aβ insult.

4.5. Conclusions

In the current study, we explored the neuroprotective potential of partridgeberry against Aβ-induced neurotoxicity. Partridgeberry polyphenols protected both primary cortical and hippocampal neurons from $A\beta_{(1-42)}$-induced neurotoxicity, and inhibited the cell death. The polyphenol-rich fractions, particularly flavan-3-ol- and flavonol-rich fractions, prevented the LDH leakage and maintained the redox homeostasis. Furthermore, partridgeberry polyphenols induced the elevation of intracellular antioxidant enzymes, SOD and CAT. Especially, all polyphenol-rich fractions suppressed the ROS expression, and hence attenuated the expression of

oxidative stress, which significantly contributes to the AD pathology. Our data further demonstrated that the flavan-3-ol- and flavonol-rich fractions of partridgeberry were capable of lowering the concentration of $A\beta_{(1-42)}$ in both neurons and their cell suspensions. The partridgeberry polyphenols are expected to exhibit neuroprotection *in vivo* by the direct effects of the absorbed parent compounds or their metabolites, which may cross the blood brain barrier. The results from the current study warrant further exploration of the partridgeberry polyphenols for treatment of AD and suggest that the antioxidant supplementation may represent a therapeutic potential to pursue AD treatment and/or prevention, particularly in view of the multiple mechanisms by which partridgeberry polyphenols exert anti-Aβ effects.

4.6. References

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Table 7. Percentage inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity by polyphenol preparations of partridgeberry (*Vaccinium vitis-idaea* L. var. minus lodd).

Polyphenol Preparation	Concentration ($\mu g\ mL^{-1}$)	AChE inhibition	IC_{50} value (AChE)	BChE inhibition	IC_{50} value (BChE)	SI for AChE
Crude	10	11.7±3.3		34.5±3.0		
	25	27.1±0.6		35.6±2.8		
	50	38.7±2.9	204.3^{d}	35.4±2.9	1014.2^{d}	4.9
	100	44.4±2.4		40.6±3.1		
	250	55.4±0.6		40.9±0.6		
	500	57.5±0.7		41.6±1.4		
	1000	64.7±2.9		49.3±3.2		
PPF1	10	34.7±3.6		24.7±3.1		
	25	40.8±1.2		38.4±0.7		
	50	49.0±4.5	109.6^{b}	42.8±2.3	772.9^{c}	7.1
	100	50.1±1.4		45.1±1.0		
	250	53.2±6.7		42.9±1.4		
	500	58.4±2.2		46.8±2.8		
	1000	62.6±2.1		52.9±1.7		
PPF2	10	17.1±1.9		24.7±3.3		
	25	35.5±1.2		36.0±2.5		
	50	40.1±1.7	192.9^{c}	49.4±0.4	255.2^{b}	1.3
	100	44.9±2.1		44.0±2.5		
	250	48.7±3.3		49.4±0.4		
	500	56.1±0.5		56.0±0.6		
	1000	68.2±2.7		62.0±3.6		
PPF3	10	26.7±2.6		18.4±0.8		
	25	34.4±2.1		30.7±1.9		
	50	42.3±4.2	97.1^{a}	35.7±1.4	241.0^{a}	2.5
	100	54.5±3.1		44.6±3.2		
	250	58.1±4.9		47.1±2.8		
	500	67.2±1.8		51.8±3.9		
	1000	70.5±0.8		67.9±1.6		

Results expressed as mean ± SD of percentage cholinesterase(s) inhibition activity with respect to an assay control (n=6). The values with different subscript for IC_{50} values are statistically different ($p \leq 0.05$). IC_{50} value is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function by fifty percent. PPF1: Anthocyanin-rich fraction; PPF2: Flavan-3-ol-rich fraction; PPF3: Flavonol-rich fraction. AChE: Acetylcholinesterase; BChE: Butyrylcholinesterase; IC_{50}: inhibitory concentration 50 is the concentration of the concerned polyphenolic preparation that can inhibit the activity of a cholinesterase by 50%; SI: Selectivity Index for AChE inhibition compared to BChE inhibition *in vitro*.

Table 8. Percentage inhibition of Aβ fibril formation *in vitro* by partridgeberry fractions as measured by the Thioflavin T assay.

Polyphenol Preparation	*In situ* analysis∞	Rat primary cortical neurons	Rat primary hippocampal neurons
Crude	63.3 ± 1.5^{b}	11.3 ± 1.1^{b}	16.8 ± 3.0^{c}
PPF1	65.2 ± 3.4^{b}	8.2 ± 0.8^{b}	13.4 ± 1.2^{d}
PPF2	82.9 ± 3.1^{a}	82.8 ± 3.5^{a}	63.5 ± 3.8^{b}
PPF3	83.3 ± 3.0^{a}	86.1 ± 3.7^{a}	68.7 ± 2.6^{a}
Vehicle	ND•	ND•	ND•

Results expressed as mean ± SD of percentage inhibition of the Aβ fibril formation activity with respect to an assay control (n=6). The values with different subscript are statistically different ($p\leq0.05$). PPF1: Anthocyanin-rich fraction; PPF2: Flavan-3-ol-rich fraction; PPF3: Flavonol-rich fraction. ∞*In situ* analysis defines the inhibition of Aβ fibril formation in the chemical reaction mixture free of cellular substrates.

*Anti amyloid activity (50 μM $A\beta_{1-42}$) was assessed using 100 μg mL^{-1} of partridgeberry fractions. Thioflavin T (ThT) dye (10 μM) was used for staining the $A\beta_{1-42}$ fibril sheets. ND• Not detected.

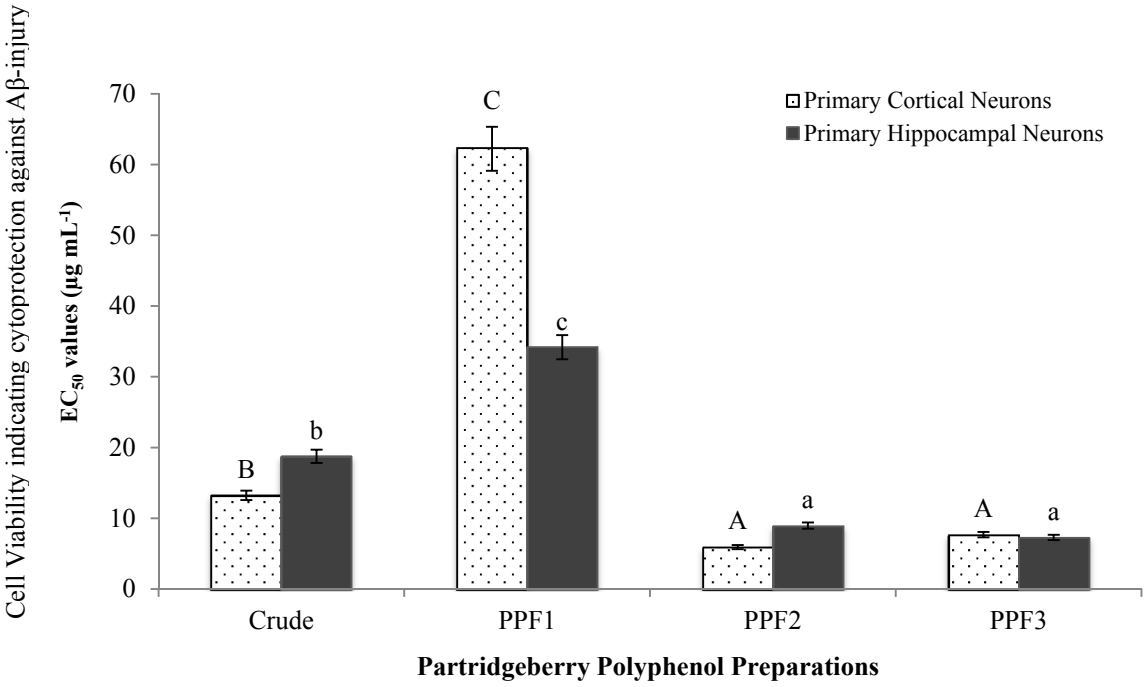

Figure 3. Inhibition of neuronal cell death as measured by MTS assay following Aβ injury by partridgeberry polyphenol preparations (100 µg mL^{-1}) in rat primary cortical and hippocampal neurons with respect to assay controls. Rat primary cortical and hippocampal neurons were treated with either 0.1 % DMSO (vehicle control), or 100 µg mL^{-1} partridgeberry polyphenol preparations, for 24 h, followed by Aβ injury (50 µM) as described under "Materials and methods". Results are expressed as EC_{50} values (half maximal effective concentration which maintains 50% cellular viability). The bars with different letters are statistically different ($p \leq 0.05$), A-C and a-c letters on bars as obtained by Tukey's test, are used to indicate statistical significance ($p \leq 0.05$) for treatment affect (Cell death inhibition) in rat primary cortical and hippocampal neurons respectively. EC_{50} values were obtained by conducting experiment at 5 different treatment concentrations. PPF1: Anthocyanin-rich fraction; PPF2: Flavan-3-ol-rich fraction; PPF3: Flavonol-rich fraction.

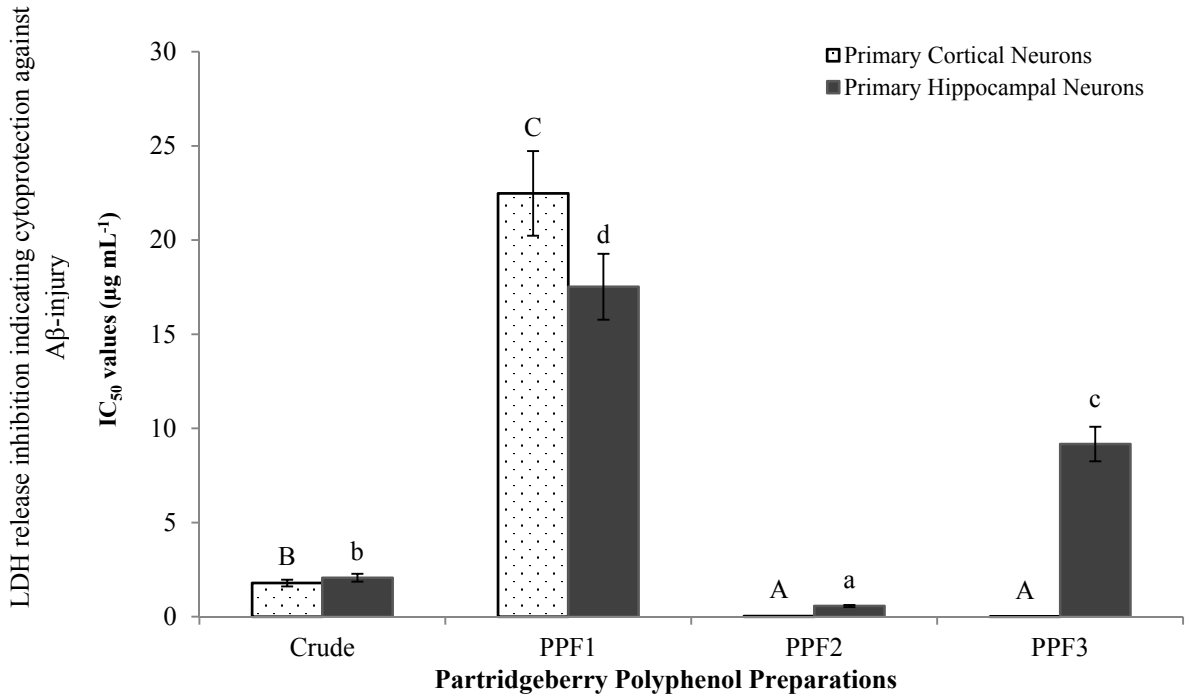

Figure 4. Inhibition of neuronal membrane damage measured by LDH release assay following Aβ injury by partridgeberry polyphenol preparations (100 µg mL^{-1}) in rat primary cortical and hippocampal neurons with respect to assay controls. Rat primary cortical and hippocampal neurons were treated with either 0.1 % DMSO (vehicle control), or 100 µg mL^{-1} partridgeberry polyphenol preparations, for 24 h, followed by Aβ injury (50 µM) as described under "Materials and methods". Results are expressed as IC_{50} values (half maximal inhibitory concentrations which inhibit 50% membrane damage), A-C and a-d letters on bars are significant different as obtained by Tukey's test, are used to indicate statistical significance ($p \leq 0.05$) for treatment affect (LDH release inhibition) in rat primary cortical and hippocampal neurons respectively. The bars with different letters are statistically different ($p \leq 0.05$). IC_{50} values were obtained by conducting experiment at 5 different treatment concentrations. PPF1: Anthocyanin-rich fraction; PPF2: Flavan-3-ol-rich fraction; PPF3: Flavonol-rich fraction.

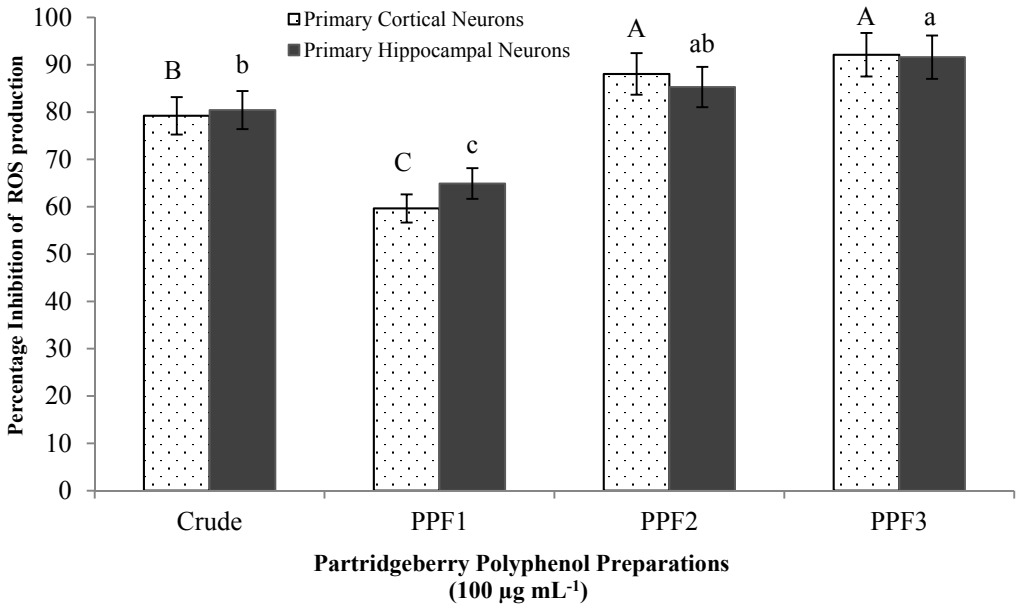

Figure 5. Percentage inhibition of reactive oxygen species (ROS) in rat primary cortical and hippocampal neurons by following Aβ injury by partridgeberry polyphenol preparations (100 µg mL^{-1}) with respect to assay controls. Rat primary cortical and hippocampal neurons were treated with either 0.1 % DMSO (vehicle control), or 100 µg mL^{-1} partridgeberry polyphenol preparations, for 24 h, followed by Aβ injury (50 µM) as described under "Materials and methods". A-C and a-d letters on bars are significant different ($p \leq 0.05$) as obtained by Tukey's test, are used to indicate statistical significance for treatment affect (ROS inhibition) in rat primary cortical and hippocampal neurons respectively. PPF1: Anthocyanin-rich fraction; PPF2: Flavan-3-ol-rich fraction; PPF3: Flavonol-rich fraction.

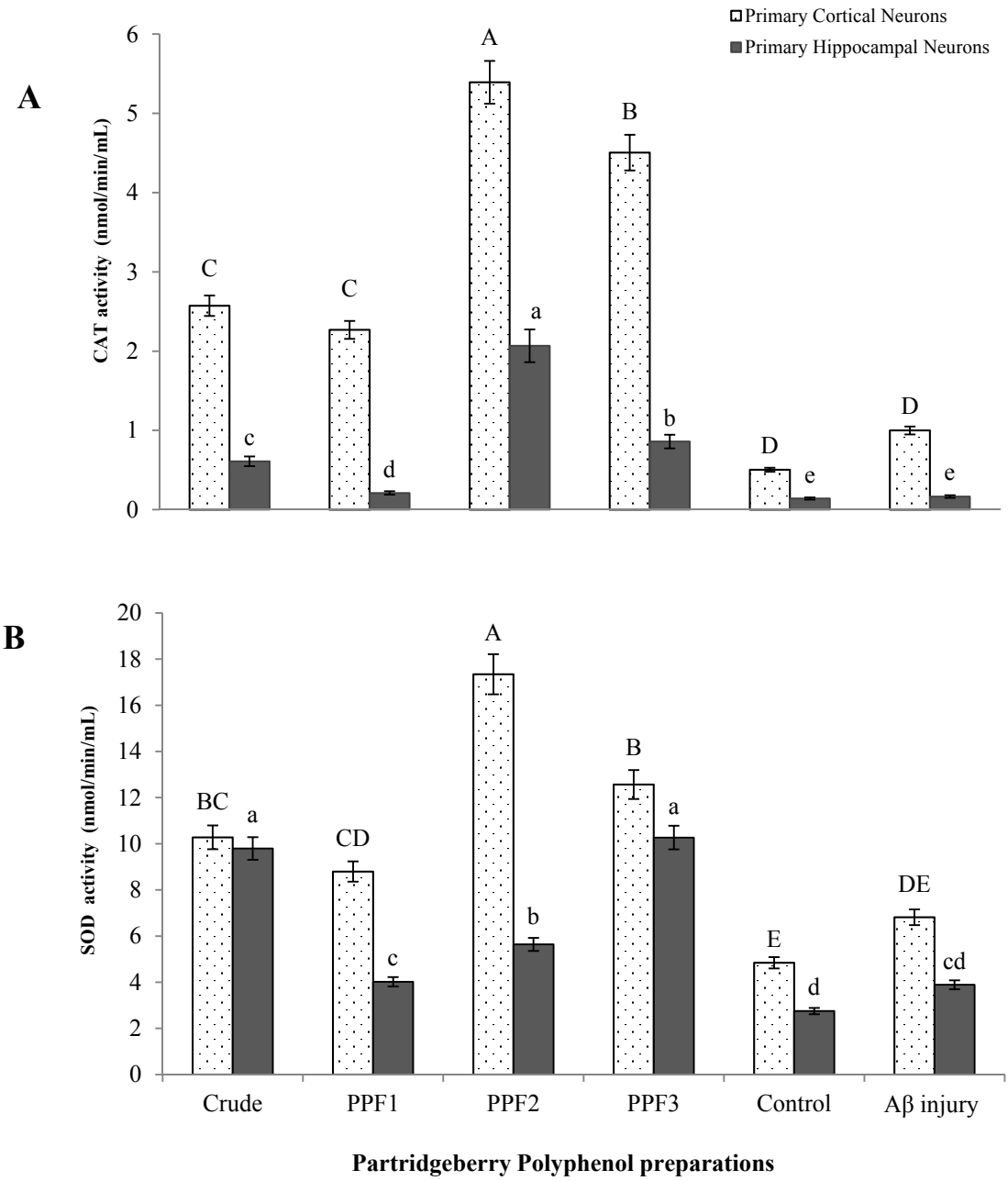

Figure 6. Activation of superoxide dismutase (SOD) (A) and catalase (B) activity following Aβ injury in relation to pre-exposure to partridgeberry polyphenol preparations (100 µg mL^{-1}) with respect to assay controls. Rat primary cortical and hippocampal neurons were treated with either 0.1 % DMSO (vehicle control), or 100 µg mL^{-1} partridgeberry polyphenol preparations, for 24 h, followed by Aβ (50 µM) as described under "Materials and methods". Results are expressed as unit/ml of antioxidant enzymes values in rat primary cortical and hippocampal neurons. The bars with different subscripts are statistically different ($p \leq 0.05$), A-E and a-d letters on bars are significant different ($p \leq 0.05$) as obtained by Tukey's test, are used to indicate statistical significance for treatment affect in rat primary cortical and hippocampal neurons respectively. PPF1: Anthocyanin-rich fraction; PPF2: Flavan-3-ol-rich fraction; PPF3: Flavonol-rich fraction. Control: cells not subjected to the Aβ injury. Aβ inujury control: the experimental control in which neurons are not treated with any antioxidant agent following the Aβ injury.

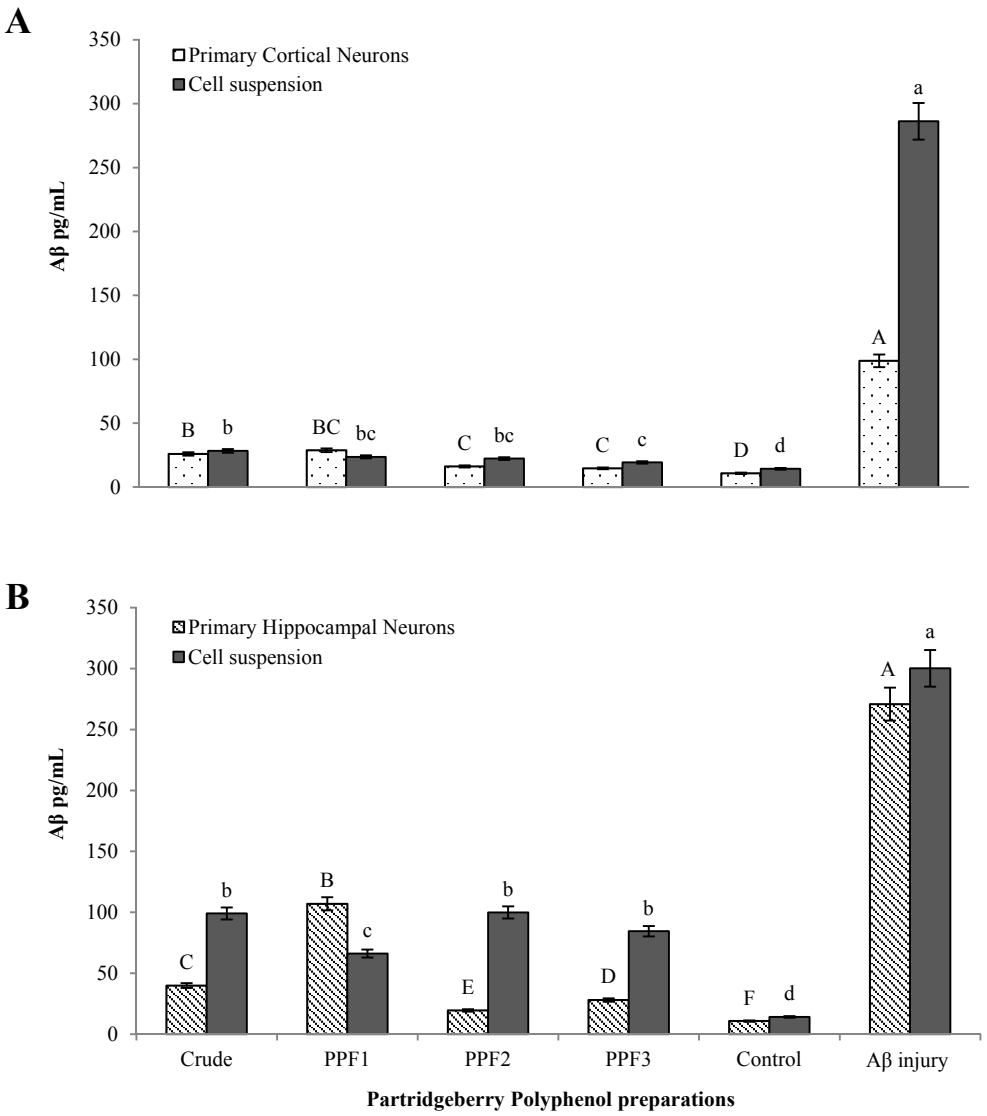

Figure 7. Quantification of $A\beta_{1-42}$ following Aβ injury by partridgeberry polyphenol preparations (100 µg mL^{-1}) in rat primary cortical (A) and hippocampal neurons (B) as measured by ELISA analysis. The bars with different subscripts are statistically different ($p \leq 0.05$), A-F and a-d letters on bars as obtained by Tukey's test ($p \leq 0.05$) are used to indicate statistical significance for treatment affect in rat primary cortical and hippocampal neurons respectively. PPF1: Anthocyanin-rich fraction; PPF2: Flavan-3-ol-rich fraction; PPF3: Flavonol-rich fraction. Control: cells not subjected to the Aβ injury. Aβ injury control: the experimental control in which neurons are not treated with any antioxidant agent following the Aβ injury.

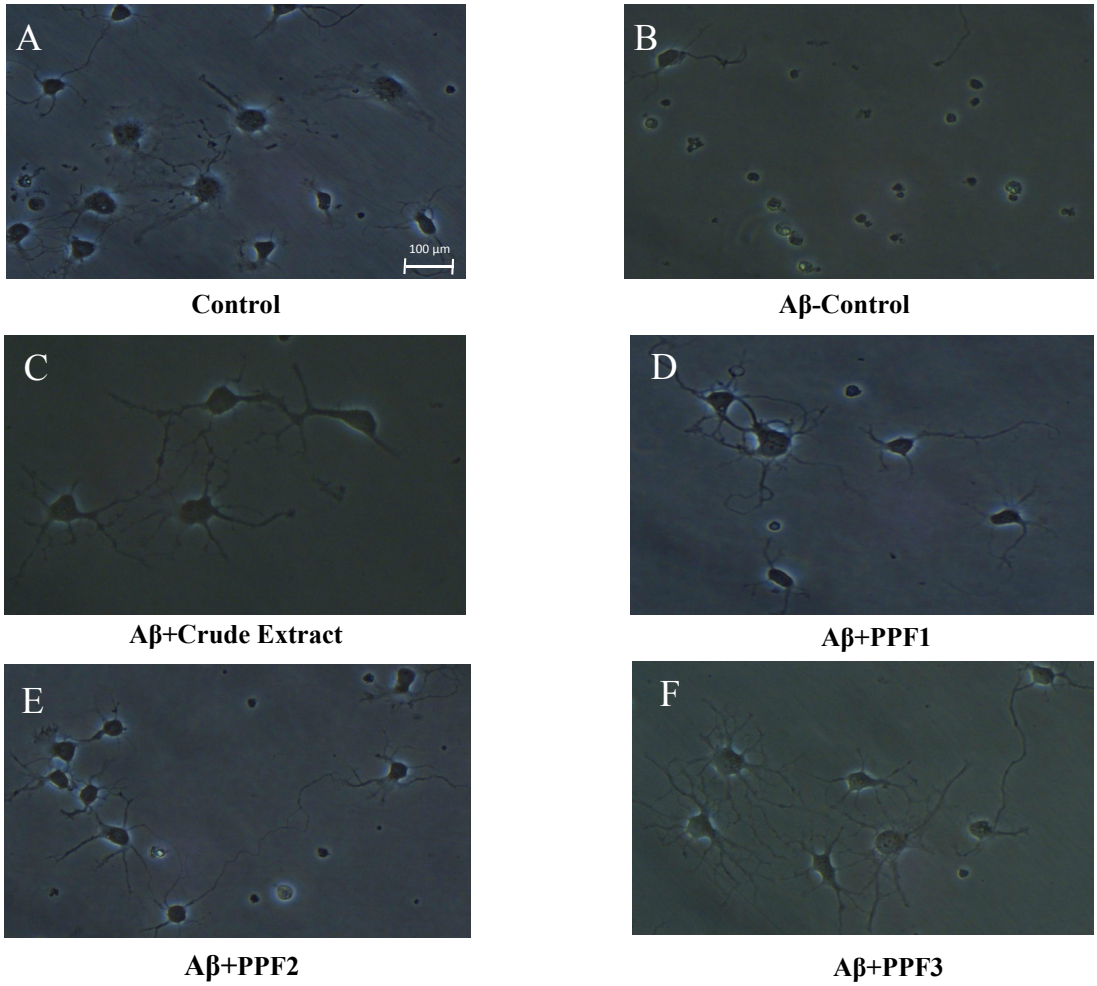

Figure 8. The morphology of neurons treated with partridgeberry polyphenol preparations (100 µg mL^{-1}) or/and $A\beta_{1-42}$ (50 µM) for 24 h (60X). (**A**) Control cells grown without $A\beta_{1-42}$ stress (**B**) $A\beta_{1-42}$ induces shrinkage of cell body and causes cell death; partridgeberry fractions prevent $A\beta_{1-42}$ induced damages on cell body and attenuates cell death. (**C**) Crude extract (**D**) Anthocyanin fraction (**E**) Flavan-3-ol fraction (**F**) Flavonol fraction.

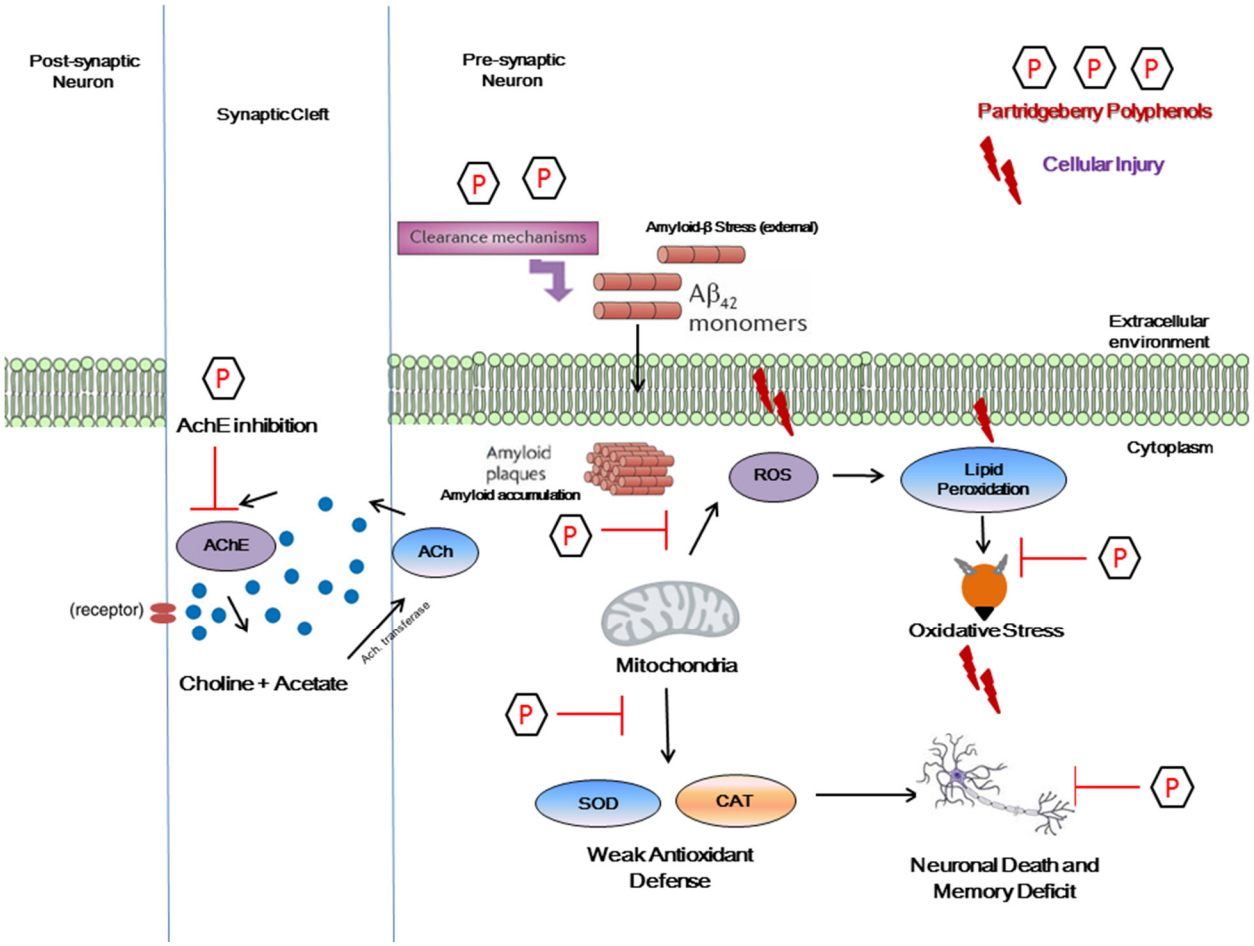

Figure 9. A schematic diagram of the neuroprotective role of the partridgeberry polyphenols, and induction of Superoxide dismutase (SOD) and Catalase (CAT) expression in $A\beta_{(1-42)}$ stressed rat cortical and hippocampal neurons. Partridgeberry polyphenols abolished $A\beta_{(1-42)}$ induced Reactive oxygen species (ROS), reductions in SOD and CAT activity, decreased $A\beta_{(1-42)}$ concentration and acetylcholinesterase (AchE) activity, leading to significant increase neuronal survival and proliferation.

CHAPTER 5: PARTRIDGEBERRY POLYPHENOLS PROTECTS PRIMARY CORTICAL NEURONS FROM OXYGEN–GLUCOSE DEPRIVATION–REPERFUSION INDUCED INJURY VIA SUPPRESSION OF INFLAMMATORY ADIPOKINES AND REGULATION OF HIF-1α AND PPARγ

5.0 Abstract

The aim of this study was to investigate the neuroprotective ability of partridgeberry polyphenols in rat primary cortical neurons against oxygen-glucose deprivation/reperfusion (OGD/R) injury *in vitro* and explore the underlying therapeutic mechanism(s). The results showed that after OGD/R, the survival rate of primary cortical neurons was significantly increased by the pre-exposure of cells to partridgeberry polyphenols in comparison to untreated cells *in vitro* ($p \leq 0.05$). The strongest activity in this regard was exhibited by partridgeberry derived PPF2 and PPF3, i.e. the flavan-3-ol- and flavonol-rich polyphenol fractions ($p \leq 0.05$). Furthermore, partridgeberry polyphenol pre-treatment reduced the membrane damage of primary neurons, as measured by the LDH release assay ($p \leq 0.05$). However, the anthocyanin-rich fraction conferred least neuroprotective activity in primary cortical neurons against OGD/R injury *in vitro* ($p \leq 0.05$) at all tested concentrations. Furthermore, PPF2 and PPF3 pre-treatment (100 μg mL^{-1}) for 24 h, before OGD/R, resulted in the strongest supression of IL-6 and TNF-α induction by OGD/R respectively, compared to the control group ($p \leq 0.05$). Additionally, the protein levels of HIF-1α and PPARγ quantified by ELISA showed a significant modulation following PPFs treatment (100 μg mL^{-1}), favorably towards neuroprotection, compared with respective controls after OGD/R *in vitro* ($p \leq 0.05$). In conclusion, partridgeberry polyphenols concentrations of 1-100 μg mL^{-1}, significantly induced a decline in OGD/R triggered apoptosis *in vitro*, suppressed the inflammatory biomarkers in neurons, and modulated the activity of HIF-1α and PPARγ after hypoxic injury.

5.1. Introduction

Cerebral ischemia, a major form of stroke, is among the leading causes of mortality and long-term disability among humans. The impediment of oxygen and glucose supply during cerebral ischemia to the antagonized brain tissues with high energy demands causes bioenergetic failure resulting in neuronal injury via oxidative stress (Baron, 2010). Oxidative stress is an important pathophysiological mechanism for host defense and plays a vital role in the nervous system. Presented by the hyperactivity of reactive oxygen and nitrogen species (ROS/RNS) following the reperfusion of oxygenated blood in ischemic brain, oxidative stress aggravates the reperfusion injury, by triggering the lipid peroxidation in neural membranes (Chen et al., 2011). Severe oxidative damage to the brain during ischemia by ROS and RNS results in the loss of neurons, memory functions, and often affects cognitive abilities (Hazelton et al., 2010). Currently, there are only a few clinically verified or FDA approved neuroprotective drugs for the prevention and/or treatment of ischemic stroke and related complications. The only available stroke therapy is thrombolysis, i.e. tissue plasminogen activator (t-PA), which is received only by about 8% of stroke patients and is accompanied by the risk of intracranial hemorrhagic transformation (Chernyshev et al., 2010). These facts suggest an alternative and complementary approach to develop nutritional intervention using natural antioxidants such as polyphenols, which may exert therapeutic defence against brain disorder(s) such as ischemic injury.

A large body of epidemiological and clinical data supports the health benefits of polyphenols in cerebrovascular diseases and suggests that a polyphenol-rich diet may prevent the onset of ischemic stroke (Cassidy et al, 2012; Liu et al., 2013). Multiple studies using cell and animal models of ischemic stroke show that a polyphenol-rich diet, administered during or after ischemic brain injury, has the potential to attenuate the neuronal injury (Simão et al., 2013; Liu

et al., 2005; Inoue et al., 2003). This pharmacological "preconditioning" by polyphenols induces exogenous antioxidants as well as triggers the endogenous antioxidant enzymes via Nrf2/ARE pathway to counter reperfusion triggered oxidative assault (Shah et al., 2014). Moreover, polyphenols are able to interfere with multiple pathways implicated in the pathophysiology of stroke, and extend neuroprotective potential against ischemic hallmarks such as inflammation and free radical damage.

As inflammatory mechanisms play a central role in the risk and pathology of brain ischemia, well known inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-6, present themselves as crucial drug targets in stroke therapy (Lambertsen, Biber, & Finsen, 2012). There is substantial evidence that elevated levels of these cytokines are attenuated by polyphenols, resulting in their acceptance as efficient neuroprotective agents that may be used for preventing and/or treating ischemic stroke (Liu et al., 2005; Marier et al., 2005). Another drug target involved in the cell signaling and neuroprotection is an oxygen-sensitive transcription factor called hypoxia-inducible factor 1-alpha (HIF-1α), which mediates cellular responses to low oxygen concentration. Apart from the adaptive response during cerebral ischemia, HIF-1α also plays an antagonistic role by engendering the pro-apoptotic genes which cause mitochondrial dysfunction leading to the cell death (Higashida et al., 2011). The pharmacological stabilization of HIF-1α by polyphenols under hypoxic conditions indicates their aptitude against the detrimental consequences of ischemia-reperfusion injury (Mandel et al., 2008).

Furthermore, anti-oxidant/inflammatory treatment with polyphenols reverses the NF-κB-mediated inflammatory signaling through activation of Proliferator-Activated Receptor Gamma (PPAR-γ) (Inoue et al., 2003). Meanwhile, numerous reports have shown the modulation of these

drug targets in neurons treated with polyphenols; however, the potential of partridgeberry derived polyphenols in the modulation of these drug targets remains unexplored. Therefore, the aim of this present study was to ascertain whether pre-exposure with partridgeberry derived polyphenol preparations exerts any neuroprotective effect against oxygen-glucose deprivation evoked oxidative damage and neural injury, using the rat primary cortical neurons.

5.2. Materials and Methods

5.2.1 Materials

Sterile 96, 24 and 6 well poly-D-lysine coated assay plates, pipettes and cell culture flasks were obtained from BD Biosciences (Mississauga, ON). CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit and CytoTox-ONE™ homogeneous membrane integrity assay kits were obtained from Promega (Madison, WI). Cell culture grade DMSO was purchased from Life Technologies Inc. (Burlington, ON).

5.2.2 Partridgeberry derived polyphenol preparations

The crude extract of partridgeberry and its polyphenol fractions (PPFs) were prepared using standardized methodology described earlier in Chapters 3 and 4. The fresh stock solutions of PPFs in sterile 5% DMSO were prepared at concentration of 1000 μg mL^{-1} and this stock solution was diluted to prepare the active assay concentrations (10-200 μg mL^{-1}) using cell culture medium. The active concentration of DMSO was maintained <0.5%, which was found to be non-toxic to the neurons *in vitro*.

5.2.3 Cell culture and treatment

Primary rat cortical neurons were obtained from Life Technologies Inc. **(**Burlington, ON**)** and cultured according to the manufacturer's instructions. Neurons were grown in neurobasal™ medium supplemented with 200 mM glutamax™-I, 50X B27 supplement (Life Technologies,

Burlington, ON) and 10% fetal calf serum, 50 units/mL penicillin and streptomycin. Poly-L-lysine-coated flasks or petri dishes (4.5 μg/cm^2) were used for cell culture. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in an incubator (VWR International, Mississauga, ON). After 24 hours of incubation, half of the growth medium was aspirated from each well and replaced with the fresh medium. Cells were fed every second day by replacing half of the medium from each well with fresh medium. PPFs stock solutions were dissolved in the complete growth media, and appropriate solvent controls were used to counter the DMSO presence in the aliquots of samples. Treated or untreated culture medium was maintained with 0.1-0.2% dimethyl sulfoxide, which was nontoxic to the cortical neuronal culture *in vitro*.

5.2.4 Oxygen glucose deprivation/reperfusion (OGD/R) model

The oxygen glucose deprivation/reperfusion injury was carried out using a modification of a previously published procedure (Tabakman, Lazarovici, & Kohen, 2002). Briefly, after incubation with crude extract and PPFs for 24 h, primary rat cortical neurons were washed once with glucose-free media previously bubbled with a mixture of nitrogen and CO_2 (19:1). The cultures were kept in the deoxygenated glucose-free medium and the plates were then placed in a hypoxia chamber (Stem Cell Technologies, Vancouver, BC) for 8 h. The hypoxia chamber was flushed with a mixture of nitrogen and CO_2 (19:1) for 20 min at a flow rate of 2 liters per min and the sealed hypoxia chamber was kept at 37°C in an incubator for 8 h (VWR International, Mississauga, ON) to initiate OGD/R injury. The OGD/R challenge was terminated by replacing assay media with the complete growth media followed by incubation in a normoxic conditions for 12 h (reoxygenation). The control cells (without hypoxia) were also cultured in a similar manner as defined in section 2.3, according to the manufacturer's instructions, but without hypoxic exposure at 37°C in the same incubator.

5.2.5 Cell viability assay

Cell viability was assessed using the CellTiter 96® AQ_{ueous} non-radioactive cell proliferation assay (Promega, Madison, WI). Briefly, 2×10^4 cortical neurons were seeded using 600 µl neurobasal media in a 24-well tissue culture plate. After the pre-treatment with PPFs with the crude extract and three partridgeberry fractions for 24 h, followed by the induction of OGD/R (section 2.4), the viability of cells was measured by pipetting MTS solution into each well of the plate containing neurons in culture medium. The plate was incubated for 3 hours at 37°C in a humidified, 5% CO_2 atmosphere and absorbance was read at 490 nm using FLUOstar OPTIMA plate reader (BMG Labtech Inc., Offenburg, Germany). The proliferation indices were normalized to the assay controls and results were expressed as EC_{50} values using regression analysis.

5.2.6 LDH release assay

Cell injury analysis was conducted using the CytoTox-ONE™ homogeneous membrane integrity assay (Promega Corporation, Madison, WI). Cells were pre-treated with the crude extract and the three partridgeberry fractions for 24 h and then subjected to OGD/R challenge as described in section 2.4. All remaining steps for membrane damage assessment assay were performed according to the manufacturer's instructions. The absorbance value was read using FLUOstar OPTIMA plate reader (BMG Labtech Inc., Offenburg, Germany) at wavelength of 490 nm. The LDH results were expressed as the percentage of LDH release with respect to the positive control and IC_{50} values were obtained using regression analysis.

5.2.7 Tumor necrosis factor-α (TNF-α) ELISA

TNF-α levels were measured using the TNF-α rat solid phase sandwich ELISA Kit purchased from Life Technologies Inc. (Burlington, ON). The ELISA was performed according to the

manufacturer's instructions and absorbance was read at wavelength of 450 nm using BioTek, Power wave XS2 spectrophotometer (BioTek, Winooski, VT). The results were expressed as pg/ml Rat TNF-α concentration in the neural lysates.

5.2.8 Interleukin (IL)-6 ELISA

IL-6 levels were measured using IL-6 (Interleukin-6) rat ELISA kit (Abcam Inc, Toronto, ON). The ELISA was performed according to the manufacturer's instructions and absorbance was read at wavelength of 450 nm using BioTek, Power wave XS2 spectrophotometer (BioTek, Winooski, VT). The results were expressed as pg/ml Rat IL-6 activity in the neural lysates.

5.2.9 Hypoxia inducible factor-1 alpha (HIF-1α) ELISA

HIF-1α levels were measured using a commercial sandwich ELISA kit (Cayman, Ann Arbor, MI). The ELISA was performed according to the manufacturer's instructions and absorbance was read at wavelength of 450 nm using BioTek, Power wave XS2 spectrophotometer (BioTek, Winooski, VT). The results were expressed as HIF-1α activity with respect to the OGD/R assay control.

5.2.10 Proliferator-activated receptor gamma (PPAR-γ) ELISA

PPAR-γ levels were measured using a commercial sandwich ELISA kit (Cayman, Ann Arbor, MI). The ELISA was performed according to the manufacturer's instructions and absorbance was read at wavelength of 450 nm using BioTek, Power wave XS2 spectrophotometer (BioTek, Winooski, VT). The results were expressed as PPAR-γ activity with respect to the OGD/R assay control.

5.2.11 Cellular morphology

Cell morphology was examined under a contrast-phase microscope (Nikon, Japan) and images were taken at different stages of cell culture.

5.2.12 Statistical analysis

All experiments were performed in hexaplicates (n=6) unless stated otherwise and the statistical significance of means was assessed by one way analysis of variances (ANOVA) using SAS software (SAS Institute, NC). The experiments were repeated twice for the cell viability and LDH release assays while the ELISA(s) were performed once using six independent replicates.

5.3. Results

5.3.1 Cell viability values

Unless otherwise stated, the primary cortical neurons were pre-treated using partridgeberry polyphenol preparations, quercetin (Q), curcumin (CUR) and ascorbate (AA) but no polyphenolic/antioxidant therapy was applied after OGD/R challenge *in vitro*. The 24-h pre-treatment with PPFs or standard antioxidant compounds showed potent defence against OGD/R challenge, while untreated cells resulted in ~100% cell death. As shown in Fig. 10, compared with known neuroprotective antioxidants like Q, CUR and AA, under hypoxic conditions, the PPFs pre-treated cells subjected to OGD/R exhibited stronger survival abilities. The strongest ability to protect rat primary cortical neurons against OGD/R was exhibited by Q and CUR *in vitro* ($p \leq 0.05$). Among the partridgeberry polyphenol preparations, the PPF2 and the PPF3 at assayed concentrations exhibited the strongest neuroprotective effect against OGD/R injury ($p \leq 0.05$). The weakest neuroprotective capacity was displayed by the PPF1 (anthocyanin-rich fraction) with ~5000 times weaker activity in comparison to PPF2 and PPF3 at assayed concentrations ($p \leq 0.05$). Meanwhile, the crude extract was a more active neuroprotective agent than PPF1, in its ability to exhibit defense against OGD/R injury in primary cortical neurons. The lower of protection against OGD/R insult by the PPF1 may be associated with the low concentration of active polyphenols in the fraction. The active therapeutic concentrations of

PPFs ranging from 1-200 μg mL^{-1} are close to the physiologically achievable plasma concentrations if the PPFs are repeatedly consumed. Overall the partridgeberry polyphenols notably enhanced the cellular viability of neurons in an *in vitro* model of neural ischemic injury.

5.3.2 LDH release values

This study determined the ability of partridgeberry polyphenol preparations to precondition primary cortical neurons cells against OGD/R mediated membrane damage *in vitro*. As shown in Fig. 11, partridgeberry polyphenol preparations and known potent antioxidants at low concentration (10-200 μg mL^{-1}) induced significant inhibition of LDH release within 24 h. Interestingly, AA at all assayed concentrations (1- 200 μg mL^{-1}) induced the strongest inhibition of LDH release ($p \leq 0.05$). Similar results were shown by the higher concentrations (200 μg mL^{-1}) of Q, CUR and PPF3, in their ability to inhibit the LDH release in primary cortical neurons subjected to OGD/R ($p \leq 0.05$). These neuroprotective candidates were followed by the PPF2 and the crude extract ($p \leq 0.05$), while the weakest ability to control neural membrane was exhibited by PPF1, with ~38 times weaker activity in comparison to AA. This suggests that the neuroprotective actions of PPF2 and PPF3, *in vitro* via inhibition of membrane damage, at least in part, may be mediated through their active polyphenol constituents such as Q, catechin and epicatechin (Chapter 3).

5.3.3 IL-6 protein concentration

We examined whether the partridgeberry polyphenol preparations and the other selected antioxidants [Q, CUR and AA (100 μg mL^{-1})] can modulate the expression of IL-6 in primary cortical neurons subjected to OGD/R using ELISA. The induction of hypoxic conditions resulted in elevated levels of IL-6, compared to the control cells ($p \leq 0.05$); causing ~41 fold elevation of the pro-inflammatory cytokine. The PPFs and selected antioxidants inhibited the OGD/R induced

IL-6 production, compared to the untreated controls ($p \leq 0.05$) (Fig. 12-A). The strongest IL-6 inhibitory activity was displayed by the PPF2 and CUR, followed by the PPF3 and quercetin ($p \leq 0.05$). However, following the earlier results, PPF2 exhibited the weakest ability to inhibit the OGD/R induced IL-6 levels in primary cortical neurons *in vitro* ($p \leq 0.05$).

5.3.4 TNF-α protein concentration

To examine the possible inhibition of the TNF-α activation in response to OGD/R challenge, primary cortical neurons were pre-treated with partridgeberry polyphenol preparations or the selected antioxidants (100 μg mL^{-1}), for 24-h. The induction of OGD/R insult resulted in ~9 fold elevation of TNF-α levels, compared to the assay control (Fig. 12-B). The 24-h pre-treatment with PPFs or standard antioxidant compounds showed an appreciable defence against OGD/R induced TNF-α ($p \leq 0.05$). The strongest anti-TNF-α activity was exhibited by PPF3, Q and CUR, followed by PPF2 and AA ($p \leq 0.05$). The weakest anti-TNF-α activity was exhibited by PPF1, which lowered the TNF-α level in primary cortical neurons subjected to OGD/R challenge only 10% ($p \leq 0.05$). Overall, Q and Q-rich PPF3, along with CUR emerged as the strongest modulators of inflammatory adipokine, TNF-α in primary cortical neurons *in vitro* ($p \leq 0.05$).

5.3.5 HIF-1α protein concentration

In this study, the effects of partridgeberry polyphenol preparations and the three antioxidants (100 μg mL^{-1}) were also assessed on HIF-1α, by measuring its levels using ELISA (Fig. 13). A trend towards decreased levels of HIF-1α with the treatment of PPFs and selected antioxidants was observed in primary cortical neurons subjected to OGD/R challenge *in vitro* ($p \leq 0.05$). The HIF-1α levels in untreated neurons were ~10 fold higher than the control cells, indicating the possible role of HIF-1α in OGD/R stress, as the same group expressed complete cell death. These elevated levels were strongly attenuated curcumin, followed by three neuroprotective

candidates, PPF2, PPF3 and Q *in vitro* ($p \leq 0.05$). The antioxidant candidates modulated the HIF-1α levels by 1.5-6 folds, in comparison to the untreated control ($p \leq 0.05$). The weakest ability to modulate HIF-1α in primary cortical neurons subjected to OGD/R injury was exhibited by the crude extract of partridgeberry, PPF1 and ascorbate *in vitro* ($p \leq 0.05$).

5.3.6 PPAR-γ protein concentration

Finally, the levels of peroxisome proliferator-activated receptor γ, PPARγ, a nuclear hormone receptor with anti-inflammatory effects were assessed in the OGD model of hypoxia *in vitro* (Fig. 14). There was a ~1.4 fold increase of PPARγ levels in the hypoxia control with respect to the vehicle, while the PPF3 (100 μg mL^{-1}), exhibited the strongest surge of PPARγ (3.5 fold) *in vitro* ($p \leq 0.05$). The PPF3, was followed by the CUR, Q and PPF2 with 3 to 2.6 fold increase in PPARγ release, while the PPF1 was the weakest activator of PPARγ (1.6 fold elevation) in primary cortical neurons subjected to OGD/R challenge *in vitro* ($p \leq 0.05$).

5.3.7 Cellular morphology

Comparative analysis regarding the effect of OGD/R challenge and antioxidant/polyphenol pre-treatment on cell morphology was assessed in primary cortical neurons on the culture plates *in vitro* (Fig. 15). In agreement with the viability and membrane damage studies, OGD/R stress led to complete breakdown of cellular morphology (Fig. 15–B), while pre-exposure with PPFs and antioxidant compounds resulted cell survival and the normal cellular form and organization (Fig. 15–C-I). In summary, the results showed that the absence of antioxidant/polyphenol therapy surged the cellular damage in neurons during OGD/R, while such impairment was attenuated by the PPFs, especially the PPF2 and PPF3, displaying limited cytoskeletal or morphological damage in neurons *in vitro*.

5.4. Discussion

Oxidative damage caused by free radicals has been associated with normal human Aging, while the amplified production of ROS and the subsequent oxidative stress have been implicated as antagonistic in cerebral ischemia reperfusion injury. The present study assessed the efficacy of partridgeberry derived polyphenol preparations against OGD/R challenge in primary cortical neurons *in vitro*. The primary advantage of "preconditioning" neurons using polyphenols is their potent antioxidant activity and their claim to be non-toxic, due to their natural origin. To our knowledge, this is the first report showing that partridgeberry polyphenols induce neuroprotection against OGD/R in an *in vitro* primary cortical neuron model relevant to ischemic stroke. In this study, we demonstrated that the pre-treatment with flavan-3-ol- and flavonol-rich fractions of partridgeberry, at the doses of 2.4-9.3 μg mL^{-1}, significantly inhibited OGD/R triggered LDH release and maintained the neural viability (50%) *in vitro*. These results, at low concentrations, are encouraging since the lipid peroxidation induced by ischemia-reperfusion causes the oxidative injury to neural membrane, leading to brain dysfunctions and cell death. This explanation is supported by the LDH release studies demonstrating that neuroprotection by partridgeberry polyphenol preparations is closely associated with attenuation of membrane damage, which can be related to partridgeberry polyphenol-mediated resistance against OGD/R damage. Further, the partridgeberry polyphenol preparations abetted the sustenance of cellular viability in primary cortical neurons and aided the resistance against cell death following the OGD/R injury. Similar to the current study, various reports have indicated the potency of polyphenol extracts and innate polyphenols against OGD/R challenge in neural cells both *in vitro* (Ha et al, 2003; Huang et al, 2014) and *in vivo* (Park et al, 2009; Sweeney et al, 2002). Our results were in agreement with these studies as PPFs, like other *Vaccinium* derived polyphenols

(Sweeney et al, 2002), rich in flavan-3-ols (Ha et al, 2003) and flavonols (Park et al, 2009) conferred protection against OGD/R injury.

Secondly, a strong surge in the expression of pro-inflammatory cytokines, TNF-α and IL-6, in primary cortical neurons was suppressed by the pre-treatment of neurons with partridgeberry polyphenol preparations (100 μg mL^{-1}). These cytokines are very active bio-molecules and occur in the brain along with their pro-forms at very low concentrations (Clausen et al, 2008). However, during stroke/ischemic injury the levels of both TNF-α and IL-6 increase by 40-60 fold, which escalates the severity of infraction in the stroke brain (Maas & Furie, 2009). Likewise, the levels of these cytokines in cerebrospinal fluid are also elevated within 24 hours of stroke (Zaremba & Losy, 2001). These features and the involvement of these cytokines in stroke pathology make them crucial drug targets. Therefore, our results depicting the attenuation of these inflammatory adipokines, present a crucial finding pertaining to the potential nutritional intervention in stroke. Our findings were in accordance with previous OGD/R studies using neurons, which indicated the neuroprotective role of polyphenols and their extracts by inhibition of inflammatory cytokines and their pathway (Keddy et al, 2012; Shao et al, 2014) along with attenuation of ROS *in vivo* (Zhang et al, 2011).

In addition, the results from the present study revealed that neurons exposed to OGD/R showed elevated levels of HIF-1α and this tendency was mitigated by the use of partridgeberry polyphenol preparations, indicating that HIF-1α suppression plays a vital role in the neuroprotective effects of PPFs. HIF-1α is implicated in regulating OGD/R injury and neural survival by activating adaptive changes via up-regulation of glucose metabolism and transport (Bergeron et al, 1999). However, continued elevation of HIF-1α causes detrimental effects in both primary neuron culture and mice models via modulation of the p53 gene which, in turn,

induces elevation of the expression of apoptotic genes, LDH release and iNOS production (Halterman, & Federoff, 1999, Banasiak & Haddad, 1998; Bergeron et al. 1999; Carmeliet et al, 1998). Therefore, one of the conceivable molecular mechanisms of the remedial effects of PPF2 and PPF3 may be related to the attenuation of HIF-1α induction, which is ascribed to higher cell viability and subsequent decrease in LDH release by partridgeberry polyphenol treated primary neurons *in vitro*. Not many polyphenolic compounds have been investigated for their ability to modulate HIF-1α in brain, but puerarin, a major isoflavonoid, has exhibited neuroprotective potential by decreasing HIF-1α levels, as measured by Western blotting (Chang et al, 2009).

Furthermore, the activation of the PPARγ signaling in OGD/R challenged primary cortical neurons indicated the wide-spectrum of neuroprotection by PPFs. PPARγ and its synthetic antagonists have shown the ability to protect brain tissue via a reduction of inflammation and apoptosis (Lin et al, 2006). PPARγ increases the neural SOD and catalase levels and reduces the ROS and apoptosis via NADPH oxidase downregulation (Khoo et al, 2013; Wu, Lin, & Wu, 2009). Therefore, PPARγ upregulation, as shown by PPFs, presents an important strategy for prevention and/or treatment of neural/endothelial ischemic injury.

Taken together, the present data indicates that PPFs attenuate the OGD/R neuronal apoptosis and rescue membrane damage at low concentrations. These therapeutic effects may be allied to PPFs' anti-oxidant/inflammatory effects of down-regulating pro-inflammatory adipokines. Among the partridgeberry derived polyphenol fractions, PPF2 and PPF3 i.e. flavan-3-ol- and flavonol-rich fractions present a strong antioxidant therapy that may confer neuroprotective benefits in prevention and/or treatment of ischemic injury. Moreover, the neuroprotective effect of these fractions is likely to be allied, at least in portion, with its inhibitory action on inflammatory markers. Furthermore, our study also indicates that the two

fractions are able to attenuate the oxidative and hypoxic damage by modulating transcription factors such as HIF-1α and PPARγ. These fractions may exhibit biological efficacy in human as the elimination of quercetin and catechin/epicatehin metabolites is very slow, with documented half-lives ranging from 11 to 24 h (Baba et al, 2000; Erlund et al, 2002; Noroozi et al, 2000). Therefore, the repeated intakes of these polyphenols may confer neuroprotective "pre-conditioning" against ischemic brain injury via an elevated accumulation of polyphenolic metabolites in plasma. The lowest activity by the anthocyanin-rich fraction, PPF1, may be related to its chemical structure and possibility exists that in media's near neutral pH, its weak hydrogen-donating ability and diverse glycosylation patterns possibly diminished its antioxidant power. Moreover, author also hypothesize that it will be of low *in vivo* activity as the metabolites of the anthocyanins, are very unstable and undergo extensive degradation during metabolism (Kähkönen, & Heinonen, 2003). The bioavailability of polyphenols varies widely and their metabolites upon reaching plasma consequentially from digestive and hepatic activities, contrast from the innate compounds. Therefore, further investigation by employing animal study using a model of focal cerebral ischemia, Western blots, and RT-PCR to understand the regulation of major drug targets and transcription factors is required to clarify the detailed mechanism of neuroprotection induced by flavan-3-ol- and flavonol-rich partridgeberry polyphenol fractions.

5.5. Conclusions

In conclusion, our data show that partridgeberry polyphenol preparations protect primary cortical neuron from OGD/R *in vitro*, and furthermore, that administration of partridgeberry polyphenols might be a potential neuroprotective strategy for the prevention of cerebral ischemia and oxidative stress-related diseases. In addition, studying the therapeutic effects of PPFs using the primary cortical neuron model, relevant to ischemic stroke provided evidence for their ability to

attenuate of inflammatory adipokines such as TNF-α and IL-6 *in vitro*. Partridgeberry polyphenol preparations attenuated OGD/R-induced LDH release and subsequently alleviated the postischemic neuronal cell death, by increasing PPAR-γ and decreasing HIF-1α levels, Besides, whether the effecatious concentration of partridgeberry polyphenol preparations used *in vitro* in this study could give rise to neuroprotective effects that needed to attain *in vivo*, requires animal model study.

5.6. References

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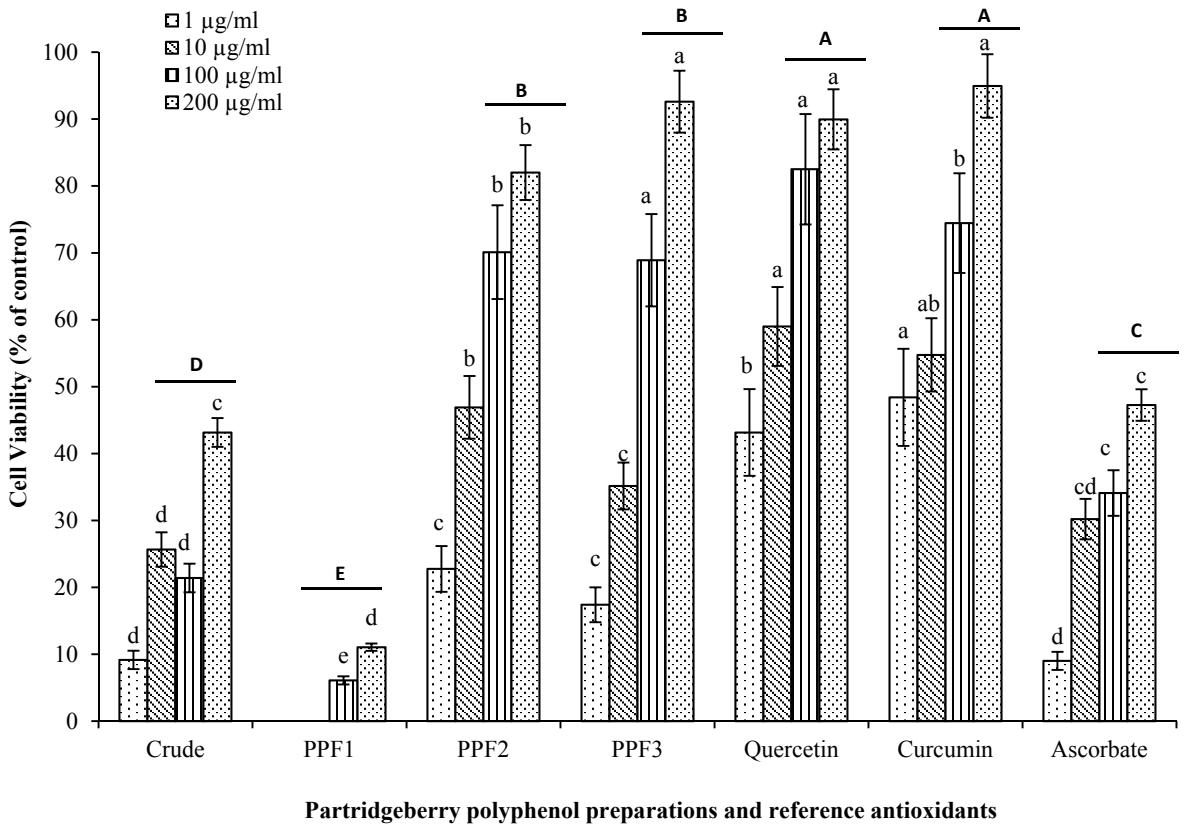

Figure 10. Plots of cell viability (MTS assay) obtained for partridgeberry polyphenol preparations (1, 10, 100, 200 µg mL^{-1}) after 24 h incubation with the rat primary cortical neurons, followed by OGD/R challenge. Data presented as percentage cell viability with respect to assay control and are the average values obtained from two independent experiments (n=6). For all bar plots, different letters represent a significance statistical difference using Tukey's *t*-test analysis ($p \leq 0.05$). A-E and a-d letters on bars as obtained by Tukey's test ($p \leq 0.05$) are used to indicate statistical significance for treatment effect on cell viability of rat primary cortical neurons. Letters A-E specifically indicates the overall stastical difference among different treatments in attenuating OGD/R injury mediated cell death in primary cortical neurons ($p \leq 0.05$). The letters a-d indicates the statistical significance among concentrations of each treatments ($p \leq 0.05$).

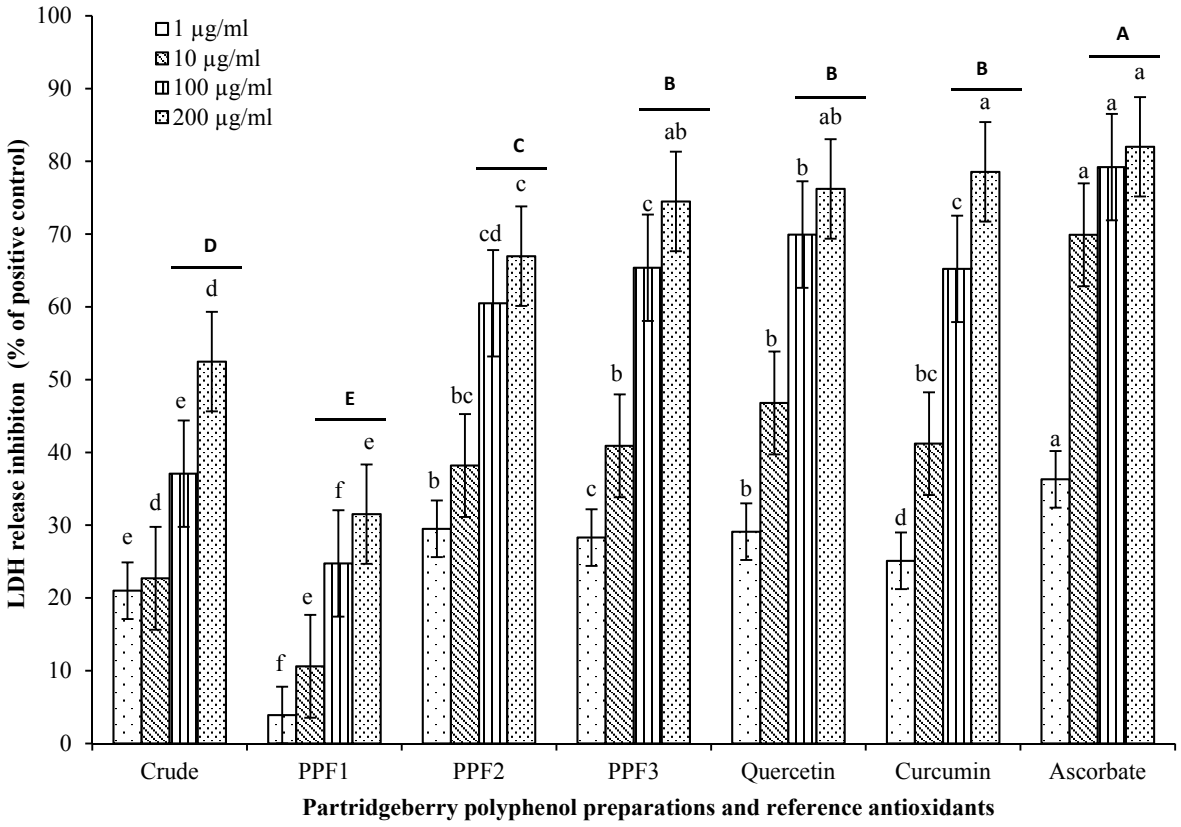

Figure 11. Plots of cell membrane damage (LDH release assay) obtained for partridgeberry polyphenol preparations (1, 10, 100, 200 µg mL^{-1}) after 24 h incubation with the rat primary cortical neurons, followed by OGD/R challenge. Data presented as % LDH release with respect to a positive assay control (OGD/R challenged neurons) and are the average values obtained from two independent experiments (n=6). For all bar plots, different letters represent significance statistical difference using Tukey's *t*-test ($p \leq 0.05$). A-E and a-f letters on bars as obtained by Tukey's test ($p \leq 0.05$) are used to indicate statistical significance for treatment effect on cell viability of rat primary cortical neurons. Letters A-E specifically indicates the overall statiscal difference among different treatments in attenuating OGD/R injury mediated LDH release in primary cortical neurons ($p \leq 0.05$). The letters a-f indicates the statistical significance among concentrations of each treatments ($p \leq 0.05$).

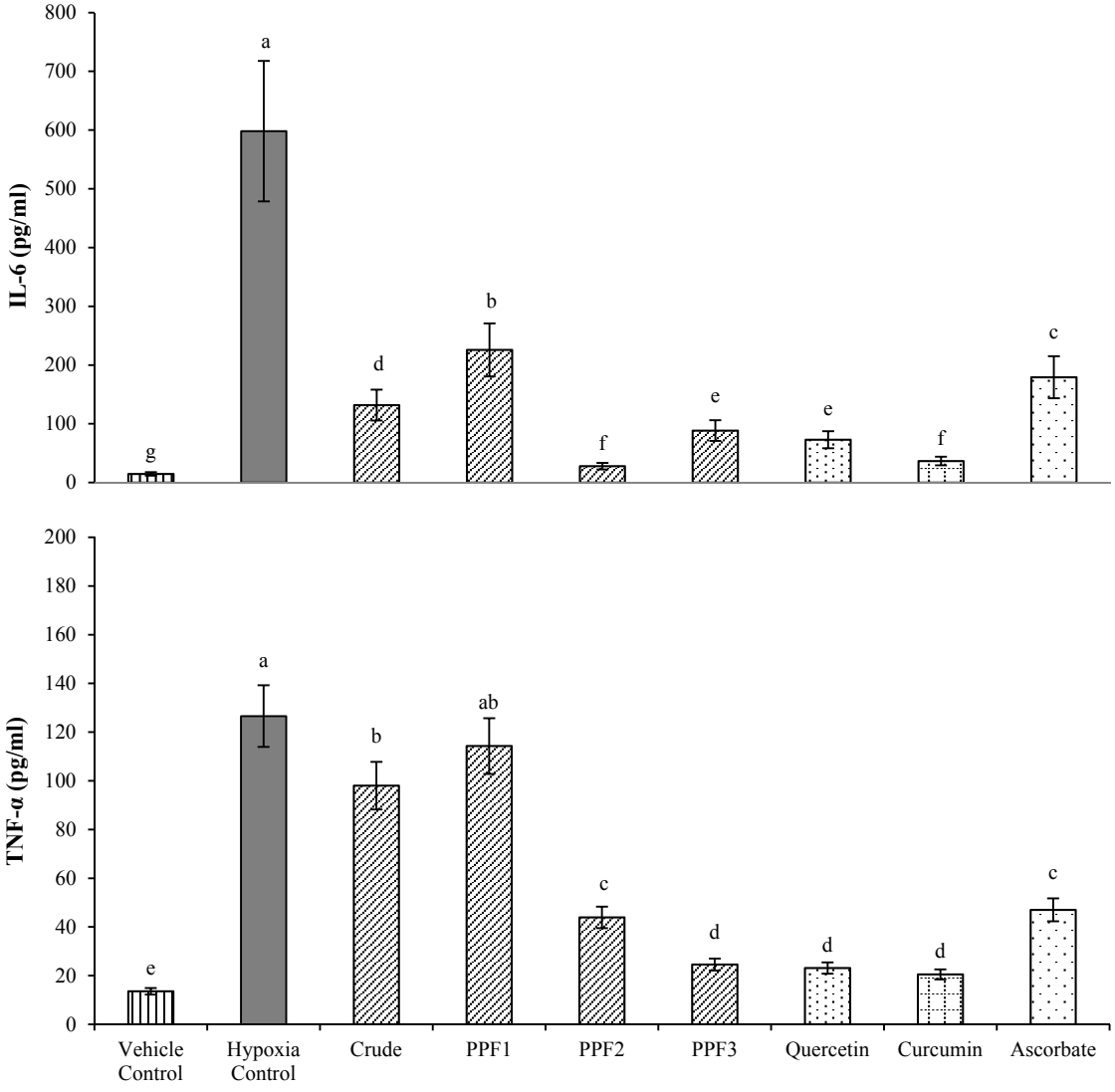

Figure 12. Effect of partridgeberry polyphenol preparations (100 µg mL^{-1}) on [A] Interleukin (IL)-6 and [B] Tumor necrosis factor-alpha (TNF-α) levels (pg/ml) in the rat primary cortical neurons after 24 h incubation, the protein levels were measured following the OGD/R challenge. Bar plots summarizing the relative IL-6 levels results from ELISA experiments after normalizing each data point against the vehicle control (n=6). For all bar plots, different letters a-e represents significance statistical difference using Tukey's *t*-test analysis ($p \leq 0.05$). Neurons not subjected to OGD/R injury are termed as vehicle control while the untreated neurons (with no antioxidant/polyphenol pre-treatment/exposure) subjected to OGD/R challenge are designated as Hypoxia control.

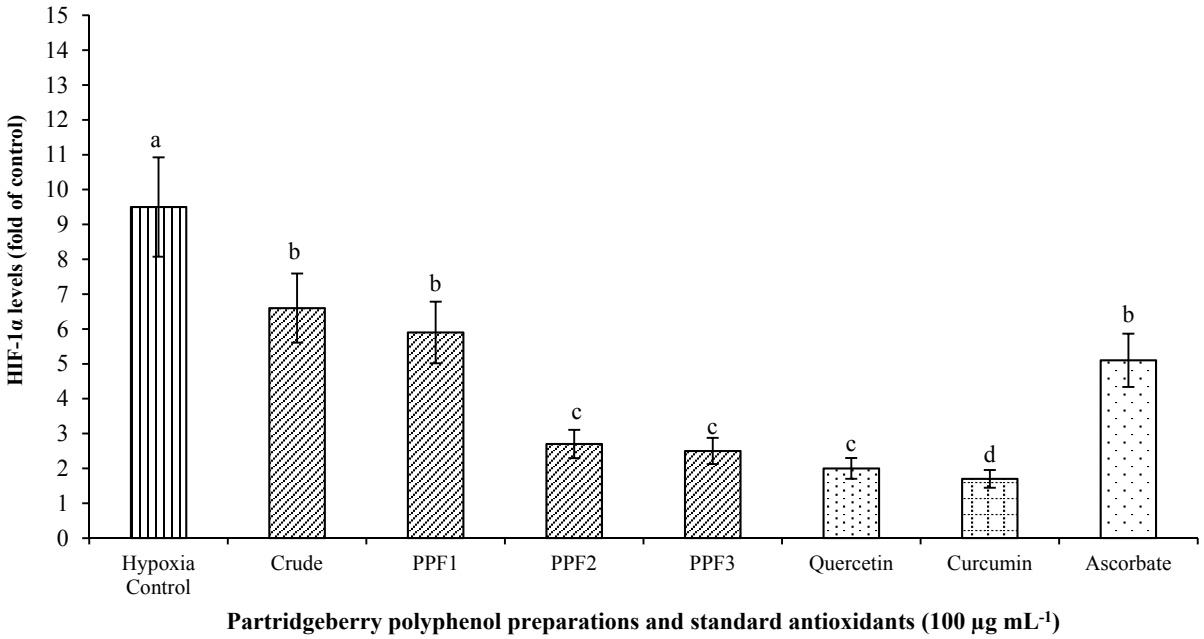

Figure 13. Fold change in the protein expression levels of hypoxia inducible factor 1, alpha subunit (HIF-1α) with respect to control cells after pre-treatment with partridgeberry polyphenol preparations (100 µg mL^{-1}). Results determined by sandwich ELISA indicate the protein levels in the rat primary cortical neurons after 24 h incubation (n=6), measured following the OGD/R challenge with respect to control (non-OGD/R challenged neurons). For all bar plots, different letters a-d represents significance statistical difference using Tukey's *t*-test analysis ($p \leq 0.05$). Neurons not given any antioxidant/polyphenol pre-treatment and subjected to OGD/R challenge are designated as Hypoxia control.

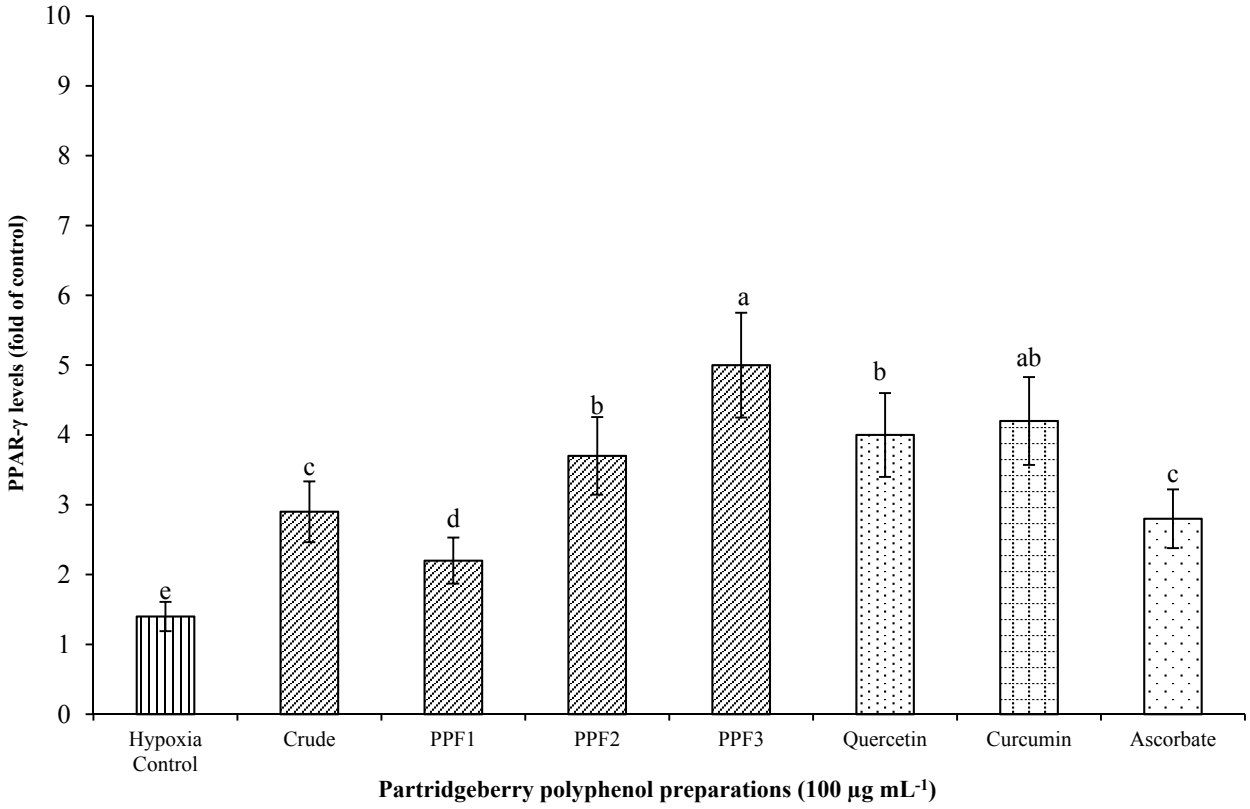

Figure 14. Fold change in the protein expression levels of peroxisome proliferator-activated receptor gamma (PPAR-γ), with respect to control cells after pre-treatment with partridgeberry polyphenol preparations (100 µg mL^{-1}). Results determined by sandwich ELISA in the rat primary cortical neurons after 24 h incubation (n=6), measured following the OGD/R challenge with respect to control (non-OGD/R challenged neurons). For all bar plots, different letters a-e represents significance statistical difference using Tukey's *t*-test analysis ($p \leq 0.05$). Neurons not given any antioxidant/polyphenol pre-treatment and subjected to OGD/R challenge are designated as Hypoxia control.

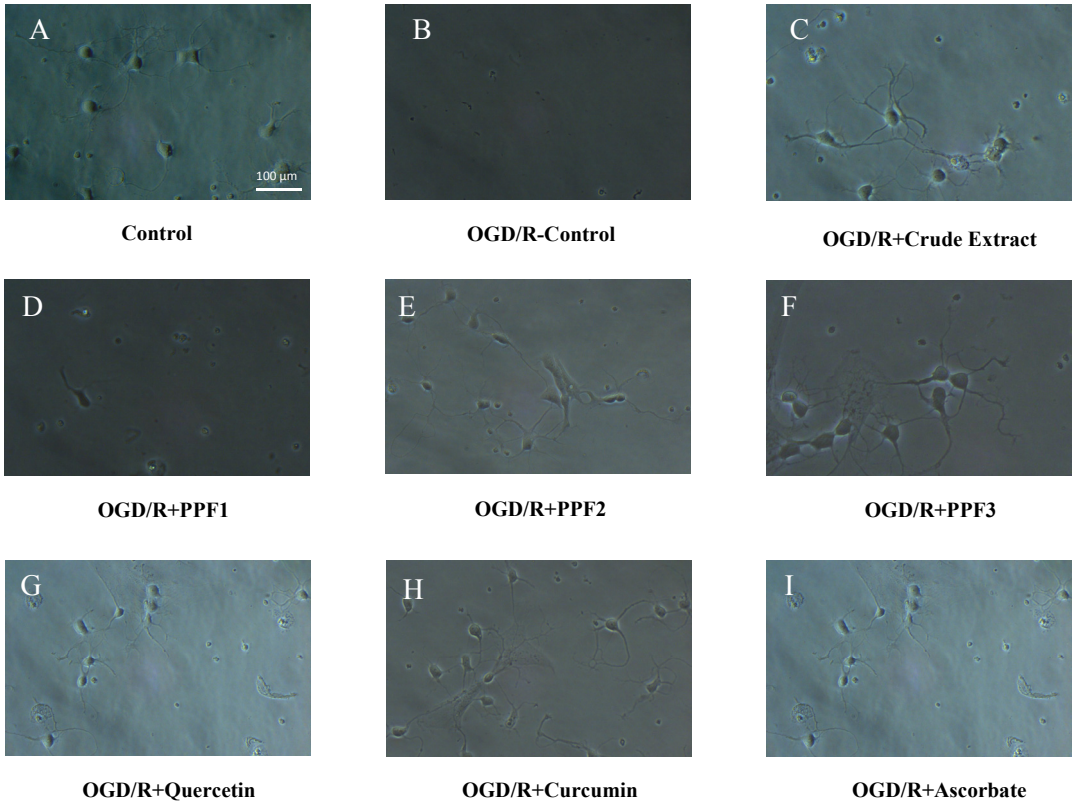

Figure 15. The morphology of neurons treated with partridgeberry fractions (100 µg mL^{-1}) or the selected antioxidants for 24 h or/and OGD/R injury (60X). (**A**) Control cells grown without OGD/R stress (**B**) OGD/R induces shrinkage of cell body and causes cell death; partridgeberry fractions and selected antioxidants prevent OGD/R induced damages on cell body and attenuates cell death. (**C**) Crude extract (**D**) Anthocyanin fraction (**E**) Flavan-3-ol fraction (**F**) Flavonol fraction (**G**) Quercetin (**H**) Curcumin (**I**) Ascorbate.

CHAPTER 6: CONCLUSIONS, SYNOPSIS AND FUTURE RECOMMENDATIONS

6.1. Conclusions

Despite years of intense research focusing on AD and ischemic stroke, the precise cure impeding the terminal outcome is absent. However, recent advances in the field of neuroscience have enabled us to comprehend the pathophysiological mechanisms of various neurological disorders, and identify novel drug targets. The contemporary research has advanced and accepted the concept that employs the role of oxidative stress, and its inhibition, as an advance manoeuvre for combating brain disorders. Especially, in the case of AD and ischemic stroke, where potential therapeutic medications are screened and designed with much of the spotlight geared towards the attenuation of free radicals. Therefore, nutritional intervention via antioxidant supplementation among patients/individuals in jeopardy of such disorders, based on the free radical hypothesis of brain disorders should be exploited. A wide range of different natural products and polyphenols have been assessed to acquire neuroprotection against AD and ischemic brain injuries. Polyphenols have shown the ability to protect brain against oxidative and ischemic injuries by their antioxidant and anti-inflammatory properties. The rationale for studying the neuroprotective effects of polyphenols is strengthened from the *in vivo* evidence that demonstrates the ability of polyphenols such as epigallocatechin gallate, naringenin, hesperetin, catechin, quercetin and resveratrol to cross the blood brain barrier (BBB) and interact with P-glycoprotein after systemic administration (Youdim, Shukitt-Hale, & Joseph, 2004). These properties help polyphenols to qualify the mandatory pharmacological prerequisite of neuroprotective agents. In light of these facts, the present study was conducted to explore the neuroprotective effects of partridgeberry polyphenols against the damaging activities of both Aβ- and OGD/R-sensitized neural injury, in rat primary cortical and hippocampal neurons.

The most significant findings of my M.Sc. thesis are the following:

- The acidified 70% acetone was the most suitable solvent for the maximum recovery of polyphenols from partridgeberry and this solvent system was successfully employed to produce three partridgeberry polyphenol fractions (PPFs 1-3), rich in either, anthocyanins, flavan-3-ols or flavonols (Chapter III).
- The PPFs protected human fibroblasts (WI-38) against the peroxy radical injury, and PPF2, PPF3-treated cultures exposed to the peroxy radical displayed enhanced survival compared to the untreated cells (Chapter III).
- The PPF2 and PPF3 amplified the Nrf2 activity in human fibroblasts *in vitro* and emerged as potent antioxidant and cytoprotective agents, which may be used in the prospective treatment and/or prevention of a wide array of oxidative stress related diseases (Chapter III).
- Aβ-sensitized neural injury was abated by pre-exposure of both rat primary cortical and hippocampal neurons to partridgeberry polyphenol preparations, especially PPF2 and PPF3 *in vitro*. The PPF-induced neuroprotection was conceivably extended through multiple mechanisms such as the improvement of antioxidant status, attenuation of membrane damage, inhibition of ROS activation and mitigation of Aβ levels (Chapter IV).
- The strong protection against Aβ-induced neural injury by PPF2 and PPF3 was related to the hyperactivity of SOD and catalase in both rat primary cortical and hippocampal neurons (Chapter IV).
- The PPF2 and PPF3 also attenuated the neuronal injury, membrane damage and cell death after OGD/R injury in both rat primary cortical and hippocampal neurons. The normalization of IL-6 and TNF-α surge and the fostered activities of both HIF-1α and PPARγ may be

among the potential mechanisms at the bottom of neuroprotection by partridgeberry polyphenols (Chapter V).

- The PPF2 and PPF3, i.e. the flavan-3-ol- and flavonol-rich fractions, emerged as key neuroprotective agents *in vitro*, and present a strong antioxidant therapy that may have confer the neuroprotective benefits in prevention and/or treatment of AD and ischemic brain injury (Chapters III-V).
- The neuroprotective effects of PPF2 and PPF3 in Aβ- and OGD/R-induced neural injury might also be related with the inhibition of inflammatory processes: a preliminary experiment using LPS-induced inflammatory responses and their attenuation by PPFs in rat primary cortical neurons supported this hypothesis (Appendix A, Note 1).

6.2. Synopsis

The complexity of AD and ischemic stroke hinders the development of neuroprotective agents; therefore, the adjunctive nutritional treatment must be developed to reduce the risks, morbidity and mortality due to these disorders. Although a number of neuroprotective drug candidates are in clinical course of development, but their repeated failure in the advance stages of the diseases has raised alarm, and calls for preventive strategy have risen (Kozauer, & Katz, 2013). To achieve this goal, more basic and analytical research is needed to screen variety of natural products and assess their performance in disease models of ischemic stress and Aβ toxicity. To this point, drugs targeting only a single key mechanism of the AD and ischemic stroke have failed to enhance therapeutic outcome in clinical trials. One credible rationale for such disappointment might be the involvement of multiple mechanisms in initiating the neuronal damage (in AD and ischemic injury). Hence, an astute strategy for the development of neuroprotective agents must incorporate the assessment of natural compounds such as

polyphenols with a wide spectrum of therapeutic action (Bhullar, & Rupasinghe, 2013). The activation of Nrf2 pathway resulting in possible diminution of neural cell death may represent such strategy, which in amalgamation with other lifestyle changes, developed over time, such as diet, exercise, avoidance or cessation of tobacco smoke and obesity constraint may serve as proficient therapeutic strategies for fighting these disorders. Overall, in order to gain essential acumens into therapy of AD and ischemic stroke, and for development of novel therapeutic and preventive strategies, different antioxidant sources such as partridgeberry must be screened to elucidate their neuroprotective actions in different contexts, as conducted in the current thesis. The knowledge acquired from the current study could be successfully extended from cell models to animal models, followed by possible therapy or preventive measure in AD and ischemic stroke patients and high risk individuals.

6.3. Suggestions for Future Work

This work has studied a few important aspects of pharmacotherapy such as the identification of pharmaceutical source and elucidation of its active molecules, partridgeberry and its polyphenols in case of the current study. This thesis has also addressed key questions regarding the composition of partridgeberry bioactives, their antioxidant and neuroprotective actions along with the possible mechanisms pertaining these therapeutic accomplishments. The importance of unravelling the molecular signalling and understanding the modulation of survival genes are becoming key factors towards the acceptance of a drug candidate or nutraceutical. Therefore, a number of additional experiments, addressing the molecular mechanisms of actions must be conducted for further understanding the biological efficacy of partridgeberry derived nutraceutical candidate(s).

One such step would be to investigate the mechanism of inhibition of apoptosis by PPF2 and PPF3 against Aβ- or OGD/R-induced cell death in primary neurons. This approach requires the therapeutic mechanism to be specified explicitly, preferably by testing pro- and anti-apoptotic genes such as Bad/Bax, Bcl-2, HO-1, cytochrome c, APAF1, JUN, MAPK, STAT, P13K, Akt along with cytokines and inflammatory targets such as IL-1β, IL-6, TNF-α and NFκB using qPCR and Western blot studies. Another possibility would be to conduct the DNA fragmentation and TUNEL assay, which would allow us to understand the impact of PPFs on DNA integrity against Aβ or OGD/R injury. This idea was presented in Chapter 4, questioning both the nature of neural damage and neuroprotection, therefore assessment of DNA damage and its possible inhibition would certainly merit the PPF2 and PPF3's neuroprotective candidacy (Appendix B, Note 2). Also, it would also be very valuable to perform a comprehensive study aimed at understanding the possible mode of action by the PPF2 and PPF3 against direct oxidative injury by peroxy radical using AAPH or H_2O_2 by PPF2 and PPF3 and their different polyphenol constituents. The study of detailed neural morphology using confocal microscopy could be an important asset as it would be interesting to see the effect of PPF2 and PPF3 on the neural membranes and structure integrity following the Aβ or OGD/R injury, as the PPF treated neural morphology (compared to vehicle) is predicted to be stable. Finally, in terms of animal studies, a mouse model of hypoxic-ischemic (HI) brain damage is under scheduling. My specific curiosity would be to use the animal model to learn the complex biochemical interactions, and use it as a mean to understand the *in vivo* mechanism of therapeutic action by PPF2 and PPF3.

6.4. Significance

This study is critical and noteworthy not only because it is the first report demonstrating the robust neuroprotective effect of partridgeberry polyphenols against the Aβ and OGD/R injury,

but also as it suggests prospective *in vivo* therapeutic potential. The verdicts from current thesis provide a foundation for the auxiliary investigation of the cellular mechanisms underlying the neuroprotective action of the PPF2 and PPF3. Additionally, and of prospective clinical importance, is that the PPF2 and PPF3 were effective at low concentrations (1-100 μg mL^{-1}) *in vitro* and are of natural non-toxic origin, raising the possibility of clinical outcome by lowering the risk of AD and ischemic stroke.

6.5. References

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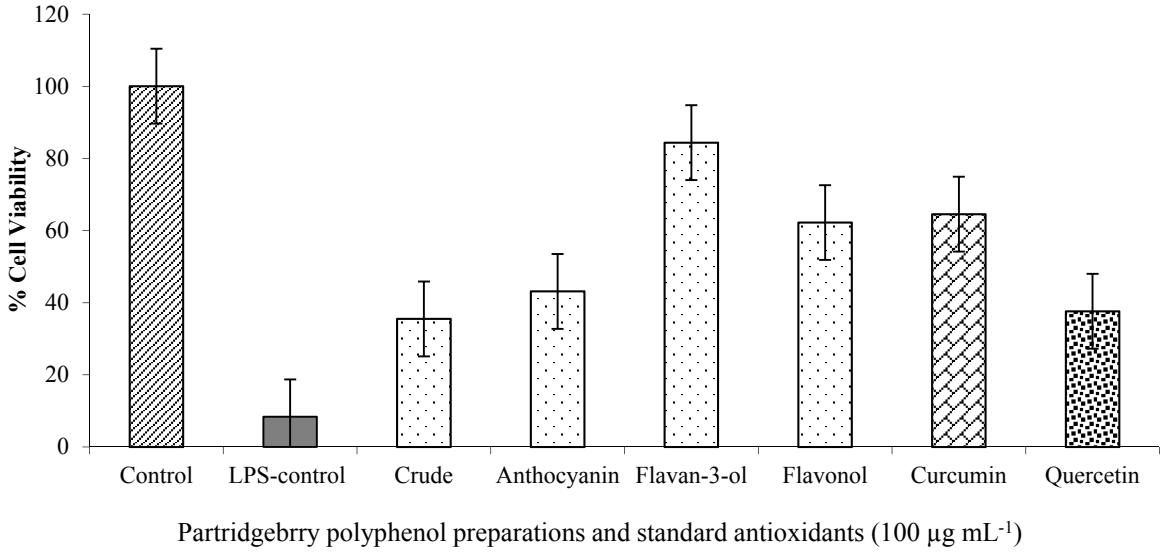
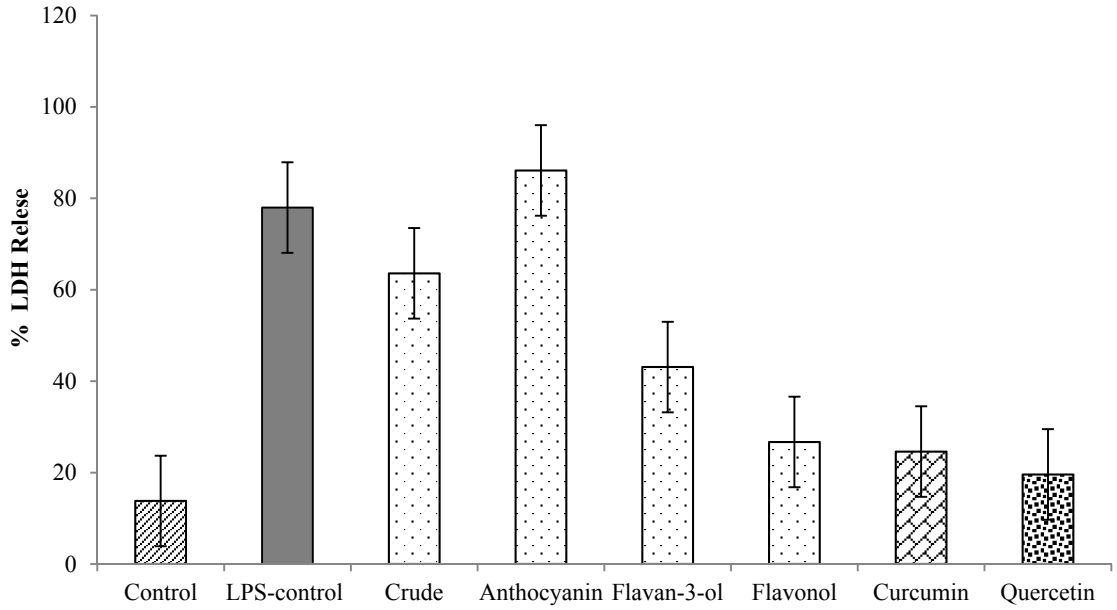
APPENDIX A Effect of partridgeberry polyphenols on the lipopolysaccharides (LPS)-sensitized neural inflammation in primary cortical neurons *in vitro*

A

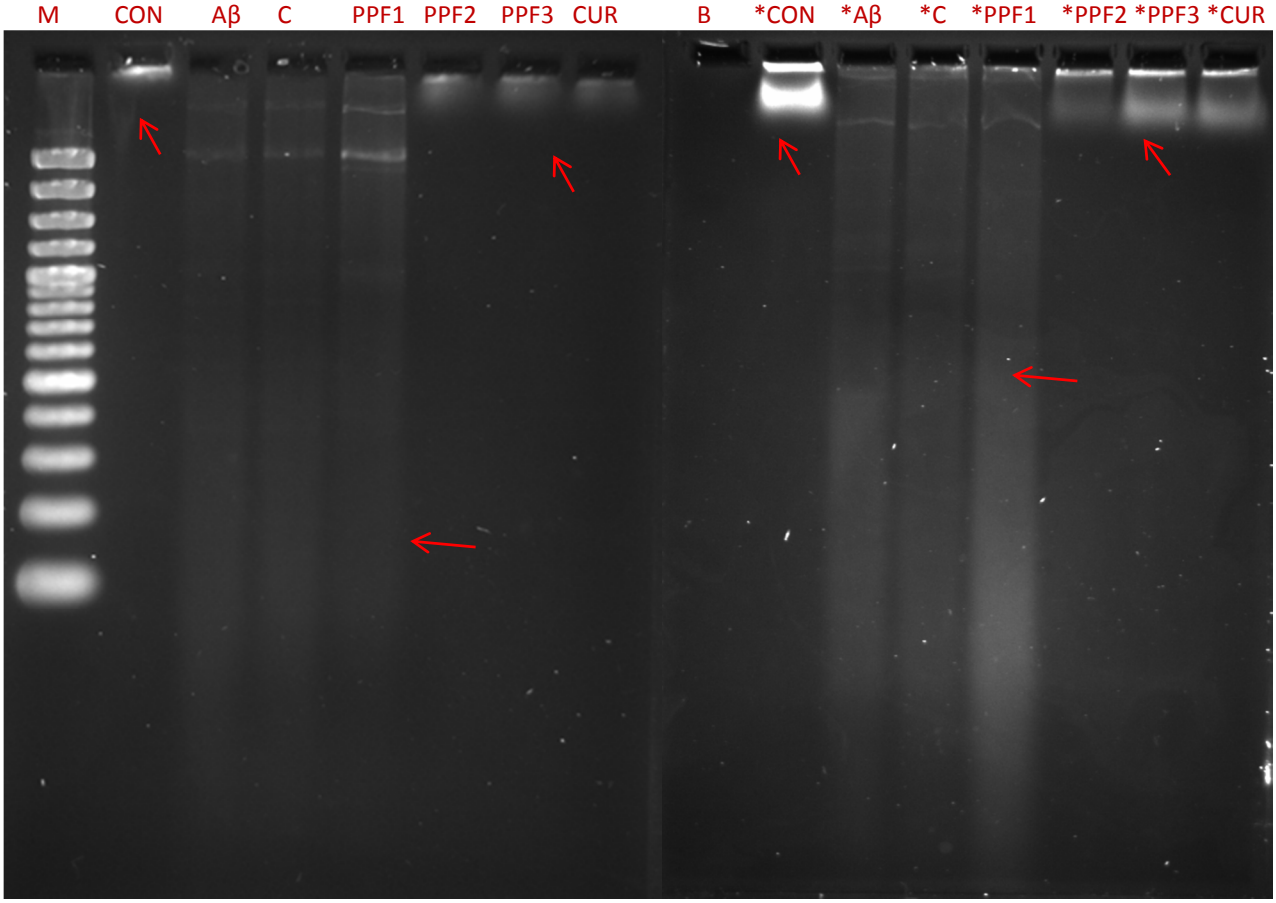


B



Note 1.The primary cortical neurons were incubated with the partridgeberry polyphenol preparations or standard antioxidants (100 µg mL^{-1}) for 24 h prior to LPS-injury *in vitro*. The cell viability [A] and LDH release [B] analysis were performed using colorimertic assays.

APPENDIX B Effect of partridgeberry polyphenols on the Aβ-sensitized neural DNA damage in primary cortical and hippocampal neurons *in vitro*



Note 2. DNA fargrentation analysis of rat primary cortical and hippocampal neurons *in vitro* (using 2% agarose gel electrophoresis); (**M**) marker DNA ladder, (**CON**) control DNA primary cortical neurons (PCN), (**Aβ**) positive control PCN in cultured neurons, (**C**) crude extract of partridgeberry, (**PPF1-3**) partridgeberry polyphenol fractions 1-3, (**CUR**) curcumin, (**B**) blank using DNA loading buffer, (*) all the abbreviations with an asterisk represent same treatments as PCN and controls in the primary hippocampal neurons (PHN) sensitized to Aβ-injury *in vitro*. The arrow pictogram (in red) highlights and compares the apoptotic DNA smears orserved in the neurons subjected to no treatment (Aβ control) or crude/PPF1 in comparison to the neurons receiving pre-exposure to flavan-3-ols- and flavonols- rich PPFs in both PCN and PHN *in vitro*. Image captured by placing the agarose gels on a UV sample tray, followed by exposure to UV light using gel Doc™ EZ System (Biorad, ON, Canada) with an exposure time of 0.29 seconds through UV transillumination mode. The primary neurons (PCN and PHN) were incubated with partridgeberry polyphenol preparations (100 µg mL^{-1}) or curcumin (100 µg mL^{-1}) for 24 h prior to Aβ-injury *in vitro*.

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