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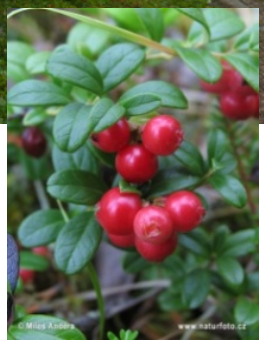
Antioxidant, Cytoprotective and Neuroprotective Properties of Wild Partridgeberry of Southern Labrador



Final Report

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Department of Natural Resources, Government of Newfoundland & Labrador

Submitted by:

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Executive Summary

The specific objectives of this Stage 1 of the investigation were to: determine optimum bioactive phytochemical extraction methods; establish fractionation technique for subclasses of polyphenols; and determine phenolic profile, antioxidant activity and cytoprotective properties of wild partridgeberry (*Vaccinium vitis-idaea* L. var. minus Lodd) grown in Southern Labrador. Polyphenols-rich extracts were derived from wild partridgeberry and subjected to *in vitro* antioxidant and cytoprotective assays. This initial assessment revealed that acidified 70% acetone was the ideal solvent for maximum recovery of various polyphenols from wild partridgeberry. Liquid-liquid based separation, column chromatography and UPLC-MS/MS were employed to produce three partridgeberry fractions that were rich in anthocyanins, flavan-3-ols and flavonols. The antioxidant capacity evaluation conducted using DPPH radical scavenging analysis showed that flavonol-rich fraction possesses 700 times greater antioxidant capacity compared to its crude extract in vitro. These results were also supported by FRAP and ORAC analysis while total phenolic, total flavonoid and total anthocyanin content assays confirmed the results. Furthermore, all the four polyphenol preparations made from partridgeberry protected lung fibroblasts (WI-38 cells) from cellular damage caused by peroxy radical-induced oxidative stress *in vitro*. The flavan-3-ol- and flavonol-rich wild partridgeberry fractions exerted the strongest antioxidant properties. The ELISA results showed that partridgeberry polyphenols could also enhance the cellular antioxidant enzyme abundance through activation of Nrf2 pathway.

Amyloid β (Aβ) deposition elicits a toxic effect on neurons and plays a crucial role in aetiology and/or progression of Alzheimer's disease (AD). Recently, a growing body of evidences suggests that polyphenols derived from plant sources may modulate the pathophysiology of AD, and extend neuroprotection through antioxidant mechanism. In the present study, we have also investigated the neuroprotective potential of wild partridgeberry and elucidated the potential mechanism by which it confers protection against Aβ toxicity. The pre-treatment of primary cortical and hippocampal neurons with partridgeberry polyphenols, significantly attenuated Aβ induced cell

death and membrane damage. The partridgeberry polyphenols also induced the activation of superoxide dismutase and catalase in a biphasic pattern, resulting in reduction of neural reactive oxygen species. We further examined that the treatment of partridgeberry polyphenols, reduced the intracellular Aβ levels, and initiated the Aβ clearance. These two events resulted in the remarkable exhibition of neuroprotection by partridgeberry polyphenols against Aβ. The in vitro anticholinesterase activity of partridgeberry polyphenols further contributed to their neuroprotective effects and potential pharmacotherapy in AD. These findings suggest that partridgeberry polyphenols, especially flavan-3-ols and flavonols, could be of importance in treatment of AD and other oxidative stress-related diseases.

Further research is warranted to assess the specific cytoprotective and neuroprotective properties associated with chronic disease such as Alzheimer's disease and to demonstrate health benefits using experimental animal model systems. However, this preliminary study revealed the potential use of partridgeberry for development of health-promoting food products, ingredients and natural health products.

BACKGROUND & RATIONAL FOR INVESTIGATION

Continuous production of reactive oxygen species (ROS) by mitochondrial respiratory chain causes damage to mitochondria, important biomolecules and initiates pathological processes in human body (Circu and Aw, 2010). The ROS including superoxide anion radical (O_2-•), hydroxyl radical (OH•), the hydroperoxyl radical (HO_2•) and the 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 cellular damage and accelerated ageing (Pan, 2011). The oxidative stress and continuous ROS production also triggers the activation of cell signaling pathways and contributes to pathology of cancer, diabetes, inflammation, neurological disorders and obesity (Reuter, Gupta, Chaturvedi, Aggarwal, 2010). With a view to attenuate the oxidative damage and its pathological manifestations, intake of dietary antioxidants especially fruit polyphenols has been used extensively to serve the purpose. Polyphenols are naturally occurring plant secondary metabolites found in fruits, vegetables, wines and other plant-based dietary sources (Cieslik, Greda, Adamus, 2006). There are over 25,000 different types of polyphenols, divided into many sub-classes including phenolic acids, flavonoids, stilbenes and lignans (Pandey and Rizvi, 2009). 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 *Vaccinium* species of berries are a good source of polyphenols and exhibit a wide range of biological activities, including antioxidant (Wang, Camp, Ehlenfeldt, 2012), anticancer (Su, 2012) and antimicrobial activities (Cote et al.,

2011). *Vaccinium* species of berries have exhibited their therapeutic potential *in vitro* and *in vivo*, both in rodent models and humans (Su, 2012). Accumulating scientific evidence on 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 to 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 antioxidant potential, phenolic profile and cytoprotective ability of these berries. In the current study we investigated the optimum extraction methods for polyphenols, their concentrations, antioxidant activity and cytoprotective properties of wild partridgeberry found in southern Labrador region of Canada.

FUNDING & PARTNERSHIPS

This project is designed with the primary aim of promoting the economic value of wild partridgeberry found in Southern Labrador through scientific investigations of potential health benefits. The primary partnership is with the Department of Natural Resources of the Government of Newfoundland and Labrador. The additional funds for this project was acquired from the funds received by Dr. Rupasinghe for conducting fundamental research of cool climate fruits through Federal funding agencies such as Canada Research Program and Discovery Grant of NSERC. Total of actual sources of funding received for this project is as follows:

Source	**$ Amount**
1. ARI Program	$19,500
2. Canada Research Program Funds to Dr. V. Rupasinghe (Stipend for M.Sc. student; $1625 x 11 months)	$17,875
3. NSERC Discovery Grant Funds to Dr. V. Rupasinghe (consumables for cell lines, media, reagents, extraction solvents, LCMS user fee, standards, ELISA kits, other facility use)	$16,500
Total Project Cost (January 2013 to March 31, 2014)	$53,875

METHODS & IMPLEMENTATION

Plant materials and chemicals

Fruit of wild partridgeberry (*Vaccinium vitis-idaea* L. var. minus Lodd) harvested during 2012 from southern Labrador area 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).

Extraction and fractionation

The wild partridgeberry fruits used in extraction were stored in -20°C freezer until extraction and further analysis. The frozen berries were removed from cold storage and 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 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 polyphenol rich fractions.

Initial extraction and solvent system selection

The solvents were selected based on literature for optimization of polyphenol extraction were: methanol (100%), methanol (70%), methanol (70% + 2% formic acid), ethanol (100%), ethanol (70%), ethanol (70% + 2% formic acid), acetone (100%), acetone (70%), acetone (70% + 2% formic acid), ethyl acetate (70% + 2% formic acid), water (100%). About 10 g of berries were weighed and mixed in 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 a ultrasonicated bath (VWR 750D, VWR International, Mississauga, ON) in three cycles of 15 minutes at 28°C. 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.

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 70% acetone + 2% formic acid using a blender (Black and Decker Type I, Applica Consumer products, Inc Miramar, FL) and then sonicated in ultrasonic cleaner (VWR 750D, VWR International, Mississauga, ON) in three cycles of 15 minutes at 28°C. Following 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 completely dried using a rotary evaporator (Buchi R-200, Buchi Corporation, New Castle, DE) and then reconstituted to 500 mL of ethyl

acetate and water solution (50:50). The ethyl acetate and water solution was then subjected to liquid-liquid separation technique using a separatory funnel. The berry extract in separatory funnel was allowed to stand for 24 h as polyphenolic constituents separated into two layers based on their polarity. The upper ethyl acetate layer and lower aqueous layer were carefully removed in different flasks by opening the PTFE stopcock. At 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 immediately 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, 250 lm, 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% measured using a digital refractometer (Model no. 300016, Sper Scientific, Scottsdale, AZ). The phenolic compounds were fractionated using step-wise gradient of varying concentration of aqueous ethanol. The phenolic 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 (Fig 1). 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 UPLC-ESI-MS/MS.

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 wild 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 (Phenomenex, Torrance, CA). 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. Electro spray ionization, in negative ion mode (ESI-), was used for the analysis of the flavonols, flavan-3-ol, and phenolic acid compounds. 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.

Preparation of partridgeberry polyphenol fractions

After selection of ideal solvent for extraction of phenolics from wild partridgeberry, the extracts were subjected to various separation and chromatography techniques for 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 PFs rich in different classes of partridgeberry polyphenols.

Total phenolic content

Total phenolic content (TPC) was measured using the Folin Ciocalteu assay as outlined by Bernaert et al (2012). The phenolic content of berry extracts was expressed as gallic acid equivalents per gram fresh weight while TPC of PFs was expressed as gallic acid equivalent per liter of PF solution.

Total flavonoid content

Total flavonoid content (TFC) was measured using the aluminum chloride colorimetric method as outlined by Bernaert et al (2012). The total content of berries was expressed as quercetin equivalents per gram fresh weight while TFC of PFs was expressed as μmole Q equivalent per liter of PF solution.

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 (PACs)

in berry extracts. The extraction solvents used to extract PACs are described in section 2.3.1. Ethanol was acidified by adding 6N H_2SO_4 (2.5 mL) 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 instead of procyanidin A2. The results (concentration of PACs) were expressed as µmole catechin equivalent per L of PF solution.

The ferric reducing ability of plasma (FRAP) assay

FRAP assay is based on ferric to ferrous ion reduction at low pH by antioxidants. The assay was performed as described by Bernaert et al (2012). The antioxidant capacity of berries extracts was expressed as Trolox equivalents per gram fresh weight while that of PFs as Trolox equivalents per L of solution.

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 Bernaert et al (2012). The antioxidant capacity of berries using ORAC assay was also expressed as µmole Trolox equivalents per L of PF solution.

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 Bernaert et al (2012) and results were expressed as IC_{50} values (inhibitory concentration of berry fractions decreasing the absorbance of DPPH solution by 50%).

Cell culture

Human fibroblasts or WI-38 cells (ATCC® CCL-75™) were obtained from Cederlane Labs (Burlington, ON) and maintained in 75 cm^2 culture flasks. Fibroblasts were grown at steady temperature of 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.

Peroxyl radical-induced cellular stress model

2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH; peroxyl radical generator) based cellular model for oxidative stress outlined by He et al (2013) was used with modification by using WI-38 cells. 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 in 96 well plates. To assess the cytoprotective effects of partridgeberry, the fibroblasts (WI-38 cells) were incubated with berry extracts before inducing oxidative insult. 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.

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).

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.

Reactive oxygen species (*ROS) analysis*

ROS were analyzed using methodology outlined by Martin et al (2008). 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. Multiwell plates were immediately placed in fluorescence plate reader (reading time 0 minutes) at an excitation wavelength of 490 nm and an emission wavelength of 510 nm. Fluorescence was quantified over period of 60 minutes and ROS were estimated with respect to fluorescein degradation.

Protein content

Total protein content was determined in the cells using the Bradford assay. Assay was performed according to the manufacturer's instructions using the Bio-Rad Protein Assay kit (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).

Peroxide detection assay

Peroxides were measured by using the Thermo Scientific Pierce quantitative peroxide assay kit (Fisher 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 four 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).

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 novel 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.

Cell culture

Primary rat cortex and hippocampus 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 200mM glutamax™-I and 50X B27 supplement (Life technologies, Burlington, ON). Cells were plated on to a poly-D-lysine (4.5 $\mu g/cm^2$) 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 fed every second day by replacing half of the medium from each well with fresh medium. The primary rat hippocampus neurons were supplemented with 25 μM L-glutamate up to the 4^{th} 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 $\mu g/cm^2$) coated plates and exposure of culture to light was avoided.

AChE and BuChE inhibition assay

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

Aβ (1-42) toxicity and treatment model

The Aβ toxicity was induced to growing cortex and hippocampus neurons on the day of the experiment after changing media. The cells were first replaced with neurobasal media, and

incubated for 2 h at 37°C in an incubator (VWR International, 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 PFs at different concentrations. The experiment was conducted twice (n=6) using treatment of PFs against $A\beta_{1-42}$ peptide toxicity.

Aβ firbril formation assay

Thioflavin T (ThT) dye fluorescence assay was used regularly to quantify the formation and inhibition of amyloid fibrils in the presence of PFs. The assay was performed as per 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.

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 hippocampus neurons were seeded using 500 µl neurobasal media in a 24 well tissue culture plate. After induction of Aβ stress, the cellular 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% CO2 atmosphere and absorbance was read at 490 nm using FLUOstar OPTIMA plate reader (BMG Labtech Inc., Offenburg, Germany). Cell injury analysis was conducted using the CytoTox-ONE™ homogeneous membrane integrity assay (Promega Corporation, Madison, WI). Cells were pre-treated with crude extract and three partridgeberry fractions for 24 h. **A**ll 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.

β-Amyloid (1-42) peptide quantification

The amount of rat beta-Amyloid (1-42) in cortex and hippocampus neurons after Aβ stress was quantified using SensoLyte® anti-rat beta-amyloid (1-42) quantitative ELISA kit (Anaspec Inc, CA). The ELISA was performed following manufacturer's instructions and absorbance was read at wavelength of 490 nm using BioTek, Power wave XS2 spectrophotometer (BioTek, Winooski, VT).

Intracellular ROS assay

Intracellular ROS were monitored in cortex and hippocampus neurons after Aβ stress using 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 reactive oxygen species were estimated with respect to fluorescein degradation.

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).

RESULTS & DISCUSSION

Initial extraction and solvent selection

The extraction of phenolic compounds from different samples is affected by the solubility of samples along with polarity of extraction solvents. Therefore, it is important to screen different solvents with varying polarity to select an ideal solvent for extraction of different classes of phenolics from partridgeberry. Eleven solvent types (**1-11**), differing in their polarity were used for the initial phenolic extraction and solvent selection. Various analytical parameters were assessed with the aim of selecting the best solvent resulting in maximum yield of a wide range of phytochemicals from partridgeberry.

Total phenolic content

Total phenolic content as estimated by Folin-Ciocalteu assay indicated the highest total phenolics in solvent **10** i.e. ethyl acetate: water: formic acid (80:28:2) extraction method (25 µM GAE/g 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**) yielded similar concentration of phenolics and were statistically similar in their total phenolic content ($p \leq 0.05$). Overall, the extraction using solvents with lower dielectric constant lead to higher yield of total phenolics.

Total flavonoid content

Total flavonoid content obtained by aluminum chloride ($AlCl_3$) colorimetric method indicated the highest flavonoid content (20 µM QE/g FW) in solvent **6** (EtOH:H_2O:FA::70:28:2) followed by solvent **9** and solvent **5** ($p \leq 0.05$) (Table 1). Unlike 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 in solvents significantly improved the flavonoid extraction from partridgeberry ($p \leq 0.05$). The acidification of solvents may improve flavonoid extraction and assist the formation of aluminum chloride assisted acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols in the $AlCl_3$ colorimetric method.

Total anthocyanin content

Total anthocyanin content was measured using the spectrophotometric pH differential method at 520 and 700 nm and results were expressed as malvidin-3-*O*-glucoside equivalents (mg/L). The assay is based on the reversible structural transformations as colored oxonium form of anthocyanin predominates at pH 1.0 while at pH 4.5 colorless hemiketal forms is dominant. Table 1 summarizes all total anthocyanin values obtained from the different extraction solvents used and indicates the possible influence of extracting solvent on total anthocyanin contents. The highest amount of the total anthocyanin were obtained by using solvent **10** [ethyl acetate: water: formic acid (80:28:2)] while solvent **11** was weakest in its ability to extract anthocyanins from partridgeberry. 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 wild partridgeberry.

Total proanthocyanidin content

The content of total proanthocyanidins in partridgeberry extracts was determined using the 4-dimethylaminocinnamaldehyde (DMAC) colorimetric assay, expressed as catechin equivalents (µmole CE/g FW). The results shown in Table 1 indicate significant difference ($p \leq 0.05$) in total proanthocyanidins contents was observed. Solvent **9** (70% acetone + 2% water) gave the highest proanthocyanidin content (5.9 µM CE/g FW) when compared with other solvents ($p \leq 0.05$), followed by solvent **1** (4.68 µM CE/g FW), solvent **4** (4.81 µM CE/g FW) and solvent **3** (4.50 µM CE/g FW) while solvents **10** and **11** exhibited the lowest amount of extracted total proanthocyanidins in wild partridgeberry extracts. Similar to earlier results, solvent **11** with the highest dielectric constant resulted in poor extraction of total proanthocyanidins from wild partridgeberry.

Ferric reducing antioxidant power (FRAP)

The total antioxidant activities of different solvent extracts was measured using FRAP assay which assesses antioxidant capacity of antioxidant candidate by reduction of Fe^{3+}-tripyridyltriazine to Fe^{2+}-tripyridyltriazine. All the solvent extracts reduced the Fe^{3+}-tripyridyltriazine to Fe^{2+}-tripyridyltriazine and emerged as potent antioxidants (Table 1). The strongest antioxidant activity ($p \leq 0.05$) was shown by solvent **9** (8.85 µM TE/g FW) followed by solvent **5** (8.28 µM TE/g FW) and solvent **1** (8.12 µM TE/g FW). Other solvent extracts exhibited the antioxidant activity in range of 7.93-6.20 µM TE/g FW. Continuing the previous trend solvent **11** exhibited the lowest antioxidant activity among all extracts which was in agreement to its phenolic and flavonoid content.

Selection of solvent system

In the current study 11 different solvent types were used to identify optimum solvent for extracting wide range of polyphenols from wild partridgeberry. All berry extracts were rich in polyphenols but the concentration of polyphenols was significantly dependent on the solvent polarity and its acidification. From the results indicated in Table 1, it is evident that solvent **11** i.e. water was the weakest among extraction solvents. On the other hand, an increase in the percentage of ethanol or methanol had no strong influence on extraction efficacy. Similar trend was observed with acetone as there was marginal difference between the extraction efficacies of its hydrous and anhydrous counterparts. However, the acidification of extraction solvents, particularly acetone indicated the strong influence on extraction procedure. Acidified hydrous acetone (**9**) emerged as ideal solvent among all assessed solvent systems as it yielded higher amount 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 wild partridgeberry. However, water is the least effective solvent for extracting the phenolic compounds despite its safety as solvent for human consumption.

Preparation of partridgeberry polyphenol fractions

Partridgeberry polyphenol fractions were prepared as described in Fig 1. 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 and then completely freeze dried

using stoppering tray dryer (Dura stop, Kinetic thermal systems, Stone Ridge, NY) for 48 hours. The powdered form of extract was weighed and stored in -20°C freezer until further analysis. The first partridgeberry polyphenol fraction (PPF1) was obtained from aqueous partition (B) as shown in Fig 1. The PPF1, rich in anthocyanins, was collected from separatory funnel and freeze dried in similar fashion as the crude extract. The powdered PPF1 was also weighed and stored in -20°C freezer. The next two fractions PPF2 (rich in flavan-3-ols) and PPF3 (rich in flavonols) were obtained after column chromatography technique was applied on the ethyl acetate partition (A) using five percent gradient to obtain fractions 1- 15.

Analysis of polyphenol fractions 1-15 using UPLC-ESI-MS/MS

Fifteen phenolic compounds were quantified by UPLC-ESI-MS/MS based on their retention time and external calibration curves. Flavan-3-ol such as the 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 ranged in fractions **1-4** while EC was also concentrated in the same fractions ($p \leq 0.05$). The highest content of both EC like catechin was in concentered in fraction **4**. All other fractions, i.e. fraction **6-15** had very low content of flavan-3-ol member ($p \leq 0.05$). These fractions (F1-F5) were pooled, the solvent was evaporated and freeze dried to prepare flavan-3-ol-rich fraction (PPF2).

Flavonols including Q, Q-3-*O*-glucoside, Q-3-*O*-galactoside, and Q-3-*O*-rutinoside were . abundant in fraction 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 ($p \leq 0.05$). Remaining fractions displayed 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 amount of the dihydrochalcones. The next group of phytochemicals analyzed using UPLC analysis 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 (PPF4). All the polyphenol preparations were weighed into new autoclaved vials and stock solution of 1000 µg/mL was prepared for *in vitro* biochemical and cell studies.

Characterization of four polyphenol preparations

Total phenolic content

The total phenolic content of partridgeberry polyphenol preparations was determined by Folin-Ciocalteu method at two different concentrations. 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). The lowest total phenolic content was shown by 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 marginally differed in their total phenolic content and exhibited almost similar concentration of total phenolic content.

Total flavonoid content

The flavonoid content in four polyphenol preparations was assayed using aluminum chloride ($AlCl_3$) colorimetric method (Table 3). The flavonol-rich fraction (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 flavonol-rich fraction exhibited ~26 times higher total flavonoid content compared to the crude partridgeberry extract. The PPF3 was followed by PPF2, with 14 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 both assayed concentrations ($p \leq 0.05$). 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.

Total anthocyanin content

Total anthocyanin content was measured by pH differential method and the results confirmed the highest anthocyanin content ($p \leq 0.05$) in PPF1 which had 8 times higher concentration of anthocyanins than that of the crude extracts (Table 3). The flavan-3-ol rich fraction, PPF2 followed by PPF3 exhibited the lowest anthocyanin content among all partridgeberry fractions ($p \leq 0.05$). The PPF2 exhibited around 42 times lower anthocyanin contents compared to PPF1, thus confirming successful separation of different phenolic compounds. Furthermore, similar results obtained at both concentrations of PPFs endorsed the efficacious liquid-liquid separation with low experimental error.

Total proanthocyanidins

Among the four polyphenol preparations, total proanthocyanidin content was greatest in the PPF2 (flavan-3-ol-rich) and PPF3 (flavonol-rich) ($p \leq 0.05$) (Table 3). The crude extract and the anthocyanin-rich fraction displayed the lower total proanthocyanidin content compared to the

other two ($p \leq 0.05$). In comparison to the crude extract and anthocyanin-rich fraction, the PPF2 and PPF3 consisted of about 4 times higher total proanthocyanidin content.

Antioxidant activity of polyphenol preparations

FRAP assay

The antioxidant capacity measured by FRAP assay was the greatest in flavonol-rich fraction (PPF3) followed by flavan-3-ol-rich fraction (PPF2). However, the lowest antioxidant capacity ($p \leq 0.05$) was exhibited by the anthocyanin-rich fraction (PPF1) followed by the crude extract. Both PPF2 and PPF3 exhibited twice higher antioxidant capacity compared to the anthocyanin-rich fraction. These results were confirmed with similar results at lower concentration (100 mg/L). The FRAP results followed the similar trend observed for total phenolic and flavonoid content of polyphenol preparations indicating the contribution of the polyphenols to the total antioxidant capacity of tested samples.

ORAC assay

ORAC assay measures the scavenging of peroxyl radical by the test samples. The strong antioxidant capacity measured by ORAC was exhibited by PPF2 and PPF3 when compared to the other two preparations (Table 3). Similar to FRAP assay results, the lowest ORAC value ($p \leq 0.05$) was observed in the anthocyanin-rich fraction (PPF1) followed by the crude extract.

DPPH radical scavenging activity

The results (Table 3) indicated that the strongest anti-radical activity was exhibited by PPF2 (flavan-3-ol-rich fraction) ($p \leq 0.05$) (Table 3). It was followed by the flavonol-rich fraction (PPF3) ($p \leq 0.05$) in its ability to scavenge free radicals. 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. The anthocyanin-rich fraction (PPF1) was also a weak scavenger of free radicals compared to the other two fractions. The antiradical activity of PPF1 was around 645 and 173 times weaker, compared to the PPF3 and PPF2, respectively. The results of anti-radical activity were in agreement with the results of FRAP and ORAC assays.

Analysis of phenolic compounds using UPLC-MS/MS

The phenolic compounds present in the polyphenol preparations were determined using UPLC-MS/MS technique (Table 4, Supplementary Data Table 1). Compared to the crude extract, anthocyanin-rich fraction (PPF1) exhibited higher concentration of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-galactoside ($p \leq 0.05$). However, the concentration of other phenolic classes in the both crude extract and PPF1 was statistically similar ($p \leq 0.05$). The flavan-3-ol fraction (PPF2) exhibited the highest concentration of flavan-3-ols among all the fractions as concentration of catechin and epicatechin exhibited 62 and 43 times higher compared to its crude counterpart. The flavonol-rich fraction, PPF3 indicated the highest concentration of quercetin and related compounds. The concentration of quercetin-3-*O*-galactoside and quercetin-3-*O*-glucoside in PPF3 was 58 and 52 times higher compared to the crude extract. Furthermore, the concentration of phloridzin, a dihydrochalcone was the highest in PPF3 ($p \leq 0.05$). Interestingly, PPF3 also exhibited higher concentration of flavonols compared to both PPF1 and PPF2.

Human cell studies

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 MTS assay (Table

5). Among the preparations, the strongest cytoprotective potential was exhibited by flavan-3-ol-rich fraction (PPF2) ($p \leq 0.05$). At the exposure to concentration of 1000 mg/L PPF2, WI-38 cells exhibited 95% cellular viability which was followed by 94 percent cell viability after PPF3 treatment. However, PPF3 treatment at 100 mg/L concentration exhibited higher cellular viability ($p \leq 0.05$) compared to PPF2 in WI-38 cells. The crude extract and anthocyanin fractions (PPF1) of partridgeberry exhibited statistically lower cell viability ($p \leq 0.05$) at both assayed concentrations compared to other fractions. The PPF2 and PPF3 possessed the greatest cytoprotective ability against peroxyl radical-induced oxidative damage.

Membrane damage

Increased extracellular lactate dehydrogenase (LDH) is a crucial biomarker of oxidative stress and disrupted cell integrity during the lipid peroxidation and oxidative stress. The LDH release assay results showed that the membrane damage in WI-38 cells ranged from 6.3% to 22.1% compared to a positive control (untreated cells) (Table 5). The results showed that among the polyphenol preparations, PPF2 exhibited the greatest inhibition of LDH release.

ROS inhibition

The increased levels of intracellular ROS in WI-38 cells by AAPH were attenuated by exposure to partridgeberry polyphenols. The results showed that flavonol-rich (PPF3) and flavan-3-ol-rich (PPF2) exhibited the highest inhibition of ROS in WI-38 cells ($p \leq 0.05$) (Table 5). These fractions at both assayed concentrations were most potent ($p \leq 0.05$) in their ability to limit the production of ROS. These fractions were followed ($p \leq 0.05$) by PPF1 and the crude extract as potent ROS inhibitors. These results followed the similar trend of cellular viability and antioxidant capacity assays, which established PPF2 and PPF3 as potent antioxidants.

Total protein content

Total protein content in WI-38 cells was assayed for 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 5). The analysis revealed that the oxidative insult decreased the level of protein content in control cells from 150 to 95.1 $\mu g\ mL^{-1}$. Subsequently, the polyphenol treatment controlled the protein damage in WI-38 cells compared to the untreated cells. The analysis showed that PPF3 was the most effective in preventing protein oxidation induced by peroxyl radical exposure. The PPF3 was followed by PPF1 in their ability to check protein oxidation ($p \leq 0.05$). Continuing the earlier trend, crude partridgeberry extract exhibited the weakest potential as antioxidant agent against protein oxidation in WI-38 cells.

Peroxide radical inhibition

The ability of the preparations to mitigate oxidative stress caused by peroxy 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 by PPF2 followed by PPF3 ($p \leq 0.05$) at both assayed concentrations (Table 5). On the other hand, PPF1 also exhibited strong ability to limit the production of peroxyl radicals in WI-38 cells following AAPH exposure. Similar to ROS inhibition results, crude extract was the weakest antioxidant candidate in comparison to the three wild partridgeberry fractions ($p \leq 0.05$).

Nrf2 ELISA

The Nrf2 ELISA analysis revealed the cellular mechanism of enhanced cytoprotective ability of polyphenol exposed WI-38 cells. The incubation of WI-38 cells with polyphenols significantly increased the intracellular levels of Nrf2 ($p \leq 0.05$). The first phase of Nrf2 studies focused on activation of antioxidant pathway in absence of oxidative stress. The phase one Nrf2 levels in

control cells i.e. without polyphenols treatment were 1.14 ng/mL while the levels of PF-treated cells ranged between 9.04-13.76 ng/mL. The highest amount of Nrf2 activation was observed in flavonol-rich fraction (12.91 mg/mL) while anthocyanin-rich fraction (PPF1) exhibited the lowest amount (9.04 ng/mL) of 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 prior to oxidative stress. In second phase, the Nrf2 levels were measured after the polyphenol treatment followed by 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 treated cells as PPF3 exhibited the highest amount (24.14 ng/mL) of intracellular Nrf2 ($p \leq 0.05$). It was followed by flavan-3-ol-rich fraction, PPF2 ($p \leq 0.05$) which exhibited 19.63 mg/mL of Nrf2. However, the anthocyanin-rich fraction maintained the lowest amount of Nrf2 among all studied fractions ($p \leq 0.05$). The results showed that the polyphenol preparations activated the antioxidant response pathway through possible translocation of Nrf2 to nucleus.

AChE and BuChE inhibition assay

The *in vitro* AChE and BuChE inhibition analysis was performed according to an earlier methodology described by Ellman, Courtney, & Featherstone (1961). The berry fractions were assayed at seven different concentrations and IC_{50} values for AChE inhibition were obtained using regression analysis (Table 6). The IC_{50} values for AChE inhibition ranged between 204.26-97.05 µg mL^{-1}. The weakest AChE inhibition was exhibited by crude extract (IC_{50} 204.26 µg mL^{-1}) of wild partridgeberry ($p \leq 0.05$) while flavonol-rich fraction (PPF3) (IC_{50} 97.05 µg mL^{-1}) was the strongest AChE inhibitor ($p \leq 0.05$). The crude extract of wild partridgeberry was closely

followed by flavan-3-ol-rich fraction (PPF2) of partridgeberry (IC_{50} 192.87 μg mL^{-1}) while the anthocyanin-rich fraction (PPF1) exhibited significantly stronger (IC_{50} 109.61 μg mL^{-1}) AChE inhibition compared to its crude and flavan-3-ol-rich counterparts ($p \leq 0.05$). Similar to AChE inhibition, fractionation significantly improved BuChE inhibition ability of wild partridgeberry. The IC_{50} values for BuChE inhibition ranged between 4240.49-241.03 μg mL^{-1}. The weakest BuChE inhibition was exhibited by the crude extract (IC_{50} 4240.49 μg mL^{-1}) while flavonol-rich fraction exhibited the strongest ($p \leq 0.05$) BuChE inhibition (IC_{50} 241.03 μg mL^{-1}). Compared to crude, the flavonol-rich fraction (PPF3) was about 18 time stronger BuChE inhibitor *in vitro*. The flavonol-rich fraction was closely followed by flavan-3-ol-rich (PPF2) fraction of partridgeberry (IC_{50} 255.23 μg mL^{-1}) while anthocyanin-rich fraction (PPF1) exhibited significantly weaker (IC_{50} 772.99 μg mL^{-1}) BuChE inhibition than the purified fractions ($p \leq 0.05$). The selectively index (SI) calculation showed that crude wild partridgeberry extract, exhibited the highest selectively index for AChE compared to all other fractions. Interestingly, both purified fractions i.e. flavan-3-ol- and flavonol-rich fraction exhibited strong AChE inhibition, but displayed low selectively index for the acetylcholinesterase.

Cell viability assay

The cell viability assay following Aβ stress in primary rat cortical and hippocampus neurons was performed using MTS assay (Promega, Madison, WI). The exposure of primary cortical and hippocampus neurons to freshly solubilised $A\beta_{1-42}$ peptides (50 μM) for 24 h resulted in the depletion of 71 and 94 percent cellular viability in the respective assay controls. The partridgeberry fractions were analysed at five different concentrations (200, 100, 50, 20, 10 μg mL^{-1}) and EC_{50} values were obtained from the emerging trends (Fig 1). All the cells treated with

wild partridgeberry fractions significantly improved the cellular viability of primary rat cortical and hippocampus neurons *in vitro*. The EC_{50} values for primary cortical cells ranged between 5.93-62.23 μg mL^{-1}. The strongest neuroprotective ability was exhibited by flavan-3-ol- and flavonol-rich fractions (EC_{50} 5.93-7.68 μg mL^{-1}) while anthocyanin-rich fraction (PPF1) was the weakest neuroprotective fraction (EC_{50} 62.23 μg mL^{-1}) of wild partridgeberry ($p \leq 0.05$). The crude extract of partridgeberry (EC_{50} 13.24 μg mL^{-1}) was closely followed the purified fractions in its ability to maintain cell viability following Aβ stress ($p \leq 0.05$). The cell viability studies using primary rat hippocampus neurons displayed EC_{50} values in range of 7.29-34.19 μg mL^{-1}. Similar to primary cortical cells, the strongest inhibition of membrane damage was exhibited by flavan-3-ol- and flavonol-rich fractions (EC_{50} 7.29-8.96 μg mL^{-1}) while anthocyanin-rich fraction (PPF1) was the weakest neuroprotective fraction (IC_{50} 34.19 ppm or μg mL^{-1}) of partridgeberry ($p \leq 0.05$). Following the previous results, the crude extract of partridgeberry (IC_{50} 18.76 μg mL^{-1}) closely followed the flavan-3-ol- and flavonol-rich fractions in its ability to maintain cell viability following Aβ stress ($p \leq 0.05$). The results showed that all berry fractions were potent neuroprotective agent *in vitro* and maintained cellular viability following Aβ stress in primary rat cortical and hippocampus neurons.

Membrane damage assay

The effect of Aβ stress on neural membrane and its attenuation by novel partridgeberry fractions was analyzed using LDH release assay (Promega, Madison, WI). Similar to cell viability studies, the crude extract and three partridgeberry fractions were analysed at five different concentrations (200, 100, 50, 20, 10 μg mL^{-1}) and IC_{50} values were obtained from the results (Fig 2). The results showed that the treatment of primary rat cortical and hippocampus 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 control in rat primary cortical cells ranged between 0.01-22.48 μg mL^{-1}. The strongest inhibition of membrane damage was exhibited by flavan-3-ol- and flavonol-rich fractions (EC_{50} 0.01-0.03 μg mL^{-1}) while anthocyanin-rich fraction (PPF1) was the weakest neuroprotective fraction (EC_{50} 22.48 μg mL^{-1}) of partridgeberry ($p \leq 0.05$). Following the earlier trend, flavan-3-ol-rich (PPF2) was the strongest inhibitor of neural membrane damage (EC_{50} 0.57 μg mL^{-1}) while anthocyanin-rich exhibited the weakest ability (EC_{50} 17.52 μg mL^{-1}) to inhibit Aβ induce membrane damage ($p \leq 0.05$). Interestingly, the flavonol-rich rich fraction (EC_{50} 9.17 μg mL^{-1}) of partridgeberry exhibited weaker neuroprotective ability than crude extract ($p \leq 0.05$) *in vitro*. Overall, all berry fractions significantly attenuated the Aβ induced membrane damage in both primary rat cortical and hippocampus neurons *in vitro*.

Intracellular ROS analysis

Reactive oxygen species (ROS) are implicated in multifactorial pathogenesis of AD as they trigger the alteration of mitochondrial function and increase lipid peroxidation. All partridgeberry berry fractions and crude extract were tested for their ability to inhibit ROS following Aβ stress in primary rat cortical and hippocampus neurons *in vitro* (Fig 3). The results showed that all partridgeberry fractions significantly attenuated the oxidative stress compared to untreated control ($p \leq 0.05$). The ROS inhibition in primary rat cortical neurons by partridgeberry fractions ranged between 59.64-92.12 % *in vitro*. The strongest inhibition of ROS was exhibited by flavonol-rich and flavan-3-ol-rich fractions (92.12-88.06 %) while anthocyanin-rich fraction exhibited weakest inhibition of ROS *in vitro* ($p \leq 0.05$). Similarly, the flavonol-rich fraction

(PPF2) also exhibited highest inhibition of ROS (91.61%) in primary rat hippocampus neurons *in vitro* ($p \leq 0.05$). The flavonol-rich fraction (PPF3) was closely followed by flavan-3-ol-rich (PPF2) and the crude extract in their ability to attenuate ROS in hippocampal neurons ($p \leq 0.05$). In like with the membrane and viability studies, anthocyanin-rich fraction also came out as the weakest antioxidant fraction ($p \leq 0.05$) in its ability to inhibit ROS following Aβ stress in neurons *in vitro*.

$A\beta_{1-42}$ firbril formation assay

Thioflavine T (ThT) staining assay was conducted to assess anti $A\beta_{1-42}$ activity of wild partridgeberry fractions (100 ppm or μg mL^{-1}) *in vitro* assay and in primary rat cortical and hippocampus neurons. The *in vitro* analysis showed that all berry fractions were potent anti $A\beta_{1-42}$ agents (Table 7). The strongest anti-Aβ activity was displayed by flavan-3-ol- and flavonol-rich fractions ($p \leq 0.05$). The purified class specific fractions (PPF2-3) were followed by anthocyanin-rich fraction (PPF1) and crude extract for anti-Aβ activity ($p \leq 0.05$). The assay was repeated for anti-Aβ activity primary rat cortical and hippocampus neurons. The anti-Aβ activity of crude extract and anthocyanin-rich fraction was significantly reduced in the cell model of Aβ stress. The crude extract of partridgeberry exhibited 6 times weaker anti-Aβ activity in rat primary cortical neurons while anthocyanin-rich fraction also lost 8 fold of its *in vitro* assay activity. The strongest anti-Aβ activity was displayed by the class specific flavan-3-ol- and flavonol-rich fractions ($p \leq 0.05$), who also maintained their strong anti-Aβ activity in cell model studies. Following the trend, both the crude extract and the anthocyanin-rich fractions (PPF1) exhibited very weak anti Aβ activity in rat primary hippocampal neurons, as they lost around 4-5 times of their *in vitro* anti-Aβ activity. However, the strongest anti-Aβ activity in rat primary hippocampus neurons was displayed by flavan-3-ol- and flavonol-rich fractions (PPF2-3) of wild

partridgeberry ($p \leq 0.05$). Overall, the class specific polyphenolic fractions of wild partridgeberry showed the strongest anti-Aβ activity both *in vitro* and in cell model experiments ($p \leq 0.05$).

Discussion

Continuous overproduction of free radicals triggers oxidative stress, which unchecked leads to cardiovascular diseases, cancer and accelerated ageing. Increased use of dietary antioxidant-rich foods has been encouraged due to their ability to contribute for multiple health benefits via scavenging of free radicals (Reuter, Gupta, Chaturvedi, Aggarwal, 2010). There is strong evidence that *Vaccinium* spp. berries are a good source of polyphenols and other bioactive phytochemicals (Wang, Camp, & Ehlenfeldt, 2012), which can be used as sources of dietary antioxidants. In the current investigation, a comprehensive study was conducted to identify appropriate solvent for maximum recovery of phenolics from wild partridgeberry. Further chromatography and analytical chemistry techniques were employed to prepare three wild partridgeberry fractions and assess their antioxidant activity and phenolic profile. As polyphenols possess strong antioxidant activity, the polyphenol fractions were evaluated for the cytoprotection ability using a cell model of peroxyl-radical induced oxidative stress of lung fibroblasts (WI-38).

In the first phase of the studies, the extraction solvents with high polarity lead to the maximum recovery of polyphenols and subsequently exhibited strong antioxidant activity. The total phenolic, flavonoid and anthocyanin content along with antioxidant activity were observed when extractions were done using solvents with higher polarity (Table 1). Acidified 70% acetone was identified as the solvent that facilitated the highest recovery of polyphenols from fresh partridgeberry. Similarly, Chavan et al. (2001) reported that acetone (70%) with or without acid

was the most efficient solvent for extraction of highest amount of polyphenols from different peas. Solvents with high polarity have been reported for extraction of polyphenols from fruits (Zhou and Yu, 2004; Yu, Ahmedna, and Goktepe, 2005). In the next phase of the study, separation of partridgeberry polyphenols into distinct three fractions was achieved by employing liquid-liquid separation and solid-phase fractionation techniques. The liquid-liquid separation was conducted using ethyl acetate and water as the two immiscible solvents. The separation is based on the principle that presence of sugar at C-3 position in anthocyanin maintains its solubility in water and helps its separation from most of other polyphenols in the dual aqueous phase. The schematic diagram (Fig 2) shows the separation of anthocyanin from remaining polyphenols leading to the formation of distinct two fractions of polyphenols. 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). The anthocyanin content was measured in both ethyl acetate and aqueous fraction and results indicated that >96% of anthocyanins migrated to aqueous phase indicating the successful separation of two classes of polyphenols (anthocyanin and non-anthocyanins). The ethyl acetate fraction was reconstituted in 50% ethanol and 15 fractions with gradient of ethyl alcohol were obtained. These fractions were subjected to HPLC analysis and were found rich in multiple phenolic classes including flavon-3-ols and flavonols (Table 2). These fractions were combined as shown in Fig 1 to obtain flavon-3-ol and flavonol-rich fractions. The distribution of the polyphenols was similar to an earlier report (Sekhon-Loodu et al, 2013) where gradient of alcohol was used to separate various phenolic classes. The flavan-3-ol and flavonol rich fractions were compared with the crude extract and anthocyanin rich fractions to understand the flavonoid structure depended antioxidant activity. The results obtained from Folin-Ciocalteu assay, aluminum chloride assay, total anthocyanin

content (pH differential method), and UPLC analysis confirmed the flavonoid sub-class wise separation of polyphenols. The next phase of study showed that four polyphenol preparations were potent antioxidant and exhibited cytoprotective abilities *in vitro*. The antioxidant ability measured by DPPH, FRAP and ORAC assays showed strong antioxidant potential of partridgeberry polyphenol fractions. The DPPH results showed that concentration of class specific polyphenols stimulated the antioxidant ability of the partridgeberry fractions (Table 3). The flavon-3-ol and flavonoid rich fractions exhibited about 700 and 190 times stronger antioxidant activity compared to its crude counterpart. The DPPH results were supported by FRAP and ORAC analysis, which also indicated similar trends. The presence and higher concentration of catechins and quercetin are strong contributory factors to the antioxidant activity of these fractions. Similar observations have been made in another report (Sekhon-Loodu et al, 2013) where the class specific fractions emerged as potent antioxidants *in vitro*.

Further, the crude extract and three partridgeberry fractions indicated cytoprotection against peroxyl radical-induced oxidative damage of WI-38 cells. Since the crude extract contains some sugars, it is expected to exhibit low cytoprotective abilities compared to three polyphenol fractions. Our cell-based assay of cytoprotection is based on induction of oxidative stress and damage of WI-38 cells by adding physiological levels of peroxyl radical generated using AAPH. Similar to the antioxidant capacity observed by in vitro assays of FRAP, ORAC and DPPH, flavan-3-ol and flavonol-rich fractions exhibited the greatest cytoprotection once the cell were pre-incubated with the fractions. The higher cytoprotective ability of these fractions may be related to individual polyphenol constituents such as quercetin glycosides (Ramyaa and Padma, 2013) and catechins (Banach, Dong, & O'Brien, 2009). The membrane damage induced by free radicals triggers lipid peroxidation and contributes to the pathology of oxidative stress.

The peroxy radical induction and membrane damage was effectively attenuated by all four polyphenol preparations of partridgeberry leading to higher cellular viability, protein content and low production of ROS in WI-38 cells. 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 have played significant role in attenuating oxidative stress *in vivo* (Banach, Dong, & O'Brien, 2009; Ramyaa and Padma, 2013). Similarly *Vaccinium* species of berries have also exhibited cytoprotective ability (Del Bo et al, 2010) leading to attenuation of lipid peroxidation and cellular damage. Finally, the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) mediated antioxidant response cascade by the polyphenol preparations uncovered the mechanism behind their strong cytoprotective ability. The Nrf2 under oxidative stress dissociates from Kelch-like ECH-associated protein 1 (Keap1) and binds with antioxidant response element (ARE) in nucleus to promoter region of antioxidant genes including NAD(P)H quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO1) (Motohashi, & Yamamoto, 2004). The activation of Nrf2 pathway by the partridgeberry polyphenol preparations confirms that these natural antioxidants not only scavenge free radicals but also trigger signal transduction pathway leading to possible activation of multiple genes involved in antioxidant response. Similar to antioxidant and cytoprotective ability, PPF2 and PPF3 were major 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 and Padma, 2013; Singh, Shankar, & Srivastava, 2011). Overall, the wild partridgeberry contained distinct polyphenol classes and exhibited antioxidant, free radical scavenging ability and cytoprotection properties in vitro.

Overall, the neuroprotective study in vitro suggests that polyphenol rich partridgeberry fractions, especially the ones rich in flavan-3-ols and flavonols, exerted a protective effect on $A\beta_{(1–42)}$ induced toxicity in cortical and hippocampus neurons *in vitro* at 0.01–8.96 μg mL^{-1} concentrations. However, the metabolic breakdown of flavan-3-ols and flavonols, during *in vivo* digestion conditions raises the question 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 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 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, we strongly aim at an *in vivo* study using mice model to further explore the bioavailability and efficacy of polyphenol rich partridgeberry extracts for neuroprotection in AD. Taken together, the results from current report provide a plausible antioxidant mechanism by which

partridgeberry polyphenols prevent cell death of cortical and hippocampus neurons following Aβ insult.

COMMUNICATIONS AND OUTREACH

1) Bhullar, K.S. and H.P.V. Rupasinghe. 2014. Cytoprotective properties of wild partridgeberry (*Vaccinium vitis-idaea* L. var. minus Lodd) polyphenols in human fibroblasts. Submitted to the 17th World Congress of Food Science and Technology and Expo (IUFoST 2014). Montreal, QC. (August 17-21, 2014).

2) Bhullar, K.S., H.P.V. Rupasinghe, and G.S. Robertson. 2014. Evaluation of wild partridgeberry polyphenols as potential natural health product to reduce the risk of Alzheimer's disease. Accepted for The 2014 Canadian Neuroscience Conference, Montreal, QC (May 25-28, 2014).

CONCLUSION & FUTURE RECOMMENDATIONS

Vaccinium species of berries are rich source of dietary polyphenols and numerous bioactives with strong antioxidant and cytoprotective properties. In current the study, acidified 70% acetone was identified as the solvent from optimum recovery of polyphenols from fresh partridgeberry. Anthocyanin-, flavan-3-ol- and flavonol-rich fractions obtained from wild partridgeberry exhibited 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. Among the three fractions, flavan-3-ol- and flavonol-rich fractions exerted the greatest antioxidants and cytoprotective properties. Wild partridgeberry polyphenols were also found to enhance antioxidant enzyme system through the activation of Nrf2 pathway.

We have also explored the neuroprotective potential of wild partridgeberry against Aβ induced neurotoxicity. The partridgeberry polyphenols protected both primary cortical and hippocampus neurons from $A\beta_{(1-42)}$ induced neurotoxicity, and inhibited the cell death. Polyphenol-rich fractions, particularly flavan-3-ol- and flavonols-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 Alzheimer's disease pathology. Our data further demonstrated that the flavan-3-ol- and flavonol-rich fractions of partridgeberry were partially successful to lower the concentration of $A\beta_{(1-42)}$ in both cells and cell suspension. This study provided new insights into the health benefits of *Vaccinium* fruits for the treatment of

Alzheimer's disease. Obviously, our recommendation is to continue this study using experimental animal models to confirm the in vitro and cell culture data. If the results are promising, natural health products derived from partridgeberry can be developed for further assessment using human clinical trials.

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REFERENCES

[1] Banach, M. S., Dong, Q., & O'Brien, P. J. (2009). Hepatocyte cytotoxicity induced by hydroperoxide (oxidative stress model) or glyoxal (carbonylation model): prevention by bioactive nut extracts or catechins. Chemico-biological interactions, 178, 324-331.
Bernaert, N., De Clercq, H., Van Bockstaele, E., De Loose, M., & Van Droogenbroeck, B. (2013). Antioxidant changes during postharvest processing and storage of leek (Allium ampeloprasum var. porrum). Postharvest Biology and Technology, 86, 8-16.

[2] Bernaert, N., De Paepe, D., Bouten, C., De Clercq, H., Stewart, D., Van Bockstaele, E., De Loose, Marc., & Van Droogenbroeck, B. (2012). Antioxidant capacity, total phenolic and ascorbate content as a function of the genetic diversity of leek (Allium ampeloprasum var. porrum). Food chemistry, 134, 669-677.

[3] Chavan, U. D., Shahidi, F., & Naczk, M. (2001). Extraction of condensed tannins from beach pea (Lathyrus maritimus L.) as a?ected by di?erent solvents. Food Chemistry, 75, 509-512.

[4] Cie?lik, E., Gr?da, A., & Adamus, K. (2006). Contents of polyphenols in fruit and vegetables. Food Chemistry, 94, 135-142.

[5] Circu, M. L., & Aw, T. Y. (2010). Reactive oxygen species, cellular redox systems, and apoptosis. Free Radical Biology and Medicine, 48, 749-762.

[6] Côté, J., Caillet, S., Doyon, G, Dussault, D., Sylvain, J. F. & Lacroix, M. (2011). Antimicrobial effect of cranberry juice and extracts. Food Control, 22, 1413-1418.

[7] Debnath, S. C & McRae, K. B. (2001). In Vitro Culture of Lingonberry (Vaccinium vitis-idaea L.), Small Fruits Review, 1, 3-19.

[8] Del Bo, C., Martini, D., Vendrame, S., Riso, P., Ciappellano, S., Klimis-Zacas, D., & Porrini, M. (2010). Improvement of lymphocyte resistance against H2O2 induced DNA damage in Sprague-Dawley rats after eight weeks of a wild blueberry (Vaccinium angustifolium) enriched diet. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 703, 158-162.

[9] Graf, B. A., Milbury, P. E., & Blumberg, J. B. (2005). Flavonols, flavones, Flavanones, and Human Health: Epidemiological Evidence. Journal of Medicinal Food, 8, 281-290.

[10] Heinonen, M. (2007). Antioxidant activity and antimicrobial effect of berry phenolics - A Finnish perspective. Molecular Nutrition and Food Research, 51, 684-691.

[11] Liu, X., Mu, T., Sun, H., Zhang, M., & Chen, J. (2013). Optimization of aqueous two-phase extraction of anthocyanins from purple sweet potatoes by response surface methodology. Food Chemistry, 141, 3034-3041.

[12] Motohashi, H., & Yamamoto, M. (2004). Nrf2-Keap1 defines a physiologically important stress response mechanism. Trends in molecular medicine, 10, 549-557.

[13] Ohkawa, H., Nobuko, O., & Kunio Y. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical biochemistry 95, 351-358.

[14] Pan, Y. (2011). Mitochondria, reactive oxygen species, and chronological aging: a message from yeast. Experimental gerontology, 46, 847-852.

[15] Pandey, K. B., & Rizvi, S. I (2009). Plant polyphenols as dietary antioxidants in human health and disease. Oxidative Medicine and Cellular Longevity, 2, 270-278.

[16] Prior, R. L., Fan, E., Ji, H., Howell, A., Nio, C., Payne, M. J., & Reed, J. (2010). Multi-laboratory validation of a standard method for quantifying proanthocyanidins in cranberry powders. Journal of the Science of Food and Agriculture, 90, 1473-1478.

[17] Ramyaa, P., & Padma, V. V. (2013). Ochratoxin-induced toxicity, oxidative stress and apoptosis ameliorated by quercetin-Modulation by Nrf2. Food and Chemical Toxicology, 62, 205-216.

[18] Ray, P. D., Huang, B. W., & Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cellular signalling, 24, 981-990.

[19] Reuter, R., Gupta, S. C., Chaturvedi, M. M., & Aggarwal, B. B. Oxidative stress, inflammation, and cancer: how are they linked? (2010). Free Radical Biology and Medicine, 49, 1603-1616.

[20] Rupasinghe, H. P. V., Kathirvel, P., & Huber, G. M. (2011). Ultra-sonication-assisted solvent extraction of quercetin glycosides from 'Idared' apple peels. Molecules, 12, 9783-9791.

[21] Sekhon-Loodu, S., Warnakulasuriya, S. N., Vasantha Rupasinghe, H. P., & Shahidi, F. (2013). Antioxidant ability of fractionated apple peel phenolics to inhibit fish oil oxidation. Food chemistry, 140, 189-196.

[22] Singh, B. N., Shankar, S., & Srivastava, R. K. (2011). Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. Biochemical pharmacology, 82, 1807-1821.
[23] Su, Z. (2012). Anthocyanins and flavonoids of Vaccinium L. Pharmaceutical Crops, 3, 7-37.
[24] Wang, S. Y., Camp, M. J & Ehlenfeldt, M. K. (2012). Antioxidant capacity and ?-glucosidase inhibitory activity in peel and flesh of blueberry (Vaccinium spp.) cultivars. Food Chemistry, 132, 1759-1768.
[25] Wu, X., Liang, L., Zou, Y., Zhao, T., Zhao, J., Li, F., & Yang, L. (2011). Aqueous two-phase extraction, identification and antioxidant activity of anthocyanins from mulberry (Morus atropurpurea Roxb.). Food Chemistry, 129, 443-453.
[26] Yu, J., Ahmedna, M., & Goktepe, I. (2005). E?ects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics. Food Chemistry, 90, 199-206.
[27] Zhou, K., & Yu, L. (2004). E?ects of extraction solvent on wheat bran antioxidant activity estimation. LWT-Food Science and Technology, 37, 717-721.

APPENDICES

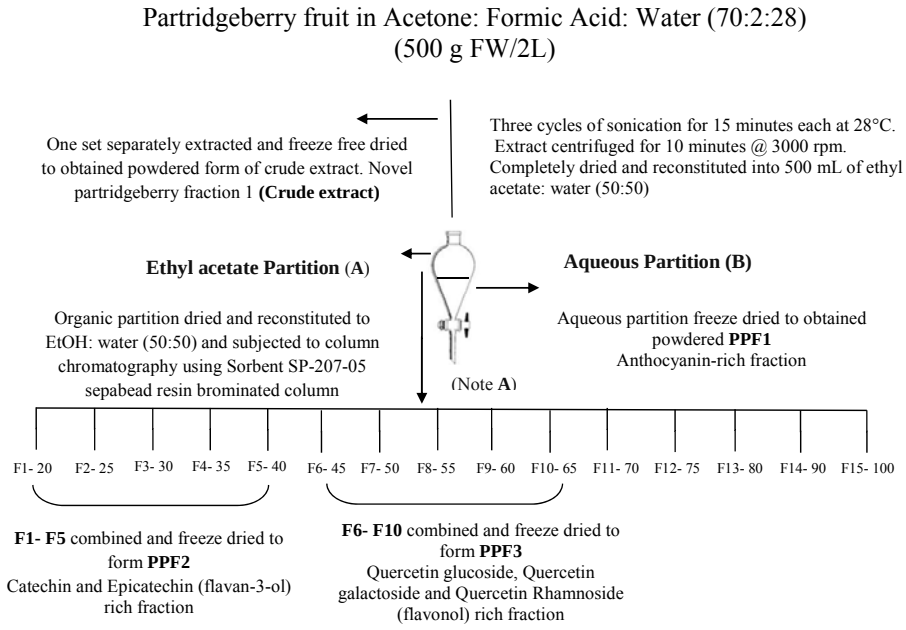


Fig. 1. Method of obtaining extracts, partitions and fractions. Crude extract was obtained using 70% Acetone + 2% Formic acid, PF 1 (Anthocyanin fraction from hydrous layer of water:ethyl acetate 1:1), PF2 (Flavan-3-ol-rich fraction obtained by merging partitions 1-5), PF3 (Flavonol-rich fraction obtained by merging fractions 6-10). Extraction solvent corresponds 70% Acetone + 2% Formic acid. Note **A**: Fractions, F-1 to with F-15 were

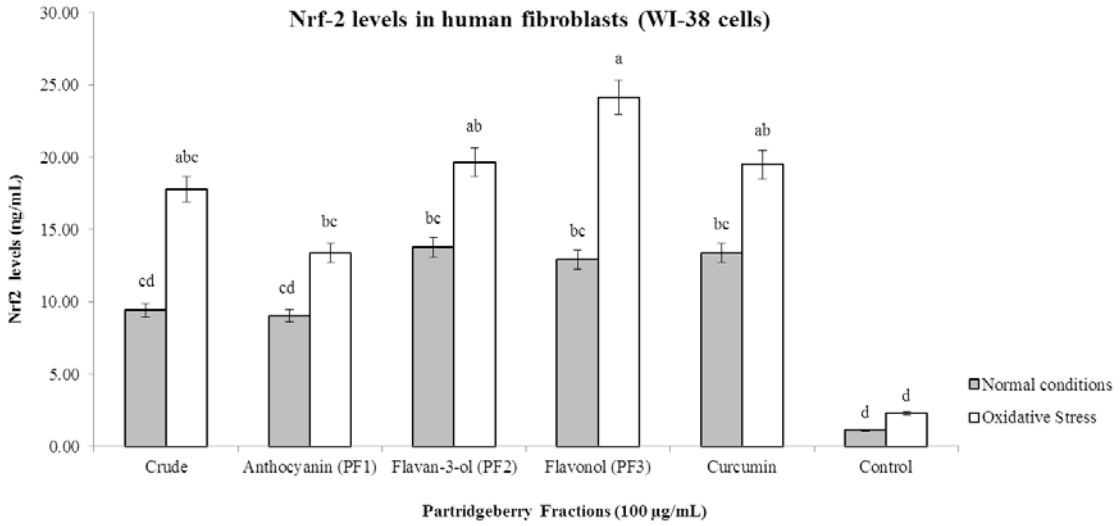


Fig. 2. Transcriptional induction of Nrf2 in fibroblasts (relative to assay control) incubated with partridgeberry fractions (100 mg/L) or curcumin (100 mg/L) for 24 h. Data is means of triplicate replicates (n=3) for each experiment.

Table 1
Total phenolics, total flavonoids, total anthocyanins, total proanthocyanains and antioxidant activity of partridgeberry (*Vaccinium vitis-idaea* L. var. minus Lodd) extracts

Solvent	Total Phenolics	Total Flavonoids	Total Anthocyanins	Total Proanthocyanidins	FRAP
	µM GAE/ g FW	µM QE/ g FW	(mg/L)	µM CE/ g FW	µM TE/ g FW
Methanol 100% (**1**)	21.42±1.02^{b}	11.18±0.12^{e}	54.04±1.53^{b}	4.68±0.13^{b}	8.12±0.22^{abc}
Methanol 70% (**2**)	22.67±0.08^{ab}	13.97±0.75^{d}	51.57±1.24^{bcd}	3.81±0.03^{cd}	7.31±0.43^{cde}
Methanol 70%+2% FA (**3**)	23.97±1.41^{ab}	12.91±0.24^{d}	30.04±0.51^{cde}	4.50±0.11^{bc}	7.93±0.12^{bcd}
Ethanol 100% (**4**)	22.81±0.69^{ab}	13.26±1.13^{d}	52.15±1.27^{bc}	4.81±0.14^{b}	6.35±0.45^{f}
Ethanol 70% (**5**)	22.75±0.53^{ab}	18.39±0.34^{abc}	37.81±2.91^{fg}	3.35±0.13^{d}	8.28±0.26^{ab}
Ethanol 70%+2% FA (**6**)	22.47±0.64^{ab}	19.84±0.25^{a}	45.75±2.13^{de}	3.31±0.24^{d}	7.07±0.37^{def}
Acetone 100% (**7**)	23.65±0.65^{ab}	17.11±0.63^{c}	41.32±1.18^{ef}	3.18±0.61^{d}	7.35±0.41^{cde}
Acetone 70% (**8**)	21.82±2.52^{ab}	17.94±0.52^{bc}	41.32±1.10^{ef}	3.56±0.47^{d}	6.85±0.35^{ef}
Acetone 70%+2% FA (**9**)	24.02±1.64^{ab}	19.32±0.24^{ab}	42.34±1.87^{ef}	5.90±0.32^{a}	8.85±0.38^{a}
EA+Water+2% FA (**10**)	25.02±0.71^{a}	7.93±0.58^{f}	80.19±3.92^{a}	2.19±0.11^{e}	6.51±0.32
Water 100% (**11**)	22.27±0.78^{ab}	10.67±0.26^{e}	34.34±3.12^{g}	2.17±0.19^{e}	6.20±0.38f

Different letters in each column indicate significant differences at $p<0.05$ as obtained by Tukey's test.

Table 2
Concentration (unit) of individual polyphenolic compounds in wild partridgeberry (*Vaccinium vitis-idaea* L. var. minus Lodd) fractions obtained

Fraction	Total Flavonol	Total Dihydrochalcones	Total Flavan-3-ols	Total Hydroxycinnamic acid
F1 (20%)	1.25±0.11^{e}	0.12±0.01^{c}	18.24±0.54^{b}	1.66±0.04^{e}
F2 (25%)	0.18±0.01^{f}	0.01±0.00^{d}	11.84±0.07^{c}	0.27±0.00^{f}
F3 (30%)	0.33±0.10^{f}	ND[1]	19.47±0.86^{a}	0.85±0.01^{f}
F4 (35%)	1.09±0.09^{e}	ND	21.57±1.9^{a}	1.04±0.00^{f}
F5 (40%)	3.12±0.21^{d}	0.03±0.00^{d}	13.15±0.45^{c}	8.38±1.01^{c}
F6 (45%)	12.63±0.34^{a}	0.19±0.00^{b}	5.67±0.25^{d}	14.39±2.15^{b}
F7 (50%)	13.88±0.23^{a}	0.31±0.00^{a}	1.25±0.92^{e}	19.73±3.22^{a}
F8 (55%)	11.66±0.1^{b}	0.39±0.10^{a}	0.36±0.05^{e}	19.77±1.81^{a}
F9 (60%)	6.51±0.21^{c}	0.21±0.00^{b}	0.21±0.01^{e}	9.84±2.00^{c}
F10 (65%)	2.05±0.01^{d}	0.04±0.00 ^{d}	0.13±0.01^{e}	2.36±3.00^{d}
F11 (70%)	0.95±0.1^{e}	0.02±0.00^{d}	0.21±0.02^{e}	0.58±0.00^{ef}
F12 (75%)	0.68±0.1^{f}	0.01±0.00^{d}	0.28±0.02^{e}	1.45±0.11^{e}
F13 (80%)	0.83±0.1^{e}	0.01±0.00^{d}	0.18±0.01^{f}	2.06±0.09^{e}
F14 (90%)	0.57±0.0^{e}	0.01±0.00^{d}	0.02±0.0^{f}	1.08±0.2^{e}
F15 (100%)	0.17±0.0^{g}	0.01±0.00^{d}	0.04±0.0^{f}	0.16±0.0^{f}

The 70% Acetone with 2% Formic acid was used as solvent for extraction of polyphenols from partridgeberry. All results are expressed as average (n=3) mg of polyphenolic compounds per 250 ml of fractionation product. Different letters in each column are significant different ($p<0.05$) 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: EGC: (-)-epigallocatechin; CAT: catechin; EC: (-)-epicatechin, Total Hydroxycinnamic acid: CA: Caffeic acid; FA: Ferulic acid; Ch.A: Chlorogenic acid;

Table 3
Phenolic composition and antioxidant activity of novel wild partridgeberry (*Vaccinium vitis-idaea* L. var. minus Lodd) fractions.

Fraction	Conc (mg/L)	TPC (µM GAE/ L)	TFC (µM QE/ L)	TAC (mg/L)	TPr.C (µM CE/ g FW)	FRAP (µM TE/ L)	ORAC (µM TE/ L)	DPPH IC_{50} (µg/mL)
Crude	1000	877.53±17.04^{f}	208.01±8.69^{ef}	3.93±0.43^{c}	634.64±27.39^{d}	1086.88±26.88^{c}	1055.25±53.72^{d}	146.31^{d}
	100	166.20±6.03^{g}	67.44±2.51^{h}	0.36±0.14^{e}	218.86±18.59^{e}	425.97±4.12^{g}	770.95±26.64^{f}	
Anthocyanin	1000	1281.53±48.06^{e}	286.89±7.20^{e}	29.05±0.91^{a}	695.51±37.19^{d}	696.80±8.84^{e}	867.87±25.43^{e}	135.57^{c}
(**PPF1**)	100	262.87±6.12^{g}	151.89±7.43^{g}	11.31±0.43^{b}	211.78±6.87^{e}	576.05±12.73^{f}	560.59±44.12^{g}	
Flavon-3-ol	1000	3060.53±59.10^{a}	2916.89±18.59^{c}	0.69±0.6^{e}	2377.41±44.30^{a}	1296.95±62.35^{b}	1773.23±33.84^{a}	0.21^{a}
(**PPF2**)	100	2044.20±36.94^{c}	1916.89±22.18^{d}	0.35±0.22^{e}	1679.60±29.45^{b}	966.65±46.52^{d}	1545.34±42.32^{b}	
Flavonol	1000	2589.20±50.93^{b}	5375.22±12.19^{a}	2.73±0.02^{d}	2346.05±31.21^{a}	1484.01±76.77^{a}	1290.54±15.57^{c}	0.78^{b}
(**PPF3**)	100	1480.20±46.97^{d}	3993.56±33.44^{b}	0.42±0.14^{e}	1560.86±31.64^{c}	1327.41±37.81^{b}	1078.13±16.93^{d}	

Different letters in each column indicate significant differences at $p<0.05$ as obtained by Tukey's test (n=6). TPC: Total phenolic content; TFC: Total flavonoid content; TAC: Total anthocyanin content; TPr.C: Total Proanthocyandin content; FRAP: Ferric reducing ability of plasma; ORAC: Oxygen radical absorbance capacity; DPPH: The 2,2-diphenylpicrylhydrazyl. 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.

Table 4
Concentration of polyphenolic sub-classes measured by UPLC-MS/MS in wild partridgeberry (*Vaccinium vitis-idaea* L. var. minus Lodd) fractions obtained through separation and chromatography techniques.

Fractions	Total Flavonol	Total Dihydrochalchones	Total Catechins	Total Anthocyanins
Crude extract	0.54±0.00^{c}	0.07±0.0^{b}	0.56±0.01^{b}	0.58±0.04^{b}
Antocyanin (PPF1)	0.32±0.02^{c}	0.06±0.0^{b}	0.23±0.01^{b}	1.55±0.00^{a}
Flavan-3-ol (PPF2)	2.15±0.01^{b}	0.08±0.0^{b}	30.67±0.01^{a}	0.07±0.00^{c}
Flavonol (PPF3)	21.99±0.23^{a}	0.36±0.01^{a}	2.76±0.01^{b}	0.11±0.01^{c}

The 70% Acetone with 2% Formic acid was used as solvent for extraction of polyphenols from partridgeberry. The analysis was performed using 100 mg/L of novel fraction solution (n=3). All results are expressed as mg of polyphenolic compounds per L of fractionation product. Different letters in each column are significant different ($p<0.05$). Total Flavonol: Total Flavonol: Quercetin Galactoside; Quercetin-Glucoside; Quercetin rutinoside; Quercetin. Total Dihydrochalchones: Phloridzin and Phloritin; Total Flavan-3-ols: EGC: (-)-epigallocatechin; CAT: catechin; EC: (-)-epicatechin, EGCG: epigallocatechin-3-gallate.Total Anthocyanins: cyanidin-3-*O*-glucoside and cyanidin-3-*O*-galactoside

Table 5
Cytoprotective and antioxidant activity of novel wild partridgeberry (*Vaccinium vitis-idaea* L. var. minus Lodd) fractions in human fibroblasts or WI-38 cells against AAPH induced oxidative stress.

Fraction	Concentration (µg/mL)	Cell Viability (%)	Membrane Damage (%)	ROS Inhibition (%)	Total Protein Content (µg)	Peroxide radical Inhibition (%)
Crude extract	1000	83.58±7.01^{a}	18.29±2.72^{A}	70.56±4.79^{b}	115.03±5.49^{b}	70.26±3.55^{d}
	100	55.93±3.62^{d}	10.94±2.73^{B}	30.11±3.56^{d}	104.19±7.46^{b}	49.78±0.77^{g}
Anthocyanin fraction	1000	80.19±2.03^{b}	13.54±4.36^{A}	72.93±3.27^{b}	112.66±6.18^{b}	77.13±4.46^{c}
(**PF1**)	100	44.03±5.43^{e}	6.32±1.94^{B}	50.51±4.69^{c}	101.77±2.55^{bc}	58.41±0.41^{f}
Flavon-3-ol fraction	1000	94.52±3.68^{a}	18.92±2.9^{A}	95.14±16.33^{a}	127.90±9.87^{b}	85.85±4.37^{b}
(**PF2**)	100	66.41±4.98^{c}	5.62±2.15^{B}	73.54±5.23^{b}	112.49±9.88^{b}	70.02±0.85^{de}
Flavonol fraction	1000	93.99±4.54^{a}	22.07±6.45A	94.44±3.57^{a}	130.43±5.97^{b}	80.03±5.60^{c}
(**PF3**)	100	81.32±4.07^{b}	12.31±2.78^{B}	78.66±3.35^{b}	115.54±2.78^{b}	65.29±1.11^{e}
Assay Control	^{2}NA	NA	NA	NA	151.32±12.12^{a}	98.02±1.20^{a}
Positive Control	NA	NA	NA	NA	95.09±6.05^{c}	NA

Different letters in each column are significant different ($p<0.05$) as obtained by Tukey's test. ^{2}NA (Not Applicable). Data Are means ± standard deviation of six independent replicates (n=6). 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 % measurement are relative to the control of no pre-incubation with polyphenol prepations.

Table 6
Percentage inhibition of AChE and BChE by novel phenolic rich fractions of **w**ild partridgeberry (*Vaccinium vitis-idaea* L. var. minus lodd).

NPF type	Concentration ($\mu g\ mL^{-1}$)	AChE inhibition	IC_{50} value (AChE)	BChE inhibition	IC_{50} value (BChE)	SI for AChE
Crude	1000	64.71±2.92		49.27±3.21		
	500	57.51±0.74		41.56±1.41		
	250	55.40±0.55	204.26^{d}	40.90±0.60	4240.49^{d}	20.76
	100	44.35±2.35		40.46±3.13		
	50	38.73±2.91		35.42±2.92		
	25	27.10±0.65		35.59±2.84		
	10	11.73±3.33		34.52±3.04		
Anthocyanin	1000	62.65±2.14		52.98±1.73		
(PPF1)	500	58.40±2.17		46.84±2.79		
	250	53.23±6.72	109.61^{b}	42.93±1.35	772.99^{c}	7.05
	100	50.09±1.36		45.08±1.02		
	50	49.01±4.54		42.81±2.34		
	25	40.79±1.16		38.41±0.65		
	10	34.70±3.61		24.68±3.13		
Flavan-3-ol	1000	68.21±2.74		62.01±3.15		
(PPF2)	500	56.11±0.46		56.02±0.59		
	250	48.67±3.30	192.87^{c}	49.41±0.41	255.23^{b}	1.32
	100	44.95±2.11		44.03±2.53		
	50	40.05±1.73		36.03±2.47		
	25	35.49±1.17		24.68±3.25		
	10	17.08±1.83		22.75±1.48		
Flavonol	1000	70.49±0.75		67.91±1.63		
(PPF3)	500	67.20±1.81		51.79±3.91		
	250	58.11±4.99	97.05^{a}	47.06±2.77	241.03^{a}	2.48
	100	54.55±3.07		44.67±3.24		
	50	42.29±4.21		35.57±1.39		
	25	34.40±2.12		30.72±1.94		
	10	26.66±2.56		18.35±0.75		

Results expressed as Mean ±SD of percentage cholinesterase(s) inhibition activity with respect to an assay control (n=6). The values with different subscript are statistically different ($p \leq 0.05$). AChE: Acetylcholinesterase; BChE: Butyrylcholinesterase; IC_{50}: inhibitory concentration 50 is the concentration of the concerned polyphenolic preparation that will inhibit the activity of a cholinesterase by 50 percent;SI: Selectivity index for Acetylcholinesterase inhibition compared to Butyrylcholinesterase inhibition *in vitro*.

Table 7

Anti $A\beta_{1-42}$ activity* of novel wild partridgeberry fractions

Fraction	*In vitro* analysis (% Inhibition)	Rat primary cortical neurons (% Inhibition)	Rat primary hippocampal neurons (% Inhibition)
Crude	63.34 ± 1.53^{b}	11.26 ± 1.12^{b}	16.88 ± 3.01^{c}
Anthocyanin	65.15 ± 3.41^{b}	8.16 ± 0.84^{b}	13.41 ± 1.94^{d}
Flavan-3-ol	82.87 ± 3.12^{a}	82.75 ± 3.47^{a}	63.53 ± 3.85^{b}
Flavonol	83.30 ± 2.97^{a}	86.08 ± 3.73^{a}	68.65 ± 2.64^{a}
Vehicle	ND•	ND•	ND•

Results expressed as Mean ±SD of Aβ fibril inhibition activity with respect to an assay control (n=6). The values with different subscript are statistically different ($p\leq0.05$).

* Anti amyloid activity (50 μM $A\beta_{1-42}$) was assessed using 100 μg mL^{-1} of novel partridgeberry fractions. Thioflavin T (ThT) dye (10 μM) was used for staining the $A\beta_{1-42}$ fibril sheets.

ND• Not detected

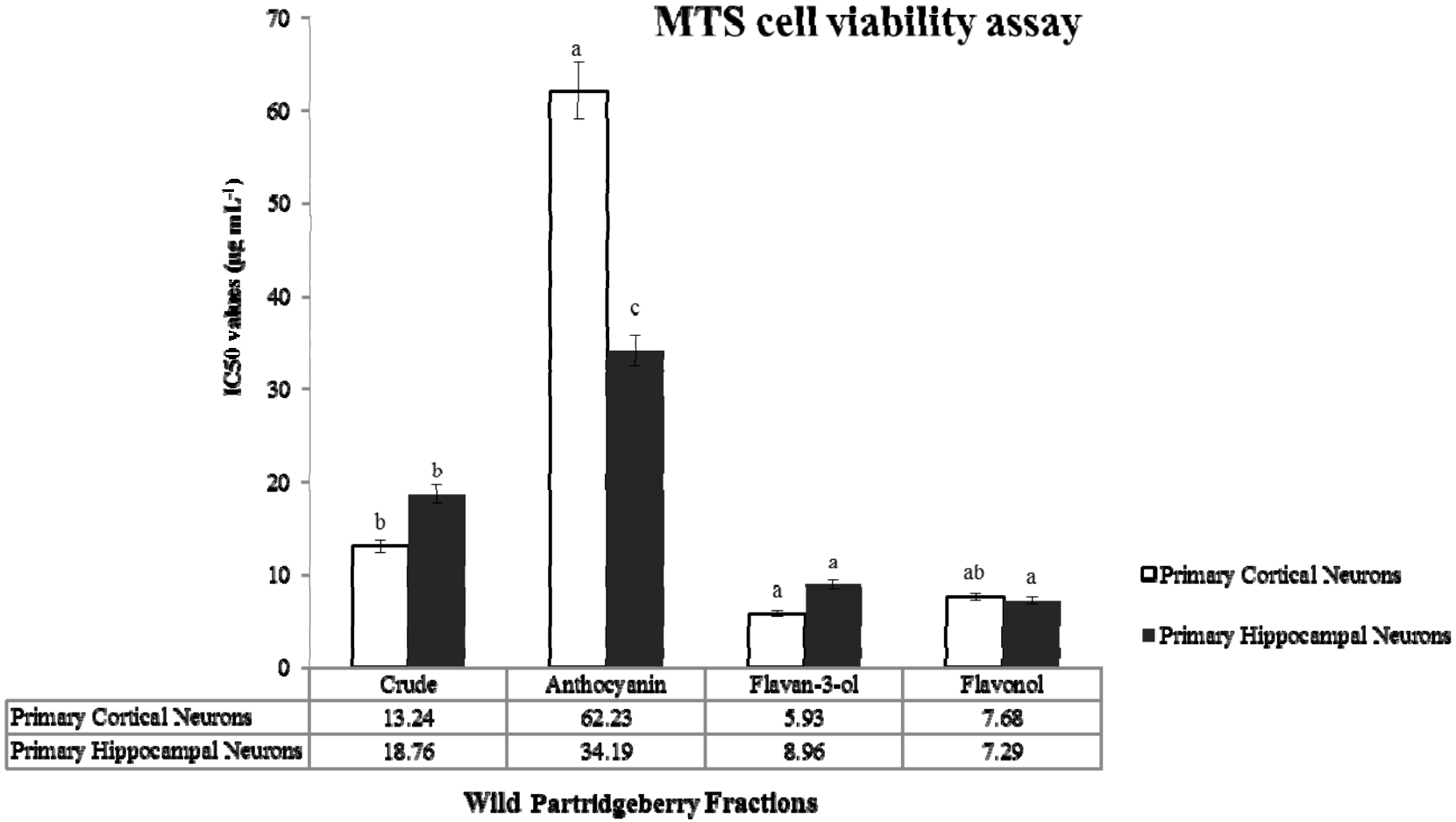


Figure 1: Inhibition of neuronal cell death following Aβ injury by novel partridgeberry fractions in rat primary cortical and hippocampal neurons with respect to assay controls. Results are expressed as EC_{50} values (Effective concentrations which maintain 50% cellular viability). The bars with different letters are statistically different ($p \leq 0.05$). EC_{50} values were obtained by conducting experiment at 5 different berry concentrations.

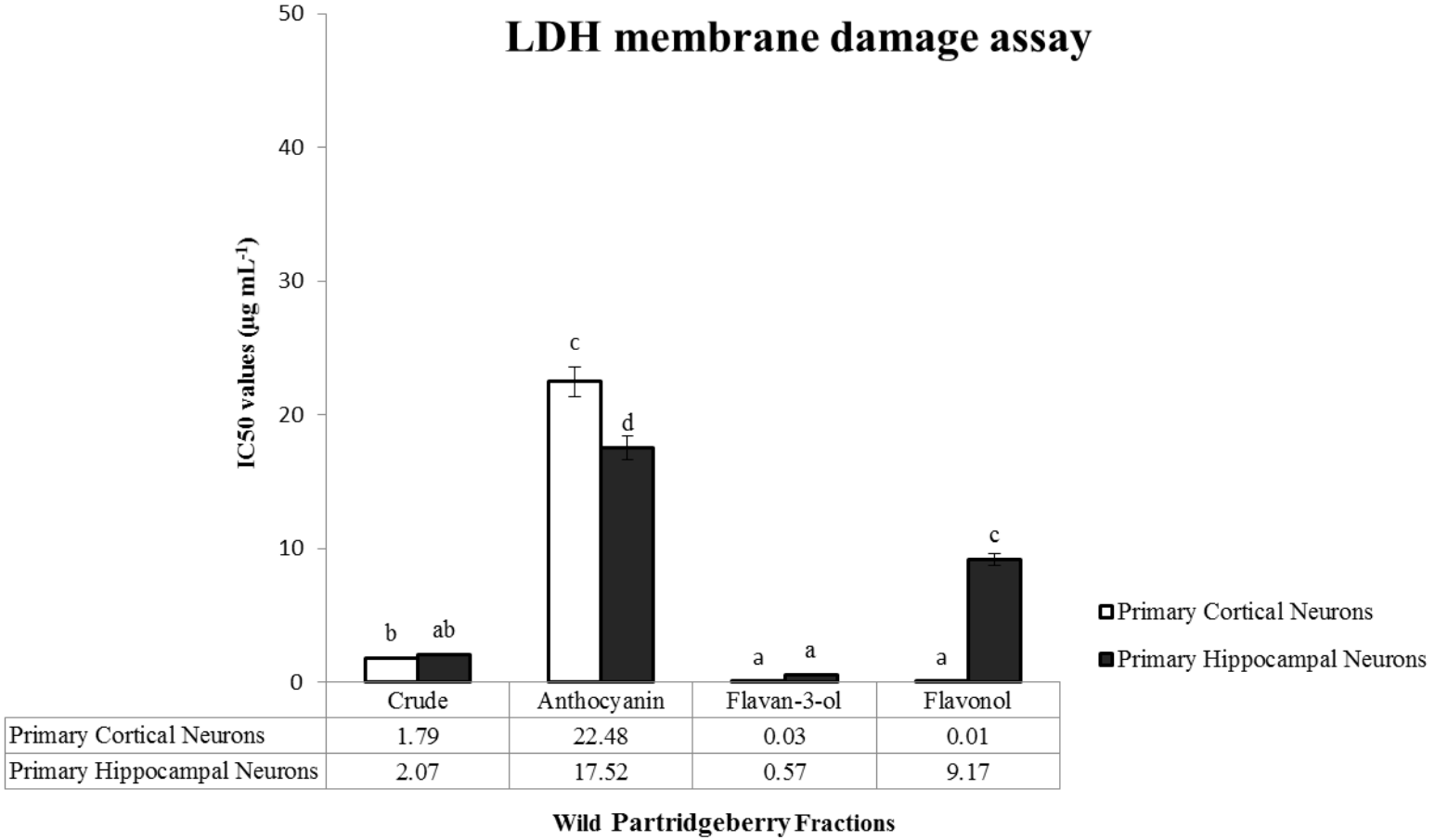
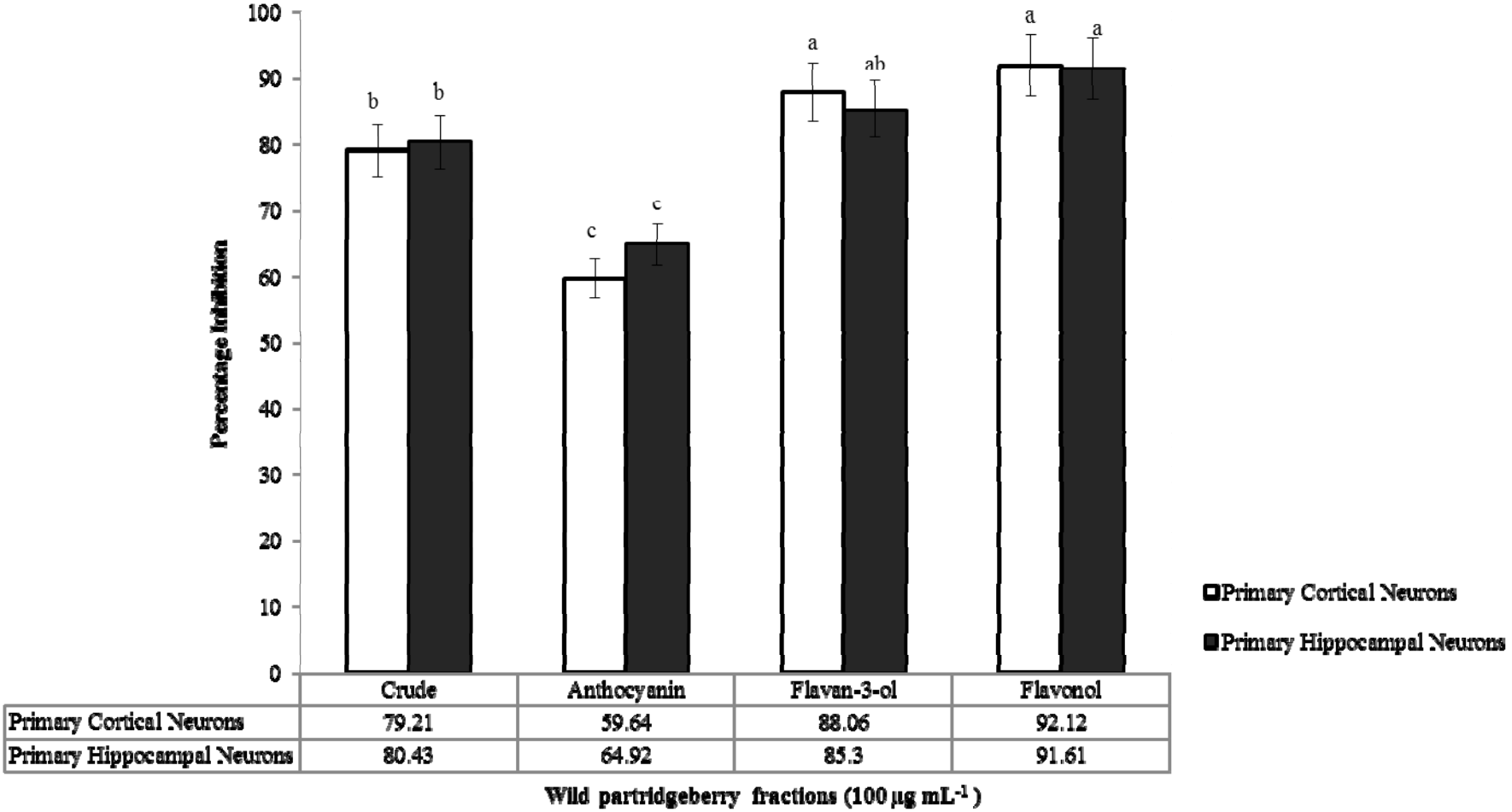


Figure 2: Inhibition of neuronal membrane damage following Aβ injury by novel partridgeberry fractions in rat primary cortical and hippocampal neurons with respect to assay controls. Results are expressed as IC_{50} values (Inhibitory concentrations which inhibit 50% membrane damage). The bars with different letters are statistically different ($p \leq 0.05$). IC_{50} values were obtained by conducting experiment at 5 different berry concentrations.



	Crude	Anthocyanin	Flavan-3-ol	Flavonol
Primary Cortical Neurons	79.21	59.64	88.06	92.12
Primary Hippocampal Neurons	80.43	64.92	85.3	91.61

Figure 3: Inhibition of reactive oxygen species (ROS) in rat primary cortical and hippocampal neurons by following Aβ injury by novel partridgeberry fractions with respect to assay controls. The bars with different letter groupings are statistically different ($p \leq 0.05$).