Partridgeberry polyphenols protect primary cortical and hippocampal neurons against β-amyloid toxicity

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ABSTRACT

β-Amyloid (Aβ) deposition elicits a toxic effect on neurons and plays a crucial role in the etiology and/or progression of Alzheimer's disease (AD). Polyphenols found in fruits are endorsed for nutritional intervention in AD, since they are known to have extensive therapeutic properties apropos of brain health owing to their antioxidative effects against Aβ and neural reactive oxygen species (ROS). The present study was aimed to investigate the neuroprotective potential of polyphenols of partridgeberry (*Vaccinium vitis-idaea* L.) and elucidate the mechanism by which they confer protection against Aβ toxicity in rat primary neurons in vitro. The pre-treatment of rat primary cortical and hippocampal neurons with partridgeberry polyphenols $(10\text{–}200~\mu g~mL^{-1})$ significantly attenuated Aβ-induced cell death and membrane damage. The flavan-3-ol- and flavonol-rich fractions of the partridgeberry exhibited the strongest ability to maintain cell viability $(EC_{50}$ $5.9~\mu g~mL^{-1})$ and prevent lactose dehydrogenase release $(IC_{50}~0.01~\mu g~mL^{-1})$ $(P \leq 0.05)$. Similar to the maintenance of cellular viability, the flavan-3-ol- and flavonol-rich fractions also amplified the greatest activity of SOD and catalase among all polyphenol preparations exposed to neurons $(P \leq 0.05)$. All four partridgeberry polyphenol preparations reduced the intracellular Aβ levels by 7–15 folds, and initiated Aβ clearance from neurons as compared to untreated cells $(P \leq 0.05)$. Partridgeberry derived polyphenol preparations; especially the flavonol-rich fraction $(IC_{50}~97.1~\mu g~mL^{-1})$ significantly modulated the apoptotic targets and in vitro acetylcholinesterase activity $(P \leq 0.05)$, indicating potential pharmacotherapy application in AD. Furthermore, the restoration of hyperactive caspases and Bcl2 family of apoptotic architects added to the neuroprotective candidacy of PPFs. These findings suggest that partridgeberry polyphenols, especially flavan-3-ol- and flavonol-rich fractions, could be of importance in prevention and/or treatment of AD.

1. Introduction

Over the last decade an increasing scientific interest has developed about berry fruits rich in polyphenols, as their consumption has been associated to a promising decrease of age-related cognitive and motor impairment (Bhullar & Rupasinghe, 2013). These therapeutic effects of berry polyphenols are credited to their pharmacological activities, including antioxidant, anti-inflammatory, and cytoprotective activities (Bhullar & Rupasinghe, 2013; Jones, Warford, Rupasinghe, & Robertson, 2012; Joseph et al., 2003). There are numerous studies indicating that the regular consumption of diet rich in polyphenols delivers protection against neurodegenerative diseases such as Alzheimer's disease (AD). AD is a progressive and fatal neurodegenerative disorder that affects millions of people worldwide through pathological decline in brain health and cognitive abilities. As of the year 2010, about 35.6 million people suffer from AD and it is predicted that the incidence

of AD may increase to 81.1 million by year 2040 (Prince et al., 2013). The pathology of this chronic disease includes a reduction in the size of temporal and frontal lobes of the brain, along with loss of memory and cognitive abilities due to degeneration of neurons. There are multiple hallmarks of AD, but principally the presence of β-amyloid (Aβ) plaques and neurofibrillary tangles (NFTs) is the major characteristics of the disorder (Bhullar & Rupasinghe, 2013). The Aβ plaques are characterized as deposits of fibrils and amorphous aggregates of Aβ peptides while the NFTs are intracellular fibrillar aggregates of the hyperphosphorylated tau protein (Takahashi, Capetillo-Zarate, Lin, Milner, & Gouras, 2010). The presence of the Aβ plaques and the NFTs in the cortical and hippocampal regions of the brain directly affect, and consequently impair, the ability of learning and trigger memory loss (Guillozet, Weintraub, Mash, & Mesulam, 2003). During the progression of AD, concentrations of neurotransmitters including acetylcholine are reduced through increased enzymatic concentrations of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). These enzymes degrade acetylcholine and create a cholinergic deficit, thus adding to pathological alterations in the brain of an AD patient (Ballard, 2002). Apart from these classical hallmarks, the brain in AD experiences increased oxidative stress and

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invasion of reactive oxygen species (ROS) leading to mitochondrial dysfunction. These mitochondrial abnormalities further initiate disruptions in energy metabolism, calcium homeostasis and trigger metal ions accumulation in Aβ plaques (Jomova, Vondrakova, Lawson, & Valko, 2010).

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

Fruits of partridgeberry also called lingonberry (*Vaccinium vitis-idaea* L.) grown in Newfoundland and Labrador, Canada are polyphenol-rich wild berry species with strong antioxidant and cytoprotective characteristics (Bhullar & Rupasinghe, 2015). Oxidative stress, cholinergic deficit and Aβ toxicity are all closely associated with the pathology of AD. As a result, the polyphenol-rich partridgeberry dietary intervention, owing to its sound antioxidant potential, is thought to be beneficial in the attenuation of the AD symptoms. However, to date, the effects of partridgeberry on Aβ toxicity and related oxidative damage have not been elucidated either in vitro or in vivo. Therefore, the present study was performed to investigate the potential of partridgeberry polyphenols against the Aβ-induced cytotoxic effects in rat primary cortical and hippocampal neurons along with its assessment as a prospective cholinesterase(s) inhibitor in vitro.

2. Materials and methods

2.1. Chemicals and reagents

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

2.2. Preparation of partridgeberry fractions

Partridgeberry polyphenol fractions (PPFs) were prepared as described recently (Bhullar & Rupasinghe, 2015). All PPFs were weighed to obtain stock solutions of 1000 μg mL^{-1} using DMSO for in vitro biochemical and cell studies.

2.3. Selection of assay concentrations

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

2.4. Cell culture

Primary rat cortex and hippocampal neurons were obtained from Life Technologies Inc. (Burlington, ON, Canada) and cultured according to the manufacturer's instructions. Briefly, the cells were cultured in neurobasal™ medium supplemented with 200 mM glutamax™-I and 50× B27 supplement (Life technologies, Burlington, ON, Canada). Cells were plated on to a poly-D-lysine (4.5 μg/cm^2) coated cell cultures flasks, and 24- and 6-well plates. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO$_2$ in an incubator (VWR International, Mississauga, ON, Canada). After 24 h of incubation, half of the growth medium was aspirated from each well and replaced with fresh medium. Cells were cultured every second day by replacing half of the medium from each well with fresh medium. The primary rat hippocampal neurons were supplemented with 25 μM L-glutamate up to the 4th day in culture for optimal growth and maintenance of neurons. Overall, the neurons were cultured using standard aseptic conditions on poly-D-lysine (4.5 μg/cm^2) coated plates and exposure of culture to light was avoided.

2.5. AChE and BuChE inhibition assay

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

2.6. Aβ (1–42) toxicity and treatment model

The Aβ toxicity was induced in growing cortex and hippocampal neurons on the day of the experiment after changing the growth media. The cells were cultured according to the standard conditions and were first given fresh neurobasal media, and incubated for 2 h at 37 °C in an incubator (VWR International, Mississauga, ON, Canada). Afterwards the cells were exposed to freshly solubilized Aβ$_{1-42}$ peptides (50 μM) for 24 h and then given fresh medium containing or not PPFs at different concentrations. The experiment was conducted using treatment of PPFs against Aβ$_{1-42}$ peptide toxicity.

2.7. Aβ fibril formation assay

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

2.8. Cell viability and injury assay

Cell viability was assessed using the CellTiter 96® AQ$_{ueous}$ non-radioactive cell proliferation assay (Promega, Madison, WI, USA). Briefly, 2×10^4 cortex and hippocampal neurons were seeded separately using 500 μL neurobasal media in a 24-well tissue culture plate. After the induction of Aβ stress, the cellular viability was measured by

pipetting 50 μL MTS solution into each well of the plate containing neurons in culture medium. The plate was incubated for 3 h at 37 °C in a humidified, 5% CO_2 atmosphere and absorbance was read at 490 nm using FLUOstar OPTIMA plate reader (BMG Labtech Inc., Offenburg, Germany). The proliferation indices were normalized to assay controls and results were expressed as EC_{50} values using regression analysis. Cell injury analysis was conducted using the CytoTox-ONE™ homogeneous membrane integrity assay (Promega Corporation, Madison, WI, USA). Cells were pre-treated with the crude extract and three partridgeberry fractions for 24 h. All remaining steps for membrane damage assessment assay were performed according to the manufacturer's instructions. The absorbance value was read using FLUOstar OPTIMA plate reader (BMG Labtech Inc., Offenburg, Germany) at wavelength of 490 nm. The LDH release assay results were expressed as the percentage of LDH release with respect to positive control and IC_{50} values obtained using regression analysis.

2.9. $A\beta_{1-42}$ peptide quantification

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

2.10. Intracellular ROS assay

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

2.11. Superoxide dismutase (SOD) and catalase assay

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

2.12. DNA fragmentation

Quantification of DNA fragmentation was performed using ApoTarget™ Quick Apoptotic DNA Ladder Detection Kit (BioSource International, Inc., Camarillo, CA, USA), according to the manufacturer's instructions. Briefly, genomic DNA from primary rat cortical and hippocampal neurons (4×10^6 cells) was extracted and equal amounts of DNA from each sample (10 μg) were electrophoresed on 2% agarose

gel and fragments visualized by Gelred staining Gelred staining (VWR International, Mississauga, ON, Canada) under UV transillumination using Biorad GelDoc system (Bio-Rad Laboratories Canada Ltd, Mississauga, ON, Canada).

2.13. Western blots

Western blotting was performed with a SDS-PAGE electrophoresis system as described previously (Izumiya et al., 2003). Briefly, 20-μg protein samples were resuspended in a reduced sample buffer, and then electrophoresed on a 4 to 20% Tris gel with Tris running buffer; blotted to nitrocellulose membrane; and sequentially probed with primary antibodies against selected targets (Cell Signaling Technology, Beverly, MA, USA) and Akt. A horseradish peroxidase-conjugated goat anti-rabbit antibody was then added, and secondary antibodies were detected using enhanced chemiluminescence (Bio-Rad Laboratories Canada Ltd, Mississauga, ON, Canada). GAPDH was used as a protein loading control.

2.14. Cellular morphology

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

2.15. Statistical analysis

All experiments were performed in hexaplicates (n = 6) unless stated otherwise and the statistical significance of means was assessed by one way analysis of variances (ANOVA) using SAS software (SAS Institute, NC, USA). The statistical significance was defined as P ≤ 0.05.

3. Results

3.1. AChE and BuChE inhibition values

Our first inquiry was whether the two cholinesterases were affected by the partridgeberry polyphenol preparations. To answer this question, we used an in vitro substrate assay to monitor changes in hydrolysis of acetylcholine in the absence or presence of four PPFs. The berry fractions were assayed at seven different concentrations and IC_{50} values for the enzyme inhibition were obtained using regression analysis (Table 1). The IC_{50} values for AChE inhibition ranged between 97.1–204.3 μg mL^{-1}. The flavonol-rich fraction (PPF3) (IC_{50} 97.1 μg mL^{-1}) exhibited the greatest AChE inhibition while the crude extract (IC_{50} 204.3 μg mL^{-1}) of partridgeberry displayed the weakest AChE inhibition (P ≤ 0.05). The crude extract of partridgeberry was closely followed by flavan-3-ol-rich fraction (PPF2) of partridgeberry (IC_{50} 192.9 μg mL^{-1}) while the anthocyanin-rich fraction (PPF1) exhibited significantly higher (IC_{50} 109.6 μg mL^{-1}) AChE inhibition compared to its crude and PPF2 counterparts (P ≤ 0.05). Similar to AChE inhibition, fractionation significantly improved BuChE inhibitory ability of PPFs in vitro (P ≤ 0.05). The IC_{50} values for BuChE inhibition ranged between 241.0–1014.2 μg mL^{-1}, indicating the stronger selectivity of PPFs towards AChE than the non-specific cholinesterase enzyme, BuChE. The strongest BuChE inhibition was exhibited by the PPF3 (IC_{50} 241.0 μg mL^{-1}) while the crude extract exhibited the strongest BuChE inhibition (IC_{50} 1014.2 μg mL^{-1}) (P ≤ 0.05). Compared to the crude extract, PPF3 was about 18-time stronger BuChE inhibitor in vitro. The PPF3 was closely followed by the PPF2 fraction of partridgeberry (IC_{50} 255.2 μg mL^{-1}) while PPF1 exhibited significantly weaker (IC_{50} 772.9 μg mL^{-1}) BuChE inhibition than PPF2 and PPF3 (P ≤ 0.05). Interestingly, both PPF2 and PPF3 exhibited strong AChE inhibition (P ≤ 0.05), but displayed low selectivity index for the BuChE. The selectivity index (SI) calculation showed that crude partridgeberry extract, exhibited the highest selectivity index for AChE compared to BuChE,

Table 1
Percentage inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity by polyphenol preparations of partridgeberry (*Vaccinium vitis-idaea* L. var. minus lodd).

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

Results expressed as mean ± SD of percentage enzyme inhibition activity with respect to an assay control (n = 6). The values with different superscripts for IC_{50} values are statistically different (P ≤ 0.05). IC_{50} value is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function by fifty percent. PPF1: Anthocyanin-rich fraction; PPF2: flavan-3-ol-rich fraction; PPF3: flavonol-rich fraction. AChE: acetylcholinesterase; BChE: butyrylcholinesterase; SI: selectivity index for AChE inhibition compared to BChE inhibition in vitro.

resulting in stronger inhibition of acetylcholine hydrolysis in comparison to butyrylcholine.

3.2. $A\beta_{1-42}$ fibril formation values

The Thioflavin T (ThT) staining assay was conducted to assess anti $A\beta_{1-42}$ activity of partridgeberry fractions (100 $\mu g\ mL^{-1}$) using real time in situ analysis and primary rat cortical and hippocampal neurons in vitro. The results showed that all berry fractions were potent anti $A\beta_{1-42}$ agents (Table 2) while classical amyloid fibrils were observed

Table 2
Percentage inhibition of Aβ fibril formation in vitro by polyphenol preparations of partridgeberry as measured by the Thioflavin T assay.

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

Results expressed as mean ± SD of percentage inhibition of the Aβ fibril formation activity with respect to an assay control (n = 6). The values with different superscripts are statistically different (P ≤ 0.05). PPF1: anthocyanin-rich fraction; PPF2: flavan-3-ol-rich fraction; PPF3: flavonol-rich fraction.
Anti amyloid activity (50 μM $A\beta_{1-42}$) was assessed using 100 $\mu g\ mL^{-1}$ of partridgeberry fractions. Thioflavin T (ThT) dye (10 μM) was used for staining the $A\beta_{1-42}$ fibril sheets. ND: no inhibition detected.
[a] In situ analysis defines the inhibition of Aβ fibril formation in the chemical reaction mixture free of cellular substrates.

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

3.3. Cell viability

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

3.4. Membrane damage

The effect of Aβ stress on neural membrane damage in vitro and its attenuation by partridgeberry fractions was next examined using LDH release assay (Promega, Madison, WI, USA). Similar to the cell viability studies, the crude extract and three partridgeberry fractions were
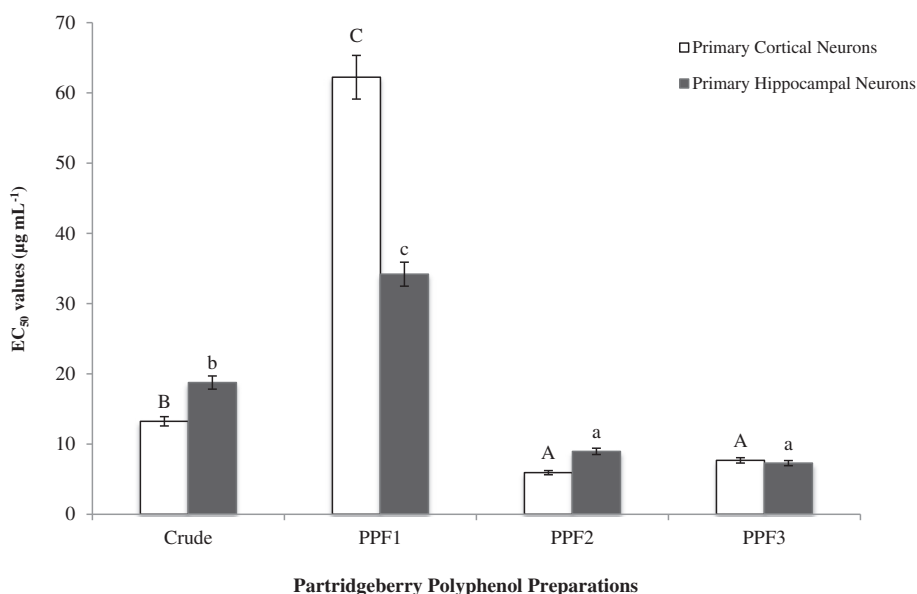
Fig. 1. EC_{50} values of partridgeberry polyphenol preparations resulting in the inhibition of neuronal cell death measured by MTS assay following β-amyloid injury in rat primary cortical and hippocampal neurons with respect to assay controls. Rat primary cortical and hippocampal neurons were treated with either 0.1% DMSO (vehicle control), or 1–1000 μg mL^{-1} partridgeberry polyphenol preparations, for 24 h, followed by Aβ injury (50 μM). Results are expressed as EC_{50} values (half maximal effective concentration which maintains 50% cellular viability). The bars with different letters are statistically different (P ≤ 0.05), A–C and a–c letters on bars as obtained by Tukey's test, are used to indicate statistical significance (P ≤ 0.05) for treatment affect (cell death inhibition) in rat primary cortical and hippocampal neurons respectively. EC_{50} values were obtained by conducting experiment at 5 different treatment concentrations. PPF1: anthocyanin-rich fraction; PPF2: flavan-3-ol-rich fraction; PPF3: flavonol-rich fraction.

analyzed at five different concentrations (10, 20, 50, 100, 200 μg mL^{-1}) and IC_{50} values were calculated from the results (Fig. 2). When Aβ$_{1–42}$ solution (50 μM) was prepared and incubated with neurons for 24 h, it was cytotoxic, and displayed around 69–71% elevation in LDH release in primary cortical and hippocampal neurons respectively. The results showed that the pre-treatment of primary rat cortical and hippocampal neurons with partridgeberry fractions prior to Aβ stress resulted in significantly lower LDH release compared to the untreated cells (P ≤ 0.05).

The IC_{50} values for LDH release inhibition in rat primary cortical cells ranged between 0.01–22.5 μg mL^{-1}. The strongest inhibition of membrane damage was exhibited by PPF2 and PPF3 (EC_{50} 0.01–0.03 μg mL^{-1}) while PPF1 was the weakest neuroprotective fraction (EC_{50} 22.5 μg mL^{-1}) of partridgeberry (P ≤ 0.05). Similar observations were made in experiments with primary hippocampal neurons, as PPF2 was the strongest inhibitor of neural membrane damage (EC_{50} 0.6 μg mL^{-1}) while PPF1 exhibited the weakest ability
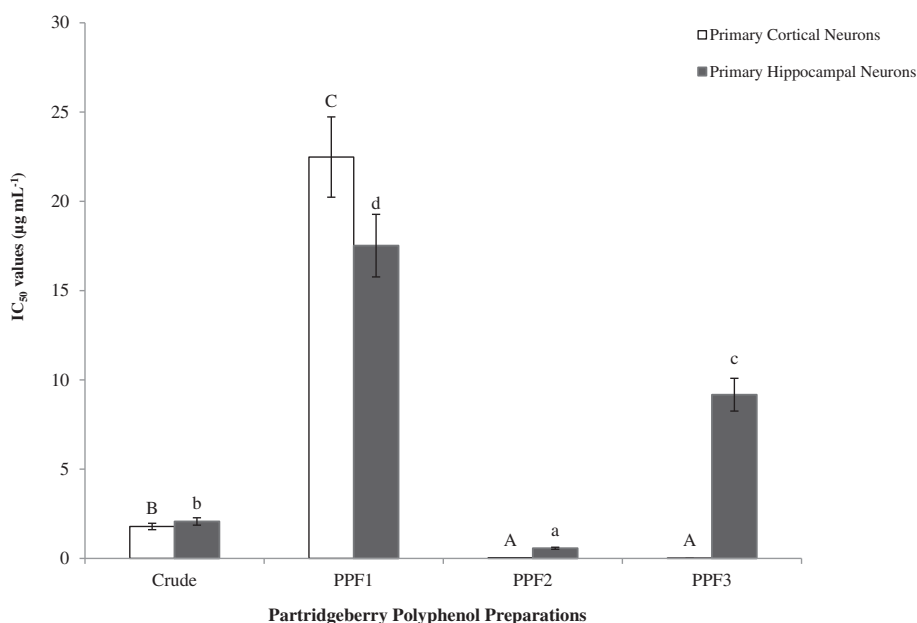

Fig. 2. IC_{50} values of partridgeberry polyphenol preparations resulting in the inhibition of neuronal cell death measured by LDH release assay following β-amyloid injury in rat primary cortical and hippocampal neurons with respect to assay controls. Rat primary cortical and hippocampal neurons were treated with either 0.1% DMSO (vehicle control), or 1–1000 μg mL^{-1} partridgeberry polyphenol preparations, for 24 h, followed by Aβ injury (50 μM). Results are expressed as IC_{50} values (half maximal inhibitory concentrations which inhibit 50% membrane damage), A–C and a–d letters on bars are significantly different as obtained by Tukey's test, are used to indicate statistical significance (P ≤ 0.05) for treatment affect (LDH release inhibition) in rat primary cortical and hippocampal neurons respectively. The bars with different letters are statistically different (P ≤ 0.05). IC_{50} values were obtained by conducting experiment at 5 different treatment concentrations. PPF1: anthocyanin-rich fraction; PPF2: flavan-3-ol-rich fraction; PPF3: flavonol-rich fraction.

(EC_{50} 17.5 μg mL^{-1}) to inhibit Aβ-induced membrane damage ($P \leq 0.05$). Interestingly, the PPF3 (EC_{50} 9.2 μg mL^{-1}) exhibited weaker neuroprotective potency in hippocampal neurons than the crude extract ($P \leq 0.05$) in vitro. In summary, all berry fractions significantly attenuated Aβ-induced membrane damage in both primary rat cortical and hippocampal neurons in vitro, while PPF2 was a highly effective inhibitor of LDH release attributable to Aβ-induced membrane damage.

3.5. Intracellular ROS

ROS are implicated in multifactorial pathogenesis of AD as they trigger the alteration of mitochondrial function and increase lipid peroxidation. All partridgeberry polyphenol fractions and the crude extract were tested at concentration of 100 μg mL^{-1} for their ability to inhibit ROS following Aβ stress in the primary rat cortical and hippocampal neurons in vitro (Fig. 3). The explosive ROS production following Aβ injury was suggestive that the binding of Aβ to the neural membrane may have triggered the intracellular ROS generation. The results showed that all partridgeberry fractions significantly attenuated the oxidative stress compared to the untreated control ($P \leq 0.05$). ROS inhibition in primary rat cortical neurons by partridgeberry fractions ranged between 59.6–92.1% in vitro. The strongest inhibition of ROS was exhibited by PPF2 and PPF3 (92.1–88.1%) while PPF1 exhibited the weakest inhibition of ROS in vitro ($P \leq 0.05$). Similarly, the PPF3 also exhibited the highest inhibition of ROS (91.6%) in primary rat hippocampal neurons in vitro ($P \leq 0.05$). The PPF3 was closely followed by PPF2 and the crude extract in their ability to attenuate ROS in hippocampal neurons ($P \leq 0.05$). Similar to the membrane and viability studies, the PPF1 showed the lowest ($P \leq 0.05$) ability to inhibit ROS following Aβ stress in neurons in vitro. The strong ROS inhibition (>90%) at 100 μg mL^{-1} suggests that either PPF3 or PPF2 preconditioning alone can dissuade the majority of ROS produced via Aβ insult, bypassing the requirement of a post injury antioxidant stimulation.

3.6. Superoxide dismutase (SOD) and catalase activity

Since ROS oxidatively activated during Aβ-injury, were significantly attenuated by PPFs, we hypothesized whether SOD and catalase were

activated in both cell types, owing to the increased concentration of exogenous activators of antioxidant pathway. In the current study, we observed that the partridgeberry fractions (100 μg mL^{-1}) protected the neurons in vitro against β-amyloid induced cell death and triggered the release of antioxidant enzyme, SOD. The release of SOD in partridgeberry extract treated primary cortical neurons ranged from 8.8–17.3 nmol/min/mL while the control and untreated cells exhibited 4.8 and 6.8 nmol/min/mL enzymatic activity respectively (Fig. 4A). The highest SOD activity was observed in PPF2 followed by PPF3 ($P \leq 0.05$), and the in vitro enzymatic activity of both the fractions was twice as compared to the untreated cells. Following the earlier trend of weak cytoprotective ability, PPF1 emerged as the weakest activator of SOD in both primary cortical and hippocampal neurons ($P \leq 0.05$), while PPF3 and the crude extract exhibited the strongest release of SOD in primary rat hippocampal cells followed by PPF2 ($P \leq 0.05$). Catalase (CAT) or glutathione peroxidase is a crucial antioxidant enzyme as it detoxifies H_2O_2 produced during metabolic processes and extends cytoprotective therapy in Alzheimer's disease and ischemia–reperfusion injury (Yan, Wang, & Zhu, 2013). In the current study, the antioxidant effect of partridgeberry fractions against Aβ through induction of catalase enzyme was studied in the primary rat cortical and hippocampal neurons. The results showed that the antioxidant candidate fractions induced the catalase enzyme in primary rat neurons against Aβ stress ($P \leq 0.05$). The total catalase activity in partridgeberry extract treated primary cortical cells ranged between 2.3–5.4 nmol/min/mL while the control and untreated neurons exhibited 1.0 and 0.5 nmol/min/mL enzymatic activity respectively (Fig. 4B). The strongest induction of catalase was exhibited by the PPF2 (5.4 nmol/min/mL) of partridgeberry ($P \leq 0.05$) while PPF3 (4.5 nmol/min/mL) followed the leading fraction in its ability to express catalase in vitro ($P \leq 0.05$). The potent antioxidants, PPF2 and PPF3 demonstrated 11 and 9 times higher catalase activity than the untreated control respectively. However, the crude extract and PPF1 demonstrated the weakest activity of catalase in primary rat cortical neurons in vitro ($P \leq 0.05$). Similarly, the primary rat hippocampal neurons were also protected against Aβ through activation of catalase. The PPF2 treated neurons, exhibited the strongest (~12 times higher) catalase activity compared to the Aβ injury control, followed by PPF3
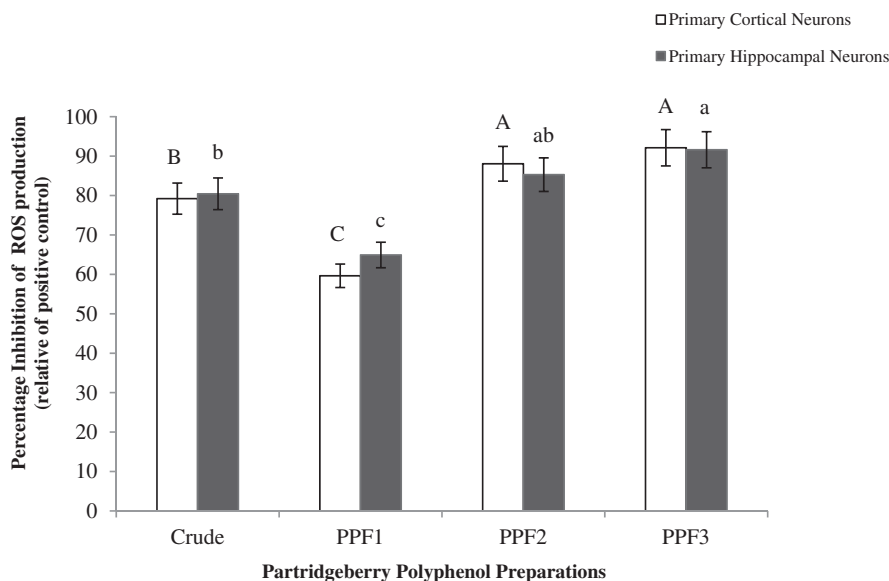

Fig. 3. Percentage inhibition of reactive oxygen species (ROS) in rat primary cortical and hippocampal neurons by following Aβ injury by partridgeberry polyphenol preparations (100 μg mL^{-1}) with respect to assay controls. Rat primary cortical and hippocampal neurons were treated with either 0.1% DMSO (vehicle control), or 100 μg mL^{-1} partridgeberry polyphenol preparations, for 24 h, followed by Aβ injury (50 μM) as described under "Materials and methods". A–C and a–d letters on bars are significantly different ($P \leq 0.05$) as obtained by Tukey's test, are used to indicate statistical significance for treatment affect (ROS inhibition) in rat primary cortical and hippocampal neurons respectively. PPF1: anthocyanin-rich fraction; PPF2: flavan-3-ol-rich fraction; PPF3: flavonol-rich fraction.
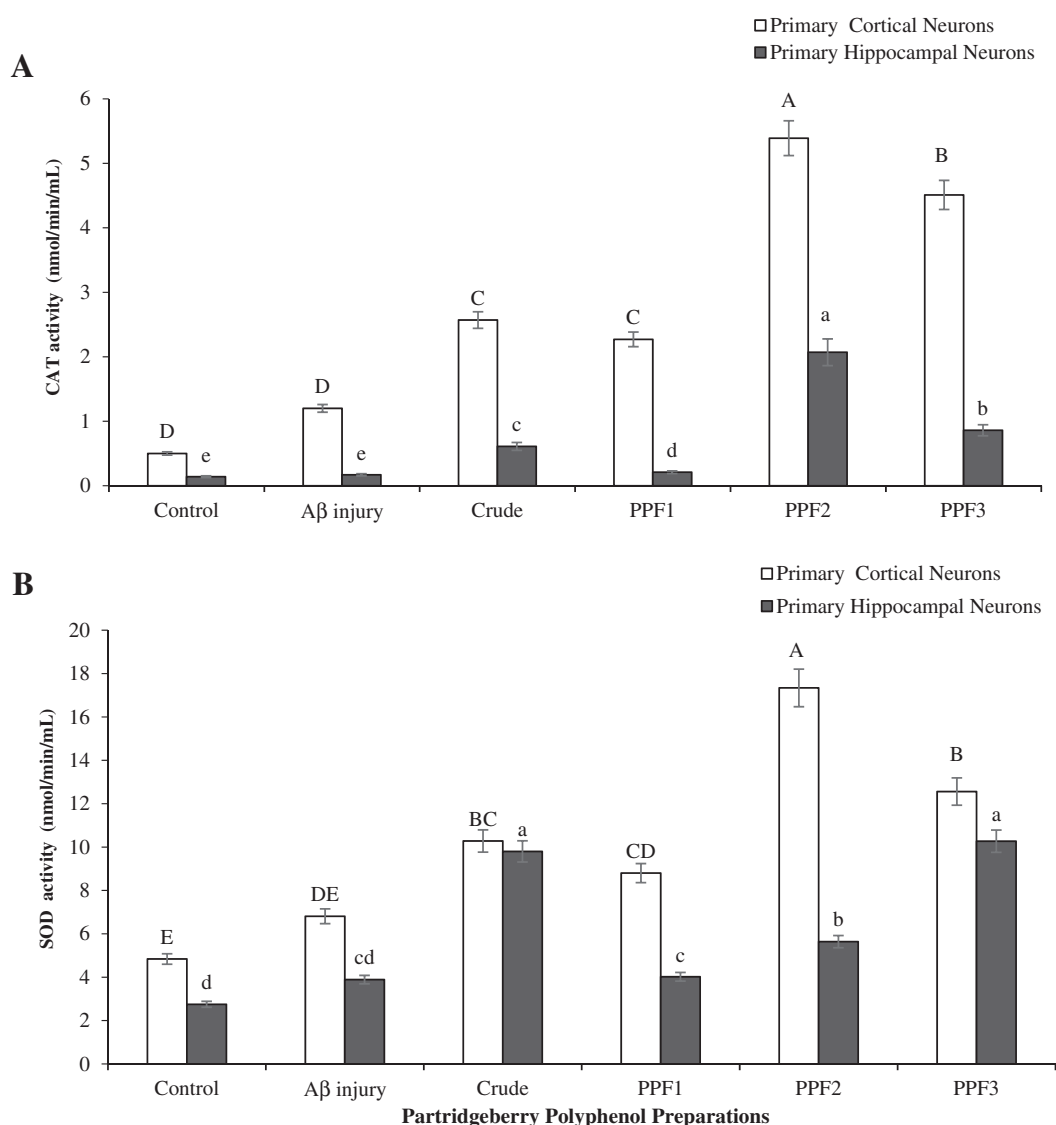
Fig. 4. Activation of superoxide dismutase (SOD) (A) and Catalase (CAT) (B) activity following Aβ injury in relation to pre-exposure to partridgeberry polyphenol preparations (100 μg mL^{-1}) with respect to assay controls. Rat primary cortical and hippocampal neurons were treated with either 0.1% DMSO (vehicle control), or 100 μg mL^{-1} partridgeberry polyphenol preparations, for 24 h, followed by Aβ (50 μM) as described under "Materials and methods". Results are expressed as unit/ml of antioxidant enzymes values in rat primary cortical and hippocampal neurons. The bars with different subscripts are statistically different (P ≤ 0.05), A–E and a–d letters on bars are significantly different (P ≤ 0.05) as obtained by Tukey's test, are used to indicate statistical significance for treatment affect in rat primary cortical and hippocampal neurons respectively. PPF1: anthocyanin-rich fraction; PPF2: flavan-3-ol-rich fraction; PPF3: flavonol-rich fraction. Control: cells not subjected to the Aβ injury. Aβ injury control: the experimental control in which neurons are not treated with any antioxidant agent following the Aβ injury.

(P ≤ 0.05). Similar to SOD results and catalase observations in cortical neurons, the PPF1 exhibited the weakest ability to trigger catalase release in vitro (P ≤ 0.05). Collectively, these results suggest that in PPF2 and PPF3-treated neurons, the possible dismutation and detoxification of superoxide anion and H$_2$O$_2$ produced during Aβ stress, are most likely caused by hyperactivity of SOD and catalase.

3.7. β-Amyloid (1–42) peptide concentration

Next, we studied whether PPFs (100 μg mL^{-1}) affect Aβ peptide levels in rat primary neuronal cells using ELISA method. As the Aβ peptides have a crucial role in the pathogenesis of AD, therefore lowering their concentrations/levels serve as a crucial drug target in AD therapy. Partridgeberry polyphenol preparations were assayed for their ability to attenuate Aβ peptides in rat primary cortical and hippocampal neurons (Fig. 5). The results showed that the treatment of cells with partridgeberry fractions (100 μg mL^{-1}) significantly lowered the concentration

of Aβ peptides in both rat cortical and hippocampal neurons (P ≤ 0.05). The strongest anti-Aβ activity in primary cortical cells was exhibited by PPF3 and PPF2 fractions of partridgeberry (P ≤ 0.05). These class specific polyphenol-rich fractions displayed ~7-fold downregulation of Aβ concentration in vitro (Fig. 5A). Compared to PPF2 and PPF3, both the crude extract and PPF1 displayed the weak attenuation of Aβ peptides in rat primary cortical neurons (P ≤ 0.05). The cell suspensions of primary cortical neurons were also analyzed for the Aβ concentration and results showed very high anti-Aβ activity of partridgeberry fractions in vitro (P ≤ 0.05). The Aβ concentration in the cell suspensions treated with partridgeberry fractions ranged between 19.28–28.31 pg/mL. The strongest anti-Aβ activity was exhibited by PPF3 (P ≤ 0.05), resulting in 15-fold downregulation of Aβ concentration than the untreated control. Following the primary cortical neurons results, the partridgeberry fractions significantly lowered the Aβ concentration (P ≤ 0.05) in the primary hippocampal cells. The strongest anti-Aβ activity in hippocampal cells was exhibited by PPF2 followed
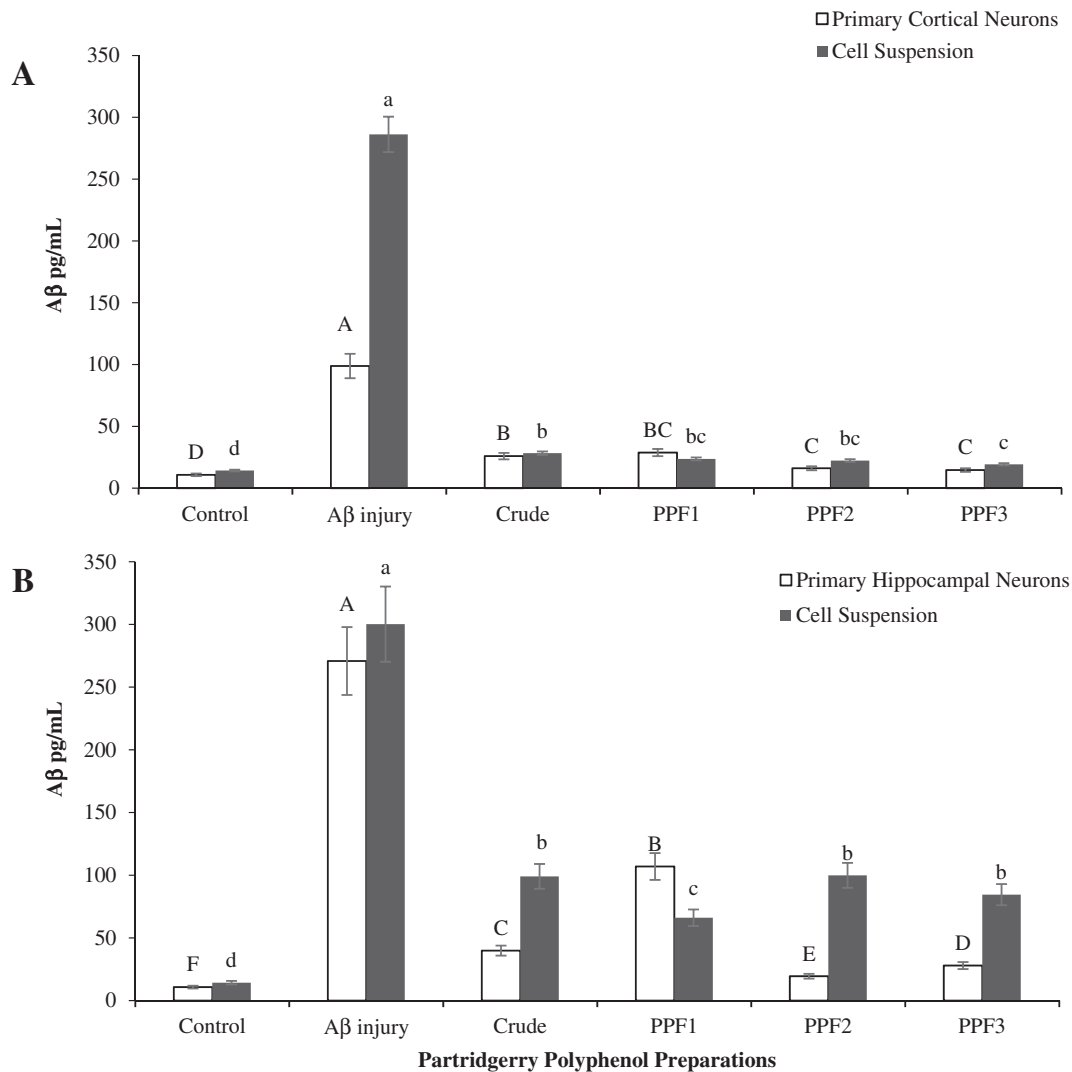
Fig. 5. Quantification of $A\beta_{1-42}$ following $A\beta$ injury by partridgeberry polyphenol preparations ($100~\mu g~mL^{-1}$) in rat primary cortical (A) and hippocampal neurons (B) as measured by ELISA analysis. The bars with different subscripts are statistically different ($P \leq 0.05$), A–F and a–d letters on bars as obtained by Tukey's test ($P \leq 0.05$) are used to indicate statistical significance for treatment affect in rat primary cortical and hippocampal neurons respectively. PPF1: anthocyanin-rich fraction; PPF2: flavan-3-ol-rich fraction; PPF3: flavonol-rich fraction. Control: cells not subjected to the $A\beta$ injury. $A\beta$ injury control: the experimental control in which neurons are not treated with any antioxidant agent following the $A\beta$ injury.

by PPF3 fraction of partridgeberry ($P \leq 0.05$). These fractions displayed 14- and 10-fold downregulation of $A\beta$ concentration in vitro respectively (Fig. 5B). Compared to PPF2 and PPF3, the PPF1 displayed the weakest anti-$A\beta$ activity in rat primary hippocampal neurons ($P \leq 0.05$). The cell suspensions analysis also showed very high anti-$A\beta$ activity of partridgeberry fractions in vitro. The $A\beta$ concentration in the cell suspensions treated with partridgeberry fractions ranged between 66.1–300.2 pg/mL. Interestingly, the PPF1 which displayed the weakest cellular anti-$A\beta$ activity, exhibited the strongest anti-$A\beta$ activity in hippocampal cell suspension followed by the PPF2 and PPF3 ($P \leq 0.05$), possibly due to higher concentration in suspension due to poor cellular uptake.

3.8. DNA fragmentation

A significant hallmark of apoptotic cell death is the fragmentation of genomic DNA into integer multiples of 180-bp units, producing a characteristic DNA ladder during its gel electrophoresis. To examine the cytotoxicity of $A\beta$ to neurons as well as the possible cytoprotective activity of partridgeberry polyphenols against $A\beta$ injury, primary rat cortical and hippocampal neurons were treated with $A\beta$ peptides (50 μM for

24 h), with or without pretreatment of partridgeberry polyphenols followed by DNA fragmentation assays. As shown in Fig. 6, DNA fragmentation was clearly observed following treatment of primary neurons with $A\beta_{1-42}$ peptides. These results demonstrate that primary neurons are sensitive to $A\beta$-induced DNA damage. However, when the neurons were pretreated with partridgeberry polyphenols for 24 h, $A\beta$-induced DNA fragmentation was dramatically inhibited in both primary rat cortical and hippocampal neurons. The cytoprotective effects observed particularly in the case of PPF2 and PPF3, possibly due to higher concentration of known cytoprotective and anti-$A\beta$ compounds such as (−)-epicatechin, catechin, epigallocatechin, isoquercitrin, phlorizin and quercetin. These results suggest that partridgeberry polyphenols inhibit $A\beta$-induced apoptosis in primary rat cortical and hippocampal neurons.

3.9. Western blots

Partridgeberry polyphenols extracts were able to attenuate the $A\beta$-induced apoptosis in primary rat cortical and hippocampal neurons as demonstrated by western blot analysis. The exposure to $A\beta$ peptides triggered the activation of caspase-3, 7 and 9 which prompts the
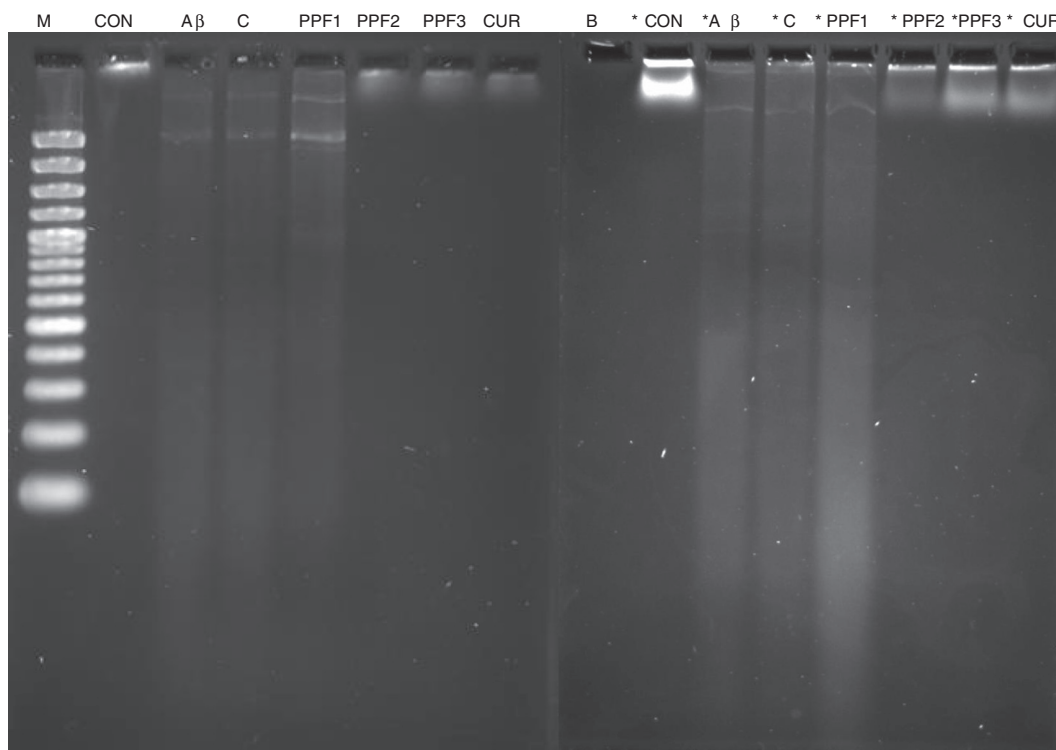
Fig. 6. Effect of partridgeberry polyphenols on the Aβ-sensitized neural DNA damage in primary cortical and hippocampal neurons in vitro. DNA fragmentation analysis of rat primary cortical and hippocampal neurons in vitro (using 2% agarose gel electrophoresis); (M) marker DNA ladder, (CON) control DNA primary cortical neurons (PCN), (Aβ) positive control PCN in cultured neurons, (C) crude extract of partridgeberry, (PPF1–3) partridgeberry polyphenol fractions 1–3, (CUR) curcumin, (B) blank using DNA loading buffer, (*) all the abbreviations with an asterisk represent same treatments as PCN and controls in the primary hippocampal neurons (PHN) sensitized to Aβ-injury in vitro. The primary neurons (PCN and PHN) were incubated with partridgeberry polyphenol preparations (100 μg mL^{-1}) or curcumin (100 μg mL^{-1}) for 24 h prior to Aβ-injury in vitro.

cleavage of poly-(ADP-ribose)-polymerase (PARP), a hallmark of cell death, leading to DNA fragmentation and apoptosis. After treatment of neurons with partridgeberry polyphenols, a decline in caspase-3, 7 and 9 cleavage was observed in both cell types (Fig. 7). Compared to Aβ control (untreated neurons), the anti-apoptotic effect was observed particularly in PPF2 and PPF3-treated neurons while the crude extract and PPF1 remained weak inhibitors of caspase 3,7 and 9 cleavage. The

activation and function of caspases are regulated by various key of molecules, such as inhibitors of apoptosis protein, Bcl-2 protein family. Multiple mitochondrial apoptotic factors such as Bad, Bim and Bax were prompted by Aβ-injury which resulted in cellular apoptosis. Similar to attenuation of caspases, these factors were inhibited by partridgeberry fractions, chiefly by PPF2 and PPF3. Interestingly, the modulation of these apoptotic targets by partridgeberry polyphenols was stronger
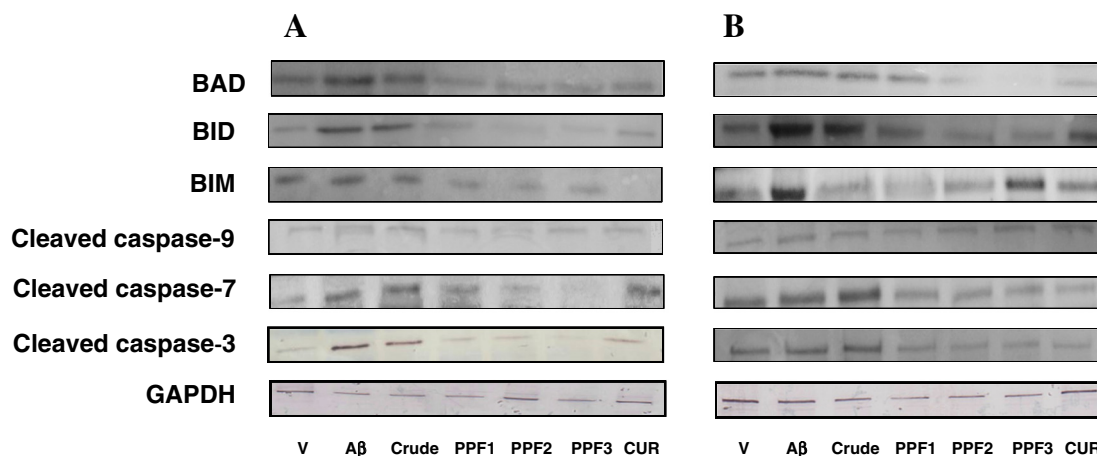

Fig. 7. Partridgeberry polyphenols modulated Aβ-induced cleavage of apoptotic mitochondrial proteins. A, Analysis of apoptotic mitochondrial factors in primary cortical neurons. B, Primary hippocampal neurons. Cells were exposed to partridgeberry polyphenols at the indicated dose (100 μg mL^{-1}) for 24 h prior to Aβ-sensitization (50 μM). Cell lysates were prepared and assayed for the apoptotic targets by western blotting. V: vehicle; Aβ: beta-amyloid control; crude: Aβ + crude extract; PPF1: Aβ + anthocyanin fraction; PPF2: Aβ + flavan-3-ol fraction; PPF3: Aβ + flavonol fraction; CUR: Aβ + curcumin.

than curcumin, a recognized inhibitor of Aβ-induced injury and cellular apoptosis.

3.10. Cellular morphology

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

4. Discussion

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

First, in the present report, an in vitro AD model was established using the primary cortical and hippocampal neurons by employing $A\beta_{(1-42)}$ stress, as the cortex and hippocampus are the key brain components involved in the memory formation and encoding of the spatial information. This model confirmed that Aβ-triggers neuronal death, increases ROS production and initiates membrane damage in neurons. The results indicate that partridgeberry polyphenols are able to protect
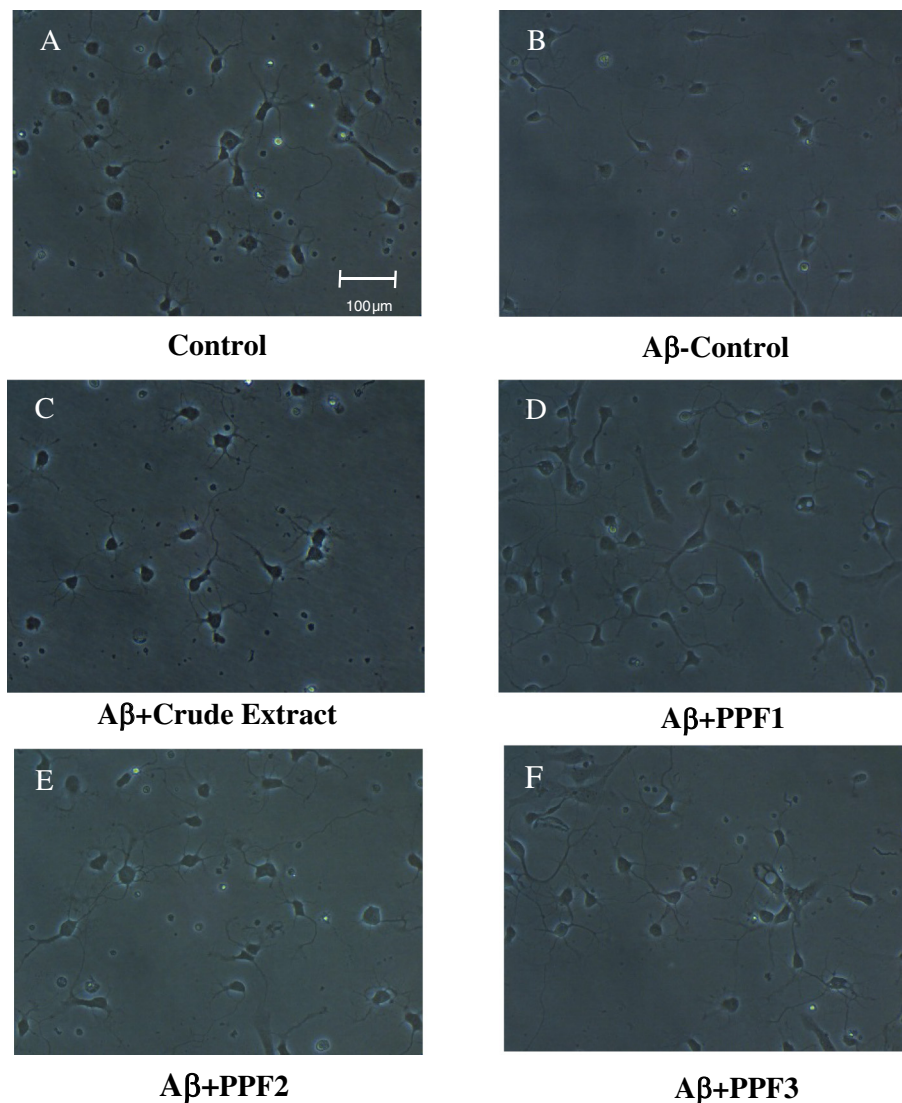

Fig. 8. The morphology of primary cortical neurons treated with partridgeberry polyphenol preparations (100 μg mL^{-1}) or/and $A\beta_{1-42}$ (50 μM) for 24 h (60×). (A) Control cells grown without $A\beta_{1-42}$ stress (B) $A\beta_{1-42}$ induces shrinkage of cell body and causes cell death; partridgeberry fractions prevent $A\beta_{1-42}$ induced damages on cell body and attenuate cell death. (C) Crude extract, (D) anthocyanin fraction, (E) flavan-3-ol fraction, (F) flavonol fraction.
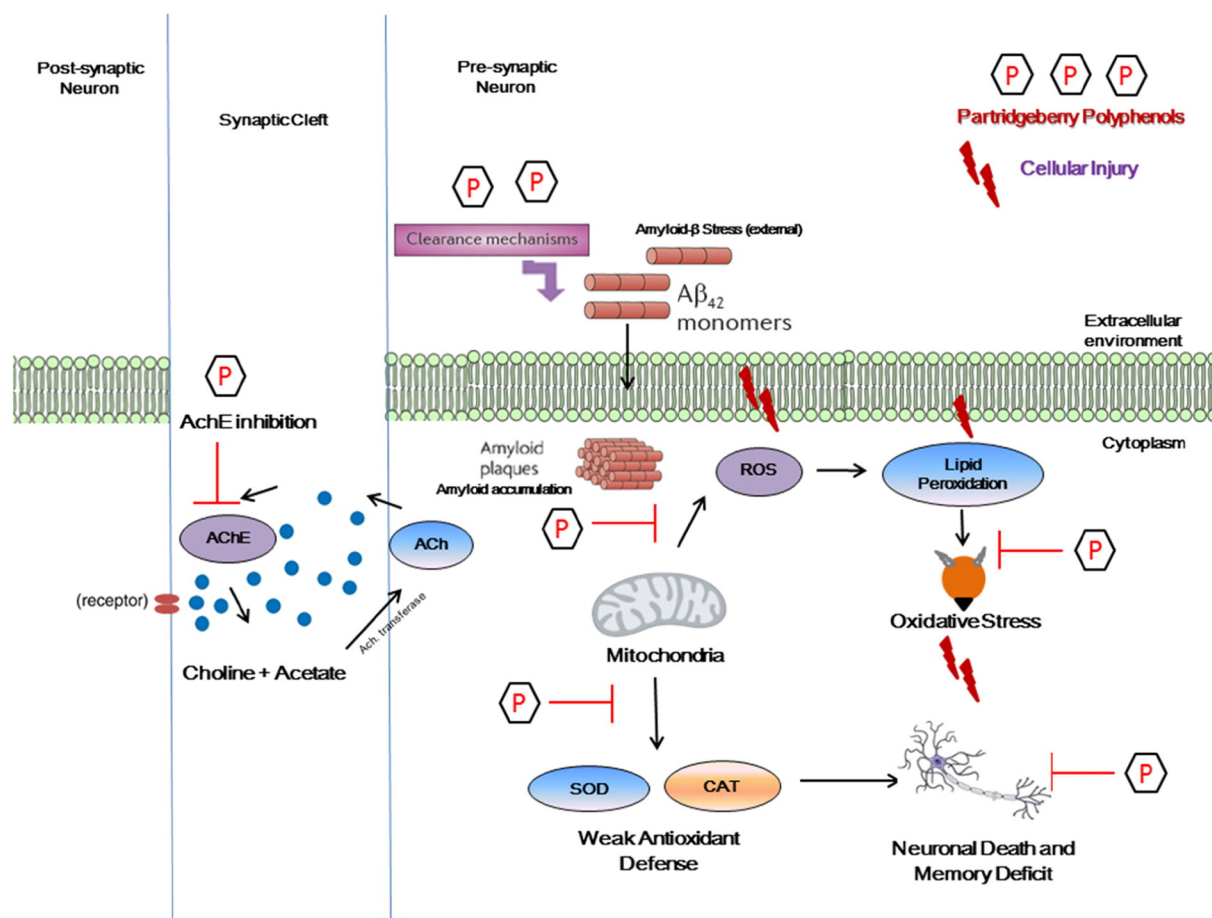
Fig. 9. A schematic diagram of the neuroprotective role of the partridgeberry polyphenols, and induction of superoxide dismutase (SOD) and catalase (CAT) expression in $A\beta_{(1-42)}$ stressed rat cortical and hippocampal neurons. Partridgeberry polyphenols abolished $A\beta_{(1-42)}$ induced reactive oxygen species (ROS), reductions in SOD and CAT activity, decreased $A\beta_{(1-42)}$ concentration and acetylcholinesterase (AchE) activity, leading to significant increase neuronal survival and proliferation.

both the cortical and hippocampal neurons exposed to the Aβ-induced toxicity in vitro. The results demonstrate that partridgeberry polyphenols can inhibit Aβ-mediated neural damage and cell death by attenuating the production of free radicals, and rejuvenating the neural antioxidant defense system. A significant feature of the current study was the strong anti-Aβ activity of partridgeberry polyphenols at low concentrations, depicting their efficacy for the reduction of Aβ pathology in primary neurons, confirming previous reports, which concluded that the polyphenols at low concentration may be useful for the attenuation of AD symptoms and/or progression (Bhullar & Rupasinghe, 2013).

Secondly, the present findings regarding cellular viability and membrane cytotoxicity were similar to the previous studies that show the ability of polyphenols to attenuate Aβ toxicity and exert strong neuroprotective effects. Brewer et al. (2010) found that 125 and 500 μg mL^{-1} of blueberry extract reduced the Aβ neurotoxicity and extended protection against ROS in a cellular model of AD. Similarly, another study found that anthocyanin-rich bilberry (*Vaccinium myrtillus* L.) and blackcurrant (*Ribes nigrum*) at 0.25 to 31 μg mL^{-1} concentrations displayed significant inhibitory effects on soluble $A\beta_{40}$ and $A\beta_{42}$ levels in human SH-SY5Y neuroblastoma cells, and also improved the spatial learning in transgenic mice (Vepsäläinen et al., 2013). A similar report has also shown that cinnamon extract administration at 20–40 μg mL^{-1} was able to rectify the neurotoxicity and neuronal damage in both neuronal PC12 cells and AD fly model (Frydman-Marom et al., 2011). Our research outcomes were also consistent with other reports, where polyphenol-rich extracts, from diverse plant sources such as walnuts (Muthaiyah, Essa, Chauhan, & Chauhan, 2011), turmeric (Shytle

et al., 2012) and green tea (Okello, McDougall, Kumar, & Seal, 2011) attenuated the Aβ-induced cognitive dysfunction in both cell and mouse models. Apart from the crude polyphenol extracts, their polyphenol constituents such as quercetin (Ansari, Abdul, Joshi, Opii, & Butterfield, 2009), catechins (Haque, Hashimoto, Katakura, Hara, & Shido, 2008), and phenolic acids (Harvey, Musgrave, Ohlsson, Fransson, & Smid, 2011) have also demonstrated potent inhibition against β-amyloid induced neurotoxicity. In line to our mechanistic findings, the increased levels of neural SOD and CAT to combat Aβ related oxidative damage has also been shown previously, which indicate the upregulation of Nrf2/ERk1/2 pathway (Hritcu et al., 2014; Zhao et al., 2012). AD is exacerbated by ROS which hamper the subsequent upregulation of neural SOD as a defense/compensatory mechanism against oxidative stress (De Leo et al., 1998). Therefore, one of the interesting findings of this study was the rise in the neural SOD, CAT activity and ROS scavenging following treatment with partridgeberry polyphenols. The antioxidant mechanism of partridgeberry polyphenols can be explained theoretically as a reaction of upregulated SOD with elevated superoxides (due to Aβ) to form diatomic oxygen and hydrogen peroxide, which is further converted to water by CAT (Yan et al., 2013). As mitochondrial dysfunction is implicated in AD, our findings showing the simultaneous upregulation of SOD and CAT by partridgeberry polyphenols presents itself as a unique therapeutic option to attenuate the oxidative pathology of AD. Thirdly, our ELISA and Aβ-fibril formation assay show that PPFs can inhibit aggregation or promote its disaggregation at low concentrations (100 μg mL^{-1}). The $A\beta_{1-42}$, a key toxic amyloidogenic form of Aβ formed fewer fibrils (as observed fluorometrically) in the presence of PPFs, whereas increased incubation of PPFs promoted clearance of Aβ-

peptides in both cell types and suspensions. Microscopic evidence, illustrating fewer non-viable neurons in PPF-treated samples, supports cytoprotective, anti-Aβ aggregation ability of PPFs, while ELISA data with a completely different method confirmed the findings (Fig. 6).

Furthermore, DNA fragmentation analysis indicated that apoptosis occurred following the Aβ injury in both primary cortical and hippocampal neurons. Based on the initial test, we demonstrated the occurrence of neural apoptosis at 12 h after administering Aβ insult by gel analysis. This assay showed that strong apoptotic positive signals were also observed in the neurons treated with the crude extract and the anthocyanin rich extract. However, the flavan-3-ol and flavonol-rich fractions of partridgeberry subdued the DNA damage. Similar results of cytoprotection against Aβ induced DNA damage have been shown by grape seed polyphenols, puerarin, and curcumin (Thomas et al., 2009; Zhang, Liu, Wang, Xu, & Hu, 2008). Western blots also unveiled the mechanistic insights into the cytoprotective action of partridgeberry polyphenols. Caspase proteinases drive apoptotic signaling and execution by cleaving critical cellular proteins solely after aspartate residues. Once activated under stress conditions, initiator caspases such as caspase 9 in turn activate the executioner caspases-3, -6, and -7 resulting in apoptosis (McIlwain, Berger, & Mak, 2013). Caspase-3 triggers early synaptic dysfunction in AD is the major executioner protease involved neural toxicity by cleaving tau protein (D'Amelio et al., 2011). Likewise, caspase-7 is also involved in the long-term overproduction of Aβ and other potential toxic manifestations in AD (Zhang, Goodyer, & LeBlanc, 2000). The inhibition of these caspases in neurons by partridgeberry polyphenols certainly augments their neuroprotective activity. Additionally, the Bcl-2 is a member of a superfamily of proteins, of which its members such as Bcl-2 is a member of a superfamily of proteins, some of which including Bim, Bad, and Bid have pro-apoptotic functions (Häcker & Vaux, 1995; Ren et al., 2010). Correspondingly, the modulation of Bcl-2 members presents a compensatory response to protect the neurons from apoptosis by maintenance of their survival (Akhtar, Ness, & Roth, 2004). Our results showed that the partridgeberry polyphenols, particularly PPF2 and PPF3 modulated the caspases and Bcl2 family members towards neuroprotection. Similar results have been shown in earlier studies where polyphenols successfully attenuated the hyperactive caspases and Bcl-2 superfamily of proteins towards neuroprotection against Aβ toxicity (Bhullar & Rupasinghe, 2013).

Finally, the restoration of cholinergic activity through AChE inhibition is also a crucial strategy of pharmacotherapy in AD, as the principal function of AChE is to terminate the nerve impulse flow in the synapse by hydrolyzing acetylcholine. The overexpression of AChE is observed in the AD brain, which causes impaired memory and learning. Drugs from plant sources such as galantamine and taurine, have been widely used for improving brain function through AChE inhibition, but no study has explored the anticholinesterase activity of partridgeberry polyphenols. Our results show that the flavonol-rich fraction (PPF2) from partridgeberry inhibited the AChE in vitro, in a dose-dependent manner. On the other hand, the crude berry extract and other fractions indicated weak AChE inhibitory activity in vitro. Conversely, all the partridgeberry polyphenol preparations, including the crude extract exhibited very weak BChE inhibition activity in vitro. As AChE is abundant in the blood and in neural synapses as compared to BuChE, therefore, the strong inhibition of the former cholinesterase by PPFs advocates the prospective use of partridgeberry polyphenols in attenuation and/or prevention of cholinergic deficit in AD. These findings are in accordance with the previous reports which indicated a strong anticholinesterase potential of polyphenol-rich Vaccinium and other fruits. For example, Papandreou et al. (2009) showed that wild blueberry (Vaccinium angustifolium) extract, abundant in polyphenols, improved the brain antioxidant status and displayed inhibition of AChE activity in adult mice. Similarly, polyphenols of rabbiteye blueberry (Vaccinium virgatum) also inhibited ROS mediated neuronal death and exhibited strong AChE inhibition in in vivo experiments (Jeong et al., 2013).

Overall, this study suggests that polyphenol-rich partridgeberry fractions, especially PPF2 and PPF3 at 0.01–8.9 µg mL^{-1} concentrations, exerted a protective effect on Aβ$_{(1–42)}$ induced toxicity in cortical and hippocampal neurons in vitro. These fractions, abundant in flavan-3-ols and flavonols respectively, exhibited the highest neuroprotection, possibly through higher cellular uptake than the crude and anthocyanin-rich fractions which are abundant in non-phenolics, especially sugars. However, the metabolic breakdown of flavan-3-ols and flavonols, during in vivo digestion raises questions regarding their efficacy (at low concentrations in plasma) for clinical use. As polyphenols, especially flavan-3-ols and flavonols, undergo extensive metabolism and biotransformation, the pharmacokinetic evidence suggests that these extracts/fractions may only attain very low-micromolar plasma levels in animal or human subjects following oral administration (Landete, 2012). The substantial amounts of polyphenols that enter the large intestine and are subjected to extensive metabolism by the intestine microflora and the metabolites are then re-absorbed into the bloodstream before excretion (Possemiers, Bolca, Verstraete, & Heyerick, 2011). Various bioavailability studies in mice show that the bulk of the polyphenolic constituents were excreted, but small amounts of metabolites reach the brain. Interestingly, these low traces of polyphenols make their way through the blood brain barrier and exhibit neuroprotective effects through multiple mechanisms (Faria et al., 2010). The re-absorption of metabolized polyphenols explains the neuroprotective potential of polyphenols, despite their low bioavailability following extensive metabolism (Landete, 2012). Authors also suggest that the synergistic effects of the absorbed intact polyphenols and their colon metabolites might present an enhanced neuroprotective effect at low concentrations. In light of these facts, an in vivo study using mice model to further explore the bioavailability and the efficacy of polyphenol-rich partridgeberry extracts for neuroprotection in AD is under development. Taken together, the results from the current report provide a plausible antioxidant mechanism by which partridgeberry polyphenols prevent cell death of cortical and hippocampal neurons following Aβ insult.

5. Conclusions

In the current study, we explored the neuroprotective potential of partridgeberry against Aβ-induced neurotoxicity. Partridgeberry polyphenols protected both primary cortical and hippocampal neurons from Aβ$_{(1–42)}$-induced neurotoxicity, and inhibited the cell death. The polyphenol-rich fractions, particularly flavan-3-ol- and flavonol-rich fractions, prevented the LDH leakage and maintained the redox homeostasis. Furthermore, partridgeberry polyphenols induced the elevation of intracellular antioxidant enzymes, SOD and CAT. Especially, all polyphenol-rich fractions suppressed the ROS, and hence attenuated the manifestation of oxidative stress, which significantly contributes to the AD pathology. Our data further demonstrated that the flavan-3-ol- and flavonol-rich fractions of partridgeberry were capable of lowering the concentration of Aβ$_{(1–42)}$ in both neurons and their cell suspensions. Furthermore, the restoration of apoptotic caspase and Bcl2 family signaling indicates the attenuation of cellular damage induced by Aβ sensitization. The partridgeberry polyphenols are expected to exhibit neuroprotection in vivo by the direct effects of the absorbed parent compounds or their metabolites, which may cross the blood brain barrier. The results from the current study warrant further exploration of the partridgeberry polyphenols for treatment of AD and suggest that the antioxidant supplementation may represent a therapeutic potential to pursue AD treatment and/or prevention, particularly in view of the multiple mechanisms by which partridgeberry polyphenols exert anti-Aβ effects.

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