Food Chemistry 168 (2015) 595–605

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Antioxidant and cytoprotective properties of partridgeberry polyphenols

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ARTICLE INFO

Article history:
Received 25 May 2014
Received in revised form 19 July 2014
Accepted 21 July 2014
Available online 30 July 2014

Chemical compounds studied in this article:
(−)-Epicatechin (PubChem CID: 72276)
Caffeic acid (PubChem CID: 689043)
Catechin (PubChem CID: 9064)
Chlorogenic acid (PubChem CID: 1794427)
Epigallocatechin (PubChem CID: 72277)
Ferulic acid (PubChem CID: 445858)
Isoquercitrin (PubChem CID: 25203368)
Phloretin (PubChem CID: 4788)
Phlorhizin (PubChem CID: 6072)
Quercetin (PubChem CID: 5280343)

Keywords:
Partridgeberry
Lingonberry
Vaccinium vitis-idaea
Antioxidant
Polyphenol
Flavonoids
Fractionation
Cytoprotection
Nrf2

ABSTRACT

Partridgeberry (*Vaccinium vitis-idaea*) is a polyphenol-rich berry of the *Ericaceous* family, grown in Newfoundland and Labrador province of Canada. The aims of this study were to identify extraction solvents for the maximum recovery of polyphenols, to establish fractionation technique for isolation of major subclasses of polyphenols, and to evaluate antioxidant and cytoprotective properties of the partridgeberry polyphenol preparations. The acidified 70% acetone was identified as the ideal solvent for the maximum recovery of polyphenols from partridgeberry. Further, aqueous two-phase extraction, column chromatography and UPLC–MS/MS were employed to produce three partridgeberry polyphenol fractions, rich in either, anthocyanins, flavan-3-ols or flavonols. All the three PPF were potent antioxidants and displayed cytoprotective activity through the activation of nuclear factor erythroid 2-related factor 2 pathway, scavenging of reactive oxygen species, and inhibition of cellular death. The current study suggests that partridgeberry has numerous potential health implications in both prevention and amelioration of various diseases involving oxidative stress.

1. Introduction

Continuous production of reactive oxygen species (ROS) by mitochondrial electron transport chain causes damage to mitochondria and important biomolecules as well as initiates pathological processes in human body (Circu & Aw, 2010). The ROS including superoxide anion radical ($O_2^{-\cdot}$), hydroxyl radical ($OH^{\cdot}$), the hydroperoxyl radical ($HO_2^{\cdot}$) 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 the cellular damage and accelerated ageing (Circu & Aw, 2010; Ray et al., 2012). The oxidative stress and continuous ROS production also trigger the activation of cell signalling pathways contributing to the 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 (Wang, Camp, & Ehlenfeldt, 2012). There are over 25,000 different types of polyphenols, divided into many sub-classes including phenolic acids, flavonoids, stilbenes and lignans (Wang et al., 2012). Since the polyphenols are of diverse structures, a highly specific analytical technique is required. One such technique is UPLC–ESI–MS/MS. The concentration of individual plant phenolics can be precisely quantified using UPLC–ESI–MS/MS (Sekhon-Loodu,

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http://dx.doi.org/10.1016/j.foodchem.2014.07.103
0308-8146/

Warnakulasuriya, Rupasinghe, & Shahidi, 2013). UPLC is an advance and rapid analyte separation technique which requires short analysis time, low solvent consumption and exhibits higher resolution in comparison to HPLC analysis (Nováková, Matysová, & Solich, 2006). The electrospray ionisation mass spectrometry (ESI–MS) technique further facilitates analyte identification and characterisation of phenolic aglycones and glycosides from variety of plant samples such as fruit extracts (Häkkinen & Auriola, 1998; Sekhon-Loodu et al., 2013).

Epidemiological studies associated with disease risk have 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, anticancer and antimicrobial activities (Su, 2012; Wang et al., 2012). *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 be related to its polyphenol constituents (Heinonen, 2007). Partridgeberry is a low (2–12 cm) evergreen *ericaceous* shrub found throughout Newfoundland and Labrador province 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 detailed phenolic profile, antioxidant potential and cytoprotective ability of partridgeberry grown in Canada. In the current study, we investigated the optimum extraction methods for polyphenols, their concentrations along with the *in vitro* antioxidant and cytoprotective properties of partridgeberry found in southern Labrador region of Canada.

2. Materials and methods

2.1. Plant materials

Fruit of partridgeberry (*V. vitis-idaea* L. var. minus Lodd) harvested during 2012 from southern Labrador area (N 51°43′, W 56°26′), was provided by the Department of Natural Resources of the Government of Newfoundland and Labrador, Canada.

2.2. Chemicals and reagents

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, USA). 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Walco Chemical Products Co Inc., (Buffalo, NY, USA). Formic acid, DMSO, 6-well and 96-well microplates were purchased from Fisher Scientific (Ottawa, ON, Canada). Sterile 96- and 6-well 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 kit was obtained from Promega (Madison, WI, USA).

2.3. Extraction and fractionation

2.3.1. Initial extraction and solvent system selection

The partridgeberry fruits used in extraction were stored in −20 °C freezer until extraction and further analysis. The frozen berries were removed from the freezer and thawed at the 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. Eleven solvent types (**1**–**11**), differing in their polarity were used for the initial extraction and solvent selection. Various analytical parameters were assessed with the aim of selecting the best solvent resulting in maximum recovery of a wide range of polyphenols from partridgeberry. The solvents selected based on literature for optimizations of polyphenol extraction were: 100% methanol, methanol:water (70:30 v/v), methanol:water:formic acid (70:28:2 v/v/v), 100% ethanol, ethanol:water (70:30 v/v), ethanol:water:formic acid (70:28:2 v/v/v), 100% acetone, acetone:water (70:30 v/v), acetone:water:formic acid (70:28:2 v/v/v), ethyl acetate:water:formic acid (80:18:2 v/v/v), 100% water. Ten grams 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, USA) and then sonicated in an ultrasonication bath (VWR 750D, VWR International, Mississauga, ON, Canada) in three cycles of 15 min at 28 °C with 15 min intervals between each cycle. Following sonication, berry extracts were centrifuged for 10 min at 315 rad/s 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 the experimental procedures below.

2.3.2. Large scale extraction and fractionation

The large scale extraction and fractionation was performed after selecting the optimum extraction solvent. Frozen berries (500 g) were extracted with 2 L of solvent (acetone:water:formic acid, 70:28:2 v:v:v) using a blender (Black and Decker Type I, Applica Consumer products, Inc Miramar, FL, USA) and then sonicated in an ultrasonication bath (VWR 750D, VWR International, Mississauga, ON, Canada) in three cycles of 15 min at 28 °C. Following the sonication, berry extracts were centrifuged for 10 min at 315 rad/s using a centrifuge (Sorvall ST16, Thermo Scientific, Hamburg, Germany). The extracts were collected in a round bottom flask and dried using a rotary evaporator (Buchi R-200, Buchi Corporation, New Castle, DE, USA) and then reconstituted to 500 mL of ethyl acetate and water solution (50:50, v:v). The ethyl acetate and water solution was then subjected to aqueous two-phase liquid–liquid separation using a 1 L separatory funnel. The berry extract in separatory funnel was allowed to stand for 24 h as polyphenols separated into two layers based on their polarity. The upper ethyl acetate layer and lower aqueous layer were carefully removed into different flasks by opening the PTFE stopcock. At the interface between two layers of polyphenol solutions, the stopcock of separatory funnel was carefully closed to prevent mixing of the layers. The separated layers were 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, USA). The upper organic layer was dried and reconstituted in 50 mL of ethyl alcohol:water mixture (50:50 v:v) and aqueous layer in 50 mL of ultrapure water to produce anthocyanin-rich fraction (PPF1). The purified organic layer in 50% ethanol was used for further fractionation in 400 g C_{18} adsorbent resin (Sorbent SP-207-05 sepabead resin brominated, Sorbent Tech., Norcross, GA, USA) 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 ultrapure water to remove sugars, until the sugar percentage reached less than 1%, measured using a digital refractometer (Model No. 300016, Sper Scientific, Scottsdale, AZ, USA). The phenolic compounds were fractionated using a step-wise gradient of varying concentration of aqueous ethanol. The polyphenols-rich fractions (F1–F15) were obtained using 500 mL of 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 90% and 100% ethanol. All the fractions were collected in amber bottles and stored at 4 °C till further analysis. The extraction and fractionation procedures were repeated 3 times to collect sufficient amounts of phenolic fractions for biochemical assays and the fractions were characterised using ultra-performance liquid chromatography electrospray ionisation tandem mass spectrometry (UPLC–ESI–MS/MS).

2.4. UPLC–ESI–MS/MS analysis

UPLC–ESI–MS/MS analysis was conducted using the methodology and parameters described by Sekhon-Loodu et al. (2013). Phenolic compounds present in partridgeberry samples were separated using the UPLC system (Model Waters Aquity CHA, Waters Corp, Milford, MA, USA) equipped with an Aquity BEH C_{18} (100 mm × 2.1 mm, 1.7 μM) column and C_{18} guard column (Waters Corp, Milford, MA, USA). The flow rate of the UPLC system was maintained at 300 μL min^{-1} with a total 12 min 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*-galactoside, 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 ionisation (ESI), in negative ion mode (ESI−), was used for the analysis of the flavonols, flavan-3-ol, and phenolic acid compounds. ESI in positive ion mode was used for anthocyanins. Mass spectrometry conditions used for the analysis were as follows: capillary voltage 3000 V, nebulizing gas (N_2) at a temperature of 375 °C at a flow rate of 0.35 mL min^{-1}. The cone voltage (25–50 V) was optimised 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.

2.5. Preparation of partridgeberry polyphenol fractions

After selection of the ideal solvent for the highest recovery of phenolics from partridgeberry, the extracts were subjected to various separation and chromatography techniques for the extraction of different classes of polyphenols. The detailed methodology as shown in Supplementary data and described in Section 3.2 and 3.2.1 was used to prepare partridgeberry polyphenol fractions (PPF) rich in three different sub-classes of polyphenols.

2.6. Determination of polyphenols

2.6.1. Total phenolic content (TPC)

TPC was measured using the Folin Ciocalteu assay as outlined by Singleton, Orthofer, and Lamuela-Raventos (1999) and with some modifications as described by Rupasinghe, Erkan, and Yasmin (2010). The phenolic content of berry extracts was expressed as gallic acid equivalents per 100 g fresh weight (FW) whilst TPC of PPFs was expressed as gallic acid equivalents per g dry weight (DW).

2.6.2. Total flavonoid content (TFC)

TFC was measured using the aluminium chloride colorimetric method as outlined by Chang, Yang, Wen, and Chern (2002). The total content of berries was expressed as quercetin equivalents per 100 g FW whilst TFC of PPFs was expressed as quercetin equivalents per g DW.

2.6.3. Total proanthocyanidin content (TPC)

An improved 4-dimethylaminocinnamaldehyde (DMAC) colorimetric method as described by Prior et al. (2010) was used with slight modifications to measure total proanthocyanidins (PAC) in berry extracts. Ethanol was acidified by adding 6 N 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 analyse samples. Catechin at 20, 100, 250, 500 and 750 μM concentrations was used as standard instead of procyanidin A2. The results were expressed as μmole catechin equivalents per 100 g FW whilst that of PPFs was expressed as catechin equivalents per g DW.

2.7. In vitro antioxidant capacity assays

2.7.1. The ferric reducing ability of plasma (FRAP) assay

FRAP assay was performed as described by Benzie and Strain (1996). Briefly, the FRAP working reagent was prepared by adding 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution and 20 mM ferric chloride in the ratio of 10:1:1. The test extracts (20 μL) and FRAP working reagent (180 μL) were added to 96-well microplates and absorbance was read at 590 nm using the FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA).

2.7.2. The oxygen radical absorption capacity (ORAC) assay

ORAC assay was performed according to previously described method by (Cao, Alessio, & Cutler, 1993) as modified for a high through-put microplate reader (Huang, Ou, Hampsch-Woodil, Flanagan, & Prior, 2002). Briefly, 35 μL of Trolox standard or test extract, 130 μL of fluorescein (0.957 μM) and 35 μL of AAPH (150 mM) were added to the black 96-well plates and pre-incubated at 37 °C for 10 min. The assay controls included a positive control with 140 μL fluorescein (0.96 μM) and 60 μL of phosphate buffer (75 mM, pH 7) along a buffer control with 200 μL of phosphate buffer. A negative control comprised of 130 μL fluorescein, 35 μL of AAPH and 35 μL of phosphate buffer. The fluorescence signal was read at excitation and emission wavelength of 485 nm and 510 nm using the FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA).

2.7.3. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

DPPH assay was performed as described by Sharma and Bhat (2009) and results were expressed as IC_{50} values (inhibitory concentration of berry fractions decreasing the absorbance of DPPH solution by 50%). Briefly, the test extracts were challenged against 0.2 mM DPPH solutions in 1:1 ratio in 96 well plates. Upon addition of both test extracts and DPPH solution, the plates were incubated at 25 °C for 10 min. The DPPH percentage inhibition was

calculated from the following equation after absorbance was measured using FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA) at 520 nm.

$$\%\ \text{DPPH Inhibition} = \frac{[\text{Abs}_{(\text{control})} - \text{Abs}_{(\text{sample})}]}{\text{Abs}_{(\text{control})}} \times 100$$

2.8. Cell culture

Human fibroblasts (WI-38 cells, ATCC® CCL-75™) were obtained from Cederlane Labs (Burlington, ON, Canada) and cultured according to the supplier's instructions. Briefly, the cells were maintained in 75 cm^2 culture flasks at temperature of 37 °C in a humidified incubator (VWR International, Mississauga, ON, Canada) supplied with 95% air and 5% CO_2. WI-38 cells were supplied with DMEM medium, supplemented with 10% FBS and 100 mg/L of penicillin and streptomycin. The growth medium of cultured cells was changed every 48 h.

2.9. Cytoprotective assays

2.9.1. Peroxyl radical-induced cellular stress model

For the peroxyl radical-induced cellular stress model, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH; peroxyl radical generator) based cellular model for oxidative stress was used as outlined by He et al. (2013). 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 polyphenols, the fibroblasts (WI-38 cells) were incubated with partridgeberry polyphenol extracts before inducing oxidative insult using peroxyl radical generated by AAPH. The effects of oxidative stress were analysed 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.

2.9.2. Cell viability assay

The cell viability assay was performed using the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA). 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, Canada). Cells were pretreated with the crude extract and three partridgeberry polyphenol fractions separately 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 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt solution (MTS) into each well of the plate containing fibroblasts in culture medium. The plate was incubated for 2 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, Durham, NC, USA).

2.9.3. LDH release assay

Cytotoxicity analysis, based on LDH release was conducted using the CytoTox-ONE™ homogeneous membrane integrity assay (Promega Corporation, Madison, WI, USA). Briefly, 2×10^4 WI-38 cells were seeded using 100 μL media in a 96-well tissue culture plate. Cells were pretreated with the crude extract and three partridgeberry fractions for 24 h and oxidative stress was induced using 30 mM AAPH for 24 h. Plates were removed from 37 °C incubator and placed at room temperature for 30 min. 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, Durham, NC, USA) at wavelength of 590 nm.

2.9.4. Reactive oxygen species (ROS) analysis

ROS were analysed using methodology outlined by Wang and Joseph (1999). Briefly, WI-38 cells were seeded in a 24-well sterile plate at density of 2×10^5 cells per well. Cells were allowed to adhere for 24 h 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 min. After 5 min, 30 mM AAPH was added to the wells of 96-well plate at temperature of 37 °C. 96-well plates were immediately placed in plate reader (BMG Labtech, Durham, NC, USA) and fluorescence was read at an excitation wavelength of 490 nm and an emission wavelength of 510 nm. Fluorescence was measured at interval of 60 min (initial reading at 0 min and final reading at 60 min) and ROS were estimated as percentage inhibition of fluorescein with respect to an assay control.

2.9.5. Protein content

Total protein content was determined in the cells using the Bradford assay (Bio-Rad Protein Assay kit). Assay was performed according to the manufacturer's instructions (Bio-Rad, Mississauga, ON, Canada). 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, Durham, NC, USA).

2.9.6. Peroxide detection assay

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

2.10. Nuclear factor erythroid 2-related factor 2 (Nrf2) ELISA

Nrf2 ELISA was performed using a commercially available kit (MyBioSource, Inc., San Diego, CA, USA) and 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 the crude extract and three partridgeberry fractions for 24 h and subjected to AAPH-induced oxidative stress for 12 h. 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 s. Cellular debris was removed by centrifuging cells at 419 rad/s and cell lysate was immediately used to perform the experiment.

2.11. Statistical analysis

Completely randomised 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 $p \leqslant 0.05$ confidence interval using the statistical analysis system (SAS Institute, Cary, NC).

3. Results

3.1. Initial extraction and solvent selection

3.1.1. Total phenolic content

Total phenolic content as estimated by Folin–Ciocalteu assay indicated the highest total phenolics in solvent **10** of ethyl acetate:water:formic acid (80:18:2 v/v/v) extraction method [25 μM GAE/g fresh weight (FW)] followed by other extraction solvents ($p \leqslant 0.05$) (Table 1). The lowest total phenolics were obtained through anhydrous methanol (**1**) and water (**11**) assisted extraction procedures ($p \leqslant 0.05$). However, all other extraction solvents (**2**–**9**) yielded similar concentration of phenolics and were statistically similar in their total phenolic content ($p \leqslant 0.05$). Overall, the extraction using solvents with lower dielectric constant lead to higher yield of total phenolics.

3.1.2. Total flavonoid content

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

3.1.3. Total anthocyanin content

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 (80:18:2)] whilst solvent **11** was the 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 partridgeberry.

3.1.4. Total proanthocyanidin content

The results shown in Table 1 indicate significant difference ($p \leqslant 0.05$) in total proanthocyanidins contents. Solvent **9** gave the highest proanthocyanidin content when compared with other solvents ($p \leqslant 0.05$), followed by solvent **1**, solvent **4** and solvent **3** whilst solvents **10** and **11** exhibited the lowest amount of extracted total proanthocyanidins in partridgeberry extracts. Similar to the earlier results, solvent **11** with the highest dielectric constant resulted in poor extraction of total proanthocyanidins from partridgeberry.

3.1.5. Ferric reducing antioxidant power (FRAP)

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

3.1.6. Selection of solvent system

In the current study, 11 different solvent types were used to identify a solvent for extracting a wide range of polyphenols from partridgeberry. All berry extracts were rich in polyphenols but the concentration of polyphenols was significantly dependent on the solvent polarity and its acidification. An increase in the percentage of ethanol or methanol had no strong influence on extraction efficacy (Table 1). 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. Amongst compared solvent systems, acidified hydrous acetone (**9**) provided the greatest recovery of total phenolics, flavonoid, proanthocyanidins and exhibited the strongest antioxidant activity. The only limiting factor in the solvent **9** assisted polyphenol extraction was the lower content of anthocyanins compared to solvent **10**. However, this limitation can be overlooked due to poor efficacy of solvent **10** in extraction of total flavonoids, proanthocyanidins along with weak antioxidant activity. These results may indicate that 70% acetone acidified with 2% formic acid is more appropriate to be used for extraction of phenolic compounds from partridgeberry. However, water (**11**) is the least effective solvent for extracting the phenolic compounds despite its safety as solvent for human consumption.

3.2. Preparation of partridgeberry polyphenol fractions

The first partridgeberry polyphenol fraction (PPF1) was obtained from aqueous partition (B) as shown in Supplementary data. The PPF1, rich in anthocyanins, was collected from separatory funnel and freeze dried in similar fashion as the crude extract. The

Table 1
Polyphenols and antioxidant capacity of various partridgeberry extracts as determined by spectrophotometric methods.

Solvent	Total phenolics (μmole GAE/100 g FW)	Total flavonoids (μmole QE/100 g FW)	Total anthocyanins (μmole C3GE/100 g FW)	Total proanthocyanidins (μmole CE/100 g FW)	FRAP (μmole TE/100 g FW)
Methanol (**1**)	21.4 ± 1.0^{b}	11.2 ± 0.2^{e}	111.5 ± 0.3^{b}	4.68 ± 0.1^{b}	8.1 ± 0.2^{a,b,c}
Methanol–water (70:30) (**2**)	22.7 ± 0.1^{ab}	13.9 ± 0.8^{d}	106.4 ± 0.3^{bcd}	3.81 ± 0.0^{cd}	7.3 ± 0.4^{cde}
Methanol–water–FA (70:28:2) (**3**)	23.9 ± 1.4^{ab}	12.9 ± 0.3^{d}	61.9 ± 0.1^{cde}	4.50 ± 0.1^{bc}	7.9 ± 0.1^{bcd}
Ethanol (**4**)	22.8 ± 0.7^{ab}	13.3 ± 1.2^{d}	107.6 ± 0.3^{bc}	4.81 ± 0.1^{b}	6.4 ± 0.5^{f}
Ethanol–water (70:30) (**5**)	22.8 ± 0.5^{ab}	18.4 ± 0.3^{abc}	77.9 ± 0.6^{fg}	3.35 ± 0.1^{d}	8.3 ± 0.3^{ab}
Ethanol–water–FA (70:28:2) (**6**)	22.5 ± 0.6^{ab}	19.8 ± 0.3^{a}	94.4 ± 0.4^{de}	3.31 ± 0.2^{d}	7.1 ± 0.4^{def}
Acetone (**7**)	23.7 ± 0.7^{ab}	17.1 ± 0.6^{c}	85.2 ± 0.2^{ef}	3.18 ± 0.6^{d}	7.4 ± 0.4^{cde}
Acetone–water (70:30) (**8**)	21.8 ± 2.5^{ab}	17.9 ± 0.5^{bc}	85.2 ± 0.2^{ef}	3.56 ± 0.5^{d}	6.9 ± 0.4^{ef}
Acetone–water–FA (70:28:2) (**9**)	24.0 ± 1.6^{ab}	19.3 ± 0.4^{ab}	87.3 ± 0.4^{ef}	5.90 ± 0.3^{a}	8.9 ± 0.4^{a}
EA–water–FA (80:18:2) (**10**)	25.0 ± 0.7^{a}	7.9 ± 0.6^{f}	165.4 ± 0.8^{a}	2.19 ± 0.1^{e}	6.5 ± 0.3^{f}
Water (**11**)	22.3 ± 0.8^{ab}	10.7 ± 0.3^{e}	70.8 ± 0.6^{g}	2.17 ± 0.2^{e}	6.2 ± 0.4^{f}

FA, formic acid; EA, ethyl acetate; FW, fresh weight; μmole, micromolar; GAE: gallic acid equivalents; QE: quercetin equivalents; total anthocyanins, expressed as μmole of cyanidin 3-glucoside equivalents (C3GE)/100 g FW; total proanthocyanins, expressed as μmole of catechin equivalents (CE)/100 g FW; FRAP, ferric reducing ability of plasma; TE, trolox equivalents.

$^{a-g}$ Different superscripts within the column indicates significant differences ($p \leqslant 0.05$).

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

Fraction (% Ethanol)	Total flavonol	Total dihydrochalcones	Total flavan-3-ols	Total hydroxycinnamic acid
F1 (20%)	2.5 ± 0.2^{e}	0.2 ± 0.0^{c}	36.5 ± 1.0^{b}	3.3 ± 0.1^{e}
F2 (25%)	0.4 ± 0.0^{f}	0.1 ± 0.0^{d}	23.7 ± 0.1^{c}	0.5 ± 0.0^{f}
F3 (30%)	0.7 ± 0.2^{f}	ND[1]	38.9 ± 1.3^{a}	1.7 ± 0.0^{f}
F4 (35%)	2.2 ± 0.2^{e}	ND	43.1 ± 2.3^{a}	2.1 ± 0.0^{f}
F5 (40%)	6.2 ± 0.4^{d}	0.1 ± 0.0^{d}	26.3 ± 0.9^{c}	16.8 ± 1.0^{c}
F6 (45%)	25.3 ± 0.7^{a}	0.4 ± 0.0^{b}	11.3 ± 0.5^{d}	28.8 ± 2.2^{b}
F7 (50%)	27.8 ± 0.5^{a}	0.6 ± 0.0^{a}	2.5 ± 1.8^{e}	39.5 ± 3.2^{a}
F8 (55%)	23.3 ± 0.2^{b}	0.8 ± 0.1^{a}	0.7 ± 0.1^{e}	39.5 ± 1.8^{a}
F9 (60%)	13.0 ± 0.4^{c}	0.4 ± 0.0^{b}	0.4 ± 0.0^{e}	19.7 ± 2.0^{c}
F10 (65%)	4.1 ± 0.0^{d}	ND	0.3 ± 0.0^{e}	4.7 ± 0.6^{d}
F11 (70%)	1.9 ± 0.1^{e}	ND	0.4 ± 0.0^{e}	1.2 ± 0.0^{ef}
F12 (75%)	1.4 ± 0.2^{f}	ND	0.6 ± 0.0^{e}	2.9 ± 0.2^{e}
F13 (80%)	1.7 ± 0.2^{e}	ND	0.4 ± 0.0^{f}	4.1 ± 0.2^{e}
F14 (90%)	0.6 ± 0.0^{e}	ND	ND	2.2 ± 0.2^{e}
F15 (100%)	0.3 ± 0.0^{g}	ND	0.1 ± 0.0^{f}	0.3 ± 0.0^{f}

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

Table 3
Polyphenol concentrations of four polyphenol preparations.

Polyphenol preparation	Spectrophotometric analyses[Z]				UPLC–ESI–MS/MS analyses[Y]			
	TPC[*]	TFC[§]	TAC[†]	TPr.C[‡]	Total flavonols	Total dihydrochalcones	Total catechins	Total anthocyanins
Crude	149.2 ± 2.9^{d}	62.9 ± 2.6^{cd}	3.9 ± 0.0^{b}	184.2 ± 7.9^{c}	9.7 ± 0.2^{c}	8.1 ± 1.0^{b}	12.3 ± 1.4^{b}	56.5 ± 7.1^{b}
PPF1	217.9 ± 8.2^{cd}	86.7 ± 2.2^{c}	29.0 ± 0.1^{a}	201.8 ± 10.8^{c}	6.6 ± 0.6^{c}	6.1 ± 0.5^{b}	7.7 ± 0.7^{b}	155.2 ± 3.4^{a}
PPF2	520.3 ± 10.0^{a}	481.6 ± 5.6^{b}	0.1 ± 0.0^{c}	689.9 ± 12.8^{b}	24.6 ± 1.1^{b}	56.9 ± 6.8^{b}	384.2 ± 2.9^{a}	7.1 ± 0.6^{c}
PPF3	440.2 ± 8.7^{b}	624.6 ± 9.6^{a}	2.7 ± 0.0^{c}	767.9 ± 9.1^{a}	271.4 ± 12.6^{a}	279.2 ± 7.6^{a}	52.7 ± 1.7^{b}	9.6 ± 1.0^{c}

[a-g] Different letters in each column indicate significant differences at $p \leqslant 0.05$ as obtained by Tukey's test ($n = 6$).
[Z] Spectrophotometric analysis.
TPC: total phenolic content ([*]Results are expressed as mg of gallic acid equivalents/g dry weight of partridgeberry fractions).
TFC: total flavonoid content ([§]Results are expressed as mg of quercetin equivalents/g dry weight of partridgeberry fractions).
TAC: total anthocyanin content ([†]Results are expressed as mg of cyanidin-3-*O*-glucoside equivalents/g dry weight of partridgeberry fractions).
TPr.C: total proanthocyanidin content ([‡]Results are expressed as mg of catechin equivalents/g dry weight of partridgeberry fractions).
Analysis was conducted at the concentration of 1000 μg/mL of assayed samples.
[Y] UPLC–ESI–MS/MS analysis: total flavonol: sum of quercetin-3-*O*-glucoside; quercetin; quercetin-3-*O*-rutinoside.
Total dihydrochalcones: sum of phloridzin and phloritin.
Total catechins: sum of (−)-epigallocatechin; catechin; (−)-epicatechin, epigallocatechin-3-gallate; (−)-epicatechin-3-gallate.
Total anthocyanins: sum of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-galactoside.
PPF1: anthocyanin-rich fraction (lower aqueous fraction after separatory funnel application to the crude extract).
PPF2: flavan-3-ol-rich fraction (composed of fractions F1–F5; see Table 2).
PPF3: flavonol-rich fraction (composed of fractions F6–F10; see Table 2).

powdered PPF1 was also weighed and stored in −20 °C freezer. The next two fractions PPF2 (rich in flavan-3-ols by pooling F1–F5) and PPF3 (rich in flavonols by pooling F6–F10) were obtained after column chromatography technique was applied on the ethyl acetate partition (A) using five percent gradient to obtain fractions 1–15 (Supplementary Data). The fractions were collected thrice and were evaporated using rotary evaporator at 40 °C and then completely freeze dried using stoppering tray dryer (Dura stop, Kinetic thermal systems, Stone Ridge, NY) for 48 h. It should be noted that the hydroxycinnamic acid could not be separated from the above two sub-groups of polyphenols and majority of hydroxycinnamic acid was mixed in PPF3.

3.2.1. Analysis of polyphenol fractions using UPLC–ESI–MS/MS

Fifteen phenolic compounds were quantified by UPLC–ESI–MS/MS based on their retention time and external calibration curves (Table 2). Flavan-3-ol such as catechin, (−)-epigallocatechin (EGC) and (−)-epicatechin (EC) were concentrated in F1–F5. The catechin was concentrated in fractions **1–5** whilst the highest amount of catechin was in fraction **4** ($p \leqslant 0.05$). The concentration of EGC was very low and ranged in fractions **1–4** whilst EC was also concentrated in the same fractions ($p \leqslant 0.05$). The highest content of both EC and catechin was found in fraction **4**. All other fractions, i.e., fraction **6–15** had very low content of flavan-3-ol compounds ($p \leqslant 0.05$). These fractions (F1–F5) were pooled, the solvent was evaporated and freeze dried to prepare flavan-3-ol-rich fraction (PPF2).

Flavonols including quercetin (Q), Q-3-*O*-glucoside, Q-3-*O*-galactoside, and Q-3-*O*-rutinoside were abundant in fraction number 6–10 which were eluted with 40–65% 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 ($p \leqslant 0.05$). Remaining fractions displayed the lowest range of the quantified flavonol ($p \leqslant 0.05$) when compared statistically. The highest amount of phloridzin, a dihydrochalcone, was detected in fractions **7** and **8** ($p \leqslant 0.05$) whilst the remaining fractions exhibited trace amount of the dihydrochalcones. The next group of phytochemicals analysed 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

Table 4
The antioxidant capacity as measured by FRAP, ORAC and DPPH assay.

Polyphenol preparation	Concentration (μg/mL)	FRAP (μmole TE/L)	ORAC (μmole TE/L)	DPPH IC_{50} (μg/mL)
Crude	100	426.0 ± 4.1^{g}	771.0 ± 26.6^{f}	146.3^{d}
	1000	1086.9 ± 26.9^{c}	1055.3 ± 53.7^{d}	
PPF1	100	576.1 ± 12.7^{f}	560.6 ± 44.1^{g}	135.6^{c}
	1000	696.8 ± 8.8^{e}	867.9 ± 25.4^{e}	
PPF2	100	966.6 ± 46.5^{d}	1545.3 ± 42.3^{b}	0.2^{a}
	1000	1296.9 ± 62.4^{b}	1773.2 ± 33.8^{a}	
PPF3	100	1327.4 ± 37.8^{b}	1078.1 ± 16.9^{d}	0.8^{b}
	1000	1484.0 ± 76.8^{a}	1290.5 ± 15.6^{c}	

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

fractions as shown in Table 2. Fractions 6–10 were combined, evaporated and freeze dried to obtain flavonol-rich fraction (PPF3), also abundant in dihydrochalcones. All the polyphenol preparations were weighed into new autoclaved vials and stock solution of 1000 μg/mL was prepared for *in vitro* biochemical and cell studies.

3.3. Spectrophotometric analysis of four polyphenol preparations

3.3.1. Total phenolic content

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

3.3.2. Total flavonoid content

The flavonoid content in four polyphenol preparations was assayed using aluminium chloride ($AlCl_3$) colorimetric method (Table 3). The PPF3 exhibited the highest flavonoid content amongst all polyphenol preparations whilst the crude extract displayed the lowest content of flavonoids ($p \leqslant 0.05$). The PPF3 exhibited ~10 times higher total flavonoid content compared to the crude partridgeberry extract. The PPF3 was followed by PPF2, with 7 times higher flavonoid content compared to the crude fraction. Similar to the total phenolic content results, crude extract and PPF1 differed marginally in their total flavonoid content at the assayed concentration ($p \leqslant 0.05$).

3.3.3. Total anthocyanin content

The results confirmed the highest anthocyanin content ($p \leqslant 0.05$) in PPF1, which exhibited ~7 times higher concentration of total anthocyanins than that of the crude extract (Table 3). The PPF2 followed by PPF3, exhibiting the lowest anthocyanin content amongst all partridgeberry fractions ($p \leqslant 0.05$). The PPF2 had only 2.4% of the anthocyanins contents compared to PPF1, thus confirming the effective separation of anthocyanins using liquid–liquid separation.

3.3.4. Total proanthocyanidins

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

3.4. Concentration of phenolic compounds measured by UPLC–ESI–MS/MS

The phenolic compounds present in the polyphenol preparations were determined using UPLC–MS/MS technique (Table 3). Compared to the crude extract, PPF1 exhibited higher concentration of anthocyanins including cyanidin-3-*O*-glucoside and cyanidin-3-*O*-galactoside ($p \leqslant 0.05$). However, the concentration of other phenolic classes in the both crude extract and PPF1 was statistically similar ($p \leqslant 0.05$). The PPF2 exhibited the highest concentration of flavan-3-ols amongst all the fractions as concentration of catechin and epicatechin was 62 and 43 times higher compared to its crude counterpart ($p \leqslant 0.05$). The PPF3 indicated the highest concentration of quercetin and related compounds as the concentration of quercetin-3-*O*-galactoside and quercetin-3-*O*-glucoside in PPF3 was 58 and 52 times higher than the crude extract. Furthermore, the concentration of phloridzin was the highest in PPF3 ($p \leqslant 0.05$) and exhibited higher concentration of total flavonols compared to both PPF1 and PPF2.

3.5. Antioxidant activity of polyphenol preparations

3.5.1. FRAP assay

The antioxidant capacity measured by FRAP assay was the greatest in PPF3 followed by PPF2 ($p \leqslant 0.05$) (Table 4). However, the lowest antioxidant capacity was exhibited by the anthocyanin-rich fraction (PPF1) followed by the crude extract ($p \leqslant 0.05$). Both PPF2 and PPF3 exhibited twice the antioxidant capacity compared to PPF1. These results were confirmed at two concentrations (100 and 1000 mg/L) and followed the similar trend observed for total phenolic and total flavonoid content of polyphenol preparations indicating the contribution of the polyphenols to the total antioxidant capacity of tested samples.

3.5.2. ORAC assay

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

3.5.3. DPPH radical scavenging activity

The results indicated that the strongest anti-radical activity was exhibited by PPF2 ($p \leqslant 0.05$) (Table 4), followed by the PPF3

($p \leqslant 0.05$) in its ability to scavenge free radicals. The lowest antiradical 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 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 obtained by DPPH assay were in agreement with the results of FRAP and ORAC analysis.

3.6. Cell studies

3.6.1. Cell viability

In order to characterise the potential cytoprotective activity of partridgeberry fractions, the cellular viability of peroxyl radical exposed WI-38 cells was evaluated using MTS assay (Table 5). Amongst the polyphenol preparations, the strongest cytoprotective potential was exhibited by PPF2 ($p \leqslant 0.05$). At the exposure to PPF2 at concentration of 1000 mg/L, WI-38 cells exhibited 95% cellular viability which was followed by 94 percent cell viability after PPF3 treatment, at the same concentration. However, the PPF3 treatment at 100 mg/L concentration exhibited higher cellular viability compared to PPF2, at the same concentration ($p \leqslant 0.05$). The crude extract and anthocyanin fraction (PPF1) of partridgeberry exhibited statistically lower cell viability at both assayed concentrations compared to the other fractions ($p \leqslant 0.05$). The PPF2 and PPF3 possessed the greatest cytoprotective ability against peroxyl radical-induced oxidative damage.

3.6.2. Membrane damage

Increased extracellular lactate dehydrogenase (LDH) is a biomarker of oxidative stress and disrupted cell integrity during the lipid peroxidation and oxidative stress. The LDH release assay results showed that the membrane damage in WI-38 cells ranged from 6.3% to 22.1% compared to a positive control (Table 5). The results showed that amongst the polyphenol preparations, PPF2 exhibited the greatest inhibition of LDH release caused by peroxyl radical at 30 mM AAPH ($p \leqslant 0.05$). Interestingly, the crude extract and PPF1 displayed strong inhibition of membrane damage in WI-38 cells *in vitro*. However, contrary to previous results, PPF3 emerged as weak inhibitor of membrane damage in comparison to the crude extract and other partridgeberry fractions ($p \leqslant 0.05$).

3.6.3. 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 PPF3 and PPF2 exhibited the highest inhibition of ROS in WI-38 cells ($p \leqslant 0.05$) (Table 5). These fractions at both assayed concentrations were most potent ($p \leqslant 0.05$) in their ability to limit the production of ROS. These fractions were followed ($p \leqslant 0.05$) 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.

3.6.4. Protein content

Total protein content in WI-38 cells was assayed for the dual purpose of ELISA protein quantification and as an index of oxidative damage. There was a significance difference ($p \leqslant 0.05$) 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 μg mL^{-1} (data not shown). Subsequently, the polyphenol treatment controlled the protein damage in WI-38 cells compared to the untreated cells. The analysis showed that PPF3 and PPF2 were most effective in preventing the protein oxidation induced by peroxyl radical exposure. They were followed by PPF1 and the crude extract in their ability to check protein oxidation ($p \leqslant 0.05$). Continuing the earlier trend, crude partridgeberry extract and PPF1 exhibited the weakest potential as antioxidants *in vitro* ($p \leqslant 0.05$).

3.6.5. Peroxide radical inhibition

The ability of the polyphenol preparations to mitigate oxidative stress caused by peroxyl radical in WI-38 cells was further examined by quantifying the inhibition of oxidative damage. The results indicated the strongest inhibition of peroxy radical damage in WI-38 cells by PPF2 and PPF3 ($p \leqslant 0.05$) at the concentration of 1000 μg/mL (Table 5). On the other hand, PPF1 also exhibited strong ability to limit the production of peroxyl radicals in WI-38 cells at the same concentration. Similar to the ROS inhibition results, the crude extract at the concentration of 100 μg/mL displayed the lowest peroxide radical scavenging capacity compared to the three partridgeberry fractions ($p \leqslant 0.05$).

3.7. Nrf2 ELISA

The Nrf2 ELISA analysis (Fig. 1) revealed the cellular mechanism of enhanced cytoprotective ability of polyphenol exposed WI-38 cells. The first phase of Nrf2 studies focused on the activation of antioxidant pathway in absence of oxidative stress. The incubation of WI-38 cells with polyphenols significantly increased the

Table 5
Cytoprotective and antioxidant activity of partridgeberry preparations in human fibroblasts (WI-38 cells) against peroxyl radical-induced oxidative stress.

Polyphenol preparation	Concentration (μg/mL)	Cell viability (%)	Membrane damage (%)	ROS inhibition (%)	Protein oxidation inhibition (%)	Peroxide radical inhibition (%)
Crude	100	55.9 ± 3.6^{d}	10.9 ± 2.7^{B}	30.1 ± 3.6^{d}	31.1 ± 1.4^{c}	49.8 ± 0.8^{e}
	1000	83.6 ± 7.0^{a}	18.3 ± 2.7^{A}	70.6 ± 4.8^{b}	24.0 ± 0.4^{bc}	70.3 ± 3.6^{bc}
PPF1	100	44.0 ± 5.4^{e}	6.3 ± 1.9^{B}	50.5 ± 4.7^{c}	32.6 ± 1.3^{c}	58.4 ± 0.4^{d}
	1000	80.2 ± 2.0^{b}	13.5 ± 4.4^{A}	72.9 ± 3.3^{b}	25.6 ± 3.0^{c}	77.1 ± 4.5^{b}
PPF2	100	66.4 ± 5.0^{c}	5.6 ± 2.2^{B}	73.5 ± 5.2^{b}	74.4 ± 2.6^{b}	70.0 ± 0.9^{bc}
	1000	94.5 ± 3.7^{a}	18.9 ± 2.9^{A}	95.1 ± 1.3^{a}	84.5 ± 2.6^{a}	85.9 ± 4.4^{a}
PPF3	100	81.3 ± 4.1^{b}	12.3 ± 2.8^{B}	78.7 ± 3.4^{b}	76.4 ± 1.8^{b}	65.3 ± 1.1^{c}
	1000	94.0 ± 4.5^{a}	22.1 ± 6.5A	94.4 ± 3.6^{a}	86.2 ± 4.0^{a}	80.0 ± 5.6^{a}

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

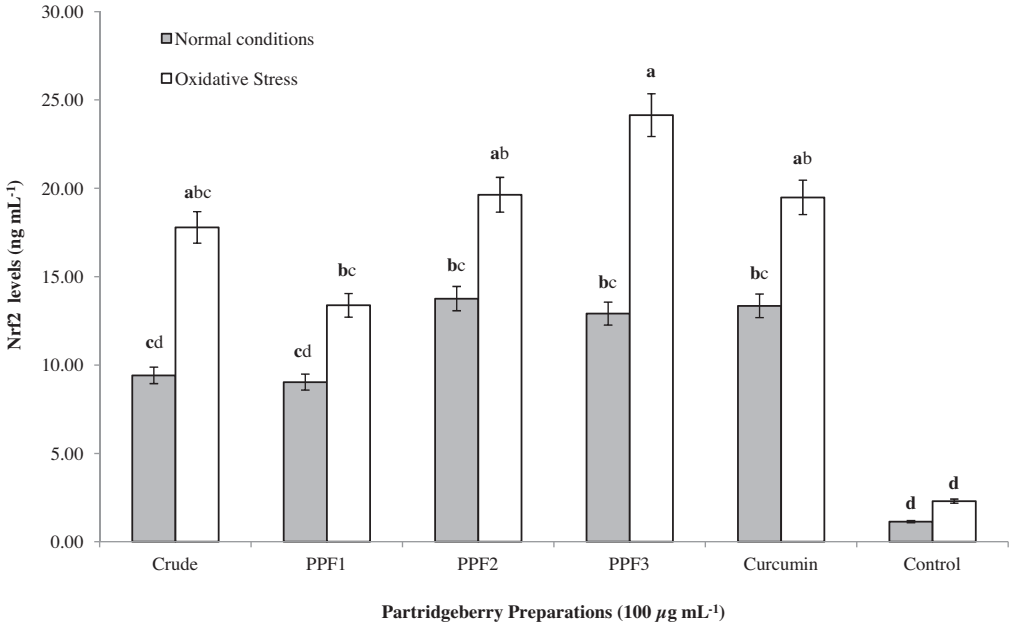

Fig. 1. Transcriptional induction of Nrf2 in fibroblasts incubated with partridgeberry fractions (100 μg/mL) or curcumin (100 μg/mL) for 24 h. Nrf2 levels are expressed as ng/mL of cell lysate. Data is representative of the six replicates ($n = 6$) for each experiment and columns with different letters (a–d) are significantly different ($p \leqslant 0.05$). PPF1: anthocyanin-rich fraction; PPF2: flavan-3-ol-rich fraction; PPF3: flavonol-rich fraction.

intracellular levels of Nrf2, compared to the vehicle control ($p \leqslant 0.05$). The Nrf2 level in control cells i.e., without polyphenols treatment was 1.14 ng/mL whilst the levels of PPF-treated cells ranged between 9.04 and 13.76 ng/mL. The highest amount of Nrf2 activation was observed in the flavonol-rich fraction (12.9 mg/mL) whilst the anthocyanin-rich fraction exhibited the lowest amount (9.0 ng/mL) of the intracellular Nrf2. Curcumin, a known Nrf2 activator was chosen for comparison and exhibited the elevated levels of Nrf2 (13.4 ng/mL) in WI-38 cells compared to control, prior to oxidative stress ($p \leqslant 0.05$). Curcumin was a stronger Nrf2 activator compared to the partridgeberry crude extract and anthocyanin-rich fraction, thus exhibiting strong antioxidant activity *in vitro* ($p \leqslant 0.05$). In the second phase, the Nrf2 levels were measured after the polyphenol treatment followed by the peroxyl radical-induced oxidative stress. The phase two Nrf2 levels in control cells i.e., without polyphenol treatment after oxidative stress were 2.3 ng/mL whilst there was a sharp increase in the levels of Nrf2 in polyphenol treated cells ($p \leqslant 0.05$). The flavonol-rich fraction exhibited the highest amount (24.1 ng/mL) of intracellular Nrf2 ($p \leqslant 0.05$), followed by flavan-3-ol-rich fraction ($p \leqslant 0.05$) which exhibited 19.6 mg/mL of Nrf2. However, the anthocyanin-rich fraction maintained the lowest amount of Nrf2 amongst all studied fractions ($p \leqslant 0.05$). Following the phase I trend, curcumin exhibited ability to activate Nrf2 and stronger antioxidant potential compared to the crude extract and anthocyanin-rich fractions ($p \leqslant 0.05$). Overall, the flavan-3-ol-rich and flavonol-rich fractions of partridgeberry exhibited the greater potential to activate Nrf2 in WI-38 cells, in comparison to curcumin ($p \leqslant 0.05$). These results showed that the polyphenol preparations may active the antioxidant response pathway through possible translocation of Nrf2 to nucleus.

4. Discussion

Increased use of dietary antioxidant-rich foods has gained much attention due to their ability to activate cytoprotective enzymes and extend health benefits via scavenging of free radicals (Reuter et al., 2010). The aim of this study was to evaluate antioxidant and cytoprotective properties of polyphenol preparations of partridgeberry. In the first phase of the studies, the extraction solvents with high polarity lead to improved recovery of polyphenols and subsequently exhibited greater antioxidant activity based on dry weight basis of extracts. Acidified 70% acetone was identified as the ideal solvent that facilitated the highest recovery of polyphenols from fresh partridgeberry. Various earlier studies have reported that the 70% acetone resulted in higher recovery of polyphenols from the plant based polyphenol sources including green tea leaves, peas and peanuts, as compared to other solvents like methanol, ethanol and water (Chavan, Shahidi, & Naczk, 2001; Drużyńska, Stępniewska, & Wołosiak, 2007). Apart from 70% acetone, 50% acetone has also displayed the capability of recovering the higher amount of polyphenols from plant sources as compared to methanol, ethanol and water (Suresh et al., 2013). Overall, our observations were in accordance with previous reports suggesting that the solvents with high polarity were optimum for extraction of polyphenols from fruits (Yu, Ahmedna, & Goktepe, 2005; Zhou & Yu, 2004). The extraction of bioactive compounds such as polyphenols from plant sources is the primary step in the preparation of dietary supplements or nutraceuticals. Selecting the right solvent affects the total amount and class of polyphenols extracted as the acidified organic solvents with weak organic acids (0.5–3%) are recommended for the maximum extraction of polyphenols. The higher amount of acids in extraction medium may result in the conflicting hydrolysis and degradation of polyphenols in plant materials (Dai & Mumper, 2010). In addition, the ultrasound-assisted extraction used in the current study, further facilitates the recovery of polyphenols, by breaking the cell wall of plant materials.

In the 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 partitioning and/or solid phase extraction helps to remove the polar non-phenolic compounds such as sugars, organic acids resulting in purification of fractionated products (Dai & Mumper, 2010). Multiple reports have used

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

In the subsequent phase of the current study, the flavan-3-ol- and flavonol-rich fractions were found to be more powerful radical scavenger and antioxidant agent *in vitro*, than the corresponding crude extract. The presence and higher concentration of catechins and quercetin glycosides are strong contributory factors to the antioxidant activity of these fractions. Our results were in line with the previous studies on partridgeberry, as the anthocyanin content observed in the current study was in agreement with an earlier report on 32 clones of partridgeberry (Debnath & Sion, 2009). Similarly, the individual compounds as obtained by UPLC–ESI–MS/MS analysis (Tables 2 and 3) were similar to an earlier study on European partridgeberry species (Ek, Kartimo, Mattila, & Tolonen, 2006). Likewise, the total proanthocyanidin and flavan-3-ol content as detected in the Canadian partridgeberry was also similar to its European counterpart (Jungfer, Zimmermann, Ruttkat, & Galensa, 2012). Furthermore, the antioxidant potential as measured by FRAP and ORAC assays was in agreement to previous observations (Mane, Loonis, Juhel, Dufour, & Malien-Aubert, 2011), however, the triterpenoid content in the Canadian partridgeberry remains unexplored (Szakiel, Pączkowski, Koivuniemi, & Huttunen, 2012).

Further, the crude extract and three partridgeberry fractions also indicated cytoprotective action against the peroxyl radical-induced oxidative damage of WI-38 cells. Similar to the antioxidant activity, flavan-3-ol- and flavonol-rich fractions exhibited the higher cytoprotective ability, which can be related to individual cytoprotective polyphenol constituents such as quercetin glycosides (Ramyaa & Padma, 2013) and catechins (Banach, Dong, & O'Brien, 2009). As phenolic acids and flavonoids are relatively easily absorbed into human circulation, compared to anthocyanins (Graf et al., 2005), the flavan-3-ol and flavonol-rich fractions may play a significant role in attenuating oxidative stress *in vivo* (Banach, Dong, & O'Brien, 2009; Ramyaa & Padma, 2013). Similar to the current observations, other berries of *Vaccinium* species have also exhibited cytoprotective ability (Del Bo et al., 2010) leading to attenuation of lipid peroxidation and cellular damage. Finally, the activation of Nrf2 pathway by the partridgeberry polyphenol preparations confirmed 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. It is important to note that Nrf2 under oxidative stress dissociates from Kelch-like ECH-associated protein 1 (Keap1) and binds with antioxidant response element (ARE) in the nucleus to trigger expression of antioxidant enzymes (Motohashi & Yamamoto, 2004). Similar to the antioxidant and cytoprotective abilities, flavan-3-ol- and flavonol-rich fractions were potent activators of Nrf2 in WI-38 cells. This may be related to the fact that these fractions contain known activators of Nrf2 such as quercetin and catechins (Ramyaa & Padma, 2013; Singh, Shankar, & Srivastava, 2011). Overall, partridgeberry contained distinct polyphenol sub-classes and exhibited strong antioxidant, free radical scavenging and cytoprotection properties *in vitro*.

5. Conclusions

Vaccinium species of berries are a rich source of dietary polyphenols with strong antioxidant and cytoprotective properties. In the current study, acidified hydrated acetone (70:28:2) provided the greatest recovery of polyphenols from fresh partridgeberry. Anthocyanin-, flavan-3-ol- and flavonol-rich fractions obtained from partridgeberry exhibited strong antioxidant and cytoprotective abilities against peroxyl radical-induced cell death in WI-38 cells. All the polyphenol fractions were potent antioxidants *in vitro* and significantly reduced the cell death and membrane damage caused by oxidative stress in WI-38 cell model systems. Amongst the three fractions, flavan-3-ol- and flavonol-rich fractions exerted relatively higher antioxidant and cytoprotective properties than the crude extract and the anthocyanin-rich fraction. Partridgeberry polyphenols were also found to enhance antioxidant enzyme system through the activation of Nrf2 pathway *in vitro*. Current study warrants further mechanistic exploration of the Nrf2-ARE pathway and suggest that the partridgeberry derived polyphenol-rich extracts may represent a potential antioxidant therapy in humans, particularly in view of its potent antioxidant and cytoprotective effects.

Acknowledgements

The authors acknowledge the funding provided by the Canada Research Chair Program, the Atlantic Innovation Funds Program of the Atlantic Canada Opportunities Agency (ACOA) and the Department of Natural Resources of the Government of Newfoundland and Labrador.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.07.103.

References

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.

Benzie, I. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry, 239*, 70–76.

Cao, G., Alessio, H. M., & Cutler, R. G. (1993). Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biology and Medicine, 14*, 303–311.

Chang, C. C., Yang, M. H., Wen, H. M., & Chern, J. C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis, 10*, 178–182.

Chavan, U. D., Shahidi, F., & Naczk, M. (2001). Extraction of condensed tannins from beach pea (*Lathyrus maritimus* L.) as affected by different solvents. *Food Chemistry, 75*, 509–512.

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

Dai, J., & Mumper, R. J. (2010). Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules, 15*, 7313–7352.

Debnath, S. C., & McRae, K. B. (2001). In vitro culture of lingonberry (*Vaccinium vitis-idaea* L.) the influence of cytokinins and media types on propagation. *Small Fruits Review, 1*, 3–19.

Debnath, S. C., & Sion, M. (2009). Genetic diversity, antioxidant activities, and anthocyanin contents in lingonberry. *International Journal of Fruit Science, 9*, 185–199.

Del Bo, C., Martini, D., Vendrame, S., Riso, P., Ciappellano, S., Klimis-Zacas, D., et al. (2010). Improvement of lymphocyte resistance against H_2O_2 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.

Drużyńska, B., Stępniewska, A., & Wołosiak, R. (2007). The influence of time and type of solvent on efficiency of the extraction of polyphenols from green tea and antioxidant properties obtained extracts. *ACTA Scientiarum Polonorum Technologia Alimentaria, 6*, 27–36.

Ek, S., Kartimo, H., Mattila, S., & Tolonen, A. (2006). Characterization of phenolic compounds from lingonberry (*Vaccinium vitis-idaea*). *Journal of Agricultural and Food Chemistry, 54*, 9834–9842.

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.

Häkkinen, S., & Auriola, S. (1998). High-performance liquid chromatography with electrospray ionization mass spectrometry and diode array ultraviolet detection in the identification of flavonol aglycones and glycosides in berries. *Journal of Chromatography A, 829*, 91–100.

He, R. R., Li, Y., Li, X. D., Yi, R. N., Wang, X. Y., Tsoi, B., et al. (2013). A new oxidative stress model, 2, 2-azobis (2-amidinopropane) dihydrochloride induces cardiovascular damages in chicken embryo. *PloS One, 8*, e57732.

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

Huang, D., Ou, B., Hampsch-Woodil, M., Flanagan, J., & Prior, R. (2002). High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *Journal of Agricultural and Food Chemistry, 50*, 4437–4444.

Jungfer, E., Zimmermann, B. F., Ruttkat, A., & Galensa, R. (2012). Comparing procyanidins in selected *Vaccinium* species by UHPLC-MS2 with regard to authenticity and health effects. *Journal of Agricultural and Food Chemistry, 60*, 9688–9696.

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.

Mane, C., Loonis, M., Juhel, C., Dufour, C., & Malien-Aubert, C. (2011). Food grade lingonberry extract: Polyphenolic composition and in vivo protective effect against oxidative stress. *Journal of Agricultural and Food Chemistry, 59*, 3330–3339.

Motohashi, H., & Yamamoto, M. (2004). Nrf2–Keap1 defines a physiologically important stress response mechanism. *Trends in Molecular Medicine, 10*, 549–557.

Nováková, L., Matysová, L., & Solich, P. (2006). Advantages of application of UPLC in pharmaceutical analysis. *Talanta, 68*, 908–918.

Prior, R. L., Fan, E., Ji, H., Howell, A., Nio, C., Payne, M. J., et al. (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.

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.

Ray, P. D., Huang, B. W., & Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular Signaling, 24*, 981–990.

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

Rupasinghe, H. P. V., Erkan, N., & Yasmin, A. (2010). Antioxidant protection of eicosapentaenoic acid and fish oil oxidation by polyphenolic-enriched apple skin extract. *Journal of Agricultural and Food Chemistry, 58*, 1233–1239.

Sekhon-Loodu, S., Warnakulasuriya, S. N., Rupasinghe, H. P. V., & Shahidi, F. (2013). Antioxidant ability of fractionated apple peel phenolics to inhibit fish oil oxidation. *Food Chemistry, 140*, 189–196.

Sharma, O. P., & Bhat, T. K. (2009). DPPH antioxidant assay revisited. *Food Chemistry, 113*, 1202–1205.

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.

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. *Methods in Enzymology, 299*, 152–178.

Su, Z. (2012). Anthocyanins and flavonoids of *Vaccinium* L. *Pharmaceutical Crops, 3*, 7–37.

Suresh, S., Guizani, N., Al-Ruzeiki, M., Al-Hadhrami, A., Al-Dohani, H., Al-Kindi, I., et al. (2013). Thermal characteristics, chemical composition and polyphenol contents of date-pits powder. *Journal of Food Engineering, 119*, 668–679.

Szakiel, A., Pączkowski, C., Koivuniemi, H., & Huttunen, S. (2012). Comparison of the triterpenoid content of berries and leaves of lingonberry *Vaccinium vitis-idaea* from Finland and Poland. *Journal of Agricultural and Food Chemistry, 60*, 4994–5002.

Wang, S. Y., Camp, M. J., & Ehlenfeldt, M. K. (2012). Antioxidant capacity and α-glucosidase inhibitory activity in peel and flesh of blueberry (*Vaccinium* spp.) cultivars. *Food Chemistry, 132*, 1759–1768.

Wang, H., & Joseph, J. A. (1999). Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radical Biology and Medicine, 27*, 612–616.

Wilson, M. A., Shukitt-Hale, B., Kalt, W., Ingram, D. K., Joseph, J. A., & Wolkow, C. A. (2006). Blueberry polyphenols increase lifespan and thermotolerance in *Caenorhabditis elegans*. *Aging Cell, 5*, 59–68.

Wu, X., Liang, L., Zou, Y., Zhao, T., Zhao, J., Li, F., et al. (2011). Aqueous two-phase extraction, identification and antioxidant activity of anthocyanins from mulberry (*Morus atropurpurea* Roxb.). *Food Chemistry, 129*, 443–453.

Yu, J., Ahmedna, M., & Goktepe, I. (2005). Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics. *Food Chemistry, 90*, 199–206.

Zhou, K., & Yu, L. (2004). Effects of extraction solvent on wheat bran antioxidant activity estimation. *LWT – Food Science and Technology, 37*, 717–721.