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Lingonberry anthocyanins protect cardiac cells from oxidative stress-induced apoptosis

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Abstract

Lingonberry grown in Northern Manitoba, Canada contains exceptionally high levels of anthocyanins and other polyphenols. Previous studies from our lab have shown that lingonberry anthocyanins can protect H9c2 cells from ischemia-reperfusion injury and anthocyanin-rich diets have been shown to be associated with decreased cardiovascular disease and mortality. Oxidative stress can impair function and trigger apoptosis in cardiomyocytes. This study investigated the protective effects of physiologically relevant doses of lingonberry extracts and pure anthocyanins against hydrogen peroxide-induced cell death. Apoptosis and necrosis were detected in H9c2 cells after hydrogen peroxide treatment via flow cytometry using FLICA 660 caspase 3/7 combined with YO-PRO-1 and then confirmed with Hoechst staining and fluorescence microscopy. Each of the three major anthocyanins found in lingonberry, cyanidin-3-galactoside, cyanidin-3-glucoside, and cyanidin-3-arabinoside, was protective against hydrogen peroxide-induced apoptosis in H9c2 cells at 10 ng mL^{-1} (20 nmol L^{-1}) and restored the number of viable cells to match the control group. A combination of the three anthocyanins was also protective and a lingonberry extract tested at three concentrations produced a dose-dependent protective effect. Lingonberry anthocyanins protected cardiac cells from oxidative stress-induced apoptosis and may have cardioprotective effects as a dietary modification.

Keywords

Lingonberry; *Vaccinium vitis-idaea*; anthocyanin; apoptosis; cardiomyocyte; oxidative stress; cyanidin; hydrogen peroxide; flow cytometry

Introduction

Lingonberry (*Vaccinium vitis-idaea* L.) is an evergreen shrub that grows in northern climates across Europe and North America. The berries contain very high levels of anthocyanins, which are the red pigments that give all berries their bright red, blue, or purple colors (Penhallegon 2006). The three main anthocyanins in lingonberry are cyanidin-3-galactoside (C-3-Gal; 58% of all anthocyanins), cyanidin-3-arabinoside (C-3-Ara; 35%), and cyanidin-3-glucoside (C-3-Glu; 7%) (Dudonné 2015; Isaak et al. 2015; Lee and Finn 2012). In addition to containing more anthocyanins per gram of fresh weight than other commonly consumed berries, lingonberry also contains other polyphenols, including quercetin glycosides, kaempferol glycosides, resveratrol, phenolic acids and proanthocyanidins (Bakowska-Barczak et al. 2007; Grace et al. 2014; Kalt et al. 2008; Rimando et al. 2004; Zheng and Wang 2003).

While polyphenols and anthocyanins can act as direct antioxidants *in vitro*, the *in vivo* mechanisms of many of their beneficial effects are still unclear (Croft 2016; Loffredo et al. 2017). Anthocyanins and their metabolites likely act by modulating the gene expression or activity levels of pro-oxidant and antioxidant enzymes in cells (Bhullar and Rupasinghe 2015) or mediating cell signaling pathways. Anthocyanins have been shown to improve the ratio of reduced and oxidized glutathione in tissue and decrease uric acid levels in plasma indicating they produce an “antioxidant effect” without directly scavenging free radicals (Alvarez-Suarez et al. 2014; Kuntz et al. 2014; Vanzo et al. 2013). These indirect antioxidant activities may be beneficial against cellular oxidative stress (Ahmed and Tang 2012; Louis et al. 2015), which plays an important role in cardiovascular diseases by promoting programmed cell death (Dey et al. 2016; Martin-Fernandez and Gredilla 2016). This study utilized hydrogen peroxide treatment as a model of oxidative stress. There are several potential sources of hydrogen peroxide in cells, including xanthine oxidase, NADPH oxidase, and superoxide dismutase, and each source may play a role in different cardiac diseases (Forstermann 2008; Harvey and Grieve 2014).

Polyphenol-rich diets have been shown to protect against cardiovascular pathologies associated with oxidative stress, such as myocardial infarct and coronary artery disease, and anthocyanin-rich diets specifically have been shown to be associated with decreased cardiovascular disease and mortality (Cassidy et al. 2013; McCullough et al. 2012; Quinones et al. 2013). However, pharmacokinetic analyses of anthocyanin metabolism have shown that after eating an anthocyanin-rich meal or consuming pure anthocyanins, the intestinal absorption rate is low (McGhie and Walton 2007). Intact anthocyanins generally do not exceed 100 nmol/L in plasma because they are rapidly taken up by the liver and kidneys where they are metabolized via methylation, glucuronidation, and degradation for excretion in feces or urine (de Ferrars et al. 2014; Vanzo et al. 2011; Vanzo et al. 2013;). This study investigated the effects of lingonberry extracts and pure anthocyanins on hydrogen peroxide-induced apoptosis in H9c2 rat cardiomyoblasts at a physiological dose, 10 ng mL⁻¹ or 20 nmol L⁻¹, which is within the reported rates of bioavailability for anthocyanins.

Methods

Sample preparation

Ultrapure water from a Milli-Q Synthesis A10 water purification system (Millipore Corp., Billerica, MA, USA) was used for all experiments. Unless otherwise stated, all chemicals of at least ACS grade were purchased from Sigma-Aldrich (Oakville, ON, Canada). Ripe fruits from wild lingonberry plants were hand-picked and immediately frozen at -20°C by a commercial berry picker on public land at Sherridon, Manitoba, Canada (55° 7' N; 101° 5' W). Frozen berries were lyophilized and ground to a fine powder. 0.5 g of lyophilized berry powder was extracted twice in 6 mL Optima-grade methanol /2% formic acid with 15 minutes sonication at 37°C followed by centrifugation for 15 minutes at 3000 x g. The supernatants from the two extractions were combined, filtered with a 0.2 µm syringe filter (Pall,

Port Washington, NY, USA), and stored at -20°C. Methanol was then removed by rotary evaporation and the remaining extract was brought back up to the original volume with ultrapure water so it could be used for cell culture studies, which produced only minimal losses in antioxidant activity (data not shown).

Antioxidant capacity assays

These assays were used for standardizing the lingonberry extracts and ensuring stability following storage, rather than for assessing quality of the sample or predicting *in vitro* bioactivity. Total phenolic content of the lingonberry extracts was determined colorimetrically as previously described (Isaak et al. 2015) by combining Folin–Ciocalteu reagent and the sample in a sodium carbonate buffer. Results were expressed as mg gallic acid equivalents per 100 g dry weight or per 100 g fresh weight using gallic acid as a standard. The oxygen radical absorbance capacity (ORAC) of the extract was determined fluorimetrically as previously described (Isaak et al. 2015) using 2,2'-azobis(2-amidinopropane) dihydrochloride (Wako Chemicals, Richmond, VA, USA) as a peroxy generator. Decline in fluorescence of fluorescein was measured kinetically for 60 minutes so the area under the curve could be calculated for each sample. Results were expressed as μmol Trolox equivalents per 100 g dry weight or per 100 g fresh weight using Trolox as a standard. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) scavenging capacity was determined colorimetrically by mixing 7 mM ABTS with 5 mM potassium persulfate to generate blue radical cations, then adding sample to reduce the radicals back to the colorless form (Re et al. 1999). Results were expressed as μmol Trolox equivalents per 100 g dry weight or per 100 g fresh weight using Trolox as a standard. The ferric reducing antioxidant power (FRAP) was determined colorimetrically by mixing 10 mM 2,4,6-Tris(2 pyridyl)-s-triazine with 20 mM ferric chloride in acetate buffer (300 mM, pH 3.6) and then adding sample (Benzie and Strain 1996). As antioxidants in the sample reduce ferric to ferrous iron, blue color develops. Results were expressed as

μmol Trolox equivalents per 100 g dry weight or per 100 g fresh weight using Trolox as a standard. All samples and standards were tested in quadruplicate. Developed color or fluorescent intensity was measured using a SpectraMax M5 microplate reader and data was analyzed using SoftMax Pro (version 6.2) software (Molecular Devices, Sunnyvale, CA, USA). Dry weight to fresh weight conversions were based on the moisture content of Manitoba lingonberries which was determined experimentally to be 85% (data not shown).

Cell culture

H9c2 rat cardiomyoblasts (CRL-1446; ATCC, Manassas, VA, USA) were maintained in high glucose (4500 mg/L) Dulbecco's modified Eagle's medium (DMEM; Hyclone, GE Life Sciences, Mississauga, ON, Canada) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% oxygen/5% carbon dioxide. Cells were subcultured when they reached 80-90% confluency.

Induction of apoptosis

Forty eight hours prior to experiments, cells were seeded into 100 mm dishes for flow cytometry or 6-well plates for fluorescence microscopy. 24 hours prior to experiments, media was aspirated and replaced with fresh media for controls or media supplemented with lingonberry extract or anthocyanins. The lingonberry extract was tested at high (H, 1:500), medium (M, 1:1000), and low (L, 1:2000) dilutions. Three anthocyanins were previously reported (Isaak et al. 2015) to be found in Manitoba lingonberry: C-3-Gal (95% purity) and C-3-Glu (95% purity), which were purchased from Chromadex (Irvine, CA, USA), and C-3-Ara chloride (>95%) which was purchased from Cerilliant Corp. (Round Rock, TX, USA). Lingonberry anthocyanins were tested at 10 ng mL^{-1} (20 nmol L^{-1} for C-3-Glu and C-3-Gal, 22 nmol L^{-1} for C-3-Ara). A combination of the three main lingonberry anthocyanins was tested using the ratio found in Manitoba lingonberry (58% C-3-Gal, 7% C-3-Glu, and 35% C-3-Ara) at 10 ng mL^{-1} . After 24 hour

pretreatment, cells were treated with $600 \mu\text{mol L}^{-1}$ hydrogen peroxide for two hours to induce apoptosis. This is the optimal concentration of hydrogen peroxide to induce apoptosis following experimental testing at 100, 200, 300, 400, 600, and $800 \mu\text{mol L}^{-1}$ hydrogen peroxide in accordance with the most commonly used concentrations found in the literature (data not shown).

Cell labeling and flow cytometry

After treatment with hydrogen peroxide, cells were washed with warm phosphate-buffered saline (PBS) and collected with 0.25% trypsin-EDTA. Cells were counted using a Countess Cell Counter (Life Technologies, Carlsbad, CA, USA) and 10^6 cells were added to each polystyrene round-bottom tube. After centrifugation at $300 \times g$ for 5 minutes, the supernatant was discarded to remove traces of trypsin and the cell pellet was resuspended in $600 \mu\text{L}$ DMEM. $10 \mu\text{L}$ FLICA 660-DEVD-FMK (ImmunoChemistry Technologies, Bloomington, MN, USA) was added and cells were incubated at 37°C for 60 minutes with agitation every 20 minutes. 1% bovine serum albumin (BSA) in PBS was then added to each tube to sequester any excess FLICA reagent. After centrifugation at $300 \times g$ for 5 minutes, the supernatant was discarded, and the cell pellet was resuspended in 1 mL 1% BSA in PBS. $3 \mu\text{L}$ YO-PRO-1 (Molecular Probes, Eugene, OR, USA) was added and cells were incubated on ice for 20 minutes prior to flow cytometry analysis.

Labeled cells were analyzed using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) with laser excitation tuned to 488 nm and 633 nm. Forward versus side scatter histograms were used to identify cardiomyocytes. FLICA 660 excites at 660 nm, emits at 690 nm, and covalently binds active caspase 3 and caspase 7. YO-PRO-1 excites at 491 nm, emits at 509 nm, and detects necrotic and late apoptotic cells because viable cells are impermeant to the compound. The fluorescence signals were separated with the standard dichroic long pass filters provided with the instrument and detected through 530/30 nm (FITC) and 712/21 nm (Alexa-700) bandpass filters. Untreated and hydrogen-

peroxide treated cells without FLICA reagent or YO-PRO-1 were used as unstained controls. FLICA labeled cells without YO-PRO-1 and YO-PRO-1 labeled cells without FLICA were used to adjust color compensation. Each treatment or control was tested on three different days and 50,000 events were recorded for each experiment. Data was analyzed using FACSDIVA software (version 8.0.1; BD Biosciences, San Jose, CA, USA).

Fluorescence microscopy

To detect chromatin condensation, Hoechst 33258, which excites at 352 nm and emits at 461 nm, was used. After treatment with hydrogen peroxide, cells were incubated with 5 $\mu\text{g mL}^{-1}$ Hoechst 33258 for 20 minutes, washed twice with PBS, fixed with 10% formalin, and viewed at 200x magnification with an Olympus IX81 microscope (Olympus, Richmond Hill, ON, Canada) equipped with X-CITE Fluorescence Illumination Series 120Q light source (Excelitas, Waltham, MA, USA). Photos of three independent experiments were analyzed using ImageJ software to count the numbers of apoptotic nuclei (bright) and non-apoptotic (dark) nuclei (Abramoff, Magalhaes, and Ram 2012). A minimum of 10,000 cells were counted for each treatment group.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Tukey multiple comparison test using PASW Statistics 18 (SPSS, New York, NY, USA). *p* values less than 0.05 were considered statistically significant.

Results

In vitro antioxidant capacity of lingonberry extract

Table 1 shows the antioxidant capacity of the lingonberry extract based on four antioxidant assays. The antioxidant activity of the lingonberry extract is likely due to the combined action of polyphenols, procyanidins, and small phenolic acids. The total phenolics assay measures the total reducing capacity of the sample, or the ability of the sample to donate electrons to the FC reagent used in the assay. The ORAC assay measures antioxidant capacity against peroxyl radicals specifically, or the ability of the sample to protect the fluorescein reagent from degradation by peroxyl radicals. The FRAP assay measures the ferric reducing capacity of the sample, or the ability of the sample to donate electrons to ferric ions. The ABTS assay measures the ability of the sample to scavenge ABTS radicals. These results are provided to demonstrate the high *in vitro* antioxidant capacity of lingonberry, in accordance with our previous results (Isaak et al. 2015) and reinforced using additional antioxidant assays.

Effects of lingonberry extract and anthocyanins on hydrogen peroxide-induced apoptosis

The two stains used, FLICA 660 and YO-PRO-1, allowed for differentiation between viable, early apoptotic, late apoptotic, and necrotic cells. Viable cells are FLICA 660 and YO-PRO-1 negative, as FLICA 660 is cell-permeant but caspase-3 and 7 would be active only at basal levels and healthy cells would be impermeant to YO-PRO-1. Early apoptotic cells are FLICA 660 positive but YO-PRO-1 negative, as caspases 3 and 7 would be strongly activated during the onset of oxidative stress-induced apoptosis, but the cell would still have an intact membrane that excludes YO-PRO-1. Late apoptotic cells are FLICA 660 and YO-PRO-1 positive, due to the activated caspase activities and loss of cell membrane integrity. Necrotic cells are YO-PRO-1 positive and FLICA 660 negative, as caspases are no longer active.

After hydrogen peroxide treatment, there was a significant decrease in the number of viable cells, with a concurrent increase in the number of late apoptotic cells, compared to the untreated group (Figure 1A and 1B). We tested the three major anthocyanins found in lingonberry at 10 ng mL⁻¹ (20 nmol L⁻¹ for C-3-Gal and C-3-Glu; 22 nmol L⁻¹ for C-3-Ara) and found them to be protective against hydrogen peroxide-induced apoptosis in rat cardiomyoblasts (Figure 1C-E). The number of necrotic, late apoptotic, and early apoptotic cells after treatment with each anthocyanin were not significantly different from the control cells, demonstrating a protective effect against hydrogen peroxide-induced cell death. A combination of the three anthocyanins was also protective (Figure 1F). There were no significant differences between the effects of each anthocyanin or combination treatment, indicating that the glycoside does not appear to affect bioactivity of the anthocyanin in cardiac cells. A lingonberry extract tested at three concentrations produced a dose-dependent effect (Figure 1G-I). A summary of the effects of each treatment on progression from viability to necrosis is shown in Figure 2.

This study provided evidence for the anti-apoptotic effects of lingonberry anthocyanins. Caspase-3 activity, a strong indicator of apoptosis, was significantly increased after hydrogen peroxide treatment, and treatment with lingonberry anthocyanins at 10 ng mL⁻¹ significantly protected against caspase-3 activation measured by flow cytometry after labeling cells with caspase 3/7-specific FLICA 660. Apoptosis was also visualized using Hoechst 33258 to detect chromatin condensation and representative images are shown in Figure 3. The hydrogen peroxide-treated group contained more brightly stained nuclei containing condensed chromatin indicative of apoptosis than the untreated group (Figure 3A and 3B). Anthocyanin-treated groups contained fewer brightly stained nuclei (Figure 3C-F), demonstrating a protective effect against apoptosis. A lingonberry extract tested at three concentrations produced a dose-dependent effect (Figure 3G-I). Chromatin condensation was quantified by counting a minimum of 10,000 nuclei per treatment group from three independent experiments and

the effects of anthocyanin and extract treatment on hydrogen peroxide-induced nuclei condensation are shown in Figure 4.

Discussion

The novel findings of this study are (1) lingonberry anthocyanins could protect cardiac cells from apoptotic cell death measured by caspase-3 activation, loss of membrane permeability, and chromatin condensation and (2) only physiologically relevant (20 nmol L^{-1}) doses of anthocyanins are needed to significantly inhibit programmed cell death and increase the number of viable cells after oxidative insult.

Oxidative stress is a hallmark of cardiovascular disease and constitutes a major mechanism of many cardiovascular pathophysiologies (Dey et al. 2016; Martin-Fernandez and Gredilla 2016). Hydrogen peroxide was used to induce oxidative stress in H9c2 cardiac myoblast cells, a model which has been extensively studied in this cell line and in adult cardiomyocytes. Reactive oxygen species can trigger many components of the apoptotic machinery, including activating mitogen-activated protein kinases like c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase, activating caspases and their downstream effectors like poly ADP-ribose polymerase, and increasing expression of pro-apoptotic mitochondrial factors like Bcl and Bax. Oxidative stress in cardiomyocytes can occur during an acute ischemia-reperfusion event, such as a myocardial infarct, when free radicals formed as a result of impaired waste removal and uncoupling of the mitochondrial electron transport chain combine with oxygen at the onset of reperfusion (Dorweiler et al. 2007; Powers et al. 2007). Leakage of electrons from the electron transport chain may be more pronounced in cardiomyocytes, where there is a particularly high abundance of mitochondria due to the high-energy demands of the heart muscle. When reactive oxygen species form but are not adequately metabolized, they can cause dysfunction in organelles like the mitochondria and endoplasmic reticulum. These organelles play major roles in calcium release and

uptake, so dysfunction at this level can lead to the formation of arrhythmias and impairment of contractility, exacerbating heart failure (Tse et al. 2016). Oxidative stress in cardiomyocytes also occurs due to environmental exposures such as air pollutants and alcohol, and after drug exposures like chemotherapy (Cosselman et al. 2015; Wang et al. 2013). If oxidative stress progresses to cardiomyocyte apoptosis, heart function can be significantly compromised, and cell death may be an important component of heart failure even if only a small number of cells undergo apoptosis, as cardiomyocytes cannot be regenerated (Pedrozo et al. 2011). Reducing cardiomyocyte cell death is therefore an important step to preserving heart function following reactive oxygen species exposure.

Antioxidant compounds have been shown to protect against oxidative stress-induced apoptosis in this *in vitro* model via several mechanisms including induction of autophagy, inhibition of mitochondrial dysfunction, and activation of antioxidant enzymes (Angeloni et al. 2007; Kim et al., 2014; Lei et al. 2015; Lv and Zhou 2012; Movahed et al. 2012; Park et al. 2003; Yang et al. 2012). Turner et al. demonstrated that JNK inhibition was protective against oxidative stress-induced apoptosis in H9c2 cells (1998), and our previous study showed that the lingonberry extract significantly inhibited JNK activation and reduced apoptosis in a model of ischemia-reperfusion injury in H9c2 cells (Isaak et al. 2015). However, this is the first study to test doses of anthocyanins that are in line with reported bioavailable concentrations.

A potential limitation of this study is that only intact anthocyanins were tested for biological effects, while *in vivo*, the beneficial effects of bioavailable anthocyanins after lingonberry consumption would also be bolstered by the presence of secondary metabolites formed after anthocyanin absorption. These metabolites, including small phenolic acids and glucuronidated or methylated metabolites (Fernandes et al. 2013), may have cardioprotective effects of their own. Thus, future studies are warranted to examine the combined beneficial effects of intact anthocyanins and metabolites in animal models of cardiovascular disease. Another limitation may be that neonatal cardiomyoblasts were used

rather than primary adult cardiomyocytes. These cells have been used extensively to model cardiac injuries and have been shown to respond similarly to adult cardiomyocytes in models of cardiac cell death, but future studies will need to test the cardioprotective effects of anthocyanins in more robust models *in vivo*.

Dietary modifications that increase the consumption of anthocyanin and antioxidant-rich berries like lingonberry may be beneficial in disease states hallmarked by oxidative stress (Louis et al. 2014). Using four antioxidant capacity assays, this study demonstrated the high antioxidant capacity of lingonberry, which is in agreement with the literature showing that lingonberry contains exceptionally high levels of anthocyanins and has higher antioxidant activity than other commonly consumed berries (Bakowska-Barczak et al. 2007; Grace et al. 2014; Isaak et al. 2015; Mane et al. 2011; Zheng and Wang 2003). The study also showed that lingonberry anthocyanins could protect cardiac cells from apoptotic cell death measured by caspase-3 activation, loss of membrane permeability, and chromatin condensation. The results of this study are especially novel because the dosages used are within the reported ranges of bioavailability, or plasma concentration, of anthocyanins indicating that lingonberry anthocyanins may have cardioprotective effects when used as a dietary modification.

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Table 1. *In vitro* antioxidant capacity of lingonberry extract using four different antioxidant assays.

	per 100 g dry weight	per 100 g fresh weight
Total phenolic content	3844 ± 433	577 ± 65
ORAC	100393 ± 4974	15059 ± 746
ABTS scavenging capacity	21556 ± 1627	3233 ± 244
FRAP	35114 ± 1851	5267 ± 278

Total phenolic content is expressed as mg gallic acid equivalents per 100 g dry or fresh weight of berries. Oxygen radical absorbance capacity (ORAC), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) scavenging capacity, and ferric reducing antioxidant power (FRAP) results are expressed as μmol Trolox equivalents per 100 g dry or fresh weight of berries. Results are shown as mean $\pm$ standard deviation and all samples were tested in quadruplicate.

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Figure Captions

Figure 1. Effects of lingonberry extract or pure anthocyanins on hydrogen peroxide-induced apoptosis.

Scatterplots of (A) untreated cells, (B) cells treated with hydrogen peroxide, and cells treated with hydrogen peroxide after pretreatment with (C) C-3-Gal at 10 ng mL⁻¹, (D) C-3-Glu at 10 ng mL⁻¹, (E) C-3-Ara at 10 ng mL⁻¹, (F) anthocyanin combo at 10 ng mL⁻¹, (G) lingonberry extract 1:2000, (H) lingonberry extract 1:1000, or (I) lingonberry extract 1:500. Changes in FLICA-660 and YO-PRO-1 fluorescence were detected using flow cytometry.

Figure 2. Summary of effects of lingonberry extract or pure anthocyanins on hydrogen peroxide-induced apoptosis.

Progression of cells from viable to necrotic was detected by flow cytometry. Percentages of cells in each stage are shown as mean ± standard deviation of three independent experiments. * denotes $p < 0.05$ compared to the untreated group.

Figure 3. Fluorescence microscopy images of H9c2 cells stained with Hoechst 33258 to detect

chromatin condensation. Representative images of (A) untreated cells, (B) cells treated with hydrogen peroxide, and cells treated with hydrogen peroxide after pretreatment with (C) C-3-Gal at 10 ng mL⁻¹, (D) C-3-Glu at 10 ng mL⁻¹, (E) C-3-Ara at 10 ng mL⁻¹, (F) anthocyanin combo at 10 ng mL⁻¹, (G) lingonberry extract 1:2000, (H) lingonberry extract 1:1000, or (I) lingonberry extract 1:500 stained with Hoechst 33258 to detect chromatin condensation and viewed under fluorescence microscope.

Figure 4. Effects of lingonberry extract or pure anthocyanins on hydrogen peroxide-induced chromatin condensation.

Percentages of apoptotic nuclei out of total nuclei are shown as mean ± standard

deviation of three independent experiments. * denotes $p < 0.05$ compared to the hydrogen peroxide-treated group.

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Fig 1

211x197mm (300 x 300 DPI)

Fig 2

49x20mm (300 x 300 DPI)

Fig 3

274x207mm (300 x 300 DPI)

Fig 4

81x52mm (300 x 300 DPI)