Journal of Functional Foods 40 (2018) 164–179

Contents lists available at ScienceDirect

Journal of Functional Foods

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

Antiproliferative efficacy of elderberries and elderflowers (*Sambucus canadensis*) on glioma and brain endothelial cells under normoxic and hypoxic conditions

Sylvie Lamy[1], Évelyne Muhire[1], Borhane Annabi*

Laboratoire d'Oncologie Moléculaire, Centre de Recherche BioMed, Université du Québec à Montréal, C.P. 8888, Succ. Centre-ville, Montréal, Québec H3C 3P8, Canada

ARTICLE INFO

Chemical compounds studied in this article:
Cyanidin 3-O-sambubioside-5-O-glucoside chloride (PubChem CID: 44256731)
Delphinidin 3-O-rutinoside chloride (PubChem CID: 44256887)
Quercetin 3-O-rutinoside (PubChem CID: 5280805)

Keywords:
Cell proliferation
Elderberry
Elderflower
Endothelial cells
Glioma
Hypoxia

ABSTRACT

Elderberries are among the richest sources of antioxidant anthocyanins with health-promoting properties. As low circulating plasma antioxidant levels contribute to solid tumor malignancy, we assessed the antiproliferative properties of two Canadian elderberry cultivar extracts, 'Kent' and 'Scotia', against human brain tumor-derived cell line models and brain microvascular endothelial cells under both normoxic and hypoxic culture conditions. Elderberry, concentrated elderberry and elderflower extracts inhibited cell proliferation in a dose-dependent manner with berry extracts being more efficient. The antiproliferative effects resulted in cell cycle arrest through alterations in cell cycle checkpoint protein expression and in apoptosis. Anthocyanins and or rutin contents of the extracts efficiently inhibited cell proliferation alone or in synergy. Given brain tumors are characterized by high hypoxic areas which contribute to therapy resistance, this is the first evidence suggesting that Canadian elderberry extracts could efficiently target both the cancer and vascular compartments associated to brain tumor development.

1. Introduction

Malignant brain tumors are among the most feared types of cancer, not only for their poor prognosis, but also because of their direct repercussions on quality of life and cognitive function. The World Health Organization (WHO) classification of tumors of the central nervous system establishes a grading of human tumors, and groups gliomas into 4 histological grades (Louis et al., 2007). WHO grade III and grade IV tumors are the most common type of primary malignant gliomas, of which glioblastoma multiforme is the most aggressive form.

Growth of gliomas is characterized by the development of a heterogeneous vascularization. Indeed, at an early phase of tumor growth, the vessels are small, sparsely branched, well organized and well perfused under normoxic conditions (Hendriksen et al., 2009). However, when the tumor increases in size, the neovascularization becomes morphologically and functionally unsuitable with irregular blood flow creating hypoxic areas (Jensen, 2009). This hypoxic microenvironment is a powerful stimulus for the expression of genes involved in tumor cell proliferation and angiogenesis including hypoxia inducible factor-1α, transforming growth factor-β and vascular endothelial growth factor (VEGF) (Kaur et al., 2005), which stimulate vasculature development in order to rapidly supply oxygen to malignant cells. These tumor cells acquire resistance to treatment and successfully treating gliomas becomes challenging. Hence, more insight into chemopreventive agent properties against tumor cell proliferation is needed.

Over the past few years, there has been a growing interest in nutraceutical intervention against different types of tumors, including brain tumors (Ramachandran, Nair, Escalon, & Melnick, 2012; Rooprai, Christidou, & Pilkington, 2003; Sheweita & Sheikh, 2011). This approach uses the chemopreventive properties of naturally occurring phytochemicals, especially those from diet-derived vegetables, spices and fruits (Surh, 2003; Wiseman, 2008). Among them, anthocyanins exhibit beneficial effects against mechanisms involved in the progression of various diseases (He & Giusti, 2010; Hou, 2003). These

Abbreviations: BBB, blood-brain barrier; Cy-3-sam-5-glu, cyanidin-3-O-sambubioside-5-O-glucoside chloride; Dp-3-rut, delphinidin 3-O-rutinoside chloride; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT-1, glucose transporter-1; HBMEC, human brain microvascular endothelial cells; PI, propidium iodide; Que-3-rut, quercetin-3-O-rutinoside; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; WHO, World Health Organization

* Corresponding author.
E-mail addresses: lamy.sylvie@uqam.ca (S. Lamy), muhire.evelyne@courrier.uqam.ca (É. Muhire), annabi.borhane@uqam.ca (B. Annabi).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.jff.2017.10.048
Received 24 March 2017; Received in revised form 16 October 2017; Accepted 30 October 2017

flavonoids are a ubiquitous group of water-soluble natural pigments responsible for the blue, purple, red and intermediate colors of leaves, flowers, vegetables and fruits (He & Giusti, 2010).

Canadian elderberry or American elder [*Sambucus nigra* L. subsp. *canadensis* (L.) R. Bolli; syn. *S. canadensis* L.] is native to eastern North America. Although its European cousin [*Sambuscus nigra* L. subsp. *Nigra* (L.), syn. *S. nigra* L] is relatively well developed as a horticultural crop, more than twenty different cultivars have been developed either at the New York Agricultural Experiment Station or at Agriculture and Agri-Food Canada in Nova Scotia for their ornamental qualities or high quality of berries (Finn, Thomas, Byers, & Serce, 2008). In fact, edible purple-black fruits of the cultivated elderberry are considered one of the richest sources of anthocyanins and phenolic compounds, which contribute to the high antioxidant activity of these berries (Ozgen, Scheerens, Reese, & Miller, 2010; Thole et al., 2006; Wu, Gu, Prior, & McKay, 2004). The fruits and flowers are used to produce juices, jams, jellies, syrup, wines, pies, colorants, and infusions (Thomas, Byers, & Ellersieck, 2009). Recently, there has been increasing demand for nonfermented elderberry juice concentrates as a nutraceutical or dietary supplement (Thomas et al., 2015).

The native *Sambucus canadensis* is well known by the Amerindian peoples and herbalists as a medicinal plant. Various parts of the plant have been used for their laxative, diuretic, expectorant, or purgative properties to treat respiratory and pulmonary disorders as well as fever and rheumatism (Charlebois, Byers, Finn, & Thomas, 2010). Recently, extracts of the berries from some varieties of both European and Canadian elderberry demonstrated significant chemopreventive potential against the initiation and promotion stages of carcinogenesis (Thole et al., 2006). However, more information is required in order to understand the mechanistic and molecular aspects of their antitumor activities. In the current study, we investigated the *in vitro* molecular effects of berry and flower extracts from two Canadian elderberry cultivars, 'Kent' and 'Scotia', developed from Agriculture and Agri-Food Canada, on human glioma and brain microvascular endothelial cell proliferation.

2. Materials and methods

2.1. Materials

Cyanidin-3-O-sambubioside-5-O-glucoside chloride (Cy-3-sam-5-glu) [purity ≥ 90%], delphinidin-3-O-rutinoside chloride (Dp-3-rut) [purity ≥ 95%] and quercetin-3-O-rutinoside also called rutin (Que-3-rut) [purity ≥ 99%] were purchased from Extrasynthese (Lyon, France). Concentrated elderberry extract (IMMUNIA™) was obtained from Fruitomed Inc. (Thetford Mines, QC). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The monoclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Advanced Immunochemical Inc. (Long Beach, CA). Polyclonal antibodies against CDK2, CDK4, p27, p21, and monoclonal antibodies against CDK6, cyclin D1 and cyclin D3 were from Cell Signaling Technology (Beverly, MA). Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-linked secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), and enhanced chemiluminescence (ECL) reagents were from Denville Scientific Inc. (Metuchen, NJ). Micro bicinchoninic acid protein assay reagents were from Thermo Scientific (Rockford, IL). All other reagents were from Sigma-Aldrich (Oakville, ON).

2.2. Plant materials

Fresh fruits and dried flowers from two genotypes of *S. canadensis*, 'Kent' and 'Scotia', were obtained from Au Versant du Sureau (Adstock, QC). Fruits were stored at −80 °C until use.

2.3. Preparation of berry and flower extracts

Berry extracts: 100 g of berries were stored at 4 °C and processed within 24 h. Extracts were prepared in a cold room by processing the berries through a hand blender. The puree obtained was clarified by centrifugation at 200,000 × *g* for 30 min at 4 °C. The supernatant was then sterilized by filtration through a 0.22 µm filter and aliquots were immediately frozen at -80 °C. Berry extract was prepared twice.

Scotia elderflower extracts: One teaspoon of dried flowers (0.8 g) was infused in 250 mL of 85 °C distilled water for 15 min (Viapiana & Wesolowski, 2017). The infusion was cooled and then filtered through a 0.22 µm filter; aliquots were immediately frozen at -80 °C. Elderflower extract was prepared twice.

2.4. Cell culture

The human glioblastoma U-87 MG and U-138 MG, and glioma Hs 683 cell lines were purchased from the American Type Culture Collection and maintained in modified Eagle's Minimum Essential Medium (Wisent, 320-036-CL) or in Dulbecco's Modified Eagle's Medium (319-005-CL), respectively, containing 10% calf serum (HyClone Laboratories, SH30541.03), 1 mM sodium pyruvate (Sigma-Aldrich, P2256), 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin (Wisent, 450-202-EL). Human brain microvascular endothelial cells (HBMEC) were from ScienCell™ Research Laboratories (Carlsbad, CA) and maintained in RPMI Medium (Wisent, 350-007-CL) containing 10% fetal bovine serum (Life Technologies, 12483-020), Nu-serum™ (VWR, CACB355500), endothelial cell growth supplement (EMD Millipore Corporation, 02-102) and 1 mM sodium pyruvate (Sigma-Aldrich, P2256). HBMEC used in this study were restricted to between 4 and 8 passages. Cells were cultured at 37 °C under a humidified 95%–5% (v/v) mixture of air and CO_2. Hypoxic conditions were performed by incubation of cells in an hypoxia workstation (BioSpherix, Parish, NY). The oxygen concentration (< 2%) was fixed with a gas mixture containing 5% CO_2 and balance of nitrogen. For experimental purposes, after 24 h of cell seeding in complete medium, cells were serum-starved under normoxic or hypoxic conditions to induce a quiescent cell state and to condition them to hypoxic conditions before treatment with extracts. After 24 h, the medium was removed and replaced by fresh complete medium containing (or lacking) extracts for 48 h.

2.5. Cell proliferation assay

Cells were seeded in 96-well plates at 5000 cells/well (Hs 683, HBMEC) or 7500 cells/well (U-87 MG, U-138 MG) in 200 µL complete medium and incubated at 37 °C under normoxic or hypoxic culture conditions according to the protocol described previously. Cells were treated with 100 µL complete medium containing berry extracts (0–200 µL/mL) or flower extracts (0–500 µL/mL) or IMMUNIA™ (0–200 µL/mL), or 200 µM of Cy-3-sam-5-glu, cy-3-gal, cy-3-rut, Dp-3-rut, Que-3-rut or a combination of these five compounds. Cell proliferation was determined by assaying the mitochondrial metabolic activity of cells, after 48 h of treatment with extracts, using the highly sensitive water-soluble tetrazolium salt (WST-1) assay. Briefly, 10 µL WST-1 reagent (Roche, Laval, QC) was added to each well and the soluble formazan dye produced by metabolically active cells was monitored for at least 45 min at 37 °C. The absorbance at 450 nm was measured using a SpectraMax Plus reader (Molecular Devices, Sunnyvale, CA). Since high concentrations of the berry extract color interfered with the assay, reference values from the samples containing extract only were deducted from the readings of the treated cell sample.

2.6. Cell cycle analysis

Cells were incubated in complete medium containing a specified

concentration of extract for 48 h as described previously. At the end of treatment, the cells were collected by mild trypsin digestion, washed with ice-cold phosphate-buffered saline (PBS) and fixed in 70% ethanol in PBS overnight at −20 °C. Cells were then centrifuged at 10,000 rpm for 10 min, followed by careful removal of the supernatant. Three volumes of staining solution in PBS, containing propidium iodide (PI, 40 μg/mL; Calbiochem, San Diego, CA) and DNase-free RNase (100 μg/mL), were added for at least 30 min at 37 °C in the dark before analysis. The proportion of the cell population in each phase of the cell cycle was determined as a function of the DNA content using a BD Accuri C6 flow cytometer (BD Biosciences, Mississauga, ON). Cell cycle analysis was performed with the BD Accuri™ C6 software (version 1.0.264.21). For each measurement, at least 10,000 cells were counted.

2.7. Western blot analysis

Cells were treated as described above for cell cycle analysis but solubilized on ice in lysis buffer [150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethyleneglycol-O, O′-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA), 0.5% (vol/vol) Nonidet P-40 and 1% (vol/vol) Triton X-100]. Lysates (25 μg) were solubilized in Laemmli sample buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 10% β-mercaptoethanol, and 0.00125% bromophenol blue], boiled for 5 min, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes which were then blocked for 1 h at 4 °C with 5% non-fat dry milk in Tris-buffered saline/Tween 20 (TBS-T; 147 mM NaCl, 20 mM Tris-HCl, pH 7.5, and 0.1% Tween 20). Membranes were further washed in TBS-T and incubated overnight with an appropriate primary antibody in TBS-T containing 3% bovine serum albumin (BSA) and 0.01% sodium azide (NaN_3), followed by a 1 h incubation with an HRP-conjugated anti-mouse or anti-rabbit secondary antibody in TBS-T containing 5% non-fat dry milk. Immunoreactive material was visualized with an ECL detection system. The immunoreactive bands were quantified using ImageJ software (NIH).

2.8. Apoptosis analysis

Apoptotic and dead cell counts were performed using the FITC Annexin V Apoptosis Detection Kit II (BD Biosciences, Mississauga, ON) and PI staining by flow cytometry. Both the floating and the adherent cells were collected by mild trypsin digestion and resuspended in the provided binding buffer (51-66121E) at a concentration of 1×10^6 cells/mL; then 100 μL of cell suspension was added to 5 μL of FITC Annexin V (51-65874X) and 5 μL of PI (50 μg/mL; 51-66211E) and mixed for 15 min in the dark at room temperature. Next, 400 μL of binding buffer (1X) was added to the solution and fluorescence was measured using a BD Accuri C6 flow cytometer. Data collection and analysis were performed with FlowJo 10.1 software. At least 10,000 cells were counted for each measurement. The following controls were used to set up gates: unstained cells, cells with FITC Annexin V only and cells with PI only.

2.9. Total RNA isolation, cDNA synthesis and real-time quantitative PCR

Total RNA was extracted from cell monolayers using TRIzol reagent (Life Technologies, Gaithersburg, MD). For cDNA synthesis, 1 μg of total RNA was reverse-transcribed into cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was stored at -80 °C prior to PCR. Gene expression was quantified by real-time quantitative PCR using Sso Fast EvaGreen Supermix (Bio-Rad, Hercules, CA). DNA amplification was carried out using a CFX Connect Real-Time System (Bio-Rad) and product detection was performed by measuring the binding of the fluorescent dye EvaGreen to double-stranded DNA. The following primer sets were provided by QIAGEN (Valencia, CA): glucose transporter-1 (GLUT-1; QT00068957), VEGF (QT01682072), ribosomal protein S18 (RPS18; QT02323251), RPS28 (QT02310203). The relative quantities of target gene mRNA against an internal control, RPS18/RPS28 RNA, were measured by following a ΔCt method employing an amplification plot (fluorescence signal vs. cycle number). The difference (ΔCt) between the mean values in the triplicate samples of target gene and those of RPS18/RPS28 RNA were calculated using CFX Manager Software version 3.0 (Bio-Rad) and the relative quantified value (RQV) was expressed as $2^{-\frac{\Delta C}{T}}$. Semi-quantitative PCR was also performed to validate single amplification products which were resolved on 1.8% agarose gels containing 4–6 μL GreenGlo™ Safe DNA Dye, 20,000X in water (Denville Scientific Inc., Holliston, MA) per 100 mL of agarose (not shown).

2.10. Characterization of phenolic compounds

The content in anthocyanins and other phenolic compounds was performed by CRIQ (Centre de recherche industrielle du Québec, Québec, QC) and INAF (Institute of Nutrition and Functional Foods, Québec, QC).

2.10.1. Anthocyanin content

The anthocyanin composition of elderberry extracts was analyzed as previously described (Loizzo et al., 2016) by reverse-phase analytical HPLC using a Waters Acquity UPLC-UV-MS system (Milford, USA). The separation was performed with a flow rate of 1 mL/min using a Zorbax SB-C8 column (250 mm x 4.6 mm, 5 μm particle size) with the following elution gradient: 0–2 min, 5% B; 2–10 min, 5–20% B; 10–15 min, 20% B; 15–30 min, 20–25% B; 30–35 min, 25% B; 35–50 min, 25–33% B; 50–55 min, 33% B; 55–65 min, 33–36% B; 65–70 min, 36–45% B; 70–75 min, 45–53% B; 75–80 min, 53–55% B; 80–84 min, 55–70% B; 84–88 min, 70–5% B; 88–90 min, 5% B (A: 5% formic acid in ultrapure water and B: methanol). Chromatographic data were acquired at 520 nm, and the quantification was performed using cyanidin-3-glucoside as standard. Individual anthocyanins were identified using UHPLC-MS/MS. Briefly, the compounds were separated using the chromatographic conditions described above. The MS analyses were carried out on a Waters Xevo TQD mass spectrometer equipped with a Z-spray electrospray interface. The analysis was performed in positive mode and data were acquired through selected ion monitoring (SIM) using Waters Masslynx V4.1 software, tracking specific *m/z* of expected compounds (Lee & Finn, 2007).

2.10.2. Phenolic acid and flavonoid content

Phenolic acids and flavonoids were characterized using a Waters Acquity UHPLC-MS equipped with an H-Class quaternary pump system, a flow through needle (FTN) sample manager system, and a column manager. The MS detector was a QDa mass spectrometer equipped with electrospray interface. The analysis was achieved using a C18 BEH column (100 mm x 2.1 mm, 1.7 μm particle size). The separation was performed at 40 °C at a flow rate of 0.4 mL/min with a mobile phase consisting of 0.2% acetic acid in ultrapure water and acetonitrile (solvent A and B, respectively) using a gradient elution as follows: 0–5 min, 5–21% B; 5–7 min, 21–24% B; 7–7.8 min, 24–37% B; 7.8–10 min, 37–41% B; 10–10.2 min, 41–90% B; 10.2–11.2 min, 90% B; 11.2–11.3 min, 90–5% B; 11.3–13 min, 5% B. Data were acquired through single ion monitoring (SIM). All compounds were quantified as gallic acid equivalent.

For identity confirmation with mass fragment, MS/MS analyses were carried out on a TQD mass spectrometer equipped with a Z-spray electrospray interface in negative mode set as follows: electrospray capillary voltage was 2.5 kV, source temperature was 140 °C, desolvation temperature was 350 °C, cone and desolvation gas flows were 80 l/h and 900 l/h, respectively. Data were acquired through multiple reaction monitoring (MRM).

2.11. Statistical analyses

Statistical analyses were assessed with Student's *t*-test when one group was compared with the control group. To compare two or more groups with the control group, one-way analysis of variance (ANOVA) with Dunnett's post hoc test was used. The significant interaction between the different cultivars or between culture conditions (normoxia vs hypoxia) or between cell lines in the same culture conditions was determined by ANOVA at the 95% confidence level using Bonferroni test. Differences with $P < .05$ were considered significant. All statistical analyses and graphs were performed using the GraphPad Prism software version 5.0b (San Diego, CA).

3. Results

3.1. Hypoxia culture condition validation in human brain tumor and brain endothelial cell lines

In order to validate our experimental normoxic and hypoxic culture conditions, we assessed the expression of two genes known to be induced under hypoxic conditions, i.e. VEGF and GLUT-1 (Chen, Pore, Behrooz, Ismail-Beigi, & Maity, 2001; Harris, 2002; Liu, Cox, Morita, & Kourembanas, 1995). VEGF is a key regulating factor of angiogenesis in cancer, provides nutrients to tumor cells for their growth, as well as access to the host vasculature to form metastases. GLUT-1 transports glucose across the plasma membrane, supporting the metabolic needs of rapidly dividing tumor cells. Cells were incubated for 24 h in complete medium. The medium was then replaced with serum-free medium and the cells were incubated under normoxic or hypoxic conditions to induce a quiescent state in these cells. After 24 h, the medium was removed and replaced by fresh complete medium for 48 h. Under these conditions, hypoxia caused a marked increase in VEGF (Fig. 1A) and GLUT-1 (Fig. 1B) mRNA levels in the brain cancer (U-87 MG, U-138 MG, Hs 683) and microvascular endothelial (HBMEC) cell lines tested, confirming that these cells were responsive to hypoxic culture conditions.

3.2. Inhibition of human glioma and brain endothelial cell proliferation by elderberry and elderflower extracts

Berry extracts from two genotypes of *S. canadensis*, 'Kent' and 'Scotia', were prepared with a hand blender and clarified by centrifugation, whereas flower extracts from the 'Scotia' variety were obtained by infusion in distilled water. These two types of extracts were then sterilized by filtration. Concentrated elderberry extract (IMMUNIA™; Fruitomed Inc.) was obtained from a proprietary mix of berries from both the 'Kent' and 'Scotia' cultivars through a cold concentration process. These four extracts were tested in human brain tumor and endothelial cell line proliferation assays under normoxic or hypoxic culture conditions. After 24 h of cell seeding in 96-well plates with complete medium, cells were serum-starved under normoxic or hypoxic culture conditions for 24 h. Then, the medium was removed and replaced by fresh complete medium containing (or lacking) extracts for 48 h. As shown in Fig. 2, all the extracts tested reduced cell proliferation in a dose-dependent manner, whereas the extent of inhibition was significantly different between the berry and flower extracts. 'Kent', 'Scotia' and concentrated elderberry extracts exhibited the greatest ability to inhibit proliferation with half-maximal inhibition concentration (IC_{50}) values ranging from 12–49 µL/mL compared to 167–277 µL/mL for elderflower extracts (Table 1). 'Kent' and 'Scotia' elderberry extracts more efficiently inhibited the proliferation of glioblastoma cells (U-87 MG and U-138 MG) than brain endothelial cells under normoxic and hypoxic conditions (Fig. 2A and B; Table 1). However, even though these two extracts also affect the proliferation of gliomas (Hs 683) under normoxic conditions, they were less effective under hypoxic conditions. Moreover, 'Scotia' extracts were the most potent inhibitors of glioma cell proliferation under normoxic conditions (Fig. 2A; Table 1), while 'Kent' extracts were better inhibitors of cell proliferation in U-138 MG and Hs 683 cells under hypoxic conditions and in HBMEC under both culture conditions (Fig. 2B; Table 1). Interestingly, flower extracts were ineffective in reducing cell proliferation under hypoxic conditions (Fig. 2C). Overall, concentrated elderberry extracts exhibited the best antiproliferative effects.

3.3. Elderberry and elderflower extracts effect on cell cycle regulation

To further investigate the molecular mechanism of extract-mediated cell growth inhibition, the effect on cell cycle regulation was evaluated by flow cytometry as described in the Methods section. FACS analyses were conducted under the same normoxic or hypoxic conditions as for cell proliferation using extract concentrations at which cell growth was strongly inhibited. Briefly, cells were treated with either 50 µL/mL concentrated elderberry extract or 100 µL/mL elderberry extract or 500 µL/mL elderflower extract, fixed, stained and subjected to flow cytometry analyses. The FACS results showed that berry extracts increased the proportion of U-87 MG cells, Hs 683 cells and HBMEC in the S phase (~22%, ~62%, ~231%) and the G2/M phase (~33%, ~102%, ~31%), and reduced the proportion of cells in the G0/G1 phase under normoxic conditions as compared to cells treated with vehicle alone (Fig. 3A; Table 2). Similar effects were observed in the G0/G1 and G2/M phases under hypoxic conditions (Fig. 3B). However, the distribution of U-87 MG and Hs 683 cells in the S phase was higher by ~78% and ~267%, respectively, whereas it was reduced by ~176% for HBMEC (Table 2). Inversely, berry extracts increased the accumulation of U-138 MG cells in the G0\G1 phase by ~21%, associated with a diminution in the G2/M phase under hypoxic conditions (Table 2). No significant changes in the S phase were observed. Moreover, berry extracts did not significantly alter the cell cycle phase of U-138 MG cells under normoxic conditions. Compared to berry extracts, flower extracts induced an increased accumulation of U-87 MG, U-138 MG and Hs 683 cells in the G2/M phase (~119%, ~60%, ~417%) associated with a diminution in the S and G0/G1 phases under normoxic conditions (Fig. 3A; Table 2). The same effects were observed under hypoxic conditions except for U-138 MG cells, where elderflower

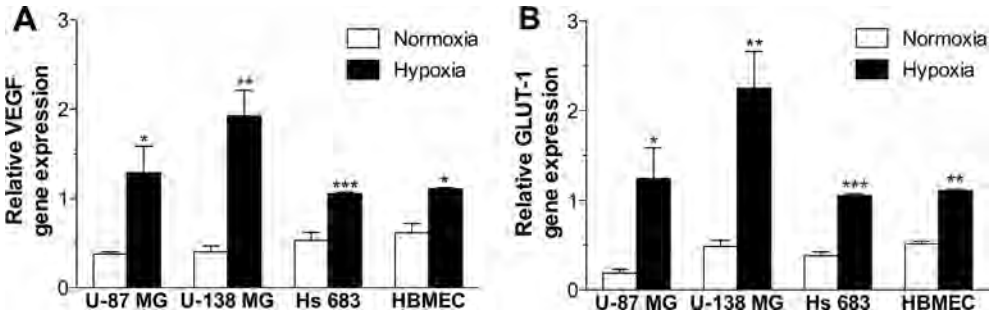

Fig. 1. Hypoxia culture condition validation in human brain tumor and brain endothelial cell lines. Cells were incubated for 24 h in complete medium and were then serum-starved under normoxic or hypoxic conditions. After 24 h, the medium was removed and replaced with fresh complete medium for 48 h. Total RNA was isolated from cells following the conditions described above, cDNA synthesis and qPCR were performed to assess (A) VEGF and (B) GLUT-1 gene expression. Values are means ± SEM of three independent experiments ($^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$ versus normoxia control).

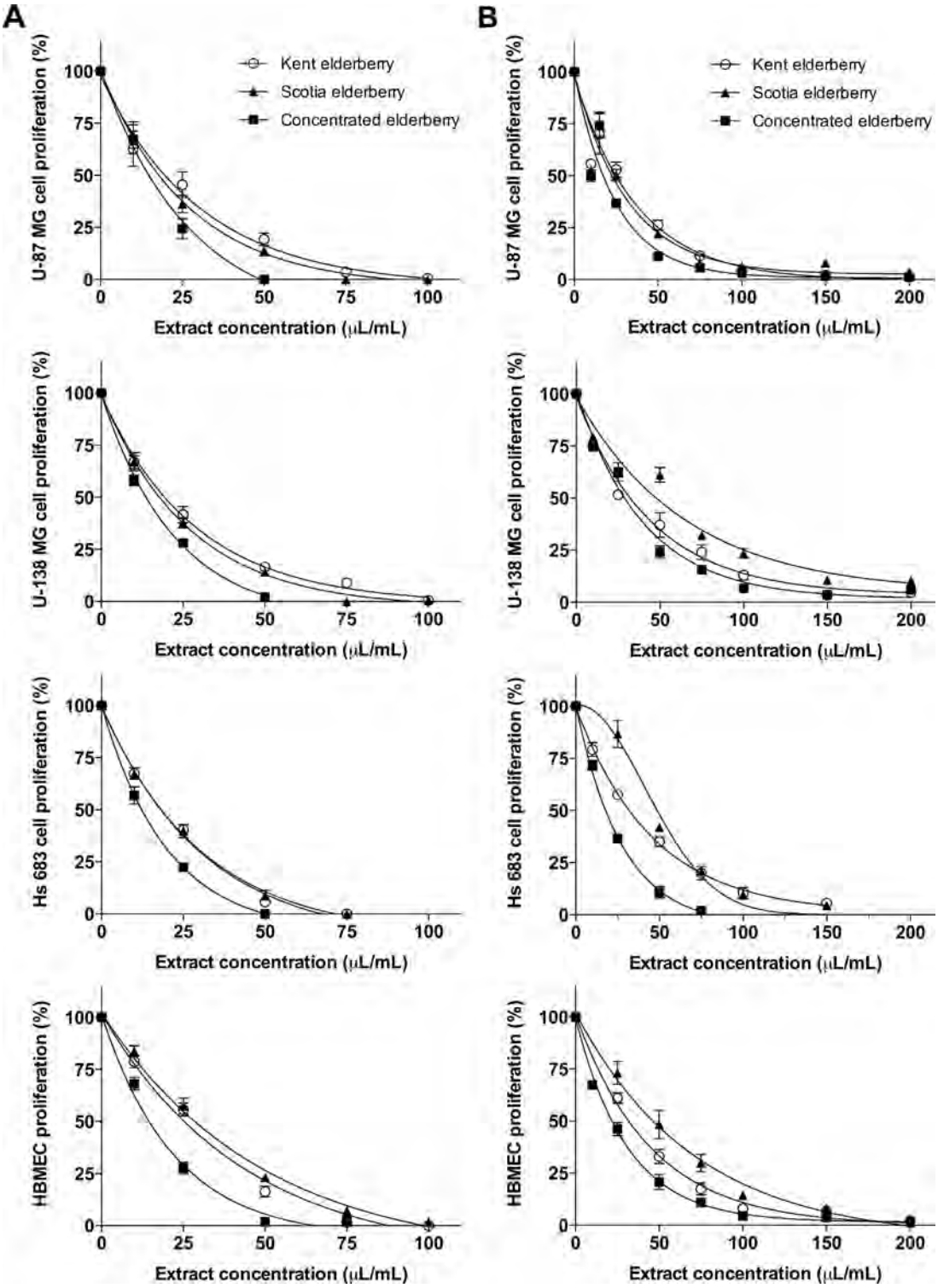

Fig. 2. Inhibition of human glioma and brain endothelial cell proliferation by elderberry and elderflower extracts. Cells were seeded into 96-well plates in complete medium. After 24 h, cells were serum-starved under (A) normoxic or (B) hypoxic conditions for another 24 h. Then the medium was removed and replaced with fresh complete medium in the presence or absence of various concentrations of the indicated (A, B) berry or (C) flower extract. After 48 h, cell proliferation was determined as described in the Methods section. Values are means ± SEM of four independent experiments performed in quadruplicate [$^{***}p < .001$ versus control for all data in panels A and B except for: Elderberry concentrate at 10 μL/mL for U-87 MG cells under normoxic conditions and for U-138 MG cells and HBMEC under hypoxic conditions as well as 'Kent' elderberry at 10 μL/mL for Hs 683 cells under hypoxic conditions ($^{**}p < .01$); 'Scotia' elderberry at 10–25 μL/mL for Hs 683 cells and 'Kent' elderberry at 10 μL/mL for HBMEC under hypoxic conditions (not significant); ($^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$ versus control) for data in panel C].

extracts induced an increased number of cells in the G0/G1 phase by ~14% and reduced the G2/M phase distribution (Fig. 3B; Table 2). No significant changes in the cell cycle phase of HBMEC were observed. Overall, these results suggest that berry and flower extracts alter cell cycle regulation, thereby inhibiting cell proliferation.

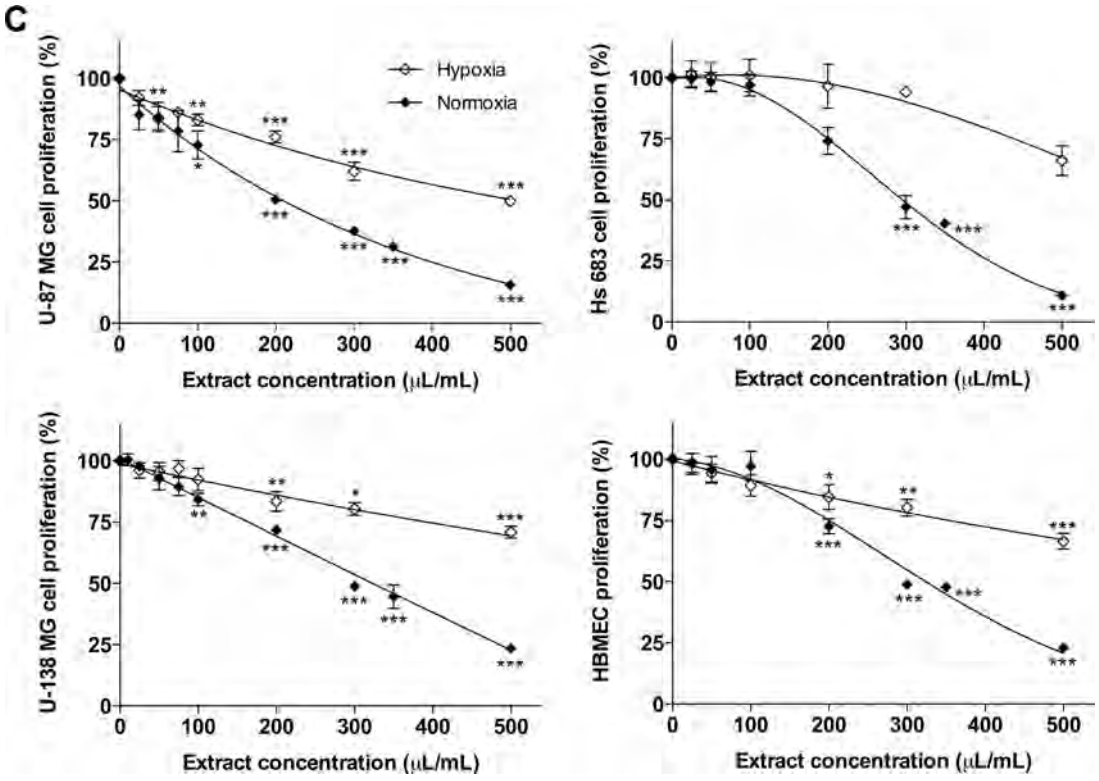

Fig. 2. (*continued*)

Table 1
Comparative overview of IC_{50} values of elderberry and elderflower extracts on cell proliferation.

	IC_{50} (μL/mL)							
	U-87 MG		U-138 MG		Hs 683		HBMEC	
Extracts	N	H	N	H	N	H	N	H
Kent elderberry	18.3 (1.1)^{a1*}	25.0 (2.6)^{a1}	19.4 (2.0)^{a2§}	30.0 (2.1)^{a2}	18.2 (0.5)^{a3¶}	31.9 (1.4)^{a3}	25.0 (0.4)^{a*§¶}	31.8 (3.7)^{a}
Scotia elderberry	17.5 (0.9)^{b1*}	22.5 (1.1)^{b1*§}	17.5 (2.3)^{b2§}	45.0 (2.2)^{ab2*§¶}	18.2 (0.5)^{b3¶}	48.8 (2.2)^{a3§¶}	28.3 (2.3)^{b4*§¶}	46.8 (2.6)^{a4*}
Concentrated elderberry	15.0 (0.7)^{c}	17.5 (1.1)^{ab*}	13.8 (1.5)^{c2}	26.2 (1.9)^{b2*§}	12.2 (0.7)^{c3}	18.0 (0.5)^{a3§}	14.7 (1.8)^{c}	21.8 (0.8)^{a*}
Elderflower	167.5 (9.9)^{abc*§}	–	245.0 (5.0)^{abc*¶}	–	276.7 (7.7)^{abc§¶}	–	275.0 (8.5)^{abc*}	–

NOTE: IC_{50} is defined as the concentration at which extracts inhibit 50% of cell proliferation.
The IC_{50} values were calculated using dose-response curves for each condition.
The same lower-case letter (within a column) indicating that the values were significantly different between the cultivars; the same lower-case number (within a line) indicating that the values were significantly different between culture conditions in the same cell line, and the same lower-case symbol (within a line) indicating that the values were significantly different in the same culture conditions between cell lines (Bonferroni test, $p < .05$). Values in parenthesis are standard errors. N = Normoxia; H = Hypoxia.

3.4. Elderberry and elderflower extracts affect the protein expression of cell cycle promoters

Cell cycle progression is regulated by cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs) (Canavese, Santo, & Raje, 2012; Musgrove, Caldon, Barraclough, Stone, & Sutherland, 2011). To investigate cell cycle arrest induced by berry or flower extracts in the four cell lines tested, the expression of cell cycle regulatory proteins was examined. We found that berry extract treatments primarily decreased the protein levels of cyclin D1/D3, CDK4/6 and CDK2 under both oxygenation conditions (Fig. 3C). On the other hand, flower extract treatment produced more variable effects on the expression levels of these proteins. Cyclin D1 expression was upregulated in U-138 MG cells and HBMEC under both oxygenation conditions as was cyclin D3 expression in U-138 MG cells under hypoxic conditions. In the other cell lines, both cyclins were downregulated. CDK4 expression was significantly downregulated in U-87 MG cells and HBMEC but slightly increased in U-138 MG and Hs 683 cells under normoxic conditions; its expression was unchanged in U-87 MG cells and downregulated in other cell lines under hypoxic conditions. The expression of CDK6 and CDK2 were generally reduced except in U-138 MG cells for CDK6 under both culture conditions. Since inhibitory mechanism of extracts on cell proliferation might also affect the expression of CKIs, we evaluated p21 and p27 protein expression, which bind to the CDK-cyclin complex to negatively regulate CDK activity (Harper et al., 1995; Koff, 2006). Treatment with berry extracts decreased the expression of p21 in U-87 MG, U-138 MG and HBMEC cells but induced it in Hs 683 cells (Fig. 3C). The results also demonstrated a downregulation of p27 protein levels, except for U-87 MG and Hs 683 cells where upregulation was observed under normoxic or hypoxic conditions, respectively. Interestingly, 'Scotia' elderberry extracts caused greater inhibition of p21 or p27 as compared to other berry extracts in U-87 MG cells. Moreover, the treatment with concentrated elderberry extract resulted in a marked increase in CKI expression in U-87 MG and U-138 MG cells. Flower extract treatments generally induced upregulation of p21 except in U-87 MG cells (hypoxic conditions) or Hs 683 cells (both culture conditions). Finally, p27 protein levels were downregulated in U-138 MG and HBMEC, and upregulated in U-

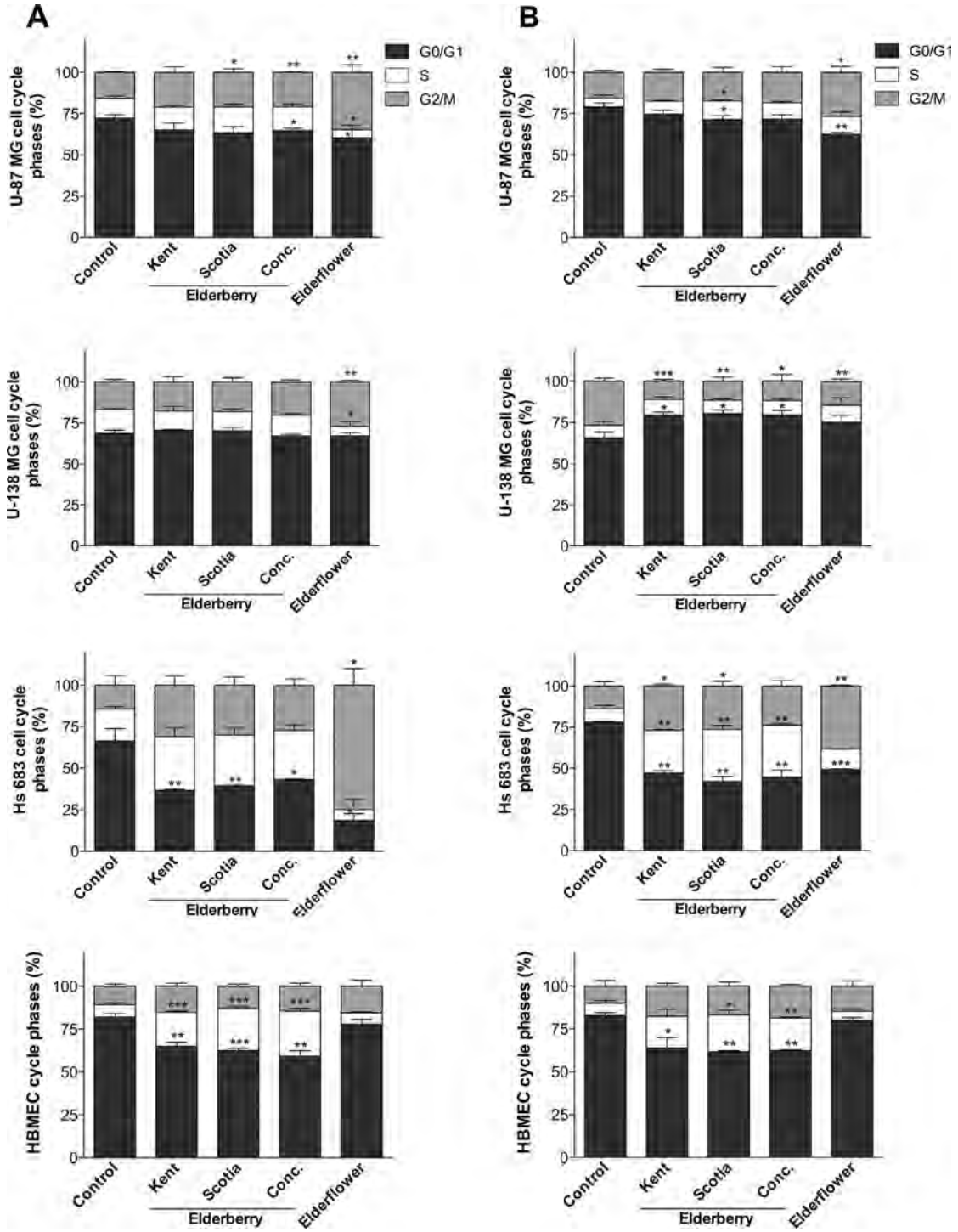

Fig. 3. Elderberry and elderflower extracts effects on cell cycle regulation. After 24 h of cell seeding in culture wells with complete medium, cells were serum-starved under (A) normoxic or (B) hypoxic conditions before treatment with extracts (50 µL/mL concentrated elderberry extract or 100 µL/mL elderberry extracts or 500 µL/mL elderflower extracts) as indicated in the Methods section. (A, B) Histogram distribution of cells in the G0/G1, S and G2/M phases as determined by flow cytometry analysis. The individual DNA content was determined by fluorescence intensity of incorporated PI. Values are means ± SEM of three independent experiments ($^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$ versus control). (C) Western blot and immunodetection of cell cycle regulatory proteins in U-87 MG, U-138 MG, Hs 683 cells and in HBMEC treated under the same normoxic or hypoxic conditions as for FACS analysis. Cells were lysed and the protein expression levels were monitored by immunoblotting using specific antibodies. Immunodetections obtained from representative samples are shown, and data are representative of three independent experiments. GAPDH expression was used as a loading control. The band intensities were analyzed by densitometry using ImageJ software. The relative levels of protein expression were normalized to those seen in the respective control (value = 1) and indicated by x-fold.

87 MG and Hs 683 cells under both culture conditions.

3.5. Elderberry extracts affect human glioma and brain endothelial cell viability

In any given cell population, cell growth is a process resulting from balance of cell proliferation and cell death. The inhibition of cell

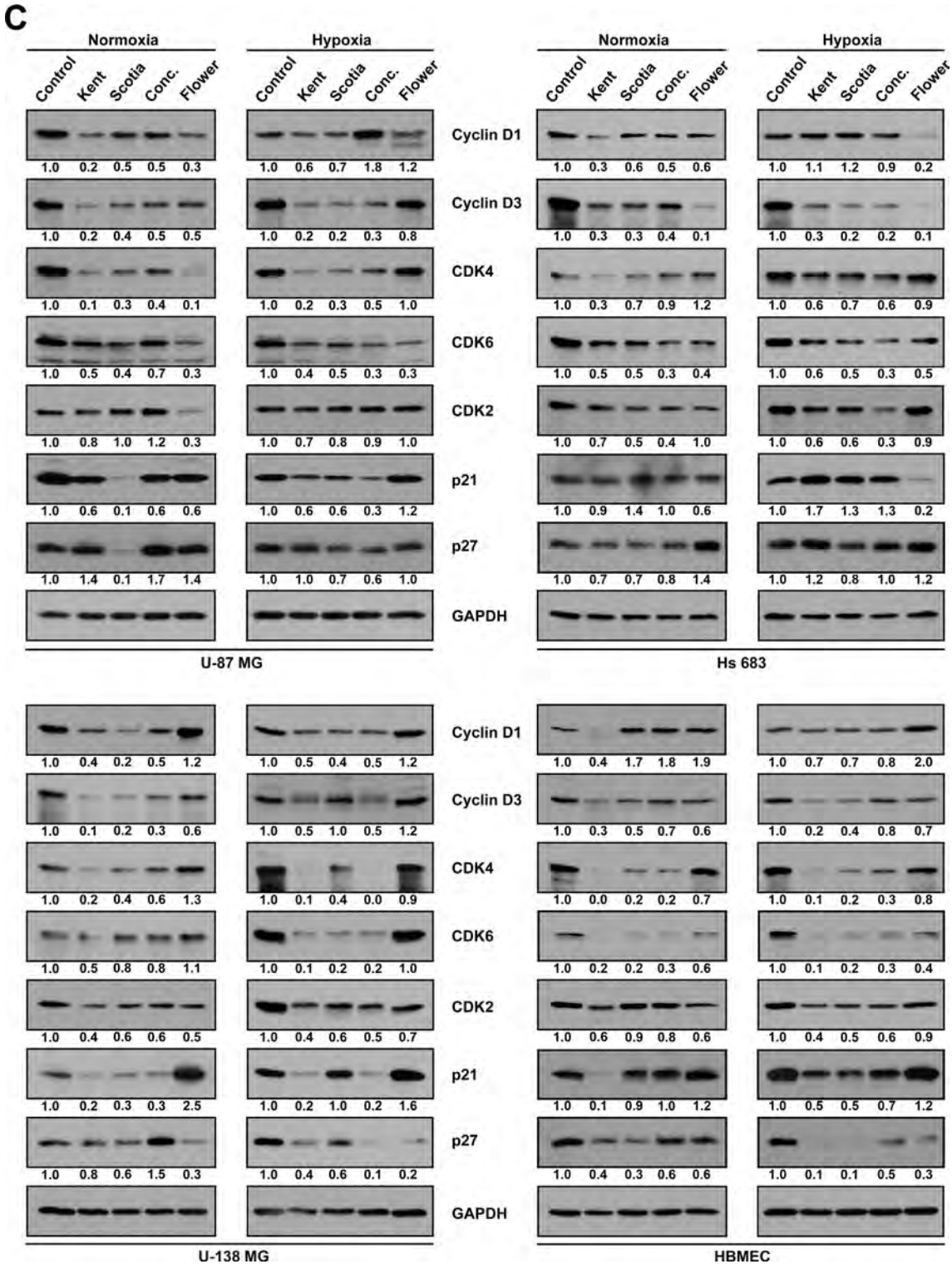

Fig. 3. (*continued*)

proliferation by elderberry and elderflower extracts could also be the result of a direct cytotoxic effect at higher concentrations. This was assayed using the FITC Annexin V Apoptosis Detection Kit II and PI staining. As shown in Fig. 4, treatment with either 50 µL/mL concentrated elderberry extract or 100 µL/mL elderberry extract for 48 h induced apoptosis under normoxic (Fig. 4A) or hypoxic conditions (Fig. 4B). However, treatment with 500 µL/mL elderflower extract did not affect cell viability as compared to control. Under normoxic conditions, treatment of U-87 MG, U-138 MG, Hs 683 and HBMEC with berry extracts resulted in 4.2-fold, 1.9-fold, 1.7-fold and 3-fold increases in apoptosis, respectively (Fig. 4A). Under hypoxic conditions, berry extracts resulted in 3.7-fold, 3.2-fold, 1.7-fold and 4-fold increases in U-87 MG, U-138M, Hs 683 and HBMEC apoptosis (Fig. 4B). Interestingly, concentrated elderberry extract had comparable effects on viability as had 'Kent' or 'Scotia' elderberry extracts.

Table 2
Percent values of cell cycle phase as monitored by flow cytometry after treatment with elderberry and elderflower extracts.

Cell lines	Cell cycle phases	Berry and flower extracts									
		Control (vehicule)		Kent elderberry		Scotia elderberry		Concentrated elderberry		Elderflower	
		N	H	N	H	N	H	N	H	N	H
U-87 MG	G0/G1	72.2 (2.9)	79.1 (3.1)	65.1 (5.7)	74.7 (3.1)^{a}	63.4 (4.4)	71.4 (2.9)*	64.8 (1.2)*	71.7 (2.6)	60.2 (6.6)*	62.4 (1.4)^{**a}
	S	12.0 (2.1)	5.4 (2.2)	13.7 (1.4)	7.8 (0.6)	15.7 (2.5)^{a}	11.3 (0.9)*	14.4 (1.6)	9.8 (0.8)	5.2 (3.4)^{*a}	11.0 (3.4)
	G2/M	15.8 (0.8)	15.5 (1.7)	21.2 (4.2)	17.5 (2.5)	20.9 (3.0)*	17.3 (3.8)	20.8 (0.4)**	18.5 (3.4)	34.6 (5.3)**	26.7 (4.8)*
U-138 MG	G0/G1	68.7 (2.7)	65.8 (4.4)	70.5 (0.6)	79.6 (2.1)*	70.2 (2.7)	80.3 (3.0)*	67.1 (1.0)	79.5 (2.9)*	67.1 (2.5)	75.1 (5.6)
	S	14.5 (1.0)	7.4 (3.0)	11.7 (3.7)	9.3 (1.9)	11.7 (1.8)	8.2 (1.1)	12.7 (1.0)	8.8 (1.2)	5.9 (3.8)*	10.3 (6.0)
	G2/M	16.8 (2.0)	26.8 (2.5)	17.8 (4.3)	11.1 (1.4)***	18.1 (3.3)	11.5 (2.9)**	20.1 (1.5)	11.9 (4.1)*	26.9 (1.4)**	14.7 (1.7)**
Hs 683	G0/G1	66.4 (7.4)	78.1 (0.4)	36.6 (0.9)^{**a}	47.1 (1.4)**	39.1 (1.1)^{**b}	42.0 (3.8)**	43.1 (0.5)^{*c}	44.6 (4.2)**	18.5 (4.0)^{abc}	49.4 (0.4)***
	S	19.2 (1.6)	8.1 (2.1)	32.6 (6.0)	26.1 (0.5)^{**a}	31.0 (5.4)	31.7 (2.6)^{**b}	29.8 (3.3)	31.4 (1.1)^{**c}	6.5 (6.1)	12.4 (0.2)^{abc}
	G2/M	14.5 (5.6)	13.8 (2.5)	30.8 (6.9)^{a}	26.8 (1.0)*	29.9 (6.3)^{b}	26.3 (3.7)*	27.1 (3.8)^{c}	23.9 (3.2)	75.0 (10.1)^{*abc}	38.3 (0.6)**
HBMEC	G0/G1	82.1 (2.6)	82.8 (1.6)	65.1 (3.0)**	63.9 (5.9)*	62.4 (1.9)^{***a}	61.7 (0.5)**	59.2 (4.1)^{**b}	62.2 (0.5)**	77.8 (2.8)^{ab}	80.2 (1.5)
	S	7.1 (1.4)	7.1 (1.5)	19.8 (0.8)^{***ab}	18.4 (4.4)	24.5 (1.4)^{***b}	21.4 (2.8)*	26.3 (2.0)^{***a}	19.1 (0.4)**	6.6 (0.6)^{ab}	5.2 (1.4)
	G2/M	10.8 (1.5)	10.1 (3.2)	15.1 (2.3)	17.8 (1.6)	13.0 (1.7)	17.0 (2.2)	14.5 (2.2)	18.7 (0.9)	15.6 (3.4)	14.7 (2.9)

NOTE: N = Normoxia; H = Hypoxia.
Extract concentration: Kent and Scotia elderberries = 100 µL/mL; Concentrated elderberry = 50 µL/mL; Elderflower concentration = 500 µL/mL.
Values are means of three independent experiments ($^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$ versus control; ANOVA with Dunnett's post hoc). The same lower-case letter (within a line) indicating that the values were significantly different between the cultivars (Bonferroni test, $p < .05$). Values in parenthesis are standard errors.

3.6. Inhibition of human glioma and brain endothelial cell proliferation by phenolic compounds

The inhibitory effects of elderberry and elderflower extracts on cell proliferation may be, in part, due to the action of specific compounds present in these extracts. Their content in anthocyanins and other phenolic compounds was analyzed by UHPLC-UV-MS and by UHPLC-MS, respectively. Individual compounds were tentatively identified by UHPLC-MS/MS. Analyses revealed that elderberry extracts contain mainly anthocyanins and other polyphenols such as cinnamic acids (neochlorogenic acid and chlorogenic acid) and flavonols while anthocyanins are absent in elderflower extracts and only rutin is found as the major compound (Table 3). Therefore, the two most prominent compounds, the anthocyanin Cy-3-sam-5-glu and the flavonol glycoside Que-3-rut (Fig. 5A), were tested alone or together at 200 µM under normoxic or hypoxic conditions. Dp-3-rut, a minor compound, was also evaluated. The choice of concentration was based on a study that had been done with some anthocyanins on the proliferation of various cell lines (Zhang, Vareed, & Nair, 2005).

Our results indicate that all of the phenolic compounds inhibited cell proliferation when compared to their vehicle controls (Fig. 5B-C). Under normoxic conditions, Cy-3-sam-5-glu was the most effective compound at inhibiting U-138 MG and HBMEC proliferation by 42.2% and 32.3%, respectively (Fig. 5B). Similar inhibitory effects were observed for Cy-3-sam-5-glu and Que-3-rut in U-87 MG and Hs 683. Under hypoxic conditions, Cy-3-sam-5-glu was also able to affect the proliferation of U-138 MG, Hs 683 and HBMEC by 29.8%, 22.0% and 22.1%, respectively (Fig. 5C). For U-87 MG cells, Que-3-rut was a better inhibitor than Cy-3-sam-5-glu with 24.4% inhibition as compared to 21.2% inhibition. For all compounds tested, Dp-3-rut was the one which showed the weakest inhibitory effect under both culture conditions, as expected (Fig. 5). Interestingly, the combination of the three compounds exhibited a more potent inhibitory effect than did the individual compounds at equal concentrations indicating that compounds present in berry extracts work synergistically at inhibiting the growth of glioma and brain microvascular endothelial cells.

4. Discussion

Malignant gliomas, and particularly glioblastomas, are characterized by aggressive invasiveness, a high proliferative rate, abundant vascularization, and the presence of extensive areas of hypoxia. Interaction with the tumor microenvironment is necessary for glioma cell growth, which is limited by the emergence of new blood vessels via angiogenesis (Dvorak, Weaver, Tlsty, & Bergers, 2011). It was reported that there is a longitudinal gradient of residual oxygen along tumor vessels leading to hypoxia at the most distal portions of the vessel (Koch et al., 2013). Hypoxia is well known to drive neovascularization resulting in migration of endothelial cells towards the tumor and proliferation to induce an angiogenic cascade (Hardee & Zagzag, 2012). Tumor hypoxia also leads to recurrence of cancers, resistance to cancer therapies and poor prognosis for patients with solid tumors such as glioblastomas (Alves et al., 2011; J. Zhou, Schmid, Schnitzer, & Brune, 2006). Depending on the oxygenation status, different areas are established within a solid tumor and a symbiotic relationship between two cancer cell populations could exist (between hypoxic and aerobic cancer cells) (Pistollato et al., 2010). Because tumors cannot grow beyond a certain size or spread without a blood supply, molecules that can block tumor angiogenesis through interfering with one of the various steps in this process such as endothelial cell proliferation will prevent or slow the growth of cancer. It is thus of great importance to find and characterize agents that are effective at targeting both hypoxic and aerobic glioma cells. Since angiogenesis in gliomas is correlated with the grade of malignancy (Lakka & Rao, 2008), we have therefore targeted the proliferation of grade IV (U-87 MG and U-138 MG) and grade III (Hs 683) glioma cells as well as of HBMEC under normoxic and hypoxic conditions. Over the years, researchers have confirmed that dietary polyphenols are capable of inhibiting cell proliferation and inducing cell cycle arrest as well as apoptosis in a number of solid tumor cell lines (Amin, Kucuk, Khuri, & Shin, 2009; Zhou et al., 2016). However, to the best of our knowledge, no studies have yet investigated the effect of elderberry, a rich source of polyphenols, on glioma and brain endothelial cell line models.

Here we documented for the first time the anticancer properties of two varieties of Canadian elderberry, 'Kent' and 'Scotia'. Berry and flower extracts caused a dose-dependent cancer and endothelial cell growth inhibition, and this antiproliferative effect appeared to be due to its ability to trigger cell cycle arrest and apoptotic cell death. Our results have shown that, of the extracts tested, concentrated elderberry extract is the most potent inhibitor of cell proliferation followed by 'Scotia' and 'Kent' elderberry under both culture conditions. However, under hypoxic conditions, concentrated elderberry extract is significantly more potent whereas 'Scotia' is less potent. Furthermore, HBMEC were less sensitive to the inhibitory effect of 'Kent' or 'Scotia'

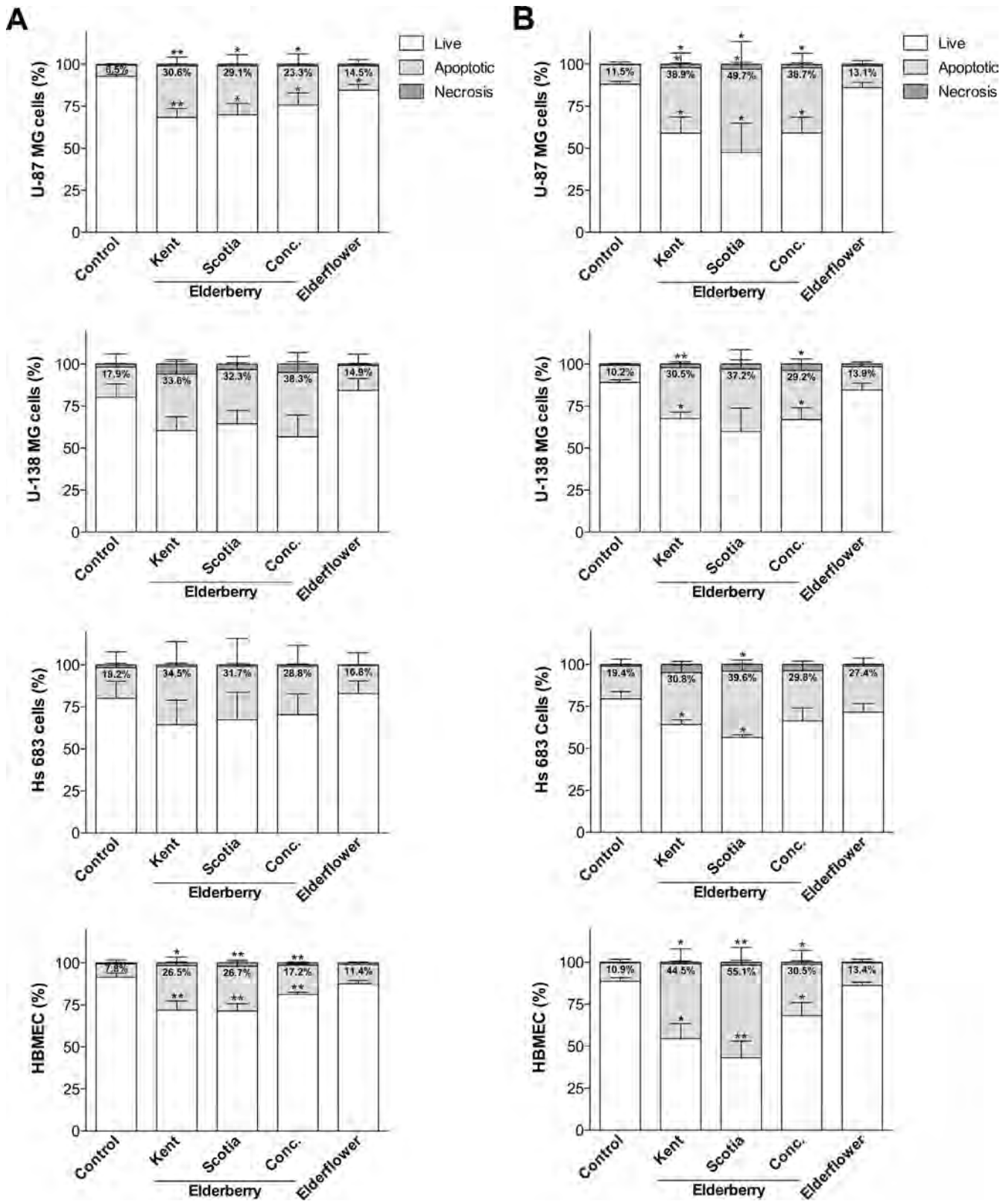

Fig. 4. Elderberry extracts affect human glioma and brain endothelial cell viability. After 24 h of cell seeding in culture wells with complete medium, cells were serum-starved under (A) normoxic or (B) hypoxic conditions before treatment with extracts (50 µL/mL concentrated elderberry extract or 100 µL/mL elderberry extracts or 500 µL/mL elderflower extracts) as indicated in the Methods section. Histogram distribution of live, apoptotic and necrotic cells as determined by flow cytometry analysis. The individual DNA content was determined by fluorescence intensity of incorporated Annexin V/PI. Values are means ± SEM of three independent experiments ($^{*}p < .05$ and $^{***}p < .001$ versus control). The proportion of apoptotic cells in the total cell population is indicated in percentage.

Table 3
Distribution of the major polyphenols present in elderberry and elderflower extracts as obtained by UHPLC.

Compound	$[M + H]^+$, *m/z*	MS/MS, *m/z*	Kent elderberry		Scotia elderberry		Concentrated elderberry		Elderflower	
Anthocyanins			mg/100 g Berries[A]	µg/0.05 mL Extract[B]	mg/100 g Berries[A]	µg/0.05 mL Extract[B]	mg/100 g Berries[A]	µg/ 0.05 mL Extract[B]	mg/0.8 g Flowers[A]	µg/0.05 mL Extract[B]
Cyanidin 3-sambubioside-5-glucoside +	743	581, 449, 287	35.4 (3.2)^{a}	35.6 (3.9)^{a}	39.5 (3.5)^{b}	37.1 (2.8)^{b}	62.6 (1.4)^{ab}	79.5 (1.4)^{ab}	ND	–
Cyanidin 3,5-diglucoside	611	449, 287								
Cyanidin 3-sambubioside +	581	287	6.2 (0.1)^{ab}	6.3 (1.3)^{ab}	7.1 (0.2)^{a}	6.7 (0.9)^{a}	7.5 (0.1)^{b}	7.1 (0.1)^{b}	ND	–
Cyanidin 3-glucoside	449	287								
Cyanidin-based anthocyanin	785	623, 449	1.0 (0.2)^{a}	1.0 (0.0)^{a}	1.0 (0.2)^{b}	0.9 (0.1)^{b}	1.8 (0.3)^{ab}	1.7 (0.3)^{ab}	ND	–
Cyanidin 3-(Z)-p-coumaroyl-sambubioside-5-glucoside +	889	727, 287	3.8 (0.4)^{a}	3.8 (0.4)^{a}	3.8 (0.3)^{b}	3.6 (0.3)^{b}	1.8 (0.0)^{ab}	1.7 (0.0)^{ab}	ND	–
Delphinidin 3-rutinoside	611	465, 303								
Cyanidin 3-(E)-p-coumaroyl-sambubioside-5-glucoside	889	727, 287	99.7 (7.0)	100.6 (12.8)	103.4 (3.7)	97.8 (12.6)	102.3 (4.0)	97.5 (3.8)	ND	–
Sum			146.0 (10.6)^{a}	147.2 (18.3)^{a}	154.8 (7.9)^{b}	140.1 (16.5)^{b}	171.3 (9.5)^{ab}	187.5 (9.1)^{ab}		
Phenolic acids and flavonoids	**$[M - H]^-$, MRM**									
Quercetin	301.2 > 151.2		2.6 (0.1)^{ab}	3.4 (0.4)^{ab}	1.6 (0.2)^{a}	2.1 (0.4)^{a}	1.6 (0.2)^{b}	2.0 (0.2)^{b}	ND	–
Quercetin 3-glucoside (Isoquercitrin)	463.2 > 300.2		13.4 (2.3)^{a}	17.8 (0.4)^{a}	13.0 (1.3)^{b}	16.3 (0.8)^{b}	22.8 (1.2)^{ab}	29.0 (1.2)^{ab}	8.2 (0.6)	2.2 (0.1)^{ab}
Quercetin 3-rutinoside (Rutin)	609.2 > 300.2		54.8 (9.4)^{a}	72.7 (1.4)^{a}	55.7 (8.4)^{b}	69.2 (0.6)^{a}	81.7 (1.6)^{ab}	103.8 (1.5)^{a}	79.8 (4.9)	21.3 (1.0)^{a}
p-Coumaric acid	163.2 > 119.0		4.9 (1.7)^{a}	6.3 (0.8)^{a}	4.7 (1.0)	5.9 (0.2)^{b}	2.6 (0.4)^{a}	3.3 (0.4)^{ab}	ND	–
Caffeoyl glucoside	341.2 > 179.2		7.6 (1.3)	10.1 (0.2)^{a}	7.5 (1.2)	9.3 (0.2)^{a}	5.6 (0.4)	7.2 (0.4)^{a}	ND	–
p-Coumaroyl glucoside	325.2 > 163.2		20.2 (3.5)^{a}	26.7 (0.6)^{a}	18.4 (2.0)^{b}	22.9 (0.9)^{a}	12.7 (0.7)^{ab}	16.1 (0.7)^{a}	0.5 (0.1)	0.1 (0.0)^{a}
3-Caffeoylquinic acid (Neochlorogenic acid)	353.1 > 191.1		3.9 (1.3)^{a}	5.1 (0.5)^{a}	4.7 (1.6)^{b}	5.7 (0.8)^{b}	15.5 (0.4)^{abc}	19.7 (0.4)^{ab}	4.0 (0.9)	1.1 (0.2)^{ab}
5-Caffeoylquinic acid (Chlorogenic acid)	353.1 > 191.1		6.2 (2.3)^{a}	8.0 (1.1)^{a}	7.8 (3.1)^{b}	9.4 (1.8)^{b}	21.3 (0.5)^{abc}	27.1 (0.4)^{ab}	11.0 (3.1)	2.9 (0.6)^{ab}
Sum			113.7 (21.8)^{a}	150.2 (1.2)^{a}	113.4 (18.4)^{b}	140.7 (0.8)^{a}	163.8 (5.2)^{abc}	208.1 (5.0)^{a}	103.5 (9.3)	27.6 (1.9)^{a}

Results are expressed as mean of duplicate from two extracts preparation. The same lower-case (within a line) indicating that the values were significantly different between the cultivars (Bonferroni test, $p < .05$). Values in parenthesis are standard errors.
ND = Not detected. Values in mg/0.8 g flower-infused were not considered in the statistical analyses. The representative chromatograms are presented in Supplementary Figs. S1A and S1B.
[A] Anthocyanins are expressed as mg cyanidin-3-glucoside 100 g^{-1} berries and quantified by UHPLC-UV-MS; other polyphenols are expressed as mg gallic acid 100 g^{-1} berries or as mg gallic acid 0.8 g^{-1} flower-infused and quantified by UHPLC-MS.
[B] Anthocyanins are expressed as µg cyanidin-3-glucoside 0.05 mL^{-1} extract; other polyphenols are expressed as µg gallic acid 0.05 mL^{-1} extract.

berries as compared to glioma cell lines. Even though flowers attenuated cell proliferation, particularly that of glioblastoma cells, berries still remained significantly effective. Remarkably, elderberries were also effective in targeting cell proliferation not only under normoxic conditions but also under hypoxic conditions. Since O_2 tension in solid tumors ranges from 2.5% to 5.3% (physiological levels) to pathological values below 0.1% in necrotic regions (Ljungkvist, Bussink, Kaanders, & van der Kogel, 2007), it is safe to speculate that the circulating plasma levels of polyphenols derived from elderberry consumption may target the mechanisms regulating glioma cell proliferation.

To investigate the relationship of cell growth inhibition by extracts to cell cycle changes, cell cycle analysis was performed. We found that elderberry causes different effects on cell cycle dependant on cell type and extract, and the cell cycle arrest was further accompanied by alterations in the levels of various cell cycle-regulated protein expression. Interestingly, the stage of cell cycle arrest triggered by extracts can indicate the molecular mechanisms of action. For example, it is well known that cells arrested in G1 phase can result from the inhibition of CDK4 and/or CDK6 complexes with cyclin D, whereas S phase arrest can be caused by inhibition of cyclin A and cyclin E complexes with CDK2 (Malumbres & Barbacid, 2009). Arrest of cell division in G2/M phase can be caused by inactivation of cyclin A with CDK2 in early G2 phase (Goldstone, Pavey, Forrest, Sinnamon, & Gabrielli, 2001) or with CDK1 in late G2 phase (Canavese et al., 2012), or also by cyclin B/CDK1 complexes through p53 activation in the early events of mitosis (Malumbres & Barbacid, 2009; Taylor et al., 1999). All these cell cycle phase arrests could result from the activation of CKIs such as p21 or p27 (Abukhdeir & Park, 2008). Since elderberry and elderflower extracts modulate the expression of several cell cycle checkpoint proteins, we hypothesize that such mechanisms could be involved in the inhibitory effect of elderberry to suppress the cell cycle progression. Additionally, several studies illustrate that CDK/cyclin complexes influence the decision of whether a cell lives or dies (Gil-Gomez, Berns, & Brady, 1998; Harvey, Blomquist, & Ucker, 1998; Kasten & Giordano, 1998; Meikrantz, Gisselbrecht, Tam, & Schlegel, 1994). Functional failure of these control points can lead to apoptosis (Pucci, Kasten, & Giordano, 2000). The fact that elderberries, but not elderflowers, induced cell apoptosis, especially in HBMEC, suggests that this mechanism may also be involved in the inhibitory action by berries. However, further investigation is essential to determine the specific molecular mechanisms of each extract.

Low antioxidant levels in the brain were reported to be correlated with the malignancy levels of brain tumors (Sheweita & Sheikh, 2011). This results in the accumulation of considerable amounts of oxidative stress products including free radicals known to damage brain tissue (Sheweita & Sheikh, 2011; Uttara, Singh, Zamboni, & Mahajan, 2009).

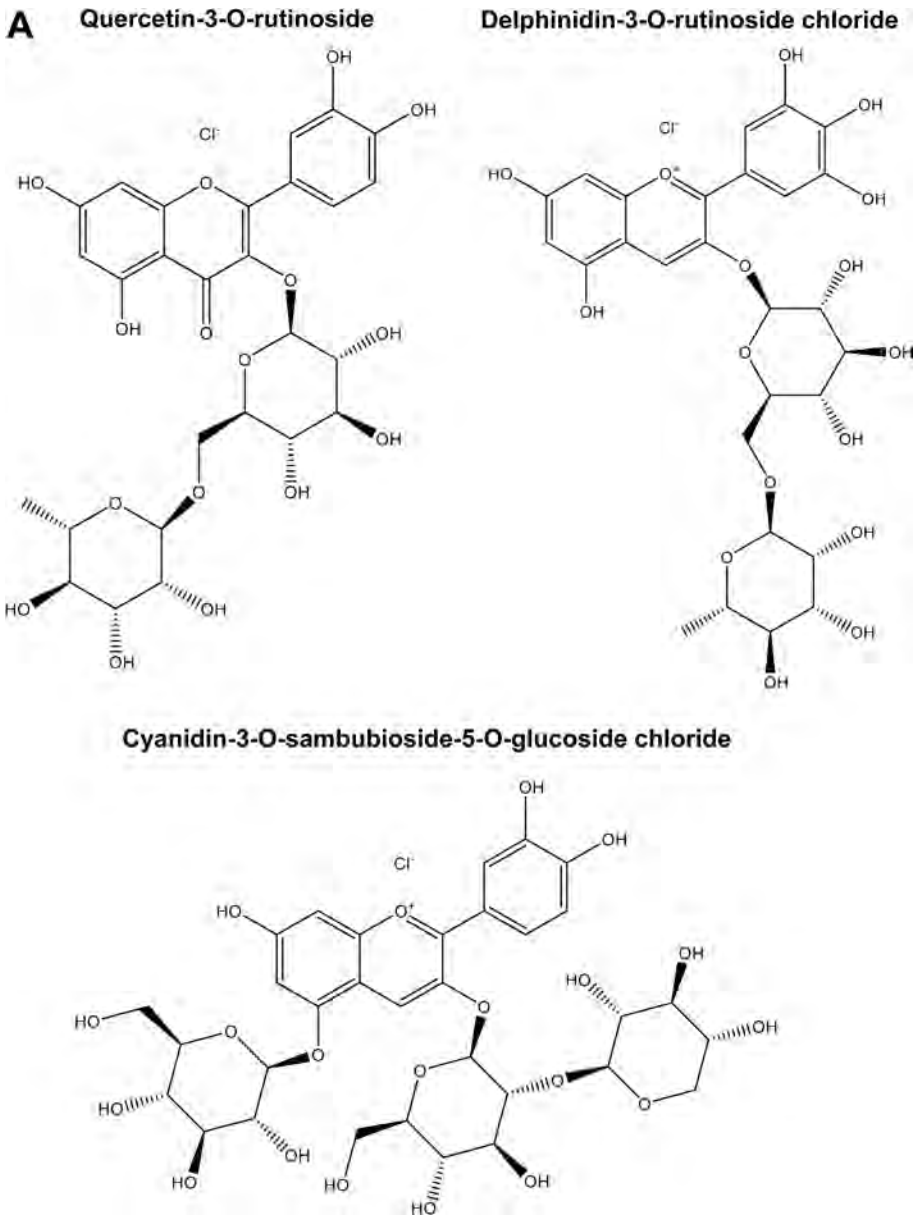

Fig. 5. Inhibition of human glioma and brain endothelial cell proliferation by phenolic compounds. (A) Chemical structures of elderberry and elderflower compounds. (B, C) Cells were seeded into 96-well plates in complete medium. After 24 h, cells were serum-starved under (A) normoxic or (B) hypoxic conditions for another 24 h. Then the medium was removed and replaced by fresh complete medium in the presence or absence of the indicated compounds or the combination of these compounds (Mix) at 200 µM. After 48 h, cell proliferation was determined by the WST-1 assay, as described in the Methods section. Values are means ± SEM of three independent experiments performed in quadruplicate ($^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$ versus control).

Dietary supplementation with antioxidants are believed to reduce these effects. Among common small fruits, elderberries are a rich source of phenolic compounds with antioxidant properties (Ozgen et al., 2010). Depending on the method used to measure antioxidative activity, some studies showed comparable activity of elderberries to blackberries, black raspberries and other dark-fleshed small fruits (Ozgen et al., 2010) or to chokeberry, and they had a much higher potential than had cranberry and blueberry, two fruits praised for their high antioxidant capacity (Wu et al., 2004). Interestingly, elderflowers are also recognized as valuable sources of bioactive polyphenols and they have moderate antioxidant activity compared to elderberries (Sroka, Marshall, & Putz, 2014).

The antioxidant properties of elderberries are mainly attributed to the presence of anthocyanins, specifically cyanidin derivatives which are major pigments (Sidor & Gramza-Michałowska, 2015). Analysis of eight different genotypes of *S. canadensis* (including 'Scotia') grown in the United States has shown the presence of Cy-3-sam-5-glu (second major pigment present), cyanidin 3 5-diglucoside, cyanidin 3-sambubioside, cyanidin 3-glucoside as well as acylated anthocyanins, namely cyanidin 3-(Z)-p-coumaroyl-sambubioside-5-glucoside, cyanidin 3-p-coumaroyl-glucoside, cyanidin 3-(E)-p-coumaroyl-sambubioside-5-glucoside (the predominant pigment present) and cyanidin 3-p-coumaroyl-sambubioside (Lee & Finn, 2007). Depending on the cultivar, cyanidin 3-rutinoside, Dp-3-rut and petunidin 3-rutinoside have been reported to be undetectable or only found in trace levels. In our study, the anthocyanin profile analysis indicates that elderberry extracts only contain Dp-3-rut. Nevertheless, the fact that cyanidin 3-(E)-p-coumaroyl-sambubioside-5-glucoside and Cy-3-sam-5-glu are also the two major anthocyanin compounds present in our elderberry extracts is consistent with this study.

Moreover, phenolic compounds such as chlorogenic acid and rutin (glycosylated conjugate quercetin) were found in greater quantities in

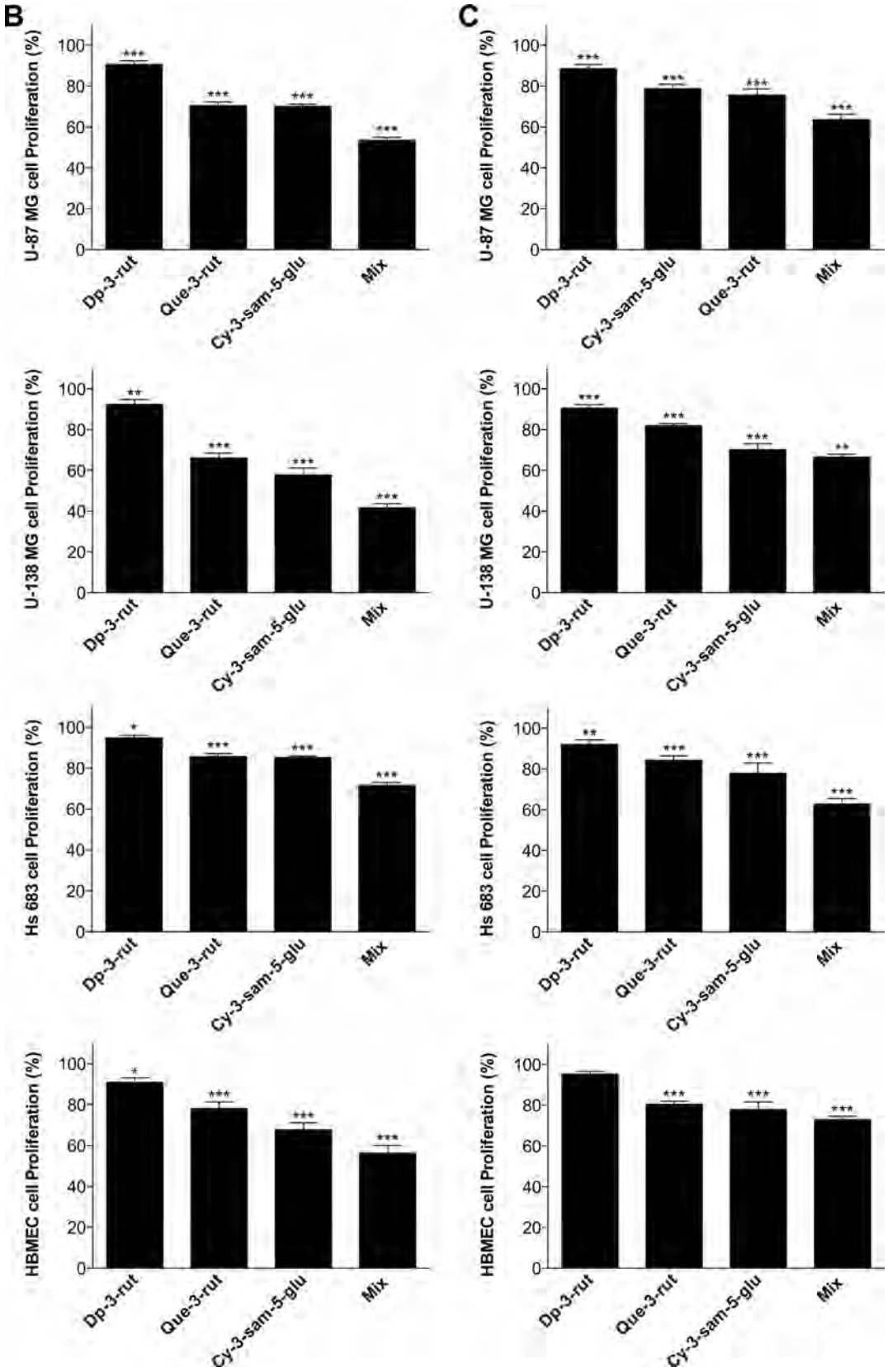

Fig. 5. (*continued*)

both *S. canadensis*. It should be noted that these compounds are also the main flavonoids found in our flower extracts, from which rutin is the major compound as reported by another study (Loizzo et al., 2016). Interestingly, isoquercetin is also another compound found in greater levels in our extracts. The variability of anthocyanin composition (and content) observed in these elderberries could be explained by the type of cultivars, degree of ripeness, growing regions as well as environmental and climatic conditions (Tomás-Barberán & Espín, 2001).

Overall, the sum of the major phenolic content present in elderberry and concentrated elderberry extracts is significantly different. Higher quantities (mg 100 g-1 berries) of phenolic content in concentrated elderberry were observed (Table 3).

It is well known that dietary anthocyanins or anthocyanin-enriched extracts reduce the actions of oxidative stress, proliferation, inflammation, and angiogenesis, all processes involved in cancer progression (Lamy et al., 2006; Lamy et al., 2008; Lin, Gong, Song, & Cui, 2016; Wang & Stoner, 2008). It has been reported that the incorporation of anthocyanins by endothelial cells enhanced their resistance to the damaging effects of reactive oxygen species (ROS) (Youdim, Martin, & Joseph, 2000). In addition, anthocyanin extracts from black soybean increased the survival of U-87 MG cells accompanied by decreased levels of ROS (Kim et al., 2012). Here, one may envision that the presence of these antioxidant molecules in our extracts could scavenge the free radicals and other ROS (Lobo, Patil, Phatak, & Chandra, 2010), thereby sensitizing glioma and brain endothelial cells to elderberry and elderflower treatment. In agreement with this, our data obtained with individual anthocyanins and rutins confirms their efficacy at inhibiting cell proliferation. For most of the cell lines tested, Cy-3-sam-5-glu and rutin were the best inhibitors, suggesting that they are involved in the inhibitory action of berry or flower extracts. The fact that there is a 2.2-fold increase of Cy-3-sam-5-glu/cyanidin 3,5-diglucoside and 1.5-fold increase of rutin contents in concentrated elderberry, as compared to 'Kent' and 'Scotia' extracts in 50 µL for 1 mL cell culture media, could explain the increased efficiency at inhibiting cell proliferation (Table 3).

When a combination of two or more compounds exhibits a more potent effect than the addition of the effects from the individual compounds at equal concentrations, the effect is considered synergistic (Tallarida, 2011). In this study, we tested whether bioactive compounds found in elderberry extracts can act synergistically to better inhibit the growth of glioma and brain endothelial cells than elderflower extracts where anthocyanins are not present. The results showed that the combination of two anthocyanin compounds (Dp-3-rut, Cy-3-sam-5-glu) and rutin exhibited a greater inhibitory effect than did the individual compounds for most cell lines tested, suggesting that the antiproliferative effects of elderberries results in part from interactions between bioactive components. In support of this, it has been reported that phytochemicals contained in fruits and vegetables often work collaboratively to achieve health benefits (R. H. Liu, 2004; Seeram, Adams, Hardy, & Heber, 2004).

Several epidemiologic and experimental studies have demonstrated the health-promoting benefits provided by regular berry consumption, including some against the incidence of certain cancers (Bishayee et al., 2016; Lee & Finn, 2007; Zafra-Stone et al., 2007). Depending on the diet, the daily intake of anthocyanins in humans has been estimated to range from several milligrams to hundreds of milligrams (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). For example, the total mean intake of anthocyanins from a Western diet was estimated at 12.5 mg/d (Wu et al., 2006) comparatively to 64.9 mg/d from a Mediterranean diet (Zamora-Ros et al., 2011). An enhanced intake of dietary anthocyanins may be occurring with the consumption of commercially available anthocyanin extract. Indeed, at least 14.5 mg of anthocyanins can be obtained in one single teaspoon (5 mL) of concentrated elderberry extract (IMMUNIA™). Oral consumption of berry phytochemicals can directly be absorbed in the oral cavity and in the stomach as well as in the gastrointestinal tract (Bishayee et al., 2016; He & Giusti, 2010; Prior & Wu, 2006). In addition, anthocyanins can be taken up by human vascular endothelial cells (Youdim et al., 2000; Ziberna et al., 2012) and have demonstrated ability to cross the blood-brain barrier (BBB) (Andres-Lacueva et al., 2005; Faria et al., 2014; Youdim, Qaiser, Begley, Rice-Evans, & Abbott, 2004). It is also worth pointing out the ability of flavonol rutin to also pass through the BBB (Habtemariam, 2016). Moreover, anthocyanins are rapidly detected in plasma after consumption of elderberry extract (Cao & Prior, 1999).

Thus, integration of elderberries in the diet might be a comprehensive strategy for preventing the molecular events involved in chronic disease progression such as cancer. Human clinical trials demonstrating the chemopreventive effects of berry preparations have focused mainly on black raspberries (Kresty, Mallery, & Stoner, 2016). However, it is likely that other berry types such as elderberries may also be effective for chemoprevention in humans. Future studies are required to better understand the molecular mechanisms of action and effectiveness *in vivo*, as elderberry and elderflower extracts have potential indications as cancer chemopreventive agents.

Acknowledgements

The authors thank the companies "Au Versant du Sureau" and "Fruitomed Inc." for providing fruits, flowers and IMMUNIA™. We thank also Annie Levert for her technical support. This work was funded by the Institutional Research Chair in Cancer Prevention and Treatment held by Dr. Borhane Annabi at UQAM (Montréal, QC). We would like to thank the Innovation and Technologies Directorate of *Ministère de l'agriculture, des pêcheries et de l'alimentation* (MAPAQ, Québec, QC) for their financial support.

Conflict of interest

The authors declare no conflict of interest.

Financial support

This study was funded by the Institutional Research Chair in Cancer Prevention and Treatment held by Dr. Borhane Annabi at UQAM and by the Innovation and Technologies Directorate of MAPAQ.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2017.10.048.

References

Abukhdeir, A. M., & Park, B. H. (2008). P21 and p27: Roles in carcinogenesis and drug resistance. *Expert Reviews in Molecular Medicine, 10*, e19. http://dx.doi.org/10.1017/S1462399408000744.

Alves, T. R., Lima, F. R., Kahn, S. A., Lobo, D., Dubois, L. G., Soletti, R., ... Neto, V. M. (2011). Glioblastoma cells: A heterogeneous and fatal tumor interacting with the parenchyma. *Life Sciences, 89*(15–16), 532–539. http://dx.doi.org/10.1016/j.lfs.2011.04.022.

Amin, A. R., Kucuk, O., Khuri, F. R., & Shin, D. M. (2009). Perspectives for cancer prevention with natural compounds. *Journal of Clinical Oncology, 27*(16), 2712–2725. http://dx.doi.org/10.1200/JCO.2008.20.6235.

Andres-Lacueva, C., Shukitt-Hale, B., Galli, R. L., Jauregui, O., Lamuela-Raventos, R. M., & Joseph, J. A. (2005). Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory. *Nutritional Neuroscience, 8*(2), 111–120.

Bishayee, A., Haskell, Y., Do, C., Siveen, K. S., Mohandas, N., Sethi, G., & Stoner, G. D. (2016). Potential benefits of edible berries in the management of aerodigestive and gastrointestinal tract cancers: Preclinical and clinical evidence. *Critical Reviews in Food Science and Nutrition, 56*(10), 1753–1775. http://dx.doi.org/10.1080/10408398.2014.982243.

Canavese, M., Santo, L., & Raje, N. (2012). Cyclin dependent kinases in cancer: Potential for therapeutic intervention. *Cancer Biology & Therapy, 13*(7), 451–457. http://dx.doi.org/10.4161/cbt.19589.

Cao, G., & Prior, R. L. (1999). Anthocyanins are detected in human plasma after oral administration of an elderberry extract. *Clinical Chemistry, 45*(4), 574–576.

Charlebois, D., Byers, P. L., Finn, C. E., & Thomas, A. L. (2010). Elderberry: Botany, horticulture, potential. *Horticultural Reviews, 37*, 213–280. http://dx.doi.org/10.1002/9780470543672.ch4.

Chen, C., Pore, N., Behrooz, A., Ismail-Beigi, F., & Maity, A. (2001). Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. *Journal of Biological Chemistry, 276*(12), 9519–9525. http://dx.doi.org/10.1074/jbc.M010144200.

Dvorak, H. F., Weaver, V. M., Tlsty, T. D., & Bergers, G. (2011). Tumor microenvironment and progression. *Journal of Surgical Oncology, 103*(6), 468–474. http://dx.doi.org/10.1002/jso.21709.

Faria, A., Meireles, M., Fernandes, I., Santos-Buelga, C., Gonzalez-Manzano, S., Duenas, M., ... Calhau, C. (2014). Flavonoid metabolites transport across a human BBB model.

Food Chemistry, 149, 190–196. http://dx.doi.org/10.1016/j.foodchem.2013.10.095.
Finn, C. E., Thomas, A. L., Byers, P. L., & Serce, S. (2008). Evaluation of American (*Sambucus canadensis*) and European (*S. nigra*) elderberry genotypes grown in diverse environments and implications for cultivar development. *HortScience, 43*(5), 1385–1391.
Gil-Gomez, G., Berns, A., & Brady, H. J. (1998). A link between cell cycle and cell death: Bax and Bcl-2 modulate Cdk2 activation during thymocyte apoptosis. *EMBO Journal, 17*(24), 7209–7218. http://dx.doi.org/10.1093/emboj/17.24.7209.
Goldstone, S., Pavey, S., Forrest, A., Sinnamon, J., & Gabrielli, B. (2001). Cdc25-dependent activation of cyclin A/cdk2 is blocked in G2 phase arrested cells independently of ATM/ATR. *Oncogene, 20*(8), 921–932. http://dx.doi.org/10.1038/sj.onc.1204177.
Habtemariam, S. (2016). Rutin as a natural therapy for Alzheimer's disease: Insights into its mechanisms of action. *Current Medicinal Chemistry, 23*(9), 860–873.
Hardee, M. E., & Zagzag, D. (2012). Mechanisms of glioma-associated neovascularization. *American Journal of Pathology, 181*(4), 1126–1141. http://dx.doi.org/10.1016/j.ajpath.2012.06.030.
Harper, J. W., Elledge, S. J., Keyomarsi, K., Dynlacht, B., Tsai, L. H., Zhang, P., ... Swindell, E. (1995). Inhibition of cyclin-dependent kinases by p21. *Molecular Biology of the Cell, 6*(4), 387–400.
Harris, A. L. (2002). Hypoxia–A key regulatory factor in tumour growth. *Nature Reviews Cancer, 2*(1), 38–47. http://dx.doi.org/10.1038/nrc704.
Harvey, K. J., Blomquist, J. F., & Ucker, D. S. (1998). Commitment and effector phases of the physiological cell death pathway elucidated with respect to Bcl-2 caspase, and cyclin-dependent kinase activities. *Molecular and Cellular Biology, 18*(5), 2912–2922.
He, J., & Giusti, M. M. (2010). Anthocyanins: Natural colorants with health-promoting properties. *Annual Review of Food Science and Technology, 1*, 163–187. http://dx.doi.org/10.1146/annurev.food.080708.100754.
Hendriksen, E. M., Span, P. N., Schuuring, J., Peters, J. P., Sweep, F. C., van der Kogel, A. J., & Bussink, J. (2009). Angiogenesis, hypoxia and VEGF expression during tumour growth in a human xenograft tumour model. *Microvascular Research, 77*(2), 96–103. http://dx.doi.org/10.1016/j.mvr.2008.11.002.
Hou, D. X. (2003). Potential mechanisms of cancer chemoprevention by anthocyanins. *Current Molecular Medicine, 3*(2), 149–159.
Jensen, R. L. (2009). Brain tumor hypoxia: Tumorigenesis, angiogenesis, imaging, pseudoprogression, and as a therapeutic target. *Journal of Neuro-Oncology, 92*(3), 317–335. http://dx.doi.org/10.1007/s11060-009-9827-2.
Kasten, M. M., & Giordano, A. (1998). PRb and the cdks in apoptosis and the cell cycle. *Cell Death and Differentiation, 5*(2), 132–140. http://dx.doi.org/10.1038/sj.cdd.4400323.
Kaur, B., Khwaja, F. W., Severson, E. A., Matheny, S. L., Brat, D. J., & Van Meir, E. G. (2005). Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis. *Neuro Oncology, 7*(2), 134–153. http://dx.doi.org/10.1215/S1152851704001115.
Kim, Y. K., Yoon, H. H., Lee, Y. D., Youn, D. Y., Ha, T. J., Kim, H. S., & Lee, J. H. (2012). Anthocyanin extracts from black soybean (*Glycine max* L.) protect human glial cells against oxygen-glucose deprivation by promoting autophagy. *Biomolecules & Therapeutics (Seoul), 20*(1), 68–74. http://dx.doi.org/10.4062/biomolther.2012.20.1.068.
Koch, C. J., Jenkins, W. T., Jenkins, K. W., Yang, X. Y., Shuman, A. L., Pickup, S., ... Evans, S. M. (2013). Mechanisms of blood flow and hypoxia production in rat 9L-epigastric tumors. *Tumor Microenvironment and Therapy, 1*, 1–13. http://dx.doi.org/10.2478/tumor-2012-0001.
Koff, A. (2006). How to decrease p27Kip1 levels during tumor development. *Cancer Cell, 9*(2), 75–76. http://dx.doi.org/10.1016/j.ccr.2006.01.020.
Kresty, L. A., Mallery, S. R., & Stoner, G. D. (2016). Black raspberries in cancer clinical trials: Past, present and future. *Journal of Berry Research, 6*(2), 251–261. http://dx.doi.org/10.3233/JBR-160125.
Lakka, S. S., & Rao, J. S. (2008). Antiangiogenic therapy in brain tumors. *Expert Review of Neurotherapeutics, 8*(10), 1457–1473. http://dx.doi.org/10.1586/14737175.8.10.1457.
Lamy, S., Beaulieu, E., Labbe, D., Bedard, V., Moghrabi, A., Barrette, S., ... Beliveau, R. (2008). Delphinidin, a dietary anthocyanidin, inhibits platelet-derived growth factor ligand/receptor (PDGF/PDGFR) signaling. *Carcinogenesis, 29*(5), 1033–1041. http://dx.doi.org/10.1093/carcin/bgn070.
Lamy, S., Blanchette, M., Michaud-Levesque, J., Lafleur, R., Durocher, Y., Moghrabi, A., ... Beliveau, R. (2006). Delphinidin, a dietary anthocyanidin, inhibits vascular endothelial growth factor receptor-2 phosphorylation. *Carcinogenesis, 27*(5), 989–996. http://dx.doi.org/10.1093/carcin/bgi279 bgi279 [pii].
Lee, J., & Finn, C. E. (2007). Anthocyanins and other polyphenolics in American elderberry (*Sambucus canadensis*) and European elderberry (*S. nigra*) cultivars. *Journal of the Science of Food and Agriculture, 87*(14), 2665–2675. http://dx.doi.org/10.1002/jsfa.3029.
Lin, B. W., Gong, C. C., Song, H. F., & Cui, Y. Y. (2016). Effects of anthocyanins on the prevention and treatment of cancer. *British Journal of Pharmacology*. http://dx.doi.org/10.1111/bph.13627.
Liu, R. H. (2004). Potential synergy of phytochemicals in cancer prevention: Mechanism of action. *Journal of Nutrition, 134*(12 Suppl), 3479S–3485S.
Liu, Y., Cox, S. R., Morita, T., & Kourembanas, S. (1995). Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. *Circulation Research, 77*(3), 638–643.
Ljungkvist, A. S., Bussink, J., Kaanders, J. H., & van der Kogel, A. J. (2007). Dynamics of tumor hypoxia measured with bioreductive hypoxic cell markers. *Radiation Research, 167*(2), 127–145.
Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Reviews, 4*(8), 118–126. http://dx.doi.org/10.4103/0973-7847.70902.
Loizzo, M. R., Pugliese, A., Bonesi, M., Tenuta, M. C., Menichini, F., Xiao, J., & Tundis, R. (2016). Edible flowers: A rich source of phytochemicals with antioxidant and hypoglycemic properties. *Journal of Agriculture and Food Chemistry, 64*(12), 2467–2474. http://dx.doi.org/10.1021/acs.jafc.5b03092.
Louis, D. N., Ohgaki, H., Wiestler, O. D., Cavenee, W. K., Burger, P. C., Jouvet, A., ... Kleihues, P. (2007). The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathologica, 114*(2), 97–109. http://dx.doi.org/10.1007/s00401-007-0243-4.
Malumbres, M., & Barbacid, M. (2009). Cell cycle, CDKs and cancer: A changing paradigm. *Nature Reviews Cancer, 9*(3), 153–166. http://dx.doi.org/10.1038/nrc2602.
Manach, C., Williamson, G., Morand, C., Scalbert, A., & Remesy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *American Journal of Clinical Nutrition, 81*(1 Suppl), 230S–242S doi: 81/1/230S [pii].
Meikrantz, W., Gisselbrecht, S., Tam, S. W., & Schlegel, R. (1994). Activation of cyclin A-dependent protein kinases during apoptosis. *Proceedings of the National Academy of Sciences USA, 91*(9), 3754–3758.
Musgrove, E. A., Caldon, C. E., Barraclough, J., Stone, A., & Sutherland, R. L. (2011). Cyclin D as a therapeutic target in cancer. *Nature Reviews Cancer, 11*(8), 558–572. http://dx.doi.org/10.1038/nrc3090.
Ozgen, M., Scheerens, J. C., Reese, R. N., & Miller, R. A. (2010). Total phenolic, anthocyanin contents and antioxidant capacity of selected elderberry (*Sambucus canadensis* L.) accessions. *Pharmacognosy Magazine, 6*(23), 198–203. http://dx.doi.org/10.4103/0973-1296.66936.
Pistollato, F., Abbadi, S., Rampazzo, E., Persano, L., Della Puppa, A., Frasson, C., ... Basso, G. (2010). Intratumoral hypoxic gradient drives stem cells distribution and MGMT expression in glioblastoma. *Stem Cells, 28*(5), 851–862. http://dx.doi.org/10.1002/stem.415.
Prior, R. L., & Wu, X. (2006). Anthocyanins: Structural characteristics that result in unique metabolic patterns and biological activities. *Free Radical Research, 40*(10), 1014–1028. http://dx.doi.org/10.1080/10715760600758522.
Pucci, B., Kasten, M., & Giordano, A. (2000). Cell cycle and apoptosis. *Neoplasia, 2*(4), 291–299.
Ramachandran, C., Nair, S. M., Escalon, E., & Melnick, S. J. (2012). Potentiation of etoposide and temozolomide cytotoxicity by curcumin and turmeric force in brain tumor cell lines. *Journal of Complementary and Integrative Medicine, 9*. http://dx.doi.org/10.1515/1553-3840.1614 Article 20.
Rooprai, H. K., Christidou, M., & Pilkington, G. J. (2003). The potential for strategies using micronutrients and heterocyclic drugs to treat invasive gliomas. *Acta Neurochirurgica (Wien), 145*(8), 683–690.
Seeram, N. P., Adams, L. S., Hardy, M. L., & Heber, D. (2004). Total cranberry extract versus its phytochemical constituents: Antiproliferative and synergistic effects against human tumor cell lines. *Journal of Agriculture and Food Chemistry, 52*(9), 2512–2517. http://dx.doi.org/10.1021/jf0352778.
Sheweita, S. A., & Sheikh, B. Y. (2011). Can dietary antioxidants reduce the incidence of brain tumors? *Current Drug Metabolism, 12*(6), 587–593.
Sidor, A., & Gramza-Michałowska, A. (2015). Advanced research on the antioxidant and health benefit of elderberry (*Sambucus nigra*) in food – A review. *Journal of Functional Foods, 18*, 941–958.
Sroka, J. T., Marshall, S. M., & Putz, F. E. (2014). Antioxidation capacity of fruit and flower extracts from American elderberry (*Sambucus canadensis*). *JUR, 15*(3), 1–5.
Surh, Y. J. (2003). Cancer chemoprevention with dietary phytochemicals. *Nature Reviews Cancer, 3*(10), 768–780.
Tallarida, R. J. (2011). Quantitative methods for assessing drug synergism. *Genes Cancer, 2*(11), 1003–1008. http://dx.doi.org/10.1177/1947601912440575.
Taylor, W. R., DePrimo, S. E., Agarwal, A., Agarwal, M. L., Schonthal, A. H., Katula, K. S., & Stark, G. R. (1999). Mechanisms of G2 arrest in response to overexpression of p53. *Molecular Biology of the Cell, 10*(11), 3607–3622.
Thole, J. M., Kraft, T. F., Sueiro, L. A., Kang, Y. H., Gills, J. J., Cuendet, M., ... Lila, M. A. (2006). A comparative evaluation of the anticancer properties of European and American elderberry fruits. *Journal of Medicinal Food, 9*(4), 498–504. http://dx.doi.org/10.1089/jmf.2006.9.498.
Thomas, A. L., Byers, P. L., & Ellersieck, M. R. (2009). Productivity and characteristics of American elderberry in response to various pruning methods. *HortScience, 44*(3), 671–677.
Thomas, A. L., Byers, P. L., Gu, S., Avery, J. D., Jr., Kaps, M., Datta, A., ... Rottinghaus, G. E. (2015). Occurrence of polyphenols, organic acids, and sugars among diverse elderberry genotypes grown in three Missouri (USA) locations. *Acta Horticulture, 1061*, 147–154. http://dx.doi.org/10.17660/ActaHortic. 2015.1061.14.
Tomás-Barberán, F. A., & Espín, J. C. (2001). Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *Journal of the Science of Food and Agriculture, 81*, 853–876. http://dx.doi.org/10.1002/jsfa.885.
Uttara, B., Singh, A. V., Zamboni, P., & Mahajan, R. T. (2009). Oxidative stress and neurodegenerative diseases: A review of upstream and downstream antioxidant therapeutic options. *Current Neuropharmacology, 7*(1), 65–74. http://dx.doi.org/10.2174/157015909787602823.
Viapiana, A., & Wesolowski, M. (2017). The phenolic contents and antioxidant activities of infusions of *Sambucus nigra* L. *Plant Foods for Human Nutrition*. http://dx.doi.org/10.1007/s11130-016-0594-x.
Wang, L. S., & Stoner, G. D. (2008). Anthocyanins and their role in cancer prevention. *Cancer Letters, 269*(2), 281–290. http://dx.doi.org/10.1016/j.canlet.2008.05.020.
Wiseman, M. (2008). The second world cancer research fund/American Institute for Cancer Research expert report. Food, nutrition, physical activity, and the prevention of cancer: A global perspective. *Proceedings of the Nutrition Society, 67*(3), 253–256.
Wu, X., Beecher, G. R., Holden, J. M., Haytowitz, D. B., Gebhardt, S. E., & Prior, R. L. (2006). Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *Journal of Agriculture and Food Chemistry, 54*(11),

4069–4075. http://dx.doi.org/10.1021/jf060300l.
Wu, X., Gu, L., Prior, R. L., & McKay, S. (2004). Characterization of anthocyanins and proanthocyanidins in some cultivars of Ribes, Aronia, and Sambucus and their antioxidant capacity. *Journal of Agriculture and Food Chemistry, 52*(26), 7846–7856. http://dx.doi.org/10.1021/jf0486850.
Youdim, K. A., Martin, A., & Joseph, J. A. (2000). Incorporation of the elderberry anthocyanins by endothelial cells increases protection against oxidative stress. *Free Radical Biology and Medicine, 29*(1), 51–60.
Youdim, K. A., Qaiser, M. Z., Begley, D. J., Rice-Evans, C. A., & Abbott, N. J. (2004). Flavonoid permeability across an in situ model of the blood-brain barrier. *Free Radical Biology and Medicine, 36*(5), 592–604. http://dx.doi.org/10.1016/j.freeradbiomed.2003.11.023.
Zafra-Stone, S., Yasmin, T., Bagchi, M., Chatterjee, A., Vinson, J. A., & Bagchi, D. (2007). Berry anthocyanins as novel antioxidants in human health and disease prevention. *Molecular Nutrition & Food Research, 51*(6), 675–683. http://dx.doi.org/10.1002/mnfr.200700002.
Zamora-Ros, R., Knaze, V., Lujan-Barroso, L., Slimani, N., Romieu, I., Touillaud, M., ... Gonzalez, C. A. (2011). Estimation of the intake of anthocyanidins and their food sources in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. *British Journal of Nutrition, 106*(7), 1090–1099. http://dx.doi.org/10.1017/S0007114511001437.
Zhang, Y., Vareed, S. K., & Nair, M. G. (2005). Human tumor cell growth inhibition by nontoxic anthocyanidins, the pigments in fruits and vegetables. *Life Sciences, 76*(13), 1465–1472.
Zhou, J., Schmid, T., Schnitzer, S., & Brune, B. (2006). Tumor hypoxia and cancer progression. *Cancer Letters, 237*(1), 10–21. http://dx.doi.org/10.1016/j.canlet.2005.05.028.
Zhou, Y., Zheng, J., Li, Y., Xu, D. P., Li, S., Chen, Y. M., & Li, H. B. (2016). Natural polyphenols for prevention and treatment of cancer. *Nutrients, 8*(8), http://dx.doi.org/10.3390/nu8080515.
Ziberna, L., Tramer, F., Moze, S., Vrhovsek, U., Mattivi, F., & Passamonti, S. (2012). Transport and bioactivity of cyanidin 3-glucoside into the vascular endothelium. *Free Radical Biology and Medicine, 52*(9), 1750–1759. http://dx.doi.org/10.1016/j.freeradbiomed.2012.02.027.