Published in final edited form as:

Nutr Cancer. 2009 November ; 61(6): 816–826. doi:10.1080/01635580903285148.

Black Raspberry Components Inhibit Proliferation, Induce Apoptosis and Modulate Gene Expression in Rat Esophageal Epithelial Cells

Nancy N. Zikri¹, Kenneth M. Riedl², Li-Shu Wang³, John F. Lechner^{3,4}, Steven J. Schwartz², and Gary D. Stoner³

¹Division of Environmental Health Sciences, College of Public Health, The Ohio State University, Tzagournis Medical Research Facility, 420 West 12th Avenue, Columbus, OH 43210

²Department of Food Science and Technology, The Ohio State University, 2015 Fyffe Road, Columbus, OH 43210

³Division of Hematology and Oncology and Comprehensive Cancer Center, The Ohio State University, 2001 Polaris Parkway, Columbus, OH 43240

⁴Maine Center for Toxicology and Environmental Health, University of Southern Maine, P.O. Box 9300, Portland, ME 04104, USA

Abstract

We have shown that a diet containing freeze-dried black raspberries (BRB) inhibits the development of chemically-induced cancer in the rat esophagus. To provide insights into possible mechanisms by which BRB inhibit esophageal carcinogenesis, we evaluated an ethanol (EtOH) extract of BRB, and two component anthocyanins (cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside) in BRB, for their effects on growth, apoptosis and gene expression in rat esophageal epithelial cell lines. The EtOH extract and both anthocyanins selectively caused significant growth inhibition and induction of apoptosis in a highly tumorigenic cell line (RE-149 DHD) but not in a weakly tumorigenic line (RE-149). The uptake of anthocyanins from the EtOH extract into RE-149 DHD cells far exceeded their uptake into RE-149 cells, which may have accounted for the selective effects of the extract on growth and apoptosis of RE-149 DHD cells. The growth inhibitory and pro-apoptotic effects were enhanced by the daily addition of the EtOH extract and the anthocyanins to the medium. Interestingly, the EtOH extract did not alter cyclooxygenase-2 (COX-2) and nitric oxide synthase (i-NOS) expression in RE-149 DHD cells whereas, both anthocyanins down-regulated the expressions of these genes. This differential effect may have been related to the relative amounts of anthocyanins in the extract versus when they were added individually to the medium. We conclude that the selective effects of the EtOH extract on growth and apoptosis of highly tumorigenic rat esophageal epithelial cells *in vitro* may be due to preferential uptake and retention of its component anthocyanins, and this may also be responsible for the greater inhibitory effects of freeze-dried whole berries on tumor cells *in vivo*.

INTRODUCTION

Esophageal cancer is the sixth leading cause of cancer death in the world and is one of the least studied cancers worldwide (1). The American Cancer Society estimates that, in 2008,

Address correspondence to: Gary D. Stoner, Ph.D., Division of Hematology/Oncology and Comprehensive Cancer Center, 2001 Polaris Parkway, Columbus, OH 43240, Phone: 614-293-3268, FAX: 614-293-5952, gary.stoner@osumc.edu.

16,470 new esophageal cancer cases will be diagnosed and 14,280 will die from esophageal cancer in the United States (2). The two principal types of esophageal cancer are squamous cell carcinoma (SCC) and adenocarcinoma, and each type has different etiological factors (3,4). The prevalence of the two types of esophageal cancer has changed in the past two decades in the Western world. Earlier, SCC accounted for more than 75% of esophageal malignancies, but during the past 20 years, the incidence of adenocarcinoma has dramatically increased and is now more prevalent than SCC. The 5-year overall survival rate from esophageal cancer is only 16% (2). Factors responsible for this low survival rate are: a) the lack of screening programs which result in the delay of cancer detection; more than 50% of esophageal cancer patients present with unresectable disease or distant metastasis, and, b) the high recurrence rate after surgery or chemoradiotherapy (5).

Epidemiologic studies have shown a protective effect of regular vegetable and fruit consumption on the occurrence of human cancer at several sites including the esophagus (6,7). We reported a protective effect of dietary freeze-dried black raspberries (*Rubus occidentalis*, BRB) on tumor initiation and progression in the rat esophagus, as well as tumor progression in the rat colon (8,9). A methanol extract of BRB was shown to inhibit benzo(a)pyrene diol-epoxide induction of activated protein-1 (AP-1) and nuclear factor- κ B (NF- κ B) (10), as well as to reduce the expression of vascular endothelial growth factor (VEGF) in JB-6 mouse epidermal cells (11). This same extract produced a dose-dependent decrease in the transformation of benzo(a)pyrene-treated Syrian hamster embryo (SHE) cells (12). An ethanol extract of BRB was shown to suppress cell proliferation, inhibit the translation of VEGF, suppress inducible nitric oxide synthase activity and induce apoptosis in human oral squamous cell carcinoma cells (13). This extract was also shown to inhibit the growth of premalignant and malignant human oral cells by targeting cell cycle regulatory proteins (14).

Black raspberries contain compounds that have chemopreventive activity against esophageal and colon cancer in experimental animals (8,9). These compounds include vitamins A, C, E, and folic acid, calcium, selenium, β -sitosterol, ellagic acid, ferulic acid and quercetin, in addition to the anthocyanins; cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, cyanidin-3-*O*-xylosylrutinoside and cyanidin-3-*O*-sambubioside (8,15). Anthocyanins are plant pigments that belong to the class of phenolic compounds known as flavonoids (16). They are found in abundance in berries, grapes, cabbages and other pigmented fruits and vegetables. In contrast to other flavonoids, anthocyanins carry a positive charge in the central ring structure and are thus cations. In plants, they are present exclusively as glycosidic compounds that have glucose, xylose, galactose, rhamnose or arabinose attached to an aglycone (anthocyanidin) nucleus (17). The anthocyanins exhibit strong free radical scavenging activity compared to other flavonoids, they are of special interest since they are present in the diet at relatively high concentrations (18). In the U.S., the daily intake of anthocyanins ranges from 180–215 mg/day whereas; the daily intake of most other dietary flavonoids, including genistein, quercetin and apigenin is estimated to be only 20–25 mg/day. The amounts of the anthocyanins in BRB (in mg/g dry weight) are: cyanidin-3-*O*-glucoside (2.1), cyanidin-3-*O*-xylosylrutinoside (12.1) and cyanidin-3-*O*-rutinoside (20.5) (19). We chose to test cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside due to their relative abundance in the extract.

Previous studies have shown the protective effects of anthocyanins against carcinogenesis *in vitro* and *in vivo* (19). Delphinidin, cyanidin and petunidin were found to inhibit 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced transformation of mouse epidermal JB6 cells as well as the induced activation of the transcription factor, AP-1 (20). Hecht et al., found that EtOH and water-soluble extracts of BRB inhibit benzo(a)pyrene diol-epoxide induction of AP-1 and NF- κ B in mouse epidermal JB6 cells (19). They fractionated both

extracts and identified the most active constituents of the subfractions to be the anthocyanins, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-xylosylrutinoside. Cyanidin and delphinidin were shown to be potent inhibitors of epidermal growth factor receptor (EGFR) resulting in a significant decrease in the proliferation of A431 human vulva carcinoma cells which over express EGFR (21). In addition, cyanidin reduced the growth of human colon cancer cell lines HT29 and HCT116 and, cyanidin 3-*O*-beta-D glucoside induced apoptosis and inhibited growth of human lymphoid leukemia Molt 4B cells (22,23).

Cyclooxygenase (COX) enzymes catalyze the synthesis of prostaglandins from arachidonic acid. There are 2 cyclooxygenases, COX-1 and COX-2. COX-1 is constitutively expressed whereas, COX-2 is induced as an early response to growth factors, oncogenes, carcinogens, and tumor promoters (24–26). COX-2 has been linked with many cancers since chronic inflammation mediated through prostaglandins predisposes to cancer development. COX-2 is over-expressed in human head and neck and esophageal squamous cell carcinomas (27,28). Our laboratory reported an up-regulation of COX-2 expression in *N*-nitrosomethylbenzylamine (NMBA)-induced rat esophageal tumorigenesis, and also demonstrated that dietary BRB inhibit both gene and protein expression of COX-2 (29).

Nitric oxide (NO) is a free radical gas that acts as an intracellular secondary messenger in multiple pathways, including vasodilatation, neuronal transmission, and inflammation-related cytotoxicity pathways (30,31). NO is synthesized by a family of isoenzymes called NO synthases (NOS) (32). There are two NOS isoenzyme groups; the calcium-dependent, constitutively-expressed group, which includes the endothelial NOS (e-NOS) and the neuronal NOS forms; and the calcium-independent-inducible NOS (i-NOS), which is involved principally in tumor-induced immunosuppression and macrophage mediated cytotoxicity. i-NOS plays an important role in several malignancies including, head and neck, breast and esophageal cancers. Our laboratory demonstrated a correlation between the up-regulation of iNOS and neoplastic progression in the rat esophagus (33,34). We also showed that dietary BRB down-regulate both the gene and protein expression of i-NOS in NMBA-induced rat esophageal tumorigenesis (29).

In the present study, we investigated the effect of the EtOH BRB extract and two component anthocyanins of BRB; i.e., cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside, on growth, apoptosis, and gene expression of caspases 3 and 7, COX-2, and iNOS, in two rat esophageal epithelial cell lines, RE-149 and RE-149 DHD. RE-149 is a spontaneously immortalized cell line with low tumorigenic potential in syngeneic hosts, and RE-149 DHD, derived from treatment of RE-149 cells with benzo(*a*)pyrene dihydrodiol, is highly tumorigenic and has a point mutation in the p53 tumor suppressor gene (35,36). The objectives of these studies were to: a) determine if the EtOH BRB extract and the two anthocyanins exhibit selective effects on growth and apoptosis of rat esophageal tumor cells; b) assess whether the effects of BRB on rat esophagus *in vivo* correlate with the effects of the EtOH extract and the two BRB anthocyanins on rat esophageal epithelial cells *in vitro*; and, c) to evaluate the contribution of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside to any growth inhibitory effect of the EtOH extract.

MATERIALS AND METHODS

Cell culture

RE-149 and RE-149 DHD cells were propagated in Ham's F12 medium supplemented with 100 U penicillin/ml, 100 µg streptomycin /ml, 0.5 µg gentamycin/ml, 4 mM L-glutamine, 5 ng epidermal growth factor /ml, 0.4 µg hydrocortisone /ml, non-essential amino acids and trace elements. Medium and supplements were obtained from GIBCO BRL, Bethesda, MD.

RE-149 DHD cells were grown in 1% serum and RE-149 cells in 5% serum, because RE-149 cells do not proliferate in medium containing only 1% serum. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and air. The medium was changed every other day. At 80% confluency, both cell lines were transferred using 0.25% trypsin:EDTA solution (GIBCO BRL, Bethesda, MD).

The cell lines were evaluated for tumorigenic potential in syngeneic newborn (one week-old) F-344 rats (Charles River Laboratories, Inc, Wilmington, MA) as follows: 10⁶ viable cells in 300 µl Ham's F-12 medium were injected subcutaneously into the back of each one week-old rat (7–8 rats per cell line), and the development of tumors was followed up by palpating the backs of the rats once a week; any visible mass bigger than 0.5 mm was considered a tumor.

Black raspberry extracts and anthocyanins

Black raspberries of the Jewel variety were obtained from a single farm in Ohio and freeze-dried as described previously (8). To prepare the extracts, a sequential soxhlet extraction process was carried out in the laboratory of Dr. Stephen S. Hecht at the University of Minnesota, as follows: In the paper thimble of a soxhlet extractor, pentane, methylene chloride and ethanol were added individually, in a serial manner, to 25 grams of freeze-dried black raspberries. The residue was removed from the thimble and sonicated three times with 100 ml portions of water, 30 min each time. The extracts from each solvent extraction were combined and the solvents were removed by rotary evaporation, except in the case of water, which was removed by lyophilization. The ethanol extract represented approximately 50% of the freeze-dried black raspberries by weight. The two anthocyanins; cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside were purchased from Indofine Inc, Hillsborough, NJ. Cyanidin-3-*O*-glucoside was 99.6% pure and cyanidin-3-*O*-rutinoside was 97.1% pure by high performance liquid chromatography (HPLC).

Cell proliferation assay

A cell counting assay (CCK-8; Dojindo, Gaithersburg, MD) was used to measure cell proliferation. The basis of this assay is the formation of formazan dye due to the reduction of tetrazolium salt by dehydrogenases present in the mitochondria of viable cells. The amount of formazan dye is directly proportional to the number of live cells. RE-149 and RE-149 DHD cells were seeded in 96 well plates at a density of 10³ cells/well. They were grown in Ham's F12 medium supplemented as described above, for 24 hours. The next day, the medium was removed and fresh medium containing EtOH extract was added to the cells at concentrations ranging from 10–100 µg/ml of medium. Cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside were added at concentrations ranging from 10–50 µg/ml. Control cells were grown in extract free medium. Every day for 4 days, after the addition of the extracts or anthocyanins, cell viability was estimated by adding the CCK-8 reagent to the cells, incubating the cells with the reagent for 2 hours, and measuring the optical density (OD) with a plate reader. The effect of adding fresh extract daily, on cell proliferation was evaluated.

Anthocyanins were tested for their effects on growth at concentrations ranging from 10–50 µg/ml. Cyanidin-3-*O*-glucoside was dissolved in 0.2% dimethyl sulfoxide (DMSO), whereas cyanidin-3-*O*-rutinoside was dissolved in a mixture of 0.2% DMSO and 0.01% hydrochloric acid (HCL) to dissolve the compound. Medium containing 0.2% DMSO or 0.2% DMSO and 0.01% HCL without the anthocyanins was added to the control cells.

To determine whether the growth inhibitory effect of the EtOH extract might be due to the generation of hydrogen peroxide from the berry phenolics in the culture medium, we treated

RE-149 DHD cells with either 100 µg EtOH extract/ml or with 100 µg EtOH extract/ml plus 30 units catalase/ml (Sigma Aldrich, Saint Louis, MO). The proliferation rates of cells grown in medium alone vs medium + EtOH extract vs medium + EtOH extract + catalase were estimated and compared.

Caspase assay

RE-149 and RE-149 DHD cells were inoculated into 25 cm² flasks at a density of 1×10^4 cells / flask, six flasks were used per cell line. Twenty four hours later, the cells were treated with 100 µg EtOH extract/ ml as previously mentioned for 96 hours. 2×10^6 cells/flask were then collected, as recommended by the manufacturer, at the highest point of the log phase of the cell growth curve (96 hours). Caspase 3 and 7 combined activity was estimated using the CaspaTag caspase-3 and 7 fluorescent *in situ* assay kit (Chemicon, Temecula, CA) according to the manufacturer's protocol. The same procedure was repeated using 50 µg cyanidin-3-*O*-rutinoside/ml since it was more growth inhibitory than cyanidin-3-*O*-glucoside.

Uptake of anthocyanins into cells

RE-149 and RE-149 DHD cells were plated at a density of 2×10^5 per 25 cm² flasks with three flasks per cell type per time point. When the cultures were approximately 50% confluent, the cells were treated for 1, 4, 12, 24 and 48 hours with either 50 or 100 µg EtOH extract/ml. At each time point, the cells were washed with phosphate buffered saline (PBS), dissociated with PET (polyvinyl pyrrolidone, EGTA, trypsin) cell dissociation solution (Athena Diagnostics, Worcester MA). The culture medium was then added to the cells to neutralize the trypsin, and the cells were centrifuged. The supernatant was discarded and the cells washed and re-suspended in 1 ml of PBS and centrifuged again. The PBS was then discarded, cells suspended in 95% methanol and 5% formic acid, and then stored at -80°C. Cells were lysed by 3 freeze-thaw cycles at room temperature and anthocyanins were purified. A reversed phase C18 column (Symmetry 4.6 × 75 mm, 3.5 µm; Waters, Beverly, MA) was used to separate the anthocyanins using 1 % (v/v) formic acid in water as (A) and 1 % (v/v) formic acid in acetonitrile as (B). The initial condition was 0 % B increased linearly to 15 % B over 8.5 minutes, to 65% by 13 minutes then returning to the initial condition. The flow was constant at 0.8 ml/min. Cell extracts in 5 % (v/v) formic acid in methanol were adjusted to 20 % (v/v) of 5 % (v/v) formic acid in water for injection.

The eluate from the HPLC column was split to divert approximately 0.08 ml/min to a triple quadrupole mass spectrometer (Quattro Ultima; Waters, Beverly, MA). The mass spectrometer was operated in positive electrospray mode to monitor fragments of the flavylum cation glycosides. Selected reaction monitoring was employed to quantify the anthocyanins utilizing the characteristic fragmentation of the glycoside groups from the cyanidin aglycone. Thus cyanidin-3-*O*-glucoside was determined as the 449>287 m/z transition, cyanidin-3-*O*-sambubioside as 581>287, cyanidin-3-*O*-rutinoside as 595>287 and cyanidin-3-*O*-xylosylrutinoside as 727>287 m/z. Cyanidin-3-*O*-glucoside was used as the standard for quantitation, and results were reported in units of cyanidin-3-*O*-glucoside.

RNA isolation and semi-quantification using Real time PCR

RE-149 DHD cells, grown in Ham's F12 cell medium containing 1% fetal bovine serum, were treated with 10, 50, or 100 µg EtOH extract/ml, or with 50 µg/ml of cyanidin-3-*O*-glucoside or 50 µg cyanidin-3-*O*-rutinoside for 4 days. The media, with or without extract or anthocyanins, were replaced daily. Total RNA was isolated from treated and untreated cells, according to the protocol supplied with the TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD). The integrity of both 18S and 28S rRNA was determined using 1.6% agarose gel electrophoresis of approximately 1 µg RNA per sample and stained with

ethidium bromide. The purity of the RNA was also determined spectrophotometrically by determining the ratio of OD260/OD280 readings.

One-Step Real-Time RT-PCR was performed using the QuantiTect SYBR Green RT-PCR kit (Gibco BRL, Bethesda, MD) according to the manufacturer's protocol, and a GeneAmp 5700 sequence detection system (Perkin-Elmer Corp, Norwalk, CT). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as the control gene. The primers for caspases 3, 7 and HPRT were purchased from Superarray, Inc. (Frederick, MD). Thermocycling conditions used were: 30 min cycle at 50°C for reverse transcription, 15 min cycle at 95°C for activation of TaqDNA Polymerase, 30 sec cycle at 95°C for denaturation, 30 sec cycle at 55°C for annealing, and finally extension at 72°C for 30 sec, for a total of 40 cycles. The primers for COX-2 and i-NOS were designed from published sequences and were purchased from Invitrogen, Bethesda, MD. The base sequences of the two genes were as follows: COX-2, sense 5'-ATGCTCTTCCGAGCTGTGCT-3' and antisense 5'-CATGGGAGTTGGGCAGTCA3' and i-NOS, sense 5'-CCACAATACAATACTACTTGC3' and antisense 5'-GGGCTATAAGTTGCTGA3'. Triplicates were used for each concentration of EtOH and for control cells. A no template control (no RNA) sample and a no amplification control sample (no QuantiTect RT Mix), were used for each run to exclude genomic DNA contamination and fluorescence contamination, respectively.

Statistical analysis

Differences in cell proliferation, caspase-3 and 7 activity, and gene expression between control and EtOH extract- or anthocyanin-treated cells were tested for statistical significance using paired Student t-test with the p-value set at <0.05.

RESULTS

Tumorigenic potential of RE-149 and RE-149 DHD cells

RE-149 and RE-149 DHD cells produced well differentiated squamous cell carcinomas after injection into syngeneic one week-old F-344 rats (Table 1). RE-149 DHD cells produced large tumors, around 4 cm² in diameter, in 100% of the rats 2 weeks after injection of the cells. RE-149 cells produced smaller tumors, around 2 cm² in diameter, in 50% of the rats after 5 weeks.

The effect of BRB extracts and anthocyanins on the growth of RE-149 and RE-149 DHD cells

Treatment of RE-149 and RE-149 DHD cells with 10, 50 or 100 µg EtOH extract/ml for 4 days, and adding fresh extract on the 3rd day, resulted in a dose-dependent growth inhibition of RE-149 DHD cells only (Table 2). The most effective inhibitory concentration of the extract was 100 µg/ml; this concentration resulted in a 45% and 16% inhibition in the growth of RE-149 DHD and RE-149 cells, respectively. The growth inhibition was significant (p<0.05) only for the RE-149 DHD cells.

The effects of the two anthocyanins, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside, on the growth of RE-149 and RE-149 DHD cells were determined at concentrations ranging from 10–50 µg/ml, using the same protocol as for the BRB extracts. Growth inhibition of the anthocyanins for RE-149 DHD cells was significant, with 50 µg/ml of cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-glucoside reducing cell proliferation by 45 and 41%, respectively, when changing the medium on the 3rd day (Tables 3 and 4). Similar to the extract data, both anthocyanins were more growth inhibitory for RE-149 DHD cells than for RE-149 cells (Tables 3 and 4).

The effect of frequent addition of fresh extract/anthocyanins on the growth of RE-149 and RE-149 DHD cells

Daily replacement of the entire medium containing either the EtOH extract or the individual anthocyanins produced significantly more growth inhibition of RE-149 DHD cells than replacing the medium on day 3 only ($p < 0.05$) (Tables 2, 3 and 4). A 60% growth inhibition of RE-149 DHD cells was observed when adding fresh medium containing 100 μg EtOH extract daily, compared to only a 45% inhibition with medium change on the 3rd day (Table 2). Fifty μg of cyanidin-3-*O*-rutinoside/ml produced a 69% inhibition in the growth of RE-149 DHD cells when changing the entire medium daily as compared to a 45% inhibition when the medium was changed on the 3rd day (Table 3). Similarly, cyanidin-3-*O*-glucoside was more effective when added in fresh medium on a daily basis (Table 4).

The effect of catalase on the growth-inhibitory effect of the EtOH extract

To determine if the growth inhibitory effect of the EtOH extract was due to the production of hydrogen peroxide from the berry phenolics into the medium, catalase (30 units/ml) was added to the medium along with 100 μg EtOH extract/ml. The results indicated that the addition of catalase did not reduce the growth inhibitory effect of the EtOH extract for RE-149 DHD cells (Fig. 1). In fact, the growth-inhibitory effect of the extract appeared to be enhanced by the addition of catalase to the medium, but this effect was not significant.

The effect of the EtOH extract and cyanidin-3-*O*-rutinoside on apoptosis in RE-149 and RE-149 DHD cells

The EtOH extract induced the activity of caspases 3 and 7 in RE-149 DHD cells, but not in RE-149 cells. RE-149 DHD cells treated with 100 μg EtOH extract/ml, with fresh medium added on the 3rd day, had 38% ($p < 0.05$) more active caspases 3 and 7 than the control cells, while RE-149 cells had only 13% ($p > 0.05$) more active caspases 3 and 7 than the control cells (Fig. 2A). When fresh extract was added daily, RE-149 DHD treated cells had 51% more active caspases 3 and 7 compared to the control cells (Fig. 2B). Fifty μg cyanidin-3-*O*-rutinoside/ml caused a 23% increase ($p < 0.05$) in caspases 3 and 7 activity in RE-149 DHD cells, but only a non-significant 5% increase in RE-149 cells (Fig. 3).

Cellular anthocyanins uptake

No anthocyanins were detected in control samples (cells not treated with berry extract). RE-149 DHD cells treated with 100 μg EtOH extract/ml showed a maximum uptake of cyanidin-3-*O*-glucoside after 1 hour while cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-xylosylrutinoside had maximal uptakes 4 hours after treatment. The levels of all three anthocyanins dropped quickly after the initial increase in uptake, with relatively small amounts of each compound remaining in the cells at 12 and 24 hours (Fig. 4A). The kinetics of anthocyanin uptake by RE-149 DHD cells treated with 50 μg EtOH extract/ml was similar to that of the same cells treated with 100 μg /ml EtOH extract (data not shown). RE-149 cells showed the same pattern of anthocyanin uptake as RE-149 DHD cells, but the levels of anthocyanins in RE-149 cells were 100 times lower than in RE-149 DHD cells (compare Figs. 4A and 4B). The level of anthocyanins measured in medium containing the EtOH extract, but no cells, reached a peak after one hour, followed by a rapid decline; the half life of the 3 anthocyanins was approximately 5 hours (Fig. 4C). These data reflect the instability of the anthocyanins in culture medium.

Gene expression

RE-149 DHD cells treated with 100 μg EtOH extract/ml for 4 days, with fresh extract added daily, revealed a significant increase in caspase 3 expression at 72 and 96 hours, and a significant increase in caspase 7 at 48, 72 and 96 hours (Figs. 5A and 5B). In contrast,

treatment of RE-149 DHD cells with 100 µg EtOH extract/ml under the same conditions led to non-significant ($p>0.05$) increases in COX-2 expression (1.67-fold) (Fig. 6A) and i-NOS expression (2.5-fold) on the 4th day (Fig. 6B). Interestingly, treatment of RE-149 DHD cells with 50 µg/ml of either cyanidin-3-*O*-glucoside or cyanidin-3-*O*-rutinoside led to significant ($p<0.05$) reductions in both COX-2 and i-NOS expression. A time point experiment designed to determine the effects of 10, 50 and 100 µg EtOH extract/ml on the daily expression of COX-2 and i-NOS showed no time dependent pattern, with mostly up-regulation of both genes (data not shown).

DISCUSSION

Although the incidence of esophageal squamous cell carcinoma has been declining in the Western world for the past twenty years, it remains a serious disease that is usually diagnosed late, and whose survival rate is low. Primary prevention of this disease involves changes in lifestyle; e.g., cessation of tobacco smoking, alcohol consumption, and eating more balanced diets with sufficient vegetables and fruit. Secondary prevention, or chemoprevention, is another approach to reduce mortality from this disease and, ideally, should involve the use of agents that influence all stages of the carcinogenesis process.

Previous studies have demonstrated the beneficial effect of black raspberries in the prevention of a number of chemically-induced cancers in rodents including esophageal, colon and oral cancer (8,9,37). Diets containing black raspberries have been shown, in our laboratory, to inhibit events associated with both the initiation and promotion/progression stages of esophageal carcinogenesis in rats (8). In the present study, we have furthered our understanding of the mechanisms of action of black raspberry compounds by demonstrating that they selectively inhibit the growth and stimulate apoptosis of highly tumorigenic rat esophageal epithelial cells (RE-149 DHD) when compared to cells with lesser tumorigenic potential (RE-149). Importantly, it appears that the basis for this selectivity is due, at least in part, to the preferential uptake of black raspberry anthocyanins in the highly tumorigenic cells. This may also be the case *in vivo* since our recent studies have shown that a synthetic diet containing 5% black raspberries has a more pronounced effect on gene expression in carcinogen-induced rat esophageal papillomas than on premalignant tissues (unpublished data). The basis for the preferential uptake and retention of black raspberry anthocyanins in RE-149 DHD cells is unknown, but may be related to the fact that tumor cells (in this case, highly tumorigenic cells) tend to be more active in pinocytosis than “normal” cells (in this case, cells with lesser tumorigenic potential) and thus, could “ingest” extracellular anthocyanins more effectively. It is also possible that the uptake of anthocyanins in cells is receptor-mediated. If RE-149 DHD cells have more receptors for anthocyanins than RE-149 cells, then one might expect more active retention of anthocyanins in RE-149 DHD cells by receptor-mediated endocytosis. It is likely that one or both of these mechanisms may be responsible for the selective effects of the EtOH extract on the growth and apoptosis of RE-149 DHD cells.

The uptake of cyanidin-3-*O*-glucoside from the EtOH extract of BRB into RE-149 DHD cells occurred optimally at 1 hour after extract treatment (Fig. 4A). In contrast, the peaks for cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-xylosylrutinoside uptake were 4 hours post extract treatment. This was followed by a rapid decline in the levels of all three anthocyanins, but the rutinoside persisted in the cells at low concentrations for up-to 48 hours. These data are in agreement with a previous study by Matsumoto et al., who showed that, in rats and humans *in vivo*, cyanidin-3-*O*-glucoside reached peak levels in the plasma within one hour post-oral administration and decreased rapidly whereas, cyanidin-3-*O*-rutinoside levels were maximal at two hours and decreased slowly (38). The authors attributed this difference in uptake and retention to the different sugar moieties (glucoside

vs. rutinose) in the two anthocyanins. They also suggested that the glucosides may be deconjugated by intestinal hydrolases, whereas the rutinose may remain intact without hydrolysis. This could explain the persistence of cyanidin-3-*O*-rutinose in cultured cells and in plasma. A pharmacokinetic study in our laboratory showed that approximately 1% of the anthocyanins in 45 grams of orally administered black raspberry powder was absorbed into the blood of healthy volunteers (39). The levels of plasma anthocyanins were maximal between 1 and 2 hours after consumption of the berry powder, and by 12 hours, the levels reached baseline. The extent of uptake of the anthocyanins into the blood paralleled their relative abundance in the berries, with cyanidin-3-*O*-rutinose taken up to the greatest extent. However, unlike the results in the study of Matsumoto, et al., there was no evidence for preferential retention of the rutinose in plasma.

Apoptosis is orchestrated by a family of cysteine proteases, the caspases, which are activated in response to pro-apoptotic stimuli (40). Caspases promote apoptosis by: (a) inducing destructive enzymes such as DNases, (b) promoting mitochondrial cytochrome *c* release via Bcl-2 family proteins, and (c) destroying key structural and regulatory proteins. Caspases are divided into two subgroups based on their roles in the process of apoptosis. The two subgroups are upstream and downstream; the upstream group includes caspases 2, 8, 9 and 10 which are responsible for initiating the caspase cascade when they receive pro-apoptotic stimuli (41). The downstream groups are the “executioner” caspases which are responsible for cell destruction; this group includes caspases 3, 6 and 7. The EtOH extract induced apoptosis significantly in RE-149 DHD cells, but not in RE-149 cells, through stimulation of the expression and activities of downstream caspases 3 and 7. Our data are in agreement with other studies indicating that the induction of apoptosis is a potential chemopreventive mechanism of action for different berry types and for the anthocyanins (16,42–44). Cyanidin-3-*O*-rutinose (100 µg/ml) did not increase caspase 3 and 7 activities as much as the EtOH extract at the same concentration (23% induction with the rutinose versus 38% for the extract), but it retained selectivity for RE-149 DHD cells. Cyanidin-3-*O*-rutinose may not be the only component in the EtOH extract responsible for its pro-apoptotic effect; other compounds might also contribute to the induction of apoptosis. The frequency of addition of fresh EtOH extract to the cells influenced the induction of apoptosis, with enhanced caspase 3 and 7 activation observed with daily extract treatment (52% induction with daily treatment versus 23% with treatment on the 3rd day).

Chen et al., reported an inhibitory effect of dietary freeze-dried black raspberries on COX-2 and i-NOS mRNA and protein expression in NMBA-induced rat esophageal tumorigenesis (29). Similarly, treatment of RE-149 DHD cells *in vitro* with either cyanidin-3-*O*-rutinose or cyanidin-3-*O*-glucoside, at 50 µg/ml medium (equivalent to 103 and 79 µM, respectively), significantly ($p<0.05$) down-regulated COX-2 and i-NOS mRNA expression after 4 days (Figs. 6A and B). In contrast, treatment of RE-149 DHD cells with the EtOH extract for 4 days increased both COX-2 and i-NOS mRNA expression, although these increases were not significant ($p>0.05$). A potential explanation for this discrepancy between treatment effects with the individual anthocyanins versus the EtOH extract may be due to differences in anthocyanin concentrations. The amounts of cyanidin-3-*O*-rutinose and cyanidin-3-*O*-glucoside in the EtOH extract are 25 to 50-fold lower than those used in experiments with the individual anthocyanins (103 and 79 µM), and perhaps too low to down-regulate COX-2 and i-NOS expression.

Flavonoids and phenolic compounds are strong reducing agents, however, in the presence of metal ions such as copper or iron, they can be pro-oxidants and generate oxidative stress, especially *in vitro* (45,46). There is some controversy as to whether these compounds are actually converted to hydrogen peroxide *in vitro*, and whether the hydrogen peroxide by itself can inhibit cell proliferation. Our data showed that treatment of RE-149 DHD cells

with EtOH extract and catalase did not reduce the growth-inhibitory effect of the extract. In fact, catalase addition appeared to have increased the inhibitory potential of the extract albeit, not significantly (68% growth inhibition with the addition of catalase versus 52% with EtOH extract only). Liu and Sun pointed out that there is a difference between the anti-proliferative and anti-oxidant activities of polyphenols; the phenolic content of flavonoids does not always correlate with their anti-proliferative activity (47). If the anti-proliferative effect of phenolics is due to solely generation of hydrogen peroxide, one might anticipate that the phenolics would produce similar inhibitory effects on multiple cell lines when added to the medium at the same concentration, and this is not the case.

In conclusion, the EtOH extract of BRB, and the component anthocyanins in BRB, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside, selectively inhibited the growth and stimulated the apoptosis of RE-149 DHD cells, an effect that was increased by adding fresh extract or anthocyanins daily. The selectivity of the extract, and the two anthocyanins, for RE-149 DHD cells might be explained by preferential uptake of the anthocyanins in RE-149 DHD cells. The individual anthocyanins were growth inhibitory only at concentrations that far exceeded (by 25 to 50-fold) their amounts in the EtOH extract suggesting that either there are other components in the extract that are responsible for its chemopreventive effects and/or the anthocyanins act synergistically with each other and with other components in the extract to produce inhibitory effects. Studies are underway to identify other inhibitory components in the EtOH extract.

Acknowledgments

This study was supported by NCI Grant CA 103180 to GDS and USDA Grant 38903-02313 to the Ohio Agricultural Research and Development Center. This research is part of the Ph.D. thesis of N.Z. The authors acknowledge Dr. Stephen Hecht and Mr. Steven Carmella for providing the berry extract.

REFERENCES

1. Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 1999;80:827–841. [PubMed: 10074914]
2. Jemal A, Siegel R, Ward E, Hao Y, Xu J, et al. Cancer statistics, 2008. *CA Cancer J Clin* 2008;58:71–96. [PubMed: 18287387]
3. Daly JM, Fry WA, Little AG, Winchester DP, McKee RF, et al. Esophageal cancer: results of an American College of Surgeons patient care evaluation study. *J Am Coll Surg* 2000;190:562–573. [PubMed: 10801023]
4. Flood WA, Furastiere AA. Esophageal cancer. *Curr Opin Oncol* 1995;7:381–386. [PubMed: 7578389]
5. Kelsen D. Preoperative chemoradiotherapy for esophageal cancer. *J Clin Oncol* 2001;19:283–285. [PubMed: 11208816]
6. Block G, Patterson B, Subar A. Fruits, vegetables and cancer prevention: a review of the epidemiological evidence. *Nutr Cancer* 1992;18:1–29. [PubMed: 1408943]
7. Vecchia CL, Tavani A. Fruits and vegetables, and human cancer. *Eur J Cancer Prev* 1998;7:3–8. [PubMed: 9511846]
8. Kresty LA, Morse MA, Morgan C, Carlton PS, Lu J, et al. Chemoprevention of esophageal tumorigenesis by dietary administration of lyophilized black raspberries. *Cancer Res* 2001;61:6112–6119. [PubMed: 11507061]
9. Harris GK, Gupta A, Nines RG, Kresty LA, Habib SG, et al. Effects of lyophilized black raspberries on azoxymethane-induced colon cancer and 8-hydroxy-2'-deoxyguanosine levels in the Fischer 344 rat. *Nutr Cancer* 2001;40:125–133. [PubMed: 11962247]
10. Huang C, Huang Y, Li J, Hu W, Aziz R, et al. Inhibition of benzo(a)pyrene diol-epoxide-induced transactivation of activated protein 1 and nuclear factor-kappaB by black raspberry extracts. *Cancer Res* 2002;62:6857–6863. [PubMed: 12460899]

11. Huang C, Li J, Song L, Zhang D, Tong Q, et al. Black raspberry extracts inhibit benzo(a)pyrene diol-epoxide-induced activator protein 1 activation and VEGF transcription by targeting the phosphatidylinositol 3-kinase/Akt pathway. *Cancer Res* 2006;66:581–587. [PubMed: 16397275]
12. Xue H, Aziz RM, Sun N, Cassady JM, Kamendulis LM, et al. Inhibition of cellular transformation by berry extracts. *Carcinogenesis* 2001;22:351–356. [PubMed: 11181460]
13. Rodrigo K, Rawal Y, Renner R, Schwartz S, Tian Q, et al. Suppression of the tumorigenic phenotype in human oral squamous cell carcinoma cells by an ethanol extract derived from freeze-dried black raspberries. *Nutr Cancer* 2006;54:58–68. [PubMed: 16800773]
14. Han C, Ding H, Casto B, Stoner G, D'Ambrosio S. Inhibition of growth of premalignant human oral cell lines by extracts and components of black raspberries. *Nutr Cancer* 2005;52:207–217. [PubMed: 15860443]
15. Tian Q, Aziz RM, Stoner GD, Schwartz SJ. Anthocyanins determination in black raspberry (*Rubus occidentalis*) and biological specimens using liquid chromatography-electrospray ionization tandem mass spectrometry. *J Food Sci* 2005;70:43–47.
16. Hou DX, Fujii M, Terahara N, Yoshimoto M. Molecular mechanisms behind the chemopreventive effect of anthocyanidins. *J Biomed Biotech* 2004;5:321–325.
17. Harborne, JB.; Baxter, H. The Handbook of Natural Flavonoids. Vol. Vol 2. Chichester: John Wiley & Sons; 1999.
18. Hertog MG, Hollman PC, Katan MB, Kromhout D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutr Cancer* 1993;20:21–29. [PubMed: 8415127]
19. Hecht S, Huang C, Stoner G, Li J, Kenney P, et al. Identification of cyanidin glycosides as constituents of freeze-dried black raspberries which inhibit anti- benzo[a]pyrene-7,8 diol-9,10-epoxide induced NFκB and AP-1 activity. *Carcinogenesis* 2006;27:1617–1626. [PubMed: 16522666]
20. Hou DX, Kai K, Li JJ, Lin S, Terahara M, et al. Anthocyanidins inhibit activator protein 1 activity and cell transformation: structure-activity relationship and molecular mechanisms. *Carcinogenesis* 2004;25:29–36. [PubMed: 14514663]
21. Meiers S, Kemeny M, Weyand U, Castpar R, von Angerer E, et al. The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth factor receptor. *J Agric Food Chem* 2001;49:958–962. [PubMed: 11262056]
22. Kang SY, Seeram NP, Nair M, Bourquin LD. Tart cherry anthocyanins inhibit tumor development in Apc^{Min} mice and reduce proliferation of human colon cancer cells. *Cancer Lett* 2003;194:13–19. [PubMed: 12706854]
23. Katsuzaki H, Hibasami H, Ohwaski S, Ishikawa K, Imai K, et al. Cyanidin 3-O-beta-D-glucoside isolated from skin of black glycine max and other anthocyanins isolated from skin of red grape induce apoptosis in human lymphoid leukemia Molt 4B cells. *Oncol Rep* 2003;10:297–300. [PubMed: 12579261]
24. Herschman HR. Prostaglandin synthase 2. *Biochim Biophys Acta* 1996;1299:125–140. [PubMed: 8555245]
25. Subbaramaiah K, Telang N, Ramonetti JT, Araki R, DeVito B, et al. Transcription of cyclooxygenase-2 is enhanced in transformed mammary epithelial cells. *Cancer Res* 1996;56:4424–4429. [PubMed: 8813136]
26. Kelley DJ, Mestre JR, Subbaramaiah K, Sacks PG, Schantz SP, et al. Benzo[a]pyrene up-regulates cyclooxygenase-2 gene expression in oral epithelial cells. *Carcinogenesis* 1997;18:795–799. [PubMed: 9111217]
27. Chan G, Boyle JO, Yang EK, Zhang F, Sack PG, et al. Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. *Cancer Res* 1999;59:991–994. [PubMed: 10070952]
28. Ratnasinghe D, Tangrea J, Roth MJ, Dawsey S, Hu N, et al. Expression of cyclooxygenase-2 in human squamous cell carcinoma of the esophagus; an immunohistochemical survey. *Anticancer Res* 1999;19:171–174. [PubMed: 10226539]
29. Chen T, Hwang H, Rose M, Nines RG, Stoner GD. Chemopreventive properties of black raspberries in *N*-nitrosomethylbenzylamine-induced rat esophageal tumorigenesis: Down-

- regulation of cyclooxygenase-2, inducible nitric oxide synthase, and c-Jun. *Cancer Res* 2006;66:2853–2859. [PubMed: 16510608]
30. Knowles RG, Moncada S. Nitric oxide synthases in mammals. *Biochem J* 1994;298:249–258. [PubMed: 7510950]
 31. Anggard E. Nitric oxide: mediator, murderer, and medicine. *Lancet* 1994;343:1199–1206. [PubMed: 7909873]
 32. Loibl S, von Minckwitz G, Weber S, Sinn HP, Schini Kerth VB, et al. Expression of endothelial and inducible nitric oxide synthase in benign and malignant lesions of the breast and measurement of nitric oxide using electron paramagnetic resonance spectroscopy. *Cancer* 2002;95:1191–1198. [PubMed: 12216084]
 33. Chen T, Nines RG, Peschke SM, Kresty LA, Stoner GD. Chemopreventive effects of a selective nitric oxide synthase inhibitor on carcinogen-induced rat esophageal tumorigenesis. *Cancer Res* 2004;64:3714–3717. [PubMed: 15150132]
 34. Chen T, Stoner GD. Inducible nitric oxide synthase expression in N-nitrosomethylbenzylamine (NMBA)-induced rat esophageal tumorigenesis. *Mol Carcinog* 2004;40:232–240. [PubMed: 15264214]
 35. Stoner GD, Babcock MS, McCorquodale MM. Comparative properties of untreated and N-nitrosomethylbenzylamine-transformed rat esophageal epithelial cell lines. *In Vitro Cell Dev Biol* 1989;25:899–908. [PubMed: 2808222]
 36. Wang D, You L, Sneddon J, Cheng SJ, Jamasbi R, et al. Frameshift mutation in codon 176 of the *p53* gene in rat esophageal epithelial cells transformed by benzo(a)pyrene dihydrodiol. *Mol Carcinog* 1995;14:84–93. [PubMed: 7576103]
 37. Casto BC, Kresty LA, Kraly CL, Pearl DK, Knobloch TJ, et al. Chemoprevention of oral cancer by black raspberries. *Anticancer Res* 2002;22:4005–4015. [PubMed: 12553025]
 38. Matsumoto H, Inaba H, Kishi M, Tominaga S, Hirayama M, et al. Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appears in the blood as the intact forms. *J Agric Food Chem* 2001;49:1546–1551. [PubMed: 11312894]
 39. Stoner GD, Sardo C, Apseloff G, Mullet D, Wargo W, et al. Pharmacokinetics of anthocyanins and ellagic acid in healthy volunteers fed freeze-dried black raspberries daily for 7 days. *J Clin Pharm* 2005;45:1153–1164.
 40. Nicholson DW. Caspase structure, proteolytic substrates and function during apoptotic cell death. *Cell Death Diff* 1999;6:1042–1048.
 41. Slee EA, Adrain C, Martin SJ. Executioner caspase-3, -6 and -7 perform distinct, non-redundant roles during the demolition phases of apoptosis. *J Biol Chem* 2001;276:7230–7236.
 42. Katsube N, Iwashita K, Tsushida T, Yamaki K, Kobori M. Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. *J Agric Food Chem* 2003;51:68–75. [PubMed: 12502387]
 43. Shih PH, Yeh CT, Yen GC. Effects of anthocyanidin on the inhibition of proliferation and induction of apoptosis in human gastric adenocarcinoma cells. *Food Chem Toxicol* 2005;43:1557–1566. [PubMed: 15964118]
 44. Lazze MC, Savio M, Pizzala R, Gazzalini O, Perruca P, et al. Anthocyanins induce cell cycle perturbations and apoptosis in different human cell lines. *Carcinogenesis* 2004;25:1427–1433. [PubMed: 15016660]
 45. Hou Z, Sang S, You H, Lee MJ, Hong J, et al. Mechanism of action of (–) epigallocatechin-3-gallate: auto-oxidation-dependent inactivation of epidermal growth factor receptor and direct effects on growth inhibition in human esophageal cancer KYSE 150 cells. *Cancer Res* 2005;65:8049–8056. [PubMed: 16140980]
 46. Lapidot T, Walker M, Kanner J. Can apple antioxidants inhibit tumor cell proliferation? Generation of H₂O₂ during interaction of phenolic compounds with cell culture media. *J Agric Food Chem* 2002;50:3156–3160. [PubMed: 12009979]
 47. Liu R, Sun J. Antiproliferative activity of apples is not due to phenolic-induced hydrogen peroxide formation. *J Agric Food Chem* 2003;51:1718–1723. [PubMed: 12617611]

Figure 1.

The addition of 30 units of catalase to the medium does not reduce the inhibitory effect of the 100 µg EtOH extract on the growth of RE-149 DHD cells. The increased antiproliferative activity of the EtOH extract in the presence of catalase was not significant compared to the antiproliferative activity of the extract alone ($p>0.05$).

Figure 2. 100 µg EtOH extract/ml induced caspase 3 and 7 activities in RE-149 DHD cells (by 38%) but not in RE-149 cells, when changing the medium on the 3rd day (A). Adding fresh extract daily increased caspase 3 and 7 activities in RE-149 DHD cells by 51% (B). *p<0.05.

Figure 3. 50 µg cyanidin-3-O-rutinoside/ml induced caspase 3 and 7 activities in RE-149 DHD cells (by 23%) but not in RE-149 cells. * $p < 0.05$.

(A) RE-149 DHD cells **(B) RE-149 cells** **(C) Anthocyanin degradation (no cells)** **Figure 4.**

(A). Uptake of anthocyanins at different time points by RE-149 DHD cells treated with 100 μ g EtOH extract/ml as measured by HPLC-MS/MS. (B). Uptake of anthocyanins at different time points by RE-149 cells treated with 100 μ g EtOH extract/ml as measured by HPLC-MS/MS. (C). Degradation of anthocyanins in medium containing 100 μ g EtOH extract/ml and no cells.

Figure 5. 100 μ g EtOH extract/ml induces the expression of caspase 3 mRNA (A) and caspase 7 mRNA (B) in RE-149 DHD cells. * $p < 0.05$

Figure 6.

Effects of 4 days of 100 µg EtOH extract/ml, 50 µg cyanidin-3-*O*-glucoside/ml and 50 µg cyanidin-3-*O*-rutinoside treatment on COX-2 (A) and i-NOS (B) gene expression in RE-149 DHD cells. * $p < 0.05$

Table 1

Tumor incidence and size produced by the injection of RE-149 and RE-149 DHD cells into one week-old rats, and latency period for tumor development.

Cell line	Number of rats	Tumor incidence (%)	Tumor size in cm ² (Mean $\pm$ S.D.)	Time between the injection of the cells to the appearance of tumors (wks)
RE-149	8	50	2 $\pm$ 2	5
RE-149 DHD	7	100	4 $\pm$ 2*	2

*
p<0.05

Table 2

Growth- inhibitory effect of EtOH extract on RE-149 and RE-149 DHD cell lines.

EtOH BRB extract concentration (µg/ml)	RE-149 growth inhibition (%) when changing the medium on the 3 rd day	RE-149 DHD growth inhibition (%) when changing the medium on the 3 rd day	RE-149 DHD growth inhibition (%) when changing the medium daily and using 1% serum in the medium
10	8 ± 0.4	14 ± 2	26 ± 7
50	15 ± 1	37 ± 3 [*]	51 ± 5 [*]
100	16 ± 1	45 ± 1 [*]	60 ± 8 [*]

*
p<0.05.

Values are Mean ± S.D.

Table 3

Growth- inhibitory effect of cyanidin-3-*O*-rutinoside on RE-149 and RE-149-DHD cells when changing the medium daily versus on the 3rd day only.

Cyanidin-3- <i>O</i> -rutinoside (µg/ml)	RE-149 growth inhibition (%) when changing the medium on the 3 rd day	RE-149 DHD growth inhibition (%) when changing the medium on the 3 rd day	RE-149 DHD growth inhibition (%) when changing the medium daily
10	6 ± 1	16 ± 1	22 ± 5
25	7 ± 0.1	24 ± 5	60 ± 15*
50	19 ± 1	45 ± 7*	69 ± 11*

*
p<0.05

Values are Mean ± S.D.

Table 4

Growth- inhibitory effect of cyanidin-3-*O*-glucoside on RE-149 and RE-149-DHD cells when changing the medium daily versus on the 3rd day only.

Cyanidin-3- <i>O</i> -glucoside (µg/ml)	RE-149 growth inhibition (%) when changing the medium on the 3 rd day	RE-149 DHD growth inhibition (%) when changing the medium on the 3 rd day	RE-149 DHD growth inhibition (%) when changing the medium daily
10	7 ± 0.3	17 ± 2	27 ± 7
25	9 ± 1	20 ± 3	50 ± 3 *
50	21 ± 1	41 ± 2 *	61 ± 9 *

*
p<0.05

Values are Mean ± S.D.