

# Delphinidin and cyanidin exhibit antiproliferative and apoptotic effects in MCF7 human breast cancer cells

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## Abstract

Fruits high in antioxidants such as berries and pomegranates have been shown to have many biological effects, including anticancer activity. We previously reported that bilberry (European blueberry) extract exhibited cytotoxic effects on MCF7-GFP-Tubulin breast cancer cells. To delve further into the mechanism of action of bilberry extract, we focused on two of the most abundant anthocyanins found in bilberry, delphinidin and cyanidin. In this study, we examined the radical scavenging activity, antiproliferative, and apoptotic effects of delphinidin and cyanidin on MCF7 breast cancer cells in comparison to Trolox, a vitamin E analog. DPPH radical scavenging activity assay showed at 50% antioxidant activity, an  $IC_{50}$  of 80  $\mu$ M, 63  $\mu$ M, 1.30  $\mu$ M for delphinidin, cyanidin, and Trolox, respectively. As determined by SRB assay, delphinidin, cyanidin, and Trolox were shown to inhibit MCF7 cell proliferation at  $IC_{50}$  of 120  $\mu$ M, 47.18  $\mu$ M, and 11.25  $\mu$ M, respectively. Immunofluorescence revealed that delphinidin, cyanidin, and Trolox caused apoptotic features such as rounding up of cell, retraction of pseudopodes, condensation of chromatin, minor modification of cytoplasmic organelles, and plasma membrane blebbing. Together, these results show that delphinidin and cyanidin have significant radical scavenging activity, inhibit cell proliferation, and increase apoptosis of MCF7 breast cancer cells.

## Introduction

Total amount of anthocyanins and oxygen radical absorbing capacity of four *Vaccinium* species was measured and blueberries were found to be one of the richest sources of antioxidant phytonutrients [1]. Bilberry (*Vaccinium myrtillus*) contains several anthocyanidins, cyanidin, which is found in highest quantity, delphinidin and petunidin found in quantities 2.5 fold lower than cyanidin, and malvidin and peonidin [2].

A public interest is growing in the health advantages of using phytochemicals such as anthocyanins in replacement of synthetic compounds used in chemotherapeutic or chemopreventive action [3,4]. Numerous anticancer effects from anthocyanins include cytotoxicity [5], antioxidation [6-12], anti-inflammation [13], cell cycle perturbations [14], active oxygen radical scavenging effect [11,15], lipid peroxidation [16], antiproliferation [5,17-19], epidermal growth factor receptor inhibition [20], and apoptosis [21-24]. In addition, pycnogenol, a preparation derived from pine bark, which contains high amounts of procyanidins, selectively induces death in human mammary cancer cells (derived from human fibrocystic mammary tissue) but not in normal human mammary MCF-10 cells [25].

Recently, we have demonstrated inhibition of proliferation, cell cycle arrest, and apoptosis-like cytotoxicity of MCF7 human breast cancer cells by bilberry extract [26]. Due to their abundance in bilberry, delphinidin and cyanidin were examined for their radical scavenging ability, antiproliferative effects, and apoptosis inducing effect in MCF7 cells. In addition, delphinidin and cyanidin are compared to Trolox, a vitamin E analog to be used as a reference compound for antioxidant capacity. DPPH radical scavenging assay, sulforhodamine B proliferation assay, and immunocytochemistry assay are used to examine the anticancer effects of delphinidin, cyanidin, and Trolox on MCF7 cells.

## Materials and methods

### Materials

All the chemicals and reagents used in this experiment were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A) unless otherwise specified. Cyanidin chloride and delphinidin chloride were dissolved by adding 30.98  $\mu$ l and 29.52  $\mu$ l dimethylsulphoxide (DMSO) to 1mg anthocyanidin, respectively, obtaining 100mM stock concentration. Trolox (6-Hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid) was purchased from Calbiochem (San Diego, CA, U.S.A.).

### Cell culture

Human Caucasian breast adenocarcinoma (MCF7) cells from American Type Culture Collection, Rockville, MD, U.S.A., were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Atlanta Biological, Atlanta, GA, U.S.A.), L-glutamine, MEM nonessential amino acid solution, sodium bicarbonate, and penicillin G (100 U/mL), streptomycin (100  $\mu$ g/mL), at 37°C in a humidified atmosphere containing 95% air and 5% CO<sub>2</sub> [27]. Culture media were changed every 3-4 days and doubling time was 36h.

### DPPH radical scavenging capacity (anti-oxidation) assay

Free radical scavenging activity of delphinidin, cyanidin, and

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Trolox (Calbiochem) were compared to each other by reaction with DPPH (2,2-diphenyl-1-picrylhydrazyl) radical. DPPH (Sigma-Aldrich) was dissolved in methanol to a final concentration of 4 mg/ml. A dilution series of concentrations for cyanidin and delphinidin were made starting at 500  $\mu\text{M}$  in DMSO. Trolox was made at 20  $\mu\text{M}$  in ethanol. Delphinidin, cyanidin, and Trolox diluted solutions were placed in 96-well microtiter plate. 100  $\mu\text{L}$  DPPH stock solution diluted 1:20 in ethanol was delivered to each well and let stand in dark room. Optical density of all wells measured at 490 nm ( $\text{OD}_{490}$ ) with microplate reader before and after DPPH addition. Absorbance values of the reaction solution were measured spectrophotometrically at 490nm at 30min and converted into the percentage antioxidant activity (AA) using the following formula:

$$\text{AA\%} = 100 - \left\{ \frac{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}) \times 100}{\text{Abs}_{\text{control}}} \right\}$$

DPPH solution and ethanol was used as negative control. A plot of % AA vs. concentration was made to establish the standard curve and calculate  $\text{IC}_{50}$  values using linear correlation formula.

### Sulforhodamine B(SRB) cell proliferation assay

MCF7 cells were seeded at a density of  $2 \times 10^5/\text{mL}$  (0.1 mL per well) in 96-well microtiter plates and allowed to attach for 24 hours before treatment. Delphinidin, cyanidin, and Trolox were serially diluted beginning at 1000  $\mu\text{M}$ , 250  $\mu\text{M}$ , and 20  $\mu\text{M}$ , respectively, in DMEM supplemented with 10% fetal bovine serum (final concentrations of ethanol were at or below 1.4%). Preliminary experiments (data not shown) were conducted to determine dose ranges. When 1.4% of 70% ethanol alone was added to the vehicle control wells, it did not affect proliferation of the cells. After incubation for 72 hr, cells were fixed with 10% (wt./vol.) trichloroacetic acid and stained with 0.4% SRB solution for 30 min, after which excess dye was removed by washing repeatedly with 1% (wt./vol.) acetic acid. The dye was eluted with Tris buffer and quantified photometrically at 490 nm. The percentage of growth inhibition was calculated as:

$$\left( \frac{\text{OD}_{\text{highest concentration}}}{\text{OD}_{\text{lowest concentration}}} \times 100 \right) / \text{OD}_{\text{lowest concentration}}$$

### Immunofluorescence microscopy

MCF7-GFP-Tubulin cells were grown on a cover glass slip in a 6-well plate and treated with delphinidin, cyanidin, and Trolox at concentrations beginning at 100  $\mu\text{M}$ , 250  $\mu\text{M}$ , and 20  $\mu\text{M}$ , respectively, for 24 hours. Immunofluorescence microscopy of actin, tubulin, and DNA staining were performed for cyanidin and Trolox drugged MCF7 cells, whereas delphinidin treated MCF7 cells were only stained for DNA and tubulin. Cells were fixed in 3.7% formaldehyde and stained with rabbit monoclonal antibody- $\alpha$ -tubulin (1:500) in 0.5% BSA-PBS or Alexa488- $\alpha$ -rabbit (1:300) in 1.0% BSA, followed by addition of FITC-conjugated anti-mouse secondary antibody to stain the microtubules and by rhodamine-conjugated phalloidin (Invitrogen, Carlsbad, CA) to stain actin filaments. Cells were mounted to the microscope slides with 40, 6-diamidino-2-phenylindole-containing ProLong Gold with DAPI medium (Invitrogen). Microscopy was performed on a spinning disk confocal microscope (model IX81 DSU, Olympus, Tokyo, Japan). Images were acquired with an Imagem camera (Hamamatsu, Shizuoka, Japan) under the control of SlideBook software (Olympus).

### Statistics

Results are reported as average values of at least three independent experiments. Means of individual groups were converted to a percentage of the control value and expressed as the mean  $\pm$  SE.

## Results

### DPPH free radical scavenging assay

The free radical scavenging capability of delphinidin and cyanidin was compared with the scavenging effects of Trolox, a reference standard antioxidant, by DPPH antioxidant assay. Concentrations of cyanidin and delphinidin (500  $\mu\text{M}$ ) were approximated to pre-determined, comparable Trolox concentrations (20  $\mu\text{M}$ ) in order to determine whether the anticancer effects are purely an antioxidant effect or some other specialized mechanism of action. Delphinidin and cyanidin were capable of scavenging DPPH radicals in a concentration-dependent manner as compared to control. After 30 minutes of incubation with DPPH, absorbance values were measured and  $\text{IC}_{50}$  values were calculated using linear correlation of antioxidant activity percentages. The  $\text{IC}_{50}$  for delphinidin, cyanidin, and Trolox were 80  $\mu\text{M}$ , 63  $\mu\text{M}$ , and 1.30  $\mu\text{M}$ , respectively (Figure 1).

### Sulforhodamine B colorimetric cytotoxicity assay

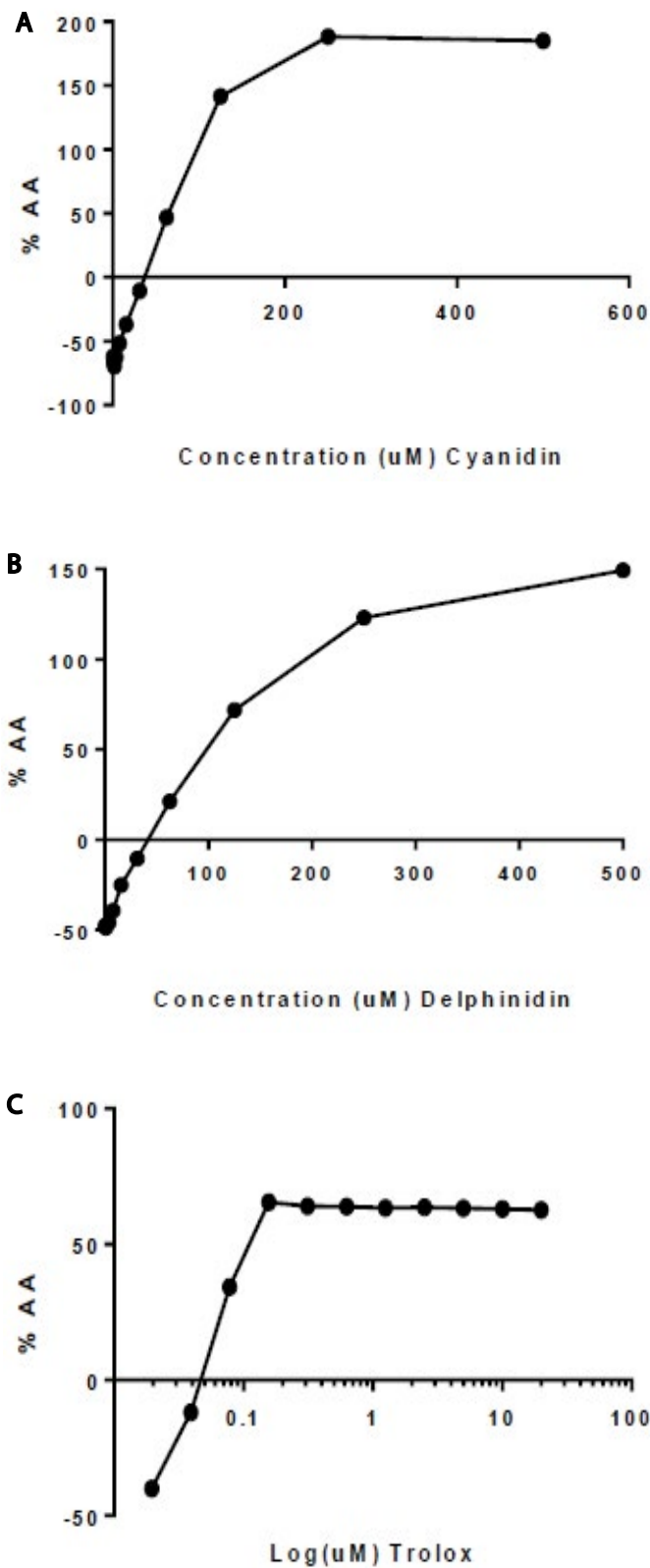
MCF7 cells were treated with delphinidin, cyanidin, and Trolox that was serially diluted beginning at 1000 $\mu\text{M}$ , 250 $\mu\text{M}$ , and 20 $\mu\text{M}$ , respectively. Treatment of delphinidin, cyanidin, and Trolox exhibited a concentration-dependent inhibition of growth of MCF7 cells after a 24 hr incubation period. The  $\text{IC}_{50}$  values for delphinidin, cyanidin, and Trolox were 120  $\mu\text{M}$ , 47.18  $\mu\text{M}$ , and 11.25  $\mu\text{M}$ , respectively (Figure 2). Trolox was more effective at DPPH radical scavenging. At the low concentrations of 1 $\mu\text{M}$  and even 0.1  $\mu\text{M}$  Trolox, 40% of MCF7 cell growth was inhibited.

### Immunofluorescence microscopy

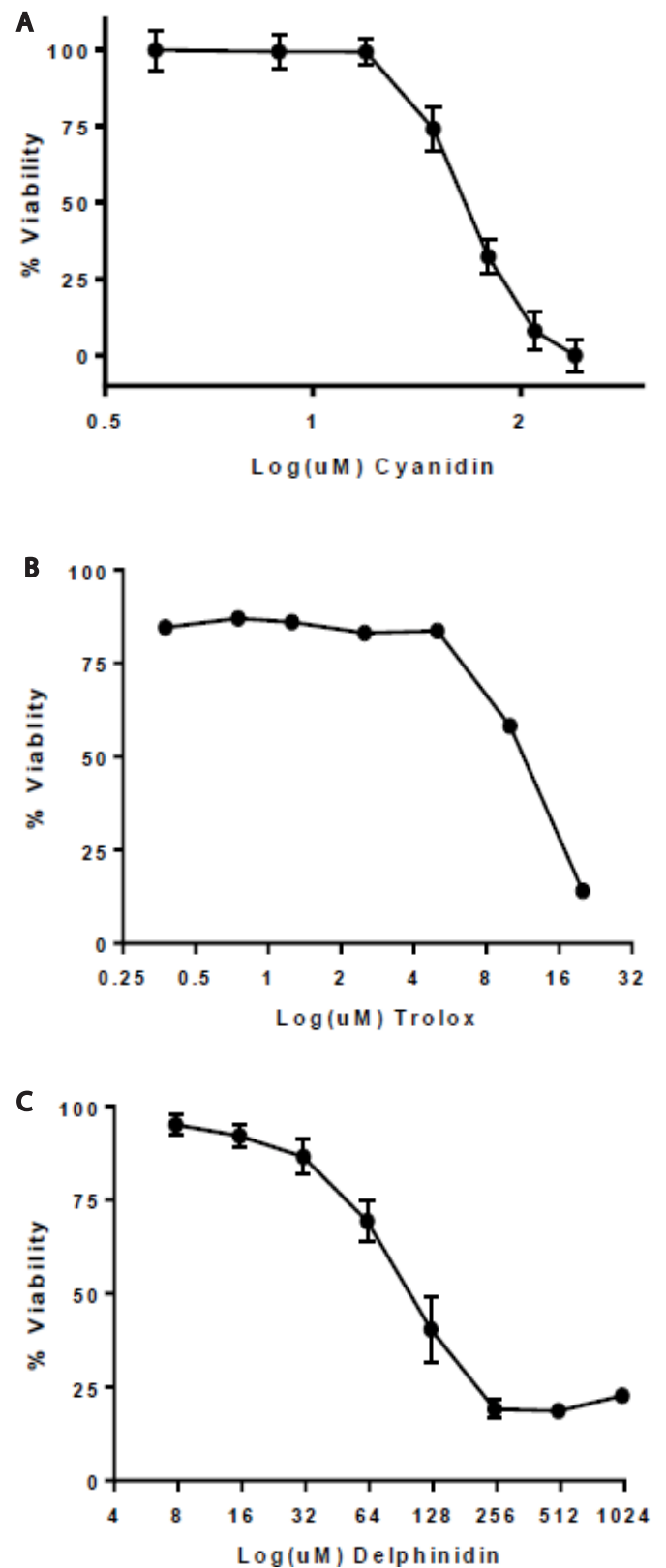
MCF7-GFP-Tubulin cells were treated with delphinidin, cyanidin, and Trolox at concentrations beginning at 100  $\mu\text{M}$ , 125  $\mu\text{M}$ , and 20  $\mu\text{M}$ , respectively, for 24 hours. Delphinidin, cyanidin, and Trolox were all capable of morphologically damaging MCF7 cells to a certain degree as determined by DAPI, tubulin and actin fluorescence microscopy. Control MCF7 cells showed round and evenly stained nuclei with intact tubulin and actin polymers that were fully extended from the nucleus (Figure 3A,3E,3I). At 24 hr of 50  $\mu\text{M}$  delphinidin treatment, rounding-up of cells and detachment from substrate is observed plus aggregated tubulin and chromatin condensation (Figure 3B). At 75  $\mu\text{M}$  and 100  $\mu\text{M}$  delphinidin, the majority of cells were either dead or detached, but the cells that were on the path to dying reveals retraction of pseudopodes, chromatin condensation, and tubulin aggregation (Figure 3C,3D). At 24 hr of 62.5  $\mu\text{M}$ , 125  $\mu\text{M}$  and 250  $\mu\text{M}$  cyanidin treatment, there is rounding-up of cells, chromatin condensation, and plasma membrane blebbing in a dose-dependent manner as seen by the heavily fragmented tubulin and actin microfilaments (Figure 3F,G,H). At the lowest concentration 1.25  $\mu\text{M}$  Trolox, only some MCF7 cells had apoptotic features and if so, were less drastic than at the higher concentrations (Figure 3J). At 5  $\mu\text{M}$  and 20  $\mu\text{M}$  treatment with Trolox, there was retraction of pseudopods, damaged plasma membrane, and chromatin condensation (Figure 3K,3L). At  $\text{IC}_{50}$ =11.25  $\mu\text{M}$  Trolox, classic apoptotic characteristics were seen in the MCF7 cells. These cells were rounded up and had chromatin condensation with plasma membrane blebbing. The cytological damage for delphinidin, cyanidin, and Trolox coincides with our approximate  $\text{IC}_{50}$  seen in SRB antiproliferation assays.

## Discussion

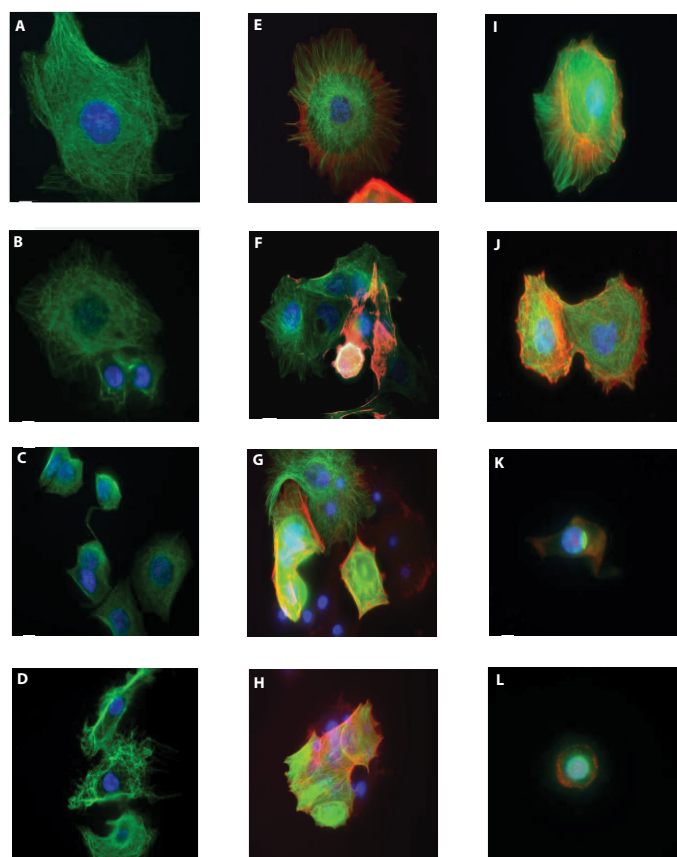
We have previously reported that bilberry extract inhibits cell proliferation and induces apoptosis in MCF7 cells [26]. In this study,



**Figure 1.** Radical scavenging activity of delphinidin, cyanidin, and Trolox. DPPH free radicals were allowed to scavenge varying concentrations of (A) Delphinidin, (B) Cyanidin, and (C) Trolox for 30 min. Percentage of antioxidant activity (AA%) was determined. At 30 min, the IC<sub>50</sub> for delphinidin, cyanidin, and Trolox were 80 uM, 63 uM, 1.30 uM, respectively.



**Figure 2.** Proliferation of MCF7 cells exposed to delphinidin, cyanidin, and Trolox. SRB stained MCF7 cells were measured at 490 nm after exposure to (A) Delphinidin, (B) Cyanidin, and (C) Trolox after 24 h. Percent inhibition is shown and the estimated IC<sub>50</sub> values for delphinidin, cyanidin, and Trolox were 120 uM, 47.18 uM, and 11.25 uM, respectively.



**Figure 3.** Cell images taken at varying concentrations of delphinidin, cyanidin, and Trolox. Effect of delphinidin, cyanidin, and Trolox on microtubule, actin, and nuclear organization in MCF7 cells shown in immunofluorescence images of microtubules (green), actin (red), and DNA (blue) in fixed MCF7 cells after incubation in the absence or presence of delphinidin, cyanidin, or Trolox for 24 hours. A-D: Delphinidin – Control, 50  $\mu\text{M}$ , 75  $\mu\text{M}$ , 100  $\mu\text{M}$ ; E-H: Cyanidin – Control, 62.5  $\mu\text{M}$ , 125  $\mu\text{M}$ , 250  $\mu\text{M}$ ; I-L: Trolox – Control, 1, 2.5  $\mu\text{M}$ , 20  $\mu\text{M}$ .

MCF7 human breast adenocarcinoma cells were used as a model to study the anticancer effects of commonly found anthocyanins in bilberry, delphinidin and cyanidin. In previous studies, the abundant amount of delphinidin and cyanidin in bilberry [15] inhibit cancer cell growth in vitro by various anthocyanin-containing extracts or purified anthocyanin fractions [20,28,29].

Given that delphinidin and cyanidin are antioxidants, the free radical scavenging capability of delphinidin and cyanidin was compared with the scavenging effects of Trolox, a reference standard antioxidant, by DPPH antioxidant assay. Delphinidin and cyanidin were capable of scavenging DPPH radicals in a concentration-dependent manner as compared to control. The  $\text{IC}_{50}$  values for delphinidin, cyanidin, and Trolox were 80  $\mu\text{M}$ , 63  $\mu\text{M}$ , 1.30  $\mu\text{M}$ , respectively (Figure 1). Cyanidin was more effective at scavenging DPPH radicals than delphinidin but Trolox was by far the most potent scavenger. Kähkönen *et al.* [6] reported a DPPH radical scavenging activity of 42  $\mu\text{M}$ , 33  $\mu\text{M}$ , and 35  $\mu\text{M}$  for delphinidin, cyanidin, and Trolox respectively. Tanaka *et al.* [30] also reported 32.3  $\mu\text{M}$  and 31.2  $\mu\text{M}$  DPPH  $\text{IC}_{50}$  values for cyanidin and Trolox. The difference is seemingly negligible as Rice-Evens and colleagues report trihydroxylation of the B ring of cyanidin as in delphinidin neither enhances nor diminishes the Trolox equivalent antioxidant capacity [25]. The tremendous potency of Trolox, despite only having two hydroxyl groups, remains to be elucidated. In general, the DPPH assay results are in accordance with previous observations

on the effect of hydroxylation and methoxylation in ring B to radical scavenging ability in aqueous phase [6].

To evaluate the anti-proliferative effects of delphinidin and cyanidin, SRB cytotoxicity assay was used to determine the  $\text{IC}_{50}$  values of these two anthocyanins. Cyanidin had a lower  $\text{IC}_{50}$  value of 47.18  $\mu\text{M}$  as compared to delphinidin with 120  $\mu\text{M}$  which correlates with the stronger radical scavenging capability of cyanidin as seen in the DPPH assay. In addition, Trolox had the lowest  $\text{IC}_{50}$  value of 11.25  $\mu\text{M}$  also correlating to the DPPH assay findings. Previous studies have proven that natural food colors and anthocyanins show a dose-dependence growth inhibition against breast, colon, stomach, central nervous system, and lung tumor cells [16,31,32]. Future studies include treating normal breast cells with delphinidin and cyanidin to discover whether the cytotoxic effects are specific to breast cancer cells. Zhao and coworkers demonstrated anthocyanin-rich extracts inhibit cancer but not normal colon cell growth [32]. Additionally, shedding light on the cellular mechanistic uptake and bioavailability of anthocyanins [33] is extremely important due to how the substitution pattern of anthocyanidins affects cell proliferation signaling cascades [34,35] and perhaps are as potent inhibitors of the epidermal growth factor receptor [20].

Concurrent with our previously published bilberry extract study, delphinidin and cyanidin at high concentrations caused depolymerization of tubulin and actin with rounding cell bodies and condensed nuclei as shown by immunofluorescence microscopy. Parallel to the DPPH and SRB assay findings, a low concentration of Trolox (5  $\mu\text{M}$ ), exhibited significant damage to microtubules. This is the first report to our knowledge that observes the effects of delphinidin and cyanidin on MCF7 microtubule and actin structures in cells.

Overall, delphinidin, cyanidin, and Trolox were able to effectively scavenge DPPH free radicals, inhibit MCF7 cell growth, and induce characteristic apoptotic features as seen in the immunofluorescence images. Strikingly, Trolox, with only 2 hydroxyl groups on its structure, was able to scavenge DPPH radicals, inhibit cell growth, and cause apoptosis at much lower concentrations than delphinidin and cyanidin, who have 6 and 5 hydroxyl groups, respectively. The purpose of these experiments was to elucidate whether anthocyanins, as antioxidants, were the key mechanism in causing cytotoxicity in MCF7 breast cancer cells. Trolox, a vitamin E derivative and an antioxidant, was more effective at inducing cytotoxic effects on the cells leading us to think anthocyanins like delphinidin and cyanidin may cause apoptosis in MCF7 cells through a different mechanism than Trolox. Future studies to examine the mechanism of action of delphinidin and cyanidin include elucidating biochemical features such as activation of proapoptotic Bcl-2 family proteins, activation of caspases, mitochondrial membrane potential, oclionucleosomal fragmentation, plasma membrane rupture, and ROS over-generation.

### Authorship and contributorship

Conception and design: Jessica Tang, Emin Oroudjev, George Ayoub, Leslie Wilson.

Development of methodology: Jessica Tang, Emin Oroudjev, George Ayoub, Leslie Wilson

Acquisition of data: Jessica Tang. Analysis and interpretation of data: Jessica Tang, Emin Oroudjev, George Ayoub. Writing, review, and/or revisions of the manuscript: Jessica Tang, George Ayoub. Study supervision: George Ayoub, Leslie Wilson.

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