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 IC50 of 80 µM, 63 µM, 1.30 µM for delphinidin, cyanidin, and Trolox, respectively. As determined by SRB assay, delphinidin, cyanidin, and Trolox were shown to inhibit MCF7 cell proliferation at IC50 of 120 µM, 47.18 µM, and 11.25 µM, 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 phytoceuticals 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 mammmary 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 µl and 29.52 µl 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 µg/mL), at 37°C in a humidified atmosphere containing 95% air and 5% CO2 [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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Troelox (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 µM in DMSO. Troelox was made at 20 µM in ethanol. Delphinidin, cyanidin, and Troelox diluted solutions were placed in 96-well microtiter plate. 100 µL 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 (OD490) with microplate reader before and after DPPH addition. Absorbance values of the reaction solution were measured spectrophotometrically at 490 nm at 30min and converted into the percentage antioxidant activity (AA) using the following formula:

\[ AA\% = 100 - \left(\frac{(Abs_{sample} - Abs_{blank})\times 100}{Abs_{control}}\right) \]

DPPH solution and ethanol was used as negative control. A plot of % AA vs. concentration was made to establish the standard curve and calculate IC50 values using linear correlation formula.

Sulforhodamine B (SRB) cell proliferation assay

MCF7 cells were seeded at a density of 2×104/mL (0.1 mL per well) in 96-well microtiter plates and allowed to attach for 24 hours before treatment. Delphinidin, cyanidin, and Troelox were serially diluted beginning at 1000 µM, 250 µM, and 20 µM, 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 repeated 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:

\[ \text{ICSG per cent} = \left(\frac{OD_{blank} - OD_{sample}}{OD_{blank}}\right) \times 100 \]

Immunofluorescence microscopy

MCF7-GFP-Tubulin cells were treated with delphinidin, cyanidin, and Troelox at concentrations beginning at 100 µM, 125 µM, and 20 µM, respectively, for 24 hours. Treatment of delphinidin, cyanidin, and Troelox was more effective at DPPH radical scavenging. At the low concentrations beginning at 1000 µM, 250 µM, and 20 µM, respectively, in DMEM supplemented with 10% fetal bovine serum. (final concentrations of delphinidin, cyanidin, and Troelox were 120 µM, 47.18 µM, and 11.25 µM, respectively (Figure 2). Troelox was more effective at DPPH radical scavenging. At the low concentrations of 1µM and even 0.1 µM Troelox, 40% of MCF7 cell growth was inhibited.

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 IC₅₀ for delphinidin, cyanidin, and Trolox were 80 µM, 63 µM, 1.30 µM, 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 IC₅₀ values for delphinidin, cyanidin, and Trolox were 120 µM, 47.18 µM, and 11.25 µM, respectively.
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µM for delphinidin, cyanidin, and Trolox respectively. Tanaka et al. reported a DPPH radical scavenging activity of 42 µM, 33 µM, and 35 µM, respectively. Trolox was by far the most potent scavenger. Kähkönen [6] was more effective at scavenging DPPH radicals than delphinidin only having two hydroxyl groups, remains to be elucidated. In general, delphinidin neither enhances nor diminishes the Trolox equivalent scavenging ability in aqueous phase [6].

To evaluate the anti-proliferative effects of delphinidin and cyanidin, SRB cytotoxicity assay was used to determine the IC_{50} values of these two anthocyanins. Cyanidin had a lower IC_{50} value of 47.18 µM as compared to delphinidin with 120 µM which correlates with the stronger radical scavenging capability of cyanidin as seen in the DPPH assay. In addition, Trolox had the lowest IC_{50} value of 11.25 µM 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 anthocyanins 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 µM), 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, oglionucleosomal 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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The technical assistance of Catherine Zheng and Brennna Duplisea is gratefully acknowledged. The use of cyanins in sunscreen applications is covered by U.S. Patent 6,783,754. The contact for licensing opportunities is: Roy Mankovitz, Director, Montecito Wellness LLC, 1482 East Valley Rd, Suite 808, Santa Barbara, CA 93108, USA, 1-805-969-4604.

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