Abstract. Rapidly accumulating laboratory and clinical research evidence indicates that anthocyanins exhibit anti-cancer activity and the evaluation of bilberry anthocyanins as chemo-preventive agents is progressing. It has previously been demonstrated that anthocyanins upregulate tumor suppressor genes, induce apoptosis in cancer cells, repair and protect genomic DNA integrity, which is important in reducing age-associated oxidative stress, and improve neuronal and cognitive brain function. Bilberry anthocyanins have pronounced health effects, even though they have a low bioavailability. To increase the bioavailability, Bilberry was encapsulated in 5.5 nm diameter liposomal micelles, called NutraNanoSpheres (NNS), at a concentration of 2.5 mg/50 µl [25% (w/w) anthocyanins]. These Bilberry NNS were used to study the apoptotic/cytotoxic effects on K562 Human Erythroleukemic cancer cells. Flow cytometric fluorescent quantification of the uptake of propidium iodide in a special cell viability formulation into dead K562 cells was used to determine the effects of Bilberry on the viability of K562 cells. The concentrations of Bilberry that demonstrated the greatest levels of percentage inhibition, relative to the control populations, were biphasic, revealing a 60-70% inhibition between 0.018-1.14 mg/ml (n=6) and 60% inhibition at 4 mg/ml. The lowest percentage inhibition (30%) occurred at 2 mg/ml. The lethal dose 50 was determined to be 0.01-0.04 mg/ml of Bilberry per 105 K562 cells at 72 h of cell culture exposure. At 48 h incubation, the highest percentage of inhibition was only 27%, suggesting involvement of a long-term apoptotic event. These levels, which demonstrated direct cytotoxic effects, were 8-40 times lower than levels required for Bilberry that is not encapsulated. The increase in bioavailability with the Bilberry NNS and its water solubility demonstrated the feasibility of using Bilberry NNS in cancer patient clinical trials.

Introduction

Anthocyanins are the chemical components that result in the intense color to many fruits and vegetables, such as bilberries, blueberries, red cabbages and purple sweet potatoes. Rapidly accumulating in vitro and in vivo evidence indicates that anthocyanins have cancer preventive and anti-cancer activity. Bilberry anthocyanins were shown to be effective cancer preventive agents in 25 colorectal cancer patients (1). Anthocyanins treated cancer cells reveal upregulation of tumor suppressor genes and intracellular-signaling cascades as common molecular targets for anthocyanins (2). Defect in apoptosis has been implicated as a major cause of resistance to chemotherapy. Anthocyanins induced apoptosis in cancer cells via activation of redox-sensitive caspase 3-pathways were observed in B-cell chronic lymphocytic leukemia (B-CLL), but had no effect in normal peripheral blood cells (3). Furthermore, Bilberry extracts exhibited strong pro-apoptotic activity through redox-sensitive caspase 3 activation-related mechanism in B-CLL cells involving dysregulation of the Bad/Bcl-2 pathway (3). Interestingly, bilberry anthocyanins also synergistically suppress growth and invasive potential of human non-small-cell lung cancer cells (4). The development of a better delivery system for Bilberry anthocyanins would significantly enhance their cancer prevention and treatment effectiveness. Herein is reported such a system utilizing the NutraNanoSphere™ (NNS) micellation of Bilberry.

Materials and methods

Cell line and media production. Experiments were performed using a chronic myelogenous leukemia K-652 cell line purchased from the American Type Culture Collection.

The tissue culture media was made by adding 5 ml of 100X penicillin-streptomycin (10,000 units penicillin with 10 mg of streptomycin/ml), 5 ml of 200 mM sterile-filtered L-Glutamine (both from Sigma-Aldrich, St. Louis, MO, USA), 5 ml of Cellgro sodium 100X bicarbonate solution (Cellgro Mediatech, Herndon, VA, USA), and 50 ml of fetal calf serum (Atlanta Biologicals, Flowery Branch, GA, USA) to 500 ml of Minimum Essential Media, Alpha 1X, with Earle's
salts without ribonucleotides, deoxyribonucleotides, and no L-glutamine (Cellgro Mediatech).

Viability stain. The viability stain used for analysis with the flow cytometer was developed by Dr Jerry Thornthwaite. The evidence that showed the viability stain, which uses a special medium and dye exclusion with propidium iodide (PI) (Sigma-Aldrich), was effective in measuring cell viability was shown in two ways. Firstly, 500 µl portions of K562 cells (5×10^6/ml) were heated as triplicates in a 56°C water bath for ≤2 h. At 5 min intervals, 500 µl of the viability reagent was added to the timed samples at room temperature. All samples were run after a 2 min incubation at room temperature on the Accuri Flow Cytometer (BD Biosciences, San Jose, CA, USA). Also, K562 cell viability was measured directly from cell cultures in which 100 µl samples from the cell culture were added to 100 µl of the viability stain. After incubation for five min at room temperature, the samples were suspended and analyzed using flow cytometry. Forward light scatter was used to gate on the K562 cells and analyze the number of viable cells within the established control viability gate in the 585±20 nm fluorescence red channel. Typical control cultures with 95-99% viability were used to establish the boundary of the viable cells, which only showed passive background staining on the cell surface.

Encapsulated reagent. Bilberry NNS were obtained from Dr Lothar Haegeler of X-Labs (Switzerland-Singapore) and had a concentration of had a concentration of 2.5 mg/50 µl or 50 mg/ml. The anthocyanin concentration in the micellized Bilberry was 25% (w/w).

Average diameter measurements of the NNS. The samples were diluted by volume in a ratio of 1:6 with DI Water and filtered by a 0.45 µm Nylon membrane to remove any dust contaminants. The Zetasizer ZSP (Malvern Instruments, Malvern, UK) was used with a backscattering angle of 173 degrees to measure the particle size by dynamic light scattering. A non-negative least squares algorithm was used to generate the size distribution by intensity, which indicated the diameter of the major population for the NNS. The intensity data was then converted to a mass or volume distribution to compare relative amounts of each size population, which indicated the percentage of the sample represented in the respective population.

Sample preparation and cell counting. In all assays, viable cell counts were obtained by mixing 100 µl of a cell culture with 100 µl of viability stain. All samples were analyzed within an hour after room temperature incubation for at least 5 min. The percentage viability was stable for ≤2 h. A 10 µl portion of each sample was run through the Flow Cytometer at a medium flow setting. The resulting number was multiplied by 200 to determine the number of viable cells/ml.

Cell growth plate preparation. The cells that were counted were then diluted with the media to a concentration of 1×10^4 cells/ml. A 500 µl portion of the cells was added to each well of the 48-well plate. The plates incubated in a Forma Scientific CO₂ water-jacketed incubator at a temperature of 37.2°C for 48 h to allow the cells to enter the exponential growth phase.

Addition of compounds. After incubation for 48 h, the stock sample compounds were diluted accordingly, by a factor of two for up to eight dilutions. A 50 µl sample was added to each well, and 56 replicates of each dilution to the wells were done. A total of 50 µl of cell culture media was added to each control well. Once finished, every well contained 550 µl. The plates were incubated for 48 h.

Cell processing, staining, and analysis. Up to six replicates at each concentration, starting with the controls that were used to set the gates for viability, were suspended with a 500 µl pipet, and 100 µl portions were added to 2 ml 96-well analysis tubes. After all of the samples were added to the tubes, 100 µl of the viability stain were added using an 8-channel multipipettor, and the tray was shaken slightly and incubated for ≥5 min. All samples were analyzed within an hour after room temperature incubation for at least 5 min. The viability-stained cells were stable for at least 2 h at room temperature. A 10 µl portion of each sample was run through the Flow Cytometer at a medium flow setting. The resulting number was multiplied by 200 to determine the number of viable cells/ml.

Percentage cell viability. Control cells were used to set the forward angle light scatter gate for the entire cell population gate for the viable cells less debris to the left of the scatter peak. The resulting fluorescent peak population of cells comprised the viable cells (95-97%) as seen as an example in the first frame of Fig. 1. Any fluorescent cells to the right of the baseline comprised the PI stained, dead cell population. The percentage viable cells were determined by dividing the number cells in the viable fluorescent cell population as established by the control cells divided number of viable + nonviable cells and multiplying by 100. Fig. 2 showed the decrease in the percentage of viable cells as the K562 cancer cell cultures progressively die because of nutritional depletion.

Data analysis. The data collected was then graphed using PSI-Plot and whose standard procedure for graphing. The
data was graphed in the form of percentage inhibition vs. concentration of each component. Proof of the quantitative ability of the viability stain is shown in Figs. 1 and 2. To calculate percentage inhibition, first the cell counts were multiplied by 200 to account for the 1:1 dilution of adding the PI stain, as well as the volume of the analyzed sample being 10 µl. Multiplication by 200 resulted in cells/ml. The values for the viable cells/ml were incorporated into the equation, \(\%\) inhibition\(=\left(1-\frac{X}{Y}\right)\times100\%\), where \(X\) was equal to the cells in a particular well, and \(Y\) was the average number of cells in the control wells. The mean of multiple replicates (4-6) ± the standard deviation were then determined.

The \(LD_{50}\) values were reported in mg/ml per well throughout this study. All well volumes were 550 µl, where 500 µl of the tumor cells had 50 µl of the supplements at different concentrations added to their respective wells in 48-well plates (Falcon Plastics, Brookings, SD, USA). A 48 h incubation started with \(5\times10^4\) cells and ended with about 6-7\(\times10^4\) cells. The initial 48 h incubation allowed the cells to enter exponential growth. The test supplements at various concentrations were added in 50 µl to the appropriate wells. Unless noted the plates were incubated for an additional 48 h, where the control population grew to 8-10\(\times10^4\) cells/ml.

**Results**

**Cell viability assay.** In the process of developing the viability assay used in this paper, K562 cells were heated at 56°C in 500 µl aliquots at 5 min intervals up to 20 min. Each sample had 500 µl of the PI-viability stain added and analyzed by flow cytometry within 30 min at room temperature (22-24°C). In Fig. 1, gating on the K562 cancer cell forward light scatter and analyzing by red fluorescence channel (585±20 nm), showed the percentage of viable K562 cells decreased in a near linear fashion as the cells were exposed to a 56°C water bath for ≥20 min.

Furthermore as shown in Fig. 2, the viability of expanded cells in long term cultures showed two populations of dead cells: Those where the cellular membranes were compromised, and a second more PI intense population where the nuclear membranes were compromised and intercalation of the PI into double stranded DNA occurred. The percentage viability was determined from the ratio of the cells in the viable cell peak (the first one) divided by the total cells times 100%. The K562 cells starting culture was \(1\times10^5\) cells/ml. The right of the vertical line was used as the cut-off of viable (left) and non-viable (right) cells, which was established for...
The control cultures (95-97% viable). The control, viable PI population curve came to a baseline on the left of the curve, which was the beginning of the viable cell population fluorescent staining. The dead cells were to the right of the vertical line. At Day 6, the cells were 98% viable, with up to 1.5-2.0x10⁶ cells/ml. At Day 10, dead cells were detected in the positive PI fluorescence portion of the histogram. By Days 14-18, two distinct populations of dead cells were seen, which showed the cells where the cell membrane was compromised (peak to the left of the vertical line) and cells where the nuclear membrane was compromised (peak to the right of the vertical line). This showed the PI had intercalated into the double-stranded nucleic acids, causing a very large increase in fluorescent yield. At Day 18, all of the cells were dead. There is over a 1,000-fold increase in fluorescence, which is indicative of the high fluorescence yield due to the intercalation of the PI into the double stained nucleic acids.

The enhanced anticancer effects of encapsulating Bilberry in the NNS. The Bilberry NNS Measurements showed a greatly enhanced potent activity when these compounds were encapsulated in the liposomal structure. Fig. 3 shows the size distributions for the Bilberry NNS. The samples were diluted by volume in a ratio of 1:6 with DI Water and filtered by a 0.45 µm Nylon membrane to remove any dust contaminants. These samples were run on the Malvern Zetasizer ZSP with

![Size distribution by volume](image)

Figure 3. Size distributions by volume of the Bilberry NutraNanoSpheres™ using the Zetasizer ZSP (Malvern Instruments) to measure the sphere sizes in nm diameter. Samples were run as six replicates. Standard deviation and mean diameters are shown.

![Bilberry NNS](image)

Figure 4. The maximum percentage inhibition results occurred after an exposure for (A) 2 days (48 h) as compared to (B) 3 days (72 h) from 0-4.54 mg/ml of Bilberry NNS. Standard Deviation bars are shown (n=6).

![Detailed range](image)

Figure 5. Detailed range from 0.01-1.14 mg/ml of Bilberry NNS. Standard deviation bars are shown (n=6). Two LD₅₀ of 0.01 and 0.04 are shown.
a backscattering angle of 173 degrees to measure the particle size by dynamic light scattering. A non-negative least squares algorithm was used to generate the size distribution by intensity.

In Fig. 4, the maximum inhibition results occurred after an exposure for 3 days (72 h) as compared to 2 days (48 h), and a subsequent decrease by day 4 (data not shown).

By Day 3, the percentage inhibition curve between 0 and 4.54 mg/ml showed two, possibly three regions of inhibition about 25, 75, and 4.54 mg/ml. A dramatic increase in the inhibition of the cancer cells was seen between Day 2 and 3. In Fig. 5, the 0.25 mg/ml inhibition region was expanded from 0.01-1.14 mg/ml for the Bilberry NNS. The LD₅₀ in two experiments were 0.01 and 0.04 mg/ml. The published data shows the LD₅₀ is ~0.3-0.4 mg/ml at Bilberry concentrations of 50 mg/ml for the stock solution (5). This shows an increase in the cytotoxicity of the Bilberry NNS of 8-40 times the published free bilberry.

Discussion

The most promising anticarcinogenic agents in plants are phenolic compounds, which are abundantly present in Bilberries (Vaccinium myrtillus), and a variety of others including lingonberry (Vaccinium vitis-idaea), and cloudberry (Rubus chamaemorus) (3.6-8). Anthocyanins are hydrophilic compounds, and a variety of others using the NNS methodology include Vitamins (C, B12, D), E, CQ, Curcumin, Artemisinin, Frankincense, Ribosome Nucleotide, Ace manan, among others. Finally, all of our NNS preparations survive the stomach and intestines and fuse with the intestinal cell walls and deposited into the bloodstream with a bioavailability exceeding 90%.

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References


