Bog Bilberry (Vaccinium uliginosum L.) Extract Reduces Cultured Hep-G2, Caco-2, and 3T3-L1 Cell Viability, Affects Cell Cycle Progression, and Has Variable Effects on Membrane Permeability

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ABSTRACT: Bog bilberry (Vaccinium uliginosum L.) is a blue-pigmented edible berry related to bilberry (Vaccinium myrtillus L.) and the common blueberry (Vaccinium corymbosum L.). The objective of this study was to investigate the effect of a bog bilberry anthocyanin extract (BBAE) on cell growth, membrane permeability, and cell cycle of 2 malignant cancer cell lines, Caco-2 and Hep-G2, and a nonmalignant murine 3T3-L1 cell line. BBAE contained 3 identified anthocyanins. The most abundant anthocyanin was cyanidin-3-glucoside (140.9 ± 2.6 μg/mg of dry weight), followed by malvidin-3-glucoside (10.3 ± 0.3 μg/mg) and malvidin-3-galactoside (8.1 ± 0.4 μg/mg). Hep-G2 LC50 was calculated to be 0.563 ± 0.04 mg/mL, Caco-2 LC50 was 0.390 ± 0.30 mg/mL and 0.214 ± 0.02 mg/mL for 3T3-L1 cells. LDH release, a marker of membrane permeability, was significantly increased in Hep-G2 cells and Caco-2 cells after 48 and 72 h compared to 24 h. The increase was 21% at 48 h and 57% at 72 h in Caco-2 cells and 66% and 139% in Hep-G2 cells compared to 24 h. However, 3T3-L1 cells showed an unexpected significant lower LDH activity (P ≤ 0.05) after 72 h of exposure corresponding to a 21% reduction in LDH release. BBAE treatment increased sub-G1 in all 3 cell lines without influencing cells in the G2/M phase. BBAE treatment reduced the growth and increased the accumulation of sub-G1 cells in 2 malignant and 1 nonmalignant cell line; however, the effect on membrane permeability differs considerably between the malignant and nonmalignant cells and may in part be due to differences in cellular membrane composition.

Keywords: anthocyanins, bog bilberry, LDH, Vaccinium uliginosum L., viability

Introduction

Bog bilberry (Vaccinium uliginosum L.) is an edible blue-colored berry classified in the same genus as the common blueberry (Vaccinium corymbosum) and bilberry (Vaccinium myrtillus L.). Bog bilberries are typically found in northern hemisphere countries such as Russia and Norway (Andersen 1987) and northern China. Bilberries and blueberries along with other pigmented fruits such as blackberry (Rubus fruticosus), cranberry (Rubus occidentalis), raspberry (Rubus idaeus), and many other anthocyanin containing fruits and vegetables have been reported to possess a wide variety of biological activities (Seeram 2008). Strong free radical scavenging antioxidant properties of berry extracts have been the focus of many reports along with an ability to inhibit the growth of variety of cultured cancer cell lines (Kong and others 2003; Elisia and Kitts 2008; Wang and Stoner 2008). Anthocyanins are responsible for the color of bog bilberries with over 15 different anthocyanins identified from bog bilberries grown in the west coast of Norway (Andersen 1987).

Reports on bog bilberry composition and bioactive properties are limited and a greater amount of literature is currently available on related species of bilberry (Vaccinium myrtillus L.). Bilberries contain cyanidin, delphinidin, pelargonidin, and malvidin glycosides making up the majority of reported anthocyanin content (Upton 2001). Anthocyanins are typically found as glycosides in berries shown by the chemical structure in Figure 1. The anthocyanin content of bog bilberry shares similar composition to other blue-pigmented fruit of the Vaccinium species (Andersen 1987). With regard to cancer cell proliferation, anthocyanin containing extracts from a variety of different berries have been reported to inhibit the growth of human colon (HT-29), prostate (LNCaP), breast (MCF-7), and oral (CAL27) cancer cell lines and stimulate apoptosis in cultured human colon cells (HT-29) (Seeram and others 2006). Specific anthocyanins such as cyanidin-3-glucoside and peonidin-3-glucoside have been shown to inhibit growth and induce apoptosis in human breast carcinoma cells (HS578T) (Chen and others 2005). There is a growing interest in the potential health promoting aspects of berries for their chemopreventative properties but the literature specifically focused on bog bilberries is scarce. Therefore, the objective of this study was to assess a bog bilberry extract propensity to selectively inhibit the growth, influence membrane permeability, and affect the cell cycle of 3 distinct cell lines. The cells included 2 human cancer cell line models, representing the colonocytes (Caco-2), hepatocytes (Hep-G2), and a nonmalignant murine fibroblast cell line (3T3-L1).

Materials and Methods

Plant material and HPLC analysis

Anthocyanin standards, cyanidin-3-glucoside, malvidin-3-galactoside, and malvidin-3-glucoside were purchased from Sigma
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(St. Louis, Mo., U.S.A.). Dried Bog bilberry anthocyanin extract (BBAE) was obtained from Hua Ye Biological Engineering (Hei Long Jiang, China). BBAE and the standards were analyzed using a reverse phase HPLC (Waters, Milford, Mass., U.S.A.) equipped with a photo diode array detector (PDA 2996). The column was a Waters Symmetry column (C18, 4.6 mm × 250 mm, 5 μm particle size). The injection volume was 20 μL with a 0.4 mL/min flow. The mobile phases consisted of 2 phases (A) and (B). Mobile phase (A) was 0.1% (v/v) TFA in water and mobile phase (B) was 0.1% TFA in water/acetonitrile (1/3, v/v). BBAE was dissolved in mobile phase (A) and filtered through a 0.22 μm membrane filter. The elution conditions were as follows: isocratic elution 10% (B), 0 to 10 min; linear gradient from 10% (B) to 30% (B), 35 min; isocratic elution 30% (B), 35 to 40 min; to 10% (B), 45 min; at 10% (B), 45 to 50 min. The column temperature was 40 °C and the detection wavelength was 525 nm.

Cell culture

The human hepatocellular carcinoma cell line (Hep-G2), colorectal adenocarcinoma cell line (Caco-2) and murine fibroblasts cell line (3T3-L1) were purchased from ATCC (Manassas, Va., U.S.A.). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Sigma) and antibiotics (100 units/mL of penicillin and 100 μg/mL of streptomycin, GIBCO, Invitrogen, Burlington, Canada). The cells were subcultured and incubated at 37 °C in a humidified atmosphere of 5% carbon dioxide incubator. Cells were maintained at a concentration between 2 × 10^5 and 1 × 10^6 cells/mL. Cells were subcultured every 2 to 3 d by total medium replacement using 0.25% (w/v) trypsin–0.53 mM EDTA solution (GIBCO). Viable cells were assessed by 0.04% trypan blue exclusion dye (MP Biomedicals, Solon, Ohio, U.S.A.) using a Neubauer hemocytometer (Blaubrand, Wertheim, Germany) and assessed in quadruplicate.

**MTT cell viability**

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT assay (Sigma), to establish an LC50 value (concentration to inhibit 50% of cells). Hep-G2 cells, Caco-2 cells, and 3T3-L1 cells were seeded separately in 96-well plates at a concentration of 1 × 10^5, 1 × 10^5, 2.5 × 10^4 cells/mL respectively, and allowed to be attached overnight. A BBAE stock solution was serial diluted and added to the cells and incubated for 72 h, untreated cells acted as control. The extract-containing medium was removed and 100 μL of 0.5 mg/mL of MTT was added and incubated for 4 h as previously described (Zhang and Popovich 2008). Formazan crystals were solubilized in 100 μL of 10% SDS in 0.01 N HCl for 4 h. The absorbance was measured at 550 nm with a reference wavelength of 650 nm using a microplate reader (Multiskan Spectrum, Thermo Electron Corp., Waltham, Mass., U.S.A.). The results were expressed as the percentage of viable cells with respect to the untreated control cells. Cell viability (%) was calculated as \[(\text{mean absorbance of the sample} – \text{reference absorbance}) / \text{mean absorbance of the control}\] × 100.

**Lactate dehydrogenase (LDH) activity**

Hep-G2, Caco-2, and 3T3-L1 cells were seeded at a concentration of 5 × 10^5, 3 × 10^5, 5 × 10^4 cells/mL in 24-well plates, respectively.

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**Figure 1** — The chemical structure of cyanidin-3-glucoside, malvidin-3-galactoside, and malvidin-3-glucoside (insert) and the general chemical formula without functional groups is C_{15}H_{11}O+. A representative HPLC chromatogram of BBAE is shown with peaks corresponding to cyanidin-3-glucoside (2), malvidin-3-galactoside (4), and malvidin-3-glucoside (5). Peaks labeled 1 and 3 are unidentified.
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BBAE was added to the cells at the LC50 concentrations determined from MTT analysis (described subsequently) and incubated for 24, 48, and 72 h. The media was removed and centrifuged (400 × g; 10 min). Two milliliters of Tris-EDTA-NADH buffer and 50 μL of media were mixed and incubated for 12 min and 200 μL of prewarmed (37 °C) pyruvate solution was added. The initial reaction velocity was recorded by continuous monitoring at 340 nm in a temperature-controlled (37 °C) microplate reader (Thermo Electron). The concentrations of the reaction components were Tris buffer, 50 mmol/L (pH 7.4, 37 °C), EDTA, 5 mmol/L, pyruvate, 1.2 mmol/L, NADH, 150 μmol/L, and previously described (Popovich and Kitts 2002).

Cell cycle analysis
BBAE-treated cells (Hep-G2, Caco-2, and 3T3-L1) were incubated separately for 24, 48, and 72 h at their respective LC50 concentrations and untreated cells served as controls. Culture media was collected and cells were removed by trypsinization, washed twice with phosphate buffer saline (PBS), and combined with the collected media and centrifuged (500 × g, 5 min). The cell pellet was vortexed vigorously and fixed in 1 mL ice-cold ethanol (70%) overnight. Ethanol was removed by centrifugation (500 × g, 5 min) and the pellet was further washed twice with PBS. The cells were incubated in the dark for 30 min with 200 μL cell cycle reagent (Guava Technologies, Hayward, Calif., U.S.A.) before data acquisition and analysis using a Guava PCA flow cytometer with Cytosoft software.

Statistical analysis
The MTT, LDH, and cell cycle analysis consisted of 3 separate experiments with 4 replicates for MTT and 3 separate experiments with 3 replicates for LDH and cell cycle analysis. A one-way analysis of variance (ANOVA) and t-test were used to analyze the experimental data. The significance was taken at a P-value of 0.05 using the Duncan post hoc multiple comparisons of observed means and values are expressed as mean ± standard deviation (SD).

Results

HPLC analysis
Representative HPLC chromatograph of BBAE is shown in Figure 1. Five peaks were detected at 525 nm and 3 were identified and quantified in relation to authentic standard curves. BBAE contained cyanidin-3-glucoside (140.9 ± 2.6 μg/mg of dry weight), malvidin-3-galactoside (10.3 ± 0.3 μg/mg), and malvidin-3-glucoside (8.1 ± 0.4 μg/mg). Peaks labeled (2), (4), and (5) correspond to cyanidin-3-glucoside, malvidin-3-galactoside, and malvidin-3-glucoside. Two peaks labeled (1) and (3) shown in the chromatogram are unknown.

MTT viability and LDH release
Dose–response curves of BBAE viability of 3 different cell lines (Hep-G2, Caco-2, 3T3-L1) are shown in Figure 2. The LC50 of BBAE in each cell line was calculated from a plot of viability (%) compared with log concentration (graph not shown) which yielded a linear equation of \( y = -90.471x + 298.75 \) (\( r^2 = 0.9815 \)), \( y = -104.45x + 320.57 \) (\( r^2 = 0.9919 \)), and \( y = -59.627x + 189.65 \) (\( r^2 = 0.9526 \)) for Hep-G2 cells, Caco-2 cells, and 3T3-L1 cells, respectively. The Hep-G2 LC50 was calculated as 0.363 ± 0.04 mg/mL, Caco-2 LC50 was 0.390 ± 0.30 mg/mL, and 0.214 ± 0.02 mg/mL for 3T3-L1 cells.

The LDH release expressed as percentage of untreated cells was significantly (\( P \leq 0.05 \)) increased in Hep-G2 cells and Caco-2 cells treated BBAE for 48 and 72 h compared to 24 h (Figure 3). The increase was 21% at 48 h and 57% at 72 h in Caco-2 cells and 66% and 139% in Hep-G2 cells compared to 24 h. However, 3T3-L1 cells showed a significant lower LDH activity (\( P \leq 0.05 \)) after 72 h of exposure corresponding to a ~21% reduction in LDH.

Cell cycle analysis
Representative DNA histograms derived from cell cycle analysis of BBAE treated cells at their respective LC50 concentrations are shown in Figure 4 and the corresponding cell cycle distribution (%) is listed in Table 1. Caco-2 and 3T3-L1 cells had significantly (\( P \leq 0.05 \)) greater accumulation of sub-G1 apoptotic cells after 48 and 72 h of treatment at their LC50. In comparison with the respective time period control, Hep-G2 cells showed a significant (\( P \leq 0.05 \)) increase of sub-G1 cells at all time periods tested. The results also indicated BBAE treatment in Hep-G2 cells resulted in a significant

\[ y = -90.471x + 298.75 \] (\( r^2 = 0.9815 \))
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\[ y = -59.627x + 189.65 \] (\( r^2 = 0.9526 \))

Figure 2—MTT cell viability curve of BBAE treated Hep-G2, Caco-2, and 3T3-L1 treated for 72 h. Values are expressed as a percentage of untreated control cells (mean ± SD).

Figure 3—Lactate dehydrogenase release (LDH) of 3 different cell lines at 24 to 72 h of treatment with BBAE at the respective LC50 concentration. Data are expressed as percentage of untreated control cells (mean ± SD) of 3 separate experiments performed in triplicate. Bars within the same cell line but different time period possessing different letters were significantly different (\( P \leq 0.05 \)).
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Reduction in cells at the G1 phase after 72 h and reductions in S phase at 24 and 48 h. Caco-2 cells showed a reduction in G1 phase after 48 h and a reduction in S phase after 72 h. In 3T3-L1 cells BBAE treatment resulted in significant (P ≤ 0.05) reductions in G1 cell phase at 72 h, S phase at 24 and an increase at 72 h. BBAE treatment did not have any effect on cells in the G2/M phase of the cell cycle in all 3 cell lines.

Discussion

We have effectively shown that a bog bilberry anthocyanin extract (BBAE) that contained 3 identified anthocyanin glycosides (cyanidin-3-glucoside, malvidin-3-glucoside, and malvidine-3-galactoside) with cyanidin-3-glucoside being the most abundant can inhibit the growth of 3 distinct cultured cell lines. In the nonmalignant embryonic murine fibroblast cell line (3T3-L1), BBAE treatment resulted in the lowest LC50 and was most sensitive to BBAE, followed by colon cancer cells (Caco-2) and hepatocarcinoma cells (Hep-G2). There are conflicting reports that anthocyanins extract may target the growth of cancer cells and not normal cells. For example, anthocyanin containing Jamun fruit extract (Eugenia jambolana Lam) has been reported to selectively target cancer cells compared to cultured normal cell lines (Li and others 2009). Jamun fruit extract was reported to contain anthocyanin glucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin and was shown to have the greatest cytotoxicity in estrogen-dependent breast cancer cell line MCF-7 followed by nonestrogen-dependent breast cancer cell line MDA-MB-231; the nontumorigenic epithelial breast cell line (MCF-10A) showed the least cytotoxicity (Li and others 2009). Furthermore, fractionated extracts of black raspberries were reported to inhibit the growth of cancer cell lines.

Table 1 — Cell cycle distribution of Hep-G2, Caco-2, and 3T3-L1 cells treated with BBAE for 24, 48, and 72 h. Three separate experiments were performed in triplicate.

<table>
<thead>
<tr>
<th></th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>BBAE</td>
<td>Control</td>
</tr>
<tr>
<td><strong>Hep-G2</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SubG1 (%)</td>
<td>0.20 ± 0.1</td>
<td>3.63 ± 0.6*</td>
<td>0.48 ± 0.1</td>
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<td>G1 (%)</td>
<td>50.11 ± 3.7</td>
<td>54.22 ± 1.7</td>
<td>51.11 ± 1.5</td>
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<tr>
<td>S (%)</td>
<td>12.36 ± 0.7</td>
<td>8.12 ± 0.4*</td>
<td>10.21 ± 0.4</td>
</tr>
<tr>
<td>G2/M (%)</td>
<td>37.33 ± 3.9</td>
<td>34.03 ± 1</td>
<td>34.16 ± 1.9</td>
</tr>
<tr>
<td><strong>Caco-2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SubG1 (%)</td>
<td>1.81 ± 0.2</td>
<td>2.78 ± 1.4</td>
<td>2.04 ± 0.8</td>
</tr>
<tr>
<td>G1 (%)</td>
<td>48.14 ± 1</td>
<td>41.10 ± 3.1</td>
<td>42.83 ± 0.9</td>
</tr>
<tr>
<td>S (%)</td>
<td>17.84 ± 0.7</td>
<td>18.62 ± 0.3</td>
<td>18.64 ± 1</td>
</tr>
<tr>
<td>G2/M (%)</td>
<td>32.1 ± 1.3</td>
<td>31.50 ± 3.4</td>
<td>36.43 ± 0.4</td>
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<tr>
<td><strong>3T3-L1</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SubG1 (%)</td>
<td>0.55 ± 0.5</td>
<td>1.02 ± 0.8</td>
<td>0.70 ± 0.1</td>
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<tr>
<td>G1 (%)</td>
<td>54.15 ± 1.2</td>
<td>54.35 ± 1.6</td>
<td>64.05 ± 1.1</td>
</tr>
<tr>
<td>S (%)</td>
<td>15.97 ± 1.3</td>
<td>13.03 ± 1.2*</td>
<td>11.72 ± 0.8</td>
</tr>
<tr>
<td>G2/M (%)</td>
<td>29.15 ± 0.5</td>
<td>31.57 ± 2</td>
<td>23.57 ± 1.8</td>
</tr>
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</table>

Values were expressed as mean ± SD.
*Significant difference (P ≤ 0.05) in same cell line and time periods compared to the respective untreated control values.
of both premalignant and malignant human oral epithelial cells but not normal cells (Han and others 2005). Weaver and others, however, reported that strawberry extracts did not show a tendency to selectively target malignant human breast and prostate cells over normal cells isolated from patients (Weaver and others 2005). In this study, we found no evidence of selective cytotoxicity with regard to cell growth, as all 3 cells lines showed a dose-dependent cytotoxic response measured by the MTT viability assay. Differences in cellular LDH release were observed between cell types. Both malignant cell lines Caco-2 and Hep-G2 showed a significant ($P \leq 0.05$) increase in LDH release over time reaching a maximum at 72 h whereas in the fibroblast 3T3-L1 LDH release did not show a similar pattern. At 72 h of exposure BBAE treatment of 3T3-L1 cells LDH release was found to be significantly lower at 72 h compared to both 24 and 48 h. This was unexpected considering 3T3-L1 was more sensitive to BBAE than both Caco-2 and Hep-G2 cells. Reduction in LDH release has been previously reported after anthocyanin treatment. Anthocyanin-rich nonalcoholic extract of Argentinean red wine has been reported to reduce LDH leakage from isolated Wistar rat hearts after ischemia-reperfusion injury (Fantielli and others 2005). Oral administration of anthocyanins from bilberry to Sprague Dawley rats administered 30 min before a forced swimming test resulted in a reduction of plasma LDH compared to nontreated animals (Marcollet and others 1969). In primary Wistar rat hepatocytes, Java plum anthocyanins (Syzygium cumini) was found to reduce LDH release after carbon tetrachloride exposure (Veigas and others 2008). LDH is found in all cells and its release is generally regarded as a marker of membrane permeability and damage (Danpure 1984). In this study, BBAE increased the release of LDH in 2 cultured cancer cells compared to the nonmalignant 3T3-L1 cells. It is possible that malignant cancer cells might be more susceptible to membrane damage induced by BBAE extract compared to nonmalignant cells. Tumor cells have different cell membrane surfaces with additional glycolipids and glycoproteins compared to the nontransformed progenitor cells (Hakomori 1985). These additions and structural changes in the cell membrane may alter membrane properties making them more susceptible to BBAE induced damage. Another possible explanation adapted from one suggested by Danpure (1984), albeit more unlikely, is that the BBAE may specifically inhibit 3T3-L1 cytoplasmic LDH activity rendering it active over time. BBAE may transverse the cell membrane directly interacting with LDH in the cytoplasm whereas in Hep-G2 and Caco-2 BBAE may become associated with additional membrane constituents of transformed cells. Cell cycle analysis did not show any major differences between the cells of these 3 distinct cell lines. Generally, the analysis showed a significant ($P \leq 0.05$) buildup of sub-G1 Hep-G2 cells consistent with the reported apoptotic cell death (Seeram and others 2006) at all time periods compared to the respective untreated control cells and a significant decrease in cells at the G1 phase at 72 h. Caco-2 cells sub-G1 cells significantly ($P \leq 0.05$) accumulated at 48 and 72 h and a decrease in G1 cells at 48 h. 3T3-L1 cells also showed an increase in sub-G1 cells at 48 and 72 h with a significant decrease G1 at 72 h compared to control cells.

**Conclusions**

An anthocyanin extract consisting of mainly cyanidin-3-glucoside derived from bog bilberry was found to dose-dependently reduce cultured cell growth in both malignant and nonmalignant cell lines and affected changes in the cell cycle. However, differences in membrane permeability and damage were observed with malignant cell showing greater damage than nonmalignant cells. Further studies are needed to determine whether anthocyanins are indeed more cytotoxic to malignant cells compared to nonmalignant cells and if this mediated through alterations in cellular membrane function.

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**References**


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