

Effects of a Bilberry Preparation on Selected Cell Lines of the Digestive System

KAMILA BOROWIEC¹, DOMINIK SZWAJGIER¹, ANNA OLEJNIK², KATARZYNA KOWALSKA²
and ZDZISŁAW TARGOŃSKI¹

¹Department of Biotechnology, Human Nutrition and Science of Food Commodities, Faculty of Food Science and Biotechnology, University of Life Sciences, Lublin, Poland; ²Department of Biotechnology and Food Microbiology, Faculty of Food Science and Nutrition Poznań University of Life Sciences, Poznań, Poland

Abstract

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Bilberry is a valuable wild forest fruit harvested in many countries in Europe. The biological activities of bilberry include antioxidant, anticancer, antiviral, antibacterial, and anticholinesterase activities. This study examines the protective effects of a bilberry (BB) preparation on IEC-6, Caco-2, and HepG2 cell lines. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to study the cytotoxicity of BB. The genotoxicity was determined using single-cell microgel electrophoresis. The Ames test was employed to assay bilberry mutagenicity. No significant effects of BB (12.5–100 µg dry mass/ml) were observed on the viability of IEC-6, Caco-2, and HepG2 cells. There were no differences in the percentage of DNA in the comet tail between the cells treated with BB (100 µg dry mass/ml) and the control cells. However, a significant reduction of oxidative DNA damage in the HepG2 cells was found. BB exhibited neither mutagenic nor promutagenic effects. Our results suggest that bilberry can be a potential tool in the prevention of chronic diseases, without any undesired effects on the cells of the gastrointestinal tract.

Keywords: *Vaccinium myrtillus* L.; cytotoxicity; functional food; genotoxicity; mutagenicity

Bilberry (*Vaccinium myrtillus* L.) fruit is well-known as a source of antioxidants, including polyphenolic compounds (ABBY *et al.* 2013). Antiviral (SEKIZAWA *et al.* 2012), antibacterial (RAUDSEPP *et al.* 2013), anticholinesterase (SZWAJGIER & BOROWIEC 2012; BOROWIEC *et al.* 2014), and antidiabetic (TÖRRÖNEN *et al.* 2013) effects of bilberry extracts have also been well documented. A phenolic extract from bilberry has been shown to inhibit proliferation of the colon cancer cells (AABY *et al.* 2013). Anthocyanidins isolated from bilberry have inhibited the growth of two aggressive non-small-cell lung cancer cell lines (KAUSAR *et al.* 2012). Regular consumption of bilberry reportedly reduces low-grade inflammation (KOLEHMAINEN *et al.* 2012) and decreases lipoprotein levels (MADIHI *et al.* 2013).

The majority of the available studies are concentrated on bilberry extracts. In this study, a bilberry (BB) preparation obtained by juice processing and ultrafiltration (molecular weight cutoff < 5 kDa) was used. Specific cell lines were selected to examine the influence of BB on the digestive system, as these are the cells that are exposed to high concentrations of compounds derived from food. The IEC-6 (normal rat small intestine epithelial) cells were chosen as a model of the small intestine. Considering the functionality of the Caco-2 (human epithelial colorectal adenocarcinoma) cells in the evaluation of passive transport of drugs and food compounds (BALIMANE & CHONG 2005), this cell line was also used as these cells are morphologically and physiologically similar to normal enterocytes. The HepG2 (human hepa-

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tocellular liver carcinoma) cells represented the metabolic function of the liver as a common tool in the study of the metabolism of many compounds (WILKENING & BADER 2003).

MATERIAL AND METHODS

Bilberry preparation. Test samples of fresh bilberry fruit in harvest maturity were purchased from a local market and frozen (−20°C) until use. Fruits were harvested in forests in the Lublin province in June 2011. The identity of the fruit samples was confirmed by Prof Kazimierz Głowniak (Medical University, Department of Pharmacognosy with Medicinal Plant Laboratory, Lublin, Poland).

Bilberry juice was obtained using a commercial juice extractor (500 g for 15 min, Thermomix TM31; Vorwerk, Wuppertal, Germany) followed by centrifugation (4°C, 13 131 g, 30 min). The supernatant solution was ultrafiltered (4°C, Vivaflow 50, 5 kDa PES membrane, Masterflex L/S Economy Drive pump; Cole-Parmer Instrument Co., Vernon Hills, USA). The ultrafiltrate solution was freeze dried (−50°C, Labconco FreeZone 2.5; Labconco, Fort Scott, USA) and frozen (−20°C) until use.

Cell cultures. The IEC-6, Caco-2, and HepG2 cell lines were purchased from the European Collection of Animal Cell Cultures (Sigma-Aldrich, Steinheim, Germany). The cells were cultured in Dulbecco's modification of Eagle's medium supplemented with nonessential amino acid solution (1 : 100, v/v), fetal bovine serum (1 : 10, v/v), and 50 mg/ml gentamicin solution (1 : 1000, v/v). In the case of IEC-6 cells, 10 mg/ml insulin (1 : 2000, v/v) was also added. Cells were maintained at 37°C, at 98% relative humidity, in the presence of 5% CO₂ and 95% air.

BB was dissolved in the complete media prepared as described above. The concentration of BB was 5 mg dry mass – DM/ml (pH 7.2). The bilberry medium solution was centrifuged (18°C, 3000 g, 10 min) and filtered (0.22 µm low-protein-binding syringe filters; Merck Millipore, Darmstadt, Germany).

Cytotoxicity assay. The cells were grown in 96-well plates at an initial density of 2.5×10^4 cells/cm². The 24-h cultures were treated with BB at concentrations ranging from 12.5 to 400 µg DM/ml and incubated for 48 h under standard culture conditions.

Cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (MOSMANN 1983) according

to the previously described procedure (OLEJNIK *et al.* 2016). Briefly, after treatment of the cells with BB, the MTT solution (0.5 mg/ml) was added to each well and the microplate was incubated at 37°C for 3 hours. Acidic isopropanol was added to each well at the end of the incubation period. The absorbance was measured at 570 nm using a Tecan M200 Infinite microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Viability of the cells treated with BB was expressed as % absorbance relative to untreated cells. The test was performed in triplicate.

Genotoxicity assay. The potential DNA damage in the IEC-6, Caco-2, and the HepG2 cells induced following the 48-h treatment with BB (100 µg DM/ml) was determined using the method described by HARTMANN *et al.* (2003) with modifications proposed by OLEJNIK *et al.* (2010). Moreover, the effect of BB on the oxidative DNA damage induced by 100 µM H₂O₂ (37°C, 30 min) was also investigated. The single-cell gel electrophoresis method was used to detect DNA damage. The slides with single cells were placed in the lysis mixture (100 mM EDTA, 5.5 M NaCl, 10 mM Tris, 1% Triton X-100, pH 10.0, 4°C, 1 h), washed and incubated in electrophoresis buffer. Electrophoresis was conducted for 40 min at 300 mA. After staining with SYBRGold (Molecular Probes, Inc., Eugene, USA), 80 comets per slide were analysed using an inverted fluorescence microscope (Axiovert 200 Zeiss; Carl Zeiss AG, Göttingen, Germany). Data were expressed as the mean percentage of DNA in the comet tail using CometScore™ software (TriTek Corp., Sumerduck, USA). At least three slides per experimental condition were analysed.

Mutagenicity assay. Mutagenicity of BB was examined using the Ames test described by MARON and AMES (1983) according to the procedure described in detail by CYPLIK *et al.* (2012). The bacterial strains (*Salmonella typhimurium*: TA98, TA100, TA1535, and TA1537) were purchased from the Polish Collection of Microorganisms, the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences (Wrocław, Poland). The reaction mixtures contained 100 µL of bacterial suspension, 10 µL of standard mutagen (a reference promutagen: 20 µg of 2-aminoanthracene (2-AA) for all tested strains or reference mutagens: 200 µg of 2-aminofluorene (2-AF) for TA98 strain; 1 µg of sodium azide (AZS) for TA100, and TA1535 strains; 30 µg of 9-aminoacridine (9-AA) for TA1537 strain), or 100–300 µL of BB (1 mg DM/ml). Optionally, 500 µL of rat liver microsomal enzymes (S9MIX; Sigma-Aldrich, Germany) was added to

the reaction mixtures containing the reference pro-mutagen or BB. PBS was added to a total volume of 1 ml. After 48-h incubation at 37°C, bacterial colonies were counted. Based on the obtained results, the mutagenic activity of BB and mutagens was calculated according to the following equation: $AM = (R_i - R_s)/R_s$, where R_i stands for the number of revertants induced by standard mutagen or by the analysed extract and R_s is the number of spontaneous revertants in the negative control. A doubling of the mutagenic activity was considered to be significant. Based on the value of AM, 2-AA, 2-AF, AZS, and 9-AA have been recognised as reference mutagens similar to other mutagenic compounds with $AM \geq 2$. The experiments were performed in triplicate.

Statistical analysis. All data are expressed as mean \pm SD. Data were assessed by analysis of variance and mean comparisons were performed according to Tukey's test using STATISTICA 6.0 (StatSoft, Kraków, Poland). A significance value of $P < 0.05$ was used to distinguish significant differences.

RESULTS AND DISCUSSION

To see any potential cytotoxic effect of BB on the IEC-6, Caco-2, and HepG2 cells, the tetrazolium reduction rate was measured in the MTT assay. No significant influence ($P < 0.05$) of BB at 12.5–400 μ g DM/ml was observed in terms of the cell proliferation and viability of the IEC-6 cells (Figure 1A). The viability of the Caco-2 and HepG2 cells was only decreased (by approximately 20%) by BB at 200 and 400 μ g DM/ml (Figures 1B and 1C). However, such high concentrations (higher than 100 μ g/ml) of BB are unlikely to be observed *in vivo*. Our results broaden our knowledge of the bilberry cytotoxicity. Admittedly, SCHANTZ *et al.* (2010) reported that the bilberry extract was not cytotoxic towards the Caco-2 cells in the alamar-Blue assay (10–500 μ g DM/ml, 24 h). In contrast, AABY *et al.* (2013) proved that the bilberry extract (75, 125, and 250 mg of gallic acid equivalents/ml, 24 h) inhibited the proliferation of the Caco-2 cells in the MTT test. Then, a daily intake of bilberry extract (100 and 400 ppm extract in drinking water) was also recommended to suppress the hepatocarcinogenic process in piperonyl butoxide-promoted hepatocarcinogenesis in rats (HARA *et al.* 2014). Last but not least, VALENTOVÁ *et al.* (2007) showed no cytotoxic effect of the bilberry concentrate in the MTT assay (100 and 500 μ g DM/ml; 4, 24, or 48 h)

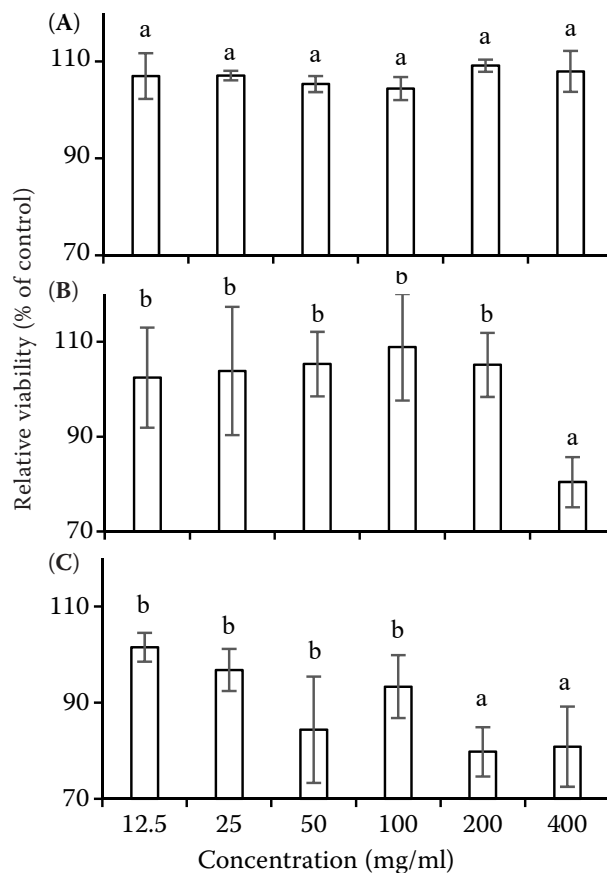


Figure 1. Effect of bilberry on the IEC-6 (A), Caco-2 (B), and the HepG2 (C) cell viability

Mean values \pm SD ($n = 3$); bars with the same letter are not significantly different ($P < 0.05$)

on primary cultures of rat hepatocytes. However, the present work has been the first attempt to examine the potential cytotoxicity of the whole bilberry fruit, not just an anthocyanin-rich bilberry extract. At the present moment, no data are available concerning the effect of either bilberry (or its preparation) or of any plant from the genus *Vaccinium* on the viability of the IEC-6 cells.

A genotoxic effect of BB on the IEC-6, Caco-2, and HepG2 cells is presented in Figure 2. There were no differences ($P < 0.05$) in the percentage of DNA in the comet tail between cells treated with BB (100 μ g DM/ml, 48 h) and control cells. No effect of BB on oxidative DNA damage caused by 100 μ M H_2O_2 on the Caco-2 and IEC-6 cells was observed. In contrast, a protective effect of BB on the HepG2 cells exposed to oxidative stress was observed. In this case, a significant reduction ($P < 0.05$) of about 12% in oxidative DNA damage was observed (Figure 2C). Therefore, it is proposed that BB has a protective ef-

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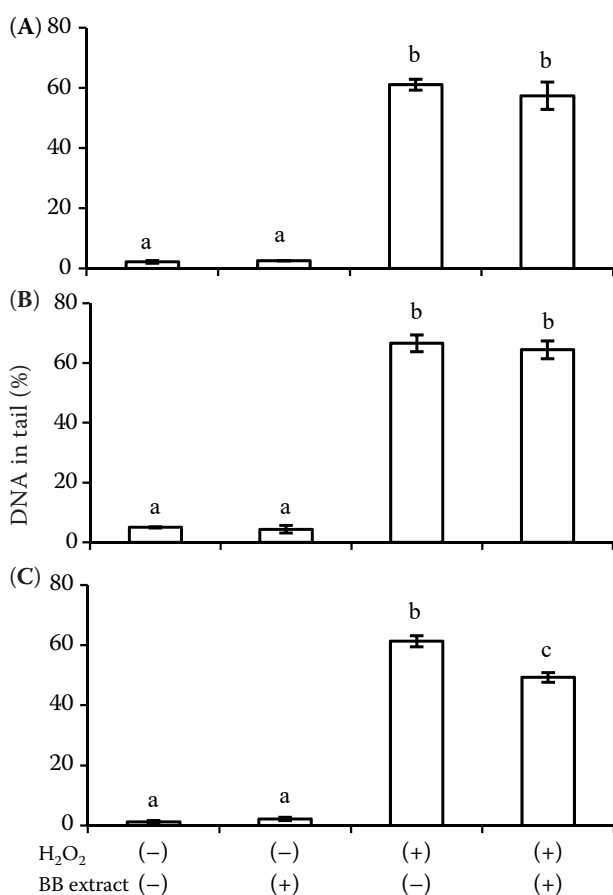


Figure 2. Effect of bilberry on DNA damage in the IEC-6 (A), Caco-2 (B), and HepG2 (C) cells untreated (–) or treated (+) with H₂O₂

Mean values \pm SD ($n = 3$); bars with same letter are not significantly different ($P < 0.05$) when compared to the control

effect against oxidative damage in hepatocytes. There are no reports on potential genotoxicity of the whole

bilberry fruit on the selected cells. VALENTOVÁ *et al.* (2007) also reported that the bilberry extract showed a significant ($P < 0.01$) dose-dependent (100 and 500 μ g DM/ml) protective activity against oxidative damage (induced by *tert*-butyl hydroperoxide and allyl alcohol) in primary cultures of rat hepatocytes. Additionally, SCHANTZ *et al.* (2010) observed a significant ($P < 0.05$) reduction of menadione-induced DNA damage (6 μ M, 1 h) in the Caco-2 cells after pretreatment with a bilberry extract (5 and 50 μ g/ml, 24 h). Likewise, JUADJUR *et al.* (2015) demonstrated that the oxidative DNA damage in the Caco-2 cells was significantly ($P < 0.05$) reduced in the presence of a commercially available bilberry extract (5 and 10 μ g/ml, 1 or 24 h).

The potential mutagenic properties of BB were determined by the Ames test. The effect of reverse mutation in altered *Salmonella typhimurium* strains (unable to synthesise histidine) was tested. In the present study, BB (100–300 μ g DM/ml) did not exert any mutagenic or promutagenic effects on all strains used (Table 1). There were no differences in the number of revertants induced by BB compared to spontaneous revertants. Additionally, the mutagenic activity was estimated to be lower than or equal to 0.5, when the mutagenic activity of the reference mutagens and 2-AA was higher than 3. The only study which focused on mutagenicity of bilberry was the test that examined Myrtocyan[®], a commercial bilberry extract (SEIFRIED *et al.* 2006). In the cited work, the authors showed that Myrtocyan[®] (100–10000 μ g/plate) caused no mutagenic lesions in *Salmonella typhimurium* TA98, TA100, TA1535, TA1537, and TA1538.

Table 1. Effect of bilberry on mutagenesis in TA98, TA100, TA1535, and TA1537 strains, with (+S9) or without (–S9) metabolic activation

Bacteria strain	Metabolic activation (+S9)/(–S9)	Spontaneously induced revertants (CFU/plate)	Induced revertants (CFU/plate)		Mutagenic activity	
			mutagen	BB	mutagen	BB
TA98	–	8 \pm 2	107 \pm 11	12 \pm 5	12.4	0.5
	+	16 \pm 5	75 \pm 6	10 \pm 2	3.7	–0.4
TA100	–	37 \pm 5	327 \pm 25	29 \pm 7	7.8	–0.2
	+	55 \pm 7	280 \pm 37	58 \pm 10	4.1	0.1
TA1535	–	13 \pm 2	76 \pm 9	11 \pm 3	4.8	–0.2
	+	10 \pm 4	47 \pm 3	12 \pm 4	3.7	0.2
TA1537	–	27 \pm 4	216 \pm 17	30 \pm 6	7.0	0.1
	+	12 \pm 3	54 \pm 12	8 \pm 4	3.5	0.3

Data were expressed as the mean number of revertants (\pm SD); mutations were induced by the mutagens: 2-AF (TA98), AZS (TA100, TA1535), 9-AA (TA1537), and the promutagen 2-AA (TA98, TA100, TA1535, TA1537)

CONCLUSION

In summary, our work suggests that a diet supplemented with bilberry fruit can be considered a potential tool in the combined therapy of many diseases, without any undesired effects on the cells present in the gastrointestinal tract. However, more extensive *in vitro* or *in vivo* assays should be carried out. We demonstrated that BB exerted no cytotoxic, genotoxic, or mutagenic effects on the Caco-2, IEC-6, and HepG2 cells. At the same time, the reduction of oxidative damage in the HepG2 cells should be stressed. Our results confirmed findings of other authors, however, unlike most of the authors, we deliberately examined potential protective effects of the whole bilberry fruit. This work has evaluated the influence of the whole bilberry fruit on the IEC-6 cells for the first time, which is important because the IEC-6 cells come into direct physical contact with a very complex mixture of food components in the intestine.

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Corresponding author:

Dr KAMILA BOROWIEC, Uniwersytet Przyrodniczy w Lublinie, Wydział Nauk o Żywności i Biotechnologii, Katedra Biotechnologii, Żywienia Człowieka i Towaroznawstwa Żywności, Skromna 8, 20-704 Lublin, Poland; E-mail: kam.borowiec@gmail.com
