

Cytoprotective effect of a bilberry extract against oxidative damage of rat hepatocytes

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Abstract

The effect of a bilberry extract (BE, 25% anthocyanins) against oxidative damage in primary cultures of rat hepatocytes, induced by *tert*-butyl hydroperoxide and allyl alcohol, was investigated. BE displayed cytoprotective effects at 100 and 500 µg/ml in the MTT viability test. It protected the cells against lactate dehydrogenase leakage and lipoperoxidation products formation. Maximum protection (58%) was noted using 500 µg/ml of BE and intoxication by allyl alcohol. The observed cytoprotective effect is probably due to the antioxidant properties of its constituents, mainly anthocyanins. BE scavenged DPPH (IC₅₀ 3.99 ± 0.14 µg/ml) and enzymatically generated superoxide radical with an activity equivalent to 108 ± 7.2 U of superoxide dismutase per mg of extract. Our results support the use of bilberry and bilberry extracts in functional foods and food supplements designed for the prevention of chronic diseases associated with oxidative stress. © 2006 Elsevier Ltd. All rights reserved.

Keywords: *Vaccinium myrtillus*; Cytoprotectivity; Radical scavenging

1. Introduction

Vaccinium myrtillus L. (bilberry) is a member of the Ericaceae family and is also known as European blueberry, huckleberry, whortleberry or blueberry. It is a shrubby perennial plant that can be found in the mountains and forests of Europe and in the northern United States. The fruit of the bilberry plant is a blue-black or purple berry and differs from the American blueberry in that the flesh of the berry is purple, rather than cream or white. Bilberry has been used as food for centuries, due to its high nutritive value, and today it represents a precious wild delicacy. Bilberry's history of medicinal use dates back to the Middle Ages but it did not become widely known to herbalists until the 16th century, when it was used for treating bladder stones, biliary disorders, scurvy, coughs and lung tuberculosis. More recently, bilberry fruit extracts have been used

for the treatment of diarrhea, dysentery, and mouth and throat inflammations (Anonymous, 2001).

The berries contain the anthocyanoside flavonoids (anthocyanins, Nakajima, Tanaka, Seo, Yamazaki, & Saito, 2004), stilbenoids (resveratrol, Lyons et al., 2003), vitamins, sugars and pectins. The anthocyanins are considered the most important of the pharmacologically active constituents. Anthocyanin concentration in the fresh fruit is approximately 0.1–0.5%, while concentrated bilberry extracts are usually standardized to 25% anthocyanins (Ichiyanagi et al., 2004; Zhang, Kou, Fugal, & McLaughlin, 2004). Phenolic compounds, including anthocyanins, are known as strong antioxidants. While in vitro antioxidant activity of bilberry extracts has been studied in various non-cellular models (Laplaud, Lelubre, & Chapman, 1997; Martín-Aragon, Basabe, Benedi, & Villar, 1998, 1999), cytoprotective activity against oxidative damage has not yet been documented. The aim of our study was to investigate the effect of a bilberry extract (BE) against oxidative cell damage induced by *tert*-butyl hydroperoxide and allyl alcohol in primary cultures of rat hepatocytes.

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2. Materials and method

2.1. Plant material

The bilberry extract was prepared as follows: Frozen bilberry fruits (harvested in 2000 in Northern and Eastern Slovakia) were extracted with an aqueous solution of phosphoric acid (0.5%) on a battery of percolators. The obtained primary extract was purified on a column filled with non-ionic polystyrene–divinylbenzene resin Sepabeads SP 825 L. The column was washed with water and the adsorbed organic compounds were desorbed by ethanol. The ethanol extract was then concentrated by evaporation and the concentrate was spray-dried using about 10% of maltodextrin as a carrier, to obtain a fine red-brown powder. The process is described in more detail in the CZ patent 292 834 (Buchta & Cvak, 2003).

2.2. Extract analysis

The extract was analysed by RP-HPLC (column: Luna C18, 250 × 4.6, mobile phase: solution A – 10% formic acid, 83% water, 7% acetonitrile; solution B – 10% formic acid, 70% water, 20% acetonitrile; start – 100% solution A, 20 min – 81% solution A and 19% solution B; 40 min – 100% solution B); as described by Goiffon, Brun, and Bourrier (1991). UV detection at 526 nm and delphinidin as external standard were used. The retention times of individual anthocyanins are presented in Table 1.

2.3. Animals

Male Wistar rats, weighing 200–250 g, were conditioned in standard boxes for 15 days before the experiments. They were fed a standard laboratory diet, provided with water ad libitum and kept on a 12/12 h light-dark cycle.

2.4. Reagents

Tert-butyl hydroperoxide (*t*BH, 70% in water), 2-thio-barbituric acid (TBA, 98%), trypan blue, dimethyl sulfoxide (DMSO) for cell cultures, Williams' medium E, MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), bovine serum and additives were purchased from Sigma–Aldrich Ltd., Czech Republic. Collagenase was from Sevapharma, Czech Republic. Other chemicals and solvents were of analytical grade from Pliva-Lachema, Czech Republic.

2.5. Rat hepatocyte primary cultures

Rat hepatocytes were isolated by two-step collagenase perfusion of rat liver (Moldeus, Hogberg, & Orrenius, 1978). The cell viability was determined by measuring trypan blue exclusion. Yields of 2–4 × 10⁸ cells/liver, with a viability greater than 80%, were routinely obtained. The hepatocytes were then dispersed in sterile conditions in

Table 1
Anthocyanin contents in the bilberry extract^a

Name	RT ^b	% Area	% Amount (w/w)
Delphinidin 3-galactoside	6.9	11.6	2.89
Delphinidin 3-glucoside	8.3	13.3	3.33
Cyanidin 3-galactoside	10.6	19.0	4.74
Cyanidin 3-glucoside	13.2	10.0	2.50
Petunidin 3-galactoside	15.1	4.02	1.00
Cyanidin 3-arabinoside	16.3	7.06	1.77
Petunidin 3-glucoside	18.4	8.99	2.25
Delphinidin	19.8	1.39	0.347
Paeonidin 3-galactoside	21.3	0.93	0.233
Petunidin 3-arabinoside	21.8	2.68	0.669
Paeonidin 3-glucoside	24.9	4.12	1.03
Malvidin 3-galactoside	25.7	3.19	0.797
Paeonidin 3-arabinoside	27.3	0.52	0.130
Malvidin 3-glucoside	28.2	8.91	2.23
Cyanidin	29.2	1.22	0.305
Malvidin 3-arabinoside	30.6	1.92	0.479
Petunidin	32.9	0.52	0.129
Paeonidin	39.7	0.20	0.049
Malvidin	41.8	0.46	0.115
Total anthocyanins		100	25.0

^a The extract was analysed by RP-HPLC (column: Luna C18, 250 × 4.6, mobile phase: solution A – 10% formic acid, 83% water, 7% acetonitrile; solution B – 10% formic acid, 70% water, 20% acetonitrile; start – 100% solution A, 20 min – 81% solution A and 19% solution B; 40 min – 100% solution B). UV detection at 526 nm and delphinidin as external standard were used.

^b Identities of cyanidin, delphinidin, malvidin and paeonidin were confirmed by comparison with standards, other compounds were assigned according to relative retention times found in literature (Goiffon et al., 1991).

Williams' medium E supplemented by penicillin (10 IU/ml), streptomycin (0.1 mg/ml), dexamethasone (1 μmol/l), insulin (0.1 μmol/l), glutamine (2 mmol/l) and bovine serum (10%) and then cultivated in collagen-coated 12-well dishes in a humidified atmosphere of 5% CO₂ and 37 °C. After a 4 h culture stabilisation, the cultivation medium was replaced by a serum-free one and the tested sample (final concentrations 100 and 500 μg/ml) was added to the incubation medium in dimethyl sulfoxide (DMSO, final concentration 0.5%).

For cytotoxicity studies, the hepatocyte monolayers were incubated with the tested extract for 4, 24 and 48 h and the viability of the cells was assessed by the MTT test (Sieuwerts, Klijn, Peters, & Foekens, 1995).

To study cytoprotective effects against *tert*-butyl hydroperoxide (*t*BH) or allyl alcohol (AA)-induced damage, the primary cultures were intoxicated by *t*BH for 1.5 h (final concentration 0.5 mmol/l) or AA for 4 h (final concentration 0.2 mmol/l) after pre-incubation with the tested extract (0.5 h). The doses and incubation times for each toxin were chosen according to our previous optimization experiments (Dvořák et al., 2003; Kosina et al., 2002; Valentová, Cvak, Muck, Ulrichová, & Šimánek, 2003). The quality of the culture was controlled by the following parameters: cell viability (MTT test), activity of released lactate dehydrogenase LDH (Bergmeyer & Bernt, 1974) and level of lipoperoxidation products (thiobarbituric acid

reacting substances, TBARS) in the medium (Buege & Aust, 1978). As the bilberry extract has a strong absorbance in acidic conditions at 535 nm, Williams' medium E, with the relevant extract concentration, was used as a blank for determination of the TBARS. All the methods were adapted for measurement in microtitre plates.

2.6. DPPH-scavenging

Antiradical activity was evaluated spectrophotometrically as the ability of the tested substances to reduce 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as described previously (Deby & Magotteaux, 1970; Joyeux, Mortier, & Fleurentin, 1995) with minor modifications. Briefly, 90 µl of a solution of the tested extract or trolox (0–100 µg/ml) were mixed with 180 µl of a freshly prepared methanolic DPPH solution (20 mg/l) in a microtitre plate well. After 30 min, absorbance at 517 nm was measured and IC₅₀ values were obtained from the inhibition curves.

2.7. Superoxide-scavenging

The superoxide-scavenging activity was determined spectrophotometrically by monitoring the effect of the tested extract or trolox on the reduction of nitroblue tetrazolium chloride (2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-3,3'-[3, 3'-dimethoxy-4,4'-diphenylene]ditetrazolium chloride, NBT) to the blue chromogen formazan by O₂^{•-}. Superoxide radicals were generated by the xanthine/xanthine oxidase (XOD) system as described previously (Lu & Foo, 2001). Briefly, 0.1 ml of aqueous superoxide dismutase (SOD) standard solutions (5, 10, 25, 50, 100 U/ml) or BE or trolox solution (1 mg/ml) were separately added to a 1.0 ml mixture of xanthine (0.4 mmol/l) and NBT (0.24 mmol/l) in a phosphate buffer (0.1 mol/l, pH 7.8) containing EDTA (0.1 mmol/l). 1.0 ml of XOD (0.05 U/ml), dissolved in the same phosphate buffer, was added and the resulting mixture was incubated at 37 °C for 20 min. The reaction was terminated by adding 1.0 ml of a sodium dodecyl sulphate (SDS) solution (69 mmol/l) and the absorbance was measured at 560 nm (Fried & Fried, 1974). Superoxide-scavenging activity was calculated as SOD equivalents (U/mg) from a SOD standard curve.

2.8. Statistics

The data were analysed with a one-way ANOVA using the StatView Statistical Package. Differences were considered statistically significant when **P* was <0.01 compared with the control.

3. Results

3.1. Extract analysis

The bilberry extract used contained 25.0% of total anthocyanins (analysed by HPLC using delphinidin as

external standard). The content of individual components are presented in Table 1. Identities of cyanidin, delphinidin, malvidin and paeonidin were confirmed by comparison with standards; other compounds were assigned according to relative retention times found in the literature (Goiffon et al., 1991).

3.2. Cytotoxicity

The cytotoxicity of BE was determined in the rat hepatocyte primary culture model, by measuring the ability of the cells to reduce 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to violet-coloured formazan after 4, 24 and 48 h of incubation. In the concentrations tested (100 and 500 µg/ml), no significant toxicity was noted (data not shown).

3.3. Cytoprotectivity

In a typical non-treated cell culture, the MTT test gave $A_{540\text{ nm}} = 1.13 \pm 0.01$, the activity of LDH was 7.74 ± 0.25 µkat/l and TBARS level in culture medium was 1.29 ± 0.07 µmol/l. After intoxication with *t*BH, these values changed to 0.22 ± 0.01 (MTT), 29.8 ± 1.6 µkat/l (LDH) and 4.87 ± 0.15 µmol/l (TBARS). If the cell culture was preincubated with 500 µg/ml of the bilberry extract before *t*BH application, $A_{540\text{ nm}}$ was 0.71 ± 0.04 , LDH activity = 18.7 ± 1.8 µkat/l and the TBARS level was 2.94 ± 0.48 µmol/l. In the case of AA, the MTT test gave $A_{540\text{ nm}} = 0.234 \pm 0.047$, LDH activity = 12.0 ± 0.25 µkat/l and the TBARS level was 4.50 ± 0.15 µmol/l. The bilberry extract at 500 µg/ml restored the control values to 0.66 ± 0.02 (MTT), 18.1 ± 2.0 µkat/l (LDH) and 2.19 ± 0.48 µmol/l (TBARS), see Table 2.

For comparison of the activity of the extract at various concentrations in cell cultures from different animals, we expressed cytoprotective effects as % of non-intoxicated controls (Figs. 1 and 2). The extract showed significant dose-dependent protective activity against oxidative damage in rat hepatocytes primary cultures induced by *tert*-butyl hydroperoxide and allyl alcohol. Maximum protection (58.16%) was noted in AA-damaged culture pre-incubated with 500 µg/ml of the extract.

Table 2
Protective effect of the bilberry extract on *tert*-butyl hydroperoxide-induced damage of rat hepatocytes primary cultures

	Non-treated	<i>t</i> BH	<i>t</i> BH + BE 500 µg/ml
MTT ($A_{540\text{ nm}}$)	1.13 ± 0.01	0.22 ± 0.01^a	0.71 ± 0.04^b
LDH (µkat/l)	7.74 ± 0.25	29.8 ± 1.6^a	18.7 ± 1.8^b
TBARS (µmol/l)	1.29 ± 0.07	4.87 ± 0.15^a	2.94 ± 0.48^b

After 30 min of preincubation with the bilberry extract (BE), the cell monolayers were treated with *tert*-butyl hydroperoxide (*t*BH, 0.5 mmol/l) during 1.5 h. Results are expressed as mean ± SD, *n* = 9.

^a *P* < 0.01 vs. non-treated cells.

^b *P* < 0.01 vs. *t*BH-treated cells.

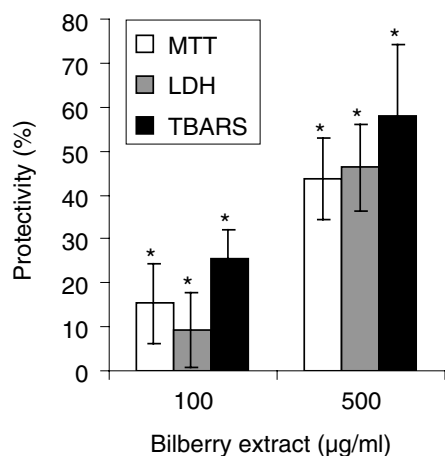


Fig. 1. Protective effect of the bilberry extract on *tert*-butyl hydroperoxide-induced damage of rat hepatocyte primary cultures. After 30 min of preincubation with tested extracts, the cell monolayers were treated with *tert*-butyl hydroperoxide (*t*BH, 0.5 mmol/l) during 1.5 h. Results are expressed as means \pm SD, $n = 9$; cytoprotective effects are expressed as % of non-intoxicated controls. Significant differences from *t*BH intoxicated controls ($*P < 0.01$) are indicated.

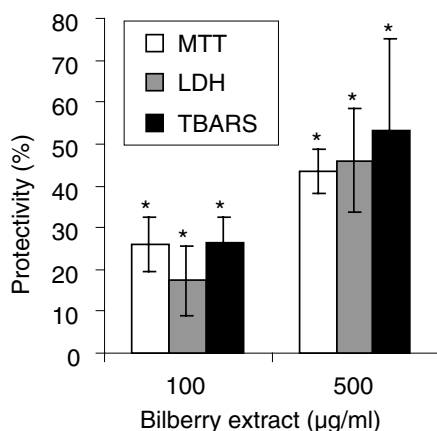


Fig. 2. Protective effect of the bilberry extract on allyl alcohol induced damage of rat hepatocyte primary cultures. After 30 min of preincubation with tested extracts, the cell monolayers were treated with allyl alcohol (AA, 0.2 mmol/l) during 4 h. Results are expressed as mean \pm SD, $n = 9$; cytoprotective effects are expressed as % of non-intoxicated controls. Significant differences from *t*-BH intoxicated controls ($*P < 0.01$) are indicated.

3.4. DPPH-scavenging

Antiradical activity of the BE was measured by the DPPH-scavenging test. The extract scavenged the DPPH radical; 50% inhibition was achieved at $3.99 \pm 0.14 \mu\text{g/ml}$ (IC_{50}). In the same system, IC_{50} of the synthetic analogue of vitamin E, trolox, was $2.15 \pm 0.06 \mu\text{g/ml}$ ($8.57 \pm 0.25 \mu\text{mol/l}$).

3.5. Superoxide-scavenging

Antioxidant activity of the BE was also assessed in the xanthine/XOD superoxide generating system. The extract

scavenged the superoxide radical and its activity was equivalent to 108 ± 7.2 units of SOD per mg of extract. In the same system, trolox had an activity equivalent to 16.4 ± 0.19 units of SOD/mg.

4. Discussion

The liver is the main organ responsible for metabolism of both endogenous and exogenous compounds and therefore it is also one of the first target organs for the toxic action of xenobiotics or their reactive metabolites. The hepatocytes represent about 65% of the liver parenchyma and they ensure almost all liver functions. They have a distinct Golgi apparatus and numerous mitochondria and are often subjected to oxidative stress. Several *in vitro* models of different degrees of complexity are used for metabolic and toxicity studies, e.g., isolated perfused liver, liver slices, liver cells – mainly hepatocytes, but also Kupfer cells and lipocytes, subcellular fractions of the cells – mitochondrial and microsomal. Hepatocytes are used as cell suspensions or primary cultures and these represent the most current and the most accepted model for biochemical, toxicological, pharmacological and molecular biological studies (Dvořák et al., 2003). After stabilisation, the hepatocytes in primary cultures regain their specific functions, metabolic processes, production of plasma proteins and biotransformation enzymes (Berry, Edwards, & Barritt, 1991).

Various experimental toxins, with different mechanisms of action, are used for the study of the hepatoprotective effects of various substances. *t*BH induces oxidative damage to the cells through hydroxyl (HO), hydrogen peroxy (HOO) or alkoxy (RO) radicals, which are formed during its spontaneous decomposition. *t*BH disrupts the cellular membranes, induces lipid peroxidation, leakage of enzymes into the extracellular space, glutathione (GSH) oxidation, and alkylation of cell macromolecules (Liu, Wang, Chu, Cheng, & Tseng, 2002; Valentao et al., 2004). Allyl alcohol (AA) is a toxin with acts indirectly through its metabolites. In the cell, AA is oxidised to acrolein by the action of cytosolic alcohol dehydrogenase. Acrolein, as a reactive aldehyde, is able to form an adduct with GSH and other biomolecules. It indirectly induces lipoperoxidation, catalysed by Fe(II) ions (Burcham & Fontaine, 2001).

In comparison with control (*t*BH or AA-intoxicated) cells, the cell cultures pre-treated with BE displayed larger amounts of formazan after the MTT viability test and lower levels of LDH and TBARS in the culture medium. Values corresponding to 58% of the non-intoxicated cell culture were noted at 500 $\mu\text{g/ml}$ of the BE. In rat hepatocyte primary cultures intoxicated by Fe(III) and Cu(II) ions, *Prunella vulgaris* organic fraction at 60 $\mu\text{g/ml}$ displayed almost 100% protective activity (Psotová et al., 2003).

The observed cytoprotective effect of the bilberry extract against oxidative damage of rat hepatocytes is probably due to the antioxidant properties of its constituents, mainly anthocyanins. The chemopreventive effect of grape seed pro-

anthocyanidin extract has been previously reported on Chang liver cells (Joshi, Kuszynski, Bagchi, & Bagchi, 2000). The antioxidant activities of various bilberry extracts and their anthocyanins have been previously found: An extract, characterised by 74.2 ± 4.9 mg of polyphenols/g, inhibited copper-induced oxidative modification of human LDL (Laplaud et al., 1997). A commercial extract scavenged superoxide anion and hydroxyl radicals, inhibited lipid peroxidation in rat liver microsomes and liver lipid peroxidation in vivo in mice (Martín-Aragon et al., 1998, Martín-Aragon, Basabe, Benedi, & Villar, 1999). Raspberry, bilberry, lingonberry and black currant extracts inhibited copper-induced protein and lipid oxidation in a lactalbumin-liposome oxidation system, bilberry extract being one of the most effective (Viljanen, Kylli, Kivikari, & Heinonen, 2004).

In our hands, BE scavenged DPPH and enzymatically generated superoxide anion radical with an efficiency comparable to trolox. The DPPH-scavenging activity of a bilberry extract (28.8% of anthocyanidins), with an IC_{50} approximately $40 \mu\text{g/ml}$, has been reported (Nakajima et al., 2004). The higher activity (IC_{50} $3.99 \pm 0.14 \mu\text{g/ml}$) in our experimental conditions was probably due to longer incubation of the reaction mixture (5 vs. 30 min). This reasoning is in accordance with the consistently lower (IC_{50} approx. $19 \mu\text{g/ml}$ vs $2.15 \pm 0.06 \mu\text{g/ml}$) trolox activity found by the above-mentioned authors (Nakajima et al., 2004). Concerning superoxide radical-scavenging, we have expressed BE activity by SOD equivalents per mg of extracts, whereas others (Martín-Aragon et al., 1998, 1999) calculated IC_{50} values and percentage of inhibition in 4 final extract concentrations. BE final concentration in our system was $47.6 \mu\text{g/ml}$ and it scavenged $25.7 \pm 0.84\%$ of superoxide radical formed. A comparison with $50 \mu\text{g/ml}$ of an anthocyanoside complex extract from *Vaccinium myrtillus* (AVM) in two different superoxide-generating systems is shown in Table 3. BE exhibited 2.5–3.5 times lower activity compared to previous findings with AVM (Martín-Aragon et al., 1998, 1999). This difference may be due, either to the higher content of active substances in AVM (only relative content of individual anthocyanosides is presented) or due to different experimental conditions (particularly 2 vs. 20 min of incubation).

Table 3
Superoxide radical-scavenging by bilberry extracts

Method	Final extract concentration ($\mu\text{g/ml}$)	Inhibition (%)	References
NADH/PMS ^a / NBT ^b	50	91.1 ± 1.60	Martín-Aragon et al. (1998)
Hypoxanthine/ XOD ^c /cyt <i>c</i> ^d	50	63.0 ± 2.11	Martín-Aragon et al. (1999)
Xanthine/XOD ^c / NBT ^b	47.6	25.7 ± 0.84	

^a Phenazine methosulfate.

^b Nitroblue tetrazolium chloride.

^c Xanthine oxidase.

^d Cytochrome *c*.

Our results support the use of bilberry and bilberry extracts in functional foods and food supplements designed for the prevention of chronic diseases associated with oxidative stress.

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