

Protective Effect of Bilberry (*Vaccinium myrtillus* L.) Extracts on Cultured Human Corneal Limbal Epithelial Cells (HCLEC)

Juxian Song,^{1†} Yiqing Li,^{2†} Jian Ge,² Yongheng Duan,² Stephen Cho-Wing Sze,¹ Yao Tong,¹ Pang-Chui Shaw,³ Tzi-Bun Ng,³ Kam Chuen Tsui,⁴ Yehong Zhuo² and Kalin Yanbo Zhang^{1*}

¹The School of Chinese Medicine, LKS Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong SAR, China

²State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, 54 South Xianlie Road, Guangzhou 510060, China

³Department of Biochemistry, The Chinese University of Hong Kong, Shatin, Hong Kong

⁴Oriental International Health Products Co., Ltd, Yip Shing Street, Kwai Chung, Hong Kong

The use of bilberry (*Vaccinium myrtillus* L.) as a food and medicine for improving human vision has a long history all over the world. However, there is lack of convincing evidence from rigorous clinical trials or scientific research. This study investigated the effects of different concentrations of bilberry extracts on the cell viability, cell cycle and the expression of hyaluronic acid and glycosaminoglycans of cultured human corneal limbal epithelial cells. The data showed that bilberry extracts had no cytotoxicity to the corneal limbal epithelial cells at a wide range of concentrations (10^{-9} – 10^{-4} M, equalized to the content of cyanidin-3-O-glucoside). Bilberry extract (10^{-6} , 10^{-5} and 10^{-4} M) increased cell viability after 48 h incubation. The number of cells decreased in G₀/G₁ phase and increased prominently in S and G₂/M phases after treatment with bilberry extracts at a high concentration (10^{-4} M). The expression of glycosaminoglycans increased prominently after incubation with bilberry extracts (10^{-7} and 10^{-4} M) for 48 h while no significant changes were observed for the expression of hyaluronic acid. The results indicated that bilberry extract may be beneficial for the physiological renewal and homeostasis of corneal epithelial cells. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: *Vaccinium myrtillus* L.; cell viability; cell cycle; hyaluronic acid; glycosaminoglycans.

INTRODUCTION

Bilberry (*Vaccinium myrtillus* L.) is a low-growing shrub of the Ericaceae family native to Europe and North America and has a long history of use in folk medicine. At present, bilberry is used in a variety of pharmaceutical and formulated food products (such as OptiBerry®) that are recommended for the treatment of vision and vascular disorders (Bagchi *et al.*, 2004; Roy *et al.*, 2002). Polyphenols, including flavonoids, and especially anthocyanins, are the main secondary components in bilberry (Faria *et al.*, 2005). Many of the health-promoting properties of bilberry are thought to be attributable to anthocyanins (Kowalczyk *et al.*, 2003), although other related flavonoids are biomedically useful (Cazarolli *et al.*, 2008). Both *in vitro* and *in vivo* studies have demonstrated the potent antioxidant capacity of bilberry extract (BE) and its anthocyanins (Faria *et al.*, 2005; Jang *et al.*, 2005; Laplaud *et al.*, 1997; Milbury *et al.*, 2007; Rahman *et al.*, 2006; Sparrow *et al.*, 2003; Wu *et al.*, 2006; Yao and Vieira, 2007; Youdim *et al.*, 2000).

Although bilberry's beneficial effects on the eye were well documented in folk medicine, contradictory clinical

results were obtained from different research groups (review in Canter and Ernst, 2004). However, experimental studies have shown some positive results. One animal study showed that long-term supplementation with BE is effective in the prevention of macular degeneration and cataract in senescence-accelerated OXYS rats (Fursova *et al.*, 2005). Anthocyanins could stimulate the regeneration of rhodopsin in frog rod outer segment membranes *in vitro* (Matsumoto *et al.*, 2003). Cell-based studies showed that anthocyanins in BE suppressed the photooxidation of A2E, a pigment that accumulates in retinal pigment epithelial (RPE) cells with age and in some retinal disorders (Jang *et al.*, 2005; Sparrow *et al.*, 2003). Anthocyanins and other flavonoids upregulated the oxidative stress defense enzymes such as HO-1 and GST-pi in RPE and protected oxidative stress-induced cell death of retinal ganglion cells (Maher and Hanneken, 2005; Milbury *et al.*, 2007).

The epithelium of the ocular surface functions as a barrier to the penetration of noxious substances into the cornea and maintains ocular surface integrity. Proliferation of corneal epithelial cells provides cells to rebuild the tissue, which maintains the protective effects of the cornea and acts to promote corneal wound healing (Capo-Aponte *et al.*, 2007; Lyu and Joo, 2005; Nagai *et al.*, 2009). Cell cycle dynamics in the corneal epithelium could reflect DNA synthesis and mitosis activity during the generation and the loss of cells. The cells stop dividing and prepare for DNA synthesis in G₀ and G₁ phases. During the S (synthetic) phase, the amount of

* Correspondence to: Dr Kalin Yanbo Zhang, The School of Chinese Medicine, LKS Faculty of Medicine, The University of Hong Kong, 10 Sassoon Road, Pokfulam, Hong Kong SAR, China.
E-mail: ybzhang@hku.hk

†The first two authors contributed equally to this work.

DNA increases and is doubled in the G₂ (tetraploid) and M (mitotic) phases of the cell cycle. Cell cycle analysis has been employed to study the corneal wound healing and re-epithelialization (Thompson *et al.*, 1991; Zagon *et al.*, 2000). Glycosaminoglycans (GAGs) are linear polymers of amino sugar uronic acid disaccharides. Sulfated GAGs were synthesized by all three cell types of the cornea, the fibroblasts, the epithelial as well as the endothelial cells (Schwager-Hubner and Gnadinger, 1976). Epithelial GAGs may modulate cell behavior such as adhesion or migration, thus contributing to corneal epithelial wound healing (Oya *et al.*, 1994; Saika *et al.*, 2000). Hyaluronic acid (HA), a GAGs, has several physicochemical and biologic functions such as space filling, lubrication and providing a hydrated matrix through which cells can migrate (Knudson and Knudson, 1993). HA is expressed in the ocular tissue as an extracellular matrix (ECM) and plays many roles in its homeostasis and pathophysiology (Fitzsimmons *et al.*, 1994; Harfstrand *et al.*, 1992; Nakamura *et al.*, 1997).

In view of limited research and contradictory results on the vision improvement effect of bilberry, further studies *in vivo* or *in vitro* are needed. Corneal epithelial cells are used widely as an *in vitro* model to study the protective effects of drugs on the eyes (Cai *et al.*, 2008; Pauloin *et al.*, 2009; Paulsen *et al.*, 2008). Although other research groups have reported the protective effects of some phenolic acids on oxidation damage and inflammation caused by ultraviolet light in corneal and conjunctival cells (Larrosa *et al.*, 2008; Lodovici *et al.*, 2003), the effects of bilberry and its anthocyanin ingredients on corneal epithelial cells, to our knowledge, have not been investigated so far. In the present study, a newly established human corneal limbal epithelial cell line (HCLEC) (Liu *et al.*, 2007) was used to study the effects of BE on the cell growth, cell cycle and the expression of HA and GAGs in corneal epithelial cells.

MATERIALS AND METHODS

Preparation of BE. Commercially available BE (bilberry extract containing 25% total anthocyanins) were supplied by Dr. Health Ltd, Hong Kong. The content of anthocyanins present in BE was quantified by HPLC using cyanidin-3-O-glucoside (Cyd-3-glu, M.W. = 449.2) as external standard (Baj *et al.*, 1983). The extract was dissolved in 0.01 M phosphate-buffered saline (PBS, pH 7.2) at 10 mg/mL and sterilized by membrane filtration through a 0.22 µm filter (Millex, Millipore) and stored at -20 °C. It was diluted with DMEM (Dulbecco's minimum essential medium) and standardized to the concentration of Cyd-3-glu (10⁻⁹-10⁻⁴ M) before the experiment.

Culture of HCLEC. HCLEC cell line (P15) used in this study was a kind gift from Professor Wang Zhichong (Zhongshan Ophthalmic Center, Sun Yat-sen University) and cultured according to the published protocols (Liu *et al.*, 2007).

Cell treatment and viability assays. HCLEC cells were seeded in 96-well culture plates at a concentration of 8 × 10³ cells/well. After 24 h, the medium was removed and the cells were incubated with fresh DMEM or BE

at various concentrations (standardized to 10⁻⁸-10⁻⁴ M Cyd-3-glu) for another 24 h or 48 h. Cell viability was then estimated via an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, which is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mosmann, 1983). After incubation, the cells were treated with MTT solution (final concentration 0.5 mg/mL) for 4 h at 37 °C. After this, the medium was removed and the dark blue formazan crystals formed in intact cells were solubilized with DMSO and the absorbance at 492 nm was measured with a microplate reader (Model 680, Bio-Rad Laboratories, UK). The optical density of the formazan generated in the control cells (no BE added) was considered to represent 100% viability. The data are expressed as the mean percentage of viable cells relative to the control.

Flow cytometry analysis of cell cycle. HCLEC cells treated with BE (10⁻⁸-10⁻⁴ M Cyd-3-glu) for 48 h were harvested, washed with 0.01 M PBS (pH 7.2) and fixed with ice-cold absolute ethanol (Sigma) for 24 h. After further being washed twice with 0.01 M PBS (pH 7.2), the cells were treated with RNase (Sigma, 0.2 mg/mL) for 30 min at 37 °C and then stained with propidium iodide (PI, 0.1 µg/mL) (Sigma) for 30 min at 4 °C. The DNA content was measured using a FACSC Aria flow cytometer equipped with Cell Quest software (Becton Dickinson, NJ, USA). A minimum of 5000 cells was counted in three independent experiments. The cell cycle phase was then grouped into G₀/G₁, S and G₂/M phases.

HA assay. HCLEC cells were seeded in 6-well culture plates at a concentration of 5 × 10⁵ cells/well. After reaching 80% confluence, the cells were incubated with/without various concentrations of BE (standardized to 10⁻⁹-10⁻⁴ M Cyd-3-glu) for 48 h. The supernatants were collected and the HA content was measured by an ELISA kit (Hyaluronan Hyaluronic Acid Assay Kit from R&D Systems Europe, Ltd) according to the protocol provided by the manufacturer (<http://www.rndsystems.com/pdf/DY3614.pdf>).

GAGs assay. HCLEC cells were seeded in 6-well culture plates at a concentration of 5 × 10⁵ cells/well. After reaching 80% confluence, the cells were incubated with/without various concentrations of BE (standardized to 10⁻⁹-10⁻⁴ M Cyd-3-glu) for 48 h. The supernatants were collected and the GAGs content was measured using Blyscan™ Assay (Biocolor Ltd, UK) in accordance with the manufacturer's protocol. (<http://www.biocolor.co.uk/manuals/blyscan.pdf>)

Data analysis and statistics. All data were expressed as mean ± standard deviation (SD) (*n* = 3-8) of three independent experiments and analysed by one-way ANOVA with SPSS15.0 (SPSS, Inc., Chicago, IL). Values of *p* < 0.05 were considered statistically significant.

RESULTS

The HCLEC cells were successfully cultured with normal morphology showing a cobblestone appearance

(Fig.1) similar to that published (Liu *et al.*, 2007). The cells were incubated with different concentrations of BE for 24 h and 48 h. The viability of the cells was measured by MTT. The cells incubated with/without different concentrations of BE were compared (Fig.2). The results showed cell proliferation rather than cytotoxicity after BE treatment. Incubation duration is a factor determining the effect of BE on cell viability. BE (10^{-5} M Cyd-3-glu) promoted cell growth to about 120% compared with the control group ($p < 0.05$) after 24 h incubation while three concentrations (10^{-6} , 10^{-5} and 10^{-4} M Cyd-3-glu) were effective in increasing cell viability to 112.9%, 130.1% and 113.8%, respectively, ($p < 0.05$) after 48 h incubation (Fig.2).

For the cell cycle assay, the proportion of cells at different stages (G_0/G_1 , S and G_2/M) was analysed by flow cytometry after incubation with BE for 48 h. As shown in Fig.3, BE at lower concentrations had no obvious effect on the ratio of different cell phases. However, when a higher concentration (10^{-4} M Cyd-3-glu) was used, the distribution of cells in G_0/G_1 phase decreased

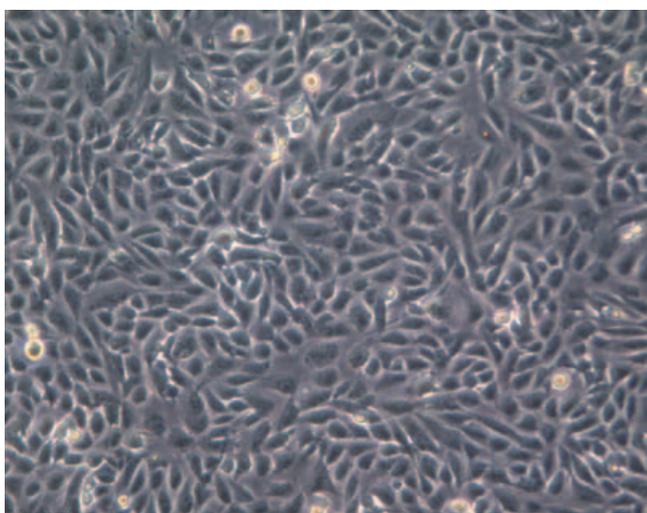


Figure 1. Representative phase-contrast micrograph of cultured human corneal limbal epithelial cells with normal morphology showing cells with cobblestone appearance (magnification $\times 100$).

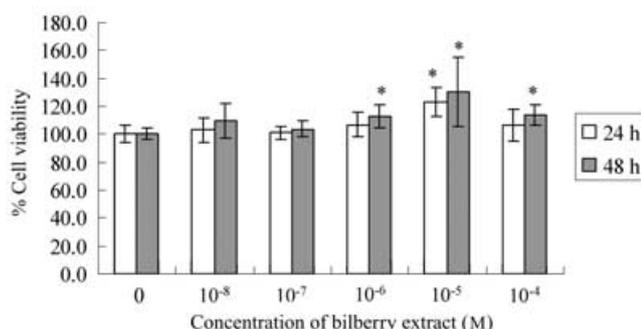


Figure 2. Effect of bilberry extract on the proliferation of cultured human corneal limbal epithelial cells. After incubation with different concentrations of bilberry extract (equalized to 10^{-8} – 10^{-4} M cyanidin-3-O-glucoside) at 37 °C for 24 and 48 h, cell viability was examined by MTT assay. The 100% value was obtained from the control (no bilberry extract added). Each value represents the mean \pm SD of eight measurements in three independent experiments. *Significant vs control (no bilberry extract added) ($p < 0.05$).

significantly and increased in S and G_2/M phases (Fig.3). Compared with the control, the amount of cells in G_0/G_1 phase decreased from 84.88% to 60.15%, while the quantity of cells in S and G_2/M phases increased from 6.18% to 18.65% and from 8.95% to 21.20%, respectively, ($p < 0.05$) after being treated with BE (10^{-4} M Cyd-3-glu) for 48 h (Fig.3).

For the HA and GAGs assay, HCLEC cells were incubated with different concentrations of BE. After 48 h incubation, the HA and GAGs contents in the supernatant were determined by ELISA kits. No significant difference was observed between cells treated with BE and the controls (Fig.4). However, the GAGs content in the supernatant of the cells increased significantly after incubation with BE for 48 h. The increase in GAGs was not dose-dependent (Fig.5). Two separate concentrations (10^{-7} and 10^{-4} M Cyd-3-glu) significantly induced the secretion of GAGs ($p < 0.05$).

DISCUSSION

The effects of BE on cytotoxicity and cytoprotectivity have been studied using different cell types (Jang *et al.*, 2005; Valentov *et al.*, 2007). Anthocyanins in BE could be incorporated into ARPE-19 cells. When incubated with anthocyanins (10^{-4} M) for up to 5 days, the ARPE-19

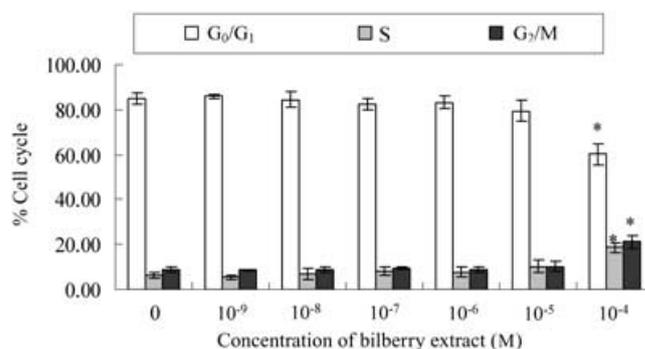


Figure 3. Flow cytometric analysis of cell cycle distribution of cells following treatment with different concentrations of bilberry extract (equalized to 10^{-9} – 10^{-4} M cyanidin-3-O-glucoside) for 48 h. Percentages of G_0/G_1 , S and G_2/M populations were measured. Each value represents the mean \pm SD of four independent experiments. *Significant vs control (no bilberry extract added) ($p < 0.05$).

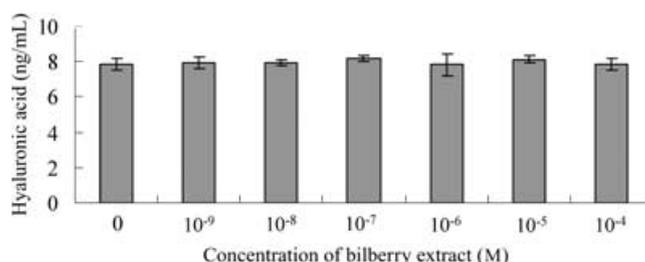


Figure 4. Hyaluronic acid assay of human corneal limbal epithelial cells treated with different concentrations of bilberry extract (equalized to 10^{-9} – 10^{-4} M cyanidin-3-O-glucoside) for 48 h. Each value represents the mean \pm SD of three independent experiments performed in triplicate.

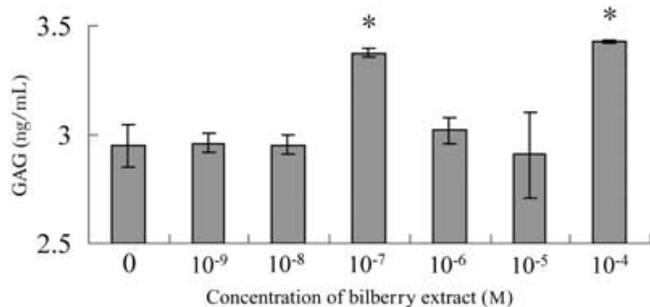


Figure 5. Glycoaminoglycans assay of human corneal limbal epithelial cells treated with different concentrations of bilberry extract (equalized to 10^{-9} – 10^{-4} M cyanidin-3-O-glucoside) for 48 h. Each value represents the mean \pm SD of three independent experiments performed in triplicate. *Significant vs control (no bilberry extract added) ($p < 0.05$).

cells showed no evidence of cell toxicity (Jang *et al.*, 2005). Also, the cytotoxicity of BE (containing 25% anthocyanins) was determined in primary cultured rat hepatocytes. In the concentrations tested (100 and 500 $\mu\text{g/ml}$), no significant toxicity was noted (Valentov *et al.*, 2007). The results from the MTT assay also verified that BE has no clear cytotoxicity at a wide range of concentrations (10^{-8} – 10^{-4} M Cvd-3-glu) and promoted cell growth at higher concentrations (Fig. 2).

Flow cytometry measures the DNA content of individual cells, which provides an accurate indication of cell cycle stage. Cell cycle dynamics have been shown to be important in the process of corneal epithelium healing (Thompson *et al.*, 1991; Zagon *et al.*, 2000). The percentage of nuclei in the G_2/M phase increased in the corneal epithelium at 36 h after experimental wounding compared with cell populations in samples from the unwounded control, which is an indication of a burst of mitotic activity (Thompson *et al.*, 1991). Mitosis is accelerated following denuding of the corneal epithelium and the distribution of mitotic cells was correlated with wounding (Zagon *et al.*, 2000). In this study, it was found that BE at a high concentration (10^{-4} M Cvd-3-glu) increased the proportion of cells in the S and G_2/M phases of the cell cycle. This result indicated that BE could promote mitotic activity and cell

division, which are important for the wound healing and re-epithelialization of the corneal epithelium. Also, it is worthwhile to note that the concentration effective on cell proliferation (i.e. 10^{-4} M) was much greater than that found *in vivo* (the maximum plasma concentration of total anthocyanins in bilberry was about 10^{-7} M after oral administration in humans (Cao *et al.*, 2001)). It is possible that anthocyanin extracts could be used topically to promote healing after corneal abrasion.

Hyaluronic acid, a naturally occurring biopolymer, is an important component of both the vitreous and aqueous humour of the eye (Durchschlag *et al.*, 1999; Swann and Constable, 1972). It could improve ocular surface health, reduce intraocular pressure during implant surgery for raised intraocular pressure and prevent desiccation of the cornea during a number of ophthalmic procedures (Price *et al.*, 2007). HA has also been used topically for the treatment of dry eye syndrome and Sjogren's syndrome (Price *et al.*, 2007). It is our hypothesis that BE may increase the expression of HA in corneal epithelial cells. However, the results showed no significant changes. It has been reported that the content of total GAGs in the aortas of rats increased by about 13% after administration of blueberries, a rich source of antioxidant compounds (Kalea *et al.*, 2006). In our cell-based study, the data showed that BE treatment also increased the total GAGs expression in epithelial cells, which may be important in corneal epithelial wound healing. However, the increase of GAGs was not dose-dependent, which may suggest that a high concentration of BE functions on GAGs expression via a different pathway from a low concentration.

Taken together, the present study demonstrated that BE and its anthocyanins could promote cell division, proliferation and increase the expression of total GAGs in corneal epithelial cells, which are important factors for their physiological renewal and homeostasis.

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