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FOOD & FUNCTION

Bilberries and their anthocyanins ameliorate experimental colitis

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Bilberries have positive effects in acute and chronic diarrhea. Patients with inflammatory bowel disease (IBD) report on improved symptoms upon ingestion. Bilberries contain approximately 10% of anthocyanins (ACs), which have anti-oxidative, anti-carcinogenic, and anti-inflammatory properties. We investigated whether experimental colitis can be ameliorated by dried bilberries or ACs. Acute and chronic dextrane sodium sulphate (DSS) colitis were induced in Balb/c mice by 2.5% DSS in the drinking water. Mice were fed with dried bilberries or ACs, respectively. Cytokines were determined in supernatants from mesenteric lymph nodes (MLNs) by ELISA and apoptosis was investigated by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling assays. Oral administration of bilberries during acute DSS-induced colitis ameliorated disease severity and reduced secretion of IFN-γ and tumor necrosis factor from mesenteric lymph node cells. Dried bilberries also improved chronic DSS-colitis. Ingestion of ACs reduced intestinal inflammation in acute and chronic DSS-colitis with decreased histological scores and cytokine secretion. Both bilberries and ACs prevented inflammation-induced apoptosis in colonic epithelial cells. Taken together, ingestion of dried bilberries had positive effects on various parameters especially in acute DSS-colitis. Oral administration of ACs resulted in an amelioration of acute colitis as well as chronic colitis. These promising results justify a clinical study on their therapeutic effect in inflammatory bowel disease patients.

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Bilberries (*Vaccinium myrtillus*) have been used to treat diarrhea in traditional medicine for hundreds of years [1]. In recent years, evidence for several protective properties of *Vaccinium* fruits was obtained. *Vaccinium* extracts blocked cellular pathways in cancer cell lines, having a growth inhibitory effect [2]. Cranberries delayed growth and inhibited metastases of human breast cancer lines grown in immunodeficient mice [3]. Furthermore, berry extracts showed a high capacity to absorb reactive oxygen species (ROS) [4].

Anthocyanins (greek: anthos-flower; kyanos-blue) are the largest group of water-soluble pigments in plants, responsible for the red, violet, blue, and purple color of fruits and

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Abbreviations: DSS, dextrane sodium sulphate; **IBD,** inflammatory bowel disease; **TNF,** tumor necrosis factor; **TUNEL,** terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling

vegetables. ACs belong to the class of flavonoids. The most common ACs are glycosides of cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin [5]. For ACs, a wide range of protective biological effects have been described, such as anti-oxidative, anti-carcinogenic, anti-microbial, and anti-inflammatory activities. Due to their phenolic structure, ACs show anti-oxidative capacity in vitro and in vivo, scavenging reactive oxygen species [6]. ACs may reduce the risk of coronary heart disease by inhibition of the oxidation of low-density lipoprotein (LDL) [7]. Delphinidin 3-sambubioside induces apoptosis in human leukemia cells (HL-60) dose dependently [8]. Furthermore, ACs showed antimicrobial activity against *Helicobacter pylori* and *Bacillus cereus* [9]. ACs reduce secretion of tumor necrosis factor (TNF) and IL-8 [10], inhibit production of cyclooxygenase 2 [11] and activation of nuclear factor κB [12]. Nuclear factor κB as well as TNF are key mediators of inflammation in inflammatory bowel disease (IBD).

Patients with IBD report on improved symptoms upon the ingestion of blueberries. Subsequently, the aim of this study was to investigate whether experimental colitis can be ameliorated by administration of bilberries or ACs.

As an animal model, we used female Balb/c mice weighing 20–22 g (Janvier, Le Genest St. Isle, France), which were housed under conventional animal facility conditions. The animal studies were approved by the Institutional Review Board.

Standard mouse feed (Ssniff NM, Ssniff GmbH, Soest, Germany) was supplemented with either 20% dried bilberries (A. Galke GmbH, Gittelde, Germany, containing 11.2% ACs) or 1 and 10% AC extract, respectively. The extract consisted of 60–70% ACs, 20% dietary fibers, 5% sugar, and 5% procyanidins extracted from bilberries (Kaden Biochemicals, Hamburg, Germany). Substances were mixed with mouse feed and pressed to pellets. Control groups received standard feed. Two weeks before induction of colitis, diets were started and continued for the duration of the experiments. Acute colitis was induced by 2.5% dextrane sodium sulphate (DSS) (MP Biomedicals, Illkirch, France) in the drinking water for 7 days. Mice were sacrificed at day 8 [13]. For chronic colitis, mice received four cycles of 2.5% DSS for 7 days interrupted by 7 days with drinking water. Four weeks later, the last cycle experiments were terminated.

For cytokine ELISA, mesenteric lymph nodes were collected in RPMI1640 (Sigma-Aldrich, Taufkirchen, Germany), with 10% fetal calf serum, 100 U/mL penicillin, and 100 μL/mL streptomycin (PAA, Cölbe, Germany) and disrupted mechanically. Tissue culture plates were coated with 2.5 μg/well monoclonal anti-CD3 antibody (clone 145.2c11, provided by W. Falk, Regensburg). A total of 2×10^5 cells/well were seeded in 200 μL culture medium. After 24 h, supernatants were collected. Secretion of IL-6 (IL-6 ELISA, R&D, Minneapolis, USA), TNF, and IFN-γ (Luminex mouse MilliplexTM Map Kit, Millipore, Billerica, MA, USA) was quantified according to the respective instructions.

For the histological analysis 1 cm of the distal colon was removed. Paraffin-embedded sections were stained with hematoxylin-eosin (Sigma-Aldrich) and scored by two blinded investigators [13].

Formalin-fixed sections were deparaffinized, rehydrated, and subjected to terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining (Calbiochem, Darmstadt, Germany). Specimens were permeabilized with proteinase K. Positive control was prepared with DNase I (Qiagen, Hilden, Germany). Slides were treated with terminal deoxynucleotidyl transferase (TdT) labeling reaction mixture and terminal deoxynucleotidyl transferase enzyme. Cell nuclei were visualized by using a filter for 4′, 6-diamidino-2-phenylindole, apoptotic cells by a standard fluorescein filter. Semi-quantitative evaluation of apoptosis was performed in a blinded fashion, ranging from 0 to 3.

Scores, colon lengths, and cytokine levels are expressed as median ± 5th/95th percentile. Statistical analyses were performed using the Student's *t*-test and Mann–Whitney rank sum test. Statistically significant differences were accepted when $p < 0.05$.

An acute DSS colitis diet with 1 or 10% ACs, respectively, resulted in a highly significant reduction of the histological score as compared with mice receiving standard feed (56 and 43% reduction, respectively, $p < 0.005$) (Fig. 1A). Bilberries also showed a moderate reduction of the histological score (35%, n.s.). Cross-sections of distal colons of mice receiving ACs displayed almost completely maintained epithelial structure with normal amounts of goblet cells and only moderate infiltration of leukocytes (Fig. 1Ba and 1Bb). In contrast, the epithelium of control animals was characterized by massive loss of crypts and severe leukocyte infiltrates (Fig. 1Bd). In total, 0.1% ACs did not ameliorate colitis (data not shown).

Diets with bilberries and 1 or 10% ACs, respectively, preserved almost normal colon length, whereas colons from DSS-control mice were significantly shortened ($p < 0.001$ for all treatment groups, Fig. 1C). Again, 0.1% ACs showed no effect (data not shown). Both bilberry- and 10% ACs-supplemented diets caused a significant reduction of IFN-γ secretion as compared with the control group (bilberries: 80% decrease, AC: 50% decrease; $p < 0.001$ for both; Fig. 1D).

Ingestion of bilberries also resulted in a significant reduced TNF secretion (64% lower compared with controls, $p < 0.001$, Fig. 1E). The effect of 10% ACs did not reach a level of significance (Fig. 1E), similar to what was seen for 1 or 0.1% (data not shown). In summary, bilberries and ACs ameliorate acute DSS-induced colitis.

The chronic DSS model reflects more the conditions of the colitis found in IBD patients. Administration of ACs during chronic DSS colitis significantly reduced histological scores as compared with controls (1% ACs: 29% reduction; 10% ACs: 41% reduction, both $p < 0.001$; Fig. 2A). In all, 0.1% AC had no influence (data not shown). In contrast to the acute DSS colitis, bilberries less ameliorated the histological score (Fig. 2A). In sections from control mice, an

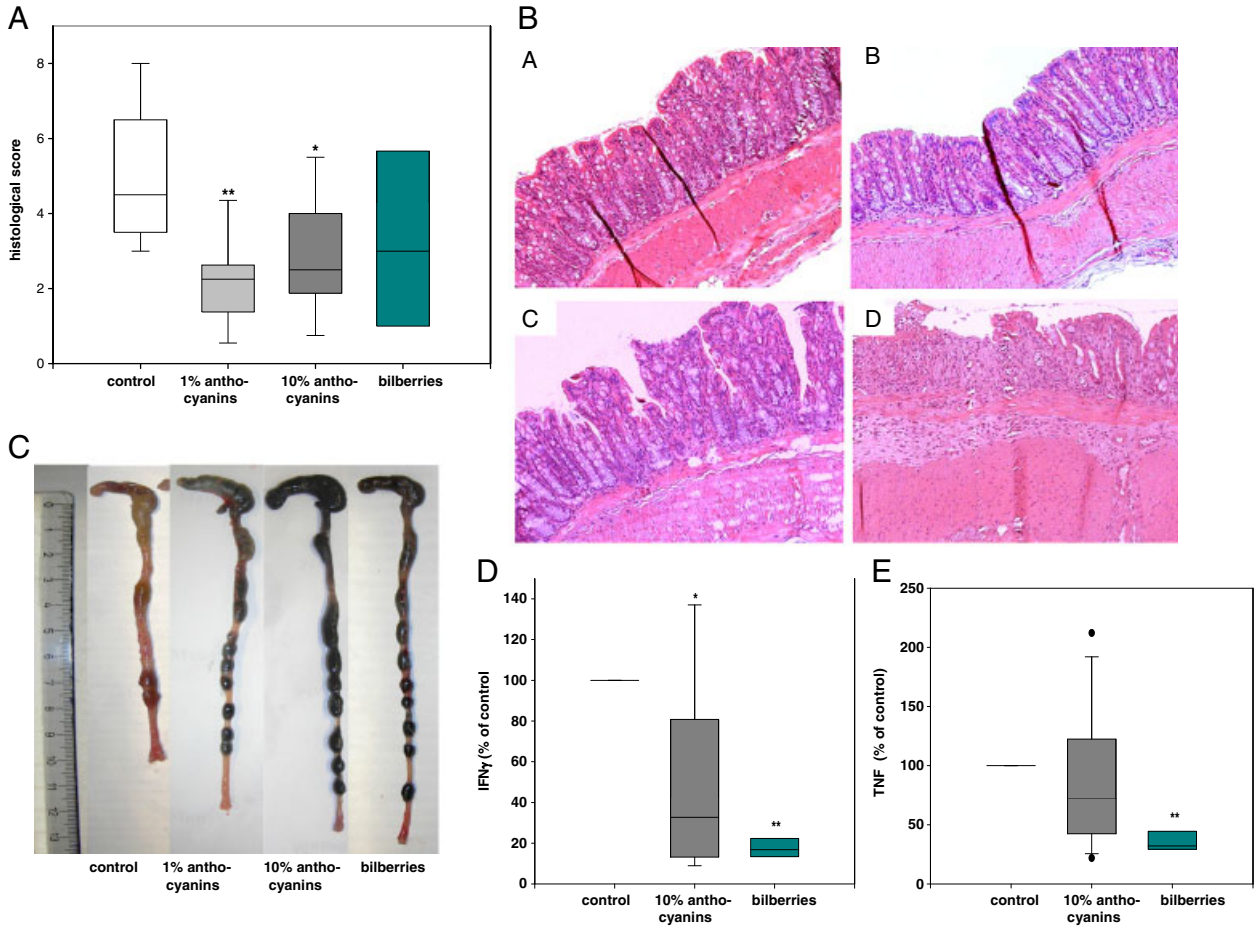

Figure 1. Bilberries and ACs ameliorate acute DSS. (A) Histological scores. (control $n = 15$; 1% ACs $n = 10$; 10% ACs $n = 14$; bilberries $n = 5$. p-Value analyzed by Student's t-test. $^{*}p = 0.001$; $^{**}p < 0.001$). (B) Representative H&E stained colon sections. (a) 1% ACs, (b) 10% ACs, (c) bilberries, and (d) control. Magnification, 100 ×. (C) Representative colons of the four groups. (D) IFN-γ secretion (mean of control: ~ 3000 pg/mL). (E) TNF secretion (mean of control: ~ 60 pg/mL). p-Value compared control analyzed by Mann–Whitney rank sum test ($^{*}p = 0.001$; $^{**}p < 0.001$; control $n = 14$; 1% ACs $n = 10$; 10% ACs $n = 13$; bilberries $n = 5$).

almost complete loss of the intestinal epithelium and a severe leukocyte infiltration was observed (Fig. 2BD). In contrast, sections from animals receiving 1 or 10% ACs showed an intact epithelial structure and low levels of leukocyte infiltration (Fig. 2BA and BB). Colons from mice fed with bilberries, 1 or 10% ACs, respectively, were significantly longer than those from control animals (Fig. 2C; $p < 0.001$ for all). A total of 0.1% ACs again had no effect on colon length.

In total, 1 and 10% ACs significantly decreased the secretion of INF-γ and IL-6, whereas bilberries had no influence (Fig. 2D and E). Briefly, 10% ACs reduced IFN-γ production by 50% ($p = 0.01$) and IL-6 secretion by 30% ($p < 0.001$). In all, 1% ACs were even more effective, reducing IFN-γ production by 70% ($p < 0.001$) and IL-6 secretion by 50% ($p < 0.001$). Bilberries had no significant effect in this setting.

Apoptosis of epithelial cells has been suggested to play an important role in the pathogenesis of intestinal inflammation in both DSS-induced colitis and human IBD. We therefore analyzed apoptotic epithelial cells by TUNEL staining of paraffin-embedded colon tissues (Fig. 3). Ingestion of bilberries caused a reduction of apoptotic epithelial cells by 35% as compared with controls group ($p < 0.05$). ACs were even more effective: 1% ACs reduced the rate of apoptotic cells by 44% ($p = 0.005$) and 10% ACs by 68% ($p < 0.001$), respectively (Fig. 3A). In specimens from bilberry-fed mice, sparse apoptotic cells in samples of mice fed with ACs hardly any apoptotic cells were detectable (Fig. 3B).

In conclusion, we demonstrate an amelioration of experimental colitis by bilberries and ACs. To investigate the influence of these substances, mice were fed with 20% bilberries or 1 and 10% ACs, respectively. With dried bilberries we observed a considerably attenuated reduction of colon length and a strongly decreased secretion of IFN-γ and TNF in acute DSS colitis; In chronic colitis, only an inhibition of the colon shortening, but no effect to

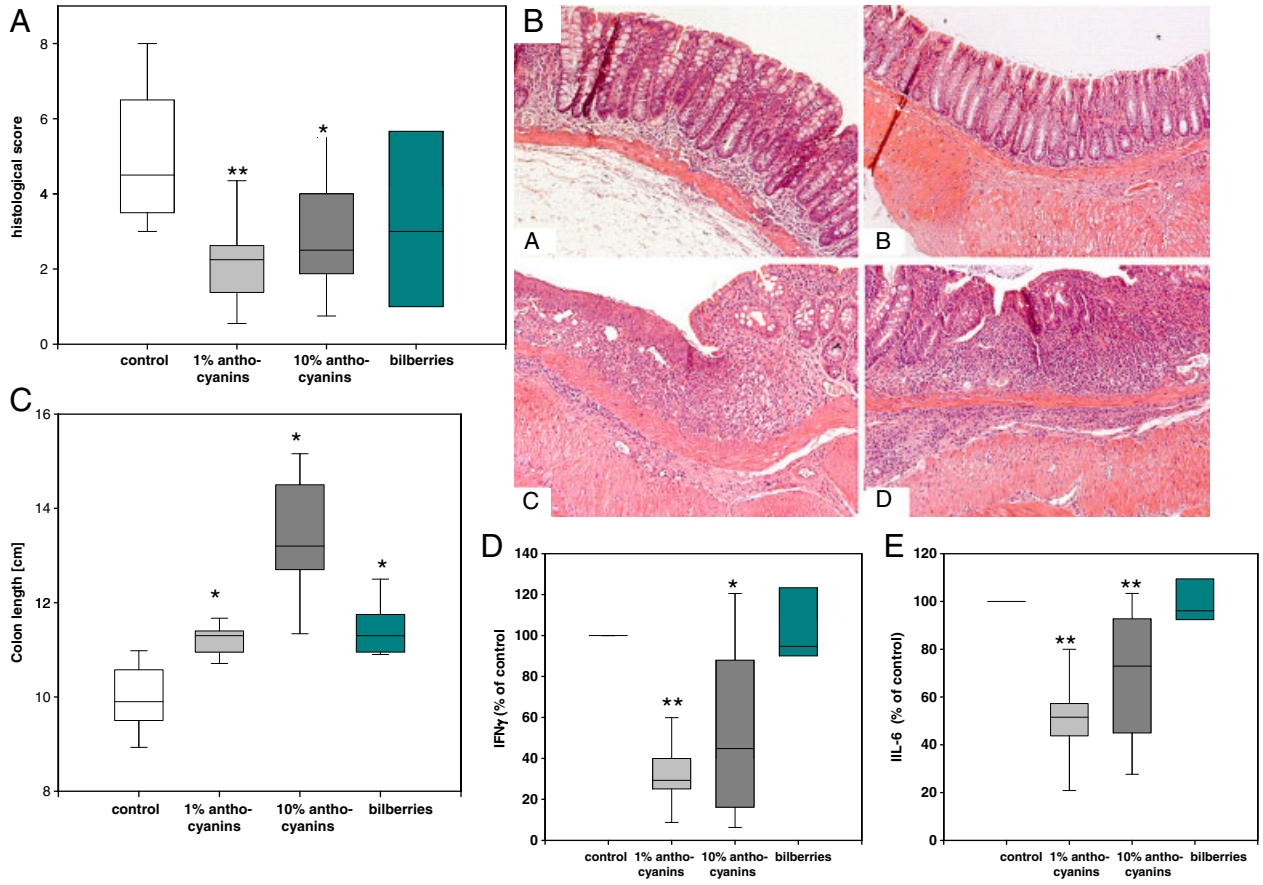

Figure 2. Bilberries and ACs ameliorate chronic DSS. (A) Histological scores. *p*-value analyzed by Mann–Whitney rank sum test ($^{*}p = 0.001$; $^{**}p < 0.001$; control $n = 19$; 1% ACs $n = 10$; 10% ACs $n = 9$; bilberries $n = 10$). (B) Representative colon sections stained with H&E (a) 1% ACs, (b) 10% ACs, (c) bilberries, and (d) control. Magnification, 100 ×. (C) Evaluation of colon length. *p*-Value analyzed using Mann–Whitney rank sum test ($^{*}p < 0.001$; control $n = 20$; 1% ACs $n = 10$; 10% ACs $n = 11$; bilberries $n = 9$). (D) IFN-γ secretion (mean of control: ~9000 pg/mL). (E) IL-6 secretion (mean of control: ~70 pg/mL). *p*-Value determined by Mann–Whitney rank sum test ($^{*}p = 0.01$; $^{**}p < 0.001$; control $n = 15$; 1% ACs $n = 9$; 10% ACs $n = 9$; bilberries $n = 7$).

proinflammatory cytokines was detectable. Ingestion of ACs resulted in a reduced inflammation in acute as well as chronic colitis demonstrated by a decreased colon shortening, an improved histological score and a lower secretion of IFN-γ, TNF, and IL-6. In general, 10% ACs were more effective in reducing the inflammation. 1% ACs were similar effective in minimizing the colon-length reduction and in amelioration of the histological score. They also reduced the secretion of IFN-γ and IL-6 in chronic colitis, but failed to do so in acute colitis. A total of 0.1% ACs had no effect.

Bilberries are composed of approximately 10% tannins, which have astringent properties and form a protective membrane on inflamed mucosa [14]. Due to their astringent and anti-inflammatory properties, tannins could be responsible for the beneficial effects we observed. In pilot studies, symptoms of patients with Crohn's disease [15] as well as traveler's diarrhea [16] were improved by tannins. However, the concentration of tannins in the ACs extract is only 3%. As ACs were considerably more effective than bilberries, it is unlikely that tannins mediated the beneficial effects. Similar is true for dietary fibers which were reported to improve the symptoms of patients with ulcerative colitis (UC) [17] as they are fermented by anaerobic bacteria to short-chain fatty acids [18]. However, the concentration of dietary fibers in the 10% ACs feed is only 0.4% higher as compared with standard feed. Still there is a chance that the interaction of fibers with ACs [19] or other health-promoting compounds beside tannins and dietary fiber such as salicylic acid or sorbic acid might play a role. Further studies with purified compounds will have to clarify that.

Ingestion of bilberries and ACs significantly reduced apoptosis in intestinal epithelial cells. This effect was observed only for chronic but not for acute DSS colitis. In the latter, toxic effects of DSS may damage the epithelium directly.

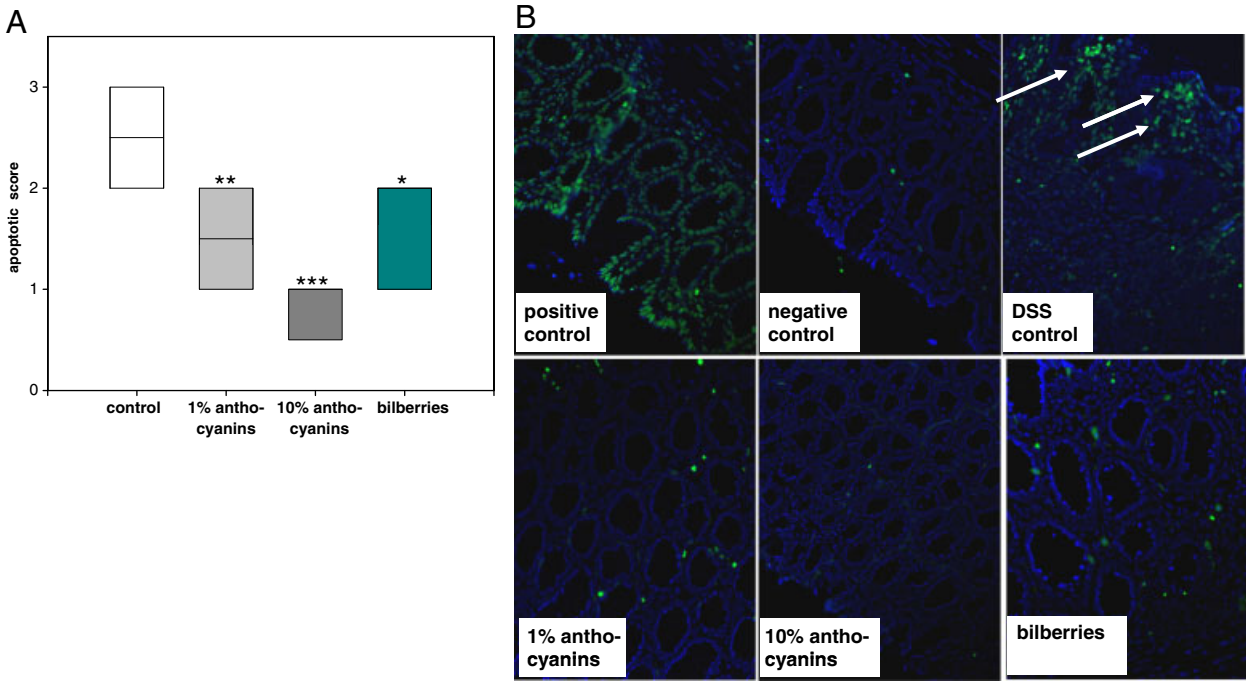

Figure 3. Bilberries and ACs inhibit epithelial cell apoptosis in chronic DSS colitis. Degree of epithelial cell apoptosis in colon tissue was evaluated by TUNEL assay. (A) Analysis of the apoptotic score. *p*-Value analyzed by Mann–Whitney rank sum test ($^{*}p<0.05$; $^{**}p=0.005$; $^{***}p<0.001$; control $n=8$; 1% ACs $n=7$; 10% ACs $n=7$; bilberries $n=5$). (B) Representative sections of TUNEL-stained colon tissues. Magnification, 100 ×.

In summary, our data show considerably positive effects of bilberries, in particular in acute DSS colitis. Furthermore, a significant improvement of acute as well as chronic colitis was induced by ingestion of ACs. We therefore conclude that clinical studies with these compounds should be undertaken due to these promising results.

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The authors have declared no conflict of interest.

References

[1] Puupponen-Pimia, R., Nohynek, L., Ammann, S., Oksman-Caldentey, K. M., Buchert, J., Enzyme-assisted processing increases antimicrobial and antioxidant activity of bilberry. *J. Agric. Food Chem.* 2008, *56*, 681–688.

[2] Katsube, N., Iwashita, K., Tsushida, T., Yamaki, K., Kobori, M., Induction of apoptosis in cancer cells by Bilberry (*Vaccinium myrtillus*) and the anthocyanins. *J. Agric. Food Chem.* 2003, *51*, 68–75.

[3] Guthrie, N., Effect of cranberry juice and products on human breast cancer cell growth. *FASEB J.* 2000, *14*, A771.

[4] Zafra-Stone, S., Yasmin, T., Bagchi, M., Chatterjee, A. et al., Berry anthocyanins as novel antioxidants in human health and disease prevention. *Mol. Nutr. Food Res.* 2007, *51*, 675–683.

[5] Kong, J. M., Chia, L. S., Goh, N. K., Chia, T. F., Brouillard, R., Analysis and biological activities of anthocyanins. *Phytochemistry* 2003, *64*, 923–933.

[6] Yan, X., Murphy, B. T., Hammond, G. B. Vinson, J. A., Neto, C. C., Antioxidant activities and antitumor screening of extracts from cranberry fruit (*Vaccinium macrocarpon*). *J. Agric. Food Chem.* 2002, *50*, 5844–5849.

[7] Frankel, E. N., Kanner, J., German, J. B., Parks, E., Kinsella, J. E., Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* 1993, *341*, 454–457.

[8] Hou, D. X., Tong, X., Terahara, N., Leo, D., Fujii, M., Delphinidin 3-sambubioside, a *Hibiscus anthocyanin*, induces apoptosis in human leukemia cells through reactive oxygen species-mediated mitochondrial pathway. *Arch. Biochem. Biophys.* 2005, *440*, 101–109.

[9] Nohynek, L. J., Alakomi, H. L., Kahkonen, M. P., Heinonen, M. et al., Berry phenolics: antimicrobial properties and mechanisms of action against severe human pathogens. *Nutr. Cancer* 2006, *54*, 18–32.

[10] Meloni, F., Ballabio, P., Gorrini, M., De Amici, M., et al., Effects of 3′-hydroxyfarrerol (IdB 1031), a novel flavonoid agent, on phagocyte products. *Inflammation* 1995, *19*, 689–699.

[11] Tsoyi, K., Park, H. B., Kim, Y. M., Chung, J. I. et al., Anthocyanins from black soybean seed coats inhibit UVB-induced inflammatory cylooxygenase-2 gene expression and PGE2 production through regulation of the nuclear factor-kappaB and phosphatidylinositol 3-kinase/Akt pathway. *J. Agric. Food Chem.* 2008, *56*, 8969–8974.

[12] Boivin, D., Blanchette, M., Barrette, S., Moghrabi, A., Béliveau, R., Inhibition of cancer cell proliferation and suppression of TNF-induced activation of NFkappaB by edible berry juice. *Anticancer Res.* 2007, *27*, 937–948.

[13] Obermeier, F., Kojouharoff, G., Hans, W., Schölmerich, J. et al., Interferon-gamma (IFN-gamma)- and tumour necrosis factor (TNF)-induced nitric oxide as toxic effector molecule in chronic dextran sulphate sodium (DSS)-induced colitis in mice. *Clin. Exp. Immunol.* 1999, *116*, 238–245.

[14] Blaschek, W., Ebel, S., *Hagers CDRom Handbuch der Drogen und Arzneistoffe*. Springer, Berlin, Heidelberg; New York 2006.

[15] Plein, K., Burkard, G., Hotz, J., Treatment of chronic diarrhea in Crohn disease. A pilot study of the clinical effect of tannin albuminate and ethacridine lactate. *Fortschr. Med.* 1993, *111*, 114–118.

[16] Ericsson, C. D., Nonantimicrobial agents in the prevention and treatment of traveler's diarrhea. *Clin. Infect. Dis.* 2005, *41*, S557–S563.

[17] Hallert, C., Kaldma, M., Petersson, B. G., Ispaghula husk may relieve gastrointestinal symptoms in ulcerative colitis in remission. *Scand. J. Gastroenterol.* 1991, *26*, 747–750.

[18] Galvez, J., Rodriguez-Cabezas, M. E., Zarzuelo, A., Effects of dietary fiber on inflammatory bowel disease. *Mol. Nutr. Food Res.* 2005, *49*, 601–608.

[19] Saura-Calixto, F., Pérez-Jiménez, J., Touriño, S., Serrano, J. et al., Proanthocyanidin metabolites associated with dietary fibre from in vitro colonic fermentation and proanthocyanidin metabolites in human plasma. *Mol. Nutr. Food Res.* 2010, *54*, 939–946.

