## Review Article

# Berries as chemopreventive dietary constituents – a mechanistic approach with the ApcMin/+ mouse

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Berries contain a number of compounds that are proposed to have anticarcinogenic properties. We wanted to see if pure ellagic acid, natural ellagitannins and three wild berries have any effect on the adenoma formation in *Apc*- mutated Min/+ mice. Min/+ mice were fed high-fat AIN93-G diets containing 10% (w/w) freeze-dried bilberry (*Vaccinium myrtillus*), lingonberry (*Vaccinium vitis-idaea*), cloudberry (*Rubus chamaemorus*), cloudberry seeds or cloudberry pulp or pure ellagic acid at 1564 mg/kg for 10 weeks. β-Catenin and cyclin D1 protein levels in the adenomas and in the normal-appearing mucosa were determined by Western blotting and immunohistochemistry. Early changes in gene expression in the normal-appearing mucosa were analyzed by Affymetrix microarrays. Three wild berries significantly reduced tumour number (15-30%, $p < 0.05$), and cloudberry and lingonberry also reduced tumour size by over 60% ($p < 0.01$). Cloudberry resulted in decreased levels of nuclear β-catenin and cyclin D1 and lingonberry in the level of cyclin D1 in the large adenomas ($p < 0.05$). Affymetrix microarrays revealed changes in genes implicated in colon carcinogenesis, including the decreased expression of the adenosine deaminase, ecto-5'–nucleotidase and PGE2 receptor subtype EP4. Ellagic acid had no effect on the number or size of adenomas in the distal or total small intestine but it increased adenoma size in the duodenum when compared with the control diet ($p < 0.05$). Neither cloudberry seed nor pulp had any effect on the adenoma formation. Berries seem to have great potential as a source of chemopreventive components.

**Key Words: Apc/Min mouse, berries, colon cancer, ellagic acid**

## INTRODUCTION

The most promising anticarcinogenic agents in plants are phenolic compounds, which are abundantly present in berries. Concomitantly, berries and their extracts have been shown to be chemopreventive at several stages of the carcinogenic process *in vitro*.[1] To screen the possible chemopreventive properties of wild berries and their effects on spontaneous intestinal tumor formation in the Min/+ mouse, we studied three berries with different phenolic profiles,[2] i.e. bilberry (*Vaccinium myrtillus*), lingonberry (*Vaccinium vitis-idaea*), and cloudberry (*Rubus chamaemorus*), that are rich in anthocyanins, proanthocyanidins and ellagic acid, respectively.[3] Because adenomatous polyposis coli (*APC*) gene is considered as a gatekeeper gene for both germline and sporadic colorectal tumors, the *Apc*-mutated Min/+ mouse is an excellent animal model for studies on the effect of diet on colon tumorigenesis. Ellagic acid, a plant phenolic compound, has been shown to decrease carcinogen-induced tumors in different organs in animal models. As regard to colon cancer, no evidence of chemoprevention by ellagic acid has, however, been found *in vivo*.[4] In foods, ellagic acid is mainly found as polymeric ellagitannins and the results of studies using free ellagic acid should be interpreted with caution. We wanted to see whether pure ellagic acid would be chemopreventive in the Min/+ mouse model. Since the 1st experiment suggested that freeze-dried cloudberry prevents adenoma formation in Min/+ mice,[3] it was also interesting to see whether cloudberry seeds and pulp have distinct effects on intestinal tumorigenesis. Ellagitannins are distributed equally between the seeds and the pulp.

## MATERIALS AND METHODS

In both experiments Min/+ mice were stratified randomly to the control or experimental diets, with 10-12 mice per group. The study designs have been described in more detail in the original publications.[3,5] In the 1st experiment, the mice were fed modified high-fat AIN93-G diets containing 10% (w/w) freeze-dried bilberry, lingonberry, or cloudberry. Concentrations of anthocyanins, flavonols and total ellagic acids in the berry diets were based on the values analyzed from the freeze-dried berries. In the 2nd experiment, pure ellagic acid (Sigma E-2250; Sigma-Aldrich Co., St. Louis, MO, USA) was administered at 1565 mg/kg in the diet, equal to its concentration in the cloudberry diet in the 1st experiment.

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In the 2nd experiment, cloudberries were freeze-dried and ground and then the seeds and pulp were separated from each other by sieving. The freeze-dried cloudberry contains 47% (w/w) seeds and 53% (w/w) pulp. In order to maintain the same concentrations of seeds and pulp in the diets than in the cloudberry diet in the 1st experiment, the seed diet contained 4.7% (w/w) seeds and the pulp diet contained 5.3% (w/w) pulp. The concentrations of ellagitannins and free ellagic acid in the seed diet were 807 and 42 mg/kg, and in the pulp diet 820 and 34 mg/kg, respectively. Based on the estimated food consumption of 2.5 g/mouse/day, the average ellagic acid intake was 3.9 mg/mouse/day in the ellagic acid group. The intakes of ellagitannins and free ellagic acid were 2.0 and 0.11 mg/mouse/day in the seed group, and 2.1 and 0.08 mg/mouse/day in the pulp group. The control diet in both experiments was a similar high-fat diet without any berry components or phenolic compounds.[3,5] All diets provided 41% of their energy from fat, 39% from carbohydrate, and 19% from protein. After 10 weeks feeding period, the mice were killed by $CO_2$ inhalation and the small intestine, caecum, and colon were removed and the number and the diameter of adenomas in the small and large intestine were counted using a dissecting microscope at 67 x magnification. The adenoma burden was calculated as a sum of the areas ($\pi r^2$) of all the adenomas for each mouse. In the 1st experiment, adenomas were categorized to small (diameter ≤ 1.0 mm) and large (diameter ≥ 1.1 mm), excised and pooled according to the size-category separately from each segment.

Western blotting and immunohistochemistry for β-catenin and cyclin D1 proteins were done as descreibed.[3] Samples were fractionated to nuclear, cytosolic, and membranous fractions for each mouse. The following primary antibodies were used for western blotting: anti-β-catenin (Sc-7199, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-cyclin D1 (RM-9104, NeoMarkers, Fremont, CA, USA). Equal loading of samples was ensured by incubating blots with β-actin antibody (A5441, Sigma-Aldrich, Europe). Tissue samples for immunohistochemistry were taken from the proximal part of the ileum and analysed as described earlier.[3] Staining and scoring for β-catenin (Transduction Laboratories, Lexington, KY, USA) and cyclin D1 (NeoMarkers) were done as described.[3] The detailed description of the Affymetrix microarray can be found in our original publication.[3]

### *Statistical analyses*

The results are expressed as the median (min-max) and tested by nonparametric methods (SPSS Inc., version 10.0) due to small number of samples. Differences were considered significant when $p < 0.05$. The body weights, adenoma data, Western blotting data, and immunohistochemical staining scores between the control and the berry groups were analyzed by Mann-Whitney U-test. The Wilcoxon signed ranked test was used to study the relation between staining data and for paired comparisons. The effects of dietary treatments on gene expression were evaluated by 1-way ANOVA with cut-off value $p < 0.05$. The resulting gene list was used for testing each berry treatment against the control by filtering for 2-fold or more increase or decrease in gene expression based on all replicates in each group and by using Student's t-test.[3]

## RESULTS

The mice grew well in all dietary groups and no adverse effects of feeding could be detected.

In the 1st experiment, all the berries significantly inhibited the formation of intestinal adenomas, which was seen as a 15-30% inhibition in tumor number ($p < 0.05$; Figure 1A). Over 80% of tumors developed in the distal part of the small intestine, explaining most of the result. No differences were found between the groups in the number of colon adenomas. However, only cloudberry and lingonberry were able to significantly ($p < 0.05$) inhibit adenoma growth. Tumor size was reduced by over 60% with both the berries in the distal small intestine (Figure 1B). Compared to the control group, the median level of nuclear β-catenin in the cloudberry group was 2.70 (0.25-6.57) vs. 5.16 (0.73-10.5) ($p = 0.065$, relative units) and that of cyclin D1 was 0.19 (0.00-0.66) vs. 0.84 (0.16-2.26) ($p < 0.05$). Even though lingonberry did not significantly reduce the level of nuclear β-catenin, it resulted in a significant decrease in the level of cyclin D1 (0.32 (0.00-1.05) vs. 0.84 (0.16-2.26) ($p < 0.05$).

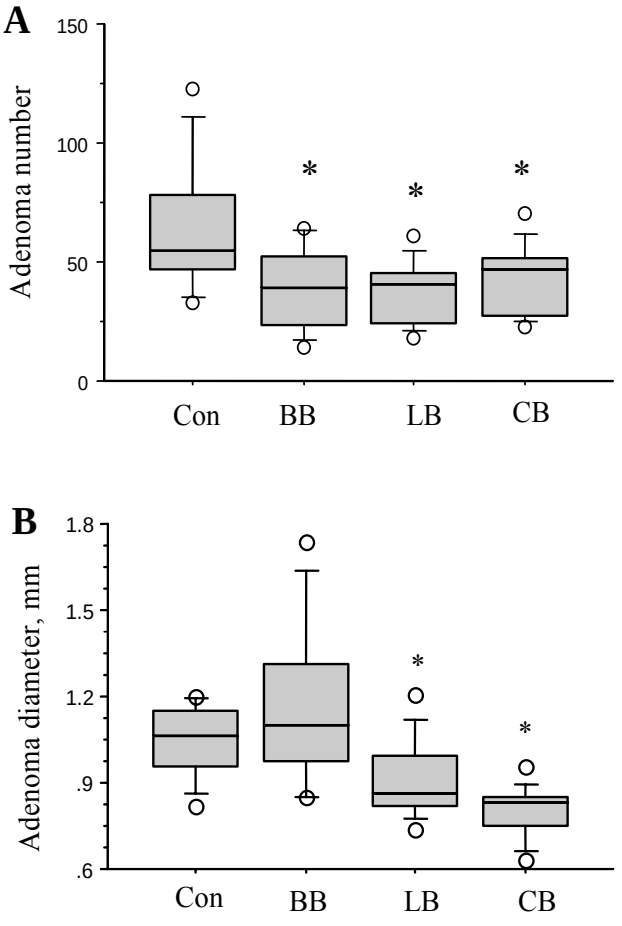


**Figure 1.** (A)Total number of intestinal adenomas and (B) adenoma diameter in the distal small intestine of Min/+ mice fed Control (Con) or bilberry (BB), lingonberry (LB) or cloudberry (CB) diet for 10 wk. Results are presented as box and whisker plots, $n$ = 10-12 per group. *Different from Con, $p < 0.05$, Mann-Whitney U-test.

No difference between the berry diets in staining intensities of β-catenin or cyclin D1 in the crypt or villus of the normal-appearing mucosa was detected. However, Affymetrix microarray approach, which was used to identify early changes in gene expression, showed changes after the berry feeding in the normal-appearing mucosa of Min/+ mice. The expression of the two genes adenosine deaminase (*Ada*) ($p < 0.05$) and 5'ecto-nucleoditase (5-NT) were consistently and similarly decreased by all the berries ($p < 0.1$). PGE2 receptor subtype EP4 expression was decreased particularly by lingonberry feeding ($p < 0.05$).

Pure ellagic acid had no effect on the number or size of adenomas in the distal or total small intestine in the 2nd experiment, but it increased adenoma size in the duodenum when compared with the control diet ($1.5 \pm 0.3$ vs. $1.2 \pm 0.3$ mm; $p = 0.029$). Either cloudberry seed or pulp had no effect on adenoma formation when compared with the control diet.

## DISCUSSION

In this study, all three berries decreased tumor number despite their different phenolic profiles, suggesting that the anticarcinogenic effects are simply not due to total amount of phytochemicals present. Importantly, no adverse effects on weight gain by any of the berries were seen. From a chemoprevention point of view, inhibiting the growth of existing tumors may be more important than preventing the initiating mutations as has been proposed by Luebeck & Moolgavkar.[6] In our study, cloudberry and lingonberry reduced the tumor burden over 60% and were able to reduce both formation and growth of tumors, supporting their strong chemopreventive capacity.

An important finding is that cloudberry and lingonberry prevented the accumulation of nuclear β-catenin and cyclin D1 in the large adenomas. Both β-catenin and cyclin D1 have been associated with tumor growth and are recognised as targets for drug development.[7,8] A decrease in nuclear β-catenin and cyclin D1 by cloudberry and lingonberry could be a significant marker of their chemopreventive activity since we have found earlier that their expression is increased by diet-induced adenoma growth[9] and also NSAIDs elucidate their effects through this pathway. Affymetrix microarray on the normal-appearing mucosa revealed a reduced expression of genes involved in adenosine and prostaglandin metabolism, which could have contributed to the chemoprevention seen in this study.

In the present study, pure ellagic acid at the concentration of approximately 1500 mg/kg diet failed to decrease the adenoma formation in the distal and total small intestine, but increased the size of adenomas in the duodenum. Rao et al. have shown[10] that ellagic acid at clearly higher concentrations of 4000 and 8000 mg/kg in diet had no effect on AOM-induced colon tumor incidence, but the higher dose, however, decreased the incidence of small intestinal adenocarcinomas. The pure ellagic acid that is not bound to the food matrix, may also have harmful effects, especially in the proximal part of the small intestine where the local concentration of ellagic acid is at its highest. This could explain the increase in the adenoma size in the most proximal fifth of the small intestine in the pure ellagic acid group. Cloudberry seeds and pulp had no effect on adenoma formation in this study. The concentration of ellagitannins in the seed diet was 807 mg/kg and in the pulp diet 820 mg/kg. The ellagitannin concentrations in these diets were equal but only half when compared with the 1565 mg/kg concentration in the whole-cloudberry diet in our 1st experiment.[3] However, we were interested to see whether the seeds and the pulp have an effect of their own on tumorigenesis. The reason why neither of these diets was able to prevent adenoma formation may be due to the lower ellagitannin concentration in these diets than in the whole-cloudberry diet.

The chemopreventive effects of whole berries and similar sources of phenolic compounds warrant more research. Further studies should focus on the responsible constituents and their underlying mechanisms.

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**AUTHOR DISCLOSURES**

Marja Mutanen, Anne-Maria Pajari, Essi Päivärinta, Marjo Misikangas, Johanna Rajakangas, Maija Marttinen and Seija Oikarinen, no conflicts of interest.

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