

RESEARCH ARTICLE

Inhibitory Effects of Low-Dose Aloe-Emodin on the Development of Colorectal Tumors in Min Mice

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Abstract

Aloe-emodin (AE), a natural anthraquinone compound, has been reported to exhibit anticancer activity in various cancer cell lines and anti-inflammatory effects in murine macrophages. In the present study, we investigated the cancer chemopreventive effects of AE in an *Apc*-deficient Min mouse model. In the first experiment, male Min mice were fed a basal diet or diets containing 5 ppm AE and 10 ppm AE for 12 weeks. The dietary administration of 5 ppm AE significantly reduced the number of colorectal tumors. In a second experiment, we investigated the effects of AE on colitis-related colon carcinogenesis in Min mouse treated with dextran sodium sulfate (DSS). Female Min mice were administered 1% DSS in their drinking water for 7 days. AE was given to mice in their diet at a dose of 5 or 50 ppm for 5 weeks. Feeding with AE significantly reduced the number of colorectal tumors. When proliferation of cells in normal-appearing colonic mucosa was assessed by monoclonal anti-rat Ki-67 antibody (MIB-5) immunohistochemistry in experiments 1 and 2, the AE treatment significantly decreased the mean MIB-5-labeling index. These results suggest that the dietary administration of low-dose AE may have chemopreventive effects against development of colorectal tumors in Min mice, possibly in part by reducing cell proliferation in colorectal mucosa.

Keywords: Aloe-emodin - colorectal tumor - *Apc*-deficient Min mice

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Introduction

Aloe-emodin (AE) is a 1,8-dihydroxyanthraquinone compound that is present in some medicinal plants such as *Cassia*, *Rheum*, and *Aloe* (Komatsu et al., 2006; Elsohly et al., 2007; Xu et al., 2012). The whole-leaf freeze-dried powder of *Aloe arborescens* Miller var. *natalensis* Berger (Japanese name Kidachi aloe), which is widely used as a component of health foods in Japan, contains aloin (barbaloin+isobarbaloin, C-glycosides; ca. 10-15 mg/g dry powder) and AE (aglycone; trace amount 0.012 mg/g dry powder) (Shimpo et al., 2001). Aloin is a C-glycoside that can be hydrolyzed in the gut to form aloe-emodin anthrone (a genuine purgative component), which, in turn, is auto-oxidized to AE (Arosio et al., 2000). However, the conversion of aloin to AE may be negligible in the large intestine (Shimpo et al, unpublished data).

Aloe extract is known to be potentially useful for cancer (Shimpo et al., 2001; 2003; 2006; Harlev et al., 2012; Chihara et al., 2013). Especially, AE has recently been shown to exhibit anticancer activity in various cancer cell lines (Pecere et al., 2000; Huang et al., 2007; Harlev et al., 2012; Suboj et al., 2012a, 2012b; Chen et al., 2014). Emodin, an analog of AE, was also capable of

inhibiting cellular proliferation, induction of apoptosis, and prevention of metastasis (Huang et al., 2007; He et al., 2012; Huang et al., 2013). However, few studies have examined the *in vivo* anti-cancer effects of AE (Pecere et al., 2000).

Park et al. (2009) previously reported that aloin and AE exhibited strong anti-inflammatory effects in murine macrophages, and showed that the anti-inflammatory effects of AE were similar to those of kampferol and quercetin. These findings indicated that AE may be responsible for the anti-inflammatory activity of aloe (Park et al., 2009). Furthermore, the dietary intake of aloe components was reported to ameliorate the intestinal inflammation response of dextran sulfate sodium (DSS)-induced ulcerative colitis in rats (Park et al., 2011). However, the effect of AE was not described in this study. To the best of our knowledge, the anti-inflammatory actions of AE have not yet been examined *in vivo*.

In the present study, we investigated the cancer chemopreventive effects of AE in an *Apc*-deficient Min mouse model. The Min mouse has a point mutation in the *Apc* gene, and is considered as an ideal model for human familial adenomatous polyposis (Yamada and Mori, 2007; McCart et al., 2008). Furthermore, Tanaka et al. (2006)

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reported that a treatment with DSS led to the intestinal mucosa inflammation and numerous colorectal neoplasms in Min mice. We also previously indicated that the dietary administration of AE, especially at 500 ppm, may have adverse effects on the proliferation of colonic epithelial cells in mice (Shimpo et al., 2009).

Therefore, we investigated the cancer chemopreventive effects of low-dose AE on the development of colorectal tumors in *Apc*^{Min/+} mice treated or not treated with DSS.

Materials and Methods

Materials

Aloe-emodin (AE) used in the present study was prepared using our established method (Chihara et al., 2005). Briefly, aloin (Sigma A-0451; from *Curacao aloe*; barbaloin content: approx. 50%; Sigma-Aldrich Japan K.K., Tokyo, Japan) was hydrolyzed in 4 mol/l hydrochloric acid containing 4% ferric chloride, and the centrifuged precipitate was washed with RO water and dried. Crude AE was extracted with chloroform-methanol-ethyl acetate (1:1:1, v/v). The concentrated solution was sequentially eluted by open silica gel column chromatography with chloroform and chloroform-ethyl acetate mixtures (3:1→2:1→1:1) as eluents. AE fractions were concentrated and dried as purified AE.

Min mouse breeding and genotyping

Male C57BL/6J-*Apc*^{Min/+} mice (Min mice) originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA) were bred with female wild-type (C57BL/6J) mice (Charles River Laboratories Japan, Inc., Yokohama, Japan). Mice were genotyped by a PCR assay for the *Apc* allele (Jacoby et al., 1996), and male and female Min mice were used in experiments 1 and 2, respectively.

Animal care and drug treatment

Min mice were kept in groups of four or five in plastic cages on woodchip bedding and fed a basal diet with the Oriental MF diet (Oriental Yeast Co., Ltd., Tokyo, Japan), in an animal facility controlled at a temperature 23±5°C, 60±5% humidity, and with a 12-h light/dark cycle. The care and use of animals followed the guidelines of the 'Care and Use of Laboratory Animals' of Fujita Health University.

Experimental procedures

Effects of AE on the development of colorectal tumors in Min mice (Experiment 1): Forty-two 5- or 6-week-old male Min mice were randomly divided into three groups with 13-15 mice per group and fed one of three diets for 12 weeks from 5 or 6 weeks of age: (a) the basal diet (Oriental MF diet) alone (Group 1), (b) the basal diet with 5 ppm AE (Group 2), and the basal diet with 10 ppm AE (Group 3).

Effects of AE on colitis-related colon carcinogenesis in Min mice treated with dextran sodium sulfate (DSS) (Experiment 2): This experiment was performed as described by Kohno et al. (2007). Thirty 5-week-old female Min mice were randomly divided into four groups with 7 or 8 mice per group and fed one of three diets for 5 weeks from 5 weeks of age: (a) the basal diet (Oriental

MF diet) alone (Groups 1 and 2), (b) the basal diet with 5 ppm AE (Group 3), and the basal diet with 50 ppm AE (Group 4). Animals in Groups 2-4 were administered 1% (w/v) DSS in their drinking water for the first week, and were then shifted to tap water for 4 weeks. Mice in Group 1 were given tap water throughout the entire experiment.

Food and water were available *ad libitum* in experiments 1 and 2. Mice were observed, food intakes were measured daily, and body weights were measured twice a week. At the end of both experiments 1 and 2, all mice were anesthetized with Nembutal, exsanguinated via the heart into heparin-coated syringes, and carefully autopsied. The intestines were removed after mice were sacrificed, opened longitudinally, spread on the filter paper with the lumen side up, and fixed in 10% buffered formalin. The number of tumors (≥0.5 mm in diameter) in the small intestine and colorectum was scored.

Determination of plasma lipid levels

Triglyceride (TG) and total cholesterol (T-Cho) levels in the plasma were enzymatically measured with Triglyceride E-Test Wako and Cholesterol E-Test Wako kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan), respectively.

Immunohistochemistry

Cell proliferation in normal colorectal mucosa was determined by a slight modification of the method described by Katsuki et al. (2006) and Muskhelishvili et al. (2003). Briefly, sections were retrieved in an autoclave in the citrate buffer (pH 6.0) for 20 min at 120°C. Endogenous peroxidase activity was blocked by incubating slides in absolute methanol containing 3% H₂O₂ for 15 min at room temperature. They were then incubated with the anti-Ki-67; MIB-5 antibody (rat anti-mouse Ki-67 antigen clone MIB-5, DakoCytomation, Denmark) at its working dilution of 1:100. After 30 min at room temperature, they were treated with the Histofine Simple Stain Mouse MAX-PO (Rat) reagent (Nichirei Bioscience Inc., Tokyo, Japan) for 30 min. Slides were washed three times with PBS after each incubation, and 3,3'-diaminobenzidine was employed as a chromogen. Nuclei were lightly counterstained with Mayer's hematoxylin solution. To determine immunostaining, epithelial cells in the colorectum were counted from the lowest point to the tip of the crypt by light microscopy using 400× magnification. The number of positively stained cells in each crypt column was recorded. The results were defined as the ratio of the number of positively stained cells to the total number of cells counted (at least 500), and then multiplied by 100.

iNOS and COX-2 mRNA expression

Total RNA was isolated from the mouse colorectal mucosa using the High Pure RNA Tissue Kit (Roche Diagnostics GmbH, Mannheim, Germany) and cDNA was amplified using the Transcriptor Universal cDNA Master (Roche Diagnostics GmbH). Messenger RNA (mRNA) expression levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were quantified by Taqman real-time quantitative PCR using the LightCycler

480 system and LightCycler 480 Probes Master (Roche Diagnostics GmbH). The housekeeping gene β -actin was used for the normalization of all analyzed genes.

Statistical analysis

All data points are given as the means \pm S.E. Statistical comparisons between groups were performed using an ANOVA followed by Dunnett's multiple comparisons test or the Kruskal-Wallis test (nonparametric ANOVA) followed by the Dunn's multiple comparisons test. These statistical procedures were performed with InStat version 3.0 for Windows (GraphPad Software Inc., San Diego, CA, USA). Differences were considered statistically significant if the p value was <0.05.

Results

Effects of AE on the development of colorectal tumors in Min mice (Experiment 1)

No significant differences were observed in final body weight between the three groups (control group: 25.7 \pm 0.7 g, 5 ppm AE group: 25.7 \pm 0.6 g, and 10 ppm AE group: 25.1 \pm 0.9 g). Furthermore, no significant differences were noted in the total number of tumors (\geq 0.5 mm in diameter) in the small intestine among the three groups (control group: 60.2 \pm 5.5, 5 ppm AE group: 67.7 \pm 5.2, and 10 ppm AE group: 59.0 \pm 5.6). However, the total number of tumors in the colorectum was significantly lower in the 5 ppm AE group (4.0 \pm 0.6; p <0.05) than in the 10 ppm AE group (6.3 \pm 0.4) and control group (6.5 \pm 0.9) (Figure 1).

The labeling index (%) of MIB-5, an immunohistochemical proliferation marker, in the 5 and 10 ppm AE groups (22.3 \pm 1.3; p <0.01 and 25.6 \pm 1.1; p <0.05, respectively) was significantly lower than that in the control group (29.9 \pm 1.3)(Figure 2).

Blood triglyceride levels were previously reported to markedly increase with age in Min mice (Niho et al., 2003). Therefore, we examined changes in plasma lipid levels in Min mice. Plasma triglyceride levels (mg/dl) in the 10 ppm AE group (614.9 \pm 95.1), but not in the 5 ppm AE group (773.9 \pm 107.1), were 21% lower than those in the control group (780.2 \pm 67.8). No significant differences were observed in total cholesterol levels in the plasma (mg/dl) among the three groups (control group: 144.6 \pm 10.1, 5 ppm AE group: 152.8 \pm 10.4, and 10 ppm AE group: 135.7 \pm 8.9).

Effects of AE on colitis-related colon carcinogenesis in Min mice treated with DSS (Experiment 2)

Final body weights in the control, DSS alone, DSS+5 ppm AE, and DSS+50 ppm AE groups were 18.2 \pm 0.3 g, 17.4 \pm 0.3 g, 17.8 \pm 0.4 g, and 18.0 \pm 0.2 g, respectively. No significant differences were observed in final body weights among the four groups. As shown Figure 3, no significant differences were noted in the total number of tumors (\geq 0.5 mm in diameter) in the small intestine among the four groups (control group: 30.7 \pm 3.5, DSS alone group: 30.3 \pm 6.0, DSS+5 ppm AE group: 43.4 \pm 5.9, and DSS+50 ppm AE group: 33.9 \pm 6.5). However, the total number of tumors in the colorectum was significantly higher in the DSS alone group (15.6 \pm 3.2; p <0.01) than

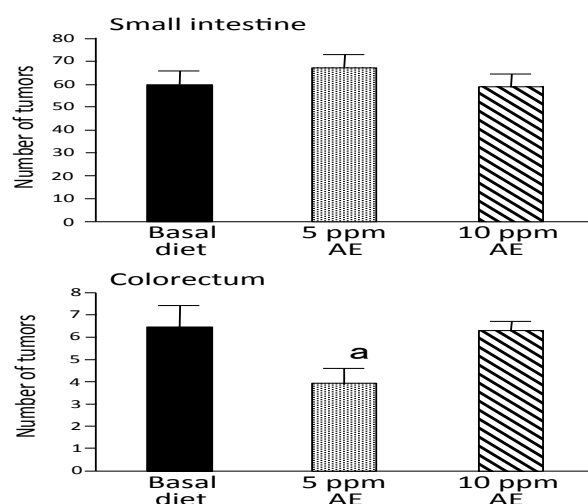


Figure 1. Effects of Aloe-Emodin on the Development of Small Intestinal and Colorectal Tumors (\geq 0.5 mm in Diameter) in Min Mice (Experiment 1). ^aSignificantly different from the basal diet group (p <0.05; Dunn's multiple comparisons test)

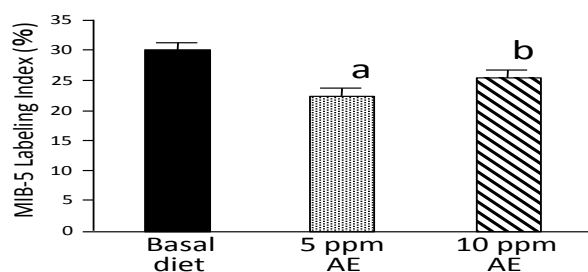


Figure 2. MIB-5 Labeling Index in Normal Colorectal Mucosa (Experiment 1). ^{a, b}Significantly different from the basal diet group (a p <0.01, b p <0.05; Dunn's multiple comparisons test)

in the control group (4.9 \pm 1.3). Feeding with the 50 ppm AE diet significantly reduced the total number of tumors in the colorectum (6.3 \pm 0.8; p <0.05) and the 5 ppm AE diet also reduced the total tumor number (7.1 \pm 0.8) by approximately half.

The labeling index (%) of MIB-5 in normal colorectal mucosa was significantly higher in the DSS alone group (33.7 \pm 1.2; p <0.05) than in the control group (28.2 \pm 0.8; p <0.01), and feeding with the 5 ppm AE diet significantly reduced the MIB-5-labeling index (27.5 \pm 0.8; p <0.05) (Figure 4).

No significant differences were observed in plasma triglyceride or total cholesterol levels among the four groups (data not shown).

DSS was previously shown to strongly enhance colon carcinogenesis in Min mice and the overexpression of iNOS and COX-2 contributed to this enhancement (Tanaka et al., 2006, Kohno et al., 2007). Therefore, we examined iNOS mRNA expression by quantitative RT-PCR in the colorectal mucosa of Min mice. The expression of iNOS mRNA in Min mice treated with DSS (DSS alone group) was higher than that in mice treated without DSS (control group) (control group: 100.0 \pm 18.6, and DSS alone group: 191.3 \pm 54.2). This increase in mRNA levels was reduced in mice fed the 5 and 50 ppm AE diets (118.6 \pm 32.2 and 136.3 \pm 35.6, respectively). On the other hand, no significant differences were observed

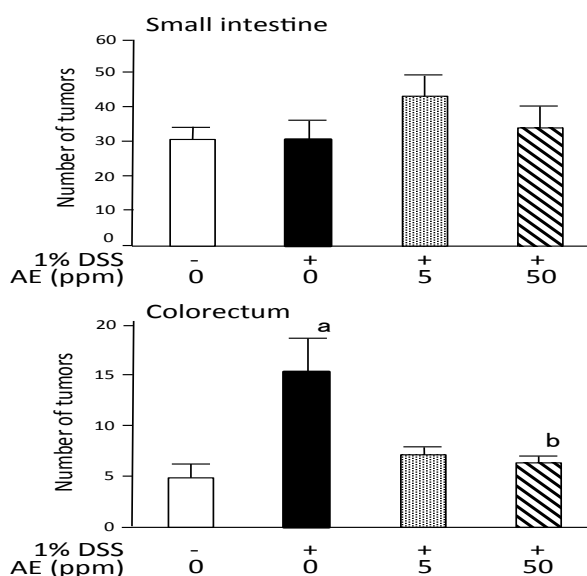


Figure 3. Effects of Aloe-Emodin on the Development of Small Intestinal and Colorectal Tumors (≥ 0.5 mm in Diameter) in Min Mice that Received DSS or DSS + Aloe-Emodin (Experiment 2). ^aSignificantly different from the basal diet group ($p < 0.01$; Dunn's multiple comparisons test). ^bSignificantly different from the DSS + basal diet group ($p < 0.05$; Dunn's multiple comparisons test)

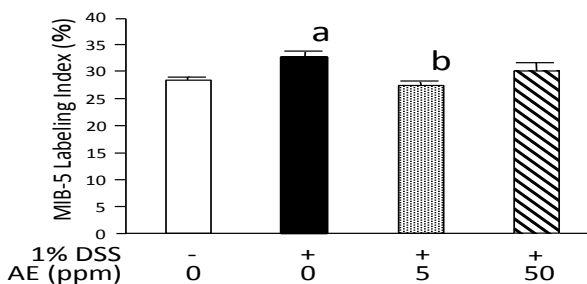


Figure 4. MIB-5 Labeling Index in Normal Colorectal Mucosa (Experiment 2). ^aSignificantly different from the basal diet group ($p < 0.05$; Dunnett's multiple comparisons test). ^bSignificantly different from the DSS+basal diet group ($p < 0.01$; Dunnett's multiple comparisons test)

in the expression of COX-2 among the four groups (data not shown).

Discussion

Using a Min mouse model treated without or with DSS (Experiments 1 and 2, respectively), we demonstrated that a low-dose of AE had inhibitory effects on the development of colorectal tumors. To the best of our knowledge, this is the first study to examine the effects of dietary AE administration in animal models of cancer.

We also assessed the effects of AE on the cellular proliferative activity of normal-appearing colonic mucosa using the monoclonal antibody MIB-5, and found that the AE treatment significantly decreased the mean MIB-5-labeling index. The regulation of cellular proliferation is known to be important for preventing cancer, and effective anti-cancer agents typically suppress cellular proliferation and inhibit the occurrence of malignant lesions (Mori et al., 1999). Therefore, a low-dose of AE may exhibit chemopreventive effects by inhibiting the proliferation

of cells during large bowel carcinogenesis in Min mice treated with or without DSS.

The mechanisms by which AE inhibits tumor development in the Min mouse colorectum have yet to be elucidated in detail. AE may inhibit the development of tumors and/or inflammation in the Min mouse model. In the present study, we demonstrated that plasma triglyceride levels in the 10 ppm AE group were slightly lower (21%) than in the control group in Experiment 1 (the Min mouse model without the DSS treatment). Niho et al. (2003) reported a hyperlipidemic state in *Apc* gene-deficient mice, such as Min mice, and the potential of peroxisome proliferator-activated receptor (PPAR) ligands to suppress both hyperlipidemia and polyp formation. However, the total number of tumors in the colorectum (but not in the small intestine) was significantly lower in the 5 ppm AE group and plasma triglyceride levels were only decreased by 21% in the 10 ppm AE group in Experiment 1. These results suggested that AE did not have a direct effect on lipid metabolism under this experimental condition

In addition, the expression of iNOS mRNA was higher in Min mice treated with DSS (the DSS alone group) than in those treated without DSS (control group), and this elevated mRNA level was reduced in mice fed the 5 and 50 ppm AE diets in Experiment 2 (using Min mice treated with DSS). On the other hand, no significant differences were observed in COX-2 mRNA expression levels between the control and treated groups. Since COX-2 and iNOS are known to be good targets in the chemoprevention of colon cancer (Watanabe et al., 2000; Budda et al., 2011; Pandurangan and Esa, 2013), the reduction observed in the expression of iNOS mRNA by the low-dose of AE may explain, at least partly, the inhibitory effects of low-dose AE on inflammation-related large bowel carcinogenesis in Min mice. However, further studies are required to fully understand the *in vivo* anti-cancer effects of AE on the development of tumors in the large intestine using the Min mouse model without or with DSS.

Anthranoid (anthraquinone, dianthrone, or anthrone)-containing laxatives, such as aloe, cascara, frangula, and rheum, may be a risk factor for the induction of colorectal tumors (Siegers, 1992; 1993; van Gorkom et al., 1999). Mori et al. (1985; 1986) reported that the synthetic 1,8-dihydroxyanthraquinone (other names used include danthron or chrysazin) induced tumors in the large intestines and livers of rodents. Danthron and emodin, the chemical structures of which are very close to that of AE and have the same genotoxicity profiles, are clearly or equivocally carcinogens in rodents, respectively (Nesslany et al., 2009).

However, we found that the dietary freeze-dried whole-leaf powder of *Aloe arborescens* (ALOE; 5% and 1% ALOE in the diet) inhibited the formation of azoxymethane (AOM)-induced aberrant crypt foci (ACF; early lesions in colorectal carcinogenesis) in the rat colorectum (Shimpo et al., 2001). Since the 5% (not 1%) ALOE dose level reduced body weights and induced soft feces in rats, we did not add a high dose level of ALOE to the diets of long-term colon carcinogenesis rat models. We previously demonstrated that ALOE (1% in diet) reduced 1,2-dimethylhydrazine (DMH)-induced colorectal

proliferative lesions (Shimpo et al., 2003). Furthermore, we examined the modifying effects of 1% and 0.2% ALOE diets on long-term AOM-induced intestinal carcinogenesis in rats, and showed that the 28-week administration of a 0.2% ALOE diet reduced the incidence of AOM-induced intestinal adenocarcinomas in rats (Shimpo et al., 2006).

Hamiza et al. (2014) very recently reported that aloin supplementation (50 or 100 mg/kg body weight, administered orally on alternate days for 14 weeks) effectively suppressed the initial phases of colon carcinogenesis, and this was attributed to the attenuation of the oxidative damage, inflammatory mediators (COX-2, iNOS, TNF- α , and IL-6), hyperproliferation (PCNA), and early neoplastic transitions (ACF and mucin-depleted foci) induced by DMH in the colons of Wistar rats.

On the other hand, Imaida and his colleagues investigated the chronic toxicity and carcinogenicity of *Aloe arborescens* in a one-year chronic toxicity study of whole-leaf *Aloe arborescens* powder at dietary concentrations up to 4.0% in Wistar Hannover rats. Diarrhea, loss of body weight gain, severe sinus dilatation of the ileocecal lymph nodes and renal tubules in both sexes receiving 4% were reported, and the no observed adverse effect level (NOAEL) for the *Aloe* powder was the 0.16% in diet (Matsuda et al., 2008). In a subsequent 2-year carcinogenicity study in rats, the whole-leaf powder of *Aloe arborescens* exerted equivocal carcinogenic potential on the colon at a 4% high dose level, and was not carcinogenic at nontoxic-dose levels (Yokohira et al., 2009). Thus, these findings suggested that the carcinogenic potential of the 4% high-dose level on the colon may have been due to irritation of the intestinal tract as a result of diarrhea (Yokohira et al., 2009).

Boudreau et al. (2012a; 2012b) reported that the *Aloe vera* whole-leaf extract was an intestinal irritant in F344/N rats and B6C3F1 mice and a carcinogen in the large intestine of F344/N rats. Pandiri et al. (2011) also demonstrated that the *Aloe vera* non-decolorized whole-leaf extract-induced large intestinal tumors in F344 rats shared several similarities with human colorectal cancer at the morphological and molecular levels.

More importantly, Nesslany et al. (2009) investigated the *in vivo* primary DNA-damaging potential of AE on both isolated kidney and colon cells using the mouse comet assay to demonstrate the possibility of organ-specific genotoxicity. The results obtained revealed that AE induced DNA primary damage between 3 and 6 h after two oral doses of 500, 1000, and 2000 mg/kg body weight. Thus, AE, which is present in the plant extract, should be considered as an *in vivo* genotoxin (Nesslany et al., 2009).

Taking our results and those of other recent studies together, the dietary or oral administration of high dose levels (induced body weight loss and diarrhea) of whole-leaf *Aloe*, such as *Aloe arborescens* and *Aloe vera*, may have potential carcinogenic or tumor-promoting activity on colonic mucosa in rodents. High doses of aloin and AE (*Aloe* anthranoids) may also induce the same phenomenon in the colons of rodents. Although we used low doses of AE in the present study, we have to examine the modifying effects of appropriate quantities of AE on animal cancer carcinogenesis models in the future.

In conclusion, the dietary administration of low-dose AE significantly inhibited colorectal tumor formation and cellular proliferative activity in Min mice treated with or without DSS. Colon carcinogenesis experiments to test modifying effects of a low-dose of AE on the development of chemically-induced colorectal cancer in rodents are now underway in our laboratory.

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