

Inhibition of Formation of Azoxymethane-induced Colonic Aberrant Crypt Foci in Rats by Edible Green Algae *Capsosiphon fulvescens* and Brown Algae *Hizikia fusiforme*

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Abstract. *Capsosiphon fulvescens* (green seaweed) and *Hizikia fusiforme* (brown seaweed) are marine algae consumed as food supplements, especially in Japan, China and Korea, and are considered traditional medicinal tonics for certain ailments. The aim of this study was to investigate the possible inhibitory effects of dietary *C. fulvescens* and *H. fusiforme* on azoxymethane (AOM)-induced colorectal cancer (CRC) in rats. F344 male rats (5 weeks, 150 g) were divided into six groups as follows. Group 1: Injected with normal saline solution and fed control diet (untreated control). Group 2: Injected with AOM and fed control diet (treated control). Group 3: Injected with AOM and fed 1% *C. fulvescens* diet. Group 4: Injected with AOM and fed 2% *C. fulvescens* diet. Group 5: Injected with AOM and fed 2% *H. fusiforme* diet. Group 6: Injected with AOM and fed 6% *H. fusiforme* diet. Test animals received subcutaneous injections of AOM (15 mg/1 ml/kg body weight) once a week for 2 weeks to induce aberrant crypt foci (ACF) in treated control and experimental groups. We evaluated the effects of dietary *C. fulvescens* and *H. fusiforme* at two different dose levels: 1 and 2% *C. fulvescens*, and 2 and 6% *H. fusiforme*, on colonic carcinogenesis by AOM in rats. Our results suggest that body weights were not significantly different amongst groups. We found that feeding *C. fulvescens* and *H. fusiforme* with a control diet significantly ($p < 0.05$) inhibited the development of ACF in experimental groups. *C.*

fulvescens and *H. fusiforme* in food also significantly ($p < 0.05$) reduced the proliferating cell nuclear antigen labeling index in the colonic tissues of experimental groups. These results demonstrate the chemopreventive potential of *C. fulvescens* and *H. fusiforme* against CRC in an AOM-induced rats.

Colorectal cancer (CRC) is the most common cancer, and a significant cause of death, in developed countries, including the United States (1, 2). Numerous factors including age, physical activity habits, environment, obesity, diabetes, diet, lifestyle, and genetics play important roles in CRC carcinogenesis (3, 4). Many genetic defects/mutations, including the adenomatous polyposis coli (*APC*) gene, kirsten ras (*KRAS*) oncogene, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) gene, phosphatase and tensin homolog (*PTEN*) gene, transforming growth factor (*TGFβ*) receptor, and tumor protein p53 (*TP53*) gene, and enzymes such as cyclooxygenase-2 and nitric oxide synthase have been suggested to play vital roles in CRC carcinogenesis (4-6). It is recognized that CRC develops sequentially from polyp to adenocarcinoma. This development may take a long time, as much as 10-17 years (4).

Azoxymethane (AOM) is a tumor-initiating chemical agent commonly used in models of colon cancer (7). Specifically, it can induce the formation of aberrant crypt foci (ACF) by epithelial cells, which then progress to adenomas and malignant adenocarcinomas, that is similar to the pathogenesis of sporadic human colon cancer (8, 9). Thus, it has been extensively used in the study of molecular biology, prevention, and treatment of colon cancer.

ACF are clusters of abnormal tube-like glands on the colonic mucosal surface, and are the earliest morphological changes after treatment with chemical carcinogens such as AOM (10). Evidence strongly suggests that ACF are pre-neoplastic lesions in the colon, and they are recognized as intermediate biomarkers

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of CRC in both rodents and humans (6, 11). Chemically-induced ACF in rodents have been used to assess new chemopreventives and diets that might prevent CRC (12).

Capsosiphon fulvescens is a green marine alga consumed as a food supplement, and is considered to possess medicinal activity and high nutritional value (13). *Hizikia fusiforme* is a brown marine alga, traditionally used as a foodstuff and medicine in Asia (14). Previous research reported that *C. fulvescens* and *H. fusiforme* exhibited beneficial medicinal activities, such as antioxidant, anti-diabetic, and antitumor effects (15).

The purpose of the present study was to identify the possible inhibitory effect on colon carcinogenesis of feeding *C. fulvescens* and *H. fusiforme* at different dose levels to F344 male rats. We investigated the effects of *C. fulvescens* at 1 and 2%, and *H. fusiforme* at 2 and 6% dose levels in the rats' diet. In addition, we immunohistochemically analyzed the expression of proliferating cell nuclear antigen (PCNA), which plays a vital role in carcinogenesis.

Materials and Methods

Chemicals. All chemicals were of the highest grade and were purchased from commercial suppliers. AOM was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Analytical-grade ethyl ether was purchased from Duksan Pure Chemicals (Ansan, Korea).

Plant materials and diets. *C. fulvescens* and *H. fusiforme* were collected from Jeonnam, Wando, South Korea, and were washed with water and dried. The seaweeds were cut into small pieces and extracted with 95% ethanol at 80°C for 2 h at 120 rpm, with ethanol at a ratio of 40 ml/g. The extract was centrifuged (8,000 ×g, 10 min), filtered, and concentrated at 60°C. The experimental diets were prepared by adding 5% brix concentrate to AIN-76A animal diet (ORIENT BIO Inc, Sangdaewon-Dong, Seongnam-si, South Korea) (Table I) with 1 or 2% of *C. fulvescens* and 2 or 6% of *H. fusiforme* by dry weight (Table II).

Animals. F344 male rats (5 weeks, 150 g) were used in these experiments (Orient Co Ltd, Seoul, Korea) and maintained in an environment with controlled temperature (23±2°C) and humidity (55±7%) under a 12 h light-dark cycle. All animal experiments were carried out in accordance with the National Institute of Health guidelines.

Experimental design. The rats were divided into six groups for experimental procedures as follows. In group 1 (untreated control), saline was injected (1 ml/kg) subcutaneously (sc.) during the first and second weeks of the experimental period. In group 2 (treated control), AOM was injected (15 mg/1 ml/kg) sc. once a week for 2 weeks. The animals in both these groups were fed the control diet throughout for 11 weeks. Groups 3, 4, 5, and 6 (experimental groups) were fed experimental diets containing 1 or 2% *C. fulvescens* or 2 or 6% *H. fusiforme*, respectively, for 8 weeks from the third week after the administration of AOM. While group 1 had eight rats, all other groups comprised 10. Body weights and food intake of the animals were measured throughout the

Table I. AIN-76A animal diet for controls and experimental groups.

Ingredients	g%	kcal%
Casein, 30 mesh	200	800
DL-Methionine	3	12
Corn starch	150	600
Sucrose	500	2000
Cellulose, BW200	50	0
Corn oil	50	450
Mineral mix S10001	35	0
Vitamin mix V10001	10	40
Choline bitartrate	2	0
Total	1000	3902

Table II. Experimental animals.

Group	Treatment	No. of animals
1	Saline + control diet	8
2	AOM + control diet	10
3	AOM + 1% <i>Capsosiphon fulvescens</i> diet	10
4	AOM + 2% <i>C. fulvescens</i> diet	10
5	AOM + 2% <i>Hizikia fusiforme</i> diet	10
6	AOM + 6% <i>H. fusiforme</i> diet	10

AOM: Azoxymethane 15 mg/kg, subcutaneous injection (1 ml/kg).

experiment. After 11 weeks, all animals were sacrificed for histopathological examination of colonic tissues (Figure 1).

Histopathological examination of colonic tumor tissues. Animals were sacrificed and colonic tumor tissues were removed immediately. The tissues were washed with normal saline and fixed in 10% neutral formalin solution (pH 7.0). After routine paraffin embedding, 6-8 µm tissue sections were cut, fixed on microscope slides, and deparaffinized, before being hydrated. Finally, sections were stained with hematoxylin and eosin (H&E) for histological examination of colonic cancer specimens.

Determination of ACF. The development of carcinoma was suggested by the detection of ACF, believed to be precancerous lesions, in colonic mucosa. Rectal crypt foci were excised, and the colon was spread, washed with potassium phosphate buffer (0.1 M, pH 7.2), and then fixed in 10% neutral formalin. After fixation and staining with 2.5% methylene blue, ACF were distinguished morphologically by their elliptical shape and increased size of crypts compared to normal crypts and measured microscopically.

Immunohistochemical evaluation. PCNA immunohistochemistry was performed to evaluate the proliferation of tumor cells. Paraffin-embedded colonic tumor tissues cut into 6-8 µm sections. These were then immersed in xylene solution to remove paraffin and rehydrate the tissues while sequentially reducing the ethanol concentration. The slides prepared in this manner were coated with mouse monoclonal antibody against PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), then coated with avidin-biotin peroxidase complex (Zymed

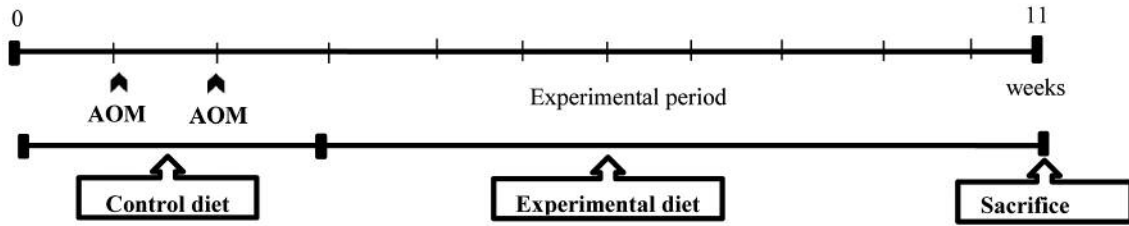


Figure 1. Experimental protocol to evaluate chemopreventive activity in F344 rats. Rats were divided into two control (untreated and treated) groups and four experimental groups. The rats were fed a control diet (AIN-76A) or diet supplemented with different doses of *Capsosiphon fulvescens* (1 or 2%) and *Hizikia fusiforme* (2 or 6%), for the 11-week experimental period. Rats in groups 2-6 received subcutaneous injections of azoxymethane (AOM; 15 mg/kg body weight) once a week for 2 weeks. Group 1 served as an untreated control (1 ml saline/kg by subcutaneous injection + control diet) and group 2 served as the treated control (15 mg AOM/kg body weight by subcutaneous injection + control diet).

Table III. Weight gain and feed intake.

Group	Treatment	Body weight at start of treatment (g)	Body weight at termination (g)	Feed intake (g/day)
1	Saline + control diet	285.56±5.7	340.14±6.7	13.17
2	AOM + control diet	268.41±5.4	323.94±4.9	13.35
3	AOM + 1% <i>Capsosiphon fulvescens</i> diet	256.05±4.8	314.44±3.4	14.16
4	AOM + 2% <i>C. fulvescens</i> diet	243.17±2.2	303.07±4.4	13.30
5	AOM + 2% <i>Hizikia fusiforme</i> diet	245.05±3.8	300.71±3.7	13.13
6	AOM + 6% <i>H. fusiforme</i> diet	259.84±4.2	315.17±4.3	14.67

AOM: Azoxymethane 15 mg/kg, subcutaneous injection (1 ml/kg).

Laboratories Inc., San Francisco, CA, USA), and incubated with diaminobenzidine (DAB, Zymed Laboratories Inc.) as a substrate. After washing, slides were examined after being counterstained with Meyer’s hematoxylin and washed with tap water. For the negative control, the primary antibody was replaced with phosphate-buffered saline. Finally, we counted proliferating tumor cells under microscopy.

Statistical analysis. Data are expressed as mean±standard error of the mean (SEM). Data were analyzed using a one-way analysis of variance (ANOVA), followed by Dunnett’s *t*-test for multiple comparisons using SPSS software (Release 12.0. SPSS Inc., Chicago, IL, USA). Statistical significance was considered at $p<0.05$.

Results

Feeding the experimental diets containing *C. fulvescens* and *H. fusiforme* did not produce any toxicity. Histological examinations revealed no morphological evidence of fatty liver (data not shown). The body weights of animals in groups 1 and 2 (control groups) were not significantly greater than those in the experimental groups (Table III) that received *s.c.* injections of AOM at 15 mg/kg body weight once a week for 2 weeks to investigate the formation of ACF in the colon. H&E staining revealed significant histopathological changes in the colonic tissues of AOM-treated rats. We found histological abnormalities such as ACF including dysplasia and hyperplasia, abnormally

shaped lumens and elongated nuclei in the colonic mucosa of the treated control group (group 2), but these findings were significantly ($p<0.05$) less frequent in the experimental groups (groups 3-6) (Figure 2). These results demonstrate that *C. fulvescens* and *H. fusiforme* suppress dysplastic cell proliferation in the colonic tissues of AOM-treated rats.

The incidence and number of colorectal tumors after 11 weeks are listed in Table IV. In group 1 (untreated control), no colonic tumors were found. However, group 2 (treated control), colonic tissues were found to contain 58 ACF (Figure 3). In the dose–response study, experimental *C. fulvescens* and *H. fusiforme* diets reduced the incidence of ACF formation ($p<0.05$) in groups 3 and 4, whereas the occurrence of ACF was significantly ($p<0.001$) lower in groups 5 and 6 compared to the treated control. These findings suggest that *C. fulvescens* and *H. fusiforme* suppress ACF formation induced by AOM in the colon of rats.

To investigate the effects of dietary *C. fulvescens* and *H. fusiforme*, we used immunohistochemistry to analyze the expression of PCNA, a marker of tumor cell proliferation and apoptosis, at week 11. The colonic adenocarcinoma PCNA labeling index was lower ($p<0.05$) in groups 4 and 5, but in groups 3 and 4 more significantly ($p<0.001$) lower than in the treated control group (Figure 5).

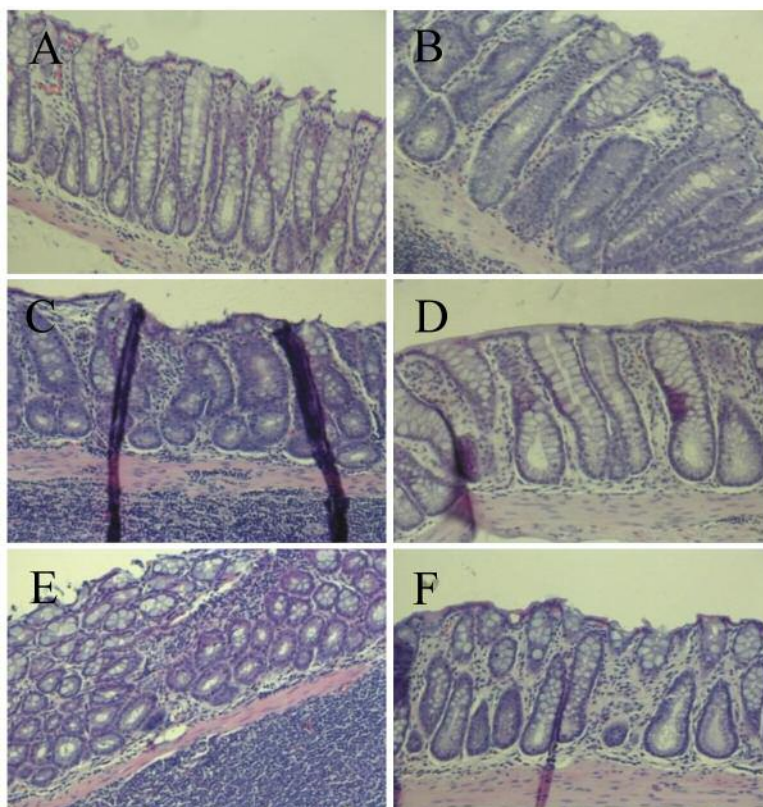


Figure 2. Histopathological examination of colonic tissues of F344 rats from group 1, saline + control diet (A); group 2, azoxymethane (AOM) + control diet (B); group 3, AOM + 1% *Capsosiphon fulvescens*-supplemented diet (C); group 4, AOM + 2% *C. fulvescens*-supplemented diet (D); group 5, AOM + 2% *Hizikia fusiforme*-supplemented diet (E); and group 6, AOM + 6% *H. fusiforme*-supplemented diet (F). Representative microscopic examination was performed with hematoxylin and eosin staining. Histological assessment showed the presence of aberrant crypt foci including dysplasia and hyperplasia, abnormally-shaped lumens and elongated nuclei in the colonic mucosa of the treated control group but a marked reduction in all experimental groups. Sections were examined at 200× magnification.

Table IV. Formation of azoxymethane (AOM)-induced aberrant crypt foci (ACF).

Group	Treatment	Number of Aberrant Crypt Foci (ACF)				Total
		1	2	3	≥4	
1	Saline + control diet	0	0	0	0	0
2	AOM + control diet	22.43±2.6	11.43±2.3	13±3.6	11±2.9	57.86±2.9
3	AOM + 1% <i>Capsosiphon fulvescens</i> diet	12.14±1.9	5.57±2.0	4.29±0.5	7.45±1.1	29.45±1.4*
4	AOM + 2% <i>C. fulvescens</i> diet	11.71±1.4	6.86±1.3	4.14±0.7	8.33±0.9	31.05±1.1*
5	AOM + 2% <i>Hizikia fusiforme</i> diet	7.22±0.9	4.88±0.5	4.11±1.3	4.97±0.3	21.18±0.8**
6	AOM + 6% <i>H. fusiforme</i> diet	6.57±1.0	4.33±0.7	3.43±0.9	6.17±1.4	20.5±1.0**

AOM given at 15 mg/kg, subcutaneous injection (1 ml/kg). Data are represented as mean±standard error of the mean. Asterisks indicate values significantly different from those in group 2: * $p < 0.05$ and ** $p < 0.01$.

Discussion

Seaweeds are rich in bioactive substances that may be useful in treating a wide spectrum of diseases. Seaweeds contain health-promoting compounds such as phytochemicals,

soluble dietary fibers, lipids, peptides, and minerals that hold potential as dietary supplements and that may reduce the risk of cancer (16, 17). Moreover, macroalgae have been used as food supplements, particularly in Japan, China, and Korea (18). The long life expectancy of Japanese people has been

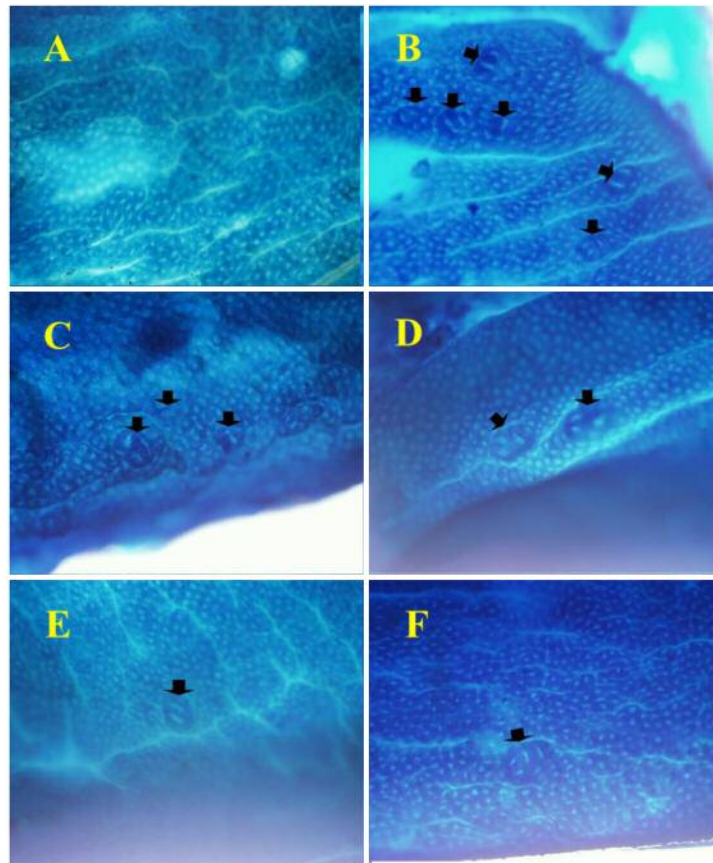


Figure 3. Histopathological sections, stained with methylene blue to identify crypts in the colonic tissues of F33 rats, and examined at 200 \times magnification. In group 1, we did not find formation of any aberrant crypt foci (ACF), while group 2 had the highest number of ACF; with groups 3-6 having significantly fewer crypts after dietary supplementation with different doses of *Capsosiphon fulvescens* (1 or 2%) or *Hizikia fusiforme* (2 and 6%), respectively. Arrows indicate ACF.

partly attributed to their dietary habits, including the regular consumption of seaweed (17).

Our experimental data suggest that *C. fulvescens* and *H. fusiforme* are effective chemopreventive agents for CRC. These chemopreventive effects may be due to the high levels of minerals such as Na, Mg, K, Ca, and Fe in both algae (15). AOM has the potential to induce colonic carcinomas in rats by promoting ACF formation. Carcinogenesis is conventionally defined by three stages: initiation, promotion, and progression (19, 20). During the initiation stage, normal cells experience DNA damage (21, 22). During the second stage, promotion, the initiated cells affect normal cells, forming preneoplastic lesions (23). Finally, in the progression stage, malignant tumors develop, leading to metastasis (24).

In the present study, we demonstrated that dietary administration of *C. fulvescens* and *H. fusiforme* caused no significant difference in body weight in groups 3-6 over the experimental period compared with that in groups 1

(untreated control group) and 2 (treated control group). Additionally, there was no significant difference in feed intake between any of the experimental groups. This suggests that *C. fulvescens* and *H. fusiforme* may both have beneficial effects (14) (Table III).

H&E staining of colonic tissues was carried out for histological analyses. Throughout the colonic mucosa of the AOM-treated rats, we observed abnormalities, consistent with molecular aberrations in the treated control group (group 2), but such histological abnormalities were not found in the experimental groups (Figure 2). This demonstrates that dietary *C. fulvescens* and *H. fusiforme* have chemopreventive effects.

The ACF assay evaluates crypts with abnormal morphology, such as those found in group 2. In groups 3-6, dietary administration of *C. fulvescens* and *H. fusiforme* resulted in significantly less ACF formation than in the treated control group (Figure 3), suggesting that these agents counteract the carcinogenic activities of AOM.

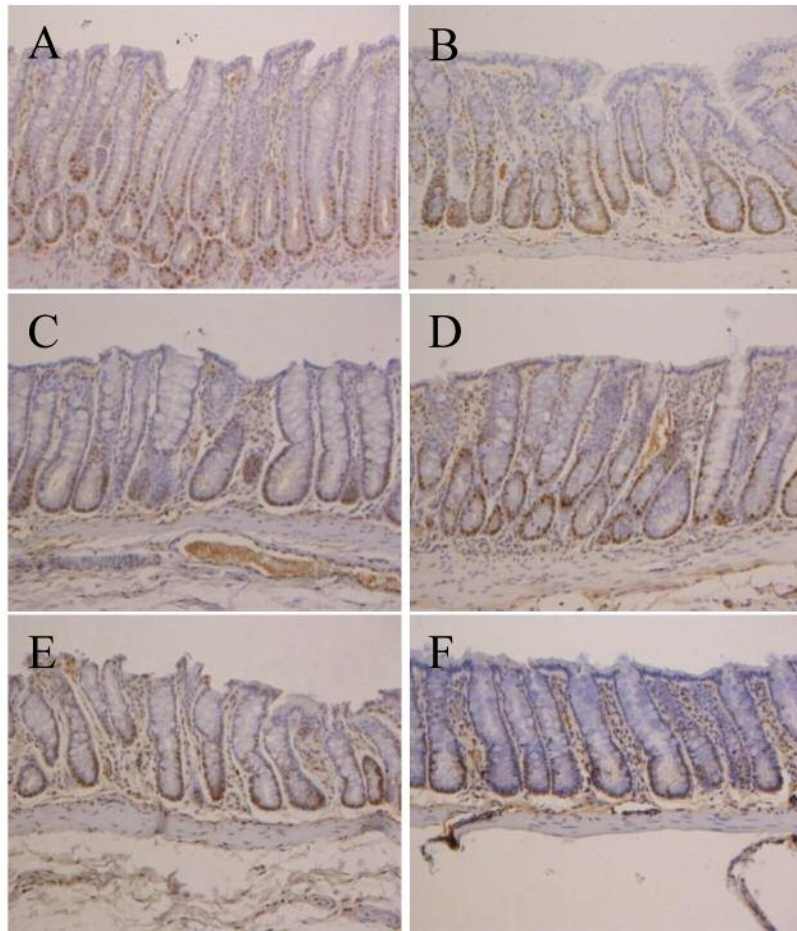


Figure 4. Immunohistochemical analysis of proliferating cell nuclear antigen (PCNA) in colonic tissue of F344 rats from groups 1-6. In group 1, we observed PCNA-negative cells (blue color); in group 2, we found PCNA-positive cells (brown color); but experimental groups 3-6 had fewer PCNA-positive cells than the treated controls (group 2). Colonic tissues were examined at 200× magnification.

Dietary administration of *C. fulvescens* and *H. fusiforme* prevented AOM-induced colorectal proliferative lesions in rats (Figure 4). Experimental groups exhibited pronounced suppression of cell proliferation compared with the treated control group. Groups 3-6 had significantly lower CRC PCNA labeling indices in comparison with that of group 2, suggesting that *C. fulvescens* and *H. fusiforme* might both modulate cell proliferation (Figure 5).

Although the mechanism of inhibition of AOM-induced ACF formation in rats by *C. fulvescens* and *H. fusiforme* has not been established, one possibility is through interference with the cytochrome P450 2E1 (CYP2E1) pathway, due to their potent antioxidant activity (14, 15, 25-27). It has been suggested that suppression of CYP2E1 inhibits chemical carcinogenesis (4, 28). *In vivo*, AOM is largely metabolized by isoform CYP2E1 (4, 29, 30). A previous study established that AOM-induced ACF formation was significantly lower in *Cyp2e1*, knockout mice (29). In the present study, we

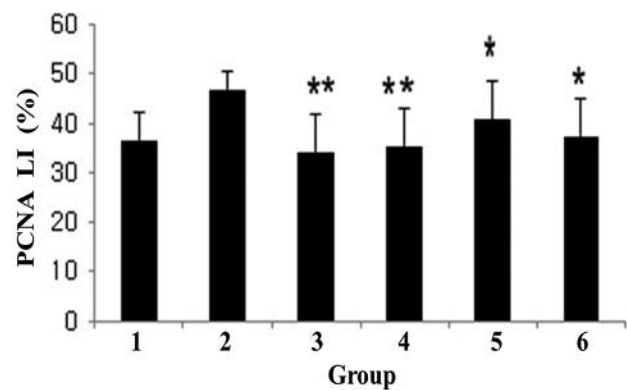


Figure 5. Proliferating cell nuclear antigen (PCNA) labeling index (%) of colonic mucosa in F344 rats. Group 1 was the untreated control, group 2 had the highest index, while groups 3-6 demonstrated the significant inhibitory effect of dietary *Capsosiphon fulvescens* (** $p < 0.01$) and *Hizikia fusiforme* (* $p < 0.05$) on PCNA labeling index compared to group 2. The data represent the mean \pm standard error of the mean.

demonstrated that dietary *C. fulvescens* and *H. fusiforme* both suppressed formation of AOM-induced ACF in rats.

In conclusion, many chemopreventive agents have been shown to exhibit inhibitory effects on cancer in preliminary studies. Our results clearly revealed the potential benefits of *C. fulvescens* and *H. fusiforme* at inhibiting ACF formation. However, the optimal dose for an anticancer effect needs to be identified. Further studies on determination of the optimum dose of *C. fulvescens* and *H. fusiforme* in inhibiting ACF formation are required.

Conflicts of interest

The Authors declare no conflict of interest in regard to this study.

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