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Chemopreventive Effects of Elm Tree Root Extract on Colonic Aberrant Crypt Foci Induced by 1,2-Dimethylhydrazine in F344 Rats

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Abstract

Cancer-preventive effects of ethanol extract of elm tree root (EEE) were investigated. In the *in vitro* cytotoxicity assay, colon cancer cells were incubated with a chloroform fraction of EEE (CF-EEE). CF-EEE significantly inhibited the proliferation of cells and induced apoptotic cell death in a dose-dependent manner. For the assessment of chemopreventive efficacy *in vivo*, male F344 rats were fed with EEE (0.5 or 1%) in diet for 8 weeks, and were subcutaneously injected with 1,2-dimethylhydrazine (DMH) to induce colonic aberrant crypt foci (ACF). EEE (0.5 and 1%) significantly decreased both the numbers of AC (1191.1/colon) and ACF (529.3/colon) induced by DMH. In addition, in the Western blot analysis on the colonic mucosa, administration of EEE triggered expression of caspase-3, a key factor of an apoptotic cascade. These results suggest that extract of elm tree root may have potential chemopreventive principles that lead to apoptosis of cancer cells, and thereby suppress colorectal carcinogenesis during the initiation stage.

Key words: elm tree root, colon cancer, aberrant crypt foci (ACF), apoptosis, caspase-3

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant cancers in Western countries, accounting for about 15% of all cancers. CRC is the second leading cause of cancer-related deaths in the USA. It has been estimated that more than 145,000 people contract and about 56,000 people die of this cancer per year (1). In Korea, the incidence and mortality of CRC has increased for 10 years, being the fourth leading cause of cancer deaths (2). Eighty to ninety percent of CRC cases occur sporadically. The major risk factor is a Western-style diet, which includes high consumption of meat and fat, and a low consumption of vegetables and fruits (3-5).

Every crypt is a self-contained proliferating unit (6). Gastrointestinal tract epithelium is characterized by active and rapid cell turnover that is closely related to high proliferative activity in basal cells. If the intestinal epithelium is exposed to a cytotoxic agent such as radiation, one of the first detectable responses will be the induction of apoptotic changes in some of the cells. The spatial distribution of those apoptotic cells along the crypt axis depends on the cytotoxic agent being used (7).

Colorectal cancer is the result of a series of genetic alternations which destroys normal mechanisms controlling cell growth (8,9). For years, a number of chemical carcinogens, 1,2-dimethylhydrazine (DMH), azoxymethane (AOM), 2-amino-3-methyl imidazole (4,5-*f*) quinoline (IQ), 2-amino-1-methyl-6-phenyl-imidazo (4,5-6) pyridine (PhIP), methylnitrosurea (MNU), *N*-methyl-*N*-nitro-N-nitrosoguanidine (MNNG), have been used to induce benign and malignant neoplasm in the colon of rodents. These agents have provided a reasonably good experimental model of human colon cancer (10). One of the chemicals, DMH, a potent and complete carcinogen, has been reliably used to induce initiation, and promotion steps of colon carcinogenesis in rodents. DMH and related compounds induce neoplasm specifically in rat colons, even after a single dose (11). Metabolic activation of DMH results in highly reactive electrophiles (methyldiazonium ion) in the liver and colon; however, the large intestine is the main target organ of DMH (12). Finally, hyperplastic proliferation induced by DMH makes aberrant crypt foci (ACF). ACF, a colon carcinoma precursor in humans and rats, is selected as one of the feasible tools, and as a sensitive, reliable,

and rapidly appearing biomarker supported by the presence of histopathological intra-epithelial preneoplasia. In the colonic mucosa, ACF has been hypothesized to represent precursor lesions of chemically induced colon cancer. Aberrant crypts can be identified by their increased size, thicker epithelial lining and increased pericryptal zone (13). Various studies have supported the concept that ACF are precancerous lesions that can be used as biologic end points of modulators of colon carcinogenesis (14-19). Pereira et al. (20) also demonstrated that the ACF might be a useful biomarker to detect possible effects of a chemopreventive agent in rat colon carcinogenesis.

Cancer can be prevented by either avoiding life style-related risk factors, such as smoking, a western diet, physical inactivity and carcinogen-containing foods, or alternatively increased exposure to beneficial influences and intake of chemopreventive agents (21). The latter will be particularly practical because they can be taken as supplements or by modulation of the current dietary status. Given difficulties in ensuring, any compound will not have any toxicity besides proven efficacy and convenience for use in the long term, the practical aim of chemoprevention should be focused those at high risk of cancer. Furthermore, there are advantages with application of natural compounds, so that regulatory approval can be facilitated (22,23). Colon cancer is one of the well-studied cancers but the progress in the field of preventing or curing this disease has not been significant. While there are chemotherapeutic drugs available for the treatment of this disease, the majority of the patients do not respond to these drugs and side effects remain problematic; Therefore, emphasis has been focused on a variety of clinical, and basic studies of chemoprevention using naturally occurring dietary substances, since they might provide useful strategies to inhibit colon cancer with minimal toxicity (24).

Elm tree (*Ulmus davidiana* var. *japonika* Nakai) is a widespread deciduous tree in Korea. The barks of stem and root of this plant have been used to treat inflammation, edema, mastitis and gastric cancer in oriental medicine literature for a long time (25). In the elm tree, the efficacy of root barks was recorded in a Korean traditional book, *Botanical List*, *Donguibogam* etc. Recently, was reported that *U. davidiana* Panch, a kind of Ulmaceae, has a strong anti-oxidative activity on lipid peroxidation and an inhibitory effect on an endogenous nitric oxide-induced apoptotic cell death (26). For a decade, it has been known that plant glycoproteins have biological active functions, including an anti-oxidative effect and an enhancing effect on immune activity in several cells (27-29). Although the stem and root barks of the elm tree have been used in oriental traditional medicine for many diseases, mechanisms of action of this species are rarely understood. Especially, there is little study about cancer prevention and only a few studies about proliferation of cancer cell lines (30). Therefore, it is important to understand how this plant extract performs disease-preventive action *in vivo*. The purpose of this study was to investigate the effects of root bark extracts of the elm tree on colon cancer cell proliferation and DMH-induced colonic ACF formation. In addition, this study also examined its effects on apoptosis and suggested possible mechanisms by these compounds induced apoptosis.

MATERIALS AND METHODS

Extraction of root bark of elm tree

Root bark of elm tree was purchased from the Eoleumgol Agricultural Cooperative Federation in Chonnam, Korea. Fresh root bark was dried in a dark well-ventilated place. The air-dried root bark was milled and extracted three times with 80% ethanol at $25^{\circ}C$, 150 rpm under reflux. The ethanol was filtered through Whatman filter paper (No. 2) and concentrated with a rotary evaporator (EYELA N-11, Tokyo, Japan) at $40^{\circ}C$. Then, the extract was further fractionated with absolute ethanol, distilled water and chloroform (0.1:0.9:1.0). After the solvent was removed by using a rotary evaporator, the chloroform fraction from ethanol extract of elm tree (CF-EEE) was used *in vitro* assay.

For the *in vivo* assay, the root bark was milled and extracted three times with 80% ethanol at $25^{\circ}C$, 150 rpm under reflux. The ethanol extract of elm tree (EEE) was filtered through Whatman filter paper (No. 2) and concentrated with a rotary evaporator (EYELA N-11, Tokyo, Japan) at $40^{\circ}C$.

Cell viability tests

Cell culture: SW 480 cells, a human colon cancer cell line, were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). SW 480 cells were incubated in RPMI 1640 medium containing 10% fetal bovine serum (FBS; Gibco, NY, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, NY, USA) at $37^{\circ}C$ and 5% CO2 atmosphere. The medium was renewed three times per week. Cells were harvested and processed for analyses of cytotoxicity and apoptosis.

Cytotoxicity by 3-(4,5-dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide (MTT) assay: To assess the cytotoxicity of CF-EEE to the cancer cells, MTT dye reduction assay was performed. Cells were plated out at

a density of 2×10^5 cells per mL into 96-well micro-plates and allowed to attach overnight. After 24 hr, the cells were treated with various concentrations (10, 25, 50, 100, 200, 500 or 1000 μg/mL) of CF-EEE for 24 hr, and were then analyzed using the MTT assay as follows: 50 μL of MTT (2 mg/mL in phpspophate-buffered saline (PBS); Sigma Co., USA) were added to each well, and further incubated at 37°C for 4 hr. After the incubation, 150 μL of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formed formazan crystals. The absorbance was recorded at 540 nm on a microculture plate reader (Benchmark, Germany). Wells containing only RPMI 1640, FBS and MTT were used as the controls. The optical density (O.D.) was measured and percent viability was calculated as (O.D. of drug-treated sample/O.D. of non-treated sample) × 100.

4,6-diamino-2-phenylindole (DAPI) staining: Apoptotic cells were identified by DAPI staining. After overnight culture in an 8-well plate (4×10^5 cells/mL), SW 480 cells were treated with various concentrations (50, 100, 200 or 500 μg/mL) of CF-EEE in fresh culture medium at 37°C for 24 hr. The cells were then fixed with 500 μL fix solution (acetic acid : methanol=1:3) for 5 min, dried, and stained with the DNA specific fluorochrome DAPI (2 μg/mL, Sigma Co., USA). Following 10 min of incubation, cells were washed with PBS, air-dried, mounted with 90 % (v/v) glycerol and observed under a fluorescence microscope at ×200 magnification (Olympus Optical Co., Japan).

Colon cancer prevention bioassay

Animal experiment: Seven week-old male F344 rats were obtained from Japan SLC Inc. (Shizuoka, Japan). Three or four rats per cage were housed in polycarbonate cages with hardwood bedding. They were fed with Teklad Global 18% protein rodent diet (Harlan, Indiana, USA). The animal room was maintained as follows; a 12-hr light/dark fluorescent light cycle, 10-times room air changes per hr, 21~24°C and 35~65% relative humidity. All animal experiments were performed in accordance with Standard Operation Procedures of Laboratory Animals that were approved by Institutional Animal Care and Use Committee (IACUC) of Laboratory Animal Research Center in Chungbuk National University (Cheongju, Korea).

Experimental design: The animals were randomly allocated into five groups (Fig. 1): Groups 1~3 had been given 4 subcutaneous injections of DMH (30 mg/kg, Sigma-aldrich Korea Co., Yongin, Korea) five times for two weeks; Groups 4 and 5 received similar injections of vehicle (1 mL/kg of saline). From the 1st initiation injection of colon carcinogenesis with DMH, the animals
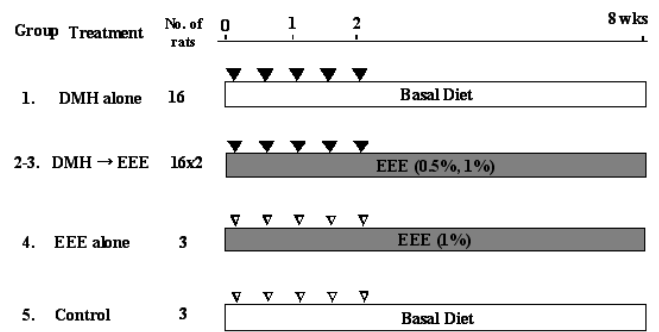

Fig. 1. Protocol of rat colon carcinogenesis. DMH, 1,2-dimethylhydrazine (30 mg/kg, 5 times for 2 weeks); EEE, ethanol extract of elm tree in diet; ▼, injection of DMH; ▽, injection of saline.

were fed on basal diet alone (Groups 1 and 5), containing 0.5% (Group 2) or 1% (Groups 3 and 4) EEE for 8 weeks. Food consumption was measured twice a week throughout the experimental period. The animals were weighed once a week. At the end of experiment at week 8, all animals were sacrificed.

AC and ACF counts: The number of ACF was counted according to Bird's criteria (14). The entire colon was removed, longitudinally opened, rinsed with 0.85% NaCl solution and fixed in 10% neutral phosphate buffered formalin for 24 hr. For ACF counting, proximal, middle and distal colons were stained with 0.5% methylene blue for 30 sec. In each ACF, the number of ACF/colon and the number of aberrant crypts (AC) were counted under a light microscope at ×40 magnification.

Western blot analysis: The colon was removed, and the mucosa was scraped from the underlying tissue with a blade. To determine apoptosis levels, the mucosa was homogenized and solubilized for 30 min at 4°C with lysis buffer containing 20 mmol/L Hepes, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 100 mmol/L NaF, 10 mmol/L sodium pyrophosphate and 1 mmol/L Na_3VO_3. The following protease inhibitors were used: 20 g/mL aprotinin, 10 g/mL antipain, 10 g/mL leupeptin, 80 g/mL benzamidine HCl and 0.2 mmol/L phenylmethylsulfonylfluoride (PMSF). The insoluble material was removed by centrifugation at 12,000 rpm for 20 minutes, and protein content was determined using the Bradford protein assay as previously described. The lysates (80 μg of protein) were resolved on one sodium dodecy sulfate-12% poly-acrylamide gel (SDS-PAGE) and transferred onto nitrocellulose membrane (Bio-Rad Laboratories Inc., CA, USA). The blots were blocked for 1 hr in 5% skim milk in TNT (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl and 0.1% Tween 20) solution and incubated overnight with either caspase-3 antibody (1:1,000, #9662, Cell

Signaling Technology Inc., MA, USA) or β-actin (1:1,000, #4970, Cell Signaling Technology Inc., USA). The blots were incubated with anti-rabbit horseradish peroxidase-conjugated antibody (1:1,000, #7074, Cell Signaling Technology Inc., MA, USA). Signals were detected by using the enhanced chemiluminescence method using WEST-ONE (iNtRON Biotechnology, Seongnam, Korea) and exposure to X-ray film.

Statistical analysis

All results were expressed as the mean ± standard deviation. One-way ANOVA and Dunnett's t-test using SPSS v 10.0 software (SPSS Inc., IL, USA) was applied, and differences with $p < 0.05$ were considered significant.

RESULTS

Cell viability

Cytotoxicity of CF-EEE: In order to examine the antiproliferative effects, SW 480 cells were treated with various concentrations (5~1000 μg/mL) of CF-EEE. After 24 hr, MTT analysis was carried out and was compared with the values obtained from the controls. However, these values are expressed as % survival.

As shown in Fig. 2, CF-EEE reduced the viability of SW 480 cells in a concentration-dependent manner, showing significant cytotoxic effect at concentrations higher than 200 μg/mL ($p < 0.01$).

Apoptotic activity of CF-EEE: To assess the features of SW 480 cells exposed to CF-EEE 100 or 200 μg/mL, and morphological changes of the cells were detected by DAPI staining. After 24 hr treatment, enhanced DNA fragmentation and apoptotic bodies were observed in a concentration-dependent manner (Fig. 3).
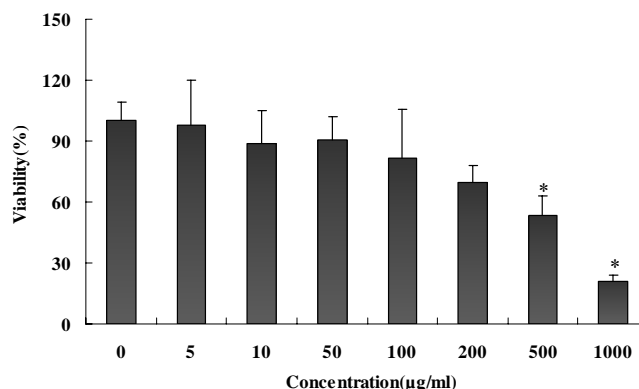

Fig. 2. Cytotoxic effect of chloroform fraction of ethanol extract of elm tree (CF-EEE) on SW 480 cells. The cells were treated with various concentrations of CF-EEE and after 24 hr, the cellular survival rate was measured using MTT assay. Data are the means ± standard deviation (n=4). *Significantly different from the control ($p < 0.01$).

Colon cancer prevention

General observations: All the rats survived the DMH challenge in good condition until the scheduled sacrifices. DMH or EEE alone did not affect body weight, but combination treatment of EEE with DMH decreased the body weight gain of rats (Fig. 4). The initial body weight gain during 2-week DMH treatment was markedly reduced by EEE.

Such decreases in the body weight gain might be due to the significant reduction of feed consumption in the groups administered with DMH and EEE (Table 1). However after 2 weeks, the feed intake and body weight gain were reduced to the control levels (Fig. 5 and Table 1).

There were no differences in either absolute or relative organ weights between control and DMH and/or EEE-
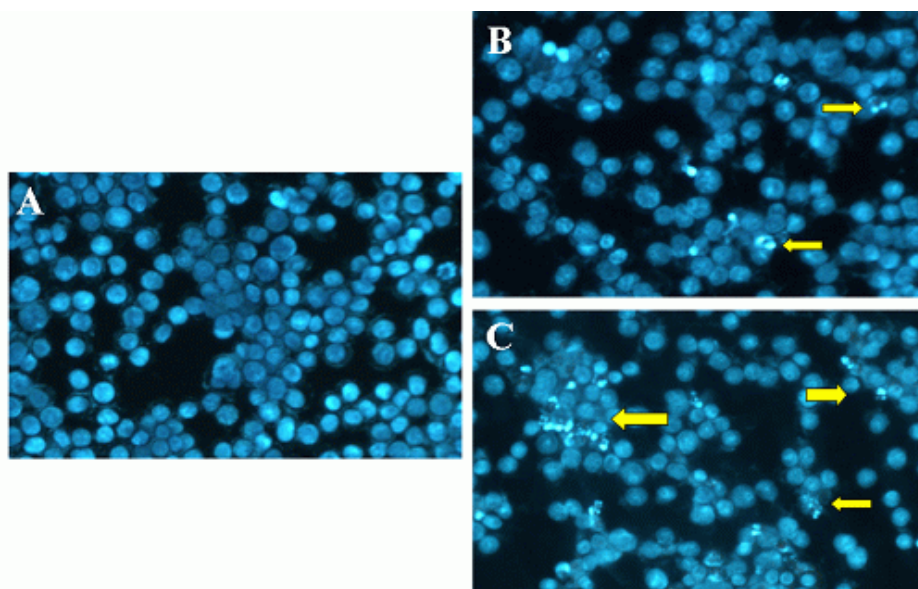

Fig. 3. Morphological analysis of SW 480 cells treated with chloroform fraction of ethanol extract of elm tree (CF-EEE). The cells were treated with 100 or 200 μg/mL for CF-EEE 24 hr, stained with DAPI and analyzed under a fluorescence microscope at ×200 magnification (arrows shows apoptotic bodies). A, control; B, 100 μg/mL CF-EEE; C, 200 μg/mL CF-EEE.
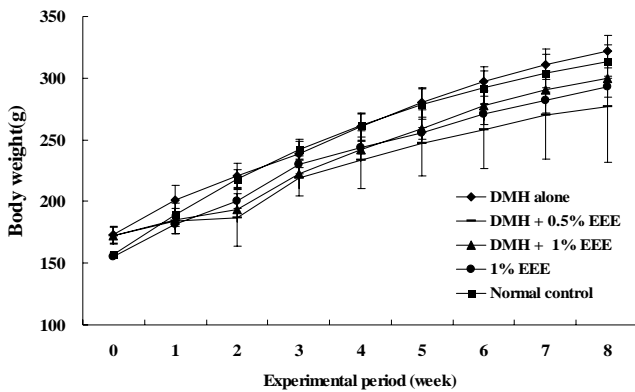
Fig. 4. Changes in body weights of rats treated with 1,2-dimethylhydrazine (DMH) and/or ethanol extract of elm tree (EEE).
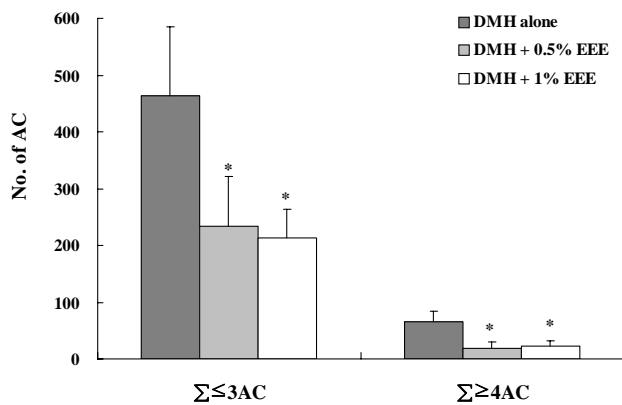

Fig. 5. Effect of ethanol extract of elm tree (EEE) on colonic aberrant crypt (AC) induced by 1,2-dimethylhydrazine (DMH). *Significantly different from the value of DMH alone (p< 0.01).

treated groups (data not shown). No gross lesions were observed in any organs of any groups, including the colon.

AC and ACF counts

Repeated doses (5 times during 2 weeks) of DMH (30 mg/kg) induced diverse ACF in 8 weeks, leading to 464.2 small ($\leq$AC) and 65.1 large ($\geq$AC) ACF (Table 2). The numbers of total ACF and AC per colon were 529.3 and 1,191.1, respectively. Treatment with 0.5% EEE reduced the ACF and AC induction by DMH to half of control levels (Figs. 5 and 6). Noteworthy, the large ACF was more greatly inhibited by EEE. Interestingly, the DMH-induced ACF was further suppressed by 1% EEE.

Caspase-3 expression: In order to investigate the mechanisms by which EEE extracts evoke apoptosis and proteins isolated from colon mucosa of the animals fed EEE were subjected to western blot analysis of caspase-3, an executor in cell apoptosis. As shown in Fig. 7,
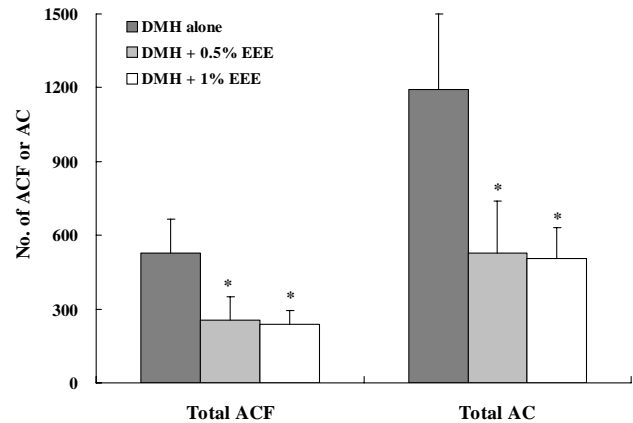

Fig. 6. Effect of ethanol extract of elm tree (EEE) on total colonic aberrant crypt foci (ACF) and aberrant crypt (AC) induced by 1,2-dimethylhydrazine (DMH). *Significantly different from the value of DMH alone (p<0.01).

Table 1. Daily feed consumption (g) of rats treated with 1,2-dimethylhydrazine (DMH) and/or ethanol extract of elm tree (EEE)

Treatments \ Weeks	1	2	3	4	5	6	7	8
DMH alone	15.99±0.81	15.96±0.81	17.10±1.24	19.12±0.57	19.73±0.75	19.68±0.80	18.89±0.89	18.61±0.82
DMH→0.5% EEE	8.09±4.04**	14.21±5.08	19.73±1.96*	16.77±5.84	17.41±6.42	17.40±6.25	16.92±6.28	16.03±6.25
DMH→1% EEE	7.39±2.15**	15.41±3.27	19.37±0.71	17.94±1.24	18.98±0.92	19.62±1.84	17.98±1.35	17.88±1.49
1% EEE alone	15.00	18.82	18.45	17.92	19.20	18.96	19.37	18.62
Normal control	17.09	17.74	18.59	18.38	20.20	19.46	19.82	19.88

*Significantly different from those of DMH alone (p<0.05). **Significantly different from those of DMH alone (p<0.01).

Table 2. Changes in aberrant crypt (AC) and aberrant crypt foci (ACF) in rats treated with 1,2-dimethylhydrazine (DMH) and ethanol extract of elm tree (EEE)

Treatments	No. of rats	No. of total ACF		Total ACF/colon (%)	Total AC/colon (%)
		$\Sigma\leq$3AC	$\Sigma\geq$4AC		
DMH alone	16	464.2±121.3	65.1±19.6	529.3±138.5 (100)	1,191.1±310.0 (100)
DMH → 0.5% EEE	16	233.9±87.8*	19.2±11.1*	253.1±97.0 (47.8)*	525.8±214.7 (44.1)*
DMH → 1% EEE	16	214.0±49.3*	23.2±9.1*	237.2±55.0 (44.8)*	507.8±122.9 (42.6)*

*Significantly different from those of DMH alone (p<0.01).

| DMH | - | - | + | + | + | + | + | + | - |
| EEE (%) | 0 | 0 | 0 | 0 | 0.5 | 0.5 | 1 | 1 | 1 |

Caspase-3   ← 35 kDa
             ← 17 and 19 kDa
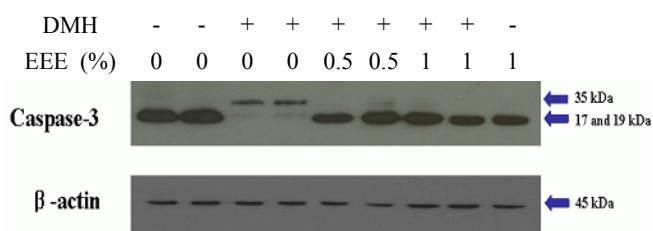
β-actin   ← 45 kDa

Fig. 7. Expression of caspase-3 detected by in colon mucosa treated with 1,2-dimethylhydrazine (DMH) alone or in combination with ethanol extract of elm tree (EEE). All samples in gels are equally loaded at 80 μg of total protein and β-actin as an internal control for equal protein loading.

activated caspase-3 (17 and 19 kDa) was expressed in normal mucosa. In contrast, the expression of activated caspase-3 was fully suppressed by DMH treatment. However, 1.5% and 1% EEE greatly reverse the suppression of activated caspase-3 expression.

DISCUSSION

Colon cancer arises from normal cells as a consequence of multistep carcinogenesis and the majority of cases occur sporadically caused by non-inherited factors like combinations of diet and other environmental factors (31). The multistage process is actually an accumulation of multiple genetic defects in somatic cells, which may be influenced by several dietary factors (32). The concern over the risk of surgical procedures and toxicity of chemotherapeutic drugs has led to a focus on alternative avenues for colon cancer prevention. Chemoprevention seems to be a promising strategy because other therapies have not been found to be effective in controlling either the high incidence, or low survival rate of colon cancers. Recently, emphasis has been on a variety of clinical and basic studies of chemoprevention using naturally occurring substances that are found in normal diets, since they might provide useful strategies to inhibit colon cancer with minimal toxicity. Moreover, modifications in dietary habits appear to be particularly influential in the prevention of colon cancer (33). Therefore, the present study was undertaken to evaluate the chemopreventive efficacy of EEE. The elm tree, especially its root barks traditionally used in Korea, is well known for its medicinal properties with varied pharmacological functions. Although elm tree has many medical effects; there are only a few studies of its biological activity. There were a few studies on the functionalities of some components several years ago (34, 35), and recently, it has been reported that glycoprotein isolated from the elm tree has an immune activity and anticancer properties (36-40).

In order to screen for cytotoxic or apoptotic activities, this study initially investigated whether CF-EEE inhibits cell proliferation or induces DNA fragmentation *in vitro* (Figs. 2 and 3). This cytotoxicity result is in agreement with a previous study showing that EEE significantly inhibits proliferation of several cancer cells (30). The colorimetric MTT assay showed that cell numbers producing formazan were decreased with increased doses of CF-EEE, in which 46.8% and 79.0% cytotoxicity were observed with 500 and 1000 μg/mL, respectively. Also, typical morphological changes of apoptosis were observed in CF-EEE-treated cells (Fig. 3). Apoptotic bodies defined by condensed nuclei were revealed by DAPI staining in a dose-dependent manner.

ACF were first reported in rodents injected with AOM by Bird in 1987 (13), and similar lesions were characterized in humans in 1991 and 1994 by Pretlow (41, 42). Thereafter, the ACF model has been the most widely used animal model system for evaluating naturally occurring compounds (flavonoids, carotenoids, green tea, etc.) as well as synthetic chemicals (cyclooxygenase-2 (COX-2) inhibitors, nitric oxide synthase (NOS) inhibitors and peroxisome proliferators-activator receptor (PPAR) γ) for their colon cancer chemopreventive efficacies. The growth dynamics and morphological and molecular features of ACF support the contention that ACF are putative preneoplastic lesions. For instance, ACF have a hyperproliferative epithelium and their size increases with time (43-45). The nuclear atypia observed in some ACF are similar to those seen in the crypts of adenocarcinomas in colons (46). Furthermore, identification of dysplasia and monoclonality strongly links this lesion to neoplastic progression (47). In this study, EEE supplementation at 0.5 and 1% doses to DMH-treated rats significantly reduced the number of ACF, AC and crypt multiplicity (Table 2, Figs. 5 and 6). Colon tumor incidence in rats correlates best with multicrypt ACF ($\geq$4 crypts/focus), which are more likely to persist, increase in size through multiplication and develop into tumors. In the present study, EEE (0.5 and 1%) were significanctly effective at reducing not only $\geq$4 crypts/focus but also $<$3 crypts/focus. This suggests that EEE is able to exert a chemopreventive effect on preneoplastic ACF development.

In this study, the initial body weight gain of rats treated with EEE in combination with DMH was significantly lower than those of animals treated with DMH alone (Fig. 4). At first, it was doubtful whether EEE has a toxic substance, but the transient decrease in body weight gain was found to be due to a decrease in feed consumption (Table 1); However, there are not any sig-

nificant differences either body weight or feed consumption in EEE alone group (Fig. 4 and Table 1). In addition, there were no significant differences in absolute and relative organ weights between rats treated with DMH alone and in combination with EEE. Furthermore, no macroscopic microscopic lesions were apparent in any organs, including the colon of animals in any groups. In blood biochemical analysis, there were no differences between groups (data not shown). So, it is suggested that EEE caused a transient decrease in feed consumption in DMH-challenged rats, leading to reduction of body weight gain.

Modulation of apoptosis provides a protective mechanism against intestinal neoplasia. Our data shows that the EEE significantly reduced the incidence of ACF. This regression of precancerous lesions induced by treatment with EEE provides indirect evidence that EEE can arrest cell growth or stimulate apoptosis. Apoptosis is a key physiological event responsible for the elimination of unwanted cells during the process of development and the removal of self-reactive lymphocytes (48). Efficient removal of these cells is important to maintain morphogenesis and homeostasis and is achieved by an intrinsic mechanism that ultimately results in cellular suicide (49). Indeed, it is well documented that AIDS, stroke, Alzheimer's diseases, Huntington's disease, amyotrophic lateral sclerosis, traumatic brain injury and spinal cord injury are associated with excess apoptosis, whereas cancers, systemic lupus erythematosus, multiple sclerosis, diabetic mellitus and rheumatoid arthritis are due to inadequate apoptosis of specific cells (50-54). The distinct morphological, and biochemical features of apoptosis include the collapse of the nucleus due to chromatin condensation, the formation of globular apoptotic bodies, increased transglutaminase activity and genomic DNA fragmentation (55). A family of specific cysteine proteases, caspases, plays a key role in the apoptotic response (56). Caspases are expressed as inactive proenzymes (procaspase) in living cells and become activated when cells receive an apoptosis-inducing signal. Once activated, they cleave a number of key substrates, resulting in their activation or inactivation (57). Among several players responsible for these features, proteolytic cleavage, and activation of caspase-3 may be functionally important in the induction of apoptosis (55). In order to elucidate the mechanism by which EEE induce apoptosis, proteins isolated from the colon scraping were analyzed by western blotting. In the present study, caspase-3 was activated in normal mucosa, but it was decreased when treated with DMH. However, following treatment with EEE, active caspase-3 was expressed as seen in normal mucosa. The results from this experiment revealed that EEE were able to increase activated caspase-3 and induce apoptosis.

Caspase-dependent processes are associated with two pathways of anti-cancer drug-induced apoptosis, a death receptor-dependent and a mitochondria-dependent pathway (58). The death receptor activation pathway is mediated with a death-inducing signaling complex, which is made of a Fas-associated death domain and a procaspase-8, activating caspase-8 (59). Caspase-8 directly activates caspase-3 and leads to apoptosis. The mitochondria-dependent pathway involves cleavage of proapoptotic proteins by caspase-8 releasing cytochrome c in the mitochondria, Related cytochrome c activates caspase-3 and caspase-9 (60) including the apoptosis with morphological changes (61). The activation and inactivation of caspases are regulated by various proteins, ions and other factors, such as Integrin Associated Protein (IAP), bcl-2 family proteins, calpain, Ca^{2+} and cytokine response modifier A (Crm A) (62). The members of the bcl-2 family are a group of crucial regulatory factors in apoptosis. According to functional and structural criteria, the members can be divided into two groups: anti-apoptotic genes and pro-apoptotic genes. Bcl-2, an anti-apoptotic gene, is known for regulating the apoptotic pathways and protecting against cell death, whereas bax, a pro-apoptotic gene of the family, is expressed abundantly and selectively during apoptosis, promoting cell death (62). It has been suggested that the bcl-2 to bax ratio determines survival or cell death. Consequently, the mechanism of how to activate caspase-3, for example, via caspase-8 or caspase-9, in addition to the bcl-2 to bax ratio, needs to be further investigated.

Our results suggested that EEE constituents may exert significant and potentially beneficial effects on decreasing the amount of precancerous lesions by inducing apoptosis in the large bowel. The ability of EEE to decrease the incidence of ACF, along with the ability of reducing crypt multiplicity to induce apoptosis in the region of the colon where most of these preneoplastic lesions accumulate, suggests that EEE could be a candidate for chemoprevention of colorectal tumors.

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