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Effect of dietary caraway (*Carum carvi* L.) on aberrant crypt foci development, fecal steroids, and intestinal alkaline phosphatase activities in 1,2-dimethylhydrazine-induced colon carcinogenesis

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Abstract

Colon cancer is one of the most common malignancies in many regions of the world and is thought to arise from the accumulation of mutations in a single epithelial cell of the colon and rectum. Caraway (*Carum carvi* L. Umbelliferae) is a shrub with a long history as a medicinal plant since ancient times. The effect of different doses of caraway (CC) on the formation of aberrant crypt foci (ACF) and the levels of fecal bile acids, neutral sterols, and alkaline phosphatase (ALP) activities were studied in 1,2-dimethylhydrazine (DMH)-induced colon cancer in rats. Animals were randomized into 6 groups. Group 1 served as control, and group 2 received 90 mg/kg body weight caraway orally everyday. Groups 3–6 rats were given subcutaneous injections of DMH (20 mg/kg body weight) once a week for the first 4 weeks to induce ACF. Rats in groups 4–6, in addition to DMH injections, received caraway at 30, 60, and 90 mg/kg body weight respectively p.o. everyday until the end of whole experimental period of 15 weeks. Caraway supplementation significantly reduced ACF development and also decreased the levels of fecal bile acids, neutral sterols, and tissue ALP activities. The histological alterations induced by DMH were also significantly improved. Overall, our results showed that all 3 doses of caraway inhibited tumorigenesis though the effect of the intermediary dose of 60 mg/kg body weight was more pronounced.

Keywords: Aberrant crypt foci; Bile acids; Caraway; Colon cancer; 1,2-Dimethylhydrazine

Introduction

Colon cancer is one of the most common malignancies in many regions of the world (Shike et al., 1998) and is thought to arise from the accumulation of mutations in a single epithelial cell of the colon and rectum (Fearon and Vogelstein, 1990). Aberrant crypt foci (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 intraepithelial neoplasia (Bird, 1987). Pereira et al. (1994) also demonstrated that the ACF might be a useful biomarker to detect possible effects of a chemopreventive agent in rat colon carcinogenesis.

In the past several years, a number of chemical carcinogens such as 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, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine have been used to induce benign and malignant neoplasm in the colon of the rodents. These agents have provided a reasonably accurate experimental model of human colon cancer (Weisburger, 1971). One such chemical 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 colon of rat even after a single dose (Ward, 1974). Metabolic activation of DMH to highly

Abbreviations: ACF, aberrant crypt foci; ALP, alkaline phosphatase; b.w., body weight; CMC, carboxymethylcellulose; DMH, 1,2-dimethylhydrazine; H and E, hematoxylin and eosin; PBS, phosphate-buffered saline.

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reactive electrophiles (methyldiazonium ion) occurs in the liver and colon. However, the main target organ of DMH is the large intestine (Brady et al., 1998).

Bile acids (Reddy et al., 1977a) and neutral sterols (Korpela et al., 1988), a family of steroidal compounds, secreted into the intestine after ingestion of a high fat diet, are known promoters of pancreatic, liver, esophageal, gastric, and colon cancer. Increased levels of bile acids in the colon may mediate the promotion of colon cancer by high fat intake. A correlation has been found between high fecal concentration of bile acids and colon cancer (Shike et al., 1998). Reddy et al. (1977b) have also shown that high amounts of fecal neutral sterols are excreted in colorectal cancer patients.

Alkaline phosphatase (ALP) is a non-specific phosphomonoester hydrolase that catalyses the hydrolysis of wide variety of organic monophosphatases (Mestrovic and Pavela-Vrancic, 2003). Previous studies in Caco-2 cells, a cell line derived from human adenocarcinoma and inflamed rat colon expressed high levels of ALP (Mulivor et al., 1978). Thus, activity of ALP parallels the epithelial damage (epitheliolysis) and may also be used as a membrane marker (Yoshida et al., 1998).

Ayurveda is an ancient form of Indian medicine, which deals with plants and plant products. This indigenous form of medicine uses the active ingredients present in plants for treating diseases (Nair and Mohanan, 1998). Caraway (*Carum carvi* L. Umbelliferae) is a shrub with a long history as a medicinal plant since ancient times (Hartmans et al., 1995). The known main constituents of caraway have been demonstrated to be carvone (40–60%), limonene, carveol, dihydrocarveol, thymol in addition to glucosides and flavanoids (Matsumura et al., 2002). The constituents of caraway possess potent chemopreventive properties, but little has been considered for its possible use as a colon cancer preventive agent.

Therefore, this study was undertaken to determine the effects of desculated doses of caraway against DMH-induced formation of ACF. The levels of fecal bile acids, neutral sterols, and intestinal alkaline phosphatase activities in association with ACF induced by DMH were also examined.

Materials and methods

Animals. A total of 72 male albino Wistar rats were obtained from Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University. The animals were kept in polypropylene cages (4 per cage) and fed standard pellet diet for 1 week. Thereafter, the animals were randomly divided into six groups each containing 12 rats and maintained under controlled conditions of temperature (24 ± 2 °C), humidity (50 ± 10%), and 12-h light/dark cycle. Commercial pellet diet containing 4.2% fat (Hindustan Lever Ltd., Mumbai, India) was powdered and mixed with 15.8% peanut oil making a total of 20% fat in the diet (Table 1). This modified powdered pellet diet was fed to rats throughout the experimental period of 16 weeks (including 1 week for acclimatization), and tap water was provided ad libitum. The animals were cared for incompliance with the principles and guidance of Ethical Committee for Animal Care of Annamalai University in accordance with the Indian National Law on Animal Care and Use (Reg. no.160/2004/CPCSEA).

Table 1
Composition of the diet

	Commercial diet 84.2%	Peanut oil 15.8%	Total
Protein	17.7	–	17.7
Fat	4.2	15.8	20.0
Carbohydrates	50.5	–	50.5
Fiber	3.4	–	3.4
Minerals	6.7	–	6.7
Vitamins	1.7	–	1.7

Tumor induction. DMH, obtained from Sigma Chemical Company, St. Louis, MO, USA, was dissolved in 1 mM EDTA, adjusted to pH 6.5 with 1 mM NaOH and administered subcutaneously in the right thigh at a dose of 20 mg/kg b.w., once a week for the first 4 weeks. All other chemicals were of high grade unless otherwise specified.

Chemopreventive agent administration. Caraway seeds were purchased from local supermarket (Chidambaram, India), dried, powdered, and used in the experiment. Caraway suspension was prepared in 1% carboxymethylcellulose (CMC) just before treatment. The low dose of caraway used here was based on the average daily intake of humans (Blumenthal et al., 1998).

Treatment schedule. The experimental design is shown in Fig. 1. Rats were randomly assorted to 6 experimental groups (12 rats/group): all the rats received a modified powdered pellet diet (20% high fat). Group 1 served as control, group 2 rats were given intragastric gavage of 90 mg/kg b.w., caraway suspended in CMC; groups 3–6 were injected with DMH (s.c. injections, 20 mg/kg b.w.,) once a week for first 4 weeks. In addition, groups 4–6 rats received 30, 60, and 90 mg/kg b.w., of caraway in 1% CMC everyday orally respectively for the total duration of 15 weeks. Body weight (every week) and food consumption (everyday) were recorded, and at the end of experimental period, all the rats were sacrificed under ketamine hydrochloride (30 mg/kg b.w., i.p.) anesthesia. In each group out of 12 rats, 6 rats were used for ACF analysis, and the remaining 6 rats were used for histopathological studies.

Pathological analysis of ACF and colon tumor. ACF were counted by the method of Bird (1995a). The colon was flushed with cold phosphate-buffered saline (PBS), opened along the longitudinal median axis, and fixed flat between 2 pieces of filter paper in 10% neutral-buffered formalin for 24 h. The colonic tissues were stained with 0.2% methylene blue in PBS for 2–3 min. They were then placed on a microscopic slide and observed through light microscope at 40× magnifications. To determine crypt multiplicity, the number of crypts in each focus was recorded. The multiplicity of ACF was expressed as the number of aberrant crypts (AC)/focus. To obtain additional information about the morphology of ACF within the colonic epithelium, sections containing ACF were marked and stained with hematoxylin and eosin (H&E) after processing.

Determination of bile acids and neutral sterols. Stool samples were collected once in 3 weeks during the period of study and were homogenized with equal volumes of water and lyophilized to a fine powder. Fecal bile acids and neutral sterols were extracted and estimated by the method of De Wael et al. (1977).

Assay of alkaline phosphatase activity. Fecal water was obtained by mixing 0.3 g feces with 0.7 g distilled water, centrifuged for 10 min at 12,000 rpm. Activity of alkaline phosphatase (ALP, EC.3.1.3.1), a membrane marker of differentiation, was measured in the fecal water using *p*-nitrophenyl phosphate as substrate (Lapre et al., 1993a). ALP activity is defined as the amount of enzyme that will produce 1 μmol *p*-nitrophenol per minute, and the protein content in the samples was estimated by the method of Lowry et al. (1951).

Statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA), and a significant difference among treatment groups was evaluated by Duncan's Multiple Range Test (DMRT). The results were considered statistically

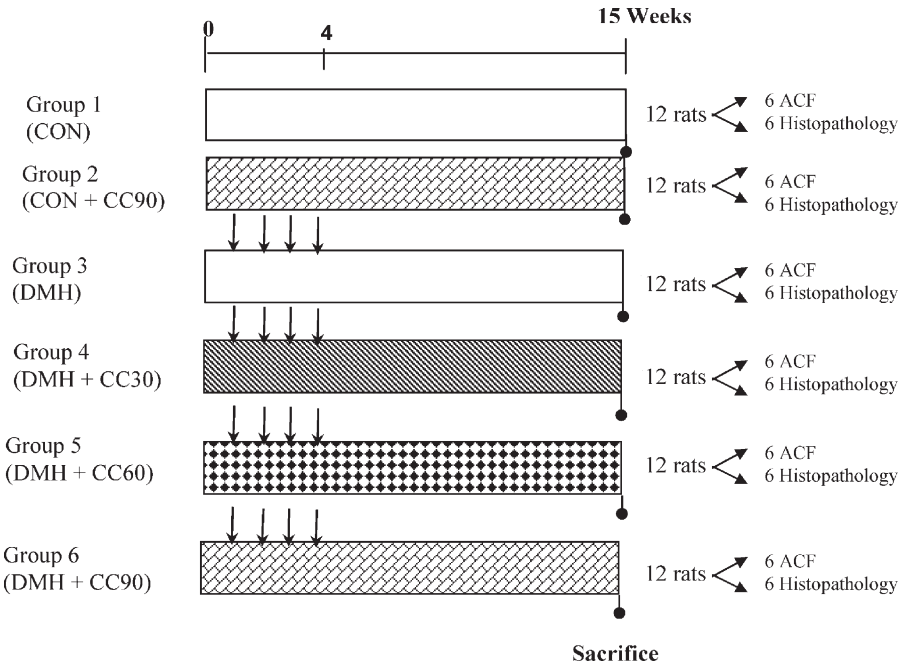

Fig. 1. Experimental protocol. ↓, 1,2-dimethylhydrazine (20 mg/kg b.w. s.c. injection). ▩—30 mg/kg body weight caraway; ▦—60 mg/kg body weight caraway; ▨—90 mg/kg body weight caraway.

significant at $P < 0.05$. All statistical analyses were made using SPSS 11.0 software package (SPSS, Tokyo, Japan).

Results

General observations

During the experiment, no difference was noted in the final body weights (g), body weight gain (g), food intake (g/day), and food efficiency (body weight gain [g/day]/food intake) between treated and corresponding control groups (data not shown). During the experimental period, no clinical signs of toxicity were observed in any of the groups.

ACF

Table 2 summarizes ACF incidence, total ACF, number of AC/ACF (crypt multiplicity), and percentage inhibition of ACF in experimental groups. A statistically significant ($P < 0.01$) reduction in total ACF, number of AC, AC/ACF were observed only in 60 mg/kg b.w., caraway supplemented animals (group 5) ($P < 0.01$). Percentage inhibitions of ACF in caraway-supplemented animals were 8.05, 53.4, and 14.2% in groups 4, 5, and 6 respectively.

Table 3 depicts the regional distribution of crypt categories (1, 2, 3, and >4). In rats treated with DMH (group 3), most of the ACF with 4 or more crypts/focus was observed in mid and distal colon. The occurrence of large ACF (>4 crypts) was prominently reduced in group 5 versus other groups ($P < 0.01$).

Histological analysis

We observed no tumors in rats from any group at week 15. Tissue sections of control rats (group 1) displayed normal crypts and colonic architecture with no signs of apparent abnormality. Enlarged nuclei and hyperchromatism with mitosis were observed in DMH-treated rats (group 3). Larger areas of thickened mucosal layer with densely packed inflammatory cell infiltrations were also noted,

Table 2
Aberrant crypt foci (ACF) formation in the colon of rats exposed to DMH and caraway

Treatment groups	ACF formation in rat colon				
	Incidence (%)	Total ACF	Total AC	Crypt multiplicity (AC/ACF)	Inhibition of ACF (%)
DMH	6/6 (100)	55.5 ± 13.5^{a}	99.5 ± 12.9^{a}	1.8 ± 0.08^{a}	–
DMH + CC 30	6/6 (100)	49.2 ± 2.2^{a}	83.7 ± 9.9^{b}	$1.7 \pm 0.08^{a,b}$	8.05
DMH + CC 60	6/6 (100)	$24.9 \pm 3.9^{b,*}$	$32.4 \pm 3.5^{c,*}$	$1.3 \pm 0.07^{c,*}$	53.4
DMH + CC 90	6/6 (100)	45.9 ± 6.9^{a}	73.4 ± 7.6^{b}	1.6 ± 0.08^{b}	14.2

Each value represents means ± SD of 6 animals in each group.
[a–c] $P < 0.05$ as compared with other groups.
* $P < 0.01$ as compared to DMH group and other DMH + caraway (CC) treatment groups.

Table 3
Distribution of altered aberrant crypt foci (ACF) category in proximal, mid, and distal colon of rats exposed to DMH and caraway

ACF category	DMH	DMH + CC 30	DMH + CC 60	DMH + CC 90
Proximal colon	1.1 ± 0.5[a]	0.5 ± 0.1[b]	–	–
1 Crypt	0.8 ± 0.4	0.5 ± 0.1	–	–
2 Crypt	0.3 ± 0.1	–	–	–
3 Crypt	–	–	–	–
>4 Crypt	–	–	–	–
Mid colon	12.0 ± 4.6[a]	10.9 ± 3.9[b]	6.5 ± 0.9[c]	8.9 ± 1.3[b,c]
1 Crypt	8.6 ± 2.0	7.6 ± 1.9	3.9 ± 0.5	4.0 ± 0.8
2 Crypt	0.9 ± 0.5	1.8 ± 1.1	2.6 ± 0.4	2.7 ± 0.3
3 Crypt	2.1 ± 1.8	0.9 ± 0.5	–	2.1 ± 0.1
>4 Crypt	0.4 ± 0.3	0.6 ± 0.4	–	0.2 ± 0.1
Distal colon	42.4 ± 8.4[a]	37.8 ± 8.2[a]	18.4 ± 3.0[b]	37.0 ± 5.6[a]
1 Crypt	13.7 ± 2.4	12.2 ± 2.2	8.8 ± 1.6	18.4 ± 1.9
2 Crypt	16.0 ± 3.3	13.5 ± 2.5	9.6 ± 1.4	11.6 ± 1.5
3 Crypt	7.8 ± 0.9	7.3 ± 1.6	–	6.1 ± 1.7
>4 Crypt	4.9 ± 1.8	4.8 ± 1.9	–	0.9 ± 0.5

Each value represents means ± SD of 6 animals.
[a–c] $P < 0.05$ as compared with other groups.

along with vascular congestion and granulation in the lamina propria. In caraway-treated rats (groups 4, 5, and 6), histology revealed no loss of nuclear polarity. Few areas showed mucosal thickenings with scattered or no infiltrations of the inflammatory cells in the mucosa. However, no changes were found in lamina propria. On histological examination, we did not find inflammation or injury to colonic mucosa in 60 mg/kg b.w., caraway-treated rats (group 5) at week15 (Figs. 2A–F). There were no observable signs of neoplasia or toxicity in rats administered 90 mg/kg caraway (group 2).

Bile acids and neutral sterols

Figs. 3A and B illustrate the levels of fecal bile acids and neutral sterols respectively during the 15-week period. Initially (until 8 weeks), there was no significant increase but a sharp elevation of fecal bile acids and neutral sterols from the 9th week was observed in DMH administered rats (group 3). Fecal bile acids and neutral sterols levels were not significantly elevated in the caraway-treated rats (groups 4–6) as compared to DMH alone treated rats. There was no significant difference in the levels of fecal bile acids and neutral sterol between caraway supplemented control rats (group 2) and unsupplemented control rats (group 1).

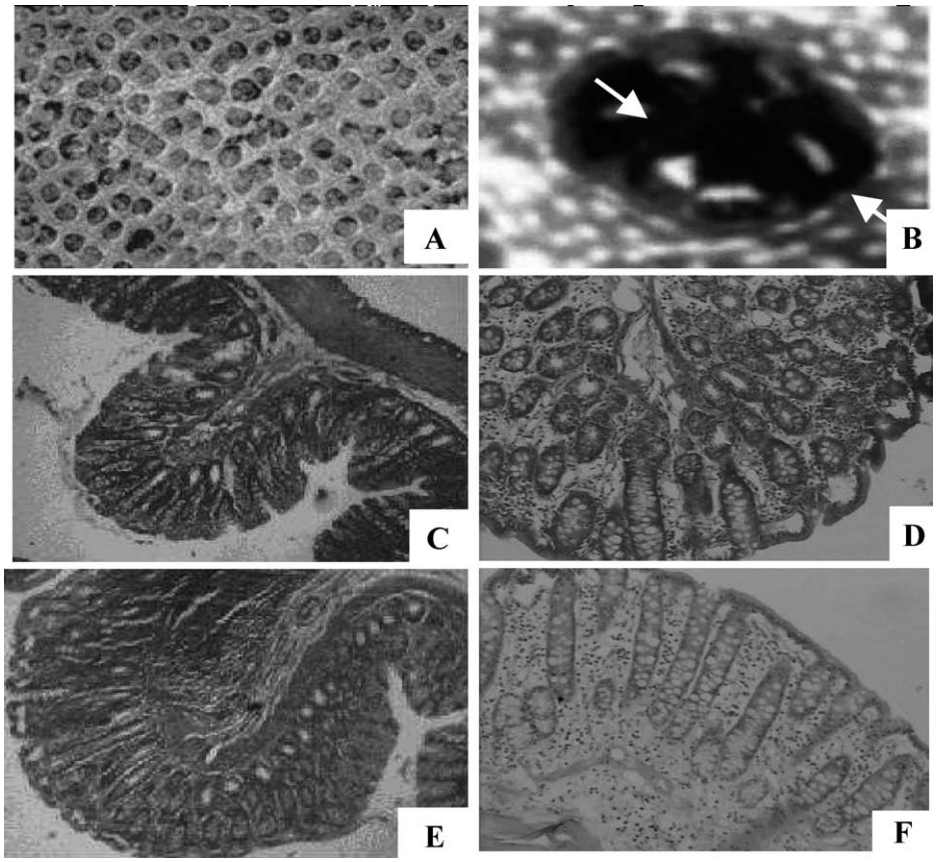

Fig. 2. (A) Topographical view of normal crypt (20×). (B) Topographical view of ACF (arrows) with 6 crypts in whole-mount colon from a rat treated with DMH (40×). (C) Longitudinal sections of normal colon. Crypts are packed with thin lamina propria and regular patterns of epithelial linings (20×). (D) Histological changes in the colonic mucosa on DMH administration shows thickened mucosa with densely packed inflammatory cell infiltration and a higher degree of hyperplasia (10×). (E) Represents the carcinogen-induced colon with vascular congestion and vascular granulation (40×). (F) Represents 60 mg/kg b.w., caraway supplemented rat colon showing mucosal thickening in few areas and scattered or no infiltration of inflammatory cells in the mucosal layer (10×).

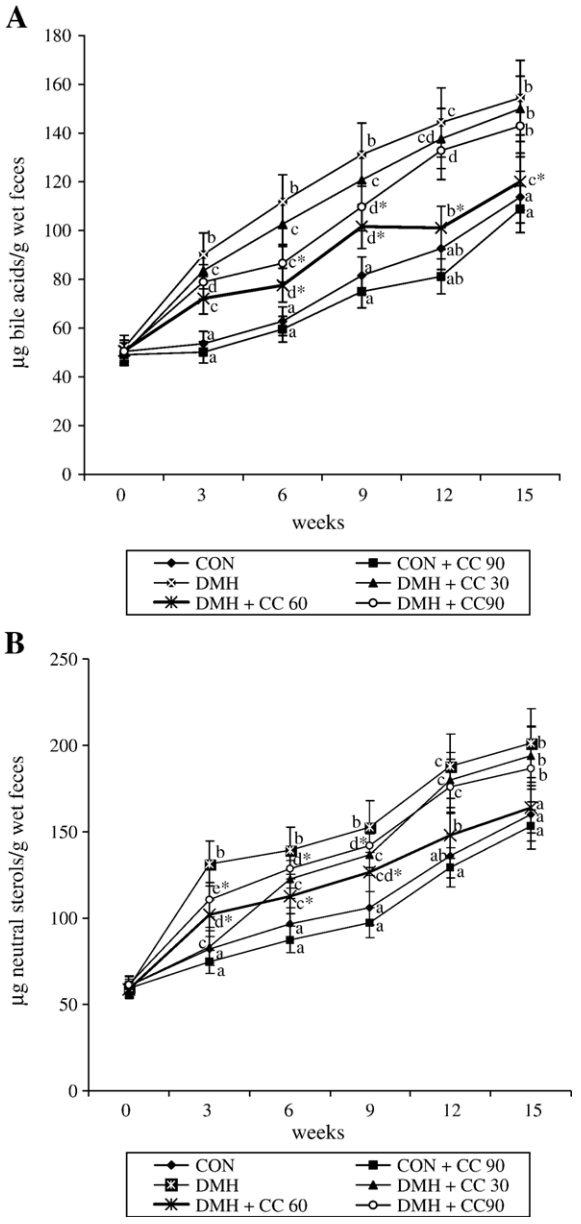

Fig. 3. (A) Effect of caraway on DMH-induced fecal bile acids excretion. Each value represents means ± SD of 12 animals. (a–d) $P < 0.05$ as compared with other groups. *$P < 0.01$ as compared to DMH group and other DMH+ caraway treatment groups. (B) Effect of caraway on DMH-induced fecal neutral sterol excretion. Each value represents means ± SD of 12 animals. (a, b) $P < 0.05$ as compared with other groups. *$P < 0.01$ as compared to DMH group and other DMH+ caraway treatment groups.

ALP, a membrane marker

Fig. 4 represents the intestinal ALP activities in the feces. The levels were significantly higher in DMH alone treated rats (group 3) and were statistically insignificant in caraway-treated control rats (group 2) as compared to the unsupplemented control rats (group 1). ALP activity was decreased significantly ($P < 0.05$) when caraway was supplemented at 30 and 90 mg/kg b.w. (groups 4 and 6), the activity being markedly reduced on 60 mg/kg b.w., caraway supplementation (group 5, $P < 0.01$).

Discussion

Certain chemicals in our environment are classified as carcinogens, co-carcinogens, and tumor promoters (Bird and Good, 2000). Hydrazine and its derivatives like DMH are such chemicals that are shown to be carcinogenic and mutagenic (Kawanishi and Yamamoto, 1991). DMH, an alkylating agent, when injected subcutaneously, is transported to the liver where it undergoes dehydrogenation and is converted to an active carbonium ion through several processes, to be excreted in the bile, where it mediates its carcinogenic activities on the mucosa while passing through the digestive tract (Fiala, 1975). Carbonium ions methylate DNA bases, induce point mutations, micronuclei and sister chromatid exchanges leading to colon-specific carcinogenesis (Choudhary and Hansen, 1998).

ACF are readily discriminable preadenomatous morphological putative lesions within the colonic mucosa of rodents and in cancer patients and may contribute to the stepwise progression to colon cancer (Mc Lellan et al., 1991). Colon carcinogenesis model using DMH with putative preneoplastic ACF as end-point marker has been used to assess the influence of modulatory factors (Bird, 1995b). Among the naturally occurring edible seeds, caraway is one, which contains relatively high amounts of flavanoids, terpenoids and steroids (De Carvalho and Fonseca, in press). Caraway and its constituents were proved to be a class of potential chemopreventive agents (Zheng et al., 1992) since their anticarcinogenic, antiinflammatory, and antiproliferative ability is correlated with enhanced repair or remodeling of precursor lesions (Eddouks et al., 2004). Caraway supplementation at all the three different doses to DMH-treated rats significantly reduced the number of

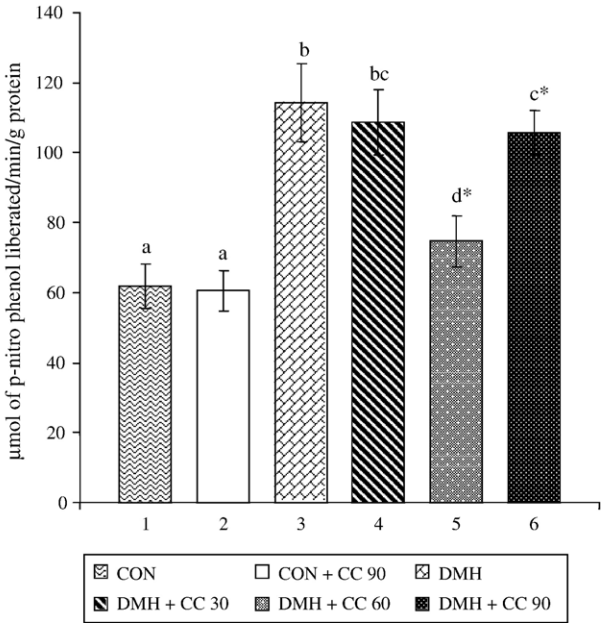

Fig. 4. Effect of caraway on DMH-induced alkaline phosphatase activity in rat feces. Each value represents means ± SD of 12 animals. Vertical lines above the bars represent SD of the means. (a, b) $P < 0.05$ as compared with other groups. *$P < 0.01$ as compared to DMH group and other DMH+ caraway treatment groups.

ACF, AC, and crypt multiplicity (Table 2). This suggests that caraway is able to exert a chemopreventive effect on preneoplastic ACF development, and the effect was more pronounced at intermediary dose of 60 mg/kg b.w. (group 5). It is evident that more the number of crypts a focus has, the more advanced it is (Shih et al., 2004). The number of ACF consisting of >4 crypts in middle and distal colon of DMH-treated group (Table 3) was significantly reduced suggesting that caraway may inhibit the ACF growth. The mechanism by which caraway inhibits the distribution of ACF not only in the middle colon, but also in the entire colon may be partly due to the reversal of some ACF to normal phenotype. Kawamori et al. (1996) demonstrated that d-limonene one of the constituents of caraway seeds inhibits the azoxymethane-induced development of colonic ACF in rats. In addition, d-limonene has been found to inhibit various stages of tumorigenesis in a number of animal models and is now being evaluated as a chemopreventive agent in humans (Uedo et al., 1999). d-limonene inhibits the development of spontaneous and chemically induced tumors in the mammary gland, skin, liver, lung, forestomach and pancreas of rodents (Chen et al., 1998). Moreover, the principal monoterpene carvone, present in caraway may exert their putative anticancer effects through interaction with the P450 1A1 system, to prevent the activation of the procarcinogen (Eddouks et al., 2004). Carvone is also known to prevent chemically induced lung and forestomach carcinoma development (Wattenberg et al., 1989). These results suggest that caraway at the dose of 60 mg/kg b.w. (group 5) provides a most stable environment to inhibit the advancement of ACF.

Bile acids are emerging as one of the major factors affecting the development of colon cancer (Roberten, 1993), by acting as tumor promoters, which is evidenced from various animal models (Reddy et al., 1977a, 1977b). Apart from bile acids, fecal neutral sterols have also been related to colorectal malignancy. Since colonic epithelium is relatively more exposed to fecal sterols in aqueous phase, it has been suggested that determination of neutral sterols in fecal water may be of great importance (Korpela et al., 1988). Several studies have shown that during DMH treatment, there is increased level of cholesterol in intestine and colon (Chirta et al., 1994), which causes the increased formation and decreased absorption of bile acids and sterols. Moreover, dietary fat intake increases bile acid secretion into the intestines. This may be the reason for observed increase in the levels of fecal bile acids and neutral sterols in DMH-treated group. Bacterially modified bile acids are known colon tumor promoters, and certain colonic bacteria are able to degrade bile acids and degraded bile acids are co-carcinogens in animal models (Hill, 1981). Thus increased fecal bile acids and neutral sterols in DMH-treated rats may explain its positive correlation with colon cancer. The aromatic seeds of caraway are rich in monoterpenes and are known to possess antibacterial activity (Oosterhaven et al., 1995). Reduced fecal bile acids and neutral sterols on caraway supplementation may be due to the modulation of the growth of one or more bacterial species of the colon by caraway, thus preventing the conversion of primary bile acids to secondary bile acids (Stadler et al., 1988).

The intestinal specific ALP expression is a marker of maturation and differentiation of the epithelial cells of intestinal tract (Yuasa et al., 1994). Measurement of changes in fecal ALP activities has also been used as a marker of epithelial cell loss in vivo (Lapre et al., 1993b). It has been shown that increased cell proliferation is linked with a higher risk to develop colon cancer (Terpstra et al., 1987). In this context, DMH alone administered group (group 3) showed increase in ALP activity. The reduced ALP activity observed on caraway supplementation may be imparted to its anti-proliferative activity (Eddouks et al., 2004).

In conclusion, dietary administration of 60 mg/kg b.w., caraway significantly suppressed the development of DMH-induced rat colonic ACF, reduced fecal bile acids, neutral sterols, and ALP activity, thus modulating cell proliferation in the intestine. Further investigations are now needed to determine long-term anticarcinogenic effects of caraway on tumor development and clarify the underlying mechanisms of action.

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