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Chemopreventive effects of caraway powder and oils to suppress 1, 2- dimethylhydrazine-induced colon carcinogenesis

[Kimyon tozu ve yağının 1,2-dimetilhidrazin ile indüklenen kolon karsinogenezini baskılamada kemopreventif etkileri]

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ABSTRACT

Objective: Caraway is native to western Asia, Europe and northern Africa with various pharmacological properties. To gain insight more into the mechanism(s) by which the caraway seed reduced colon premalignancies, in this study, for the first time, we decided to consider the chemopreventive effects of the whole caraway seed in comparing with its essential oils at higher dose (0.2% in diet).

Methods: Colon cancer was induced by 1, 2-dimethylhydrazine (DMH) in rats (20 mg/kg body weight for 5 weeks) and groups of animals were given caraway preparations (0.2% in diet). After 16 weeks, the colon tissue biopsies were collected and analyzed for pathological observation of aberrant crypt (AC) and aberrant crypt foci (ACF) formations. In parallel, hepatic cytochrome P4501A1 (CYP1A1) and glutathione S-transferase (GST) activities were measured. In addition, colonic β-catenin was measured either at protein or mRNA levels through ELISA and RT-PCT-ELISA assays, respectively.

Results: The results showed that DMH-induced changes in hepatic CYP1A1 and GST activities were recovered in animals treated with caraway preparations. Also, elevated colonic β-catenin both at protein and mRNA levels were diminished in animals treated with caraway seed powder as well as caraway essential oils. These results are in parallel with decreased ACF formations in histopathological biopsies in caraway treated groups.

Conclusion: From this study, it is obvious that one of the mechanism(s) of colon chemopreventive effects applied by caraway powder and its oils may be the decreased of colonic β-catenin at protein and mRNA levels through modulation of DMH metabolism(s).

Key Words: Caraway seed, Essential oil, β, - catenin, DMH, Colon carcinogenesis

Conflict of Interest: This research was conducted by the research deputy grant of Qom Branch, Islamic Azad University.

ÖZET

Amaç: Kimyon, batı Asya, Avrupa ve kuzey Afrika için birçok farmakolojik özellikleri ile yöresel bir üründür. Bu çalışmada, kimyon tohumunun kolonun premalignitelerini azaltmaya yönelik mekanizmalarını ilk defa detaylı anlamak için, bütün kimyon tohumunun ve diette alınan yüksek doz (%0.2) yağının karşılaştırılarak kemopreventif etkilerinin değerlendirilmesine karar verildi.

Metod: Ratlarda kolon kanseri 1, 2-dimetilhidrazin (DMH)'in 20 mg/kg/tüm vücut ağırlığına 5 hafta boyunca uygulanması ile oluşturuldu ve gruplara diette %0.2 olacak şekilde kimyon preparatları verildi. 16 hafta sonunda kolon doku örnekleri alınarak aberant kript (AC) ve aberant kript odak (ACF) oluşumları patolojik olarak değerlendirildi. Eş zamanlı olarak hepatik sitokrom P4501A1 (CYP1A1) ve glutatyon-S-transferaz aktiviteleri ölçüldü. Ayrıca kolon β-katenin tayini protein ve mRNA düzeyinde ELIZA ve RT-PCT-ELIZA yöntemleri ile ölçüldü.

Bulgular: Kimyon preparatları ile tedavi edilen hayvanlarda, DMH indüklenmesi ile hepatik CYP1A1 ve glutatyon-S-transferaz aktivitelerinde gözlenen değişikliklerin düzeldiği görüldü. Ayrıca hem protein hem de mRNA düzeyinde artmış β-katenin düzeyi kimyon tohumu tozu ve aynı zamanda kimyon esansiyel yağı ile tedavi edilen grupta azaldı. Bu sonuçlar, kimyon ile tedavi edilen gruplarda histopatolojik biyopsilerde azalan ACF oluşumu ile de desteklendi.

Sonuç: Bu çalışmanın sonucunda, kimyon tozu ve yağı ile elde edilen kolona ait kemopreventif etkilerden birinin DMH metabolizmasında ki düzenleyici etki veya etkilerinin kolon β-katenin protein ve mRNA düzeyinde azalmaya neden olarak oluştuğu görülmüştür.

Anahtar Kelimeler: Kimyon tohumu, esansiyel yağ, β- katenin, DMH, kolon karsinogenezi

Çıkar Çatışması: Bu araştırma İslami Azad Üniversitesi (Qum Branch) ödeneği ile gerçekleştirilmiştir.

Introduction

Colorectal cancer (CRC) is the third most common malignant neoplasm in the world and it remains an important cause of death, especially in western countries [1]. Also, CRC with age-adjusted rate of 6–7.9 per 100,000 people per year is the fourth most common cancer in Iran [2, 3].

Certain chemicals in our environment are classified as carcinogens, co-carcinogens and tumor promoters [4]. One such chemical dimethylhydrazine (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 [5]. DMH is a colon specific carcinogen and an alkylating agent. It is believed to form active intermediates including azoxymethane and methylazoxymethanol in the liver gone throughout metabolism by cytochrome P450 isoensymes such as CYP1A1. These active products subsequently transported into the colon through bile. Methylazoxymethanol is decomposed to form methyldiazonium ion, which methylate cellular components and in turn produce tumors in the colon [6-9]. Meanwhile, hepatic detoxification enzymes such as glutathione S-transferase (GST) can be an efficient factor in promoting DMH detoxifications [10].

DMH–induced aberrant crypt foci (ACF) are preneoplastic lesions found in most colon cancers [11]. ACF represents a precancerous change within the colon. Based on the proposition that these lesions are early preneoplastic events of colorectal cancer, ACF is being used as a potent preneoplastic marker for long-term bioassay to identify modulators of colon carcinogenesis [12, 13].

In the progression of DMH carcinogenesis, the reactive metabolite causes the methylation of DNA in colonic epithelial cell leaded to β-catenin gene mutation [14-19]. β-catenin is a cadherin-binding protein that also functions as a transcriptional activator when complexes in the nucleus with members of the TCF/LEF family [20]. In normal epithelial cell, cytosolic β-catenin interacts with adenomatous polyposis coli (APC), Axin, glycogen synthase kinase-3β (GSK 3β) and other proteins leading to phosphorylation of Ser and Thr in the N-terminal region of β-catenin, followed by ubiquitination and proteosomal degradation [16,20,21]. Mutation in β-catenin or APC prevents the phosphorylation and consequently β-catenin proteosomal degradation in cytoplasm leading to increased level of cytoplasmic β-catenin. This is resulted in accumulation of β-catenin/TCF/LEF complexes in the nucleolus and activation of downstream target oncogenes such as c-myc, c-jun and cyclin D1 leaded to colon cancer [16].

Most colorectal cancers should be preventable through increased surveillance, improved lifestyle and the use of dietary chemopreventive agents such as spices or herbs containing bioactive phenolic substances [22-24]. Some natural compounds such as mangiferin, furan-2-yl-3-pyridin-2-yl-propenone (FPP-3), curcumin should also be useful [25-28].

Caraway [*Carum carvi L.*, (Umbelliferae)] known as "meridian fennel" or "Persian cumin" has been commonly used like a whole seed in phytomedicine as antibacterial, antiproliferative and laxative agent [29-31]. The caraway seeds contain many ingredients such as essential oils and aqueous extract that likely work together to produce the desired medicinal effect. The aqueous extract of caraway contains 19 monoterpenoids, including eight stereoisomers of *p*-menthane-2,8,9-triol and five stereoisomers of *p*-menthane-1,2,8,9-triol isolated from the water-soluble fraction of the seeds. In addition, aromatic compounds, aromatic compound glycosides, alkyl glycosides, glucides, nucleoside and flavonoids have been identified in aqueous extract of caraway seeds [32]. Chromatography on cellulose column also indicated quercetin 3-glucuronide, isoquercitrin, kaempferol 3-glycoside and quercetin 3-O-caffeylglucoside as the flavonoid constituents of caraway extract [33]. The major constituents of the essential oils extracted in this study were *(Z)-ß-Ocimene* (32.7%), *γ-Terpinene* (22.03%) and p-*Cymene* (8.03%) [34,35].

Our in vitro and in vivo studies indicated that the essential oils of caraway seeds have various pharmacological properties. We showed the suppressive effects of caraway extracts on cytochrome P-4501A1, a phase-I xenobiotic metabolizing enzyme over-expressed with dioxane in hepatoma cell line [36]. More freshly, we have proved the in vitro antioxidant and antibacterial properties of caraway oil preparation [37,38]. In addition, we indicated that caraway essential oils modulate the parameters related to oxidative liver and lung injuries i.e. myeloperoxidase (MPO) activity, thiobarbituric acid reactive substances (TBARS) and gluthathione (GSH) levels in cecal ligation and puncture (CLP) rat model [39,40]. In our recent studies including colon carcinogenesis, we indicated that caraway essential oils at low doses (0.01 and 0.1% in diet) inhibited colon premalignant lesions induced by DMH [34,35].

Accordingly, in regards to the medicinal importance of different caraway preparations, considering the chemopreventive effects of whole caraway seed powder in compare to one of its active constituent's i.e essential oils in the same conditions cannot be groundless. In this way, we decided to consider, for the first time, the expression of the beta-catenin oncogene in regard to DMH detoxification enzyme activities in whole caraway seed powder and essential oil treated rats.

Materials and methods

Chemicals

1, 2-Dimethylhydrazine (DMH) was from Sigma Chemical Co. St. Louis, MO, USA. DMH was dissolved in 1 mM EDTA just before use and the pH was adjusted to 6.5

with 1 mM NaOH to ensure the stability of the chemical. β-catenin ELISA kit was from Assay Design Co., U.S.A. RNA extraction kit (Nucleospin RNA-II) was purchased from Macherey-Nagel (MN) Co., Germany. RNasin, Reverse transcriptase (moloney murine leukemia virus (M-MuLV), oligo-dt mixture and Taq DNA polymerase and diethylpyrocarbonate (DEPC) were from Sina-gene, Tehran, Iran. Digoxigenin-11-dUTP and PCR ELISA (Dig-Detection) kit was from Roche Co., Germany. Primers and probes for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and β-catenin genes were prepared by Gen-fanavaran Co., Tehran, Iran. All the other chemicals and reagents used were analytical grade.

Preparation of caraway seed powder and essential oils

The whole fresh caraway seed was powdered in mixer. The essential oil was extracted from caraway seeds using a Clevenger-type apparatus as described previously [40]. The extraction was carried out for 2 h and the oil was stored in dark glass bottles in freezer (-20°C) until further use.

Animals and treatments

A total of 40 male Wistar rats weighing 100±20 g were obtained from Pasteur Institute of Iran. The animals were randomly divided into 4 groups each consisting of 10 rats and maintained at 22 ± 2°C with a 12-h light/12-h dark cycle. Animal studies were approved by the Medical Ethics Committee of Tarbiat Modares University. This Ethics Committee was based on the World Medical Association Declaration of Helsinki (adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964).

The rats in group 1 (control group) received 0.5 ml of EDTA- the vehicle of DMH once a week for five weeks. Rats in group 2 received DMH dissolved in EDTA (20 mg/kg b.w) injection (s.c) once a week for a period of five weeks. Groups 3 and 4 were given DMH (20 mg/kg b.w) for 5 weeks and fed with diet containing 0.2 % of caraway seed powder and its oil and considered as treated groups. The diet containing caraway was started simultaneously with DMH treatment and continued for 16 weeks. At the end of the experiment (16 weeks), the animals were anesthetized and the blood was collected by heart puncture. Then, animals were scarified and colon tissues were removed and processed for histological and biochemical assays.

Pathological observation of AC and ACF formations

The pathological analysis of AC and ACF formations were described previously [34,35]. Briefly, after a total experimental period of 16 weeks, the animals were sacrificed and the colons were removed, cut open along the longitudinal axis from cecum to anus and were flushed with PBS solution. The colons were assessed for the macroscopic changes. The total number of the ACF in the distal colon was determined in a 2 cm section (1-1.2 cm2) of the colon starting from the distal to the proximal end of the colons. The specimens were sandwiched between filter papers, fixed in 10% neutral buffered formalin and stained with 0.2% methylene blue in saline for 2-3 minutes and then placed mucosal slides up on a microscopic slide [41]. The specimens were examined grossly and topographically at 40× magnification under a light microscope. The number of ACF (aberrant crypt foci) and aberrant crypts (AC) were determined. Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, slit-like opening, thick and darker stained epithelia and pericryptal zone. After the number of ACF and AC had been determined, for histopathologic examination of the colonic epithelium changes, the tissues were previously fixed in 10% buffer formalin, embedded in paraffin blocks and were cut into serial sections of 6 microns wide strips and stained with hematoxylin and eosin (H&E). Hyperplasia was assessed by counting the number of cells per crypt columns (crypt height). For assessment of malignancy of the ACF, a grading of nuclear atypia on the basis of nuclear stratification and elongation (mild, moderate and severe) and intact basement membrane were pointed out. All of aberrant crypts were distinguished with a relatively nuclear morphology and mild nuclear atypia considered as hyperplastic ACF and aberrant crypts with a moderate to severe cellular atypia considered as a higher degree of hyperplasia.

GST Activity

Liver cytosolic GST activity were measured by spectrophotometrically using CDNB as substrate as described by Habig et al. (1974) [42]. The specific activity was calculated based on the nmol/min/mg protein in samples which was measured by Bradford assay (Bradford 1976) [43].

Cytochrome P450 1A1 Activity (EROD Assay)

Ethoxyresorufin-o-deethylase (EROD) assay was performed on liver preparations according to the procedure described by Burke and Mayer (1974) [44].

Measurement of β-catenin at protein levels

The concentration of β-catenin in colon preparations was measured by ELISA using a commercially available kit (Assay Design Co., U.S.A). The assays were performed according to the manufacture's instruction.

β-catenin expression at mRNA levels using RT-PCR-ELISA

RT-PCR technique was used to compare β-catenin expression in colon biopsies obtained from different experimental groups. For this purpose, tissue homogenate was subjected to total RNA isolation using a commercial kit (Macherey-Nagel Co.). RNA purity was controlled by measuring optical density (OD) at 260/280 nm.

The first strand cDNA was synthesized from the isolated RNA using oligo-dt primers. The reaction mixture contained 5 µg RNA; 1 µl oligo-dt (100 µmol) which reached to 11 µl by deionized water. The mixture was mixed, incubated for 5 min at 70°C and kept on ice. In order to prepare cDNA from total RNA, 4 µl of PCR buffer (5X), 2

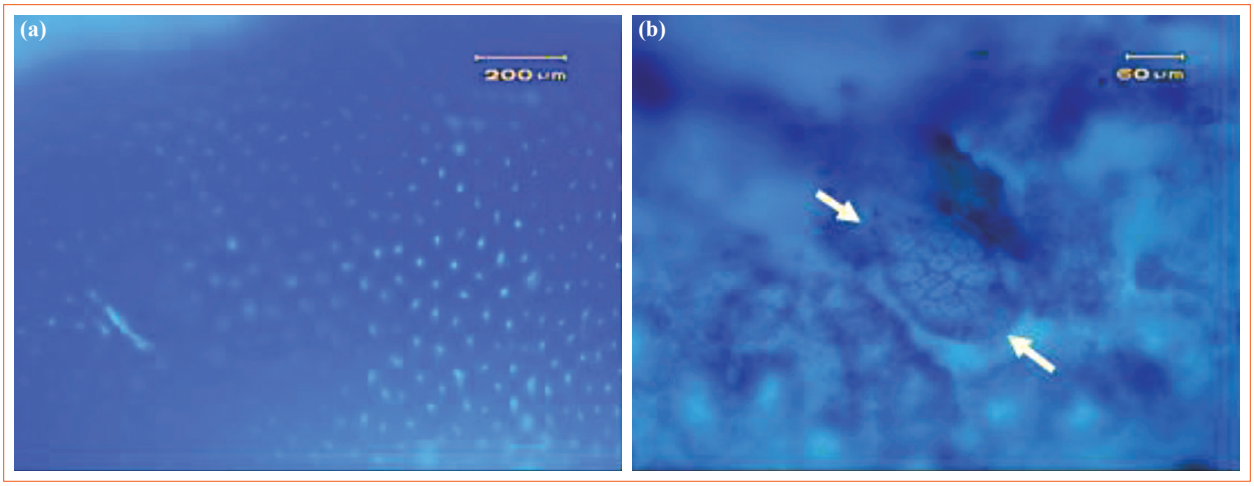

Figure 1. Topographical view of ACF. **(a)** Topographical view of normal crypt (methylene blue ×100). **(b)** Topographical view of a ACF (arrows). The focus has 10-12 aberrant crypts in whole mount crypts from a rat treated with DMH (methylene blue ×200).

µl (10 mM) dNTP mixture, 0.5 µl RNasin (40 U/µl) were mixed and reached to 19µl by deionized water. The mixture incubated for 5 min at 37°C. Then, 1 µl of M-MuLV reverse transcriptase (200 U/µl) was added to the reaction mixture and incubated for 60 min at 42°C followed by incubation at 70°C for 5 min.

PCR- ELISA

Specific primers for β-catenin and GAPDH (internal standard) gene amplification were designed using OLIGO 6 and AlleleID Software programs. The sequences of these primers are as follows:

β-catenin rat)F(: 5' GGTGCTGTCTGTCTGCTC 3'

β-catenin rat)R (: 5'CCATCCCTTCCTGCTTAG 3'

GAPDH rat)F(: 5' TGCCAGCCTCGTCTCATAG 3'

GAPDH rat)R(: 5' ACTGTGCCGTTGAACTTGC 3'

The probes for ELISA were also designed by OLIGO 6 and AlleleID Software programs. The probes used are as follows:

β-catenin: 5'-Biotin- TCAGCGACTTGTTCAAAACT-3'

GAPDH: 5'-Biotin- GCTGCCTTCTCTTGTGAC- 3'

Both primers and probes were synthesized in Expedite DNA synthesizer (TAG Copenhagen A/S., Denmark). The primers and probes were purified using reverse phase chromatography (RP-column) and reverse phase-High Pressure Liquid Chromatography (HPLC), respectively (MALDI-TOF, Denmark).

Preparation of DIG-labeled β-Catenin and GAPDH specific PCR products

First strand cDNA was directly used to amplify the target sequence (β-catenin) and internal control (GAPDH) genes for each sample. The 25 µl PCR mixture contained 15.2 µl of deionized water, 2.5 µl 10x reaction buffer, 2 µl MgCl2 (25 mM), 1 µl of each primers (20 pM), 0.95 µl dNTP mixture (10 mM) together with 0.05 µl Digoxigenin-11-dUTP (1 mM), 0.3 µl Taq DNA polymerase (5 U/µl) and 2 µl DNA template. The reaction mixture was subjected to the following amplification protocol; 1 cycle at 94°C for 2 min (initial denaturation) followed by 30 cycles; 94°C for 30 s (denaturation), 56°C for 45s (annealing) and 72°C for 75s (extension). The reaction continued for 5 min at 72°C for final extension.

Detection of PCR products by ELISA

ELISA was performed according to the manufacturer's instructions (Roche Co.). Briefly, 20 µl of DIG-labeled GAPDH and β-catenin-specific PCR products were placed into separate microfuge tubes. 20 µl of denaturation solution was added to each tube and incubated at room temperature for 10 min, followed by the addition of 210 µl of hybridization solution. After mixing an aliquot (200 µl) of the mixture was added to streptavidin-coated microtiter plate (MTP). The plates were incubated at 37°C for 3 h. Then the wells were washed five times with 250 µl of washing solution and then 200 µl of diluted anti-DIG POD (1:2000) was added to each well and incubated for 30 min at 37°C. The wells were washed five times with 250 µl washing solution, 200 µl of ABTS-substrate solution was added to each well and the plate was incubated in dark for 30 min at 37°C to allow enzymatic reaction. Also, 200 µl of ABTS substrate solution was added to an empty well to measure intrinsic extinction of the ABTS solution. Absorbance of each sample was measured at 405 nm using ELISA reader (TECAN, GmbH Co. Austria). The GAPDH OD405 in the sample was used as normalization factor.

Analysis of PCR products

The PCR products were also analyzed semi-quantitatively by band densitometry analysis. The band densitometry analysis was performed as follows, 10 µl of each PCR product was electrophoresed on agarose gel and stained with ethidium bromide. The pictures were captured using a Gel documentation instrument (UVI tec. UK). The den-

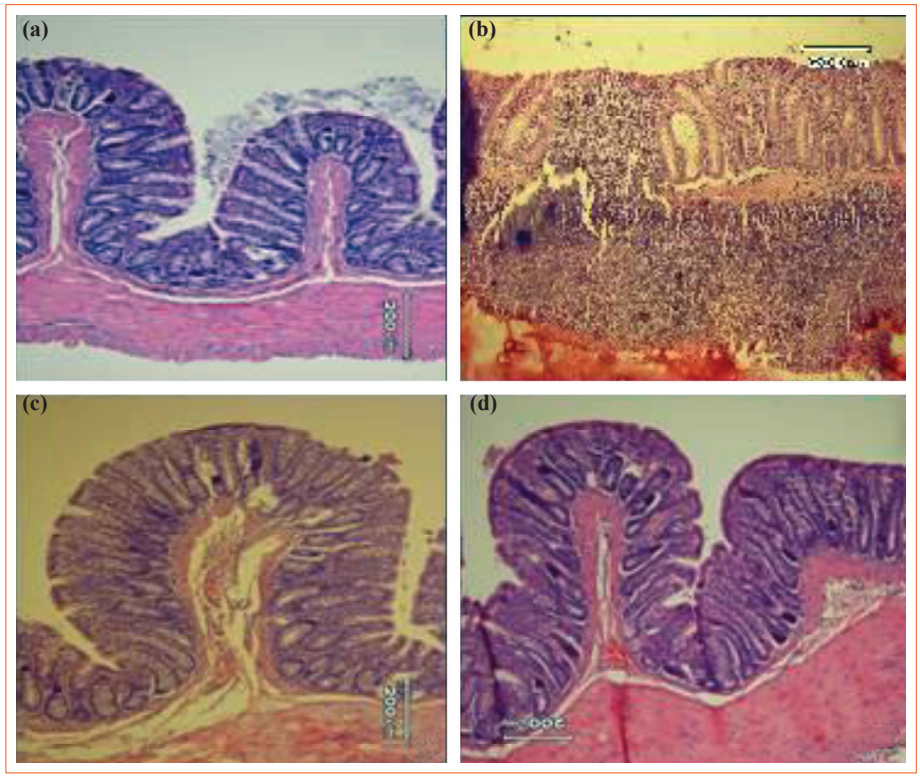

Figure 2. Histopathological changes in the colon of rats. **(a)** A histological section of normal colon shows normal colonic architecture with no signs of abnormality (H&E ×100). **(b)** A microscopic section of colon from a DMH-treated rat. The thickened mucosal layer with severe infiltration of inflammatory cells in the lamina properia along with lymphoid aggregates in the submucosa and higher degree of hyperplasia in the glands are present (H&E ×100). **(c&d)** Histological sections of caraway essential oil & powder supplemented rat colons. The colons show mild infiltration of inflammatory cells in the lamina properia, normal submucosa, intact basement membrane and muscularis mucosa and lower degree of hyperplasia (H&E ×100).

sitometric evaluation of bands was performed on a computer using densitometric analysis program (UVIdoc., version 12.4). The program determines the optical density of the ethidium bromide stained band by using a grey scale threshold operation. The amount of β-catenin specific mRNA amplified is explained as relative β-catenin mRNA expression (%) that was obtained after normalization with the GAPDH internal control.

Statistical analysis

Data are presented as means ± standard error of mean. The results were subjected to one-way ANOVA followed by Tukey's HSD using SPSS (version 19.0) software. Significant levels were defined as $P<0.05$.

Results

Effect of caraway seed powder and its essential oils on ACF formation

No tumor was observed macroscopically and microscopically in any experimental groups at 16th week. In the DMH-treated group (group 2), the size of ACs were two to three times larger than the crypts observed in the surrounding normal tissue with a slit-like opening (Figs. 1a and 1b, respectively). The crypts were exhibited thickened epithelia that stained darker than normal crypts. They were elevated and a large pericryptal zone was present around them (Fig. 1b). In the caraway treated rats (groups 3 and 4), the formation of large ACFs was suppressed and the ACFs were smaller and contained 2-3 aberrant crypts in each focus. There was no dysplasia, adenoma or adenocarcinoma in ACs in all the experimental groups.

The quantitative results of the total number of ACF and AC, crypt multiplicity (AC/ACF) and percentage inhibition of ACF in all experimental groups are shown in Table 1. All the ACFs were large and contained up to four or more aberrant crypts per focus. ACF formations were found to be significantly higher in DMH- treated group (group 2) as compared to the control group (group 1). Administrations of dietary caraways (0.2%) mixed in pellet to DMH-treated animals for 16 weeks caused a significant ($P<0.05$) decrease in the total number of ACF in the colon of rats. Dietary seed powder and its essential oils also resulted in 65 and 80% inhibition in the ACF formation, respectively ($P<0.05$). Also, the total number of AC and crypt multiplicity were significantly decreased ($P<0.05$) in animals treated with the both caraways (Table 1).

Table 1. Effect of caraway powder and its essential oils on DMH-induced aberrant crypt foci (ACF) formation in rat colon.

Treatment	Total number of ACF	Total number of aberrant crypt (AC)	Crypt multiplicity (AC/ACF)	Inhibition of ACF (%)
Control	0	0	0	–
DMH	8±2.2*	6.75±2.1*	0.84±0.95*	–
DMH+ 0.2% essential oil	2.8±0.5**	0±0**	0**	65**
DMH+ 0.2 % powder	1.6±0.4**	1.6±0.4**	1±1**	80**

ACF: Aberrant Crypt Foci; AC: Aberrant Crypt; The multiplicity of ACF was expressed as the number of aberrant crypts (AC)/focus; Values are mean± S.E.M. obtained from six animals in each group and carried out in duplicate; *P<0.05 is considered significantly different from control group within each parameter; **P<0.05 is considered significantly different from DMH-treated group within each parameter.

Table 2. Effect of caraway powder and its essential oils on xenobiotic metabolizing enzymes in liver of DMH-treated rats.

Treatment	GST (nmol/min/mg protein)	CYP1A1 (pmol resorufin/min/mg protein)
Control	1213±48	14.6±8.5
DMH	939±54*	21.8±3.7*
DMH+ 0.2% essential oil	1152±26**	14.3±1.1**
DMH+ 0.2 % powder	1621±72**	13.0±4.3**

Values are mean± S.E.M. obtained from six animals in each group and carried out in duplicate; *P<0.05 is considered significantly different from control group within each parameter; **P<0.05 is considered significantly different from DMH-treated group within each parameter.

Histopathological observations

In the control group (group 1), the colons showed normal mucosa, ordinary submucosa layers and normal colonic architecture with no signs of apparent abnormality (Fig. 2a). This group share common morphological structures in which there were no microscopically observable changes including ACF. On histological examination, we did not observe ACF formation, inflammation or injury to colonic mucosa in control group at 16th week (Fig. 2a). There were no histological evidences of neoplasia or toxicity in this group. The histopathological study showed that ACFs were observed only in the colons of animals of the groups that received DMH. In DMH-treated rats (group 2) with higher degree of hyperplasia, crypts showed moderate to marked pleomorphism (atypia). The nuclei were enlarged and hyperchromatic. Also, the nucleoli were prominent and cytoplasm and mitotic figures were scant. The larger areas of thickened mucosal layer with severe infiltration of inflammatory cells, along with vascular congestion in the lamina properia and lymphoid aggregates in the submucosa were also noted (Fig. 2b). These observations revealed the formation of histopathological intraepithelial neoplasia. The cells did not infiltrate the wall and there was no tumor extension beyond the muscular mucosa in any of the groups.

In the DMH-treated rats, following the essential oil treatments (groups 3 and 4), the colon of rats were showed normal submucosa with lymphoid aggregates. In these groups, the basement membrane and muscularis mucosa were intact. The size and shape of the cells were also uniform. In these groups, histopathological examinations revealed no loss of nuclear polarity (Figs. 2c and 2d). The number of cells in the crypt column (crypt height) was decreased in these groups as compared to the DMH-treated rats. In groups 3 and 4, almost all of the aberrant crypts were histopathologically distinguished with a relatively normal nuclear morphology and mild nuclear atypia considered as hyperplastic ACF. There were no aberrant crypts with severe nuclear atypia (as dysplastic ACF) without any break down of basement membrane. Few areas showed mucosal thickening with scattered or no infiltrations of the inflammatory cells in the mucosa. As mentioned above, no changes were found in lamina properia.

In all groups, paneth cells were rarely present between epithelium of aberrant crypts. However, all sections were carefully examined and there were no evidence of invasion through the basement membrane and muscularis mucosa. No macroscopic changes were observed in the colons. No tumor was observed macroscopically and microscopically in rats from any group at week 16.

Effect of caraway seed powder and its essential oils on hepatic detoxification enzyme activities (GST & CYP450 1A1)

As shown in Table 2, the activity of CYP1A1 in liver of DMH-treated animals is significantly increased (P<0.05). Oral administration of caraway seed powder as well as caraway essential oils could significantly reduce the activity of hepatic CYP450 (P<0.05). Similarly, GST activity significantly decreased in liver of DMH-treated rats when compared to untreated control (P<0.05). Oral administration of both caraways to DMH-treated rats significantly increased the activities of hepatic GST as compared to DMH group only (P < 0.05) (Table 2).

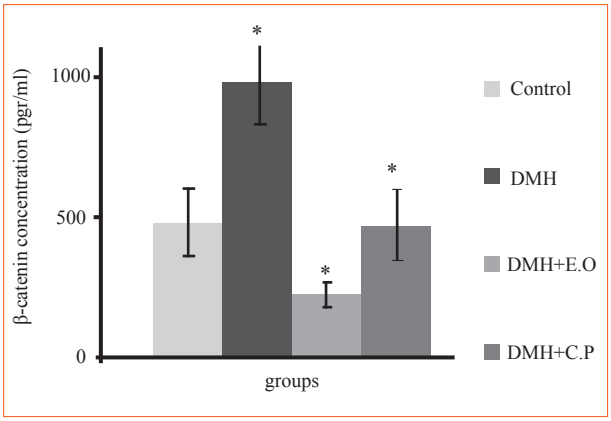

Figure 3. Effect of caraway powder and essential oils on colonic beta-catenin at protein levels in rats treated with DMH. Values are mean± S.E.M. obtained from six animals in each group and carried out in duplicate. *P<0.05 is considered significantly different from control group within each parameter. **P<0.05 is considered significantly different from DMH-treated group within each parameter.

Effect of caraway seed powder and its essential oils on colonic β-catenin in DMH-induced colon carcinogenesis

β-catenin at protein levels

As shown in Fig. 3, DMH treatment significantly increased (2 folds) the levels of β-catenin in colon tissue (P<0.05). Dietary administration of caraway seed powder (0.2% in feed) to DMH-treated rats significantly decreased the level of colonic β-catenin (P<0.05) as compared to rats treated with DMH alone. Also, the caraway essential oils could significantly inhibited the colonic β-catenin (P<0.05) (Fig. 3).

β-catenin expression at mRNA levels

The expression of β-catenin-specific mRNA determined by semi-quantitative RT-PCR ELISA was markedly elevated in premalignant colon biopsies of the rats treated with DMH as judged by densitometric analysis of the PCR products (Fig. 4a-b). Similarly, the results of OD ratio of β-catenin/GAPDH of ELISA detection of PCR products showed a significant increase (~2 folds) in β-catenin mRNA expression in premalignant tissues as compared with control group (P<0.05) (Fig. 4c). Dietary caraway essential oils as well as caraway seed powder (0.2%) resulted in a significant suppression in the β-catenin gene expression (P<0.05) (Fig. 4).

Discussion

In our recent studies, we demonstrated that feeding long term of low dose caraway essential oils could effectively inhibit aberrant crypt foci (ACF) formation in rat colon during the DMH-induced carcinogenesis by modulating

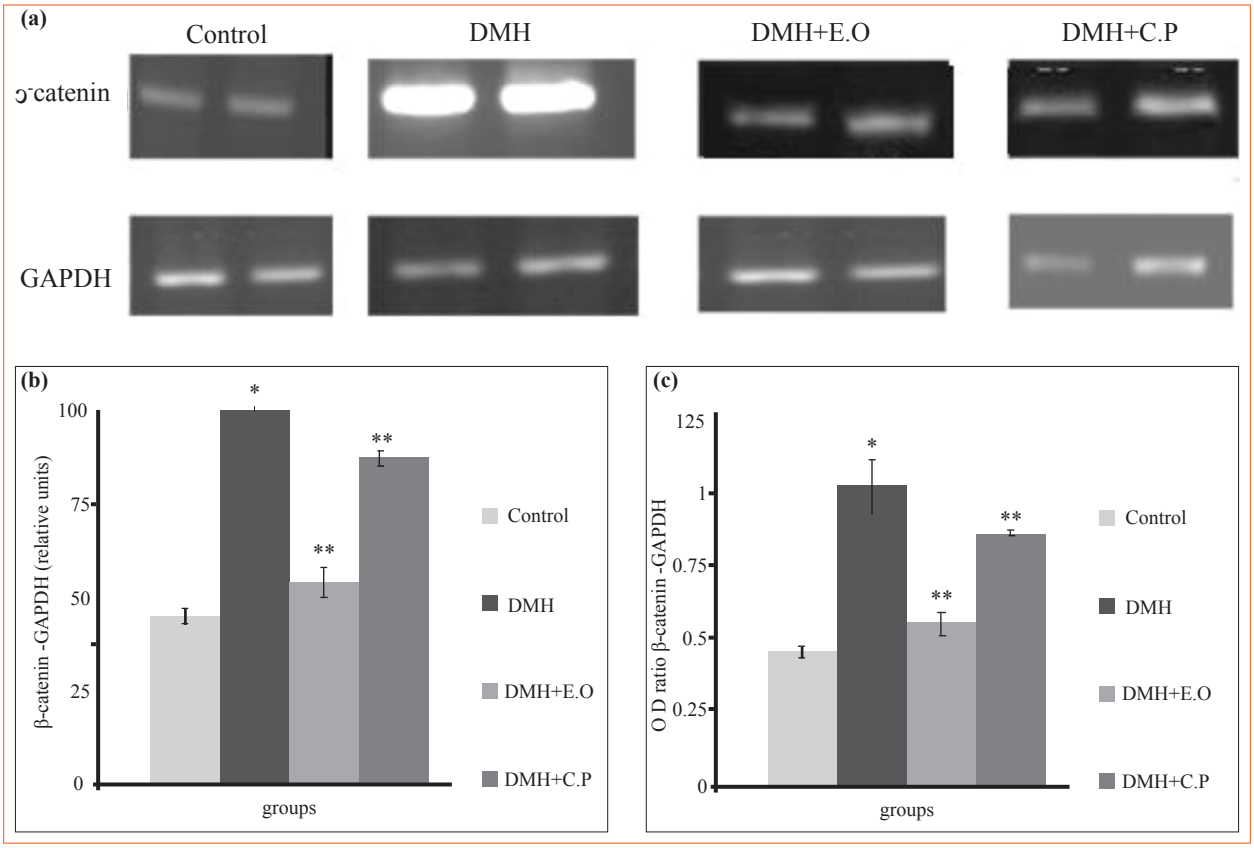

Figure 4. RT-PCR ELISA for beta-catenin specific mRNA in the colon of rats. Panel A depicts representative bands (196 bp for GAPDH and 187 bp for beta-catenin) from each group, Panel B indicate densitometry analysis for transcripts of beta-catenin, and Panel C shows the analysis of related optical density of beta-catenin /GAPDH mRNA expression. Beta-catenin gene expressions were normalized to the expression of GAPDH. Values are mean ± S.E.M. of 6 samples carried out in duplicate. *P<0.05 is considered significantly different from control group within each parameter. **P<0.05 is considered significantly different from DMH-treated group within each parameter.

the activities of hepatic detoxification enzymes i.e. GST and CYP1A1 leading to the decreased expression of colonic β-catenin [34,35]. So, to find out more about the mechanism(s) by which the caraway reduced colon premalignancies, in the present study we decided to consider the target of DMH metabolite i.e β-catenin at protein and mRNA levels induced by DMH in whole caraway seed treated animals concomitant with its essential oil at higher same dose (0.2% in diet).

The results indicated that DMH treatment ensued ACF formation accompanied with increase (~2 folds) in colonic β-catenin at protein and mRNA levels ($P<0.05$) (Table 1 and Fig. 2-4). In addition, the main detoxification enzymes involving in xenobiotic metabolisms were disturbed in DMH treated groups (Table 2).

Oral administration of caraway seed powder and its essential oils (0.2% in diet) to DMH-treated rats significantly inhibited colonic ACF developments ($P<0.05$) (Table 1 and Fig. 2). Our biochemical data indicated that the mechanism(s) by which both caraways inhibited ACF formation is by modulating the DMH detoxification pathways in the liver which mediate detoxification and metabolic disposal of carcinogen leading to inhibition of carcinogenic process. Increase in CYP450 activity in DMH-treated rats confirming its role in detoxification of DMH as a carcinogen [6-9]. Also, induction of GST activity, one of the main biotransformation enzymes in phase II detoxification pathway, in liver of DMH treated rats may be due to its effective role in detoxification of carcinogenic metabolite of DMH [10,45]. In this connection, caraway seeds as well as oils decrease the hepatic CYP450 elevated in DMH treated rats. Likewise, the increased hepatic GST activity in DMH treated rats is compensated by both caraways (Table 2).

In the process of carcinogenesis, reactive oxygen radicals may damage specific genes that control the growth and differentiation of cells [46]. DMH metabolite produced by CYP450 may alter beta-catenin gene that control the growth and differentiation of the cells [16]. Treatment of rats with diet containing 0.2% caraway essential oils could successfully reverse the β-catenin levels in cooperation with decreased ACF formation (Table 1, Fig 2-4). Also, treatment of rats with diet containing 0.2% caraway seed powder could successfully inhibit the β-catenin expression in cooperation with decreased ACF formation (Table 1, Fig 2-4).

In regards to the effect of DMH metabolite produced by CYP450 on beta-catenin gene expression [15,16,18,19], the data of this research together with our previous results [34,35] imply that feeding of rats with caraway essential oils and powder could modulate the enzymes interfere with DMH metabolism (GST and CYP1A1) leading to the decreased level of β-catenin gene expression. These entire events, finally leaded to the decreased level of ACF formation terminating in decreased progression of colon carcinogenesis.

Other studies also indicate that natural products such as black tea polyphenols inhibit 1, 2-dimethylhydrazine induced colorectal carcinogenesis by inhibiting cell proliferation via Wnt/beta-catenin pathway [24]. One study demonstrates the chemoprotective effect of lutein against colon cancer by modulating the proliferative activity of K-ras, PKB, and β-catenin proteins [14]. Also, dammarane-type triterpene sapogenin (20(S)-25-OCH3-PPD; PPD25) isolated from the leaves of *Panax notoginseng* exerts its anticancer effect by targetting β-catenin signaling, suggesting that PPD25 may have potential as a chemotherapeutic and/or chemopreventive agent for colon and lung cancer [47]. A plant extract of *Eleutherine palmifolia* led to a significant decrease in the level of nuclear beta-catenin protein, resulted in the inhibitory effect on the transcription of TCF/beta-catenin in SW480 colon cancer cells in a dose-dependent manner together with selective cytotoxicity against three colorectal cancer cell lines [48]. Luteolin-7-O-Glucoside induced apoptosis by scavenging ROS and suppressing the expression of β-catenin in COLO 320 DM cells and effectively inhibited ACF development in DMH-induced experimental carcinogenesis [15]. Silibinin supplementation to DMH-treated rats restored the levels of GSH-dependent enzymes and decreased the levels of β-catenin, PCNA, argyrophilic nucleolar organizer regions and cyclin D1 [49]. Long-term feeding of various fat diets modulates azoxymethane-induced colon carcinogenesis through Wnt/beta-catenin signaling in rats [50].

In conclusion, these data indicate that caraway essential oils as well as caraway whole seed powder exert its chemopreventive effects by modulating the activities of hepatic xenobiotic enzymes leading to decreased level of colonic β-catenin at protein and mRNA expression. Of course, further studies are needed to understand the exact mechanism(s) by which caraway essential oils prevent DMH-induced colon carcinogenesis.

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