RESEARCH COMMUNICATION

Dietary Cardamom Inhibits the Formation of Azoxymethane-induced Aberrant Crypt Foci in Mice and Reduces COX-2 and iNOS Expression in the Colon

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Abstract

Recently, considerable attention has been focused on identifying naturally occurring chemopreventive compounds capable of inhibiting, retarding, or reversing the multi-step carcinogenesis. The primary aim of the present study was to identify the effects of a commonly consumed spice, viz., cardamom against azoxymethane (AOM) induced colonic aberrant crypt foci (ACF) in Swiss Albino mice. The secondary aim, was to explore the ability of cardamom to modulate the status of proliferation and apoptosis, and to understand its role in altering cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression. Male Swiss albino mice were injected with AOM (dose: 5mg/Kg body weight) or saline (Group 1) weekly once for two weeks. The AOM-injected mice were randomly assigned to two groups (Groups 2 and 3). While all the groups were on standard lab chow, Group 3 received oral doses of 0.5% cardamom, in aqueous suspension, daily for 8 weeks. Following treatment, significant reduction in the incidences of aberrant crypt foci ($p<0.05$) was observed. This reduction in ACF was accompanied by suppression of cell proliferation (mean Brdu LI in carcinogen control=13.91$\pm$3.31, and 0.5%cardamom=2.723$\pm$0.830) and induction of apoptosis (mean AI in carcinogen control=1.547$\pm$0.42 and 0.5% cardamom= 6.61$\pm$0.55). Moreover, reduction of both COX-2 and iNOS expression was also observed. These results suggest that aqueous suspensions of cardamom have protective effects on experimentally induced colon carcinogenesis. Cardamom as a whole and its active components require further attention if the use of this spice is to be recommended for cancer prevention.

Key Words: Cardamom - azoxymethane - aberrant crypt foci - COX-2 - iNOS

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Introduction

Colorectal cancer is still a leading cause of cancer deaths in the United States and is increasing at an alarming rate in developing countries due to changing dietary habits and life style (Greenlee et al., 2000; Yeole et al., 2001). Plants, vegetables, herbs and spices used in folk and traditional medicine are currently being recognized as cancer chemopreventive agents (Gupta, 2001); because of their antioxidant and anti inflammatory properties that plausibly contribute to their anticarcinogenic and antimutagenic activities (Ann et al., 2001). Some of them have many other beneficial effects like hypocholesterolaemic, hypoglycaemic, and antimicrobial properties (Krishnaswamy & Polasa, 2001).

Elettaria cardamomum [Family.Zingiberaceae], Cardamom, is a sweet spice and is employed as a medicinal flavouring agent. Cardamom has been reported to possess antioxidant properties. The existing use of this spice as antioxidant and anti-inflammatory agent in traditional and folklore medicine, prompted us to use it as treatment agent in our experiment.

The present study was designed to observe the inhibiting effect of aqueous suspension of cardamom on azoxymethane (AOM) induced colon carcinogenesis in Swiss Albino mice in terms of incidences of ACF. Enhancement of cell proliferation, and inhibition of apoptosis are considered to be the risk factors for tumour development as well as for multistep carcinogenesis in colon (Jenab et al., 2001).Thus, in the present study the ability of cardamom suspension to modulate the level of cell proliferation and apoptosis, was assessed.

Cyclooxygenase (COX)-1 and COX-2 are isoforms responsible for the production of prostaglandin (PG) from arachidonic acid. While COX-1 is constitutive .COX-2 is the inducible form and is overexpressed in colon tumors (Ferrandez et al., 2003; Ota et al., 2002). Similarly, inducible nitric oxide synthase (iNOS), a generator of cellular nitric

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oxide is also overexpressed in colon tumors (Barrachina et al., 2001; Watanabe et al., 2000). It is well known that the inhibition of COX-2 and iNOS activities lead to the prevention and/or control of colon cancer (Watanabe et al., 2000; Ricchi et al., 2003). Thus, in this study we also have aimed to test if suspension of cardamom modulates the expression of COX-2 or iNOS proteins.

Materials and Methods

Male Swiss albino mice, (obtained from animal colony of our Institution) were maintained in plastic cages (~6 mice / cage) at an ambient temperature of 22-25˚C on a 12 hour light / dark cycle with access to drinking water and pellet diet (Lipton India Ltd) ad libitum. Use of animals was under strict animal care ethics guidelines of the institute.

AOM (Sigma Chemicals Co. MO, USA) was injected intraperitoneally once a week for two weeks (5mg/kg b. w.). Cardamom was bought from local market. The spice was powdered in a mixer and diluted with distilled water so as to make a 0.5% (w/v) aqueous suspension of Cardamom. The suspensions were prepared fresh everyday before oral administration at a dose of 100µl / mouse per day. In our previous studies (unpublished), we used three doses, viz. 0.25%, 0.5% and 1% (w/v) solutions of cardamom in order to observe their effects on ACF formation during AOM induced colon carcinogenesis. Treatment with the selected dose (0.5% (w/v) aqueous suspension of Cardamom) was found to be most effective in reducing the incidences of ACF without any toxic manifestations.

Experimental Design

The experiment was designed in four sets according to the experimental parameters used. Each set, having 30 mice, was divided into 3 groups - Normal, Carcinogen Control and Cardamom treated groups. Each group in a set consisted of 10 mice. All the mice were injected with azoxymethane (AOM, dose: 5mg/Kg body weight) or saline (Group-1), weekly once for two weeks. The AOM-injected mice were randomly assigned to two groups (Groups2 and 3). While all the groups were on standard lab chow, Group 3 received oral dose of 0.5% cardamom, in aqueous suspensions, respectively; daily for 8 weeks. The treatment was commenced from the first day of AOM injection in Group 3. The different parameters were studied thereafter.

Set I: Assessment of ACF

After 8 weeks of 1st AOM injection, the mice in this set were sacrificed to assess the incidences of colonic ACF, identified, by staining with 2% methylene blue (Qualigens ltd.), on glass slides with mucosal side up under the light microscope at 100X magnification, by their large and elliptical luminal opening (Mclellan et al., 1991; Bird, 1987) .The total number ACF was counted for each mouse.

Set II: Detection of In situ Cell Proliferation

Cell proliferation in colon was measured using 5-bromo-2′-deoxyuridine labelling (Davidson et al., 2000) and a Detection Kit II, procured from Roche Molecular Biochemicals. The Proliferative Index (PI) was determined by dividing the number of labelled cells by the total cells counted and multiplying by 100.

Set III: In situ Cell Death detection (Apoptosis)

Apoptotic cells in colon were visualized using the terminal deoxynucleotidyl transferase (TdT) - mediated dUTP- biotin nick end labelling (TUNEL) method with the help of in situ cell death detection kit, AP (Roche Molecular Biochemicals). Apoptotic Index (AI) was determined as the percentage of the labelled nuclei with respect to the total number of nuclei counted (Caderni et al., 2000).

Set IV: SDS-PAGE Western blotting for COX-2 and iNOS Expression

Distal 5cms of colon tissues were removed, washed in PBS at 0˚C, cut into pieces and homogenized in 5 volumes of ice-cold homogenizing buffer (0.1M NaCl, 0.01M Tris-Cl, 0.001M EDTA) containing 1mM PMSF, 1µg/ml Aprotinin, 0.1 mM Leupeptin. After centrifugation at 13000 rpm for one hour at 4˚C, the supernatants were estimated for their protein content using Bovine Serum Albumin (BSA) as a standard (Lowry et al., 1951).

SDS-PAGE and Western blotting were carried out essentially as described by Laemmli. (1970). Two separate gels, one 10% (for COX-2) and another 8% (for iNOS) were run simultaneously. Electrophoretically resolved proteins were transblotted onto Immobilon-P (PVDF) membranes and subsequently blocked with 5% TBS blotto A (Santa Cruz Biotech. Inc., Santa Cruz, California) and incubated with the anti-COX-2 and anti-iNOS rabbit polyclonal antibodies (Santa Cruz Biotech. Inc., Santa Cruz, California) diluted 1:500 in TBS containing 0.5% TBS blotto A (non fat dried milk). After extensive washing, blots were reincubated with HRP-conjugated anti-rabbit secondary antibody (Santa Cruz Biotech. Inc., Santa Cruz, California). The protein bands were then visualized using Luminol reagent (Santa Cruz Biotech. Inc. Santa Cruz, California) and applied for exposure of X-ray film.To demonstrate equal loading of samples onto the gel, the PVDF membranes were stained with Coomassie Brilliant blue (Tao et al., 2002).

Statistical Analysis

The differences in mean values among different groups were tested and the values were expressed as mean ± SD. Data obtained from incidences of ACs, AI and Brdu LI were analyzed using Students-t-test. All the statistical calculations were carried out using Microsoft Excel and a P-value < 0.05 was considered significant.

Results

ACF

At the end of 8 weeks, in comparison to the carcinogen controls, total number of ACF was significantly lower by

Table 1. Effects of Aqueous Suspension of Cardamom on AOM Induced ACF in Mice Colon

Groups	Total ACF (mean±SD)	% of inhibition after 8 weeks	ACF (4 or more AC) (mean±SD)	% of inhibition after 8 weeks
Normal	0	-	0	-
Carcinogen control	97.75 ± 6.53		56.75 ± 8.01	
0.5% Cardamom	50.5 ± 6.73**	48.33	1.5 ± 0.5*	97.3

Significant differences have been indicated by the asterisks on the respective values (*p<0.001, **p<0.005). Percentage inhibition has been calculated with respect to the Carcinogen control values.

48.33 % in the cardamom (p<0.005) treated group (Table 1). Mean numbers of ACF, consisting of 4 or more aberrant crypts in each group, are also presented in TableI. The numbers of foci consisting of 4 or more crypts were significantly lower (p<0.001) in mice treated with aqueous suspension of cardamom. No aberrant crypts were visible in the colons of normal mice.

Cell Proliferation and Apoptosis

Sections from distal 5cm of colons were chosen and the cells were counted. Significant reduction in Brdu LI (Figure 1) and significant induction in AI (Figure 2) was observed in the treatment group. The Brdu LI increased significantly in the carcinogen control group (13.91±3.31; p<0.001) from that observed in the normal group (2.911±0.335). Cardamom significantly reduced Brdu LI (2.723±0.830) in comparison to the carcinogen control group (p<0.001).

AI was found to decrease in the carcinogen control group (1.547±0.421; p<0.001) with respect to the normal group (2.996±0.603). Following treatment, the AI increased significantly in 0.5% cardamom (6.61±0.55) as compared to the carcinogen control group (p<0.001).

Cyclooxygenase-2 Expression

Expression of COX-2 protein in Normal, Carcinogen control and Cardamom treated animals has been represented in Figure 3. COX-2 immunoreactivity with polyclonal anti-COX-2 antibody was not detectable in colonic fraction of Normal mice. In contrast, dark immuno reactive bands of COX-2 at a position of 72KD were observed in animals treated with AOM only (Carcinogen control). In case of the cardamom treated animals, all the lanes showed negligible expression of COX-2.

Inducible Nitric Oxide Synthase Expression

Data for expression of iNOS are presented in Figure 4. As with COX-2 expression, there was no detectable iNOS immunoreactivity with polyclonal anti-iNOS antibody in colonic fractions of normal mice. Whereas immunoreactive bands at a position of 117KD were clearly observed in carcinogen control mice, they were only very faint in the treated group.

Discussion

The major aim of the present study was to elucidate the inhibitory role of cardamom against AOM induced colonic

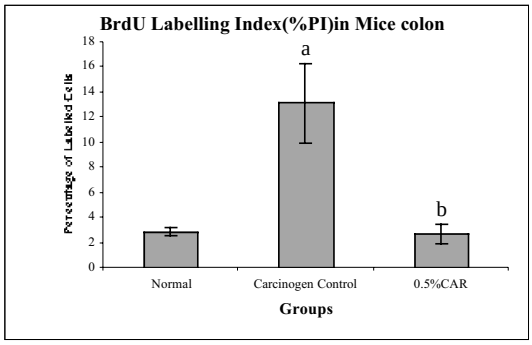

Figure 1. Cell Proliferation is Expressed as % PI, which is calculated as percentages of Brdu labeled cells with respect to the total no. of cells counted throughout the distal 5 cm of colons from each group. a: p<0.01 w.r.t. the Normal; b: p<0.001 in comparison with the Carcinogen control value.

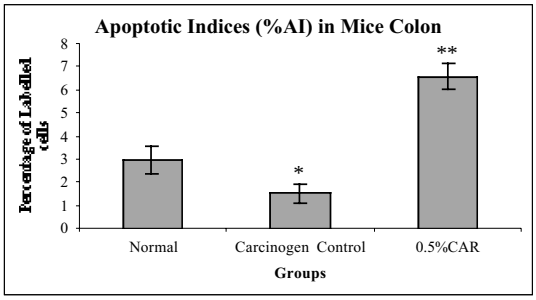

Figure 2. Apoptosis is Expressed Through AI, calculated as the percentage of TdT-labeled cells w.r.t the total number of cells counted throughout the distal 5 cm of colons from each group. * : p<0.01 w.r.t. the Normal; **: p<0.001 in comparison with the Carcinogen control value.

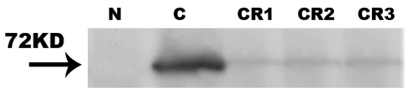

Figure 3. Expression of COX-2 Protein After Immunoblotting in Different Groups of Animals. Lane N represents the proteins from Normal group and C from Carcinogen Control group. CR1, CR2 and CR3 show the COX-2 expression in cardamom-treated animals.

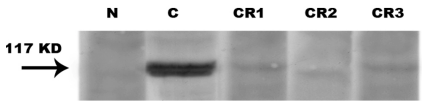

Figure 4. Expression of iNOS Protein After Immunoblotting in Different Groups of Animals. Lane N represents protein from Normal group and C from Carcinogen Control group. CR1, CR2 and CR3 show the iNOS expression in cardamom-treated animals

ACF. ACF are recognized as early preneoplastic lesions of the colon (Bird, 1995; Mclellan et al., 1991). They have consistently been observed in experimentally induced colon carcinogenesis in laboratory animals (Mclellan et al., 1991). Pretlow et. al. (Pretlow et al, 1992) have also shown that these lesions are present in the colonic mucosa of patients with colon cancer. ACF have been proposed as biomarkers for short term bioassays for assessing the chemopreventive potential of drugs, diets and naturally- occurring compounds (Dashwood, 1999; Michael et al., 1994). The results obtained in this study shows that aqueous suspension of cardamom was able to reduce the incidence of ACF in the colon of male Swiss Albino mice.

In the present study cell proliferation as reflected by Brdu LI in colon was decreased significantly and apoptosis as shown by AI was increased significantly by cardamom treatment. This observation is of significance because the balance between cell proliferation and apoptosis is important in the genesis of colon carcinoma (Bedi et al., 1995). As our results suggest, the ability of cardamom to induce apoptosis during the early stages of colon carcinogenesis could possibly be related to its ability to lower total ACF. However, specific studies are warranted to understand if apoptosis is occurring in the ACF per se.

The results of the present study also demonstrate that administration of cardamom also induced AI significantly. This may be due to its active ingredient- 1, 8- cineole which has been shown to trigger apoptosis in human leukaemia cells (Moteki et al., 2002). In various studies it has already been reported that monoterpenes, especially limonene (a cyclic monoterpene), which is also present in cardamom has antiproliferative activity. D-limonene causes G1 cell cycle arrest thus inhibiting proliferation (Belanger, 1998; Hudes et al., 2000; Ripple, 2000; Reddy et al., 1997; Kaji et al., 2001; Uedo et al., 1999; Crowell et al., 1994; Asamoto et al., 2002).This corresponds well with the result obtained in this study, where a reduction in PI has been observed in the cardamom treated group.

One previous study observed a marked anti-inflammatory activity of the oil extracted from commercial *Elettaria cardamomum* seeds, in doses of 175 and 280 microliters/kg against acute carrageenan-induced planter oedema in male albino rats (al-Zuhair et al., 1996).In our present study also, the anti-inflammatory activity of cardamom has been proven by a decrease in the COX-2 expression in the cardamom treated group, as determined by Western blot analysis. The inducible NOS produces much greater amounts of NO than constitutive NOS and appears to be the "pathophysiological"form of the enzyme because high concentration of nitric oxide is associated with inflammatory diseases, mutagenesis, and formation of carcinogenic N nitrosamines (Moncada et al., 1991; Moncada & Higgs, 1995; Marletta, 1993; Schimdt et al., 1993). The results obtained in this experiment suggest that cardamom was able to reduce the expression of iNOS as compared to the carcinogen control group. As the involvement of COX-2 and iNOS in colon carcinogenesis is well known, the observed reduction in ACF in the treatment group may be due to this inhibition in COX-2 and iNOS expression as determined by Western Blot analysis in our experiment.

In conclusion, our preliminary study on reduced risk of colon cancer associated with dietary consumption of cardamom is of great importance particularly in relation to the global prevalence of this cancer. This beneficial spice, therefore, is worthy of serious consideration for further study. Future studies should me aimed at elucidating the metabolism, pharmacokinetics, and toxicity of the active compounds of cardamom. Clinical trials could be useful for assessing the ability of cardamom to prevent human colon cancer or pre-neoplastic lesions.

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