Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(6):490-496

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Dietary essential oil protects gastrointestinal tract against 1,2-dimethylhydrazine and dextran sodium sulfate induced ulcerative colitis associated colon carcinogenesis

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ABSTRACT

Colorectal cancer (CRC) is the third common malignancy and a major cause of morbidity and cancer-related death worldwide. Carvacrol is a dietary essential oil possesses several pharmacological effects such as antioxidant, anti-inflammatory, anti-proliferative in experimental animal models. The present study is aimed to analyze the effect of carvacrol on matrix degrading lysosomal enzymes, collagen accumulation and glycoconjugate levels during 1, 2 dimethylhydrazine (DMH) plus dextran sodium sulfate (DSS) induced inflammation associated colon carcinogenesis. Ulcerative colitis associated colon cancer was developed in male Fischer rats (F344 rats) by three subcutaneous injection of DMH (40 mg/kg body wt) in the first week and free access to drinking water containing 1% DSS for next one week followed by 7-14 days of water as three cycles. Dietary supplementation of carvacrol was given before and after induction period through orally at a concentration of 50mg/kg body weight. The increased level of collagen deposition was observed by histopathological staining in DMH/DSS-induced rats were decreased upon carvacrol treatment. Furthermore, the increased activities of matrix degrading lysosomal enzymes in DMH/DSS-induced rats were regulated upon carvacrol supplementation. Carvacrol also reduced the mucus depletion due to ulcer in tumor bearing rats. In conclusion, Carvacrol may be act as a potent chemopreventive agent against experimental colon carcinogenesis.

Key words: Colon cancer, Carvacrol, Mucus, collagen, Lysosomal enzymes

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INTRODUCTION

Gastrointestinal (GI) tract mucosa acts as an important barrier to genotoxic chemicals and protects an individual from different antigenic and inflammatory reactions [1]. Epidemiological reports states that over 25% of all human cancer cases are related with chronic inflammation [2]. The relationship between inflammation and malignant diseases is increasing the risk of CRC in patient with prolonged inflammation. Animal models are still considered as suitable model for better understanding of the chemotherapeutic efficacy for inflammation associated colon carcinogenicity. Our laboratory have identified as inbred Fischer 344 rat is moderately susceptibility to the combination of DMH/DSS within short period of 10 weeks [3]. Tumors induced in rats exposed to DMH/DSS treatment accurately recapitulate the pathogenesis observed in human IBD [4].

Mucus secreting goblet cells are the major source of mucin and responsible for the viscous nature of the mucus gel in the GI tract. Mucins are the major glycoproteins of the mucus layer that coats and barrier against infection and toxic chemicals [5]. On the surface of the gastrointestinal tracts, glycoprotein bearing carbohydrate moiety in the

mucosal tissues is responsible for cell adhesion, proliferation and transport of metabolites across the cell membranes in the colonic epithelium [6]. The mucopolysachacride were displayed in neoplastic cells of tumor bearing animals [7]. Dysfunction of mucin glycosylation has been documented to cause increased intestinal permeability and increased susceptibility to DSS-induced colitis in experimental animals [8].

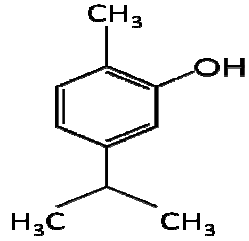

Fig.1 Chemical Structure of Carvacrol

Carvacrol

Carvacrol, (5-isopropyl-2-methylphenol) is the major compound of essential oils produced by numerous aromatic plants and spices of the family Lamiaceae [9], which includes the genera Origanum and Thymus [10]. Essential oils are recognized as a safe food additive and it is consumed with flavoring agent in sweets, beverages, and chewing gum [11]. Carvacrol is recommended as safe for consumption by the Federal Drug Administration (FDA) for its uses in food industry [12]. Due to its lipophilic or hydrophobic nature, carvacrol has an effective impact on biological membranes. Cytotoxicity of carvacrol has been documented as median lethal dose to experimental rats at LD50 910 mg/kg [13]. However, there are no detailed reports regarding the gastroprotective role of carvacrol against ulcerative colitis related colon carcinogenesis.

EXPERIMENTAL SECTION

Animals

Male Fischer 344 Rats (5 weeks old) procured from the National Center for Laboratory Animal Science (NCLAS), National Institute of Nutrition (NIN), Hyderabad, India. The experiments were conducted in accordance with the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines (**IAEC No:07/01/2012**). Animals were maintained in polystyrene cages at an ambient temperature of 25 ± 2ºC and 12/12 h of light-dark cycle in animal cages with six animals per cage. The animals were fed with commercial pellet diet (Hindustan Lever Ltd., Bangalore, India) and water ad libitum.

Chemicals

Dextran sodium sulfate was purchased from MP Biomedicals, USA and 1, 2-dimethylhydrazine and Carvacrol was purchased from Sigma Aldrich, St. Louis, MO, USA. All other chemicals used were of analytical grade.

Induction of colitis associated colon cancer

Colitis associated colon cancer model was developed previously described methods [3]. Briefly, male F344 rats (n=6) received three subcutaneous injections of DMH (40 mg/kg b.wt) in the first week of the experimental period. DSS was dissolved in normal drinking water at a concentration of 1% (w/v). The rats received 1% DSS orally followed by DMH as three cycles which consist of 7 days of DSS and 14 days of normal drinking water. The control rats were given subcutaneous dose of saline alone.

Experimental procedure

Animals were divided into five groups, each group consisting of 6 animals;

Group I: Received standard diet and pure drinking water **(Control)**

Group II: Rats were received Carvacrol, 50mg/kg b.w (this dose of carvacrol was fixed based on the effective dosage fixation studies) for the entire period of the experiment **(Carvacrol alone)**

Group III:Rats were treated with DMH in the first week and DSS in three cycles for 10 weeks **(DMH/DSS-Induced).**

Group IV:Rats were received Carvacrol, (50mg/kg b. w) for one week prior to the induction **(Pre- treatment)**

Group V: Rats were received Carvacrol, (50mg/kg b. w) for one week after the induction period **(Post-treatment)**

After the end of the experimental period of 12 weeks, all the animals were killed by cervical dislocation after an overnight fast.

Assay of lysosomal hydrolases

Colonic lysosomal enzymes were separated according to the method of Wattiaux et al. [14].The activity of β-D-Glucuronidase was determined by the method of Kawai and Anno [15]. B-D-galactosidase was determined by the method of Conchie et al. [16]. β-N-Acetyl glucosaminidase was determined by the procedure of Moore and Morris [17]

Periodic acid-schiff's reagent-Alcian Blue (PAS-AB)

Histopathological analysis of glycoconjugates using PAS-AB staining was carried out by previously described method [18]. Briefly, deparaffinized colonic tissue sections of 4μm thickness were re- hydrated using graded ethanol solutions and distilled water. The sections were stained using 0.5% of Alcian blue followed by 2% periodic acid and Schiff's reagent in dark for 20 min. The slides were then visualized under a light microscope (Nikon XDS- 1B).

Picrosirius Red staining

Tissue sections were stained with picrosirius red solution to identify collagen fibers as described earlier [19]. Staining of extra cellular matrix in 4 μm-thick colonic tissue sections was performed by picrosirius red staining solution for 30-45 min and washed in two changes of acidified water. Tissue sections were cleared in two changes of 100% xylene and mount in a DPX medium.

Mucicarmine staining

Paraffin embedded colon tissue sections of 4 μm thickness were rehydrated first in xylene and then in graded ethanol solution and distilled water each for 5 min [20]. The tissue sections were incubated in freshly prepared Weighert iron haematoxylin solution for 10–15 min, washed in running tap water and differentiated in acid alcohol. The tissue sections in the glass slides were then incubated in mucicarmine solution for 1hr preceded by running tap water wash and followed by distilled water rinse. The slides were then subjected to metanil yellow staining for 10 min followed by a wash with distilled water and alcohol treatment. The tissue sections in the glass slides were finally dipped in xylene, air dried and mounted using DPX. The appearance of deep rose red color showed the presence of mucin content. The images of the tissue sections were captured at 20X magnification.

Statistical analysis

All the grouped data were significantly evaluated with SPSS/10 software. Hypothesis testing method included one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test $p < 0.05$were considered to indicate statistical significance. All the results were expressed as Mean ± SD for six rats in each group.

RESULTS

Carvacrol alleviates hyperactivity of matrix-degrading lysosomal enzymes

The activities of matrix-degrading lysosomal enzymes in serum and colon of control and experimental groups of animals are presented in Tables 1 and 2. A significant elevation in the activities of these enzymes was evident in DMH/DSS-induced rats when compared to control rats. Administration of Carvacrol to tumor bearing rats altered the activities of these enzymes towards normal values.

Table.1 Carvacrol effects on the activities of matrix degrading lysosomal enzymes in the serum of control and experimental groups of rats

Experimental Groups	β-d-Glucuronidase	β-d-Galactosidase	N-acetyl- β-d-glucosaminidase
Control	34.17±2.69	37.24±2.86	40.69±3.342
Carvacrol alone	33.53±2.75^{ns}	37.79±3.04 ^{ns}	41.04±3.53 ^{ns}
DMH/DSS Induced	53.16±5.08^{a}	49.67±4.89 ^{a}	65.72±6.05 ^{a}
Carvacrol+ DMH/DSS	39.42±3.64^{b}	41.42±3.81 ^{b}	46.75±4.29 ^{b}
DMH/DSS+Carvacrol	42.39±3.56^{c}	43.56±3.09 ^{c}	48.67±4.35 ^{c}

Values are given as mean ±SD for 6 rats in each group. Values not sharing a common superscript letter (ns, a, b, c) differ significantly. Values are statistically significant at p < 0.05.

ns, non-significant

^{ns} Carvacrol alone vs control

^{a} DMH/DSS vs control

^{b} Carvacrol + DMH/DSS (Pre-treatment) vs DMH/DSS

^{c} DMH/DSS + Carvacrol (Post-treatment) vs DMH/DSS

Table.2 Carvacrol effects on the activities of matrix degrading lysosomal enzymes in the colonic tissue of control and experimental groups of rats

Experimental Groups	β-d-Glucuronidase	β-d-Galactosidase	N-acetyl- β-d-glucosaminidase
Control	12.43±0.72	16.02±0.89	21.43±1.31
Carvacrol alone	11.52±0.69[ns]	16.87±1.07[ns]	22.08±1.34[ns]
DMH/DSS Induced	18.76±1.54[a]	27.63±2.25[a]	29.59±2.30[a]
Carvacrol+ DMH/DSS	14.05±0.87[b]	20.74±1.56[b]	24.26±1.69[b]
DMH/DSS+Carvacrol	16.08±0.92[c]	22.41±1.72[c]	26.35±1.78[c]

Values are given as mean ± SD for 6 rats in each group. Values not sharing a common superscript letter (ns, a, b, c) differ significantly. Values are statistically significant at
$p < 0.05$.
ns, non-significant
[ns] Carvacrol alone vs control
[a] DMH/DSS vs control
[b] Carvacrol + DMH/DSS (Pre-treatment) vs DMH/DSS;
[c] DMH/DSS + Carvacrol (Post-treatment) vs DMH/DSS.

Effect of Carvacrol on collagen deposition

Fig.2 shows the effects of DMH/DSS and carvacrol on collagen deposition. We found that prominent level of collagen deposition was observed in the colon of DMH/DSS induced rat which was identified by picrosirius red staining (Fig.2c). Alternatively, Carvacrol administrated groups were extensively diminished type IV collagen accumulation in the extra cellular matrix of colonic tumors and improves colon histology when compared with DMH/DSS induced group of rats. Statistically significant differences ($p < 0.05$) were observed between the control group and the DMH/DSS group in the histopathological examination of the colon tissue sections. An increased amount of collagen accumulation was noticed in the colon tissues of DMH/DSS-induced group (Fig.2c). Strong red staining which was evident in DMH/DSS-induced group was considerably reduced in carvacrol-treated group (Fig. 2d&e). But, in corresponds to control (Fig.2a) and Carvacrol alone groups (Fig.2b) there was negligible amount of collagen deposition were observed.

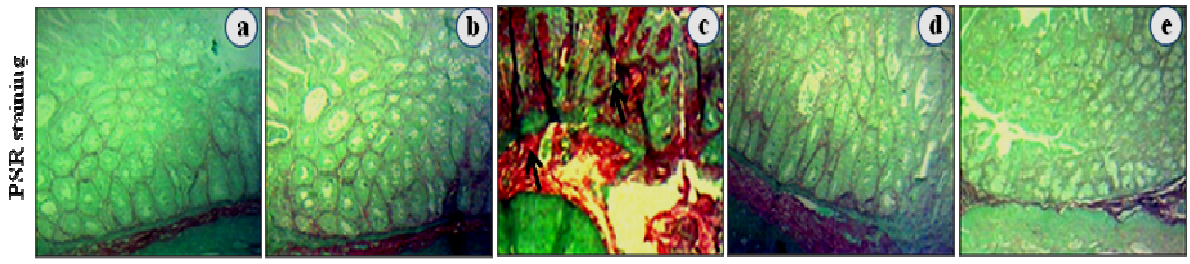

Fig.2 Picrosirius red (PSR) staining in the colon of control and experimental groups of rats (20X). Arrow shows collagen deposition (a) Control (b) Carvacrol alone. (c) DMH/DSS induced group. (d) Carvacrol + DMH/DSS (pre-treated) (e) DMH/DSS + Carvacrol (post-treated)

Effect of carvacrol on glycoconjugates

Using the Periodic Acid Schiff staining, glycoconjugates is detected in the colon of control and experimental rats (Fig.3c). Carvacrol treated to colon cancer bearing rats showed a mild decrease in glycoconjugates with degradation of tumor cells (Fig.3d & e). There were no significant changes were observed in control (Fig.3a**)** and carvacrol alone treated group (Fig.3b) of animals.

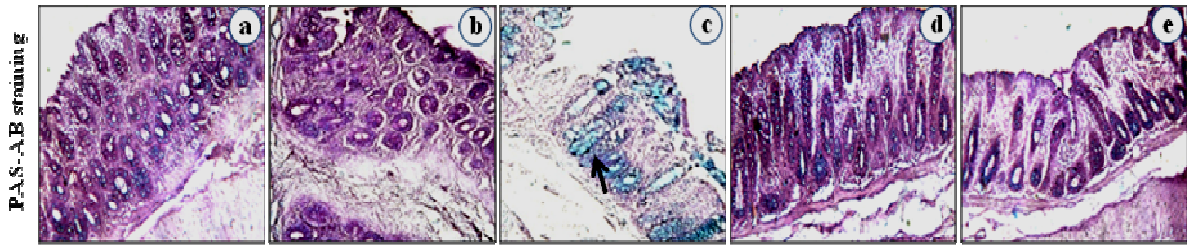

Fig.3 Periodic Acid Schiff–Alcian blue (PAS-AB) staining in the colon of control and experimental groups of rats (20X). Arrow shows Glycoconjugates levels (a) Control (b) Carvacrol alone. (c) DMH/DSS induced group. (d) Carvacrol + DMH/DSS (pre-treated) (e) DMH/DSS + Carvacrol (post-treated)

Effect of carvacrol on mucin depletion

Analysis of mucin content changes in the colonic sections was done by mucicarmine staining. (Fig.4). Colon of control and carvacrol alone-supplemented rats (Group I and II) showed strong and intense mucicarmine staining. The mucosa of DMH/DSS-exposed rats showed faint and weak mucicarmine staining (Fig.4c) and progressive loss of mucin. Carvacrol supplemented to DMH/DSS-induced rats showed increased staining, as well as an obvious increase in the mucus cell size and number as compared to those found in the mucosa of the induced rat (Group III). This obvious increase in mucus content was more pronounced in mucosa of rats supplemented with carvacrol (Fig.4d & e) as compared to DMH/DSS induced rats (Group III).

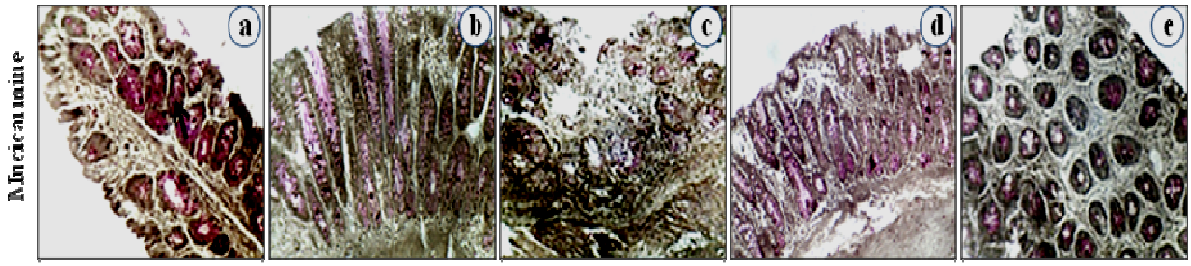

Fig.4 Mucicarmine staining in the colon of control and experimental groups of Rats (20X)

Arrow shows mucus producing goblet cells (a) Control (b) Carvacrol alone (c) DMH/DSS induced group; Damaged &reduced level of goblet cells. (d) Carvacrol + DMH/DSS (pre-treated) (e) DMH/DSS + Carvacrol (post-treated).

DISCUSSION

Collagen comprised the scaffold of tumor microenvironment and affects microvascular niches such that it regulates extra cellular matrix remodeling by collagen degradation and deposition. Additionally, thickening and linearization of collagen fibers facilitate active tissue invasion and tumor vasculature. Several collagens, including collagen I, II, III, V and IX, show increased deposition during tumor progression [21, 22]. The basement membrane has the capacity to deposit type IV collagen in the extra cellular matrix during tumor progression [23]. However, collagen degradation is associated with the extensive microvascular proliferation of neuroblastoma cells [24]. Collagen detection in histological samples represents the pathological conditions results from an imbalance state of collagen due to chronic inflammatory disorders [25]. This abnormal deposition of collagen reflects extra cellular matrix damage in the DMH/DSS induced inflammation related tumorigenesis. Carvacrol treated rats showed limited amount of collagen accumulation which substantiate the protective role of carvacrol on mucosal epithelial barrier through regulating extra cellular matrix degradation.

Glycoconjugates are the specialized markers for cell surface membrane proteins in colonic epithelium and responsible for colon epithelial cell proliferation and metastasis [7]. Elevations of glycoconjugates serve as a classical marker in tumorigenic process via alter the stiffness of cell membrane with higher proliferative activity in neolpastic diseases [26]. Dysregulation of cell surface protein glycosylation is correlated with ulcerative colitis mediated colon carcinogenesis [8]. Glycoconjugates bearing carbohydrate moiety plays a critical role in mediating cell surface function, such as cell–cell interaction and transport of metabolites across the cell membranes [27]. Carvacrol supplementation significantly ($p<0.05$) reduced the level of glycoconjugates in DMH/DSS exposed rats.
Lysosomes are considered as the garbage bags in which waste material is disposed to regulate cellular homeostasis [28]. Lysosomal enzymes of macrophages have an ability to trigger heterocytolysis to destruct the malignant cells [29]. Glycosidase are major source of lysosomal enzymes responsible for the degradation of carbohydrate subunit in the glycoproteins, glycolipids, glycosaminoglycans and polysaccharides [30]. In this study, the N-acetyl-β-D-glucosaminidase level was elevated than the other enzymes. The activity of N-acetyl-β-D-glucosaminidase has been reported as a marker in malignant tissues. Transformation of neoplastic cell in colonic epithelium leads to increases in lysosomal volume and enhanced secretion of lysosomal proteases [31]. Carvacrol supplementation normalized the levels of lysosomal enzymes in DMH/DSS exposed tumor bearing rats. Thus, the results of this study revealed that carvacrol attenuates disease progression by inhibiting the release of lysosomal enzymes.

Mucin is a complex glycoprotein secreted by the intestinal goblet cells [32] and protects the epithelial barrier against invading foreign agent such as microorganism and toxic chemicals [18]. Goblet cells are gradually decreased in number and size during ulcerative colitis. Recent studies in rodent models of colitis substantiate the importance of

mucus in maintaining the integrity of protective epithelial barriers whose degradation can leads to ulcerative colitis. The histopathological staining of mucus using mucicarmine dye revealed that restoration of mucus layer was observed in carvacrol treated colitis induced rats. Accumulating evidence indicates that mucin play a crucial role in several stages of colon carcinogenesis [33]. The data presented in this study indicates depletion of mucus secreting goblet cells in DMH/DSS-induced rats which could be due to hydrolysis of mucus barrier by exalting activity of mucinase. Carvacrol treatment reduced mucinase activity and abrogates mucin hydrolysis [34]. Similarly, the results of this study suggest that carvacrol supplementation effectively protected the mucosal epithelial layer of the gastrointestinal tract against DMH/DSS induced colon carcinogenesis.

CONCLUSION

In conclusion, we have used a reliable animal model to evaluate the protective role of carvacrol against gastrointestinal tumor in F344 rats. We found that carvacrol potentially restored the architecture of mucus epithelial layer, reduced glycoconjugate levels and decreased collagen deposition in DMH/DSS induced rats. Taken together, these results suggest that carvacrol may serve as a potential dietary essential oil to treat the ulcerative colitis mediated digestive diseases.

Acknowledgment
KA is a recipient of BSR Research fellowship (UGC-BSR/2013/RF) from University Grants Commission (UGC), New Delhi.

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