Methanolic extract of *Terminalia chebula* protects against DMH-induced colon damage in Wistar rats by restoring antioxidant enzyme activities and suppressing inflammation

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Abstract:

Present study deals with the chemopreventive efficacy of *T. chebula* against DMH induced colon toxicity and its role in inflammatory response. *T. chebula* is a popular traditional medicine not only used in India but also in other countries of Asia and Africa. This is used in traditional medicine due to the wide spectrum of pharmacological activities associated with the biologically active chemicals present in this plant. In the present study, we report the chemopreventive effects of *T. chebula* against DMH induced colon oxidative stress and inflammatory response. Colon toxicity was initiated by single i.p injection of DMH 20 mg/kg BW for 16 wk. To ascertain the molecular mechanism implicated in the anti-inflammatory activity of *T. chebula* its effect was investigated on inflammation: iNOS and COX-2 expression, and on levels of nitrite and myeloperoxidaes. Pretreatment of animals with *T. chebula* at both doses (50 and 100 mg/kg body weight) markedly inhibited all. Further, DMH enhances colonic lipid peroxidation, with concomitant reduction in colon glutathione content (GSH), antioxidant enzymes, and phase II metabolizing enzymes. Treatment with *T. chebula* ameliorate the damaging effects induced by DMH through a protective mechanism that may involve reduction of increased oxidative stress markers, alleviate the expressions, levels and activities of COX-2, iNOS, TNFa , PCNA, MPO, NO and Mast cell infiltration. From the results, it could be concluded that from results suggest *T.chebula* as an effective chemopreventive agent having the capability to obstruct DMH induced colon toxicity and acts reasonably by inducing anti-apoptotic, anti-inflammatory, anti-proliferative and anticancer effects in the rat model.

Keywords: DMH - inflammation - oxidative stress - tumor promotion and *T. chebula*

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Introduction:

Colon cancer is one of the diseases causing cancer and deaths in both men and women throughout the world. The etiology of colorectal cancer is very complex and may be attributable to combined actions of environmental, life styles and inherited factors (1). Epidemiological studies indicated that colon cancer is strongly associated with the diet, and thus the occurrence of colorectal cancer may be prevented by dietary modifications (2).

1, 2 Dimethyl hydrazine (DMH), is a effective colon carcinogen, inducing cancer in different organ specially in colon and rectum in experimental animals (3, 4) and is the most widely used model of chemically induced colon carcinogenesis. DMH and its metabolites are widely used agents for the induction of colorectal carcinogenesis in rodents. DMH is metabolically activated in the liver by a series of reactions through intermediates AOM and methylazoxymethanol (MAM) to the ultimate carcinogenic metabolite, highly reactive methyldiazonium ion (5). MAM can be excreted into the bile and transported to the colon (the development of small intestinal tumors distal to the

entrance of the bile duct into the intestine is ascribed to this path) or enter directly into epithelial cells of the colon from blood circulation (5). Some studies have also demonstrated that rat colon epithelial cells are capable of metabolizing DMH into the carcinogenic metabolite without previous metabolism by other tissues or colon bacteria (6, 7). The ultimate carcinogenic metabolite of DMH is responsible for methylation of the DNA bases of various organs, including epithelial cells in the proliferative compartment of the crypts, which result in a great loss of colonic cells by apoptosis, an increase in proliferation, and an apparent increase in mutations of colonic epithelial cells (8).

Previous studies have demonstrated that cyclooxygenase-2 (COX-2), which catalyzes the adaptation of arachidonic acid to prostaglandins, and inducible nitric oxide synthase (iNOS), which stimulates nitric oxide (NO) synthesis, are up-regulated during pro-inflammatory and inflammatory of bowel disease (9). COX-2 selective inhibitors are believed to suppress colonic damage induced by Azoxymethane (AOM), a carcinogenic metabolite of 1,2-dimethylhydrazine (DMH), and inhibit colon tumor formation (10).

T. chebula is a popular traditional medicine not only used in India but also in other countries of Asia and Africa. This is used in traditional medicine due to the wide spectrum of pharmacological activities associated with the biologically active chemicals present in this plant. It is used for the treatment of number of diseases like cancer, paralysis, cardio vascular diseases, ulcers, leprosy, arthritis, gout, epilepsy etc. It has been reported as antioxidant (11), antidiabetic (12), antibacterial (13), antiviral (14), antifungal, anticancerous, antiulcer, antimutagenic, wound healing activities

etc. The present investigation pertains to protective action of *Terminalia chebula Retzius* (*T. chebula Retz.*) (Combretaceae) against DMH induced colon toxicity. *T. chebula* commonly known as ``black myroblans'' in English and ``Harad'' in Hindi The phytochemical investigation of *T. chebula* shows the presence of several phenols such as gallic acid, ellagic acid, tannic acid, ß-sitosterol, ethyl gallate, chebulic acid, and mannitol. It is also one of the richest sources of ascorbic acid (15). These compounds responded to various cell lines basically by inhibition of proliferation and induction of cell death: apoptosis and necrosis. The antioxidant property of *T. chebula* due to the presence of active phenols we propose that *T. chebula* may protect against DMH induced colon toxicity in Wistar rats.

Materials and Methods

Chemicals

Reduced glutathione (GSH), nicotinamide adenine dinucleotide phosphate reduced (NADPH, ethylene diamine tetra-acetic acid (EDTA), nicotinamide adenine dinucleotide reduced (NADH), thiobarbituric acid (TBA), trichloroacetic acid (TCA), bovine serum albumin (BSA), 1,2-dithio-bis-nitrobenzoic acid (DTNB), Terminalia chebula (T. Ch) SAIBA Industries PVT. LTD. India, 1-chloro-2,4-dinitrobenzene (CDNB), 1,2- Dimethylhydrazine (DMH), glutathione reductase were obtained from Sigma (Chemical Co.,St Louis, MO), ferric nitrate, ammonium thiocyanate, hydrogen peroxide, magnesium chloride, di-sodium hydrogen phosphate, sodium di-hydrogen phosphate and sodium hydroxide were purchased from E. Merck Limited, India. Primary antibodies were rabbit anti-COX-2 (dilution 1:200, Santa Cruz, USA), rabbit anti-iNOS

(1:500, Jackson ImmunoResearch laboratories, INC.) and PCNA (dilution 1:300,).

Animals and Treatment schedule

The experiments were carried out using male Albino Wistar rats, 120-150 g, obtained from the Central Animal House of Hamdard University, New Delhi, India. Thirty animals were randomly divided into five groups of six animals each. The rats were kept in polypropylene cages and were kept in room maintained at 25±2°C with a 12 h light/dark cycle. They were given free access to diet and water. The rats of Group I control group received distilled water orally three times a week for whole study. Group II received DMH subcutaneous at the dose of 20 mg/kg body weight once a week. Group III (dose 1 of *T. chebula*) received *T. chebula* orally at the dose of 50 mg/kg body weight five times a week for 15 consecutive weeks. Group IV and V (dose 2 of *T. chebula*) received *T. chebula* at the dose of 100 mg/kg body weight three times a week for 15 consecutive weeks. Group II, III and IV were given a five time injection of DMH (dissolved in 1mM EDTA solution pH a adjusted to 6.5 with 1mM NaOH) at the dose 20 mg/kg body weight, subcutaneously in the groin start after two week from administration of treatment *T. chebula*. Group I and V rats also received subcutaneous injection of 1mM EDTA solution, (pH adjusted to 6.5 with 1mM NaOH), which was used as the vehicle for DMH. All the rats were anaesthetized with mild anesthesia and sacrificed by cervical dislocation after 12 hours of orally in both dose of *T. chebula.* The doses of *T. chebula* were selected on the basis preliminary studies performed in our laboratory and also on the basis of available literature (15). Our study was approved by the Institutional Animal Ethics Committee (IAEC) of the university with registration no 599. (Figure 1)

Figure 1: Treatment protocol
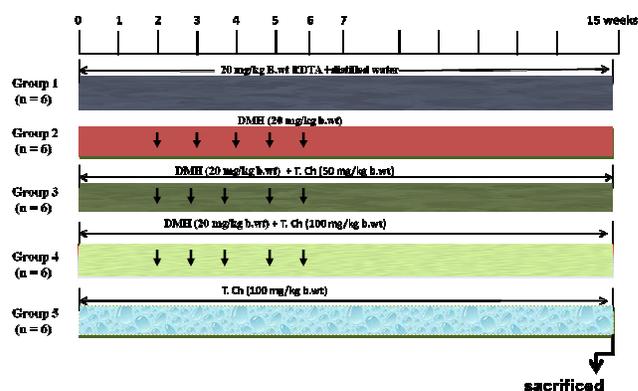
Post-mitochondrial supernatant (PMS) preparation and estimation of different parameters

Colons were removed quickly, cleaned free of irrelevant material and immediately perfused with ice-cold saline (0.85% sodium chloride). The colons (10% w/v) were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) using a Potter Elvehjen homogenizer. The homogenate was filtered through muslin cloth, and were centrifuged at 3000 rpm for 10 min at 4°C by Eltek Refrigerated Centrifuge (model RC 4100 D) to separate the nuclear debris. The aliquot so obtained was centrifuged at 12000 rpm for 20 min at 4°C to obtain PMS, which was used as a source of various enzymes (16).

Measurement of reduced glutathione level (GSH)

The GSH content in colon was determined by the method of Jollow (17) in which 1.0 ml of PMS fraction (10%) was mixed with 1.0 ml of sulphosalicylic acid (4%). The samples were incubated at 4°C for at least 1 h and then subjected to centrifugation at 1200 g for 15 min at 4°C. The assay mixture contained 0.4 ml filtered aliquot, 2.2 ml phosphate buffer (0.1 M, pH 7.4) and 0.4 ml DTNB (10mM) in a total volume of 3.0 ml. The yellow color developed was read immediately at 412 nm on spectrophotometer

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(Milton Roy Model-21 D). The GSH content was calculated as nmol of DTNB conjugate formed/g tissue using molar extinction coefficient of 13.6×10^3 M^{-1} cm^{-1}.

Measurement of Malondialdehyde (MDA)

Lipid peroxidation as evidenced by the formation of thiobarbituric acid reactive substances (TBARS) was estimated by the method described by Ohkawa (18). To 0.2 ml of tissue homogenate, 0.2 ml of 8.1% Sodium dodecylsulphate, 1.5ml of 20%acetic acid and 1.5ml of 0.8% TBA were added. The mixture was made upto 4.0 ml with distilled water and then heated in a boiling water bath at 95°C for 60 min. After cooling, 1.0 ml water and 5.0 ml n-butanol/pyridine mixture were added and shaken vigorously. After centrifugation at 600×g for 10 min, the pink coloured chromogen formed by the reaction of 2-thiobarbituric acid with the breakdown products of lipid peroxidation was read at 535 nm. The results were expressed as the nmol MDA formed/min/gram tissue by using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Measurement of glutathione peroxidase (GPx) activity

The GPx activity was calculated by the method of Mohandas (19). A total of 2 ml volume consisted of 0.1ml EDTA (1 mM), 0.1ml sodium azide (1 mM), 1.44ml phosphate buffer (0.1M, pH 7.4), 0.05 ml glutathione reductase (1 IU/ml), 0.05 ml reduced glutathione (1 mM), 0.1 ml NADPH (0.2mM) and 0.01ml H_2O_2 (0.25mM) and 0.1ml 10% PMS. The depletion of NADPH at 340 nm was recorded at 25°C. The enzyme activity was calculated as nmol NADPH oxidized/min/mg protein with the molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

Measurement of Glutathione reductase (GR) activity

The GR activity was measured by the method of Carlberg (20). The assay system consisted of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml oxidized glutathione (1.0 mM), 0.1 ml NADPH (0.1 mM) and 0.1 ml of 10% PMS in a total volume of 2.0 ml. The enzyme activity was assessed at 25°C by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

Measurement of glutathione-S-transferase (GST) activity

The GST activity was measured by the method of Habig (21). The reaction mixture consisted of 2.4 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml reduced glutathione (1.0 mM), 0.2 ml CDNB (1.0 mM) and 0.2 ml of cytosolic fraction in a total volume of 3.0 ml. The changes in absorbance were recorded at 340 nm and the enzyme activity was calculated as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6×10^3 M^{-1} cm^{-1}.

Measurement of Catalase activity

The Catalase activity was measured by the method of Claiborne (22). In brief, the assay mixture consisted of 2.0 ml phosphate buffer (0.1 M, pH 7.4), 0.95 ml hydrogen peroxide (0.019 M) and 0.05 ml of PMS (10%) in a final volume of 3.0 ml. Changes in absorbance we are recorded at 240 nm. The Catalase activity was calculated in terms of nmol H_2O_2 consumed/min/mg protein.

Measurement of SOD activity

The SOD activity was measured by the method of Marklund and Marklund (23). The reaction mixture consisted of 2.875 ml Tris–HCl buffer (50 mM, pH 8.5), pyrogallol (24mM in 10mM HCl) and 100 µl

PMS in a total volume of 3 ml. The enzyme activity was measured at 420 nm and was expressed as units/mg protein. One unit of enzyme is defined as the enzyme activity that inhibits auto-oxidation of pyrogallol by 50%.

Assay for hydrogen peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) was assayed by H_2O_2-mediated horseradish peroxidase-dependent oxidation of phenol red by the method of Pick and Keisari (24). 2.0 ml of supernatant, suspended in 1.0 ml of solution containing phenol red (0.28 nm), horse radish peroxidase (8.5 units), dextrose (5.5 nm) and phosphate buffer (0.05 M, pH 7.0) was incubated at 37°C for 60 min. The reaction was stopped by the addition of 0.01 ml of NaOH (10N) and then centrifuged at 800 × g for 5 min. The absorbance of supernatant was recorded at 610 nm against a reagent blank. The quantity of H_2O_2 produced was expressed as nmol H_2O_2/h/gm tissue based on the standard curve of H_2O_2 mediated oxidation of phenol red.

Nitrite estimation

Nitrite assay was done using Griess reagent by the method of Green et al. (25) with some modifications. In brief, 100 ll of Griess reagent (1:1 solution of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in water) was added to 100 ll of PMS incubate for 5–10 min at room temperature protected from light. Purple/magenta color began to form immediately. Absorbance was measured at 546 nm, nitrite concentration was calculated using a standard curve for sodium nitrite, and nitrite levels were expressed as l mol/mg protein

Myloperoxidase quantification (Marker for early inflammation)

The Myloperoxidase (MPO) activity or direct neutrophil quantification was determined by the method of Bradley et al (26). With some modifications and expressed as units/ min/mg protein. One unit of MPO activity was defined as that degrades 1 mmol of peroxide per min.

Protein estimation

The protein concentration in all samples was determined by the method of Lowry et al. (27) using bovine serum albumin (BSA) as standard.

Mast Cell Staining

For detection of mast cells, colon was fixed in methacarn solution (methanol: chloroform: glacial acetic acid:: 4:2:1) overnight at 4ºC and then fixed in 4% neutral buffered formalin for additional 24 hours. The colonic sections of 4μm were cut from formalin-fixed, paraffin-embedded tissue blocks and mounted on poly-l-lysine coated microscopic slides. Paraffinized sections were dewaxed in xylene and rehydrated through graded series of ethanol to water. The sections were stained with 0.1% toluidine blue (pH 2.3) in 1% sodium chloride solution for 5min. The slides were then washed three times in distilled water and dehydrated quickly in alcohol, clear in xylene and mounted by using mounting media. The slides were then evaluated under the light microscope (Olympus BX51). Staining with toluidine blue permits the identification of mast cells because mast cell granules stain metachromatically, resulting in deep purplish-blue granular cytoplasmic staining.

Assay for TNF-a level

TNF-a protein level was measured by enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, Inc., San Diego., USA). Analysis was performed according to the manufacturer's instruction.

Histopathology

The colon were excised, flushed with saline, cut open longitudinally along the main axis, and then

again washed with saline. These colonic sections fixed in 10% buffered formalin for at least 24 h and after fixation, the specimens were dehydrated in ascending grades of ethanol, cleared in benzene, and embedded in paraffin wax. Blocks were made and 5µm thick sections were cut from the distal colon. The paraffin embedded colonic tissue sections were deparaffinized using xylene and ethanol. The slides were washed with phosphate buffered saline (PBS) and permeabilized with permeabilization solution (0.1M citrate, 0.1% TritonX-100). These sections stained with haematoxylin and eosin and were observed under light microscope at 10X and 40X magnifications to investigate the histoarchitecture of colonic mucosa.

Immunohistochemical staining for detection of (iNOS, COX-2 and PCNA)

Section of 4µm were cut from formalin-fixed, paraffin-embedded tissue blocks and mounted on poly-L-lysine coated microscopic slides. The protocol followed it was according to (28) Paraffinized sections were dewaxed in xylene and rehydrated through graded series of ethanol to water followed by antigen retrieval in sodium citrate buffer (10mM, pH 6.0). The slides were then allowed to cool for 15 minutes and washed 3 times with tris-buffered saline (TBS) for 5 minutes each. Slides the incubated in 3% H_2O_2 in methanol for 10 min to reduce the endogenous peroxidase activity and then subject to Ultra block (UltraVision Plus Detection System, Thermo Scientific) for 10 min to block non-specific binding. After rinsing the sections in TBS, the slides were incubated overnight at 4°C with primary antibody inside humidified chamber and then were washed in TBS. The sections were incubated with biotinylated goat anti-polyvalent secondary antibody (UltraVision Plus Detection System,

Thermo Scientific) for 20 min and then were rinsed in TBS. The sections were again incubated with streptavidin peroxidase plus (UltraVision Plus Detection System, Thermo Scientific) for 30 min. The sections were washed in TBS and developed with 3, 3'-Diaminobenzidine (DAB) solution (UltraVision Plus Detection System, Thermo Scientific) until sections become brown. Counterstained the sections with Mayer's haematoxylin, mounted by using mounting media and then visualized under the light microscope (Olympus BX51). Primary antibody of rabbit ant dilution was used.

Results:

Effect of prophylactic treatment of *T. chebula* against DMH-induced lipid peroxidation

The level of MDA was significantly enhanced (P,0·01) in Group II as compared to Group I. *T. chebula* pretreatment significantly decreased the level of MDA in Group III (P,0·05) and Group IV (P,0·01), respectively, as compared to Group II. No significant difference was found in the level of MDA between Group I and Group V (Tab. 2).

Effect of prophylactic treatment of *T. chebula* against DMH-induced reduced glutathione depletion in the colon

The level of GSH was depleted significantly (P, 0·001) in the DMH-treated group (Group II) as compared to the control group (Group I). *T. chebula* pretreatment showed a significant increase in the level of GSH in Group III (P, 0·05) and Group IV (P, 0·05) when compared with Group II. No significant difference was found in the level of GSH between Group I and Group V (Tab. 1)

Effect of *T. chebula* supplementation and DMH on the activities of glutathione-dependent enzymes in the colon

DMH treatment caused a significant decrease in the activities of GPx (P, 0·001), GST (P, 0·001) and GR (P,0·001) in Group II as compared to Group I. *T. chebula* supplementation at the dose of 50 mg/kg b.wt. significantly increased the activity of GST only (P, 0·05) but not other enzymes in Group III as compared to Group II. But the higher dose of T. chebula (100 mg/kg b.wt.) significantly attenuated the activities of GPx (P, 0·01), GST (P, 0·05), GR (P, 0·001) and G6PD (P, 0·001) in Group IV as compared to Group II. However, the activities of these enzymes in Group V did not change significantly as compared to Group I (Table 1)

Effect of *T. chebula* supplementation and DMH on the activities of antioxidant enzymes in the colon

The activities of CAT, and SOD were decreased significantly (P, 0·05, P, 0·001 and P,0·001, respectively), in Group II as compared to Group I. *T. chebula* pretreatment at the dose of 50 mg/kg b.wt. significantly augmented the activities of CAT (P, 0·05), QR (P, 0·01) and SOD (P, 0·001) in Group III as compared to Group II. The higher dose of T. chebula (100 mg/kg b.wt.) also showed significant increase in the activities of CAT (P, 0·05), QR (P, 0·001) and SOD (P, 0·001) in Group IV as compared to Group II. However, the activities of these enzymes in Group V did not change significantly as compared to Group I (Table 2).

## Effect of *T. Chebula* pretreatment and DMH on the H2O2 in colon

The activity of H2O2 was significantly increased (P,0·001) in Group II as compared to Group I. *T. chebula* pretreatment significantly decreased the activity of H2O2 in Group III (P,0·001) and Group IV (P,0·001) as compared to Group II. Group V

exhibited no significant change in the level of H2O2 as compared to Group I (Tab. 2).

Effect of *T. chebula* and DMH on mast cell infiltration

In DMH treated group (Group II), there is intense infiltration of mast cells in the sub-mucosal layer below the lamina propria within the colonic section. Treatment with T. chebula attenuated the infiltration of mast cells in Group III and IV as compared to Group II. There is no mast cells infiltration in colonic sections of Group I and Group V. (Figure 2)
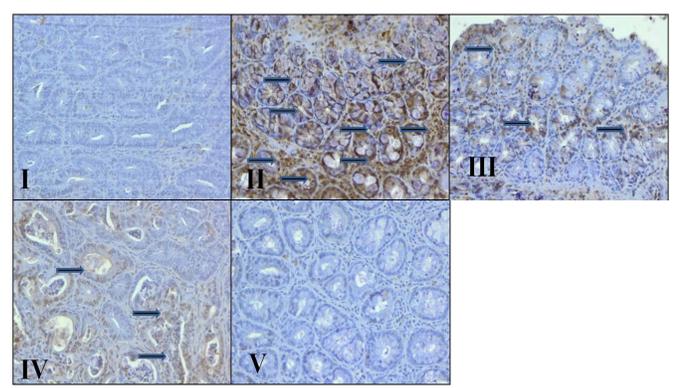
Figure 2: Mast cells staining.
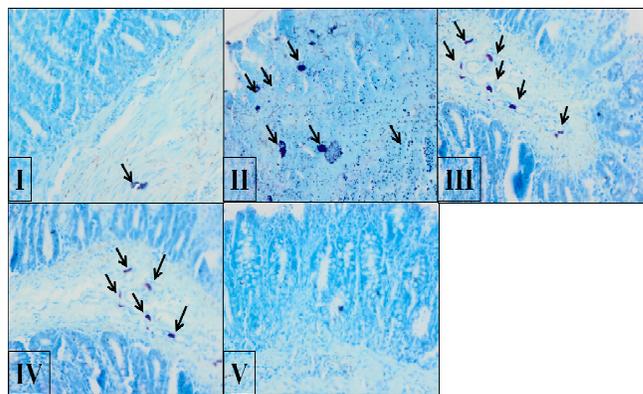
(I) only vehicle (II) DMH only (III) DMH (20 mg/kgBW) + *T chebula* (50 mg/kg BW) (IV) DMH (20 mg/kg BW) + *T chebula* (100 mg/kg BW) (V) *T chebula* (100 mg/kg BW). Dark colour cells indicates mast cells and blue colour indicates haematoxylin staining. DMH treated group (group II) shows more mast cells (arrows) as compared with vehicle treated group (group I). *T Chebula* pretreatment (groups III and IV) reduces infiltrating mast cells as compared to group II. However there was no visible difference in the mast cell infiltration in group V as compared to group I.

Effect of *T. chebula* pretreatment and DMH on the expression of COX-2, iNOS and PCNA in the colon

In Group II (DMH only) the COX-2 and iNOS proteins showed more positive staining compared to group I (Control). In pre-treatment group III *T. chebula* at dose (50 mg/kg b.wt) the COX-2, iNOS and PCNA expression appeared slightly decrease in colon tissue compared to group II (DMH only). In pretreatment (Group IV) *T. chebula* at dose (100

mg/kg b. wt) showed substantial decrease in COX-2, iNOS and PCNA protein expression in colonic tissue as compared to group II (DMH only). No significant difference was observed in staining pattern in group V as compared group I (control). In case of post-treatment in group VI, TA at dose (50 mg/kg b.wt) showed slight decrease in COX-2, iNOS and PCNA expression. In post-treatment in group VII TA at dose (100 mg/kg b.wt) showed considerable decrease in COX-2, iNOS and PCNA expression as compared to group II (DMH only) but decrease was not as effective as in case of pre-treatment. (Figure 3-5).

Figure 3: Effect of *T. chebula* pretreatment on DMH-induced colonic expression of COX-2
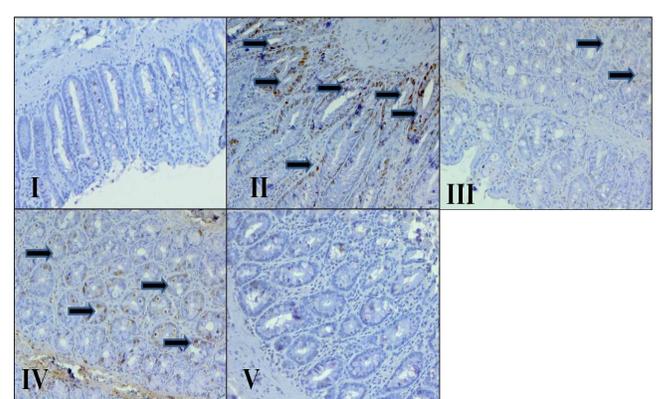

(I) only vehicle (II) DMH only (III) DMH (20 mg/kg BW) + *T chebula* (50 mg/kg BW) (IV) DMH (20 mg/kg BW) + *T chebula* (100 mg/kg BW) (V) *T chebula* (100 mg/kg BW). Brown colour indicates COX-2 specific staining and blue colour indicates haematoxylin staining. DMH treated group (group II) shows more COX-2 immunopositive staining (arrows) as compared with vehicle treated group (group I). *T chebula* pretreatment (groups III and IV) reduces COX-2 expression as compared to group II. However there was no visible difference in the COX-2 immunostaining in group V as compared to group I.

Figure 4: Effect of *T chebula* pretreatment on DMH-induced colonic expression of iNOS
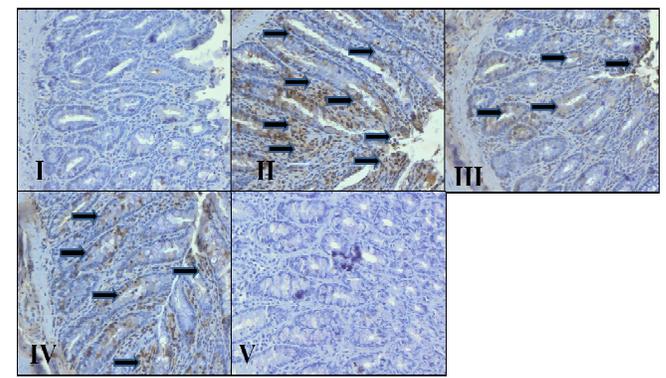
(I) only vehicle (II) DMH only (20 mg/kg BW). (III) DMH (20 mg/kg BW) + *T chebula* (50 mg/kg BW). (IV) DMH (20 mg/kg BW) + *T chebula* (100 mg/kg BW) (V) *T chebula* only (100 mg/kg BW). Brown colour indicates iNOS specific staining and blue colour indicates haematoxylin staining. DMH treated group (group II) shows more iNOS immunopositive staining (arrows) as compared with vehicle treated group (group I). *T chebula* pretreatment (groups III and IV) reduces iNOS expression as compared to group II. However there was no marked difference in the I-NOS immunostaining in group V as compared to group I.

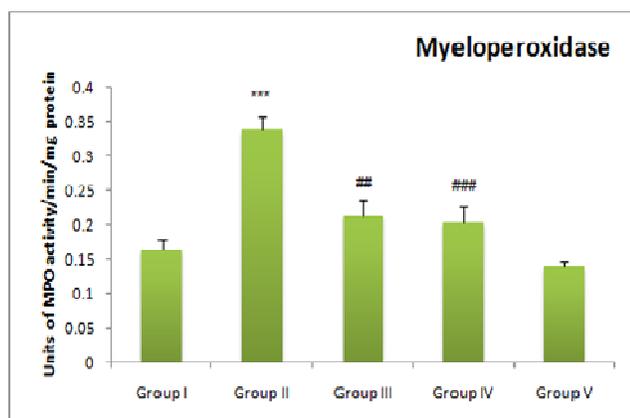**Figure 5:** Effect of *T. chebula* pretreatment on DMH-induced colonic expression of PCNA

(I) only vehicle (II) DMH only (III) DMH (20 mg/kg BW) + *T chebula* (50 mg/kg BW) (IV) DMH (20 mg/kg BW) + *T chebula* (100 mg/kg BW) (V) *T chebula* only (100 mg/kg BW). Brown colour indicates PCNA specific staining and blue colour indicates haematoxylin staining. DMH treated group II shows more PCNA immunopositive staining (arrows) as compared with vehicle treated group (group I). *T chebula* pretreatment (group III and IV) reduces PCNA expression as compared to group II. However, there was no significant difference in the PCNA immunostaining in group V as compared to group I.

**Myloperoxidase activity and nitrite level restored by *T. chebula***

Treatment with T.Ch resulted in significant increase in the MPO activity when compared with control. Both Pre-treatment and post-treatment of *T. chebula* significantly brought the level MPO to normal (Figure 6) *T. chebula* alone did not show any significant difference as compared to control. Similar pattern as above was observed in nitric oxide estimation. (Figure 7)

Figure 6: Effect of *T Chebula* pre-treatment on DMH induced neutrophil infiltration

Values are expressed as mean±SEM (n = 10). ***p < 0.001 shows significant difference in group II (DMH 20 mg/kg body weight) when compared with only vehicle treated group I. #p < 0.05, ##p < 0.01, ###p < 0.001 shows the significant difference in the groups III (DMH 20 mg/kg BW + *T Chebula* 50 mg/kg BW) and group IV (DMH 20 mg/kg BW + *T Chebula* 100 mg/kg BW) when compared with group II.

**Figure 7:** Effect of *T. Chebula* pre-treatment on DMH induced Nitrite levels
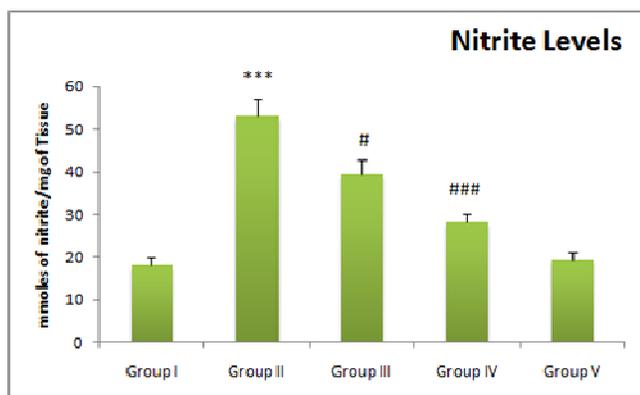
Values are expressed as mean±SEM (n = 10). ***p < 0.001 shows significant difference in group II (DMH 20 mg/kg body weight) when compared with only vehicle treated group I. #p < 0.05, ##p < 0.01, ###p < 0.001 shows the significant difference in the groups III (DMH 20 mg/kg BW + *T Chebula* 50 mg/kg BW) and group IV (DMH 20 mg/kg BW + *T Chebula* 100 mg/kg BW) when compared with group II.

Effect of *T. chebula* on colon tissues proinflammatory cytokines (TNF-a)

We have assessed the effect T. chebula on DMH induced abnormal increase in TNF-a level (Figure 8). We found that there was a significant increase in the level of proinflammatory cytokines in DMH (20 mg/kg b. wt) treated group compared with control group I (p<0.001). Pre-treatment with *T. chebula* (50 mg/kg b. wt) significantly inhibited abnormal increase in the group III when compared with the only DMH treated group II (p<0.05). Pre-treatment with T. chebula (100 mg/kg b. wt) significantly inhibited increase in TNF-a in the group IV when compared with the only DMH treated group II (p<0.01) There was no significant difference in levels of this proinflammatory cytokine between group I and group V.

**Figure 8:** Effect of *T. chebula* treatment on DMH induced TNF-a
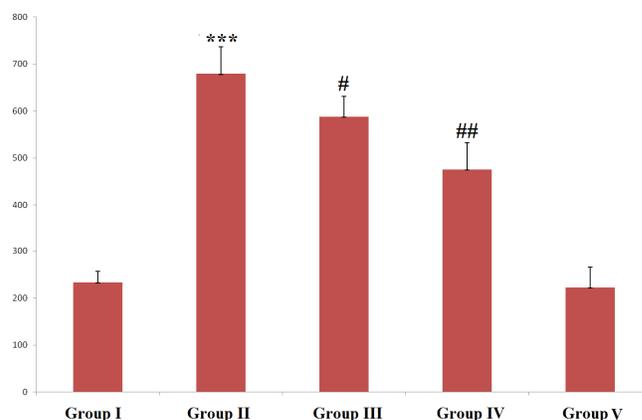
In DMH-treated (group II), the level of TNF-a was increased significantly (***p<0.001) as compared to control group. While treatment with *T. chebula*

significantly attenuated the level of TNF-α in Group III (#p<0.05) and IV (##p<0.01) as compared to Group II. There was no significant difference in the level of TNF-α in Group V as compared to Group I.

Histological finding

In control group showed normal histology of the rat colon with mild inflammatory cells infiltration (normal appearance of the mucosal crypts, submucosa, and muscular layers). (II) Toxicant Group showed Irregular glandular structure with intense inflammatory cells infiltration in mucosal and submucosal layers along with crypt ablation were observed in DMH treated group. (III, IV and V) *T. chebula* supplementation prevents the mucosal damage and ameliorates inflammatory cells infiltration as well severity of the submucosal edema. Insets at the right panel show a magnified view (40X magnifications). (Figure 9)

Figure 9: Histopathology Finding
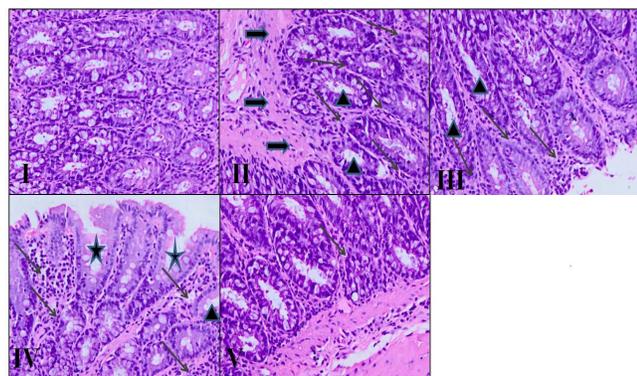
(I) only vehicle, (II) DMH only (20 mg/kg BW), (III) DMH (20 mg/kg BW) + T chebula (50 mg/kg BW), (IV) DMH (20 mg/kg BW) + T chebula (100 mg/kg BW) and (V) T chebula only (100 mg/kg BW). (I) Normal histology of the rat colon with mild inflammatory cells infiltration (normal appearance of the mucosal crypts, submucosa, and muscular layers). (II) Irregular glandular structure with intense inflammatory cells infiltration in mucosal and submucosal layers along with crypt ablation were observed in DMH treated group. (III, IV and V) T chebula supplementation prevents the mucosal damage and ameliorates inflammatory cells infiltration as well severity of the submucosal edema. Insets at the right panel show a magnified view (40X magnifications).

Discussion

In this study, we have observed the protective effects of *T. Chebula* against DMH-induced colon toxicity in Wistar rats. The DMH-induced colon toxicity is well documented (28-30). The mechanism underlying DMH-induced colon toxicity is well documented and ROS generation is recognized as a key player leading to the condition of oxidative stress. Therefore, the natural compounds with antioxidant properties are gaining much attention. The present investigation was carried out to elucidate the effect of *T. chebula* on DMH-induced colon toxicity in rats and to assess its role in modulation of inflammation and tumor promotion.

T. chebula Retz. commonly known as harad or black myroblans belongs to Combretaceae family (31). It is an integral ingredient of famous ayurvedic formulation in India known as ''Triphala'' which contains equal parts of *T. chebula, T. Cellerica and E. Officinalis* and has been scientifically proven to promote immunity, health and longevity. Organic and aqueous extracts of *T. chebula* exhibit antioxidant, antimicrobial, anti-anaphylactic, antidiabetic, antimutagenic, anticancerous, apoptotic, antibacterial, antifungal and antiviral (32-37, 15).

DMH is a well known carcinogen and on metabolic activation it results in the formation of methyl free radical which is known to induce oxidative stress. Oxidative stress is implicated in colon toxicity (38). DMH induced toxicity is mainly dependent on bio-activation, which occurs by two pathways, glutathione (GSH) conjugation and cytochrome P450 dependent oxidation (38). Major biochemical markers of oxidative stress response were found to be altered including Lipid

peroxidation (LPO), decrease in levels of reduced glutathione (GSH) and related redox cycle enzymes like SOD, Catalase, GR and GPX was observed (38- 41). Lipid peroxidation is one of the important indicators of oxidative damage and high level of lipid peroxidation product (MDA) has been found after treatment with DMH (35, 37 and 38). Consistent with previous reports our results also showed remarkable increase in the level of MDA after DMH treatment. *T. chebula* pre-treatment significantly attenuated elevated levels of MDA, our results are in agreement with recently published report from our laboratory where *T.. chebula* shown to be effective against chemically induced colon toxicity (42, 28).

DMH is reported to inhibit the activity of antioxidant enzymes (glutathione reductase, glutathione-S transferase, and catalase) in colon tissue of rats (38, 43). In agreement with previous studies from our and other labs, DMH exposure leads to a depletion in glutathione content and inhibits the activities of the anti-oxidant enzymes glutathione reductase, catalase, superoxide dismutase, glutathione peroxidase, and glutathione S-transferase (28, 29). GSH is a low molecular weight antioxidative tripeptide and has essential role in the detoxification of chemotherapeutic drugs, toxicants, metabolism of nutrients and regulation of various pathways to maintain cellular homeostasis. Enzymatic (Catalase, SOD, GR, GPX etc) and endogenous non-enzymatic antioxidant (GSH) were significantly restored to normal levels in *T. chebula* treated groups (Table 1). DMH treatment preserved the level of all these antioxidant enzymes as previously reported by (10, 36)

Inflammation is related to oxidative stress and is documented to influence tumor initiation and promotion (38, 25 and 14). COX-2 an important inflammatory marker is induced during DMH mediated inflammation and is up-regulated in adenomas and over-expressed in colon cancer (44, 45). COX- 2 has been reported to play an important role in colon toxicity and polyp formation and its inhibition has proven to be an important strategy for chemopreventive treatment of colon related disorders (46). We observed that DMH-treated rats showed increased expression of COX-2 and pre-treatment of *T. chebula* efficiently inhibited the expression of COX-2, iNOS, another inflammatory marker has been shown to be up-regulated in DMH-induced colon Tumorigenesis (47, 48), and its inhibition is yet another important approach to prevent colon cancer (49). DMH treated group showed similar pattern of i-NOS expression as in COX-2 and *T. chebula* inhibited this over-expression of i-NOS in pre-treatment condition.

Activation of transcription factor NFκB by pro-inflammatory cytokine TNF-α is one of the many actions of TNF-α that causes genes to generate potentially cell damaging oxidative enzymes such as NADP oxidase, cyclooxygenase (COX-2) and iNOS as well as further release of TNF-α and other pro-inflammatory cytokines (50, 51). Therefore, targeting TNF-α is an effective approach for the treatment of colon cancer (52). Previous reports have shown ellagic acid and tannic efficiently reduced the expressions of TNF-α in DMH-induced colonic tumors and thus exhibited its anti-inflammatory activity (28). Our results confer that *T. chebula* effectively contain the production of cytokines which implicates its possible role in chemoprevention of colon cancer.

In the present strategy of inducing colon injury by DMH administration, our results are in complete accordance with the previous reports that the levels of oxidative and inflammatory markers (TNF-

a, iNOS and COX-2) increased in the rats treated with DMH. Furthermore, *T. chebula* administration in the present study has been found to restore these markers.

NO (nitric oxide) is another important mediator in the pathogenesis of inflammatory diseases (53, 16). Thus, we also evaluated the effect of *T. chebula* on colon l NO in DMH induced colon toxicity (Figure 6); DMH exposure elevated the level of NO. However, pretreatment with *T. chebula* prevented DMH induced NO production in a concentration-dependent manner. Further, MPO is an enzyme formed mainly by polymorphonuclear leucocytes, and is linked to the degree of neutrophil infiltration in a given tissue (16). Following DMH treatment, MPO activity was markedly elevated (Figure 5).This increase in MPO activity was significantly prevented by *T. chebula* administration.

PCNA is already reported to be an important biomarker in gastrointestinal cancer (54, 55). Hall et al. (56) reported PCNA, a 36 kDa co-factor of DNA polymerase- δ, is one of the downstream effectors of the activation of MAPK/ERK1/2 signaling and is very useful molecular biomarker of hyperproliferation. In this study, DMH administered rats showed amplified expression of PCNA in the colon thereby indicating the hyper-proliferative activity of colon cells. A decrease observed in PCNA positive cells following *T. chebula* pretreatment indicated suppression induced by *T. chebula* in cellular proliferation and hence tumor promotion.

Mast cells, one of the inflammatory cells, play an important role in carcinogenesis and also form an important tumor-promoting component of the cellular stroma which promotes tumor growth (57, 58). Incursion of mast cells is the sign of initiation of inflammation. In this study, it was observed that there is intense influx of mast cells in the sub mucosal layer in DMH treated group while there is no mast cells influx in control group. Treatment with *T. chebula* markedly reduced the influx of mast cells within in the sub-mucosal layer in group III and group IV as compared to group II (DMH treated group). No influx of mast cells in group V (only *T. chebula* treated group). A number of studies have recognized the contribution of oxidative stress and inflammation to tumor promotion. In this study we presented data demonstrating that *T. chebula* inhibits DMH induced colon toxicity in animal model. *T. chebula* treatment resulted in marked decline in colonic oxidative stress, protein expression of PCNA, iNOS, COX-2 and secretion of proinflammatory cytokines, all of which are traditional markers of inflammation and tumor promotion. Therefore *T. chebula* may prevent the tumor promoting property of DMH through antioxidant and anti-inflammatory properties but the exact mechanism of *T. chebula* is not fully defined yet. Hence, further studies are needed to elucidate the exact mechanism of action of *T. chebula.*

Table 1: Results of pretreatment of *T chebula* on antioxidant enzymes like GSH, GST, GR and GPX on DMH induced imbalance in colonic tissue

Treatment regimen per group	GSH (n mol GSH/g tissue)	GST (n mol CDNB Conjugate formed/min/mg protein)	GR (n mol NADPH Oxidized/min/ mg protein)	GPX (n mol NADPH Oxidized/min/ mg protein)
Group I	0.080±0.0073	250.0±10.11	320.9±18.5	414.5±25.5

Group II	0.031 ± 0.0031***	59.0 ± 5.10***	103.6 ± 13.5***	265.2 ± 19.41***
Group III	0.0043 ± 0.006#	133.6 ± 13.17###	233.5 ± 11.5##	310.1 ± 31.08ns
Group IV	0.063 ± 0.0041###	220.7 ± 10.01###	312.8 ± 19.3###	392.3 ± 17.63###
Group V	0.079 ± 0.0061	245.2 ± 12.50	313.4 ± 22.2	402.4 ± 30.6

Results represent mean ± SE of six animals per group. Results obtained are significantly different from group I (***$P < 0.001$). Results obtained are significantly different from group II (#$P < 0.05$), (##$P < 0.01$) and (###$P<0001$). Group I – control; group II (toxicant) – DMH; group III – DMH + *T chebula*; group IV – DMH + *T chebula*; group V – *T chebula*.

Table 2: Effects of *T chebula* and DMH on the activities of Catalase (CAT), Superoxide dismutase (SOD), H_2O_2 and Lipid peroxidase (LPO)

Treatment regimen per group	Catalase (nmol H_2O_2 consumed /min/mg protein)	H_2O_2 (nmoles of H_2O_2/g tissue)	SOD (Unit per mg/ protein)	MDA (nmoles of MDA formed/g tissue)
Group I	22.71 ± 0.76	186.5 ± 9.9	21 ± 1.0	6.88 ± 0.91
Group II	10.12 ± 0.32**	575.5 ± 31.1***	14 ± 2.9**	18.12 ± 1.3***
Group III	15.3 ± 0.13#	310.4 ± 19.4###	17 ± 1.3ns	14.31 ± 1.9#
Group IV	18.11 ± 2.1##	230.1 ± 21.70###	16 ± 1.2##	9.12 ± 1.02###
Group V	19.81 ± 3.5	171.6 ± 22.41	20 ± 2.2	7.15 ± 0.7

Results represent mean ± SE of six animals per group. Results obtained are significantly different from group I (***$P < 0.001$). Results obtained are significantly different from group II (#$P < 0.05$), (##$P < 0.01$) and (###$P<0001$). Group I – control; group II (toxicant) – DMH; group III – DMH + *T chebula*; group IV – DMH + *T chebula*; group V – *T. chebula*.

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