

Article

Piperine Regulates Nrf-2/Keap-1 Signalling and Exhibits Anticancer Effect in Experimental Colon Carcinogenesis in Wistar Rats

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Abstract: Colon cancer is the most common cancer in men and women globally, killing millions of people annually. Though there widespread development has been made in the management of colorectal cancer, still there is an urgent need to find novel targets for its effective treatment. Piperine is an alkaloid found in black pepper having anticancer, anti-inflammatory activities, safe and nutritive for human consumption. Nuclear factor-erythroid 2–kelch-like ECH-associated protein 1(Nrf-2/Keap-1)/Heme-oxygenase1 (HO-1) signaling pathway plays a vital part in shielding cells from intracellular oxidative stress and inflammation. A potential cross-talk between the Nrf-2 and NF-κB pathways is recognized during cancerous growth and expansion. We studied this pathway extensively in the present study to discover novel targets in the prevention of chemically induced colon cancer with piperine to simulate human colon cancer pathology. Animals were divided into four groups. Groups 1 and 2 were used as a negative control and positive control where 1,2-Dimethylhydrazine, DMH was administered in group 2, while group 3 and 4 were prevention groups where piperine at two different doses was given two weeks prior to DMH and continued until end of experiment. We found that piperine inhibited NF-κB by the activation of Nrf-2, blocking downstream inflammatory mediators/cytokines (TNF-α, IL-6, IL-1β, Cox-2, PGE-2, iNOS, NO, MPO), triggering an antioxidant response machinery (HO-1, NQO-1, GSH, GR, GPx, CAT, SOD), scavenging ROS, and decreasing lipid peroxidation. Histological findings further validated our molecular findings. It also downregulates CEA, MDF and ACF, markers of precancerous lesions in colon, alleviates infiltration of mast cells and depletes the mucous layer. Our results indicate that piperine may be an effective molecule for the prophylactic treatment of colon carcinogenesis by targeting the NF-κB/Nrf-2/Keap-1/HO-1 pathway as a progressive strategy in the preclusion and effective treatment of colorectal cancer.

Keywords: piperine; colon carcinogenesis; NF- κ B/Nrf-2/Keap-1/HO-1 signalling pathways; ACF; MDF

1. Introduction

According to GLOBOCAN 2018 data from Saudi Arabia, Colorectal cancer (CRC) is documented as the top most common cancer in men, third most common cancer-caused death in women, and fourth most frequently occurring cancer throughout the world (GLOBOCAN 2018). Surgery, radiotherapy, and chemotherapy are the conventional strategies of treatment for CRC, which fail because patients either develop drug resistance or excessive adverse effects which become fatal in themselves [1,2]. Hence, there is an imperative to mitigate adverse effects of the present treatment regimen. In this sense, environmental factors, explicitly dietary trends, contribute significantly towards the aetiology of colon cancer incidences in various populations. Drug discovery paradigms have shown remarkable results by using natural products. Therefore, natural compounds possess various organically potential molecules which have been used to either converse, inhibit, or avert early cancer stages or the promotion of precancerous cells like inflammation, transformation, and proliferation to metastatic disease. Nevertheless, many natural product-based drugs have been isolated from nature due to their lower toxicity, easy availability, affordability and targeted nature. A number of drugs have been isolated from natural sources owing to their great chemical diversity, which may be a consequence of millions of years of evolutionary process to combat biotic and abiotic stresses. In the past, plants have been used as an enriched source of drugs and will further deliver state-of-the-art chemical frameworks for mitigation of diseases [3].

Epidemiological reports demonstrate that CRC cases are commonly sporadic, which may be associated with nutrition and diet-influencing oxidation reactions in cells and tissues, by activating a cascade of molecular events in them. Oxidative stress deregulates normal functioning of cells by inducing DNA damage, mutations, peroxidation of lipids and proteins. Moreover, a close association between biomarkers of oxidative stress, nitrosative stress and progression of CRC has been unraveled by clinical studies [4–7]. Inflammation is a normal biological response of our body at the time of infection or damage. It stimulates the immune system to release chemical molecules called pro-inflammatory signals which protect our body. Nevertheless, too much inflammation is a bad representation as well. There is an association between carcinogenesis and inflammation as inflammation is one of the hallmarks of cancer. Cancer progression is exacerbated by inflammatory processes via averting the differentiation of cells and promoting tumor formation and expansion [8]. In the present study, we used DMH to induce colon carcinogenesis where DMH is metabolized in the liver to azoxymethane and, after subsequent metabolization, forms methylazoxymethanol (MAM). Glucuronic acid conjugates with MAM, forming glucuronide MAM which, with the help of blood or bile, is released into the colon. Following deconjugation by intestinal microbial enzymes like β -glucuronidase, electrophilic methyldiazonium ion is produced as a by-product of MAM metabolization. Thereafter, the methyldiazonium ion produces carbonium ion which methylates DNA and other biomolecules in the epithelial layer of colon tissue, leading to procarcinogenic events resulting from inflammation and tumor promotion [9].

Piperine is an interesting compound present in the fruits and roots of *Piper nigrum* L. (black pepper) and *Piper longum* (long pepper) containing 3–9% content of black pepper. It is the most prevalent dietary amide alkaloid, having anti-inflammatory, immunosuppressive, anti-cancer, neuroprotective, and anti-oxidant potential. Lately, piperine was found to suppress the promotion of human colon cancer cells, prostate cancer cells, cytotoxic for human and murine melanoma cells [10]. It was also found to enhance the efficacy of anti-cancer drugs by alleviating drug resistance remarkably [11]. In traditional Indian medicine, black pepper is used extensively and is beneficial because of the piperine in it [12,13]. The bioavailability of the drug, inhibition of the drug transporter and cytochrome P450 results in an increase in absorption caused by Piperine. Piperine was found to decrease protein damage

and impede cell proliferation in benzo(a)pyrene-induced lung cancer in an animal model. It also repressed stem cell renewal in breast tissue. It was found to be cytotoxic to human rectal cancer cells (HRT-18), androgen-dependent prostate cancer cells (LNCaP), androgen-independent prostate cancer cells (PC-3), HER2-overexpressing breast cancer cells (SKBR3), mouse mammary carcinoma cells (4T1), and mouse melanoma cells (B16F-10) [14]. Moreover, it is reported to suppress angiogenesis and regulates multidrug resistance in breast and lung cancer cells to respond to traditional anti-cancer drugs. The current study was done to explore the role of piperine, keeping in mind the cytotoxic, anti-proliferative and anti-cancer potential, using Dimethyl hydrazine (DMH)-induced colon cancer in Wistar rats [10,14,15], thereby highlighting the efficacy of piperine in various cancer cells including colon cancer. However, the exact mechanism of anti-carcinogenic action of piperine is unknown and more research needs to be done to elucidate it. As per our knowledge, our study is the first to decipher the role of piperine in mitigating experimental colon cancer in Wistar rats by regulating cross-talk between the Nuclear factor kappa light chain enhancer of activated B cells (NF- κ B-p65) and Nuclear factor erythroid 2-related factor 2 (Nrf-2) pathway to discover novel targets for the prevention of chemically induced-colon cancer which simulates human colon cancer pathology. We extensively studied inflammatory mediators and markers of inflammation like NF- κ B, Tumor Necrosis Factor Alpha (TNF- α), Interleukin 6 (IL-6), cyclooxygenase-2 (Cox-2), prostaglandinE-2 (PGE-2), inducible nitric oxide synthase (iNOS), nitric oxide (NO), myeloperoxidase (MPO); markers of oxidative stress like reactive oxygen species, lipid peroxidation; depletion of anti-oxidant machinery, activation of Nrf-2 pathway and its downstream molecules, which trigger antioxidant response machinery like Heme oxygenase-1 (HO-1), NADP(H) quinone oxidoreductase-1 (NQO-1), reduced glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), which has never been done before using piperine in a colon cancer model. We are the first to unravel the downregulation of markers of precancerous lesions in the colon like carcinoembryonic antigen (CEA), mucin-depleted foci (MDF), aberrant crypt foci (ACF), mast cells expansion and the depletion of mucous layer with prophylactic treatment of piperine. Therefore, the current work was planned to assess the beneficial properties of piperine on colon cancer in vivo to further illuminate its molecular mechanism involving the Nrf-2/Keap-1/NF- κ B pathway.

2. Materials and Methods

2.1. Chemicals

Piperine, 1,2 dimethyl hydrazine (DMH) and other chemicals were obtained from Sigma Aldrich and were of greatest purity grade.

2.2. Animals

For the animal study, 4–6-week-old male Wistar rats, weighing 120–150 g were obtained from Institutional Animal Housing Facility. Rats were kept under typical laboratory environment with 45–55% relative humidity, 23–25 °C temperature and 12 h light/12 h dark period, having free access to standard diet and tap water during the experimental tenure. All procedures for using experimental animals were checked and proper permission was obtained from the “Institutional Animal Ethical Committee (IAEC)” (Approval No, Au/FVS/PS-57/9713) which is fully accredited by the Committee for Purpose of Control and Supervision on Experiments on Animals (CPCSEA), New Delhi, India.

2.3. Preparation of Carcinogen

A total of 1 mM EDTA was used as a vehicle to dissolve DMH in distilled water. The pH was adjusted to 6.5 with 1 M NaOH solution to check the steadiness of the chemical before administering it to rats.

2.4. Experimental Regimen

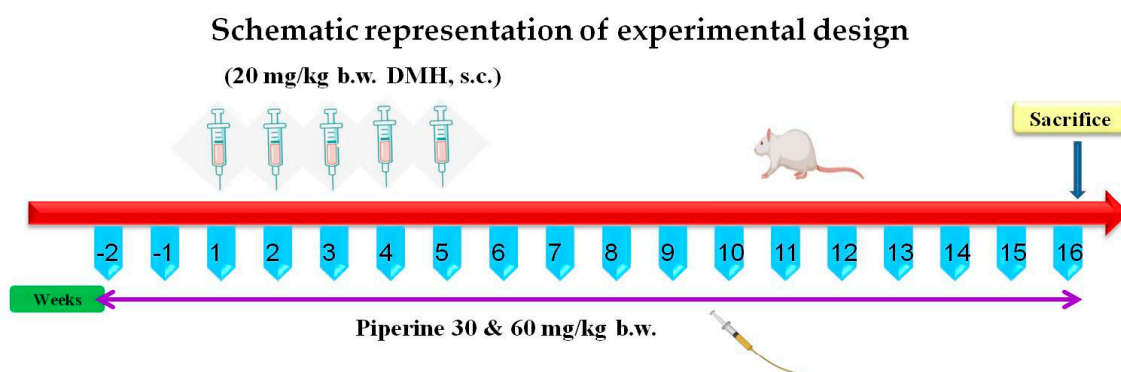
Group I: Rats had access to standard diet and drinking water along with physiological saline given orally.

Group II: In the current group, rats had access to standard diet and drinking water. Rats were given 20 mg/kg b.wt. (b.wt.) DMH injection given subcutaneously in the groin once weekly for 5 weeks.

Group III: Piperine and DMH treated group. In this group, rats were prophylactically treated with Piperine (30 mg/kg b.wt.) two weeks prior to the start of DMH injection, which then continued five times a week along with 20 mg/kg b.wt. subcutaneous DMH injection as given in group II. Piperine treatment was continued till the end of 16th week.

Group IV: Piperine and DMH treated group). In this group, rats were prophylactically treated with Piperine (60 mg/kg b.wt.) two weeks prior to the start of DMH injection, which then continued five times a week along with 20 mg/kg b.wt. subcutaneous DMH injection as given in group II. Piperine treatment was continued till the end of 16th week.

All the animals were sacrificed at the end of the 16th week (Figure 1). The chemo-preventive potential of Piperine was assessed for mitigating colon tissue injury and the initiation of precancerous lesions/events/biomarkers of colon carcinogenesis via checking ROS measurement, anti-oxidant armory, carcinoembryonic antigen (CEA), mucin-depleted foci (MDF), aberrant crypt foci (ACF), NF- κ B /Nrf-2/Keap-1/HO-1 pathway proteins were measured, and histological alterations were studied.



Group I: physiological saline orally

Group II: 20 mg/kg b.w. DMH injection (s.c.) in the groin once weekly for 5 weeks

Group III: Prophylactic treatment of Piperine orally (30 mg/kg b.w.) five times a week, two weeks prior to the start of DMH injection until 16th week +20 mg/kg b.w. DMH injection (s.c.) in the groin once weekly for 5 weeks

Group IV: Prophylactic treatment of Piperine orally (60 mg/kg b.w.) five times a week, two weeks prior to the start of DMH injection until 16th week +20 mg/kg b.w. DMH (s.c.) in the groin once weekly for 5 weeks

↓All the animals were sacrificed at the end of 16th week

s.c: subcutaneously; b.w: body weight

Figure 1. Represents treatment schedule of the study.

2.5. Post-Mitochondrial Supernatant (PMS) Preparation

Colons were cleaned immediately and perfusion was done by cold saline. Colons were homogenized in a homogenizer in chilled phosphate buffer (0.1 M, pH 7.4) (10% w/v). To separate the nuclear debris, centrifugation was done at 700× g in cooling centrifuge to homogenized colons for 10 min. PMS in the form of aliquot, thus obtained, was used as a repertoire of numerous enzymes [16].

2.6. Estimation of Carcinoembryonic Antigen (CEA)

CEA was measured by commercially available kit from USCN Life Science Inc. CEA enzyme levels in serum was measured by commercially available kit ARCHITECT CEA enzyme chemiluminescent microparticle immunoassay test kit (Abbott, Ireland Diagnostic Division, Sligo, Ireland) as per the manufacturer's protocol.

2.7. Estimation of Aberrant Crypt Foci (ACF)

ACF were measured by the method [15]. Randomly, colons were selected and stained for 6 min in a 0.05% filtered solution of methylene blue. ACFs were counted under light microscope at 40× magnification per colon.

2.8. Estimation of Mucin-Depleted Foci (MDF)

MDF were measured by the method [15]. After measuring ACF, Colons were stained with high-iron diamine-alcian blue (HID-AB) to assess mucin production. MDF were counted per colon under light microscope at 40× magnification.

2.9. Immuno-Histochemical Staining pNF-kB-p65, Nrf-2, Keap-1, HO-1 and NQO-1

Immunohistochemical staining protocol was followed as described [17]. We used the following anti-bodies: anti-rat NF-kB-p65 rabbit antibody (dilution 1:100), anti-rat Nrf-2 rabbit antibody (dilution 1:100), anti-rat keap-1 polyclonal antibody (dilution 1:200), anti-rat HO-1 polyclonal antibody (dilution 1:200) and anti-rat NQO-1 (1:250) overnight at 4 °C. The same procedure was followed as previously described. Finally, slides were visualised under light microscope.

For semi quantitative Evaluation of pNF-kB-p65, Nrf-2, Keap-1, HO-1 and NQO-1, the tissue sections were categorized as 0 (no staining), 1 (staining, 25%), 2 (staining between 25% and 50%), 3 (staining between 50% and 75%), or 4 (staining >75%), depending upon diffusivity of the DAB. As regards intensity of staining, colon tissue sections were classified as follows: 0 (nil staining) 1 (staining is detectable, but weak), 2 (staining is distinct), or 3 (staining is intense). Scores for immunohistochemical staining were determined by adding the diffusivity and scores of staining intensities. Slides were seen by two independent observers who did not know about the experimental protocol. The slides with variable evaluations were re-evaluated, and a consensus was achieved. Measurements were carried out using an Olympus BX51 microscope having objectives of magnification 10 and 40×.

2.10. Estimation of Reactive Oxygen Species (ROS)

ROS was estimated as explained [18]. Based on the oxidation of 2'7' dichlorodihydrofluorescein diacetate to 2'7'-dichloro-fluorescein, ROS levels were measured. ROS levels were measured based on the oxidation of 2'7' dichlorodihydrofluorescein diacetate to 2'7'-dichloro-fluorescein.

2.11. Estimation of MDA

Lipid peroxidation (LPO) was measured by the following method [19]. The reaction mixture contained 580 µL phosphate buffer of 0.1 M, pH 7.4, 200 µL PMS, 200 µL ascorbic acid of 100 mM, and 20 µL ferric chloride of 100 mM, forming a total volume of 1 mL. This reaction was kept in a shaking water bath for 1 h to incubate at 37 °C. By adding 10% of 1 mL, the trichloroacetic acid reaction was stopped, followed by the addition of 0.67% of 10 mL thiobarbituric acid (TBA), and all the reactions in tubes/vials were placed in a boiling water bath for a period of 20 min. The tubes/vials of the above-mentioned reaction mixture were transferred to an ice bath and then centrifuged at 2500× g for 10 min. The optical density of the supernatant at 535 nm was measured by the amount of malondialdehyde (MDA) formed in each of the samples. The results were expressed as nmol TBA formed/h per g tissue at 37 °C using a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

2.12. Estimation of Antioxidant Enzyme Armory

2.12.1. Measurement of Superoxide Dismutase Activity (SOD)

SOD was evaluated by method [20]. The total reaction mixture is 3 mL with 2875 μL Tris-HCl buffer of 50 mM of pH 8.5, pyrogallol with 24 mM in 10 mM-HCl, and 100 μL PMS. Finally, enzyme activity was expressed as units/mg protein at 420 nm.

2.12.2. Measurement of Catalase Activity (CAT)

CAT was determined by method [21]. The reaction mixture contains 3 mL with 1950 μL phosphate buffer of 0.1 M with pH 7.4, 1000 μL H_2O_2 of 0.10 mM, and 50 μL of 10% PMS. Absorbance changes are measured at 240 nm and enzyme activity is calculated as nmol H_2O_2 consumed/min/mg protein.

2.12.3. Measurement of Reduced Glutathione (GSH)

GSH in colon was measured by the method [22]. A total of 10% PMS fraction of 1000 μL was combined with 1000 μL of 4% sulphosalicylic acid. The reaction mixture was incubated at 4 $^\circ\text{C}$ for 60 min at least and then centrifuged at $1200\times g$ for 15 min at 4 $^\circ\text{C}$. The reaction mixture consisted of total volume of 3 mL with 400 μL filtered aliquot, 2200 μL phosphate buffer of 0.1 M with physiological pH and 400 μL DTNB of 10 mM. The yellow color developed was read immediately at 412 nm.

2.12.4. Measurement of Glutathione Reductase (GR) Activity

GR activity was determined by method [23]. The reaction mixture contained a total volume of 2 mL with 1650 μL phosphate buffer of 0.1 M, at pH 7.6, 100 μL EDTA of 0.5 mM, 50 μL oxidized glutathione of 1.0 mM, 100 μL NADPH of 0.1 mM and 100 μL of 10% PMS in a total volume of 2.0 mL. The enzyme activity was assessed at 25 $^\circ\text{C}$ by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/ mg protein using a molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

2.12.5. Measurement of Glutathione Peroxidase Activity (GPx)

GPx was calculated by method [24]. The reaction mixture contained 100 μL EDTA of 1 mM, 100 μL sodium azide of 1 mM, 1440 μL phosphate buffer of 0.1 M with pH 7.4, 50 μL glutathione reductase (1 IU/mL), 50 μL reduced glutathione of 1 mM, 100 μL NADPH of 0.2 mM and 10 μL H_2O_2 of 0.25 mM and 100 μL of 10% PMS. The consumption of NADPH at 340 nm was measured at room temperature. GP x was calculated as nmol NADPH oxidized/min/mg protein with a molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

2.13. Estimation of Nitric Oxide (NO)

NO content was determined by method [25] At room temperature, various concentrations of piperine in phosphate buffer solution of pH 7.4 were incubated for 5 h with an equal volume of sodium nitroprusside solution of 5 mM in phosphate buffer of pH 7.4. Similarly, various concentrations of ascorbic acid (25–400 $\mu\text{g}/\text{mL}$) in phosphate buffer of pH 7.4 were also incubated with an equal volume of sodium nitroprusside solution (5 mM) in phosphate buffer (pH 7.4). Controlled experiments had no test compound, but instead an equal volume of buffer was used. Once incubation was done, 500 μL of incubation mixture was combined with 500 μL of Griess' reagent (sulphanilamide 1%, *o*-phosphoric acid 2%, and naphthyl ethylene diamine dihydrochloride 0.1%), and the absorbance was taken at 546 nm.

2.14. Estimation of Hydrogen Peroxide

Hydrogen peroxide (H_2O_2 standard curve) was assessed by method [26]. A total of 2000 μL microsomes were suspended and incubated at 37 $^\circ\text{C}$ for 60 min in 1000 μL reaction mixture containing

phenol red of 0.28 nm, horseradish peroxidase, dextrose of 5.5 nm, and phosphate buffer of 0.05 M with pH 7.0. A total of 10 μ L of NaOH with 10 N was used to stop the reaction and then centrifuged at $800\times g$ for 5 min. The absorbance of the supernatant was recorded at 610 nm against a blank. The quantity of H_2O_2 produced was expressed as nmol H_2O_2 per hour per g tissue based on the standard curve of H_2O_2 -oxidized phenol red.

2.15. Protein Estimation

The protein estimation was done by method [27] using BSA as standard.

2.16. Estimation of Myeloperoxidase (MPO)

The neutrophil quantification was measured as a level of MPO activity and was carried out using the Bradley et al. method [28].

2.17. Estimation of Tumor Necrosis Factor Alpha (TNF- α), Interleukin 6 (IL-6), Cyclooxygenase-2 (Cox-2), Prostaglandin E-2 (PGE-2), and Inducible Nitric Oxide Synthase (iNOS)

TNF- α , IL-6, PGE-2, iNOS and Cox-2 were measured by using commercial kit (eBioscience, Inc., San Diego, CA, USA). Assays were performed as per the instructions of the manufacturer provided with the kit on an Elisa Plate Reader.

2.18. Alcian Blue–Neutral Red (AB-NR) Staining for Mucin Analysis

This procedure was done as described [15]. Four-millimetre colon sections were cut from formalin-fixed, paraffin-embedded tissue blocks and mounted on poly-L-lysine coated microscopic slides. Dewaxing of paraffinized sections in xylene and rehydration in graded series of ethanol to water was done, followed by staining with 1% Alcian blue of pH 2.5 in 3% acetic acid solution for half an hour and then rinsing for 1 min in 3% acetic acid solution to prevent nonspecific staining followed by washing in distilled water and the sections are then counterstained with neutral red (0.5% aqueous solution) for 20 s, then dehydration in alcohol and mounting using mounting media is completed, and finally slides are visualized under a light microscope.

2.19. Statistical Analysis

The data are presented as the mean \pm standard error of the mean (SEM) from all the individual groups. Analysis of variance (ANOVA) has been used to show the differences between groups followed by Tukey–Kramer multiple comparisons test, and the minimum criterion for statistical significance is set at $p < 0.05$ for all comparisons.

3. Results

3.1. Piperine Treatment Mitigates CEA Production

There was an increase in CEA in tumor group rats when compared with control rats (** $p < 0.001$). However, treatment with Piperine treatment diminished levels of CEA in group II and IV significantly ($\#\# p < 0.01$ and $\#\#\# p < 0.001$), as shown in Figure 2.

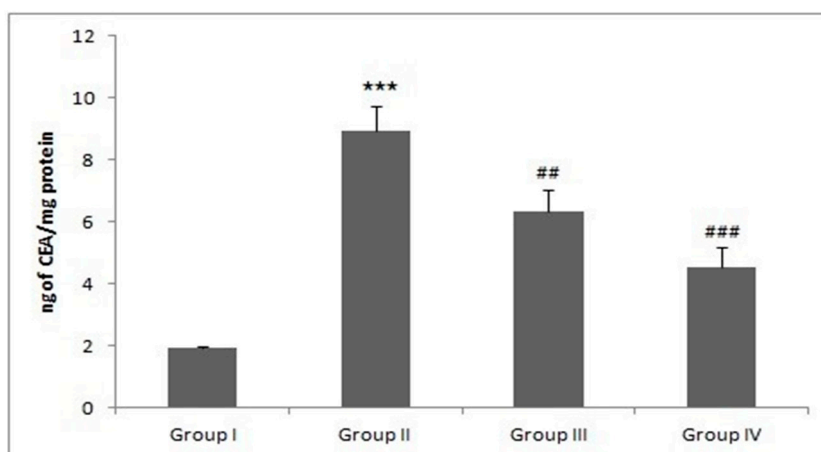


Figure 2. Piperine treatment mitigates CEA production. In group-II, the CEA level was increased significantly (** $p < 0.001$) as compared to control group. Treatment with Piperine (30 and 60 mg/kg b. wt.) significantly attenuated CEA level in group III (** $p < 0.01$) and group IV (*** $p < 0.001$) as compared to group II ($n = 10$).

3.2. Piperine Treatment Mitigates ACF and MDF, Precancerous Lesion Markers

ACF and MDF developed by DMH administration are early events which lead to colon carcinogenesis. In the current study, there was increased ACF and MDF in the tumor group when compared with control group, although treatment with piperine reduced ACF and MDF scores in group III and group IV animals significantly, as shown in Figure 3A,B.

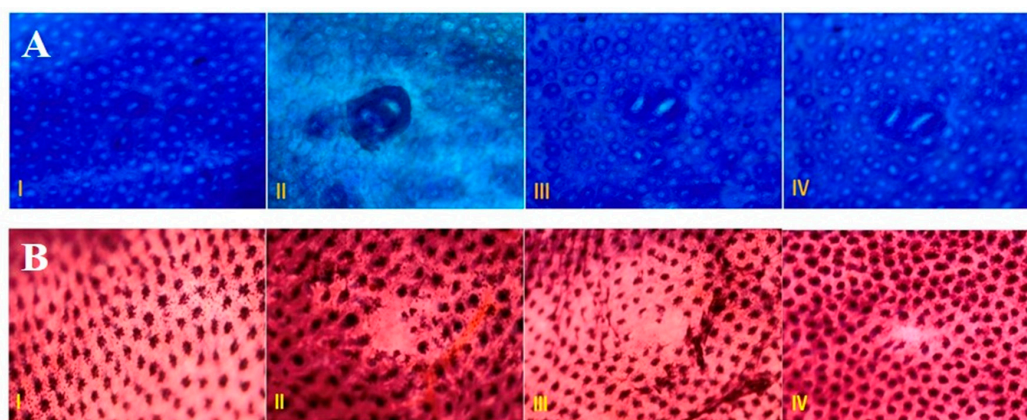


Figure 3. Effect of piperine treatment on ACF and MDF. (A) Piperine attenuates ACF per rat colon in DMH administered groups as compared to tumor group. ACF visualized by methylene blue (MB) staining are detectable under microscope. The colons here were opened and stained with high iron diamine (HID) and Alcian blue (AB) as we can see in the picture. (B) Piperine attenuates MDF per rat colon in DMH administered groups as compared to tumor group. MDF are the dysplastic crypts lacking mucin formation found in the colons of chemical carcinogen rodent studies. The colons here were opened and stained with high iron diamine (HID) and Alcian blue (AB) as we can see in the picture ($n = 10$).

3.3. Piperine Treatment Regulates Nrf-2/Keap-1/HO-1/NQO-1 Pathway

The immunohistochemical slides show strong expression of Keap-1 (Figure 4B) and lower expression of Nrf-2 (Figure 4A), NQO-1 (Figure 4C) and HO-1 (Figure 4D) in DMH group as compared to control. However, piperine treatment induced Nrf-2, NQO-1 and HO-1 expression in colon tissue in rats as compared to group II, where no significant staining was observed. Moreover, Piperine (60 mg/kg

b.wt.)-treated rats showed lesser staining of Keap-1 as compared to DMH-treated rats which infers the anti-inflammatory potential of Piperine and increased Nrf-2, NQO-1, and HO-1, causing anti-oxidant machinery to function and shutting down NF- κ B and its downstream elements (Figure 4A–D).

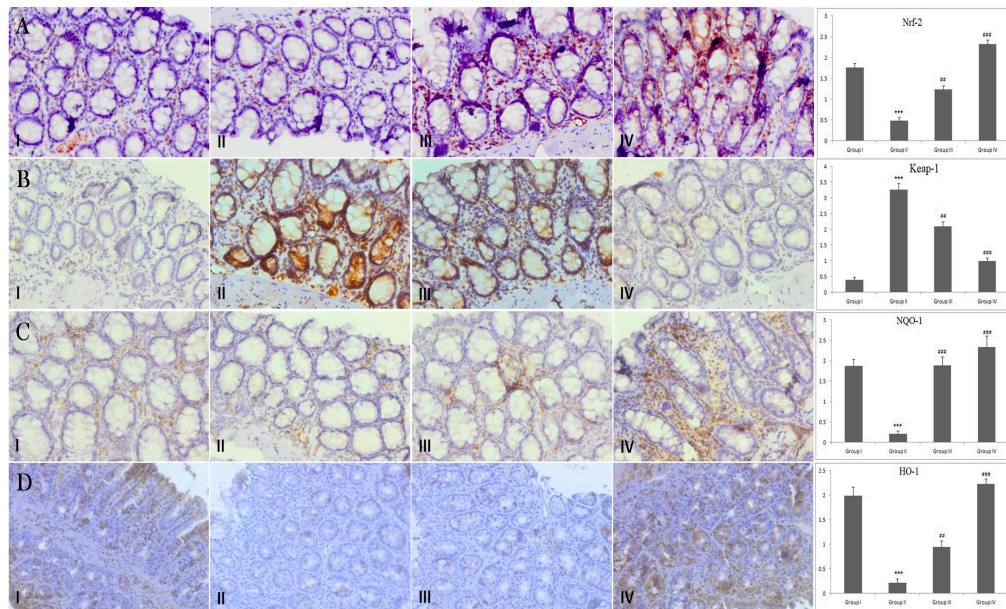


Figure 4. Effect of piperine treatment on Nrf-2, Keap-1, NQO-1 and HO-1 expression. Photomicrographs of colon sections depicting immunohistochemical analyses. Adjacent to photomicrographs are four panels which show quantitative evaluation of Nrf-2, Keap-1, NQO-1 and HO-1 expression immunostaining. Significant differences were indicated by *** $p < 0.001$ when compared with group I and (## $p < 0.01$), (### $p < 0.001$) when compared with group II (A) Brown color indicates specific immunostaining of Nrf-2, and light blue color indicates counter-staining by hematoxylin. The colonic section of DMH-administered group-II has decreased immunopositive staining of Nrf-2, as indicated by brown color, as compared to control group-I, while treatment of Piperine (30 and 60 mg/kg b. wt.) in groups-III and IV increased Nrf-2 compared to group II. Piperine significantly activated Nrf-2 in group III and IV, respectively, (## $p < 0.01$) and (### $p < 0.001$), when compared with DMH-administered group-II. (B) Photomicrographs of colon sections depicting immunohistochemical analyses; brown color indicates specific immunostaining of Keap-1 and light blue color indicates counter staining by hematoxylin. The colonic section of DMH-administered group-II has more Keap-1, as indicated by brown color, as compared to control group I, while treatment of piperine (30 and 60 mg/kg b. wt.) in groups III and IV reduced Keap-1 immunopositive as compared to group II. Piperine significantly suppressed Keap-1 in group III and IV, respectively, (## $p < 0.01$) and (### $p < 0.001$), when compared with DMH-administered group-II. (C) Photomicrographs of colon sections depicting immunohistochemical analyses; brown color indicates specific immunostaining of NQO-1 and light blue color indicates counter-staining by hematoxylin. The colonic section of DMH-administered group-II has decreased immunopositive staining of NQO-1, as indicated by brown color, as compared to control group I, while treatment of piperine (30 and 60 mg/kg b. wt.) in groups III and IV increased NQO-1 as compared to group II. Piperine significantly upregulated NQO-1 in group III and IV, respectively, (### $p < 0.001$) when compared with DMH-administered group-II. (D) Photomicrographs of colon sections depicting immunohistochemical analyses; brown color indicates specific immunostaining of HO-1 and light blue color indicates counter-staining by hematoxylin. The colonic section of DMH-administered group-II has decreased immunopositive staining of HO-1, as indicated by brown color, as compared to control group I while treatment of piperine (30 and 60 mg/kg b. wt.) in groups III and IV increased HO-1 as compared to group II. Piperine significantly upregulated HO-1 in group III and IV, respectively, (## $p < 0.01$) and (### $p < 0.001$), when compared with DMH-administered group-II. All images have original magnification of 40 \times ($n = 10$).

3.4. Piperine Treatment Mitigates ROS

ROS was pointedly higher ($*** p < 0.001$) in group II (DMH group) when compared with group I, suggesting that DMH increases oxidative stress by initiating ROS. Piperine treatment reduced ROS in colon tissue in group III ($## p < 0.01$) and group IV animals, respectively ($#### p < 0.001$) (Figure 5).

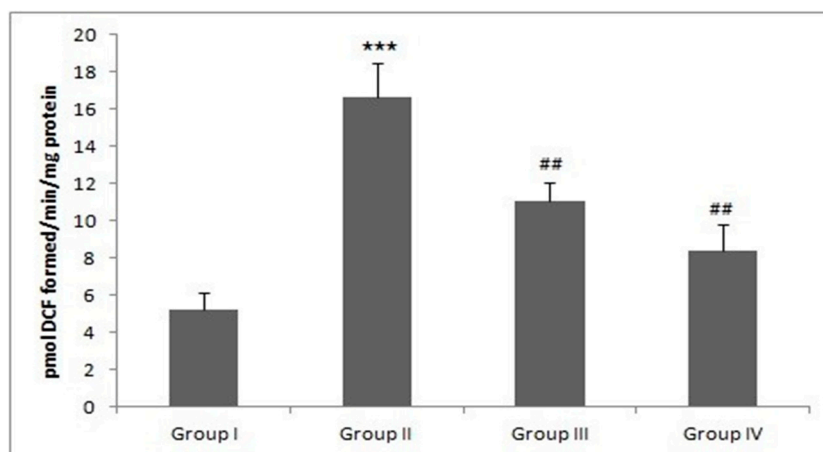


Figure 5. Piperine treatment mitigates ROS. In DMH-administered group-II, a tremendous amount of ROS was produced ($*** p < 0.001$) as compared to group-I. Treatment with piperine (30 and 60 mg/kg b. wt.) significantly mitigated ROS levels in group III ($## p < 0.01$) and group IV ($## p < 0.01$) as compared to group II ($n = 10$).

3.5. Piperine Treatment Alleviates MDA Levels

MDA is a by-product and hallmark of lipid peroxidation. There was a sharp rise in MDA in the DMH-administered/tumor group as compared to control ($*** p < 0.001$). However, treatment with piperine decreased unusually high LPO levels in group III and IV ($## p < 0.01$ and $#### p < 0.001$) (Figure 6).

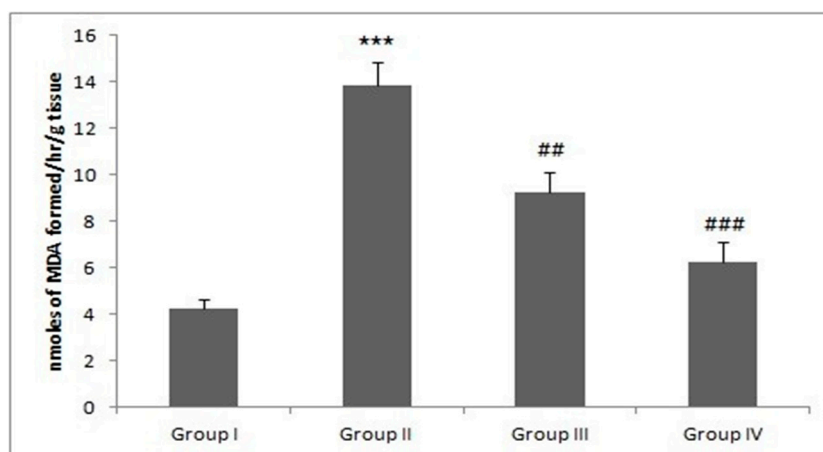


Figure 6. Piperine treatment alleviates MDA levels. In DMH-administered/tumor group-II, the MDA level was increased significantly ($*** p < 0.001$) as compared to control group. Treatment with piperine (30 and 60 mg/kg b. wt.) significantly alleviated MDA levels in group III ($## p < 0.01$) and group IV ($#### p < 0.001$) as compared to group II ($n = 10$).

3.6. Piperine Treatment Alleviates Anti-Oxidant Armory

Piperine's effect on DMH-induced exhaustion of different antioxidants was studied. The results are shown in Table 1. There was a significant difference ($*** p < 0.001$) in the activity of antioxidants

between control and the DMH-administered group. However, piperine treatment reinstated the activity of all antioxidant enzymes to normal ($^{\#} p < 0.05$, $^{\#\#} p < 0.01$ and $^{\#\#\#} p < 0.001$) (Table 1).

Table 1. Effect of Piperine on Glutathione and Antioxidant Armory.

	Group I	Group II	Group III	Group IV
Reduced Glutathione (GSH; nmol mg protein)	214.72 ± 11.2	92.32 ± 8.31 ***	141.39 ± 11.3 [#]	197.52 ± 15.8 ^{\#\#\}
Oxidized Glutathione (GSSG; nmol mg protein)	31.02 ± 3.23	79.86 ± 3.03 ***	62.03 ± 4.91 [#]	41.52 ± 5.02 ^{\#\#\}
GSH/GSSG Ratio	6.921 ± 0.82	1.156 ± 0.21 ***	2.279 ± 0.33 [#]	4.759 ± 0.61 ^{\#\#\}
GPx (nmol/ min/mg protein)	201.23 ± 17.1	82.43 ± 7.25 ***	160.28 ± 14.7 ^{\#\#\}	182.46 ± 17.3 ^{\#\#\}
GR (nmol min/min/mg protein)	201.44 ± 19.5	84.25 ± 9.22 ***	139.12 ± 17.8 ^{\#\#\}	189.23 ± 20.9 ^{\#\#\}
SOD (units/ min/mg protein)	10.63 ± 1.93	4.01 ± 0.41 ***	7.11 ± 0.73 ^{\#\#\}	9.03 ± 0.97 ^{\#\#\}
Catalase (nmol H ₂ O ₂ consumed/min/mg protein)	10.23 ± 1.62	4.82 ± 0.48 ***	5.94 ± 0.33 [#]	8.32 ± 0.72 ^{\#\#\}
H ₂ O ₂ (nmol of H ₂ O ₂ /g tissue)	185.2 ± 17.4	403.5 ± 32.1 ***	256.9 ± 21.3 ^{\#\#\}	205.7 ± 19.3 ^{\#\#\}

Group-I: Control; Group-II: DMH-administered group (20 mg/kg BW); Group-III: DMH rats treated with Piperine (30 mg/kg b.wt.); Group-IV: DMH rats treated with Piperine (60 mg/kg b.wt.). Data are represented as mean ± S.E.M ($n = 10$).

3.7. Piperine Treatment Mitigates H₂O₂ Levels

H₂O₂ levels in colon tissue in group II increased significantly ($^{***} p < 0.001$) when compared with group I. However, treatment with piperine in groups III and IV caused a marked decrease in H₂O₂ at both doses ($^{\#\#} p < 0.01$ and $^{\#\#\#} p < 0.01$) (Table 1) ($n = 10$).

3.8. Piperine Treatment Regulates pNF-κB

There was an increase in pNF-κB expression in group II when compared with group I. However, Piperine treatment to group III and group IV animals decreased pNF-κB expression significantly, inhibiting pNF-κB and its downstream elements (Figure 7).

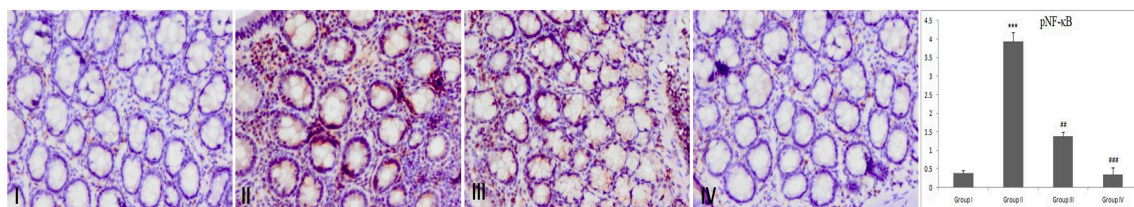


Figure 7. Effect of piperine treatment on pNF-κB expression. Photomicrographs of colon sections depicting immunohistochemical analyses; brown color indicates specific immunostaining of pNF-κB and light blue color indicates counter-staining by hematoxylin. The colonic section of DMH-administered group-II has more pNF-κB immunopositive staining, as indicated by brown color, as compared to control group I, while treatment of piperine (30 and 60 mg/kg b. wt.) in groups III and IV reduced pNF-κB immunopositive staining as compared to group II ($n = 10$). Piperine significantly downregulated pNF-κB in group III and IV, respectively, ($^{\#\#} p < 0.01$) and ($^{\#\#\#} p < 0.001$), when compared with DMH-administered group-II ($n = 10$). Original magnification: 40×.

3.9. Piperine Treatment Mitigates Cox-2 and Inflammatory Mediators

Levels of inflammatory cytokines like TNF- α , PGE-2, IL-6 and Cox-2 were also increased significantly in the DMH-treated group II in comparison to untreated control group I (** $p < 0.001$). However, Piperine treatment in groups III and IV attenuated TNF- α , PGE-2, and IL-6 and Cox-2 levels significantly (Table 2) at both the doses ($\# p < 0.05$, $\#\# p < 0.01$ and $\#\#\# p < 0.001$), respectively (Table 2).

Table 2. Effect of Piperine on Serum Levels of Inflammatory Cytokines (IL-6, TNF- α , Cox-2, iNOS and PGE-2).

	Group-I	Group-II	Group-III	Group-IV
IL-6 (pg/mL)	834.12 \pm 42.34	2023.32 \pm 187.4 ***	1342.07 \pm 146.6 #	1054.83 \pm 94.50 $\#\#\#$
TNF- α (pg/mL)	522.83 \pm 40.27	1443.62 \pm 138.2 ***	987.83 \pm 105. #	774.31 \pm 55.49 $\#\#$
Cox-2 (pg/mL)	921.62 \pm 63.11	1998.23 \pm 92.3 ***	1532.13 \pm 144.1 $\#\#$	1098.23 \pm 68.70 $\#\#\#$
iNOS (pg/mL)	774.52 \pm 87.63	1638.65 \pm 106.1 ***	1310.22 \pm 119.4 $\#\#$	897.35 \pm 77.42 $\#\#\#$
PGE-2 (pg/mL)	814.89 \pm 99.10	1547.86 \pm 165.2 ***	1211.17 \pm 113.4 $\#\#$	998.64 \pm 94.71 $\#\#\#$

Group-I: normal control; Group-II: DMH-administered group (20 mg/kg BW); Group-III: DMH rats treated with Piperine (30 mg/kg b.wt.); Group-IV: DMH rats treated with Piperine (60 mg/kg b.wt.). Data are represented as mean of 10 rats \pm S.E.M.

3.10. Piperine Treatment Mitigates NO and iNOS Production

There was a significant rise in NO and iNOS production in group II as compared with the group I animals (** $p < 0.001$). However, treatment with Piperine effectively reduced NO and iNOS formation in group III and IV when compared with group II ($\#\# p < 0.01$ and $\#\#\# p < 0.001$) (Figure 8 and Table 2).

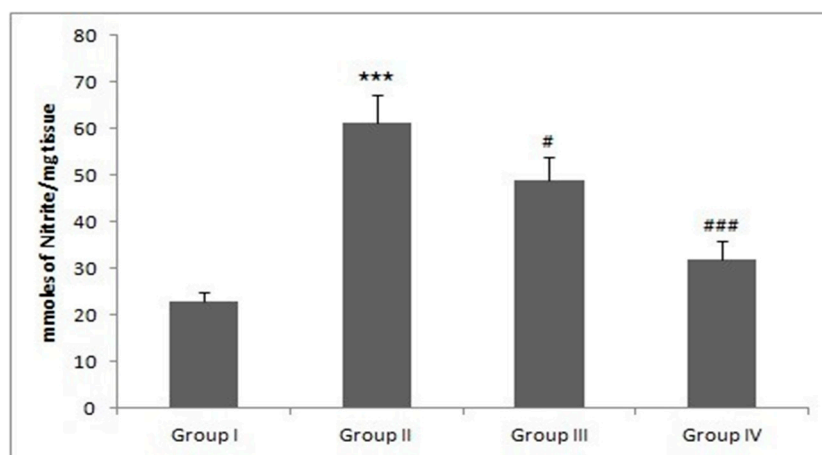


Figure 8. Effect of piperine and DMH-administered group on nitrite levels. In DMH administered group-II, the nitrite levels were significantly increased (** $p < 0.001$) as compared to control group-I. Treatment with piperine significantly (30 and 60 mg/kg b. wt.) attenuated nitrite levels in group III ($\# p < 0.05$) and group IV ($\#\#\# p < 0.001$) as compared to group-II ($n = 10$).

3.11. Piperine Treatment Mitigates MPO Production

There was a significant rise in MPO production in group II as compared with the group I animals (** $p < 0.001$). However, treatment with Piperine effectively reduced MPO formation in groups III and IV when compared with group II ($\#\# p < 0.01$) (Figure 9).

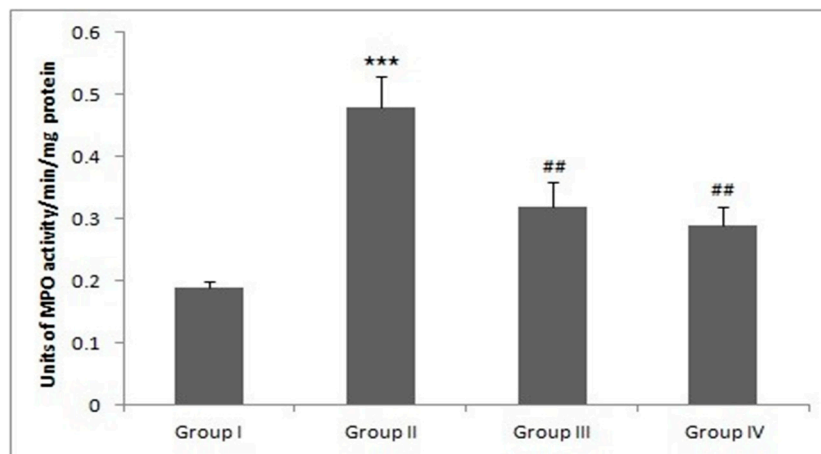


Figure 9. Effect of piperine and DMH-administered group on MPO levels. In DMH-administered group-II, the MPO levels were significantly increased (***) $p < 0.001$ as compared to control group-I. Treatment with piperine significantly (30 and 60 mg/kg b. wt.) attenuated MPO levels in group III (## $p < 0.05$) and group IV (### $p < 0.01$) as compared to group-II ($n = 10$).

3.12. Piperine Treatment Attenuates Mucin Staining in Colonic Tissue

There is negligible blue staining in control group, which indicates mucin integration as compared to the DMH-administered group. However, piperine treatment decreased disintegration of the mucous layer in groups III and IV (Figure 10).

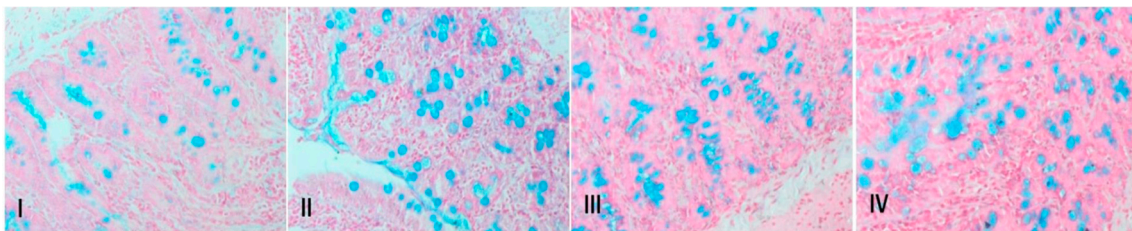


Figure 10. Photomicrographs showing mucin staining. There is decrease in mucin in mucous layer in DMH administered group, which is depicted by blue staining, when compared with control. Treatment with piperine increased mucous layer, as we can see from the lesser appearance of blue staining pattern in III and IV slides, representing groups III and IV ($n = 10$).

3.13. Effect of Piperine on the Colon Histology

Histological evaluation revealed that group I rats showed normal histological architecture with no signs of visible malformation/anomaly (Figure 11). There was disintegration of histoarchitecture like irregular colonic crypts, goblet cells disintegration, penetration of inflammatory cells, erosion of the mucous membrane, and gross inflammation was observed in DMH-administered rats. However, treatment with piperine restored cryptic architecture and goblet cell integration. Piperine also repealed adenoma formation and intrusion of inflammatory cells, hence regulating inflammation, which forms the basis of carcinogenesis (Figure 11).

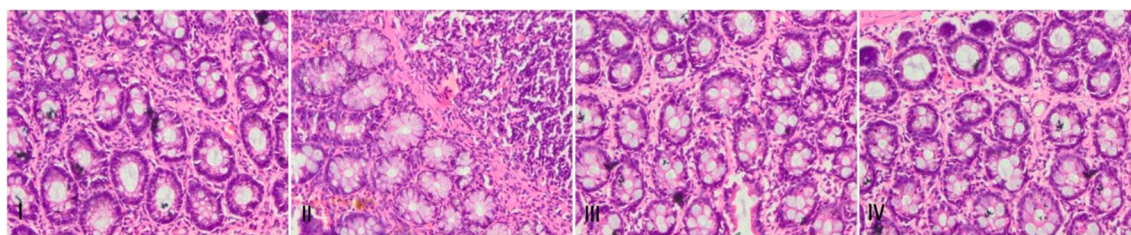


Figure 11. Effect of Piperine treatment on DMH-administered pathological changes in rat colon tissues. Photomicrographs of H&E staining of histological sections of colon tissue depicting different experimental groups: group-I indicate normal histo-architecture of colon sections. Group-II shows extensive disintegration of normal architecture in DMH administered group. In groups III and IV piperine treatment showed protection against piperine-induced pathological changes. Both the doses of piperine maintained the integrity of mucous membrane, goblet cells and colonic crypts ($n = 10$), magnification: 40 \times .

4. Discussion

Colon cancer process involves varied steps starting from pathological modifications extending from disconnected microscopic mucosal lesions of ACF and MDF to progression of tumor. Likewise, oxidative stress and inflammation are critical in the pathogenesis of cancer growth and promotion at molecular level. DMH was used as a carcinogen in the present study which mimics the human colon cancer pathology and does not only rely on extrapolating data from *in vitro* models due to the intricate complexity of genetic and epigenetic processes taking place inside humans which contribute to colon cancer. Hence, piperine was tested to find its anti-cancer efficacy at molecular, cellular and morphological levels, which have shown anti-colon cancer activity *in vitro* previously [6–10].

We first studied the influence of piperine on biomarkers of colon cancer. CEA is one of the most used tumour markers with predictive implication in early stages of colon cancer. It mediates adhesion intercellularly, promotes cell accretion, and controls immunity and signalling. Consequently, it shows tumour invasiveness and metastatic activity [29]. We did an investigation of the same biomarker in our experimental colon cancer study as well. It is not astonishing that CEA level was raised in DMH administered group significantly, as reports suggest that CEA is elevated in gastric and colon cancer. Nevertheless, the CEA level was decreased by the piperine treatment, possibly due to its anti-cancerous activities, resulting in the prevention of DMH-induced colon carcinogenesis. We also studied ACF and MDF, considerable pre-cancerous lesions found in colorectal cancer in carcinogen-administered rats as well in high-risk human cases. Because of their similarities in genotypic and phenotypic descriptions in animal and humans, ACF and MDF are standard biomarkers to detect and analyse pathogenesis of colon carcinogenesis at an early stage. The development of ACF and MDF due to DMH administration in experimental rodents was also used as a short-term model to investigate the protective mechanisms of organic compounds [15]. We found that piperine reduced ACF and MDF scores in group III and IV rats, respectively, when compared with the tumor group, which implies a role of piperine in inhibiting early events of colon carcinogenesis, which has been supported by earlier studies [30].

Next, we studied a robust signalling pathway, namely 2 (Nrf-2)/Keap-1, against oxidative stress for cellular protection. Nrf-2, a protective transcription factor, controls downstream antioxidant machinery or detoxification system molecules found in the cells. The instigation of Nrf-2 during the initial stage of inflammation-mediated tissue damage impedes the formation of proinflammatory modulators like pro-inflammatory cytokines, chemokines, and cell adhesion molecules [31]. The development and destruction of Nrf-2 are in balance under normal physiological conditions. However, activation of Nrf-2 is blocked by Keap-1 protein under stress conditions. Upon activation and dissociation of Keap-1, Nrf-2 accrues and binds with the antioxidant responsive elements (AREs) via translocating into the nucleus in the promoter region to activate the transcription and defensive activities of downstream antioxidant machinery like HO-1 and glutathione-dependent enzymes. In the current study, Nrf-2 expression was

diminished in DMH administered animals and treatment with piperine upregulated Nrf-2 expression and further activated regulatory enzymes downstream, as reported previously [32]. This was further validated and supported by a study where in Nrf-2- (−/−) mice were additionally vulnerable to dextran sulfate sodium (DSS)-induced colitis [33]. This demonstrated that Nrf-2 activation is an auxiliary therapy, possibly useful for colitis and colon cancer patients. The augmented sternness of colonic damage in DMH group animals may be correlated with a decrease in phase II detoxification enzymes, as reported previously [34]. In Nrf-2-deficient mice, proinflammatory modulators like interleukin 1 β (IL-1 β), interleukin 6 (IL-6), and tumor necrosis factor α (TNF- α) were prominently elevated as compared with the colon tissues of wild-type Nrf-2 mice [33,35]. Moreover, Nrf-2-deficient (Nrf-2-/-) mice are at an increased risk of having various cancers like stomach, CRC and skin because they are increasingly vulnerable to oxidative injury-instigated ailments and DNA damage caused by chemicals as compared to wild-type mice. Khor et al. testified that Nrf-2 knockout mice with azoxymethane and DSS administration showed greater tumor incidence (80% versus 29%, respectively) as well as an augmentation of inflammatory markers like Cox-2, 5-lipoxygenase, PGE-2, and leukotriene levels in colon as compared with azoxymethane and DSS-treated wild-type (WT) mice [36]. In the present study, there was decreased expression of Nrf-2 and HO-1 in DMH group animals. However, treatment with piperine increased Nrf-2 and HO-1 expression in treatment groups. Thereby, alleviating ROS, and hence DNA damage, resulting in the protection of cells against potentially harmful entities may be the fundamental process through which Nrf-2 shields against chemical-induced carcinogenesis [37].

Keap-1 is a protein that keeps Nrf-2 in an inhibited state in cytoplasm. Dissociation and amendment of Nrf-2–Keap-1 complex is necessary for the transfer of Nrf-2 in the nucleus, which leads to the activation of Nrf-2–ARE-dependent signalling, and numerous cell transduction pathways. ARE inducers like piperine treatment downregulate Keap1 level in treatment groups as compared to the DMH group, and due to this, Nrf-2 translocation occurs, activating the downstream pathway. Various pathological events induce stress and HO-1 is a crucial protein that helps in cellular adaptation. It has been found to be a potent beneficial target for various oxidant and inflammatory diseases. The HO enzyme family provides carbon monoxide (CO) and free iron (Fe²) by breaking down heme to biliverdin and bilirubin. Both bilirubin and CO may shield molecules against oxidative injury via a reduction in superoxide anions and lipid peroxidation. Additionally, reports indicate pro-apoptotic and anti-proliferative activities of HO-1 in prostate, breast and oral cancer, although the exact process of the act is unknown [14,38].

Nrf-2 is also found to control several phase II detoxification molecules like HO-1, NQO1, glutathione S-transferase (GST), UDP-glucuronosyltransferase (UGT) and glutamate-cysteine ligase (GCL) [39]. NQO1 is an important target of Nrf-2, is involved in detoxification process, and its upregulation thwarts the ability to amplify IL-1 β and TNF- α [36]. In the present study, we found downregulation in HO-1 and Keap-1 in DMH group. However, treatment with piperine upregulated expression of HO-1 and Keap-1 at both the doses, signifying activation of Nrf-2. Nonetheless, activated Nrf-2 triggers ARE resulting in the activation of anti-inflammatory and anti-oxidant responses, activating protective responses in cells, as reported previously [40].

Oxidative stress and chronic inflammation are two of the life-threatening features intricately associated both in the commencement and progression of cancerous growth by modulating tumor microenvironment which, in ROS, acts as a secondary messenger to dysregulate various signaling pathways. The deficit of antioxidant machinery or detoxification system enzymes, as well as up-surfing in ROS and RNS, is detrimental to colonic tissue homeostasis. We found that there was an increase in ROS production in the DMH-administered group as compared to control group. Treatment with piperine attenuated ROS levels in both groups, as reported previously [39]. MDA is a hallmark of oxidative stress produced by ROS and is a carcinogenic agent attributing to the formation of cancers in humans. We observed augmented levels of MDA in DMH-induced CRC as reported previously [41]. Piperine treatment repressed the production of MDA levels, which may be due to the alkaloids present in piperine scavenging free radicals due to its potent anti-oxidant activity attenuating the MDA level

in treatment groups. Enzymatic and non-enzymatic antioxidants are the primary defenders against cytotoxic oxygen radicals by scavenging intermediates of oxygen reduction [42]. Some anti-oxidants like SOD and CAT protect against lipid peroxidation in tissues by directly eliminating reactive oxygen metabolites like superoxide ($O_2^{2\bullet}$) and hydroxyl ions (OH^\bullet), providing one of the most efficient defensive mechanisms in the biological system against diseases [43]. CAT converts H_2O_2 to H_2O and O_2 , hence averts oxidative injury. In the present study, SOD and CAT activities were diminished in DMH-induced CRC, whereas these activities were enhanced after the administration of piperine, which may be because of its free-redox-trapping activity [11]. GSH constitutes the major non-protein thiol in mammalian cells, which helps in many cellular activities like the regulation of protein synthesis. GSH acts as a substrate for many xenobiotic and free radical elimination reactions. Augmented GSH may stimulate other GSH-dependent enzymes like GPx, which has four selenium cofactors that catalyse the breakdown of H_2O_2 and organic hydroperoxides [41]. GR is another glutathione-restoring enzyme that catalyses oxidized glutathione (GSSG) to GSH by the oxidation of NADH to NAD [44]. In the current study, GSH was diminished and GSSG increased in DMH group in eliminating free radicals, hence the levels of reduced GSH were exhausted. However, piperine supplementation increased GSH levels and decreased GSSG in treatment groups due to its anti-oxidative potential. Moreover, the detoxification of xenobiotics, carcinogens, free radicals and peroxides occurs primarily through GPx and GR by conjugating xenobiotics with GSH, resulting in cellular protection against mutagen-induced toxicity. In the present study, there were decreased activities of GSH and GSH-dependent enzymes like GPx and GR in DMH-induced rats, whereas treatment with piperine increased the antioxidant armory, which may be because of its free radical scavenging potential [45]. Earlier reports recommend that alterations in redox balance and signaling are hallmarks of the initiation and promotion of carcinogenesis and resistance to treatment [46]. Hence, agents that upregulate antioxidant enzyme machinery like CAT, SOD, GR, GPx, which inactivate ROS, have a massive potential to avert the initiation and promotion of cancer, as observed in the current study. Therefore, the upregulation of antioxidant machinery and the preclusion of DMH-induced colon carcinogenesis by prophylactic treatment of piperine may possibly be due to the presence of alkaloids which resulted in boosting endogenous anti-oxidant machinery because of its anti-oxidant, anti-inflammatory and various pharmacological activities attributed to it [11,12,29,39,47].

In the instigation and development of cancer, a potential cross-talk between Nrf-2 and NF- κ B pathways is recognized. One of the principle ways through which chronic inflammation leads to the formation of neoplasm/growth is by the production of ROS via inflammatory cells. This Nrf-2 intermediated anti-cancer response is accomplished by not only an increase in antioxidant armory, but, additionally confirmed by the repression of inflammatory pathway mediators smoothed through the NF- κ B signaling pathway. Correspondingly, an upregulation of cytokines occurs due to Nrf-2 deficiency as a result of NF- κ B activation or the activation of Nrf-2 attenuates NF- κ B and downstream signalling, as reported previously [47,48]. Carini et al. reported that the dysregulation of ROS may alter DNA assembly, and consequentially modify proteins and lipids, and stimulate numerous stress-activated transcription elements including NF- κ B, the formation of pro and anti-inflammatory cytokines which contribute to carcinogenesis via oxidative insults [38,49]. However, antioxidant agents like piperine impede IL-1beta, iNOS, Cox-2, NO, NF- κ B production and, henceforth, carcinogenesis [40,50].

Chronic inflammation contributes to 25% of human cancers. One of the most important pathways involved in inflammation is NF- κ B. NF- κ B is a redox specific transcription factor which activates immune and cell detoxification systems, endorses the development of pro-inflammatory cytokines like TNF- α , IL-1, IL-6, and IL-8, and promotes tumor growth [51]. NF- κ B's enhanced expression may be attributed to chronic inflammation followed by cyclic administration with carcinogen; DMH contributes to colon carcinogenesis. Other studies also decipher that colon cancer cell lines have remarkably abnormal NF- κ B expression and lower I κ B levels, showing that dysregulated NF- κ B is a major contributor to colon cancer, which is in accordance with our results as well [52]. Our results further validate that the piperine inhibits NF- κ B activity, as reported previously, and strongly recommend

that the bioactivity of piperine against colon carcinogenesis may possibly be due to the inactivation of NF- κ B, which is further evident due to the inhibition of downstream pathway player proteins [11,53,54].

NF- κ B has a critical connection amongst inflammation and cancer since it increases levels of tumor-stimulating cytokines downstream, like IL-6, TNF- α , PGE-2, Cox-, as well as survival genes such as Bcl-extra-large (Bcl-xL). Similar results were obtained in the current study. ROS is released by the activation of inflammatory cells, resulting in oxidative injury to DNA and proteins. There are cumulative indications which decipher that the chances of colon carcinogenesis increase by enhancement of pro-inflammatory cytokines like TNF- α , IL-1 α , IL-1 β and IL-6, which further increase the secretion of PGE-2, an inflammatory mediator. However, piperine treatment attenuated the levels of the above-mentioned pro-inflammatory cytokines, possibly by the inhibition of NF- κ B and anti-inflammatory activity of piperine, as reported previously [54–56].

Cox-2 is triggered in inflammatory or hypoxic environments and is upregulated in various cancers, including colon cancer, unlike in normal cells, thus making it a potential therapeutic target. The major downstream mediator of Cox-2 is PGE-2, which enhances cellular growth and angiogenesis, obstructs apoptosis, augments invasiveness, and regulates immunosuppression in colonic mucosa. Additionally, enhanced Cox-2 expression is directly proportional to PGE-2, resulting in increased production of malondialdehydes, forming DNA adducts in colonic tissue, and quickens the process of carcinogenesis. In the current study, we found an increase in Cox-2 and hence PGE-2 in the DMH group, whereas treatment with piperine decreased Cox-2 and hence PGE-2 in both the groups, thereby precluding the process of carcinogenesis and not allowing its acceleration [56].

NO, an RNS, an inflammatory mediator, plays an essential role in colon tumorigenesis in both humans and investigational experiments. NO is formed by three isoforms of nitric oxide synthase (NOS), under normal physiological conditions. iNOS produces micromolar concentrations of NO which last for hours or days, which is a greater amount than in NO production by other isoforms of NOS, resulting in DNA damage, deficiency of DNA repair, cancerous growth proliferation and promotion. Consequently, tumor malignancy is enhanced by variations brought about by NO formation in cells by damage/mutation in DNA, dysregulating signalling pathways and instability in genome DNA repair pathways are negatively impacted by high NO concentrations, leading to carcinogenesis [9]. iNOS is produced by proinflammatory agents and the tumor micro-environment and does not depend on calcium. Femia et al. reported that DMH-induced colorectal cancer has high pro-inflammatory enzyme iNOS expression [57]. We obtained similar results in our current study, which is supported by earlier findings. However, over-production of NO contributes by iNOS damaging DNA repair and promoting cancer growth. Therefore, mitigating iNOS, and hence NO, at early stages of colon cancer in humans can be beneficial strategy to diminish development of cancer. In addition, carcinogens may increase the activities of Cox-2 and iNOS on the mucosa of the colon, which also causes the promotion of cancer occurrence. Both Cox-2 and iNOS are upregulated by NF- κ B. It was indicated that by reducing NF- κ B expression, the occurrence of cancer can be reduced and decrease the induction of NOS. Thereby, decreasing NO further alleviates DNA damage and hence reduces cancer growth and promotion [10,11].

MPO is another enzyme found in neutrophils for H₂O₂ production. It has been reported that in the inflamed tissues, MPO level is directly proportional to the neutrophil concentration and infiltration. Thus, acute intestinal inflammation is evaluated by MPO measurement, which acts as a quantitative and sensitive assay. Inflammation is associated with oxidative stress and promotes tumor initiation and promotion. Chronic intestinal inflammation and colon cancer are related to each other. The present study found that the colon cancer group showed an elevation in the intestinal inflammatory markers like MPO and Cox-2 through the NF- κ B-mediated response. Piperine treatment alleviated MPO levels through suppression of NF- κ B, henceforth suppressing the infiltration of neutrophils and chronic inflammation leading to colon cancer [15,58].

The histology of colon tissue sections of control did not show any anomaly in crypt morphology and architecture, with no signs of inflammation. DMH group animals showed infiltration of inflammatory

cells, aberrant crypt formation, depletion of mucin, tissue necrosis, ulcer formation and cellular injury. However, Piperine treatment decreased hemorrhage formation, infiltration of inflammatory cells, edema in sub mucosal layers, and ulceration, as reported previously [59–62]. We discovered a similar pattern in the present study after treatment with piperine, as it restored the histo-architecture, similar to that of normal colon.

Therefore, the results of the current study indicate a novel bifunctional role of NF- κ B suppression, i.e., deactivating the NF- κ B modulated inflammatory pathways and in the upregulation of Nrf-2 facilitated anti-oxidative/anti-inflammatory pathways. Thus, Piperine looks like a substantially potential bioactive phyto-product with imminent scientific application in prophylactic treatment of colon carcinogenesis. However, the immunohistochemical results could be further explored through quantitative estimations which would further support/clarify our immunohistochemical, histological and biochemical data. However, in future studies, clarity and certainty about mechanisms could be revealed through how piperine impacts colon cancer at preclinical and clinical levels.

5. Conclusions

It is imperative to find innovative ways for effective treatment of colorectal cancer. Even though widespread improvement has been made in the treatment of colorectal cancer, yet millions of people die annually due to colon cancer worldwide. Nrf-2 and its downstream mediators protect cells from extracellular and intracellular oxidative damage and maintain redox homeostasis while NF- κ B contributes to survival and resistance of colon cancer cells. We show that prophylactic treatment of piperine activates Nrf-2 pathway which triggers antioxidant response machinery mediators like HO-1, NQO-1 GSH, GR, GPx, CAT, SOD, scavenges ROS; decreases lipid peroxidation; blocks NF- κ B and its associated downstream signalling molecules which include Cox-2, PGE-2, TNF- α , IL-6, NO, iNOS and other inflammatory mediators like NO, MPO. Our histological findings and pre-cancerous markers of colon further validate beneficial effects of piperine on alleviating DMH induced colon carcinogenesis. Hereafter, mitigation of Nrf-2/Keap-1 pathway and pre-cancerous events by piperine may possibly be a potential mechanism. Henceforth, piperine may be a promising molecule for the prophylactic treatment of colon carcinogenesis.

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Conflicts of Interest: The authors declare that there are no conflicts of interest.

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