Anti-Cancer Activity of Piperine Against Colon Carcinogenesis Via Modulation of NF-κB/Nrf-2/Keap1/HO-1 Signalling Pathways

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Research

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Abstract

Background: Colon cancer is the most common cancer in men and women globally, killing millions of people annually. Though widespread development has been made in the management of colorectal cancer, still there is an urgency to find novel targets for effective treatment of these patients. Piperine is an alkaloid found in black pepper having anticancer, anti-oxidant and anti-inflammatory activities. Nuclear factor-erythroid 2–kelch-like ECH-associated protein 1(Nrf-2/Keap1)/ Heme-oxygenase1 (HO-1) signaling pathway plays a vital part in shielding cells from the injury of intracellular oxidative stress and inflammation. A potential cross-talk between 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 prevention of chemically induced colon cancer with piperine.

Results: We found that NF-κB inhibition occurs by the activation of Nrf-2 blocks downstream inflammatory mediators/cytokines (TNF-α, IL-6, IL-1β, Cox-2, PGE2, iNOS, NO, MPO) and triggers antioxidant response machinery (HO-1, NQO-1 GSH, GR, GPx, CAT, SOD), scavenges ROS, decreases lipid peroxidation. Histological findings further validated our defensive effects of piperine on colon carcinogenesis. Piperine down-regulates CEA, MDF and ACF, markers of precancerous lesions in colon.

Conclusion: Our results indicate that piperine may be an effective molecule for the prophylactic treatment of colon carcinogenesis by targeting NF-κB/Nrf-2/Keap1/HO-1 pathway as a progressive strategy in preclusion and effective treatment of colorectal cancer.

Introduction

According to Globocon 2018 data from Saudi Arabia, Colorectal cancer (CRC) is documented as the top most cancer in men, third commonest cancer-causing death in women, and fourth most frequently occurring cancer throughout the world (Globocon 2018). Surgery, radiotherapy, and chemotherapy are the conventional strategies of treatments for CRC which fail because patients either develop drug resistance or excessive adverse effects which become fatal in itself [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 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 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 least 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 attributed as a consequence of millions of years of evolutionary process to combat biotic and abiotic stresses. In the past, plants have aided 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 majorly 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, close association between biomarkers of oxidative stress, nitrosative stress and progression of CRC has been unravelled by clinical studies. Inflammation is a normal biological response of our body at the time of infection or damage. It stimulates 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 differentiation of cells and promoting tumor formation and expansion [4]. 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, 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 [5].

Piperine is an interesting compound present in the fruits and roots of Piper nigrum (black pepper) and Piper longum (long pepper) containing 3–9% content of black pepper. It is most prevalent dietary amide alkaloid having anti-inflammatory, immunosuppressive, anti-cancer, neuroprotective, and anti-oxidant potential. Lately, piperine was found to suppress promotion of human colon cancer cells, prostate cancer cells, cytotoxic for human and murine melanoma cells [6]. It is also found to enhance efficacy of anti-cancer drugs by alleviating drug resistance remarkably [7]. In traditional Indian medicine, black pepper is used extensively and is beneficial because of having piperine in it. Bioavailability of the drug, inhibition of the drug transporter and cytochrome P450 results in increase in absorption caused by Piperine. Piperine was found to decrease protein damage and impeded cell proliferation in benzo(a)pyrene-induced lung cancer in animal model. It also repressed stem cell renewal in breast tissue, was found to be cytotoxic to human rectal cancer cells HRT, LNCaP and PC-3 (androgen-dependent androgen independent human prostate cancer cells, SKBR3 HER2-overexpressing breast cancer cells, mouse mammary carcinoma cells 4T1, mouse melanoma cells B16F-10 [8]. 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 view the cytotoxic, anti-proliferative and anti-cancer potential using Dimethyl hydrazine (DMH) induced colon cancer in Wistar rats [9]. Thereby, highlighting the efficacy of piperine in various cancer cells including colon cancer. Though, the exact mechanism of anti-carcinogenic action of piperine is unknown yet and more research needs to be done to elucidate it. Therefore, the current work was planned to assess beneficial properties of piperine on Colon cancer in vivo to further illuminate its molecular mechanism involving Nrf-2/Keap1/NF-κB pathway.

Materials And Methods

Chemicals

Pieprine, 1,2 dimethyl hydrazine and other chemicals were obtained from Sigma Aldrich and were of greatest purity grade.

Animals

Animal study, 4–6 weeks-old male Wistar rats weighing120–150 g was gotten from Institutional Animal Housing Facility. Rats were kept under typical laboratory environment having 45–55% relative humidity with 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) that is fully accredited by the Committee for Purpose of Control and Supervision on Experiments on Animals (CPCSEA), New Delhi, India.

Preparation of Carcinogen

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

Experimental Regimen

Group I, Negative Control group. Rats had access to standard diet and water along with drinking water given orally.

Group II, Tumor group. In the current group, Rats had access to standard diet and drinking water along with 20 mg/kg body weight DMH injection given subcutaneously in the groin once weekly for 5 weeks.

Group III, Piperine and 1,2-dimethylhydrazine (DMH) treated group (Prevention Group I). In this group, rats were prophylactically treated with Piperine (30 mg/kg body weight) two weeks prior to the start of DMH administration and then continued daily along with 20 mg/kg body weight subcutaneous DMH injection as given in tumor group and Piperine treatment is continued till the end of the research.

Group IV, Piperine and 1,2-dimethylhydrazine (DMH) treated group (Prevention Group I). In this group, rats were prophylactically treated with Piperine (60 mg/kg body weight) two weeks prior to the start of DMH administration and then continued daily along with 20 mg/kg body weight subcutaneous DMH injection as given in tumor group and Piperine treatment is continued till the end of the research.

All the animals were sacrificed after 16 weeks. The chemo preventive potential of Piperine was assessed for mitigating colon tissue injury and initiation of precancerous lesions/events/biomarkers of colon carcinogenesis via checking ROS measurement, anti-oxidant armory, CEA, MDF, ACF, NFkB/Nrf-2/Keap1/HO-1 pathway proteins were measured and also histological alterations were studied.

Post–mitochondrial Supernatant (PMS) Preparation

Colon was cleaned immediately and perfusion was done by cold saline. Colon was homogenized in a homogenizer in chilled phosphate buffer (0.1M, pH 7.4). To separate the nuclear debris, centrifugation was done at 700 g in cooling centrifuge to homogenized colon for 10 minutes. PMS in form of aliquot so obtained was used as repertoire of numerous enzymes [10].

Estimation of Carcinoembryonic Antigen (CEA)

CEA was measured by commercially available kit form USCN Life Science Inc. Cloud-Clone Corp, USA. All the instructions and materials to carry out the experiment was provided in the kit.

Estimation of Aberrant crypt foci (ACF)

ACF were measured by the method[9]. Randomly colons were selected and stained for 6 min in a 0.05% filtered solution of methylene blue. ACF were counted under light microscope at 40x magnification per colon.

Estimation of Mucin depleted foci (MDF)

MDF were measured by the method [9]. 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 40x magnification.

Immuno-histochemical Staining pNF-kB-p65, Nrf-2, Keap1, HO-1and NQO-1

Immunohistochemical staining protocol was followed as described [10]. We used the following antibodies as 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 4^0C. Rest same procedure was followed as described. Finally, slides were visualised under light microscope.

Estimation of ROS

ROS was estimated as explained [11].

Estimation of MDA

Lipid peroxidation (LPO) was measured by following the method [12].

Estimation of Antioxidant Enzyme Armory

Measurement of Superoxide Dismutase Activity

SOD was evaluated by method [13].

Measurement of Catalase Activity (CAT)

CAT was determined by method [14].

Measurement of GSH

GSH was determined as done by method [10].

Measurement of Glutathione Reductase (GR) Activity

GR activity was determined as described by method [10].

Measurement of Glutathione Peroxidase Activity (GPx)

GPx was calculated by method [15].

Estimation of NO

NO was determined by method [16].

Estimation of hydrogen peroxide

Hydrogen peroxide (H_2O_2 standard curve) was assessed by method [17].

Protein Estimation

The protein estimation was done by method [18] using BSA as standard.

Estimation of Tumor Necrosis Factor Alpha (TNF-α), Interleukin 6 (IL-6), Cox-2, PGE2, iNOS and Myeloperoxidase (MPO)

TNF-α, IL-6. PGE2, iNOS and Cox-2 were measured by using commercial kit (eBioscience, Inc., San Diego., USA). Assays were performed as per the instructions of the manufacturer provided with the kit on an Elisa Plate Reader.

Alcian blue-neutral red (AB-NR) staining for mucin analysis

This procedure was done as described [9].

Statistical Analysis

The data is presented as the mean ± 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 minimum criterion for statistical significance is set at $p < 0.05$ for all comparisons.

Results

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 level of CEA in group II and IV significantly ($^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$) as shown in Fig. 1.

Piperine treatment mitigates ACF and MDF, precancerous lesion markers

ACF and MDF developed by DMH administration promotes or are early events which lead to colon carcinogenesis. In the current study, there was increased ACF and MDF in tumor group whne compared with control group. Although treatment with piperine reduced ACF and MDF scores in group III and group IV animals significantly as shown in Figs. 2 and 3.

Piperine treatment regulates Nrf-2/keap1/HO-1/NQO-1 pathway

The immunohistochemical slides show strong expression of Keap-1(Fig. 3B) and less expression of Nrf-2 (Fig. 3A), NQO-1 (Fig. 3C) and HO-1(Fig. 3D) 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 anti-inflammatory potential of Piperine and increased Nrf-2, NQO-1, and HO-1 executing anti-oxidant machinery to function and shutting down NF-κB and its downstream elements (Fig. 3A-D).

Piperine treatment mitigates ROS

ROS was pointedly higher (**$P < 0.001$) in group II (DMH group) when compared with group I, deciphering 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 ($^{\#\#\#}P < 0 .001$) (Fig. 4).

Piperine treatment alleviates MDA levels

MDA, by product and hallmark of lipid peroxidation and hence oxidative injury. There was a sharp rise in MDA in DMH administered/tumor group as compared to control (**$P < 0.001$). However, treatment with piperine decreased unusually risen MDA levels in group III and IV ($^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.01$) (Fig. 5).

Piperine treatment alleviates anti-oxidant armory

Piperine's effect was studied on DMH-induced exhaustion of different antioxidants was studied. The results are shown in Table 2. There was a significant difference (***$P < 0.001$) in the activity of antioxidants between control and DMH administered group. However, piperine treatment reinstated back 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_2O_2 consumed/min/mg protein	10.23 ± 1.62	4.82 ± 0.48***	5.94 ± 0.33#	8.32 ± 0.72###
H_2O_2 (nmol of H_2O_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/tumor group (20 mg/kg BW); Group-III: DMH rats treated with Piperine (30 mg/kg bw/day); Group-IV: DMH rats treated with Piperine (60 mg/kg bw/day). Data are represented as mean ± S.E.M.

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 (Fig. 6).

Piperine treatment mitigates Cox-2 and inflammatory mediators

Also, levels of inflammatory cytokines like TNF-α, PGE-2, IL-6 and Cox-2 were increased significantly in DMH treated group II in comparison to untreated control group I ($P < 0.001$). However, Piperine treatment to 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 X on serum levels of inflammatory cytokines (Cox-2, TNF-α and IL-6)

	Group-I	Group-II	Group-III	Group-IV
IL-6 (pg/ml)	834.12 ± 42.34	2023.32 ± 187.4***	1342.07 ± 146.6$^{\#}$	1054.83 ± 94.50$^{\#\#\#}$
TNF-α (pg/ml)	522.83 ± 40.27	1443.62 ± 138.2***	987.83 ± 105.4$^{\#}$	774.31 ± 55.49$^{\#\#}$
Cox-2 (pg/ml)	921.62 ± 63.11	1998.23 ± 92.3***	1532.13 ± 144.1$^{\#\#}$	1098.23 ± 68.70$^{\#\#\#}$
i-NOS (pg/ml)	774.52 ± 87.63	1638.65 ± 106.1***	1310.22 ± 119.4$^{\#\#}$	897.35 ± 77.42$^{\#\#\#}$
PGE-2 (pg/ml)	814.89 ± 99.10	1547.86 ± 165.2***	1211.17 ± 113.4$^{\#\#}$	998.64 ± 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 bw/day); Group-IV: DMH rats treated with Piperine (60 mg/kg bw/day). Data are represented as mean of 6 rats ± S.E.M.

Piperine treatment mitigates NO and iNOS production

There was a significant rise in NO and iNOS production in the 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 the group II ($^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$) (Fig. 7 and Table 2).

Piperine treatment mitigates MPO production

There was a significant rise in MPO production in the group II as compared with the group I animals ($^{***}P < 0.001$). However, treatment with Piperine effectively reduced MPO formation in group III and IV when compared with the group II ($^{\#\#}P < 0.01$) (Fig. 8).

Piperine treatment attenuates mucin staining in colonic tissue

There is negligible blue staining in control group which indicates mucin integration as compared to DMH administered group. However, piperine treatment decreased disintegration of the mucous layer in group III and IV respectively (Fig. 9).

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 (Fig. 13). 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 (Fig. 10).

Discussion

Colon cancer process involves varied steps starting from pathological modifications extending from disconnected microscopic mucosal lesions of ACF, MDF to progression of tumor. Likewise, oxidative stress and inflammation are critical role players 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 not to rely only on extrapolating data from in vitro models due to 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 level which has shown anti-colon cancer activity in vitro [6].

We first studied the influence of piperine on biomarkers of colon cancer. CEA is one of the utmost used tumor marker with predictive implication in early stage of colon cancer. It mediates adhesion intercellularly, promotes cell accretion, controls immunity and signalling. Consequently, it shows tumor invasiveness and metastatic activity [19]. We did investigation of 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, CEA level got decreased by the treatment of piperine possibly due to its anti-cancerous activities resulting in prevention of DMH-induced colon carcinogenesis. We also studied ACF and MDF, considerate 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. Also, development of ACF and MDF due to DMH administration in experimental rodents is used as a short-term model to investigate protective mechanism of organic compounds [9]. We found that piperine reduced ACF and MDF scores in group III and IV rats respectively when compared with tumor group which implies role of piperine in inhibiting early events of colon carcinogenesis, which has been supported by earlier studies [20].

Next, we studied a robust signalling pathway namely Nuclear factor erythroid 2-related factor 2 (Nrf-2)/Kelch-like ECH-associated protein 1(Keap1) 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 initial stage of inflammation-mediated tissue damage impedes the formation of proinflammatory modulators like pro inflammatory cytokines, chemokines, and cell adhesion molecules [21]. The development and destruction of Nrf-2 are in balance under normal physiological conditions. However, activation of Nrf-2 is blocked by Keap1 protein under stress conditions. Upon activation and dissociation of keap1, Nrf-2 accrues and binds with the antioxidant responsive elements (AREs) via translocating into the nucleus in the promoter region to activate 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 [22]. This was further validated and supported by a study where in Nrf-2- (-/-) mice were additionally vulnerable to dextran sulfate sodium- (DSS-) induced colitis [23]. Therefore, demonstrating that Nrf-2 activation is an auxiliary therapy possibly for colitis and colon cancer patients. The augmented sternness of colonic damage in DMH group animals may be correlated with decrease in phase II detoxification enzymes as reported previously [24]. In Nrf-2 deficient mice, proinflammatory modulators like interleukin 1β (IL-1β), 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 [23, 25]. Moreover, Nrf-2- deficient (Nrf-2−/ −) mice are at an increased risk of having various cancers like stomach, CRC, 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) while as there was 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 [26]. 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 protection of cells against potentially harmful entities may be the fundamental process through which Nrf-2 shields against chemical-induced carcinogenesis [27].

Keap-1 is a protein that keeps Nrf-2 in inhibited state in cytoplasm. Dissociation and amendment of Nrf-2–Keap1 complex is necessary for transfer of Nrf-2 in nucleus which leads to the activation of Nrf-2–ARE-dependent signalling, and numerous cell transduction pathways. ARE inducers like piperine treatment downregulates Keap 1 level in treatment groups as compared to DMH group due to which Nrf-2 translocation occurs activating downstream pathway. Various pathological events induce stress and HO-1 is a crucial protein that helps in cellular adaptation. It has been found as 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 reduction of superoxide anions and lipid peroxidation. Additionally, reports indicate pro-apoptotic and anti-proliferative activities of HO-1 in prostate, breast and oral cancer. Although exact process of the act is unknown [28, 29].

Nrf-2 is also found to control several phase II detoxification molecules like HO-1, NADP(H), quinone oxidoreductase-1 (NQO1), glutathione S-transferase (GST), UDP-glucuronosyltransferase (UGT) and glutamate-cysteine ligase (GCL) [30]. NQO1 is an important target of Nrf-2, is involved in detoxification process and its upregulation thwarts amplified IL-1β and TNF-α [26]. In the present study we found downregulation in HO-1 and Keap1 in DMH group. However, treatment with Piperine upregulated levels of HO-1 and Keap1 at both the doses respectively signifying activation of Nrf-2 which in turn switches on the rest of ARE response elements which involves theses enzymes resulting in activation of anti-inflammatory and anti-oxidant response, activating protective response in cells as reported previously [31].

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 where in ROS acts as secondary messenger to dysregulate various signaling pathways. The deficit of antioxidant machinery or detoxification system enzymes as well as up surging in ROS and RNS is detrimental to colonic tissue homeostasis. We found that there was an increase in ROS production in DMH administered group as compared to control group. Treatment with piperine attenuated ROS levels in both groups as reported previously [30]. 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 [32]. Piperine treatment repressed the production of MDA levels which may be due to alkaloids present in piperine scavenge free radicals due to its potent anti-oxidant activity attenuating MDA level in treatment groups. Enzymatic and non-enzymatic antioxidants are the primary defenders against cytotoxic oxygen radicals by scavenging intermediates of oxygen reduction. Some of the 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 [10]. 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 got enhanced after administration of piperine which may be because of its free redox trapping activity [7]. GSH constitutes the major nonprotein thiol in mammalian cells which helps in many cellular activities like regulation of protein synthesis. Reduced glutathione 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 [32]. GR is another glutathione restoring enzyme that catalyses oxidized glutathione (GSSG) to reduced glutathione (GSH) by the oxidation of NADH to NAD [33]. In the current study, GSH got diminished and GSSG got 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, detoxification of xenobiotics, carcinogens, free radicals and peroxides occurs primarily by GPx and GR by conjugating xenobiotics with GSH, resulting in cellular protection against mutagen-induced toxicity. In the present study, there was decreased activities of GSH and GSH dependent enzymes like GPx and GR in DMH induced rats whereas treatment with

pieprine increased antioxidant armory which may be because of its free radical scavenging potential [34]. Earlier reports recommend that alterations in redox balance and signalling are hallmarks of initiation and promotion of carcinogenesis and resistance to treatment [35]. Hence, agent that upregulates antioxidant enzyme machinery like CAT, SOD, GR, GPx that inactivate ROS, have massive potential to avert initiation and promotion of cancer as observed in the current study. Therefore, 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 of endogenous anti-oxidant machinery because of its anti-oxidant, anti-inflammatory and various pharmacological activities attributed to it [7, 8, 20, 30, 36].

In the instigation and development of cancer a potential cross-talk between Nrf-2 and NF-κB 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 increase in antioxidant armory. But additionally, confirmed by the repression of inflammatory pathway mediators smoothed through NF-κB signalling pathway. Correspondingly, upregulation of cytokines occurs due to Nrf-2 deficiency as a result of NF-κB activation or the activation of Nrf-2 attenuates NF-κB and downstream signalling as reported previously [36, 37]. Carini et al. reported that dysregulation of ROS may alter DNA assembly, consequentially modify proteins and lipids, stimulate numerous stress-activated transcription elements including NF-κB, formation of pro and anti-inflammatory cytokines which contribute to carcinogenesis via oxidative insults [38]. Whereas antioxidant agents like piperine impede IL-1beta, iNOS, COX-2, NO, NF-κB production and henceforth, carcinogenesis [39, 31].

Chronic inflammation contributes to 25% of human cancers. One of the most important pathways involved in inflammation is Nuclear factor kappa light chain enhancer of activated B cells (NF-κB). NF-κB 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, promoting tumor growth [40]. NF-κB's enhanced expression may be attributed to chronic inflammation followed by cyclic administration with carcinogen, DMH contribute to colon carcinogenesis. Other studies also decipher that colon cancer cell lines have remarkably abnormal NF-κB expression and less IκB levels, unravelling that dysregulated NF-κB is a major contributor of colon cancer which is in accordance to our results as well [41]. Our results further validate that the piperine inhibits NF-κB activity as reported previously and strongly recommend that the bioactivity of piperine against colon carcinogenesis may possibly be due to inactivation NF-κB which is further evident due to inhibition of downstream pathway player proteins [7, 42, 43].

NF-κB has a critical connection amongst inflammation and cancer since it increases levels of tumor stimulating cytokines downstream to it like interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), PGE-2, COX-2 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 of DNA and proteins. There are cumulative indications which decipher that chances of colon carcinogenesis

increase by enhancement in pro-inflammatory cytokines like TNF-α, IL-1α, IL-1β and IL-6 which further increase the secretion of PGE-2, an inflammatory mediator. However, piperine treatment attenuated the levels of above mentioned pro inflammatory cytokines possibly by inhibition of NF-κB and anti-inflammatory activity of piperine as reported previously [44, 45, 43].

COX-2 is triggered in inflammatory or hypoxic environment and is upregulated in various cancers including colon cancer unlike in normal cells and thus making it a potential therapeutic target. The major downstream mediator of COX-2 is PGE-2 that 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 DMH group whereas treatment with piperine decreased COX-2 and hence PGE-2 in both the groups respectively. Thereby precluding the process of carcinogenesis and not allowing its acceleration [45].

Nitric oxide (NO), an RNS, an inflammatory mediator plays an essential character in colon tumorigenesis in both humans and in investigational experiments. NO is formed by three isoforms of nitric oxide synthase (NOS), Under normal physiological conditions. Inducible (NOS) produces micromolar concentrations of NO which lasts for hours or days which is greatest amount unlike 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 by NO formation in cells by damage/mutation in DNA, dysregulating signalling pathways and instability in genome Also, DNA repair pathways are negatively impacted by high NO concentrations, leading to carcinogenesis [46]. iNOS is produced by proinflammatory agents and tumor micro environment and does not depend on Calcuim. Femia et al. reported that DMH-induced colorectal cancer has high pro-inflammatory enzyme inducible nitric oxide synthase (iNOS) expression [47]. We got similar results in our current study which is support by earlier findings. However, over production of NO is contributed by iNOS damaging DNA repair and promoting cancer growth. Therefore, mitigating iNOS and henceforth 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-κB. It was indicated that by reducing NF-κB expression, the occurrence of cancer can be reduced and decrease in induction of NOS hence decreasing NO which further alleviates DNA damage and cancer growth and promotion [6, 7].

Myeloperoxidase (MPO) is another enzyme found in neutrophils for H_2O_2 production. It has been reported that in the inflamed tissue, 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. Also, chronic intestinal inflammation and Colon cancer are related to each other. The present study found that colon cancer group showed an elevation in the intestinal inflammatory markers (MPO and COX-2) through NF-κB mediated response. Piperine treatment alleviated MPO levels

through suppression of NF-κB and henceforth suppressing infiltration of neutrophils and chronic inflammation leading to colon cancer [9, 48].

Histology of colon tissue sections of control did not show any anomaly in crypt morphology and architecture. DMH group animals showed infiltration of inflammatory cells, aberrant crypt formation, and depletion of mucin. Though treatment with piperine restored the histoarchitecture back to the control animals.

Conclusion

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 animals and humans die annually due to colon cancer worldwide. Nrf-2 and its downstream mediators protect cells from extracellular and intracellular oxidative damage and maintains redox homeostasis while NF-κB 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-κB and its associated downstream signalling molecules which include Cox-2, PGE-2, TNF-α, IL-6, IL-1β, 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 NF-κB/Nrf-2/Keap1/HO-1 pathway and pre-cancerous events by piperine may possibly be a potential mechanism and piperine may be a promising molecule for the prophylactic treatment of colon carcinogenesis.

Declarations

Ethics approval and consent to participate:

The processes for use of animals for various experiments were checked and appropriate approval was taken from the Institutional Animal Ethics Committee (IAEC) having sanction number Au/FVS/PS-9713), which is completely accredited by the committee for purpose of control and supervision on experiments on animals (CPCSEA), New Delhi, India.

Consent for publication:

All the authors give consent for publishing of this manuscript in this journal.

Availability of data and material:

On request to corresponding author

Competing interests:

All the authors declare that there are no competing interests.

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Authors' contributions:

Conceptualization, Muneeb U Rehman, Summya Rashid and Shahzada Mudasir Rashid; Data curation, Summya Rashid and Shahzada Mudasir Rashid; Formal analysis, Muneeb U Rehman Summya Rashid; Funding acquisition, Muneeb U Rehman; Investigation, Muneeb U Rehman, Summya Rashid, Ajaz Ahmad, Shahzada Mudasir Rashid and Sheikh B Ahmad; Methodology, Summya Rashid, Azher Arafah, Wajhul Qamar and Shahzada Mudasir Rashid; Project administration, Muneeb U Rehman, Summya Rashid and Sheikh B Ahmad; Resources, Azher Arafah, Rana M Al Saffar and Nada M Almatroudi; Software, Wajhul Qamar and Ajaz Ahmad; Supervision, Sheikh B Ahmad; Validation, Muneeb U Rehman; Visualization, Wajhul Qamar and Rana M Al Saffar; Writing – original draft, Muneeb U Rehman and Summya Rashid; Writing – review & editing, Azher Arafah, Wajhul Qamar, Rana M Al Saffar, Ajaz Ahmad, Nada M Almatroudi and Saeed M A Alqahtani.

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Figures

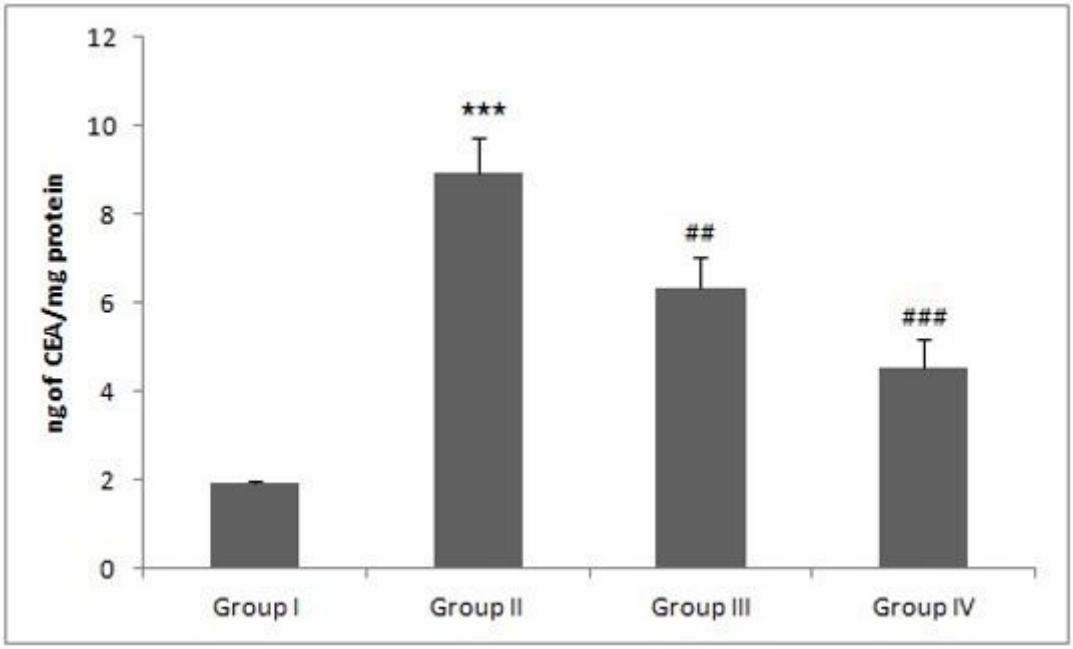

Figure 1

Piperine treatment mitigates CEA production. In DMH administered/tumor 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.

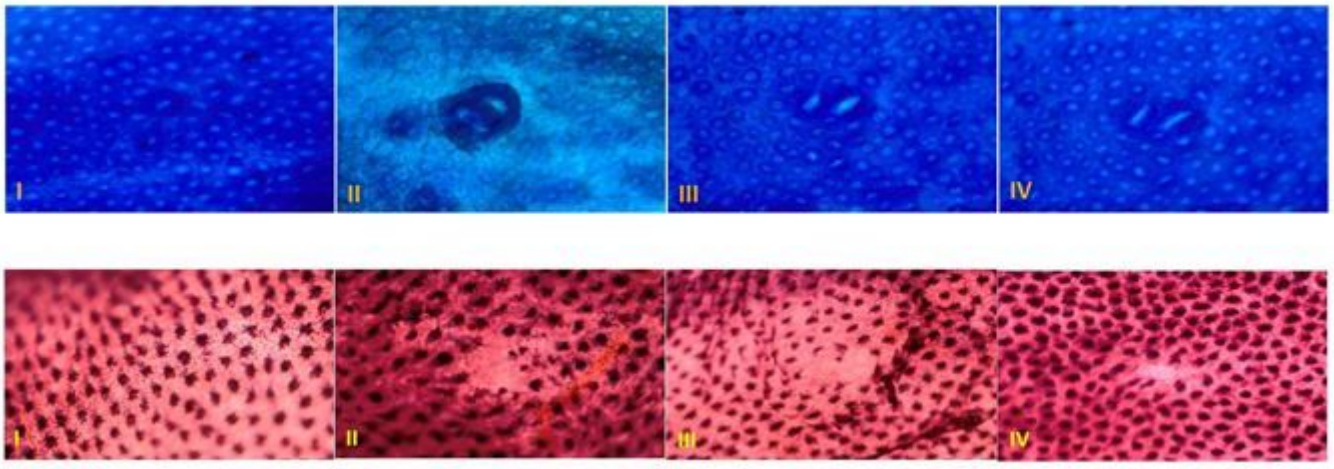

Figure 2

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.

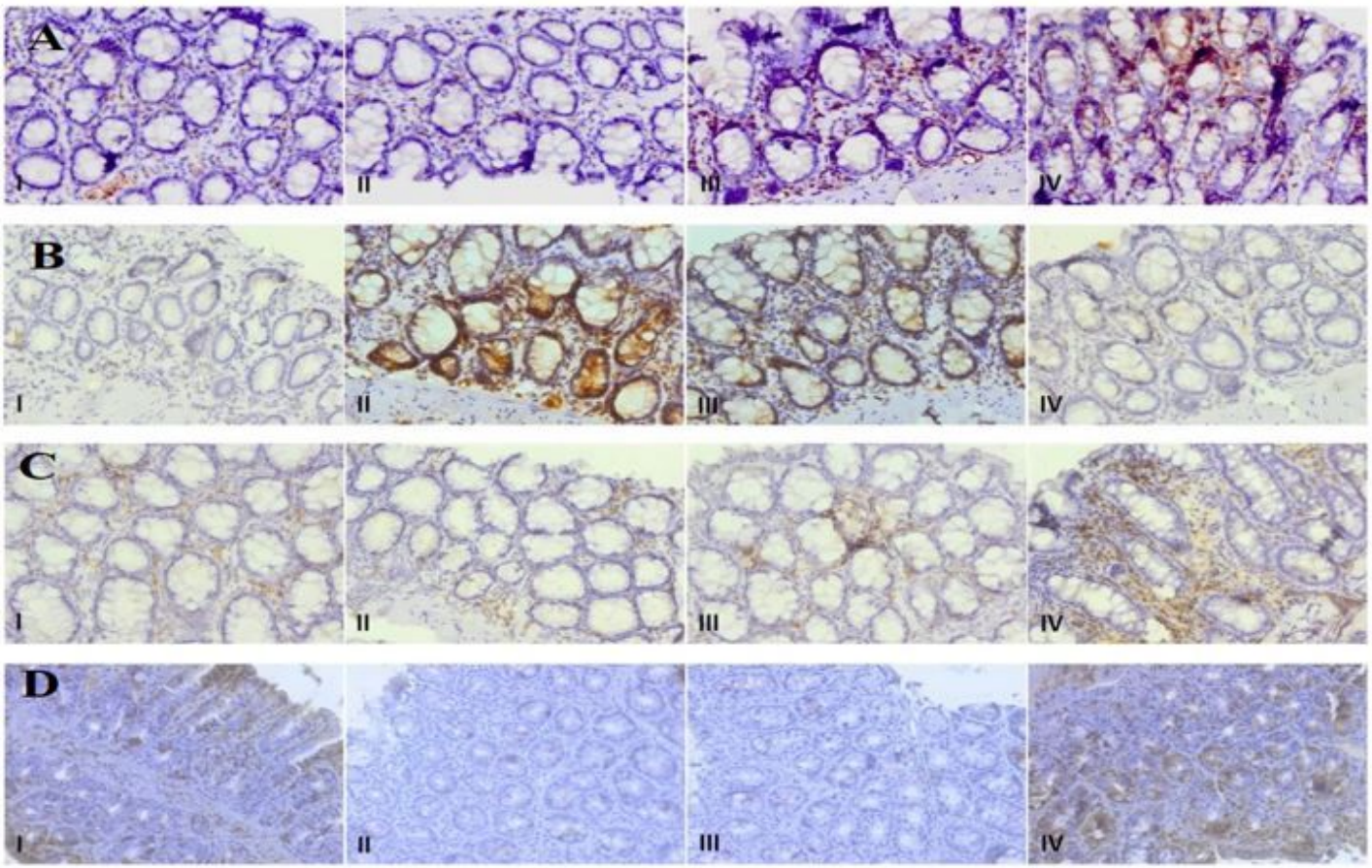

Figure 3

Effect of piperine treatment on Nrf-2, Keap-1, NQO-1 and HO-1expression. (A) Photomicrographs of colon sections depicting immunohistochemical analyses, 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. (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. (C) Photomicrographs of colon sections depicting immunohistochemical analyses, brown color indicates specific immunostaining of NQO-1and 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 HO-1 as compared to 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. All images have original magnification of 40×.

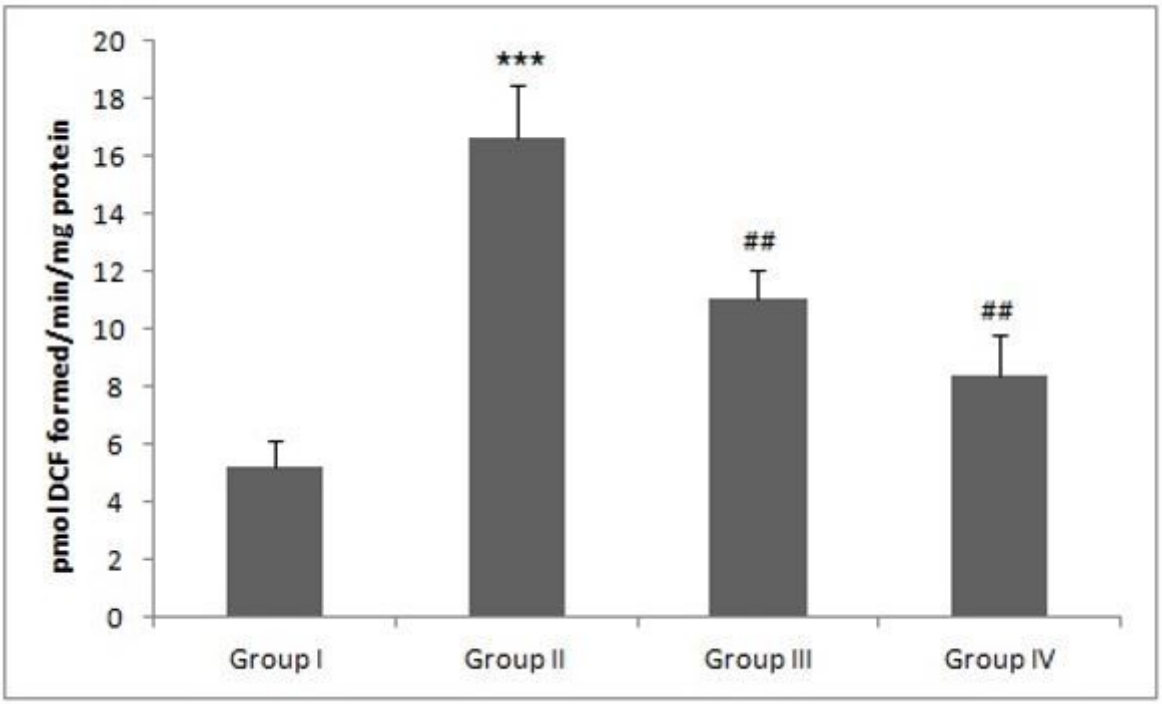

Figure 4

Piperine treatment mitigates ROS. In DMH administered group-II, 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 control.

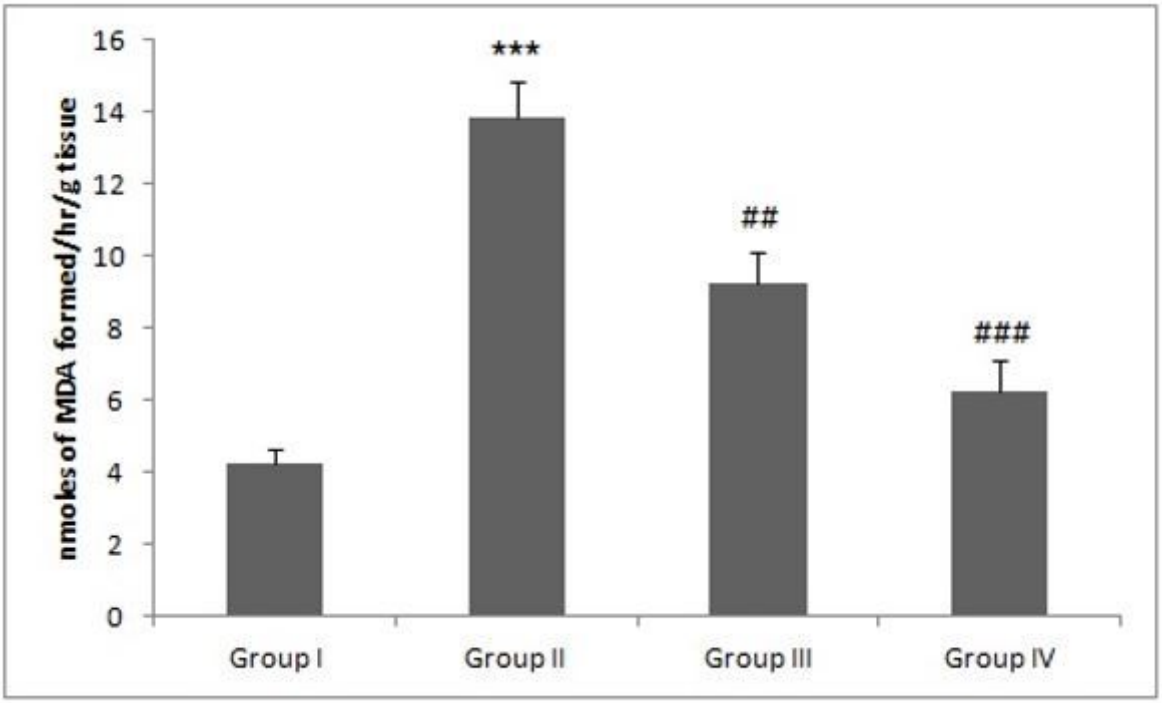

Figure 5

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 level in group III (##p<0.01) and group IV (###p<0.001) as compared to group II.

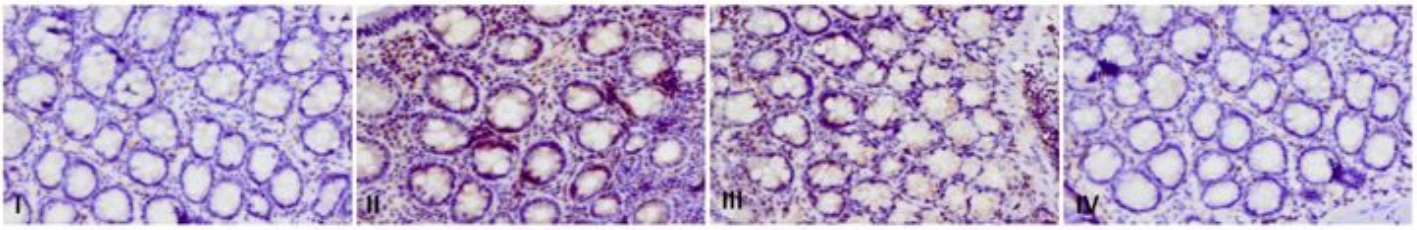

Figure 6

Effect of Piperine treatment on pNF-kB expression. Photomicrographs of colon sections depicting immunohistochemical analyses, brown color indicates specific immunostaining of pNF-kB and light blue color indicates counter staining by hematoxylin. The colonic section of DMH-administered group-II has more pNF-kB 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-kB immunopositive as compared to group II. Original magnification: 40×.

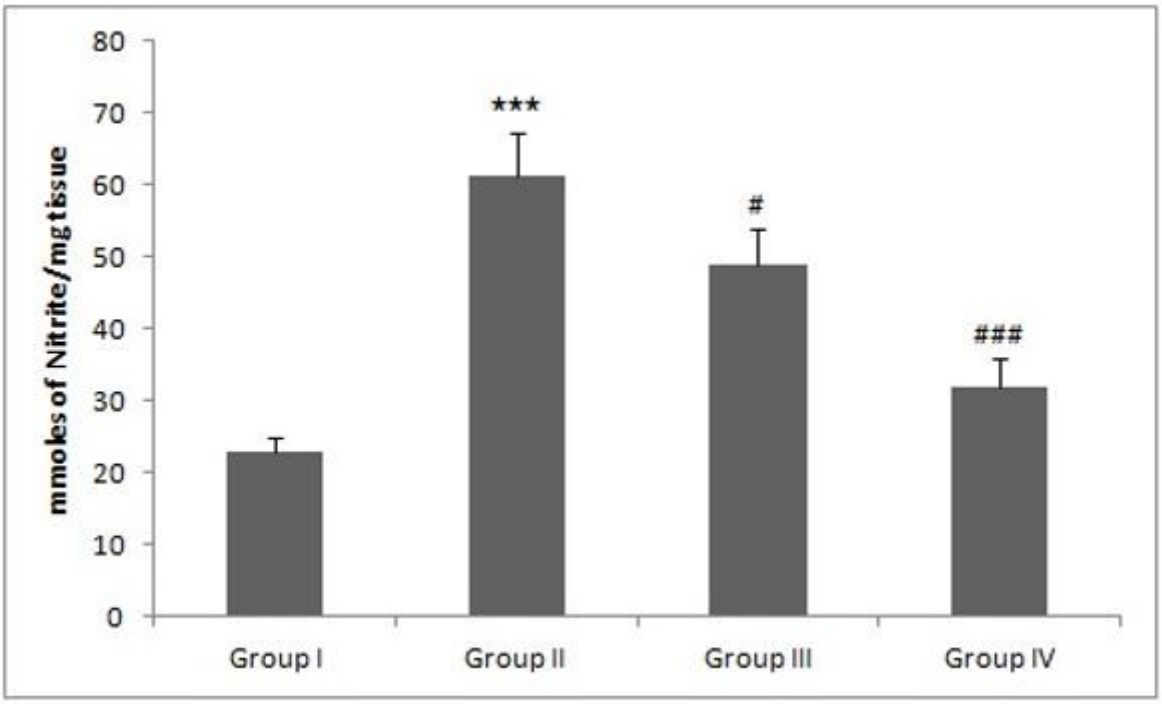

Figure 7

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.

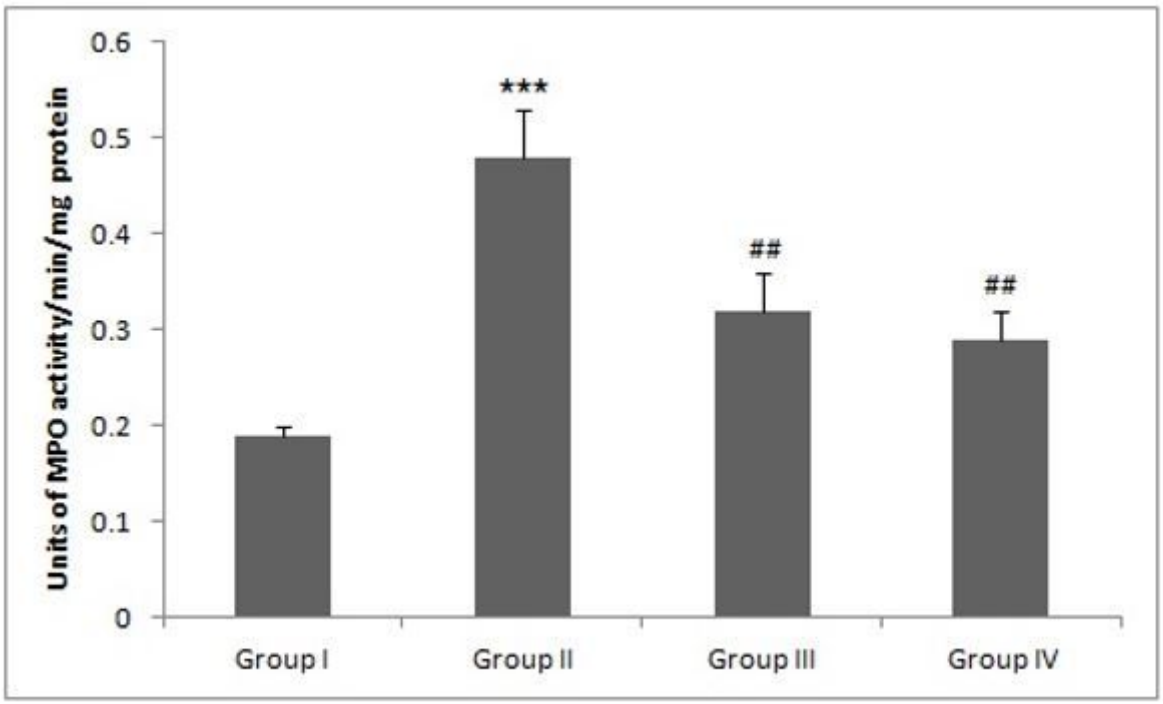

Figure 8

Effect of Piperine and DMH adminsitered group on MPO levels. In Dox-treated 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.

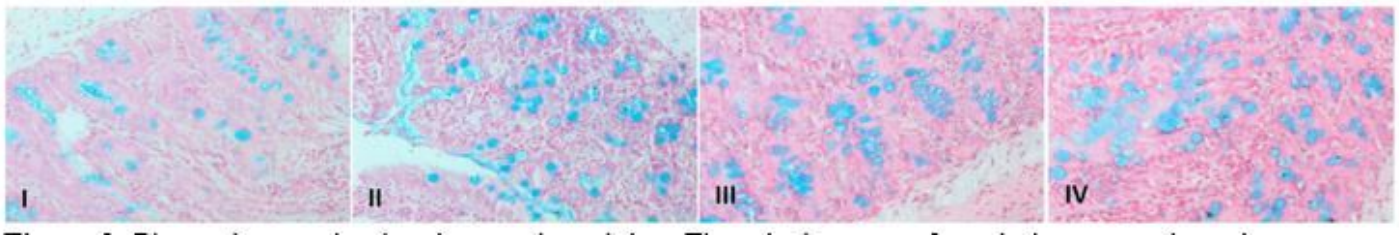

Figure 9

Photomicrographs showing mucin staining. There is decrease of mucin in mucous layer in In DMH administered group which is depicted by blue staining when compared with control. Treatment with Piperine increased mucous layer mucous as we can see from less blue staining pattern in III and IV slides representing group III and IV.

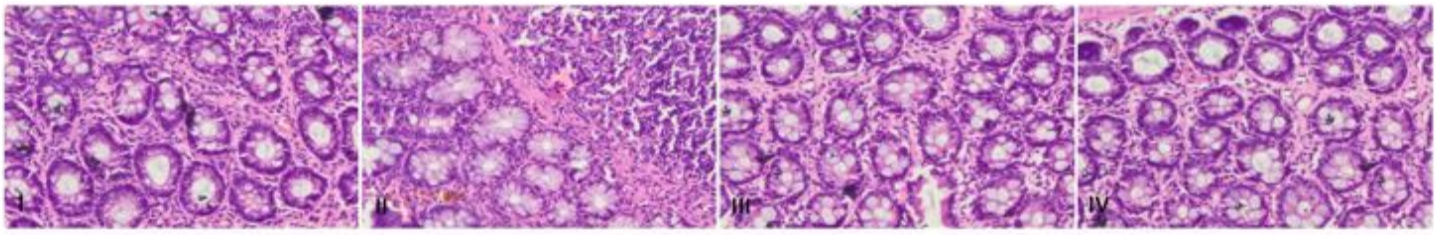

Figure 10

Effect of Piperine treatment on DMH-administered Pathological changes in rat colon Photomicrographs of H&E staining of histological sections of colon tissue depicting different experimental groups, group-I indicate normal histo-architecture of liver sections. Group-II shows extensive disintegration of normal architecture in DMH- administered group. In groups III & 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. magnification: 40×.