RESEARCH PAPER

# Piperine-Mediated Modulation Of Cancer Stem Cell Markers And Apoptotic Pathway Via Caspase-9 In Colorectal Cancer-Induced Mice

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**Abstract**
**Background:** Colorectal cancer (crc) is one of the most prevalent malignancies and a leading cause of cancer-related mortality worldwide. Oxidative stress, resulting from an imbalance between reactive oxygen species (ros) and antioxidant defense systems, plays a crucial role in crc development and progression. Piperine, a bioactive alkaloid derived from piper nigrum, is known for its antioxidant, anti-inflammatory, and anticancer properties; however, its role in modulating oxidative stress in experimental crc models remains inadequately explored.
**Aim:** To investigate the effect of piperine on oxidative stress markers and antioxidant enzyme status in an aom/dss-induced colorectal carcinogenesis model in swiss albino mice.
**Materials and Methods:** Swiss albino mice were randomly divided into experimental groups, including normal control, carcinogen control, standard drug-treated, and piperine-treated groups at two different doses. Colorectal carcinogenesis was induced using azoxymethane (aom) and dextran sulfate sodium (dss). Following the experimental period, serum levels of antioxidant enzymes (sod, cat, and gpx) and oxidative stress markers (mda and inos) were assessed using elisa-based methods. Statistical analysis was performed using one-way anova followed by duncan's multiple range test, with significance considered at $p < 0.05$.
**Results:** The carcinogen-induced group exhibited a significant reduction in antioxidant enzyme activities along with elevated levels of oxidative stress markers, indicating enhanced oxidative damage and inflammation. Piperine treatment at both doses significantly restored the activities of sod, cat, and gpx, while markedly reducing mda and inos levels. The effects were more pronounced at the higher dose and were comparable to the standard drug treatment.
**Conclusion:** Piperine exhibits potent antioxidant and anti-inflammatory effects in experimental colorectal carcinogenesis by enhancing endogenous antioxidant defenses, reducing lipid peroxidation, and suppressing inflammatory mediators. These findings highlight its potential as a promising chemopreventive agent for crc, although further studies are required to elucidate its molecular mechanisms and clinical applicability.
**Keywords:** Piperine, Colorectal Cancer, Oxidative Stress, Antioxidant Enzymes, Chemoprevention.
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## INTRODUCTION

Colorectal cancer (CRC) is among the most commonly diagnosed malignancies and represents the second leading cause of cancer-related mortality worldwide. According to GLOBOCAN 2022 estimates, approximately 1.93 million new CRC cases and 0.94 million deaths were reported globally, with projections suggesting that the incidence may rise to 3.2 million cases by 2040 (1,2). The burden of CRC varies significantly across regions and is closely linked to socioeconomic status, with higher incidence rates observed in countries with elevated Human Development Index (HDI) (3,4). Major risk factors include unhealthy dietary patterns, obesity, sedentary lifestyle, smoking, alcohol consumption, genetic predisposition, and chronic intestinal inflammation. Despite advancements in screening and therapeutic strategies, high recurrence rates and the development of drug resistance continue to limit clinical outcomes, highlighting the urgent need for safer and more effective therapeutic interventions.

Oxidative stress, defined as an imbalance between reactive oxygen species (ROS) generation and the antioxidant defense system, plays a pivotal role in CRC pathogenesis. While ROS are essential for normal cellular signaling and homeostasis, their excessive accumulation leads to oxidative damage of DNA, proteins, and lipids, thereby promoting genomic instability and tumor initiation (5,6). Furthermore,

oxidative stress contributes to CRC progression by activating oncogenic signaling pathways, enhancing inflammatory responses, inhibiting apoptosis, and promoting angiogenesis and metastasis (7,8). The cellular antioxidant defense system, comprising enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), serves as a critical protective mechanism against ROS-induced damage. SOD catalyzes the conversion of superoxide radicals into hydrogen peroxide, which is subsequently detoxified by CAT and GPx, thereby maintaining redox balance (9,10). Alterations in the activity and expression of these enzymes have been widely reported in CRC, contributing to increased oxidative stress and tumor progression (11). In contrast, malondialdehyde (MDA), a byproduct of lipid peroxidation, is commonly used as a biomarker of oxidative damage and is significantly elevated in CRC patients (12). Additionally, inducible nitric oxide synthase (iNOS) is overexpressed in colorectal tumors, leading to excessive nitric oxide production, which contributes to DNA damage, tumor angiogenesis, and inflammation (13,14). Elevated iNOS expression has also been associated with poor prognosis and increased metastatic potential in CRC (15).

The azoxymethane/dextran sulfate sodium (AOM/DSS) model is a well-established experimental model for studying colitis-associated colorectal carcinogenesis. This model effectively mimics the multistep progression of human CRC by inducing chronic inflammation, oxidative stress, and dysplastic changes in the colon (16). As such, it provides a reliable platform for evaluating the chemopreventive and therapeutic potential of novel compounds. 5-Fluorouracil (5-FU), a pyrimidine analog, remains a cornerstone in CRC chemotherapy due to its ability to inhibit thymidylate synthase and disrupt DNA and RNA synthesis. However, its clinical application is often limited by toxicity, adverse effects, and the emergence of chemoresistance, necessitating the exploration of alternative or adjunctive therapeutic agents (17).

Piperine (1-piperoylpiperidine), a bioactive alkaloid derived from *Piper nigrum* and *Piper longum*, has gained considerable attention due to its diverse pharmacological properties, including antioxidant, anti-inflammatory, and anticancer activities. Accumulating evidence indicates that piperine exerts anticancer effects through multiple mechanisms, such as induction of apoptosis via ROS-mediated mitochondrial pathways and endoplasmic reticulum stress, cell cycle arrest, and autophagy induction through modulation of the AKT/mTOR pathway (18,19). Moreover, piperine has been shown to inhibit key oncogenic signaling pathways, including PI3K/Akt/mTOR, Wnt/β-catenin, NF-κB, and STAT3/Snail-EMT, thereby suppressing tumor growth, invasion, and metastasis (20,21). It also enhances antioxidant defense mechanisms and reduces lipid peroxidation in various experimental models. Notably, piperine acts as a bioavailability enhancer and chemosensitizer, improving the efficacy of conventional anticancer drugs and overcoming multidrug resistance (22). Despite these promising findings, the specific effects of piperine on oxidative stress markers and antioxidant enzyme systems in the AOM/DSS-induced CRC model remain insufficiently investigated. Therefore, the present study aims to evaluate the protective role of piperine at two dose levels (50 and 100 mg/kg body weight) on oxidative stress biomarkers (MDA and iNOS) and antioxidant enzymes (SOD, CAT, and GPx) in AOM/DSS-induced colorectal cancer in Swiss albino mice.

## MATERIALS AND METHODS

### Reagents and Chemicals

Piperine, Azoxymethane (AOM), Dextran Sulphate Sodium (DSS) and 5- Fluorouracil (5-FU) were purchased from Sigma Alderich chemical sciences company, India.

### Animals

In this study, 6-8 weeks-old Swiss Albino mice (n=4) weighing 60-80g were used, which was procured from Laboratory Animal Medicine, Centre for Animal Health Studies, Tamil Nadu Veterinary & Animal Sciences University (TANUVAS), Chennai - 600 051, Tamil Nadu. The standard environment and food condition were provided to all the animals and were maintained at 45–55% relative humidity and temperature of 24 ± 3ºC with 12h of light / 12h of dark cycle along with free access to water and standard diet. The experiments were performed according to CPCSEA guidelines (IAEC approval No: BRLULAC/SDCH/SIMATS/IAEC/802021/077).

### Blood and tissue collection

After 10 weeks animals were anesthetized with Ketamine (50–100 mg/kg) and Xylazine (5–10 mg/kg), and blood was collected from the retro-orbital plexus and serum was separated for examination of biochemical and ELISA analysis. The colon samples were collected and washed with normal saline and treated with phosphate buffer saline for analysis of antioxidants and biomarkers.

### Assessment of CEA (Carcinoembryonic Antigen), CD133, and CD44 markers

Serum CEA, CD133, CD44 levels were quantified using a sandwich ELISA kit following the manufacturer's protocol. Standards and samples were added to antibody-coated wells and incubated to allow antigen binding. After washing, enzyme-conjugated secondary antibody was added, followed by substrate solution for color development. Absorbance was measured at 450 nm using a microplate reader, and concentrations were calculated from the standard curve.

### Assessment of CAS-9 markers

Serum Caspase-9 levels were quantified using a commercially available sandwich ELISA kit according to the manufacturer's instructions. Briefly, standards

and samples were added to microplate wells pre-coated with anti-Caspase-9 antibodies and incubated to allow antigen binding. After washing, HRP-conjugated detection antibody was added, followed by substrate solution for color development. The reaction was stopped, and absorbance was measured at 450 nm using an ELISA microplate reader, and Caspase-9 concentrations were calculated from the standard curve.

**STATISTICAL ANALYSIS**

Data was subjected to statistical analysis using one-way analysis of variance and Duncan's multiple range test to assess the significance of individual variations between the control and treatment groups using a computer- based software (SPSS 23 for Windows student version) and expressed as mean + standard error of the mean. In Duncan's test, the significance was considered at the level of $p < 0.05$.

**RESULTS**

**Effect of Piperine on colorectal cancer stem cell expressing biomarker- CD133**

CD133 expression was significantly elevated in the CRC-induced group compared to the normal control, indicating the enrichment of cancer stem cell (CSC) populations during colorectal carcinogenesis. Treatment with piperine at both 50 and 100 mg/kg body weight markedly reduced CD133 expression levels, suggesting its inhibitory effect on CSC maintenance and proliferation. Notably, the higher dose of piperine (100 mg/kg) showed a more pronounced reduction, comparable to the standard drug 5-fluorouracil (5-FU). These findings indicate that piperine effectively suppresses CD133-positive cancer stem cells, thereby potentially limiting tumor initiation and progression.

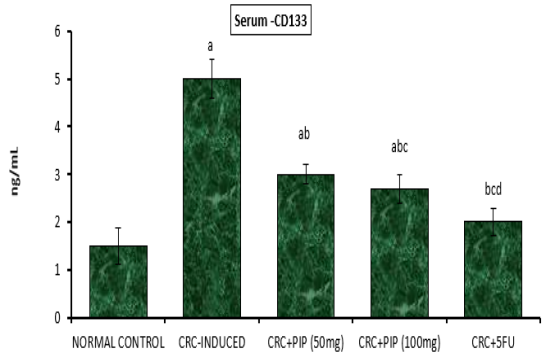


**Figure 1:** Piperine attenuates the expression of cell surface biomarkers CD133 in AOM induced carcinogenesis groups, III and IV. CRC induced models expressed high level of CD133 than normal and treated groups. The five groups are Normal control, CRC induced group, CRC plus 50 mg/kg b.w.t Piperine treated group, CRC plus 100 mg/kg b.w.t Piperine treated group and CRC plus 25 mg/kg b.w.t 5FU standard group.

**Effect of Piperine on colorectal cancer stem cell expressing biomarker- CD44**

The CRC-induced group exhibited a significant increase in CD44 expression compared to the normal control group, confirming enhanced cancer stemness and tumor aggressiveness. Piperine treatment at both doses significantly attenuated CD44 expression, indicating its role in reducing CSC-associated properties such as cell adhesion, migration, and invasion. The reduction was dose-dependent, with 100 mg/kg showing greater efficacy and effects comparable to 5-FU. This suggests that piperine may inhibit tumor progression by targeting CD44-mediated signaling pathways involved in colorectal cancer stem cell survival.

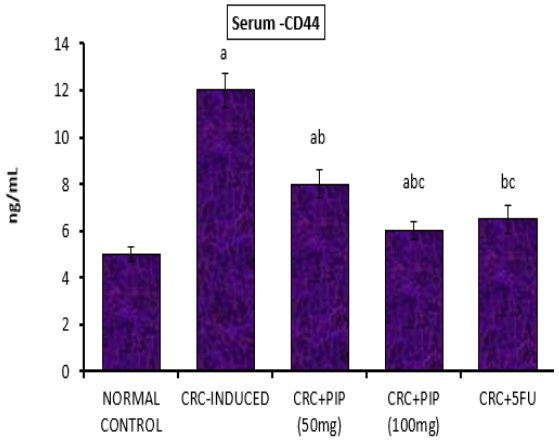


**Figure 2:** Piperine attenuates the expression of cell surface biomarkers CD44 in AOM induced carcinogenesis groups, III and IV. CRC induced models expressed high level of CD44 than normal and treated groups. The five groups are Normal control, CRC induced group, CRC plus 50 mg/kg b.w.t Piperine treated group, CRC plus 100 mg/kg b.w.t Piperine treated group and CRC plus 25 mg/kg b.w.t 5FU standard group.

**Effects of Piperine on CEA levels in serum**

Serum carcinoembryonic antigen (CEA) levels were significantly elevated in the CRC-induced group, reflecting tumor burden and disease progression. Piperine treatment at 50 and 100 mg/kg body weight significantly reduced CEA levels, indicating its antitumor activity and potential to suppress tumor growth. The reduction in CEA was more prominent at the higher dose and was comparable to the standard drug 5-FU. These findings suggest that piperine effectively reduces tumor-associated biomarkers and may serve as a potential therapeutic agent in colorectal cancer management.

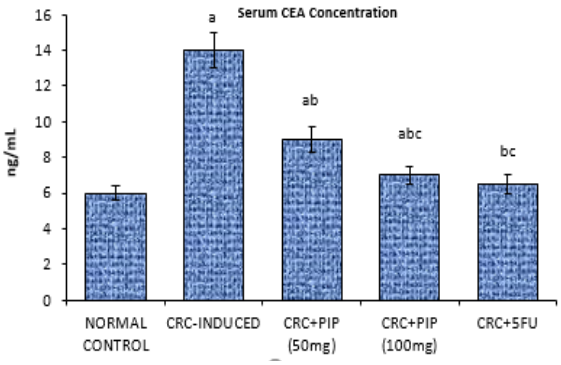

**Figure 3:** Piperine diminishes the generation of CEA which indicates the tumor inhibition. The CRC group shows high levels of CEA. The Piperine significantly alleviates CEA levels in 50 and 100 mg/kg b.w.t of Piperine treated groups.

**Effect of Piperine on Caspase-9 marker**

CRC-induced group showed a significant decrease in caspase-9 expression compared to the normal control, indicating suppression of the intrinsic apoptotic pathway during carcinogenesis. Treatment with piperine at both 50 and 100 mg/kg body weight significantly increased caspase-9 expression, suggesting induction of apoptosis via the mitochondrial pathway. The effect was dose-dependent, with the higher dose exhibiting greater activation, comparable to 5-FU treatment. These results indicate that piperine promotes apoptosis in colorectal cancer cells by activating caspase-9, thereby contributing to tumor suppression.

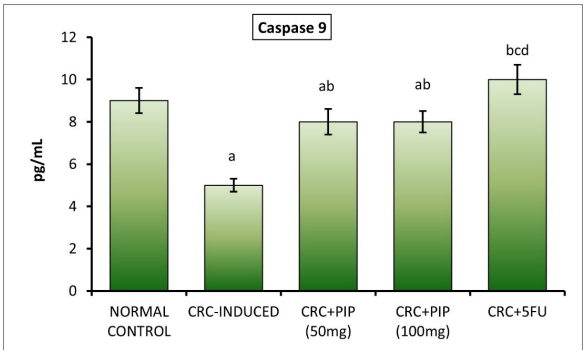


**Figure 4:** Piperine significantly increased caspase-9 expression in AOM-induced colorectal cancer groups compared to the CRC control, indicating activation of the intrinsic apoptotic pathway. The effect was dose-dependent, with 100 mg/kg showing results comparable to the standard drug 5-fluorouracil (5-FU).

**DISCUSSION**

The present study evaluated the impact of piperine on oxidative stress markers and antioxidant enzyme status in an AOM/DSS-induced colorectal carcinogenesis model in Swiss albino mice. The findings clearly demonstrate that piperine administration at doses of 50 and 100 mg/kg body weight significantly enhanced antioxidant enzyme activities (SOD, CAT, and GPx), reduced lipid peroxidation as indicated by decreased MDA levels, and suppressed inflammatory responses evidenced by reduced iNOS levels compared to the carcinogen-treated group. These results strongly support the chemopreventive potential of piperine through modulation of oxidative stress and antioxidant defense mechanisms. In the carcinogen-induced group, a marked decline in the activities of SOD, CAT, and GPx was observed, which aligns with previous reports indicating that carcinogenesis is associated with impaired antioxidant defense systems (11). These enzymes constitute the primary cellular defense against reactive oxygen species (ROS), where SOD converts superoxide radicals into hydrogen peroxide, which is further detoxified by CAT and GPx (12). Their depletion facilitates ROS accumulation, leading to oxidative damage, genomic instability, and tumor progression (6,7). Reduced SOD activity, in particular, has been linked to cancer development and resistance to therapy (23).

Piperine treatment significantly restored the activities of these antioxidant enzymes, suggesting its ability to reinforce endogenous defense systems. These findings are consistent with earlier studies demonstrating the antioxidant potential of piperine in various experimental models. Selvendiran et al. (2003) reported that piperine administration (100 mg/kg) enhanced SOD, CAT, and GPx activities in benzo[a]pyrene-induced lung cancer in mice (24). Additional studies have confirmed that piperine mitigates oxidative stress in diabetes and hepatotoxicity models by restoring antioxidant enzyme activities (25,26). Mechanistically, the antioxidant effect of piperine may be attributed to activation of the Nrf-2/Keap1/HO-1 signaling pathway. Piperine has been shown to upregulate antioxidant and cytoprotective genes through Nrf-2 activation, thereby enhancing cellular resilience against oxidative damage (27,28). Furthermore, piperine possesses intrinsic free radical scavenging properties, which further contribute to its protective effects (29). The present study also demonstrated a significant elevation in MDA levels in the carcinogen-treated group, indicating enhanced lipid peroxidation and oxidative membrane damage. Elevated MDA levels are a hallmark of CRC and reflect increased ROS-mediated injury (30). Piperine treatment markedly reduced MDA levels, suggesting its efficacy in preventing lipid peroxidation and protecting cellular integrity. Similar reductions in MDA levels following piperine treatment have been reported in radiation-induced colon and lung injury models, as well as in renal and hepatic oxidative stress conditions (31-33). This reduction may be attributed to both direct scavenging of free radicals and indirect enhancement of antioxidant enzyme activity.

Inflammation is a key driver of colorectal carcinogenesis, and iNOS plays a crucial role in mediating inflammatory and nitrosative stress. The elevated iNOS levels observed in the carcinogen group in this study are consistent with its reported overexpression in colorectal tumors and its association with poor prognosis (34-36). Piperine treatment significantly reduced iNOS levels, indicating its potent anti-inflammatory activity. This effect is supported by studies showing that piperine suppresses iNOS expression and nitric oxide production through inhibition of NF-κB signaling (37,38). Additional evidence suggests that piperine modulates inflammatory cytokines and signaling pathways, including NF-κB, MAPK, and HIF-1α, thereby attenuating inflammation and tumor progression (39-41). Comparison with the standard drug 5-fluorouracil (5-FU) revealed that while 5-FU effectively improved

oxidative stress parameters, piperine exhibited comparable efficacy, particularly at the higher dose. This is noteworthy given piperine's natural origin and favorable safety profile. Moreover, piperine has been reported to enhance the efficacy of conventional chemotherapeutic agents and overcome drug resistance through its chemosensitizing properties (42). For instance, Srivastava et al. (2021) demonstrated that piperine synergistically enhances the anticancer effects of celecoxib via modulation of the Wnt/β-catenin pathway (21).

Collectively, the findings of this study suggest that piperine exerts its chemopreventive effects through a multi-targeted mechanism involving enhancement of antioxidant defenses, reduction of lipid peroxidation, and suppression of inflammatory pathways. These mechanisms are interconnected, as improved antioxidant capacity reduces ROS levels, thereby limiting activation of pro-inflammatory signaling pathways such as NF-κB. In addition, previous studies have shown that piperine also targets multiple oncogenic pathways, including PI3K/Akt/mTOR, Wnt/β-catenin, and STAT3 signaling, while inducing apoptosis and autophagy in cancer cells (43-45). Future studies should focus on evaluating the expression of key signaling molecules such as Nrf-2, NF-κB, and related downstream targets, as well as conducting long-term studies to assess tumor incidence and progression. Finaly, the present study provides strong evidence that piperine possesses significant antioxidant and anti-inflammatory properties, contributing to its chemopreventive potential in colorectal cancer.

## CONCLUSION

Piperine exerts significant chemopreventive effects against AOM/DSS-induced colorectal carcinogenesis in Swiss albino mice by enhancing antioxidant defenses (SOD, CAT, GPx), reducing lipid peroxidation (MDA), and suppressing inflammation through downregulation of iNOS, thereby restoring redox balance and inhibiting key processes involved in tumor progression. The observed efficacy, comparable to 5-fluorouracil, highlights piperine's potential as a safe, naturally derived therapeutic or adjunct agent in colorectal cancer management. Future research should focus on elucidating the precise molecular mechanisms underlying its action, particularly involving pathways such as Nrf-2 and NF-κB, along with detailed tissue-level and gene expression studies. Additionally, dose optimization, pharmacokinetic profiling, long-term efficacy studies, and well-designed clinical trials are necessary to establish its safety, bioavailability, and translational potential in human colorectal cancer prevention and therapy.

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