

RESEARCH ARTICLE

Chemoprevention of DMH Induced Colon Carcinogenesis by Combination Administration of *Lactobacillus acidophilus*, Calcium and *Moringa oleifera* Leaves Extract

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ABSTRACT

Colon cancer has become one of the most prevalent and deadliest type of cancer in the globe in recent years. In order to prevent colon cancer triggered by DMH (1,2-dimethyl hydrazine hydrochloride), this study was to investigate the potential chemopreventive effects of calcium citrate, *Lactobacillus acidophilus*, and *Moringa oleifera* leaf extract. Sprague Dawley rats were randomized into 10 different groups and received subcutaneous injections of DMH (30 mg/kg) for 8 weeks of duration. Treatment groups were served for 4 weeks, 5-Fluorouracil (20 mg/kg) was dosed with two intraperitoneal injections in a week and *L. acidophilus* (1×10^9 cfu/kg p.o.), calcium citrate (2 mg/kg p.o.) and hydroalcoholic extract of *M. oleifera* leaves (100 and 200 mg/kg p.o.) were used for daily. All animals were killed after the completion of treatment, and colonic tissues were assessed for biochemical and histopathological parameters. DMH treatment-induced cellular and systemic oxidative stress results in depletion of cellular antioxidant enzyme levels with an alteration in hematological counts. The colonic tissues had a loose histoarchitecture with severe cellular infiltration, edema in the epithelium, and a moderate nuclear/cytoplasmic ratio, goblet cells. Combination treatments of *L. acidophilus* (LA), Calcium citrate (CC), and hydroalcoholic extract of *M. oleifera* leaves (ME) showed positive improvements in antioxidant enzyme levels, hematology count, and colonic tissue histoarchitecture. From outcomes of the present work concluded that a combination treatment of *L. acidophilus*, calcium citrate and a hydroalcoholic extract of *M. oleifera* leaves showed chemo-preventive effects against colon cancer triggered by DMH.

Keywords: Carcinogenesis, DMH, Antioxidant, Calcium, *Lactobacillus acidophilus*, *Moringa oleifera*.

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INTRODUCTION

Colon carcinogenesis is one of the most frequent and deadliest cancers globally, accounting for 500,000 deaths and 1-million new cases annually. Carcinoma of the colon is more widespread in nations that are industrialized as well as developing. One of the main causes of the rise in colon cancer cases among patients is a recent dietary modification that is characterized by a diet high in fat, high in protein, and low in carbohydrates and fiber.¹ Worldwide, each year around 11 million patients are diagnosed with colon carcinogenesis. Almost 12.5% of fatalities globally are attributable to cancer.² Regular consumption of high animal fat diet, exposure to cancer-causing chemicals, radiation, X-rays and other sources are major causative factor for the increased incidence and mortality due to colon carcinogenesis in recent years.³

Potentially effective treatment strategies like radiation and chemotherapy are available for cancer treatment, but they

have poor efficacy with serious systemic side effects and low patient survival with cancer, and most cancers are detected at last phases, when signs become more obvious.⁴ Therefore, much attention has been concentrated on reducing cancer threat by improving dietary habits with regular consumption of probiotics, antioxidant supplements and natural plant extracts with potential antioxidant and anti-inflammatory effects.⁵

Regular dietary and excess dietary calcium consumption can protect against the development of colon carcinogenesis. Calcium inhibits cellular proliferation of cancerous cells, along with lowered hyper-proliferation of other cells and tissues.⁶ A number of studies have reported that calcium has the potential to diminish cellular oxidation and DNA disfigurement and prevent cancer cell progression by modulating colon homeostasis with a reduction in colonic cell hyperproliferation.⁷ Calcium has the ability to form bile acids and indissoluble soaps with ionized fatty acids and reducing proliferative momentum in colonic mucosa for preventing colon carcinogenesis.⁸

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As a preventive dietary supplement, Probiotic microorganisms are a health protagonist with significant potential advantages in the management of colon cancer. Probiotics have received considerable attention due to their potential therapeutic effect and mode of action specifically for colon cancer prevention and treatment.⁹ *Lactobacillus* bacteria have potential effects on the gut microbiota, including immunomodulation, sustaining the equilibrium of the intestinal tract and reducing pathogen-induced inflammation.¹⁰ *Lactobacillus* strain bacteria are used to prevent and as a prophylactic against different gastrointestinal disorders like diarrhea (infectious and travelers), irritable bowel disorders, ulcerative colitis, gastric ulcers, and *Helicobacter pylori* infection. *Lactobacillus acidophilus*, *L. rhamnosus*, and *L. casei* species could alleviate colonic carcinogenesis¹¹ via compromising cellular defense systems and promoting apoptosis.¹²

M. oleifera lam (Moringaceae) is familiar as the drumstick tree, and tree of life. It is mostly planted in worldwide along with different states of India, Pakistan, and Africa. *M. oleifera* extracts possess significant pharmacological activities; its leaves possess antioxidant, hepatoprotective, cardioprotective, nephron-protective, antiproliferative, and lowering the expression of inflammatory cytokines, and anti-inflammatory action.^{13,14} The ability of extracts from *M. oleifera* leaves to suppress the proliferation of malignant epithelial cells has been extensively investigated as it relates to its anticancer abilities. The antiproliferative effects against pancreatic cancer, leukemia, and breast and colon cancer cell lines have also been confirmed through different research findings. The aforementioned advantages are accomplished by improving programmed cell death and suppressing the normal inflammatory mediators signaling pathway.¹⁵ Different studies showed that *M. oleifera* leaf extracts suppress colonic cancers through mitigating inflammation and oxidative stress.

DMH (1,2-dimethylhydrazine) is a potent carcinogen for colonic mucosal tissue. It undergoes enzymatic metabolism and forming azoxymethane (AOM) are pro-carcinogen further converted into methylazoxymethanol (MAM). Active metabolites are transferred to the colon via the bloodstream and bile. They produce cellular oxidative stress in inflamed tissue through reactive oxygen species production, which can

lead to the development of colonic tumors.^{16,17} Similarity in the progression of laboratory rat and human colonic carcinoma involves the abnormal cumulation epithelial cells with the possible redundant proliferation, avoidance of apoptosis, and cellular uncertainty.¹⁸ In this work, we explored the chemopreventive effect of hydroalcoholic extracts of *M. oleifera* leaves, calcium, and *L. acidophilus*, both individually and in combination, against colonic cancer triggered by DMH.

MATERIAL AND METHODS

The carcinogen DMH (1,2-dimethyl-hydrazine hydrochloride) was obtained from BLD Pharmatech Pvt. Ltd., Hyderabad, India. Hydroalcoholic extract of *M. oleifera* leaves was supplied by Ayurvedic Medical College, Ch. Sambhajinagar, India; Calcium citrate granules and *L. acidophilus* capsules (Inlife Healthcare, India) purchased from Medical Store, Ch. Sambhajinagar, India. A 5-Fluorouracil injection (RMPL Pharma LLP, Mumbai) and other reagent-grade chemicals were procured from Sigma Aldrich Chemicals Co. USA.

Animals

For the DMH-induced colon cancer model, 60 SD rats with a body weight varying from 180 to 220 gm and a sex of both were employed, and animals were procured from Lacsmi Biofarms, Alephata, Narayangaon, Pune, Maharashtra, India (CCSEA-registered). All the animals were placed in experimental animal house facility with 12-hour light/dark cycles, conventional laboratory temperature of $22 \pm 3^\circ\text{C}$ and 50 to 60% humidity, with a continuous supply of food and water in PP cages. Animals were housed in a test facility for a week to acclimatize with an animal house before the commencement of the treatment. The Institutional Animals Ethics Committee (IAEC), Trans Genica Services Pvt. Ltd., Maharashtra, India, consented to the experimental protocol (TRS/PT/022/025). The guidelines promulgated by the Committee for Control and Supervision of Experiments on Animals (CCSEA, New Delhi, India) were followed in the conduct of all laboratory experiments.

Method

Colon Carcinogenesis induction model

The procedure for initiation of colon carcinogenesis by using 1, 2 dimethyl hydrazine modified method from Juca's *et al.* and

Table 1: Animals grouping and treatment protocol in DMH induced Colon carcinogenesis

Group I	:	Negative control (Distilled water 10 mL/Kg p.o.)
Group II	:	Cancer control (DMH 30 mg/kg s.c. 5 times in a week for 8 weeks)
Group III	:	Cancer control + 5-Fluorouracil 20 mg/Kg i.p.
Group IV	:	Cancer control + Calcium citrate 2 mg/Kg p.o.
Group V	:	Cancer control + <i>L. acidophilus</i> 1×10^9 cfu/Kg p.o.
Group VI	:	Cancer control + Calcium citrate 2mg/Kg p.o. + <i>L. acidophilus</i> 1×10^9 cfu/Kg p.o.
Group VII	:	Cancer control + Calcium citrate 2 mg/Kg p.o. + <i>L. acidophilus</i> 1×10^9 cfu/Kg p.o. + Hydroalcoholic extract of <i>M. oleifera</i> leaves 100 mg/Kg p.o.
Group VIII	:	Cancer control + Calcium citrate 2mg/Kg p.o. + <i>L. acidophilus</i> 1×10^9 cfu/Kg p.o. + Hydroalcoholic extract of <i>M. oleifera</i> leaves 200 mg/Kg p.o.
Group IX	:	Cancer control + Hydroalcoholic extract of <i>M. oleifera</i> leaves 100 mg/Kg p.o.
Group X	:	Cancer control + Hydroalcoholic extract of <i>M. oleifera</i> leaves 200 mg/Kg p.o.

Rekha R. *et al.* were used.^{19,20} Healthy 60 laboratory rats of the Sprague-Dawley strain (180–220 g) were allocated into 10 groups ($n = 6$). As shown in Table 1 Animals were treated with DMH subcutaneously at 30 mg/kg dose five times a week for an 8-week induction period. A solution comprising 1.5% EDTA with 0.9% NaCl, pH 6.5, was utilized to dissolve DMH. After accomplishment of carcinogen treatment, animals were served with calcium citrate, *L. acidophilus*, hydroalcoholic extract of *M. oleifera* leaves daily for 4 weeks, and Standard 5-Fluorouracil twice a week for 4 weeks during the treatment period.

After completion of last dose, all animals were sacrificed humanely, and an EDTA tube was used for the collection of blood samples by heart puncture for hematological analysis, entire colon tissue was collected and fecal matter was removed by washing with 0.9% saline solution, then colon tissue was scrutinized for number of tumors and tumor size, entire colon tissue weight and length were measured to calculate the colon length/weight ratio, and colon tissue was processed for histopathological examination and for estimation of different antioxidant enzymes in tissue homogenate (Superoxide dismutase, Catalase, Glutathione peroxidase and Lipid peroxidation).²¹

Evaluation Parameters

Body weight measurement

Throughout the study period, the body weights of the animals in the *L. acidophilus*, hydroalcoholic extracts of *M. oleifera* leaves, calcium citrate treatment groups and negative, cancer control groups were measured. The body weight of the animals was assessed at the beginning of the experiment and thereafter at the 4th, 8th and 12th weeks of the study.

Colonic Tumors Characterization

After sacrifice, the entire gastrointestinal tissue (small and large intestine tissue) was grossly necropsied and scrutinized for any lesions and tumors (polyps). Tumor size and number were quantified. The dimensions of the tumors were determined using a Vernier caliper by using a formula $[(\text{length} + \text{width}) \times 0.5]^3$.²¹

Colon length-weight ratio

The entire colon tissue was excised and weighed (gm) and determined length (cm), calculate ratio by using formula:²²

Colon length-weight ratio = Length (centimeters)/ weight (grams)

Estimation of Hematological Parameters

Following sacrifice, each animal's heart was punctured to obtain blood samples, which were then placed in labeled EDTA tubes. A hematology analyzer was used to estimate the amount of platelets, red blood cells, and white blood cells.

Estimation of Antioxidant Markers in Homogenized Colon Tissue

Tissue homogenization

Instantly after the sacrifice, colon tissue was cleaned with saline. Small sections of colon tissue were chopped and homogenized in 10% (w/v) buffer (Tris-Hydrochloride, pH 7.4).

After centrifuging tissue lysate at 8000 g for 15 minutes, the clear supernatant was utilized to measure the activity of antioxidant enzymes.^{23,24}

Catalase

A specific amount of time was given for the catalase (CAT) preparation to dissolve with H₂O₂ based on the activity of the catalase enzyme. Upon adding the dichromate acetic acid reagent to stop the reaction, the resulting mixture was heated in a water bath for fifteen minutes. Chromic acetate produced at 570 nm was measured to determine the resting H₂O₂, and CAT enzyme activity is demonstrated as μmol of H₂O₂ utilized per minute per milligram of protein.²⁵

Reduced Glutathione

Reduced glutathione (GSH) enzyme activity was measured using tissue homogenate (supernatant), 20% TCA was mixed with equal volumes, and the precipitate was centrifuged. 0.25 mL of tissue homogenate were combined with 2ml of Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid). Phosphate buffer was used for final volume make up to 3 mL. Color developed samples were analyzed at 412 nm against reagent blank, and values were demonstrated as μmol . of NADPH oxidized per minute per mg protein.^{26,27}

Superoxide Dismutase

Tissue homogenate was used to assess the superoxide dismutase (SOD) activity, and the homogenate supernatant was combined with nitro blue tetrazolium (NBT), sodium pyrophosphate buffer, and phenazine methosulphate (PMT). NADH was added, and that started the reaction. Following that, for 90 seconds, the reaction mixture was left at 30°C. During that time, glacial acetic acid (1-mL) was added. At 560 nm, the chromogen's absorbance was measured. The amount of enzyme needed was determined to be one unit of SOD activity for 50% inhibition of NBT reduction per minute per mg protein.^{28,29}

Lipid Peroxidation

The formation of thiobarbituric acid-reactive molecules in colonic tissue homogenate was employed to determine the amount of lipid peroxidation. 0.5 mL of colon tissue lysate was diluted with 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid, and 1.5 mL of 0.8% thiobarbituric acid and purified water was added to make volume 4 mL, and the resulting mixture was heated at 95°C in boiling water bath. Upon cooling down 1-mL of purified water and 5.0 mL of n-butanol were added, and the final mixture was gently agitated. The degradation results of lipid peroxidation and 2-thiobarbituric acid developed a pink chromogen, which was detected at 535 nm and quantified as m.mol/mg tissue after a 10-minute centrifugation at 800 g.^{30,31}

Histopathological Examinations

All animals were sacrificed after the last dose was administered. Microscopically, colon tissue was examined for tumors and other pathological changes. Tissue with abnormal morphology was preserved in 10% neutral buffered formalin, dried using 90% alcohol, and then inserted into wax made from paraffin

for further preservation. Histological assessments using hematoxylin and eosin (H and E) stains were carried out to ensure any presence and type of tumors. Tissue abnormality was semi-quantitative measured in the form of structural irregularity, nuclear discrepancy, depletion of goblet cells and epithelial stratification.³²

Statistical Analysis

One-way analysis of variance (ANOVA) was employed to analyze the statistical significance of the data, and Dunnett's test was applied to determine any significant differences between the cancer control group and treatment groups. At $p < 0.05$, the statistical significance of the data was assessed. Minitab statistical software was utilized for all statistical analyses.

RESULTS

Effect of Calcium Citrate, *L. acidophilus*, Hydroalcoholic Extract of *M. oleifera* Leaves on Body Weight Measurements

Animals were treated with calcium citrate, *L. acidophilus*, and hydroalcoholic extract of *M. oleifera* leaves for 4 weeks. Body weights were significantly improved in group IV (Calcium citrate), group V (*L. acidophilus*), group VI (CC + LA), group VII (CC + LA+ME100), group VIII (CC + LA+ME200), group IX (ME100), and group X (ME200) as compared with group II (Cancer disease control) ($p < 0.05$) on the 12th week of the treatment period (Figure 1).

Effect of Calcium Citrate, *L. acidophilus*, Hydroalcoholic extract of *M. oleifera* leaves on Characterization of Tumor Parameters

After 4 weeks of individual and combined treatment with calcium citrate, *L. acidophilus*, and a hydroalcoholic extract of

M. oleifera leaves, tumor numbers were significantly lowered in group III (5-FU), group V (*L. acidophilus*), group VI (CC + LA), group VII (CC + LA+ME100), group VIII (CC + LA+ME200), group IX (ME100), group X (ME200) (Figure 2), and tumor size in group VI (CC + LA), group VII (CC + LA+ME100), group VIII (CC + LA+ME200), group IX (ME100), group X (ME200) (Figure 3)

Effect of Calcium Citrate, *L. acidophilus*, Hydroalcoholic Extract of *M. oleifera* Leaves on Colon Length-weight Ratio

Colon length-weight ratio decreased as a result of mucosal small tumors that developed after DMH therapy. Pathological alterations were noticed in the positive control group in the form of moderate colonic atrophy with tumors to the colonic mucosa (microadenomas, adenomas) during gross necropsy of the colonic mucosa. Mild atrophy in the colonic tissue of the treatment groups was observed in groups IV to X (Figure 4). The cancer control group and the treatment groups did not significantly differ from one another.

Effect of Calcium Citrate, *L. acidophilus*, Hydroalcoholic Extract of *M. oleifera* Leaves on Hematological Parameters

In the cancer control group, Hb and RBC counts were lowered with regard to individual and combined treatment of calcium citrate, *L. acidophilus*, and *M. oleifera* extract were repaired Hb and RBCs count, after 4 weeks of treatment, significant improvements were observed ($p < 0.05$) in group VI (CC + LA), group VII (CC + LA+ME100), group VIII (CC + LA+ME200) and group X (ME200) (Figures 5, and 6).

Due to tumor development, animals become immunocompromised and susceptible to infection. A gradual rise in WBCs and platelet count was observed. After 4 weeks of

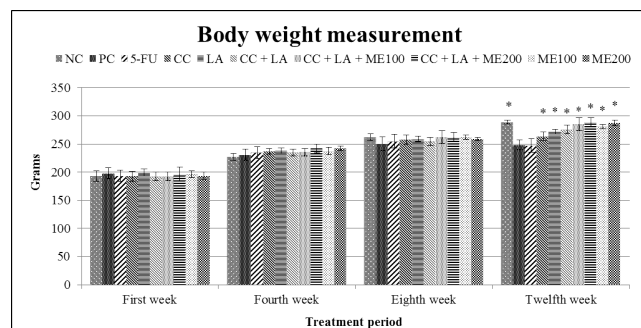


Figure 1: Body weight measurement

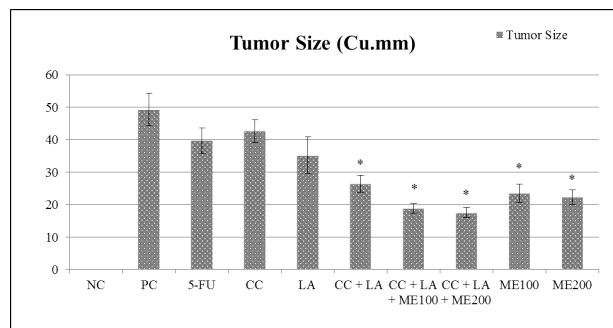


Figure 3: Characterization of tumor: Tumor size (Cu. mm)

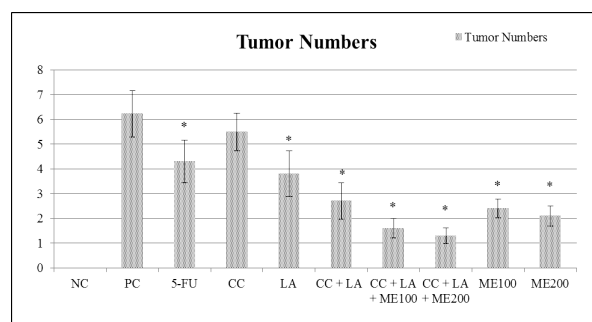


Figure 2: Characterization of tumor: Tumor numbers

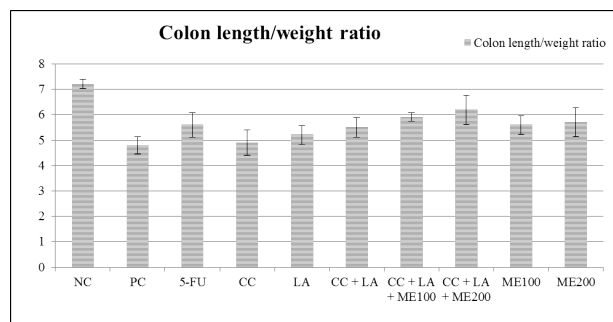


Figure 4: Colon length/weight ratio

calcium citrate, *L. acidophilus*, and *M. oleifera* leaves extract treatment, significant decreases in WBCs and platelet count ($p < 0.05$) were observed in group VI (CC + LA), group VII (CC + LA + ME100), group VIII (CC + LA + ME200), group IX (ME100), and group X (ME200). All data were compared with cancer control (Group II) (Figures 7 and 8).

Effect of Calcium Citrate, *L. acidophilus*, Hydroalcoholic Extract of *M. oleifera* Leaves on Antioxidant Enzymes Activity Estimation

After DMH exposure, there was a reduction in antioxidant enzyme activity, i.e., catalase, reduced glutathione, superoxide dismutase, and lipid peroxidation due to excess cellular

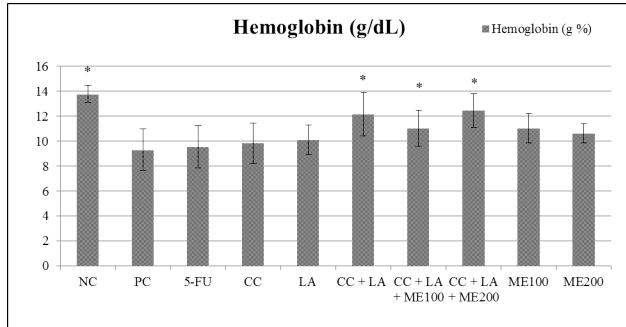


Figure 5: Hematological parameters: Hemoglobin

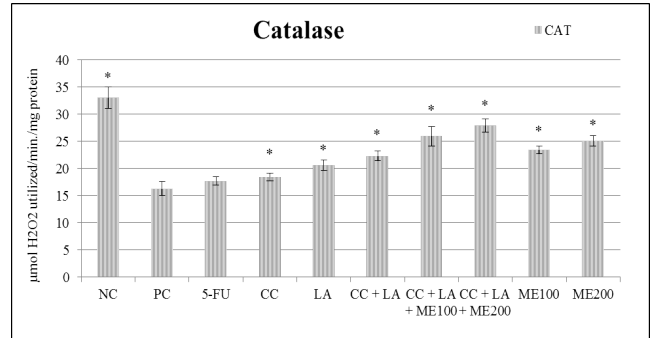


Figure 9: Antioxidant marker: Catalase

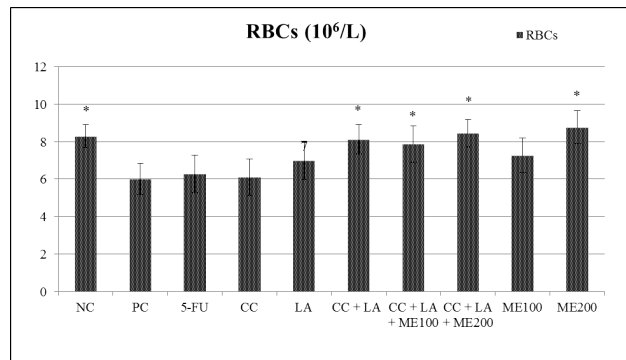


Figure 6: Hematological parameter: Red blood cells

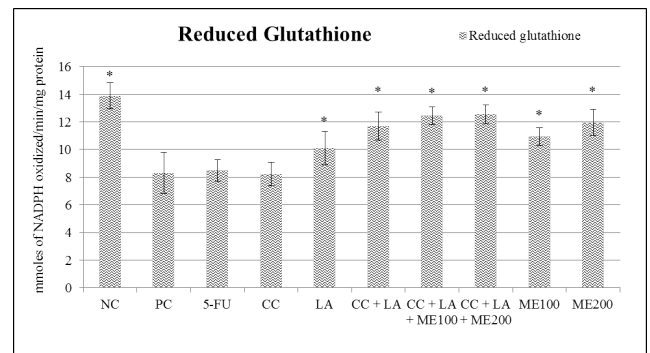


Figure 10: Antioxidant marker: Reduced glutathione

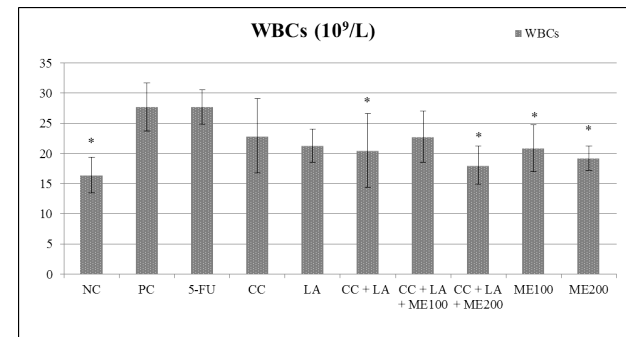


Figure 7: Hematological parameter: White blood cells

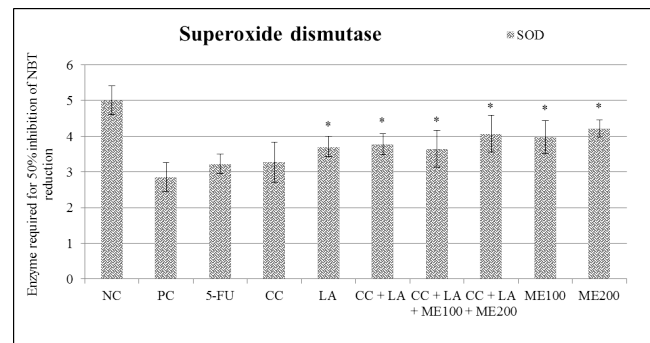


Figure 11: Antioxidant marker: Superoxide dismutase

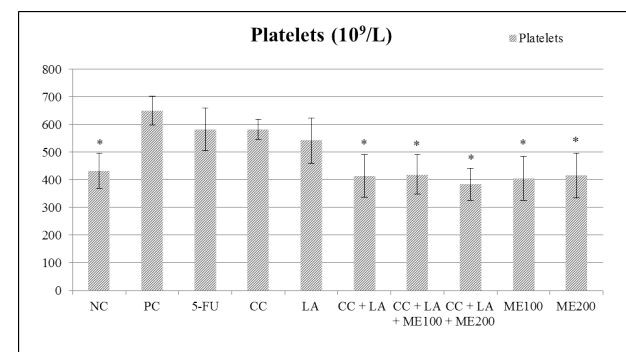


Figure 8: Hematological parameter: Platelets

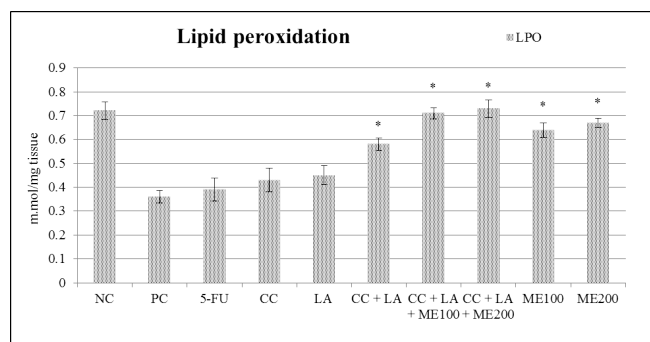


Figure 12: Antioxidant marker: Lipid peroxidation

production of reactive oxygen species. After treatment with calcium citrate, *L. acidophilus*, and hydroalcoholic extract of *M. oleifera* leaves, cellular oxidation was lowered through its antioxidant effect of it. Catalase, superoxide dismutase, reduced glutathione, and lipid peroxidation enzyme activity were elevated significantly in group VI (CC + LA), group VII (CC + LA + ME100), group VIII (CC + LA + ME200), group IX (ME100), and group X (ME200) as compared ($p < 0.05$) with group II (Cancer control) (Figures 9-12).

Effect of Calcium Citrate, *L. acidophilus*, Hydroalcoholic Extract of *M. oleifera* Leaves on Colonic Histoarchitecture

The DMH metabolites promoted cellular oxidative stress, which in consequence triggered cellular infiltration, hemorrhage with an altered nuclear/cytoplasm ratio, goblet destruction, and aberrations in colonic epithelial cells (Figure 13B). Improvements in colonic cellular infiltration with a normal nuclear/cytoplasmic ratio and goblet cells were observed by the combined treatment of calcium citrate, *L. acidophilus*, and hydroalcoholic extract of *M. oleifera* leaves extract. (Figures 13F, G and H) and individual treatment was showed a moderate recovery in colonic histoarchitecture (Figures 13D, E, I, J).

DISCUSSION

The incidence of colon cancer has increased in economically developing countries. Colon carcinogenesis is one of the most prevalent malignancies worldwide and it is a lethal, complex cancer that is triggered by dietary and environmental factors as well as the adoption of a western lifestyle.^{33,34} A high-fat diet, changes in lifestyle, and less physical activity may be linked to at least one-third of all human colon cancer cases. Treatment is perhaps less efficient or, in rare cases, not possible, as evidenced by the escalating death rate associated with the huge number of colon cancer cases that are in advanced or incurable stages. Treatments include use of chemotherapeutic drugs, radiation and surgery³⁵ are often only used to treat late stages of colon cancer and come with detrimental effects as severe as anemia, alopecia, fatigue, and weight loss. Certain drugs have the ability to harm the liver, kidneys, nervous system, heart, and other organs.^{36,37}

DMH is a potent colon carcinogen was used for induction of colonic carcinogenesis and animals were treated with DMH at 30 mg/kg s.c. dose for 8 weeks and for chemopreventive effect assessment, treatment groups were treated with individual and combined treatment of Calcium, *L. acidophilus*, and a hydroalcoholic extract of *M. oleifera* leaves for 4 weeks of the treatment period. DMH enters the intestines by bile and blood after being metabolized by the hepatocytes. It forms methyl-diazonium ions and hydroxyl radicals, which trigger oxidative stress by methylating biomolecules in the colon's epithelial cells. Oxidative stress is an occurrence that illustrates a disproportion between molecules of reactive oxygen and the body's defense system of antioxidants,³⁸ this is followed by a change in the normal epithelium and hyperproliferation, which encourages the development of colon cancer.^{39,40}

Upon DMH exposure, an increase in polyp stress-induced cachexia results in no significant improvements in body

weights of treatment groups were noticed in SD rats. Treatment of calcium citrate, *L. acidophilus*, and a hydroalcoholic extract of *M. oleifera* leaves by oral gavage revealed a significant gain in body weight by the 12th week of the experimental period by efficient reversal of detrimental abnormalities imposed by

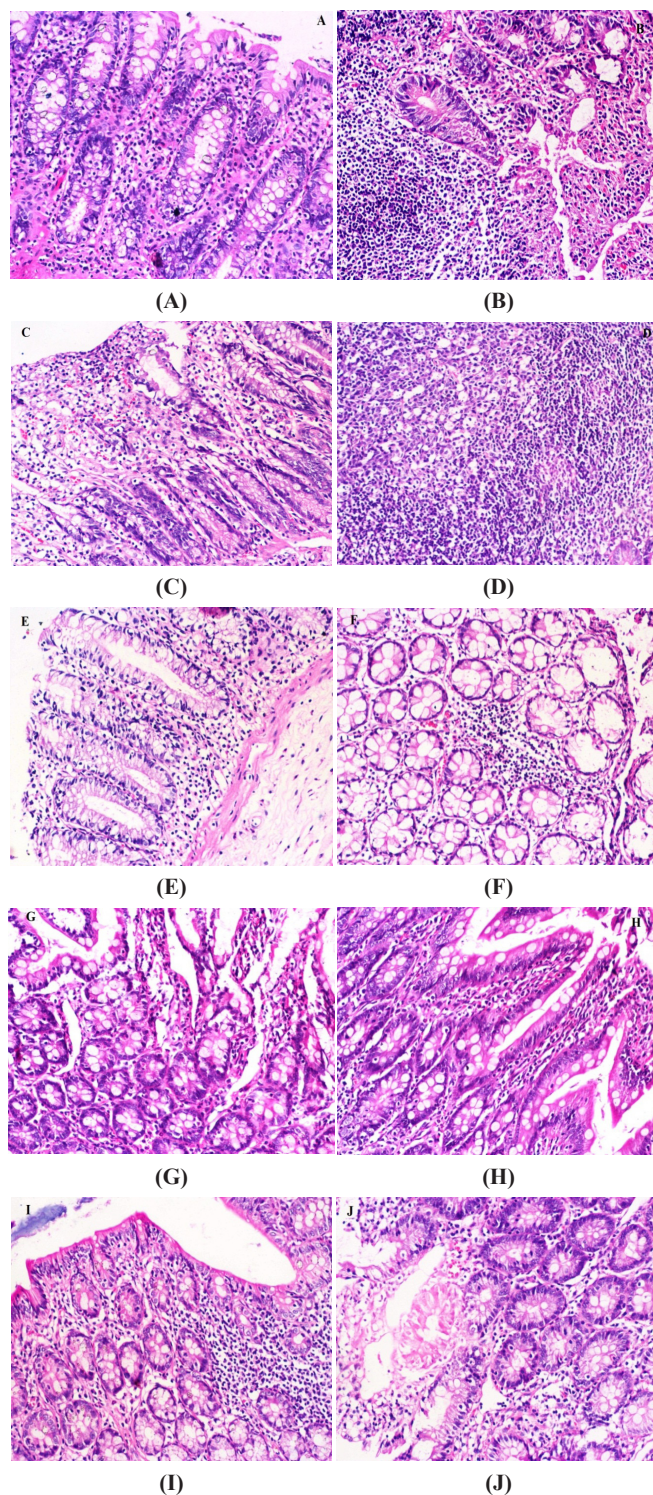


Figure 13: (A) Negative control, (B) Cancer control, (C) 5-FU group, (D) CC treated group, (E) LA treated group, (F) CC + LA group, (G) CC + LA + ME100 group, (H) CC + LA + ME200 group, (I) ME100 group, (J) ME200 group.

the cancer. This suggests the possibility of using combination therapies to impede chemically induced carcinogenesis and avoid cachexia (Figure 1).

Carcinogen exposure encourages small colonic tumors with tissue atrophy that are easily distinguishable in the inflamed mucosa. There was a decrease in the numbers and size of tumors along with the improvements in colon length/weight ratio (Figures 2-4) in combined treatment groups of hydroalcoholic extract of *M. oleifera* leaves extract, Calcium citrate and *L. acidophilus* by minimizing cellular reactive oxygen species and tumor cell proliferation.^{41,42}

Circulatory oxidative stress is increased by DMH metabolites that cause an excess of systemic reactive oxygen species. RBCs and hemoglobin are prone to circulatory oxidative stress, and a decrease in RBCs and Hb were noted, indicating anemic pathology, along with an upsurge in platelet and WBC levels, signifying the body's defensive mechanisms is activated.⁴³ Leukocytosis and thrombocytosis are expected to promote tumor development. After combined treatment of calcium citrate, *L. acidophilus*, and *M. oleifera* leaves extract were showed improvements in erythrocyte oxidative stress and hematological abnormalities were observed,^{44,45} while individual treatments showed slight amelioration in circulatory oxidative stress and hematological alterations. (Figures 5-8)

DMH metabolites cause cellular oxidative stress^{46,47} and methylation of DNA, which causes mutations in epithelial cells of colonic mucosa.⁴⁸ Antioxidant enzyme and non-enzymatic antioxidant levels were significantly reduced after being exposed to carcinogens.

In the current research investigations, the positive control group showed a significant drop in the levels of enzymes that scavenge free radicals following DMH administration. Colon tissue treated with a combination of calcium citrate, *L. acidophilus*, and a hydroalcoholic extract of *M. oleifera* leaves demonstrated significantly higher levels of SOD, catalase, GSH, and LPO. This could be due to the possible antioxidant effects of calcium, *L. acidophilus*, and *M. oleifera* leaves extract (Figures 9-12).

DMH metabolites initiated cellular oxidative stress responsible for mutations in colonic epithelial cells, resulting in the induction of carcinogenesis, histological abnormalities, i.e., cellular infiltration, hemorrhage, and eruption in the epithelial mucosal layer with an increased nuclear/cytoplasm ratio, and the depletion of goblet cells (Figure 13B).⁴⁹ Combined treatment of calcium citrate, *L. acidophilus*, and hydroalcoholic extract of *M. oleifera* leaves extract suppressed colonic cellular infiltration with a normal nuclear/cytoplasmic ratio and goblet cells, and clear improvements were observed in the histoarchitecture of the colonic mucosa (Figures 13F-H). Besides, individual treatment showed moderate improvements in colonic histoarchitecture (Figure 13D-J).⁵⁰ Thus, calcium citrate, *L. acidophilus*, and hydroalcoholic extract of *M. oleifera* leaves treatment have the potential to act as chemopreventive agents by preventing the growth of cancerous cells. In the current investigation, we assessed the chemopreventive potential of a hydroalcoholic extract of *M. oleifera* leaves,

calcium and *L. acidophilus* in reducing the growth of tumors in the colon triggered by DMH and managing the proliferation of malignant mutations in the colon, they can also aid in the removal of carcinogens from an animal's body by controlling the activities of enzymatic antioxidants.⁵¹ Compared to the previously described individual treatments, the combination of Calcium, *L. acidophilus*, and the hydroalcoholic extract of *M. oleifera* leaves had a greater capacity to scavenge free radicals.

CONCLUSION

Findings of the present study indicated a combination treatment of *L. acidophilus*, calcium citrate and hydroalcoholic extract of *M. oleifera* leaves showed chemopreventive measures against colon cancer caused by DMH.

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