RESEARCH PAPER

Studies on the Beneficial Effects of Methanolic Fruit Extract of Muntingia Calabura (MFMC) in Combination with Probiotic Kefir Against 1,2-Dimethylhydrazine and High-Fat Diet-Induced Colorectal Carcinogenesis in Male Wistar Rats

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ABSTRACT

Colorectal cancer (CRC) is the main cause of tumour-associated morbidity and mortality globally, which is closely associated with high-fat diet consumption, chronic inflammation, excessive oxidative stress, and gut microbiome dysbiosis. The current study was aimed to evaluate the chemo preventive potential of the methanolic fruit extract of Muntingia calabura (MFMC) used in combination with probiotic kefir against 1,2-dimethylhydrazine (DMH) and high-fat diet (HFD)-induced colorectal carcinogenesis in male Wistar rats. The animals were randomly assigned to four groups (n = 5 in each group): Control, DMH + HFD and Kefir-fed, MFMC + kefed- fed for up to 12 weeks. The treatment effects were determined through growth performance, mortality, haematological and biochemical variables, inflammatory and oxidative stress makers, fecal microbial enzyme activities, aberrant crypt foci (ACF) count in colon, serum carcinoembryonic antigen (CEA), histopathological and immunohistochemical alterations in colonic tissue. DMH + HFD exposure remarkably decreased body weight gain, modified the haematological indices and upregulated liver enzymes; increased C-reactive protein levels, oxidative stress, fecal microbial enzyme activity and ACF formation as well as β-catenin dysregulation. Interventions of kefir relieved these changes to some extent; and the combined treatment of MFMC + kefir had more protection on them. The integrated regimen effectively enhanced antioxidant status, with a concomitant decrease in inflammatory markers and had less effect on intestinal microflora enzyme activity; ACF formation incidence was worse attenuated; serum CEA level decreased and physiological structure of colonic epithelium (β-catenin expression) was well preserved.

In conclusion, the present results revealed that MFMC along with probiotic kefir plays a major role in prophylactic activities against dietary-induced colorectal cancer through its antioxidant, anti-inflammatory, hepatoprotective, immunomodulatory and modulation of gut microbiota. Such a mixture of food may be an efficient dietary approach for the prevention of colorectal cancer.

Keywords: *Colorectal cancer; Muntingia calabura; Kefir; 1,2-Dimethylhydrazine; High-fat diet; Oxidative stress; Aberrant crypt foci; Probiotics; β-catenin.*

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INTRODUCTION

Colorectal cancer (CRC) is a common cause of cancer-related morbidity and mortality globally, and its occurrence is closely linked to dietary practices, lifestyle and chronic inflammation. Intake of high-fat diet (HFD) has been reported to exacerbate colorectal carcinogenesis69-70 through increasing oxidative stress, dysregulation of lipid metabolism and intestinal inflammation [1]. Experimental models with 1,2-dimethylhydrazine (DMH)-induced and HFD-induced CRC are closely related to human sporadic CRC and mostly used to explore the chemo preventive efficacy of natural compounds [2]. Inflammation and oxidative stress are the major player in the development of CRC. DMH metabolism produces ROS, resulting in lipid peroxidation, DNA damage and inhibition of endogenous antioxidant system [3]. These changes also promote the formation of ACFs, a known precursor and reliable marker of early colorectal carcinogenesis. In addition, levels of the inflammatory mediator, C-reactive protein (CRP) and

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dysregulation of cancer-related pathways such as β-catenin, are strongly associated with tumor development and poor prognosis [4]. Bioactive compounds from plants have received much interest as chemo preventive agents because they may present antioxidant, anti-inflammatory, and anticancer propensities. *Muntingia calabura* L. (Jamaican cherry) has been used historically for the management of inflammatory and gastrointestinal conditions as a folk remedy[5]. It was found that the fruits of M. calabura are rich sources of flavonoids, phenolic acids and various other bioactive agents which are well known for their strong antioxidant and anti-proliferative potentials. On the other hand, systematic description on methanolic fruit extract of M. calabura (MFMC) against dietary colorectal carcinogenesis is still sparse [6]. Probiotics have gained popularity as a dietary approach to maintaining gut health and preventing colon cancer. Kefir, which is fermented milk with beneficial bacteria and yeasts, has been found to modulate gut microbiota, inhibiting activity of deleterious microbial enzyme hydrolyzing genotoxic agents/fibers and improve antioxidant status reduce intestinal inflammation [7]. The potential synergistic effect of the combined application of plant-based bioactives and probiotics against other mechanisms in colorectal carcinogenesis such as oxidative stress, inflammation, gut microbiota dysbiosis and tumor marker expression has been proposed [8].

Therefore, prophylactic potential of MFMC with probiotic kefir on DMH and HFD induced colorectal carcinogenesis was studied in male Wistar rats. This study was designed to evaluate the growth performance, mortality and haematological and biochemical variables, systemic inflammatory and antioxidant status, fecal microbial enzyme in vivo activity (EIA), microscopical vs histopathological colonic abnormalities including aberrant crypt foci (ACF) formation, serum levels of Carcinoembryonic Antigen (CEA), as well as histopathology and immunohistochemistry of colon. This systemic strategy was employed to clarify the chemopreventive efficacy and underlying mechanisms of MFMC and kefir in CRC prevention.

MATERIALS AND METHODS

Carcinogen 11,2-Dimethylhydrazine (DMH) and all HPLC grade chemicals were purchased from commercial suppliers. Authentic fruit was used to prepare methanolic fruit extract of Muntingia calabura (MFMC). The probiotic culture, milk kefir, was prepared freshly before ingestion. Pellet chow diet and high-fat diet were obtained from an authorized feed supplier. Biochemical and inflammatory tests were analyzed using commercial diagnostic kits, whereas antioxidant, oxidative stress, fecal microbial enzyme, histopathological and immunohistochemical analyses were performed using standard reagents. Statistical analyses were performed using GraphPad Prism software (version 8.4.2). Fresh melkefir with added probiotics was given orally.

Preparation of Methanolic Fruit Extract

The shade-dried fruits of Muntingia calabura were pulverized to obtain a coarse powder and then extracted with methanol by Soxhlet extraction. Extraction was performed for 6–8 h, until the solvent in the siphon tube was colorless. The filtered extract, was evaporated under vacuum at reduced pressure using rotary evaporator to eliminate the solvent. The dry extract was put away at 4 °C in a sealed container until used for further applications. [9].

Animals

Adult male Wistar rats (13–14 weeks old, 130–140 g) were maintained under controlled conditions (25 ± 2 °C; humidity 45–55%; with a 12:12 h light/dark cycle) with standard pellet diet and water ad libitum. Animals were habituated for 72 h before the test. The experiments are approved by the Andhra University College of Pharmacy, Vishakhapatnam, Institutional Animal Ethics Committee(IAEC-16/AU-Pharm/2025-26) and performed according to guidelines of CPCSEA [10].

Experimental Design

Twenty-four weight-matched rats were randomly divided into four groups (n = 6) [11]:

- Control: Vehicle and normal diet
- DMH + HFD: DMH (40 mg/kg, i.p., twice weekly) and high-fat diet
- Kefir: DMH + HFD with milk kefir (5 ml/kg, p.o.)
- MFMC + Kefir: MFMC (200 mg/kg, p.o.) + DMH + HFD + milk kefir

All treatments were administered for 12 consecutive weeks (91 days).

Induction of Colorectal Carcinogenesis

To induce CRC, DMH (40 mg/kg body weight in saline, twice weekly) was injected intraperitoneally, and mice were fed HFD daily. Concentration-dependent inhibitory effect of DMH was compared with butyrate using 1 g/L of D-glucose as a substrate (data not shown).Freshly prepared DMH in EDTA saline was administered [12].

Body Weight and Growth Assessment

Body weight were measured at the start and end of the experiments. Body weight gain and growth rate (%) were calculated [13].

Procedure for Haematological analysis

On the last day of the study (day 91), blood was collected via retro-orbital plexus. Hemoglobin level, total RBC cells, WBC cells, platelets count, neutrophils and lymphocytes were determined by an automated hematology analyzer [14].

Determination of Serum biochemistry and inflammatory biomarkers

The serum was separated by means of centrifugation, and used for the determination of SGPT, SGOT, and ALP by monitoring substrate conversion using colorimetric

enzyme assays. Protein content was determined using Biuret. Serum C-reactive protein was measured by a turbidimetric immunoassay [15].

Determination of the antioxidant and oxidative stress parameters

Homogenates of colon tissue and serum were used for the assay of glutathione in form of reduced GSH by Ellman's reagent, GSH peroxidase (GPx) through the rate of consumption of GSH, catalase (CAT) by its ability to decompose hydrogen peroxide and lipid peroxidation (LPO) by thiobarbituric acid reactive substances method using TBARS [16-19].

Fecal microbial enzyme assay treatments 5.

The pH of homogenates of faeces was determined by the method described and it was tested for by a digital pH meter. The β-glucuroni-dase and β-glucosidase were determined in vitro using p-nitrophenyl substrates spectrophotometrically and mucinolytic activity by monitoring reducing sugars193 liberated [20].

Aberrant Crypt Foci (ACF) investigations The procedure of ACF analysis

Colons were excised, fixed, and stained with 0.2% methylene blue under the microscope. The total ACF number and crypt multiplicities were determined in entire colon lengths [21].

Histopathology and Immunohistochemistry

Colon samples were fixed, paraffin–embedded, and sectioned for the hematoxylin and eosin (H&E) staining. Immunohistochemical staining for β-cateninwas also evaluated. [22-23]

Statistical Analysis

The data was subjected to statistical analysis with the help of GraphPad Prism version8. 4. 2. Difference between groups was assessed by One- way analysis of variance (ANOVA) test with Dunnet's test. Data were presented graphically as means ± SEM and significance level was regarded at $p < 0.05$.

RESULTS

Effect of MFMC and Kefir on Body Weight, Weight Gain, and Growth Rate

The impacts of MFMC and Kefir on certain body weights for DMH and HFD-induced CRC rats. Disease control (DMH+HFD) final body weight, weight gain and growth rate were significantly decreased when compared to normal controls reflecting a deterioration in growth caused by carcinogen and high fat diet. The body weight gain and growth rate were increased by a significant dose of Kefir compared to the disease group. The growth performance was further ameliorated in the MFMC + kefir group, which was closer to normal level, indicated a protective and synergistic effect of MFMC together with Kefir on growth performance. (Table 1& Figure 1).

Table 1 Prophylactic effects of MFMC, HSHD along with Kefir on Body weights in DMH and HFD-induced CRC in Male Wistar rats.

Group Name (n=6)	Initial body weight (g)	Final body weight (g)	Weight gain (g)	Growth rate (%)
Control	133.02 ± 1.98	293.03 ± 6.42	160.01 ± 7.69	120.3
DMH+HFD	132.20 ± 3.25	241.27 ± 8.22	109.07 ± 7.37	82.5
Kefir	134.65 ± 2.69	254.64 ± 6.58	119.99 ± 8.47	89.1
MFMC+Kefir	136.08 ± 2.29	260.80 ± 7.89	124.72 ± 8.23	91.7

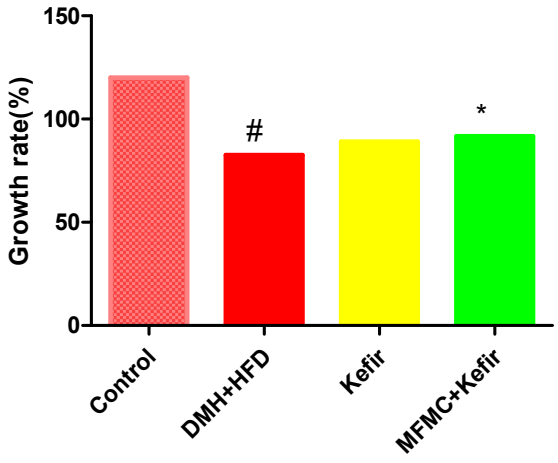

Figure 1: Prophylactic effects of MFMC, HSHD along with Kefir on Body weights in DMH and HFD-induced CRC in Male Wistar rats. All the values were expressed as Mean ± SD where n=6. The data were analysed by one-way ANOVA, followed by Dunnett's test. The data represent the initial body weight, final body weight, weight gain, and growth rate across all groups. The disease group showed a significant ($P<0.05^{\#}$) reduction in weight gain and growth rate compared to the control group. The kefir group and the MFMC + Kefir groups, exhibited significantly ($P<0.05^{*}$) improved weight gain, and growth rate when compared with the disease group.

Effect of MFMC and Kefir on Mortality Rate in DMH and HFD-Induced CRC Rats

Each experimental group mortalities. Mortality was the most severe in DMH+HFD-exposed mice (41.6%). Mortality was down to 30% after Kefir administration, reduced to 20% after MFMC + Kefir treatment. Our results indicate the protective effect of Kefir and increased survival advantage on co-administration with MFMC against DMH-HFD induced colorectal carcinogenesis. (Table 2&Figure 2).

Table 2: Prophylactic effects of MFMC, HSHD along with Kefir on % Mortality in DMH and HFD-induced CRC in Male Wistar rats.

Group Name	No. of Initial animals	No. of deaths	% of Mortality
Control	10	0	0
DMH+HFD	12	5	41.6
Kefir	10	3	30.0
MFMC + Kefir	10	2	20.0

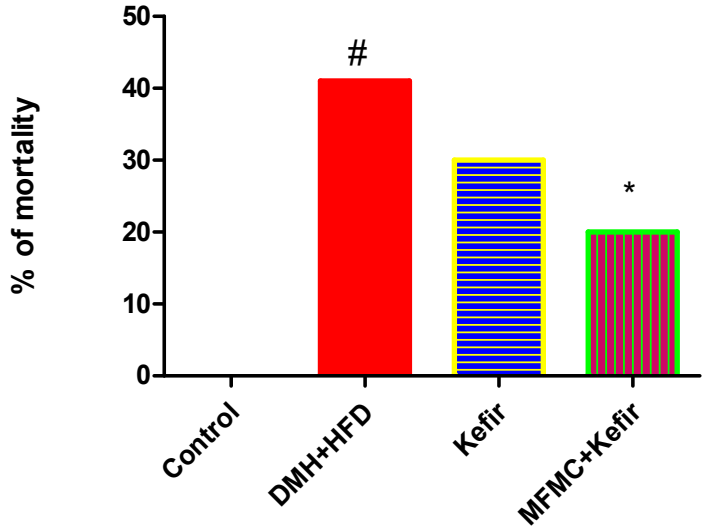

Figure 2: Prophylactic effects of MFMC, HSHD along with Kefir on % Mortality in DMH and HFD-induced CRC in Male Wistar rats.All the values were expressed as Mean ± SD where n=6. The data were analysed by one-way ANOVA, followed by Dunnett's test. The data represent the initial number of animals, number of deaths, and percentage of mortality across different groups. The disease group showed a higher mortality rate (41.6%). Probiotic and treatment groups exhibited reduced mortality (30% and 20%, respectively) compared to the disease group.

Effect of MFMC and Kefir on Hematological Parameters

Effects of MFMC and Kefir on hematological parameters. The number of RBCs and Hb was significantly lower, while the WBCs and platelets were higher in the group with disease compared to the control group indicative of anemia and systemic inflammation. Kefir therapy markedly corrected these factors to normal values. The hematological indices of the MFMC + Kefir group were more recovered, which implies a better recovery in hematopoietic function and lower inflammatory condition (Table 3&Figure 3).

Table 3: Prophylactic effects of MFMC along with Kefir on Total RBC count, Total WBC count, Total Hemoglobin count, and Total Platelets count in DMH and High-fat diet-induced CRC in Male Wistar rats.

Group name (n=6)	RBC ($\times 10^6/\mu l$)	WBC ($\times 10^3/\mu l$)	HGB(g/dl)	Platelets(($\times 10^3/\mu l$)
Control	6.58±0.17	9408.50±314.43	13.00±0.62	982.33±37.25
DMH+HFD	6.04±0.14	11194.33±507.82	10.29±0.39	1367.83±89.58
Kefir	6.16±0.18	10229.50±258.09	12.08±0.51	1156.33±55.32
MFMC+Kefir	6.31±0.18	9412.17±272.83	12.91±0.76	1041.50±56.91

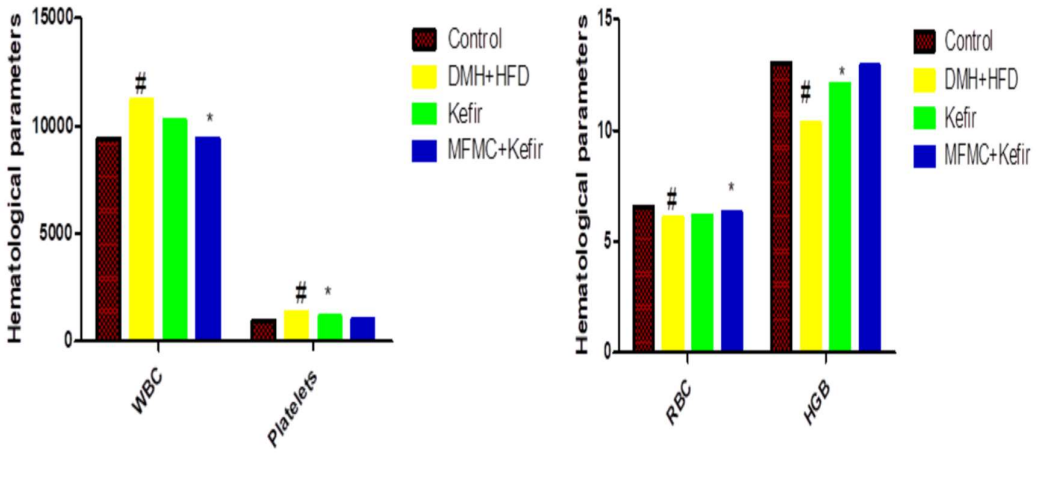

Figure 3: Prophylactic effects of MFMC along with Kefir on Haematological parameters: All the values were expressed as Mean ± SD where n=6. The data were analyzed by one-way ANOVA, followed by Dunnett's test. The disease group showed significant alterations in hematological parameters compared to the control group, including a decrease in RBC and Hb levels ($P<0.05^{\#}$) and an increase in WBC and Platelets count ($P<0.05^{\#}$) when compared with the control group. The Kefir group significantly improved these parameters ($P<0.05^{*}$) and the MFMC + Kefir group significantly showed a more pronounced improvement in these parameters, ($P<0.05^{*}$) when compared with the disease group.

Effect of MFMC and Kefir on Neutrophil and Lymphocyte Counts

The change of immune cells count between groups. The number of neutrophils in the disease group was elevated and that of lymphocytes was decreased, indicating an inflammatory and immunosuppressed state. Kefir treatment significantly diminished neutrophils and enhanced lymphocytes. The immunomodulatory effects were more pronounced in the MFMC + K group, which indicates a better balance in immunity and less inflammation. (Table 4&Figure 4).

Table 4: Prophylactic effects of MFMCalong with Kefir on Neutrophil and Lymphocyte count in DMH and High-fat diet-induced CRC in Male Wistar rats.

GROUPS	Neutrophil Count	Lymphocyte Count
Control	5785.83±988.09	7506.67±846.18
DMH+HFD	12456.67±1153.42	4156.33±352.78
Kefir	11413.83±731.27	6971.50±698.64
MFMC+Kefir	8859.50±544.41	8807.67±520.29

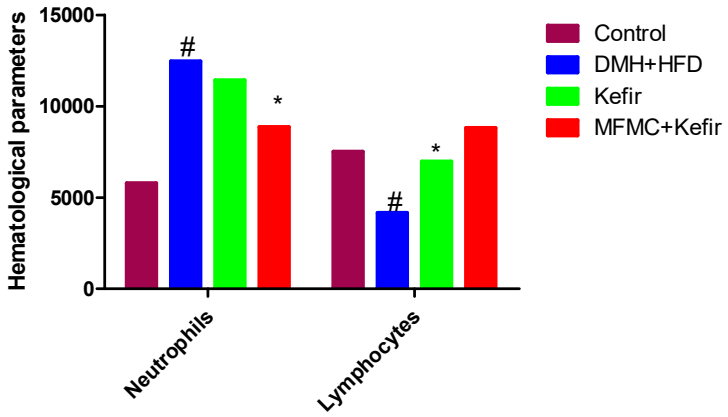

Figure 4: Prophylactic effects of MFMCalong with Kefir on Neutrophil and Lymphocyte count .All the values were expressed as Mean ± SD where n=6. The data were analysed by one-way ANOVA, followed by Dunnett's test. The disease group showed a significant increase in neutrophil count ($P<0.05^{\#}$) and a decrease in lymphocyte count ($P<0.05^{\#}$) compared to the control group, indicating an inflammatory response. The kefir group, and the MFMC + Kefir group, ($P<0.05^{*}$) showed a reduction in Neutrophils and an improvement in lymphocyte count when compared with the disease group.

Effect of MFMC and Kefir on Serum Biochemical (Liver Function) Parameters

The DMH+HFD group also had a significantly increased SGPT, SGOT and ALP as well as decrease in total protein levels, suggestive for hepatic damage and diminished protein synthesis. However, Kefir treatment has significantly decreased liver enzyme activities and increased total protein concentration. MFMC + Kefir group demonstrated more hepato protection in biochemical parameters that achieved to near normal control values. (Table 5&Figure 5).

Table 5: Prophylactic effects of MFMC along with Kefir on serum biochemical markers in DMH and High-fat diet-induced CRC in Male Wistar rats.

GROUPS	SGPT(U/L)	SGOT(U/L)	ALP(U/L)	Total Protein (mg/ml)
Control	51.67±4.69	72.67±5.08	169.33±13.09	7.50±0.36
DMH+HFD	110.50±7.02	121.33±4.89	273.17±10.29	4.88±0.42
Kefir	82.67±7.35	98.67±5.17	228.67±9.69	7.00±0.40
MFMC+Kefir	72.83±4.09	86.00±4.31	187.17±11.62	7.18±0.32

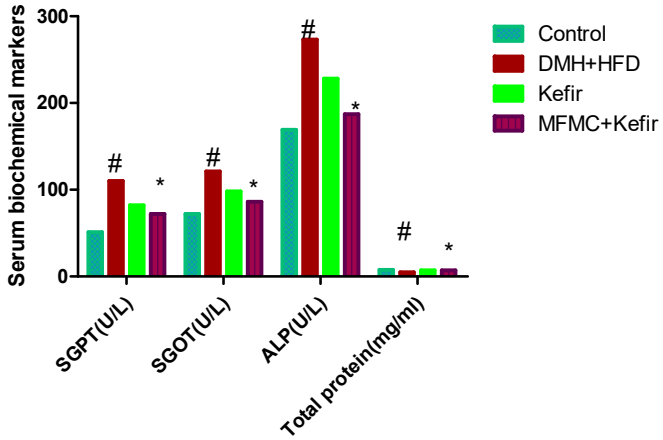

Figure 5: Prophylactic effects of MFMC along with Kefir on serum biochemical markers .All the values were expressed as Mean ± SD where n=6. The data were analysed by one-way ANOVA, followed by Dunnett's test.

The disease group showed a significant elevation ($P<0.05^{\#}$) in SGPT, SGOT, and ALP levels compared with the control group indicating liver damage. The kefir group and the MFMC + Kefir group, significantly decreased ($P<0.05^{*}$) the levels when compared with the disease group.

Effect of MFMC and Kefir on Serum Inflammatory Marker (C-Reactive Protein)

CRP levels were significantly higher in the patients with the disease compared to control group, indicating increased inflammatory response. CRP levels were significantly decreased by Kefir treatment, and the reduction was greater for the MFMC + Kefir group, confirming the anti-inflammatory activity of combined therapy (Table 6&Figure 6).

Table 6: Prophylactic effects of MFMC along with Kefir on serum Inflammatory marker in DMH and High-fat diet-induced CRC in Male Wistar rats.

GROUPS	Serum C- reactive protein (mg/dl)						
	R1	R2	R3	R4	R5	R6	Mean±SEM
Control	12	15	18	17	16	17	**15.83±0.96**
DMH+HFD	42	38	51	36	48	47	**43.67±2.66**
Kefir	36	23	38	41	35	29	**33.67±2.93**
MFMC+Kefir	34	27	31	28	33	26	**29.83±1.48**

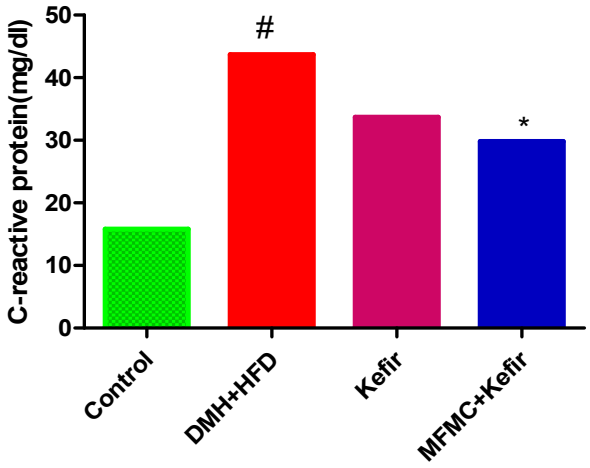

Figure 6: Prophylactic effects of MFMC along with Kefir on serum Inflammatory marker .All the values were expressed as Mean ± SD where n=6. The data were analysed by one-way ANOVA, followed by Dunnett's test. The disease group showed a significant ($P<0.05^{\#}$) increase in CRP levels compared with the control group indicating a heightened inflammatory response. The kefir group significantly ($P<0.05^{*}$) reduced them and the MFMC + Kefir group showed a more pronounced reduction ($P<0.05^{*}$) when compared with the disease group

Effect of MFMC and Kefir on Colon Tissue Antioxidant Status

Significant reduction in GSH, GPx and CAT levels observed in the disease group, point towards oxidative stress with impaired antioxidant defense. Kefir treatment increased the antioxidant enzyme activities and decreased lipid peroxidation. MFMC + Kefir group exhibited the best level of antioxidant restoration, indicating a powerful protection activity against the oxidative damage in the colon. (Table 7&Figure 7).

Table 7: Prophylactic effects of MFMC along with Kefir on Colon tissue homogenate Anti-oxidant markers in DMH and High-fat diet-induced CRC in Male Wistar rats.

GROUPS	GSH (μM/g tissue)	GPx (µgm GSH utilized/min/mg protein)	LPO (nmol MDA/ml plasma)	CAT (units/mg protein
Control	5.06±0.38	36.52±2.21	0.332±0.03	0.090±0.00
DMH+HFD	1.64±0.13	19.82±1.44	0.103±0.01	0.038±0.00
Kefir	4.38±0.23	26.90±1.95	0.170±0.01	0.048±0.00
MFMC+Kefir	4.44±0.19	39.07±1.97	0.257±0.02	0.070±0.01

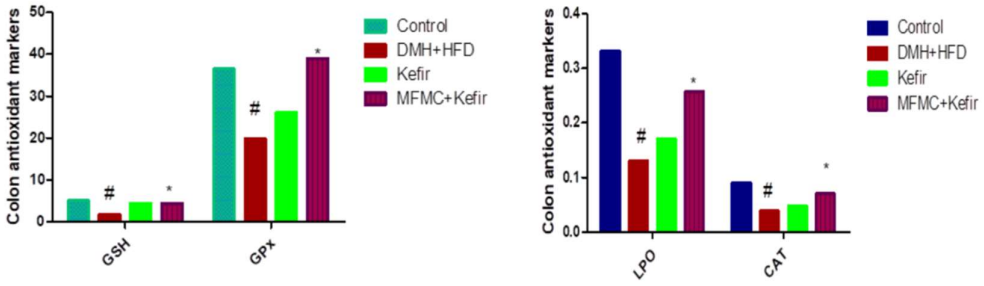

Figure 7: Prophylactic effects of MFMC along with Kefir on Colon tissue homogenate Anti-oxidant markers .All the values were expressed as Mean ± SD where n=6. The data were analyzed by one-way ANOVA, followed by Dunnett's test. The data represent levels of glutathione (GSH), glutathione peroxidase (GPx), lipid peroxidation (LPO), and catalase (CAT) activity across different groups. The disease group exhibited a significant decrease in GSH ($P<0.05^{\#}$), GPx levels ($P<0.05^{\#}$) and CAT activity ($P<0.05^{\#}$) but a significant decrease in LPO levels ($P<0.05^{\#}$) compared to the control group, indicating oxidative stress and impaired antioxidant defense. The Kefir group significantly ($P<0.05^{*}$) increased the levels of GSH, GPx, and catalase activity along with significantly ($P<0.05^{*}$) reducing the LPO levels and the MFMC + Kefir group, showed more pronounced effects ($P<0.05^{*}$) compared with the disease group. If P>0.05 then it was considered not significant.

Effect of MFMC and Kefir on Serum Antioxidant Enzyme Levels

DMH+HFD treatment led to marked reduction in GSH, GPx, and CAT activities and an increase in lipid peroxidation indicative of systemic oxidative stress. A partial restoration of the levels of antioxidants and a decrease in LPO were observed in Kefir-treated animals. The additional benefit of the MFMC + Kefir group may reflect greater systemic antioxidant defense. (Table 8&Figure 8).

Table 8: Prophylactic effects of MFMC along with Kefir on serum Anti-oxidant markers in DMH and High-fat diet-induced CRC in Male Wistar rats

GROUPS	GSH (*μ*M/g tissue)	GPx (μgm GSH utilized/min/mg protein)	LPO (nmol MDA/ml plasma)	CAT (units/mg protein
Control	7.80±0.33	13.59±0.93	0.083±0.01	0.384±0.02
DMH+HFD	2.86±0.24	7.42±0.60	0.414±0.03	0.070±0.01
Kefir	4.88±0.35	8.63±0.46	0.230±0.02	0.091±0.01
MFMC+Kefir	5.68±0.30	12.33±0.62	0.135±0.01	0.215±0.04

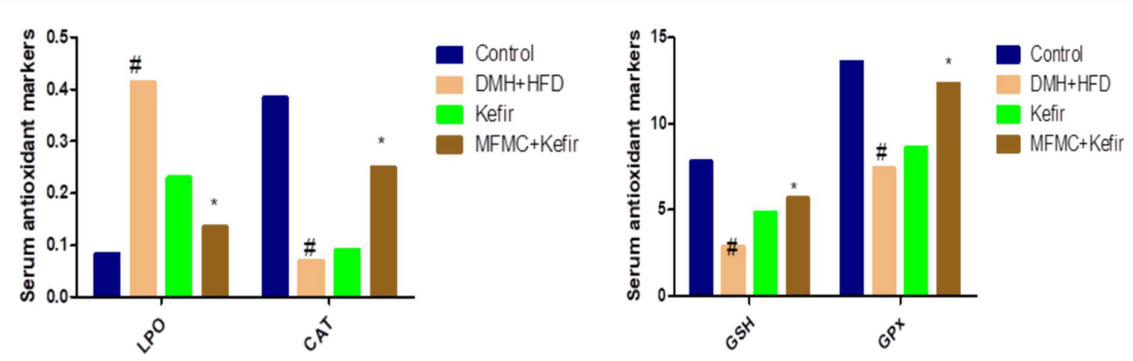

Figure 8: Prophylactic effects of MFMC along with Kefir on serum Anti-oxidant markers. All the values were expressed as Mean ± SD where n=6. The data were analyzed by one-way ANOVA, followed by Dunnett's test. The data represent levels of glutathione (GSH), glutathione peroxidase (GPx), lipid peroxidation (LPO),

and catalase (CAT) activity across different groups. The disease group exhibited a significant decrease in GSH ($P<0.05^{\#}$), GPx levels ($P<0.05^{\#}$) and CAT activity ($P<0.05^{\#}$) but a significant decrease in LPO levels ($P<0.05^{\#}$) compared to the control group, indicating oxidative stress and impaired antioxidant defense. The Kefir group significantly ($P<0.05^{*}$) increased the levels of GSH, GPx, and catalase activity along with significantly ($P<0.05^{*}$) reducing the LPO levels and the MFMC + Kefir group, showed more pronounced effects ($P<0.05^{*}$) compared with the disease group. If $P>0.05$ then it was considered not significant

Effect of MFMC and Kefir on Fecal pH and Microbial Enzyme Activity

The disease group had higher fecal pH values and higher activities of β-glucuronidase, β-glucosidase, and mucinase that play a role in colorectal carcinogenesis. Oral Kefir also decreased activity of these enzymes and reversed fecal pH. The MFMC + Kefir group exhibited a higher modulation of microbial activity in the gut, which may indicate better intestinal health and lower carcinogenic risk (Table 9&Figure 9).

Table 9: Prophylactic effects of MFMC along with Kefir on Fecal Microbial enzymes in DMH and High-fat diet-induced CRC in Male Wistar rats.

GROUPS	Fecal Matter pH	ß-Glucorunidase	ß- Glucosidase	Mucinase
Control	6.05±0.49	5.10±0.48	12.38±0.51	3.60±0.37
DMH+HFD	8.13±0.39	8.64±0.27	15.82±0.58	7.66±0.30
Kefir	7.11±0.27	6.68±0.36	14.01±0.78	6.85±0.30
MFMC+Kefir	5.77±0.30	5.41±0.27	13.73±0.67	4.73±0.39

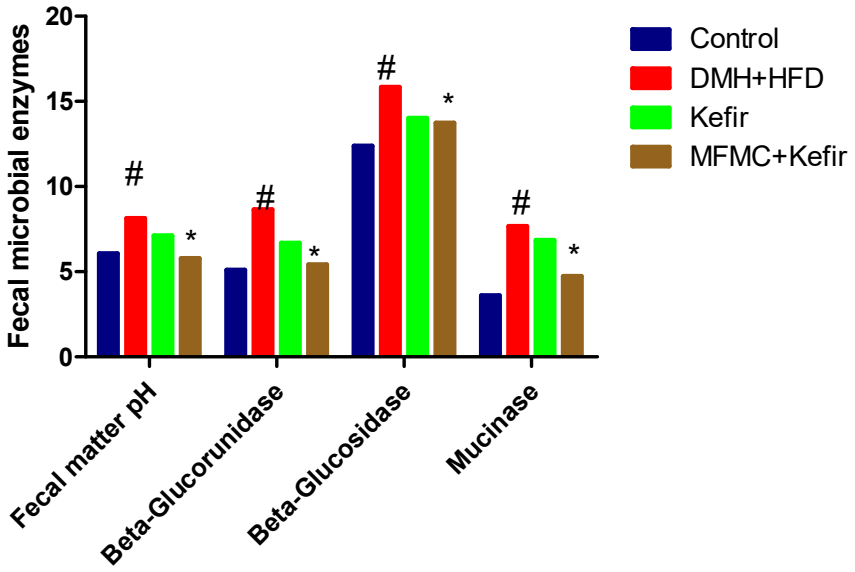

Figure 9: Prophylactic effects of MFMC along with Kefir on Fecal Microbial enzymes .All the values were expressed as Mean ± SD where n = 6. The data were analyzed by one-way ANOVA followed by Dunnett's test. The disease group (DMH + HFD) showed a significant increase (#P < 0.05) in fecal pH, β-glucuronidase, β-glucosidase, and mucinase activities when compared with the control group. Treatment with kefir and MFMC + kefir significantly reduced (P < 0.05) fecal pH and microbial enzyme activities when compared with the disease group. If P > 0.05, the difference was considered not significant.

Effect of MFMC and Kefir on Aberrant Crypt Foci Formation in Colon Tissue

CF were not detected in the control group, but increase in total ACF number and crypt multiplicity were both significantly higher in disease group. Kefir suppressed the incidence and the multiplicity of ACF. The MFMC + Kefir group presented additional reductions, which suggests the induction of potent chemo preventive effects against early colorectal cancer lesions. (Table 10&Figure 10&11).

Table 10: Prophylactic effects of MFMC, HSHD along with Kefir on Aberrant crypt Foci count in DMH and High-fat diet-induced CRC in Male Wistar rats.

Group Name (n=6)	Number of aberrant crypts per ACF				Total no. ACF	% of incidence
	1 crypt	2 crypts	3 crypts	>4 crypts		
CONTROL	NIL	NIL	NIL	NIL	NIL	NIL
DISEASE	22.80±5.2	17.33±3.44	14.0±1.18	22±1.58	76.13±6.538	100
KEFIR	10.98±2.8*	9.21±1.63*	9±0.98*	14±2.02*	43.19±3.940	56.73
MFMC+*KEFIR*	8.33±1.5*	5.83±0.81*	6.32±1.09*	11.34±1.18*	31.82±2.33	41.79

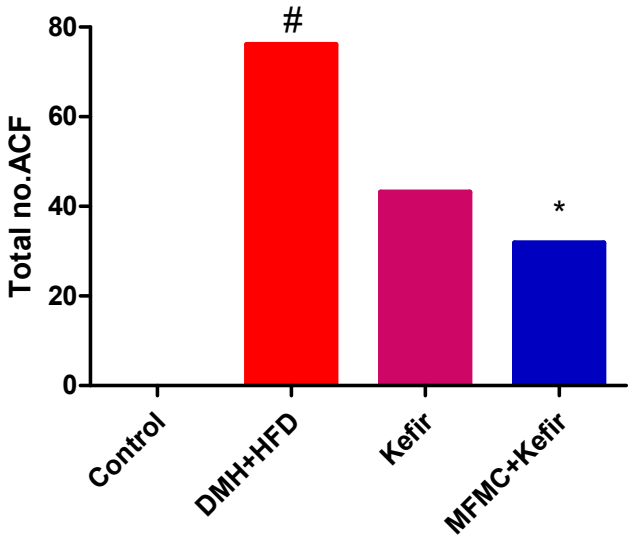

Figure 10: Prophylactic effects of MFMC, HSHD along with Kefir on Aberrant crypt Foci count. All the values were expressed as Mean ± SD where n=6. The data were analyzed by one-way ANOVA, followed by Dunnett's test. The p-value for the kefir group and the MFMC & HSHD+ Kefir group was ($P<0.05^*$) when compared with the disease group. If the p-value was P>0.05 then it was considered as not significant.

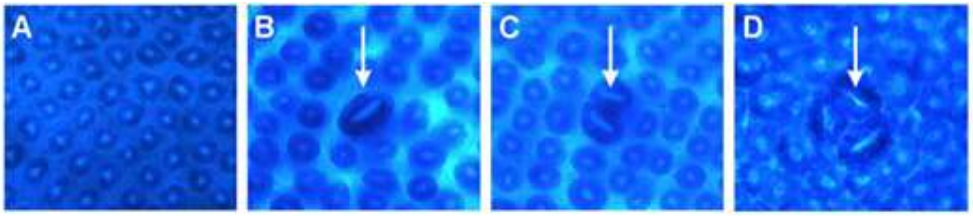

Figure 11: Prophylactic effects of MFMC along with Kefir on Aberrant crypt Foci count in DMH and High-fat diet-induced CRC in Male Wistar rats**.** Aberrant crypt foci (ACF) were observed under a light microscope after staining the colon with methylene blue (magnification of 10). The images show the whole mount colon of animals in (A) the control group and (B) the disease group (DMH &HFD) showing a topographic view of ACF indicated by a white arrow, with one crypt, (C) with two crypts or (D) with three crypts.

Effect of MFMC and Kefir on Serum Carcinoembryonic Antigen (CEA) Levels

The disease group presented a higher CEA level than the control group, which suggested aggravation of tumor. CEA levels in serum decreased markedly on Kefir treatment and CEA levels increased toward the normal range for both MFMC + Kefir and HSHD + Kefir groups, suggesting efficient control of tumor growth. (Table 11&Figure 12).

Table 11: Prophylactic effects of MFMC along with Kefir on serum tumor marker CEA in DMH and High-fat diet-induced CRC in Male Wistar rats.

Group Name (n=6)	Serum CEA (ng/ml)
CONTROL	0.32±0.02
DISEASE	0.49±0.01$^{\#}$
KEFIR	0.42±0.02*
MFMC+*KEFIR*	0.34±0.01*

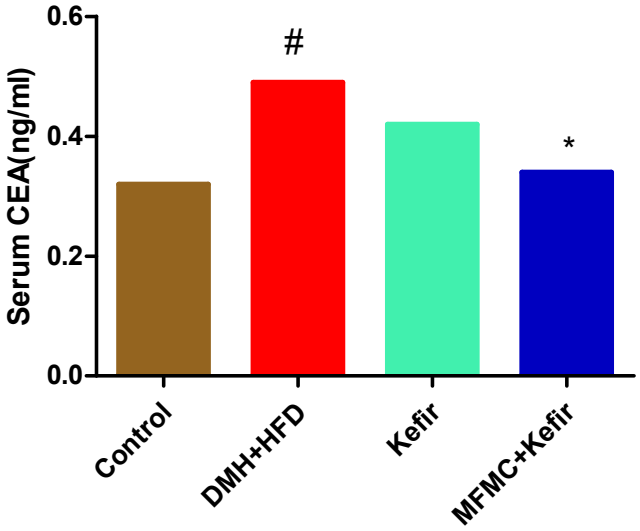

Figure 12: Prophylactic effects of MFMC along with Kefir on serum tumor marker .All values are expressed as Mean ± SD, where n = 6. Data were analyzed using one-way ANOVA followed by Dunnett's test. The data in the graph represent a significant increase in serum CEA levels($P<0.05^{\#}$) in the disease group compared with control group.

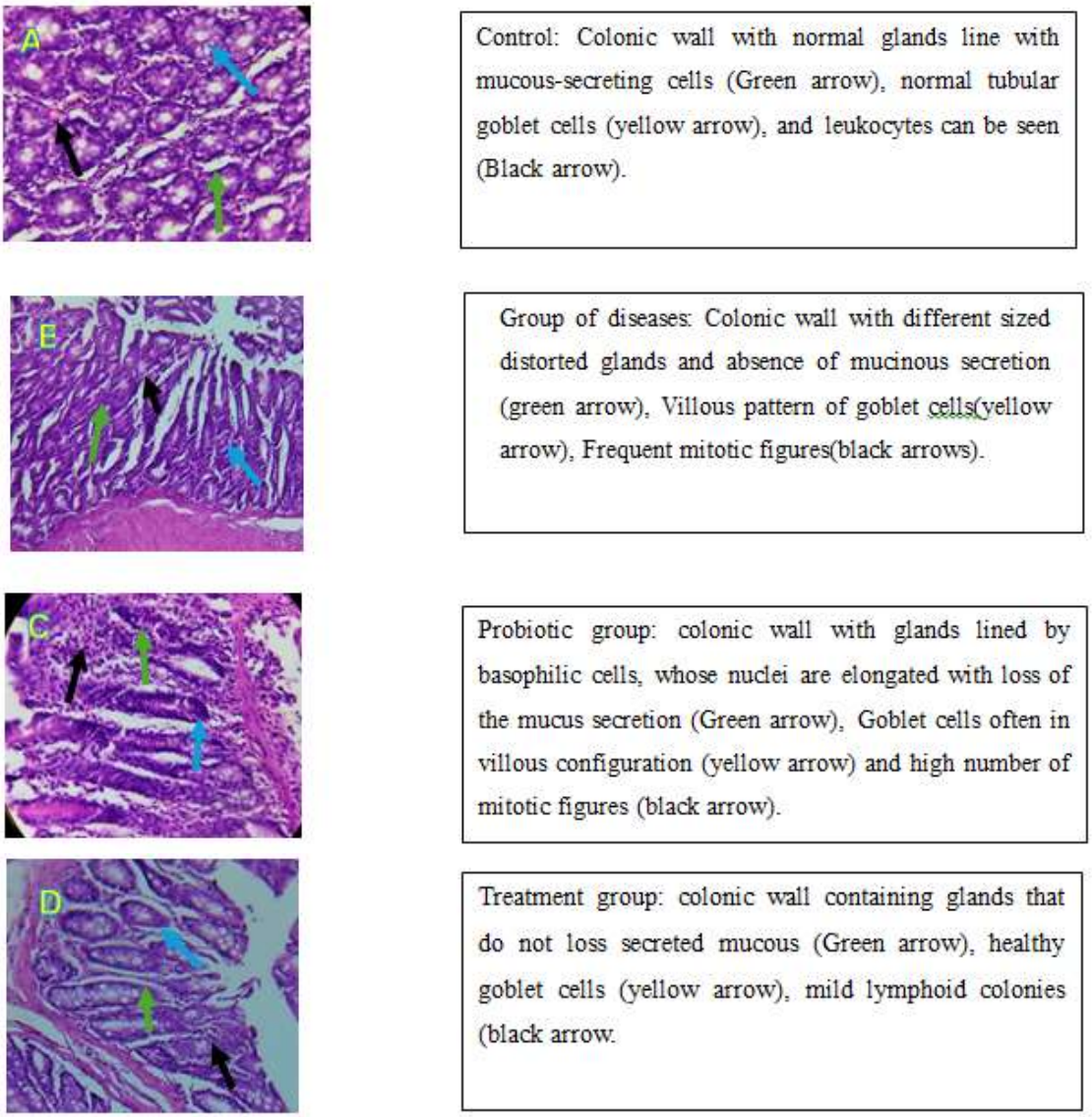

Figure:13. Prophylactic effects of MFMC along with Kefir on Histopathological changes of colon tissue in DMH and High-fat diet-induced CRC in Wistar rats.

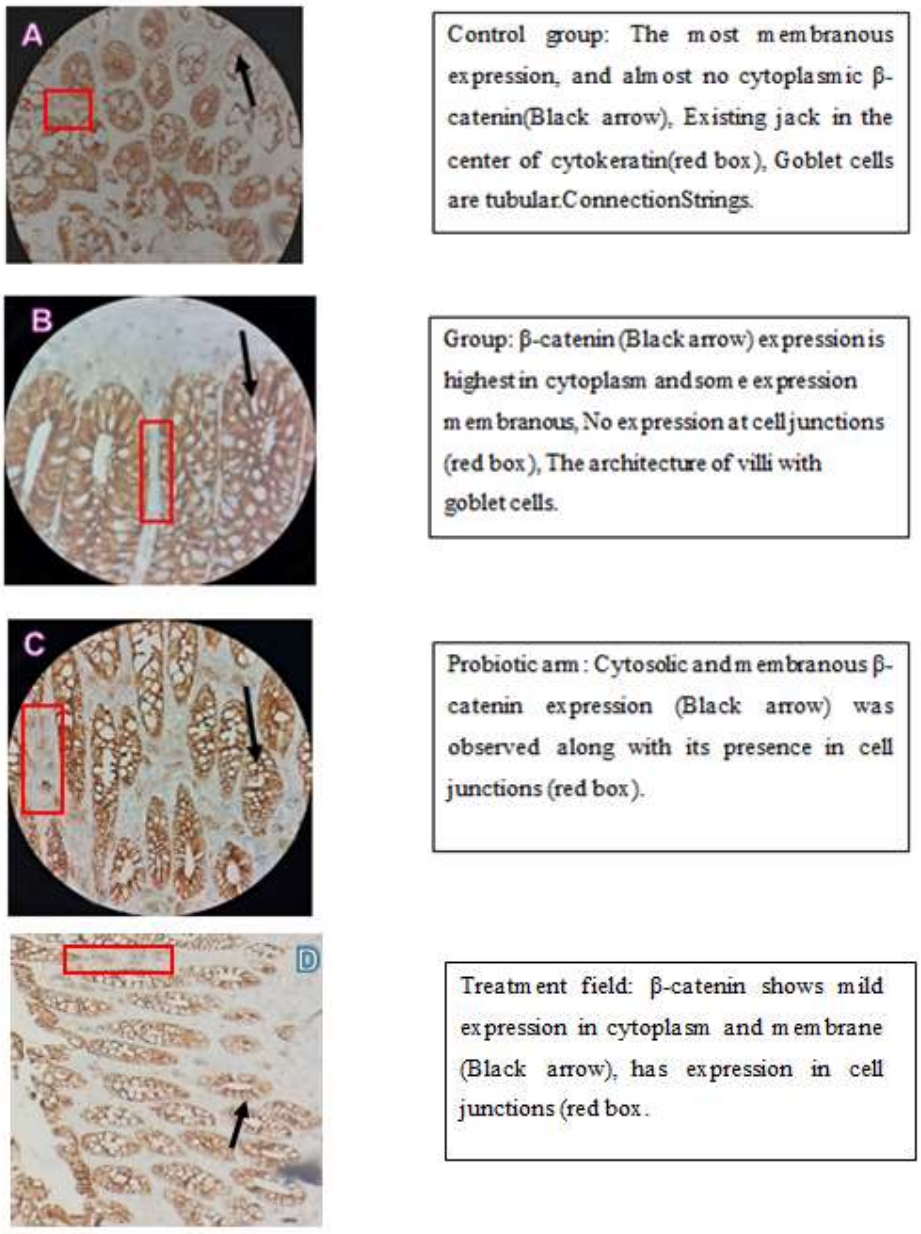

Figure 14: Prophylactic effects of MFMC along with Kefir on Immunohistochemical analysis of β-catenin in colon tissue in DMH and High-fat diet-induced CRC in Male Wistar rats.

DISCUSSION

The present study focused on chemopreventive potential of methanolic fruit extract of Muntingia calabura (MFMC) in association with probiotic kefir against 1,2-dimethylhydrazine and high-fat diet–induced colorectal carcinogenesis in male Wistar rats. Results show that the simultaneous treatment of MFMC + kefir significantly diminished 1,2-dimethulhydrazine-induced colorectal carcinogenesis through improvement in growth performance and survival rate; restoration of hematological and biochemical homeostasis associated with suppression of inflammation and oxidative stress; modulation of gut bacterial enzyme activity, prevention of ACF formation along with improvement in histopathological alterations. Diminished weight gain and growth rate in DMH+HFD are characteristics of systemic toxicity, metabolic dysfunction, and cancer-related cachexia. Treatment with kefir reversed them to some extent, which suggested the enhancement of nutrient uptake and metabolic homeostasis. 2.1MFMC in combination with kefir resulted in a more significant improvement, which can be interpreted as a synergistic protective effect. The antioxidant and anti-inflammatory phytoconstituents of MFMC in conjunction with the gut-

modulating effects of kefir may account for this. As expected, mortality was the greatest in disease group, while kefir and MFMC + kefir treatments significantly declined mortality, suggesting increased survival and resistance to the disease.

Blood disorders such as a chronic inflammatory reaction and ineffective erythropoiesis are often observed during the development of colorectal cancer. On the other hand, WBC and platelet were higher while RBC and hemoglobin were lower in the disease group, which indicate anemia and systemic inflammatory response. Kefir therapy normalized these parameters, MFMC with kefir produced near normalization; which suggests amelioration of hematopoietic function and decreased inflammation. The disease group also displayed raised neutrophils and reduced lymphocytes, which indicated with immune imbalance. Regeneration of the reduced immune cell subsets after MFMC + kefir treatment is indicative of its immunomodulatory capacity. Liver dysfunction is also a known effect of DMH metabolism, as shown by the increase in serum SGPT, SGOT and ALP levels in the disease group. These changes correlate with hepatocellular injury and oxidative stress. The serum levels of liver enzymes were significantly decreased in the kefir-supplemented group, and when MFMC was combined with kefir the hepatoprotective effect was more prominent. Total protein recovery also suggests the enhanced liver synthetic function and systemic metabolic recovery on treatment.

'Inflammation' Is a key factor in the initiation and progression of colorectal tumours. High levels of serum C-reactive protein (CRP) in the disease group indicate an increased level of inflammation. Both kefir and MFMC+kefir markedly decreased CRP compared to the HC group, and the combined treatment led to the highest level reduction ($p < 0.05$). This decrease in systemic inflammation can help explain the observed attenuation of tumor development. One of the primary mechanisms in DMH-induced colorectal carcinogenesis is oxidative damage. The hepatic disease group had depleted levels of endogenous anti-oxidants such as GSH, GPx and CAT, and an enhanced level of lipid peroxidation in colon tissue and serum. Kefir reduced partially the oxidative stress, while MFMC + kefir was effective in increasing antioxidant enzyme levels and decreasing lipid peroxidation. These results suggest that MFMC increases the antioxidant activity of kefir, leading to enhanced oxidative stability in colonic tissue. In the stomach, the spontaneous formation of acetoxy-gluco conjugates is temperature-dependent, naturally providing a rationale for a gastric-acid-neutralizing effect as well; and in the colon altered gut microflora and increased activity of some microbial enzymes, eg β-glucuronidase, beta-glucosidase and mucinase - all contributing to carcinogenesis by aiding in carcinogen activation or degradation of mucosa. Elevated fecal pH and enhanced microbial enzyme activity were found in the disease group, indicating deysbiosis. Kefir reduced them all, both in MFMC and were normalized to a greater extent by MFMC + kefir for pH and enzymatic activity in fecal samples indicating the beneficial modulation that increased microbial balance ameliorated risk of carcinogenicity. ACF is a well-established early biomarker of colorectal cancer. No ACF were found in control animals, whereas their number was significantly increased in the diseased group confirming efficient carcinogenesis. Treatment with ACF of kefir suppressed the total number of ACF and crypt multiplicity and MFMC + kefir resulted in much more reduction, suggest that its very strong chemopreventive effect against early neoplastic lesions. The level of serum carcinoembryonic antigen (CEA) which is a useful clinical tumor marker, could be increased with the disease and progression. Decrease of CEA levels after MFMC+ kefir treatment was also indicative of the inhibition of tumor growth. Moreover, histopathological observation corroborates these biochemical data, as severe glandular distortion/congestion pansinusitis and loss of mucous secretion were alleviated in response to treatment relative to the diseased group with disease-induced increased mitotic activity being greatly ameliorated. Immunohistochemistry showed that abnormal cytoplasmic staining of β-catenin was observed in the disease-group and suggested activation of Wnt/β-catenin signaling, an important cancer-promoting pathway for colorectal cancer. MFMC + kefir decreased cytoplasmic β-catenin expression with restoration of membranous localization, implying regulation of oncogenic signaling pathways and maintenance of normal epithelial structure.

To conclude, the current data indicate that MFMC along with probiotic kefir provide marked protection against DMH and HFD-mediated colorectal carcinogenesis. The anticancer trait was attributed to antioxidant/anti-inflammatory/hepatoprotective and gut microbiota-regulating effects, in addition to detrimental effect on early neoplastic changes, tumour associated markers and immunomodulation. These results provide support for the promise of probiotics combined with plant bioactives as an efficacious dietary prophylactic strategy against colorectal cancer.

CONCLUSION

Results The results of the present study have showed that MFMC supplemented with probiotic kefir could significantly prevent 1,2-dimethylhydrazine (DMH) and high-fat diet–induced colorectal carcinogenesis in male Wistar rats. MFMC/K treatment significantly increased many fish growth parameters, survival and also restored both haematological and biochemical lesions induced by the carcinogen. The combined therapy attenuated systemic inflammation and improved antioxidant defense, while decreasing oxidative stress in colon tissues as well as serum. Additionally, MFMC and kefir supplementation altered gut microbial enzymatic activity, which resulted in fecal pH normalization and the decrease of carcinogen-activating enzymes. A significant reduction in formation of crypt foci and level of serum carcinoembryonic antigen was found, which reflected suppression at early-stage

neoplasia as well as tumor promotion. Histopathological and immunohistochemical studies also corroborated that MFMC / kefir protects against DMBD induced changes by maintaining the morphological architecture of colon with down regulation of β-catenin.

In summary, our data indicate that MFMC, in combination with probiotic kefir warrants further investigation as a novel dietary strategy for the prevention of colorectal cancer via inhibition of multiple pathological processes involved in cancer formation.

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CONFLICT OF INTEREST
All authors are declared no conflict of Interest

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