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The preventive effects of diosmin alone or combined with irinotecan on 1,2-dimethylhydrazine-induced colon cancer in rats

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Abstract. – OBJECTIVE: Colorectal cancer, one of the most frequently diagnosed cancers worldwide, has a high mortality rate. Thus, our research aims to examine the preventive effects of diosmin (DIO) alone and in conjunction with the anti-cancer drug irinotecan (camptothecin-11, CPT-11), on 1,2-dimethylhydrazine (DMH)-induced colon cancer (CC) in male Wistar rats.

MATERIALS AND METHODS: Fifty adult male Wistar rats were categorized into five groups. Group I (Normal) received saline 0.9 orally % as a vehicle once a week for 14 weeks. Group II (DMH) received DMH (20 mg/kg/week) orally dissolved in 0.9% saline for 14 weeks and 1% carboxymethylcellulose (CMC) every other day for the final 10 weeks. Group III (DMH+DIO) received DMH orally for 14 weeks and DIO (10 mg/kg, suspended in 1% CMC) every other day for the final 10 weeks. Group IV (DMH+CPT-11) received DMH orally for 14 weeks and intraperitoneal injection of CPT-11 (3 mg/kg) twice a week for the final 10 weeks. Group V (DMH+DIO+CPT-11) orally received DMH for 14 weeks and both DIO and CPT-11.

RESULTS: All treated groups showed a significant reduction ($p<0.05$) in their elevated serum malondialdehyde levels and significant amelioration ($p<0.05$) of their lowered activities of colon glutathione-S-transferase (GST) and glutathione reductase (GR) as well as serum glutathione level (GSH). In addition, simultaneous treatment with DIO and CPT-11 led to a significant decrease ($p<0.05$) in the elevated serum levels of carcinoembryonic antigen (CEA) in rats administered with DMH, as well as a reduction in the colon expression levels of the inflammatory mediator (NF-κB), cell proliferator protein (Ki-67), and proapoptotic protein (p53).

CONCLUSIONS: These findings suggest DIO, CPT-11, and their combination have anticarcinogenic effects against DMH-induced CC by suppressing oxidative stress, simulating the antioxidant defense system, attenuating the inflammatory effects, and reducing cell proliferation.

Key Words:
Diosmin, Irinotecan, 1,2-dimethylhydrazine, Oxidative stress, Ki-67, *Nrf2*, p53, NF-κB.

Introduction

Colorectal cancer (CRC) ranked as the third most detected cancer and the second most lethal malignancy worldwide, posing a serious health problem[1]. In 2020, CRC contributed to roughly 9.4% of cancer-related fatalities[2]. Various risk factors have been identified as potential contributors to CRC, such as diet, environmental triggers,

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a sedentary lifestyle, and genetic susceptibility[3]. Colon cancer is a multifaceted and progressive disease that involves multiple causes, stages, mechanisms, linkages, and genetic alterations. Nevertheless, the initial growth of the subject is rather sluggish, and the outlook is promising[4,5]. Hence, it is crucial to investigate the causes and progression mechanisms of colon cancer to facilitate timely detection and intervention[6].

Despite advances in chemotherapy, cancer drug approvals were associated with statistically significant deaths[7], and the majority of existing chemotherapeutic therapies have detrimental side effects. Consequently, there is a need to investigate alternative anticancer agents for treating CRC. The use of plant constituents as novel chemotherapeutic agents with minimal side effects is gaining popularity[8]. Furthermore, natural products can potentially be used as drugs and molecular probes[9]. Due to the abundance of vegetables and fruits in flavonoids and carotenoids, which have been proven to possess anticarcinogenic, antimetastatic, and immunomodulatory properties, they could be advantageous for the treatment and prevention of cancer[10].

1,2-dimethylhydrazine (DMH) is frequently utilized as a cancer-causing agent to promote CRC in animal models since the lesions it causes are identical to human precancerous and cancerous lesions. Additionally, investigations on colon carcinogenesis triggered by DMH in rodent models reveal details on the molecular, biochemical, and histological mechanisms underlying various stages of colon carcinogenesis. DMH enters the body through ingestion, where it passes through a number of metabolic processes and eventually reaches the colon as a carcinogen. There, it releases reactive oxygen species (ROS), which induce alkylate DNA and trigger the development of CRC[11,12].

Oxidative stress is summarized as a state when there is an unequal balance between the generation of ROS and the ability of a biological system to protect against the impacts of reactive free radicals or repair oxidative damage[13]. Elevated ROS levels also deteriorate the antioxidant defense system, leading to damaged DNA, lipids, and protein[14]. Oxidative damage may be initiated by the reduced efficacy of the antioxidant defense system[15]. The extramitochondrial NAD(P)H oxidase (Nox) system or mitochondria can produce ROS, including hydroxyl radicals, peroxides, and superoxides[16,17]. Various factors, for instance, ROS, oxidative stress, and reactive nitrogen species (RNS), can cause chronic inflammation, which is well-established as a crucial factor in 15-20% of cancers, including CRC[18].

Multiple studies[19,20] have demonstrated that inflammation leads to chromosomal instability, increased growth of cancer cells, restructuring of tissues, and promotion of angiogenesis. Oxidative stress induces the stimulation of nuclear factor kappa B (NF-κB), which worsens inflammation by blocking the activity of anti-inflammatory interleukins, nuclear factor erythroid 2-related factor 2 (*Nrf2*), and peroxisome-activated receptors (PPARs)[21-24]. *Nrf2* is a transcription factor that becomes activated upon encountering cellular stress[25]. In accordance with the cellular context and environment, it can either contribute to cancer progression or prevention. *Nrf2* acts as a key protection mechanism against radiation and chemicals that can disrupt DNA integrity and initiate carcinogenesis[26]. Furthermore, it was presently investigated as a novel objective for CRC chemoprevention[27]. *Nrf2* is expressed in all cell types; however, it is concealed in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1). The process of transcribing target genes that encode proteins participating in redox regulation, protein homeostasis, iron metabolism, apoptosis resistance, xenobiotic effux, and DNA repair is stimulated during elevated cellular stress, and *Nrf2* translocates into the nucleus[28]. Therefore, it is thought that *Nrf2* promotes several anti-inflammatory effects, including inhibiting NF-κB and reducing the expression of several inflammatory mediators[29-31].

The *Nrf2*/*Keap1* pathway is a key controller of cellular defense response to exogenous and endogenous stresses resulting from electrophiles and ROS. It also protects cells from inflammation and oxidative stress[32,33]. *Nrf2* has been reported to regulate irritable bowel disease *via* facilitating redox regulation and reducing inflammation and tissue damage[33-35]. Consequently, attention attracting in the ROS scavenging impact of phytochemicals and the capacity to eliminate carcinogens through the activation *Nrf2* signaling[36].

Irinotecan (camptothecin-11, CPT-11) is a cytotoxic drug approved for CRC treatment[37]. High doses of CPT-11 induce gastrointestinal toxicity (diarrhea, vomiting, nausea, and abdominal cramps) and hematological toxicity (neutropenia)[38]. Furthermore, CPT-11 induces a high level of oxidative stress in cells, resulting in cellular dysfunction and tissue injury[39].

There are extensive investigations on boosting the efficiency of a medication while minimizing its adverse effects. One of these studies[39] revealed that enhancing the therapeutic effectiveness of CPT-11 by combining it with other agents is critical in the treatment of CRC to prevail CRC resistance to a single treatment and reduce toxic side effects. One example is therapy with CPT-11 in combination with flavonoids, such as quercetin[40].

Flavonoids are prominent phytoconstituents of vegetables, fruits, wine, and tea. They possess diverse pharmacological properties and are beneficial to health[41]. Furthermore, they can potentially act as both chemopreventive and chemotherapeutic agents[42]. Diosmin (DIO) is a flavonoid phytochemical compound that possesses antioxidant, antiangiogenic activities, and anti-inflammatory[43-45] and has recently been extensively studied for additional beneficial effects, such as anticancer activity and treating premenstrual syndrome, diabetes, and colitis[46].

DIO exhibits anticancer and chemopreventive properties in various *in vitro* models in multiple types of cancers, including colon[47,48] and prostate[49]. Additionally, the combined treatment of DIO and interferon-alpha (IFN-α) is a novel therapeutic regimen used to treat metastatic pulmonary melanoma[50]. Accordingly, our study evaluated the anticarcinogenic effects of DIO+CPT-11 *vs.* CPT-11 alone on CRC induced by DMH in rats. It also investigated the potential role of the antioxidant defense system, NF-κB, *Nrf2*, and apoptosis in the mechanisms participating in the anticarcinogenic effect of DIO, CPT-11, and their combination.

Materials and Methods

Animals and Housing

Adult male Wistar rats (90-120 g) were purchased from the Animal House of the Egyptian Holding Company for Biological Products and Vaccines (VACSERA, Animal House Facilities, Helwan, Egypt). The animals were exposed to a 2-week period of observation to allow for acclimatization before beginning the experiment and housed in standard cages under a controlled 12 h light/dark cycle at 60%±10% humidity and a temperature of 25°C±2°C. The animals were provided with standardized daily diet and access to water *ad libitum*.

The Experimental Animal Ethics Committee of the Faculty of Science for the Care and Use of Animals at Beni-Suef University in Egypt approved the research protocol and all experimental procedures (approval number: BSU/FS/2018/12). All precautions were implemented to ensure the utilization of the least feasible quantity of animals and to mitigate their distress and unease.

Chemicals

DMH and DIO were obtained from Sigma Aldrich (St. Louis, MO, USA) and preserved at 2°C-4°C. Irinotecan hydrochloride (CPT-11, Campto injection) was provided by Pfizer (Perth) Pty Ltd. (Bentley, Australia). Ki-67, NF-κB and p53 primary antibodies were purchased from ABclonal Technology (Wuhan, China). All extra chemicals utilized in the experimentation and assays were of analytical grade.

Experimental Design

The present study divided 50 adult male Wistar rats into five groups (n=10) (Figure 1). Rats in Group I (Normal) received vehicles only, namely, saline (0.9%, orally) once a week for 14 weeks, carboxymethylcellulose (CMC; 1% w/v, orally) every other day, and intraperitoneal injection of saline (0.9%) twice a week for the final 10 weeks. Rats in Group II (DMH) received DMH orally (20 mg/kg/week) dissolved in 0.9% saline for 14 weeks)[51], along with 1% CMC every other day for the final 10 weeks. Rats in Group III (DMH+DIO) received DMH orally for 14 weeks and DIO (10 mg/kg, suspended in 1% CMC)[52] every other day for the final 10 weeks. Rats in Group IV (DMH+CPT-11) received DMH orally for 14 weeks and intraperitoneal injection of CPT-11 (3 mg/kg)[53] twice a week for the final 10 weeks. Rats in Group V (DMH+DIO+CPT-11) received DMH orally for 14 weeks and both DIO and CPT-11 (as per the dosages described above).

Blood and Colon Tissue Sampling

At the end of the experiment, the rats were euthanized under anesthesia using diethyl ether, and blood samples were collected. For the purpose of analyzing tumors and oxidative stress biomarkers and tumors, serum was separated from the blood samples. The colon was excised and cleansed with a cold saline solution. Three portions (3 mm^3) were cut from each colon. The first portion was fixed in 10% neutral buffered formalin for 24 h prior to sectioning for histopathological analysis

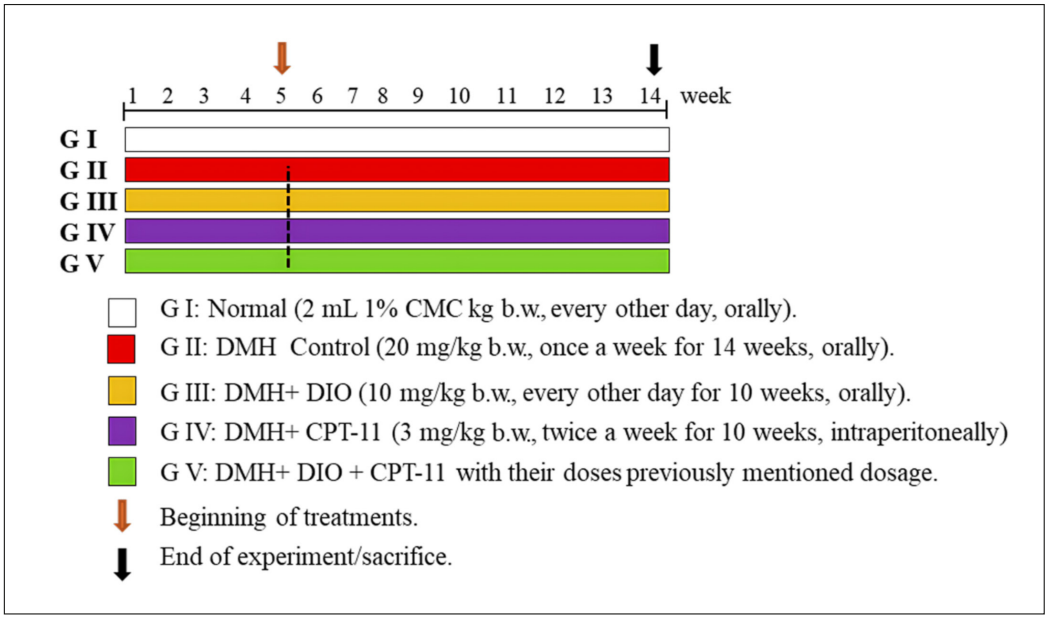

Figure 1. Schematic figure of experimental design and categorization of animals.

and immunohistochemical detection of Ki-67, NF-κB, and p53. The second portion was homogenized in phosphate buffer saline at 25% w/v and then centrifuged at 3,000 rpm for 15 min at -4°C. The supernatants were maintained and retained at -80°C for consecutive analysis of colon glutathione reductase (GR) and glutathione-S-transferase (GST). The third portion was stored at -80°C until RNA isolation for the identification of *Nrf*2 by reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Assessment of Serum Carcinoembryonic Antigen (CEA) Levels

The serum level CEA levels were determined using ELISA kits (R&D Systems, Minneapolis, MN, USA) as per the manufacturer's instructions.

Assessment of Oxidative Stress Biomarkers

The serum levels of malondialdehyde (MDA) – an indicator of lipid peroxidation (LPO) – and glutathione (GSH) were determined using the methodology of Preuss et al[54] and Beutler et al[55], respectively. Additionally, the colon tissue homogenates were assayed for GST and GR, which are enzymatic indicators of the antioxidant status, following the methods of Mannervik and Guthenberg[56] and Goldberg and Spooner[57], respectively.

RNA Isolation and RT-PCR Analysis

The total RNA was extracted from the colon tissue samples using the Chomczynski and Sacchi technique (1987)[58] utilizing a Qiagen tissue extraction kit from the United States to produce cDNA and carry out RT-PCR. A UV spectrophotometer (Photometer 5010, Robert Riele GmbH & Co KG, Berlin, Germany) was used to examine the quality and quantity of the resulting PCR products. Ratios ranging from 1.8 to 2.0 for A260/A280 are considered acceptable as an indication of pure RNA, and hence it was used in subsequent procedures. Subsequently, the isolated RNA was transformed into cDNA, and the cDNA was amplified using a My Taq One-Step RT-PCR Kit (Bioline, Meridian Bioscience, Memphis, TN, USA) along with particular primers (LGC Biosearch Technologies, Petaluma, CA, USA) (Table I). The PCR products obtained were examined by electrophoresis in a 1× Tris Borate EDTA buffer (pH 8.3-8.5) on a 1.5% agarose gel that was treated with ethidium bromide for staining. A gel documentation system was utilized to observe the electrophoretic pattern. The gene expression data were standardized relative to *β-actin*.

Table I. Primers used for RT-PCR.

Gene	GeneBank accession number	Sequence (5′–3′)
β-actin	NM_031144.3	F: AGGAGTACGATGAGTCCGGC R: CGCAGCTCAGTAACAGTCCG
Nrf2	NM_031789.2	F: TTGTAGATGACCATGAGTCGC R: TGTCCTGCTGTATGCTGCTT

F, forward; R, reverse; *Nrf2*, nuclear factor erythroid 2-related factor 2.

Histology and Immunohistochemistry

The colon samples from each rat were preserved in a 10% buffered formalin solution for 24 hours. After that, they were dehydrated using a sequence of increasing alcohol concentrations, cleared with xylene, and finally embedded in paraffin wax. Next, the embedded sections were cut into sections that were 4 μm thick using a sled microtome. These sections were then treated with hematoxylin and eosin (H&E) stain for observation under a microscope. Subsequently, the colon samples that were embedded in paraffin were cut into sections that were 5 μm thick. These sections were then placed on slides with a positive charge (Thermo Fisher Scientific, Pittsburgh, PA, USA) and subjected to immunostaining, as previously described[59]. Overall, after deparaffinization, rehydration, antigen retrieval, and sealing, the sections were incubated in a 3% H_2O_2 solution for 15 minutes. Subsequently, the samples were obstructed and cultured with Ki-67, NF-κB, and p53 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:200 and kept at a temperature of 4°C overnight. Following the washing step with phosphate-buffered saline, the sections treated with the peroxidase-labeled secondary antibody (diluted 1:200) were left to incubate for a duration of 30 minutes. The reaction with the 3,3-diaminobenzidine substrate allowed for the visualization of the bound antibody complex and hematoxylin was used to counterstain the slides (ABclonal Inc., Wuhan, China). The immunohistochemically stained sections were subsequently examined at high power (×400) using light microscopy. A brown color indicated a positive reaction. We used ImageJ 1.54d (http://imagej.org; Wayne Rasband and Contributors, National Institutes of Health, Bethesda, MD, USA) to assess the integrated positive reaction intensities to measure the intensities of the Ki-67 and p53 positive reactions.

Statistical Analysis

The obtained data were statistically analyzed using SPSS v. 20 (IBM Corp., Armonk, NY, USA). The results were displayed as the mean ± standard error of the mean (SEM). Statistical comparisons were carried out using one-way analysis of variance. The level of significance was determined at $p<0.05$.

Results

Treatment Effects on Serum CEA Levels

DMH administration significantly increased serum CEA when compared with the rats in the Normal group ($p<0.05$). Furthermore, the treatment of DMH-administered rats with DIO, CPT-11, and DIO+CPT-11 significantly improved serum CEA levels in comparison to the DMH-administered group ($p<0.05$) (Table II).

Treatment Effects on Colon Oxidative Stress and Antioxidant Defense System

The DMH-administered group exhibited a significant increase in serum LPO ($p<0.05$) and a significant decrease in GSH content and activ-

Table II. Effects of CPT-11 and DIO on serum CEA levels of DMH-administered rats.

Parameter	Normal	DMH	DMH+DIO	DMH+CPT-11	DMH+DIO+CPT-11
CEA (ng/ml)	1.95 ± 0.07^{a}	11.51 ± 0.31^{d}	3.62 ± 0.22^{b}	5.70 ± 0.23^{c}	3.56 ± 0.42^{b}

The data are presented as mean ± SEM (n=6). Means with distinct superscript symbols (^{a}, ^{b}, ^{c}, and ^{d}) exhibit significant differences with $p<0.05$. DMH; 1,2-dimethylhydrazine. CEA, carcinoembryonic antigen; DIO, diosmin; CPT-11, irinotecan hydrochloride.

Table III. Effects of CPT-11 and DIO on serum MDA and GSH levels and colon GST and GR activities in DMH-administered rats.

Parameter	Normal	DMH	DMH+DIO	DMH+CPT-11	DMH+DIO+ CPT-11
MDA (nM/100 ml/h)	6.04±0.66^{a}	17.26±0.23^{c}	7.00±0.54^{ab}	9.43±1.50^{b}	8.46±0.63^{ab}
GSH (nM/100 ml)	25.90±1.69^{bc}	15.93±0.45^{a}	22.92±1.04^{b}	21.55±1.23^{b}	30.10±3.07^{c}
GST (U/100 mg tissue)	951.08±12.74^{d}	707.07±26.87^{a}	925.52±18.69^{cd}	831.75±8.70^{b}	887.68±7.48^{c}
GR (mU/100 mg tissue)	115.91±2.07^{b}	56.34±8.88^{a}	119.58±7.63^{b}	110.12±11.11^{b}	125.50±11.17^{b}

The data are presented as mean ± SEM (n=6). Means with distinct superscript symbols (^{a}, ^{b}, ^{c}, and ^{d}) exhibit significant differences with $p<0.05$. DMH, 1,2-dimethylhydrazine; GR, glutathione reductase; GSH, glutathione; GST, glutathione-S-transferase; MDA, malondialdehyde; DIO, diosmin; CPT-11, irinotecan hydrochloride.

ities of GST and GR when compared with the rats in the Normal group. On the other hand, serum LPO ($p<0.05$) of rat groups treated with DIO, CPT-11, and DIO+CPT-11 were significantly decreased while significantly increasing GSH content and the activities of GST and GR when compared with the DMH-administered group (Table III).

Treatment Effects on Nrf2

DMH-administered rats showed significant downregulation of *Nrf2* mRNA expression in comparison to the Normal group ($p<0.05$). The mRNA expression of *Nrf2* was considerably elevated in rats treated with DIO, CPT-11, and DIO+CPT-11 after DMH administration ($p<0.05$) (Figure 2).

Histopathological Results

Light microscopy examination of H&E-stained colon sections displayed that the Normal group (Figure 3A) had nearly normal histological layers from the mucosa through to the submucosa and musculosa. Meanwhile, colon sections from DMH-administered rats (Figure 3B-C) showed polypoid hyperplasia with mitotic figures and hyperplasia of tumor cells between the glandular acini, infiltration of the connective tissue of the submucosa by tumor cells, and goblet cell hyperplasia. However, the sections of the colon from DMH-administered rats treated with DIO exhibited moderate hyperplasia (Figure 3D). The colon sections from DMH-administered rats treated with the CPT-11-treated group (Figure 3E) showed nearly normal villi except for mild

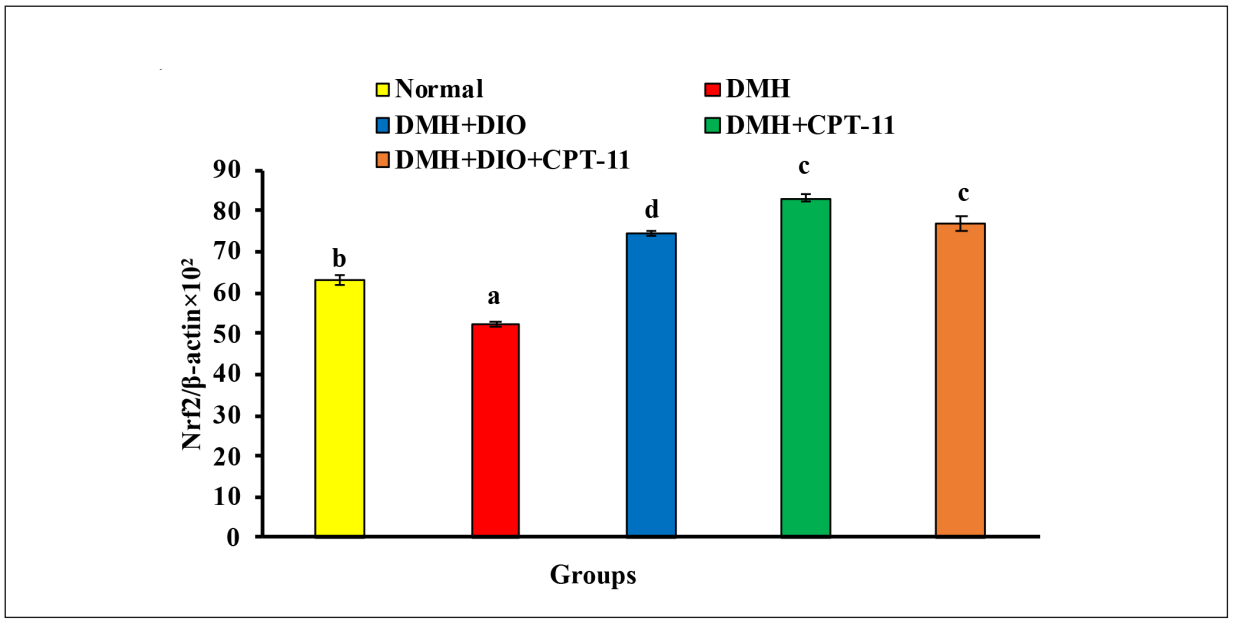

Figure 2. Effect of DIO and CPT-11 on colon mRNA *Nrf2* expression relative to mRNA *β-actin* expression in DMH-administered rats. Means with distinct superscript symbols (^{a}, ^{b}, ^{c}, and ^{d}) exhibit significant differences with $p<0.05$.

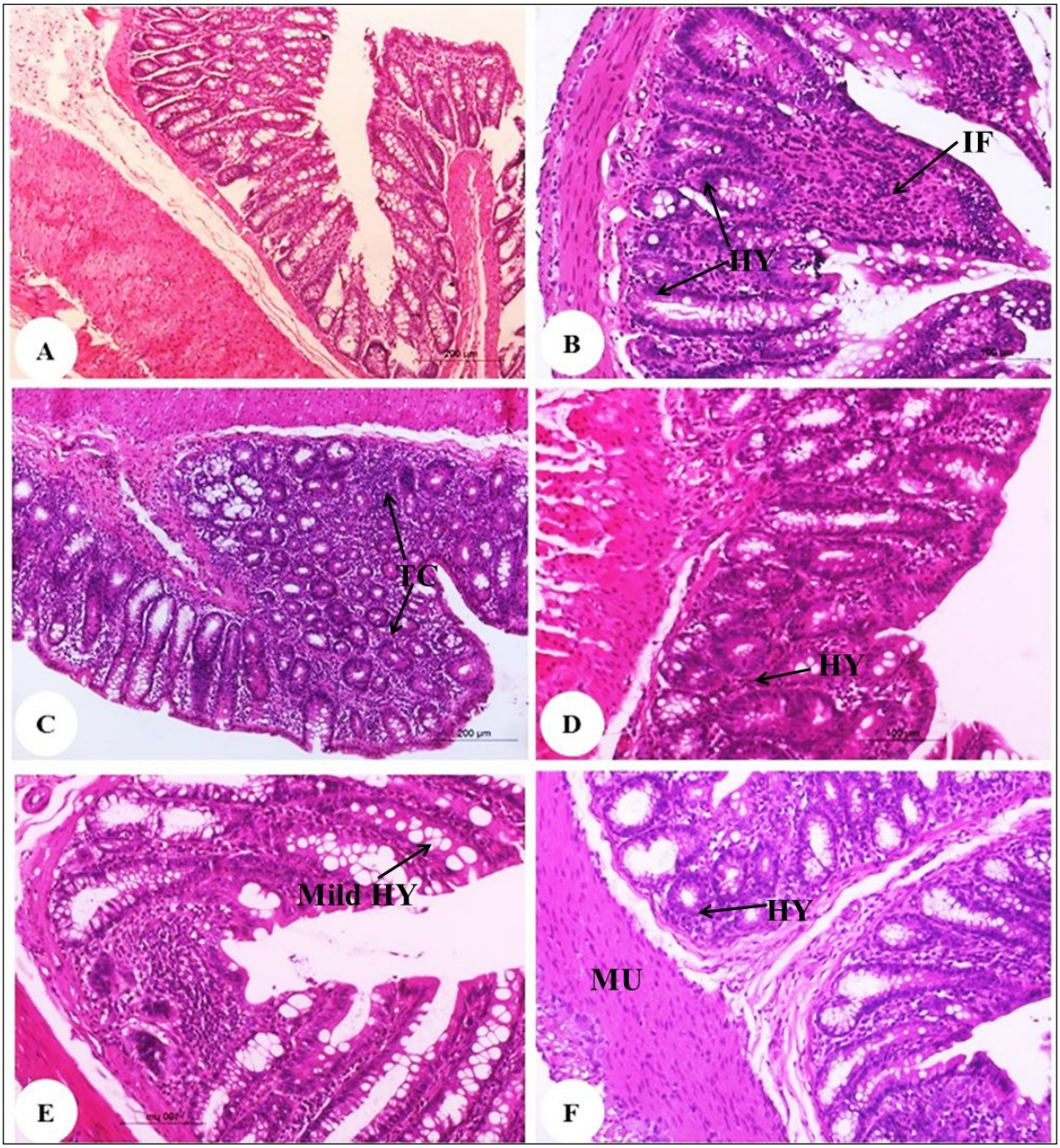

Figure 3. Photomicrographs of colon sections of the different groups stained using H&E (n=6). **A**, Colon tissue from the rats in the Normal group demonstrated nearly normal histological layers from the mucosa through to the submucosa and musculosa (magnification: ×100). **B**, Colon from the cancer-induced rats (DMH-administered group) exhibited marked changes, including polypoid hyperplasia with mitotic figures (magnification: ×200). **C**, Colon tissue from the cancer-induced rats also presented hyperplasia of the tumor cells between the glandular acini, the connective tissue of the submucosa shows tumor cell infiltration and goblet cell hyperplasia is evident (magnification: ×100). **D**, Photomicrograph of a colon section from a cancer-induced rat treated with DIO demonstrated moderate hyperplasia (magnification: ×200). **E**, Photomicrograph of a section of a cancer-induced rat treated with CPT-11 displayed nearly normal villi except for mild hyperplasia (magnification: ×200). **F**, Photomicrograph of a colon section from a cancer-induced rat treated with DIO+CPT-11 revealed mild polypoid hyperplasia, musculosa, as well as submucosa, are free from tumor (magnification: ×200). HY, hyperplasia; IF, infiltration; TC, tumor cells; MU, musculosa.

hyperplasia. Moreover, colon sections from the DMH-administered rats supplemented with DIO+CPT-11 (Figure 3F) showed mild polypoid hyperplasia and no tumors in the musculosa or submucosa.

Immunohistochemical Results

Effect on proliferation marker Ki-67

Ki-67 immunostaining of glandular cells of the mucosa layer showed a moderate positive

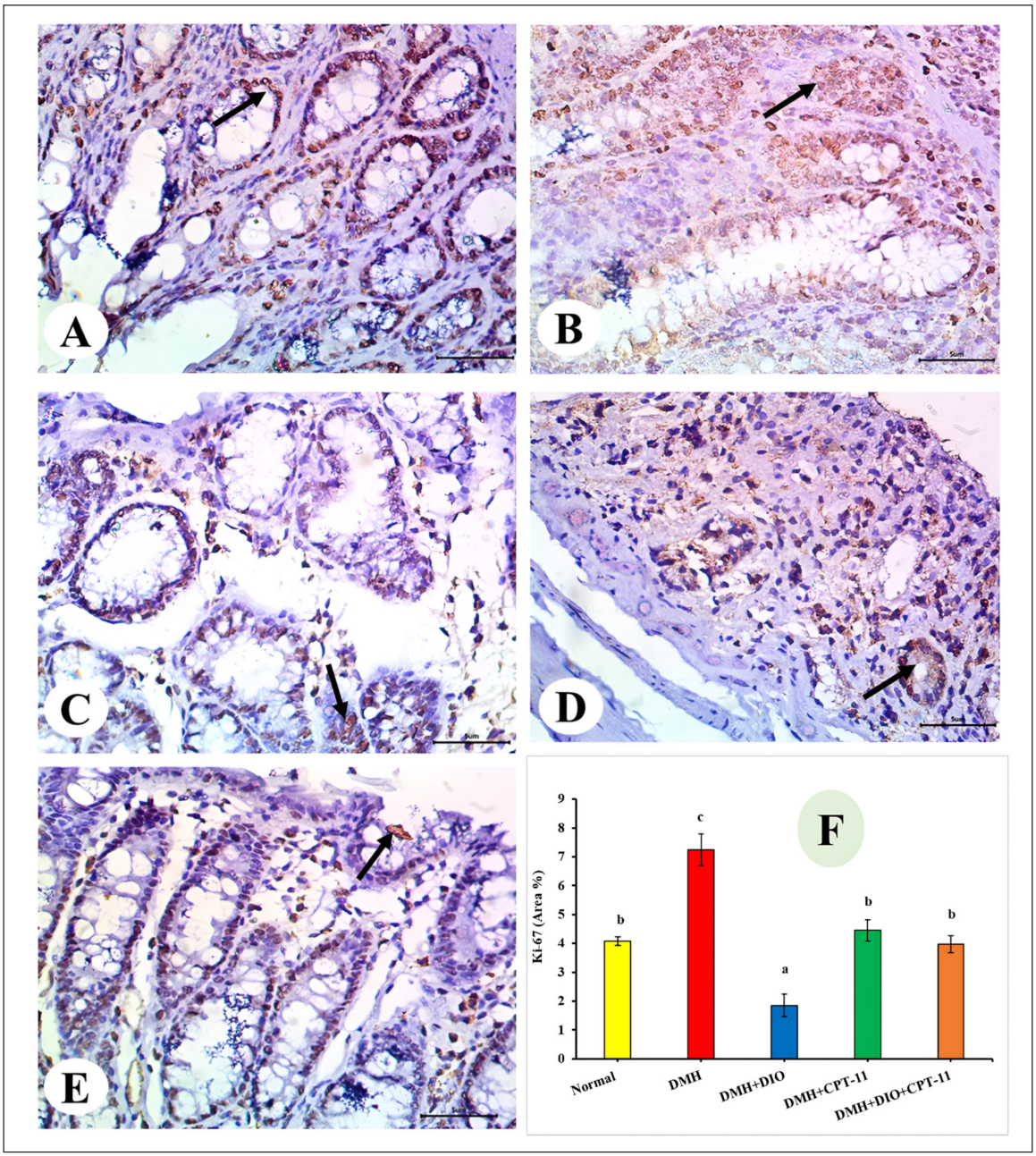

Figure 4. Photomicrographs of colon sections immunostained with Ki-67 from various groups (magnification: ×400) (n=4). **A**, The colon tissue of rats in the Normal group showed the typical anti-Ki-67 reaction of mucosal gland cells. **B**, Colon tissue section from the cancer-induced rats presented a substantial increase in anti-Ki-67 reaction in the cells of mucosal glands. **C**, The colon tissue of cancer-induced rats treated with DIO revealed a significant improvement in the anti-Ki-67 reaction in mucosal gland cells. **D-E**, Photomicrographs of a section of colon tissue from a cancer-induced rat treated with CPT-11 only (**D**) and DIO+CPT-11 (**E**) exhibited moderate improvement in the anti-Ki-67 reaction in mucosal gland cells. **F**, Image and statistical analysis indicated a significant increase in Ki-67 expression in DMH-administered rats and subsequent treatment with DIO, CPT-11, and DIO+CPT-11 resulted in a significant decrease. Means with different superscript letters (^{a}, ^{b} and ^{c}) indicate significant differences ($p<0.05$).

reaction in the colon tissue of rats in the Normal group (Figure 4A). There was a strong expression of Ki-67 in mucosal glands in the colon samples from the rats in the cancer-induced (DMH-administered) group (Figure 4B). A marked reduction in Ki-67 positive reactions in the glandular cells of the mucosa layer was seen after treatment with DIO (Figure 4C), CPT-11 (Figure 4D), or DIO+CPT-11 (Figure 4E). While administration of CPT-11 alone or DIO+CPT-11 produced moderate expression of Ki-67 in the glandular cells of the mucosa layer (Figure 4D-E), administration of DIO induced mild expression (Figure 4C). Immunohistochemical analysis demonstrated that DMH-administered rats showed a significant increase in expression of Ki-67 ($p<0.05$). The treatment with DIO, CPT-11, and DIO+CPT-11 significantly reduced the expression of Ki-67 in the colon cells in DMH-administered rats ($p<0.05$) (Figure 4F).

Effect on proapoptotic factor p53

Immunohistochemical staining of colon tissue from rats in the Normal group showed a moderate p53-positive reaction in glandular cells of the mucosa layer (Figure 5A). In the cancer-induced group (DMH-administered group), we observed a substantial increase in p53 positivity in the mucosal glands (Figure 5B). Subsequent treatment with DIO (Figure 5C), CPT-11 (Figure 5D), and DIO+CPT-11 (Figure 5E) remarkably decreased the expression of p53. Figure 5F revealed a significant elevation ($p<0.05$) in p53 in the colon mucosal glands of the DMH-administered group and subsequent treatment with DIO, CPT-11, and DIO+CPT-11 led to a significant decrease ($p<0.05$).

Effect on inflammatory marker NF-κB

The colon sections from the rats in the Normal control group had typical histological structure (Figure 6A), but colon sections from the DMH-administered group showed intense expression of NF-κB in mucosal glands in the colon samples (Figure 6B). DIO (Figure 6C), CPT-11 (Figure 6D), or DIO+CPT-11 (Figure 6E) treatment resulted in a significant decrease in NF-κB positive reactions in the glandular cells of the mucosa layer. While CPT-11 alone or in combination with DIO provoked significant expression of NF-κB in the glandular cells of the mucosa layer (Figure 6D-E), DIO produced very mild expression (Figure 6C). Immunohistochemical assessment revealed that DMH-treated rats had a substantial increase in expression of NF-κB ($p<0.05$). The treatment with DIO, CPT-11, and DIO+CPT-11 significantly reduced the expression of NF-κB in the colon cells in DMH-administered rats ($p<0.05$) (Figure 6F).

Discussion

CRC is the third most frequent disease worldwide, with 1.1 million new cases diagnosed each year and the second largest cause of cancer death[60]. Although novel drugs are accessible, systemic therapy remains the preferred treatment for almost 25% of patients with metastatic disease[61]. Due to the cytotoxicity and development of agent resistance, chemotherapeutic therapy of CRC is problematic[62]; it is crucial to research and generate novel substances with anticancer activity and low toxicity.

We studied the impact of the flavanone DIO on a rat model of CC induced by DMH. DIO was previously proved to exert a potential anticancer effect by inducing apoptosis, inhibiting cell proliferation, and suppressing inflammatory responses and oxidative stress[63,64]. The combination of DIO with different chemotherapeutics can enhance their therapeutic effectiveness by diminishing drug resistance and functioning as a chemosensitizer. Furthermore, DIO has shown anticarcinogenic activity in a variety of cancers, including CRC[42,65-67]. Considering this information, we assessed the capability of DIO to enhance the effectiveness of CPT-11 against adverse histological and molecular changes in DMH-induced CRC in rats.

Tumor markers can be used as preventive screening approaches and are frequently utilized for early cancer detection[68]. For instance, CEA is the best marker for CRC diagnosis and as well as the tumor-associated antigen with the best clinical and analytical characterization[69,70]. Benign adenomas do not increase serum CEA levels, making it the most useful tumor marker for distinguishing benign from invasive colon carcinomas[70]. In line with previous research[71,72], the serum levels of CEA were substantially elevated in DMH-administered rats compared with the Normal group, owing to increased CEA production by malignant cells. Another study[73] showed that the level of CEA has increased in colon cancer. The administration of DIO alone, CPT-11 alone, and DIO+CPT-11 showed a successful

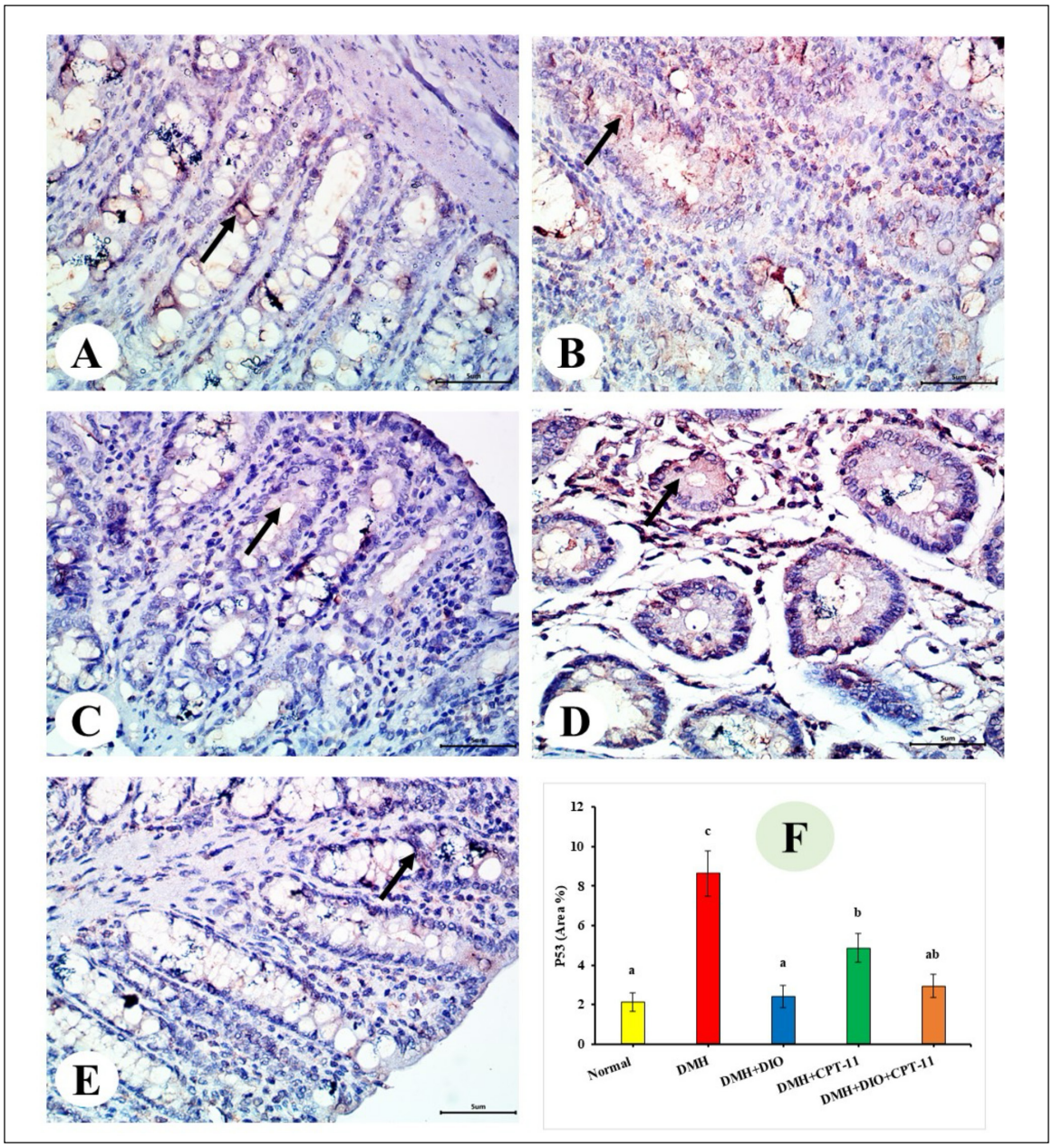

Figure 5. Photomicrographs of colon sections from various groups immunostained with p53 (magnification: ×400) (n=4). **A**, Colon tissue section from rats in the Normal group showed the typical anti-p53 reaction in the cells of mucosal glands. **B**, Colon tissue section from the cancer-induced rats presented a substantial elevation in anti-p53 reaction in the cells of mucosal glands. **C**, Colon tissue section of cancer-induced rats treated with DIO exhibited an obvious improvement in anti-p53 reaction in the cells of mucosal glands. **D**, A photomicrograph of a section of colon tissue from a cancer-induced rat treated with CPT-11 demonstrated mild improvement in anti-p53 reaction in the cells of mucosal glands. **E**, A section of colon tissue from a cancer-induced rat treated with DIO+CPT-11 revealed significant improvement in anti-p53 reaction in the cells of mucosal glands. **F**, Changes indicated a significant increase in p53 expression in DMH-administered rats and subsequent treatment with DIO, CPT-11 and DIO+CPT-11 resulted in a significant decrease. Means with different superscript letters (^{a}, ^{b} and ^{c}) indicate significant differences ($p<0.05$).

improvement in CEA levels compared with the rats administered DMH alone. Similar findings have shown that DIO and CPT-11 treatment reduced the serum levels of CEA[74]. In contrast to the result of the present study, it was reported that irinotecan-based chemotherapy has the potential to cause a CEA spike[75,76]. In this regard, it was indicated that early CEA elevation following

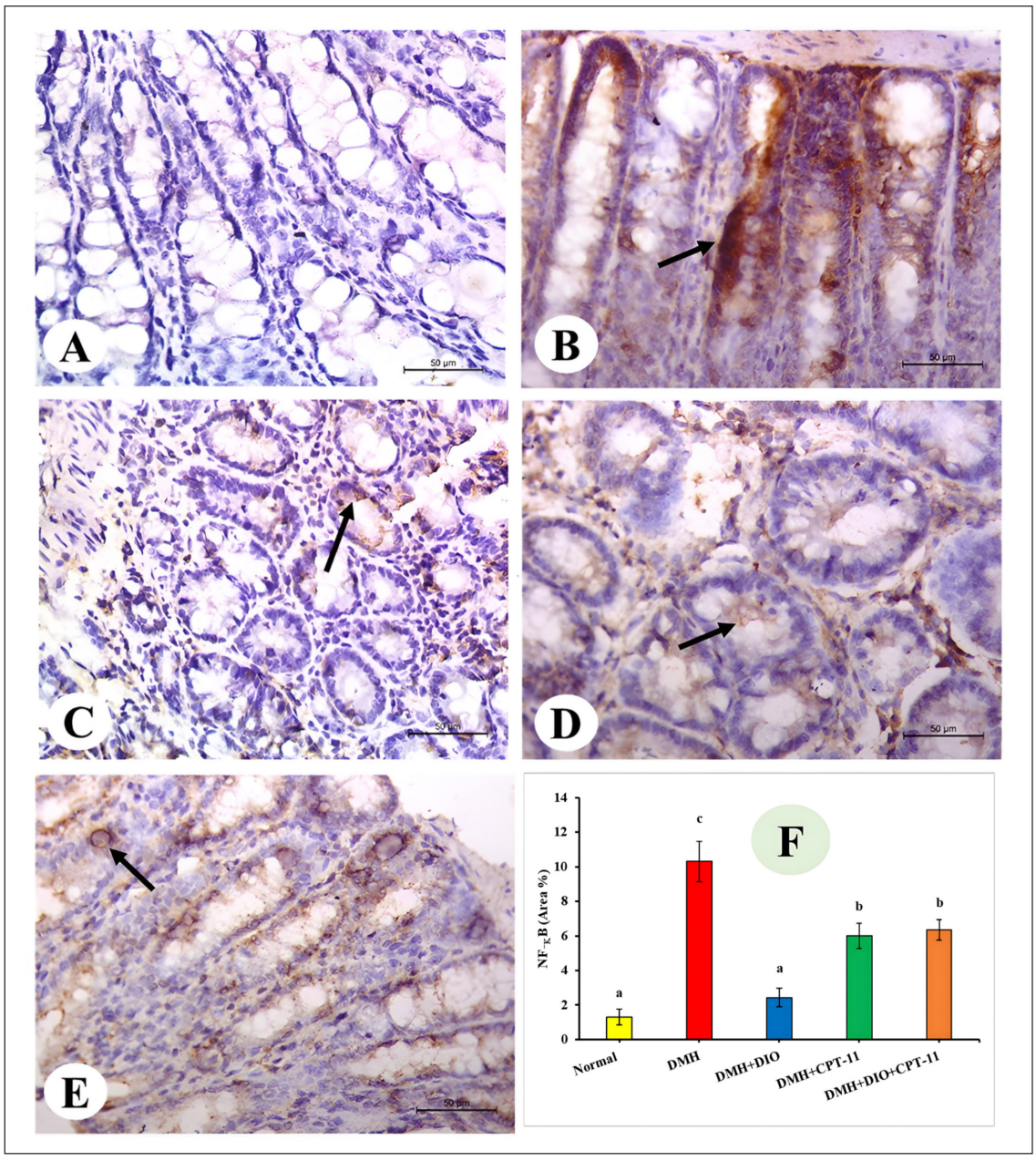

Figure 6. Photomicrographs of colon sections immunostained with NF-κB from various groups (magnification: ×400) (n=4). **A**, The colon tissue of rats in the Normal group showed the typical anti-NF-κB reaction of mucosal gland cells. **B**, Colon tissue section from the cancer-induced rats presented a substantial increase in anti- NF-κB reaction in the cells of mucosal glands. **C**, the colon tissue of cancer-induced rat treated with DIO displayed a significant improvement in the anti-NF-κB reaction in mucosal gland cells. **D-E**, Photomicrographs of a section of colon tissue from a cancer-induced rat treated with CPT-11 only (**D**) and DIO+CPT-11 (**E**) exhibited moderate improvement in the anti-NF-κB reaction in mucosal gland cells. **F**, Image and statistical analysis indicated a significant increase in NF-κB expression in DMH-administered rats and subsequent treatment with DIO, CPT-11 and DIO+CPT-11 resulted in a significant decrease. Means with different superscript letters (^{a}, ^{b} and ^{c}) indicate significant differences ($p<0.05$).

irinotecan-based chemotherapy is not typically a sign of disease progression or therapy failure and should not necessitate switching to another chemotherapy regimen[75,76].

These increases in CEA serum levels were supported by the histopathological findings in the colon tissue sections of rats in the DMH-treated group. We observed the development of hyper-

plastic lesions, hyperchromatic staining, ulceration, and erosion, as well as dense lymphocytic infiltration in the submucosal layer of colon tissue and mitotic figures, confirming CRC induction. Similar results have been reported[77,78]. However, the pathological alterations in our study were restored by treatment with DIO more than treatment with CPT-11 or DIO+CPT-11 since rats treated with DIO showed decreased hyperplasia, dysplasia, mucosal ulceration, and mild hyperplasia.

DMH is a procarcinogen. After undergoing metabolic activation, it forms methyl free radicals that can cause oxidative stress. In the presence of metal ions, DMH also generates hydroxyl radicals or hydrogen peroxide, which may contribute to LPO initiation[79,80]. Elevated ROS generation and LPO have been assessed in the blood, serum, liver, and colon of DMH-administered rats[81,82]. These previous findings correlate with the observations in our study of remarkably elevated levels of serum ROS and MDA in response to DMH administration.

Additionally, our results reported that DIO reduced the level of MDA in DMH-administered rats. Generally, flavonoids contain a number of hydroxyl groups (DIO contains eight hydroxyl groups). It has been suggested that the quantity of hydroxyl groups substituted on ring B directly correlates with free radical scavenging activity[83]. Thus, DIO exhibited protective efficacy against LPO in serum, as mentioned in a previous study[84].

GSH is a natural thiol that demonstrates both anti-carcinogenic and antioxidant characteristics[85]. In our study, DMH significantly decreased the level of serum GSH. Therefore, the deficiency of circulating GSH observed in the DMH-administrated rats in our study may be caused by the increased use of GSH to prevent LPO. The previous findings are in line with the findings of this study, which revealed that a drop in the serum level of GSH correlates with our observation of a significant reduction in colon GST and GR activity[86,87].

We found that the treated groups (DIO, CPT-11, or DIO+CPT-11) exhibited a significant increase in antioxidant levels compared with the DMH-administered group. GSH content was significantly higher in the DIO+CPT-11- administrated group than in the DIO- administrated group or the CPT-11-treated group. Furthermore, GST activity increased considerably in the DIO-treated group than in the DIO+CPT-11-treated group or the CPT-11-treated group. However, when it came to GR activity, all treated groups increased, but there were no statistically significant differences between them. Treatment with DIO and/or CPT-11 resulted in the enhancement of the antioxidant-defense systems in the rats that were administrated with DMH. This improvement was linked to the restoration of the colon's histological features to almost normal levels and the absence of cancer cells. Consequently, we proposed the inhibition of oxidative stress and the improvement of the antioxidant defense system play an essential role in generating the anticarcinogenic effects of DIO or/and CPT-11 in DMH-induced colon carcinogenesis in Wistar rats (Figure 7).

Nrf2 is crucial in shielding cells from damage and preventing different diseases, including cancer. Keap1 regulates *Nrf2* production and prevents its cytoplasmic depletion. Therefore, the *Nrf2*/*Keap1* pathway is vital to maintaining homeostasis in cells[88]. Our study revealed a significant downregulation in *Nrf2* expression in the DMH-administered rats in comparison to the Normal group, whereas the CPT-11-treated group exhibited significant upregulation of *Nrf2* expression compared with the DMH group.

Nrf2 is downregulated in a variety of cancer types and DMH-administered rats, and most reported therapeutic modalities promote *Nrf2* and its target antioxidants[89-92]. Similarly, *Nrf2* downregulation by DMH administration has been reported in mice[93]. Extreme and persistent ROS generation frequently results in the downregulation of *Nrf2* signaling[94].

The NF-κB signaling pathway regulates genes both inside and outside the immune system. As such, it plays an important role in the carcinogenic process. As a result, it could impact a variety of diseases, including CRC[95]. Consequently, it serves as a molecular target for the therapy of numerous malignancies, including those of the bladder, colon, kidney, and pancreatic[96-99].

NF-κB has been claimed to act antagonistically on *Nrf2*[100]. In our study, NF-κB expression in the DMH-induced group was significantly increased compared with the Normal group, which was in accordance with previous studies[101,102]. In line with another study[103], we found that DIO supplementation decreased NF-κB expression. Subsequently, we investigated the role of p53 in order to comprehend the signaling mechanism associated with the chemotherapeutic effectiveness of DIO+CPT-11 in the context of colon carcinogenesis. There is a claim that mutations in the

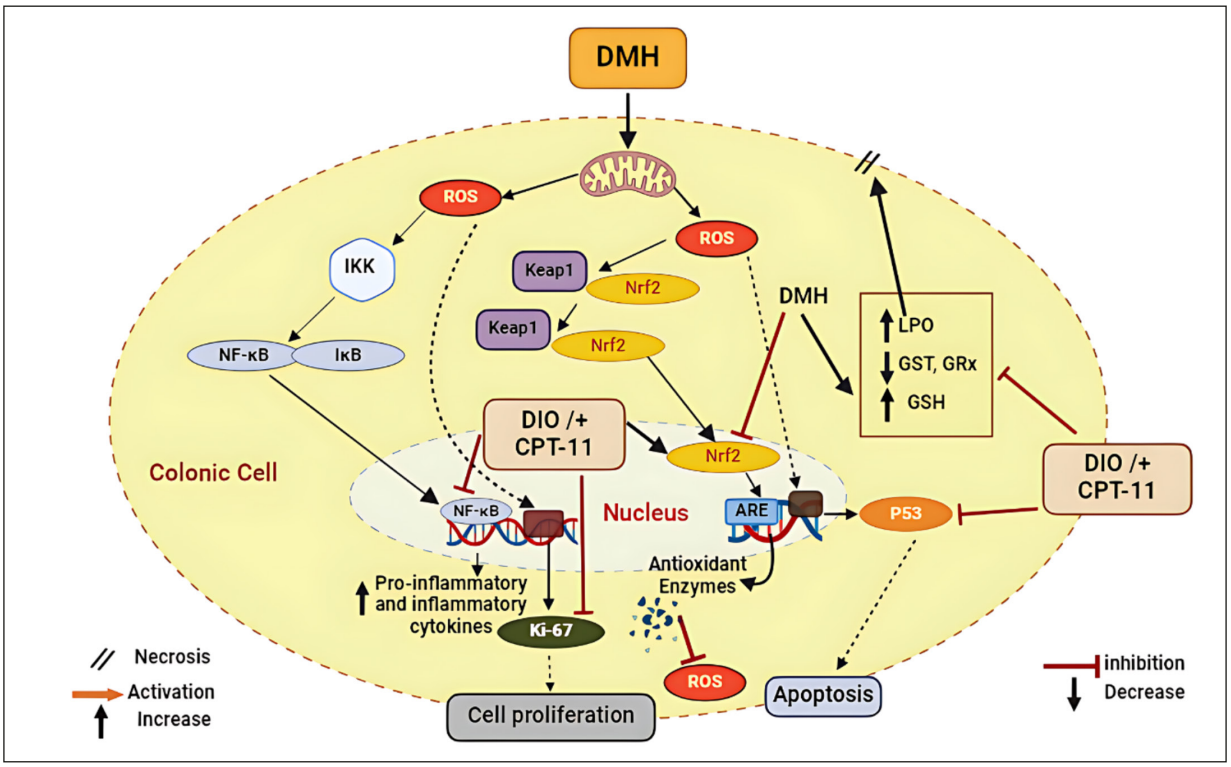

Figure 7. Schematic figure showing the effects of DIO and irinotecan (CPT-11) on oxidative stress, antioxidant defense system, *Nrf2*, cell proliferation, inflammation, and apoptosis.

p53 gene result in the development of malignant growths, which is a characteristic of the tumor suppressor pathway (Figure 7)[104]. Inducing *p53* expression reduces the number of cells by promoting apoptosis[105].

Apoptosis is an active form of mitochondrial-mediated programmed cell death that involves cell death receptors. Meanwhile, DMH administration caused an imbalance in the expression of Bcl2 family members that are pro- and antiapoptotic. This imbalance might result in ROS generation and consequent activation of *p53* gene expression[106,107]. Accordingly, Dong et al[108] proved that the protein level of p53 was increased in CRC cells. We found that treatment with DIO and CPT-11 significantly decreased p53 expression, suggesting activation of the intrinsic apoptotic pathway in our rat CC model (Figure 7).

The aforementioned findings have been confirmed by the immunohistochemical analysis of nuclear antigen Ki-67, a nonhistone protein and a biomarker for cancer staging[109]. It is present in all cell cycle phases except for the resting phase (G0) and has a crucial function in cell proliferation[110]. Owing to its high sensitivity, the evaluation of cancer cell proliferation frequently relies on the utilization of Ki-67 expression[111,112]. Cell proliferation has been associated with elevated cancer risk[113]. Additionally, Ki-67 is frequently used in pathological investigations to evaluate cellular proliferation in various malignancies[114-116]. Although benign tumors may exhibit low levels of Ki-67, malignant lesions often display high amounts of this protein. Elevated Ki-67 levels are associated with distant metastasis and are indicative of a poor prognosis for patients. The current study revealed a significant increase in Ki-67 expression in rats treated with DMH alone compared to normal rats, which aligns with the findings reported by Alazzouni et al[17].

The DMH-administered rats in our investigation showed markedly enhanced Ki-67 protein expression, consistent with previous findings[118-120], demonstrating that Ki-67 expression is elevated in CRC. Furthermore, the treatments by DIO in DMH-administered rats used in this study induced a significant and effective reduction in the expression of Ki-67, indicating that it ultimately reduces carcinogenesis through its effects on antioxidant capacity and detoxification[64,103].

Conclusions

In conclusion, our study results revealed that DIO alone or in combination with CPT-11 exerted an inhibitory effect on precancerous pathological colorectal lesions induced by DMH administration. Based on our findings, it appears that reducing cell proliferation, oxidative stress and inflammation are important mechanisms for preventing CC. However, further studies are required to determine other mediators in the signaling pathways of apoptosis, inflammation and cell proliferation to scrutinize the complete profile for the mechanisms of actions of DIO and CPT-11 and their combination.

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Ethics Approval
The Experimental Animal Ethics Committee of the Faculty of Science for the Care and Use of Animals at Beni-Suef University in Egypt approved the research protocol and all experimental procedures (approval number: BSU/FS/2018/12).

Informed Consent
Not applicable.

Availability of Data and Materials
All data generated or analyzed during this study are included in the article.

Authors' Contributions
ASAA, and OMA proposed the research plan. ASAA, MAA, HIS, AMA and OMA managed the research work. KHM performed the experimental work and carried out the investigations and statistical analysis under supervision of ASAA, MAA, HIS, AMA and OMA. KHM, ASAA, and OMA wrote the original draft of the manuscript. KHM, ASAA, MAA, HIS, AMA and OMA revised the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest
The authors declare no conflict of interest.

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AI Disclosure
We confirm that we did not use artificial intelligence or assisted technologies in the production of the study. Only Figure 7 was generated with the aid of Biorender (www.biorender.com).

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