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Orlistat and Hydroxycitrate Ameliorate Colon Cancer in Rats: The Impact of Inflammatory Mediators

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Abstract Colon cancer (CC) is the third type of cancer worldwide. This study aimed to investigate the possible protective effects of orlistat and hydroxycitrate (HCA) against dimethylhydrazine (DMH) and high-fat diet (HFD)-induced CC in adult male rats. The rats were divided into ten groups including the normal group. The rats were treated with the DMH/HFD, orlistat (32 mg/kg), orlistat/DMH, orlistat/DMH/HFD, HCA (135 mg/kg), HCA/DMH, HCA/DMH/HFD, DMH (40 mg/kg), and HFD (20%) for 16 weeks. Administration of orlistat and HCA improved the measured markers of a colon tumor, inflammatory mediators, oxidative stress, as well as caspase-3 (an apoptotic marker) in the colon tissue compared with the CC group. Furthermore, orlistat and HCA corrected the histopathological lesions in the colon lining. These findings revealed that orlistat and HCA had a positive chemopreventive efficacy and could be viewed as a possible clinical solution for colon cancer.

Keywords: *colon cancer, orlistat; hydroxycitrate, high-fat diet, dimethylhydrazine*

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1. Introduction

Colon cancer (CC) is considered a common cancer worldwide [1]. Furthermore, treatment with traditional chemotherapeutic agents is typically ineffective, either due to the progression of drug resistance or severe systemic toxicity [2]. The Western way of life is strongly connected to colon cancer. The incidence of colon cancer is affected by lifestyle and dietary habits. Among dietary factors, a high fat and carbohydrate intake raises the risk of developing CC [3,4]. The risk of colorectal cancer may be associated with obesity [5].

One of the potent carcinogens that induce colon tumors is Dimethylhydrazine (DMH) [6]. The DMH as well as its azoxymethane (AOM) metabolite are considered carcinogens that develop DNA-reactive products when activated metabolically via N-oxidation and hydroxylation steps forming methyl-azoxy-methanol (MAM) [7,8].

Orlistat is a saturated lipstatin derivative that is a potent natural pancreatic lipase inhibitor isolated from *Streptomyces toxytricini*. However, due to its relative simplicity and stability, orlistat was preferred as an anti-obesity drug over lipstatin [9]. Orlistat has also been found to inhibit the fatty acid synthase (FAS) thioesterase domain, an enzyme that is involved in cancer cell proliferation but not in normal cells [10]. Another anti-obesity medication is HCA, which is a citric acid derivative found in many tropical plants, which grow in Sri Lanka and India as *Hibiscus subdariffa* and *Garcinia cambogia* [11]. The HCA inhibits lipogenesis, a mechanism in which carbohydrate is transformed to fat in the body [12]. The mechanism of the development of CC in obese people is still unknown. Previous research has shown that obesity causes inflammation and extracellular matrix remodeling [13]. Different cytokines induce the expression of IL-32, which increase the expression of IL-8, IL-6, IL-1, and TNF-α in various cell types by activating the classic pro-inflammatory mediators as nuclear factor B (NF-B) and p38 constituting angiogenesis and apoptosis [12-14].

Based on the foregoing, this study aimed to determine the potential chemoprotective effect of anti-obesity drugs including orlistat and hydroxycitrate on colon cancer induced in albino rats.

2. Materials and methods

2.1. Animals

We used male Wistar rats (200±20 g), obtained from the animal facility (El-Nile Co., Cairo, Egypt), and were housed two weeks before the experiment and during experimentation in the animal facility at the Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt. All experimental animals were housed in cages with food and water (*ad libitum*) in 12h/12h light/dark cycles.

2.2. Ethical Approval

The animal experiment was conducted following the National Research Council (US) Guide for the Care and Use of Laboratory Animals (2011) [15], and

approved by an independent ethics committee (No, bio._9med.research_0000009) at the Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt.

2.3. Chemicals

The DMH solution (Sigma-Aldrich Chemical Co., USA) was administrated to rats at a dose of 40 mg/kg as verified by our pilot study (data not shown). Orlistat (Sigma pharmaceutical industries agency, Quesna, Egypt) and Hydroxycitrate (puritan's pride, USA) were dissolved in normal saline and administered to rats orally at doses of 32 and 135 mg/kg/day, p.o., respectively. The previous rat doses were equivalent to the human clinical doses for obesity treatment [16,17] based on body surface area conversion ratios [18]. In the present study, we used the following ELISA kits: Cancer embryogenic antigen (CEA) (Roche Diagnostics Co., Basel, Switzerland); rat interleukin-6 (IL-6) (Promokine Co., Heidelberg, Germany); NRF2 transcription factor and rat TNF-α (Abcam UK); cancer antigen 19-9 (CA19-9) (Roche Diagnostics Co., Switzerland); nuclear factor κB P65 (NFκB-P65) (MY-Bio-Source Co., San Diego, USA); Caspase-3 (Biovision, San Francisco, USA).

2.4. Design of the Experiment

We used one hundred rats (10 groups, 10 rats in each group) as the following:

Group 1: Control group (normal rats,16 weeks).

Group 2: DMH (40 mg/kg i.p., two times/week/16 weeks).

Group 3: HFD (20%, supplied daily for 16 weeks).

Group 4: DMH and HFD in combination as in groups 2 & 3.

Group 5: Orlistat (32 mg/kg/day, p.o.; 16 weeks).

Group 6: DMH and orlistat in combination as in groups 3 & 5.

Group 7: DMH, HFD, and orlistat in combination as in groups 2, 3 & 5.

Group 8: Hydroxycitrate (135 mg/kg/day, p.o.; 16 weeks).

Group 9: DMH and hydroxycitrate in combination as in groups 2 & 8.

Group 10: DMH, HFD, and hydroxycitrate in combination as in groups 2, 3 & 8.

2.5. Induction of Tumor (CC)

To induce colon cancer in the experimental animals, the rats were treated with DMH (40 mg/kg two times per week) and/or HFD (20% daily) for 16 weeks. The DMH was freshly prepared. The model was established in our lab in a pilot study (data not shown).

2.6. Serum and Colon Tissue Sampling

Animals were placed under light anesthesia one week after the last dose. Before sacrifice, the retro-orbital plexus blood was centrifuged at 3000 rpm (10-15 min) and the serum was separated and preserved at -80 °C. The rats were sacrificed shortly after blood collection, and the colon tissues were excised, washed in saline, and divided into two sections. For the histopathological study, the first section was fixed in formalin solution (10%). The other section was frozen immediately (-70°C, liquid nitrogen) for ELISA assays and biochemical analysis [19].

2.7. Estimation of Physical Characteristics and Mortality Rate

The animals were weighed before and after treatment and then euthanized by cervical dislocation.

The weight gain= Final body mass-Initial body mass.

The growth rate= (Final mass -Initial mass)/ days of the experiment.

Mortality (%) in each group= (The no. of dead animals in each group x 100)/ Total no. of animals in each group.

Aberrant crypt foci (ACF%) incidence (histopathological study) = (Animals with ACF X 100)/The no. of animals examined.

2.8. Biomarkers Estimation

We measured caspase-3 in the colon tissues of the rats and estimates CEA and CA19.9 in the rat sera according to the ELISA kits instructions (sandwich technique). In addition, we used an ELISA assay to measure NRF2 and other oxidative stress parameters, as well as inflammatory parameters (IL-6, TNF-α, and NF-B) in the tissues of the colon. All biomarkers were determined using the methods outlined in the diagnostic kits.

2.9. The Histopathological Study

The colon tissue samples were fixed, processed, sectioned (4 μm), and stained with Hematoxylin and Eosin (H&E) [20].

2.10. Statistical Analysis

GraphPad Prism version 5.0 was used to perform statistical analyses on the results. For multiple compression between classes, we performed a one-way analysis of variance (ANOVA) test, followed by a post-hoc t-test (Tukey-Kramer). The degree of significance was set at $p \leq 0.05$, and the results were expressed as means ± standard deviations (SD).

3. Results

3.1. Effect of Orlistat and Hydroxycitrate on Physical Characteristics and Mortality Rate of the Treated Rats (Table 1)

The rats treated with DMH, HFD, and DMH/HFD show significantly reduced weight gain by 46%, 39%, and 19%, respectively as compared to the control rats. Moreover, Table 1 shows an increased ACF incidence and mortality rate in the previous groups comparing to the control rats. However, the treated rats supplemented for 16 weeks with orlistat (32 mg/kg/day) record an increase in weight gain significantly in comparison with rats treated with DMH only. Furthermore, orlistat and hydroxycitrate (135 mg/kg/day) decreased ACF incidence and mortality rate in the groups treated with DMH and/or HFD.

3.2. Effect of Orlistat and Hydroxycitrate on Serum CEA and CA19-9 Tumor Markers of the Treated Rats (Table 2)

The rats treated with DMH and DMH/HFD record an increase in serum CEA levels significantly compared to the control group. On the contrary, the treated rats coadministred with orlistat and hydroxycitrate show significantly reduced serum CEA levels compared to the DMH treated group. Similarly, the treated rats supplemented with orlistat and hydroxycitrate show significantly reduced serum levels of CEA compared to the rats treated with DMH/HFD. In addition, the rats treated with DMH and/or HFD show increase serum levels of CA19-9 significantly compared with control rats. The treated rats supplemented with orlistat and hydroxycitrate, on the other hand, show substantially reduced serum CA19-9 levels compared to the group treated with DMH. Similarly, supplementation with orlistat and hydroxycitrate significantly reduced serum CA19-9 levels compared to the groups treated with DMH and/or HFD.

Table 1. Effect of treatment with orlistat or hydroxycitrate (HCA) on growth rate, % of mortality, incidence of tumor and % of ACF incidence of DMH and/or HFD-treated rats

Items / Groups	Initial body weight (g)	Final body weight (g)	Weight gain (g)	Growth rate	No. of initial rats/group	No. of dead rats/group	% of mortality	No. of tumor bearing rats/group	% of ACF incidence
Control	179.3±11.02	396.3±22.60	217.0±9.58	1.94	7	0	0	0	0
DMH	215.5±8.41[a]	332.9±27.13[a]	117.1±8.40[a]	1.04	12	6	50	4	100
HFD	188.0±8.00[a,b]	318.7±50.00[a,b]	131.3±39.37[a,b]	1.17	7	3	43	0	80
DMH+HFD	209.7±10.22[a,b,c]	385.6±18.40[a,b,c]	175.9±5.90[a,b,c]	1.57	12	6	50	5	100
Orlistat	211.4±4.879[a]	381.4±40.06[a]	170±30.20[a]	1.52	7	0	0	0	0
Orlistat+DMH	205.4±9.160[a,b]	364.0±36.34[a,b]	168.6±22.52[a,b]	1.42	12	1	8	0	9
Orlistat+DMH+HFD	192.3+15.16[a,d]	331.6±52.38[a,d]	139.3±33.20[a,d]	1.24	12	2	17	0	10
HCA	214.0±11.40[a]	341.2±45.00[a]	127.2±22.54[a]	1.14	7	0	0	0	0
HCA+DMH	209.3±22.61[a,b]	330.0±46.72[a,b]	120.7±17.89[a,b]	1.08	12	2	17	0	10
HCA+ DMH+HFD	208.6±18.03[a,d]	353.3±34.70[a,d]	144.7±13.85[a,d]	1.29	12	1	8	0	18

Data are expressed as means ± SD of six rats per group.
1,2 N,N-Dimethylhydrazine (DMH) was given in a dose of 40 mg/kg, i.p., twice weekly for 16 consecutive weeks.
Each of orlistat and hydroxycitrate (HCA) was given in a dose of 32 and 135 mg/kg/day, p.o., respectively for 16 consecutive weeks.
Growth rate= (Final body weight-Initial body weight)/ Total No. of experimental days.
% of ACF incidence = (No. of rats with ACF X 100)/ No. of rats examined (ACF count was measured by histopathological study).
Each of orlistat and hydroxycitrate (HCA) was given in a dose of 32 and 135 mg/kg/day, p.o., respectively for 16 consecutive weeks.
a: Significantly different from control group**; b:** Significantly different from DMH-treated group; **c:** Significantly different from HFD-treated group; **d**: Significantly different from DMH+HFD-treated group at P ≤0.05 using one-way ANOVA, followed by Tukey-Kramer as a post-ANOVA test for multiple comparisons between groups.

Table 2. Effect of treatment with orlistat or hydroxycitrate (HCA) on serum tumor marker levels of CEA and CA19-9 of DMH and/or HFD-treated rats

PARAMETERS / GROUPS	CEA (ng/ml)	CA19.9 (U/ml)
Control	0.12±0.012	0.57±0.04
DMH	1.60±0.350[a]	16.00±2.60[a]
HFD	0.18±0.016[b]	2.40±0.45[a,b]
DMH+HFD	2.00±0.350[a,b]	17.00±2.80[a,c]
Orlistat	0.13±0.028	0.73±0.19
Orlistat +DMH	0.17±0.012[b]	1.40±0.43[b]
Orlistat+DMH+HFD	0.18±0.008[d]	1.60±0.65[d]
HCA	0.13±0.022	1.40±0.46
HCA+DMH	0.17±0.012[b]	3.80±3.30[a,b]
HCA+DMH+HFD	0.18±0.008[d]	3.60±0.83[a,d]

Data are expressed as means ± SD of six rats per group.
1,2 N,N-Dimethylhydrazine (DMH) was given in a dose of 40 mg/kg, i.p., twice weekly for 16 consecutive weeks.
Each of orlistat and hydroxycitrate (HCA) was given in a dose of 32 and 135 mg/kg/day, p.o., respectively for 16 consecutive weeks.
a: Significantly different from control group; **b:** Significantly different from DMH-treated group; **c**: Significantly different from HFD-treated group; **d**: Significantly different from DMH+HFD-treated group at P ≤ 0.05 using one-way ANOVA, followed by Tukey-Kramer as a post-ANOVA test for multiple comparisons between groups.

Table 3. Effect of treatment with orlistat or hydroxycitrate (HCA) on caspase-3 content as an apoptotic marker in the colonic tissues of DMH and/or HFD-treated rats

PARAMETERS / GROUPS	Caspase 3 (pg/g)
Control	10.5±0.58
DMH	28.0±8.65[a]
HFD	11.7±8.15[a,b]
DMH+HFD	25.2±4.35[a,b,c]
Orlistat	9.2±4.60[a]
Orlistat + DMH	14.6±6.40[a,b]
Orlistat+ DMH +HFD	18.4±6.80[a,d]
HCA	16.8±9.60[a]
HCA+ DMH	28.0±6.70[a]
HCA+ DMH +HFD	17.0±6.70[a,d]

Data are expressed as means ± SD of six rats per group.
1,2 N,N-Dimethylhydrazine (DMH) was given in a dose of 40 mg/kg, i.p., twice weekly for 16 consecutive weeks.
Each of orlistat and hydroxycitrate (HCA) was given in a dose of 32 and 135 mg/kg/day, p.o., respectively for 16 consecutive weeks.
a: Significantly different from control group; b: Significantly different from DMH-treated group; c: Significantly different from HFD-treated group; d: Significantly different from DMH+HFD-treated group at P ≤ 0.05 using one-way ANOVA, followed by Tukey-Kramer as a post-ANOVA test for multiple comparisons between groups.

3.3. Effect of Orlistat and Hydroxycitrate on the Tissue Caspase-3 Apoptotic Marker in the Treated Rats (Table 3)

The rats treated for 16 weeks with DMH and/or HFD recorded a significant increase in caspase-3 content in the tissues of the colon compared with control rats. In contrast, orlistat reduced the caspase-3 content in the tissues of the colon significantly compared with the group treated with DMH only. Also, the treated rats supplemented with orlistat and hydroxycitrate show a decrease in the caspase-3 tissue content in the tissues of the colon significantly compared with the groups treated with DMH/HFD.

3.4. Effect of Orlistat and Hydroxycitrate on the Inflammatory Markers in the Tissues of the Colon of the Treated Rats (Table 4)

The TNF-α content in the tissues of the colon in rats treated with DMH and/or HFD was increased significantly compared with normal control rats. On the contrary, orlistat and hydroxycitrate administration to the treated rats substantially reduced the colonic TNF-α tissue content as compared with the rats treated with DMH/HFD. Similarly, supplementation with orlistat and hydroxycitrate decreased the TNF-α content in the tissues of the colon significantly as compared with the rats treated with DMH/HFD.

Compared with control rats, the NF-κ_B content in the tissues of the colon in rats treated with DMH and/or HFD was increased significantly. In contrast, the groups that received co-treatment of orlistat and hydroxycitrate record a significant reduction in the NF-kB content in the tissues of the colon as compared with the group treated with DMH. In addition, supplementation with orlistat and hydroxycitrate decreased the NF-κ_B content significantly in the tissues of the colon of the rats as compared with the rats treated with DMH/HFD.

The rats treated for 16 weeks with DMH and/or HFD show a significant increase in the IL-6 content in the tissues of the colon in comparison with the normal rats. The groups that received orlistat and hydroxycitrate, on the other hand, greatly reduced the colonic IL-6 tissue content significantly as compared with the group treated with DMH. Similarly, the treated rats supplemented with orlistat and hydroxycitrate show decreased IL-6 content significantly in the tissues of the colon as compared with the rats treated with DMH/HFD.

3.5. Effect of Orlistat and Hydroxycitrate on the Oxidative Stress Markers in the Tissues of the Colon of the Treated Rats (Table 5)

In comparison with the normal control rats, the MDA content was increased significantly in the tissues of the colon in groups treated with DMH and/or HFD. The treated rats received orlistat and hydroxycitrate, on the other hand, show a significant reduction in the colonic MDA tissue content as compared with the group treated with DMH. Similarly, the groups supplemented with orlistat and hydroxycitrate show a significant reduction in the colonic MDA tissue content as compared with the rats treated with DMH/HFD.

Meanwhile, the NRF2 content shows a significant reduction in the tissues of the colon in groups administered with DMH and/or HFD as compared with the control group. On the contrary, the treated rats supplemented with orlistat and hydroxycitrate show a significant increase in the colonic NRF2 content as compared to the groups treated with DMH and/or HFD.

Moreover, the CAT, SOD, GSH, and GST contents were significantly decreased in the tissues of the colon in rat groups treated with DMH and/or HFD as compared with the control group. On the contrary, the treated rats supplemented with orlistat and hydroxycitrate show an increase in the colonic CAT content as compared to the groups treated with DMH and/or HFD.

Finally, the total NO content in the tissues of the colon in rats treated with DMH and/or HFD was increased as compared with the normal control rats. In contrast, the treated rats supplemented with orlistat and hydroxycitrate show a significant reduction in the colonic NO content as compared with the group treated with DMH. Similarly, the treated rats supplemented with orlistat and hydroxycitrate show a significant reduction in the colonic NO content as compared with the groups treated with DMH/HFD.

Table 4. Effect of treatment with orlistat or hydroxycitrate (HCA) on inflammatory markers (IL-6, TNF-α and NF-κ_B) content in the colonic tissues of DMH DMH and/or HFD-treated rats

PARAMETERS / GROUPS	TNF-α (pg/g)	NF-k_B (pg/g)	IL-6 (pg/g)
Control	82.6±1.85	36.6±3.43	221.2±21.50
DMH	246.0±4.10[a]	194.3±2.65[a]	613.0±12.80[a]
HFD	113.4±10.00[a,b]	70±5.10[a,b]	251.5±9.20[a,b]
DMH+HFD	271.3±7.60[a,b,C]	217±4.15[a,b,c]	659.8±14.50[a,b,c]
Orlistat	104.5±3.40[a]	65.3±6.06[a]	275±31.00[a]
Orlistat +DMH	174.7±4.20 [a,b]	125.4±3.94 [a,b]	337.7±6.65 [a,b]
Orlistat+ DMH+HFD	190.9±3.20 [a,d]	137.6±1.60 [a,d]	379.3±10.40 [a,d]
HCA	124.1±2.10[a]	69.0±5.17[a]	305.6±7.59[a]
HCA+DMH	215.6±3.5 [a,b]	153.2±3.24 [a,b]	454.8±8.79 [a,b]
HCA+DMH+HFD	232.6±4.20 [a,d]	128.8±2.42 [a,d]	414.1±9.22 [a,d]

Data are expressed as means ± SD of six rats per group.
1,2 N,N-Dimethylhydrazine (DMH) was given in a dose of 40 mg/kg, i.p., twice weekly for 16 consecutive weeks.
Each of orlistat and hydroxycitrate (HCA) was given in a dose of 32 and 135 mg/kg/day, p.o., respectively for 16 consecutive weeks.
a: Significantly different from control group; **b:** Significantly different from DMH-treated group; **c**: Significantly different from HFD-treated group; **d**: Significantly different from DMH+HFD-treated group at $P \leq 0.05$ using one-way ANOVA, followed by Tukey-Kramer as a post-ANOVA test for multiple comparisons between groups.

Table 5. Effect of treatment with orlistat or hydroxycitrate (HCA) on the oxidative stress parameters in the colonic tissues of DMH and/or HFD-treated rats

Parameters / Groups	MDA (mmol/g)	NRF2 (pg/g)	CAT (mmol/g)	SOD (U/g)	GSH (nmol/g)	GST (µmol/g)	Total NO (nmol/g)
Control	50.2 ± 3.50	47.7±21.50	73.7±3.14	68.7±2.12	127.4 ± 4.27	1.298± 0.012	152.9±1.55
DMH	296.7 ± 3.53[a]	31.0±11.50[a]	13.5±2.11[a]	10.6±1.16[a]	44.0± 4.17[a]	0.579± 0.021[a]	497.3±1.52[a]
HFD	105.5± 6.60[a,b]	42.4±2.60[a,b]	34.0±3.13[a,b]	32.2±2.93[a,b]	77.2±4.36[a,b]	0.925±0.030[a,b]	217.7±4.77[a,b]
DMH+HFD	307.5±4.50[a,b,c]	24.8±7.80[a,b,c]	15.2±2.85[a,b,c]	9.9±1.93[a,b,c]	58.8±2.73[a,b]	0.644±0.045[a,c]	501.3±2.64[a,b]
Orlistat	74.8± 3.32[a]	56.1±1.94[a]	44.0±2.61[a]	46.2±2.79[a]	96.3± 3.50[a]	1.025± 0.059[a]	174.3±4.13[a]
Orlistat +DMH	146.5±5.35[a,b]	51.3±1.33[a,b]	57.3±5.24[a,b]	38.7±4.41[a,b]	85.4±3.64[a,b]	0.9467±0.084[a,b]	215.8±3.31[a,b]
Orlistat+DMH+HFD	167.5±5.02[a,d]	38.8±2.30[a,d]	51.7±3.98[a,d]	37.5±4.93[a,d]	90.7±9.18[a,d]	1.030±0.094[a,d]	228.7±1.86[a,d]
HCA	82.2±6.28[a]	7.2±2.50[a]	36.3±5.16[a]	41.3±4.41[a]	93.0± 7.59[a]	1.065±0.083[a]	274.3±4.13[a]
HCA+DMH	160.8±7.14[a,b]	45.3±1.02[a,b]	53.3±5.09[a,b]	36.3±3.08[a,b]	79.3±5.06[a,b]	0.835±0.044[a,b]	315.8±3.31[a,b]
HCA+DMH+HFD	153.1±5.91[a,d]	45.0±1.31[a,d]	48.5±3.27[a,d]	40.5±3.83[a,d]	74.2±6.88[a,d]	0.877±0.051[a,d]	346.8±5.12[a,d]

Data are expressed as means ± SD of six rats per group.
1,2 N,N-Dimethylhydrazine (DMH) was given in a dose of 40 mg/kg, i.p., twice weekly for 16 consecutive weeks.
Each of orlistat and hydroxycitrate (HCA) was given in a dose of 32 and 135 mg/kg/day, p.o., respectively for 16 consecutive weeks.
a: Significantly different from control group; **b:** Significantly different from DMH-treated group; **c**: Significantly different from HFD-treated group; **d**: Significantly different from DMH+HFD-treated group at $P \leq 0.05$ using one-way ANOVA, followed by Tukey-Kramer as a post-ANOVA test for multiple comparisons between groups.

Table 6. Effect of treatment with orlistat and/ or hydroxycitrate (HCA) on histopathological findings of colonic tissues of DMH and/or HFD-treated rats

items / Groups	Intact mucosa	Reduction mucous secretion	interstitial inflammatory infiltrate	lymphoid hyperplasia	Muscularis mucosa (thickened)	congested blood vessels	Dysplasia ACF	pleomorphic cell (tumor cell)
Control	-	-	-	-	-	-	-	-
DMH	++	+++	+++	+++	+++	++	+++	++
High fat diet 20%	-	+	++	+	+	+	++	-
DMH + HFD	++	+++	++	+++	++	++	+++	+++
Orlistat	-	-	-	-	-	-	-	-
Orlistat + DMH	+	+	+	+	+	+	-	-
Orlistat + DMH + HFD	+	+	+	+	+	+	-	-
HCA	-	+	+	-	+	+	-	-
HCA + DMH	+	+	+	+	+	+	-	-
HCA + DMH + HFD	+	+	+	+	+	+	-	-

(-) None, (+) mild, (++) moderate and (+++) severe
1,2 N,N-Dimethylhydrazine (DMH) was given in a dose of 40 mg/kg, i.p., twice weekly for 16 consecutive weeks.
Each of orlistat and hydroxycitrate (HCA) was given in a dose of 32 and 135 mg/kg/day, p.o., respectively for 16 consecutive weeks.

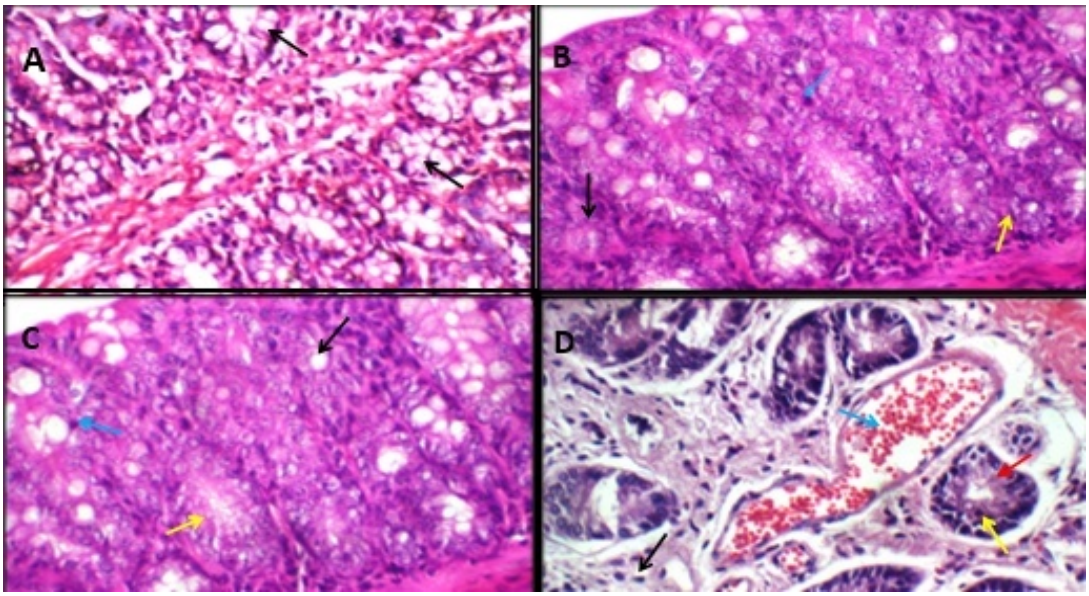

Figure 1.1. Histology of colon of the control (saline), DMH group, HFD group, DMH+HFD group. **(A) Control:** colonic wall show glands lined by tall columnar mucous-secreting epithelial cells (black arrow); **(B) DMH group:** colonic wall showed glands lined by epithelial cells with loss of mucous secretion (black arrow), focal nuclear stratification with prominent nucleoli (yellow arrow), and few mitotic figures (blue arrow), ACF; adenoma with moderate to severe dysplasia; **(C) HFD group:** colonic wall showing glands with reduction of mucous secretion of epithelial lining with focal nuclear stratification (black arrow) and prominent nucleoli (yellow arrow), and scattered mitotic figures (blue arrow), ACF; adenoma with mild to moderate dysplasia; **(D) DMH/HFD group:** colonic wall showing glands lined by epithelial cells with reduction of mucous secretion and elongated nuclei (red arrow), frequent mitotic figures (yellow arrow), mildly edematous interstitium (black arrow), and markedly dilated congested blood vessels (blue arrow), ACF; adenoma with moderate to severe dysplasia

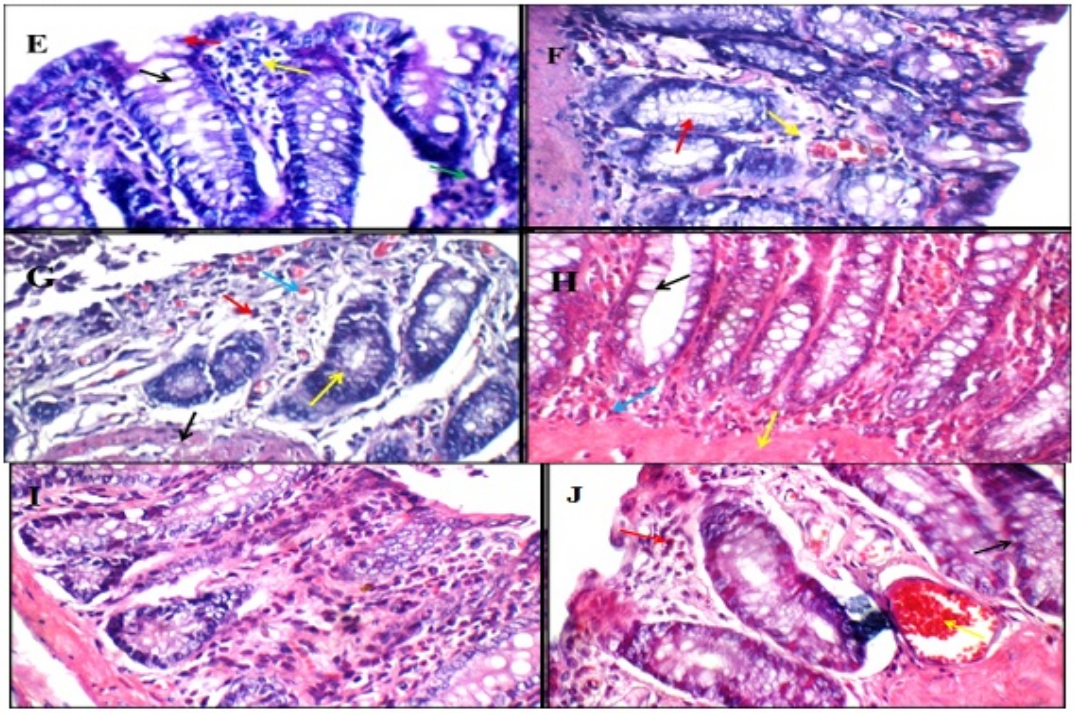

Figure 1.2. Histology of colon samples of the orlistat group, orlistat/DMH group, orlistat/DMH/HFD group, hydroxycitrate (HCA) group, HCA/DMH group, HCA/DMH/HFD group, thymol group, thymol/DMH group, thymol/DMH/HFD group. **(E) Orlistat group:** colonic wall showing glands with serrated luminal configuration (black arrow) with single cell lining (red arrow), scattered mitotic figures (green arrow), and mild interstitial inflammatory infiltrate (yellow arrow); **(F) Orlistat/DMH group:** colonic wall showing glands lined by mucous-secreting epithelium with normal-looking goblet cells (red arrow), and mild interstitial inflammatory infiltrate with scattered eosinophils (yellow arrow); **(G) Orlistat/DMH/HFD group**: colonic wall showing glands lined by slightly basophilic cells with focal nuclear stratification (yellow arrow), mild inflammatory infiltrate (red arrow) and mildly dilated congested blood vessels (blue arrow), and thickened muscularis mucosa (black arrow); **(H) HCA group:** colonic wall showing wide glands lined by single cell layer with normal-looking goblet cells (black arrow) and mild inflammatory infiltrate with scattered eosinophils (blue arrow), and thickened muscularis mucosa (yellow arrow).; **(I) HCA/DMH group:** colonic wall showing glands lined by single cell layer with regular nuclei and scattered mitotic figures (yellow arrow), and moderate interstitial inflammatory infiltrate with scattered eosinophils (red arrow), and average muscularis mucosa (blue arrow).; **(J) HCA/DMH/HFD group:** colonic wall showing glands with moderately apoptotic epithelial lining (black arrow) and mild interstitial inflammatory infiltrate with scattered eosinophils (red arrow) and moderately dilated congested blood vessel (yellow arrow), and thickened muscularis mucosa (blue arrow).Hematoxylin-eosin staining, magnification: X400

3.6. Effect of Orlistat and Hydroxycitrate on the Colon Tissue Histopathology of the Treated Rats

Table 6 and Figure 1 show the histopathological findings in colon tissues. The control colon is shown in Figure 1.1A. In contrast, The DMH-treated rats show a colonic wall with average musculosa, mildly edematous submucosa, and ulcerated mucosa, the colon wall also shows aberrant crypt foci (ACF) which are clusters of abnormal tube-like glands (hyperplastic polyp) and considered as one of the marked changes that lead to cancer as seen in Figure 1.1B which demonstrate adenoma with dysplasia. The group of rats treated with a high-fat diet shows a colon with an average musculosa, submucosa displayed mild edema, and intact mucosa and the colon wall appeared with hyperplastic polyp and show adenoma with dysplasia (ACF) as depicted in Figure 1.1C. In addition, the group of rats treated with the DMH/HFD shows a colon with average musculosa, average submucosa, intact mucosa, and adenoma with dysplasia (ACF) (Figure 1.1D). On the contrary, as shown in Figure 1.2E-J, the tissues of the colon in rats supplied with orlistat and hydroxycitrate reveal improved tissue architecture.

4. Discussion

Obese people have a lot of adipose tissue that secretes growth factors and cytokines that can increase the risk of colon cancer [21]. Dimethylhydrazine (DMH), potent carcinogen was used as a standard *in vivo* model to induce malignant neoplasms in rodent colons [8]. The DMH undergoes metabolic enhancement, forming free radicals causing oxidative stress and initiates lipid peroxidation cascade [22,23]. The nitric oxide (NO) produced by iNOS, on the other hand, mediates inflammation and induces tumors in the colon [24]. Excess NO development suppresses DNA repair and inhibits apoptosis causing induction of colon cancer [25].

The CEA marker is used in the clinical diagnosis and monitoring of colon cancer and is considered as the colon cancer best marker with a well-characterized antigen [26,27]. Furthermore, CA19-9 has been linked to a poor prognosis in colorectal cancer [28]. As a result, combining CEA and CA19-9 markers led to a better diagnosis of colorectal cancer [29,30].

The present results showed increased plasma levels of CEA and CA 19-9 in the groups treated with DMH and/or HFD as compared with the control group, this was owing to increased development in the malignant cells. These

results agreed with a previous study [31]. The treated groups supplied with orlistat and HCA record a significant decrease in the levels of the plasma CEA and CA 19-9 as compared to the groups treated with DMH and/or HFD only as approved in other studies [32-34]. These findings indicate the anticancer activity of orlistat and/or HCA, as indicated by the marked reduction in the levels of CEA, and CA 19-9.

The present study records significantly the increased caspase-3 content in the colon tissue of groups treated with DMH and/or HFD comparing to the control group, which indicate an apoptosis-induction process [22,29]. Caspase-3 is an important apoptotic marker enzyme in the apoptosis process [24]. These data are in approval with Hassan *et al.* (2017) [35] who found that caspase-3 is elevated in DENA-induced hepatocellular carcinoma in rats. on other hand, supplementation of animals with orlistat, HCA, and thymol led to a reduction in the colon caspase-3 content in comparison with rat groups treated with DMH and/or HFD and did not receive the previous supplementation. In addition, our results are consistent with previous work that reported increased apoptosis in isogenic human colon adenocarcinoma cell lines [36].

The NF-κB and TNF-α are considered inflammatory mediators that create a positive feedback loop causing cellular and DNA damage in the sites of inflammation and share in cancer progression [37]. NF-κB control genes are involved in inflammation, proliferation, and apoptosis [38]. There is substantial evidence that NF-κB regulates inducible enzymes as well as TNF-α, IL-2, and IL-6 expression [39,40]. The NF-κB response genes in the groups treated with DMH and/or HFD may share in the process of inflammation and tumor growth as recorded in the present study. As a result, the anti-inflammatory activity of orlistat and HCA is the key mechanism that may be associated with the reduced expression of NF-κB in the groups treated with DMH and/or HFD that may cause the prevention of colorectal cancer.

It has been documented that the chronic inflammation's long duration is produced due to activation of NF-B that leads to increased expression of NF-κB and IL-6 [41]. As a result, treatment of colon cancer is associated with the blocking or suppression of TNF-α and IL-6 genes [42]. The present data record that orlistat and HCA can inhibit the activity of NF-B and modulate the inflammatory processes by suppressing inflammation in the groups of rats treated with DMH and/or HFD, which is consistent with previous research [43].

In the present study, the treatment with DMH significantly increases the lipid peroxidation as indicated by increased MDA concentration (tumor promoter) which leads to a state of oxidative stress [43]. Colorectal cancer can develop due to DNA mutation and cell proliferation caused by lipid peroxidation [44].

The current study records significantly increased levels of MDA and NO in the colon tissues in groups of animals treated with DMH and/or HFD as compared with the control group. The administration of DMH causes the release of the ROS that react with the cell membrane lipid bilayers and start a cascade of peroxidation that release products as MDA causing disintegration in the cell membranes and mutations that favor cancer growth [45,46].

Furthermore, rats treated with DMH recorded a significant decrease in the antioxidant parameters (SOD, CAT, GSH, GST, and NRF2). The current study also recorded that the supplementation of the treated rats with orlistat and HCA causes a significant reduction in the colon MDA and NO in contrast to a significant increase in the colonic SOD, CAT, GSH, GST, and NRF2 antioxidant contents. This indicates that orlistat and HCA have antioxidant effects as they can scavenge free radicals and prevent lipid peroxidation [45,47]. Finally, the histopathological analysis of the colon lining shows that the groups of animals treated with DMH and/or HFD recorded marked cancerous features which were ameliorated in the groups supplemented with orlistat and HCA.

5. Conclusion

Our results in the present study evidenced that the use of orlistat and hydroxycitrate can potentially ameliorate the pathological lesions in the colon and reducing oxidative stress, inflammation, and apoptosis which are considered critical mechanisms for preventing colon cancer caused by DMH and/or HFD.

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Conflict of Interest

The authors declare that they do not have any conflict of interest.

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Ethical Approval

The animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011) and the use of animals was reviewed and approved by an independent ethics committee of the Faculty of Pharmacy, Al-Azhar University in Cairo, Egypt, with the reference number “bio._9med.research_0000009”.

Availability of Data and Materials

The data and materials are available with the corresponding author at any time you request.

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