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Original Article

Ameliorative effects of combination therapy with metformin and crocin in experimental colitis in rats

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Abstract

Background and purpose: Inflammatory bowel disease (IBD) is characterized by aberrant immune responses in the colon, leading to the inflammatory cascades. Metformin (MTF) and crocin demonstrated beneficial effects in reducing inflammation and oxidative stress, as the main triggers of IBD. Considering the coloprotective properties of MTF and crocin alone, the present study aims to investigate the possible therapeutic effects of combination therapy with MTF and crocin on the acetic acid-induced colitis model.
Experimental approach: Acute colitis was induced in Wistar rats by intrarectal administration of acetic acid. Nine study groups were assessed, including normal group received normal saline, control group received normal saline after colitis induction, dexamethasone as reference (1 mg/kg), MTF-treated groups received 100, 150, 200 mg/kg of drug, crocin-treated received 20 and 30 mg/kg of crocin, and combination therapy received MTF (150 mg/kg) + crocin (20 mg/kg). Colon tissues were collected to assess macroscopic, microscopic, and biochemical parameters.
Findings/Results: Our data revealed that the combination therapy, crocin-treated and MTF-treated (at the higher dose) groups, ameliorated disease severity by decreasing myeloperoxidase activity and malondialdehyde level, compared to the control group. Combination therapy was also effective in attenuating macroscopic parameters, including ulcer index as well as wet weight of the colon. Histopathological scores considerably decreased in all groups.
Conclusion and implications: MTF, crocin, and their combined administration exerted ameliorative effects in the colons of acetic acid-induced colitis rats, which is probably due to their anti-inflammatory and antioxidant properties. Further experimental studies are required to elucidate the underlying mechanisms.

Keywords: Crocin; inflammation; Inflammatory bowel disease; Metformin; Rat; Ulcerative colitis.

INTRODUCTION

Inflammatory bowel disease (IBD) is a group of chronic relapsing immune disorders of the gastrointestinal tract (GI) with multifactorial and polygenic pathogenesis, the details of which are still controversial (1). In general, IBD encompasses two principal categories: ulcerative colitis (UC) and Crohn's disease (CD) (2). In both subtypes, excessive and persistent inflammation leads to oxidative stress and the overproduction of pro-inflammatory cytokines, which are responsible for clinical manifestations such as bloody stool, diarrhea, weight loss, fatigue, and abdominal cramps (3). According to population-based studies, the prevalence of IBD continues to rise globally, particularly in newly industrialized regions (4).

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In recent years, there has been significant progress in the development of therapies for IBD; however, the conventional therapeutics, including immunosuppressants and corticosteroids, result in different side effects such as bone marrow suppression, diarrhea, infections, and neoplastic disorders. These side effects primarily arise from their systemic long-term administration (5,). Thus, there is a crucial need for the development of highly effective therapeutic agents or a combination of them that offer favorable side effect profiles while achieving remission.

Metformin (MTF, *dimethyl biguanide*) has been a breakthrough in managing type 2 diabetes worldwide since its discovery over five decades ago. Beyond its established metabolic role, various evidence suggest pleiotropic effects of MTF extending from anti-inflammatory (7), anti-aging (8), anticancer (9) and impacts on counteracting cardiovascular disorders (10). Considering the significant impact of oxidative stress and abnormal immunity response in the etiology of IBD, numerous studies have explored the potential role of MTF in alleviating inflammatory signals in the gut, particularly in experimental UC. For instance, it has been reported that by enhancing 5'-adenosine monophosphate-activated protein kinase (AMPK) and down-regulating interleukin-6 (IL-6) expression, MTF inhibits mucosal inflammation in dextran sulfate sodium (DSS)-induced colitis (11). MTF supplementation enhanced goblet cell differentiation and improved the function of the epithelial barrier in IL-10-deficient (IL10KO) mice (12) and alleviated UC in mice through alterations in microbiota structure (13).

It is well known that plant-based compounds rich in antioxidants can prevent or mitigate IBD due to their ability to scavenge reactive oxygen species (ROS) and maintain homeostasis in the gut microbiota (14-17). Crocin, the primary constituent of *Crocus sativus L.* extract, is a chemical diester composed of the dicarboxylic acid crocetin and disaccharide gentiobiose. It is responsible for the color of saffron and gardenia (18,19). Several studies have indicated beneficial pharmacologic properties of crocin, including anti-inflammatory, antioxidant, anti-apoptotic, and cardioprotective effects (20,21). Multiple findings suggest that crocin could potentially exert anti-inflammatory properties against UC by suppressing the production of various cytokines, particularly ILs, improving the antioxidant defenses in the colon, and decreasing the level of tumor necrosis factor alpha (TNF-α) (22,23). Furthermore, saffron administration notably suppressed elevated serotonin levels in the colon tissue of DSS-induced colitis mice (24).

Accumulating evidence has demonstrated that combination therapies are successful in controlling patient outcomes and reducing drug resistance, probably due to blocking more than a single pathway. So, simultaneous administration of available drugs and natural antioxidants that target various pathways related to disease pathogenesis could be beneficial. For instance, the combination of mesalamine with a natural antioxidant, curcumin, significantly reduced relapse rates by modulating inflammatory pathways (25). Based on the established antioxidant and anti-inflammatory properties of crocin, we hypothesized that its coadministration with MTF may offer enhanced protection against both inflammation and oxidative damage in UC. Therefore, this research aimed to evaluate this combined therapeutic approach.

MATERIAL AND METHODS

Drugs and chemicals

MTF was purchased from Mahban Darou Company (Tehran, Iran). Crocin (powder form with purity of 95%) was obtained from Sami Saz Pharmaceutical Company (Mashhad, Iran). The purity of crocin powder was determined and confirmed using a high-performance liquid chromatography (HPLC) method by the manufacturer. Dexamethasone powder was received as a gift from Raha Pharmaceutical Company (Isfahan, Iran). O-dianisidine dihydrochloride (ODZ) and hexadecyl-trimethyl ammonium bromide (HTAB) were purchased from Sigma-Aldrich (Germany). Formaldehyde and glacial acetic acid were obtained from Merck Company (Darmstadt, Germany). The normal saline was purchased from a local pharmacy.

Ethical consideration

The animal procedures were conducted in compliance with the national guidelines and recommendations outlined by the Iranian National Committee for Ethics in Biomedical Research under the code IR.MUI.RESEARCH.REC.1401.024.

Experimental animals

Seventy-two male Wistar rats weighing 160-210 g were obtained from the animal house of the School of Pharmacy and Pharmaceutical Sciences, Isfahan, Iran. The animals underwent a one-week acclimatization period before the start of the experimental protocol. All of the rats were kept in clear polypropylene cages (3 rats/cages) under a standard environmental situation with a 12/12-h light/dark cycle, relative humidity ranging from 30% to 50%, and a temperature maintained between 20-23 °C. The researchers made efforts to minimize the possible distress during the experimental processes.

Experimental groups

The rats were randomly allocated into 9 groups (n = 8) as outlined below:

Group I served as the normal group: the rats received a vehicle consisting of normal saline/tween 20 by gavage (5 mL/kg, p.o.), 2 h before intra-rectal instillation of normal saline (2 mL/rat) and continued for 5 days.

Group II (control group): the rats received vehicle, 2 h before intra-rectal instillation of 2 mL acetic acid (3%), and continued for 5 days (26).

Groups III, IV, and V (MTF groups): the rats received MTF alone (100, 150, and 200 mL/kg, p.o.), 2 h before the induction of colitis, and continued for 5 days.

Groups VI and VII (crocin groups): the rats received crocin alone (20 and 30 mL/kg, p.o 2 h before the induction of colitis and continued for 5 days (23,27).

Group VIII (MTF + crocin): the rats received MTF 150 mL/Kg and crocin 20 mL/kg, p.o. 2 h before the induction of colitis and continued for 5 days.

Group IX: dexamethasone group (reference group): the rats received dexamethasone (1 mg/kg intraperitoneally) 2 h before the induction of colitis and continued for 5 days (28).

Induction of UC

After a 24-h fasting period, the animals were anesthetized with ketamine/xylazine (75/10 mg/kg)/ and a suitable catheter (2 mm in diameter and 8 cm in length) was used to instill 2 mL acetic acid (3%) into the colon *via* the rectum (2). Before removing the catheter, the rats were positioned in a head-down position for 30 s to prevent the acid solution from leaking out. Following the colitis induction, administration of drugs was carried out once daily for 5 consecutive days (29). On the sixth day after receiving acetic acid, animals were sacrificed by CO_2 inhalation in a suitable chamber. Before sacrificing animals, blood samples were collected, and the obtained serum was kept at -80 °C for quantifying malondialdehyde (MDA) levels.

Assessment of colon macroscopic damage

Eight centimeters of the colon were excised and longitudinally opened, and washed in ice-cold Dulbecco's phosphate-buffered saline (DPBS). Then the samples were fixed on a clean board and photographed with a mobile phone camera for subsequent macroscopic investigation. The severity of colonic damages was determined by calculating the ulcer index = ulcer score + ulcer area as follows: the score of colonic ulcer severity (0-3) was assessed as 0: no ulcer; 1 (inflammation, edema, thickness, and superficial erosions); 2 (bleeding, hemorrhagic spots and severe ulcers); 3 (necrosis and/or perforation). Ulcer area was also measured using Fiji-win 32 software (29).

Finally, colon tissues were cut into different pieces: one of them was fixed in 10% buffered formalin for histopathological analysis, and the others were stored at -80 °C for biochemical experiments.

Histopathological evaluations

For histopathological evaluation, appropriate tissue samples were collected from the colon segments and were then fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 µm thickness, and stained with hematoxylin and eosin for light microscopic examination. Description and scoring of lesions were performed according to the grading criteria explained previously by El-Akabawya and El-Sherifa (30). Briefly, an assessment of histopathological colonic lesions was performed with consideration for the following parameters: glandular atrophy, edema and hyperemia, morpho-architectural distortion of crypts, cryptitis, surface ulceration and necrosis, and sub-mucosal. Each of these parameters was scored according to the following scale: 0, absent; 1, mild

(present in less than 10% of examined tissue); 2, moderate (present in 10%-50% of examined tissue); and 3, intense (present in over 50% of examined tissue). The final scores for each sample were calculated as the sum of those scores. The total score for the degree of colonic inflammation was determined as follows: 0, absent; 1, restricted to the mucosa; 2, mucosa and submucosa; and 3, traversal of the entire length of the colon wall.

Evaluation of myeloperoxidase activity in colonic tissues

The myeloperoxidase (MPO) activity in colon tissues, as a great marker of leukocyte migration, was evaluated according to the *Lefkowitz et al.* method (31). Briefly, 100 mg of colon tissue was weighed and homogenized in one mL of potassium phosphate solution (50 mM, pH 6) containing 0.5% hexadecyl trimethyl-ammonium bromide (HTAB) using a micro smash for three cycles of 45 s. Subsequently, the homogenate was sonicated in an ice bath for 10 s and centrifuged at 13400 rpm for 6 min, 4 °C. Then, 7 µL of supernatant was added to each well, and H_2O_2 (0.1 mM) and o-dianisidine dihydrochloride (0.167 mg/mL) were added to each sample. The absorbance change at 450 nm was measured using a UV/VIS spectrophotometer at 0 and 3-min time intervals. MPO activity was measured and reported as U/100 mg of colon tissue.

Lipid peroxidation (MDA) assay in serum

As a representative of lipid peroxidation in inflammatory diseases, MDA levels were quantified by the Kiazist kit (Kiazist company, Iran) based on the manufacturer's protocol. After serum sample preparations according to the kit's instructions, thiobarbituric acid solution was added to serum samples to produce a colored compound. Following centrifugation, the absorbance of the supernatants was recorded at 532 nm. Finally, the standard curve was plotted, and MDA concentration was reported as (nmol/mL).

Statistical analysis

Statistical data analysis was done using SPSS version 26.0. Data presented in the tables are expressed as mean ± SEM, whereas graphical data are expressed as mean ± SD. Animal weight changes were assessed using a Student's paired t-test. Parametric data were evaluated by one-way ANOVA followed by the Tukey post hoc test. The Mann-Whitney U test was also applied for scoring data. A *P*-value of < 0.05 was considered statistically significant.

RESULTS

Whole body weight changes

According to the results (Table 1), rat weight loss induced by acetic acid is evident across all study groups, which is significant_in the colitis group, dexamethasone group, and MTF 100 and 150 mg/kg groups. On the other hand, our results showed that combination therapy halted the weight loss trend observed in the MTF 150 mg/kg group.

Table 1. Effect of MTF (100, 150, and 200 mg/kg), crocin (20 and 30 mg/kg), MTF + crocin (150 mg/kg + 20 mg/kg), and dexamethasone (1 mg/kg, i.p.) on body weight change in Wistar rats with acetic acid-induced colitis. Normal group treated with normal saline/tween (5 mL/kg, p.o.); control group treated with normal saline/tween (5 mL/kg, p.o.) after colitis induction. Data are expressed as mean ± SEM. Student's paired t-test was used for analysis; ***P* < 0.05** and ****P* < 0.01** indicate significant differences between the groups.

Study groups/doses	Before treatment	After treatment	*P*-value
Normal	186.65 ± 21.10	192.68 ± 15.6	> 0.05
Control	188.7 ± 10.5	172.61 ± 9.7	< 0.01**
MTF (100 mg/kg)	190.7 ± 16.3	176.9 ± 12	< 0.01**
MTF (150 mg/kg)	193 ± 12.17	181.03 ± 14.25	< 0.05*
MTF (200 mg/kg)	194.6 ± 9.6	189.18 ± 7.76	> 0.05
Crocin (20 mg/kg)	182.8 ± 11	179.48 ± 12.6	> 0.05
Crocin (30 mg/kg)	197.95 ± 13.6	194.81 ± 11.3	> 0.05
Combination therapy (MTF 150 mg/kg + crocin 20 mg/kg)	200.8 ± 14	197.82 ± 11.3	> 0.05
Dexamethasone (1 mg/kg)	181.44 ± 13.8	171.25 ±18.1	< 0.01**

MTF. Metformin.

Effects of MTF, crocin, and combination therapy on macroscopic parameters in rats

The data analysis of colon tissue has shown that acetic acid resulted in severe ulceration and mucosal damage both macroscopically and histologically, and caused an increase in the wet weight of colon compared to the normal group (Fig. 1A and B). In the normal group, no sign of colitis induction, such as ulceration, wall thickening, bleeding, or necrosis, was observed. (Fig. 1A and Table 2). MTF at doses of 100, 150, and 200 mg/kg reduced the severity of colonic lesions and colon wet weight compared to the colitis control group (Fig. 1C-E, Table 2), except for the dose of 100 mg/kg, which did not significantly reduce the colon weight. The groups treated with crocin (20 and 30 mg/kg) also diminished macroscopic features, including ulcer area, ulcer score, ulcer index, and the wet weight of colon compared to the colitis control group (Fig. 1F and G and Table 2). In the combination therapy group (MTF 150 mg/kg + crocin 20 mg/kg), we observed a significant reduction in ulcer area, ulcer score, and ulcer index, as well as the wet weight of colon compared to that of the colitis control group (Fig. 1H and Table 2). Our findings indicated that the administration of dexamethasone (1 mg/kg) effectively mitigated ulcer area, ulcer score, ulcer index, and wet weight of the colon compared to that of the acetic acid colitis group.

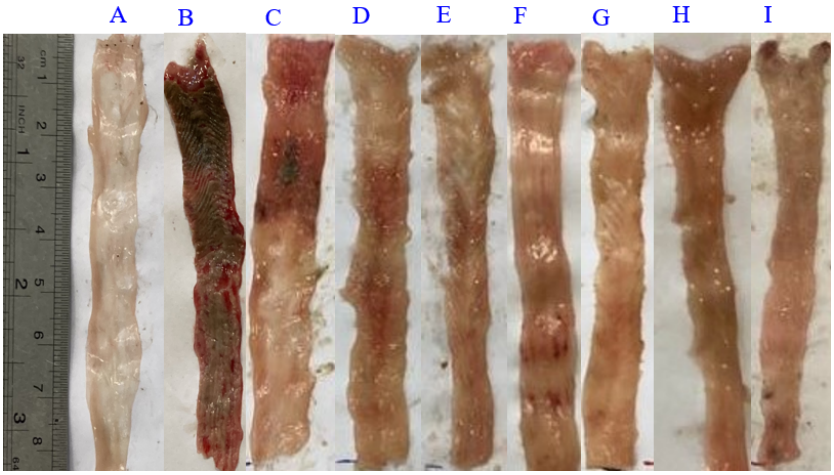


Fig. 1. Macroscopic illustration of acetic acid-induced colitis in Wistar rats. (A) Normal group treated with normal saline/tween (5 mL/kg), (B) control group, treated with normal saline/tween (5 mL/kg) after colitis induction, (C-E) oral administration of MTF at 100, 150, and 200 mg/kg), (F and G) oral administration of crocin at 20 and 30 mg/kg, (H) combination therapy with MTF (150 mg/kg oral) + crocin (20 mg/kg), and (I) dexamethasone (1 mg/kg, i.p.). MTF, Metformin.

Table 2. Effects of different doses of MTF, crocin, and their combination (MTF 150 mg/kg + crocin 20 mg/kg) on macroscopic parameters of experimental colitis. Normal group treated with normal saline/tween (5 mL/kg, p.o.); control group treated with normal saline/tween (5 mL/kg, p.o.) after colitis induction. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate significant difference in comparison with control; $^{###}P < 0.001$ versus combination therapy group.

Study groups/doses	Ulcer area (cm^2)	Ulcer score (0-3)	Ulcer index (0-8)	Colon weight (mg/8 cm)
Normal	$0.0 \pm 0.0^{***}$	$0\ (0\text{-}0)^{***}$	$0.0 \pm 0.0^{***}$	$585.3 \pm 13.4^{***}$
Control	4.9 ± 1.4	$3\ (2\text{-}3)$	7.6 ± 1.6	1188.6 ± 29.7
MTF (100 mg/kg)	$3.5 \pm 1^{*}$	$2.5\ (1\text{-}3)$	$5.5 \pm 1.8^{**}$	1116.6 ± 31.7
MTF (150 mg/kg)	$2.4 \pm 1.2^{***, ###}$	$1\ (0\text{-}3)^{*}$	$3.4 \pm 0.8^{***, ###}$	$996.9 \pm 40.5^{**, ###}$
MTF (200 mg/kg)	$0.7 \pm 0.35^{***}$	$1\ (0\text{-}1)^{**}$	$1.6 \pm 0.25^{***}$	$973.2 \pm 37.2^{***}$
Crocin (20 mg/kg)	$0.35 \pm 0.17^{***}$	$1\ (0\text{-}2)^{**}$	$1.1 \pm 0.6^{***}$	$916.8 \pm 30.2^{***}$
Crocin (30 mg/kg)	$0.3 \pm 0.2^{***}$	$0.5\ (0\text{-}1)^{**}$	$0.9 \pm 0.5^{***}$	$891 \pm 28.8^{***}$
Combination therapy (MTF 150 mg/kg + crocin 20 mg/kg)	$0.2 \pm 0.12^{***}$	$0.5\ (0\text{-}1)^{**}$	$0.7 \pm 0.5^{***}$	$809.6 \pm 36.5^{***}$
Dexamethasone (1 mg/kg)	$0.6 \pm 0.14^{***}$	$1\ (1\text{-}2)^{**}$	$1.6 \pm 0.15^{***}$	$749.4 \pm 21.6^{***}$

MTF, Metformin.

The rats treated merely with MTF at 150 mg/kg exhibited a significant reduction in ulcer area, ulcer index, and the wet weight of colon in comparison with those that received combination therapy, while relating to ulcer score, the difference between the mentioned groups was not meaningful. Moreover, comparison between the combination therapy group and crocin (20 mg/Kg) group did not reveal a significant difference in terms of macroscopic features (Table 2).

Effects of MTF, crocin, and their combination on microscopic parameters

Colons of all animals in the normal group showed typical architecture including mucosa with simple tubular colonic crypts covered with intact epithelium and extended down to the muscularis mucosae, submucosa with infiltration of slight number of inflammatory cells, muscularis and serosa layers (Fig. 2A). In contrast, intracolonic administration of acetic acid caused crypt damage and ulceration in mucus and sub-mucosal layers and necrosis of epithelium layers.

The control group exhibited extensive ulcerative and desquamated areas in the mucosa extending through the muscularis mucosa, severe mucosal necrosis, and crypt disarray with goblet cell depletion, crypt abscesses, lymphoid follicular hyperplasia, marked sub-mucosal edema, severe hemorrhages, and inflammatory cell infiltration (mainly neutrophils and plasma cells) to the mucosa, submucosa, muscularis, and serosa layers. In this group, depth of lesions was observed in the serous layer, but there was no evidence of fibrosis (Fig. 2B-D).

In detail, the colon of rats treated with MTF revealed moderate to severe focal ulceration and was accompanied by diffuse moderate inflammatory cell infiltration and mild to moderate edema and hemorrhages in the mucosa, submucosa, and muscularis layer (Fig. 3A-C). In the groups treated with crocin, mild to moderate inflammatory cell infiltration with mild focal ulceration and mild edema and hyperemia in the mucosa, submucosa, and muscularis layer, as well as more regular epithelium and crypts were observed in the colon tissues (Fig. 3D and E). Among the treated groups that received MTF and crocin, the most therapeutic effects were observed with the administration of MTF (150 mg/kg) + crocin (20 mg/kg). This combination resulted in minimal focal inflammatory changes, ulceration, and sub-mucosal edema hyperemia, as well as more improvement of the crypt architecture and epithelial arrangement (Fig. 3F). The dexamethasone group also showed a therapeutic effect in the damaged colon, similar to that seen in the groups treated with crocin (Fig. 3G).

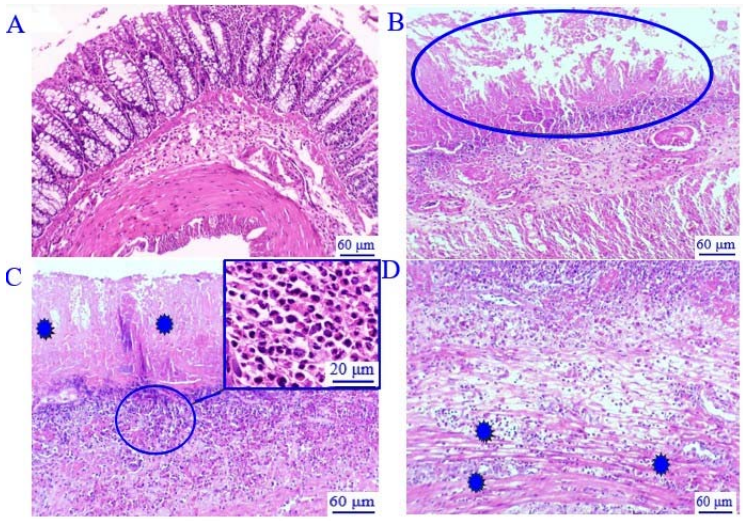
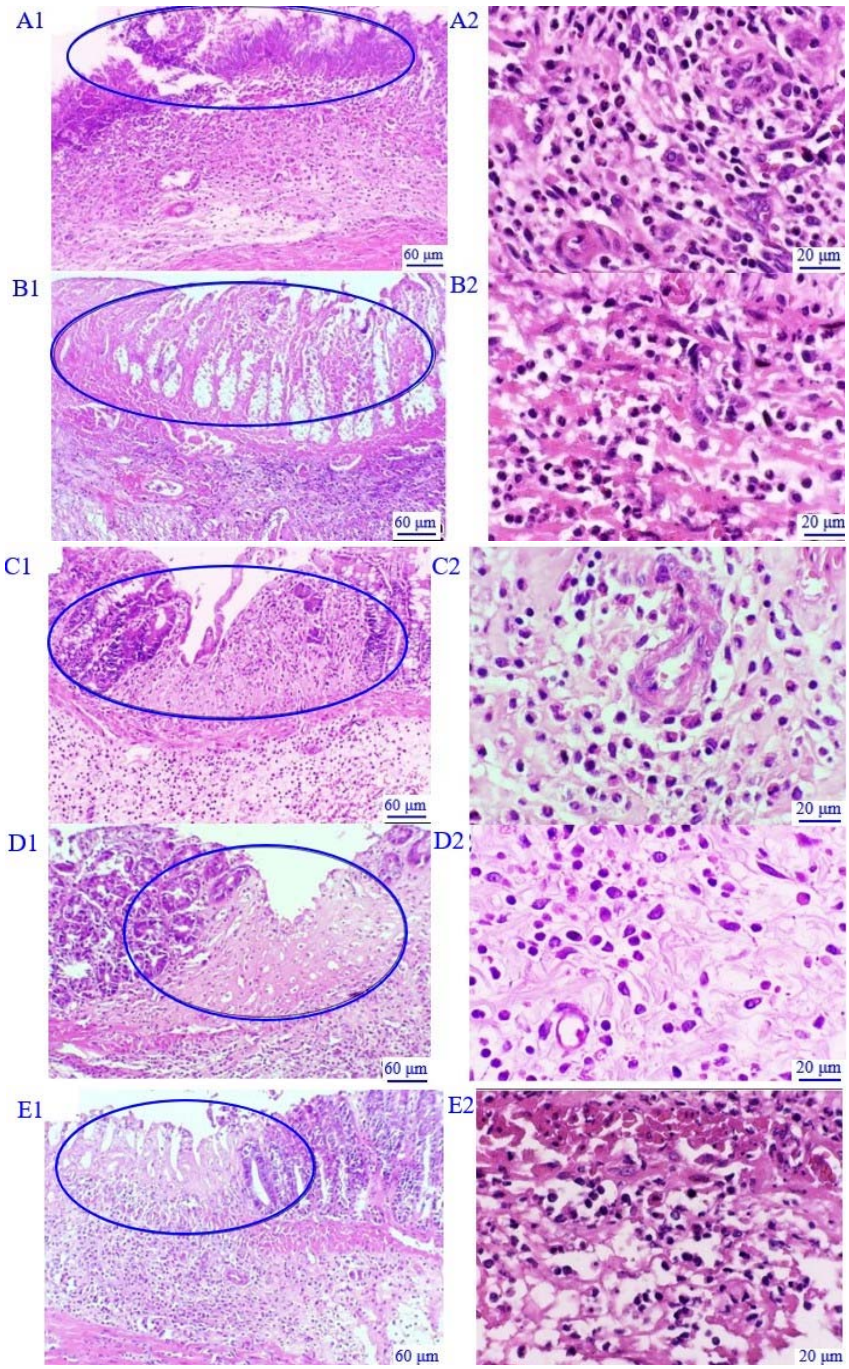


Fig. 2. Histopathological lesion in the colon tissue of normal and control groups (H&E staining; magnification: ×720). (A) Normal group, normal colon treated with normal saline/tween (5 mL/kg), normal architecture of colon tissue; (B) control group, treated with normal saline/tween (5 mL/kg) after colitis induction, focal ulceration, crypt destruction in the mucosal layer (circle); (C) control group, severe necrosis in the mucosal layer and severe infiltration of mono- and polymorphonuclear cells in the submucosal layer (circle); (D) control group, necrosis of muscle tissue and infiltration of inflammatory cells in the muscularis layer (stars).

In terms of pathological scores, treatment with MTF and crocin considerably attenuated the histopathological scores of colonic lesions compared to the acetic acid group. Our data showed that treatment with MTF at 200 mg/kg had its highest efficiency. On the other hand, comparison between the combination therapy group and MTF 150 mg/kg showed marked alleviation in combination therapy, which was not significant between this group and the group that received crocin at 20 mg/kg (Table 3).



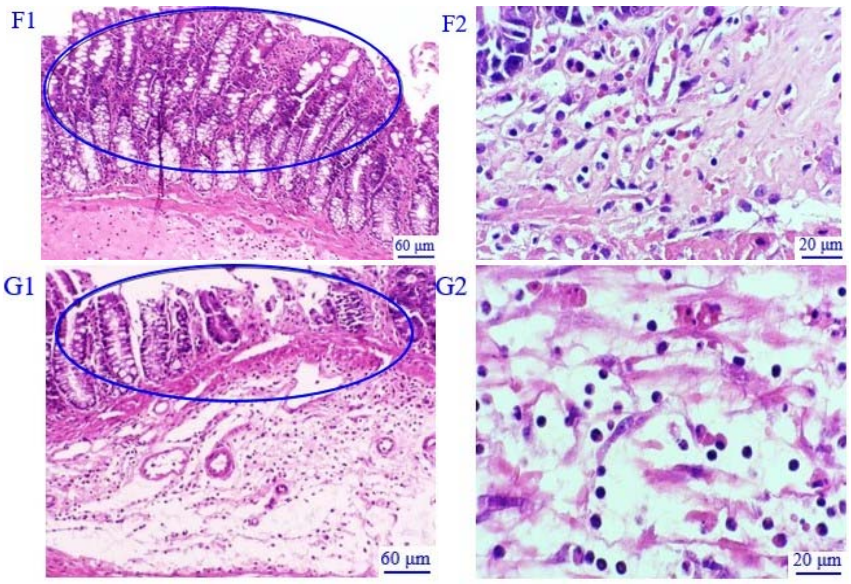


Fig. 3. Histopathological lesions in the colon tissue of different treated groups (H&E staining; magnification: ×720 for A1-G1 and ×2800 for A2-G2). (A1, A2) MTF (100 mg/kg), (B1, B2) MTF (150 mg/kg), (C1, C2) MTF (200 mg/kg), (D1, D2) crocin (20 mg/kg), (E1, E2) crocin (30 mg/kg); (F1, F2) MTF 150 mg/kg + crocin 20 mg/kg, (G1, G2) dexamethasone (1 mg/kg). Severe to moderate focal ulceration and necrosis in the groups treated with MTF (circle) (A1, B1, C1) accompanied with moderate inflammation in the submucosa layer (A2, B2, C2); mild focal ulceration and necrosis in the groups treated with crocin (circle) (D1, E1) and moderate to mild inflammation in the submucosa layer (D2, E2); minimal focal ulceration and necrosis in the combination group (circle) (F1) with a few inflammatory cell infiltrations in in the submucosa layer (F2); moderate focal ulceration and necrosis in the group treated with dexamethasone (circle) (G1) and mild inflammation in the submucosa layer (G2). MTF, Metformin.

Table 3. Histopathological and inflammatory response scores of colons in different groups. Normal group treated with normal saline/tween (5 mL/kg, p.o.); control group treated with normal saline/tween (5 mL/kg, p.o.) after colitis induction; The values are the median (range) and mean ± SEM for each group. $^{*}P < 0.05$ indicates significant difference compared to the normal group; $^{\#}P < 0.05$ versus control group.

Study groups/doses	Histopathological scores (0-15)		Inflammatory response scores (0-3)	
	Mean ± SEM	Median (min-max)	Mean ± SEM	Median (min-max)
Normal	0.26 ± 0.15	0 (0-2)	0.13 ± 0.09	0 (0-1)
Control	13.06 ± 0.22*	13 (11-14)	2.73 ± 0.11*	3 (2-3)
MTF (100 mg/kg)	9.80 ± 0.29$^{*,\#}$	10 (8-12)	2.53 ± 0.13*	3 (2-3)
MTF (150 mg/kg)	8.86 ± 0.33$^{*,\#}$	9 (7-11)	2.26 ± 0.11*	2 (2-3)
MTF (200 mg/kg)	6.66 ± 0.28$^{*,\#}$	7 (5-9)	2.20 ± 0.10$^{*,\#}$	2 (2-3)
Crocin (20 mg/kg)	3.53 ± 0.19$^{*,\#}$	4 (2-5)	1.46 ± 0.13$^{*,\#}$	1 (1-2)
Crocin (30 mg/kg)	2.93 ± 0.18$^{*,\#}$	4 (2-4)	1.33 ± 0.12$^{*,\#}$	1 (1-2)
Combination therapy (MTF 150 mg/kg + crocin 20 mg/kg)	2.20 ± 0.17$^{*,\#}$	2 (1-3)	1.13 ± 0.09$^{*,\#}$	1 (1-2)
Dexamethasone (1 mg/kg)	4.26 ± 0.20$^{*,\#}$	4 (3-6)	1.53 ± 0.13$^{*,\#}$	2 (1-2)

MTF, Metformin.

Colonic MPO activity

As presented in Fig. 4, in the control group, an almost fourfold increase in MPO activity in the colon tissues of the colitis rats group was observed compared with the normal group. Moreover, results of the current study demonstrated that in the group treated with MTF at 200 mg/kg, crocin-treated (20 and 30 mg/kg) groups, as well as the combination therapy group (150 mg/kg + 20 mg/kg), MPO

activity was declined considerably as compared to the control group. In doses of 100 and 150 mg/kg of MTF, this reduction was not significant. Dexamethasone (1 mg/kg), as a reference treatment, lowered the enzyme activity; however, it did not return to the normal level found in the normal group. Moreover, it seems that in the combination therapy group, MPO activity was considerably reduced compared to the group that merely received MTF (150 mg/kg); however, this difference was not significant between the combination therapy group and crocin (20 mg/kg) treatment group.

Serum MDA levels determination

Given the findings of the current study, MTF at doses of 100 and 150 mg/kg decreased the MDA level in the serum of rats, though not significantly. In contrast, administration of MTF at 200 mg/kg significantly reduced the MDA level in comparison with the colitis control group. In addition, both doses of crocin (20 and 30 mg/kg) and combination therapy with MTF and crocin, as well as dexamethasone, caused a notable reduction in serum MDA concentration (Fig. 5). Also, combination therapy, markedly declined the serum MDA level compared to MTF therapy group at the dose of 150 mg/kg, however the difference was not remarkable between combination therapy and crocin (20 mg/kg) groups.

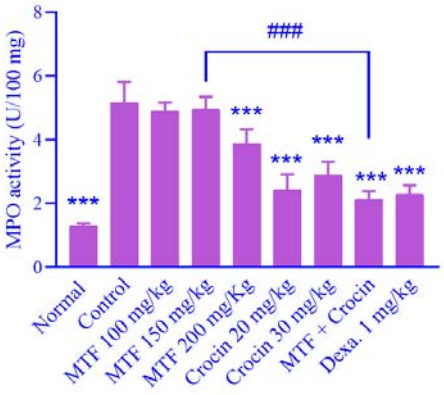


Fig. 4. MPO activity in colon tissues of rats. Data are shown as mean ± SD. ***P < 0.001 indicates significant differences compared to the control group; $^{###}P$ < 0.001 demonstrates a significant difference between the designated groups. Normal group treated with normal saline/tween (5 mL/kg, p.o.); control group treated with normal saline/tween (5 mL/kg, p.o.) after colitis induction. MTF, Metformin; Dexa, dexamethasone; MPO, myeloperoxidase.

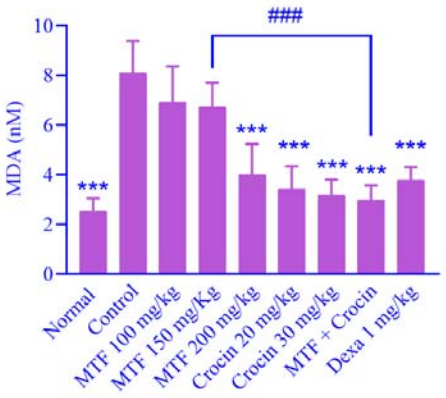


Fig. 5. Serum MDA levels of rats. Data are shown as mean ± SD.***P < 0.001 indicates significant differences compared to the control group; $^{###}P$ < 0.001 demonstrates a significant difference between the designated groups. Normal group treated with normal saline/tween (5 mL/kg, p.o.); control group treated with normal saline/tween (5 mL/kg, p.o.) after colitis induction. MTF, Metformin; Dexa, dexamethasone; MDA, malondialdehyde.

DISCUSSION

Among the animal models of UC, acetic acid-induced colitis is a reproducible chemical model that mimics human UC in terms of histopathological features, mechanism of pathogenesis, and inflammatory responses (32). Over recent decades, numerous compounds with antioxidant and anti-inflammatory properties, particularly administered as combination therapies, have shown promise in improving UC outcomes (25,33). In this study, macroscopic, microscopic, and biochemical analyses indicated that combination therapy with MTF and crocin effectively ameliorated acetic acid-induced colitis in rats. Our macroscopic analysis revealed significant weight loss and severe damage to colon tissue, characterized by edema, increased thickness, and necrosis, as a result of intrarectal administration of acetic acid. Additionally, histopathological assessments revealed pronounced crypt damage and ulceration in the mucosal and submucosal layers. Moreover, elevated colonic MPO activity and serum MDA levels observed in the control group suggested increased macrophage and neutrophil infiltration, driving oxidative stress and lipid peroxidation. Data analysis indicated that although MTF reduced the trend of weight loss, most effectively at the 200 mg/kg dose, it did not result in weight

gain as expected. This outcome may be attributed to MTF's potential effects on weight loss. MTF promotes weight loss through multiple metabolic mechanisms, including appetite suppression, improved insulin sensitivity, reduced hepatic gluconeogenesis, and increased energy expenditure. Its anorectic effect, potentially mediated by hypothalamic pathways, decreases caloric intake, while enhanced insulin sensitivity in peripheral tissues (*e.g.,* skeletal muscle and adipose tissue) facilitates glucose uptake and utilization, reducing blood glucose levels and fat storage (34,35). MTF at a dosage of 200 mg/kg appeared to stabilize the animals' condition by reducing inflammation, resulting in more effective weight maintenance compared to lower doses. Moreover, dexamethasone, as a glucocorticoid, exerts a catabolic effect, which is probably responsible for the animal weight loss in the reference group. On the other hand, individual doses of crocin and its combination with MTF considerably abrogated body weight loss value, with the lowest percentage of weight loss seen in the combined therapy group. Concerning the macroscopic parameters, data analysis indicated that all doses of MTF and crocin, except for MTF at 100 mg/kg, significantly reduced the ulcer index and colon weight, the primary indicators of macroscopic parameters.

It is well-established that neutrophil recruitment to the colon tissue triggers the production of superoxide anion, initiating a chain reaction that generates various reactive species. The accumulation of these reactive species can cause significant tissue damage and exacerbate mucosal dysfunction in the gut (36). Our findings demonstrated that the increased MPO activity in distal colon segments, along with elevated serum MDA levels in the colitis control group following intrarectal acetic acid administration, were diminished in MTF and crocin-treated groups. Except for the 100 and 150 mg/kg doses of MTF, other treatment groups, including both doses of crocin, 200 mg/kg of MTF, combination therapy, and dexamethasone, effectively reduced MPO activity and MDA levels. It is noteworthy that no dose-dependent relationship regarding MPO activity was detected in either the groups treated with crocin or those treated with MTF. Additionally, histopathological scores showed that treatment with MTF and crocin significantly reduced the severity of colonic lesions. This effect exhibited a dose-dependent relationship, with the most effective outcomes observed in the combination therapy group. Furthermore, the combination therapy group demonstrated greater efficacy across all investigated parameters compared to dexamethasone as the reference drug.

Based on our observations in the monotherapy groups, two optimum doses of MTF and crocin (150 and 20 mg/kg) were selected for combination therapy to clearly demonstrate the superiority of combination therapy over monotherapy.

Combination therapy with MTF and crocin in our study offered a promising approach, likely due to the complementary mechanisms through which each compound addresses inflammation, oxidative stress, and cellular repair. A study carried out by Pandey *et al*. showed that MTF resulted in a decrease in mucosal injury, ulcer score, and MPO activity. They reported that through suppressing ROS production, inflammatory mediator release, such as TNF-α, MTF exerts its protective effects in UC (37). Another experiment performed by Chen *et al*. reported that the phosphorylated form of AMPK (Thr_{172}) in colonic epithelial cells decreased following colitis induction. They observed that MTF administration alleviated UC outcomes, likely by activating AMPK, which promotes oxidative energy metabolism essential for anti-inflammatory activity in cells such as M2 macrophages and regulatory T cells. AMPK activation also suppresses inflammation through various signaling pathways such as NF-kB and mitogen-activated protein kinases (MAPK). They also demonstrated that inflammation-related factors, including IL-1β, IL-6, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2), were significantly reduced following MTF treatment (38). Additionally, research by Xue *et al.* indicated that MTF supplementation reduced intestinal permeability in IL10KO mice, though, as observed in our study, it did not promote weight gain. Supplementation with MTF in IL10KO mice reduced the pro-inflammatory activity of macrophages, as evidenced by decreased expression levels of the pro-inflammatory cytokines IL-1β, TNF-α, and interferon gamma (IFN-γ). Consistent with our results, El-Shahat *et al.* found that a 200 mg/kg dose of MTF significantly improved weight loss,

colon shortening, and histological scores in DSS-induced colitis. They concluded that MTF's protective effects in UC may occur through the modulation of metallothioneins, NF-κB expression, and reduced apoptosis (12,39). Furthermore, the beneficial effects of MTF, alone and in combination with alpha-lipoic acid, on body weights, survival rates, disease activity index, colonic oxidative stress markers, and TNF-α levels in a chronic model of colitis were reported earlier (40). On the other hand, Rezaei *et al*. reported that crocin pre-treatment in DSS-induced colitis mice attenuated disease activity index, fibrosis, and cell proliferation and generated anti-inflammatory responses in colon tissues of colitis mice and subsequently decreased the risk of colitis-associated colorectal cancer, as one of the most serious consequences of UC (22). Other investigations also reported the anti-ulcerogenic and colo-protective effects of crocin in animal models (23,41). In Khorasani *et al.'s* experiments, the pollen of *Crocus sativus* extract administration resulted in an improvement in wound healing in rat models. Histopathologic data showed re-epithelialization of the epidermis and mild inflammatory cell infiltration (42). In accordance with the above-mentioned studies, we observed that crocin treatment significantly reduced the ulcer area, ulcer score, and ulcer index compared to the control group.

Taken together, combination therapy likely led to a more comprehensive therapeutic effect. It seems that, while MTF decreased inflammation through the abovementioned mechanisms, crocin complemented this effect by directly reducing oxidative stress and inflammatory mediator levels. This multifaceted approach not only enhanced mucosal healing but also improved overall gut health, resulting in a significantly better treatment outcome compared to monotherapy with MTF. Such findings highlight the potential of this combination therapy as a novel strategy for managing UC effectively. The measurement of inflammatory mediators such as IL-6, IL-1β, and TNF-α, and oxidative stress indicators such as catalase, superoxide dismutase activities, and total antioxidant capacity, as well as intestinal microbiota evaluations, which were not possible due to limitations in this study, would enhance our understanding of the mechanisms underlying this effect.

CONCLUSION

In conclusion, the present study demonstrated that combination therapy with MTF and crocin significantly alleviated acetic acid-induced colitis in rats, outperforming both monotherapy and dexamethasone across multiple parameters. This combined treatment effectively reduced macroscopic damage, including ulcer index and colon weight, as well as histopathological scores and biochemical markers of inflammation and oxidative stress. It seems that complementary mechanisms of MTF and crocin, targeting inflammation *via* AMPK activation and NF-κB inhibition while concurrently reducing oxidative stress, highlight their potential to enhance mucosal healing and improve overall gut health. These findings suggest that MTF and crocin in combination therapy may offer a promising, multifaceted approach to managing UC and related inflammatory conditions. Further research is warranted to explore its translational potential in clinical settings.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

B. Koohshekan executed all of the experiments and interventions under the supervision of the supervisor and the adviser professors. MH. Aarabi presented the idea of research, designed, and supervised the project. M. Minaiyan supervised all of the parts related to the grouping of animals, determining the doses of drugs, and the induction of colitis. M. Hashemnia supervised all procedures involving tissue sampling, preparation, and histopathological evaluation. All authors contributed to the study design, data analysis, manuscript preparation, and final approval.Each author has fulfilled the authorship criteria and affirmed that this article represents honest and original work.

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