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RESEARCH ARTICLE

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Crocin synergistically enhances the antiproliferative activity of 5-flurouracil through Wnt/PI3K pathway in a mouse model of colitis-associated colorectal cancer

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Abstract

Colorectal cancer (CRC) is the third most common cause of cancer-related death, and hence there is a need for the identification of novel-agents to improve the efficacy of existing therapies. There is growing evidence for the antitumor activity of crocin, although its activity and molecular mechanisms in CRC remains to be elucidated. Here we explored the therapeutic application of crocin or its combination with 5-flurouracil in a mouse model of colitis-associated colon cancer. The antiproliferative activity of crocin was assessed in two-dimensional and three-dimensional cell-culture models. The migratory behaviors were determined, while the expression levels of several genes were assessed by quantitative reverse transcriptase polymerase chain reaction/Western blot analysis. We examined the anti-inflammatory properties of crocin by pathological evaluation and disease-activity index as well as oxidative or antioxidant markers: malondialdehyde (MDA) and total-thiols (T-SH) levels and superoxide dismutase (SOD) and catalase (CAT) activity. Crocin suppressed cell-growth and the invasive behavior of CRC cells through modulation of the Wnt-pathway and E-cadherin. Moreover, administration of crocin alone, or in combination with 5-FU dramatically reduced the tumor number and tumor size in both distal/mid-colon followed by reduction in disease-activity index. Crocin also suppressed the colonic

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inflammation induced by dextran-sulfate-sodium and notably recovered the increased levels of MDA, decreased thiol levels and activity of CAT levels. Crocin was able to ameliorate the severe inflammation with mucosal ulcers and high-grade dysplastic crypts as detected by inflammation score, crypt loss, pathological changes and histology scores. We demonstrated an antitumor activity of crocin in CRC and its potential role in improvement of inflammation with mucosal ulcers and high-grade dysplastic crypts, supporting the desireability of further investigations on the therapeutic potential of this approach in CRC.

KEYWORDS

antitumor effect, colitis-associated colorectal cancer gastrointestinal, colorectal cancer, crocin

1 | INTRODUCTION

Colorectal cancer (CRC) remains the third most common type of malignancy globally.[1] Recently, both clinical and basic science studies have made considerable progress in the therapeutic strategies used in CRC; these currently include surgery, radiotherapy, and, most notably, chemotherapy. However, the overall survival rate of patients with CRC remains disappointing due to drug resistance, severe side effects, and high recurrence rate.[2] 5-fluorouracil (5-FU) treatment, alone or in combination with other chemical cytotoxic drugs, is among the routinely used standard therapies in CRC.[3-6] Despite its therapeutic benefits, 5-FU has also shown limited efficacy, high resistance, and a low response rate in advanced CRC patients.[7,8] These limitations have necessitated further research for developing more efficient alternative or potential combinational agents in chemopreventive and chemotherapeutic strategies to enhance effectiveness, while reducing toxicity and resistance.

Recently, crocin has emerged as a novel anticancer agent in the treatment of several tumor types, including hepatic, pancreatic, prostate, breast, and colorectal cancers.[9-13] There are already studies showing the therapeutic value of crocin as anticonvulsant, anti-inflammatory, antidepressant and antitumor molecule.[14] Hoshyar et al[15] showed that crocin could induce apoptosis in gastric adenocarcinoma cells via increasing Bax/Bcl-2 ratio in stimulated cells. Bathaie et al explored the beneficial activity of saffron extract on gastric cancer in an in vivo experiment. They showed that an aqueous extract of saffron, inhibited the progression of cancer in rats dose dependently in gastric cancer.[16,17] Moreover, a study in diethylnitrosamine-treated rats showed that saffron potently inhibited nodular and foci of altered hepatocyte formation in the rat liver. The authors found that this inhibition was correlated with induced apoptosis, decreased cell proliferation, reduced oxidative stress and downregulation of some inflammatory markers including nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), inducible nitric oxide synthase (iNOS) and COX-2.[18] Furthermore, Aung et al[13] reported that *Crocus sativus* extract and crocin possess remarkable antiproliferative effects on CRC cells. Similarly, it has been shown that long-term treatment with crocin elevated survival rate in rats with CRC.[19]

In this current study, we assessed the antitumor activity of crocin alone to investigate its potential as a chemotherapeutic compound, and also in combination with 5-FU to explore whether crocin treatment could improve the antitumor activity of 5-FU both in in vitro and in colon cancer mice models. Moreover, we investigated the antioxidant activity of crocin. Next, to investigate the underlying molecular changes within anticancer activity of crocin, alone and in combination with 5-FU, we evaluated the expression of related specific key molecular markers in monolayer and three-dimensional cultured cells to imitate the tumor microenvironment.

2 | MATERIALS AND METHODS

2.1 | Drugs and chemicals

Crocin was obtained from Faculty of Pharmacy, Mashhad University of Medical Sciences (Mashhad, Iran). 5-FU was purchased from MUMS and dissolved in ethanol or sterile water. Dulbecco modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin (50 IU/mL) and streptomycin (50 μg/mL) were purchased from Gibco (Gaithersburg, MD). Azoxymethane (AOM), and dextran sodium sulfate (DSS; molecular weight = 40 kD) were purchased from Sigma–Aldrich (St Louis, MO).

2.2 | Cell culture

CT-26 cell line was originally purchased from Pasteur Institute, Tehran, Iran. The cells were grown in DMEM containing 10% heat-inactivated FBS and 1% streptomycin/penicillin. The cells were maintained at 37°C in 5% CO_2 atmosphere. Regular passaging was performed in their exponentially growing phase, at 70% to 80% confluency, by using trypsin-EDTA.[20]

2.3 | Growth inhibition studies

The cell-growth inhibitory effects of crocin, 5-FU, and their combination were evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay before and after 24-hour to 72-hour treatment. CT-26 cells were treated for 24 hours with crocin (1–500 μM), 5-FU (1 to 500 nM), and simultaneous combination at a fixed ratio based on IC_{50} (ie concentration of a drug required for 50% inhibition of cell growth) of each drug. The plates were then processed for MTT as described previously.[21]

2.4 | Evaluation of synergistic/antagonistic interaction with 5-FU

The pharmacological interaction between crocin and 5-FU was evaluated by the median drug effect analysis method as described previously.[22] Briefly, the combination index (CI) was calculated to compare cell growth inhibition of the combination and each drug alone. Data analysis was carried out using CalcuSyn software (Biosoft, Oxford, UK).

2.5 | Multicellular spheroids

The spheroids were formed by seeding 10^5 cells/mL in DMEM/F12 + GlutaMAX-I (1:1) in agarose coated 96-well plates. The cell attachment, growth, and cytotoxic effects were determined for 10 days under the inverted phase contrast microscope Leica-DMI300B (Leica, Wetzlar, Germany). Spheroid volume (V) was defined from the geometric mean of the perpendicular diameters $D = (D_{max} + D_{min})/2$, as follows: $V = (4/3) \times \pi\ (D/2)3$.[23]

2.6 | In vitro invasion assay

Cell invasion assays were carried out using transwell chambers with polycarbonate membranes and 8-μm pores.[22,24] The transwell filters were coated with 100 μL of 0.1 mg/mL collagen I solution. Briefly, 10^5 cells were plated on the upper face of the filter and incubated with crocin at 2 mM in serum free media. Migratory cells attached to the polycarbonate membranes were fixed with paraformaldehyde solution for 24 hours. The filters were then photographed and counted after visualization with Giemsa stain.

2.7 | Migration assays

The ability of crocin and its combination with 5-FU to inhibit the migratory behavior of CT-26 cells was investigated by in vitro migration assay, as described previously.[25] The cells were exposed to the drug at their 2 mM. Images were taken at the beginning of the exposure (time 2), with those taken after 7, 11, 13, 23, and 32 hours.

2.8 | Quantitative reverse transcriptase polymerase chain reaction

Total RNAs were extracted from the cells before and after treatment with crocin at IC_{50} (3 mM) and using the RNXPLOS (CinaColon, Tehran, Iran), according to the manufacturer's protocol. cDNA was synthesized by cDNA synthesis kit (CinaColon, Tehran, Iran). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed with specific primers for cyclin D1, survivin, MMP-2, MMP-9, and E-cadherin (Macrogene Co, Seoul, Korea). The cDNA was amplified by ABI-PRISM StepOne instrument (Applied Biosystems, Foster City, CA). Gene expression data were normalized to glyceraldehyde 3-phosphate dehydrogenase, using a standard curve of cDNAs purchased from Quantitative PCR Human Reference RNA (Stratagene, La Jolla, CA), as described.[26,27]

2.9 | Cell cycle assay

To further investigate the antiproliferative effect of crocin on cell cycle progression, CT-26 cells were treated with two concentrations of crocin (3 and 5 mM) for 24 hours and were analyzed using flow cytometry, as described previously.[25,28]

The effect of drugs on modulation of cell cycle was assessed in the cells treated for 24 hours with two concentrations of crocin at IC_{50} concentrations. Cells were stained by propidium iodide (PI) and cell cycle modulation was determined using a FACSCalibur flow cytometer (Becton Dickinson, San José, CA), equipped with the CELLQuest (Becton Dickinson and Company, Asia Pacific Division, Singapore) software for data analysis. The ability of crocin and its combination with 5-FU to induce cell death was explored by measuring sub-G1 regions during cell cycle analysis, as described previously.

2.10 | Western blot analysis

Western blot analyses were performed as described previously.[29] Briefly, 40 μg of proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene difluoride membrane (Immobilion-FL; Millipore, Billerica, MA). The membrane was incubated overnight with rabbit anti-cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PI3K, anti-p-Akt, and anti-p-GSK3α/β (Abcam, Cambridge, UK), and anti-β-actin (1:10 000; Sigma-Aldrich). The secondary antibody was goat anti-rabbit (1:10 000; Westburg, Leusden, The Netherlands). The membrane was then proceeded for visualization via chemiluminescence detection.[30,31]

2.11 | Animal experiment

The animal experiments were approved by Ethical Committee of animal experiment of the Mashhad University of Medical Science. Eight-week old female C57BL/6 mice were supplied by Pasteur Institute (Tehran, Iran) and divided into four groups (n = 6 each): control, treated with 5-FU, or crocin, or a 5-FU/crocin combination. Mice were given a single intraperitoneal injection of AOM (10 mg/kg body weight followed by three cycles of 1.5% DSS in drinking water for 1 week and normal drinking water for 2 weeks.[32] Starting one week after the second DSS exposure, groups 3, 4, and 5 received diets containing crocin (200 ppm in drinking water), 5-FU (35 mg/kg once weekly[33] and crocin + 5-FU, respectively). Group 1 was served as an untreated control. All animals were killed at week 10. For macroscopic and histological examination of tumors, the colons were isolated and washed by ice-cold phosphate-buffered saline (PBS) and opened longitudinally and assessed tumor number and tumor size with digital caliper. Tumor localization was also determined. Then, distal colon samples were placed into 10% formalin solution for histopathological examination. The histopathological examination was conducted on paraffin-embedded 5 μm sections which were stained with hematoxylin and eosin (H&E). The middle parts of colons were stored at − 70°C for evaluation of oxidative/antioxidative markers. For this purpose, colonic tissues were homogenized on ice in tissue-lysis buffer. Homogenates were then centrifuged at 4°C for 1 hour at 10 000 rpm, and the supernatants were used for oxidative/antioxidative markers.

2.12 | Scoring inflammation

The inflammation was scored in the large bowel. Large intestinal inflammation was graded with respect to the morphological criteria[34] as following: normal appearance (grade 0); shortening and loss of the basal 1/3 of the actual crypts together with mild inflammation in the mucosa (grade 1); loss of the basal 2/3 of the crypts and moderate inflammation in the mucosa (grade 2); loss of all of the crypts plus severe inflammation in the mucosa and submucosa, while the surface epithelium retained (grade 3); the mucosal ulcer presentation with sever inflammation in the mucosa, submucosa, muscularis propria, and/or subserosa (grade 4). The scoring was carried out on the entire colon with or without proliferative lesions and was considered as a mean score/mouse.[35]

2.13 | Measurement of MDA

Malondialdehyde (MDA) level is a marker of lipid peroxidation and was used to evaluate the antioxidant activity of crocin. Shortly, 1 mL of 10% homogenates mixed with 2 mL of TBA + TCA + HCL solution for 45 minutes in a boiling water bath and centrifuged for 10 minutes. Then, the absorbance at 535 nm was read and MDA was calculated by $C\ (\mathrm{M}) = (A/1.65 \times 10^5)$.[35]

2.14 | Measurement of total thiol groups (SH)

Total thiol was measured using di-thio nitrobenzoic acid (DTNB) reagent. A 1 mL of Tris-EDTA buffer (pH = 8.6) was added to tissue homogenate. The specimen absorbance read at 412 nm against Tris-EDTA buffer alone (A_1). Then 20 μL of DTNB reagents were added to this solution and stored for 15 minutes at room temperature. Then, the sample absorbance was record again (A_2). The absorbance of DTNB reagent was recorded lonely as a blank (B). Total thiol concentration (mM) was calculated from the following formula (total thiol concentration $(\mathrm{mM}) = (A_2 - A_1 - B) \times (1.07/0.05 \times 13.6)$.[36]

2.15 | Measurement of SOD and CAT activity

Superoxide dismutase (SOD) was assessed by Ransod kit (Randox Laboratory, London, UK), while catalase (CAT) activity was evaluated as described by Aebi.[37] The principle of this method was based on the hydrolyzation of H_2O_2 in phosphate buffer, pH 7.0, and essential volume of sample and reducing absorbance at 240 nm. Enzyme response velocity can be measured by the conversion of H_2O_2 to H_2O and O_2 in 1 minute below standard condition.[35]

2.16 | Statistical analysis

All as mean values ± SEM and analyzed by Student t test or analysis of variance followed by Tukey's

multiple comparison test. Data were analyzed by SPSS v.20 statistical software (IBM, Chicago, IL). Statistical significance was set at $P < 0.05$.[38]

3 | RESULTS

3.1 | Crocin inhibits colon cancer cell growth in monolayer cell culture

To investigate the antiproliferative activity of crocin alone and its combination with 5-FU, CT-26 cells were exposed to increasing concentrations of crocin (1 nM to 10 mM) and 5-FU (1 to 50 mg/mL) for 24 hours. This analysis showed that crocin and 5-FU inhibited cell growth in a dose-dependent manner (Figure 1A). Results clearly showed that cotreatment of crocin and 5-FU decreased the IC_{50} value of 5-FU. The median drug effect analysis, comparing cell growth inhibition of the combination and each drug alone revealed a slight synergism between crocin and 5-FU (Figure 1B).

3.2 | Crocin causes tumor shrinkage in CT-26 cells

To further assess whether crocin could be active in three-dimensional models of cell culture and overcome the major obstacles in conventional cell culture method, CT-26 spheroids were developed and treated with crocin at IC_{50} (3 mM) and $5\times IC_{50}$ values. Tumor shrinkage was detected after 8 days as shown in Figure 1C.

3.3 | Crocin and its combination with 5-FU inhibit invasive behavior of CRC cells

The effect of crocin on cell invasion was explored by the invasion assay in the cells treated with crocin at IC_{50} (3 mM) and $5xIC_{50}$ values. Our data showed that the invasion of CT-26 cells, treated with crocin and 5-FU in combination, was significantly reduced as compared with control group (Figure 1D). To further investigate the mechanism of the inhibitory effect of crocin on CRC invasive behavior, expression of E-cadherin, a cell adhesion molecule, was analyzed in the presence and absence of crocin in these cells.

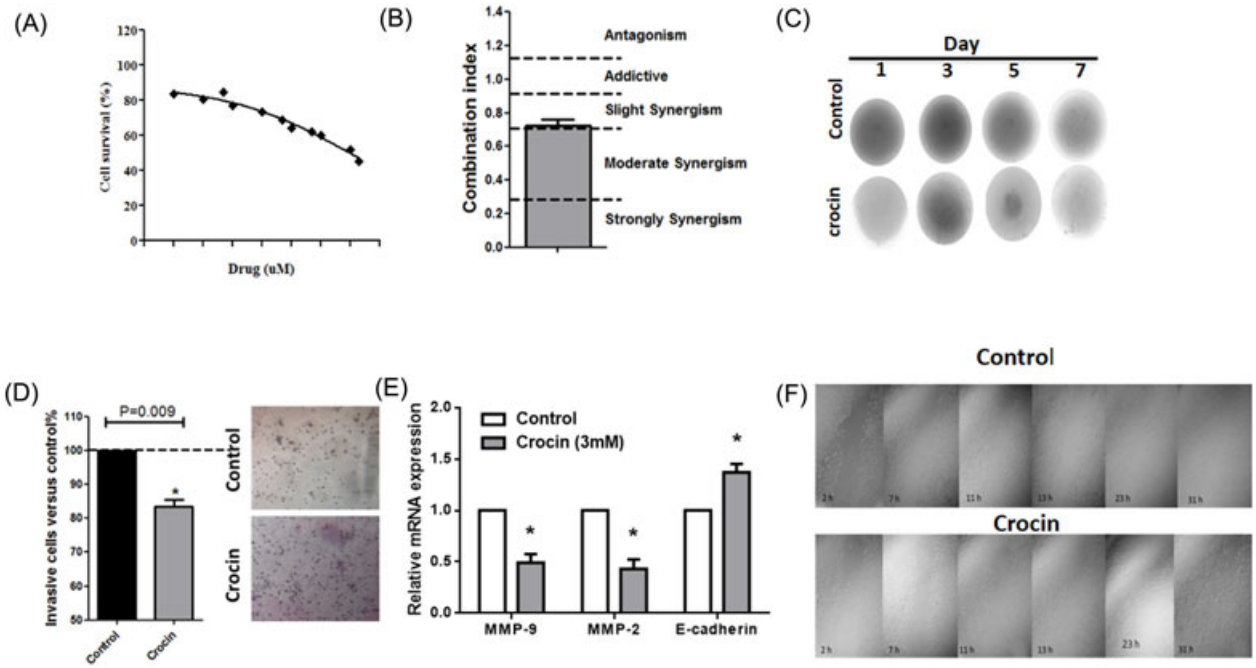

FIGURE 1 Crocin inhibits cell proliferation and invasion of colon cancer cells. A, Growth inhibitory effects of crocin (μM) after 72 hours exposure to crocin in CT-26 cells. B, Mean combination index (CI) of the crocin or 5-FU treatment. CI values at fractional effect analysis (FA) of 0.5, 0.75, and 0.9 were averaged for each experiment, and this value was used to calculate the mean between experiments. C, Effect of crocin on the CRC spheroids. D, Results of invasion experiment in the CRC cells exposed to crocin at IC_{50} values for 24 hours. E, Modulation of E-cadherin, MMP-2, and MMP-9 mRNA levels in CRC cells after 24 hours exposure to crocin (3 mM) as determined by qRT-PCR. F, The inhibitory effect of crocin on the migration of CT-26 cells. Columns or points, mean values obtained from three independent experiments; scale bar, SEM. *Significantly different from controls. MMP, matrix metalloproteinase; mRNA, messenger RNA; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction

Results showed that crocin significantly increased expression of E-cadherin in crocin-stimulated cells (Figure 1E).

3.4 | Crocin and its combination with 5-FU inhibited the migratory behavior of colorectal cancer cells

To investigate the effects of crocin on migratory behavior, a scratch mobility assay was carried out in CT-26 cells (Figure 1F). The cells were exposed to crocin in IC_{50} concentration (3 mM) and they showed a significant reduction of migration starting after 20 hours exposure to crocin compared to control.

To further confirm the antimetastatic behavior of the cells treated with crocin, we evaluated the expression of matrix metalloprotease-2 and -9 (MMP-2 and MMP-9), two gelatinases which facilitate cell invasion by breaking down the extracellular matrix.[39] As shown in Figure 1E, crocin significantly suppressed the expression of MMP-2 and MMP-9 suggesting the mechanism by which crocin decreased cellular migration and metastasis in CRC cells.

3.5 | Crocin suppresses cell cycle progression in CRC cells

To further explore the regulatory effect of crocin on cell cycle progression, CT-26 cells were treated with two concentrations of crocin (3 and 5 mM) for 24 hours and were analyzed by flow cytometry method. Our results indicated that crocin significantly increased the percentage of sub-G1 population in dose-dependent manner (Figure 2A-D). As shown in Figure 2, when cells were treated with crocin (3 mM), the percentage of Sub-G1 population showed a marked increase (23.1%) compared with the control group (1.1%). These data suggest that crocin induces CRC cell apoptosis by arresting cells in G0/G1 phase of cell cycle.

3.6 | Crocin suppresses tumor growth in a mouse model of colitis-associated colorectal cancer

We first develop colitis-associated colorectal cancer models, as the workflow outlined in Figure 3A. Mice

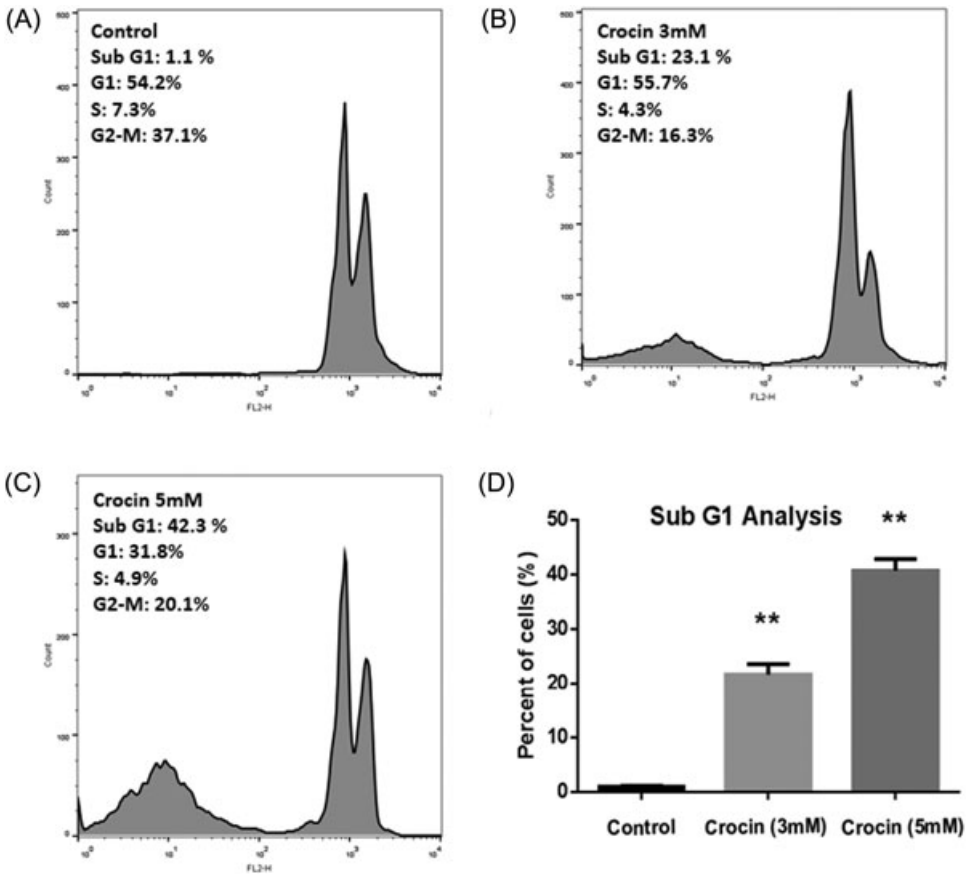

FIGURE 2 Cell cycle distribution of the colon cancer cells upon crocin treatment. CT-26 cells were treated with two concentrations of crocin (3 and 5 mM) for 24 hours and after staining with propidium iodide (PI), analyzed by flow cytometry for cell cycle and sub-G1 analysis (A-D), respectively. $^{**}P < 0.01$

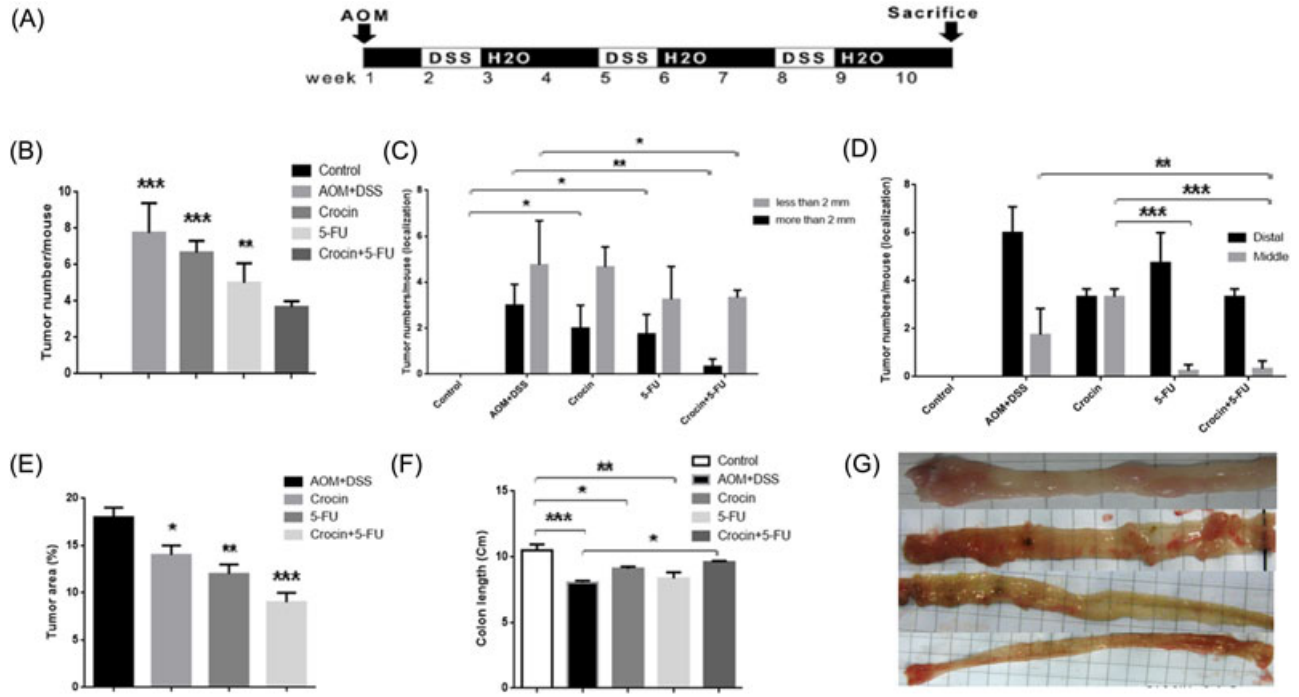

FIGURE 3 Crocin suppresses tumor growth in a mouse model of colitis-associated colorectal cancer. A, The treatment schedules and development of colitis-associated colorectal cancer models. Mice were given 10 mg/kg body weight AOM. One week after AOM injection, they received 1.5% DSS/oral gavage for 7 days, followed by 2 weeks rest. One week after DSS exposure groups 3, 4, and 5 were treated with crocin, 5-FU, crocin + 5-FU for 15 weeks, respectively. B, Colitis-associated colorectal cancer mice were treated with different reagents as indicated and number of tumors were analyzed in each group. C, The same as B, except that tumor size was analyzed. D, The same as B, except that tumor location was analyzed in each group. E, Colitis-associated colorectal cancer mice were treated as explained in B and tumor area was analyzed. F, The same as B, except that colon length was analyzed. G, Picture of colons, indicating tumors in four groups, AOM/DSS, 5-FU, crocin, and 5-FU + crocin. Columns or points, mean values obtained from three independent experiments; scale bar, SEM. *Significantly different from controls. AOM, azoxymethane; DSS, dextran sodium sulfate; 5-FU, 5-fluorouracil. $^{**}P < 0.01$; $^{***}P < 0.0001$

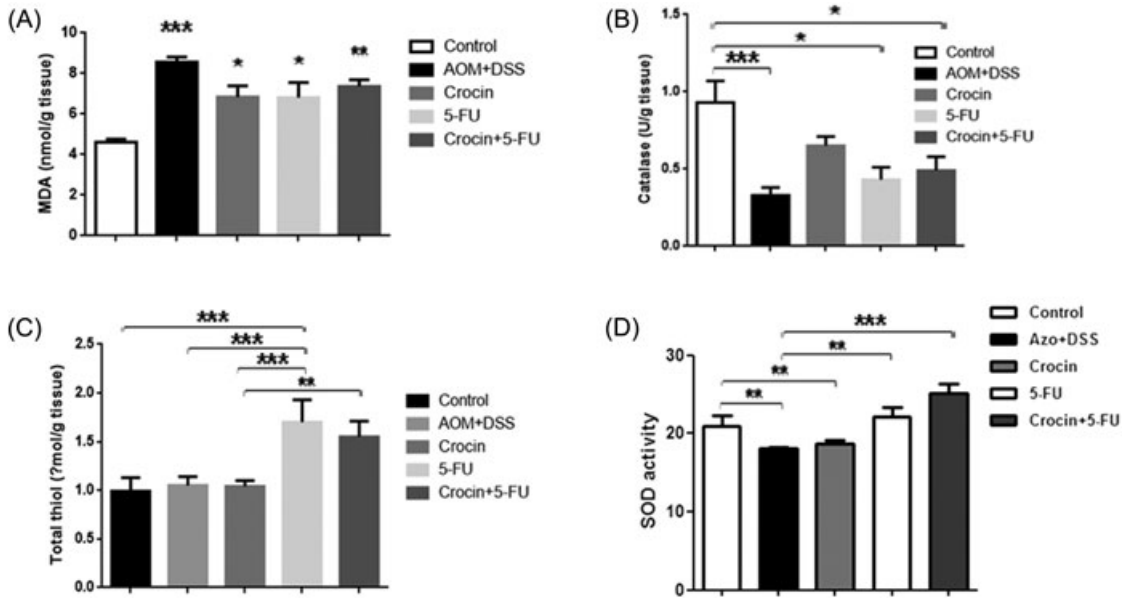

FIGURE 4 Crocin regulates oxidative stress in homogenized colon samples. Mice were treated ad indicated and following collection of colon samples. MDA (A), catalase (B), total thiol concentration (C) and SOD activity (D) were measured in colon tissues. Columns or points, mean values obtained from three independent experiments; scale bar, SEM. *Significantly different from controls. AOM, azoxymethane; DSS, dextran sodium sulfate; 5-FU, 5-fluorouracil; SOD, superoxide-dismutase

were given 10 mg/kg body weight AOM. One week after AOM injection, they received 1.5% DSS/oral-gavage for 7 days, followed by 2 weeks rest. One week after DSS exposure groups 3, 4, and 5 were treated with crocin, 5-FU, crocin and 5-FU for 15 weeks, respectively. The possible physiological significance of antiproliferative effect of crocin was demonstrated by findings that crocin potently decreased CRC growth in animal model too. As shown in Figure 3B, the tumor numbers were significantly reduced in crocin and 5-FU groups. Interestingly this reduction was much higher in combination group (Figure 3B). Similar results were also observed for tumor size and tumor number based on the location with respect to the colon area and we observed treatments had more impact on larger tumor (more than 2 mm in diameter (Figure 3C and 3D). Moreover, our data showed that tumor area with respect to the colon length was markedly decreased in combination group, compared to crocin or 5-FU groups or control group (Figure 3E). Also crocin was able to improve the colon length, compared to control mice which they did not received therapy (Figure 3F and 3G).

3.7 | Crocin affects MDA, thiol, and CAT activities

To determine the role of crocin on oxidative stress, the levels of SOD, CAT, and MDA and thiol were analyzed in homogenized colon samples and results presented in Figure 4A-D. In particular the level of MDA and CAT

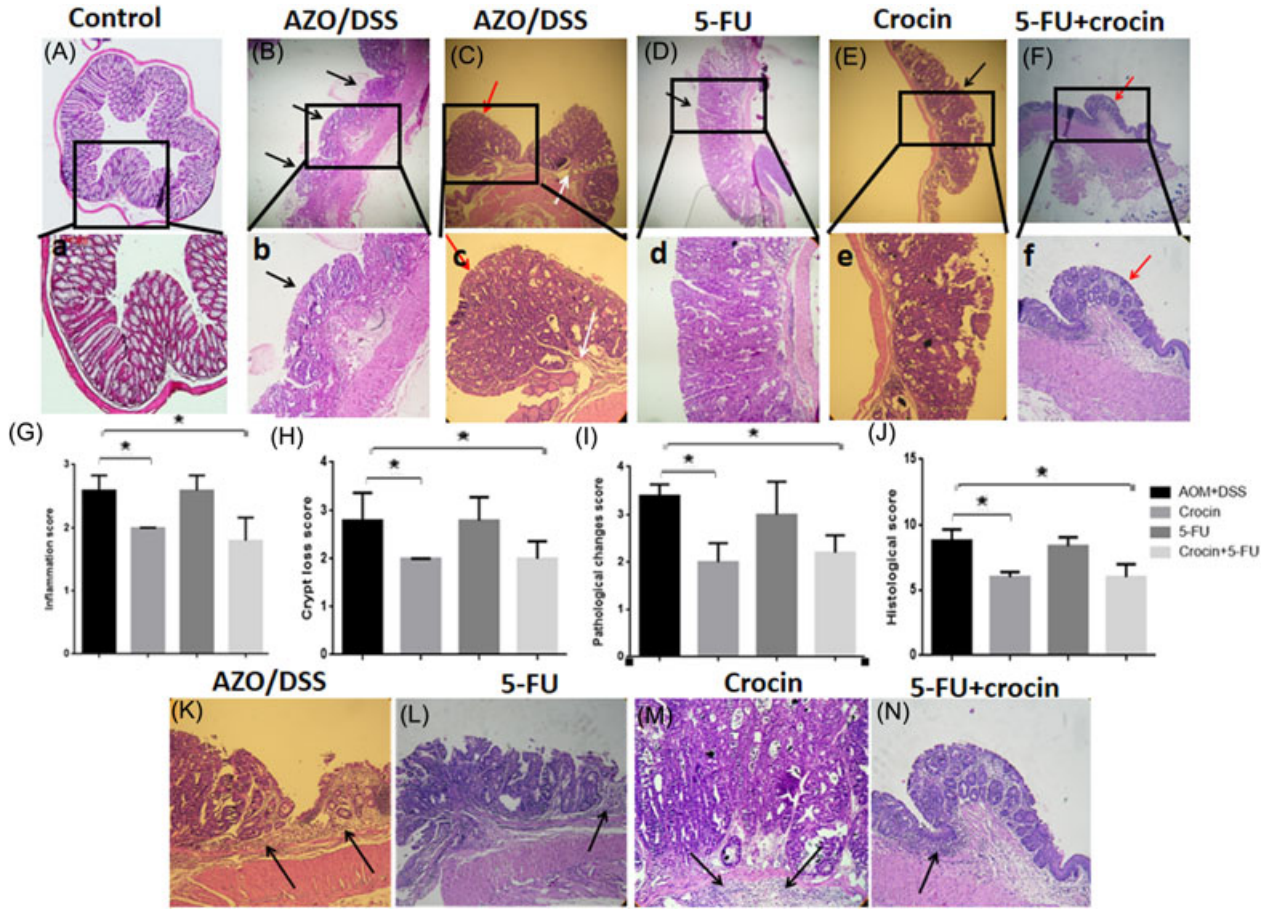

FIGURE 5 Crocin ameliorates multiplicity of severe inflammation with mucosal ulcers and high-grade dysplastic crypts. A, a, tissue from group 1 (mice without AZO/DSS treatment). B-C, b-c, tissue from group 2 (mice with AZO/DSS treatment), polypoid well-differentiated adenocarcinoma (black arrows) and pedunculated adenocarcinoma (red arrows) with invasion to muscular layer (white arrow); In situ adenocarcinoma (black arrows) and tubular adenoma/adenocarcinoma (red arrows). D-F, d-f, effect of crocin, 5-FU, and crocin + 5-FU on colon. G, total inflammation score. H, Crypt loss score. I, Pathological changes score. J, Histology score. Columns or points, mean values obtained from three independent experiments; scale bar, SEM; *significantly different from controls. K-N, A representative picture of inflammation with respect to effect of crocin, 5-FU and crocin + 5-FU. The H&E stained histopathological examination illustrated that there were no pathological changes in ventricular muscle structure in the control group (A, a). In contrast, in LPS group, increased infiltration of inflammatory cells and disarrangement of myofibers were observed (B-C, b-c). Administration of corcin and its combination with 5-FU illustrated improvement of pathological changes). AZO, azoxymethane; DSS, dextran sodium sulfate; 5-FU, 5-fluorouracil; H&E, hematoxylin and eosin

were significantly increased and decreased, respectively, in mice only treated with AOM and/or DSS (group 2) (Figure 4A and 4B). Our data showed that mice treated with crocin or its combination with 5-FU was able to reduce the level of MDA, while increasing the CAT or thiol activities, suggesting that crocin attenuates inflammatory responses by regulating oxidative stress in tumors.

3.8 | Crocin ameliorates multiplicity of severe inflammation with mucosal ulcers and high-grade dysplastic crypts

The H&E staining of resected tumors revealed an organized structure of tumor cells nests within stromal tissue. These tumors also showed the presence of Polypoid well-differentiated adenocarcinoma and pedunculated adenocarcinoma with invasion to muscular layer. Also AOM and/or DSS treatment resulted in the occurrence of veracious colorectal lesions, such as colitis with mucosal ulcers, dysplastic crypts, tubular adenoma, and tubular adenocarcinoma. The incidences and multiplicity of severe colorectal inflammation with mucosal ulcers, the inflammation score, and the presence of dysplasia are shown in Figure 5A-C. In particular our data showed that the total score of inflammation, crypt loss, pathological changes and histology score of mice treated with crocin were significantly reduced and improved, compared to control group (Figure 5 D-J). Moreover, crocin was able to ameliorate multiplicity of severe inflammation with mucosal ulcers and high-grade dysplastic crypts as detected by total score of inflammation, Crypt loss, pathological changes and histology scores (Figure 5G-N).

3.9 | Crocin suppresses PI3K/Akt and Wnt/β-catenin pathways in colorectal cancer

To further investigate the molecular mechanisms of antiproliferative activity of crocin, the effects of this pharmacologically active component on PI3K/Akt and Wnt/b-catenin signaling pathways were analyzed. It has been reported that PI3K/Akt signaling pathway regulated several down-stream signaling pathways involved in cellular proliferation, survival and apoptosis.[40] Thus, we examined the inhibitory effect of crocin on protein levels of PI3K, cyclin D1 and phosphorylated levels of Akt and glycogen synthase kinase 3α/β (GSK3α/β) by Western blot method. Several studies indicated that the oncogenic

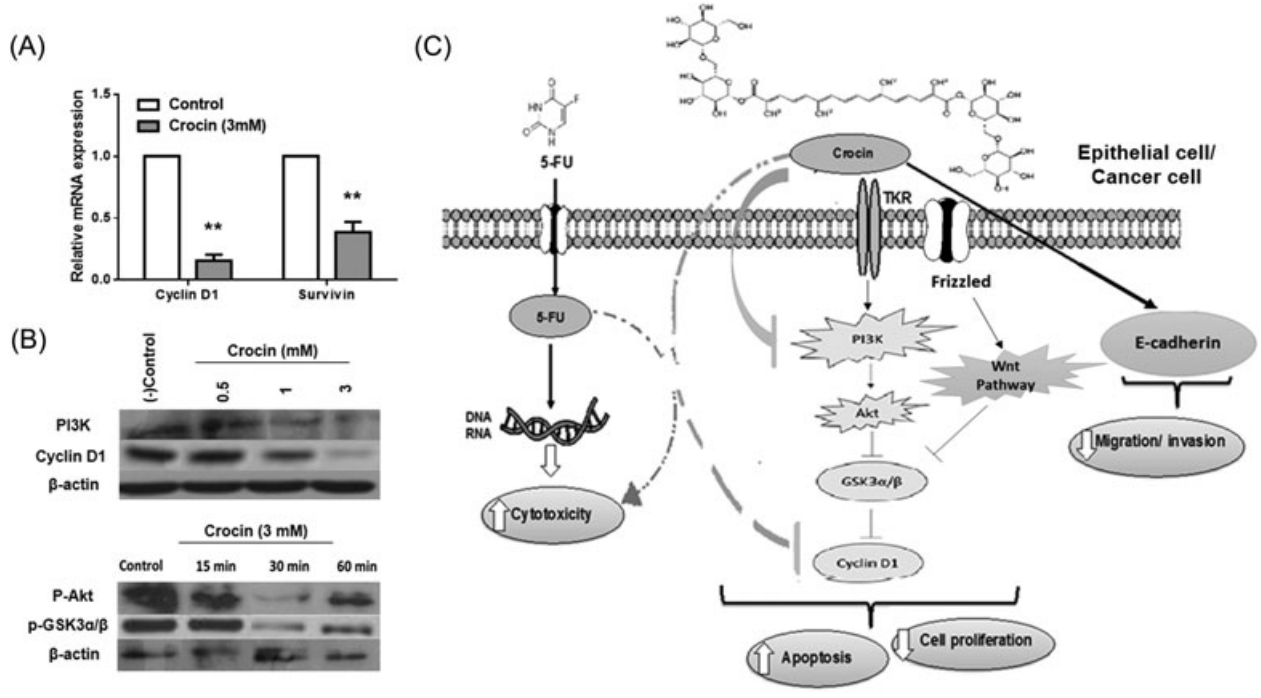

FIGURE 6 Schematic representation of the molecular mechanisms involved in the synergistic effects of crocin on 5-FU. A, , Modulation of cyclin D1 and surviving at mRNA levels and PI3K, cyclin D1, p-Akt and p-GSK3α/β at protein level in CRC cells after 24 hours exposure to crocin (3 mM) as determined by qRT-PCR and Western blot analysis. Columns mean values obtained from three independent experiments; scale bar, SEM. **Significantly different from controls. C, Crocin inhibits PI3K/Akt and Wnt activation and enhances the growth inhibitory effects of 5-FU through its pronounced antiproliferative and anti-invasive effects, as well as the attenuation of inflammation and oxidative stress status. 5-FU, 5-fluorouracil; mRNA, messenger RNA; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction

PI3K/Akt signaling induces Wnt/b-catenin signaling by phosphorylation and inactivation of GSK3α/β. GSK3α/β is the key negative regulator of Wnt signaling pathway which is suppressed upon phosphorylation and then the activated pathway increases expression of its down-stream targets including cyclin D1. Cyclin D1 is one of the Wnt/β-catenin targets genes, mainly involved in regulation of the G1 to S phase in the cell cycle.[41] Our results showed that crocin inhibited expression of PI3K and its down-stream targets like cyclin D1 and survivin in CRC cells in a concentration-dependent manner (Figure 6A and 6C). We also showed that crocin suppressed phosphorylation of Akt and GSK3α/β (Figure 6C), suggesting that the antiproliferative function of crocin is at least partially mediated by attenuating Wnt/PI3K signaling pathway activation in crocin-stimulated cells.

4 | DISCUSSION

To the best of our knowledge this is the first study evaluating the combination of crocin and 5-FU in two-dimensional and three-dimensional cell culture models of CRC cells as well as in a mouse model of colitis-associated colon cancer. Our results showed that crocin synergistically enhanced the antiproliferative activity of 5-FU in both in vitro and in vivo systems (Figure 6B). Moreover, crocin had an antioxidant activity in homogenized colon samples. Moreover, our data showed that crocin and especially its combination therapy with 5-FU could dramatically reduce the tumor number and tumor size. More specifically, the tumor number was also considerably decreased in both distal and middle parts of the colon. Crocin also ameliorated multiplicity of severe inflammation with mucosal ulcers and high-grade dysplastic crypts in mice colon cancer model, which the tumors showed the presence of Polypoid well-differentiated adenocarcinoma and pedunculated adenocarcinoma with invasion to muscular layer. To further support the protective effect of crocin on colon cancer pathology, we showed that crocin improved colon length, disease-activity index score, as well as tumor number/colon size ratio. Taken together, our results not only support the importance of crocin as an alternative or adjuvant compound in cancer therapy,[42] but also are in harmony with the assumption that crocin could improve the 5-FU chemosensitivity.

Consistent with our findings, there are studies showing that the over-activation of the oncogenic PI3K/Akt and Wnt pathways play important roles in cell proliferation, differentiation and migration in CRC.[43-45] Chikazawa et al[46] reported that silencing of β-catenin, component of Wnt signaling, in CRC cells leads to an elevation in their sensitiveness to paclitaxel and irinotecan. In agreement with this study, blocking Wnt activity can be considered as a strategy to reverse the drug-sensitivity of CD133-positive cells to 5-FU.[47] In line with this, overexpression of cyclin D1 is well-documented in CRC.[48] In a study on *N*-nitroso-*N*-methylurea-induced breast cancer in rats, crocin induced cell cycle arrest via suppression of cyclin D1.[49] Zhao et al[50] have also reported the potential activity of crocin in the downregulation of cyclin D1 in bladder cancer. Consistent with these findings, we showed that crocin suppressed cyclin D1 expression in CRC. Thus it can be hypothesized that crocin could increase CRC cells sensitivity to 5-FU via triggering PI3K/Akt and Wnt pathway, as evidenced by downregulation of p-Akt, p-GSK3α/β, PI3K, and cyclin D1.

Inflammation regulates development of colon carcinogenesis.[51] It has been evidenced that the risk of developing inflammatory bowel disease-associated CRC is highly correlated with the extension and duration of inflammation.[52] There are studies supporting the anti-inflammatory activity of crocin.[32] To further investigate the anti-inflammatory effect of crocin, the antioxidant activity of crocin in CRC was analyzed by assessing MDA, CAT and total thiol levels in tissue homogenate samples. Results showed that DSS administration led to a remarkable elevation and reduction in MDA content and CAT activity, respectively. Although the total thiol concentration did not alter significantly in azoxymethane-induced colon cancer rats as compared with control group, but considerable alteration in MDA and CAT levels highlights the involvement of oxidative stress in CRC. Interestingly, crocin and 5-FU combined treatment of azoxymethane-induced colon cancer rats notably recovered the increased levels of MDA and decreased activity of CAT, as well as the decline in total thiol concentration. These data are compatible with the observations of other investigators using saffron and its constituents to improve oxidative damage in injury and ischemia in rats.[53] Increasing evidence showed that oxidative stress plays crucial roles in development and progression of CRC.[54] In particular Chang et al[55] found that oxidative stress level was increased in CRC subjects. Similarly, several studies have reported that lipid peroxide levels, as an indicator of oxidative stress, are higher in malignant colorectal tissues compared to normal tissue.[56,57] Accordingly, as our result showed that crocin may play an important role in CRC prevention and also therapy by increasing antioxidant levels while decreasing oxidants. These findings suggest that anti-inflammatory responses of crocin plays important role in the chemopreventive functions of crocin in colorectal cancer.

In summary, our results support that crocin may potentiate the therapeutic efficacy of 5-FU in CRC

therapy via attenuating cancer cell proliferation and inflammation. These findings, not only suggest that crocin can be considered as a potential anticancer compound for CRC treatment, but also could sensitize cancer cells to 5-FU which is at the forefront of drugs in CRC clinical therapy. Although both our in-vitro and in-vivo investigations successfully brought us to this conclusion but still further preclinical investigations are warranted to explore this therapeutic combination as well as its detailed antitumor mechanisms.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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