

# Crocin has pharmacological effects against the pathological behavior of colon cancer cells by interacting with the STAT3 signaling pathway

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**Abstract.** The aim of the present study was to investigate changes in proliferation, apoptosis, inflammation and chemokine release of colon cancer cells after treatment with crocin, as well as to investigate the signaling pathway that is regulated by crocin. The inhibition rates of different doses of crocin on the proliferation of HCT116 cells were measured by MTT assay. The IC<sub>50</sub> was calculated from the inhibition rates at 48 h. Proliferation curves of HCT116 cells were plotted after treatment with 271.18  $\mu$ M (high-dose group) or 135.6  $\mu$ M (low-dose group) crocin. Flow cytometry and Hoechst 33342/propidium iodide double staining were used for detecting apoptosis. ELISA was used to measure the levels of macrophage inflammatory protein 2, interleukin (IL)-8, monocyte chemoattractant protein 1, tumor necrosis factor- $\alpha$ , IL-6 and IL-1 $\beta$  in the supernatant from cultured HCT116 cells following both high- and low-dose crocin treatment. Phosphorylated (P)-STAT3/STAT3 in HCT116 cells were measured by western blotting. Crocin inhibited the proliferation of HCT116 cells in a dose-dependent manner and the high-dose treatment with crocin resulted in a lower rate of proliferation. Additionally, crocin increased the apoptosis of HCT116 cells and the high-dose treatment with crocin led to a higher level of apoptosis. Notably, crocin decreased the secretion of chemokines and inflammatory factors from HCT116 cells and the high-dose treatment with crocin caused the greatest reduction in secretion of the factors. Crocin reduced the ratio of P-STAT3/STAT3, and thereby reduced the release of cytokines. The present study demonstrated that crocin may have pharmacological effects against the pathological

behavior of colon cancer cells, and its mechanism of action may be related to the STAT3 signaling pathway.

## Introduction

Crocin is an effective water-soluble active monomer extracted from *Crocus sativus*, a plant that is used in traditional Chinese medicine (1). It is reported that crocin has a number of beneficial properties, such as for the treatment of myocardial ischemia and hypoxia; improving behavior and cognition; and anti-lipid peroxidation, anti-atherosclerosis and antitumor effects (1-4). Proteoglycans extracted from *C. sativus* can promote the activity of macrophages and promotes immune regulation and invasion resistance (5). Crocin can effectively inhibit the activity of free radicals and xanthine oxidase, thereby acting as an antioxidant (6). In addition, crocin has anti-inflammatory effects and has been used as an adjuvant for various inflammatory diseases (7,8).

The incidence of colorectal cancer has increased in China, with only lung cancer and gastric cancer showing higher incidences (9). Early colorectal cancer lacks clear and typical symptoms and most patients with colorectal cancer are already at an advanced stage and at risk of metastasis upon diagnosis. As such, the optimal point for beginning treatment has already passed and the prognosis is not as favorable (10). Inflammation is involved in the occurrence and development of colon cancer (11). For example, interleukin (IL)-1 $\beta$ , IL-6 and tumor necrosis factor (TNF)- $\alpha$  are involved in all aspects of colon cancer (12,13). However, the anticancer and anti-inflammatory functions of crocin in colon cancer cells have not been investigated.

In the present study, proliferation and apoptosis of colon cancer cells, inflammatory responses in colon cancer cells, as well as chemokine release from colon cancer cells was investigated following treatment with crocin. The signaling pathways that are regulated by crocin were also examined.

## Materials and methods

**Cells.** HCT116 cells (The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) were cultured in McCoy's 5A (Modified) Medium (16600082; Thermo Fisher

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Scientific, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) at 37°C and 5% CO<sub>2</sub>. Prior to western blot analysis, HCT116 cells were treated with Stattic (HY-13818; MedChemExpress), according to the manufacturer's instructions, to inhibit the activity of the STAT3 signaling pathway.

**MTT assay.** HCT116 cells in the logarithmic growth phase were digested, resuspended at a density of 2x10<sup>4</sup> cells/ml and seeded into 96-well plates at 37°C and 5% CO<sub>2</sub>. To test the effect of crocin on the proliferation of HCT116 cells, cells were treated with either a high or low dose of crocin. The cells in the high-dose group were treated with 271.18 μM crocin and those in the low-dose group were treated with 135.6 μM crocin at 37°C for 24, 48 or 72 h. To test the inhibitory rate of crocin, after adhesion of the cells to the surface of the plate at 37°C for 8 h, the medium was replaced with McCoy's 5A (Modified) Medium containing 10% FBS and varying concentrations of crocin (50, 100, 200, 400, 800 and 1,600 μM; ES-0329; Extrasynthese) following previous studies (14,15). The control group was cultured in medium without drugs. Each concentration was examined in triplicate. After culture at 37°C and 5% CO<sub>2</sub> for 24, 48, 72, 96, 120 or 144 h, the medium was replaced with serum-free medium. In the dark, 5 mg/ml MTT solution (20 μl) was added onto the cells which were then incubated at 37°C for 4 h. Subsequently, the medium was discarded, and DMSO (150 μl) was added into each well before shaking at 37°C in the dark for 5 min. Then, the absorbance was read at 570 nm using a microplate reader (DG5033A; Nanjing Huadong Electronics Co., Ltd.), and this reflected cell viability or number. The following formula was used: Inhibition rate of drugs on cell proliferation (%) = (1 - absorbance of drug group / absorbance of control group) x 100%. Growth curves were plotted using time (h) as the x-axis, and absorbance as the y-axis. The IC<sub>50</sub> was calculated from inhibitory rates at 48 h.

**Transwell assay.** To test invasion ability, Matrigel® (BD Biosciences) was thawed at 4°C overnight and diluted with serum-free medium (dilution, 1:2). The mixture (50 μl) was evenly applied to the upper chambers of Transwell plates (Merck KGaA) on ice and incubated at 37°C for 1 h for solidification. HCT116 cells (2x10<sup>5</sup> cells/well) from the control group or the crocin treatment groups were seeded into the upper chamber containing 200-μl serum free medium at 37°C. A total of 500 μl medium supplemented with 10% FBS was added into the lower chamber. After 24 h of incubation at 37°C, the chamber was removed and the cells in the upper chamber were wiped off. After being fixed with 4% formaldehyde at room temperature for 10 min, the membrane was stained with Giemsa at room temperature for 15 min and observed using a light microscope (in 5 random fields (magnification, x200)). The number of invading cells was counted to evaluate the cell invasion ability.

**Hoechst 33342/propidium iodide (PI) double staining.** After being treated with crocin for 24 h, HCT116 cells were subjected with Hoechst 33342/PI double staining (cat. no. C1056; Beyotime Institute of Biotechnology). The cells were first washed with PBS twice, and 5 μl Hoechst stain and 5 μl PI stain were added onto the cells before incubating at 4°C for 20-30 min. After staining, the cells were washed with PBS

twice before observing red (Hoechst) and blue (PI) fluorescence under a fluorescence microscope (Axio Scope A1; Carl Zeiss AG) at a magnification of x100.

**Flow cytometry.** Cells (1x10<sup>6</sup>) in each group were washed with pre-cooled phosphate-buffered saline twice and subjected to flow cytometry using Annexin V-FITC/PI Apoptosis Detection kit (A211-01/02; Vazyme) following the manufacturer's protocol to detect cell apoptosis. Cells with Annexin V-positive values were considered early apoptotic cells, those with PI-positive values were considered necrotic, and those with double positive values were considered late apoptotic.

**ELISA.** Cell supernatant was centrifuged at 3,000 x g and 4°C for 10 min to eliminate cell debris. IL-6 (cat. no. ab46027), TNF-α (cat. no. ab181421), IL-1β (cat. no. ab46052), macrophage inflammatory protein (MIP)-2 (cat. no. ab184862), monocyte chemoattractant protein (MCP)-1 (cat. no. ab100586), and IL-8 (cat. no. ab46032) ELISA kits (Abcam) were used to determine the concentrations of respective proteins in the cell supernatant. In 96-well microplates, standards (50 μl) and samples (10 μl serum and 40 μl diluent) were added into predefined wells, while blank wells were left empty. In the wells for standards and samples, horseradish peroxidase-labelled conjugates (100 μl) were added before sealing the plates for incubation at 37°C for 1 h. After washing the plates five times, substrates A (50 μl) and B (50 μl) were added into each well. After incubation at 37°C for 15 min, stop solution (50 μl) was added into each well, and absorbance of each well was measured at 450 nm using a microplate reader within 15 min.

**Reverse transcription-quantitative PCR (RT-qPCR).** Cells (3x10<sup>6</sup>) were directly lysed with 1 ml TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was extracted using phenol chloroform method. The concentration and quality of RNA was measured using ultraviolet spectrophotometry (NanoDrop™ ND2000; Thermo Fisher Scientific, Inc.). Subsequently, cDNA was obtained by RT from 1 μg RNA and stored at -20°C. RT of mRNA was performed using TIANScript II cDNA First Strand Synthesis kit (Tiagen Biotech Co., Ltd.) according to the manufacturer's protocol. SuperReal PreMix (SYBR-Green) kit (Tiagen Biotech Co., Ltd.) was used to detect mRNA expression, using GAPDH as an internal reference. The reaction system (20 μl) was composed of 10 μl SYBR Premix EX Taq, 0.5 μl forward primer (STAT3, 5'-GGAGGAGGCATTCGGAAAG-3'; β-actin, 5'-AACGGC TCCGGCATGTGCAA-3'), 0.5 μl reverse primer (STAT3, 5'-TCGTTGGTGTACACAGAT-3'; β-actin, 5'-CTTCTG ACCCATGCCCACCA-3'), 2 μl cDNA and 7 μl ddH<sub>2</sub>O. The following thermocycling conditions were used: Initial denaturation at 95°C for 5 min; 46 cycles of denaturation at 95°C for 20 sec and annealing at 55°C for 20 sec; and a final extension at 72°C for 30 sec (iQ5 system; Bio-Rad Laboratories, Inc.). The 2<sup>-ΔΔC<sub>q</sub></sup> method was used to calculate the relative expression of target mRNA against GAPDH (16). Each sample was tested in triplicate.

**Western blotting.** Before lysis, cells (1x10<sup>6</sup>) were trypsinized and collected. Then, the cells were lysed with precooled

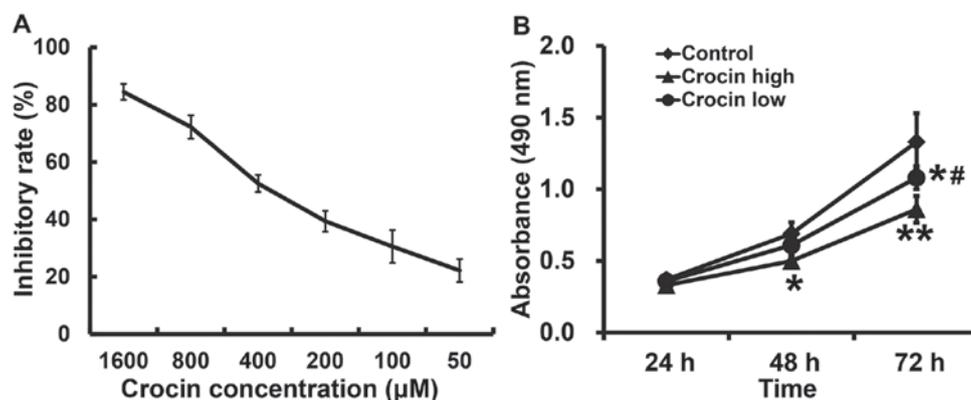


Figure 1. Effect of crocin on the proliferation of HCT116 cells. (A) Inhibition rate curve used for the determination of  $IC_{50}$  of crocin on the proliferation of HCT116 cells. The cells were treated with 50, 100, 200, 400, 800 or 1,600  $\mu$ M crocin for 48 h before the MTT assay. (B) Proliferation of HCT116 cells treated with crocin high-dose (271.18  $\mu$ M) or low-dose (135.6  $\mu$ M) for 24, 48 or 72 h. Absorbance was recorded at 490 nm. \* $P < 0.05$ , \*\* $P < 0.01$  vs. the control group; # $P < 0.05$  vs. crocin high.

RIPA lysis buffer (600  $\mu$ l; 50 mM Tris-base, 1 mM EDTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% sodium deoxycholate; Beyotime Institute of Biotechnology) for 30 min on ice. The mixture was centrifuged at 11,000  $\times$  g and 4°C for 10 min. The concentration of protein within the supernatant was determined by BCA protein concentration determination kit [RTP7102, Real-Times (Beijing) Biotechnology Co., Ltd.]. The samples were then mixed with 5X SDS loading buffer before denaturation in a boiling water bath for 10 min. Afterwards, the samples (20  $\mu$ g) were subjected to 10% SDS-PAGE at 100 V. The resolved proteins were transferred to PVDF membranes on ice (100 V, 2 h) and blocked with 5% skimmed milk at room temperature for 1 h. Then, the membranes were incubated with rabbit anti-human phosphorylated (P)-STAT3 (1:1,500; ab30647; Abcam), STAT3 (1:1,000; ab68153; Abcam) or  $\beta$ -actin (1:5,000; ab129348; Abcam) monoclonal primary antibodies at 4°C overnight. After extensive washing with PBS with Tween-20, three times for 15 min, the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:3,000; ab6721; Abcam) for 1 h at room temperature before washing with PBS with Tween-20, three times for 15 min. Then, the membrane was developed with an ECL kit (Sigma-Aldrich; Merck KGaA) for imaging. Image Lab v3.0 software (Bio-Rad Laboratories, Inc.) was used to acquire and analyze imaging signals. The relative amounts of target proteins were normalized against  $\beta$ -actin.

**Statistical analysis.** The results were analyzed using SPSS 20.0 statistical software (IBM Corp.). The data are shown as the mean  $\pm$  SD. Multigroup measurement data were analyzed using one-way ANOVAs, followed by Student-Newman-Keuls post-hoc tests. Comparisons between two groups were carried out using the Student's t-test. Three repeats were performed for each experiment.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*Crocin inhibits the proliferation of HCT116 cells in a dose-dependent manner and a high dose of crocin results in a*

*lower level of proliferation.* To calculate the  $IC_{50}$  of crocin on the proliferation of HCT116 cells, the cells were treated with 50, 100, 200, 400, 800 or 1,600  $\mu$ M crocin for 48 h and an MTT assay was performed. These data showed that crocin inhibited the proliferation of HCT116 cells in a dose-dependent manner and the  $IC_{50}$  was 271.18 $\pm$ 21.83  $\mu$ M (Fig. 1A). The absorbance of HCT116 cells in both the low- and high-dose groups was significantly lower than that in the control group ( $P < 0.05$  for both), and that in low-dose group was significantly higher than that in high-dose group after 72 h ( $P < 0.05$ ; Fig. 1B). The results suggested that crocin inhibited the proliferation of HCT116 cells in a dose-dependent manner, with higher doses of crocin resulting in lower levels of proliferation.

*Crocin does not affect the invasion ability of HCT116 cells.* To evaluate the invasion of HCT116 cells, Transwell assays were carried out. These data showed that number of invasive cells in either the high-dose crocin group or the low-dose crocin group was not different from that of the control group ( $P > 0.05$ ; Fig. 2). These results indicated that crocin did not affect the invasive capabilities of HCT116 cells.

*Crocin increases the apoptosis of HCT116 cells and a high dose of crocin leads to a higher level of apoptosis.* To examine the effect of crocin on the apoptosis of HCT116 cells, flow cytometry and Hoechst/PI staining were carried out after treatment with high-dose and low-dose crocin for 72 h. These data showed that both high- and low-dose crocin treatment induced significant apoptosis of HCT116 cells, and the apoptotic rate in the low-dose group was significantly lower than that in the high-dose group ( $P < 0.05$ ; Fig. 3A). Hoechst/PI staining showed a similar trend to the flow cytometry data (Fig. 3B). The results indicate that crocin increased the apoptosis of HCT116 cells and a higher dose of crocin led to a higher level of apoptosis.

*Crocin decreases the secretion of chemokines and inflammatory factors from HCT116 cells and a high dose of crocin causes reduced secretion of these factors.* To examine how crocin influences the mRNA levels of chemokines (MIP2, IL-8 and MCP-1) and inflammatory factors (TNF- $\alpha$ , IL-6 and IL-1 $\beta$ ), as well as the secretion in the culture supernatant of

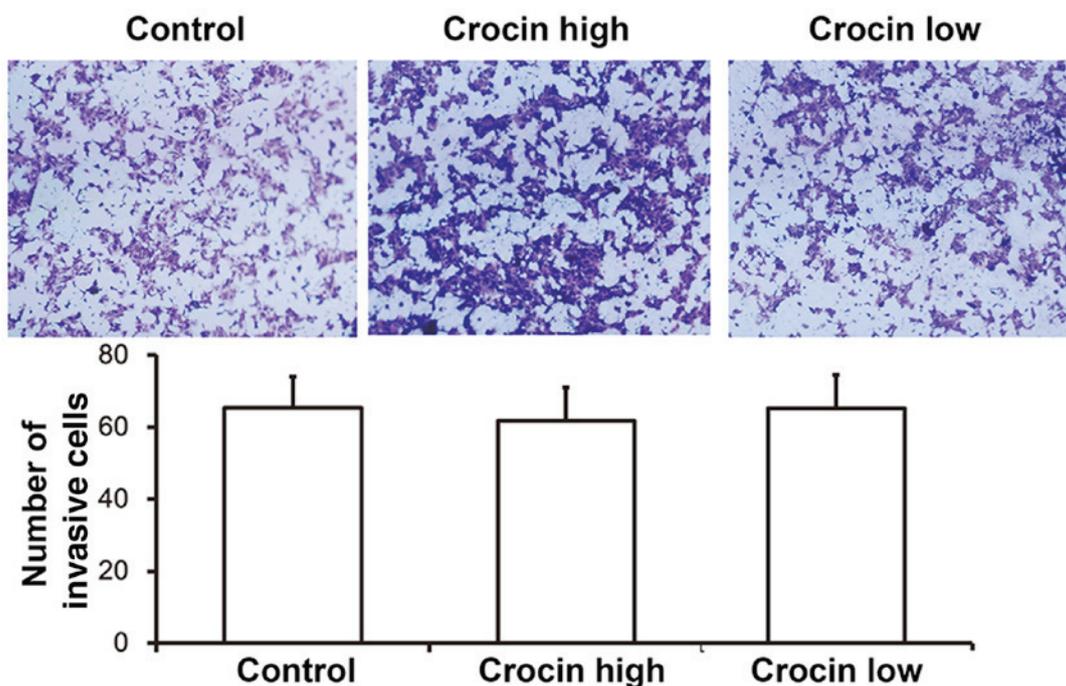


Figure 2. Effect of crocin on the invasion of HCT116 cells. A Transwell assay was used to determine the invasion ability of HCT116 cells.

these factors from HCT116 cells at 72 h following treatment with crocin, RT-qPCR and ELISA were employed. These data showed that the mRNA expression and secretion of MIP2, IL-8, MCP-1, TNF- $\alpha$ , IL-6 and IL-1 $\beta$  in both the high- and low-dose crocin groups were significantly lower than that in the control group ( $P < 0.05$ ), and those in the low-dose group were significantly higher than those in the high-dose group ( $P < 0.05$ ) (Fig. 4). The results suggested that crocin decreased the secretion of chemokines and inflammatory factors from HCT116 cells, and a high-dose of crocin had the most significant effect.

*Crocin reduces the levels of P-STAT3, and thereby reduces the release of cytokines.* The secretion of chemokines and inflammatory factors is regulated by the STAT3 signaling pathway (17). To examine the expression of proteins related to the STAT3 signaling pathway, western blotting was used. The data showed that the expression of P-STAT3 in the Statcic group was significantly reduced compared to the control group ( $P < 0.05$ ). Similarly, the expression of P-STAT3 in the high- and low-dose crocin treatment groups was also significantly lower than that in control group ( $P < 0.05$ ), and that in the low-dose group was significantly higher than that in the high-dose group ( $P < 0.05$ ) (Fig. 5). These results indicated that crocin reduces the levels of P-STAT3, and thereby reduced the release of cytokines.

## Discussion

At present, surgical treatment combined with radiotherapy, chemotherapy and molecular targeted therapy, is the main treatment procedure for colorectal cancer, but the surgical cure rate and postoperative survival rate is still low (18,19). Therefore, finding effective drugs to treat colorectal cancer is crucial. Crocin is reported to have anti-myocardial ischemia

and anti-atherosclerotic properties, to regulate the immune system, protect the liver and gallbladder, and regulate blood lipid levels (20). *In vitro* experiments show that crocin has a strong cytotoxic effect on tumor cells. For example, crocin and its liposomal form can induce apoptosis in HeLa and MCF-7 cells, and the liposomal form of crocin has increased cytotoxicity compared with crocin (21). Additionally, crocin inhibits the proliferation of tongue squamous cell carcinoma cells and inhibits their nucleic acid synthesis, as well as inducing apoptosis (22). Cells treated with crocin show extensive cytoplasmic vacuolar regions and cytoplasmic reduction, but the sensitivity to crocin varies between cell lines (23). Animal experiments show that crocin can reverse tumor-like pathological changes in mice and is a potential antitumor agent (24). According to a previous report, it was found that crocin may have a dose-dependent effect on tumors (25); therefore, the present study tested the effect of low- and high-dose treatments of crocin on colon cancer cells. The results showed that crocin inhibited the proliferation of HCT116 cells. After obtaining the  $IC_{50}$  value, the cells were treated with high (271.18  $\mu M$ ) and low (135.6  $\mu M$ ) doses of crocin in the following experiments. Flow cytometry showed that crocin induced apoptosis of HCT116 cells in a dose dependent manner. This further demonstrated that crocin has an inhibitory effect on the survival of colon cancer cells.

Chemokines and inflammatory factors released by colon cancer cells are some of the important factors affecting the progression of the disease (26). In the present study, the quantity of common inflammatory factors (IL-6, IL-1 $\beta$  and TNF- $\alpha$ ) (27) and chemokines (MIP2, MCP-1 and IL-8) (28,29) secreted by HCT116 cells was examined. MIP2, MCP-1 and IL-8 are reported to promote the aggregation of neutrophils to tumor sites, and thus are deemed biomarkers for the chemotactic and metastatic capability of cells (30-33). As stimulating factors, IL-6, IL-1 $\beta$  and TNF- $\alpha$  further promote the transformation

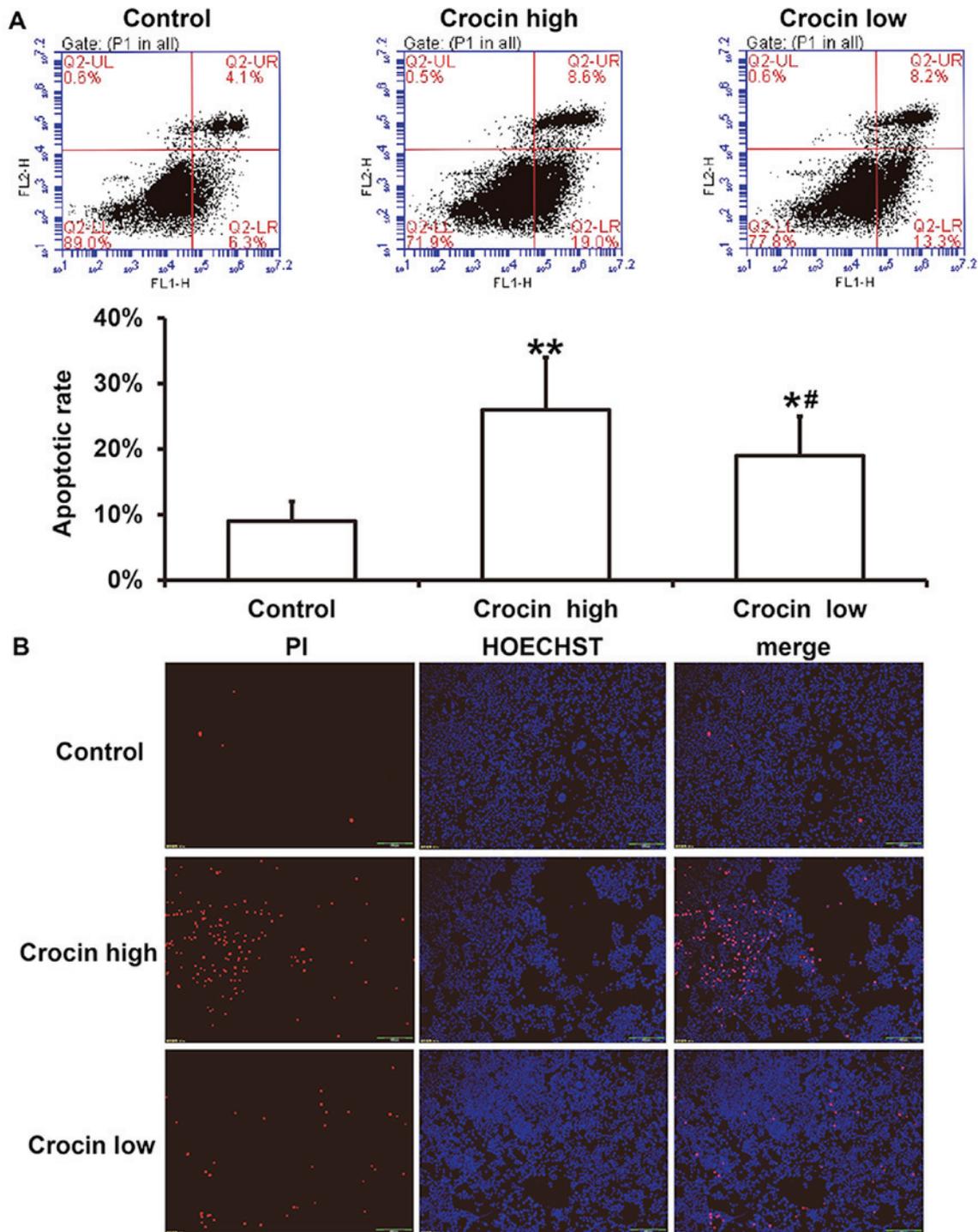


Figure 3. Apoptosis of HCT116 cells in the crocin high- and low-dose groups. (A) Apoptotic rate of HCT116 cells determined by flow cytometry. (B) Images of HCT116 cells double-stained with Hoechst/PI. Magnification, x100. \* $P < 0.05$ , \*\* $P < 0.01$  vs. the control group; # $P < 0.05$  vs. crocin high. PI, propidium iodide.

from inflammation to colon cancer (12,13). The results showed that crocin treatment reduced the levels of MIP2, MCP-1, IL-8, IL-6, IL-1 $\beta$  and TNF- $\alpha$  in the supernatant from cultured HCT116 cells. It has been demonstrated that activation of the STAT3 signaling pathway is important for cell proliferation, migration and survival, and can also lead to the release of chemokines and inflammatory factors (17). The results of the present study showed that crocin treatment reduced the expression of P-STAT3 in HCT116 cells, suggesting that crocin may affect the proliferation and apoptosis of HCT116 cells and that

crocin may also affect the release of chemokines and inflammatory factors from HCT116 cells, by inhibiting the activity of the STAT3 signaling pathway. A limitation of the present study is that only one cell line was used. Further studies should extend the number of cell lines used to confirm these observations.

In conclusion, the present study demonstrated that crocin has pharmacological effects against the pathological behavior of colon cancer cells, and its mechanism of action may be related to the STAT3 signaling pathway. However, the exact mechanism of action still requires further investigation.

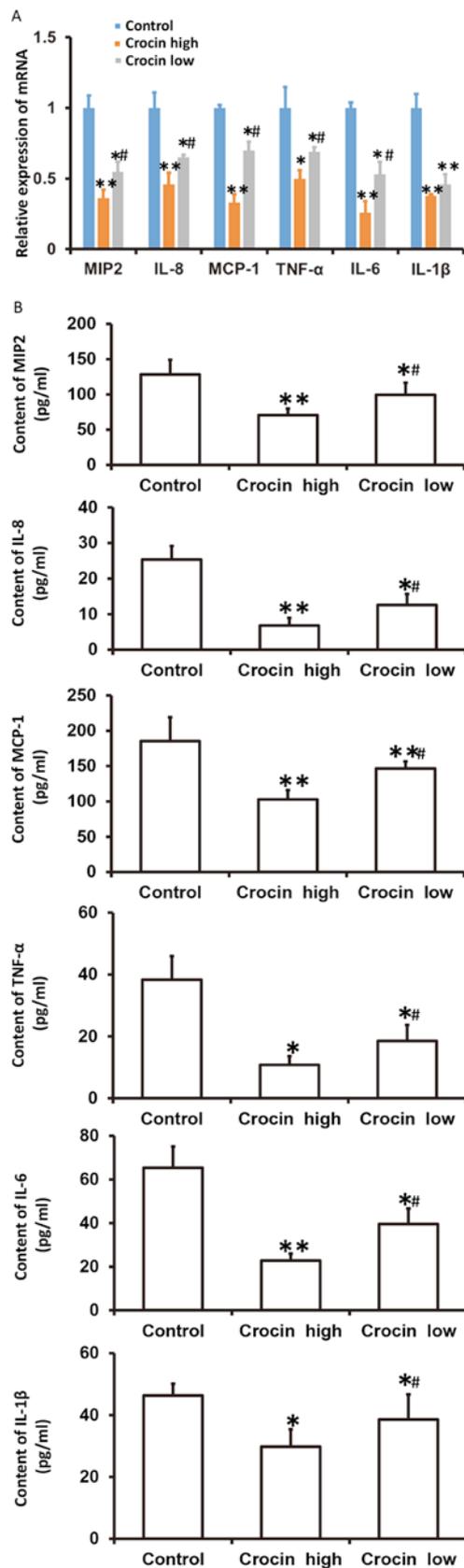


Figure 4. Expression of chemokines and inflammatory factors. (A) mRNA expression and (B) secretion of chemokines (MIP2, IL-8 and MCP-1) and inflammatory factors (TNF- $\alpha$ , IL-6 and IL-1 $\beta$ ) by HCT116 cells in the crocin high- and low-dose groups. Reverse transcription-quantitative PCR was used to measure intracellular mRNA expression and ELISA was performed to determine the content of the factors in supernatant of HCT116 cells. \* $P$ <0.05, \*\* $P$ <0.01 vs. the control group; # $P$ <0.05 vs. crocin high. IL, interleukin; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; TNF, tumor necrosis factor.

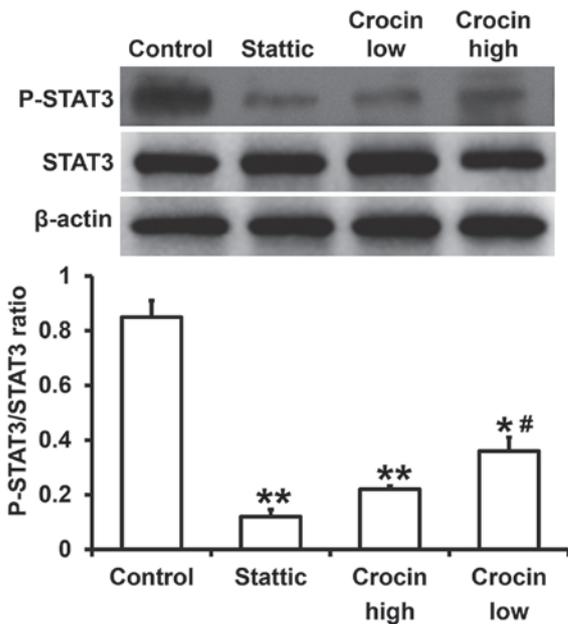


Figure 5. Expression of proteins related to the STAT3 signaling pathway in HCT116 cells of the Static, crocin high-dose and low-dose groups. Static was reported to inhibit the activity of the STAT3 signaling pathway. Western blotting was used to determine the expression of P-STAT3 and STAT3 and the ratio of P-STAT3/STAT3 was calculated and compared. \* $P$ <0.05, \*\* $P$ <0.01 vs. the control group; # $P$ <0.05 vs. crocin high. P, phosphorylated.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

The final version of the manuscript has been read and approved by all authors, and each author states that the manuscript represents honest work. JW and TS collaborated to design the study. JW, YK and TS were responsible for performing experiments. JW and TS analyzed the data. All authors collaborated to interpret results and develop the manuscript.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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