Crocetin induces apoptosis of BGC-823 human gastric cancer cells

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Abstract. Gastric cancer (GC) is one of the most common types of gastrointestinal tumors worldwide, and the side effects of chemotherapeutic drugs and the resistance to chemotherapy remain problematic in its clinical treatment. Therefore, safe and effective novel agents are urgently required. The purpose of the present study was to investigate the crocetin-sensitive treatment of GC and its possible mechanisms. BGC-823 human GC cells were treated with crocetin. The effects of crocetin on the viability of the cells were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Hoechst 33258 dyeing and Rh123 staining were used to detect cell apoptosis. The BGC-823 cells were subjected to western blotting analysis for detection of cytochrome *c* and cleaved caspase-3 protein expression. Crocetin inhibited the proliferation of the GC cell line in a dose- and time-dependent manner. Apoptotic BGC-823 cells induced by crocetin were stained by Hoechst 33258 and observed under a light microscope for cell membrane staining of dense nuclei, nuclear pyknosis, fragmentation, chromatin condensation and highlighted nuclear membrane staining. This revealed a decline in the mitochondrial membrane potential of the BGC-823 cells. Crocetin also induced caspase-3 activation and cytochrome *c* translocation into the cytosol from the mitochondria. The results of this study indicate that crocetin induces the apoptosis of BGC-823 cells, and may be used as an effective agent in the treatment of GC.

Introduction

Gastric cancer (GC) remains the most common type of cancer and the second major cause of mortality from cancer in the world (1,2). Despite the significant decline in GC cases over the last decades, the mortality rates of GC in Asia and other developing countries are considerably higher than in developed countries (3,4), which is largely due to a lack of timely diagnosis and effective treatment. Drug-assisted surgical treatment has been widely used in clinics, but the side-effects of chemotherapy drugs and the resistance to chemotherapy are underlying problems in its treatment (5). Therefore, the requirement for novel agents to enhance the effects of chemotherapeutic drugs and reduce their resistance remains critical.

Chinese herbal antitumor agents, including assolanine (6), an extract from *Rhodiola rosea* rhizomes (7), Gleditsioside E from *Gleditsia sinensis* (8) and saponins from *Gleditsia sinensis* (9), exert significant effects against a variety of tumors and have been confirmed to be effective antitumor agents. Saffron is a derived plant product from the dried stigma of the *Crocus sativus* flower (family, Iridaceae), which has been used for several hundred years in the treatment of cardiovascular diseases, inflammatory diseases and several other tumors (10,11). Extracts of saffron contain multiple biologically active compounds, including crocin, crocetin, saffron bitter element, saffron aldehyde, vitamins and flavonoids. Crocetin, a major active ingredient of saffron extract, has been revealed to possess potent antiproliferative and antioxidative characteristics. Moreover, the antitumor activity of crocetin has been observed to enhance the apoptosis of several types of cancer cells *in vitro* and inhibit the growth of tumors *in vivo*, including human liver (12), colorectal (13), pancreatic (14) and breast (15) cancer cells.

However, the effect of crocetin on human gastric cells and its mechanism have never been investigated to date. BGC-823 human GC cells have been widely used in antitumor drug research (16,17). Therefore, the present study was designed to investigate the effects of crocetin on the proliferation of BGC-823 cells and its mechanism *in vitro*.

Materials and methods

Materials. The following reagents were used: Crocetin (MP Biomedicals, Solon, OH, USA; $C_{20}H_{24}O_4$; formula weight, 328.4; purity, >98%), docetaxel (Jiangsu Hengrui Medicine Co., Ltd., Nanjing, China), trypsin (Gibco, Carlsbad, CA, USA), fetal bovine serum (Gibco), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; MP Biomedicals, Santa Ana,

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CA, USA) and TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). The Rh123 and Hoechst 33258 dyes were purchased from Sigma (St. Louis, MO, USA), and an inverted microscope obtained from Olympus (Tokyo, Japan) was used. Cleaved caspase-3 and cytochrome *c* antibodies were purchased from Cell Signaling Biotechnology (Beverly, MA, USA). Horseradish peroxidase-conjugated anti-rabbit antibody was shipped from Boster (Wuhan, China).

Crocetin preparation. Crocetin was diluted in phosphate-buffered saline (PBS), filter sterilized (GE Healthcare, Greenwich, CT, USA) and stored at 4˚C or -20˚C (for a maximum of 2 months) in the dark.

Cell line and cultures. The GC BGC-823 cell line was obtained from the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). The GC cell line was cultured in RPMI-1640 medium supplemented with 10% fetal-bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin. The cell culture dish was placed in a humidified atmosphere with 5% CO_2 at 37˚C. The medium was changed every 2-3 days and the cells were trypsinized, harvested and seeded in a novel plate once the cells had reached 80-90% confluence.

Cell viability assays. The effect of crocetin on cell proliferation was determined by the MTT uptake method. The cells ($1x10^5$/ml/well) were incubated with varying concentrations of crocetin in a 96-well plate and then incubated for 24, 48 or 72 h at 37˚C. MTT solution (10 μl) was added to each well and incubated for 4 h at 37˚C. Subsequently, the medium was replaced by 150 μl dimethylsulfoxide (DMSO) per well to dissolve the formazan crystals. The absorbance value of each well was determined by the enzyme-linked immunosorbent assay (wavelength, 570 nm).

Experimental groups and morphological analysis. The following groups were used: Group A, the blank control group; group B, the 200-μmol crocetin group; group C, the docetaxel 5-μmol group (18); and group D, the 0.5% DMSO group. The four groups were supplemented with $2x10^6$/ml BGC-823 cells for 48 h, and changes in cell morphology were observed by inverted fluorescence microscopy (Leica DMI4000B, Leica, Mannheim, Germany).

Hoechst 33258 staining. DNA staining by Hoechst 33258 dyes were used to evaluate the chromosomal condensation and morphological changes. The BGC-823 cells were plated in 6-well plates. Following treatment, the BGC-823 cells were fixed with 4% paraformaldehyde for 20 min. Hoechst 33258 (5 mg/ml in PBS; pH 7.4) was added to the cells for 10 min, followed by washing with PBS three times. The nuclear morphology was observed using a fluorescence microscope (Leica DMI4000B). The intensity of the mean fluorescence was analyzed using ImageJ 1.41o software (NIH, Bethesda, Maryland, USA).

Measurement of the mitochondrial membrane potential (MMP). The MMP was measured using Rh123, a cationic fluorescent dye, as previously described (19). In the four groups, Rh123 was added to the cultures to a final concentration of 2 μM for 30 min at 37˚C. Following incubation, the cells were rinsed with PBS and images were captured by Confocal Laser Scanning Microscopy (LSM 710; Zeiss, Oberkochen, Germany) immediately afterwards. The intensity of the mean fluorescence was analyzed using ImageJ 1.41o software (NIH).

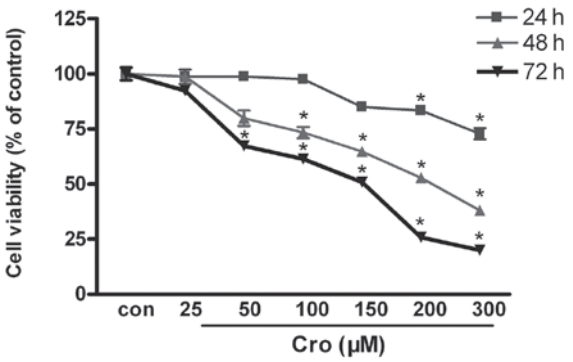

Figure 1. BGC-823 cells were treated with the indicated concentrations (25-300 μM) of crocetin (Cro) for 24, 48 and 72 h. IC_{50}, 200 μM and processing time, 48 h (n=6, *P<0.05 vs. control).

Western blotting. To confirm the apoptosis mechanisms induced by crocetin in the BGC-823 cells, the processing of cytochrome *c* and cleaved caspase-3 was analyzed by western blotting. The cells were treated as aforementioned and then washed three times with cold PBS (pH 7.4) and lysed in ice-cold lysis buffer [1X PBS, 1% NP40, 0.1% SDS, 5 mM EDTA, 0.5% sodium deoxycholate and 1% phenylmethanesulfonyl fluoride (PMSF)]. The lysate was centrifuged at 12,000 x g for 5 min at 4˚C, then the supernatant was collected and the protein concentration was quantified using a bicinchoninic acid protein assay kit (Bioworld, Dublin, OH, USA). Equal quantities of protein were separated on 15% SDS-polyacrylamide gels (SDS-PAGE). The proteins were transferred to polyvinylidene difluoride (PVDF) membranes, which were blocked with 5% skimmed milk in Tris-buffered saline with Tween-20 (TBST) for 1 h at room temperature. The membranes were incubated overnight with different primary antibodies for cytochrome *c* (1:1,000) and cleaved caspase-3 (1:1,000) at 4˚C. Following three washes with TBST, the blots were incubated with the secondary horseradish peroxidase-conjugated anti-rabbit antibody at room temperature for 1 h followed by washing again three times using TBST buffer. Subsequently, the antibody-bound proteins were detected by an Enhanced chemiluminescence substrate (Vector Labs, Burlingame, CA, USA) and exposed to X-ray films. ImageJ 1.41o software (NIH) was used to quantitatively analyze the expression levels of the protein.

Statistical analysis. All the experiments were repeated three times. All the data are presented as the mean ± standard error of the mean. Statistical significance was determined using a one-way analysis of variance followed by Duncan's multiple range test, utilizing a SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA). The differences between groups were compared with the least significant difference test. $P<0.05$ was considered to indicate a statistically significant difference.

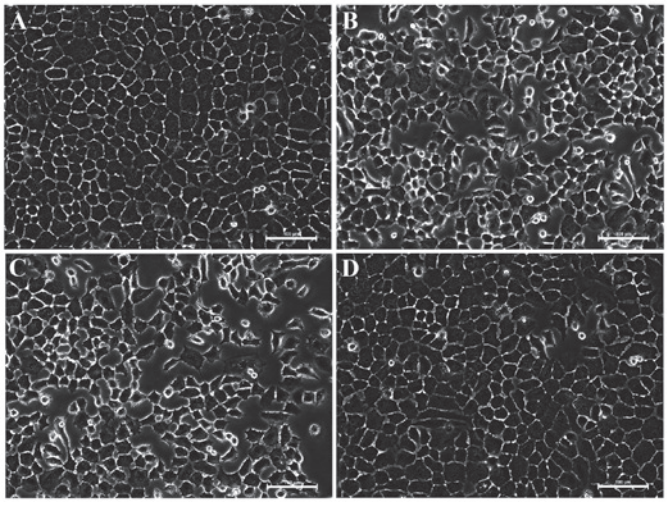

Figure 2. Morphological characteristics of cultured BGC-823 cells as visualized by microscopic analysis. (A) The control group, (B) the 200-μM crocetin group, (C) the 5-μM docetaxel group and (D) the DMSO group (scale bar, 100 μm).

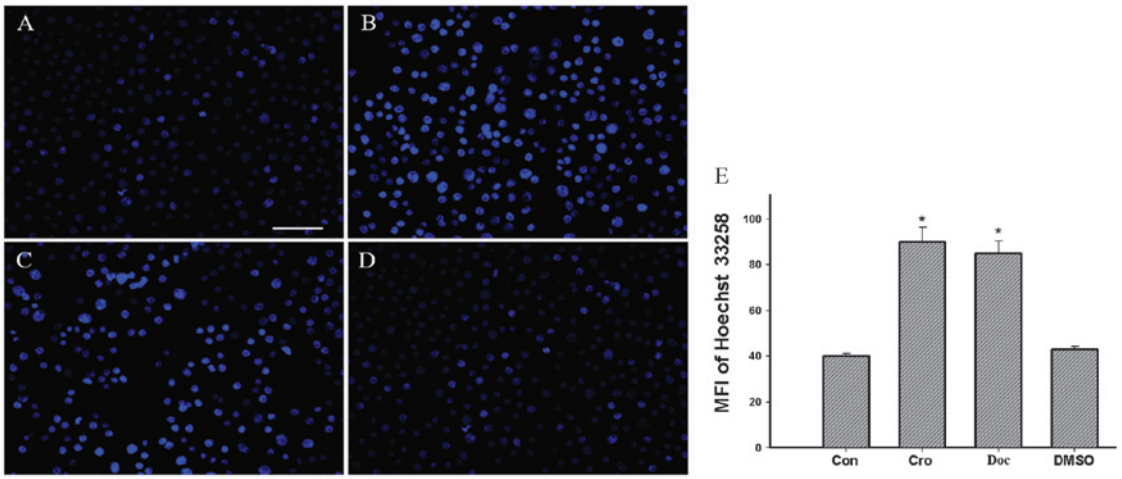

Figure 3. Crocetin-induced apoptosis in BGC-823 cells. Apoptotic nuclei were visualized by Hoechst 33258 staining. (A) Control (Con), (B) crocetin (Cro), (C) docetaxel (Doc) and (D) dimethylsulfoxide (DMSO) groups (scale bar, 100 μm), and (E) quantitative analysis of the fluorescence intensity (*P<0.01 vs. the control group). MFI, mean fluorescence intensity.

Results

Cytotoxic activities of crocetin on BGC-823 cells. The MTT assay demonstrated that crocetin significantly inhibited the viability of the BGC-823 cells (Fig. 1). The cells were incubated in the absence or presence of various concentrations of crocetin (25-300 μM) for 24, 48 and 72 h. The MTT assay revealed that the crocetin-treated cells exhibited a significant reduction in cell proliferation in a concentration- and time-dependent manner, and that the MTT absorbance value was significantly reduced, demonstrating a significant difference compared with the control group (P<0.05). The 50% maximal inhibitory concentration (IC_{50}) was 200 μM in the 48 h group (Fig. 1).

Morphology of the BGC-823 cells. The cells treated with vehicle (Fig. 2A) were healthy, as they had networks of cell processes and vacuole-free cell bodies. Connections between BGC-823 cells were clearly observed. Nucleus fragmentation and shrinkage of the cell bodies were observed when the BGC-823 cells were exposed to 200 μM crocetin (Fig. 2B) or docetaxel (Fig. 2C) for 48 h. DMSO itself did not interfere with the BGC-823 cells (Fig. 2D).

BGC-823 cell apoptosis is induced by crocetin. The nuclear morphological changes associated with apoptosis were observed by Hoechst 33258 staining. The control group (Fig. 3A and E) revealed intact and relatively large nuclei, whereas the crocetin- or docetaxel-treated BGC-823 cells exhibited an increase in condensed nuclei (Fig. 3B, C and E). DMSO itself did not interfere with the BGC-823 cells (Fig. 3D and E).

Crocetin-induction decreases the MMP. In this study, the effects on the 200 μM crocetin-induced changes of the MMP in the BGC-823 cells were investigated with the fluorescent dye, Rh123, a cell permeable cationic dye, which preferentially enters into the mitochondria based on the highly nega-

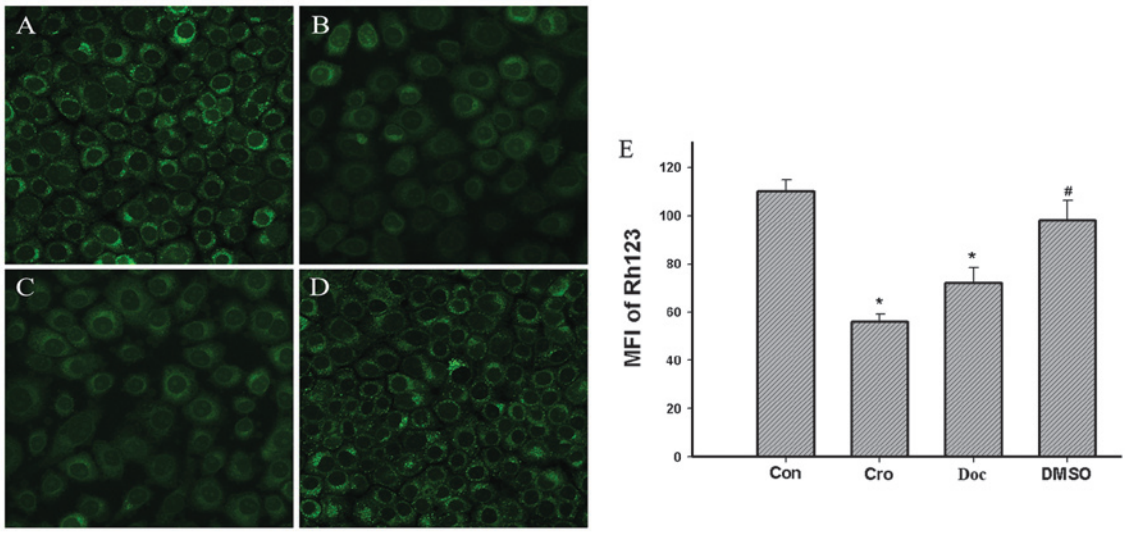

Figure 4. Effect of loss of MMP in BGC-823 cells induced by crocetin (magnification, x400). (A) Control (Con), (B) crocetin (Cro), (C) docetaxel (Doc) and (D) dimethylsulfoxide (DMSO) groups, and (E) quantitative analysis of the fluorescence intensity (*P<0.01 vs. the control group). MMP, mitochondrial membrane potential; MFI, mean fluorescence intensity.

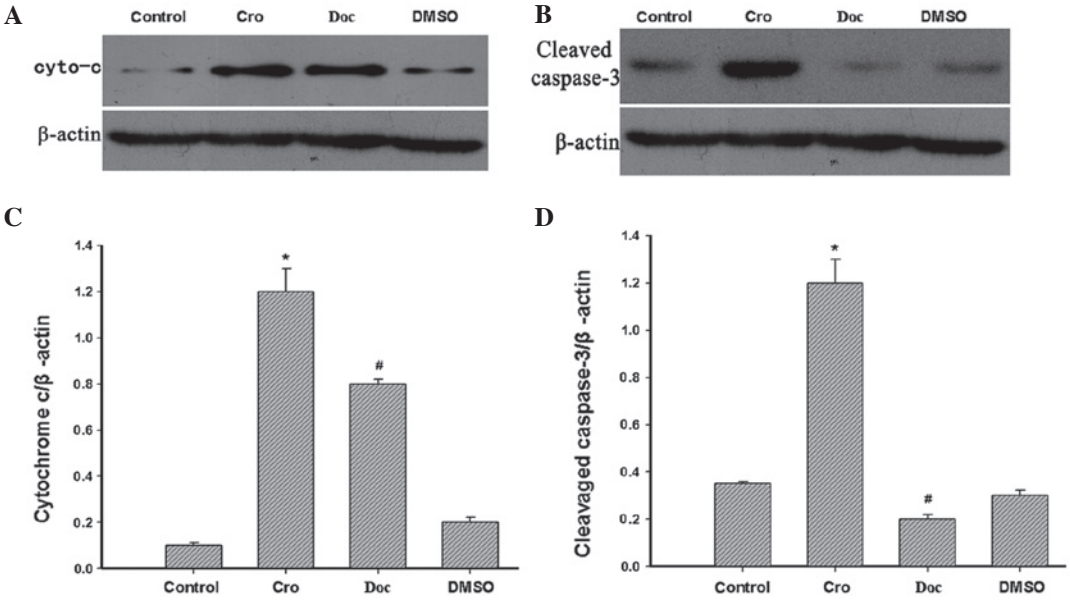

Figure 5. Effects of crocetin on cytochrome *c* and cleaved caspase-3 proteins induced by crocetin. (A) Cytochrome *c* and (B) cleaved caspase-3 proteins were detected by western blotting. A statistical comparison was made using a one-way analysis of variance followed by Dunnett's test. Grayscale analysis demonstrated that pre-treatment with 200 μM crocetin decreased the (C) cytochrome *c* and (D) cleaved caspase-3 levels. (*P<0.01 and #P<0.01 vs. the control group).

tive MMP. Following the exposure to 200 μM crocetin for 48 h, the mitochondrial uptake of Rh123 was significantly reduced (Fig. 4B) and the intensity of the mean fluorescence was significantly decreased (P<0.01) by 50% compared with the control (Fig. 4E).

Apoptosis-related protein changes in crocetin-treated BGC-823 cells. Cytochrome *c* is a component of the electron transport chain in the mitochondria, and it is also involved in the initiation of apoptosis. Therefore, the levels of cytochrome *c* and cleaved caspase-3 in cortical neurons were examined in the present study by western blotting. The levels of cytochrome *c* and cleaved caspase-3 were shown to be markedly increased in the cells that were incubated with 200 μM crocetin for 48 h compared with the control group (Fig. 5A-D).

Discussion

GC is one of the most common types of malignant tumors, with mortality rates in the forefront of cancer-related mortality. Previous studies regarding the pathogenesis of GC demonstrated that the incidence and progression of cancer have a close correlation with cell apoptosis (20). Apoptosis is programmed cell death and is distinguished from cell necrosis by morphological and biochemical changes with its own specific characteristics (20). A number of the chemotherapeutic agents, including crocetin and crocin,

the principal active ingredients of saffron, have potential antiproliferative effects (5,21,22) and have been revealed to suppress the growth of a variety of cancer cells via arresting the cell cycle and inducing cell apoptosis. To the best of our knowledge, the present study observed for the first time that crocetin suppressed the proliferation of BGC-823 cells in a dose- and time-dependent manner.

In the present experiments, a preliminary study demonstrated the antitumor effect of crocetin in an *in vitro* experimental BGC-823-cell model. Among the cytotoxicity detection experiments, crocetin revealed significant antiproliferative effects on the BGC-823 cells in a dose- and time-dependent manner. It is noteworthy that accumulated data concerning the toxic effect of crocetin has been reported, but there are different standpoints with regard to its effect (14). However, one previous *in vitro* study observed that crocetin had no cytotoxic effect in the normal cells (23). This difference may be due to the different cell lines and culture conditions used. Therefore, it was hypothesized that there may be different mechanisms behind the effects of crocetin on different cell lines, and for this purpose there was a requirement to conduct the present study. Considering the clinical value, the results imply that crocetin may be used as a potential candidate for future drug development.

DNA damage and cell membrane death receptor aggregation have long been considered to be a starting point for the induction of MMP changes or the direct activation of apoptosis (24). In the present study, the apoptosis of BGC-823 cells induced by crocetin and stained by Hoechst 33258 was observed, as well as the cell membrane staining dense nuclei, nuclear pyknosis, fragmentation, chromatin condensation and highlighted nuclear membrane staining under a light microscope. The MMP of the BGC-823 cells was reduced by crocetin, which indicated that crocetin induced apoptosis through a mitochondrial damage pathway. In the latter part of the study, crocetin was demonstrated to increase the activation of cleaved caspase-3 and cytochrome *c*, as revealed by western blotting. Increased cytoplasmic cytochrome *c* levels demonstrated that cytochrome *c* released from the disruptive mitochondria results in a decrease in the mitochondrial transmembrane potential. The high level expression of cytochrome *c* resulted in apoptosis of the BGC-823 cells through the caspase-9 and caspase-3 pathways (25).

Caspase-3, one of the key factors of apoptosis, uses the mitochondrial-initiated intrinsic pathway to increase its activity as cleaved caspase-3 and to lead to poly ADP ribose polymerase cleavage, DNA damage and fragmentation, nuclear condensation and ultimately induce apoptosis (26-29). These results were consistent with other agents previously reported, including docetaxel (30), γ-tocotrienol (31), magnolol (32), milk fermented by *Propionibacterium freudenreichii* (33), triptolide and cisplatin (34).

Therefore, in the present study, it was shown that mitochondrial damage, cytochrome *c* release and caspase-3 activation may be significant mechanisms for the crocetin-induced apoptosis of BGC-823 cells. The underlying mechanism for crocetin involved in signaling pathways modulating BGC-823 cell apoptotic responses remains to be elucidated.

The data from the present study revealed that crocetin suppressed the proliferation of the BGC-823 cells. This inhibition was associated with reduced cell proliferation through the downregulation or upregulation of certain apoptotic proteins, including cytochrome *c* and cleaved caspase-3 expression. In addition, a number of pharmacological studies have demonstrated crocetin to have multiple activities, including anti-inflammatory (35,36) and anti-oxidant (37,38) effects. Therefore, it was hypothesized that the anti-oxidant and anti-inflammatory effects of crocetin may also be considered to be involved in several synergistic antitumor mechanisms (39,40). Further *in vivo* studies are required to investigate in greater detail the mechanisms and pharmacokinetics of crocetin and to provide an experimental basis for the clinical application of the drug. In conclusion, the extant results endorse our hypothesis that crocetin has antitumor potential and may be considered as a novel drug candidate for the treatment of GC and to reduce chemotherapy side effects.

Acknowledgements

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