Experimental Research

ANNALS OF MEDICINE & SURGERY

OPEN

Crocin inhibit the metastasis of MDA-MB-231 cell line by suppressing epithelial to mesenchymal transition through WNT/β-catenin signalling pathway

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Background: Triple-negative breast cancer has the poorest prognosis and survival rates compared to other breast cancer subtypes due to its invasive behaviours. This type of cancer does not respond to biological therapies and exhibits resistance to available treatment options. Therefore, it is imperative to discover new therapeutics to address this challenge.

Methods: In this study, a TNBC cell line was utilized to investigate the anti-metastatic effect of crocin on the Wnt/β-catenin pathway. Cell proliferation was assessed using the MTT assay, and the effects of crocin on migration were monitored through transwell and wound healing experiments. The expression of specific epithelial-mesenchymal transition marker genes was evaluated using real-time polymerase chain reaction, and β-catenin expression was also examined through real-time polymerase chain reaction.

Results: The findings revealed that crocin significantly inhibits cell proliferation and migration of tumour cells in a dose-dependent manner. Moreover, crocin decreased the expression of Vimentin, Snail, Zeb-1, and β-catenin. Additionally, crocin increased the expression of E-cadherin in the MDA-MB-231 cell line.

Conclusions: The results demonstrated an association between crocin and the Wnt/β-catenin signalling pathway. In conclusion, this study establishes that crocin holds promise as a potential therapeutic option for triple-negative breast cancer.

Keywords: Crocin, epithelial-mesenchymal transition (EMT), metastasis, triple-negative breast cancer (TNBC), β-catenin

Introduction

According to reports from GLOBOCAN 2020, breast cancer stands out second most frequently diagnosed malignancy among the general population and it is the most commonly diagnosed cancer in the majority of the countries[1].

Triple-negative breast cancer (TNBC) is defined by tumours that lack expression of the human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR) and oestrogen receptor (ER). As a result, it is unresponsive to medications that specifically target these receptors[2]. This tumour type behaves more aggressively and includes for ~15% of invasive breast cancers[3]. Treatment options due to the absence of these receptors are limited and current effective therapies such as antibody therapies and hormone are not effective to this patient population. In most patients after chemotherapy tumour relapse occurred in higher rates in first 3 years after treatment and chemo resistance tend to develop, which causes reduction chemotherapy successfulness for TNBC treatment[4,5]. TNBC is distinguished by its aggressive cell invasion and tendency to spread to organs, notably the brain, lungs, and liver. On average, patients with TNBC have a survival time of ~18 months. TNBC has a higher propensity to metastasize to the central nervous system and internal organs, including the liver, bones, and lungs. Once distant metastases occur, patients with TNBC experience a significantly reduced survival period. Typically, TNBC is detected at an advanced stage in the body[6,7].

At present, there is no established standard treatment plan for TNBC, and chemotherapy is the main systemic treatment

HIGHLIGHTS

- In this study, results show that crocin inhibits triple-negative breast cancer metastasis.
- Treatment of MDA-MB-231 cells with crocin causes the prevention of epithelial-mesenchymal transition.
- E-cadherin, a key molecule in cell junction and polarity, upregulated after crocin treatment.
- Crocin decrease the expression of β-catenin in MDA-MB-231 cells.

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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Annals of Medicine & Surgery (2024) 86:1401–1407

Received 23 September 2023; Accepted 27 December 2023

Published online 8 January 2024

http://dx.doi.org/10.1097/MS9.0000000000001691

option[7]. The urgent need for new treatments TNBC, especially those derived from natural compounds, arises from the limitations of current therapies and the potential of natural products to target cancer stem cells (CSCs) and reduce toxic side effects. Natural compounds derived from plants have shown potential in inhibiting tumour metastasis in TNBC. These compounds, such as sulforaphane, curcumin, genistein, resveratrol, lycopene, and epigallocatechin-3-gallate, have been found to inhibit the epithelial-mesenchymal transition (EMT) that plays a crucial role in metastasis. Additionally, they have been shown to target pathways important CSCs, such as NF-κB, PI3K/Akt/mTOR, Notch 1, Wnt/β-catenin, and YAP, which are also involved in metastasis. Clinical trials have demonstrated varying degrees of effectiveness of these compounds, and further research is needed to explore their full potential. The use of natural compounds, either alone or in combination with other drugs, holds great promise in improving TNBC treatment efficacy and patient outcomes[8–10].

Cancer metastasis is a highly orchestrated process involving cellular and molecular alterations in cancer cells and the tumour microenvironment. During this process, the EMT plays a fundamental role in tumour formation and metastasis. In physiological conditions, EMT is involved in embryonic development, organogenesis, reconstruction of fibrosis, and healing of wounded tissues. However, in cancer, this process transforms non-mobile, adhesive, polar epithelial-like tumour cells into cells with a non-polar mesenchymal-like phenotype that is invasive and mobile, allowing them to migrate from the initial tumour site to other organs[11]. In this state, molecular biomarkers of epithelial cells such as E-cadherin and claudin are downregulated, while markers of mesenchymal cells such as vimentin and N-cadherin are upregulated. Studies have revealed that certain transcription factors, known as EMT-activating transcription factors (EMT-TFs), regulate the EMT process by repressing the expression of E-cadherin. These EMT-TFs primarily include the Snail, Slug, ZEB, and Twist families. Additionally, numerous signalling pathways, including Wnt/β-catenin, transforming growth factor-β (TGF-β), Hedgehog (Hh), and Notch, are involved in this process[12].

In recent decades, along with the unceasing exploration of traditional medicine, naturally occurring dietary substances have gained distinctive attractiveness in the treatment of cancers. They possess unique characteristics, including low toxicity, well-tolerance in the human body, and easy availability[13].

Saffron is an ancient herbaceous species gain from dried stigma of the *Crocus sativus L*. Among the main bioactive compounds that have been identified in saffron, crocin, a carotenoid pigment, possesses the most potent anti-cancer and anti-metastatic properties[14–16]. Arzi *et al.*[17] showed that it has an inhibitory effect on the metastasis of triple-negative breast cancer (TNBC) by interfering with the Wnt/β-catenin pathway in murine models. Crocin has also been shown to inhibit tumour invasion and reduce cell mobility in TNBC cells, as well as induce cell cycle arrest and apoptosis[18]. In addition, crocin has been found to target microtubules in breast cancer cells, inhibiting cell proliferation and perturbing mitosis in cancer cells[16]. These findings suggest that crocin has potential as an anti-metastatic agent for TNBC and other forms of cancer. Further research is needed to explore the full potential of crocin and its mechanisms of action.

Although many studies have shown the anti-cancer and anti-metastatic properties of crocin, the molecular mechanism of anti-metastatic properties of this valuable compound is still unclear[19].

In this research, we evaluated the effects of crocin on the cell proliferation, migration, invasion, and EMT of TNBC breast cancer cells. Finally, we explored the WNT signalling cascade as the mechanism underlying the effects of crocin on TNBC breast cell lines.

Material and methods

Cell culture

Human breast cancer cell line (MDA-MB-231) were obtained from the Pasteur Institute Cell Bank of Iran. The cells were cultured and maintained in RPMI1640 medium containing 10% FBS and 1% penicillin/streptomycin and incubated in a humidified atmosphere of 5% CO_2 at 37°C. All assays were done using cells in exponential growth phase. Twenty-four hours after seeding, the cells were treated with culture medium containing different concentrations of crocin. Crocin of high purity (>99%) was purchased from Sigma-Aldrich and stored as a powder at +4°C in the dark. The stock solution of crocin (10 mM) was prepared in PBS, split in aliquots, and stored at −20°C in the dark. In each experiment a fresh diluted solution of crocin was prepared. All experiments were performed in triplicate.

Cell cytotoxicity assay

In vitro, cell proliferation and cytotoxicity were monitored by standard MTT assay for both treated and untreated control MDA-MB-231 cell line. MTT experiment is a colorimetric method with highly accuracy and broadly used to determine cell viability and cytotoxicity, especially in the development of novel therapeutics.

MDA-MB-231 breast cancer cells were harvested at exponential growth phase with 0.05% trypsin-EDTA and seeded in 96-well plates (SPL) at a density of 5000 cells per well. After 24 h incubation, cells were treated with 0, 5, 10, 20,40, 80, 160, 320, and 640 μM concentration of crocin. After 24, 48 and 72 h of incubation period, the supernatant culture medium was eliminated and MTT solution (0.5 mg/ml, 50 μl) (Sigma) were added and cells were incubated for another 4 h. Then, 200 μl of DMSO (dimethyl sulfoxide) was added to each well to dissolve formazan crystals and the absorbance of each well was obtained using a 3200 statfax ELISA plate reader (statfax, USA) at 570 nm (with a reference wavelength of 650 nm). The IC50 value (Concentrations that reduce the cell population up to 50%) were calculated by means of GraphPad Prism 6.01 software (GraphPadSoftware Inc.).

Cell migration assay

The CytoSelect TM 24-well cell migration and invasion assay (Cell Biolabs, Inc.) was utilized for the cell transwell migration assay. The assay used an 8-μm pore size and a colorimetric format. It was applied to assess the effect of crocin on the migration and invasion of MDA-MB-231 cells. The assay was performed according to the manufacturer's protocol.

In brief, the cells were treated with 60, 120, and 240 μM of crocin for 48 hours. After the treatment period, a cell suspension of 500 000 cells/ml was prepared and transferred into the upper chamber of the plate. The bottom chamber was loaded with

150 µl of complete RPMI1640 medium containing 10% FBS as a chemoattractant. The plate was then placed in a humidified incubator for 24 h.

In response to the chemoattractant, migratory cells passed through the polycarbonate membrane pores and invaded the bottom of the membrane. Finally, the migrated cells were stained, extracted, and quantified at an absorbance of 560 nm as outlined in the manufacturer's protocol.

Wound healing assay

The wound healing assay was used to assess the effect of crocin on the migration of MDA-MB-231 cells. A six-well plate was seeded with 5×10^5 cells in fresh medium and incubated in a humidified incubator until a monolayer with 100% confluency was formed. Subsequently, a straight scratch was generated in each replicate well of the monolayer cells using a sterile yellow pipette tip. To remove detached cells and debris, the plates were washed twice with PBS. Then, the plates were incubated with fresh RPMI1640 complete medium in the absence or presence of crocin for 48 hours[20].

The width of the wound was monitored using an inverted microscope (Olympus) within a specific time frame and photographed. ImageJ software (National Institute of Health) was used to measure the area of the wound. The relative area of the wound was calculated for each triplicate treatment, and the data were presented as mean ± SD.

Quantitative real-time polymerase chain reaction (PCR)

After 48 h of crocin treatment, the expression of desired genes was assessed by real-time PCR (qRT-PCR). Total RNA extraction was performed using RNX-plus reagent (SinaClon) following the manufacturer's protocol. cDNA synthesis was carried out using the Pars toos RT Reagent kit with 1 µg of total RNA (Pars toos).

qRT-PCR was performed in triplicate for each sample using specific primers for β-catenin, E-cadherin, Vimentin, Snail, and Zeb-1 (Table 1) in a 20 µl reaction mixture containing 1 µL of 0.5 mM primer, 10 µl of SYBR Green master mix, 5 µl of DW, and 4 µl of cDNA in a PCR microtube. The amplification reaction was carried out using the Rotor gene 6000 system (Corrbet).

The fold change in expression level of each mRNA sample was normalized against β-actin mRNA and quantified using the comparative $2^{-\Delta\Delta Ct}$ method.

Western blot assay

After 48 h of crocin treatment, expression of E-cadherin and β-catenin proteins was assessed by western blot. To begin, the protein was extracted from the lysed cells. The cells were collected and subjected to centrifugation at 1300 rpm for 10 min. The resulting supernatant was discarded, and 100 µl of RIPA buffer was added to the cell tube. After incubating at – 20°C for 1 hour, the mixture was centrifuged again at 1300 rpm for 10 min at 4°C. The supernatant, which contained the protein, was carefully transferred to a new microtube.

Next, SDS-PAGE was conducted using a resolving gel with a concentration of 28% and a stacking gel with a concentration of 22%. The protein obtained from SDS-PAGE was then transferred onto a PVDF membrane. The membrane was subsequently blocked with a blocking solution consisting of 5% skim milk in 0.05% PBS-Tween 20. This blocking process took place at – 4°C for a duration of 12 h. Following the blocking step, the membrane was washed three times with PBS-Tween 20 for 5 min each.

Subsequently, the membrane was incubated at 25°C for 90 min with monoclonal antibodies (Padza Co) at a concentration of 10 µg/ml. After the primary antibody incubation, the membrane underwent another round of washing with PBS-Tween 20 three times for 5 min each. Then, the membrane was incubated for 1 h at 25°C with an HRP secondary antibody (Padza Co) at a concentration of 0.4 µg/ml. The secondary antibody was diluted in a mixture of 1:2500 with 3% skim milk in 0.05% PBS/Tween.

Finally, the protein bands on the membrane were visualized using the ECL detection system[21].

Table 1

Primer sequence

Gene	Forward (5′–3′)	Reverse (5′–3′)
E-cadherin	GTGCCTGAGAACGAGGCTAA	CTGCATCTTGCCAGGTCCTT
Snail	CGAGTGGTTCTTCTGCGCTA	CTGCTGGAAGGTAAACTCTGGA
Vimentin	ACCCGCACCAACGAGAAGGT	ATTCTGCTGCTCCAGGAAGCG
ZEB-1	TGCACTGAGTGTGGAAAAGC	TGGTGATGCTGAAAGAGACG
β-catenin	GATTTGATGGAGTTGGACATGG	TGTTCTTGAGTGAAGGACTGAG
β-actin	TCCCTGGAGAAGAGCTACG	GTAGTTTCGTGGATGCCACA

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.01 software. The results were presented as the mean ± standard deviation (SD). The unpaired Student's *t*-test was used to assess statistical differences, and a p value of less than 0.05 was considered significant.

Results

Crocin inhibit cell proliferation in dose-dependent manner

The results showed that crocin changed the morphology of cells and decreased the number of cells in the treated groups. To confirm this observation, cell proliferation was determined using the MTT assay. The results indicate that crocin significantly inhibited the proliferation of MDA-MB-231 cells in a dose-dependent manner (Fig. 1). The inhibitory effect of crocin was much stronger at 48 h of incubation compared to 24 and 72 h. The IC50 value at 48 hours of incubation was 248 µM, and the crocin concentration for the subsequent experiments was selected based on this IC50 value.

Crocin suppress breast cancer cell migration

A wound healing assay was performed to investigate the inhibitory effect of crocin on the migration of the MDA-MB-231 cell line. The results indicated that crocin suppressed cell migration into the denuded zone in a dose-dependent manner within the first 24 h after treatment, and this effect continued for more than 48 h. Figure 2 demonstrates this effect and shows that the untreated group displayed a high degree of cell migration, while the treated cells exhibited less closure of wounds and a slower migration rate.

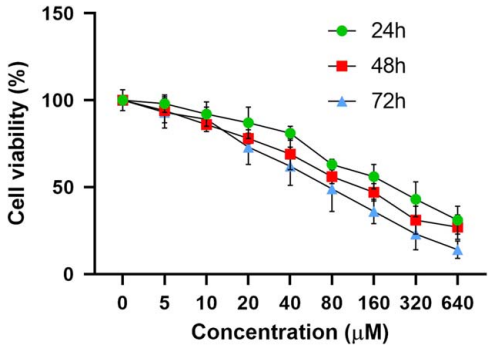

Figure 1. Crocin inhibits cell proliferation in triple-negative breast cancer cells. Different concentration ranges from 0 to 640 μM of crocin were used for MDA-MB-231 cells treatment for 24, 48 and 72 h. MTT assay were used for evaluating cytotoxic and antiproliferative effect of crocin on MDA-MB-231 cell line. Each assay repeated in three independent experiments and the data are presented as mean ± SD.

Crocin inhibits MDA-MB-231 cells invasion

The dispersion of cancer cells from the primary tumour to distant organs requires transmission through the extracellular membrane. A Transwell migration assay was performed to evaluate the inhibitory effect of crocin on invasion. The crocin-treated cells demonstrated a significant inhibitory effect on invasion and migration compared to the untreated controls. Treatment with 60, 120, and 240 μM crocin resulted in inhibition rates of 32.4%, 61.3%, and 83.6%, respectively. Compared to the untreated group, both the 120 and 240 μM crocin-treated cells exhibited a reduction in migration rate of up to 50% (Fig. 3). These findings provide evidence that crocin inhibits cell invasion and migration in the breast cancer TNBC cell line.

Crocin upregulate E-cadherin and decreased expression of genes involved in EMT

To determine if crocin reduces β-catenin expression, we treated MDA-MB-231 cells with 60, 120, and 240 μM of crocin for 48 h. In the previous sections, we examined the effects of crocin on the migration and invasion of the TNBC breast cancer cell line.

As the EMT process is linked to cancer metastasis, we also investigated the expression of epithelial (E-cadherin) and mesenchymal markers (Snail, Vimentin, and ZEB-1) in breast cancer cells[22]. Compared to the untreated control group, the group of MDA-MB-231 cells treated with crocin showed an increase in mRNA expression of E-cadherin, while the expression of Snail, Vimentin, β-catenin, and ZEB-1 mRNA was reduced (see Fig. 4). Furthermore, the crocin treatment group exhibited an oval cell morphology, while the untreated control group displayed a spindle-like mesenchymal phenotype (data not shown). These findings suggest that crocin has the ability to suppress the invasiveness of breast cancer cells by downregulating EMT markers and the β-catenin pathway.

Crocin upregulate E-cadherin protein and decreased expression of β-catenin

The levels of E-cadherin and β-catenin proteins were examined through western blot analysis (Fig. 5). The results from both western blot and real-time PCR analyses indicated that β-catenin exhibited a decrease in expression, while E-cadherin showed an increase in expression in MDA-MB-231 cell line.

Discussion

In women diagnosed with TNBC, systemic chemotherapy beside surgery and radio therapy remains the mainstay regimen in the treatment of these patients[6]. Despite the progress in our understanding and treatment of breast cancer, attempts to cure TNBC have often failed due to the absence of hormone receptors and other therapeutic targets. This type of breast cancer is associated with high rates of metastasis, recurrence, and mortality compared to other molecular subtypes. As metastasis is the leading cause of death in TNBC, it is crucial to develop new treatment approaches that target molecular networks and inhibit cancer metastasis in order to improve the poor prognosis of this disease[23].

The use of traditional herbal medicines has gained momentum in the development of anti-cancer drugs due to their significant

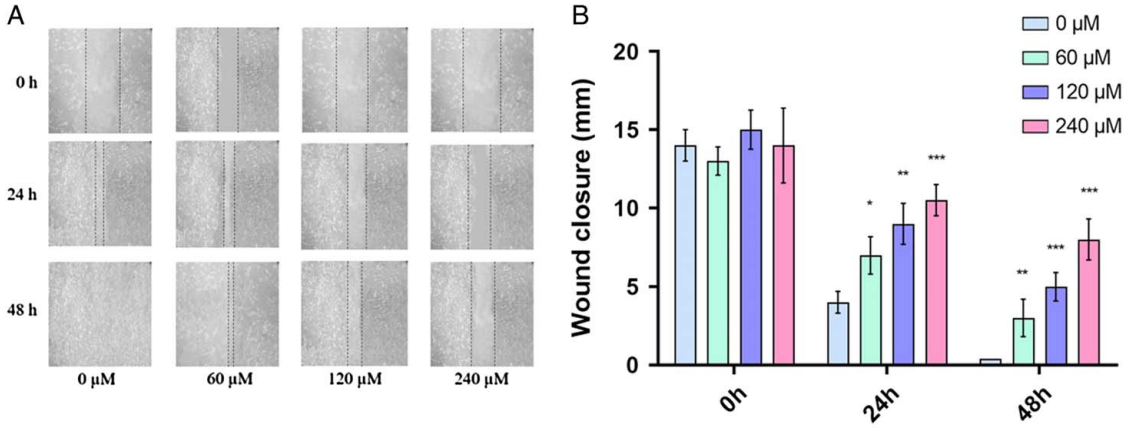

Figure 2. Wound healing assay exhibit that crocin possess inhibitory effect on the MDA-MB-231 cells migration. (A) Raw microscopic image of the scratched region in control and treated groups. (B) Wound closure distances were quantified by ImageJ software and its histogram chart was plotted. The data obtained from the triplicate experiment and shows a significant difference compared to the control group. Data are presented as mean ± SD (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. untreated control).

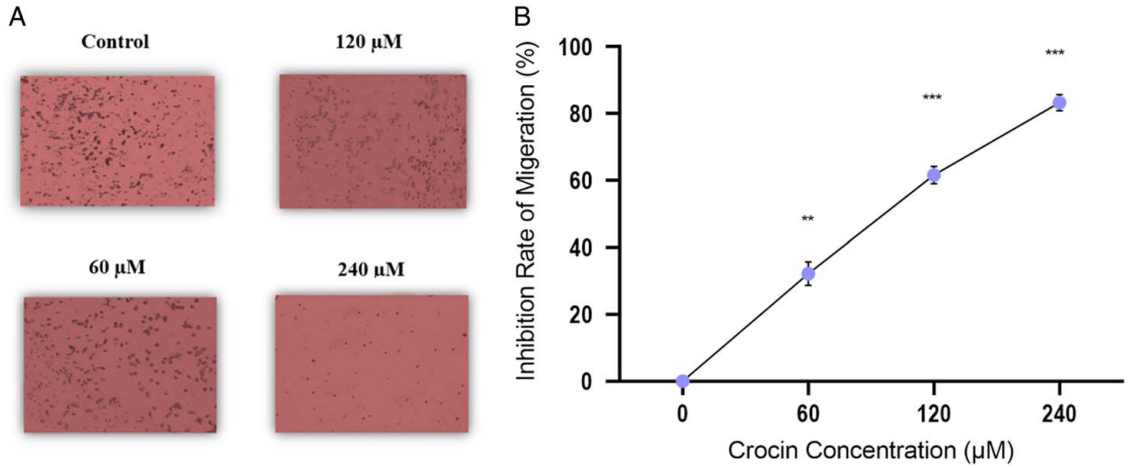

Figure 3. Transwell assay were used for evaluation effect of crocin on MDA-MB-231 cells migration. MDA-MB-231 cells were treated with different concentrations of crocin (0, 60, 120 and 240 µM) for 48 h. (A) Microscopic image of cell migration. (B) The experiment performs in three independent experiments and the chart depicts the crocin inhibition rate of migration of the MDA-MB-231 cell line. Data are presented as mean ± SD (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. untreated control).

anti-cancer potential and fewer adverse effects. Recent studies have shown that saffron and its main carotenoids, crocin and crocetin, possess anti-cancer and anti-metastatic properties. This ancient spice has been found to have anti-migratory, anti-invasion, and anti-angiogenic effects in various types of cancers. Previous research has also shown that crocin is more effective in inhibiting metastasis compared to saffron extract and crocetin[24]. In this study, we utilized crocin as a potential inhibitor of metastasis in TNBC tumour cells.

Results of this study revealed that crocin markedly inhibited the migratory effect of TNBC cells. The abundant studies confirm these anti-metastatic results of crocin. Amerizadeh *et al.* showed that crocin retards cellular migration via downregulation of several genes involved extracellular matrix include MMP-2 and MMP-9 and also genes involved WNT/ PI3K signalling pathway in murine breast cancer model[25]. Zhou *et al.*[26] reported the crocin inhibit migration, invasion, and EMT in gastric cancer via KLF5/HIF-1α signalling. Bakhshi and colleagues showed that crocin inhibited angiogenesis and metastasis in colon cancer via the TNF-α/NF-kB/VEGF pathways. The anti-angiogenic activity of crocin was found to hamper tumour growth and metastasis in colon cancer cells. The study suggested that crocin may inhibit colon carcinoma-induced angiogenesis through the TNF-α/NF-kB/VEGF pathways[27].

In the process of metastasis, EMT plays a crucial role and contributes to tumour development. EMT is characterized by the

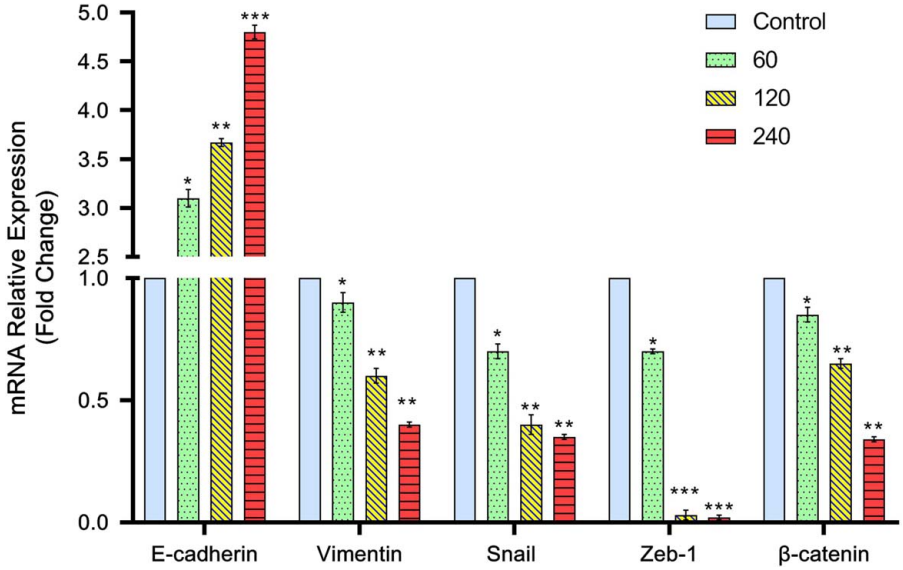

Figure 4. Crocin effects on expression of epithelial-mesenchymal transition markers and β-catenin in triple-negative breast cancer cell line. Real-time polymerase chain reaction assays were used for determining mRNA levels. β-actin housekeeping gene was used for qPCR data normalization. Each sample was repeated in triplicate and the data were displayed as mean ± SD. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. untreated control).

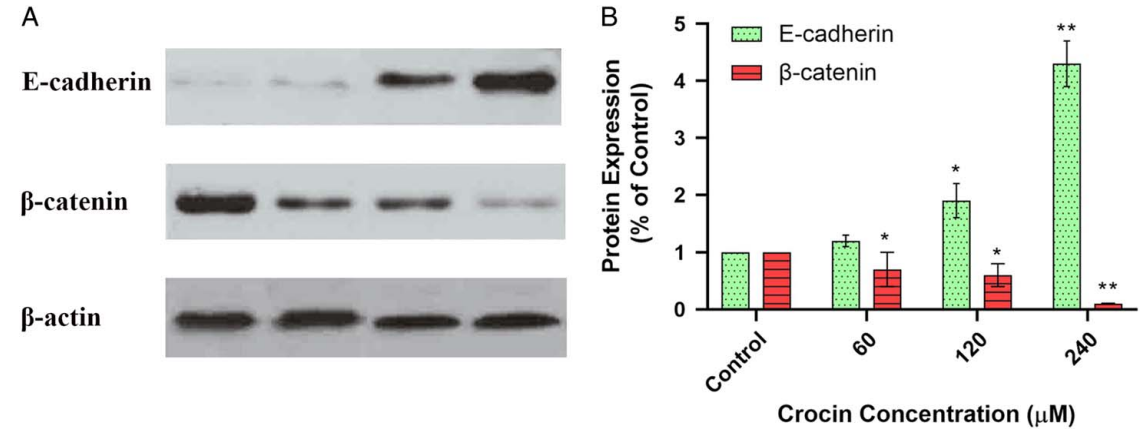

Figure 5. Quantification of E-cadherin and β-catenin protein levels in MDA-MB-231 cells incubated with crocin. (A) Shows the expressions of E-cadherin and β-catenin in cell lysates analyzed by western blotting. The cells were treated with crocin in 60, 120 and 240 μM of croin for 48 h. β-actin protein was used as control. (B) Indicates the densitometric ratio of E-cadherin and β-catenin / β-actin for each treatment. The results are representative of three independent experiments. Data were quantified using ImageJ software.

loss of intracellular junctions and cell polarity, as well as the acquisition of mesenchymal features. The relationship between EMT and metastasis is closely linked to the poor prognosis of patients with TNBC cancer[28]. In the present study, we assessed the expression of epithelial marker E-cadherin and mesenchymal markers Snail, ZEB-1, and vimentin in TNBC cancer cells. Our findings showed a significant increase in E-cadherin expression and a drastic decrease in Snail, ZEB-1, and vimentin expression in MDA-MB-231 cells treated with crocin. This suggests that crocin may have the ability to reverse EMT (Fig. 4).

The loss of E-cadherin affects cell junctions and polarity. This molecule plays a crucial role in the process of EMT[29]. one possible mechanism involved in the dysfunction and loss of expression of E-cadherin may be through β-catenin signalling. In its normal state, E-cadherin serves as the primary binding partner of β-catenin and plays a crucial role in stabilizing and functioning of this molecule[30]. Our findings indicate that the expression levels of β-catenin were significantly reduced in a dose-dependent manner when cells were treated with varying doses of crocin. This is consistent with a study by Arzi and colleagues, which demonstrated the anti-metastatic effects of bioactive carotenoids from saffron through the Wnt/β-catenin pathway in 4T1 cells. Our study also revealed that crocin has anti-migratory and anti-metastatic properties that are linked to the regulation of β-catenin[18].

In addition to EMT role in metastasis, it is increasingly recognized as a crucial process in tissue fibrogenesis during disease and normal aging. The interplay between EMT activity, senescence, cellular senescence, and the involvement of resident mesenchymal stem cells (MSCs) in tissues represents a complex and evolving area of research. Persistent inflammation and tissue fibrosis, commonly observed in aging, are influenced by EMT. Tissue-resident MSCs also contribute to tissue regeneration following cell turnover, apoptosis, or necrosis. However, aging modifies the characteristics of tissue-resident MSCs, resulting in reduced proteasome activity and heightened oxidative stress, ultimately leading to cellular senescence[31]. Given that this study demonstrated some extent of crocin's ability to modulate the expression of genes involved in EMT, further investigation is warranted to explore its effects in this domain.

Our study has several limitations that should be considered when interpreting the results. Firstly, we only utilized a single cell line (MDA-MB-231) to investigate the effect of crocin on metastasis through the WNT/β-catenin signalling pathway. Therefore, it is possible that our findings may not be generalizable to other cell lines or cancer types. Secondly, we did not perform additional tests such as Matrigel invasion assays to further support the anti-metastatic effects of crocin. This limits the strength of our conclusions regarding the ability of crocin to inhibit metastasis. Finally, we only measured the expression of E-cadherin and β-catenin proteins through Western blot analysis and did not investigate other proteins that may be involved in the WNT/β-catenin signalling pathway. Therefore, further studies are needed to validate our findings and explore the underlying molecular mechanisms in more detail.

Conclusion

In conclusion, our findings suggest that crocin exhibits inhibitory effects on cell proliferation and metastasis in the triple-negative MDA-MB-231 breast cancer cell line by suppressing the β-catenin signalling pathway. While these results provide promising insights, it is important to acknowledge the limitations of our study. The use of a single cell line and the absence of additional tests, such as matrigel invasion assays, may restrict the generalizability and comprehensive understanding of crocin's anti-metastatic potential. Further investigations involving diverse cell lines and additional experimental validations are warranted to fully elucidate the therapeutic efficacy of crocin in controlling metastatic triple-negative breast cancer.

Ethical approval

Ethics Committee of Lorestan University of Medical Sciences approved this research (IR.LUMS.REC.1398.096).

Consent

None of the authors in this study worked on human samples.

Sources of funding

This study was funded by Deputy of Research and Technology (grant No. 1019), Lorestan University of Medical Sciences, Khorramabad, Iran.

Author contribution

H.D. designed the study and V.G. wrote the manuscript. K.A.H. ALJAF collected and analyzed data, and H.M. revised the manuscript. All authors read and approved the final submitted manuscript.

Conflicts of interest disclosure

The authors declare there is no conflict of interest.

Research registration unique identifying number (UIN)

Not applicable.

Guarantor

Hassan Dariushnejad.

Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

Provenance and peer review

We agree.

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