Crocin Inhibit the Metastasis of MDA-MB-231 cell line by Suppressing Epithelial to Mesenchymal Transition through WNT/β-catenin Signaling Pathway

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Research Article

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Abstract

Breast cancer is divided into different subtypes based on molecular characteristics, among these subtypes, Triple-negative breast cancer, has the poorest prognosis and survival with invasive. In this study, TNBC cell line was used to explore crocin anti-metastatic effect on the Wnt/β-catenin pathway. Cell proliferation assessed by MTT assay and effects of crocin on migration monitored by transwell and wound healing experiments. Expression of certain epithelial-mesenchymal transition (EMT) markers genes was evaluated by real-time PCR. β-catenin expression also examined by real-time PCR. Findings revealed crocin significantly inhibits cell proliferation and migration of tumor cells in a dose-dependent manner. Moreover, crocin decreased the expression of Vimentin, Snail, Zeb-1 and β-catenin. Also, crocin increased the expression of E-cadherin in MDA-MB-231 cell line. Results showed an association between crocin and Wnt/β-catenin signaling pathway. In conclusion, this study establishes that crocin can be a promising therapeutic for triple-negative breast cancer.

Introduction

According to reports from GLOBOCAN 2018, breast cancer stands out second most frequently diagnosed malignancy for the general population and it is the most commonly diagnosed cancer in the majority of the countries (Bray et al., 2018).

Triple-negative breast cancer (TNBC) is defined by tumors that lack expression of the human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR)and estrogen receptor (ER). This tumor type behaves more aggressively and includes for approximately 15% of invasive breast cancers (da Silva et al., 2021). 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 tumor relapse occurred in higher rates in first three years after treatment and chemo resistance tend to develop, which causes reduction chemotherapy successfulness for TNBC treatment (Dass et al., 2021; Lashgarian et al., 2020). In view of these reasons and limited clinical treatment options, finding new therapies is a necessity that has attracted many studies today.

Cancer metastasis is highly orchestrated of cellular and molecular alterations in cancer cells and the tumor microenvironment. During this process Epithelial-to-mesenchymal transition (EMT) plays a fundamental role in tumor formation and metastasis. In physiological condition, EMT involved in embryonic development, organogenesis, reconstruction of fibrosis and wounded tissues. In return, this process transform non-mobile, adhesive, polar epithelial like tumor cells into cells with a non-polar mesenchymal-like phenotype, invasive, mobile which provides to migrate from initial tumor site to other organs (Singh et al., 2018). In this state epithelial cell molecular biomarkers like E-cadherin and claudin are downregulated and mesenchymal cells markers such as vimentin and N-cadherin altered in an opposite way and up regulate. Studies revealed that some transcriptional factors (TFs) so-called EMT-activating transcription factors (EMT-TFs) regulate EMT process by repressing the encoding of E-cadherin. These EMT-TFs mainly include Snail, Slug, ZEB and Twist families and numerous signaling

pathways like Wnt/β-catenin, transforming growth factor-β (TGF-β), Hedgehog (Hh) and Notch are involved in this process (Majidpoor and Mortezaee, 2021).

In recent decades, along with the unceasing exploration of traditional medicine, naturally dietary substances have presented distinctive attractiveness in the treatment of cancers. They possess unique characteristics include, low toxicity, well-toleration in human body and easy to be obtained (Sferrazza et al., 2020).

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 anticancer and anti-metastatic properties (Dariushnejad et al., 2020; Ghorbanzadeh et al., 2017; Veisi et al., 2020).

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

Material And Methods

In-silico analysis

The cBioPortal for Cancer Genomics database is an open-access resource at http://www.cbioportal.org used for genomic alteration, visualization, analysis and correlation of the mRNA levels between individual genes (CTNNB1, SANI1, ZEB1, VIM and CDH) and Pearson correlation analysis was used to attain their correlation coefficients.

The bcGenExMiner v4.1 database at http://bcgenex.ico.unicancer.fr/ was used to evaluate the relationship among the expression of β-catenin, E-Cadherin, Vimentin, SNAIL and ZEB1.

The Kaplan Meier Plotter (http://kmplot.com/analysis/) used for evaluating the biological relationships between survival information and gene expression levels. This tool provides powerful platform prognostic values of each selected genes.

Cell culture

Human breast cancer cell line (MDA-MB-231) were purchased from the Pasteur Institute Cell Bank of Iran. Cells were cultured and maintained in RPMI1640 medium containing 10% FBS and 1% penicillin/streptomycin. Cells were incubated in a humidified atmosphere of 5% CO2 at 37 ◦C. All assays were done using cells in exponential cell growth phase. Twenty-four hours after seeding, the cells were treated with culture medium containing different concentrations of crocin.

Cell cytotoxicity assay

In-vitro, cell proliferation and cytotoxicity were monitored by standard MTT assay for 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, chiefly 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, Korea) at a density of 5,000 cells per well. After 24h 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, Germany) were added and cells were incubated for another 4 h. Then, 200 µL of DMSO (dimethyl sulfoxide) was added to each well for 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., USA).

Cell migration assay

CytoSelect TM 24-well cell migration and invasion assay utilize for Cell trans-well migration assay (8-µm pore size; colorimetric format; Cell Biolabs, Inc., USA) this test applied for assessment of crocin effect on the migration and invasion of the MDA-MB-231 cells. Assay was performed According to the manufacturer's protocol. In brief, cells treating with 60, 120 and 240 µM of crocin for 48 h. After treatment period, a 500,000 cells/ml cell suspension was prepared and transferred into the upper chamber of the plate. 150 µl complete RPMI1640 medium containing 10 % FBS as chemoattractant loaded to the bottom chamber and plate were placed for 24 h in humified incubator. In response to chemoattractant, migratory cells passed through polycarbonate membrane pores and invaded to the bottom of the membrane. Lastly, migrated cells were stained, extracted, and quantified at A560 nm as pronounced in the manufacturer protocol.

Wound healing assay

wound healing assay were used for assess crocin effect on migration of MDA-MB-231 cells. Six-well plate seeded with 5×10^5 cells in fresh medium and incubated in humified incubator to forming the monolayer with 100 % confluency. Subsequently, in each replicate well of monolayer cells a straight scratch was generated using a sterile yellow pipette tip. To remove detached cells and debris, plates were washed twice with PBS. Then plates incubated with fresh RPMI1640 complete medium in the absence or presence of crocin for 48 h (Grada et al., 2017).

The width of the wound was monitored by inverted microscope (Olympos, Japan) within a desired time frame and photographed. ImageJ software (National institute of Health, Bethesda, MD, USA) were used for measurement of area of wound. Relative area of wound was calculated from each triplicate treatment and the data were presented as mean ± SD.

Quantitative Real-Time PCR

After 48 h of crocin treatment, desired gene expressions were assessed by Real-time polymerase chain reaction. Extraction of total RNA was performed using RNX-plus reagent (SinaClon, Iran) according to the manufacturer's protocol. cDNA was synthesized using Pars toos RT Reagent kit with 1 µg total RNA (Pars toos, Iran). qRT-PCR was performed in three replicates of each sample with specific primers for β-catenin, E-cadherin, Vimentin, Snail and Zeb-1(Table 1) in a 20 µL reaction mixture microtube containing, 1 µL of 0.5 mM of primer, 10 µL SYBR Green master mix, 5 µL DW and 4 µL of cDNA in PCR micro tube. Rotor gene 6000 system (corrbet, Australia) were used for amplification reaction. Expression level fold change of each mRNA sample normalized against the β-actin mRNA and quantified based on of the comparative $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Statistical analyses performed by GraphPadPrism 6.01 software. Results were demonstrated as the mean ± standard deviation (SD). Unpaired student t-test for assessment of statistical differences were used; and P value less than 0.05 was considered significant.

Results

β-catenin is associated with the expressions of Vimentin and key mesenchymal markers in TNBC

β-catenin play key role in the WNT signaling pathway and EMT process. Also, this molecule part of adherents junctions and links E-cadherin to the cytoskeleton. On the other hand this molecule contributed to chemoresistance in multiple malignancies include breast cancer (Xu et al., 2020).

After analysis of depicted Heat-map by bcGenExMiner v4.1 database, we found that among selected EMT markers, Vimentin showed highest correlation to β-catenin in breast cancer and the rest of the genes showed lower degrees of correlation with β-catenin. (Fig1a)

Since CDH1 is crucial for breast cancer EMT, we evaluated genetic alteration of cadherin and EMT transcription factors. For this means, genomic alteration of selected genes analyzed in TCGA datasets via cBioPortal.

Percentages of alterations in CTNNB1, SNAI1, ZEB1, VIM and CDH1 genes among all tumors samples of the Metastatic Breast Cancer Project (Provisional, February 2020) varied from 3-14% in between individual genes (CTNNB1, 6%; CDH1,17%; VIM, 6%, ZEB1, 3%, SANI1, 14%). CDH1 Showed the highest genomic alteration percentage between desired genes. of note that mRNA low-level expression is relevant to the alteration events (mutations or deletions) in the Cadherin profile. (Fig1B)

The prognostic significance of cadherin and EMT-TF genes determined by Kaplan-Meier survival curve. Kaplan–Meier Plotter analysis showed that the level of CDH1 mRNA expression was positively correlated

with overall survival in breast cancer patients (HR = 1.2, p = 0.00036), the result of survival curve of EMT-TF genes showed in Fig1c.

Crocin inhibit cell proliferation in dose-dependent manner

Result showed crocin changes morphology of cells and decreased number of cells in treated groups. To confirm this observation, cell proliferation was determined by MTT assay. Results indicate that crocin significantly inhibited the proliferation of MDA-MB-231 cells in dose-dependent manner. (Fig2) the inhibitory effect of crocin was much stronger at 48h incubation than that at 24 and 72 h. The IC50 value at 48h incubation was 248 μM and crocin concentration of the subsequent experiments was selected according to this IC50 value.

Crocin suppress breast cancer cell migration

Wound healing assay was performed to investigate inhibitory effect of crocin on migration of MDA-MB-231 cell line. The results indicated that crocin suppress cell migration to the denuded zone at dose-dependent manner in early 24h after treatment and this effect continued more than 48h. Fig 3 demonstrates this effect and showed the untreated group displayed a high degree of cell migration and treated cells showed less closure of wounds and more slow migration rate.

Crocin inhibits MDA-MB-231 cells invasion

Cancer cells dispersion from the primary tumor to distant organs require transmission from extracellular membrane. Transwell migration assay was performed to evaluate the invasion inhibitory effect of crocin. Crocin-treated cells showed an appropriate invasion and migration inhibitory effect in comparison to untreated controls. Treatment with 60, 120, and 240 μM crocin resulted in 32.4, 61.3, and 83.6 %, respectively. In comparison with the untreated group both 120 and 240 μM crocin-treated cells exhibited migration rate reduction up to 50 % (Fig. 4). These findings proved that crocin inhibits cell invasion and migration in breast cancer TNBC cell line.

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

To investigate whether crocin functions by reducing β-catenin expression, MDA-MB-231 cells treated with 60, 120 and 240 μM of crocin for 48h.

In above sections, we investigated the crocin effects on the migration and invasion of TNBC breast cancer cell line. Given that the EMT process related to cancer metastasis, we also explored the expression of the epithelial (E-cadherin) and mesenchymal markers (Snail, Vimentin and ZEB-1) in breast cancer cells (Valizadeh Otaghsara et al., 2020). Compared with the untreated control group, crocin-treated MDA-MB-231 cells had increased mRNA expression of E-cadherin, whereas the expression of Snail, Vimentin, β-catenin and ZEB-1 mRNA was reduced (Fig 5). Additionally, crocin treatment group showed oval cells morphology while untreated control group showed spindle-like mesenchymal phenotypes (Data not

shown). Taken together, these results indicate that crocin can suppress invasiveness of breast cancer cells through downregulating EMT markers and β-catenin pathway.

Discussion

In women diagnosed with TNBC, systemic chemotherapy beside surgery and radio therapy remains the mainstay regimen in the treatment of these patients. Despite of progress in our knowledge and breast cancer treatment procedure, Due to the absence of hormone receptors and other therapeutic targets, attempts to cure TNBC usually failed and this type of breast cancer associate with metastasis, high recurrence risk and mortality compared with other molecular subtypes. Since metastasis is a majority cause of TNBC death, therefore new treatment approaches based on new molecular networks and inhibiting cancer metastasis required to advance the poor prognosis of TNBC (Wang et al., 2021).

Traditional herbal medicines, possessing significant anticancer potential and less adverse effects, have received increasing momentum for anticancer drug design. In the last decades, studies revealed that saffron and its main carotenoids, crocin and crocetin possess anti-cancer and anti-metastatic potential. This ancient spice demonstrated the anti-migratory, anti-invasion, anti-angiogenic effect in various types of cancers. According to previous studies, in comparison with saffron extract and crocetin, crocin displayed more effective anti-metastatic potency (Arzi and Hoshyar, 2021). Therefore, in this study, we applied crocin as a metastatic potential inhibitor for TNBC tumor cell line.

Here, we did an in-depth analysis of public databases including TCGA, BcGenExMiner v4.1 and Kaplan–Meier-plotter. The results revealed a strong link between β-catenin and vimentin mRNA expression, which also demonstrated an intimate connection with major EMT markers in TNBC. This remarkable association was also verified by Transwell assay and QPCR results (Figs. 4 and 5)

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 signaling pathway in murine breast cancer model (Amerizadeh et al., 2018). Zhou et al. reported the crocin inhibit migration, invasion, and EMT in gastric cancer via KLF5/HIF-1α signaling (Zhou et al., 2019).

In Metastasis process, EMT play crucial role and contributes to tumor development. EMT is characterized by loss of intracellular junctions and cell polarity and acquirement of mesenchymal features. EMT and metastasis are closely associated to the poor prognosis of patients with TNBC cancer (Koleckova et al., 2021). In present study, we evaluated the expression of epithelial marker (E-cadherin) and mesenchymal markers (Snail, ZEB-1 and vimentin) in TNBC cancer cells. Our results revealed that expression of E-cadherin was significantly increased, while expression of Snail, ZEB-1 and vimentin was radically reduced in MDA-MB-231 cells treated with crocin, suggesting that crocin could reverse EMT (Fig. 5).

Loss of E-cadherin is influencing cell junction and polarity. This molecule is one of the important key molecules involved in EMT (Aban et al., 2021). One of the probable mechanisms involved in E-cadherin dysfunction and loss of its expression could be through β-catenin signaling. In normal state, E-cadherin is the main binding partner of β-catenin and play crucial role in stabilization and function of this molecule (Ma et al., 2021). Our results showed that when cells were treated with different doses of crocin, the expression levels of β-catenin significantly decreased in a dose-dependent manner. In consist of study Arzi et al. that show bioactive carotenoids of saffron show their anti-metastatic effect through Wnt/b-Catenin pathway genes in 4T1 cells, we showed anti-migratory and anti-metastatic effects of crocin are associated with the β-catenin (Arzi et al., 2018).

In summary, we have described here that crocin inhibits cell proliferation and metastasis of the triple negative MDA-MB-231 breast cancer cell line through suppression of β-catenin signaling pathway. These data suggest that crocin might be an experimental therapeutic for control TNBC metastatic cancer in clinic.

Declarations

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Data availability The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Author Contribution H. D: wrote the manuscript, K. A: conceived the original Idea, developed the theory, H. W: verified the analytical methods, L. P: analyzed the data, V.GH: analyzed the data, performed the methods and supervised the findings of this work. All the authors contribute to the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare there is no conflict of interest.

Consent to participate All authors have seen the manuscript and approved to submit the manuscript.

Consent for publication All authors consent to the publication of the manuscript.

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

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Tables

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

Figures

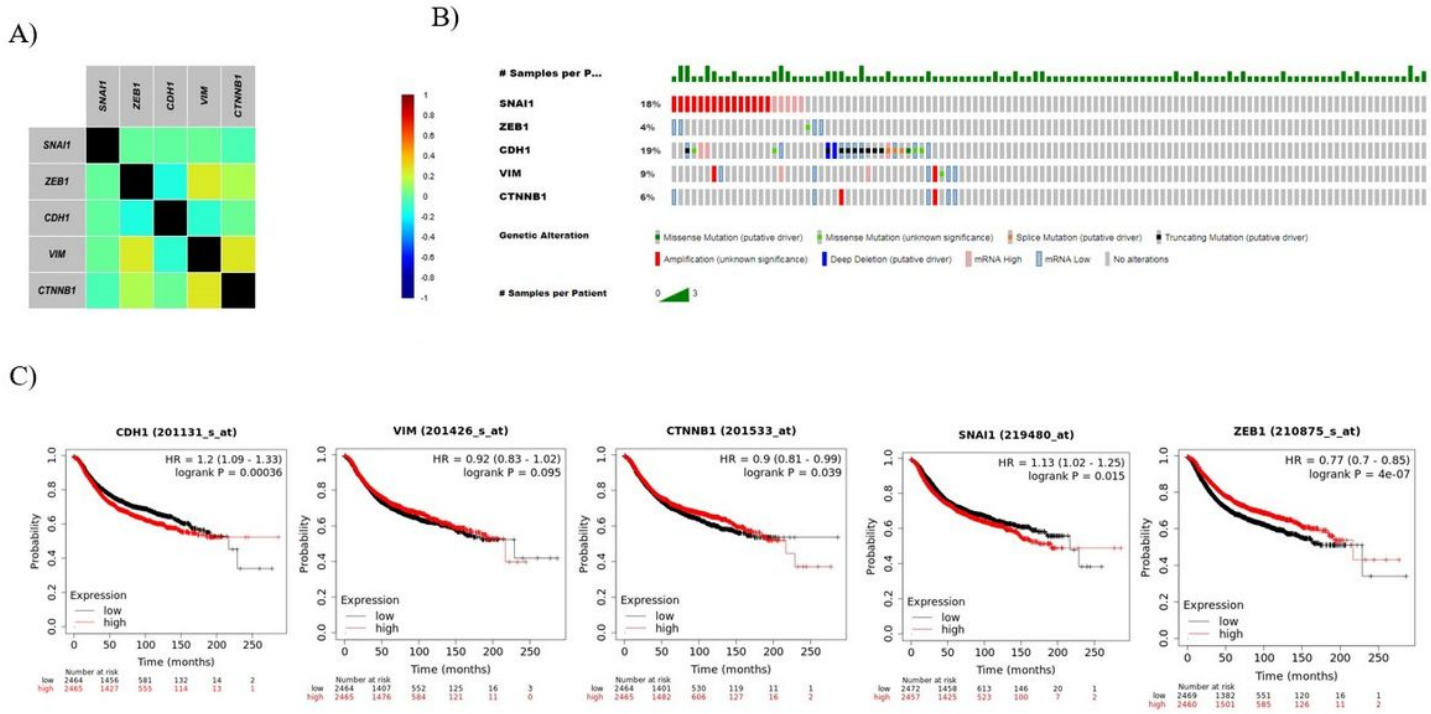

Figure 1

Bioinformatic analysis of expression correlation in breast cancer. (A) Heat-map representing the correlation between CDH and some EMT markers. (B) Oncoprint analysis indicate summary of genetic alterations in CTNNB1, SANI1, ZEB1, VIM and CDH across patients with breast cancer from the study of the Metastatic Breast Cancer Project (Provisional, February 2020). (C) The Kaplan–Meier survival curves of high and low expressions of CTNNB1, SANI1, ZEB1, VIM and CDH expression in all subtypes of breast cancer. Kaplan Meier-plotter were used for data analyzing.

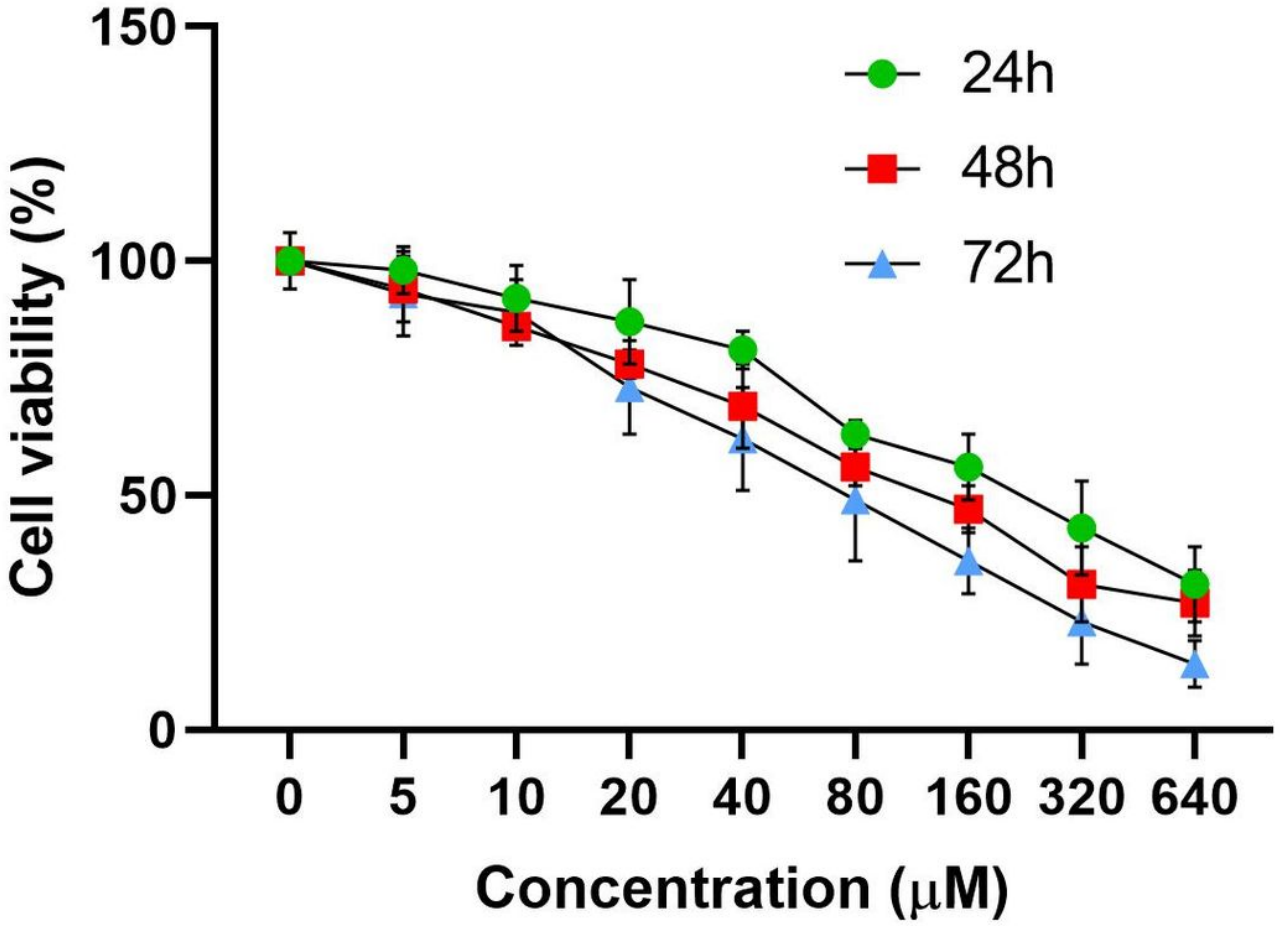

Figure 2

Crocin inhibits cell proliferation in TNBC cells. Different concentration ranges from 0 to 640 μM of crocin were used for MDA-MB-231 cells treatment for 24,48 and 72h. 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.

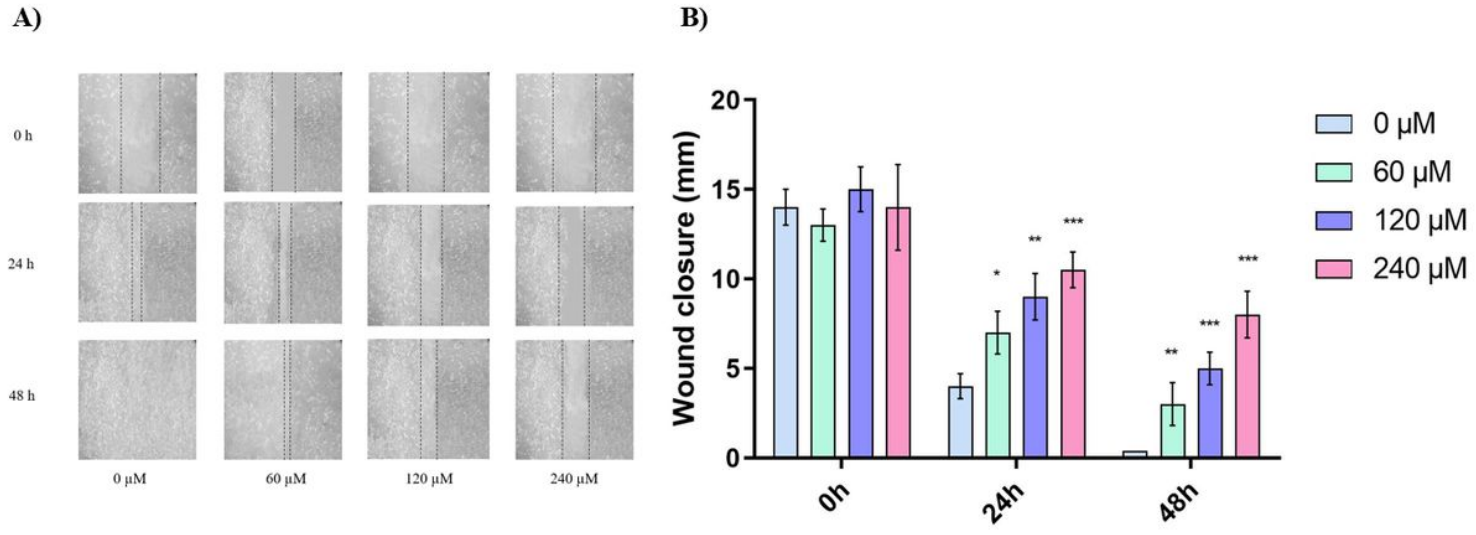

Figure 3

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).

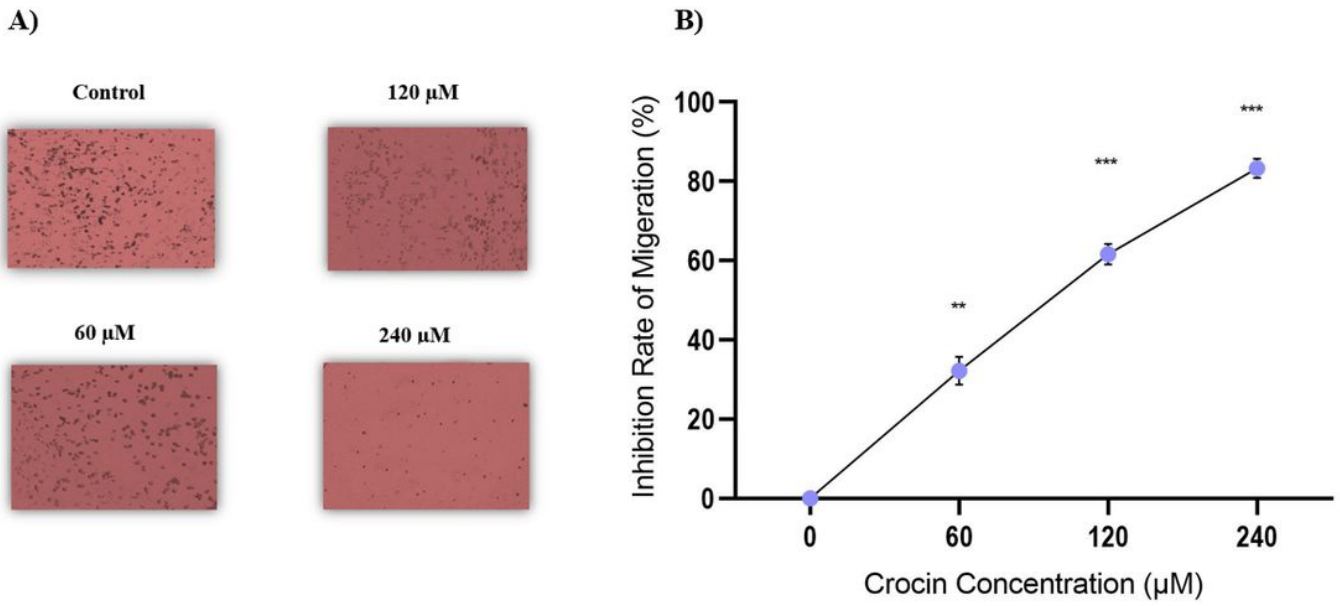

Figure 4

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).

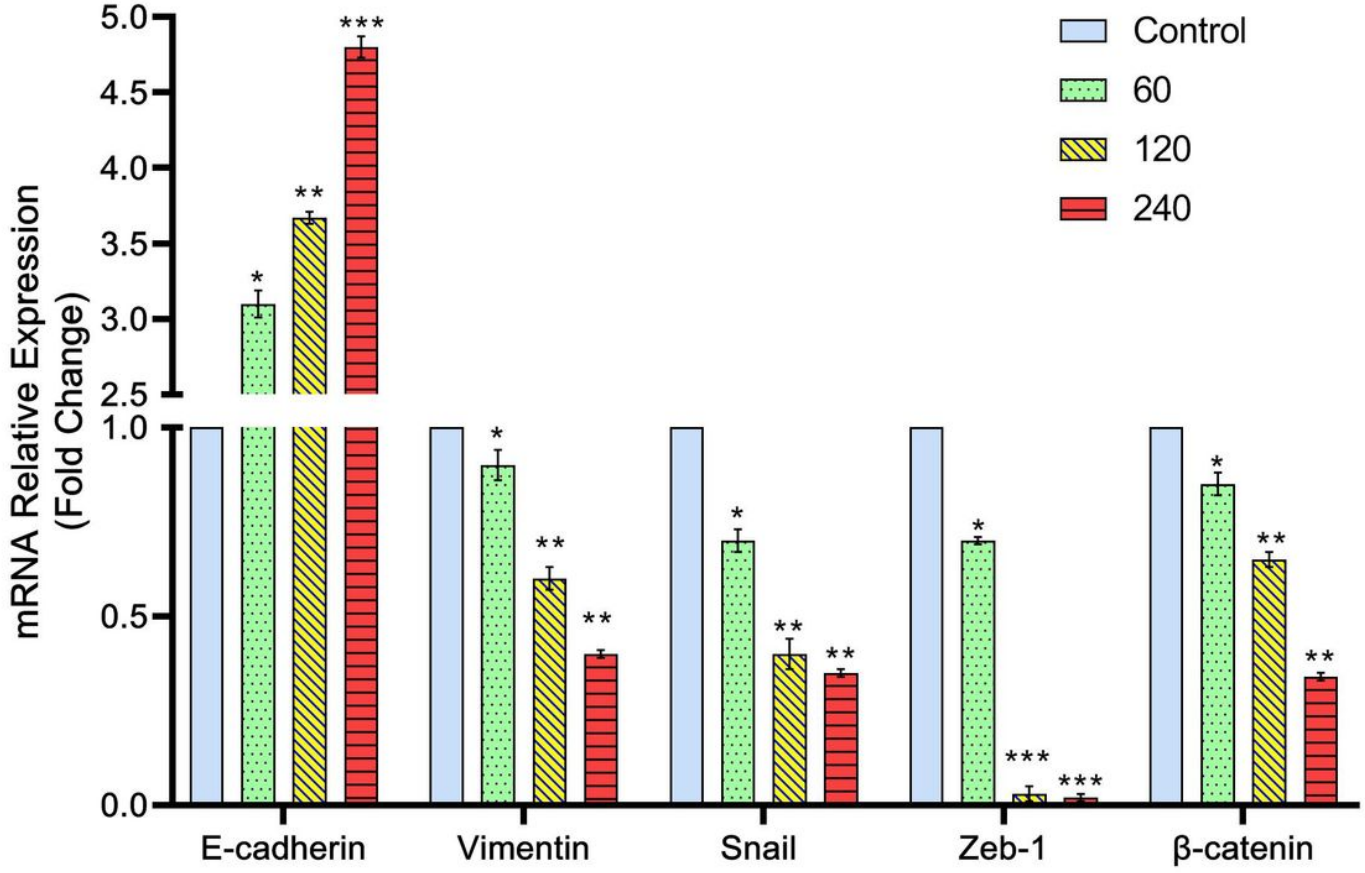

Figure 5

Crocin effects on expression of EMT markers and β-catenin in TNBC cell line. Real-time PCR 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).