BT-474 Breast Cancer Cell Apoptosis Induced by Crocin, a Saffron Carotenoid

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ABSTRACT

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Background: Saffron carotenoids have been known as powerful phytochemicals in breast cancer treatment. The purpose of this study was to investigate the anti-cancer properties of an important saffron carotenoid, crocin, on BT-474 which is a known HER2+ breast cancer cell line.

Methods: The effect of crocin on cell viability was investigated using MTT assay. Apoptosis induction was studied via flow cytometry and Western blotting of caspase-9 and cleaved caspase-9. Involvement of unfolded protein response (UPR) was also investigated via RT-PCR study of the XBP1 gene.

Results: The results showed that crocin treatment decreases the viability of BT-474 cells and induces early and late apoptosis in these cells. The mechanism of crocin action was through the induction of caspase-9 expression and cleavage. Furthermore, we found that crocin induced XBP1 gene splicing in these cells.

Conclusion: The present study provides important evidence that crocin induces apoptosis in BT-474 cells. In addition, the activation of UPR may play a role in the anticancer effects of crocin.

Keywords: HER2+; MTT Assay; Flow Cytometry; UPR; Caspase-9; Anticancer.

INTRODUCTION:

Based on a report published in 2007, breast cancer incidence in Iranian women was 22 per 100,000. In this population, prevalence was 120 per 100,000[1] and mortality rate was approximately 2.5 per 100,000[2]. Although it has now become easier than ever to diagnose breast cancer, and treatment protocols have progressed, cancer-related mortality among women is still high[3]. The current focus of anticancer therapy is to identify compound(s) that induce programmed cell death or apoptosis in breast tumors, and this topic has become the focus of considerable research interest[4]. Apoptosis is the main considerable cell response to chemotherapeutic agents[5].

The epidermal growth factor (HER2) protein plays an important role in normal cell growth, differentiation and tumorigenesis[6]. HER2 receptors are overexpressed and/or amplified in almost half of all breast tumor cases[7-9], and this overexpression is associated with lymph node-positivity and high proliferative activity[6]. A HER2+ cell line called the BT-474 cell line[10] can be used as a model of HER2+ breast cancer to investigate the effect of possible candidates for use in cancer therapy.

Apoptosis, also known as a programmed cell death mechanism, is responsible for the maintenance of cell and tissue homeostasis. It is also responsible for the removal of problematic, cancerous and virally-infected cells. Apoptosis is characterized by blebbing of the cellular membrane, cell shrinkage, chromatin condensation, DNA fragmentation and apoptotic body formation[11-14].

After apoptosis induction, a number of aspartate-specific proteases known as caspases are activated, working to amplify the apoptotic signal(s)[15]. A proteolytic network is formed and initiator caspases - such as caspase-9 - are activated, resulting in the propagation of a proteolytic caspase cascade. Many cellular proteins, with various functions, are cleaved by caspases, eventually leading to the morphological manifestations of apoptosis[16,17].

It has been known that the ribosomes bound to the endoplasmic reticulum (ER) membrane are responsible for the synthesis of secretory and membrane-bound proteins, and their folding is continued and accelerated in the ER. The quality control systems in the ER detect unfolded and misfolded proteins[18]. Under any type of ER stress, the unfolded protein response (UPR) is induced, and can result in an adaptive or apoptotic response. Activation of UPR reduces protein translation and induces transcription factors to increase the number of molecular chaperones. The overall result of this process is decreased protein load and an increased protein folding capacity in the ER. If the ER stress is prolonged, cells can enter the apoptotic process[19]. The X-box binding protein 1 (XBP1) is an ER stress sensor[18], which can turn into the spliced XBP1 (XBP1s) by the removal of a 26bp intron. XBP1s with a different C terminus are a more effective transcription factor than the unspliced form[20-22]. Previous studies indicated a relationship and crosstalk between UPR and apoptosis in cancer cells[23]. In addition, persistent or severe ER stress can shift the cytoprotective functions of UPR into mechanisms promoting cell death[24].

Saffron is derived from the dried red stigma of the plant that is scientifically identified as Crocus Sativus L. It belongs to the iris family (Iridaceae) and is grown in many countries such as Iran, Greece, India, and Spain. The medicinal properties of saffron components, and the therapeutic uses (both traditional and modern) of saffron and its constituents are covered in our review papers[25,26]. Crocin as a water-soluble carotenoid, is the main and basic element of the antioxidant activity of saffron, and is capable to produce a variety of pharmacological effects, including an anti-cancer effect[27]. It has shown antitumor and chemopreventive activity

both in vitro and in vivo[28-31].

Our previous in vitro studies have shown that crocin directly interacts with telomeric DNA structures, G-quadruplex and i-motif[32]. It also reduces H1–DNA complex formation; a model of chromatin, which is decondensed in the presence of crocin[33]. Crocin also induces a conformational transition of B- to C-DNA through binding with the DNA minor groove[34].

We have previously shown that crocin induces apoptosis in the human gastric cancer cell line (AGS), through activation of caspases and alteration of Bax/Bcl-2 balance[31]. Crocin treatment also induces cell cycle arrest in chemical-induced breast cancer in rats. This effect was effected through changing cyclin D1 expression and alteration in p53 and p21 expression[30]. Since the apoptotic effect of crocin on all human breast cancer cell lines and its mechanism of action has not yet been elucidated, in this study we aim to investigate the effect of crocin on the BT-474 cell line, and its molecular mechanism of action. Therefore, we studied the impact of crocin on cell death induction, caspase-9 expression and caspase-9 activation (or cleavage). In addition, we assessed the possibility of UPR activation following treatment by crocin.

METHODS:

Reagents and Antibodies

The BT-474 cell line was prepared at the Iranian Biological Resource Center (IBRC). Dulbecc's modified Eagle's medium (DMEM-F12); Fetal Bovine Serum (FBS) and Penicillin/Streptomycin were obtained from Gibco-BRL (Paisley, UK). MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazoliun bromide was purchased from Sigma- Aldrich (St. Louis, MO). Saffron (Crocus Sativus L.) was procured from Ghaenate, Iran. All other materials were of analytical grade.

RiboExTM kit and HyperscriptTM RT Mastermix Kit were purchased from GeneAll (GeneAll®, Korea). Taq DNA Polymerase Master Mix, Red was obtained from Ampliqon (Skovlunde, Denmark).

The antibodies against β-actin (ab227387, Abcam, Cambridge, UK), caspase-9 (C3465, Sigma- Aldrich St. Louis, MO) and cleaved caspase-9 (9502S Cell Signaling, Danvers, US) were bought from the aforementioned companies.

Crocin Preparation

Crocin was extracted and purified from the Iranian saffron using column chromatography, as was previously explained in detail. The procedure was followed by UV-Vis spectroscopy and TLC, to verify crocin purity[35].

Cell Culture

The human breast cancer cell line BT-474 was purchased from the Iranian Biological Research Center (IBRC, Tehran, Iran) and cultured in Dulbecco's Modified Eagle's Medium: Nutrient Mix F-12 (D-MEM/F-12 1:1) medium supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 100 mg/ml streptomycin in a 37°C incubator with 5% CO2. The cells were passaged by harvesting with trypsin/ EDTA and seeding into 25cm2 flasks. Cell viability was assessed via MTT assay.

Crocin Treatment and Cell Growth Assay

About 10 mg/ml solution of crocin was prepared and dissolved in DMEM/F12 prior to the experiments. The effects of crocin on cell growth were determined using MTT assay. Cells were seeded at 8×10^3 cells/well in 200μl of medium in 96-well plates and incubated for 24h at 37°C. The cells were treated in the presence or absence of different concentrations of crocin, ranging from 0 to 5 mg/ml, and at different time intervals (0-72 h). The anti-proliferative effect of crocin was evaluated by a colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, in comparison with the control group (0 mg/ml of crocin). The cells were incubated with 20μl of MTT reagents (0.5

mg/ml) in the dark at 37°C for a duration of 4h. To end the reaction, the culture supernatant was removed, and 150µl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the resulting formazan crystals completely. The optical density of the product was recorded using an ELISA plate reader (Magellan, U.S.) at 570nm. According to the obtained data, a crocin IC50 of 3 mg/ml was obtained, which was subsequently used in all other experiments.

Flow Cytometry Analysis

Annexin V-FITC Apoptosis Detection Kit (eBioscience) and propidium iodide (PI) staining was used to indicate apoptosis in the BT-474 line following crocin treatment. The cells were treated with 3 mg/ml of crocin for 6, 12 and 24h. Afterwards, the cells were trypsinized, pelleted by centrifugation at room temperature at 1200rpm for 5min and washed twice with cold PBS. Cell suspensions were incubated with Annexin V- FITC and PI for 15min at room temperature in the dark and analyzed using BD FACSCantoTM II Flow Cytometer (USA). The obtained raw results were analyzed with the help of Flowing Software Version 2.4.1.

RT-PCR/ RNA extraction and reverse transcriptase polymerase chain reaction

Total RNA was isolated from the crocin-treated BT-474 cells using the protocol supplied alongside the RiboExTM kit (GeneAll®, Korea) following the procedure described by the manufacturer. The concentration of the RNA samples was determined by measuring absorbance at 260nm using Nanodrop 2000 (Thermo Scientific). The ratio of absorbance at 260 and 280 nm was 1.8 or higher (Data not shown). The cDNA was synthesized from 2µg of total RNA as a template using the HyperscriptTM RT Mastermix Kit (GeneAll, Korea). The cDNA was stored at - 20°C until use. RT-PCR was performed using Taq DNA Polymerase Master Mix, Red and the cDNA template by amplification of the XBP-1 gene mRNA primer pair sets: XBP-1 (forward primer: TTACGAGAGAAAACTCATGGCC and reverse primer: GGGTCCAAGTTGTCCAGAATGC). PCR was conducted on a total volume of 20µl of master mix containing 2µl of cDNA and 0.2µM primers. The sequencing involved 30 cycles at 95°C, 15s for denaturation, 60°C, 5 s for annealing, and 72°C, 10s for extension. The resulting amplified fragments were resolved in 1-1.5% agarose containing ethidium bromide (EB) along with DNA Ladder (SMOBIO DM2300 ladder). The experiment was performed by three independent experimenters in triplicate. The intensity of the PCR product bands was assessed with the Image J software (NIH approved) and semiquantitative data was obtained.

Protein extraction and Western blotting

Cellular levels of caspase-9, and β-actin (used as a control for equal protein loading) were determined in untreated and crocin-treated cells using Western blotting. Then, the BT-474 cells were plated and treated with suitable concentrations of crocin for 6, 12 and 24h. The medium was added to the control cells. At the end of the incubation time, cells were washed twice with ice-cold PBS buffer and were lysed with lysis buffer (RIPA) containing protease inhibitor and phosphatase inhibitor cocktails and centrifuged at 12,000g for 15min at 4°C. Following that, supernatants were collected and stored at –80°C until analysis by gel electrophoresis. Protein concentration in the samples was determined using the Bradford method, while BSA was used as a standard. For Western blot analysis, 20-40 µg of whole cellular protein extraction were loaded onto each lane of a 12.5% SDS-polyacrylamide gel, separated, and then electrotransferred to polyvinyl difluoride (PVDF) membrane. Blocking was performed in Tris-buffered saline containing 5% skim milk and 0.05% Tween-20 for 2h at room temperature. The membranes were subjected to immunoblot analysis, while purified polyclonal human anti-caspase-9 and

anti-β-actin antibodies were used as primary antibodies (1:1,000 and 1:3,000, respectively) and followed by incubation with the corresponding secondary antibody (1:8000) at room temperature for 1h, and then visualized by the enhanced chemiluminescence (ECL) reagents according to the manufacturer's instructions. The intensity of the bands was assessed using Image J software and semiquantitative data was obtained.

Statistical Analysis

All experiments were indepently repeated 3 times, unless otherwise indicated. The values are reported as mean ± SD. All statistical analyses were carried out using SPSS software (version 19.0). To compare the significance of the data between different groups (control and treated cells) we used one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. Statistical significance was defined as $P < 0.05$ (95% confidence limit).

RESULTS:

In vitro cytotoxicity assay of crocin

The results of the cell viability (MTT) assay on BT-474 breast cancer cells are shown in **Figure 1**. Crocin exhibits an inhibitory effect on cancer cell growth and significantly reduces the viability of BT-474 cells in a dose- and time-dependent manner. The IC50 values of crocin for the BT-474 cell line were 3.5 mg/ml at 24 h.
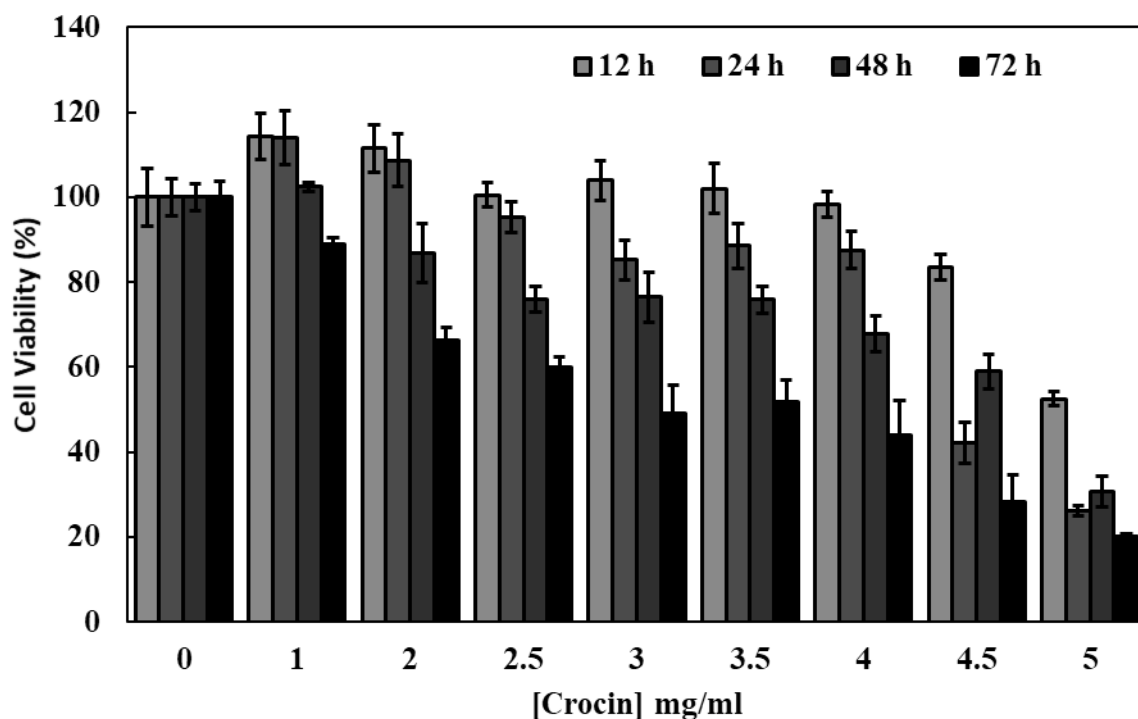

Figure 1. **ACytotoxic effect of crocin on the BT-474 cell line, at different times.** BT-474 cancer cells were incubated with crocin (1– 5 mg/ml) at 0, 12, 24, 48 and 72 h. Cell viability was estimated using MTT assay. Each value is represented as the mean± SD of three independent experiments.

Apoptosis induction in crocin treated cells

To better understand the anticancer effect of crocin and its role in apoptosis induction, Annexin V/ PI staining was used. As shown in **Figure 2A**, the percentage of early apoptosis fraction (Annexin V +/PI - cells and upper left quadrant) was significantly increased (p=0.000) to 6.16% after 6h of treatment. The total apoptosis fractions (both early and late) further increased with time, reaching more than 70 % after 24h of treatment (**Figure 2B**).

The effect of crocin on XBP1 mRNA splicing

Semiquantitative RT-PCR was carried out to determine the effect of crocin on XBP1 mRNA splicing. The results
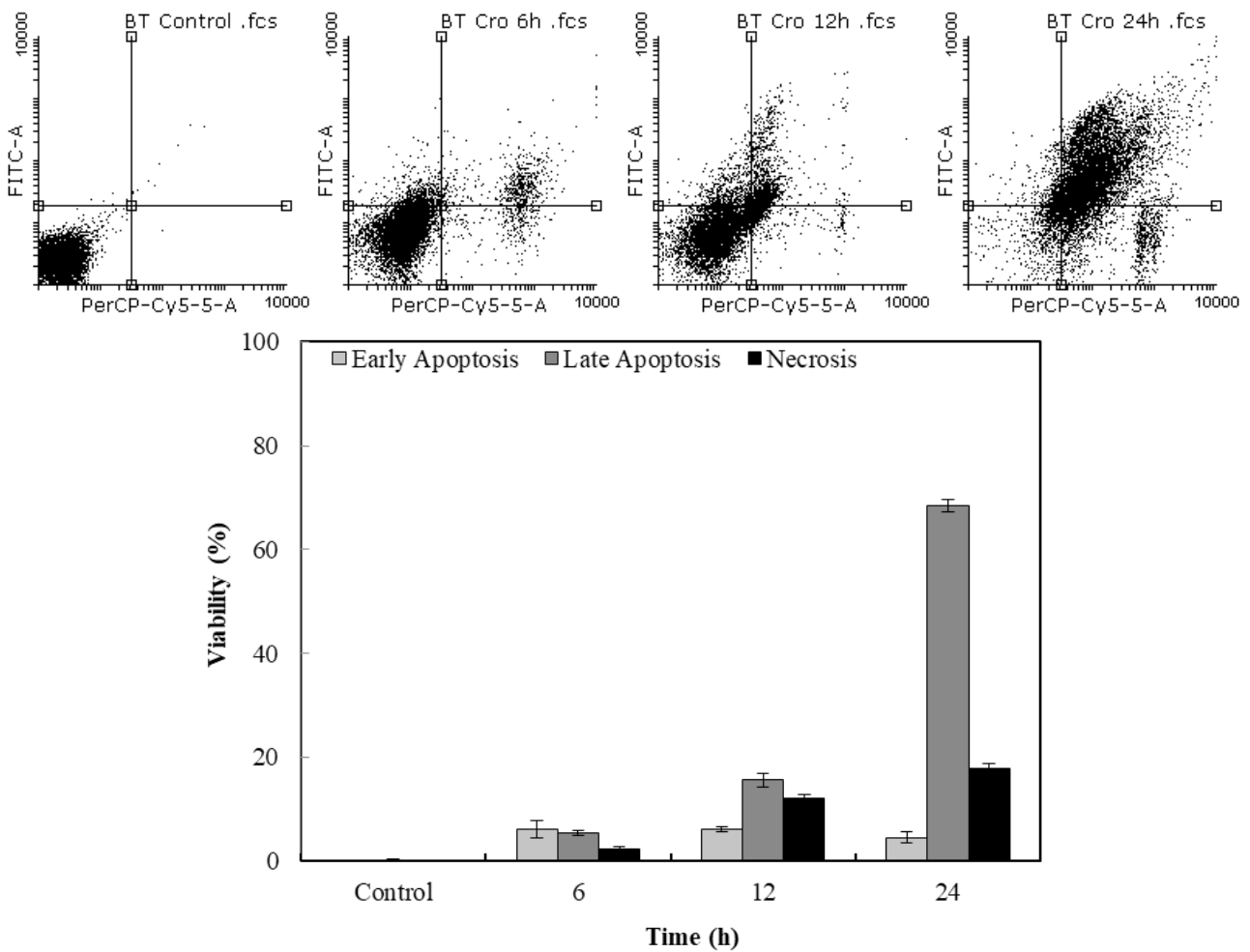

Figure 2. **Flow Cytometric analysis of BT-474 cell apoptosis induced by crocin**. Untreated (control) (A) and crocin treated (3.5 mg/ml) at 6 h (B), 12 h (C), and 24 h (D). BT-474 breast cancer cell lines were incubated with annexin V-FITC and PI and analyzed using flow cytometry. In each panel the lower left quadrant shows cells negative for both PI and annexin V-FITC staining. The upper left quadrant shows only annexin positive cells, which are early apoptotic cells. The lower right quadrant shows PI positive cells (necrotic) and the upper right quadrant shows annexin and PI positive cells (late apoptotic cells). (E) The percentage of necrotic, early and late apoptotic cells is presented in the histogram. *P = 0.000.
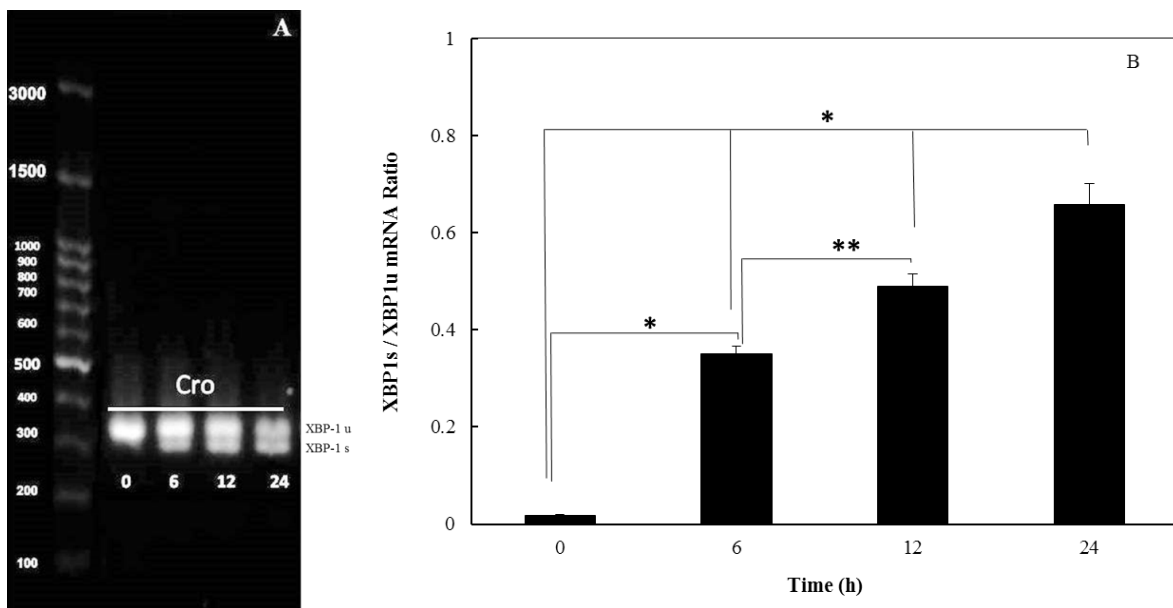
Figure 3. **Stimulation of XBP-1 splicing in BT-474 cells by crocin treatment.**
A) Splicing of XBP1 mRNA by using RT-PCR analysis in the presence of crocin (3.5 mg/ml) at different times. The upper and lower bands represent the spliced and unspliced RNA, respectively. The left lane represents the molecular weight marker.
B) Semiquantitative analysis of the RT-PCR data, by using ImageJ software.
*P < 0.001 between control and crocin treated cells at 6 and 24h.
**P < 0.005 between crocin treated cells at 6h versus 12h.

are shown in **Figure 3A**. It demonstrates crocin-spliced XBP1 (XBP1s), which is obviously visible below the unspliced form (XBP-1u). The splicing was a time dependent process and increased from 6 to 12 and up to 24h. Semiquantitative data (**Figure 3B**) confirmed the significance of the ratio of XBP1s/ XBP1u mRNA ratio in the BT-474 cells after 24h crocin treatment (p=0.000).

Caspase-9 expression and cleavage

The level of caspase-9 expression and cleavage (activation) in the presence and absence of crocin are shown in **Figures 4A and 4B**. The results indicate that crocin (3.5 mg/ml) significantly increased caspase-9 protein expression. The level of cleaved or activated caspase-9 was also elevated following crocin treatment. The semiquantitative analysis of these data (**Figure 4B**)

indicates the obvious (p=0.000) increase in cleaved caspase-9/caspase-9 ratio in a time-dependent manner.

DISCUSSION:

In the present study, crocin suppressed the growth and proliferation of a triple positive breast cancer cell line, BT-474. This behavior was evaluated by MTT assay. Flow cytometry data using annexin/PI staining indicated apoptosis induction in these cells. The populations of early and late apoptotic cells increased with time (from 6 up to 24h). The increases in the expression of caspase-9 and its cleavage are affirmative for apoptosis induction via the intrinsic pathway. XBP1 splicing also increased with time; indicating the possible involvement of UPR in this process.

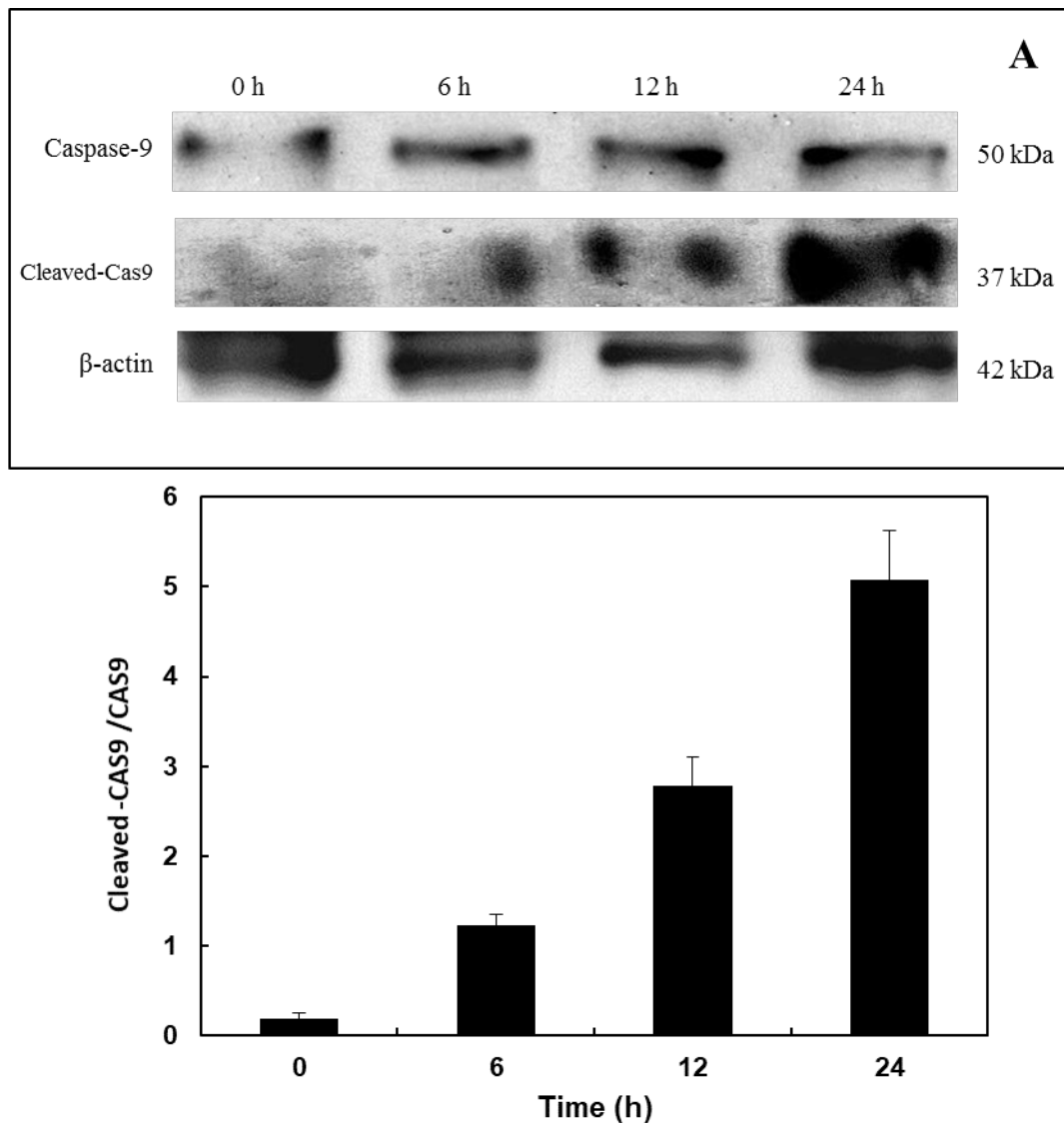The safety profile and lower toxicity of ''natural prod-

Figure 1. **Effect of crocin on Caspase-9 expression and cleavage.** A) Western blot results of procaspase-9 and cleaved caspase-9. B) The ratio of cleaved caspase-9/ caspase-9 at different time points during the experiment. P = 0.000 at all time points.

ucts''/ phytochemicals, and their application in chronic diseases such as cancer has attracted attention over the past few decades[36,37]. Previous studies indicated that crocin, a unique water soluble carotenoid of saffron with a wide range of pharmaceutical properties[25], is safe for normal cells and normal animals, and even

for humans[30,31,38,39].

Hyperactivation or overexpression of HER2 is associated with uncontrolled cell growth and proliferation, leading to cancer development[7,8,10]. The BT-474 human breast cancer cell line has been introduced as a HER2 positive model[10]. Previous studies indicate the

induction of apoptosis by crocin in different breast cancer cell lines[40], while its molecular mechanism of action and the intermediary role of hormones are not clearly known. Therefore, in this study, the apoptotic effect of crocin, as a pharmacologically active component of saffron, was evaluated in this HER2 positive breast cancer cell line.

Apoptosis, as a form of programmed cell death, is a natural and gene-regulated way that is triggered by many stimuli including chemotherapeutic agents[41,42]. Two pathways are involved in apoptosis; an intrinsic (or mitochondrion-mediated) and extrinsic (or death receptor-mediated) pathway[43]. Caspase-9 plays a critical role in the mitochondrial death pathway. Induction of stress signals lead to the release of cytochrome C from mitochondria and activation of the apoptosome, which in turn cleaves the pro-enzyme of caspase-9 into the active form (cleaved caspase-9). Afterwards, the executioner caspase is cleaved by this initiator caspase. Thus, in addition to caspase-9 activation, caspase-9 cleavage has been also suggested as a marker for apoptosis[44]. Our results showed an increase in the capase-9/β-actin ratio, which indicate the activation of the intrinsic pathway of apoptosis. The observed increase in the caspase-9/cleaved caspase ratio was also an affirmation for the activation of this pathway. Similar results were previously observed following treatment with saffron extract and crocin in the MCF-7[45,46] and MDA-MB-468 breast cancer cell lines[40]. These indicate the independent role of these phytochemicals in hormonal function.

Flow-cytometry data were also applied to interpret our findings, since flow-cytometry is a mechanism of cell death in tumor cells[47]. As shown in the results, crocin treatment increased the percentages of Annexin V-positive and PI-negative cells (indicators of the early apoptotic cells) and both Annexin and PI positive cells (indicating the late apoptotic cells) in the BT-474 breast cancer cell line after 24h. Increased Annexin V

binding with time indicated the phosphatidylserine externalization, which is due to the apoptotic changes in the cell membrane. This phenomenon is accompanied with an increase in the shrinkage of the membrane and decrease of PI binding.

XBP1 mRNA splicing is a common marker of UPR activation in cells under stress conditions[19,48]. Therefore, the effect of crocin was evaluated on the mRNA splicing of XBP1 in the BT-474 breast cancer cell line. It seems that crocin regulates the mRNA splicing of XBP1 in BT-474 cells effectively, and in a time-dependent manner; with the spliced form (XBP1s) gradually increasing with time (from 6 to 24h) after crocin treatment, while the unspliced form (XBP1u) decreased in concentration.

In conclusion, our results demonstrate that crocin exerts a cytotoxic and anticancer effect against BT-474 cells. It inhibits cancer cell growth and viability by increasing the activation and expression of caspase-9. It also triggers UPR by splicing the XBP1 mRNA in the BT-474 breast cancer cell line. All of our findings suggest that, regarding the anti-cancer properties of crocin, and its safety for both animals and humans, it can be used as a natural anticancer component against breast cancer in women.

ACKNOWLEDGMENT:

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