Original Article

Crocetin regulates cell cycle progression in *N-nitroso-N*-methylurea(NMU)-induced breast cancer in rat: cyclin D1 suppression, $p21^{Cip1}$ and p53 induction

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ABSTRACT

Objectives: *Crocetin*, a saffron-derived carotenoid, inhibits tumor growth in some cancer types. However, its mechanism of action still needs to be clearly understood. Here, the *Crocetin* effect on the expression of some cell cycle regulators was investigated.

Methods: The N-nitroso-N-methylurea (NMU)-induced rat mammary carcinomas were induced in a group of 35-day-old female Wistar Albino rats. Then, the expression of several cell cycle regulators was studied using reverse transcription-polymerase chain reaction (RT-PCR) and Western blot. After the tumor appearance, these rats were treated with *Crocetin* through weekly, intraperitoneal injections of 100 mg/kg body weight for four weeks. A control group has received the vehicle only. In the end, rats were sacrificed, and tumors were divided into two parts. A part was for pathologic investigation, and a part was retained at -70 °C to determine desired parameters.

Results: Before *Crocetin* treatment, the tumor volumes were 13.27 ± 3.77 and 9.44 ± 4.39 cm^3, which was changed to 23.66 ± 8.82 and 4.71 ± 2.44 cm^3 at the end of the experiment in the untreated and treated groups, respectively. The results showed that *Crocetin* markedly decreased the increased expression of cyclin D1 due to NMU administration. However, it further increased the expression of p53 and $p21^{Cip1}$, with no significant effect on $p27^{Kip1}$ expression. We previously showed Crocin-induced cell cycle arrest through a p53-dependent mechanism.

Conclusion: *Crocetin* induced the cell cycle arrest in NMU-induced breast cancer in rats through a p53-independent mechanism. It is the second mechanism additional to apoptosis induction in cancer cells.

Keywords: Saffron Carotenoid; Cell Cycle; p53; Cyclin D1; p21; p27.

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Introduction

Breast cancer is the most prevalent cancer worldwide. However, the risk of death decreased after the 1930s, when surgery (specifically, radical mastectomy) was the primary mode of treatment, and after the 1970s, when early detection programs were implemented in various countries. Moreover, survival rates improved beginning in the 1990s, when early detection programs were combined with comprehensive treatment, including surgery, radiotherapy, and effective medication (1). Consequently, there is growing interest in discovering new drugs for therapeutic strategies with greater therapeutic potential and fewer side effects. Nowadays, the use of naturally occurring compounds has garnered significant attention in chemoprevention and adjuvant therapy. *In vitro* experiments using various cell lines and *in vivo* studies using animal models of various diseases are considered to study the effectiveness of new compounds. In this regard, animal models of various diseases, especially breast cancer, are considered an effective tool for studying the processes of carcinogenesis and treatment.

The anticancer effect of saffron (*Crocus sativus* L.) dried stigmas and its primary carotenoid, *Crocin*, has been demonstrated previously (2, 3). Pharmacokinetic studies have revealed that the glycolytic pathway of *Crocin* in the digestive tract leads to its conversion to *Crocetin*, and approximately two hours post oral administration of *Crocin*, *Crocetin* appears in the serum (4). The role of *Crocin* and *Crocetin* in inducing apoptosis in various cancer cell lines (5) and in a rat model of gastric cancer (6) has been demonstrated by us and other research groups (7). Both of these 20-carbon atoms (C20) carotenoids are safe for normal cells, animals (8), and humans (9), even at high concentrations. However, they induce cell death in cancer cells and tumor tissues. Additionally, low concentrations of these natural carotenoids have been effective in decreasing Alzheimer's Disease (AD) markers, as studied in differentiated PC12 cells treated with Aβ-peptides, serving as a cellular model of AD (10). Another critical aspect of the mechanism of action of these natural carotenoids is their effective doses. Due to the difference in the effective anticancer doses of these carotenoids (11), which is primarily related to their distinct structures, we attempted to compare their mechanism(s) of action in the present study. We previously reported a p53-dependent *Crocin*-induced cell cycle arrest in breast cancer in rats (2). In this study, we investigated the effect of *Crocetin* in the same breast cancer model in rats.

Materials and methods

Crocetin separation and purification

Crocetin was extracted and purified from the dry stigmas of *Crocus Sativus* L. using the method we previously described (12) and the registered patent (54960, Nov. 25, 2008) in Iran. Briefly, two processes of alkaline and acidic hydrolysis of the methanolic extract of saffron were followed by a subsequent extraction with benzene. Then, additional purification was performed using dimethyl sulfoxide. *Crocetin* was characterized using various spectroscopic methods and by determining the melting temperature.

Animals and treatment

Forty 35-day-old female Wistar Albino rats were procured from the Pastor Institute, Tehran, Iran. Throughout the experiment, all rats were housed in a controlled environment with 12-hour light/dark cycles and a temperature of 23 ± 2ºC. They were given a commercial diet with tap water ad libitum and weighed weekly. After a 2-week acclimatization period, the rats were divided into two groups: one group of rats (#26) received three intraperitoneal injections of NMU (Sigma, St. Louis, MO), freshly dissolved in 0.9% NaCl adjusted to pH 4.0 with acetic acid, at a dose of 50 mg/kg body weight, at 50, 65, and 80 days of age. The remaining rats, the control groups, received only the vehicle.

The number, sizes, and volumes of tumors were determined weekly by palpation starting four weeks after the NMU injection until the end of the experiment. Tumor volume was calculated according to the formula $V = (4/3)\pi R_1^2 R_2$ (radius $R_1 < R_2$) (2). When the tumor size reached 10 to 15 mm in the largest dimension, the NMU-treated and control groups of rats were divided into two subgroups; one subgroup received *Crocetin* and the other received only the vehicle. The groups were named as follows: C1, control group with no treatment (# 7); C2, control group receiving *Crocetin* (# 7); T1, NMU-treated control group (# 7); and T2, NMU-treated group receiving *Crocetin* (# 7). The rats without tumors were excluded from the study. Four doses of 100 mg/kg body weight of *Crocetin*, dissolved in the minimum volume of DMSO and diluted in serum physiologic, were intraperitoneally injected into groups T2 and C2 at 7-day intervals. Groups T1 and C1 were intraperitoneally injected with only the vehicle. The Tarbiat Modares University Ethics Committee approved the animal care and treatment protocol.

Tissue sample preparation

The rats were euthanized under anesthesia seven days after the final *Crocetin* injection. The mammary tumors and normal mammary glands from all groups were dissected as follows: half of the tumors were fixed in 10% formalin, embedded in paraffin wax, and subsequently stained with hematoxylin and eosin (H & E) for histological examination. The normal mammary glands and the other half of the tumors were immediately

Table 1: Experimental conditions for RT-PCR. The primer sequences, annealing temperatures, and expected size of PCR products.

Gene	Primer sequence	Product size	Annealing temperature (ºC)
Cyclin D1	F: 5'-CAGACCAGCCTAACAGATTTC-3' R: 5'-TGACCCACAGCAGAAGAAG-3'	208	56
p21	F: 5'-CTGGATGCTAGAGGTCTGC-3' R: 5'-AGAGTTGTCAGTGTAGATGC-3'	105	58
GAPDH	F: 5'-CAAGGTCATCCATGACAACTTTG-3' R: 5'-GTCCACCACCCTGTTGCTGTAG-3' F: 5'-AACGACCCCTTCATTGAC-3' R: 5'-TCCACGACATACTCAGCAC-3' F: 5'-AACGACCCCTTCATTGAC-3' R: 5'-TCCACGACATACTCAGCAC-3'	500, 190, 160	58, 60
p53	F: 5′-GTGGCCTCTGTCATCTTCCG-3′ R: 5′-CCGTCACCATCAGAGCAACG-3′	290	60
p27	F:GAGGGCAGATACGAGTGGCAG R:CTGGACACTGCTCCGCTAACC	238	60

frozen in liquid nitrogen and stored at -70°C for RT-PCR and Western blot analysis.

Reverse transcription (RT)-polymerase chain reaction (PCR)

According to the manufacturer's recommendations, total RNA from excised rat mammary tissues was isolated using the TRIZOL extraction reagent (Invitrogen, CA, USA). The integrity of mRNA was confirmed by electrophoresis in a 1% agarose gel and a Thermo Scientific Nanodrop 2000C spectrophotometer. The cDNA was synthesized using a Revert AidTM H Minus first-strand cDNA synthesis kit, as described in the manufacturer's instructions (Fermentas, Inc., USA). The PCR was carried out by amplifying genes together with the reference gene (GADPH) using an equivalent cDNA template, PCR master mix (Fermentas, Inc., USA), and specific primers in an MJ Mini™ Personal Thermal Cycler (BioRad, USA). PCR conditions for cyclin D1 amplification were as follows: 30 cycles of 95 °C for 30 s, 56 °C annealing for 45 s, and 72 °C extension for 45 s. For $p21^{Cip1}$, the conditions were as follows: 30 cycles of 95 °C for 30 s, 58 °C annealing for 45 s, and 72 °C extension for 45 s. For p53 and $p27^{Kip1}$, the conditions were 30 cycles of 95 °C for 30 s, 60 °C annealing for 45 s, and 72 °C extension for 45 s. The primer sequences, product sizes, and annealing temperatures are given in Table 1. The primer sequences of p53, $p27^{Kip1}$, and GADPH were taken from the literature and the cDNA synthesis kit. Cyclin D1 and $p21^{Cip1}$ primers were designed using Oligo 6, Generunner, and AlleleID 07 software. Then, reaction products were separated on a 2% agarose gel and visualized by ethidium bromide staining. The bands were quantified by densitometric analysis through image-capturing system software. GAPDH normalized the relative target mRNA expression level in the same sample.

Western blot analysis

For Western blotting, approximately 100 mg of frozen mammary tissues, whether tumor or normal, were homogenized in lysis buffer (containing 150 mM NaCl, 50 mM EDTA, 1 mM NaF, 10 mM $Na_4P_2O_7$, 0.1% SDS, 100 mM Tris-HCl, 1% glycerol, and 1% Triton X-100), which contained a cocktail of protease and phosphatase inhibitors (Sigma, St. Louis, MO). Equivalent amounts of protein were applied to 12% SDS-polyacrylamide gels, separated by electrophoresis, and electrotransferred to 0.45 µm pore size polyvinylidene difluoride (PVDF) membranes (Roche, Germany). Membranes were immersed in blocking solution (5% non-fat dry milk, 0.05% Tween 20 in PBS) and were incubated overnight at 4 °C. Primary incubation of the membranes was carried out sequentially, using 1:100 and 1:200 dilution of mouse monoclonal anti-$p21^{Cip1}$ and anti-cyclin D1 antibodies (#450 and #271610 Santa Cruz Biotechnology, Inc.) for 2 h at room temperature (RT) in 3% non-fat dry milk. After washing with PBS-0.05% Tween 20 (PBST), filters were incubated for 1 h at RT with the horseradish peroxidase-conjugated secondary antibody (1:7000 dilution; goat anti-mouse, #2005 Santa Cruz Biotechnology, Inc.). After washing, protein bands were visualized on radiographic films using an ECL advanced Western blotting detection kit (Amersham, GE Healthcare, Little Chalfont, UK). β-actin was assessed as an internal control. Thus, a mouse monoclonal anti-β-actin antibody(#81178 Santa Cruz Biotechnology, Inc.) was applied to detect the β-actin bands in the membranes. Then, Image J was used to obtain semi-quantitative data of the bands.

Statistical analysis

Data were shown as mean ± standard deviation. For gene expression, differences between the data obtained

in all groups, and for tumor volume analysis, one-way ANOVA was used. All comparisons with p-values below 0.05 were considered significant.

Results

Treatment response and pathology of mammary tumors

Breast tumors were observed only in the NMU-injected rats (Groups T1 and T2). Before *Crocetin* treatment, the mean volumes of tumors were approximately 13.27 ± 3.77 and 9.44 ± 4.39 in the T1 and T2 groups, respectively. At the end of the experiment, they were 23.66 ± 8.82 and 4.71 ± 2.44 in these groups (T1 and T2, respectively). As demonstrated in Fig.1, *Crocetin* administration markedly (P<0.05) suppressed tumor growth in the NMU-treated rats.

The H & E staining of the tissue sections (Figs. 2A and 2B) after analysis by a pathologist revealed that the majority (about 91.9%) of tumors from the NMU-treated groups were malignant adenocarcinomas of different types, including papillary and comedo carcinoma. About 8.1% were benign epithelial neoplasms, including lactating adenomas (with milk-like substances in the lumen) and papillary adenomas.

Expression of cyclin D1, $p21^{Cip1}$, p53 and $p27^{Kip1}$

The expression of cyclin D1 at both mRNA and protein levels is depicted in Figs.3A and 3B. These figures indicate a significant increase in the mRNA and protein levels of cyclin D1 in the NMU-induced tumors (T1), which significantly decreased (p<0.05) after treatment with *Crocetin* (T2). Fig. 3B and its corresponding histogram display the Western blot results for the expression of Cyclin D1 in these samples.

Fig. 4A shows the mRNA expression of p53 in different groups. Fig. 4B, the histogram, indicates a significant increase in the p53/GAPDH ratios in groups T1 and T2 due to the NMU treatment and the subsequent *Crocetin* treatment, respectively.

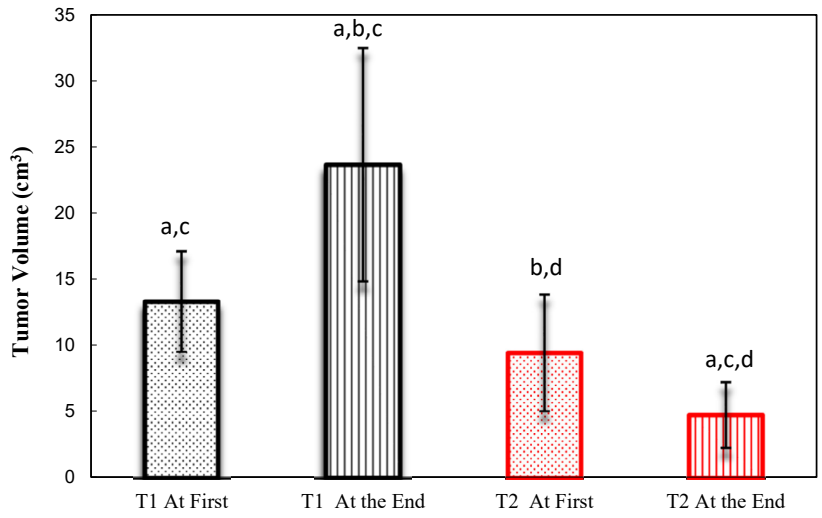

Figure 1: Tumor volume in T1 and T2 groups before and after treatment. Analysis was performed by one-way ANOVA analysis. Values are presented as mean ± SD and the same letters indicates the significance (p< 0.05) of the data that compares with another.

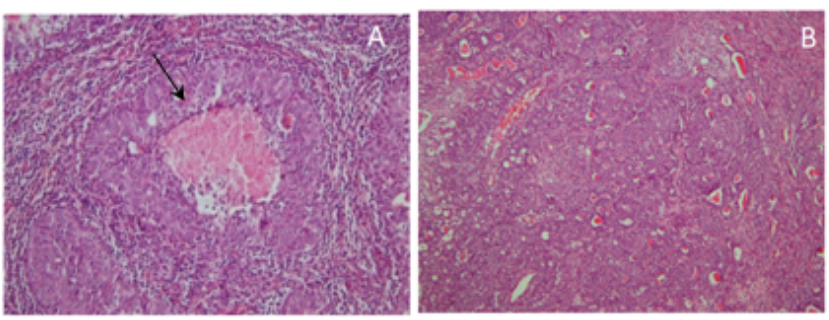

Figure 2: Histopathology images of tumors. (A) Comedo type tumor from T1 group with areas of "necrosis" (arrow pointed) which is debris from dead cancer cells; this indicates that this tumor is growing so fast that papillary tumor cells and (B) Papillary type tumor from T2 group with slower growth than comedo and without areas of necrosis. H & E staining of mammary tumors shows invasive intraductal carcinoma.

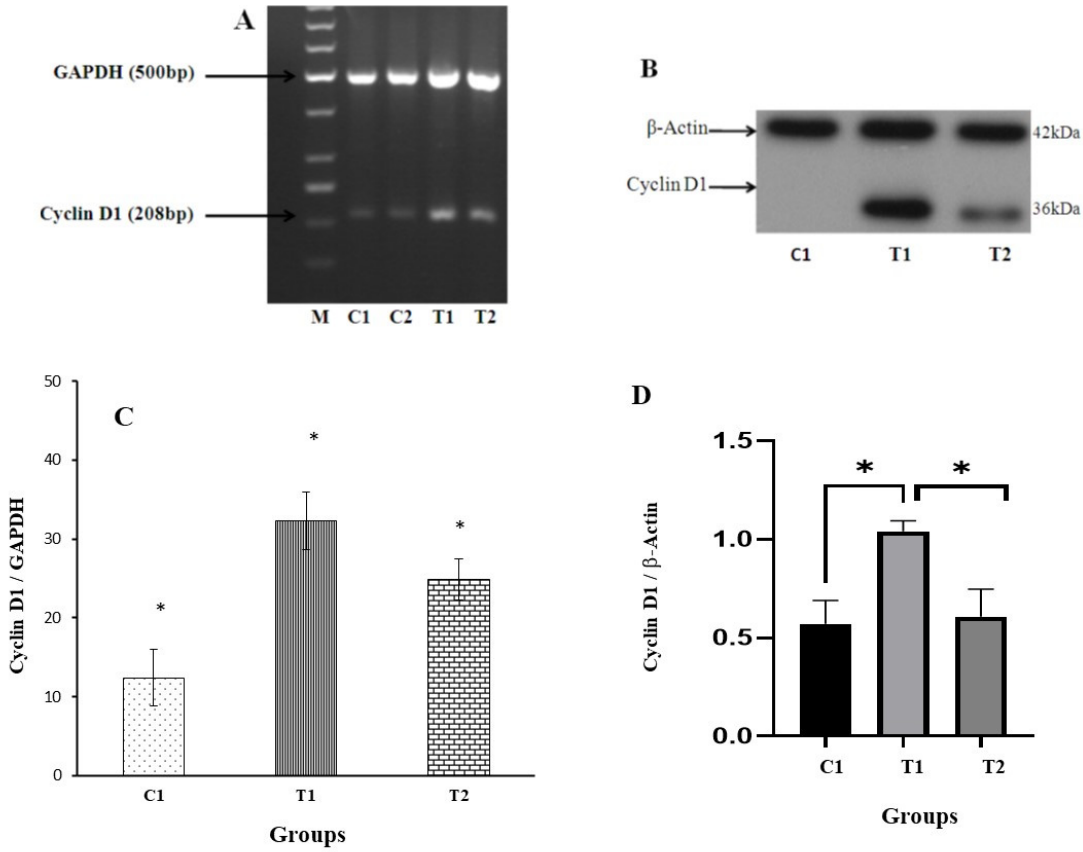

Figure 3: The results of cyclin D1 mRNA and protein expression. (A) RT-PCR and (B) Western blot results of the rat tumors without or with treatment by *Crocetin* compared to the normal mammary gland. (C) Densitometric analysis of cyclin D1 mRNA expression. (D) Histogram of cyclin D1 protein expression after analyzing the Western blot spots by Image J.
Values are presented as mean ± SD of at least three independent repeats. The significant data ($p < 0.03$) were indicated with asterisk.
Abbreviations: M: DNA marker; C1 and C2: control groups without or with *Crocetin* treatment, respectively; T1 and T2: NMU-injected groups without or with *Crocetin* treatment, respectively.

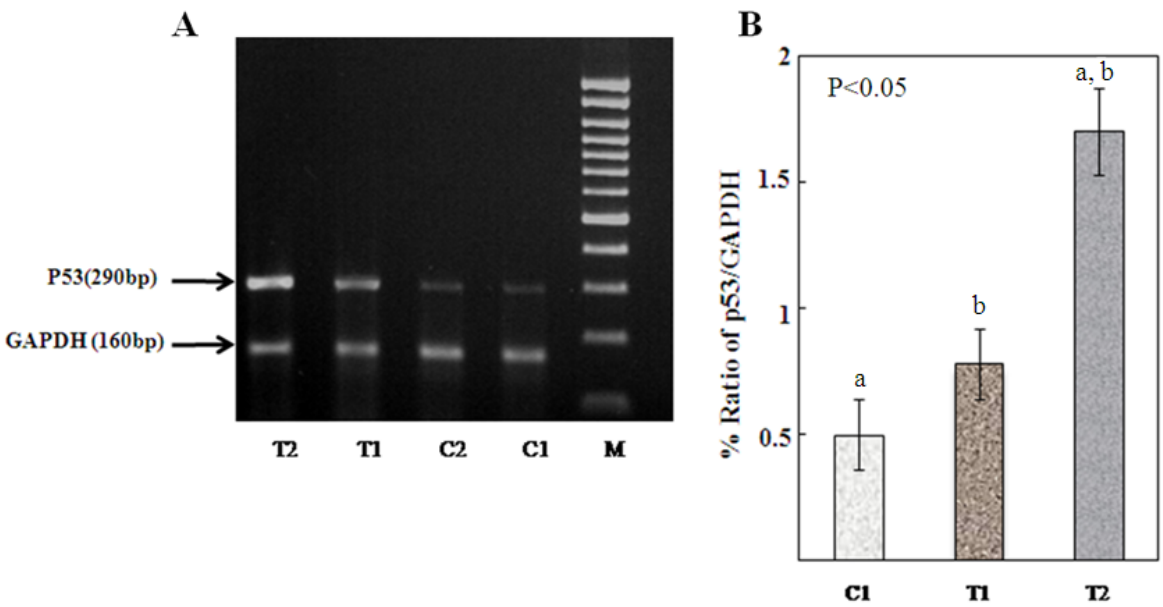

Figure 4: The results of p53 mRNA expression. (A) RT-PCR and (B) Densitometric analysis of P53 mRNA expression.
Values are expressed as mean ± SD and the same letters indicates the significance of the data that compares with another.
Abbreviations: M: DNA marker; C1 and C2: control groups without or with *Crocetin* treatment, respectively; T1 and T2: NMU-injected groups without or with *Crocetin* treatment, respectively.

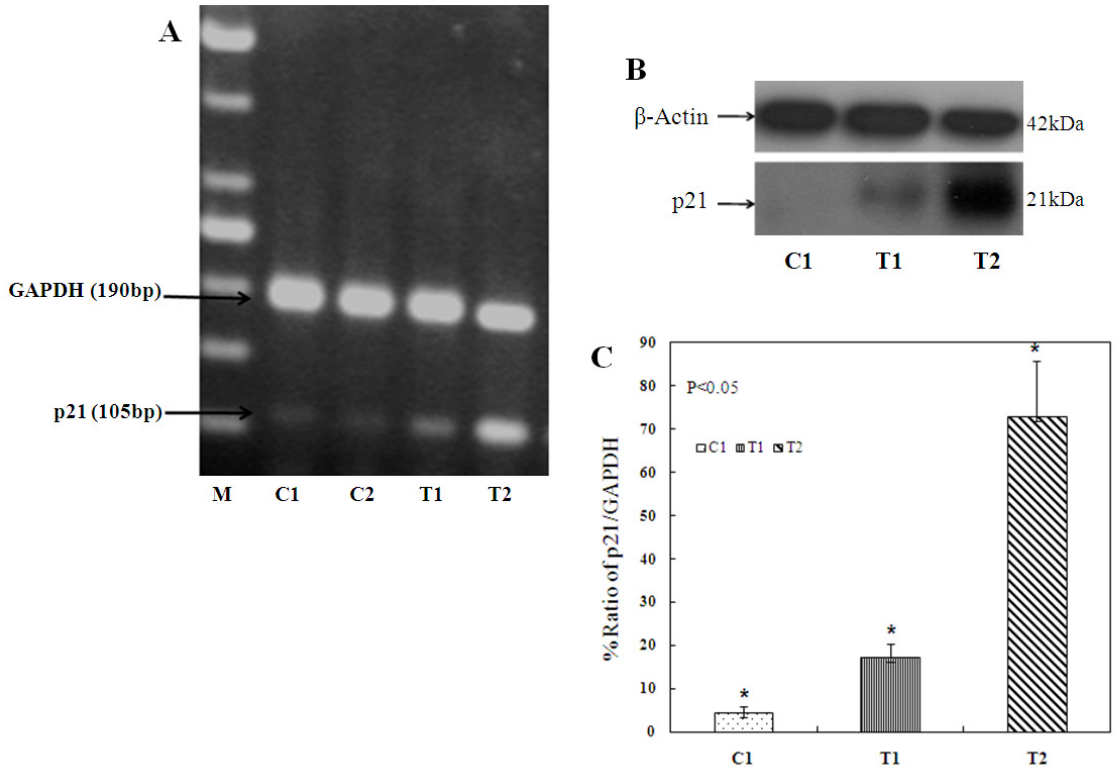

Figure 5: The results of p21^{Cip1} mRNA and protein expression. (A) RT-PCR and (B) Western blot results of the rat tumors without or with *Crocetin* treatment in comparison with the normal mammary gland. (C) Densitometric analysis of p21^{Cip1} mRNA expression. Values are expressed as mean ± SD and the significant data ($p< 0.05$) were indicated with asterisk.
Abbreviations: M: DNA marker; C1 and C2: control groups without or with *Crocetin* treatment, respectively; T1 and T2: NMU-injected groups without or with *Crocetin* treatment, respectively.

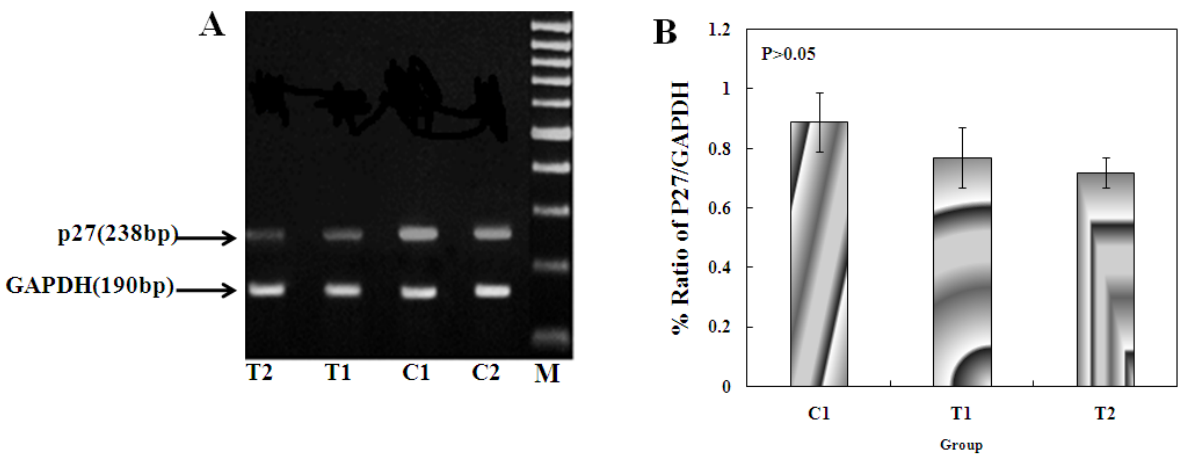

Figure 6: The results of p27^{Kip1} mRNA expression. (A) RT-PCR and (B) Densitometric analysis of P27^{KIP1} expression. Values are expressed as mean ± SD of at least three independent experiment.
Abbreviations: M: DNA marker; C1 and C2: control groups without or with *Crocetin* treatment, respectively; T1 and T2: NMU-injected groups without or with *Crocetin* treatment, respectively.

Figs. 5A and 5C also demonstrate a significant increase in both mRNA and protein levels of p21^{Cip1} after tumor induction, which was further increased due to the *Crocetin* administration (T2 compared to T1). Fig. 5B is the histogram of p21^{Cip1}/GAPDH ratios, indicating significant changes ($p< 0.05$) in different groups.

Fig. 6A shows a decreasing trend in the mRNA levels of p27^{Kip1} due to the NMU injection and the subsequent appearance of tumors. This decrease was even more pronounced after the administration of *Crocetin*.

Discussion

Our preliminary study demonstrated the antitumor effect

of saffron carotenoids (*Crocin* and *Crocetin*) against NMU-induced breast cancer in rats (13). Subsequently, we showed their effectiveness as preventive agents against breast cancer in vivo (11). The present study reveals a significant decrease in tumor volume after *Crocetin* treatment. In addition, the tumor growth rate was twice as high in the NMU-treated rats with no further treatment than in the similar group receiving *Crocetin*. This suggests that *Crocetin* suppressed tumor growth and induced cell death, causing the tumors to shrink. We then studied some cell cycle markers in all tumors. The results indicated a significant suppression of Cyclin D1 in tumors after *Crocetin* treatment.

Breast cancer is the second leading cause of death among cancers in women, worldwide, with lung cancer being the first. Current surgical methods and systemic therapies are not sufficient to cure breast cancer. Therefore, for patients who do not prefer to undergo any surgical method, non-surgical methods (14) have been suggested. New surgical strategies using personalized medicine (15) have also been applied. In addition, alternative or complementary therapies using phytochemicals to increase the efficiency of current treatments have garnered much attention (16-18).

Saffron, a commonly used spice and food additive, is recognized for its various therapeutic properties, including anticancer and antitumor effects (19, 20). The suppression of tumor growth through the induction of caspase-dependent apoptosis following the administration of saffron (21), *Crocin* (5, 22), and *Crocetin* (7, 23, 24) has been reported in some types of cancer, both *in vitro* and *in vivo*. Various mechanisms, such as the involvement of antioxidant properties and free radical scavenging (25), and alterations in some cyclins in tumor cells (24), have also been reported. We have also demonstrated the involvement of cyclin D1, $p21^{Cip1}$, and p53 in the anticancer effect of *Crocin* (2). Therefore, we were interested in investigating the involvement of these cell cycle regulators in the anticancer effect of *Crocetin* in NMU-induced breast cancer rats.

The cyclin D family comprises critical proteins that facilitate the entry of cells into the cell cycle and progression from G1 to S phase and act as the allosteric regulator of the cyclin-dependent kinases 4 and 6 (CDK4 and CDK6). Normal cells carefully regulate Cyclin D1 expression. In contrast, its activity is increased in cancer cells to various degrees (26). The overexpression of cyclin D1 and the activation of CDKs in the G1 phase may be key to shortening this phase, increasing the cell proliferation rate, and oncogenesis (27). In humans, the cyclin D1 gene is amplified in approximately 20% of mammary carcinomas, and its protein is overexpressed in more than 50% of cases (27). Cell cycle control, adhesion, invasion, and tumor/stroma/immune-system interplay in cancer are all linked by Cyclin D1's molecular bridge function (26). In the present study, RT-PCR and Western blot analysis indicate that cyclin D1 expression was increased in the NMU-treated rats, but *Crocetin* treatment significantly suppressed its overexpression. Previous studies have shown that decreased cyclin D1 expression was associated with the anticancer potential of cow ghee due to the presence of conjugated linoleic acid, n-3 polyunsaturated fatty acid docosahexaenoic and eicosapentaenoic acid (28). A similar effect was reported due to the treatment with curcumin (29), and tamoxifen (30) in mammary tumors; and black and green tea polyphenols and *Crocin* on bladder cancer (31).

Furthermore, cyclin D1 is a target gene of the NFκB pathway. It has been reported that alkylating agents such as NMU significantly upregulate NFκB activation (32, 33). Therefore, the observed overexpression of cyclin D1 in NMU-induced breast cancer may be related to NFκB activation. Since the anti-inflammatory activity of saffron and *Crocetin* due to the down-regulation of NFκB activation has been reported (34), it can be deduced that a part of the decreased cyclin D1 expression in *Crocetin*-treated tumors occurred through the suppression of the NFκB pathway. This is a subject that needs to be examined.

p53 has been recognized as a tumor suppressor. Its biological consequences include development, differentiation, gene amplification, DNA recombination, chromosomal segregation, cellular senescence, apoptosis induction, and cell-cycle regulation since 1990 (35). The critical role of p53 in all types of human cancers, especially breast cancer, has also been identified (36). In addition, it is known that $p21^{Cip1}$, a critical downstream mediator of wild-type p53, regulates several cell cycle proteins including cyclin D1, inhibits CDKs, and induces cell cycle arrest (37). Increased expression of $p21^{Cip1}$ in mammary tumors induced by DMBA has been reported (38). We have also shown the induction of $p21^{Cip1}$ after NMU administration in rats (39). An increased expression of p53 has also been reported after NMU administration to Sprague-Dawley rats (40). Recently reported data in 30 breast cancer tissues and 10 normal breast specimens have shown positive IHC reactions for p53 in 27/30 (90%) breast cancer tissues, compared with 2/10 (20%) normal breast tissues. In that study, those ratios for $p21^{Cip1}$ were 28/30 (93.33%) in cancerous tissues and 3/10 (30%) in control tissues. For cyclin D1, those ratios were 25/30 (83.33%) in cancerous tissues and 1/10 (10%) in control tissues (41). These data indicated high expression rates of $p21^{Cip1}$, cyclin D1, and p53 in malignant breast cancer cells (41). Our data in Wistar Albino rats also indicated an increased expression of these three markers after NMU administration.

In contrast, other reports indicated a paradoxical effect of $p21^{Cip1}$ expression on human breast cancer, which has been related to the subcellular localization of $p21^{Cip1}$. Thus, nuclear or cytoplasmic $p21^{Cip1}$ has an essential

role in its functional regulation as either a cell growth inhibitor or an antiapoptotic molecule (42).

It has also been reported that abnormal cells increase $p21^{Cip1}$ in an attempt to "break" the process of cellular proliferation at the G1 checkpoint (43). Our RT-PCR and Western blot results showed that *Crocetin* markedly induced $p21^{Cip1}$ and p53 expression in the tumor tissue of the T2 group compared to T1. Given the role of $p21^{Cip1}$ in cell cycle arrest, the suppression of tumor growth in the presence of *Crocetin* is significant. The upregulation of $p21^{Cip1}$ through p53-dependent or -independent pathways has been reported previously in different cancer types (44).

$p27^{Kip1}$ is known as a cell cycle regulator that inhibits G1 progression via CDK2 inhibition. In most cancers, mitogens stimulate the elimination of $p27^{Kip1}$ by decreasing translation and increasing ubiquitin-directed degradation (45), leading to lower expression of this cell cycle inhibitor in tumor tissues. Our results indicated a decreasing trend of $p27^{Kip1}$ expression in T1 and T2 tumors, compared to the normal breast tissue of the C1 group. The expression of $p27^{Kip1}$ is mainly controlled by the rate of its mRNA translation and degradation rather than by changes in transcriptional activities (46). We also studied this protein at the mRNA level. In future studies, $p27^{Kip1}$ should be analyzed at the protein level.

Conclusions

The results of the present study indicated the involvement of cell cycle regulators in terms of Cyclin D1, p53, and $p21^{Cip1}$ in the anticancer effect of *Crocetin* against breast cancer. So, *Crocetin* suppressed tumor growth by downregulating cyclin D1 and upregulating p53 and $p21^{Cip1}$. The observed changes in the $p27^{Kip1}$ mRNA indicated a decreasing trend after *Crocetin* treatment.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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