RESEARCH ARTICLE

Crocetin Induces Cytotoxicity in Colon Cancer Cells Via p53-independent Mechanisms

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Abstract

Objective: Crocin has been proposed as a promising candidate for cancer chemoprevention. The purpose of this investigation was to investigate the chemopreventive action and the possible mechanisms of crocin against human colon cancer cells in vitro. Methods: Cell proliferation was examined using MTT assay and the cell cycle distribution fractions were analyzed using fow cytometric analysis after propidium iodide staining. Apoptosis was detected using theTUNEL Apoptosis Detection Kit with laser scanning confocal microscope. DNA damage was assessed using the alkaline single-cell gel electrophoresis assay, while expression levels of p53, cdk2, cyclinA and P21 were examined by Western blot analysis. Results: Treatment of SW480 cells with crocetin (0.2, 0.4, 0.8 mmol/L) for 48 h signifcantly inhibited their proliferation in a concentration-dependent manner. Crocetin (0.8 mmol/L) signifcantly induced cell cycle arrest through p53-independent mechanisms accompanied by P21 induction. Crocetin (0.8 mmol/L) caused cytotoxicity in the SW480 cells by enhancing apoptosis and decreasing DNA repair capacity in a time-dependent manner. Conclusions: This report provides evidence that crocetin is a potential anticancer agent, which may be used as a chemotherapeutic drug.

Keywords: Crocetin - cell cycle - apoptosis - DNA damage - p21 - neoplasm

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Introduction

Cancer continues to represent the largest cause of mortality in the world and claims over 6 million lives each year (Abdullaev et al., 2000). An extremely promising strategy for cancer prevention today is chemoprevention, which is defined as the use of synthetic or natural agents (alone or in combination) to block the development of cancer in human beings (Abdullaev et al., 2004).It is a hotspot for cancer chemopreventive drug research to look for safe and effective antitumor drugs from plants, vegetables, herbs.

Saffron is a naturally derived plant product from the dried stigma of the Crocus sativus flower (family Iridaceae) that may have biologically useful properties. In fact, saffron extract and its biologically active compounds, including crocin, crocetin, carotene, and safranal, have been shown both in vitro and in vivo to possess antioxidant, anticancer, anti-inflammatory, and memory-improving properties (Abdullaev et al., 2002; Abdullaev et al., 2004; Das et al., 2010). Crocetin (8, 8-diapo-8, 8-carotenoic acid), which is the major ingredient of saffron that is responsible for its coloring property, is a low-molecular-weight carotenoid compound characterized by a diterpenic and symmetrical structure with seven double bonds and four methyl groups. This compound exhibits antioxidant (Kanakis et al., 2009; Ordoudi et al ., 2009), antihyperlipidemic (Lee et al., 2005), antiatherosclerotic (Zheng et al., 2005; He et al., 2007), cardioprotective (Cai et al., 2009), hepatoprotective (Dhar et al., 2005), and neuroprotective effects (Ahmad et al., 2005) in vitro and in vivo. Crocetin has recently gained considerable interest for its capacity to interfere with cancer at initiation and promotion stages as well as for cancer treatment (Abdullaev et al., 2002). Although crocetin causes cell growth inhibition or induces cell death in several malignant cells including human rhabdomyosarcoma (RD) cells (Jagadeeswaran et al., 2000), pancreatic cancer cells (Dhar et al., 2009), and breast cancer cells(Chryssanthi et al., 2007)in vitro studies,to date, the exact mechanism of anticancer effect of saffron is not clear. Studies showed it induces cancer cell death could via p53-dependent and independent mechanisms.

In order to understand the mechanisms of chemopreventive action of saffron, this study used a p53 gene mutation of Sw480 cells to study the mechanism of the anticancer action of saffron by evaluating its antioxidant, proapoptotic, antiproliferative effects.

Materials and Methods

Reagents

Crocetin and Propidium iodide (PI) were purchased from Sigma (St Louis, MO, USA).MTS(3-(4,5-

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dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)were purchased from Promega (Madison, WI, USA).The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL)was purchased from Roche (Basel, Switzerland). Anti-bodies against cdk2, poly (ADP-ribose) polymerase (PARP) were purchased from BD Biosciences (San Diego, CA, USA). Anti-p53 were purchased from Cell Signaling (Beverly, MA, USA). Anti-β-actin was purchased from Proteintech (Chicago, IL, USA). Anti-P21 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).The bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA).

Cell culture and Treatment

SW480, NIH3T3 cell lines were obtained from the American Type Culture Collection and cells were cultured in Dulbecco's modified Eagle's medium (DMEM;Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone) at 37 °C in 5% CO_2. Cells were plated on 35-mm dishes and cultured in DMEM containing 10% FBS until the cells had reached 70% confluence. The crocin-treated Cells were incubated with several concentrations of crocetin and the control cells were treated with PBS.

MTT Cell Proliferation and Cytotoxicity Assays

The MTS an improved tetrazolium cell proliferation assay reagent was used in the SW480 cell line to assess the effect on cell proliferation of a range of concentrations of crocin. Cells (5×10^3) were plated and grown in 200uL of growth medium in 96-well microtiter plates. After an overnight attachment period, cells were treated with varying concentrations of crocin (0.2, 0.4, 0.8 mmol/L) prepared from a 50 mmol/L stock solution dissolved in ddH2O for 48h. The crocin-treated cells were incubated with MTS at 37 °C in a humidified 5% CO_2 atmosphere for 2 hours and product was measured at 570 nm in a microplate reader (Bio-Rad).

Cell-Cycle Analysis by Flow Cytometry

The crocin-treated cells in 0.8 mmol/L were harvested at 6, 24, 48 hours, washed with 4 °C PBS, fixed in 70% ethanol and stored at 4 °C over-night, cells were then treated with RNase A solution (500 U/ml) at 37 °C for 15 min and stained with propidium iodide (PI, 50 μg/ml) at room temperature. DNA content was determined by flow cytometry (Flow CytoMeter, Millipore, USA) using CytoSoft 5.3 software (Millipore). The fractions of cells in G0/G1, S and G2/M phases were determined using FCS Express (version 3, Denovo, USA).

TUNEL Assay

Cells were cultured on 6 well plates with glass slice until the cells had reached 70% confluence, and cells were treated with 0.8 mmol/L crocin.After 6, 24, 48 hours, the crocin - treated cell grown on slices were fixed with 4% paraformalde-hyde and were pre-incubated in 0.1% Triton X-100 (0.01M PBS). Then the slices were incubated with TUNEL reaction mixture (Roche; Switzerland) for 60 min at 37 °C in a humidified atmosphere in the dark. The slices were rinsed 3 times with PBS, coverslipped using VectaSHIELD mounting medium containing DAPI (Vector Laboratories, catalog no. H-1200), and analyzed by laser scanning confocal microscope (Olympus FV1000 Viewer). Excitation wave lengths were in the range of 450-500 nm and detection wave lengths in the range of 515-565 nm.

DNA Damage Assay

DNA damage was assessed using the alkaline single-cell gel electrophoresis assay (comet assay). After the crocetin treatments mentioned above, cells were washed in PBS, then incubated in normal medium for 3 h. Cells were then harvested and mixed with low-melting agarose and fixed onto slides. The cells were then lysed and DNA was denatured using an alkaline (pH >14) solution at 4 °C. Slides were then placed on a horizontal electrophoresis device which allowed damaged and/or broken DNA to migrate away from the nucleus. Slides were then stained with ethidium bromide, visualized by fluorescence microscopy, and analyzed using the Komet 5comet assay analysis software which quantitatively determines the extent of DNA damage in each sample using the mean Olive tail moment calculation as previously described.

Western Blotting

The crocin-treated cell groups (treated with 0.8 mmol/L crocin) and the control cell groups (treated with PBS) were harvested after 48 hours and cells were lysed with cold RIPA lysis buffer containing protease inhibitors, and proteins were collected by centrifugation. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) Immunoblots were incubated with the following antibodies: $p21^{WAF1/Cip1}$, P53, cdk2, CyclinA and β-actin. Blots were developed using Supersignal WestPico chemiluminescent substrate (Pierce), imaged and analyzed by photodensitometry with an imaging system (Bio-Rad).

Statistical Analysis

SPSS statistical program (SPSS Inc., Chicago, IL) was used to carry out a one-way analysis of variance (ANOVA) on our data. When significant differences by ANOVA were detected, analysis of differences between the means of the treated and control groups were performed by using Dunnett's t test.

Results

Crocin Induces Growth Arrest and Apoptosis

To test the effect of crocin on cell growth, we carried out the MTS assay. The MTS test showed that crocin significantly reduced the viability of SW480 cells in a dose-dependent manner (Figure 1). For further studies,a crocin concentration of 0.8 mmol/L was used. The effect of crocin on cell cycle progression was also assessed using flow cytometric analysis. crocin-treated SW480 cells displayed an accumulation of the cell population at the S phase starting from 6 hours and in a time-dependent manner (Figure 2). These findings were further supported by measuring the apoptotic cell of green fluorescence

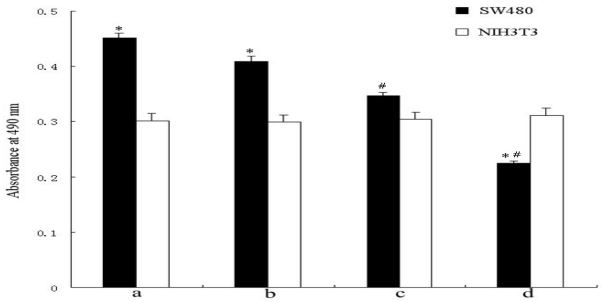

Figure 1. Growth-inhibitory Effect of Crocin on Cells Detected by MTT Assay. Cells were untreated (a) or treated with 0.2 mmol/L(b), 0.4 mmol/L(c), 0.8 mmol/L(d); Absorbance at 490 nm showed a significant decrease in the growth of crocin treated cells compared with control cells (* means $P < 0.001$, # means $P < 0.05$)

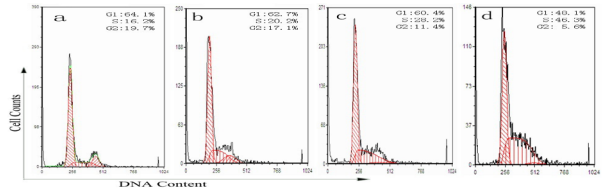

Figure 2. Crocetin Induces Cell Cycle Arrest in Cancer Cells. Cells were untreated (a) or treated with 0.8 mmol/L crocin for 6(b), 24(c), and 48(d) hours, harvested, and DNA was stained with PI for flow cytometric analysis and and cell phases were determined using the Watson pragmatic algorithm. The cells(d) had a significantly increased number of SW480 cells (46.3%) in the S phase compared with those of the SW480 cells at 6h(b)(20.2%, p=0.001) and the SW480 cells at 24h(c) (28.2 %, P=0.003)

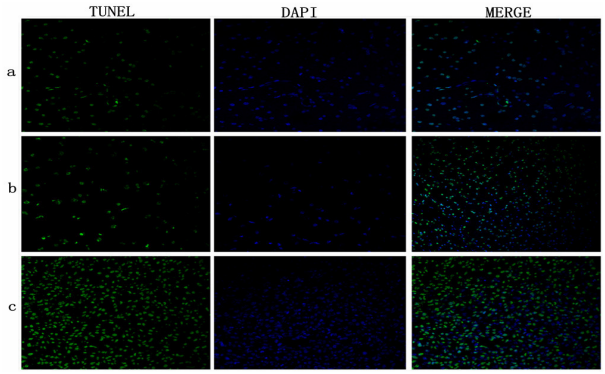

Figure 3. SW480 Cells were Treated with Crocetin for Different Time and Stained with TUNEL and DAPI to Detect Apoptosis; cells were treated with 0.8 mmol/L crocin for 6(a), 24(b), and 48(c) hours. The proportion of apoptotic cells at 48 h(c) after crocetin treatment, was significantly higher than at 24 h(b) after treatment (60.14% vs 35.32%, P =0.027), at 6 h(a) after treatment (60.14% vs 13.17%, P =0.012)

after crocin treatment using Tunel assay. Crocin induced apoptosis in SW480 as early as 6 hours after treatment (Figure 3). The apoptosis induction further increased in a time dependent manner reaching 60.14% after 48 hours. The comet assay analysis indicated cells treated with 0.8mmol/L crocin had significantly longer mean tail moments starting from 6 hours (Figure 4) and in a time-dependent manner.

Effect of crocin on on the expression of apoptosis-related genes

We choose the cells treated in 0.8 mmol/L and 48 hours

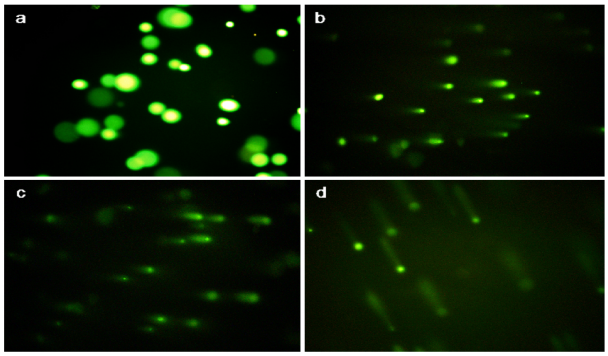

Figure 4. DNA Damage Repair Capacity Assessed by the Comet Assay. SW480 cells were untreated (a) or treated with 0.8 mmol/L crocin for 6(b), 24(c), and 48(d) hours; representative cell images from each of the four groups. Mean Olive tail moment determined from 50 cells from each of the four treatment groups was 0.202±0.017 for untreated(a), 0.603±0.246 for 6 hours(b), 0.904±0.102 for 24 hours(c), and 1.804±0.151 for 48 hours(d). The cells 0.8 mmol/L for 48 hours had a significantly longer mean tail moment ($p<0.001$), indicating decreased DNA repair capacity compared with cells with untreated

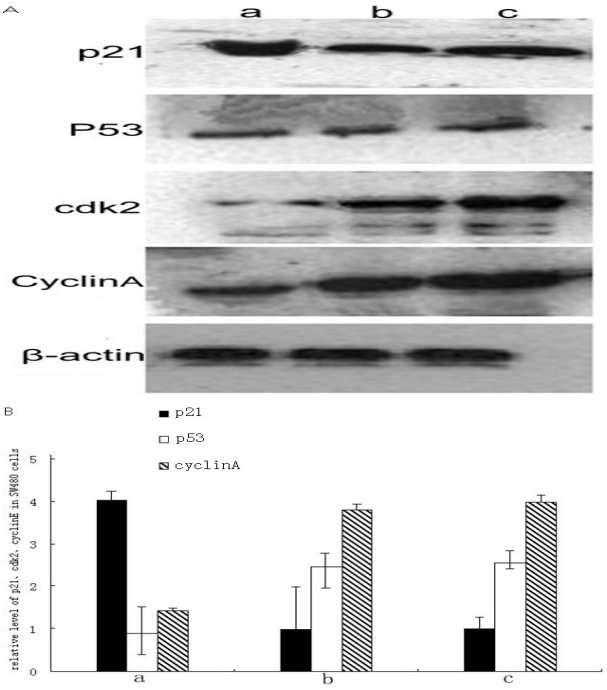

Figure 5. Relative Expression of p21, p53, cdk2, CyclinA Within the Crocin-treated SW480 (a), PBS-treated SW480(b), Normal SW480(c). (A) The level of p21, P53, cdk2, CyclinA protein were detected by Western blot analysis and were normalized to the level of β-actin using a Molecular Dynamics densitometer. (B) The ratio of p21, cdk2, CyclinA signal to β-actin signal in the cells prepared from normal SW480 were arbitrarily set as 1.0. Error bars indicate standard error of the mean (SEM) (n = 3)

to study the mechanism underlying the tumor suppressive effects of crocin. After protein normalization to actin levels, western blot analysis also demonstrated that the level of P53 in crocin-treated SW480 cells no significant difference between crocin-treated NIH3T3, PBS-treated SW480, PBS-treated NIH3T3, normal SW480, normal NIH3T3; there was an apparent decrease in the levels of cdk2, CyclinA protein in crocin-treated SW480 cells, compared with other cells; the level of p21 protein was upregulated, as shown in Figure 5.

Discussion

Natural products have long been used to prevent and treat diseases including cancers and might be good candidates for the development of anti-cancer drugs (Liu et al., 2004). Saffron, a commonly-used spice and food additive, is known for its anti-cancer and anti-tumor properties (Mousavi et al., 2009). Altough most of the in vivo studies were interested in the isolated bioactive compounds of saffron, little research has been done to examine anti-cancer mechanisms of saffron. Crocetin which is the major ingredient of saffron, had been certified two distinct anticancer functions of crocetin as follows: to inhibit cell proliferation at early time by inducing cell cycle arrest in different human cancer cell types via p53-dependent and -independent P21 mediated mechanisms and to kill cancer cells via apoptosis (Zhong et al., 2011). Because p53 is mutated in approximately 50% of human tumors and p53 gene is involved in cell cycle regulation, controlling DNA repair and apoptosis (Aizat et al., 2011; Heah et al., 2011), we selected SW480 human colonic cancer cell lines with mutation of p53 to study the anticancer effects of crocetin.

The present results were in agreement with previous reports indicating that saffron and its ingredients possessed anti-tumor and anti-cancerogenic activities and have no cytotoxic effect on non-malignant cells (Aung et al., 2007; Tavakkol et al., 2008; Yanatatsaneeji et al., 2010; Harrison et al., 2011).

The results show that crocetin inhibits cell proliferation by inducing S arrest. The proportion of SW480 cells in the S phase at 48 h after crocetin treatment (46.3%) was significantly higher than at 24 h after treatment (P = 0.003), and significantly higher than at 6 h after treatment (p=0.001). No such association between crocetin treatment and proportion of cells in S phase was seen in NIH3T3 cells. These results also show an association between DNA damage and apoptosis level following crocetin treatment, the proportion of apoptotic cells at 48 h after crocetin treatment, when had a significantly longest mean tail moment, was significantly higher than at 24 h (P =0.027) or 6h (P =0.012) after treatment. Consistent with our previous study on SW480 cells, Crocetin induced a significant toxicity start from 6 h and the toxicity seemed to grow stronged over time. The observed apoptotic induction in SW480 cells obviously resulted from DNA damage as reflected by comet assay, suggesting an additional role of Crocetin in sensitizing cancer cells to the effects of other chemotherapeutics.

Progression through the cell cycle is monitored by cell cycle checkpoints that ensure proper replication and segregation of genetic material between daughter cells (Elledge et al., 1996). The eukaryotic cell cycle is controlled by different cyclins and their associated kinases. In mammalian cells, levels of CyclinA and its associated kinase, cdk2, rise in late G1/early S-phase when DNA replication is initiated .The high level of CyclinA may accelerate the progress of S/G2 stage. Without synthesis of CyclinA prior to the S/G2 transition, the cell cannot enter mitosis and the cell cycle will arrest at S phase. In present results , the expression levels of P21 protein increased significantly in the SW480 cells treated with crocetin for 48h, but not in SW480 cells treated with PBS or normal. In contrast, there was an apparent decrease in the levels of CyclinA and cdk2 protein in SW480 cells treated with crocetin for 48h, compared with treated with PBS or normal, whereas there was no significant difference in the level of p53 protein between SW480 cells treated with crocetin and the control cells.

P21, the product of the WAF1/CIP1/SDI1 gene, is an inhibitor of cyclin-dependent kinases and is activated through p53-dependent or p53-independent pathways and it has been reported that P21may contribute to G1 arrest in crocetin-treated cancer cells via inhibits the activity of cyclin dependent kinases (Cdks) or proliferating cell nuclear antigen (PCNA) (Nakano et al., 1997; Lee et al., 1998). Based on these results, it is likely that crocetin decreases CDK2 and cyclins levels in the cells that are sensitive to crocetin, which contributes to increased p21accumulation and crocetin activates p21 through a p53-independent mechanism which could be the one of the main mechanisms of antitumour.

Tavakkol-Afshari and colleagues (Tavakkol et al., 2008) reported that 96% ethanol saffron extract is selectively cytotoxic against epithelial-like human hepatocellular carcinoma cell125s (HepG-2) as well as human cervical carcinoma cells (HeLa) but nontoxic towards normal mouse fibroblast cells (L929). There were the three major carotenoids that are derived from saffron (crocin, crocetin, and dimethylcrocetin) and the additive and synergistic effects among them may enhance its anti-carcinogenic properties (Aung et al., 2007). Amr Amin et also confirmed these findings that HepG2 cells treated with the saffron extract contained crocin and safranal increased cleavage of caspase-3, as well as DNA damage and cell cycle arrest (Amin et al., 2011).

Taken together, we suggest three major carotenoids of saffron having the anti-tumor activity but serving different mechanisms and may have synergism anti-tumor activity to some extent.

References

Abdullaev F (2002). Cancer chemopreventive and tumoricidal properties of saffron (*Crocus sativus* L.). *Exp Biol Med*, **227**, 20-5.

Abdullaev F, Espinosa J (2004). Biomedical properties of saffron and its potential use in cancer therapy and chemoprevention trials. *Cancer Detect Prev*, **28**, 426-32.

Abdullaev F, Rivera R, Roitenburd V, et al (2000). Pattern of childhood cancer mortality in Mexico. *Arch Med Res*, **31**, 526-31.

Ahmad AS, Ansari MA, Ahmad M (2005). Neuroprotection by crocetin in a hemi-parkinsonian rat model. *Pharmacol Biochem Behav*, **81**, 805-13.

Aizat AAA, Shahpudin SNM, Mustapha MA, et al (2011). Association of Arg72Pro of P53 polymorphism with colorectal cancer susceptibility risk in Malaysian population. *Asian Pac J Cancer Prev,* **12**, 2909-13.

Amin A, Hamza AA, Bajbouj K, et al (2011). Saffron: a potential candidate for a novel anticancer drug against hepatocellular carcinoma. *Hepatology*, **54**, 857-67.

Aung HH, Wang CZ, Ni M (2007). Crocin from *Crocus sativus* possesses significant anti-proliferation effects on human

colorectal cancer cells. *Exp Oncol*, **29**, 175-80.
Bajbouj K, Schulze LJ, Diermeier S (2012). The anticancer effect of saffron in two p53 isogenic colorectal cancer cell lines. *BMC Complement Altern Med*, **12**, 69.
Cai J, Yi FF, Bian ZY (2009). Crocetin protects against cardiac hypertrophy by blocking MEK-ERK1/2 signalling pathway. *J Cell Mol Med*, **13**, 909-25.
Chryssanthi DG, Lamari FN, Iatrou G (2007). Inhibition of breast cancer cell proliferation by style constituents of different Crocus species. *Anticancer Res*, **27**, 357-62.
Das I, Das S, Saha T: Saffron suppresses oxidative stress in DMBA-induced skin carcinoma: A histopathological study. *Acta Histochem*, **112**, 317-27.
Dhar A, Cherian G, Dhar G (2005). Molecular basis of protective effect by crocetin on survival and liver tissue damage following hemorrhagic shock. *Mol Cell Biochem*, **278**, 139-46.
Dhar A, Mehta S, Dhar G (2009). Crocetin inhibits pancreatic cancer cell proliferation and tumor progression in a xenograft mouse model. *Mol Cancer Ther*, **8**, 315-23.
Elledge SJ (1996). Cell cycle checkpoints: preventing an identity crisis. *Science*, **274**, 1664-72.
Harrison S, Benziger H (2011). The molecular biology of colorectal carcinoma and its implications: A review. *Surgeon*, **9**, 200-10.
Heah KG , Hassan MI, Huat SC (2011). p53 Expression as a marker of microinvasion in oral squamous cell carcinoma. *Asian Pac J Cancer Prev*, **12**, 1017-22.
He SY, Qian ZY, Wen N (2007). Infuence of Crocetin on experimental atherosclerosis in hyperlipidamic-diet quails. *Eur J Pharmacol*, **554**, 191-5.
Jagadeeswaran R, Thirunavukkarasu C, Gunasekaran P (2000). In vitro studies on the selective cytotoxic effect of crocetin and quercetin. *Fitoterapia*, **71**, 395-99.
Kanakis C, Tarantilis P, Pappas C (2009). An overview of structural features of DNA and RNA complexes with saffron compounds: Models and antioxidant activity. *J Photochem Photobiol B*, **95**, 204-12.
Lee I, Lee J, Baek N (2005). Antihyperlipidemic effect of crocin isolated from the fructus of *Gardenia jasminoides* and its metabolite Crocetin. *Biol Pharm Bull*, **28**, 2106-10.
Lee SJ, Ha MJ, Lee J (1998). Inhibition of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase pathway induces p53-independent transcriptional regulation of p21WAF1/CIP1 in human prostate carcinoma cells. *J Biol Chem*, **273**, 10618-23.
Liu RH (2004). Potential synergy of phytochemicals in cancer prevention: Mechanism of action. *J Nutr*, **134**, S3479-85.
Mousavi SH, Tavakkol AJ, Brook A (2009). Role of caspases and Bax protein in saffron-induced apoptosis in MCF-7 cells. *Fd Chem Toxicol*, **47**, 1909-13.
Nakano K, Mizuno T, Sowa Y (1997). Butyrate activates the WAF1/Cip1 gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. *J Biol Chem*, **272**, 22199-206.
Ordoudi S, Befani C, Nenadis N (2009). Further examination of antiradical properties of Crocus sativus stigmas extract rich in crocins.*J Agric Food Chem*, **57**, 3080-6.
Tavakkol AJ, Brook A, Mousavi SH (2008). Study of cytotoxic and apoptogenic properties of saffron extract in human cancer cell lines. *Fd Chem Toxicol*, **46**, 3443-7.
Yanatatsaneeji P, Kitkumthorn N, Dhammawipark C, er al (2010). Codon72 polymorphism in the p53 tumor suppressor gene in oral lichen planus lesions in a Thai population. *Asian Pac J Cancer Prev*, **11**, 1137-41.
Zheng S, Qian Z, Tang F (2005). Suppression of vascular cell adhesion molecule-1 expression by crocetin contributes to attenuation of atherosclerosis in hypercholesterolemic rabbits. *Biochem Pharmacol*, **70**, 1192-9.
Zhong YJ, Shi F, Zheng XL (2011). Crocetin induces cytotoxicity and enhances vincristine-induced cancer cell death via p53-dependent and -independent mechanisms. *Acta Pharmacol Sin*, **32**, 1529-36.