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Research Article

# *In Vitro* Cytotoxicity of Iranian Saffron and Two Main Components as a Potential Anti-Cancer Drug

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**Keywords** *Crocus sativus* L; Crocin; Picrocrocin; TC-1; Cytotoxicity; Apoptosis

## Abstract

Natural products are good candidates for the development of anti-cancer drugs. Saffron and its components have been proposed as a promising candidate for cancer chemoprevention. The more powerful components of saffron are carotenoids and monoterpene aldehydes. In the current study, cytotoxic and apoptogenic effects of saffron extract and two main components, crocin and picrocrocin, were evaluated in malignant TC-1 and non-malignant COS-7 cell lines. We showed that the aqueous extract of saffron and its ingredients decreased cell viability in malignant cells as a concentration and time-dependent manner. Furthermore, our data demonstrated that cytotoxic effect of saffron and its components is mediated via apoptosis. Although, picrocrocin is capable of inhibiting the growth of TC-1 cells *in vitro*, its high IC50 along with low percentage of apoptotic effects, indicate that the cytotoxic activity detected in saffron extract is mostly due to crocin. The solubility of crocin and picrocrocin due to their glycosylated state together with their cytotoxic effects on malignant cells, make them the most appropriate saffron compounds to be evaluated in cancer treatment. This study provides evidence that saffron and its components exert a significant chemo preventive effect against tumor through inhibition of cell proliferation and induction of apoptosis.

## Introduction

A large and increasing number of patients in the world use medicinal plants and herbs for health purposes [1]. Saffron, the dried stigmas of *Crocus sativus* L., is used mainly as a herbal medicine, food coloring and flavoring agent in different parts of the world [2]. The recent studies indicate its potential as an anti-cancer agent [3,4]. Saffron originally grew in India, Iran, Spain, Greece and various places in China, especially Tibet [2, 5]. Iran is the world's largest producer of saffron (about 85% of world saffron production) [6]. The value of saffron is determined by the existence of three main secondary metabolites: crocin, responsible for color; picrocrocin, responsible for taste; and safranal responsible for odor [3]. Some studies reported the anti-cancer activity of saffron extract and its purified components against a wide spectrum of murine and human cancerous cell lines [7]. Regarding to previous studies, carotenoids possess anti-carcinogenic, anti-mutagenic and immuno-modulating effects. Saffron is also an important spice rich in carotenoids so-called as crocin and its derivatives (crocetin and dimethyl-crocetin) [7,8]. The induction of apoptosis in tumor cells is considered very useful in the management and therapy as well as in the prevention of cancer. A wide variety of natural substances have been recognized to have the ability to induce apoptosis in various tumor cells [9]. It is thus considered important to screen apoptotic inducers from plants, either in the form of crude extracts or as components isolated from them. In the present study, we isolated two main components from Iranian saffron (picrocrocin as a monoterpene aldehyde and crocin as a natural carotenoid). The structures of these components are shown in Figure 1. Then, the effects of these molecules were investigated on *in vitro* growth of TC-1 tumor cell line. TC-1 murine model was prepared from primary C57BL/6 mice lung epithelial cells by co-transformation with HPV16 E6, HPV16 E7 and ras oncogenes [10]. Human Papilloma Virus (HPV), particularly HPV16, is associated with a majority of cervical cancers and a subset of head and neck cancers. HPV16 E7, one of its oncoproteins, is essential for the induction and maintenance of cellular transformation [11]. The present study is to show toxicity of saffron extract and also its components on TC-1 malignant cell line in which apoptosis plays an important role.

## Materials and Methods

### Plant materials

Dry stigmas of pure 'Ghaenat' saffron (*Crocus sativus* L.), picrocrocin and crocin were provided as described previously [12,13]. Briefly, picrocrocin and crocin were extracted by adsorption chromatography with neutral aluminum oxide 90 active and detected at 250 nm and 440 nm using a spectrophotometer, respectively.

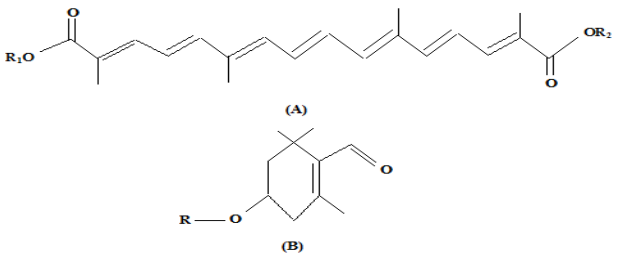


**Figure 1:** Structures of different saffron components: (A) crocin and (B) picrocrocin; R1=R2=R=β-D-gentiobiosyl.

### Cell culture

Malignant (TC-1, ATCC number: CRL-2785) and non-malignant (COS-7, fibroblast-like cell line, ATCC number: CRL-1651) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, and incubated at 37˚C in a humidified atmosphere containing 5% $CO_2$.

### MTT cell proliferation and cytotoxicity assays

The MTT proliferation assay was used to assess the cytotoxic effects of a range of concentrations of saffron extract (0.25-10 mg/ml), crocin (0.5-4 mM) and picrocrocin (0.5-4 mM) in the malignant (TC-1) and non-malignant (COS-7) cell lines for 24 and 48 hours. Cell growth was quantified by the ability of living cells to reduce the yellow dye MTT (5 mg/ml in PBS) to a purple formazan product. The absorbance was measured at 570 nm in an ELISA reader.

### Detection of apoptotic cell death using saffron and two main components

The apoptotic effects of saffron extract, crocin and picrocrocin on malignant cells were determined at 24 and 48 h post-incubation using a FITC-conjugated-annexin V/ Propidium Iodide (PI) apoptosis kit (BioVision) following the manufacturer's instructions. The apoptosis of TC-1 cells was analyzed by a Partec flow cytometer. In this experiment, for each time course study, there was a control sample which remained untreated and received the equal volume of medium. All different treatments carried out in duplicate.

### Flow cytometric analysis of DNA content

One day before treatment, cells were seeded at a density of $1\times10^6$ cells per plate. At 24 and 48 h post-treatment, the cells were harvested by trypsin release, washed twice with PBS, fixed with 70% ethanol, treated with 1% ribonuclease and finally stained with PI (50µg/ml). Distribution of cell cycle phases with different DNA contents was determined using a Partec flow cytometer.

### Statistical analysis

Statistical analysis was performed using Prism 3.0 software (Graph Pad, California and USA). Observed differences in cytotoxicity and apoptosis between untreated and treated cells were evaluated using student's t test. All results were presented as mean ± Standard Deviation (SD). A probability level of $p < 0.05$ was considered statistically significant.

**Table 1:** Doses inducing 50% cell growth inhibition (IC50) of aqueous saffron extract, crocin and picrocrocin against TC-1 (A) cell lines.

| Time / Compounds | 24 h | 48 h |
|---|---|---|
| Saffron extract | 5 mg/ml | 4 mg/ml |
| Crocin | 2 mM | 1.5 mM |
| Picrocrocin | 4 mM | 3 mM |

**Table 1:** Doses inducing 50% cell growth inhibition (IC50) of aqueous saffron extract, crocin and picrocrocin against COS-7 (B) cell lines.

| Time / Compounds | 24 h | 48 h |
|---|---|---|
| Saffron extract | 6 mg/ml | 5 mg/ml |
| Crocin | 4 mM | 3 mM |
| Picrocrocin | 8 mM | 7 mM |

## Results

### Cytotoxicity of saffron extract and its isolated compounds

The isolation and purification of two main components of Iranian saffron (crocin and picrocrocin) were performed by adsorption column chromatography using aluminum oxide. Their identity was established by their spectral and chromatographic features. The *in vitro* cytotoxicity of saffron and its components showed a concentration and time-dependent manner. Doses including 50% cell growth inhibition (IC50) against TC-1 and COS-7 cells are presented at different times in Table 1. Regarding to our results, picrocrocin indicated high IC50 and low cytotoxicity as compared to treatment with crocin. In addition, the cell viability was higher for COS-7 against TC-1 cells at 24 h and 48 h after treatment by saffron and its components ($p < 0.05$). The effects of aqueous saffron extract on cell viability of TC-1 and COS-7 cells have been indicated in Figure 2.

### Saffron and its components induce apoptosis *in vitro*

In order to determine the effects of chemotherapy on TC-1 tumor cells, we incubated TC-1 tumor cells with different doses of saffron, crocin and picrocrocin. The cells were then characterized for apoptotic cell death using annexin-V and PI staining. The results

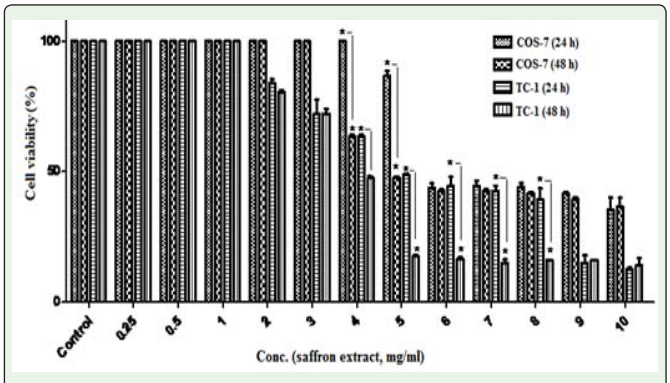


**Figure 2:** Growth inhibition of TC-1 (A & B) and COS-7 (B & C) cells by an aqueous extract of saffron. Viability was quantitated by MTT assay. Values represent the mean of three replicates ± SD (*$p < 0.05$). The asterisks are indicator of statistical differences obtained separately at different time points.

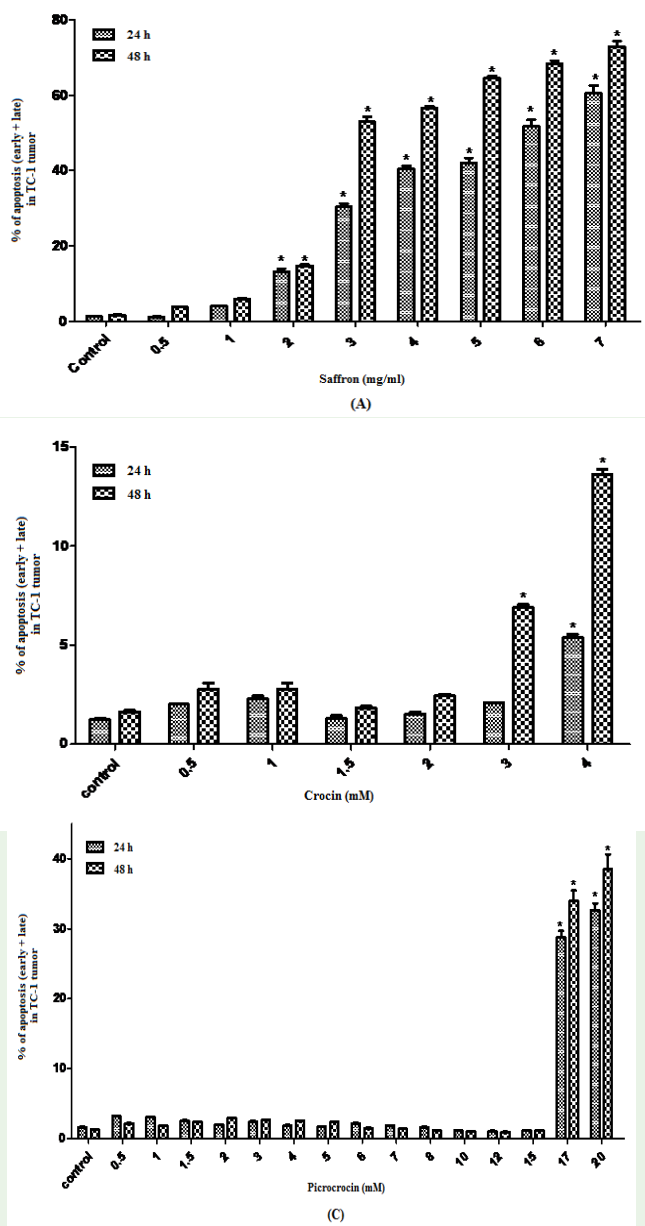


**Figure 2:** Growth inhibition of TC-1 (A & B) and COS-7 (B & C) cells by an aqueous extract of saffron. Viability was quantitated by MTT assay. Values represent the mean of three replicates ± SD (*$p < 0.05$). The asterisks are indicator of statistical differences obtained separately at different time points.

showed that treatment of TC-1 tumor cells with saffron extract, crocin and picrocrocin enhances the apoptotic tumor cell death *in vitro* in a dose-dependent manner. As shown in Figure 3, we observed that TC-1 tumor cells treated with the higher doses of saffron, crocin and/or picrocrocin demonstrated a greater degree of apoptotic tumor cell death (early + late apoptosis) compared to control untreated TC-1 tumor cells at 24 and 48 hours following treatment ($p < 0.05$).

### Flow cytometric analysis of DNA content

DNA content measurement can be used to detect apoptotic cells, which have diminished DNA content. The effect of saffron extract, crocin and picrocrocin on cell cycle progression was assessed using flow cytometric analysis. Saffron-treated TC-1 cells displayed an accumulation of the cell population at the S phase starting from 24 hours confirmed by measuring the apoptotic cell fraction using annexin-PI staining. The apoptosis induction further increased in a time dependent manner. These findings are in agreement with the observed sub-G1 cell population which showed a progression in the induced apoptosis by the accumulation of DNA in cells treated with saffron (> 3 mg/ml), crocin (> 3 mM) and picrocrocin (> 8 mM). All three compounds induced a sub-G1 peak (one of the reliable biochemical markers of apoptosis) in flow cytometry histogram of treated cells compared to control indicating apoptotic cell death is involved in saffron/crocin/picrocrocin-induced toxicity (Data not shown).

## Discussion

Many fruits, vegetables, herbs and spices contain protective factors against various diseases especially cancer [14, 15]. Recently, extracts from natural products and saffron have also been shown to exhibit anti-cancer activity [16]. In present study, we reported the anticancer activity of saffron extract and its main components (crocin and picrocrocin) against TC-1 tumor cell lines. Crocin and picrocrocin are responsible for the color and flavor of the spice, respectively [17]. We isolated these molecules by adsorption chromatography of saffron extracts. The studies have shown that the mechanisms of saffron action are based on their carotenoid-like action. Saffron possesses the richest source of carotenoids as well as riboflavin [7]. Carotenoids are well tolerated even at high doses, and numerous studies have supported their use in cancer chemoprevention and chemotherapy [18]. Recently, it was shown that carotenoids from saffron either as crocins or purified derivatives (dimethyl-crocetin) were very effective in inhibiting the proliferation of HL-60 leukemia cells. The concentrations that produced 50% inhibition in cell growth were 1.2, 5.0, and 6.6 mM for dimethyl crocetin, crocetin and crocins, respectively during three days in culture. Longer incubations in culture up to five days decreased the effective concentration of the drug to produce the same effect [8]. In addition, Abdullaev and Frenkel detected a dose-dependent decrease in colony formation of A549 lung adenocarcinoma, cervical epithelioid carcinoma and HeLa cells using saffron [19]. The IC50 values against the A549 cell lines were determined as 1.2 and 0.65 mg/ml after 24 and 48 h, respectively [20]. This observation was proved in our studies using TC-1 tumor cell line. We found that the concentration inducing 50% cytotoxicity (IC50) on TC-1 cells was 4 mg/ml, 1.5 mM and 3 mM at 48 hours after treatment with saffron, crocin and picrocrocin, respectively. In addition, the aqueous extract of saffron and its purified components decreased cell viability in malignant cells as a concentration and time-dependent manner. Extracts of saffron have been previously reported to inhibit cell growth of human tumor cells [14]. Doses inducing 50% cell growth inhibition on HeLa cells were 2.3 mg/ml for an ethanolic extract of saffron dry stigmas, 3mM for crocin, 0.8 mM for safranal and 3 mM for picrocrocin. Cells treated with crocin exhibited wide cytoplasmic vacuole-like areas, reduced cytoplasm, cell shrinkage and pyknotic nuclei, suggesting apoptosis induction [14]. In addition, the

IC50 values against the lung cancer cell line were determined as 1.5 and 0.565 mg/ml after 24 and 48 h treatment with the ethanolic extract of saffron, respectively [21]. *In vitro* and *In vivo* cytotoxic assays have also shown that saffron extracts inhibit growth and cellular nucleic acid synthesis of tumor cells, whereas, interestingly, non-tumor cells are less sensitive or even insensitive to the extracts [21-23]. Regarding to our results, picrocrocin is capable of inhibiting the growth of TC-1 cells *in vitro*, but its high IC50 indicates that the growth inhibitory activity detected in saffron extract is mostly due to crocin. This observation has been previously supported by other studies [14]. Indeed, water-solubility and high inhibitory growth effect of crocin make them the most appropriate saffron compounds to be evaluated in cancer treatment. The reports demonstrated that *Crocus sativus* extract and its major constituent, crocin, significantly inhibited the growth of colorectal cancer cells, while not affecting normal cells [24]. Recently, cell proliferation inhibition of different Crocus species was shown in MCF-7 and MDA-MB-231 breast cancer cells [25]. Different hypotheses for anti-tumor effects of saffron and its ingredients have been proposed, including inhibition of nucleic acid and free radical chain reactions and interaction of carotenoids with topoisomerase II [26,27,28]. Despite these studies, the mechanisms of saffron-induced toxicity are still unknown. Our data demonstrated that cytotoxic effect of saffron as well as its components (crocin and picrocrocin) is mediated via apoptosis. Other studies have also indicated that saffron-induced apoptotic cell death of the A549 cells is involved in a concentration-dependent manner and consequently, in the toxicity of saffron. Therefore, saffron could cause cell death in the A549 cells, in which apoptosis plays an important role [20]. Our previous study indicated that chemotherapy using saffron and its components followed by immunotherapy generates different anti-tumor effects [29]. Briefly, natural products have been used to prevent and treat many diseases including cancer. In present study, the cytotoxic and apoptogenic effects of aqueous saffron extract, crocin and picrocrocin in TC-1 cell line were investigated. Our data confirmed that saffron extract and its ingredients have cytotoxic activity against TC-1 cell line more than non-malignant cells (COS-7) which is consistent with previous studies indicating that saffron and its ingredients possess anti-tumor and anti-carcinogenic activities. We indicated that saffron and two main components induce apoptosis. These data could provide further knowledge to mechanisms involved in their toxicity. Therefore, saffron and its components especially crocin could be considered as a promising chemotherapeutic agent in cervical cancer treatment, in future.

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