

# Cytotoxic Effect of Saffron Stigma Aqueous Extract on Human Transitional Cell Carcinoma and Mouse Fibroblast

Behzad Feizzadeh,<sup>1</sup> Jalil Tavakkol Afshari,<sup>2</sup> Hassan Rakhshandeh,<sup>3</sup> Alireza Rahimi,<sup>1</sup> Azam Brook,<sup>2</sup> Hassan Doosti<sup>4</sup>

*Keywords: Crocus sativus, antineoplastic agents, transitional cell carcinoma, fibroblast*

<sup>1</sup>Department of Urology, Ghaem Hospital, Mashhad University of Medical Sciences, Mashhad, Iran

<sup>2</sup>Immunology and Cell Culture Laboratory, Immunology Research Center, Bu-Ali Research Institute, Mashhad, Iran

<sup>3</sup>Department of Pharmacology, Medical Plants Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

<sup>4</sup>Department of Statistics, Ferdowsi University, Mashhad, Iran

Corresponding Author:  
Behzad Feizzadeh, MD  
Department of Urology, Ghaem Hospital, Mashhad, Iran  
Tel: +98 511 841 7404  
Fax: +98 511 841 7404  
E-mail: behzadfeizzadeh@yahoo.com

Received May 2007  
Accepted June 2008

**Introduction:** Saffron has been suggested to have inhibitory effects on tumoral cells. We evaluated the cytotoxic effect of aqueous extract of saffron on human transitional cell carcinoma (TCC) and mouse non-neoplastic fibroblast cell lines.

**Materials and Methods:** Human TCC 5637 cell line and mouse fibroblast cell line (L929) were cultivated and incubated with different concentrations of aqueous extract of saffron stigma (50 µg/mL to 4000 µg/mL). Cytotoxic effect of saffron was evaluated by morphologic observation and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay after 24, 48, 72, and 120 hours in each cell line.

**Results:** After 24 hours, morphological observations showed growth inhibitory effects at saffron extract concentrations higher than 200 µg/mL for L929 cells and at concentrations of 50 µg/mL to 200 µg/mL for the TCC cells. These changes became more prominent after 48 hours. However, significant growth inhibitory effects of the extract were shown at concentrations of 400 µg/mL and 800 µg/mL. Higher concentrations of saffron correlated inversely with cell population of both cell lines. Significant reduction of the survived cells was seen at concentrations of 400 µg/mL and 2000 µg/mL for TCC and L929 cell lines, respectively. After 120 hours, decrease in the percentage of survived cells at higher concentrations of saffron extract was seen in both cell lines. At a concentration of 800 µg/mL, the survived L929 cells plummeted to less than 60% after 120 hours, while no TCC cells survived at this time. No L929 cells survived at 2000 µg/mL.

**Conclusion:** Saffron aqueous extract has inhibitory effects on the growth of both TCC 5637 and normal L929 cell lines. This effect is dose dependent.

*Urol J. 2008;5:161-7.  
www.uj.unrc.ir*

## INTRODUCTION

Efforts to find any therapeutic options for cancers have guided the investigators to consider even herbal medicine to be tested. Treatment of bladder cancer was the subject of our interest that led us to study alternative therapies such as the use of herbs. Bladder cancer is the 5th

most common cancer with a high rate of mortality and morbidity.<sup>(1)</sup> Transitional cell carcinoma (TCC) is the most common bladder tumor which can be induced directly by cigarette smoking and environmental factors.<sup>(2)</sup> Occupational exposure risk factors include aromatic amines, industrial



**Figure 1.** Saffron and its stigma.

dyes and solvents, plastic painting, rubber, heavy metals, mixtures of polycyclic aromatic hydrocarbons, etc.<sup>(3)</sup>

We sought to investigate saffron and its potential effect on cancerous cells. Saffron (*Crocus Sativus* L) is one of the worthiest perennial flowers with a violet color and usually 3 golden petal stigma in the Iridaceae family (Figure 1). It has been used as a food spice since the ancient times.<sup>(4,5)</sup>

Some of saffron's chemical ingredients are carbohydrates, minerals, vitamins (especially riboflavin and thiamin), and pigments including crocin, anthocyanin, carotene, and lycopene.<sup>(5)</sup> Anticarcinogenic activity of saffron was reported in the beginning of 1990 and research on this subject has increasingly continued during the past decade.<sup>(6)</sup> Saffron and its main ingredients have shown antitumor and anticarcinogenic activities both *in vitro* and *in vivo*.<sup>(6,7)</sup> To date, however, there has not been any report in literature on saffron effects in bladder cancer. We studied *in vitro* cytotoxic effect of saffron aqueous extract on TCC cell line proliferation and non-neoplastic fibroblast cells of mouse as a normal cell line.

## MATERIALS AND METHODS

### Preparation of Saffron Extract

Saffron harvested from saffron farms of Ghaen (a city in the northeast of Iran) was used in this study. Aqueous extract was prepared with 15 g

of its ground petal stigma and 400 mL of distilled water in a Soxhlet extractor for 18 hours. The prepared extract was concentrated to 100 mL with a rotatory evaporator in low pressure and filtered through a 0.2-mm filter to be sterilized. The resultant solution was stored at 4°C to 8°C. Various concentrations of saffron (50 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL, 800 µg/mL, 1000 µg/mL, 2000 µg/mL, and 4000 µg/mL) and a control solution without saffron extract were prepared immediately and refrigerated before the experiments.

### Morphologic Observation of Cell Lines

Human transitional carcinoma cells (TCC 5637) and mouse fibroblast cell line (L929) were provided from the *National Cell Bank of Iran*. The TCC cell line 5637 is an epithelial-like adherent cell line originally taken from the primary bladder carcinoma and L929 cell morphology is similar to fibroblast derived from mouse C3H/An connective tissue. Both of the cell lines were retrieved and cell passage was done. The viability of cells was determined by trypan blue test. Six well plates for TCC 5637 cells and similar plates for L929 cells were used. In each well,  $5 \times 10^5$  neoplastic cells or  $2 \times 10^5$  normal cells were placed. The cells were cultivated in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St Louis, Missouri, USA) with 10% fetal calf serum (Gibco, Paisley, UK). The media were supplemented with 100 IU/mL of penicillin and 100 IU/mL of streptomycin (Jaberebn-e-Hayan, Tehran, Iran). The cells were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 24 hours. Then, exposing the cells to saffron extract was started: first, the media (2 mL capacity) were replaced with similar new media. Then the plates were incubated with different concentrations of saffron extract (zero to 4000 µg/mL) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 24, 48, 72, and 120 hours, and the cells were observed under the light inverted microscope for morphological alterations. The observation was done 3 times for each of the extract concentrations to check its reliability. Viability of cells throughout the experiment was always higher than 95% as determined by trypan blue.

## Quantitative Assessment

In vitro cytotoxicity of saffron aqueous extract was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. This method was first described by Mosmann in 1983 and modified by Alley and colleagues.<sup>(8,9)</sup> A total of 2000 normal cells and 5000 neoplastic cells from both cell lines were cultured after cellular passage and viability test in five 96-well plates. Every 3 well groups were marked for each concentration of extract and recorded in files as case and similarly as control groups. The plates were incubated for 24 hours, and thereafter, the cells were exposed to the extract as follows: the first culture medium of each plate was changed by fresh culture medium (200  $\mu$ L). Different concentrations of extract were prepared and added to 3 well groups, which contained TCC 5637 or L929 cell lines. No extract was added to the control group. Plates were incubated in a humidified 5% CO<sub>2</sub> atmosphere, and after 24 hours, 1 plate was chosen randomly and growth medium was removed. For each 200  $\mu$ L of the growth medium, 25  $\mu$ L of MTT solution was added (Sigma, Missouri, USA) and incubated for 4 hours. After removing the growth medium and shaking microplates for 2 to 3 minutes, dissolution of crystals in 200  $\mu$ L of dimethyl sulfoxide and 25  $\mu$ L of glycine buffer was achieved. The absorbance of formazan dye was recorded at 570 nm using enzyme-linked immunosorbent assay plate reader. The last stages were reported in the same manner for the second, third, and fourth plates after 48, 72, and 120 hours.

The optical density read from the extract treated wells was converted to a percentage of living cells against the control using the following formula:

$$\text{Surviving cells (\%)} = \frac{\text{optical density of treated cells in each well} \times 100}{\text{mean optical density of control cells}}$$

## Statistical Analyses

Data were analyzed by 1-way analysis of variance (ANOVA), followed by the Tukey multiple range tests for significant differences. The SPSS software (Statistical Package for the Social Sciences, version

11.5, SPSS Inc, Chicago, Ill, USA) was used for analyses. *P* values less than .05 were considered significant.

## RESULTS

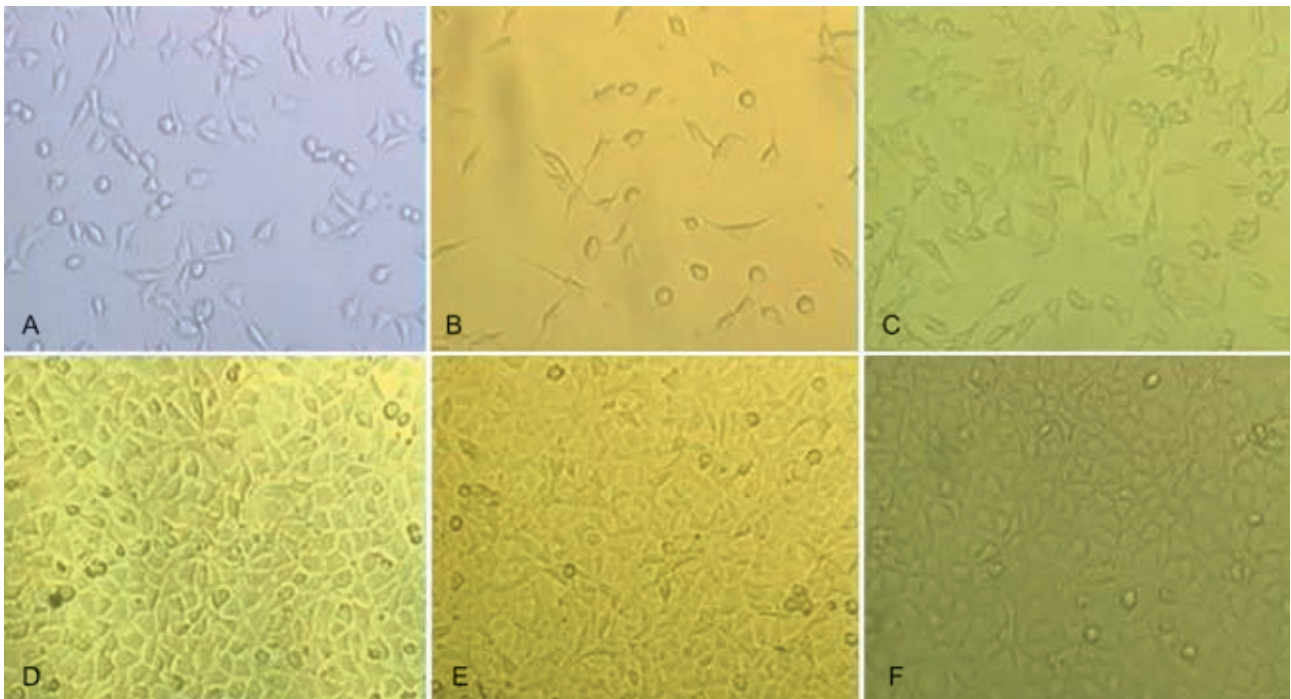
### Morphological Alterations

**L929 Cell Line.** After 24 hours, saffron extract did not affect significantly the normal cells and they were intact in morphologic view. There were no changes in number, cytoplasm, and nucleus of the cells. However, in higher concentrations (> 200  $\mu$ g/mL) decreased intercellular connections as the only notable alteration was apparent. After 48 hours, cell population increased at concentrations of 50  $\mu$ g/mL, 100  $\mu$ g/mL, and 200  $\mu$ g/mL. At higher concentrations (400  $\mu$ g/mL and 800  $\mu$ g/mL), cytotoxic effects were prominent. Nearly, all cells were granulated, cell proliferation stopped, and cellular detachment was significant. After 72 to 120 hours, the process was followed similarly to the second day (Figure 2).

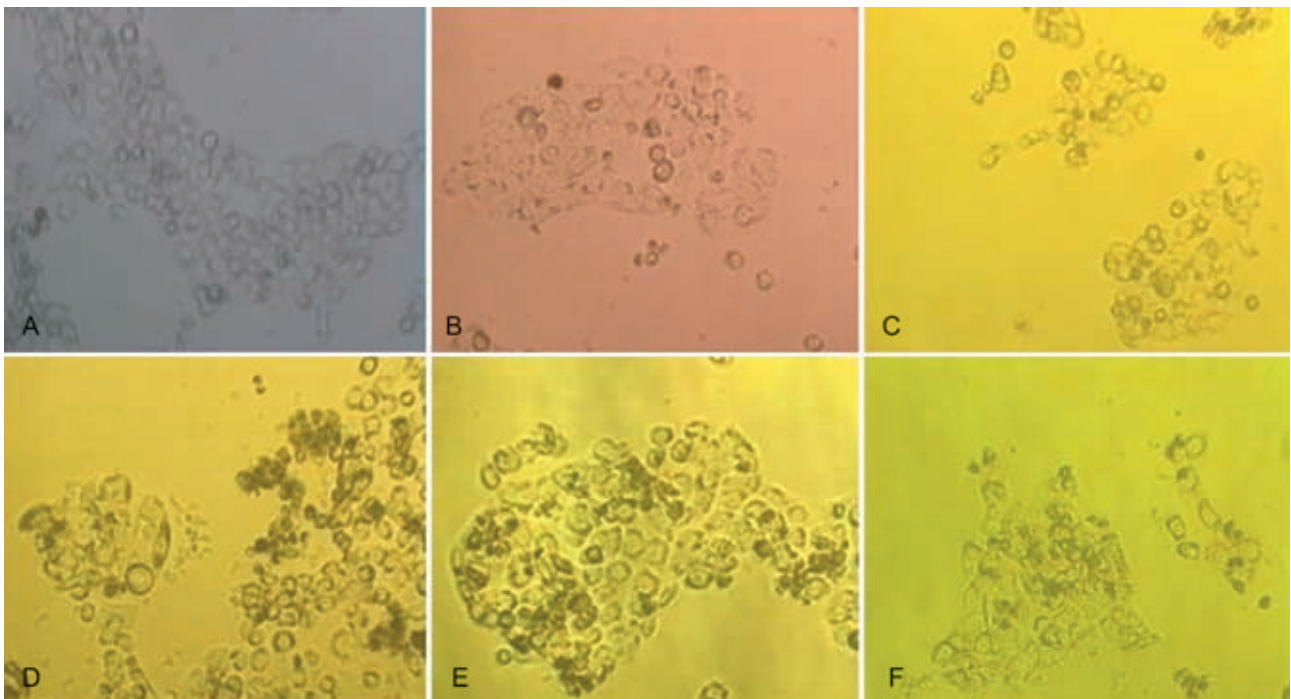
**TCC 5637 Cell Line.** After 24 hours, at the saffron extract concentrations of 50  $\mu$ g/mL, 100  $\mu$ g/mL, and 200  $\mu$ g/mL, cell population decreased compared with the control group, and intercellular connections were also disrupted. At the concentrations of 400  $\mu$ g/mL and 800  $\mu$ g/mL, pigmentation increased and cellular detachment and vacuolization was apparent. At the concentration of 2000  $\mu$ g/mL, 85% to 90% of cells were destroyed. After 48 hours, decrease in cell population and intercellular disruption, vacuolation, and pigmentation were apparent at concentrations of 50  $\mu$ g/mL, 100  $\mu$ g/mL, and 200  $\mu$ g/mL. At the concentration of 800  $\mu$ g/mL, most of the cells were destroyed. After 72 and 120 hours, all these alterations were significant (Figure 3).

### Quantitative Results

Quantitative assessment was done by MTT assay. The results of the absorbance according to the extract concentration and cell population after 120 hours are shown in Figure 4. There was a significant correlation between the increasing extract concentration and decreasing of cell population. No survived cell was detected after



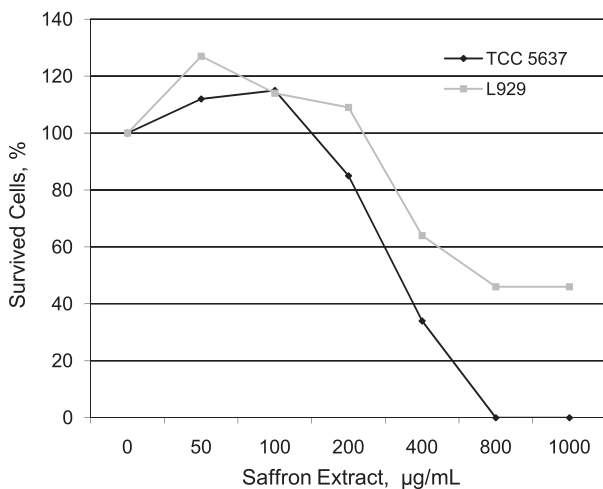
**Figure 2.** Effect of aqueous extract of saffron on L929 morphology at a concentration of 400 µg/mL. **A** is the control L929 and **B** to **F**, are the L929 cells after 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours of exposure to saffron extract, respectively.



**Figure 3.** Effect of aqueous extract on TCC 5637 morphology at a concentration of 400 µg/mL. **A** is the control TCC 5637 and **B** to **F**, are the TCC 5637 cells after 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours of exposure to saffron extract, respectively.

120 hours at saffron extract concentrations of 2000 µg/mL and 400 µg/mL in the L929 and the TCC 5637 cell lines, respectively. In similar extract concentrations higher than 50 µg/mL, the

percentage of survived TCC cells was less than survived L929 cells. Also, gradient of both curves in Figure 4 are the same after concentrations of 50 µg/mL up to 1000 µg/mL and 2000 µg/mL.



**Figure 4.** Effect of aqueous extract of saffron on the percentage of survived TCC 5637 and L929 cells after 120 hours.

In the beginning of the study, no significant difference was seen in cell population between the two cell lines. Therefore, both cell lines were enrolled to our study in similar conditions. Data analysis showed a good correlation between extract concentrations and cell viability in both cell lines during the study. Of course, this was more significant in the TCC cell line on the first day of the study ( $r = 0.9$ ,  $P = .001$ ). After 24 hours, cell population decreased in correlation with increasing extract concentrations, but the decrease was more significant in the TCC cells compared to the L929 cells. In both cell lines, correlation between increasing concentration and percentage of survived cells was significant ( $P = .001$ ,  $R^2 = 0.88$ , and  $\beta = -0.00003$  for TCC;  $P = .004$ ,  $R^2 = 0.771$ , and  $\beta = -0.00006$  for L929). After 120 hours, decrease in the percentage of the survived cells due to increasing concentration of saffron extract was seen in both cell lines, and at the concentrations of 800 µg/mL and 1000 µg/mL, the relation was linear. There was a significant correlation between increasing concentration and the percentage of the survived cells in both cell lines ( $P < .001$ ,  $R^2 = 0.453$ , and  $\beta = -0.0001$  for TCC;  $P = .001$ ,  $R^2 = 0.398$ , and  $\beta = -0.0002$  for L929).

## DISCUSSION

Treatment of bladder cancer is based on the stage of cancer. In lower stages, treatment includes

resection and also intravesical therapies, especially with bacillus Calmette-Guerin. Other antitumor agents can be helpful, but extensive research is required to confirm their clinical applicability. We demonstrated in vitro cytotoxic effect of saffron on human TCC cell lines. In a review of the literature, we found no report of saffron extract effects on TCC cell lines.

Saffron is a plant which grows mostly in Spain and Iran, and in a smaller scale in Greece, Turkey, India, and some other countries.<sup>(10)</sup> Saffron is usually used as a food spice, but some other effects such as anticarcinogenic effect, decreasing blood pressure, and controlling tonic-clonic and absence seizures have been reported so far.<sup>(5)</sup> In addition, saffron is used in cosmetic products.<sup>(11)</sup> There are several reports on the anticarcinogenic effects of saffron.<sup>(6,7)</sup> In a research on ethanolic extract of saffron on Hela cells (cervix epitheloid carcinoma cells), Abdullaev and Frenkel documented the a significant inhibitory effect of colony formation and intracellular DNA and RNA synthesis.<sup>(12)</sup> They performed another study on A549 cells (lung adenocarcinoma cells), WI-38 (normal lung fibroblast-like cells), and VA-13 (WI-38 cells which were transformed by SV-40 viruses), and showed that malignant cells were more sensitive to the inhibitory effect of saffron on DNA and RNA synthesis in comparison with normal cells.<sup>(13)</sup>

The involved chemical ingredients in the antitumor effect of saffron has been investigated by some researchers. In one study, it was shown that crocin isolated from saffron inhibits PC-12 (rat's pheochromocytoma cell line) cell growth with increased synthesis of glutathione.<sup>(14)</sup> It was also shown that saffron inhibited the carcinogenesis caused by chemical substances in mouse's skin, which was probably due to modulator of phase 2 of detoxification enzymes such as glutathione peroxidase, catalase, and superoxide desmutase.<sup>(15)</sup> In one research on chemoprevention,<sup>(16)</sup> saffron ingredients were separated by high performance liquid chromatography and photodiode array methods; 12 chemical ingredients were tested by colony formation assay: crocin-1, crocin-2, crocin-3,

trans-crocin-2, trans-crocin-3, trans-crocin-4, cis-crocin-3, 4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde-diglycosyl-kaempferol, picrocrocin, acid form of picrocrocin, safranal, and crocetin. In vitro inhibitory effect of some extracted ingredients on different types of human malignant cells was observed. In addition, no toxic or mutagenic effect was seen, and the authors concluded that saffron could be used as a chemopreventor in clinical studies.<sup>(16)</sup>

Antitumor mechanism of saffron is not well understood to the present time; however, different hypotheses have been proposed for its mechanism, eg, inhibitory effects of free radical chain reactions. Saffron includes carotenoid ingredients which are fat solvable and can act as free radical inhibitors.<sup>(17)</sup> Inhibition of intracellular DNA and RNA synthesis without any effect on protein synthesis has been reported as a role for carotenoid ingredients of saffron.<sup>(18)</sup> Researchers have suggested transformation of carotenoid to retinoid, interaction of carotenoid with topoisomerase II (the enzyme which interferes in DNA-protein interactions),<sup>(19)</sup> and absorption of extracellular fluid due to swelling and local membrane invagination.<sup>(20)</sup> Saffron also contains lectins which may cause antitumor effects of saffron.<sup>(21)</sup> In addition, apoptosis is induced by crocin.<sup>(22)</sup> In a research on PC-12 cells, crocin caused inhibition of cell growth by its effects on tumor necrosis factor- $\alpha$ .<sup>(14)</sup> However, our knowledge on the exact mechanisms of antitumor effect of saffron needs to be expanded to weigh up its clinical usage.

## CONCLUSION

Our study showed that saffron aqueous extract has an in vitro inhibitory effect on the proliferation of human TCC and mouse L929 cells which is dose dependent.

## ACKNOWLEDGMENT

We would like to thank the research Deputy of Mashhad University of Medical Sciences for financial support and Mrs Yaghoti, the secretary of department of urology, for her kind cooperation.

## CONFLICT OF INTEREST

None declared.

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