Iranian Journal of Pharmaceutical Sciences
Autumn 2008: 4(4): 261-268
www.ijps.ir

Original Article

Study of Cytotoxic Effects of Saffron in MCF-7 Cells

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Abstract

Current therapies for breast cancer are often limited by short-term efficacy due to the emergence of drug resistance. There has been increased interest in the use of naturally occurring compounds with chemopreventive and chemotherapeutic effects in the treatment of cancers. Saffron, dry stigmas *Crocus sativus* L., used in Iran as a spice, is known for its anticancer properties. In this study, the cytotoxic and apoptogenic effects of saffron in MCF-7 cells as an *in vitro* model for breast cancer study were investigated. Meanwhile role of caspases were studied in its toxicity. MCF-7 and L929 cell lines were cultured in DMEM medium and incubated with different concentrations of saffron extract (100 to 2000 µg/ml). Cell viability was assessed by MTT assay. Apoptotic cells were determined using PI staining of DNA fragmentation by flow cytometry (sub-G1 peak). Role of caspase were studied using the pan-caspase inhibitor, z-VAD-fmk. Saffron extract decreased cell viability in MCF-7 cells as a concentration- and time-dependent manner. Doses of saffron inhibited 50% cell growth (IC_{50}) against MCF-7 was 400 µg/ml after 48 h of incubation. Saffron could induce apoptosis in MCF-7 cells in which apoptosis was dependent on caspase activation. It might be concluded that saffron could cause MCF-7 cell death, in which apoptosis or programmed cell death play an important role. Saffron could be also considered as a promising chemotherapeutic agent in breast cancer treatment.

Keywords: Apoptosis; *Crocus sativus*; Cytotoxicity; Saffron.
Received: January 16, 2007; *Accepted:* April 11, 2008.

1. Introduction

Breast cancer continues to be a major health problem for women worldwide. Globally, breast cancer is the most common type of cancer and the leading cause of cancer-related mortality in women. Approximately one-third of women with breast cancer develop metastases and ultimately die from the disease [1-3].

Current systemic therapies for breast cancer are often limited by their nonspecific mechanism of action, unwanted toxicities on

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normal tissues, and short-term efficacy due to the emergence of drug resistance [4].

There has been increased interest in the use of naturally occurring compounds with chemopreventive and chemotherapeutic effects in the treatment of cancers. The most recent advances that provide new insights into the molecular mechanisms suggest the apoptotic potential of nutritional supplements and herbs [5].

Saffron, the dry stigmas of the plant *Crocus sativus* L., belongs to the Iridaceae family and principally grows in Iran and Spain [6, 7]. Although it is currently used as a spice and food colorant, however, traditional medicine has used saffron in the treatment of numerous illnesses including cough, colic, insomnia, chronic uterine hemorrhage, cardiovascular disorders and tumors [8, 9]. In the last few years, the antitumor properties of crude saffron extracts, both *in vitro* and *in vivo*, have been demonstrated. It has been a dose-dependent inhibitory effect on carcinoma, sarcoma, leukemia, and several other malignant cells *in vitro*. Saffron increased life span of treated tumor-bearing mice compared to untreated animals by 45-120%. Different hypotheses for anticarcinogenic and antitumor effects of saffron and its ingredients have been proposed, including inhibition of nucleic acid and free radical chain reactions and interaction of carotenoids with

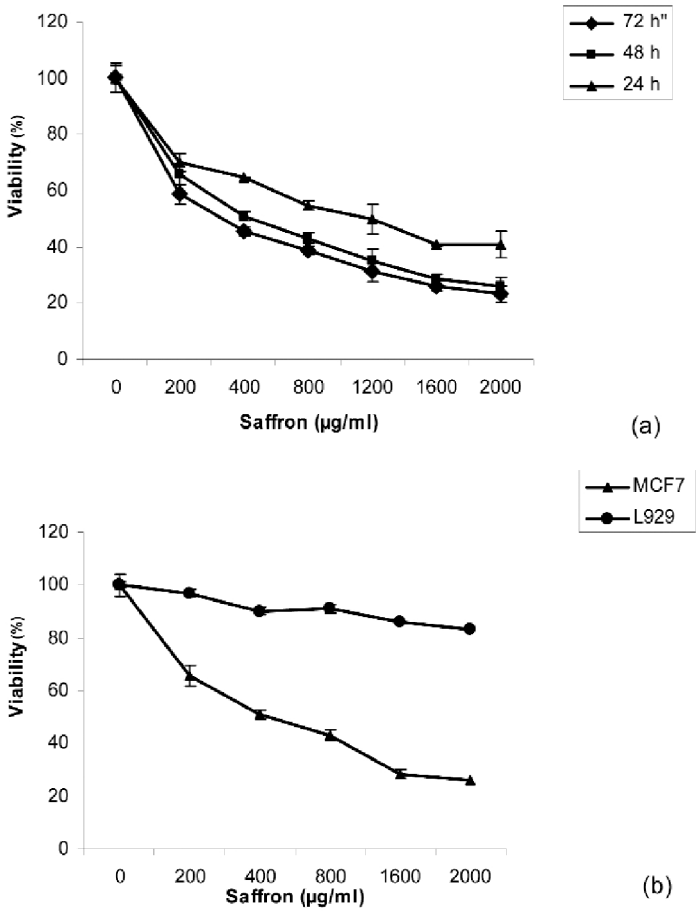

Figure 1. Effect of saffron extract on cell viability of MCF-7 (a) and L929 (b) cells, (Mean±SEM; n=3).

topoisomerase II. It was also reported that saffron was nontoxic and had no effect on growth of normal cells [7, 9].

Despite these studies, role of apoptosis in saffron-induced toxicity has not been fully understood. Therefore, in this study, the cytotoxic and apoptogenic effects of hydro-alcoholic extract of saffron in MCF-7 cells as a prominent model system for the study of breast cancer [10] was studies.

2. Material and methods

2.1. Reagents

The general caspase inhibitor, Z-Val-Ala-Asp (Ome)-CH2F (z-VAD-fmk), was purchased from R&D system. The fluorescent probe 2',7'-dichlorofluorescein diacetate (DCF-DA), propidium iodide (PI), sodium citrate, 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), and triton X-100 were purchased from Sigma. DMEM and FCS were purchased from Gibco.

2.2. Saffron extracts preparation

Saffron was supplied by Novin Zaferan Co (Mashhad, Iran) and was identified by the Pharmacological Research Center of Medicinal Plants. The stigma's extract was prepared as follows: Dried and milled stigmas (20 g) were extracted with 300 ml ethanol (80%) by suxhelat apparatus. The solvent was then removed by evaporation in temperature between 35 and 40 °C.

2.3. Cell culture

MCF-7 and L929 cells were obtained from Pasteur institute (Tehran, Iran). Cells were maintained at 37 °C in a humidified atmosphere (90%) containing 5% CO_2. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 5% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. L929cells were cultured in DMEM containing 10% FCS. Cells were seeded overnight, and then incubated with

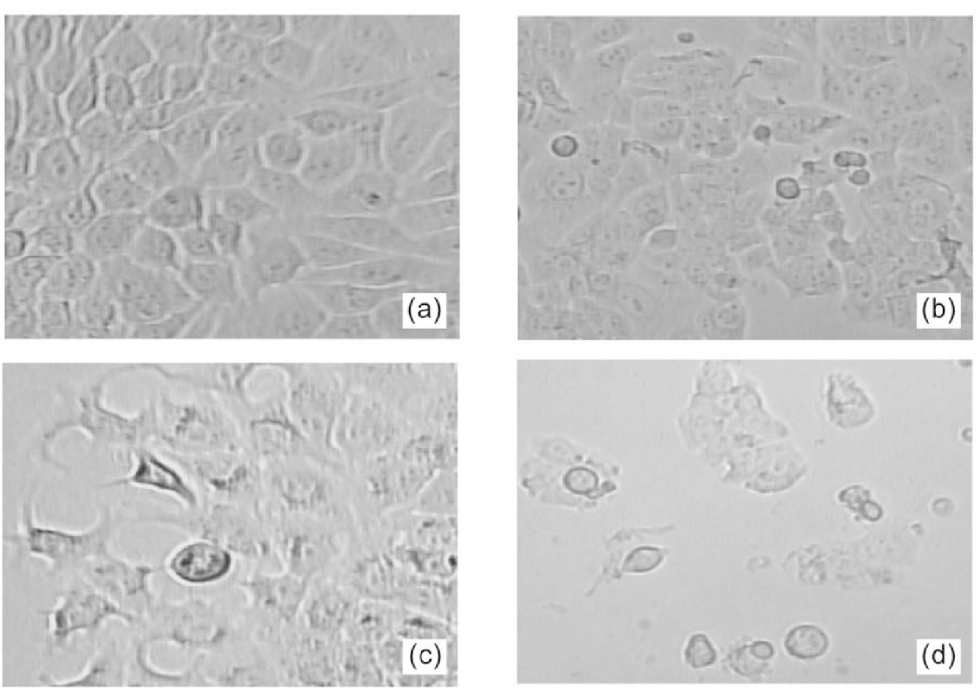

Figure 2. Saffron induces morphologic changes and affects the viability of cultured MCF-7 cells. a: control, b: saffron 400 µg/ml, c: saffron 800 µg/ml, d: saffron 1600 µg/ml. Cells were incubated with saffron for 48 h.

various concentrations of saffron extract (100 to 2000 µg/ml) for 24 h, 48, and 72 h. For each concentration and time course study, there was a control sample which remained untreated and received the equal volume of media. All different treatment carried out in duplicate.

2.4. Cell viability

The cell viability was determined using a modified 3-(4, 5 -dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT) assay [11, 12]. Briefly, cells were seeded at 5000/well onto flat-bottomed 96-well culture plates and allowed to grow 24 h followed by treatment with saffron extract (100 to 2000 µg/ml). After removing the medium, cells were then labelled with MTT solution (5 mg/ml in PBS) for 4 h and resulting formazan was solubilized with DMSO (100 µl). The absorption was measured at 570 nm (620 nm as a reference) in an ELISA reader.

2.5.Apoptosis

Apoptotic cells were determined by staining using the PI method [13]. In brief, MCF-7 cells were cultured overnight in a 24-well plate and treated with saffron for 48 h. Floating and adherent cells were then harvested and incubated overnight at 4 °C in the dark with 750 µl of a hypotonic buffer (50 µg/ml PI in 0.1% sodium citrate plus 0.1% triton X-100) before flow cytometric analysis using a FACScan flow cytometer (Becton Dickinson).

2.6. Inhibition of caspase activity

In brief, MCF-7 cells were cultured overnight in a 24-well plate and caspases were inhibited using the z-VAD-fmk, which were added to the medium at a concentration of 50 and100 µM, 1 h before the addition of saffron extract (1600 µg/ml). After 48 h, cells were harvested and processed for the detection of apoptotic cells. The apoptotic cells were determined by staining using the PI method as above.

2.7. Statistical analysis

All results were expressed as mean±SEM. The significance of difference was evaluated with ANOVA and Bonfrroni's test. A probability level of $p<0.05$ was considered statistically significant.

3. Results

3.1. Effect of saffron on cell viability

MCF-7 and L929 (as non-malignant control cells) were incubated with various concentrations of saffron extract (100-2000 µg/ml) for 24, 48 and 72 h. The impact of saffron extracts on cell viability was assessed by MTT assays. As shown in Figure 1, saffron extract decreased cell viability in MCF-7 but not in L929 cells, as a concentration- and

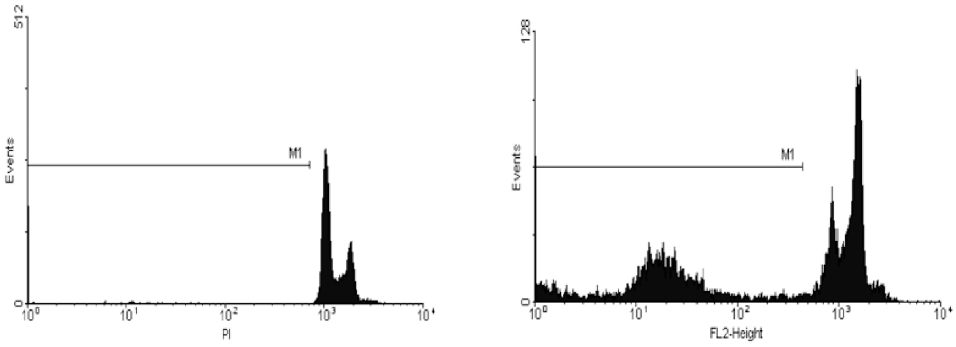

Figure 3. Saffron induces apoptosis in MCF-7 cells. Flow cytometry histograms of apoptosis assays by the PI method. The proportion of apoptotic cells was measured with Sub-G1 peak. MCF-7 cells were treated with 1600 µg/ml of saffron for 48 h.

time-dependent manner. This toxicity was consistent with morphologic changes including reduction in cell volume and rounding. No morphological changes were shown in L929 cells. (Figure 2). Doses inducing 50% cell growth inhibition (IC_{50}) against MCF-7 was 400 µg/ml after 48 h incubation with saffron.

3.2. Role of apoptosis

The proportion of apoptotic cells was measured with PI staining of DNA fragmentation by flow cytometry. Sub-G1 is one of the reliable biochemical markers of apoptosis.

There was sub-G1 peak in flow cytometry histogram of saffron-treated but not in control cells indicating apoptotic cell death is involved in saffron -induced toxicity in MCF-7 cells (Figure 3).

To determine the role of caspase activation in Saffron-induced apoptosis, MCF-7 cells were treated with the pan-caspase inhibitor, z-VAD-fmk (50 and 100 µM), 1 h before adding saffron extract (1600 µg/ml) for 48.

As shown in Figure 4, z-VAD inhibited saffron-induced apoptosis in MCF-7, in a concentration-dependent manner. At concentration of 100 µM, z-VAD significantly inhibited apoptosis of MCf-7 induced by saffron.

4. Discussion

Natural products have long been used to prevent and treat many diseases, including cancer and thus they are good candidates for the development of anticancer drugs [14]. Saffron, used extensively as a spice in Iran, is known for its anti-cancer and anti-tumor properties.

In this study, the cytotoxic and apoptogenic effects of saffron in MCF-7 cells, which to our knowledge is the first report on saffron-induced apoptosis in this cell line, was investigated. *In vitro* cell proliferation inhibition test using MTT viability assay confirmed that hydro-alcoholic extract of saffron has cytotoxic activity against MCF-7 cell line but not in non-malignant cells tested. The present results obtained were consistent with previous studies indicating that saffron and its ingredients possesses antitumor and anticancerogenic activities and has no cytotoxic effect on non-malignant cells [8, 15].

In the present study, saffron-induced apoptosis was involved in induction of cell death. Apoptosis is a gene regulated phenomenon which is induced by many chemotherapeutic agents in cancer treatment [16, 17]. It is characterized by distinct morphological features including; chromatin condensation, cell and nuclear shrinkage, membrane blebbing and oligonucleosomal DNA fragmentation [18]. The induction of

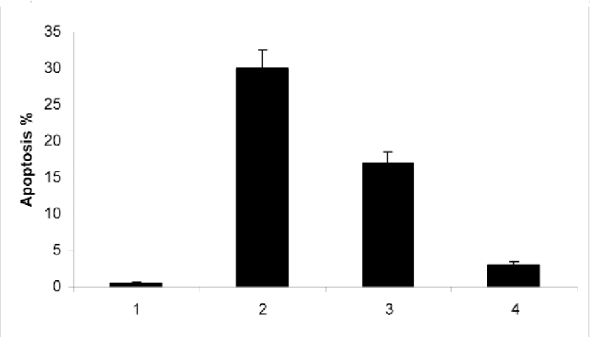

Figure 4. Effects of inhibition of caspases on saffron-induced apoptosis in MCf-7 cells after 48 h. 1: z-VAD 100 µM, 2: Saffron 1600 µg/ml, 3: Saffron+z-VAD 50 µM, 4: Saffron+z-VAD 100 µM. Results are Mean±SEM; n = 3.

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 [19]. It is thus considered important to screen apoptotic inducers from plants, either in the form of crude extracts or as components isolated from them [5].

Activation of caspases appears to be directly responsible for many of the molecular and structural changes in apoptosis. These include degradation of DNA repair enzyme poly (ADP)ribosepolymerase (PARP) and DNA dependent protein kinase (DNA-PK), and cleavage of chromatin at inter-nucleosomal sites mediated by caspase-activated DNase (CAD) [20]. In this study, inhibition of caspases, could block saffron-induced apoptosis in MCF-7 cells (Figure 4). It is indicating caspase-dependent pathways were induced by saffron in MCF-7 cells and some factors other than caspases such as apoptosis inducing factor (AIF) might not to be markedly involved.

As shown in Figure 1, while saffron at 1600 µg/ml affected the viability of most of MCF-7 cells, apoptosis only partially contributed in its toxicity. It might be concluded that non-apoptotic cell death to be also involved in saffron-induced toxicity in MCF-7 cells.

In addition to inducing apoptosis, a number of chemotherapeutic agents have been reported to induce non-apoptotic forms of cell death. The significance of non-apoptotic forms of cell death in chemotherapy and the mechanism(s) by which they are induced by chemotherapeutic drugs remain, largely unclear. It is however noteworthy that the non-apoptotic cell death is often observed under conditions in which apoptosis is inhibited [21].

Taking together, the present study is the first to show toxicity of saffron in MCF-7 cell line in which apoptosis or programmed cell death play an important role. It could provide further knowledge to mechanisms involved in this toxicity. Saffron could be also considered as a promising chemotherapeutic agent in breast cancer treatment.

Acknowledgements

The authors would like to thank Research Affairs of Mashhad University of Medical Sciences for financial support of this work and Mrs Tabasi for her assistance in flow cytometry.

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