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Suppression of Pulmonary Tumor Promotion and Induction of Apoptosis by *Crocus sativus* L. Extraction

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Abstract *Crocus sativus* L., commonly known as saffron, is the raw material for one of the most expensive spice in the world, and it has been used in folk medicine for centuries. We investigated the potential of the ethanolic extract of saffron to induce cytotoxic and apoptosis effects in carcinomic human alveolar basal epithelial cells (A549), a commonly used cell culture system for in vitro studies on lung cancer. The cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum treated with different concentrations of the ethanolic extract of saffron for two consecutive days. Cell viability was quantitated by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. Apoptotic cells were determined using annexin V–fluorescein isothiocyanate by flow cytometry. Saffron could decrease the cell viability in the malignant cells as a concentration- and time-dependent manner. The IC_{50} values against the A549 cell lines were determined as 1,200 and 650 μg/ml after 24 and 48 h, respectively. Saffron-induced apoptosis of the A549 cells in a concentration-dependent manner, as determined by flow cytometry histogram of treated cells that induced apoptotic cell death, is involved in the toxicity of saffron. It might be concluded that saffron could cause cell death in the A549 cells, in which apoptosis plays an important role. Saffron could also be considered as a promising chemotherapeutic agent in lung cancer treatment in future.

Keywords A549 · Annexin V · Apoptosis · *Crocus sativus* L. (Iridaceae) · MTT

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Introduction

Lung cancer is the most common cancer with a high rate of mortality and morbidity. Lung cancer is the leading cause of cancer deaths in the world [1]. Approximately 40% of lung cancers are adenocarcinomas that belong to the subgroup of the nonsmall cell lung cancers, the most common type of lung cancer in the USA and Asia [2]. A carcinomic human alveolar basal epithelial cell (A549) is the most common and widely studied cell lines in lung cancer [3]. The A549 tumor cell line, initiated from a human alveolar cell carcinoma, has morphologic and biochemical features of the pulmonary alveolar type II cells. A549 cells were chosen because they display many differentiated features of lung alveolar cells [4].

Current treatment options include surgical resection, platinum-based doublet chemotherapy, and radiation therapy alone or in combination. Unfortunately, despite these therapies, the disease is rarely curable and prognosis is poor, and these therapies can frequently cause undesirable side effects [5]. Therefore, new drugs without significant side effects are absolutely required. For this aim, using medicinal plants with good efficacy and few side effects is a good strategy in comparison with allopathic medicine [6].

Chemotherapy is the administration of drugs that can regulate the uncontrolled proliferation of abnormal cancer cells. The majority of chemotherapeutic drugs can be divided into alkylating agents, antimetabolites, and anthracycline [7]. Currently, chemoprevention strategies are very attractive and have earned serious consideration as a potential means of controlling the incidence of cancer. Chemopreventive agents such as retinol, beta-carotene, synthetic retinoids, and alpha-tocopherol are known to have antitumor potential against lung cancer [8]. Efforts to find any therapeutic options for cancers have guided the investigators to consider even herbal medicine to be tested. There is accumulating evidence that the overall dietary intake of phytochemicals reduces the risk of cancer [9].

Saffron, the dry stigmas of the plant *Crocus sativus* L., belongs to the Iridaceae family and principally grows in Iran and Spain [10, 11]. Since ancient times, saffron which is harvested from the dried, dark red stigmas of *C. sativus* L. flowers, has not only been used as a spice for flavoring and coloring food and as a perfume but also for treating several diseases. Recent data show that the saffron extract and its components possess anticarcinogenic (inhibition of chemical carcinogenesis) and antitumor (inhibition of tumor growth) in vivo and in vitro activities [12–15].

Several studies have shown cells to be sensitive to saffron and its components. The differences in sensitivity to the effect of saffron and its main ingredients in normal and the malignant cells [16–18] could be due to the existence of distinct cell surface receptors, intracellular retention transport, and differences in the uptake of certain drugs or in the methods used for the extraction and assessment of toxicity. It was also demonstrated that the saffron extract inhibited cellular nucleic acid synthesis but had no effect on protein synthesis in tumor cells [19, 20]. Interestingly, there was a stimulatory or supporting effect of the saffron extract on the nonspecific proliferation of lymphocytes in vitro and on colony formation of the normal human lung cells [21].

Characteristic compounds of saffron include crocin, safranal, picrocrocin, crocetin, and β-carotene [22]. It was shown that these saffron ingredients inhibit different types of tumor cell growth, although crocetin having no effect on colony formation of tumor cells, but it had a dose-dependent inhibitory effect on DNA, RNA, and protein synthesis of different human malignant cells [23]. It was recently shown that a novel glycoconjugate, isolated from saffron corms and calluses, possesses cytotoxic activity against tumor cells [24]. Another study reported that the saffron extract itself was also nonmutagenic [25]. Although

there are some studies about anticancer effect of saffron, the mechanism of saffron-induced toxicity is still largely unknown yet.

Meanwhile, apoptotic inducers were extracted from plants, but role of apoptosis has not been studied in saffron-induced toxicity. Apoptosis is a gene-regulated phenomenon induced by many chemotherapeutic agents in cancer treatments [26, 27]. The induction of apoptosis in tumor cells is considered very useful in the management and therapy as well as in the prevention of cancer. Treatment of lung cancer was the subject of our interest that led us to study alternative therapies such as the use of herbs. The present study provides an updated overview of experimental in vitro investigation on the biological activities of saffron (*C. sativus* L.), especially focusing on cytotoxicity toward the A549 lung cancer cell line.

Material and Methods

Chemicals and Reagents

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Amerso (USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL (Grand Island, NY, USA). Annexin V/fluorescein isothiocyanate (FITC) was obtained from Invitrogen Corporation (Camarillo, CA, USA). Fetal bovine serum was purchased from PAA Laboratories GmbH, Austria.

Preparation of the Saffron Extracts

Saffron was supplied by SaharKhiz Co. (Mashhad, Iran) and was processed in the Pharmacological Research Center of Medicinal Plants. The part of *C. sativus* that is being used as additive and also as herbal medicine is the stigma. The stigma's part of saffron was air-dried in the shade before extraction. After grinding, 1 g weight of the dried stigma was extracted with 10 ml ethanol (96%), for 2 h in an ultrasonic bath. The extract was filtered and concentrated in a vacuum evaporator. The extract then was concentrated to dryness. Then the resulting powder was kept at 2–6 °C (the yield of extraction was around 30%). The powder dissolved in phosphate-buffered saline (PBS) prior to applying to the cell cultures.

Cell Culture

The human nonsmall lung cancer cells, A549, were obtained from Pasteur Institute (Tehran, Iran). The cells were grown either in 96-well tissue (TC) plate (NUNC, Wiesbaden, Germany) or in 25-cm^2 TC flasks (NUNC, Wiesbaden, Germany), cultured in DMEM medium supplemented with 10% fetal bovine serum (Gibco–Invitrogen), 100 U/ml of penicillin (Gibco–Invitrogen), and 100 μg/ml streptomycin (Gibco–Invitrogen). A549 cells were cultured in CO2 incubator MCO-17AI (Sanyo Electric Co., Ltd, Japan) at 37 °C in 95% humidified atmosphere enriched by 5% CO_2 and subcultured every 3 to 4 days.

Cell Viability Assay

Cell viability was measured using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases [28]. Briefly, A549 cells were plated at a density of (1×10^3 cells/ml) in 96-well plates and allowed to attach for 24 h to

keep the log-phase growth at the time of drug treatment. Saffron (100 μl) at different concentrations (400, 800, 1,200, and 1,600 μg/ml) was added to the wells for 24 and 48 h. After treatment with saffron for 24 and 48 h, 10 μl MTT was added into each well. After 4 h incubation at 37 °C, this solution was removed, and the produced formazan was solubilized in 100 μl dimethyl sulfoxide. Absorbance was measured at 550 nm using an automated microplate reader (Bio-Rad 550). Cell viability was expressed as a percentage of the control culture (PBS) value. The cytotoxic effects of the saffron extract on cell line (A549) were expressed as the IC_{50} value (the drug concentration reducing the absorbance of treated cells by 50% with respect to untreated cells). All experiments were carried out in triplicate.

Morphological Studies of Cell Lines Using the Normal Inverted Microscope

Morphological studies using the normal inverted microscope were carried out to observe the morphological changes of cell death in A549 cell line elicited by the ethanolic extract of saffron. Different concentrations of (400, 800, 1,200, and 1,600 μg/ml) of the saffron extract for 24 and 48 h were used for the morphological studies. The untreated cells served as the negative control. The morphological changes of the cells were observed under the normal inverted microscope after 24 and 48 h posttreatment.

Assessment of Apoptosis by Annexin V–FITC

Apoptotic cell death of saffron was measured using FITC-conjugated annexin V/PI assay kit by flow cytometry [29], briefly 5×10^5 cells were washed with ice-cold PBS, resuspended in 100 μl binding buffer, and stained with 5 μl of FITC-conjugated annexin V (10 mg/ml) and 10 μl of PI (50 mg/ml).The cells were incubated for 15 min at room temperature in the dark, then 400 μl of binding buffer was added, and analyzed by a FACScan flow cytometry (Becton–Dickinson, USA). For analysis, the A549 cells were gated separately according to their granularity and size on forward scatter versus side scatter plot. Early apoptosis and late apoptosis were evaluated on fluorescence 2 (for propidium iodide) versus fluorescence 1 (for annexin) plots. The percentage of cells stained with annexin V only was evaluated as early apoptosis; the percentage of cells stained with both annexin V and propidium iodide was evaluated as late apoptosis or necrotic stage.

Statistical Analysis

All results were expressed as mean ± SEM. The significance of difference was evaluated with ANOVA and Bonferroni's test [30]. A probability level of $P<0.05$ was considered statistically significant.

Results

Effects of Saffron on Cell Viability

Incubation of the A549 cells with various concentrations of the saffron extract for 24 and 48 h was shown in Fig. 1. The impact of the saffron extract on the cell viability was quantitated by the MTT assay. Figure 1 shows that exposure of A549 cells for 24 and 48 h with the ethanolic saffron extract inhibits significantly proliferation of human lung cancer

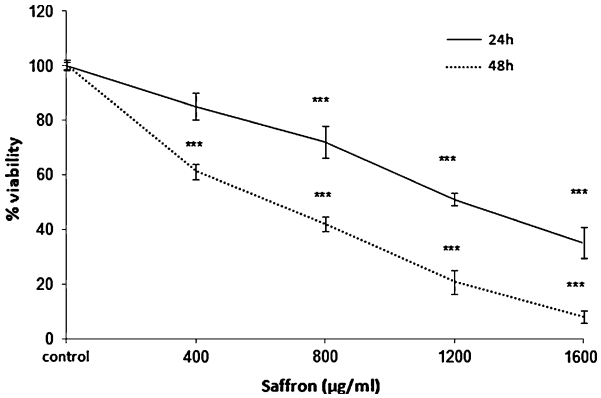

Fig. 1 Exposure of A549 cells for 24 and 48 h with the ethanolic saffron extract

cell line in a concentration- and time-dependent manner ($p<0.001$). Exposure of the A549 cell line for 24 h decreased the number of viable cells at higher doses of saffron (800, 1,200, and 1,600 μg/ml; $p<0.001$), but not at concentration of 400 μg/ml ($P<0.4$). On the other hand, treatment of the A549 cell line for 48 h with different concentrations of saffron (400, 800, 1,200, and 1,600 μg/ml) resulted in marked reduction of number of viable cells ($p<0.001$).

Table 1 presented the IC_{50} value (the dose inducing 50% cell growth inhibition) determined from the graphs of the various concentrations of saffron extracts for 24 and 48 h on the A549 cell line. The extract showed potent cytotoxic effect with the IC_{50} value of 1,200±15.2 and 650±13.8 μg/ml against the malignant cells (A549) after 24 and 48 h, respectively.

Morphological Evaluation

As shown in Fig. 2 although the morphological features were not significantly changed after 24 h of incubation with low dose of saffron (400 μg/ml; data were not shown), however, after 48 h of incubation with concentrations of 400 and 1,200 μg/ml of saffron, morphological changes were observed using the normal inverted microscope in comparison with control. As clearly shown in Fig. 2b, the saffron extract (400 μg/ml) inhibited the growth of A549 cell line after 48 h in comparison with control (Fig. 2a); however, Fig. 2c shows that treated cells with saffron (1,200 μg/ml) had more prominent growth inhibition and shrinkage of the cells compared with treated cell with a lower dose of saffron (400 μg/ml) which consisted of induction in number of living cells, volume, and rounding and confirmed our MTT results.

Quantification Studies for Apoptosis by Saffron

As it can be seen in Fig. 3, the malignant cells were treated with concentrations of 400 and 1,200 μg/ml saffron for 48 h, then the cells were harvested, and apoptosis was examined by

Table 1 IC_{50} value determined from the graphs of the various concentrations of saffron extracts on the A549 cell line

IC_{50} (h)	24	48
A549 (μg/ml)	1,200±15.2	650±13.8

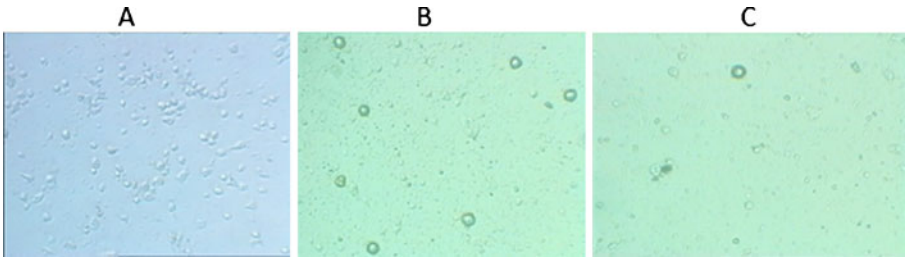

Fig. 2 **a**–**c** Saffron extract

flow cytometry. The cells were treated with 400 and 1200 μg/ml saffron for 48 h(symbol Π,ΠΙ) or media (control symbol I), and apoptosis was examined with flow-cytometry after Annexin V-PI double staining. The necrotic cells lost cell membrane integrity that permits PI entry. Viable cells exhibit Annexin V (-)/PI (-); early apoptotic cells exhibit Annexin (+)/PI (-); late apoptotic cells or necrotic cells exhibit Annexin V (+)/PI (+). To study roles of saffron in apoptosis, the ethanolic extract of saffron was used to setup apoptosis system on

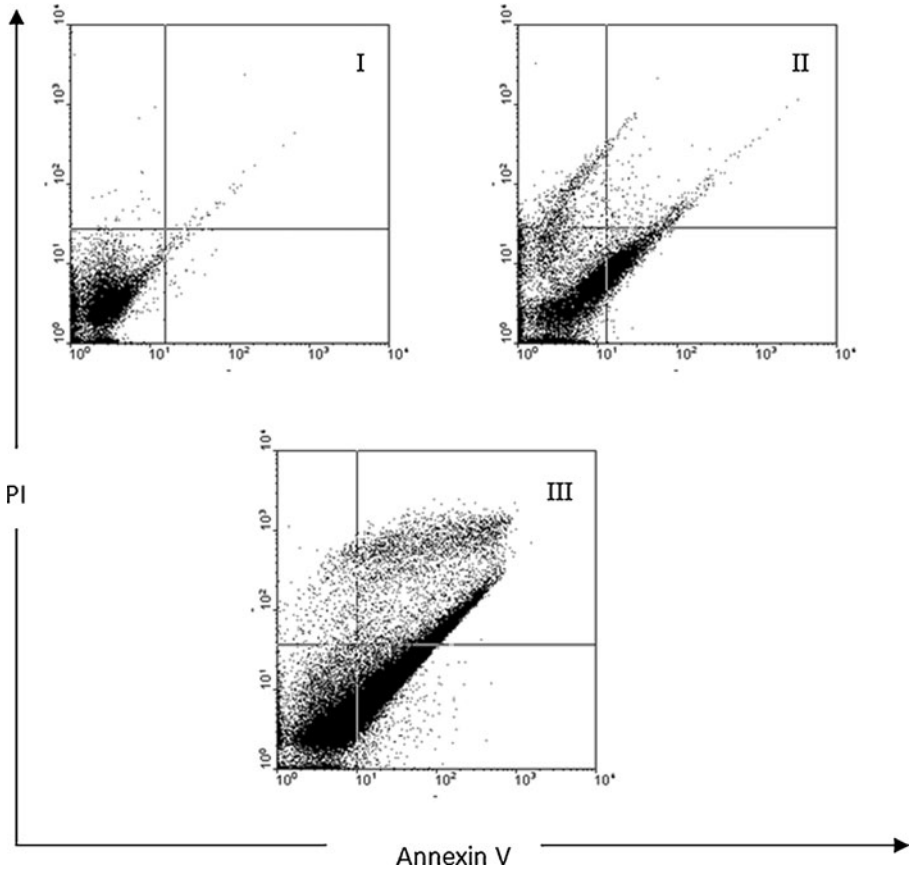

Fig. 3 Malignant cells treated with concentrations of 400 and 1,200 μg/ml saffron for 48 h, the cells harvested, and apoptosis examined by flow cytometry

the A549 cells. Quantitative analysis using annexin V/PI assay further showed that the proportion of early-stage apoptotic cells (annexin V+/PI−) increased significantly from 7.56% to 28.70%, while proportion of late stage apoptotic cell (annexin V+/PI+) increased significantly from 9.31% to 33.54%. Apoptosis induced from 400 and 1,200 μg/ml of saffron was statistically higher than control, and the percentage of the early and late apoptotic cells significantly increased by increasing saffron concentrations ($p<0.001$).

The results related to assessment of apoptosis by annexin V/PI on the carcinomic human alveolar basal epithelial cells are plotted in Fig. 4. Although no significant difference was detected between percentage of early and late apoptotic cells at concentration of 400 μg/ml, however, the number of late apoptotic cells versus to early apoptotic cells at concentration of 1,200 μg/ml saffron treated cells was statically significant ($p<0.001$); thus, percentage of the late apoptotic cells increased significantly versus percentage of the early apoptotic cells.

Discussion

In the present study, we investigated the potential of the ethanolic saffron extract for inhibition of cell growth and induction of apoptosis in the A549 cell line. Our data showed that saffron inhibits significantly proliferation of human alveolar basal epithelial cells in a concentration- and time-dependent manner. Morphological features also confirmed these results. We also showed that apoptosis induced by saffron was significantly higher than control, and the percentage of both the early and late apoptotic cells statically increased by increasing saffron concentration. On the other hand, although no significant difference was detected between percentage of the early and late apoptotic cells at lower concentration, however, the number of the late apoptotic cells versus the early apoptotic cells at higher concentrations of the saffron treated cells was statically significant.

Wide varieties of natural food and products have been recognized to induce apoptosis in various tumor cells. There is strong evidence supporting the positive role of medicinal plants in oncology and that they affect all phases of cancer process [31]. It is thus considered important to screen apoptotic inducers and good candidate for development of

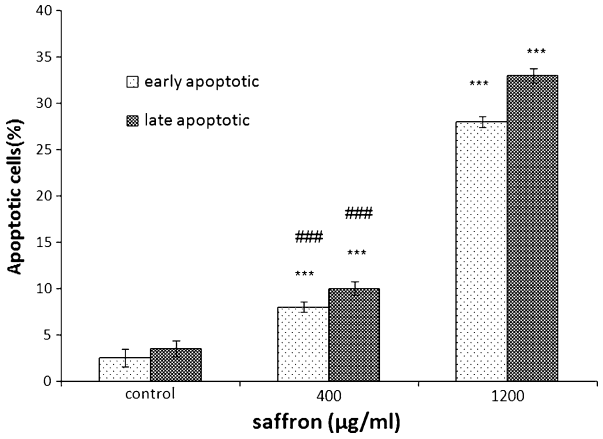

Fig. 4 Results related to assessment of apoptosis by annexin V/PI on the carcinomic human alveolar basal epithelial cells

anticancer drug from plants either in the form of crude extract or as components isolated from them, so that it is significant that over 60% of the currently used anticancer agents are derived from natural sources including plants, marine organisms, and microorganisms [32, 33] and offering an opportunity to study the molecular mechanism of tumor genesis [34].

Characteristic compounds of saffron include crocin, safranal, picrocrocin, crocetin, and β-carotene. It was shown that these saffron ingredients inhibited different types of tumor cell growth, with crocetin having no effect on colony formation of tumor cells, although it had a dose-dependent inhibitory effect on DNA, RNA, and protein synthesis of different human malignant cells [31].

In this study, the cytotoxic and pro-apoptotic effects of the ethanolic saffron extract in A549 cells were investigated. To the authors' knowledge, this is the first report on saffron-induced apoptosis in human alveolar basal epithelial cells. Our data confirmed that saffron extract has cytotoxic activity against carcinomic human alveolar epithelial cells, which is consistent with previous studies indicating that saffron and its ingredients possess antitumor and anticarcinogenic activities [35, 36]. Different studies have shown the antiproliferative activity of the ethanolic extract of saffron on human breast cell line (MCF7) [37]. The ability to induce tumor cell apoptosis is an important property of a candidate anticancer drug, which discriminates between anticancer drugs and toxic compounds [38]. The MTT assay is thought to be produced by the mitochondrial enzyme succinate dehydrogenase and can be dissolved and quantified by measuring the absorbance of the resultant solution. The absorbance of the solution is related to the number of live cells. A multiwell spectrophotometer assay can be semiautomated to process a large number of samples and provide a rapid object measurement of cell number [39]. Our study showed that the ethanolic extract of saffron exerted a significant proliferation inhibitory activity against A549 cells in a dose-dependent manner (Fig. 2). Much effort has been directed toward the effect of saffron on apoptosis and understanding their mechanisms of action. The apoptosis evoked by saffron was confirmed by the annexin V–FITC (Fig. 3). In the present study, saffron-induced apoptosis was involved in cell death. Apoptosis is characterized by distinct morphological features including chromatic condensation, cell and nuclear shrinkage, membrane blabbing, and oligonucleosomal DNA fragmentation [40]. As shown in Figs. 3 and 4, saffron at 400 and 1,200 g/ml induced significant cell toxicity in the A549 cells in dose-dependent manner. Apoptosis only partially contributed in this toxicity, and it might be conducted that nonapoptotic cell death can also be involved in saffron-induced toxicity in these cells. Although the significant of nonapoptotic cell death in chemotherapy remains largely unclear, it is believed that the nonapoptotic cell death is important under conditions in which apoptosis is inhibited [41, 42].

A number of in vivo and in vitro experiments indicate that saffron and its main ingredients have the potential to reduce the risk of developing several types of cancer. The saffron plant has been shown to be a source of bioactive compounds with cytotoxic, antitumoral, chemopreventive, antimutagenic, and immunostimulating properties. Crocins, the major carotenoid components of saffron stigma, demonstrated antitumor properties, promoting tumor growth inhibition and increasing the life span of treated tumor-bearing animals. Crocins are well tolerated and present no or minor side effects. These, together with their water solubility, make them suitable for chemotherapeutic use. Crocins and crocetins (the deglycosylated forms) were also found to be potent inhibitors of carcinogenesis as well as attenuators of the toxicity of some anticancer agents [43]. Crocins inhibit skin tumor promotion in mice (i.e., with benzo(a)pyrene). They have an inhibitory effect on the intracellular nucleic acid and protein synthesis in malignant cells as well as on protein kinase C and proto-oncogene in INNIH/3T3 cells, which is most likely

due to their antioxidant activity [44, 45], although the antioxidant of free radical scavengers properties of saffron and its ingredients (crocin) has been shown in previous studies [46, 47]. However, carotenoids at high concentrations may act as pro-oxidants in biological systems [48]. Therefore, it seems likely that potential compounds responsible for the inhibitory effect of saffron on tumor cell growth are its carotenoid ingredients. With respect to the mechanism(s) that may be involved, the intracellular level of sulfhydryl (SH) compounds in tumor cells may be important factors partaking in the relative sensitivity of malignant cells to the effect of saffron [49] because it has been shown that the pretreatment of tumor cells with saffron resulted in a doubling of the intracellular SH compound levels. Thus, these results reveal that the saffron extract is nontoxic and that it possesses cytotoxic activity against the human lung cancer cell line.

Overall, this study showed that saffron may contain bioactive compounds that inhibit the proliferation of human alveolar cell line (A549) with the involvement of apoptosis or programmed cell death. Further studies are needed to fully recognize the mechanism involved in cell death, and saffron could be considered as promising chemotherapeutic agent in lung cancer treatment.

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Conflicts of interest No competing financial interests exist.

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