

RESEARCH ARTICLE

Distinct Pro-Apoptotic Properties of Zhejiang Saffron against Human Lung Cancer Via a Caspase-8-9-3 Cascade

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Abstract

Lung cancer is the leading cause of cancer-related death worldwide. Here we investigated the antitumor effect and mechanism of Zhejiang (Huzhou and Jiande) saffron against lung cancer cell lines, A549 and H446. Using high performance liquid chromatography (HPLC), the contents of crocin I and II were determined. *In vitro*, MTT assay and annexin-V FITC/PI staining showed cell proliferation activity and apoptosis to be changed in a dose- and time-dependent manner. The inhibition effect of Jiande saffron was the strongest. *In vivo*, when mice were orally administered saffron extracts at dose of 100mg/kg/d for 28 days, xenograft tumor size was reduced, and ELISA and Western blotting analysis of caspase-3, -8 and -9 exhibited stronger expression and activity than in the control. In summary, saffron from Zhejiang has significant antitumor effects *in vitro* and *in vivo* through caspase-8-caspase-9-caspase-3 mediated cell apoptosis. It thus appears to have more potential as a therapeutic agent.

Keywords: Saffron extract - A549 cell - H446 cell - pro-apoptotic - caspase

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Introduction

Lung cancer is the leading cause of cancer-related death worldwide. The incidence ratio of non-small cell lung cancer to small cell lung cancer is about 4:1 (Shepherd et al., 2011). The majority of chemotherapeutic drugs used in hospitals can be divided into alkylating agents, antimetabolites and anthracycline (Coate and Shepherd, 2010). But there was a lot of side effects and poor prognosis. The use of molecular targeting drugs such as the tyrosine kinase activator imatinib is also increasing (Grimaudo et al., 2013), but there are few drugs that achieve a complete recovery in cancer patients, and the failure of conventional chemotherapy to effect a major reduction in mortality indicates that the development of more effective chemotherapeutic drugs is essential for the treatment of cancer worldwide.

Herbs have been considered natural and valuable sources for anticancer drug discovery with chemopreventive and chemotherapeutic properties. Saffron, the dry stigmas of the plant *Crocus sativus* L, has been used in the treatment of numerous illnesses (Schmidt et al., 2007; Joukar et al., 2010; Rezaee and Hosseinzadeh, 2013). In the recent past, saffron is found to have the superiority of antitumor with broad spectrum, low toxicity and few side effects (Zhang et al., 2013). Saffron has been introduced into China since the 60's last century. Huzhou and Jiande regions in Zhejiang province is one of the first saffron cultivation bases in China. However, there is no evidence on the therapeutic

effects of Zhejiang saffron on the lung cancer. Therefore, the aim of the present study was to assess the potential antitumor effects and potential mechanism of Zhejiang saffron against human lung cancer cell lines, A549 and H446, compared with Iranian saffron. It would facilitate the spread and utilization of Zhejiang saffron.

Materials and Methods

Reagents

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl (MTT), Annexin V-FITC apoptosis detection kit were purchased from KeyGEN Biotech Inc. (Nanjing, China). Caspase 3 activity assay kit was purchased from Beyotime institute Biotech (Jiangsu, China). Anti-caspase-8, anti-caspase-9 and anti-caspase-3 antibodies were obtained from Cell Signaling Technology (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Sunub Bio-Tech Development Inc. (Shanghai, China). Saffron was purchased from Tarvand Saffron Co. (Tehran, Iran) and Hengfa Saffron Co. (Jiande, Zhejiang, China). Other chemicals were of the highest, commercially available quality.

Preparation of Saffron Extract

Saffron from Iran, Huzhou and Jiande of Zhejiang province was processed in the Experimental and Research Centre of Zhejiang Medical College. The stigma part of

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saffron was air dried in the shade before extraction. After grinding, a 1 g weight of the dried stigma was extracted with 10 ml ethanol (75%) for 30 min in an ultrasonic bath, which was repeated for 3-5 times until the extract was clear and transparent. All the extract was filtered, concentrated in a vacuum evaporator and kept at 4°C (refrigerator). The yield of extraction was around 30%.

HPLC Analysis

For HPLC analysis, we used a Lumtech K501 liquid chromatographic system equipped with a Lumtech K2501 UV detector (Lumtech, Germany). The saffron extract was carried out on a Diamonsil C₁₈ (250mm×4.6mm, 5μm) (Dikmatech, Beijing). The guard column was a Diamonsil EasyGuardIIC₁₈ column. The detector was set at 440 nm. For the mobile phase, mixture of methanol and water (48:52) was used. The flow rate of the mobile phase was 1.0 ml/min, and the injection volume was 10 μl. All solutions were filtered through a 0.2 μm hydrophilic polypropylene membrane (Merck Millipore, Billerica, MA) before use. Saffron extract was accomplished at 30°C. Five different concentrations of crocin I and II solutions were prepared to determine the calibration curve. The calibration curve was constructed with crocin I and II content versus peak area (crocin I: $y=51.313x+1352.9$, $R^2=0.9994$, linear range: 10 - 220 ug/ml; crocin II: $y=150.72x+125.59$, $R^2=0.9998$, linear range: 10 - 120 ug/ml). The content of crocin in saffron extract was calculated using the standard curve of crocin I and II, and determinations were repeated 3 times.

Cell Culture

The human non-small lung cancer cells (A549) was obtained from Research Centre of Zhejiang Chinese Medical University and the human small lung cancer cells (H446) from KeyGEN Biotech Inc. (Nanjing, China). Cells were maintained at 37°C in a humidified atmosphere (90%) containing 5% CO₂ and subcultured every 3-4 days. The Malignant cells were cultured in DMEM with 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100μg/ml streptomycin.

Cell Proliferation Assay

The cell viability was determined using a modified 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT) assay. Briefly, cells were seeded (5000 cells/well) onto flat-bottomed 96-well culture plates and allowed to attach for 8-10 h to keep the long-phase growth at the time of drug treatment. Saffron extract (150 μl), at different concentrations (0.25, 0.50, 1.0, 2.0, 4.0 and 8.0 mg/ml), was added to the wells and allowed to grow for 24 and 48 h. For each concentration and time course study, there was a control sample that remained untreated and received an equal volume of serum-free medium. After removing the medium, cells were then labeled with 20μl MTT solution (5mg/ml in PBS) for 4 h and the resulting formazan was solubilized with DMSO (150 μl). Absorbance was measured at 492 nm using an automated microplate reader (Bio-Rad 550, Illinois, USA). Cell viability was expressed as a percentage of the control culture value. Experiments for each extract were carried out in triplicate, including untreated cell control and a blank cell-free control.

The inhibitory effect of the saffron extract on the lung cancer cell lines was expressed as IC₅₀ values (the drug concentration reducing the absorbance of treated cells by 50% with respect to untreated cells).

Flow Cytometric Analysis of Apoptosis

Apoptotic cell death of saffron was measured using FITC-conjugated Annexin V/PI assay kit by flow cytometry. After treatment with saffron extract (0.50, 2.0 and 8.0mg/ml) for 24 h, cells were collected by trypsinization, washed with ice-cold PBS twice, resuspended in 500μl binding buffer, and stained with 5μl of FITC-conjugated Annexin V (10mg/ml) and 5μl of PI (50mg/ml). The cells were incubated for 5-15 min at room temperature in the dark, and analyzed by a flow cytometry (Beckman Coulter, USA). For analysis, the A549 and H446 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 PI) versus fluorescence 1 (for FITC) plots. The cells positive for FITC only was evaluated as early apoptosis; the cells positive for both FITC and PI was evaluated as late apoptosis or necrotic stage.

Animal Xenograft Model

A total of 32 males BALB/c nude mice (5 weeks old) were purchased from Shanghai Slac Laboratory Animal Co. (Shanghai, China), Chinese Academy of Sciences. Animals were maintained under standardized, sterilized conditions in specific pathogen-free (SPF) laboratory, and were fed a regular nude mice chow. The mice were acclimatized to the housing condition for 1 week. All the experiments were conducted under the guidelines of laboratory animal use and care of China.

H446 cells (~2×10⁶) were injected into the back of the right hind leg of each mouse for the development of transplanted tumor. Immediately after the injection of H446 cells, the mice were randomly divided into 4 groups:

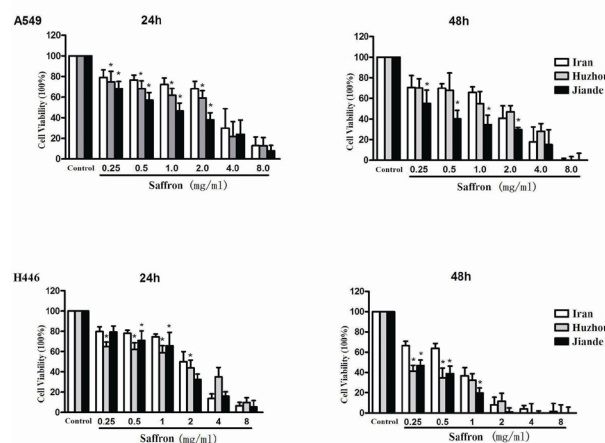


Figure 1. Effect of Saffron Extract from Three Places on Cell Viability of A549 and H446 Cell Lines Middle concentrations (1.0 and 2.0mg/ml) and high concentrations (4.0 and 8.0mg/ml) of saffron extract apparently decreased the cell viability after 24h (* $p<0.05$). After 48h, low concentrations (0.25 and 0.50mg/ml) of saffron extract also significantly inhibited cell proliferation (* $p<0.05$)

control group was treated with 0.2ml/d saline by orally administration; 3 experimental groups were administered 100 mg/kg/d saffron from Iran, Jiande and Huzhou (diluted in saline to 0.2ml) for 28 days, respectively. Tumor size and body weight were monitored daily throughout the experiment, and its volume calculated as $V=ab^2/2$, where a is length and b is width. All mice were sacrificed after 28 d treatment, and tumors were taken out and stored at -80°C .

Western Blotting Analysis

Western blot analysis was performed as described (Zou et al., 2012), with modifications. Protein extracts were prepared using the protein extraction kit (Panomics Inc., Santa Clara, USA). Following BCA assay, extracts were separated by SDS-PAGE, transferred to a PVDF membrane. Membranes were blocked overnight at 4°C with 5% nonfat dry milk in Tris-buffered saline Tween (TBST, 20 mM Tris-HCl, pH 7.6, 150mM NaCl, 0.05% Tween-20), incubated with a primary antibody of caspase-8, caspase-9 and caspase-3 for 1h at room temperature in 5% nonfat dry milk/TBST, washed three times with TBST, and incubated with HRP-linked secondary antibodies in 5% nonfat dry milk/TBST for 1 h at room temperature. After washing with TBST, protein bands were visualized by chemiluminescence on X-ray films.

Caspase-3 Activity Analysis

The activity of caspase-3 assay was carried out according to Caspase-3 Activity Assay Kit (Beyotime, Haimen, China). The caspase-3 activity assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*-NA), after its cleavage from the labeled substrate, acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-*p*NA). Briefly, per 3-10 mg tumor tissue was added 100 μl lysate. Assays were performed in Eppendorf tubes by incubating 10 μl total protein of tumor lysate per sample and add reaction buffer (1% NP-40, 20 mM Tris-HCl (pH 7.5), 137 mM Nad, and 10% glycerol) to a total volume of 90 μL , containing 10 μL caspase-3 substrate (Ac-DEVD-*p*NA) (2mM). Lysates were incubated at 37°C for 12 h.

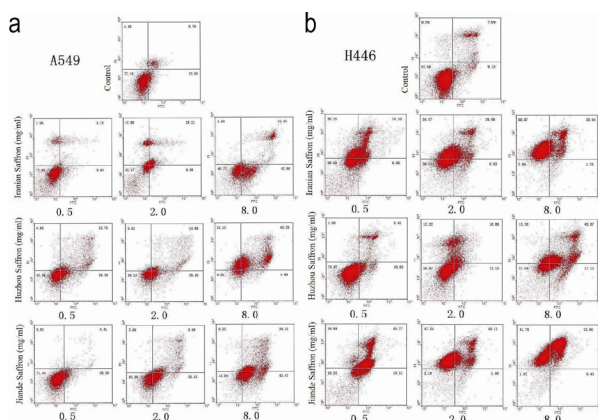


Figure 2. Flow cytometry histograms of apoptosis assays by annexin V-FITC/PI staining method in A549 (A) and H446 (B) cells The proportion of early and late stage apoptotic cells increased significantly by increasing saffron concentrations ($p<0.01$). Apoptosis induced from Zhejiang saffron was statistically higher than Iran and control ($p<0.01$)

Samples were measured with an enzyme-linked immunosorbent assay (ELISA) reader at an absorbance of 405 nm and the caspase activities were expressed as the the output of *p*NA. All the experiments were performed in triplicate.

Statistical Analysis

All results were expressed as mean \pm SD. The significance of difference was evaluated with ANOVA and LSD test. A probability level of $p<0.05$ was considered statistically significant.

Results

HPLC Analysis of Crocin

To identify the different crocin molecules, we subjected the saffron extract to HPLC analysis. Because crocin derivatives showed a maximum absorption near 440 nm, we selected this wavelength for the assay. Crocin has been identified as the main components in saffron extract by HPLC. Table 1 shows retention times, peak areas and contents for crocinI and II in saffron extract, respectively.

Effect of Saffron on Cell Viability

Cells were incubated with different concentrations of saffron extract (0.25, 0.50, 1.0, 2.0, 4.0 and 8.0mg/ml) for 24 and 48h, and their growth inhibitory effects were compared. The impact of the saffron extract on cell viability was quantitated by the MTT assay. The saffron from Jiande and Huzhou of Zhejiang province showed significantly higher growth inhibitory effects on both A549 and H446 in a concentration- and time- dependent manner compared with Iranian saffron ($*p<0.05$). As shown in Figure 1, middle concentrations (1.0 and 2.0 mg/ml) and high concentrations (4.0 and 8.0 mg/ml) of saffron extract apparently decreased the cell viability after 24 h. This

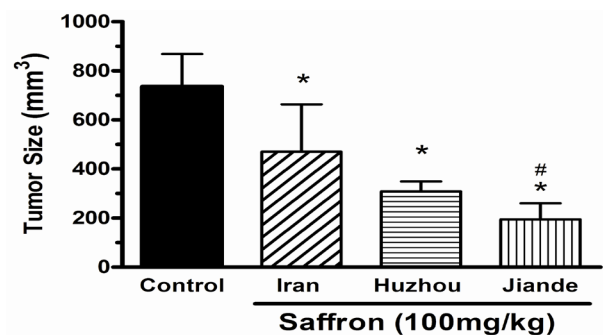


Figure 3. Tumor Volume of H446 Xenograft in Nude Mice ($\bar{x}\pm s, n=8$) Tumor size in mice treated by saffron extract from three places was all significantly inhibited compared with the control group ($p<0.05$). Among the three experimental groups, tumor size of Jiande group was statistically smaller than that of Iran ($p<0.05$)

Table 1. Determination of Crocins in Saffron Extracts

Assay	Retention time (min)	Peak Area	Concentration(ug/ml)	Content (%)
Crocin I	Iran	13.696	5785.3	86.38
	Huzhou	13.811	4341.6	58.24
	Jiande	12.955	5748.3	85.66
Crocin II	Iran	26.517	2457.9	17.14
	Huzhou	26.71	2161.8	15.18
	Jiande	25.1656	3062.2	21.15

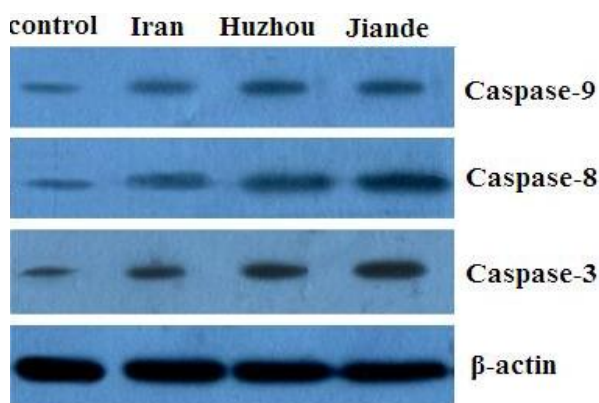


Figure 4. Western Blotting Analysis of Caspases in Tumors. The expression of caspase-3, -8 and -9 were all increased in saffron treated tumor tissues compared with the control. The increasing of caspase 8 was more significant than caspase 9. So caspase 8 dependent exogenous death receptor pathway may play a more important role in cell apoptosis induce by saffron.

toxicity was consistent with morphologic changes. After 48 h, low concentrations (0.25 and 0.50 mg/ml) of saffron extract also significantly inhibited cell proliferation. Doses inducing 50% cell growth inhibition (IC_{50}) against A549 and H446 after 24 and 48 h are presented in Table 2.

Assessment of Apoptosis by Annexin V-FITC

As it can be seen in (Figure 2), the A549 and H446 cells were treated with concentration of 0.5, 2.0 and 8.0mg/ml saffron for 24h, then the cells were harvested, 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 compare roles of saffron from Zhejiang and Iran in apoptosis, the saffron extract was used to setup apoptosis system on the cells. Quantitative analysis using Annexin V/PI assay further showed that the proportion of early and late stage apoptotic cells increased significantly by increasing saffron concentrations ($p<0.01$). Apoptosis induced by Zhejiang saffron was statistically higher than Iran and control ($p<0.01$). For the same saffron extract, the late apoptotic and necrotic cell percentage of H446 is obviously higher than A549, but the early apoptotic cell percentage is quite low.

Antitumor Efficacy of Saffron in Vivo

According to the experimental results *in vitro*, H446 was chosen to carry out *in vivo* assay. At the time of receiving H446 cells, there was no significant difference in the body weight among the four groups. As the tumors grew, there was no treatment-related death of mice.

The tumor formation rate of the control and experiment groups (Iran, Huzhou, Jiande saffron extract) was 100%, 88%, 75%, and 75%, respectively. There was no significant difference in the tumor formation rate among the four groups. At the end of the study, the transplanted tumor was excised from each sacrificed mouse, and tumor volume was calculated (Figure 3). Tumor size in mice treated with saffron extract from three places was all

Table 2. Doses Inducing 50% Cell Growth Inhibition (IC_{50}) of saffron extracts against A549 and H446 Cell Lines

IC_{50} (mg/ml)	24 h		48 h	
	A549	H446	A549	H446
Iranian saffron	1.82	1.4	1.2	0.59
Huzhou saffron	1.2	1.22	1.05	0.37
Jiande saffron	1.03	1.13	0.54	0.33

Table 3. Caspase-3 Activity of H446 Xenograft Tumor (n=8, $\bar{x}\pm s$)

Groups	Product of pNA (μ M)	Enzyme activity
Control	14.97 \pm 0.07	935.6 \pm 0.00
Iranian group	33.82 \pm 2.78	2113.7 \pm 0.17*
Huzhou group	26.2 \pm 3.24	1637.5 \pm 0.20*
Jiande group	69.67 \pm 4.94	4354.3 \pm 0.31*#

*Compared with the control, * $p < 0.05$; compared with Iranian group, # $p < 0.05$

significantly inhibited compared with the control group ($p<0.05$) (Figure. 3). Among the three experimental groups, tumor size of Jiande group was statistically smaller than that of Iran ($p<0.05$).

To investigate the possible mechanism underlying saffron-induced apoptosis in lung cancer, western blotting analysis of caspase-8, caspase-9 and caspase-3 in xenograft tumors was conducted. The expression of caspase-3, -8 and -9 were all significantly increased in saffron treated tumor tissues. The increasing of caspase 8 was more greatly than caspase 9 (Figure 4). It is consistent with the ELISA analysis of Caspase-3 Activity (Table 3). Caspase-3 is considered a key protease that is activated during the early stages of apoptosis. Compared with the control, caspase-3 activity was increased in saffron treated tumors ($p<0.05$). Among the three experimental groups, caspases expression and caspase-3 activity of Jiande group was statistically strongest ($p<0.05$). All these suggest caspase-8-caspase-9-caspase-3 dependent pathway mediated cell apoptosis is one of the important factors affecting tumor size.

Discussion

In the present study, for the first time, the antiproliferative and pro-apoptotic effects of Zhejiang saffron extracts on human non-small cell and small cell lung cancer cell lines, A549 and H446, were investigated. Our results indicate that the saffron extract had a dose and time dependent inhibitory effect on the growth of the human lung cancer cell lines *in vitro*, which was consistent with previous studies (Tavakkol et al., 2008; Bakshi et al., 2009; Dhar et al., 2009; Samarghandian et al., 2010; Samarghandian et al., 2011; Sun et al., 2013), indicating that saffron and its ingredients possess antitumor activities. FACS analysis after Annexin-V FITC/PI double staining showed along with the increase of saffron concentration, the apoptosis percentage of tumor cells gradually elevated, which was in agreement with the change of cell proliferation inhibitory rate. But A549 cells exhibited apoptotic change mainly in early stage, and H446 cells presented apoptotic increase mainly in late stage, which

may be related with the different sensitivity of tumor cells to saffron extract. Tavakkol et al. suggested most of the apoptotic cells were inhibited in G1 period (Tavakkol et al., 2008; Bakshi et al., 2009). Saffron-induced cytotoxicity in the lung cancer cells was involved in the induction of morphological changes. The morphological features characteristic rounding of dying cells by treatment with saffron for 24h compared with untreated controls (Samarghandian et al., 2010). It is clear that the rise of cell apoptosis after saffron treatment is the first cause of cell proliferation reduction. The antiproliferative and pro-apoptotic effects of Zhejiang saffron on lung cancer cells are all higher than that of Iranian *in vitro*, and Jiande saffron is the highest. The content of effective ingredients in saffron extract from the three places may explain the results. Crocin I and II were found to be most abundant in Jiande saffron extract through HPLC. They were 38.81% and 9.59%, which were two times higher than Iran saffron. The crocin content of Huzhou saffron is only next to the Jiande. Crocins, the major carotenoid components of saffron stigma, demonstrated antitumor properties with broad spectrum (Sun et al., 2011), promoting tumor growth inhibition and increasing the life-span of treated tumor-bearing animals. Crocins are well tolerated and present no or few side-effects. These, together with their water-solubility, make them suitable for chemotherapeutic use.

To confirm our *in vitro* results, we employed nude mice transplanted model to evaluate the *in vivo* antitumor effect. Our results showed the saffron extract had strong inhibitory effect on A549 and H446 cells growth in nude mice, the tumor size of Jiande group is the smallest and is significantly smaller than that of Iranian group. There was no accidental death throughout the course of the animal experiment, indicating the safety of Zhejiang saffron in treatment. The growth and development of tumor is associated with the unbalance between cell growth cycle and apoptosis (Dziegiel et al., 2004). Thus one of the important antitumor ways is to induce tumor cells into apoptosis. Cell apoptosis involves a series of proteases and their response to apoptotic signals. The cleavage and degradation of the subunit of proteases would result in cell death. Caspase-3, also called death protein, is one of the deeply studied proteases. It targets at N-terminal ascorbase site of substrate to enzymolysis related proteins. The activation of caspase-3 promotes cells into an irreversible apoptosis pathway. So caspase-3 is the executor of cell apoptosis and the important target to regulate apoptosis (Jiang et al., 2012). Hoshyar et al. showed that crocins initiated cell apoptosis by increasing caspase activity in human gastric adenocarcinoma cell line AGS (Hoshyar et al., 2013). It is well known that caspase-8 and caspase-9 are essential proteases of extrinsic and intrinsic apoptotic pathways, while caspase-3 acts as a downstream effector of these two pathways (Chen et al., 2014). Thus, our data suggested that saffron-induced apoptosis is caspase dependent and may involve in both the endogenous mitochondrial pathway and the exogenous death receptor pathway. But the exogenous way mediated by caspase 8 may play a more important role in cell apoptosis induced by saffron.

The antitumor activity of saffron extract is also

associated with cytotoxicity of all the effective components. Except for crocin, the characteristic chemical constituents include safranal, crocetin, picrocrocin and β -carotene et al. 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, anti-tumor, chemopreventive, anti-mutagenic and immuno-stimulating properties. Recently crocin is found to have potential function in scavenging free radicals and in inhibiting lipid peroxidation (Bakshi et al., 2009). Crocins inhibit skin tumor promotion in mice. They have an inhibitory effect on the intracellular nucleic acid and protein synthesis in malignant cells as well as on protein kinase C (PKC) and pro-oncogene in INNIH/3T3 cells (Giaccio et al., 2004), which is most likely due to their antioxidant activity. Crocetin has no function in colony forming of tumor cells, but it could inhibit DNA, RNA and protein synthesis of human malignant tumor cells in a dose dependent manner (Ashrafi et al., 2005), which may be related to p53-dependent mechanisms (Li et al., 2012). In addition, crocetin could reduce interaction between histone H1 and DNA *in vitro*, interfere gene transcription (Magesh et al., 2006). In the Annexin-V FITC/PI assay, the number of PI positive cells rose with the increase of saffron concentration. These necrotic cells may have direct relationship with cytotoxicity of saffron extract.

In summary, the antitumor effect of Zhejiang saffron is distinguished and it may serve as a promising cancer therapy agent. Meanwhile, due to the versatile ingredients and complicated mechanisms, further research is needed. Data from this study confirmed the anti-proliferative and pro-apoptotic effects of Zhejiang saffron on lung cancer cells *in vitro* and *in vivo*. The potential mechanism was discussed in cell apoptosis. It would lay an essential foundation for the utilization of Zhejiang saffron in prevention and treatment of tumors.

Acknowledgement

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