

Original Research Paper

The *Crocus Sativus* Stigma Extract as Anticancer for SKOV-3 Cell Line

Rusul Hammoodi Hasan Kabla¹
Jinan M. Abed-al-Zahra²

^{1,2} Department of biology, Faculty of Science, University of kufa, Kufa, Iraq.

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*Corresponding Author:

Email:

rusulh.kabla@student.uokufa.edu.iq,

Abstract: Cancer is an uncontrolled growth and division of abnormal cells with the capacity to spread to other body regions (metastasis). The aim of this study is to investigate the effect of saffron aqueous extract an anticancer on the ovarian cancer the material and method included preparation the saffron aqueous extract and SKOV-3 after maintained of cell cultures in RPMI-1640 supplemented with 10% Fetal bovine serum, penicillin and streptomycin. Next, three methods were used to investigate the impact of saffron. One of these methods involves flow cytometry (FACSCalibur flow cytometer) was used to measure the generation of ROS in cells, measuring the impact of saffron on the mitochondrial activity of SKOV-3 cells using the fluorescent dye Rhodamine (Rh123). Furthermore, the activation statuses of p53 and caspase-3 were evaluated using a fluorescein caspase-3 and p53 staining kit. Eventually, the results were promising because the saffron extract caused damage in mitochondria and a significant increase by caspase-3 and p53 that caused to apoptosis SKOV-3 cells without effect on normal cells. Finally, the usage of saffron supplements in addition to established treatment procedures may benefit people with ovarian cancer since saffron extract triggers cancer cell apoptosis through several indications that manifest differently.

Keywords: *Crocus Sativus* Stigma, Anticancer, SKOV-3 Cell Line, and fluorescent dye Rhodamine (Rh123).

1. Introduction

Cancer is the disease most widespread in the world, it is the second cause of death following cardiovascular diseases. The cancer arises when group of cells begin to abnormally grow in size and increase in number. Consequently, these cells lose control of performing their vital functions [1,2].

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The ovarian cancer is the third most common gynecologic malignancy worldwide, its malignant tumor arises from the ovarian, and in case it is left untreated, it becomes able to spread to another part of the body (metastasis)[3]. The danger of ovarian cancer is in its diagnosis at advanced stages caused by its symptoms that shares similarities with symptoms of other diseases (that is why it is also called as the silent killer)[4]. On the other hand, complicated chemotherapy and extensive surgical intervention are used to treat ovarian cancer. However, the nonselective affliction of cells, including normal tissue cells, in various therapy approaches results in considerable adverse effects[5]. For this reason, many researchers resorted to the traditional/complementary

medicine by using herbs to find a better treatment for cancer with fewer side effects.

One of the herbs adapted for that purpose is *Crocus sativus* (as called as saffron). There are many experiments by researchers that proved the positive effect of *Crocus sativus* stigma extract over the ovarian cancer. Those experiments showed that the *Crocus sativus* stigma extract can be an effective anticancer where it can induce the apoptosis in the cancer cells[6]. The recent studies tested the effect of major bioactive components that existing in the *Crocus sativus* by FTIR technique that is an analysis technique used to identify organic, polymeric, and in some cases, inorganic materials. The bioactive components include crocin, crocetin, picrocrocin, and safranal. Nevertheless, it is interesting that saffron's major ingredients impact synergistically the against malignant cells. As results, the saffron extract more effective than its components separately [7].

By flow cytometry (FACSCalibur flow cytometer) was used to measure the generation of ROS in cells, ROS relies on measuring the end products that are formed when they react with particular substances [8]. By employing a test for mitochondrial membrane potential, we examined the impact of saffron aqueous extract on the ovarian cancer cell line SKOV-3. The mitochondrial membrane potential was measured using rhodamine (Rh123) fluorescent dye before and after saffron therapy at an IC₅₀ concentration[9]. Moreover, we used a fluorescein caspase-3 and p53 staining kit to evaluate the levels of caspase-8 and p53 in SKOV-3 both before and after treatment with saffron at an IC₅₀ concentration [10,11]. Our research produced astounding results: saffron causes mitochondrial malfunction in SKOV-3 cells, which in turn causes p53 and caspase-3 release and SKOV-3 demise. Moreover, caspase-3 is essential for nucleic acid fragmentation and plays a critical role in apoptosis [12,13,14]. There are several types of cancer as colorectal cancer, prostate cancer, breast cancer, lung cancer and ovarian cancer. We deal with ovarian cancer cell line (SKOV-3) in our study.

2. Methodology

Plant Collection

A sample of commercial saffron was collected from the local market in Al-Najaf governorate, Iraq. The stigma and styles of the herb and pulverized via mechanical grinder to a fine powder and kept at room temperature.

Saffron Extraction

The first day included taking 40g from the dried stigma and styles *Crocus sativus*, then putting it in a conical

flask and dissolving it with 400 ml of distilled water. Thereafter, we put it on a magnetic stirrer hotplate for 1 hour for better mixing, then put it in a cooling centrifuge to 4500rpm at 4 °C. On the second day, we filtered the solution with medical gauze. Thereafter, we filtered it with filter paper 11µm, followed by taking the water extract and putting it in the oven at 40°C until dried. Last but not least, collected the extraction powder, using the powdered crude saffron extract for the experiment.

FTIR Analysis

FTIR (Fourier Transform Infrared Spectroscopy) is a powerful analytical technique used to identify and characterise chemical compounds based on their absorption of infrared light. The FTIR instrument consists of several key components, including a source of infrared radiation (usually a heated filament or a laser), a beamsplitter, an interferometer, and a detector. The interferometer modulates the incoming IR beam and producing an interferogram that contains information about the sample's absorption of IR radiation. The sample is exposed to infrared radiation, and the resulting spectrum is recorded. This spectrum represents the absorption of different wavelengths of infrared light by the sample. The spectrum is typically plotted as percent transmittance %T or absorbance $\log 1/(\%T)$ versus wavenumber cm^{-1} . Once the spectrum is obtained, various data analysis techniques can be applied to extract additional information from the data. This may include peak fitting, baseline correction, and spectral subtraction to remove unwanted signals or artefacts.



Figure 1. FTIR Instrument.

Ovarian Cancer Cell Culture

The SKOV-3 cell lines are maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Capricorn, Germany), 100 units/mL penicillin, and 100 µg/mL streptomycin. The cells are passaged using trypsin-EDTA, cultivated at 37 °C, and then reseeded twice a week at 80% confluence. We used the identical method as that found in[15,16].

ROS Determination

Flow cytometry (FACSCalibur flow cytometer) was used to measure the generation of ROS in cells. SKOV-3 cells were plated on six-well plates at density 1×10^6 . Cells were then incubated overnight and treated with saffron at IC₅₀ concentration for 8 hr. Then, the ROS probe (DCFH-DA) (15 μ M) was added to a fresh medium and incubated in darkness for 30 minutes. Cells were harvested and analysed for fluorescence intensity using a flow cytometer (BD Biosciences).

Mitochondrial Membrane Potential Assay

Mitochondrial activity SKOV-3 cells was tested in relation to saffron using the fluorescent dye Rhodamine (Rh123). Testing the mitochondria membrane potential with the dye both before and after saffron treatment. In summary, 24 hours after the cells in 96-well plates were seeded, they underwent saffron treatment and were stained for two hours at 37°C using 5 M Rh123 dye. A 0.2 mL solution of 5% trypsin–EDTA was then used to separate the cells, and the mixture was centrifuged for five minutes at 300 rpm. Following that, cells were evaluated using a flow cytometry test, resuspended in FACS buffer, and histograms were produced.

Measurement of Caspase-8 and p53 Levels (Flow Cytometry Assay)

The activation states of caspase-3 and p53 were evaluated using a fluorescein caspase-3 and p53 staining kit (Thermo Fisher Scientific, USA). In summary, 4×10^4 cells were cultured using 5 mL of medium.mL⁻¹ of SKOV-3 cancer cells, followed by a 24-hour incubation period at 37°C. The incubated cells were then exposed to saffron for 24 hours at an IC₅₀ concentration. Following the incubation period, the treated cells were taken out and given two rounds of ice-cold PBS washing. After gathering the pellets, growth media was used to reset the cell density to 1×10^6 cells.mL⁻¹. The generated cells were then exposed to 1 mL of FITC-IETD-FMK for 60 minutes at 37°C. This was done in order to detect caspase-8 and FITC-anti-p53. The cells were subjected to two washes with wash buffer (0.5 mL) after the incubation period. The cells were put through flow cytometry after being moved into tubes. BD Accuri C6 was utilized to compute and examine the data.

Statistical Analysis

Graph-Pad Prism 6 was used to do a statically analysis of the acquired data using an unpaired t-test [17]. The data was shown as the mean $\pm$ SD of three measurements in triplicate [18]

3. Results and discussion

Bioactive Components of Saffrons

The distinctive qualities of saffron come from its rich and adaptable composition of proteins (10–14%), carbohydrates (12–15%), fibers (4–5%), ashes (4%), and volatile compounds (0.3–0.8%) [19].

Three primary chemical components make up this mixture: picrocrocin, the glycoside of safranal, which imparts the bitter taste; safranal (monoterpene aldehyde), which is the source of the distinctive saffron aroma; and the glycosyl esters of crocetin, which are members of the carotenoid family and give saffron stigmas their vivid yellow-red color[20]. One of the few families of water-soluble carotenoids found in nature, crocins (C₄₄H₆₄O₂₄) are responsible for the distinctive and unusual color of saffron [21]. It is believed that picrocrocin (4 - (β - D - glucopyranosyl xy) - 2,6,6 - trimethyl - 1- cyclohexene - 1- carboxaldehyde, C₁₆H₂₆O₇) is the primary cause of saffron's bitter flavor[22]. In relation to the volatile portion of saffron that gives it its scent, over ninety different chemicals were found. But among all of them, safranal C₁₀H₁₄O was the most important chemical [23].

This chemical is the end product of the conversion of picrocrocin by either direct thermal degradation or a two-step enzymatic or dehydration process involving the intermediate HTCC (4-hydroxy-2,6,6-trimethyl-1-cyclohexen-1-carboxaldehyde) [24].

Safranal can be produced by subjecting some carotenoids to a thermal process, although it has only been found in a few number of plants. It typically ranges in concentration from 0.1% to 0.6% in saffron. Compositionally, most of saffron's bioactive properties have been associated with its main compounds: crocins, safranal, and picrocrocin (called carotenoids). The carotenoids are the reasons behind the widespread application of this herb as a spice, and in traditional and complementary medicine. These compounds are highly soluble in water and have antioxidant activity. The number of carotenoids is insignificant in fresh saffron, but it rises during drying and storage [25]. Several studies have documented the effect of saffron aqueous extract and two of its main ingredients, crocin and crocetin, on several types of cancer, such as colorectal cancer, prostate cancer, breast cancer, lung cancer, and gastro cancer[26, 27]. Saffron bioactive components have a synergistic effect, preferably using saffron extract more than using each component individually[28]. Many studies showed the pharmacological and medical effects of the saffron extract, such as anti-inflammatory, antioxidant, anticonvulsant, antidepressant, anticancer, and other medical activities [29].

The FTIR results of saffron extract during this study were matched for all the above-mentioned, as shown in Figure 2 and Table 1.

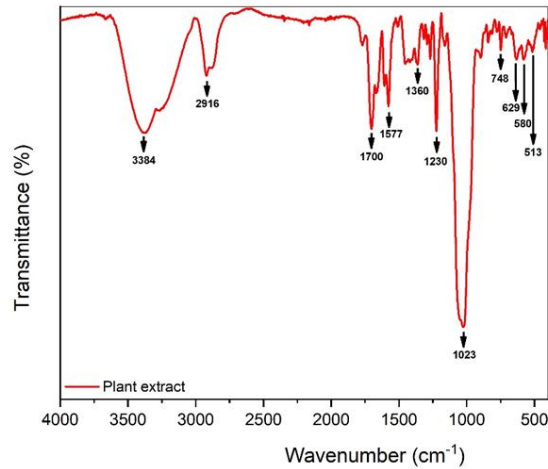


Figure 2. FTIR Results of Saffron extract

Peak No.	X (cm-1)	Group	Compound Class
1	3384	N-H stretching	N-H stretching
2	2916	C-H stretching	C-H stretching
3	1700	C=O stretching	conjugated aldehyde
4	1577	N-O stretching	nitro compound
5	1360	O-H bending	Phenol
6	1230	C-O stretching	vinyl ether
7	1023	CO-O-CO stretching	anhydride
8	748	C=C bending	Alkene
9	629	C-I stretching	halo compound
10	580	-	-
11	513	-	-

Table 1: Chemical composition of saffron

Saffron Induces ROS Generation in SKOV-3 Cells

In aerobic organisms, the formation of reactive oxygen species (ROS) is an important signaling molecule that plays a role in transmitting signals and maintaining cellular redox balance. In addition to being effective therapeutic agents for the treatment of cancer, ROS also

act as boosters of tumor cells. In the current investigation, the SKOV-3 cell line's intracellular ROS generation was found utilizing a DCFH-DA probe. After 24 hours of treatment with saffron, flow cytometry showed that saffron significantly increased ROS generation, as indicated in Figure 2.

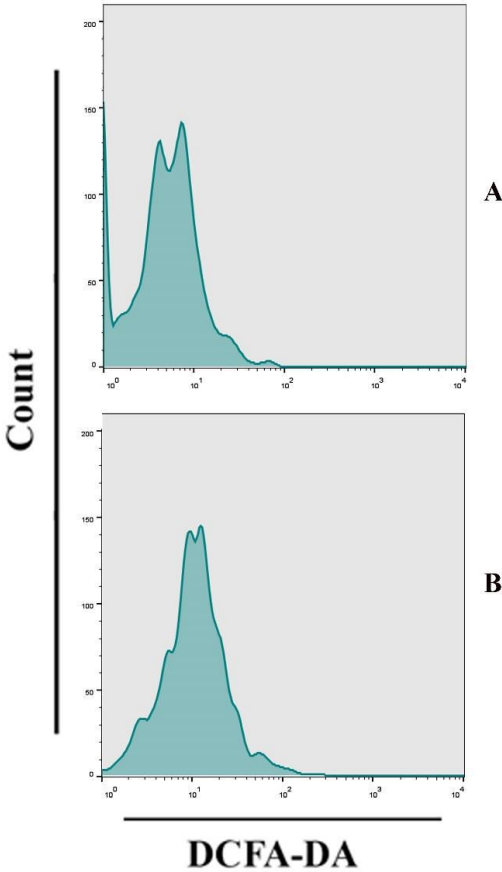


Figure 3. Saffron induces ROS generation in SKOV-3 cells. A, Control untreated cells. B, SKOV-3 cells after being treated with saffron

A study was conducted, and its results showed the effects of saffron on human colorectal cancer cells through an increase in ROS and cell cytotoxicity, which leads to programmed cell death (apoptosis)[30]. Reactive oxygen species (ROS) are essential in killing tumor cells by mediating oxidative stress and triggering programmed cell death. The sensitivity of cell therapy is related to the amount of reactive oxygen species within the cell. Reactive oxygen species are usually found in high concentrations in cancer cells and these cells may be specifically targeted for destruction[31].

Saffron Induce Mitochondrial Dysfunction of SKOV-3 Cell

Matrix metalloproteinases (MMPs) are important markers and potential therapeutic targets for cancers

because they induce cells to undergo apoptosis, which is a key symptom of mitochondrial dysfunction. Through various cellular death stimuli, mitochondria are essential for inducing apoptotic processes. When the cytochrome c protein is released into the cytoplasm and the membrane potential ($\Delta\psi_m$) of this organelle is lost, the caspase-9 pathway causes caspase-3 to be upregulated[32,33]. In the current study, we identified MMPs using a flow cytometry test, in accordance with the manufacturer's recommendations. A significant indicator of apoptotic cell death is a decrease in the mitochondrial membrane's potential. After staining the SKOV-3 cells with the Rh123 probe, flow cytometry was used to measure the mitochondrial membrane potential.

The secret of saffron is that it has a selective effect, where it affects cancer cells without affecting healthy cells[34]. This effect of Saffron comes as a result of the carotenoid components' existence[35]. A study was conducted about effect of saffron on glioblastoma and rhabdomyosarcoma cell lines. The findings showed that the carotenoids cause the cancer cells to undergo apoptosis, which then starts apoptotic pathways through various entry sites (such the mitochondria, which are known as the intrinsic or mitochondrial pathway). Proapoptotic proteins Bax, p53, and FasR receptor (FAS receptor)/APO-1 (apoptosis antigen 1) are altered after the carotenoid enters the cell as a result of saffron metabolism. In the meantime, the mitochondrial apoptotic pathway is triggered by Bax and BID's disruption of the mitochondrial membrane potential, which integrates apoptotic signals. In addition, they facilitate the permeabilisation of the mitochondrial outer membrane and release apoptogenic factors to the cytosol, which eventually results in apoptosis[36].

Our experiments clearly showed the same effect. Following the Rh123 probe staining of the SKOV-3 cells, the mitochondrial membrane potential was ascertained by flow cytometry. We investigated the percentage of SKOV-3 cells that experienced apoptosis after being treated with saffron. Figure 1 demonstrates very well the substantial rise in apoptosis observed throughout these medicines' therapy. In comparison to the untreated control SKOV-3 cells, the Rh123 staining of the treated SKOV-3 cells dramatically dropped after a 24-hour saffron treatment, suggesting a decrease in their mitochondrial membrane potential.

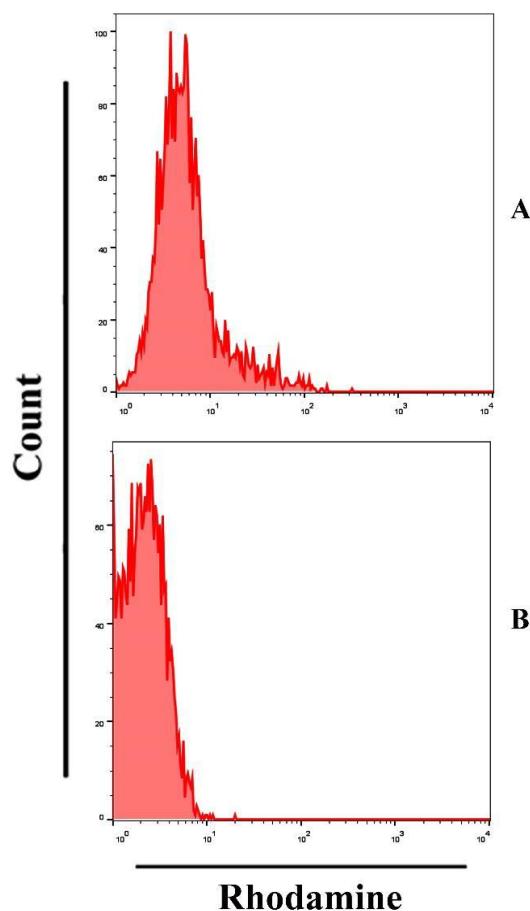


Figure 4. Saffron induces mitochondrial dysfunction SKOV-3 cells. **A**, Control untreated cells, shifted to right, mitochondria unavailable. **B**, SKOV-3 cells after being treated with saffron, shifted to left, mitochondria available due to apoptosis.

Saffron Induces Secretion of Caspase-3 and p53

The results of the earlier experiment unequivocally shown that saffron can have an apoptotic impact that is dependent on mitochondria. Two main processes are needed to induce apoptosis: the extrinsic system, which is driven by death receptors, and the intrinsic pathway, which is controlled at the mitochondrial level[37]. Signals on the surface molecules (Fas/FasL) via the extrinsic pathway aid in the recruitment of the Fas-associated death domain (FADD). The FADD–Fas complex has the potential to activate procaspase-8. The dynamic important complex (DISC) is another name for the death-inducing signaling complex. Moreover, possesses the capacity to activate and trigger caspase-3, which triggers apoptosis[38]. Using flow cytometry, the activities of p53 and caspase-3 were examined. The cells treated with saffron showed a considerable shift toward

the right for both caspase-3 and p53, which were defined as the apoptosis, according to the flow cytometry results displayed in Figures 2 and 3. Apoptosis can be seen in apoptotic events, which cause the cell to disassemble in a methodical manner. We assessed the expression levels of caspase-3 and p53 in this study. The study demonstrated that, in comparison to the control, SKOV-3 cells treated with saffron significantly increased the expression of both P53 and caspase-3. Thus, caspase-3 was activated in the apoptotic events as a result of the extrinsic and intrinsic apoptotic pathways. Caspases 3 is specifically needed for the fragmentation of nucleic acids and mimic a crucial role in apoptosis.

A study showed the effect of saffron on glioblastoma and rhabdomyosarcoma cell lines. The results imply that saffron and its active constituents induce apoptosis via activation and/or increasing the synthesis of caspases. The caspases, including caspase 3, are essential for both the beginning and end of apoptosis. Carotenoids have been shown to stimulate the expression of the proapoptotic proteins Bax, p53, and FasR receptor (FAS receptor)/APO-1 (apoptosis antigen 1) in a variety of cancer cell lines. By upregulating the expression of the Fas/APO-1 death receptor and inducing additional apoptotic proteins of the BCL-2 family (Bax, Puma, and Noxa) to stimulate mitochondria-mediated apoptosis, the tumor suppressor protein p53 initiates the death receptor/extrinsic apoptosis pathway[36].

Our findings are based on flow cytometry assessment of the p53 and caspase-3 activity. Both caspase-3 and p53 showed a significant shift to the right in the saffron-treated cells, which was identified as apoptosis. Furthermore, in comparison to the control, the SKOV-3 cells treated with saffron showed a considerable increase in both p53 and caspase-3. Thus, caspase-3 was activated in the apoptotic events as a result of the extrinsic and intrinsic apoptotic pathways. Caspase 3 is specifically needed for the fragmentation of nucleic acids and mimic a crucial role in apoptosis.

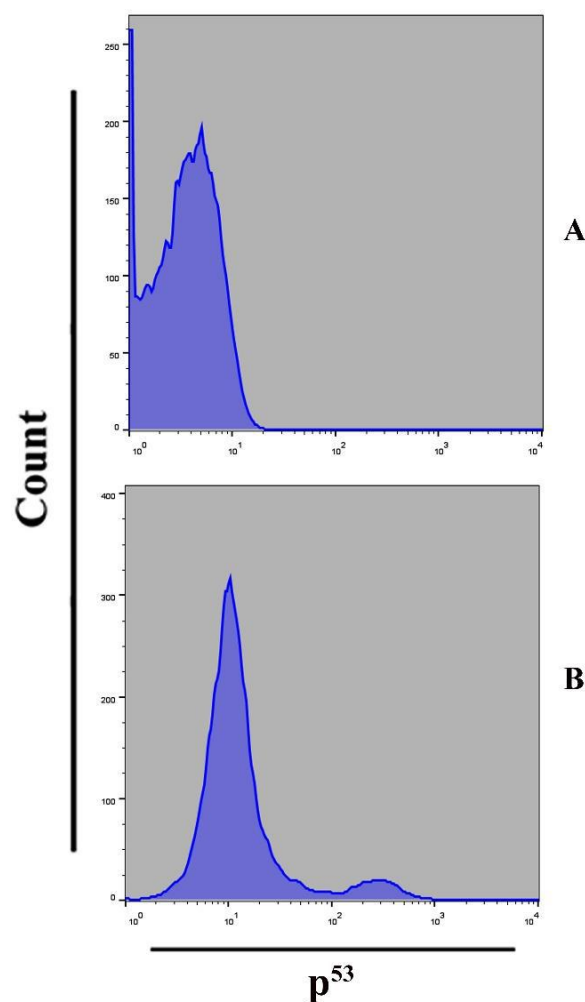


Figure 5. The saffron induces p53 expression in SKOV-3 cells. A, Control untreated cells. B, SKOV-3 cells after been treated with saffron exhibited a marked shift toward the right referred to apoptosis.

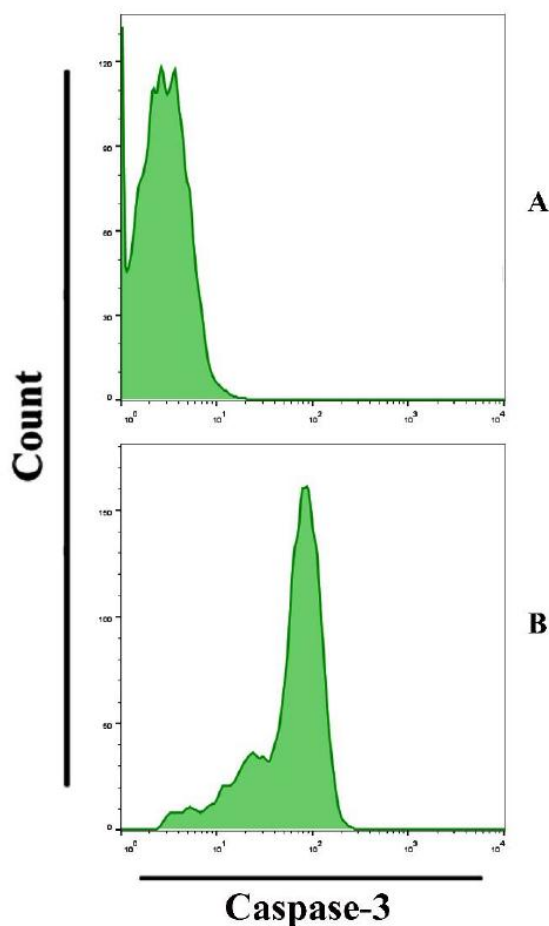


Figure 6. The saffron induces of caspase-3 secretion in SKOV-3 cells. **A**, Control untreated cells. **B**, SKOV-3 cells after been treated with saffron.

Conclusions

Based on the abovementioned rationale, this study adapts the Saffron as an herbal treatment for remedy the ovarian cancer. The extract of saffron stimulates the apoptosis of cancer cells by some signs that occurs in different ways. The extract molecules reduce the efficiency of the mitochondrial membrane of cancer cells compared to healthy cells. The extract stimulates caspase-3 and p53 in cancer cells, which leads to apoptosis.

Suggestion for future works, conducting experiments on the effect of *C. sativus* extract on other types of cell lines and on in vivo tumors. studying the extract's active ingredient at the nanoscale. carrying out a molecular analysis of the extract's active ingredient.

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Authors' Contributions

Rusul Hammoodi Hasan Kabla: Conceptualization, methodology, formal analysis, writing - original draft preparation, investigation, visualization, resources, methods and materials. Dr. Jinan M Abed-al-Zahra: Supervision, project administration, writing - review & editing

Ethics

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the human and animal Ethics Committee at the university of kufa.

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