RESEARCH
O&G Forum 2024; 34-3s: 2209-2214

THE CYTOTOXICITY EFFECT OF SAFFRON AQUEOUS EXTRACT ON SKOV-3 CELL LINE

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Abstract
Cancer is the uncontrolled growth of abnormal cells in the body. Ovarian cancer (OC) is one type of cancer, and it is the third most common female cancer in the world (According to WHO in 2022). The aim of the current study was to estimate the effect of saffron aqueous extract on ovarian cancer cell lines (SKOV-3), the material and method was conducted after maintaining of cell cultures in RPMI-1640 supplemented with 10% Fetal bovine serum, penicillin and streptomycin. Cells were passages using Trypsin-EDTA reseeded at 80% confluence twice a week, and incubated at 37 °C. To determine the cytotoxic effect of saffron, by MTT assay and acridine orange -ethidium bromid staining. The result of the study shows that there was morphological changes in SKOV-3 cell lines after being treated by the extract by using a fluorescent microscope like unregulated shape and low density. Moreover, the saffron has a highly cytotoxic effect against SKOV-3 cell line in addition it is can induce apoptosis in SKOV-3, which is caused by the induction of a programmed cell death mechanism that affects gene expression levels.
The conclusion of this study for using the saffron extract has high effect on the SKOV-3 and the effect increase with increasing the concentration of the extract and yield beneficial effects for individuals with ovarian cancer.

1. Introduction

Cancer is a genetic disorder of normal cells that results from mutation leading to abnormal cells, can metastasis to other body parts through the bloodstream to cause unregulated growth of cells called neoplasm or tumor [1]. The causes of this mutation include lifestyle decisions such extensive alcohol consumption, cigarette smoking, consuming a lot of red meat and fat, and consuming little fiber and genetic predisposition and exposure to carcinogens such as chemicals, radiation, and heavy metals [2]. Cancer symptoms vary depending on the cancer site, size, sex, and age [3]. There are several types of cancer including colorectal, lung, prostate, breast and ovarian cancer [4].

This paper specifically focuses on ovarian cancer (SKOV-3). Ovarian cancer is a rapid division of cells formed in the ovary, and it is called a silent killer due to the absence of any noticeable symptoms, and the symptoms masquerade as other problems (e.g., gastrointestinal disorder) [5,6]. The symptoms of ovarian cancer include abdominal bloating, prolonged pain (as the pressure in the abdomen and pelvis and/or lower back pain), fatigue, weight loss, difficulty eating (quick feeling of fullness after eating), changes in bowel movements such as constipation, and change in bladder function (frequent urination)[6]. Risk factors for ovarian cancer increased in female with advancing age, along with having a family history, inherited gene changes including BRCA (breast cancer susceptibility gene), nulliparity, estrogen exposure, and obesity [7]. The main type of ovarian cancer is epithelial ovarian cancer (EOV), which is the most common and has several subtypes according to the type of tissue into mucinous, serous, endometrioid, clear cell and Brenner tumor[8]. The traditional methods of treating the ovarian cancer include surgery, chemotherapy and radiotherapy [9]. Nevertheless, the treatment methods of this cancer cause damage to healthy cells as a side effect [10]. Fortunately, the researchers were able to find herbal treatment reduces the side effect of cancer treatment style, moreover, speeds up recovery through studies of traditional/complementary medicine.

One of Complementary/traditional medicine treatments is *Crocus sativus*, that do not effected on healthy cells during using it with cancer traditional treatment [11]. *Crocus sativus* (as called as saffron) is the stigma of the purple *Crocus sativus* flower from family *Iridaceae*. *Crocus sativus* stigmas and styles are carefully picked, dried and used as a spice for food. Compositionally, the saffron contains active compositions including crocin, picrocrocin, and safranal [12]. In addition, many researches have shown that saffron has antioxidant, anti-inflammatory, and anti-cancer properties [13]. Geographically, saffron is found in many countries including Iran, Turkey, India, Spain, and Greece [14].

The effects of saffron on a few other cancers, such as nervous system, prostate, breast, and colorectal cancer, should also be taken into consideration, given its documented benefits on the elimination of cancer cells. [15].

2 Materials and Methods

2.1 Plant Collection

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

2.2 Saffron Extraction

The first day included taking 40g from the dried stigma and styles *Crocus sativus*, then put it in a conical flask and dissolved it with 400 ml of distilled water. Thereafter, we put it on magnetic stirrer hotplate for 1 hour for better mixing, then put it in 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 40C° until dried. Last but not least, collected the powder of the extraction, using the powdered crude saffron extract for experiment.

2.3 Preparation of Saffron extract concentration

The IC_{50} was obtained from Graph-Pad Prism 6 and it value was 21.03 µg/ ml for SKOV-3, it was 73377.06 µg/ ml for normal cell.

2.4 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 100µg/ mL streptomycin. THE cells are passaged using Trypsin-EDTA reseeded at 80% confluence twice a week, and incubated at 37 °C. We used the same method that was used in[16,17].

2.5 Cytotoxicity Assays

To determine the cytotoxic effect of saffron, the MTT assay was done using 96-well plates [18,19]. The cell line were seeded at 1 × 104cells/well. 24 hour later, until the confluent monolayer was achieved A saffron treatment was given to SKOV-3 cells. However, after treatment for 24 and 48 hours, the media was removed, 28µL of a 2 mg/mL MTT solution was added, and the cells were then incubated for 2.5 hours at 37°C. This enabled the measurement of cell viability. Upon extracting the MTT solution, 130µL of Dimethyl Sulphoxide (DMSO) was added to the wells to solubilize the residual crystals. This was then incubated for 15 minutes at 37°C while being shaken [20]. As part of a triple assay, the absorbency was measured at 492 nm on a micro-plate reader. Equation (1) was used to calculate the percentage of cytotoxicity, or the inhibition rate of cell growth. n [21,22]:-

Inhibition rate (cytotoxicity) = A- B/A*100

where **A** is the optical density of control, and **B** is the optical density of the samples [23].

To visualize the shape of the cells under an inverted microscope, the cell were seeded into 24-well micro-titration plates at a density of 1×105 cells mL−1 and incubated for 24 hour at 37°C. Therefor, cells were exposed to saffron for 24hr. After exposure time, the plates were stained with crystal violet stain and incubated at 37°C for 10–15min [21]. The stain was washed off gently with tap water until the dye was completely removed [24].

2.6 Acridine Orange–Ethidium Bromide (AO-EB) Staining

The saffron-induced death of SKOV-3 cells was assessed using the AO-EB (Sigma–Aldrich, USA) staining method. In brief, 24 hour after the seeding of SKOV-3 cells in 24- well plates, they were treated with saffron at IC_{50} concentration 10 and then incubated for an additional 20 hour. The cells were washed twice with phosphate-buffered saline. The dual fluorescent dyes (10µL) were added into the wells at an equal volume of cells for 2min. Finally, the cells were observed using a fluorescent microscope [25,26].

2.7 Statistical Analysis

The obtained data were statically analyzed using an unpaired t-test with Graph-Pad Prism 6 [27]. The values were presented as the mean ± SD of triplicate measurements [28].

3 Results

3.1 The Cytotoxic Effect of Saffron

The cytotoxic effect of saffron against SKOV-3 cells was studied. The anti-proliferative activity of the saffron was tested by studying its ability to inhibit cell proliferation. The results demonstrated that the saffron has a highly cytotoxic effect against the SKOV-3 cell line as shown in Figure (1). The results showed that the saffron made clear morphological changes in SKOV-3 cell lines after treated, as shown in figures (2, 3, 4).

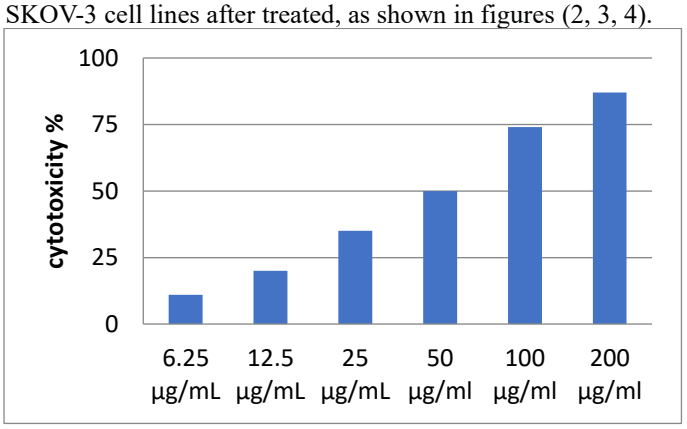

Fig. 1. The cytotoxicity effect of saffron Increases in SKOV-3 cells with increase concentration. IC_{50}=21.03µg/ml.

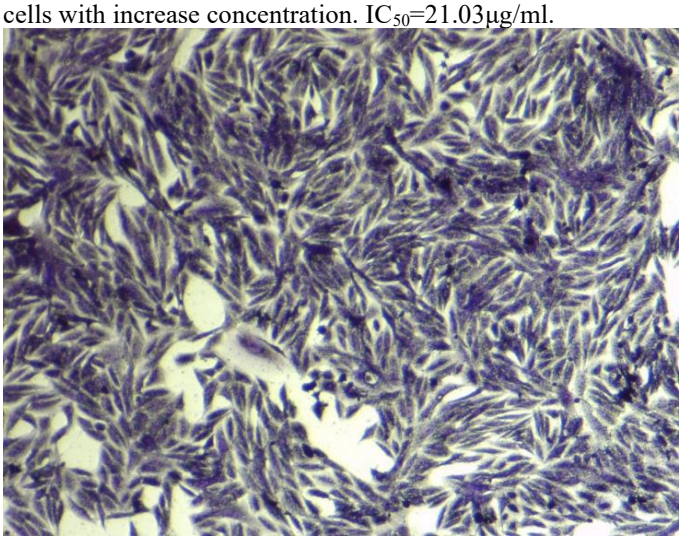

Fig. 2. Control untreated SKOV-3 cells, its shape as normal epithelial cells with clear extensions and high density, magnification power 10x.

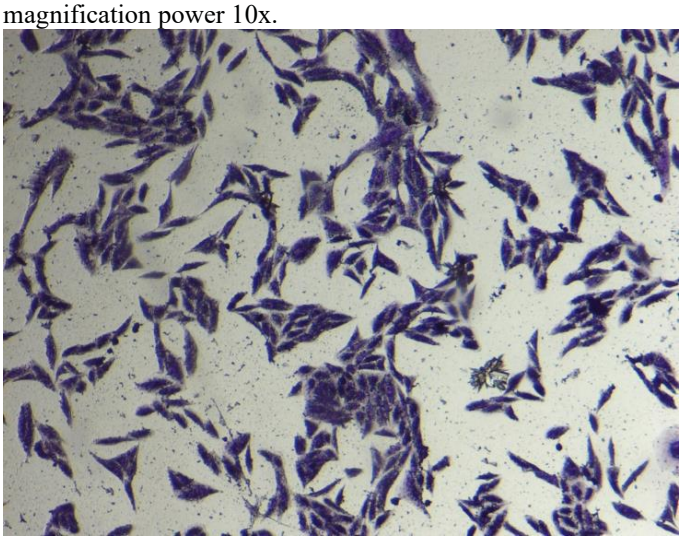

Fig. 3. Morphological changes in SKOV-3 cells after treated with saffron extract at 200µg/ml concentration, its unregulated shape and low density, magnification power 10x.

3.2 Saffron Induce SKOV-3 Cell Death

The findings of this work prove that the Saffron can induce apoptosis in the Ovarian cancer cells.by reduction the cell growth often involves the modification of various important

signaling pathways, which is caused by the induction of apoptosis, that affects gene expression levels. Moreover, the nuclear morphology of treated cells was evaluated using acridine orange–ethidium bromide (AO-EB) dual staining. Apoptotic cells were evaluated based on DNA damage. In this study, the saffron effectiveness is also investigated. The AO-EB staining was used to examine the different apoptotic features of the nuclear alterations. The non-apoptotic cells appeared green in color, and apoptotic cells appeared orange or red in color after staining with AO-EB, as shown in Figure 5.

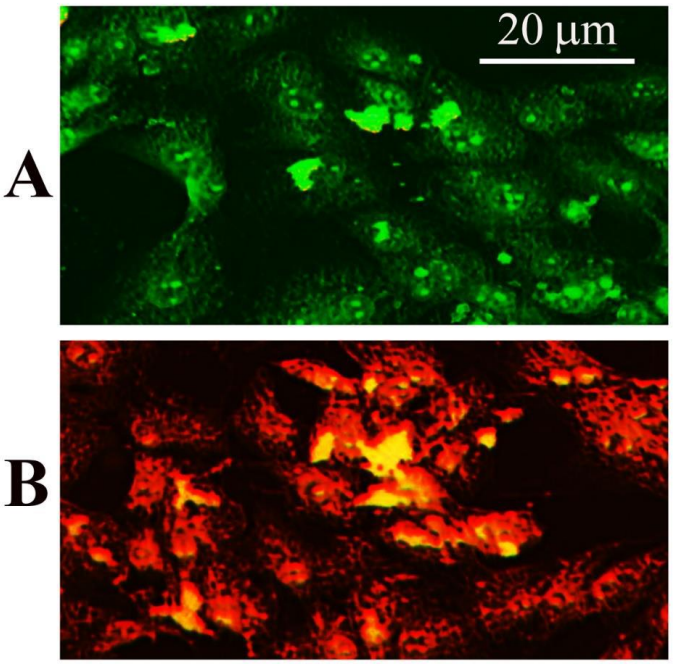

Fig. 5. Apoptosis markers in SKOV-3 cells following treatment with saffron. SKOV-3 cells treated as indicated. **A**, Control untreated cells. **B**, SKOV-3 cells after been treated with saffron. Control untreated cells are shown normal structure of cell without significant apoptosis or necrosis, after treated, red color indicated apoptosis by using AO-EB staining.

4 Discussion

4.1 Extraction Bioactive Components of Saffron

Saffron is one of the herbals that used as spice (as called as king of condiments) which applied in many food products, because of its aromatic, flavor and coloring properties [29,30]. Saffron called red gold, because of its medicine properties as antitussive, antioxidant, anti-inflammatory and anti-nociceptive, anti-depressing, memory enhancing and treatment of Alzheimer's disease [31,32,33].

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 [34,35]. Researchers have proved the effect of saffron aqueous extract on ovarian cancer [36,37].

4.2 Cytotoxicity Assay

As proven in the related works, the Saffron aqueous extract can play a vital role several cell lines of cancer, this work contributes to investigate and measure the effect of Saffron aqueous extract on SKOV-3 cell line. There are two techniques used in this work: MTT assay and acridine orange-ethidium bromide AO/EB staining.

Cell lines are cultures of animal cells that can be further subcultures repeatedly and sometimes indefinitely (called continuous cell lines) [38]. They arise from primary cell cultures, primary cultures are taken directly from the donor cells, tissues, or organs human or animals and are typically used in experiments within a few days [38]. The primary cell cultures are often isolated from mammalian cells because of the relevance of this to the mechanisms of cellular senescence and the origins of tumor cells for use during practical study (for use in drug screening and for biological assays) [40,41].

SKOV-3 is a cell line of human ovarian cancer with epithelial-like morphology, that was isolated from the ovary of a 64-year-old, Caucasian female with ovarian adenocarcinoma [42,43].

We used MTT assay technique to detection the cytotoxicity effect of saffron with IC_{50} concentration on SKOV-3 cell line. Thereafter, we observed the saffron has anti-proliferative activity was tested by studying their ability to inhibit the cells proliferation. Furthermore the results showed that the saffron has a highly cytotoxic effect against SKOV-3 cell line, but has no effect on normal human cells. The saffron cytotoxicity effect is efficacious, potent, and safe in cancer treatment through its action mechanism by blocking G-2/M. Furthermore, it arrests the cell cycle and anti-proliferation induces programmed cell death (apoptosis) caused by target-specific cancer treatment. As a result, this study proved that saffron is better herbal formulation for chemo-protective [44].

A study was performed, the lung cancer cells were influenced after it treated by saffron, where appeared morphology changes resulting by bioactive compounds of saffron because of its antitumor, anti-mutagenic and cytotoxicity properties [45]. Carotenoid is a major components (as crocin) in saffron are promoting tumor growth inhibition, i.e., components witless any side effects on normal cells [46]. In addition, the crocins and crocetins potent are inhibitors of carcinogenesis where inhibited nucleic acid and protein synthesis inside malignant cells [47].

There is a study, showed up the effect of saffron against osteosarcoma cells, which proved the morphology changes that gained by cells undergoing apoptosis are distanced cell shrinkage, chromatin condensation, nuclear fragmentation and membrane bleeding, all that was mentioned, indicate the saffron possesses potent biological and pharmacological properties [48].

4.3 Acridine Orange–Ethidium Bromide Staining

We also used another technique, acridine orange–ethidium bromide (AO-EB) dual staining, that reveal the modification of various important signalling pathways, that is caused by the induction of a programmed cell death mechanism which affects gene expression levels. Cancerous cells are more sensitive to the inhibitory effect of saffron on DNA, RNA and protein synthesis than healthy cells [49]. Nonetheless, the saffron exerts selective toxicity on the cancerous cell mechanisms including programming cell death (apoptosis), capturing cell cycle progression, tumor cell metabolism modulation, DNA fragmentation, LDH activity, antioxidant activity, and self-renewal gene expression reduction. In addition, it prevents tumor formation by reacting with and scavenging free radicals [50].

Croctin (one of the major three carotenoids available in saffron) is more interactive, binding directly with DNA and RNA from malignant cells and causing damage to them [51].
Recently, molecular study proved, that the saffron effect on gene-expression in malignant cells to cause DNA damage which leads to a blocking cell-cycle, it result in cell undergoes apoptosis [52]. Interestingly, that matches our findings during using acridine orange–ethidium bromide dual staining, where the cells were observed using a fluorescent microscope.

5 Conclusion

Our conclusion from the experiment and studies mentioned earlier, the saffron has a highly cytotoxic effect against SKOV-3 cell line, the saffron concentration of 200 µg/ml kills 87% of cancer cells, and the half-maximal inhibitory concentration (IC_{50}) is equal to 21.03 µg/ml, the extract causes damage to DNA which leads to the apoptosis of cancer cells by some signs that occur in different ways, moreover, it works to release free radical molecules in a way that kills cancer cells.

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