Evaluation of the Antiproliferative Effect of Safranal in C-4 I Cervical Cancer Cell Line

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ABSTRACT

Aim: Safranal is a bioactive compound responsible for the aroma propriety of Crocus sativus. Many studies have shown the antioxidant activity of safranal besides some pharmacological properties, including its anti-inflammatory effect. Our study aimed on identifying the cytotoxic effects of safranal on the C-4 I cell line of cervical cancer.

Methods: The cytotoxic effect of safranal on the C-4 I cell line was determined after incubating the cells for certain durations (2 to 72 hours) and concentrations (25 to 800 µM). After incubation, cell viability and anti-proliferation effect of safranal were determined respectively by Mitochondrial Dehydrogenase and Lactate dehydrogenase enzymes activities assays. Additionally, morphological changes occurring during incubation in cells were examined with inverted and optical microscope using Giemsa staining.

Results: According to the results, compared to Control group, the % viability of treated cells was decreased depending on concentration and the incubation time, and safranal significantly inhibited the growth of C-4 I cells ($p<0.05$). Some morphological changes such as nuclear condensation, and apoptotic and pyknotic cells were examined under light microscopy with Giemsa staining.

Conclusion: In conclusion, based on these results safranal has an antiproliferative effect against cervical cancer C-4 I cell lines.

Keywords: C-4 I, Safranal, Cervical cancer, Cytotoxicity, Antiproliferative

Safranal'ın Servikal Kanseri C-4 I Hücre Hattı Üzerindeki Antiproliferatif Etkisinin Değerlendirilmesi

ÖZET

Amaç: Safranal, Crocus sativus'un aroma özelliğinden sorumlu bioaktif bir bileşendir. Safranalın antiinflamatuar etkisi dahil olmak üzere farmakolojik özelliklerinin yanı sıra antioksidan aktivitesi birçok çalışmada gösterilmiştir. Çalışmamızda, safranalın serviks karsınoma kökenli C-4 I hücre hattı üzerindeki sitotoksik etkilerini tespit etmek amaçlanmıştır.

Yöntem: Safranalın C-4 I hücre hattı üzerindeki sitotoksik etkisini belirlemek için hücreler belirli sürelerde (2-72 saat) ve konsantrasyonlarda (25-800µM) inkübe edildi. İnkübasyon süresi bitiminde hücrelerin viabilitesi ve safranal antiproliferatif etkisi sırasıyla Mitokondriyal Dehidrojenaz ve Laktat dehidrojenaz enzimlerinin aktiviteleri ile belirlendi. Bununla birlikte, inkübasyon süresince meydana gelen morfolojik değişiklikler Faz kontrast ve ışık mikroskopları aracılığıyla Giemsa boyama kullanılarak gözlemlendi.

Bulgular: elde edilen sonuçlara göre, safranal ile tedavi edilen hücreler kontrol grubu ile karşılaştırıldığında hücrelerin canlılık yüzdesi doza ve inkübasyon süresine bağlı olarak azaldı ve safranal C-4 I hücrelerinin çoğalmasını anlamlı bir şekilde inhibe etti ($p<0.05$). Nükleer yoğunlaşma, apoptotik ve piknotik hücreler gibi bazı morfolojik değişiklikler ışık mikroskobu altında Giemsa boyama ile incelendi.

Sonuçlar: Elde edilen bulgulara dayanarak safranalın serviks kanseri kökenli C-4 I hücre hatlarına karşı antiproliferatif bir etkiye sahip olduğu söylenebilmektedir.

Anahtar Kelimeler: C-4 I, Safranal, Serviks karsinoma, Sitotoksisite, Antiproliferatif

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INTRODUCTION

Cancer encompasses a group of diseases characterized by uncontrolled proliferation and invasiveness which constitutes a major public health problem worldwide. Cancer also ranks second as the leading cause of death (Gavas et al., 2021). Cancer incidence and mortality rates are increasing by approximately 1% each year and this rate is expected to cause approximately 26.4 million new cases and 1.7 million cancer deaths by 2030 (Siegel et al., 2020). Cervical cancer is one of the most common malignant tumours among women, ranking second as the cause of death worldwide. This cancer is a virus-borne disease caused by human papillomavirus (HPV) which integrates with a high risk into the host's genome (González Martín, 2007). The treatment methods used in patients with cervical cancer are regional treatments such as surgery, radiotherapy, and chemotherapy (Liontos et al., 2019). These therapeutic methods which are used commonly in clinics cause side effects during and after treatment.
Safranal, abundant in Crocus sativus essential oil, is a bioactive compound with monoterpene aldehyde properties responsible for the aroma and odor of the plant. Safranal has antioxidant activity (Rezaee and Hosseinzadeh, 2013; Cerdá-Bernad et al., 2022) as well as pharmacological effects such as anti-inflammatory, antidepressant, anxiolytic, anti-asthmatic, antihypertensive, anticonvulsant, antitussive and antigenotoxic (Mollazadeh et al., 2015; Nanda and Madan, 2021; Esmaeilzadeh et al., 2023). Many kinds of research have shown the therapeutic effect of safranal against some diseases such as diabetes (Maeda et al., 2014), cardiovascular diseases (Toledo-ibelles and Mas-Oliva, 2018), malignant diseases, and Alzheimer's (Samarghandian et al., 2017). Additionally, the antitumor activity of safranal has been demonstrated in some cancer cell lines (Samarghandian and Shabestari, 2013; Jabini et al., 2017; Chaiboonchoe et al., 2018; Zarrineh et al., 2022; Burak and Güven, 2023).
The primary goal of most cancer treatment research is finding an effective therapeutic agent capable of neutralizing cancer cells without damaging normal cells or minimizing the damage observed during and after treatment. In this context, our study aimed to identify the cytotoxic effects of safranal on the human cervical cancer C-4 I cell line. We focused on the effects of safranal treatment, particularly on its cytotoxic effect on C-4 I, such as viability, proliferation, and cell morphology.

METHODS

Cell Culture

The culture of C-4 I cells (ATCC, CRL1594) was carried out with Waymouth MB 752/1 (Gibco) medium supplemented %10 Fetal Bovine Serum (Gibco) and %1 of 100 µg/ml of penicillin and streptomycin at 37°C in 95% humid atmosphere containing 5% CO_2 (Ozcan et al., 2016).

Evaluation of Cell Morphologies

C-4 I cells (120 x 10^4 cells/800 µl) were seeded in a 24-well plate. After incubation of the control and experimental groups (24-72 h) with safranal (25-800µM), cells were fixed with Carnoy's fixative

(1:3) for 10 minutes. It was then washed twice for five minutes with 70% alcohol and left to dry. Afterwards, fixation, cells were stained with Giemsa at +4°C for 6mn. Morphological changes in cells were observed under the inverted and light microscopies (10X25, 10X40) (Ozcan et al., 2016).

Mitochondrial Dehydrogenase (MTT) Assay

C4-I cells were seeded in 96-well cell plates at 3 x 10^4 cells/200 µl. After 24 hours, cells were incubated with safranal (Sigma, CAS: 116-26-7, Indian). After incubation time, 40µl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Duchefa Biochemie) were added to the cells. In order to dissolve the formazan crystals formed after a 4 h incubation period with MTT, 160 µl of dimethylsulfoxide (DMSO; BioFroxx, CAS: 67-68-5) was supplemented and incubated overnight at 37°C in 95% humid atmosphere containing 5% CO2. After incubation, absorbance values were measured at 570 nm with reference to the 690 nm in the µQuant ELISA plate reader (µQuant, Bio-tek Instruments, INC.) (Ozcan et al., 2016; Ozsoylemez and Ozcan, 2018).

Lactate Dehydrogenase (LDH) Assay

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme found in almost every cell in the body. When cells are exposed to a toxic substance, the integrity of their plasma membrane is disrupted and the LDH enzyme escapes from the cells and passes into the medium. LDH is a very important biomarker to show the presence of secondary apoptosis and necrosis (Tokur and Aksoy, 2017; Erkekoğlu and Baydar, 2021). In our study, an LDH kit (Cytotoxicity Detection Kit, Roche, Ref:11644793001; Lot:51751400) was used to measure the amount of LDH released into the medium as a result of the disruption of plasma membrane integrity in secondary apoptotic/necrotic cells to examine the cytotoxic effects of safranal in C-4 I cell lines. The cells used in the experiments were seeded to 96 well plates at 3 x 104 cells/200 µl per well. Cells were treated with safranal for 24-72 h. After incubation, 100µl of the medium was withdrawn from the medium whose cells were incubated and transferred to a new 96-well plate. 100µl of the freshly prepared reaction mixture in the kit was added to the wells and incubated for 30 minutes at room temperature in the dark. At the end of this period, absorbance values at 490-492 nm were read on the µQuant ELISA Plate reader (µQuant, Bio-tek Instruments, INC.)

Statistical Analysis

All results are expressed as mean with the mean and standard deviation of the average of all 3 independent analyses. Significant data were determined statistically with ANNOVA test: (ANNOVA: Single Factor) according to the value of $p<0.05$.

FINDING AND DISCUSSION

Morphological Assessment

Some morphological changes were observed under an inverted microscope in C-4 I cells during treatment (24-72 hours) with safranal (25µM, 50µM, 100µM, 200µM, 400µM and 800µM) depend on concentration and incubation time (**Figure 1**). In addition, These changes included cell shrinkage, nuclear condensation, pyknosis, and apoptotic bodies were clarified with Giemsa staining under light microscope. It was also observed also a decrease in cell density along with an increase in cell condensation and the number of apoptotic cells (**Figure 2**)..

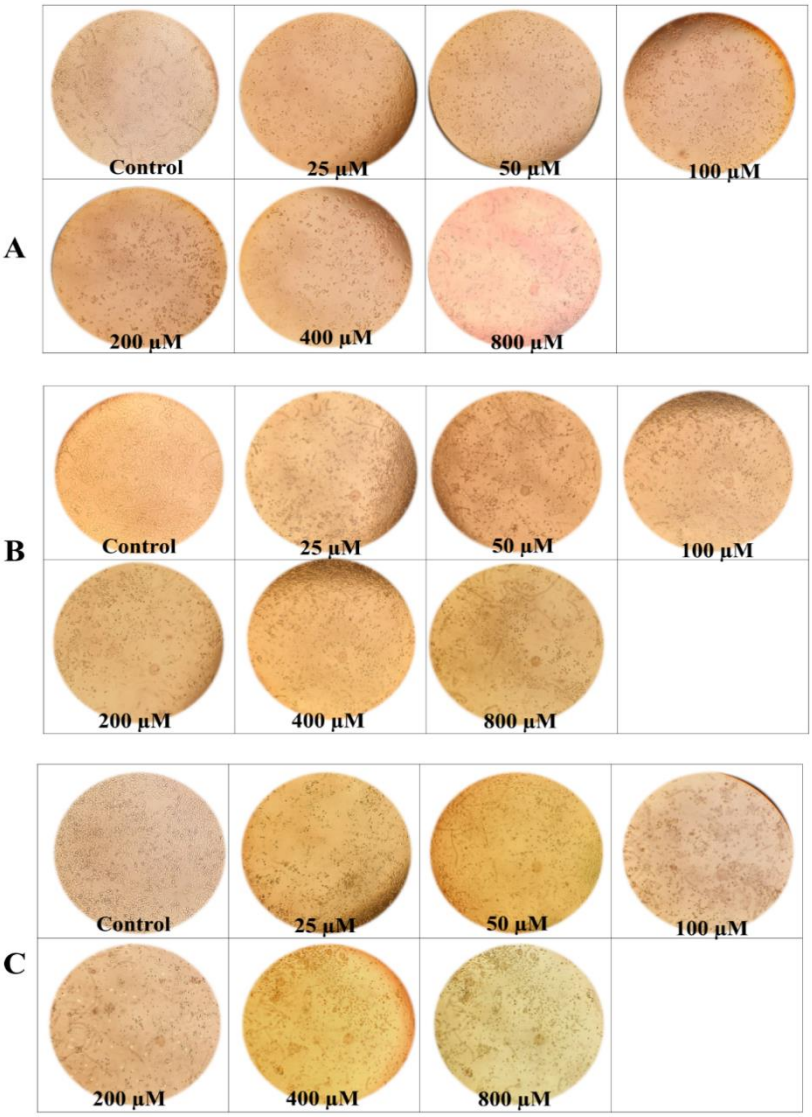

Figure 1. Inverted microscopy images of Control group and treated groups for 24 (**A**), 48 (**B**), and 72 (**C**) hours (10x25).

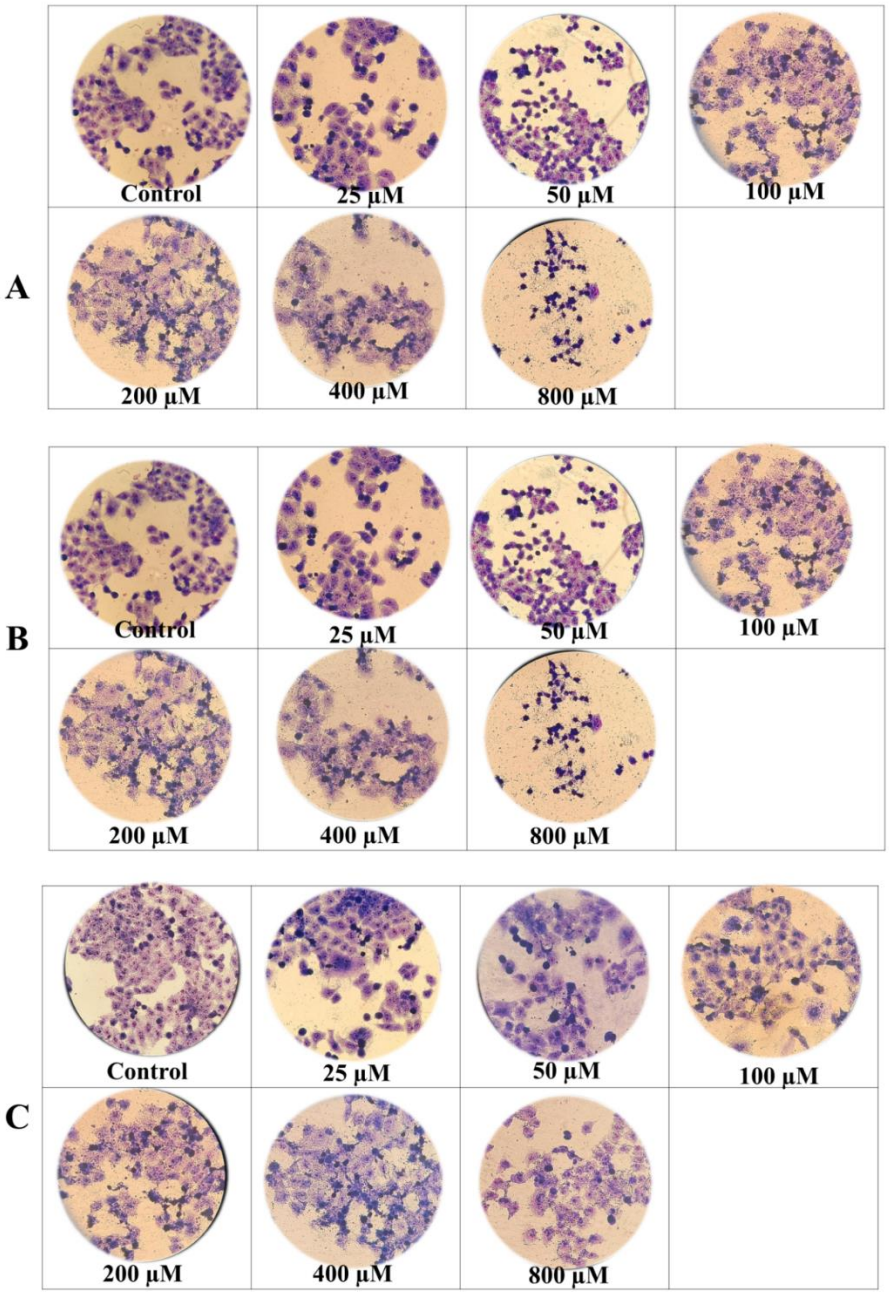

Figure 1. Giemsa staining images of Control group and treated groups for 24 (A), 48 (B), and 72 (C) hours (Light microscopy; 10X40).

Mitochondrial Dehydrogenase Assay

MTT assay was applied for assaying cytotoxic effect of safranal on C4-I cells were treated for 24, 48, and 72 hours with a range of concentrations (25-800 µM). The % viability values were calculated based on absorbance values determined after the incubation period. The viability of cells treated with safranal compared to the control group was decreased depending on concentration and time (*$p<0.05$) (**Figure 3, Table 1**).

Table 1. Absorbance values of C- 4 I treated with safranal at 24, 48, and 72 h (D1=25µM, D2= 50µM, D3=100µM, D4=200µM, D5=400µM, D6=800µM) (* $p<0.05$, compared to Control group).

	Absorbance values (570 – 690 nm). Mean ±SD		
Groups	24 h	48 h	72h
Control	$635.1x10^{-3} \pm 0.02$	$795.5x10^{-3} \pm 0.01$	$768.07x10^{-3} \pm 0.01$
D1	$510.2\ x10^{-3} \pm 0.03$*	$548\ x10^{-3} \pm 0.02$*	$390.28\ x10^{-3} \pm 0.02$*
D2	$441.7x10^{-3} \pm 0.01$*	$389x10^{-3} \pm 0.01$*	$338.07x10^{-3} \pm 0.01$*
D3	$444.7\ x10^{-3} \pm 0.01$*	$310.1x10^{-3} \pm 0.02$*	$291.88x10^{-3} \pm 0.02$*
D4	$373.9\ x10^{-3} \pm 0.03$*	$290.3x10^{-3} \pm 0.02$*	$260.47\ x10^{-3} \pm 0.02$*
D5	$329.7\ x10^{-3} \pm 0.03$*	$215\ x10^{-3} \pm 0.02$*	$200.27\ x10^{-3} \pm 0.02$*
D6	$267.3\ x10^{-3} \pm 0.01$*	$199\ x10^{-3} \pm 0.01$*	$156.4x10^{-3} \pm 0.01$*

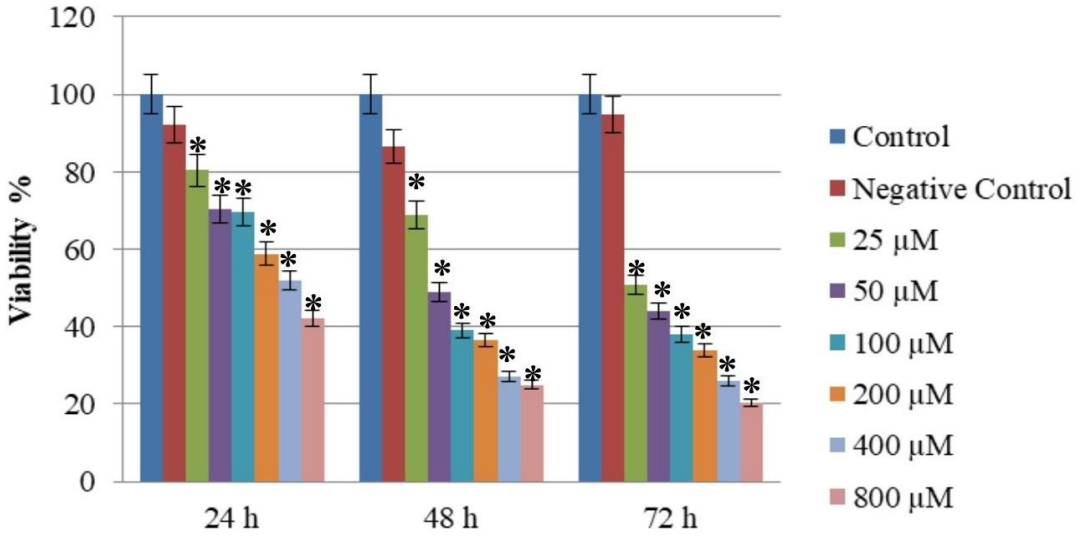

Figure 3. Viability values (%) of C-4 I treated with safranal for 24, 48 and 72 hours (negative control = DMSO; *$p<0.05$).

After applying safranal to C-4 I cells, a significant reduction was observed in the viability rate values compared to the control group. The decrease was concentration and time-dependent. For 24 hours the viability values were decreased to 80.33%, 70.38%, 69.59%, 58.87%, 51.91%, and 42.09% at doses D1, D2, D3, D4, D5, and D6, respectively. Similarly, for 48 hours, the viability values were reduced to 68.89%, 48.9%, 38.98%, 36.49%, 27.03%, and 25.02% at doses D1, D2, D3, D4, D5, and D6, respectively. Finally, at 72 hours, the viability values of cells decreased to a minimum of 20.31% at D6.

Lactate Dehydrogenase Assay

When C4- I cells were exposed, the LDH enzyme is released from the cytoplasm. LDH is released when cells are dead at seconder apoptosis and necrosis. LDH assay was applied to approve the results obtained in MTT assay. The percentage of inhibition of proliferation and growth of cells treated

with safranal is calculated based on the determined absorbance values (**Figure 4**). It was observed that safranal inhibited the proliferation and growth of cervical cancer C-4 I cell line by seconder apoptosis and necrosis.

Table 2. Absorbance values of C- 4 I treated with safranal at 24, 48, and 72 h (D1=25µM, D2= 50µM, D3=100µM, D4=200µM, D5=400µM, D6=800µM) (* p<0.05, compared to control group).

Absorbance values (490-492 nm). Mean ±SD			
Treatment groups	24 h	48 h	72h
Control	$111.27x10^{-3} \pm 0.02$	$632.9 \times 10^{-3} \pm 0.02$	$1107.9x\ 10^{-3} \pm 0.02$
D1	$173.97 \times 10^{-3} \pm 0.04$*	$1010.6\ x10^{-3} \pm 0.01$*	$2340.4 \times 10^{-3} \pm 0.03$*
D2	$222.37\ x10^{-3} \pm 0.04$*	$1182.3 \times 10^{-3} \pm 0.04$*	$2845.7x\ 10^{-3} \pm 0.03$*
D3	$256.43 \times 10^{-3} \pm 0.02$*	$1271.1 \times 10^{-3} \pm 0.01$*	$3021.83 \times 10^{-3} \pm 0.02$*
D4	$290.03\ x10^{-3} \pm 0.04$*	$1377 \times 10^{-3} \pm 0.02$*	$3342.9 \times 10^{-3} \pm 0.36$*
D5	$325.73\ x10^{-3} \pm 0.03$*	$1413.1 \times 10^{-3} \pm 0.02$*	$3844.6x\ 10^{-3} \pm 0.01$*
D6	$364.6\ x10^{-3} \pm 0.02$*	$1445\ x10^{-3} \pm 0.02$*	$4245.86x10^{-3} \pm 0.01$*

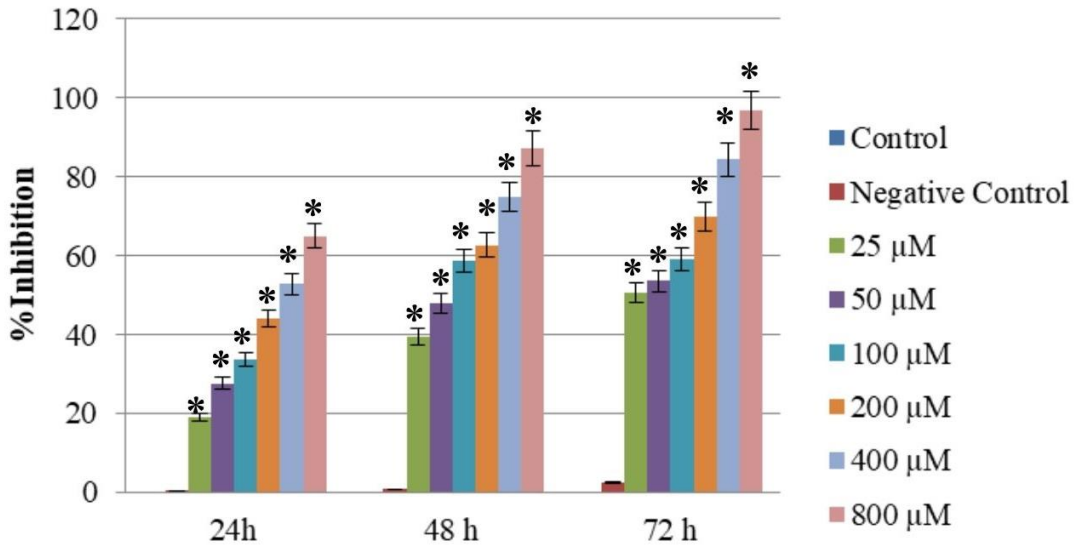

Figure 4. Inhibition values (%) of C-4 I treated with safranal for 24, 48 and 72 hours (Negative control = DMSO; *p<0.05)

According to Figure 4, the inhibition rates depend on concentration and time; after 24 hours, the inhibition rate was increases with the concentration and reached 65.04% at the last concentration (800 µM) compared to the control. Over 48 hours, the inhibition rate increased further and reached 87.26% at the last dose. Finally, for 72 hours, the inhibition was reduced to a maximum rate of 96.79% at the last concentration.

Medicinal plants are the main source of treatment in traditional medicine. Their natural antiseptic properties make them important in medical research on the study of the pharmaceutical and therapeutic properties of plant extracts for the treatment of many diseases, including cancer. In many

studies, plant species focusing on compounds contained in plants and exhibiting anti-cancer properties have been identified in developing countries (Chermahini et al., 2010). Plants are considered one of the main sources for the development of new cancer chemopreventive drugs due to their potential to inhibit oxidative stress (Greenwell and Rahman, 2015). *Crocus sativus L.* (Saffron) is a rootless annual herbaceous plant of the famous Iridaceae family whose flower stigmas, properly named saffron, are one of the most expensive aromatic herbs in the world (Mollazadeh et al., 2015). Safranal, one of the important compounds of Saffron, is a bioactive compound responsible for the aroma and odor proprieties of this plant which has potential antioxidant properties as well as photoprotective properties. Studies have demonstrated that, pharmacologically, safranal has the ability to eliminate free radicals. However, its protective effect against oxidants produced by ischemia-reperfusion injury has also been determined (Hosseinzadeh and Sadeghnia, 2005; Cerdá-Bernad et al., 2022). In addition, the antitussive and anticonvulsant activities of safranal have been mentioned in numerous studies (Malaekeh-Nikouei et al., 2013; Moratalla-Lopez et al., 2019).

Our research focused on examining the cytotoxic properties of safranal on the Cervical C-4 I cancer cell line. We observed a decrease in cell density at 24, 48, and 72 hours after treatment, along with some morphological changes such as nucleic condensation, cell shrinkage, and cell fragmentation under light and inverted microscopies (**Figures 1** and **2**). These morphological changes are considered indicators of apoptosis and necrosis. The results of the MTT assay also indicated a decrease in the viability rate of cells treated with safranal (**Figure 3**). Additionally, this effect is approved by LDH assay's findings which showed that safranal has the potential to inhibit the growth and proliferation of C-4 I cells (**Figure 4**). Our study demonstrated that safranal can initiate apoptosis and necrosis in C-4 I cells by inhibiting proliferation. The data obtained in this study confirmed that safranal has a cytotoxic effect on C-4 I cells, which is dependent on the dose and incubation time.

Several studies have shown its antitumor activity in some cell lines. Many of these studies demonstrated that saffron extract and its derivatives exhibit selective toxicity between cancer cells and normal cells, meaning that saffron and its derivatives do not have a toxic effect on healthy cells (Milajerdi et al., 2016). . The investigation of Samarghandian and Shabestari (2013) on human prostate cancer cells concluded that safranal has a cytotoxic effect and the ability to initiate apoptosis against prostate PC-3 cancer line cells by inhibiting the proliferation and growth of PC-3 cancer cells. In Jabini et al. (2017) study, safranal has been shown on oral squamous cancer cell lines NIH 3T3 (Healthy) and KB (Cancer). This cytotoxicity effect against oral squamous cancer cells acts by inhibiting the growth of KB cell lines. It was determined that this effect was partially selective against KB cells because it was biologically very low on health (NIH 3T3) cells. Cheriyamundath et al. (2018) study showed that safranal has the potential to inhibit HeLa cell viability. In Zarrineh et al. (2022) study, the antiproliferative effect of safranal was investigated in breast cancer. They found that Safranal induces a significant decrease in the viability of MDA-MB-231, MCF10A, and MDA-MB468 cell lines in different concentrations. According to Hatziagapiou et al. (2022), the Saffron carotenoids (Crocin

Extracts and Dimethylcrocetin) have been found to have anticancer properties against glioblastoma and rhabdomyosarcoma cell lines through apoptosis death pathways. This study showed that this apoptotic effect was upregulated by BAX and BID, while it was downregulated by MYCN and BCL-2, SOD1, and GSTM1. Bakshi et al. (2022) study demonstrated that crocin has a significant antiproliferative effect on colon cancer cells (HT-29, Caco-2) and human umbilical vein endothelial cells (HUVEC) by inhibiting angiogenesis, migration, invasion, and metastasis of colon carcinoma cells through the down-regulation of VEGF via the NF-kB pathway. The investigation of Xu et al. (2022) showed that crocin exhibited anti-inflammatory and anti-proliferative effects on breast cancer cells by inhibiting the PRKCQ/NF-κB pathway. This study revealed that crocin suppressed cell viability and proliferation and led to a reduction in the levels of TNF-α and IL-1β.

The results of our study correlate with previous studies which demonstrated that safranal has an antiproliferative effect against cancer cells. But they are not sufficient to confirm the type of safranal death pathway on C- 4 I cells. To clarify the type of death (apoptosis or necrosis) and the molecular pathways under these effects, more staining assays and molecular analysis should be applied. The current findings do not provide enough evidence to determine the type of safranal death pathway on cells. In order to identify inhibition caused by safranal on cells, and to understand the molecular pathways involved, additional staining assays and molecular analysis must be conducted.

CONCLUSION

According to the results obtained in our study, it can be said that safranal has an anti-proliferative effect against cervical cancer C-4 I cell lines. This antiproliferative effect can be induced by cell death types such as apoptosis and necrosis. However, further research is needed to fully clarify the molecular mechanism of anticancer effects of safranal. In future studies, we will be focusing on identifying the molecular pathways involved in this effect through molecular assays and analyses.

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Conflict of Interest

The authors declare that they have no competitive interests.

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