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ANTI-PROLIFERATIVE, PRO-APOPTOTIC, AND CHEMOSENSITIZING EFFECTS OF CARTHAMUS TINCTORIUS (SAFFLOWER) EXTRACT ON BREAST CANCER CELL LINE

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ABSTRACT – ***Objective:*** Breast cancer is one of the most common types of cancer and one of the major causes of cancer deaths in women. Considering the increasing prevalence of this cancer and the ineffectiveness of common treatments, finding new treatment strategies seems necessary. In this study, the anti-proliferative properties of aqueous, methanolic, and ethanolic extracts of Carthamus tinctorius (safflower) were evaluated. Then, the pro-apoptotic, and chemosensitizing effects of the most cytotoxic extract were tested.

Materials and Methods: The aqueous, methanolic, and ethanolic extracts of the plant were prepared and their effects on the viability of MCF-7 breast cancer cells were investigated after 24, 48, 72, and 96 hr treatment by MTT assay. The extract with the most effect was selected. The effect of the extract on apoptosis was estimated using Annexin V-FITC/PI staining and Real-time PCR. Then, the effect of co-treatment with the extract and tamoxifen was investigated using compusyn software.

Results: Among aqueous, methanolic, and ethanolic extracts, ethanolic extract had the greatest effect in reducing the viability of cancer cells. This extract significantly increased apoptosis and Bax/Bcl-2 expression ($p<0.05$). The co-treatment with extract and tamoxifen showed synergistic toxicity.

Conclusions: The safflower plant has anti-proliferative and pro-apoptotic effects on breast cancer cells and can synergistically intensify the toxic effect of tamoxifen.

KEYWORDS: Safflower, Carthamus tinctorius, Tamoxifen, Breast cancer, Bax/Bcl-2.

INTRODUCTION

Breast cancer is one of the most common cancers affecting women across the world. In 2012 around 1.7 million patients were diagnosed with breast cancer and 522,000 deaths were reported[1]. Tamoxifen has been the first choice for adjuvant therapy for breast cancer since its discovery in 1970, but 20-30% of tumors are resistant to tamoxifen therapy, which was either present before the treatment (Inherent resistance) or develops during the therapy (acquired resistance). These resistances are still one of the major hurdles in the effective management of breast cancer. Several biochemical factors and molecular pathways had been ob-

served as key factors for tamoxifen resistance. The resistance leads to the development of other cancers like uterine cancer[2]. Also, like many cancer drugs, tamoxifen has many adverse effects associated with it[3]. Plant extracts comprise a complex mixture of natural compounds with diverse biological properties including anticancer activities. Therefore, the study of these extracts has received much attention in cancer research. In addition, plants' active constituents such as polyphenols could confer protective effects against damage by free radicals as well as lessen the toxicity of chemotherapeutic drugs on normal cells. Besides enhancing the therapeutic efficacy, this has also been proven to reduce the dosage of chemotherapeutic drugs used, and hence overcome multiple drug resistance and minimize treatment side effects[4].

Safflower (Carthamus tinctorius L.) is a plant of the Compositae family, which is mainly used to produce edible oil and as bird feed. The flower flag of this plant is used to color and make food products desirable and also as medicine. This plant is cheaper than saffron, and for this reason, its flowers are widely used to color traditional Iranian dishes, especially in restaurants, instead of saffron. Safflower seed oil is also very welcome as a low-harm liquid vegetable oil. It has many medicinal effects such as protective effects on the heart and nerves as well as anti-tumor activity[5-7]. The beneficial effects of safflower on the urinary system have also been reported[8-10]. It is a native plant of Iran that is cultivated in the western and central regions of this country[11] and despite the wide use of this plant, very few studies have been done on its therapeutic and beneficial properties. This study was conducted to determine the effect of different extracts of safflower plant alone and together with tamoxifen on breast cancer cells.

MATERIALS AND METHODS

Preparation of plant materials and extracts

The plant was collected from gardens around Kermanshah city (the capital of Kermanshah Province, in the western part of Iran) and confirmed by a botanist. The aerial parts of the plant were first dried in the shade and then crushed to a fine powder. Then, 0.1 g of the sample was mixed in 10 ml of distilled water or 80% ethanol (Sigma-Aldrich, St. Louis, MO, USA) or 80% methanol (Sigma-Aldrich) and placed in a Bain-Marie apparatus at 70°C for an hour. Then, it was placed in a centrifuge for 15 min at 2000 rpm and the resulting mixture was smoothed. Finally, the obtained sample was dried at room temperature and stored at -20°C. At the time of use, it was dissolved in a serum-free medium.

This research was approved in agreement with the Helsinki declaration with permission from the Kermanshah University of Medical Sciences Ethics Committee (Ethics No. IR.KUMS.MED.REC.1401.078)

Cell culture

MCF-7 breast cancer cell line (Pasteur Institute, Tehran, Iran) was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NJ, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco) without antibiotics. The cultures were kept in a humidified incubator at 37°C.

Viability evaluation

MTT colorimetric assay was used to investigate the toxicity effect of the extracts on the growth and proliferation of cancer cells and to determine the IC50 values. The cells were seeded (15×10^3 cells/well) in 96 well plates. After 24 hr, they were treated with increasing concentrations (6.25, 12.5, 25, 50, 100, 200, 400, and 800 µg/ml) of extracts. After 24, 48, 72, and 96 hr, 30 µl of MTT solution (Sigma-Aldrich) was added to each well. After 4 hr incubation in the dark, the supernatant solution from each well was replaced with 200 µl of dimethyl sulfoxide (Sigma-Aldrich). Finally, the absorption was measured at 540 nm by an ELISA reader. The percentage of cell viability was expressed relative to the control cell control. The IC50 values were calculated using GraphPad Prism version 6 software (La Jolla, CA, USA).

Apoptosis evaluations

The cells were treated with an IC50 concentration of ethanolic extract. After 24 hr, they were trypsinized and suspended (5 ×105 cells) in 500 µl of binding buffer (BioLegend, London, UK). Then, 5 µl of annexin V-FITC (Abcam

Inc., Cambridge, MA, USA) and 5 µl of propidium Iodide (PI; Sigma-Aldrich) were added. Samples were incubated in the dark at room temperature for 5 min. Finally, 10,000 cell events in each sample were analyzed by DML program. Also, the percentage of DNA fragmentation was determined by diphenylamine assay as described by Cohen and Duke, and the absorbance of samples was measured at 600 nm using a spectrophotometer.

Gene expression evaluation

Real-time PCR technic was used to determine the Bax/Bcl-2 expression in control and treated cells. After 24 hr treatment with IC50 concentration of ethanolic extract, RNA was isolated from 1×10^6 cells with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The purity and integrity of RNA were evaluated using a NanoDrop spectrophotometer and gel electrophoresis. Complementary DNA (cDNA) was synthesized according to the cDNA synthesis kit (Vivantis Technologies kit, Selangor DE, Malaysia) procedure. Real-time PCR was conducted by SYBR Premix Ex Taq Technology (TaKaRa Bio Inc., Otsu, Shiga, Japan) and according to the manufacturer's protocol. The fold changes were obtained based on the comparative Ct ($2^{-\Delta\Delta ct}$) method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control. All the primer sequences were designed by GeneRunner software (Hastings Software, Hastings, NY, USA) version 3.05. The primer sequences were as follows:

Bax Forward: 5´-CCTGTGCACCAAGGTGCCGGAACT-3´ and Reverse: 5´-CCACCCTGGTCTTGGATCCAG-CCC-3´.

Bcl-2 Forward: 5´-TTGTGGCCTTCTTTGAGTTCGGTG-3´ and Reverse: 5´-GGTGCCGGTTCAGGTACT-CAGTCA-3´.

Median effect analysis

The cells were treated with increasing concentrations of tamoxifen (0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 µg/ml) for 24 hr. Then, the viability was measured using MTT assay. After calculating the IC50 of tamoxifen, the co-treatment was done by combining tamoxifen and ethanolic extract simultaneously in two higher and two lower concentrations than IC50 values, and the MTT assay was repeated. 5 co-treatment groups were used, including Group 1: 98.33 µg/ml of extract + 1.07 µg/ml tamoxifen; Group 2: 196.67 µg/ml of extract+ 2.15 µg/ml tamoxifen; Group 3: 393.34 µg/ml of extract+ 4.31 µg/ml tamoxifen; Group 4: 786.68 µg/ml of extract+ 8.62 µg/ml tamoxifen; and Group 5: 1573.36 µg/ml of extract+ 17.24 µg/ml tamoxifen.

Determination of combination indexes (CI) and dose reduction indexes (DRI) were performed by CompuSyn software (NJ, USA). CI was determined based on the equation CI = (D)1 / (Dx)1 + (D)2 / (Dx)2, where (Dx)1 and (Dx)2 are the dose of extract and tamoxifen alone that inhibits x% and (D)1 and (D)2 are the amounts of extract and tamoxifen in the combination that inhibit the experimentally observed x. A CI < 1 indicates synergy, a CI = 1 indicates additivity and a CI > 1 indicates antagonism. In addition, DRI was calculated according to the equation (DRI)1 = (Dx)1/(D)1 and (DRI)2 = (Dx)2/(D)2. DRI value defines the degree to which the concentration of a drug is decreased when combined with another drug to maintain an equal efficacy, and Fa is the fraction of cell death, varying from 0 (no death) to 1 (100% death).

Statistical Analysis

The tests were done in triplicate and the results were expressed as means ± Standard error of the mean (SEM). One-way analysis of variance with Tukey's test correction was used to determine significant differences between groups, with $p<0.05$ considered statistically significant.

RESULTS

The results of MTT assay showed that both aqueous and methanolic extracts of Safflower significantly reduced cell viability in concentrations higher than 50 µg/ml after 24 hr ($p<0.05$). After 48 hr, reduction in cell viability was significant in concentrations higher than 12.5 µg/ml ($p<0.05$). Also, after 72 and 96 hr, this reduction was significant in all the concentrations ($p<0.05$) (Figure 1 A and B).

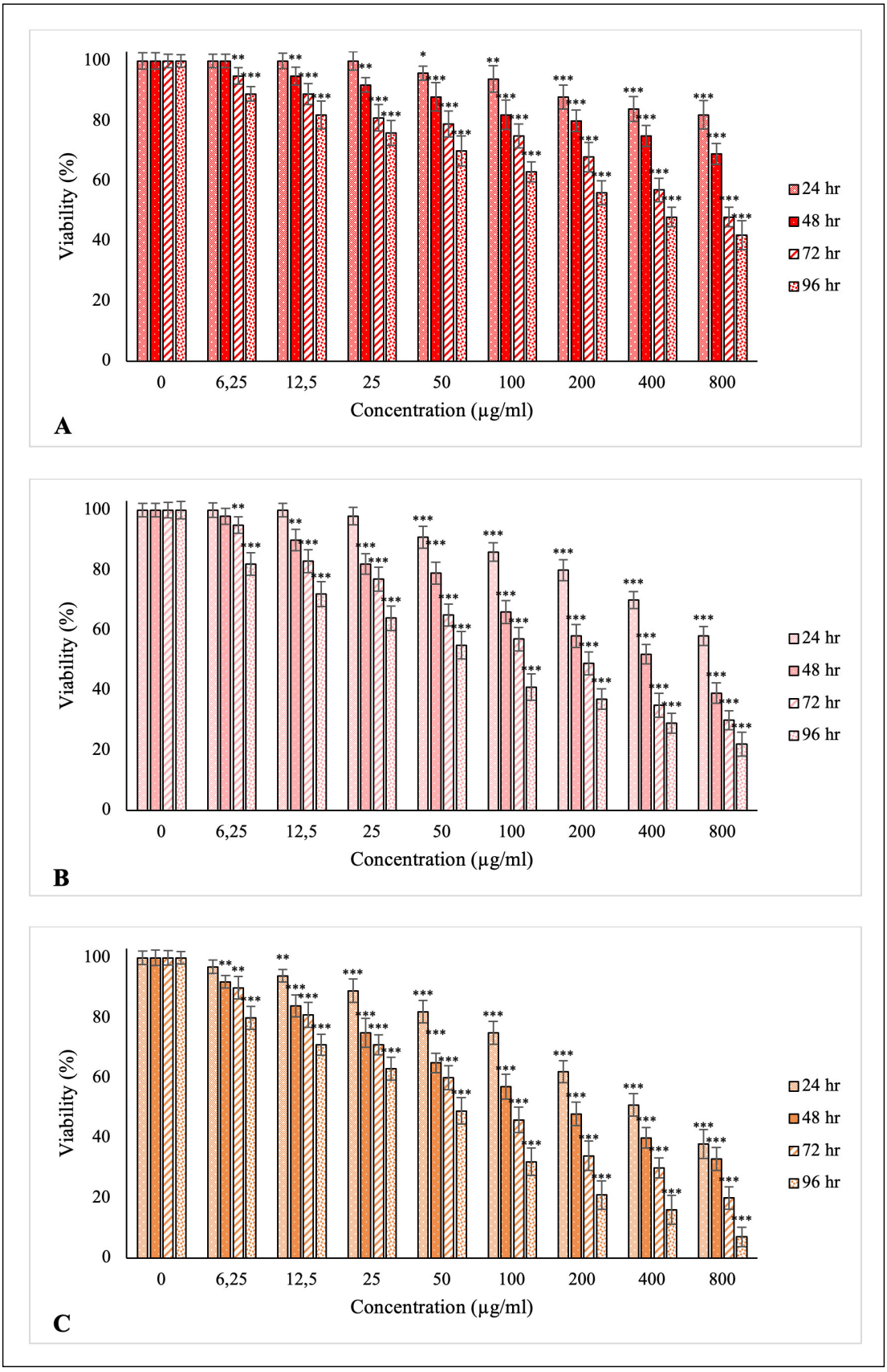

Figure 1. The effect of safflower aqueous (A), methanolic (B), and ethanolic (C) extracts on the viability of breast cancer cells. After 24, 48, 72 and 96 hr treatment viability was evaluated by MTT test. The control cells of received the same volume of medium without extract. *indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$ compared to the control.

The ethanolic extract of Safflower significantly reduced cell viability in concentrations higher than 12.5 µg/ml after 24 hr (p<0.05). Also, after 48, 72, and 96 hr, this reduction was significant in all the concentrations (p<0.05) (Figure 1 C). All of these extracts reduced breast cancer cell viability in a concentration and time-dependent manner.

The IC50 values for each extract are given in Table 1. For each treatment period, the aqueous extract has the highest IC50, which indicates the lowest effectiveness and toxicity. The methanolic extract has an IC50 between aqueous and ethanolic extracts, and the lowest IC50 corresponds to an ethanolic extract, which had the highest effectiveness and toxicity. The ethanolic extract of Safflower was used in the next tests.

Table 1. IC50 values for aqueous, methanolic, and ethanolic extracts of safflower.

	24 hr	48 hr	72 hr	96 hr
Aqueous extract (µg/ml)	6511.02±300.25	3220.48±217.37	642.59±45.28	335.78±73.92
Methanolic extract (µg/ml)	932.60±67.12	329.01±20.45	173.39±21.76	73.60±9.78
Ethanolic extract (µg/ml)	393.34±13.64	189.38±11.95	98.27±8.56	40.74±6.97

Effect of Safflower ethanolic extracts on breast cancer cell apoptosis

The percentage of intact cells, early apoptosis, late apoptosis, and necrotic cells after treatment with IC50 concentration for 24 hr showed that there were significant differences between treated and control cells. The results showed that 97.6% of the control cells were live, 0.34% were early apoptotic cells, and 1.19% were late apoptotic cells. The treatment group showed a significant increase in early (61.4%) and late (5.81%) apoptotic cells (Figure 2).

The results of the diphenylamine assay showed that extract significantly induced apoptosis compared to control cells (p<0.05) (Figure 3).

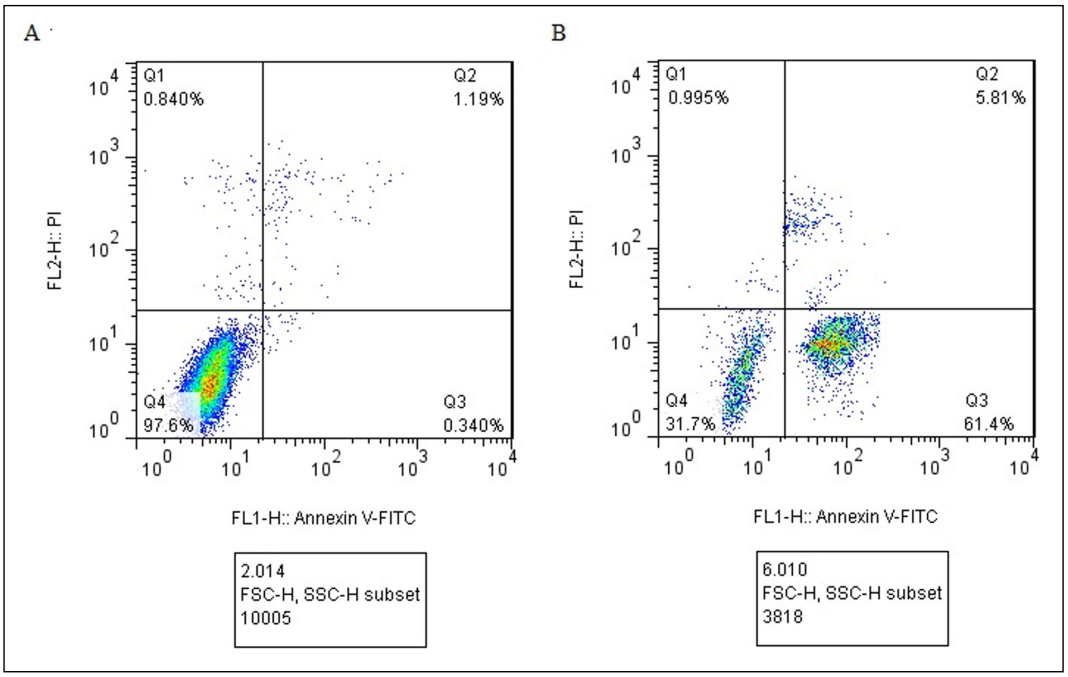

Figure 2. The effect of safflower ethanolic extract on apoptosis of breast cancer cells. After 24 hr treatment, apoptosis was evaluated by annexin V and propidium iodide staining. The control cells of received the same volume of medium without extract.

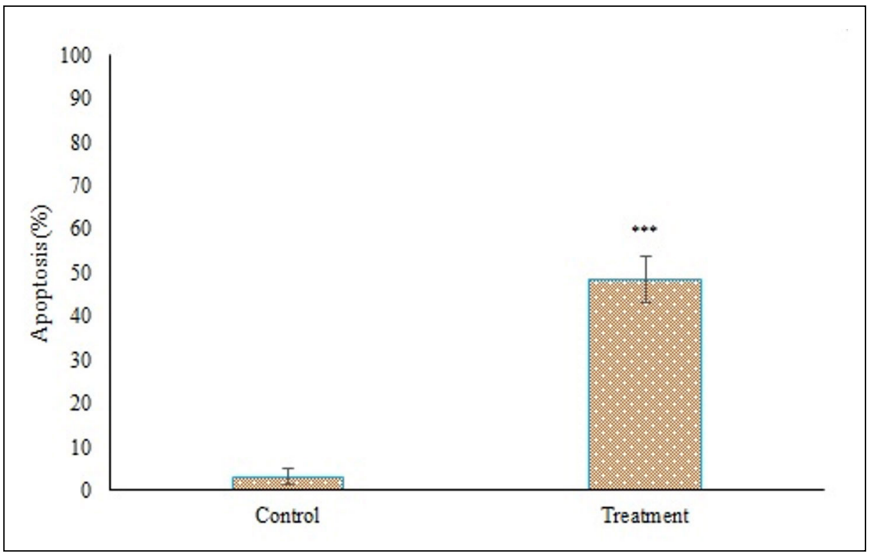

Figure 3. The effect of safflower ethanolic extract on apoptosis of breast cancer cells. After 24 hr treatment, apoptosis was evaluated by diphenylamine test. The control cells of received the same volume of medium without extract. *** indicates $p < 0.001$ compared to the control.

Effect of Safflower ethanolic extracts on Bax/Bcl-2 in breast cancer cells

The results of Real-time PCR showed that, after 24 hr of treatment with the IC50 concentration of ethanol extract of safflower, there were a significant increase in Bax and a significant decrease in Bcl-2 expression ($p<0.05$) (Figure 4 A and B). So, treatment with extract reduced Bax/Bcl-2 expression.

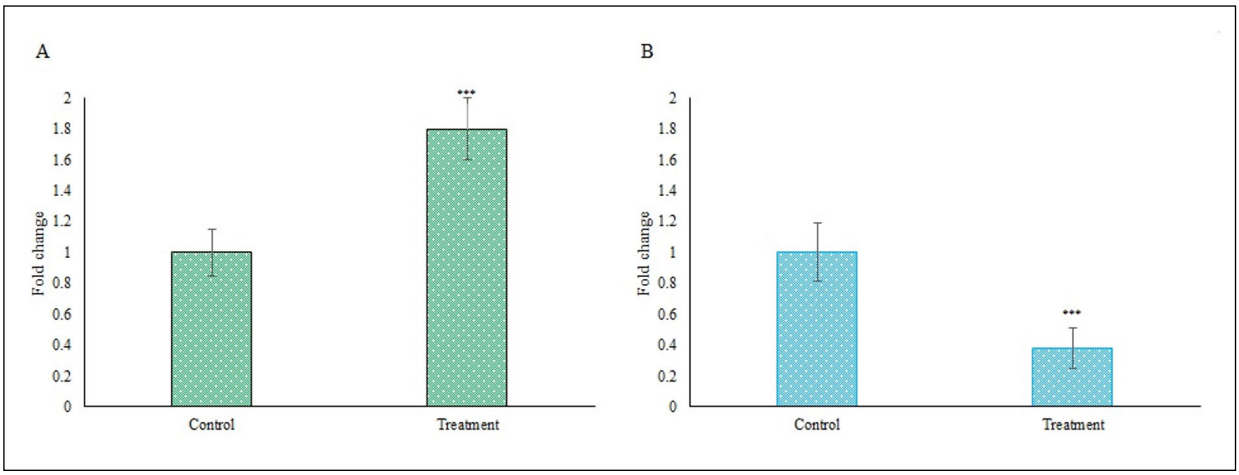

Figure 4. The effect of safflower aqueous on Bax (A) and Bcl-2 (B) expression in breast cancer cells. After 24 hr treatment, gene expression was evaluated by Real-time PCR test. The control cells of received the same volume of medium without extract. *** indicates $p < 0.001$ compared to the control.

Effect of co-treatment with Safflower ethanolic extracts wand tamoxifen on breast cancer cell viability

Tamoxifen significantly decreased the cell viability at 0.75, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 µg/ml ($p<0.05$) (Figure 5). This decrease was in a concentration-dependent manner. The IC50 value for tamoxifen was 4.31 µg/ml. The CI values were presented in Table 2. The obtained values were less than 1 (synergistic effect). The DRI values were greater than 1 demonstrating a dose reduction for a given therapeutic effect in both.

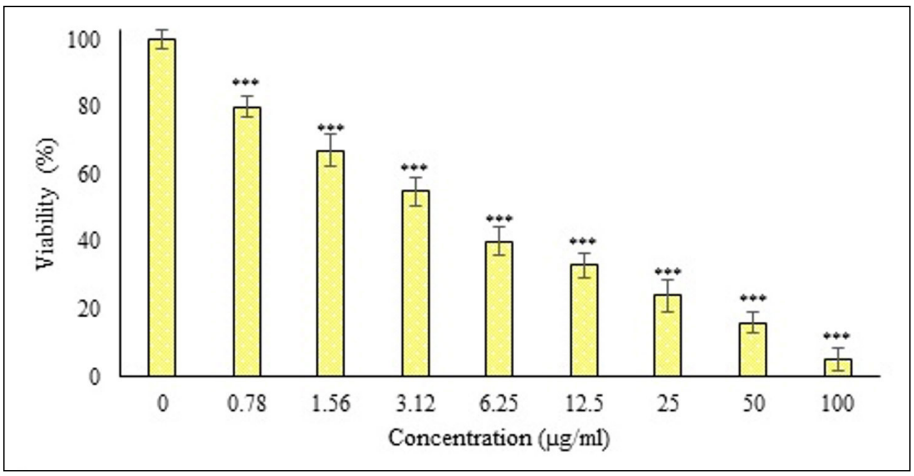

Figure 5. The effect of tamoxifen on the viability of breast cancer cells. After 24 hr treatment, viability was evaluated by MTT test. The control cells of received the same volume of medium without extract. *** indicates $p < 0.001$ compared to the control.

Table 2. Fraction affected (Fa), Combination index (CI) and dose reduction index (DRI) values for safflower ethanolic extracts and tamoxifen combination.

Co-treatments groups	Fa	CI	DRI extract	DRI tamoxifen
1	0.39±0.02	0.86±0.05	2.30±0.05	2.30±0.02
2	0.55±0.01	0.77±0.04	2.57±0.01	2.58±0.04
3	0.68±0.03	0.77±0.02	2.56±0.06	2.57±0.04
4	0.81±0.5	0.65±0.02	3.06±0.05	3.10±0.01
5	0.95±0.03	0.20±0.05	9.94±0.08	10.34±0.05

Group 1: 98.33 µg/ml of extract + 1.07 µg/ml tamoxifen; Group 2: 196.67 µg/ml of extract+ 2.15 µg/ml tamoxifen; Group 3: 393.34 µg/ml of extract+ 4.31 µg/ml tamoxifen; Group 4: 786.68 µg/ml of extract+ 8.62 µg/ml tamoxifen; and Group 5: 1573.36 µg/ml of extract+ 17.24 µg/ml tamoxifen.

DISCUSSION

In this project, the effect of three aqueous, methanolic, and ethanolic extracts of safflower on the viability of breast cancer cells was investigated. The results showed that after 24, 48, 72, and 96 hr treatment with aqueous, methanolic, and ethanolic extracts, the viability of cells was decreased in a concentration- and time-dependent manner. The IC50 values obtained from the MTT test showed that among these three extracts, the ethanolic extract had the greatest effect in reducing cell viability.

The presence of different bioactive chemical agents in the extracts may be the reason of the different anticancer properties of this plant. Hydroxyl safflower yellow A (a water-soluble chalcone glycoside extracted from safflower) is active as anti-tumor. It is highly soluble in water, while hardly dissolve in lipophilic solvents such as ethyl-acetate, ether, benzene, and chloroform[12].

The anticancer effects of safflower have been confirmed in previous studies. Safflower seed significantly inhibits the proliferation of human colon cancer cells (HCT116, SW480, LoVo, and HT-29). It has antiproliferative activity by inducing proteasomal degradation of cyclin D1 through ERK1/2-dependent threonine-286 phosphorylation of cyclin D1[13]. Also, a safflower polysaccharide significantly inhibits the proliferation of the human breast cancer cell line (MCF-7). It also induces cell apoptosis through the downregulation of Bcl-2 expression and upregulation of Bcl-2-related protein X expression. Furthermore, the expression of matrix metalloproteinase-9 was significantly decreased, and the expression of tissue inhibitor of metalloproteinase-1 was increased in MCF-7 human breast cancer cells treated with safflower polysaccharide. Therefore, it inhibits the metastasis of MCF-7 breast cancer cells[14]. Also, the effect of this polysaccharide on tongue squamous cell carcinoma (TSCC) has been investigated. This polysaccharide may inhibit the development of TSCC by regulating the expression of Bcl-2, COX-2, and Bax and inhibiting caspase-3[15]. The antitumor effect of HSYA on hepatocellular carcinoma and its effect on tumor immune microenvironment have been investigated. HSYA may also be suitable as a new treatment for patients with this type of cancer[16]. The results of these studies are consistent with the present study.

Also, our results showed that after 24 hr treatment with tamoxifen, there was a significant decrease in cell viability. Simultaneous treatment with Safflower extract and tamoxifen showed that the combination of these two agents had more toxic effects on cancer cells than each one alone. The CI values were less than 1, indicating a synergistic interaction between the extract and tamoxifen in reducing cell viability. The DRI values for the extract and tamoxifen were greater than 1, indicating a dose reduction to produce a specific therapeutic effect in both cases.

Despite the recent advances in cancer treatment, there is still no improvement in the life span and quality of life of patients with breast cancer. One of the main causes of this problem is drug resistance. Therefore, it is necessary to develop new strategies to overcome this problem. Recently, the use of drug combinations is the most widely used treatment method for deadly diseases such as cancer and acquired immunodeficiency syndrome. The main goal of this strategy is to achieve a synergistic therapeutic effect, reduce dosage and toxicity, and minimize or delay the development of drug resistance. The benefits of reducing toxicity and minimizing resistance can be the result of synergistic interactions. Today, the use of several anticancer drugs from different groups is widely used in the treatment of all types of cancer[17].

Molecular studies showed that after 24 hr treatment with the IC50 concentration of ethanol extract of safflower plant, there was a significant increase in Bax expression and a significant decrease in Bcl-2 expression.

Many proteins with anti-apoptotic and pro-apoptotic activity have been reported in the cell. The ratio between these proteins plays an important role in regulating cell death. The Bcl-2 family of proteins consists of anti-apoptotic and pro-apoptotic proteins, which plays an important role in regulating apoptosis, especially from its internal pathway. The mentioned proteins mainly act at the mitochondrial level. All members of the Bcl-2 family are located in the outer membrane of mitochondria, where they dimerize and are responsible for membrane permeability by forming ion channels or by creating barriers in the membrane. Bcl-2 is the first known protein of this family, which is encoded by the BCL-2 gene located on chromosome 18q21, which inhibits apoptosis without affecting cell proliferation. Bax protein is a member of Bcl-2 family and promotes apoptosis. Bax/Bcl-2 ratio determines the occurrence of apoptosis in the cell. In many human cancers, the expression of Bcl-2 is increased, while the expression of Bax is decreased. This factor causes the resistance of most cancer cells to stimuli such as chemotherapy drugs[18].

CONCLUSIONS

Among the aqueous, methanolic, and ethanolic extracts of safflower, the ethanol extract had the greatest effect in reducing the viability of cancer cells. This extract together with tamoxifen had synergistic toxicity on cells and induced apoptosis in cancer cells by increasing Bax/Bcl-2 ratio.

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Author's Contributions:

D. R.; Designed experiments and supervised the research. H. N,; Analyzed data and wrote the manuscript. F. D.; Performed the cell culture experiments. C. J.; Done gene expression. All authors read and approved the final manuscript.

Conflict of interest:

The authors declare that there is no conflict of interest.

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