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Research Article

Evaluation of anti-proliferative effects of *Crocus pallasii* subsp. *Haussknechtii* on MCF-7 cells

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ABSTRACT

Background: Breast cancer is the most common cancer and is the second leading cause of death among women. It is necessary to pay attention to the role of natural antioxidant compounds along with chemotherapy that can prevent or reduce the rate of progression of cancer and the complications of synthetic drugs. Species of the *Crocus* genus has been proved to have anti-proliferative and apoptosis inducing properties against various cancerous cells. The purpose of this study was to evaluate the anti-proliferative effects of *Crocus pallasii* subsp. *Haussknechtii* on MCf-7 cells as a natural product with fewer side effects.

Materials and Methods: After cell culture, treatment was performed with the extract of 0.15, 0.20 and 0.25 mg / ml, and 0.1 doses of doxorubicin as a positive control for 24 hours. Then the cells proliferation and viability of cells were measured using the MTT assay and staining of acridine orange-propidium iodide by a spectrophotometer and fluorescence microscope, respectively.

Results: After analyzing the data, it was observed that the amount of proliferation and survival in the cancerous cells treated with *Crocus pallasii* subsp. *Haussknechtii* was significantly reduced compared to the normal group.

Conclusion: It seems that the extract had less anti-proliferative effects compared to doxorubicin, but the extract could be considered as a natural remedy.

Keywords: *Crocus pallasii* subsp. *Haussknechtii*, anti-proliferative, viability, MCF-7

INTRODUCTION

Breast cancer is the most prevalent cancer among women worldwide, and accounts for the second cause of women deaths due to cancer. 23% of all new cancer occurrences and 14% of deaths because of cancer are associated with breast cancer. Almost half of the cases of breast cancer and approximately 60% of deaths due to this type of cancer occur in developing countries (1). Chemotherapy has played an essential role in conventional approaches adopted to control the

progression of the breast cancer (2). But this kind of treatment has some adverse effects on healthy body organs, brings about irreversible damages to tissues, and generates drug resistance due to unselective toxicity of the consumed pharmaceuticals (3). Thus, it seems necessary to explore more deeply for developing novel drugs in studies related to breast cancer.

Recently, several studies have been focused on investigating the anti-proliferative effects of a wide range of herbal drugs on cancerous cells in vitro or in animal models. The reported results suggest a promising solution to prevent cancer through exploiting some natural compounds existed in diets that are naturally capable of impeding the progression of cancer (4).

Epidemiological studies indicate that a high intake of carotenoids may have protective effects against breast cancer progression (5,6,7). Species of the *Crocus* genus is deemed highly significant because of containing a wide range of carotenoids, particularly crocin, and having a special place in dietary habits of inhabitants of some areas. Compounds found in *Crocus*, particularly crocetin and picorcrocin, exert therapeutic effects through inducing apoptosis (8). Crocetin affects cancerous cells by suppressing the synthesis of nucleic acids, enhancing anti-oxidative system, inducing apoptosis, and inhibiting growth factors of the signaling paths. Furthermore, ethanol extract of *Crocus* makes a profound and time-dependent decrease in viability of existing cells within malignant tumors (9).

Crocus genus encompasses about 100 species around the world from southwest of Europe to southwest of Asia. *C. sativus*, among these species, has been widely studied, whereas the *Crocus pallasii* species which is one the close relatives of *C. sativus* and common in certain regions from the Balkan peninsula to Iran and from Crimea to south of Jordan has remained an exception to this trend (10,11).

Considering the current growing trend towards using novel natural compounds along with chemotherapy in order to prevent or hinder the cancer progression (12), the present study aims at examining the anti-proliferative effects of hydroalcoholic extract of *Crocus pallasii* subsp. *Haussknechtii*, as a newly found compound compared with doxorubicin, on cancerous cells at MCF-7 level from both quantitative perspective through MTT test and qualitative perspective using fluorescence microscopy.

In case of yielding desirable results verified by the experiments, the current research is an attempt to validate the potentiality of using this extract as an adjunct therapy together with chemotherapy pharmaceuticals.

MATERIALS AND METHOD:

Collecting and preparing the herbal hydroalcoholic extract:

Wild species of *Crocus pallasii* subsp. *Haussknechtii* were collected from the mountains of Kordistan in western region of Iran during fall, 2016. Once the collecting process was done by a qualified botanist, the collected samples were validated and designated by voucher number: 12543 in herbarium of Kurdistan Center for Research and Education on Natural Resources and Agriculture (HKS). Extraction process was carried out in accordance with the proposed approach by Sayyah et al. (13).

The collected herbs were dried and grinded in a dark room, and then were subjected to the percolation process for the purpose of producing the hydroalcoholic extract. For the purposes of the required preparations, a 100-gram mass of *Crocus pallasii* subsp. *Haussknechtii* root was kept in contact with 900 ml of 80% ethanol inside a decanter funnel for 48 hours, which was accomplished in three stages with addition of 300 ml per each.

Once the liquid inside the decanter was collected, the ethanol was separated, by a rotary device, from the extract which was refrigerated after being concentrated.

The dose used is according to a previous study by Sayyah et al., Which reported a dose of 0.25 g / kg as non-toxic (13).

Culturing the MCF-7 cells

MCF-7 cells were acquired from Iran cell bank (Pasteur Institute, Tehran, Iran). The cells were cultured on a RPMI-1640 medium containing 10% FBS together with 5% CO_2 within an incubator at 37°C (14). These cells grow in a single layer in a flask. The medium was changed three times a week and the trypsin- EDTA solution was used to remove the cells.

MCF-7 cells treatment with hydroalcoholic extract of *Crocus pallasii* subsp. *Haussknechtii*

Once the cells occupied 70% of the flask surface, they were collected from the flask bottom with the aid of trypsin, and were transferred onto a 96-cell plate afterwards. 0.15, 0.20, and 0.25 mg concentrations were prepared from the dried extract of *Crocus pallasii* subsp. *Haussknechtii*. Doxorubicin with a concentration of 0.1 millimolar was used as a positive control. The first group as a negative control received no treatment, second group as a positive control received doxorubicin, and third, fourth, and fifth groups were treated with the extract of *Crocus pallasii* subsp. *Haussknechtii* with concentrations of 0.15, 0.20, and 0.25 mg respectively.

Examining the cell killing rate using the MTT method

MTT method using tetrazolium dye is an appropriate experimental approach to determination of cells viability. 20 micro liters of the prepared MTT solution (5 mg/ml PBS) was added to each pit 48 hours after treatment. The plates were incubated for 4 hours. Then the remaining was expelled from the medium, and 100 micro liters dimethyl sulfo oxide (DMSO) was added to each pit so that the resultant formazan was solved. The plated were shaken for 20 minutes, and then the absorbance of the formazan was read at 492 nanometers (10).

Examining the rate of apoptosis

Perhaps fluorescent painting and counting cells under a microscope is the simplest and fastest method to detect alive and dead cells, provided that it is done carefully. Fluorescent dyes used in the present study were acridine orange and propidium iodide (PI). Acridine orange is a vital dye that is absorbed by living cells. Acridine orange permeates into DNA of the living cells and gives a green tint to their chromatin as being examined under a microscope, whereas PI only paints dead cells. PI permeates into DNA of the dead cells and gives an orange tint to their chromatin as being examined under a fluorescent microscope. Thus, living cells will be seen green and dead cells will be seen orange or brown as the result of applying the PI-acridine orange solution. To do this, 100 microgram in ml of each dye was added to 1 ml PBS solution with equal proportions. Then 10 microliter of the obtained solution was mixed into 250 microliter of the cellular suspension. Next, 10 microliter of the mix was put on a clean lam and covered with a lamel. Finally, at least 200 cells were examined and counted for being alive or dead under a fluorescence microscope through 40x or 50x lens. The number of green cells (living) divided by total sum of the green cells (living) and dead cells (orange) equals percentage of the living cells (15).

Data analysis

The collected data was fed into and analyzed by Excel and SPSS programs. Once the core indices of mean and standard deviation dispersion were calculated, statistical tests including Student t-test and ANOVA were used to analyze the data. Then the results were reported as mean ± fault or standard assuming $p<0.05$ as the significance level.

RESULTS

Comparing the obtained result from MTT, designed for assessing the viability percentage of cancerous cells treated with hydroalcoholic extract of *Crocus pallasii* subsp. *Haussknechtii,* indicated that the average percentage of viability among treated group was significantly different ($p<0.05$) from that of control group. The average viability of cancerous cells in the groups treated with doxorubicin was lower compared with hydroalcoholic extract. 100 cells was counted, and living, early apoptotic, late apoptotic, and necrotic

cells were determined as described by Table 1 below.

Propidium Iodide	Acridine orange	Cell types
Negative	Positive and normal core	living
Negative	Positive and dense core	Early apoptosis
Positive	Positive and dense core	Late apoptosis
Positive	Positive and swollen core	Necrosis

Table 1: Comparison among living, early apoptotic, late apoptotic, and necrotic cells

Similarly, the obtained results from acridine orange and propidium iodide dyeing showed that the rate of apoptosis among treated cells with *Crocus pallasii* subsp. *Haussknechtii* is higher than the negative control group ($p<0.05$) but lower than doxorubicin group (see Table 2).

Studied Group	Proliferation Rate (MTT)	Percentage of Apoptosis
Negative Control	0/704±0/004	22/66±0/577
0.15	0/526±0/002*	31/33±1/527*
0.20	0/459±0/002*	49/33±0/577*
0.25	0/395±0/001*	56/33±3/055*
Doxorubicin	0/201±0/002*	66/33±0/577*
Probability (P)	0.001	0.001

Table 2: The results of proliferation and apoptosis rates among MCF-7 cells following treatments with microgram/ml of saffron extract and millimolar concentration of doxorubicin

DISCUSSION

Important aspects of biomedical research are to provide a practical approach to identify an effective inhibitor of tumor progression and study the molecular mechanism of the tumor (16). More than 60% of drugs which have anticancer activity have been isolated from natural sources in clinical trials, thus the development of natural products as one of the most important sources of anticancer is considered as a growing and promising strategy (17). Natural compounds, especially those derived from plants, are of considerable significance due to their studied abilities to induce apoptosis, cell proliferation, and modulation of signal transduction (18). Different research results show that resurrection to natural products such as medicinal plants, along with the use of synthetic drugs, can be a positive approach in controlling and treating a wide range of diseases, including cancers (19,20).

One of the reasons of using natural products, along with synthetic drugs and chemotherapy, is escalating recurrence and toxic side-effects of synthetic therapies that greatly reduce their efficacy (8). To date, extensive studies have been conducted on the anticancer effects of saffron and other constituents of *Crocus sativus* L., but as far as we know, no studies has been raised on the anticancer properties of *Crocus pallasii* subsp. *Haussknechtii*. In this study, we investigated the anti-proliferative effects of *Crocus pallasii* subsp. *Haussknechtii* on MCF-7 cells.

Various studies have related the inhibitory and antiproliferative effects of *Crocus sativus* to Crocin. The studies have shown that Crocin in the species of *Crocus sativus* can exert anti-proliferative effects by transforming cells in the stage (G1) and creating apoptosis (18). Researchers have proposed 3 main mechanisms for the anti-tumor effects of carotenoids of *Crocus sativus* L., mechanisms which include inhibitory effect on the synthesis of DNA and RNA, protein, and free radical chain interactions (21).

Old studies have shown that carotenoids of *Crocus sativus* L. have growth-inhibitory activity against tumor cells (21, 22). These findings are aligned with newer studies in which alcoholic extract of saffron reduces the survival of malignant cancer cells and has anti-proliferative and cytotoxic effects on them. The aforementioned studies confirm the results of the present study, in which *Crocus pallasii* subsp. *Haussknechtii* causes cancer cells to disappear (23).

The results of a study done by Escribano et al. Suggest that proteoglycan extracted from *Crocus sativus* L. corm has a significant cytotoxic activity against various cancer cells (24). Also, the results of a study, which were subsequently carried out

on the same extract obtained from *Crocus sativus* L. corm, in order to evaluate the antitumor and immuno-modulator properties of it, proved that this glycoconjugate significantly increases the activity of macrophages (25).

The results indicate that this proteoglycan existed in the extract *of Crocus sativus* L. corm exhibits more cytotoxic effects on malignant cells in relation to the normal cell line by introducing damages to the plasma membrane of the cancerous cells, thus selective cytotoxic behavior of the extract to cells can be deduced (26). This selective cytotoxic effect is an important criterion because existing drugs currently target normal cells and lead to side effects. The results of this study revealed that the proliferation of MCF-7 cancer cells after treatment with hydroalcoholic extract of *Crocus pallasii* subsp. *Haussknechtii* was reduced compared to untreated control group; however, it showed a lower reduction compared to doxorubicin.

In the present study, the study of cells with fluorescence microscopy showed that apoptosis of cancerous cells treated with *Crocus pallasii* subsp. *Haussknechtii* had a significant increase compared to the control group, but was lower than that of doxorubicin.

All of these results indicate that treatment with this extract can induce the death of breast cancer cells through apoptosis.

There may be antioxidant properties of the substances found in *Crocus pallasii* subsp. *Haussknechtii* that is inhibiting the advancement of cancer, while the potential cytotoxicity leads them to apoptosis and cell death (16).

CONCLUSION

Based on the results of this study, the extract of *Crocus pallasii* subsp. *Haussknechtii* which is abundant in different parts of the world has anti-proliferative effects on MCF-7 cells and can be used as a natural compound without side effects along with traditional synthetic drugs for treatment of breast cancer.

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DECLARATIONS

Conflict of Interest

There are no conflicts of interests to declare.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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