Research Journal of Pharmacognosy (RJP) 6(3), 2019: 51-59
Received: 6 Mar 2019
Accepted: 3 June 2019
Published online: 23 June 2019
DOI: 10.22127/rjp.2019.89461

Original article

Cytotoxic and Antioxidant Activities of *Crocus pallasii* subsp. *haussknechtii* Corms Extracts Compared with *Crocus sativus*

Raheleh Shakeri[1*], Jalal Khorshidi[2], Tayebeh Radjabian[3], Ali Lashkari[4], Maliheh Safavi[5]

[1]Department of Biological Science and Biotechnology, Faculty of Science, University of Kurdistan, Sanandaj, Iran.
[2]Department of Horticultural Science and Engineering, Research Center of Medicinal Plants Breeding and Development, University of Kurdistan, Sanandaj, Iran.
[3]Immunoregulation Research Center, Shahed University, Tehran, Iran.
[4]Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran.
[5]Department of Biotechnology, Iranian Research Organization for Science and Technology, Tehran, Iran.

Abstract

Background and objectives: The goal of this study was to compare the cytotoxic and antioxidant activities of the corms extracts of two *Crocus* species (*C. sativus* L. and *C. pallasii* subsp. *haussknechtii*). **Methods:** *n*-Hexan, chloroform and methanol extracts of the corms were prepared. Cytotoxic activities of the extracts against human breast cancer cell lines MDA-MB-231 and MCF-7 were evaluated via MTT assay and dual acridine orange/ethidium bromide fluorescent staining test. Antioxidant activities of the extracts were assessed by DPPH method. **Results:** The results showed that the methanol extracts of the corms from both species had cytotoxic activity against the breast cancer cells. Cytotoxic activity of the corms extract of *C. sativus* L. was significantly higher than *C. pallasii* subsp. *haussknechtii*. *n*-Hexan and chloroform extracts from the corms of *C. pallasii* subsp. *haussknechtii* and *Crocus sativus* L. showed no anti-proliferative activity which indicated the polar nature of the cytotoxic agent(s) in the extracts. The results of dual acridine orange/ethidium bromide fluorescent staining test revealed that the methanol extracts of both species corms destroyed cancer cells through apoptosis. Moreover, the methanol corm extracts of the two species showed little antioxidant activity. **Conclusion:** It can be concluded that *C. sativus* L. may possess more significant apoptotic properties compared to *C. pallasii* subsp. *haussknechtii.*

Keywords: antioxidant; apoptosis; breast cancer; *Crocus*

Citation: Shakeri R, Khorshidi J, Radjabian T, Lashkari A, Safavi M. Cytotoxic and antioxidant activities of *Crocus pallasii* subsp. *haussknechtii* corms extracts compared with *Crocus sativus*. Res J Pharmacogn. 2019; 6(3): 51-59.

Introduction

Breast cancer has been the second leading cause of cancer death in women after lung cancer [1]. Chemotherapy is the most widely used therapeutic option to suppress the proliferation of cancer cells, but unfortunately the efficacy of chemotherapy is limited because of drug resistance [2]. For this reason, finding new agents with high impact against cancer cells is valuable. Medicinal plants are important natural sources for new cytotoxic agents [3]. *Crocus sativus* L. which belongs to Iridaceae family, commonly known as saffron, is a medicinal plant with various bioactivities including antioxidant, anticancer and antibacterial properties [4]. *Crocus pallasii* subsp. *haussknechtii* is another plant which is a very close relative species to *C. sativus* L. This plant grows in various regions of Iran such as Kurdistan province [5]. Despite extensive studies over anticancer properties of saffron, the cytotoxic properties of *C. pallasii* subsp. *haussknechtii* are not well understood. To

*Corresponding author: r.shakeri@uok.ac.ir

date, few studies have investigated the anticancer properties of this species. Mosaddegh et al. reported the anti-angiogenesis properties of *C. pallasii* subsp. *haussknechtii*. It has been shown that the methanol extract of the corms of *C. pallasii* subsp. *haussknechtii* showed cytotoxic activity against HUV-EC-C cells [6]. Further, the methanol, ethanol and chloroform extracts of the species showed cytotoxicity for A-549 human lung carcinoma cell [7]. In the present study, we investigated the anti-proliferative activity of *n*-hexan, chloroform and methanol extracts prepared from the corms of *C. sativus* L. and *C. pallasii* subsp. *haussknechtii* against two breast cancer cell lines MCF-7 and MDA-MB-231. The main objective of this study was to examine the cytotoxic activities of the corm extracts of *C. pallasii* subsp. *haussknechtii* in comparison with *C. sativus* L.

Material and Methods

Ethical considerations

Ethical approval for this study was granted by the Ethical Committee of Shahed University, Tehran, Iran (ethics committee reference number: IR.Shahed.REC.1396.577, 2017).

Chemicals

The cell culture medium (RPMI 1640), fetal bovine serum (FBS) and penicillin–streptomycin were purchased from GibcoBRL (Life Technologies, Scotland). The culture plates were obtained from Nunc (Roskilde, Denmark). Dimethyl sulfoxide (DMSO), methanol, and chloroform were purchased from Carlo Erba, France. Ethanol and *n*-hexane were obtained from Merck (Germany) and Biochem (France), respectively. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1-diphenyl-2-picryl-hydrazyl (DPPH) and phosphate buffer saline (PBS) tablet were obtained from Sigma-Aldrich (USA). MDA-MB-231 and MCF-7 human breast cancer cell lines were purchased from Pasteur Institute of Iran (Tehran).

Plant material

The corms of *Crocus sativus* L. were purchased locally in Qaen, South Khorasan Province, Iran (2017). *Crocus pallasii* subsp. *haussknechtii* (whole plant) was collected from natural habitats in Sanandaj, Kurdistan, Iran in autumn 2017. *Crocus pallasii* subsp. *haussknechtii* was authenticated at the Research Center of Agricultural and Natural Resources of Sanandaj, Kurdistan, Iran (voucher specimen number: 5641). The corms of the both plants were carefully separated from the surrounding fibers and dried in shade.

Extraction

The powdered corm samples were sequentially macerated with *n*-hexane, chloroform and methanol and left for 24 h in a dark place (5g: 100 mL). The extracts were collected, filtered and evaporated using rotary evaporation. The concentrated extracts were completely dried. The powdered extracts were dissolved in DMSO and diluted with distilled water to obtain 50, 100, 200 and 400 µg/mL concentrations [8]. It should be noted that the final percent of DMSO in the diluted extracts and the cell culture mediums were 40% and 1%, respectively.

MTT assay

The cell lines were seeded in 96-well cell culture plates (15000 cells/well) and left in CO_2 incubator. After 16 h, the cells were treated with different concentrations of the extracts for 24 or 48 h. The viable cells were assessed by MTT test [9]. MTT powder was dissolved in sterile phosphate-buffered saline. Each culture well of the plates was filled with MTT solution and placed in CO_2 incubator for 3 h. Final concentration of MTT in the wells was 0.5 mg/mL. The viable cells convert MTT solution to formazan crystals. The supernatant fluid was removed and formazan crystals (pellets) were dissolved in 100 µL DMSO. Finally, the absorbance of each well was read using a microplate reader (BioTek, USA) at 492 nm. For each extract, the concentration corresponding to a survival rate of 50% of cancer cells (IC_{50} value) was calculated from concentration-response curves by regression analysis.

Dual acridine orange/ethidium bromide (AO/EB) fluorescent staining

The cells were cultured in 6-well plates and treated with IC_{50} concentration of the extracts. After 12 h, the cells were harvested and washed with cold PBS three times. They were suspended in 100 mL of acridine orange and ethidium bromide mixture (1:100 mg/mL). Ten µL of the stained cell suspension was pipetted on to a clean microscope slide and covered with a coverslip. The apoptotic and healthy cells were observed

under a microscope fluorescent (Zeiss, Germany) [10].

DPPH test

DPPH was dissolved in methanol as a stock solution (100 µM) and diluted to its working concentration (60 µM) with methanol immediately before use. Twenty five µL of different concentration (50-100 µg/mL) was mixed with 975 µL of DPPH working solution and incubated for 10 min in a dark place. Finally, the absorbance was measured at 517 nm using spectrophotometer (AnalytikJena's spectrophotometer SPECORD 210, Germany). Ascorbic acid was used as the reference standard [11].

Statistical analysis

The results were expressed as mean ± SD of three independent experiments. Data were statistically analyzed by two-tailed unpaired Student's t test using Microsoft Excel software. Differences were considered significant at $p< 0.05$.

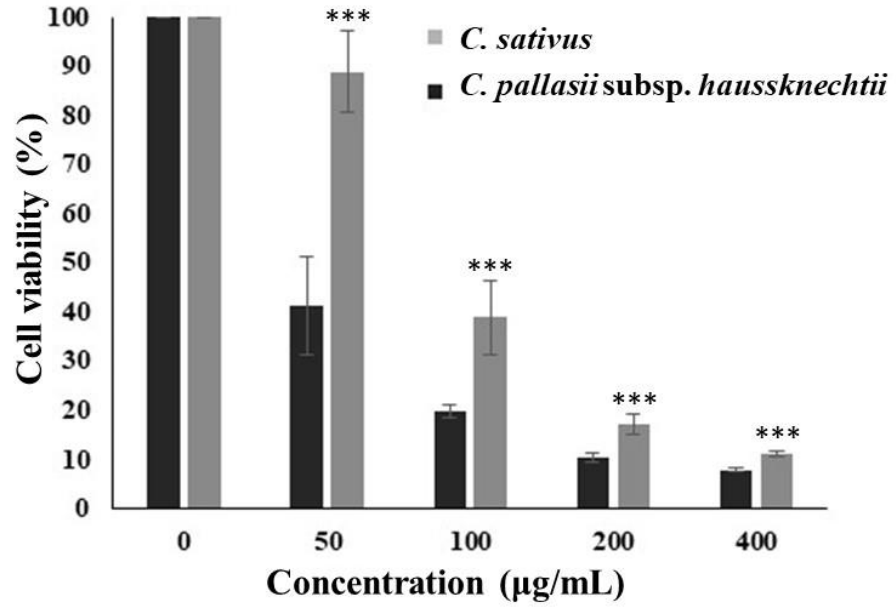
Results and Discussion

Cell viability of two human breast cancer cell lines including MDA-MB-231 and MCF-7 was quantitated by MTT assay in the presence of *n*-hexane, chloroform and methanol extracts of *C. sativus* L. and *C. pallasii* subsp. *haussknechtii* corms. No cytotoxicity was detected in MDA-MB-231 and MCF-7 cancer cells treated with *n*-hexane and chloroform extracts of the corms of both examined species within 24 and 48 h. As shown in figures 1 and 2, the methanol extracts of the corms of both species exhibited cytotoxicity against the breast cancer cell lines. A number of studies have demonstrated anti-proliferative effect of *C. sativus* L. corms on tumor cells in vitro [12-14]. Hamzeloo-Moghadam et al. [7] investigated the cytotoxic activity of two methanol extract prepared from the fiber cover and nut of *C. pallasii* subsp. *haussknechtii* against various human cancer cell lines. They indicated that the cytotoxic activity of the corm covering fibers methanol fraction was higher than that of the corm nut. The IC_{50} value of methanol extract of *C. sativus* L. corms was lower than that of *C. pallasii* subsp. *haussknechtii* (table 1). The cell viability of MDA-MB-231 and MCF-7 cell lines was also assessed within 24 and 48 h treatment with the methanol extracts. The results showed that the IC_{50} value obtained from the methanol extract of *C. sativus* L. corms decreased with increasing time of treatment. This process was similar to doxorubicin as the positive control. The IC_{50} value for the methanol extract of *C. pallasii* subsp. *haussknechtii* against MDA-MB-231 cell line decreased following 48 h of treatment, but no changes was observed for MCF-7 cell line. It has been reported that the corm aqueous extract of *C. sativus* L. contains a proteoglycan with remarkable cytotoxic activity against HeLa and MDA-MB-231 human cancer cell lines [13,14]. The corm of *C. pallasii* subsp. *haussknechtii* may be containing similar proteoglycan which has cytotoxic activity against human breast cancer cells. Further analysis is required to identify the cytotoxic compound(s) present in the species.

Apoptosis is a type of cell death with unique morphological features which is different from other types of cell death such as necrosis [15,16]. Chemotherapeutic drugs destroy tumor cells by inducing apoptosis. Detection of apoptosis-associated changes in the presence of cytotoxic agents is of great importance [17]. In order to observe the morphological changes occurring during cell death induced by the cytotoxic extracts, MDA-MB-231 and MCF-7 cells were treated with the IC_{50} values (48 h) of *C. sativus* L. and *C. pallasii* subsp. *haussknechtii* corms methanol extracts for 12 h (early stage of apoptosis) and subsequently were analyzed by dual AO/EB fluorescent staining. As seen in figures 3 and 4, the methanol extracts of the corms induced apoptosis in MDA-MB-231 and MCF-7 cell lines. Therefore, dual AO/EB fluorescent staining results confirmed the MTT data. Although MTT assay is a colorimetric methods to quantitate viable cells in the presence of cytotoxic agents, but it is unable to distinguish between apoptotic and necrotic cells [18,19]. The intact plasma membrane integrity is a major characteristic morphological sign of apoptotic cells [20,21]. Unlike apoptosis, necrosis results in a loss of cell membrane integrity [21]. Dual AO/EB fluorescent staining is a convenient method to detect apoptosis-associated changes of cell membranes during apoptosis. AO enters cells with intact membrane and emitting green fluorescence when bound to DNA. EB only penetrates cells with damaged membranes, binds to the DNA and emits orange-red fluorescence [19]. Thus, AO stains both live and early apoptotic cells and EB stains both late apoptotic

cells and necrotic cells. Early apoptotic cells (shown as dashed arrows in figures 3 and 4), in contrast to live cells (shown as solid arrows in figures 3 and 4), contain bright green dots in the nuclei as a results of chromatin condensation and nuclear fragmentation. Late apoptotic cells (shown as dotted arrows in figures 3 and 4), unlike necrotic cells, contain orange dots in the nuclei as a consequence of DNA fragmentation and chromatin aggregation, which do not happen to the nuclei of necrotic cells [22].

(a)



(b)

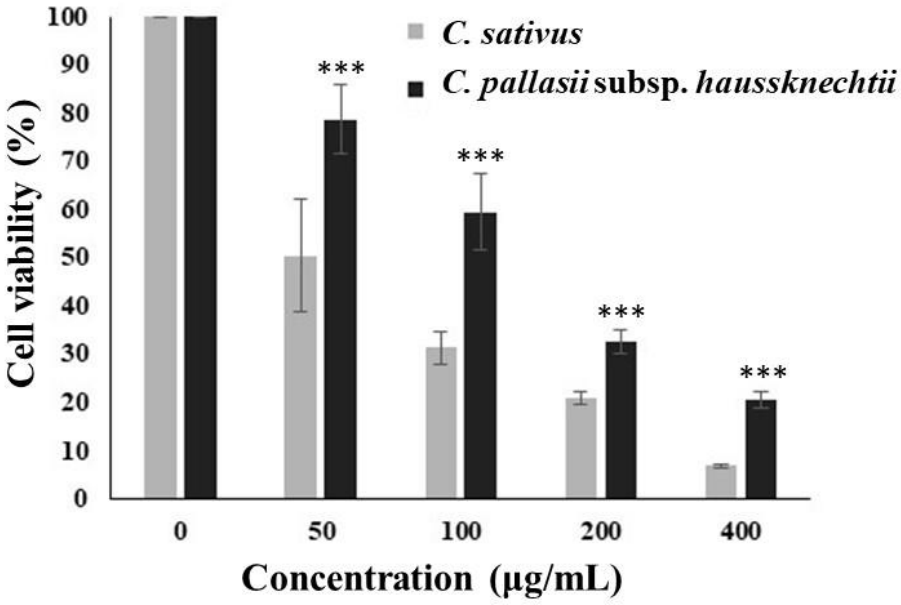


Figure 1. Cell viability of MDA-MB-231 cell in the presence of methanol extracts of *Crocus sativus* and *Crocus pallasii subsp. haussknechtii* after 24 (a) and 48 hours (b). Data are means of three independent replicates ± SD; ***: P< 0.001 in comparison to *Crocus sativus* L.

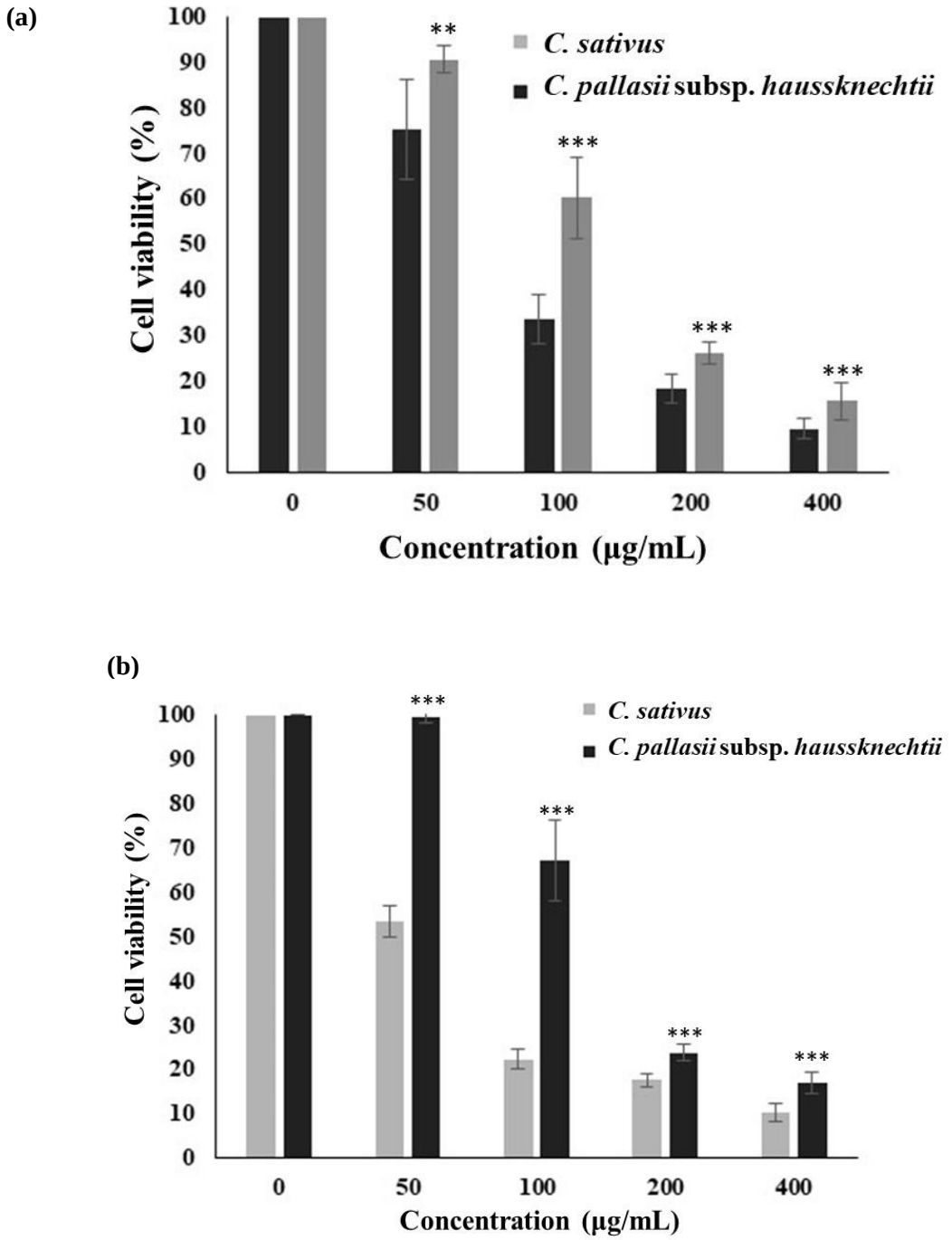


Figure 2. Cell viability of MCF-7 cell in the presence of methanol extracts of *Crocus sativus* L. and *Crocus pallasii* subsp. *haussknechtii* for 24 (a) and 48 hours (b). Data are means of three independent replicates ± SD; ***: P< 0.001 and **: P < 0.01 in comparison to *Crocus sativus* L.

Antioxidant activity of the methanol extracts was assessed by DPPH test. The results showed that both methanol extracts were able to reduce DPPH at concentrations of 50-400 µg/mL. DPPH radical scavenging activity of *C. sativus* L. corms methanol extract at concentration of 200 µg/mL was significantly higher than *C. pallasii* subsp. *haussknechtii*. Ascorbic acid was used as the positive control (IC_{50} = 2.25 ± 0.01). *n*-Hexan and chloroform extracts of both species showed no antioxidant activity in scavenging DPPH radicals. This is the first report of antioxidant activity of *C. pallasii* subsp. *haussknechtii* corms. It has been shown that the methanol extract of *C.*

sativus L. dormant corms at a concentration of 3750 ppm reduced DPPH to 29 ± 0.854 % [23]. Baba et al. measured free radical scavenging activity of ethanol and aqueous extracts of *C. sativus* L. corms by DPPH test. The IC_{50} values for ethanol and aqueous extracts were 246.22±5.60 and 456±3.52 µg/mL, respectively [24].

Antioxidant activity of ethanol extract of *C. sativus* L. corms was higher that the aqueous extract. Based on the results of antioxidant activity, antioxidants agents in *C. sativus* L. are extracted by polar solvents such as methanol, ethanol and water. Polar extracts contain high amount of phenolic contents [25]. It has been found a strong association between total phenolic content and antioxidant activity in fruits, vegetables, and medicinal plants [26].

Table 1. IC_{50} values of *Crocus sativus* and *Crocus pallasii* subsp. *haussknechtii* extracts against MDA-MB-231 and MCF-7 human breast cancer cell lines

Sample	**IC_{50} (µg/mL)**			
	MDA-MB-231		**MCF-7**	
	24 h	**48 h**	**24 h**	**48 h**
Crocus sativus (H)	-	-	-	-
Crocus pallasii (H)	-	-	-	-
Crocus sativus (CL)	-	-	-	-
Crocus pallasii (CL)	-	-	-	-
Crocus sativus (M)	32.81±4.22***	53.04 ± 3.23***	82.14 ± 13.8***	40.54 ± 6.65***
Crocus pallasii (M)	102.55 ±12.23***	150.58 ±5.44***	132.58 ± 6.7***	145.75 ± 9.58***
Doxorubicin (Positive control)	11.45 ± 1.38	0.99 ± 0.1	8.39 ± 0.99	0.67 ± 0.12

H: *n*-hexane extract; CL: chloroform extract; M: methanol extract; -: No cytotoxicity; Data are means of three independent replicates ± SD. Stars indicate that the IC_{50} value of *C.sativus* was significantly different (*** $P < 0.001$) from the IC_{50} value of *C. pallasii* subsp. *haussknechtii*.

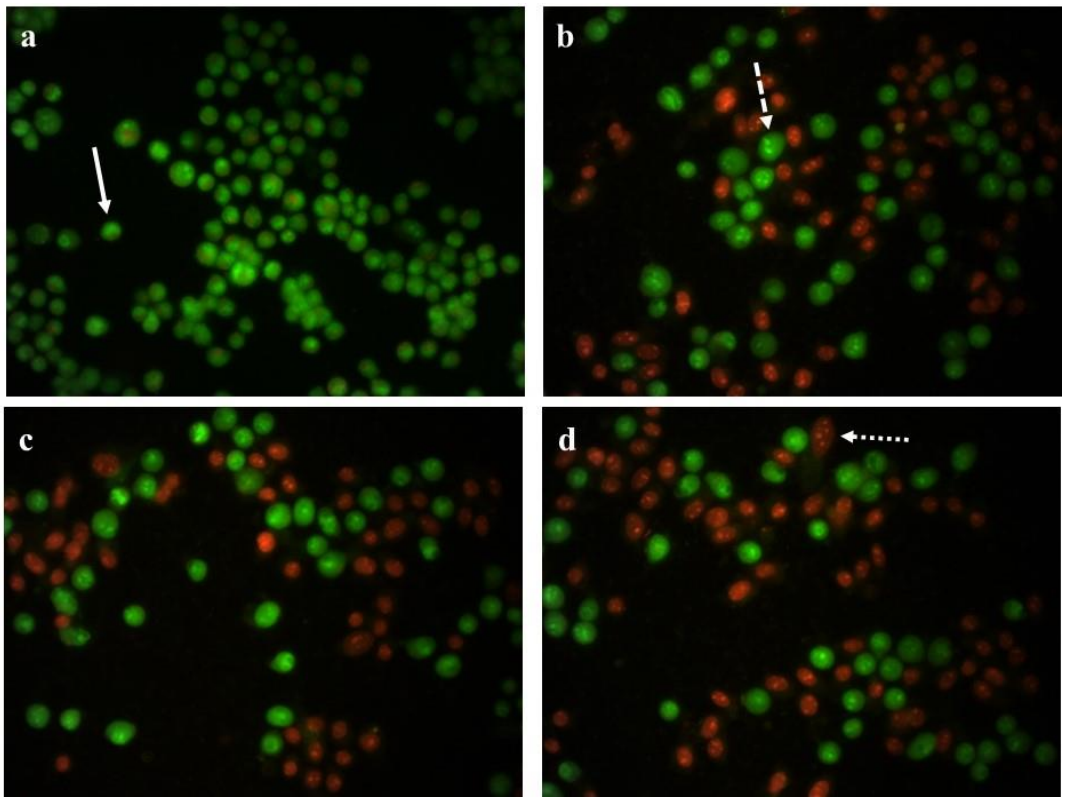


Figure 3. Dual acridine orange/ethidium bromide fluorescent staining of MDA-MB-231 cells. The cells were treated with (a) DMSO 1% as control, (b) IC_{50} concentration of doxorubicin as the positive control, (c) IC_{50} concentration of corm methanol extract of *Crocus sativus* and (d) IC_{50} concentration of methanol extracts of *Crocus pallasii* subsp. *haussknechtii*. Solid arrow indicates live cells, dashed arrow indicates early apoptotic cells and dotted arrow indicates late apoptotic cells. The images were taken with a fluorescence microscope at 400×.

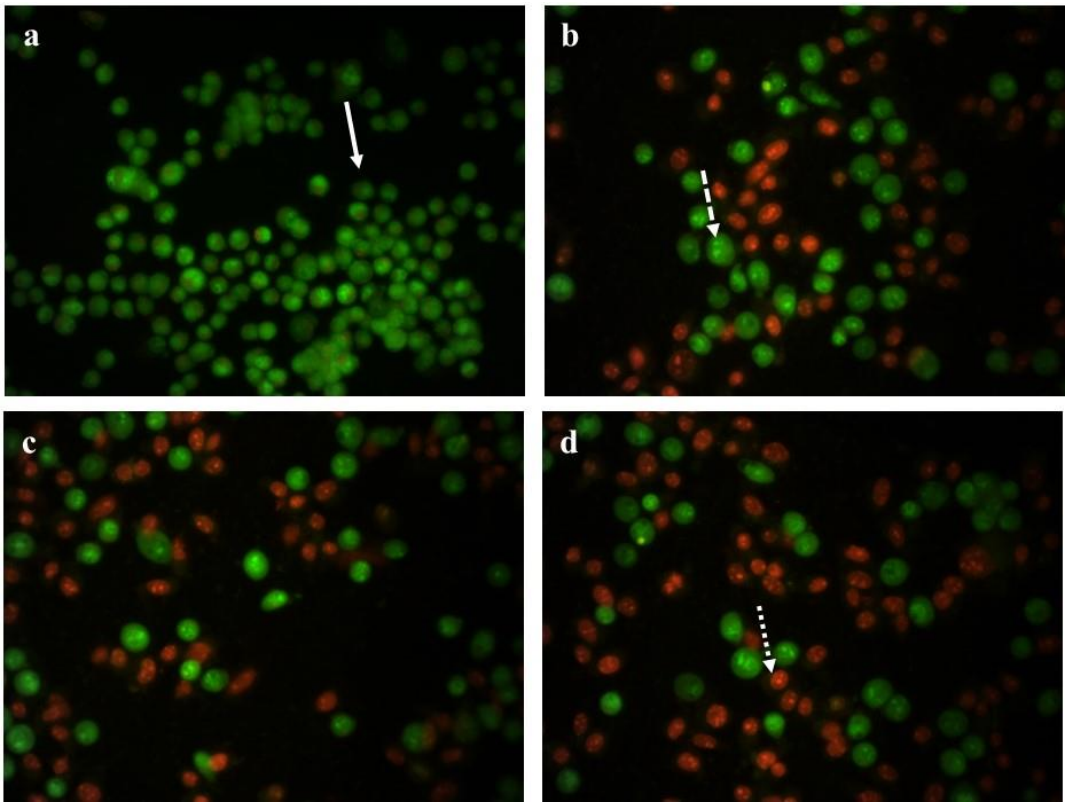


Figure 4. Dual acridine orange/ethidium bromide fluorescent staining of MCF-7 cells. The cells were treated with (a) DMSO 1% as control, (b) IC_{50} concentration of doxorubicin as thepositive control, (c) IC_{50} concentration of methanol extract of *Crocus sativus* and (d) IC_{50} concentration of methanol extract of *Crocus pallasii* subsp. *haussknechtii*. Solid arrow indicates live cell, dashed arrow indicates early apoptotic cell and dotted arrow indicates late apoptotic cell. The images were taken with a fluorescence microscope at 400×.

Gentisic acid and gallic acid were the highest and lowest phenolic compound in dormant corms of *C. sativus* L., respectively [23]. The antioxidant activity of *C. pallasii* subsp. *haussknechtii* corms may be attributed to the presence of phenolic compound(s) in the methanol extract which needs to be further examined.

It can be concluded that the methanol extracts of *C. sativus* L. and *C. pallasii* subsp. *haussknechtii* corms contain cytotoxic agent(s) against human breast cancer cells and it may possess higher apoptotic activity compared to *C. pallasii* subsp. *haussknechtii*.

Acknowledgments

This research was supported financially by the Foundation of Immunoregulation Research Center, Shahed University, Tehran, Iran. We also thank the Universities of Tehran and Kurdistan for their support.

Author contributions

Raheleh Shakeri and Tayebeh Radjabian conceived and planned the study; Raheleh Shakeri, Ali Lashkari, and Maliheh Safavi performed the experiments; Jalal Khorshidi collected and identified specimens; Raheleh Shakeri analyzed and wrote the manuscript with support from Tayebeh Radjabian.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

References

[1] DeSantis CE, Ma J, Sauer AG, Newman LA, Jemal A**.** Breast cancer statistics, 2017, racial disparity in mortality by state. *CA Cancer J Clin*. 2017; 67(6): 439-448.

[2] Goldman B. Multidrug resistance: can new drugs help chemotherapy score against cancer? *J Natl Cancer Inst.* 2003; 95(4): 255-257.

[3] Balunas MJ, Kinghorn AD. Drug discovery from medicinal plants. *Life Sci*. 2005. 78(5): 431-441.

[4] Khorasanchi Z, Shafiee M, Kermanshahi F, Khazaei M, Ryzhikov M, Parizadeh MR, Kermanshahi B, Ferns GA, Avan A, Hassanian SM. *Crocus sativus* a natural food coloring and flavoring has potent anti-tumor properties. *Phytomed*. 2018; 43: 21-27.

[5] Sanei M, Rahimyan H, Agayev Y, Soheilivand S. New cytotype of *Crocus pallasii* subsp. *haussknechtii* from west of Iran. *Acta Hortic*. 2007; 739: 107-111.

[6] Mosaddegh M, Esmaeili S, Eslami-tehrani B, Kermatian B, Mohebby S, Hamzeloo-moghadam M. Anti-angiogenesis properties of *Crocus pallasii* subsp. *haussknechtii*, a popular ethnic food. *Res J Pharmacogn*. 2015; 2(3): 37-42.

[7] Hamzeloo-moghadam M, Shahrestani R, Keramatian B, Mohebby S. Cytotoxic activity of *Crocus pallasii* subsp. *haussknechtii* in human cancer cell lines. *Int J Pharm Sci Res*. 2019; 10(1): 117-120.

[8] Azmir J, Zaidul I, Rahman M, Sharif K, Mohamed A, Sahena F, Jahurul M, Ghafoor K, Norulaini N, Omar A. Techniques for extraction of bioactive compounds from plant materials: a review. *J Food Eng*. 2013; 117(4): 426-436.

[9] Van Meerloo J, Kaspers GJ, Cloos J. Cell sensitivity assays: the MTT assay. *Methods Mol Biol*. 2011; 731: 237-245.

[10] Smith SM, Ribble D, Goldstein NB, Norris DA, Shellman YG. A simple technique for quantifying apoptosis in 96-well plates. *Methods Cell Biol*. 2012; 112: 361-368.

[11] Sharma OMP, Bhat TK. DPPH antioxidant assay revisited. *Food Chem*. 2009; 113(4): 1202-1205.

[12] Escribano J, Díaz-guerra MJM, Riese HH, Ontañón J, García-olmo D, García-olmo DC, Rubio A, Fernández JA. In vitro activation of macrophages by a novel proteoglycan isolated from corms of *Crocus sativus* L. *Cancer Lett*. 1999; 144(1): 107-114.

[13] Escribano J, Ríos I, Fernández JA. Isolation and cytotoxic properties of a novel glycoconjugate from corms of saffron plant (*Crocus sativus* L.). *Biochim Biophys Acta Gen Subj*. 1999; 1426(1): 217-222.

[14] Escribano J, Diaz-guerra M, Riese HH, Alvarez A, Proenza R, Fernández JA. The cytolytic effect of a glycoconjugate extracted from corms of saffron plant (*Crocus sativus*) on human cell lines in culture. *Planta Med*. 2000; 66(2): 157-162.

[15] Shakeri R, Kheirollahi A, Davoodi J. Apaf-1: Regulation and function in cell death. *Biochimie*. 2017; 135: 111-125.

[16] Hengartner MO. The biochemistry of apoptosis. *Nature*. 2000; 407(6805): 770-776.

[17] Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell*. 2002; 108(2): 153-164.

[18] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983; 65(1-2): 55-63.

[19] Liu K, Liu PC, Liu R, Wu X. Dual AO/EB staining to detect apoptosis in osteosarcoma cells compared with flow cytometry. *Med Sci Monit Basic Res*. 2015; 21: 15-20.

[20] Vermes I, Haanen C, Reutelingsperger C. Flow cytometry of apoptotic cell death. *J Immunol Methods*. 2000; 243(1-2): 167-190.

[21] Zhang Y, Chen X, Gueydan C, Han J. Plasma membrane changes during programmed cell deaths. *Cell Res*. 2018; 28(1): 9-21.

[22] Kasibhatla S, Amarante-mendes GP, Finucane D, Brunner T, Bossy-wetzel E, Green DR. Acridine orange/ethidium bromide (AO/EB) staining to detect apoptosis. *CSH Protoc*. 2006; Article ID pdb.prot4493.

[23] Esmaeili N, Ebrahimzadeh H, Abdi K, Safarian S. Determination of some phenolic compounds in *Crocus sativus* L. corms and its antioxidant activities study. *Pharmacogn Mag*. 2011; 7(25): 74-80.

[24] Baba SA, Malik AH, Wani ZA, Mohiuddin T, Shah Z, Abbas N, Ashraf N. Phytochemical analysis and antioxidant activity of different tissue types of *Crocus sativus* and oxidative stress alleviating potential of saffron extract in plants, bacteria, and yeast. *S Afr J Bot*. 2015; 99: 80-87.

[25] Barchan A, Bakkali M, Arakrak A, Pagán R, Laglaoui A. The effects of solvents polarity on the phenolic contents and antioxidant activity of three *Mentha* species extracts. *Int J Curr Microbiol App Sci*. 2014; 3(11): 399-412.

[26] Dorman HD, Koşar M, Kahlos K, Holm Y, Hiltunen R. Antioxidant properties and composition of aqueous extracts from Mentha species, hybrids, varieties, and cultivars. *J Agric Food Chem*. 2003; 51(16): 4563-4569.

Abbreviations

AO/EB: dual acridine orange/ethidium bromide; DMSO: Dimethyl sulfoxide; DPPH: 1-diphenyl-2-picryl-hydrazyl; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide