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Original article

# Anti-angiogenesis properties of *Crocus pallasii* subsp. *haussknechtii*, a popular ethnic food

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## Abstract

**Background and objectives:** Angiogenesis is essential for tumor survival. Inhibiting angiogenesis could be a mechanism for hindering tumor development. Numerous studies have now been focused on agiogenesis inhibitors and many of such studies have targeted plant materials. In the present study, *Crocus pallasii* subsp. *haussknechtii* has been evaluated for anti-angiogenesis properties. **Methods**: Anti-angiogenesis activity of the plant extracts and fractions has been investigated through wound healing assay in HUV-EC-C cells. The cytotoxic activity has also been evaluated by MTT assay. **Results:** The methanol extract and the methanol fraction of the corm along with the chloroform fraction of the aerial parts demonstrated to be cytotoxic to HUV-EC-C cells with $IC_{50}$ values of 27.2, 74.1 and 60.0 µg/mL, respectively while the chloroform fraction of the corm showed the most considerable anti-angiogenesis property among the samples in wound healing assay. **Conclusion:** Regarding the results of the present study, *Crocus pallasii* subsp. *haussknechtii* is suggested for further studies in cancer research evaluations.

**Keywords:** anti-angiogenesis, *Crocus pallasii* subsp. *haussknechtii*, HUV-EC-C, MTT assay, wound healing assay

## Introduction

During angiogenesis, new blood vessels are produced from pre-existing ones; while angiogenesis is also involved in some pathological disorders like rheumatoid arthritis, diabetic retinopathy and inflammatory disorders [1]. Angiogenesis has been found to be an important factor in tumor invasion and metastasis and it is a vital process in growth and invasion of many solid tumors which makes it a proper target for focusing treatments [2]. Tumor growth is greatly dependent to formation of new blood vessels which lead to entering of cancer cells to blood circulation and spreading throughout the body [3]. By anti-angiogenic therapy pro-angiogenesis factors would be suppressed and anti-angiogenesis parameters would be activated.

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Several natural products are under investigations for anti-angiogenesis properties in cancer research studies [2]. Cell-based angiogenesis assays like wound healing assay could be used as primary examinations for evaluating angiogenesis/anti-angiogenesis properties of materials. In the present study *Crocus pallasii* subsp. *haussknechtii* extracts and fractions whose peeled corm is widely popular as a spring nut in western provinces of Iran, has been evaluated for its anti-angiogenesis properties.

## Experimental

*Plant material*

*Crocus pallasii* subsp. *haussknechtii* whole plants was collected from Kermanshah Province, Kermanshah, Iran (2011) and was authenticated by botanists of Traditional Medicine and Matria Medica Research Center, Shahid Beheshti University of Medical Sciences (TMRC), Tehran, Iran. The aerial parts and corms were carefully separated and dried in shade and ground.

*Chemicals and reagents*

Dulbecco's modified Eagle medium (DMEM), Fetal Bovine Serum (FBS), Ham-F12 (Gibco, Auckland, New Zealand), penicillin-streptomycin, MTT ([3-(4, 5-dimethylthiazol-2-yl) -2, 4-diphenyl tetrazolium bromide]), PBS (Sigma, St. Louis, MO, USA) and DMSO (dimethyl sulfoxide) (Merck, Hohenbrunn, Germany), were used in the cell culture evaluations and solvents (Mojalali, Iran), were used for extraction and fractionation.

*Extraction*

Aerial parts and the corms were separately macerated with methanol (1:10, 5 g each) for 24 h with continuous shaking. The mixture was filtered afterwards and the residue was macerated again with fresh solvent (thrice). The concentrated filtrate was kept in refrigerator for further evaluations.

*Fractionation*

Fractionation for the aerial parts and corms (1:10, 15 g each) was carried out in three successive steps using *n*-hexane, chloroform and methanol with the same method as described above for extraction.

*Cell line*

HUV-EC-C cells (human umbilical vein endothelial cells) were provided from Pasture Institute of Iran, Tehran, Iran. They were cultured in DMEM with 10% FBS and 25% Ham-F12 to maintain the desired growth in a humidified incubator at 37 °C in an atmosphere containing 5% $CO_2$. They were further treated with 1% penicillin-streptomycin.

*Sample preparation*

The stock solutions of the extracts/fractions were prepared in DMSO (10 mg/mL), serial two fold concentrations were prepared from the above stock solutions (6.25-100 µg/mL) in a way that the final DMSO concentration did not exceed 1%.

*MTT assay*

The toxicity of samples to HUV-EC-C cells was assessed in a Microculture formazan assay (MTT assay) [4,5] to find the concentrations which did not induce cytotoxicity to the cells and almost all cells retained their viability. Briefly, the cells were seeded in 96-plates, after 24 h incubation at 37 ºC, they were treated with samples for 72 h. The medium was then replaced with fresh medium containing MTT, with a final concentration of 0.5 mg/mL. Following 4 h incubation, the medium containing MTT was removed and the formazan crystals were dissolved in DMSO. The absorbance was recorded at 570 nm with an ELISA reader (TECAN). The relative cell viability (%) related to control wells was calculated as:

[A]samples/[A]control × 100.

Where [A] samples is the absorbance of test sample and [A] control is the absorbance of wells containing cells+medium+DMSO 1%. To calculate $IC_{50}$ values, viability (%) versus log concentrations was graphed using the Microsoft Excel program.

*Wound healing assay*

Wound healing assay is based on evaluating cell migration into a wound in a monolayer of cells

[6]. The cells were seeded in 6-well plates ($5\times10^5$ in each well). After 24 h a straight scratch, simulating a wound was made with a sterile yellow pipette tip in the center of each well. Then samples at concentration of 12.5 µg/mL (the concentration that almost all cells retained their viability) were exposed to cells. Control wells received DMSO 1%. The wells were photographed at the start of the experiment in alternate intervals for 4 days.

**Results and Discussion**

For building new blood vessels, the endothelial cells should move from the basement membrane and migrate to the stimuli from tumor cells, activated lymphocytes, or wound-associated macrophages. They should also reproduce rapidly to supply new blood vessels [7]. There are a number of assays for measuring cell proliferation and one of them is the wound healing assay. In the present study, the anti-angiogenesis property of *Crocus pallasii* subsp. *haussknechtii* extract and fractions have been evaluated in HUV-EC-C cells by wound healing assay (figures 1-3). The cytotoxicity of these extracts and fractions were investigated through MTT assay prior to the anti-angiogenesis evaluation (table 1).

**Table 1.** Cytotoxici activity of extracts and fractions of *Crocus pallasii* subsp. *Haussknechtii* on HUV-EC-C cells

| | $IC_{50}$ (µg/mL) | |
|---|---|---|
| **Sample** | **Aerial parts** | **Corm** |
| *Crocus palasii* (M) | - | 27.2 |
| *Crocus palasii* (H) | - | - |
| *Crocus palasii* (CL) | 60.0 | - |
| *Crocus palasii* (ME) | - | 74.1 |

M: methanol extract; H: *n*-hexane fraction; CL: chloroform fraction; ME: methanol fraction

The results demonstrated that the corm extract and methanol fraction and the aerial parts chloroform fraction were toxic to HUV-EC-C cells with $IC_{50}$ values of 27.2, 74.1 and 60.0, respectively, which suggested the polar and semi polar constituents of the species to be responsible for the observed activity. The cells maintained

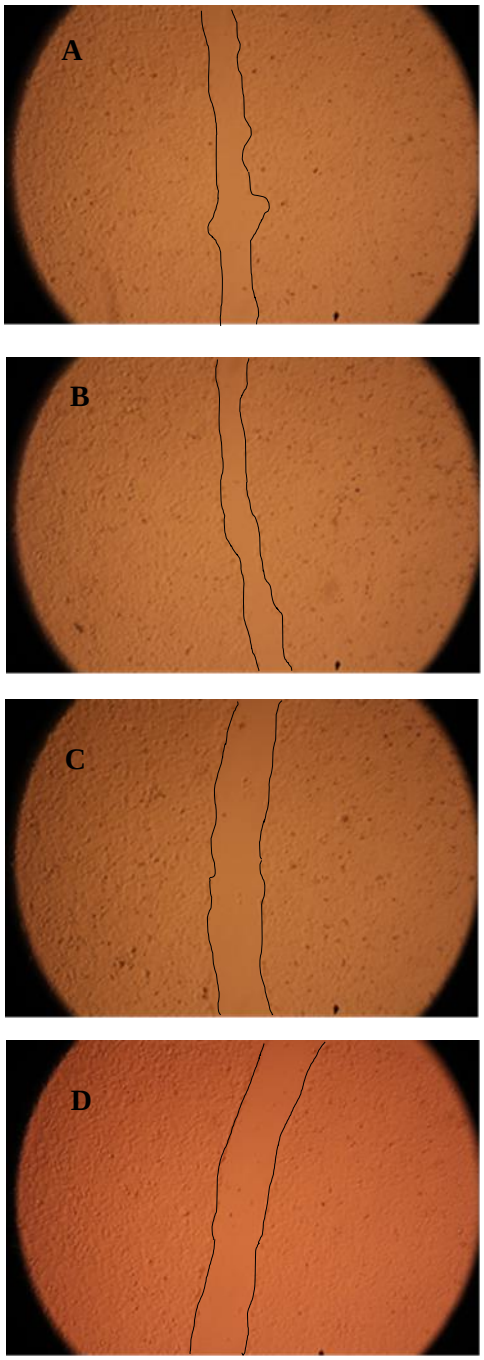


**Figure 1**. Anti-angiogenesis activity of the chloroform fraction of the aerial parts (A), methanol extract of the corm (B), cholorom fraction of the corm (C) and control (D); day 1, hour 4

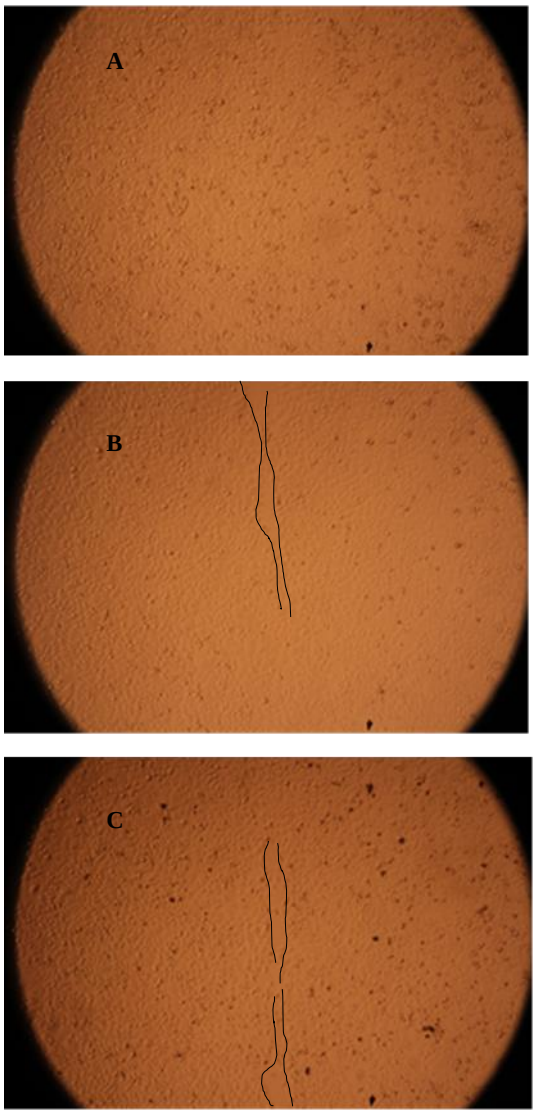


**Figure 2**. Anti-angiogenesis activity of the chloroform fraction of the aerial parts (A), methanol extract of the corm (B) and cholorom fraction of the corm (C); day 2, hour 4

their viability in concentration of 12.5 µg/mL for all extracts and fractions; thus the anti-angiogenesis activity was assessed in 12.5 µg/mL for all samples to find whether the samples could prevent angiogenesis in the concentrations that were not toxic to the HUV-EC-C cells.

Evaluating the anti-angiogenesis activity was performed for 4 days and the activity (regarding the size of the scratch) was observed in the following order in the second day of the experiment:

Chloroform fraction of the corm> methanol extract of the corm> methanol extract of the aerial parts. In the third day the scratch could still be observed for the chloroform fraction of the corm; while all wounds had been closed in the 4$^{th}$ day. The wound for the control cells was closed in the second day.

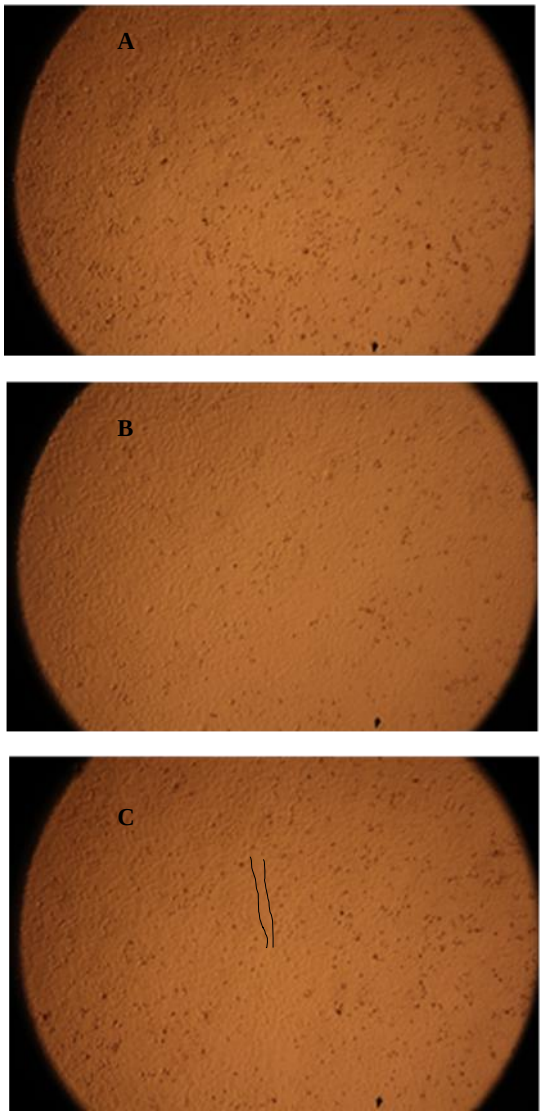


**Figure 3**. Anti-angiogenesis activity of the chloroform fraction of the aerial parts (A), methanol extract of the corm (B) and cholorom fraction of the corm (C); day 3

*Crocus sativus* L. (saffron) is a well-known species of the same genus of the plant in our study, which has shown to be able to inhibit angiogenesis in several studies. The aqueous extract of *Crocus sativus* has shown anti-angiogenesis properties in cut aorta of Wistar rat cultured in the collagen matrix, in a dose dependent manner. 200 and 300 μg/mL of the extract have significantly decreased the average number and length of vessels [8]. The extract has also demonstrated anti-angiogenesis potential by decreasing the expression of vascular endothelial growth factor receptor (VEGFR2) in MCF-7 cells as a synergic result of combination with electromagnetic field as demonstrated by Mousavi *et. al*. [9]. In another study in chick chorioalantoic membrane, saffron aqueous extract showed a dose dependent inhibitory activity on the average length of blood vessels [10]. Considering that crocetin which is an aglycone of crocin (a carotenoid found in *Crocus sativus)*, has suppressed the VEGF-induced angiogenesis in previous studies [11], isolation of compounds and evaluating their activity for anti-angiogenesis properties could be of interest for further evaluations. Our findings suggest that the effects of the corm extracts might be more considerable than the aerial parts in the preliminary wound healing assay. More specific examinations could be useful in better understanding the mechanism of the anti-angiogenesis property of *Crocus pallasii* subsp. *Haussknechtii* and its components.

**Acknowledgments**

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**Declaration of interest**

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

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