# The Synergic Effects of *Crocus Sativus L.* and Low Frequency Electromagnetic Field on VEGFR<sub>2</sub> Gene Expression in Human Breast Cancer Cells

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#### Abstract

**Background:** Angiogenesis, which is required for embryonic development and many physiological events, plays crucial role in many pathological conditions such as tumor growth and metastasis. Recent studies indicate anticancer and antitumor properties of saffron against human cancers. Many processes are affected by Electromagnetic Field (EMF) and its effect on proliferation and gene expression were examined. In this experimental study, the synergic effects of saffron and EMF on VEGFR<sub>2</sub> gene expression in MCF7 cells were investigated.

**Methods:** Saffron was extracted using freeze dryer. MCF7 cells were grown in RPMI 1640 medium supplemented with 10% FBS and incubated at 37 °C with 5% CO<sub>2</sub>. After 24 *hr* cells were treated with saffron extract at concentrations of 100, 200, 400 and 800  $\mu g/ml$ . Forty eight *hr* after treatment all flasks were exposed with EMF (50 *Hz*, 0.004 *T*). Then total RNA was extracted and cDNA was synthetized using specific primer. Synthetized products were analyzed by Real Time PCR to determine expression level of VEGFR<sub>2</sub>. Data were analyzed by SPSS (ANOVA & Tukey).

**Results:** Critical inhibitory effect on VEGFR<sub>2</sub> gene expression was 20% at 400  $\mu g/ml$ . Synergic use of EMF and saffron extract showed most reduction (38%) at 100  $\mu g/ml$ . On the other hand synergic use of 200, 400 and 800  $\mu g/ml$  saffron aqua extract and EMF decline noticeably the VEGFR<sub>2</sub> level of gene expression to 29, 35 and 36%, respectively. EMF itself also reduced VEGFR<sub>2</sub> up to 25% in comparison with control group which is remarkable at p<0.001.

**Conclusion:** Results indicate a decrease in the expression of vascular endothelial growth factor receptor in the treated samples with saffron extract compared to control. This reduction in VEGFR<sub>2</sub> level induced by synergic treatment of saffron and EMF which reveals induction of inhibitory effects of saffron on angiogenesis and could be also considered as a promising chemotherapeutic agent in breast cancer treatment.

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# Introduction

Tumor angiogenesis which is related to Folkman's hypothesis explains about the growth of solid tumors as a result of blood vessel development. Also, it is regulated by expression of specific mediators which initiate a cascade of events leading to the formation of new micro vessels <sup>1</sup> At least five VEGF-related ligands are identified. Among these types, Vasular Endothelial Growth Factor-A (VEGF-A) is thought to be the most important angiogenesis simulator which is a hypoxia regulated gene<sup>2</sup>. The biological effects of VEGF binding to three receptor tyrosine kinases, VEGFR-1, VEGFR-2, and VEGFR-3 are fairly well known. Research shows that VEGFR-2 is the primary receptor involved in angiogenesis, and its activation is considered critical in endothelial cell proliferation and survival <sup>3</sup>. The search for antiangiogenic strategies in cancer treatment indicated that targeting VEGF-A/VEGFR2 pathway resulted in significant inhibition of neovascularization and tumor growth <sup>4</sup>.

Saffron, the stigma of *Crocus sativus L*. (Iridaceae), currently used as spice, is the source of food additives and colorants and a component of traditional medicines <sup>5</sup>. This biomedical herb contains many constituents such as crocetin, picrocrocin and volatile compounds including safranal, crocin and crocetin <sup>6,7</sup>. Saffron was evaluated for its pharmacological activities such as anticancer and antitumor and its constituents showed antioxidant activity in different organs such as muscle, kidney and hippocampus. Recent studies demon-

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strated the antitumor properties of saffron both in vitro and in vivo<sup>8</sup>. However, use of more effective treatments with less thread was considered by researchers during the recent years. One of these methods is using the electromagnetic field. Results of different studies showed different effects on organisms according to type of electromagnetic field, amplitude, frequency and time of exposure. Several mechanisms, both thermal and nonthermal, are well established by which electromagnetic fields can interact with biological systems <sup>9</sup>. It is also shown that static magnetic fields impair angiogenesis and growth of solid tumors in vivo. Furthermore, DNA, cell membrane and microtubules could be affected by EMF<sup>10</sup>. Therefore, the aim of this study was to investigate the synergic effect of EMF and saffron extract on VEGFR<sub>2</sub> expression in MCF<sub>7</sub> cell line. The study investigated whether 0.04 T electromagnetic field could increase inhibitory effects of saffron on VEGFR<sub>2</sub> expression in MCF7 cell line.

# **Materials and Methods**

# Saffron sample preparation

Original Iranian saffron (*Crocus sativus L.*) which is widely grown and gathered in autumn in south of Khorasan province was purchased from Novin Zaferan Co. (Mashhad, Iran). The plant was identified by a plant taxonomist from the Herbarium Division of the College of Ferdowsi University. The stigma's aqueous extract was prepared as follows: 3 g of dried stigmas was extracted with 250 ml of sterile distilled water by Soxhlet apparatus. The mixture was transferred to rotary to remove water. In order to dry the extract, lyophilization was done by using freeze dryer.

## Cell culture

MCF7 cells were obtained from Pasteur Institute. Cells were cultured in RPMI medium (Biosera, Iran) with 10% fetal bovine serum (Gibco, USA), 100 *units/ml* penicillin and 100  $\mu g/ml$  streptomycin (Sigma, France) and also 1 *ml* L-glutamine (Sigma, France) and incubated at 37 °C with 5% CO<sub>2</sub>. Twenty four *hr* after cell culture and after being confident about cell adhesion to flask, cells were treated with aqueous extract of saffron at concentrations of 100, 200, 400 and 800  $\mu gm/$ *ml*. Then to evaluate the viability, Trypan blue (Sigma, France) test was used and the pictures were captured with invert microscope (Dinocaptre) with a digital camera for 5 days to investigate cell morphological changes.

#### **Exposing to EMF**

After 48 hr of successful cell culture and treatment, samples prepared for testing the synergic effect were exposed to EMF. Flask was placed in 50 Hz, 0.04 T EMF for 1 hr (made in Islamic Azad University of Mashhad, Iran).

## **RNA** extraction

RNA was extracted by total RNA purification kit (Bioscience, Germany). After 48 hr of treatment, total RNA was purified and stored at -20°C until cDNA synthesis. The amount of RNA Nanodrap was measured with wavelengths 260, 280 and 320 nm and data were obtained and analyzed. These data indicated the concentration of the extracted RNA which was used for cDNA synthesis. RNA concentration=(OD 260-OD 320)×40 ×100.

#### cDNA synthesis

cDNA was synthesized by Bioneer Kit (Korea) and the temperatures for synthesis are shown in table 1.

# Synthesis of primers

The sequences of genes were received from NCBI site and the bioinformatic validation of RT-PCR primers were done by PRIMER BLAST and delivered to Bioneer Company (Korea). Primers were designed according to table 2.

# Evaluation of gene expression

Process of real time PCR to study gene expression was done by Applied Biosystems according to the protocol of Bioneer kit (Korea). Based on the protocol, firstly, Master Mix was prepared and added to strip cap microtube and then the cDNA was added to it. The temperatures were determined according to Melting Temperature (Tm) of designed primers and the characteristics of different phases in polymerase chain reaction.

Gene expression levels of VEGFR<sub>2</sub> in samples treated with aqueous extract of saffron and the control samples were analyzed by SPSS (V.16). A p-value of less than 0.05 and CI (Confidence interval) of 95% were considered significant. In this project, we used relative quantities which were based on expression of target gene to the reference gene via comparing the target gene efficiency with control sample and also using their threshold cycle (CT). The primers and the temperature of

Table 1. The temperature required for the synthesis of cDNA

Step	Temperature	Time (min)	
Primer annealing	Tm of specific primer	1	
cDNA synthesis	42-70°C	10-60	
Heat inactivation	95°C	5	

Gene	Forward $5' \rightarrow 3'$	Reverse 5'→3'	ТМ	Chromosomal location
β-Actin	CCC GCC GCC AGC TCA CCA TGG	AAG GTC TCA AAC ATG ATC TGG GTC	64.5	7p22
Vascular endothelial growth factor receptor	TAT GTC TAT GTT CAA GAT TAC	AAG TTT CTT ATG CTG ATG CTT	42.9	4q11-q12
	RQ 18. Target 1.1 1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0 Beta Actin Legend ■ control	RQ 18. Target 1.1 1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0 VEGFR2 Target Legend Control	-	

Table 2. Sequences of genes

Figure 1. Housekeeping gene ( $\beta$ -Actin) expression in comparison with target gene (VEGFR<sub>2</sub>)

binding primer were essential factors in the optimization of real time PCR reaction. So conditions of reaction were optimized and no nonspecific products were produced. This optimized condition observed by mono peak in melting curve and also electrophoresis in agar gel by looking single sharp band. The mean±SEM was determined for each study group. Data were analyzed by ANOVA and Tukey multiple comparison. The p<0.05 was considered statistically significant.

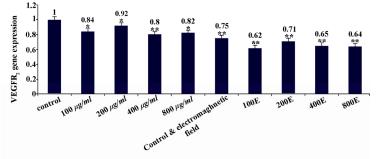
## **Results**

Since the PCR reaction efficiency between the target gene and the housekeeping gene ( $\beta$ -Actin) was the same (Figure 1), the comparative (CT) was used in this project. Amplification curve of samples treated with aqueous extract of saffron was obtained as well. Gene expression studies showed a significant reduction (p<0.05) in gene expression levels of VEGFR2 treated with concentrations of 100, 200, 400 and 800  $\mu g/ml$  in comparison with the control group.

Data analysis showed that inhibitory effects of

saffron extract in concentrations of 100, 200, 400 and 800  $\mu g/ml$  on VEGFR<sub>2</sub> gene expression were 16, 8, 20 and 18%, respectively in MCF7 cell line in comparison with the control group (p < 0.05). As data indicate, significant inhibitory effect on gene expression of VEGFR<sub>2</sub> was 20% in 400  $\mu g/ml$  of saffron extract (p<0.001). EMF itself could reduce VEGFR<sub>2</sub> up to 25% in comparison the with control group (p < 0.001).

Synergic use of EMF and saffron extract resulted in most amount of reduction in concentration of 100  $\mu g/ml$  with inhibitory effect of 38% on VEGFR<sub>2</sub> level (p=0.001). Moreover, the synergic use of saffron aqua extract in concentrations of 200, 400 and 800  $\mu g/ml$  and in electromagnetic field of 400 Gauss noticeably reduced VEGFR<sub>2</sub> level of gene expression to 29, 35 and 36%, respectively (p<0.001). The difference between concentrations of 400 and 800  $\mu g/ml$  was not significant (p>0.05). Figure 2 represents the level of VEGFR<sub>2</sub> gene expression in comparison with the control group after being normalized to β-Actin. It was also examined whether the CT variance was scaled



Concentration of saffron extract µg/ml

Figure 2. VEGFR<sub>2</sub> gene expression in samples treated with saffron aqueous extract in different concentrations and samples treated with saffron aqueous extract µg/ml exposed to electromagnetic field for one hr. \* p<0.05, \*\*p<0.001



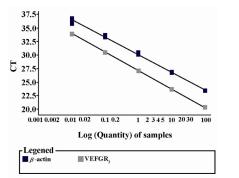


Figure 3. CT scaling down by using standard dilution samples with quantities of 100, 10, 0.1, and 0.01.

down by using standard dilution samples with quantities of 100, 10, 0.1, and 0.01 or not. Data analysis revealed that by dilution of samples CT changed and was scaled down. Acceptable data with high efficiency are represented in figure 3.

#### Discussion

In this research, we investigated synergic effect of low frequency (50 Hz) and 0.04 T intensity electromagnetic field and saffron extract on VEGFR<sub>2</sub> level in human breast cancer cells. It has been demonstrated that saffron's main components including crocin, crocetin and safranal could inhibit neuron erosion and depression <sup>6</sup>.

Results of a research in 2009 revealed that the most important point in curing cancers with synthetic medication and chemotherapy is the acquisitive resistance of cells to chemical drugs because of high mutation and genetic variation in tumor cells comparing with other cells. Therefore their resistance to synthetic drugs is higher and angiogenesis inhibition with natural products such as saffron seems to be a useful and promising method for having less resistance <sup>7</sup>.

There are several mechanisms for the antitumor effect of saffron and its components, including inhibition of nucleic acid, scavenging free radical, its effect on the expression of topoisomerase 2, induction of programmed cell death. Furthermore, it could reduce the possibility of developing cancer, decline the rate of tumor cells and significantly increase the lifespan of animals <sup>11</sup>. It was indicated that saffron in different tumors, including leukemia, ovarian and breast carcinoma has an anticancer and selective cytotoxtic effect on different malignant cells <sup>5</sup>. Moreover, saffron had cancer-preventive and antigenotoxic potential. Therefore, it can be used in combination with chemotherapy since some properties in saffron have the potential against lipid peroxidation and at the same time increase the level of enzymatic antioxidants like superoxide dismutase and catalase and non-enzymatic antioxidants such as liver glutathione <sup>12</sup>. In addition, safranal increases tissue oxygen which has sweeping effect on free radicals and can inhibit oxidative stress of genotoxic compounds and safranal has protective effect on lipid peroxidation. Also, angiogenesis shows direct relation with tissue oxygen and hypoxia is one of the most important simulators of angiogenesis. Therefore, increasing tissue oxygen accompanied with saffron treatment may define some part of antiangiogenic effect of this herb <sup>13</sup>.

Results of another research represented that saffron has antitumor effects on Transitional Cell Carcinoma (TCC) cell line (related to bladder cancer) which is time and dose-dependent in the sense that in high concentrations, the percentage of vital cells declines dramatically <sup>14</sup>. Results of these researches are consistent with the findings of the present project. Influential potential of saffron on the induction or inhibition of gene expression in few cases has been studied so far. However, Mousavi et al had studied the effect of saffron extract on the level of protein which was related to apoptosis such as Bax protein and influential enzymes like caspase in breast cancer cells. They represented that using the extract could reduce cell viability based on the dose applied <sup>15</sup>. The obtained results on the induction of apoptosis in saffron extract justifies that it can have significant cytotoxic effects on hepatocarcinoma and cervix cells <sup>16</sup>. These results coincided with our findings and cancer cell growth was inhibited by the extract in a concentration-dependent manner.

In this research, the reduction in the level of VEGFR<sub>2</sub> and decline in gene expression by saffron extract demonstrated the conformity in our findings with other data that saffron has cytotoxic and anticancer effects. EMF was applied as the treatment for some specific pathologic conditions like bone fractures, skin ulcers, and migraine <sup>17</sup>. Delle et al showed that some important functions of human micro vascular endothelial cells (in vitro) like proliferation, migration and tube formation are increased under the influence of EMF (1 mT, 50 Hz). Furthermore, they demonstrated that EMF increased the degree of endothelial cell proliferation and tubule formation and a significant increase in phosphorylation as well as the overall expression of VEGF receptor 2 was observed <sup>18</sup>. Chen et al found the inhibitory effects of nanosecond pulsed electric fields on survival of mice with hepatocellular carcinoma. Also they observed the decrease in vascular endothelial growth factor expression and micro-vessel density and apoptosis induction besides inhibition of angiogenesis <sup>19</sup>. Obviously, time of exposure, amplitude, intensity and type of electromagnetic fields and genetic characteristics of samples which were used in different researches were not the same leading to various findings. In addition, Wang et al reported that gradient static magnetic fields might inhibit or prevent new blood vessel formation and could be helpful for the treatment of some diseases relevant to pathological angiogenesis <sup>20</sup>. According to this research, we observed the inhibitory effects of EMF on angiogenesis by reduction of VEGFR<sub>2</sub> level in human breast cancer cells. Moreover, synergic use of both saffron extract and EMF could decline VEGFR<sub>2</sub> gene expression which may account for antitumor property of saffron and novel antiangiogenic characteristic of EMF.

### Conclusion

The results of this study indicated the reduction of VEGFR<sub>2</sub> gene expression in breast cancer cells treated with saffron aqueous extract and electromagnetic field. The study revealed both the antiangiogenic potential of this medical herb and therapeutic effects of EMFs as a promising chemotherapeutic agent in treatment of breast cancer, and its potential on prevention of angiogenesis.

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#### References

- 1. Bhat TA, Singh RP. Tumor angiogenesis-A potential target in cancer chemoprevention. Food Chem Toxicol 2008;46(4):1334-1345.
- Lyons JM 3rd, Schwimer JE, Anthony CT, Thomson JL, Cundiff JD, Casey DT, et al. The role of VEGF pathways in human physiologic and pathologic angiogenesis. J Surg Res 2010;159(1):517-527.
- 3. Otrock ZK, Hatoum HA, Musallam KM, Awada AH, et al. Is VEGF a predictive biomarker to anti-angiogenic therapy? Crit Rev Oncol Hematol 2010;79(2):103-111.
- Roodink I, Leenders WP. Targeted therapies of cancer: Angiogenesis inhibition seems not enough. Cancer Lett 2010;299(1):1-10.
- Melnyk JP, Wang S, Marcone MF. Chemical and biological properties of the world's most expensive spice: saffron. Food Res Int 2010;43(8):1981-1989.

- Ochiai T, Shimeno H, Mishima K, Iwasaki K, Fujiwara M, Tanaka H, et al. Protective effects of carotenoids from saffron on neuronal injury in vitro and in vivo. Biochim Biophys Acta 2007;1770(4):578-584.
- Premkumar K, Thirunavukkarasu C, Abraham SK, Santhiya ST, Ramesh A. Protective effect of saffron (Crocus sativus L.) aqueous extract against genetic damage induced by anti-tumor agents in mice. Hum Exp Toxicol 2006;25(2):79-84.
- Hosseinpour Chermahini S, Abd F, Sarmadi M, Taghizadeh E, Salehnezhad S. Impact of saffron as an anticancer and anti-tumor herb. Afr J Pharm Pharmacol 2010;4(11):834-840.
- Rochalska M. The effect of electromagnetic fieds on living organism: plants, birds and animal. Med Pr 2007; 58(1):48-37.
- Berg H, Günther B, Hilger I, Radeva M, Traitcheva N, Wollweber L. Bioelectromagnetic field effects on cancer cells and mice tumors. Electromagn Biol Med 2010;29 (4):132-143.
- 11. Schmidt M, Betti G, Hensel A. Saffron in phytotherapy: Pharmacology and clinical uses. Wien Med Wochenschr 2007;157(13-14):315-319.
- Ochiai T, Soeda S, Ohno S, Tanaka H, Shoyama Y, Shimeno H. Crocin prevent the death of PC-12 cells through sphingo myelinaseceramide signaling by increasing glutathione synthesis. Neurochem Int 2004;44(5):321-330.
- Holloway GM, Gainer JL. The carotenoid crocetin enhances pulmonary oxygenation. J Appl Physiol 1988;65 (2):683-686.
- Feizzadeh B, Afshari JT, Rakhshandeh H, Rahimi A, Brook A, Doosti H. Cytotoxic effect of saffron stigma aqueous extract on human transitional cell carcinoma and mouse fibroblast. Urol J 2008;5(3):161-167.
- Mousavi SH, Tavakkol-Afshari J, Brook A, Jafari-Anarkooli I. Role of caspases and Bax protein in saffron-induced apoptosis in MCF-7 cells. Food Chem Toxicol 2009;47(8):1909-1913.
- Tavakkol-Afshari J, Brook A, Mousavi SH. Study of cytotoxic and apoptogenic properties of saffron extract in human cancer cell lines. Food Chem Toxicol 2008;46 (11):3443-3447.
- McKay JC, Prato FS, Thomas AW. A literature review: the effects of magnetic field exposure on blood flow and blood vessels in the microvasculature. Bioelectromagnetics 2007;28(2):81-98.
- Delle Monache S, Alessandro R, Iorio R, Gualtieri G, Colonna R. Extremely low frequency electromagnetic fields (ELF-EMFs) induce in vitro angiogenesis process in human endothelial cells. Bioelectromagnetics 2008;29 (8):640-648.
- Chen X, Zhuang J, Kolb JF, Schoenbach KH, Beebe SJ. Long term survival of mice with hepato cellular carcinoma after pulse power ablation with nanosecond pulsed electric fields. Technol Cancer Res Treat 2012;11(1):83-93.
- Wang Z, Yang P, Xu H, Qian A, Hu L, Shang P. Inhibitory effects of a gradient static magnetic field on normal angiogenesis. Bioelectromagnetics 2009;30(6):446-453.

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