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Anti-angiogenesis effect of *crocous sativus* L. extract on matrix metalloproteinase gene activities in human breast carcinoma cells

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A R T I C L E   I N F O

A B S T R A C T

Introduction: There is an interest in *Crocous sativus* L. (Saffron) mainly because of its biological properties. Biomedical research has focused on saffron as a powerful antioxidant, anti-inflammatory and anti-tumor, but its mechanism has not yet been thoroughly clarified. In this study, the effects of saffron aqua extract on matrix metalloproteinases (MMP) gene expression were investigated.

Methods: In this experimental study, the saffron was extracted using water as solvent. MCF-7 cells in RPMI1640 medium were supplemented with 10% FBS and incubated at 37 °C with 5% CO2. After 24 h, the cells were treated by saffron extract at concentrations of 100, 200, 400 and 800 μg/ml. 48 h after treatment, total RNA was extracted and cDNA was synthesized using specific primer. Synthesized products were analyzed by Real Time PCR to determine expression level of MMP.

Results: Data analysis showed inhibitory effect of saffron at concentrations of 100 to 800 μg/ml on MMP gene expression in comparison with control group. Reduction for 100, 200, 400 and 800 μg/ml were 5%, 18%, 15%, 11%, respectively. According to data analysis treating MCF-7 cells with saffron at concentration of 200 μg/ml caused the highest decrease, with 18% reduction in gene expression (P<0.001).

Conclusion: Results indicate decrease in the expression of MMP compared with controls revealing induction of inhibitory effects of saffron on angiogenesis which might be also considered as a promising chemotherapeutic agent in breast cancer treatment.

Implication for health policy/practice/research/medical education:
In this study saffron decreased the expression of MMP revealing an inhibitory effect on angiogenesis. This might be considered as a promising chemotherapeutic agent in breast cancer treatment.

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Introduction

First blood vessels in a process, known as vasculogenesis, is formed from endothelial progenitor cells with novel specific patterns and gradually begin to spread, develop and form new branches (1). Formation of new blood vessels in adults is controlled precisely than previous vessels and angiogenesis just occurs in a physiological conditions such as pregnancy and specific pathological conditions such as wound healing, diabetic retinopathy, rheumatoid arthritis or cancer. Since angiogenesis is happened in pathological condition such as tumor growth and metastasis and plays an important role, it can also be targeted for antitumor therapy. In general, the process of angiogenesis is considered to have 10 stages

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which one or more sequential steps could be targeted by angiogenesis inhibitors or simulators (2). This process is extensively depend on interactions between different cells and molecules and also is controlled by a variety of peptides and moderating factors (3). Several factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), placental transforming growth factor (PTGF), signal transducer and activator of transcription 3 (STAT 3) are involved in this pathway (4). VEGF binding to vascular endothelial growth factor receptor 2 (VEGFR-2) triggers the specific activation of tyrosine amino acid residues within intra cytoplasmatic tail of the receptor inducing multiple signaling networks that result in endothelial cell survival, proliferation, migration, focal adhesion turnover, actin remodeling and vascular permeability. Signaling through mitogen-activated protein kinase and phosphatidylinositol 3' kinase is a common receptor tyrosine kinase activation pattern. Additional signaling pathways are also triggered upon VEGFR-2 activation, i.e., phospholipase C gamma, protein kinase focal adhesion kinase and T Cell-Specific Adaptor-Src kinase which are linked to migration and vascular permeability (5,6). Under hypoxic conditions, reduction of oxygen pressure is crucial and tissues start synthetizing and releasing angiogenic factors such as endothelial cell growth factors after binding to their special receptors, activating them which may lead to secretion of metalloproteinase from endothelial cells to digest and decompose basement membrane in the region. By reaching this aim endothelial cells would be able to migrate and proliferate. In addition, the molecule of adhesion such as integrins $\alpha_7\beta_5$ and $\alpha_3\beta_7$ are helpful in the process of pulling forward and bud blood vessels develop. In the next steps in the process of angiogenesis, matrix metalloproteinases (MMP) degrade extracellular matrix and start the production or reconstruction. By the interaction of the Tie-2 with angiopoietin tube formation begins at a later stage, EphB (ephrin B) also regulates formation of tubes, finally smooth muscle cells and pericytes as well as inducing the stabilization of newly formed blood vessel structure (7,8).

MMPs degrade and modify the extracellular matrix (ECM) as well as cell-ECM and cell-cell contacts, facilitating detachment of epithelial cells from the surrounding tissues (9). MMPs play key functions in embryonic development and mammary gland branching morphogenesis, but they are also up-regulated in breast cancer, where they stimulate tumorigenesis, cancer cell invasion and metastasis (10).

Rising cancer resistance to chemical therapies increased effort to find out new drugs in cancer therapy with fewer side effects and resistance (11). Cancer is growing in the world, and causes death of more than 6 million people each year, and breast cancer has the highest rates of mortality at ages of 40 (12,13). This cancer is hormone-dependent, which many of risk factors are known (14). Some of these risk factors are: geographic diversity, age, family history,

pregnancy history, pregnancy, benign breast disease, exogenous estrogens, oral contraceptive drugs, obesity, high-fat diet, alcohol consumption, smoking and radiation chest out (15,16).

Interfering with normal function of at least 4 to 6 genes by genetic and epigenetic mechanisms can lead to breast cancer, which disturb the balance between proliferation, apoptosis and differentiation, and also regulation of steroid receptors, binding cells and angiogenesis (17,18). Saffron, the stigma of *Crocus sativus* L. (Iridaceae), is currently used as spice, source of food additives, colorants and as a component of traditional medicines (19). This biomedical herb contains many constituents such as crocetin, picrocrocin and volatile compounds including safranal, crocins and crocetin (20,21). Saffron has been evaluated for its pharmacological activities such as anti-cancer, and antitumor and its constituents showed antioxidant activity in different organs such as muscle, kidney and hippocampus. Recent studies demonstrated the antitumor properties of saffron both *in vitro* and *in vivo* (22).

As regards to identification an effective strategy by using new agents from natural sources to treatment cancers, and since matrix metalloproteinase (MMP) is potent endothelial mitogen that up regulated in number of tumor types, including breast cancer, the aim of this study was to investigated the effect of saffron aqueous extract on level of MMP in human breast cancer cell line (MCF-7).

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

Cell culture and treatment
MCF7 cells were obtained from Pasteur Institute, Iran. Cells were cultured in RPMI medium (Biosera, Iran) with 10% fetal bovine serum (Gibco, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma, France) and also 1 ml L-glutamine (Sigma, France). Then, it was incubated at 37 °C with 5% CO2. 24 h after cell culture and insurance about cell adhesion to flask, cells were treated with aqueous extract of saffron at concentrations of 100, 200, 400 and 800 micrograms per ml. Then to evaluate the viability, Trypan blue (Sigma, France) test was used and pictures captured with a digital camera with invert microscope (Dinocapture) for 5 days to investigate cell morphological changes.

RNA extraction

RNA was extracted by total RNA purification kit (Bioscience, Germany). After 48 h of treatment, the total RNA was purified and stored at -20 °C until cDNA synthesis. To measure the amount of RNA, Nanodrop was used by wavelengths 260, 280 and 320 nm and data were measured and analyzed. These data indicate the concentration of the extracted RNA which was used for cDNA synthesis as follow:

RNA concentration = (OD 260 - OD 320) $\times$ 40 $\times$ 100

cDNA synthesis

cDNA was synthesized by Bioneer Kit (Korea), the temperature of synthesis was according to Table 1.

Synthesis of primers

The sequence of genes was received from NCBI site and the bio-informatic validation of RT-PCR primers were done in PRIMER BLAST and delivered Bioneer Company (Korea). Primers were designed according to Table 2.
Evaluation of gene expression using real time PCR
Process of real time PCR to study gene expression was done according to the protocol by Bioneer kit (South Korea) by Applied Biosystem. According to the protocol, Master Mix firstly was prepared and added to strip cap microtube and then the cDNA was added to it. Application temperature according Tm of designed primers and the characteristics of the different phases in polymerase chain reaction were determined.

Gene expression levels of MMP in samples exposed low frequency electromagnetic field in comparison with control samples were analyzed by SPSS (V.16). P-value of less than 0.05 and CI (Confidence Interval) 95% were accepted as significant. In this project, we used relative quantitative based on expression of target gene to the reference gene via comparing the target gene efficiency with control sample. The primer and the temperature of binding primer are essential factors in the optimization of Real Time PCR reaction. So condition of reaction was optimized in which no nonspecific products

produced. This optimized condition observed by mono peak in melting curve and also electrophoresis in agar gel by looking single sharp band. The mean ± SD were determined for each study group. Data were analyzed by ANOVA & Tukey multiple comparison procedure to calculate the significance. P<0.05 value between study groups was taken as statistically significant.

Results

Gene expression studies showed a significant reduction in the gene expression levels of MMP gene treated with concentrations of 100 (P<0.05), 200 (P<0.001), 400 (P<0.05) and 800 (P<0.05) micrograms per ml of cells in comparison with the control group (Figure 1). Also data analysis showed inhibitory effect of saffron extract at concentrations of 100, 200, 400 and 800 μg/ml on MMP gene expression with 5%, 18%, 15% and 11% respectively in MCF-7 cell line in comparison with control group. As data indicate significant inhibitory effect on gene expression of MMP was 18% in 200 μg/ml of saffron extract.

Since the PCR reaction efficiency between the target gene and the housekeeping gene (beta-actin) was the same, the comparative threshold cycle (CT) was used in this project. It was examined whether the CT variance scale down by using dilution standard samples with quantity of 100, 10, 0.1, and 0.01 or not? Data analysis revealed that by dilution of samples CT changed and scale down. Acceptable data with high efficiency are represented in Figure 2.

Discussion

In this research, we investigated the effect of saffron extract on MMP level in human breast cancer cells. Since extracellular proteolysis mediates tissue homeostasis and in cancer, altered proteolysis leads to unregulated tumor growth, tissue remodeling, inflammation, tissue invasion, and metastasis, so the MMPs represent the most prominent family of proteinases associated with tumorigenesis. Also while MMPs regulate signaling pathways that control cell growth, inflammation, or angiogenesis, the study of MMP function may be new approaches to cancer therapy (23).

Table 1. The temperature required for the synthesis of cDNA

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

Table 2. Sequences of genes

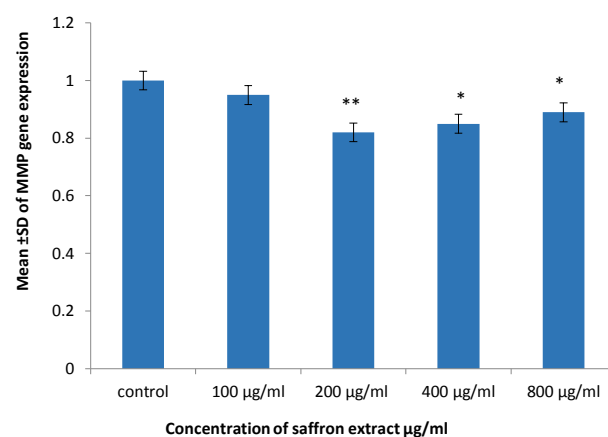| Gene	Forward 5'→3'	Reverse 5'→3'	Chromosomal location
Beta actin	CCC GCC GCC AGC TCA CCA TGG	AAG GTC TCA AAC ATG ATC TGG GTC	7p22
MMP	CTG CAT CCT CAG CAG GTTG	GTC TCG GAT AGT CTT TAT CC	1q 26422762-26426467bp

Figure 1. MMP gene expression in samples treated with saffron aqueous extract in different concentration compared with control group; * P<0.05, **P<0.001.
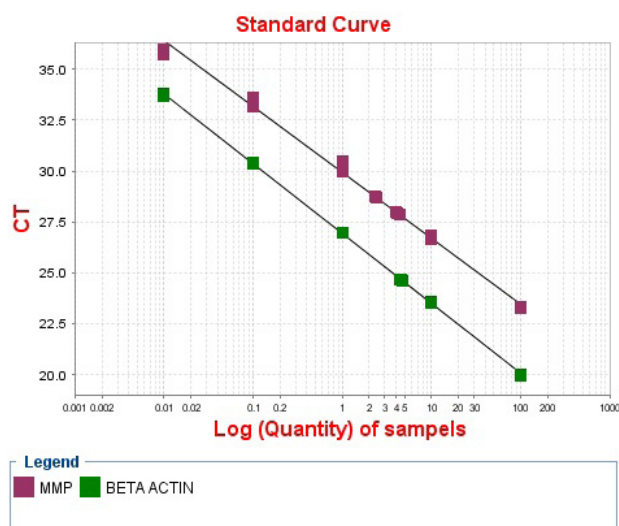
Standard Curve

Legend
■ MMP ■ BETA ACTIN

Figure 2. CT scale down by using dilution standard samples with quantity of 100, 10, 0.1, and 0.01.

Previously it was revealed that inhibition of MMP with two murine models could lead to decrease angiogenesis and MMPs may be a target in cancer treatment and inhibition of metastasis (24). Another research in 2009 indicated EGFR activation led to enhanced MMP expression and/or function, and suggested that modulating the expression or activity of the EGFR and/or matrix metalloproteinases offers opportunity for targeted intervention in patients with metastatic disease (25).

It has been demonstrated that saffron's main component such as crocin, crocetin and safranal could inhibit neurons erosion and depression (21). Result of a research in 2009 revealed that angiogenesis inhibition with natural products seemed to be useful and promising method in order to encounter less resistance (22). There are several mechanisms for the antitumor effect of saffron and its components, including inhibition of nucleic acid, scavenging free radical, effect on the expression of topoisomerase 2, and induction of programmed cell death. Furthermore, it could reduce cancer, decline the rate of tumor cell and significantly increase the lifelong of animal (26). It is indicated that saffron in different tumors, including leukemia, ovarian and breast carcinoma had anticancer and selective cytotoxic effect on different malignant cells (27). Moreover, saffron had cancer-preventive and anti genotoxic potential, so it would be used in combination with chemotherapy. Saffron has been shown reduce potential against lipid peroxidation and at the same time increase the enzymatic antioxidants like superoxide dismutase and catalase and non-enzymatic antioxidants such as liver glutathione regeneration (28). In addition, safranal increased tissue oxygen which have sweeping effect on free radicals and could inhibit oxidative stress of genotoxic compounds. Safranal also had protective effect on lipid peroxidation; since angiogenesis shows direct relation with tissue oxygen and hypoxia is one of the most important simulator of angiogenesis, increasing tissue oxygen accompanied. Saffron treatment may explain some part of anti-angiogenic effect of this herb (29). Results of another research represented that saffron had antitumor effects on TCC cell line (related to bladder cancer) in a time- and dose-dependent manner in a way that in high concentration the percentage of vital cells declined dramatically (30). Results of these researches were 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.* studied the effect of saffron extract on the level of protein which was related to apoptosis such as bax protein and enzyme as influential as caspase in breast cancer cells and represent that using saffron extract could reduce cell viability dose-dependently (31). The result on the induction of apoptosis of saffron extract depicts that on hepatocarcinoma and cervix cells extract showed significant cytotoxic effects (32). These results were coincided with our findings and cancer cell growth inhibited by the extract on concentration-dependent manner. In this research, saffron extract could reduce the level of MMP and gene expression decline which shows conformity with other data that reveals cytotoxic and anticancer effect of saffron.

Conclusion
The results of this study indicate reduction in MMP gene expression in breast cancer cells treated with the saffron aqueous extract. This may show the anti-angiogenic potential of this medical herb as a promising chemotherapeutic agent, and its potential in prevention of angiogenesis and metastasis.

Authors' contributions
JB designed and supervised the experiments, analyzed the data and wrote the paper; MM performed experiments, analyzed data and wrote the paper; MAS designed, analyzed data and wrote and edited the paper.

Conflict of interests
The authors declare no conflict of interest.

Ethical considerations
Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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