The Effect of Piperine on MMP-9, VEGF, and E-cadherin Expression in Breast Cancer MCF-7 Cell Line

Zahra Zare1, Tina Nayerpour Dizaj2, Armaghan Lohrasbi3,4, Zakieh Sadat Sheikhalishahi5, Mohammad Panji6, Fahimeh Hosseinabadi7, Vajiheh Najafi8, Omid Abazari9, Mojtaba Abbasi10,11, Parisa Khanicheragh12*

ABSTRACT

Background: Vascular endothelial growth factor (VEGF), matrix metalloproteinase-9 (MMP-9), and E-cadherin play a vital role in the behavior of angiogenesis, metastasis, and invasion of breast tumor cells. Piperine, the main component of Piper Nigrum, has shown anti-cancer properties in various malignancies. This Study investigates the potential effect of piperine on MMP-9, E-cadherin, and VEGF expression in breast cancer MCF-7 cell line.

Methods: MTT assay was applied to assess the viability of MCF-7 cells. The mRNA levels of MMP-9, VEGF, and E-cadherin were assayed by qRT-PCR. Western blot was performed to identify the protein level of MMP-9.

Results: MTT assay results showed that piperine treatment (5, 10, 25, 50, 75, and 100 μM) for 24 hours effectively inhibited cell viability of MCF-7 cells as compared with the control group. Furthermore, the gene expression of VEGF, MMP-9, and E-cadherin was dose-dependently suppressed by piperine treatment (5, 10, and 25 μM) (P<0.05; P<0.01). The results also indicated that piperine (5, 10, and 25 μM) significantly suppressed MMP-9 protein expression after 24 hours of piperine treatment (P<0.01).

Conclusion: These results suggest that piperine may prevent angiogenesis, migration, and invasion of MCF-7 cells by suppressing MMP-9 and VEGF, and by inducing E-cadherin expression. Hence, it may be a suitable candidate for designing new drugs in cancer therapy.

Keywords: Piperine, MCF-7, MMP-9, VEGF, E-cadherin
INTRODUCTION:

Although medical knowledge about cancer therapy is developing, breast cancer remains the second major cause of cancer-related death among women in both developing and developed countries (1). The metastasis and invasion of breast tumor cells are responsible for many deaths from breast cancer (2). Metastasis involves the migration of cancer cells from primary tumor tissue to another tissue of the body. This process is an essential factor in poor clinical prognosis in patients with breast cancer (3). The degradation and remodeling of the extracellular matrix (ECM) play a critical role in the migration and metastasis of tumor cells (2). Matrix metalloproteinase-9 (MMP-9) is a sub-family of zinc-containing endopeptidases with an essential role in the remodeling of ECM, angiogenesis, migration, and invasion mechanism (4). E-cadherin is a cell-adhesion molecule whose primary function is to mediate cell-matrix and cell-cell contacts and leads to ECM maintenance and integrity. The loss of E-cadherin is one of the first events in the initiation of metastasis and tumor invasion (5-7). Tumor growth relies on the new blood vessels formation for carrying nutrients and oxygen. The vascular endothelial growth factor (VEGF) plays a fundamental role in tumor growth and metastasis by inducing the angiogenesis process (8). VEGF signaling inhibitors are confirmed as anti-cancer drugs for solid tumor treatment, which prevents blood vessel formation and reduces the metastatic process (9, 10).

Different types of phytochemical compounds have been identified as critical natural sources for novel anti-tumor therapy (11, 12). Piperine is a bioactive constituent extracted from Piper Nigrum with a range of therapeutic properties such as anti-inflammatory, anti-oxidant, and anti-cancer activity (13). Recently, the anti-tumorigenic activities of piperine against many types of tumor cells have been investigated (11, 14). In recent years, numerous experimental studies have reported that the anti-neoplastic property of piperine may partly be due to its effects on angiogenesis and tumor metastasis (15-17). However, the anti-tumor impact of piperine on cell growth, angiogenesis, and metastasis has not yet been fully understood. In this study, we examined the anti-angiogenic and anti-metastatic effects of piperine on MMP-9, E-cadherin, and VEGF expression in breast cancer cells.

METHODS

Materials

Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), penicillin (100 units/ml)/streptomycin (100 μg/ml), and trypsin-EDTA were obtained from Invitrogen (Island, NY, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Abcam Company (Cambridge, MA, USA). Piperine (>95% purity), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and Horseradish peroxidase (HRP)-conjugated secondary antibody were obtained from Sigma Aldrich (St. Louis, MO, USA). The primary anti-MMP-9 antibody was purchased from Abcam Company (Cambridge, MA, USA). Forward and reverse primers were purchased from Takapouzist (Tehran, Iran).

Cell culture and treatment

The human breast cancer MCF-7 cell line was obtained from the Pasteur Institute of Iran, Tehran. MCF-7 cells were maintained and grown in DMEM medium supplemented with 10% FBS and 1% antibiotic and incubated in 5% CO2 incubator at 37°C. Before cell treatments, MCF-7 cells were seeded onto 96-well and 6-well plates and then starved with DMEM involved 0.5% FBS and 1% antibiotic for 24 hours at 37°C.

For cell treatment, MCF-7 cells were exposed to different concentrations of piperine (0, 5, 10, 25, 50, 75, and 100 μM) for 24 hours, and subsequently, cell viability
was examined using the MTT assay method. Next, cells were treated with piperine (5, 10, and 25 µM) for 24 hours, and then the mRNA levels of MMP-9, VEGF, and E-cadherin and the protein level of MMP-9 were investigated.

**Cell viability assay**

For measuring the impact of piperine on the viability of MCF-7 cells, the MTT assay method was applied. MCF-7 Cells (8×10³ cells/well) were plated into 96-well plates and grown for 24 hours at 37°C and were treated with various concentrations of piperine (0, 5, 10, 25, 50, 75 and 100 µM) for 24 hours at 37°C. At the end of incubation time, the media was removed from the cultured cell, and subsequently, 150 µM serum-free media and 20 µl MTT solution (5 mg/ml in PBS) was added into each well of the 96-well plates and incubated for 4 hours. The optical density at 540 nm was measured using a microplate reader (BioTek® ELx800, USA).

**Quantitative RT-PCR (qRT-PCR)**

For exploring the gene expression of metastatic and angiogenic markers, total RNA was isolated from the MCF-7 cell line using the Yekta-Tajhiz kit (Iran) according to the standard producer’s protocol. One µg of total RNA was used to synthesize cDNA, applying the superscript II reverse transcriptase kit (Takara, Japan) according to the standard producer’s protocol. The mRNA expression was amplified and detected using SYBR Green PCR Master Mix (Yekta-Tajhiz, Iran) and Applied Biosystem 7500 Fast Real-Time PCR System (USA).

The specific forward and reverse primers used for gene expression of metastatic markers were organized in Table 1.

The mRNA levels of experimental groups were normalized using GAPDH (a housekeeping gene), and fold-changes were derived using the comparative CT method.

**Western blot analysis**

Firstly, the treated MCF-7 cells with various concentrations of piperine were collected and lysed in a lysis buffer. Then, the concentration of protein in each experimental group was quantified by the Bradford assay method according to the standard protocol. The protein samples (40 µg/well) were resolved by 10% SDS-polyacrylamide gel electrophoresis and then electro-blotted onto a polyvinylidene difluoride (PVDF) membrane. The blots were blocked with a blocking buffer (5% non-

---

### Table 1. Sequences of primers

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
<td>5′-CACCTGGAGAGGCGCATGT-3′</td>
<td>5′-TGGGAAACAT-GAGCAGCCTCT -3′ (18)</td>
</tr>
<tr>
<td>VEGF</td>
<td>5′-TGCCCGCTG CTTGTC TAAT-3′</td>
<td>5′-TCTCCGCTCTGA GCAAGG -3′ (19)</td>
</tr>
<tr>
<td>MMP-9</td>
<td>5′-GATGCGTGAGAGCTCGAAAT-3′</td>
<td>5′-CACAAACTGGATGACGATG-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-TTGAGGCTCAATGAAGGGGTC-3′</td>
<td>5′-GAAGGTGAAGGTCCGAGTCAGA-3′ (20)</td>
</tr>
</tbody>
</table>
fat milk) at room temperature for 1 hour. Then, blocked blots were exposed to a specific primary antibody (anti-MMP-9 antibody) at 4°C overnight, followed by a secondary antibody conjugated with HRP at room temperature for 1 hour and enhanced chemiluminescence (ECL) detection kit. Image J software (Bio-Rad) was performed for the densitometry analysis of the blots. All experiments were performed in the Clinical Biochemistry laboratory at Lorestan University of Medical Sciences (Khorramabad, Iran) and Farhangian University of Tehran in a multi-centric manner.

**Ethical Considerations**
The study was approved following the Ethical recommendations of Lorestan University of Medical Sciences. The authors have observed ethical issues.

**Statistical analysis**
All data were shown as the mean ± standard error of the mean (SEM). Statistical significances between individual experiments were evaluated by one-way analysis of variance (one-way ANOVA) followed by least significant difference (LSD) posthoc analysis. Probability values *P<0.05 and **P<0.01 were reported as a statistically significant difference.

**RESULTS:**
**Piperine exerts cytotoxicity effect on MCF-7 breast cancer cells**
The chemical structure of piperine was represented in Figure 1A. For exploring the cell viability, MCF-7 cells were exposed to different concentrations of piperine (0, 5, 10, 25, 50, 75, and 100 µM) for 24 h at 37°C. MCF-7 cell viability was analyzed using the MTT assay method. The results obtained from the MTT assay showed that cell treatment with 25, 50, 75, and 100 µM of piperine significantly resulted in decreased viability of cultured MCF-7 cells compared to the control group (*P<0.05; **P<0.01; ***P<0.001) in a dose-dependent manner (Figure 1B). Therefore, 5, 10, and 25 µM of piperine were used for further experiments.

**The effect of Piperine on MMP-9, VEGF, and E-cadherin gene expression in MCF-7 cells**
To investigate the mRNA expression of metastatic and angiogenic markers, we explored MMP-9, VEGF, and E-cadherin in MCF-7 cells treated with piperine.

---

**Figure 1. Piperine inhibits cell viability of human Breast cancer MCF-7 cells. A) The chemical structure of piperine. B) The effect of piperine (0, 5, 10, 25, 50, 75 and 100 µM) on cell viability of MCF-7 at 24 hours. Cell viability was examined using the MTT assay. All results are shown as mean ± SEM of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared with the control group.**
(5, 10, and 25 µM). The qRT-PCR revealed that 5, 10, and 25 µM of piperine downregulated the mRNA levels of MMP-9 as compared to the untreated group (P<0.01) (Figure 2A). Our results also demonstrated that following piperine treatment, the mRNA levels of proangiogenic VEGF was significantly suppressed compared to the control (P<0.01; Figure 2B). Piperine (5, 10, and 25 µM) augmented the expression levels of E-cadherin compared to the control group (Figure 2C) (P<0.05; P<0.01).

**The effect of Piperine on MMP-9 protein expression in MCF-7 cells**

We also investigated the protein expression levels of MMP-9 in MCF-7 cells by Western blot analysis. Our Western blot results demonstrated that following treatment with 5, 10, and 25 µM of piperine for 24 hours, MMP-9 protein expression was significantly decreased compared with the control group (P<0.01; Figure 3).

**DISCUSSION:**

In the present research, we reported that piperine could inhibit breast cancer cell angiogenesis, invasion, and metastasis through the upregulation of E-cadherin and downregulation of MMP-9 and VEGF in MCF-7 cells. Breast cancer is the second most common malignancy in females (1). Angiogenesis has a significant role in the metastasis and progression of breast cancer (21). Therefore, growth factors and markers that regulate angiogenesis and tumor progression are essential targets for the development of targeted molecular therapies (21-23). It is now assumed that some phytochemical compounds can play a protective role in the growth of various tumors. Piperine, a bioactive component of black pepper, has been indicated to be a potential therapeutic factor in various cancers. It involves a diversity of mechanisms, including cell growth, apoptosis, cell cycle, metastasis, and angiogenesis (15, 24, 25). The upregulation of MMP-9 in breast cancer cells is correlated with tumor metastasis and associated with...
a poor prognosis in cancer patients (4). Thus, MMP-9 inhibitors may prevent tumor metastasis and consequently decrease cancer mortality (26). In our study, piperine treatment decreased the MMP9 gene and protein expression levels in the MCF-7 cell line in a concentration-dependent manner. These results are consistent with Zeng et al., who reported that piperine represses cell migration and metastasis by downregulating MMP-9 in prostate cancer DU145 cells (16). Zhang et al. indicated that MMP2/9 expression was suppressed in response to piperine treatment in human osteosarcoma cells (15).

On the other hand, the formation of new blood vessels around the solid tumor is required for growth and metastasis of tumor cells. VEGF, as a proangiogenic growth factor, is a critical regulator of angiogenesis during the process of tumor progression and metastasis in many types of cancers (27-29). The upregulation of VEGF can be used as a predictor of lung metastasis in patients with osteosarcoma (27). In MCF-7 cells, we reported that piperine downregulates VEGF gene expression in a dose-dependent manner.

Several studies have reported that E-cadherin is responsible for cell-matrix and cell-cell adhesion, and loss of E-cadherin participates in tumor cell metastasis (5). Hence, inhibition of E-cadherin is a vital hypothesis in preventing tumor formation, migration, and invasion (30, 31). Very little data is available regarding the effect of piperine on E-cadherin in breast cancer cells. Our qRT-PCR results showed that piperine suppresses the E-cadherin expression in a concentration-dependent manner in the MCF-7 cell line.

**CONCLUSION:**

In conclusion, our finding indicated that piperine has an inhibitory effect on cell viability of the MCF7 breast cancer cell line in comparison with the control group. The anti-cancer effects of piperine may partly be due to its roles in angiogenesis, invasion, and metastasis through the upregulation of E-cadherin and
downregulation of MMP-9 and VEGF in breast cancer cells. These findings suggest that piperine may be considered as a capable anti-cancer candidate for new drug design in cancer treatment.

ACKNOWLEDGMENT:
Notably, the clinical biochemistry department of Lorestan University of Medical Sciences would be greatly acknowledged for their support.

CONFLICT OF INTERESTS:
The authors declare that there is no conflict of interests associated with this work.

FUNDING/SUPPORT:
The present research was not supported by any funding organization of the commercial, nonprofit or public sectors.

AUTHORS’ CONTRIBUTION
Contribution of each author made to the manuscript included:
(1) Participation in study design: Zahra Zare, Parisa Khanicheragh, Fahimeh Hosseinabadi
(2) Participation in the implementation of methods: Zahra Zare, Parisa Khanicheragh, Tina Nayerpour Dizaj, Zakieh Sadat Sheikhalishahi,
(3) Participation in data analysis: Mohammad Panji
(4) Contribution in the writing of the manuscript: Zahra Zare, Parisa Khanicheragh, Armaghan Lohrasbi, Mojtaba Abbasi
(5) Edit of the manuscript: Omid Abazari
(6) Photograph Processing: Vajiheh Najafi
(7) Responsible for overall supervision of the authors: Parisa Khanicheragh
(8) Administrative support: Mojtaba Abbasi
(9) All authors performed editing and approving the final version of this paper for submission, also participated in the finalization of the manuscript, and approved the final draft.

REFERENCES:
14. Samykutty A, Shetty AV, Dakshinamoorthy G, Bartik MM,


