

Eugenol Suppressed Metastasis of Breast Carcinoma Cells and Migration by Regulation of MMP-9 & Paxilin Gene Expression

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Abstract: Eugenol, the principal chemical component of clove oil, has been long well known for its local anesthetic, anti-inflammatory, antiseptic, and antibacterial effects. However, its anti-metastasis effect has not yet been fully explored. This study was performed to investigate the anti-metastasis effect of eugenol in human breast cancer cells (MCF-7). To ascertain the molecular mechanisms implicated in the anti-metastasis effect of eugenol on MCF-7 cell line was cultured and tests was performed using MTT assay, scratch-wound healing assay and evaluation transcriptional expression of MMP-9 and Paxilin expression by RT-PCR semi-quantification. Product percentage of relative density was calculated with Image J software. Data were analyzed with SPSS-16, ANOVA ($P < 0.05$). Eugenol decreased cell viability dose and time dependently. MTT assay results represented that inhibitory concentration (IC-50 value) was 1.5 µg/ml after 48h of incubation. Microscopic examination of eugenol treated cells showed cell shrinkage, membrane blebbing and apoptotic body formation. Scratch-wound healing assay which was conducted to investigate anti-migration effects of eugenol represented significantly inhibition of cell migration at 1.5 µg/mL ($P < 0.05$) and suppressing metastasis of MCF-7 cells. In this study we examined inhibitory effects of eugenol on downregulation of MMP-9 and paxilin mRNA expression which was remarkable with 34.3% and 13.7 reduction ($P < 0.05$) in the gene expression. Taken together our finding may provide a new approach to use natural product for metastasis therapy and clinical use although further investigation are required.

Keywords: Eugenol, Metastasis, Migration

INTRODUCTION

Phenolic phytochemicals are a broad class of nutraceuticals found in plants which have been extensively researched by scientists for their health-promoting potential. One such a compound which has been comprehensively used is eugenol which is the active component of *Syzygium aromaticum* (cloves)[1]. Aromatic plants like nutmeg, basil, cinnamon and bay leaves also contain eugenol. Eugenol has a wide range of applications like perfumeries, flavorings and in medicine as a local antiseptic and anesthetic[2]. 4-Allyl-2-methoxyphenol (eugenol) is widely used and well known for its medicinal properties[3]. Traditional uses of clove oil include use in dental care, as an antiseptic and analgesic. It is active against oral bacteria associated with dental caries and periodontal disease and effective against a large number of other bacteria[4]. Previous studies have reported biological activities of eugenol including antifungal, anti-carcinogenic, anti-allergic, anti-mutagenic activity, antioxidant and insecticidal properties[3]. Eugenol has been demonstrated to inhibit prostaglandin biosynthesis

and to block COX-2 activity with an IC50 value of 129 micro-mol[5]. It was also inhibited proliferation different tumor cell lines dose and time dependently but anti-metastasis effect of eugenol had not been studied[3]. eugenol induced apoptosis via the mitochondrial pathway by modulating the Bcl-2 family proteins, Apaf-1, cytochrome C, and caspases and inhibiting invasion, and angiogenesis as evidenced by changes in the activities of MMPs and the expression of MMP-2, VEGF, VEGFR1, TIMP-2 and RECK[6].

Matrix metalloproteinases (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. 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[7].

Since 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, in the present study, anti-metastasis potential and inhibitory effects of eugenol on MCF-7 cell proliferation and suppression of migration by evaluation MMP-9 and paxilin gene expression was investigated.

MATERIALS AND METHODS

Chemicals

MCF-7 was purchased from NCBI (National Cell Bank of Iran). RPMI-1640 medium, FBS (fetal bovine serum), trypsin-EDTA and antibiotic (Penicillin-Streptomycin) were obtained from Gibco-USA. Ethanol, dionizedwater was purchased from Merck (Germany). MTT (3-[4,5- dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was prepared from Applichem (USA). The RNA isolation kit was purchased from Roshe, Germany, the cDNA synthesis kit and RT-PCR kit were purchased from Pars Tous, Iran.

Eugenol was purchased from Sigma-France.

Cells and culture

MCF-7 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin and maintained at 37 °C with 5% CO₂ in a humidified atmosphere.

Cell viability assay

The effect of eugenol on the cell survival was evaluated by MTT test. 1.5×10⁴ cells/well was cultured into 96-well plate .After overnight incubation, the cells were treated with different concentrations of eugenol (0.25, 0.5, 0.75, 1, 1.5 and 3µg/ml).

The effects of eugenol on cell viability were measured 24 and 48hoursafter treatment and estimated by a MTT assay using 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide; 10 µL of MTT solution was added to each well and the plate was incubated for two hours in dark conditions, then 100 µL DMSO was added to solubilize the MTT. In the last part, the absorbance of each well was measured at 570 nm with a microplate spectrophotometer. Concentrations of eugenol showing 50% reductions in cell viability (i.e., IC₅₀ values) were calculated and the

percent mitochondrial activities of treated cells against untreated cells (as control group) were determined.

Morphological observation by inverted microscopy

In order to evaluate the effect of eugenol on the cell morphology, MCF-7 cells were plated 10⁵ cells/well. After 24h adherence of cells, treated with different concentration of eugenol. After 48h, the morphological changes were observed by inverted microscope (Bio Photonic, Brazil).

Scratch-wound healing assay

The effect of eugenol on migration of MCF-7 cells was investigated via scratch healing assay. The cells were culture in 6-well plate, after 48h the cells reached approximately 80% confluence, then a wound track created across the center of each well with a sterile pipet tip and cells were treated with different concentrations of eugenol. Finally, the edge of the wound areas and the number of cells migrated around wound margin was photographed using light microscope. The areas of the gaps were measured using Image J software. The pictures of gaps taken at different time points were opened in Image J. The unit of length was set as “mm”. The shapes of the gap areas were selected using the “freehand selections” and the area was measured[8].

Reverse transcription-polymerase chain reaction of MMP-9 & Paxilin

In order to assess the anti-metastasis effect of eugenol, the changes in the expression of MMP-9 and Paxilin mRNA were examined by the reverse transcription-polymerase chain reaction methods. RNA of treated MCF-7 cell was isolated using the High Pure RNA Isolation kit (Roche, Germany) according to the manufacturer’s protocols and stored at -80°C. cDNA was synthesized using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer’s instruction. The produced cDNA (5 µg) was added to 10µl Taq premix, 2µl froward primer, 2µl reverse primer (sequence of primer represented in table 1) and distilled water (Parstous, Iran). Ultimately, RT-PCR was performed with 1 cycle at 94 °C/5 min, 30 cycles at 94 °C/30 s for denaturation, 56 °C/30 s for annealing, 68 °C/45 s for extension and 1 cycle 5 min at 72 °C according to the manufacturer’s protocol.

Table1. The sequences of primers and annealing temperatures

Gene	Forward 5'→3'	Reverse 5'→3'	TM
GAPDH	5' GGCCAAGAT CAT CCA TGA CAA CT3'	5' ACCAGGACATGAGCTTGA CAA AGT3'	57
MMP-9	5'GCCTGCACCACGGACGGTCTGCTCC3'	5'GAGGTGCCGGATGCCATTCACGTC3'	59
Paxilin	5' AGGGACTGGGGTTTCTGG3'	5'AAATCACAGGAATTGAAATGGG3'	56.5

Following amplification, PCR products were run in 2% agarose electrophoresis gel and visualized by green viewer staining using UVITEC (Cambridge, UK).

The PCR products were analyzed by 1.5% agarose gel electrophoresis and the gels were observed using gel documentation (UV TEC Cambrege, Uk). The experiments were reputed in triplicate; the percentage of band relative densities was calculated using ImageJ software.

Statistical analysis

Statistical evaluation of the data was performed using one-way analysis of variance (ANOVA), Tukey test used for multiple comparisons as a post-test with the help of SPSS software. The results

are shown as mean \pm SD and $p < 0.05$ accepted as the minimum level of significance.

RESULTS

Effect of eugenol on MCF-7 cell viability and cell morphology

The MTT assay and morphological analysis indicated that eugenol exerted cytotoxic and inhibitory effect on the cell viability of the MCF-7 cells dose dependently. The IC_{50} value was $1\mu\text{g/ml}$ after 24h and $1.5\mu\text{g/ml}$ after 48h which was similar to IC_{50} that reporting with other researchers [9, 10]. Figure 1 represented MTT assay results from treatment of MCF-7 cell with various concentration of eugenol in 24 h and 48 h.

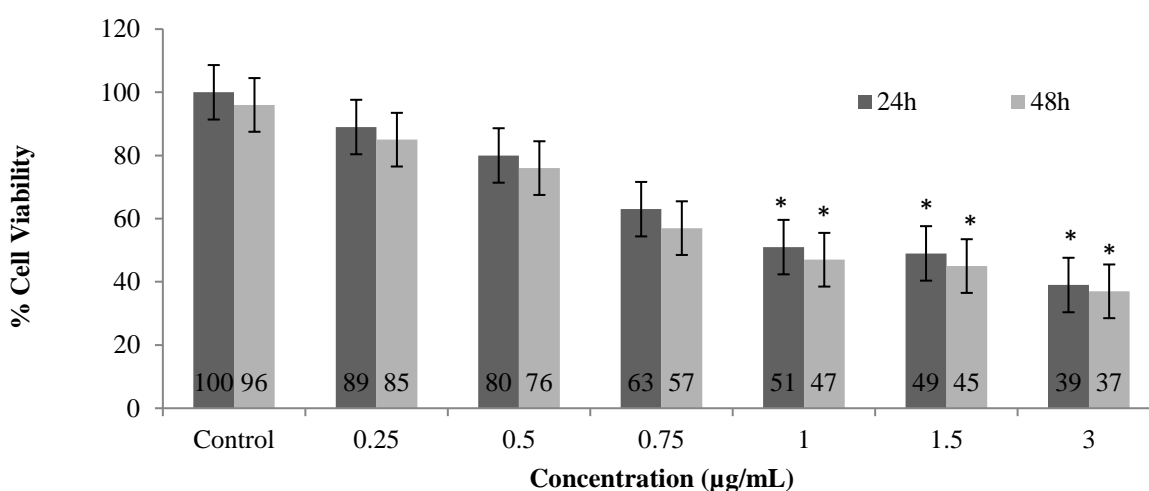


Fig-1: MTT assay results from treatment MCF-7 cell with various concentration of eugenol in 24 h and 48 h, Mean \pm SD, * P < 0.05.

Figure 2 revealed the cytotoxic effect of eugenol on MCF-7 cells which induced clear morphological changes such as a cell shrinkage, cytoplasm blebbing and alteration of cell shape and size. Eugenol-treated cells displayed condensed and

fragmented nuclei compared to the typical round nuclei of the control which coincide to previous findings [11, 12]. These observations verified that eugenol exhibited the cytotoxicity against MCF-7 cells and inhibited their proliferation which made them available to migration.

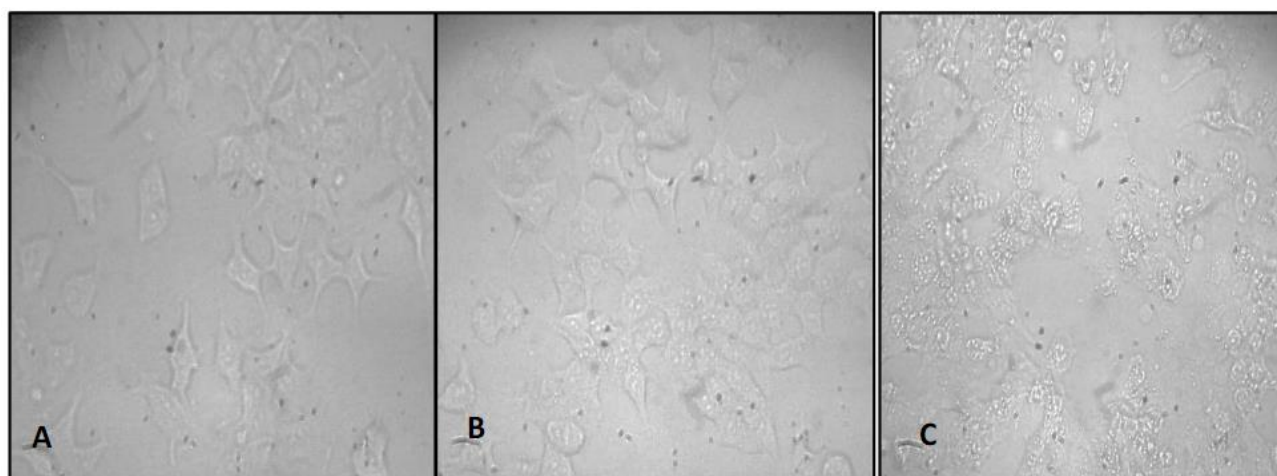


Fig-2 –Cytomorphological alterations MCF-7 cells treated with eugenol for 48h. A) Control. B) MCF-7 Cells treated with 0.25 µg/ml C) MCF-7 Cells treated with 1.5 µg/ml

Inhibitory effect of eugenol on migration of MCF-7 cells

The scratch wound healing assay was conducted to determine the effect of eugenol on the migration of MCF-7 cells. As exhibited in figure 3, MCF-7 cell migration was significantly decreased at concentrations

of 1.5µ/ml (P<0.05). At a concentration of 0.25µ/ml eugenol could not suppress migration while using a higher dosage of eugenol promisingly was available to inhibit migration and metastasis of MCF-7 cells. These results confirmed that eugenol may be considered for suppressing migration of MCF-7 cells.

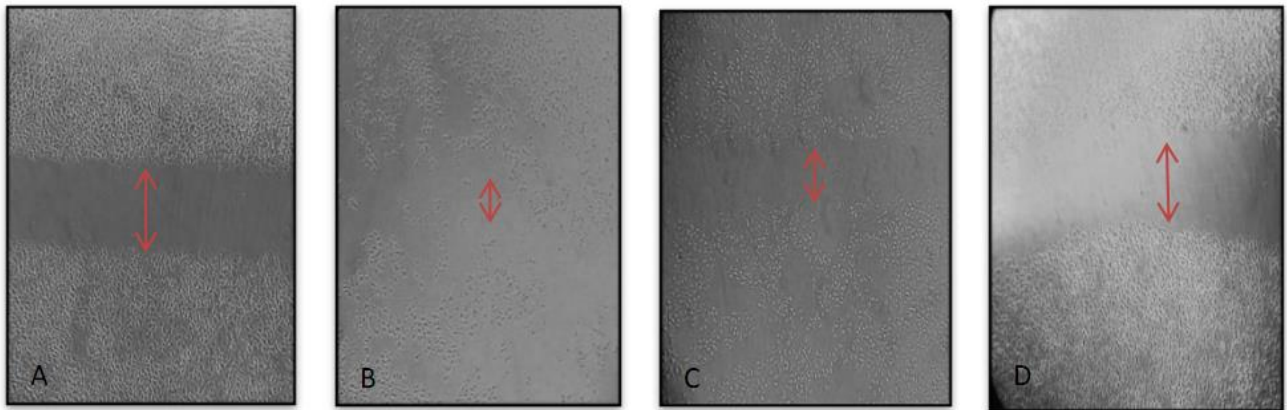


Fig-3: Inhibitory effect of eugenol on cell migration of MCF-7 cells by scratch healing assay 200X.

A) untreated cells 24h after scratch assay. B) Experimental groups with 0.25 µg/ml eugenol C) Experimental groups with 0.75 µg/ml eugenol D) Experimental groups with 1.5 µg/ml eugenol indicated that this treatment effectively inhibited cell migration of MCF-7 cells. Magnification.

The effect of eugenol on the expression of MMP-9 and Paxilin in MCF-7 cells

Analysis of RT-PCR data represented a reduction (P<0.05) in gene expression levels of MMP-9 and Paxilin treated with eugenol at concentrations of

1.5µg/ml in comparison with the control group. Figure 4 shows images of RT-PCR products in gel electrophoresis and figure 5 revealed the percentage of relative density which was calculated using Image J software.



Fig-4: MCF-7 cells were treated with EUGENOL and the mRNA expressions of MMP-9 and Paxilin were evaluated by RT-PCR analysis that displayed a suppressing effect of eugenol on mRNA expressions of pro-metastasis factors.

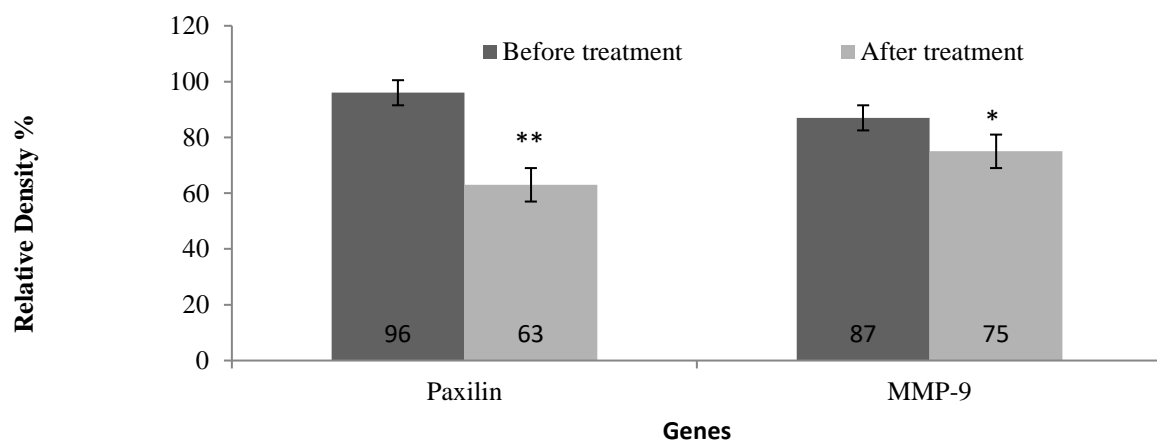


Fig-5: Percentage of relative density for Paxilin and MMP-9
*P<0.05 and **P<0.001

DISCUSSION & CONCLUSION

In recent years anti-cancer activity of eugenol was an important topic for researchers while chemical synthesized anti-cancer drugs represent different side effects. It was showed that eugenol can reduce the side effects on normal cells[13].

Related to metastasis suppression potential of eugenol it was reported that eugenol could inhibit cell apoptosis, cancer cell invasion and angiogenesis by regulating Bcl-2 protein family, apoptotic protease activating factor 1 (Apaf-1), cytochrome C and cysteine aspartic protease and changes the activity of matrix metalloproteinase-2 (MMP-2), vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor-1 (VEGFR-1), tissue metallo-protease inhibitor-2 (TIMP-2)[6][14].

Kim et al. demonstrated eugenol-induced apoptosis in human melanoma cells[12]. Eugenol's cytotoxic effects were observed in G361 cells in the range of 0.5 to 2 mM. Cell staining also showed that eugenol induced a change in nuclear morphology[12]. Data of present study also coincide to their results, eugenol could inhibit cell proliferation and migration of MCF-7 cells dose and time dependently. On the other hand eugenol-treated cells displayed condensed and fragmented nuclei compared to the control group which match to previous researches.

Ghosh et al. explored eugenol and isoeugenol as an antiproliferative agent against malignant melanoma cells. Eugenol was also found to inhibit the colony formation of melanoma cell lines[15]. Additionally, they tested the effect of eugenol against B16 melanoma xenograft. Eugenol caused significant tumor decrease (almost 40%). Moreover, 50% of animals developed non-treatment-related metastases while in the treatment group there was no sign of invasion or metastasis[16]. As data represented results of current research on metastasis inhibition in MCF-7 cell line match to other findings on different cell lines.

Eugenol at low dose (2 μ M) has specific toxicity against different breast cancer cells. This effect was mediated mainly through inducing the internal apoptotic pathway and strong down-regulation of E2F1 and its downstream anti-apoptosis target survivin, independently of the status of p53 and ER α [17]. Eugenol inhibited also several other breast cancer related oncogenes, such as NF- κ B and cyclin D1. Moreover, eugenol up-regulated the versatile cyclin-dependent kinase inhibitor p21 WAF1 protein, and inhibited the proliferation of breast cancer cells in a p53-independent manner[18].

Hyanget al, investigated the direct scavenging effects of eugenol on DPPH radical, hydrogen peroxide, reducing power, lipid peroxidation and genomic DNA damage related to oxidative stress were evaluated in cell

free system. They were observed that eugenol specifically exhibited higher inhibitory effect on hydrogen peroxide than other reactive oxygen species, and also blocked DNA oxidation and lipid peroxidation induced by hydroxyl radical. They also were tested the inhibitory effects of eugenol on the activity and expression of MMP using gelatin zymography and western-blot previously. Their data showed that eugenol inhibited MMP activities in PMA-stimulated HT1080 cells and exerts inhibitory effects on MMP via inactivation of ERK. Therefore, these results suggest that eugenol could be available as an excellent agent for prevention of metastasis related to oxidative stress[19].

In conclusion, for the first time we established that eugenol could suppress breast cancer cell migration which was mediated mainly through decreasing the MMP-9 and Paxilin gene expression.

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