Reports of Biochemistry & Molecular Biology
Vol.13, No.2, Jul 2024

Original article

www.RBMB.net

3,4 Dihydroxyphenylethanol May Inhibit Metastasis in HepG2 Cells by Influencing the Expression of miR-21 and Genes Associated with Metastasis

Mahdi Alaee[1], Gholamreza Shahsavari[2], Mohammad Yazdi[1], Maryam Hormozi*[2]

Abstract

Background: Hepatocellular carcinoma (HCC) is one of the lethal malignancies with a poor prognosis due to metastatic complications. Matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs), have an important role in metastasis. MicroRNA-21 (miR-21) is significantly overexpressed in nearly all types of human cancers, including HCC. Targeting miR-21 pharmacologically could be a promising therapeutic approach for HCC. 3,4-dihydroxyphenylethanol (DHPE), a phenolic phytochemical compound found in olive, has potent antioxidant and anticancer properties. This study aimed to investigate the effect of DHPE on the expression of miR-21 with genes associated with metastasis (MMP-2, MMP-9, TIMP-1, and TIMP-2) and their correlation with miR-21 in HepG2 cells.

Methods: This experimental study had four groups, including a control, and three groups of treatment with different concentrations of DHPE (50, 100, and 150 µM) for 24 hours. The expression levels of genes were determined by RT-qPCR.

Results: The results showed that the treatment of cells with DHPE significantly reduced the expression of miR-21, MMP-2, MMP-9, and TIMP-1 but increased TIMP-2 compared to the control group; additionally, there was a negative correlation between miR-21 and TIMP-2 but a positive correlation between miR-21 with MMP-2, MMP-9, and TIMP-1.

Conclusion: The results showed that DHPE, likely by reducing the expression of miR-21, can increase TIMP-2 and reduce MMP-2, MMP-9, and TIMP-1 gene expression and may play a role in inhibiting cell migration in HepG2 cells.

Keywords: HepG2 cells, miR-21, Matrix Metalloproteinases, Tissue Inhibitor of Metalloproteinases, 3,4-Dihydroxyphenylethanol.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and commonly metastasizes to different tissues such as the lungs, lymph nodes, adrenal gland, and bones (1). Although there are high-quality techniques to fight against cancer, including surgery, radiation therapy, and chemotherapy, metastasis is the most important cause of failed treatment (2). The overexpression of matrix metalloproteases (MMPs) by cancer cells leads to the degradation of the extracellular matrix (ECM) and basement membrane (BM), which facilitates cell migration (3). Elevated levels of MMP-2 and MMP-9, members of the MMP family, are associated with a poor prognosis in HCC patients (4).

1: Student Research Committee, Lorestan University of Medical Sciences, Khorramabad, Iran.
2: Razi Herbal Medicines Research Center, School of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran.
***Corresponding author: Maryam Hormozi; Tel: +98 6633120133; E-mail: hormozi.maryam@lums.ac.ir.**
Received: 11 Apr, 2024; Accepted: 19 Aug, 2024

[DOI: 10.61186/rbmb.13.2.254]

Tissue inhibitors of metalloproteases (TIMPs) are endogenous proteins that play an important role in regulating MMP activity (3). TIMPs interact preferentially with MMP-2 and MMP-9, with TIMP-1 and TIMP-2 showing a stronger preference (5). MicroRNAs (miRNAs) are small, non-coding RNAs, approximately 19–25 nucleotides in length, present in all eukaryotic cells. Studies have shown that miRNAs can modulate several cancer-related processes like cell proliferation, migration, invasion, and apoptosis, thus influencing cancer initiation and progression (6). MicroRNA 21 (miR-21) is upregulated in most known human cancers, including multiple myeloma, lung cancer, breast cancer, colorectal cancer, and gastric cancer (7, 8).

3,4-Dihydroxyphenylethanol (DHPE), or hydroxytyrosol, a phytochemical compound found in olive leaves and oil, exhibits beneficial effects in various types of cancer (9). For example, it triggers the cellular antioxidant system and reduces the level of cellular interleukin (IL)-6 by suppressing the nuclear factor-κB (NF-κB) pathway. It induces G2/M cell cycle arrest, leading to inhibition of proliferation and induction of apoptosis in the HCC cell line HepG2 (10). It has been shown that DHPE can decrease tumor cell migration and invasion by targeting epithelial-mesenchymal transition (EMT) markers (11). Another study indicated that DHPE and oleuropein may suppress the invasion of cells in HCC by activating autophagy (12). Although the anticancer effects of DHPE have been shown in various studies, whether DHPE also inhibits metastasis remains poorly investigated. The aim of this study was to investigate the effect of DHPE on the gene expression levels of miR-21 and genes related to metastasis (MMP-2, MMP-9, TIMP-1, and TIMP2) and their correlation with miR-21 in HepG2 cells. Understanding the roles of these key molecules and their correlation in cancer progression is essential for developing novel clinical strategies in cancer therapy.

Materials and Methods

RPMI-1640, Trypsin/EDTA 0.25 % (1726653), and Fetal Bovine Serum (FBS) (42Q7363K) were obtained from Gibco (Maryland, USA). Blue Tetrazolium Blue (MTT) (M2128) powder, DHPE (H4229), and phosphate buffer saline (PBS) tablets (SLBJ2117V) were achieved from Sigma-Aldrich (St. Louis, MO, USA). dimethylsulfoxide (DMSO) (K44917952) were obtained from Merck (Merck, Germany). RNA extraction kit (Yekta-Tajhiz Azma, Iran)**,** cDNA synthesis kit (Sinaclon, Iran)**,** cDNA synthesis kit for *miR-21* (RNAbiotech, Iran). SYBR-green master mix (Yekta-Tajhiz Azma, Iran)**.**

Cell line and culture

The HepG2 cell line was purchased from the Iran's National Cell Bank, Pasteur Institute in Tehran. Cells were cultured in RPMI-1640 containing 10% FBS with 1% penicillin and streptomycin 1% at 37 °C with 5% CO2.

MTT assay for viability

The MTT (3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay was performed to determine the cell viability. Briefly, cells (100 μL containing 10^4 cells per well) were seeded into 96-well plates and treated with various concentrations of DHPE (0, 10, 20, 40, 50, 100, 150, 200, 250, and 300 μM). The plates were incubated for 24, 48, and 72 hours at 37 °C with 5% CO2. Then, 20 µl of MTT (0.5 mg/ml) was added to each well and incubated for 4 hours at 37 °C. Afterward, 100 µl of dimethyl sulfoxide (DMSO) was added to each well. The plates were shaken for 15 minutes, and the absorbance of the plates was measured at 570 nm. Finally, the cell viability of the samples was determined for 24, 48, and 72 hours.

RNA extraction and cDNA synthesis

The RNA of the cells was extracted using an RNA extraction kit, and the concentration of the extracted RNA was determined by a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Then, mRNA was

[DOI: 10.61186/rbmb.13.2.254]

converted to cDNA using a cDNA-synthesis kit according to the manufacturer's instructions. For miR-21, cDNA was synthesized based on stem-loop method using cDNA synthesis kit.

Evaluation of gene expression using RT-qPCR

The expression of miR-21, MMP-2, MMP-9, TIMP-1, and TIMP-2, genes was analyzed using RT-qPCR (MIC, BMS Corbett Research) and SYBR-green kit. GAPDH was used (glyceraldehyde-3-phosphate dehydrogenase) for the normalization of gene expression MMP-2, MMP-9, TIMP-1, and TIMP-2. U6 (small nuclear RNA U6) was used to normalize miR-21 expression. The sequence of primers used is shown in Table 1.

Table1. Primer sequences used in Real-time PCR.

Genes	GenBank accession number	Forward sequence	Reverse sequence	Product size (bp)
MMP-2	NC_000016	CTCATCGCAGATGCCTGGAA	TTCAGGTAATAGGCACCCTTGAAGA	104
MMP-9	NC_000020	ACGCACGACGTCTTCCAGTA	CCACCTGGTTCAACTCACTCC	94
TIMP-1	NC_000023	AAGGCTCTGAAAAGGGCTTC	GCAGGATTCAGGCTATCTGG	105
TIMP-2	NC_000017	GAAGCATTTGACCCAGAGTG	CCTTTCAGACCGAACCTACT	165
GAPDH	NC_000012	CTCTCTGCTCCTCCTGTTCG	ACGACCAAATCCGTTGACTC	114

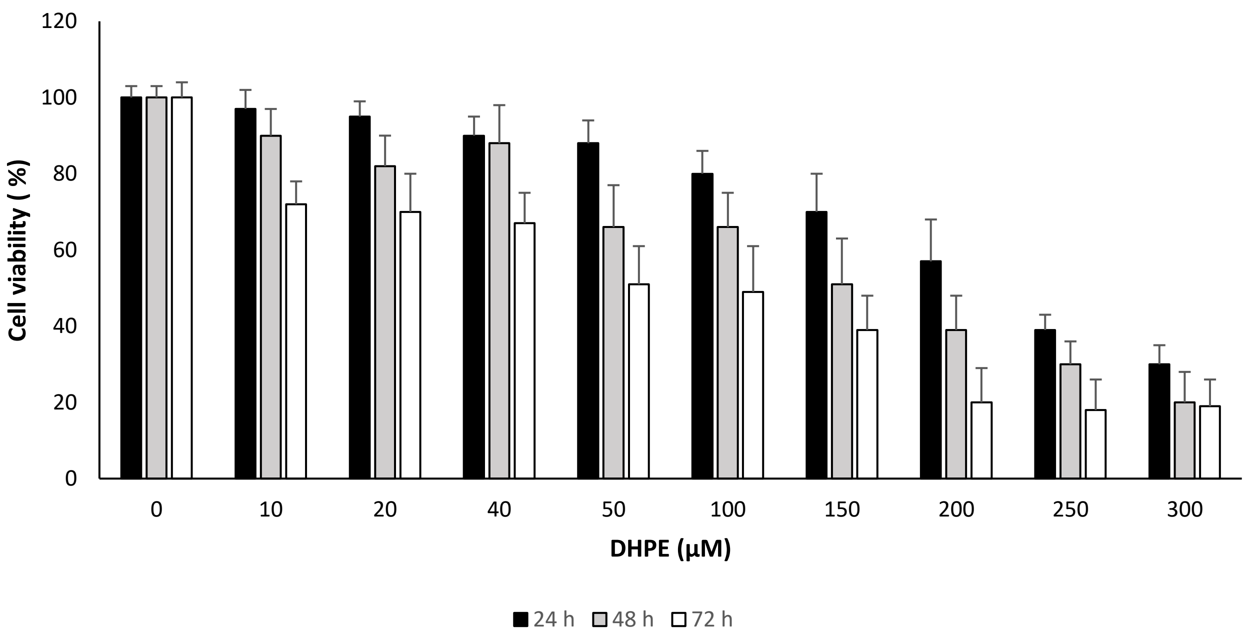


Fig. 1. Cell viability of HepG2 cells after treatment with different concentrations of DHPE (0-300 µM) at 24, 48, and 72 h by MMT assay test. The findings are presented as Mean ± SD of three separate measurements.

Data analysis

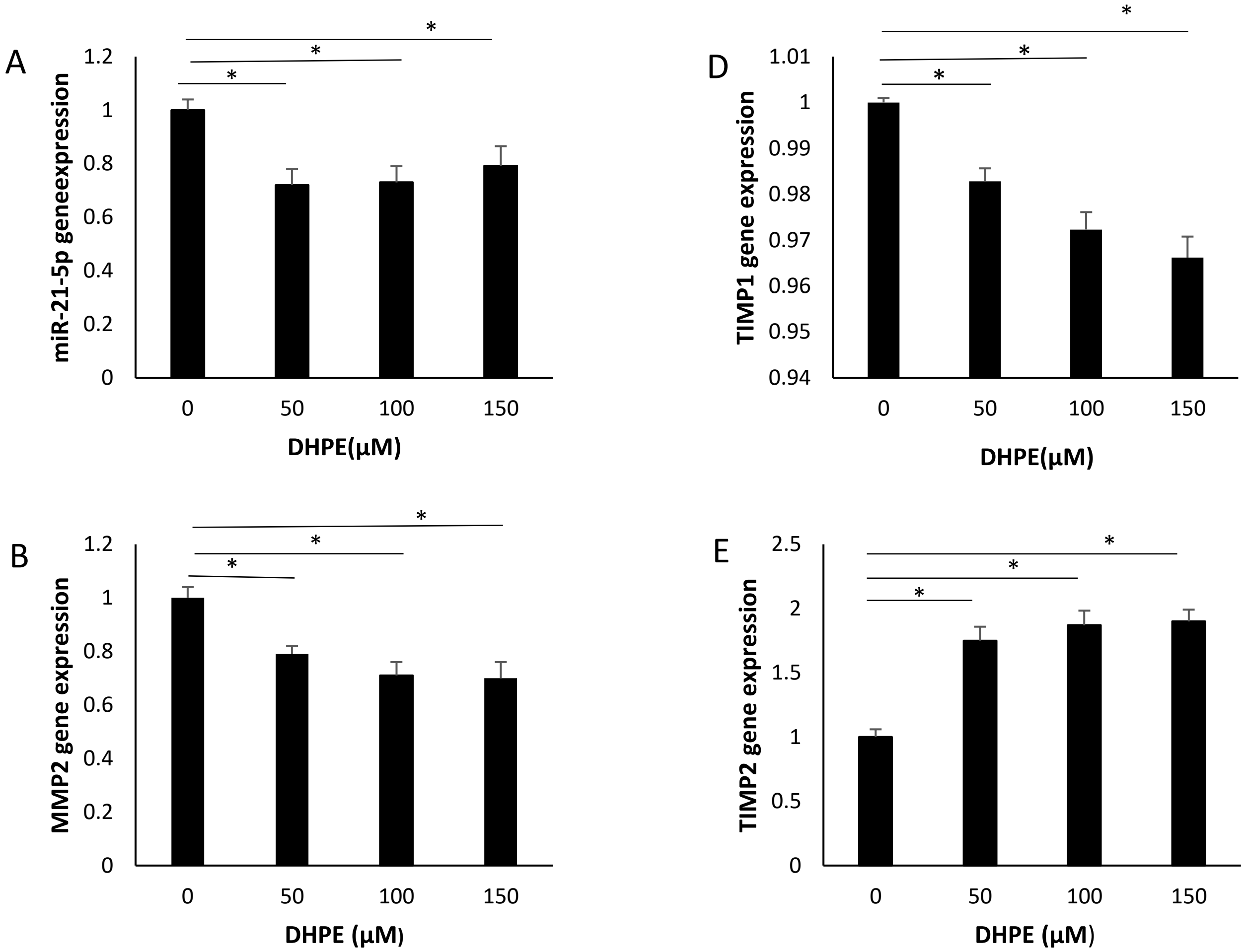
The analysis of gene expression using real-time PCR was performed by REST software (13). The relationships between gene expression levels were calculated with the Pearson correlation test. A p-value of <0.05 was considered significant.

Results

MTT Cytotoxicity assay

According to the results obtained by MTT, the time of 24 h and concentrations of 50, 100, and 150 µM of DHPE, at which the percentage of cell survival was above 70%, were selected for treatment (Fig. 1).

[DOI: 10.61186/rbmb.13.2.254]



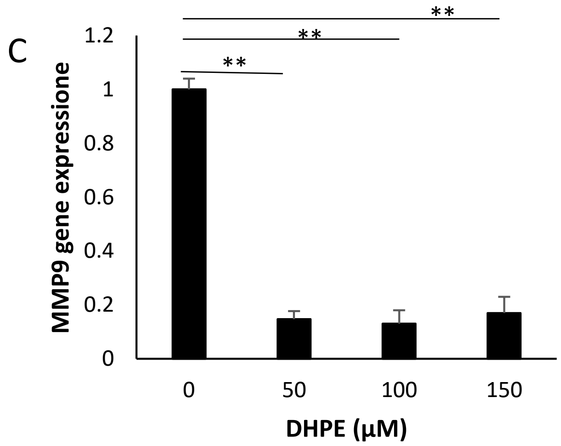


Fig. 2. Effect of DHPE on the expression levels of Mir-21-5p (A), MMP-2 (B), MMP-9 (C), TIMP-1 (D) and TIMP-2 (E) in the HepG2 cell line. The HepG2 cells were treated with 50,100 and 150 µM concentrations of DHPE. Relative expression of genes measured by RT-qPCR. The mean expression of each gene was normalized to GAPDH. The findings are presented as Fold change $2^{-\Delta\Delta Ct}$ compared to the control group. *P< 0.05 and **P< 0.01.

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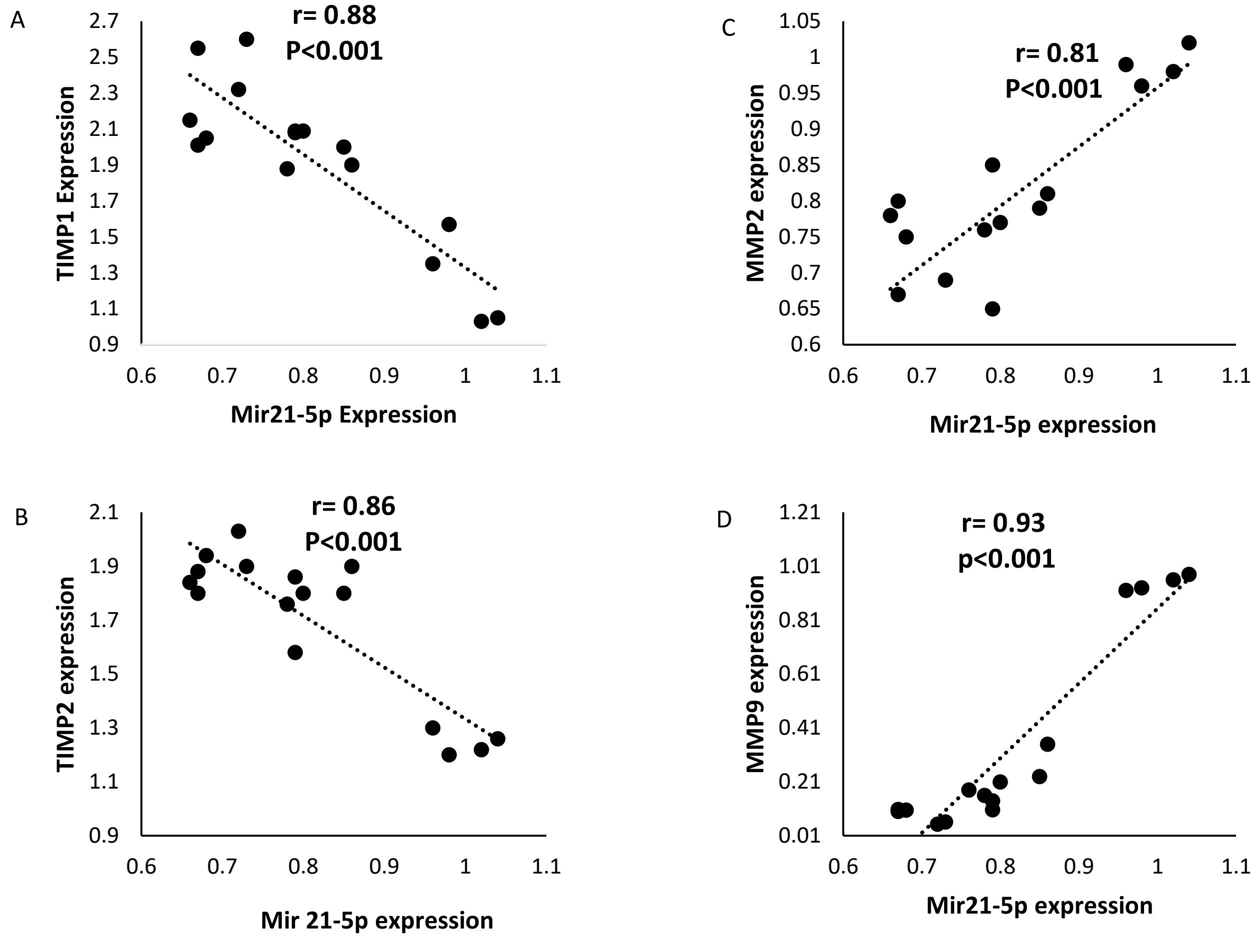

Fig. 3. Gene expression correlation analyses. Pearson's correlation analysis showing correlation between Mir-21-5p and TIMP-1 **(A)**, Mir-21-5p and TIMP-2 **(B)**, Mir-21-5p and MMP-2 **(C)**, Mir-21-5P and MMP-9 **(D)** gene expression in Hep 2 cells. Pearson's correlation coefficient (r) and p-values are shown for each analysis.

Evaluation of Gene Expression

The miR-21, MMP-2, MMP-9, TIMP-1, and TIMP-2 gene expressions in HepG2 cells were assessed. The assessed genes were under treatment with DHPE (50, 100, and 150 µM) for 24 hours. The results showed that the gene expression level of miR-21 was significantly decreased in each of 50 (p= 0.019), 100 (p= 0.02), and 150 µM (p= 0.03) treatments with DHPE (Fig. 2A). MMP-2 gene expression was remarkably reduced at all concentrations of DHPE: 50 (p= 0.017), 100 (p= 0.01), and 150 µM (p= 0.012) (Fig. 2B). The gene expression of MMP-9 was significantly decreased at all concentrations treated with DHPE: 50 (p= 0.001), 100 (p= 0.001), and 150 µM (p= 0.001) (Fig. 2C). TIMP-1 gene expression was remarkably reduced at different concentrations of DHPE: 50 (p= 0.045), 100 (p= 0.03), and 150 µM (p= 0.025) (Fig. 2D). TIMP-2 gene expression was markedly increased in all treated groups compared to the control group: 50 (p= 0.014), 100 (p= 0.012), and 150 µM (p= 0.011) (Fig. 2E).

The Pearson correlation test showed a positive correlation of miR-21 with TIMP-1 (r= 0.45, p=0.04) (Fig. 3A), MMP-2 (r= 0.81, p=0.014) (Fig. 3C), and MMP-9 (Fig. 3E) (r= 0.93, p=0.01) gene expression (P< 0.05), and a negative correlation of miR-21 with TIMP-2 (Fig. 3B) (r= 0.86, p=0.013).

[DOI: 10.61186/rbmb.13.2.254]

Discussion

The results showed that DHPE decreased the gene expression of miR-21, MMP-2, MMP-9, and TIMP-1 compared to the control group, but TIMP-2 gene expression was increased in the HepG2 cells. Invasion and migration are the most important biological features related to tumor malignancy (14). MMPs and their inhibitors, TIMPs, play an important role in the degradation of the extracellular matrix and basal membrane, closely associated with tumor invasiveness (14). Among the different MMPs, MMP-2 and MMP-9 play an important role in tumor invasion and metastasis, showing increased expression levels in many human tumors (3). TIMPs are endogenous inhibitors of MMPs that regulate the breakdown of the extracellular matrix. Several studies have revealed that deregulated TIMP levels in tissue and blood are associated with unfavorable outcomes in nearly all types of human cancer (3, 14).

MicroRNAs are small molecules that play an important role in regulating various cellular processes essential for cancer progression. One such microRNA, miR-21, is significantly overexpressed in nearly all types of human cancers, including HCC, and is believed to play a key role in driving cancer development (7). Thus, targeting miR-21 pharmacologically could be a promising therapeutic approach for HCC.

Many studies have extensively documented the beneficial effects of various natural compounds derived from fruits and vegetables in preventing the initiation and progression of cancer (15). Phenolic phytochemicals, including DHPE, exhibit a remarkable range of structural diversity, leading to a broad therapeutic spectrum. They have antioxidant, anti-inflammatory, anti-angiogenic, and anti-metastatic properties. These bioactive compounds can modulate the functions of specific proteins and enzymes involved in the degradation of extracellular matrix proteins. (16, 17).

The results showed that DHPE at all concentrations decreased the expression of miR-21 in the HepG2 cells compared to the control. MicroRNA-21 (miR-21) is known as onco-miR and is consistently and significantly up regulated in various human cancers and autoimmune diseases, such as systemic lupus erythematosus (SLE) (18, 19). It targets many known tumor suppressors, and its overexpression leads to inhibition of apoptosis and an increase in cellular proliferation, which shifts the balance between proliferation and apoptosis (18).

A study has reported a significant reduction in the expression of miR-21 in the oleuropein-treated MCF-7 breast cancer cell line, depending on the dose. It is proposed that oleuropein could reduce the expression of miR-21 by decreasing NF-KB as a mechanism (17). Fan et al. showed that miR-21 contributes to the invasion and migration of cancer cells through up-regulation of MMP-2 and MMP-9 in renal cell carcinoma by the programmed cell death 4/activation protein-1 (PDCD4/AP-1) signaling pathway (20, 21). It has been demonstrated that the miR-21 inhibitor repressed cell migration and invasion by inhibiting the protein levels of MMP-2 and MMP-9 and significantly changing the expression of phosphatase and tensin homolog (PTEN), phosphatidylinositol-3-kinase (PI3K), and phosphorylated-AKT (p-AKT) (22).

In this study, DHPE significantly decreased the expression of MMP-2 and MMP-9 at all concentrations in comparison with the control group. Coccia et al. showed that DHPE and other compound extracts from olive oil can prevent the metastatic potential of bladder cancer cell lines by inhibiting cell migration and invasion through the downregulation of MMP-2 expression (23). In addition, Scoditti et al. showed that virgin olive oil polyphenols such as DHPE inhibit endothelial tube formation and migration of human vascular endothelial cells by regulating NF-KB and the matrix metalloproteinase-9/cyclooxygenase2 (MMP-9/Cox2) axis (24).

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The results of this study showed that the gene expression of TIMP-1 was reduced by DHPE in the HepG2 cells. Different studies have shown that most cancer patients with high TIMP-1 serum levels have a poor prognosis. This may be explained by another ability of TIMP-1, which is an inhibitor of various MMPs, to function as a growth factor by binding to the cell surface ligand CD63. This binding causes the activation of intracellular focal adhesion kinase (FAK), which may promote cancer progression (25). It has been shown that some proteins involved in metastasis, **such as** TIMP-1, were regulated through PDCD4 via miR-21 (22). The expression of TIMP-1 may have decreased through inhibition of the NF-KB pathway by some antioxidants, such as DHPE (16, 26).

The results of this study showed that DHPE increased TIMP-2 gene expression in HepG2 cells. Among the members of the TIMP family, TIMP-2 has a unique position as an inhibitor that not only exhibits a correlation with matrix remodeling and the suppression of angiogenesis but also actively contributes to the intricate mechanisms underlying tumor growth, inflammation, and various other diseases. The inhibitory effects of TIMP-2 on the invasion and migration of HCT-116 cells have been shown through the regulation of MMP-9 (14). Kaplan-Meier survival analysis showed that low expression of TIMP-2 in tumor tissues was associated with poor overall survival in CRC patients (14). Many studies have shown that TIMP-2 can act as an anticancer agent (27, 28). TIMP-2 expression could be implicated in many cancers, such as lung, breast, ovarian, bladder, and cervical cancers. It has been shown shown that the low expression of TIMP-2 in colorectal cancer (CRC) tumor tissues is closely related to pathological classification, depth of invasion, metastasis to lymph nodes, and TNM stage (14).

The anti-tumor properties and low toxicity of DHPE make it a suitable candidate for cancer treatment (29, 30). Since the results showed a positive relationship between miR-21 expression and MMPs (MMP-2 and MMP-9) as well as TIMP-1 but a negative relationship between miR-21 expression and TIMP-2, DHPE, through the reduction of the expression of miR-21, helps to reduce the expression of MMP-2, MMP-9, and TIMP-1, and increasing TIMP-2 may inhibit metastasis, although this hypothesis needs more studies to be confirmed.

The results of this research indicate that DHPE may have the potential to inhibit the invasion and migration of HepG2 cells. This effect is probably achieved through the downregulation of miR-21-5p, MMP-2, MMP-9, and TIMP-1 and the up-regulation of TIMP-2 in HepG2 cells.

Funding information
This work was supported by the Lorestan University of Medical Sciences under grant number 1397-1-99-1988.

Ethical Consideration
This study was approved by the ethics committee of Lorestan University of Medical Sciences with registration number IR.LUMS.REC.1400.069.

The results of this research are taken from the master's thesis of Lorestan University of Medical Sciences Mehdi Alaee with scientific code 1397-1-99-1988.

Conflicts of interest
The authors declare that they have no conflicts of interest.

Acknowledgments
We would like to express our gratitude to the staff of the Biochemistry Department, School of Medicine, Lorestan University of Medical Sciences, for providing us with the required accommodations and research equipment.

[DOI: 10.61186/rbmb.13.2.254]

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[DOI: 10.61186/rbmb.13.2.254]