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Original Article

Evaluation of the Effect of Curcumin on the Expression of Matrix Metalloproteinase Genes in RAW264.7 Cell Line Treated with Diethylhexyl Phthalate

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Abstract

Introduction: Cancer is a complex disease influenced by genetics and environmental factors, registering a high annual mortality rate worldwide. Despite significant advances in cancer treatment, conventional drug therapies for various cancers have many side effects. Today, the use of complementary medicine is prevalent. Curcumin, as a polyphenol, has many biological activities such as antioxidant, anti-inflammatory, antimicrobial, and antiviral activity. In recent years, its anti-cancer potential has been recognized by scientists worldwide. In the present study, the toxicity of diethylhexyl phthalate (DEHP) and the inhibitory effect of curcumin on the expression of matrix metalloproteinases (MMPs) 1, 8, 13, and 18 as genes involved in metastasis in RAW264.7 cell line were investigated.

Materials and Methods: In this study, the effect of different doses of DEHP and curcumin, were respectively, studied on the cancer cell line of RAW264.7 by MTT assay and AO/EB staining. The RT PCR was employed to determine the gene expression of MMPs 1, 8, 13, and 18.

Results: The results of the MTT assay and AO/EB staining indicated that the optimal dose of DEHP was 200 µM, and the optimal dose of curcumin was 25 µM. The combination group selected the same dose (optimal dose of DEHP and curcumin) The results of Real-time PCR showed a decrease in the expression of curcumin-influenced MMPs 1, 8, 13, and 18 genes.

Conclusions: The *in vitro* inhibitory effect of curcumin on the expression of MMPs genes, which are very important in cancer cell metastasis and establishment, encourages us to continue this project in the form of *in vivo* research.

Keywords: Curcumin, Diethylhexyl Phthalate, Matrix Metalloproteases, Cancer, Metastasis

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Introduction

Cancer is known as the excessive proliferation of cells with dysregulated signaling pathways. I Up to 500 genes suffering from signaling pathway dysregulation are estimated to be involved in cancer. Usually, gene dysregulation takes = up to three decades, and then the cancer syndromes begin to demonstrate[1] Cancer is recognized as a significant concern in global public health, and annually it costs millions of dollars to governments, public healthcare systems, and patients.[2,3] Cancer is a complex disease affected by environmental factors and genetic disorders. There are about 100,000 chemicals in the human environment. Three hundred chemicals are directly involved in the production of cancer; many of these substances have not been studied.[4] One of these chemical agents is DEHP, which is known to be a carcinogen.[5] This chemical compound is responsible for the flexibility and transparency of plastics. It accounts for 10 to 60% of the weight of many plastics because phthalate esters are not covalently attached to polymers and can penetrate foods, beverages, or other materials.[6] This substance has been identified in amniotic fluid[7] and breast milk.[8] DEHP is generally considered an endocrine disruptor, and the mechanisms involved in its toxicity are not yet well understood.[9] Today, DEHP a potential human carcinogen.[10,11] Matrix metalloproteinases (MMPs) are a large family of proteolytic enzymes that can degrade several extracellular matrix components. In 1962, the first vertebrate MMP was identified for tadpole tail analysis. Today, this enzyme is referred to as MMP-1.[12,13] The MMP family is classified into six protease groups, of which MMP-1, MMP-8, and MMP-13 belong to the group of collagenases, MMP-3 stromelysins.[14] There is ample evidence that MMPs are involved in tumor invasion, neoangiogenesis, and cancer cell metastasis, so

MMPs are ideal drug targets for cancer treatment.[15] In cancer, most patients die from secondary and metastatic tumors. The molecular mechanisms of tumor metastasis are complex and involve numerous interactions between tumor cells and their microenvironment. Prevention of metastasis and possible treatment is essential for curing and treating of cancer. Cancer cells invade the surrounding tissue during the metastatic process; proteolytic enzymes are essential for the metastatic process. These enzymes destroy the extracellular matrix (ECM) and allow the tumor to spread.[16,17] Chemotherapy costs expensive for patients, and its production for companies is expensive and time-consuming; besides, this procedure always needs to be more successful. Hence the use of combination therapy seems good. Most anti-cancer drugs are initially obtained from natural herbs.[1,3,18] Due to this, curcumin, a critical phytochemical, is used as an anti-cancer natural herb.[19] Curcumin is taken from turmeric (Curcuma longa), an ancient Persian herb which been recognized since 4,000 BC in ancient Persia. Curcuma is rooted in the Persian word Curcumin meaning saffron.[20,21] Curcumin , as the primary yellow-colored substance taken from rhizomes belonging to turmeric, has shown its anti-cancerous and anti-tumoral effects in *in vitro* and clinical trial investigations.[22,23] The present study aimed to investigate the toxicity of DEHP and the inhibitory effect of curcumin on the gene expression of MMPs 1, 8, 13, and 18 on the cancer cell line of RAW264.7.

Materials and Methods

Cell Culture

The RAW264.7 cell line (a murine macrophage cell line) cultured in DMEM was purchased from the Pasteur Institute of Iran (Tehran-Iran). The cells were then cultured in supplemented Dulbecco's modified eagle's minimum essential medium (DMEM) (Gibco-Ireland) with 10% Fetal Bovine Serum (FBS) (Gibco-Ireland), Penicillin (100 u/ml), and Streptomycin (100 µg/ml) (Sigma Aldrich-USA). Finally, they were kept at 37 °C within an incubator containing 5% (V/V) CO_2 overnight.[24,25] When the cells reached a density of 80%, the supernatants were aspirated and then washed with PBS. Than 0.25% trypsin was added to the cells and incubated at 2 °C for 2-5 minutes within an atmosphere comprising 5% (V/V) CO_2.

Cell Viability

A suspension of 1×10^5 cells per ml[26] was prepared to be treated by five (0 (DMSO/control), 10, 25, 50, and 75 µM) and eight (0 (DMSO/control), 10, 50, 100, 150, 200, 250, and 300 µM) different concentrations of curcumin and phthalates, respectively. These samples were all added to a 24-well plate and treated to a performance of MTT assay. After 48 h, the viability of RAW264.7 cell line was measured by (5 mg/ml) 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma Aldrich-USA). The absorbance of each plate was assessed by an ELISA reader (BioRad-USA) at the wavelength of 570 nm.[27,28]

Acridine Orange/Ethidium Bromide Staining

The cell viability was evaluated by the fluorochrome staining of Acridine Orange/Ethidium Bromide (AO/EB) (Sigma Aldrich-USA). The cultured cells in 24-well plates (3×10^4 cells) were exposed to various concentrations of curcumin and phthalates for five days. 100 µl of AO/EB dye mixture was added to each well previously washed with PBS. After 5 minutes, the dye was removed from the well and washed with PBS; then, each well was observed under a fluorescent microscope (Leica 090-135002, Germany)[28]

Table 1. The Sequences of Used Primers

Gene	Primer Sequences (5′→3′)	Product Size (bp)	Ref
Glyceraldehydes-3-phosphate dehydrogenase (GAPDH)	F: CAAGTTCAACGGCACAGTCA R: CCCCATTTGATGTTAGCGGG	150	(30)
MMP1	F: TTACGGCTCATGAACTGGGT R: GTTGGCTGGATGGGATTTGG	162	(31)
MMP8	F: TGGTGATTTCTTGCTAACCCC R: TACACTCCAGACGTGAAAAGC	139	(32)
MMP13	F: AACATCCATCCCGTGACCTT R: TTCTCAAAGTGAACCGCAGC	154	(31)
MMP18	F: GCTCCTGTCTATGCTGGCTA R: TCTCGGTCTCTTCCTCCTCA	121	Designed in this study

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Test

The total cellular RNA was extracted using a kit (GeneAll, Korea, Catalog No. 001-300). Then, a nanodrop spectrophotometer (OD = A260/A280) checked the purity of extracted RNAs. This ratio for DNA is ~1.7-2.0, and RNA is ~ 2.0.[18,19] and was run on the 1% agarose gel electrophoresis. The RNA molecules were reverse transcribed into complementary DNA or cDNA by the cDNA synthesis kit (Yekta Tajhiz kit, Iran, with catalog number YT4500).[29] The MMPs 1, 8, and 13 the primers were obtained from different papers (Table 1) and then were checked by BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi) while the MMP 18 forward and reverse primers were designed in silico software tools of oligo7 and primer3. For running the PCR, cDNA (1 µl), low rox Master mix real-time (Ampliqon, Denmark) (10 µl), and primers F and R (1 µl) were added into the RT-PCR microtubes, and then, the left amount was reached to 20 µl

by adding double-distilled water into the tube. The RT-PCR (Corbett, Australia) program was done as follows: denaturation at 95 °C for 15 min (40 cycles at 95 °C for 20 s), annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. The PCR products were checked by running on 2% agarose gel electrophoresis. Ethidium bromide was used as the fluorochrome.

Statistical Analysis

In this study, all tests were repeated four times for each sample, and the data were reported as mean ± standard deviation (SD). The SPSS software (version 17) was used for a one-way analysis of variance (ANOVA) to determine the significance of the results. In this regard, the $p \leq 0.05$ was considered statistically significant. Microsoft office excels software (2010) was also used for sketching the plots.

Results

MTT Assay Result

The use of MTT assay enables us to evaluate the inhibitory effect of different doses of curcumin and the accumulative effect of different doses of phthalate on the survival rate of Raw264.7 cell lines. In the present study, the optimal doses (IC_{50}) of curcumin and phthalate are 25 µM and 200 µM, respectively. At optimal doses, the cells were normal while at doses higher than optimal ones, most cells were damaged and lost (Figure 1, A and B).

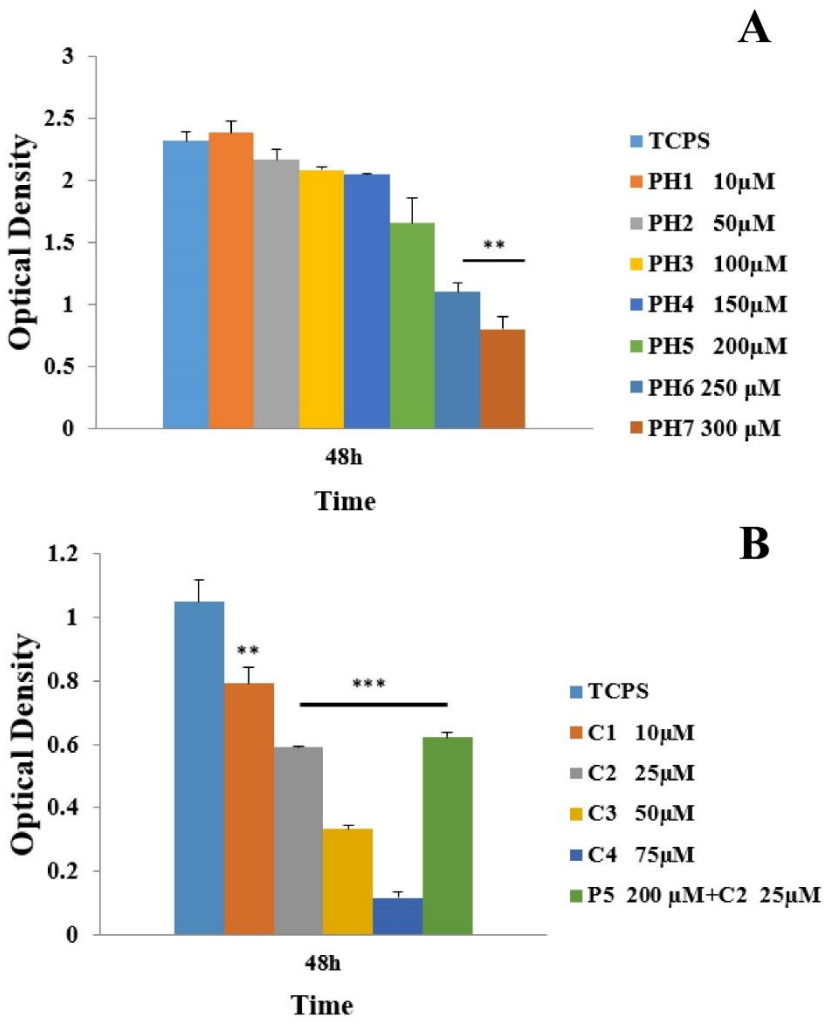

Figure 1. A) MTT diagram shows different doses of phthalate and control group (TCP). The optimal dose of diethyl hexyl phthalate is recognized as 200 (The PH depicts diethylhexyl phthalate). **B)** MTT diagram shows different doses of curcumin (C), complex group (including optimum doses of phthalate and curcumin), and control group (TCP). The optimal dose of curcumin is recognized as 25 µM (C stands for curcumin). The orange bar shows the result of the complex group compared to the other groups. The three and two-star signs indicate a significant difference between the two groups with $p \leq 0.001$, and $p \leq 0.01$ respectively.

Acridine Orange Test

As a quantitative assay, the Acridine Orange/Ethidium Bromide (AO/EB) test is used to evaluate the survival and death of Raw264.7 cell lines affected by curcumin and phthalates. As results show, the Raw264.7 cells survive at low concentrations of both phthalates and curcumin. They were compared with the control group (Figure 2, Figure 3). Figure 2 shows the condition of Raw264.7 cells in the presence

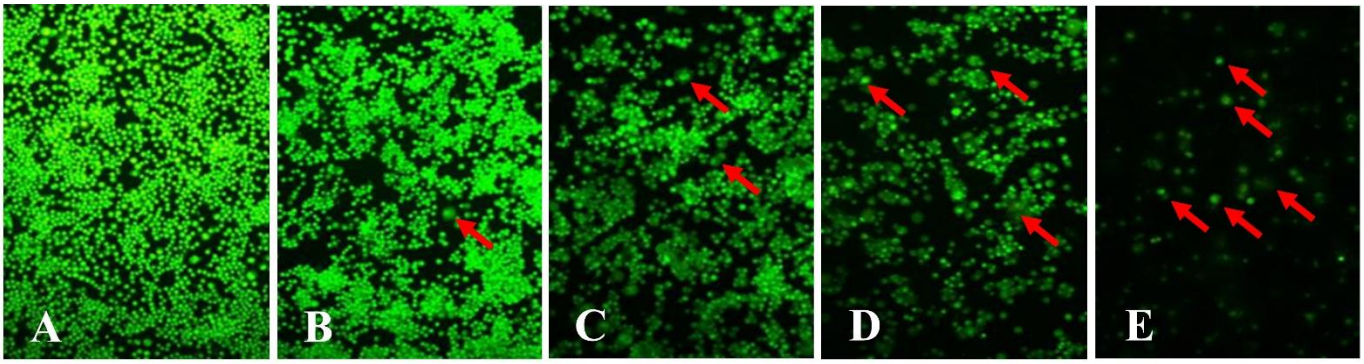

Figure 2. AO/EB Microscopy Imagery. The Bioassay of Raw264.7 Cells treated with different doses of curcumin: **A)** control group; **B)** 10 µm curcumin; **C)** 25 µm curcumin; **D)** 50 µm curcumin; **E)** 75 µm curcumin. (magnification: 100x). The cells in the control group (A) are entirely healthy. The nuclei and cytoplasm are normal. By increasing the concentrations of curcumin, the cells progress to cell death. As shown by the red arrow, as the concentration of curcumin increases, the cell density decreases, and the cells disintegrate.

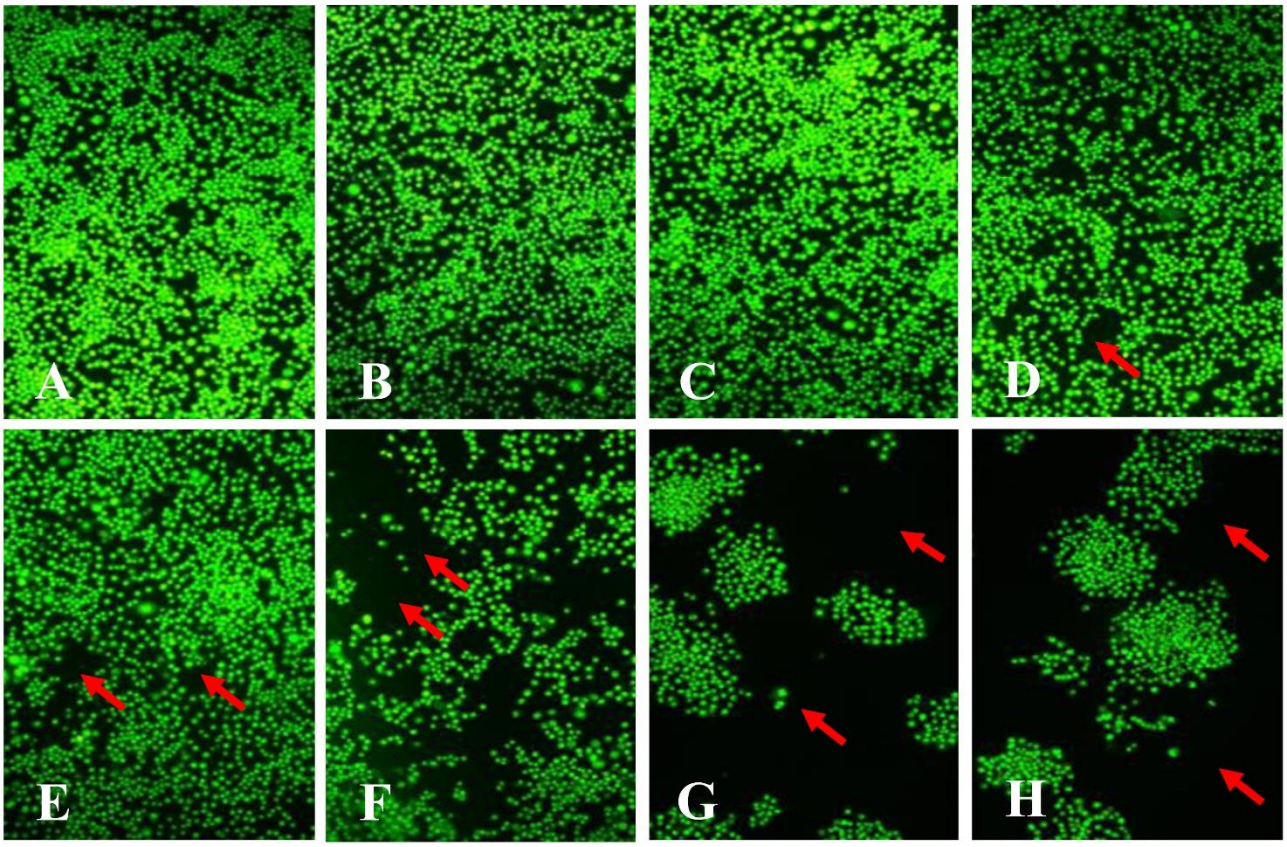

Figure 3. AO/EB Fluorescence Microscopy Images. The bioassay of RAW264.7 cells treated with different doses of Phthalates: **A)** control group; **B)** 10 µm Phthalate; **C)** 50 µm Phthalate; **D)** 100 µm phthalate; **E)** 150 µm Phthalate; **F)** 200 µm phthalate dose; **G)** 250 µm phthalate does; **H)** 300 µm phthalate dose. The cells in the control group are entirely healthy. The nuclei and cytoplasm are normal (A). By increasing the concentrations of phthalate, the cells progress to cell death. As the concentration of phthalate increases, the density of the cells decreases, and the cells disintegrate. As can be seen in the picture, the empty parts of the cell are shown with a red arrow (magnification: 100x).

of different doses of curcumin. Figure 3 Shows the condition of Raw264.7 cells in the presence of different doses of DEHP. Figure 4 shows the cell survival assay for a complex group of the Raw264.7 cell line. In these figures, healthy cells have a normal nucleus and cytoplasm. Show natural morphology. However, the dead cells have defective nuclei and cytoplasm and are out of normal, reducing and the number and density of cells.

Quantitative Analysis of Gene Expression in Real-time PCR System

Real-time PCR results show that MMP1 gene expression increased 3.724 times in the treated group with the optimum dose of DEHP compared with the control group. This increase significantly correlates with the dose of phthalate (p<0.001). Moreover, the expression MMP1 gene in the curcumin-treated group decreased 0.912 times compared with

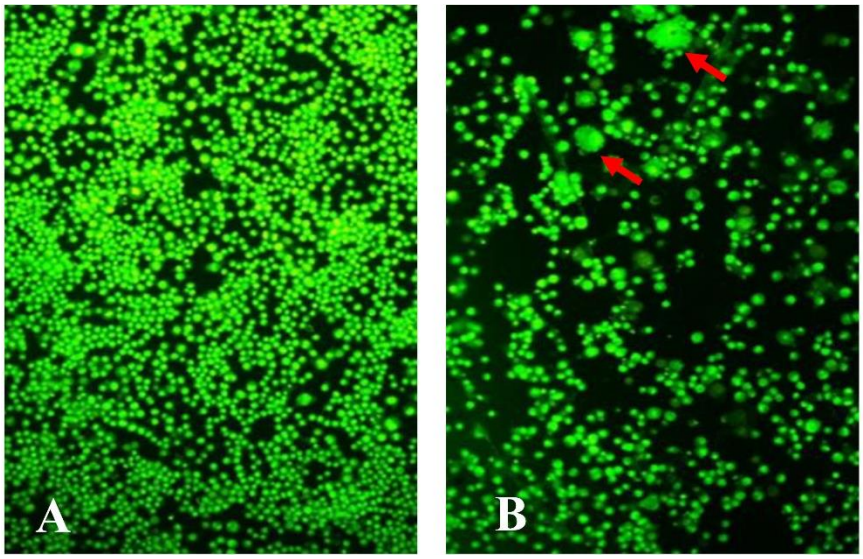

Figure 4. AO/EB Fluorescence Microscope Images. The cell viability assay regarding a complex group of Raw264.7 cell line. The complex group contains a combination of optimal doses of phthalate and curcumin (magnification: 100x) **A)** control group; **B)** complex group. The red arrow shows that some cells have lost their normal structure by adding complexes (disrupting the structure of the nucleus and cytoplasm).

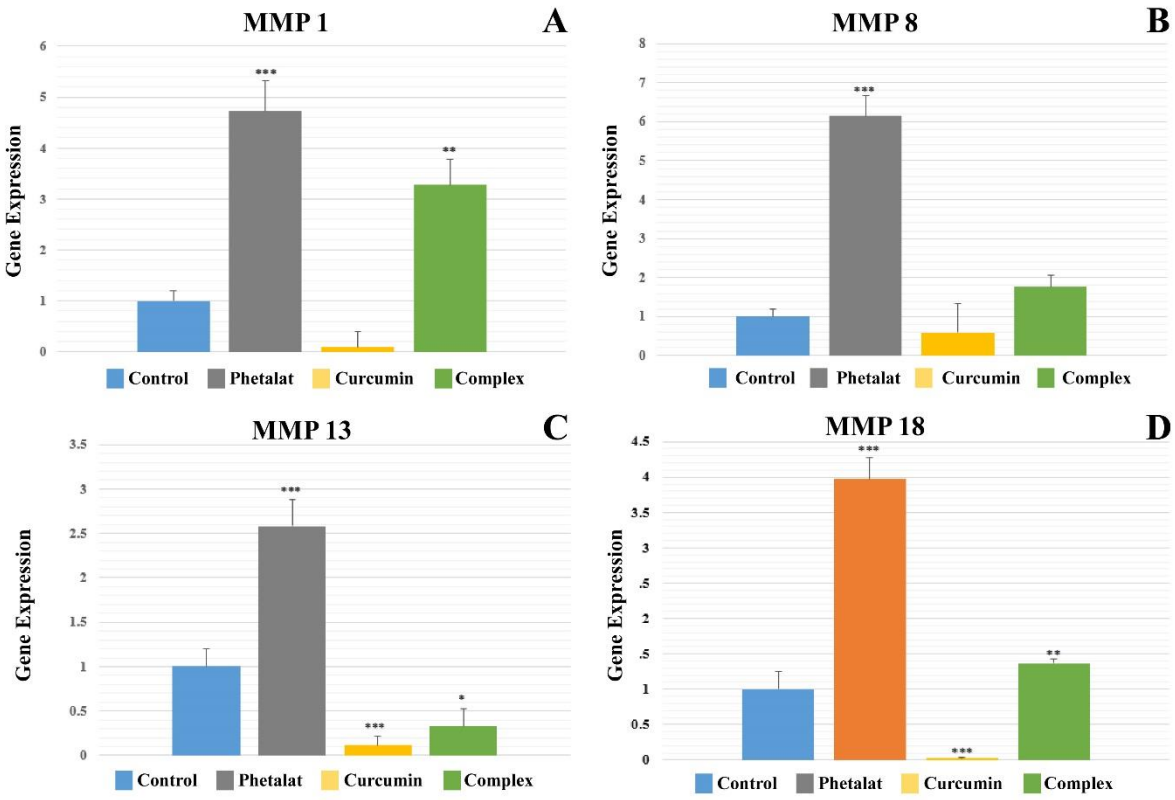

Figure 5. A) MMP1 Gene Expressions in Control, Complex, Phthalate- and Curcumin-treated Groups. **B)** MMP8 gene expression in control, complex, phthalate-treated, and curcumin-treated groups. **C)** MMP13 gene expression in control, complex, phthalate-treated, and curcumin-treated groups. **D)** MMP18 gene expression in control, complex, phthalate-treated, and curcumin-treated groups. The three, two and one-star signs indicate a significant difference between the two groups with $p \leq 0.001$, $p \leq 0.01$, and $p \leq 0.05$ respectively.

the control group. It showed a 2.272 folded increase in MMP1 gene expression within the complex with the control group. This increase has a significant correlation with the presence of phthalate (p = 0.003). (Figure 5A). The MMP8 gene expression was increased 5.112 times within the optimal dose of DEHP compared with the control group. The increase in MMP8 gene expression significantly correlates with the optimal dose of DEHP (p<0.001). The MMP8 gene expression had a 0.397-fold decrease in the curcumin-treated group compared with the control group.

Moreover, the MMP8 gene expression showed a reduction of 0.765-fold in the complex group compared with the control group. In other words, curcumin suppresses the effect of phthalate (Figure 5B). The MMP13 gene expression was increased 1.585-fold within the optimal dose of DEHP compared with the control group. This increase significantly correlates with the phthalate dose (p<0/001), while the expression of the MMP13 gene in the curcumin-treated group compared with the control group had a reduction of 0.885-fold. This reduction significantly correlates with the phthalate dose (p<0.001). In this regard, curcumin has reduced the expression of the MMP13 gene significantly. Besides, the MMP13 gene expression was considerably reduced in the complex-treated group compared to the control group. This feature indicates that curcumin suppresses the phthalate effect in the complex treated group which may lead to a reduction of MMP13 gene expression (Figure 5C). As results show, the MMP18 gene expression had a 2.972 folded increase within the optimal dose of DEHP in compared to the control group. This increase in MMP18 gene expression significantly correlates with the dose of phthalate (p<0.001). In contrast, the expression of the MMP18 gene in the curcumin-treated group had a 0.973 folded reduction compared to the control group This reduction also significantly correlates with the dose of curcumin (p<0.001). The MMP18 gene expression has a reduction of 0.366 folded in the complex group compared to the control group. This reduction significantly correlates with the curcumin suppression effect on phthalate (p<0.01). Hence, DEHP and curcumin increase and decreases the expression of MMP1, MMP8, MMP13, and MMP18 genes, respectively. Moreover, curcumin suppresses DEHP regarding the expression of these four genes (Figure 5D).

Discussion

Cancer rates are rising worldwide. Prevention of this disease is important but sometimes impossible or complicated.[33] Due to the side effects of standard cancer treatments such as chemotherapy, radiation therapy, etc., today, we should look for safer ways to treat this disease.[34,35] The molecular pathways involved in disease and the genes involved in cancer progression must be examined to find more effective ways to treat cancer. The molecular pathways involved in disease and the genes involved in cancer progression must be examined to find more effective ways to treat cancer.[36] Cancer treatment seems to be more effective if it affects the molecular pathways of cancer with some effective compounds. Cancer cells can affect their ECM and metastasis to nearby tissues The use of complementary medicine has received much attention today, especially the role of curcumin, a hydrophobic polyphenol with several therapeutic properties.[37] Researchers consider the role of environmental factors in cancer development very important; one of the compounds that play a role in the occurrence and progression of cancer is DEHP. This substance, in different doses, increases the risk of cancer.[38] Numerous studies have shown that curcumin can interfere with the apoptosis of cancer cells by interfering in the regulation of cellular connections[39] and inhibiting the mechanism of NF-κB activation.[40] Curcumin has also been shown to exert its anti-inflammatory effect by inhibiting the production of MMP-9 in blood mononuclear cells[41] In addition, it is speculated that increased MMP-3 activity may be involved in Fas-mediated apoptosis, where curcumin may modulate MMP-3 expression.[42] In addition, curcumin can significantly inhibit the activity of the MMP-2 matrix.[43] The fact that MMPs 1,8,13 play an important role in the establishment, angiogenesis, and metastasis of cancer cells is well established. The role of these genes as anti-target *in vivo* has also been confirmed.[15,44] Zheng et al., (2019) showed that the expression of MMP3 is inhibited by curcumin, thereby reducing cell viability, inhibiting cell proliferation, increasing apoptosis, and eventually reducing inflammation of osteoarthritis.[45] In addition, it has been proven that curcumin analog suppresses the proliferation, migration, and invasion of the human gastric cancer cell line HGC-27.[46] In 2020, Xiang et al., demonstrated the antitumor effects of curcumin on the proliferation, migration, and apoptosis of human colorectal carcinoma HCT-116 cells. In this study, curcumin was found to reduce the expression level of the MMP9 gene.[47] Curcumin also reduces the expression levels of matrix MMP 2 and 9, inhibiting oral squamous cell carcinoma.[48] The present study showed that curcumin, a well-known herbal active ingredient, can suppress the expression of MMP1, 8, 13, and 18 genes. Our research has shown that curcumin can suppress the expression of MMP genes in phthalate-treated cells. Because, that the expression of MMP in cancer cells is high and DEHP also causes overexpression of these genes, the proper effect of curcumin in reducing the expression of the MMP gene is very important and shows the effectiveness of curcumin composition. Considering the very important role of MMPs in cancer progression and especially metastasis, it can be hoped that if curcumin can be delivered to cancer cells using targeted drug delivery systems, it is possible to see a reduction in cancer progression and metastasis.

Conclusion

In this study, it was shown that curcumin could suppress the expression of MMPs genes. Given that MMPs play a crucial role in cancer development, especially metastasis, it can be hoped that curcumin can positively prevent the tumor from entering the metastatic stage. Further research *in vivo* will confirm these results.

Authors' Contributions

Conducting experiments: SFH and MG, and PB; Acquiring

and analyzing data: SFH and MG, and MG; Drafted the manuscript: SFH, MG, PB, and MG; Edited and approved the final version of the manuscript: MG and MG. All authors read and approved the final manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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