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The Inhibition Effect of Curcumin on MCF-7 Breast Cancer Cells via GSK-3beta and VEGF Signals

Kurkumin'in MCF-7 Meme Kanseri Hücreleri Üzerine GSK-3beta ve VEGF Sinyali Aracılı İnhibitör Etkisi

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Öz

Giriş ve Amaç: Günümüzde kanser tedavisinde destekleyici ve alternatif tedavilere yönelim söz konusudur. Bu nedenle, çalışmada kurkuminin hücre canlılığı ve hücre göçü üzerine etkisi MCF-7 meme kanseri ve L929 fibroblast hücrelerinde GSK-3beta ve VEGF molekülleri aracılığı ile araştırılması amaçlandı.
Gereç ve Yöntemler: Deneyde, sitotoksisite düzeyini MTT yöntemi ile belirlemek amacıyla, MCF-7 meme kanseri ve L929 fibroblast hücrelerine kurkumin beş farklı konsantrasyonda (5, 10, 20, 40 ve 80 µM) 24 ve 48 saat süre ile uygulandı. İmmunositokimya boyaması için, hücreler 5µM, 20µM ve 80µM kurkumin ile 48 saat muamele edildi. GSK-3beta ve VEGF ekspresyonları immunositokimyasal olarak belirlendi. İmmunositokimya boyanma sonuçları H-skor yöntemi ile değerlendirildi. Hücre göçü için, çizik yara modeli üç konsantrasyonda 48 saat süre ile muamele edilerek gerçekleştirildi, ve yara kapanma yüzdesi hesaplandı. Tüm sonuçlar istatistiksel olarak analiz edildi.
Bulgular: MTT analizi sonrasında, kurkuminin L929 hücreleri ile karşılaştırıldığında, MCF-7 kanser hücreleri üzerinde doza bağlı toksik etkiye sahip olduğu gözlendi. GSK-3beta ve VEGF'nin immunositokimyasal dağılımları, MCF-7 hücrelerinde kurkumin uygulanması ile belirgin bir şekilde azaldı. Bununla birlikte, kurkumin, kontrol grubu MCF-7 hücreleri ve L929 hücreleri ile karşılaştırıldığında hücre göçü üzerinde belirgin bir inhibitör etki gösterdi.
Sonuç: Kurkuminin moleküler etki mekanizmalarının bilinmesi, kanser tedavisinde kullanımı açısından güvenilirliğini destekleyen bir faktördür. Etki mekanizmasının tamamen ortaya konulabilmesi için *in vivo* çalışmalara ve gelişmiş tekniklere ihtiyaç vardır.

Anahtar kelimeler: Hücre Göçü, Hücre Çoğalması, Kurkumin,Meme Kanseri, Sitotoksisite.

Abstract

Objective: Nowadays, there is a tendency towards supportive and alternative therapies in cancer treatment. So, the aim was to search the effect of curcumin on the cell viability and migration via GSK-3beta and VEGF in MCF-7 breast cancer cells and L929 fibroblast cells.
Materials and Methods: In the experiment, MCF-7 breast cancer cells and L929 fibroblast cells were treated with curcumin at five concentrations (5, 10, 20, 40 and 80µM) for 24 and 48 hours, and MTT assay was used to detect the cytotoxicity level of curcumin. For immunocytochemical staining, both cells were exposed with 5µM, 20µM and 80µM of curcumin for 48 hours. The immunocytochemistry method was performed to evaluate the expressions of GSK-3beta and VEGF. The immunocytochemical results were evaluated using H-score. For cell migration, the scratch wound assay was done at three concentrations for 48 hours, and the percentage of wound closure was calculated. All data were analyzed statistically.
Results: After MTT assay, it was observed that curcumin had a dose-dependent toxic effect on MCF-7 cancer cells compared to L929 cells. The immunocytochemical distributions of GSK-3beta and VEGF were significantly decreased with the curcumin treatment in MCF-7 cells. However, curcumin showed a marked inhibitory effect on the cell migration in comparison with the non-treated MCF-7 cells and L929 cells.

Conclusion: Knowing the molecular effect mechanisms of curcumin used in cancer treatment is a factor that supports their reliability in terms of use. In vivo studies and advanced techniques are needed to fully reveal the mechanism of action.

Keywords: Breast Cancer, Cell Migration, Cell Proliferation, Curcumin, Cytotoxicity.

1. Introduction

Cancer is the common cause of deaths in the world. Despite all the developments in the field of health, its incidence is increasing. Breast cancer is one of the cancers that threaten women [1,2]. Many factors such as environmental factors, signal molecules and hormone receptors play a role in the emergence and progression of breast cancer [2,3]. Especially the presence of some signal molecules and hormone receptors, estrogen and progesterone receptors, makes the treatment difficult [4]. The glycogen synthase kinase-3beta (GSK-3beta) and vascular endothelial growth factor (VEGF) are molecules that promote the progression of cancer [5,6]. GSK-3beta primarily modulates the glycogen metabolism, also it has been found that it is involved in many mechanisms such as cell differentiation and proliferation, protein synthesis, inflammation and immune response. Therewithal, it is expressed in various cancer types such as breast, ovarian, liver, pancreatic and colon cancers [6]. Similarly, the metastatic effect of VEGF in many cancers is known [7-11]. A few studies have shown a relationship between GSK-3beta and VEGF, the downregulation of GSK-3beta suppresses the activation and expression of VEGF [12-15].

Since many molecules and factors play a role in the formation and metastasis mechanism of cancer, applications for treatment may be insufficient. Therefore, it is common to use alternative applications or agents to support the treatment. Curcumin is one of them, and it is a polyphenol isolated from turmeric (Curcuma longa L.) widely used in daily life. Its synonymous is diferuloylmethane ((1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) [16]. It has been established that it gives a positive result in many diseases (cardiovascular diseases, diabetes, etc.) due to its antioxidant and anti-inflammatory properties. However, curcumin exhibits a anti-tumor effect in many types of cancer, such as colorectal, breast, head and squamous carcinomas by inhibiting the signal molecules, inflammatory cytokines, protein kinases 17]. So, in the current study, it was aimed to detect the cytotoxic effect of curcumin on MCF-7 breast cancer cell line via GSK-3beta and VEGF molecules..

2. Materials and Methods

2.1. Cell Culture

The MCF-7 breast cancer cell line and L929 fibroblast cells were purchesed from Şap Institute, Ankara, Turkey. The both cells were grown in 25T flasks containing RPMI-1640 (350-000-CL, Wisent, Canada) medium with 10 % fetal bovine serum (S1810, Biowest, France), 200mM L-glutamine (XC-T1715; Biosera, France) and 100UI/ml. penicillin/streptomycin (PS-B, Capricorn Scientific GmbH, Germany) in an incubator providing 370C, 5 % CO2 and 70% humidified atmosphere. At 70-80% confluency, cells were passaged for the experiments.

2.2. MTT Assay

The cytotoxicity level of curcumin was detected using 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT, 20395.02; Serva Electrophoresis GmbH, Germany) assay. MCF-7 and L929 cells were placed into 96-well plate (45x103 cells/well) and grown for the confluency of 70-80 %. Curcumin (9469.3, Carl Roth, Germany) was commercially available, and applied to the cells at five concentrations, 5 µM, 10 µM, 20 µM, 40 µM and 80 µM. At the end of 24 and 48 hours, the media removed and fresh media (100 µL) and MTT solution (10 µL) put into each well. After four hours of incubation, media with MTT was discharged. Then dimethyl sulphoxide (DMSO, A3672, AppliChem, Darmstadt, Germany) was added into the each well. The values of absorbance were measured using an UV visible microplate reader (EPOCH2, BioTek, USA) at a wavelenght of 570 nm. The MTT assay was performed thrice [18].

2.3. Cell Migration Assay

The scratch wound model was used to determine the effect of curcumin on cell migration. For this purpose, MCF-7 and L929 cells were put into the 24-well plate (2.5x105 cells/well), and seeded for the confluency of 90-95 %. The wells were scratched vertically with a 200 µL pipette tip. Cells were exposed to the concentrations of 5 µM, 20 µM and 80 µM curcumin for 48 hours. At the end of 48 hours, the scratched fields were photographed from each cell. The scratched areas were measured by ImageJ.1.47 software, and percentage of closure was calculated for each group [19].

2.4. Immunocytochemistry Assay

The both cells were passaged into the 8-well chamber slides (2.5x104 cells/per well) for 24 h. And cells were applied with the three concentrations of curcumin (5 µM, 20 µM and 80 µM) for 48 hours. Then cells were fixed with 4% paraformaldehyde for 30 minutes and washed in phosphate buffer saline (PBS). The permeabilization was performed with 0.1% Triton X-100 (A4975, AppliChem, Darmstadt, Germany). After washing in PBS, endogenous peroxidase activity was eliminated using 3% hydrogen peroxide (1 08600, Merck, Darmstadt, Germany). The cells were treated with primary antibodies: anti-GSK-3beta (NBP1-47470, Novus, USA) and anti-VEGF (NB100-664, Novus, USA) at +4oC overnight. Samples for negative control were not treated with antibody. Followed washing in PBS, the secondary antibodies, biotinylated secondary antibodies and peroxidase-conjugated streptavidin (Histostain kit, 85-9043, Zymed, Carlsbad, USA), were carried out for 30 minutes. To make the immunoreactivities visible, diaminobenzidine/hydrogen peroxide (DAB, 00-2014, İnvitrogen, CA, USA) was used and cells were stained

with Mayer's hematoxylin (800-729-8350, ScyTek, UT, USA) for counterstaining. Samples were mounted using aqueous medium (K002, DBS, Pleasanton, USA), and were examined under camera attached (SC50, Olympus, Germany) light microscope (IX71 inverted-florescence-phase microscope) (Olympus, Japan). The immunocytochemical staining was repeated three times, and was evaluated using H-score method. The immunoreactivities were assigned as weak (+), moderate (++) and strong (+++) respectively. For each intensity, the number of cells were identified in five different fields choosen randomly. The H-score formula was used: Σ Pi (intensity of staining + 1). Pi is the percentage of stained cells for each intensity, and varies from 0% to 100% [18].

2.5. Statistical Analysis

The data was analyzed by repeated-measures of the ANOVA test on GraphPad software (California, USA). The differences between the groups were established using the Tukey-Kramer multiple comparisons test. The results were given as mean ± standard deviation. The P values of < 0.05 were considered statistically significant [18].

3. Results and Discussion

3.1.Results

Curcumin exhibited a cytotoxic effect on L929 fibroblast cell line and MCF-7 breast cancer cell line *in vitro* conditions for 24 and 48 hours (Figure 1). After MTT assay, the IC_{50} doses were determined as and 125.50 µM (24 h) and 127,50 µM (48 h) for L929 cells (data not shown), whereas IC_{50} doses were for MCF-7 cells as 22,07 µM (24 h) and 25,30 µM (48 h) (Figure 1b).

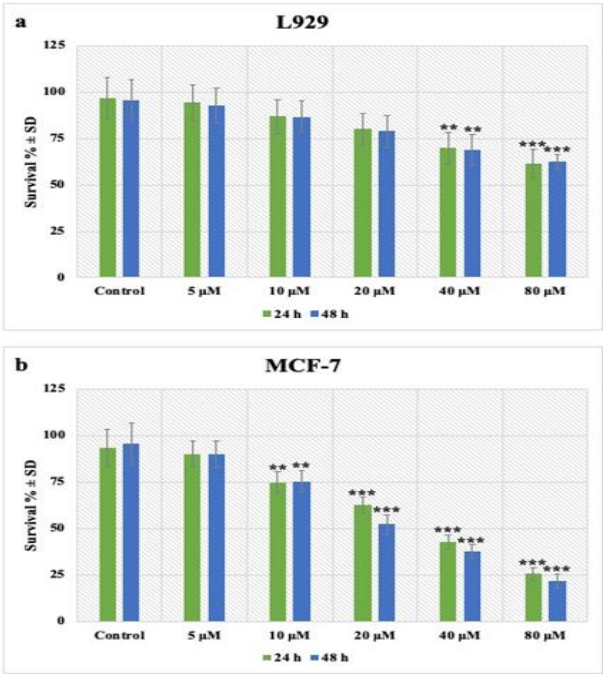


Figure 1. The cytotoxicity levels of curcumin on L929 fibroblast cell line (a) and MCF-7 breast cancer cell line (b) for 24 and 48 hours via MTT assay.

Also, curcumin inhibited the cell migration of MCF-7 at the concentrations of 20 µM and 80 µM significantly (**P<0.001) compared to L929 cells. The rates of scratch wound closure were 35,10 % at 20 µM and 15,40 % at 80 µM. In L929 cells, the percentages of scratch wound closure were 76,45 % at 20 µM and 64,60 % at 80 µM, respectively (Figure 2). At 5 µM of curcumin, there was no inhibition effect in both L929 and MCF-7 cells (Figure 3).

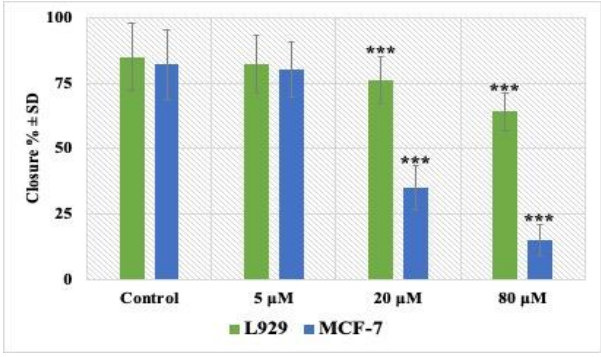


Figure 2. The percentages of scratch wound closure in L929 fibroblast cell line and MCF-7 breast cancer cell line after application of curcumin for 48 h.

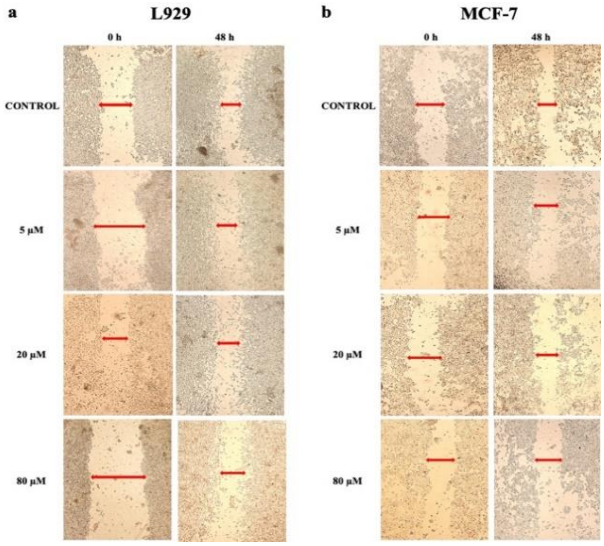


Figure 3. The cell migration images of L929 fibroblast cell line (a) and MCF-7 breast cancer cell line (b) in the presence of curcumin at 0 h and 48 h. Magnification: x100.

The immunoreactivities of GSK-3beta and VEGF were evaluated at the concentrations of 5 µM, 20 µM and 80 µM curcumin after 48 hours in both cells. A pronounced staining was detected in the control groups of L929 and MCF-7 cells. The expressions of GSK-3beta and VEGF were significantly different in L929 and MCF-7 cells. In the control groups, GSK-3beta was 198,45 ± 16,50 in L929 cells and 355,45 ± 25,15 in MCF-7 cells. The immunoreactivities of VEGF were 210,55 ± 17,76 in L929 cells and 348,32 ± 26,21 in MCF-7 cells. These results suggested that MCF-7 cells expressed these two molecules at a high rate (Figure 4, 5 and 6).

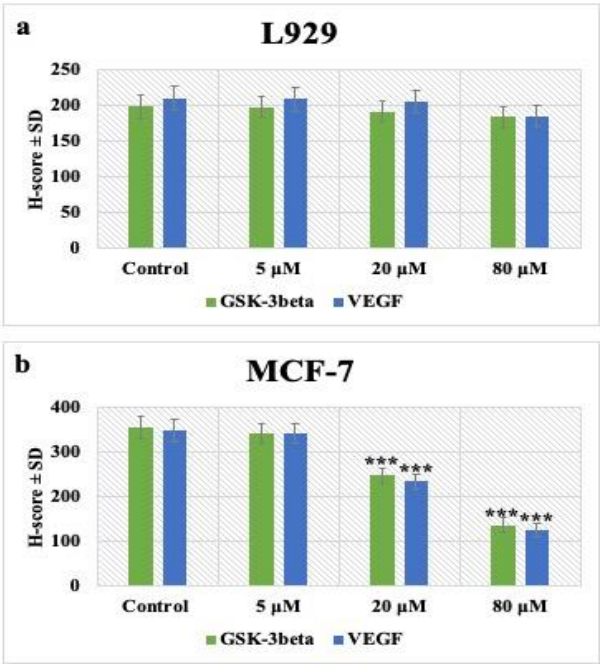


Figure 4. The H-score results of GSK-3beta and VEGF immunoreactivities in L929 fibroblast cell line (a) and MCF-7 breast cancer cell line (b) in the presence of curcumin for 48 hours.

The immunoreactivities of GSK-3beta (197,17 ± 15,21) and VEGF (208 ± 16,34) at 5 µM were close to the control groups in L929 cells, 198,45 ± 16,50 and 210,55 ± 17,76, respectively (Figure 4a). Also, there was no a prominent difference between the control group and group of 20 µM (191,42 ± 14,40) and 80 µM (183,23 ± 14,22) in L929 cells in terms of GSK-3beta immunoreactivity (P>0.05) (Figure 5). The immunoreactivity of VEGF was not changed with the 5 µM curcumin application. The levels of VEGF were 205 ± 15,11 at 20 µM and 184, 68 ± 14,60 at 80 µM.

In MCF-7 cells, the application curcumin decreased the immnuoreactivities of GSK-3beta and VEGF in comparison with the control group and group of 5 µM (***P<0.001) (Figure 4b). The H-score values of GSK-3beta were 247,50 ± 17,12 at 20 µM, and 135,35 ± 16,24 at 80 µM, whereas it was 355,45 ± 25,15 in the control group. However, VEGF was diminished with the curcumin application. The levels of VEGF were 348,32 ± 26,21 in the control group, 233,55 ± 15,70 at 20 µM, and 125,67 ± 15,25 at 80 µM. There was no a significant difference between the control group and group of 5 µM (P>0.05) (Figure 6).

3.2.Discussion

In this study, MCF-7 and L929 fibroblast cells were used for breast cancer and control, respectively. Curcumin has shown to have a toxic effect on MCF-7 breast cancer cell line by inhibiting the expressions of GSK-3beta and VEGF significantly in comparison with the non-treated group and L929 fibroblast cell line. Though, the decreases of GSK-3beta and VEGF supported the inhibition of migration of MCF-7 cells.

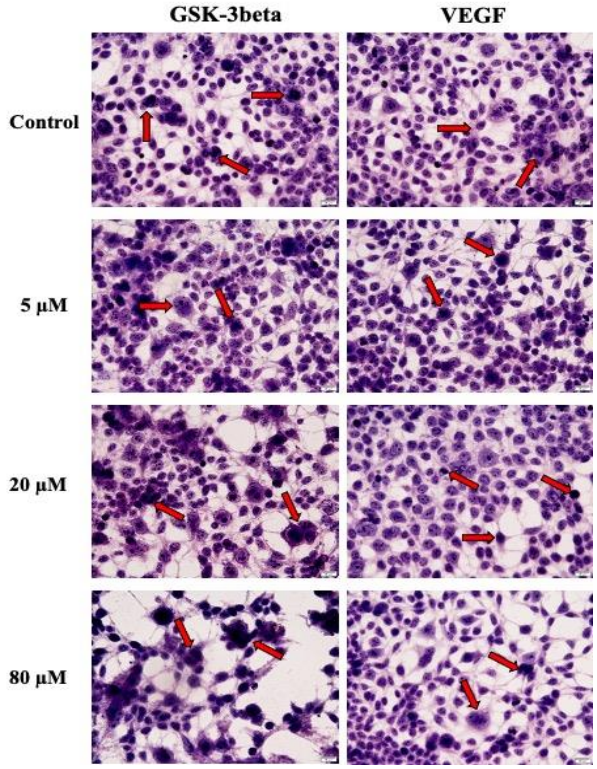


Figure 5. The immunocytochemical distributions of GSK-3beta and VEGF in L929 fibroblast cell line in the presence of curcumin for 48 hours. Arrows: Immunopositive cells, Scale bars: 20µm.

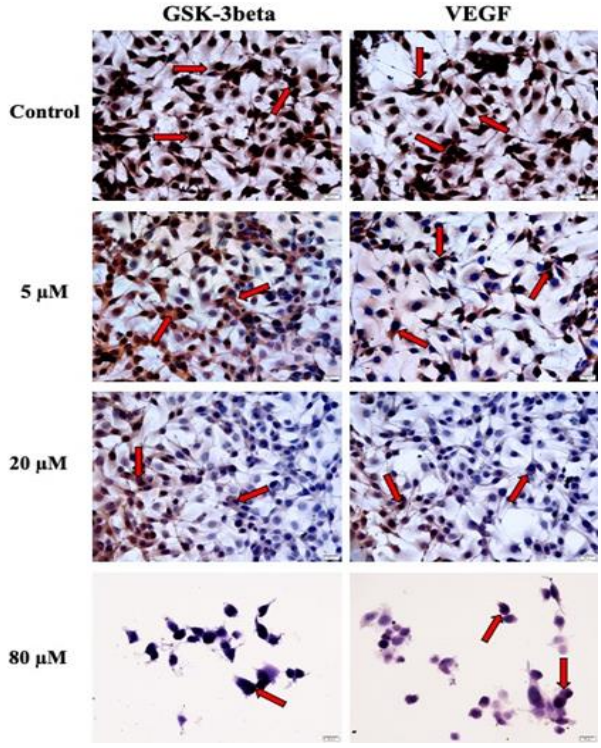


Figure 6. The immunocytochemical distributions of GSK-3beta and VEGF in MCF-7 breast cancer cell line in the presence of curcumin for 48 hours. Arrows: Immunopositive cells, Scale bars: 20µm

Curcumin has been shown to have an anti-tumor and anti-proliferative effect *in vitro* and *in vivo* conditions. Schmidt et al., stated that curcumin reduced the cell proliferation of MCF- breast cancer cells, its IC_{50} doses were observed as 24,5 μM for 24 h and 38,3 μM for 48 h by MTT assay [20]. In another *in vitro* study with MCF-7 cells, the IC_{50} doses of curcumin were identified as 25 μM for 24 h and 15 μM for 48 h [21]. Hasan et al., recognized that the inhibitory effect of curcumin at 12 μM and 20 μM, while 5 μM was not toxic in MCF-7 cells [22]. In the current experiment, the findings were similar to these studies, IC_{50} doses were determined as 22,07 μM for 24 h and 25,30 μM for 48 h. There was no cell death at 5 μM significantly. Unlike these studies, L929 fibroblast cells were used as a control in the experiment. The cytotoxicity of curcumin was seen at high concentrations, IC_{50} doses were 125.50 μM for 24 h and 127,50 μM 48 h, respectively.

The inhibition effect of curcumin on cell migration has been demonstrated using MDA-MB-231 breast cancer cell line. The concentrations of 20 nM and 40 nm ST09, a curcumin derivative, have applied to the these cells in transwell migration model *in vitro* condition. After 24 h, it was seen that cell migration was suppressed approximately five-fold in the ST09 treated group in comparison with the non-treated group. It has been assigned that the inhibition of cell migration occured due to dow-regulation of matrix metalloproteinase 1 and 2 (MMP1 and MMP2) and Vimentin molecules [23]. In this experiment, inhibition of cell migration searched at 5 μM, 20 μM and 80 μM in the scratch wound model in both L929 and MCF-7 cells. While no inhibition effect was seen in L929 cells, a significant inhibition of cell migration was detected in MCF-7 cells at 20 μM and 80 μM.

It is reported that in many studies curcumin showed its cytotoxic effect by inhibiting the expression of different signal molecules in MCF-7 cells. For example, it enhanced the expression of pro-apoptotic Bcl-2-associated X protein (Bax), while it diminished the B-cell lymphoma 2 (Bcl-2) expression. Also it caused the decrease of NF-kB. Thus, it has been reported that apoptosis was triggered in MCF-7 cells and cell proliferation was suppressed. In that research, it has been stated that the inhibitory effect of curcumin initiated by the concentration of 20 μM [24]. Hajigholami et al., reported the similar results with the curcumin application in MCF-7 cells, they ascertained that curcumin reduced the level of anti-apoptotic protein Bcl-2 and increaed the Bax level [25]. It has also been found to trigger cell death by causing a decrease in levels of Toll-like receptor 4 (TLR4), interferon regulatory factor 3 (IRF3), and an increase in the p53 and p21 in MCF-7 cells [26,27]. In the literature, it is stated that curcumin exerts toxic effects by inhibiting many metastatic and proliferative signal molecules, but there is no study examining its effects on the mechanisms of GSK-3beta and VEGF in MCF-7 cells.

On the other hand, there are various experiments revealing the role of GSK-3beta and VEGF in cancer growth and metastasis in MCF-7 breast cancer cell line and other types of tumor cell lines. These studies showed that down-regulation of the GSK-3beta and VEGF molecules in the presence of different inhibitory agents triggered the apoptosis in tumor cells. The cell migration is also inhibited due to down-regulation of these signal molecules [28-34]. In the current study, it was revealed that curcumin reduced the expressions of GSK-3beta and VEGF apparently in MCF-7 breast cancer cell line compared to L929 fibroblast cells.

4. Conclusion

As a result, it has been revealed that curcumin inhibited the cell proliferation and migration by supressing the expressions of GSK-3beta and VEGF molecules in MCF-7 breast cancer cell line. *In vivo* animal and clinical studies are required for the use of curcumin in breast cancer cases where these molecules are highly expressed.

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