ARAŞTIRMA MAKALESİ / *RESEARCH ARTICLE*

The Effects of Engeletin on Cell Proliferation and Invasion in the Human Breast Cancer Cell Line (MCF-7)

İnsan Meme Kanseri Hücre Hattında (MCF-7) Engeletin'in Hücre Proliferasyonu ve İnvazyonu Üzerindeki Etkileri

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ABSTRACT

Aim: *Belonging to the group of flavonoids, Engeletin is a molecule with strong anti-inflammatory, antioxidant and anticancer properties. However, the effect of this molecule on breast cancer cells has not been studied yet. For this purpose, the effectiveness of Engeletin (ENG) on cell proliferation, invasion, and apoptosis in the human breast cancer cell line (MCF-7) was investigated in this study.*

Material and Method: *ENG was studied at 1, 10, and 100 μM doses in the MCF-7 cell line. In the study, cell proliferation was analyzed by MTT cell viability test, its effectiveness on invasion was analyzed by Transwell assay, and cellular viability and apoptotic evaluation were analyzed by fluorescence staining method.*

Results: *It was determined that engeletin reduced MCF-7 cell proliferation. The ENG 100 μM dose was found to be the most effective dose. While ENG application decreases the number of viable cells, it causes an increase in the number of apoptotic cells. In addition, it was determined that ENG application significantly reduced the number of invasive cells in a dose-dependent manner compared to the control group (p<0.001).*

Conclusion: *Engeletin is a molecule with anti-carcinogenic, antiproliferative activity on MCF-7 cells. In addition, ENG shows an anti-invasive activity in MCF-7 cells, demonstrating that it is a molecule with anti-metastatic activity.*

Key words: *engeletin; cell proliferation; cell viability; invasion; MCF-7*

ÖZET

Amaç: *Flovonoidler grubunda yer alan engeletin, güçlü antiinflamatuar, antioksidan ve antikanser özellikleri olan bir moleküldür. Ancak bu molekülün meme kanseri hücrelerinde etkisi henüz araştırılmamıştır. Bu amaçla bu çalışmada hücre kültüründe engeletinin (ENG) meme kanseri hücrelerinin (MCF-7) proliferasyon, invazyon ve apoptozisle olan etkisi araştırılmıştır.*

Materyal ve Metot: *MCF-7 hücre hattında ENG 1, 10 ve 100 μM dozlarında çalışıldı. Araştırmada hücre proliferasyonu MTT hücre canlılık testi ile invazyon üzerindeki etkinliği Transwell deneyi ile, hücresel canlılık ve apoptotik değerlenirilmesi ise floresans boyama yöntemi ile analiz edildi.*

Bulgular: *Engeletinin MCF-7 hücre proliferasyonunu azalttığı tespit edildi. ENG 100 μM dozu en etkin doz olduğu görüldü. ENG uygulaması canlı hücre sayısını azaltırken apoptotik hücre sayılarınında artışa neden olmaktadır. Ayrıca ENG uygulamasının doza bağlı olarak invaze olan hücre sayısını kontrol grubuna göre anlamlı şekilde azalttığı belirlendi (p<0,001).*

Sonuç: *Engeletin MCF-7 hücreleri üzerinde anti-kanserojen, antiproliferatif etkinlik gösteren bir moleküldür. Buna ilaveten ENG, MCF-7 hücrelerinde anti-invaziv bir etkinlik göstererek anti-metastatik etkinlik gösteren bir molekül olduğunuda ortaya koymaktadır.*

Anahtar kelimeler: *engeletin; hücre proliferasyonu; hücre canlılığı; invazyon; MCF-7*

Introduction

Cancer, known as the plague of the century, leads to the death of thousands of people or suffering from disease every day around the world. Breast cancer is the most common cancer in women, responsible for approximately 1 in 3 cancer types[1]. In addition, the incidence of breast cancer is increasing day by day in the world[2]. Despite the chance of success against breast cancer, technological development, advanced diagnosis and treatment options, increased social awareness, and early diagnosis in recent years, the emergence of metastatic cancer types in delayed cases cannot be prevented[3]. Although the chemotherapeutic agents cause cell death in tissue, the inability to suppress the invasion ability of the cells leads to metastatic cancers[3]. In this respect, new therapeutic

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drugs aiming to inhibit the migration of breast cancer cells may lead to the recovery of patients from the disease and increase their life expectancy and comfort.

Many native herbs have been used for medicinal purposes throughout history. Also, some chemotherapeutic drugs used for cancer treatment are extracted from plant origin, especially flowers, leaves, fruits, fungi, and lichens[4]. Moreover, some plants used in breast cancer treatment include ginseng, goldenseal, ginkgo, Echinacea, garlic, saw palmetto, and aloe vera[5]. These plants used for medicinal purposes contain aromatic-essential oils, carotenoids, and flavonoid compounds[6]. Flavonoids are a large group of heterogeneous polyphenol molecules with various health benefits[7,8]. These compounds almost find in everything from vegetables to fruits, from wine to tea; they are a large family of molecules divided into six classes consisting of flavonols (kaempferol, Quercetin), flavones (luteolin, apigenin), flavanones (naringenin, hesperidin), flavans (catechin, theaflavin), anthocyanidins (cyanidin) and isoflavones (daidzein, genistein)[9]. In recent years, research on cancer pathways such as anti-cancer[10], anti-invasion[11] ve anti-metastasis[12] has increased interest in these molecules. Engeletin, in the group of flavonoids, is a glycoside compound obtained from wine, Hymenaea martianada, Petiveria alliacea, and Engelhardia roxburghiana. Recent studies have shown that engeletin (ENG) has a strong anti-inflammatory effect[13,14]. In addition, Huang et al.[15] showed in their study that ENG is a powerful antioxidant and protects neuron cells against oxidative stress. Studies have also shown that ENG may have an anticancer effect[16]. ENG does this by inhibiting Nuclear Factor kappa B (NF-kB) in cervical carcinoma[16].

This suggests that inhibition of NF-kB[17], which is required for epithelial-mesenchymal transition and metastasis in breast cancer development, may play an anti-invasive role in breast cancer. Thus, this study was designed to investigate the possible effect of ENG on proliferation, apoptosis, and invasion of MCF-7 cells in cell culture was investigated in this study.

Materials and Methods

The cell culture, cell viability, and invasion tests of this study were carried out in the Central Research Laboratory of Kafkas University.

Preparation of MCF-7 Culture Medium

The human MCF-7 breast cancer cell line was obtained from the ATCC (American Type Culture Collection). The cancer cell line was incubed at 95% humidity and 5% CO_2, and 37°C temperature. It was fed in Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermo Fisher Scientific) consisting of 10% Fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific) and 1% antibiotics (Penicillin, Streptomycin, Amphotericin, Gibco, Thermo Fisher Scientific). The medium was renewed every 24 hours until the cells reached the desired numerical density in the culture medium. Cell count was calculated manually using a toma slide. For calculation, cells were first removed with the trypsin enzyme. Then, at the end of the centrifugation process, the remaining cells on the tube wall were collected in a 1 ml medium. Finally, 10 µl cells and 10 µl 0.2% Trypan blue dye were mixed in a tube, and 10 µl of the mixture was added to a toma slide and calculated. Engeletin (CAS Number: 572–31–6, MedChemExpress, USA) was applied to the cells at concentrations of 1, 10, and 100 µM.

Cell Viability Test

The antiproliferative effects of Engeletin on MCF-7 cells were investigated by MTT method[18]. After determining the appropriate doses, cells were cultered into a 96-well plate. After 24 hours, different concentrations of ENG were applied to the cells. In this study, the 3-(4.5-dimetiltiyazol2-yl)-2.5-difeniltetrazolyum-bromür (MTT) (CAS Number: M5655, Sigma, Germany) method was applied to the cells 24 hours after the application of Engeletin[19], Then, absorbance values at 570 nm wavelength were calculated with a microplate reader spectrophotometer (Thermo Scientic Multisican, Singapore). Cell viability rates were analyzed by comparison with control wells.

Fluorescent Staining Method

Twenty-four well plates were seeded as 2.5×10^4 cells in 200 µL medium for each well plate. At the end of the standard 24-hour incubation, the medium was withdrawn and washed twice with PBS. Then, 4% formaldehyde (200µL) was added to all wells for fixation and incubated for 4 minutes at room temperature. At the end of the time, formaldehyde was removed by washing with PBS. To ensure the permeabilization of the cells, 99.9% methanol (200µL) was added to the wells and kept at room temperature for 20 minutes. It was again washed with PBS. Fluorescent diacetate (FDA) was dissolved in 1 mg/mL dimethyl sulfoxide (DMSO). Propidium iodide (PI) was dissolved in 1

mg/mL purified water 5µL of PI and FDA were added to each well and visualized with an inverted microscope (Invitrogen Evos FL) with fluorescence attachment after 10 minutes of incubation.

Transwell Invasion Test

The invasion abilities of MCF-7 cells were tested with a Transwell (Corning Incorporated NY, USA) plate. This test procedure was performed as follows. Transwell wells with custom membrane 8 microns a pore width were placed in a 24-well cell plate. Matrigel solution (BD, Bioscience) was added to the membranes and incubated for 16 hours. Before transferring cells to Transwell wells, they were kept in a serum-free medium for 12 hours. After counting from the stock cell solution, cells (2.5×105 in 1 mL) were seeded into Transwell wells, including a 200 µl serum-free medium. 750 µl medium containing 10% Fetal Bovine Serum (FBS) was placed in the well under the Transwell well. Afterward, ENG 1, 10, and 100 µM doses were applied to the Transwell wells for the drug groups, and the plate was left to incubate at 37°C for 16 hours. At the end of the incubation, the medium in the Transwell wells was removed and after washing twice with PBS (Phosphate Buffer Saline). Then, 3.7% formaldehyde was added to the wells to fix the cells in the Transwell. After 2 hours, formaldehyde was removed, and 99.9% methanol was added for cell permeabilization in the Transwell. At 20 minutes, methanol was withdrawn, the Transwell wells were washed twice with PBS, and 0.1% Crystal Violet (200 µl) (Sigma Aldrich, Germany) dye was applied[20]. Traswell wells, kept in the dark for 15 minutes, were washed with PBS and cleaned with a sterilized cotton swab. Then, cells in the invading traswell membrane were counted and pictured with a light microscope (Zeizz Primostar). Graphs were drawn according to the average cell numbers, with three replicates for each group.

Statistical Analysis

Statistical analyses were performed with SPSS 20.0 software (IBM, USA), and standard error bars were added to the graphs. Analysis results were done with one-way ANOVA and Tukey multiple comparative tests. Significant differences were determined by comparing all groups among themselves. If the character used in the columns are the same, they are statistically insignificant; if they are different, they are statistically significant ($p<0.05$)

Results

Cell Viability

According to the results of the cell proliferation MTT test, it was found that there was a significant increase in the cells of the control group after 24 hours ($p>0.05$). On the other hand, ENG administration significantly reduced the number of MCF-7 cells ($p>0.05$). The best effective dose of ENG was determined as 100 µM (Fig. 1).

Results of Fluorescent Staining

According to the results of fluorescence staining, which investigated the antiproliferative and apoptotic effects of engeletin on MCF-7 cells, it is seen that the viability of MCF-7 cells in the control group is quite high (Fig. 2). In the ENG-administered groups, it is seen that the number of living cells decreases while the number of apoptotic cells increases. Depending on the dose, engeletin has been shown to have an antiproliferative and apoptotic effect on MCF-7 cells, and the best effect is shown at a dose of 100 µM (Fig. 2).

Transwell Invasion Results

Transwell wells invasion test results are shown in Fig. 3. It was found that MCF-7 cells in the control group were more invasive to the membrane base compared to other groups. As shown in Fig. 4, it was determined that ENG administration significantly reduced the number of invasive cells depending on the dose compared to the control group ($p<0.001$). When the ENG groups were compared, it was seen that the number of cells in the ENG 100 µM group was significantly less than in the other groups ($p<0.001$).

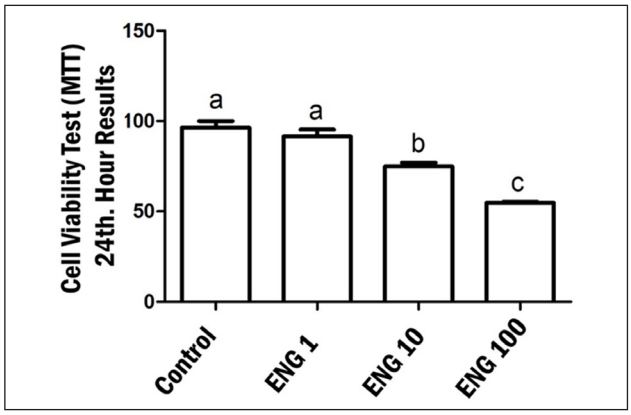

Figure 1. *Cell Viability Test (MTT) 24th hour results.*

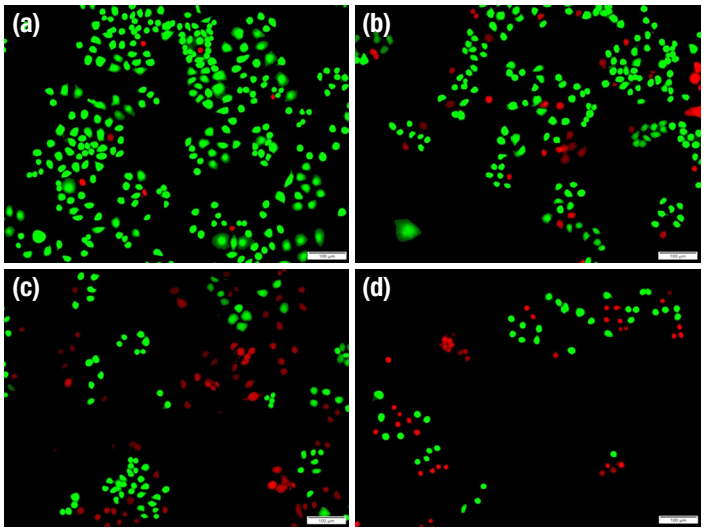

Figure 2. a–d. *FDA and PI fluorescent staining findings (**a:** Control group, **b:** ENG 1 Um group, **c:** ENG 10 Um group, **d:** ENG 100 Um group, red cells: PI (apoptotic cells), green cells: FDA (healthy cells), magnifications: 10×).*

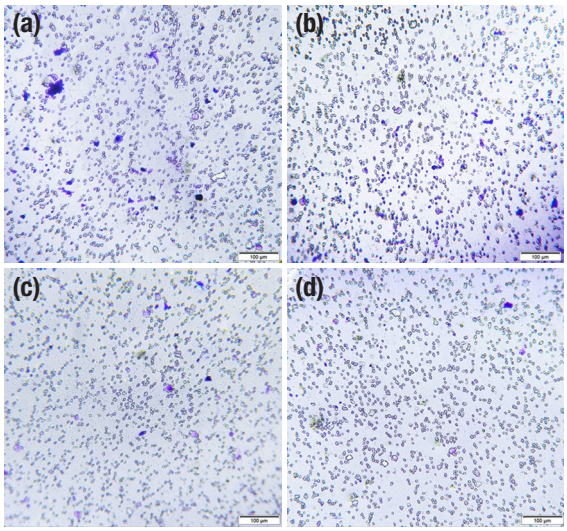

Figure 3. a–d. *Transwell membrane invasion images (**a:** Control group, **b:** ENG 1 Um group, **c:** ENG 10 Um group, **d:** ENG 100 Um group).*

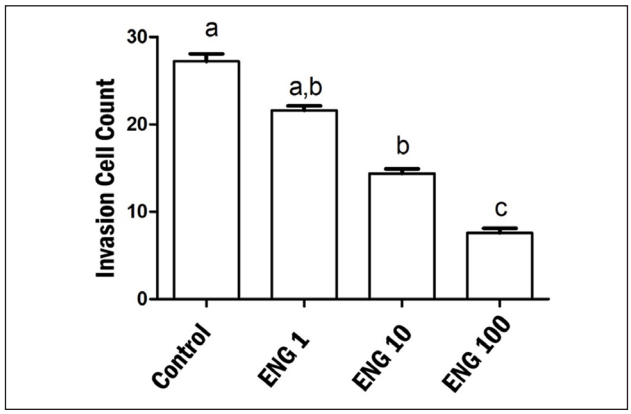

Figure 4. *Findings of Transwell membrane invasion cell count.*

Discussion

Metastasis is defined as moving to another area at the end of the body in certain conditions and processes of cancer cells, difficulties in clinical treatment with adverse effects on other tissues and organs in cancer patients. Unfortunately, metastasis is also seen in breast cancer[21]. Moreover, metastasis is responsible for most of the deaths from breast cancer[22]. This feature of breast cancer cells stems from their ability to invade. From this point of view, it can be a treatment protocol for suppressing the invasion abilities of cancer cells, especially in early cancer treatments. Thus, adding anti-invasion properties to existing chemotherapeutic drugs may support the development of new generation drugs.

Recent studies, the trial of natural biological products in treating many diseases, reveal the potential of such compounds[23,24]. Flavonoids, at the top of these molecules, have been reported as potential anti-tumor compounds by inducing apoptotic cell death, oxidative stress, and ER stress[25]. ENG, which is from the group of flavonoids, has anti-inflammatory[13,14], anti-antioxidant[15], and anticancer effects[26]. However, there are no studies on ENG's anti-proliferative and anton human breast cancer cells.

For this purpose, we first performed the MTT assay to test the antiproliferative effect of ENG on MCF-7 cells. The MTT assay measures cellular metabolic activity, indicating cell viability, proliferation, and cytotoxicity. This colorimetric method is based on converting yellow tetrazolium salt, an MTT solution, into purple formazan crystals by living and active cells. This test, one of the most preferred tests in cell culture studies, is used to test the effectiveness of active substances in cell culture. In a study investigating ENG activity on lung cancer cells, similar to our study, the MTT method was used, and its antiproliferative activity was demonstrated[26]. Another study by Wungsintaweekul et al.[27], showed that ENG has antiestrogenic activity. MCF-7 breast cancer cells are estrogen receptor-positive cells. Therefore, Briand et al.[28] has been shown that MCF-7 cells are sensitive to estrogen and antiestrogenic substances reduce the proliferation of MCF-7 cells. This important condition contributes to its antiproliferative effect on MCF-7 cells, as the blocker has antiestrogenic activity.

In our study, we applied the fluorescent staining method to show the apoptotic effect of ENG in MCF-7 cells. This method, widely used to test cell viability, is based on staining target cells with various fluorescent dyes. FDA, one of these dyes, passes passively through the phospholipid bilayer of the cell and gives a green glow in fluorescence microscope[29]. Another important dye we use is PI. Unlike FDA, PI cannot cross the undamaged plasma membrane and can only bind in the DNA of cells where the plasma membrane is compromised/permeable. These cells, seen as red in the fluorescence microscope, give us information about the cells that started the apoptotic cascade[30,31]. In our findings, while intense FDA-positive cells detected high viability in the culture medium in the control group, it was determined that these FDA-stained cells decreased in the ENG applied groups. On the other hand, the shines of PI dye in ENG applied groups proves that ENG is apoptotic and anticancer. In a study on nanoparticles on MCF-7 cells, the viability and apoptotic properties of MCF-7 cells were tested, as we observed in our findings[32]. This result confirms the method and findings of our study. These also results explain the ENG anti-proliferative effect that we observed in our MTT results by apoptosis mechanism.

In recent years, research on the mechanism underlying the invasion abilities of cancer cells and the inhibition of this metastatic cellular behavior has attracted much attention. One of these research methods, the Transwell cell invasion test method, is based on measuring the chemotactic ability of cells against an attractive chemical[33]. In our research, we tested the invasion effect of ENG on MCF-7 cells with this method. In our findings, MCF-7 cells invaded the Transwell membrane base more in the control group compared to the other groups. This result explains the metastatic behavior of MCF-7 cells. In the study of Li et al.[34], in which they investigated the effectiveness of calicosine substance on invasion and migration in human breast cancer cells, they showed that MCF-7 cells significantly penetrated the traswell membrane. This study supports our result. When ENG was applied to MCF-7 cells, it was determined that the number of invasive cells decreased significantly compared to the control group, depending on the dose. This result reveals that blockade on MCF-7 cells can show an anti-invasive effect.

A study by Bai et al.[16], determined the anti-invasive effect of ENG through NF-κB inhibition in cervical carcinoma cells. Moreover, in another study by Wu et al.[35], they showed a role in the inhibition of NF-kB via TLR4 in the lipopolysaccharide-induced endometriosis model; this explains the reason for the anti-metastatic activity of the ENG.

Engeletin is a molecule with anti-carcinogenic, antiproliferative activity on MCF-7 cells. In addition, it has been an anti-invasive activity on MCF-7 cells and anti-metastatic activity. In conclusion, it is important to prevent metastasis in breast cancer. In this respect, ENG may be a chemotherapeutic drug that can be used to treat breast cancer.

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Conflicts of Interests

The authors report no conflicts of interest.

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