RESEARCH ARTICLE / *Araştırma Makalesi*

Ganoderma Lucidum Fruiting Body Dry Extract Inhibits Cell Proliferation and Induces Apoptosis in Breast Cancer Cells by Activating Both Caspase-8 and Caspase-9

Ganoderma Lucidum Fruiting Body Dry Extract, Hem Kaspaz-8 Hem De Kaspaz-9'u Aktive Ederek Hücre Proliferasyonunu Inhibe Eder ve Meme Kanseri Hücrelerinde Apoptozu Indükler

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Abstract

Introduction: Breast cancer is one of the most prominent causes of mortality among women worldwide due to factors such as aggressive behavior of the cancer and resistance to chemotherapeutic agents. Resistance to chemotherapeutic drugs in cancer treatment is a common phenomenon, especially in progressive diseases with a high prevalence. Therefore the current study aimed to demonstrate the anti-cancer effects of Ganoderma Lucidum (GL) on MDA-MB-231 and MCF-7 cell lines.

Materials and Methods: We showed the effect of GL on cell proliferation using the MTS method, its effect on clone formation with a clonogenic test, and we also evaluated whether the apoptotic pathway was activated by western blot.

Results: GL significantly inhibited cell proliferation depending on the dose in MDA-MB-231 and MCF-7 cell lines and it significantly reduced the number of colonies compared to non-treated cells. In addition, GL induced the initiation of apoptosis in MDA-MB-231 and MCF-7 cells, as evidenced by an enhanced level of caspase-8 and caspase-9 and decreased expression of PARP.

Conclusion: These results demonstrated the molecular mechanism underlying the anti-cancer effects of GL, suggesting that GL might be useful in anticancer therapy. Novel products, such as GL, undoubtedly have promise for the future.

Keywords: Ganoderma Lucidum, Breast cancer, apoptosis, natural products

Öz

Amaç: *Meme kanseri, agresif davranışı ve kemoterapötik ajanlara direnç gibi faktörler nedeniyle dünya çapında kadınlar arasında en önemli ölüm nedenlerinden biridir. Kanser tedavisinde kemoterapötik ilaçlara direnç, özellikle prevalansı yüksek olan ilerleyici hastalıklarda sık görülen bir olgudur. Bu nedenle mevcut çalışma, Ganoderma Lucidum (GL)'un MDA-MB-231 ve MCF-7 hücre hatları üzerindeki anti-kanser etkilerini göstermeyi amaçladı.*

Yöntem ve Gereçler: *GL'nin hücre proliferasyonu üzerindeki etkisini MTS yöntemi ile, klon oluşumu üzerindeki etkisini klonojenik test ile gösterdik ve ayrıca apoptotik yolun aktive edilip edilmediği western blot yöntemi ile değerlendirdik.*

Bulgular: *GL, MDA-MB-231 ve MCF-7 hücre hatlarında doza bağlı olarak hücre proliferasyonunu önemli ölçüde inhibe etti ve tedavi edilmemiş hücrelere kıyasla koloni sayısını önemli ölçüde azalttı. Ek olarak, GL'nin, MDA-MB-231 ve MCF-7 hücrelerinde apoptozu indüklediği artan kaspaz-8 ve kaspaz-9 seviyesi ve azalmış PARP ekspresyonu ile kanıtlandı.*

Sonuç: *Bu sonuçlar, GL'nin antikanser etkilerinin altında yatan moleküler mekanizmayı gösterdi ve GL'nin antikanser tedavisinde faydalı olabileceğini düşündürdü. GL gibi yeni ürünler şüphesiz gelecek vaat ediyor.*

Anahtar Kelimeler: *Ganoderma Lucidum, meme kanseri, apoptoz, doğal ürünler*

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INTRODUCTION

In recent years, the increasing number of cases and deaths has made breast cancer the world's most prevalent malignancy.[1,2] According to Global Cancer Statistics (GLOBOCAN), worldwide there are almost 19.3 million new cancer diagnoses and approximately 10 million deaths from cancer. Breast cancer (BC) is the most common type of cancer with an estimated 2.3 million new diagnoses.[3] BC is generally diagnosed by the presence or absence of three receptors identified as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Triple-negative breast cancer (TNBC) is a subtype of BC that lacks expression of the three receptors. TNBC accounts for approximately 15%–20% of all breast cancer cases.[2,4] In the treatment of breast cancers that are positive for one of the three receptors, appropriate hormone treatments according to the receptor they carry usually gives effective results. TNBC shows a more aggressive clinical behavior and poor prognosis versus other types of BC, and survival rates tend to be lower due to widespread metastasis or drug resistance in addition to the absence of effective targeted therapies.[2,4-6] These results mean that the hormones estrogen and progesterone, or the HER2 protein, do not induce the growth of TNBC. Hence, they do not respond to hormonal therapy targeting ER and PR or medicines that target HER2 protein receptors. In recent years, many therapeutic options have emerged in the fight against metastatic breast carcinoma. However, these therapies frequently fail due to the development of resistance. Therefore, there is an urgent need for the development of alternative and more effective therapeutic strategies for the treatment of BC.[2,4,7,8]

Ganoderma Lucidum (GL) is a type of mushroom that is widely used in traditional treatments in China and Asian countries and is also known as the mushroom of immortality due to its superior therapeutic properties.[9] Additionally, it has recently attracted great attention due to its anti-tumorigenic effects in various types of cancer and tumor models.[10,13] GL has a large number of pharmacological actions, such as anti-oxidative, immunity-boosting, anti-inflammatory, and antitumor properties. The effect of GL against cancer cells has been summarized in a limited number of studies and has been shown to affect many cancer cell lines via apoptosis with activation of the caspase cascade.[14,15] Moreover, according to the information obtained from the studies, it is thought that GL may serve as a practical anticancer agent by inducing caspases in various cancer types.[16-18] Similarly, Ganoderma extracts or components from GL have previously been reported to possess antitumor activities for breast cancer.[19-22] Although these and other studies have reported the anti-cancer effects of GL in BC cells[16,23], the molecular mechanisms of the anti-proliferative effects of GL in MDA-MB-231 and MCF-7 cells have not been characterized in detail. Therefore, we aimed to examine the molecular mechanisms of the effects of GL on these cell lines.

MATERIALS and METHODS

This prospective study took place in Erciyes University, Faculty of Medicine, Genome Stem Cell Center (GENKOK) in 2019. MDA-MB-231 and MCF-7 breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA).

Cell culture and GL reagents (GL Fruiting Body Dry Extract)

GL Fruiting Body Dry Extract was obtained from Sigma-Aldrich (Sigma Aldrich, St. Louis, USA). According to the manufacturer, this sample contains NLT 0.3% of ganoderic acid D and ganoderenic acid D. This was prepared at six different doses (5, 10, 20, 40, 80 and 100 μM) using an adequate volume of dimethylsulfoxide (DMSO, Sigma-Aldrich). In addition to these groups, in order to compare the experimental groups, the DMSO group, which was applied to the cells in the percentage of GL dissolved, and the NT (Non-treatment) group, which was treated with nothing, were formed. The stock solution of GL was stored at 4˚C and diluted in FBS-free DMEM and applied to MDA-MB-231 and MCF-cells at certain concentrations

for 24 h or 48 h.

Cell culture

Cell lines were cultured in DMEM medium (Sigma Aldrich, St. Louis, MO) with addition of penicillin (100 units/ml), streptomycin (100 μg/ml) and 10% heat-inactivated fetal bovine serum (FBS). Cells were incubated at 37°C in a humidified incubator with 5% CO2. They were evaluated for viability and contamination using an inverted microscope. When the cells covered 80-90% of the flask area in which they were seeded, they were removed with trypsin-EDTA and passaged. When sufficient cells were obtained for the study, cells were counted on a Thoma slide with trypan blue.

Cell viability and proliferation assays

Cells treated with GL were evaluated for cell viability and proliferation using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, Madison, WI). MDA-MB-231 and MCF-7 cells were seeded in appropriate quantities in each well of a 96-well plate (1.5×10^3 cells/ well) and incubated at 37°C overnight. After incubation, the cells were treated with different doses of GL (5μM, 10 μM, 20 μM, 40 μM, 80 μM and 100 μM) for 24 and 48 hr. After 48 hours, a mix containing MTS and phenazine metho-sulfate (20:1 v/v) was supplemented to the cells and they were incubated at 37°C for 30 minutes. The results were obtained by measuring absorbance at 450 nm using an ELISA Reader (Promega Glomax Multi Detection System).

Colony formation assays

A clonogenic test was performed to examine the effect on colony formation of GL for MDA-MB-231 and MCF-7 cells. For this, the cells were seeded in six-well plates (1.5×10^3 cells/well) and incubated overnight at 37°C. After incubation, the cells were treated with different doses of GL and were kept in an incubator for approximately 2 weeks to follow the growth of the cells. After incubation, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS) by removing the medium, and the colonies were made visible with crystal violet. The numbers of colonies were counted using the Image J Software program.

Western Blot analysis

Cells were seeded in 25-cm^2 culture flasks (3.5×10^5 cells/4 ml medium) for western blot analyses. After GL treatment, the cells were collected with trypsin-EDTA and washed twice with DPBS. Cells were lysed by adding lysis solution to the cell pellet after centrifugation. The total protein concentration of samples was defined with a detergent-compatible protein assay kit (DC kit; Bio-Rad, Hercules, CA). Protein values at 40 μg for each sample were determined using absorbance measurement at 750 nm and accordingly, aliquots containing loading buffer and distilled water were prepared for each sample. Samples were loaded on a gel and were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with a 4% a 20% gradient for protein separation. The SDS-PAGE gel was electrotransferred to polyvinylidene difluoride membranes (PVDF) with the western blot method. Blocking buffer was used to block the membrane for 60 min at room temperature and then membranes were washed with TBS-T. After washing, the membranes were treated with the following primary antibodies diluted in TBS-T containing 5% dry milk: cleaved-caspase-8 (cell-signaling, USA), caspase-8 (protein tech, USA), cleaved-caspase-9 (cell-signaling, USA), caspase-9 (protein tech, USA) and PARP (protein tech, USA) and were incubated overnight at 4°C. After incubation, the membranes were washed with TBS-T and were treated with suitable secondary antibodies. Chemiluminescence detection refers to a detection method that exploits the interaction of an antibody and antigen and was performed with Clarity Western ECL Substrate (Biorad). The ChemiDoc MP Imaging System (Biorad) was used for visualized blots.

Statistical Analysis

All experiments were repeated 3 times to minimize the

margin of error and the data was summarized as group means with standard deviations (SD) using Graphpad PRISM (Graphpad Software Inc., Version 8.0d) program for statistical analysis. Results in terms of statistical significance were analyzed using the Student t-test and one-way ANOVA. The p value less than 0.05 was regarded as statistically significant.

RESULTS

GL inhibits breast cancer cell proliferation and colony formation

To determine the effects of GL on cell proliferation of MDA-MB-231 and MCF-7 cells, we performed an MTS assay after 24 h and 48 h treatment with GL at doses ranging between 5 and 100 µM. Cell viability showed a decrease at increasing GL concentrations in both breast cancer cell lines and there was no significant change between the two-time points (24 and 48 h).

This assay demonstrated that GL had almost no cytotoxic effects at the 5 and 10 µM concentrations in MDA-MB 231 cells. The cell viability seemed to decrease at concentrations higher than 20 µM and showed a statistically significant decrease in viability at the 40 µM concentration of GL treatment (Figure 1A, $p< 0.01$). However, the treatment concentration became more effective as the exposure time increased. The MDA-MB-231 cell line did not show a significant change in cell viability when exposed to 20 µM GL at 24 h of treatment while it significantly decreased at 48 h, as shown in Fig 1A. In addition, we formed a DMSO group to test the effect of DMSO, which we used to dissolve GL, on cells. There was a significant difference in cell viability between the NT group, that is, the cell group to which we did not apply anything, and the DMSO group ($p< 0.05$), so we compared the GL doses with the DMSO group. In MCF-7 cells, GL had almost no cytotoxic effects at the 5 and 10 µM concentrations. The cell viability showed a statistically significant decrease at the 20 µM ($p< 0.01$) and 40 µM ($p< 0.0001$) concentrations, as shown in Fig 1B. On the other hand, the 80 µM and 100 µM concentrations of GL led to an almost complete elimination of MCF-7 viable cells. In MCF-7 cells, there was no significant change between the two-time points (24 and 48 h). Moreover, a significant difference was not observed between the NT group, that is, the cell group to which we did not apply anything, and the DMSO group (Figure 1A).

We performed a clonogenic assay to determine the effects of GL on colony formation for 10 days in MDA-MB-231 cell lines (Figure 2A). We found that there was a significant reduction in the number of colonies at doses of 5 µM and 10 µM but we did not observe any colony formation at doses of 15 µM and 20 µM (Figure 2B). These results were significant compared to NT and DMSO groups. In MCF-7 cells, there was a significant reduction in the number of colonies at doses of 10 µM and 15 µM and we did not observe any colonies at a dose of 20 µM (Figure 3 A-B). These results were statistically significant ($p<0.001$).

GL stimulates both intrinsic and extrinsic apoptotic-death of BC cells

To assess whether GL contributes to the apoptosis-related death of BC cells, we analyzed the ability of GL to induce apoptotic cascades for 24h and 48h treatment using western blot analysis (Figure 2). GL treatment induced activation of the initiator caspase-9 (as evidenced by an increase of the 35 kDa cleaved form) in the intrinsic pathway and activation of the initiator caspase-8 (as evidenced by an increase of the 18, 41, 43 kDa cleaved forms) in the extrinsic pathway. PARP expression was significantly decreased in both MDA-MB-231 and MCF-7 BC cells treated with GL for 24h and 48h treatments (Figure 4-5).

Our results suggest that activation of caspases comes from specific cleavage of the precursor protein, so we examined the expression of caspase-8, caspase-9, cleavage of caspase-8, and cleavage of caspase-9 using Western analysis 24h and 48h after GL treatment (Figure 4-5). In this study, with the decrease in caspase 8 and caspase 9 expression together with the increase in cleaved-caspase 8 and

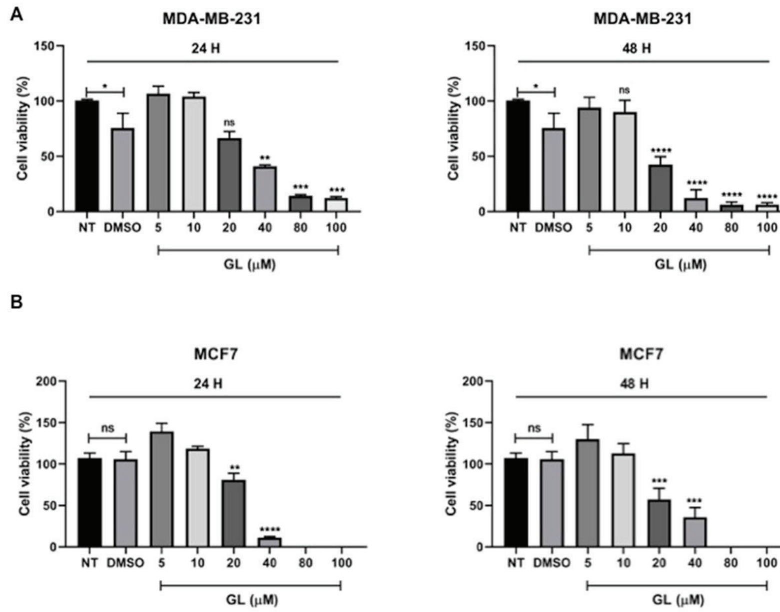

*Fig. 1: Cell viability determined with MTS assay after treatment of MDA-MB-231 (A) and MCF-7 (B) cell lines with serial doses (5μM, 10 μM, 20 μM, 40 μM, 80 μM and 100 μM) of GL for 24h and 48h. Data were measured after 24 h and 48 h. Values less than 0.05 compared to control were considered significant (**p < 0.01, ***p < 0.001, ****p < 0.0001).*

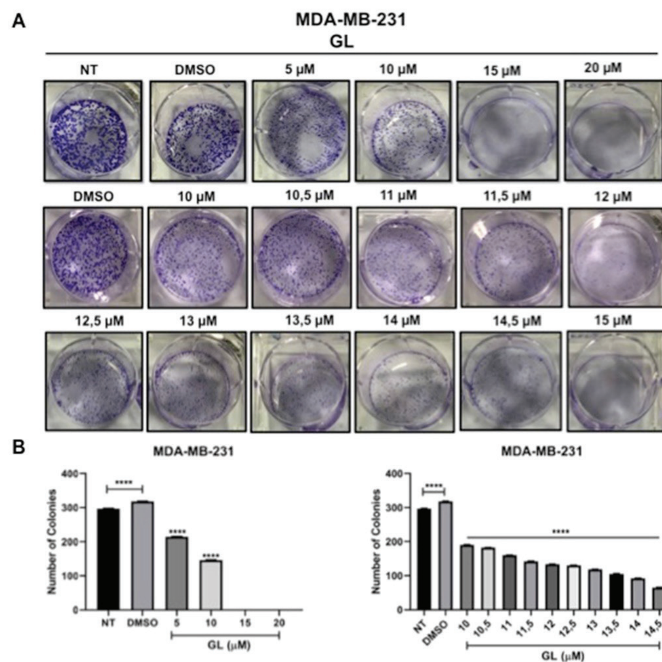

*Fig. 2: Effect of GL on colony formation in MDA-MB-231. MDA-MB-231 cells were assessed for colony formation by staining with crystal violet and colony areas were counted with image J at the end of 14 days for MDA-MB-231 and MCF-7. Data are presented as mean ± SD (****p < 0.0001).*

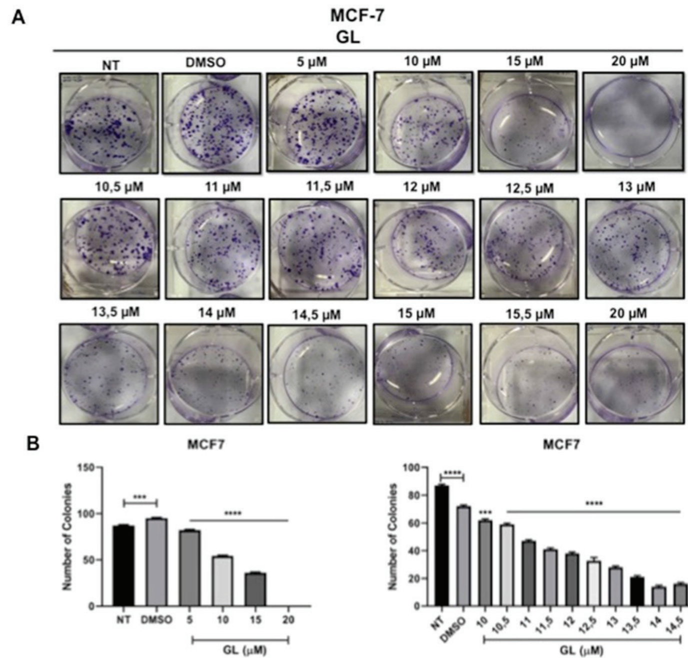

*Fig. 3: Effect of GL on colony formation in MCF-7 cells. MCF-7 cells were assessed for colony formation by staining with crystal violet and colony areas were counted with image J at the end of 14 days in MDA-MB-231 and MCF-7. Data are presented as mean ± SD (***p < 0.001, ****p < 0.0001).*

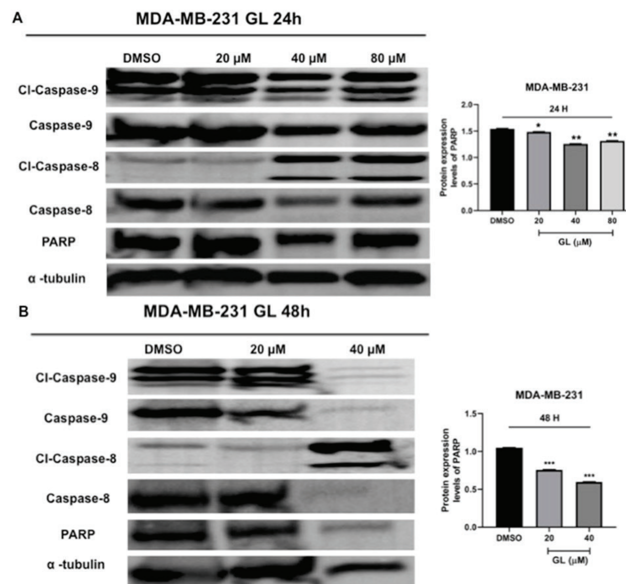

*Fig. 4: Effects of GL on apoptosis in MDA-MB-231 cells. MDA-MB-231 cells were cultured with different concentrations of GL for 24 and 48h and Western blot assays were carried out to examine the effects of GL on the expression of apoptosis pathway markers in MDA-MB-231 cells after 24 (A) and 48 h (B) of GL treatment. Protein α-tubulin was used as internal control. Measurements were repeated 3 times independently of each other. The data are presented as mean ± SD (*p < 0.05, **p < 0.01, ***p < 0.001).*

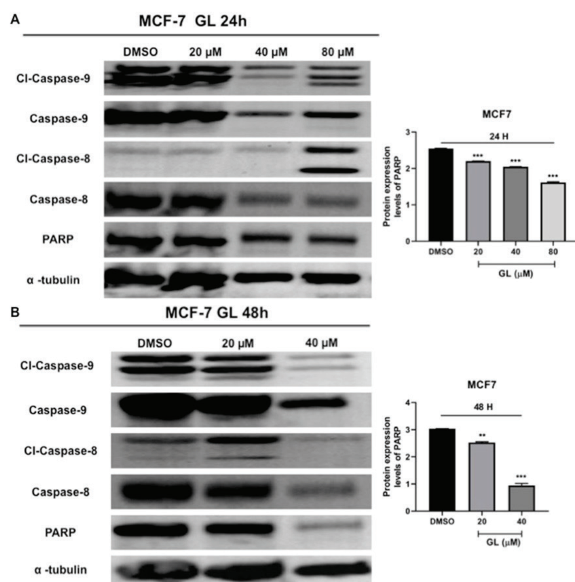

*Fig. 5: Effects of GL on apoptosis in MCF-7 cells. MCF-7 cells were cultured with different concentrations of GL for 24 and 48h and Western blot assays were carried out to examine the effects of GL on expression of apoptosis pathway markers in MCF-7 cells after 24 (A) and 48 h (B) of GL treatment. Protein α-tubulin was used as internal control. Measurements were repeated 3 times independently of each other. Data are presented as mean ± SD (**$p < 0.01$, ***$p < 0.001$).*

cleaved-caspase 9 expression indicates that GL treatment activates the apoptosis pathway.

DISCUSSION

Chemotherapy remains an essential treatment for patients with breast cancer despite the side effects.[24] Cancer patients undergoing chemotherapy treatment are more likely to get infections due to their weakened immune systems.[25] Chemotherapy can damage the immune system by reducing the amount of infection-fighting white blood cells and making the body more vulnerable. Therefore, patients often are not able to continue treatment because of the side effects of the drugs.[26] For this reason, natural product research has promise for discovering biologically active compounds from different sources such as fungi or plants with anti-cancer potential.[27]

GL is a popular mushroom that is called the "Mushroom of Immortality" and has been known for more than 4000 years for health promotion. It has also been used to prevent or cure various diseases, including cancer, in traditional Chinese medicine.[28] Martinez-Montemayor and et al. showed that GL compounds had significant anti-cancer activity against triple-negative breast cancer models.[29] Similarly, in recent years, GL polysaccharides (GLP) extracted from GL were shown to inhibit cell proliferation, invasion, and metastasis, and induced tumor cell apoptosis and suppressed drug resistance in BC cells. Although the composition of GLP has not yet been fully identified, recent studies have found that GL contains more bioactive compounds than extracts of unbroken spores.[16,30-32] Jiang and et al. showed that GL obstructed the proliferation of MCF-7 and MDA-MB-231 cells through regulation of the estrogen receptor (ER) and NF-kappaB signaling. In this study, while GL suppressed the expression of ER alpha in MCF-7 cells, it did not affect the expression of ER beta in MCF-7 and MDA-MB-231 cells. Overall, emerging data suggest that GL has estrogenic activity on breast cancer

cells.[19] Similarly, Ye and et al. showed the binding ability of GL-1, a component of GL, to estrogen receptor by computer-aided simulation. The results showed that GL-1 could bind to estrogen receptor β, and had estrogen-like effects, which might induce secretion of estrogen and expression of ERβ by binding to ERs. In this way, Ye et al. reported the effects of GL-1 on the proliferation of estrogen-induced MCF-7 cells.[33]

Tumor metastasis is a multistep process with formation of new vessels (angiogenesis), tissue invasion, and formation of new colonies and is often responsible for major death in patients with cancer. Prevention of colony formation is an important part of the treatment process.[34,35] Zhong et al. showed that GL reduces the number of colonies and prevents the formation of new colonies in MCF-7 cells.[35] Similarly, in this study, we showed that GL was effective on colony formation in MDA-MB-231 and MCF-7 cells with crystal violet staining.

Wu and et al. noted that GL had anti-proliferative and apoptotic effects in BC cells by Hoechst staining, DNA fragment assay, and Western blot analysis. Additionally, in the same study, it was demonstrated by the Comet method that GL caused DNA damage to breast cancer cells.[20] It is clearly understood in the literature that GL has anti-proliferative, anti-tumorigenic, and apoptotic effects on BC cells. However, even if some authors indicate direct cytotoxicity of GL on cancer cells,[30,36,37] the pathway mediating the anticancer functions of GL is not yet known. Therefore, in our study, we wanted to show that GL induces apoptotic pathways, both the mitochondrial intrinsic pathway via caspase 8 and the extrinsic pathway via caspase-9 in destroying breast cancer cells. Poly (ADP-ribose) polymerase (PARP) is a type of enzyme involved in many cellular processes including DNA repair, genomic stability, and programmed cell death.[38] In cancer treatment, blocking PARP can eliminate cancer cells by preventing them from repairing their damaged DNA, causing them to die.[39-41]. Therefore, the agents that block PARP may be crucial for cancer treatment. In our study, we noticed that GL significantly decreased the expression of PARP.

CONCLUSION

Our findings indicate that GL induced apoptotic cell death through activation of caspase-8, the initiator caspase of the intrinsic pathway, and caspase-9, the initiator caspase of the extrinsic pathway. Additionally, PARP, active in DNA repair, was significantly decreased in MDA-MB-231 and MCF-7 cells with GL treatment. These results could suggest GL supplementation as a potential anti-cancer agent against BC cells. Moreover, it could be a guide for the discovery of new drugs to use instead of drugs with high side effects and may be promising for patients with BC.

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Conflict of Interest

No conflict of interest was declared by the authors.

Author Contributions

Concept and Design: Ö.G., B.Y Supervision: B.Y Materials: Ö.G., V.Ç., Z.H Data and Analysis: Ö.G., V.Ç., Z.H Writing: Ö.G Revision: Z.H., B.Y.

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