



Gossypin Suppresses Cell Growth by Cytotoxic Effect and Induces Apoptosis in MCF-7 Cells

Gossypin, Sitotoksik Etki ile Hücre Büyümesini Baskılar ve MCF-7 Hücrelerinde Apoptozu İndüklemektedir

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Abstract

Aim: Today, breast cancer is a disease that is encountered commonly in women with limited options for treatment. It is needed to find new agents that can be effective in preventing or managing this disease. It has been demonstrated that gossypin inhibits tumor growth. In our study, it has been targeted to examine the effects of gossypin regarding both anticancer activity and apoptosis in MCF-7 cells.

Material and Method: MCF-7 cells were treated with different doses of gossypin and with 50 µM cisplatin for 24, 48, and 72 hours. The MTT analysis, Caspase-3, Caspase-9, and NF-κB mRNA expressions of those MCF-7 cells which were treated with gossypin were also conducted in order to evaluate the apoptosis or necroptosis-induced cell death.

Results: In MTT experiments, it has been observed that the administration of 100 µM dose of gossypin had similar effects to the routine cisplatin administration, caused a significant decrease in cell proliferation, and increased apoptosis in the evaluations of Hoechst staining and morphology. It has been put forth that gossypin decreases the expression of CASP-3 and CASP-9 mRNA and increases the expression of NF-κB.

Conclusion: Our results demonstrate that for the breast cancer cells, the 100 µM of gossypin positively affects cell death pathways due to apoptosis.

Keywords: Gossypin, Apoptosis, Caspase-3, Caspase-9, NF-κB

Öz

Amaç: Meme kanseri günümüzde kadınlarda sık görülen ve tedavi seçenekleri kısıtlı olan bir hastalıktır. Bu hastalığı önlemede veya yönetmede etkili olabilecek yeni ajanların bulunması gerekmektedir. Gossypin'in tümör büyümesini engellediği gösterilmiştir. Çalışmamızda MCF-7 hücrelerinde hem antikanser aktivitesi hem de apoptoz üzerine gossypin'in etkilerinin incelenmesi hedeflenmiştir.

Materyal ve Metot: MCF-7 hücreleri, 24, 48 ve 72 saat boyunca farklı dozlarda gossypin ve 50 uM sisplatin ile muamele edildi. Apoptoz veya nekroptoz kaynaklı hücre ölümünü değerlendirmek için, gossypin ile tedavi edilen MCF-7 hücrelerinin MTT analizi, Caspase-3, Caspase-9 ve NF-κB mRNA ekspresyonları da yapıldı.

Bulgular: MTT deneylerinde 100 µM dozda gossypin uygulamasının rutin sisplatin uygulamasına benzer etkiler gösterdiği, Hoechst boyaması ve morfolojisi değerlendirmelerinde hücre proliferasyonunda anlamlı azalmaya ve apoptoz artışına neden olduğu gözlemlendi. Gossypin'in CASP-3 ve CASP-9 mRNA ekspresyonunu azalttığı ve NF-κB ekspresyonunu arttırdığı ileri sürülmüştür.

Sonuç: Sonuçlarımız meme kanseri hücreleri için 100 µM gossypinin apoptozla bağlı hücre ölüm yollarını olumlu etkilediğini göstermektedir.

Anahtar Kelimeler: Gossypin, apoptosis, Caspase-3, Caspase-9, NF-κB

INTRODUCTION

Breast cancer is one of the most common malignant diseases in women and its emergence is increasing rapidly due to the stress of modern life (1). Because surgical

resection, radiation therapy and chemotherapy are limited options for breast cancer treatment, there is a need to find new chemotherapeutic agents that can be effective in preventing or managing breast cancer pathology (2).

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For the treatment of cancer and some diseases, natural products that have antioxidant, antimutagenic, and anticarcinogenic properties are utilized as an important resource regarding the development of modern drugs (3,4). When these compounds' properties are considered regarding their low toxicity, fewer side effects and high efficiency as chemopreventive and chemotherapeutic agents in cancer, they are widely used in cancer treatment (5). Gossypin has been isolated from plants of the Malvaceae family, in particular *Hibiscus vitifolius*. It has been reported to suppress the β amyloid-induced toxicity through its strong antioxidant activity, analgesic, and anti-inflammatory activities (6,7). Recently, it has been reported that gossypin is a potential anticarcinogenic agent and inhibits cell proliferation and tumor progression in some tumor cells (8). Nuclear factor KB (NF-kB) is both inducible for genes that are involved in cell viability, inflammation, adhesion, growth and differentiation, and plays a role in the regulation of critical genes in the early and late stages of cancers. Additionally, apoptosis suppressor proteins, genes required for metastasis, and angiogenesis are regulated by NF-kB (9). In a recent study, it was shown that gossypin induces apoptosis of cancer cells through inhibition of Aurora kinase A and p90 ribosomal S6 kinase-2 proteins in HGC27 and AGS cancer cells. Inhibition of these proteins resulted in the induction of cleavage of caspase-3, caspase-9 and PARP and induced cytochrome c expression (10). Even though gossypin has been demonstrated to inhibit various stages of tumor growth in recent studies, its molecular mechanism regarding anticancer activity and apoptosis has not been fully defined. In our study, it has been targeted to examine in MCF-7 cells whether there is an apoptotic, anticarcinogenic, and proliferative difference or not between different doses of cisplatin, which is utilized as a routine chemotherapeutic in cancer, and gossypin, a natural bioflavonoid. This study happens to be the first research report in the literature that examines the anticancer activity of gossypin on MCF-7 cells.

MATERIAL AND METHOD

MCF-7 Breast Cancer Cell Proliferation and Viability Analysis

The MCF-7 cell line was obtained from ATCC (USA). Cell lines stored in Cyrotube at -80°C were thawed by incubating in a 37°C water bath. Dissolved cells were taken into T75 cm² flasks. Cells were counted after 48 hours at 2×10^5 cells/well in DMEM medium containing 10% FBS. These cells were seeded in a 96-well plate and incubated at 37°C in 5% CO₂. After 24 h, cells were exposed to 50 μM cisplatin as a positive control with different concentrations of gossypin (5-100 $\mu\text{g}/\text{ml}$). Then, MTT method was performed to the cells for 24 h, 48 h and 72 h and measurements at 620 nm absorbance value were made with a spectrophotometer (Epoch Microplate Spectrophotometer, BioTek, USA) in 3 repetitions. Cell viability rates were analyzed by comparison with control wells.

Determination of Gene Expressions in MCF-7 Cell Lines

MCF-7 cells were seeded at 200,000/well in 6-well plates and then incubated at 37°C in a 5% CO₂ environment. After removing the MCF-7 cells from the plates, they were homogenized by trypsinization using a Tissue Lyser II (Qiagen) device (by adding 350 μl of RLT buffer to 1×10^5 cells). RNA extraction was maintained in the QIAcube RNA isolation device according to the instructions for use.

Performing Reverse Transcriptase Reaction and Obtaining cDNA Synthesis

cDNA was obtained from Total RNA using the High Capacity cDNA Reverse Transcription Kit. All reactions were performed with 10 μl of RNA. Veriti 96 Well Thermal Cycler (Applied Biosystem) device was used for cDNA synthesis. The amount of cDNA was determined by nanodrop spectrophotometry (EPOCH Take3 Plate, Biotek) and stored at -20°C .

Detection of mRNA Expressions using Real-Time PCR

The CASP-3 (Hs00234387_m1), CASP-9 (Hs00962278_m1), and NF-kB (Hs01042014_m1) mRNA expression was quantified through the utilization of the Taq Man Gene Expression Master Mix kit. The amplification and quantification process was conducted in StepOne Plus Real-Time PCR System (Applied Biosystems) device. As the reference gene, β -actin (Hs01060665_g1) was used. For the 200 ng cDNA, the TaqMan® Gene Expression Assays, which are represented as a table below, were pipetted again as demonstrated below, and 40 cycles were run. Ct values are automatically converted to $\Delta\Delta\text{CT}$. Statistical evaluation of our findings was made in IBM SPSS 20.0 package program.

Fluorescent Staining (Hoechst 33342)

With the help of trypsin, cells were removed from the 75 cm² flask and were counted. The calculation was made as there would be 5,000 cells in each well. The process of seeding was conducted on 96-well plates as there would be 3 repetitions for 24 hours. After 24 hours, gossypin and cisplatin were administered. Hoechst stain was prepared. Hoechst was prepared for application by calculating as 5 $\mu\text{g}/\text{ml}$ from the base stock. 48 hours after the application, the medium was removed from the wells and the wells were washed with PBS and Hoechst (5 $\mu\text{g}/\text{ml}$) dye (Thermo Fisher) was applied. It was incubated in the dark for 30 min. Visualized with a fluorescent microscope (Leica, DMIL LED).

Statistical Analysis

For statistical analysis, the results obtained using SPSS 20.0 software (IBM, USA) for all data were shown as mean \pm SD. Data analysis was conducted first through the utilization of a one-way analysis of variance (ANOVA), then through Dunnett's test. $p < 0.05$ was considered significant.

RESULTS

Anti-proliferative/cytotoxic effect of Gossypin on MCF-7 cells

The ability of Gossypin to inhibit MCF-7 cell proliferation at different concentrations was determined by MTT assay for 24, 48 and 72 hours. As represented in Figure 1, it has been observed that gossypin inhibits the cell viability of breast cancer cells according to the time and dose. In addition, it has been observed that 50 μM concentration of cisplatin (CISP), which is utilized as a routine chemotherapeutic, significantly reduces the cell activity in MCF-7 cells at 24, 48, and 72 hours. Excluding the gossypin's group of

concentration at 100 $\mu\text{g}/\text{ml}$, it was determined that the other dose groups did not have a significant effect on cell viability in MCF-7 cells (Figure 1). After 48 hours, on the other hand, decreases in cell viability were observed in concentrations at 25 $\mu\text{g}/\text{ml}$, 50 $\mu\text{g}/\text{ml}$, 75 $\mu\text{g}/\text{ml}$, and 100 $\mu\text{g}/\text{ml}$ (Figure 1). It has been observed that the optimal effect was in 72 hours of gossypin administration at 10 $\mu\text{g}/\text{ml}$, 25 $\mu\text{g}/\text{ml}$, 50 $\mu\text{g}/\text{ml}$, 75 $\mu\text{g}/\text{ml}$, and 100 $\mu\text{g}/\text{ml}$ concentrations (Figure 1). It was observed that after 48 and 72 hours, the gossypin dose group that was at 100 $\mu\text{g}/\text{ml}$ concentration demonstrated the same cell viability effect as that of CISP (Figure 1).

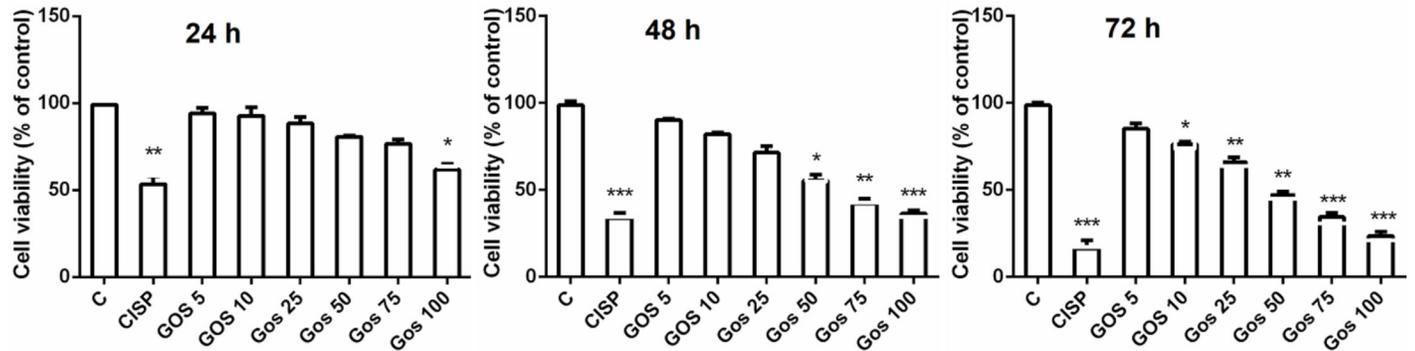


Figure 1. Effects of gossypin and cisplatin on the viability of MCF-7 cells. (a. 24 hours, b. 48 hours c. 72 hours). * MTT shows significant differences between experimental groups compared to control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$).

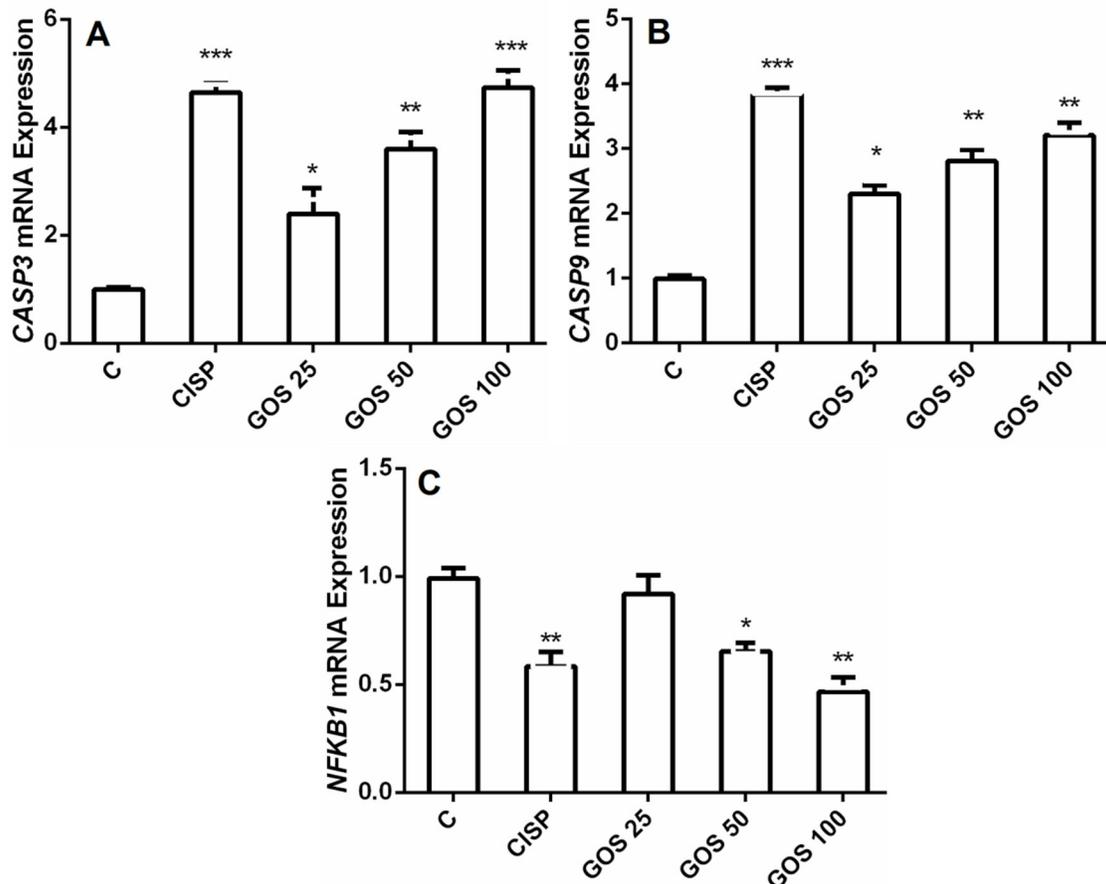


Figure 2. 24-hour CASP-3, CASP -9 and NF- κ B mRNA expression levels of all experimental groups. * shows significant differences between caspase-3, caspase-9 and NF- κ B mRNA expression levels compared to control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$).

Analyses results of mRNA expression via RT-PCR

It has been observed that in MCF-7 cells, CISP prompted a significant increase in the expression of CASP-3 and CASP-9 mRNA levels, whereas a significant decrease in the expression of NF- κ B mRNA level. At the same time, it has been determined that there were a significant increase in NF- κ B mRNA expression levels and a decrease in CASP-3 and CASP-9 mRNA expression levels due to the utilization especially at the concentration of 100 μ g/ml of gossypin, which is utilized as therapeutic, when compared to the control group ($p < 0.05$) (Figure 2).

Cell death analyzed via the Hoechst (33342) staining

Immunofluorescent Hoechst staining was used in order to determine the apoptotic level (Figure 3). No apoptotic nucleus or commonly stained area was observed in the control cell groups. The presence of apoptotic cell nuclei was observed in the evaluation of a comparison between the cells treated with 50 μ g/ml CISP and the control group. In therapeutic gossypin applications, apoptotic cell nuclei characterized by concentrated chromatin, nuclear shrinkage, and irregular fragmentation were determined in gossypin dose groups of 50 and 100 μ g/ml. This situation demonstrated that in MCF-7 cells, apoptosis was significantly induced in the 50 and 100 μ g/ml of gossypin dose groups (Figure 3).

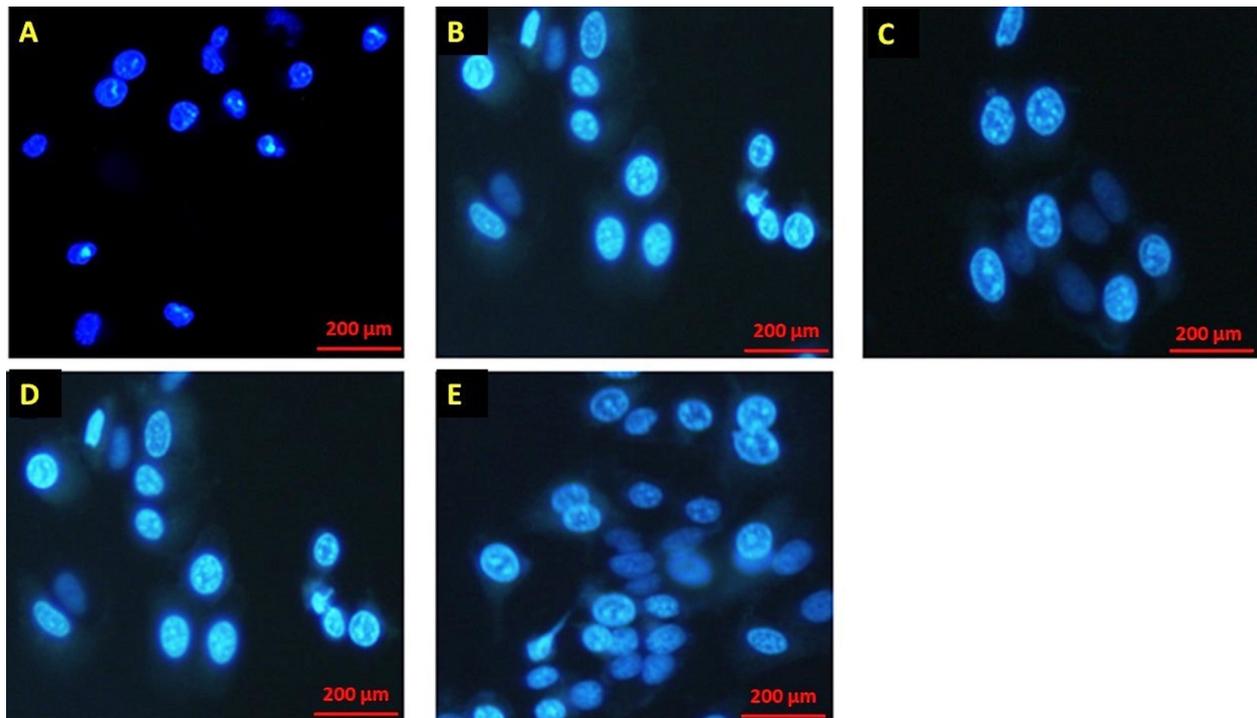


Figure 3. Detection of cell apoptosis and cell morphological changes with Hoechst 33342 staining of MCF-7 cells. Fluorescence photomicrographs of cells stained with Hoechst 33342 at magnification. (A) Control, B) Cisplatin 50 μ M C) Gossypin 25 μ g / ml D) Gossypin 50 μ g/ml, E) Gossypin 100 μ g / ml).

DISCUSSION

Breast cancer is cancer that is encountered most commonly in women, and its incidence worldwide is increasing by an average of 0.4% each year. This frequent increase of breast cancer and the economic burden it brings on society augments the search for a new, effective, and fruitful treatment procedure (11). It is known that available chemotherapeutic drugs restrict the growth of cancer and induce the apoptosis of the cancer cell. Yet, these drugs are not sensitive to some patients and cause negative side effects on healthy cells (12). For this reason, it is necessary to find an effective and non-toxic therapeutic agent from natural bioflavonoids in order to treat breast cancer. In our study, it has been

aimed to examine the effect of the natural compound gossypin, which is obtained from *Hibiscus vitifolius*, on MCF-7 breast cancer cells, and it is also the first authentic research article within this scope in the literature. Tumor cell formation is associated with reduced cellular apoptosis and uncontrolled cell proliferation. Therefore, the use of cytotoxic drugs that activate apoptotic pathways and inhibit cell proliferation is one of the most valid methods in cancer treatment (13). After the use of chemotherapeutic drugs, serious side effects such as drug resistance, kidney and liver damage, allergic reactions and gastrointestinal system disorders are encountered (14). Recently, researchers have conducted studies for the utilization of natural compounds due to the serious side effects of chemotherapeutics and determined that these

compounds demonstrate fewer side effects with high efficiency and low toxicity (5). Since natural compounds are known to be the main source of apoptosis-inducing agents, they are utilized in many studies in order to induce apoptosis in human cancer cells (15,16). Gossypin is conventionally used for the treatment of diabetes and is known to have anti-inflammatory, antioxidant, and anticancer activities (10). In a study conducted regarding the cancer cell lines, it was reported that gossypin has an antiproliferative effect (17). In recent cancer research, it was demonstrated that gossypin inhibits cell growth and cell migration (10). In this study, it has been targeted to assess the apoptotic, antiproliferative, and cytotoxic effects and activities of different doses of gossypin, a natural bioflavonoid, on breast cancer cells. In this context, gossypin was administered to MCF-7 cells on different concentrations, and the MTT viability test was assessed according to the dose and time. Gossypin has demonstrated an antiproliferative effect at concentrations of 25 µg/ml, 50 µg/ml, 75 µg/ml, and 100 µg/ml for 48 and 72 hours periods. When compared to cisplatin, a routinely used chemotherapeutic, the effect of gossypin, especially in the dose groups of 100 µg / ml, was found to be almost the same. Intracellular signaling pathways and secondary messengers that suppress the apoptosis mechanism are selected as targets for treatment in cancerous cells (18). Caspases affect the release of regulatory factors in the cell by participating in immunological functions, cell proliferation and migration. In addition, since the expression of caspases is associated with the frequency of metastasis of cancer cells, their use as a prognostic factor has become widespread (19). In order to prove the anti-cancer activities of natural compounds, the apoptotic properties and mechanisms of these compounds are assessed. It is defined as the form of controlled cell death, adjusted by the organism's own autonomic mechanism, and genetically programmed in the nucleic cells of the organism ever since the fetal development to adult tissue homeostasis (20). In mitochondria-mediated apoptosis, through the release of cytochrome c to the cytosol, the apoptosis complex consisting of cytochrome c/Apaf-1/ATP/procaspase-9 activates first caspase-9, and then caspase-3 (21). For this reason, the induction of apoptosis in cancer cells can easily be demonstrated by evaluating the protein expression of caspase enzymes or mRNA expression levels (22). Compared to the literature, it has been observed that a significant increase in CASP-3 and CASP-9 mRNA expression levels occurred in gossypin treated MCF-7 cells compared to the control group. At the same time, it has been detected that when compared to the cisplatin administration, the gossypin dose group of especially 100 µg/ml had the same effect as the cisplatin dose administration of 50 µg/ml. NF-κB is a protein complex that plays a role in the regulation of DNA transcription, initiates the transcription of cytokines and chemokines, and is known as an apoptosis inhibitor (23). Activation of the NF-κB protein can promote the growth of cancer cells in breast tissue by modulating the expression of proteins associated with the mechanism of

tumorigenesis (24). When considered within this scope, the suppression of NF-κB activity can induce apoptosis. In our study, it has been determined that compared to the control group, NF-κB expression was decreased significantly in the MCF-7 cells that were administered with especially 50 and 100 µg/ml of gossypin. When compared to cisplatin, which is a routine chemotherapeutic, it has been observed that the dose group at 100 µg/ml demonstrated the same effect as cisplatin. In accordance with our research findings, it has been observed that gossypin can suppress the apoptosis inhibitory activity of NF-κB via preventing translocation from the cytoplasm to the nucleus of MCF-7 cells. Our study findings suggest that gossypin can induce apoptosis in cancerous tissue by suppressing the NF-κB-related anti-apoptosis signaling pathway. Since the NF-κB activation is responsible for the transcription of cytokines and chemokines, we think that its effects on both inflammation and cancer cells may be stemming from the inhibition of gene expression that is regulated by NF-κB. On the other hand, in order to analyze the apoptosis, Hoechst staining was performed. In untreated cells, the cell wall remained intact and Hoechst dye could not pass through the cell membrane. Thus, the staining of the cells decreased. On the other hand, the cells treated with Gossypin showed that the cell walls of the cancer cells were damaged, so the Hoechst dye penetrated the cells and apoptosis of MCF-7 cells was observed. The results demonstrate that the high dose (100 µg/ml) of gossypin administration in cells leads to the gradual increase in the morphological change to reach the maximum. Additionally, the cell size and number were significantly reduced, thus it was clearly seen that gossypin caused cell separation and change in morphology.

CONCLUSION

In conclusion, we showed that gossypin inhibited cell growth and induced apoptosis in MCF-7 breast cancer cells. Among the tested doses of gossypine, the dose concentration at 100 µg/ml is the most potent inhibitor of MCF-7 cell growth, and apoptosis stimulant. It has been observed that gossypin's ability to inhibited MCF-7 breast cancer cell growth was in a relationship with the activation of CASP-3 and CASP-9 and inhibition of NF-κB activity. Our immunofluorescent staining results also support this situation. As a result, it has been shown that gossypin can demonstrate proliferation damage, anti-inflammatory, and anti-carcinogenic activity in MCF-7 cells. In this regard, we think that in breast cancer, gossypin can be utilized as an anti-carcinogenic natural compound for cancer patients, or at least it may be of benefit.

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Conflict of Interest: *The authors declare that they have no competing interest.*

Ethical approval: *Ethics committee approval is not required as it is a cell study.*

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