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ARAŞTIRMA / RESEARCH

Effect of *Melissa officinalis* L. on human breast cancer cell line via apoptosis and autophagy

Melissa officinalis L. bitkisinin apoptoz ve otofaji yoluyla insan meme kanser hücre hattı üzerine etkisi

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Abstract

Purpose: The aim of this research is to see how *Melissa officinalis* L. affects the death pathways of MCF-7 breast cancer cells in vitro.
Materials and Methods: In order to determine the cytotoxic activity and IC50 value of the 70% methanolic extract prepared from the aerial parts of *Melissa officinalis*, on the MCF-7 breast cancer cell line; the extract was applied at different concentrations and times and MTT analysis was performed. Apoptotic effects of *Melissa officinalis* on breast cancer cells at the determined IC50 dose were revealed by TUNEL method, and autophagic effects were revealed using Beclin-1 and LC3 immunofluorescence staining method.
Results: When the effects of *Melissa officinalis* on MCF-7 breast cancer cells were evaluated, it was observed that the cell viability of the MCF-7 breast cancer cell line was decreased in a dose- and time-dependent manner. In addition, it was observed that it induced apoptosis and increased the immunoreactivity of autophagy markers Beclin-1 and LC3.
Conclusion: Findings from the study showed that *Melissa officinalis* has a cytotoxic effect on MCF-7 breast cancer cells through both apoptosis and autophagy cell death pathways. These data will lead to further *in vitro* studies necessary to elucidate the antitumor mechanism of action.
Keywords:. MCF-7 cell line, *Melissa officinalis* L., apoptosis, autophagy

Öz

Amaç: Bu araştırmanın amacı, *Melissa officinalis* L. bitkisinin *in vitro* olarak MCF-7 meme kanseri hücrelerindeki ölüm yollarını nasıl etkilediğini görmektir.
Gereç ve Yöntem: *Melissa officinalis* bitkisinin topraküstü kısımlarından hazırlanan %70 metanolik ekstrenin sitotoksik aktivitesi ve IC50 değerini belirlemek için MCF-7 meme kanseri hücre hattı farklı konsantrasyonlar da ve sürelerde *Melissa officinalis*' e maruz bırakılarak MTT analizi yapıldı. *Melissa officinalis*' in belirlenen IC50 dozunda meme kanseri hücreleri üzerindeki apoptotik etkileri TUNEL yöntemi ile, otofajik etkileri Beclin-1 ve LC3 immünofloresan boyama yöntemi kullanılarak ortaya çıkarıldı.
Bulgular: *Melissa officinalis*' in, MCF-7 meme kanseri hücreleri üzerindeki etkileri değerlendirildiğinde, MCF-7 meme kanseri hücre hattındaki hücre canlılığını doza ve zamana bağlı bir şekilde azalttığı görüldü. Bunun yanı sıra, apopitozu indüklediği ve otofaji belirteçleri olan Beclin-1 ve LC3 immunreaktivitesini de artırdığı gözlendi.
Sonuç: Çalışmadan elde edilen bulgular, *Melissa officinalis*'in hem apoptoz hem de otofaji hücre ölümü yolları üzerinden MCF-7 meme kanseri hücreleri üzerinde sitotoksik bir etkiye sahip olduğunu göstermiştir. Bu veriler, antitümör etki mekanizmasını aydınlatmak için gerekli olan daha ileri *in vitro* çalışmalara öncülük edecektir.
Anahtar kelimeler: MCF-7, *Melissa officinalis* L., apoptoz, otofaji

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INTRODUCTION

Breast cancer is an important type of cancer with high malignancy and mortality in women[1]. Although surgical resection, chemotherapy and radiotherapy methods are used to treat breast cancer, the mortality rate is still high today[2].

Since breast cancer is a very heterogeneous group of diseases with various morphological, histopathological and prognostic features, it responds to treatments in different ways[3]. Understanding the characteristics of breast cancer and developing specific drugs to improve treatment response is critical for the disease's prognosis[4].

MCF-7 is a breast cancer cell line frequently used in preclinical laboratory experiments[5]. MCF-7 cells are widely used in estrogen receptor (ER) positive breast cancer cell assays and research where many subclones represent different ER classes and represent different (ER+) tumor classes with varying nuclear receptor expression levels[6].

Melissa officinalis L. (Lemon balm) is a member of the Lamiaceae family with a characteristic lemon-like fresh odor. It's a perennial herb that grows wild and/or cultivated in the Mediterranean countries, including Turkey. *Melissa officinalis* is a traditional medicinal plant comprising various pharmacological effects to heal gastrointestinal system disorders, nervous system disorders, cardiovascular diseases and asthma[7]. Essential oil and different classes of phenolic compounds, including flavonoids and phenolic acids, have been reported from *Melissa officinalis*[8].

Aside from the existence of a variety of natural chemicals, effective cancer therapy is still a work in progress. Furthermore, devising a successful treatment requires an understanding of the interactions of such natural compounds with their recognized biological targets.

Melissa officinalis has been shown to have an inhibitory effect on a variety of cancer cells, including breast cancer cells, in both *in vivo* and *in vitro* experiments[9-12]. While the intracellular targets of *Melissa officinalis*, which have significant anti-tumor activity, are believed to have different molecular pathways, the exact mechanisms of this activity are not clear[9]. The hypothesis of this study is to show how *Melissa officinalis* influences death pathways on MCF-7 breast cancer cells in vitro.

Beclin-1 is one of the regulatory proteins of autophagosomal membrane formation and plays a critical role in the initiation of autophagy[13]. Microtubule-associated protein light chain 3 (LC3), located in autophagosome membranes, is very important for autophagosome formation and function[14]. Targeting autophagy and apoptosis with natural products is considered a possible therapeutic method for the prevention and treatment of a variety of diseases. Several studies show that the anti-cancer effects of *Melissa officinalis* are related to its ability to induce apoptosis in cancer cells[9,10,15,16]. Along with these studies showing the apoptosis-inducing effects of *Melissa officinalis*, studies investigating the effects of *Melissa officinalis* on different apoptotic pathways are continuing to determine its molecular targets. The aim of this study was to learn more about the putative molecular effects of *Melissa officinalis* on the mechanisms of apoptosis and autophagy in the MCF-7 breast cancer cell line, in addition to what was already known from the literature.

MATERIALS AND METHODS

This study was carried out at Erciyes University Betül-Ziya Eren Genome and Stem Cell Center. Since the study did not include experimental animals or human patients, there is no need for an ethics permission.

Cell culture

For the culture of the MCF-7 cell line, 10% fetal bovine serum (FBS), 1% L-glutamine and 1% Penicillin-Streptomycin were added to Dulbecco's Modified Eagle's Medium (DMEM). Cells inoculated in fresh medium in 25 cm^2 flasks were placed in an incubator at 37 °C with 5% partial CO_2 pressure and a humid environment. Cells were cultured at regular intervals until sufficient numbers were reached.

Preparation of *Melissa officinalis*

The *Melissa officinalis* extract was provided by Dr. Perihan Gürbüz. The extraction procedure and the detailed phytochemical characterization of the extract were reported in a previous study[8].

The lyophilized 70% methanolic extract of *Melissa officinalis* was used in the current study. *Melissa officinalis* was dissolved in Dimethyl Sulphoxyde (DMSO) and a 25 mg/5mL stock *Melissa officinalis* solution was prepared, then filtered and sterilized.

Then, different concentrations of *Melissa officinalis* solutions were prepared by diluting the medium to determine the dose.

MTT cell viability assay

The Methylthiazole diphenyl tetrazolium (MTT) test is frequently used in cell culture drug application studies to determine cytotoxic or proliferative effects[17].

MTT cell viability test was applied to determine the IC50 dose of *Melissa officinalis*. For this purpose, 5 μg, 10 μg, 20 μg, 40 μg, 80 μg, 100 μg and 250 μg/mL doses were selected in accordance with the doses in the literature in the first MTT trial. In addition, analyses were performed for 24 and 72 hours to observe the time-dependent change in dose administration. Since the IC50 value in cell viability could not be determined exactly after the first trial, the test was repeated by changing the dose range. For this purpose, doses of 5 μg/mL - 50 μg/mL were applied to the cells for 24 hours. To begin, a suitable quantity of cells ($5x10^3$ cells/well) were seeded into a 96-well plate. The next day, freshly prepared *Melissa officinalis* solutions at the determined doses were prepared by diluting them with the medium. Three different wells were applied for each dose. No drug was administered to the control group. Then, the cells were cultured for 24 and 72 hours in 37 °C, 5% CO_2 incubator. 10 μL of MTT solution was added to the cells in the relevant plate at the end of the stipulated time and incubated for 4 hours at 37 °C, in a 5% CO_2.

DMSO was added to the cells in all wells to dissolve the formed formazan crystals. The absorbance values of the solutions on the plates were measured in a spectrophotometer at a wavelength of 570 nm at the conclusion of the period. In light of the data obtained, the study groups were formed after determining the dose (IC50) at which cell viability was inhibited by 50% for each group.

Control group: Cell line group incubated only with the medium without any drug application,

Melissa officinalis 1 group (IC50): The group in which the IC50 dose of Melissa officinalis is applied to the cells,

Melissa officinalis 2 group (IC50/2): The group in which half of the IC50 dose of Melissa officinalis was applied to the cells,

D 1 group: The group in which the DMSO ratio used in the preparation of Melissa officinalis is applied to the cells in the Melissa officinalis 1 group

D 2 group: In the Melissa officinalis 2 group, the DMSO ratio used in the preparation of Melissa officinalis is applied to the cells.

After the groups were formed, 2 mL of cells were seeded at $5x10^4$ cells/well on 12-well plates to determine antibody expression, and *Melissa officinalis* and DMSO were added at the determined doses for a 24-hour application. After the time period, the cells were fixed in a 10% formaldehyde solution after washing with Dulbecco's phosphate-buffered saline (DPBS).

TUNEL Method

The TUNEL (terminal deoxynucleotide transferase-mediated20-deoxyuridine 50-triphosphate nick end-labeling) method was used to determine apoptosis. The ApopTag® Fluorescein In Situ Apoptosis Detection Kit (S7110, EMD Millipore, Darmstadt, Germany) was studied on cell-inoculated coverslips according to the manufacturer's instructions. Phosphate Buffered Saline (PBS) was used to wash the coverslips. Then equilibration buffer for 5 minutes. It was kept waiting and then the TUNEL mixture was applied without washing and incubated for 1 hour. At the end of the period, a stop/wash buffer was added to the coverslips and kept at 37 ^{0}C. Then washing is done and 30 minutes in the anti-digoxigenin conjugate solution at room temperature. Nuclear staining was performed by treating the coverslips with DAPI by washing them again. Afterward, the coverslips were covered on the slide with a water-based sealer by washing them again. Slides were evaluated under an Olympus BX51 fluorescence microscope (Olympus BX51, Tokyo, Japan). After randomly selecting images from ten microscopic fields in each group at 400x original magnification to detect apoptosis, the immunoreactivity intensities of TUNEL positive cells were measured using Image J software (ImageJ, Bethesda, USA).

Immunofluorescent staining method

The immunofluorescence staining method was applied to determine the expression of Beclin-1 and LC-3 in the cell line. The prepared coverslips were washed with PBS and incubated with goat serum for 60 min. incubated. Then, the cells were incubated

with Beclin-1 (NB500-249, Novus Biologicals, USA) and LC-3 (12741, Cell Signaling, USA) antibodies separately for 1 night at $+4^0$C. It was cleaned twice with PBS the next day. The cells were then treated at room temperature with secondary antibodies. Then, nuclei staining was performed by applying 4' 6'-diamino-2-phe-nylindole (DAPI) to the cells washed with PBS. After the cells were washed with PBS again, they were covered with an occlusive medium. Slides were examined under an Olympus BX51 fluorescence microscope (Olympus BX51, Tokyo, Japan). Images were taken from ten unique microscopic fields for each group at 400x original magnification and analyzed with Image J software to measure the intensity of Beclin-1 and LC-3 immunoreactivity (ImageJ, Bethesda, USA).

Statistical analysis

The GraphPad Prism Version 8.0 statistical software tool was used for statistical analyses. The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to assess the data's compliance with the normal distribution. The MTT variables for 24 and 72 hour were evaluated by the One Sample t test. Kruskal Wallis test was used for those with no normal distribution in multiple comparisons for apoptosis and autophagy variables. For the non-normal distribution variables, comparisons between groups were made by Kruskal-Wallis analysis; if there was a difference, multiple comparisons were made using the Dunn's test. The significance level was accepted as $p<0.05$.

RESULTS

The Cell viability was determined using to MTT analysis. First, different doses of *Melissa officinalis* (5 μg, 10 μg, 20 μg, 40 μg, 80 μg, 100 μg and 250 μg/mL) were applied to the cells and cell viability was measured at 24 and 72 hour intervals (Figure 1A, 1B). Then, starting from the first data, doses of 5 μg, 10 μg, 20 μg, 40 μg, and 50 μg/mL were applied to MCF-7 cells for 24 hours. According to the results of the MTT analysis, the IC50 dose at the 24th hour was determined as 20 μg/mL. It was observed that the cell viability of MCF-7 cell groups treated with *Melissa officinalis* decreased at the end of 24 hours due to the dose increase compared to the control group. This research showed that *Melissa officinalis* has a negative effect on cell viability and proliferation inhibitory activity. As a result, MCF-7 cells treated with *Melissa officinalis* showed that 24-hour application may be sufficient for a possible change in the autophagy mechanism while going into apoptosis.

The possible effect of applying different doses of *Melissa officinalis* to the MCF-7 cell line on apoptosis was evaluated by the TUNEL method. TUNEL-positive apoptotic bodies were observed as green reflections in the fluorescent microscope (Figure 2).

At the 24th hour after *Melissa officinalis* application, a significant increase was observed in the IC50 group compared to the control group ($p<0.001$). Similarly, there was a statistically significant difference between the

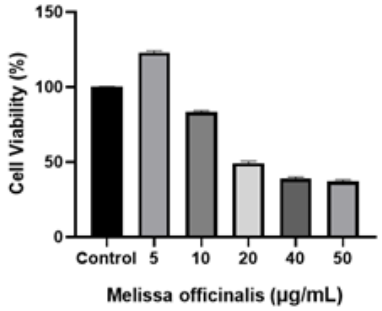

Figure 1. A. 24-hour cytotoxicity of *Melissa officinalis* on MCF-7 cells by MTT assay.

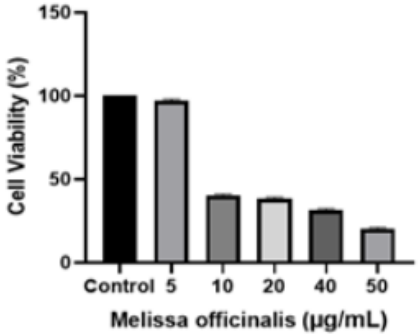

Figure 1. B. 72-hour cytotoxicity of Melissa officinalis on MCF-7 cells by MTT assay.

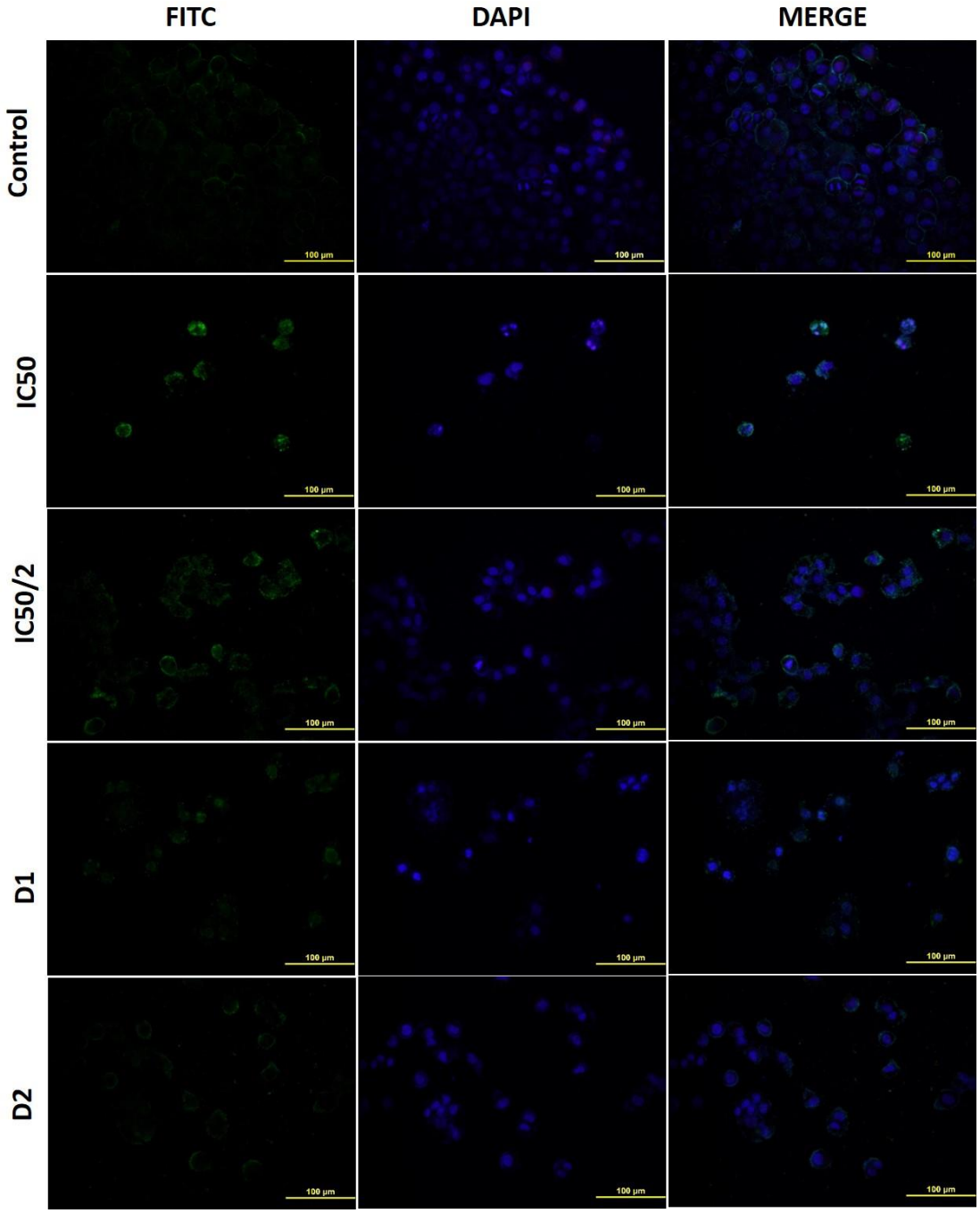

Figure 2. 24th hour TUNEL images of MCF-7 breast cancer cell line treated with *Melissa officinalis*. Apoptotic bodies formed in the cells are seen as green reflections in the fluorescent microscope (x40).

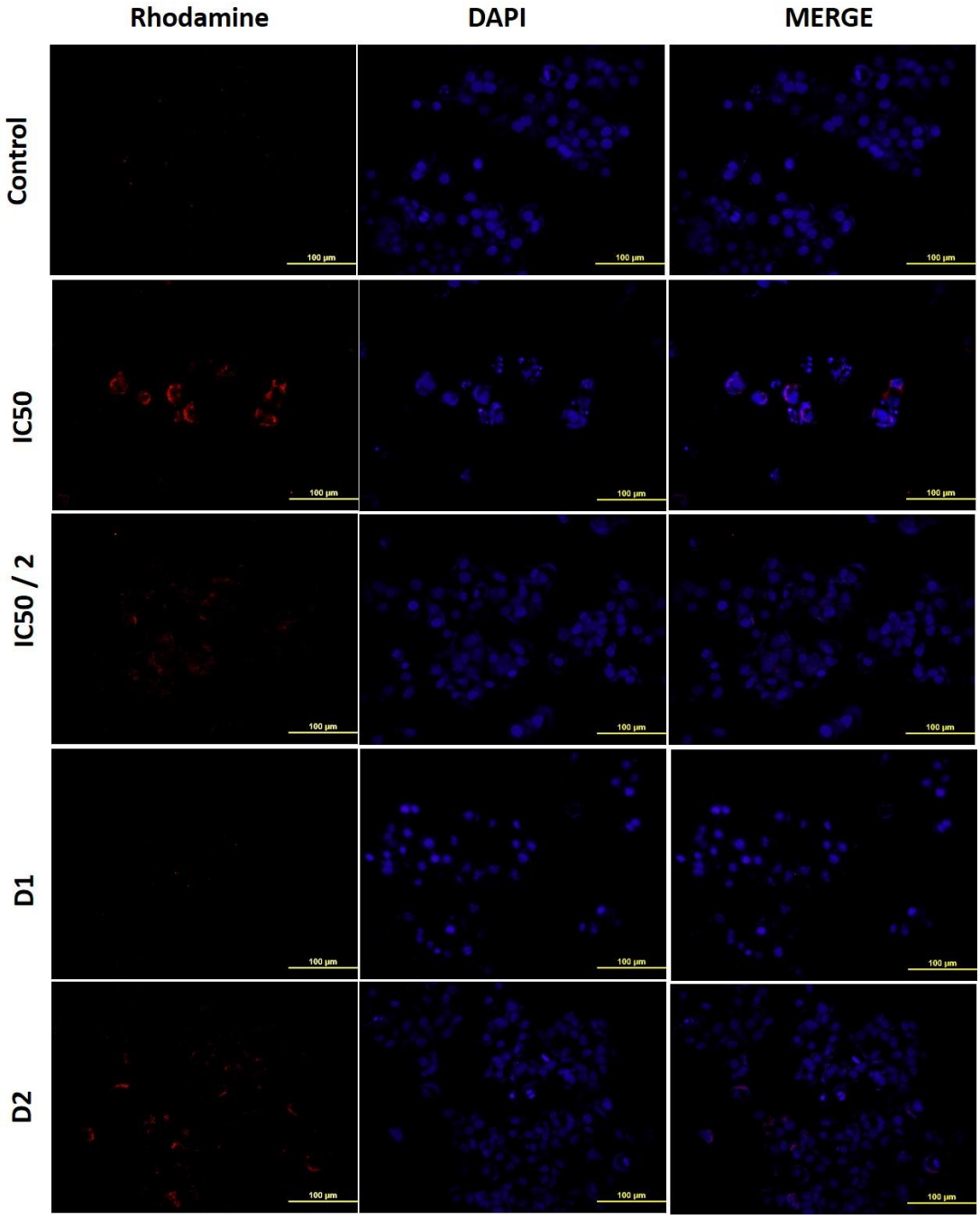

Figure 3. 24th hour Beclin-1 expressions of MCF-7 breast cancer cell line treated with *Melissa officinalis*. Beclin-1 is seen as red reflections in the cell (x40).

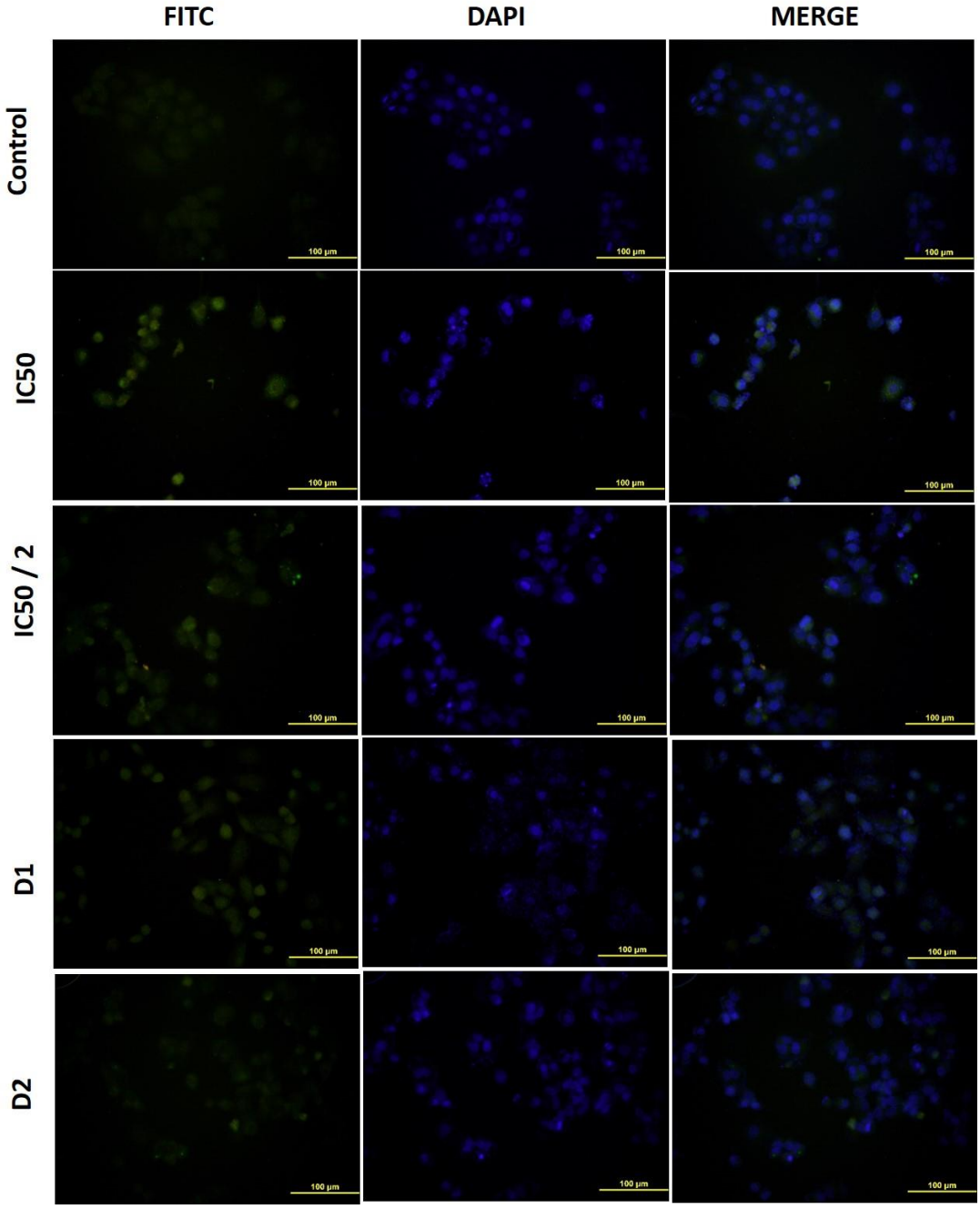

Figure 4. 24th hour LC3 expressions of MCF-7 breast cancer cell line treated with *Melissa officinalis.* LC3 expression is seen in the fluorescent microscope as green fluorescent reflections (x40).

IC50 group and the D1 group (p<0.001). The number of TUNEL+ apoptotic cells in the IC50 group was statistically significantly higher than in the IC50/2 group (p<0.001) (Table 1).

When the immunoreactivity of both Beclin-1 and LC3 autophagy markers was evaluated in the effect of *Melissa officinalis* on the MCF-7 breast cancer cell line, it was observed that the highest immunoreactivity was in the group in which the IC50 dose was applied. Beclin-1 expression was evaluated in the cell cytoplasm by red reflections under a fluorescent microscope (Figure 3). It was observed that the intensity of Beclin-1 immunoreactivity of the IC50 group was significantly different from both the control group and the D1 group (p<0.001). The difference between the control group and the IC50/2 group, on the other hand, was found to be insignificant (p>0.01) (Table 2).

LC3 expression was observed in the fluorescent microscope as diffuse green fluorescent reflections in the cell cytoplasm (Figure 4). When the immunoreactivity intensity of LC3 was examined between the IC50 group and the control group, there was a statistically significant difference (p<0.01). It was determined that there was an increase in LC3 expression in the IC50 group compared to the IC50/2 group, but this increase was not statistically significant (p>0.001) (Table 2).

Table 1. Apoptosis statistical analysis results at 24 hours of MCF-7 breast cancer cell line treated with *Melissa officinalis*

MCF-7	Groups					
	Control	IC50	IC50/2	D1	D2	p
Apoptosis	1.3 (1.2-1.6)^{a}	3.5 (2.9-8.0)^{b}	1.17 (0.9-1.5)^{ac}	1.3 (1.1-1.6)^{a}	0.8 (0.6-0.9)^{c}	<0.0001

Data are expressed as median (1st quartile-3rd quartile). p: indicates the significance of the difference between groups. The same lowercase letters on the same line indicate similarity between groups, and different letters indicate difference (a, b, c). IC50: Cytotoxic dose; D: DMSO ratio.

Table 2. 24-hour statistical analysis results of autophagy markers of MCF-7 breast cancer cell line treated with *Melissa officinalis.*

MCF-7	Groups					
	Control	IC50	IC50/2	D1	D2	p
Beclin-1	0.4 (0.2-0.5)^{a}	1.6 (1.3-1.9)^{b}	0.3 (0.2-0.7)^{a}	0.3 (0.1-0.8)^{a}	0.4 (0.2-0.6)^{a}	<0.05
LC3	3.4 (2.5-4.1)^{a}	4.4 (4.1-4.5)^{bc}	3.6 (2.4-4.2)^{ac}	3.2 (2.9-3.7)^{a}	3.1 (2.8-3.5)^{a}	<0.05

Data are expressed as median (1st quartile-3rd quartile). p: indicates the significance of the difference between groups. The same lowercase letters on the same line indicate similarity between groups, and different letters indicate difference (a, b, c). IC50: Cytotoxic dose; D: DMSO ratio.

DISCUSSION

The necessity for an effective medicine in the treatment of breast cancer is evident, given the high morbidity and mortality rates, and the discovery and development of less toxic and more effective chemotherapeutic drugs is critical for a prospective therapy approach in breast cancer[18,19].

Today, a cancer diagnosis may be delayed and surgery and chemotherapy may be insufficient in its treatment. For this reason, studies have been continuing in recent years to include supportive factors or agents for classical treatment among the clinical applications. The use of traditional and medicinal plants is one of them, and it is a common situation among people. However, when it is done without determining the effectiveness of the plants in

the studies, it can have a negative effect on the patient. For this reason, the therapeutic efficacy of the plant or product to be used must be proven experimentally. In our study, the anticarcinogenic effect of *Melissa officinalis*, which is consumed as a herbal tea or standard extract for its medicinal properties, has preserved its importance throughout history and is still used as a folk remedy in the treatment of many diseases, on breast cancer cell lines was investigated.

Both *in vivo* and *in vitro* investigations have shown the inhibitory effect of *Melissa officinalis* on various cancer cells, including breast [9-12]. In their studies with breast cancer cell lines, Saraydn SU et al. looked at apoptosis with annexin V staining and flow cytometry analysis and stated that *Melissa officinalis* has antitumor activity[9]. Weidner, C et al. To determine the apoptotic effect of *Melissa officinalis* on the colon cancer cell line, they evaluated the activity of caspase 3 and 7 by immunoblotting and fluorescence microscopy and showed that they induced apoptosis by inhibiting proliferation[10]. Enclada, MA et al. also demonstrated the antiproliferative effect of *Melissa officinalis* in colon cancer cells by MTT analysis [11]. Jahanban-Esfahlan A et al. also demonstrated the antiproliferative effect of *Melissa officinalis* on lung, breast, ovarian and prostate cancer cell lines by MTT analysis[12]. While the intracellular targets of *Melissa officinalis*, which have significant anti-tumor activity, are believed to have different molecular pathways, the exact mechanisms of this activity are not clear[9]. Natural products that target autophagy and apoptosis are being evaluated as a potential therapeutic strategy for the prevention and treatment of a variety of diseases. Several studies show that the anti-cancer effects of *Melissa officinalis* are related to its ability to induce apoptosis in cancer cells[9,10,15,16]. Along with these studies showing the apoptosis-inducing effects of *Melissa officinalis*, studies investigating the effects of *Melissa officinalis* on different apoptotic pathways are continuing to determine its molecular targets. However, no study has been found showing cell death through the autophagy pathway of *Melissa officinalis*. This study aims to look into the probable molecular effects of *Melissa officinalis* on the mechanisms of apoptosis and autophagy in the MCF-7 breast cancer cell line, in addition to what was already known from the literature. The possible effect of *Melissa officinalis* on apoptosis (TUNEL staining) and autophagy mechanisms (Beclin-1 and LC3) is discussed. In this study, which we modeled on the MCF-7 breast cancer cell line, the effect of 70% methanolic extract of *Melissa officinalis* on cells at 24, 48 and 72 hours were evaluated. It was observed that there was a significant decrease in cell viability depending on the dose increase per hour. In addition, it was observed that the expression of the autophagy markers Beclin-1 and LC3 increased. In breast cancer cells, our findings show a bidirectional link between the apoptotic and autophagy pathways. These findings show that apoptosis and autophagy mechanisms are activated together after *Melissa officinalis* application in the MCF-7 breast cancer cell line.

Several studies have been undertaken to learn more about the composition of this plant in order to better understand its anticancer action. *Melissa officinalis* comprises phenolic compounds such as caffeic acid derivatives (mainly rosmarinic acid), flavonoids, and essential oils[20]. More recently, molecular processes have been studied all pointing to lemon balm's great potential as a useful source of phytocompound-rich extracts with anti-cancer effects[21]. *Melissa officinalis* extracts have anticancer potential against breast tumors in the cell culture line due to the presence of phenolic acids[10]. Rosmarinic acid is one of the main components of *Melissa officinalis* and may interfere with aromatase activity and thus estrogen synthesis[22]. Regarding rosmarinic acid, which was tested as a pure compound, it was stated in a study that it increased the cell viability of breast cancer cells, especially estrogen-dependent cells (MCF-7)[23]. Another phenolic component, Salvianolic Acid B, was reported to effectively inhibit the growth of human breast cancer MCF-7 cells *in vitro* in a concentration- and time-dependent manner, according to the results of the MTT assay[24]. It has been stated that Salvianolic Acid B can exhibit better chemoprotective activity by showing pro-oxidative and pro-apoptotic properties as well as antiproliferative properties[25].

According to the results, *Melissa officinalis* 70% methanolic extract which was chemically characterized in a previous study (8) and found to be rich in rosmarinic acid and salvianolic acids, had a cytotoxic impact by killing MCF-7 breast cancer cells by both apoptosis and autophagy. The study's limitation is the inability to compare the effects of *Melissa officinalis* with other extracts and on different breast cancer cell lines.

In conclusion, further studies are needed to elucidate the antitumor action mechanism of *Melissa officinalis* due to its current components and multi-targeted mechanism of action.

Yazar Katkıları: Çalışma konsepti/Tasarımı: NB, MB, GÖÖ, PAS, AY; Veri toplama: NB, MB, GÖÖ, PAS, PG, AY; Veri analizi ve yorumlama: NB, MB, GÖÖ, PAS, PG, AY; Yazı taslağı: NB; İçeriğin eleştirel incelenmesi: NB, MB, GÖÖ, PG, AY; Son onay ve sorumluluk: NB, MB, GÖÖ, PAS, PG, AY; Teknik ve malzeme desteği: NB, AY, PG; Süpervizyon: NB, MB, GÖÖ; Fon sağlama (mevcut ise): yok.
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