

The Effect of *Lysimachia sarrani* on the Breast Cancer Cells in Cell Culture Conditions Compared to Normal Cells

Lysimachia Sarranii'nin Hücre Kültürü Ortamında Normal Hücrelere Göre Meme Kanseri Hücrelerine Etkisi

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Abstract: The breast cancer has become a threat to women's lives at an increasing rate. The inadequate treatments of surgical procedures and chemotherapy affect negatively the quality of patient life. Therefore, there has been an increase in demand for alternative and supportive agents, especially for the use of herbal products. The purpose of this study is to evaluate the *in vitro* anti-tumor activity of *Lysimachia sarranii* in breast cancer cell lines compared to normal adipose tissue derived mesenchymal stem cells. The adipose tissue derived mesenchymal stem cells as normal cells with MCF-7 and, MDA-MB-231 breast cancer cells were cultured. IC₅₀ doses of *Lysimachia sarranii* extract for each cells were determined via 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. The immunocytochemical staining was performed for the oxidative stress by eNOS, whereas apoptosis were analyzed by TUNEL method. The data exhibited with H-score and apoptotic index. The plant extract inhibited the proliferation and survival in the breast cancer cells compared to the stem cells. *Lysimachia sarranii* extract caused the increase of eNOS staining for oxidative stress and the TUNEL labeling for apoptosis. The further molecular techniques should be used to determine the biologic activity of *Lysimachia sarranii* extract and its content should be investigated.

Keywords: *Lysimachia sarranii*, breast cancer, proliferation, cytotoxicity.

Öz: Meme kanseri, giderek artan oranda kadınların yaşamları için bir tehdit haline gelmiştir. Yetersiz cerrahi işlemler ve kemoterapi tedavileri hasta yaşam kalitesini olumsuz yönde etkilemektedir. Bu nedenle, özellikle bitkisel ürünlerin kullanımı için alternatif ve destekleyici ajanlara olan talep artmıştır. Bu çalışmanın amacı, meme kanseri hücre dizinlerinde *Lysimachia sarranii*'nin *in vitro* anti-tümör aktivitesini, normal yağ doku kaynaklı mezenkimal kök hücrelerle karşılaştırarak araştırmaktır. Yağ dokusu kaynaklı mezenkimal kök hücreler, MCF-7 ve MDA-MB-231 meme kanseri hücreleri, *in vitro* ortamda kültüre edildi. Her bir hücre için *Lysimachia sarranii*'nin IC₅₀ dozları MTT metodu ile belirlendi. Oksidatif stres için eNOS ile immünohistokimyasal boyama yapılırken, apoptosis TUNEL metodu ile analiz edildi. Veriler H-skor ve apoptotik indeks ile gösterildi. Bitki ekstraktının, meme kanseri hücrelerinde, proliferasyon ve canlılığı, kök hücrelere kıyasla inhibe ettiği görüldü. *Lysimachia sarranii* ekstraktı, oksidatif stres için eNOS ve apoptoz için TUNEL boyanmasında artışa neden oldu. *Lysimachia sarranii* ekstraktının biyolojik aktivitesini belirlemek için başka moleküler teknikler kullanılmalı ve içeriği araştırılmalıdır.

Anahtar Kelimeler: *Lysimachia sarranii*, meme kanseri, proliferasyon, sitotoksosite.

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Introduction

In the health area, the cancer is the second most common cause of death after cardiovascular disease. Especially in women, breast cancer is a

major health problem because of its high mortality and morbidity. Therefore, intensive research is needed to treat this type of cancer (Torre et al.,

2016, DeSantis et al., 2016). With the increase in breast cancer cases in recent years, the status of metastasis negatively affects the treatment. It is reported that the risk of metastasis is higher in breast cancer, especially estrogen receptor independent. Because of metastasis, cancer treatment may be inadequate, and therefore there is a tendency to seek alternative or complementary treatment options. The herbal products are one of alternative or complementary treatment agents. *In vitro* and *in vivo* studies have shown that many chemotherapeutic agents and herbal products are used for their cytotoxic effect and antitumorigenic properties on breast cancer (Hack et al., 2015, Kim et al., 2015, Zheng et al., 2016, Tariq et al., 2017).

The reactive oxygen species (ROS) play a role in the metabolic events of the cells, but the accumulation of ROS causes cell death by oxidative stress (Trachootham et al., 2009). In the cancer treatment, the aim is to trigger cell death of tumor cells without damaging healthy cells. The apoptosis is desired because it is programmed cell death (Sumalatha et al., 2017).

Lysimachia savranii is a new species belonging to the Primulaceae family and the biological effects of this specie have not been studied. The other species of Primulaceae, such as *Lysimachia capillipes*, *Lysimachia punctata*, *Lysimachia fortunei*, *Lysimachia parvifolia*, *Lysimachia nummularia* and *Lysimachia clethroides* have been reported to have analgesic, antibacterial, antiinflammatory and cytotoxic effects (Podolak et al., 2005, Podolak et al., 2013, He et al., 2013, Liang et al., 2013, Fei et al., 2014). In the current experiment, we aimed to define the cytotoxic effect of *Lysimachia savranii* extract on adipose tissue derived mesenchymal stem cells (ADSCs), MCF-7 and MDA-MB-231 breast cancer cell lines by oxidative stress and cell death.

Material and Methods

Preparation of Plant Extract

Lysimachia savranii was gathered in term of July 2013 from the Bolkar Mountains within the boundaries of Niğde province. Its taxonomic classification was made by biologist Ahmet Savran.

In the current study, the leaf parts of the plant were used for extraction. The extraction method was performed according to the previously described protocol (Atila et al., 2019). Briefly, the dried plant materials were powdered using electric blender. Twenty gram (20.0 g) of *Lysimachia savranii* powdered samples were extracted with 500 ml of solvents (95% ethanol) for 3 h by using Soxhlet apparatus. The extract were concentrated using a rotary evaporator at 40 °C.

Cell Culture

The breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from DSMZ- German Collection of Microorganisms and Cell Cultures, Germany. As control group, adipose tissue derived mesenchymal stem cells (ADSC), in a previous study (Ethics approval number: 17/09/2014/77.637.435-32, Manisa Celal Bayar University, Animal Experiments Local Ethics Committee) frozen, were used. MCF-7 and MDA-MB-231 were grown in RPMI-1640 (F1213, Biochrom, Berlin Germany) containing 10% fetal bovine serum (S0113, Biochrom, Berlin Germany), 200mM L-glutamin, 100UI/ml penicillin/streptomycin (A2213, Biochrom, Berlin, Germany), at 37°C in 5 % CO₂ in a humidified atmosphere. ADSCs at passage 3 were taken from -80°C and were dissolved at 37°C. Then the cells were cultured in alpha-MEM (F0915, Biochrom, Berlin, Germany), 10% fetal bovine serum µg/ml gentamisin (A2712, Biochrom, Berlin, Germany), 100UI/ml penicillin/streptomycin (A2213, Biochrom, Berlin, Germany), 100 UI/ml amphotericin B (A2612, Biochrom, Berlin, Germany) and 200 mM L-glutamin (K0282, Biochrom, Berlin, Germany). The medium was changed every two days.

Cytotoxicity Assay

To detect IC₅₀ of *Lysimachia savranii* extract for each cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, M5655, Sigma, Steinheim, Germany) assay was performed. All cells were put into 96-well (2.5x10⁵ cells/well) plates and seeded for 24 h. The cells were treated with an increasing concentration of plant extract

in ethanol (0, 0.1, 1, 10, 30, 100 µg/ml) for 24 hours. Then the media containing extract was removed and 100 µL of fresh medium and 10 µL MTT (5 mg/ml in distilled water) were added. After the incubation with MTT at 37°C for 4 h, media containing MTT was discharged, and 100 µL of dimethyl sulphoxide (DMSO, A3672, AppliChem, Darmstadt, Germany) was put into each well. The absorbance was measured at a wavelength of 570 nm using an UV visible spectrophotometer multiplate reader (ELx800UV, BioTek). The IC₅₀ dose of plant extract was calculated for each cell (Deliloglu-Gurhan et al., 2006).

Immunocytochemistry

The immunocytochemistry staining was performed to detect the level of oxidative stress marker endothelial nitric oxide synthase (eNOS). Following the treatment with IC₅₀ dose of plant extract for 24 h, cells were fixed with 4% paraformaldehyde (1.04004, Merck, Darmstadt, Germany) in phosphate buffered saline (PBS, Invitrogen) at +4°C for 30 min and were washed in PBS for 5 min. The cells were treated with 0.1% Triton X-100 (A4975, AppliChem, Darmstadt, Germany) at +4°C for 15 min for permeabilization and the endogenous peroxidase activity was inhibited by 3% hydrogen peroxide (1 08600, Merck, Darmstadt, Germany). Then primary antibody anti-eNOS (sc-654, Santa Cruz Biotechnology) was applied to the each cell at +4°C overnight. For negative control, some samples were not put the primary antibody. Then the biotinylated secondary antibodies and peroxidase-conjugated streptavidin (Histostain kit, 85-9043, Zymed, Carlsbad, USA) were used. The diaminobenzidine/hydrogen peroxide (DAB, 00-2014, Invitrogen, CA, USA) and counterstaining was performed using Mayer's hematoxylin (800-729-8350, ScyTek, UT, USA). The cell samples were mounted using aqueous media (K002, DBS, Pleasanton, USA) (Özdemir-Kurt et al., 2016). Under light microscope (BX53, Olympus, Japan) camera attached (SC50, Olympus, Germany), the images of stained cells were obtained. The immunocytochemistry assay was performed triplicate. The staining was evaluated as weak (+),

moderate (++) and strong (+++) respectively, in five different microscopic areas. The cells were counted for each intensity and the respective score was calculated by the following formula H-Score = Pi (intensity of staining + 1). Pi is the percentage of stained cells for each intensity, varying from 0% to 100%. The H-score was evaluated by at least two observers blinded to study independently (Ozbilgin et al., 2015).

TUNEL assay

After the plant extract application, apoptotic cells were detected by terminal Transferase dUTP Nick End Labeling (TUNEL Promega G7130) method was used to detect the apoptotic cells. For fixation, 4% paraformaldehyde was used and for permeabilization, the cells were treated with 0.1% Triton X-100 for 15 min. After washing in PBS, endogenous peroxidase activity was eradicated by 3% hydrogen peroxide. After equilibration buffer process, cells were incubated with terminal deoxynucleotidyl transferase enzyme (Tdt-enzyme) for 1 h at 37°C. Then cells were applied with 2× SCC solution for 15 min. The apoptotic cells were made visible by DAB staining and cells were stained with Mayer's haematoxylin for counterstaining. Cells were mounted with aqueous media. The TUNEL staining procedure was repeated three times (Tuğlu et al., 2010). The apoptotic cells were counted using light microscope (BX53, Olympus, Japan) by two blinded observer. The TUNEL positive and negative cells were counted in 10 randomly chosen areas, and the apoptotic index was revealed as: Apoptotic index = (number of apoptotic cells / total number of cells) × 100 (Pourheydar et al., 2016).

Statistical analysis

The results were statistically analyzed in GraphPad Software (San Diego, CA, USA) using repeated-measures of the ANOVA test and the data was given as mean ± SD values. The differences between groups was determined by The Tukey-Kramer multiple comparisons test and values for P<0.05 were defined as significant (Metel et al., 2016).

Results

In our study, the IC₅₀ doses of *Lysimachia sarranii* extract determined to be 16.25 µgr/ml for ADSC,

13.75 µgr/ml for MCF-7, and 12.5 µgr/ml for MDA-MB-231 by MTT assay. The plant extract was found to be significantly more toxic for MDA-MB-231 cells (Figure 1).

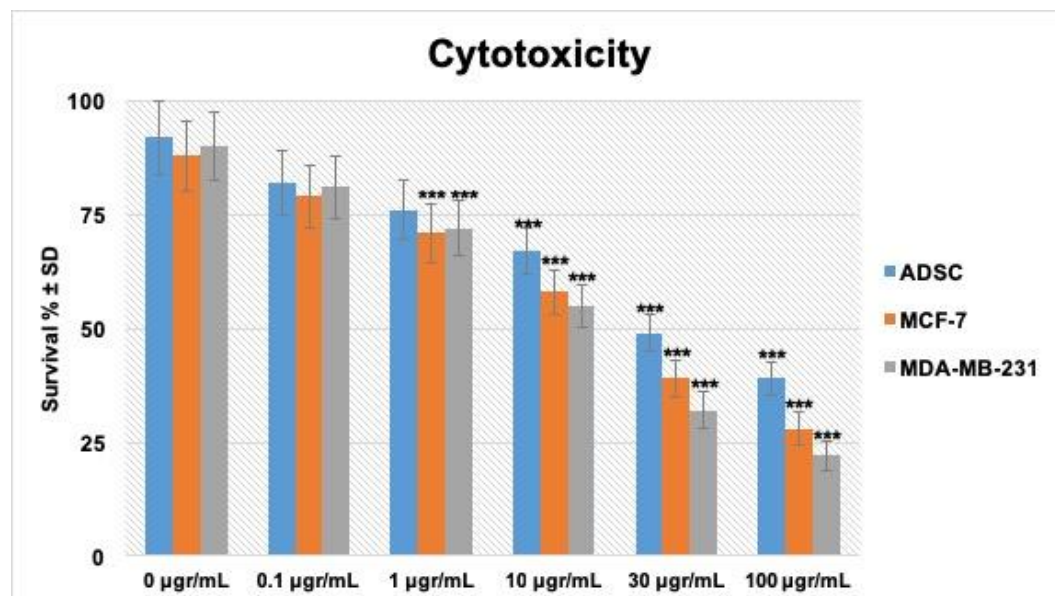


Figure 1. The cytotoxicity level of *Lysimachia sarranii* extract on ADSCs, MCF-7 and MDA-MB-231 breast cancer cell lines for 24 h (**P<0.01, ***P<0.001).

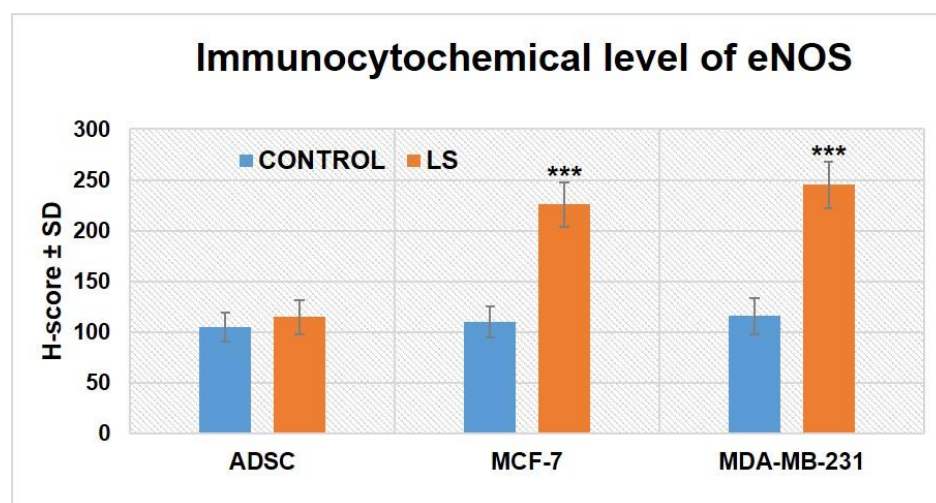


Figure 2. H-score analysis of immunocytochemical staining of eNOS in ADSCs, MCF-7 and MDA-MB-231 breast cancer cells after application of *Lysimachia sarranii* extract (IC₅₀ dose) for 24 h (**P<0.01, ***P<0.001).

By immunocytochemistry staining, oxidative stress marker, eNOS was evaluated after IC₅₀ doses of plant extract application by H-score (Figure 2).

There was not a significant difference between plant extract treated ADSCs and non-treated

ADSCs (P<0.05). The immunoreactivity of eNOS was higher in MDA-MB-231 and MCF-7 cells than treated with plant extract ADSCs (**P<0.01, ***P<0.001). However, immunoreactivity was found to be increased in MDA-MB-231 cells (**P<0.01, ***P<0.001) than MCF-7 cells (Figure 3).

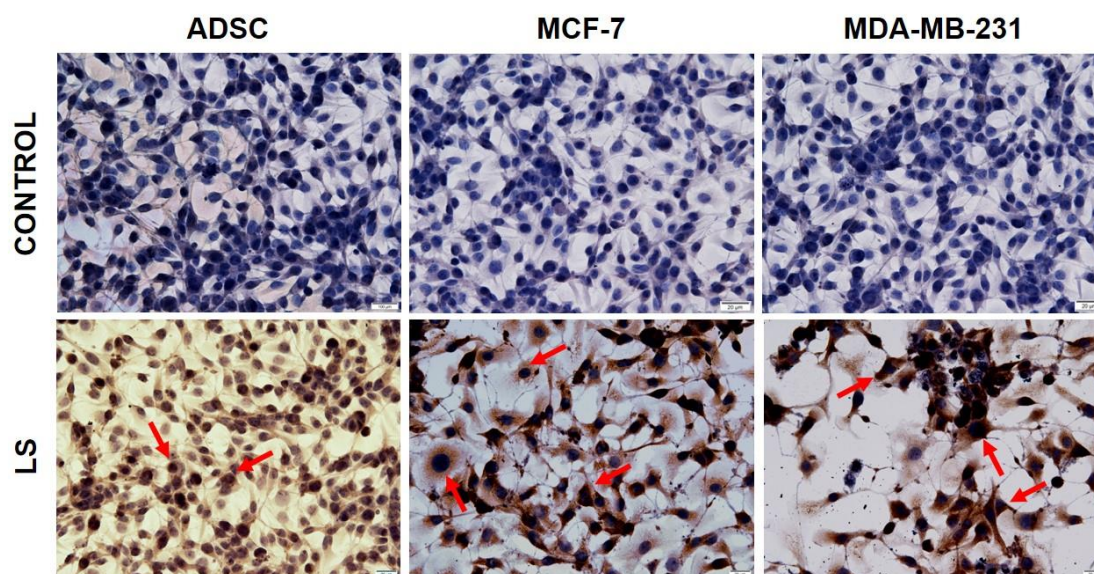


Figure 3. The distribution of immunocytochemical staining of eNOS in ADSCs, MCF-7 and MDA-MB-231 breast cancer cells after application of *Lysimachia savranii* extract (IC₅₀ dose) for 24 h. Arrows: Immunopositive cells, Scale bars: 20µm.

Table 1. The apoptotic index in ADSCs, MCF-7 and MDA-MB-231 breast cancer cells after application of *Lysimachia savranii* extract (IC₅₀ dose) for 24 h.

| CELL | CONTROL | LS |
|------------|----------|----------|
| ADSC | 9,8±1,1 | 10,0±1,2 |
| MCF-7 | 10,6±1,2 | 12,0±1,5 |
| MDA-MB-231 | 10,1±1,3 | 13,2±1,7 |

The apoptotic index were given in Table 1. In ADSCs, the increase of apoptotic cell number was not statistically significant with the application of plant extract (P<0.05). However, a significant increase in MDA-MB-231 cells was noted compared to non-treated cells (**P<0.01) (Figure 5). Similarly, in treated MCF-7 cells, there was an increase in apoptotic cells compared to non-treated cells (**P<0.01). The number of apoptotic cells was the highest in the treated MDA-MB-231 cells.

Discussion

The current study has showed that *Lysimachia savranii* extract has a cytotoxic effect on breast cancer cell lines MCF-7 and MDA-MB-231. The antiproliferative effect was revealed by the MTT, immunocytochemical and TUNEL assays. The distribution of eNOS was enhanced by the application of *Lysimachia savranii* extract, and also apoptotic cell count was also increased. The specie we used in our study, *Lysimachia savranii*, have not been investigated in the previous studies, whereas the other species of *Lysimachia* genus were studied *in vivo* and *in vitro* experiments.

Capilliposide C agent in the saponins group isolated from *Lysimachia capillipes* showed no cytotoxic effect below 1µg / ml in the TE-1 and TE-2 esophageal squamous carcinoma cells. After 48 hours of administration, the IC₅₀ dose for TE-1 cells was reported to be 5.43 ± 0.63 µM and 6.64 ± 0.91 µM for TE-2 cells. When capilliposide C combined with oxaliplatin, it has been observed that antiproliferative effect is increased by synergistic effect and thus apoptosis is triggered. This effect is shown by PI3K/Akt/mTOR pathway which plays an important role in proliferation and metastasis, and also the increase

of Cas3 and Bax from apoptotic markers are supported the antiproliferative effect (Shen et al., 2017).

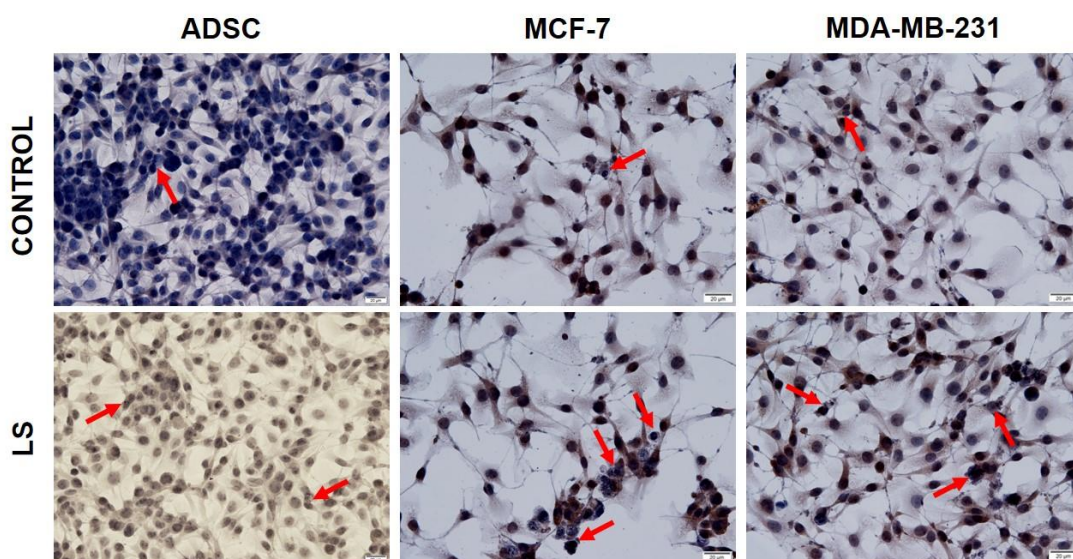


Figure 5. TUNEL staining for apoptosis in ADSCs, MCF-7 and MDA-MB-231 breast cancer cells after application of *Lysimachia savranii* extract (IC₅₀ dose) for 24 h. Arrows: Apoptotic cells, Scale bars: 20µm.

Another study with Capilliposide from *Lysimachia capillipes* examined its antitumorigenic effect on the lung cancer cell lines and experimental lung cancer model. It has cytotoxic effect against the lung cell lines A549, H1299 and H460 cells, and after 24 hours of treatment, the IC₅₀ dose was calculated as 4.13µg / mL for A549 cells, 3.76 µg / mL for H1299 cells and 2.85 µg / mL for H460 cells (Fei et al., 2014). In the study with PC3 and DU145 prostate cancer cell lines, IC₅₀ dose of Capilliposide C was found to be 5.53 ± 0.05 µMol / L for PC3 cells and 9.99 ± 1.98 µMol / L for MT3 cells. Annexin V-FITC / PI staining showed that apoptosis was enhanced with the increasing dose of Capilliposide C in PC3 cells, and this apoptotic effect was supported by the increase in Cyt-C and Bax and decrease in proCas3 with Western blot analysis (Li et al., 2014). In our study, we used the extract of *Lysimachia savranii*, not a compound. Whether the toxic effect of this extract is due to the whole or a compound it contains should be investigated. Similar to the content of our study, in a previous experiment, the cytotoxic effect of *Lysimachia nummularia* extract on PC3, DU145 prostate cancer cells and PNT2 normal

prostate cell, A-375 and BLM malignant melanoma and HSF skin fibroblast cells and U373 glioblastoma cells was reported. The IC₅₀ dose of *Lysimachia nummularia* extract determined as 7.4 ± 1.1 µgr/mL for PC3, 1.2 ± 0.3 µgr/mL for DU145, and 30.0 ± 3.2 µgr/mL for PNT2. While the extract showed toxic effect in prostate cancer cells at low doses, it was found that the dose increased for toxic effect in normal prostate cells. The IC₅₀ dose for A-375 was 23.2 ± 1.2 µgr/mL, BLM was 17.5 ± 1.6 µgr/mL, for HSF was 21.3 ± 1.8 µgr/mL, and for U373 glioblastoma cells was 6.0 ± 1.3 µgr/mL. Furthermore *Lysimachia nummularia* extract in prostate and glioblastoma cells was found to be toxic at lower doses compared to skin cancer cells (Podolak et al., 2013). In the current study, the IC₅₀ dose of *Lysimachia savranii* extract was detected as 16.25 µgr/ml for ADSCs, 13.75 µgr/ml for MCF-7, and 12.5 µgr/ml for MDA-MB-231. Besides, cytotoxic effect was supported with the increase of apoptosis and oxidative stress.

In our study, we used only the leaves of *Lysimachia savranii*. In previous studies, the aerial parts of

Lysimachia genus plants were used, such as *Lysimachia foenum-graecum* and *Lysimachia fortunei*. The several compounds were isolated from aerial parts of these species, and their cytotoxic effects against to the varied cancer cell lines were reported (Dai et al., 2017, Zhang et al., 2018). Our aim is to emphasize that different parts of the plant may contain different compounds and therefore their effect on cancer cells may change.

Conclusion

In our experiment, *Lysimachia savranii* extract was found to have toxic effect on breast cancer cells. The toxic effect was demonstrated by MTT, immunocytochemical staining and apoptosis. These findings should be supported by molecular techniques and further studies of *in vitro* and *in vivo* models.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest: The authors have no conflicts of interest to declare.

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