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Boswellia serrata Ekstraktının Karaciğer Kanseri (HepG2) ve Meme Kanseri (MCF7) Hücre Hatlarında Hücre Canlılığı, Apoptotik ve Nekrotik Aktivitelerinin Belirlenmesi

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ÖZ

Kanser, insanlık tarihinde çok uzun zamandır var olan ve günümüze kadar gelerek insanlık için büyük bir tehdit oluşturan çok önemli bir hastalıktır. Bilim dünyası birçok yenilikçi tedavi yaklaşımı ortaya çıkarmıştır ancak, kanser hücrelerinin karmaşıklığı ve tedaviye direnç gösterebilmesi nedeniyle kanserle mücadele için kesin bir tedavi henüz bulunamamıştır. *Boswellia serrata*, antik çağlardan günümüze kadar dünyanın birçok yerinde çeşitli rahatsızlıklar için kullanılmıştır, Burseraceae familyasından gelir ve genellikle beyaz sakız olarak bilinir. *B. serrata*, çölde 4-6 metre yüksekliğinde küçük bir ağaç veya çalı şeklinde yetişen bir bitki türü olduğu için çok küçük ve az yapraklı bir reçine ağacıdır. Bu çalışmada, *B. serrata* reçinesi Gaziantep ilinden ticari olarak elde edilmiş ve metanol ve hekzan çözücülerinde ekstrakte edilmiştir. *B. serrata* reçine ekstraktının sitotoksik aktivitesi MTT Assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) ile belirlenmiştir. Apoptotik/nekrotik aktivite ikili boyama (Hoechst 33342 ve propidium iyodür) ile belirlenmiştir. Bu deneylerde insan meme kanseri (MCF7) ve insan karaciğer kanseri (HepG2) hücre hatları kullanılmıştır. Hücre canlılığı deneyi sonucu HepG2 hücre hattında en iyi etkiyi %31.63 canlı hücre *ile B. serrata*'nın hekzan 400 µg/ml ekstresi verirken MCF7 hücre hattında ise en iyi etkiyi %13.71 canlı hücre ile *B. serrata* hekzan 800 µg/ml ekstresi vermiştir. İkili boyama sonuçları incelendiğinde HepG2 hücre hattına uygulanan metanol ekstraktı hem nekroz hem de apoptoza neden olurken MCF7 hücrelerinde ise dağınık çekirdekli ve membranlı hücreler gözlenmiştir. Antioksidan aktivite deney sonuçları incelendiğinde ise her iki çözücünün birbirine yakın sonuçlar verdiği gözlemlenmiştir.

Determination of Cell Viability, Apoptotic and Necrotic Activities of Boswellia serrata Extract on Liver Cancer (HepG2) and Breast Cancer (MCF7) Cell Lines

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ABSTRACT

Cancer is a very important disease that has existed for a very long time in human history and poses a great threat to humanity today. The scientific world has come up with many innovative treatment approaches, but due to the complexity of cancer cells and their resistance to treatment, a definitive treatment for the fight against cancer has not yet been found. *Boswellia serrata* has been used for various ailments in many parts of the world from ancient times to the present day, comes from the Burseraceae family, and is commonly known as white gum. *B. serrata* is a resin tree with very small and few leaves, as it is a plant species that grows as a small tree or shrub 4-6 meters high in the desert. In this study, *B. serrata* resin was obtained commercially in Gaziantep province and extracted in methanol and hexane solvents. The cytotoxic activity of *B. serrata* resin extract

was determined by MTT Assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Apoptotic/necrotic activity was determined by double staining (Hoechst 33342 and propidium iodide). Human breast cancer (MCF7) and human liver cancer (HepG2) cell lines were used in these experiments. As a result of the cell viability assay, hexane 400 µg/ml extract of *B. serrata* gave the best effect in HepG2 cell line with 31.63% viable cells, while in MCF7 cell line, *B. serrata* hexane 800 µg/ml extract gave the best effect with 13.71% viable cells. When the double staining results were examined, methanol extract applied to the HepG2 cell line caused both necrosis and apoptosis, while cells with disorganized nuclei and membranes were observed in MCF7 cells. When the results of antioxidant activity experiments were analyzed, it was observed that both solvents gave similar results.

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1. Introduction

Cancer is a word derived from the meaning of "crab", which means crab. The Greek physician Hippocrates made this definition by observing the spread of the disease from the area where it started to other organs (Health (US) and Study, 2007). Cancer is a biological problem that occurs when cells lose control of their natural growth and a process of clonal spread occurs. It is one of the most common and complex somatic genetic diseases. The occurrence and development of cancer can be directly related to factors such as characteristics, diet, and family history (Yokuş and Çakır, 2012). Cancer represents a significant public health issue and is currently the second most frequent cause of death worldwide. (Sung et al., 2021). Globally, an average of 14 million cancers are diagnosed annually and cancer is responsible for about 20 percent of all deaths. The most common types of cancer in women are breast, uterine corpus, and colorectal cancer. (Siegel, Naishadham, et al., 2012). According to a study conducted by the World Health Organization, the number of people diagnosed with cancer is increasing daily, and it is anticipated that approximately 22 million new cancer cases will be diagnosed within the next decade (Sung et al., 2021). There are several reasons for the high mortality rate from cancer, the main one being inadequate intervention (Baykara, 2016; Özgüç et al., 2018). Scientists have achieved significant results and advances that cannot be ignored in the fight against cancer, but despite all this research, the desired success in cancer treatment has not yet been achieved (Markham et al., 2020). The fact that treatment costs cannot be covered by many people and that many treatment methods have various side effects makes the treatment process very difficult (Erkurt et al., 2009; Prasad et al., 2017; Turkoglu et al., 2020). Another is that cancer is a highly lethal pathology due to inadequate surgical interventions, excessive risk, and failure to achieve the desired survival rate (Siegel, DeSantis, et al., 2012).

B. serrata, also known as white gum from the Burseraceae family, has been used throughout the world in the past and present to treat various health problems (Siddiqui, 2011). White gum is a small tree or shrub of 4-6 meters in size, usually growing in the desert, so it is a very small and low-leaved resin tree. The bark of the tree is deeply scratched and the resin is collected from the area. Its composition includes 30-60% resin, 5-10% essential oils, and 30-40% polysaccharides (Efferth and Oesch, 2020). The resin with essential oil and gum is used in bronchial asthma, respiratory system disorders, arthritis

(osteoarthritis), joint pain and swelling caused by rheumatoid arthritis, inflammation in the bowel caused by Crohn's disease or ulcerative colitis, and as a remedy for flatulence. *B. serrata* is usually powdered or mixed with water and consumed orally. Although there is not much detailed research on *B. serrata*, it generally stands out with its antioxidant effect and asthma (Liu et al., 2015; Zhou et al., 2015; Roy et al., 2016; Sarkate and Dhaneshwar, 2017). *B. serrata* resin contains bioactive compounds known as boswellic acids, which have been reported to exhibit anti-inflammatory, immunomodulatory and anti-cancer activities (Sahu and Chawla, 2022). These activities have been attributed to triterpenes present in *B. serrata*, which show potential to inhibit tumour growth and induce apoptosis in cancer cells (Bonilla et al., 2022). Furthermore, Boswellic acids were found to modulate the production of pro-inflammatory cytokines and inhibit the activation of NF-κB, an important regulator of inflammation and cancer progression (Sengupta et al., 2009). *B. serrata* has also been shown to protect dopaminergic neurons from neurotoxicity and exhibit antioxidant activity in the cerebrovascular system (Shadfar et al., 2022)

In many studies, anticancer, antidiabetic and antioxidant activities of some active substances obtained from *B. serrata* plant have been shown. However, these studies focused on a specific active substance, and the activity of this substance was evaluated. This study aimed to determine the apoptotic and necrotic activity of the resin extract obtained directly from the natural plant.

2. Methods and Materials

2.1. Obtaining and preparation of the extract

The resin of the *B. serrata* plant used in this study was purchased from an herbalist in the Nizip district of Gaziantep province in southern Turkey. Commercially obtained *B. serrata* was pulverized using a porcelain mortar and pestle and weighed 1 g for each solvent using a precision balance. Then, each 1 g of extract was treated separately with 10 ml of hexane or methanol respectively, and incubated in an oven with shaking for 72 hours. After 72 hours, samples were filtered into a petri dish with the help of Whatman No 1 filter paper and a glass funnel and allowed to dry for 48 hours at the end of the elapsed time, each petri dish was treated with 10 ml DMSO and the samples were taken into different falcon tubes and stored at +4°C. The extracts obtained were measured according to the following formula.

$$\%\ \text{Yield} = \frac{\text{Amount remaining after extraction(g)}}{\text{Dry amount before extraction(g)}}\ x100 \quad (1)$$

2.2. MTT Assay Application

The MTT test is a method used to measure cell viability, and toxic effects on cell reproduction and cell. The MTT method relies on the idea that functioning mitochondria in living cells diminish a tetrazolium salt named MTT, which enters the cell membrane, by catching electrons internally and transforming into purple formazan crystals that cannot be dissolved in water. After the incubation period, we tested how the plant extracts affected the cells using the MTT technique. We added 20µl of 5mg/ml MTT

solution from the supply to each well of the plate containing the cells. We maintained the plate in a dark oven with 5% CO_2 at 37°C for 4 hours. The solution also contained sterile phosphate buffer with a pH of 7.2. We used a plate reader at a wavelength of 570 nm to measure the absorbance value (Dalkılıç et al., 2023; Riss and Moravec, 2004). The average of the absorbance values of the control wells was calculated and this value was accepted as 100% of viable cells. Percent viability was calculated by comparing the absorbance values of the regions containing the plant extract with the absorbance value of the control (Coskun et al., 2021; Dalkılıç et al., 2021). Absorbance readings were taken with a plate reader and compared to control groups. To confirm the accuracy of the results, a 2.5 µg/ml doxorubicin positive control and a cell-treated medium were used as negative controls.

2.3. Determination of apoptotic/necrotic activity

In the double staining method, 10000 cells were transferred as 100 µl into a 6-well plate and incubated for approximately 5 hours for the cells to adhere to the wells, then 2 ml of DMEM was transferred into the wells and left to incubate at 37°C and 5% CO_2 atmosphere for 24 hours. After 24 hours, the medium on the plate was drained and extracted in hexane or methanol solvents to add 2 ml of medium containing *B. serrata* resin at a concentration of 800 µg/ml. It was then incubated for 48 hours. After 48 hours of incubation, 1 ml of a solution containing 10 µg/ml Hoeschst and 1 µg/ml Propidium Iodide (PI) dyes prepared with 1x PBS was added to the wells (1 ml of medium was pipetted from the well before adding the dye). The plate was kept in the dark for 30 minutes at 37°C in a 5% carbohydrate medium and after incubation, cell morphology was examined under a fluorescence microscope in comparison with the control group (Vanden Berghe et al., 2013).

2.4. Antioxidant determination

The antioxidant capacity of *B. serrata* plants was tested using the DPPH method. A stock solution was made from the extracts and diluted with methanol to a concentration of 800 µg/ml. The samples were supplemented with 0.3 mM DPPH methanol solution. The plate was placed in a dark environment for 30 minutes, and subsequently, absorbance measurements were conducted using a spectrophotometer at wavelengths of 492 nm and 540 nm. As the absorbance decreases, we establish the leftover DPPH as the activity of scavenging free radicals (Flieger and Flieger, 2020; Huang et al., 2005).

The results are calculated according to the formula $\% = \frac{\text{ControlABS} - \text{SampleABS}}{\text{ControlABS}} x100$ (2)

2.5. Statistical analysis

Differences between groups were evaluated using SPSS (version 22) software. The data was analyzed using ANOVA. Results were significant if $p<0.05$.

3. Results

3.1. Cell viability

Among the *B. serrata* extracts extracted with hexane and methanol, the compounds generally showed significant cytotoxic activity against the cell line at high concentrations. The results of the MTT assay with 6 replicates are given below (Figure 1 and Figure 2).

When the results are examined, it is seen that the hexane extract of *B. serrata* showed the best effect at a concentration of 400 µg/ml in HepG2 cell line, followed by the methanol extract, which also showed the best effect at a concentration of 400 µg/ml. When the effect of the plant extract on MCF7 cancer cell line was examined, it was determined that the cytotoxic effect of the methanol extract was the best at a concentration of 800 µg/ml and the hexane extract also showed the best activity at a concentration of 800 µg/ml. This shows that methanol and hexane extracts exhibited the best activity at the same concentration in both cell lines.

In the HepG2 cell line, the hexane compound was used at a concentration of 400 µg/ml to obtain the maximum cytotoxic effect, while the concentration required for this effect in the MCF7 cell line was 800 µg/ml. No strong effect of the methanol compound was observed except the 80.36% cytotoxic effect of 800 µg/ml concentration in MCF7 cells. In the HepG2 cell line, the hexane compound was used at a concentration of 400 µg/ml to obtain the maximum cytotoxic effect as a result of six replicates, while the concentration required for this effect in the MCF7 cell line was 800 µg/ml. No strong effect of the methanol compound was observed except the 80.36% cytotoxic effect of 800 µg/ml concentration in MCF7 cells.

Methanol and hexane are two different solvents commonly used in laboratories. Their chemical structures are different; methanol has a polar structure and hexane has an apolar structure. This means that methanol is soluble in solvents such as water and can dissolve many ionic and polar compounds, while hexane is soluble in compounds such as oils and waxes. Both compounds are reported to have less toxicity than many solvents used in experimental studies, such as dichloromethane, which is important for cell culture studies because the clinical applicability and similarity of cell culture studies to real-world situations are enhanced by minimizing toxic effects. These solvents, which are preferred for in vitro studies such as cell culture, are economical. It is also important that experimental work in a cancer study environment achieves its goal and minimizes harm to researchers and the environment as much as possible.

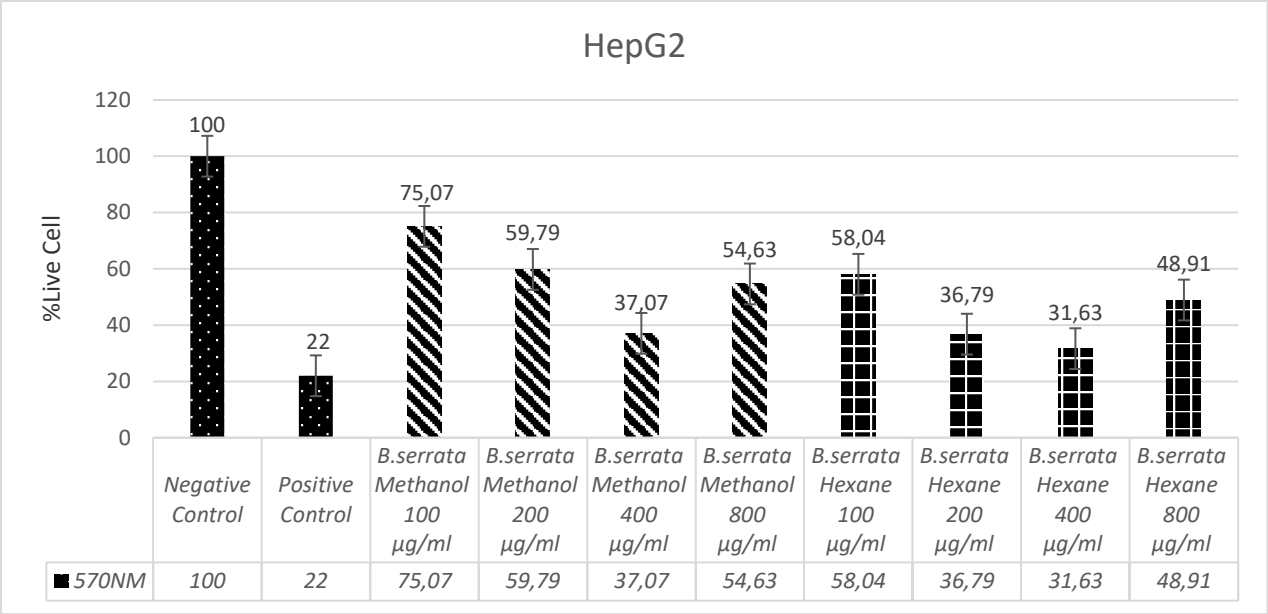

Figure 1. Measurements of cytotoxic activity of the extracts on HepG2 cell line at 570 nm.

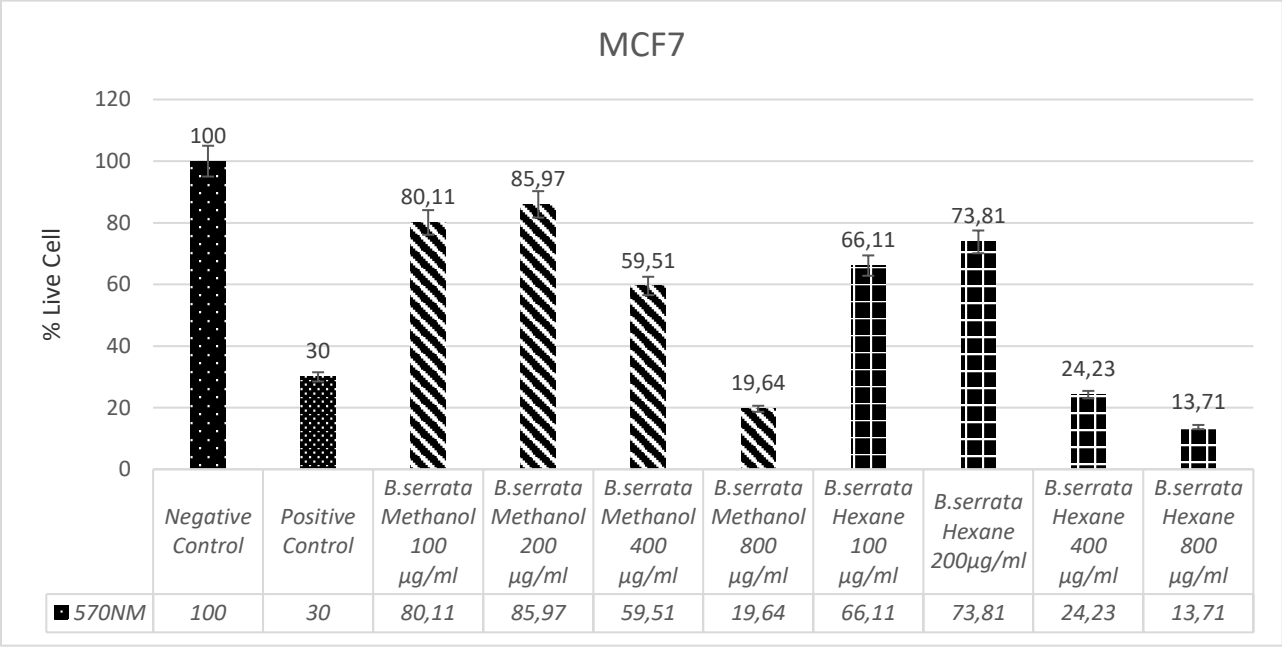

Figure 2. Measurements of cytotoxic activity of the extracts on MCF7 cell line at 570 nm.

3.2. Double staining (Hoechst 33342 and Propidium Iodide) method

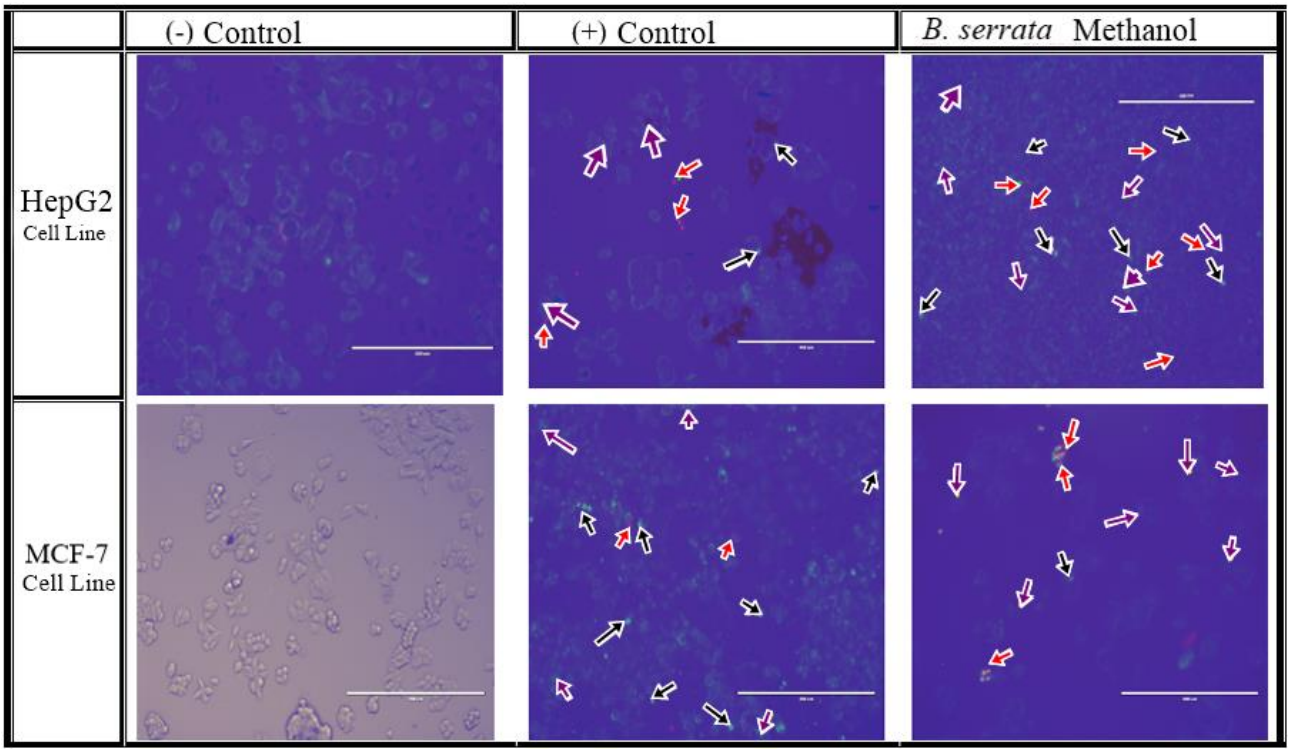

Figure 3. According to the results of Hoechst 33342 and Propidium Iodide staining method, cells were processed with Hoechst 33342 and PI stains.

Images of *B. serrata* extract prepared with methanol at a concentration of 800 µg/ml after treatment in MCF7 and HepG2 cell lines for 48 hours are shown in the figure above (Figure 3). In the visual analysis, black arrows represent pyknosis, purple arrows represent fragmented nuclei and red arrows represent necrotic cells. Doxorubicin 2.5 µg/ml was used in the positive control group, while untreated cells were used in the negative control group. Hoechst and PI fluorescent dyes were used to assess cell integrity. Hoechst stain can stain all cells, while propidium iodide stain is a fluorescent stain that can only stain the membranes of damaged cells. Dying cells can be stained by both staining methods, but propidium iodide is a differential stain used to identify dead cells. In all cancer cell lines used in the trial, no negative changes were detected in the negative control classes and no decrease in numbers was observed. Hoechst stain was able to pass through all membranes. Cells in negative controls were stained, but the integrity of their membranes was disrupted. Other hand, propidium iodide stain, which can stain necrotic cells, was not effective in this group. Consistent with these results, the cells remained viable and proliferated. Similarly, a noticeable reduction in the appearance of positive control cells was detected and often seen by positive staining with propidium iodide, suggesting that doxorubicin kills through necrosis. In addition, it appears to cause complete lysis of necrotic cells and apoptotic cells and nuclei. In this cell line, apoptotic cells were observed together with necrotic cells (Figure 3). Methanol extract applied to the HepG2 cell line caused both necrosis and apoptosis in the cells. In MCF7 cells, the effect of methanol extract was similar to that in the positive control group and an image in which apoptotic cells were

predominant was obtained. In the analysis of the results, cells with scattered nuclei and membranes were observed, as in other cells. These cells may be cells undergoing early apoptosis or cells undergoing secondary necrosis.

3.3. Determination of antioxidant activity

The antioxidant activity of 2,2-diphenyl-picrylhydrosyl (DPPH) in two different solvents (methanol, hexane) and at the highest concentration (800 µg/ml) at 492 and 540 nm wavelengths, which are the most suitable ranges to determine the purple color of DPPH solution, was examined according to the radical scavenging capacity method (Figure 4) and the results were analyzed. As a result, a high similarity was observed at two different wavelengths. In terms of the activity ratio of the extracts, *B. serrata* hexane extract gave the highest result with 41.44% at 492 nm wavelength, while methanol extract closely followed the hexane extract with 41.06%. At 540 nm wavelength, *B. serrata* hexane extract showed the highest effect with 36.58%, while the result of methanol extract was observed as 35.72%.

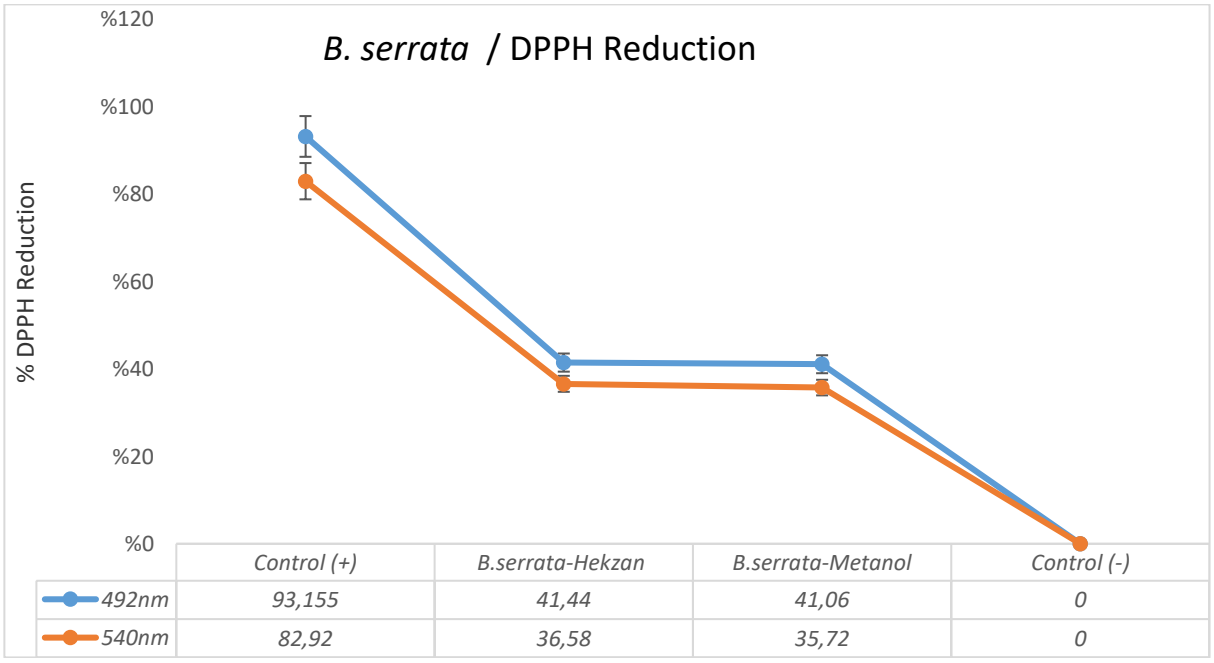

Figure 4. Antioxidant Effect of *B. serrata* methanol and hexane extracts measured at 492 and 540 nm

4. Discussion

In studies on *B. serrata*, it was reported that boswellic acid obtained from the resin of the plant showed cytotoxic and apoptosis-suppressing effects in many different human cancer cells (Ahmed et al., 2015; Conti et al., 2018; Schmiech et al., 2019; Wang et al., 2020). However, in these studies, the biological activity of Boswellic acid, a component of the resin, was investigated. In this study, the whole resin obtained from a plant frequently used among the people was extracted and the anticancer and antioxidant activities of this extract were investigated.

In one study, HT-29 cells were exposed to different concentrations of *B. serrata* plant resin and cell viability was measured by MTT assay, while apoptosis was assessed by the percentage of cells in the G1 phase. PGE2 levels and caspase-3 effect were measured by ELISA. Tube formation ability and migration of HT-29 cells were evaluated by 3D vessel formation assay and scratch assay. As a result, *B. serrata* extract significantly increased the number of cells in the G1 phase and the number of cells in the proliferation phase, and cell viability and cell migration were significantly decreased in HT-29 treated with *B. serrata* compared to the control group. When the MTT results were analyzed, it was reported that the methanol extract of *B. serrata* was studied between 1-200 μg/ml concentrations, and more effective results were obtained at high concentrations compared to low concentrations (Ranjbarnejad et al., 2017). Comparing the study by Ranjbarnejad et al. with our study, it appears that there is a positive correlation between *B. serrata* concentration and the observed cytotoxic activity, although the studies were conducted in different cell lines. In another study, different concentrations of *B. serrata* extracts were used and these extracts were tested for cytotoxicity on HepG2 and Hep3B cell lines and it was reported that *B. serrata* had a visible cytotoxic activity in both cell lines (Khan et al., 2014). Again, when examining the results of the study, it was observed that there was a direct proportional relationship between *B. serrata* concentration and cytotoxic activity, which supports the results of our study. In one study, 4T1 cells, a breast cancer cell line derived from mouse mammary gland tissue of the BABL/c strain, were subjected to MTT assay using *B. serrata* resin extracts, and the 4T1 cell line showed a decrease in cell viability in response to BSE (*B. serrata* gum resin alcoholic extract) treatment. As a result, *B. serrata* induced cell-specific cytotoxicity in breast cancer cells and suppressed cell proliferation, angiogenesis, and metastasis rate (Alipanah and Zareian, 2018). Although the alcoholic extract of *B. serrata* was used in the study, it is evident that the anticancer effect of the substance increased with increasing concentration. This result shows that *B. serrata* can be an effective agent in cancer research and supports the results of our studies. In another study, methanol and dichloromethane extracts *of B. serrata* were tested on human breast cancer cell line MCF7 and it was found that 100 μM solution of methanol extract of these three extracts was the most effective in suppressing cell proliferation. As a result, it was reported that *B. serrata* could be a possible chemotherapeutic agent for breast cancer (Poornima and Deeba, 2020). Comparing the results of the study conducted by Poornima et al. with the results of the study conducted by our team, the results of the studies using the same cell line and solvent support each other. In another study, to evaluate the antioxidant activity of *B. serrata* plant extracts in an experimental model of acute ulcerative colitis in rats induced by acetic acid administration, *B. serrata* extract was administered orally with 4 ml of 4% acetic acid at a dose of 34.2 mg/kg/day two days before and two days after colitis induction. As a result, lipid peroxidation and nitric oxide levels were significantly reduced by both pretreatment and *B. serrata* treatment. It has also been reported that *B. serrata* has an antioxidant effect and reduces tissue damage in animals with ulcerative colitis (Hartmann et al., 2014). Looking at the results of this study and other similar studies, it is reported that *B. serrata* has a strong antioxidant effect and these results support the results of our study.

5. Conclusion

Cytotoxic, apoptotic and necrotic activities of *B. serrata* were investigated against MCF7 and HepG2 cell lines. In the antioxidant study, the highest effect was found in *B. serrata* extract prepared in methanol. When the cytotoxic activity was examined, the highest cytotoxic activity in the HepG2 cell line was reported at 400 µg/ml concentration of the hexane extract, while the highest concentration of *B. serrata* in the methanol extract was observed in MCF7 cells. As a result of all these studies, it has been observed that *B. serrata* has an important cytotoxic, necrotic and antioxidant activity and will play an effective role in the fight against cancer.

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Conflict of İnterest

No potential conflict of interest was reported by the authors.

Researchers' Contribution Rate Declaration Summary

The authors of this article are all equal contributors to this article.

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