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Mentha pulegium Extracts Showed Strong Antimicrobial And Cytotoxic Effects in Vitro.

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ABSTRACT

Mentha pulegium is a medicinally important and well-known plant and used for various purposes such as medicinal, nutritional and spice. We are analyzed to observe the antimicrobial, antioxidant and cytotoxic properties of *M. pulegium* extracts in this study. The antimicrobial activity of *M. pulegium* was tested using the agar well method. MIC, MBC and antimicrobial activity were tested on *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 700603), *Escherichia coli* (ATCC 25322), *Bacillus megaterium* (DSM32) and *Candida albicans* (FMC17) microorganisms. Clindamycin (2 µg) and Amoxicillin (30 µg) were used as positive control and Dimethylsulfoxide (DMSO) as negative control. Cytotoxic activity of extracts at different concentrations obtained from solvents such as acetone, chloroform and methanol using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test method. Cell death patterns after exposure to different concentrations of the extracts on human breast cancer (MDA-MB-231), human pancreatic cancer (PANC1), human ovarian cancer (OVCAR3) and human lung cancer (A549) cell lines were determined accordingly. As a result, it was determined that *M. pulegium* extract has a strong antimicrobial activity spectrum and cytotoxic effect.

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Mentha pulegium Ekstraktının Güçlü Antimikrobiyal ve Sitotoksik Etkisinin Invitro Olarak Gösterilmesi

ÖZET

Mentha pulegium tıbbi açıdan önemli ve iyi bilinen bir bitkidir ve tıbbi, beslenme ve baharat gibi çeşitli amaçlar için kullanılır. Bu çalışmada *M. pulegium* ekstraktının antimikrobiyal, antioksidan ve sitotoksik özelliklerini görmek için analiz edildi. *M. pulegium*'un antimikrobiyal aktivitesi agar kuyucuk yöntemi kullanılarak test edildi. MIC, MBC ve antimikrobiyal aktivite *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 700603), *Escherichia coli* (ATCC 25322), *Bacillus megaterium* (DSM32) ve *Candida albicans* (FMC17) mikroorganizmaları üzerinde test edildi. Pozitif kontrol olarak Klindamisin (2 µg) ve Amoksisilin (30 µg), negatif kontrol olarak Dimetilsülfoksit (DMSO) kullanıldı. 3-(4,5-dimetiltiyazol-2-il) -2,5-difenil tetrazolyum bromür (MTT) test yöntemi kullanılarak aseton, kloroform ve metanol gibi çözücülerden elde edilen farklı konsantrasyonlarda ekstraktların sitotoksik aktivitesi; ekstraktların farklı konsantrasyonlarına maruz kaldıktan sonra µg/ml sitotoksik aktivite ve hücre ölüm şekilleri; Aseton, kloroform ve metanol gibi çözücülerden 3-(4,5-dimetiltiyazol-2-il) -2,5-difenil tetrazolyum bromür (MTT) test yöntemi kullanılarak aseton, kloroform ve metanol insan meme kanseri (MDA-MB-231), insan pankreas kanseri (PANC1), insan yumurtalık kanseri (OVCAR3) ve insan akciğer kanseri (A549) hücre hatları buna göre belirlendi. Sonuç olarak M. pulegium ekstraktının güçlü bir antimikrobiyal aktivite spektrumuna ve sitotoksik etkiye sahip olduğu belirlenmiştir.

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INTRODUCTION

Since ancient times, drugs of natural origin have formed the basis of traditional medicine in different cultures, and today medicinal plants occupy the most important place as a source of new drug molecules (Hassan and Ullah, 2019; Sofowora et al., 2013). It has been reported that approximately 400,000 plant taxa have been identified to date, and approximately 30,000 have been used in traditional medicine for the treatment of many diseases (Top et al., 2019; Erarslan et al., 2020).

Cancer is a complex disease that occurs as a result of disorders in the mechanisms regulating the basic functions of the cell and seriously affects the human population (Şekerli et al., 2017; Greenwell and Rahman, 2015). There are many treatment methods for this life-threatening disease. These are: radiotherapy, surgery, chemotherapy-hormone therapy, immunotherapy, gene therapy, check-point inhibitors and angiogenesis inhibitors (Hasham et al., 2018). Due to the fact that these treatment methods have some side effects (such as chemotherapy) and treatments such as immunotherapy and gene therapy are expensive, interest in researches such as the development of new plant-derived drug candidate molecules and the discovery of new bioactive molecules and the development of new treatment methods is increasing and investments are made in this subject (Kurt et al., 2013; Pucci et al., 2019).

The lack of easy access to primary health care and veterinary services in many rural areas of the world has increased the need for traditional medicine to treat both humans and animals and has helped maintain the use of medicinal plants (McGaw and Eloff, 2008). The need for new, effective and affordable drugs to treat microbial infections is increasing, especially in developing countries of the world, where up to half of deaths are caused by infectious diseases, which is seen as a major challenge in global healthcare (Awouafack et al., 2013).

The development of antibiotic-resistant bacterial strains is due to a number of factors, such as the widespread and inappropriate use of antibiotics and the increase in antibiotic-resistant pathogenic bacteria (Lowy, 2003). According to the data obtained, the problem of antibiotic resistance in humans and animals will continue for a long time (Andersson and Hughes, 2011). However, the development of alternative drug classes is necessary to treat such infectious diseases. Communicable diseases represent an important cause of death, especially in developing countries. For this reason, pharmaceutical companies have focused on developing new antimicrobial drugs in recent years, especially due to the continuous emergence of microorganisms resistant to antimicrobials. A synergy between traditional medicines and products derived from medicinal plants has also been reported (Nascimento et al, 2000). As bacteria resistant to conventional drugs are becoming more common, medicinal plants represent an alternative to antimicrobial action and infection treatment (Sakagami and Kajimura, 2002).

According to the researches of the World Health Organization (WHO), due to the great biological and structural diversity of medicinal plant components used for therapeutic purposes, it constitutes an effective resource for the discovery of antibacterial, antifungal and antiparasitic compounds (Faydaoğlu and Sürücüoğlu, 2013; Ugboko et al., 2020). Plant extracts showing antimicrobial activity are used as preservatives, medicinal purposes, analgesic, anti-inflammatory properties in foods and show a good effect against pathogens (Swamy et al., 2016; Winska et al., 2019). Nowadays, people resort to medicinal plants because of the unavoidable proliferation of diseases and the inadequate treatments (Erdoğan et al., 2013). The genus *Mentha* is from the Labiatae family and is used as an aromatic and medicinal plant (Gonzalez-Tejero et al., 2008). *M. pulegium* species is also called pennyroyal, watermelon and filiskin . It is a perennial, pungent-smelling plant that grows in Western, Southern and Central Europe, Asia, Iran, and Arab countries and grows in a wide area in Turkey (Çöteli et al., 2013). It is also used as a preservative in the food industry (Ahmed et al., 2018). In addition, it is known that it is loved and consumed among the people as a natural flavor and because it is thought to be medicinal.

The Labiatae family is one of the large plant families used as a basis for assessing the formation of some secondary metabolites (Wink, 2003). Plants of the genus *Mentha* are a source of essential oils found in terpenes, various phenolic compounds, tannins, terpenoids, quinones, coumarins, flavonoids, alkaloids, sterols, and saponins, especially in the epidermal glands of leaves, stems, and reproductive structures (Dorman et al., 2003). *M. pulegium* is rich in essential oils in its structure and its essential oil yield varies between 1.90% and 6.20% according to dry weight. The majority of essential oils are composed of a substance called Pulegon (Benlarbi et al., 2014; Gülçin et al., 2020). At the same time, *M. pulegium* has traditionally

been used in medicine for digestive problems, colds, sinusitis, cholera, bronchitis, tuberculosis, carminative, expectorant (Mamadalieva et al., 2020). It is also used topically to kill germs, repel insects and treat skin diseases, gout, venomous bites and mouth sores (Salem et al., 2017).

Considering the problems mentioned in this study, cytotoxic activity of *M. pulegium* species from Labiatae family was determined on human breast cancer (MDA-MB-231), human pancreatic cancer (PANC1), human ovarian cancer (OVCAR3) and human lung cancer (A549) cell lines. intended to be examined. In addition, it was aimed to determine the apoptotic/necrotic activity by double staining (Hoechst 33342 and Propidium Iodide) method. Although the blooming aerial part of *M. pulegium* is widely used for its antiseptic properties, studies on the antimicrobial activity of this plant have been reported so far. (Chalchat et al., 2000; Mamadalieva et al., 2020) It was aimed to investigate the antioxidant activity of the extract obtained from the aerial part of the *M. pulegium* plant and antimicrobial activity experiments with the agar-well method using five different microorganisms and the MIC and MBC values of the applied extract in addition to these.

MATERIAL and METHODS

In this study; The plant *M. pulegium* was obtained naturally. Gram positive (+) in the study *Staphylococcus aureus* ATCC 25923, *Bacillus megaterium* DSM32 and gram negative (-) *Escherichia coli* ATCC 25322, *Klebsiella pneumoniae* ATCC 700603 bacteria and *Candida albicans* FMC17 as fungus were used. Microorganism cultures were obtained from the culture collection of Fırat University, Faculty of Science, Department of Biology, Microbiology Laboratory.

Preparation of Extract and Test of Antimicrobial Effect

The *M. pulegium* plant grown in Diyarbakır and its surroundings was collected in April-May. Above-ground parts of *M. pulegium* were pounded in a porcelain mortar and powdered, then weighed on a precision scale and 1 g of dried *M. pulegium* was taken and dissolved with 10 ml of acetone, 10 methanol and 10 ml of chloroform solvents. After keeping it at room temperature for 72 hours, it was passed through a 0.45 µm minipore filter (Oxoid). Then, the main stock was prepared by performing the extraction process at 55-60˚C in a rotary evaporator for 4-6 hours and dissolving the obtained extract in DMSO (Dimethyl sulfoxide). The extracts were stored at +4 ˚C (Salem, et al., 2017; Dalkılıç et al., 2020). % efficiency calculation was made for the extract obtained (Chalchat et al., 2020). Polar solvents are very effective in separating polyphenols from plant tissues. For this reason, acetone, methanol and chloroform solvents, which are polar solvents, were preferred for the extraction of *M. pulegium* (Wink, 2003; Darman et al., 2003).

Formula 1.

% Efficiency calculation:

$$\%\,Yield = \frac{\text{Amount remaining after extraction (g)}}{\text{Dry amount before extraction (g)}}\ \text{X}\ 100$$

All the materials used were sterilized in an autoclave and the necessary labels were made. Before the experimental study, bacteria were grown in Nutrient Broth (Difco) and yeast were grown in malt extract broth (Difco). The agar well method was used to test the antimicrobial activity of *M. pulegium* plant, acetone, methanol and chloroform extracts. Mueller Hinton Agar (Oxoid) was prepared for the bacteria used in the study and Sabouraud Dextrose Agar was prepared for the fungus and after the sterilization process was completed in the autoclave (15 min at 121°C), bacteria and fungi were inoculated with the help of the loop and according to Mc Farland 0.5 (10^8 microorganism/ml). After it was adjusted and shaken well, 25 ml was placed in sterile petri dishes of 9 cm diameter and the medium was homogeneously distributed. Petri dishes were left to solidify for 5-15 minutes at room temperature. Solidified Müeller Hinton Agar and Sabouraud Dextrose Agar were sterilized and each petri dish was well drilled with cork borer except for the negative and positive controls. Extract at different concentrations (5 mg/ml, 25 mg/ml, 50 mg/ml and 100 mg /ml) was inoculated under aseptic conditions, as 100 µl for each well. After the inoculation process, the petri dishes inoculated with bacteria were incubated at 37±0.1°C for 24 hours, and the petri dishes inoculated with fungi for 48 hours at 25±0.1°C. Standard discs (Amoxicillin 30 µg/disc, Clindamycin 2 µg/disc) were used for positive control and DMSO was used as negative control. The diameters of the inhibition zones formed at the end of the incubation period were evaluated and recorded. This study was carried out in 3 replications.

Minimal Inhibition Concentration

The minimum inhibitory concentration (MIC) is the lowest concentration of a herbal extract or chemical that markedly inhibits the growth of bacteria. Serial dilutions of the *M. pulegium* were prepared at concentrations ranging from 4 to 10,000 µglmI. Nutrient broth (100 µl) was added to all wells of the 96-well microtitration plate. 40 µl of *M. pulegium* was added to the A1-H1 wells and serial dilution was made by taking 100 µl. Each well was inoculated (4 µl) of tested bacteria and the 96-well microtiter plate was then incubated (37°C for 24 hours) so far as the growth requirement of each organism (Hemeg et al., 2020).

Minimal Bactericidal Concentration

Mueller Hinton Agar was prepared and autoclaved (121°C for 15 minutes) and then poured into petri dishes. Each petri dish was divided into four sections and each section was divided into 12 wells. 3µl of each well of the samples in the 96-well microtiter plate, in which the MIC was made, was taken from each well and added to the medium containing Mueller Hinton Agar to its own number. Then incubated in an oven at 37°C for 24 hours. The lowest medium concentration without growth in the subculture on the agar was accepted as MBC. (Dalkılıç et al., 2020).

Statistical Analysis

It was checked whether the results showed a normal distribution, and a paired t-test was applied on the results. For all values, $p<0.05$ was considered statistically significant. All statistical analyzes were done with SPSS version 22.

Cytotoxic Activity

MTT Assay assay: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay is a method used to measure cell viability, proliferation and cytotoxicity. MTT method; It is based on the principle of reducing MTT, a tetrazolium salt that can pass through the cell membrane, by the active mitochondria in living cells by gaining electrons inside the cell and transforming it into purple colored water-insoluble formazan crystals. Then, formazan crystals are dissolved with suitable solvents and the resulting color change is measured by spectrophotometric methods and the number of viable cells is determined (Talbaoui et al., 2016; Stockert et al., 2012).

Application of MTT Assay

After the cells grown in 25cm² flasks were 90% confluent, the medium in the flask was removed and washed with 5 ml of sterile PBS (Phosphate-bufferedsaline) solution. 1 ml of Trypsin-EDTA was added to the flasks and incubated for 2 minutes at 37˚C in an oven with 5% CO_2. After the cells were separated from the surface, Trypsin-EDTA was inactivated with 5 ml of medium. The cells were taken out of the flask and centrifuged at 1500 rpm for 5 minutes, then the supernatant was removed, the cell pellet was dissolved with 1000 µl of DMEM and the cell count was made with the Countess II automatic cell counting device. After the calculations were made, cell dilution was prepared with standard DMEM and 100 µL DMEM was seeded on 96-well plates with $5x10^3$ cells in each well. Only DMEM was used as a blank in the first row, 2.5 µg/ml Doxorubicin was used as positive control and only medium was used as negative control. Then it was incubated for 24 hours in an oven with 5% CO_2 at 37˚C. After incubation, the medium in the wells was removed and methanol, acetone and chloroform extracts of *M. pulegium* prepared in DMEM were added to the cells in 4 different concentrations of 1000, 500, 250,125 µg/ml 6 repetitions and 5% CO_2 at 37˚C for 72 hours. It was left to incubate in an oven. After the incubation period, 20 µl of MTT solution (5 mg/ml) was added to the wells containing the cells and incubated for 4 hours at 37˚C in a dark environment containing 5% CO_2. After incubation, the medium was removed and formazan crystals were dissolved with 100 µl of DMSO (dimethylsulfoxide). The color change expected to occur afterwards was measured with the ELISA micro-plate reader device at a wavelength of 570 nm. Since no significant effect was observed when the measurement was made, the concentrations were changed. The study was repeated by changing the concentrations to 1000, 500, 250, 125 µg/ml, taking into account the molecular weight of the plant we used in our study and the compounds in its content, and significant results were observed.

Antioxidant Activity

2,2-Diphenyl-1-Picrylhydrazil (DPPH) Radical Scavenging Capacity Method

Antioxidant activity of 100, 50, 25 and 12.5 mg/ml concentrations of *M. pulegium* methanol extract 2,2-diphenyl-1-picrylhydrazil (DPPH) was determined according to the radical scavenging capacity method. The solution was prepared in methanol at concentrations of 100, 50, 25 and 12.5 mg/ml from the lyophilized drug. 100 µl of the prepared solution was taken and 100 µl of DPPH solution was added. 100% Ascorbic acid was used as positive control and 100% methanol was used as negative control. Each concentration was studied in 3 repetitions, then mixed until a homogeneous mixture was obtained and 30 minutes after the mouth was closed. The same procedure was repeated for all concentrations. At the end of this period, the absorbances of each mixture were read in the spectrophotometer at 570, 540 and 492 nm, and the % inhibition values were calculated.

Apoptotic/Necrotic Activity

Double staining (Hoechst 33258 and Propidium Iodide) method

This step; apoptotic and necrotic activity was tested using dual staining (Hoechst 33258 and Propidium Iodide) technique.

The basis of this method is; It is based on the ability of fluorescent dyes to bind to DNA, making the chromatin and thus the nucleus of the cell visible. Hoechst 33258 (HO) dye is a dye that can bind to DNA and thus penetrate through the cell membrane. It is used to stain the nuclei of living and dead cells. Propidium iodide (PI) stain can only be taken up by cells with impaired membrane integrity, thus allowing detection

of late apoptotic / necrotic cells. For apoptotic cells, features such as smaller (pycnotic) and/or fragmented nuclei are sought compared to normal cells, while for necrotic cells, the nucleus is slightly larger than normal cells and has less staining. Thus, in the absence of pycnotic and/or fragmented nuclei: cells with HO (+) / PI (-) observed; Alive. In the presence of pycnotic and/or fragmented nuclei: cells with HO (+) / PI (-) observed; early apoptotic. In the presence of pycnotic and/or fragmented nuclei: cells with HO (+) / PI (+) observed; late apoptotic or secondary necrotic. In the absence of pycnotic and/or fragmented nuclei: cells with HO (+) / PI (+) observed; It was evaluated as necrotic (HO dye blue, PI dye red) and apoptotic/necrotic activity was determined in this context (Cevatemre, 2012).

Application of the double staining (Hoechst 33258 and Propidium Iodide) method

A549 cell line, $10x10^3$ cells in 2 ml DMEM (1% L-Glutamine, 1% Penicillin-Streptomycin and 10% FBS (Fetal Bovine Serum)) in a 6-well plate planted. Then, it was incubated for 24 hours at 37˚C under 5% CO_2 atmospheric conditions. After the incubation, the medium in the wells was emptied and 2 ml of medium containing 1000 µg/ml *M.pulegium* extract was added and left to incubate for 48 hours. After incubation, the medium was removed from the wells and the cells were washed with PBS, then 2 ml of the solution prepared with 5-10 µg/ml HO and 1 µg/ml PI dyes (the dye solution was prepared in 1x PBS (without Ca/Mg)) was added. The plate was then incubated at 37 ˚C in the dark with 5% CO_2 5 for 30 minutes. After incubation, cell morphologies were compared with control groups (negative control: no drug-treated cells, positive control: 2.5 µg/ml Doxorubicin-treated cells) and evaluated under a fluorescent microscope.

RESULTS

Since the blooming aerial part of *M. pulegium* is widely used due to its antiseptic properties, this study was carried out to use methanol, acetone and chloroform extracts of the aerial parts of *M. pulegium* as antioxidant, antibacterial, investigated its antifungal and anticancer properties.

Antimicrobial activity

The antimicrobial activity of *M. pulegium* extract is shown in tables 1, 2 and 3.

In the acetone extract of *M. pulegium*, the highest inhibition zone was observed in *K. pneumoniae* with 13±0.44 mm at 100 mg/ml concentration and in *C. albicans* as 13±0.55. The lowest inhibition zone of *C. albicans* was measured as 10 mm at a concentration of 10±0.32 mg/ml (Table 1).

Table 1. Antimicrobial effect of *M. pulegium* acetone extract (zone diameters mm)
Çizelge 1. *M. pulegium* aseton ekstraktının antimikrobiyal etkisi (zon çapları mm)

Microorganisms	100 mg/ml	50 mg/ml	25 mg/ml	5 mg/ml	Clindamycin	Amoxicillin
E. coli	9±0.13	11±0.11	-	-	22 ± 1.65	-
K. pneumoniae	13±0.44	-	-	-	21 ± 1.24	11 ± 1.21
S. aureus	11±0.16	-	-	-	23 ± 1.88	-
B. megaterium	-	-	-	-	20 ± 1.63	-
C. albicans	13±0.55	10±0.32	-	-	-	-

In the chloroform extract of *M. pulegium*, the highest inhibition zone was seen in *C. albicans* as 11±0.13 mm at 100 mg/ml concentration, and the lowest inhibition zone was 9±0.08 mm in *E. coli* (Table 2).

It was observed that the methanol extract of *M. pulegium* formed effective zone diameters in all microorganisms at 4 different concentrations. While the highest inhibition zone was observed as 25±1.75 mm at 100 mg/ml concentration in *B. megaterium*, the lowest inhibition zone was measured as 13±0.17 mm at 5 mg/ml concentration (Table 3).

Table 2. Antimicrobial effect of *M. pulegium* chloroform extract (zone diameters mm)
Çizelge 2. *M. pulegium* kloroform ekstraktının antimikrobiyal etkisi (zon çapları mm)

Microorganisms	100 mg/ml	50 mg/ml	25 mg/ml	5 mg/ml	Klindamisin	Amoksisilin
E. coli	9±0.08	-	-	-	22 ± 1.65	-
K. pneumoniae	-	-	-	-	21 ± 1.24	11 ± 1.21
S. aureus	-	-	-	-	23 ± 1.88	-
B. megaterium	-	-	-	-	20 ± 1.63	-
C. albicans	11±0.13	10±0.11	-	-	-	-

It has been observed that *M. pulegium* methanol extract has an effective antimicrobial activity on the tested microorganisms and at certain concentrations. Among the positive controls, clindamycin; While it created a zone of 22±1.65 mm diameter against *E.coli*, amoxicillin did not show any effect against the same

bacteria. Clindamycin; formed a zone of 21 ± 1.24 mm against *K. pneumoniae* and amoxicillin with a diameter of 11 ± 1.21 mm against the same bacterium (Figure 1).

Table 3. Antimicrobial effect of M. pulegium methanol extract (zone diameters mm)
Çizelge 3. M. pulegium metanol ekstraktının antimikrobiyal etkisi (zon çapları mm)

Microorganisms	100 mg/ml	50 mg/ml	25 mg/ml	5 mg/ml	Klindamisin	Amoksisilin
E. coli	24±0.57	21±1.15	20±1.32	10±0.09	22 ± 1.65	-
K. pneumoniae	22±0.57	20±0.81	19±1.17	8±0.13	21 ± 1.24	11 ± 1.21
S. aureus	23±1.75	20±1.17	15±0.14	9±0.15	23 ± 1.88	-
B. megaterium	25±1.75	17±0.11	15±0.17	13±0.17	20 ± 1.63	-
C. albicans	19±0.47	16±0.10	16±0.67	12±0.14	-	-

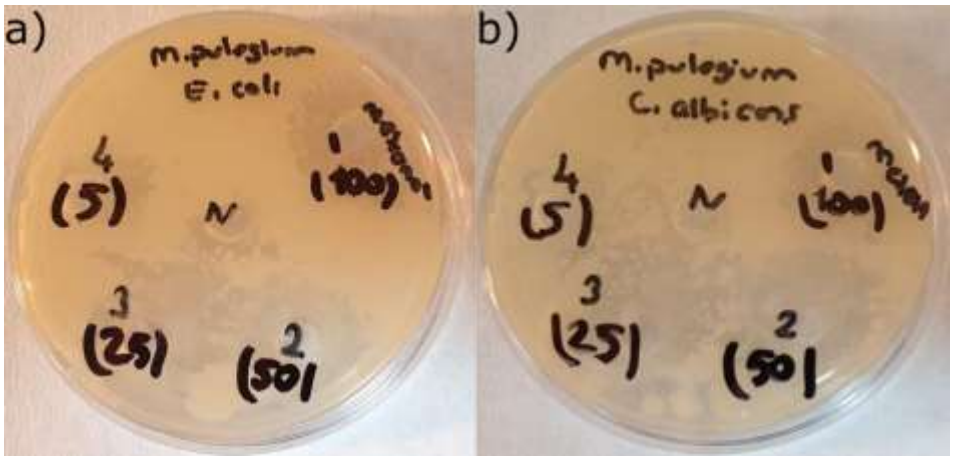

Figure 1. (a) Zones of inhibition of *M. pulegium* extract of *E.coli* in methanol solvent (b) Zones of inhibition of *M. pulegium* extract of *C. albicans* in methanol solvent.
Şekil 1. a) *M. pulegium* ekstraktının *E.coli*'nin metanol çözücüsündeki inhibisyon zonları (b) *M. pulegium* ekstraktının *C. albicans*'ın metanol çözücüsündeki inhibisyon zonları.

MIC and MBC activity

The most significant MIC value (512 µg/ml) in *M.pulegium* extract was observed on *E.coli with acetone and chloroform extracts*. The best MIC value (512 µg/ml) in the extract with methanol was seen in *K. pneumonia*. The MIC value (512 µg/ml) in the chloroform extract had a great effect on inhibiting the growth of *B. megaterium*. As a result of the antimicrobial study of *M. pulegium*, it was observed that different extracts gave an effective result even at low concentrations (Table 4).

Table 4. Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values of *M. pulegium* extracts (µg/ml)
Çizelge 4. *M. pulegium* ekstraktlarının minimum inhibisyon konsantrasyonu (MIC) ve minimum bakterisidal konsantrasyon (MBC) değerleri (µg/ml)

Microorganism	*E.coli*		*K.pneumonia*		*S.aureus*		*B.megaterium*		*C.albicans*	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Acetone extract	512	1024	256	1024	128	256	256	512	512	1024
Chloroform extract	512	512	256	512	256	512	512	1024	512	2048
Methanol extract	64	512	512	512	32	256	32	64	256	1024

Antioxidant activity

The antioxidant activity of *M. pulegium* reduced DPPH by approximately 15% at 570 and 540 nm and 62% at 492 nm at a concentration of 100 mg/ml. It reduced DPPH by approximately 33% at 50 mg/ml concentration, and by approximately 20-34% at 25 and 12.5 mg/ml concentrations, that is, it showed antioxidant activity (Figure 2).

Cytotoxic activity

Medicinal plants are important sources of chemotherapeutic drugs and are used in the prevention and treatment of cancer. (Top et al., 2019) For this reason, the cytotoxic activity of *M.pulegium* species from the Labiatae family was investigated on human breast cancer (MDA-MB-231), human pancreatic cancer (PANC1), human ovarian cancer (OVCAR3) and human lung cancer (A549) cell lines.

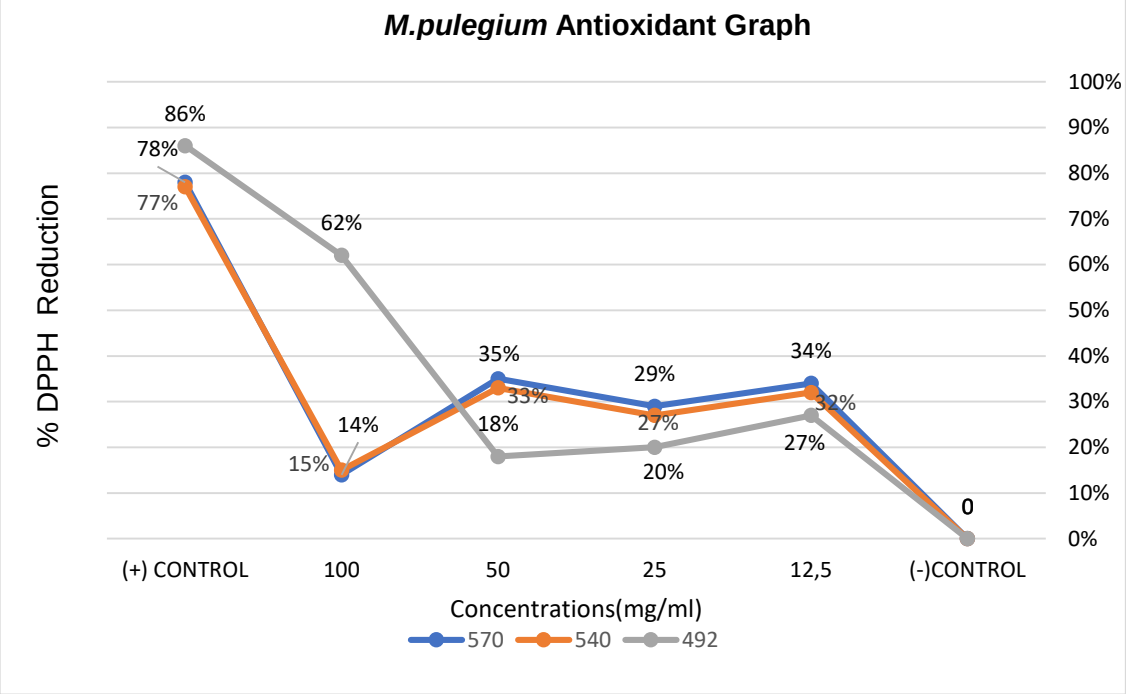

Figure 2. The result of DPPH analysis with *M. pulegium* methanol solvent measured at 3 different nm. (+ control: Ascorbic acid, - control methanol)

Şekil 2. *M. Pulegium* metanol çözücüsü ile 3 farklı nm' de ölçülmüş DPPH analizi sonucu. (+ control: Askorbik asit, -control:metanol)

The results showed that *M. pulegium* extract has a cytotoxic effect against breast cancer cell line. The most effective cytotoxicity was determined as 29.5% total viable cell percentage at 1000 µg/ml concentration prepared with chloroform. The lowest viable cell rate was observed as 110.7% at 250 µg/ml concentration. In the extract prepared with acetone, 50.9% cell viability was determined at 250 µg/ml. The best result of *M. pulegium* methanol extract on MDA-MB 231 cell viability was 51.8% at 1000 µg/ml concentration (Figure 3).

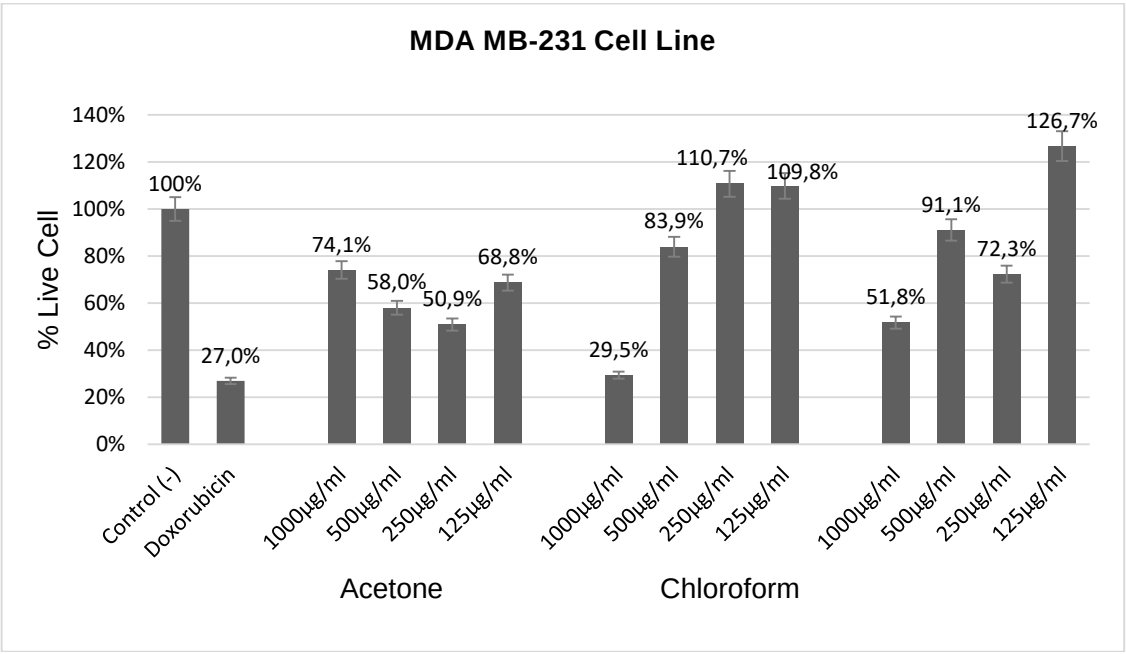

Figure 3. Cytotoxic activity of *M. pulegium* on MDA MB-231 cell line

Şekil 3. MDA MB-231 hücre hattı üzerinde *M. pulegium*'un sitotoksik aktivitesi.

The results showed that *M. pulegium* extract has cytotoxic effect against OVCAR3 cell line. The best cell viability rate in the extract prepared with acetone solvent was determined as 9.9% total viable cell percentage at 250 µg/ml concentration. In chloroform extract, the best cell viability rate was determined as 4.4% total viable cell percentage at 1000 µg/ml concentration. In the extract of *M. pulegium* prepared with methanol solvent, cell viability was observed depending on the dose. The percentage of total cell viability was determined as 9.4%, 24.3%, 53.6% and 78.5% at 1000, 500, 250 and 125 µg/ml concentrations, respectively (Figure 4).

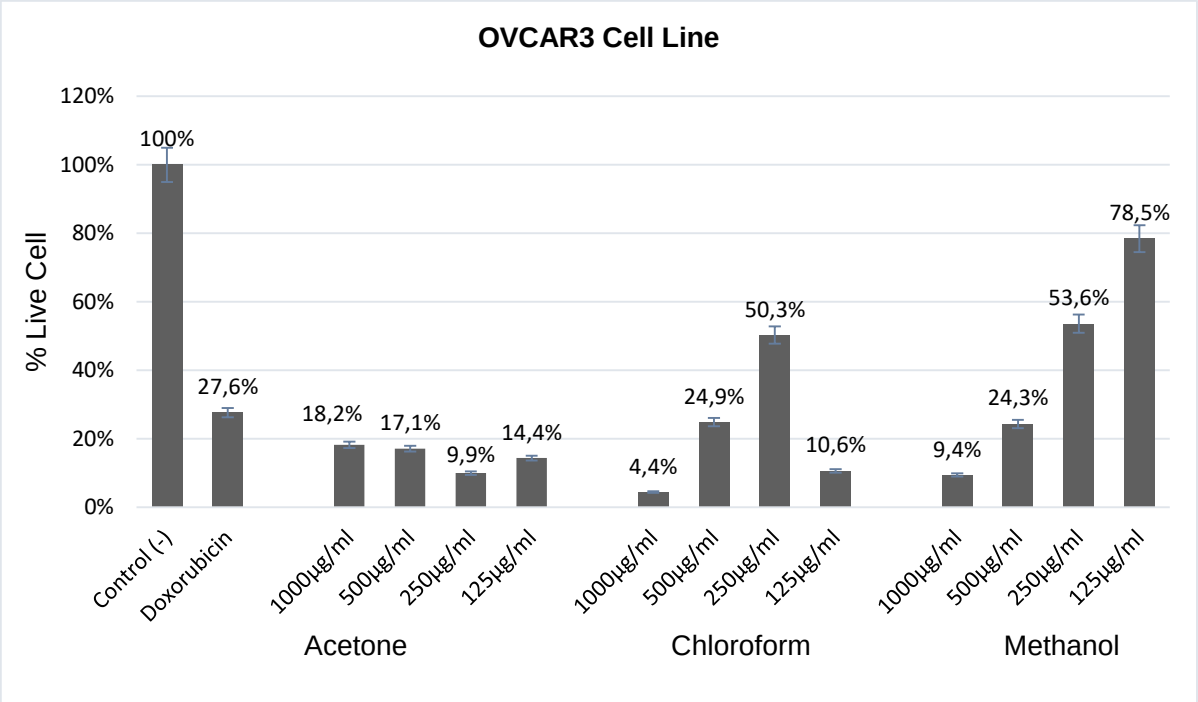

Figure 4. Cytotoxic activity of *M. pulegium* on the OVCAR3 cell line
Şekil 4. *M. pulegium*'un OVCAR3 hücre hattı üzerindeki sitotoksik aktivitesi

In the extract of *M. pulegium* prepared with chloroform solvent, dose-dependent cell viability was observed against the PANC1 cell line. While 43.0% cell viability was observed at 1000 µg/ml concentration of chloroform, it was observed as 97.3% total viable cell percentage at 125 µg/ml concentration. In the extract prepared with acetone, approximately 42% of total cell viability was observed. The best cytotoxic activity in the methanol extract was observed at a concentration of 1000 µg/ml with a total viable cell ratio of 46.2% (Figure 5).

The best cytotoxic activity in acetone solvent against A549 cell line of *M. pulegium* was seen as 20.4% total viable cell percentage at 500 µg/ml concentration. The best cell viability percentage in chloroform was seen as 5.7% at 250 µg/ml concentration. In the methanol extract, on the other hand, the best cytotoxic effect was observed at 500 µg/ml concentration as a percentage of 16.1% total viable cells (Figure 6).

The best cytotoxic activity in acetone solvent against A549 cell line of *M. pulegium* was seen as 20.4% total viable cell percentage at 500 µg/ml concentration. The best cell viability percentage in chloroform was seen as 5.7% at 250 µg/ml concentration. In the methanol extract, on the other hand, the best cytotoxic effect was observed at 500 µg/ml concentration as a percentage of 16.1% total viable cells (Figure 6).

Apoptotic/Necrotic Activity Results

The result of the double staining experiment showed parallelism with the MTT test and a decrease in cells was observed. The results of this test performed to determine the mechanism of this decrease in cells are given in Figure 7.

In line with these results; In the A549 cell line, (-) control cells remained healthy and their number increased. HO stain (-), a dye that can pass through all membranes and stain the nucleus, stained the cells in the control group, but the PI dye, which can stain cells with impaired membrane integrity and necrosis, could not stain this group. For this reason, the cells remained viable and reproduced as expected. Likewise, the cells in the (+) control group, that is, the cells treated with doxorubicin drug, were clearly decreased in number and density and stained with PI dye, which gave us the result that doxorubicin killed the cells by necrosis.

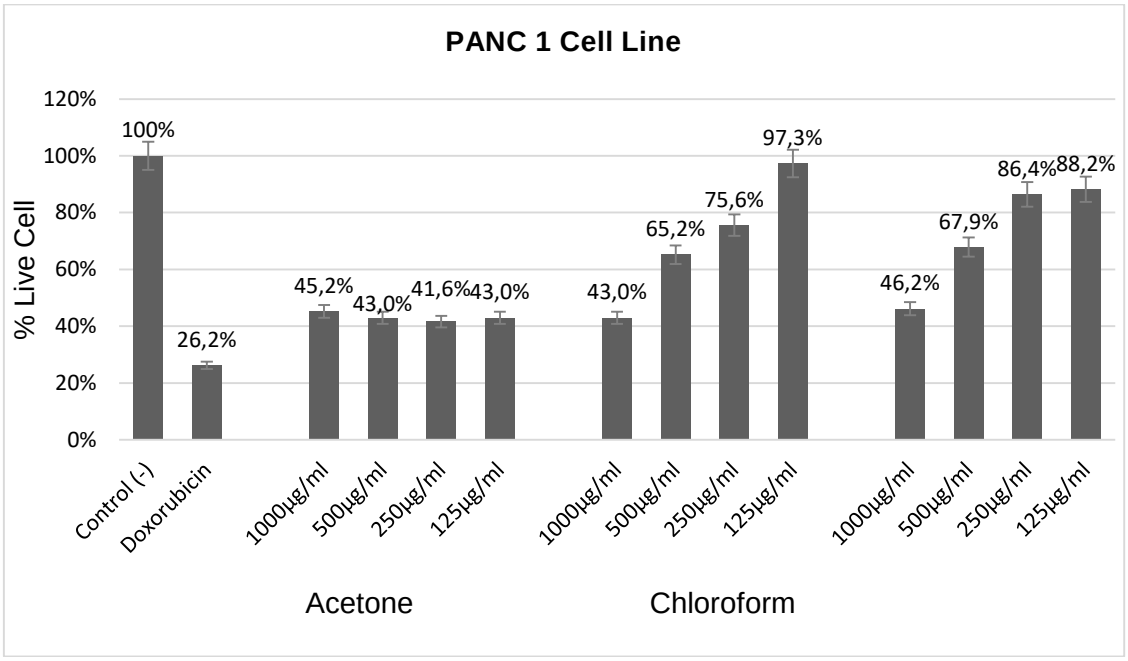

Figure 5. Cytotoxic activity of *M. pulegium* on PANC 1 cell line
Şekil 5. *M. pulegium*'un PANC 1 hücre hattı üzerindeki sitotoksik aktivitesi

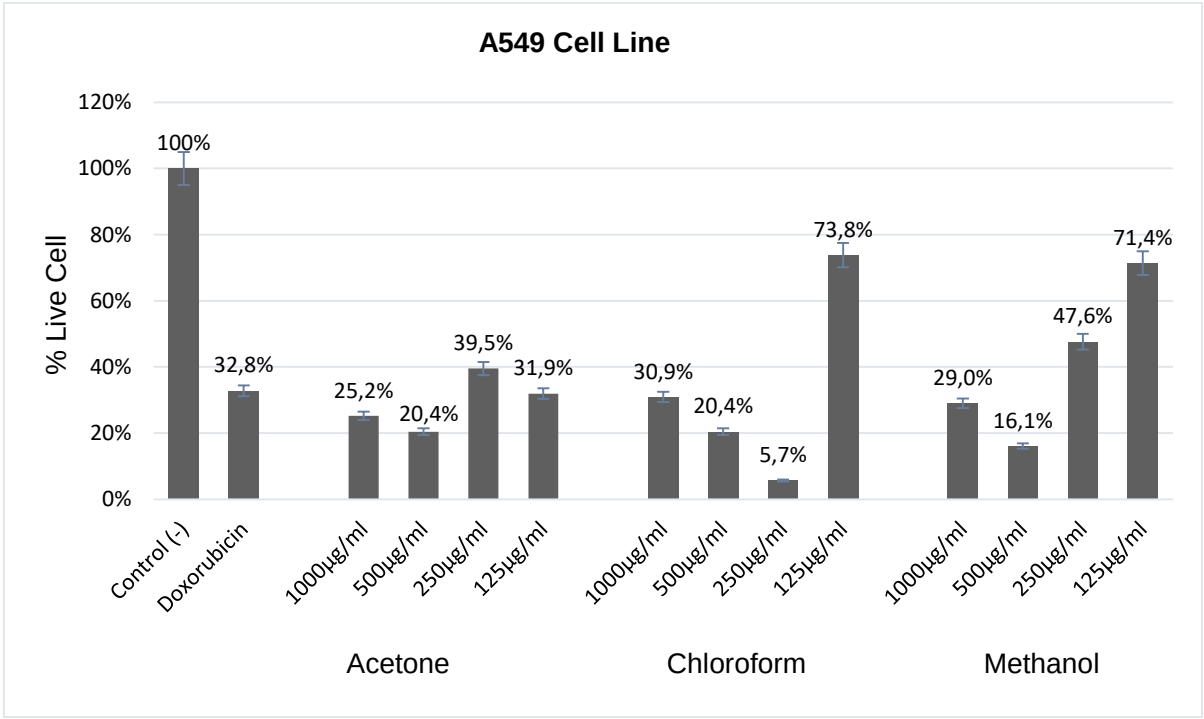

Figure 6. Cytotoxic activity of *M. pulegium* on A549 cell line
Şekil 6. *M. pulegium*'un A549 hücre hattı üzerindeki sitotoksik aktivitesi

Table 5. MDA-MB 231 cell IC50 results
Çizelge 5. MDA-MB 231 hücresi IC50 sonuçları

	MDA-MB 231 cells		
	Mentha pulegium		
	Aseton ekstrat	**Kloroform ekstrat**	**Metanol ekstrat**
IC_{50}	452 µg/ml	125 µg/ml	235µg/ml

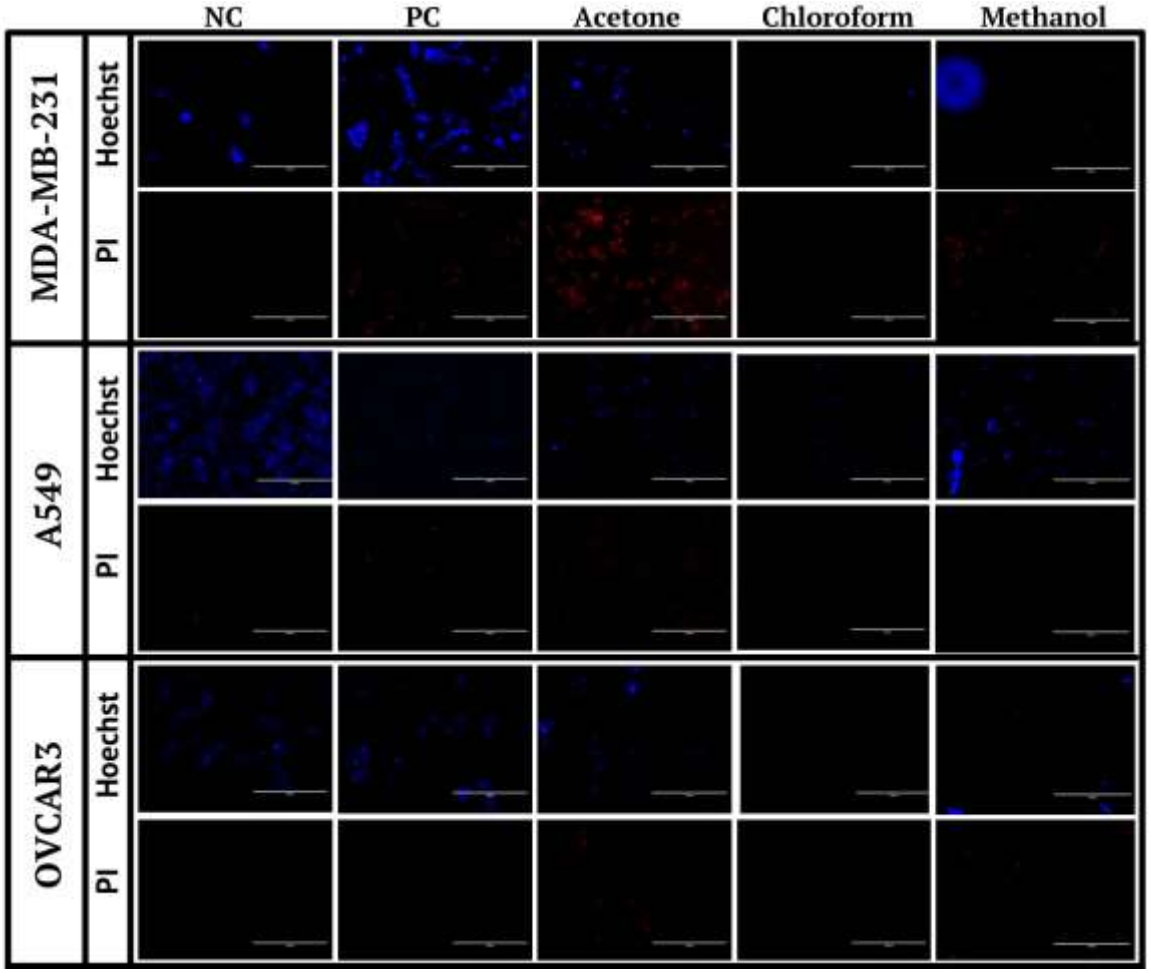

Figure 7. Dual staining apoptic/necrotic activity assay images of *M. pulegium* on MDA-MB 231, A549 and OVCAR3 cell lines

Şekil 7. MDA-MB 231, A549 ve OVCAR3 hücre hatları üzerinde M. pulegium'un ikili boyama apoptik/nekrotik aktivite deneyi görüntüleri

Table 6. OVCAR3 cell IC50 results

Çizelge 6. OVCAR3 hücresi IC50 sonuçları

	OVCAR3 cells		
	Mentha pulegium		
	Aseton ekstrat	**Kloroform ekstrat**	**Metanol ekstrat**
IC_{50}	156 µg/ml	482 µg/ml	483 µg/ml

Table 7. IC50 results for cell PANC1

Çizelge 7. PANC1 hücresi için IC50 sonuçları

	PANC1 cells		
	Mentha pulegium		
	Aseton ekstrat	**Kloroform ekstrat**	**Metanol ekstrat**
IC_{50}	129 µg/ml	500 µg/ml	272 µg/ml

Table 8. IC50 results for cell A549

Çizelge 8. A549 hücresi için IC50 sonuçları

	A549 cells		
	Mentha pulegium		
	Aseton ekstrat	**Kloroform ekstrat**	**Metanol ekstrat**
IC_{50}	225 µg/ml	303 µg/ml	488 µg/ml

In *M. pulegium* extracts applied to cells, the most prominent result was seen in methanol extract. The vesicle shapes and dispersed membrane seen in the cells represent apoptotic morphology. Likewise, not staining with PI dye showed that these cells died by apoptosis. In addition, the cells stained with HO dye in the acetone extract did not give apoptotic morphology and were not stained with PI dye, indicating that the cells remained viable or were early apoptotic cells. On the other hand, the cells to which chloroform extract was applied decreased in number but were not fully stained with either dye, indicating that the cells could be secondary necrotic or viable cells.

IC50 values (50% inhibition of cell growth) were calculated based on the MTT assay results as shown in table 5, table 6, table 7 and table 8. The lowest IC50 value in MDA-MB231 cell line was observed in chloroform extract (Table 5.), while the lowest IC50 value in OVAR3, PANC1 and A549 cell lines was given by acetone extract (Table 6., Table 7., Table 8.).

DISCUSSION and CONCLUSION

Plant species were used as traditional medicines for the treatment of many diseases, especially infectious diseases (Gonzalez-Tejero et al., 2008). Considering the biological potential of plants as a source of antimicrobial drugs, the antimicrobial activity of *M. pulegium* was tested with acetone, chloroform and methanol solvents in this study. In this study, antimicrobial effects of acetone, chloroform and methanol extracts of *M. pulegium* against *K. pneumoniae*, *E. coli*, *S. aureus*, *B. megaterium* and *C. albicans* were determined with agar wells. In addition to antimicrobial activity, minimal inhibition concentration and minimal bactericidal concentration levels were also examined.

Of the five Lamiaceae species; It has been reported that *Mentha piperita, Mentha pulegium, Lavandula angustifolia, Satureja montana* and *Salvia lavandulifolia* have significant antimicrobial effects against some bacteria (*Streptococcus mutans, Streptococcus sanguis, Streptococcus salivarius, Streptococcus pyogenes, Pseudomonas aeruginosa, Lactobacillus acidophilus,* and *Enterecoccus feacalis*) (Nikolic et al., 2014).

In another study, Marzouk et al. (2008) defined the chemical composition of fresh and dried *M. pulegium L.* essential oils. All oils were found to be rich in oxygen monoterpene hydrocarbons. In *M. pulegium* applied to microorganisms, gram-positive species are more sensitive to essential oils from fresh leaves collected in the vegetative state than dried ones (Morzouk et al., 2008). Marzouk et al. found the maximum inhibition zones of fungal species sensitive to the essential oil of *M. longifolia* and *M. pulegium* in the range of 9-15 and 8-25 mm, respectively.

Some researchers investigating the antimicrobial effect of plant extracts have used a wide variety of solvents for different parts of different plants (Cowan, 1999). The main solvents among these are solvents such as acetone, ethanol, n-hexane, methanol, chloroform and methylene chloride (Caceres et al., 1990). It is not possible to make a preference order for all plants and plant parts among these solvents. However, considering the studies in the literature, acetone, chloroform and methanol were preferred as solvents in this study, which is generally used extensively and considering the positive results. In this study, *E. coli* formed an inhibition zone of 24, 21, 20 and 10 mm in length in the methanol extract of *M. pulegium* at concentrations of 100, 50, 25 and 5 mg/ml, respectively. The highest inhibition zone was found to be 25 mm in *B. megaterium*. Based on these results, it is possible to conclude that the essential oil has a stronger antimicrobial activity spectrum than the acetone and chloroform extract compared to the methanol extract.

In many studies, DPPH has been widely used to determine antiradical/antioxidant capacities (Soylu and Çebi, 2017; Mensor et al., 2001; Sharma and Bhat, 2009). In the present study, the antioxidant activity of *M. pulegium* essential oil and methanol extract was determined by three different test systems: DPPH, ascorbic acid and reducing power. It was concluded that *M. pulegium* plant has high bioactive components and depending on these values, it can be used as a potential antioxidant source in the pharmaceutical and food industries. It is important to carry out more studies on the use of this plant in the prevention and treatment of diseases, rich in bioactive components, which have important effects on health, and also to ensure the continuity of the researches towards industrial applications by determining the contributions of this plant in food compositions.

Since cancer is a disease for which there is no cure yet, the development of therapeutic drugs is considered in a special status by international health organizations. Medicinal plants have long been a fruitful resource for the treatment of cancer, which is predicted to be the main cause of death this century (Shoeb, 2006; Tariq et al., 2017). In the study, the cytotoxic activity of three extracts (acetone, chloroform, methanol) obtained from the above-ground part of *M. pulegium* was determined by MTT test in MDA MB-231, PANC-1, OVCAR3 and A549 cell lines. It was determined that *M. pulegium* extract had a cytotoxic effect against MDA-MB-231, PANC1, OVCAR3 and A549 cells at applied concentrations. It was determined that especially *M. pulegium* methanol extracts had a significant cytotoxic effect on the OVCAR3 cell line. It was observed that the solvent that showed the most important effect

among the solvents was the chloroform solvent.

Ovarian cancer (OC) is responsible for the highest tumor-related mortality among gynecological malignancies (Woopen and Shrouli, 2009). In most OC patients diagnosed with advanced stages (III and IV), this creates panic and provokes an emergency to explore a new therapeutic strategy. Plants with medicinal properties attract attention because they are enriched with various chemical compounds that have the potential to treat various diseases. Providing innovative and important clues to a range of pharmacological targets for a human disease management system is a long process (Ebona et al., 2018; Dutta et al., 2018). Although the development of a new drug has faced challenges and difficulties, the emergence of combinatorial chemistry provides a new glimmer of hope, and the effort to discover the drug and a chemical compound has been quite successful (Liu et al., 2001). By incorporating new cytotoxic agents, the standard platinum-based treatment for ovarian cancer Numerous studies have been conducted to increase its effectiveness (Buys et al., 2005). The combination of natural compounds with standard chemotherapeutic drugs may exert additive or synergistic effects in killing cancer cells, thus achieving better therapeutic effect or allowing lower and safer drug doses to be administered (Coşkun, 2017).

As a result of the study, it was observed that *M. pulegium* extract may have active substances that can be used in the development of new drugs and that it has a cytotoxic effect. Obtained IC50 values are 452 µg/ml, 125 µg/ml and 235 µg/ml in MDA-MB 231 cells, 225 µg/ml, 303 µg/ml and 488 µg/ml in A549 cells, 129 µg/ml , 500 µg in PANC1 cells /ml and 272 µg/m were determined as 165 µg/ml, 482 µg/ml and 272 µg/ml in OVCAR3 cells, showing that *M. pulegium* has a promising growth inhibitory effect on cancer cells.

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Contribution Rate Statement Summary of Researchers

The authors declare that they have contributed equally to the article.

Conflict of Interest Statement

The authors of the article declare that there is no conflict of interest between them.

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