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Anticancer potential of *Origanum munzurense* extract against MCF-7 breast cancer cell

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ABSTRACT

Origanum munzurense (*O. munzurense*) is an endemic species of Tunceli region of Turkey. In this study, we investigated *in vitro* anticancer effect of aqueous extract of *O. munzurense* (OME) on breast cancer cells (MCF-7). *In vitro* cytotoxic effect of OME was evaluated in MCF-7 cells by MTT assay. The wound-healing assay was used to examine the inhibition of migration. Annexin V/propidium iodide staining, cell-cycle distribution was assessed by flow cytometry for MCF-7 cells treated with OME. MTT results show that OME demonstrated in vitro cytotoxicity with 600 mg at 48 h on MCF-7. Doses of 400 µg/mL and 600 µg/mL OME significantly suppressed the migration rate of MCF-7 cells. OME significantly decreased the percentage of live cancer cells and showed an apoptotic effect after 48 h of incubation. These results show that OME is effective against breast cancer when administered at high doses and for a long time.

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KEYWORDS
Origanum munzurense; MCF-7; cytotoxicity; apoptosis; anticancer

1. Introduction

Phytotherapy is a discipline of medicine that uses plants to treat disease or as health-healing agents. It is known as herbalism in Western medicine. This treatment method is used in a wide area, for instance, after chemotherapy treatment, respiratory infections, symptom relief in cold and influenza, symptom inflammatory, neuropathic pain, depression and many chronic illnesses (Efferth et al. 2017). Traditional use in phytotherapy preserves the general composition of the source plant. Thus, the whole plant or desired components can be used for medicinal purposes. The more traditional use of phytotherapy often includes the whole part of the plant as a herb tea, whereas Western herbal medicine more commonly uses single herbs standardized to a component of the extract. These whole plants or their extract are used in many fields of medicine.

Most of the plants of the Lamiaceae family are used for the treatment of various diseases in alternative medicine as a therapeutic agent. There are 200 genera and 4000 species of the Lamiaceae family being worldwide. This family is represented by 46 genera and 575 species in Turkey. *Origanum, Satureja* and *Thymus* genera are added to foods for their organoleptic properties and are often consumed as herbal tea in Turkey in the treatment of indigestion, infectious diseases, asthma, cramping, diarrhoea, muscle pain, nausea and rheumatism. According to several preclinical studies, many species of the Origanum genus evidenced their pharmacological potential as antiproliferative or anticancer, antidiabetic,

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antihyperlipidemic, anti-obesity, renoprotective, anti-inflammatory, vasoprotective, cardioprotective, antinociceptive, insecticidal, and hepatoprotective properties (Sharifi-rad et al. 2021). *O. munzurense* is an endemic species found in Turkey. There are five genera (*Origanum L., Satureja L., Coridothymus Rchb.f., Thymbra L. and Thymus L.*) of the Lamiaceae family, which are commonly known as thyme, and the majority of the thyme taxa belong to the genus *Origanum. O. munzurense* is rich in chemical components. Carvacrol, thymol and cymene, known as active constituents that have beneficial chemical effects, were detected in the extracts of *O. munzurense* (OME) (Erdal et al. 2020). In recent years, many studies have been performed on the anticancer effect of these active ingredients. Ezz-Eldin et al. (2020) reported that, as a natural antioxidant compound, carvacrol has inflammatory relief effects, antimicrobial, antihepatotoxic, antitumor, neuroprotective and cardioprotective properties (Ezz-Eldin et al. 2020).

It has been reported that carvacrol has strong cytotoxic, genotoxic and apoptotic effects against cancer cells also and has effects on cell invasion by reducing metalloproteases 2 and 9 expressions (Fan et al. 2015).

In another study examining thymol and thyme herb, it has been reported that this plant and its volatile oil have long used for a long time in the treatment of upper respiratory tract infections, bronchitis symptoms and parasitic infections etc (Kowalczyk et al. 2020). Additionally, thymol also shows antimicrobial, antioxidant, anti-carcinogenic, anti-inflammatory, and antispasmodic activities (Salehi et al. 2018). According to Santos et al. (2019), cymene, which has an antinociceptive effect in oncological diseases, also shows anti-inflammatory and anticarcinogenic properties (Santos et al. 2019).

Worldwide, cancer deaths are the second leading cause of death after cardiovascular diseases. Breast cancer is the most common female malignancy and consists of 24% of cancers in women. As in the world, breast cancer is the most common type of cancer in women in Turkey and has a high mortality rate (Ergin et al. 2019).

Today, surgical methods, chemotherapy and radiotherapy are used in cancer treatment. These methods have many disadvantages such as nausea, vomiting, hair loss, blood cell disorders caused by damage to the bone marrow, severe weakness, skin disorders, etc. Natural herbs produced as alternative medicines are preferred for including lesser side effects. Many herbs and herb oil play an important role in medicine and herbals, which contain biologically active compounds, have been shown to have potent therapeutic effects in various cancer research. These compounds have gained significant recognition in the field of cancer therapy as anticancer agents. Medicinal plants are one of the main sources of active compounds that are an excellent source of new drugs and do not have major side effects compared to synthetic drugs (Kemp 1994; Dias et al. 2012; Cordell and Colvard 2012).

This study aimed to investigate the effect of OME, an endemic species, which is a candidate for use in medicine, on breast cancer. The choice of MCF-7 is that breast cancers are among the major cancers in Turkey. For this purpose, cytotoxic, apoptotic and wound-healing effects of OME were investigated in MCF7 cells.

2. Material and methods

2.1. Plant extract preparation

The extraction method and the solvent used are among the most important factors in the extraction of various matrices (Yabalak 2018; Erdal et al. 2020; Yabalak et al. 2020). For the extraction of *O. munzurense*, Soxhlet was used as the extraction method and distilled water as solvent. 15 g of grounded and dried *O. munzurense* sample was extracted with 350 mL distilled water for 3 h. The extract obtained was concentrated to 17.5 mL using a rotary evaporator and stored at 4 °C for further steps. The density of the concentrated extract was determined as 49.2 mg/mL dry *O. munzurense*.

2.2. Cell culture

MCF-7 cells (human breast cancer, ATCC HTB-22^{TM}) were used for anticancer activity tests. MCF-7 cells were incubated in RPMI 1640 medium with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum and 2.5 mM L-glutamine (Gibco®, Invitrogen™ GmbH, Karlsruhe, Germany). Incubations were conducted at 37 °C in a humidified atmosphere (5% CO_2, 95% air). The culture medium was changed three times each week.

2.3. MTT assay

The colorimetric MTT assay was performed in triplicate the MCF-7 cells to evaluate the OME cytotoxicity. The MCF-7 cells were incubated into 96 well-plates (5×10^3 cell/well) and cured for 24, 48 and 72 h with OME concentrations of 50 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL and 600 µg/mL. 20 µL MTT solution was added to each well and the plate was wrapped with foil then incubated for 2 h at 37 °C After 2 h, MTT solution was removed from the well plate. The blue-violet formazan formed in the wells was dissolved in 150 µL dimethylsulfoxide (DMSO). DMSO containing plate was kept in dark for 30 min. Absorbance values were read in an Elisa Multiskan Ascent Microplate Reader (Thermo Labsystems, Franklin, MA, USA) set at 570 nm. Untreated cells were used as a negative control of cytotoxicity (representing 100% of mitochondrial activity).

The percentage of mitochondrial activities of cells in each well was calculated using the following equality.

Cell viability (%) = (absorbance of sample/absorbance of control) x 100 (Xing et al. 2019)

2.4. Annexin V-APC/PI staining analysis apoptosis assay

MCF-7 cells were seeded in 6-well plates (1×10^6 cells/well) overnight and treated with OME (12,1 mg/mL) and various concentrations (400 µg/mL and 600 µg/mL) for 48 h and 72 h. The cells (1×10^5 cells/100 µL) were incubated with 5 µL Annexin V-APC of and 10 µL of PI for 15 min in the dark, according to the manufacturer‘s instructions (BioLegend Cat No: 640932). When the incubation process is completed, 400 µL device by adding 1x binding buffer and analysis was performed on BD FacsARIA III Cell Sorter (Becton Dickinson BD, USA) (Al Dhaheri et al. 2013).

2.5. Cell migration effect

The capacity of cell migration was evaluated using an in vitro wound-healing assay. MCF-7 cells were cultured with RPMI-1640 (10% FBS) in a 6-well plate at a concentration of 5×10^6 cells/mL. The cells left on the plate were starved for 24 h to eliminate the effect of proliferation. Micropipette tips were used to scratch to create linear gaps on the plate base. After the scratch, the cells were washed three times with 3 mL of PBS to remove any suspended cells. OME doses (400 µg/mL and 600 µg/mL) were added to the wells in RPMI-1640 (1% FBS) culture medium. The experiments were repeated 3 times. The images were recorded between 0 and 72 hours according to the character of the cell, doubling time and wound closure. The rate of wound healing (%) was calculated using the following equality.

Wound healing (%) = [1 - (scratch with of the OME group/scratch with of the control group)] x 100% (Xing et al. 2019)

2.6. Statistical analysis

All bioactivity studies were carried out in triplicate and results were expressed as means ± SD. Statistical significance between groups was evaluated using one-way ANOVA comparisons.

$p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Cytotoxic effect of OME

Cytotoxic response of OME was evaluated by the MTT assay at 24th, 48th and 72nd hours. MTT assay results are shown in Figure 1. According to our findings both OME doses (400 µg/mL and 600 µg/mL) decreased the percentage of cell viability depending on the time duration. The average of cell viability in 400 µg/mL is 59.7% at 48 h, 77.1% at 72 h and in 600 µg/mL is 45.3% at 48 h and 55.6% at 72 h. It was determined that the highest cytotoxic effect was found in the 600 µg OME dose at 48 h.

Yabalak et al. (2020) reported that OME oil extract contains biologically active substances such as carvacrol, thymol and cymene (Erdal et al. 2020). In this study, the effect of the same extract was investigated. Similar results on the cytotoxicity of the other origanum species such as *Origanum Vulgare* (*O. Vulgare*) against triple-negative breast cancer cell lines have been reported. - Rojoet al. compared the proliferation index of *O. Vulgare* and carvacrol, and they reported that the proliferation index in *O. Vulgare* reduced by 5.95% and in carvacrol reduced by 6.57% (Rojo et al. 2020).

Although there is no study on the cytotoxic effect of OME, there are studies on *O. Vulgare* in which the effect of *O. Vulgare* was investigated on different cancer cell lines, as in colon cancer (HT-29), melanoma (A375) and hepatocarcinoma (HepG2) and significant decline in cell viability was found in a dose-dependent (Vokou et al. 1993; Arunasree 2010; Thomford et al. 2018). It has been reported that breast cancer cell lines are more susceptible to origanum cytotoxicity. It was observed that cell viability decreased in breast cancer cells in a study reported by Makrane et al., in which cytotoxic effect of *Origanum majorana* (*O. majorana*) compared to colon cancer cells (Makrane et al. 2018). Our MTT findings show that OME is effective at 600 mg at 48 h on MCF-7 cells.

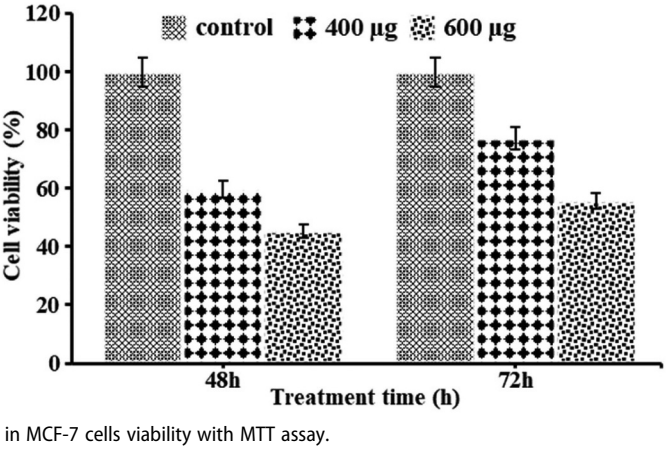

Figure 1. Effect of OME in MCF-7 cells viability with MTT assay.

Table 1. Mean values of cells, primary apoptosis, secondary apoptosis and necros for 48 h live.

Groups (n=3)	Control	400 µg/mL	600 µg/mL	p_{48}
Live Cells	94.60 ± 5.26	45.70 ± 5.65	31.67 ± 5.26	<.001*
Primary apoptosis	0 ± 0	1.37 ± 0.64	1.30 ± 0.10	0.007*
Secondary apoptosis	0.07 ± 0.12	19.73 ± 2.65	32.37 ± 9.80	0.002*
Necrosis	5.30 ± 5.40	13.50 ± 4.71	34.60 ± 5.63	0.002*

Data are presented as mean ± SD values. *p < 0.05 is significantly different., p_{48}: One Way ANOVA.

Table 2. Mean values of cells, primary apoptosis, secondary apoptosis and necros for 72 h live.

Groups (n=3)	Control	400 µg/mL	600 µg/mL	P_{72}
Live Cells	91.53 ± 7.62	83.63 ± 3.61	83.07 ± 4.15	0.183
Primary apoptosis	0.20 ± 0.20	0.23 ± 0.06	0.50 ± 0.10	0.062
Secondary apoptosis	1.17 ± 1.15	2.67 ± 1.70	3.93 ± 1.62	0.160
Necrosis	7.17 ± 6.35	13.50 ± 4.71	12.47 ± 2.89	0.303

Data are presented as mean ± SD values. *p < 0.05 is significantly different, p_{72}: One Way ANOVA.

3.2. Apoptotic effect of OME

For subsequent experiments, only OME with the highest concentrations (400 µg/mL and 600 µg/mL) was used (Tables 1 and Tables 2). For the MCF-7 cells, all tested OME significantly decreased the percentage of live cancer cells and an apoptotic effect was recorded after 48 h of incubation. (Table 1). During the 48 h experiment, the percentage of MCF-7 cells with live phenotype decreased significantly and all OME induced apoptosis but 72 h MCF-7 cell, there was a not significant decrease in the percentage of live cells, primary apoptosis, secondary apoptosis and necrosis in other groups compared to control (Table 2). After 72 h treatment, increase in viable cell mean and decrease in secondary apoptosis mean in the 400 µg/mL group and the 600 µg/mL group, the increase in the mean percentage of living cells, the decrease in the percentage of primary apoptosis, secondary apoptosis and necrosis were found to be significant compared to 48 h (Table 3). These findings indicate that during the 48-h experiment, the percentage decrease in the number of viable phenotype MCF-7 cells and apoptosis was seen to be significantly induced by the whole OME doses.

Apoptosis can be induced either by the extrinsic pathway or by the intrinsic pathway (Konopleva et al. 1999). In the activation of death receptor-mediated apoptosis, ligands such as TNF-α and Fas must interact with transmembrane receptors. This ligand–receptor interaction activates effector caspase 8 and caspase 3. The intrinsic mitochondria-mediated apoptosis pathway is associated with alteration in the expression of anti-apoptotic Bcl2 family members and pro-apoptotic members. Activation of Bax, caspase 9 and consequent activation of caspase 3 leads to apoptosis (Zou et al. 1999). Al Dhaheri et al. reported that *O. majorana* extract was able to inhibit the viability of MDA-MB-231 cells in a time- and concentration-dependent manner. Low concentrations of *O. majorana* extract (150 and 300 µg/mL) caused the accumulation of apoptotic resistant cell population that was

Table 3. Mean values of cells, primary apoptosis, secondary apoptosis and necros for live 48 h and 72 h. .

	48 h	72 h		48 h	72 h		48 h	72 h	
Groups (n=3)	Control	Control	P_{time}	400 µg/mL	400 µg/mL	P_{time}	600 µg/mL	600 µg/mL	P_{time}
Live Cells	94.60 ± 5.26	91.53 ± 7.62	0.658	45.70 ± 5.65	83.63 ± 3.60	0.004*	31.67 ± 5.26	83.07 ± 4.15	0.010*
Primary apoptosis	0 ± 0	0.20 ± 0.20	0.225	1.37 ± 0.64	0.23 ± 0.06	0.079	1.30 ± 0.10	0.50 ± 0.10	0.015*
Secondary apoptosis	0.07 ± 0.12	1.17 ± 1.15	0.212	19.73 ± 2.65	2.67 ± 1.70	0.020*	32.37 ± 9.80	3.93 ± 1.62	0.048*
Necrosis	5.30 ± 5.40	7.17 ± 6.35	0.760	13.50 ± 4.71	13.5 ± 4.71	0.065	34.60 ± 5.63	12.47 ± 2.89	0.010*

Data are presented as mean ± SD values. *p < 0.05 is significantly different, p_{72}: One Way ANOVA. ptime: Paired t-test, ptimexgroup: repeated measurement ANOVA

mitotically arrested and overexpress the cyclin-dependent kinase inhibitor p21 and the apoptosis inhibitor survivin, while higher concentrations (450 µg/mL and 600 µg/mL) caused the accumulation of *O. majorana* extract, induced massive apoptosis along the extrinsic pathway, including activation of tumor necrosis factor-α (TNF-α), caspase 8, caspase 3 (Al Dhaheri et al. 2013). Benhalilou et al. showed that OME triggered abortive autophagy and activated a caspase 3 and 7-dependent extrinsic apoptotic pathway, most likely through a mechanism involving the TNFa pathway in colorectal cancer (Benhalilou et al. 2019). In this study, it was demonstrated OME induces the apoptotic pathway using flow cytometry analysis. Our results are in line with those studies with OME, higher concentrations were more effective in decreasing the proliferation of MCF-7 cells. At the cell proliferation level, 400 µg/mL and 600 µg/mL OME blocked in 48 h. Moreover, the apoptotic and necrotic cell levels were comparable between the groups and in line with the cell proliferation results, higher concentrations of OME were the more effective treatment. However, further studies are needed to determine through which pathway of apoptosis OME induces apoptosis in MCF-7 cells.

The previous study has been shown that *O. majorana* extract exerts an anti-proliferative, anti-metastatic and anti-tumor growth against the human colorectal cancer cells (Benhalilou et al. 2019). Al Dhaheri et al. reported that *O. majorana* concentrations of 450 and 600 µg/mL along the extrinsic pathway, including cleavage of tumor necrosis factor-a (TNF-a), caspase 8, caspase 3, and PARP massive apoptosis is triggered in the breast cancer cell (MDA-MB-231) (Al Dhaheri et al. 2013). Our data reveal the concentration and time duration effect of OME on cell cycle progression of the MCF-7 cells.

3.3. Cell migration effect

Wound healing is a complex cellular and biochemical process that includes dynamic interactions between cells, interaction with extracellular matrix molecules, and regulated production of soluble modulators and cytokines (Grada et al. 2017).

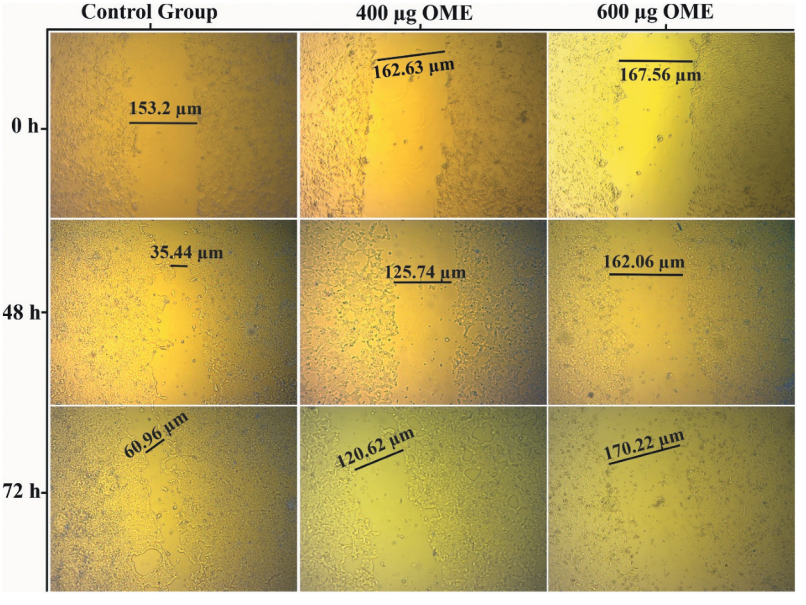

Figure 2. Treatment with OME for 0 h, 48 h and 72 h inhibits the migration of breast cancer cells in a wound-healing assay in a dose-dependent manner.

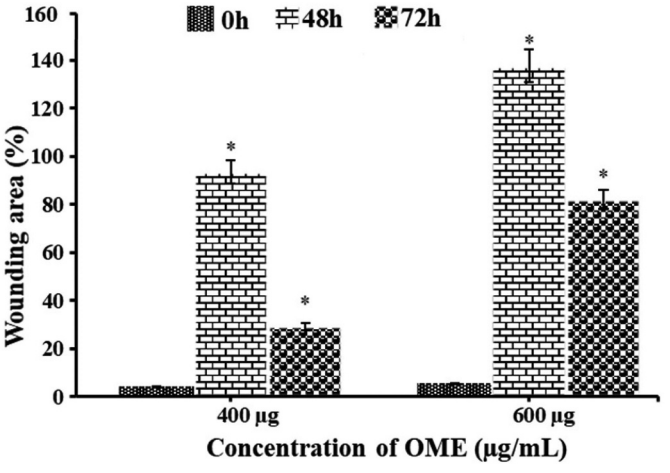

Figure 3. Effect of OME in MCF-7 wound healing assay. *p < 0.05 compared to the control.

Migration and invasion are generally known as important factors in the process of breast cancer metastasis. When observing the migration and invasion of cancer cells, cells undergo remarkable morphological changes (Xing et al. 2019). In this study, the effect of OME on the migratory ability of MCF-7 cells was investigated by wound healing assay. Wound area results at 48 and 72 h after MCF-7 cells were treated with OME are shown in Figure 2 and Figure 3.

According to the results, it was determined that the wound closure of the MCF-7 cells was earlier in the control group than in the 400 µg/mL and 600 µg/mL OME groups. Doses of 400 µg/mL and 600 µg/mL OME significantly suppressed the migration rate of MCF-7 cells. It showed the highest effect at 48 h at 600 µg/mL OME. In vitro cell migration analysis is a useful assay for measuring changes in cell migration capacity of bioactive materials.

Spyridopoulou et al. (2019) examined the anticancer effect of *Origanum onites L.* essential oil in the colorectal cancer cell line (HT-29) and colon tumor (CT26) with wound healing assay. This study's results reported that the *Origanum onites L.* essential oil doses in both HT-29 and CT26 cells, the open area (wound) closed earlier in control cells compared to the cells that were treated (Spyridopoulou et al. 2019).

In another study, Pereira et al. (2020) reported that with wound healing assay the antitumor effect of *Linneu* essential oil extract in vitro on bladder cancer cell RT24. This study demonstrated that the treatment of *Linneu* essential oil extract reduced the process of cell migration in RT4 cells (Pereira et al. 2020). The present study provides novel evidence on the anti-migratory of OME against breast cancer.

4. Conclusion

Origanum species contain a range of secondary metabolites flavonoids, phenolic compounds and numerous essential oil compounds that are known for their antitumor effects. The assays performed in this study demonstrated that OME both has high cytotoxicity against breast cancer cells and induces apoptosis and inhibits the migration and invasion of breast cancer cells. OME has an antitumor effect against breast cancer with the effect of a single compound in its content or the synergistic or strengthening effect of all compounds. Based on the findings discussed above, it should be concluded that OME could be used as a clinical drug or adjuvant drug to help treat breast cancer metastasis.

Highlights

- Anticancer effect of *Origanum munzurense extract on breast cancer cells was investigated.*
- Soxhlet extraction method was performed to obtain aqueous extract of *Origanum munzurense.*
- MTT assay was used to evaluate in vitro cytotoxic effect of OME in MCF-7 cells.
- The inhibition of migration was examined by the wound-healing assay.
- Extracts decreased the percentage of live cancer cells and showed an apoptotic effect.

Abbreviations

FBS: fetal bovine serum
MCF-7: human breast cancer cell line
MTT: 3-4,5-dimethyl-thiazole-2,5-diphenyltetrazolium bromide
OME: extract of Origanum munzurense
PBS: phosphate buffer saline

Disclosure statement

No potential conflict of interest was reported by the author(s).

Authors' contributions

Şeyma Gülnaz Yarlılar Writing, reviewing & editing. Formal analysis, discussing the results, commenting on the manuscript and cell culture analysis.

Erdal Yabalak Writing , reviewing & editing. Formal analysis, discussing the results and commenting on the manuscript , visualisation. Preparing the plant and performing the extraction.

Derya Yetkin: Cell culture analysis.

A. Murat Gizir Review of the initial manuscript

Birgül Mazmancı Designing and supervising Commenting on the manuscript. All authors were informed of the content of the manuscript and agreed to submit it.

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