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Comparison of the cytotoxic effects of different *Origanum majorana* extracts on lung cancer cells

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ABSTRACT: The determination that compounds used for chemotherapeutic purposes in cancer treatment cause cytotoxic effects on healthy cells as well as target cells has led researchers to work on reducing treatment-related toxicity, and accordingly, research on the effectiveness of herbal resources in cancer treatment has gained importance. In our study, ethanol, methanol and chloroform extracts were obtained from the aboveground parts of *Origanum majorana* L. (*O. majorana*) and their cytotoxic effects on A-549 cells (non-small cell lung cancer (NSCLC)) were investigated using MTT test. Additionally, the free radical scavenging activities of extracts were demonstrated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) test. It has been shown that cytotoxic effects of extracts increased depending on concentrations on A-549 cells. It was determined that methanol, ethanol and chloroform extracts of *O. majorana* caused an increase in the activity of lactate dehydrogenase (LDH), and apoptotic enzyme, caspase-3 activity, compared to the control. It has been demonstrated that methanol, ethanol, and chloroform extracts of *O. majorana* had membrane damaging and apoptotic effects on A-549.

KEYWORDS: *Origanum majorana*; apoptosis; antioxidant; DPPH.

1. INTRODUCTION

Lung cancer, one of the most dangerous diseases today, is the cancer that causes the most deaths in men and women [1-3]. More than one million people die from lung cancer every year. Lung cancer accounts for half of cancer-related deaths in men and women [4]. The number of lung cancer cases has increased by 44% in men and 76% percent in women since 1985 [3]. Deaths in other types of cancer other than lung cancer have decreased over the years, the situation is exactly the opposite in lung cancer and it has been observed that there is a 3-fold increase in deaths [5]. The prognosis of lung cancer appears to be worse compared to other types of cancer. The five-year survival rate is less than 15% [6].

There are many different types of lung cancer. However, the most histologically different from each other are Small Cell Lung Cancer (SCLC) and Non-Small Cell Lung Cancer (NSCLC). This distinction between the two cancer subtypes is due to their differences in clinical presentation, metastatic ability, and response to treatment [7].

SCLC is a progressive tumor with a poor clinical course. However, it responds very well to chemotherapy and radiotherapy [8]. Treatment approaches for NSCLC include surgery, radiotherapy, chemotherapy and photodynamic therapy, but all these treatment approaches generally fail [9]. While 5-year survival rate of an NSCLC patient in the 4th stage of the disease is approximately 1%, the average survival time is approximately 7 months [10, 11]. A-549 (NSCLC) we used in our study are adenocarcinoma human alveolar basal epithelial cells.

Türkiye has a rich flora in terms of medicinal plants. *Lamiaceae* is one of the families that make up this flora. *Lamiaceae* family consists of approximately 230 genera and 7100 species worldwide. Many species of this family are of great importance with their use in medicine, food industry and cosmetics. *Origanum majorana* L. *(O. majorana)* belongs to the *Lamiaceae* family. There are different components such as tannins, hydroquinone, flavonoids, phenolic glycosides, phenolic terpenoids, sitosterol, acids (oleanolic acid), triacontan, and cis-sabinene hydrate in the content [12-14]. *O. majorana* oil is safe according to Food and Drug Administration (FDA). It has been reported that extracts from *O. majorana* alleviated the effects of acetylcholine, histamine, serotonin and nicotine [15]. Ursolic acid and *O. majorana* essential oil (especially

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due to its thymol and carvacrol components) have been shown to have different features like antiviral, bactericidal, antiseptic and antifungal [16, 17]. In traditional medicine, *O. majorana* oil is used to cure many distinct diseases such as dizziness and gastrointestinal disorders [18]. Ursolic acid obtained from *O. majorana* leaves is also used for Alzheimer's [19]. It has been reported in various studies that *O. majorana* has antioxidant and antitumor effects [20-23].

It is known that compounds used for chemotherapeutic and chemopreventive purposes in cancer treatment not only have undesirable effects, but also have cytotoxic effects on healthy cells. For this reason, in recent years, approaches to reduce treatment-related toxicity as well as increasing the effectiveness of treatment have come to the fore in studies aimed at developing new strategies in cancer prophylaxis and treatment. The fact that 63% of anticarcinogenic drugs are of plant origin [24] and the anticarcinogenic effects of various herbal extracts in current studies have brought use sources of derived from plants as alternative/supportive agents in cancer treatment to the agenda. The American National Cancer Institute (NCI) evaluated the potential anticancer activities of approximately 35,000 plant species and reported that 3,000 plant species among them may be effective in cancer treatment [25]. Studies on active ingredients of plant herbal sources have increased highly recently, and their cancer therapeutic effects are particularly focused on and studies are carried out on this subject.

The antioxidant and apoptosis stimulating properties of chloroform, ethanol and methanol extracts obtained from *O. majorana* on lung cancer cells were demonstrated by comparison in this study.

2. RESULTS

2.1. Antioxidant Activity

The radical scavenging activities of chloroform, ethanol, and methanol extracts of *O. majorana* were tested with 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay. The antioxidant features of methanol, ethanol, and chloroform extracts were compared with positive controls. EC_{50} values were calculated for each extract scavenging 50% DPPH to reveal antioxidant activity. Low EC_{50} value indicates high DPPH radical scavenging activity. As shown in Table 1, each extract exhibited significant DPPH radical-scavenging activity. The antioxidant activity of *O. majorana* chloroform extracts (EC_{50} = 0.17 mg/mL) was found to be higher than the methanol, and ethanol extracts. EC_{50} values of α-tocopherol, ascorbic acid, and BHT at 0.007, 0.004 and 0.017 mg/mL, respectively.

Table 1. Antiradical activities of methanol, ethanol, and chloroform extracts and positive controls.

Samples	DPPH assay (EC_{50}, mg/mL) Mean ± SD
Methanol extracts	0.71 ± 0.93[a]
Ethanol extracts	0.41 ± 0.26[b]
Chloroform extracts	0.17 ± 1.27[c]
BHT	0.017 ± 0.4
Ascorbic acid	0.004 ± 0.62
α-Tocopherol	0.007 ± 1.4

Bars with the different letter indicate significant difference ($p < 0.05$). SD, standard deviation. BHT, butylated hydroxytoluene.

2.2. Effect of *O. majorana* Extracts on the Cell Viability

Cytotoxicity of *O. majorana* extracts on A-549 cells was investigated for 48 h, and according to the experimental results obtained cytotoxicity increased depending on the concentration. IC_{50} (extract concentration that kills 50% of the cells) values were calculated for each extract. As a result of 48 h incubation of A-549 cells with different methanol, ethanol, and chloroform extracts concentrations, IC_{50} and IC_{70} concentrations for methanol extract was calculated as 252 µg/mL and 362 µg/mL, respectively, IC_{50} and IC_{70} concentrations for ethanol extract was calculated as 291 µg/mL and 410 µg/mL, respectively, while IC_{50} and IC_{70} concentrations for the chloroform extract was calculated as 333 µg/mL and 462 µg/mL, respectively (Figure 1).

O. majorana methanol extract showed more cytotoxicity than ethanol, and chloroform extracts. The cytotoxicity of all treatment were found to be statistically distinct from control groups (treated with untreated cells with medium only) ($p < 0.05$). It was found that the 0.5% DMSO concentration we used to dissolve the extracts did not affect the cytotoxicity.

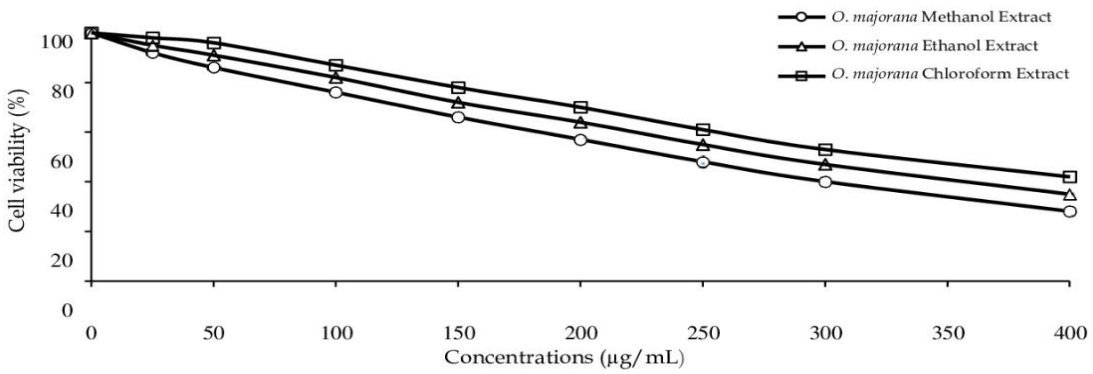

Figure 1. Dose-dependent cytotoxicity of *O. majorana* extracts.

2.3. Effect of *O. majorana* Extracts on LDH Activity

One of the indicators of cytotoxicity is LDH release. LDH is an enzyme released from the cytoplasm into the culture medium after damage to the cell membrane [26].

LDH enzyme activities were determined after IC_{50} concentration of *O. majorana* extracts were applied to A-549 cells for 48 h. The maximum increase in cells was seen after chloroform extract treatment. It was observed that LDH activity in cells reated with chloroform extract was higher (1.4 times) than in the control (Figure 2). The LDH enzyme activity of all treatment were found to be statistically distinct ($p < 0.05$) from control groups.

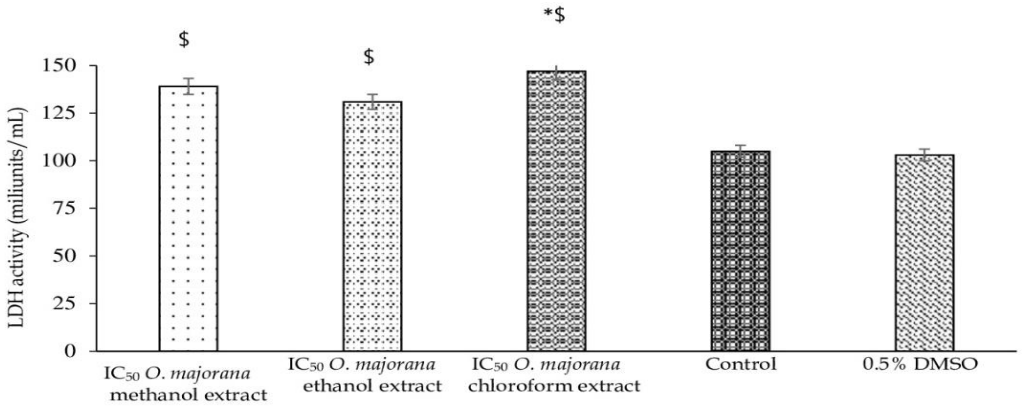

Figure 2. Changes in LDH activities after treated with *O. majorana* extracts.
* Significantlydistinct from the other two treatments ($p < 0.05$).
$ Significantly different from controls and %0.5 DMSO ($p < 0.05$).

2.4. Methanol, Ethanol, and Chloroform Extracts of *O. majorana* Increases GPx Activity in A-549 Cells Indicative of Enhanced Oxidative Stress

After methanol, ethanol, and chloroform extracts of *O. majorana* (IC_{50} concentration) were applied to A-549 cells, changes in Glutathione peroxidase (GPx) activity, which is a marker of oxidative stress, were laid out. When the cells exposed to chloroform extracts of *O. majorana* GPx activity increased approximately 3.2 times according to control and this increase was determined statistically distinct ($p < 0.05$) (Figure 3). GPx activity in cells to which chloroform extracts of *O. majorana* was applied was approximately 1.6 times higher than GPx activity in cells to which methanol extracts of *O. majorana* was applied.

2.5. Measuring Effects of *O. majorana* Extracts on Caspase-3/7 Enzyme Activity

After *O. majorana* extracts were applied to A-549 cells for 48 h, their potential to stimulate apoptosis has been detected by measuring the changes in caspase-3 activity. The enzyme activity in cells was approximately 3 fold higher compared to control at the end of 48 h of chloroform extract of *O. majorana* incubation, and when methanol extract of *O. majorana* was applied, it increased approximately 2 fold compared to the control (Figure 4). After all three types of *O. majorana* extracts applications, caspase-3 activity was found to be statistically distinct from each other and from the control ($p < 0.05$).

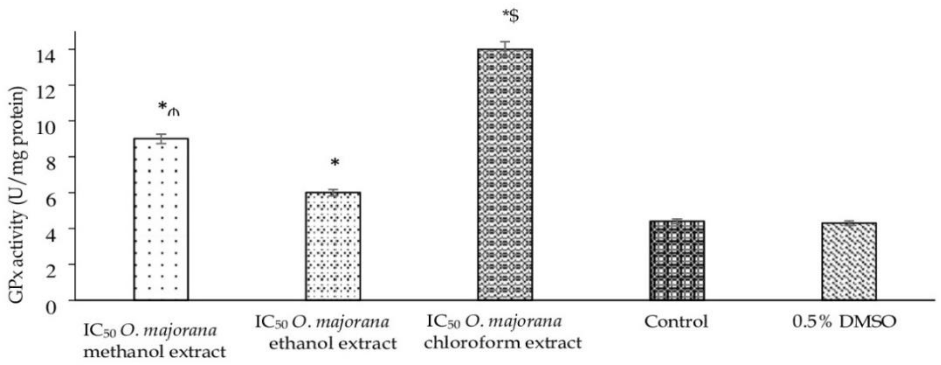

Figure 3. Effect of *O. majorana* extracts of on glutathione peroxidase (GPx) activity.
* Significantly distinct from %0.5 DMSO and control ($p < 0.05$).
$ Significantly distinct from IC50 *O. majorana* methanol and ethanol extracts ($p < 0.05$).
ᵑ Significantly distinct from IC_{50} *O. majorana* ethanol extract ($p < 0.05$).

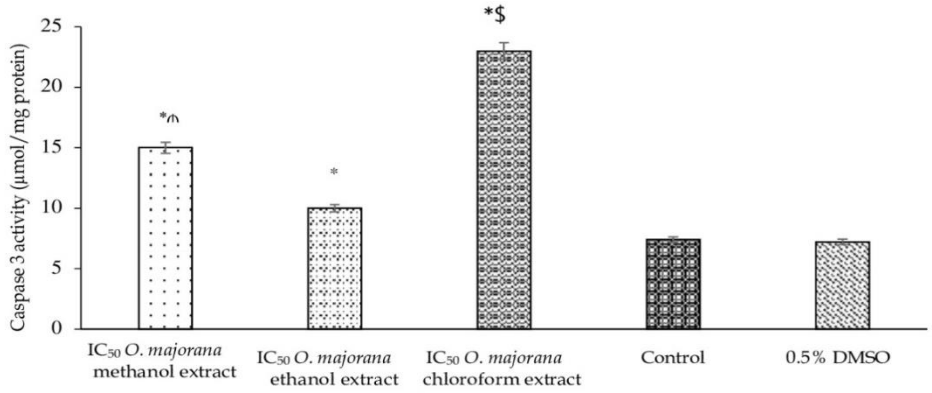

Figure 4. Changes in caspase-3 activities after treated with *O. majorana* extracts. Different asterisks show significant distinct ($p < 0.05$).
* Significantly distinct from 0.5%DMSO and control ($p < 0.05$).
$ Significantly distinct from IC_{50} *O. majorana* methanol and ethanol extracts ($p < 0.05$).
ᵑ Significantly distinct from IC_{50} *O. majorana* ethanol extracts ($p < 0.05$).

3. DISCUSSION

For centuries, people have tried to find cures for diseases with the plants they obtained. Methods of treating diseases with plants have yielded very successful results. Therefore, the use of plants in treatment has continued until today.

With the intense consumption of medicinal and aromatic plants by the public and their increasing use in various sectors, researching the chemical components and bioactivities of these plants has become very important scientifically and economically. The medicinal and aromatic plants have begun to be widely used in many industrial areas, especially in the pharmaceutical, food, cosmetics and perfume industries [27]. Naturally sourced substances that have fewer side effects than synthetic drug active substances are preferred. Today, interest in antioxidants has increased, as it has been scientifically proven that consuming foods rich in antioxidants and/or using natural antioxidants in nutritional supplements delays or prevents cellular aging and the formation of many chronic diseases. Determining that synthetic antioxidants and their by-products can cause cancer has increased research on finding natural antioxidants that can be used instead [28].

It is known that plants have been used for medicinal purposes since ancient times. In Türkiye, which has a rich vegetation, plants used to treat patients are examined chemically and biologically [29]. As a result of these studies, many new secondary metabolites were found and the activities of some of them were determined [30].

Lamiaceae family is important as one of the families that contain most essential oils used in perfumery and medicine. *Lamiaceae* family, which is represented by 46 genera and 758 taxa in Türkiye, is the third

richest family in Türkiye and the endemism rate in Türkiye has been determined as 45% [31]. *O. majorana* is one of the mint family *Lamiaceae*.

In one of our studies, we observed that *O. majorana* essential oil had a cytotoxic effect on A-549 and A-431 cells. According to the MTT assay, after 24, 48 and 72 hours essential oil incubations, IC_{50} values were calculated to be 266, 222 and 182 µg/mL in A-549 cells, and 218, 187 and 140 µg/mL in A-431 cells, respectively. It has been shown that it causes membrane damage with an increase in LDH activity. The apoptosis-stimulating effect was demonstrated by revealing changes in caspase-3/7 activity in both cells [32]. If we compare the results of our previous study with the *O. majorana* essential oil and our current study results, it has been observed that the essential oil showed more cytotoxicity than methanol, ethanol, and chloroform extracts of *O. majorana* on A-549 cells. In another study, ethanol extract from the same plant was shown to reduce cell proliferation in a human lymphoblastic leukemia cell lines and kill the cells by activating apoptosis. *O. majorana* extracts had an IC_{50} of approximately 8 and 5 mg/mL in Jurkat cells at 48 and 96 h respectively with a significant decrease in viability of 78% at 48 h and 67% at 96 h as compared to the control. In addition, it has been demonstrated that no significant cytotoxic effects were noted when the concentrations studied were tested on activated fresh human mononuclear lymphocytes indicating that *O. majorana* extracts preferentially inhibited leukemia cells [33]. The apoptosis-stimulating effect was revealed by looking at changes in the expression of p53 and Bcl-2. Accordingly, it was found that the expression of p53 increased and the expression of Bcl-2 decreased. In another similar study, it was reported that extracts of the same plant stopped the proliferation of different hepatoma cells [20].

In our study, the antioxidant activity of three different extracts of *O. majorana* were investigated using the DPPH test. DPPH radicals found in this test are very useful in the preliminary determination of antioxidant molecules [34]. In our study, chloroform extract exhibited higher antioxidant activity than methanol, and ethanol extracts. In another study, the EC_{50} value of *O. majorana* essential oil was calculated as 89.2 µg/mL, demonstrating its radical scavenging activity [35].

It has been suggested in another study that *O. majorana* acetone extract may be a good anticancer agent because it can stop the cell cycle in the G2/M phase, stimulate the apoptotic pathway, and increase the expression of p53, a pro-apoptotic gene. In this study IC_{50} value of *O. majorana* leaf extract was shown to be at the concentration of 90 µg/mL [36]. In a different study, the biological activities (antioxidant and anticancer) of ZnO nanoparticles (ZnO-NPs) produced using *Origanum majorana* leaf extract were evaluated. It has been demonstrated that spherical nanoparticles with a size of 32 nm have antioxidant and cytotoxic effects. After 48 h of incubation, IC_{50} concentration for human breast (MCF-7) cells was calculated as 16.8 µg/mL, while IC_{50} concentration for colon (HT-29) cancer cells was calculated as 194.3 µg/mL. Additionally, it was shown that it stimulated apoptosis in MCF-7 cells by increasing the expression of Bax, a pro-apoptotic gene, and decreasing the expression of Bcl 2, an anti-apoptotic gene [37]. It has been reported that *Origanum majorana* extracts have anticancer effects in HT-29 (colon cancer cell line) cells by activating apoptotic mechanisms, causing damage to DNA and also stopping cell growth. The IC_{50} values of *O. majorana* ethanol extract was calculated as approximately 450 µg/mL at 24 h, 250 µg/mL at 48 h, and 300 µg/mL at 72h treatment [38]. It has been shown that *Origanum majorana* extracts inactivate NFKB pathways at concentrations that do not show cytotoxic effects. Thus, it was revealed that the migration and invasion of cells were blocked [39]. It has also been shown that after the application of two low concentrations of *Origanum majorana*'s ethanolic extracts, 150 and 300 µg/mL, to MDA-MB-231 cells, apoptosis-resistant cell populations accumulate by keeping them in mitosis, and the expression of survivin, one of the targets of treatment in breast cancer, increases [40]. On the contrary, when MDA-MB-231 cells were incubated with two high concentrations of 400 and 600 µg/mL, it was shown that survivin expression decreased and apoptosis was stimulated by activation of the caspase and PARP pathway. The IC_{50} was calculated as approximately 350 mg/mL at 24 h and 400 mg/mL at 48 h treatment [40]. In another study, antioxidant activity was demonstrated by showing that cerium oxide nanoparticle (CeO-NP) synthesized using *Origanum majorana* L. leaf extract scavenged DPPH and ABTS free radicals, and this antioxidant activity was found to be lower than the antioxidant activity of Butylated hydroxyanisole (BHA), which is a standard antioxidant. It was found that the cytotoxic effect of CeO-NP was more effective in MDA-MB-231 cells, a cancer cell line, than in HUVEC cells, a normal cell line. Evaluation of the viability of MDA-MB-231 cells demonstrated an IC_{50} value of 125, 60 and 50 µg/mL at 24, 48 and 72 h after treatment, respectively. Additionally, it has been shown that CeO-NP application increases the expression of catalase and superoxide dismutase, which are antioxidant genes [41].

In this study, it was revealed that methanol, ethanol, and chloroform extracts of *O. majorana* has antiradical activities. When A-549 cells were treated with *O. majorana* extracts, it has been observed that the

extracts have a cytotoxic effect on cells. These results were supported by investigating the effect of extracts on membrane damage. With studies that investigate the effects of *O. majorana* on cells in detail, new cancer treatment strategies that have little or no side effects on healthy cells can be identified.

4. CONCLUSION

In this study, it was revealed that all three extracts had antiradical activity by DPPH test, and it was determined that the chloroform extract was determined to have the highest antioxidant activity. All three extracts prepared in this study had a cytotoxic effect on A-549 cells. It was found that the 48 h cytotoxic effects of *O. majorana* extracts on A-549 cells increased depending on the concentration. This study may lead to contribute to the economy by producing new antioxidant drugs by revealing the antioxidant properties of extracts in vitro, using them in food and beverages as an alternative to synthetic antioxidant substances, reducing the toxic effects caused by the use of synthetic antioxidant substances, and utilizing natural resources. Showing that *O. majorana* extracts have an apoptotic effect on lung cancer cells, causing the cells to die, may increase interest in cancer treatment research of natural-derived compounds. A more detailed explanation of the intracellular mechanisms of action of methanol, ethanol and chloroform extracts of *O. majorana* may lead to the production of natural anticancer drugs.

5. MATERIALS AND METHODS

5.1. Plant Material

The extracts we used in our experiments were prepared using *O. majorana*, which we collected from Türkiye (Alanya-730 m) during the flowering period. The plants we used in the preparation of the extracts were identified by experts in the field.

5.2. Preparation of Plant Extracts

Aerial parts of the collected plants were dried in an airy area away from sunlight. To increase the extraction efficiency, the dried plants were ground into fine powder. Powdered plant samples were added to methanol, ethanol and chloroform and kept at room temperature, away from light, for 24 h. After the maceration period, the mixture was filtered through filter paper, the extracts obtained by evaporating the solvents at the appropriate temperature with the help of a rotary evaporator were stored at -20 °C until further analysis.

5.3. DPPH Antioxidant Assay

The radical scavenging capacities of the studied extract samples were measured by determining the color changes in the purple methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). While determining the antioxidant activity of the samples with this test, spectrophotometric measurements are made using DPPH, a stable radical, as a reagent [46, 47]. In determining the most suitable DPPH radical concentration for measurements; methanol solution of DPPH radical at different concentrations was prepared. Equal volumes of extract samples and standards (50 µL) were added to each different concentration of (w/v) methanol solution of DPPH solution (5 mL). The extract samples are mixed in the vortex for 30 sec and left at room temperature and in the dark for 30 min. At the end of the period, the absorbance was read against a blank at 517 nm on the UV Spectrophotometer. Inhibition free radical DPPH in percent (I%) was calculated in following way:

$$I\ \% = (A_{control} - A_{sample}/A_{control}) \times 100$$

Where $A_{control}$ shows the absorbance value of the all reagents except the extract samples, and A_{sample} shows the absorbance value of the extract samples. The concentrations (EC_{50}) of methanol, ethanol and chloroform extracts of *O. majorana* and standards that provide 50% DPPH radical scavenging were calculated from the graphs created using the experimental results. Tests were performed in triplicate.

5.4. Cell Line and Cell Culture

The human non-small-cell lung cancer (NSCLC) cell line A-549 that we used in our experiments was purchased from the American Type Culture Collection (ATCC) and cultured under appropriate conditions. The cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640) containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 units/ml) in a humidified environment with 5% CO_2 at 37 °C. Once the cells occupied approximately 80% of the culture vessel, the cells were subcultured or treated with different extracts to perform experiments.

5.5. Cytotoxicity Assay

The cells were incubated with methanol, ethanol, chloroform extracts of *O. majorana* for 48 h. The cytotoxic effect of different extracts on lung cancer cells after 48 h of incubation was revealed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. In this test, tetrazolium salts such as MTT are metabolized by mitochondrial dehydrogenases to form a blue formazan dye that is used to measure cytotoxicity. Test reagents were added to the culture medium and incubated at 37 °C for 2 h. Then, solubilizing/stopping solutions (dimethyl sulfoxide) was added to each well for a 1 h incubation. The absorbance of all extracts samples was measured at 490 nm [42]. Eight wells were replicated for each concentration. To determine the cytotoxic effect, the percentage of viable cells was calculated by dividing the number of viable cells by the control cells (containing only the culture medium without the test sample). IC_{50} concentrations (extract concentration that kills 50% of the cells) were calculated for each of the methanol, ethanol and chloroform extracts of *O. majorana*.

5.6. Measurement of Lactate Dehydrogenase (LDH) Release

To determine whether methanol, ethanol and chloroform extracts of *O. majorana* (IC_{50}) caused any damage to the membranes of lung cancer cells, the cells were treated with extract samples for 48 h and changes in LDH activity were determined. After incubation, cell supernatant was prepared and changes in LDH activity were determined using the LDH Activity Assay Kit (MAK066, Sigma-Aldrich) according to the manufacturer's instructions at 450 nm. Tests were performed in triplicate. LDH activity was calculated using the following formula provided by the instructions.

LDH Activity = The amount of NADH that occurs between the first and last measurement (nmol) × Sample Dilution Factor/Reaction Time× Sample volume (mL)

5.7. Glutathione Peroxidase (GPx) Activity

After incubation with methanol, ethanol, and chloroform extracts of *O. majorana* (IC_{50}), cell supernatant was prepared. Cells were washed twice with phosphate buffered saline (PBS) and then lysed and centrifuged. Cell debris was collected and homogenized by sonication in 50 mM potassium phosphate, pH 7.2, containing 1 mM fluoride and 1 µg/mL leupeptin. The supernatant was then collected by centrifuging the homogenate (150,000 g, 45 min, 4 °C). Activity of GSH-Px was determined according to Flohe and Gunzler [43] with tert-butyl hydroperoxidase as substrate. Tests were performed in triplicate. Protein was determined by the Bradford method with bovine serum as a standard [44].

5.8. Caspase-3 Activity

Caspase-3 activity were determined using commercially available colorimetric Caspase-3 Activity Assay Kit (Elabscience) according to the manufacturer's protocol after 48 h of application of methanol, ethanol, and chloroform extracts of *O. majorana* (IC_{50}). The plates were read at 405 nm using the microplate reader. Tests were performed in triplicate. Results are given as Unit/mg protein.

5.9. Data Analysis

The results of the replicates were pooled and expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was carried out. The one-way ANOVA was used to determine whether there were any significant differences between the means of three or more independent (unrelated) groups on some variable. Tukey multiple comparisons tests were used. Statistical differences were considered significant at $p < 0.05$ [45]. Statistical analyses were performed using the Minitab programm (http://www.minitab.com/products), release 13.0.

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