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Evaluation of Cytotoxic and Apoptotic Effects of Methotrexate and Carvacrol Combination in Lung Cancer Cancer Cells

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Akciğer Kanseri Hücrelerinde Metotreksat ve Karvakrol Kombinasyonunun Sitotoksik ve Apoptotik Etkilerinin Değerlendirilmesi

Ayşe ERDOĞAN*

Alanya Alaaddin Keykubat University, Rafet Kayış Faculty of Engineering, Dept. of Genetics and Bioengineering, Türkiye

Abstract

The aim of this study is to compare the cytotoxic and apoptotic effects of methotrexate (MTX),one of chemotherapy drug, alone application with the combined application of MTX and carvacrol, a naturally occurring monoterpenoid phenol, in lung cancer cells. Cell viability was assessed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) assay after A-549 cells were treated with MTX alone and in combination with carvacrol for 48 h. Lactate dehydrogenase (LDH) activity test was employed to assess whether MTX alone or in combination with carvacrol treatments, induced membrane damage by detecting changes in LDH activity in A-549 cells. Caspase-3 enzyme activity changes in cells were measured to show the apoptotic effect. By examining the alterations in glutathione peroxidase activity, an attempt was made to determine whether MTX, alone and in combination with carvacrol, played a role in inducing oxidative stress. It was observed that the combined application of MTX and carvacrol for 48 h inhibited cell proliferation more than MTX alone treatment, demonstrating a stronger cytotoxic effect. Additionally, it caused a greater increase in LDH activity, a marker of membrane damage, and also led to a higher increase in caspase-3 activity, an enzyme involved in the apoptotic pathway. The results of this study show that the combined application of MTX and carvacrol leads to a greater increase in LDH activity and caspase-3 activity compared to MTX alone treatment, indicating more significant membrane damage and apoptotic effects. Therefore, combined treatment could be considered a strategy for alleviating the side effects associated with MTX in lung cancer.

Keywords: *Lung cancer; Carvacrol; Methotrexate, Combined treatment, Apoptotic effect.*

Öz

Bu çalışmanın amacı, bir kemoterapi ilacı olan metotreksatın (MTX) tek başına ve doğal olarak oluşan bir monoterpenoid fenol olan karvakrol ile birlikte uygulamasının akciğer kanseri hücrelerindeki sitotoksik ve apoptotik etkilerini karşılaştırmaktır. Hücre canlılığı, A-549 hücrelerine MTX tek başına ve karvakrol ile birlikte 48 saat uygulandıktan sonra 3-(4,5-dimetil-2-tiyazolil)-2,5-difenil-2Htetrazoliumbromid (MTT) testi ile değerlendirilmiştir. Laktat dehidrogenaz (LDH) aktivite testi, MTX tek başına veya karvakrol ile kombinasyon halinde uygulandıktan sonra, A-549 hücrelerinde LDH aktivitesindeki değişiklikler tespit edilerek membran hasarına yol açıp açmadığı değerlendirilmiştir. Hücrelerdeki kaspaz-3 enzim aktivitesindeki değişiklikler, apoptotik etkiyi göstermek için ölçülmüştür. Ayrıca, MTX'in, tek başına ve karvakrol ile birlikte, oksidatif stresin uyarılmasında bir rol oynayıp oynamadığını belirlemek amacıyla glutatyon peroksidaz aktivitesindeki değişiklikler incelenmiştir. Çalışmada, MTX ve karvakrolun kombinasyon halinde 48 saatlik uygulamasının, MTX tek başına uygulamasından daha fazla hücre proliferasyonunu engellediği ve daha güçlü bir sitotoksik etki gösterdiği gözlemlenmiştir. Ayrıca, bu kombinasyon uygulaması membran hasarının bir göstergesi olan LDH aktivitesinde ve apoptoz yolunda görev alan bir enzim olan kaspaz-3 aktivitesinde daha fazla artışa yol açmıştır. Bu çalışmanın sonuçları, MTX ve karvakrol kombinasyonunun, MTX tek başına ugulamasına göre LDH aktivitesinde ve kaspaz-3 aktivitesinde daha büyük bir artışa neden olarak daha belirgin membran hasarı ve apoptotik etki oluşturduğunu göstermektedir. Bu nedenle, kombinasyon uygulaması akciğer kanserinde MTX ile ilişkili yan etkilerin hafifletilmesi için bir strateji olarak değerlendirilebilir.

Anahtar Kelimeler: *Akciğer kanseri; Karvakrol; Metotreksat; Kombine tedavi; Apoptotik etki.*

1. Introduction

Lung cancer, despite all the advancements in early detection and screening methods, continues to be a significant global threat and remains one of the most commonly diagnosed cancers in both women and men that requires treatment. The combined treatment approaches developed today, in addition to monotherapies such as surgery, chemotherapy, hormone therapy, and radiotherapy, have gained significant importance in the treatment of lung cancer because of

*Sorumlu Yazar/Corresponding Author: Ayşe Erdoğan e-posta/e-mail: ayse.erdogan@alanya.edu.tr

the high rates of tumor reappearance and disease advancement. Therefore, there is great attention towards investigating the cancer-fighting effects of biologically active plant compounds derived from therapeutic herbs when paired with traditional treatment methods (Newman and Cragg 2020). Although significant advancements have been made in the detection and therapy methods of lung cancer in recent times, the outlook for patients with late-stage disease is still not at the desired level. Therefore, the identification of potent new substances for the treatment of lung cancer is of great importance (Siegel et al. 2022). Scientists have been drawn to natural products in the creation of antitumor medications because of their established effectiveness and safety. Naturally derived substances can provide a source of new antitumor compounds. Carvacrol is one of the natural compounds that has been evaluated in medicine for centuries (Marchese et al. 2018a).

Methotrexate (MTX) is a known chemotherapy agent and immunosuppressant. MTX, commonly used as an anticancer drug, is primarily a folic acid antagonist. It is used for treating various cancers, including breast cancer, leukemia, lung cancer, lymphoma, bladder cancer, gestational trophoblastic disease, and osteosarcoma. Initially, it was preferred in cancer treatment due to being less toxic compared to existing treatments, but several side effects have been observed with prolonged use. Frequent side effects include nausea, tiredness, fever, heightened infection risk, reduced white blood cell count, and damage to the mucous membranes in the mouth (Howard et al. 2016). Additional side effects could involve liver and lung conditions, lymphoma, and severe skin eruptions. It has been noted that lower doses may be required, especially in patients with kidney problems. The precise mechanism behind the toxicity caused by MTX is still unidentified. It is thought that MTX inhibits the cellular antioxidant mechanism, leading to oxidative stress-induced damage in liver cells (Ebrahimi et al. 2019).

Tetrahydrofolate is crucial for the production of thymidine and purines, both of which are necessary for DNA synthesis (Howard et al. 2016). The inhibition of tetrahydrofolate synthesis by methotrexate leads to the inability of cells to divide and difficulty in protein production (Howard et al. 2016). Disruptions in the normal functioning of apoptosis can contribute to the onset of several human diseases, including cancer. The identification of apoptosis mechanisms, effector proteins, and genes that regulate apoptosis have opened up a new possibility for the discovery and development of novel compounds that can enhance the susceptibility of cancer cells to apoptosis or lower their apoptotic threshold. For these reasons, apoptosis has become one of the most important molecular targets in drug discovery and development, particularly for the management of conditions like cancer (Pistritto et al. 2016).

Carvacrol, also called 5-isopropyl-2-methylphenol, is a liquid phenolic monoterpene identified in the essential oils of plants such as thyme (Origanum vulgare), (Thymus vulgaris), wild bergamot (Citrus aurantium bergamia), and black pepper (Lepidium flavum). Carvacrol, based on current research, exhibits a broad spectrum of biological effects. These include bacteria-fighting and fungus-fighting properties (Sharifi-Rad et al. 2018, Suntres et al. 2015), antiviral (Gilling et al. 2014), antioxidant (Suntres et al. 2015, Aristatile et al. 2011, Aristatile et al. 2009), anticarcinogenic (Arunasree 2010), reduction of oxidative damage and radical scavenging activity (Samarghandian et al. 2016), hepatoprotective (Suntres et al. 2015, Aristatile et al. 2009), antibiofilm and antimicrobial effects (Marchese et al. 2018b), as well as anticancer, anti-inflammatory, and spasmolytic properties, among others (Suntres et al. 2015).

Given the defensibility and antineoplastic properties of plant-derived compounds such as carvacrol, these monoterpenoid phenolic compound could offer a reliable and affordable approach to mitigating chemoresistance in lung cancer patients. Thus, it may contribute to strategies aimed at reducing MTX related side effects in patients. No data is available regarding the potential anticancer effects of MTX and carvacrol on lung cancer cells. This study aimed to provide a combination of phytotherapeutic agents such as carvacrol and low-dose chemotherapy drugs, and the combination of MTX and carvacrol could be beneficial in the future for lung cancer patients undergoing conventional chemotherapy but failing to achieve the desired treatment outcomes.

2. Materials and Methods

2.1 Chemicals and drugs

Methotrexate (MTX) used in the experiments was mixed with the medium in suitable ratios for dilution. Carvacrol is available for purchase with a purity of 98% (282197, Sigma Chemical Co.). The caspase activity kit (E-CK-A311) was sourced from Elabscience Biotechnology Co. Ltd, USA. The kit (MAK066) used to measure Lactate Dehydrogenase Activity was acquired commercially from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Cell Lines and cultivation

A-549 cell line (human non-small cell lung cancer (NSCLC)) employed in our experiments was sourced from the American Type Culture Collection (ATCC). The cells were

grown in Roswell Park Memorial Institute 1640 medium (RPMI 1640) (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA), along with penicillin (100 units/mL) and streptomycin (Gibco, USA) (100 units/mL), under a humidified atmosphere with 5% CO_2 at 37 °C. Once the cells reached an adequate density (over 75%) in the culture vessel, experimental groups were established, and then MTX ($<IC_{50}$) and the natural monoterpenic phenol (carvacrol) ($<IC_{50}$) were administered to the cells for 48 h.

2.3 Cell viability test

After the cells in the flask were treated with trypsin, a total of 10^4 A-549 cells per well were plated in a medium, ensuring a final volume of 200 µL per well. The cells were exposed to MTX alone or in combination with carvacrol for 48 h. The cytotoxic effects of MTX (500-9000 µg/mL) and carvacrol (20-70 µg/mL) on A-549 cells were assessed over a 48 h period. In addition, the cells were treated simultaneously with MTX ($<IC_{50}$) and carvacrol ($<IC_{50}$) for a duration of 48 h. The cytotoxicity of the treatments was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) method.

In this assay, mitochondrial dehydrogenases metabolize tetrazolium salts like MTT to produce a blue formazan dye, which is then used to assess cytotoxicity. We prepared a stock solution at 5 mg/mL by dissolving MTT (Gibco, USA) in PBS and vortex the solution until the MTT powder is fully dissolved. We used a 0.22 µm sterile filter to remove any undissolved particles and prevent contamination. We divided into small aliquots stored at 4°C for short-term use.. At the end of the process, MTT solution was added at a final concentration of 0.5 mg/mL per well and the samples were placed in an incubator at 37 °C for 2 h. Next, solubilizing/stop solutions (dimethyl sulfoxide) (Merck) were added to each well, and the plates were incubated for 1 h. The optical density of all samples was recorded at 490 nm (Mosmann 1983). Each concentration was replicated in eight wells.

The concentrations below IC_{50} ($<IC_{50}$) were determined separately for MTX and carvacrol. The combination treatments were then carried out using the calculated combination index (CI) values ($<IC_{50}$). To determine whether the combined application of MTX and carvacrol in cells exhibited additive, synergistic, or antagonistic effects, the combination index (CI) was calculated. The combination index (CI) was determined using the formula: $CI = (Ea+b) / (Ea + Eb - Ea \times Eb)$ (Huang et al. 2014). In the formula, Ea+b represents the inhibition rate when carvacrol and MTX were applied in combination, while Ea represents the inhibition rate of carvacrol alone, and Eb represents the inhibition rate of MTX alone. The effect of drug interaction was categorized into four types:

i) Additive (+) when the CI is between 0.85 and 1.15;

ii) Synergistic (++) when the CI falls within 1.15 to 2.0;

iii) Subtractive (-) when the CI ranges from 0.85 to 0.55;

iv) Antagonistic (—) when the CI is less than 0.55.

Based on the cytotoxicity results, the optimal combination concentration was identified after applying MTX ($<IC_{50}$) and carvacrol ($<IC_{50}$) together. The optimal combination concentrations were also applied in other ongoing experiments. Cells treated with only the medium or 0.1% DMSO were regarded as the control group.

2.4 Lactate dehydrogenase (LDH) activity test

LDH activity was measured after exposing A-549 cells to MTX alone (IC_{10}) and combination of MTX (IC_{10}) with carvacrol (IC_{10}) concentrations, which demonstrated the most significant cytotoxic effects, for 48 h. LDH activity changes were assessed to evaluate whether either treatment induced membrane damage in lung cancer cells. LDH activity in each sample was measured using the protocol provided in the commercially available kit (Sigma-Aldrich). The equation used to determine LDH activity is provided below.

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

2.5 Glutathione peroxidase (GPx) enzyme activity

After exposing the cells treated with MTX only (IC_{10}) and the combination of MTX (IC_{10}) and carvacrol (IC_{10}), which demonstrated the strongest cytotoxic effects, for a duration of 48 h, the cell supernatant was collected for assessment of GPx activity. GPx activity was evaluated using tert-butyl hydroperoxide as the substrate, following the specified method (Flohe and Gunzler 1984) Protein concentration was measured using Bradford assay, with bovine serum as the standard (Bradford 1976). The tests were conducted in three replicates.

2.6 Caspase-3 enzyme activity

Caspase-3 activity was evaluated after A-549 cells were exposed to MTX (IC_{10}) individually and together with carvacrol (IC_{10}), the concentrations that showed the highest cytotoxic effects, for 48 h. Apoptotic enzyme performance was assessed by utilizing the colorimetric Caspase-3 Activity Assay Kit (Elabscience), following kit's instructions, after treating the cells with MTX alone and in combination with carvacrol. The plates were analyzed

at 405 nm with a microplate spectrophotometer. The experiments were carried out in three replicates, and the results are presented as Units per milligram of protein.

2.7 Analysis of data

The replicate results were combined and expressed as the average ±standard deviation (SD). A statistical analysis using ANOVA was conducted. One-way ANOVA was employed to evaluate if there were any notable differences between the averages of three or more unrelated groups for a specific variable. Tukey multiple comparisons tests were applied. Statistical importance was accepted at $p<0.05$. Statistical evaluations were conducted using the Minitab software (http://www.minitab.com/products), version 13.0.

3. Results

3.1 Cytotoxic effect of methotrexate alone and in combination with carvacrol on lung cancer cells

The cells were exposed to methotrexate (MTX) and carvacrol alone for 48 h, and the IC concentrations for both MTX and carvacrol were calculated separately using the MTT assay. IC_{10}, IC_{20}, IC_{30}, IC_{40}, and IC_{50} concentrations (the concentration that induces a 50% cell death rate) determined for both MTX and carvacrol were used to proceed with the other experiments (Figure 1 and 2). It was noted that the cytotoxicity observed on A-549 cells after the incubation of both MTX and carvacrol increased with the concentration (Figure 1 and 2).

IC_{10}, IC_{20}, IC_{30}, IC_{40}, and IC_{50} concentrations for MTX were determined as 1112, 2316, 3521, 4726, and 5931 µg/mL, respectively, while for carvacrol, IC_{10}, IC_{20}, IC_{30}, IC_{40}, and IC_{50} concentrations were calculated as 11 µg/mL, 21 µg/mL, 31 µg/mL, 41 µg/mL, and 51 µg/mL, respectively.

After determining concentrations lower than IC_{50} concentrations ($<IC_{50}$) for both MTX and carvacrol, the combination of MTX and carvacrol was applied, and the most effective cytotoxic effect was identified by assessing cell viability at different combination concentrations. Based on the cytotoxicity results after the that experiments of our study, the most effective combinati on concentrations, IC_{10} MTX and IC_{10} carvacrol, were used.

The results are shown as the viability ratio relative to the control group (cells treated only with medium). Data are presented as the mean of three independent experiments with three replicates ± standard deviation (SD) (ANOVA, $p<0.05$).

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The synergistic effect of applying IC_{10} MTX and IC_{10} carvacrol in A-549 cells was also confirmed by calculating CI value of 2.1. Since the calculated CI value was 2.1, which falls within the synergistic interaction range as defined in the materials and methods section, and represents the highest synergistic CI value, further experiments were conducted using these combination concentrations (IC_{10} MTX + IC_{10} carvacrol). In the following experiments of our study, the most effective combination concentrations, IC_{10} MTX and IC_{10} carvacrol, were used (Figure 3).

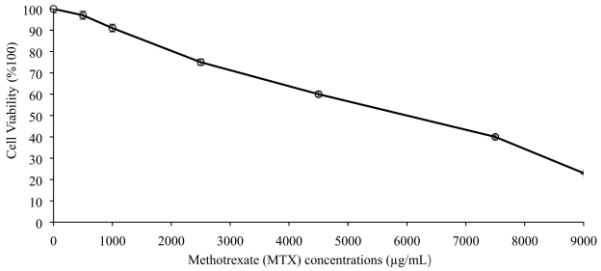

Figure 1. The cytotoxic effects of methotrexate (MTX) (500-9000 µg/mL) over 48 h on A-549 cells were evaluated using MTT assay.

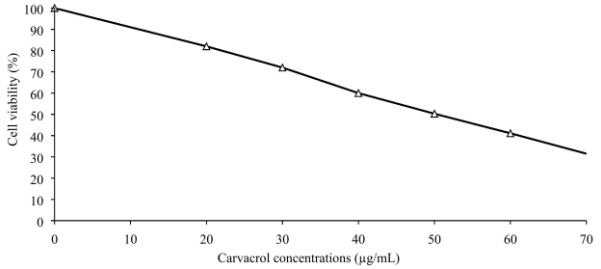

Figure 2. The cytotoxic effects of carvacrol (20-70 µg/mL) over 48 h on A-549 cells were evaluated using MTT assay.

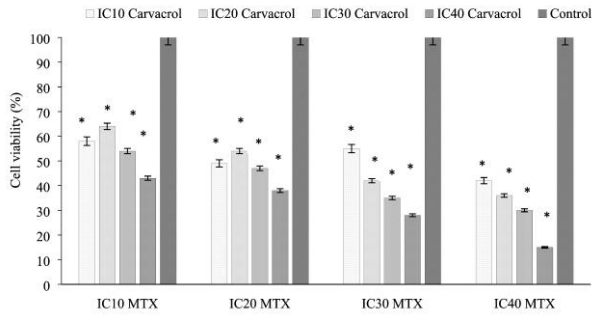

Figure 3. Combined cytotoxic effects of methotrexate (MTX) and carvacrol on A549 cells after 48 h.* Significantly different from control (untreated cells) ($p<0.05$).

3.2 Evaluation of the membrane damaging effect of methotrexate alone and in combination with carvacrol

Lactate dehydrogenase (LDH) is an enzyme and it plays a crucial role in converting lactate to pyruvate during cellular metabolism, specifically in anaerobic glycolysis. Measuring LDH activity is a common assay used to assess

cell membrane integrity and cytotoxicity, as LDH is released into the extracellular space when cells are damaged or lysed. Elevated LDH levels in culture supernatants can indicate cellular injury or death. A-549 cells were incubated for 48 h with MTX alone and also in combination with carvacrol (IC_{10} MTX + IC_{10} carvacrol) (the most effective cytotoxic combination concentrations). Changes in LDH activity were then assessed to evaluate the membrane damaging effects of both treatments.

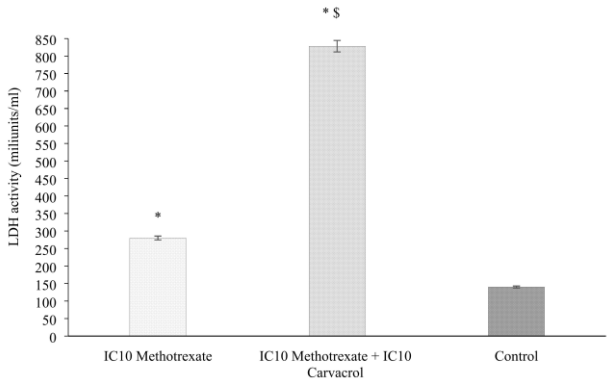

Figure 4. Alterations in LDH activity following treatment with methotrexate alone and in combination with carvacrol.*Significantly distinct from control ($p<0.05$). $^{\$}$Significantly distinct from methotrexate alone treatment ($p<0.05$).

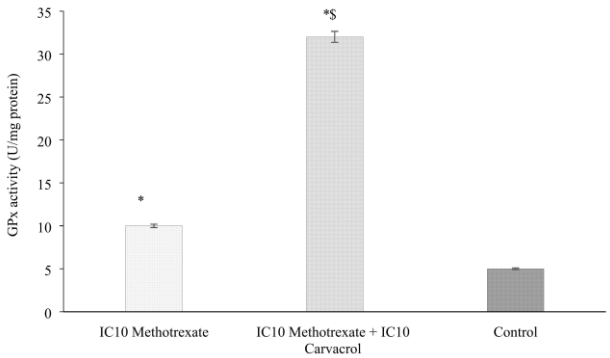

Figure 5. The impact of methotrexate alone and in combination with carvacrol on glutathione peroxidase (GPx) activity. *Significantly distinct from control ($p<0.05$). $^{\$}$Significantly distinct from methotrexate alone treatment ($p<0.05$).

Both MTX treatment alone and the combined treatment were found to cause an increase in LDH enzyme activity compared to the controls (cells located in the medium consisting solely of culture medium components). The combined application of MTX and carvacrol was found to cause more membrane damage compared to MTX treatment alone (Figure 4). While MTX treatment alone caused a 2-fold increase in LDH activity compared to the controls after 48 h of incubation, the combined application of MTX and carvacrol resulted in approximately a 6-fold increase compared to the controls. It was found that the LDH activity observed in the cells after the combined application of MTX and carvacrol was statistically different from the LDH activity determined after MTX treatment alone and the controls ($p<0.05$) (Figure 4).

3.3 Assessment of the impact of methotrexate alone and in combination with carvacrol on glutathione peroxidase activity

Glutathione peroxidase (GPx) is a vital antioxidant enzyme that safeguards cells from oxidative damage by converting hydrogen peroxide (H_2O_2) and organic peroxides into harmless substances, using glutathione (GSH) as a cofactor. It plays a crucial role in maintaining the balance of cellular redox reactions and preventing oxidative stress, which is implicated in the development of various conditions, such as cancer and neurodegenerative diseases. Assessing GPx activity is an important method for evaluating oxidative stress and the antioxidant potential of cells.

After treating A-549 cells with MTX alone and in combination with carvacrol for 48 h, changes in GPx activity were measured to compare the potential of the treatments to induce oxidative stress. MTX treatment alone caused a 2-fold increase in GPx activity compared to the controls. However, it was found that the combined treatment (IC_{10} MTX + IC_{10} carvacrol) resulted in a 6.4-fold greater increase in GPx activity compared to the controls. The increase in GPx activity observed after both MTX treatment alone and the combined treatment, compared to the controls, was found to be statistically significant ($p<0.05$) (Figure 5). It was found that the combined treatment was more effective in inducing oxidative stress compared to MTX treatment alone.

3.4 Evaluation of the apoptotic effect of methotrexate alone and in combination with carvacrol

Caspase-3 is an essential enzyme involved in apoptosis (programmed cell death). It is part of the cysteine-aspartic protease family (caspases), which are key players in the execution phase of apoptosis, as they break down various cellular components, ultimately leading to cell death. Monitoring caspase-3 activity is a widely used method for assessing apoptosis induction in cells.

In our study, changes in caspase-3 enzyme activity were determined after 48 h incubation following the application of MTX alone and in combination with carvacrol, in order to assess the apoptotic effects of both treatments.

It was found that the combined treatment (IC_{10} MTX + IC_{10} carvacrol) (the most effective cytotoxic combination concentrations) induced a greater apoptotic effect compared to MTX treatment alone. It was found that the

caspase-3 activity measured after the combined treatment was 3.1 times higher than the caspase-3 activity measured after MTX treatment alone (Figure 6). Additionally, it was found that the increase in caspase-3 activity measured after the combined treatment was statistically different from the increase in caspase-3 activity measured after MTX treatment alone ($p<0.05$). Both treatments caused a statistically significant increase in caspase-3 activity compared to the controls.

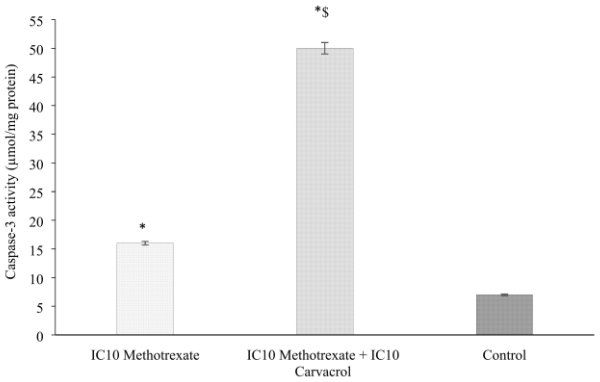

Figure 6. Effect of methotrexate alone and in combination with carvacrol on caspase-3 enzyme activity. *Significantly distinct from control ($p<0.05$). $^{\$}$Significantly distinct from methotrexate alone treatment ($p<0.05$).

4. Discussions

Methotrexate (MTX) is employed for the treatment of various conditions, such as cancer, rheumatoid arthritis, psoriasis, uveitis, asthma, granulomatosis with polyangiitis, sarcoidosis, primary biliary cirrhosis, and inflammatory bowel disease (Dawson et al. 2004, Bara et al. 2003). Methotrexate is associated with several known side effects, including pancytopenia, birth defects, infertility, liver damage, kidney toxicity, and neurological toxicity (Rondon et al. 2011, Fan et al. 2011, Bourré-Tessier et al. 2010, Schmıegelow 2009). Neurotoxicity associated with methotrexate is a significant clinical issue in cancer patients (Brock and Jenning 2004, Krajinovic et al. 2005, Harila-Saari et al. 2001, Linnebank et al. 2005). The duration of treatment, MTX dosage, type of disease, as well as genetic, molecular, and apoptotic factors all contribute to the development of the small intestine toxicity induced by MTX. MTX has a cytotoxic effect not only on cancer cells but also on other rapidly proliferating cells in the body, particularly those in the gastrointestinal mucosa (Dadhania et al. 2010, Chen et al. 2013). Intestinal mucositis is a severe adverse effect of MTX treatment. Naturally occurring compounds and their derived substances, which are high in antioxidant properties, help mitigate the harmful impacts of various substances (Gurib-Fakim 2006). The search in support of and use of naturally occurring antioxidants, particularly the ones derived originating from plants, has significantly grown significantly in recent years, as some man-made antioxidants are believed to have carcinogenic properties (Madhavi and Salunkhe 1995). Laboratory-based studies have shown that carvacrol has various curative properties, including anti-inflammatory, antithrombotic, and anti-carcinogenic effects, in both lung and breast tissues (Edris 2007).

In a study, researchers aimed to explore the impact of carvacrol, a monoterpene flavonoid, in conjunction with the chemotherapy agent 5-FU. In this study, Chou and Talalay combination index method was used in the analysis of drug-drug interactions using the data obtained from MTT analyses. The presence of synergy between carvacrol and 5-FU in an in vitro breast cancer model was shown. MTT assay results indicated that the combined treatment of cells with carvacrol and 5-FU significantly reduced the required 5-FU concentrations. The simultaneous treatment with carvacrol and 5-FU led to a greater increase in the proportion of apoptotic cells compared to individual treatments (Azimi et al. 2022). In another study, treatment of HeLa and HCT116 cells with carvacrol/topotecan was shown to result in 7.70- and 5.71-fold decrease respectively, in the half-maximal inhibitory concentration (IC_{50}) of topotecan, respectively, compared to topotecan alone. In contrast, treatment of MCF-7, HepG2, SKOV3 and A549 cancer cells with carvacrol/topotecan was shown to result in 1.49-, 1.33-, 1.50- and 1.26-fold increases in IC_{50} of topotecan, respectively, compared to topotecan alone (Bayoumi et al.2021). In a different study, according to the results of MTT test, carvacrol and 5-FU showed dose-dependent inhibitory effect. Data of apoptosis test showed that combining carvacrol with 5-FU increased the apoptotic effect of 5-FU by 6.7 times compared to the control group. In the current study. Its ability to increase apoptosis was shown to be greater than the combination of verapamil, a widely recognized P-gp inhibitör, and 5-FU (4.26-fold) (Ghorbanzadeh Akhlaq et al. 2022). Another study showed that the combination group of carvacrol nanoparticles with doxorubicin exerted greater dose-dependent growth inhibition of both MCF-7 and HeLa cells compared to the single carvacrol nanoparticles and doxorubicin. In this study, it was found that the combination index values of carvacrol nanoparticles and doxorubicin combination group showed a strong synergistic effect, 0.31 for MCF-7 and 0.34 for HeLa cells. Carvacrol nanoparticles used together with doxorubicin in MCF-7 cells reduced the dose by 16.32-fold for carvacrol nanoparticles and 4.09-fold for doxorubicin at IC_{50} of 6.23 µg/mL, while in HeLa cells, this combination reduced the

dose by 13.18-fold for carvacrol nanoparticles and 3.83-fold for doxorubicin at IC_{50} of 9.33 µg/mL, as reported in the same study (Akhlaq et al. 2023).

Sorafenib is among the few effective drugs for advanced hepatocellular carcinoma (HCC) treatment, but its clinical effectiveness is hindered by resistance and cardiotoxicity. A study examined the impact of carvacrol, an inhibitor of transient receptor potential melastatin 7 (TRPM7), in counteracting sorafenib resistance and cardiotoxicity in thioacetamide induced HCC in rats. According to the results of the study, carvacrol/ sorafenib combination significantly improved survival rate and liver functions, reduced Alpha-Fetoprotein level and slowed down hepatocellular carcinoma progression compared to sorafenib group. Carvacrol/sorafenib combination suppressed drug resistance and stem cell proliferation by downregulating ATP-binding cassette subfamily G member 2, NOTCH1, Spalt-like transcription factor 4, and CD133. Carvacrol was reported to enhance the antiproliferative and apoptotic activities of sorafenib by decreasing cyclin D1 and B-cell leukemia/lymphoma 2 and increasing BCL2-Associated X and caspase-3 (Yousef et al. 2023).

No research has been carried out on synergistic cytotoxic and apoptotic effects of carvacrol and MTX application on A-549 cells. In this study, greater membrane damage and increased apoptotic effects were observed in lung cancer cells treated with both MTX and carvacrol compared to cells treated with MTX alone. Our study is the first to demonstrate that carvacrol enhances MTX cytotoxic effect at low doses ($<IC_{50}$) in lung cancer cells. In our study, MTX and carvacrol reduced cancer cell viability at elevated concentrations. Following the treatment of MTX ($<IC_{50}$) in combination with various carvacrol concentrations ($<IC_{50}$), we determined the combined concentration that enhanced cytotoxicity on A-549 cells. Consequently, the combination treatment of MTX with carvacrol exhibited an enhanced cytotoxic effect in A-549 cells compared to MTX alone. Lower carvacrol concentrations may have exhibited stronger cytotoxic effects in A-549 cells when combined with MTX, as these concentrations might have more effectively activated the cells' antioxidant mechanisms. LDH enzyme activity was assessed after A-549 cells was treated with IC_{10} MTX alone and in combination with IC_{10} carvacrol concentration that exhibited the strongest cytotoxic effect for 48 h. It was observed that LDH enzyme activity increased more in A-549 cells treated with the combination concentrations compared to MTX treatment alone. Our LDH activity findings align with the cell viability results. In our study, treatment with MTX, both alone and in combination with carvacrol, was found to elevate GPx activity in A-549 cells. This is because MTX, alone and in combination with carvacrol, triggered oxidative stress by generating reactive oxygen species in the cells. Treatment with MTX, both alone and in combination with carvacrol, resulted in increased caspase-3 activity, thereby promoting a stronger apoptotic effect. Based on our findings, the combination treatment of MTX and carvacrol in cells induced greater apoptosis compared to MTX treatment alone.

Oxidative damage plays a crucial role in the onset and advancement of several diseases, while antioxidants are crucial in defending to protect from oxidative damage (Toullec et al. 2010). MTX possesses anti-inflammatory and immunosuppressive effects as it promotes the generation of reactive oxygen species (ROS) (Phillips et al. 2003). The reactive oxygen species (ROS) caused by MTX treatment increase both its therapeutic efficacy and its toxicity (Phillips et al. 2003, Devrim et al. 2005). Experimental investigations have revealed that MTX treatment causes a rise in production of oxygen free radicals, which may result in mitochondrial damage (Miyazono et al. 2004). It has been shown that MTX increases the levels of hydrogen peroxide, which triggers the release of free radicals that cause cellular damage (Phillips et al. 2003). Other studies have demonstrated that methotrexate reduces the effectiveness of the antioxidant system (Babiak et al. 1998, Miketova et al. 2005, Uzar et al. 2006a). Malondialdehyde (MDA) is a highly reactive oxidative by product resulting from the degradation of unsaturated fatty acids in cell membranes. Therefore, MDA serves as a dependable biological marker for lipid degradation in rats (Uzar et al. 2006b). The application of MTX leads to elevated MDA concentrations in the blood, liver, and kidneys in rats (Jahovic et al. 2004, Demiryilmaz et al. 2012).

In another study, pretreatment with carvacrol or pomegranate extract significantly reduced MTX-induced lipid peroxidation, as evidenced by the lower MDA levels observed in the blood plasma and lung tissue. Enhanced lipid degradation can be caused through the direct or indirect impact of increased ROS resulting from MTX-triggered damage. In the mentioned study indicated that MTX-induced injury may be caused due to oxidative damage, and that both intraperitoneal carvacrol and pomegranate extract administered via oral gastric administration provide a shielding effect against MTX-induced oxidative lung harm (Şen et al. 2014). The study showed that carvacrol and pomegranate extract offer protection against MTX-induced lung damage in rats (Şen et al. 2014). In our study, it was observed that the

combined application of carvacrol and MTX induced more oxidative stress in cells compared to MTX alone.

In another research, it was shown that caspase-3 activity was notably raised after MTX-induced intestinal damage (Ciralik et al. 2006, Koppelmann et al. 2012). Previous studies have shown that carvacrol and pomegranate treatment alleviated MTX-induced damage and oxidative stress in the lung and kidney (Bozkurt et al. 2014, Şen et al. 2014).

5. Conclusions

The outcomes of our study demonstrate that the combined application of carvacrol and MTX in A549 lung cancer cells leads to more membrane damage, an increase in GPx activity, and an apoptotic effect compared to MTX treatment alone. The fact that MTX showed more effective cytotoxic activity in combination with carvacrol at low doses ($<IC_{50}$) can be considered for reducing or eliminating the side effects associated with MTX use. Therefore, new approaches that could be applied in the treatment of lung carcinoma will be identified. Moreover, a better response to treatment can be attained, thereby reducing the economic burden on patients. Indirectly, benefits can be gained for the country's economy.

Declaration of Ethical Standards

The author declares that they comply with all ethical standards. The study does not need ethics committee approval as it does not involve any human or animal subjects.

Credit Authorship Contribution Statement

Author: Conceptualization, methodology/study design, software, validation, formal analysis, investigation, resources, data curation, writing-original draft, writing-review and editing, visualization, supervision

Declaration of Competing Interest

The author has no conflicts of interest to declare.

Data Availability Statement

All data generated or analyzed during this study are included in this published article.

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