DOI: 10.29409/ijcmg.v17i1.346

Cancer Research

Iraqi Journal of Cancer and Medical Genetics

Synergistic effect of essential oil of thyme extract (EOT) with chemotherapy in esophageal cancer cells

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Abstract

Background: Esophageal cancer is one of the most common causes of cancer-related death in various countries around the world, and thus represents a major global health challenge. This cancer is most often found in the upper and middle parts of the esophagus but can occur anywhere along the length of the esophagus. Aim of study: Essential oils have activity that targets cancer cells and their ability to increase the effectiveness of commonly used chemotherapy, including cisplatin, as they act as antioxidants when given to a cancer patient. Methods: In this study, the volatile oil of Thymus was extracted from the dry leaves of the plant and EOT was detected by the gas chromatography mass spectrometry (GS-MS) method. Cytotoxicity was evaluated by two experiments, one to determine the effect of volatile oil on the esophageal cancer cell line (SKG-4) and to compare chemotherapy when combined with the extract.

Results: Essential oil cytotoxicity in 48 hours showed a significant percentage ($P < 0.05$) at all diluted volumes used, while the highest toxicity (89.016%) was in the diluted volume of 2.5 µl/ml. The same results were also observed for the combination of EOT, and chemotherapy cisplatin used during the trial. A significant percentage ($P < 0.05$) within 72 hours was observed to show the highest cytotoxicity (70.191%) in the diluted volume of 10 µl/ml, and no difference appeared in cytotoxicity. Conclusions Through this experiment, we concluded that the extract has a toxic effect on the cancer cell line and does not affect the effectiveness of chemotherapy and that the aim of this study is to mitigate the negative effects of chemotherapy.

Keywords: *Cisplatin, esophageal squamous cell carcinoma (ESCC), Gas Chromatography-Mass (GC-MS), esophageal cell lines (SKG-T4), essential oil of thyme (EOT)*

Introduction

Esophageal cancer is one of the deadliest types of tumors, ranked sixth in the world, and as observed in the past 20 years, there has been a marked increase in the incidence of esophageal cancer (1). Surgical treatment for esophageal cancer is a vital and complex procedure that involves removing the esophagus and lymph nodes (2). Due to its seriousness, this surgery still suffers from an unacceptable high rate of complications and deaths (3), so doctors often resort to other treatment methods and make it the last option for treatment, especially for patients with esophageal cancer, which highlights the need for less risky methods and more effective treatments (4), but in cases of more advanced tumors, it becomes necessary to use neoadjuvant chemotherapy. Cisplatin-based treatment plans are usually used for this purpose. (5) For esophageal cancer that cannot be operated on, the standard approach includes chemoradiotherapy, which It may be an option in cases where the tumor is curable (6). Cisplatin is usually used as first-line chemotherapy for patients with various types of cancer, including leukemia, lymphoma, breast, testicular, cervical, and sarcomas. Its mechanism of action involves entering the cell and exerting cytotoxic effects by binding to DNA, forming DNA adducts within the cell, and inhibiting DNA synthesis and cell growth. Despite its effectiveness, some patients may relapse due to the development of drug resistance (7). Contemporary cancer chemotherapy often results in multidrug resistance, significant adverse reactions, and high costs. However, the effectiveness of chemotherapy can vary between tumors, mainly due to the development of drug resistance (8). This highlights the urgent need for more effective and less toxic interventions. The plant kingdom has consistently provided an attractive re-

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pository of therapeutic options, with many natural products being incorporated into existing treatment protocols to address the shortcomings of chemotherapy (9). Components of natural essential oils play an important role in cancer prevention and treatment (10). Thyme (Thymus vulgaris) is widely cultivated and is an aromatic plant used in folk medicine. (11), and thyme enjoys a prominent status as a popular medicinal plant in Asia, with a history of use in traditional medicine in Africa and Europe (12). Essential oil derived from Thymus vulgaris, found in most types of thyme studied, has antibacterial and antifungal properties (13). Furthermore, the use of thyme oil as a supplement has been associated with maintaining elevated levels of activity in superoxide dismutase, glutathione peroxidase, and general antioxidant status (14). It is also distinguished when added at nontoxic concentrations. Thyme extract is considered one of the natural substances that are used to prevent mutations, particularly DNA restoration, when used in recommended amounts (15). Of the ten essential oils tested, thyme essential oil showed the highest level of toxicity against three different types of cavity squamous cell carcinoma (OCSCC) in human cancer cells (16). The study aims to confirm the anticancer efficacy of this natural product in vivo by comparing the laboratory cytotoxic activity of thyme volatile oil in defiance of human esophageal cancer (SKG) that was resistant to treatment.

Materials and Methods

Preparation of Plant Extract

Using a Clevenger apparatus, 30 grams of dried Thymus vulgaris leaves were placed in a 2000 ml glass beaker and topped with 1000 ml of distilled water until the sample was completely submerged. This process has been used to extract essential oils. The essential oil was extracted from the aqueous extract after repeating the extraction process several times over a period of 5 hours. The oils were then stored in small opaque glass tubes that were tightly closed and placed in the refrigerator at 4 °C (17).

Preparation of Diluted Plant Extract

Thyme oil was dissolved with dimethyl sulfoxide (DMSO) to make a solution and then a volume of 10 µl were added to 1 ml of serum free medium to stock.

Chemical analysis of thyme

Gas chromatography-mass spectrometry analysis of essential oil (EOT) was performed in the laboratories of the Ministry of Science and Technology using a Shimadzu GC-2010 plus gas chromatograph (Shimadzu, Japan). The capillary tube was DB-5. GC received two microliter injections of EOT. The GC device was heated to 60 °C for 4 minutes for calibration, 150 °C for 4 minutes, and 250 °C for the final 4 minutes. The sample material was transferred directly from the injector to the detector at a flow rate of 1.35 ml/min using helium as a carrier gas mass spectrometer (MS) in electron impact (EI) mode (18).

Cell culture

The Iraqi Center for Cancer and Medical Genetics Research (ICCMGR) at Mustansiriyah University in Baghdad provided the esophageal squamous cell lines (SKG-T4) used in the experimental therapy department. After incubation in the assigned RPMI medium, we supplemented the medium with 10% FBS, 100 mg/ml streptomycin, 100 IU/ml penicillin and L-glutamine to create a complete growth environment under standard conditions (37 °C, 5% CO2, humid atmosphere).

Cytotoxicity assay

The cytotoxic effect of the extract solution after culture was evaluated in the SKG-4 cell line in humans in 96-well plates for 24 hours. Each 96-well plate was filled with 200 µl of a single-cell suspension containing 104 cells per well. The cells were then treated with thyme oil extract in solution dilutions in sequential quantities (1, 2.5, 5, and 10 µl/ml) in culture medium. Furthermore, the previously mentioned dilution was included in a separate set-up combination with the chemotherapy cisplatin dilution (Celplat 50 mg) dilution of 0.2 µl/ml. Four identical copies of this are inserted into each well. The duration of exposure was between 48 and 72 hours. After the medium was removed from the plates, the wells were washed with distilled water. To assess cytotoxicity by cell staining, a volume of 50 µl of Crystal Violet dye (Sigma Aldrich, USA) was added to each well and incubated for 30 min at 37 °C. Finally, the plates have read using a microplate reader (Biochrom, UK) at a wavelength of 592 nm to measure the absorbance. The percentage of cytotoxicity was determined using standard formula (19).

Morphology and quantitative images analysis

Using a 200 X magnification inverted light microscope (Leica Microsystems, Germany) and a digital color camera (Leica- Microsystems, Germany), the treated and untreated cells were taken in four randomly selected fields for cultivation. The ImageJ program (http://rsb.info.nih.gov/ij/) was used to analyze the images. Each picture was undergoing a triple quantitative measurement in preparation for statistical analysis.

statistical analysis

Means ± standard error of the mean is used to display the data. The approach used to compare the dilution sequence of the data was a one-way analysis of variances. When $P < 0.05$ was determined, the data differences were considered statistically significant. We used GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA) for this analysis.

Results

Table 1: show the GS-MS technology of essential oil of thyme extract (EOT) leaves.

NO	Name	Conc.%	H /A	Compounds EOS	Mechanisms
2	(1R)-2,6,6-Trimeth-ylbicyclo	2.81	7.35	1R-alpha-Pinene, 2,6,6-Trimethylbicy-clo, mom-terpene	Anticancer (20)
5	Beta-Myrcene	8.50	6.49	Carvacrol acetate, 5-Isopropyl-2-meth-ylphenyl acetate	Anti-inflammation (21)
7	1,3-Cyclohexadiene, 1-met	9.87	19.14	Tran-Geranylacetone, 5,9-Undecadien-2-one,3,6,10-dimethyl	Antioxidant (22)
12	2,3,5-Trimethylani-zole	32.02	39.01	2-Pentadecanone, 6,10,14-trimethyl, Hexahydrofarnesylacetone	Antiproliferative (23)
16	Beta-Bisabolene	8.06	8.77	octahydro-1-naphthalenol, Tetrameth-yl-1-octahydronaphthalen	Cytotoxicity (24)
21	Caryophyllene oxide	3.49	6.02	2-Propenoic acid, Octyl methacrylate	Antioxidant anticancer (25)
30	Benzylsulfonyl-2,2,6-tirm	2.26	4.05	Caryophyllenyl alcohol, -Tetramethyl-bicyclo	Anti- inflammatory (4)

According to Table 1, seven active compounds were detected: trimethylanizole had the highest extraction peak area per minute (32.02%), while benzylsulfonyl-2,2,6-tirm had the lowest area (2.26%).

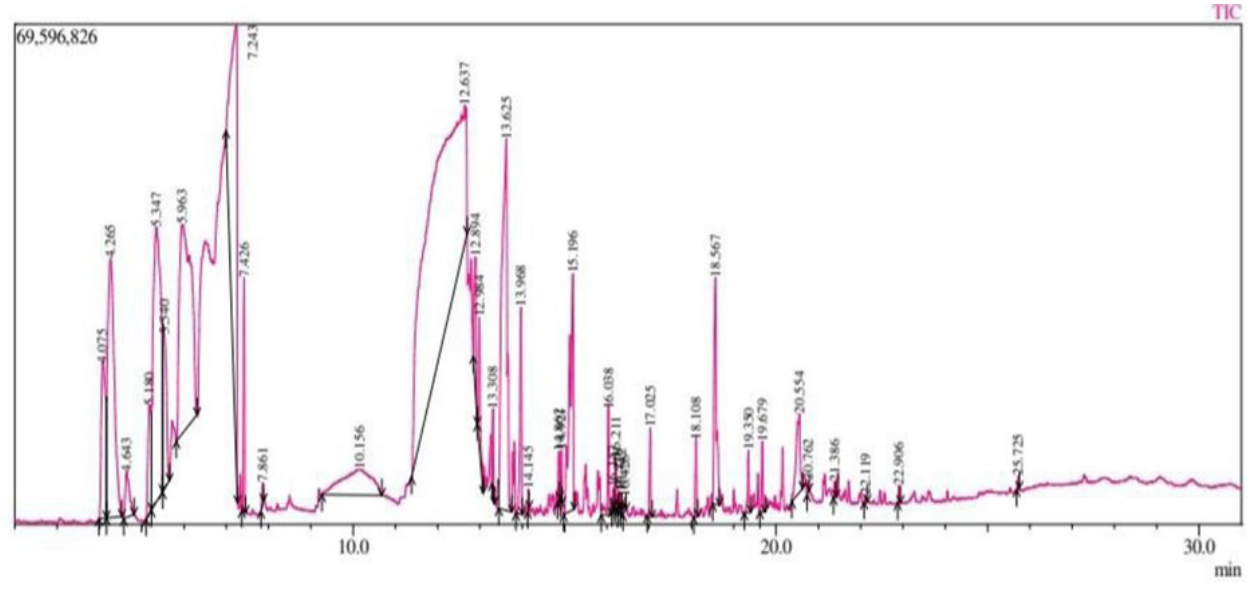

Figure 1: Results of the GC-MS analysis of the aqueous extract of thymus leaves.

Table 2: cytotoxicity percentage of cisplatin chemotherapy (CISP) in Esophageal cancer (SKG-4) in vitro evaluated by cytotoxicity assay 48 hours.

Dilution	Chemotherapy (cisplatin)
0.1µL	87.93%
0.2 µl	90.49%
0.3 µl	94.78%
0.4 µL	95.19%

The effect of cisplatin on growth was evaluated using a cytotoxicity assay (Table 2). The rate of growth inhibition on the SKG cell line for 48 hours at different dilutions (0.1, 0.2, 0.3, and 0.4 µmol/mL) is shown. After that, the results were observed to decrease the number of cancer cell adhesions at very high rates when examining the rate of cytotoxicity.

Table 3: percentage of cytotoxicity of the combination of chemotherapy cisplatin (CISP) and thyme extract dilution essential oil (EOT) in esophageal cancer (SKG-4) in vitro evaluated by cytotoxicity assay 48 hours.

Dilution	SKG-4 EOT	SKG-4 Combination - EOT, CISP
1µl	85.8195 %	88.66 %
2.5 µl	89.016 %	88.45 %
5 µl	68.3955 %	76.62 %
10 µl	59.8245 %	75.09 %

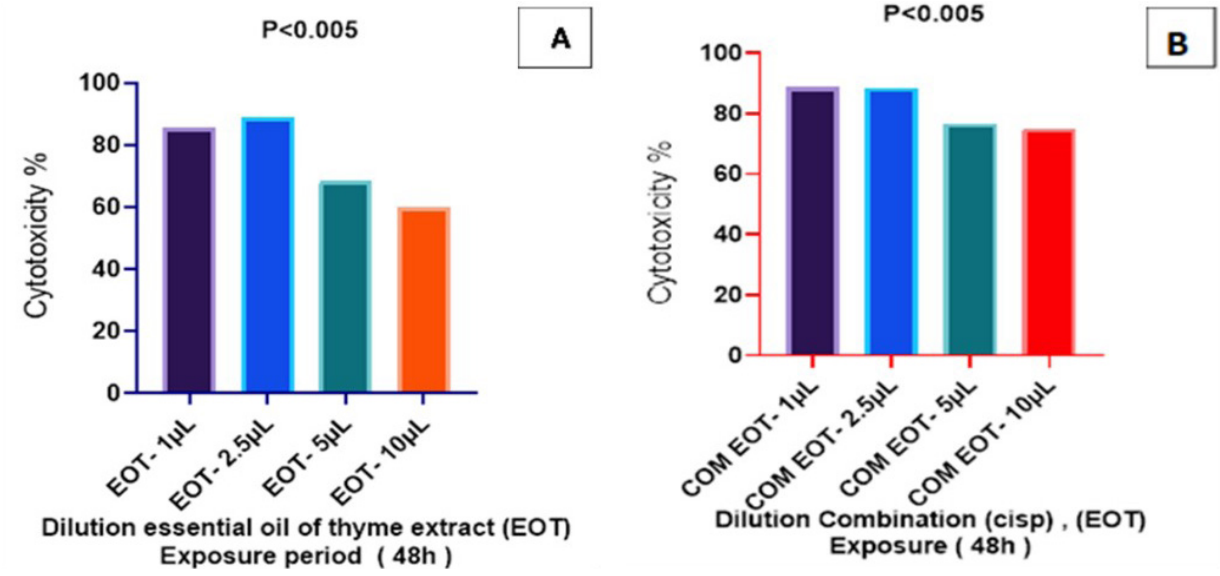

Figure 2: Cytotoxicity rate of esophageal cancer (SKG-4) cells in vitro. (A) Cytotoxicity percentage 48 hours after exposure to various dilutions of thyme essential oil extract. (B) Cytotoxicity percentage 48 hours after exposure to a combination of cisplatin and thyme essential oil extract

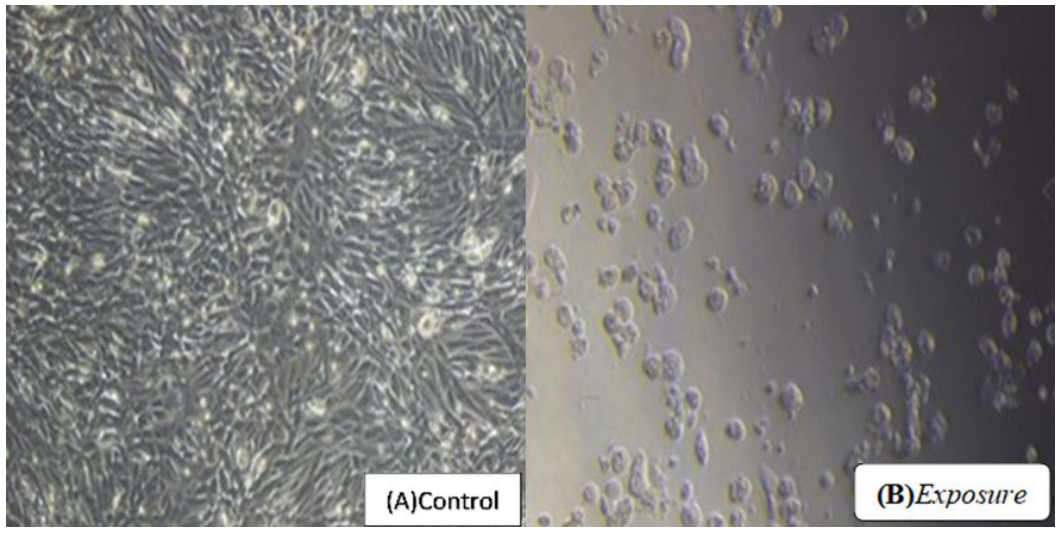

Figure 3: Microscopic image of the SKG-4 cell line after 48 hours of cytotoxicity assay. (A) Image control of the SKG cell line. (B) Image of SKG cell line inhibition at a dilution of 2.5 µl/ml. (Microscope magnification power 200 X).

The effect of thyme on growth was evaluated by a cytotoxicity assay (Table 3). It shows the growth inhibition rate of the thyme extract in the SKG cell line over 48 h at different dilutions (1, 2.5, 5, and 10 µl/ml). Subsequently, the results were observed to decrease the number of cancer cell adhesions in the cytotoxicity rate assay, where the highest percentage of inhibition was at a 2.5 l / ml dilution of 2.5 µl/ml and the lowest percentage of inhibition was 10 µl/ml. Comparing the inhibition rate between chemotherapy (cisplatin) and essential oils (thyme) in the SKG cancer cell line showed the highest inhibition rate (2.5 µl/ml) and the lowest inhibition rate (10 µl/ml).

Table 4: Cytotoxicity percentage of the combination of cisplatin (CISP) and thyme essential oil extract (EOT) in esophageal cancer (SKG-4) cells in vitro, evaluated by a cytotoxicity assay after 72 hours (Microscope magnification: 200X)

Dilution	EOT SKG-4	SKG-4 Combination - EOT, CISP
1µL	62.928 %	47.137 %
2.5 µl	58.489 %	61.429 %
5 µl	66.783 %	76.362 %
10 µL	70.191 %	71.748 %

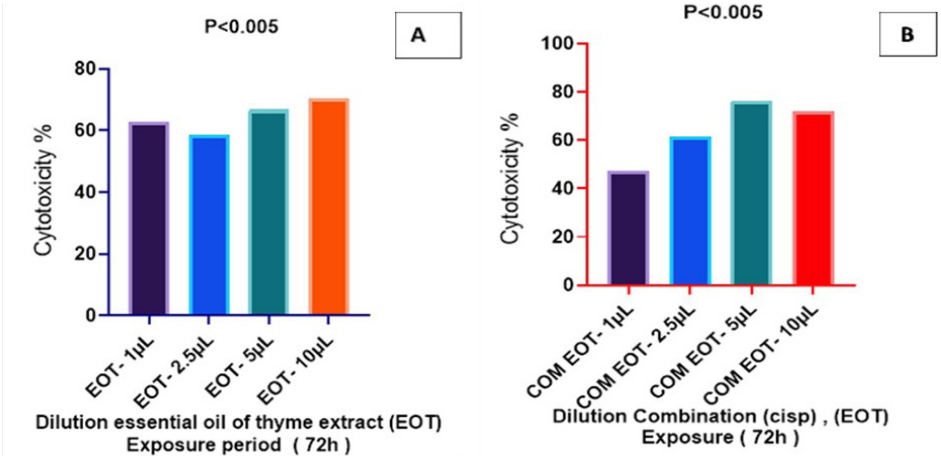

Figure 4: Cytotoxicity rate of esophageal cancer (SKG-4) cells in vitro. (A) Cytotoxicity percentage 48 hours after exposure to various dilutions of thyme essential oil extract (EOT). (B) Cytotoxicity percentage 48 hours after exposure to a combination of cisplatin and thyme essential oil extract.

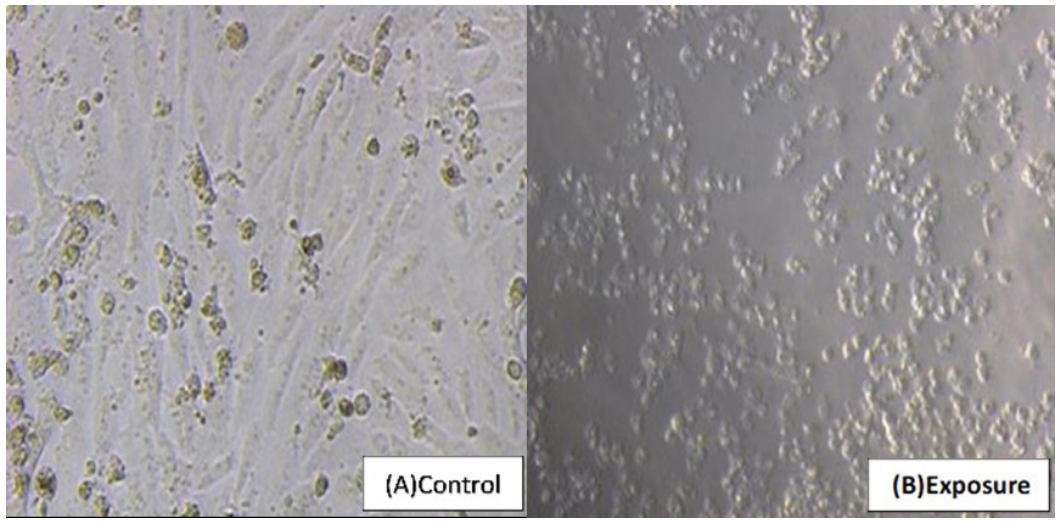

Figure 5: Microscopic image of the SKG-4 cell line after 72 hours of cytotoxicity assay. (A) Microscopic image control of the SKG cell line. (B) Microscopic image of SKG cell line inhibition was at a dilution of 10 µl/ml (Microscope magnification power 200 X).

The effect of thyme on growth was evaluated by a cytotoxicity assay (Table 4). It shows the growth inhibition rate of the thyme extract in the SKG cell line over 72 h at different dilutions (1, 2.5, 5, and 10 µl/ml). Subsequently, the results were observed to decrease the number of cancer cell adhesions in the cytotoxicity rate assay, where the highest percentage of inhibition was at a dilution of 10 µl/ml and the lowest percentage of inhibition was 2.5 µl/ml. Comparing the inhibition rate between chemotherapy (cisplatin) and essential oils (thyme) in the SKG cancer cell line showed the highest inhibition rate (5 µl/ml) and the lowest inhibition rate (1 µl/ml).

Discussion

Due to the increased rate of cell proliferation in cancer, conventional chemotherapy medications are more harmful to cancer cells. However, their mechanism of action poses challenges related to tumor cell selectivity and associated cytotoxicity to healthy cells (27). Thyme is now believed to be one of the aromatic compounds isolated from Thymus vulgaris with oily components and has an antiproliferative impact on human esophageal tumor cells when treated with chemotherapy and surgery as combination treatment techniques (28). The results of chemical analysis (GS-MS) showed the presence of many active compounds; the most concentrated were pentadecagon, 6,10,14-trimethyl, and hexahydrofarnesylacetone (conc. 32%), which according to (23) have an antiproliferative effect. Thymol is one of its most important components and is considered to have protective properties. They also have antibacterial and anti-inflammatory properties (29). These compounds are important for their chemopreventive properties, as well as their biological activity, including anticancer, antioxidant, antibacterial, and anti-inflammatory properties. (30). The research aims to compare the in vitro cytotoxic activity of thyme essential oil against human esophageal cancer (SKG), which is resistant to chemotherapy, and to investigate the anticancer efficacy of this natural product in vivo. The use of EO extracts as single agents has been shown in various in vitro studies to specifically target cancer cells, with absent or markedly less cytotoxicity exhibited toward healthy cells with a range of mechanisms of action. The results of the cytotoxicity analysis in 48 hours showed that the highest inhibition rate was for the thyme extract (2.5 µl) at 89% and the highest inhibition rate was for the combination of EOT and CISP at 88%, which is consistent with the fact that Thyme has been linked to different pharmacological effects, including antioxidants (31). Specific EO constituents have been shown to enhance the cytotoxic activity of chemotherapy drugs in various cell lines. Thus, increasing the therapeutic window, that is, lowering the required drug concentrations while providing the same effect. 10 µL concentrations of thyme oil have the lowest inhibition rate. The components of natural essential oils are involved in multiple mechanisms, including but not limited to antioxidant, antimutagenic, antiproliferative, enhancement of immune function and surveillance (32), promotion of enzyme induction and detoxification, modification of multidrug resistance, and synergistic mechanisms of volatile components. Aromatic herbs and food plants have several constituents found in their essential oils, such as monoterpenes, sesquiterpenes, oxygenated monoterpenes, oxygenated sesquiterpenes, phenols, and others (33). In 72 hours, 10 µL gave the best result of 70% for EO and 5 µL for combination with cisplatin, and it was observed that the inhibition rate increases with increasing concentrations of the extract (34). This suggests that the concentration of the extract affects the survival of cancer cells because the cytotoxicity of thyme may be due to the accumulation of molecules. We also

conducted another trial that included a combination of different concentrations of thyme extract with fixed dilutions of cisplatin, a common cancer chemotherapy, to reduce the side effects of chemotherapy. The comparative study showed that the death rate increases with increasing dilutions of thyme extract within 72 hours and the death rate also increases. This shows that thyme essential oil extract affects the inhibitory ratio in the presence of chemotherapy (35). The purpose of this test was to compare the cytotoxic activity of thyme volatiles against human esophageal cancer (SKG) resistant to chemotherapy, and thyme has been linked to an effect on cisplatin as chemotherapy (36). It may be clinically useful as adjuvant therapy during chemotherapy for cancer patients or immunocompromised patients to overcome leukopenia. Thymol was the most abundant component in present-day thyme essential oil. Thymol has shown cytotoxic activity against various human tumor models (37). Studies show the importance of essential oil extracts as anticancer treatments, including T. vulgaris. It has antiproliferative activity against breast and cervical cancer cells and works to regulate p53 gene expression, which could be a potential treatment for cancer patients, as the research aims to compare the in vitro cytotoxic activity of thyme essential oil against human esophageal cancer (38). Many studies related to our study have important points that involve safety, selectivity, and significantly reduced activity toward normal human cells.

Conclusions

From this experiment we determined that the extract is toxic to the tumor cell line SKG-4 and has no effect on the effectiveness of chemotherapy; rather, it reduces the severity of symptoms caused by chemotherapy.

Authors contribution

The volatile oil Thymus was extracted by Budoor Sattar Abbas and Ola H. Fadhil. Visualization, Ola H. Fadhil. Methodology, Amer T. Tawfeeq, Cytotoxicity, Budoor Sattar Abbas. Software, Abbas A. Mohammed. Writing Peper, Budoor Sattar Abbas, and Ayat Subhi Jadou. Under supervision of Amer T. Tawfeeq.

Acknowledgments

The authors thank everyone who made this research possible. The project was funded by Mustansiriyah University's Iraq Center for Cancer and Medical Genetic Research. All authors have read and approved the published version of the manuscript.

Conflict of Interest

There were no revelations of the author's conflicts of interest.

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