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Evaluating the *in vitro* Cytotoxicity of *Thymus vulgaris* Essential Oil on MCF-7 and HeLa Cancer Cell Lines

Alsafa N. Khalaf*, Ibrahim J. Abed
Department of Biology, College of Science, University of Baghdad, Iraq

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Abstract

Thyme essential oil (TEO) was extracted from dried leaves of *Thymus vulgaris.* The air-dried aerial parts of the plant produced 1.0% yield of TEO. The detection of this essential oil's compounds was performed by GC-MASS. The cytotoxic activity of TEO was evaluated against two human cancer cell lines, namely HeLa (human epithelial cervical cancer) and MCF-7 (human breast carcinoma). Cells grown in 96 multi-well plates were treated with six concentrations of EO (6.25, 12.5, 25, 50, 100, 200 ppm) and incubated at 37 °C for 72 hrs. Cancer cell lines elicited various degrees of sensitivity to the cytotoxic effect of essential oil. The TEO exhibited significant differences ($p \leq 0.01$) between the effects of all concentrations against these two human cell lines. The results showed the highest toxicity of TEO on HeLa cell line (78.67%) and MCF-7 cell line (83.60%) at 200 ppm concentration. Also the values of half-maximal inhibitory concentration (IC50) of TEO against HeLa and MCF-7 cell lines were 34.63 and 27.66 ppm, respectively. Cells treated with the IC50 of TEO showed a significant difference ($p \leq 0.01$) in *p53* fold expression between HeLa cell line (4.33±0.41 folds) and MCF-7 cell line (5.10±0.32 folds). In general, a dose-dependent decrease the survival of the two cell lines was observed. In addition, MCF-7 cell line revealed higher sensitivity against TEO than HeLa cell line.

Keywords: *T. vulgaris*, HeLa cell line, MCF-7 cell line, TEO, *p53*.

تقييم الفعاليه السميه للزيت العطري لنبات الزعتر على بعض خطوط الخلايا السرطانيه

الصفا نصر خلف*, ابراهيم جابر عبد
قسم علوم الحياة, كلية العلوم, جامة بغداد, العراق

الخلاصه

استُخلص الزيت الاساسي من الاوراق الجافة لنبات الزعتر. وانتجت الاوراق الجافة لهذا النبات 1% من الزيت الطيار. وقد تم الكشف عن مركبات الزيت الطيار للزعتر بطريقه GC–MASS. قُيمت السمية الخلوية لهذ الزيت لدراسة فعاليتها في خطين خلويين سرطانيين للإنسان (خلايا سرطان عنق الرحم , خلايا سرطان الثدي) اذ حضنت الخطوط الخلوية بدرجة 37 درجه مئوية لمدة 72 ساعه واستعلمت تراكيز مختلفة من الزيت الطيار(6,25, 12,5, 25, 50, 100, 200جزء في المليون). اظهرت خطوط الخلايا السرطانية درجات مختلفة من الحساسية للسمية الخلوية للزيت الاساسي. أظهر الزيت الاساسي لنبات الزعتر فروق معنوية كبيرة (P≤0.01) في جميع التركيز المستخدمة ضد هذين الخطين من الخلايا البشرية. بينت النتائج ان أعلى سمية

*Email: safanassr66@gmail.com

للزيت كانت على خط خلايا سرطان عنق الرحم (78.67٪) وخط خلايا سرطان الثدي(83.60٪)بتركيز 200 جزء في المليون، وكذلك كانت نسبةIC50الخطوط خلايا سرطان عنق الرحم وخلايا سرطان الثدي هي 34.68جزء في المليون و 27.66 جزء في المليون على التوالي. كان للخلايا المعالجة بتركيز IC50من الزيت الاساسي لنبات الزعتر تعبير معنوي كبير لجين *p53*(P≤ 0.01) وصل إلى 4.33 ± 0.41 ضعف في خط خلية سرطان عنق الرحم و 5.10 ± 0.32 ضعف في خط خلايا سرطان الثدي. بشكل عام, لوحظ ان بقاء الخطوط الخلوية قيد الدراسة يعتمد على جرع الزيت الطيار المستخدمة. بالإضافة الى ان الخلية السرطانية للثدي اظهرت حساسيه عالية تجاه الزيت الطيار مقارنة مع خلية سرطان عنق الرحم

Introduction

Breast cancer is considered as one of the common and well known cancers and is the main cause of death in woman throughout the world. Statistics in 2012 reported this kind of cancer as one of the three most ordinarily diagnosed cancer types amongst women. It is predicted that breast cancer will reach a prevalence value of 29 % (226,870 cases) of all cancer types between women. On the other hand, cervical cancer, caused by Human Papilloma Virus, is the fourth most common cause of death due to cancer (266,000 deaths) among women, especially in less developed countries [1]. The most common genetic variations in many human cancers are mutations in *p53* gene. It was recorded that more than half of human malignancies have 5-80% mutation relying on the kind, stage, and etiology of cancer. Many studies found relations between *p53* mutations and frequent genetic alterations in breast cancer, such as that observed in 30% of breast carcinomas [2] and also is hardly linked with cervical cancer [3, 4]. The traditional treatments applied nowadays (e.g. chemotherapy, surgery and radiotherapy) are obstructed by side effects that include tumor resistance, vomiting, nausea, mouth soreness, fatigue, hair loss, reduced resistance to infections, decrease in weight, bleeding, and diarrhea [5]. The discovery of new and successful treatments against breast and cervical cancers is thus a real confrontation to science. As novel candidates [6], increasing attention has been paid to develop anticancer agents from medicinal plants. The anticancer potential of bioactive plant ingredients is based on precise targeting of aberrant epigenetic changes that are known to have carcinogenic characteristics [7, 8]. In recent years, the use of plant-based natural antioxidant compounds, such as phenolics (flavonoids, phenolic acids), as well as preventive and therapeutic medicinal products in foods, has gained considerable popularity [9]. Indeed, over half of the prescriptions of drugs available at present are derived from plants [10, 11].One of several common plants that have been shown to possess therapeutic capability is *Thymus vulgaris* L. This plant is native to the Western Asia, Southern Europe, and Middle East, especially the Mediterranean region [12]. *T. vulgaris* L. is a plant that is rich in essential oils and its main chemical components are the oxygenated monoterpenes and monoterpene hydrocarbons. In particular, carvacrol, thymol, p-cymene, borneol, trans-caryophyllene, and cis-sabinene hydrate are present at the highest concentrations. In addition, the plant contains phenolics, represented by derivatives of rosmarinic acid,

and flavonoids. Thus, T. vulgaris is categorized amongst plants with the highest antioxidant activity [13]. There are a few preclinical studies that point to the anticancer potentials of T. vulgaris. For example, it has demonstrated substantial free radical scavenging and proapoptotic effects in the BC T47D cell line [14]. T. vulgaris L. also displayed important chemo-preventive and therapeutic effects against clinical breast carcinoma [15]. Therefore, this study aimed to evaluate the possible cytotoxic effects of TEO on breast and cervical cancer cell lines, as well as its effects on the expression of p53 gene in these cells.

Materials and Methods

Maintenance of cell line cultures

Human cervical cell line (HeLa) and breast carcinoma cell line (MCF-7) were cultured and maintained using RPMI-1640 media supplemented with 10 % fetal bovine serum (Capricorn, Germany), 100 µg/mL streptomycin and 100 U/mL penicillin. Using Trypsin- EDTA, the cell passage was then reseeded at 80 % confluence twice a week and incubated at 37° C in 5 % CO2 [16].

T. vulgaris essential oil extraction

The air-dried leaves (250 g) of thyme were applied in a Clevenger apparatus for the extraction of essential oil, using the hydrodistillation method, and boiled for three hours with distilled water, then the extracted oil was stored at 4°C until use [17].

Chemical composition of *T. vulgaris* essential oil

In the Ministry of Science and Technology laboratories, Gas Chromatography-Mass (GC-MS) analysis of TEO was performed using a Shimadzu gas chromatograph GC-2010 plus (Shimadzu, Japan). The type of capillary column used was DB-5 (25 m long, 0.2 mm inner diameter, 0.25µm thickness). Two microliters of TEO were injected into the GC apparatus. The temperature of the GC apparatus was calibrated for four minutes at 60°C, increased to 150°C for four minutes, and then raised to 250°C. The flow rate was 1.35 ml / min and the carrier gas was helium, bringing the sample material straight from the injector to the detector. The electron impact (EI) mode was applied with mass spectrometry (MS) [18].

Cytotoxicity assays for TEO

The MTT cell viability assay was conducted using 96-well plates to assess the cytotoxic effects of thyme EO on HeLa and MCF-7 cell lines. Cell lines were seeded at 1 x 104 cells / well. After 24 hrs., or when a confluent monolayer was obtained, cells were treated with the tested EO at various concentrations. After 72 hrs. of treatment, cell viability was determined by removing the medium, adding 28 µL of 2 mg / mL of MTT solution, and incubating the cells at 37° C for 2.5 hrs. The crystals remaining in the wells were solubilized after the removal of the MTT solution by the addition of 130 µL of DMSO (Dimethyl Sulphoxide) accompanied by 37 °C incubation for 15 minutes with shaking [19]. The absorbency was determined on a microplate reader at 492 nm; The assay was carried out in triplicate. The inhibition rate of cell growth (the percentage of cytotoxicity) was determined as in the following equation [20]:-

Cytotoxicity = A-B / A * 100

where A and B are the optical density values of control and tested samples, respectively.

200 µL of cell suspensions were seeded into 96-well micro-titration plates at 1x104 mL-1 cell concentration, visualized under an inverted microscope, and incubated at 37 °C for 48 hrs. After 24 hrs., the medium was withdrawn and the oil at the IC50 dose was added. The plates were treated with crystal violet stain at 50 µL and incubated at 37 °C for 15 minutes. The stain was gently cleaned with tap water. The cells were examined under a 100x magnification of inverted microscope and filed and captured with a digital camera [21].

Quantitative real-time PCR test for the expression of *p53* gene

According to the manufacturer's instructions, RNAs from HeLa and MCF-7 cell lines were collected using Trizol (Invitrogen). Using RevertAid TMH Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario, Canada), cDNA synthesis was achieved with 2 µg of total RNA. The primers used to express the p53 gene were 5-TTGAGGTGCGTGTTGTG-3 forward and 5-CTTCAGTGGCTGGGAGTG-3 reverse. GAPDH (housekeeping gene) primers, forward 5-ACCACAGTCCATGCCATCAC-3 and reverse 5-TCCACCACCCTGTTGCTGTG-3, were used for the normalization of qPCR [22]. Each reaction consisted of 7.5 µl of SYBR green (SYBR ® Premix Ex Taq package, USA), 0.3 µl of cDNA (1 µl), 0.3 µl of each associated primer, and completed with a volume of up to 15 µl distilled water. The PCR reactions were detected using the ABI PRISM 7500 sequence detection method (Applied Biosystems , USA). The quantification of *p53* gene expression was carried out using the ΔΔCT calculation and the fold of gene expression was given as $2^{-\Delta\Delta CT.}$

Statistical Analysis

To detect the effects of differential factors on study parameters, the Statistical Analysis Software- SAS [23] was used. The least significant difference (LSD) and the Analysis of Variance (ANOVA) T test were used in this research to substantially compare the means.

Results and Discussion

The air-dried aerial parts of *T. vulgaris* produced 1.0% yield of EO. All biochemical compounds in the TEO were analyzed using GC-MS, as shown in Figure-1. TEO showed 42 peaks in GC-MS chromatogram, which were characterized and listed based on their retention time (RT) and peak area (Area %). The essential oil was found to be rich in caryophellene (11.18%), caryophellene oxide (8.11%), linalol (8.00%), gamma.-Terpinen (7.07%), thymol (6.99%), 1H-Cycloprop[e]azulene (6.51%), ledene (5.13%), 2-(4a,8-Dimethyl (4.89%), Thymolacetate (3.61%), (+)-4-Carene (3.59%), Durenol (3.55%), alpha.-Pinene (3.39%), 4-Terpineol (3.01%), alpha.-Terpieol (2.22%), Thymol methyl ether (2.06%), Cineole (1.98%), (-)-.beta.-Pinene (1.92%), Terpinolen (1.87%), 1-Cyclohexyl-2-buten-1-ol (c,t) (1.37%), Camphene (1.34%), 1,4,7,-Cycloundecatriene (1.27%), Bergamol (1.26%), 3-Benzylsulfonyl-2,6,6-trimethylbicyclo(3.1.1) (1.05%), 2,5-Diethylphenol (0.87%), decahydro-1,1,7-

trimethyl-4-methylene (0.81%), Isoaromadendrene epoxide (0.80%), 3-Benzylsulfonyl-2,6,6-trimethylbicyclo(3.1.1) (0.74%), 10,12-Pentacosadiynoic acid (0.72%), 3-Thujene (0.70%), 1-Heptatriacotanol (0.68%), alpha.-Methyl-.alpha.-[4-methyl-3-pentenyl]oxiranemethanol (0.62%), beta.-Guaiene (0.44%), Cuminic alcohol (0.35%), Cycloheptane (0.30%), trans-Decalin (0.30%), Carvacrol (0.27%), Ocimene (0.21%), Borneol (0.19%), trans-Dihydrocarvone (0.19%), 2-Hexenal (0.19%), Hexahydrofarnesyl acetone (0.12%), and delta.-Cadinene (+) (0.10%). Similar results were obtained by a previous study [24]. These studies confirmed that the major component of *T. vulgaris* is caryophellene. It also contains volatile oil compounds with various species of dominantchemotype, as was also reported earlier [25].

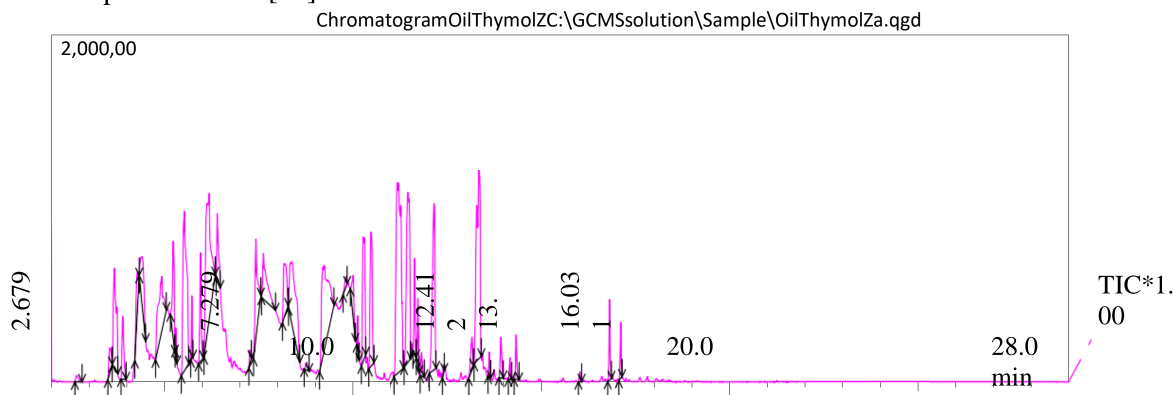

Figure 1- GC-MASS analyses chromatogram of *T. vulgaris* essential oil

Cytotoxic effects of TEO on MCF-7 cell line

Cytotoxic effects of the essential oil extracted from *T. vulgaris* on breast cancer (MCF-7ve) cell line were investigated using the MTT assay. The results shown in Figure-2 that appears there was significant difference (P≤ 0.01) of cytotoxicity effect on essentialoil extract. The results of the MTT assay are given in Figure-2, compared withthe viability of MCF-7 breast cancer cells after 24, 48, and 72 hrs. have revealed meaningful differences among groups treated with six various concentrations of TEO. These results indicate that, as the concentration of the EO is increased, the viability of MCF-7 cells is significantly decreased. This result is in agreement with that described by a previous study [26], which reported the effect of essential oils on the viability of cells. This result is also similar to that reported by another research group [27], which tested the effects of *Thymus caramanicus* extract at different concentrations (10-300µg/ml) on MCF-7 cell line, showing a decrease in cell viability in a dose dependent manner.

At the 200 ppm concentration, the higher inhibition rate (82 percent) was seen, while at the 6.25 ppm concentration, the lowest rate (5 percent) was seen. The concentration that contributed to a 50 percent rate of growth inhibition (IC50) was 27.66 ppm.

According to the GC-MS analysis results, caryophellene (11.18%) was the most abundant component of the oil. The cytotoxic effect of the thyme EO on the breast cell line suggests that the presence of caryophellene as the major component inhibited cell proliferation, without affecting the normal cells.

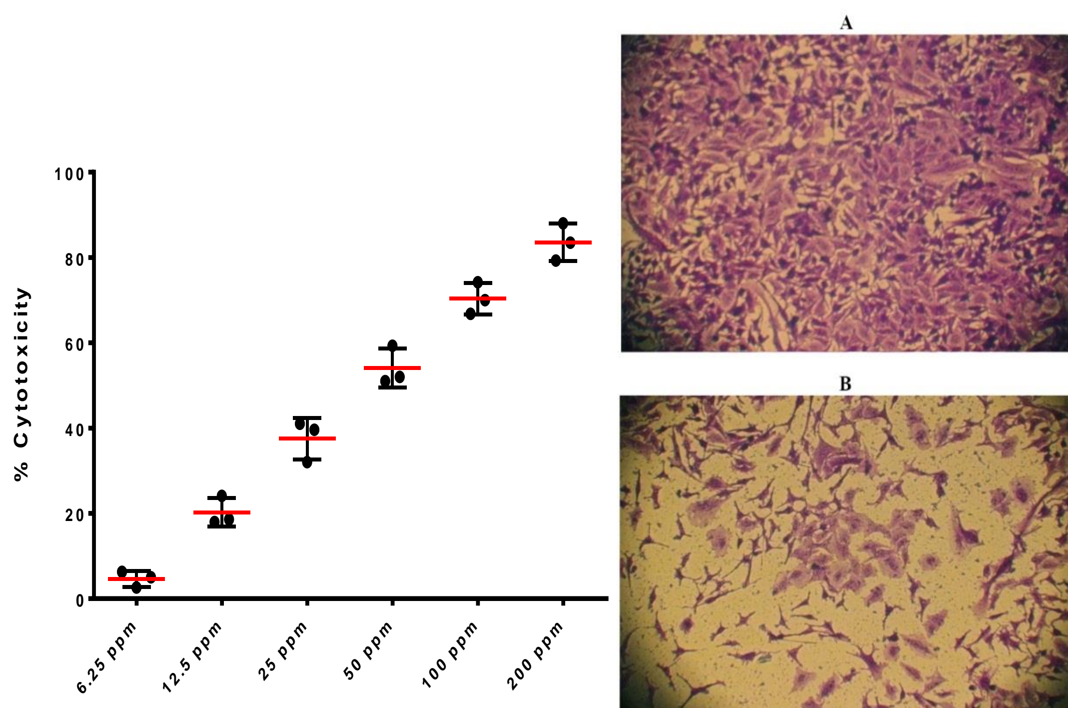

Figure 2- Cytotoxicity of Thyme EO against MCF-7 cell line.

The left scale represents the cytotoxicity which is dose dependent, while the IC50 = 27.66 ppm. The results were represented as the mean ± SEM of triplicate measurements. A represents the untreated cells while B shows antiproliferative effects of thyme EO on cancer cells at the concentration of IC50; scale bar = 10 µm.

These results are in agreement with those of a previous work [28], which demonstrated the anti-proliferative activity of the extract of *M. persicum*, showing that the inhibition of MCF-7 cells growth by the extract was in dose and time dependent manner. Another study [29] found that all treated cell lines showed a significant reduction in cell viability in response to the increasing seed oil concentrations (0.15 to 1 mg/mL) of *M. oleifera*, which has potent cytotoxic activities against cancer cell lines. Another *in vitro* research also demonstrated the antiproliferative and proapoptotic impacts of *T. vulgaris* essential oil (0.12 µg/mL) on MCF-7 cell line [15].

Cytotoxic effects of TEO on HeLa cell line

The results also showed significant differences ($p \leq 0.01$) in the percentage of inhibition of the growth of HeLa cell line, depending on the concentration of thymol. The concentrations used ranged 6.25-200 ppm, which induced inhibition values that ranged 4.06-80.25 %. The concentration that contributed to a 50 percent of growth rate of inhibition (IC50) was 34.63 ppm (Figure- 3).

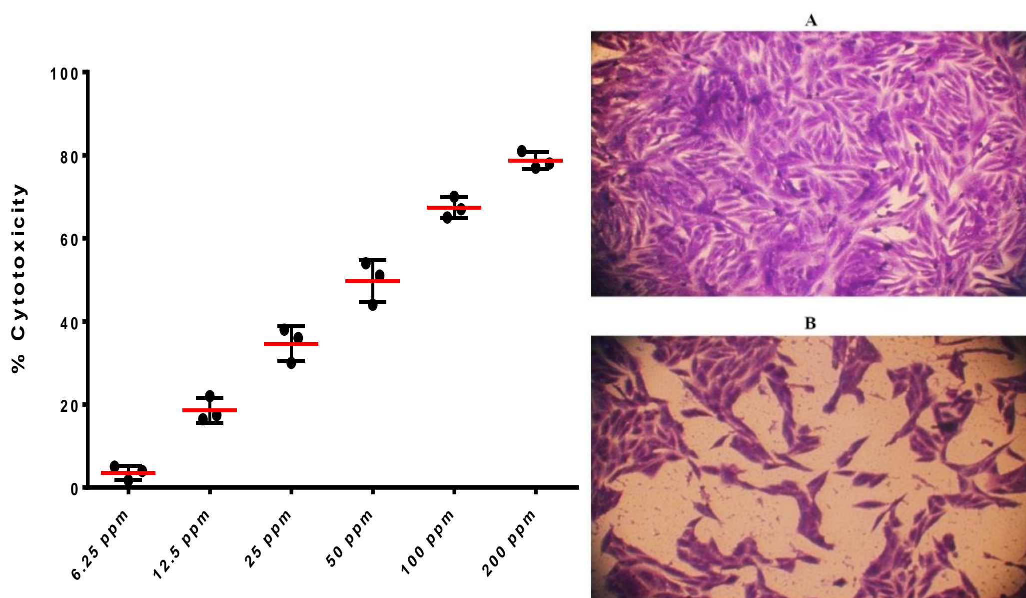

Figure 3- Cytotoxicity of thyme EO against HeLa cell line.

The left scale represents the cytotoxicity, which is dose dependent; the IC50 = 34.63 ppm. The results were represented as the mean ± SEM of triplicate measurements. A represents the untreated cells while B shows the antiproliferative effects of thyme EO on treated cells at the concentration of IC50' scale bar = 10 µm.

These results are in accordance with those of an earlier study [30] which reported that the essential oil of *Pinuseldarica* significantly reduced the viability of HeLa and MCF-7 cells in a concentration dependent manner. These results were attributed to the essential oil contents of terpenes and phenolic compounds. The synergistic effect was revealed to be more significant than that of isolated compounds. This is because of the notion that the activity of the main components can be modified by other minor molecules, which can lead to better distribution of the essential oil [31]. According to a recent study that tested the cytotoxicity of 10 essential oils (grapefruit, mint, lavender, cinnamon, ginger, lemon, jasmine, chamomile, thyme, and rose), thyme was identified as the most effective on human lung carcinoma (A549), human prostate carcinoma (PC3), and human breast cancer (MCF7) cell lines [32].

The extract of *T. vulgaris* showed a dose-dependent inhibition of HeLa cells, but this inhibitory effect didn't reach 50% [33]. In the culture of Vero cells, this extract showed stimulatory effects at concentrations lower than 400 µg/ml. The highest stimulatory effect was observed at 50 µg/ml, while at 400 µg/ml, cell proliferation was markedly decreased. Another study found that the active compound Alpha-terpineolfound in many plants, such as *T.* vulgaris, showed significant cytotoxicity against HeLa and other cell lines [34]. Also, thymol was an abundant active compound in thyme essential oil, showing cytotoxic activity against different human tumor models, including human hepatoma cells (HepG2), human colonic cells (Caco-2), and hamster lung cells (V79) [35].

The applied cytotoxicity assay is based on the ability of living cells to reduce the yellow water-soluble XTT into an insoluble formazan product. The IC50 values were used to determine the selectivity index (SI) values of each extract, which represents the overall activity. SI values were calculated as the IC50 of Vero / IC50 of HeLa [36].

This result is deals with [37] The results showed a significant differences ($p<0.05$) in the percentage of inhibition of HeLa cell line, dependent on the concentration of thymol. Thus, the percentage of inhibition ranged 87.25-74.06 % , while the concentration ranged 30.5-244 ng/ml. The highest inhibition rate of 87.25% was recorded at the concentration of 30.5 ng/ml after 72 hrs. of exposure.

Thyme essential oil's cytotoxicity might be due to its lipophilic compounds that accumulate in cancer cell membranes and increase their permeability, resulting in leakage of enzymes and metabolites [35].

The effects of TEO on the expression of *p53* in MCF-7 and HeLa cell lines

Using qRT-PCR, the changes in *p53* gene expression was investigated in thyme EO treated and untreated cancer cells. *P53* has been efficiently expressed and quantified compared to the GAPDH

gene, which is not surprising because GAPDH is a widely used housekeeping gene assisted by data on gene expression.

. MCF7 cells treated with the TEO IC50 dose of 27.66 ppm had a significantly higher ($p \leq 0.01$) value of *p53* fold expression (5.10±0.32) in comparison to the untreated cells (1.03±0.04) (Figure 4- A). Furthermore, HeLa cells treated with thyme EO showed a significantly higher ($p \leq 0.01$) *p53* fold expression (4.33±0.41) than untreated cells (1.00±0.11), as illustrated in Figure 4-B. From the results, it is concluded that the effects of TEO on the expression of *P53* in MCF-7 cell line was higher than that in HeLa cell line.

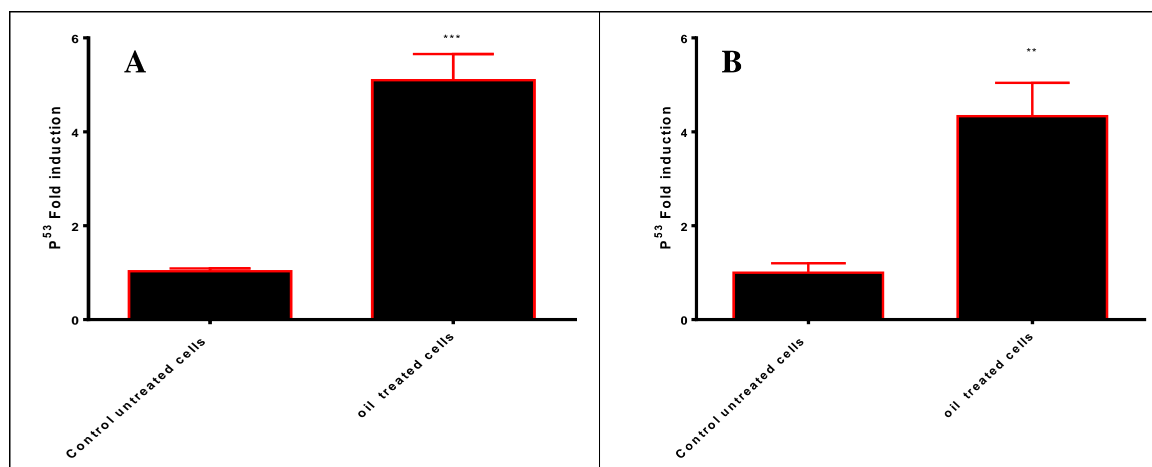

Figure 3- Thyme EO upregulation of *p53* expression in MCF-7 (A) and HeLa (B) cancer cells. Data are represented as mean ± SD.

The activation of upstream regulatory proteins, such as *p53*, leads to the activation of downstream genes that trigger either cell repair (cycle arrest) or cell death via apoptosis. The different pathways are achieved using different mechanisms, including transactivation of unique genes, independent transcription mechanisms, and downregulation of special genes [38]. This is the first report that describes the upregulation of *p53* in HeLa and MCF-7 cancer cell lines following treatment with TEO. The effect of the plant extract on *p53* gene expression in various cancer cell lines has been documented by other plant extracts. *Euryale ferox* Salisb extract demonstrated apoptotic behavior and cell cycle arrest by *Akt* downregulation and *p53* upregulation in A549 lung cancer cells [39]. While several studies have explored the significance of plant extracts and essential oils as anticancer drugs for promising cancer treatment, before they engage in clinical trials, they need to be tested via other studies, including safety and toxicity studies.

Furthermore, it was previously found that *Artemisia vulgaris* L. essential oil induces apoptosis in a *p53*-independent manner. The pro-apoptotic protein *p53* plays a major role in cellular responses to DNA damage and, when activated, leads to cell cycle arrest and DNA repair or to apoptosis. *p53* protects cells against cancer transformation and, therefore, many apoptosis inducing chemotherapeutics depend on a functional *p53* [40].

Recently, the active compound thymol of *T. vulgaris* was shown to induce toxicity, apoptosis, and cell cycle arrest in MDA-MB231 BC cells. The intrinsic apoptosis pathway by thymol involved increased reactive oxygen species levels, leading to the loss of mitochondrial membrane potential, activation of caspase-3, and DNA damage that resulted in S-phase cell cycle arrest. Similarly, the other studied compound, ruthenium(II) *p*-cymene complex, induced *p53* protein expression in MCF-7 cells and reduced their ability to invade other tissues. Moreover, the activation of the p53 pathway after treatment increased other apoptotic mRNA levels of p63, p73, PUMA, BAX, and NOXA genes, while gene expressions of cyclinD1, MMP3, and ID1 growth promoters were significantly decreased [41]. The treatment of MCF-7 cells with essential oil of *Tarchonanthus camphoratus* at 12.5 and 25 µg/mL for 24 h caused a dose-dependent decrease in the gene expression of the anti-apoptotic *Bcl-2* and the upregulation of *p53*, *Bax* and *caspases 8, 9, 3* [42].

The flavonoid quercetin was found to suppress the viability of HeLa cells in a dose-dependent manner by inducing cell cycle arrest at the G2/M phase and mitochondrial apoptosis through a *p53*-dependent mechanism [43].
In general, a dose-dependent decrease in the survival of the two cancer cell lines was observed. In addition, MCF-7 cell line revealed higher sensitivity to TEO than HeLa cell lines.

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