

The Effects of Pre-Treatment and Post-Treatment of Thymol against tert-Butyl Hydroperoxide (t-BHP) Cytotoxicity in MCF-7 Cell Line and Fibroblast Derived Foreskin

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Abstract

Background: Some recent studies have reported anti-tumor activity for Thymol, but the findings are inconsistent. This study aimed to investigate and compare Thymol's effects on MCF-7 cancer cells and fibroblasts while treated with tert-Butyl hydroperoxide (t-BHP).

Methods: In the pre-treatment, MCF-7 and fibroblast cells were treated with various Thymol concentrations and incubated for 24 h. Then, t-BHP was added to a final concentration of 50 μ M, and the cells were incubated for one h. In the post-treatment, cells were incubated first with 50 μ M t-BHP for one h and then treated with Thymol. Cell viability was tested by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Thymol's antioxidant capacity was measured by DPPH and FRAP assays, and lipid peroxidation levels were determined by the TBARS method.

Results: The thymol effects were dose-dependent, and despite their antioxidant properties, at concentrations of 100 μ g/ml or more, increased t-BHP toxicity and reduced cancer cell viability. MTT assay result showed that pre-treatment and post-treatment with Thymol for 24 hours effectively reduced MCF-7 and fibroblast cell viability compared with the untreated control group. Both pre- and post-treatment of Thymol, normal fibroblast cell viability was significantly greater than that of the MCF-7 cells.

Conclusions: Our finding showed that Thymol appears to be toxic to MCF-7 cells at lower concentrations than fibroblasts after 24 hours of incubation. Pre-treatment with Thymol neutralized the oxidative effect of t-BHP in fibroblasts but was toxic for MCF-7 cells.

Keywords: Breast Cancer, MCF-7 Cells, Oxidative Stress, tert-Butyl Hydroperoxide, Thymol.

Introduction

Breast cancer is the most frequently occurring malignancy among females worldwide, with about 2.1 million new cases in 2018 (1). A combination of environmental and genetic factors plays a significant role in breast cancer (2). These risk factors may exert their effects through oxidative stress generation(3). Oxidative stress occurs due to an imbalance between the overproduction of reactive oxygen species (ROS)

in the body and their removal by antioxidant defense systems (4). Under stress oxidative, normal cellular processes are impaired by ROS, including cell metabolism pathways, signaling pathways regulating gene expression, cell proliferation, differentiation, and apoptosis (3). During carcinogenesis, stress oxidative in cancer cells causes oxidative damage to the structure of lipids, proteins, and DNA, which play an essential role in the initiation and progression of breast

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cancer (5, 6). For controlling the overproduction of ROS, the cells are protected against oxidative stress by non-enzymatic antioxidants (reduced glutathione, vitamins A, vitamin E, vitamin C, flavonoids, and metabolites of polyphenols) and various antioxidant enzymes (catalase, glutathione peroxidase, superoxide dismutase) (5).

Numerous epidemiological and experimental studies have demonstrated that natural phenolic products with antioxidant activity are associated with reducing the risk of various tumors, including breast cancer (7). Among many natural phenolic compounds, Thymol (2-isopropyl-5-methyl phenol) is a dietary monoterpene phenol which found in the essential oils of various plant sources such as thyme (*Lamiaceae*), oregano, and other plant species (8). Some of thymol biological activities such as antioxidant, free radical scavenging, anti-microbial, anti-fungal, anti-inflammatory, and immunomodulatory effects have been studied (9-11). It has also been reported that Thymol exerts anticancer properties in different human cancer cell lines depending on the cell types and their concentration (9). Thymol was shown to protect against ROS-induced cytotoxicity and DNA damage in cancer cell lines depending on its concentration (12). Thymol's central anticancer mechanisms include the induction of apoptosis and inhibition of growth, angiogenesis, and migration of cancer cells (13-15).

t-BHP is an organic hydroperoxide that is used as a toxic and acute oxidant to study oxidative stress-mediated cellular damage (16). A previous *in vitro* study reported that Thymol could protect Chang liver cells against t-BHP-induced-oxidative damage through their ability to prevent liver cell death (16).

Given the contradictory results in studies and lack of study on the effect of high thymol concentrations on MCF-7 cells, we investigated the effect of thymol pre- and post-treatment on t-BHP toxicity in MCF-7 and fibroblast cells.

Materials and methods

Preparation of Cells MCF-7 and foreskin fibroblast cells were used in this study. The cells were grown in 24- and 96-well plates. After reaching the desired confluency, cells were treated with Thymol and t-BHP.

Treatment of cells with thymol and t-BHP

MCF-7 and fibroblast cells were seeded at 10×10^3 and 12×10^3 cells per well, respectively, and incubated for 24 h at 37 °C. When the confluency reached 80%, in the pre-treatment group, cells were first treated with 0, 50, 100, 200, 400, or 800 µg/ml of Thymol for 24 h; then with 0 or 50 µM t-BHP for one h. In the post-treatment group, cells were first incubated with 50 µM t-BHP for one h, then treated with Thymol as above.

The control groups received neither Thymol nor t-BHP. After 24 hours, the culture medium was collected for DPPH, ferric reducing antioxidant power (FRAP), and thiobarbituric acid reactive substances (TBARS) tests and the cells were used for 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. All assays were performed in triplicate.

Cytotoxicity and percentage of viability by MTT assay

After incubations, the cell supernatants were removed, 50 µl of MTT solution (5 mg/ml in PBS) were added to each well, and plates were placed in a CO₂ incubator at 37 °C for three h. After that, the supernatants were removed, and the DMSO solvent solubilized water-insoluble formazan crystals before the colorimetric process. Finally, the samples' optical absorption was read at 570 nm on an RT2100 model ELISA reader (Poway Medical Lab Equipment Co. Ltd., China) (17).

Measurement of oxidative stress parameters

DPPH method

The specimens' free-radical scavenging activity was measured using the DPPH assay, which is commonly known as an antioxidant assay. The method is based on the ability to stabilize 2, 2-diphenyl-1-picrylhydrazyl free radicals in reaction with hydrogen donors. In this method, 25 µl of the sample was added to 975 µl of DPPH at a concentration of 4×10^{-5} µM. After 30 min, the DPPH solution's adsorption was measured on a UNICO UV/VIS 2100 spectrophotometer at 517 nm. Data were analyzed using SPSS software version 22 and ANOVA test.

FRAP method

The total antioxidant capacity of the specimens was measured using the FRAP assay. 150 µL of

FRAP reagent (prepared by mixing 25 ml of 300 mM acetate buffer pH 3.6, 2.5 ml of 10 mM TPTZ solution, and 2.5 ml of 20 mM FeCl₃ solution in a 10:1:1 ratio) was added to all wells and plates were incubated for 5 min at 37 °C. Subsequently, 20 µL of standard and the treated cell supernatant samples were added to the wells and mixed, and the plates incubated for 15 min at 37 °C. Color intensity at 593 nm was measured with the RT2100 ELISA reader. The obtained OD values were converted to concentrations using the standard curve.

TBARS method

Malondialdehyde (MDA), a biomarker of lipid peroxidation, was measured using the TBARS method. Under acidic conditions at 95 °C, a malondialdehyde molecule reacts with two molecules of thiobarbituric acid (TBA) to form a pink complex with a maximum absorbance at 532 nm) (18). In this method, 800 µl of HCl-TBA-TCA solution was mixed with 200 µl of the sample. The samples were placed in a boiling water bath for 45 min, centrifuged at 1500 rpm for 10 min, and then the supernatant absorbance was read at 532 nm.

Statistical Analysis

Data were analyzed using SPSS software version 22 and ANOVA test. Statistical significance between various experiments was determined by one-way analysis of variance (one-way ANOVA) followed by Tukey post-hoc analysis. Probability values (p-value) *p < 0.05, **p < 0.01 and ***p < 0.001 were performed as a statistically significant difference.

Results

Effect of t-BHP on MCF-7 and fibroblast cell survival

The MTT assay determined the toxicity of various concentrations of t-BHP (0, 12.5, 25, 50, and 100 µM) on MCF-7 and fibroblast cell survival. After one h of t-BHP treatment, MCF-7 cell viability decreased from 57.19 ± 10.9% to 18.9 ± 9.1% in a dose-dependent manner (p < 0.01 and p < 0.001) (Fig. 1A). The viability of fibroblast cells treated with 50 or 100 µM of t-BHP (1 h) was significantly less than that of the untreated controls (p < 0.05, p < 0.01 and p < 0.001); however, 12.5 and 25. µg/ml of t-BHP had no significant effect on fibroblast cell viability (Fig. 1B) (p ≥ 0.05).

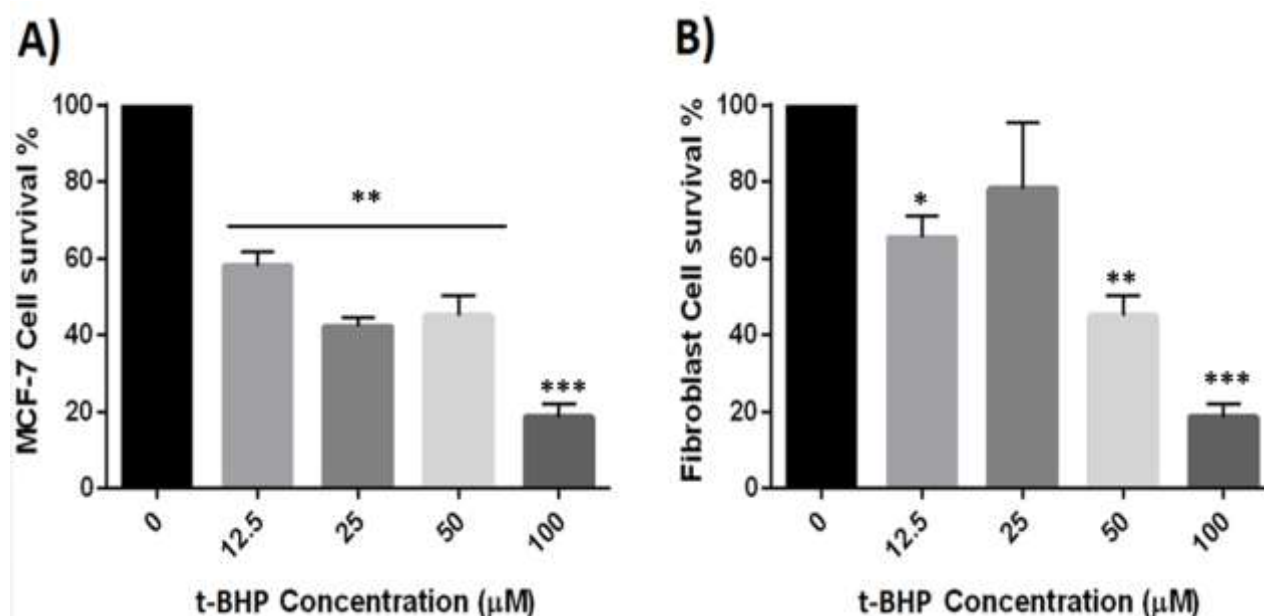


Fig. 1. A) MCF-7 cell and B) fibroblast survival percentages after 1 h of t-BHP treatment (0, 12.5, 25, 50 and 100 µM) in culture medium. Cell survival was determined by MTT assay. All results were shown mean ± SD of three separated experiments. *p < 0.05, **p < 0.01 and ***p < 0.001 compared with the untreated control group.

Effect of Thymol on MCF-7 and fibroblast cell survival

The toxicity of increasing thymol concentrations on MCF-7 and fibroblast cell survival after 24 h was also determined by MTT assay. Thymol (50-800 $\mu\text{g/ml}$) significantly inhibited MCF-7 cell viability at all

concentrations (Fig. 2A) ($p < 0.01$ and $p < 0.001$). In contrast, thymol had no apparent toxic effect on fibroblasts at 50 or 100 $\mu\text{g/ml}$; however, at 200, 400, and 800 $\mu\text{g/ml}$ it significantly inhibited cell viability (Fig. 2B) ($p < 0.01$ and $p < 0.001$).

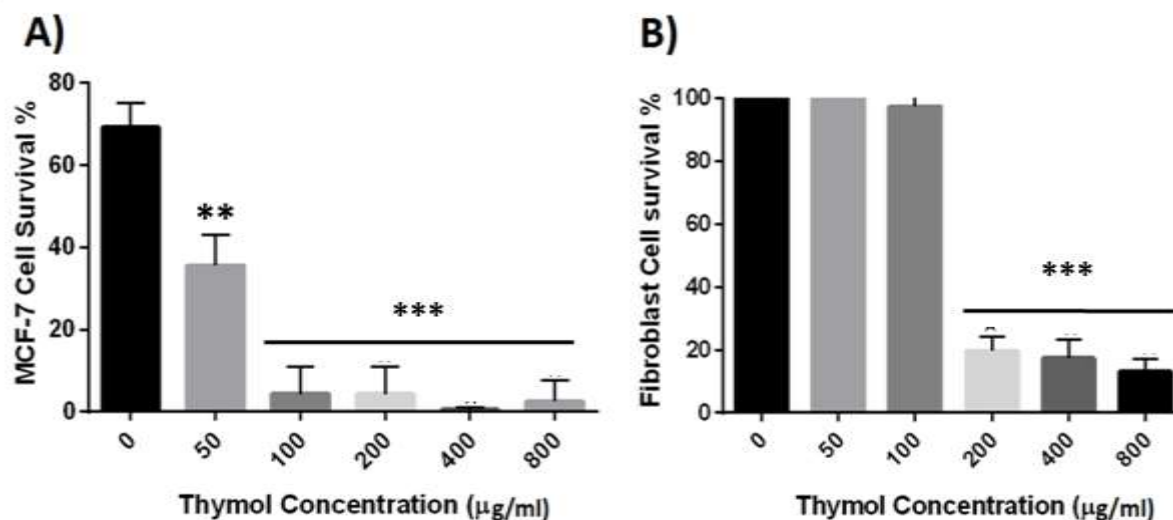


Fig. 2. A) MCF-7 cell and B) Fibroblast survival percentages after 24 h of thymol treatment (0, 50, 100, 200, 400 and 800 $\mu\text{g/ml}$) in the culture medium. Cell survival was determined by MTT assay. Data was shown mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the untreated control group.

The effect of thymol pre-treatment on MCF-7 cell and fibroblast viability

In the pre-treatment group, the cells were exposed to increasing thymol concentrations for 24 h, 50 μM t-BHP was added for one hour, and MTT assays were performed. MCF-7 cell viability in the thymol pre-treatment groups (50-800 $\mu\text{g/ml}$) was significantly less than in the untreated group ($p < 0.05$, $p < 0.01$ and $p < 0.001$) (Fig. 3A). When MCF-7 cells were pre-incubated with different concentrations of Thymol (50-800 $\mu\text{g/ml}$) in the presence of 50 μM of t-BHP for one h, the cell viability significantly decreased compared to the untreated group ($p < 0.01$ and $p < 0.001$)

(Fig. 3A). Interestingly, the addition of 50 $\mu\text{g/ml}$ of Thymol in the presence of 50 μM of t-BHP reduces the oxidative effect of t-BHP compared to the t-BHP group alone, but it was not statistically significant ($p \geq 0.05$).

Fibroblast cell viability in the pre-treatment with Thymol at 200-800 $\mu\text{g/ml}$ was significantly less than the untreated group ($p <$

0.001) Fibroblast cell viability in the pre-treatment group showed that 50 $\mu\text{g/ml}$ of Thymol in the presence of 50 μM of t-BHP increased the cell viability compared to t-BHP. However, it was not statistically significant ($p \geq 0.05$) (Fig. 3B).

The effect of thymol post-treatment on MCF-7 and fibroblast cell survival

The cells were treated for one h with 50 μM t-BHP, then incubated with increasing doses of Thymol (50-800 $\mu\text{g/ml}$) for 24 h, and the MTT assay was performed. The viabilities of both cell types were significantly less than controls when treated with 200, 400, or 800 $\mu\text{g/ml}$ of Thymol ($p < 0.001$), while viabilities of cells treated with 50 $\mu\text{g/ml}$ of Thymol were not significantly different from controls ($p \geq 0.05$) (Fig. 4).

FRAP index in cell culture medium

After pre- and post-treatment with Thymol (0-800 $\mu\text{g/ml}$), the FRAP test was performed on the collected supernatants when MCF-7 cells

Effects of Thymol in MCF-7 and Fibroblast Cell Lines

were pre-treated with 200 and 400 $\mu\text{g/ml}$ of Thymol (24 h) in the presence of 50 μM of t-BHP, the FRAP index was significantly less than the non-treated control ($p < 0.05$). In fibroblasts treated with 400 $\mu\text{g/ml}$ of Thymol (24 h), the FRAP level was significantly less than the untreated control group ($p < 0.05$).

In the post-treatment, no significant differences were observed in MCF-7 cells (Table 1) ($p \geq 0.05$), while the FRAP indexes in the fibroblast cells treated with 100, 200, 400, and 800 $\mu\text{g/ml}$ of Thymol were significantly more significant than the controls ($p < 0.05$) (Table 1).

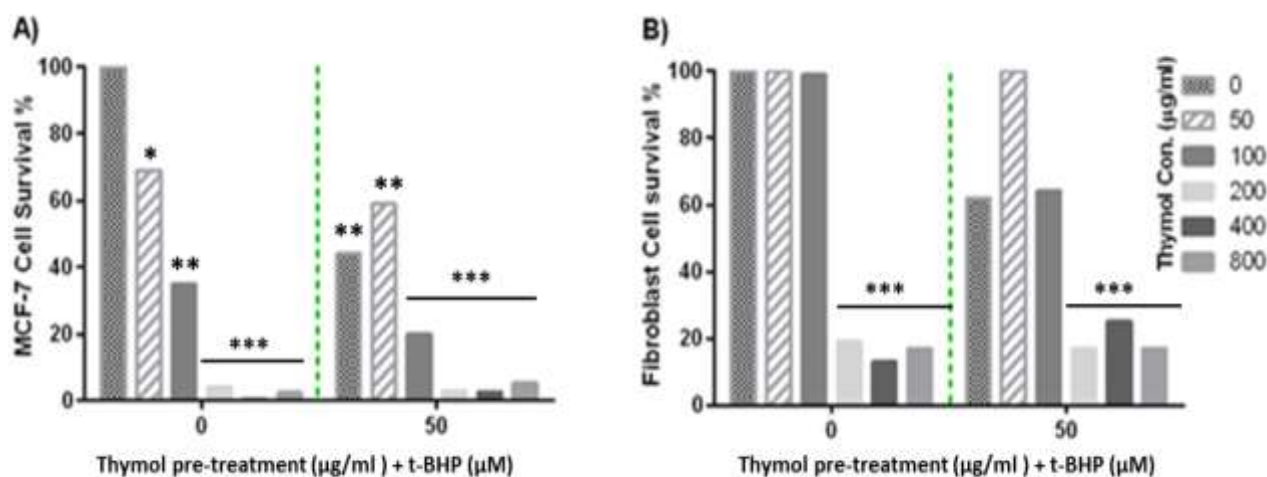


Fig. 3. A) MCF-7 cell and B) Fibroblast survival percentages after 24 h of pre-treatment with Thymol (0, 50, 100, 200, 400 and 800 $\mu\text{g/ml}$) and then one h of incubation with 50 μM t-BHP in the culture medium. Cell survival was determined by MTT assay. All results were shown mean \pm SD of three separated experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the untreated control group.

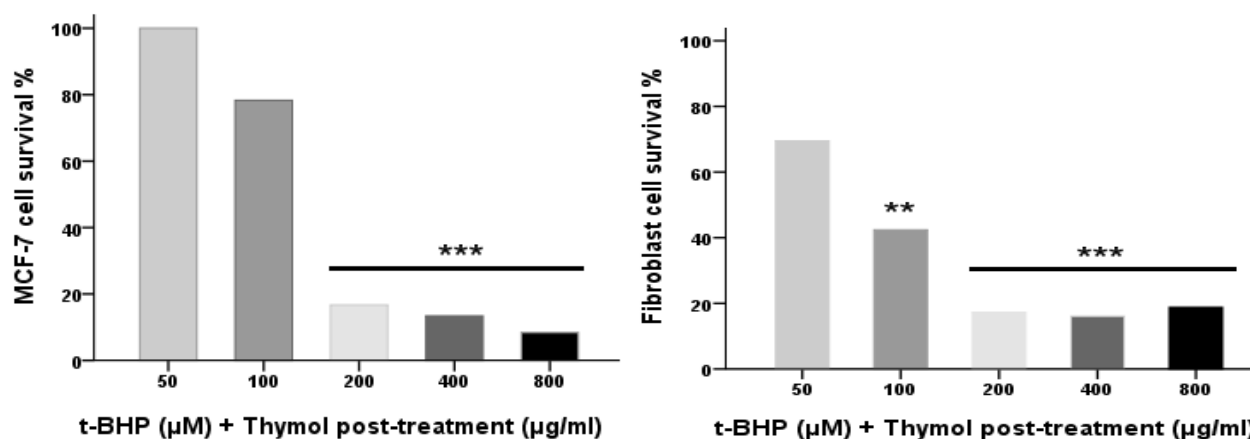


Fig. 4. MCF-7 cell and fibroblast survival percentages after one h of incubation with 50 μM t-BHP and then 24 hours with thymol post-treatment (0, 50, 100, 200, 400 and 800 $\mu\text{g/ml}$) in culture medium. Cell survival was determined by MTT assay. Data was shown mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the control group.

Table 1. FRAP values in pre- and post-treatment groups.

Treatment	Cell line	Thymol (µg/ml) t-BHP (µM)	0	50	100	200	400	800
Pre-treatment	MCF-7	0	243±0.01	150±0.04	155.2±0.01	137.6±0.01	182.3±0.05	208.09±0.06
		50	382.1±0.03	203.3±0.03	174.2±0.01	125.2*±0.01	125.7*±0.04	135.7±0.03
	Fibroblast	0	205.2±0.01	180±0.01	213.3±0.01	257.6±0.02	319.5±0.04	523.8±0.19
		5	353.5±0.04	166.1±0.03	175.7±0.01	150.9±0.05	123.3*±0.01	131.9±0.01
Post-treatment	MCF-7	0	240±0.03	150±0.04	155.2±0.01	137.6±0.01	182.3±0.05	208±0.06
		50	382.1±0.03	194.7±0.008	206.6±0.05	274.2±0.03	370.4±0.09	545.7±0.1
	Fibroblast	0	240±0.03	180±0.01	213.3±0.01	257.6±0.02	319.5±0.04	523.8±0.19
		50	353.5±0.04	232.3±0.01	340.4*±0.02	422.3**±0.03	683.3**±0.05	807.1***±0.00

* In the pre-treatment group, MCF-7 and fibroblast cells were pre-treated with 0, 50, 100, 200, 400, or 800 µg/ml of thymol for 24 h; then t-BHP was added (0 or 50 µM) for 1 h. In the post-treatment group, MCF-7 and fibroblast cells were first incubated with 50 µM t-BHP for 1 h, then Thymol was added at 0, 50, 100, 200, 400, or 800 µg/ml for 24 h. After 24 h incubation, the culture medium was collected for FRAP test by ELISA reader. All results were shown mean ± SD of three independent experiments. *p< 0.05, **p< 0.01 and ***p< 0.001 compared with the untreated control group.

TBARS Index in cell culture medium

The TBARS test was also performed on cell supernatants from the pre- and post-treated cells. No significant differences were seen between the thymol pre- and post-treated cells and controls (p> 0.05) (Table2).

DPPH index in cell culture medium

Pre-treatment of MFC-7 and fibroblast cells

with Thymol, either with or without t-BHP, did not affect DPPH indexes, nor did thymol post-treatment of MCF-7 cells. However, fibroblast cells treated first with 50 µM t-BHP and then post-treated with Thymol at concentrations of 100 µg/ml and greater showed DPPH values significantly more significant than the untreated and t-BHP control groups (p< 0.01) (Table 3).

Table 2. TBARS values in Pre-treatment and Post-treatment groups.

Treatment	Cell line	Thymol (µg/ml) t-BHP (µM)	0	50	100	200	400	800
Pre-treatment	MCF-7	0	49.3±0.05	53.7±0.03	44.8±0.01	30.5±0.03	34.8±0.03	32.5±0.02
		50	32.8±0.01	54.03±0.03	47.7±0.6	36.5±0.03	34.5±0.03	32±0.03
	Fibroblast	0	59.2±0.03	39.7±0.01	49.7±0.01	37.5±0.01	43.5±0.01	46.8±0.03
		50	57.5±0.01	79.7±0.06	79.7±0.05	86.2±0.05	79.7±0.02	69.7±0.05
Post-treatment	MCF-7	0	42.9±0.00	53.7±0.03	44.8±0.01	30.5±0.03	34.8±0.03	32.5±0.02
		50	32.8±0.01	31.3±0.03	30.5±0.02	24.3±0.02	28.5±0.01	24.8±0.01
	Fibroblast	0	59.2±0.03	39.7±0.01	49.7±0.01	37.5±0.01	43.5±0.01	46.8±0.03
		50	57.5±0.01	51.8±0.01	55.5±0.00	56.3±0.01	59.03±0.01	61.7±0.07

*In the pre-treatment group, MCF-7 and fibroblast cells were pre-incubated with thymol (0, 50, 100, 200, 400, or 800 µg/ml) for 24 h; then t-BHP was added (0 or 50 µM) for 1 h. In the post-treatment group, MCF-7 and fibroblast cells were first exposed with 50 µM t-BHP for 1 h, then Thymol (0, 50, 100, 200, 400, or 800 µg/ml) was added for 24 h. After 24 h incubation, the culture medium was collected for TBARS assay by ELISA reader. All results were shown mean ± SD of three separated experiments.

Table 3. DPPH values in Pre-treatment and Post-treatment groups.

Treatment	Cell line	Thymol ($\mu\text{g/ml}$) t-BHP (μM)	0	50	100	200	400	800
Pre-treatment	MCF-7	0	17.64 \pm 0.02	17.23 \pm 0.08	15.75 \pm 0.03	15.53 \pm 0.04	16.27 \pm 0.01	20.76 \pm 0.1
		50	15.81 \pm 0.09	17.74 \pm 0.07	17.04 \pm 0.10	17.78 \pm 0.17	16.84 \pm 0.04	19.38 \pm 0.09
	Fibroblast	0	17.8 \pm 0.04	15.75 \pm 0.06	17.7 \pm 0.04	16.57 \pm 0.03	17.1 \pm 0.04	16.50 \pm 0.03
		50	16.1 \pm 0.04	17 \pm 0.02	18.1 \pm 0.05	17.47 \pm 0.07	17.68 \pm 0.03	17.89 \pm 0.08
Post-treatment	MCF-7	0	16.12 \pm 0.1	17.23 \pm 0.08	15.75 \pm 0.03	15.53 \pm 0.04	16.27 \pm 0.01	20.76 \pm 0.1
		50	15.81 \pm 0.09	15.89 \pm 0.09	15.01 \pm 0.08	14.97 \pm 0.04	15.02 \pm 0.03	14.53 \pm 0.05
	Fibroblast	0	15.81 \pm 0.05	15.75 \pm 0.06	17.7 \pm 0.04	16.57 \pm 0.03	17.1 \pm 0.04	16.5 \pm 0.03
		50	16.15 \pm 0.04	17.9 \pm 0.06	55.5 \pm 0.00* *///	56.3 \pm 0.01**/# #	59.03 \pm 0.01 **///	61.7 \pm 0.07**/ ///

*In the pre-treatment group, MCF-7 and fibroblast cells were first exposed with thymol (0, 50, 100, 200, 400, or 800 $\mu\text{g/ml}$) for 24 h; then t-BHP was added (0 or 50 μM) for 1 h. In the post-treatment group, MCF-7 and fibroblast cells were first exposed with 50 μM t-BHP for 1 h, then Thymol (0, 50, 100, 200, 400, or 800 $\mu\text{g/ml}$) was added for 24 h. After 24 h incubation, the culture medium was collected for DPPH assay using a spectrophotometer at 517 nm. ** $p < 0.01$ compared with the untreated control group. /// $p < 0.01$ compared with the t-BHP control group.

Discussion

In this manuscript, we reported that pre- and post-treatment of MCF-7 and fibroblast cells with Thymol accompanied by t-BHP had significantly more cytotoxicity compared with both untreated and t-BHP groups in a dose-dependent manner.

Many studies have recently been done on the protective effects of natural antioxidants against oxidative stress in both *in vitro* and *in vivo* (19-21). However, several studies have shown that some antioxidants can act as a pro-oxidant under certain conditions, increasing ROS production, and resulting in cell damage (22, 23). The oxidation effects of these antioxidant compounds may vary depending on their dosage, exposure time, and intracellular interference (24). Among the known antioxidants, Thymol, a monocyclic monoterpene, has shown various biological activities, including antioxidant, anti-inflammatory, and anti-microbial anti-diabetic, and neuropharmacological effects (12, 13). *In vitro* studies have shown that thymol treatment suppressed oxidant-induced DNA damage, cell cycle arrest, and apoptosis in different cancer cells. These reports revealed that Thymol is a potent anti-tumor agent (26-28). However, there is no study on Thymol's effect on cytotoxicity and oxidative stress in MCF-7 cells and foreskin fibroblasts. Based on previous research, t-BHP, as

an acute oxidant, was used to evaluate the antioxidant and anti-proliferation activity of Thymol in cellular models (16). Here, we used increasing concentrations of Thymol to investigate its effects on MCF-7 and fibroblast cells in the presence and absence of t-BHP. Thymol significantly reduced cell viability of MCF-7 cancer cells at all concentrations tested. In the normal fibroblasts, thymol only at concentrations of 200 $\mu\text{g/ml}$ and above led to cell viability reduction. Accordingly, Thymol appears to be more toxic to MCF-7 than fibroblast cells after 24 hours of incubation. Also, pre- and post-treatment with Thymol in the presence of t-BHP significantly reduced the MCF-7 cell and fibroblast viability compared to controls in a dose-dependent manner. Therefore, thymol pre-treatment seems to be more effective than post-treatment for cell survival.

Thymol has free radical scavenging activity to reduce oxidative stress (29, 30). A study using Chang cells at 12.5, 25, and 50 $\mu\text{g/ml}$ of Thymol and 80 μM t-BHP showed that Thymol increased cell survival. Thymol protects Chang cells against oxidative stress by preventing mitochondrial membrane potential (MMP) loss and inhibiting overproduction of ROS and lipid peroxidation (MDA levels) resulting from oxidative stress (16).

Here, we used different cell lines and different doses of Thymol (50 µg/ml and higher) and t-BHP (50 µM). Pre- and post-treatment with Thymol and t-BHP showed no significant differences in MDA levels in the MCF-7 and fibroblast cells. Compared to Chang cells, our different results could be due to differences in Thymol doses and the type of cells. At high concentrations, it appears Thymol may have been unable to protect the cells from the oxidative damage caused by t-BHP.

A study that examined the protective effects of Thymol on lipid peroxidation and non-enzymatic antioxidants in isoproterenol-induced myocardial infarction in rats reported that Thymol could trap and regenerate the superoxide anion, hydroxyl, in a dose-dependent manner (31). In 2016, Palabiyik *et al.* examined the protective effect of Thymol on HepG2 cells. They reported that Thymol at concentrations of 25, 50, 100 µM reduced oxidative damage and improved the antioxidant enzyme activity after 24, 48, and 72 h. At higher concentrations, this phenolic compound may oxidize and release free radicals (32, 33). Thymol (10–400 µM) had cytotoxic, genotoxic, and apoptotic effects on

AGS gastric cancer cells by producing ROS and reducing glutathione (34). In another report, Thymol at 400 mg/L was shown to exhibit anti-proliferation effects, whereas at 19, 25, and 50 mg/L after thymol treatment, the total antioxidant capacity was increased in cultured neuroblastoma cells (N2a cells) (25).

In our study, with increasing concentration in pre-treatment with Thymol, no significant change was observed in Thymol's free-radical scavenging activity in both cell lines. This can be attributed to the presence of t-BHP as an oxidant. In post-treatment with Thymol, free-radical scavenging activity increased in fibroblast cells compared to MCF-7 cell line. We also observed that pre- and post-treatment with Thymol in the presence of t-BHP affected the total antioxidant capacity in both MCF-7 and fibroblast cells.

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The authors declare that there is no conflict of interest.

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