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Effects of Thymol on B16-F10 Melanoma Cells

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Supporting Information

ABSTRACT: Aromatic monoterpene, thymol, shows several beneficial activities, such as an antioxidative effect. However, the mechanism of its toxicity remains to be fully defined. In preliminary studies, thymol was characterized as a melanin formation inhibitor in an enzymatic system; however, thymol showed moderate cytotoxicity but not an antimelanogenic effect on B16-F10 melanoma cells. Thymol exhibited cytotoxicity, with an IC_{50} value of 400 μM (60.09 μg/mL). This moderate toxic effect was suppressed with the addition of vitamin C and vitamin D, and 20 and 40% of cell viability was increased, respectively. Subsequently, the treatment of L-cysteine on thymol-treated melanoma cells reversed the toxic effect of thymol. Moreover, a significant oxidative stress condition was observed when B16 melanoma cells were cultured with thymol. In conclusion, the antioxidant actions of thymol generate a stable phenoxy radical intermediate, which generates reactive oxygen species and quinone oxide derivatives. Thus, it is proposed that the primary mechanism of thymol toxicity at high doses is due to the formation of antioxidant-related radicals.

KEYWORDS: *Thymol, thyme oil, oxidative stress, cytotoxicity, melanogenesis*

INTRODUCTION

Melanogenesis is a bioprocess of melanin production by melanocytes within the skin and hair follicles and is mediated by several enzymes, such as tyrosinase, TRP-1, and TRP-2.[1] In various living systems, melanin is responsible for pigmentation and other functions, such as chelation. However, undesirable browning is a considerable problem in food industries or for cosmetic users. Furthermore, toxic oxidized products are commonly generated in the melanin synthetic pathway; for instance, high lipid content fruits, such as avocado, develop toxic oxide products in the browning process.[2] Thus, antibrowning reagents are frequently used. Previously, the most widespread method for antibrowning was the use of sulfiting agents,[3] but the U.S. Food and Drug Administration (FDA) has banned sulfate agents for fruits and vegetables.[4] Hence, an immediate finding of replacement antibrowning reagents is essential. For these applications, naturally occurring substances are usually more favorable than synthetic substances. The most common natural antibrowning agent is ascorbic acid. However, the effect of ascorbic acid against enzymatic oxidation is temporary because it is chemically oxidized to a nonfunctional form, dehydroascorbic acid.[5] These problems prompt us to search for safer and more stable melanin formation inhibitors from natural sources.

In our continuing search for melanin formation inhibitors from natural sources, aromatic monoterpenes, such as thymol (5-methyl-2-isopropylphenol) (**1**; see Figure 1 for the structure), were active principles.[6] Thymol was a redox inhibitor, which disrupts the redox reaction between leukodopachrome and dopaquinone. Thymol or other monoterpenes, such as carvacrol (5-isopropyl-2-methylphenol) (**2**) and *p*-cymene (1-methyl-4-(1-methylethyl)benzene) (**3**), are found in the essential oil fraction of thyme, *Thymus vulgaris* or *Thymus zygis* L. var. *gracilis* Boissir (Lamiaceae). The main constituent of thyme oil is thymol (up to 80%). Thyme essential oil has been commercially available as a part of mouthwashes for more than 100 years. In addition to its well-known broad antimicrobial activity,[7−10] thymol was previously reported to inhibit phospholipid peroxidation and lipid autoxidation without prooxidant effects.[11−13] As a result of preliminary studies and wide usage of thymol as a spice or food preservative, thymol is expected to function as a multifunctional antibrowning reagent. This prompted us to investigate the effect of thymol on B16-F10 murine melanoma cells.

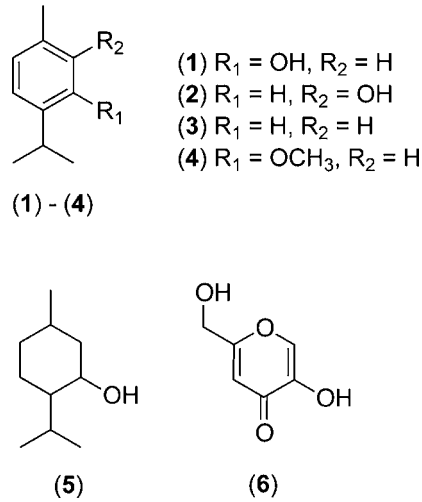

Figure 1. Chemical structures of thymol and its related compounds: thymol (**1**), carvacrol (**2**), *p*-cymene (**3**), thymol methyl ether (**4**), menthol (**5**), and kojic acid (**6**).

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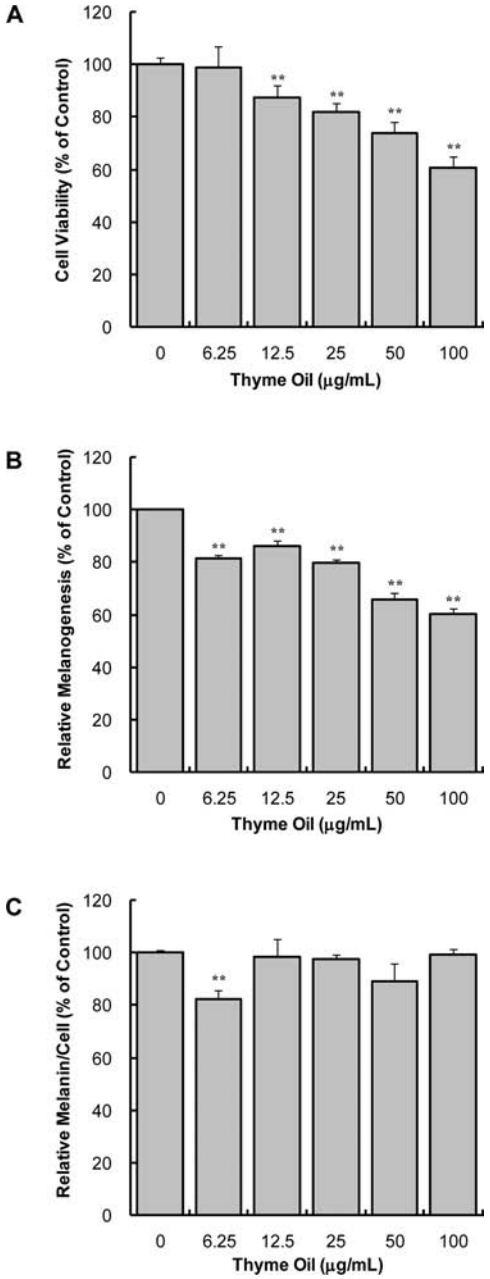

Figure 2. (A) Viabilities of B16-F10 melanoma cells following treatment with thyme oil for 72 h. The cell numbers were determined with the trypan blue assay. Data are expressed as a percentage of the number of viable cells observed with the control, and each column represents the mean ± standard deviation (SD) of four determinations. (B) Total melanin content in B16 melanoma cells following treatment with thyme oil for 72 h. Data are expressed as a percentage of the melanin content per well observed with the control, and each column represents the mean ± SD of four determinations. (C) Cellular melanin content in B16 melanoma cells following treatment with thyme oil for 72 h, measured as a percentage of the melanin content per cell observed with the control, and each column represents the mean ± SD of four determinations. The statistical significance of differences was evaluated using Student's or Welch's *t* test. Significantly different from the control value: (*) $p < 0.05$ and (**) $p < 0.01$.

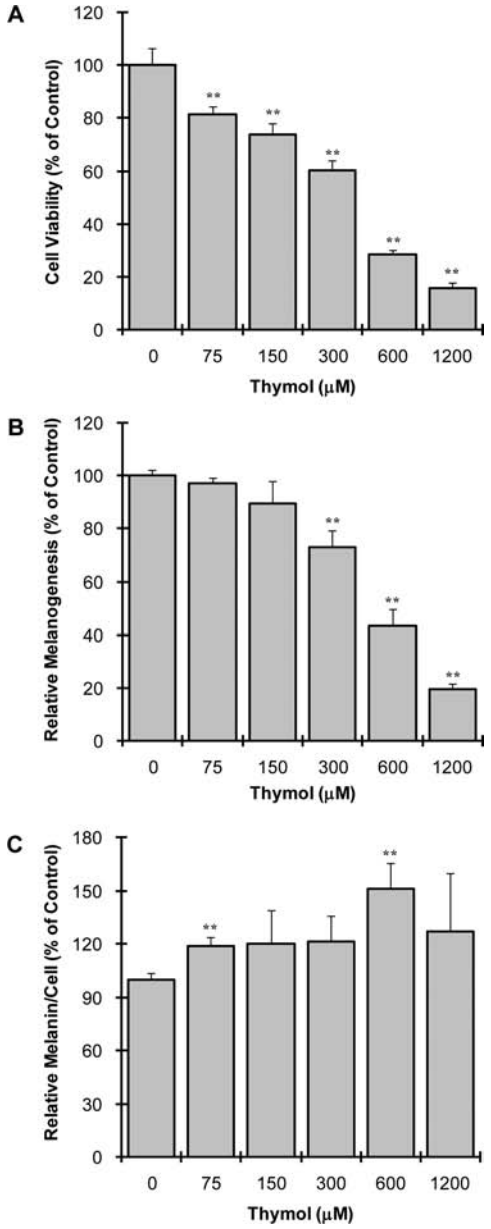

Figure 3. (A) Viabilities of B16-F10 melanoma cells following treatment with thymol for 72 h. Data are expressed as a percentage of the number of viable cells observed with the control, and each column represents the mean ± SD of four determinations. (B) Total melanin content in B16 melanoma cells following treatment with thymol for 72 h. Data are expressed as a percentage of melanin content per well observed with the control, and each column represents the mean ± SD of four determinations. (C) Cellular melanin content in B16 melanoma cells following treatment with thymol for 72 h, measured as a percentage of melanin content per cell observed with the control, and each column represents the mean ± SD of four determinations. The statistical significance of differences was evaluated using Student's or Welch's *t* test. Significantly different from the control value: (*) $p < 0.05$ and (**) $p < 0.01$.

MATERIAL AND METHODS

General Procedures. General procedures were the same as in previous works.[14−16] All assays were performed in triplicate on separate occasions as long as not specified. Cells were viewed in 96-well plates approximately 72 h after treatment with thymol or its related products.

Materials. Thyme oil, manufactured by NOW Foods (Bloomingdale, IL) was purchased from a local grocery store. Thymol, carvacrol, *p*-cymene, menthol, and kojic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). L-Tyrosine, L-3,4-dihydroxyphenylalanine

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(L-DOPA), L-cysteine, L-ascorbic acid (vitamin C), butylated hydroxyanisole, butylated hydroxytoluene, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO). 1-Methyl-3-methoxy-4-isopropylbenzene (thymol methyl ether) was purchased from SASF Supply Solutions (St. Louis, MO). α-Tocophenol (vitamin E) was purchased from Lancaster Synthesis, Inc., Pelham, NH). Glutathione was purchased from Eastman Kodak Co. (Rochester, NY). *N*-Acetyl-L-tyrosine was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS), trypsin, Dulbecco's modified Eagle's medium (DMEM), and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay kit were purchased from American Type Culture Collection (ATCC) (Manassas, VA).

Instrumentation: Gas Chromatography−Mass Spectrometry (GC−MS). The composition of thyme oil was analyzed by a GC−MS system (GC-17A/QP5050; Shimadzu Co., Ltd., Kyoto, Japan) equipped with a DB-5 column (30 m × 0.25 mm inner diameter, 0.25 μm film thickness, J&W Scientific, Inc.). The temperature program was as follows: 45 °C for 8 min, followed by increases of 2.5 °C/min to 180 °C and 10 °C/min to 250 °C, and holding for 3 min. The other parameters were as follows: injection temperature, 250 °C; ion source temperature, 250 °C; ionization energy, 70 eV; carrier gas, helium (He) at 1.7 mL/min; injection volume, 1 μL (90 μg/mL Et_2O); split ratio, 1:20; mass range, m/z 50−450.

Enzyme Assay. The mushroom tyrosinase (EC 1.14.18.1) used for the bioassay was purchased from Sigma Chemical Co. and was purified by anion-exchange chromatography using DEAE-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) as previously described.[17] The experiment was subjected to use the purified tyrosinase. Although mushroom tyrosinase differs somewhat from those of other sources, this fungal enzyme was used for the entire experiment because it was readily available. Throughout the experiment, L-DOPA or L-tyrosine was used as a substrate. In most experiments, L-tyrosine was used as a substrate because it is a natural substrate of tyrosinase. In a spectrophotometric experiment, the enzyme activity was monitored by dopachrome formation at 475 nm with a SpectraMAX Plus Microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at 30 °C. All samples were first dissolved in DMSO and used for the experiment after dilution. The final concentration of DMSO in the test solution was always 3.3% (v/v). The assay was performed as previously reported, with slight modifications.[18] First, 100 μL of a 3 mM L-DOPA or L-tyrosine aqueous solution was mixed with 2.1 mL of filtered distilled H_2O and 600 μL of 67 mM phosphate buffer (pH 6.8) and incubated at 30 °C for 5 min. Then, 100 μL of the sample solution and 100 μL of the purified mushroom tyrosinase (1 μg/mL) in the same phosphate buffer solution were added in this order to the mixture. Results were recorded as absorbance at 475 nm, and all data were processed with SigmaPlot 10 software (Systat Software, Inc.).

Cell Culture. B16-F10 mouse melanoma cells (CRL-6475) were obtained from ATCC (Manassas, VA) and cultured in continuous log phase growth in DMEM containing 10% FBS. Cells were seeded in 96-well plates (2000 cells/well) and incubated at 37 °C in 5% CO_2 for about 24 h before chemical treatment. Each chemical was applied in duplicate with a final content of 0.1% DMSO, and treated cells were cultured for 72 h before assays.

Cell Viability Assays. Cell viability was determined by trypan blue exclusion and MTT cell proliferation assays. Both bioassays provided comparable results, but the concentration leading to 50% viable cells lost (IC_{50}) was established by the trypan blue assay for steady comparison. The appropriate concentrations of the test chemicals were selected by microscopic observation of the preliminary cell viability assay using a Nikon Diaphoto TMD (Nikon, Tokyo, Japan).

Trypan Blue Method. Cells were washed with phosphate buffered saline (PBS) and dispersed by trypsinization. Trypsin was obtained from ATCC, and 25 μL of trypsin (1.3× diluted from the original concentration) was added after the washing of the cells with FBS. An aliquot of the cells was mixed with a half volume of DMEM containing 10% FBS, and then mixed with trypan blue solution (final content of 0.1%) at room temperature. Unstained cells (viable cells) were counted using a hemocytometer within 10 min after mixing with trypan blue solution.

MTT Method. Cells were washed with PBS and dispersed with trypsinization (the same procedure listed in the Trypan Blue Method), and an aliquot of the cells was seeded in 96-well plates and incubated with DMEM containing 10% FBS at 37 °C in 5% CO_2 for 16−24 h. At the end of the period, 10 μL of MTT reagent was added to each well, which was then incubated at 37 °C in 5% CO_2 for 4 h. Then, 100 μL of detergent reagent (provided by the MTT kit obtained from ATCC) was added to each well. The plate was kept at room temperature in the dark for 2 h, and the relative amount of MTT reduction was determined on the basis of the absorbance at 570 nm using a SpectraMax Plus spectrophotometer and SoftMax Pro software (Molecular Devices). The obtained absorbance was normalized with the control values (DMSO-treated cells). Using control cells as 100%, we compared the cell viability of each concentration.

Melanin Assay. The melanin content was determined as previously described,[19,20] with minor modification. Cells were washed with PBS, harvested by trypsinization (the same procedure listed in the Trypan Blue Method), and centrifuged for 10 min at 1500*g*. The cell pellets were then dissolved in 1.0 M NaOH containing 10% DMSO for 2 h of incubation at 80 °C. The melanin content was measured at 475 nm using a SpectraMax Plus spectrophotometer and SoftMax Pro software (Molecular Devices, Union City, CA).

DCFH-DA Method. The level of intracellular reactive oxygen species (ROS) in B16-F10 melanoma cells was measured by the DCFH-DA method. General procedures were performed as previously described,[21,22] with minor modifications. Briefly, the cultured cells were incubated with the sample and DCFH-DA reagent for 1 h at 37 °C in the dark. After the incubation, cells were removed from the plate with trypsinization to measure fluorescence. The amount of formed DCF was measured using fluorolog-3 with DataMax (Instruments S.A., Inc. Edison, NJ) at the excitation wavelength of 485 nm and the emission wavelength of 520 nm.

Statistical Analysis. The statistical significance of differences was evaluated by either Student's or Welch's t test after examining the variances using the F test, and $p < 0.01$ (marked as **) was considered to be statistically significant.

RESULTS AND DISCUSSION

In preliminary experiments using mushroom tyrosinase (EC 1.14.18.1), thymol (**1**) inhibited the tyrosinase-catalyzed dopachrome formation from L-tyrosine.[6] In fact, thymol did not interact with tyrosinase; however, thymol inhibited a redox reaction in the melanin synthesis pathway. Tyrosinase-catalyzed dopachrome formation is a key step in melanogenesis, and therefore, inhibitors of dopachrome formation are expected to inhibit cellular melanin production. Thus, the investigation was extended to test for the effects of thymol on murine B16-F10 melanoma cells.

The initial goal was to test whether thymol inhibits melanogenesis in cultured melanocytes without affecting cell growth. Hence, their cell viability was examined first. In this regard, cell viability was determined on the third day for melanocytes using both trypan blue dye exclusion and MTT colorimetric assays. The same result was usually observed by both assays, but the concentration leading to 50% viable cells lost (IC_{50}) was established by the trypan blue assay for comparison. The specificity of melanogenesis inhibition was assessed by dividing the melanin content by the number of cells determined by trypan blue exclusion. Appropriate concentrations of the test chemicals were selected by microscopic observation of the preliminary cell viability assay. Kojic acid (**6**) was also tested as a reference compound for comparison.

The investigation began with thyme essential oil because it contains thymol, and it is also commercially available. The

components of thyme oil were characterized using a GC−MS system. The major composition of thyme oil was thymol (75.71%) and carvacrol (5.47%) (see the Supporting Information). The examined highest concentration of thyme oil was 100 μg/mL, and it was the maximal concentration tested because of a problem in solubility. The thyme oil concentration dependently suppressed cell viability (Figure 2A), with an estimated IC_{50} of 150 μg/mL. The cell viability above 12.5 μg/mL was significantly different ($p < 0.01$) from the control. The total melanin production was significantly suppressed ($p < 0.01$), up to 100 μg/mL, in a concentration-dependent manner (Figure 2B). However, thyme oil did not show any effect on cellular melanin production (Figure 2C). Further experiments focused on thymol, which is the major component of thyme oil and which has been reported to have various biological activities.

Both thymol and carvacrol were noted to exhibit moderate cytotoxicity when they were cultured with murine B16-F10 melanoma cells. The highest concentration of thymol was 1200 μM. Cell viability of thymol-treated cells was suppressed in a concentration-dependent manner (Figure 3A). Observed IC_{50} was 400 μM (60.09 μg/mL), and almost complete lethality was observed at 1200 μM. Cell viability above 75 μM was significantly different ($p < 0.01$) compared to the control. The total melanin production was also suppressed in a concentration-dependent manner (Figure 3B), and this suppression above 300 μM was significant ($p < 0.01$). The cellular melanin production was not suppressed but slightly increased. In the case of carvacrol, similar results were obtained in cell viability (Table 1) and melanin assays (data not shown). Unfortunately, neither thymol nor carvacrol inhibited melanogenesis in cultured melanocytes, even though they inhibited dopachrome formation. Maeda and Fukuda[23] previously reported that a correlation was not seen between the inhibition of mushroom tyrosinase activity with that of cellular tyrosinase or melanin formation in cultured melanocytes. However, the toxicity mechanism of these monoterpenes is poorly understood, and clarification of the mechanism of toxicity is important for further usage of thymol as a food additive and for scientific purposes. Thus, investigation was focused on identifying the mechanism of toxicity of thymol or carvacrol.

Table 1. Summary of the Cytotoxic Effect of Thymol and Its Related Compounds on B16-F10 Melanoma Cells

tested compounds	IC_{50} (μg/mL)	log P^a
thyme oil	N/A (~150)	N/A
thymol	400 μM (60.09)	3.37
carvacrol	550 μM (82.62)	3.37
thymol methyl ether	>200 μM (>32.84)	3.63
p-cymene	>1000 μM (>134.22)	3.76
menthol	>1000 μM (>156.27)	2.75
kojic acid	20 μM (0.28)	−2.45

[a]log *P* was calculated with ChemBioDraw Ultra 12.

p-Cymene (**3**), thymol methyl ether (**4**), and menthol (**5**) were tested for structure−activity relationship (SAR) studies. Among the tested compounds, none of the compounds showed any significant cytotoxicity on B16 melanoma cells up to designated concentrations (Table 1). It should be noted that thymol methyl ether had relatively less solubility; hence, the tested highest concentration of thymol methyl ether was 200 μM. The results suggested that the blocking or lacking of the hydroxyl OH group in monoterpene phenol decreased cytotoxicity against B16-F10 melanoma cells. As concluded in a previous report, the phenolic hydroxyl group of thymol or carvacrol is essential for biological activities.[24] Subsequently, menthol also did not show significant toxicity up to 1000 μM. It indicates that the aromaticity was essential for the toxicity.

Cytotoxicity of thymol observed at 400 μM (approximately equivalent to IC_{50}) was suppressed by 20% in cell viability with the addition of an equivalent concentration of vitamin C (Figure 4A). As a result, intracellular transformation of thymol to either a toxic oxide by intracellular oxidation or a stable phenoxy radical to generate oxidative stress was involved in the mechanism of toxicity of thymol. To determine this postulate, the effect of vitamin E was also tested. Vitamin E reversed the effect on cell viability by approximately 40% compared to the control (Figure 4B). Subsequently, butylated hydroxyanisole

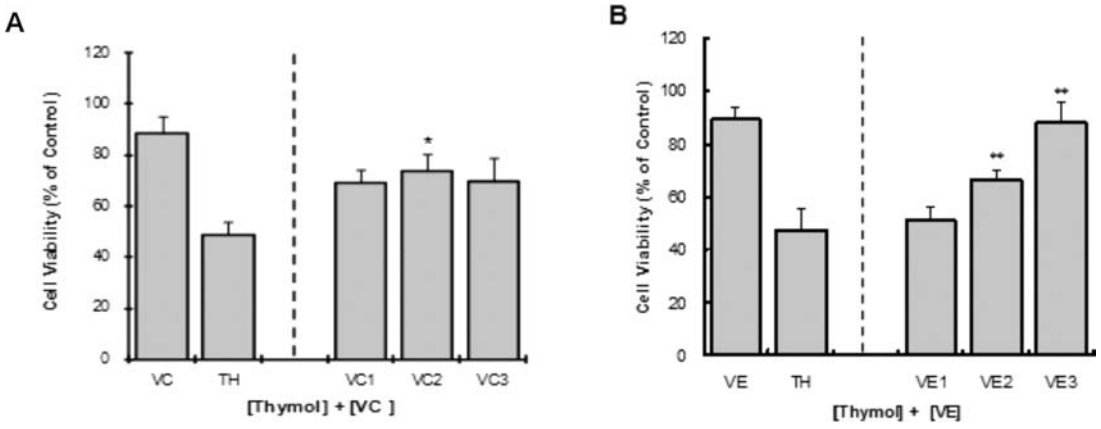


Figure 4. (A) Viabilities of B16 melanoma cells following treatment with 400 μM thymol in the presence (VC1−VC3) or absence (TH) of vitamin C for 72 h. The concentrations of vitamin C applied to thymol-treated cells were chosen at 200 μM (VC1), 400 μM (VC2), and 800 μM (VC3). Cells are also treated with 800 μM vitamin C without thymol (VC). (B) Viabilities of B16 melanoma cells following treatment with 400 μM thymol in the presence (VE1−VE3) or absence (TH) of vitamin E for 72 h. The concentrations of vitamin E applied to thymol-treated cells were chosen at 100 μM (VE1), 200 μM (VE2), and 400 μM (VE3). Cells are also treated with 400 μM vitamin E without thymol (VE). Data are expressed as a percentage of the number of viable cells observed with the TH control, and each column represents the mean ± SD of four determinations. The statistical significance of differences was evaluated using Student's or Welch's *t* test. Significantly different from TH control value: (*) $p < 0.05$ and (**) $p < 0.01$.

(BHA) and butylated hydroxytoluene (BHT) were examined. Both treatments reduced the cytotoxicity of thymol by 17 and 11% in cell viability, respectively (Table 2). Additionally,

Table 2. Summary of Mechanisms of Tested Treatments on Thymol-Applied B16 Melanoma Cells

tested treatments	maximum recovery (%)	mechanism[a]
vitamin C	23	two e^- reduction/radical scavenging
vitamin E	40	radical scavenging
BHA	17	radical scavenging
BHT	11	radical scavenging
GSH	22	two e^- reduction/radical scavenging
L-cysteine	15	nucleophilic addition

[a]Possible mechanism to mask thymol cytotoxicity.

glutathione (GSH) was tested in the same manner. GSH treatment suppressed the toxic effect of thymol by 22% in cell viability on B16-F10 melanoma cells. Because vitamin E, BHA, BHT, and GSH are well-known radical scavengers, their defensive effects against thymol toxicity on B16 melanoma cells explained that a radical-based toxicity was, at least partly, involved in the mechanism of thymol toxicity. A surprising result was observed with the treatment of L-cysteine on thymol-treated cells. The toxicity of thymol on B16-F10 melanoma cells were reduced 15% by the treatment of L-cysteine (Table 2). In fact, L-cysteine does not act as either a reducing agent or a radical scavenger, but it acts as a nucleophile to react with a Michael-addition receptor, such as quinone-related compounds. This result suggested that thymol was converted to reactive electrophilic compound(s), such as quinone. Thus, both radical formation and toxic oxide(s) formation may be involved in the mechanism of thymol toxicity.

Intracellular oxidative stress was measured with the DCFH-DA assay. Intracellular ROS oxidizes DCFH to a highly fluorescent compound, DCF, after esterase cleaves two acetate groups.[25] After 1 h of incubation of B16-F10 melanoma cells with thymol and DCFH-DA, cells were assayed to measure intracellular ROS. Within 75−300 μM, the total ROS were increased, even though cellular viability was decreased (panels A and B of Figure 5). The cellular ROS generation above 75 μM was significantly increased ($p < 0.01$) in a concentration-dependent manner (Figure 5C). The highest increase of ROS was observed when 600 μM thymol was applied to the cells. About 2 times higher ROS was generated in comparison to the control. It appeared that thymol acted as a prooxidant above 75 μM rather than as an antioxidant. Thus, oxidative stress was generated with a high concentration of thymol and was involved in the mechanism of toxicity.

Cellular morphological changes of thymol-treated melanoma cells were microscopically observed. Figure 6A represents the cellular morphology of control (DMSO-treated) cells, while Figure 6B is that of thymol-treated cells (panels A and B of Figure 6). As seen in these panels, the density of melanoma cells was significantly reduced by the treatment of 400 μM thymol. Further, the individual cells were enlarged in comparison to DMSO treatment. As expected, after the treatment of intracellular antioxidant, GSH, to thymol-treated cells, the morphological change of the cells was similar to the control (Figure 6C).

Antioxidants work in various ways, including direct quenching of ROS, inhibition of enzymes involved in the production of the ROS, chelation of low valent metal ions, such as Fe^{2+} or Cu^{2+}, and regeneration of membrane-bound antioxidants, such as

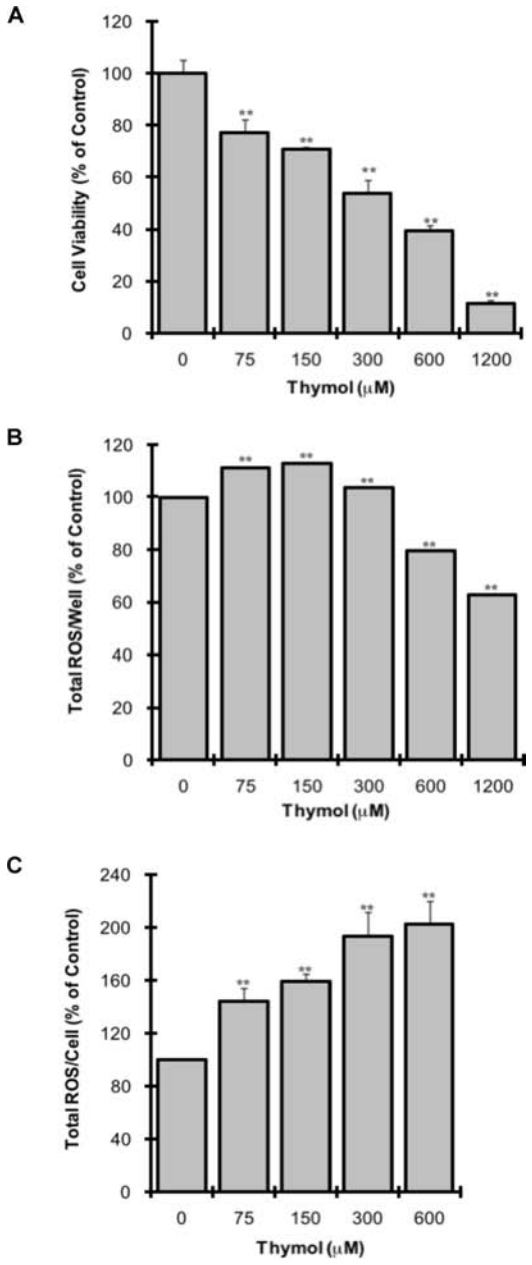


Figure 5. (A) Viabilities of B16-F10 melanoma cells following treatment with thymol for 1 h. Data are expressed as a percentage of the number of viable cells observed with the control, and each column represents the mean ± SD of four determinations. (B) Total ROS contents in B16 melanoma cells following treatment with thymol for 1 h. Data are expressed as a percentage of ROS content per well observed with the control, and each column represents the mean ± SD of four determinations. (C) Cellular ROS contents in B16 melanoma cells following treatment with thymol for 1 h, measured as a percentage of ROS contents per cell observed with the control, and each column represents the mean ± SD of four determinations. The statistical significance of differences was evaluated using Student's or Welch's t test. Significantly different from the control value: (*) $p < 0.05$ and (**) $p < 0.01$.

α-tocophenol;[26] however, antioxidant-related radicals are usually formed during the action. The fate of newly formed radicals from antioxidant action to humans is still unclear. Because of this property, antioxidants are also known as a double-edged sword. All of the obtained data suggested that

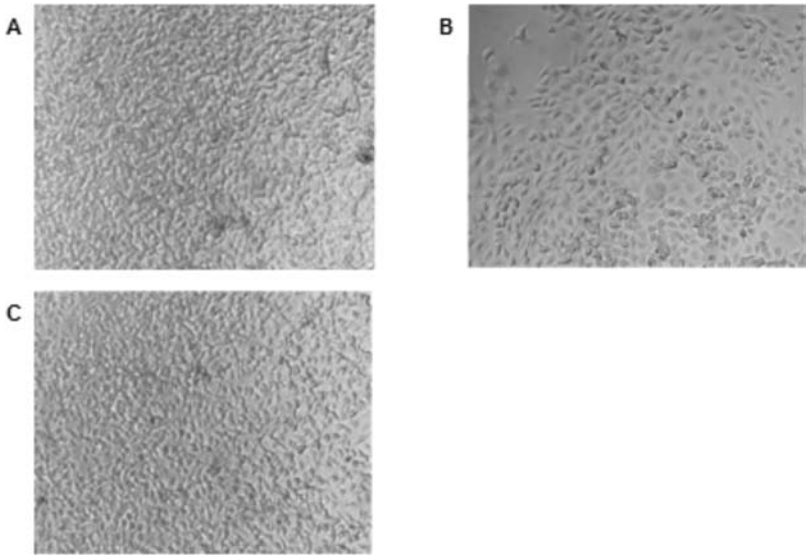
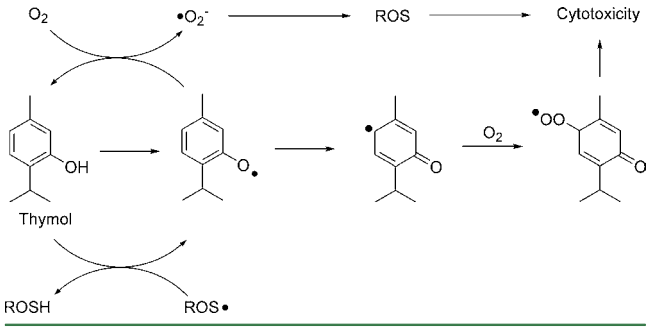


Figure 6. Cellular morphological change of B16-F10 melanoma cells. Panel A represents cellular morphology of 0.1% DMSO-treated melanoma cells, while panel B indicates morphological change of 400 μM thymol-treated B16 melanoma cells. Panel C shows that morphology of melanoma cells treated with thymol (400 μM) and glutathione (400 μM).

thymol showed moderate toxicity without any effects on melanogenesis. It is proposed that the mechanism of thymol toxicity is due to the intracellular transformation to corresponding toxic phenoxy radical and quinone. Because of its lipophilicity and small structural feature, thymol passively dissolves in the cell through the lipid bilayer. Once inside the cell, thymol first acts as an antioxidant, which scavenges free radicals from the environment.[11−13] However, the phenoxy radical is newly formed through this reaction. The contribution of the electron-donating methyl or isopropyl group in the *ortho* position of the phenolic hydroxyl group helps thymol form a relatively stable phenoxy radical.[27] It has previously been reported that the electron-donating isopropyl and methyl groups contribute to the stability of the phenoxy radical and their antioxidative activity.[28] Moreover, the resonance because of the aromaticity is also involved for the stabilization of the phenoxy radical based on the nontoxicity data of menthol. It is proposed that this thymol-derived phenoxy radical oxidizes environmental oxygen to generate ROS; at the same time, the phenoxy radical itself is further oxidized to form quinone-oxide-related compounds (Scheme 1). Excess levels of free radicals

Scheme 1. Postulated Mechanism of the Cytotoxicity of Thymol on B16-F10 Melanoma Cells



and quinone-related compounds are known to lead to oxidative stress in cells, which causes serious damage, such as diabetes mellitus and coronary arteriosclerosis,[29,30] as well as being linked with aging and carcinogenesis.[31]

It is logical to conclude that the oxidative-stress-related toxicity mechanism is the major mechanism of thymol toxicity. The moderate cytotoxicity of thymol or thyme oil is, interestingly, non-specific to all organisms; for example, it is cytotoxic to bacterial strains, including *Escherichia coli*, *Aeromonas hydrophila*, and *Staphylococcus aureus*,[32,33] and fungal strains, such as *Candida albicans* and *Saccharomyces cerevisiae*.[34,35] For all organisms, from humans to microorganisms, oxidative phosphorylation is an essential metabolic mechanism to create energy for their activities; however, ROS, such as superoxide and hydrogen peroxide, are usually propagated in this process. Thus, the excess amounts of thymol administration leads to the excess level of proton-donating/radical-scavenging action to eliminate these ROS; this leads to the generation of toxic levels of phenoxy radical intermediates. Therefore, thymol acts as a prooxidant rather than an antioxidant at high concentrations.

Safety is the primary concern for the purpose of the additives in industries. Thymol is commonly used in mouthwashes and food for preservatives or flavors. Despite its beneficial biological effects, few toxicological studies on thymol have been performed. Our investigations on thymol toxicity provide evidence that supports caution in the usage of thymol as a food additive.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Composition of thyme oil analyzed with the GC−MS system (Supplemental Table 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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