ORIGINAL RESEARCH

Effects of Thymol, a Natural Phenolic Compound, on Human Gastric Adenocarcinoma Cells In Vitro

Ayse Günes-Bayir, DVM; Abdurrahim Kocyigit, MD; Eray Metin Güler, PhD; Huriye Senay Kiziltan, MD

ABSTRACT

Context • Alternative and complementary medicine has gained importance in anticancer treatment, reflecting a movement toward an integrated approach to treating various diseases. Natural products originating from plants can contain biologically active substances. Thymol is a major component of many plants from the family Lamiaceae that are often used for medicinal and culinary purposes in Mediterranean countries.

Objective • The purpose of the present study was to investigate thymol's cytotoxic, genotoxic, apoptotic effects on gastric adenocarcinoma (AGS) cells, including measurement of reactive oxygen species (ROS) and glutathione (GSH) levels at the same time.

Design • The research team studied thymol's anticancer potential in vitro.

Setting • The research was conducted at the Laboratory of Biochemistry of the Faculty of Medicine at Bezmialem Vakif University (Istanbul, Turkey).

Intervention • Caucasian human AGS cells were exposed to 7 concentrations of thymol—10, 20, 30, 50, 100, 200, and 400 μM—prepared from a stock solution of 600 μM of thymol in dimethyl sulfoxide (DMSO), and after 24 h of incubation, the results were analyzed. The thymol was obtained commercially. The study used a negative control prepared in a concentration of 1:1000 from the stock solution of DMSO.

Outcome Measures • Cytotoxicity was determined using (1) the adenosine 5'-triphosphate cell viability assay; (2) the dichloro-dihydro-fluorescein diacetate assay to evaluate the generation of ROS; (3) the luminescence-based, total GSH assay to determine the GSH levels; and (4) the comet assay to study genotoxicity. Apoptotic induction of thymol was detected (5) by acridine orange/ethidium bromide staining and (6) by Western blotting using a value below the half maximal inhibitory concentration (IC_{50}).

Results • Thymol showed significant cytotoxic, genotoxic, apoptotic, ROS-generating, and GSH-reducing effects, in a dose-dependent manner ($P \leq .001$). A close negative relationship existed between cell viability and the ROS level.

Conclusions • After researchers have confirmed thymol's anticarcinogenic effects in vitro on healthy cell lines and in vivo, it may be found to be a novel and strong therapeutic agent against gastric cancer. The study's results suggest that thymol may have therapeutic power when developed from natural components of the diet for treatment of the disease. (*Altern Ther Health Med.* 2018;25(2):12-21.)

Ayse Günes-Bayir, DVM, is an assistant professor in the Department of Nutrition and Dietetics, Faculty of Health Sciences, Bezmialem Vakif University, in Istanbul, Turkey. Abdurrahim Kocyigit, MD, is a professor doctor, and Eray Metin Güler, PhD, is a lecturer; both are in the Department of Medical Biochemistry, Faculty of Medicine, Bezmialem Vakif University. Huriye Senay Kiziltan, MD, is a lecturer in the Department of Radiation Oncology, Faculty of Medicine, Bezmialem Vakif University.

Corresponding author: Ayse Günes-Bayir, DVM
E-mail address: agunes@bezmialem.edu.tr

Cancer is a health problem that is still increasing worldwide, being the second leading cause of mortality after heart disease.[1] Gastric cancer (GC) is the fourth most-common cancer among cancer cases. In the United States alone, 26 370 new GC cases and 10 730 deaths had been projected for 2016.[2] The incidence of GC is high in East Asia, Eastern Europe, parts of Central and South America, and in Western countries. Gastric adenocarcinoma (AGS) is the most common type in such cases.[3]

The etiology of GC is multifactorial, including dietary factors such as consumption of high quantities of salt and preserved meat products.[4] Conversely, GC is preventable by a dietary change that includes a high intake of fruit and

vegetables. Populations with Mediterranean-type diets have a lower incidence of GC.[5] In Mediterranean countries, a wide range of aromatic plants, especially those of the family *Lamiaceae*, are used for culinary and medicinal purposes.[6] The health benefits of these plants include anti-inflammatory, antibacterial, antifungal, antioxidant, and antitumor activities.

Thymol—2-isopropyl-5-methylphenol—is one of the main phenolic compounds of essential oils from the family *Lamiaceae*.[7] It has been widely used in medical practice as an antiseptic; in agriculture against nematodes; in cosmetics for bath, skin, personal care, and hair products and for hair dyes and mouthwash; and in the food industry.[8]

Some research has revealed that thymol provides antioxidant, antimicrobial, antifungal, and anti-inflammatory benefits.[9-12] In addition, Aydin et al[13] reported a genotoxic effect for thymol when its concentration was above 0.1 mM in human lymphocytes. Research has focused on this phenolic compound to determine its further benefits, such as anticancer activities that might include cytotoxic and genotoxic effects on human hepatoma (HepG2) and human colon carcinoma (Caco-2) cells.[14-16]

Researchers have found cytotoxic effects for 150 to 900 μM of thymol on human hepatoma carcinoma, 150 to 900 μM on human colon carcinoma, 200 to 800 μM on human glioblastoma, and 25 to 100 μM on acute promyelotic cancer cells.[14,15,17] However, Llana-Ruiz-Cabello et al[16] found no cytotoxic effects on Caco-2 cells for 0 to 250 μM of thymol after 24 and 48 hours of its exposure. In the mentioned study, cells exposed the thymol showed the ultrastructural changes such as lipid degeneration, mitochondrial damage, nucleolar segregation, and apoptosis.

Oxygen-containing molecules produce radical and nonradical oxygen species through mitochondrial metabolism in cells, called reactive oxygen species (ROS).[18] They have a role in signaling and modifying functions in cells. Overproduction of ROS has been seen in the onset and progression of many diseases, including cancer.[19] It can lead to the permeabilization of the mitochondrial membrane and, thus, induce apoptosis by releasing apoptotic proteins and destroying macromolecules, such as DNA, proteins, and lipids.

Although many health-promoting effects and anticancer activities have been found for thymol for a few cancer types, its toxic mechanism has not been completely clarified due to the limited research so far. Deb et al[17] and Kang et al[20] have explained that mechanism as being the result of apoptotic induction following mitochondrial membrane damage, which the researchers found was caused by overproduction of ROS in human promyelotic cancer (HL-60) and AGS cells.

At 5 to 100 μM in one study, thymol did not show any cytotoxic effects on peripheral blood mononuclear cells (PBMCs; ie, healthy cells), whereas it induced a significant increase in ROS production in HL-60 cells, an acute promyelocytic cancer-cell line.[17] However, Kang et al[20] investigated the effects of 0 to 400 μM of thymol on adenocarcinoma (AGS) cells and found no statistically significant results. Conversely, in another study 100 μM of thymol was shown to cause a decrease in intracellular ROS in the V79 cell line (ie, Chinese hamster lung fibroblast cells).[21]

Plants that increase production of ROS, and so activate the apoptosis of cancer cells, are expected to be ideal anticancer treatments.[22] In addition, glutathione (GSH) has a 3-amino-acid peptide formula—gamma glutamyl-cysteinylglycine—which is the most-generated antioxidant produced by cancer cells to protect themselves from oxidative stress; so, the cells can remove ROS.[23] However, reduced GSH in cells can induce intracellular ROS production, which causes apoptosis of cells. In addition, GSH is an indicator for cell health through its defense against ROS.

Apoptosis is a genetically directed process of cell self-destruction that (1) is marked by the fragmentation of nuclear DNA; (2) is activated either by the presence of a stimulus or removal of a suppressing agent or stimulus; (3) is a normal physiological process eliminating DNA-damaged, superfluous, or unwanted cells; and (4) when halted—as by genetic mutation—may result in uncontrolled cell growth and tumor formation, also called cell suicide, and programmed cell death. Apoptosis differs from necrosis.[22] It is organized by protein families, which are the upstream Bcl-2 family (eg, the anti-apoptotic Bcl-2 and the proapoptotic Bax) and the downstream caspase family (eg, caspase-3 and capase-9). It has been revealed that anticancer or cancer-preventive agents induce an apoptotic mechanism.

In the recent past, the anticarcinogenic and antiproliferative effects of thymol have gained the attention of investigators. Most of the studies have been performed in in vitro systems, and the clinical significance of the effects of the compound on the normal stomach or other organs of the body is still unknown. The anticancer activity of thymol has been investigated and reported in vitro in different cancer models: human glioblastoma, promyelotic cancer, AGS, colon carcinoma, hepartoma, and lung carcinoma.[15-17,20,24,25]

After researchers have confirmed the bioactivity of thymol in in vitro and in vivo systems on normal and cancer cells, its potential usefulness may be found in clinical applications. The in- vitro cytotoxic, genotoxic, and antioxidant potential of thymol has been investigated on some normal cells: human blood, human lymphocytes, V79 Chinese hamster lung fibroblasts, mouse cortical neurons, and PBMCs.[13,17,21,26-28]

An in vivo study reported that 100 mg of thymol per kg of body weight induced a genotoxic effect on bone marrow cells in rats.[27] In addition, a pharmacodynamic study demonstrated that thymol was almost completely absorbed in the stomach and the proximal small intestine of pigs.[28] Given those studies, it is of great interest as to whether thymol has a therapeutic potential against gastric cancer.

The Caucasian human AGS cell line is a widely accepted cell model for human stomach research.[29] However, no studies have been performed with 0 to 600 μM of thymol in human AGS cells using cytotoxicity, apoptosis, and genotoxicity

assays, including measurement of ROS and GSH levels at the same time. Therefore, the aim of the current study was to investigate the cytotoxic, genotoxic, and apoptotic activities of thymol on the AGS cells. To the best of the research team's knowledge, the current study is the first to evaluate the in vitro genotoxicity of thymol exposure on human AGS cells. Furthermore, production of ROS and GSH in human AGS cells was also investigated after 24 hours of exposure to thymol.

METHODS

Materials

The research team studied thymol's anticancer potential in vitro. The research was conducted at the Laboratory of Biochemistry of the Faculty of Medicine at Bezmialem Vakif University (Istanbul, Turkey).

The thymol (T0501) used in the study was obtained from Sigma-Aldrich (St Louis, MO, USA). The purity of the thymol was reported as ≥98.5% in manufacturer's instructions. The PubChem substance identification of the thymol used was 57654643. The AGS cells (CRL-1739) were purchased from the American Type Culture Collection (Manassas, VA, USA).

Procedures

The AGS cells were cultured in the Ham's F-12 (Kainghn's) medium, the growth medium, which was obtained from Gibco Invitrogen (Carlsbad, CA, USA). It was supplemented with 10% fetal bovine serum (FBS), the phenol red and antibiotics (ie, 100 U/mL of penicillin and 100 µg/mL of streptomycin), which were supplied by Life Technologies (Carlsbad, CA, USA). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. The cells were grown to a number near confluence in 75-cm^2 plastic flasks, and they were harvested weekly. For performing all tests, the AGS cells were plated in a 96-well plate at a density of 15×10^3 cells/mL and in a 6-well plate at a density of 18×10^4 cells/mL, which were calculated according to area of related well plates.

Intervention

Test Solutions. For determination of the value for the half maximal growth inhibitory concentration (IC_{50}), a range of concentrations of thymol was prepared according to the procedures indicated by LIana-Ruiz-Cabello et al.[16] Seven test solutions—10, 20, 30, 50, 100, 200, and 400 µM—of thymol were prepared from a stock solution of 600 µM of thymol in dimethyl sulfoxide (DMSO). The final concentration of the DMSO (Sigma-Aldrich) was below 0.1 %. The AGS cells treated with these 7 solutions became the 7 intervention groups studied.

Negative control solution was prepared as 1:1000 in a concentration from the stock DMSO. The AGS cells treated with this solution became the negative control group.

Outcome Measures

Cell Viability Assay. The determination of the viable cells in a culture was achieved using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA). The AGS cells in both the negative control group and in the 7 intervention groups were included in the test, which was intended to determine whether the thymol was cytotoxic to the AGS cells. The method was based on quantitation of the adenosine 5'-triphosphate (ATP) present.

A total of 100 µL of Ham's growth medium and 15×10^3 AGS cells were placed in each well of a 96-well microtiter plate. After 24 hours of incubation of the AGS cells at 37°C, CellTiter-Glo Reagent was added to each well. The amount of the ATP was measured using a luminometer, a fluorescent multiplate reader (Varioskan Flash Multimode Reader, Thermo, Waltham, MA, USA).

The light emitted in the presence of ATP was quantitated in relative light units. The intensity of emitted light quants was directly related to the ATP content in the tested samples. The cell viability of the 7 intervention groups was expressed as percentages compared with the negative control group's, designated as 100%.[30] The value for the half maximal growth inhibitory concentration (IC_{50}) was calculated from the concentration-response curves by nonlinear regression analysis. All tests were repeated 3 times, and the standard deviation (SD) was within 5%.

2', 7'-Dichloro-Dihydrofluorescein-Diacetate Assay. This assay is reliable and efficient and functions to quantify the potency of pro-oxidants and the cellular oxidative stress.[32] The oxidant-sensitive, fluorescent probe, dichloro-dihydro-fluorescein diacetate (DCFH-DA) from Sigma Chemical (St Louis, MO, USA), was used to measure the intracellular generation of ROS. A total of 200 µL of Ham's growth medium and 15×10^3/mL AGS cells were placed into a 96-well plate.

After 24 hours of growth in the culture plates, the original growth medium containing 10% FBS was replaced with growth medium containing reduced FBS (5%, v/v). Plates were incubated in the absence or presence of the 7 test solutions for another 24 hours. The cells were washed with cold phosphate buffered saline (PBS) from Gibco/Invitrogen and incubated with 100 mM of DCFH-DA for 30 minutes at 37°C. DCF fluorescence intensity was measured using the luminometer (Thermo) at Ex/Em = 488/525 nm. The estimations were carried out in triplicate, ensuring each time that the number of cells per group had the same reproducibility. The results were expressed as percentages of the relative fluorescence of the 7 intervention groups in comparison with the control group.

GSH/GSSG-Glo Assay. The assay detects reduced (GSH)/oxidized (GSSG) GSH directly, with a property of minimizing oxidation in samples.[32] Therefore, the assay (Promega) was used to determine and quantify the GSH level in the AGS cells. The assay was based on luminescence measurements in a multiwell plate. A total of 100 µL of Ham's growth medium containing 10% FBS and AGS cells at a density of 15×10^3/mL were placed in each well of a 96-well microtiter plate. Both the negative control group and the 7 intervention groups with test solutions of thymol were tested.

After 24 hours of incubation of the AGS cells at 37°C, the growth medium was removed and the total GSH Lysis Reagent from the assay was added to each well. Luciferin Generation Reagent was added to all wells, and the solutions in the wells were mixed. After 30 minutes of incubation, Luciferin Detection Reagent was added to all wells, and the solutions in the wells were mixed again and incubated for 15 minutes. The amount of GSH was measured using the luminometer (Thermo). The light emitted in the presence of GSH was quantitated in relative light units. The intensity of emitted light quants was directly related to GSH content in the tested samples. All tests were repeated 3 times, and the SD was within 5%.

Acridine Orange/Ethidium Bromide Staining Assay. The apoptotic effects of thymol at the cellular level were investigated using acridine orange/ethidium bromide (AO/EB) fluorescent dyes (Sigma-Aldrich), which show nuclear changes and formation of apoptotic bodies in cells. Apoptotic, viable, and necrotic cells were differentiated and quantified under a fluorescence microscope.[33]

After placing 18 × 104/mL AGS cells in a 6-well plate, 10 to 100 µM of thymol was added, and the cells were incubated for 24 hours. The cells were harvested and washed twice with PBS. Finally, a solution of AO/EB stain was added to the cell suspension, and the nuclear morphology was evaluated using the fluorescence microscope.

Multiple photos were taken at randomly selected areas (Leica DM1000, Solms, Germany), and a minimum of 100 cells were counted. According to the method, the viable cells have normal green nuclei, apoptotic cells have green nuclei with fragmented chromatin, and necrotic cells have orange/red nuclei. All tests were carried out in triplicate.

Western Blotting. The apoptotic activity of thymol at the protein-expression level was determined by Western blotting. The AGS cells for the control group and for 4 of the intervention groups—those with 10, 30, 50, and 100 µM of thymol—were lysed in radioimmunoprecipitation assay buffer and a proteinase inhibitor cocktail (Roche, Mannheim, Germany). The buffer included 50 mM of Tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl), pH = 7.4; 150 mM of sodium chloride (NaCl); 5 mM of ethylenediaminetetraacetic acid (EDTA), 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1% aprotinin; 50 mM of sodium fluoride (NaF); and 0.1 mM of sodium orthovanadate (Na_3VO_4).

The lysates were centrifuged at 13 000 g for 15 minutes at 4°C. The pellet was discarded and the supernatant containing the protein was transferred into a clean tube. The cell protein was measured using the Bradford assay with Coomassie brilliant-blue-dye (Sigma-Aldrich).[34] Samples containing 30 µg of protein, together with the molecular weight marker (BenchMark Pre-Stained Protein Ladder; Invitrogen, Grand Island, NY, USA), were subjected to 10% SDS polyacrylamide gel electrophoresis (Sigma-Aldrich) under reducing conditions.

The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After the membranes at nonspecific sites were blocked with 5% powdered skim milk in 0.05%, Triton X-100/Tris-buffered saline with Tween (TBS-T) from Sigma-Aldrich for 1 hour. Blots were incubated overnight with an immunoglobulin G (IgG)-purified, rabbit, polyclonal Bcl-2, Bax, caspase-3, and caspase-9 antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) in a solution containing 5% powdered skim milk and 0.05% Triton X-100/TBS. The blots were washed 3 times in TBS-T for 10 minutes each and were incubated with a peroxidase-conjugated, goat, antirabbit IgG at a concentration of 1 µg/mL in 5% powdered skim milk in 0.05% TBS-T. All samples were also blotted for β-actin with clone AC-15 (Santa Cruz Biotechnologies) to normalize protein amounts. The expression levels of proteins were detected using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ, USA) and captured with an imaging system (Vilber Lourmat Sté, Collégien, France).

Alkaline Single Cell Gel Electrophoresis (Comet Assay). The genotoxic effects of thymol on AGS cells was assessed using the comet assay (Sigma-Aldrich) according to the method of Singh et al,[35] with slight modifications. Approximately 2×10^5 cells per well were seeded into 6-well plates with Ham's growth medium and incubated at 37°C in 5% CO_2. After 24 hours of incubation, the thymol was added to the cells at below IC_{50} concentrations, and the cells were incubated for a further 24 hours at 37°C.

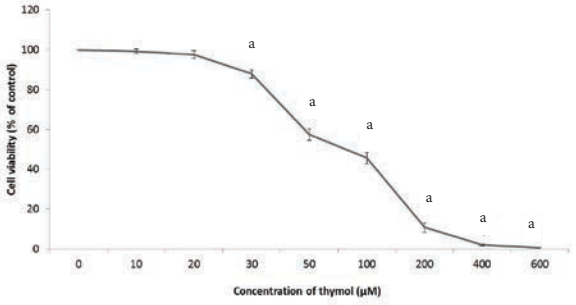
DMSO (1%) was used as a negative control, and 50 µM if H2O2 was applied as a positive control. After incubation, the cells were washed with PBS, harvested using 0.25% trypsin/EDTA (Life Technologies), and collected for centrifugation at 400 g for 5 minutes at 4°C. The supernatant was discharged, and the cell density was adjusted to 2×10^5 cells/mL, using cold PBS. Ten microliters of resuspended cells were placed into centrifuge tubes for the comet assay, as described later. All tests were repeated in triplicate.

Before performing the comet assay, the trypan blue exclusion test (Sigma-Aldrich) was carried out for determination of the viable number of cells in a cell suspension.[36] Ten microliters of cell suspension were mixed with 90 µL of 0.6% low-melting agarose (Sigma-Aldrich) and added to slides precoated with 1% normal melting agarose (Sigma-Aldrich). After solidification of the agarose, the slides were immersed in a lysis solution (Sigma-Aldrich)—2.5 M of NaCl; 100 mM of disodium EDTA (Na_2EDTA); and 10 mM of Tris-HCl, pH = 10, 1% Triton X-100 and 10% DMSO—at 4°C for 1 hour to remove cellular proteins.

After removal from the lysis solution, the slides were washed with cold PBS and placed in a horizontal electrophoresis box. The DNA was allowed to unwind for 40 minutes in a freshly prepared alkaline electrophoresis buffer containing 300 mM of sodium hydroxide and 10 mM of Na_2EDTA (pH > 13). Then, electrophoresis was run at 300 mA for 25 minutes at 4°C under minimal illumination to prevent further DNA damage.

The slides were washed 3 times with a neutralization buffer (0.4 M Tris, pH = 7.5) from Sigma-Aldrich for 5 minutes at 4°C and treated with ethanol for 5 minutes before

Figure 1. Cytotoxic Effects of 0 to 600 µM of Thymol on AGS Cells After 24 Hours of Incubation, as Tested Using the ATP Cell Viability Assay

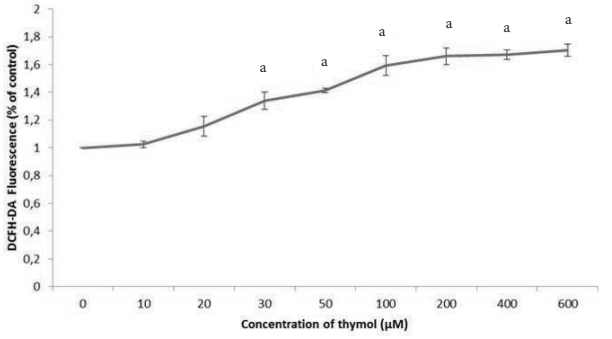


Note: Thymol induced cytotoxicity in a dose-dependent manner. All values are expressed as means ± SDs.

[a]Significantly different from controls ($P \leq .001$).

Abbreviations: AGS, Caucasian human gastric adenocarcinoma cell line; ATP, adenosine 5'-triphosphate; SD, standard deviation.

Figure 2. Production of Reactive Oxygen Species in AGS Cells After 24 Hours of Exposure to 0 to 600 µM of Thymol, Detected by the DCFH-DA Assay



Note: Thymol induced ROS generation in a dose-dependent manner. All values are expressed as means ± SDs.

[a]Significantly different from controls ($P \leq .001$).

Abbreviations: AGS, Caucasian human gastric adenocarcinoma cell line; DCFH-DA, dichloro-dihydro-fluorescein diacetate; ROS, reactive oxygen species; SD, standard deviation.

staining. Dried microscopic slides stained with ethidium bromide (EB) from Sigma-Aldrich—2 µg/mL in distilled H_2O; 70 µL/slide—were covered with a coverslip. The slides were analyzed using a fluorescence microscope (Leica) at a 200× magnification with an epiflourescence-equipped rhodamine filter and with an excitation wavelength of 546 nm and a barrier of 580 nm.

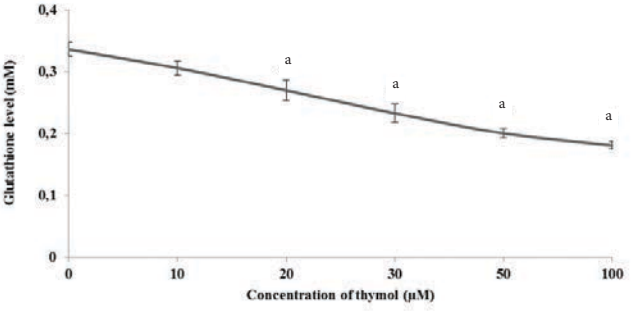
A computerized image analysis system (comet assay IV; Perceptive Instruments Ltd, Bury St Edmunds, Suffolk, UK) was used. The percentage of DNA tail was used as a parameter for measurement of DNA damage (ie, DNA strand breaks).[37] A total of 100 comets were scored per each sample in each electrophoresis.

The same biochemistry staff performed all tests. In addition, DNA damage was identified by a single observer, who was not aware of the cell's status.

Statistical Analyses

The results are presented as means ± SDs and calculated as percentages in relation to the control cells. Statistical analyses were carried out using 1-way analysis of variance (ANOVA) test, and post hoc analysis of different concentrations was performed by the least significant difference and Tukey's tests for comparison of the results. A P value of ≤.05 was considered to be statistically significant. Data were analyzed using the Statistical Package for the Social Sciences, version 16.0 (IBM, Armonk, NY, USA). In addition, a nonlinear regression analysis was performed for the estimation of the IC_{50} value of thymol in AGS cells.

Figure 3. Gluthatione Content in AGS Cells After 24 Hours of Exposure to 0 to 100 µM of Thymol



Note. All values are expressed as means ± SDs.

[a]Significantly different from controls ($P \leq .001$).

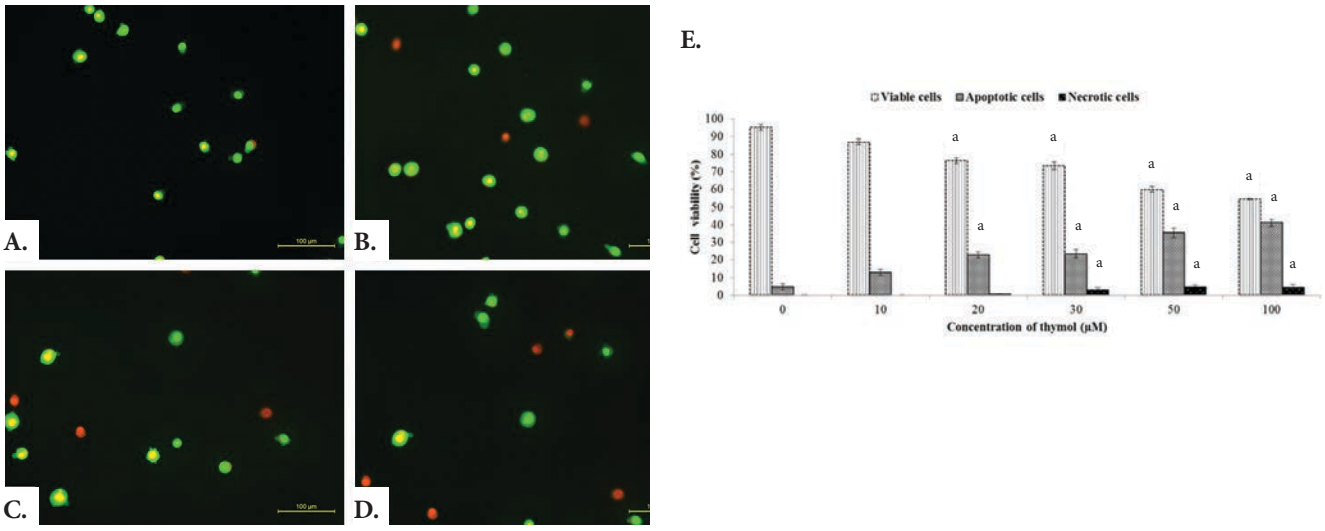
Abbreviations: AGS, Caucasian human gastric adenocarcinoma cell line; SD, standard deviation.

RESULTS

Effects on AGS Cell Viability

After 24 hours of incubation, the effects of thymol on the AGS cells were investigated using the ATP cell viability assay. The cell viability of the AGS cell cultures was greater than 95%, before adding thymol. After thymol exposure, the cell viability of the treated cells was significantly reduced, in a dose-dependent manner, with $P \leq .001$ (Figure 1). Thymol showed significant toxicity at the 50-µM dose. The cells exposed to 30 to 600 µM of thymol showed significant differences in their decreases in cell viability compared with

Figure 4. Apoptotic Effects of 0 to 100 μM of Thymol on AGS Cells, Detected Using a Fluorescence Microscope Following AO/EB Staining



Note. The apoptotic effect of thymol was in a dose-dependent manner after 24 hours of incubation. The figures show cells exposed to 0 μM (Figure 4A), 10 μM (Figure 4B), 30 μM (Figure 4C), and 100 μM (Figure 4D) of thymol. The apoptotic effects of thymol on cell viability are presented in percentages in Figure 4E. All values are expressed as means ± SDs.

[a]Significantly different from controls ($P \leq .001$).

Abbreviations: AGS, Caucasian human gastric adenocarcinoma cell line; AO/EB, acridine orange/ethidium bromide; SD, standard deviation.

the control cells after 24 hours ($P \leq .001$) (Figure 1). The concentration-response curve demonstrated significant decreases in cell viability, with 87.8% of the cells being viable at 30 μM of thymol and only 0.66% at 600 μM, in comparison with the untreated control cells.

The IC_{50} value was calculated from the concentration response curve and was found to be 75.63 ± 4.01 μM. Therefore, further tests assessing apoptosis and genotoxicity of thymol were performed using the 10 to 100 μM thymol.

Effects on ROS Generation

ROS generation was studied using the oxidation-sensitive, fluorescent probe DCFH-DA. Figure 2 shows the measured relative fluorescence as percentages. Thymol was found to induce ROS generation in the AGS cells. The intracellular ROS levels in cells were significantly increased after thymol exposure at 30, 50, 100, 200, 400, and 600 μM ($P \leq .001$). A positive correlation was found between the relative ROS levels in AGS cells and an increasing dose of thymol (Figure 2). The results indicated that the increases in intracellular ROS production in the AGS cells after 24 hours of exposure to thymol were significantly different compared with the control cells ($P \leq .001$).

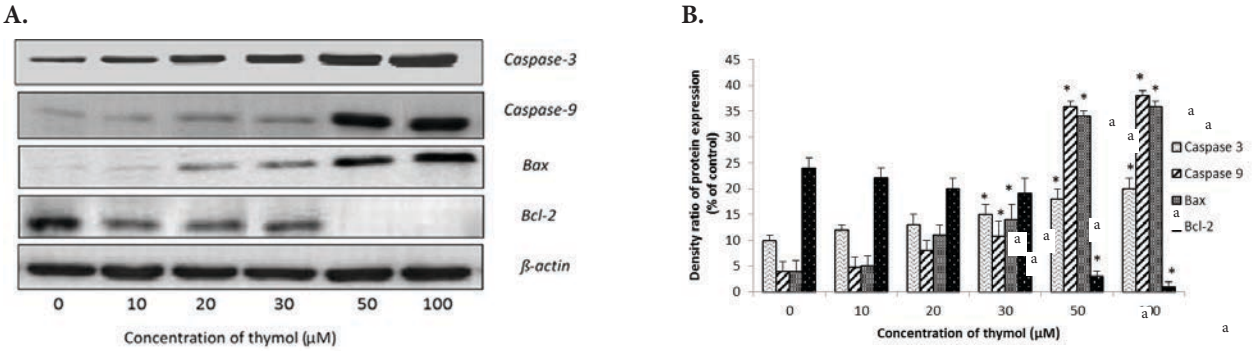
Effects on GSH Levels

GSH levels in the AGS cells were detected using the GSH/GSSG-Glo Assay. Figure 3 shows the measured relative luminescence in percentages for the 10 to 100 μM doses of thymol. GSH levels were significantly decreased after 24 hours of thymol exposure at 50 μM ($P \leq .001$). A negative correlation was found between the relative GSH level and increasingly larger doses of thymol. Significant differences in GSH levels were observed between the control cells and the cells exposed to 20 to 100 μM of thymol ($P \leq .001$) (Figure 3).

Apoptotic Effects—AO/EB Staining Assay

Since thymol has a cytotoxic effect on AGS cells, the cause of those effects should be determined. This AO/EB method allows the visualization and quantification of apoptotic, necrotic, and viable cells under a fluorescence microscope after staining.[33] Apoptosis is characterized by nuclear changes and apoptotic body formation. AGS cells exposed to 10 to 100 μM of thymol were seen to be morphologically abnormal, with shrinkage of the cell's nucleus and progression of chromatin condensation (Figure 4A-D). The apoptotic effects of thymol occurred in a dose-dependent manner. Thymol showed significant apoptotic effects at a dose of 50 μM. When the AGS cells were exposed to thymol, the number of apoptotic cells in the groups exposed to 20 to 100 μM and the number of necrotic cells in the groups exposed to 30 to 100 μM significantly increased, and the number of viable cells in the groups exposed to 20 to 100 μM of thymol significantly decreased, with $P = .001$ (Figure 4E). Significant differences were found between the controls and cells exposed to 20 to 100 μM of thymol ($P \leq .001$).

Figure 5. Apoptotic Effects of 0 to 100 μM of Thymol on AGS Cells Investigated by Western Blotting

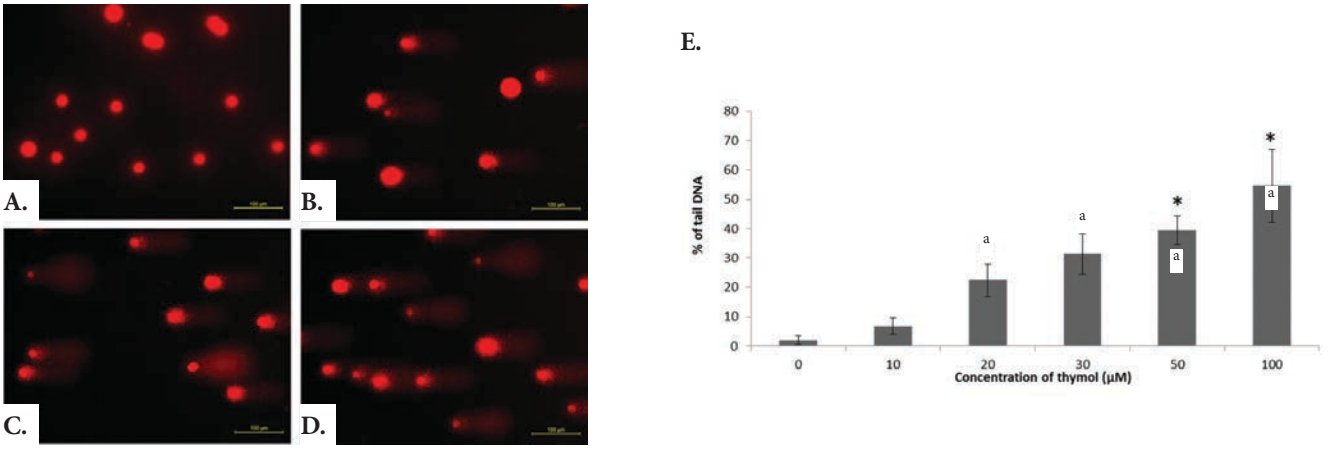


Note. Figure 5A shows Bax, Bcl-2, caspase-3, and caspase-9 proteins detected after 24 hours of exposure to 0 to 100 μM of thymol. β-actin was used as an internal control. Figure 5B shows the density ratio of expressed proteins, presented in percentages. All values are expressed as means ± SDs.

[a]Significantly different from controls ($P \leq .001$).

Abbreviations: AGS, Caucasian human gastric adenocarcinoma cell line; SD, standard deviation.

Figure 6. Genotoxic Effects of 0 to 100 μM of Thymol on AGS Cells Detected by the Comet Assay After 24 Hours of Incubation



Note. Cells were exposed to 0 μM (Figure 6A), 10 μM (Figure 6B), 30 μM (Figure 6C), and 100 μM (Figure 6D) of thymol. Increased comet formation shows increased DNA damage (Figure 6E). Thymol induced DNA damage in a dose-dependent manner. All values are expressed as means ± SDs.

[a]Significantly different from controls ($P \leq .001$).

Abbreviations: AGS, Caucasian human gastric adenocarcinoma cell line; SD, standard deviation.

Apoptotic Effects: Western Blotting

Western blot analysis was performed to detect apoptotic cells at the protein-expression level. Expressional analyses of apoptotic—caspase-3, caspase-9, pro-apoptotic (Bax), and antiapoptotic (Bcl-2)—as well as β-actin proteins were studied by Western blotting (Figure 5A). It is known that these proteins have a role in the apoptotic pathway,[22] and the current study evaluated AGS cells exposed to 0 to 100 μM of thymol after 24 hours of incubation (Figure 5A). Significant differences existed between the negative controls and the cells exposed to 10 to 100 μM of thymol ($P \leq .001$; Figure 5B). The results also showed that the density ratios of the 3 proteins caspase-3, caspase-9, and Bax increased significantly in a dose-dependent manner when the AGS cells were exposed to 30-100 μM, with $P = .001$ (Figure 5B). The density ratio of Bcl-2 protein decreased significantly at 50 to 100 Mm of thymol ($P \leq .001$). The significant apoptotic effects of thymol first became evident at a dose of 50 μM.

It was shown that thymol induces apoptosis by decreasing Bcl-2 protein levels, and increasing Bax, caspase-3, and caspase-9 protein levels. β-actin was used as a positive control, and it was positive for all samples.

Genotoxic Effects

The comet assay is a sensitive genotoxicity assay for detection of DNA damage,[37] which is seen as a comet formation. The assay was used to detect genotoxic effects in AGS cells exposed to 0 to 100 µM thymol, after 24 hours of incubation (Figure 6A-D). Figure 6E presents the percentages of AGS cells with DNA damage. Cells exposed to 20 to 100 µM of thymol induced significantly greater DNA damage in a dose-dependent manner when compared with the negative control cells ($P \leq .001$). A positive correlation was found between the DNA damage and an increasing dose of thymol in AGS cells.

DISCUSSION

Worldwide, GC is still the third leading cause of cancer mortality for both genders, accounting for 8.8% of all deaths.[38] Surgical resection of the stomach and peri- and postoperative chemo- or chemoradiation therapy are the current treatments for GC, However, patients who receive these treatments at later stages of cancer may show poor prognoses, with 5-year survival rates overall of less than 25%.[39]

Surgery remains the only curative therapy, whereas perioperative and adjuvant chemotherapy as well as chemoradiation therapy can improve the results from resectable GC with extended lymph node dissection.[40] Therefore, surgical treatment of GC has been preferred to chemotherapy; however, intensive treatment with anticancer curatives or genetic engineering may offer GC treatments that can inhibit the proliferation and differentiation of cancer cells.[41,42]

For the purpose of making cancer treatment more effective, novel anticancer drugs must be developed. The current research team's strategy is to evaluate the biological functions of naturally occurring phenolic monoterpenes, especially those of thymol, and to determine whether they have the ability to inhibit cell proliferation and induce apoptosis in human AGS cells.

Plants from the family Lamiaceae, such as origanum and thyme, are the most-used spices in the Mediterranean diet, which is associated with a low risk of gastric cancer.[5] The chemical composition of thyme indicates that phenols are the major components, followed by terpene and sesquiterpene. Thymol has been reported to be one of the major phenolic compounds of the essential oils of thyme and oregano.[7] However, the chemical composition and the amount of thymol in these plants depends on the soil and climate, methods of cultivation, growing and harvesting seasons, and geographical regions of the plants.[43,44] Therefore, the use of thymol seemed to be a better solution in the current study than the use of an extract or essential oil of thyme or oregano.

Recently, Kang et al[20] reported that thymol can induce apoptotic effects on human AGS cells, but their study was performed only for thymol up to 400 µM, and the mechanisms, such as genotoxicity, were not fully elucidated except for the apoptotic pathway. In another study, human glioblastoma cells were incubated with 100 to 800 µM of thymol for 24 hours, and 200 to 600 µM doses of thymol significantly reduced cell viability in a dose-dependent manner.[15] In addition, the IC_{50} value of thymol was found to be 60.01 µM in human hepatocellular carcinoma HepG2 cells, 497 µM in human lung carcinoma H1299 cells, and 400 or 700 µM in human glioblastoma cells, depending on the calcium ionen effect.[15,24,25] Therefore, the current research team prepared and investigated thymol up to 600 µM and found that the IC_{50} value of thymol in human AGS cells was 75 µM. The differences between the prior and current studies may be related to the types of cells used and the high absorption rate of thymol in pigs' stomachs in one of the studies.[28]

Overgeneration of ROS has been seen in the onset and progression of cancer cases.[45] However, it is known that phenolic compounds can have either antioxidant or pro-oxidant effects.[47] Those effects depend on such conditions as cell resistance, concentration, and time.[47] Pro-oxidants can induce oxidative stress, either by generating ROS or by inhibiting the antioxidant status in cells.[46] However, the toxic mechanism in thymol seems to be related to damage to the mitochondrial membrane by permeabilization and to activation of apoptosis.[15-17,20] It appears to be dependent on the overgeneration of ROS, which induces the depolarization of the mitochondrial membrane potential following intracellular stress.

A decrease in the mitochondrial-membrane can cause an increase in Bax and a decrease in Bcl-2 levels, release of cytochrome C, and the subsequent activation of caspases.[17,20] Caspases play an important role in the initiation and execution of caspase-dependent apoptosis. Conversely, mitochondrial protein can also induce apoptosis through caspase-independent pathways.

By the increase of cellular stress, an apoptosis-inducing factor translocates to the nucleus, causing DNA damage followed by apoptosis.[17] It has been reported that ROS do not affect the apoptosis of human promyelotic cancer (HL-60) cells directly, unlike their inducement of the molecular cascade for release of cytochrome c from the mitochondria. In addition, an increase in ROS activity has been found when HL-60 cells were treated with 25 to 100 µM of thymol.[17]

Another study reported that thymol at lower concentrations (ie, $<IC_{50}$) can protect cells against H_2O_2-induced membrane and DNA damage, but at higher concentrations (ie, $>IC_{50}$) can damage the cell membrane and DNA, including damage at 60.01 µM of thymol in human hepatoma (HepG2) cells and damage at 497 µM of thymol in lung cancer (H1299) cells.[24,25] In contrast, another study found that thymol at concentrations of 0 to 250 µM, showed no change after 24 hours of exposure in ROS levels in human colon carcinoma (Caco-2) cells, whereas a concentration of 100 µM could protect such cancer cells.[47]

Although interest exists in the antiproliferative and apoptotic effects of thymol, limited information exists on its pro-oxidant activity in cancer cells. Therefore, in the current study, the effects of thymol on intracellular ROS generation were investigated within the study's wide range of test solutions. ROS generation was significantly higher at 600 µM of thymol compared with controls. In addition, a positive relationship was

found between cell death and ROS generation after 24 hours of exposure to thymol at higher concentrations (ie, $<IC_{50}$).

In the current study, the detection of the cytotoxic and apoptotic effects of thymol at the cellular level in human AGS cells used the ATP cell viability assay and AO/EB staining, respectively. The AGS cells were evaluated after 24 hours of exposure to thymol. The ATP cell viability assay differentiates between viable and dead cells, whereas apoptotic, necrotic, and viable cells are distinguishable after AO/EB fluorescence staining.

The results of the present study revealed that thymol showed cytotoxic and apoptotic effects on the AGS cells in a dose-dependent manner. Other studies have also reported cytotoxic and apoptotic effects for thymol on human promyelotic leukemia, glioblastoma, hepatoma (HepG2), and non-small-cell lung cancer (H1299) cells.[14-17,24,25] No cytotoxic effects for thymol have been reported on human colon carcinoma (Caco-2) cells using different cytotoxicity assays, but the apoptosis of such cells has been observed under an electron microscope.[16] That result may have occurred because of the sensitivity of the methods used, which in that study were less sensitive and were colorimetric compared to the current study's luminometric ATP cell viability assay.[48]

Other studies also have reported that 200 to 800 µM and 100 to 400 µM of thymol can induce apoptosis and necrosis, respectively, in a dose-dependent manner, as detected by an Annexin-V/PI double staining assay on human glioblastoma and AGS cells.[15,20] These studies support the current study's results that apoptosis as well as necrosis occurred after 24 hours of exposure to 20 to 100 µM of thymol.

Conversely, in another study no apoptotic or necrotic effects for 75 to 250 µM of thymol were detected by the Annexin-V/PI double staining assay, but the cells were observed only under an electron microscope.[16] In addition, it is known that phenolic compounds may have pro-oxidant properties and, therefore, can affect the cellular redox status.[46] Consequently, increasing levels of ROS generation call the results of the cytotoxic, apoptotic and/or necrotic and genotoxic effects of plant polyphenols.[46,49] The mechanisms for the cytotoxicity of thymol are not known, but different pathways generally have been associated with plant products' cytotoxicity such as proteasome inhibition, topoisomerase inhibition, inhibition of fatty acid synthesis, accumulation of p53, induction of cell cycle arrest, inhibition of phosphatidyl-inositol 3-kinase, or enhanced expression of c-Fos and c-Myc.[50]

It has been reported that anticancer drugs kill the cancer cells by stimulation of apoptotic pathways.[22] Apoptosis, programmed cell death by different proteins, was also investigated in the current study at the protein-expression level in AGS cells treated with 0 to 100 µM of thymol. Because caspase-9 protein initiates the cascade of apoptosis, and Bcl-2 protein has been found to play an important role in cancer cases,22 the current research team studied protein expression of apoptosis-related proteins, such as Bax, Bcl-2, caspase-3, and caspase-9, using Western blotting. The study found that AGS cells exposed to 0 to 100 µM of thymol (ie, $<IC_{50}$) showed downregulation of Bcl-2 protein and dose-dependent upregulation of Bax, caspase-3, and caspase-9 proteins in comparison with the control cells.

Likewise, another study demonstrated that 50 to 100 µM of thymol induced apoptosis in human promyelocytic leukemia (HL-60) cells, involving both caspase-dependent and caspase-independent pathways in a dose-dependent manner.[17]

The current study was also supported by Kang et al,[20] who investigated 100 to 400 µM of thymol in AGS cells, but those researchers provided no comparisons nor presented analyses showing statistically significant differences between the different concentrations of thymol. To the research team's knowledge, the current study is the first reporting the Bax, Bcl-2, caspase-3, and caspase-9 activity of thymol doses below the IC_{50} in AGS cells. The downregulation of Bcl-2 gene expression and the upregulation of Bax, caspase-3, and caspase-9 gene expression found in the current study draw attention to the potential of thymol to be an ideal chemopreventive or therapeutic agent for AGS.

The comet assay is a commonly applied method to demonstrate genotoxicity in terms of DNA damage.[35,37] Polyphenols can have antioxidant and pro-oxidant effects depending on their concentrations.[46] Their higher concentrations can damage and permeabilize the mitochondrial membranes and oxidize the antioxidants. The change from antioxidants to pro-oxidants happens at higher concentrations of polyphenols.[49]

Polyphenols may also induce DNA damage through their pro-oxidant effects on cells. Kang et al[20] reported no testing for the genotoxic effects of thymol on AGS cells. Therefore, the current research team investigated the genotoxic effects of 0 to 100 µM of thymol (ie, $< IC_{50}$) on AGS cells. Thymol at 10 to 100 µMs showed genotoxic effects on AGS cells in a dose-dependent manner.

To the research team's knowledge, the current study represents the first time that the genotoxic effect of thymol has been evaluated in AGS cells. The current findings are in accordance with previous studies that have been performed in human hepatoma (HepG2) cells and non-small lung cancer (H1299) cells exposed to thymol.[24,25] Thymol at 25 µM also has been shown to cause genotoxicity in the V79 Chinese hamster, lung fibroblast cells.[21] As opposed to those results, 0 to 600 µM of thymol have shown cytotoxic effects on HepG2 and Caco-2 cells, but the results were not associated with a DNA-damaging effect.[14]

The limitations of this study included the inadequate project budget; because of this, we cannot perform the investigations on more than 1 cell culture, either cancer or healthy cells. However, with a new budget after a submission of another project, we would like to study all these effects also on a healthy cell line and to compare these effects with the cancer cell line.

CONCLUSIONS

This study is the first to examine the cytotoxic, apoptotic, and genotoxic activities of thymol, including measurements of both ROS and GSH levels in human AGS cells. Thymol induced cytotoxicity, apoptosis, DNA damage, increased

ROS activities, and decreased GSH levels in a dose-dependent manner. A close negative relationship existed between cell viability and the ROS level.

The results reveal that the dose-dependent effect of thymol on AGS cells may be related to the generation of ROS, via its pro-oxidant activity. The study also demonstrates that thymol-induced apoptosis in AGS cells is regulated by the increased expression of Bax, caspase-3, and caspase-9 and the decreased expression of Bcl-2 proteins. Taken together, these data show the possibilities of using components of a natural diet in the development of therapeutic agents for cancer treatment. Furthermore, the possibility is raised that thymol may find application in treatment of human AGS. Therefore, the current study's results call for further investigations of thymol in vitro in healthy cell lines and in vivo.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist for the research team.

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