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Type

Research paper

Keywords

lung cancer, Apoptosis, HPLC, thymol, Thymbra spicata

Abstract

Introduction
Alternative medicine is important in cancer treatment. The apoptotic effect of Thymol and extracted Thymol from Thymbra spicata on non-small-cells lung cancer was studied.

Material and methods
Thymol was evaluated in Thymbra spicata extract by HPCL. Cell viability was assessed by MTT method. DCF and flu3-AM probe was used for ROS and cai2+ analysis, respectively. Western blotting was performed to measure NOX2 and Bax/Bcl-2 ratio.

Results
Obtained data showed that Thymol was 1.51 mg/g in Thymbra spicata extract. Treatment with Thymol and extracted Thymol from Thymbra spicata resulted in cell death at high concentrations [LC50= 111±4.5 and 119±5.2 µM, respectively]. Subsequently, Thymbra spicata extract and its bioactive component increased ROS and Cai2+ production, NOX2, and Bax/Bcl-2 ratio.

Conclusions
This study revealed the anticancer effects of Thymol and Thymbra spicata extract on non-small-cells lung cancer and at least part of that effect was related to the increase in the NOX2 and Bax/Bcl-2 ratio. Our results demonstrated that TSE and Thymol at high concentrations (180, 120, and 80 µM) decreased the growth of A549 cells. It appeared that cytotoxic activity was exerted through activation of NOX2, ROS generation, increase in Cai2+, and Bax/Bcl-2 ratio. Present results demonstrated that TSE and thymol may be potential therapeutic agents for human lung cancer.

The effect of *Thymbra spicata* extract and its bioactive component Thymol on non-small-cells lung cancer cell line A549

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keywords: *Thymbra spicata*, Thymol, Lung cancer, Apoptosis, HPLC.

Introduction

One of the most widespread cancers which cause death is lung cancer [1]. Non-small cell lung cancer (NSCLC) is the most frequent type of cancer [2]. ROS (Reactive oxygen species) has a significant role in the mitochondrial membrane depolarizing and this phenomenon to go causes apoptosis [3]. There are several sources of intracellular ROS production such as the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (NOX family) [4]. ROS production leads to an increased Bax/Bcl-2 ratio which is one of the causes of apoptosis [5]. Chemotherapy drugs such as cyclophosphamide, doxorubicin, and etoposide are used to treat NSCLC and small-cell lung cancer [6]. Herbal medicines have a long history of being used in the treatment of cancer and are a major source of new medicine. Even the ingredients of medicinal plants such as vincristine obtained from the *Vinca rosea* and paclitaxel (Taxol) from the leaves of various Taxus species have an important place in the treatment of cancer today [7].

Also, some medicinal herbs such as *Cordyceps militaris, Selaginella tamariscina, and Crocus sativus* L. are traditional herbs with antimetastatic effects against lung cancer cells [8]. It has been demonstrated that the active components of medicinal plants such as myricetin [9], berberine [10], and luteolin [11] have shown anti- lung cancer effects.

Thymus species have been used as a group of medicinal plants to treat various diseases. Monoterpene Thymol has been found in the oils of many medicinal plants, such as *Thymus ciliates*, *Trachyspermum ammi*, *Thymus vulgaris*, *Nigella sativa*, *Monarda fistulosa*, and *Thymbra spicata* [12]. It is known that Thymol activates apoptosis in a dose-dependent manner in various cancers. Thymol uses to treat malignancies is newly appreciated while the mechanism of apoptosis induction is unknown [13].

In this study, possible mechanisms of cell death in lung cancer were investigated in addition to the identification of the active ingredient of Thymol in the *Thymbra spicata* plant.

Material and methods

Thymbra spicata extraction and Thymol identification by HPLC

The *Thymbra spicata* specimens were collected from the surrounding environment of Ilam, Iran, in May 2019. The plant materials were authenticated by the Horticulture Department, School of Agriculture, Ilam University (voucher specimens, 596). The plant's powder (25 g) was extracted with water-methanol for 24 h by the soxhlet apparatus. Solvents were evaporated (rotary, Germany, IKA HB 10). The extract percentage was 7.21%, and after lyophilization, it was kept stored in the freezer (-20^{o} C). Then, the dissolved samples in methanol were filtered by a syringe (0.22 μm) [14].

To detect Thymol, a reversed-phase high-performance liquid chromatography (Smart line; Knauer, Germany) with a C18 HPLC Columns (Knauer, Nucleosil H.P., 100 Å pore size, internal diameter 25 cm * 0.46 cm, particle size 3 μm,) and a UV detector (Knauer, K-2600, Well chrome) was calibrated. Mobile phase (methanol (A) and water with 0.1% formic acid (B) at range from 5-70%), column temperature (25°C), injection volume (1 μL), detection wavelength (284 nm), and flow rate (1 ml/min) was set. The standard solution of Thymol was injected under similar conditions. The obtained peak from the extract of *Thymbra spicata* (TSE) was compared to the standard of Thymol. A prepared Thymol standard stock solution in methanol (0.1 mg/ml) was diluted to obtain 5, 10, 20, 40, 80,120, and 180 μM [15].

Cell culture

The cells (A549) were cultured in Dulbecco's Modified Eagle Medium with 1% antibiotic and 10% FBS. In an incubator at 37 ºC, containing 5% CO2 cells were incubated. Then, cells were used between the 2nd and the 5th passages.

Cell viability assay

Cell viability and cytotoxic effects of different concentrations of TSE and Thymol were evaluated by MTT. In this technique, the color change by mitochondrial dehydrogenases from tetrazolium salt (yellow) to formazan crystals (purple) was considered [16]. Cells were cultured with different concentrations (5, 10, 20, 40, 80, 120 , and 180 μM) of Thymol in TSE and free Thymol. For 24, 48, and 72h, cells were incubated. Then, after MTT reagent (10 μl) addition, for 4 h plates were incubated. Formazan was soluble with gentle shaking at 37◦C in DMSO (100 μL), and with an ELISA Plate Readers absorbance was read at 595 nm (USA, Molecular Devices; Spectra MAX).

Assessment of ROS

To measure intracellular ROS levels, DCFH-DA (a fluorescent probe) was used [17]. The cells were incubated with DCFH-DA (10 μM, 37°C, 30 min) and analyzed in a bioassaymulti-detection (the USA, plate reader, FLx800-Biotek). Dichlorofluorescein was read at emission 520 nm and excitation 485 nm. ROS production was detected from a 10–200 nM H_2O_2 standard curve.

Intracellular calcium (Cai^{2+}) measurement

Intracellular calcium (Cai^{2+}) was determined by Fluo-3-acetoxymethyl ester (Fluo-3/AM, 10 mM, Interchim) dissolved in DMSO [18]. Two types of buffer solutions were prepared for washing: buffer 1 (Phenol red-free DMEM containing 10 mM HEPES (4-(2-hydroxyethyl) piperazine-1- ethane sulfonic acid, pH7.0) and buffer 2 [DMEM containing 10-mM HEPES,

5%fetal calf serum, and pH 7.4)]. First, aliquots of 1 mL cell suspensions (1×10^6 cells/mL) were washed and resuspended in buffer 1. Then, 0.4 µL of Fluo 3-AM (1.0 M in DMSO) was added and for 30 min, cells at room temperature were incubated and before performing the assay, washed with buffer 2. Cai^{2+} was analyzed by a FAC scan caliber™ flow cytometer (USA, California, Becton Dickinson).

Investigating the expression of NOX2 and Bax/Bcl-2 ratio by Western Blot

The cells were lysed in lysis buffer (20 mMTris–HCl ,137 mM NaCl (pH 8.0), 1%NP40, glycerol 10%, aprotinin 10 µg/mL, phenylmethyl sulfonic fluoride 1 mM, sodium vanadate 0.5 mM, and leupeptin 1µg/mL). For 20 min, homogenates were centrifuged at 12,500 rpm (4°C), and then, at -70°C supernatants were stored [19]. Based on the micro BCA kit protocol (USA, Rockford, Pierce. IL), total protein content was measured. Equal protein amounts (3,000 µg) from every sample were loaded on gels and electrotransferred to PVDF membranes. Thereafter for 1 h, blocked at room temperature and then, incubated PVDF with primary antibodies followed by appropriate secondary antibodies anti-primary IgG horseradish peroxidase-conjugated. Using the ECL kit (USA, Piscataway, Biotech Inc., Amersham Pharmacia NJ), according to the manufacturer's instructions, chemiluminescence detection of immunocomplexes was done and by using software (Gel-Pro analyzer, USA, Bio-Rad), the results were quantified. Analysis of protein was done by anti-human ILK (Priab 1/5000, ab-76468, Sec ab 1/2000), VEGF, BDNF (SAB4300702, Sigma,1: 750), NGF (Priab 1/500, ab- P5498, Sigma). (Germany, C-2, Santa Cruz, Heidelberg) at a 1:1000 dilution ratio.

Statistical analysis

Experiments were repeated at 3 different times. Statistical analyses were performed on different groups using ANOVA and t-tests. All the results were expressed as mean ± standard deviation.

The term half-maximal lethal concentration (LC 50) refers to a drug or toxicant that induces a response between the baseline and the maximum. P values of less than 0.05 were considered significant.

Results

Extraction and identification

Thymol Standard was read at a wavelength of 284 nm with retention time at 5.200 min, and in the same condition, Thymol in TSE had retention time at 5.267 min (Figure 1a, b). Figure 1 showed the peak of Thymol in TSE in comparison to standard Thymol. Quantitative analysis showed that thymol was dominant in every fractionated methanol (Thymol 19.14 mg/g) of TSE. All methods were validated and showed a linear calibration curve:

$y = 827.68\ x - 12599$ $R^2 = 0.9942$

Effects of Thymol and *Thymbra spicata* extract on cell viability

Figure 2 showed no evidence of cell death in free Thymol or TSE at concentrations of 5, 10, 20, and 40 μM. However, incubation at high concentrations (180 (18 and 24 vs 100 ,control, **P<0.001), 120 (35 and 41 vs 100, **P<0.001), and 80 μM (75 and 82 vs 100, (*P<0.05)), with free Thymol or TSE respectively, led to a decrease in cell viability.

Effects of Thymol and *Thymbra spicata* extract on ROS generation

Treatment with Thymol and TSE (5, 10, 20, and 40 μM) did not increase ROS levels in cells. However, free Thymol and TSE at concentrations of 80 (125 and 129 vs. 100, control, (*P<0.05), 120 (143 and 147 vs. 100, **P<0.001), and 180 (161 and 182 vs. 100, **P<0.001) μM significantly increased intracellular ROS (Figure 3).

Effects of Thymol and *Thymbra spicata* extract on intracellular calcium

Cai^{2+} significantly increased in Thymol and TSE treated samples (180 (137 and 151 vs. 100 ,control, **P<0.001), 120 (128 and 135 vs. 100, **P<0.001), and 80 μM (115 and 118 vs. 100, (*P<0.05)) treated samples. Thymol and TSE at low concentrations of 5, 10, 20, and 40 μM did not increase Cai^{2+} (Figure 4).

Effects of Thymol and *Thymbra spicata* extract on NOX_2 protein expression

The apoptotic capacity of Thymol and TSE was performed by the NOX_2 expression. Notably, a NOX_2 expression increase has been observed in Thymol and TSE at high concentrations (Figure 5).

Effects of Thymol and *Thymbra spicata* extract on Bax/Bcl-2 ratio expression

Finally, the apoptotic capacity of Thymol and TSE was performed by the whole-mount immunofluorescence of Bax/Bcl-2 ratio expression by western blotting analyses. Notably, it was observed a decrease in the expression of the anti-apoptotic (Bcl-2) in line with the Bax expression increasing at high concentrations Thymol and TSE exposed (180 (171 and 192 vs. 100, control, **P<0.001), 120 (153 and 157 vs. 100, **P<0.001), and 80 μM (135 and 139 vs. 100, **P<0.001)) (Figure 6).

Discussion

In this study, Thymol present in TSE was induced cell death due to activation of NOX_2, ROS generation, increase in Cai^{2+}, and Bax/$Bcl\text{-}_2$ ratio. It found that high concentrations of TSE and Thymol in it caused cell death in lung cancer cells. There are some compounds in TSE, among them Thymol was 18.64 mg/g. Previously, It has been shown that 55.35% of thyme extract was Thymol [20]. But another study documented that the amount of Thymol in *Thymbra spicata* extract was varying from 0.4-37.5%. Studies showed that agronomic practices under cultivation

conditions, environmental, and genetic had effects on essential oils, chemical compositions, and agronomic characteristics of *Thymbra spicata* [21].

In this study, high concentrations (80, 120, and 180 μM) of Thymol and TSE increased the rate of cell death in a concentration-dependent manner. LC_{50} values of Thymol and TSE were 111±4.5 and 119±5.2 μM, respectively. But, cell death was not observed with low concentrations (40, 20, 10, and 5 μM) of Thymol and TSE. In one study, the LD_{50} value of Thymol for evaluating the human malignant tumor cells was 18.9 μM [22]. Also, it was shown that LC50 of Thymol for different cell lines such as T24 and SW280 were 90.1 ± 7.6 and 108.6 ± 11.3 μM, respectively [23]. In another study, it was shown that the LD_{50} value for *Thymbra spicata* extract was 109±4.3 μM [24].

As reported previously, apoptosis was induced by ROS production in various types of cancer cells [25]. An important source of reactive oxygen species is nicotinamide adenine dinucleotide phosphate (NADPH) Oxidases (NOXs) [26]. Evidence suggests the role of ROS-related signaling in the survival, proliferation, and other phenotypic behaviors of cancer cells [27]. Results of some studies showed that compounds such as aloin inhibit the proliferation and migration of gastric cancer cells by NOX2–ROS-mediated activation of Akt/mTOR, Stat3, and NF-κB signaling pathways [28]. NOX_2, a respiratory burst oxidase, generates $O_2^{\bullet-}$ in activated neutrophils [29] and is a promising therapeutic target for oxidative stress-related diseases [30]. Our data suggested that high concentrations (80, 120, and 180 μM) of Thymol and TSE caused an increase in the generation of NOX_2 and ROS as compared to untreated cells during apoptosis. Incubation of cells with low concentrations (40, 20, 10, and 5 μM) of Thymol and TSE prevented the production of NOX_2 and ROS from increasing. An investigation was performed on Korean red ginseng to see if it exerted an anti-inflammatory effect on A549 cells via the suppression of

the generation of ROS and downregulation of NF-kB activation [31]. Moreover, another study, showed that autophagic cell death was induced by Derron through intracellular ROS and stable ERK phosphorylation in A549 cells [32]. In one study, thymoquinone generated cellular ROS, including hydrogen peroxide and superoxide radicals, particularly at a higher concentration (25 μM). Reactive oxygen species scavenger (NAC (N-acetyl-cysteine)) treatment inhibited thymoquinone-induced Caki-1 cell death, implying cancer cell death through its pro-oxidant property. The main sources of cellular reactive oxygen species comprise the mitochondrial respiratory chain component, XO (xanthine oxidase), and NOXs [33]. Data indicated the efficacy and safety profile of terpenoids of *Celastrus orbiculatus***,** a multi-terpenoid-based remedy against PMA-induced NETosis via NOX signaling pathway, which provided initial evidence for *Celastrus orbiculatus* as an anti-inflammatory medication [34].

Increased intracellular calcium (Cai^{2+}) promotes cell death in many different types of cells, including lung cells [35]. Our data indicated that Cai^{2+} significantly increased for samples treated with Thymol and TSE (180, 120, and 80 μM), but low concentrations of 5, 10, 20 and 40 μM did not increase Cai^{2+}. The apoptotic effect of Thymol induced a [Cai^{2+}] rise in a concentration-dependent manner in human glioblastoma, osteosarcoma, and renal tubular cells [36-38]. It was shown that there are some cytotoxic effects on lung NCI-H292 cells due to increasing Cai^{2+} induced by ursolic acid, which is a triterpene compound present in natural plants [39]. A study showed that terpenoids in *Zingiber officinale* (Ginger) caused cell death in endometrial cancer cells by increasing intracellular calcium [40]. Also, it was demonstrated that Asiatic acid, a pentacyclic triterpene isolated from the *Centella Asiatica*, was successfully tested for its apoptotic effects in HepG2 cells by an increase in intracellular calcium levels [41].

Bcl-2 proteins often form heterodimer complexes with Bax proteins, which result in the release of cytochrome c from the mitochondria and subsequent induction of cell death [42]. Hence, one of the major markers of pre-apoptosis is an increase in the ratio of Bax/Bcl-2 [43]. In this study, it has been shown that in A549 cells, high concentrations (180, 120, and 80 µM) of Thymol and TSE increased Bax/Bcl-2 ratio protein expression as compared to untreated cells during apoptosis while low concentrations (40, 20, 10 and5 µM) of Thymol and TSE did not. It was demonstrated that *Thymus Caramanica Jalas* (Lamiaceae family), which is one species of Thymus, significantly increased cell damages, activated caspase 3, and Bax/Bcl-2 ratio [44]. Also, *Origanum majorana* containing phenolic terpenoids (thymol and carvacrol) increased cell death against breast cancer cell lines by increasing the Bax/Bcl-2 ratio [45]. In another study, Thymol showed an increase in Bax protein levels with a concomitant decrease in Bcl2 protein expression in a dose-dependent manner [46]. Also, it has been shown that treatment with the *Salvia aurea* (Lamiaceae family) led to the activation of pro-apoptotic protein Bax, shifting the Bax/Bcl-2 ratio in favor of apoptosis [47].

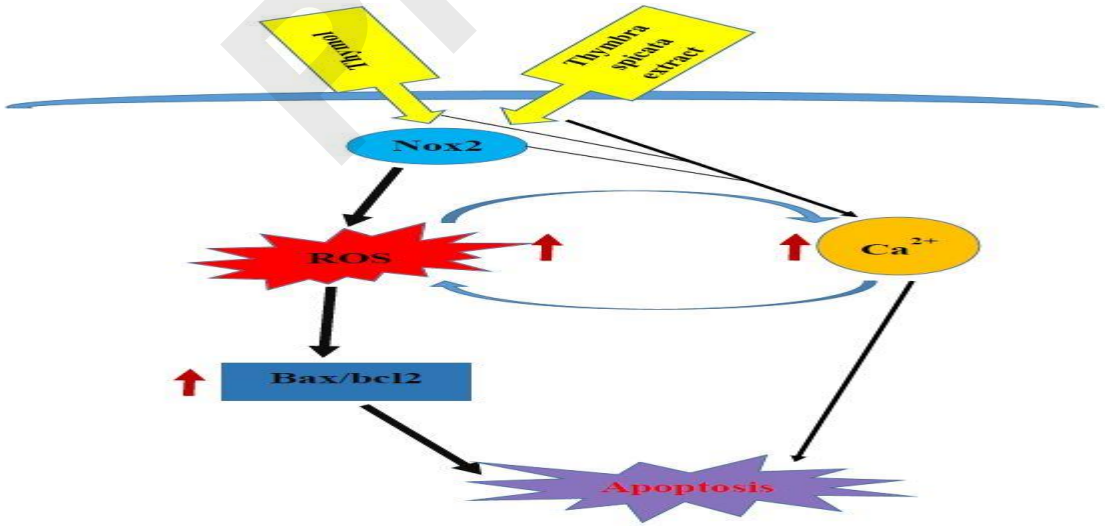

Diagram of anti-cancer action mechanism of the *Thymbra spicata* extract and Thymol (active metabolite)

Conclusions

This study focused on the cytotoxicity assay of *Thymbra spicata* extract (TSE) and its bioactive component, Thymol, on non-small-cells lung cancer A549 and its mechanism of cell death. Our results demonstrated that TSE and Thymol at high concentrations (180, 120, and 80 μM) decreased the growth of A549 cells. It appeared that cytotoxic activity was exerted through activation of NOX_2, ROS generation, increase in Cai^{2+}, and Bax/Bcl-2 ratio. Present results demonstrated that TSE and thymol may be potential therapeutic agents for human lung cancer.

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Conflict of interest

None stated.

Authors' contributions

All authors designed the study. AM: designed, took the lead in writing the manuscript, Sh M: contributed to the interpretation of the results, NA: contributed to the interpretation of the results, AA: performed the experiments, HGH: derived the models and analyzed the data, EK: took the lead in writing the manuscript. All authors have read and approved the manuscript.

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Legends of figures

Figure 1. Standard Thymol (a) and extract of *Thymbra spicata* (b) HPLC chromatogram. The states were the same for both Thymol and extract of *Thymbra spicata*.

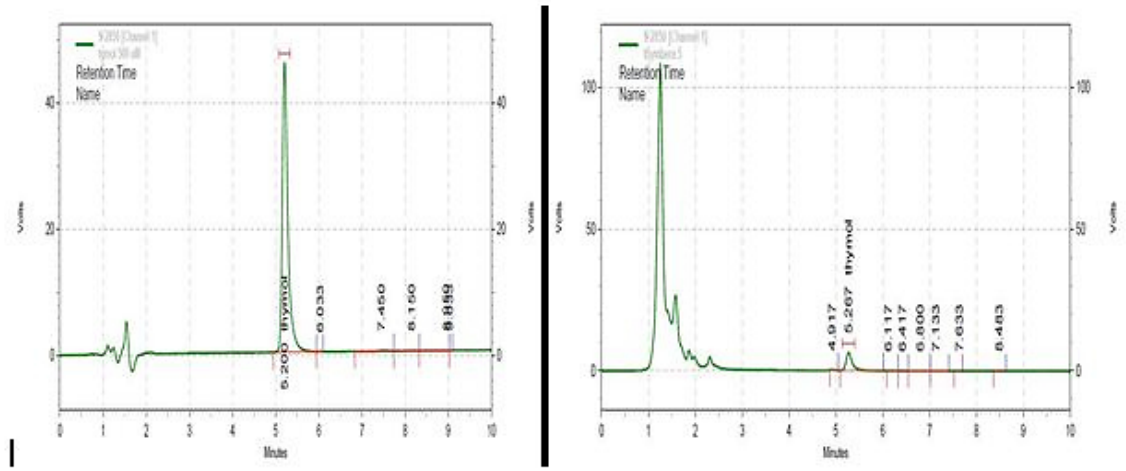
Figure 2. Thymol and TSE concentrations effects on A549 cell viability. Cells were treated with different concentrations of Thymol and TSE for 48 h, and by the MTT method, cytotoxicity was determined (*P<0.05 vs control, **P<0.001 vs control).

Figure 3. Thymol and TSE concentrations affect the ROS generation in A549 cells. DCF relative fluorescence was determined at excitation 485 nm and emission 520 nm (*P<0.05 vs control, **P<0.001 vs control).

Figure 4. Effects of different concentrations of Thymol and TSE on intracellular calcium (Cai^{2+}). (*P<0.05 vs control, **P<0.001 vs control).

Figure 5. Effect of Thymol and TSE on NOX_2 in A549 cells. NOX_2 expression (%) in exposed A549 cells to TSE (NOX E) and Thymol (NOX T). Data are shown as mean±SEM.

Figure 6. Effect of Thymol and TSE on the Bax/Bcl-2 ratio in A549 cells. Bax expression (%) in A549 cells exposed to TSE (BAX E), Thymol (BAX T), and Bcl-2 expression (%) in A549 cells exposed to TSE (Bcl-2 E), Thymol (Bcl-2 T). Data are shown as mean±SEM.



a b

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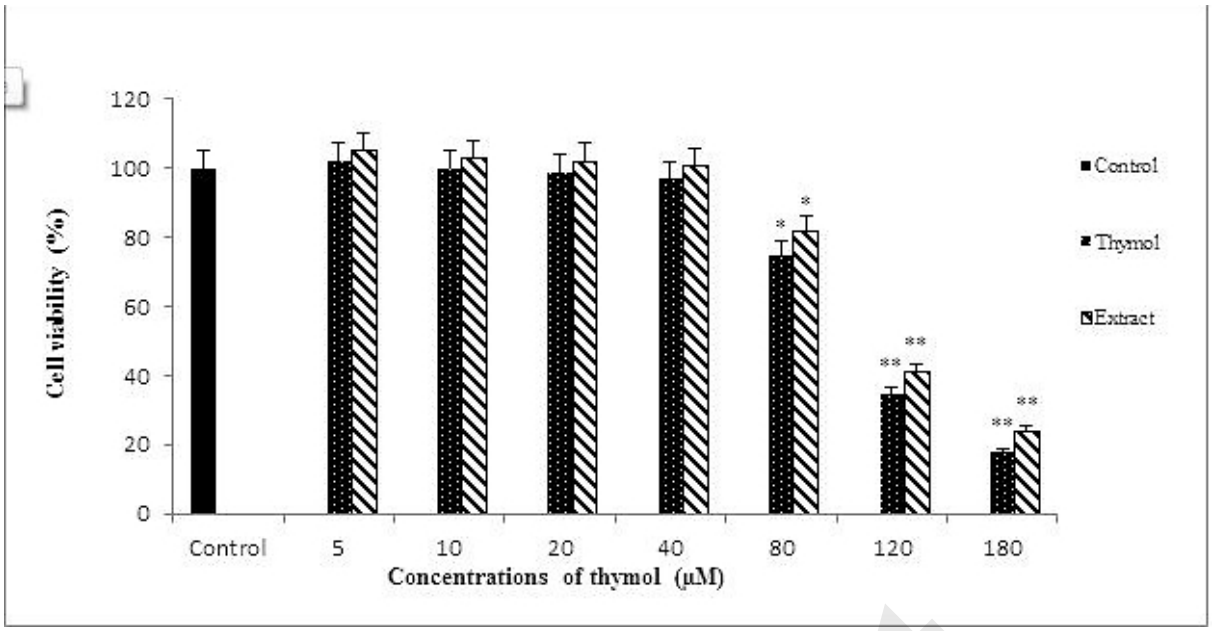

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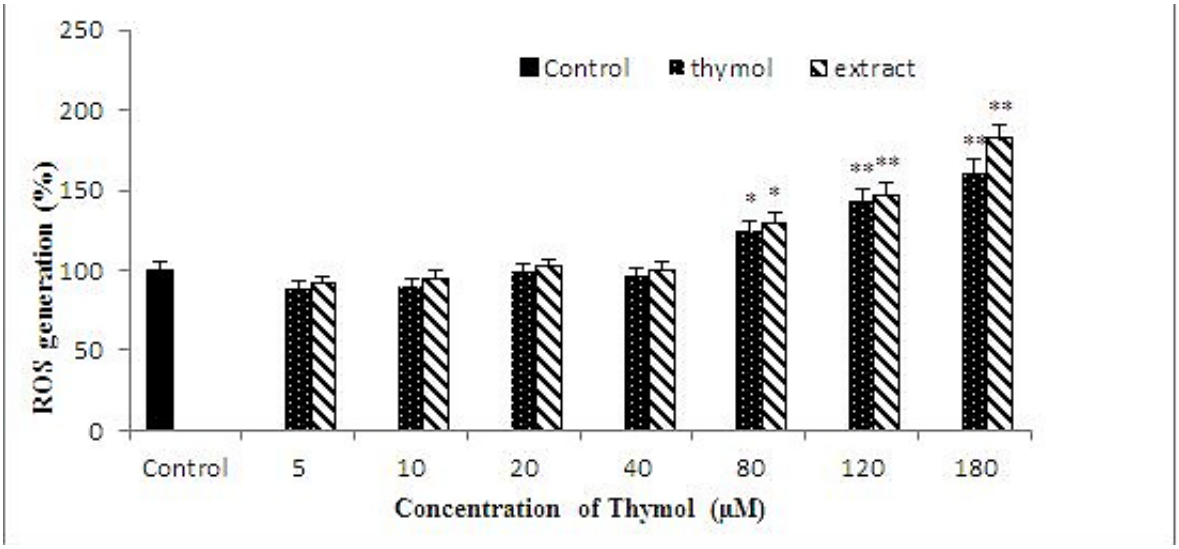

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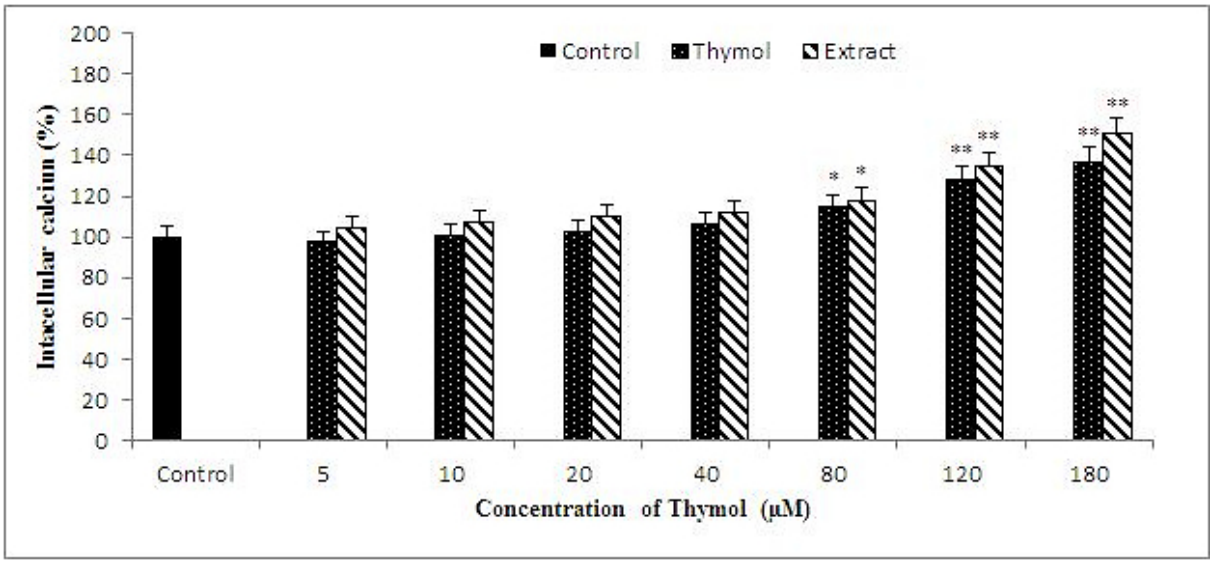

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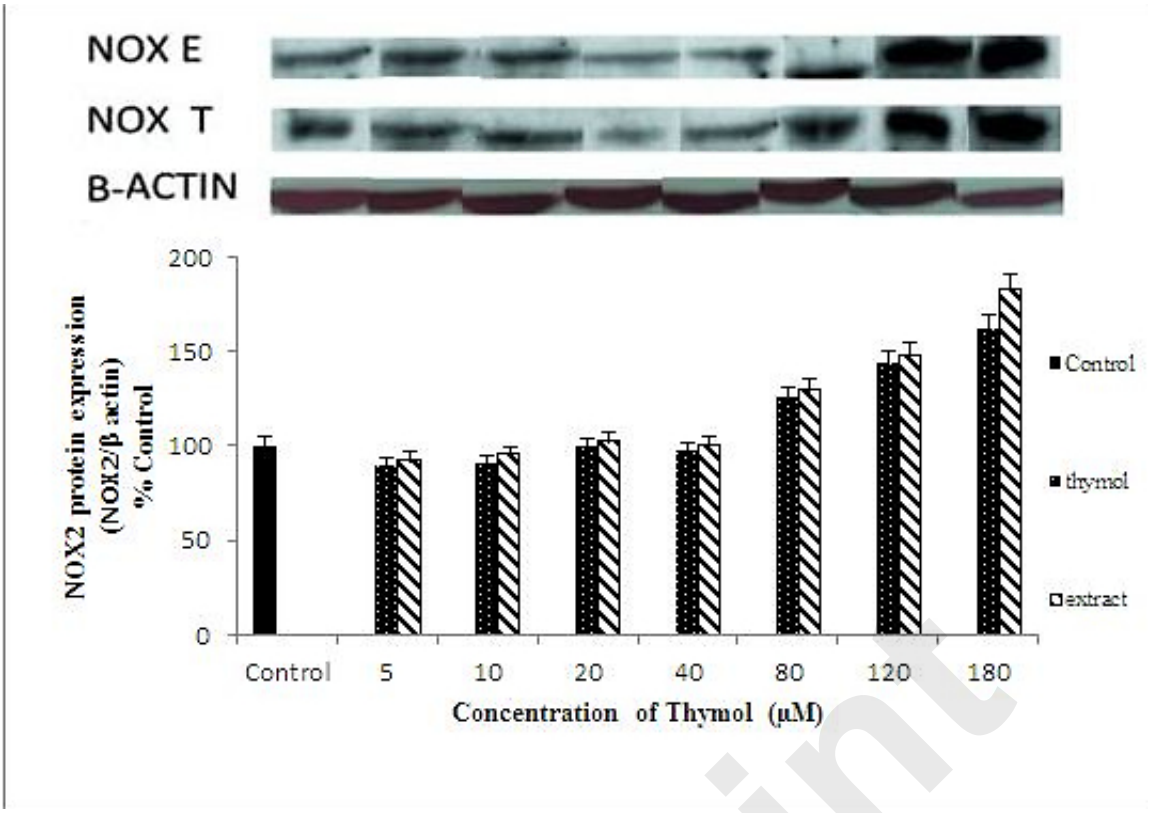

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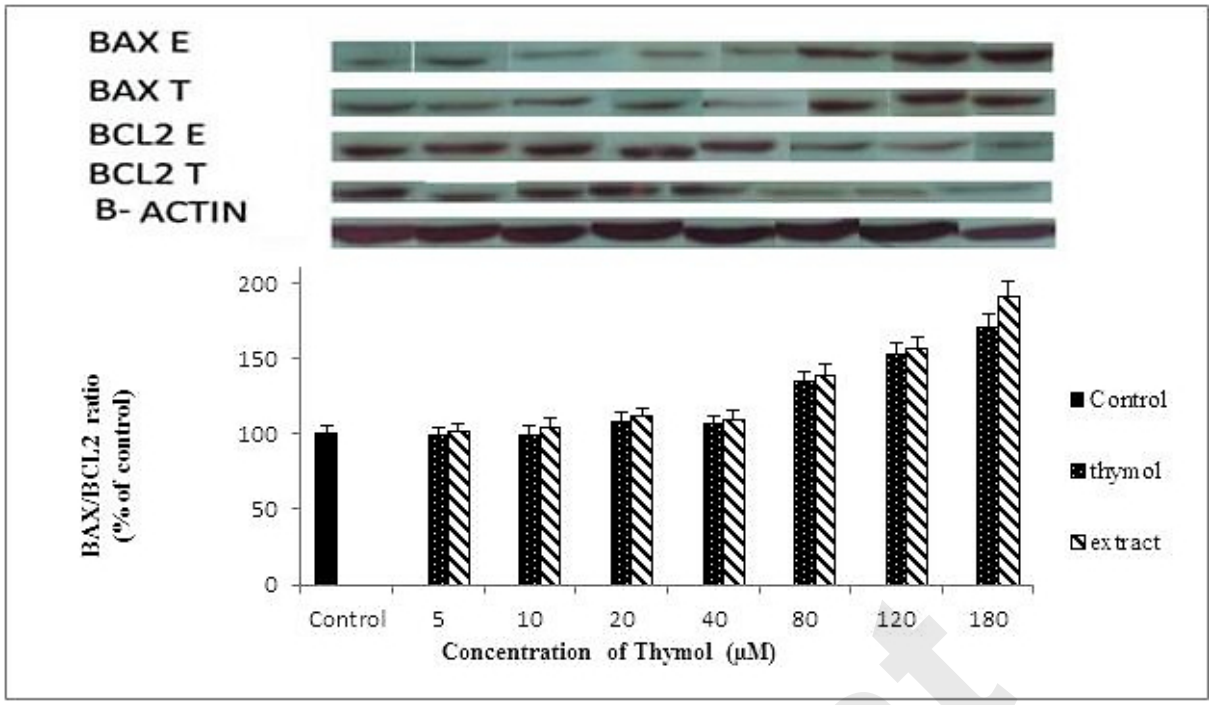

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