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In vitro Elucidation of Antiproliferative and Apoptotic Effects of Thymol against Prostate Cancer LNCaP Cells

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Abstract: Recent research suggested the role of plant-derived bioactive compounds as potent anticancer agents. Thymol, a monoterpene phenol, possesses numerous pharmacological properties such as antioxidant, anti-inflammatory, and antitumor effects. However, the inhibitory potential of thymol on prostate cancer cells still elusive. Therefore, the purpose of this study is to explore the antiproliferative and apoptotic effects of thymol against prostate cancer LNCaP cells. Our results indicated dose-dependent growth inhibitory effects of thymol on prostate cancer LNCaP cells. Morphological analysis and DAPI staining revealed that thymol induces marked morphological and nuclear alterations in LNCaP cells. Moreover, thymol could induce significant apoptosis in LNCaP cells through caspase-3 activation and modulation of mRNA expression of apoptotic-related genes. Overall, these findings showed that thymol could offer a novel therapeutic approach against prostate cancer.

Keywords: thymol; prostate cancer; anticancer; apoptosis; caspase-3.

1. Introduction

Prostate cancer is considered the most common form of malignancy in males. Various factors are considered the primary causes of increased cancer incidence rate, including race stress, age, and a high-fat diet [1]. However, Asian countries have lower incidences of cancers than Western countries due to their dietary habits, including tea, soy, fish, vegetables, and fruits [2,3]. However, adopting Western eating habits like the consumption of red meat and a high-calorie diet resulted in a rapid increase in cancer incidence in Asian countries. Effective therapeutic strategies with the least side effects and a multi-targeted approach are needed to relieve the increasing burden of cancer incidence worldwide.

Many research studies have reported that the uses of herbs, plants, and their bioactive compounds have been effective against several cancer cell lines *in vitro* and animal models via multiple pathways, ultimately leading to cell growth inhibition and cell death [4, 5]. While there are high risks related to conventional therapies used for cancer treatment, therapies using natural compounds are suggested to have lower risks due to their specific targeting effect against cancer cells. Several plant-derived bioactive compounds such as catechins, curcumin,

genistein, and rutin have anticancer activities [6, 7]. For example, various compounds like auriculacin, celastrol, and carvacrol have recently been shown to induce apoptosis and inhibit the growth of prostate cancer cells [8-10].

Thymol is a monoterpene phenol commonly found in the essential oils of numerous plants, like *Thymus vulgaris* and *Carum copticum* [11, 12]. Thymol has been reported to have several pharmacological activities, including antiseptic, antibacterial, anthelmintic, antifungal, antiviral, antioxidant, antispasmodic, anti-rheumatic, anticancer, anti-hyperglycemic, and anti-hyperlipidemic effects [13–15]. Recently, it has gained substantial attention due to its potential antitumor properties. Nevertheless, very less is known about the antitumor effects of thymol against prostate cancer. Thus, the present study was aimed to explore the antiproliferative and apoptotic effects of thymol against prostate cancer cells.

2. Materials and Methods

2.1. Chemicals and reagents.

Thymol and 4',6-diamidino-2-phenylindole (DAPI) staining dye were obtained from Sigma-Aldrich (St. Louis, MO, USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], Fetal bovine serum, RPMI (Roswell Park Memorial Institute)-1640 media, and antibiotics were procured from Himedia India, Ltd. (Mumbai, India). Caspase-3 assay kit was purchased from BioVision U.S.A. cDNA synthesis kit (Product name: Verso), and qPCR Kit (DyNAmoColorFlash SYBR Green) were procured from Thermo Scientific, USA.

2.2 Cell culture.

The human prostate cancer LNCaP cell line and normal HaCaT cells were obtained from the National center for cell science, Pune (India) and maintained in RPMI medium with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (Himedia, India).

2.3. In vitro cell viability assay.

MTT cell viability assay was carried out to examine the cytotoxic effect of thymol on prostate cancer LNCaP cells and normal HaCaT cells as described by Khan *et al.*, 2020 [16]. Approximately $5x10^3$ cells per well were seeded in 96-well plates and grown for 24h. After that, cells were exposed to various concentrations of thymol (50, 100, 150, 200, and 250 µM). Thereafter, in each well 20 µL, MTT dye (5 mg/ml in PBS) was added for 3 h at 37°C. Subsequently, formazan crystals were solubilized by DMSO (dimethyl sulfoxide) addition. The absorbance was then analyzed at 540 nm in a microplate reader to estimate percent cell viability (Bio-Rad, USA).

2.4. Assessment of morphological changes.

The growth inhibitory effect of thymol on prostate cancer LNCaP cells was evaluated by observing morphological changes in treated cells. Cells were seeded at a density of 5×10^3 cells/well in 96-well plates and allowed to grow for 24 h. After incubation, cells were treated with various concentrations of thymol (50, 100, 150, 200, and 250 µM) for 24 h, and morphological alterations were observed by fluorescence microscope (Floid cell Imaging Station, Life Technologies, USA).

2.5. Apoptosis detection by DAPI staining.

The effect of thymol on nuclear morphology related to apoptotic induction was evaluated by DAPI staining [17]. Thymol-treated LNCaP cells were washed twice with PBS, detached from the surface, and pellet down by centrifugation. Thereafter, cells were fixed with ice-cold methanol (10 min), and the cell membrane was permeabilized with Triton X -100. Finally, DAPI dye was added to LNCaP cells for 10 minutes and examined under a fluorescence microscope (Floid cell Imaging Station, Life Technologies, USA).

2.6. Estimation of caspase-3 activity.

To monitor the effects of thymol on caspase-mediated apoptosis, caspase-3 activity was assessed by Colorimetric Assay Kits (BioVision, USA). Briefly, cells (3x106) were lysed in cell lysis buffer (ice-cold) for 10 minutes, and then cell lysate was centrifuged to collect the supernatant. Further, 50 µl lysate was transferred into a 96-well plate with 50 µl of reaction buffer, comprising 10 mM DTT. Afterward, 5 µl of the 4 mM substrate of each caspase was added in each well, incubated at 37°C for 1 h, and absorbance recorded at 405 nm on a microtiter plate reader. Finally, percent caspase-3 activity was calculated by comparing the data with the control sample.

2.7. Analysis of caspase inhibitor effect on cell viability.

To illustrate the growth inhibitory effects of thymol, LNCaP cells were pre-treated with 50µM of caspase-3 inhibitor (Z-DEVD-FMK) for 2h. Subsequently, LNCaP cells were treated with various concentrations of thymol (50, 100, 150, 200, and 250 µM) for 24h, and MTT cell viability assay was carried out to elucidate the role of caspase activation underlying the antiproliferative effect of thymol.

2.8. Real-time qPCR analysis.

The RNA extraction of treated and untreated cells was done using the HiPurATM Total RNA Miniprep Purification Kit (Himedia, India). The cDNA synthesis was carried out, and the mRNA expression level of target genes was estimated using SYBR Green qPCR Kit (Thermoscientific, USA). The primer sequences targets for this study were as following: Bax;Forward: 5’-AAGAAGCTGAGCGAGTGT-3’ Reverse: 5’-GGAGGAAGTCCAATGTC-3’, Bcl-2; Forward: 5’-TCCATGTCTTTGGACAACCA-3’, Reverse: 5’-CTCCACCAGTGTTCCCATCT-3’, and GAPDH; Forward: 5'-GAAGGTCGGAGTCAACGGATTTGGT-3', Reverse Primer: 5'-CATGTGGGCCATGAGGTCCACCAC-3'. GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) gene was used as an internal control and the data evaluated by the comparative 2–ΔΔCt threshold cycle.

2.9. Statistical analysis.

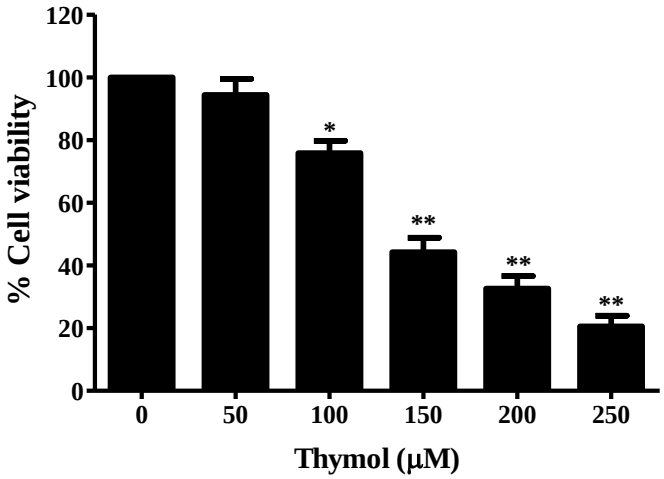
Experimental data from three independent experiments, performed in triplicate, and represented as the mean±S.E.M. Statistical analysis was done using one-way ANOVA coupled with Dunnett's *post hoc* test for multiple comparisons with control (*p<0.01, **p < 0.001 represent significant difference compared with control).

3. Results and Discussion

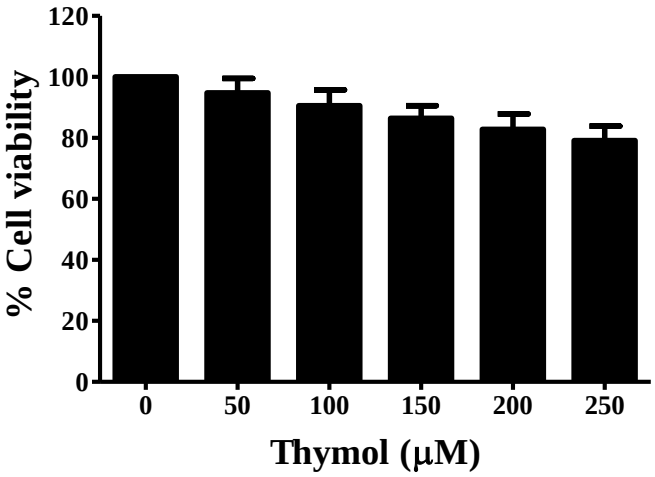
3.1. Results.

3.1.1. Thymol inhibited cell growth in LNCaP cells

Human prostate cancer LNCaP cells were treated with/without varying concentrations of thymol for 24 h, and then cell viability was estimated by MTT assay. Thymol treatment resulted in a significant reduction in the growth of human prostate cancer LNCaP cells in a dose-responsive manner. Results shown that after 24 h of thymol (50, 100, 150, 200, and 250 µM) treatment, cell viability of LNCaP cells reduced by 94.38%, 75.86%, 64.48%, 44.2%, 32.61% and 20.58%, respectively (Figure 1A). However, insignificant cytotoxic effects of thymol were observed on normal HaCaT cells were found as compared to prostate cancer cells (Figure 1B).



(A)



(B)

Figure 1. Cytotoxic effect of thymol on normal HaCaT cells and human prostate cancer LNCaP cell line assessed by MTT assay (**A**) Percent cell viability of normal HaCaT cells exposed to various concentrations of thymol (50-250 µM) for 24; (**B**) Percent cell viability of LNCaP cells exposed to various concentrations of thymol (50-250 µM) for 24. Results were represented by mean±SEM and significant differences were compared to the control (*p<0.01 and *p<0.001).

3.1.2. Thymol-induced morphological changes in LNCaP cells.

To investigate the visible effects of thymol on LNCaP cells, phase-contrast microscopic analysis was performed. As clearly evident from the figure, exposure of thymol (50-250 µM) on LNCaP prostate cancer cells exhibited significant morphological alterations in a dose-dependent manner compared to control. The morphological changes involve cell shrinkage and a decrease in cell count, which was more pronounced with increasing concentrations (Figure 2A).

3.1.3. Thymol-induced nuclear condensation in LNCaP cells.

Analysis of preliminary events of apoptotic induction in thymol treated cells, DAPI staining was done. After treatment with thymol (100, 150, and 200 µM) for 24 h, marked nuclear changes were observed in LNCaP cells. Results of fluorescence micrographs clearly suggested that thymol induced nuclear condensation and fragmentation in a dose-dependent manner, while normal cells displayed normal cell morphology (Figure 2B).

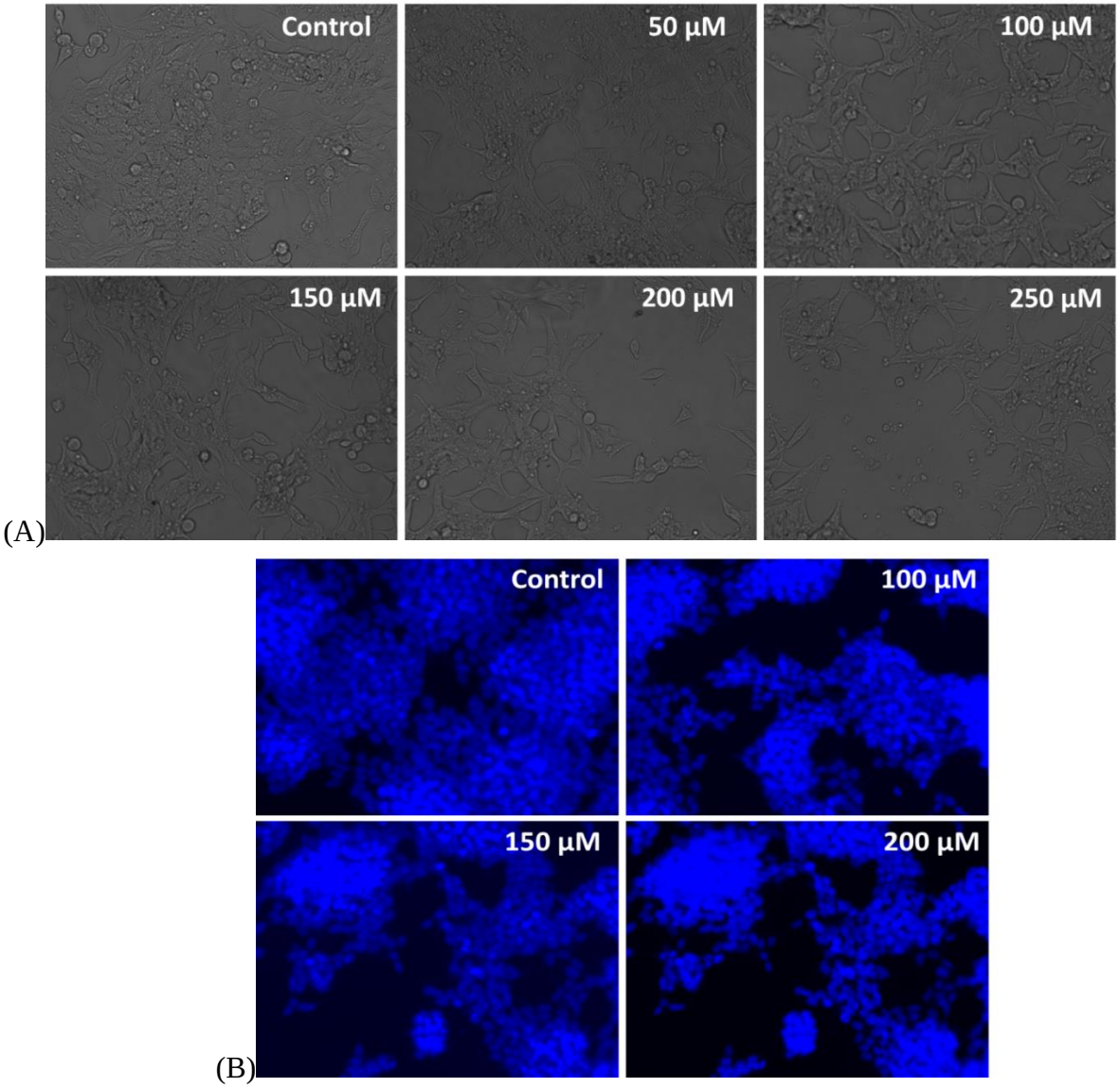
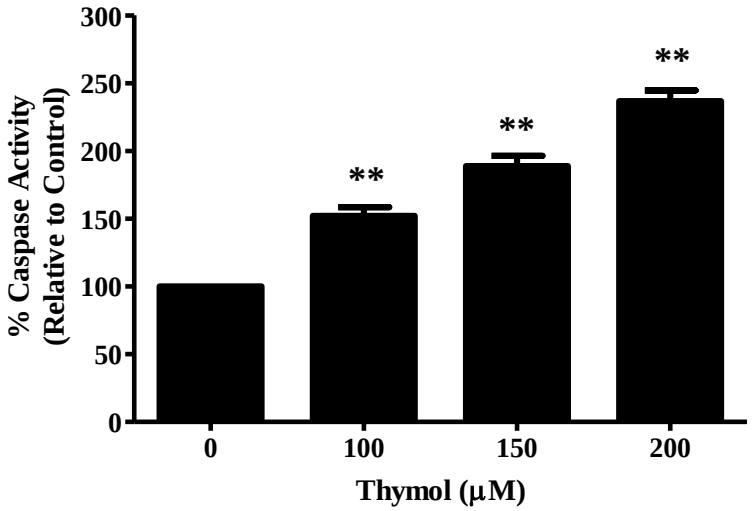


Figure 2. (**A**) Phase-contrast photomicrograph of LNCaP cells exposed with various doses of thymol (50-250 µM) for 24 h; (**B**) Thymol induced nuclear morphological changes as evaluated by DAPI-stained nuclei of LNCaP cells treated with different concentrations of thymol (100, 150, 200 µM) for 24 h.

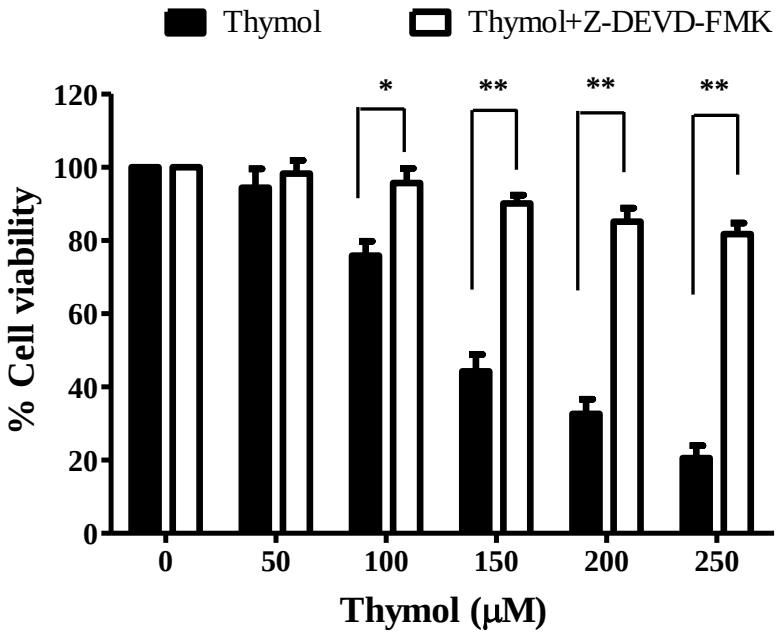
3.1.4. Thymol induced caspase-3 activity in LNCaP cells.

To understand the role of caspase activation in apoptosis induction, we determined caspase-3 activity in the treated and untreated control. Results revealed significant caspase-3 activation in LNCaP cells after treatment with various concentrations of thymol for 24 h (Figure 3A). Caspase-3 activity was markedly increased as compared to control by 52.03%, 88.87%, and 136.79% at a concentration of 100, 150, and 200 µM of thymol, respectively.

Additionally, to further confirm whether caspase activation played a role in thymol-induced cytotoxicity in LNCaP cells, a cell viability assay was performed after pre-treatment with a caspase inhibitor. The results suggested that caspase inhibitor pre-treatment resulted in significant attenuation in the thymol-induced cytotoxicity in prostate cancer LNCaP cells (Figure 3B).



(A)

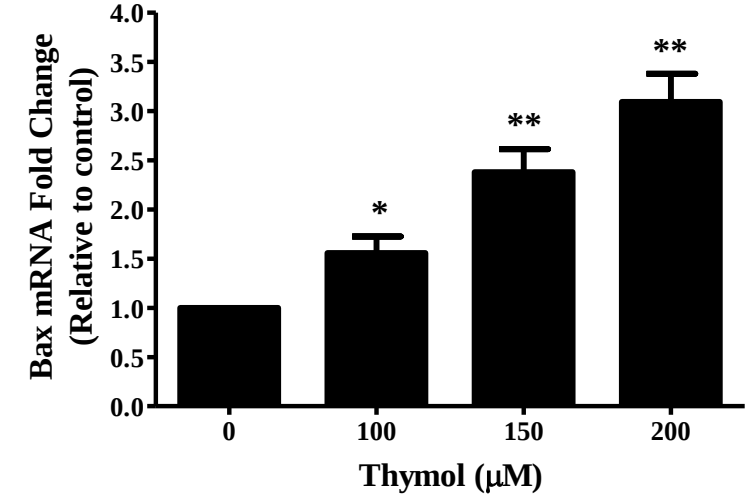


(B)

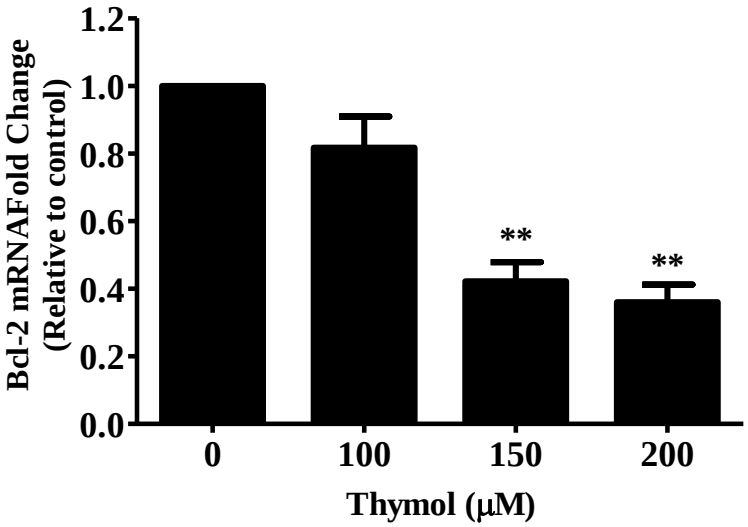
Figure 3. Dose-dependent apoptosis induction via caspase-3 activation in thymol-treated LNCaP cells (**A**) Percent caspase-3 activation in LNCaP cells exposed with various concentrations of thymol (100, 150, and 250 µM) for 24 h; (**B**) Percent cell viability of LNCaP cells pre-treated with Z-DEVD-FMK (caspase-3 inhibitor) and then exposed with various doses of thymol (0-250 µM) for 24 h evaluated by MTT assay. Results were represented by mean±SEM, and significant differences were compared to the control (*$p<0.01$ and *$p<0.001$).

3.1.5. Thymol modulates mRNA expression of apoptosis-associated genes.

To explore the antiproliferative mechanism associated with apoptotic induction in the thymol-treated LNCaP cells, RT-qPCR evaluated the mRNA expression of Bcl-2 family proteins. Results demonstrated that thymol treatment significantly elevated Bax mRNA expression level in prostate cancer LNCaP cells (Figure 4A). Moreover, the mRNA expression of the anti-apoptotic Bcl-2 gene was decreased in a concentration-dependent manner after thymol treatment (Figure 4B).



(A)



(B)

Figure 4. Effect of thymol on modulation of apoptotic related Bcl-2 mRNA expression in human prostate cancer LNCaP cells. (**A**) Upregulated mRNA expression of pro-apoptotic Bax gene; (**B**) Downregulated mRNA expression of anti-apoptotic Bcl-2 gene in thymol-treated LNCaP cells examined by quantitative real-time PCR. Results were represented by mean±SEM, and significant differences were compared to the control (*p<0.01 and *p<0.001).

3.2. Discussion.

Natural phytocompounds have already gained increasing consideration in cancer therapeutics due to their biologically responsive nature and low toxicity in normal cells [18-21]. One of such phenolic monoterpene is thymol which is mostly found in thyme species plants. It is one of the major constituents of thyme essential oil [23]. Essential oil containing thymol has long been used in folk medicine as antiviral, expectorant, antiseptic agents,

antibacterial, anti-inflammatory, and the management of the upper respiratory system [24-28]. The current research focused on thyme essential oil and thyme for finding novel biologicals or therapeutic compounds as natural plant substances. However, few studies are still reported that evaluate thymol's anticancer potential against various types of cancer. Based on these shreds of evidence, we demonstrated the growth inhibitory and apoptotic inducing effects of thymol in prostate cancer LNCaP cells. As shown in Figure 1A, thymol treatment can inhibit prostate cancer cell growth, while insignificant cytotoxicity was seen in normal HaCaT cells, as detected by the MTT cell viability assay (Figure 1A and B). Moreover, the morphological analysis demonstrated the cell membrane disruption and cell shrinkage of prostate cancer cells after thymol treatment (Figure 2A). These results are in accordance with the data published by other researchers [29-31].

Apoptosis is a crucial event characterized by the cells' distinct cellular and morphological variations, including cellular detachment, shrinkage, nuclear fragmentation, and apoptotic body formation [32]. Our results were also per the above notion where condensed and fragmented nuclei were observed in LNCaP cells, suggesting that thymol induced apoptosis in prostate cancer cells (Figure 2B) [33].

Caspase-3 is considered one of the main apoptosis execution components, which is directly involved in the proteolytic cleavage of many target cellular proteins [34]. Thus, we examined caspase-3 activity in treated and untreated cells, and results showed that thymol treatment-induced caspase-3 activation, which further leads to apoptotic cell death (Figure 3A). While pre-treatment with caspase-3 inhibitor (Z-DEVD-FMK) markedly attenuates thymol-induced cytotoxicity in prostate cancer LNCaP cells, confirms caspase-3 activation during thymol-induced apoptosis (Figure 3B).

Several studies suggested the regulatory involvement of Bcl-2 family proteins during apoptotic induction [35, 36]. To gain insight into the molecular mechanism related to apoptosis induction by thymol, mRNA expression of the key apoptotic genes Bcl-2 and Bax was analyzed in LNCaP cells. The results clearly demonstrated that thymol upregulates Bax mRNA expression and downregulates Bcl-2 mRNA expression in a dose-dependent manner suggesting the association of Bcl-2 family proteins with apoptotic induction stimulated by thymol in LNCaP prostate cancer cells (Figure 4A and B).

4. Conclusions

Overall, it may be proposed that thymol has significant anticancer and apoptotic potential against prostate cancer LNCaP cells. Based on the above results and findings, we could suggest that thymol-mediated cell growth inhibition is related to caspase-mediated apoptotic induction in prostate cancer cells. To the best part of my knowledge, this study is the first to illustrate the growth inhibitory and apoptotic inducing effects of thymol in human prostate cancer LNCaP cells. More studies are still needed, including *in vivo* animal and clinical studies, to verify further whether thymol treatment is effective against prostate cancer and could be used as a chemotherapeutic agent against prostate cancer.

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Conflicts of Interest

The authors declare no conflict of interest.

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