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Original Research Article

Targeting chemo-resistance, inhibiting proliferation and inducing apoptosis in prostate cancer cells using *Thymus vulgaris* extract and its main monoterpene phenol (thymol): An in vitro study

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Abstract

Objective: Drug resistance remains a major challenge in prostate cancer treatment, reducing the efficacy of docetaxel. Identifying natural agents that can enhance the response to chemotherapy is of growing interest. This study aimed to evaluate the antioxidant, antiproliferative, apoptosis-inducing, and chemosensitizing effects of the aerial parts of *Thymus vulgaris* hydroalcoholic extract and its major monoterpene phenol, thymol, on prostate cancer cells.

Materials and Methods: The hydroalcoholic extract was phytochemically characterized, and its antioxidant activity was measured via DPPH and ABTS assays. Prostate cancer (LNCaP) and normal fibroblast cell lines were treated with the extract, thymol, and their combination with docetaxel. Cell viability (MTT), apoptosis (TUNEL, Annexin V/PI, and DNA fragmentation), mitochondrial membrane potential (JC-1), and cytochrome C levels were assessed. Gene expression levels of *Bax*, *Bcl-2*, *p53*, and *caspase-3* were quantified using RT-PCR. The combination index (CI) and dose reduction index (DRI) were also calculated.

Results: The extract contained polyphenols, flavonoids, alkaloids, and saponins and showed antioxidant activity. Both extract and thymol selectively reduced prostate cancer cell viability (IC_{50}: 44.6 and 16.7 µg/ml, respectively) while sparing normal fibroblasts. Apoptosis was confirmed by DNA fragmentation, increased *Bax*, *caspase-3*, and *p53* expression, loss of mitochondrial membrane potential, and elevated cytochrome C release. Combination with docetaxel resulted in synergistic cytotoxicity (CI<1) and substantial dose reduction for both agents (DRI >1).

Conclusion: *Thymus vulgaris* extract and thymol exhibit selective anticancer activity and enhance docetaxel efficacy *in vitro*. These findings highlight their potential as adjunct agents in prostate cancer therapy, although further *in vivo* validation is required.

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Introduction

Prostate cancer is one of the most prevalent malignancies among men and represents the second leading cause of cancer-related death worldwide, accounting for approximately 33% of all newly diagnosed male cancers (Khazaei and Pazhouhi 2019). Standard treatments include surgery, radiotherapy, and chemotherapy. Among chemotherapeutic agents, docetaxel is widely used in advanced prostate cancer management but its use is limited by adverse effects and the frequent development of resistance (Varaprasad et al. 2023; Rizzo et al. 2021). Overcoming docetaxel resistance and improving therapeutic efficacy remain urgent clinical needs.

Resistance to chemotherapy is often associated with evasion of apoptosis, one of the hallmarks of cancer progression (Hanahan 2022). Strategies that can reactivate apoptotic pathways in tumor cells are being intensively explored. In this context, natural compounds have gained substantial attention for their potential to induce apoptosis selectively in cancer cells (Rajabi et al. 2021).

Thymus vulgaris, commonly known as thyme, is a widely used medicinal plant belonging to the Lamiaceae family. It has been traditionally employed for its antimicrobial, antioxidant, anti-inflammatory, and anticancer properties (Hammoudi Halat et al. 2022). Its major monoterpene phenol, thymol, has demonstrated anticancer activities including induction of apoptosis and mitochondrial dysfunction, and enhancement of response to chemotherapy in several cancer models (Islam et al. 2019; Hassan et al. 2021). Previous studies have shown that thymol and thyme extracts can inhibit proliferation and induce apoptosis in breast, colorectal, and bladder cancer cells (Zeng et al. 2020; Li et al. 2017). However, only limited studies have addressed their potential in prostate cancer, and little is known about their ability to overcome docetaxel resistance. To our knowledge, no prior report has systematically compared *T. vulgaris* extract and thymol in prostate cancer cells while also evaluating their interaction with docetaxel.

Although chemo-resistant prostate cancer sublines such as DU145 or PC3 are frequently used to study drug resistance, LNCaP cells were chosen in this study because they represent an androgen-sensitive prostate cancer model, allowing us to assess early mechanisms of chemosensitization before resistance develops. Moreover, LNCaP cells provide a well-characterized system for evaluating apoptosis and response to docetaxel

Therefore, the present *in vitro* study aimed to (i) evaluate the antioxidant, antiproliferative, and apoptosis-inducing effects of the aerial parts of *T. vulgaris* hydroalcoholic extract and thymol, (ii) assess mitochondrial dysfunction and pro-apoptotic gene regulation, and (iii) investigate their potential to enhance the efficacy of docetaxel in prostate cancer LNCaP cells.

Materials and Methods

Plant material and extract preparation

Thymus vulgaris was collected from the region of Kermanshah, Iran, in June 2024, and authenticated by a botanist (Dr. Seyed Mohammad Masouni, Razi University) (voucher No: 1135 (RUHK)). The dried aerial parts were powdered, and 15 g of the powder was extracted with 150 ml of 70% ethanol (Merck, Germany) by maceration in the dark for 48 hr. The extract was filtered (Whatman No. 42), air-dried, and reconstituted in serum-free RPMI-1640 (Gibco, Germany). The solution was sterilized using a 0.22 µm syringe filter.

Phytochemical screening

Qualitative phytochemical analysis was performed to detect the presence of alkaloids (Mayer's, Wagner's, and Dragendorff's reagents), tannins (ferric chloride), terpenes/sterols (Liebermann–Burchard test), saponins (Froth test),

mucilage (ethanol precipitation), coumarins (NaOH test), polyphenols ($FeCl_3$), and flavonoids (cyanidine test) (Hayat et al. 2020).

Antioxidant activity assays

The antioxidant capacity was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging assays. Various concentrations of the extract (50–800 µg/ml) or thymol (Sigma, USA) were mixed with either DPPH or $ABTS^{\bullet+}$ solutions. Absorbance was measured at 517 nm (DPPH) and 734 nm (ABTS) using a spectrophotometer. IC_{50} values were calculated from dose-response curves (Hayat et al. 2020).

Cell culture

LNCaP prostate cancer cells (Pasteur Institute, Iran) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Germany) at 37°C in a humidified incubator with 5% CO_2. Human dermal fibroblasts (normal cells) were maintained in DMEM/F12 medium under the same conditions (Malekara et al. 2020).

Cell viability (MTT assay)

Cells were seeded into 96-well plates (15000 cells/well) and treated with various concentrations of extract, thymol, or docetaxel (Sigma, USA) for 24 hr. MTT solution (5 mg/ml) was added and incubated for 3 hr at 37°C. DMSO (100 µl) was added to dissolve formazan crystals, and absorbance was read at 570 nm using a microplate reader. Cell viability (%) was calculated as:

Cell viability (%) = (OD treated / OD control) × 100.

IC_{50} values were calculated using GraphPad Prism v6.

Combination index analysis

Combination treatments were performed using fixed ratios based on IC_{50} values (The IC_{50} values of *T. vulgaris* extract, thymol, and docetaxel were first determined using MTT assays. For combination experiments, treatments were performed at the IC_{50} concentration as well as at two concentrations above and two concentrations below the IC_{50} in a fixed-ratio design. This approach allowed evaluation of synergistic, additive, or antagonistic effects across a range of clinically relevant concentrations of the extract, thymol, and docetaxel. After 24 hr treatment, MTT assay was conducted. Combination Index (CI), Dose Reduction Index (DRI), and Fraction Affected (FA) were calculated using CompuSyn software. CI values <1 indicate synergism (Chou 2010; Pazhouhi et al. 2016).

Apoptosis assays

Apoptosis was assessed using the TUNEL assay (Elabscience, China), Annexin V-FITC/PI staining (Abcam, USA), and the diphenylamine assay for DNA fragmentation (Tanourlouee et al. 2023; Khazaei and Pazhouhi 2017; Rezazadeh et al. 2023). Fluorescence microscopy and spectrophotometry (600 nm) were used for quantification.

Mitochondrial membrane potential (ΔΨm)

After 24 hr treatment with IC_{50} concentrations, JC-1 dye (10 µM, Sigma, Germany) was added for 30 min at 37°C. Fluorescence intensity was measured using excitation/emission wavelengths of 492/520 nm (monomer) and 544/590 nm (aggregates). The 590/520 nm ratio was used to quantify ΔΨm (Malekara et al. 2020).

Cytochrome C release

Cytosolic cytochrome C levels were measured using a Cytochrome C ELISA kit (Abcam, USA) according to the manufacturer's instructions. Absorbance was measured at 450 nm (Malekara et al. 2020).

Gene expression analysis

Total RNA was extracted, and cDNA was synthesized. Expression levels of Bax, *Bcl-2*, *p53*, and *Caspase-3* were determined using real-time PCR with SYBR Green. Primers were designed using GeneRunner and verified via NCBI Primer-BLAST. PCR protocol: 50°C for 15 min, 95°C for 10 min, then 40 cycles of 95°C for 15 sec and 60°C for 45 sec (Malekara et al. 2020).

Statistical analysis

All experiments were conducted in triplicate. Results are presented as mean ± standard deviation (SD). Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test (SPSS v16.0). A p-value <0.05 was considered statistically significant.

Results

Phytochemical composition

Phytochemical screening of the hydroalcoholic extract of *T. vulgaris* revealed the presence of catechic tannins, polyphenols, flavonoids, alkaloids, and saponins. Gallic tannins, mucilage, coumarins, resin, or terpenoids were not detected (Table 1).

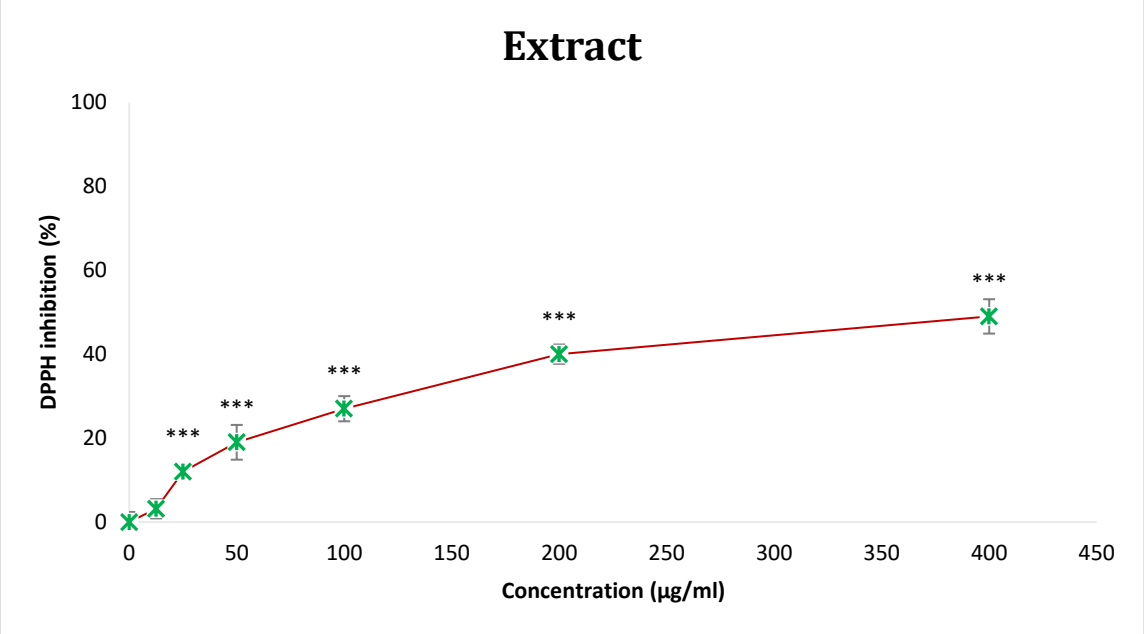
Antioxidant activity

Both the extract and thymol exhibited concentration-dependent free radical scavenging activity. The IC_{50} values for DPPH and ABTS radical inhibition were 413.48 ± 4.22 µg/ml and 375.16 ± 1.78 µg/ml for the extract, and 86.63 ± 2.83 µg/ml and 55.35 ± 3.65 µg/ml for thymol, respectively (Figure 1A and 1B).

Table1 . Phytochemical screening results of *Thymus vulgaris* hydroalcoholic extract

Chemical components									
Alkaloids	Gallic tannins	Catechic tannins	Terpenoids	Mucilage	Coumarine	Polyphenols	Resin	Saponins	Flavonoides
+	-	+	-	-	-	+	-	+	+

A



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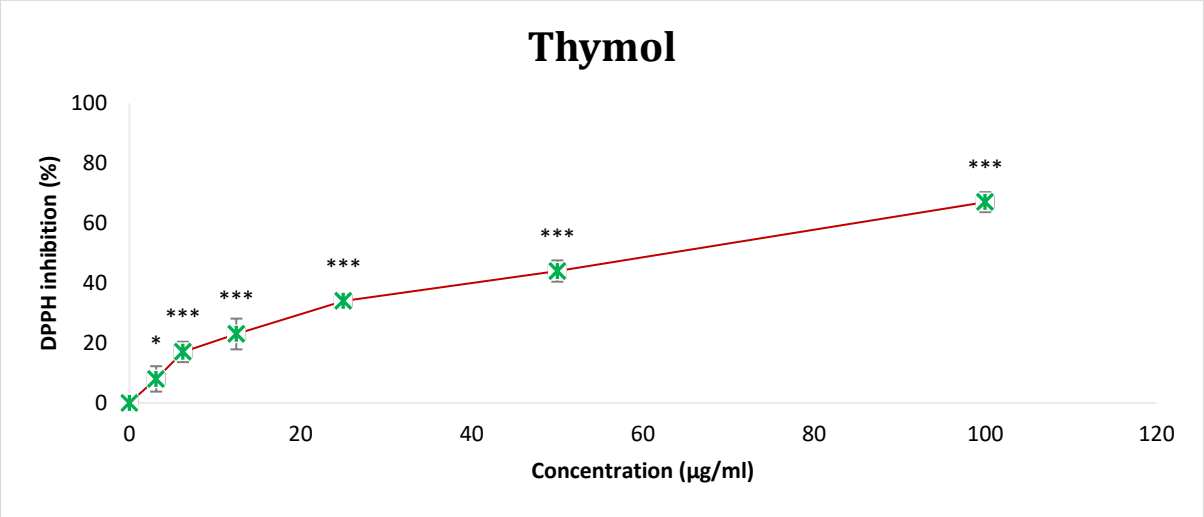

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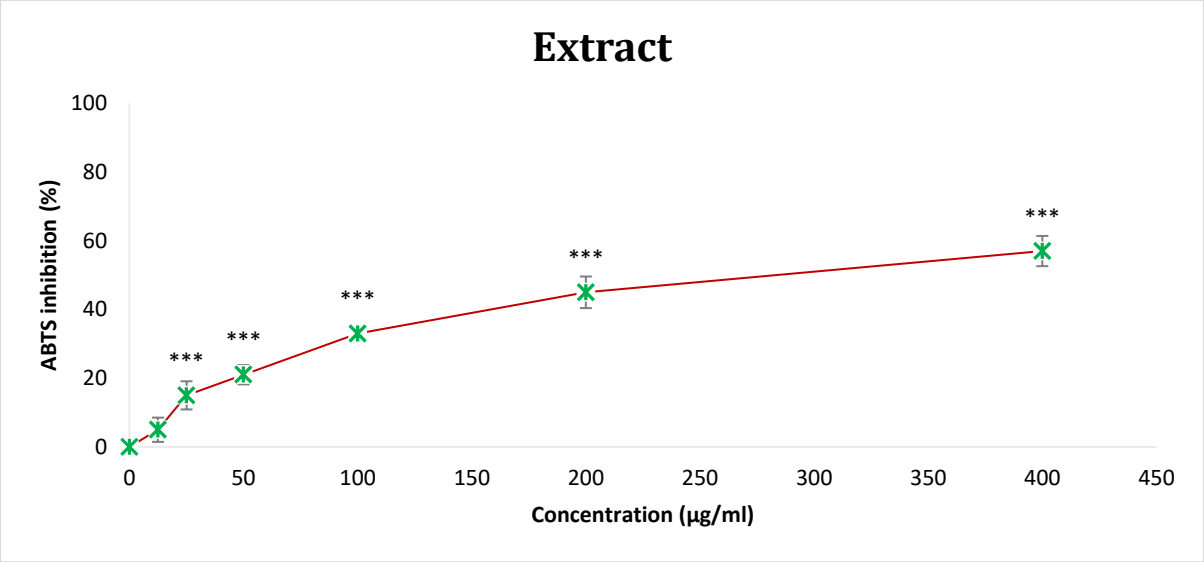

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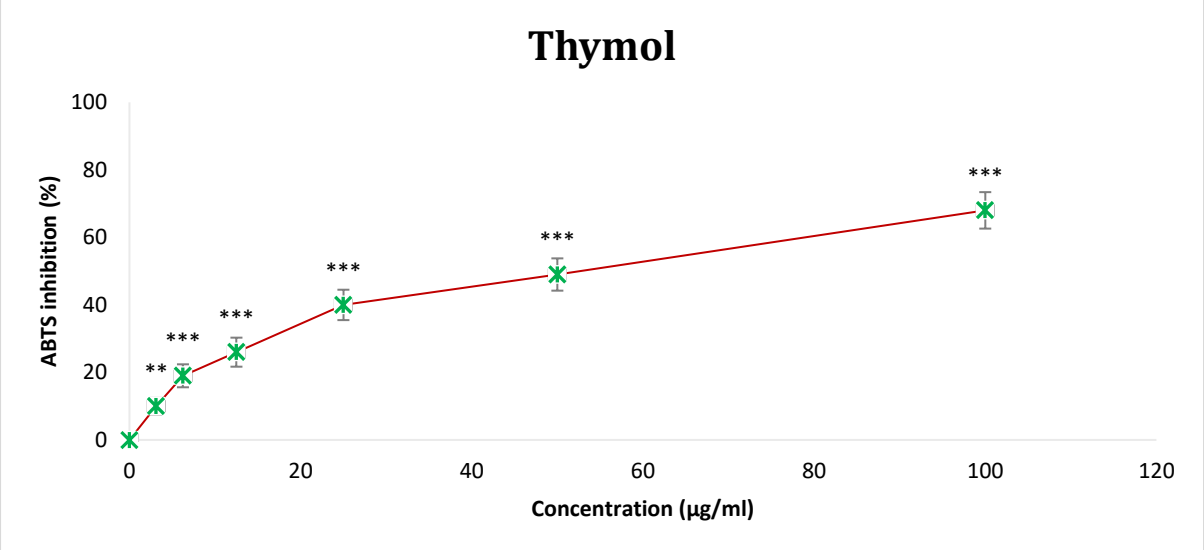


Figure 1. Antioxidant activity of Thymus vulgaris hydroalcoholic extract and thymol measured by (A) DPPH and (B) ABTS radical scavenging assays. Data are expressed as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. *p<0.05, **p<0.01, and ***p<0.001 vs. control.

Cytotoxic effects on prostate cancer and normal fibroblasts

Treatment with increasing concentrations of the extract, thymol, and docetaxel for 24 hr significantly reduced LNCaP cell viability in a dose-dependent manner ($p<0.05$) (Figure 2). The IC_{50} values were 44.60 ± 1.32 µg/ml for the extract, 16.72 ± 1.88 µg/ml for thymol, and 0.88 ± 0.28 µg/ml for docetaxel (Table 2).

In normal fibroblasts, cytotoxicity was observed only at high concentrations: 200–400 µg/ml for the extract and 50–100 µg/ml for thymol (Figure 3), with IC_{50} values of 2754.85 ± 11.79 µg/ml and 633.41 ± 9.11 µg/ml, respectively, indicating selective toxicity toward cancer cells.

Synergistic effects with docetaxel

Combination treatment of the extract or thymol with docetaxel significantly enhanced cytotoxicity in LNCaP cells. Combination Index (CI) values were <1 across most combinations, indicating synergism. Dose Reduction Index (DRI) values >1 suggested that the effective doses of both agents could be reduced when used in combination (Tables 3 and 4).

Table 2. The IC50 values of hydroalcoholic extract of *Thymus vulgaris*, thymol, and docetaxel for 24 hr treatment against LNCaP and fibroblast cell.

	Extract	**Thymol**	**Docetaxel**
LNCaP	44.60 ± 1.32 µg/ml	16.72 ± 1.88 µg/ml	0.88 ± 0.28 µg/ml
Fibroblast	2754.85 ± 11.79 µg/ml	633.41 ± 9.11 µg/ml	-
p-values	≤ 0.001	≤ 0.001	-

Table 3. Fraction affected (Fa), combination index (CI), and dose reduction index (DRI) values for the combination of *Thymus vulgaris* hydroalcoholic extract and docetaxel.

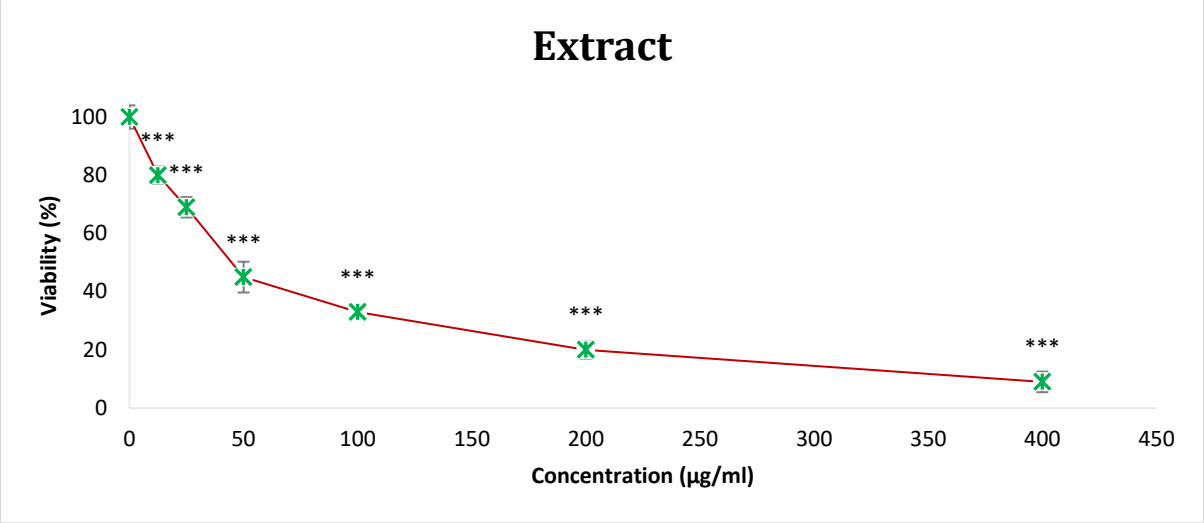
Combination treatment groups	**Fa**	**CI**	**DRI Extract**	**DRI Docetaxel**
1	0.47 ± 0.08	0.95	1.99	1.90
2	0.57 ± 0.05	0.88	2.44	3.70
3	0.68 ± 0.05	0.69	2.36	4.55
4	0.88 ± 0.09	0.42	3.12	5.98
5	0.91 ± 0.12	0.40	3.17	6.64

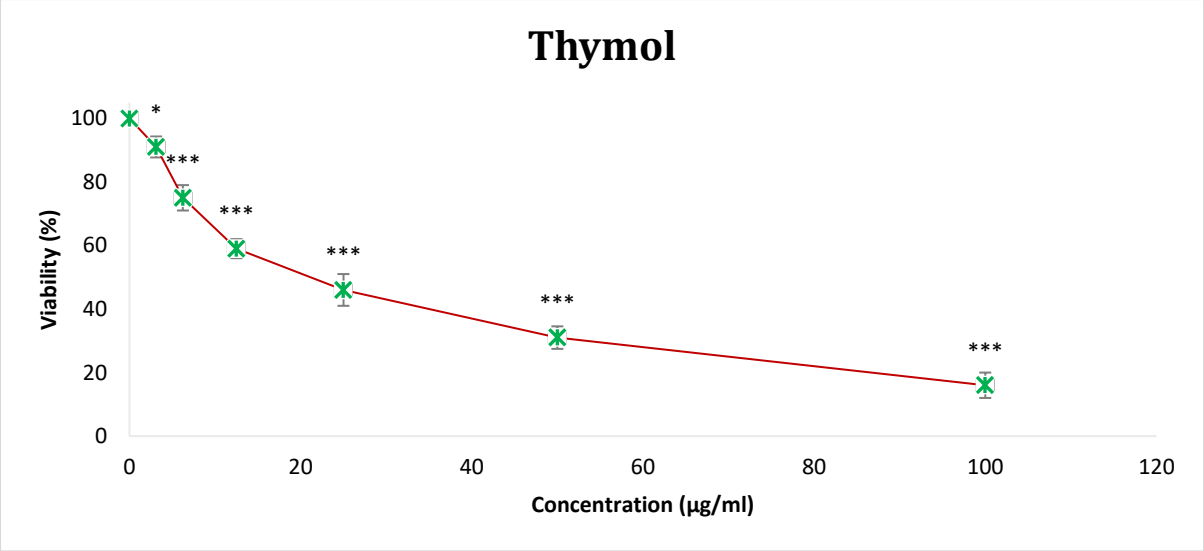
Group 1: 11.15 µg/ml of Extract + 0.22 µg/ml Docetaxel; Group 2: 22.3 µg/ml of Extract + 0.44 µg/ml Docetaxel; Group 3: 44.60 µg/ml of Extract + 0.88 µg/ml Docetaxel; Group 4: 89.2 µg/ml of Extract + 1.76 µg/ml Docetaxel; and Group 5: 178.4 µg/ml of Extract + 3.52 µg/mlDocetaxel.

Table 4. Fraction affected (Fa), combination index (CI), and dose reduction index (DRI) values for the combination of thymol and docetaxel.

Co-treatments groups	**Fa**	**CI**	**DRI Thymol**	**DRI Docetaxel**
1	0.58 ± 0.06	0.55	3.15	3.02
2	0.65 ± 0.09	0.45	3.07	3.27
3	0.82 ± 0.14	0.35	4.98	4.62
4	0.95 ± 0.05	0.40	4.33	4.99
5	0.98 ± 0.06	0.11	10.15	12.93

Group 1: 4.18 µg/ml of thymol + 0.275 nM Docetaxel; Group 2: 8.36 µg/ml of thymol + 0.55 nM Docetaxel; Group 3: 16.72 µg/ml of thymol + 1.10 nM Docetaxel; Group 4: 33.44 µg/ml of thymol + 2.20 nM Docetaxel; and Group 5: 66.88 µg/ml of thymol + 4.40 nM Docetaxel.





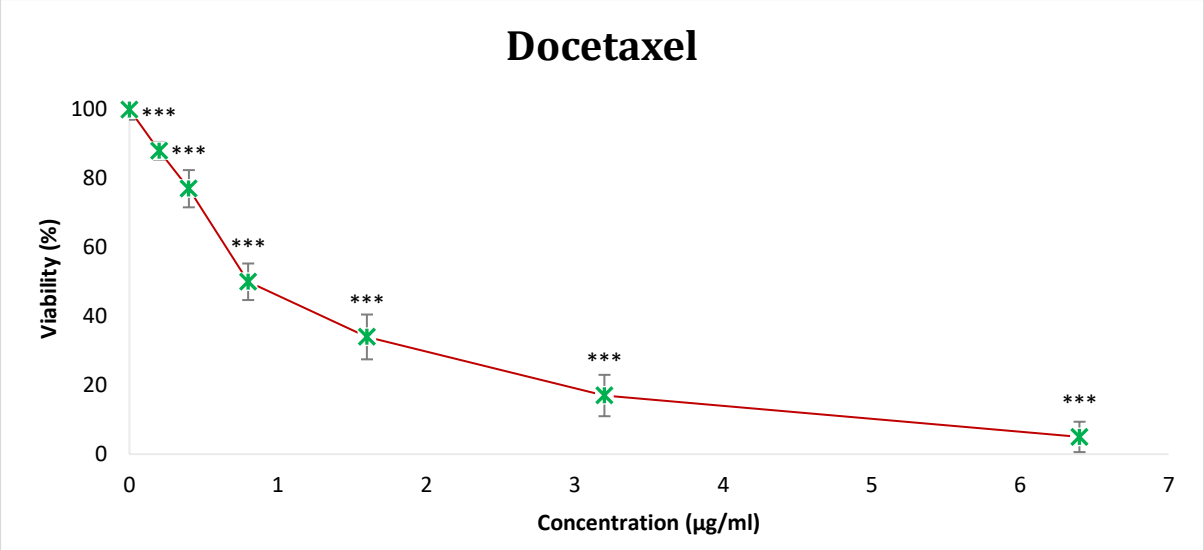


Figure 2. Effect of *Thymus vulgaris* extract, thymol, and docetaxel on the viability of LNCaP prostate cancer cells measured by MTT assay after 24 hr. Data are expressed as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. *p<0.05 and ***p<0.001 vs. control.

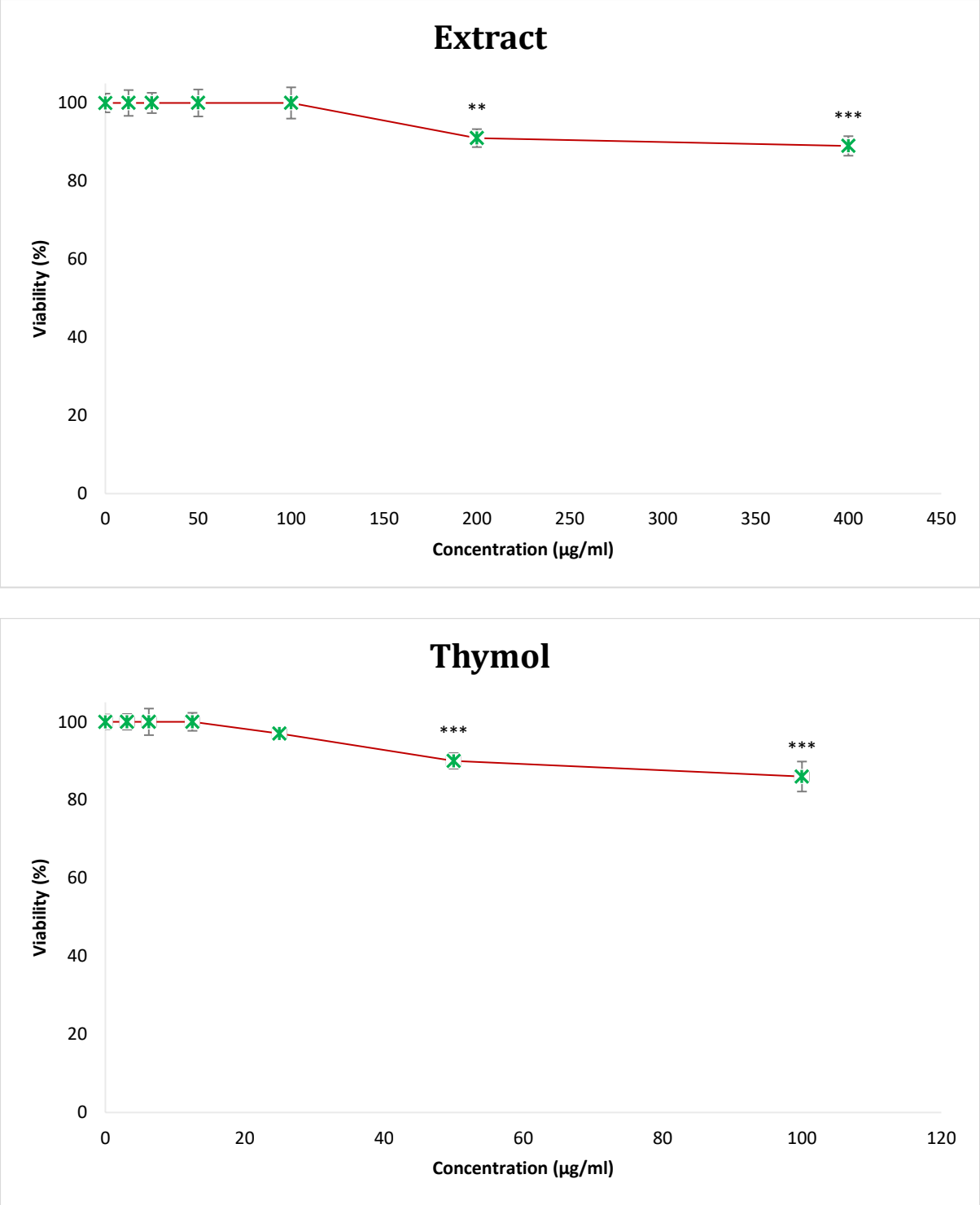


Figure 3. The effect of hydroalcoholic extract of *Thymus vulgaris* and thymol on fibroblast cell viability was measured by MTT test. Data are expressed as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. ***p<0.001 vs. control.

Induction of apoptosis

TUNEL staining revealed an increased number of apoptotic cells in extract- and thymol-treated groups compared to control (Figure 4A). Annexin V/PI staining showed a rise in early and late apoptotic cells: 24.2% (extract) and 25.65% (thymol), vs. 4.46% in control (Figure 4B). DNA fragmentation increased significantly (~11.66- to 12.03-fold) in treated groups ($p \leq 0.001$, Figure 4C).

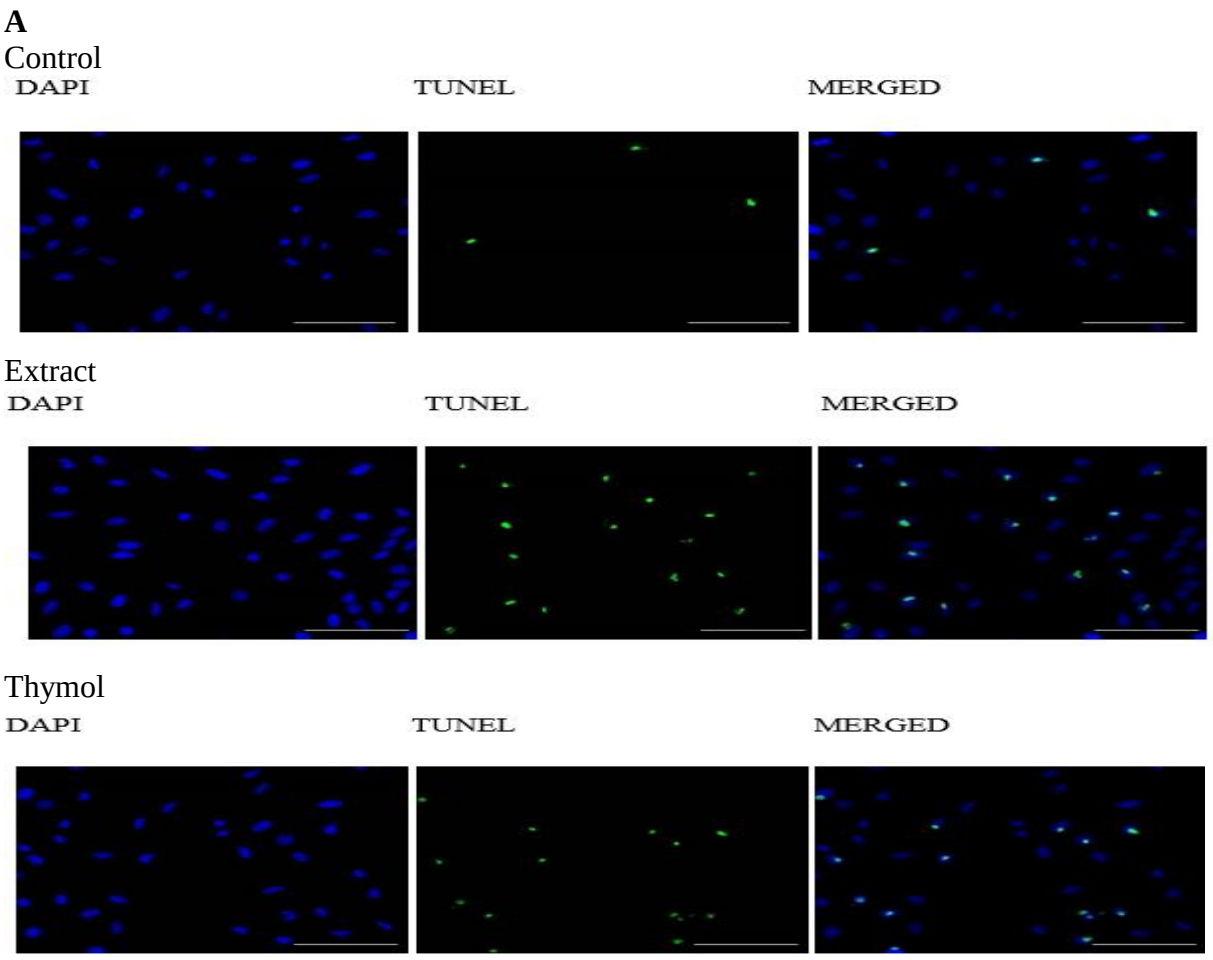


Figure 4. Apoptosis induction by *Thymus vulgaris* extract and thymol in LNCaP cells. (A) TUNEL staining (green) with DAPI counterstain (blue). Scale bar = 50 µm. (B) Annexin V/PI staining quantified by flow cytometry. (C) DNA fragmentation assay. Data are expressed as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. ***p<0.001 vs. control.

Effect on mitochondrial membrane potential and cytochrome C

JC-1 staining showed a significant decrease in mitochondrial membrane potential (ΔΨm) after treatment with extract and thymol ($p<0.05$, Figure 5A). Correspondingly, cytochrome C levels in the cytosol were significantly elevated in both groups ($p\leq0.001$, Figure 5B).

Gene expression analysis

Real-time PCR results demonstrated significant upregulation of *Bax*, *p53*, and *caspase-3* mRNA levels following treatment with the extract or thymol ($p<0.05$), with no significant change observed in *Bcl-2* expression. This indicates activation of intrinsic apoptotic pathways (Figure 6A–D).

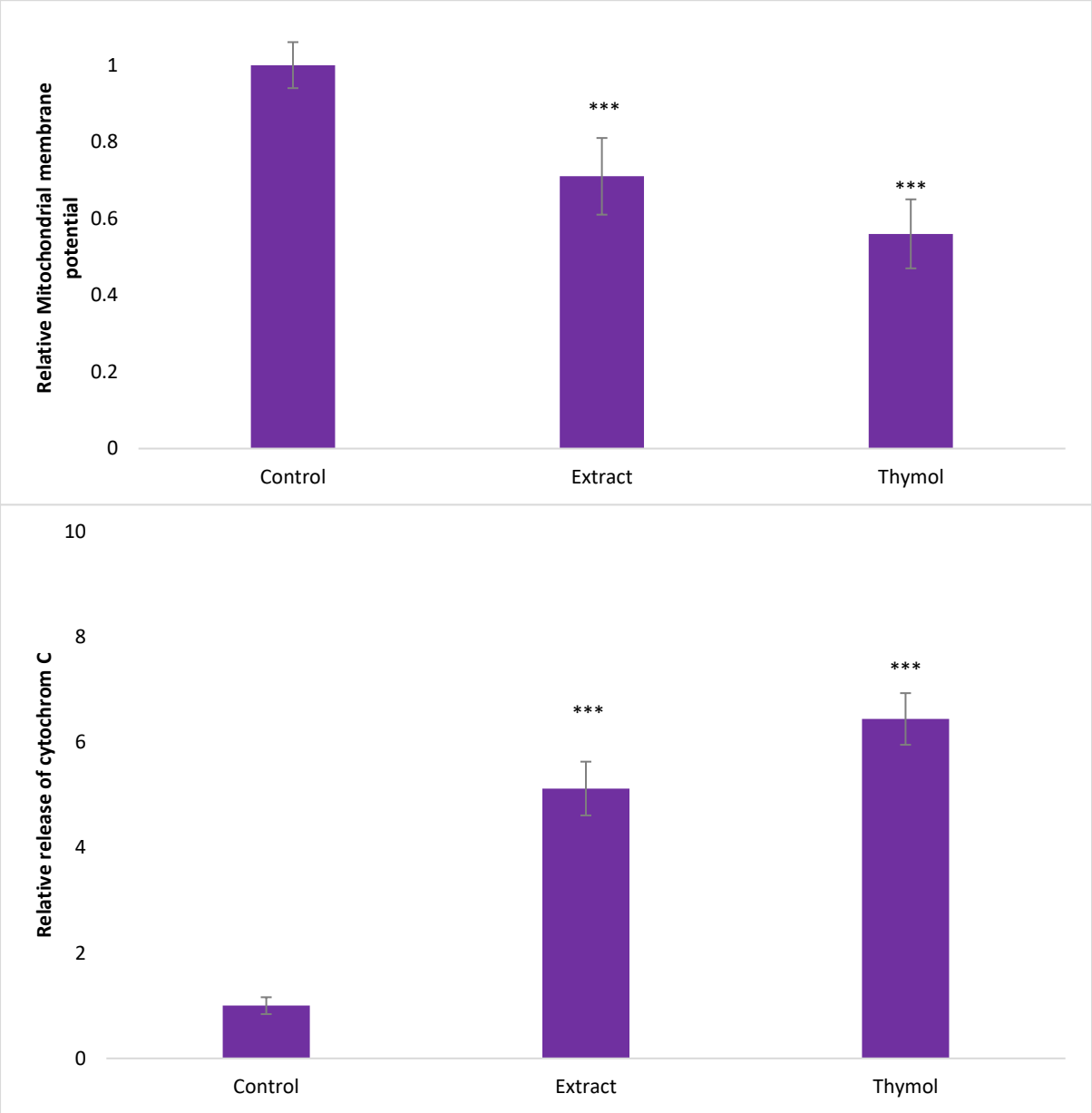


Figure 5. Effects of *Thymus vulgaris* extract and thymol on (A) mitochondrial membrane potential (ΔΨm, JC-1 assay) and (B) cytosolic cytochrome C levels (ELISA). Data are mean ± SD (n = 3). Data are expressed as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. ***$p<0.001$ vs. control.

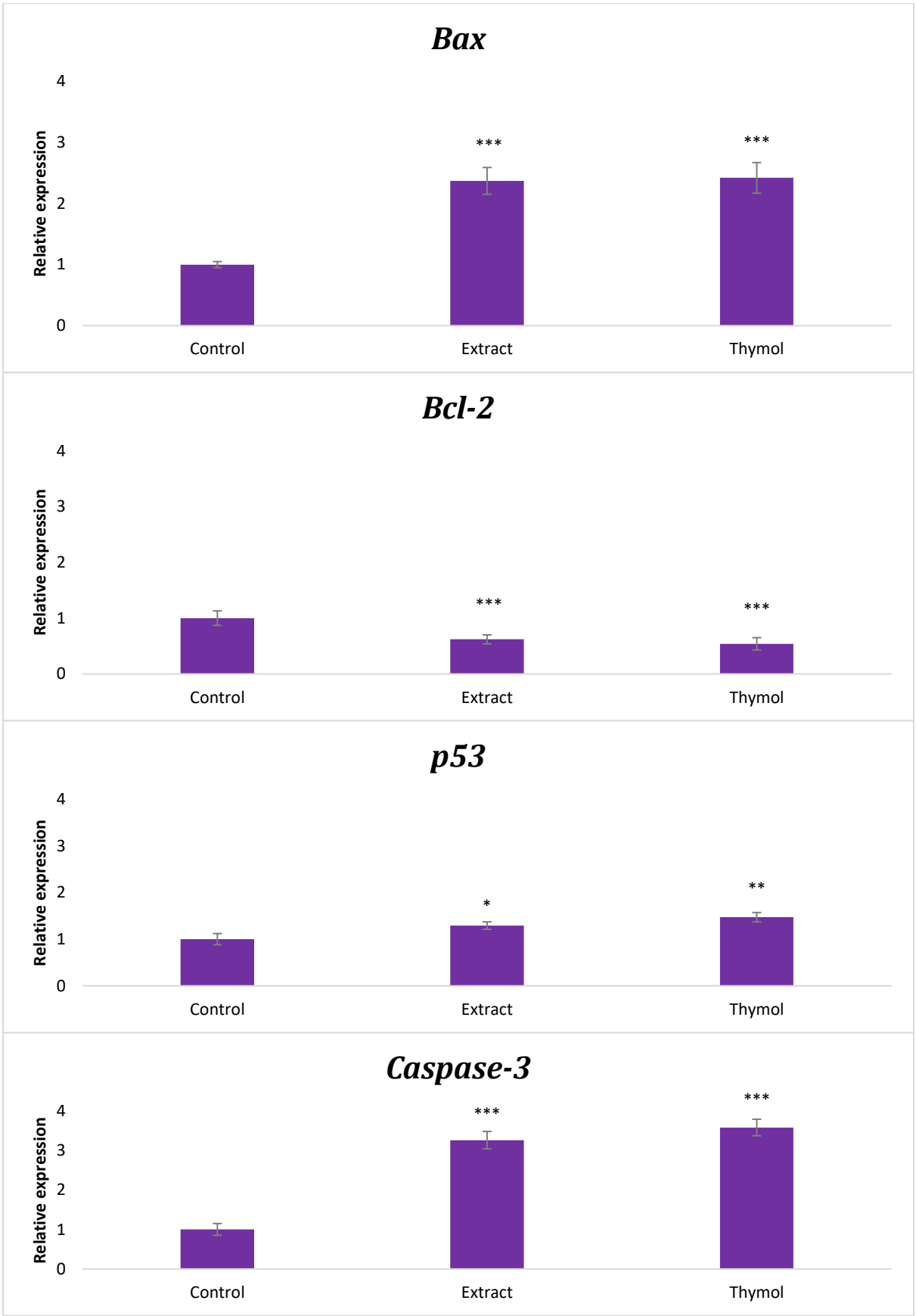


Figure 6. Relative mRNA expression of (A) *Bax*, (B) *Bcl-2*, (C) *p53*, and (D) *Caspase-3* after treatment with *Thymus vulgaris* extract and thymol. Expression levels were normalized to GAPDH. Data are expressed as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. *p<0.05, **p<0.01, and ***p<0.001 vs. control.

Discussion

Natural products are widely recognized as valuable sources of therapeutic compounds, and many modern pharmaceuticals are derived from traditional herbal remedies (Atanasov et al. 2021; Liu et al. 2014). In this study, we investigated the phytochemical composition and biological effects of the hydroalcoholic extract of *T. vulgaris* and its major active component, thymol. Our findings demonstrated their antioxidant, antiproliferative, apoptosis-inducing, and chemosensitizing properties in prostate cancer cells. Our results are consistent with previous reports on thymol pro-apoptotic effects in breast, colorectal, and bladder cancer cells (Li et al. 2017; Islam et al. 2021). However, few studies have examined these effects in prostate cancer, and to our knowledge, this is the first study to evaluate both extract and thymol in combination with docetaxel in LNCaP cells. This aspect represents the main novelty of our work.

Phytochemical analysis revealed the presence of catechic tannins, polyphenols, flavonoids, alkaloids, and saponins, while gallic tannins, mucilage, coumarins, resin, or terpenoids were absent. These results are consistent with previous findings indicating that *T. vulgaris* contains several bioactive compounds with therapeutic potential, such as p-hydroxybenzoic acid, rosmarinic acid, and kaempferol (Selim et al. 2023). The antioxidant activity observed in our study aligns with prior reports linking the antioxidant potential of *T. vulgaris* to its high phenolic and flavonoid content (Köksal et al. 2017; Abu Bakar et al. 2009; Yassin et al. 2022; Alnasser 2023).

The selective cytotoxicity of thymol and *T. vulgaris* extract toward prostate cancer cells, while sparing normal fibroblasts, underscores their therapeutic potential. Numerous phytochemicals have been shown to preferentially target cancer cells via various mechanisms (Singh et al. 2016), and our findings support the idea that thymol is among them (Elbe et al. 2020; Nagoor Meeran et al. 2017; Pakdemirli et al. 2020). In addition, our data are in line with *in vivo* studies demonstrating reduced tumor volume and mitotic index following treatment with *T. vulgaris* extracts (Al-Menhali et al. 2015; Niksic et al. 2021; Sertel et al. 2011).

Thymol has previously been reported to exert dual effects—protecting normal cells through antioxidant activity while inducing oxidative stress in cancer cells (Günes-Bayir et al. 2020; Aydın et al. 2017). Studies have shown that thymol reduces tumor markers, arrests the cell cycle at G2/M, and promotes intrinsic apoptosis via cytochrome C release and caspase activation (Hassan et al. 2021; Li et al. 2017). The minimal cytotoxic effects noted in normal cells during our research enhance its therapeutic index.

Importantly, our combination studies revealed a synergistic interaction between thymol or *T. vulgaris* extract and docetaxel. This synergy, reflected by CI values below 1 and favorable DRI values, suggests that these natural agents can potentiate chemotherapeutic efficacy while possibly allowing dose reduction—addressing a major challenge in overcoming drug resistance and minimizing systemic toxicity (Lin et al. 2020). The synergistic effects observed in combination with docetaxel highlight the potential role of natural agents as chemosensitizers. Such combinations could provide dual benefits: enhancing efficacy against resistant cells and allowing lower doses of chemotherapy, thus reducing adverse effects.

Mechanistically, we observed significant loss of mitochondrial membrane potential and increased cytosolic cytochrome C in treated prostate cancer cells, indicating activation of the mitochondrial apoptotic pathway. The depolarization of $\Delta\Psi m$ is a key event in apoptosis initiation, leading to caspase cascade activation and cell death (Lim et al. 2019; Forresto 2015; Bagkos et al. 2014; Jiang and Wang 2004; Wang and Youle 2009).

Additionally, both treatments upregulated *Bax*, *p53*, and *caspase-3*, while *Bcl-2* expression remained unchanged. The *Bax/Bcl-2* ratio is widely considered a determinant of mitochondrial outer membrane permeabilization and apoptosis initiation (H N et al. 2020). The elevated expression of *p53*, a key tumor suppressor, further suggests transcriptional activation of pro-apoptotic genes and suppression of cell survival mechanisms. One interesting finding was that *Bcl-2* expression remained unchanged, despite marked upregulation of *Bax*, *caspase-3*, and *p53*. The absence of Bcl-2 downregulation may suggest that apoptosis induction in our model was driven primarily by pro-apoptotic signaling (*Bax/p53*) rather than suppression of anti-apoptotic pathways. This is in line with evidence exhibiting that apoptosis can be triggered by *Bax* activation and mitochondrial membrane depolarization even in the absence of significant changes in *Bcl-2* (Hanahan 2022).

Another notable observation was the imbalance between *p53* (~1.5-fold increase) and *caspase-3* (~3.5-fold increase). While *p53* is a central regulator of apoptosis, *caspase-3* can also be activated through *p53*-independent mechanisms, including reactive oxygen species (ROS) generation and mitochondrial cytochrome C release (Wang and Youle 2009). Therefore, our results suggest that thymol and *T. vulgaris* extract may activate apoptosis via multiple converging pathways.

These findings are consistent with earlier research exhibiting that thymol induces apoptosis through mitochondrial disruption, ROS generation, activation of caspases, and inhibition of angiogenesis and tumor-promoting pathways (Elbe et al. 2020; Nagoor Meeran et al. 2017; Ahmad et al. 2021; Islam et al. 2021).

The present study was limited to *in vitro* experiments, which may not fully capture the complexity of drug resistance and tumor microenvironment *in vivo*. Extract standardization was also limited, as the precise thymol content of the crude extract was not quantified. Furthermore, we did not investigate other potential molecular targets or pathways that may contribute to the observed effects. Future *in vivo* and clinical studies are required to validate these findings and establish their translational relevance.

In summary, the hydroalcoholic extract of *T. vulgaris* and its primary active compound, thymol, demonstrated potent antiproliferative, pro-apoptotic, and chemosensitizing effects in prostate cancer cells. Their selective cytotoxicity, mitochondrial-mediated apoptosis induction, and synergistic enhancement of docetaxel activity highlight their potential as adjunct therapies. These findings provide a foundation for future preclinical and clinical studies aimed at validating their efficacy and safety *in vivo*.

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Conflicts of interest

The authors declare no conflict of interest for this study.

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Code of Ethics

The study was approved by the Ethical Committee of Kermanshah University of Medical Sciences, Kermanshah, Iran (code: IR.KUMS.MED.REC.1403.054).

Authors' Contributions

Study concept and design: C. J.; Acquisition and analysis and interpretation of data: M. P.; Drafting of the manuscript: A. J. S.; Critical revision of the manuscript for important intellectual content: Z. B.

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