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Research Article

Teucrium persicum Methanolic Extract Alters Cellular Homeostasis and Regulates the Expression of *MMP-2*, *MMP-9*, *TP53*, and *β-catenin* Genes in PC-3 Cells

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Abstract

Reactive oxygen species (ROS) are highly reactive molecules that play a key role in cellular physiology.in *Teucrium persicum*, an Iranian endemic plant native to the southern regions of Iran, particularly the Fars province. It has long been used in traditional medicine for the treatment of headaches, abdominal pain, diabetes, inflammation, and hyperlipidemia. Our previous findings demonstrated that treatment of PC-3 prostate cancer cells with *T. persicum* extract induces significant cytotoxicity and programmed cell death. In the present study, we aimed to further explore the effects of the methanolic extract of *T. persicum* on lactate dehydrogenase (LDH) activity, intracellular ROS species levels, mitochondrial membrane potential, and the expression of *Matrix metalloproteinase-2 (MMP-2)*, *Matrix metalloproteinase-9* (*MMP-9)*, *Tumor protein 53 (TP53)*, and *β-catenin* (*CTNNB1*) genes in PC-3 cells. For this purpose, PC-3 cells were treated with both sublethal concentrations and concentrations above the IC_{50} value of the extract. The results revealed that *T. persicum* treatment caused marked mitochondrial membrane damage and increased LDH release. These effects were associated with decreased intracellular ROS levels and loss of mitochondrial membrane potential, which correlated with reduced cellular uptake of rhodamine 123 in a dose-dependent manner. Furthermore, gene expression analyses showed that exposure of PC-3 cells to the extract at the IC_{50} concentration led to significant downregulation of *MMP-2*, *MMP-9*, and *CTNNB1* genes, accompanied by upregulation of *TP53*. Collectively, these findings suggest that the methanolic extract of *T. persicum* can inhibit the proliferation and survival of PC-3 prostate cancer cells through multiple molecular pathways. Further comprehensive studies are warranted to elucidate the precise mechanisms underlying these anticancer effects.

Keywords**:** *Teucrium persicum*; PC-3 cells; ROS, Lactate dehydrogenase, Cell death, Invasion

Introduction

Reactive oxygen species (ROS) are highly reactive molecules derived from oxygen metabolism that play dual roles in cellular physiology (Renaudin, 2021). At moderate levels, ROS function as signaling molecules that regulate processes such as cell proliferation and differentiation. However, excessive ROS accumulation induces oxidative stress, leading to damage of cellular macromolecules including DNA, proteins, and lipids. DNA damage caused by ROS can result in mutations, genomic instability, and activation of oncogenes or inactivation of tumor suppressor genes, thereby promoting carcinogenesis. Moreover, ROS can activate pro-survival signaling pathways such as NF-κB and MAPK, which enhance cell proliferation and inhibit apoptosis. Collectively, these mechanisms illustrate how dysregulated ROS levels contribute to cancer initiation and progression (Jomova et al., 2023; Iqbal et al., 2024). Targeting oxidative stress pathways offers a promising strategy for anticancer therapy (Hajam et al., 2022).

Antioxidants, which significantly have a pivotal role in cellular defense, possess the remarkable capability to counteract oxidative damage induced by ROS within biological systems (Hajam et al., 2022). These molecular guardians act as scavengers, neutralizing free radicals and they prevent the detrimental effects on cellular structures, including lipids, proteins, and nucleic acids. The intricate balance between oxidative stress and antioxidant defenses plays a critical role in maintaining cellular homeostasis and influencing the overall health (Pizzino et al., 2017). Notably, the synergistic interactions among various antioxidants contribute to the overall efficacy of this defense mechanism, highlighting the intricacies of cellular redox regulation (Di Giacomo et al., 2023). Understanding

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the nuanced interplay between oxidative stress and the antioxidant defense system holds significant implications for designing therapeutic strategies to mitigate oxidative damages and associated pathologies, offering promising avenues for advancing preventative and therapeutic interventions in diverse medical contexts (Abnosi and Hosseini, 2020).

Various scientific investigations have robustly elucidated the role of plants as a significant reservoir of exogenous antioxidants (Mohadjerani et al., 2016; Najafi et al., 2016). Contemporary research indicates that there is an inverse relationship between antioxidant-rich food consumption and the incidence of human diseases (Medina-Vera et al., 2012). This intricate interplay underscores the potential health benefits, derived from a diet rich in plant-derived antioxidants, highlighting the multifaceted impact of phytochemical compounds on human well-being.
Experimental investigations had revealed that the presence of various compounds in the essential oils or extracts of plants may be responsible of their biological properties (Licá et al., 2017). *Teucrium* species are known for a diverse range of secondary metabolites, considering amounts of sesquiterpenes, phenolic and flavonoid compounds that play a crucial role in the plant's defense mechanisms and contribute to its potential medicinal properties (Hao et al., 2013; Zlatić et al., 2017).

Teucrium persicum, an Iranian endemic plant, belongs to the *Teucrium* genus, Lamiaceae family, and grows in southern parts of Iran, local to the Fars province, and is called *Marv-e-talkh* (Miri et al., 2012). In the Iranian traditional medicine, it is used to treat headaches and abdominal pain as well as diabetes, inflammation, and hyperlipidemia. In our previous study, we identified 70 different compounds in methanolic extract of *T. persicum* that the sesquiterpenes were dominant (Miri et al., 2015). There are a few studies that consider the biological properties of the *T. persicum*. Our previous studies revealed that the *T. persicum* methanolic extract shows inhibitory and anticancer effects on different cancer cell lines (Hajipour et al., 2022; Tafrihi et al., 2023).

Although we did not perform a comprehensive phytochemical profiling in the current study, our earlier research on the same plant species demonstrated that its extract is abundant in bioactive compounds, particularly polyphenols and flavonoids. Notably, key constituents such as phytol, calarene epoxide, and 1-docosene were identified (Tafrihi et al., 2023). These compounds are well known for their strong antioxidant potential, which operates through multiple complementary mechanisms, including direct neutralization of free radicals, metal ion chelation, and stimulation of the body's own antioxidant defense systems (Cheng et al., 2015; Abd El-Gawad et al., 2019; Kim et al., 2022).

Advancing in this way, we decided to evaluate whether the *T. persicum* methanolic extract influences ROS levels, and also its effects on the expression of *MMP-2*, *MMP-9*, *CTNNB1*, and *TP53* genes in PC-3 cells.

Materials and Methods

Extract preparation

T. persicum plants were gathered from the southern regions of Iran and were authenticated by Dr. Alireza Naqinezhad from the Department of Plant Biology, Faculty of Sciences, University of Mazandaran (9008-HUMZ). Subsequently, 50 gr of the powdered aerial parts of the *T. persicum* were immersed in 100 mL of methanol (Merck, Germany) and were shaken for 48 h. The methanolic solvent was completely removed from the extract using a rotary evaporator followed by freeze-drying (Christ, Germany). The resulting dried extract contained no residual methanol and was reconstituted in the appropriate culture medium prior to cell treatment. Therefore, no solvent (methanol) vehicle control was required. The dried extract was dissolved in dimethyl sulfoxide (DMSO, Merck, Germany) to a final concentration of 25 µg/mL and stored at -20 °C for subsequent use.

Cell culture

PC-3 human prostate cancer cells were selected for this study based on our previous investigations, in which we examined the effects of the extract on these cells. Using the same cell line allows for direct comparison and continuity with our earlier findings, facilitating a more consistent evaluation of the extract's biological effects. So, PC-3 cells were purchased from a national cell bank (Pasteur Institute, Tehran, Iran) and were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) supplemented with 10% fetal bovine serum (Invitrogen, USA), 100 µg/ml of streptomycin, and 100 unit/ml of penicillin (Sigma, Germany).

Lactate dehydrogenase assay

PC-3 cells were counted using a Neubauer hemocytometer and seeded at a density of 7.5×10^3 cells per well in 96-well plates. Cells were then incubated under standard culture conditions until they reached approximately 70% confluency prior to

treatment. The treatment concentrations of the methanolic extract of *T. persicum* were selected based on our previously determined IC_{50} value obtained from dose–response experiments (Tafrihi et al., 2014). Accordingly, the cells were then treated with 50, 100, 142, and 150 µg/ml of the methanolic extract of the *T. persicum* for 48 h. Subsequently, the supernatant was collected and used to measure the LDH activity. The LDH mixture was prepared according to the manufacturer's instructions (Kiazist, Iran), and a volume equal to twice the volume of the discarded medium was added to each well. The reaction was then incubated at 37 ºC for 30 min. The absorbance then was measured using an ELISA microplate reader (BioTek, USA) at a wavelength of 520 nm.

Dichloro-dihydro-fluorescein diacetate-Reactive oxygen species Assay

PC-3 cells were seeded at a density of 7.5×10^3 cells per well in 96-well plates and allowed to grow until approximately 70% confluency. Cells were then treated with 50, 100, 142, and 150 µg/mL of the methanolic extract of *T. persicum* for 48 h. After treatment, the culture medium was removed, and cells were incubated with the ROS detection reagent prepared according to the manufacturer's instructions (Teb Pazhouhan Razi, Iran). Plates were incubated at 37 °C for 60 min, and intracellular ROS levels were measured using a BioTek fluorescence microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Determination of mitochondrial membrane potential

The PC-3 cells were seeded at a density of 7.5×10^3 cells/well in a 96-well plate and were allowed to grow until they reached approximately 70% confluency. The cells were then treated with 50, 100, 142, and 150 µg/mL of the methanolic extract of *T. persicum* for 48 h. Subsequently, the Rhodamine-123 fluorescent dye (Sigma, Germany) was added into the wells and was left at 37 ºC for 30 min. The cells were then washed with PBS and examined under a fluorescence microscope (Nikon, Japan).

Real-Time Quantitative PCR assay

RNA was extracted from the cells using the RNX-Plus kit (Sinaclon, Iran), and the cDNA samples were synthesized using a cDNA synthesis kit (Pars Tous, Iran). The expression of the *CTNNB1*(β-catenin), *MMP-2*, *MMP-9,* and *P53* genes was measured using SYBR® Green PCR Master Mix QuantiFast (QIAGEN). The primer sequences are listed as follows: *β-actin*: (Forward: 5′- CTTCCTTCCTGGGCAT-3′, Reverse: 5′-GTCTTTGCGGATGTCCA-3′); *CTNNB1* (β-catenin): (Forward: 5′-AAGGTGCTATCTGTCTGCT-3′, Reverse: 5′-TCCATCCCTTCCTGTTTA-3′); *MMP-2*: (Forward: 5′-GCTCGTGCCTTCCAAG-3′, Reverse: 5′-AGTCCGTCCTTACCGTC-3′); *MMP-9*: (Forward: 5′-CGGACCAAGGATACAGTTT-3′, Reverse: 5′-CTCAGTGAAGCGGTACATAG-3′); *TP53*: (Forward: 5′-GGAGTATTTGGATGACAGAAA-3′, Reverse: 5′-GATTACCACTGGAGTCTT-3′).

Statistical analysis

Statistical analyses were performed using SPSS software (version 16). All experiments were conducted in three independent biological replicates, each carried out on different experimental days and using cells from distinct passages. Within every biological replicate, each treatment condition was assessed in triplicate as technical replicates. Data are presented as mean ± standard deviation (SD). The appropriate statistical tests were applied, including one-way ANOVA followed by a suitable post-hoc multiple comparison test to determine differences among groups. A P value < 0.05 was considered statistically significant.

Results

Effect of the methanolic extract of the *T. persicum* on the LDH activity in the PC-3 cells

In our earlier investigation, we determined the IC_{50} value of the methanolic extract to be 142 µg/ml (Tafrihi et al., 2014). Accordingly, PC-3 cells were treated with 50, 100, 142, and 150 µg/ml of the extract for 48 h. LDH assay results demonstrated a significant, concentration-dependent increase in LDH release compared with the untreated control (Figure 1). LDH activity increased from 16.8 ± 1.2 U/L at 50 µg/ml to 27.9 ± 1.5 U/L at 100 µg/ml, 31.4 ± 2.0 U/L at 142 µg/ml, and 35.8 ± 2.3 U/L at 150 µg/ml. All treated groups showed a statistically significant increase in LDH release compared with the control group (***$p < 0.001$), indicating dose-dependent membrane damage and cytotoxicity induced by the extract.

Effect of the extract on the Reactive oxygen species levels in PC-3 cells

After 48 hof treatment with different concentrations of the methanolic extract of *T. persicum*, intracellular reactive oxygen species (ROS) levels were quantified using a fluorescence

microplate reader. The untreated control group showed a fluorescence intensity of approximately 58.6 ± 6.1 arbitrary units. Treatment with the extract significantly reduced ROS levels to 14.2 ± 3.0, 22.1 ± 4.2, and 18.7 ± 3.5 at concentrations of 50, 100, and 142 µg/ml, respectively (***p < 0.001 vs. control). In contrast, exposure to hydrogen peroxide (H_2O_2) as a positive control markedly increased ROS production to 102.4 ± 7.8 (***p < 0.001). These results indicate that the extract significantly suppresses intracellular ROS generation in PC-3 cells (Figure 2).

Effect of the extract on the mitochondrial membrane potential

To investigate the effect of the methanolic extract of the *T. persicum* on the PC-3 cells, the loss of mitochondrial integrity was investigated, and for that purpose, the rhodamine assay was performed. According to this assay, the cells were exposed to the rhodamine 123 dye, and its intensity in the cells was assessed. As results showed, the reduction in the mitochondrial membrane potential correlated with a decline in the cellular uptake of the rhodamine 123 in the treated cells compared to the control cells, leading to a subsequent decrease in the fluorescent signals (Figure 3).

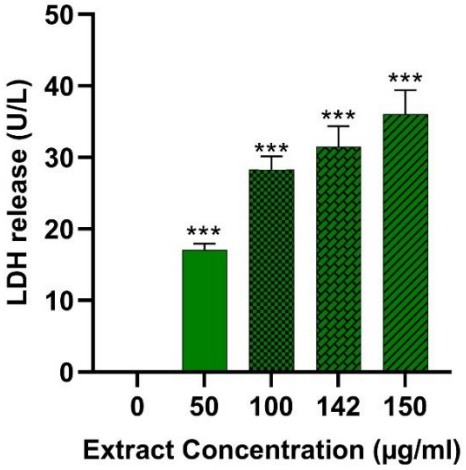

Figure 1. LDH release in PC-3 cells treated with different concentrations of methanolic extract of T. persicum for 48 h. According to the kit instructions, the LDH release were evaluated. The shown data is the mean±SD of three independent experiments (* p < 0.001).

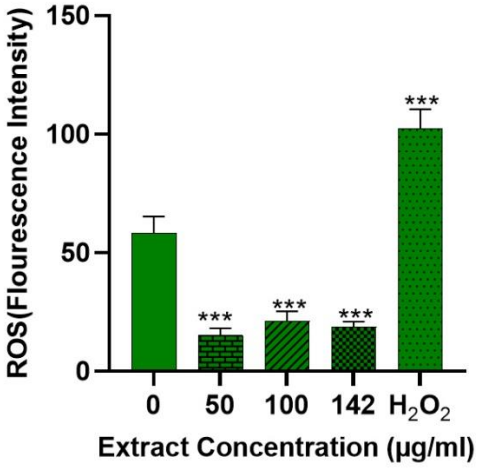

Figure 2. The ROS levels in PC-3 cells. The PC-3 cells were seeded in 96-well plates and were allowed to grow to 70% confluency. The cells were then treated with treated with different concentrations of the methanolic extract of T. persicum for 48 h and the ROS levels was measured. The shown data is the mean±SD of three independent experiments (* p < 0.001).

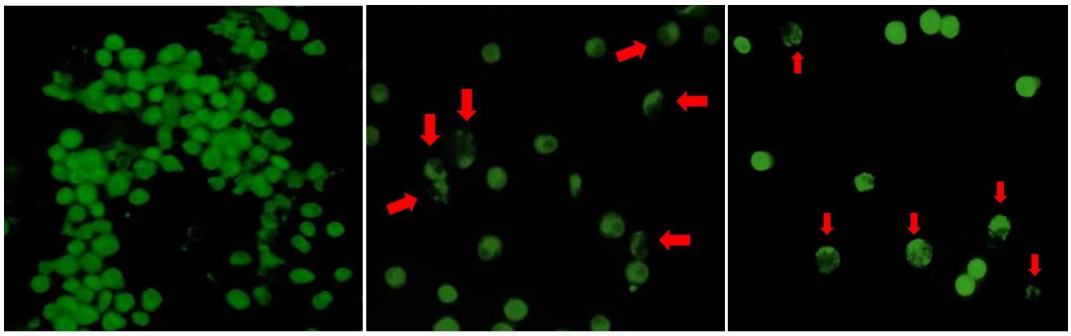

Figure 3. Analysis of mitochondrial membrane potential using rhodamine 123 in PC-3 cells treated with T. persicum methanolic extract for 48 h. Arrows indicate cells with damaged mitochondrial membrane and a decline in the cellular uptake of the rhodamine 123. The images were taken using light microscope (magnification 40×, for control cells, 20×).

Effect of the methanolic extract on the expression of *TP53*, *CTNNB1*, *MMP-2*, and *MMP-9* genes

To further characterize the cellular response to the methanolic extract of *T. persicum*, we examined the expression levels of four genes involved in proliferation, invasion, and survival pathways (*MMP-2*, *MMP-9*, *TP53*, and *CTNNB1*). PC-3 cells were treated with sub-lethal concentrations of the extract (25 and 50 µg/ml) for 48 h. As shown in Figure 4A, *MMP-2* expression decreased from 1.00 ± 0.05 in the control group to 0.38 ± 0.04 at 25 µg/ml and 0.29 ± 0.03 at 50 µg/ml (*p < 0.05). Similarly, *MMP-9* expression was reduced from 1.00 ± 0.06 to 0.89 ± 0.05 at 25 µg/ml and 0.66 ± 0.04 at 50 µg/ml (*p < 0.05; Figure 4B). In contrast, *TP53* expression showed a concentration-dependent increase, rising from 1.00 ± 0.04 in the control to 1.11 ± 0.06 at 25 µg/ml and reaching 1.78 ± 0.08 at 50 µg/ml (*p < 0.05; Figure 4C). Expression of *CTNNB1* displayed a biphasic pattern, increasing to 1.52 ± 0.07 at 25 µg/ml (*p < 0.05) but decreasing to 0.88 ± 0.05 at 50 µg/ml (**p < 0.01; Figure 4D). These results indicate that *T. persicum* extract modulates the transcription of key genes associated with invasion (*MMP-2*, *MMP-9*), tumor suppression (*TP53*), and cell signaling (*CTNNB1*), in a concentration-dependent manner.

Discussion

In this study, we explored the anticancer potential of the methanolic extract derived from *T. persicum*, an Iranian endemic plant. Given its widespread application in the Iranian traditional medicine for managing conditions such as diabetes, abdominal pain, inflammation, and hyperlipidemia, and considering our prior investigations into the chemical composition, antioxidant potential, and cytotoxic effects of the *T. persicum* extract on various cancer cell lines (Tafrihi et al., 2014; Naeimi et al., 2022), our objective was to examine its inhibitory impact on the PC-3 cancer cells using diverse methodologies.

In this study, we investigated the effect of the extract on the LDH enzyme activity in the PC-3 cells. LDH is a cytoplasmic enzyme that is released from membrane-damaged cells, so it has been established as a cell death biomarker (Feng et al., 2018). The results of the LDH assay represented the considerable cytotoxic effects of higher concentrations of the extract on the PC-3 cells (Figure 1), which are consistent with the viability test results of our previous studies (Hajipour et al., 2022; Tafrihi et al., 2014).

Analyzing the chemical constituents of the methanolic extract of the *T. persicum* confirmed the presence of the phenolic compounds, flavonoids, and other bioactive compounds, underscoring its substantial antioxidant and cytotoxic potential (Tafrihi et al., 2023). Thus, it may be inferred that the *T. persicum* emerges as a valuable natural reservoir, exhibiting considerable promise for diverse therapeutic applications, including the development of cancer treatments. In the next step, we assessed the extract's capacity to mitigate intracellular levels of ROS, a crucial element in cancer progression. The results of the ROS assay revealed that all applied concentrations of the extract significantly reduced the intracellular ROS levels, potentially hindering cancer progression (Figure 2).

Studies have shown that various anticancer drugs induce intracellular ROS formation, which is a key factor in mediating their therapeutic effects. This oxidative stress can contribute to the induction of

cell death through mechanisms such as apoptosis (Schumacker, 2006; Marioli-Sapsakou and Kourti, 2021; Slika et al., 2022). On the other hands, there are some anticancer agents that reduce ROS levels, which can disrupt cancer cells' survival mechanisms (Trachootham et al., 2009; Gorrini et al., 2013). Understanding how anticancer agents can both induce and reduce ROS levels is crucial for developing effective therapeutic strategies.

By reducing ROS, these agents can interfere with ROS-dependent signaling pathways such as MAPK, PI3K/AKT, and NF-κB, weakening signals that promote cancer cell proliferation (Liu et al., 2014; Liu et al., 2018). Lower ROS levels can also decrease oxidative damage to DNA, helping to limit mutation rates and slow cancer progression. In addition, they can impair mitochondrial function, a major source of ROS, leading to energy stress within the cells. Together, these disruptions can shift the oxidative balance enough to induce cell cycle arrest or trigger apoptosis, ultimately reducing the viability of cancer cells (Trachootham et al., 2009; Panieri and Santoro, 2016).

The results of LDH and ROS experiments prompted us to survey the effects of the extract on the mitochondrial membrane potential. The Rhodamine 123 is a fluorescent cationic dye that enters the live cells but not the dead ones and accumulates into the mitochondria (Baracca et al., 2003; Jouan et al., 2014). Our findings revealed the extract-induced mitochondrial damage that resulted in cell death (Figure 3). The antioxidant properties and cancer cell selectivity of the *T. persicum* extract were found to be associated with its potential to inhibit migration and invasion, which are correlated with a reduction in intracellular ROS levels. This was further supported by the upregulation of *TP53* and *CTNNB1* and the downregulation of *MMP-2* and *MMP-9* genes (Figure 4). Surveying the literature shows that these genes play key roles in cancer development and progression. *TP53* is a well-known tumor suppressor that helps regulate the cell cycle, repair DNA damage, and trigger apoptosis, that are often disrupted in cancer (Vousden and Lane, 2007).

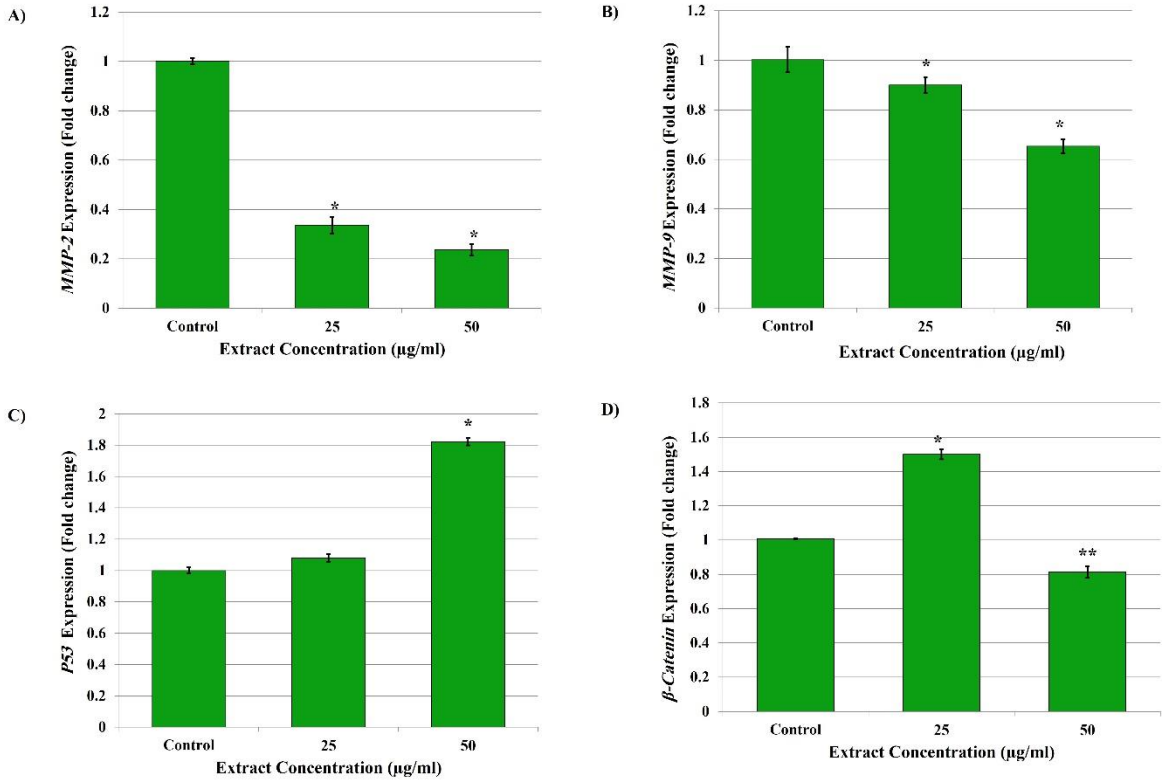

Figure 4. Real-time PCR experiments to measure the mRNA levels of the MMP-2, MMP-9, TP53, and β-catenin genes in the PC-3 cells treated with the methanolic extract of T. persicum. PC-3 cells were treated with 25 and 50 µg/ml of the extract for 48 h. Total RNA was collected from different sets of cells and was used for real-time PCR experiments. Gene expression data are presented as fold change relative to the untreated control using the $2^{-}\Delta\Delta Ct$ method. All values represent the mean ± SD of three independent biological experiments, each performed in technical triplicate. Statistical analysis was carried out using one-way ANOVA followed by Dunnett's post-hoc test. Asterisks indicate statistically significant differences compared with the control (* $p < 0.05$ and ** $p<0.01$).

CTNNB1, which encodes β-catenin, is a central player in the Wnt/β-catenin signaling pathway, controlling cell proliferation and survival, and its dysregulation is closely linked to tumor progression (Liu et al., 2022). On the other hand, MMP-2 and MMP-9 are enzymes that break down the extracellular matrix, facilitating cancer cell invasion and metastasis (Song et al., 2021). By examining these genes, we aimed to capture both the impact of the extract on cell survival and apoptosis (*TP53*, *CTNNB1*) as well as its potential effects on the invasive behavior of cancer cells (*MMP-2*, *MMP-9*). This combination provides a clearer picture of how the plant extract might influence multiple aspects of cancer cell biology.

Cell invasion and metastasis suppression are desirable approaches in cancer treatment. Surveying the literature revealed that in vitro wound healing is dependent on both cell proliferation and migration (Zanca et al., 2022). Our previous studies showed that sublethal concentrations of the *T. persicum* extract did not reduce the viability of different cancer cells, but significantly decreased the rate of wound healing of the cell sheets (Tafrihi et al., 2014; Naeimi et al., 2022). So, it can be concluded that any reduction in healing the wound in monolayers might be due to a decrease in cell proliferation and invasion. This possibility was very well supported by the results of the gene expression assays that showed the downregulation of *MMP-2* and *MMP-9* genes in the PC-3 cells treated with 25 and 50 µg/ml of the extract. These genes encode gelatinases that belong to the matrix metalloproteinase family involved in ECM degradation, cell migration, and cancer cell metastasis (Webb et al., 2017).

The transcription factor P53 functions as a sensor for a variety of cellular stress signals, including DNA damage. As a response to these stimuli, P53 triggers cell cycle arrest and apoptosis (Feroz and Sheikh, 2020). Our previous investigation revealed that the extract induces DNA degradations and apoptosis in the PC-3 cells (Tafrihi et al., 2014). The current study indicates the upregulation of P53 and β-catenin encoding genes. Surveying the literature suggests a β-catenin-dependent upregulation of *TP53* in the cancer cells (Dannheisig et al., 2020; Xiao et al., 2022). Thus, our combined findings suggest that treatment with the extract leads to widespread genomic damage and upregulation of β-catenin and P53 encoding genes in PC-3 cells, ultimately suppressing cancer cell proliferation and migration.

Conclusion

In summary, it appears that the effects of inhibitory potential of *T. persicum* extract can be attributed to its effects on the intracellular levels of ROS, the mitochondrial membrane potential, and the expression pattern of genes associated with cell proliferation, invasion, and apoptosis.

Acknowledgments

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Conflict of interest

The authors declare that they have no competing interests for this publication.

Data availability

The data produced in this research are ready by the corresponding author on request.

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