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The Potential Role of Plant-Derived *Ziziphora tenuior* Extract in the Treatment of Endometrial Cancer

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Abstract

In recent years, there has been a growing interest in the potential effects of plant-derived compounds in cancer treatment. Within this context, *Ziziphora tenuior (Z. tenuior)*, known for its antioxidant, antimicrobial, and antiproliferative properties, has emerged as a notable medicinal plant. This study aimed to evaluate the cytotoxic effects of *Z. tenuior* extract and its influence on the colony-forming ability of Ishikawa cells, a human endometrial cancer cell line. Cell viability was assessed using the CCK8 assay, followed by colony formation assays to investigate long-term proliferative effects. Treatment with various concentrations of *Z. tenuior* extract revealed a dose-dependent cytotoxic response. The highest level of cell death was observed at a specific time point, and the corresponding extract concentration significantly suppressed cell proliferation. Moreover, colony formation assays demonstrated a marked reduction in the clonogenic capacity of *Z. tenuior*-treated cells compared to the control group. These findings indicate that *Z. tenuior* extract exerts both short- and long-term antiproliferative effects on endometrial cancer cells in a dose-dependent manner. The results suggest that *Z. tenuior* may serve as a promising plant-based therapeutic agent for the treatment of various cancers, particularly hormone-dependent malignancies.

Keyword: Endometrial cancer, *Ziziphora tenuior*, Ishikawa cells, Antiproliferative activity, Wnt/β-catenin

Özet

Son yıllarda bitkisel kökenli bileşiklerin kanser tedavisindeki potansiyel etkileri üzerine yapılan çalışmalar artış göstermektedir. Bu kapsamda, antioksidan, antimikrobiyal ve antiproliferatif özelliklere sahip olduğu bilinen *Ziziphora tenuior (Z. tenuior)*, dikkat çeken tıbbi bitkilerden biridir. Bu çalışmanın amacı, *Z. tenuior* ekstresinin endometrial kanser hücre hattı olan Ishikawa hücreleri üzerindeki sitotoksik ve koloni oluşturma kapasitesine etkilerini değerlendirmektir. Araştırmada, hücre canlılığı CCK8 testi ile belirlenmiş; ardından koloni formasyon analizi ile uzun vadeli proliferatif etkiler değerlendirilmiştir. *Z. tenuior* ekstresinin farklı konsantrasyonlarda uygulanmasıyla doz bağımlı bir sitotoksik etki gözlenmiştir. En etkili hücre ölümünün belirli bir zaman noktasında ortaya çıktığı ve bu etkiye karşılık gelen ekstrakt dozunun hücre proliferasyonunu anlamlı şekilde baskıladığı görülmüştür. Koloni oluşum analizinde, *Z. tenuior* ile muamele edilen hücrelerin koloni oluşturma kapasitesinde anlamlı bir azalma tespit edilmiştir. Elde edilen veriler, *Z. tenuior* ekstresinin endometrial kanser hücrelerinde hem kısa hem uzun vadeli antiproliferatif etkiler gösterdiğini ve bu etkilerin doz bağımlı olduğunu ortaya koymaktadır. Bulgular, *Z. tenuior*'un hormon bağımlı kanserler başta olmak üzere çeşitli kanser türlerinde kullanılabilecek potansiyel bir bitkisel tedavi ajanı olabileceğini göstermektedir.

Anahtar kelime: Endometriyal kanser, *Ziziphora tenuior*, Ishikawa hücreleri, Antiproliferatif aktivite, Wnt/β-katenin

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1. INTRODUCTION

Endometrial cancer is the most common gynecological malignancy worldwide, with approximately 417,000 new cases reported in 2020. This makes it the sixth most frequently diagnosed cancer among women (Crosbie et al., 2022). The main risk factors for endometrial cancer include obesity, anovulation, prolonged estrogen exposure, and diabetes. These factors may increase cellular proliferation in the endometrium, disrupt hormonal balance, and consequently raise the likelihood of malignancy development (Sorosky, 2012).

Treatment options include local approaches such as surgery and radiotherapy, as well as systemic therapies such as chemotherapy, targeted therapy, hormonal therapy, and immunotherapy. However, the limited efficacy of current treatments and variable responses among molecular subtypes present significant clinical challenges (Rütten et al., 2021; Anca-Stanciu et al., 2025).

Several signaling pathways play a role in the molecular pathogenesis of endometrial cancer. One of these important pathways is the Wnt signaling pathway. This pathway is a signaling system that regulates fundamental biological processes such as cellular proliferation, differentiation, apoptosis, and tissue homeostasis. The Wnt signaling pathway is divided into two types: β-catenin-dependent canonical (Wnt/β-catenin) and β-catenin-independent non-canonical pathways. The canonical pathway is particularly effective in regulating gene transcription, whereas non-canonical pathways control processes such as cell polarity and intracellular calcium balance (Yu et al., 2022; Tian et al., 2023).

The Wnt/β-catenin signaling pathway functions in a wide range of physiological processes, from embryonic development to the maintenance of stem cell homeostasis in adult tissues. This pathway activates the expression of genes associated with the cell cycle. Dysregulation of this pathway may disrupt the balance between cellular growth and programmed cell death, thereby triggering tumor development (Pandey et al., 2023).

Studies have shown that abnormal activation of the Wnt/β-catenin pathway plays an important role in the pathogenesis of many cancer types, including tumors, hematological malignancies, and sarcomas. This abnormal activation is often caused by mutations in pathway components. For example, mutations in the CTNNB1 gene, which encodes β-catenin, are particularly common in endometrioid-type endometrial cancers and have been closely associated with disease prognosis (Tian et al., 2023; Song et al., 2024). The central role of the Wnt/β-catenin signaling pathway in cancer development has increased interest in therapeutic strategies targeting this pathway.

In conclusion, the Wnt/β-catenin signaling pathway stands out as a critical regulator in both the maintenance of normal physiological processes and cancer development. A better understanding of the molecular mechanisms in this pathway will significantly contribute to the development of future targeted cancer therapies. This has increased interest in agents capable of modulating the Wnt/β-catenin pathway, particularly natural compounds.

Medicinal plants have been used for thousands of years in the treatment of various diseases, and their potential is still widely investigated today. The evaluation of new molecules and plant-derived compounds is of great importance for the development of alternative therapeutic strategies (Van Wyk and Wink, 2004).

One of these plants is *Ziziphora tenuior* L., belonging to the Lamiaceae family. This annual aromatic species can grow up to 5–15 cm in height. In addition to its high essential oil content, its richness in phenolic, polyphenolic, and flavonoid compounds provides it with significant pharmacological potential (Sonboli et al., 2006). Although the main component of the essential oil varies among populations, pulegone is generally dominant, accompanied by monoterpenes such as menthone, 1,8-cineole, limonene, thymol, and β-caryophyllene (Azimi et al., 2021; Sonboli et al., 2006).

The main active component, pulegone, has been reported to exhibit analgesic, anti-inflammatory, anti-stress, hypolipidemic,

and antidiabetic effects (Zarei et al., 2022). In addition, the phenolic and volatile compounds of *Z. tenuior* demonstrate antioxidant, anti-inflammatory, antibacterial, and antiviral properties (Van Wyk and ,Wink 2004; Wink, 2008). Recent studies have shown that *Z. tenuior* exhibits cytotoxic and apoptotic effects on cancer cells (Azimi et al., 2021; Nasiri and Nasri, 2020). In particular, it has been reported that the essential oil induces apoptosis by activating caspase-3 and caspase-9 and causes significant cytotoxicity in HT-29 colorectal and A549 lung cancer cell lines (Azimi et al., 2021; Bateni et al., 2020).

When all these findings are considered together, it becomes important to investigate the potential effects of *Ziziphora tenuior* on malignancies such as endometrial cancer. However, the effects of this plant on Ishikawa endometrial adenocarcinoma-derived cells have not yet been clearly demonstrated. Therefore, in this study, cytotoxic effects and proliferation in Ishikawa cells treated with *Z. tenuior* extract were evaluated using cytotoxicity assays and colony formation analysis; furthermore, the effect of the extract on the Wnt signaling pathway was investigated by qPCR analysis.

2. MATERIAL AND METHODS

2.1 Preparation of plant extract

The leaves of *Ziziphora tenuior L.* (Lamiaceae) were dried in a shaded, well-ventilated laboratory. The dried samples were cut into small pieces and prepared for extraction. A total of 30 g of the dried leaves were extracted with 300 ml of methanol using a Soxhlet apparatus for 6–8 hours. After extraction, the solution was filtered and the solvent removed using a rotary evaporator to obtain the methanolic extract (ZTE).

2.2. Cytotoxicity Assay

The effects of ZTE on the viability and proliferation of endometrial cancer cells were evaluated using a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium]-based cell viability assay. This method relies on the reduction of WST-8 to a water-soluble orange formazan product by mitochondrial dehydrogenases, providing a sensitive measure of cell viability (Martin et al., 2024).

Ishikawa cells were seeded into 96-well plates at a density of 2×10^3 cells per well and allowed to attach for 24 hours. After attachment, the cells were treated with different concentrations of ZTE (2.5, 10, 15, 20, 30, 40 µg/mL) in DMEM for 24, 48 and 72 hours. Following the incubation, the medium was removed, and 90 µL of DMEM and 10 µL of WST-8 reagent were added to each well. The plates were incubated for 4 hours, and absorbance was measured at 450 nm with a reference wavelength of 650 nm using a microplate reader. Cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = (\text{Absorbance of experimental group} / \text{Absorbance of control group}) \times 100$$

The half-maximal inhibitory concentration (IC_{50}) of ZTE was determined using GraphPad Prism 8.0.2 software.

2.3 Gene Expression Analysis

Total RNA from the control and treatment groups in the experimental set established with Ishikawa cell lines was isolated using RNA extraction buffer (GeneAll, 301-001), and RNA quality and quantity were determined using a spectrophotometer. Following RNA isolation, cDNA synthesis was performed using a cDNA synthesis kit (Bio-Rad, 170-8891) according to the manufacturer's protocol. The effects of Z. tenuior on the expression levels of CTNNB1, APC, GSK-3β, HIF-1α, and PGK-1 (components of the Wnt/β-catenin signaling pathway) in Ishikawa cells were evaluated by quantitative real-time PCR (qPCR) using BrightGreen 2× qPCR MasterMix–ROX (ABM, MasterMix-R). GAPDH was

employed as the reference gene for normalization.

The total reaction volume of 10 µL consisted of 5 µL qPCR mastermix, 0.5 µL forward and reverse primers, 2.5 µL nuclease-free water, and 2 µL cDNA. The prepared reaction mixtures were dispensed into 96-well plates and subsequently loaded into a real-time PCR instrument.

The thermal cycling conditions were set as follows: initial enzyme activation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s and combined annealing/extension at 60 °C for 1 min. Ct values obtained from qPCR were normalized to the expression level of the housekeeping gene GAPDH. Relative changes in gene expression were calculated using the 2^−ΔΔCt method. Statistical differences between the control and ZTE-treated groups were assessed using an unpaired Student's t-test and one-way Anova (Graphpad Prism 8.0.2) Statistical significance was set at $p < 0.05$.

2.4. Colony Formation Assay

The long-term effects of ZTE on cell proliferation were evaluated using a colony formation assay. Ishikawa cells were seeded into six-well plates at a density of 2×10^3 cells per well and incubated for 24 hours to allow cell attachment. Subsequently, the culture medium was replaced with fresh DMEM containing the indicated concentrations of ZTE for the treatment groups, while the control group received fresh DMEM alone. The cells were then allowed to grow and form colonies for approximately 14 days. At the end of the incubation period, the colonies were fixed with 100% methanol, washed with PBS, and stained with 0.1% crystal violet solution. Excess dye was removed with distilled water, the plates were air-dried, and the number of colonies was counted. Colony formation analysis was performed using an unpaired t-test through the GraphPad Prism (8.0.2) software.

2.5. Statistical Analysis

To ensure the reliability of the results, all experiments were performed in at least three independent biological replicates. Data were expressed as mean ± standard deviation (SD). Statistical differences between the control and ZTE-treated groups were assessed using an unpaired Student's t-test, one-way Anova and unpaired t test (Graphpad Prism 8.0.2). Statistical significance was set at $p < 0.05$.

3. FINDINGS

In this study, the potential therapeutic effects of *Ziziphora tenuior* L. methanolic extract effects were investigated for its effect against human endometrium cancer cells. For this, the cells were treated with the extract in a dose-and time- dependent manner. Following the treatment, a cytotoxicity assay was performed to evaluate the viability of the Ishikawa cell line, and a colony formation assay was conducted to assess its proliferative capacity.

As a result of cytotoxicity analysis, the *Ziziphora tenuior* extract decreased viability rates of Ishikawa cells depending on the applied doses and exposure time. The dose ranges used in the study were treated as 2.5, 10, 15, 20, 30, and 40 µg/mL, and the effects of these doses on cell viability were evaluated at 24, 48, and 72 hours. The IC_{50} values in the Ishikawa cell line at 24, 48, and 72 hours were determined as 28.99 µg/mL, 19.94 µg/mL, and 11.78 µg/mL, respectively. In the study, the IC50 value was determined as 19.95 µg/mL at 48 hours (Figure 1).

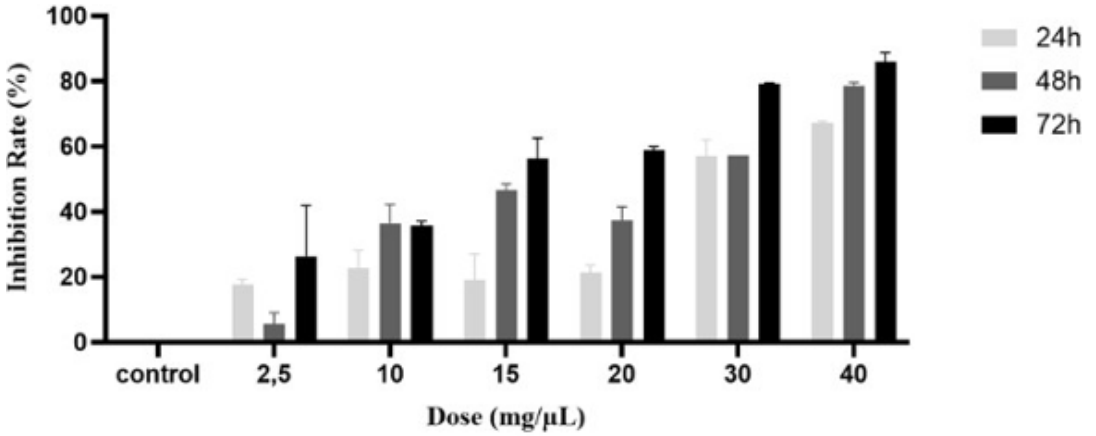

Figure 1. Dose- and time-dependent cytotoxic effects of *Ziziphora tenuior* extract on Ishikawa endometrial cancer cells

The effect of *Z. tenuior* on the Wnt/β-catenin signaling pathway in the endometrial cancer cell line Ishikawa was evaluated at the molecular level using qRT-PCR analysis. Following treatment with *Z. tenuior*, the mRNA expression levels of Wnt/β-catenin pathway–related genes, including **PGK-1, CTNNB1, HIF-1α, APC, and GSK-3β**, were analyzed by qRT-PCR.After 48 hours of treatment with *Z. tenuior* at the IC_{50} concentration in Ishikawa cells, the expression levels of **PGK-1**(2.28-fold decrease; $p < 0.000001$), **CTNNB1** (3.94-fold decrease; $p = 0.000262$), **HIF-1α** (5.54-fold decrease; $p = 0.006742$), **APC** (4.63-fold decrease; $p = 0.004586$), and **GSK-3β** (8.06-fold decrease; $p = 0.000006$) were significantly reduced (Figure 2).

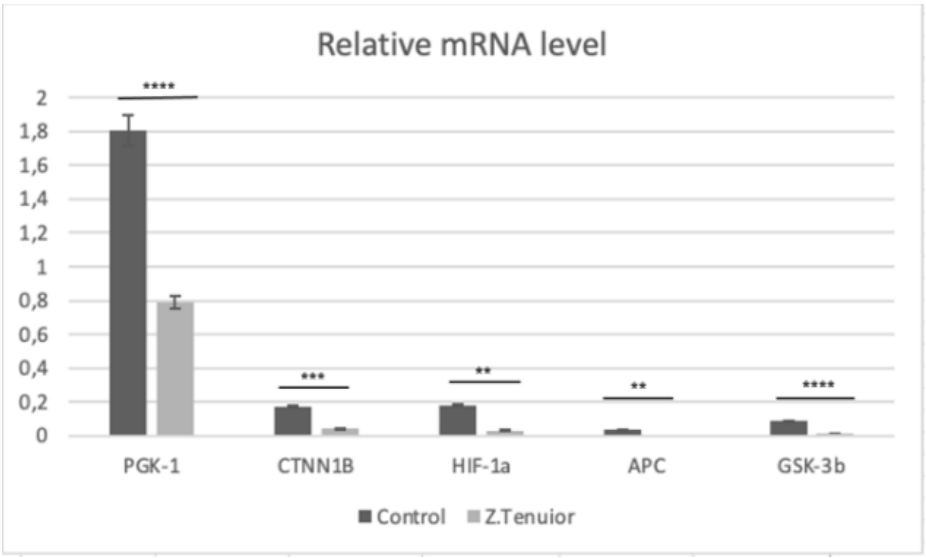

Figure 2. The effects of *Z. tenuior* on Wnt/β-catenin pathway gene expressions in Ishikawa cells

In our study, a colony formation assay was performed to evaluate the effects of *Z. tenuior* treatment on the colony-forming ability of Ishikawa cells. The findings demonstrated that *Z. tenuior* treatment significantly affected the clonogenic potential of the cells. The cells were treated with *Z. tenuior* at a concentration of 19.94 µg/ml, which was determined to be the most effective dose based on cytotoxicity analysis. As a result, a statistically significant reduction in colony formation capacity was observed in *Z. tenuior*-treated cells compared to the control group (Figure 3A-B).

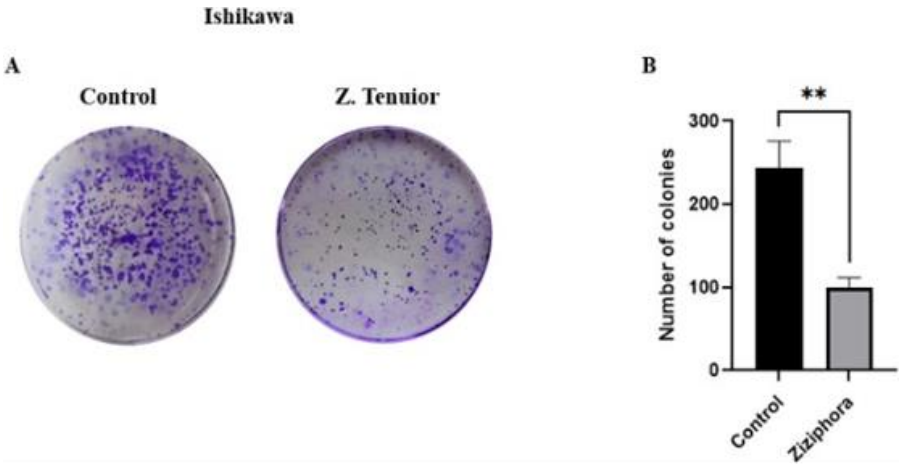

Figure 3. The effects of *Z. tenuior* on colony formation in Ishikawa cells

4. DISCUSSION

Azimi et al. (2021) reported that treatment with *Ziziphora tenuior* in the HT-29 colorectal cancer cell line resulted in the highest cell death at 24 hours post-treatment with a dose of 200 µg/mL, demonstrating a dose-dependent effect. Similarly, Bateni et al. (2020) calculated the IC_{50} values for *Z. tenuior* treatment in the non-small cell lung cancer cell line A549 as 9.06, 6.32, and 6.48 µg/mL at 24, 48, and 72 hours, respectively, noting significant differences between the control and treated groups especially at 72 hours. These findings indicate that *Z. tenuior* extract inhibits cell growth and proliferation in a dose-dependent manner. In our study, the highest cell death in the Ishikawa endometrial cancer cell line was observed at 48 hours, with an IC_{50} value of 19.94 µg/mL following *Z. tenuior* treatment. Moreover, similar to its effects in colorectal and lung cancers, *Z. tenuior* exhibited a dose-dependent cytotoxic effect in endometrial cancer cells. In a related study by Nasiri and Nasri (2020), *Z. tenuior* was applied to HeLa cells, a cervical cancer cell line, and the IC_{50} value was determined to be 100 µg/mL at 48 hours. These findings align with our results and further support the dose-dependent cytotoxic effect of *Z. tenuior* on gynecological cancer cells. The consistency between these two studies suggests that *Z. tenuior* may exert broad-spectrum antitumor activity across different types of hormone-related cancers, including both cervical and endometrial cancers.

In the study conducted by Sonboli et al. (2006), the extract obtained from *Ziziphora clinopodioides* subsp. *bungeana* was reported to exhibit antibacterial activity against *Staphylococcus epidermidis*, *S. aureus*, *Escherichia coli*, and *Bacillus subtilis*, indicating its significant pharmacological potential. In contrast, our study focused on *Ziziphora tenuior* and primarily investigated its cytotoxic effects on cancer cells. Consistent with the findings of Sonboli et al. (2006), our results suggest that the extract of *Z. tenuior* possesses anticancer potential and may serve as a promising pharmacological candidate.

Also, the changes in the mRNA expression levels of key genes involved in the Wnt/β-catenin signaling pathway—namely PGK-1, CTNNB1, HIF-1α, APC, and GSK-3β, which play critical roles in cancer development—were investigated in Ishikawa endometrial cancer cells following treatment with Z. tenuior.The results confirmed that treatment with *Z. tenuior* significantly suppressed the expression of these genes in endometrial cancer cells. In particular, the observed decrease in APC and GSK-3β expression indicates a marked inhibition of the Wnt/β-catenin signaling pathway. These findings suggest that *Z. tenuior* exhibits antiproliferative and antiangiogenic effects in endometrial cancer.

In our study, when evaluating whether *Z. tenuior* affects the colony-forming capacity of the Ishikawa cell line, the number of colonies was found to be 90 ± 10 in the Z. tenuior-treated group, whereas it was 240 ± 10 in the untreated control group. Based on these findings, *Z. tenuior* reduced the colony-forming capacity of the cells by 62.50%. These results, which are

consistent with the cytotoxicity data, indicate that the *Z. tenuior* extract is effective in suppressing the proliferative potential of the cells.

4. CONCLUSION

In summary, our study demonstrates that *Ziziphora tenuior* extract induces a dose-dependent cytotoxic effect on the Ishikawa endometrial cancer cell line. Analysis revealed that the highest and most effective cell death occurred at 48 hours post-treatment with an IC_{50} concentration of 19.94 µg/mL. Consistent with findings from previous studies on colorectal, lung, and cervical cancer cell lines, our results confirm the anticancer effects of *Z. tenuior* extract across various cancer types.

Moreover, the colony formation assays and PCR results following *Z. tenuior* treatment indicate that *Z. tenuior* may exert antiproliferative and anti-angiogenic effects on the cells.

Collectively, these findings suggest that *Z. tenuior* extract is a promising pharmacological candidate for the development of novel therapeutic strategies against endometrial cancer, particularly hormone-dependent cancers. We believe that further in vivo studies and molecular investigations are necessary to elucidate the underlying mechanisms of action and to evaluate the clinical potential of *Z. tenuior* extract.

AUTHORS' CONTRİBUTİONS

SP: Data acquisition, data analysis and manuscript preparation. **SM:** Data acquisition, data analysis and manuscript preparation. **HIK:** Data acquisition, data analysis. **GI:** Data acquisition, manuscript preparation. **HV:** Study conception and design, Supervisor.

CONFLICTS OF INTEREST

The authors have declared no conflict of interest.

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