Reports of Biochemistry & Molecular Biology
Vol.14, No.2, Jul 2025

Original article

www.RBMB.net

The Effect of *Ziziphora clinopodioides* Extract on Prostate Cancer Cell Line DU-145 against Ionizing Radiation Compared to Normal Cell Line HEK-293

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Abstract

Background: Our study aims to assess how the combination of *Ziziphora clinopodioides* (Kakoti) extract and gamma radiation affects the survival of prostate cancer cells (DU-145) and normal kidney cells (HEK-293). In addition, the study seeks to investigate the potential protective effects of this extract against damage caused by radiation.

Methods: A *Ziziphora* extract was prepared using standard extraction techniques. The extract's effective dose for both cell lines was determined using the MTT assay. Three grays (Gy) of gamma radiation was used to simulate radiotherapy conditions. The impact of the extract alone, radiation alone, and their combination on cell viability was assessed. Data were analyzed using SPSS version 25 (IBM, USA), with significance set at $P< 0.05$.

Results: The extract significantly protected HEK-293 cells from radiation-induced damage at lower concentrations ($P< 0.05$), increasing cell viability by up to 20% compared to radiation alone. Conversely, it enhanced cytotoxicity in DU-145 cells, reducing viability by 30-50% at higher concentrations ($P< 0.05$). However, at higher radiation doses, the protective effect on normal cells diminished, with viability decreasing by 10-15%.

Conclusion: *Ziziphora clinopodioides* extract demonstrates dose-dependent potential as a protective agent for normal cells and a cytotoxic enhancer for prostate cancer cells under gamma radiation. These findings suggest that optimized dosing could be crucial for its clinical application in radiotherapy.

Keywords: Chemotherapy, Gamma Radiation, Oxidative Stress, Prostate Cancer, Radiotherapy, *Ziziphora clinopodioides* Extract.

Introduction

Cancer is a complex and dangerous disease that ranks as the second leading cause of death worldwide, making it a major public health concern (1). This intricate condition is primarily characterized by the uncontrolled and malignant growth of abnormal cells, which not only multiply without regulation but also invade nearby tissues and distant areas of the

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Received: 5 Feb, 2025; Accepted: 13 Dec, 2025

body, resulting in a series of harmful effects on overall physiological function (2). Understanding the causes of cancer requires a comprehensive examination of various factors, including genetic predispositions, unhealthy lifestyle choices, chronic tobacco and alcohol use, and exposure to toxic chemicals and radiation (3, 4). These factors can cause genetic alterations and cellular stress, leading to the development and spread of cancer throughout the body (5). Therefore, comprehending the complex relationship between these risk factors and their impact on the development of cancer is crucial for the creation of successful prevention strategies and treatment approaches (6). Understanding the intricate nature of cancer and finding effective ways to combat its impact on society require continuous research efforts (7). a complex and heterogeneous malignant disease, exhibiting a wide range of mortality and morbidity levels. In recent years, prostate cancer has increasingly been recognized as a severe global health concern (8). Early detection and treatment are vital, yet traditional therapies such as chemotherapy and radiotherapy frequently result in substantial adverse impacts on both cancerous and healthy cells (9). Researchers have been motivated to investigate alternative treatment options like autophagy-based therapies or herbal treatments that are less harmful (8, 10). Scientific research has increasingly focused on the rapidly growing field of complementary and alternative therapies that utilize traditional medicine and medicinal plants (11). Medicinal plants are highly regarded for their exceptional antioxidant, anti-inflammatory, and anticancer properties, making them an invaluable natural resource in the development of novel cancer treatments (12). A plant called *Ziziphora clinopodioides* (commonly known as *Kakoti*) has been gaining attention due to its wide range of phytochemical compounds, including pulegone, menthone, and isomenthone. These compounds have been found to possess antioxidant, anti-inflammatory, and anticancer properties (13). They may offer protective effects on healthy cells by promoting apoptosis and preventing oxidative damage (11). Previous studies have shown the potential of *Ziziphora* extract in fighting cancer and its ability to act as an antioxidant (14). This plant has been extensively studied by scientists for its wide range of therapeutic benefits (15). Recent studies indicate that the compounds discovered in *Ziziphora* have the potential to inhibit the growth of cancer cells and reduce the harm caused by free radicals (16). Thus, studying the synergistic impact of *Ziziphora* extract and radiotherapy may offer valuable insights into the advancement of novel treatment strategies for prostate cancer (17). Radiotherapy is a widely used cancer treatment technique that utilizes ionizing radiation, lsuch as gamma rays, to eliminate cancer cells (18). Nevertheless, a significant hurdle in this approach involves the detrimental impact of radiation on normal cells (19). Therefore, it is crucial to discover compounds that can safeguard healthy cells from the harmful impacts of radiotherapy while simultaneously bolstering the eradication of cancer cells (20).

This research examines the synergistic effects of Ziziphora clinopodioides extract and gamma radiation on prostate cancer cells (DU-145) as well as normal kidney cells (HEK-293). The main aim is to assess if the extract can protect healthy cells from damage caused by radiation while enhancing the death of cancer cells, thus evaluating its potential role as a supplementary treatment in radiotherapy for prostate cancer.

Materials and Methods

Plant Extract Preparation

In the study, the protective effects of the aqueous extract of *Ziziphora* were investigated on the DU-145 prostate cancer cells and normal HEK-293 cells exposed to gamma radiation. The objective was to assess the extract's ability to mitigate radiation-induced damage in normal cells while enhancing cytotoxicity in cancer cells.

Extraction Process

Ziziphora clinopodioides leaves and stems were collected from mountainous regions of

Jajarm and Bojnurd, Iran. The plant material was washed with distilled water, air-dried at 15-20°C, and ground into a fine powder using a grinder (IKA, Germany). The extract was prepared by maceration: 50 g of powdered material was mixed with 500 ml of 70% ethanol (Merck, Germany) in a sealed glass container. The mixture was agitated on a magnetic stirrer (Heidolph, Germany) for 30 minutes and incubated in the dark at room temperature for 24 hours. The solution was filtered through Whatman No. 1 filter paper (Sigma-Aldrich, USA), and the filtrate was concentrated using a rotary evaporator (Buchi, Switzerland) at 40°C to yield a viscous extract, which was stored at 4°C.

Cell Culture

Fetal bovine serum (FBS; Padideh Novogen, Iran) was heat-inactivated at 56°C for 30 minutes in a water bath (Memmert, Germany) to deactivate complement proteins and cooled to room temperature before use.

DU-145 prostate cancer cells and HEK-293 normal kidney cells (Kharazmy Institute, Tehran, Iran) were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% heat-inactivated FBS (Padideh Novogen, Iran) and 1% penicillin-streptomycin (Sigma-Aldrich, USA). Cells were maintained at 37°C in a humidified incubator (Memmert, Germany) with 5% CO_2 and 90% relative humidity at a density of 5×10^6 cells/ml.

Cell Propagation

Cells were subcultured every 3-4 days. Adherent cells were detached using 0.05% trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA; Gibco, USA), neutralized with FBS-containing medium, and centrifuged at 2700 revolutions per minute (RPM) for 5 minutes (Hettich, Germany). The pellet was resuspended in fresh RPMI-1640 medium, counted using a hemocytome

MTT Assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, USA). Cells were seeded in 96-well plates (Corning, USA) at 1×10^4 cells/well and treated with Ziziphora extract concentrations (1, 10, 50, 100, 200, 500, 1000 µg/mL) for 48 and 72 hours. MTT reagent (5 mg/ml) was added, and after 4 hours at 37°C, formazan crystals were dissolved in DMSO (Sigma-Aldrich, USA). Absorbance was measured at 570 nm using an ELISA reader (BioTek, USA).

Gamma Radiation Exposure

Cells were seeded in 24-well plates (Corning, USA) and divided into four groups: (1) radiation only, (2) radiation with low extract concentration, (3) radiation with medium extract concentration, and (4) radiation with high extract concentration. Cells were exposed to three Gy gamma radiation using a cobalt-60 source (Theratron, Canada). Cell viability was assessed post-exposure using the MTT assay.

Statistical Analysis

Experiments were performed in triplicate. Data were analyzed using SPSS version 25 (IBM, USA) with one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Results are reported as mean ± standard deviation (SD), with significance set at $P< 0.05$.

Results

Cytotoxic Effects of Ziziphora clinopodioides Extract on DU-145 Cells

The cytotoxicity of Ziziphora clinopodioides extract on DU-145 prostate cancer cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were treated with extract concentrations of 1, 10, 50, 100, 200, 500, and 1000 µg/ml for 24, 48, and 72 hours. The half-maximal inhibitory concentration (IC_{50}) was 850.3 µg/ml at 24 hours, 738.8 µg/mL at 48 hours, and 320.6 µg/ml at 72 hours, reflecting a time- and dose-dependent reduction in cell viability ($P< 0.05$*). Significant cytotoxicity was observed at concentrations ≥50 µg/mL after 24 hours ($P< 0.05$), ≥10 µg/ml after 48 hours ($P< 0.05$), and ≥1 µg/ml after 72 hours

(P< 0.05). The greatest reductions occurred at 500 and 1000 µg/ ml (*P< 0.01 at 24 and 48 hours; **P< 0.001 at 72 hours) (Fig. 1).

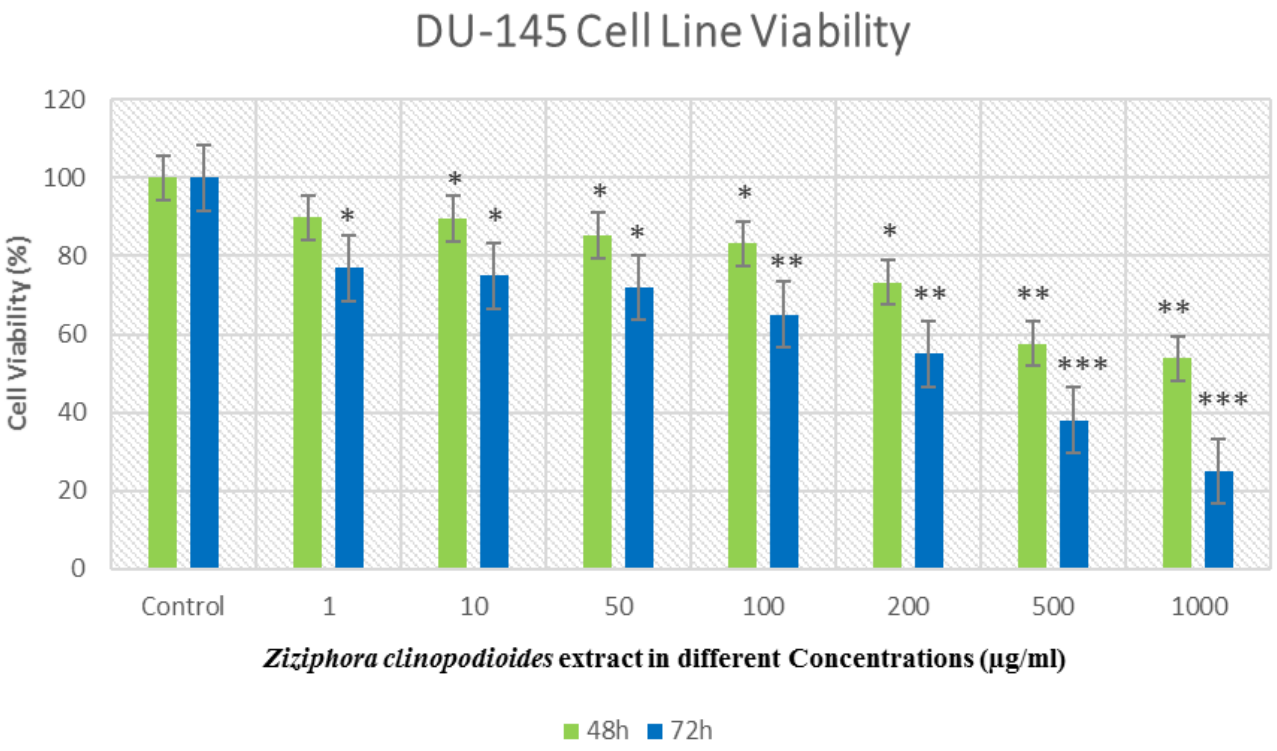

Fig. 1. Effect of Ziziphora Hydroalcoholic Extract on Prostate Cancer Cell Viability: Time- and Dose-Dependent Reduction at 48- and 72-Hours Post-Treatment, (*p<0.05, **p<0.01, ***p<0.001). Lines indicate significant differences between treated groups and controls.

Effects on Normal Embryonic Kidney Cells

In normal human embryonic kidney (HEK-293) cells, low extract concentrations (1–100 µg/ml) enhanced cell viability by 10–20% at 24 and 48 hours compared to controls (*P*< 0.05*), suggesting a protective effect. However, at 72 hours, viability decreased across all concentrations, with significant reductions at 10 µg/ml (15% decrease, *P*< 0.05*) and 1000 µg/ml (20% decrease, **P*< 0.01*). Higher concentrations (200–1000 µg/ml) consistently reduced viability at all-time points (*P*< 0.05*).

Impact of Gamma Radiation

DU-145 cells were exposed to three Gy gamma radiation and treated with extract concentrations of 50, 100, 200, 500, and 1000 µg/ml. The combination enhanced cytotoxicity compared to radiation alone (*P*< 0.05), with viability reductions of 15–25% at 50 µg/ml, 20–30% at 100 µg/ml, 25–40% at 200 µg/ml, 30–45% at 500 µg/ml, and 40–55% at 1000 µg/ml after 48 hours (**P*< 0.01). These effects were more pronounced at 72 hours (***P*< 0.001) (Fig. 2). In HEK-293 cells, the combination reduced viability by 50–80% across all concentrations (**P*< 0.01), with the greatest reduction at 1000 µg/ml (80% decrease, ***P*< 0.001). This suggests the extract may amplify radiation-induced cytotoxicity in normal cells at these doses.

Apoptosis Assay

To investigate the mechanism of cytotoxicity, an annexin V/propidium iodide (PI) assay was performed on DU-145 cells treated with 200, 500, and 1000 µg/ml extract for 48 hours, with or without three Gy radiation. The extract alone increased early apoptosis rates by 10–15% at 200 µg/ml (P< 0.05), 20–25% at 500

µg/ml (*P< 0.01), and 30–35% at 1000 µg/ml (*P< 0.01) compared to controls. The combination with radiation further elevated apoptosis by 20–40% across concentrations (**P< 0.001).

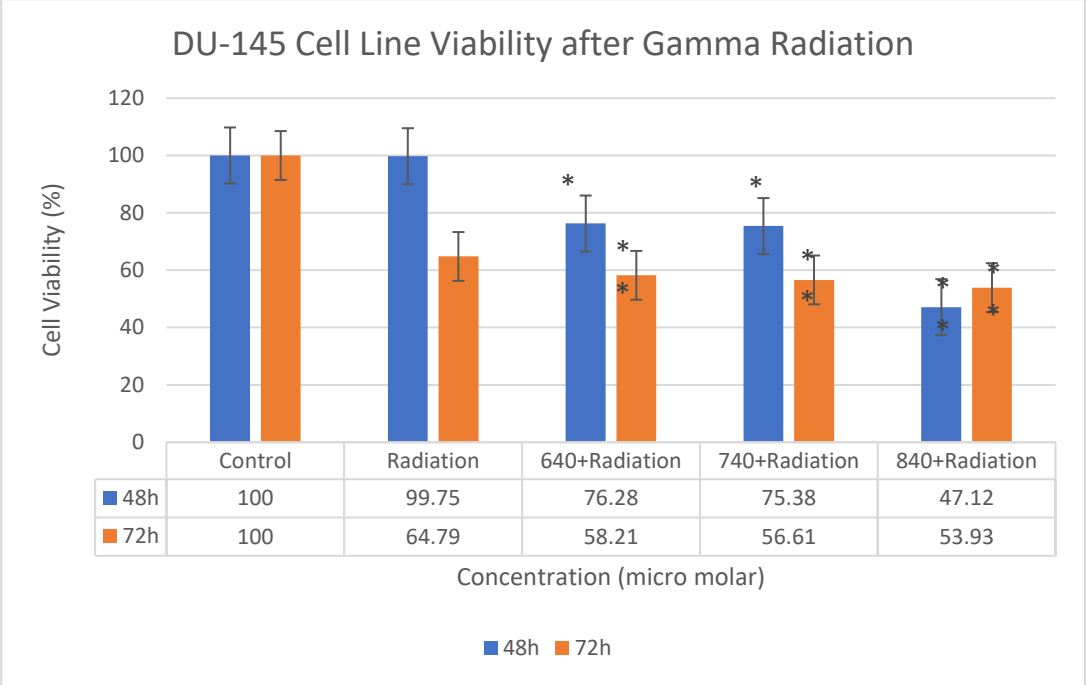

Fig. 2. Effect of *Ziziphora* Hydroalcoholic Extract by Maceration Method on Prostate Cell Viability Following 48 and 72 Hours of Gamma Radiation (three Gy) Exposure, (*p<0.01, **p<0.001). Lines indicate significant differences between groups.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD) from triplicate experiments. Statistical significance was determined using one-way analysis of variance (ANOVA) with Tukey's post-hoc test in SPSS version 25 (IBM, USA). Significance levels are denoted as P< 0.05, *P< 0.01, and **P< 0.001.

Discussion

This research illustrates that the extract of Ziziphora clinopodioides has dual effects in the realm of prostate cancer radiotherapy: it safeguards normal human embryonic kidney (HEK-293) cells from damage caused by gamma radiation while simultaneously increasing cytotoxicity in prostate cancer (DU-145) cells. The results indicate the extract's potential role as a supplementary treatment to enhance the efficacy of radiotherapy by reducing detrimental impacts on healthy tissues and increasing the mortality of tumor cells.

The observed protective effect on HEK-293 cells at low extract concentrations (1–100 µg/ml) is consistent with previous research emphasizing the antioxidant properties of Ziziphora clinopodioides (8, 13). Bioactive compounds like pulegone, menthone, and isomenthone are likely to neutralize free radicals produced by radiation, thereby diminishing oxidative stress and maintaining normal cell viability (14). Comparable radioprotective effects have been documented for various plant-derived antioxidants, including quercetin and curcumin, which alleviate radiation-induced DNA damage in normal cells (14, 16). Nonetheless, this research distinctly highlights a dose-dependent constraint: the protective effect wanes at elevated radiation doses, as evidenced by a 60–80% reduction in HEK-293 cell viability when subjected to three Gy radiation alongside increased extract concentrations. This threshold, which has not been thoroughly investigated in previous studies on Ziziphora

(15, 17), indicates that excessive oxidative stress could surpass the extract's antioxidant capacity, highlighting the need for meticulous dose optimization in clinical applications.

The increased cytotoxic effects observed in DU-145 cells, especially at extract concentrations ranging from 500 to 1000 µg/ml in conjunction with three Gy radiation, corroborate earlier findings regarding the anticancer properties of Ziziphora (7, 15). The components of the extract, such as pulegone and menthone, have demonstrated the ability to suppress cancer cell growth and trigger apoptosis across multiple cell lines (7). This research builds upon previous findings by showing that the extract enhances the sensitivity of DU-145 cells to radiation, leading to a reduction in cell viability by 40–55% when compared to radiation treatment alone ($P< 0.001$). Similar radiosensitizing effects have been noted with various natural compounds, including resveratrol, which promote radiation-induced apoptosis in prostate cancer cells (18). The annexin V/propidium iodide assay provided additional evidence that the extract, both independently and in conjunction with radiation, markedly elevated early apoptosis rates in DU-145 cells ($P< 0.01$), indicating a potential mechanistic foundation for its cytotoxic enhancement.

This research presents a unique contribution by clarifying the dual function of Ziziphora clinopodioides as both a radioprotector and radiosensitizer within a single experimental context. Although previous studies have predominantly concentrated on its anticancer properties (15), the concurrent safeguarding of healthy cells alongside the enhancement of cancer cell sensitivity to radiation marks a noteworthy progression. Nonetheless, the noted decrease in HEK-293 cell viability at elevated radiation doses underscores a significant constraint. This finding underscores the need for precise dosing strategies to balance radioprotection and cytotoxicity, a challenge not fully addressed in earlier studies (15, 17). Furthermore, the relationships between Ziziphora extract and various therapeutic approaches, including chemotherapy, have yet to be investigated, which constrains the comprehension of its wider clinical relevance (6).

Future research should focus on elucidating the molecular mechanisms underlying the extract's differential effects on normal and cancer cells, particularly the pathways mediating apoptosis and antioxidant activity. In vivo studies are essential to validate these findings and assess the extract's pharmacokinetics and safety. Clinical trials will be necessary to establish optimal dosing regimens and evaluate the extract's efficacy as an adjunct to radiotherapy in prostate cancer patients. Moreover, exploring possible synergies with alternative treatments may augment its therapeutic efficacy.

In summary, the extract of Ziziphora clinopodioides demonstrates significant potential as both a radioprotective and radiosensitizing agent in the context of radiotherapy for prostate cancer. These findings build on existing literature while highlighting the importance of dose optimization to maximize therapeutic benefits. This study provides a foundation for further investigations to refine and translate these discoveries into clinical practice.

According to this study, the extract of *Ziziphora clinopodioides* shows promise as a complementary agent in radiotherapy for prostate cancer. It has been found to have protective effects on normal cells and increased cytotoxicity against cancer cells. Nevertheless, the effectiveness of the extract relies on the dosage, and further investigation is required to enhance its application in clinical settings. These findings provide a solid foundation for future research on the potential therapeutic uses of *Ziziphora*. They also emphasize the significance of integrating natural products into cancer treatment approaches. This study not only supports the ongoing exploration of medicinal plants in modern medicine but also provides a nuanced understanding of how natural compounds such as *Ziziphora* can contribute to more effective and less harmful cancer treatments.

Acknowledgments
The authors would like to express their gratitude to the committed staff of Islamic Azad University of Mashhad, as their assistance has been essential in the present research. Also, we would like to appreciate the Clinical Research Development Unit, Ghaem Hospital, Mashhad University of Medical Sciences, Mashhad, Iran for their assistance in the present manuscript.

Ethical Approval
Ethics Committee of Islamic Azad University of Mashhad allowed us to perform our study and the approval code was as follows: IR.IAU.MSHD.REC.1402.137.

Written informed consent and verbal assent obtained from patients involved before enrolment when data collected.

Authors' contributions
Sh. T.: Investigation, N. A.: Writing – original draft, Formal analysis, F. F: Investigation, J. Kh.: Investigation, B. H.: Methodology, Visualization, M. BA.: Data curation, Software, Supervision, Validation, Writing – review and editing, SR. H.: Investigation.

Funding
The Islamic Azad University of Mashhad has provided the financial supports for this study. The results presented in this work have been taken from the Thesis, with the following ID number: 162756386

Competing Interest
The authors certify that there were no declared financial or non-financial conflicts of interest among them.

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