

Enhancement of radiation response by scorpion venom in colorectal cancer cells: An in vitro study

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ABSTRACT

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Keywords: *Mesobuthus eupeus* venom (MEV), colorectal cancer (CRC), radiation, cell viability, apoptosis.

Background: Colorectal cancer (CRC) is a highly prevalent malignancy, with conventional treatments often challenged by resistance. *Mesobuthus eupeus* venom (MEV), recognized for its cytotoxicity against cancer cells, was investigated in this study as a novel adjuvant to CRC radiotherapy, with the hypothesis of enhancing its effectiveness. **Materials and Methods:** MEV was extracted and fractionated, and its protein content was determined. Human colorectal carcinoma (HT-29) cells were then exposed to varying concentrations of MEV fractions, both alone and combined with X-radiation (2, 4, and 6Gy). Cell viability was assessed using the MTT assay, and clonogenicity was analyzed via clonogenicity assays. Apoptosis induction was analyzed using flow cytometry. **Results:** MEV significantly reduced HT-29 cell viability in a dose-dependent manner, achieving an IC₅₀ value of 0.2µg/ml at 48 hours. Crucially, combining MEV with radiation led to a marked decrease in colony formation compared to radiation alone. For instance, the combination of MEV with 6Gy radiation resulted in a colony formation rate of 38.9 ± 8.7%, indicating approximately a 30% increase in efficacy compared to radiation alone. Flow cytometry further confirmed a significant increase in apoptosis in CRC cells when MEV was combined with radiation (approximately 48.3% apoptosis in the MEV+2Gy group vs. 27.6% with 2Gy alone). This underscores a synergistic interaction between the two treatments. **Conclusion:** MEV holds promise as a potential adjuvant to radiotherapy for CRC. The observed synergistic effect suggests MEV could enhance radiotherapy efficacy by sensitizing cancer cells and potentially overcoming treatment resistance. Further investigation into its underlying mechanisms and utility in preclinical and clinical settings is warranted.

INTRODUCTION

CRC is the third most common malignancy worldwide and the second leading cause of cancer-related deaths. Compounds derived from venomous animals have been pivotal in the search for novel therapeutic agents targeting cancer cells⁽¹⁾. Despite advancements in conventional treatments like chemotherapy, radiotherapy, immunotherapy, and surgery, many patients still face challenges such as treatment resistance, side effects, and disease recurrence^(2,3).

Immunotherapy, especially through the use of immune checkpoint inhibitors (ICIs), has transformed CRC treatment by leveraging the immune system to target cancer cells. However, the effectiveness of ICIs is limited by specific genetic profiles, such as dMMR/MSI-H, and significant side effects often accompany their application^(3,4).

The effectiveness of radiation can be compromised by the development of radioresistance in metastatic colorectal cancer, along with the overproduction of reactive oxygen species (ROS) in the surrounding healthy tissue, which further diminishes its therapeutic efficacy^(5,6). Due to the side effects associated with these therapies, research is ongoing to develop novel drugs from natural sources by pharmaceutical companies, such as venoms extracted from snakes, spiders, or scorpions. Venoms have been used as medicinal and therapeutic agents from ancient times to the present day^(7,8). Recent studies have identified scorpion venom as a promising source of bioactive compounds with therapeutic potential for developing effective drugs against various cancer types and other diseases. Its anti-proliferative and cytotoxic effects are primarily attributed to its ability to induce apoptosis in cancer cells^(9,10).

The venom of *M. eupeus* (a member of the Buthidae family commonly known as the spotted yellow scorpion) exhibits significant therapeutic potential, particularly its anti-tumor properties. Notably, the venom of Iranian *M. eupeus* has shown efficacy against various cancers, including chronic lymphocytic leukemia and CRC (7). The lesser Asian scorpion, *M. eupeus*, is one of the most extensively distributed species within the *Mesobuthus* genus, and its venom has been the focus of numerous studies (11). However, the venom's significant complexity means that many active compounds remain insufficiently explored (12). Recent studies on MEV have demonstrated that crude MEV can selectively induce cell death by activating the formation of reactive oxygen species (ROS). Additionally, it has been found that the toxic effects of MEV on cell membranes in HT-29 cells may be associated with their inherent sensitivity.

Additionally, MEV was observed to elevate ROS levels, leading to programmed cell death. Morphological alterations were also noted in HT-29 cells after MEV exposure, as previously mentioned. These changes not only served as indicators of apoptotic activity but also highlighted significant damage to the cellular core (13).

Contemporary oncology increasingly embraces combination treatment strategies to overcome the limitations of single-modality therapies. Scorpion venom peptides, particularly those from *Mesobuthus eupeus*, have demonstrated radioprotective and stress-modulating properties. Their ability to enhance cellular responses under conditions such as radiation-induced damage is well-documented (14,15). While the DNA-damaging capacity of ionizing radiation underpins its clinical efficacy in cancer treatment, its impact is often limited by resistance mechanisms in certain malignancies, including CRC.

This study presents a pioneering investigation that addresses a critical gap in CRC research: to date, no studies have examined the combined use of MEV with irradiation for CRC. The central innovation lies in exploring the synergistic potential of MEV to enhance radiation-induced apoptosis and reduce cellular resistance. By investigating both the therapeutic promise and the underlying mechanisms of this unique combination, this work offers a novel and biologically grounded approach to improving radiotherapy outcomes in CRC.

MATERIALS AND METHODS

Collecting scorpion venom

Scorpions were collected in the Khuzestan province of Iran, and their identification was confirmed at the Razi Institute's scorpion reference laboratory (16). To extract venom from the *M. eupeus* scorpion, we applied a gentle electric shock (20 Volts,

500 mA) and dissolved the venom in sterile distilled water. The solution was centrifuged at $8000 \times g$ for 15 minutes at 4°C , after which the supernatant was freeze-dried and stored at -20°C for later use. Subsequently, we dissolved the raw venom in Dulbecco's Modified Eagle Medium (DMEM)- Thermo Fisher Scientific (Gibco, USA), and its protein content was measured using the Bradford method.

Fractionation of MEV

To isolate specific components of *M. eupeus* scorpion venom, we utilized gel filtration with Sephadex, prepared in a 0.02 M ammonium bicarbonate buffer (Merck, Sigma-Aldrich, USA) (pH 7.0). The Sephadex gel- Cytiva (Sweden) was packed into columns of various sizes depending on the amount of venom we had, and the gel was washed with the same ammonium bicarbonate buffer. During this process, we collected 5 ml of liquid from each wash cycle of the Sephadex gel columns, resulting in a total of 240. Subsequently, we applied 100 mg of crude scorpion venom to the columns and performed gradient elution. Initially, we used 1000 ml of 0.05 M ammonium acetate (pH 6.0) with 1% acetic acid, followed by 2000 ml of 0.4 M ammonium acetate (pH 8.0, adjusted with ammonia water) from Thermo Fisher Scientific (Invitrogen, USA). This washing procedure was continued until the Fractions were obtained. The second washing process involved using 1000 ml of 0.4 M ammonium acetate (pH 8.0) in the mixing vessel, followed by 1000 ml of 1.0 M ammonium acetate (pH 10.0) in the tank. During the process, protein elution was tracked by measuring absorbance at 280 nm with a spectrophotometer. Based on these absorbance data, we generated a concentration profile in Excel, which yielded seven distinct fractions. These fractions were then freeze-dried and dialyzed for 48 hours to remove residual ammonium acetate. Finally, the protein concentrations of these fractions were measured by the Bradford method with a NanoDrop spectrophotometer (NanoDrop One C, Thermo Fisher -USA). The prepared fractions were subsequently ready for further analysis.

Radiation conditions

Cells cultured as a monolayer in 96-well flasks were irradiated with 2Gy using a 6 MV clinical linear accelerator (Elekta Compact™, Sweden) at 200 cGy/min in a $30 \times 30 \text{ cm}^2$ field. To ensure proper dose build-up, a 1.5 cm-thick plexiglass sheet was placed beneath the flasks. Flasks were positioned at the beam isocenter, with the gantry set to 180° to deliver radiation from beneath. Dose calculations and verification were performed under the supervision of a medical physicist in the Radiotherapy Department of Golestan Hospital, Ahvaz, Iran. Control cells were transported to the accelerator without irradiation to maintain identical environmental conditions.

Cell culture

HT-29 cells (sourced from the Iranian Biological Resource Center) were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS)- Thermo Fisher Scientific (Gibco, USA), 100 U/ml streptomycin- Merck (Sigma-Aldrich, USA), and 100 U/ml penicillin G- Merck (Sigma-Aldrich, USA). The cells were maintained in a CO₂ incubator at 37°C with 5% CO₂ in a humidified environment.

Assessing cell viability

To evaluate the impact of MEV on cell viability, we conducted bioassays employing the MTT method. Cells were seeded in 96-well plates at a density of 8×10^3 cells per well and allowed to adhere for 24 hours in an incubator set at 37 °C with 5% CO₂. Subsequently, cells were exposed to varying concentrations of the F2 fraction of MEV toxin (0.025, 0.05, 0.1, 0.2, and 0.4 µg/ml) for 24 and 48 hours, corresponding approximately to one and two complete cell cycles of the HT-29 cell line (doubling time \approx 24 h), respectively, to assess both early and cumulative cellular responses. Untreated cells cultured in DMEM served as controls. Following each treatment, 100 µL of 0.5 mg/mL MTT solution (Sigma-Aldrich, USA) was added to each well and incubated for 4 hours. After incubation, the cells were washed with PBS (Thermo Fisher Scientific (Gibco, USA), and 100 µL of dimethyl sulfoxide (DMSO)- Merck (Sigma-Aldrich, USA) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA)- (USA) plate reader. Cell viability was calculated using the following formula:

$$\% \text{ viability} = \frac{\text{absorbance of treated cells}}{\text{absorbance of control cells}} \times 100\%$$

Additionally, we determined the IC₅₀ value for cancer cells to gauge effectiveness. Experiments were repeated five times to ensure accuracy.

Clonogenicity assessment

A colony formation assay was performed to assess the effect of MEV on the growth of HT-29 cells. Initially, 1000 cells were seeded into each well of four separate 6-well culture plates and allowed to grow for 24 hours. The first set served as untreated controls with 1000 cells seeded per well. The second, third, and fourth sets were irradiated with X-rays at doses of 2, 4, and 6Gy, respectively, with 100, 150, and 200 cells seeded per well, and subsequently subjected to the same culture conditions. After treatment, the cells were rinsed and incubated in a complete medium for 10 days. After incubation, the cells were washed with 1× PBS, then fixed with 4% paraformaldehyde at room temperature for 20 to 30 minutes, and stained with a 0.1% crystal violet/PBS solution for 10 minutes. Colonies containing a minimum of 50 cells were counted in triplicate wells for each treatment group. Results were quantified as

the percentage of colony formation in comparison to the untreated control group. The number of colonies was normalized to the plating efficiency of the control group, and the results were expressed as the surviving fraction (SF), representing the ratio of colonies formed after treatment to those formed in the untreated control.

Flow cytometry analysis

For flow cytometry analysis, cells were initially cultured and subjected to their respective treatment protocols, which included a control group, MEV, 2 Gy (used in conventional radiotherapy), and MEV+2 Gy. Following the treatment, the cells were collected and washed two times with cold PBS. Approximately 1×10^6 cells were resuspended in 100 µL of 1× binding buffer. To assess apoptosis, 5 µL of Annexin V-FITC (fluorescein isothiocyanate-Immunostep, Spain) and 5 µL of PI (propidium iodide) were added to the suspension. The cells were incubated in the dark at room temperature for 15 minutes. After staining, 400 µL of 1× binding buffer was added to each sample, and the cells were promptly analyzed using flow cytometry (BD FACS Calibur, USA). Data analysis was performed with FlowJo software (BD Life Sciences, USA).

Statistical analysis

GraphPad Prism v8.0 software was used for statistical analysis. Continuous variables are reported as mean \pm standard deviation (SD) and were analyzed with one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Protein concentration in MEV fractions

We measured the protein concentrations in the fractions obtained from scorpion venom using the NanoDrop device and then converted them to original values using a standard curve derived from the Bradford solution (table 1).

Table 1. Protein concentration in MEV fractions (µg/ml).

Fraction	protein concentration Measured (µg/ml)	Total protein concentration in the fraction (µg/ml)
F1	0.19	8.0
F2	0.45	19.2
F3	0.18	7.6
F4	0.06	2.4
F5	0.10	4.1
F6	0.11	4.5
F7	0.14	5.8

Fi: ith Fraction

Table 1 shows the protein concentrations for the seven distinct fractions (F1 through F7) obtained from MEV following gel filtration fractionation. The Optical Density (OD) was measured for each fraction at 280 nm using a spectrophotometer to track protein elution. The measured protein concentration (µg/ml) represents the protein content determined using the Bradford method with a NanoDrop spectrophotometer. These measured values were then converted to the Total protein concentration in the fraction (µg/ml) using a standard curve derived from the Bradford solution. MEV refers to Mesobuthus eupeus venom. µg/ml denotes micrograms per milliliter.

Effect of F2 of MEV on HT-29 cell viability using MTT assay

Figure 1 demonstrates the toxicity of different concentrations of F2 MEV on HT-29 cells using MTT assay in 24 and 48 hours. The results indicate a dose-dependent reduction in HT-29 cell viability due to MEV, with IC50 values of 0.3µg/ml at 24 hours and 0.2µg/ml at 48 hours, demonstrating increased cytotoxicity over time. Consequently, since the venom's full effects necessitate more time, further analyses were conducted at the 48-hour time point.

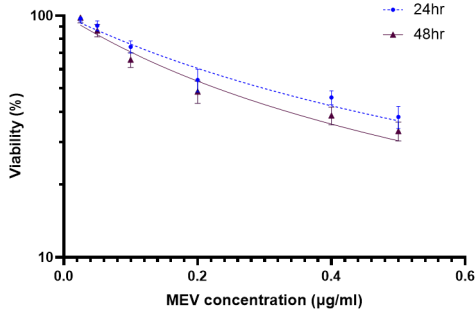


Figure 1. Viability of HT-29 cells treated with different concentrations of F2 MEV after 24 and 48 hours.

Clonogenic assay

Based on figure 2, the clonogenic assay evaluated the colony-forming ability of HT29 cancer cells under different X-ray doses (2, 4, and 6Gy) with/without MEV. "The group treated with MEV at an IC50 concentration of 0.2 µg/ml in combination with radiation exhibited a significant reduction in colony formation. In more detail, the MEV-treated group alone reduced colony formation to 71 ± 10%. The groups treated with radiation alone showed further reductions: 65 ± 9.9% for 2 Gy, 59 ± 8.1% for 4 Gy, and 52 ± 8.1% for 6 Gy. The combination of MEV with radiation resulted in even greater reductions in colony formation, with rates of 50 ± 7.2%, 47 ± 6.8%, and 38.9 ± 8.7% for MEV with 2, 4, and 6 Gy, respectively. This suggests a synergistic effect of MEV and radiation, leading to approximately a 30% increase in efficacy compared to radiation alone. The significant reduction in colony formation in the combination groups indicates a potential dose enhancement effect, which can be further evaluated by calculating the dose enhancement factor (DEF) for each radiation dose.

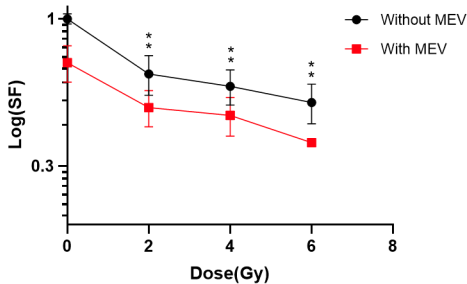


Figure 2. Colony formation of HT-29 cell groups treated with MEV (0.2 µg/ml) and exposed to 0, 2, 4, or 6 Gy X-rays. Statistical comparisons indicated by asterisks (**p < 0.01) represent differences between MEV-treated and untreated groups under the same radiation dose.

Flow cytometric analysis

Flow cytometry analysis was conducted following Annexin-PI staining to investigate the apoptosis rate in each treatment group. As presented in figure 3, the control group displayed high cell viability, with over 93.8% of cells remaining viable, indicating minimal apoptosis in the absence of treatment. In contrast, irradiation alone induced significant apoptosis, with 27.6% of cells undergoing apoptosis. The MEV-treated group showed approximately 80% cell viability, with 15.35% of cells undergoing apoptosis. This suggests a lesser cytotoxic effect of MEV treatment alone compared to irradiation. However, the combination of MEV and irradiation significantly increased apoptosis, with the MEV+ irradiation group exhibiting approximately 48.3% apoptosis, indicating a markedly enhanced cell death compared to either treatment alone.

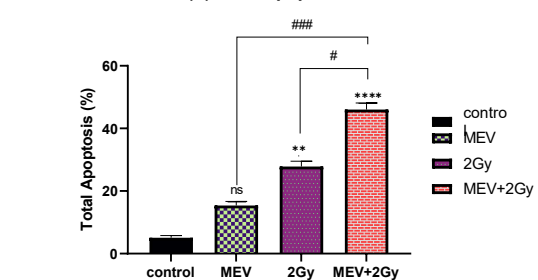
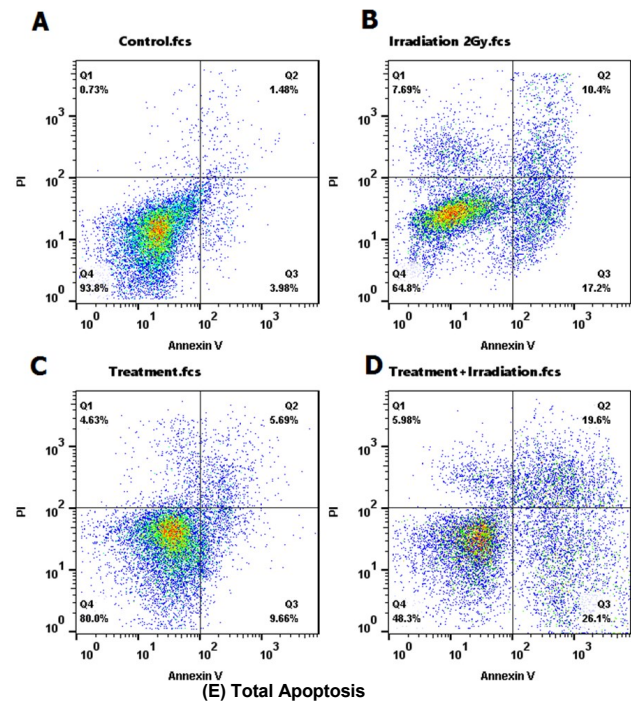


Figure 3. The apoptosis rate of HT-29 cells based on Annexin V/PI staining in (A) control group, (B) irradiation-treated group, (C) MEV group, and (D) MEV+ irradiation group. (E) Percentage of total apoptosis in control, MEV treatment, 2Gy irradiation, and MEV treatment + 2Gy irradiation groups. [ns: not significant, **: p<0.01, ****: p<0.0001, compared to the control group] [#: p<0.05, ###: p<0.001, compared to single treatment groups].

DISCUSSION

Our findings demonstrate that MEV possesses significant cytotoxic activity against HT-29 colorectal cancer cells, an effect which is synergistically enhanced when combined with radiotherapy. These results underscore the importance of MEV not only as an independent cytotoxic agent but also as a potential adjuvant for increasing the sensitivity of cancer cells to radiation. This synergistic effect could help mitigate major limitations in colorectal cancer treatment, particularly cellular radioresistance, and lay the groundwork for developing targeted combination therapies. Furthermore, these findings open avenues for more detailed investigation into the molecular mechanisms underlying the synergy between MEV and radiotherapy, which is crucial for optimizing treatment and reducing potential side effects⁽¹³⁾.

The present study revealed the notable cytotoxic effects of MEV on the HT-29 colorectal cancer cell line, characterized by a dose-dependent reduction in cell viability and specific IC50 values⁽¹³⁾. These findings are consistent with previous studies on MEV, which have confirmed its anticancer properties through the induction of apoptosis, increased production of reactive oxygen species (ROS), and disruption of mitochondrial function⁽⁷⁾. Notably, the significance of our study is reinforced by similar findings indicating that MEV can selectively target HT-29 cancer cells while showing no significant effects on normal cells⁽¹³⁾. This selectivity is of paramount importance and aligns with the urgent need for therapeutic agents with high precision and specificity in cancer treatment, a major goal in oncology⁽¹⁾.

A key finding of this study is the ability of MEV to potentiate the effects of radiation on HT-29 cells, as evidenced by a significant reduction in colony formation⁽⁶⁾. This synergy suggests that MEV can reduce the radioresistance of colorectal cancer cells, a well-known challenge in treating this malignancy⁽¹⁷⁾. The potential mechanisms behind this synergism are corroborated by a marked increase in apoptosis in cells treated concurrently with MEV and radiation⁽³⁾. These observations align with other studies on scorpion venoms, including that of *Leiurus quinquestriatus*, which have demonstrated that venom components can modulate oxidative stress and apoptotic pathways critical for tumor cell death⁽¹⁵⁾. Consequently, our study provides further evidence supporting the potential of scorpion venom to enhance the sensitivity of cancer cells to radiation-induced DNA damage⁽¹⁷⁾.

This study is the first to investigate the combined use of MEV and radiotherapy for colorectal cancer, thereby providing novel insights into the mechanisms underlying this synergistic interaction, particularly the enhancement of oxidative stress and apoptosis by MEV in combination with radiation⁽¹³⁾.

Given the challenges of conventional colorectal cancer therapies, such as treatment resistance and side effects, along with the need for agents with high specificity and sensitivity, our findings propose a promising complementary therapeutic strategy. The ultimate goal is to develop potent and selective adjuvant therapies that can significantly improve patient survival and quality of life^(1, 18, 19).

However, this study has limitations, including its *in vitro* design and the use of only one cell line (HT-29), which may not fully reflect the complex tumor microenvironment present *in vivo*⁽¹³⁾. Therefore, to confirm efficacy, optimize dosing, and ensure the safety of MEV combined with radiotherapy, future research must encompass comprehensive studies in animal models and, ultimately, clinical trials⁽¹⁷⁾. Moreover, a more detailed investigation into the molecular pathways through which MEV exerts its synergistic effects with radiation—including its impact on apoptotic pathways and oxidative stress—is essential for a complete understanding of its mechanism of action⁽¹⁴⁾. These efforts will be a fundamental step towards establishing MEV as an integral component of combination treatment regimens for colorectal cancer⁽¹³⁾.

CONCLUSION

The present study reveals the remarkable potential of MEV as an adjuvant therapy in the radiotherapy of CRC. The findings demonstrated that MEV effectively reduced the viability of HT-29 colorectal cancer cells and, more importantly, synergistically enhanced the apoptotic effects of irradiation. This remarkable synergistic interaction between MEV and irradiation not only suggests a way to increase the sensitivity of cancer cells to radiation-induced damage but also may help overcome therapeutic resistance, which is one of the major challenges in the treatment of colorectal cancer. As the first study to investigate the combined application of MEV and irradiation for CRC, this research is a pioneering step in the discovery of novel therapeutic strategies.

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