Yüksek İhtisas Üniversitesi
Sağlık Bilimleri Dergisi
Yüksek İhtisas University Journal of Health Sciences
ISSN 2717-8439
E-ISSN 2717-9257

ARAŞTIRMA MAKALESİ / RESEARCH ARTICLE

YIU Saglik Bil Derg 2026;7(2):65–71
https://doi.org/10.51261/yiu.2026.1908432

Determination of the Effect of Androctonus crassicauda (Oliver, 1807) Crude Venom on Bcl-2 and Bax Genes Expression Levels in Colon Cancer Cell Line (HT-29) Using qPCR Method

Androctonus crassicauda (Oliver, 1807) Ham Zehirinin Kolon Kanseri Hücre Hattı (HT-29) Üzerindeki Bcl-2 ve Bax Genleri Expresyon Düzeylerine Etkisinin qPCR Yöntemi ile Belirlenmesi

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ABSTRACT

Introduction: Colorectal cancer (CRC) is one of the most common malignancies worldwide and remains a leading cause of cancer-related mortality, particularly when diagnosed at advanced stages. The search for novel anticancer agents derived from natural products has attracted increasing attention, with scorpion venoms representing a promising source of biologically active compounds.

Material and Methods: This study investigated the in vitro cytotoxic effects of crude venom obtained from Androctonus crassicauda (Oliver, 1807) on human colorectal cancer cells. Human colorectal adenocarcinoma cells (HT-29) were used as the experimental model, whereas human embryonic kidney cells (HEK-293) served as the non-cancerous control.

Results: Cell viability was assessed using the MTT assay, and the half-maximal inhibitory concentration (IC_{50}) values were determined to be 40.54 mg/mL for HT-29 cells and 57.30 mg/mL for HEK-293 cells. The expression levels of apoptosis-related genes were further analyzed by quantitative real-time PCR (qPCR). The analysis of gene expression levels using the qRT-PCR method showed that Bax gene expression decreased by approximately 0.25-fold in HT-29 cells compared with control group, whereas it increased approximately fivefold in HEK-293 cells. Similarly, Bcl-2 expression decreased to approximately 0.5-fold in HT-29 cells while increasing approximately 2.5- fold in HEK-293 cells.

Conclusion: The lower IC_{50} value observed in HT-29 cells indicates that Androctonus crassicauda crude venom exerts greater cytotoxic activity against colorectal cancer cells than against non-cancerous cells. Although the observed changes in apoptosis-related gene expression suggest that the venom modulates cellular death pathways, further studies are required to elucidate the underlying molecular mechanisms and to identify the bioactive venom components responsible for these effects. Overall, these findings support the potential of A. crassicauda venom as a promising natural source of bioactive molecules for the development of novel anti-cancer therapeutics.

Keywords: Androctonus crassicauda, scorpion venom, colorectal cancer, cytotoxicity, apoptosis, HT-29, HEK-293, qPCR

ÖZ

Giriş: Kolorektal kanser, dünya çapında en sık görülen malignitelerden biridir ve özellikle ileri evrelerde teşhis edildiğinde kansere bağlı ölümlerin önde gelen nedenlerinden biri olmaya devam etmektedir. Doğal ürünlerden elde edilen yeni anti-kanser ajanların aranması giderek artan bir ilgi görmektedir ve akrep zehirleri, biyolojik olarak aktif bileşikler için umut vadeden bir kaynak olarak öne çıkmaktadır.

Materyal ve Metodlar: Bu çalışma, Androctonus crassicauda (Oliver, 1807)'den elde edilen ham zehirin insan kolorektal kanser hücreleri üzerindeki in vitro sitotoksik etkilerini araştırmıştır. Deneysel model olarak insan kolorektal adenokarsinom hücreleri (HT-29) kullanılırken, kontrol olarak non-kanseröz insan embriyonik böbrek hücreleri (HEK-293) kullanılmıştır.

Bulgular: Hücre canlılığı MTT testi kullanılarak değerlendirilmiş ve yarı maksimum inhibitör konsantrasyon (IC_{50}) değerleri HT-29 hücreleri için 40,54 mg/mL ve HEK-293 hücreleri için 57,30 mg/mL olarak belirlenmiştir. Apoptoz-ilişkili genlerin ekspresyon düzeyleri, kantitatif gerçek zamanlı PCR (qPCR) ile daha ayrıntılı olarak analiz edilmiştir. qRT-PCR yöntemi kullanılarak yapılan gen ekspresyon düzeyleri analizi, Bax gen ekspresyonunun HT-29 hücrelerinde kontrol grubuna kıyasla yaklaşık 0,25 kat azaldığını, HEK-293 hücrelerinde ise yaklaşık beş kat arttığını göstermiştir. Benzer şekilde, Bcl-2 ekspresyonu HT-29 hücrelerinde yaklaşık 0,5 kat azalırken, HEK-293 hücrelerinde yaklaşık 2,5 kat artmıştır.

Sonuç: HT-29 hücrelerinde gözlemlenen daha düşük IC_{50} değeri, Androctonus crassicauda ham zehirinin, kanserli olmayan hücrelere kıyasla kolorektal kanser hücrelerine karşı daha yüksek sitotoksik aktivite gösterdiğini göstermektedir. Apoptoza bağlı gen ekspresyonundaki gözlemlenen değişiklikler, zehirin hücresel ölüm yolaklarını modüle ettiğini düşündürse de, altta yatan moleküler mekanizmaları aydınlatmak ve bu etkilerden sorumlu biyoaktif zehir bileşenlerini belirlemek için daha fazla çalışmaya ihtiyaç vardır. Genel olarak, bu bulgular, A. crassicauda zehrinin yeni anti-kanser terapötiklerin geliştirilmesi için umut vadeden doğal bir biyoaktif molekül kaynağı olarak potansiyelini desteklemektedir.

Anahtar Sözcükler: Androctonus crassicauda, akrep zehiri, kolorektal kanser, sitotoksisite, apoptoz, HT-29, HEK-293, qPCR

Cite this article as: Perktaş Bağcı N, Yiğit Kayhan N, Çorak Özel İ. Determination of the Effect of Androctonus crassicauda (Oliver, 1807) Crude Venom on Bcl-2 and Bax Genes Expression Levels in Colon Cancer Cell Line (HT-29) Using qPCR Method. YIU Saglik Bil Derg 2026;7(2):65–71. https://doi.org/10.51261/yiu.2026.1908432

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Geliş Tarihi/Received: 16.03.2026, **Kabul Tarihi/Accepted:** 03.08.2026, **Çevrimiçi Yayın Tarihi/ Available Online Date:** 27.08.2026

Introduction

Cancer remains one of the leading global health challenges and is characterized by the uncontrolled proliferation of abnormal cells that invade surrounding tissues and metastasize to distant organs, resulting in high morbidity and mortality. Despite substantial advances in cancer therapy, the development of novel therapeutic strategies capable of overcoming drug resistance and improving treatment efficacy remains a major research priority (1).

Among the natural products investigated for anticancer applications, scorpion venom has emerged as a promising source of bioactive molecules. All scorpion species produce venom, a highly complex and heterogeneous secretion synthesized in specialized venom glands located within the metasoma. Scorpion venom contains a diverse array of biologically active compounds, including peptides, proteins, enzymes, and other low-molecular-weight molecules with a wide range of pharmacological activities (2). In particular, venom-derived peptides and proteins have attracted considerable attention because of their selective cytotoxicity toward cancer cells and their diverse mechanisms of action. These bioactive molecules have been reported to exert anticancer effects through apoptosis induction, inhibition of angiogenesis, immune modulation, oxidative stress regulation, DNA damage, and cell cycle arrest (3). Recent studies have further demonstrated the diagnostic and therapeutic potential of scorpion venom peptides against several malignancies, including glioma, breast, prostate, and colorectal cancers. Consequently, well-characterized venom-derived peptides are increasingly recognized as promising candidates for the development of selective anticancer agents that may enhance therapeutic efficacy while minimizing adverse effects (4). Among these, chlorotoxin (CTX) and neopladin-1/2 have shown remarkable anticancer activity by targeting specific signaling pathways, suppressing tumor cell migration and metastasis, and promoting apoptosis (1).

Several studies have evaluated the cytotoxic activity of venoms obtained from scorpion species belonging to the family Buthidae, which includes some of the world's most medically significant scorpions. These venoms have been tested against a variety of normal and cancer cell lines, including MRC-5 (normal human lung fibroblasts), MIA PaCa-2 (pancreatic carcinoma), HeLa (cervical adenocarcinoma), MCF-7 (breast adenocarcinoma), and A375 (human melanoma) cells. Although certain venoms exhibited limited or no cytotoxic effects in some cell lines, they induced pronounced, dose-dependent cytotoxicity in others, suggesting that specific venom components may display selective activity against particular cancer types (5). These findings highlight the potential of scorpion venoms as valuable resources for the discovery of novel anticancer compounds and underscore their growing importance in cancer biology research (6).

Colorectal cancer (CRC) is one of the most prevalent malignancies worldwide and remains a major cause of cancer-related mortality. According to GLOBOCAN 2023 statistics, CRC ranks among the three most frequently diagnosed cancers and is one of the leading causes of cancer deaths globally (7). Although surgery, radiotherapy, and chemotherapy constitute the cornerstone of current treatment strategies, therapeutic resistance, tumor recurrence, and treatment-associated toxicity continue to compromise clinical outcomes and patients' quality of life (7). Therefore, the identification of novel anticancer compounds from natural sources has become an important area of investigation. In this context, a recent study demonstrated that venom obtained from Scorpio fuscus significantly reduced the viability of human colorectal cancer cells in a dose-dependent manner while also suppressing cell migration and colony formation, highlighting its potential as a promising therapeutic candidate for colorectal cancer treatment (8).

Growing evidence indicates that animal venoms and venom-derived biomolecules represent an important reservoir of novel therapeutic agents for cancer diagnosis and treatment (9). Continued investigation of these naturally occurring compounds may facilitate the development of innovative, selective, and more effective anticancer therapies. Therefore, the present study aimed to investigate the cytotoxic activity of crude venom obtained from *Androctonus crassicauda* (Oliver, 1807) against human colorectal cancer cells and to evaluate its potential as a source of novel anticancer compounds.

Material and Methods

Venom Extraction

Crude venom was obtained from *Androctonus crassicauda* (Oliver, 1807) one of the most medically important scorpion species, using a mild electrical stimulation (12 V) method. Immediately after collection, the venom was transferred to sterile microcentrifuge tubes and stored under appropriate conditions until use.

Cell Culture

Human colorectal adenocarcinoma cells (HT-29) and human embryonic kidney cells (HEK-293) were used in this study. HEK-293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), and 100 U/mL penicillin–streptomycin. HT-29 cells were cultured in DMEM supplemented with 2 mM L-glutamine, 10% FBS, 100 U/mL penicillin–streptomycin, and 1% Eagle's non-essential amino acids (NEAA). Both cell lines were maintained at 37°C in a humidified incubator containing 5% CO_2.

Cell Count

Cell viability and concentration were determined using the trypan blue exclusion method. Briefly, 10 µL of the cell suspension was mixed with an equal volume of 0.4% trypan

blue solution. The mixture was loaded onto a hemocytometer (Thoma chamber), and viable cells were counted under a light microscope. Cell numbers were calculated by considering the dilution factor, and the final cell concentration was adjusted for subsequent experiments.

Determination of the Protein Content of Crude Scorpion Venom

Crude venom was dissolved in double-distilled water and centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant containing soluble venom proteins and peptides was carefully collected for protein quantification. Protein concentration was determined using the bovine serum albumin (BSA) standard curve method. A BSA stock solution (10 mg/mL) was serially diluted to prepare standards at 0.156, 0.312, 0.625, 1.25, 2.5, 5.0, and 10.0 mg/mL. Absorbance values were measured using a spectrophotometer, and a standard calibration curve was generated from the absorbance values of the BSA standards. Protein concentrations were calculated from the linear regression equation.

MTT Test

Cell viability was evaluated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. HT-29 and HEK-293 cells were seeded into 96-well plates at a density of 1×10^4 cells per well and incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. The cells were then treated with various concentrations (0.310 mg/ml, 0.775 mg/ml, 0.775 mg/ml, and 1.550 mg/ml) of *A. crassicauda* crude venom and incubated for an additional 24 h. Subsequently, 10 µL of MTT solution was added to each well and incubated for 4 h. The resulting formazan crystals were dissolved in 100 µL dimethyl sulfoxide (DMSO), and absorbance was measured at 570 nm using a microplate reader (BioTek Instruments, USA). Cell viability was expressed as a percentage relative to untreated control cells, and the half-maximal inhibitory concentration (IC_{50}) values were calculated.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from treated and untreated cells using a standard RNA isolation protocol according to the manufacturer's instructions. RNA concentration and purity were determined spectrophotometrically before complementary DNA (cDNA) synthesis. cDNA was synthesized from the isolated total RNA using the NucleoGene cDNA Synthesis Kit according to the manufacturer's instructions. Briefly, the reaction mixture containing total RNA, 5× reaction buffer, reverse transcriptase enzyme mix, and nuclease-free water was prepared according to the kit protocol. Reverse transcription was carried out in a thermal cycler (Eppendorf-Mastercycler) under the recommended reaction conditions to generate first-strand cDNA. The synthesized cDNA was subsequently stored at −20°C until use in quantitative real-time PCR (qRT-PCR) analyses. Quantitative real-time PCR (qRT-PCR) was performed using a SYBR Green detection system to evaluate gene expression levels. The expression levels of the apoptosis-related genes Bax and Bcl-2 were analyzed. GAPDH (housekeeping gene) was used as the endogenous reference gene for normalization. The primer sequences used for quantitative real-time PCR (qRT-PCR) analysis are presented in Table 1. Relative gene expression levels were calculated using the 2^−ΔΔCt method, with GAPDH serving as the endogenous reference gene. Gene expression levels were normalized to the control group, which was assigned a value of 1.

Statistical Analysis

All experiments were performed in triplicate (n = 3) and the results are presented as the mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Differences among groups were analyzed using two-way analysis of variance (two-way ANOVA). A p-value of less than 0.05 was considered statistically significant.

Results

The Effect of *A. crassicauda* (Oliver 1807) Crude Venom on Cell Viability

The effects of *A. crassicauda* (Oliver 1807) crude venom at concentrations of 0.31 mg/ml, 0.775 mg/ml, and 1.55 mg/ml on HT-29 colon cancer and HEK-293 human embryonic kidney epithelial cell lines were evaluated. A significant decrease in cell viability was observed after 24 hours of treatment with crude venom. The MTT cytotoxicity assay revealed that the highest concentration of cell death occurred at the highest venom concentration of 1.55 mg/ml. The experiment was conducted with three repetitions.

Table 1. Primer sequences used for qRT-PCR analysis.

Gene	Primer	Sequence (5′→3′)	Amplicon (bp)	Annealing (°C)
GAPDH	Forward	GAAATCCCATCACCACTTCCAGG	138	60
GAPDH	Reverse	GAGCCCCAGCCTTCTCCATG	138	60
Bax	Forward	GATGCGTCCACCAAGAAGC	152	60
Bax	Reverse	AAGTCCAATGTCCAGCCCAT	152	60
Bcl-2	Forward	TGTGGATGACTGAGTACCTGAACC	147	60
Bcl-2	Reverse	CAGCCAGGAGAAATCAAACAGAG	147	60

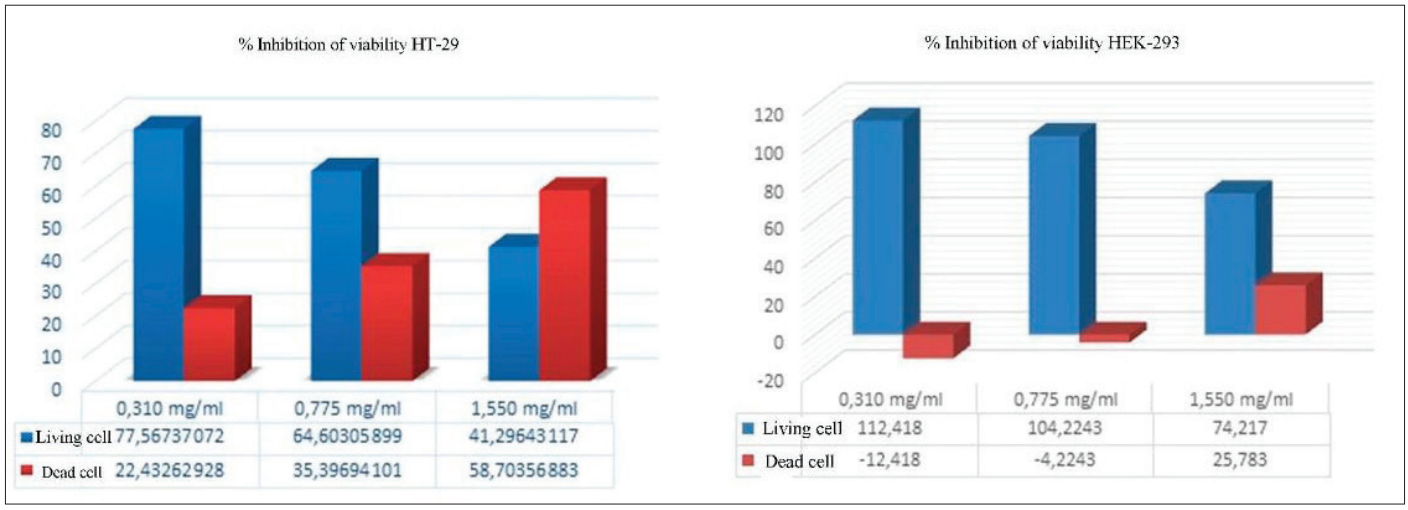

Figure 1. The effect of *A. crassicauda* crude venom on the viability of HT-29 colon cancer and HEK-293 human embryonic kidney epithelial cells

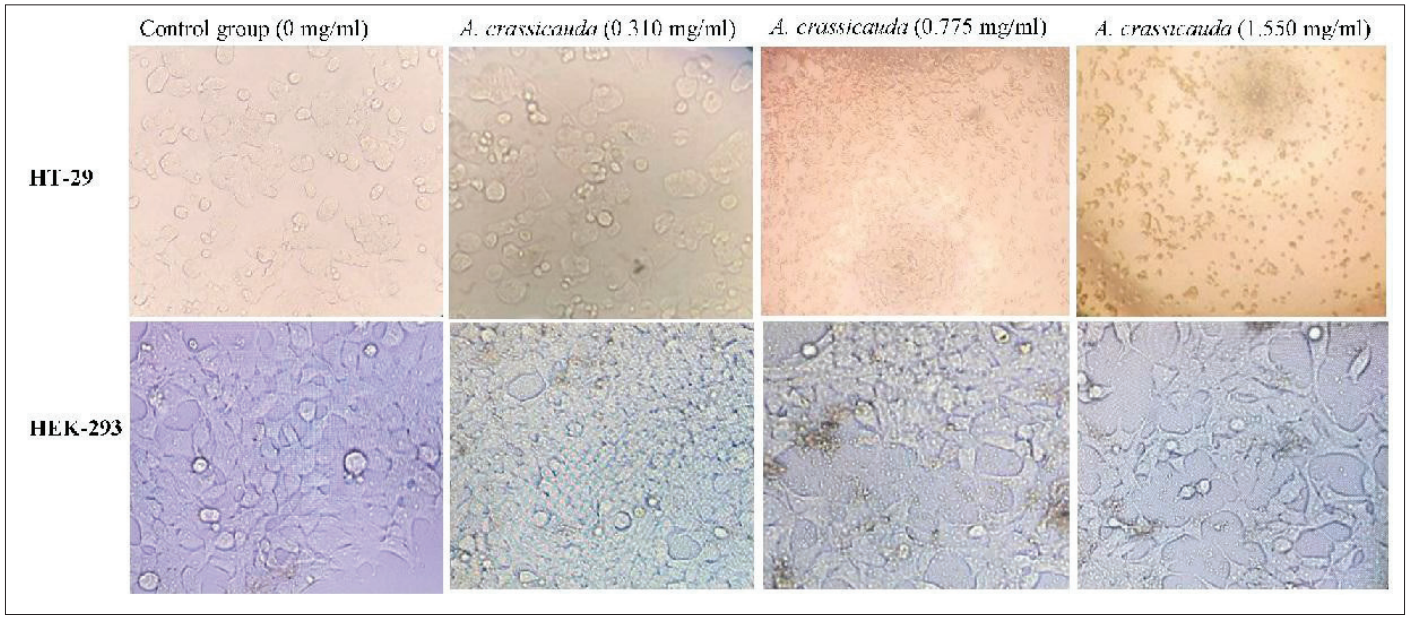

Figure 2. Inverted microscope images of cell lines treated with different concentrations of *A. crassicauda* scorpion crude venom for 24 hours.

MTT Test Results

The MTT test was used to determine the cytotoxic effect of *A. crassicauda* crude venom on HT-29 colon cancer and HEK-293 human embryonic kidney epithelial cell lines. The absorbance values obtained from the ELISA plate reader have been calculated. Crude venom samples were applied at different concentrations (0.310 mg/ml, 0.775 mg/ml, 0.775 mg/ml, and 1.550 mg/ml). Cell viability in the HT-29 colon cancer cell line was found to be 77.5%, 64.6%, and 41.29%, respectively (Figure 1). Similarly, in the HEK-293 human embryonic kidney epithelial cell line, cell viability was found to be 112.41%, 104.22%, and 74.21% respectively. According to these results, the crude venom of *A. crassicauda* is more cytotoxic against HT-29 colon cancer cells than against HEK-293 human embryonic kidney epithelial cells.

Statistical Analysis of the MTT Assay

The MTT assay results were statistically analyzed using two-way analysis of variance (two-way ANOVA) in GraphPad Prism. Statistical significance was set at $p<0.05$. Comparisons among treatment groups demonstrated significant differences in cell viability following exposure to *Androctonus crassicauda* crude venom. In particular, treatment with 0.775 mg/mL crude venom for 24 h significantly reduced the viability of HT-29 cells compared with HEK-293 cells ($p<0.05$). The statistical analysis was performed at a 95% confidence level, and the results are presented in Figure 2.

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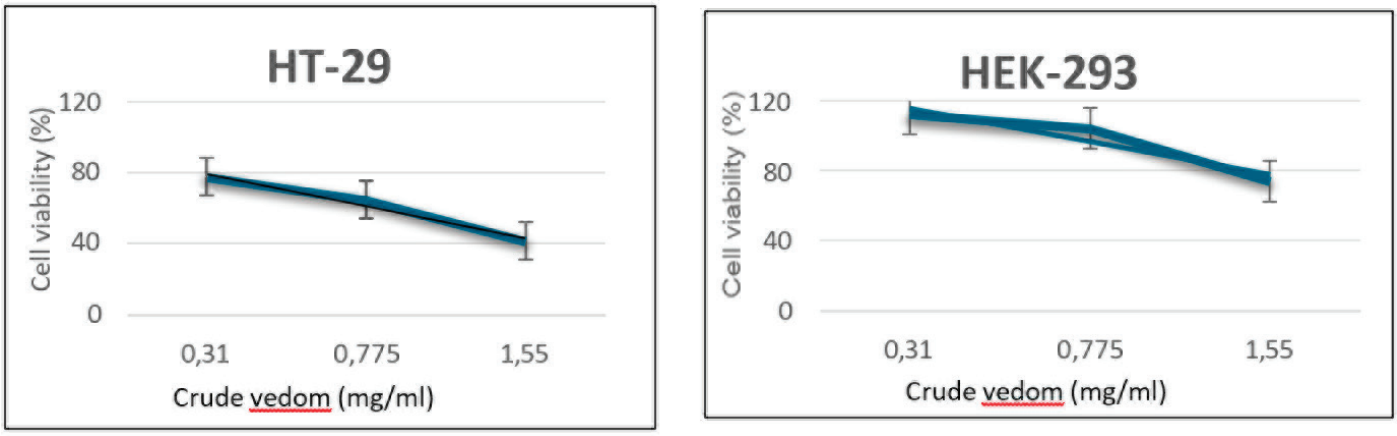

Figure 3. Effect of *Androctonus crassicauda* crude venom on the viability of HT-29 and HEK-293 cells after 24 h of treatment. Cell viability was determined using the MTT assay. Data are presented as mean ± SD.

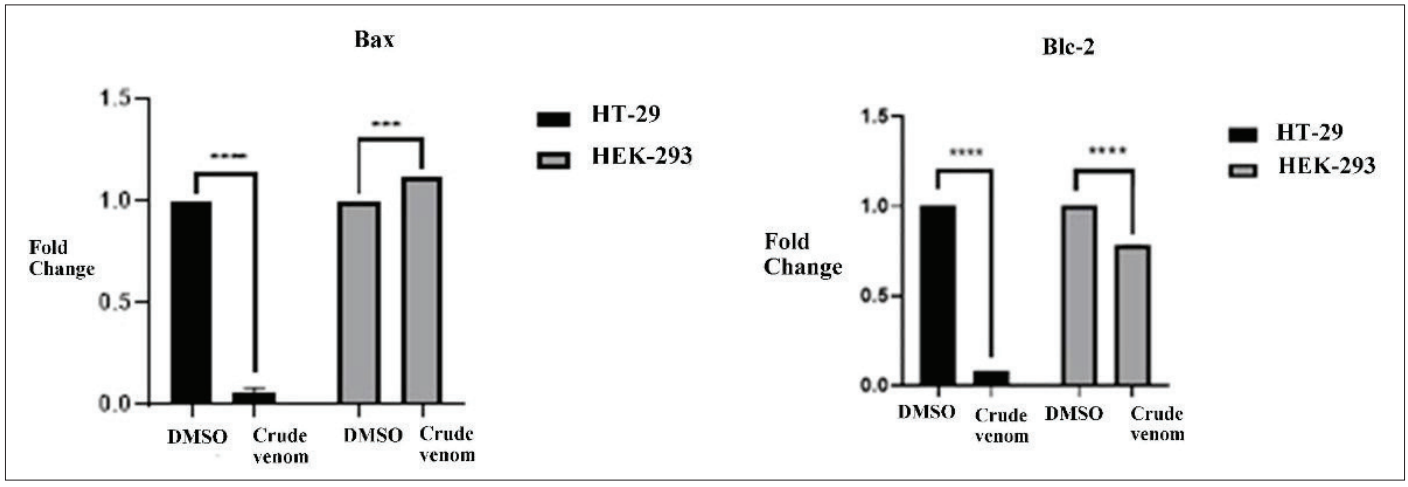

Figure 4. Evaluation of Bax and Bcl-2 gene expression by RT-qPCR in HT-29 (IC50: 40.54 mg/ml) and HEK-293 (IC50: 57.3 mg/ml) cell lines treated with crude venom from *A. crassicauda* scorpion (1.55 mg/ml).

cell viability following exposure to *Androctonus crassicauda* crude venom. In particular, treatment with 0.775 mg/mL crude venom for 24 h significantly reduced the viability of HT-29 cells compared with HEK-293 cells ($p<0.05$). Statistical analyses were performed at a 95% confidence level. The effects of crude venom on the viability of HT-29 and HEK-293 cells are shown in Figure 3.

RT-qPCR Analysis of Apoptosis-Related Gene Expression

Following treatment of normal and cancer cell lines with crude *A. crassicauda* venom, changes in apoptosis-related gene expression were evaluated using RT-qPCR in order to investigate the apoptotic pathways induced by the venom. Based on the MTT assay results, the appropriate venom concentration was selected, and the expression levels of Bax and Bcl-2 genes were analyzed to determine which apoptotic pathways were activated. Gene expression levels were normalized to the GAPDH reference gene and calculated relative to the untreated control group.

Relative quantification was performed using the 2^−ΔΔCT method. The results showed that Bax expression decreased by approximately 0.25-fold in the HT-29 cell line compared with the control group, whereas it increased by approximately 5-fold in the HEK-293 cell line. Similarly, Bcl-2 gene expression decreased by approximately 0.5-fold in the HT-29 cell line relative to the control, while a 2.5-fold increase was observed in the HEK-293 cell line.

Overall, treatment with the venom resulted in reduced mRNA levels of both Bcl-2 and Bax genes in cancer cells, whereas an increase in the mRNA expression of both genes was observed in venom-treated normal cells (Figure 4).

Discussion

Cancer remains one of the leading causes of mortality worldwide despite considerable advances in diagnosis and treatment. The emergence of drug resistance and the adverse effects associated with conventional therapies have stimulated the search for novel

anticancer compounds from natural sources. Among these, scorpion venoms have attracted increasing attention because they contain a diverse repertoire of biologically active peptides and proteins capable of modulating multiple cellular pathways involved in tumor progression.

In the present study, the in vitro cytotoxic activity of crude venom obtained from *Androctonus crassicauda*, one of the most medically important scorpion species distributed in southeastern Türkiye, was evaluated against HT-29 human colorectal adenocarcinoma cells and HEK-293 human embryonic kidney cells. The results demonstrated a concentration-dependent reduction in cell viability, with HT-29 cells exhibiting greater sensitivity to the venom than HEK-293 cells. The lower IC_{50} value obtained for HT-29 cells (40.54 mg/mL) compared with HEK-293 cells (57.30 mg/mL) suggests that the crude venom displays greater cytotoxic activity toward colorectal cancer cells than toward non-cancerous cells.

The anticancer potential of scorpion venoms has been documented in numerous studies. Venoms from species belonging to the family Buthidae contain peptides capable of interacting with ion channels and intracellular signaling pathways involved in cell proliferation, migration, and apoptosis. For example, peptides isolated from *Buthus martensii* have been reported to inhibit the proliferation of malignant glioma cells by blocking ion channels and inducing apoptotic cell death (10). Similarly, venom-derived compounds from other buthid scorpions have demonstrated cytotoxic activity against several human cancer cell lines, highlighting the therapeutic potential of these natural toxins.

To further investigate the molecular response to venom treatment, the expression of the apoptosis-related genes Bax and Bcl2 was analyzed by qRT-PCR. Both genes showed reduced expression in HT-29 cells following venom exposure, whereas their expression increased in HEK-293 cells. Members of the Bcl-2 protein family play central roles in regulating mitochondrial membrane permeability and apoptotic signaling. While Bcl-2 proteins generally promote cell survival, Bax proteins facilitate mitochondrial outer membrane permeabilization and apoptosis (11). The simultaneous downregulation of both Bax and Bcl-2 observed in the present study suggests that *A. crassicauda* venom modulates apoptosis-related gene expression; however, these transcriptional changes alone are insufficient to establish activation or inhibition of apoptosis. Additional studies examining protein expression, caspase activation, mitochondrial membrane potential, and Annexin V/propidium iodide staining are required to clarify the mechanisms underlying the cytotoxic effects of the venom.

Previous studies have also demonstrated the anticancer potential of *A. crassicauda* venom components. For example, the peptide Acra3, isolated from *A. crassicauda*, exhibited marked cytotoxicity against mouse brain tumor cells, with an IC_{50} value of 5 µg/mL (12). Likewise, venom from *Mesobuthus eupeus* significantly inhibited the proliferation of HT-29 colorectal cancer cells and altered the expression of numerous genes associated with apoptotic pathways (13). In addition, venom obtained from *Androctonus amoreuxi* has been shown to suppress tumor growth through cytotoxic, apoptotic, and anti-angiogenic mechanisms (14). These studies collectively support the growing evidence that scorpion venoms contain bioactive molecules with selective anticancer properties.

Recent studies have further highlighted the therapeutic potential of scorpion venoms against colorectal cancer. Using an experimental rat model, *Leiurus quinquestriatus and Androctonus bicolor* venoms were shown to exert significant antitumor activity by modulating cell proliferation, apoptosis, and the expression of genes associated with colorectal carcinogenesis (15). Similarly, demonstrated that scorpion venom possesses anti-angiogenic properties by suppressing neovascularization required for tumor growth and reducing the expression of angiogenesis-related biomarkers, thereby limiting tumor progression (16). More recently, *Scorpio fuscus* venom was reported to reduce the viability of colorectal cancer cells, induce apoptosis, and suppress tumor growth in vitro, supporting its potential as a promising source of anticancer bioactive compounds (8). Collectively, these findings, together with the results of the present study, strengthen the growing evidence that scorpion venoms contain biologically active molecules with considerable potential for the development of novel therapeutic strategies against colorectal cancer.

Although the crude venom exhibited greater cytotoxicity toward HT-29 cells than HEK-293 cells, the relatively high IC_{50} values indicate that the crude venom is less potent than purified venom-derived peptides reported in the literature. This difference is expected because crude venom contains a complex mixture of biologically active and inactive components that may reduce the apparent activity of individual anticancer molecules. Consequently, purification and characterization of the active venom constituents are likely to enhance their therapeutic potential.

Overall, the present findings demonstrate that crude *A. crassicauda* venom exerts concentration-dependent cytotoxic effects against human colorectal cancer cells while exhibiting comparatively lower toxicity toward non-cancerous cells. Although the precise molecular mechanisms remain to be elucidated, these results support the potential of *A. crassicauda* venom as a valuable natural source of bioactive molecules for anticancer drug discovery. Future studies should focus on the purification of active venom peptides, identification of their molecular targets, and validation of their efficacy through comprehensive mechanistic investigations and in vivo experimental models.

Ethical Considerations: Ethical committee approval was not required for this study, as it involved only in vitro experiments using established cell lines and did not involve human participants or animals.

Peer-review: Externally peer-reviewed.

Conflict of Interest: No conflict of interest was declared by the authors.

Funding: This research was funded by Scientific Research Projects Coordination Unit of Kırıkkkale University, grant number 2023/039.

Consent of Patients: The participants were informed in detail, and informed consent was obtained.

Data Availability Statement: All relevant data are within the paper and they are available from the corresponding author on reasonable request.

Author Contributions: Concept- NPB, NYK; Design- NPB, NYK; Supervision- NPB, NYK; Providing Resources and Funding- NPB, NYK; Materials- NPB, NYK; Data Collection and/or Processing- NPB, NYK, İÇÖ; Analysis or Interpretation- NPB, NYK; Literature Search-NPB; Writing- NPB; Critical Review- NPB, NYK.

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