

The Effect of the Persian Gulf Jellyfish (*Cassiopea andromeda*) Venom on the Expression of P15, P21, P53, DNMT1, and Bcl-2 in Acute Lymphoblastic Leukemia Jurkat Cells

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Received: 04, Mar, 2021

Accepted: 19, Jul, 2021

ABSTRACT

Background: One of the acute hematologic malignancies is acute lymphoblastic leukemia (ALL), which is formed in B or T lymphocyte stem cells. Regarding the increasing tendency to herbal and marine studies and unclear characteristics of *Cassiopea andromeda* Venom, this study was performed to determine its effects on Jurkat cells as a model for T-ALL.

Materials and Methods: In this experimental study, the cells were treated with a variety of concentrations of *Cassiopea andromeda* venom at different periods and times. Growth inhibition and toxic effects of *Cassiopea andromeda* Venom were evaluated by methyl thiazole tetrazolium salt reduction (MTT test). The flow cytometry analysis was carried out using 7-aminoactinomycin D (7AAD) and Annexin V stains to evaluate the venom's effect on apoptotic pathways. Besides, Real-Time PCR was performed to evaluate the relative gene expression.

Results: *Cassiopea andromeda* venom inhibited the growth of Jurkat cells in a concentration and time manner. Jurkat cell growth was inhibited by 48.9% after 72 hours of treatment with 250µg/mL *Cassiopea andromeda* venom. The venom increased the apoptotic process through the upregulation of p15INK4b and P53 proteins and downregulation of Bcl-2, p21^{WAF1/CIP1}, and DNMT1 in the Jurkat cell line.

Conclusion: Considering the growth inhibitory property of *Cassiopea andromeda* Venom, we recommend it as a part of combinational medication for treating ALL in animal trials and for other leukemias in vitro studies.

Keywords: Acute lymphoblastic leukemia; Apoptosis; Cnidaria

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy¹. Many studies on the treatment and prevention of cancers have

focused on cell cycle, apoptosis, epigenetics, and their association with cancer. Gene expression has also been a significant issue. In this regard, the p53 transcription factor has been considered in studies

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due to its essential role in apoptosis, DNA repair, and cell cycle². Moreover, a protein called p15 (also known as CDKN2B, INK4B, p15INK4B) captures the cell cycle in the G1 phase by inhibiting cyclin-dependent kinase 4/6 (CDK4 / 6)³. Another important cell cycle protein that has received much attention is p21, a major member of cyclin-dependent kinase inhibitors, operating dependently or independently of p53⁴.

DNA methylation is one of the notable mechanisms of epigenetic changes in eukaryotic cells, occurring by adding a methyl group to the 5-position of cytosine rings in CpG islands. Because of the multiplicity and functional diversity of DNA methyltransferase (DNMT) enzymes, which regulate cell cycle methylation and DNA repair gene promoters under other cell physiological processes, DNA methylation patterns are also diverse⁵. Moreover, it is now well established that the major regulators of apoptosis are the Bcl-2 family proteins. BCL-2 is the first proto-oncogene discovered with an anti-apoptotic function, which is associated with several cancers⁶.

Marine ecosystems have long been described as an unfinished biologically active resource with extensive medicinal properties. Hence, the recognition and introduction of new compounds with selective toxicity can be useful in basic science research. Sea-derived compounds, due to their chemical complexity and biological diversity, can be considered as major candidates for the discovery of new therapeutic agents⁷. Jellyfish is one of the Cnidarians family that have been introduced pharmacologically active sea-compound. Recent studies have shown that different species of jellyfish can play important roles, including antimicrobial activity, antioxidant effects, and cytotoxicity^{8,9}. Furthermore, its antitumor property and growth inhibitory effect have been shown in several studies. In particular, Jellyfish extract venom has shown to be apoptotic and could arrest the cell cycle in chronic myelogenous leukemia K562 cell line¹⁰. Cassiopea Andromeda, is another member of the Cnidaria family, called the "upside-down jellyfish"¹¹. Although this species was native to the eastern Mediterranean, it was first observed in 2014 in the coastal waters of the Persian Gulf of

Bushehr-Iran, Nayband Bay¹². Several studies have shown that C. Andromeda venom can act as a harmful compound. For instance, the potential of C. Andromeda venom to cause apoptosis and cytotoxicity through mitochondrial pathway in human breast adenocarcinoma tissue have been shown in previous studies¹³.

Since no studies have examined the effect of C. Andromeda venom on lymphoblastic leukemia, the present study was performed to evaluate the effect of C. Andromeda venom on survival, apoptosis, and necrosis of the Jurkat cell line as a representative of T-ALL.

MATERIALS AND METHODS

The study protocol was approved by the Ethics Committee of Bushehr University of Medical Sciences with a code number of IR.BPUMS.REC1397.042.

The lymphoblastic leukemia cell line was purchased from the National Cell Bank of Iran-Pasteur Institute. The cells were stored in RPMI-1640 purchased from CBSA co., (IRAN) with 10% fetal bovine serum and antibiotics in a humid atmosphere of 5% CO₂ in air at 37 ° C. Bloom et al. (1998) method was used to separate the tentacles¹³. In short, immediately after capture, the tentacles were manually separated from the specimens and placed directly into small glass containers filled with seawater. They were then placed in ice bags to be transferred to the laboratory of the Persian Gulf Marine Biotechnology Research Center from the Persian Gulf, Bushehr University of Medical Sciences, Iran. Subsequently, after homogenization (IKA homogenizer, Germany), the samples were kept at 4 ° C for two days for autolysis. After the release of tissue toxins, the sample was centrifuged at 4 ° C for 15 minutes at 12,000 g to separate sediments (Eppendorf, Germany).The resultant supernatant was freeze-dried (Christ, UK) and kept at -80°C until further analysis¹⁴.

Cell culture and cell viability

Acute Lymphoblastic leukemia and peripheral blood mononuclear cells (PBM's) were cultured in RPMI medium supplemented with 10% Fetal Bovine

Serum (FBS) and antibiotics at 37°C in 5% CO₂. During successive cultures, cell survival reached more than 90%, then the cells were counted by the Neubauer slide and transferred to the 96-well plate (2×10^4 cells per well). After 24 hours, the medium was replaced with an experimental medium containing different concentrations of C. Andromeda venom (31, 62, 125, 250, 500, and 1000 µg/mL prepared in culture medium) to evaluate the effect of the venom. The control groups received RPMI only. The Methylthiazol tetrazolium (MTT) assay was used to evaluate cell viability after a period of 24, 48, and 72 hours, according to standard protocols. A cell-free culture medium with the mentioned concentrations of venom was used as blank control. Finally, the optical density was detected by a microplate reader at a wavelength of 570 nm and 690 nm for cells and blank control wells, respectively. Each experiment was repeated three times (triplicates). The percentage of cytotoxicity was calculated by the following equation using optical density (OD):

$$\text{Cytotoxicity} = \frac{[(\text{OD}_{\text{Test}} - \text{OD}_{\text{Blank}}) - (\text{OD}_{\text{Control}})] \times 100}{(\text{OD}_{\text{Control}})}$$

Cell apoptosis assay

The Jurkat cells were treated (2×10^6 cells/well) with C. Andromeda venom (250 µg/mL) for 72 h to determine apoptosis and necrosis of cells. After incubation, cell suspension of each well was transferred to microtube and centrifuged at 600g for 5 minutes, and the supernatant was removed at last. Thereafter, the cells have been washed by Phosphate buffered saline (PBS), and finally, Annexin-V-(FITC) and 7-Amino-Actinomycin-D (7AAD) (Biolegend, USA) were used to determine the apoptotic cells by FACSCalibur flow cytometry (Becton Dickinson, Heidelberg, Germany).

Real-time Polymerase Chain Reaction (qRT-PCR)

The RNA extraction was performed using Trizol after treating the cells with concentrations of 250 µg/mL (72 hours) of venom. The purity and concentration of the extracted RNA was evaluated at 260/280 nm and 260/230 nm, using NanoDrop DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE USA). Then, cDNA was synthesized

using the kit based on the Oligo-dT method (BonYakhteh Co., Iran) as recommended in the manufacturer's instructions. The qRT-PCR was performed in triplicate using SYBR Green Master Mix (Yektatajhez CO., Iran) and specific primers for p15INK4b, p21^{WAF1/CIP1}, p53, DNMT1, and Bcl-2 genes (BonYakhteh Co., Iran) on an ABI StepOnePlus RT-PCR system (Applied Biosystems, USA). Non-template control (NTC) was used for each primer to ensure the target gene amplification. The relative quantification of gene expression were calculated using $2^{-\Delta\Delta C_t}$ method. The primer sequences are illustrated in Table 1.

Statistical analysis

Frequency, mean and standard deviation were reported for descriptive data. One-way ANOVA was used to compare the means between groups, and ANOVA test with repeated measures was used to compare the means of groups at three-time points. IBM SPSS version19 (SPSS Inc., Chicago, IL, USA) and Graph Pad Prism 8.4.3 (GraphPad prism Software, Inc. San Diego CA, USA) were used for data analysis and charting, respectively. Significance level was considered less than 0.05 for each test.

Table 1: Primer sequences used in the present study

Primer name	Primer sequences (5' to 3')	Product length
p53 Reverse	AGGACAGGCACAAACACGCACC	464
p53 Forward	TAACAGTTCCTGCATGGGCGGC	
p21 Reverse	CAGCCGGCGTTTGGAGTGGTAGAA	1425
p21 Forward	GAGGCCGGGATGAGTTGGGAGGAG	
p15 Reverse	GAGCAAAGGCCAGCATCCT	82
p15 Forward	GATGTGCAAGCGACGACAGA	
Bcl-2 Reverse	CGGTTTCAGGTAAGTCAATCC	89
Bcl-2 Forward	GGTGGGGTTCATGTGTGTGG	
DNMT1 Reverse	CCTAGCCCCAGGATTACAAGG	1791
DNMT1 Forward	ACTCATCCGATTTGGCTCTTTC	
HGPRT Reverse	ATAGCCCCCTTGAGCACAC	88
HGPRT Forward	GGACAGGACTGAACGTCTTG	

RESULTS

Inhibiting proliferation and inducing cytotoxicity

The effect of *C. Andromeda* Venom on Jurkat cells proliferation was investigated. The results of the MTT method revealed that *C. Andromeda* Venom in the Jurkat cell line caused cell death in a concentration and time-dependent manner. According to the evaluations performed between 24, 48 and 72 hours in Jurkat cell lines and normal lymphocytes, the difference between mean survival rates was significant ($P < 0.001$). It was shown that concentration was an effective factor in creating differences between time shifts, and the changes are that in both normal lymphocytes and Jurkat cell line, the survival rate decreases with increasing concentration.

After treatment of the cells in different periods and concentrations, the difference in survival in normal lymphocytes between concentrations at the time interval of 48 and 72 was found to be significant ($P = 0/004$ and $0/049$ respectively), but it was not statistically significant at 24 hours of time interval ($P = 0.07$). However, in the Jurkat cell line, the difference in survival between different

concentrations at 24 and 48 hours of time interval was found to be not significant ($P = 0.066$, $0/99$, respectively), while it was found to be statistically significant within 72 hours ($P < 0.001$) (Figure 1).

A comparison of changes in cell viability showed that the normal lymphocyte cells were able to regenerate 100% of their killed cells during the three times exposed to the venom compared to the survival of the first treatment (24 hours), While the Jurkat cell line was unable to regenerate killed cells during three exposure times to the venom.

Notably, according to the results, it was found that the amount of venom required to inhibit biological processes by 50% in the studied cells (IC50) for Jurkat cells is $250 \mu\text{g/mL}$ within 72h (Figure 2). In a comparison between cell viability of the Jurkat cell line and normal lymphocytes at IC50 concentration, it was found that this difference in the Jurkat cell line was significantly different from the survival rate at the same concentration in the normal lymphocytes ($P = 0.015$).

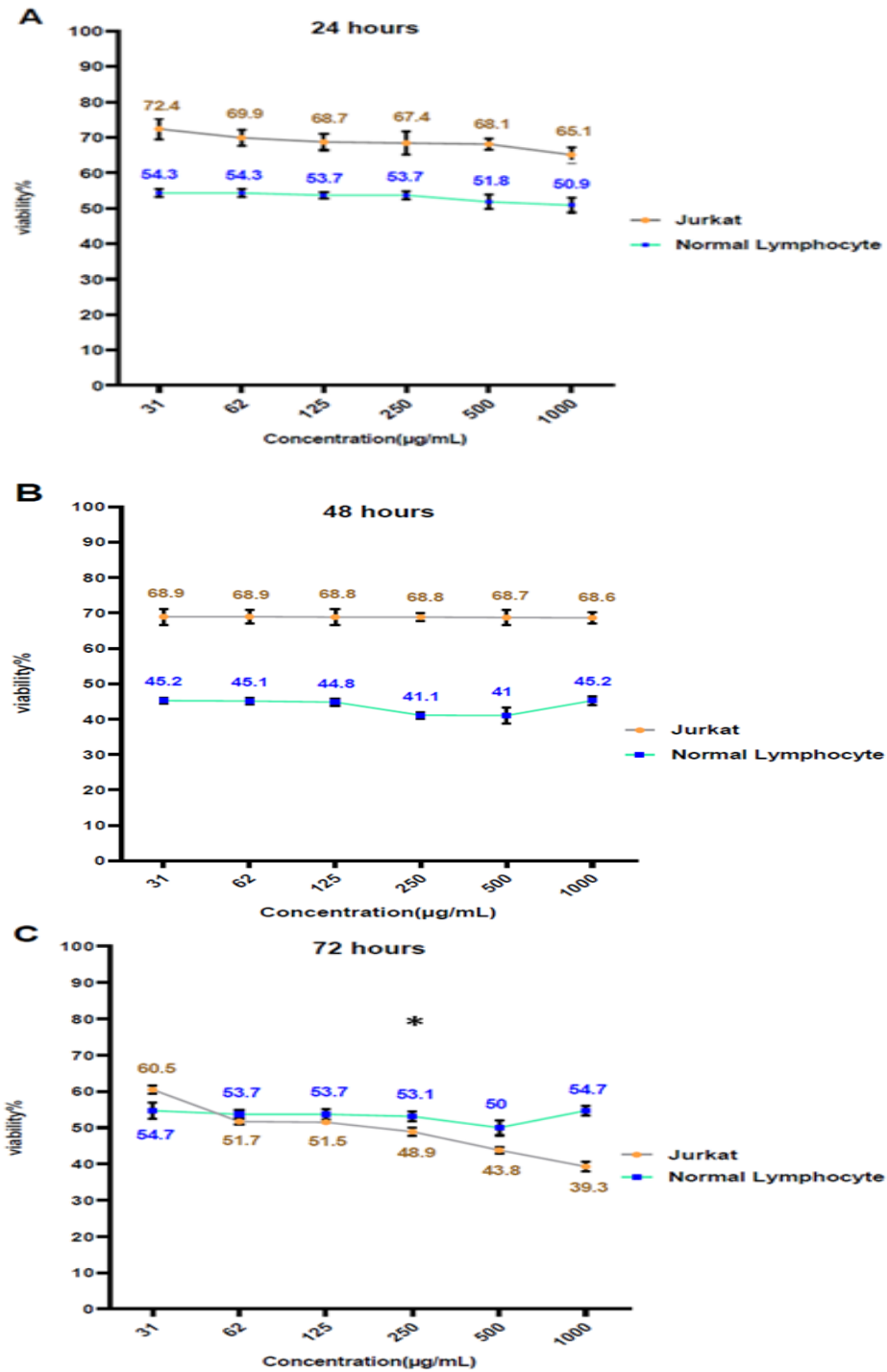


Figure 1. A) Cell survival comparison in Jurkat and normal lymphocytes at different concentrations in 24h. The difference in survival rate between the different concentrations of Jurkat ($P = 0.066$) and normal lymphocytes ($P = 0.071$) were not significant. B) Comparison of Jurkat cell survival and normal lymphocytes at different concentrations in 48h. The difference in survival rate between different concentrations of cell Jurkat ($P = 0.999$) was not significant and was significant in normal lymphocytes ($P = 0.004$). C) Comparison of Jurkat cell survival and normal lymphocytes at different concentrations in 72h. The difference in survival rate between the different concentrations of Jurkat ($P < 0.001$) and normal lymphocytes ($P = 0.049$) were significant.

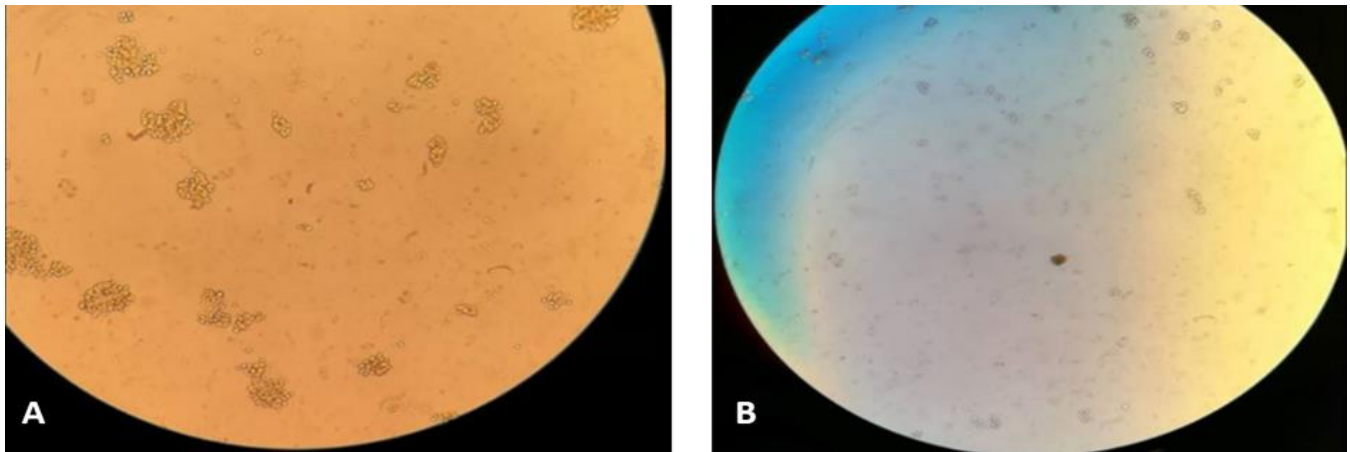


Figure 2. Comparison of the effect of IC50 concentration with Cassiopea andromeda Venom in Jurkat cells. (x40)
 A: Jurkat control cell, B: Jurkat cell after 72 hours of treatment with 250 µg/mL concentration

Measurement of cell apoptosis by flow cytometry

After treatment of the Jurkat cells with a concentration of 250µg/mL of the C. Andromeda Venom for 72 hours, the results were considered apoptosis or necrosis. By comparing the two groups of control and treated Jurkat cells, it turned out that

89.5% of control cells were AnnexinV(neg)/7AAD(neg) and 48.5% of treated cells were AnnexinV(pos)/7AAD(pos), indicating the apoptotic effect of C. Andromeda Venom on Jurkat cells (Figure 3).

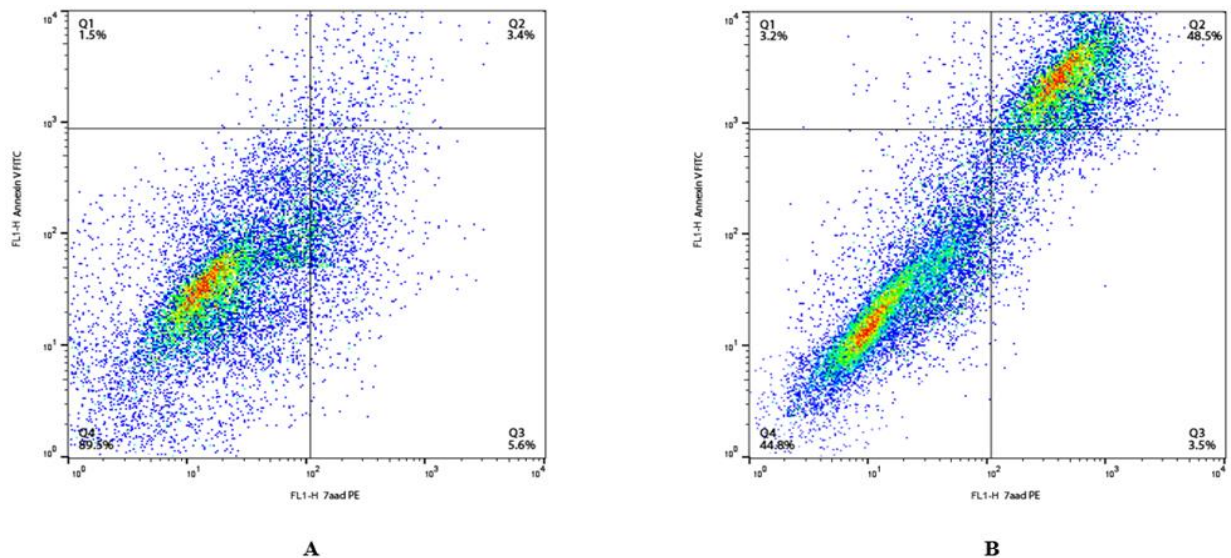


Figure 3. The apoptotic effect of Cassiopea andromeda Venom (250 µg/mL) on Jurkat cell versus control group after 72 hours. AnnexinV(neg)/7AAD(neg), AnnexinV(pos)/7AAD(neg), AnnexinV(neg)/7AAD(pos), and AnnexinV(pos)/7AAD(pos), were considered as viable, early apoptotic, necrotic and late apoptotic, respectively. A) Control Jurkat. B) Treated Jurkat.

Gene expression analysis by real-time PCR technique

Relative quantification of the expression of defined target genes was calculated using the $2^{-\Delta\Delta Ct}$ method, and cycle thresholds (Ct) values were normalized to the HGPRT gene as an internal control. According to the results, it was found that after treating the

Jurkat cell line with venom, the expression level of p15INK4b and P53 genes increased 22.78 and 675.5 times, respectively, or in other words, 2278% and 67550%. Moreover, the expression of Bcl-2, p21^{WAF1/CIP1}, and DNMT1 were decreased by 0.57, 0.64, 0.056 times or 43%, 36%, and 99.94%, respectively (Figure 4).

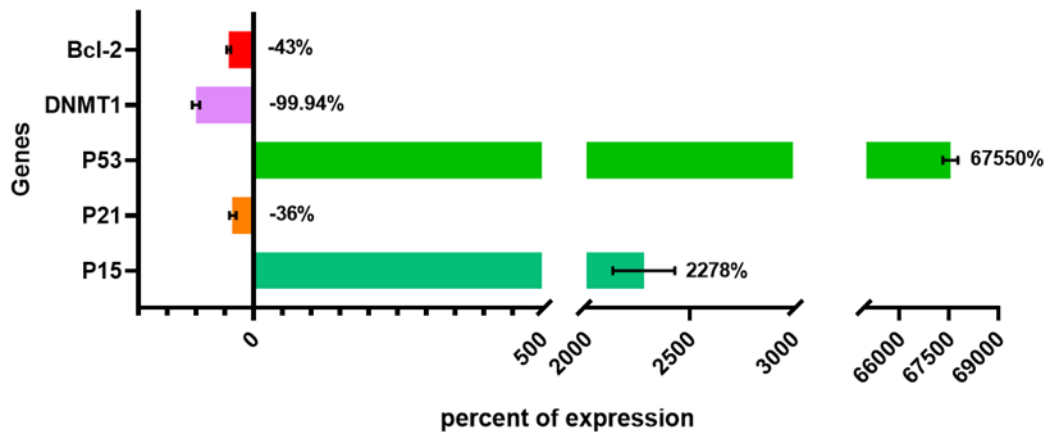


Figure 4. Gene expression fold change in Jurkat cells

DISCUSSION

Despite therapeutic and clinical care improvements, cancer remains one of the major causes of death worldwide. In recent years, the role of the marine ecosystem in the production of drugs, especially anti-cancer drugs, has been extensively studied¹⁵. In this scenario, cytarabine (Cytosar) which is widely used in the treatment of leukemia, was the first marine-based drug approved by the Food and Drug Administration (FDA)¹⁶. In the present study, we found that *C. Andromeda* venom could also play a possible inhibitory role in the cancer process. We report that *C. Andromeda* venom affects epigenetics, apoptosis, and cell cycle by decreasing the expression of DNMT1, P21, and BCL-2 and increasing the expression of P15 and P53, leading to increased apoptosis and decreased cell line proliferation.

Although the cytotoxicity of jellyfish venom has been well established, the exact mechanism is unknown. However, mitochondrial defects and oxidative stress have been proposed as the main mechanisms for cell damage and death caused by jellyfish venom¹⁷. In line with this issue, it has been indicated that *C. Andromeda* venom enforced

cytochrome c release in the mitochondria of the cells obtained from patients with adenocarcinoma of the breast¹³. In the present study, it was shown that *C. Andromeda* venom inhibits cell growth and induces apoptosis in the Jurkat cell line. Hence, the effect of this venom on the mitochondrial apoptotic pathway can be considered.

Bcl-2 is known as an anti-apoptotic protein, which is associated with various cancers, so its decreased expression causes prognosis improvement¹⁸. In the present study, it was shown that *C. Andromeda* venom can reduce Bcl-2 gene expression and increase cell apoptosis. In line with our study, it was shown that star fish steroids can also inhibit Bcl-2 and CDK-4/Cyclin D1¹⁹. Disruption in the apoptotic pathway and mutations in cell cycle control points are the most important causes of cancer²⁰. It has already been demonstrated that the p53 increases can cause cell apoptosis, hence many treatments attempt to increase p53 expression²¹. In the present study, *C. Andromeda* venom increased the expression of the p53 gene, resulting in apoptosis of Jurkat cells. Previous studies have shown a significant relationship between p15INK4b hypermethylation or reduced expression and the

incidence of malignancies²². Besides, if there is an increase in p15INK4b expression, the prognosis will be better, and the effect of chemotherapy will be greater²³. On the other hand, if the expression of p53 and p21 WAF1/CIP1 increases at the same time, the cell cycle arrests²⁴, but if p53 or p21 WAF1/CIP1 expression predominates, apoptosis or cell cycle arrest occurs, respectively^{24,25}. Due to the increased expression of p15INK4b in the present study, C. Andromeda venom can likely arrest Jurkat cells in phase G1 of the cell cycle by inhibiting CDK4 / 6 (cyclin-dependent kinase 6.4).

Aberrant DNA methylation in the promoter of tumor suppressor genes is another important cause of carcinogenesis, so the decreased expression of DNMT1 causes the hypomethylation of key genes such as p15INK4b, p53 genes and leads to prevent cancer progression^{26,27}. In the present study, gene expression assessment was shown that C. Andromeda venom upregulated p15INK4b and p53 and the DNMT1 gene expression in the Jurkat cell line. According to the decreased expression of DNMT1, it seems that due to the decrease in DNMT1 expression, p15 INK4b and p53 methylation are also reduced, and by increasing their expression, C. Andromeda venom causes apoptosis of Jurkat cells.

It should be noted that this study is the first report of the C. Andromeda venom effect on p15INK4b, p21 WAF1/CIP1, p53, Bcl-2, DNMT1 gene expression, apoptosis, and cell growth inhibition induction in acute lymphoblastic leukemia Jurkat cell line. Therefore, it is the novelty of the current work.

CONCLUSION

In conclusion, C. Andromeda venom can cause an apoptosis increase through upregulation of p15INK4b and P53 proteins and downregulation of Bcl-2, p21 WAF1/CIP1, and DNMT1 in the Jurkat cell line. Therefore, it can be concluded that C. Andromeda venom in the Jurkat cell line or acute lymphoblastic leukemia reduces the number of cells by inducing apoptosis and can be considered as a new treatment for this disease. In this regard, it is recommended that the synergistic properties of

chemotherapeutic drugs with C. Andromeda venom be investigated on cell lines of different leukemias.

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