Original Article

Treatment with 2-methyl- 3-pentyl-6-methoxyprodiginine isolated from serracia marcescens decreases cell viability and induces appoptosis in acute lymphoblaastic leukemia cells

Sommayeh Ghoreishi[1], *Mohammad Reza Sam*[2*]

Received: 25 May, 2017; Accepted: 23 Aug, 2017

Abstract

Background & Aims: Acute lymphoblastic leukemia (ALL) is the most common malignancies in the world. Despite advances in treatment of patients with ALL, a subset of patients will have recurrent disease or refractory to chemotherapy and hematopoietic stem cell transplant. Consequently, assessment of the effectiveness of natural compounds with high efficacy and minimal side effects is warranted. In this regard, it has been shown that some of bacterial pigments such as prodigiosin isolated from cell wall of *Serratia marcescens* have dramatic anti-cancer activities. The aim of this study was to evaluate the effects of prodigiosin on the cell viability and cell number, cell proliferation and apoptosis in CCRF-CEM cell line that serves as a model for ALL cells.

Materials & Methods: Malignant cells were treated with 100, 200 and 400 nM prodigiosin for 24, 48 and 72 h and cell proliferation-rates were measured by performing WST-1 assay. Furthermore, malignant cells were treated with the indicated concentrations of prodigiosin for 48 h and cell viabilities and cell numbers along with apoptotic-rates were determined by trypan blue staining method and flow cytometer respectively.

Results: Treatment of cells with increasing concentrations of prodigiosin significantly decreased

Proliferation-rates in a dose- and time-dependent manner compared to untreated cells. Specifically, after 72 h treatments with 100, 200 and 400 nM prodigiosin, proliferation-rates were measured to be%77.3 ± %1.5, %63 ± %2, and%46.3 ± %3.2 respectively as compared to untreated cells. Furthermore, following 48 h treatments with indicated concentrations of prodigiosin, the cell numbers and viabilities were decreased in a dose-dependent manner. Specifically, treatment with 400 nM prodigiosin resulted in 44% (4.5×10^5 cells) and 63% for cell number and viability respectively as compared to untreated cells. At the same conditions, apoptotic-rates (Early + Late) were measured to be 33.8% to 72.8% at the indicated prodigiosin concentrations ranging.

Conclusion: Prodigiosin decreased cell number and viability as well as cell proliferation-rates. This compound also increased apoptosis in CCRF-CEM cells. Therefore, this compound with high pro-apoptotic capacity represents an attractive anti-leukemic agent in ALL.

Keywords: Acute lymphoblastic leukemia (ALL), Prodigiosin, *Serratia marcescens*, Cell proliferation, Apoptosis

Address: Department of Cellular and Molecular Biotechnology, Institute of Biotechnology, Urmia University, Shahid Beheshti Street, Urmia, Iran. P. O. Box: 165.

Tel: +98-4433440199

Email: m.sam@urmia.ac.ir; s_mohammadreza@yahoo.com

[1] *Department of Cellular and Molecular Biotechnology, Institute of Biotechnology, Urmia University, Urmia, Iran*

[2] *Department of Cellular and Molecular Biotechnology, Institute of Biotechnology, Urmia University, Urmia, Iran (Corresponding Author)*

Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy in Iran and the world. This type of leukemia is the most common pediatric cancer that some patients do not have an appropriate response to chemotherapy and hematopoietic stem cell transplant despite many advances in its treatment, and the disease is re-opened. Since most anti-cancer available and commercial drugs are chemically synthesized and have many side effects on the patient, the researchers focused on natural ingredients with the aim of replacing them with chemical kinetics drugs. Including natural anti-cancer drugs, the red pigment extracted from various microorganisms such as Prodigiosin cell wall of Serratia marcescens bacteria. It has been shown that this pigment leads to a significant reduction in cell proliferation and the induction of apoptosis in several types of cancer cells. It has been shown that prodigiosin cause loss of cancer cells through induction of different conditions, these conditions are acidifying the cytoplasm, stop the cell cycle progress with increased expression of P21WAF / CIP1, damage to DNA molecules by blocking the function of I and II enzymes topoisomerase, decline in the mitochondrial ATP production. It is also shown that it has the high apoptosis induction in cancer cells. Since the favorable conditions of an anti-cancer drug is to induce apoptosis rather than cell necrosis, the property of prodigiosin is important. In this study, cell line CCRF-CEM (T cell type and acute lymphoblastic leukemia) has been treated with different concentrations of Prodigiosin and the number of cells, their survival rates and cell proliferation, as well as induction of apoptosis have been studied in these cells.

Materials and Methods

RPMI-1640culture medium, fetal bovine serum, penicillin and streptomycin were purchased from Austrian company (PAA). WST-1 Solution, 2-methyl-3 pentyl -6-methoxy prodigiosin and dimethyl sulfoxide (DMSO) were obtained from German Sigma Company. CCRF-CEM cell line was purchased from Pastor Institution. Trypan Blue was purchased from Merck, 6 and 96-part plates as well as cell culture flasks were purchased from Jet Biophyle Company, Korea. Staining Kit of Annexin / PI was purchased from Roche, Germany.

Prodigiosin preparation and cell treatment:

Prodigiosin was dissolved in ethanol for preparation of Stoke 20µg / ml. Then stored at -80 ° C. 100 to 400 nano-molar concentrations of Prodigiosin stock solution were prepared for all tests.

Cell culture:

CCRF-CEM cell line was cultured in RPMI-1640 medium containing 10% fetal bovine serum (Culture medium) in T-25 culture flask and kept in CO_2 incubator (5%) and 90% humidity at 37 degrees centigrade.

Treatment and Cell Count:

$10^5 \times 5$ of CCRF-CEM cells were added to a 6-part plate wells in 2 ml culture medium. Cell culture medium replaced with supplemented mediums with 100, 200 and 400 micro-molars of prodigiosin after 24 hours, respectively, and 2 wells were considered as controls which contained the untreated cells. Then the plate was placed in incubator for 48 hours at 37 ° C with 90% humidity and 5% CO_2. At the end of the treatment, the supernatant of each well was removed and the number of cells were counted using neobar lam by providing a uniform cell suspension.

Determination of cell Viability:

The viability of cancer cells treated with prodigiosin was determined using trypan blue. Live cells were impermeable to trypan blue entrance, while the dead cells absorb the color of trypan blue. Preparation of a uniform cell suspension, 50 ml of the suspension was added to 50 ml Trypan blue and the number of viable cells were counted after a few minutes using the slide

neobar and the viability rate was calculated by the following formula.

Viability= A/B × 100

A : The number of live cells

B :The total number of cells (live + dead)

Testing cell proliferation:

For testing cell proliferation, 10^4 cells in 200 ml of culture medium were added to 96-well 3-plate wells. After 24 hours, the cell culture medium was replaced with fresh medium with the mentioned concentrations of Prodigiosin and plates were incubated for 24, 48 and 72 hours in an incubator with 5% CO_2 at 37 ° C. At each stage after incubation, 100 microliters of medium were removed from each well and 10 microliters of WST-1 solution was added on 100 ml culture medium to each well, and plates were placed at 37 ° C for 4 hours. Then, the medium became homogeneous slowly with a Sampler. The intensity of the yellow color was recorded by ELISA reader at 492 nm and the growth rate and cell proliferation was calculated by the following formula.

Cell proliferation (%) = $A_{Sample} - A_{Blank} / A_{Control} - A_{Blank}$

Flow cytometry test:

$10^5 \times 5$ cells were poured in 2 ml of culture medium in each part of 6-part well. After 24 hours, Prodigiosin was added to each part at concentrations of 100, 200 and 400 micromoles per liter. Then the plates were incubated with 5% CO2for 48 hours at 37 °. After incubation, the cells were collected separately and sediment cell suspensions were obtained by centrifugation. The supernatant was discarded and precipitated cells were washed with sterile phosphate buffered. Then, the supernatant was discarded and 2 ml of Annexin solution and 100 ml cell binding buffer was added to the sediment and set 15 minutes on ice and incubated in the dark. Centrifugation was performed and the supernatant was discarded. Next, 1.5 ml of sterile PBS buffer plus 2 micro-liters of PI color was added to the cells and the results were recorded by flow cytometry in less than 5 minutes. The cells stained with Annexin are at the Early apoptosis stage and cells stained with PI necrosis and cells that have attracted both colors have been experiencing Late apoptosis. In this method, the cell without the above treatments have been painted in the same manner and the results were recorded by flow cytometry.

Results

The effect of treatment with Prodigiosin on the number of cells and their viability rates:

CCRF-CEM cells were treated with different concentrations of Prodigiosin. The number of cells at concentrations of 100, 200 and 400 nM were 10260000, 370000, 330000, 287000, respectively (Figure 1). Also the cell viability at concentrations of 100, 200 and 400 nmol per liter were 77.3%±1.5, 72.7% ± 1.5 and 63% ± 4, compared to the alive control. Based on these results, it was found that the number of cells and the viability rate depend on the concentration of the drug. As with an increase in drug concentration, the cell number and survival rates will be reduced (Figure 1a: and b).

Prodigiosin effect on cell growth and proliferation Assay:

Based on the results of cell proliferation, time and dose-dependent proliferation of malignant cells will be decreased. After 24 hours of treatment with Prodigiosin at concentrations of 100, 200 and 400 nM, the cells growth was 92% ±2.5, 82% ± 4 and 64% ± 3, respectively, compared to untreated control cells. At 48-hour treatment of listed Prodigiosin concentrations, cells growth was 85% ±1, 72% ± 3.8, 52%± 10.1 compared to untreated control and at 72-hour treatment the growth rates were 77% ±3.2, 64% ±2, 46%±3.2 compared to untreated control. Based on the results, the maximum reduction in cell growth and proliferation have been recorded within 72 hours after treatment (Figure 2). It's worth noting that no

cytotoxic effects on PBMCs cells was observed in the listed prodigiosin concentrations.

The effect of prodigiosin on apoptosis induction:

In order to analyze the effect of prodigiosin on apoptosis induce, cancer cells were prepared for flow cytometry analysis after treatment for 48 hours at prodigiosin concentrations of 100, 200 and 400 nM. Based on the results, prodigiosin induces apoptosis in CCRF-CEM cells and the necrosis created at different concentrations was very low. Apoptosis (primary + secondary) in the prodigiosin concentration ranges of % 33.8-72.8% was calculated. The highest rate of apoptosis was seen in 400nano molar of prodigiosin (Figure 3 and Table 1). Thereby, reduction in cell proliferation in this study was due to the induction of apoptosis in CEM - CCRF cells.

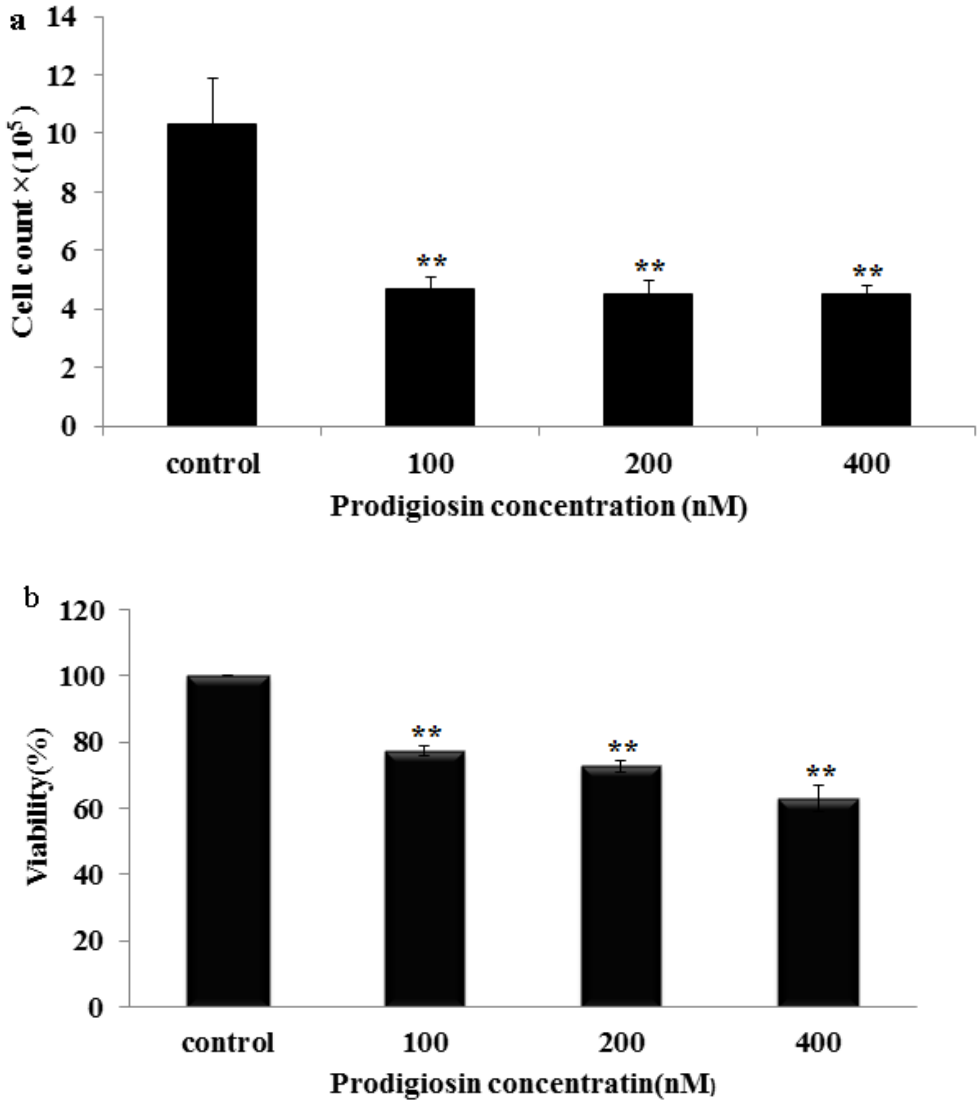

Figure 1: The effect of 48-hour treatment with prodigiosin on the number of cells and viability of CCRF-CEM cells. Cells were treated at Prodigiosin concentrations of 100, 200 and 400 nanomoles per liter. After 48 hours, the number of cells (Figure a) and survival rates (Figure b) were calculated compared with control cells without treatment. The results are the mean from two experiments, each with 3 to 4 reps. A significant amount is calculated as $P< 0.01$ ** compared to control cells without treatment.

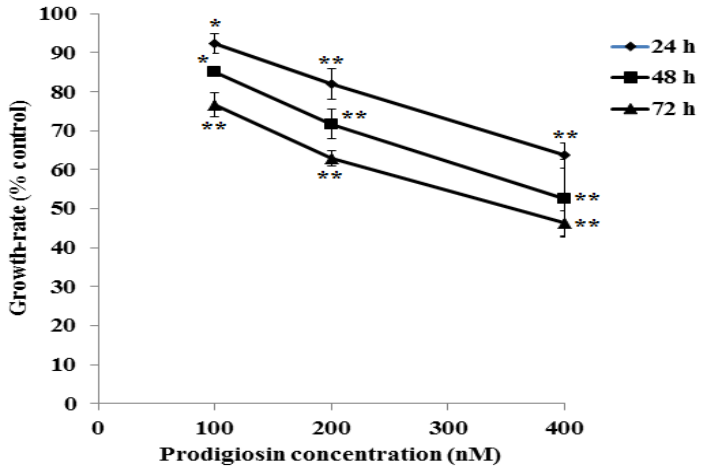

Figure 2: The growth and proliferation rate of CCRF-CEM cells treated with Prodigiosin. cells treated with above mentioned prodigiosin concentrations and the rate of cell growth and proliferation in intervals of 24, 48 and 72 hours was calculated after treatment compared with untreated control cells. The mean results are for two experiments, each with 3 repeat. A significant amount is calculated as * P < 0.05 and P <** 0.01 compared to control.

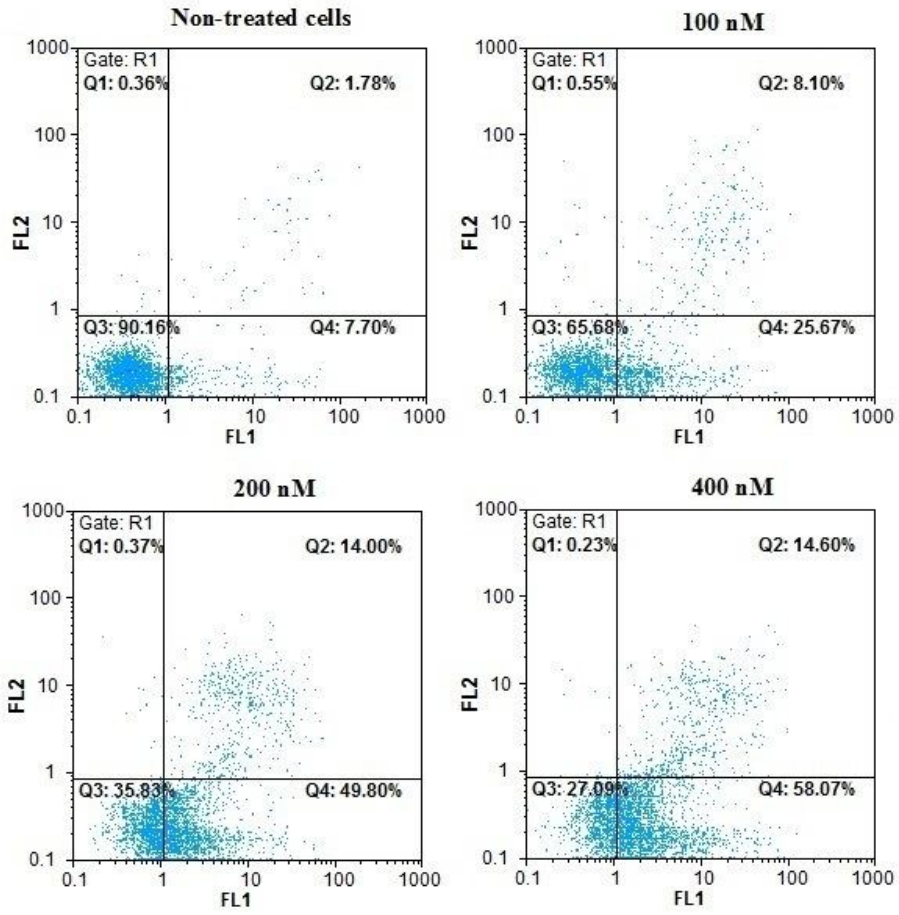

Figure 3: Flow cytometry plots of apoptosis and necrosis in CCRF-CEM cells treated with various concentrations of Prodigiosin after 48 hours. Q1: necrotic cells, Q2: apoptosis delay, Q3: living cells and Q4: initial apoptosis

Table 1. Created apoptosis and necrosis in CCRF-CEM cells treated with various concentrations of prodigiosin after 48 hours

Control (untreated cells)	Results		Mean ± SD
Live cells	90.1%	92.4%	91.3% ±1.6
Primary apoptosis	7.7%	4.8%	6.3% ±2.1
Delayed apoptosis	1.8%	2.2%	2% ±0.3
Necrosis	0.4%	0.42%	0.3% ±0.1
100nM prodigiosin			
Live cells	65.7%	61.3%	63.5% ±3.1
Primary apoptosis	25.7%	37.1%	31% ± 8
Delayed apoptosis	8.1%	2%	5% ±4.3
Necrosis	0.6%	0.4%	0.5% ±0.1
200nM prodigiosin			
Live cells	35.8%	46.2%	41% ±7.3
Primary apoptosis	49.8%	45.5%	47.6% ±4.5
Delayed apoptosis	14%	8%	11% ± 4%
Necrosis	0.4%	0.38%	0.4% ±0.1
400nM prodigiosin			
Live cells	27.1%	37%	32.1% ±5.6
Primary apoptosis	58.1%	51.5%	54.6% ±5.7
Delayed apoptosis	14.6%	10.7%	12.4% ±3.1
Necrosis	0.2%	0.8%	0.5% ±0.1

Discussion

Despite recent advances in cancer treatment, the disease is remained as one of the deadliest ones of human life. Chemotherapy and hematopoietic stem cell transplantation is the main strategy for the treatment of acute lymphoblastic leukemia. However, these methods have limitations including resistance to chemical drugs and medication side effects on the tissue and healthy cells as well as the lack of appropriate response of patients to hematopoietic stem cell transplant. So researchers are interested to find drug compounds with high performance and fewer side effects. Recently, bacterial natural components including prodigiosin family have attracted the attention in therapy and have become a potential source of new drugs. The results of cell counting and viability were indicative of the fact that prodigiosin has been led to a significant reduction in the number of malignant cells and in survival rates. On this basis, it can be used as a drug to inhibit the cell cycle in malignant cells. In this context, it has been shown that prodigiosin, depending on the cell type and structure, leads to cell cycle stop in late G1-phase or G1-S phase. Induction of the cyclin-dependent kinase inhibitor of p27, Cyclin E, Cyclin D and retinoblastoma

protein phosphorylation is inhibited by End Seel prodigiosin that leads to cell cycle stop in B and T lymphocytes.

Apoptosis is a programmed death of cells without inflammation leading the tissue homeostasis. Defects in this program can cause the cancer and its progression. Since one of the goals of anti-cancer drugs is to induce apoptosis and necrosis in the cell, this effect of prodigiosin in lymphoblastic leukemia cells is important. Related to our study, different researchers showed that the treatment of colorectal cancer cell lines (HT-29), liver (HepG2), Blood HL (60and Jurkat), Breast (T47D) with prodigiosin leads to the induction of apoptosis in these cells, and decreases necrosis of the cells. Based on these observations, it can be concluded that prodigiosin activates similar molecular mechanisms in the different types of malignant cells. The result of their activity is to induce apoptosis rather than necrosis in these cells. Further tests are absolutely necessary in this area to deny or prove the hypothesis mentioned above.

Since Cell proliferation test has been designed based on the metabolic activity of mitochondria, reduction in cell proliferation in WST-1 test may indicate this fact that the composition causes defects in these activities by hitting the crucial mitochondrial activity which results in reducing the proliferation of malignant cells. In a study by Montaner and colleagues on leukemia cancer cells, it was shown that the metabolic activity of live cells after 4-hour treatment with Prodigiosin had been reduced. This reduction was Prodigiosin dose-dependent.

Given our results and other researchers and fact that the toxic material does not effect on normal cells [15], this compound has potential to be used in patients with acute lymphoblastic leukemia beside other chemotherapy drugs. However, there must be considered appropriate and complete tests on animal models prior to human tests.

Acknowledgments

This research has been done supported by National Science and Technology Fund. Authors would like to thank the respected authorities.

References

1. Iran Cancer Registration Report, 2008. Tehran: Ministry of Health and Medical Education, Centre for Disease Control; 2011.
2. Inaba H, Greaves M, Mullighan CG. Acute lymphoblastic leukaemia. Lancet 2013; 381 (9881): 1943-55.
3. Sumathi C, MohanaPriya D, Swarnalatha S, Dinesh, GM, Sekaran G. Production of prodigiosin using tannery fleshing and evaluating its pharmacological effects. Sci World J 2014; 2014:1–8.
4. Francisco R, Pe´rez-Toma´s R, Gime`nez-Bonafe´ P, Soto-Cerrato V, Gime´nez-Xavier P, Ambrosio S. Mechanisms of prodigiosin cytotoxicity in human neuroblastoma cell lines. Eur J Pharmacol 2007; 572:111–9.
5. Campàs C, Dalmau M, Montaner B, Barragán M, Bellosillo B, Colomer D, et al. Prodigiosin induces apoptosis of B and T cells from B-cell chronic lymphocytic leukemia. Leukemia 2003; 17(4):746–50.
6. Soto-Cerrato V, Viñals F, Lambert JR, Pérez-Tomás R. The anticancer agent prodigiosin induces p21 WAF1/CIP1 expression via transforming growth factor-beta receptor pathway. Biochem Pharmacol 2007; 74(9):1340–9.
7. Hassankhani R, Sam MR, Esmaeilou M, Ahangar P. Prodigiosin isolated from cell wall of Serratia marcescens alters expression of apoptosis-related genes and increases apoptosis in colorectal cancer cells. Med Oncol 2015; 32(1):366.
8. Sam S, Sam MR, Esmaeillou M, Safaralizadeh R. Effective Targeting Survivin, Caspase-3 and MicroRNA-16-1 Expression by Methyl-3-pentyl-6-methoxyprodigiosene Triggers Apoptosis in Colorectal Cancer Stem-Like Cells. Pathol Oncol Res 2016; 22(4):715-23.

9. Chang C-C, Chen W-C, Ho T-F, Wu H-S, Wei Y-H. Development of natural anti-tumor drugs by microorganisms. J Biosci Bioeng 2011; 111(5):501–11.
10. Songia S, Mortellaro A, Taverna S, et al. Characterization of the new immunosuppressive drug undecylprodigiosin in human lymphocytes: retinoblastoma protein, cyclin-dependent kinase-2, and cyclin-dependent kinase-4 as molecular targets. J Immunol 1997; 158(8):3987-95.
11. Denicourt C, Dowdy SF. Targeting apoptotic pathways in cancer cells. Science 2004; 305(5689):1411-3.
12. Yenkejeh RA, Sam MR, Esmaeillou M. Targeting survivin with prodigiosin isolated from cell wall of Serratia marcescens induces apoptosis in hepatocellular carcinoma cells. Hum Exp Toxicol 2017; 36(4):402-11.
13. Montaner B, Navarro S, Piqué M, Vilaseca M, Martinell M, Giralt E, et al. Prodigiosin from the supernatant of Serratia marcescens induces apoptosis in haematopoietic cancer cell lines. Br J Pharmacol 2000; 131(3):585-93.
14. Dalili D, Fouladdel S, Rastkari N, Samadi N, Ahmadkhaniha R, Ardavan A, et al. Prodigiosin, the red pigment of Serratia marcescens, shows cytotoxic effects and apoptosis induction in HT-29 and T47D cancer cell lines. Nat Prod Res 2012; 26(22):2078-83.
15. Campas C, Dalmau M, Montaner B, Barragán M, Bellosillo B, Colomer D, et al. Prodigiosin induces apoptosis of B and T cells from B-cell chronic lymphocytic leukemia. Leukemia 2003; 17(4):746-50.