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# The Antitumoral Activity of *Zataria Multiflora* Methanolic Extract on Acute Promyelocytic Leukemia Cell Line; NB4

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**ABSTRACT**

**Background & Objective:** *Zataria multiflora* is a plant that belongs to *Laminaceae* family. It is traditionally believed to have several therapeutic effects. Acute promyelocytic leukemia is a distinct subtype of acute myeloid leukemia with dominancy of promyelocytes in bone marrow and blood stream. The aim of this study is to investigate the anticancer effects of *Z. multiflora* extract on acute promyelocytic leukemia cell lines.

**Materials & Methods:** Viability of NB4 cells was determined by trypane blue test after treatment with 20, 40 and 80µg/mL of *Z. multiflora* extract for 24 hours. Then, the metabolic activity of cells was determined by MTT assay after 24 hours of treatment with 80 µg/mL *Z. multiflora*. Finally, Real-time PCR was employed to study the effect of *Z. multiflora* extract on the expression of hTERT gene.

**Results:** *Z. multiflora* extract decreased the viability of NB4 cells in a dose-dependent manner. Metabolic activity of NB4 cells significantly decreased following treatment with 80 µg/mL *Z. multiflora*. Gene expression analysis showed 59%±4% decrease in the expression of hTERT gene after treatment with 80 µg/mL of *Z. multiflora*.

**Conclusion:** *Z. multiflora* extract significantly decreased the viability and metabolic activity of NB4 cells. It also led to significant downregulation of hTERT gene compared to the control group. Therefore, *Z. multiflora* methanolic extract potentially has anticancer effect on acute promyelocytic leukemia cells through down regulation of hTERT. Further investigations are needed to explore other mechanisms of actions and the active ingredients.

**Keywords:** *Zataria multiflora*, Tumor Suppressor, Leukemia, Acute Promyelocytic Leukaemia, NB4, hTERT gene



## Introduction

Global efforts have been made to reduce the incidence of cancers, but it has been the most important cause of death in the last 50 years. Since the incidence of cancers is increasing in developed and developing countries, immediate need to find a more effective remedy for them is vital (1). Herbal medicines refer to any product derived from plants that has therapeutic benefits. The use of herbal medicines in developing and industrialized countries is increasing. About 65-80% of the population around the globe take herbal medicines for primary health care (2).

Acute myeloid leukemias (AMLs) are malignant neoplasms that are responsible for a huge number of cancer-related deaths (3). A distinct subtype of AML is Acute promyelocytic leukemia (APL), morphologically known as M3 in French-American-British (FAB) classification. APL is recognized by the bilateral translocation between the chromosomes 15 and 17. This translocation leads to the fusion of promyelocytic leukemia (*PML*) and retinoic acid receptor α (*RARα*) genes (4).

The management and outcome of APL have been revolutionized since the introduction of all-transretinoic acid (ATRA) and arsenic trioxide. However, Cure of APL is dependent on peculiar aspects that are related to the management and supportive measures that are central to counteract life-threatening complications associated with the molecularly targeted treatment and disease biology (5).

Natural products with plant origin have been medicinally used for many years (6) and they play an undeniable role in the treatment of diseases (7) including cancer (6, 8). *Zataria multiflora Boiss* is a plant belonging to the *Laminaceae* family and is well-known for various therapeutic effects (9, 10). The Persian name for this plant is Avishan Shirazi (11-13). It grows in Iran,

Afghanistan and Pakistan and has traditionally been used as antiseptic, anti-anesthetic, anticonvulsant and anti-seizure medication (14). Its benefit for employment in inflammatory and immunomodulatory diseases has been demonstrated (15). It also diminishes tumor growth by inhibiting angiogenesis and migration (16).

The aim of the current study is to investigate the effects of *Z. multiflora* methanolic extract on acute promyelocytic leukemia. The expression of hTERT gene was also investigated.

## Materials and Methods

This study was approved by the ethical committee of Kerman University of Medical Sciences. The Ethic approval Cod is IR.KMU.REC.1395.875.

### Preparation of *Zataria Multiflora* Extract

Fresh aerial segments of *Z. Multiflora* were prepared and desiccated at room temperature. Then, they were grinded by a hammer mill. A sequential extraction was performed using petroleum ether, chloroform, and methanol. Then, rotary evaporator was employed to evaporate the methanolic extract at 45°C. Finally, the desiccated extract was gathered and kept 4°C (11).

### Cell Culture

Acute promyelocytic leukemic cell line, NB4 (Pasteur Institute, Iran), was cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY, USA) and 1% penicillin–streptomycin (Gibco Laboratories, Grand Island, NY, USA) at 37°C and 5% $CO_2$.

### Viability Assay

NB4 cells were treated with different concentrations of *Z. multiflora* methanolic extract (20, 40, 80 µg/mL) for 24 hours. Cell viability was determined by trypan blue exclusion test (Sigma-Aldrich). For this end, cell suspension was mixed with trypan blue 0.04% in a 1:1 ratio and incubated for 2 minutes. Viability was calculated after counting the number of live and dead cells under light microscope using neubauer hemocytometer. The results were presented as percentage.

### Metabolic Activity

NB4 cells (10,000 cells/well) were treated with *Z. multiflora* methanolic extract (80 µg/mL) in a 96-well plate. Metabolic activity was determined using MTT assay. MTT dye 0.5 mg/mL was added to each well and incubated at 37°C with 5% $CO_2$ for 3-4 hours. Then, 100 µL dimethyl sulfoxide (DMSO) was added to each well and the plate was incubated at room temperature in darkness for 30 minutes. The Absorbance was measured at 570 nm by ELISA reader. Metabolic activity was calculated using $OD_{\text{treated group}}/OD_{\text{control}}$ multiply by 100. Finally, the results were expressed as percentage.

### RNA Extraction and cDNA Synthesis

RNA was extracted 24 h after treating NB4 cells with *Z. multiflora* by RNA extraction kit according to the manufacturer protocol (Yektatajhiz, Iran). Then cDNA was synthetized using cDNA Synthesis kit (TaKaRa, Japan). The reaction contained nuclease-free water (9-µl), Random Hexamer Primer (1 µL), 5× Reaction Buffer (4 µL), dNTP Mix 10 mM (2 µL), RiboLock RNase Inhibitor 20 U/µL (1 µL), and 1 µL RevertAid M-MuLV Reverse Transcriptase (200 U/µL), and 2 µL of total RNA (1 µg per reaction). The temperature profile for reverse transcription contained 5 min incubation at 25°C, followed by 60 min incubation at 42°C. The reaction was terminated upon heating at 70°C for 5 min.

### Quantitative Real-Time PCR

Real-time PCR was performed using 5 µL of RealQ Plus 2X Master Mix Green (Ampliqon), 80 ng of the cDNA product, 5 pmol of each forward and reverse primers, and nuclease-free water in total volume of 10 µL. Temperature profile contained an initial activation step at 95°C for 15 min that was followed by 40 cycles. Each cycle included a denaturation step at 95°C for 30 s and a combined annealing/ elongation step at 65°C for 60 s. The reactions were performed in Rotor Gene 6000 Real-time PCR System (QIAGEN). The specificity of the products was approved by analyzing melt curves. The expression of hTERT gene was compared with the control group by computing the fold changes using the $2^{-\Delta\Delta CT}$ formula. β actin gene was used as the house-keeping gene for normalizing the results. The sequences of the specific primers are presented in Table1.

**Table 1. Sequences of primers**

| Reverse | Forward | |
|---|---|---|
| 5'- CCAACCGCGAGAAGATGA-3' | 5'- TCCATCACGATGCCAGTG-3' | ß-actin |
| 5'-CACTGTCTTCGACAAGTTCAC-3' | 5'- TGACACCTCACCTCACCCAC-3' | hTERT |

### Statistical Analysis

Statistical analysis was performed by SPSS 22 (SPSS Inc., Chicago, Ill. USA). One-way ANOVA and Tukey`s post hoc test was used to compare the results. P-value less than 0.05 was considered statistically meaningful.

## Results

### Investigation of Viability

Viability of the NB4 cells was evaluated 24 hours following treatment with different concentrations of *Z. multiflora* methanolic extract. The viability was

decreased in a dose dependant pattern. The viability of NB4 cells was decreased to 81.99 and 79.32% after treatment with 20 and 40 µg/mL of *Z. multiflora* methanolic extract respectively. The decreases were not statistically significant ($P$>0.05). Therefore, low concentrations *of Z. multiflora* methanolic extract did not significantly affect the viability of NB4 cells. Cell viability demonstrated a significant decrease at the dose of 80 µg/mL ($P$<0.001).

### Investigation of Metabolic Activity

Metabolic activity of NB4 cells was evaluated 24 hours after treatment with 80 µg/mL *Z. multiflora* methanolic extract. Metabolic activity was decreased to 73.66 %±5.78 after treatment in comparison with the control group. The reduction in metabolic activity was meaningful ($P$<0.01).

### Investigation of Gene Expression

The expression of hTERT gene was evaluated 24 hours after treatment with *Z. multiflora* methanolic extract. As shown in Figure 3, the expression of hTERT gene in NB4 cells significantly decreased following treatment with 80 µg/mL *Z. multiflora* methanolic extract ($P$<0.01).

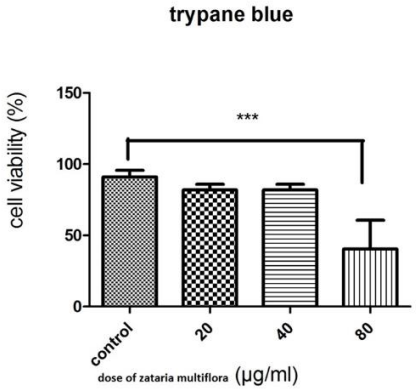


**Figure 1. Viability of NB4 cells 24 hours following treatment with different concentrations of *Z. multiflora* methanolic extract**

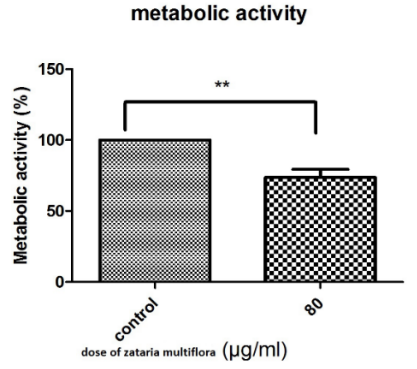


**Figure 2. metabolic activity of NB4 cells 24 hours following treatment with *Z. Multiflora* methanolic extract**

## Discussion

Cancer is considered as the most prevalent disease and a human tragedy in the world. Therefore, identifying new strategies to treat such deadly diseases is essential (17). Of the cancers, APL is a distinct subtype of AML (18), constituting 4.8%-34% of AML cases with a high prevalence in Iran (19). Standard cares for APL are ATRA, arsenic trioxide (ATO) and chemotherapy with the cure rate exceeding 80% (14, 15). ATRA accompanies some potentially severe complications including ATRA syndrome, thrombosis, pulmonary distress syndrome, and hepatotoxicity. ATO poses complications as well; the most prevalent complications are hepatotoxicity, gastrointestinal symptoms, and damage to nervous system (17). Recently, plant-derived medicines have gained popularity since they have fewer side effects and have natural origin (20). Moreover, studies have indicated that Chinese herbal medicines in combination with chemo- or radio-therapy enhance the efficacy of chemo- and radio-therapy and diminish the complications in cancer treatment (21). Throughout the study, the anti-cancer effects of *Z. multiflora* methanolic extract as a herbal medicine was investigated on acute promyelocytic leukemia cells NB4. It was shown that *Z. multiflora* methanolic significantly decreased the viability and metabolic activity of NB4 cells. Therefore, this study suggests that *Z. multiflora* methanolic extract has cytotoxic effects on NB4 cells.

The study conducted by Maryam Janitermi *et al.* in 2015 on breast cancer cells, showed that *Z. multiflora* extract had an inhibitory effect on cancer cells while not affecting normal fibroblast cells and it can be used as a potential drug for the treatment of cancer; however, further studies should be conducted to evaluate its effect on other cancer cells (22). In 2015, they also showed that *Z. multiflora* extract had potent inhibitory effect on gastric cancer and cervical cancer cells (23, 24). The maximum cytotoxicity of *Z. multiflora* extract was seen after 24 hours (23). In full accord with previous studies, this study showed that *Z. multiflora* extract had potent anticancer effects on acute promyelocytic leukemia cells.

One of the hallmarks of cancer is the activation of hTERT enzyme, which prevents shortening of the chromosomes end by adding repetitive sequences (20). Resultantly, the expression of hTERT was investigated throughout this study. It was demonstrated that *Z. multiflora* methanolic extract significantly diminished the expression of hTERT gene, compared with the control group. Thus, down-regulation of hTERT can be suggested as a possible anticancer mechanism for *Z. multiflora* extract. Baharara *et al.* in 2018 also reported p53 activation and resultantly apoptosis induction *by Z. multiflora* (25). More studies should be conducted to further approve the anticancer effects of *Z. multiflora* extract and unveil other anti-cancer mechanisms. Hosseinimehr *et al.* showed that *Z. multiflora* extract

had chemoprotective effects on murine bone marrow cells **(26).** However, the effect of *Z. multiflora* extract on normal human hematopoietic cells should be studied in future investigations. Meanwhile, Methanolic extract of *Z. multiflora* was used throughout this study, which contains several therapeutic compounds. Further investigations are recommended to unveil the pure anticancer compounds and their effects on other cancer cell lines.

## Conclusion

The results of current study demonstrated that *Z. multiflora* methanolic extract decreased the cell viability and metabolic activity of acute promyelocytic leukaemia cells in a dose dependant manner. It was also shown that the expression of hTERT was significantly downregulated. Therefore, *Z. multiflora* can potentially have anti-cancer effects on acute promyelocytic leukemia cells. However further investigations are needed to approve the underlying mechanism and unveil the active ingredients.

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## Conflict of Interest

Authors declare no conflict of interests.

## References

1. Li W, Li N, Tang Y, et al. Biological activity evaluation and structure-activity relationships analysis of ferulic acid and caffeic acid derivatives for anticancer. Bioorga Med Chem Lett. 2012; 22(19): 6085-8. [DOI:10.1016/j.bmcl.2012.08.038] [PMID]
2. Laelago T, Yohannes T, Lemango F. Prevalence of herbal medicine use and associated factors among pregnant women attending antenatal care at public health facilities in Hossana Town, Southern Ethiopia: facility based cross sectional study. Arch Public Health. 2016; 74(1): 7. [DOI:10.1186/s13690-016-0118-z] [PMID] [PMCID]
3. Wang ZY, Chen Z. Acute promyelocytic leukemia: from highly fatal to highly curable. Blood. 2008; 111(5): 2505-15. [DOI:10.1182/blood-2007-07-102798] [PMID]
4. Sanz MA, Grimwade D, Tallman MS, et al. Management of acute promyelocytic leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. Blood. 2009; 113(9): 1875-91. [DOI:10.1182/blood-2008-04-150250] [PMID]
5. Haque MA, Jantan I, Arshad L, Bukhari SNA. Exploring the immunomodulatory and anticancer properties of zerumbone. Food Function. 2017; 8(10): 3410-31. [DOI:10.1039/C7FO00595D] [PMID]
6. Xu J, Wold E, Ding Y, Shen Q, Zhou J. Therapeutic potential of oridonin and its analogs: from anticancer and antiinflammation to neuroprotection. Molecules. 2018; 23(2): 474. [DOI:10.3390/molecules23020474] [PMID] [PMCID]
7. Cheng YY, Hsieh CH, Tsai TH. Concurrent administration of anticancer chemotherapy drug and herbal medicine on the perspective of pharmacokinetics. J Food Drug Anal. 2018; 26: 288-S95. [DOI:10.1016/j.jfda.2018.01.003] [PMID]
8. Hosseinzadeh H, Ramezani M, Salmani G. Antinociceptive, anti-inflammatory and acute toxicity effects of Zataria multiflora Boiss extracts in mice and rats. J Ethnopharmacol. 2000; 73(3): 379-85. [DOI:10.1016/S0378-8741(00)00238-5]
9. Sakhaee E, Emadi L, Azari O, Khanaman FS. Evaluation of the beneficial effects of Zataria multiflora Boiss in halothane-induced hepatotoxicity in rats. Adv Clin Exper Med. 2011; 20: 23-9.
10. Ahmadipour A, Sharififar F, Najafi A, Atashbar J, Karami-Mohajeri S. Preventive effect of methanolic extract of Zataria Multiflora Boiss on liver toxicity of paracetamol in rats. J Med Life. 2015; 8: 270-4.
11. Fatemi F, Asri Y, Rasooli I, Alipoor SD, Shaterloo M. Chemical composition and antioxidant properties of γ-irradiated Iranian Zataria Multiflora extracts. Pharmaceut Biol. 2012; 50(2): 232-8. [DOI:10.3109/13880209.2011.596208] [PMID]
12. Sharififar F, Moshafi M, Mansouri S, Khodashenas M, Khoshnoodi M. In vitro evaluation of antibacterial and antioxidant activities of the essential oil and methanol extract of endemic Zataria multiflora Boiss. Food control. 2007; 18(7): 800-5. [DOI:10.1016/j.foodcont.2006.04.002]
13. Shokrzadeh M, Azadbakht M, Ahangar N, Naderi H, Saravi SS. Comparison of the cytotoxic effects of Juniperus sabina and Zataria multiflora extracts with Taxus baccata extract and Cisplatin on normal and cancer cell lines. Pharmacogn Mag. 2010; 6(22): 102-5. [DOI:10.4103/0973-1296.62894] [PMID] [PMCID]
14. Khazdair MR, Ghorani V, Alavinezhad A, Boskabady MH. Pharmacological effects of Zataria multiflora Boiss L. and its constituents focus on their anti-inflammatory, antioxidant, and immunomodulatory effects. Fundament Clin Pharmacol. 2018; 32(1): 26-50. [DOI:10.1111/fcp.12331] [PMID]
15. Norooznezhad AH, Keshavarz M, Norooznezhad F, Mansouri K. Inhibition of angiogenesis: A Novel effect of Zataria multiflora. Int J Hematol Oncol Stem Cell Res. 2017; 11(2): 96-101.
16. Safarzadeh E, Sandoghchian Shotorbani S, Baradaran B. Herbal Medicine as Inducers of Apoptosis in Cancer Treatment. Adv Pharmaceut Bullet. 2014; 4: 421-7.
17. Huang M, Ye Y, Chen S, et al. Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. Blood. 1988; 72(2): 567-72.
18. Sheikhrezaei Z, Heydari P, Farsinezhad A, et al. A new indole derivative decreased SALL4 gene expression in acute promyelocytic leukemia cell line (NB4). Iran Biomed J. 2018; 22(2): 99-106.
19. Modak M, Dixit P, Londhe J, Ghaskadbi S, Devasagayam TPA. Recent advances in Indian herbal drug research guest editor: Thomas Paul Asir Devasagayam Indian herbs and herbal drugs used for the treatment of diabetes. J Clin Biochem Nut. 2007; 40(3): 163-73. [DOI:10.3164/jcbn.40.163] [PMID] [PMCID]
20. Qi F, Li A, Inagaki Y, et al. Chinese herbal medicines as adjuvant treatment during chemoor radio-therapy for cancer. Bio Sci Trends. 2010; 4(6): 297-307.

21. Janitermi M, Nemati F. Cytotoxic effect of Zataria multiflora on breast cancer cell line (MCF-7) and normal fibroblast cells. Cumhuriyet Sci J. 2015; 36(3): 1895-904.

22. Janitermi M, Nemati F, Mohammadpour G. Cytotoxic effect of Zataria multiflora on gastric cancer cell line (AGS) and normal fibroblast cells. Cumhuriyet Sci J. 2015; 36(3): 1876-84.

23. Janitermi M, Nemati F. Cytotoxic effect of Zataria multiflora on cervical cancer cell line (HeLa) and normal fibroblast cells. Cumhuriyet Sci J. 2015; 36(3): 1885-94.

24. Baharara J, Ramezani T, Hosseini N, Mousavi M. Silver nanoparticles synthesized coating with Zataria Multiflora leaves extract induced apoptosis in hela cells through p53 activation. Iran J Pharmaceut Res. 2018; 17(2): 627.

25. Hosseinimehr SJ, Ahmadashrafi S, Naghshvar F, Ahmadi A, Ehasnalavi S, Tanha M. Chemoprotective effects of Zataria multiflora against genotoxicity induced by cyclophosphamide in mice bone marrow cells. Integ Cancer Ther. 2010; 9(2): 219-23. [DOI:10.1177/1534735409360361] [PMID].

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