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Effect of Estradiol on miR-21& miR-155 Expression in promyelocytic leukemia-derived cell line NB4

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Abstract

Background: Due to the estrogen participation in modulating the proliferation and commitment of stem cells and the effects of miR-21 and miR-155 expression on reduced proliferation and colony formation of acute myeloid leukemia (AML), the aim of the present study was to evaluate the effect of estradiol on expression of miR-21 and miR-155 in the NB4 cell line, as an acute promyelocytic leukemia (APL).

Materials and Methods: In the present experiment, NB4 cells were treated with different quantities of estradiol (5, 25, 50, 75, 100, 150, 200, 250 µg/ml) and vehicle control for 24 and 48 hours. Viability, apoptosis, and cellular proliferation were estimated by trypan blue exclusion, flow cytometry, and MTT assays, respectively. The level of miR-155 and miR-21 expression was studied using absolute quantitative real-time PCR.

Results: Results showed that estradiol in the effective dose (200 µg/ml) led to decreased cellular viability (in a dose dependent manner, $P = 0.004$) and apoptosis of NB4 cells. In addition, the expressions of miR-155 and miR-21 were significantly and dose-dependently decreased ($p<0.05$).

Conclusion: Estradiol at the effective dose caused apoptosis in NB4 cell line. This substance can be used as a drug for the treatment of APL. However, further assessments are needed to support the effectiveness of estradiol in the treatment of APL.

Keyword: Acute Myeloid Leukemia (AML), Estradiol, MiR-155, MiR-21

Introduction

Leukemia as a most common hematologic malignancy is known by atypical proliferation of stem cells and development of leukocytes and their precursors in blood and bone marrow (1). Acute myeloid leukemia (AML), a group of heterogeneous clonal disorders of hematopoietic progenitors, is driven by numerous genetic perturbations that affect cell proliferation, differentiation, and apoptosis. As a result, immature blasts of myeloid lineage stack up in the bone marrow, the generation of mature end stage cells is blocked and finally fatal infections and hemorrhages are occurred (2). Based on the morphology of promyelocytic cells, the acute promyelocytic leukemia (APL) is identified as a M3 subtype of AML. The characteristic features of this abnormality are hemorrhagic episodes, differentiation arrest at the promyelocytic stage, and sensitivity to the differentiation response to all-trans-retinoic acid (ATRA). Cytogenetically, a balanced reciprocal translocation between chromosomes 15 and 17 and production of PML/RARA fusion protein are responsible of APL. Interestingly, current experiments reported that microRNAs are contributed to the pathogenesis of APL. Mature miRNAs are

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non-protein encoding RNAs that range from 19-25 nucleotides in size, regulate gene expression post-transcriptionally (3), and play important roles in a diversity of biologic processes and differentiation of stem cells into RBC lineage (4). They are critical regulators of cellular activities such as differentiation, development, proliferation, and apoptosis. Dysregulation of miRNAs can cause cancer and miRNA expression profile has been used to classify cancers. MiR-155, an important miRNAs involved in hematopoiesis (control both myelopoiesis and erythropoiesis), is expressed in high and low levels in normal hematopoietic stem-progenitor cells (HSPCs) and mature hematopoietic cells, respectively. However, the exact regulation mechanisms of normal myeloid commitment have not been obviously realized until now. Negative regulation of apoptosis, increase of the proliferation rate of myeloid progenitors, and promotion of HSPCs commitment to the common myeloid progenitors are probable functions of this microRNA. MiR-155 was frequently overexpressed in solid tumors as well as in hematological malignancies, such as diffuse large B-cell lymphoma (DLBCL), follicular lymphoma, Hodgkins lymphoma, primary mediastinal B-cell lymphoma, chronic lymphoid leukemia, and acute myeloid leukemia (AML) (5). Depending on the tissue and malignancy type, it can be either oncogene or tumor-suppressor.

In addition, it was found to be presented in CD34+ human HSPCS, and guess that may hold these cells at an early stem-progenitor stage, inhibiting their transformation into more mature cells (6). High expression of miR-155 in AML cell lines causes reduced proliferation and colony formation (7). On the other hand, MiR-21 functions as an oncogene and often over-expressed in various malignancies and has been participated in various malignancy-related processes such as uncontrolled proliferation, inhibition of apoptosis, and promotion of invasion and metastasis (8). MiR-21 is frequently overexpressed in AML blasts, and its expression is particularly increased in NPM1 mutant AMLs (9).

Estradiol, namely 17β-Estradiol (E2), is the most potent mammalian estrogenic steroids that circulates endogenously throughout the body and acts as the major female sex hormone. Its effects are mediated through Estrogen Receptor (ER) that is found in varied tissues such as in breasts, uterus, ovaries, bone, skin, fat, prostate, and brain. This hormone can bind to two types of Estrogen Receptors, Alpha (ERα) and Beta (ERβ) (10). Studies have indicated that Ets-1/miR-126 possibly mediated the protective effect of estradiol against atherogenesis (10). Increasing evidence indicates that estrogen has role in modulation of stem cell niche (11) as well as proliferation and lineage commitment of adult and pluripotent stem cells. In the current study, the effect of estradiol on miR-21 and miR-155 expression in AML cell line (NB4) was investigated by the researchers.

Materials and Methods

Cell cultivation

NB4 cells (purchased from Pasteur Institute, Tehran, Iran) were cultured at 37°C along with 5% CO_2 in RPMI 1640 enriched with fetal bovine serum (FBS, 10%), penicillin (100 units/ml), and streptomycin (100 µg/ml). All components of culture medium were provided from Gibco Life Technologies, USA. Dimethyl sulfoxide (DMSO; Merck, Germany) was used for preparation of NB4-stock suspension.

Hormones

17β-Estradiol Hormone (Sigma,USA) was purchased from Sigma. Using methanol, as diluents, hormone was formulated as 10-3 M stock suspensions and stored at -20°C.

In vitro treatment with estradiol

Treatment was done in three groups. NB4 cells were cultured for 1 to 3 days under the following conditions: i. Blank: NB4 did not receive any treatment (untreated

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group), ii. Estradiol: NB4 cells were treated with Estradiol and iii. Methanol: NB4 cells were treated with methanol (negative control group). Each groups had three samples. Two-five×10^5 cells/ml were seeded in culture flasks containing antibiotic-supplemented RPMI enriched with FBS. Next, NB4 cells were treated with 5, 25, 50, 75, 100, 150, 200, 250 µg/ml of estradiol, and control groups were treated with methanol as diluents. After 24, 48, and 72 hours of treatment, enumeration of viable and dead cells was performed with Neubauerhemocytometry and Trypan blue stain.

Cell viability measured by Trypan Blue Exclusion Assay

As described above, to investigate the estradiol effect on the cell viability, cells were seeded into a 12-well culture plate (2×10^5 cells/well) and treated with concentrations of estradiol.After mixing (1:1 ratio) of cellsuspension with 0.4% trypan blue solution (Sigma-Aldrich, USA) and incubation at room temperature (1-2 minutes), the clear (viable) and blue (dead) cells were enumerated on neubauer hemocytometer, and the viability percentage was determined.

MTT assay

MTT (Merck, Germany) colorimetric method was applied for evaluation of estradiol effect on metabolic activity of NB4 cells. The cells were seeded into a 96-well plate (10^4cells/well) and treated with concentrations of estradiol. After 24, 48, and 72 hours, cells were exposed (4 hours) to MTT solution (5 mg/ml PBS) at 37°C. The resulting absorbance after solubilization of formazan crystals (using 100 µLDMSO) was measured at 570 nm by an enzyme-labeled analyzer. The metabolic activity (%) of estradiol-treated cells was calculated relative to control cells (set as 100%).

Apoptotic assay

The cells were cultured (2×10^5 cells/well of 6-well plate) in Estradiol-containing RPMI media, according to manufacturer protocol. Detection of viability and apoptosis was performed with Annexin V-FITC/Propidium iodide kit (B.D, USA). After 1 day, the cells were centrifuged, the resulting pellet was washed with cold PBS (2×) and then resuspended in binding buffer (1×), 5 µl of FITC-Annexin V and 5 µlof PI. Upon 15minutes incubation at room temperature in dark, the cells were analyzed by flow cytometer (FloMax software V2.0).

RNA Extraction

After treatment with Estradiol, total RNAs from NB4 cells were isolated by Trizol RNA extraction kit (Invitrogen, USA) based on the manufacturer protocol. Agarose gel electrophoresis and optical density measurement (A260/A280 ratio, Nano Drop 1000 Spectrophotometer) were applied for evaluation of quality and purity of extracted RNA, respectively.

cDNA synthesis and Real-time PCR

Aftersynthesis of cDNA using corresponding synthesis kit (Thermo, USA), the expression levels of miR-155 and miR-21 was investigated by stem-loop real-time PCR (Tables I, II, and III; Rotor-Gene Q Real-time PCR System, Qiagen, USA). Annealing temperatures and concentrations of MgCl2 were optimized to establish a one-peak melting curve. The relative expression levels of miRNAs was determined from the threshold cycle (Ct) by $2^{-\Delta\Delta Ct}$ method and normalized to the expression of endogenous control U48.

Statistical Analysis

Two-tailed Student's t test by SPSS 22 software was used to determine the significant differences between studied groups. P-value <0.05 was considered statistically meaningful

Results

Effects of estradiol on cell viability

Viability of the NB4 cells was investigated 1, 2, and 3 days after exposure to concentrations of estradiol using trypan blue exclusion assay. As shown in Figure 1.A, the cellular viability decreased in a dose-dependent but not in a time-dependent manner and demonstrates a

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considerable decrease at the dose of 200µg/ml (P = 0.004).

Effect of estradiol on metabolic activity

Metabolic activity of cells was assessed 24, 48, and 72 hours after treatment with estradiol. As presented in Figure1.B, this factor decreased in a dose-dependent but not time-dependent manner ($p<0.03$ and $p>0.7$, respectively). In addition, metabolic activity reduced to 50 % at 24 hours after treatment with the dose of 200 µg/ml (P = 0.001).

Effect of estradiol on apoptosis

The cells were exposed to estradiol for 24 hours, and then the apoptosis-inducing effects of estradiol on NB4 cells were checked out with Annexin V/PI approach (Figure 2). As depicted, estradiol dramatically promotes apoptosis in the studied cells.

MicroRNAs expression

The expression level of miR-155 and miR-21 was assessed 24 hours after treatment with 200 µg/ml of estradiol.

MiR-155

Estradiol had obvious effect on miR-155 expression. Namely, the miR-155 level in test group was decreased comparing with control group ($p<0.05$; Fig 3.A).

MiR-21

In comparison with control group, 200 µg/ml of estradiol decreased the miR-21 expression in test group ($p<0.05$; Figure 3.B).

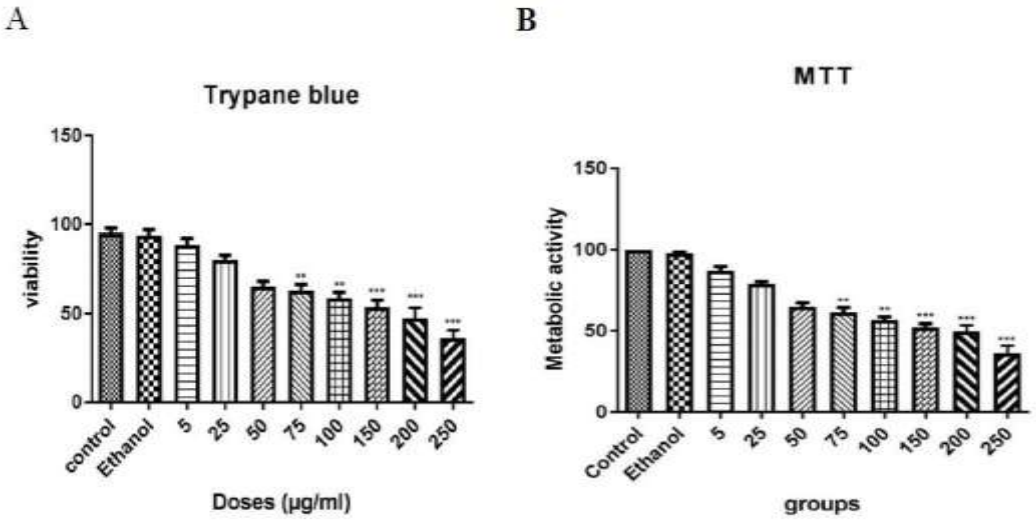

Figure1. A: The NB4 cells were incubated 24, 48, and 72 hours with various concentrations of estradiol (5, 25, 50, 75, 100, 150, 200, and 250 µg/ml) and the cell viability was assessed by trypan blue exclusion assay. Estradiol decreases the cell viability of cells in a dose- but not in a time-dependent manner ($p<0.03$ and $p>0.7$, respectively). B: The NB4 cells were cultured for 24, 48, and 72 hours in the presence of different amounts of estradiol (5, 25, 50, 75, 100, 150, 200, 250 µg/ml) and metabolic activity was assessed using MTT assay. The metabolic activity of cells was significantly reduced after exposure to the different concentrations of estradiol.

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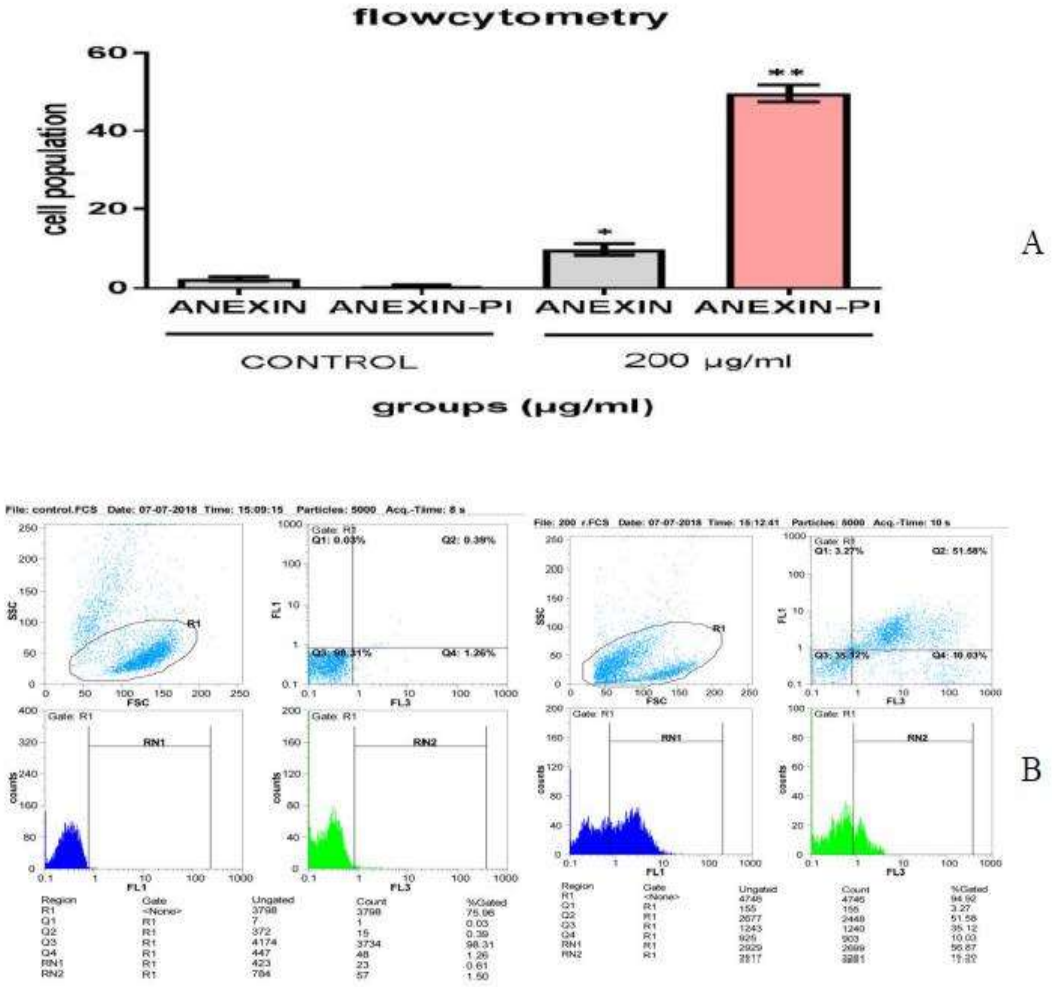

Fig2: A: Estradiol significantly promotes apoptosis in studied cells. NB4 cells were incubated with estradiol for 24 h. Then, cells were analyzed for Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) uptake by flow cytometry (B). One representative experiment of three independent experiments is shown. The different subpopulations were defined as UL (upper-left): Annexin V-negative but PI-positive, i.e. necrotic cells; UR (upper-right): Annexin V/PI-double positive, i.e. late apoptotic cells; LL (lower-left): Annexin V/PI-double negative, i.e. normal live cells; and LR (lower-right): Annexin V-positive but PI-negative, i.e. early apoptotic cells.

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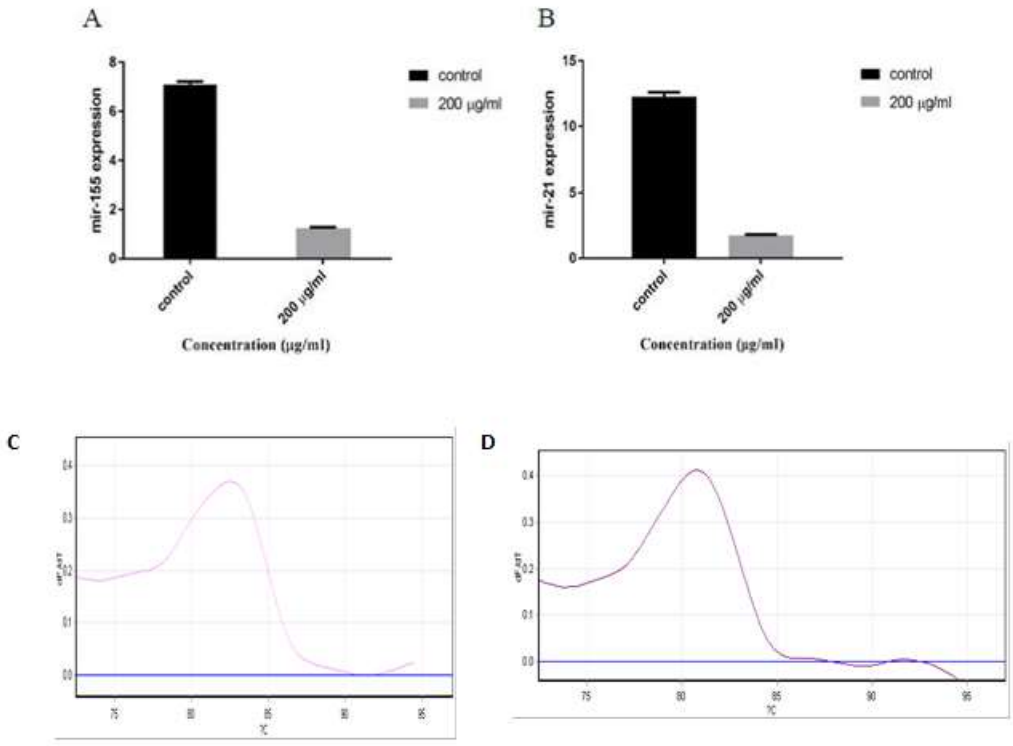

Figure 3. A: MiR-155 expression following treatment with estradiol 200 µg/ml for 24 h. Following 24h of culture cells were harvested and subjected to mRNA expression. The cDNA was synthetized and Real-Time PCR was carried out. The mRNA expression was then calculated against U48 (housekeeping gene). B: Expression of miR-21 following treatment with estradiol 200 µg/ml for 24 h. After 24h, the cells were harvested and subjected to mRNA expression. The cDNA was synthetized and Real-Time PCR was carried out. The mRNA expression of miR-21 was then calculated against U48 (housekeeping gene). C: Melting curve of miR-21. D. Melting curve of miR-155.

Discussion

The majority of APL patients are currently treated with the molecularly targeted therapy with all-trans retinoic acid (ATRA) and arsenic trioxide (ATO). About 90% of these patients ameliorate after treatment. Nevertheless, some defeats such as hemorrhage (heavy bleeding) during the treatment's initial phases, resistance to cure, and the return of APL (relapse) can occur (2).

MiRNAs are small and noncoding RNAs that regulate many cellular functions and events such as development, proliferation, differentiation, and apoptosis (3). In addition, they control gene expression by degrading or suppressing target messenger RNAs (mRNAs) at the post-transcriptional level. Unusual expression of miRNAs has been discovered in both solid and hematopoietic tumors by various genome-wide techniques (12). Considering oncogenic and tumor suppressor activities of miRNAs, recent study has reported that miRNAs additionally play an important role in the APL pathogenesis (3). Additionally, miRNAs expression profile is changed during ATRA treatment and, on the other hand, the inhibition of some miRNAs also changes the gene expression pattern in the APL cell lines. Accordingly, the discovery of these RNAs as potential diagnostic and prognostic markers will open a new insight into the APL management (12). It has been reported that miR-155, an "oncomiR", is the most significantly up-regulated miRNA in

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breast cancer and its expression is significantly higher in ERα (+) breast tumors. High expression of this microRNA was found in different lymphomas, such as Hodgkin, pediatric Burkitt, and diffuse large B-cell. Furthermore, it has been reported that miR-155 play a unique role in myeloid leukemogenesis (12), as occurs in AML. On the other hand, miR-21 through modulating multiple genes can provoke growth of cancer cells, including breast, colon, lung, pancreas, prostate, stomach, and cervical carcinomas. Similar to miR-155, miR-21 is often overexpressed in AML blasts and particularly increased in NPM1 mutant AMLs (9).

A plethora of experiments have revealed the effects of estradiol on differentiation, cell-cycle arrest, and apoptosis of cancer cells (11). Additionally, estrogen therapy has long been applied to decrease the risk of cardiovascular disorders in postmenopausal period. It has been discovered that endothelial miR-126-3p expression, via Ets-1, was increased by E2. Next, binding of miR-126-3p to VCAM-1 and Spred1 mRNA, leading to silencing and degradation of proteins as well as triggers endothelial proliferation, migration, tube formation, and impedes monocyte adhesion. The most effective cure for vasomotor symptoms and other symptoms of climacteric is Menopausal hormone therapy (MHT) (16). Transdermal Estradiol therapy for advanced prostate cancer has an effective tumor response. Transdermal Estradiol therapy costs a tenth of prevalent therapy costs, with the potential for notable economic saving over common hormone therapies (12).

The nature of the mechanisms regulating hematopoiesis is unknown. Two specific hallmarks of stem cells are Self-renewal and Differentiation, which are controlled by numerous intrinsic and extrinsic elements. Growing evidences illustrate that estrogen participates in management of proliferative capability and lineage commitment of adult/pluripotent stem cells as well as modulating the stem cell niche (10). Estrogen signaling plays important roles in regulating reproduction, bone density, lactation, cardiovascular function, immune function, neuronal signaling, and homeostasis (13). Mechanisms by which E2 and other estrogens regulate various physiological effects are both genomic and non-genomic ones including ERα, ERβ, and GPR30. It seems that E2 regulates miRNA expression in a cell type-dependent manner (13). Some studies on miRNA analysis have shown that E2 regulates a variety of miRNAs in ERa (+) cells. E2 is promoted breast cancer progression/development possibly via up- and down- regulation of miR-155 and TP53INP1, respectively (14).

Through a regulatory site in the producing gene (MIRN21) promoter region, 17ß-estradiol down-regulates the expression of miR-21. E2 treatment of MCF-7 cells reestablishes JAG1 levels via reducing the expression of miR-21 (15).

In the present study, it was demonstrated that estradiol led to the downregulation of miR-155 and miR-21 in NB4 cells; therefore, it can be proposed as another underlying mechanism involving in anticancer effects of estradiol.

Conclusion

Throughout the present study, it was found that Estradiol led to the apoptosis of NB4 cell line. Additionally estradiol led to significant down-regulation of miR-21 and miR-155. Therefore, it can be considered as an effective agent in the treatment of promyelocytic leukemia. However, further investigations are needed to approve the efficacy of estradiol in the treatment of promyelocyticleukemia, others signal pathways and its anticancer properties on other cancer cells.

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Iran (ethical code: IR.BPUMS.RES.1395.188).

Conflict of interest

The authors declare that they have no competing interests.

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